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Original Paper

Evaluation of neuroprotective efficacy of *Terminalia bellerica* and *Terminalia catappa* fruit extracts in zebrafish model

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Abstract

The present study evaluated the neuroprotective efficacy of *Terminalia bellerica* and *Terminalia catappa* fruits through dietary application of different solvent extracts (aqueous, methanol, ethanol, hexane) at various concentrations (0.2, 0.4, 0.8 g kg^{-1} feed) in an aluminium-induced neurotoxic zebrafish model. Aluminium exposure significantly elevated brain acetylcholinesterase activity, monoamine oxidase activity, and glial fibrillary acidic protein levels in comparison to the control, indicating neurodegeneration. Dietary administration of both plant materials reduced the level of all three biochemical parameters. *T. bellerica* aqueous extract and *T. catappa* ethanol extract, both at the concentration of 0.8 g kg^{-1} feed, showed the best results, restoring acetylcholinesterase, monoamine oxidase, and glial fibrillary acidic protein levels close to those in the control fish. Both *T. bellerica* and *T. catappa* fruits may be used as therapeutic agents against neurodegenerative diseases, however, their efficacy depends on the extraction solvent and concentration of dietary supplementation.

Key words: *Danio rerio*, dietary administration, neurodegeneration, plant extract, *Terminalia bellerica, Terminalia cattalpa*, therapeutic potential.

Abbreviations: AChE, acetylcholinesterase; GAE, gallic acid equivalents; GFAP, glial fibrillary acidic protein; LC_{50}, lethal concentration 50%; MAO, monoamine oxidase; RE, rutin equivalents; TFC, total flavonoid content; TPC, total phenolic content.

Introduction

Neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease and different forms of dementia have emerged as pressing global health concerns due to their escalating prevalence and profound impact on individuals and healthcare systems (Zahra et al. 2020). The World Health Organization predicts neurological disorders to be the second leading cause of human demise in the next 20 years (Palanisami et al. 2023). The intricate pathophysiology of these disorders, characterized by progressive neuroinflammation, synaptic dysfunction and cognitive decline, underscores the urgency for potential therapeutic interventions (Rekatsina et al. 2020).

A significant number of studies have used brain acetylcholinesterase (AChE) activity, monoamine oxidase (MAO) activity, and glial fibrillary acidic protein (GFAP) level as standard biomarkers for elucidating the mechanisms of neurotoxicity and screening plant-based neuroprotective agents (Naoi et al. 2010; Moreira et al. 2022; Clement et al. 2024). The role of AChE in the pathogenesis of neurodegenerative diseases is mediated by regulating the inflammatory response, apoptosis, oxidative stress and aggregation of pathological proteins (Walczak-Nowicka, Herbet 2021). MAO mediates the metabolism of different monoamine neurotransmitters in the brain, and sudden elevation in its activity may directly impact the form and function of mitochondria, thereby impacting the health of surviving neurons (Banerjee et al. 2024). The intermediate filament protein GFAP levels in brain astrocytes are correlated with clinical severity of pathological conditions such as traumatic brain injury, making it a potential prognostic indicator of neurodegeneration (Kamada et al. 2024). Consequently, these three biomarkers may also be used to evaluate the effectiveness of different therapeutic agents against neurological disorders.

In the quest for therapeutic strategies against neurodegenerative disorders, phytochemicals derived from medicinal plants have gained prominence due to their potential safety advantage over synthetic drugs (Ara et al. 2022). Several natural products, mainly plant extracts, have been used in traditional medicine for memory-enhancing functions (Goel, Maurya 2019). The efficacy of most of these active plant-derived compounds was identified through research inspired by ethnobotanical applications (Najmi et al. 2019). Bioactive constituents in various plant parts include steroids, terpenoids, carotenoids, flavonoids, alkaloids, tannins, and glycosides (Ingle et al. 2017). Qualitative phytochemical screening reveals a diverse array of secondary metabolites produced by

plants, and their quantification is crucial for extraction, purification, and identification of bioactive compounds with potential human health benefits (Mukherjee et al. 2019). Securing adequate yields is fundamental to obtain a rich concentration of phytochemicals (Sheneni et al. 2018). The extraction solvent can influence the isolation of such bioactive phytoconstituents, and different solvent extracts from the same plant may demonstrate a range of neuroprotective activities (Nampoothiri et al. 2011). Furthermore, the functional efficacy of the plant extracts may also vary depending on the dose of administration (Mukherjee et al. 2019). Consequently, determining the optimal extraction solvent and administration dose is crucial to maximize the functional potential of the plant material.

The genus *Terminalia* (family Combretaceae) comprises approximately 250 species of medium to large flowering trees widely distributed throughout the tropical and subtropical regions of Asia, Australia, and Africa (Zhang et al. 2019). Asian *Terminalia* species are particularly diverse and among them, *Terminalia arjuna, Terminalia chebula, Terminalia bellerica* and *Terminalia catappa* are arguably the most versatile, with documented uses for their various therapeutic properties (Cock et al. 2015). The neuroprotective potential of *T. arjuna* bark extract and *T. chebula* fruit extract has already been demonstrated in a myriad of scientific studies (Chang, Lin 2012; Shen et al. 2017; Suganthy et al. 2018; Das et al. 2020). A recent study investigated the neuropharmacological and behavioural effects of *T. bellirica* leaf extract, demonstrating its antiepileptic activity in a chronic pentylenetetrazole-induced kindling mice model (Saleem et al. 2024). Antidepressant-like effects of hydrolysable tannins from *T. catappa* leaf extract was reported, mediated through modulation of hippocampal plasticity and regulation of monoamine neurotransmitters in a chronic mild stress model. (Chandrasekhar 2017). In another study, effect of *T. catappa* leaf hydroalcoholic extract on cognitive functions and cholinesterase activity was evaluated in scopolamine-induced amnesia in rats (Joshi, Malviya 2017). A number of *in vitro* and *in vivo* experiments have indicated their bioactivity, including antitumor, anti-inflammatory, antiviral, antioxidant, antifungal, antimicrobial and analgesic effects (Zhang et al. 2019; Das et al. 2020). However, limited research has investigated the neuroprotective potential of *T. bellerica* fruits. Methanolic extract of *T. bellerica* fruits was screened for its neuroprotective activity against aluminium chloride and haloperidol-induced amnesia in Swiss albino mice (Reddy et al. 2020). *T. bellirica* fruit pulp aqueous extract was observed to enhance cognition in rodents (Rajaduraivelpandian et al. 2024). Gallic acid extract from *T. bellirica* fruits was tested on chronic mild stress-induced depression-like activity in a mouse model, and the extract was found to have an antidepressant-like activity (Yadavalli et al. 2020). However, there have been no reports about the effects of *T. catappa* fruits against neurodegenerative diseases. Thus, further evidence-based experimental validation is required to affirm the neuroprotective efficacy of *T. bellerica* and *T. catappa* fruits.

Zebrafish (*Danio rerio*) is a frequently exploited model for research on human neurological disorders, offering a practical means of studying neurotoxicity and neurodegeneration (Palanisami et al. 2023). Also, the model is ideal for drug investigations as medication can be easily supplemented to the feed.

Among various environmental factors implicated in neurodegeneration, aluminium (Al) has garnered significant attention owing to its pervasive presence and neurotoxic potential (Exley et al. 2016). Chronic exposure to aluminium has been associated with oxidative stress, neuroinflammation, and disruption of neurotransmitter systems, all of which are hallmarks of neurodegenerative processes (Dey et al. 2022). The use of aluminium as a neurotoxicity model is supported by discernible functional damages resulting from neurodegeneration in the zebrafish brain (Palanisami et al. 2023). Aluminium-induced neurotoxicity in a zebrafish (*D. rerio*) model has been observed to cause synaptic dysfunction and behavioural deficits, mirroring aspects of human neurodegenerative conditions (Senger et al. 2011; Mani et al. 2018; Capriello et al. 2021). Hence, this model has often been used to screen efficacy of novel neuroptotective curatives (Haridevamuthu et al. 2023; Boopathi et al. 2024).

The present study was carried out to validate the use of *T. bellerica* and *T. catappa* fruits as therapeutic strategies against neurodegenerative diseases using an Al-induced neurotoxic zebrafish model by assessing AChE, MAO and GFAP levels. Dietary administration with different solvent extracts of both plant materials at various concentrations was carried out to infer about the solvent and concentration rendering the best functional efficacy for each plant.

Materials and methods

Collection and acclimatization of test fish

Adult (about 6- to 8-month old, 0.5 ± 0.03 g in weight, 2.5 ± 0.7 cm in length) wild-type zebrafish (*Danio rerio*) of both sexes were used in this study. The fish were obtained from a commercial supplier (Saha Enterprise; Reg No. 1828/ PO/Bt/S/15/ CPCSEA) and acclimated in the laboratory for at least 2 weeks. Fish were kept on a 14/10 h light/dark cycle at a temperature of 27 ± 2 °C. All procedures for the use of animals were in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and permitted by the institutional animal ethics committee (Department of Zoology, University of Calcutta; Registration No. 885/GO/Re/S/05/CPCSEA; Project No. CAL/ZOO/SBC/2024-2) under 'Committee for the purpose of Control and Supervision of Experiments on Laboratory Animals', Ministry of Environment and Forest, Government of India.

Acute toxicity test with aluminium chloride

For Al toxicity tests on zebrafish, an acute semi-static bioassay was conducted with seven nominal concentrations (0, 80, 90, 100, 110, 120, 130 mg L^{-1}) of $AlCl_3$ according to the OECD 203 Guideline for Testing of Chemicals, Fish, Acute Toxicity Test (OECD 1992). Three replicates of 10 fishes (kept in 5 L glass aquaria with maximum loading of 1.0 g fish per L tanks) for each concentration was used. Stock standard $AlCl_3$ solution was prepared in HPLC-grade deionized water at a concentration of 200 mg L^{-1} and stored at –20 °C, which was found to be stable for at least 1 month. The stock solution was diluted in the water of the experimental aquaria to prepare the final nominal concentrations. The respective nominal concentrations of $AlCl_3$ were renewed every 24 h. Fish mortality was recorded at 24, 48, 72 and 96 h. Feeding to fish was stopped 24 h before the toxicity test, and fish were not fed throughout the four days of the experiment. The results of acute toxicity test data were analysed using probit analysis with the SPSS 22.0 statistical tool to determine the 96 h LC_{50} value. A sublethal dose of $1/10^{th}$ of the 96 h LC_{50} value was selected as the treatment dose for the subsequent experimental studies (Kavitha et al. 2012; Kumar et al. 2024). The administered concentration was within the range of $AlCl_3$ exposure reported in previous investigations (Capriello et al. 2021).

Plant extract preparation

Terminalia catappa L. and *Terminalia bellirica* (Gaertn.) Roxb. fruits were procured from a commercial plant supplier at Gariahat Market, Kolkata, and identified and authenticated at the Department of Botany, University of Calcutta, West Bengal, India. The plant materials were then washed in sterile distilled water, air-dried in shade and powdered. The powdered plant materials (50 g) were extracted separately with 200 mL solvents such as hexane, methanol, ethanol in a percolator for 48 h, and filtered with Whatman filter paper (Grade 42). The extracts were then evaporated to dryness under pressure at 45 °C using a rotary evaporator (Roteva ASP-8763.RD0.000, ASP-EQUITRON-ASP, India) and stored at –20 °C in an amber glass bottle. The aqueous extract was prepared by boiling 18 g powder in 1500 mL distilled water for 30 min and then filtering it with Whatman filter paper (Grade 42) twice (Ghosal et al. 2015).

The yield from different solvent extracts on a dry weight basis was calculated using the formula:

$$Yield\ (\%) = (W1 \times 100) / W2\ ,$$

where $W1$ is weight of extract after evaporation of the solvent and $W2$ is dry weight of the plant powder.

Qualitative phytochemical analysis

Qualitative phytochemical analyses were carried out using standard procedures (Mukherjee et al. 2018). Briefly, a few drops of $FeCl_3$ were added to 2 mL of the extracts, and the formation of a bluish colour indicated the presence of tannins. A drop of $NaHCO_3$ was added to 5 mL of the extract and shaken vigorously. It was left undisturbed and the formation of honeycomb-like froth after 3 min indicated the presence of saponins. A few drops of Mayer's reagent were added to 1 mL of the extract, and the appearance of a pale-yellow precipitate indicated the presence of alkaloids. Benedict's reagent (5 mL) was added to 0.5 mL of the plant extract, mixed, and boiled for 5 min. The formation of brick-red precipitate indicated the presence of carbohydrates. Aqueous NaOH (1 mL) was added to 1 ml of the extract, and the appearance of a pale-yellow precipitate indicated the presence of glycosides. Ten drops of dilute HCl were added to 1 mL of the extract, followed by a small piece of zinc. The development of a reddish-pink colour indicated the presence of flavonoids. Glacial CH_3COOH (1 mL) was mixed with 1 mL of the extract followed by addition of 1 mL concentrated H_2SO_4 along the wall of the test tube over ice. The formation of brown, green, and red colouration indicated the presence of terpenoids, steroids, and triterpenoids, respectively.

Quantitative phytochemical analysis

The total phenolic content (TPC) and the total flavonoid content (TFC) of different plant extracts were determined using standard protocols (Mukherjee et al. 2018). Briefly, total phenolic content of the plant extracts was determined using the Folin–Ciocalteu reagent. The absorbance was measured using a UV–VIS spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan) at 765 nm and the total phenolic content was expressed as milligrams of gallic acid equivalents (GAE) per gram of dry weight of the plant material. Total flavonoid content was estimated using a colourimetric assay. The absorbance was measured spectrophotometrically at 425 nm and the total flavonoid content was expressed as milligrams of rutin equivalents (RE) per gram of dry weight of the plant.

Preparation of feed

Each plant extract was dissolved in dimethyl sulfoxide and added individually to the finely ground (< 500 to 1000 μm) basal diet (Artificial floating fish feed, Tokyu® Fish Food Spirulina, Tokyu, Japan, 32 % crude protein). The control feed was prepared by adding only dimethyl sulfoxide to a finely ground artificial diet. The feed was then mixed thoroughly, and dried at room temperature. The fish were fed respective diets twice daily at the rate of 2% body weight (De et al. 2022).

Acute toxicity studies of plant extracts

An acute oral toxicity test with the plant extracts on zebrafish was first conducted according to OECD 423 guidelines (OECD 2011). Zebrafish were fed individual diets fortified with aqueous, methanol, ethanol and hexane

extract of *T. catappa* fruit and *T. bellirica* fruit at seven different concentrations (0, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0 g kg^{-1}) with three replicates per concentration (n = 10 fish per replicate) for 4 days. The fish were kept in glass aquaria of 5 L capacity with a maximum loading of 1.0 g fish per L tanks. The test concentrations were selected based on previous studies (Meneses et al. 2020). The cumulative mortality at 96 h in different extract concentrations was recorded to determine the sub-lethal concentration (LD_{50}) for each plant extract. As no mortality was observed during the toxicity test for any of the extracts, 1/5th (0.8 g kg^{-1} feed), 1/10th (0.4 g kg^{-1} feed) and 1/20th (0.2 g kg^{-1} feed) of the maximum tolerated concentration was selected for a subsequent study (Singh et al. 2014).

Dietary treatment with different solvent extracts of plants

In the subsequent feeding experiment, zebrafish from the acclimated stock were divided into three groups (Fig. 1). The first group was not exposed to Al and was fed a control diet without any plant extract supplementation for 28 days (negative control). The second group was exposed to Al (9.97 mg L^{-1} $AlCl_3$) and was fed a control diet without any plant extract supplementation for 28 days (positive control). The third group was exposed to Al (9.97 mg L^{-1} $AlCl_3$) and was simultaneously fed diets fortified with different solvent extracts at different concentrations of the plant materials for 28 days. Accordingly, the third experimental group was sub-categorized in 2 × 4 × 3 factorial design (Ghosal et al. 2015): the first factor was plant material (*T. catappa* fruit, *T. bellirica* fruit), the second factor was extraction solvent (aqueous, methanol, ethanol, hexane), and the third factor was extract concentration for dietary treatment (0.2, 0.4, 0.8 g kg^{-1}). Three replicates of 30 fish (kept in 15-L glass aquaria with a maximum loading of 1.0 g fish per litre tanks) for each treatment group were used in this experiment.

The experiment was conducted under semi-static exposure conditions (Banaee et al. 2019) and water was renewed daily to remove excess diet and detritus. The concentration of Al in the treatment aquarium was maintained through the daily addition of the $AlCl_3$ solution after the renewal of water. Constant water temperature (27 to 28 °C), pH (7.3 to 7.8), dissolved oxygen (5.0 to 6.0 mg L^{-1}) and photoperiod (14 h light / 10 h darkness) were maintained throughout the experiment. Fish in each treatment group were fed their respective diet ad libitum twice daily during the 28 days of the experiment, and feeding was stopped 24 h before the final sampling on the 30th day. Fish from all groups were anaesthetized with phenoxy-ethanol (1 : 20 000, *v*/*v*), sacrificed and the brain tissue was quickly dissected out. Brain tissue from all 30 fish in a replicate for a treatment group was pooled together. Thus, three pools of brain tissue samples were obtained from three replicates for each treatment group. Pooled tissue was stored at −20 °C for biochemical analysis.

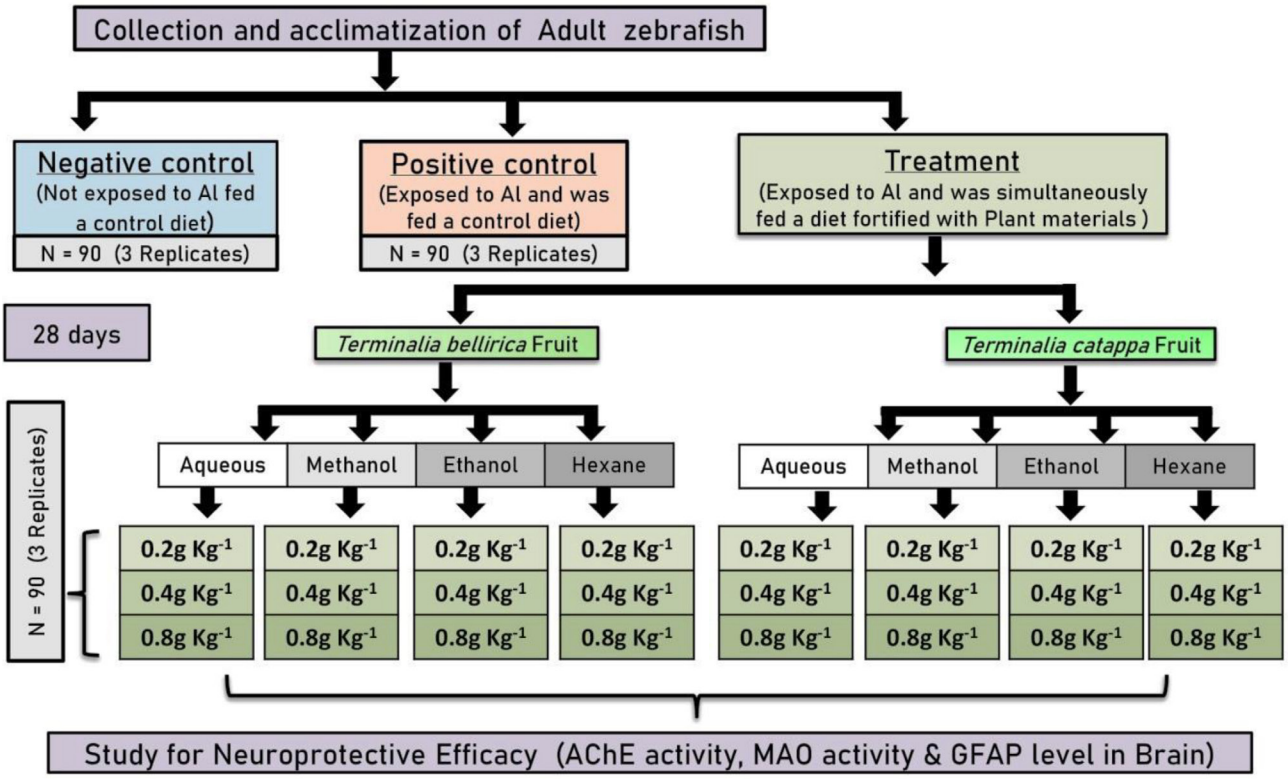

Fig. 1. Experimental design to study the neuroprotective efficacy of *T. bellerica* and *T. catappa* fruits (prepared using different solvents and concentrations) in an aluminium-induced neurotoxic zebrafish model.

Sample preparation for biochemical analysis

Pooled brain tissue samples (100 mg, $n = 3$) from different treatment groups were homogenized with Tris buffered saline (1:10 *w*/*v* tissue/buffer ratio; 10 mM Tris-HCl, 0.1 mM NaEDTA, 10 mM sucrose, 0.8% NaCl, pH 7.4), centrifuged at 20 000 rpm for 30 min at 4 °C and the supernatant was stored at –20 °C for further analysis of different biochemical parameters. Protein concentrations in the supernatants were determined using a rapid colourimetric assay, using 10 µL of sample with 190 µL of Bradford reagent followed by measuring the absorbance at 595 nm and calculating concentrations based on a bovine serum albumin standard curve (Bradford et al. 1976).

Determination of GFAP level

GFAP level in the brain tissue was quantified in microplate reader using a commercial ELISA kit (MyBioSource, San Diego, USA, catalogue No. MBS032098; intra-assay coefficient of variation < 15%, inter-assay coefficient of variation < 15%, sensitivity 0.1 pg mL^{-1}, detection range 1.56 to 50 pg mL^{-1}) following the manufacturer's protocol. The ELISA kit has cross-reactivity in fish, and no significant cross-reactivity or interference between the fish protein and analogues is observed according to the manufacturer's protocols. The kit was a sandwich ELISA kit, where the provided microplate was pre-coated with a GFAP-specific antibody. Samples were added to the wells along with a biotin-conjugated GFAP antibody. After incubation with horseradish peroxidase-conjugated Avidin, 3,3',5,5'-tetramethylbenzidine substrate was added. The reaction was terminated with sulphuric acid, and the colour change was measured at 450 nm using a microplate reader (Varioskan LUX Multimode, Thermo Scientific) at 450 nm. GFAP levels were determined by comparing the sample absorbance to a standard curve, and the data were expressed in pg per mg protein.

Measurement of activity of brain enzymes

The determination of AChE activity was carried out using the method described by Ellman et al. (1961). The reaction mixture included crude enzyme extract, 0.5 mM acetylthiocholine iodide, 0.33 mM 5,5'-dithiobis-(2-nitrobenzoic acid), and 92.7 mM phosphate buffer (pH 8.0) (Jeon et al. 2016). AChE activity was measured spectrophotometrically at 412 nm by quantifying the thiocholine produced from acetylcholine hydrolysis, and expressed as nnmol of thiocholine released per minute per microgram of protein.

MAO activity was assayed by the method of Tabor et al. (1955) based on the oxidative deamination of benzylamine to benzaldehyde. The reaction mixture contained 3.7 mM benzylamine solution, 0.25 M phosphate buffer (pH 8.0), distilled water and the crude enzyme extract (Razygraev et al. 2016). The progress of the reaction (formation of benzaldehyde) was monitored at 250 nm, and MAO activity was expressed as nmol benzaldehyde produced per milligram of protein per minute.

Statistical analysis

All data are expressed in terms of mean ± SE ($n = 3$ pooled samples). Normal distribution and equal variance of the data were analysed by Shapiro–Wilk test and Levene's test, respectively. The total phenolic content and the total flavonoid content of different plant extracts were compared by one-way ANOVA followed by a post hoc Tukey test (p-value 0.05). The treatment effect of Al exposure and dietary administration of different solvent extracts of individual plant material at different concentrations was evaluated by one-way ANOVA. A post hoc Tukey test (p-value 0.05) was performed for separating treatment means where significant differences were found. Moreover, a multivariate factorial ANOVA was performed to analyze the influence and interaction effect of independent variables (plant materials, extraction solvents, extract concentrations for dietary administration) on different brain biochemical parameters of the Al-exposed fish. All analyses were carried out using SPSS version 22 for Windows.

Results

LC_{50} of aluminium chloride was 99.79 mg L^{-1} (Y = 17.745 X = 30.476, fiducial limits lower = 9.811, upper = 25.679, $p = 0.00342$). A sublethal concentration of $1/10^{th}$ LC_{50} value (9.97 mg L^{-1}) was selected to induce Al-induced neurotoxicity in zebrafish in the present study.

Dietary administration of both *T. bellirica* and *T. catappa* fruit extract during the evaluation of acute toxicity resulted in no mortality. Hence, $1/5^{th}$ (0.8 g kg^{-1} feed), $1/10^{th}$ (0.4 g kg^{-1} feed) and $1/20^{th}$ (0.2 g kg^{-1} feed) of the highest dose for all solvents of *T. bellirica* and *T. catappa* fruit extract were selected for the subsequent study of neuroprotective efficacy.

The yields of the extracts from *T. bellirica* and *T. catappa* fruit with different solvents were estimated on a dry weight basis to exclude interference with the plant's moisture content. The highest yield for both *T. bellirica* and *T. catappa* fruits was obtained from the aqueous extract (15.04 and 15.52%, respectively). Hexane extract showed the minimum yield percentage for *T. bellirica* and *T. catappa* fruits (1.40 and 2.12%, respectively) while ethanol (4.87 and 3.82%, respectively) and methanol (11.00 and 3.05%, respectively) extracts showed intermediate yield percentages.

Qualitative analysis of phytochemicals revealed the presence of tannins, flavonoids and alkaloids in all the solvent extracts for both plants (Table 1). Terpenoids were present in aqueous and methanol extracts of *T. bellirica* and ethanol and methanol extracts of *T. catappa*. Glycosides were only found in the ethanol extract of *T. bellirica* and ethanol and aqueous extracts of *T. catappa*. Saponin was

Table 3. Qualitative and quantitative analysis of phytochemicals in different solvent extracts of *Terminalia bellirica* and *Terminalia catappa* fruits. '+' indicates presence and '–' indicates absence of the phytochemical group in qualitative analysis. Different superscripts indicate a significant difference ($p < 0.05$) in mean values of total phenolic content and total flavonoid content between different plant extracts following post hoc Tukey's test [η^2 = total phenolic content (0.975), total flavonoid content (0.928)]

Plant extracts	Qualitative parameters Phytochemical groups							Quantitative parameters	
	Tannins	Saponins	Carbohydrates	Flavonoids	Terpenoids	Glycosides	Alkaloids	Total phenolic content (mg GAE g^{-1} dry weight)	Total flavonoid content (mg RE g^{-1} dry weight)
T. bellirica water	+	+	+	+	+	–	+	19.016 e	505.15 cd
T. bellirica methanol	+	–	+	+	+	–	+	17.370 d	509.88 d
T. bellirica ethanol	+	+	+	+	–	+	+	13.550 bc	497.01 bcd
T. bellirica hexane	+	–	+	+	–	–	+	12.780 b	419.01 a
T. catappa water	+	–	–	+	–	+	+	17.035 d	478.15 bc
T. catappa methanol	+	+	+	+	+	–	+	14.300 c	468.02 b
T. catappa ethanol	+	+	+	+	+	+	+	17.180 d	481.75 bcd
T. catappa hexane	+	–	–	+	–	–	+	11.095 a	432.42 a

found in ethanol and aqueous extracts of *T. bellirica* and ethanol and methanol extracts of *T. catappa*. Carbohydrates were present in all extracts except the hexane extract of *T. bellirica* and the aqueous extract of *T. catappa*.

The aqueous extract of *T. bellirica* showed the highest total phenolic content and it was significantly higher compared to all other solvent extracts for both plant species (Table 1). *T. bellirica* methanol extract exhibited the highest flavonoid content among all the solvent extract for both plant species. For *T. catappa* fruits, however, the ethanol extract showed significantly higher total phenol and total flavonoid content than other solvents. On the other hand, hexane extract of both plant materials showed significantly lower total phenol and total flavonoid content than other solvents. Hexane extracts of *T. catappa* exhibited the lowest total phenolic content, while *T. bellirica* hexane extract exhibited the lowest flavonoid content among all the solvent extracts for both plant species. There was a moderate correlation ($R^2 = 0.5972$) between the total phenol content and the total flavonoid content.

Fish fed the control diet and exposed to Al for 28 days (positive control) showed significantly higher AChE activity, MAO activity and GFAP level than the fish fed the control diet and without Al exposure (negative control) (Table 2). This positive control group showed the highest AChE activity, MAO activity and GFAP level in the brain at the end of the experimental period. The dietary administration of different solvent extracts of *T. bellirica* and *T. catappa* fruits at different concentrations reduced all three brain biochemical parameters in the Al-exposed fish compared to the positive control group. For dietary administration of *T. bellirica* fruit extracts, the best results for all three brain biochemical parameters were obtained with the aqueous extract at a concentration of 0.8 g kg^{-1} feed (Table 2). For dietary administration of *T. catappa* fruit extracts, the best results for all three brain biochemical parameters were obtained with the ethanol extract at the concentration of 0.8 g kg^{-1} feed. Moreover, both these treatment groups showed statistically homogenous AChE activity, MAO activity and GFAP level to the negative control group. Interestingly, dietary administration of *T. catappa* fruit ethanol extract at 0.8 g kg^{-1} feed concentration yielded lower, but non-significant, AChE activity, MAO activity and GFAP level than the *T. bellirica* fruit aqueous extract at 0.8 g kg^{-1} feed concentration group (Table 2).

Multivariate test results revealed significant effect of plant material [Pillai's trace = 0.661, $F_{(3, 46)} = 29.94$, $p = 0.000$], solvent [Pillai's trace = 1.094, $F_{(9, 144)} = 9.189$, $p = 0.000$], concentration [Pillai's trace = 1.124, $F_{(6, 94)} = 20.092$, $p = 0.000$], and significant interaction effect was observed between plant material and solvent [Pillai's trace = 2.311, $F_{(9, 144)} = 53.716$, $p = 0.000$], plant material and concentration [Pillai's trace = 0.907, $F_{(6, 94)} = 12.988$, $p = 0.000$], solvent and concentration [Pillai's trace = 1.590, $F_{(18, 144)} = 9.016$, $p = 0.000$], and plant material, solvent and concentration

Table 2. Acetylcholinesterase (AChE) activity (nmol μg^{-1} min^{-1}), monoamine oxidase (MAO) activity (nmol mg^{-1} min^{-1}), glial fibrillary acidic protein (GFAP) level (pg mg^{-1}) in the brain of adult zebrafish from different treatment groups. Data are means ± SE; $n = 3$ pooled samples. Different superscripts indicate a significant difference ($p < 0.05$) in mean values within columns following post hoc Tukey's test. Negative control – fish not exposed to Al and fed a control diet without any plant extract supplementation for 28 days. Positive control – exposed to Al (9.97 mg L^{-1} $AlCl_3$) and fed a control diet without any plant extract supplementation for 28 days. Al-exposed + plant extract fed – exposed to Al (9.97 mgL^{-1} $AlCl_3$) and simultaneously fed diets fortified with different solvent extracts at different concentrations of the plant materials for 28 days [η^2 = AChE activity (0.944), MAO activity (0.848), GFAP level (0.989)]

Treatment group	Plant material	Extraction solvent	Extract concentration (g kg^{-1})	AChE activity (nmol μg^{-1} min^{-1})	MAO activity (nmol mg^{-1} min^{-1})	GFAP level (pg mg^{-1})
Negative control				0.298 ± 0.014 a	0.858 ± 0.026 a	0.088 ± 0.010 a
Positive control (Al-exposed)				0.514 ± 0.019 gh	1.334 ± 0.025 f	0.587 ± 0.027l m
Al-exposed + plant extract fed	*T. bellirica* fruits	Water	0.2	0.452 ± 0.005 cdefg	0.993 ± 0.055 bcd	0.357 ± 0.005 ghij
			0.4	0.426 ± 0.026 cdefg	1.109 ± 0.059 bcdef	0.163 ± 0.007 abc
			0.8	0.301 ± 0.012 ab	0.924 ± 0.047 abc	0.141 ± 0.014 ab
		Ethanol	0.2	0.429 ± 0.018 cdefg	1.220 ± 0.018 def	0.238 ± 0.033 cde
			0.4	0.490 ± 0.022 fg	1.305 ± 0.043 ef	0.341 ± 0.018 fghi
			0.8	0.390 ± 0.031 bcde	1.162 ± 0.154 cdef	0.497 ± 0.031 kl
		Methanol	0.2	0.364 ± 0.023 abcd	1.154 ± 0.068 cdef	0.389 ± 0.001 hij
			0.4	0.400 ± 0.030 cdef	1.130 ± 0.084 cdef	0.607 ± 0.036 m
			0.8	0.359 ± 0.016 abc	1.301 ± 0.020 ef	0.778 ± 0.019 n
		Hexane	0.2	0.441 ± 0.001 cdefg	1.153 ± 0.044 cdef	0.215 ± 0.009 bcd
			0.4	0.358 ± 0.011 abc	0.989 ± 0.041 abcd	0.204 ± 0.004 bcd
			0.8	0.468 ± 0.033 efg	1.098 ± 0.057 bcdef	0.774 ± 0.019 n
	T. catappa fruits	Water	0.2	0.365 ± 0.035 abcd	1.075 ± 0.006 bcde	0.227 ± 0.013 bcde
			0.4	0.419 ± 0.002 cdefg	1.323 ± 0.001 ef	0.433 ± 0.007 jk
			0.8	0.588 ± 0.002 hi	1.328 ± 0.019 ef	0.614 ± 0.014 m
		Ethanol	0.2	0.380 ± 0.004 abcde	0.997 ± 0.001 bcd	0.233 ± 0.022 cde
			0.4	0.407 ± 0.018 cdef	1.109 ± 0.000 bcdef	0.241 ± 0.004 cde
			0.8	0.287 ± 0.013 ab	0.735 ± 0.018 a	0.093 ± 0.009 a
		Methanol	0.2	0.591 ± 0.001 hi	1.270 ± 0.011 ef	0.404 ± 0.00 1ij
			0.4	0.612 ± 0.001 i	1.327 ± 0.001 ef	0.576 ± 0.008 lm
			0.8	0.728 ± 0.002 j	1.179 ± 0.001 def	0.977 ± 0.012 o
		Hexane	0.2	0.416 ± 0.002 cdef	1.213 ± 0.001 def	0.311 ± 0.008 efgh
			0.4	0.392 ± 0.003 bcde	1.297 ± 0.001 ef	0.279 ± 0.013 defg
			0.8	0.457 ± 0.001 defg	1.236 ± 0.001 def	0.252 ± 0.001 cdef

[Pillai's trace = 1.484, $F_{(18, 144)} = 7.834$, $p = 0.000$] for all three brain biochemical parameters taken together.

Discussion

Neurological disorders account for 3% of the total global burden of disease (Vrellingiri, 2024). Available synthetic drugs for the treatment of neurodegenerative ailments are mainly intended to alleviate the symptoms of the diseases. In recent times, significant research has focused on the use of natural plant extracts containing antioxidant secondary metabolites as a potential option for mitigating neurological disorders (Vrellingiri 2024). In this context, the present study aspired to ascertain the therapeutic potential of *T. catappa* and *T. bellirica* fruits in an Al-induced neurotoxic zebrafish model. Also, the study intended to determine the best solvent-concentration combination for each plant material to induce neuroprotection.

The aluminium-induced neurotoxic zebrafish model is a cost-effective and useful tool for pharmacological screening (Nadiga et al. 2024). The concentration of aluminium in natural waters can vary widely depending on various physicochemical and mineralogical factors (Driscoll et al. 2020). Aluminium concentration in aquatic environments is reported to be highly variable, ranging from 0.001 – 0.05 mg L^{-1} at near-neutral pH levels to as high as 50 mg L^{-1} in polluted acidic water (Capriello et al., 2021). The average concentration of aluminium in the Hooghly River was even recorded to reach 78 mg L^{-1} (Mitra et al. 2018). Exposure of adult zebrafish to 11 mg L^{-1} of Al was observed to induce behavioural disorders (Capriello et al. 2021). Consequently, the concentration administered in the present study to induce Al-induced neurotoxicity in zebrafish (9.97 mL^{-1}) was within both the environmentally and experimentally relevant range for the metal as reported in several earlier investigations (Capriello et al. 2021; Closset et al. 2021).

Zero mortality in zebrafish during the toxicity test with the plant extracts indicated that dietary treatment with *T. bellirica* and *T. catappa* fruit extracts had no adverse effects on general fish health. In the subsequent feeding experiment, standard biomarkers such as brain AChE activity, MAO activity, and GFAP level were used to assess the neuroprotective potential of *T. bellirica* and *T. catappa* fruit extracts. AChE regulates synaptic transmission at cholinergic synapses by modulating acetylcholine activity, while MAO is essential for the metabolism of catecholaminergic neurotransmitters (Aksoz et al. 2020). GFAP, a principal cytoskeletal component of glial cells, serves as a key biomarker for astrogliosis, with its expression reported to be altered in the zebrafish brain following exposure to metals (Monaco et al. 2016). Aluminium interacts with the cholinergic system and increases AChE activity in the brain. The metal may also elevate MAO activity by interfering with dopaminergic metabolism, thus affecting neurocognitive functions (Senger et al. 2011; Hegazi, Elebshany 2019). Elevated GFAP levels were observed to correlate positively with increased aluminium exposure (Tykhomyrov et al. 2016). The significant increase in AChE activity, MAO activity and GFAP levels in positive control fish compared to the negative control, as observed in the present study (Table 2), is consistent with previous observations, which showed aluminium exposure to be associated with neurotoxic effects (Prakash et al. 2013; Bais et al. 2018; Liu et al. 2020). On the other hand, inhibition of AChE activity is recognized as a therapeutic strategy for managing neurological disorders such as Alzheimer's disease (Jiang et al. 2017). Methanolic extract of *T. bellirica* fruits was erstwhile found to improve cognitive function in mice by inhibiting AChE activity (Reddy et al. 2020). MAO inhibitors are also considered a class of clinical antidepressant drugs used extensively to manage neuropathologic conditions (Banerjee et al. 2024).

Several neuroprotective drugs have been noted to decrease GFAP levels in the brain (Pablo et al., 2018; Zhang et al., 2024). Hence, a reduction in the level of all three biomarkers in the brains of Al-exposed fish fed diets fortified with different solvent extracts of either *T. bellirica* or *T. catappa* fruits compared to the positive control group indicated the neuroprotective efficiency of both plant materials (Table 2). The present results conform with earlier studies showing the antioxidant and neuroprotective properties of different plants belonging to the genus *Terminalia* (Chang, Lin 2012; Joshi, Malviya 2017).

Crude plant extracts are known to potentiate endogenous antioxidant responses and modulate neuroinflammatory pathways, thereby conferring neuroprotection (Moura et al. 2023). Biological activity detected in plant extracts is mostly caused by secondary metabolites, which occur in plants at a higher level of structural diversity and number. Bioactive compounds such as phenolics, flavonoids, alkaloids, and terpenoids are commonly implicated in the neuroprotective functions of plant extracts (Reddy et al. 2020). The therapeutic efficacy of such extracts is influenced by additive and synergistic actions of these phytochemicals present in the crude extract (Chaachouay et al. 2025). The decreased AChE and MAO activity in plant extract-fed fish may be due to the altered affinity for free-SH groups and consequential inhibition of their hyperactivity by the bioactive phytoconstituents in the extracts (Liu et al. 2013). Plant secondary metabolites such as polyphenols may inhibit AChE binding to its receptors and reduce activity of the enzyme to restore cholinergic levels and promote neuroprotection (Wang et al. 2022). Polyphenols are associated with the regulation of the mitochondrial apoptosis system involved in neuroprotective functions (Naoi et al. 2017). Polyphenols can also chelate Al ions, thereby reducing the availability of the metal that disrupts neuronal membranes (Wang et al. 2017). Similar to the present observations, phenolic and organic acids present in *T. chebula* have earlier been reported to alter neurotransmitter activity and reduce oxidative stress to promote neuroprotection (Kumar, Khanum 2012; Lin et al. 2022). Moreover, bioactive compounds such as gallic acid, eugenol and ferulic acid possess inhibitory effects on MAO-A and MAO-B activity (Chandrasekhar et al. 2017). These polyphenols may also show neuroprotective effects by modulating inflammatory responses through attenuation of astrocyte activation and GFAP gene expression (Dornelles et al. 2020). Restoration of GFAP expression by hydroalcoholic extract of *Moringa oleifera* in aluminium-induced neuroinflammation models (Ekong et al. 2017) further corroborates the neuroprotective relevance of plant-based interventions, as reflected in the current study.

The nature and amount of phytoconstituents may also differ with the extracting solvent, and such differences may affect the neuroprotective efficacy of the extract (Dhawan et al. 2017). The present study indicated that the neuroprotective efficacy of *T. bellirica* and *T. catappa* fruits depended on the extracting solvent. A polar solvent, such as water, excels at extracting polar compounds like tannins, flavonoids, and glycosides. In contrast, a non-polar solvent such as hexane is better suited for extracting non-polar compounds like some terpenoids and saponins (Jiang et al. 2016). High concentrations of flavonoids and phenolics in the aqueous extract of both plant materials corroborate with a better polarity match between the extracting solvent and the bioactive phytoconstituents in the extracts (Ali et al. 2022). Thus, the high concentrations of flavonoids and phenolics in the aqueous extract of *T. bellirica* and the ethanol extract of *T. catappa* fruits (Table 1) might be responsible for the highest efficacy of those two extracts for the respective plant species against neurotoxicity (Table 2).

The extraction yield often depends on the type of plant and the solvent used. Similar to the present observation, the aqueous extract of *Basella alba* leaves and *Tribulus terrestris*

seeds showed the highest yield percentages (Ghosal et al. 2015). The high yield from aqueous extracts for both plant materials may be attributed to the polarity of water, which facilitates the extraction of polar bioactive compounds (Nawaz et al. 2020).

The concentration of the plant extract is one of the key factors for the treatment success. Concentration comparison studies with plant extracts must be conducted for a true test of their relative efficacies (Moura et al. 2023). The present results also indicated the importance of the concentration of plant extracts for dietary administration on their neuroprotective efficiency. Similar to the present observation, a dose-dependent anti-convulsive effect of *Orthosiphon stamineus* ethanolic leaf extract was observed in pentylenetetrazol-challenged zebrafish (Choo et al. 2018). Dietary administration at a concentration of 0.8 g kg^{-1} feed for both *T. bellirica* fruit aqueous and *T. catappa* fruit ethanolic extracts exhibited the most pronounced neuroprotective outcomes in the zebrafish model (Table 2). However, further research with variable concentrations of the respective plant extract in a mammalian model will be required to standardize their subsequent clinical application.

Conclusions

The findings of this study suggest that the fruits of *Terminalia bellirica* and *Terminalia catappa* possess potential as natural therapeutic agents for alleviating neurotoxicity. Moreover, their neuroprotective efficacy appears to be influenced by both the extraction solvent used and the concentration administered. Polar solvents such as water and ethanol are likely to enhance the extraction of phenolic compounds and flavonoids, which are key antioxidant phytoconstituents contributing to the neuroprotective effects of the plant extracts. Notably, dietary supplementation with aqueous extract of *T. bellirica* fruit and ethanolic extract of *T. catappa* fruit at a concentration of 0.8 g kg^{-1} feed yielded the most pronounced neuroprotective outcomes, with *T. catappa* administration showing better therapeutic efficacy than *T. bellirica*. Further research in the mammalian model is necessary to standardize the concentration of *T. bellirica* fruit aqueous extract and *T. catappa* fruit ethanolic extract during their clinical application. In addition, isolation and characterization of the specific bioactive compounds, and elucidation of the molecular mechanisms underlying their neuroprotective actions are also warranted.

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