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Cinnamomum burmannii EXTRACT AMELIORATES HIGH GLUCOSE-INDUCED BRAIN APOPTOSIS IN ZEBRAFISH EMBRYOS THROUGH INHIBITION OF PROCASPASE-9 : IN SILICO AND IN VIVO STUDY

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ABSTRACT

Background: Brain is an organ that is prone to oxidative stress and subsequent apoptosis due to high aerobic metabolism and relatively low antioxidants, especially under hyperglycemic condition. *Cinnamomum burmanii* (CB) is a species that is abundant in Indonesia, therefore it is of special concern for researchers to identify the anti-apoptotic effect of CB.
Objective: This study was initiated to determine the effect of CB extract on the inhibition of brain apoptosis in zebrafish embryos exposed to high glucose and to investigate its anti-apoptosis mechanism by molecular docking approach.
Methods: Molecular docking was conducted to determine the interaction between several CB extracts main constituents with target protein procaspase-9, compared to control ligand Saxagliptin. Zebrafish embryos were used to assess the effect of 4% glucose exposure and three doses of CB extract treatment (1.25, 5, and 10 µg/ml) on apoptosis in brain region. High-glucose condition in zebrafish embryo was confirmed with overexpression of Phosphoenolpyruvate carboxykinase (PEPCK). Apoptosis was evaluated by performing acridine orange (AO) staining and quantified by ImageJ software.
Results: Molecular docking study indicated that main CB compounds, namely epicatechin, displayed stronger molecular interactions with procaspase-9 compared to control ligand Saxagliptin. There were increased numbers of apoptotic cells seen around brain region in glucose-treated group. Meanwhile, supplementation of CB extract at dose of 10 µg/ml resulted in decreased amount of apoptotic cells in brain region.
Conclusion: The results suggest that CB extract protects from hyperglycemic-induced apoptosis in zebrafish embryos brain by modulating procaspase-9.

Keywords: cinnamon, brain apoptosis, zebrafish, molecular docking, procaspase-9

Introduction

Hyperglycemia in pregnancy can have detrimental impact on the health of the mothers and their fetuses.[1] Hyperglycemia is associated with high level of reactive oxygen species (ROS), inducing oxidative stress. Persistent oxidative stress triggers apoptosis which is characterized by DNA damage, protein and lipid oxidation.[2] The brain is an organ that is susceptible to oxidative stress due to high aerobic metabolism and relatively low antioxidants, especially under hyperglycemic conditions.[3]

Apoptosis is executed by series of caspases activation.[4] Caspases are formed as inactive zymogens (procaspases) and activated in response to apoptotic stimuli.[5] Procaspase-9 is the precursor of initiator caspases (caspase-9) which is an important trigger of apoptosis by mitochondrial pathway.[6] Saxagliptin, an oral antidiabetic drug of class DPP4 inhibitor, has been shown to reduce apoptosis by binding to caspases 3, 8, and 9.[7] Zebrafish is gaining popularities as experimental animals in the study of various physiological and pathological conditions such as diabetes and its secondary complications including impaired brain function in vertebrates. Zebrafish ability to mimic chronic hyperglycemia as experienced by diabetic human was also reported.[8] In terms of organs and genetics, zebrafish (*Danio rerio*) embryos are similar to humans. Zebrafish embryo at the age of 6-30 hours post fertilization (hpf) corresponds to human's 3-4 weeks old embryo which is prone to any detrimental milieu.[9] Therefore, zebrafish embryos are ideal animal model to investigate fetal response to

hyperglycemia. Phosphoenolpyruvate carboxykinase (PEPCK) is a key enzyme involved in gluconeogenesis in the liver which recently became an ideal marker of hyperglycemia in zebrafish embryo.[10]

Cinnamomum burmannii (CB) extract has been shown to be effective in the treatment of diabetes in a variety of diabetic animal models.[11] Several studies have also reported the anti-apoptotic effect of *Cinnamomum sp.* in cell cultures and animal models exposed to chemotherapeutic agents and heavy metals.[12–15] Cinnamon extracts' anti-diabetic and anti-apoptosis activity has been attributed to potent antioxidant profile of its constituents, including polyphenols and cinnamaldehyde.[16,17] In the meantime, there is still limited evidence regarding the effect and mechanism of cinnamon extract in preventing brain apoptosis.

Therefore, we intend to investigate the effect of CB extract on brain apoptosis in hyperglycemic-induced zebrafish embryos. We also aim to determine the potential interaction between several CB extracts main compounds and procaspase-9 by molecular docking approach.

Methods

Study Design

This research is a true experimental laboratory study with a randomized post-test only design using zebrafish embryos model. This study also used *in silico* approach to investigate the potential interaction of cinnamon main compounds with target protein Procaspase-9.

Molecular Docking

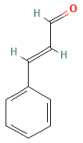
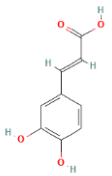
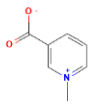
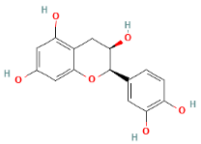
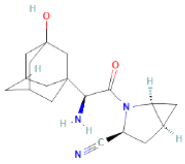
Ligands derived from CB extract were chosen based on previous studies.[16,18] The 3D structure of cinnamaldehyde (PubChemID 637511), caffeic acid (PubChemID 689043), epicatechin (PubChemID 72276), and trigonelline (PubChemID 5570) were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/) (Table 1). The control ligand used in this study is an established and FDA-approved oral hypoglycemic drug of the dipeptidyl peptidase-4 inhibitor (DPP-IV inhibitor) class, i.e. Saxagliptin (SAX, PubChemID 11243969) which was generated through previous study.[7] The tested ligand compounds were analyzed for pharmacokinetic profiles using SwissADME (http://www.swissadme.ch/index.php) by entering the SMILES formula of each active substance. Lipinski's Rule of Five analysis was carried out to assess the pharmacokinetic properties of the compounds.[19]

Procaspase-9 target protein[20] (PDB ID: 3YGS) was downloaded from Protein Data Bank (www.rscb.org) and optimized by removing water molecules and adding hydrogen atoms in PyMol software 2.5. Ligand compound's energy was minimized in order to achieve optimum binding strength. Chosen ligands were then docked specifically with procaspase-9 using PyRx 0.9.5 software on a grid of 36.125 x 36.344 x 36.037 Å (x, y, z). Ligand and target protein complexes were then visualized and analyzed for their interaction using the Discovery Studio 2021 software.

Ethical clearance

This study was ethically approved by the Research Ethic Committee of Faculty of Medicine, Universitas Brawijaya, Indonesia (No. 192/EC/KEPK-S2/07/2019).

Table 1. Ligands with Their Molecular and Structural Formula

Compound Name (PubChem CID)	Molecular Formula	Structural Formula
Cinnamaldehyde (637511)	C_9H_8O	
Caffeic Acid (689043)	$C_9H_8O_4$	
Trigonelline (5570)	$C_7H_7NO_2$	
Epicatechin (72276)	$C_{15}H_{14}O_6$	
Saxagliptin (11243969)	$C_{18}H_{25}N_3O_2$	

Experimental Animals

This study used zebrafish (*Danio rerio*) embryo aged 3 hours post fertilization (hpf) obtained from *wild-type* adult zebrafish fertilization in Hydrology Laboratory, Faculty of Fisheries and Marine Science, Universitas Brawijaya. The adult wild-type zebrafish were kept at 28ºC with photoperiodicity of 14 h light and 10 h dark and were fed with brine shrimp twice per day (*Artemia Sp.*) to promote breeding.[21] Male and female adult zebrafish (1:2) were separated the night before and then reunited in the morning, thus triggering spawning. Fertilized eggs will be deposited at the bottom of the tank.[22] Healthy embryos were selected, washed, and placed in 12-wells plate, each containing 15 embryos in 5 mL of embryonic medium (EM). The ingredients of EM used with 10 times concentration is 0,815 gr MgSO4, 0.25 gr CaCl2, 0,15 gr KCl and 5 gr NaCl, diluted in 500 mL of aquadest.[23]

Cinnamomum burmannii extraction

Cinnamon bark quills (Materia Medica, East Java, Indonesia) were cleansed and pulverized. *Cinnamomum burmannii* extraction using ethanol 96% as solvent was carried out in accordance with previous research.[17] Firstly, 100 g of cinnamon powder was immersed in 96 percent

ethanol in a percolator for 24 hours. The extracted sample was then filtered and evaporated at 40°C using vacuum rotary evaporator until semisolid ethanolic extract was obtained. Pure extract was then diluted in EM for the desired concentration.

Exposure of zebrafish embryos

Three hpf-old zebrafish embryos were grouped into 5 experimental groups: control group (C) with EM, glucose group (G) with 4% glucose, and 4% glucose with CB extract concentration of 1.25 (CB1), 5 (CB2) and 10 (CB3) µg/ml. Glucose solution was made by diluting 4 g of D-Glucose in 100 mL EM. Glucose powder were obtained from Makmur Sentosa Ltd., Malang, Indonesia. For the three latter groups, extract solution was co-administered with 4% glucose in volume proportion of 1:1. All the solution was renewed every 24 hours for 3 days.

PEPCK Expression Analysis

We performed semi-quantitative conventional reverse transcriptase polymerase chain reaction (RT-PCR) in a thermal cycler (BIO-RAD T100, California, USA). 20 zebrafish embryos (72 hpf) of each well were homogenized and then underwent RNA isolation using TRIzol (Tiangen Biotech). Synthesis of cDNA was performed using a reverse transcriptase kit according to the manufacturer's protocols. The PEPCK primers used in this study is as follows (QIAGEN): Forward 5'-GAG AAT TCT CAC ACA CAC ACA CGT GAG CAG TA-3'; Reverse 5'-GTA AAA GCT TTC CGC CAT AAC ATC TCC AGC AGAA-3'.[24] Thermal cycle of PEPCK PCR was carried out in the following: denaturation at 94°C for 3 minutes, 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 45 seconds with a final extension of 7 minutes at 72°C. The amplified PCR products were then visualized by agarose gel electrophoresis with ethidium bromide staining. Gel Doc 2000 fluorescent gel documentation system was used for gel visualization and quantification (Bio-Rad, USA). PEPCK expression was normalized against β–actin as the housekeeping gene.[25]

Apoptosis Analysis Using Acridine Orange Staining

All embryos (72 hpf) were anesthetized using tricaine 0.016% and then stained with acridine orange (Sigma-Aldrich) by soaking them in a 5 µg/mL solution of the dye for 30 minutes in room temperature.[26] The embryos were then washed three times with EM before transferred into 12-well plates filled with EM. The plates were wrapped in aluminium foil to prevent bleaching. Embryos were placed on object glass and positioned as needed using 1% agarose.[27] Apoptotic cells were observed using inverted fluorescence microscope (Olympus, Japan). All images were captured using identical exposure parameters.

ImageJ software was used to manipulate and analyze the images (National Institutes of Health, NIH). A uniform color threshold value (84) was set during this procedure to facilitate measuring light only from apoptotic cells.[28] By using the tracing tool, ImageJ can calculate the mean grey value in outlined areas representing the region of interest. As a result, the integrated density (IntDen) of selected fluorescent cells could be calculated by multiplying the mean grey value (MGV) to outlined cell area. After measuring the background mean grey value (BMGV), the following equation could be used to measure Correlated Total Cell Fluorescence (CTCF): CTCF = IntDen – (Area of selected cells * BMGV).[29]

Statistical Analysis

Differences between groups were determined by one-way ANOVA test and continued with Tukey post-hoc comparison using SPSS 25.0 (SPSS,Chicago,USA). The result is considered statistically significant at p value of < 0.05.

Results

Pharmacokinetic Analysis

SwissADME was used to analyze pharmacokinetic profiles of selected compounds. The oral regiment's absorption capacity and permeability are assessed using Lipinski's 'Rule of Five' criterion. Our findings as shown in Table 2 indicated that all four chosen ligands as well as control ligands have met Lipinski criteria without any violation.

Molecular Docking Result of *C. burmannii* Compounds

Protein target procaspase-9 was docked three times with its control ligand and selected compound for accuracy, and then the average binding affinity was calculated and compared. Negative binding affinity value suggests how likely the ligand will form complex with the target protein. The more negative the binding affinity correlates with higher possibility to form a bond.[30] The average binding affinity of 4 chosen CB compounds were ranging from -4.1 to -6.2 kcal/mol (Table 3). The binding affinity score was as follow: trigonelline (-4.1 kcal/mol), cinnamaldehyde (-4.7 kcal/mol), caffeic acid (-5.1kcal/mol), epicatechin (-6.2 kcal/mol) compared to the control ligand Saxagliptin (-5.6 kcal/mol). Epicatechin was shown to be the only compound that has higher binding affinity than control ligand Saxagliptin.

We analyzed the molecular interactions between chosen ligands and target protein using Discovery Studio software. As shown in Figure 1, cinnamaldehyde, caffeic acid, epicatechin, and trigonelline have the same binding pocket interaction compared with control (Saxagliptin). Trigonelline forms hydrogen bonds in Ala96 and hydrophobic interactions in Ala5, Arg94, and Leu9 (Figure 1A). Cinnamaldehyde has hydrophobic interactions to Arg8, Arg12 and Leu9 (Figure 1B). Caffeic acid creates hydrophobic interactions with Arg8, Arg12 and Leu9 and hydrogen bonds in Asn93 and Asp6 (Figure 1C). Saxagliptin forms hydrogen bond interaction with procaspase-9 at Asn93 and Ala5, as well as hydrophobic interactions at Arg12 and Leu9 (Figure 1E). Epicatechin forms hydrogen bonds with Arg12 and hydrophobic interactions with Arg8, Arg12, Arg94, Leu9, and Ala5 (Figure 1D), with similar amino acid residues to Saxagliptin with procaspase-9.

PEPCK Expression Pattern of Zebrafish Embryo

We investigated the expression of PEPCK in zebrafish embryo to prove that hyperglycemic condition was achieved. Based on Figure 2, exposure to 4% glucose significantly increased PEPCK expression in zebrafish embryo, approximately 1.6 times compared to control group. Exposure of *Cinnamomum burmannii* significantly decreased the expression of PEPCK dose dependently.

Table 2. Pharmacokinetics Profiles of Chosen Ligands

Compound	Mol. Weight (g/mol)	H Bond Donor	H Bond Acceptor	Log P	Fulfill Lipinski Criteria
Saxagliptin	315.41	2	4	1.15	Yes
Cinnamal-dehyde	132.16	0	1	1.899	Yes
Caffeic Acid	180.16	3	4	1.196	Yes
Epicatechin	290.27	5	6	1.546	Yes
Trigonelline	137.14	0	2	-0.330	Yes

Table 3. Molecular Docking Result of Cinnamon Compound Ligands Compared to Saxagliptin (Control) Ligand

Compound	Binding Affinity (Kcal/mol)	Interaction with Amino Acid	
		Hydrophobic Bond	Hydrogen Bond
Saxagliptin	-5.6	**ARG12, LEU9**	**ASN93**, ALA5
Epicatechin	-6.2	ARG8, **ARG12**, ARG94, **LEU9**, ALA5	ARG12
Caffeic Acid	-5.1	ARG8, **ARG12**, **LEU9**	**ASN93** AND ASP6
Cinnamaldehyde	-4.7	ARG8, **ARG12, LEU9**	-
Trigonelline	-4.1	ALA5, ARG94, **LEU9**	ALA96

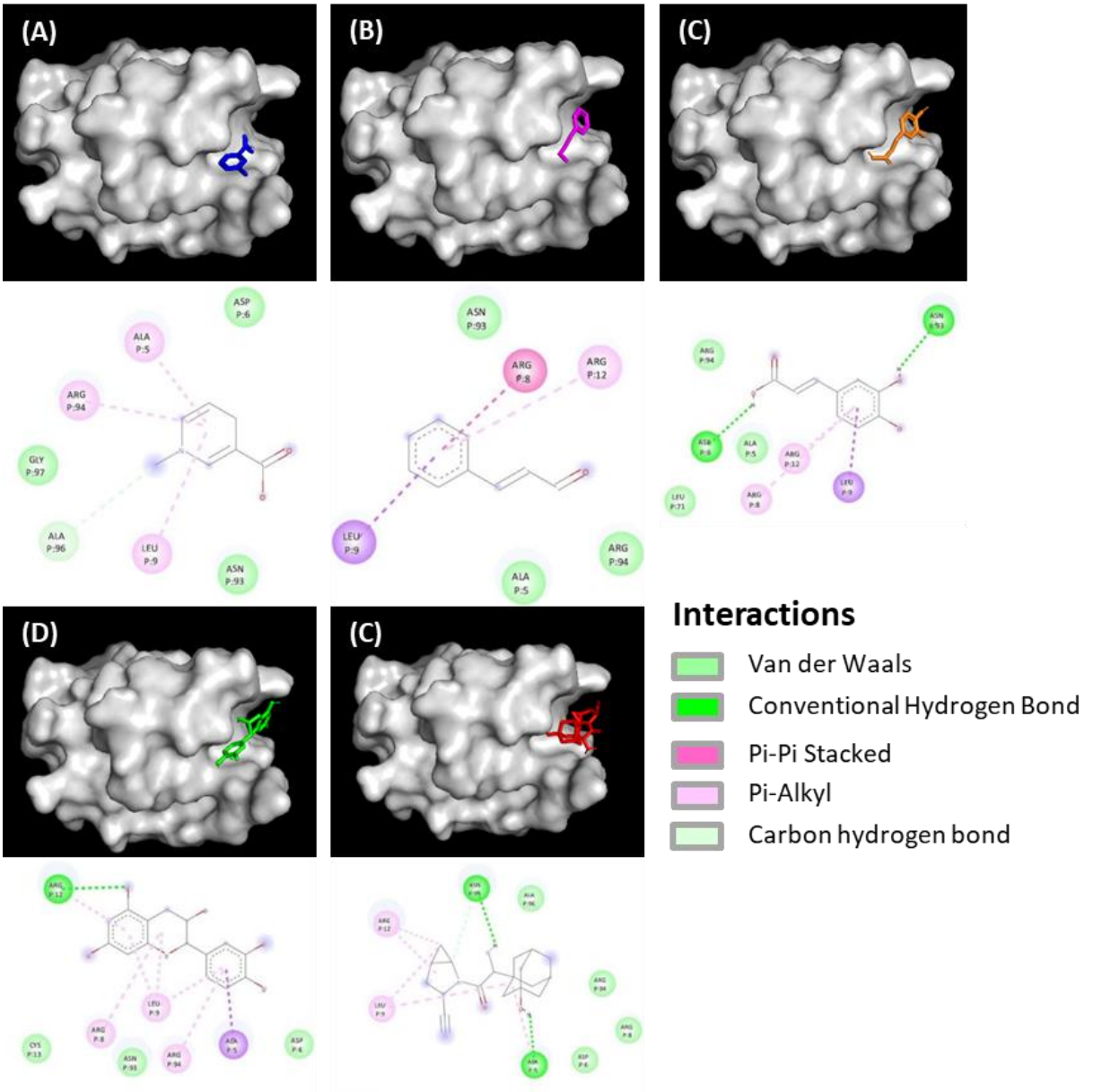


Figure 1 Interaction Visualization and Active Site Residues Between Ligands and SIRT1: (A) Trigonelline; (B) Cinnamaldehyde; (C) Caffeic acid; (D) Epicatechin; (E) Saxagliptin (Control)

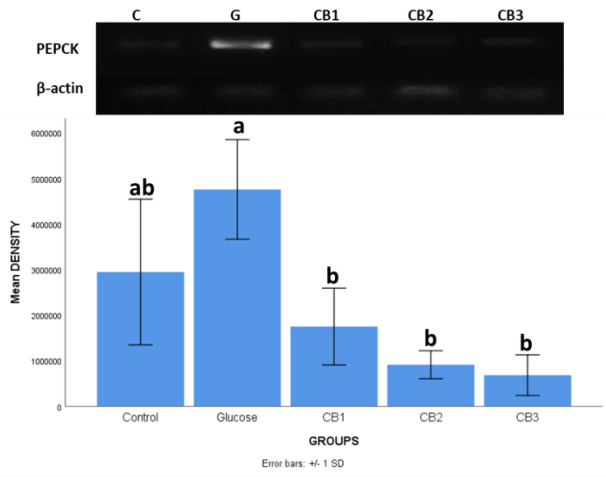


Figure 2. PEPCK Expression in zebrafish embryos. PEPCK expression is upregulated in glucose exposed group. Different annotation (bar chart) signifies difference by Tukey Post-Hoc test ($p<0,05$)

Effect of *C. burmannii* extract on Apoptosis in Brain Area

To investigate the role of apoptosis in hyperglycemic zebrafish embryopathy, acridine orange staining was performed. Acridine orange generates green fluorescence once bound to DNA and is widely used for detecting apoptosis in zebrafish. The control group had barely visible apoptotic cells, whereas there were significant numbers of apoptotic cells seen around the brain region in glucose-treated group (Figure 3B). Meanwhile, supplementation of CB extract showed reduced amount of apoptotic in brain region. CB extract at the dose of 10 µg/ml was the most effective in reducing apoptotic cells.

Discussion

In silico pharmacokinetics analysis results of CB active compounds indicate that cinnamaldehyde, caffeic acid, trigonelline and epicatechin are predicted to be a drug like molecule based on Lipinski's Rule of Five. The rules of Lipinski are molecular weight ≤500 daltons, high lipophilicity ≤5, hydrogen bond donor ≤5, hydrogen bond acceptor ≤10, and molar refractivity of 40-130.[31] From the results of molecular docking, it was found that epicatechin possess higher binding affinity to procaspase-9 than three other chosen CB compounds and control ligand Saxagliptin. Epicatechin was also shown to form interaction with amino acid residues that closely resembles Saxagliptin interaction with procaspase-9. Saxagliptin is an antidiabetic drug of the DPP-4 inhibitor which is approved and widely prescribed as monotherapy or combination therapy with other oral antidiabetic agents.[32] Recent molecular docking study reported that saxagliptin reduced apoptosis and showed high binding affinity to several caspases (caspase-3, caspase-8, and caspase-9) thus it is suitable to be used as control ligand.[7] Our findings are also supported by previous study. Earlier study reported that epicatechin reduced p-JNK, p38 of MAPKs, and cleaved caspase-3 protein expression which increases post radiation exposure and was shown to prevent cell death in fibroblasts cell culture.[33] This result suggests that the components of the CB extract (especially epicatechin) have potential as an antiapoptotic agent based on their interaction with procaspase-9.

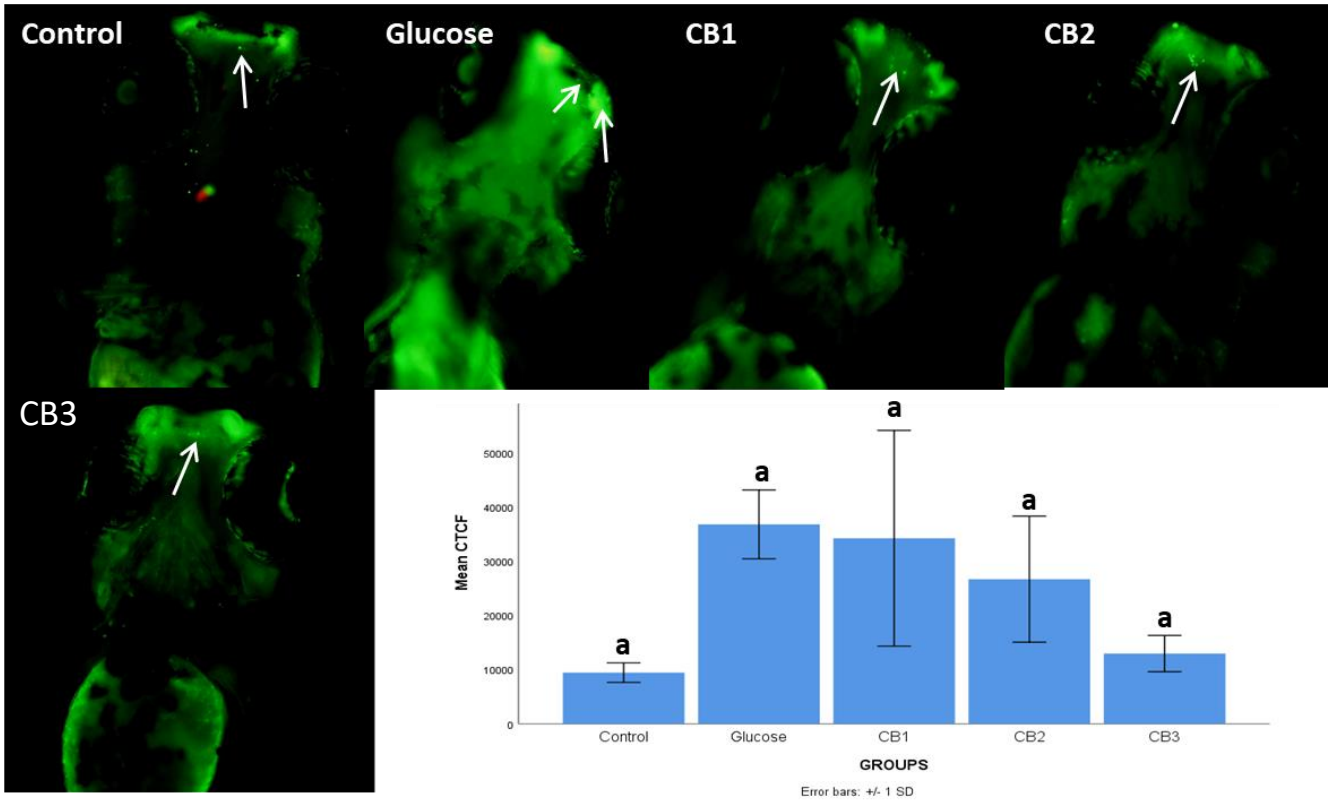


Figure 3. Visualization and Quantification of Apoptosis by Acridine Orange Staining. White arrow indicates apoptotic cells. Different annotation (bar chart) indicates statistically different by Tukey Post-Hoc test ($p<0,05$)

We analyzed PEPCK to confirm hyperglycemic condition in zebrafish embryo. The result showed that exposure to 4% glucose significantly increased PEPCK expression compared to control group. Increased expression of PEPCK may indicate the occurrence of insulin resistance and/or insulin deficiency.[24]

This study evaluates the effect of high-glucose exposure in early life stage of zebrafish. The results clearly showed that there was an increase in apoptotic cells around brain region in the group exposed to 4% glucose. This can be explained by the fact that hyperglycemia cause an increase in oxidative stress induced by ROS, which then affects several molecular pathways required for the development of various organs.[2] Normal embryonic development requires a balance between cell proliferation and apoptosis. The brain is an organ that is susceptible to oxidative stress due to high aerobic metabolism and relatively low antioxidants, especially in conditions of hyperglycemia.[3] Hyperglycemia disrupt the integrity of blood brain barrier by damaging tight junctions and transporters, thereby reducing mitochondrial function which accelerates cell apoptosis and induces neuronal loss.[34,35] Besides, hyperglycemia also triggers an increase in advanced glycation end products (AGE) which can accumulate and cause damage to vascular endothelial cells in the brain vascular.[36] Earlier study also reported that high high glucose treatment in animal model leads to increased reactive oxygen species in the brain and subsequent neuronal deaths.[37–39]

Present study investigated that supplementation of CB showed protective effect against high-glucose induced apoptosis in zebrafish embryos brain. Several earlier studies have reported anti-apoptotis effect of *Cinnamomum sp.* both in cells and animals model.[12,13,40] The anti-apoptotic ability of C. burmannii is thought to come from its strong antioxidant potency.[12,15] *Cinnamomum burmannii* extract contains several compounds, e.g. cinnamic acid which can inhibit palmitic acid that is known to induce lipotoxicity and accelerate apoptosis.[41] Cinnamaldehyde has antioxidant activity through its ability to be a free radical scavenger.[16] Epicatechin, catechin, and procyanidin B2, are also proved to neutralize several free radicals, including hydrogen peroxide, nitric oxide, and lipid peroxide by donating hydrogen ions.[11]

Conclusion

Cinnamomum burmannii extract shows protective effect from hyperglycemic-induced apoptosis in zebrafish embryo brain through probable inhibition of procaspase-9. Our study provide evidence for future phytopharmaceutical development, specifically *Cinnamomum burmannii* as anti-apoptosis and anti-diabetic agent.

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