Integrated molecular docking, dynamic simulations and *in vivo* analysis of ethanol extract *Citrus sinensis* peel as an antioxidant and neurotrophic agent for ameliorating motor and cognitive functions in traumatic brain injury

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Abstract. Traumatic brain injury (TBI) is the primary injury to the brain caused by mechanical forces. It is one of the leading causes of mortality in mid-life. Increased levels of inflammation and oxidation, and decreased levels of neurotrophic factor lead to a worsening of the condition. In previous studies, the authors demonstrated that the ethanol extract of *Citrus sinensis* peel (CSPE) functioned as an antioxidant using molecular docking and dynamic simulations. In the present study, in order to confirm these findings, a Marmarou weight drop model which included rats with TBI. Molecular docking against c-Jun N-terminal kinase 3 (JNK3) was also performed. The rats were divided into the sham-operated, control and treatment groups. The treatment groups received CSPE at 50, 250 and 500 mg/kg body weight (groups 1, 2 and 3, respectively) orally once a day for 7 days following the induction of TBI. Superoxide dismutase (SOD)1, SOD2, and brain-derived neurotrophic factor (BDNF) expression levels were analyzed using immunohistochemistry. Surviving neurons were analyzed using hematoxylin and eosin staining. Barnes' maze and wire grip tests were performed on the 1st, 3rd and 7th days following TBI. The results revealed a significant increase in the numbers of surviving neurons and in BDNF expression in groups 2 and 3, a higher expression of SOD1 in group 3, and a higher expression of SOD2 in all the treatment groups. Wire grip duration was significantly longer on the 7th day following TBI in group 3, and Barnes maze latency was significantly shorter on the 3rd and 7th days following TBI compared to the previous time in all treatment groups. Molecular docking analysis also revealed that CSPE compounds, particularly chanoclavine and nootkatone, interacted with JNK3 in the same binding pocket. On the whole, the present study demonstrates that CSPE helps protect the brain from damage by functioning as an antioxidant, increasing neurotrophic activities and inhibiting apoptosis. This reduces the overall neurological issues that are associated with TBI. However, further studies on the efficacy and safety of CSPE are required in order to determine its full potential in improving motor and cognitive functions following TBI.

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Abbreviations: TBI, traumatic brain injury; BDNF, brain-derived neurotrophic factor; SOD, superoxide dismutase; CSPE, *Citrus sinensis* peel extract; IHC, immunohistochemistry; H&E, hematoxylin and eosin; LC-HRMS, liquid chromatography high-resolution mass spectrometry; PBS, phosphate-buffered saline; T, Retention time; m/z, mass to charge; HMF, heptamethoxyflavone; NMDA, *N*-methyl-D-aspartate; cAMP, cyclic adenosine monophosphate; CREB, cAMP response element-binding protein; JNK3, c-Jun N-terminal kinase 3; Keap1, Kelch like ECH associated protein 1; Nrf2, nuclear factor erythroid 2-related factor 2; BDNF, brain-derived neurotrophic factor

Key words: traumatic brain injury, superoxide dismutase, brain-derived neurotrophic factor, molecular docking

Introduction

Traumatic brain injury (TBI), occurs due to primary damage to the brain induced by mechanical forces on the head and brain through various mechanisms. Secondary injury due to TBI involves a variety of complex biochemical cascades, such as oxidative stress, glutamate excitotoxicity and neuroinflammation, leading to neuronal cell death (1). Mitochondrial dysfunction has been reported to be a key pathway for neuroinflammation, resulting in the accumulation of reactive

oxygen species (ROS) (2). Another manifestation of TBI is an impairment in fine motor control and coordination, and difficulty with balance. TBI often leads to disability, with motor weakness as its main symptom (3).

Brain-derived neurotrophic factor (BDNF) comprises a family of neurotrophic proteins secreted by autocrine factors, which are essential for the survival, differentiation and regeneration of neuronal cells. BDNF has been reported to reduce secondary brain injury through neuroprotection mechanisms and to restore connectivity (4). Previous studies have reported clinical improvement with BDNF-related treatment in TBI. Studies increasing BDNF expression using protein kinase-like endoplasmic reticulum kinase antagonist (5), the direct use of collagen-domain bond BDNF (6) and the activation of BDNF receptor using 7,8-dihydroxyflavone have demonstrated the promotion of neuron growth and an improvement in overall neurological function (7,8).

There are three types of superoxide dismutase (SOD) in humans; SOD1 is present in various parts of mammalian cells, while SOD2 is localized to the mitochondria and efficiently eliminates superoxide, and has been reported to be induced in several inflammatory conditions in the central nervous system (9). Neurological and clinical improvements have been reported with SOD-related treatment in previous studies. Previous studies treating animals with TBI with SOD mimetic (10), exogenous lecithinized SOD (11) and the transgenic increment of SOD activity have revealed an improvement in hemodynamic recovery, more viable neurons, and an improvement in neurological function following TBI (12).

c-Jun N-terminal kinase (JNK)3 has been found to play a key role in the etiology of brain damage. JNK3 is activated in the brain in response to TBI, stroke and ischemia, resulting in the promotion of apoptosis, inflammation and oxidative stress. JNK3 activation can induce the production of pro-inflammatory cytokines and chemokines, which can aggravate brain damage by boosting immune cell recruitment and the activation of microglia. JNK3 can also trigger the generation of ROS, which can further harm neurons and other brain cells (13).

Citrus sinensis (*C. sinensis*) has various compounds, such as flavonoids, that have been identified in citrus. Flavonoids have a variety of biological benefits, such as antioxidant and anti-inflammatory effects (14). A previous study by the authors demonstrated the potential antioxidant effects of *C. sinensis* extracts using molecular docking analyses and dynamic simulations (15). However, the neurotrophic, antioxidant and anti-apoptotic potential of *C. sinensis* extract has not been investigated to date, at least to the best of our knowledge. The present study thus aimed to investigate the neurotrophic, antioxidant and anti-apoptotic effects of *C. sinensis* peel extract (CSPE) using *in vivo* and *in silico* analyses.

Materials and methods

Phytochemical extraction. CSPE was used according to a previous study by the authors (15). The metabolite compound of CSPE was published at MetaboLights (https://www.ebi.ac.uk/metabolights/) with the identifier no. MTBLS5785.

Molecular docking analysis. Molecular docking analysis was performed using Pyrx 9.5 (https://pyrx.sourceforge.io/) as previously described (15). In the present study, JNK3 (PDB ID: 7KSK) was targeted using several compounds, such as linoleic acid (CID: 5280450), tangeretin (CID: 68077), nootkatone (CID: 1268142), alminoprofen (CID: 2097), chanoclavine (CID: 5281381) and scoparone (CID: 8417) that have been previously pharmacokinetically analyzed (15). THE 2D visualization of compounds and protein target interaction was performed using Discovery Studio (https://discover.3ds.com/discovery-studio-visualizer-download). The strongest interaction was analyzed using molecular dynamics (16,17).

Molecular dynamic analysis. We performed molecular dynamic simulations of the complex interaction of chanoclavine and nootkatone with JNK using the OpenMM engine and the AMBER force field for protein and ligand systems in the Google Collaboratory (https://colab.research.google.com/?utm_source=scs-index). The model was thoroughly solvated with TIP3P water models108 in a 12x12x12 water box and neutralized by the addition of NaCl at a concentration of 150 mM. The simulation was performed using the force field ff19SB. The system was minimized for 1,000 steps and equilibrated in the isothermal-isobaric (NPT) ensemble for 20 nsec with a timestep of 2 fsec to achieve temperatures and pressures in the 310 K and 1 atm ranges, respectively. The temperature was controlled using the modified Berendsen thermostat with a coupling time of 0.1 psec, while the pressure was controlled by Parrinello-Rahman with a reference pressure of 1.0 bar and a compressibility of 4.5Xe-5 bar -1. The periodic electrostatic interactions were computed using particle mesh Ewald (PME) summation with a grid spacing <1 Å. The protein was unconstrained during the MD simulation (18). In the present study, the Radius gyration (Rg), root mean square distance (RMSD) and root mean square fluctuation (RMSF) were evaluated.

Experimental and animal design. A total of 30 *Rattus norvegicus* rats aged 8-12 weeks, weighing 300-450 g, were sourced from the Animal Research Laboratory, Medical Faculty, Brawijaya University, Malang, Indonesia. The weight and age data of the rats are presented in Table SI. The animals were housed in a controlled environment and acclimatized at room temperature (21-25˚C) and humidity (45-50%) with 12-h light/dark cycle for 2 weeks (19). The rats were monitored once daily and were provided with standard food and water *ad libitum*. The rats were randomly divided into 5 groups using simple randomization, as shown in Table I. The minimum sample size was 20 rats in total (at least 4 rats in each group), calculated using the Federer formula. CSPE was orally administered for 7 days following the induction of TBI. The animals were subjected to wire grip and Barnes maze tests on the 1st, 3rd and 7th days following the induction of TBI. Following treatment, the rats were sacrificed using ketamine (40-100 mg/kg) and xylazine (5-13 mg/kg) as anesthetics, followed by decapitation. Various criteria, such as self-mutilation, amputation or crush injury of the limbs and tail, generalized dermatitis, sepsis and severe dehydration, were also used to determine the indication of euthanasia. The death of the animals was verified by the absence of respiration, the absence of a heartbeat, pupillary dilation, the absence of reflexes, the poor color of extremities and mucous membranes, and dry and opaque corneas. The study experimental design is

Table I. Experimental animal groups in the present study.

Group	Treatment
Sham	Normal diet + normal saline for 7 days
TBI	TBI induction + normal diet + normal saline for 7 days
Treatment group 1	TBI induction + normal diet for 7 days + CSPE administered orally at a dose of 50 mg/kg body weight for 7 days
Treatment group 2	TBI induction + normal diet for 7 days + CSPE administered orally at a dose of 250 mg/kg body weight for 7 days
Treatment group 3	TBI induction + normal diet for 7 days + CSPE administered orally at a dose of 500 mg/kg body weight for 7 days

TBI, traumatic brain injury; CSPE, *Citrus sinensis* peel extract.

illustrated in Fig. 1A. Brain samples were stored and placed in a buffered formalin solution for fixation. Further examinations were performed using histological analysis and immunohistochemistry (IHC). The present study was conducted under international ethical principles and the National Institutes of Health's Guide for the Care and Use of Laboratory Animals of Medical Faculty, Brawijaya University.

Induction of TBI. TBI was induced according to a previously developed weight drop model to produce mild brain injury (16). Briefly, the rats were anesthetized using ketamine (40-100 mg/kg) and xylazine (5-13 mg/kg). The scalp was cleaned with 10% povidone-iodine, and 10% lidocaine HCl was then injected as a local anesthetic. The scalp was incised longitudinally at the midline of the skull with a diameter of 5 mm to expose the skull underneath, between the lambda and bregma sutures. A steel plate (2-mm-thick) was fixed on the exposed skull. Subsequently, 175 g of weight was dropped onto the steel plate through a 30-cm-long cylinder, and the scalp of the rats was stitched back, as shown in Fig. 1B, and as previously described (20,21). The tats in the sham-operated (sham) group only underwent scalp incision and stitching.

Spatial memory and motor test. Barnes maze test, adapted from Krishna *et al* (7), was performed to analyze the rat's spatial memory, as illustrated in Fig. 1C. The diameter of the circular platform was 160 cm, with 20 circular holes equally sawed 5.5 cm from the platform's edge. The holes were each 8 cm in diameter and were equally distanced at 16 cm from each other. The escape box was made from plexiglass (30x15x15 cm) and positioned under the selected escape hole (7).

The wire grip test was performed as illustrated in Fig. 1D. A wire grip apparatus with a large plastic box (55x40x35 cm) and a 2-mm-thick wire attached to two vertical stands. Bedding material with a distance of 35 cm below the wire was used to prevent injury to the animal (22). The investigator allowed the rat to hang from the wire using only its forelimbs. As soon as the rat held the wire properly, the timer began. The time taken for the rat to keep its limbs on the wire was recorded. If the rat fell, the timer stopped.

Surviving neuron cell count using hematoxylin and eosin staining (H&E). The rat brains were fixed in 4% paraformaldehyde (Path Chem, BBC Biochemical) for 24 h at 24˚C. The fixed brain tissues were embedded in paraffin and sliced coronally. The sliced brain tissues were then stained with hematoxylin for 20 min and eosin for 3 sec at 24˚C (Leica Biosystems Inc.). Stained slides were analyzed under a light microscope at x400 magnification (BX51, Olympus Corp.). The numbers of surviving neurons per high power field (hpf) were counted. Non-surviving neurons were identified by their shrunken and thickened nuclei (23).

IHC. The brain tissues were deparaffinized and stored for 24 s at room temperature prior to use. The brain tissues were then washed using phosphate-buffered saline (PBS) and then incubated with normal rabbit serum at room temperature. The incubated brain tissues were washed with PBS and subsequently incubated at room temperature for 30 min with a secondary antibody (AMF080, ScyTek Laboratories, Inc.), washed with PBS, and developed with 0.05% diaminobenzidine tetrahydrochloride. The primary antibodies used for IHC were anti-BDNF (cat. no. sc-65514,), anti-SOD1 (cat. no. sc-101523), and anti-SOD2 antibody (cat. no. sc-137254) (1:50; all from Santa Cruz Biotechnology, Inc.). The expression levels of BDNF, SOD1, and SOD2 were observed under a light microscope at x1,000 magnification in 20 fields of view (BX51, Olympus Corp.), as previously described (24).

Statistical analyses. Statistical analyses were performed using SPSS 26.0 software (IBM Corp.). All results are presented as the mean ± SD. The normality of the data was assessed using the Shapiro-Wilk test. The analyses of BDNF, SOD1, SOD2, Barnes maze latency and wire-closure time data were performed using one-way ANOVA with the Bonferroni post-hoc test.

Results

Molecular docking analysis of CSPE against JNK. Previously, the authors analyzed various compounds in CSPE that can penetrate the brain blood-barrier (BBB) (15). Herein, the binding affinity of the compounds with JNK protein was analyzed. It was found that chanoclavine and nootkatone had a stronger binding affinity compared to 1-(trans-4-{[7-oxo-8-(propan-2-yl)-7,8-dihydropyrido[2,3-d]pyrimidin-2-yl]

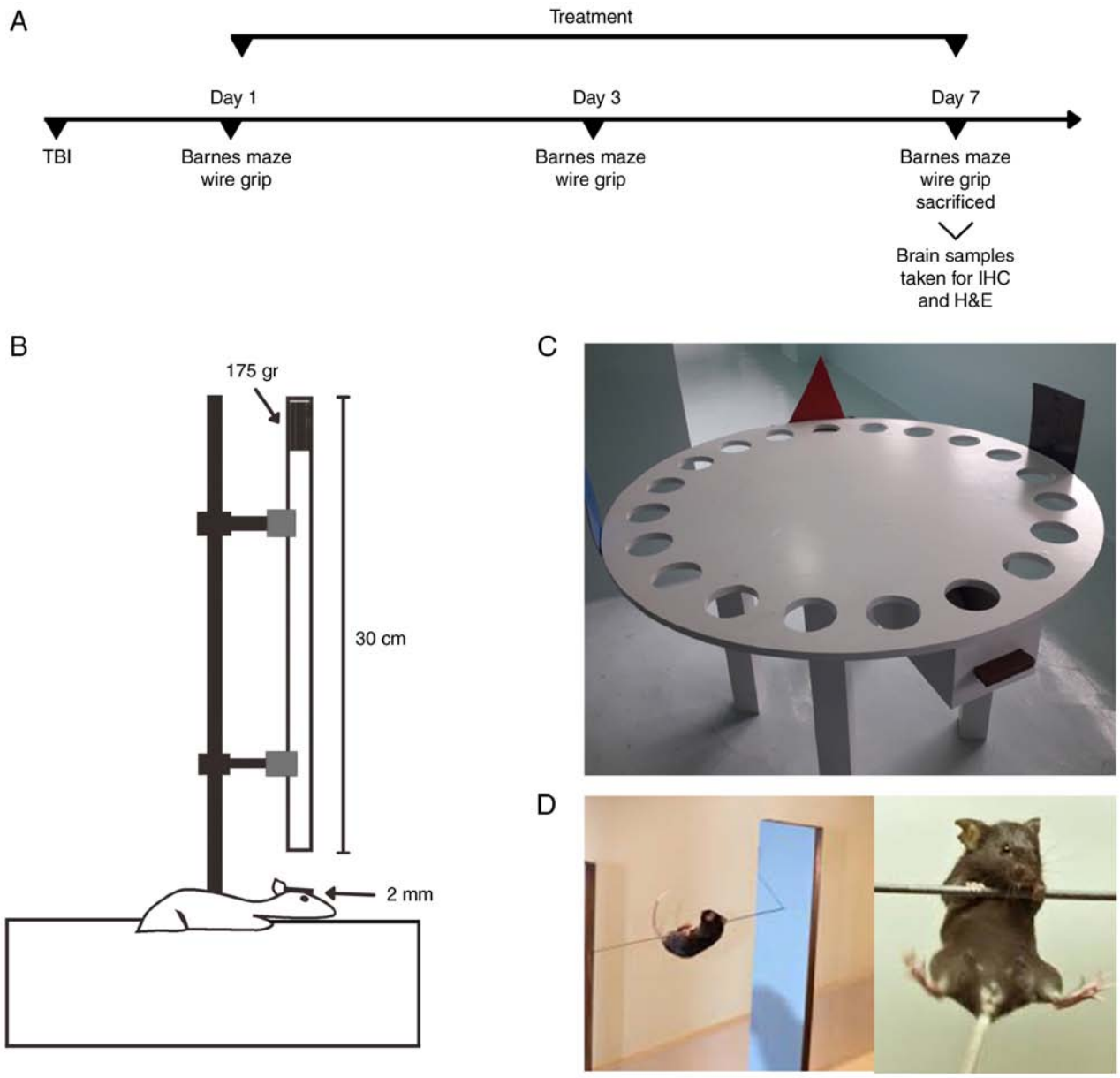

Figure 1. Experimental procedure and tools used in the present study. (A) Study timeline, (B) rat-induced traumatic brain injury illustration, (C) Barnes maze instrument, (D) wire grip test. TBI, traumatic brain injury; IHC, immunohistochemistry; H&E, hematoxylin and eosin.

amino}cyclohexyl)-3-propan-2-ylurea as a control, as shown in Table II. Molecular docking analysis identified that chanoclavine bound to JNK3 with the highest affinity compared to the control ligand and other ligands. Chanoclavine exhibited a binding affinity of -6.8 Kcal/mol, whereas nootkatone had a binding affinity of -6.6 Kcal/mol. The molecule with the lowest bond energy will have a constant temperature and pressure, and this is known as a stable molecule (19,23,24). The amino acid residues affect the binding domain of the target protein, as well as the sort of chemical interplays in the binding domain.

The different types of bonds found between chanoclavine, nootkatone and the target protein were analyzed. The type of interaction between the ligand and protein target is a critical parameter in predicting the effect of interactions (25,26). Chanoclavine attaches to JNK via six hydrophobic bond interactions, (ILE70, VAL78, ALA91, MET146, VAL196 and LEU206). Furthermore, nootkatone interacts with eight amino acid residues. Nootkatone created hydrogen bonds with ASN152 and hydrophobic bonds with the amino acids ILE70, ALA80, LEU148, MET149, VAL196, and LEU206, as shown in Fig. 2. The present study investigated the interaction with the control ligand and found similar amino acid residue interaction, as shown in Table II.

Molecular dynamic simulations analysis of CSPE against JNK. The Rg (radius of gyration), which is defined as the root mean square distance of the collection of atoms from their shared center of gravity, defines the general dispersion of the molecule. The radius of gyration values for protein-ligand complexes, illustrated in Fig. 3A, reveal that while chanoclavine was found to affect the secondary structure of the proteins, the radius of gyration of JNK remained constant following contact. The nootkatone Rg value was higher than that of chanoclavine, indicating that chanoclavine is more

Table II. Binding affinity interaction of CSPE compounds and c-Jun N-terminal kinase.

Compound	Target protein (PDB ID)	Binding affinity energy (kcal/mol)	Interacting residues	Type of bond	Grid box
Chanoclavine	JNK (4Y5H)	-6.8[a]	**Ile70**	Hydrophobic	Coordinates
			Val78	Hydrophobic	X: 1.161
			Ala91	Hydrophobic	Y: -29.628
			Met146	Hydrophobic	Z: -30.545
			Val196	Hydrophobic	
			Leu206	Hydrophobic	Radius
					X:10
					Y: 10
					Z: 10
Nootkatone		-6.6[a]	**Ile70**	Hydrophobic	
			Val78	Hydrophobic	
			Ala91	Hydrophobic	
			Met146	Hydrophobic	
			Met149	Hydrophobic	
			Asn152	Hydrogen	
			Val196	Hydrophobic	
			Leu206	Hydrophobic	
Tangeretin		-6.3	**Ile70**	Hydrophobic	
			Val78	Hydrophobic	
			Ala91	Hydrophobic	
			Lys93	Hydrophobic	
			Met146	Hydrophobic	
			Asn152	Hydrogen	
			Val196	Hydrophobic	
Alminoprofen		-5.5	**Ile70**	Hydrophobic	
			Ala80	Hydrophobic	
			Leu148	Hydrophobic	
			Met149	Hydrogen	
			Asn152	Hydrogen	
			Val196	Hydrophobic	
			Leu206	Hydrophobic	
Scoparone		-5.8	Val78	Hydrophobic	
			Ala91	Hydrophobic	
			Gln155	Hydrogen	
			Val196	Hydrophobic	
			Leu206	Hydrophobic	
Linoleic acid		-5.5	**Ile70**	Hydrophobic	
			Val78	Hydrophobic	
			Ala91	Hydrophobic	
			Leu148	Hydrophobic	
			Met149	Hydrophobic	
			Asp150	Hydrogen	
			Gln155	Hydrogen	
			Val196	Hydrophobic	
			Leu206	Hydrophobic	
1-(trans-4-{[7-oxo-8-(propan-2-yl)-7, 8-dihydropyrido[2,3-d] pyrimidin-2-yl]amino} cyclohexyl)-3-propan-2-ylurea (control)		-6.5	Ile70	Hydrophobic	
			Val78	Hydrophobic	
			Ala91	Hydrophobic	
			Met146	Hydrogen	
			Glu147	Hydrogen	
			Met149	Hydrophobic	

Table II. Continued.

Compound	Target protein (PDB ID)	Binding affinity energy (kcal/mol)	Interacting residues	Type of bond	Grid box
			GLn155	Hydrogen	
			Val196	Hydrophobic	
			Leu206	Hydrophobic	

[a]Indicates a binding affinity stronger than the control. Bold font indicates same amino acid residue interaction compared with the control. CSPE, *Citrus sinensis* peel extract

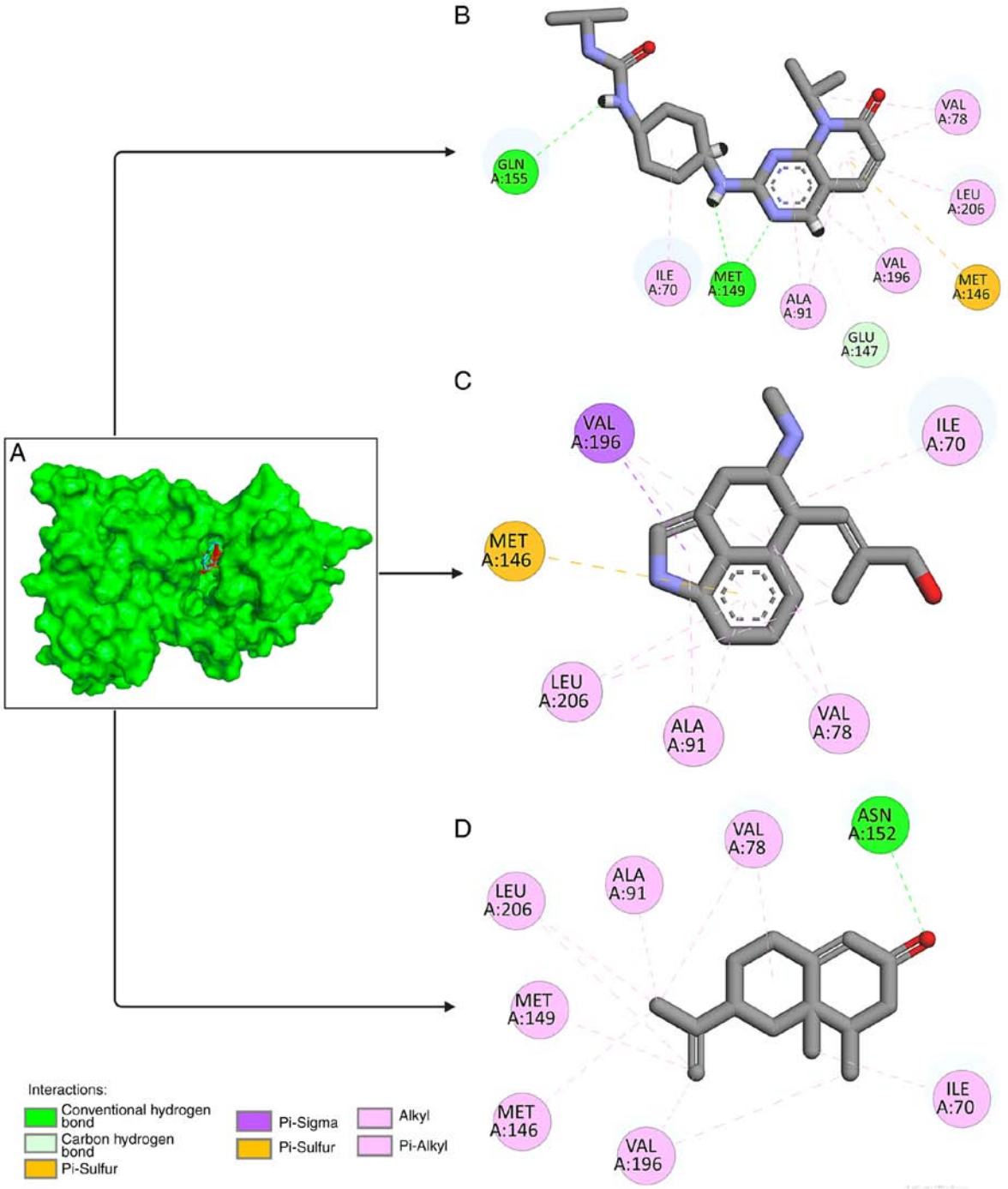

Figure 2. Amino acid residue interaction of the *Citrus sinensis* peel extract compound and JNK. (A) Binding pocket interaction of chanoclavine (red), nootkatone (orange) and the control (cyan) in the same binding pocket. (B) Amino acid residue of the control-JNK complex. (C) Amino acid residue of the chanoclavine-JNK complex. (D) Amino acid residue of the nootkatone-JNK complex.

Table III. Effects of traumatic brain injury and treatment with *Citrus sinensis* peel extract on wire grip duration and Barnes maze latency.

	Groups (mean ± SD)				
Parameter	Sham	TBI	Group 1	Group 2	Group 3
Wire grip duration (sec)					
D1	22.83±11.64	5.33±2.50	5.83±2.32	7.67±4.46	5.17±2.79
D3	26.50±12.82	12.00±4.00	16.67±8.82	13.67±4.97	14.33±6.65
D7	28.33±11.91	14.67±6.22	21.17±3.97	26.00±11.82	27.83±10.72[a]
Barnes maze latency (sec)					
D1	47.67±43.50	172.50±8.39	173.17±8.95	173.33±5.24	170.33±8.17
D3	42.67±38.90	114.00±38.58[a]	87.50±46.45[a]	83.00±27.49[a]	88.67±39.05[a]
D7	40.67±37.30	73.50±54.17	49.00±65.07[a]	37.17±24.91[a]	33.17±23.24[a]

[a]P<0.05 vs. previous time within the same group. TBI, traumatic brain injury; sham, sham-operated; group 1, 50 mg/kg body weight CSPE; group 2, 250 mg/kg body weight CSPE; group 3, 500 mg/kg body weight CSPE.

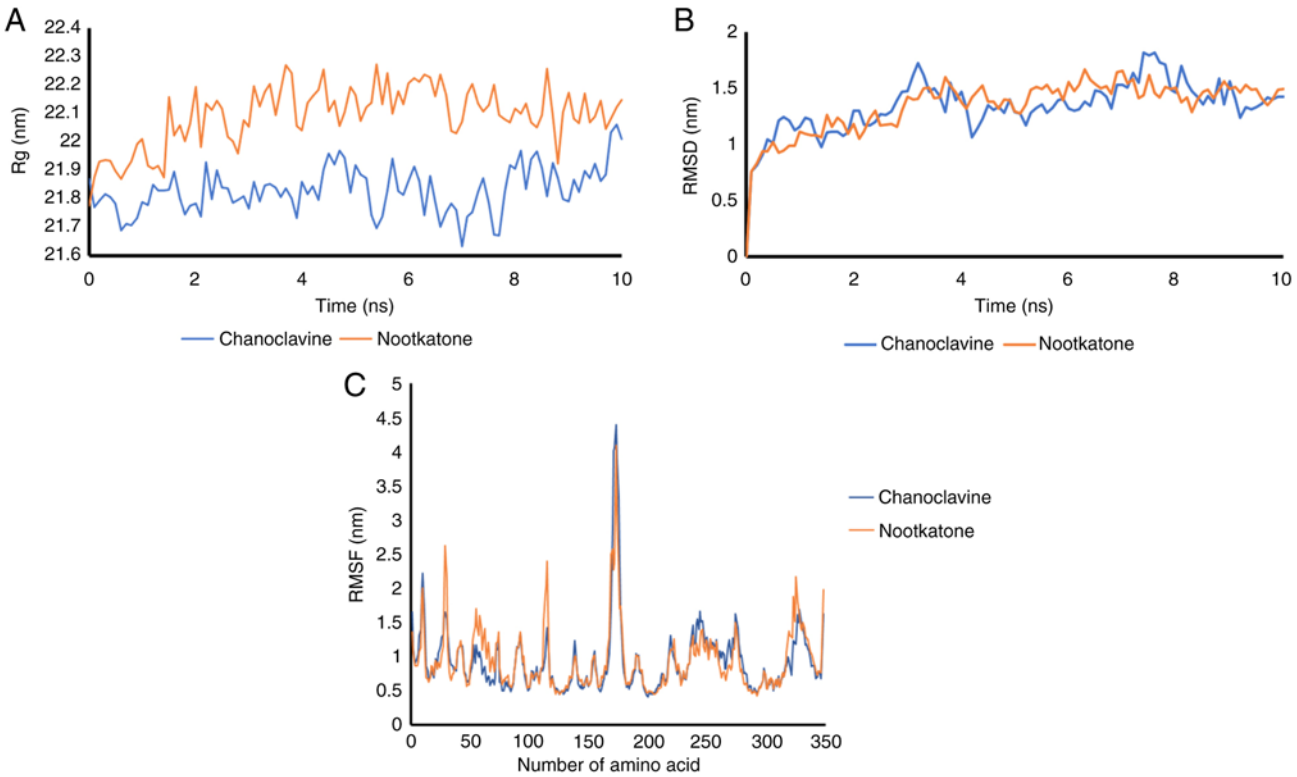

Figure 3. Molecular dynamic simulations of chanoclavine and nootkatone. (A) Radius gyration; (B) RMSD; (C) RMSF. RMSF, root mean score distance; RMSF, root mean square fluctuation.

compact than nootkatone. A lower radius of gyration indicates that the polymer is relatively compact, implying that the polymer spends the majority of its time folded along its course.

The JNK protein complex with chanoclavine had average RMSD fluctuations of ~1.2 nm, with an equilibrium of 6 nsec. The RMSD of he nootkatone and JNK complex had an unstable fluctuation from 0 to 10 nsec. Similarly, substantial RMSD deviations were reported for the nootkatone-Kelch like ECH associated protein 1 (Keap1) complex, indicating that the complex generated was unstable, as shown in Fig. 3B.

Subsequently, the RMSF was investigated. RMSF was monitored at 10-nsec intervals to calculate residual flexibility. The complex interaction combination had a wavelength ~0.75 nm for each residue, whereas it had the most unstable residue at residue number 170-177 with a RMSF value of >2.5 nsec, as shown in Fig. 3C.

Effect of CSPE on spatial memory and motor performance. A total of 30 rats were used in the present study, and no deaths occurred during the study period. The results revealed

Table IV. Effects of traumatic brain injury and treatment with *Citrus sinensis* peel extract on surviving neurons, and on SOD1, SOD2 and BDNF expression.

	Groups (mean ± SD)					
Parameters	Sham	TBI	Group 1	Group 2	Group 3	P-value
Surviving neurons	42.50±20.95	17.67±11.09	25.00±13.84	30.67±5.85[b]	41.00±14.04[b]	0.024
SOD1	49.67±15.31	60.67±11.20	64.33±5.39	72.33±7.92[a]	82.00±17.55[a,b]	0.002
SOD2	56.17±13.18	60.67±9.67	76.33±11.22[a,b]	77.50±11.08[a,b]	93.17±19.95[a,b]	0.001
BDNF	53.00±13.33	68.83±9.75	75.33±8.50[a]	86.67±13.10[a,b]	113.83±11.27[a,b]	<0.001

[a]P<0.05 vs. the sham group; [b]P<0.05 vs. the TBI group; TBI, traumatic brain injury; BDNF, brain-derived neurotrophic factor; SOD, superoxide dismutase; sham, sham-operated; group 1, 50 mg/kg body weight CSPE; group 2, 250 mg/kg body weight CSPE; group 3, 500 mg/kg body weight CSPE.

a significant difference in wire grip duration within group 3 (Table III). The difference was observed on the 3rd day following TBI compared to the 7th day following TBI. The mean latency in all groups treated with CSPE improved progressively from the 1st to the 7th day following the induction of TBI.

A downward pattern in the mean Barnes maze latency values was observed in all groups, gradually decreasing from the 1st to the 7th day following TBI. In the sham group, there was no significant difference in the Barnes maze latency between each test within the group. Significant differences were found in all groups treated with CSPE, with significantly lower Barnes maze latency values on the 3rd day compared to the 1st day following TBI, and on the 7th day compared to the 3rd day following TBI. The results of the Barnes maze test are presented in Table III.

Surviving neuron cell count. Neuron survival was assessed by cell counting using H&E-stained brain samples. Non-surviving neurons were identified by shrunken and thickened nuclei. The number of surviving neurons in groups 2 and 3 was significantly higher than those in the TBI group (P<0.05; Table IV). Staining images of the surviving neurons are presented in Fig. S1.

BDNF and SOD expression. The results of BDNF and SOD expression analyses are shown in Table IV. An increasing pattern in BDNF expression was observed with the increasing CSPE dosage. The results revealed a significantly higher BDNF expression in groups 2 and 3 compared to the TBI group (P<0.05). There was an increasing pattern of SOD1 and SOD2 expression with increasing CSPE dosage. The results revealed a significantly higher SOD1 expression in group 3 compared to the TBI group, and a significantly higher SOD2 expression in all groups treated with CSPE compared to the TBI group (P<0.05). Staining images of BDNF, SOD1 and SOD2 expression are presented in Figs. S2, S3 and S4.

Discussion

The present study demonstrated the potential of CSPE to attenuate various neurological injuries following TBI. A significant increase in BDNF expression was found following treatment with CSPE. This is consistent with the findings of previous studies, as indicated below. Several compounds contained in CSPE have been examined for their effects in inducing neurotrophins, such as BDNF. Auraptene, a compound found in citrus, has been proven to activate the cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) pathway, resulting in the increased production of various neurotrophic factors, including BDNF (27). A significant increase in BDNF mRNA levels has been found in mouse neuroblastoma neuro2a cells (27). Sawamoto *et al* revealed a promoting effect of the heptamethoxyflavone (HMF) active compound in citrus on BDNF via the cAMP/ERK/CREB pathway. In their study, treatment of C6 glioma cells with 10 μM HMF resulted in an increase in cAMP levels, CREB phosphorylation and BDNF expression (28). Another citrus flavonoid, hesperidin, was previously found to stimulate BDNF (29). Hesperidin was used to treat zebrafish in a pentylenetetrazole-induced convulsion paradigm, which significantly increased seizure latency and decreased hyperactive responses. Hesperidin was found to have a good *N*-methyl-D-aspartate (NMDA) receptor binding affinity, according to an *in silico* investigation. NMDA receptor activation stimulated the CREB/BDNF pathway (29).

In the present study, a significant increase in SOD1 and SOD2 expression was found following treatment with CSPE. Previous studies have also shown the antioxidant effects of CSPE via increments in the levels of antioxidant enzymes. For example, a significantly higher SOD level was previously reported in rats with alcohol-induced peptic ulcers pre-treated with 200 and 400 mg/kg CSPE (30). The levels of other antioxidant enzymes, such as glutathione peroxidase and catalase were significantly increased in the rats pre-treated with similar dosages. A significant decrease in the levels of oxidative stress, indicated by a decrease in the levels of malondialdehyde and hydrogen peroxide was also observed in rats pre-treated with 100, 200 and 400 mg/kg CSPE (30). An increase in the levels of antioxidant enzymes has also been found in rats treated with CSPE in their diet. Erukainure *et al* (31) examined rats fed a diet consisting of 35% CSPE for 6 weeks. A significant increase in the levels of SOD and glutathione was observed following 6 weeks of treatment (31). In another study, in Wistar rats treated orally with a combination of *Citrus aurantifolia* and *Cinnamomum burmannii* at 100, 300 and 500 mg/kg, exhibited an increase in the levels of SOD following treatment (32).

In addition to inducing antioxidative enzymes, previous studies have shown that CSPE exerts antioxidant effects via various other mechanisms, such as oxidative enzyme inhibition, radical scavenging and metal chelating activity. The study by Malterud and Rydland (33) demonstrated an inhibitory effect on lipoxygenase (LOX) by various flavones of CSPE. In their study, various flavones that inhibited LOX could penetrate the BBB, such as sinensetin and tangeretin (33). Another study by Pepe *et al* (34) demonstrated that CSPE exerted an inhibitory effect on nitric oxide synthase.

Several compounds, such as vitamin E and carotenoid contained in CSPE are also known as singlet oxygen ($^1O^2$) scavengers (35). CSPE has the most radical scavenging ability compared to other citrus species, such as *Citrus aurantifolia* and *Citrus limonum* (36). In addition to its antioxidant effects, neuronal growth promoted by neurotrophic agents has been proven to be beneficial in TBI. Various studies have shown that treatment with SOD and its related pathways in TBI can lead to better neurological and clinical recovery, demonstrating the importance of oxidative stress control in TBI (10-12). In addition, previous studies using BDNF treatment or treatment with its related pathways have demonstrated that this treatment leads to an improvement in neurological recovery and better clinical function, highlighting the importance of neurological recovery accelerated by neurotrophic agents (5,7,8).

In the present study, the significant increase in the numbers of surviving neurons in rats in treatment groups 2 and 3 may be attributed to increased levels of antioxidants and neurotrophic expression. Significantly higher levels of antioxidant enzymes prevent further cellular damage from oxidative stress, while increased levels if neurotrophins lead to more rapid neuron repair and growth. However, a previous study by Chen *et al* (37) provided insight into another pathway. Their study demonstrated that the treatment of mice with optic nerve injury with various citrus flavones, such as naringenin, nobiletin and hesperidin led to increased retinal ganglion survival (37). In addition, in the same study, *in vitro* experiments using 293T cells, in which the JNK-JUN apoptotic pathway was activated by incubation with 500 mM sorbitol, revealed that treatment with 2.5 μM of naringenin resulted in a significant decrease in JUN phosphorylation and its subsequent apoptotic pathway (37).

In the present study, subsequent molecular docking analysis confirmed the potential effects of CSPE on the JNK pathway, with nookatone and chanoclavine exhibiting a strong binding energy, and similar amino acid interactions compared to the control. In its various isoforms, JNK is crucial for brain development, in regulating various functions such as cell death in early brain development, and neuronal migration and axon maintenance in later brain development. The most critical function of JNK in relation to TBI is its function in mediating stress-induced neuronal cell death in the adult brain. A previous study revealed that JNK knockout (KO) mice exhibited resistance to neuronal cell death induced by a neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (38). Other studies have also indicated that JNK KO mice are resistant to ischemia-induced neuronal cell death, and excitotoxicity-induced neuronal cell death by kainic acid (39,40). The JNK-mediated apoptotic pathway involves the nuclear translocation of JNK, activating various transcription factors such as c-Jun, resulting in the activation of various pro-apoptotic genes, and in the subsequent release of apoptotic proteins (41).

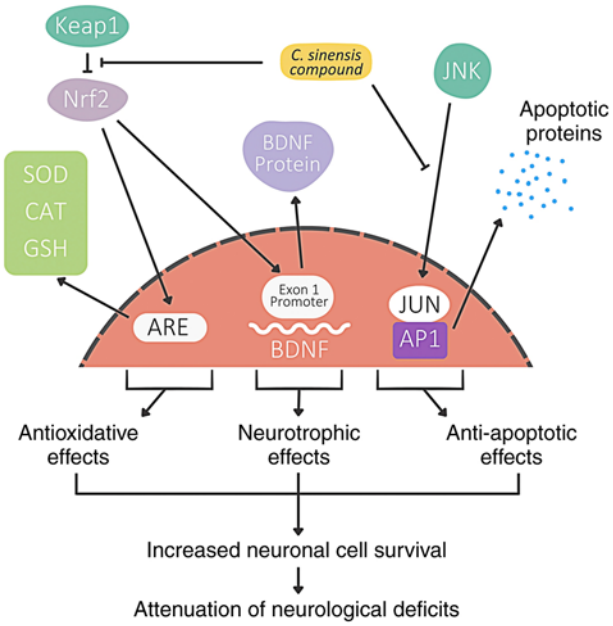

Figure 4. Various mechanisms of the protective effects of *Citrus sinensis* peel extract on traumatic brain injury. The figure was created using BioRender, licensed by Mokhamad Fahmi Rizki Syaban.

A previous study by the authors found that CSPE has potential to inhibit Keap1 activity (15). In the present study, the pathways suggested to be involved in the various mechanisms of the effects of CSPE on TBI are illustrated in Fig. 4. CSPE exerts antioxidant effects by inducing an increase in the levels of antioxidant enzymes, radical scavenging and metal chelating activity. CSPE also exerts neurotrophic activity via the activation of exon 1 promoter in the BDNF gene and CREB-BDNF pathway. Its antioxidant activity, combined with neurotrophic activity, and potential anti-apoptotic activity, lead to an increased neuronal cell survival and the consequent attenuation in motor and visuospatial memory deficits, as revealed herein.

In the presents study, motor and cognitive aspects assessed using wire grip and Barnes maze tests revealed clinical improvements following CSPE treatment. This finding is in accordance with the findings of previous studies. For example, a previous study by Liu *et al* (42) demonstrated a significant improvement in a grip strength test in model rats with spinal cord injury treated with trihydroxyethyl rutin. Another study by Zhu *et al* (43) revealed a significant improvement in Morris water maze escape latency in rats with TBI treated with docosahexaenoic acid (43). These findings prove the beneficial effects of neuroprotective treatment on clinical motor and cognitive improvements.

In the present study, various effects of CSPE on TBI were demonstrated by analyzing related components, such as SOD1, SOD2 and BDNF, which revealed the antioxidant and neurotrophic effects of CSPE. Molecular docking analysis and dynamic simulation also revealed the anti-apoptotic activity of the extract. While the present study delved into the antioxidant and neurotrophic effects of CSPE by analyzing components such as SOD1, SOD2 and BDNF, it should be noted that it

focused on a specific subset of pathways. There are numerous other pathways involved in antioxidant, neurotrophic and anti-apoptotic responses. The limited focus of the present study on these particular components suggests that other significant factors and interactions that may also have an impact on the effects of CSPE in TBI may have been overlooked, which limits the findings presented herein. The analysis of other component pathways needs to be conducted in the future in order to better understand the additional pathways involved in the effects of CSPE on TBI. Toxicity analysis should also be conducted in future studies in order to elucidate the aspects of the toxicity of different doses of CSPE.

In conclusion, the present study demonstrated the potential antioxidant effects of CSPE on TBI by significantly increasing the levels of SOD1 and SOD2, enhancing neurotrophic activities by significantly increasing BDNF, and attenuating overall neurological deficits, as evidenced by improvements in the wire grip and Barnes maze tests. Chanoclavine and nootkatone can also inhibit JNK to induce apoptosis, as revealed using molecular docking and dynamic simulation analyses. Future research however, is warranted to evaluate the efficacy and safety of CSPE in improving motor and cognitive functions in mouse models of TBI.

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Availability of data and materials

The data that support the findings of this study are available on the MetaboLights Compound Database (https://www.ebi.ac.uk/metabolights/MTBLS5785).

Authors' contributions

WMS, HS, and GFAP were involved in the conception and design of the study, data collection and analysis, and in the writing, revising and reviewing of the manuscript. MFRS, RAV, JPK and ISM were involved in the conception and design of the study, and in the revising and reviewing of the manuscript. WMS and HS 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 (Approval no. 84/EC/KEPK/04/2020), Faculty of Medicine, Brawijaya University, Malang, Indonesia.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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