

## The Effects of *Clitoria ternatea* Extract on Zebrafish Model of Alzheimer's Disease: A Neurobehavioural Study

(Kesan Ekstrak *Clitoria ternatea* pada Model Ikan Zebra bagi Penyakit Alzheimer: Suatu Kajian Tingkah Laku Neuron)

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### ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disease that is currently affecting 40-50 million people worldwide. It is generally recognized from its main symptom dementia, in which the patient undergoes a progressive decline in their cognitive memory. Recent studies have shown that medicinal plants such as *Clitoria ternatea* equipped with antioxidant properties has high potential in treating AD. The study was conducted using zebrafish model of AD induced with aluminium chloride for 28 days. The treatment dose of *C. ternatea* extract (4.34 mg/L) was then given for 14 days. The behaviour of the zebrafish were evaluated through memory testing by using a T-maze test and novel tank diving test. Histological studies were also performed. 50% of the zebrafish tested showed improvement in memory through the T-maze test after treatment with *C. ternatea* extract. Zebrafish model of AD treated with *C. ternatea* extract also shows a decrease in anxiety in the novel tank diving test. A significant increase of purkinje cells were also observed from the histological study after treatment with *C. ternatea* extract. Nucleus elongation of oligodendrocytes from zebrafish model of AD induced with aluminium chloride were improved when treated with the *C. ternatea* extract. In conclusion, it was found that *C. ternatea* extract exhibits strong potential for treating zebrafish model of AD induced with aluminium chloride.

Keyword: Alternative treatment; Alzheimer's disease; *Clitoria ternatea*; neurodegenerative; Zebrafish model

### ABSTRAK

Penyakit Alzheimer adalah penyakit kemerosotan neuron yang kini menjejaskan 40-50 juta orang di seluruh dunia. Ia secara amnya dikenali melalui gejala utamanya iaitu demensia, apabila pesakit mengalami kemerosotan dalam ingatan kognitif mereka. Kajian baru-baru ini telah menunjukkan bahawa tumbuhan perubatan seperti *Clitoria ternatea* yang dilengkapi dengan sifat antioksidan mempunyai potensi yang tinggi dalam merawat penyakit Alzheimer. Penyelidikan ini dijalankan dengan menggunakan model ikan Zebra yang telah diberikan penyakit Alzheimer menggunakan aluminium klorida selama 28 hari. Dos rawatan menggunakan tumbuhan *C. ternatea* sebanyak 4.34 mg/L kemudiannya diberikan selama 14 hari. Tingkah laku ikan Zebra telah dinilai melalui ujian ingatan dengan menggunakan ujian pagar sesat dan ujian penyelaman tangki. Kajian histologi juga telah dilakukan. 50% daripada ikan Zebra yang diuji menunjukkan peningkatan dalam ingatan melalui ujian pagar sesat selepas rawatan dengan ekstrak *C. ternatea*. Ikan Zebra yang dirawat dengan ekstrak *C. ternatea* juga menunjukkan penurunan tahap kegelisahan dalam

ujian penyelaman tangki. Peningkatan bilangan sel purkinje juga dapat diperhatikan daripada kajian histologi selepas dirawat dengan ekstrak *C. ternatea*. Pemanjangan nukleus 'oligodendrocytes' di dalam sel otak ikan Zebra yang dijangkiti penyakit Alzheimer telah bertambah baik apabila dirawat dengan ekstrak *C. ternatea*. Kesimpulannya, kajian ini telah mendapati bahawa ekstrak *C. ternatea* menunjukkan potensi yang tinggi untuk merawat ikan Zebra yang telah dijangkiti dengan penyakit Alzheimer.

Kata kunci: *Clitoria ternatea*; kemerosotan neuron; model ikan Zebra; Penyakit Alzheimer; rawatan alternatif

## INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease that is currently affecting 40-50 million people worldwide. It is generally recognized from its main symptom dementia, in which the patient undergoes a progressive decline in their cognitive memory affecting the patient's daily activities (Wu et al. 2017). An alarming discovery made by researchers in 2019 had found that the number of AD cases is expected to rise exponentially in the next 30 years. This is due to the increasing number of elder people with age 65 years and above in the coming years who are the main demographics for this disease (Halim Shukri & Noriszura 2019).

The main cause of the disease is still unclear however, several theories have been suggested by researchers in the field. One of the most popular theory is that AD is caused by biochemical alterations in the brain such as the formation of beta-amyloid peptide plaques and tau tangles as well as significant changes in the nerve cells itself. The cell may have been undergoing mutation and changes that leads to astrogliosis, microgliosis, neuronal cell loss, and dystrophy (De Strooper & Karran 2016).

Although numerous studies have been performed on this disease, no complete cure has been obtained up to this date. Current treatments for this disease only soothes the symptoms by using drugs such as donepezil, galantimine, and rivastigmine. These drugs helped in inhibiting cholinesterase activity in the brain. However, the medications cause patients to experience side effects including nausea, vomiting, diarrhoea, and insomnia. The side effects cause patients in opting out from the drugs due to the possibility of it disturbing their daily activities (Birks & Evans 2015; Birks & Harvey 2018).

A study had shown the potential of medicinal plants in becoming an alternative treatment for Alzheimer disease plant extracts such as the *Clitoria ternatea*. The flower part of this plant was discovered to contain high contents of flavonoids and catechins which are known to prevent inflammation, oxidative stress and neurodegeneration. The *C. ternatea* also

has antioxidant, antimicrobial, anti-inflammatory, anti-cancer, analgesic antipyretic, neuro-protective and several other properties that is beneficial towards human health (Md Bakhtiar et al. 2017). The *C. ternatea* efficacy to help boost memory is similar to the drug pyritinol due to the similar significant increase in cholinergic activity (Taranalli & Cheeramkuzhy 2000). Previous studies of using *C. ternatea* extract against rodent model of AD induced with aluminium chloride had discovered the potential of *C. ternatea* of having neuroprotective ability against oxidative stress through the recovery of antioxidant enzymes in the hippocampus of the brain (Durga Mahalakshmi et al. 2015).

The model used for this AD study is zebrafish. Zebrafish can portray significant similarities towards human Alzheimer's disease pathology and symptomology using several methods such as transgenesis, beta-amyloid injection, and induction using substances such as aluminium chloride to present short term amnesia (Caramillo et al. 2017). Zebrafish exposed to aluminium chloride can also change behaviour and increase the acetylcholinesterase activity in the brain of the zebrafish. This compound can be used to replicate symptoms of AD by causing nerve and locomotive impairment (Senger et al. 2011).

This study aims to identify the potential of using *C. ternatea* flower in treating zebrafish model of AD induced with aluminium chloride. A further understanding of the plant extract may help create alternative medications that carries a higher efficacy in inhibiting the progress of neurodegeneration of AD without any potential life-threatening side effects towards the affected patients.

## MATERIALS AND METHODS

### STUDY DESIGN

The study design used for this experiment was a cross-sectional study using zebrafish model of AD induced using aluminium chloride and treated using *C. ternatea*

flower extract. Several parameters were observed to identify the effects of using the *C. ternatea* flower extract to treat the zebrafish model of AD. The study was conducted at the Management & Science University, located at University Drive, Off Persiaran Olahraga, 40100 Shah Alam, Selangor Darul Ehsan, Malaysia.

#### PREPARATION OF *Clitoria ternatea* EXTRACT

*C. ternatea* flowers were collected from a nearby plantation and washed using distilled water. The petals of the flowers were separated from the sepal before being coarsely chopped and dried in a 40 °C oven for 24 h. The dried petals were milled repeatedly until a fine powder was obtained. Using a Soxhlet apparatus, the *C. ternatea* powder was then mixed with ethanol solvent using a 1:10 ratio of extract powder to solvent. The mixture was left for 24 h. The extract was then filtered with Whatman filter paper, No.1 (Surechem Sdn. Bhd., Malaysia) before being evaporated using a rotary evaporator at 55 °C for 30 min. The solution was then constantly mixed to allow cooling before sonication for 15 min. The extract was filtered and dried using vacuum desiccators before freeze-dried and stored at -20 °C until used (Kumar 1998).

#### ANIMAL

Adult zebrafish used as the animal model for this study and were obtained from a certified fish vendor. The subjects were 4-5 months old and only same size subjects were selected for the study. The zebrafishes were housed in a proper aerated tanks with sufficient supply of oxygen using a bubble sparger. A filtration system was also put in place to keep the water clean and safe for the zebrafishes. The temperature of the tank system was maintained at 28 °C with a water pH range of 7.0 to 8.0. The photoperiod of the tank was set at 14 h: 10 h (light: dark). The zebrafish were fed twice daily and the water tank system and quality was checked and maintained regularly. Maintenance was done once a week by replacing the filter and the water in the tank (Kim et al. 2017).

#### EMBRYO BREEDING

Zebrafish breeding were performed with a ratio of 2:1 male to female zebrafish. The embryos obtained from the zebrafish were collected into a petri dish with distilled water and methylene blue. The fertilized embryos were then selected and separated into another petri dish

before being used for experimental procedures (Kim et al. 2017).

#### SAMPLE DIGESTION

120 specimens of adult zebrafish (60 males and 60 females) were divided into three equal groups (control, negative control, and treatment) containing 40 zebrafish. The negative control group and treatment group was induced with 50 µg/L of aluminium chloride (Mutiarra Saintifik, Malaysia) by dissolving the compound into the tank water for 28 days. The tank water is replaced with new aluminium chloride water every 7 days. After 28 days of immersion in the aluminium chloride tank water, the treatment group was treated with *C. ternatea* extract for 14 days. Treatment was administered to the zebrafishes using a similar method of adding in the *C. ternatea* extract into the tank water. The negative control group was placed in unchlorinated water without any *C. ternatea* extract added in. The normal group was left untouched throughout the period of study (Muhammad Danial et al. 2019; Senger et al. 2011).

#### EMBRYO TOXICITY TEST

Healthy embryos obtained from breeding were incubated at 28.5 °C with different concentrations of the *C. ternatea* extract using a 96-well plate. The concentrations of *C. ternatea* extract used were obtained using a 2-fold serial dilution starting with the dose 50 mg/L, 25 mg/L, 12.5 mg/L, 6.25 mg/L, 3.12 mg/L, 1.56 mg/L, 0.78 mg/L, and 0.39 mg/L. The control group was exposed to only distilled water. 30 embryos were used for each group of concentration and control group throughout triple replications of the study. The embryos were observed at the 48 h post fertilisation (hpf) time point for their mortality rate and heart beat rate per 20 s. The observation was performed using a Leica MZ16F stereoscopic microscope (Sandra et al. 2019).

#### NOVEL TANK DIVING TEST (TEST)

After treatment, three adult zebrafish from each groups were individually placed in three novel tank apparatus (measurements 7 cm width × 33 cm length × 15 cm height) filled three quarters full with aquarium treated water. The tank was divided horizontally into two equal sized regions to indicate top and bottom region. The zebrafish swimming behaviour was recorded for three minutes using a side-mounted camera. The parameters observed include the number of entries and time spent (s)

in the bottom area, number of entries and time spent (s) in the top area, the distance travelled (m) and maximum speed (m/s) of the zebrafish. The zebrafish exhibits anxiety by a decrease number of entries in the top region as well as a longer latency in reaching the top region. The zebrafish swimming recordings were then analysed using the SMART-software (Nunes et al. 2017).

#### T-MAZE TEST

The apparatus consists of a large T-shaped water maze made of transparent acrylic glass sheet with dimensions of one long arm (50 cm length × 10 cm width × 10 cm height) and two short arms (20 cm length × 10 cm width × 10 cm height) and a start box (10 cm length × 10 cm width × 10 cm height) located at the foot of the stem. The short arms on the left and right were covered using green and red coloured sleeves, respectively. The maze was filled with aquarium treated water up to 6 cm in height and the water temperature to be maintained at 28 °C throughout the experiment. The zebrafish was first trained for a period of two weeks to familiarise themselves to the apparatus and identify the correct alternation to obtain their food reward. The food rewards were given in the green sleeve while a punishment of disturbances were given in the red sleeve. The training proceeds daily until all the zebrafish enters the correct alternation of the green sleeve.

The trained zebrafish were then given aluminium chloride treatment for 28 days. Every seven days, the zebrafish was placed into the T-maze apparatus to observe the effect of the compound to the zebrafish memory. After 28 days, half of the zebrafish were treated with *C. ternatea* extract while another half was left untreated for 14 days. All the zebrafish were placed in the T-maze apparatus to observe the treatment effect of the *C. ternatea* extract on every seven days. All the sessions were done without any food placement in the green sleeve. The number of entries to both sleeves were recorded (Pilehvar et al. 2020).

#### HISTOLOGY

Five adult zebrafish specimens were taken from each group to study the histological changes in the zebrafish brain. The adult zebrafish were fixed in formalin for 24 h before extracting the brain out of the zebrafish. The brains then underwent tissue processing before being mounted in paraffin blocks. The hardened blocks were then cut into five µm thick sections using a microtome.

The cut sections were then fished onto glass slides before dried for 24 h. The dried glass slides were stained with Hematoxylin and Eosin staining. The stained glass slides were then viewed for cellular morphological changes using a comparison microscope (Tayebbeh et al. 2020).

#### DATA ANALYSIS

The data obtained from embryo toxicity test and novel tank diving test were analysed with a one-way ANOVA and a Tukey's post hoc test using the SPSS software. The variance evaluated between each sample to be considered relevant results was P0.05. T-maze data obtained were analysed using a two-way ANOVA and a Bonferroni post-test with P0.05 as relevant significance (Huang et al. 2016).

#### RESULTS AND DISCUSSION

The *C. ternatea* extract was seen to have high potential in treating AD. The damages caused by the inducing of aluminium chloride in attempt to replicate AD in zebrafish can be seen to improve with the administration of the *C. ternatea* extract treatment.

The toxicity levels of the *C. ternatea* were discovered to be relatively low from the embryo toxicity study. All embryos incubated in concentration 50 mg/mL, 25 mg/mL, and 12.5 mg/mL were found dead by the 48 hours' time point. Several dead embryos were also seen in concentrations 6.25 mg/mL to 0.78 mg/mL. Only embryos incubated in 0.39 mg/mL survived throughout all three repetitions of the test. The average percentage of dead embryos from each concentration in all three replications were used to produce a concentration-mortality curve. The curve was then used to obtain the LC<sub>50</sub> for *C. ternatea* extract which was 4.34 mg/mL or 4340 µg/mL (Figure 1).

The LC<sub>50</sub> value is used to determine the toxicity activity level of a compound. The toxicity activity of a compound is weak when the LC<sub>50</sub> is placed between 500 and 1000 µg/mL, moderate if placed between 100 and 500 µg/mL and considered as strong when LC<sub>50</sub> ranged from 0 to 100 µg/mL (Padmaja et al. 2002). The LC<sub>50</sub> obtained from this study supports the low toxicity activity of the *C. ternatea* extract. A similar study on *C. ternatea* extract toxicity effects on brine shrimps supports the notion of the plant having low toxicity levels. The LC<sub>50</sub> was 0.49 mg/mL on the 48 h mark of *C. ternatea* extract exposure to the brine shrimps indicating a moderate level of toxicity towards the brine shrimps (Kamilla et al 2012).

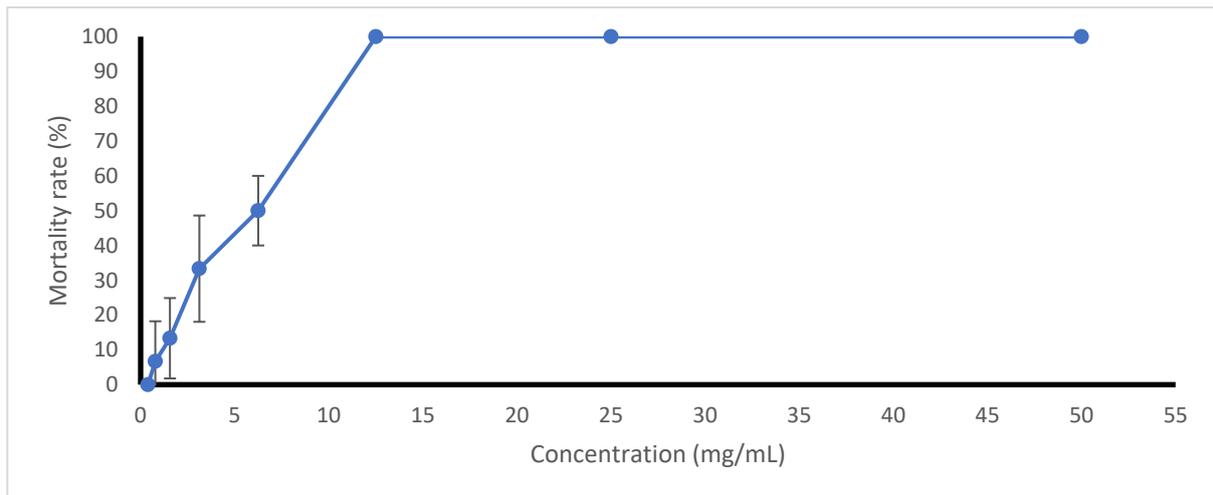


FIGURE 1. Effect of *Clitoria ternatea* on zebrafish embryo mortality rate. Data represent means  $\pm$ SD of eight different concentration groups performed in triplicate. Line represents the mortality rate of the zebrafish

The survival rate of the zebrafish embryos was seen to correlate with the concentration of the *C. ternatea* extract. This may be due to the weakening or damaging of the embryo's chorion (protective layer) from the exposure of high concentration *C. ternatea* extract. This leads to an increased accessibility of the extract into the embryo, leading to the observed mortality rate (Ali et al. 2017). The breachment of the *C. ternatea* extract into the chorion may have also cause side effects on the cardiovascular system of the zebrafish embryo.

Zebrafish embryo in concentrations 6.25 mg/mL and 3.12 mg/mL can be seen having a distinct lower heart beat rate compared to control zebrafish. Low heart beat rates can also be seen in the heart beat rate of zebrafish embryo in concentrations 1.56 mg/mL, 0.78 mg/mL, and 0.39 mg/mL, however, the data for the concentration groups mentioned does not exhibit significant differences compared to control zebrafish (Figure 2). No heart beat rate was observed in zebrafish embryos incubated in concentrations 50 mg/mL to 12.5 mg/mL due to the embryo's mortality.

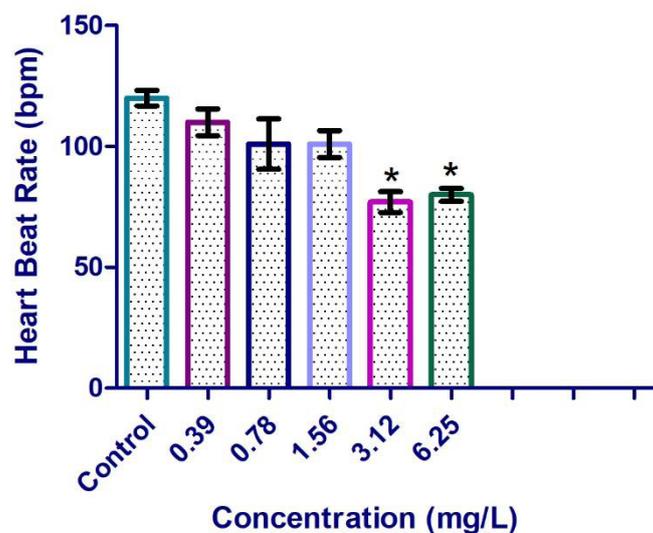


FIGURE 2. Effects of *Clitoria ternatea* extract on zebrafish embryo heartbeat rate. Data represent means  $\pm$ SD of six different groups performed in triplicate. No data was obtained from concentration groups 12.5 mg/mL and 50 mg/mL due to full mortality. Asterisk represents significant differences towards the control group. Data analysis was performed using one-way ANOVA with a Tukey's post hoc test using  $P < 0.05$  as significant

Based on the results obtained, there is a possible concentration-dependant relationship between the heartbeat rate of the embryo and the concentration of the *C. ternatea* extract. The higher the concentration of the *C. ternatea* extract, the lower the heart beat rate of the zebrafish embryo. A low heart beat rate indicates a possibility of abnormalities in the heart of the embryo that may be caused by changes in a molecular and cellular level from the extract used. No prominent abnormalities can be seen at the heart of the zebrafish embryo suggesting a possible internal effect from the *C. ternatea* extract that could not be seen using the naked eye. A similar study using *Sophora alopecuroides* extract shows that the plant has a strong toxicity level due to the  $LC_{50}$  of the extract being 3  $\mu\text{g/mL}$ . The *Sophora alopecuroides* extract also causes morphological abnormalities towards the zebrafish embryo, affecting the heart beat rate of the zebrafish embryo. The heart beat rate was seen to decrease the higher the concentration of the *Sophora alopecuroides* extract. The abnormalities in the heart caused by the extract may cause changes in the way the blood flows through the heart to all parts of the zebrafish body. This can be observed through the lower number of heart beats from the zebrafishes (Thangal et al. 2015). Comparing both the extracts, the *C. ternatea* can be seen to have a lower chance on inflicting heart related side effects such as upon consumption. Further studies need to be done to understand the mechanism of the *C. ternatea* extract on the cardiovascular system of the zebrafish embryo.

The behaviour of the zebrafish model of AD were able to be observed and quantified through the novel tank diving test and T-maze test. The effects of aluminium chloride on the zebrafish can be seen more clearly through their behaviours and how the introducing *C. ternatea* extract helped gradually improve the zebrafish behaviour. Ideally in a novel tank diving test, zebrafish will dive towards the bottom of the tank and dwell for the initial few minutes, before gradually swimming towards the upper region of the tank. This behaviour can be seen similarly to rats, where the rats were seen to spend some time being close to the container walls. This phenomenon is known as thigmotaxis (Ruchi Jakhmola et al. 2018).

In this study, the control zebrafish preferred the bottom region of the tank while the zebrafish model of AD induced with aluminium chloride preferred the upper region of the tank. The zebrafish model of AD treated with *C. ternatea* extract was seen to have similar preferability as the control zebrafish (Figure 3). The

total number of entries and latency into the bottom region of the tank decreases in zebrafish model of AD induced with aluminium chloride. Zebrafish model of AD induced with aluminium chloride has also decreased their distance travelled by 40% by the end of day 28. The maximum speed of the zebrafish declined by 50%. The exposure of aluminium chloride affects the natural responses of zebrafish of being a bottom dweller. The aluminium chloride caused altered thigmotaxis and shoaling behaviour. The thigmotaxis phenomenon were reduced and gradually causing a decline in the zebrafish preferential towards the bottom region of the tank. Exposure to aluminium chloride also caused a decrease in anxiety levels reflecting low awareness of threats. The toxic compound may have decreased the production of stress hormones in the zebrafish neuroendocrine system leading to low anxiety and threat awareness level (Egan et al. 2009).

*C. ternatea* treatment shows a significant improvement towards the zebrafish model of AD swimming pattern and behaviour. Treatment with *C. ternatea* extract was seen to improve the number of entries and latency to the bottom region similar to the control zebrafish. When comparing the distance travelled and maximum speed of the AD zebrafishes group and the 14 days of treatment zebrafish group, a 20% significant improvement can be observed (Figure 4). The *C. ternatea* treated zebrafish model of AD were seen to have a gradual increase of region preference towards the bottom region similar to the control zebrafish. The treatment may have had also improved the neuroendocrine system production of stress hormones in the zebrafish. A study done by Ruchi Jakhmola et al. (2018) using Quercetin against zebrafish model of AD induced with aluminium chloride resulted in a similar outcome in which the control zebrafishes were observed to swim in the lower region of the tank while AD zebrafishes were swimming more towards the upper region. The Quercetin treatment was found to have gradually modulate the zebrafishes behaviour with time. Another study was done on the effects of *C. ternatea* extract towards the cognitive behaviour, anxiety, stress, convulsions, and depression in rat models induced using Pentylene tetrazol and maximum electroshock. The anxiolytic activity of the extract was tested using a light/dark exploration test and an elevated plus maze test. The study had showed a reduction of anxiety in the rats treated with *C. ternatea* extract by 157% in a light/dark exploration test and a 60% increase of occupancy in the open arm of the elevated plus maze test (Jain et al. 2003).

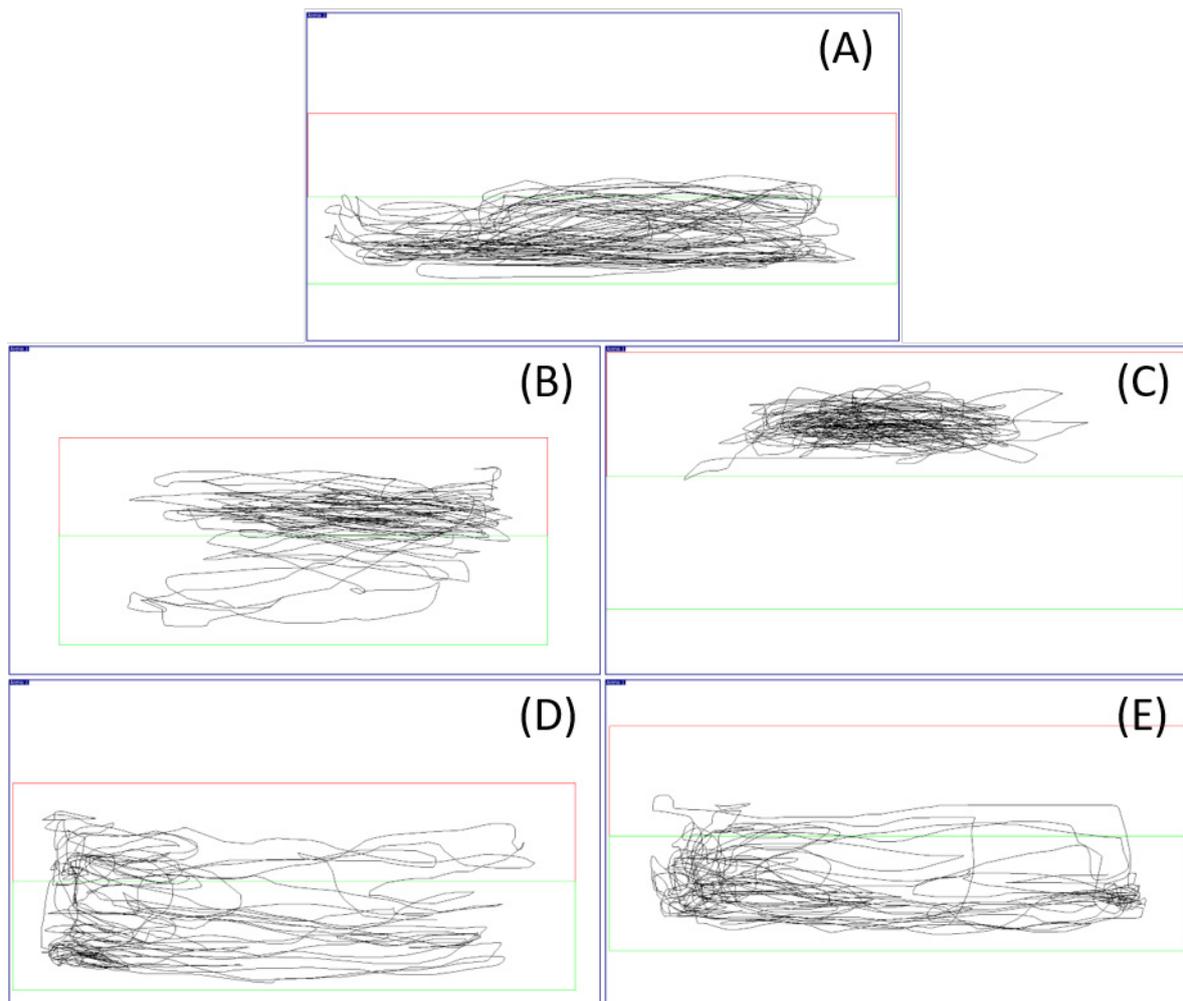


FIGURE 3. Representative trace of adult zebrafish model of AD behaviour in novel tank diving test. (A) Control day 0, (B) AD induced with aluminium chloride day 14, (C) AD induced with aluminium chloride day 28, (D) *Clitoria ternatea* extract treatment day 7, and (E) *Clitoria ternatea* extract treatment day 14

In the T-maze test, the zebrafish model ability in recognition, spatial navigation and memory retention were evaluated. The zebrafish were trained for two weeks to recognize and memorise the correct alternation to obtain food reward. The colours red and green were used on the sleeves due to the natural preferences of the zebrafish towards both of the colours and its relativity to colours from their natural habitat (Avdesh et al. 2012). Adding a food reward as a positive motivation is similar to how humans use associations to rewards in improving their visual working memory (Kim et al. 2017). The trained zebrafish were able to swim through the correct alternation even without food rewards by the end of the

training period. The combination of the food rewards, preferred colours as well as repeated training helped in obtaining this result. This proves the zebrafish ability to recognise, learn and memorise after training.

In the present study, the number of entries from zebrafish model of AD induced with aluminium chloride into red sleeves increases as the days progressed. By day 28, all of the trained zebrafish model of AD were seen entering and remained in the red sleeves (Figure 5). Aluminium chloride were seen to cause a decline in cognitive and memory retention of the zebrafish from the T-maze test results. Previous studies on aluminium chloride suggested its potential in triggering the natural

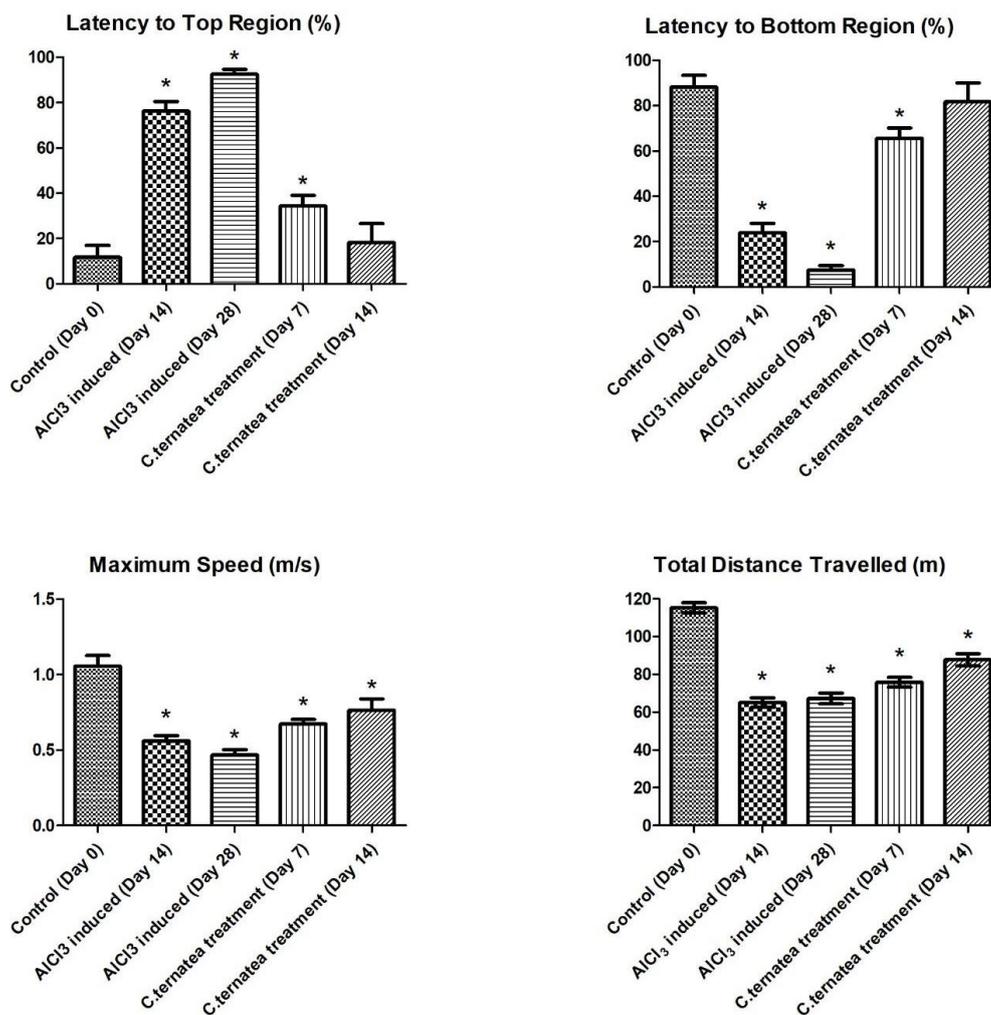


FIGURE 4. Behavioural effects of aluminium chloride and *Clitoria ternatea* extract on adult zebrafish model seen through novel tank diving test. Data represent means  $\pm$ SD of five different groups performed in triplicate. Asterisk represents significant differences towards the control group. Data analysis was performed using one-way ANOVA with a Tukey's post hoc test using  $P < 0.05$  as significant

ageing process of the body. This process proceeded to cause a dysfunction in the cholinergic activity and brain cognition leading to oxidative stress (Samaila et al. 2019). Cholinergic activity dysfunctions are related to the decline in the brain's locomotive activity, memory retainment and the performance of spatial memory tasks (Stungaru et al. 2019).

The zebrafish model of AD was then treated with *C. ternatea* extract for 14 days and assessed again using the T-maze every seven days. The number of entries of zebrafish into the green sleeves were seen to progressively increase and reached more than 50% improvement by

day 14. A comparison on the zebrafish model of AD treated with *C. ternatea* and the negative control zebrafish sees a significant improvement of up to 30% (Figure 5). The *C. ternatea* extract causes a better performance in the T-maze test. More than 50% of the zebrafish model of AD were seen to use the correct alternation by day 14 of treatment. Comparing the results to negative control results, a 30% significant improvement can be observed from the *C. ternatea* treatment. The results suggest the ability of *C. ternatea* in improving the damages caused by the aluminium chloride. The *C. ternatea* may have the ability to increase the acetylcholine production in

Neurodegeneration occurs in an interconnected neural network due to the auto-spreading of neurotoxic

agents along neural pathways connecting distributed nodes to functional modules. In the present study, the aluminium chloride exposure were seen to cause changes

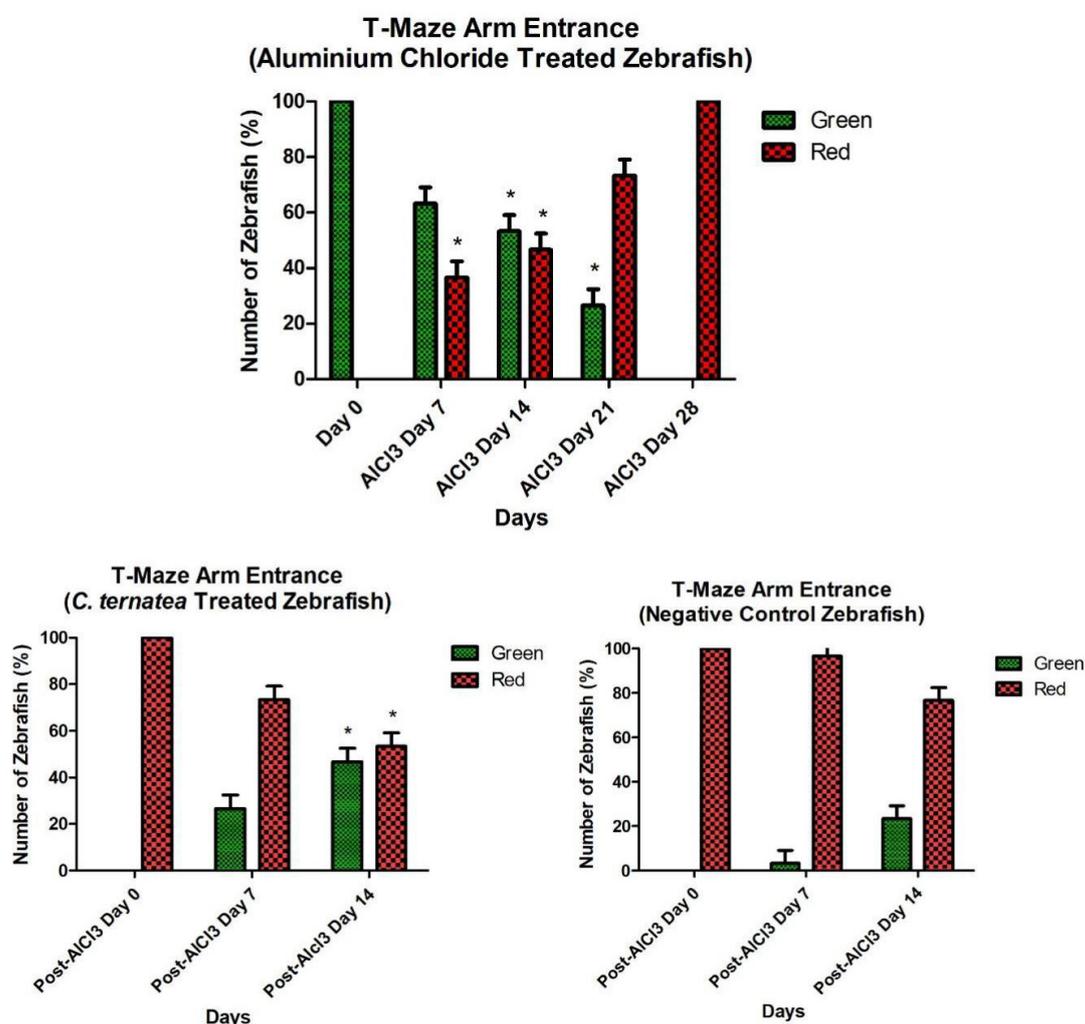


FIGURE 5. Number of zebrafish (%) entering green and red sleeves of T-Maze. (A) Zebrafish model of AD treated with *Clitoria ternatea*, and (B) Negative control. Data represent means  $\pm$ SD of one sample with two outcomes performed in triplicate. Asterisk represents significant differences between the column factors. Data analysis was performed using two-way ANOVA with a Bonferroni post hoc test using  $P < 0.05$  as significant

the brain causing improvements in acetylcholinesterase activity. The increase of the neurotransmitter is theorized to indicate an improvement of their learning and memory retention ability (Rai et al. 2002).

After 28 days of exposure to aluminium chloride and 14 days of *C. ternatea* extract treatment, the brains of the zebrafish were collected and processed to observe cellular morphological changes in the brain cells. Using Hematoxylin and Eosin staining, the Purkinje cells and

Oligodendrocytes in the Lateral Pallium region were seen to have abnormalities in zebrafish model of AD induced with aluminium chloride. The observation shows a lower density in Purkinje cells lining the grey matter and an elongated nuclei of the Oligodendrocytes when compared to the control zebrafish. The zebrafish model of AD treated with *C. ternatea* exhibited neurogenesis of the Purkinje cells and improves the nuclei shape of the Oligodendrocytes similar to control zebrafish (Figure 6).

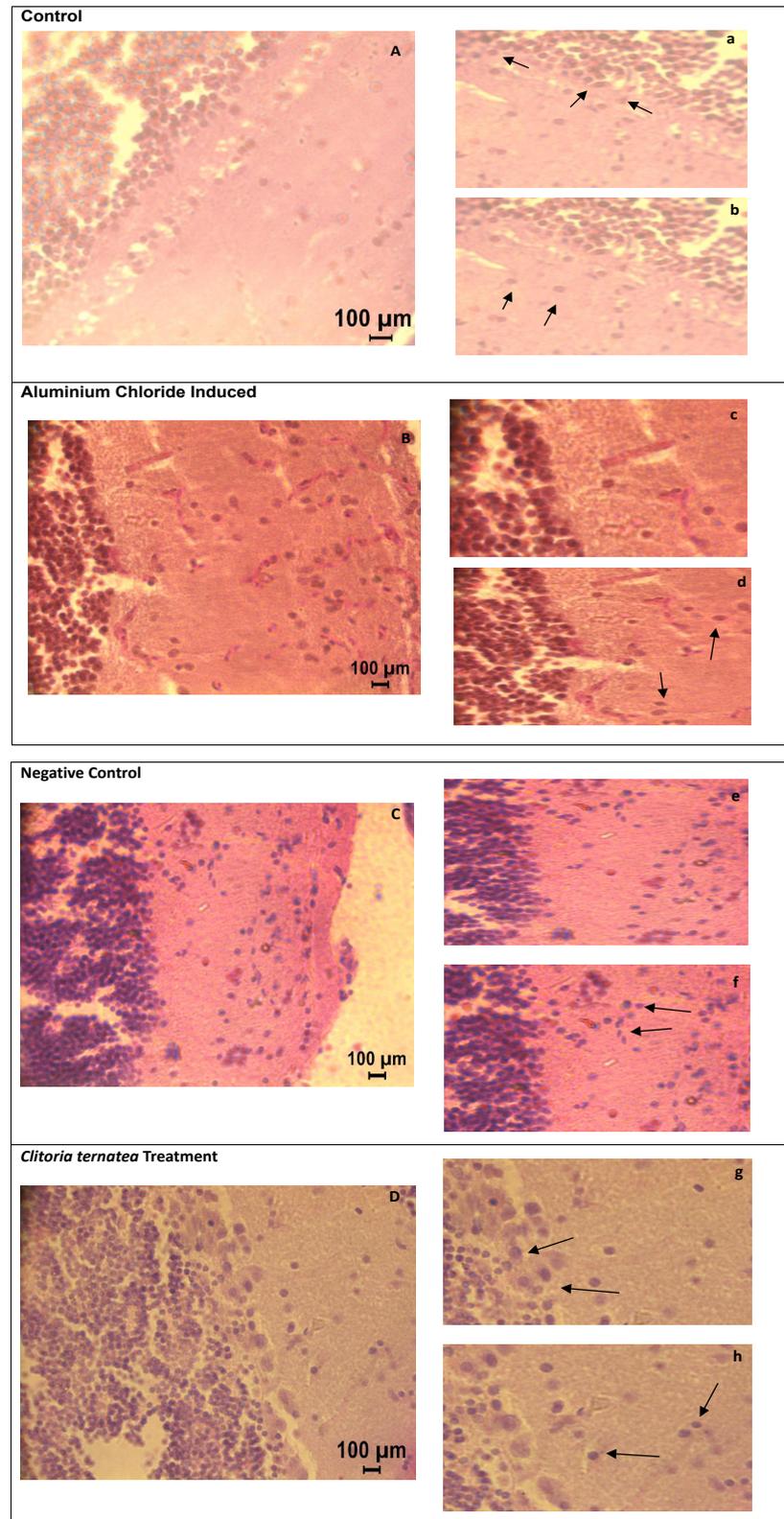


FIGURE 6. Histological analysis of adult zebrafish brain with H&E staining. (A) Control. (B) Zebrafish model of AD induced with aluminium chloride day 28. (C) Negative control day 14. (D) Zebrafish model of AD treated with *Clitoria ternatea* extract day 14. Purkinje cells (↑) can be seen in control (a) and treatment with *Clitoria ternatea* extract day 14 (h). No Purkinje cells were found in zebrafish model of AD induced with aluminium chloride day 28 (c) and negative control (e). Oligodendrocytes in control (b) were seen to have normal round nuclei but zebrafish model of AD induced with aluminium chloride shows elongated nuclei (d). Negative control (f) Oligodendrocytes shows no improvement in nucleus elongation. Zebrafish model of AD treated with *C.ternatea* shows improvement in Oligodendrocyte nuclei shape

in the cellular morphology in the Lateral Pallium region of the zebrafish brain. The Purkinje cells aligning the grey matter were seen to decrease in numbers while the nuclei of the Oligodendrocytes were elongated compared to control zebrafish. Purkinje cells play a key role in the process of memory and learning, as well as in the control of motor response, especially the response of optics. Recent studies show correlations in the loss of Purkinje cells and changes in Purkinje cell dendrites, synaptic ageing in particular, linked to AD pathogenesis (Fan et al. 2018). Oligodendrocytes is a major player in the development of myelin sheaths. Myelin sheaths are essential for helping neuron signalling while providing trophic and metabolic support for neuron axons against disruptions and neurodegeneration. Loss of myelin sheaths can cause disruption in the delivery of synaptic signalling (Butt et al. 2019).

*C. ternatea* treatment were seen to have mediated the Oligodendrocyte nuclei changes and increased the number of Purkinje cells. The nuclei were seen to gradually become similar towards the Oligodendrocyte nuclei in control zebrafish. The results reflect the capability of the *C. ternatea* in treating neurological damages to the brain. The *C. ternatea* were found to contain neurostimulants that are able to push the regeneration of dendrites and triggers the release of the release of corticotrophin-releasing hormone in the hippocampus to improve the efficacy of the synaptic process (Hemamalini & Rao 2018). Further studies are needed to identify the mechanisms of the *C. ternatea* extract efficacy in neurogenesis and neurostimulation.

#### CONCLUSION

The study successfully showed the potential of *C. ternatea* in becoming an alternative treatment in zebrafish model of AD induced with aluminium chloride. Exposure to aluminium chloride was used to replicate similar symptoms of AD in humans on the zebrafish model used. The aluminium chloride was seen to damage the zebrafish spatial memory, learning ability and memory retention. The behavior response of zebrafish from anxiety was also affected by the administration of aluminium chloride. Through histological studies, the Purkinje cells and Oligodendrocytes in the zebrafish brain was severely damaged by the aluminium chloride inducing. The treatment using *C. ternatea* extract for the zebrafish model of AD was seen to help in neurogenesis and improving the zebrafish swimming behavior from the results of the T-maze test. This reflects an improvement in the zebrafish's ability in learning and memory retention.

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