Toxicity evaluation and anti-ischemic stroke activity of selected natural extracts in zebrafish

NI MADE DWI MARA WIDYANI NAYAKA[1,2], ARI MUKTIAH[3], RIFAT ADRIANA IBRAHIM[3], I KETUT ADNYANA[1], KUSNANDAR ANGGADIREDJA[1], HAN WANG[4] and INDRA WIBOWO[3]

[1]Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, Institut Teknologi Bandung, Bandung 40132, Indonesia; [2]Department of Natural Medicine, Faculty of Pharmacy, Universitas Mahasaraswati Denpasar, Denpasar 80236, Indonesia; [3]Physiology, Animal Development and Biomedical Science Research Group, School of Life Sciences and Technology, Institut Teknologi Bandung, Bandung 40132, Indonesia; [4]Center for Circadian Clocks, Soochow University, Suzhou, Jiangsu 215123, P.R. China

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Abstract. Natural products are used in the discovery of novel therapeutic agents. Ischemic stroke is a neurological disorder caused by the obstruction of cerebral blood vessels, sometimes leading to paralysis and potentially death. Despite the complexity of this condition, therapeutic options are limited and typically associated with severe side effects, including intracranial hemorrhage. The present study aimed to explore the toxicity and anti-ischemic stroke activity of aqueous extracts from the aerial parts of gotu kola (*Centella asiatica*; CA), moringa leaves (*Moringa oleifera*; MO), turmeric rhizomes (*Curcuma longa*; CL), black pepper seeds (*Piper nigrum*; PN), and snakehead fish fillets (*Channa striata*; CS) in zebrafish. Toxicity tests were conducted in zebrafish embryos for 96 h. Ischemic stroke was induced in zebrafish larvae incubated in ponatinib (Pon) solution. In total, three concentrations of each extract, namely ¼ of the 10% lethal concentration (LC_{10}), ½ LC_{10} and LC_{10}, were derived from toxicity testing and applied in anti-ischemic stroke assays. All extracts were considered non-toxic as their LC_{50} values were >100 μg/ml. At certain concentrations, the extracts decreased hatching (>625 μg/ml CA and CL, >250 μg/ml MO, and >125 μg/ml CS) and survival rates (>625 μg/ml CA, >250 μg/ml MO, >156.25 μg/ml, >125 μg/ml PN and CS) and resulted in morphological deformity. Moreover, CA, MO, CL and CS, especially at their highest concentrations, significantly decreased the area of cerebral thrombosis compared with the Pon group. CA, MO, PN and CS ameliorated locomotor deficits following ischemia, as evidenced by significant improvements in average speed and total distance traveled. Among all extracts, CS at 29 μg/ml showed the greatest potential for development as an ischemic stroke treatment, exhibiting the strongest effects in preventing blood vessel blockage and restoring locomotor function following ischemia.

Correspondence to: Dr Indra Wibowo, Physiology, Animal Development and Biomedical Science Research Group, School of Life Sciences and Technology, Institut Teknologi Bandung, Jl. Ganesha 10, Bandung 40132, Indonesia
E-mail: indra.wibowo@itb.ac.id

Mrs. Ni Made Dwi Mara Widyani Nayaka, Department of Pharmacology and Clinical Pharmacy, School of Pharmacy, Institut Teknologi Bandung, Jl. Ganesha 10, Bandung 40132, Indonesia
E-mail: 30723004@mahasiswa.itb.ac.id

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Introduction

Natural products have long been used in traditional medicine based on empirical knowledge (1). Researchers have made efforts to provide scientific evidence supporting the use of traditional medicine (2). Critical steps in drug discovery include toxicity and pharmacological studies (3). In preclinical study, these two assessments are typically performed in animal models (3). Toxicological evaluations and pharmacological assessments aim to establish safety profiles and drug candidate effectiveness, respectively (4).

Stroke is a major cause of morbidity and mortality worldwide, with ischemic stroke accounting for 62.4% of stroke cases globally in 2019 (5). The number of patients with stroke is predicted to increase, and the number of people with stroke risk factors, such as hypertension, obesity and diabetes mellitus, is also expected to grow (6). Pharmacological treatments for ischemic stroke are associated with severe side effects, including intracranial hemorrhage (7). Therefore, the development of novel therapies for ischemic stroke is needed.

Widely used natural sources known for their health-promoting properties include gotu kola (*Centella asiatica*; CA), moringa (*Moringa oleifera*; MO), turmeric (*Curcuma longa*; CL), black pepper (*Piper nigrum*; PN) and snakehead fish (*Channa striata*; CS) (8-12). Numerous studies have examined the therapeutic potential of these natural resources for ischemic stroke in rodent models (10,13-17). Ethanolic extracts from CA decrease infarct size and prevent neuronal damage and motor

dysfunction in rats with stroke (13). Studies on ischemic stroke in mouse models have shown that MO leaf ethanolic extract decreases cerebral infarct volume and oxidative stress (14,15). Curcumin, derived from CL, decreases reactive oxygen species (ROS) in the basilar artery wall, thereby delaying the onset of stroke (10). PN contains piperine, which shows neuroprotective effects in an ischemic stroke rat model (16). Moreover, CS has been reported to induce cerebral angiogenesis in a rat model of ischemic stroke (17).

Many studies have used zebrafish as a model to determine the toxicological and pharmacological effects of natural products (18,19). The zebrafish model offers several advantages, including low cost, ease of maintenance, genetic similarity to humans and the ability to observe living organisms, which can be used for screening new drugs. Finally, the present study aimed to explore the toxicity and pharmacological potency of aqueous extracts from the aerial parts of CA, MO leaves, CL rhizomes, PN seeds and CS fillets for ischemic stroke and to highlight the advantages of using zebrafish in drug discovery, particularly for studies using natural extracts.

Materials and methods

Materials. Ponatinib (Pon; lot no. #13771), aspirin (lot no. #323026) and edaravone (Eda; lot no. #35508) were purchased from MedChemExpress. O-dianisidine (lot no. #SHBP1768) was obtained from Sigma-Aldrich (Merck KGaA). Water extracts of the aerial parts of CA (lot no. #24ENLC02), MO leaves (lot no. #24DDLC01), CL rhizomes (lot no. #24DQLC03), PN seeds (lot no. #24DTLC01) and CS fillets (lot no. #24BWLC01) were purchased from PT. Sari Alam Sukabumi, which complies with good manufacturing processes for producing extracts. Stock solutions of the drugs were dissolved in either 100% dimethyl sulfoxide (DMSO) or 0.9% sodium chloride. Stock solutions of extracts were prepared in either deionized water or 1% DMSO. All stock solutions were diluted with E3 medium (Sigma-Aldrich; Merck KGaA) before each experiment.

Chemical profile analysis. High-resolution mass spectrometry (MS) analysis was performed using a Waters ACQUITY UPLC® H-Class system combined with an Xevo G2-S QT of MS (Waters Corporation). The analysis used a C18 column (1.8 μm, 2.1x100 mm; ACQUITY UPLC® HSS, Waters Corporation) maintained at 50˚C, while the room temperature was maintained at 25˚C. Liquid chromatography was performed using a step gradient with a mobile phase consisting of water containing 5 mM ammonium formate (A) and acetonitrile containing 0.05% formic acid (B), at a flow rate of 0.2 ml/min over a 23-min run time. The injection volume was 5 μl and samples were prefiltered using a 0.2-μm syringe filter. Electrospray ionization in the positive mode was used for MS, scanning a mass range of 50-1,200 m/z. The source temperature was 100˚C and the desolvation temperature was maintained at 325˚C. The cone gas flow rate was 0 l/h, while the desolvation gas flow rate was 794 l/h. A collision energy gradient of 4-60 eV was applied. Data acquisition, analysis and instrument control were performed using MassLynx software v4.1 (20).

Zebrafish care and maintenance. Adult wild-type zebrafish (54 male and 36 female; age, 4-5 months, 0.4-0.6 g) were purchased from a local breeder from Bogor, Indonesia. The zebrafish species (*Danio rerio*) was confirmed at the Museum Zoology, School of Life Sciences and Technology, Institut Teknologi Bandung, Bandung, Indonesia (specimen no. 3939/IT1.C11.2/TU/2024). Before the experiment, adult zebrafish were acclimatized for ≥14 days. The zebrafish were maintained under standard laboratory conditions, including a temperature of 26-28˚C, 14/10-h light/dark cycle, continuous water filtration and aeration, pH maintained at 6.9±0.2 and conductivity within a standard range (21). They were fed three times daily with commercial pellets. Any dead animals were removed immediately to maintain water quality. The fish were handled carefully to minimize stress. Animal health and behavior were monitored at least twice daily. Observations included swimming behavior (hypoactivity and erratic movement), feeding response, morphological abnormality and signs of distress (surface gasping and loss of righting reflex). In total, nine adult zebrafish that exhibited severe distress or abnormal behavior were promptly separated and closely monitored throughout the experimental period.

Zebrafish eggs were obtained from breeding adult fish in a 6:4 male: female ratio. The fertilized eggs were collected and transferred to E3 medium. For thrombosis analysis, 10 h after embryo collection, 3% propylthiouracil (PTU; v/v) was added to inhibit melanin formation in the zebrafish embryos. The experimental procedures were conducted over 4 days for the toxicity study and 5 days for the anti-ischemic stroke study, including breeding, treatment exposure and endpoint assessment. At the end of the experiments, euthanasia of zebrafish larvae was performed using 0.4% tricaine solution. Death was confirmed based on established criteria for zebrafish, including absence of opercular (gill) movement, lack of heartbeat (observed under stereomicroscope) and no response to gentle tactile stimulation (22). To ensure accuracy, observations were conducted for ≥5 min before confirming mortality. All procedures were approved by the Animal Research Ethics Committee at the Institut Teknologi Bandung (Bandung, Indonesia; approval nos. KEP/I/2024/II/H211223ND/TAAZ for the toxicity test and KEP/I/2024/VI/H110624NM/ANSZ for the anti-ischemic stroke test).

Toxicity test. The fish embryo acute toxicity test, based on the Organization for Economic Co-operation and Development protocol no. 236, was conducted to evaluate toxicity (23). E3 medium and 4 μg/ml 3,4-dichloroaniline solution were used as negative and positive controls, respectively. Toxicity testing was performed on zebrafish embryos <6 h post-fertilization (hpf). The embryos were placed in a 24-well plate, with one embryo/well. Subsequently, the embryos were incubated with CA (156.28-5,000.00 μg/ml), MO (31.25-1,000.00 μg/ml), CL (156.28-5,000.00 μg/ml), PN (31.25-1,000.00 μg/ml) and CS (31.25-1,000.00 μg/ml) for 24-96 h at room temperature. Observations of embryonic abnormality, including embryo coagulation, imperfect somite formation and tail bud release and absence of heartbeat, were carried out every 24 h (24). The 10% (LC_{10}) and 50% lethal concentration (LC_{50}) were calculated by probit analysis using a Microsoft Excel 2021-based template (25). The experiments were performed in triplicate.

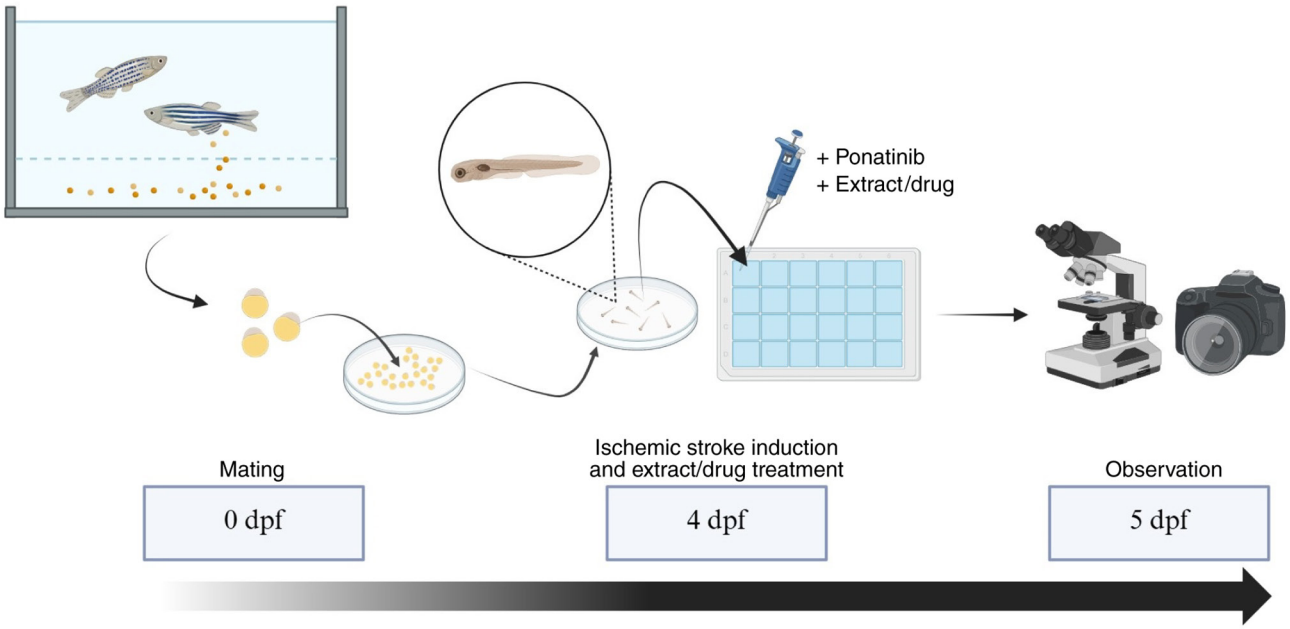

Figure 1. Schematic of the experimental design for anti-ischemic stroke evaluation using a zebrafish model induced by ponatinib. Created with BioRender.com. dpf, days post-fertilization.

Ischemic stroke induction in zebrafish larvae. Fig. 1 presents a schematic diagram of the experimental design. The method for producing ischemic stroke in zebrafish larvae was adapted from a previous study (19). Zebrafish larvae at 4 days post fertilization (dpf) were placed into 24-well plates, with each well containing 5 larvae. Larvae were incubated for 24 h (room temperature) in a mixture of 3 μg/ml Pon solution and sample solutions (¼ LC_{10}, ½ LC_{10} and LC_{10} of each extract) or reference drug solutions (12.5 μg/ml Asp and Eda). Larvae incubated in E3 or E3 + PTU were used as the control group.

Analysis of the area of thrombosis. Following ischemic stroke induction, the larvae were euthanized with 0.4% tricaine solution, and whole larvae were stained in room temperature with 5.85 mmol/l o-dianisidine solution, sodium acetate buffer (0.1 M, pH 4.5), deionized water and 30% (v/v) hydrogen peroxide (20:5:20:1) for 15 min in the dark. Larvae were washed three times with 100% DMSO and mounted on glass slides with low-melting point agarose. The zebrafish larvae were observed under a light microscope. The area of cerebral thrombosis was characterized by a dark brown color and was quantified using ImageJ software (version 1.54g) (26).

Analysis of locomotion. Following induction, zebrafish larvae were placed in a Petri dish containing 3 ml incubation medium and recorded individually. Zebrafish larvae were subjected to light/dark stimulation, comprising a 1 min light period followed by a 1 min dark period. Larval locomotion was recorded for 3 min using a camera. If the larvae did not exhibit movement at the beginning of the recording period, tactile stimulation by touching the head or tail was performed to trigger movement (27). The video recordings were analyzed with AnimalTA software v3.2.2 (28). Locomotor parameters used to assess the anti-ischemic stroke effect included total distance traveled, average swimming speed and swimming path (29).

Statistical analysis. Data were analyzed with GraphPad Prism 8.0 (Dotmatics) and are presented as the mean ± SEM of ≥3 independent experimental repeats. Data were analyzed by one-way ANOVA or Kruskal-Wallis test followed by Dunnett's test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Chemical compound profiling. Fig. 2 shows the chromatogram and predicted metabolites of CA, MO, CL, PN and CS. CA comprised various metabolites classified into alkaloid, phenolic and terpenoid groups. MO contained chemical components belonging to the phenolic, flavonoid and glycoside classes. Additionally, phenolic and terpenoid groups were detected in CL. PN exhibited compounds classified into alkaloid and phenolic classes. Amino acids, fatty acids and peptides were detected in CS.

Effect of the extracts on larval survival. To evaluate the toxicity of the extracts on zebrafish embryonic development, survival rate was measured 24-96 hpf following exposure. No surviving embryos were found at 72 hpf following exposure to 5,000 μg/ml CA or CL, 2,500 μg/ml CL or 1,000 μg/ml CS (Fig. 3). PN at 1,000 μg/ml exhibited a strong lethal effect, with all embryos dying by 24 hpf. Additionally, more than half of the embryos remained viable at 96 hpf following exposure to ≤1,250 μg/ml CA, ≤500 μg/ml MO, ≤1,250 μg/ml CL and ≤250 μg/ml PN and CS. Compared with the control, embryos exposed to 156.25-625 μg/ml CA, 31.25-250 μg/ml MO, 156.25-625 μg/ml CL and 31.25-125 μg/ml PN and CS did not show a significant difference in survival rate. These results

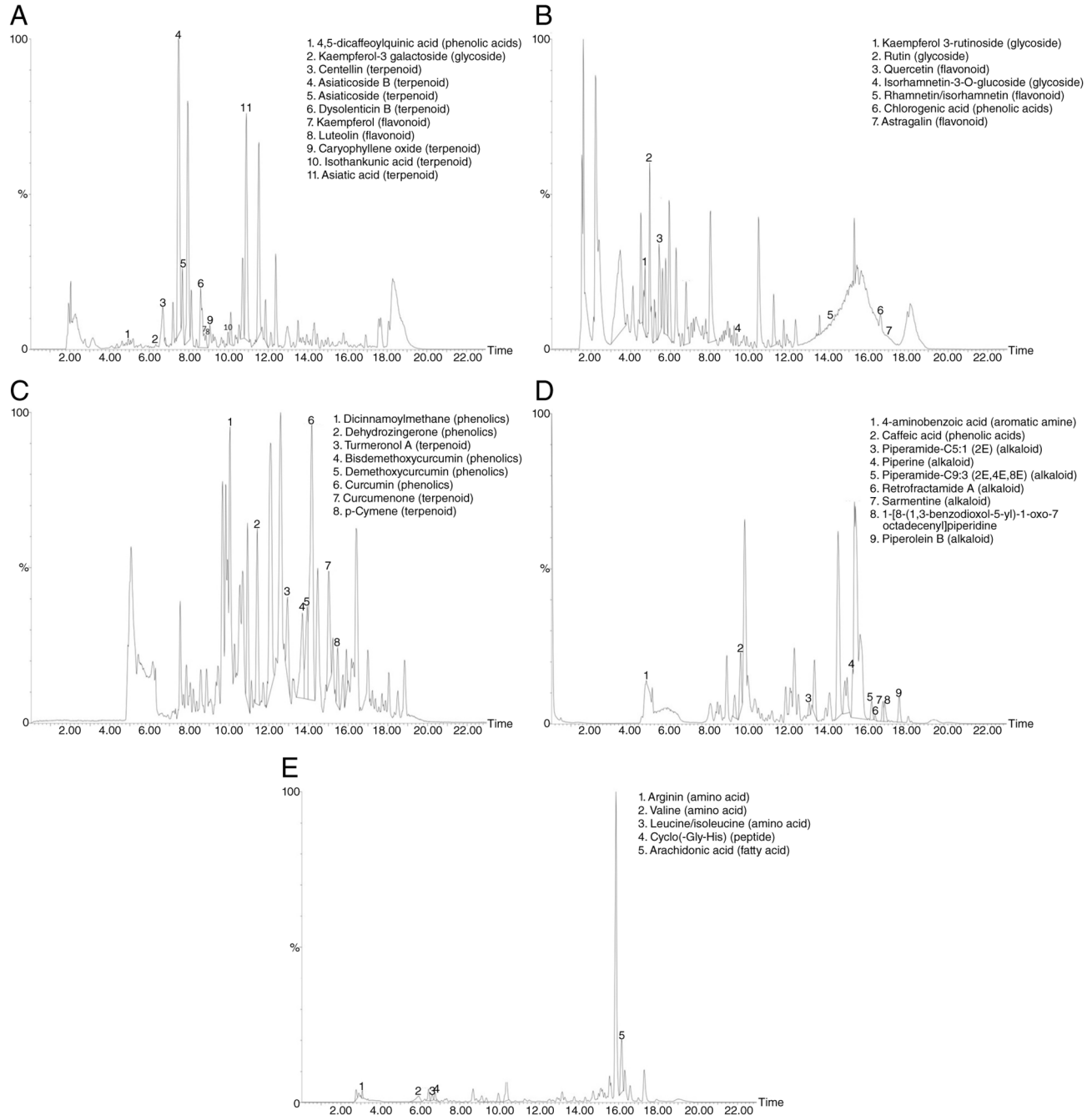

Figure 2. Chemical characterization of extracts. UPLC-Q-TOF-MS chromatogram and chemical composition of (A) *Centella asiatica*, (B) *Moringa oleifera*, (C) *Curcuma longa*, (D) *Piper nigrum* or (E) *Channa striata* extract.

indicate that lower extract concentrations were associated with higher embryo survival.

Effect of the extracts on embryo hatching. Hatching rate was determined by quantifying the number of embryos that emerged from the chorion between 24 and 96 hpf. Embryos showed no evidence of hatching at 24 h, with a significant increase observed at 48 h, particularly following exposure to lower concentrations of extracts (Fig. 4). Control embryos (incubated in E3) hatched normally at 48 hpf. Exposure to 5,000 μg/ml CA, 1,000 μg/ml MO, 1,250-5,000 μg/ml CL and 1,000 μg/ml PN and 1,000 μg/ml CS resulted in delayed hatching of all embryos. Conversely, more than half of the embryos completed hatching by 96 hpf when exposed to ≤625 μg/ml CA, ≤500 μg/ml MO, ≤312.5 μg/ml CL, ≤125 μg/ml PN and ≤250 μg/ml CS. Lower extract concentrations, such as 156.25-625 μg/ml CA, 31.25-250 μg/ml MO, 156.25 μg/ml CL and 31.25-125 μg/ml PN and CS, did not induce any effect on the hatching rate compared with the control group. These findings suggest that embryos exposed to lower extract concentrations exhibited greater hatching success.

Effect of the extracts on morphological abnormality. To assess the toxic effects of extracts, morphological alterations in embryonic development were observed every 24 h (Figs. S1-S5). After 24 h extract exposure, high

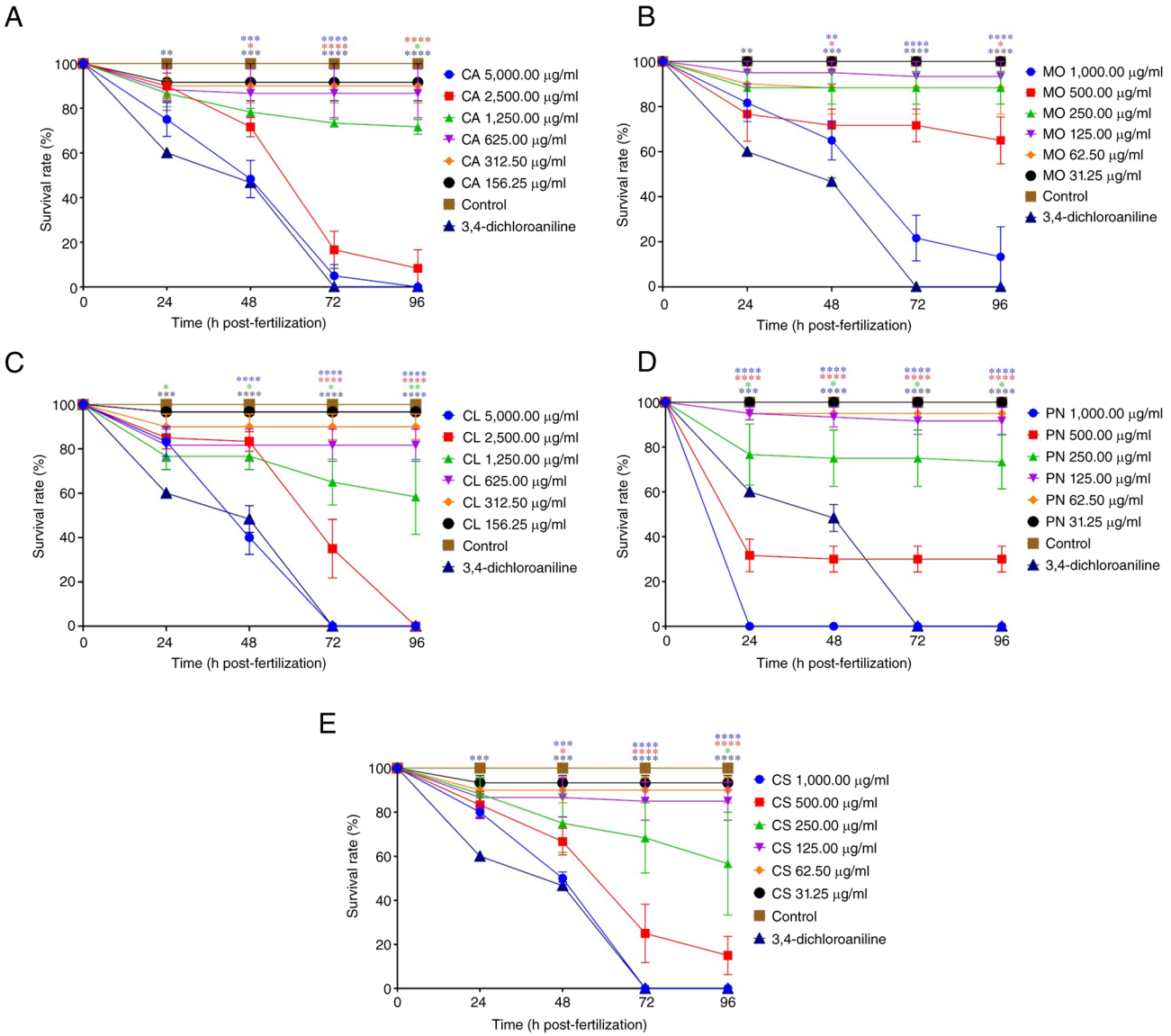

Figure 3. Effects of analyzed extracts on the survival rate of zebrafish embryos. Effect of (A) CA, (B) MO, (C) CL, (D) PN and (E) CS extracts on survival rate of zebrafish embryos. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs. control. CA, *Centella asiatica*; MO, *Moringa oleifera*; CL, *Curcuma longa*; PN, *Piper nigrum*; CS, *Channa striata*.

concentrations of the extracts (≥2,500 µg/ml for CA and CL, and 1,000 µg/ml for MO, PN and CS) caused delayed gastrulation, absence of somites and coagulation. At lower concentrations, exposure to certain extracts caused pericardial edema (72 and 96 hpf following exposure to 625 µg/ml CA and CL, respectively), yolk sac edema (96 hpf following exposure to 1,250 µg/ml CA or 500 µg/ml MO or CS) and spine malformation (48 and 72 hpf following exposure to 312.5 µg/ml CA and 125 µg/ml PN, respectively). Additionally, delayed embryonic development was observed, including the lack of eye bud development (24 hpf after exposure to 1,250 µg/ml CA, 500 µg/ml MO and 125 µg/ml CS and 72 hpf after exposure to 2,500 µg/ml CA) and reduced body size (96 hpf after exposure to 2,500 µg/ml CA, 500 µg/ml MO and 625 µg/ml CL). By contrast, embryos exposed to lower extract concentrations (156.25 µg/ml CA, 31.25 µg/ml MO, 156.25-312.5 µg/ml CL and 31.25-62.5 µg/ml PN and CS) exhibited normal developmental progression.

Lethal concentration of the extracts on zebrafish embryos. To evaluate the mortality effects of the extracts on embryos, lethal concentrations were calculated using probit analysis (Fig. 5; Table I). The mortality effect order was as follows: CS > PN > MO > CL > CA. Based on the Globally Harmonized System of Classification and Labeling of Chemicals, all extracts were classified as non-toxic based on their LC_{50} values (LC_{50}>100 µg/ml) (30). Therefore, these extracts were used at concentrations corresponding to ¼ LC_{10}, ½ LC_{10}, and LC_{10} for subsequent experiments in the zebrafish ischemic stroke model (Table I).

Effect of the extracts in decreasing the area of thrombosis. The anti-ischemic stroke effects of the extracts were evaluated by measuring cerebral thrombosis. Following 24 h incubation with Pon, the zebrafish larvae exhibited an increased area of cerebral thrombosis compared with the control group (Fig. 6). By contrast, 114.50 and 229.00 µg/ml CA, 75.00 µg/ml MO,

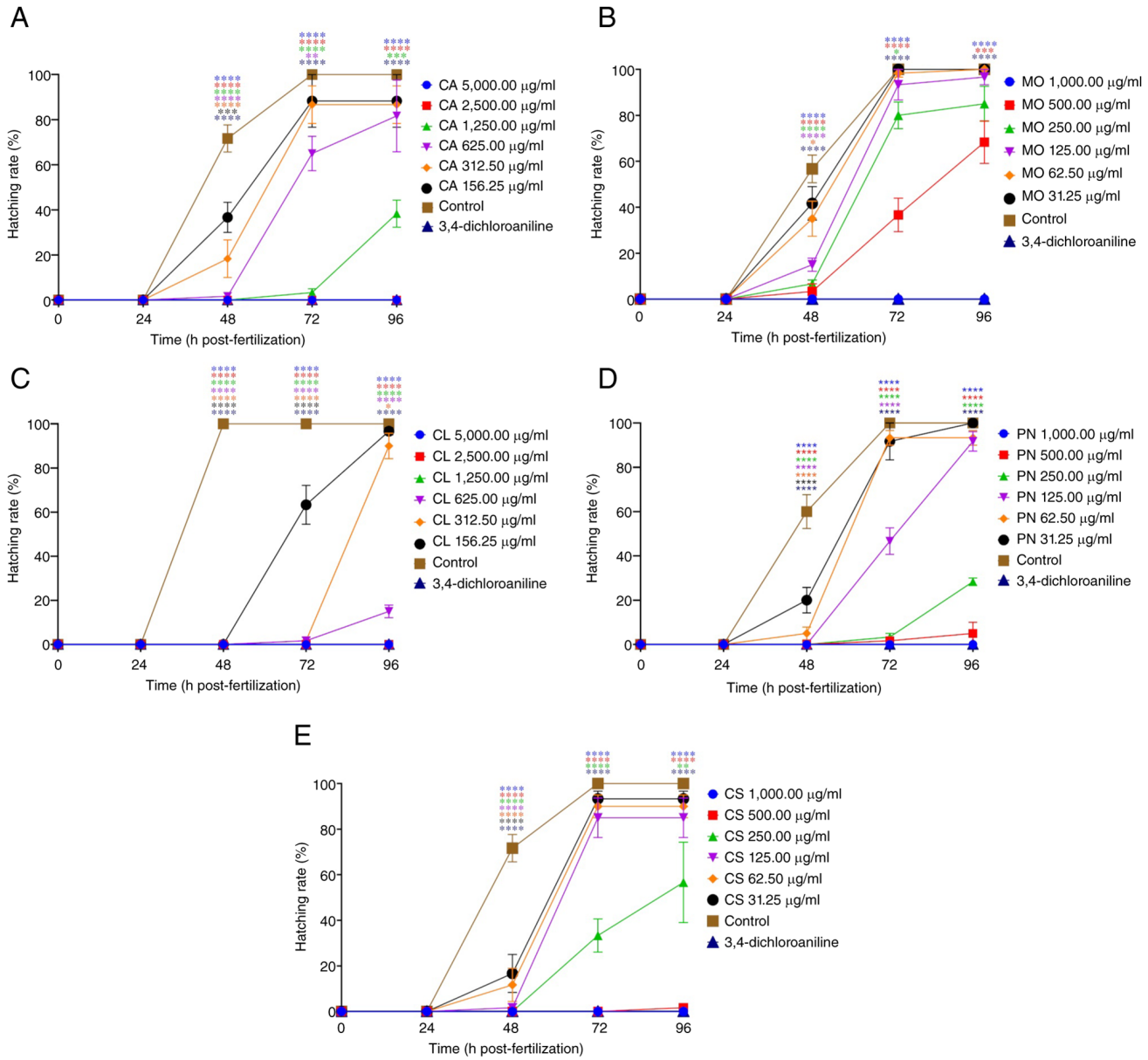

Figure 4. Effects of extracts on the hatching rate of zebrafish embryos. Embryos were incubated in (A) CA, (B) MO, (C) CL, (D) PN and (E) CS. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs. control. CA, *Centella asiatica*; MO, *Moringa oleifera*; CL, *Curcuma longa*; PN, *Piper nigrum*; CS, *Channa striata*.

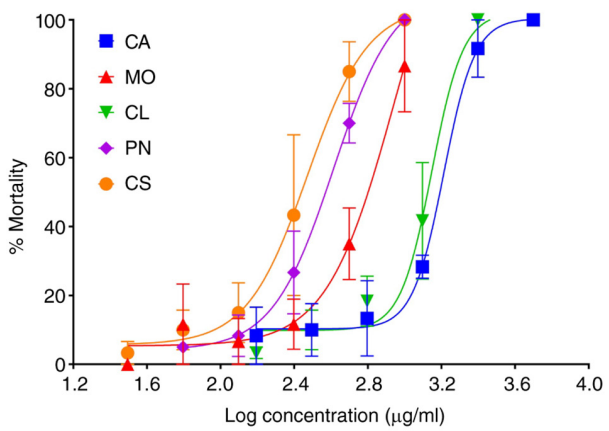

Figure 5. Concentration-response curve of CA, MO, CL, PN and CS extracts. CA, *Centella asiatica*; MO, *Moringa oleifera*; CL, *Curcuma longa*; PN, *Piper nigrum*; CS, *Channa striata*.

275.00 µg/ml CL and 14.50-58.00 µg/ml CS demonstrated significant anti-brain thrombosis activity. The effects of CA, MO and CL were dose-dependent, with lower extract concentrations resulting in larger cerebral thrombosis areas than higher concentrations. These three extracts decreased the thrombosis area by 1.85-2.51-fold compared with the Pon group. Conversely, none of the PN concentrations significantly decreased the area of cerebral thrombosis. CS showed a comparable anti-thrombosis effect between concentrations. Moreover, CS resulted in a 1.4-3.2-fold decrease in cerebral thrombosis area relative to the Pon group, with the most pronounced decrease at 29 µg/ml CS, suggesting potent anti-thrombotic activity.

Effect of the extracts on improving locomotor function. Locomotor function in zebrafish larvae following extract treatment was assessed based on the average swimming speed, total

Table I. LC of extracts.

Extract	¼ $LC_{10,\mu g/ml}$[a]	½ $LC_{10,\mu g/ml}$[a]	$LC_{10,\mu g/ml}$[a]	$LC_{50,\mu g/ml}$
CA	57.25	114.50	229.00	1285.00
MO	18.75	37.50	75.00	547.52
CL	68.75	137.50	275.00	1187.89
PN	26.25	52.50	105.00	374.08
CS	14.50	29.00	58.00	240.14

[a]Concentrations used for experiment in the zebrafish ischemic stroke model. CA, *Centella asiatica*; MO, *Moringa oleifera*; CL, *Curcuma longa*; PN, *Piper nigrum*; CS, *Channa striata;* LC, lethal concentration.

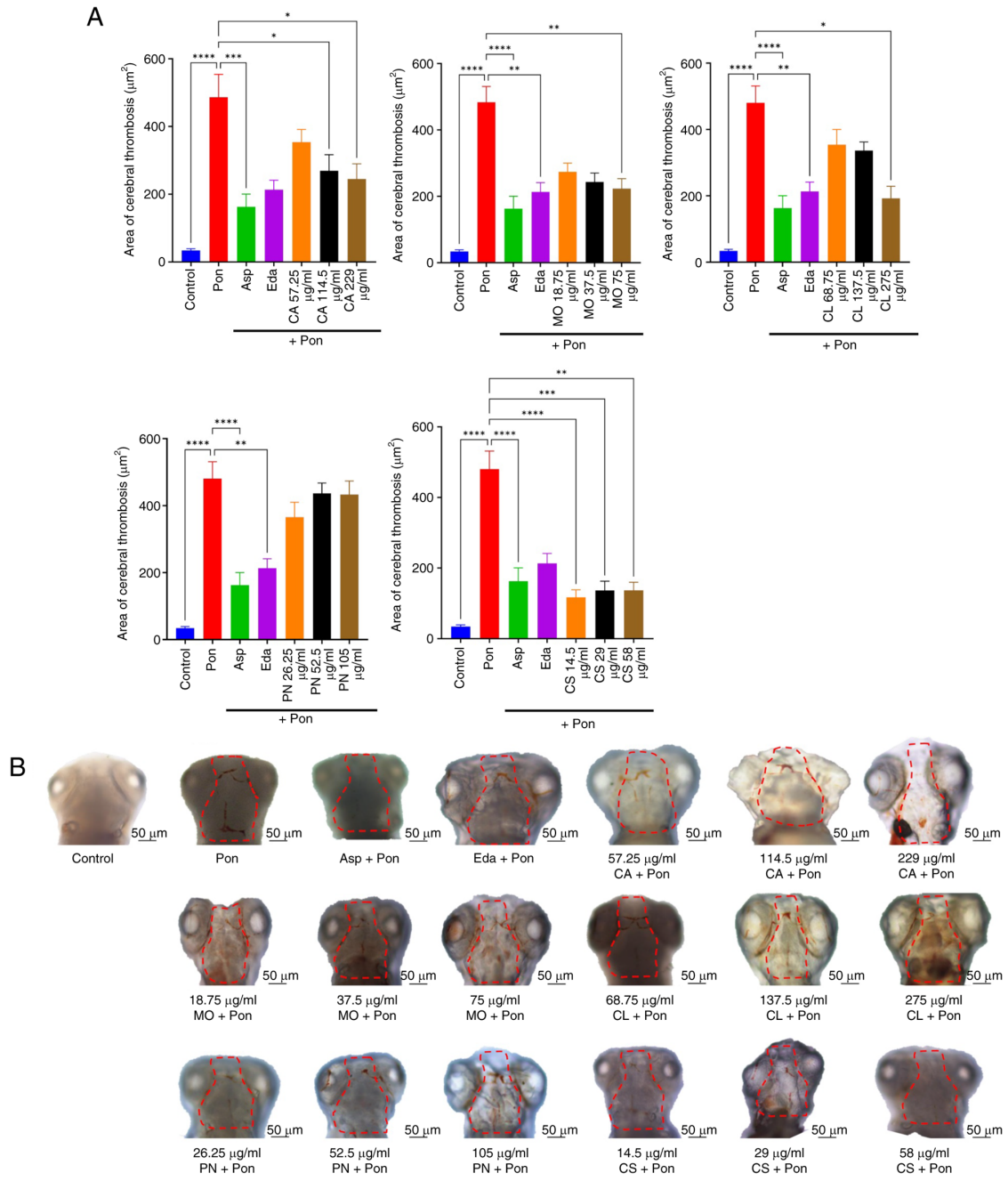

Figure 6. Effects of analyzed extracts on the area of cerebral thrombosis of zebrafish larvae. (A) Area of cerebral thrombosis and (B) representative images of the zebrafish larvae (5 days post-fertilization). Area inside the dashed lines indicates the observed area. Scale bar, 50 μm. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. CA, *Centella asiatica*; MO, *Moringa oleifera*; CL, *Curcuma longa*; PN, *Piper nigrum*; CS, *Channa striata;* Asp, 12.5 μg/ml aspirin; Eda, 12.5 μg/ml edaravone; Pon, 3 μg/ml ponatinib.

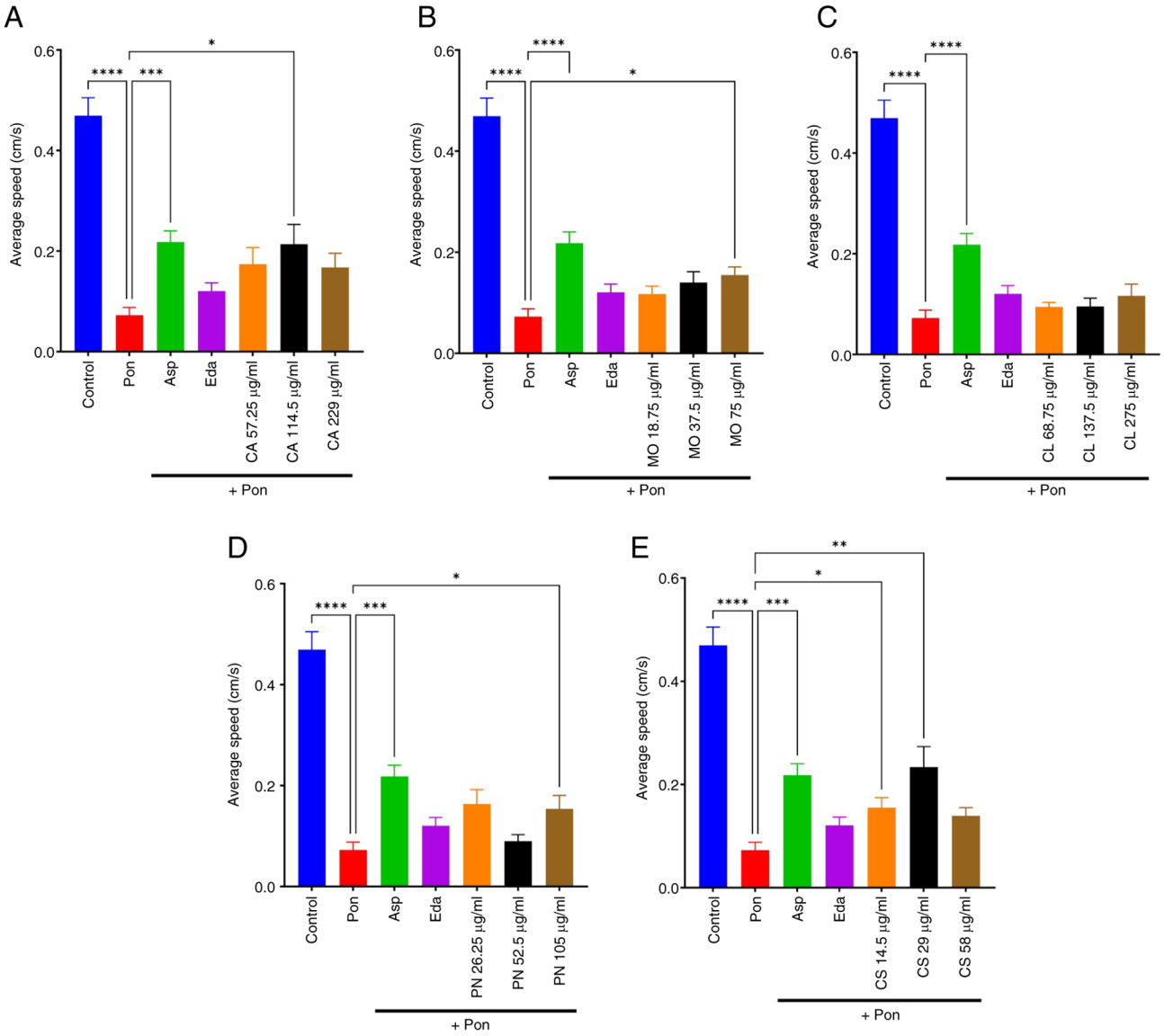

Figure 7. Effects of extracts on the total distance traveled of zebrafish larvae. Total distance traveled by zebrafish larvae (5 days post-fertilization) exposed to Pon and (A) CA, (B) MO, (C) CL, (D) PN and (E) CS extracts. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. CA, *Centella asiatica*; MO, *Moringa oleifera*; CL, *Curcuma longa*; PN, *Piper nigrum*; CS, *Channa striata;* Asp, 12.5 μg/ml aspirin; Eda, 12.5 μg/ml edaravone; Pon, 3 μg/ml ponatinib.

distance traveled and swimming trajectory. After Pon induction, notable locomotor impairment was observed compared with the control group in larvae treated with 114.5 μg/ml CA, 75 μg/ml MO, 26.25 and 105 μg/ml PN and 14.5 and 29 μg/ml CS (Figs. 7-9). CL had no effect on the restoration of locomotor damage in an ischemic environment. CA and CS at their highest concentrations demonstrated a reduced effect compared with lower concentrations. Conversely, a moderate PN concentration (52.5 μg/ml) showed a weaker effect than both low (26.25 μg/ml) and high (105 μg/ml) concentrations. These findings indicated that each extract exerts its effect at a specific optimal dose.

MO was the only extract that showed a dose-dependent effect. Furthermore, among all tested concentrations, CS at 29 μg/ml demonstrated the greatest effect, increasing the average speed and total distance traveled by ~3.22 and 2.97-fold, respectively, compared with the Pon group. This indicates its high potency in restoring locomotor damage following ischemia.

Discussion

In this study, we examined the toxicity and anti-ischemic stroke activity of CA, MO, CL, PN and CS in the zebrafish models. The chemical compounds of the extracts (Fig. 2) were also analyzed using UPLC-Q-TOF-MS, and they were reported to be consistent with other studies (31-36). Survival and hatching rates of zebrafish embryos exposed to CA, MO, CL, PN and CS were time- and concentration-dependent, with longer exposure times and higher concentrations leading to decreased embryo survival and hatchability. The extracts contained compounds that may induce embryonic death, such as asiatic acid and kaempferol in CA, rutin and quercetin in MO, curcumin in CL, piperine in PN and arginine in CS (37-41). Since zebrafish embryonic stages are sensitive to external stimuli, elevated levels of compounds present in higher extract concentrations may create a toxic environment, leading to disrupted organogenesis and embryo mortality (42-44).

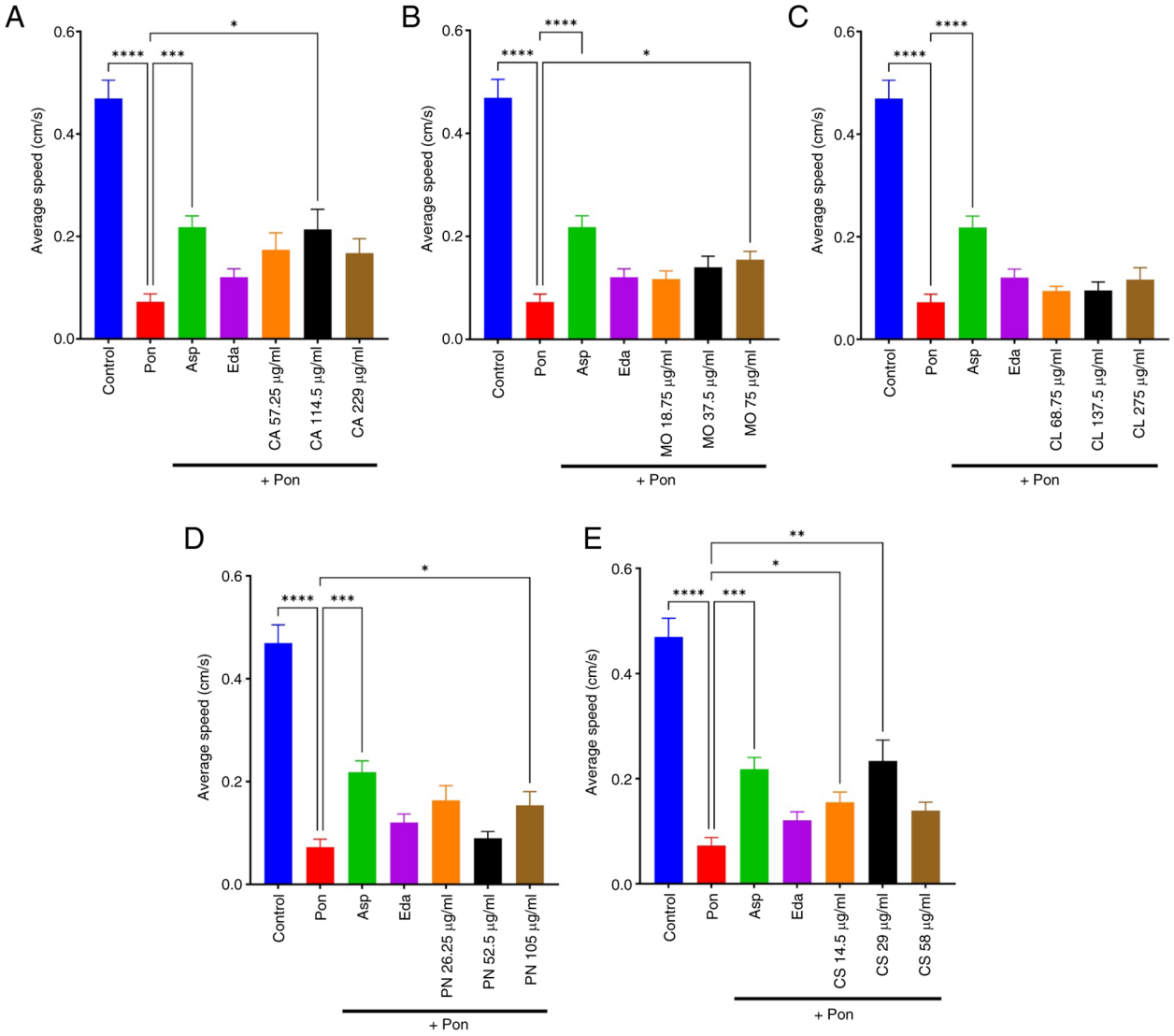

Figure 8. Effect of extracts on the average speed of zebrafish larvae. Average speed of zebrafish larvae (5 days post-fertilization) exposed to Pon and (A) CA, (B) MO, (C) CL, (D) PN and (E) CS extracts. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. CA, *Centella asiatica*; MO, *Moringa oleifera*; CL, *Curcuma longa*; PN, *Piper nigrum*; CS, *Channa striata;* Asp, 12.5 μg/ml aspirin; Eda, 12.5 μg/ml edaravone; Pon, 3 μg/ml ponatinib.

Extended exposure to high concentrations of the present extracts can impair or damage the chorion, decreasing its ability to protect the embryo, consequently leading to delayed hatching or embryo coagulation. The decreased hatching rate caused by exposure to CA, MO, CL and PN extracts has also been reported previously (40,45-47). Furthermore, bioactive compounds within the extracts may interact with components on the chorion surface, resulting in delayed hatching. For example, piperine in PN interacts with the hatching enzyme 1a (He1a), which serves a key role in regulating chorion hardening during the hatching process (40).

Moreover, the present extracts induced sublethal toxicity. In a normal embryo, the eye bud is clearly visible at 24 hpf and fully developed at 48 hpf (48). By contrast, lack of an eye bud formation was observed following exposure to low and high concentrations of CA, MO and CS at 24-72 hpf. Spinal malformations were observed at 72 and 96 hpf in hatched embryos exposed to MO, CL, PN and CS, whereas growth inhibition was detected at 96 hpf following exposure to CA, MO and CL. These results are likely attributable to the ability of extract constituents to interact with key cell proteins that regulate development and physiological metabolism, following their accumulation on the chorion surface and penetration into embryos via chorion pores (40). However, low extract concentrations (156.25 μg/ml CA, 31.25 μg/ml MO, 156.25-312.5 μg/ml CL and 31.25-62.5 μg/ml PN and CS) did not induce morphological deformities in zebrafish embryos.

In the present study, yolk sac edema was observed at 96 hpf after exposure to 1,250 μg/ml CA and 500 μg/ml MO and CS. In normal development, the yolk sac provides key nutrients to the developing embryo and decreases in size by 96 hpf (49). A swollen yolk indicates abnormal nutritional absorption (44). This may result from impaired osmoregulation and toxin accumulation in the yolk sac caused by the extracts (50). The abnormal organ development reported in the present study is consistent with other studies (40,41,46).

Table II shows the LC_{50} values of the analyzed extracts from published studies (40,45,51-58). Currently, research on the toxic effects of these extracts, particularly PN and CS, in zebrafish models remains limited. Previous studies on PN in

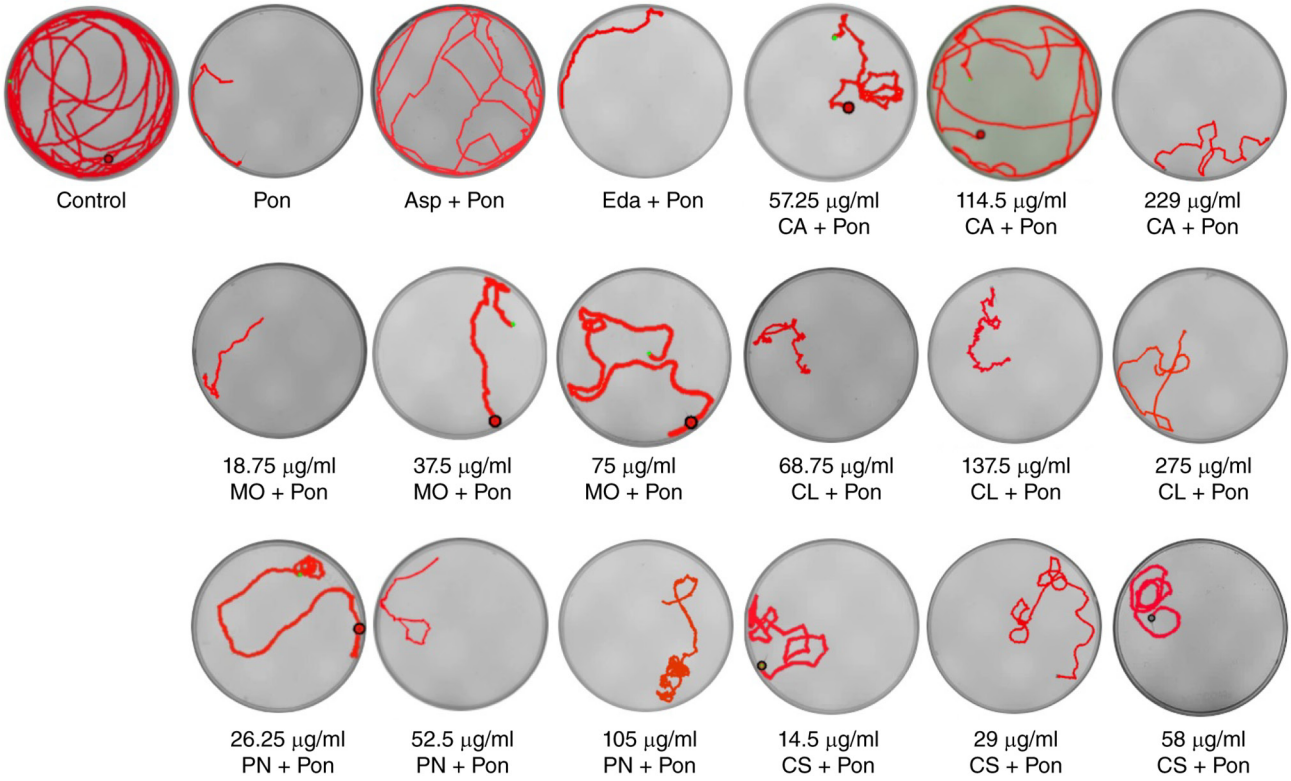

Figure 9. Representative swimming path of zebrafish larvae (5 days post-fertilization). CA, *Centella asiatica*; MO, *Moringa oleifera*; CL, *Curcuma longa*; PN, *Piper nigrum*; CS, *Channa striata;* Asp, 12.5 µg/ml aspirin; Eda, 12.5 µg/ml edaravone; Pon, 3 µg/ml ponatinib.

zebrafish used essential oils or piperine as test samples (59,60). Moreover, there are currently no published studies on the toxic effects of CS in the zebrafish models. The majority of toxicity studies on CA, PN, CL, PN, and CS using varied solvents have been conducted in rodent models, and all studies have reported the extracts to be considerably safe (9,61-64).

Previous studies (Table II) have demonstrated toxic effects comparable to those in the present study (LC_{50}>100 µg/ml). Conversely, several studies have reported conflicting results (40,45,52,53,55,57,58), potentially due to differences in extraction methods and toxicity testing protocols, limiting the relevance of direct comparisons with other studies. Unlike most published studies that have employed ethanolic extracts (51,54,55,57,58,65), the present study used water as the extraction solvent. Water extracts typically exhibit lower toxicity and greater compatibility with *in vivo* models (57,66,67). They are also environmentally friendly and inexpensive, which is suitable for large-scale production (68). Extraction with organic solvents results in higher toxic effects than those of water extracts (57). Patel *et al* (40) adopted a modified fish embryo acute toxicity test based, which prolongs the exposure time up to 120 h. This longer duration of extract exposure produces a lower LC_{50}, indicating a higher toxic effect (65).

Ischemic stroke-induced brain injury results from a decrease in cerebral blood flow, typically due to the presence of a thrombus or embolus within the cerebral vasculature (7). In the present study, Pon was used to create an ischemic stroke model in zebrafish larvae. This chemical compound has been widely used in other studies to mimic ischemic stroke in zebrafish larvae (21,29,69). The method is easy to perform and time-saving; thus, it is used in drug screening (69). Pon administration in the present study was performed on zebrafish larvae at 4 dpf, when blood vessels and the swim bladder have developed, supporting its use in anti-thrombotic and locomotion tests (19,27).

In the present study, the anti-thrombosis effect of the extracts was demonstrated by a decrease in the cerebral thrombosis area. This effect may be useful for anti-thrombotic treatment. Thrombolytic therapy is key in treating acute ischemic stroke as it may restore blood flow and decrease the infarct area and brain damage. Among the extracts, CS showed the strongest anti-thrombotic effect. Nasution *et al* (17) reported that CS extract induces angiogenesis in a rat model of ischemic stroke by enhancing the expression levels of vascular endothelial growth factor, nitric oxide (NO) and vascular endothelial growth factor receptor 2. Moreover, cyclo(-Gly-His), a peptide in CS, exhibits anti-thrombotic effects by reducing thrombin activity, decreasing fibrin formation and inhibiting platelet aggregation (70).

CA, MO and CL also significantly decreased the cerebral thrombosis area. Asiaticoside in CA decreases the levels of endothelin-1, intercellular adhesion molecule 1, vascular cell adhesion molecule 1 and E-selectin and increases NO and cyclic guanosine monophosphate production under hypoxic conditions (71). This supports blood vessel vasodilation and hinders platelet adhesion and aggregation, inhibiting thrombus formation (71). MO contains numerous bioactive compounds, such as quercetin, kaempferol and rutin. Some studies have shown that quercetin and kaempferol suppress thrombin activity and tissue factor activity and prevent clot production by fibrin, as well as platelet adhesion and aggregation (72,73). A study

Table II. LC_{50} values of extracts from previous studies.

Solvent	Toxicity study protocol	$LC_{50,\mu g/ml}$	(Refs.)
A, *Centella asiatica* leaves			
70% ethanol	OECD Test No. 203	1,250.00	(51)
96% ethanol	OECD Test No. 236	808.81	(52)
Ethyl acetate	OECD Test No. 236	26.61	(52)
Methanol	OECD Test No. 236	39.56	(53)
B, *Moringa oleifera* leaves			
70% ethanol	OECD Test No. 203	1,231.00	(54)
80% ethanol	OECD Test No. 236	30.04	(55)
80% methanol	OECD Test No. 236	163.87	(55)
80% ethanol	Modified OECD Test No. 236	445.10	(65)
C, *Curcuma longa* rhizomes			
80% methanol	OECD Test No. 236	56.68	(45)
Hexane	Modified OECD Test No. 236	5.00	(57)
Ethyl acetate	Modified OECD Test No. 236	12.00	(57)
Ethanol	Modified OECD Test No. 236	14.00	(57)
Methanol	Modified OECD Test No. 236	7.00	(57)
Water	Modified OECD Test No. 236	>1,000.00	(57)
Ethanol	Modified OECD Test No. 236	72.00	(58)
D, *Piper nigrum* seeds			
Water	Modified OECD Test No. 236	35.60	(40)

OECD, Organization for Economic Co-operation and Development; LC, lethal concentration.

in a rat model demonstrated that rutin has anti-thrombotic effects via delayed platelet aggregation time (74). Additionally, the curcumin in CL inhibits platelet adhesion in the cerebral vascular endothelium through endothelial regulation (75).

In humans, ischemic stroke leads to oxidative stress and neuronal inflammation, causing neuronal damage in the brain. Paresis is one of its primary clinical manifestations (76). In the zebrafish model, the impaired swimming ability of the larvae represents the motor disorder caused by the ischemic insult. The present study showed that all extracts except CL improved locomotor function in Pon-induced larvae. Despite the anti-thrombotic activity of CL (275 μg/ml), it failed to inhibit locomotor damage. Conversely, studies on CL bioactive compounds, such as curcumin and hexahydrocurcumin, via intraperitoneal injection in a rat model have showed restoration of the motor impairment caused by brain ischemia (77-80). This discrepancy suggests that the route of drug administration serves a key role, as the neuroactive compounds in CL administered via bath immersion in the present study may have low bioavailability, decreasing their pharmacological efficacy. Ciubotaru *et al* (70) reported that, following immersion exposure, the concentration of curcumin in the zebrafish brain is lower than that of its derivative, mitocurcumin.

Additionally, the locomotor improvement following CA, MO, PN and CS treatments may be associated with their neuroprotective components. Asiaticoside in CA decreases apoptosis and improves neurological function in transient middle cerebral occlusion in rats (81). Quercetin, rutin and kaempferol, present in MO, decrease ROS production and the levels of cytokines and proinflammatory mediators, protecting against neurodegeneration and inhibiting neuronal cell death (82-84). Moreover, flavonoids and polyphenols in ME decrease oxidative stress in the brain due to ischemia (85). Piperin, a bioactive compound in PN, improves neurological function, postural reflex and balance in rats following cerebral ischemia (16). Valine, leucine and isoleucine in CS increase mitochondrial biogenesis and stimulate the activity of antioxidant enzymes, including superoxide dismutase and glutathione peroxidase, thereby protecting neurons from ischemia-induced damage (86). Alleviation of oxidative stress promotes improved muscle physiology, leading to enhanced swimming ability in zebrafish larvae (87).

The severity of motoric impairment is associated with the extent of the cerebral thrombosis area (88). This supports the hypothesis that anti-thrombotic agents may decrease motor deficits in cerebral ischemia. In the present study, a contradictory result was observed: 26.25 μg/ml PN did not significantly decrease the area of cerebral thrombosis but improved locomotor dysfunction. These findings suggested that, at certain concentrations, the extract may exert a greater effect on locomotor recovery than on thrombosis-associated outcomes.

In conclusion, the present study demonstrated the safety and therapeutic potential of CA, MO, CL, PN and CS for ischemic stroke treatment. Among the tested extracts, CS exhibited the most promising potential as an anti-ischemic stroke agent, as it effectively decreased the cerebral thrombosis area and improved locomotor function following ischemia. The present study was limited to organism-level observations. Further research is needed to elucidate the cell and molecular mechanisms by which each extract contributes to recovery from ischemic brain injury. The anti-ischemic effects of the extracts examined in the present zebrafish model are consistent with findings reported in rat models (14,17,89). The zebrafish model may serve as a tool for screening novel pharmacological agents for the treatment of ischemic stroke.

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Availability of data and materials

The data generated in this are available upon reasonable request from the corresponding author.

Authors' contributions

NMDMWN, IW, KA and IKA conceived the study. NMDMWN, IW and HW designed the study. NMDMWN, AM and RAI analyzed and interpreted the data. IW and IKA confirm the authenticity of all the raw data. NMDMWN wrote the manuscript. HW, IW, KA and IKA supervised the study and revised the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All procedures in this study were approved by the Animal Research Ethics Committee-Institut Teknologi Bandung, Bandung, Indonesia (approval no. KEP/I/2024/II/H211223ND/TAAZ for the toxicity test and KEP/I/2024/VI/H110624NM/ANSZ for the anti-ischemic stroke test).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Use of artificial intelligence tools

During the preparation of this work, AI tools were used to improve the manuscript's readability and language. The authors revised and edited the content produced by the AI tools as necessary, and they take full responsibility for the final content of the manuscript.

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