Borneo Journal of Resource Science and Technology (2023), 13(2): 79-91
DOI: https:doi.org/10.33736/bjrst.5669.2023

The Mechanism of Anxiolytic Effects of *Moringa oleifera* Leaf Extracts Associated with Significant Differential Expression of *Crhb*, *Faah2a*, *Mao*, and *Pah* Genes in *Danio rerio*

MUHAMMAD FAIQ NAJMI[1,2], MOHD SALLEH ROFIEE[1,3], TEH LAY KEK[1,4], FARIDA ZURAINA MOHD YUSOF[1,2] & MOHD ZAKI SALLEH*[1]

[1]Integrative Pharmacogenomics Institute, Universiti Teknologi MARA Selangor, Puncak Alam Campus, Bandar Puncak Alam, 42300 Selangor, Malaysia; [2]Faculty of Applied Science, Universiti Teknologi MARA, Shah Alam Campus, 40450 Shah Alam, Selangor, Malaysia; [3]Faculty of Health Sciences, Universiti Teknologi MARA Selangor, Puncak Alam Campus, Bandar Puncak Alam, 42300 Selangor, Malaysia; [4]Faculty of Pharmacy, Universiti Teknologi MARA Selangor, Puncak Alam Campus, Bandar Puncak Alam, 42300 Selangor, Malaysia
*Corresponding author: zakisalleh@uitm.edu.my
Received: 8 May 2023 Accepted: 10 August 2023 Published: 31 December 2023

ABSTRACT

The search and development of new therapeutic agents from medicinal plants to alleviate anxiety is well justified due to the increasing cases of anxiety disorder and lack of effective treatment. *Moringa oleifera* has been used traditionally to treat anxiety. However, there is still lack of understanding on the mechanism for its anxiolytic effect. The purpose of this study was to investigate the anxiolytic effects and the mechanism of ethanolic extracts of the leaves of *M. oleifera* (MOLE) by observing behavioural changes of the *Danio rerio* and the differential gene expression analysis using custom RT^2 Profiler PCR array. A 14-day chronic behaviour study was conducted using three concentrations of MOLE (500 mg/L, 1000 mg/L and 2000 mg/L) fluoxetine as the positive control. Stress-induced *D. rerio* treated with 1000 mg/L MOLE showed the lowest level of anxiety compared to other groups as evidenced by a decrease in freezing episodes and freezing time, increased entries into the light region. The fish also showed significant changes in the expression of *crhb, faah2a, mao,* and *pah* genes. MOLE with the presence of quercetin and kaempferol are believed to exert its anxiolytic effects through differential expression of gene (i) modulating the function of GABAA receptor (crhb), (ii) inhibiting the expression of nitric oxide synthase (NOS) and the production of nitric oxide, (iii) increasing the AEA levels in the brain (faah2a), (iv) increasing the level of dopamine levels in the brain (mao). These findings provide valuable insights into the potential use of MOLE as a treatment for anxiety-related disorders as well as the significance of the molecular pathways involved in its anxiolytic properties.

Keywords: Chronic behaviour study, *Danio rerio,* gene expression, *Moringa oleifera,* RT^2 profiler PCR array

INTRODUCTION

Anxiety disorder is one of the global mental health problems. There were 260 million people who lived with anxiety in 2017 and the number was expected to increase every year (World Health Organization, 2017). Even in Malaysia, according to the survey conducted by the Institute for Public Health Malaysia in 2017, about 40% of Malaysians have depression and anxiety disorder. Anxiety is the body's natural response to stress and it appears to be a normal process for everyone. However, if the feeling of fear or nervousness reach an intense level, the individual will be diagnosed with anxiety disorder. The wide spectrum of the anxiety disorder consists of generalised anxiety disorder (GAD), phobia disorder, obsessive-compulsive disorder (OCD), separation anxiety disorder (SAD) and post-traumatic stress disorder (PTSD) and panic disorder (Baldwin *et al.*, 2014). Chronic anxiety without treatments may lead to suicidal thoughts. The two methods to treat anxiety disorder properly were psychopharmacological drugs and psychological therapy (Bandelow *et al.*, 2008; National Institute for Health and Clinical Excellence (NICE), 2011; Baldwin *et al.*, 2014).

Most patients prefer taking anxiolytic drugs as it is more effective compared to psychological therapy. However, many of these commonly

used drugs such as buspirone, benzodiazepines, and pregabalin may cause side effects such as headache, stomachache, nausea and even abnormal heart rate caused by the increased dopamine level (Davis, 2017). Moreover, some drugs may have delayed onset in their actions and this can cause extended risk due to unsuccessful treatment outcomes (Falcon *et al.,* 2015). Thus, most people prefer using herbal medicine as the alternative medicine for anxiety treatment, such as *Tilia europeae, Salvia guaranitica* and *T. tormentosa* (Lakshmi *et al.,* 2014). *Moringa oleifera* or the miracle tree is rich in minerals and nutrients that have various nutritional and medicinal values. Traditionally, the plant was said to be able to treat anxiety and epilepsy (Khawaja *et al.,* 2013). Based on previous study, the extract showed significant anxiolytic effect, however, the study was conducted using different doses of ethanolic extract (50, 100, 200 and 400 mg/kg) and different animal models (albino mice) (Bhattacharya *et al.,* 2014). Furthermore, information on the mechanism of action of *M. oleifera* extract is still lacking as previous study only analysed the behaviour of the animal. Due to limited understanding, zebrafish model was chosen to further delineate the underlying mechanism. Thus, this study aims to determine the potential anxiolytic effect of the *M. oleifera* leaf extract on chronically stressed zebrafish (*Danio rerio*). Zebrafish, were used in this study due to their simple and easy maintenance and its high similarity with human genetics (Howe, 2013). Moreover, zebrafish are commonly used in neuroscience research (Piato *et al.,* 2010) since they possess all of the conventional neurotransmitters found in vertebrates (Mueller *et al.,* 2004). The scopes of the study include acute limit test of the *M. oleifera* leaf extract and the behavioural tests of the extract on the zebrafish. Zebrafish were induced with stressors daily after being treated with different concentrations of the *M. oleifera* leaf extract and an anxiolytic drug, fluoxetine, was used as the positive control. The behaviour changes in zebrafish was observed using the novel tank test (NTT) and light-dark test (LDT). Finally, fish from different treatment groups were sacrificed and their brains were dissected for RNA extraction. The differential genes expression was analysed through a high sensitivity custom RT^2 profiler PCR array to assess the expression profile of the chronically stressed zebrafish after being treated with *M. oleifera.*

MATERIALS AND METHODS

Plant Extract

The extract was prepared in a Good Manufacturing Practice (GMP) certified company named Mitomasa Sdn. Bhd. It was prepared using extraction solvent, with a ratio of 70:30 (ethanol: water). The ethanolic extract of *M. oleifera* leaves (MOLE) was prepared in double-distilled water. From 16,000 mg/L of MOLE, the stock was diluted to three different concentrations: 500 mg/L, 1000 mg/L and 2000 mg/L using the source of water from the same reservoir used for husbandry.

Profiling of MOLE Using Liquid Chromatography-Mass Spectrometry Time of Flight (LC MS/QTOF)

The separation was performed with the mobile phase containing solvent A and B in liner gradient, where A was water with 0.1% ammonium formate and B is acetonitrile with the following gradient: from 5% to 95% of mobile phase over 36 minutes at a flow rate of 0.25 mL/min. Before the injection, the extract powder (1 mg/mL) was dissolved in the mobile phase using a ZORBAX Eclipse Plus C18 column (100 mm x 2.1 mm x 1.8 µm, Agilent Technologies, SA, USA) maintained at 40 °C. For each analysis, the total runtime was 48 minutes. The eluent was monitored by electrospray ion (ESI) scanned from 100 to 1000 mass/charge (m/z). ESI was conducted with V Cap 4000 V, skimmer 65 V, and fragmentor 125 V. High-purity nitrogen (99.999%) was used as dry gas and at a flow rate of 12 L/min and capillary temperature at 350 °C. Nitrogen was used as nebulizer at 45 psig. The range of data collected in negative ESI mode was from 100 to 1000 m/z. The MS data were processed using and analysed using Agilent MassHunter Qualitative Analysis B.05.00 software (Agilent Technologies, Santa Clara, CA, USA). The molecular features based on mass/charge (m/z) were extracted from the profiles using the data analysis software (Agilent MassHunter Qualitative Analysis B.05.00 software). The accurate masses and chemical formulas were annotated using PlantCyc database. Using PlantCyc, the compounds identified were filtered by monoisotopic masses and all the compounds were listed in Table 1.

Table 1. Predicted compound that can be found in *Moringa oleifera* leaves extract

Search Mass	Monoisotopic MW	Compound Name	Chemical Formula
432.1055	432.1056	kaempferol	$C_{21}H_{19}O_{10}$
448.1004	448.1005	quercetin	$C_{21}H_{19}O_{11}$
192.0266	192.0270	citrate	$C_6H_5O_7$
104.0112	104.0109	malonate	$C_3H_2O_4$
104.0112	104.0109	hydroxypyruvate	$C_3H_3O_4$
116.0108	116.0109	maleate	$C_4H_2O_4$
148.0372	148.0371	citramalate	$C_5H_6O_5$
292.0905	292.0906	EDTA	$C_{10}H_{14}N_2O_8$
340.0809	340.0794	daphnin	$C_{15}H_{16}O_9$
102.0318	102.0316	acetoacetate	$C_4H_5O_3$
120.0419	120.0422	threose	$C_4H_8O_4$
197.0328	197.0324	TRIBOA	$C_8H_6NO_5$
342.1165	342.1162	trehalose	$C_{12}H_{22}O_{11}$
342.1165	342.1162	sucrose	$C_{12}H_{22}O_{11}$
342.1165	342.1162	lactose	$C_{12}H_{22}O_{11}$
120.0435	120.0435	purine	$C_5H_4N_4$
135.0541	135.0544	adenine	$C_5H_5N_5$
244.0702	244.0695	uridine	$C_9H_{12}N_2O_6$
267.0961	267.0967	adenosine	$C_{10}H_{13}N_5O_4$
268.0813	268.0807	inosine	$C_{10}H_{12}N_4O_5$
283.0904	283.0916	guanosine	$C_{10}H_{13}N_5O_5$
312.1046	312.1056	primeverose	$C_{11}H_{20}O_{10}$
312.1046	312.1056	vicianose	$C_{11}H_{20}O_{10}$
610.1532	610.1533	rutin	$C_{27}H_{30}O_{16}$
200.1776	200.1776	laurate	$C_{12}H_{23}O_2$
228.2086	228.2089	myristate	$C_{14}H_{27}O_2$
294.2188	294.2194	etheroleate	$C_{18}H_{29}O_3$
296.2341	296.2351	vernolate	$C_{18}H_{31}O_3$
298.2502	298.2507	ricinoleate	$C_{18}H_{33}O_3$
302.2247	302.2245	levopimaric acid	$C_{20}H_{29}O_2$
344.2341	344.2351	dihydrosorgoleone	$C_{22}H_{32}O_3$
372.1424	372.1420	syringin	$C_{17}H_{24}O_9$
432.1075	432.1056	genistin	$C_{21}H_{20}O_{10}$
456.2257	456.2260	vindoline	$C_{25}H_{33}N_2O_6$
256.2392	256.2402	palmitate	$C_{16}H_{31}O_2$
280.2398	280.2402	linoleate	$C_{18}H_{31}O_2$
304.2397	304.2402	kauralexin A1	$C_{20}H_{31}O_2$
304.2397	304.2402	arachidonate	$C_{20}H_{31}O_2$
592.2684	592.2685	pheophorbide a	$C_{35}H_{34}N_4O_5$

Animal and Housing

The project protocol was approved by the UiTM Committee on Animal Research and Ethics (UiTM Care, Ref: 284/2019).

Adult zebrafish were maintained in a recirculating habitat system (ZebTEC Active Blue Stand Alone, Techniplast, Italy), at 28 °C with a 14 light:10 dark photoperiod at Integrative Pharmacogenomics Institute (iPROMISE). The water system was monitored for nitrites (<0.2 ppm), nitrates (<50 ppm) and ammonia (0.01 – 0.1 ppm), while the pH was maintained at 7.0 and conductivity was maintained at 700 Sm, respectively. The fish were fed twice a day at 9 am and 5 pm with adult zebrafish irradiated diet (Ziegler™, Gardner, PA, USA).

Experimental Design

A total of 72 adult zebrafish were divided into six experimental groups (12 fish in each group): (i) control without stress; (ii) stress-induced control without treatment; (iii) stressed induced zebrafish treated with fluoxetine and stressed induced zebrafish treated with three different concentrations; (iv) 500 mg/L, (v) 1000 mg/L, and (vi) 2000 mg/L of MOLE. The zebrafish behaviour was observed following the exposure

stressors and treatment for 14 days. Each fish was placed in a separate tank, exposed to the treatment for 15 minutes, and proceeded to two random stressors before the behavioural testing started. The zebrafish were subjected to a wide spectrum of stressors, which are warm stress (WS); heating tank water up to 33 °C for five minutes, tight space stress (TS); fishes were put in a small glass bottle, predator stress (PS); predator (*Archocentrus nigrofasciatus* fish) close exposure for five minutes without direct contact and out of water stress (OS); exposure of the fish whole body out of water for five minutes. The fishes were induced to the stressors twice a day for 14 days randomly. The fish were subjected to unpredictable stress exposure by altering the time and arrangement of stressors during the 14 days of the behaviour study to avoid the fish becoming familiarised with the stressors. Each stressor was used to induce stress on all the fishes for five minutes. Subsequently, the fish were transferred into the other tank for two behavioural tests; novel tank test (NTT) and light-dark test (LDT). Next, the behaviour of the fish was tracked and analysed using the tracking software (ANY-maze, Wood Dale, IL, USA). The video tracking files were stored for further analysis. The zebrafish were immediately euthanized using cold water at the end of treatment on the 14th day. The brain tissues of the fishes were processed for RNA extraction f (n = 12) and then reverse transcribed to cDNA before performing gene expression analysis using the custom RT2 profiler PCR array.

Acute Limit Test of MOLE

The acute toxicity test of MOLE on zebrafish were conducted based on the limit tests by OECD 203 guideline (OECD, 2019). This test was conducted with a static system where there will be no change in the solution for 96 hours. Two groups of zebrafish (ten per group) were used; control group and treatment group (fish exposed to 100 mg/L MOLE). This acute limit test was conducted over 96 hours. One litre of water to one gram of fish mass was used as the water to fish ratio. *M. oleifera* leaf extract was diluted using system water and the extract was poured into large plastic tanks to allow the waterborne exposure of the extract to the fishes. Moreover, important parameters such as water pH, temperature, and dissolved oxygen percentage were recorded using a multiparameter meter (Hanna Instruments, Model HI9829) and the mortality of the fishes were recorded every day. This test was to make sure that the extract is safe to be used for further experiment since the LC_{50} was higher.

Behavioural Testing

Novel tank test (NTT)

After exposure to the stressors, zebrafish were placed individually into a plastic tank (11.5 cm × 20 cm × 11 cm) which was fully filled with the water from the habitat. The novel tanks were divided into two equal horizontal portions for the upper and lower area (Stewart *et al.*, 2011). Once relocated to the novel tanks, zebrafish swimming behaviour was recorded using an action camera (Xiaoyi, YI 4K+ Plus Action Camera, Shanghai, China) over a 15 min period at 30 frames per second. The first one minute was the habituation and the remaining 14 minutes was the test time. Using our own setup (Figure 1), we were able to record four fish in separate tanks, at the same time.

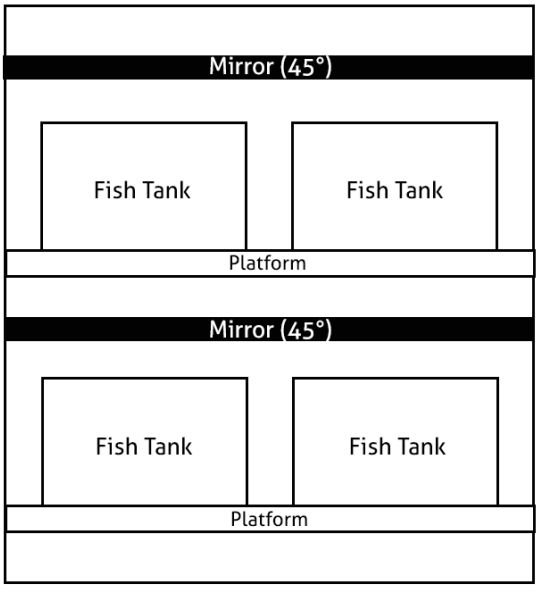

Figure 1. Recording setup used to record the fish behaviour

Light-dark test (LDT)

The tanks for the experiment were designed using a plastic tank (11.5 cm × 20 cm × 11 cm) by dividing the tank into two equal vertical portions for light and dark areas (Stewart *et al.*, 2011). The light area of the tank was exposed to background lights, and black coloured corrugated plastic boards were used to cover the other half of the tank (front, back, side and bottom) to define the dark area, respectively. The tank was filled with system water before the test started. Videos were recorded for 15 minutes using an action camera (Xiaoyi, YI 4K+ Plus Action Camera, Shanghai, China) at 30 frames per second, after the fishes were transferred to the tank. The first one minute was the habituation time for the fish and the remaining nine minutes were the test duration. Using our own setup (Figure 1), we were able to record four fishes in separate tanks, at the same time.

Behavioural analysis

The video was compressed using VLC software (version 3.0.11), to reduce the file size for video processing while maintaining video quality. Subsequently, automated tracking software, specifically ANY-maze software (version 4.99), was utilised to track the fish's behaviour. The study measured anxiety endpoints, (i) the duration of preference for the top zone(s) in the depth preference test and (ii) the duration of preference for the light zone(s) in the light/dark test.

Gene Expression Analysis

RNA isolation and cDNA synthesis

Zebrafishes were euthanised in an ice bath and the brains were dissected. All brain tissue specimens to be used for RNA isolation were preserved in a microcentrifuge tube containing RNAlater (Ambion, Austin, USA) and stored in a 20 °C chiller for a day to make sure that the samples were fully immersed with the RNAlater solution. After removing the solution, the samples were stored at -80 °C until further use. Dry ice was used to freeze before brain samples were homogenised. Whole brain samples were disrupted and homogenised with homogenisation pestle in a microcentrifuge tube with 1 mL QIAzol lysis reagent. Total RNA was extracted using RNeasy Lipid Mini Kit (Qiagen Inc., CA, USA), following the manufacturer's instructions. The protocols included treatment with RNase-free DNase set (Qiagen, Inc., CA, USA) for efficient on-column digestion of DNA. Next, RNA measurement of the absorbance at 260 and 280 nm was conducted using the Nanodrop® ND-1000 UV-Vis spectrophotometer (Peqlab, Erlangen, Germany) in order to determine RNA yield and purity. The integrity of RNA was tested by electrophoresis in 1% agarose gels. RNA purity was determined using Bioanalyzer outsource from Neoscience. Next, 0.8 µg of total RNA were reverse-transcribed using RT^2 First Strand kit (Qiagen Inc., CA, USA) according to the manufacturer instructions.

RT^2 Profiler PCR Array

The RT^2 Profiler Zebrafish Custom PCR Arrays for genes related to anxiety were obtained from Qiagen (Valencia, CA, USA) in a Rotor-Disc® 100 format, which included triplicates of 26 genes with functions previously related to stress, anxiety and depression and standard controls (housekeeping genes, reverse transcription controls and positive PCR controls). Housekeeping genes (*actb2*, *b2m* and *gapdh*) were used to normalize the data. The expression of genes encoding for corticotropin releasing hormone (*crhb*), monoamine oxidase (*mao*), fatty acid amide hydrolase 2a (*faah2a*), and phenylalanine hydroxylase (*pah*) were determined in RNA derived from brain samples of control and treated fish with quantitative RT^2 profiler PCR array using RT^2 SYBR Green Mastermixes. The PCR array was performed on QIAGEN's Rotor-Gene Q real time PCR cycler (Qiagen® GmbH, Hilden, Germany) using the following cycling condition: 10-min incubation at 95 °C, followed by 40 cycles for 15 seconds and 60 °C for 30 seconds. Fluorescence changes were monitored with SYBR Green after every cycle. Results were evaluated with the web-based data analysis software by Qiagen at https://geneglobe.qiagen.com/my/my-geneglobe. Fold change determined was the $\log_2$ value of the $[2^{-\Delta\Delta C_T}]$. This is the normalised gene expression $[2^{-\Delta C_T}]$ in the test samples divided by the normalised gene expression in the control samples. Fold change values greater than one (positive value of $\log_2$) indicates up-regulation; while fold change values less than

one negative value of log_2) indicates down-regulation.

Statistical Analysis

Group data were expressed as mean ± standard error of the mean. The data were analysed using Student's t-test at a confidence level of 95%. ANOVA was used for statistical comparisons of the behavioural observational data, followed by pairwise post hoc comparisons. Statistical analysis was performed using IBM® SPSS®.

RESULTS

Profiling of MOLE Using Liquid Chromatography-Mass Spectrometry Time of Flight (LC MS/QTOF)

Table 1 shows the compounds that were identified in the MOLE extract. Quercetin and kaempferol were among the flavonoids identified.

Behavioural Analysis

Novel tank test (NTT)

Based on Figure 2a), stress induced zebrafish which were treated with 1000 mg/L had the lowest number of freezing episodes compared to other groups; while, zebrafish treated with 2000 mg/L MOLE showed the highest number of freezing episodes among other groups. Meanwhile in Figure 2b), it showed that 2000 mg/L MOLE treatment group has a higher total time of freezing compared to other treatment groups.

Light-dark test (LDT)

Based on Figure 2c, stress induced zebrafish which were treated with 1000 mg/L had significantly ($p < 0.05$) higher number of entries to the light zones when compared to stressed zebrafish without any treatment and those received 500 mg/L and 2000 mg/L MOLE. Among the groups, zebrafish treated with 2000 mg/L showed the lowest number of entries to the light region.

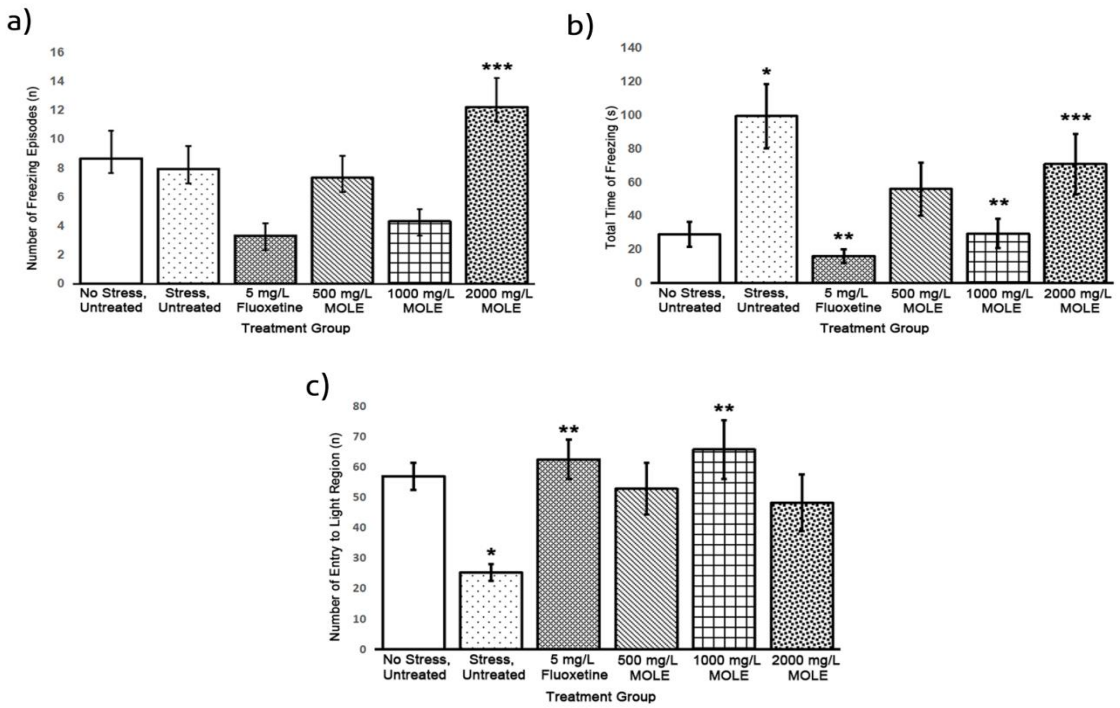

Figure 2. Behavioural effects of induced anxiety on zebrafish after 14 days in novel-tank test (NTT). a) Number of freezing episodes and b) total time of freezing c) number of entries to light region. Data are expressed as the mean ± SEM and analyzed by ANOVA followed by Tukey Test. *p<0.05 vs. no stress untreated group, **p<0.05 vs. stress untreated group, ***p<0.05 vs. 5 mg/L Fluoxetine treatment group

Gene Expression Analysis

Four genes were differentially regulated and showed significant fold changes, which were *crhb, faah2a, mao* and *pah* genes. In comparison to the control group, zebrafish that were not stressed and received no treatment, the stressed zebrafish that were treated with 1000 mg/L of MOLE exhibited notably lower expression levels of *crhb, faah2a*, and *mao* genes, except for the *pah* gene, when compared to the other groups. (Figure 3). Down-regulation in the expression of *crhb, faah2a mao* and *pah* genes have resulted in lower levels of anxiety that may be contributed by the anxiolytic compounds that are present in *M. oleifera* leaves.

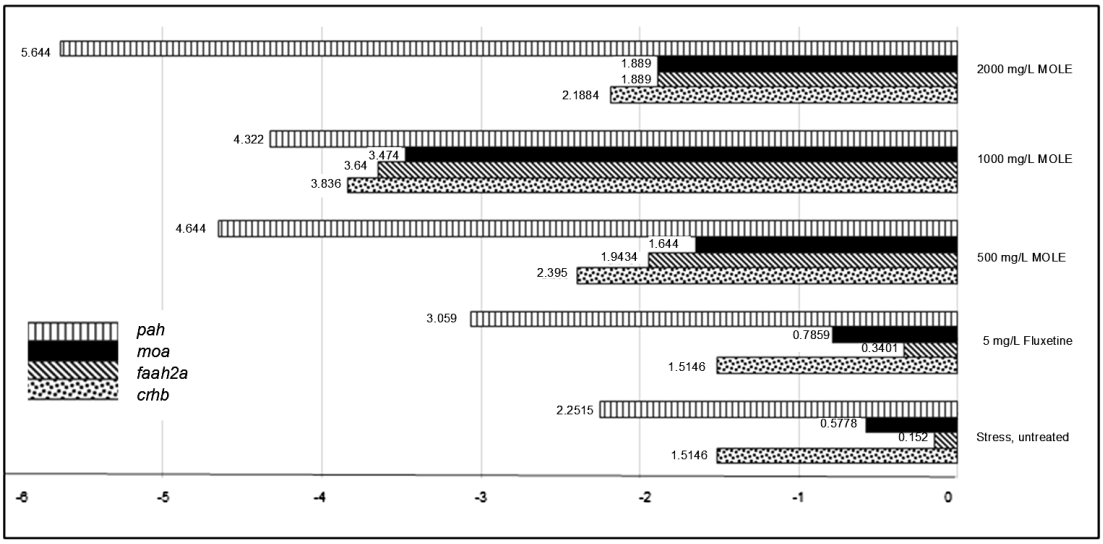

Figure 3. Differential expression of *crhb*, *faah2a*, *mao* and *pah* genes in stress induced zebrafish treated with different modalities. Fold change (log_2) of the different treatment groups in comparison to the control group, with no stress without treatment

DISCUSSION

The freezing responses of zebrafish were assessed in order to evaluate their anxiety levels. Previous studies have suggested that freezing in zebrafish is associated with anxiety rather than fear (Blaser *et al.*, 2010). Freezing was defined as complete immobilisation lasting more than two seconds, accompanied by an increased opercular beat, a higher number of freezing episodes indicates a higher level of anxiety (Kalueff *et al.*, 2013). Continuous and automatic recording was desired and ANY-Maze software was used to automatically measure and record the total duration of freezing and the number of freezing episodes.

In this study, zebrafish induced with chronic stress but not given any treatment displayed disrupted preference in the swimming zone. Zebrafish treated with 1000 mg/L of the treatment had the lowest number of freezing episodes compared to other groups. Conversely, the group treated with 2000 mg/L of MOLE showed the highest number of freezing episodes. This suggests that the higher dosage of the treatment may have increased the stress response in zebrafish.

Furthermore, the study observed the swimming behaviour of zebrafish in a novel tank in response to stressors. This active swimming behaviour seen in treated stress induced zebrafish suggests that zebrafish can effectively cope with stressors by maintaining an exploratory swimming pattern.

In our study, anxious zebrafish exhibited higher total freezing time, as observed in the untreated stress group and the 2000 mg/L MOLE treatment group. The most effective concentration which reduced zebrafish anxiety-like behaviour was 1000 mg/L of MOLE, as it demonstrated the lowest duration of total freezing time. This is in line with observation by Egan *et al.* (2009) who reported that zebrafish with lower level of anxiety had significantly higher exploration behaviour such as lower freezing behavior, more entries to the top, shorter duration of freezing.

On the other hand, zebrafish that displayed higher anxiety tended to freeze more compared

to less anxious fish (Cachat *et al.,* 2010; Müller *et al.,* 2017). When anxiety levels rise, there is a notable reduction in movement, leading to a freezing response characterized by frequent periods of immobility (freezing episodes) that continue for an extended duration (freezing) (Campanari *et al.,* 2020). The group treated with 2000 mg/L MOLE showed the highest number of freezing episodes and the longest total freezing time compared to the other MOLE concentration groups. This indicates a dose-dependent anxiolytic effect of the treatment.

The light-dark test (LDT) reveals the inner struggle faced by zebrafish when deciding whether to remain in "safe" areas like the dark compartment or to follow their inherent curiosity and venture into new environments. The dark environment offers the zebrafish a sense of protection from predators due to reduced light reflection of the dorsal distribution of melanophores. Despite this internal conflict, adult zebrafish consistently show a distinct preference for the dark region, actively avoiding the light region (Maximino *et al.,* 2010). In this study, the group of zebrafish treated with 1000 mg/L of MOLE exhibited the highest number of entries into the light region. This suggests that this group of fish had a lower level of anxiety compared to the other groups. Treatment with 1000 mg/L of MOLE and 5 mg/L of fluoxetine successfully altered zebrafish behaviour by alleviating anxiety. Based on the parameters observed during the behavioural test, zebrafish treated with 1000 mg/L of MOLE showed a more efficient reduction in anxiety-like behaviour compared to other MOLE dosages. This result is consistent with previous studies where zebrafish displaying a higher preference for the light region, characterised by increased time spent and more frequent entries into the light area, suggested a decrease in their anxiety levels (Collymore *et al.,* 2015; Benneh *et al.,* 2017; Duarte *et al.,* 2019).

The main polyphenols identified in the leaves of *M. oleifera* were quercetin, kaempferol and myrecytin (Sultana *et al.,* 2008; Coppin *et al.,* 2013). Previous studies have reported that anxiolytic compounds such as quercetin and kaempferol present in *M. oleifera* leaves may affect the expression of these genes modulating the production and functions of neurotransmitters (Bhutada, 2010). Quercetin and kaempferol have been identified as flavonoids potentially contributing to the anxiolytic effect of MOLE (Pu *et al.,* 2007). In our study, quercetin and kaempferol were detected which potentially contribute to the anxiolytic effect of MOLE. The gene expression analysis revealed the anxiolytic properties of 1000 mg/L of MOLE were related to expression of *crhb*, *faah2a*, *mao* genes and *pah*. Zebrafish treated with this concentration showed the lowest expression of *crhb*, *faah2a*, and *mao* genes, and higher expression of *pah*. Quercetin has been shown to have an inhibitory effect on genes linked to anxiety disorders and decreases *crhb* expression in the brain (Bhutada, 2010). It affects behaviour by modulating neurotransmitter systems such as Gamma-Aminobutyric Acid (GABA), nitric oxide, and serotonin (Filho *et al.,* 2008). The GABAA receptor antagonist, bicuculline, was found to suppress the behavioural effect of quercetin, indicating its influence on crhb-induced anxiety and depression through the GABAA receptor.

Quercetin also modulates nitric oxide systems by inhibiting the expression of nitric oxide synthase (NOS) and the production of nitric oxide (Kao *et al.,* 2010; Romero *et al.,* 2010). NOS inhibitors were reported to have antidepressant and anxiolytic properties (Volke *et al.,* 2003; Sevgi *et al.,* 2006; Spolidorio *et al.,* 2007; Jesse *et al.,* 2008). By reducing NOS and nitric oxide production, quercetin affects the nitrergic system, suppressing crhb-induced anxiety and depression-like effects via the serotonergic and/or nitrergic systems (Hsieh *et al.,* 2010). In addition, quercetin influences the expression of monoamine oxidase (*mao*). *M. oleifera* leaf extract has been shown to suppress monoamine oxidase type B (MAO-B) activity in the brains of stressed rats (Prabsattroo, 2015). The lower expression of mao results from the suppression activity of quercetin, which increases dopamine levels in the brain (Glover *et al.,* 1977; Sriraksa, 2019).

Another compound that was also proved to have anxiolytic properties, which is contained in *M. oleifera* leaves extract is kaempferol. Kaempferol, a natural flavone presents in a variety of plants (apples, beans, brussels sprouts, broccoli, cabbage, citrus fruits, gooseberries, grapes, grapefruit, kale, strawberries and tomatoes), has been studied for a variety of therapeutic effects (Ahmad *et al.,* 2020). Through modulation of molecular pathways

such as NF-kB, PI3k/AKT, MAPK, *Bcl2*, *Caspase 3* and VEGF, it is found to be particularly efficient against inflammation (Alam *et al.,* 2020), cancer (Imran *et al.,* 2019), and oxidative stress (Kim *et al.,* 2003 & Ren *et al.,* 2019). It is also worth noting that kaempferol has been shown to improve central nervous system illnesses like depression (Park *et al.,* 2010), anxiety (Grundmann *et al.,* 2009), and cognitive deficits (Babaei *et al.,* 2018). Moreover, they have previously been discovered to interact with fatty acid amide hydrolase (FAAH) and the endocannabinoid (eCB) system (Lenman, 2007).

The principal enzyme responsible for degrading anandamide (AEA) is fatty acid amide hydrolase (FAAH) (Haller *et al.,* 2009), which results in a decrease in endocannabinoids and an increase in anxiety-like behaviour in individuals. Anxiolytic effects have been linked to pharmacologic regulation of endocannabinoids, with FAAH inhibitors and anandamide transport inhibitors being discovered as having anxiolytic effects via boosting endogenous anandamide levels (Bitencourt *et al.,* 2008; Gunduz-Cinar *et al.,* 2012; Haller *et al.,* 2013; Burman *et al*., 2016). As a result, lower *faah2a* expression raises AEA levels in the brain, causing anxiolytic-like effects in zebrafish.

Meanwhile, higher expression of *pah* genes were also related with lower anxiety level. Phenylalanine hydroxylase was produced by the instructions provided by the PAH gene (Waters, 2003). Phenylalanine hydroxylase enzyme is responsible to catalyse the hydroxylation of phenylalanine, a building block of proteins (an amino acid) in the the diet to another amino acid, tyrosine (Waters, 2003). Tetrahydrobiopterin (BH_4) works with the enzyme to conduct this chemical reaction (Scriver, 2007). Tyrosine is well known as a producer for several types of hormones, neurotransmitters, and a melanin pigment, which determined the colour of hair and skin (https://medlineplus.gov/genetics/gene/pah/ Accessed on 2 Dec 2022). Lower expression of *pah* genes elevates the concentrations of amino acid phenylalanine in the blood. A recent study reported that higher anxiety level significantly associated with the hyperphenylalaninemia severity and the changes of blood phenylalanine concentration (Didycz & Bik-Multanowski, 2018). Therefore, we concluded that 1000 mg/L MOLE might have the potential to increase the expression of *pah* genes and decrease the blood phenylalanine concentration.

CONCLUSION

This study focuses on understanding the mechanism of MOLE which had been reported to be an anxiolytic agent. We replicated the study using stress induced anxious zebrafish and MOLE was able to reduce the anxiety level of the zebrafish at a dose-dependent effect. MOLE at 1000 mg/L showed the highest efficacy in reducing stress in the zebrafish; the number of freezing episodes, the total time of freezing and as well as the number of entries to the light region were reduced. MOLE caused lower expression of three genes which were *crhb*, *faa2ha*, *mao* and higher expression of *pah*. Changes in the expression of these genes are the underlying mechanism that contributed to the decrease of anxiety level in zebrafish.

ACKNOWLEDGEMENTS

This study was conducted at the Integrative Pharmacogenomics Institute (iPROMISE). The authors acknowledge the funding provided by Univesiti Teknologi MARA [Bestari Grant UiTM (600-IRMI/DANA 5/3/BESTARI (066/2017)] and Zebrafish Facility at iPROMISE. The authors thank Mitomasa Sdn. Bhd. for providing MOLE, which was used in this study.

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