7,8-Dihydroxyflavone functions as an antioxidant through the inhibition of Kelch-like ECH-associated protein 1: Molecular docking and an *in vivo* approach in a rat model of ischemia-reperfusion brain injury

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Abstract. Stroke is a medical condition characterized by a sudden, localized neurological impairment caused by damage to the central nervous system due to either a blockage or bleeding in the blood vessels. This process leads to increased oxidation, which can exacerbate brain damage. Flavonoids are compounds that are widely found in nature and are considered to have numerous health benefits. 7,8-dihydroxyflavone (7,8-DHF) is a flavonoid that has tropomyosin receptor kinase B agonist activity. However, the other beneficial activities of 7,8-DHF remain to be explored, particularly in neuron-related diseases. The present study aimed to explore 7,8-DHF as an antioxidant agent using *in vivo* and *in silico* analyses. An *in vivo* experiment was conducted using *Rattus novergicus*, in which ischemia-reperfusion brain injury was induced. A total of 30 rats were divided into the sham-operated, control and treatment groups. The treatment groups were administered doses of 10, 20 and 50 mg/kg body weight of 7,8-DHF orally, once daily for a duration of 14 days following the induction of ischemia-reperfusion brain injury. Subsequently, blood and brain tissues were collected for the examination of nuclear factor E2-related factor 2 (Nrf2) expression, glutathione peroxidase-1 (GPX-1) expression and the malondialdehyde (MDA) level. Molecular docking analysis and dynamic simulations of 7,8-DHF and Kelch like ECH-associated protein 1 (Keap1) protein interactions were performed using AutoDock Vina and AMBER. The visualization of the interaction was performed using ChimeraX and Discovery Studio. The drug-like properties of 7,8-DHF were predicted using the SwissADME web server, and the bioactivity of the compounds was evaluated using the PASS online server. The results revealed that 7,8-DHF exhibits antioxidant activity through the inhibition of Keap1, thereby increasing Nrf2 and GPX-1 expression and decreasing the levels of MDA. *In silico* analysis revealed that 7,8-DHF can bind Keap1 protein, thereby disrupting the interaction between Nrf2 and Keap1. Molecular dynamics also demonstrated that 7,8-DHF has good stability compared to the control. On the whole, as demonstrated in the present study

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Abbreviations: BDNF, brain-derived neurotrophic factor; SOD, superoxide dismutase; IHC, immunohistochemistry; PBS, phosphate-buffered saline; GPX, glutathione peroxidase; CAT, catalase; MDA, malondialdehyde; Keap1, Kelch like ECH-associated protein 1; Nrf2, nuclear factor erythroid 2-related factor 2; GSH, glutathione; ARE, antioxidant response element; 7,8-DHF, 7,8-dihydroxyflavone; TNF, tumor necrosis factor; ROS, reactive oxygen species; RNS, reactive nitrogen species; MCAO, middle cerebral artery occlusion; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; TrkB, tropomyosin receptor kinase B; MEP, molecular electrostatic potential; MESP, molecular electrostatic potential surface; ECA, external carotid artery; ICA, internal carotid artery; CCA, common carotid artery; HPLC, high performance liquid chromatography; DAB, diaminobenzidine; ELISA, enzyme-linked immunosorbent assay; ADMET, absorption, distribution, metabolism, excretion and toxicity; RMSD, root mean square distance; TYR, tyrosine; ARG, arginine; SER, serine; GLU, glutamic acid; ALA, alanine; GLN, glutamine; GLY, glycine; MD, molecular dynamics

Key words: 7,8-dihydroxyflavone, reperfusion ischemic brain injury; Keap1, GPX-1, MDA, *in silico*, *in vivo*

7,8-DHF has promising biological activity as an antioxidant agent in the rat model of ischemia-reperfusion brain injury. Further studies on the effects of 7,8-DHF are required to clarify its potential as a novel therapeutic agent for cerebral infarction.

Introduction

Cerebral infarction, a critical medical condition, occurs when blood flow to a specific region of the brain is blocked. This obstruction results in damage to the affected tissue and the possibility of neurological impairment (1). Stroke is a medical condition characterized by a sudden, localized neurological impairment caused by damage to the central nervous system due to either a blockage or bleeding in the blood vessels. Stroke is a critical public health concern due to its significant impact on both mortality and disability statistics. An obstruction of blood flow to the brain characterizes ischemic stroke cases, which comprise ~87% of all stroke cases. The obstruction of blood flow is often attributed to the presence of atherosclerosis and thrombosis within the cerebral arteries. Cerebral infarction is characterized by a complex interaction of molecular processes. Inflammation and oxidation play a central role in the state of cerebral infarction (2,3).

The increased production of reactive oxygen species (ROS) and inflammation creates a detrimental feedback loop, as ROS have the ability to initiate pro-inflammatory pathways and stimulate the secretion of cytokines, thereby exacerbating tissue injury and inflammation associated with brain injury. Oxidative stress, along with malondialdehyde (MDA), is recognized as a major contributor to the complex pathophysiology of brain injury (2). The decrease in MDA levels may be associated with the upregulated expression of nuclear factor E2-related factor 2 (Nrf2), glutathione S-transferase (GST) and glutathione peroxidase (GPX), which are antioxidant enzymes activated by the Kelch like ECH-associated protein 1 (Keap1)/Nrf2 pathway (4). Researchers have previously found that the blocking of Keap1 increases the production of Nrf2. Nrf2 is a key factor in protecting neurons from ischemic stroke and reducing brain damage caused by oxidative stress. This process of activating the Keap1/Nrf2 pathway has also been linked to higher levels of GPX, an enzyme that plays a critical role in eliminating ROS and lipid hydroperoxides. The Keap1/Nrf2 pathway has exhibited potential in mitigating oxidative stress and providing neuroprotection in stroke and several neurodegenerative diseases (5-7).

The Keap1/Nrf2 pathway controls the antioxidant response and has been shown to influence inflammation in a number of diseases, including ischemic stroke (8). Reactive nitrogen species bind to a key regulatory cysteine thiol in the Keap1 protein during oxidative-nitrosative stress (9). When this chemical reaction occurs, Keap1 adducts are formed that break the link between Keap1 and Nrf2. The disruption prevents Keap1 from degrading Nrf2, thereby facilitating the accumulation of Nrf2 in the nucleus. This localization allows Nrf2 to initiate the activation of genes associated with the antioxidant response. It has also been shown that a few small compounds can prevent Keap1 from functioning by S-alkylating the important thiol group. This results in the suppression of the ability of Keap1 to degrade Nrf2 and instead promotes the activation of antioxidant defense pathways (9,10).

For the prevention of oxidative damage in cardiovascular tissue, an expanding array of medicinal herbs are employed as innate antioxidants in clinical settings (11). Antioxidant activity can be extracted from several medicinal plants, yielding active compounds (12,13). Natural antioxidant medications have the ability to reduce the generation of harmful free radicals by boosting both particular and general immune responses, or by directly limiting the cellular and tissue harm caused by free radicals. Flavonoids, a class of organic chemicals found naturally in plants, have exhibited great promise as agents with anti-inflammatory properties. This is attributed to their ability to modulate pro-inflammatory genes and inhibit the function of enzymes involved in the inflammatory cascade (14). One particular flavonoid that has been the subject of extensive research for its anti-inflammatory properties is 7,8-dihydroxyflavone (7,8-DHF). Previous research has documented the robust anti-inflammatory capabilities of 7,8-DHF (15). Through the downregulation of the nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) pathways in lipopolysaccharide-treated RAW264.7 cells, the study by Park *et al* (15) demonstrated that 7,8-DHF exhibited anti-inflammatory properties. 7,8-DHF directly suppressed the inflammatory response in an obese adipose cell line by disrupting the paracrine loop between adipocytes and macrophages. This is evidenced by the reduction in pro-inflammatory cytokines and the release of non-esterified fatty acids (16-18). 7,8-DHF may also decrease the activity of inflammatory mediators by inhibiting the JNK and NF-κB signaling pathways. Furthermore, 7,8-DHF has been shown to reverse the decreased absorption of glucose in the combined culture of adipocytes and macrophages, along with an elevation in the phosphorylation of Akt (19). Thus, 7,8-DHF treatment is also effective in reducing blood glucose levels.

The unusual properties of 7,8-DHF have attracted considerable attention in the fields of neuroscience and neurology. It has been found that the compound in question binds to and activates the tyrosine kinase receptor B (TrkB), which is linked to brain-derived neurotrophic factor (BDNF). A notable feature of 7,8-DHF is its ability to cross the blood-brain barrier (BBB); thus, it holds promise for use in neurological applications (16). Thus, it is worthy to investigate the role of 7,8-DHF in stroke. Numerous studies have been conducted to investigate the antioxidant properties of 7,8-DHF (17,18,20); however, further investigations are required to fully elucidate the underlying mechanisms responsible for its antioxidant effects. It is important to know the potential of 7,8 DHF for the treatment of ischemic stroke.

The present study aimed to explore 7,8-DHF as an antioxidant agent using *in vivo* and *in silico* analyses. The present study demonstrates the potential of 7,8-DHF as an antioxidant using molecular docking techniques and *in vivo* experiments in a rat model of ischemia-reperfusion brain injury.

Materials and methods

Experimental animals. A total of 25 male rats (*Rattus norvegicus*), aged between 7 and 9 weeks and weighing at least 200 g from the Animal House Faculty of Medicine Universitas

Table I. Experimental animal groups.

Group	Treatment administered
Sham	Operation without MCAO + normal saline for 14 days
MCAOP	MCAO induction + normal saline for 14 days
MCAO10	MCAO induction + normal diet + 7,8-DHF administered orally at dose 10 mg/kg weight body for 14 days
MCAO20	MCAO induction + normal diet + 7,8-DHF administered orally at dose 20 mg/kg weight body for 14 days
MCAO50	MCAO induction + normal diet + 7,8-DHF administered orally at dose 50 mg/kg weight body for 14 days

MCAO, middle cerebral artery occlusion; 7,8-DHF, 7,8-dihydroxyflavone.

Brawijaya (Malang, Indonesia), were used in the present study. The duration of the study was 4 months. These rats were divided into five different groups, with each group consisting of 5 rats, as demonstrated in Table I. The initial total number of experimental animals used was 30 rats, with 6 rats per group. At the final stage of the experiment, there were 2 rats that died naturally and 3 rats that died following middle cerebral artery occlusion (MCAO) due to complications associated with this procedure. A total of 25 rats were used for parameter analysis. The selection of these groups was made to evaluate the efficacy of 7,8-DHF treatment compared to the placebo and to normal rats. The rats were placed in a suitable housing habitat and allowed to acclimatize at room temperature (22-28˚C). The humidity in the room was maintained at 45-50%, with a 12-h light-dark cycle. Food and water were provided *ad libitum*. Each week, the experimental animals were examined and monitored concerning overall health, neurological functions, animal behavior and physical parameters, such as body weight and body condition score. The indication for euthanasia was determined based on various factors, including self-mutilation, the amputation or crushing of limbs and tail, generalized dermatitis, infection and severe dehydration. Death was determined based on the absence of respiration, absence of heartbeat, dilated pupils, the absence of reflexes, poor pigmentation of the extremities and mucous membranes, and the presence of dry and opaque corneas. The research was carried out following internationally recognized ethical principles and the guidelines outlined in the National Institutes of Health's Guide for the Care and Use of Laboratory Animals of the Medical Faculty, Brawijaya University (No. 160/EC/KEPK/09/2020).

MCAO. The experimental procedure involved the induction of ischemia-reperfusion brain injury in the rats by performing MCAO. In the present study, the rats were injected with ketamine (40-100 mg/kg) and xylazine (5-13 mg/kg) to induce and maintain anesthesia. The experimental setup included the use of a heat lamp and a heating pad to maintain body temperature at a constant level of 37˚C. A longitudinal incision of ~2 cm was made along the midline of the neck. The right common carotid artery (CCA) was carefully dissected from the vagus nerve and briefly occluded with cotton thread. The left external carotid artery was ligated at its bifurcation into the internal and external carotid arteries. Following a delicate ligation procedure on the right internal carotid artery (ICA), a 3-0 monofilament nylon was carefully inserted into the ICA through the CCA incision. The nylon material exhibited signs of detachment over a 90-min occlusion period. Upon the completion of the experimental procedure, the rats were transferred to the incubator for specialized and attentive medical care (21,22). In the sham-operated (sham) group, the rats underwent an identical surgical technique but without the induction of MCAO. Following 14 days of treatment, the rats were euthanized with intraperitoneal overdoses of ketamine (40-100 mg/kg body weight) and xylazine (5-13 mg/kg body weight), followed by decapitation. Blood and brain tissue samples were collected. Brain tissue samples were fixed overnight by immersion in a 10% formalin solution.

7,8-DHF treatment. 7,8-DHF was obtained from the manufacturer, Tokyo Chemical Industry Co., Ltd. (product no. D1916; lot no. OQ5VB). The 7,8-DHF isolate has a purity of 99.5%, as shown by high performance liquid chromatography and 100% by neutralization titration (data not shown). This isolate was dissolved using a concentration of 10% dimethyl sulfoxide (Cas No. 67-68-5, MilliporeSigma) for the dissolution (23). Isolate preparations of 7,8-DHF were prepared every 5 days to prevent oxidation and damage. The administration of 7,8-DHF was performed by a professional assistant using a sonde/feeding tube (Thermo Fisher Scientific, Inc.) as doses, as demonstrated in Table I.

Immunohistochemical staining. Immunohistochemistry (IHC) was used to measure the amount of Nrf2 and GPX-1 protein in the brain tissues of rats in the model of ischemic stroke. The kit used was from Bioss with the following antibodies: [Nrf2, cat. no. bs-2013R; (1:75), and GPX-1, cat.no. bs-3882R; (1:75)]. The calculation was based on the amount of Nrf2, and GPX-1 protein found in the cytoplasm and nucleus of brain tissue in the cortical lesion area. This included neurons, and glia and Schwann cells observed under a microscope at x400 magnification. Briefly, the brain tissue samples were deparaffinized and stored at room temperature for 24 h prior to use. Following deparaffinization, the slides were immersed in a sodium citrate buffer solution (pH 6.0) and then washed with phosphate-buffered saline (PBS) (P-5119, MilliporeSigma) three times for 5 min. Blocking was performed with a blocking buffer (skimmed milk) (Blocker BLOTTO in Tris-buffered saline; cat. no 37530, Thermo Fisher Scientific, Inc.) followed by incubation for 60 min at room temperature. Nrf2 polyclonal antibody, unconjugated (bs-2013R), GPX-1 polyclonal antibody,

unconjugated (bs-3882R) at a ratio of 1:75 were applied overnight at 4˚C. Secondary antibodies (rabbit anti-rat IgM antibody (H+L), biotin-conjugated; 1:200; bs-0346R-Biotin; Bioss) were used and incubated with the samples for 60 min at room temperature, then washed three times for 5 min with PBS. Streptavidin HRP was then incubated with the samples for 40 min at room temperature. The chromogen diaminobenzidine (DAB; AMF080, ScyTek Laboratories, Inc.) ratio was 1:40 (DAB chromogen: DAB buffer) and the samples were incubated for 5 min at room temperature. Mayer's hematoxylin (cat. no. 109249, MilliporeSigma) (Mayer's hematoxylin: distilled water, 1:3) was added, incubated for 2 min at room temperature, then washed with distilled water and mounted with Entella. The final result was observed under a confocal microscope (BX51, Olympus Corporation) at x400 magnification. To minimize examination bias, IHC calculations were conducted by two observers under the supervision of a pathologist. The counting of positive cells was performed in a single-blind manner, with codes known only to one individual to confirm the results.

Enzyme-linked immunosorbent assay (ELISA). To assess the extent of oxidative damage in the brain tissue, the concentration of MDA, a reliable indicator of lipid peroxidation, was quantified in the brain samples. This was performed using a commercially available ELISA kit (MDA Elabscience® ELISA kit, cat. no. E-EL-0060, Elabscience Biotechnology, Inc.) according to the manufacturer's instructions.

Absorption, distribution, metabolism, excretion and toxicity (ADMET) and drug-likeness. The ADMET of 7,8-DHF was evaluated using SwissADME (http://www.swissadme.ch/) by inserting the SMILES formula of 7,8-DHF. Lipinski's rule of 5 (LR5) was used to evaluate the pharmacokinetic potential of the compounds. A chemical can be classified as a drug if it meets the LR5 criteria. Finally, the OSIRIS software tool (https://www.organic-chemistry.org/prog/peo/) was used to predict the human safety of a compound based on its toxicity analysis (24,25).

Biological activity and molecular potential prediction. To derermine the potential activity of 7,8-DHF, the biological activity was measured using the prediction of activity spectra for substances (PASS) online web server (http://www.way2drug.com/index.php). The purpose of the web server is to predict the biological activity spectrum of organic compounds by analyzing their structural formulas. It covers a wide range of >4,000 biological activities and achieves an average accuracy rate of >95%. The predictions are obtained by studying structure-activity relationships in a training set consisting of structure and biological activity data for >300,000 organic molecules (26,27). Briefly, a canonical molecule from PubChem was entered into the PASS online web server. The biological activity prediction of a 7,8-DHF was then calculated. Graph visualization was performed using GraphPad Prism version 10 software (https://www.graphpad.com/features), while the potential molecular electrostatics were measured using Gaussian 09W and Gaussian View 6 software (https://gaussian.com/gaussview6/). The molecular electrostatic potential (MESP) was also predicted using Chimera (https://www.cgl.ucsf.edu/chimerax/).

Molecular docking. In the present study, molecular docking simulation was performed using the Keap/Nrf2 complex structure (PDB ID: 2FLU). 7,8-DHF was downloaded from PubChem (https://pubchem.ncbi.nlm.nih.gov/compound/7_8-Dihydroxyflavone) with code CID: 1880. Molecular docking was performed using PyRx (https://pyrx.sourceforge.io/) on a personal computer running Windows. Protein stabilization was performed using PyMOL by removing water atoms and introducing hydrogen atoms. To verify the accuracy and reliability of the molecular docking process, a redocking procedure was performed. The original ligand was repositioned into the target binding site using the PyRx with accurate grid coordinates. After the redocking process, an evaluation was performed to determine the position of the ligand. Ligands were required to have a root mean square deviation (RMSD) of <2.0. The lattice and docking parameters were modified according to the docking validation results. The interaction visualization was performed using the Protein ChimeraX (https://www.cgl.ucsf.edu/chimera/) and Discovery Studio (https://discover.3ds.com/discovery-studio-visualizer-download) (28,29).

Molecular dynamics (MD) simulations. MD simulations were used to determine how 7,8-DHF and Keap1 interact. The OpenMM engine and the AMBER force field were used for both the protein and ligand systems. The simulations were performed within the Google Collaboratory platform (https://colab.research.google.com/?utm_source=scs-index) as described in a previous study (30). In the present study, the model was subjected to thorough solvation using the TIP3P water model with 108 water molecules contained in a cubic water box of 10x10x10 units. A solution of sodium chloride was introduced at a concentration of 150 mM. The simulation was performed using the ff19SB force field. The system underwent 1,000 steps of minimization followed by 20 nsec of equilibration in the isothermal-isobaric (NPT) ensemble using a 2-femtosecond time step. Temperatures are set at 310 Kelvin, and pressures are in the range of 1 atmosphere. The present study focuses on the investigation of the RMSD.

Statistical analysis. Statistical analysis in the present study was conducted using GraphPad Prism 9 software (Dotmatics). Data are presented as mean ± SD. Normality testing was performed using the Shapiro-Wilk test due to the sample size being <50 subjects. The analysis of the Nrf2, GPX-1 and MDA parameters was carried out using a one-way ANOVA parametric test to assess the overall efficacy of administering the 7,8-DHF treatment, followed by post-hoc Tukey analysis to examine the differences within each group. P-values <0.05 were considered to indicate a statistically significant difference.

Results

Antioxidant effects of 7,8-DHF in brain tissue of rats with ischemia-reperfusion injury. To determine the antioxidant effects of 7,8-DHF, Nrf2 expression was examined.

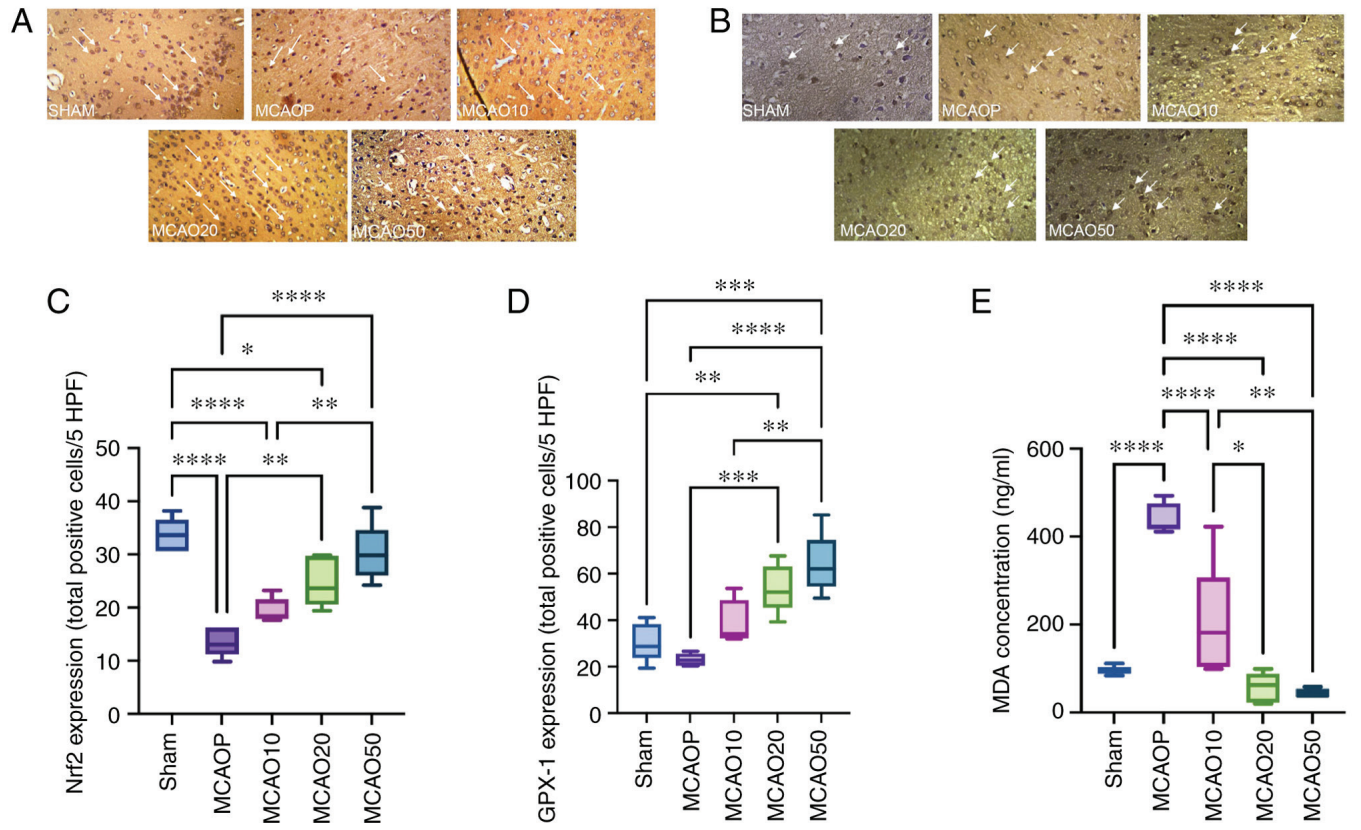

Figure 1. Antioxidant effects of 7,8-DHF in a rat model of ischemia-reperfusion brain injury. (A) Nrf2 expression in the brain tissue in each group. Arrows indicate Nrf2 expression. (B) GPX-1 expression in the brain tissue in each group. Arrows indicate GPX-1 expression (C) Statistical analysis of Nrf2 expression in each group. (D) Statistical analysis of GPX-1 expression in each group. (E) MDA level in each group. *P≤0.05, **P≤0.01, ***P≤0.001 and ****P≤0.0001. The rat groups were as follows: Sham, sham-operated group; MCAOP, control (MCAO induction and normal saline); MCAO10, MCAO induction and 7,8-DHF at 10 mg/kg weight body; MCAO20, MCAO induction and 7,8-DHF at 20 mg/kg weight body; MCAO50, MCAO induction and 7,8-DHF at 50 mg/kg weight body. 7,8-DHF, 7,8-dihydroxyflavone; Nrf2, nuclear factor erythroid 2-related factor 2; GPX, glutathione peroxidase; MDA, malondialdehyde; MCAO, middle cerebral artery occlusion.

7,8-DHF was found to increase the expression of Nrf2 in dose-dependent manner. The data demonstrated that 7,8-DHF at 20 and 50 mg/kg body weight (MCAO20 and MCAO50, respectively) significantly increased Nrf2 expression compared with the control, as shown in Fig. 1A and C.

GPX-1 expression was also examined using IHC. The results revealed a significant increase in GPX-1 expression beginning at the dose of 20 mg/kg body weight compared with the control. The most significant increase in GPX-1 expression was observed in the MCAO50 group, as illustrated in Fig. 1B and D.

Furthermore, the level of MDA was lower in the MCAO20 group than in the control (MCAOP group). The most notable decrease level in the MDA level was found in the MCAO20 and MCAO50 groups compared with the other groups. There was no significant difference in the MDA level between the MCAO20 and MCAO50 groups, as shown in Fig. 1E.

ADMET and drug-likeness of 7.8-DHF. To examine the characteristics of 7,8-DHF as a drug, ADMET, drug-likeness and toxicity analyses were performed. In drug absorption, lipophilicity and solubility are critical molecular characteristics. This property is critical to the drug feasibility requirements, particularly for orally administered drugs (31). This drug feasibility test is also known as the drug likeliness criteria. Numerous criteria have been established, including the Lipinski criteria. A drug-like compound that can be taken orally should have no more than one violation of the criteria outlined by Lipinski's rule: No more than 5 hydrogen bond donors, no more than 10 hydrogen bond acceptors, a molecular weight of no more than 500 Da, and an octanol-water partition coefficient (log P) of no more than 5 (28). The results revealed that 7,8-DHF fulfilled the LR5 and had a good ADMET, as presented in Table II. This indicates that the 7,8-DHF compound has sufficient oral bioavailability to be administered orally.

7,8-DHF has a BBB score of -0.057, indicating low permeability. BBB permeability is interpreted as logBBB >0.3, indicating that molecules can easily pass through the BBB, whereas molecules with logBB-1 are poorly distributed in the brain. The difference between BBB permeability and central nervous system (CNS) permeability lies in their respective properties. For CNS permeability, logPS units accurately predict the compound administered into the carotid artery. Compounds with logPS >-2 can penetrate the CNS, whereas compounds with logPS-3 cannot penetrate the CNS. The 7,8-DHF score logPS was-1.927, indicating that it can penetrate the CNS (Table II). Finally, these data were confirmed with BOILED-Egg analysis prediction using SwissADME (http://www.swissadme.ch/), demonstrating that 7,8-DHF

Table II. Pharmacokinetic prediction of 7,8-dihydroxyflavone.

		Computationally predicted values for the phytochemicals (ADMET profile)		
Pharmacokinetic property	Model name	Reference value	7,8-DHF	Measurement units
Lipinski rule of 5	Molecular weight	<500	254.24	Numeric (g/mol)
	Acceptor hydrogen	<10	4	Numeric (AH)
	Donor hydrogen	<5	2	Numeric (DH)
	LogP	<5	2.87	Numeric (MLogP)
Absorption	Water solubility	<0.4 mol/l (low) 0.4-41 mol/l (medium) >41mol/l (high)	-3.605	Numeric (mol/l)
	Caco2 permeability	>0.90	1,048	Numeric (Papp in 10^{-6} cm/sec)
	Intestinal absorption (human)	Low: 0-20% Medium: 20-70% High: 70-100%	94,22	Numeric (% absorbed)
	Skin permeability	Likely skin permeable: >-2.5	-2.751	Numeric (log K_p)
	P-glycoprotein substrate	Yes	Yes	Categorical (yes/no)
	P-glycoprotein I inhibitor	No	No	Categorical (yes/no)
	P-glycoprotein II inhibitor	No	No	Categorical (yes/no)
Distribution	VDss (human)	Low: <-0.15 High >2.81	0,063	Numeric (log L/kg)
	Fraction unbound (human)	-	0,146	Numeric (Fu)
	BBB permeability	Low: <0.1 Medium: 0.1-0.2 High: >0.2	-0,057	Numeric (log BB)
	CNS permeability	>-2 (penetrate BBB) <-3 (Cannot penetrate BBB)	-1,927	Numeric (log PS)
Metabolism	CYP2D6 substrate	No	No	Categorical (yes/no)
	CYP3A4 substrate	No	No	Categorical (yes/no)
	CYP1A2 inhibitor	No	Yes	Categorical (yes/no)
	CYP2C19 inhibitor	No	Yes	Categorical (yes/no)
	CYP2C9 inhibitor	No	Yes	Categorical (yes/no)
	CYP2D6 inhibitor	No	No	Categorical (yes/no)
	CYP3A4 inhibitor	No	No	Categorical (yes/no)
Excretion	Total clearance	-0.002	0.229	Numeric (ml/min/kg)
	Renal OCT2 substrate	No	No	Categorical (yes/no)
Toxicity	AMES toxicity	No	No	Categorical (yes/no)
	hERG I inhibitor	No	No	Categorical (yes/no)
	hERG II inhibitor	No	No	Categorical (yes/no)
	Hepatotoxicity	No	No	Categorical (yes/no)
	Skin sensitization	No	No	Categorical (yes/no)
	Tetrahymena pyriformis toxicity	Non-toxic: >0.5 Toxic: <0.5	0,654	Numeric (log μg/l)
	Minnow toxicity	Non-toxic: >-0.3 Toxic: <-0.3	1,178	Numeric (log mM)
	Mutagenicity	No risk 100% Medium risk 80% High risk 60%	60%	Numeric (%)
	Tumorigenicity		100%	Numeric (%)
	Irritating effects		100%	Numeric (%)
	Reproductive effects		100%	Numeric (%)

7,8-DHF, 7,8-dihydroxyflavone; ADMET, absorption, distribution, metabolism, excretion and toxicity; BBB, blood-brain barrier.

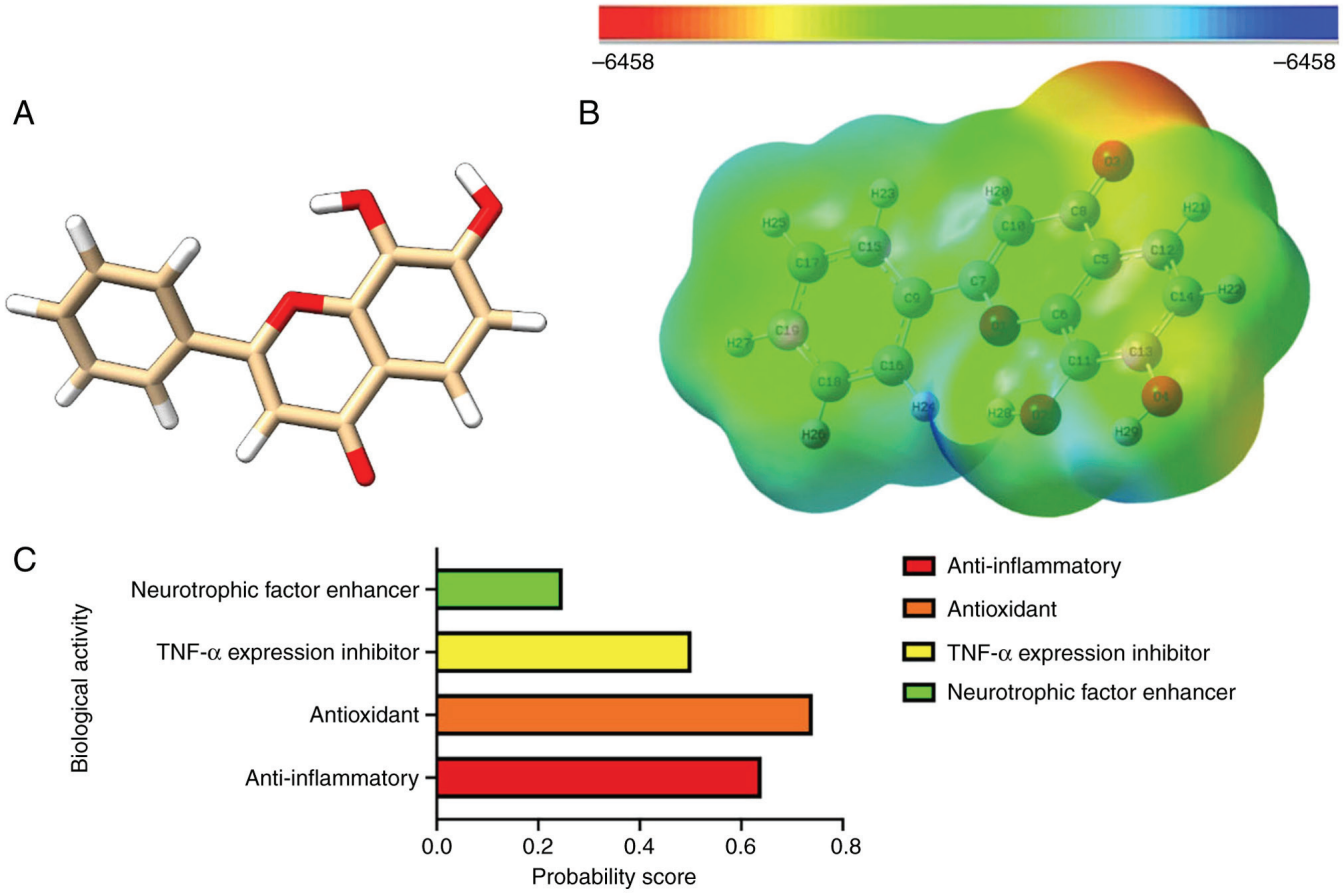

Figure 2. Characteristic and biological activity of 7,8-DHF. (A) The 3D structure of 7,8-DHF was downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/compound/7_8-Dihydroxyflavone) and visualized using UCSF ChimeraX (https://www.cgl.ucsf.edu/chimerax/). (B) Molecular electrostatic potential maps density of 7,8-DHF. (C) Prediction of biological activity of 7,8-DHF based on Canonical SMILES from the PASS online webserver. 7,8-DHF, 7,8-dihydroxyflavone; TNF, tumor necrosis factor.

can penetrate the BBB, as shown in Fig. S1. As shown by the results, the 7,8-DHF compound has moderate potency based on its distribution profile (32).

The toxicity of 7,8-DHF was also predicted using the biocomputational approach, an important parameter for safety in the human body. Several parameters are evaluated to determine toxicity. The Ames test is a widely used technique to determine the mutagenic potential of substances using bacteria (33). The Ames test used in the present study was a prediction test using OSIRIS (https://www.organic-chemistry.org/prog/peo/). The OSIRIS toxicity prediction model uses structural features and substructures associated with toxic effects to determine the potential toxicity of a chemical molecule. OSIRIS evaluates the submitted chemical structure by comparing it to a database of recognized toxicophores (substructures associated with toxic effects) and structural alerts. Toxicophores refer to distinct chemical properties that have been associated with various forms of toxicity. OSIRIS provides toxicity predictions for several domains, including mutagenicity, tumorigenicity, irritation, reproductive effects and others. Each category can have different warnings associated with it (28). The data demonstrated that 7,8-DHF was mostly safe, although there was a moderate risk of mutagenicity, as shown in Table II.

7,8-DHF activity and molecular electrostatic potential prediction. 7,8-DHF has a molecular structure consisting of two aromatic rings connected by a single rotatable bond. It has four acceptor sites and two donor hydrogen bonds. The flavone skeleton contains two hydroxyl groups at positions 7 and 8 with a *trans* configuration. The biological activity of the molecule is influenced by several functional groups, with the hydroxyl groups being critical for protein-receptor interaction. The planarity and stability of the flavone backbone are due to the presence of a carbonyl group at position 4 and a double bond between positions 2 and 3. A representative 3D structure of 7,8-DHF is presented in Fig. 2A.

To determine the characteristics of the structure, MESP was performed. The MESP surface is a tool used to identify electrophilic and nucleophilic regions of a molecule. Different shades on the surface represent different levels of electrostatic potential. The ability of a molecule to bind to biomolecules can be determined by calculating its electrophilicity index (34). Different colors represent the electrostatic potential levels at the surface in the following order: red-orange-yellow-green-blue. The color coding for these maps in the identified compound varies from -6.457 (deepest red) to +6.457 (deepest blue) (blue indicates the most electronegative and red is the most electropositive). The most electronegative region of 7,8-DHF in a hydrogen atom is shown in Fig. 2B.

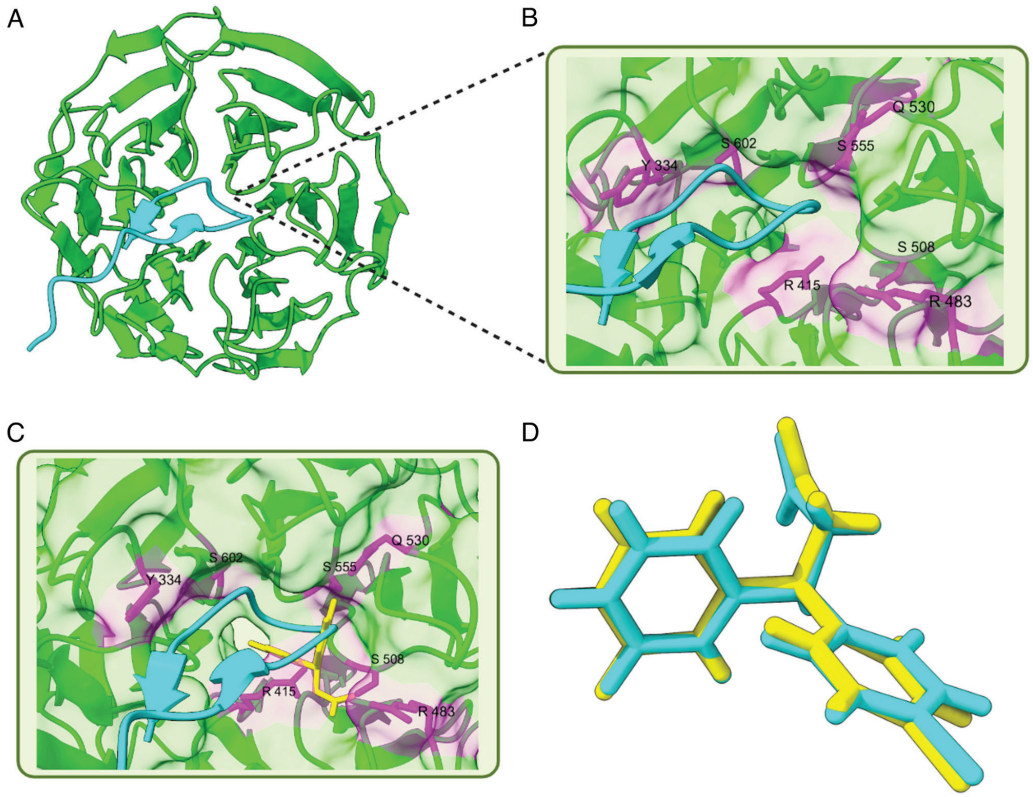

Figure 3. The complex interaction between Keap1/Nrf2 and their small molecule inhibitor. (A) the complex interaction between Keap1 (green) and Nrf2 (cyan). (B) The important residue (magenta) in the interaction between Keap1 and Nrf2. (C) Superimposed small molecule inhibitor of Keap1 (control). (D) Redock analysis to verify the molecular docking protocol. Keap1, Kelch like ECH-associated protein 1; Nrf2, nuclear factor erythroid 2-related factor 2.

To determine the biological activity of 7,8-DHF based on chemical structure, the biological activity was predicted using a PASS online web server. PASS is a computer program designed to predict the biological activities of chemical substances. The PASS online tool allows users to predict the pharmacological, biological and toxicological properties of organic substances by analyzing their chemical structures. This can be beneficial in the drug discovery and development process (35). The biological activity prediction of 7,8-DHF shows that it has the probability of anti-inflammatory and antioxidant activity using PASS online. The highest predicted score is antioxidant activity with a score of 0.741, followed by anti-inflammatory activity with a score of 0.640, indicating that 7,8-DHF has promising anti-inflammatory and antioxidant activity. Notably, the prediction also specified 7,8-DHF as an inhibitor of tumor necrosis factor (TNF)-α expression with a score of 0.502, as shown in Fig. 2C. This suggests a possible mechanism of anti-inflammatory activity through the inhibition of TNF-α expression. Finally, this prediction also consists of a neurotrophic factor enhancer with a score of 0.248. Neurotrophic factor enhancer activity is important in the pathogenesis of stroke, which is reduced in stroke conditions. It is important to recognize that while PASS and other similar predictive methods are valuable for screening large databases of chemical compounds, they have limitations. Predictions are derived from the current information contained in the training data set, and the accuracy of these predictions depends on the quality and variety of the data used to develop the model. Experimental validation is critical to verifying and improving the expected biological activity of compounds.

Molecular docking of 7,8-DHF against Keap1. To determine the antioxidant effect related to the molecular prediction mechanism, molecular docking was performed by targeting Keap1. The interaction of the Keap1/Nrf2 complex is illustrated in Fig. 3A. Nrf2 appears to interact with the binding pocket of Keap1 in the middle of the protein. There are several key amino acid residues that play critical roles in the complex interaction between Keap1 and Nrf2. These are tyrosine (TYR)334, arginine (ARG)415, ARG483, serine (SER)508, glutamic acid (GLU)30, SER555 and SER602, as shown in Fig. 3B. To clarify whether the binding of 7,8-DHF can inhibit Keap1, a small-molecule inhibitor [(3~{S})-3-(4-chlorophenyl)-3-(2-oxidanylidene-1~{H}-pyridin-4-yl)propanoic acid] of Keap1 was used as a control in the present study (36). It was found that this small-molecule inhibitor can bind to the binding pocket site of the Keap1-Nrf2 interaction, thereby directly disrupting the Keap1/Nrf2 interaction, as shown in Fig. 3C. Before beginning molecular docking, redocking was performed to clarify the molecular procedure. The redocking result was good as it had the same configuration, which has an RMSD value <2 Å, as shown in Fig. 3D.

Following successful validation, molecular docking simulations were performed between 7,8-DHF and Keap1 proteins, whose value data, or ΔG (kcal/mol), are presented in Table III. The results of molecular docking revealed that 7,8-DHF can bind to Keap1 with a binding affinity

Table III. Binding affinity interaction of 7,8-DHF and Keap1.

Target protein (PDB ID)	Substance	Binding affinity (kcal/mol)	Number of clusters	Residue interaction	Grid box	Dimension	RMSD	Validation (<2 Å)
Keap1	7,8-DHF	-7.4	6	GLY364* ARG415* ILE461* GLY462* ARG483* GLY509* TYR525* GLN530* SER555* ALA556* TYR572* SER602 GLY603*	X: 16.212 Y: 63.988 Z: 27.113	X: 21 Y: 21 Z:21	1.8	Valid
	Control	-7.8	1	GLY462* GLY364* ARG415* ILE461* PHE478 ARG483* SER508 GLY509* TYR525* GLN530* SER555* ALA556* TYR572* GLY603*			1.2	Valid

Similar residues are indicated by an asterisk (*). 7,8-DHF, 7,8-dihydroxyflavone; Keap1, Kelch like ECH-associated protein 1; RMSD, root mean square distance; TYR, tyrosine; ARG, arginine; SER, serine; GLU, glutamic acid; ALA, alanine; GLN, glutamine; GLY, glycine; ILE, isoleucine; PHE, phenylalanine.

of -7.4 Kcal/mol. This binding affinity is lower than that of the control (-7.8 Kcal/mol). To confirm whether the binding is similar to the control at important residues, visualization was performed using ChimeraX and Discovery Studio. It was found that 7,8-DHF has a bond at the same site as the control ligand, as shown in Fig. 4A. This interaction appears to have an interaction with an important amino acid in Keap1, as shown in Fig. 4B. The control was found to be able bind to SER508, ARG483 and glutamine (GLN)530 in a hydrogen bond manner; isoleucine (ILE)461, phenylalanine (PHE)478, GLY462, glycine (GLY)509, GLY364, GLY603, SER555 and TYR572 with van der Waals; and TYR525, alanine (ALA)556, and ARG415 in a hydrophobic bond manner. While 7,8-DHF can bind to the Keap1 protein via GLY364, ILE461, GLY462, ARG483, GLY509, TYR525, SER555, TYR572, SER602 and GLY603 in a hydrogen bond manner, while ARG415, GLN530 and ALA556 have hydrophobic bond modes. Almost all bonds formed between 7,8-DHF and Keap1 are similar to the control, except for SER602, as shown in Fig. 4C.

MD simulations 7,8-DHF against Keap1. Based on the molecular dynamic analysis of 7,8-DHF and control against Keap1. It was found that 7,8-DHF was slightly more unstable than the control. The data revealed that the Keap1 protein complex against the control had an average RMSD fluctuation of 0.8 angstrom (Å) with an equilibrium of 5 nsec, while 7.8 DHF has an RMSD fluctuation from 2 to 6 ns and an equilibrium from 6 to 9, with an average value of 0.81 Å, as shown in Fig. 5. This indicates that there is no notable difference in the stability interaction between 7,8-DHF and the control.

Discussion

As aforementioned, the Keap1/Nrf2 complex plays a critical role in stroke-ischemic situations by regulating cellular defense against oxidative stress and promoting the activation of genes involved in antioxidant and detoxification pathways, such as superoxide dismutase (SOD), catalase (CAT) and glutathione or GST (37,38). Under conditions

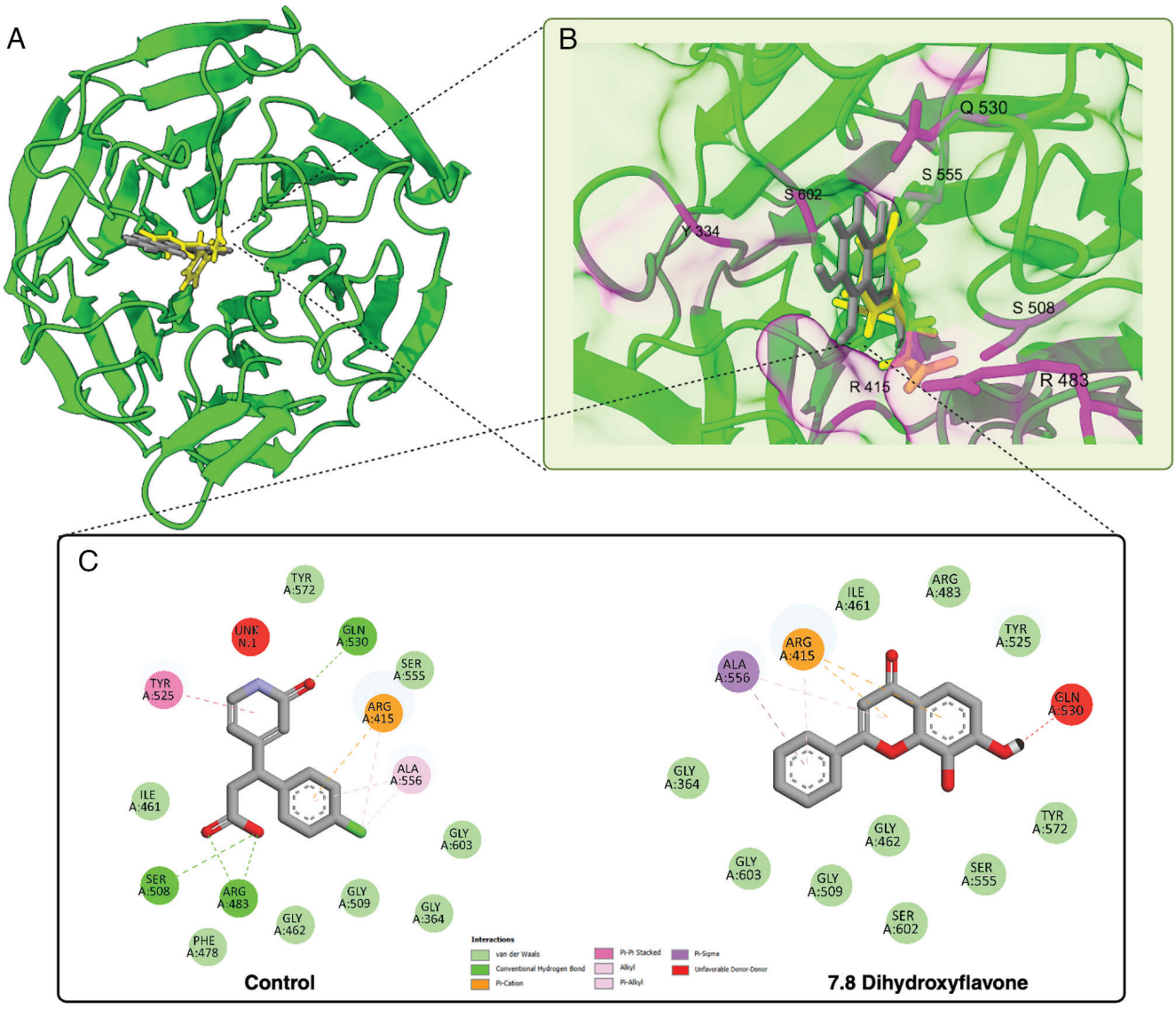

Figure 4. The binding interaction of 7,8-DHF with Keap1. (A) Superimposed image of 7,8-DHF (grey) and control (yellow) in Keap1. 7,8-DHF has a similar binding with the control. (B) Detailed visualization interaction 7,8-DHF with important residue in Keap1 protein. 7,8-DHF has near interaction with a key residue in Keap1. (C) Comparison of the interaction of 7,8-DHF and the control. 7,8-DHF has several similar residues in interaction with the control. 7,8-DHF, 7,8-dihydroxyflavone; Keap1, Kelch like ECH-associated protein 1; Nrf2, nuclear factor erythroid 2-related factor 2.

of oxidative stress, BDNF is also modulated by activated Nrf2 (39). BDNF has been found to play a role in regulating neural function, plasticity and neurogenesis in individuals who have survived ischemic stroke (40). The data of the present study demonstrated that the administration of 7,8-DHF increased the expression of Nrf2. Nrf2 translocates to the nucleus and induces the production of antioxidants. Antioxidant response elements (AREs) are specific enhancer sequences found in gene regulatory regions that Nrf2 targets. These AREs play a critical role in facilitating the recruitment of key transcription factors (41). The Nrf2 protein has a significant impact on the regulation of ~500 genes. These genes are responsible for encoding various proteins that play key roles in redox homeostasis, detoxification processes, stress response mechanisms and metabolic enzyme activities (42,43).

The result of increasing Nrf2 expression will increase the antioxidant regulatory genes such as SOD, CAT and GPX-1, which may improve oxidative stress conditions in ischemic stroke (44). GPX-1 plays a crucial role as an antioxidant enzyme in reducing intracellular hydrogen peroxide accumulation. This enzyme is ubiquitous within cellular structures, including the cytosol, mitochondria and certain cells. Previous studies have demonstrated that it is superior to CAT in removing intracellular peroxides in a number of physiological conditions (45,46). The effects of GPx-1 on various processes involving oxidants have been extensively studied. GPX-1 has been studied in the context of disease and tissue injury processes, such as atherogenesis, drug toxicity and ischemia-reperfusion injury (44).

Based on the measured MDA levels in the present study, the administration of 7,8-DHF also improved the oxidative stress condition, as evidenced the significant decrease in MDA levels in the MCAO20 and MCAO50 groups. According to the study by Wei *et al* (47), MDA levels as a marker of oxidative stress are inversely proportional to BDNF and

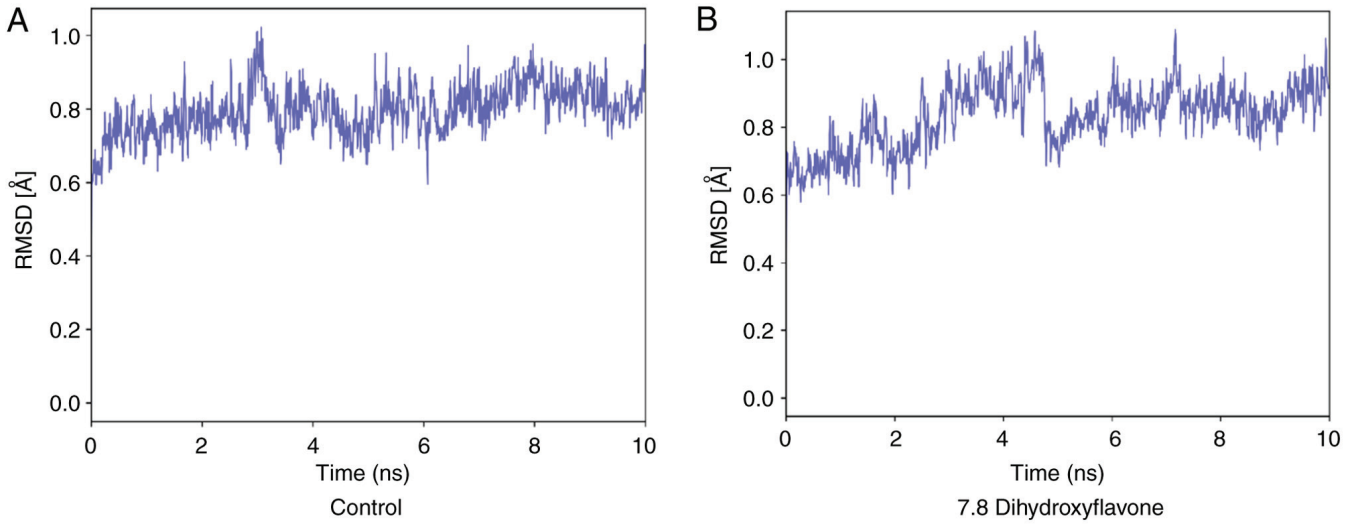

Figure 5. Molecular dynamic simulations of 7,8-DHF and Keap1. (A) Root mean square distance of control and Keap1. (B) Root mean square distance of 7,8-DHF and Keap1. 7,8-DHF, 7,8-dihydroxyflavone; Keap1, Kelch like ECH-associated protein 1; Nrf2, nuclear factor erythroid 2-related factor 2.

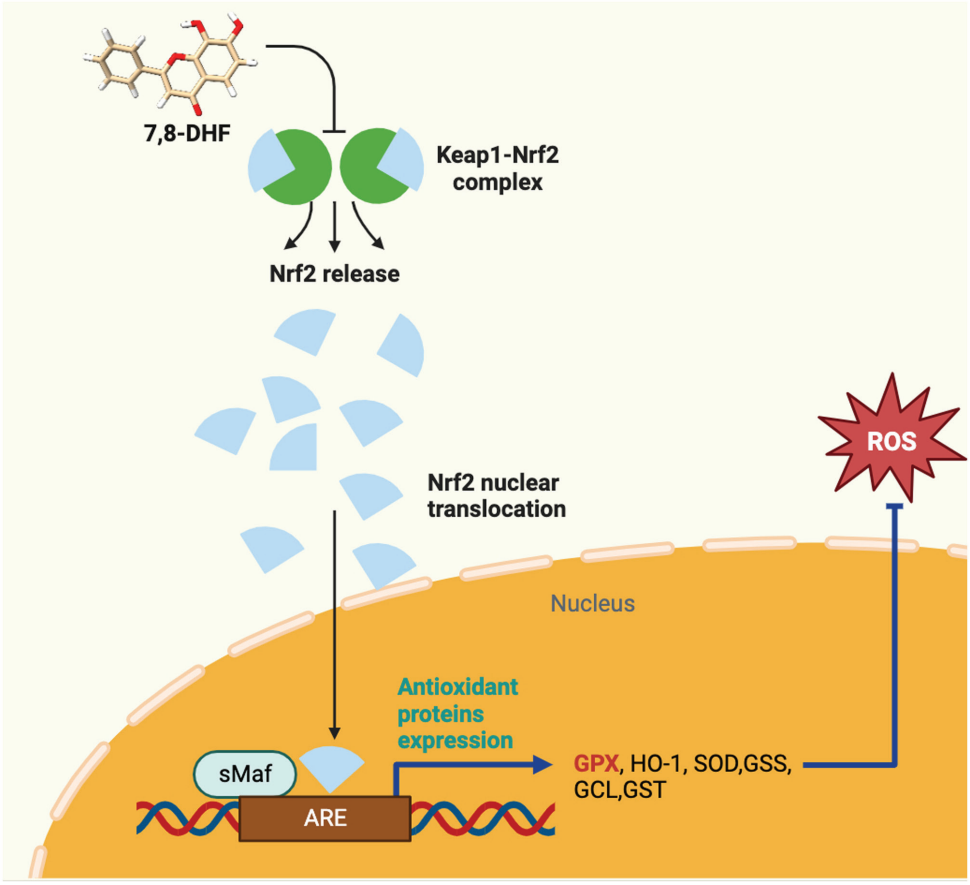

Figure 6. Proposed mechanism of 7,8-DHF as an antioxidant in ischemic reperfusion brain injury rat model. 7,8-DHF inhibits the Keap1 interaction, thereby stabilizing Nrf2, which accumulates in the cytoplasm and then translocates to the nucleus. Nrf2 interacts with the ARE, which then activates the transcription of genes involved in antioxidant defense, detoxification, and cell protection, such as HO-1, GPX, SOD and various phase II detoxification enzymes. 7,8-DHF, 7,8-dihydroxyflavone; Keap1, Kelch like ECH-associated protein 1; Nrf2, nuclear factor erythroid 2-related factor 2; ARE, antioxidant response element; HO-1, heme oxygenase-1; GPX, glutathione peroxidase; SOD, superoxide dismutase; GST, glutathione S-transferase; GCL, glutamate-cysteine ligase; GSS, glutathione synthetase.

SOD levels in patients with chronic schizophrenia, and the expected compensation does not occur. If there is a condition of abnormal BDNF levels accompanied by a disturbance in redox homeostasis (oxidative stress), this condition will also cause disruptions in neurotrophic processes and neuron development (47). From the aforementioned result, the dose of

50 mg/kg body weight 7,8-DHF was the optimal dose among all parameters. Thus, 7,8-DHF has antioxidant activity by increasing Nrf2 and GPX-1 expression, while decreasing MDA levels.

The prediction of biological activity was in agreement with the experimental result. This prediction was also in agreement with the findings of previous studies. The study by Park *et al* (15) demonstrated that 7,8-DHF exhibited anti-inflammatory properties via the downregulation of the NF-κB and MAPK pathways in lipopolysaccharide-treated RAW264.7 cells. In addition, 7,8-DHF functions as a potent TrkB agonist and a potent antioxidant through direct free radical scavenging and increased SOD activity. It significantly inhibits 6-hydroxydopamine-induced oxidative damage, preventing cell death, apoptosis and mitochondrial dysfunction (48).

Molecular docking revealed that 7,8-DHF could bind to the Keap1 protein. 7,8-DHF can bind to key amino acid residues in Keap 1. 7,8-DHF had amino acid residue similarity with the control (>80%), indicating it has a biological function (49). The molecular dynamic result indicates that there is no notable difference in stability interaction between 7,8-DHF and control, suggesting that it has an equal stability interaction with Keap 1. It was expected that 7,8-DHF may function as a Keap1 inhibitor similar to the control.

7,8-DHF has been shown to function as a TrkB agonist (16). It activates TrkB receptors and induces downstream signaling pathways that promote cell survival, growth, differentiation and plasticity. Additionally, 7,8-DHF has demonstrated neuroprotective properties and has been found to restore motor function deficits in a mouse model of Parkinson's disease (11,19,48). 7,8-DHF has also been reported to upregulate heme oxygenase 1 (HO-1) to protect PC12 cells against 1-methyl-4-phenylpyridinium-induced cytotoxicity (48). The data of the present study demonstrated that 7,8-DHF administration increased GPX-1 expression, thus contributing to decreased oxidation processes in pathological conditions. This supported the data demonstrating that it also upregulated GPX-1. Thus, it is possible that 7,8-DHF regulates the antioxidant mechanism at the upper site of GPX-1 and HO-1. Thus, it is suggested that a mechanism responsible for the antioxidant effects of 7,8-DHF may be the inhibition of Keap1, which allows the translocation of Nrf2 to induce antioxidant production, as shown in Fig. 6.

A limitation of the present study is that the analyses performed were not sufficient to judge that 7,8-DHF can directly inhibit the interaction of the Keap1/Nrf2 complex; thus, further studies are warranted to clarify the underlying mechanisms in a more in-depth manner. However, the present study demonstrated that 7,8-DHF increases Nrf2 and GPX-1 expression, while decreasing the MDA levels. Molecular docking elucidated the possible mechanisms and interactions that occurred in the *in vivo* results. Further follow-up studies, such as the experimental identification of interactions and the visualization of the model using X-ray or cryo-EM, are required to clarify these results and confirm the potential of 7,8-DHF as a novel therapy for ischemic stroke through the inhibition of Keap1. In addition, experimental toxicity studies are required to further investigate the safety of 7,8-DHF.

In conclusion, from the findings presented herein, it can be concluded that 7,8-DHF has antioxidant activity through the Keap1 inhibition, thereby increasing Nrf2 expression. 7,8-DHF can decrease the levels of MDA. It can also increase the expression of GPX-1 in brain tissue. Molecular docking also revealed a promising result; 7,8-DHF can inhibit the interaction of the Keap1/Nrf2 complex. However, further studies are warranted to clarify the mechanisms of 7,8-DHF in ameliorating other biomarkers of stroke. Therefore, further more advanced studies on 7,8-DHF are required to clarify the findings of the present study.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YY and MFRS were involved in the conception and design of the study, in data collection and analysis, and in the writing, revising and reviewing of the manuscript. DBK, AAA, DMA, YNS, RFM and BA were involved in data collection and analysis, and in the writing of the manuscript. AHV, SDA, AH, EN, YF, AM, KAS, URZ, DYP and SU were involved in data analysis, and in the writing, revising and reviewing of the manuscript. YY and MFRS confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was conducted by following the University of Brawijaya animal ethics guidelines and was approved by the Ethics Committee (No. 160/EC/KEPK/09/2020), Faculty of Medicine, Brawijaya University, Malang, Indonesia.

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 (QuillBot Premium; https://quillbot.com/) were used to improve the

readability and language of the manuscript or to generate images, and subsequently, the authors revised and edited the content produced by the AI tools as necessary, taking full responsibility for the ultimate content of the present manuscript.

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