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KEAP1/NRF2-INDEPENDENT ANTIOXIDATIVE ACTIVITY OF *Phyllanthus amarus* EXTRACT IN ZEBRAFISH

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SUMMARY

The Keap1 protein (Kelch-like ECH-related protein 1) and the Nrf2 transcription factor (NF-E2-related factor 2) are important systems for maintaining homeostasis, redox, and metabolism. Based on the Keap1/Nrf2 pathway, the antioxidative mechanism of *P. amarus* extract (PAE) was predicted. In this paper, we evaluated the protective effects of PAE on the oxidative toxicity induced by sodium arsenite ($NaAsO_2$) and hydrogen peroxide (H_2O_2) in zebrafish larvae. We first determined that the LC_{50} values for $NaAsO_2$, H_2O_2, and PAE at 3.5 days postfertilization (dpf) were 1 mM, 3 mM, and 200 µg/mL, respectively. Then, to assess the antioxidant effects of *P. amarus*, 3.5 dpf zebrafish larvae were pretreated with PAE at concentrations of 0, 50, 75, and 100 µg/ml for 12 h, and then the PAE solution was replaced with 1 mM $NaAsO_2$ or 3 mM H_2O_2 to assess challenge survival within 48 h. Interestingly, all three concentrations, 50, 75, and 100 µg/mL PAE, increased the survival rate of zebrafish larvae compared to those larvae exposed to only 1 mM $NaAsO_2$. Similarly, PAE at concentrations of 75 and 100 µg/mL protected zebrafish larvae after exposure to 3 mM H_2O_2. Real-time qPCR analysis was performed after 3.5 dpf zebrafish larvae were exposed to 100 µg/mL PAE for 12 h to verify whether the increasing antioxidative activity is depended on the Nrf2 pathway. The expression of the Nrf2 target genes glutathione-S-transferase Pi 1 (*gstp1*) and peroxidase 1 (*prdx1*) was assessed using real-time qPCR. However, the expression of this gene was not significantly different between control larvae and PAE-treated larvae. Thus, PAE induces antioxidant activity in zebrafish in a Keap1/Nrf2-independent manner.

Keywords: arsenite, antioxidant, hydrogen peroxide, Keap1/Nrf2, *Phyllanthus amarus*, zebrafish

INTRODUCTION

A key function of Keap1 (Kelch-like ECH-associated protein 1) and Nrf2 (NF-E2-related factor 2) is the regulation of redox, metabolism, and protein homeostasis as well as inflammation.

By activating Nrf2 through Keap1, cells can be protected from various stressors, including chemical-induced oxidative stress, which causes chronic pulmonary and liver diseases, autoimmune diseases, neurodegenerative diseases, and metabolic disorders in humans (Bauer *et al.*, 2013; Namani *et al.*, 2014; Harder *et al.*, 2015; Lu *et al.*, 2016; Mukaigasa *et al.*, 2018).

Nrf2 is a transcription factor that regulates antioxidative stress proteins and detoxification enzymes. Keap1 is a cytosolic protein that interacts with Nrf2 to promote its ubiquitination and proteasomal degradation. Interestingly, Keap1 can also sense a variety of stress and chemicals and changes its protein conformation to disrupt the proper Keap1-Nrf2 interaction for ubiquitination, leading to stabilization of Nrf2 and transactivation of its target genes. Previously, the Nrf2 target gene was explored in a zebrafish model and showed that the systematic regulation was based on the same molecular mechanism as that in vertebrates (Nakajima *et al.*, 2011; Hahn *et al.*, 2014). In addition, an analysis of the mutation of zebrafish Nrf2, *nrf2a*^{fh318}, (Mukaigasa *et al.*, 2012) revealed that the antioxidant and biological protective role of Nrf2 was conserved in zebrafish. My former laboratory member previously searched Nrf2 target genes by microarray analysis using Nrf2 overexpressing embryos. In total, 115 genes were found to be more than 1.5-fold up-regulated by over-expressing Nrf2 embryos. Among these genes, glutathione-S-transferase Pi 1 (*gstp1*) and peroxidase 1 (*prdx1*) are the completely Nrf2-dependent genes that have been used to analyze stressors (Fuse *et al.*, 2016; Nguyen *et al.*, 2016, 2020).

Scientists are currently developing Nrf2 activators, one of which has been approved by a physician for clinical use. The development of electrophilic substances, such as sulforaphane (Kensler *et al.*, 2012; Takaya *et al.*, 2012), extracted from broccoli, modulated cysteine-sensors of Keap1, and inhibitors of Keap1's interaction with Nrf2, are currently underway. However, it remains difficult to overcome challenges related to the specificity of the target gene, pharmacodynamic properties, efficacy, and safety.

Phyllanthus amarus is a plant that can be used as a medicinal herb, whether it is dried or used fresh. It is usually found on coasts, large islands, midlands, and provinces. In traditional medicine, *P. amarus* is often used to treat liver diseases and detoxify the liver (Phu *et al.*, 2021). It also has a high oxidation resistance (Huong *et al.*, 2010). However, there is no evidence that Keap1/Nrf2 plays a role in the antioxidant mechanism of *P. amarus*.

The zebrafish is commonly used as a model for toxicological and human disease research (Zhang *et al.*, 2003; Xu, Zon 2010; Fuse, Kobayashi 2017). A recent study employed zebrafish to assess the protective effects of phytochemicals on acute H_2O_2 and $NaAsO_2$ toxicity (Endo *et al.*, 2020; Nguyen *et al.*, 2020; Watanabe *et al.*, 2022). This study provides evidence of the protective effects of PAE against oxidative stress induced by H_2O_2 and $NaAsO_2$. Furthermore, we examined whether the PAE enhancement of antioxidative function in zebrafish is in Keap1/Nrf2-dependent manner.

MATERIALS AND METHODS

Zebrafish and chemical testing

We obtained AB zebrafish strains from Professor Makoto Kobayashi's laboratory at Tsukuba University (Japan) and maintained them at the Fisheries Laboratory of the Biotechnology Center of Ho Chi Minh City.

P. amarus was collected in the EaKly Commune of KrongPak District, DakLak Province, Vietnam. In the experiments, sodium arsenite (Xiya Reagent, China), 35% hydroperoxide (Xilong Scientific, China), sulforaphane (Sigma, USA), and dimethyl sulfoxide (Sigma, USA) were used.

P. amarus extraction process

The stems and leaves of dried *P. amarus* were pureed and 200 g dried powder was

dissolved in 2000 mL of 70% ethanol. The jar was soaked at room temperature for 3 days. The soaked fluid was pureed from the soaking jar to the filter container through the filter bag to filter the residue and medicinal residue, then, vacuum pump/filtration system was used to purify medicinal residues before extraction. Ethanol extraction was carried out automatically using Rotavapor® R-300 vacuum spinner (Buchi AG, Switzerland) under these following conditions: rotation pressure of 150 – 250 mBar (alcohol collection phase); 45 – 65 mBar (water collection phase); revolutions: 100 – 150 rpm; heating tank temperature: 60 – 65°C; and condensation temperature: 10°C. The potion container was allowed to cool for approximately 5 min, and then the jar was slowly removed from the device. A specialized spoon was used to scrape, collect the extract and place it in a 15 mL Falcon. Extract was diluted in DMSO to 100 mg/mL and store at -20°C until use.

Survival assay and chemical LC_{50} value determination

The median lethal concentration (LC_{50}) was determined using survival assay procedures. The LC_{50} values were determined by exposing 3.5–4 dpf zebrafish larvae to 25–200 µg/mL PAE, 1–4 mM $NaAsO_2$ and 1–5 mM H_2O_2 for 72 h. A survival assay was conducted following by Nguyen *et al.* (2020).

Using a survival rescue experiment, we tested whether PAE protects against acute toxicity caused by $NaAsO_2$ and H_2O_2. In each well of a 6-well plate (3 cm in diameter), larvae at 3.5 dpf were placed in a solution of PAE and 5 mL E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$ and 0.1 mg/L methylene blue). Following 12 h of exposure, all solutions were drawn out and exposed to $NaAsO_2$ or H_2O_2. For a period of 48 h, dead fish larvae were collected every 12 h. The larvae were not fed during the experiment.

Gene expression analysis

Isolation of RNA was achieved using the TRIzol™ Reagent kit (Invitrogen, USA). Total RNA (300 ng) was generated using the ReverAid First Strand cDNA Synthesis Kit (ThermoFisher, USA). We conducted a real-time qPCR experiment using the Roche Life Science Real-time LightCycler® 96 system and Maxima SYBR Green/ROX qPCR Mastermix (2X) (ThermoFisher, USA). Gene expression levels (*gstp1* and *prdx1*) were normalized to *ef1α*. According to Nguyen et al. (2020), the primer sequences were as follows: *ef1a (*5'-CGTGGTAATGTGGCTGGAGA and 5'-CTGAGCGTTGAAGTTGGCAG)*; gstp1* (5'-CAACGCCATGCTGAGACATC; and 5'-GAAGATCTTCAACGCCGCCGTCG), and *prdx1* (5'-GTCCCACTGAGATCATCGCCCCCC; and 5'-AACCACCTTTTTTTTTTTGGGGT).

Statistical analysis

Statistical analysis of survival data was conducted using the log-rank test. A two-tailed Student's *t* test was used to analyze gene expression levels. The significance of the results was determined using a *p* value of 0.05.

RESULTS

Extraction yields of *P. amarus*

To prepare the *P. amarus* extract for the rescue experiments, 200 g of dried *P. amarus* stems and leaves were soaked in 2 L of 70% ethanol for 3 days. The extraction was then performed on the Buchi system using 1 L of immersion fluid. As a result, 15.05 g (yield: 15%) PAE was obtained (Table 1).

Toxicity of $NaAsO_2$, H_2O_2, and *P. amarus* extract

A survival test was performed on zebrafish larvae to determine the LC_{50} values of $NaAsO_2$, H_2O_2 and PAE to select the appropriate concentration of chemicals for the experiments. $NaAsO_2$ or H_2O_2 exposure occurred at 4 dpf in zebrafish larvae for 72 h (Figure 1A). The larvae exposed to 4 mM arsenite died after 12 h (n = 135). A dose-dependent survival pattern was observed during exposure to sodium arsenite at concentrations in the range of 1 to 2 mM,

indicating that 1 mM was an appropriate concentration to test the acute toxicity of sodium arsenite in zebrafish (Figure 1B). In a similar fashion, all larvae (n = 135) died after 12 h of exposure to 5 mM hydrogen peroxide. Despite its acute toxicity, zebrafish survival was dose-dependent from 1 to 4 mM hydrogen peroxide, suggesting that a concentration of 3 mM was suitable for evaluating the acute toxicity of hydrogen peroxide in zebrafish (Figure 1C).

Table 1. Yield of *P. amarus* extracts by 70% ethanol.

	Parts used	Mass dried medicinal herbs (g)	Solvent volume (mL)	Extraction volume (mL)	Mass obtained (g)	Yield (%)
Phyllanthus amarus	Stems and leaves	200	2000	1000	15.05	15%

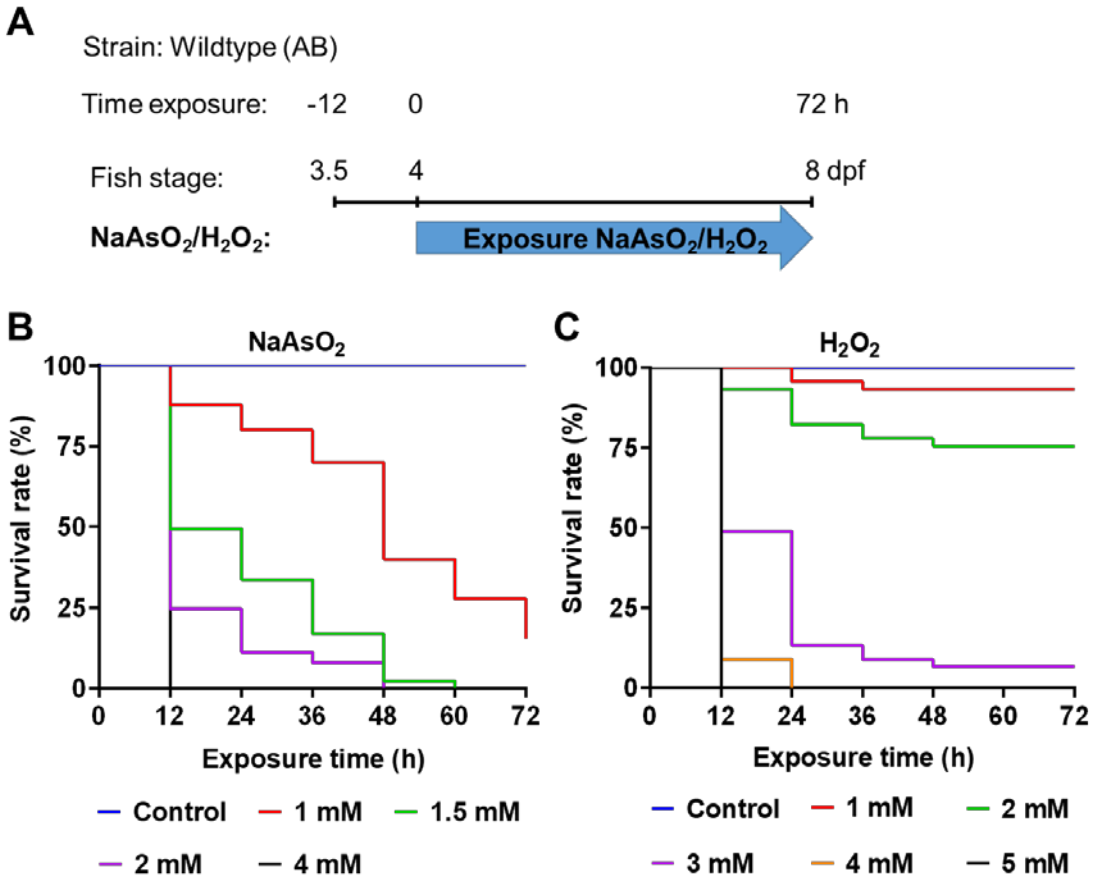

Figure 1. Determination of the average lethal concentration (LC50) value of $NaAsO_2$ and H_2O_2 on zebrafish larvae. A: Schematic representation of the $NaAsO_2/H_2O_2$ processing experiment. B: The LC_{50} value for $NaAsO_2$. C: Estimate of the LC_{50} value for H_2O_2. The data were collected from three separate experiments. The number of samples for each test was n = 135.

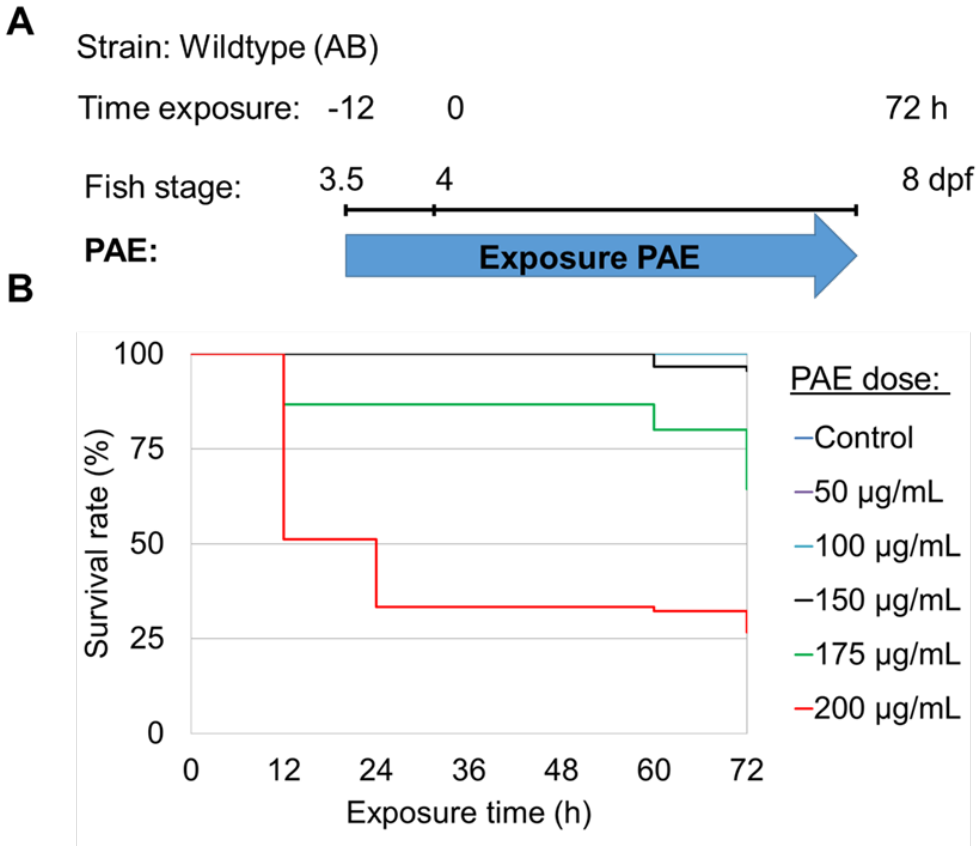

Figure 2. Analyze the effects of *P. amarus* extracts on zebrafish larvae at an average lethal concentration value (LC_{50}). **A.** A diagram describing the PAE exposure experiment. **B.** Zebrafish larval survival rate following exposure to 50–200 µg/mL PAE. Three independent experiments were conducted. The sample size for each test is n = 135.

Furthermore, it was important to determine the LC_{50} value of PAE for the rescue experiments to determine the safe dose of PAE to be used. Zebrafish larvae were administered 25–200 µg/mL PAE for 72 h at 3.5 dpf (Figure 2A). As shown in Figure 2B, survival was dose-dependent between 150 µg/mL and 200 µg/mL. The LC_{50} value was identified at 200 µg/mL PAE at 12 h, suggesting that 50–100 µg/mL PAE was a safe concentration for zebrafish rescue experiments (Figure 2B).

PAE pretreatment suppresses oxidative stress

We pretreated zebrafish larvae with 50-100 µg/mL PAE at 3.5 dpf to confirm that PAE protects against oxidative stress induced by arsenite or hydrogen peroxide (Figure 3A, B). A log-rank test revealed that pretreatment with 50 and 100 µg/mL PAE significantly improved survival in AB zebrafish larvae exposed to 1 mM $NaAsO_2$ (Figure 3B).

Furthermore, larvae that were exposed to 3 mM H_2O_2 showed significantly improved survival when treated with 75 or 100 µg/mL PAE (Figure 3C, log-rank test, $p < 0.001$). The presence of 50 µg/mL PAE did not result in any protection against hydrogen peroxide (Figure 3C). These results suggest that PAE pretreatment improves the survival of larval zebrafish exposed to arsenite and hydrogen peroxide.

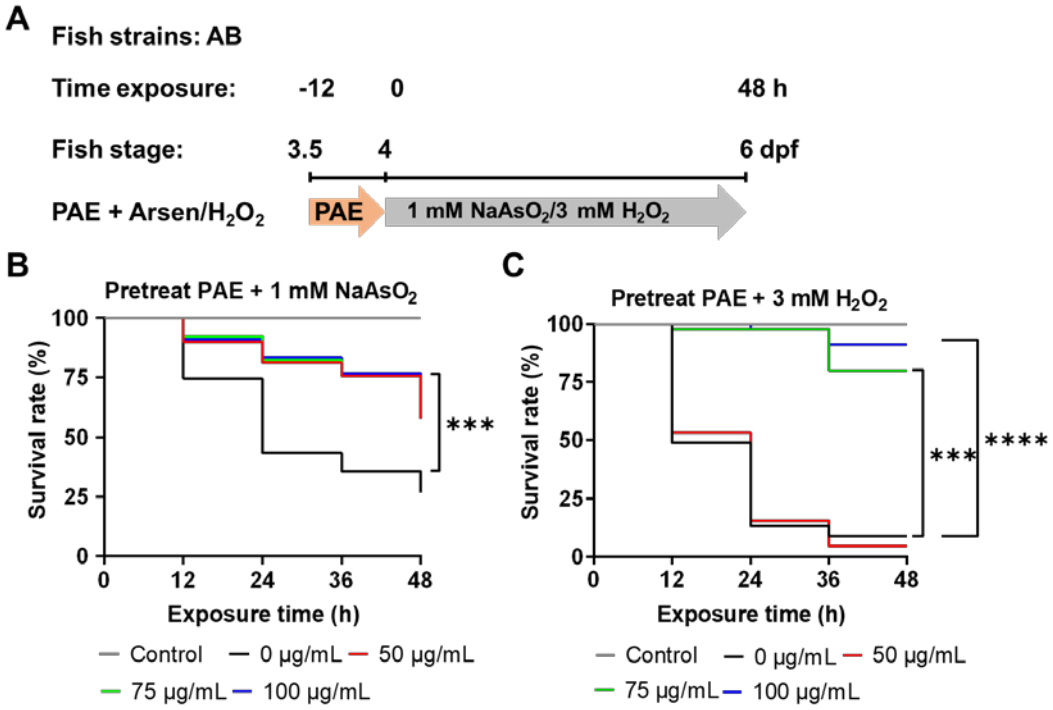

Figure 3. Protective effect of PAE on the acute toxicity of arsenite and hydrogen peroxide. A: Schema describing the survival test. B: Protective effect of 50 to 100 µg/mL PAE against 1 mM $NaAsO_2$ toxicity. C: Protective effects of 50–100 µg/mL PAE on 3 mM H_2O_2 toxicity. Three repetitions of the experiment were carried out. The asterisk indicates a statistically significant difference (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; and ns: no statistical significance). Sample size n = 135.

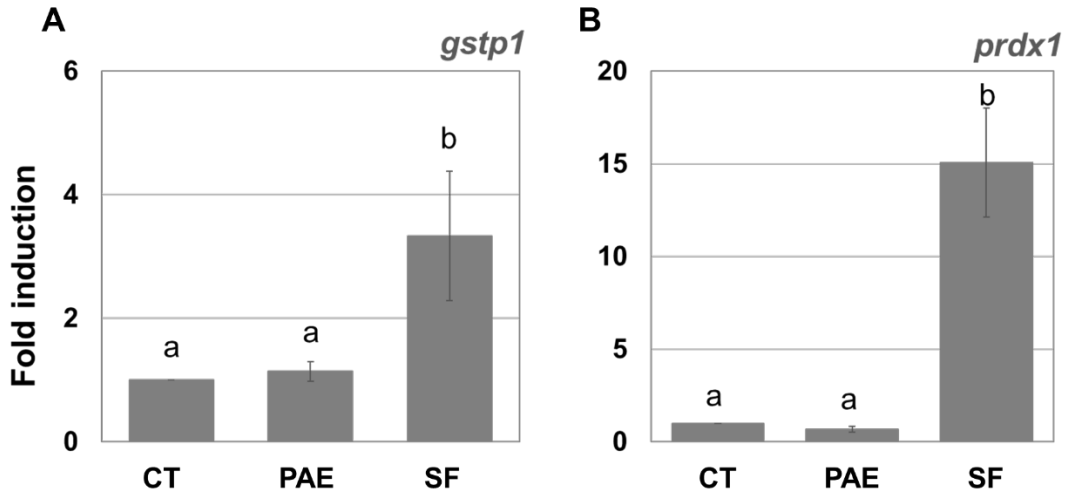

Figure 4. Expression of Nrf2 target genes in 100 µg/mL PAE- and 40 µM sulforaphane-treated larvae. A: Expression of *gstp1* in 100 µg/mL PAE- and 40 µM sulforaphane-treated larvae. B: Expression of *prdx1* in 100 µg/mL PAE- and 40 µM sulforaphane-treated larvae. It was normalized to 1 in the control group (CT). The experiments were conducted at least twice with duplicate samples in each experiment. The letters "a" and "b" indicate significant differences (p<0.05).

PAE did not induce Nrf2 target gene expression

Keap1/Nrf2-dependent protection against oxidative stress in zebrafish was elucidated by examining the expression of the *prdx1* and *gstp1* genes (Nguyen *et al.,* 2016; Fuse, Kobayashi 2017).

We exposed 3.5 dpf zebrafish larvae to 100 μg/mL PAE and 40 μM sulforaphane (SF), a positive Nrf2 activator derived from broccoli (Fuse et al. 2016), for 12 h. As shown in Figures 4A and 4B, SF-treated larvae were strongly induced to express *gstp1* and *prdx1*, whereas PAE-treated larvae did not show this induction. These findings suggest that the upregulated antioxidant activity of PAE may not be mediated by Keap1/Nrf2.

DISCUSSION

Our study examined the protective effect of *P. amarus* extract on zebrafish larvae in response to oxidative stress caused by $NaAsO_2$ and H_2O_2. In the investigation of Nrf2 target gene expression, we found that oxidation resistance in PAE may occur via a Keap1/Nrf2-independent pathway.

It has been shown that *P. amarus* contains a high level of phenolic content and has good antioxidant activity (Nhu *et al.,* 2018; Phu *et al.,* 2021). The aqueous extract of the leaves of *P. amarus* was found to be significant in protecting against DNA destruction by lymphocytes and improving oxidative status (Karuna *et al.,* 2009). A study by Rajeshkumar et al. (2002) on a mouse model also demonstrated that the high-water extract of *P. amarus* leaves possesses strong anticancer activity against chemicals that promote the growth of sarcomas.

Considering our results, PAE may be suitable for supporting acute detoxification from arsenite, a substance known for its ability to cause oxidative stress, damage DNA and increase the risk of cancer-causing abnormal cell formation (Ventura-Lima *et al.,* 2011; Fuse *et al.,* 2016, 2018). Furthermore, arsenite depletes liver and kidney functions in humans, resulting in serious health effects and even death (Susan *et al.,* 2019). It must be noted, however, that PAE may be independent of oxidation via the Nrf2 pathway, so treatment is a strategy that has side effects such as "double-edged sword" effects due to Nrf2 activation (Tebay *et al.,* 2015; Deshmukh *et al.,* 2017). Molecular biology studies must determine the exact mechanism by which PAE affects arsenic detoxification and resistance to oxidation (in addition to Keap1/Nrf2). The result of this process is the preparation of the most effective drugs and the reduction of unwanted ingredients in herbs.

CONCLUSION

Phyllanthus amarus extract showed protective effects against oxidative stress induced by arsenite and hydrogen peroxide. In response to *P. amarus* extract treatment, zebrafish larvae did not demonstrate increased expression of the Nrf2 target genes (*gstp1* and *prdx1*). This suggests that the oxidation resistance of *P. amarus* extract may not be mediated by Keap1/Nrf2.

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