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Original Article

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Vitexin, Isovitexin and Other Biochemical Constituent of *Ficus Deltoidea* Leaf Extract in 80% Methanol Inhibits Cholinesterase Enzymes on Javanese Medaka (*Oryzias Javanicus*) Model

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Abstract

***Background & Aims*:** More than 80% of people in the developing countries rely on phytomedicine for primary healthcare in both human and livestock. Traditionally, herbal medicinal practice and treatment of cognitive disorders or neurodegenerative diseases such as Alzheimer's disease (AD) and other memory-related disorders have been achieved with numerous plant products. The aim of this study is to evaluate the anticholinesterase properties of *Ficus deltoidea* leaf extract in 80% methanol on Javanese medaka.

***Materials & Methods*:** *Ficus deltoidea* leaf was extracted with 80% methanol. Crude extract was then evaluated for toxicity effect on adult Javanese medaka. The neuroprotective effect of the crude extract was also evaluated using anticholinesterase assay. Identification of phytochemical constituents were carried out using High Performance Liquid Chromatography (HPLC) and Liquid chromatography/mass spectrometry (LCMS) techniques.

***Results*:** Results disclosed low toxicity effect of the crude extract with LC_{50} of 59.34 ± 0.1 (Sub-acute toxicity test) and 44.67 ± 0.7 (Chronic toxicity test). High anti-cholinesterase activities with significant differences at $p<0.001$ was recorded in this study. Vitexin and isovitexin were identified in the crude extract using HPLC and LCMS.

***Conclusion*:** This study shows that *Ficus deltoidea* has high neuroprotective potential due to the high vitexin, isovitexin and several other bioactive components that are yet to be identified. Hence, it could be developed and used as new neuroprotective supplement/herbal product.

***Keywords*:** Antioxidant, anti-cholinesterase, Ficus deltoidea, Javanese medaka, Neuroprotective, Toxicity

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Introduction

Undeveloped nations are predisposed to heavy metal toxicants that adversely affect the life and wellbeing of humans and animals, leading to the development of several disease conditions. Many health problems associated with heavy metal intoxication has resulted in the deaths of more than 220,000 individuals annually (1).Toxicity in warm-blooded animals mostly results from overuse of synthetic chemicals for agriculture and veterinary medicine purposes. The cumulative effects of some of these heavy metals such as insecticides and pesticide include inhibition of the activities of some of the biochemical agents that neutralize acetylcholine, resulting in the increased and persistent effects at neuromuscular junctions (2). Acetylcholine (ACh) is responsible for action potential at vicinities that uses ACh as their neurotransmitter. In mammals, it mediates signal passage between motoneurons of muscular-skeletal junction, as well as between fibers that undertake connections in the central nervous system (CNS) and ganglionic neurons (3). Alzheimer's disease mostly results from pathophysiological changes leading to cholinergic dysfunction (4). The application of anticholinesterase agents (e.g. donepezil, rivastigmine, and galantamine) in patients with Alzheimer's disease or dementia yield several positive results, including reversal of altered cognition, abnormal behaviors and disappearance of psychological symptoms (5).

A local wild evergreen tree called *Ficus deltoidea* or Jack (Moraceae) is popular in Malaysia and other parts of Asia. It is also called Emas Cotek and or Mas Cotek in Malay (6). Different parts of this tree have been used for the treatment of several diseases. *Ficus deltoidea* leaf extract has been used widely in pharmaceutical society to cure diabetes, hyperlipidemia, and hypertension (7). Evaluation of this extract revealed that it has high potential to boost libido as well as enhances aphrodisiac effects in both male and female mammals (8). It's use as a medication after parturition to enhance uterine involution, especially in case of prolonged labor or dystocia has also been reported (9). Several extracts from different parts of this plant show high potential and are used as hepatoprotective agent (10), anti-hypertensive agent (11), anti-inflammation agent (12), anti-ulcer agent (13), wound healing agent (14), agent for inhibition of carbohydrate hydrolyze by enzyme (15), and anti-nociceptive agent (16).

However, despite the health benefits of *Ficus deltoidea* plant as a supplement and/or medicinal agent, there is no sufficient information on its anticholinesterase effects on both the central and peripheral nervous system. In order to investigate the toxicity and anticholinesterase activity of *Ficus deltoidea* plant, *Javanese medaka* fish was procured and used as an experimental model. The scientific name of the fish species is *Oryzias javanicus,* and they are commonly found in the tropical regions, including Malaysia (17). They are small in size, have a translucent body which enables visualization of it interior organs and can grow to around 3-4 cm long (18). The species of *Javanese medaka* also contain some sub-species such as *Oryzias* latipes which was identified as one of the experimental models for several studies. They have been mostly used in molecular studies, genomic sequence, and informatics and transgenic studies (19). Hence, the present study may shed light on the toxicity and anticholinesterase effect of the methanolic extract of *Ficus deltoidea*.

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Materials & Methods

Ethical Approval:

This study was conducted in compliance with the internationally accepted principles for laboratory animal use and care as well as ethical clearance by Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia (Ref. No. UPM/IACUC/AUP-R005/2016).

Plants Collection and Identification:

The leaf of *Ficus deltoidea* was collected from Taman Pertanian Universiti (TPU) Universiti Putra Malaysia (UPM), Selangor, Malaysia. Authentication of the leaf was conducted by a botanist at the Institute of Bioscience (IBS), UPM, and voucher numbers were given (Table 1).

Table 1: Plant samples purchase and it voucher numbers

No.	Scientific name	Local/ Common name	Part	Voucher No.	Sources
1	*Ficus deltoidea*	Mas cotek	Leaf	SK2892/15	UPM

Plant Extraction:

The leaf of *Ficus deltoidea* was washed and chopped into small pieces with the aid of an anvil prunner, (UK). The leaf was then air-dried for two weeks at room temperature (26±1 ^{o}C) in the Biotechnology 2 laboratory, UPA, and later grounded to reduce its size (40–60 mesh). 200 g of the leaf sample was soaked in 1000 mL of 80% methanol for 3 days in a flat bottom flask (Sigma Aldrich, USA). The mixture was routinely agitated for three other days to obtain high crude extract; this procedure was repeated three times. The obtained extract was filtered with Whatman filter paper (1.5 Sigma Aldrich, USA) and solidified with a rotary evaporator (IKA® RV 10, USA) at 42 °C. The crude extract obtained was then weighed and kept at -4 °C in sample bottles until required.

The percentage yield was calculated as the weight of the filtrate divided by the total weight of the ground powder.

Yield (%) = [wt of extract (g)/wt of plant material (g)] x 100.

Crude Extract Dilution:

The crude extract stock solution was prepared by dissolving 100 mg of *Ficus deltoidea* (leaf) in 1 mL of 100% DMSO. DMSO was used to solubilize the crude extract, and the sub-stock was constituted in a microliter (µg/mL) by diluting the stock solution with distilled water to the concentration of interest (7.81-1000 µg/mL) using two-fold serial dilution.

Evaluation of the Sub-Acute Effect of Arsenic:

A 48-hour sub-acute toxicity test of *arsenic* was carried out on adult J. medaka (*Oryzias javanicus*) for 6 months. Five fishes were fed prior to the commencement of the experiment. The fishes were treated with a 0.5–4 mM dose of arsenic in a 5-litre aquarium filled to 1/2 level with de-chlorinized water. Four different concentrations (0.1, 1, 2 and 4 mM) were prepared using 2 fold dilution method and used alongside with the control groups in this experiment. The choice of the concentration was based on high toxicity effects of arsenic in other aquatic animals. Mortality was monitored throughout the experimental period; signs of deaths include the inability to move the operculum covering the gills and lack of response to touch. Dead fishes were removed from the tank immediately after it was noticed and water was replaced after every 24 hours. Behaviors of the experimental fish were monitored regularly by taking note of any abnormal movement or response during the study.

Evaluation of the Sub-Chronic Effect of Arsenic:

A 7 day **s**ub-chronic toxicity test of arsenic was carried out on the six-month-old adult J. medaka fishes. Different concentrations (0.05, 0.15, 0.75, 1.75, 2.75, 3.75 mM) of arsenic in 5-liter capacity rectangular glass aquarium half-filled with de-chlorinized water. Three replicates were also used in this experiment along with control groups that are maintained in tap water. Observation of death was carefully carried out, following signs like the inability to respond to touch or lack of operculum movement. Dead fishes were immediately removed from the tank and water was replaced after every 24 hours. Feed was given to the fishes before not during the experiment. Behaviors of the experimental fish were monitored regularly by taking note of any abnormal movement or respond during the study.

Evaluation of Sub-Acute Toxicity Effect of Crude Extract:

A 2 days sub-acute toxicity test of crude extract on adult *O. javanicus* (months) was carried out in de-chlorinized water. Fishes were treated with a 62.5–1000 mg/L concentration of the crude extract in an aquarium

filled to the ½ level containing five fishes in each aquarium. In this experiment, each concentration had replicated along with control groups. Continuous monitoring for dead fishes, removal and replacement of the water was also carried out.

Evaluation of the Sub-Chronic Effect of Crude Extract:

A 2 weeks sub-chronic toxicity test of crude extract on adult *Oryzias javanicus* fishes was carried out in de-chlorinized water. Fishes were treated with various concentrations of the crude extract (35, 45, 55, 65, 75, 85, and 95 mg/L) including the replicates and control groups. Dead fishes were removed and the water were replaced, similarly.

Experimental Design:

Assay of the various concentrations of anticholinesterase enzyme due to the crude extract was evaluated in adult Javanese medaka fishes. Fishes were separated into four groups with 25 fishes per group. They were initially acclimatized for two weeks at the Biotechnology 2 Laboratory, UPM. An aerated 14 liter tank was used for this experiment, and water was changed every 24 hrs. Fishes were initially exposed to the safe concentration of the crude extract (45 mg/L) for 24 hours, and arsenic 0.15 mM was added later to the experimental tank. These concentrations were chosen from the results of toxicity studies. Untreated fishes were kept under similar conditions on tap water. The experiment lasted for 10 days.

Sample Preparation:

At the end of the experiment, all fishes were cryo-anesthetized by exposing them to ice for 20 seconds. The fish head was separated from the body, and the brain was gently dissected out and removed without any damage. It was then washed with 50 mM Tris–HCl buffer weighed and homogenized with the aid of tissue homogenizer (Polytron PT-6100, USA) in scope bottle. The buffer was constituted with 1% Triton X and 0.1% Phenylmethylsulfonyl fluoride (Sigma Aldrich) at pH 7.4; this buffer was used as the homogenizing solvent. The homogenized sample was then centrifuged at 12,000 g for 20 minutes with high-Speed Refrigerated Centrifuge (GRACE India). The supernatant was transferred into a separate tube and used as source of enzymes.

Total Protein Estimation:

The total protein content of the homogenized Javanese medaka brain was measured after exposure to the crude extracts and the arsenic using the Bradford technique. Albumin from bovine serum (BSA) was used as a reference. One milliliter stock solution consisting 1000 µg BSA and 200 uL Tris–HCl (10 mg/200 mL) in distilled water was prepared and stored in the refrigerator until needed. The sample was later thawed and diluted with Tris–HCl at different concentrations from 0 to 1000 µg/mL, and 200 uL of sterile phosphate buffered saline (PBS) was then added to each well. After running the assay, the standard curve was plotted. The same procedure was repeated with 200 uL supernatant from the homogenized fish brain. The plotted standard curve was used to determine the concentration of samples according to the optical density OD values.

Evaluation of Enzyme Inhibitory Effect of Crude Extract:

Determination of enzyme activity was evaluated using Ellman's method. Acetylcholinesterase was harvested from the experimental fish brains. Ellman's reagent (DTNB), Acetylcholine, Butyrylcholine and propionyl choline were prepared individually in Tris–HCl buffer (50 mM) at pH7.4. Tris–HCl 210 μL, 0.1 mM Ellman's reagent 20 µL, and acetylcholinesterase (AChE) 10 µL were mixed in 96-well plates and incubated for 15 min at 28°C. This was followed by the addition of 10 μL of 2.5 mM Acetylcholine, Butyrylcholine and propionyl choline separately to the preparation and kept in an incubator at 28°C for ten minutes. Hydrolysis of the substrate acetyl-thiocholine by the enzyme ChE results in the formation of thio-choline. Thiocholine combined with DTNB to form 2-nitrobenzoate-5-mercaptothiocholine. The measurement was fulfilled with microplate reader (Tecan multimode microplate U.K.) at fluorescent 485 nm and 535 nm (excitation and emission).

Phytochemical Examination of the Secondary Metabolites:

Plant bioactive compounds (vitexin and isovitexin) were prepared as standard and used in this experiment. Two standard series were prepared at the concentration of 70 to 4.4 µg/mL and 97 to 6.1 µg/ mL. To identify the compounds, the crude extract was constituted at one milligram per milliliter concentration. Phytochemical constituents were then identified using HPLC and Liquid LC/MS.

Phytochemical Studies using HPLC:

High-Performance Liquid Chromatographic system (Waters, USA) comprising of a six hundred pump with an automated injector was set up. The system also has 2998 photodiode array detector from 200 to 500nm. This was used to determine the bioactive compounds (vitexin and isovitexin) present in the crude extract. The separation was carried out using 4.6 x 250 mm ODS 3, 3 mm column (UK) with temperature regulated at 40 ^{o}C. A gradient technique with methanol and deionized water was utilized for the division. At 0 min, the mobile phase was set at 10% methanol in deionized water and expanded to 90% methanol in deionized water for a duration of 45 minutes. The 90% methanol in deionized water was kept for further 15 minutes. The peaks were integrated at the wavelength of 337 nm.

Phytochemical Studies using LC/MS:

To confirm the presence of the bioactive compounds and identified many other compounds of biological importance, liquid chromatography/mass spectrometry (LC/MS) was used with the aid of quadrupole ion traps MS (Bruker Esquire LC/MS, Billerica, MA, USA). The column used was symmetrical (Waters) C18 (250 × 4.6 mm). A 25 µL sample volume was automatically injected using the system's auto-injector. Solvent A is mixed with 5% formic acid in the water, and solvent B is mixed with HPLC-grade methanol (Malhado et al., 2016). The UV response during LC/MS was monitored at 360 nm and the highest absorbance wavelength for each set of components was determined. The LC/MS was operated in the positive-ion mode using the electrospray ionization (ESI) source, regarding the manufacturer's recommended operating conditions.

Statistical Analysis:

Each study in this research was carried out three times separately. Results were gathered, processed and interpreted as mean ± standard error of the mean (mean ± SEM). Survival rate and anticholinesterase effect of the crude extract on Oryzias javanicus were analyzed using statistical software GraphPad Prism version 5.0. Statistical analysis using One-way analysis of variance and Dunnett's post hoc test was carried out to calculate the p-value.

Results

Crude Extract Yield:

The percentage yield of *Ficus deltoidea* leaf following extraction, evaporation and concentration with a rotary evaporator at 42 ^{o}C was 12.79%.

Sub-Acute Effect of Arsenic:

The result of the two days' sub-acute toxicity test of arsenic on Javanese medaka fishes shows high mortality at concentrations above 0.15 mM. Only 20% of the fishes survive the second day at 0.15 mM, and all the fishes died after day two of the experiment. Only 20% of the fishes survived the second days at 0.5 mM while all died after day two of the experiment. All fishes at 1.2 mM concentration died by day 1. (Figure 1).

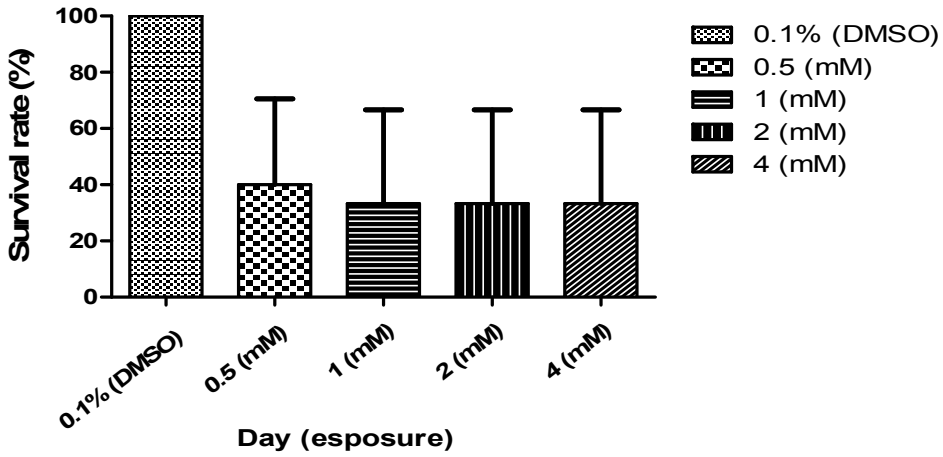

Fig 1: Sub-acute toxicity effect of arsenic on the survival rate of adult Javanese medaka (*Oryzias javanicus*) treated with different concentrations (0.05-4 mM). The percentage of survival rate (n=3) is shown versus concentration of the tested sample. The values represented as mean ± SE from three independent experiments.

Sub-Chronic Effect of Arsenic:

Results of the sub-chronic effect of arsenic on fishes show that almost all fishes exposed to 1.75 mM, 0.75 mM dead at day seven. There was less than 50% mortality in groups that were exposed to 0.15 mM. There was a significant difference ($p< 0.01$) between the groups that were maintained in tap water and the groups that were exposed to 0.15 mM of arsenic. Significant difference ($p< 0.001$) was also observed between the control groups and the groups that were exposed to 0.75 and 1.75 mM of arsenic (Figure 2).

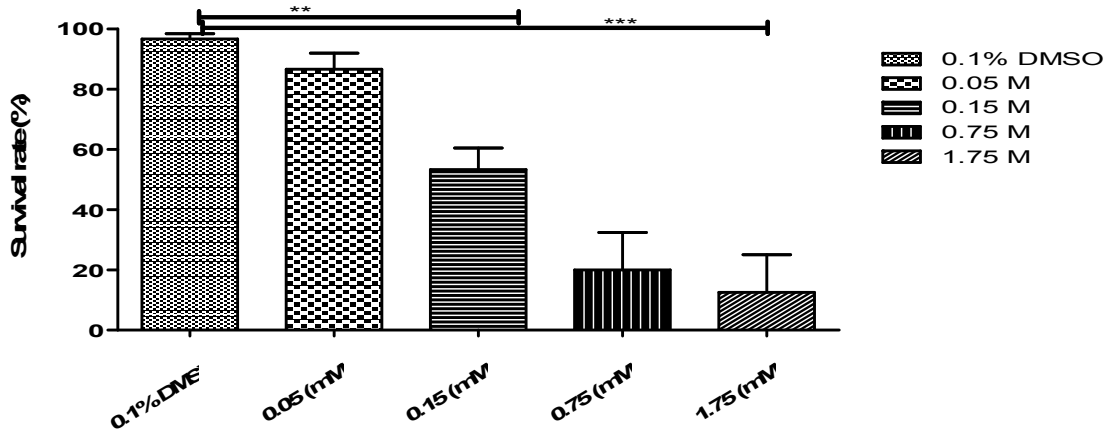

Fig 2: Sub-chronic toxicity effect of arsenic at different concentrations (0.05-1.75 M) tested on the survival rate of adult Javanese medaka (*Oryzias javanicus*). The percentage of survival rate (n=3) is shown versus concentration of the tested sample. The values represent mean ± SD from two independent experiments with LC_{50} of 0.14 ± 0.8 M.

Sub-Acute Toxicity Effect of Crude Extract:

Result of the sub-acute effect of the crude extract on the fishes revealed high mortality at concentrations above 125 mg/L. At the concentration of 250 mg/L, only 35.5% of the fishes survived on day 1 of the experiment and later all died on day 2. All the fishes exposed to 500 and 1000 mg/L died by day 1 of the experiment (Figure 3).

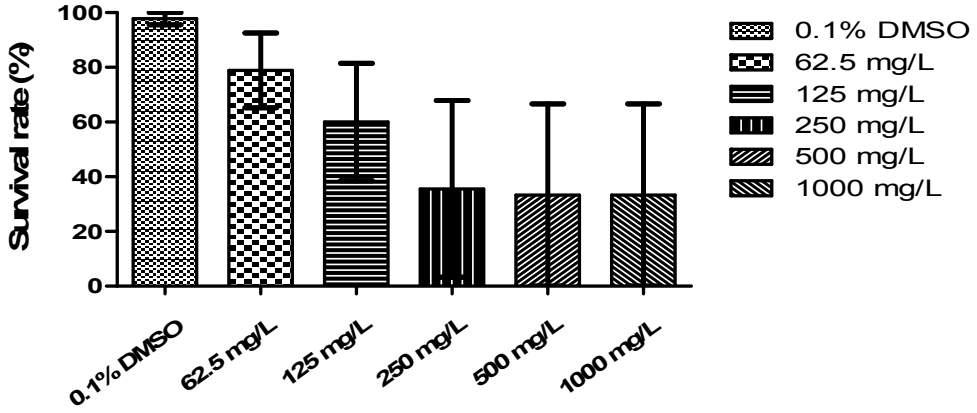

Fig 3: Sub-acute toxicity effect of *Ficus deltoidea* leaf extract on the survival rate of Adult J. medaka treated with different concentrations (62.5 – 1000 mg/L) of plant crude extract. Percentage of survival (mean ± SD), (n=3) is shown versus concentration of the tested sample

Sub-Chronic Effect of Crude Extract:

Result of sub-chronic toxicity effect of crude extract on the fishes revealed high mortality at 55 mg/L with a survival rate of 38.7%. All the fishes at a concentration of 65 mg/L died on day 8 while at 75 mg/L all the fishes survived until day 5 of the experiment before they died. Fishes exposed to 85 and 95 mg/L died on day two of the experiment. There was a significant difference ($p<0.01$) between the group that were exposed to 45 mg/L and the control group. Significant difference ($p<0.001$) was also observed between the fishes that were maintained in tap water and those that were exposed to 55, 65, 75, 85 and 95 mg/L. Hence 35 mg/L was taken as the safe concentration to be used in the experimental neuroprotective assay (Figure 4).

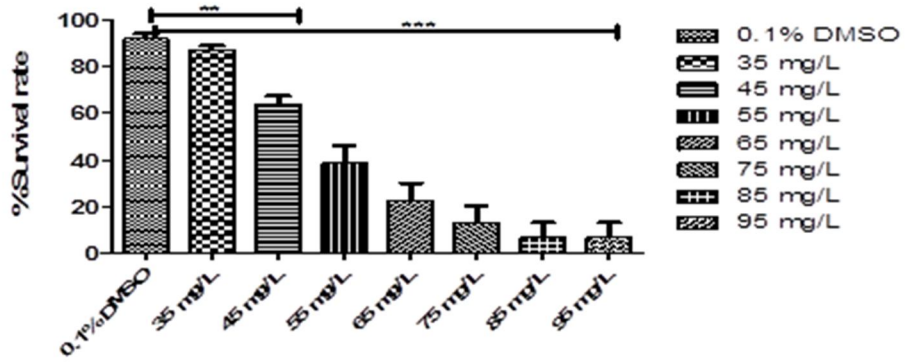

Fig 4: Chronic toxicity effect of Ficus deltoidea leaf extract on the survival rate of adult Javanese medaka treated with different concentrations (35–95 mg/L) of plant crude extract. Percentage of survival (mean ± SD), (n=3) is shown versus concentration of the tested sample.

Total Protein Content:

Result of total protein assay showed high protein content in groups that were exposed to the crude extract only. Increased protein content was also observed in groups that were maintained in tap water as well as the groups that were treated with crude extract and exposed to arsenic compared to the groups that were exposed to arsenic only. There was a significant difference ($p<0.001$) between the fishes that were exposed to

arsenic and the remaining groups (groups maintained in tap water, groups treated with crude extract only, and groups treated with crude extract followed exposure to arsenic) (Figure 5).

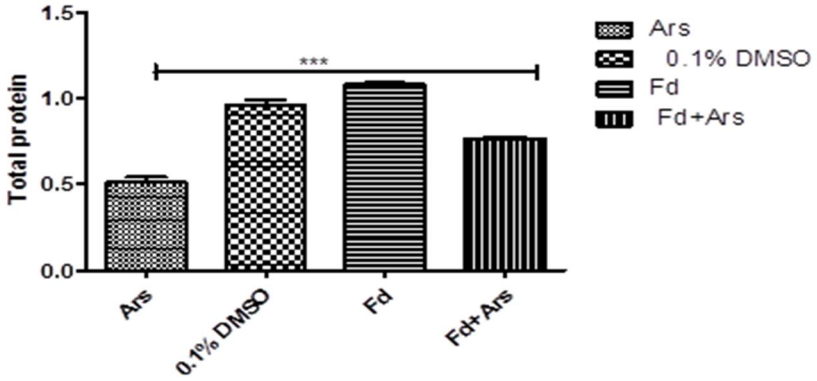

Fig 5: Effects of Ficus deltoidea (leaf) 35 µg/mL extract and arsenic (0.15 M) on total protein content extracted from the brain of adult Javanese medaka (Oryzias javanicus). ***P<0.001 represented significantly different values from an arsenic-treated group. The values represent mean ± SEM from three independent experiments. Ars = Arsenic and Fd=Ficus deltoidea leaf extract.

Acetyl-cholinesterase inhibitory assay:

Result of acetylcholinesterase inhibitory assay shows high activities in groups that were exposed to crude extract only. Increased activities were also observed in groups that were treated with crude extract and exposed to arsenic as well as the groups that are maintained in tap water compared to the groups that were exposed to arsenic only. There was a significant difference ($p<0.001$) between the fishes that were exposed to arsenic and remaining groups (groups maintained in tap water, groups treated with crude extract only, and groups that are treated with crude extract followed by exposure to arsenic) (Figure 6).

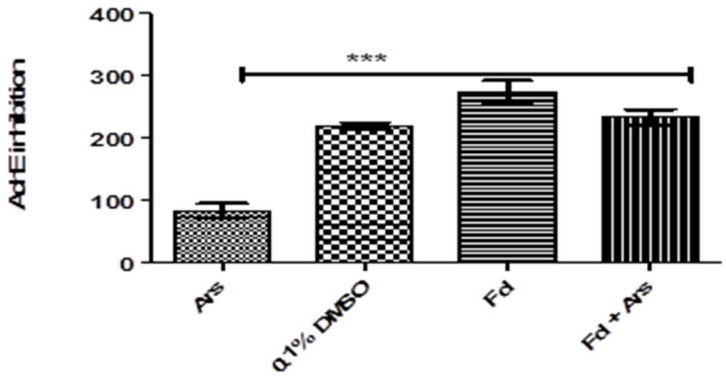

Fig 6: Acetyl cholinesterase inhibitory effects of *Ficus deltoidea* leaf extracts against Arsenic was measured using DTNB, Acetylcholine iodide substrate with a microplate reader at 405 nm. ***P<0.001 represented significantly different values from an Arsenic-treated group. The values represent mean ± SEM from three independent experiments. Ars = Arsenic and Fd = Ficus deltoidea leaf extract.

Butyryl-Cholinesterase Inhibitory Assay:

Result of Butyryl-cholinesterase inhibitory assay shows high activities in groups that were exposed to crude extract only. Similarly, increased activities were also observed in groups that were treated with crude extract and exposed to arsenic as well as the groups that were maintained in tap water compared to the groups

that were exposed to arsenic only. There was a significant difference ($p<0.001$) between the fishes that were exposed to arsenic and remaining groups (groups maintained in tap water, groups treated with crude extract only, and groups that were treated with crude extract and then exposed to arsenic) (Figure 7).

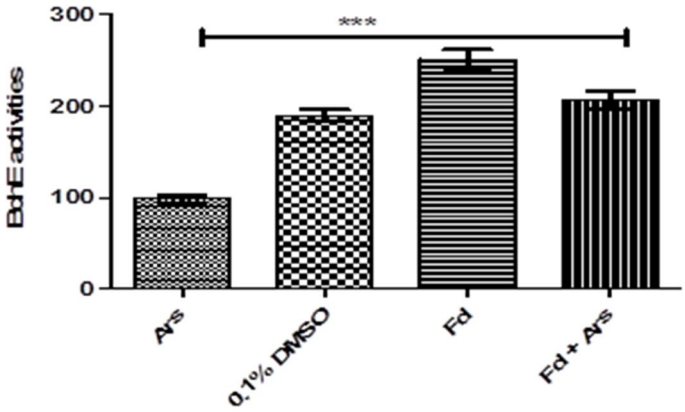

Fig 7: Butyryl cholinesterase inhibitory effects of *Ficus deltoidea* leaf extracts against Arsenic was measured using DTNB, Butyrylcholine iodide substrate with a microplate reader at 405 nm. ***P<0.001 represented significantly different values from an Arsenic-treated group. The values represent as mean ± SEM from two independent experiments. Ars = Arsenic and Fd = *Ficus deltoidea* leaf extract.

Propionyl-Cholinesterase Inhibitory Assay:

Result of Propionyl-cholinesterase inhibitory assay shows high activities in groups that were exposed to crude extract only, followed by the groups that were treated with crude extract and exposed to arsenic. Increase activities were also observed in groups that were maintained in tap water compared to the groups that were exposed to arsenic only. There was a significant difference ($p<0.01$) between the fishes that were exposed to arsenic and remaining groups that were maintained in tap water. Significant difference ($p<0.001$) was also observed between the groups that were exposed to arsenic and the fishes that were treated with crude extract only as well as between the groups that were treated with crude extract and then exposed to arsenic (Figure 8).

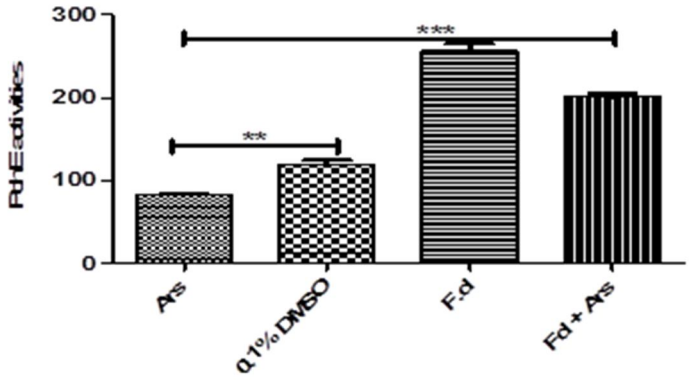

Figure 8: Propionyl cholinesterase Protective effects of Ficus deltoidea leaf extracts against Arsenic was measured using DTNB, Propionyl choline iodide substrate with a microplate reader at 405 nm. **P<0.01 and ***P<0.001 represented significantly different values from an Arsenic-treated group. The values represent as mean ± SEM from two independent experiments. Ars = Arsenic and Fd = Ficus deltoidea leaf extract.

HPLC:

Phytochemical constituents (vitaxin and isovitaxin) were identified in the crude extracts using HPLC method (Figure 9). Both peak areas identified in the crude extract were compared with the two standards (vitexin) at 21.834 (retention time) and (isovitexin) at 23.002 (retention time) (Figure 9 a-c). The peaks that correspond with the standard were colored red with a computer to justify the presence of vitexin and isovitexin.

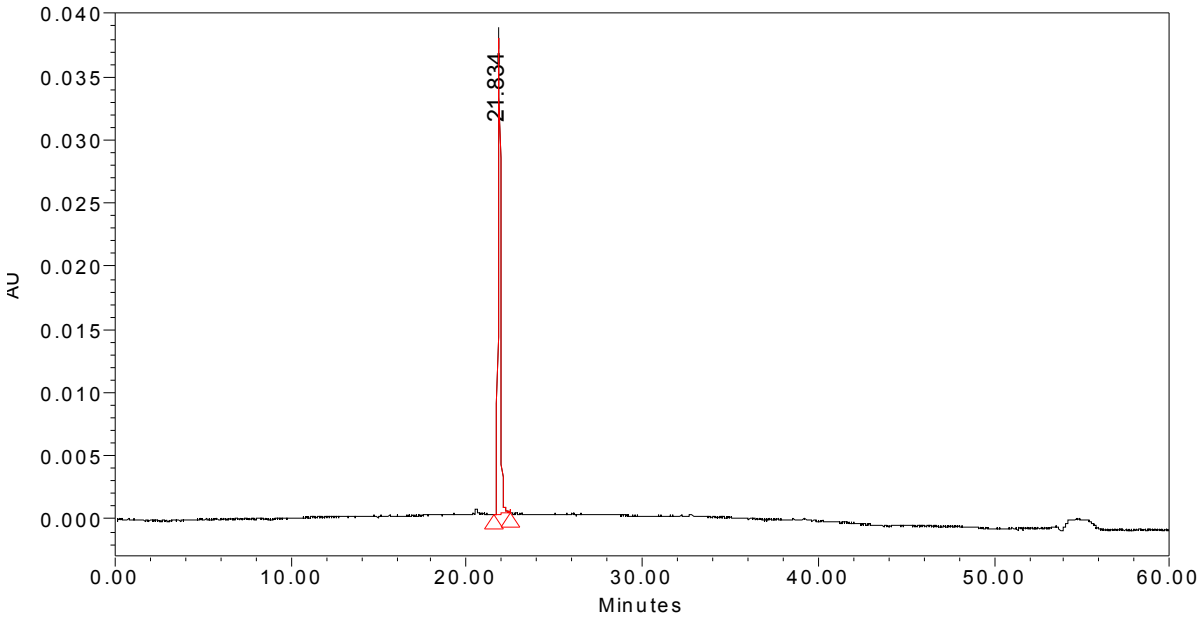

Figure 9a: Normal phase HPLC profile of vitexin (standard) identified at retention time of 21.834 min

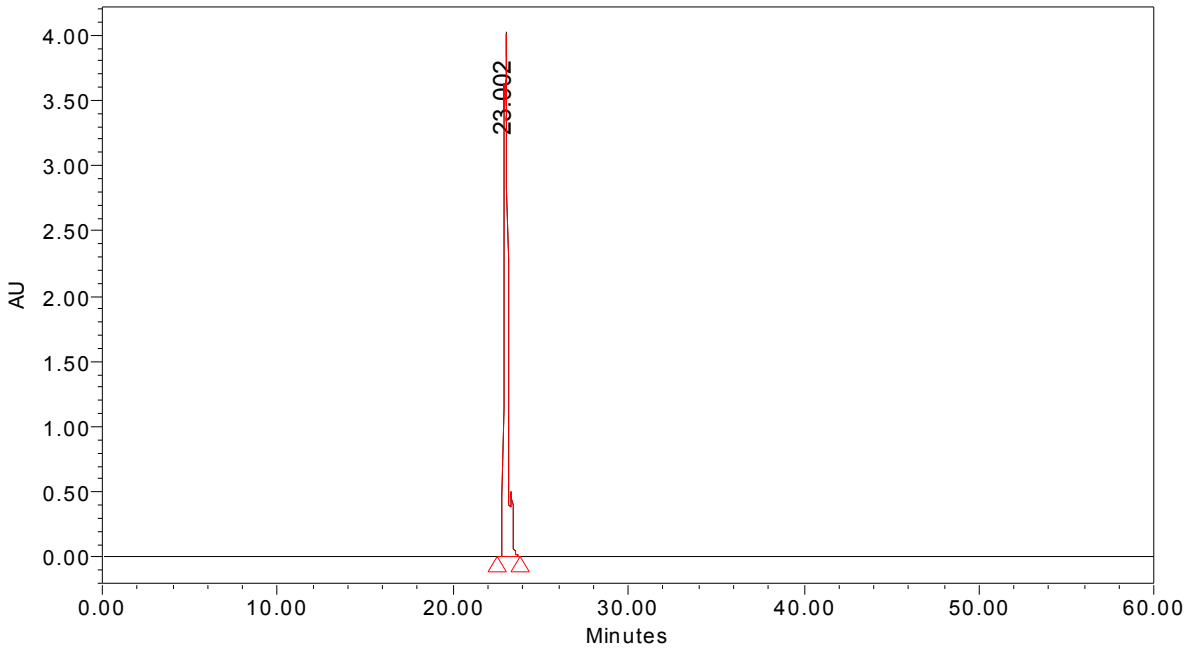

Figure 9 b: Normal phase HPLC profile of isovitexin (standard) identified at retention time of 23.002 min

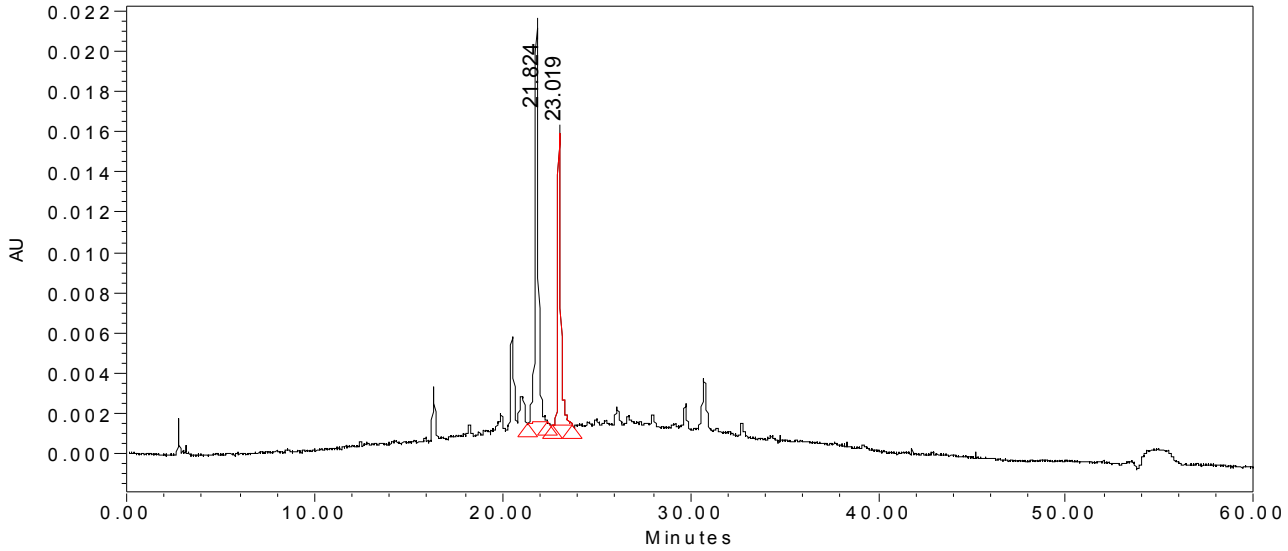

Figure 9c: Normal phase HPLC profile of vitexin and isovitexin identified in *Ficus deltoidea* (leaf) at retention time of 21.834 and 23.002 min

LCMS:

Dimethylamine, methylamine, vitexin**,** and glycine were identified in the extract with HPLC and LCMS at a retention time of 7.88, 10.24, 11.49, 12.07, and 13.27 min (Figure 10). Following the experimental study, results were obtained by online analysis using Mass Bank that is financially supported by the National Bioscience Database Center, Japan Science and Technology Agency (2011-2013). Identification of amino acid was made by the aid of computer-assisted evaluation of the resulting data, by searching against the spectral library.

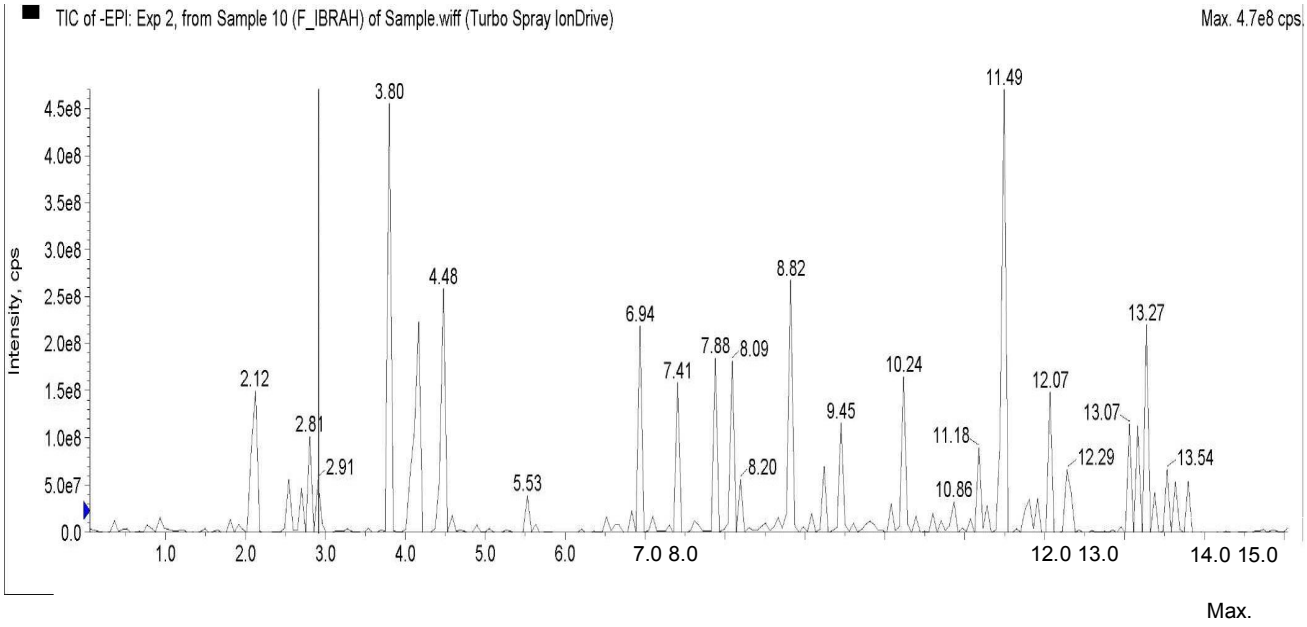

Figure 10: Normal phase LCMS profile of dimethylamine, methylamine, vitexin, and glycine vitexin identified in Ficus deltoidea (leaf) at retention time of 7.88, 10.24, 11.49 mn 12.07 and 13.27 min

Discussion

Mental and neurological disorders are a serious public health challenge globally, particularly in developing countries where cultural factors and limited access to standard healthcare have led to a reliance on traditional medicines (20). In order to overcome the high cost of treatment and prolong drug administration and/or drug side effects, *Ficus deltoidea* 80% leaf extract was evaluated to overcome toxicity and anticholinesterase activities on Javanese medaka. Considering the ability of methanol in the extraction of most quantities of plant and it's commonly use by traditional herbalist, 80% methanol was chosen as the extraction solvent in this research.

Methanol, ethanol, acetone and ethyl acetate have previously been used and recommended for extraction in others studies to obtain high yield of crude extract (21). Although solvent polarity influences the presence of secondary metabolite in herb during extraction, phenolic content of crude extracts and their antioxidant power were observed to increase in solvent polarity (22). High crude extracts were reported in the extraction of fruit and vegetable with acetone instead of methanol as the solvent (23). This might be because of the capacity of acetone to separate a large portion of the nonpolar part of the plant sample. Similarly, high return of extract was observed in the extraction of the phenolic compound from horseradish roots with ethanol, and ethanol/water solutions (24). However, the report by Dhawan and Gupta, (2016) (25) revealed increased outcome of the secondary metabolites in leaves extracted with methanol compared to the leaf that was extracted with other solvents (acetone, hot water, and chloroform). Increase yield of phenolic compounds because of extraction was also documented by Chigayo et al. (2016) (26) but may not necessarily be as a result of extraction power of acetone solvent alone. Extraction time technique may have played a major role in yielding high phenolic compounds in the extract. A few proteins and substances that are added as cancer prevention agents (for example, superoxide dismutase, catalase, glutathione peroxidase, polyphenols, mineral elements likeselenium, copper, chromium, Zinc, and vitamins like A, C and E are abundant in fruits and vegetables than the stem and root using methanol as extracting solvent have been documented (27).

There was a variation in the forms and content of bioactive compounds available in different herbs extracts as shown in the mortality rate of Javanese medaka. Plant toxicity leading to mortality and other side effects may be attributed to the presence and concentration of bioactive compounds that are used as immunity to the plants (28). Herbs, in general, have different ways of defense and overcome environmental pathogens due to their stationary autotrophic nature (29). Medicinal importance of some evaluated herbs, for example, *Digitalis purpurea*, *Hyoscyamus niger*, *Atropa belladonna*, *Physostigma venenosum*, *Podophyllum peltatum,* and *Solanum nigrum* are attributed to their therapeutic bioactive compounds. These compounds may also be responsible for their toxic effects (30). Several studies show that alkaloids are among the common bioactive compound with high medicinal values, but they have the potential to impair respiration system (31). Alteration of osmoregulation through increase stimulation of opercula beat on gills by this compound was also reported (31, 32).The interferences of normal function of nerves and/or neurotransmitters by several bioactive constituents available in the herbs are the main causes of abnormal changes in the movement of fish as well as altered physiology of essential animal body part leading to mortality (33). Some findings also reported obstruction of the metamorphosis process and skin formation in developing larvae resulting in increased mortality rate and morphological defects caused by the cumulative effects of unknown lipids available in the herb extract (34). The scientific findings also implicate anthraquinones present in most of the crude extract to be harmful and altered embryogenesis during pregnancy as well as predisposes to different forms of the teratogenic defect (35).

The enzymes that lyse choline-based esters are referred to as cholinesterases or choline esterases. These enzymes neutralized neurotransmitters acetylcholine or acetyl choline-like substances. Therefore, they catalyze the breakdown and hydrolysis of the cholinergic

neurotransmitters to choline and acetic acid (36). The biochemical pathway involved is very vital since it permits a cholinergic neuron to come back to its resting state after excitation. Contraction in muscle triggers the release of acetylcholine present at neuromuscular junctions to enhance contraction, and this allowed locomotive movement of the body organs (37). Cholinesterase enzymes neutralize the neurotransmitter acetyl choline and hence allowe the contractile muscle to relax for a while (38). Pseudocholinesterase, or plasma cholinesterase that also referred to as Butyryl-cholinesterase (BChE, BuChE), is among the nonspecific cholinesterase enzyme choline-based esters (39). It is synthesized in the liver and found mainly in the blood plasma, and encoded by the BCHE gene in mammals. There is a high similarity between this esterase and neuronal acetylcholinesterase, which is also known as RBC or erythrocyte cholinesterase (39). Propionyl-cholinesterase is another pseudocholinesterase present in the plasma that is less commonly compared to Acetyl-cholinesterase and Butyryl-cholinesterase (40).

Antioxidants, as the therapeutic potential of *Ficus deltoidea* are due to the present of vitaxin, isovitaxin, and many unknown secondary metabolites (flavonoids and phenolic compounds). Several findings reported the use of *Ficus deltoidea* as a medicinal plant for curing many illnesses. Some of this finding includes the use of aqueous, ethanolic, or methanolic extract of this plant in curing induced diabetes in lab animals (41)., Others showed the inflammatory inhibitory effect, the anti-melanogenic effect, and anti-photoaging potentials of *Ficus deltoidea* extracts (42), Others are the antioxidant effect (43), wound healing effect (44), the antibacterial effect (45), the anticancer effect (46) and antithrombotic potentials of *Ficus deltoidea* extract (47). The potential of this plant to cure several diseases may be attributed to the presence of several secondary metabolites (flavonoid, tannins, triterpenoids, proanthocyanins, phenols, flavonoids, phenols, and tannins) that strongly naturalized oxidative free radicals that are implicated as the major causes of these diseases (48).

Examples os dimethylamine and methylamine are NG,NG-dimethylarginine (ADMA) and NG,NG-dimethylarginine (SDMA) that obstruct nitric-oxide synthase (NOS) in the body tissue (49). The nervous system mostly uses glycine to inhibit several neurotransmitters, and it is very vital in preventing neurological diseases (50). Glycine could be applied to the management of unknown neurological symptoms (51). This amino acid also playes a unique role in ATP production in the body (52). It also found that it could be synthesized in the prostate fluid of the male's mammals and plays a vital role to enhance prostate gland function (53). Glycine is a constituent of glutathione, and a coenzyme played diverse role biochemical reactions. Among the physiological effect of glutathione is the maintenance of the integrity of the cell by protecting –SH group of hemoglobin, catalase and lipoproteins of the cell's membrane (54).

Conclusion and Recommendation:

It is concluded that availability of vitaxin and isovitaxin in this plant contributed in its neuroprotective and antioxidative potential. Cholinesterase-inhibitory effect of the crude extract justified its potential as chemotherapeutic agents. Toxicity screening of this crude extract on mammals such as mice and rat to reaffirm their toxicity profile are recommended. Antioxidant screenings as well as isolation of bioactive compounds such as vitaxin and isovitaxin are strongly recommended.

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Conflict of interest

The authors have no conflict of interest in this study.

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