Molecular docking analysis and dynamics simulation of ethanol extract of *Citrus sinensis* as a Keap1 and NMDA inhibitor in brain injury

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Abstract. Traumatic brain injury is a significant cause of mortality in young adults and disability in all age groups. This is followed by secondary injury involving various processes, such as oxidative stress which induce neuronal apoptosis. The present study aimed to analyze the active compounds of *Citrus sinensis* and predict its antioxidative activity in traumatic brain injury. Liquid chromatography/high resolution mass spectrometry (LC/HRMS) was used to identify compounds in the ethanol extract of *Citrus sinensis* peel. We analyzed pharmacokinetics, blood-brain barrier (BBB) permeability and the toxicity of active compounds in *Citrus sinensis* peel using SwissADME and OSIRIS. The compounds with a good BBB permeability were subjected to molecular docking analysis to identify the molecular interaction with Kelch-like ECH associated protein 1 (Keap1) and N-methyl-D-aspartate (NMDA) proteins using PyRx, PyMol and Discovery Studio software. The results of the LC/HRMS analysis revealed 16 active compounds contained in the ethanol extract of the orange peel. Nootkatone, alminoprofen, linoleic acid, chanoclavine, scoparone and tangeretin were predicted to pass the BBB. The active compounds of *Citrus sinensis* strongly bound against Keap1 and NMDA, particularly scoparone and nootkatone. The strong binding affinity of scoparone-Keap1 was -5.0, more than that of the control ligand, and that of nootkatone-NMDA was -7.8, similar to the control ligand. The active compounds of *Citrus sinensis* peel exhibited inhibitory potential, good pharmacokinetics and toxicity profiles against Keap1 and NMDA. On the whole, these findings suggest that ethanol extract of *Citrus sinensis* peel has potential for use as an oxidative stress inhibitor in the treatment of brain injury.

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Introduction

Brain injury, such as stroke and traumatic brain injury (TBI), is one of the main causes of mortality worldwide. It is a substantial cause of mortality and disability among young and middle-aged individuals worldwide (1,2). Brain injury includes molecular cascades that disseminate to neighboring tissues, a condition known as secondary brain injury. Primary injury in the brain is irreversible; however, secondary damage that subsequently develops is responsive to therapeutic interventions (3,4).

Brain injury is a molecular pathophysiological sequence linked to excitotoxicity, cell death and oxidative stress. Brain injury destroys the blood-brain barrier (BBB), causing excessive glutamate release, which activates N-methyl-D-aspartate (NMDA) receptors and induces neuronal depolarization. However, shear and strain forces from a head injury may activate NMDA receptors. Excessive NMDA receptor activation causes excessive Ca^{2+} and Na^{+} entry into the cell. Excessive Ca^{2+} influx in the cytosol leads to mitochondrial dysfunction and reactive oxygen species (ROS) generation. Activating mitochondrial protein apoptosis-inducing factor (AIF) and cytochrome *c* leads to apoptotic cell death (4-6).

Brain injury-induced oxidative radicals reduce nuclear factor erythroid 2-related factor 2 (Nrf2) signaling, interfering with Kelch-like ECH-associated protein-1 (Keap1)-Nrf2 connections. Keap1 is a Cullin3 (Cul3)-based ubiquitin E3 ligase substrate adaptor component. Nrf2 is a transcription factor that regulates antioxidant genes and protects against oxidative damage. Keap1 interacts with Nrf2 in the cytoplasm. The Cul3-Rbk1 complex ubiquitinates Nrf2, inducing proteasome degradation. Consequently, oxidative stress alters the structure of Keap1, affecting Keap1-Nrf2 interactions and limiting Keap1-mediated ubiquitination (7,8). To activate downstream signaling pathways encoding detoxification enzymes and antioxidant proteins, such as nicotinamide adenine dinucleotide phosphate (NADPH), NADPH quinone

dehydrogenase 1 (NQO1), heme oxygenase 1 (HO-1), superoxide dismutase (SOD) and glutathione peroxidase, newly released Nrf2 from the Keap1-Nrf2 complex translocates from the cytoplasm into the nucleus where it sequentially binds to the antioxidant response element (9,10). These findings suggest that the interaction of the Keap1-Nrf2 complex may be a downstream target for therapeutic agents in their role as protective factors against oxidative stress.

Orange (*Citrus sinensis*) is a major fruit commodity which is widely consumed, although the peel is generally regarded as waste. Previous studies have demonstrated that the antioxidant content of orange peel is higher than that of the juice bag (11,12). The antioxidant function of orange peel is mainly due to its high content of flavonoids, polyphenols and other bioactive components (13-16).

Previous studies on the antioxidant effects of orange peel have been carried out. Orange peel extract has been reported to exert antioxidant effects against chemotherapy-induced toxicity, promote the upregulation of the Nrf2 signaling pathway in arthritis models, and attenuate seizure-induced oxidative stress (12-14). However, the identified specific compounds that play a critical role in antioxidant signaling are still lacking, and the process of developing clinical compounds takes longer. In this case, screening potential compounds as drug candidates through molecular docking is an option for predicting the interaction mechanisms between ligands and target proteins (17). The present study aimed to predict the interaction of potential compounds in tangerine peel (*Citrus sinensis*) with the target protein, Keap1, in the upregulation of the Nrf2 signaling pathway and NMDA receptor against brain injury-induced oxidative stress and neuronal apoptosis.

Materials and methods

Citrus sinensis extraction. The samples used were *Citrus sinensis* peels obtained from fruit suppliers in Malang, East Java, Indonesia. The peels were dried in an oven at 60˚C for 24 h and then ground into a fine powder using a mill. The resulting powder was then diluted with 96% ethanol in a 12.5:100 (sample:solvent) ratio with a total volume of 1,300 μl. The samples were then vortexed at 2,000 rpm for 2 min. The supernatants were taken, filtered using a syringe filter of 0.22 μm, and injected into the liquid chromatography/high resolution mass spectrometry (LC/HRMS) instrument (Thermo Fisher Scientific, Inc.).

Analysis of active compounds of citrus sinensis peels. Compounds inside ethanol extract of *Citrus sinensis* peel were analyzed with high-performance liquid chromatography (HPLC) using the Dionex Ultimate 3000 system (Thermo Fisher Scientific, Inc.) without any additional structural analysis or protein profiling analysis. The solvents used were 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The Hypersil GOLD column (Thermo Fisher Scientific, Inc.) with a flow speed of 40 μl/min was used for the analysis. HRMS was performed using Q Exactive Orbitrap (Thermo Fisher Scientific, Inc.). Data were processed using Compound Discoverer software with mzCloud MS/MS Library (Thermo Fisher Scientific, Inc.). The results of the active compounds obtained have been uploaded to the Metabolights database (Study Number: MTBLS5785).

Active compounds and ADME of Citrus sinensis peels. Compounds were selected by filtering Lipinski's criteria and analyzing the BBB permeability of compounds found by LC-HRMS analysis. A total of 16 compounds were found to have good BBB permeability, as shown in Table SI. Three-dimensional structures were obtained from PubChem (pubchem.ncbi.nlm.nih.gov). Additionally, the pharmacokinetics of these drugs were evaluated for compliance with Lipinski's rule of five and ADME using the SwissADME website (swissadme.ch). The toxicity of each compound was also evaluated each using the OSIRIS website (18,19).

Retrieval of protein samples. The target proteins in the present study were NMDA, a glutamate receptor, and Keap1. Keap1 is a transcription factor involved in synthesizing antioxidants, such as SOD1, SOD2 and glutathione, as well as neurotrophins, such as brain-derived neurotrophic factor (20). The PDB protein code for the NMDA protein used was 4KFQ, whereas that of Keap1 protein was 5WIY from the Protein Data Bank (PDB) (rcsb.org). The three-dimensional structure of this protein is available for download in.pdb format. The residual water molecules and existing control ligands were subsequently separated from the proteins using PyMOL 2.0 (https://pymol.org/2/). Molecular docking was performed on a specific site, and grid box dimensions were established by fixing x, y, and z, as shown in Table I.

Molecular docking simulation and visualization. The molecular docking procedure was performed using PyRx 0.9 software (https://pyrx.sourceforge.io/) on a personal computer with the Windows 10 operating system, 8GB RAM, 500GB NVME SSD and an AMD Athlon 3150U CPU. Molecular docking revealed the site of interaction and binding affinity interaction between the ligands and the target proteins. The interaction of the amino acid residues of the target protein with the ligands was visualized using Pymol and Discovery Studio 2021 software (https://discover.3ds.com/discovery-studio-visualizer-download) to discover the binding location and the type of molecular interactions that occur.

Molecular dynamics simulation. Molecular dynamics simulation was performed using WebGro webserver (https://simlab.uams.edu/ProteinWithLigand/index.html). Before the simulation process, ligand preparation was performed using the PRODRG2 webserver (http://davapc1.bioch.dundee.ac.uk/cgi-bin/prodrg/submit.html). Subsequently, the prepared proteins and ligands were uploaded to Webgro with GROMOS96 43a1 as force field parameters, 0.15M NaCl solvent, 5,000 steps energy minimization, 310 K temperature with 1 bar pressure, MD integrator leapfrog, 100 nsec simulation time, and limitations frames per MD simulation fixed at 1,000.

Results

Compound analysis of Citrus sinensis peel extract. Several compounds were found in the LC-HRMS analysis. Compound

Table I. Grid box docking dimension of the study.

Protein target	Center_X	Center_Y	Center_Z	Size_X (Ao)	Size_Y (Ao)	Size_Z (Ao)
Keap1	432.216	74.894	112.592	10	10	10
NMDA	26.917	35.357	46.858	10	10	10

Keap1, Kelch-like ECH-associated protein-1; NMDA, N-methyl-D-aspartate.

Table II. Pharmacokinetic profile of chosen compounds in *Citrus sinensis* peels extract.

Compound Name	MW (Da)	Hydrogen donor	Hydrogen acceptor	LogP	MR	BBB permeability
Stachydrine	143.18	0	2	-1.10	41.35	No
Choline	104.17	1	1	-1.38	29.69	No
Nobiletin	402.39	0	8	3.02	106.87	No
Tangeretin	372.37	0	7	3.02	Yes	Yes
Proline	115.13	2	3	-0.92	32.52	No
Pipecolic acid	129.16	2	3	-0.61	37.33	No
Triethanolamine	149.19	3	4	-0.66	37.34	No
Cetrimonium	284.54	0	0	3.51	95.82	No
Hesperidin	610.56	8	15	-0.72	141.41	No
Scoparone	206.19	0	4	1.84	Yes	Yes
Alminoprofen	219.28	2	2	2.57	Yes	Yes
Limonin	470.51	0	8	2.55	11.17	No
Naringin	580.53	8	14	-0.79	134.91	No
Linoleic acid	280.45	1	2	5.45	Yes	Yes
Nootkatone	218.33	0	1	3.58	Yes	Yes
Chanoclavine	256.34	3	2	2.39	Yes	Yes

BBB, blood-brain barrier; MR, molar refractivity; MW, molecular weight (Da).

intensities were ranked based on peak area. Data for retention time, compound formula (Table SI), 2D structure, and 2D chemical formulas were also obtained (Fig. S1). Based on LC-HRMS findings, the pharmacokinetic profiles of each compound were further analyzed. Several compounds with good pharmacokinetic profiles based on Lipinski's rule of five and BBB permeability were identified, as shown in Table II.

Pharmacokinetic profile of selected compounds. According to Lipinski's rule of five, substances with good pharmacokinetics, particularly oral administration, have a molecular weight of <500 Daltons, a hydrogen acceptor value of <10, a hydrogen donor value of <5, a water partition coefficient (logP) <5, and a molar refractivity of 40-130 (21). Compounds that meet Lipinski's rule of five have good pharmacokinetic and bioavailability profiles as they are similar to drugs. In the present study, the pharmacokinetic investigation of selected compounds found in *Citrus sinensis* peel extract exhibited good results. Nootkatone, alminoprofen, linoleic acid, chanoclavine, scoparone and tangeretin all fulfill Lipinski's rule of five criteria and exhibit adequate BBB permeability, allowing them to cross the BBB, as shown in Table II. Nootkatone (CID: 1268142), alminoprofen (CID: 2097), linoleic acid (CID: 5280450), chanoclavine (CID: 5281381), tangeretin (CID: 68077) and scoparone (CID: 8417). The three-dimensional structures of these chemicals were retrieved in sdf format. It was also found that the selected compounds had good pharmacokinetics and bioavailability when administered orally. The parameter of physicochemistry lipophilicity must be evaluated, while designing new pharmaceuticals, since it has been found to significantly influence a number of pharmacokinetic features such as absorption, distribution, permeability and drug clearance processes (22).

Toxicity analysis. Toxicity identification is an essential parameter in determining compound safety for human use. The present study used the OSIRIS website to predict toxicity in some parameters. There were a mutagenic, tumorigenic, irritant and reproductive effects. Mutagenic and tumorigenic parameters predict the effect of a compound to become mutagenic and induce tumor (18). Toxicity analysis revealed various risk profiles for each compound that had been selected based on its good pharmacokinetic profile. The only compound with a high toxicity risk was tangeretin, with a predicted high risk in mutagenic and tumorigenic properties. Scoparone, chanoclavine and alminoprofen were predicted to have a medium toxicity risk in the reproductive system. Nootkatone and linoleic acid were predicted to have no toxicity risk in the

Table III. Binding affinity of ligands and protein receptor.

Compounds	Binding affinity (Kcal/mol)	
	Keap1	NMDA receptor
Nootkatone	2.9	-7.8[a]
Alminoprofen	-2.4	-6.9
Linoleic acid	-1.6	-6,3
Chanoclavine	3.0	-7.5
Scoparone	-5.0[a]	-7.0
Tangeretin	-2.1	-0.4
4-Amino-1,7-dihydro-6H-pyrazolo[3,4-d]pyrimidine-6-thione (control)	-4.2	
1-Sulfanyl[1,2,4]triazolo[4,3-a]quinoxalin-4(5H)-one (control)		-7.8

[a]Indicates the strongest binding affinity.

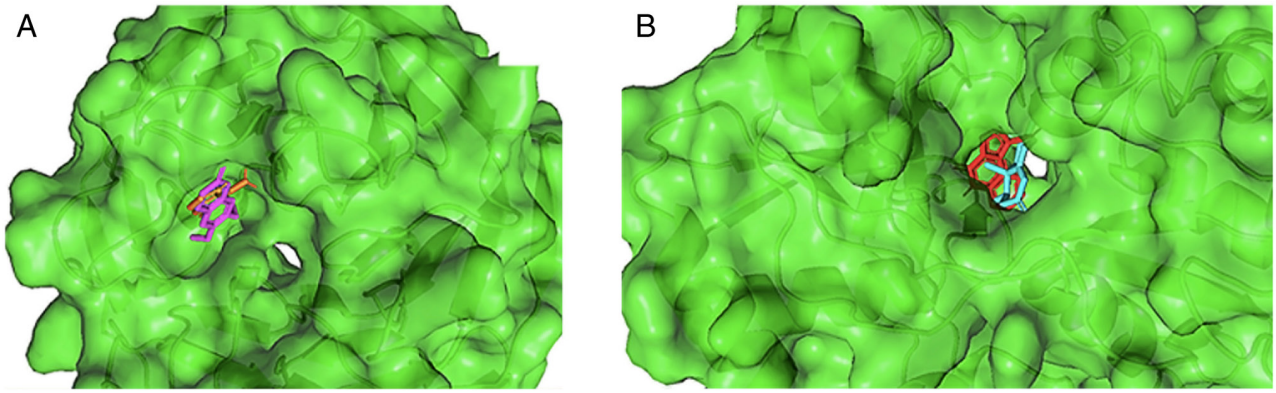

Figure 1. Binding pocket interaction. (A) Keap1 interaction, (B) NMDA interaction, nootkatone (cyan), scoparone (magenta) with Keap1 (A) and NMDA control (red). Keap1, Kelch-like ECH associated protein 1; NMDA, N-methyl-D-aspartate.

reproductive system, mutagenic and tumorigenic properties, or as an irritant (Table SII).

Molecular interaction. The present study analyzed nootkatone, alminoprofen, linoleic acid, chanocalvine, scoparone and tangeretin, since they have good BBB permeability. The molecular docking analysis identified that scoparone bound to Keap1 with the highest affinity compared to the control ligand and other ligands, whereas nootkatone attached to the NMDA receptor with the highest affinity compared to other ligands, but had a similar affinity to the control ligand. Scoparone exhibited a binding affinity of -5.0 Kcal/mol for NMDA receptors, whereas nootkatone had a binding affinity of -7.8 Kcal/mol (Table III). The molecule with the lowest bond energy will have 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.

Molecules interact when a bond is formed between the ligand and the protein. These bonds are located on certain amino acids to affect a particular reaction or function (25,26). Scoparone and nootkatone attach to the same binding pocket as the control ligand, as illustrated in Fig. 1. Scoparone binds to Keap1 by forming five bond interactions, three hydrogen bonds and two hydrophobic bonds. These interactions occur via hydrogen bonds in ARG415, SER508 and GLN530, whereas they occur via hydrophobic bonds in TYR525 and ALA556 (Fig. 2). Moreover, nootkatone interacted at five amino acid residues and formed two types of bonds. Nootkatone formed hydrogen bonds with GLN144, and hydrophobic bonds with the PRO124, PHE250, TRP223 and PHE92 amino acids (Fig. 3). Compared to the control, some amino acids in Keap1 interacted with scoparone were similar compared to control ligand (TYR525 and SER508). Interaction between NMDA receptor and nootkatone also showed similar interaction of amino acids compared to control ligand (PRO124 and PHE92). This suggested that scoparone interaction with Keap1 and nootkatone interaction with NMDA receptor might have similar inhibitory results compared to control ligands.

Molecular dynamics simulation. Molecular dynamics simulation is a technique to simulate the whole protein-ligand system due to the course of a certain time to analyze the confirmation changes. These parameters are the radius of gyration, root-mean-square deviation (RMSD), root-mean-square fluctuation (RMSF) and a number of hydrogen bonds (27).

The average RMSD fluctuations for the protein and ligand in the Keap1 complex were 0.7 nm, with an equilibrium since

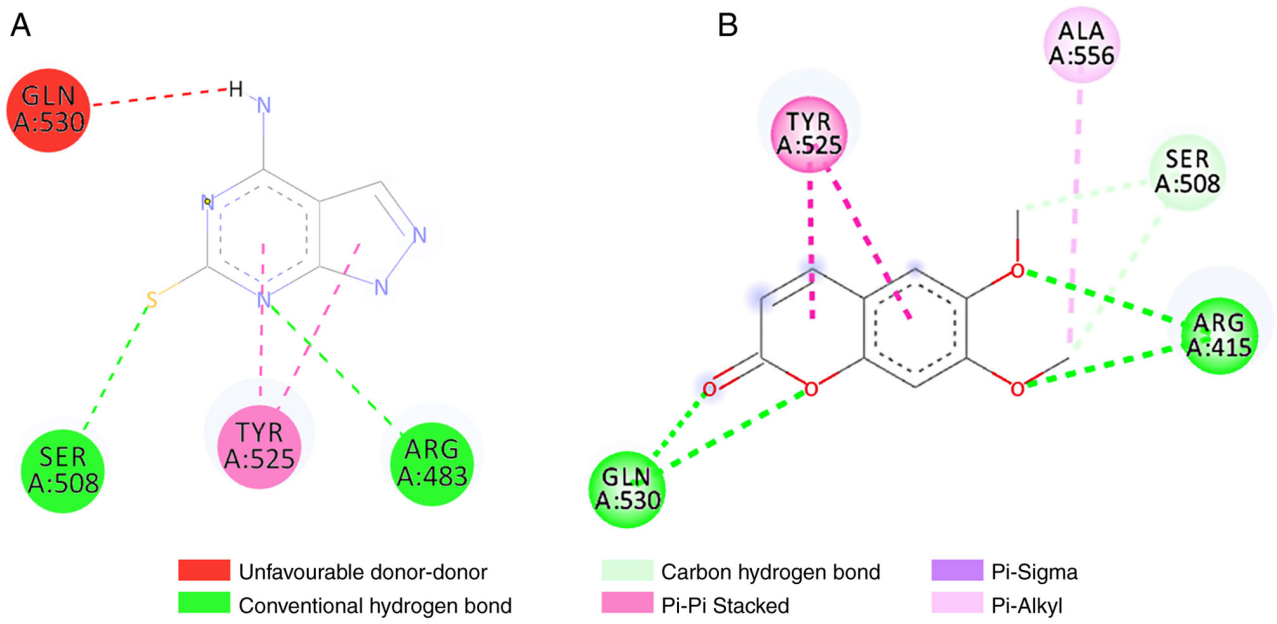

Figure 2. Amino acid residue interaction. (A) Control-Keap1, (B) scoparone-Keap1. Keap1, Kelch-like ECH associated protein 1.

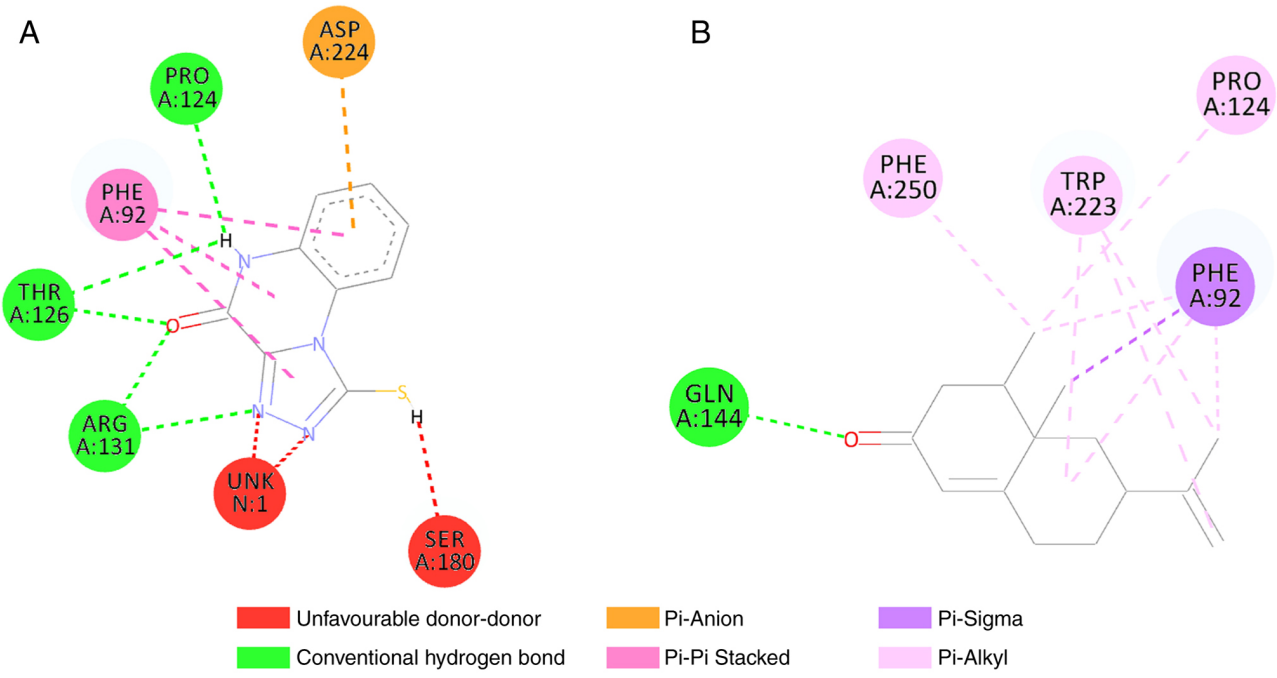

Figure 3. Amino acid residue interaction. (A) Control-NMDA, (B) nootkatone-NMDA. NMDA, N-methyl-D-aspartate.

4 nsec. The RMSD of the scoparone-Keap1 complex was found to be stable, first from 4 to 85 nsec and later from 86 to 100 nsec. Similarly, large deviations in RMSD were observed for the control-Keap1 complex, indicating an unstable nature of the complex thus formed. Furthermore, the RMSD value of the nootkatone-NMDA complex was 0.6 with equilibrium since 22 nsec. The RMSD of control-NMDA was more stable than that of nootkatone, as shown in Fig. 4A and B.

RMSF was measured to further compute the residual flexibility over a period of 100 nsec. The RMSF scoparone-Keap1 complex was <0.45 nm for each residue, the same as that of the control. But the mean of RMSF Scoparone-Keap1 was more stable than the control. Moreover, the RMSF of the nootkatone-NMDA complex was <0.81 nm for each residue, although that of the control was more stable, with a score of <0.6 nm for each residue (Fig. 4C and D).

Hydrogen bonds are regarded as a powerful dipole-dipole interaction. There are two varieties: Conventional and non-conventional. Hydrogen and elements other than N, O and F may form conventional hydrogen bonds. For the protein-ligand complex, hydrogen bonding and their number are more crucial. In terms of the average number of hydrogen

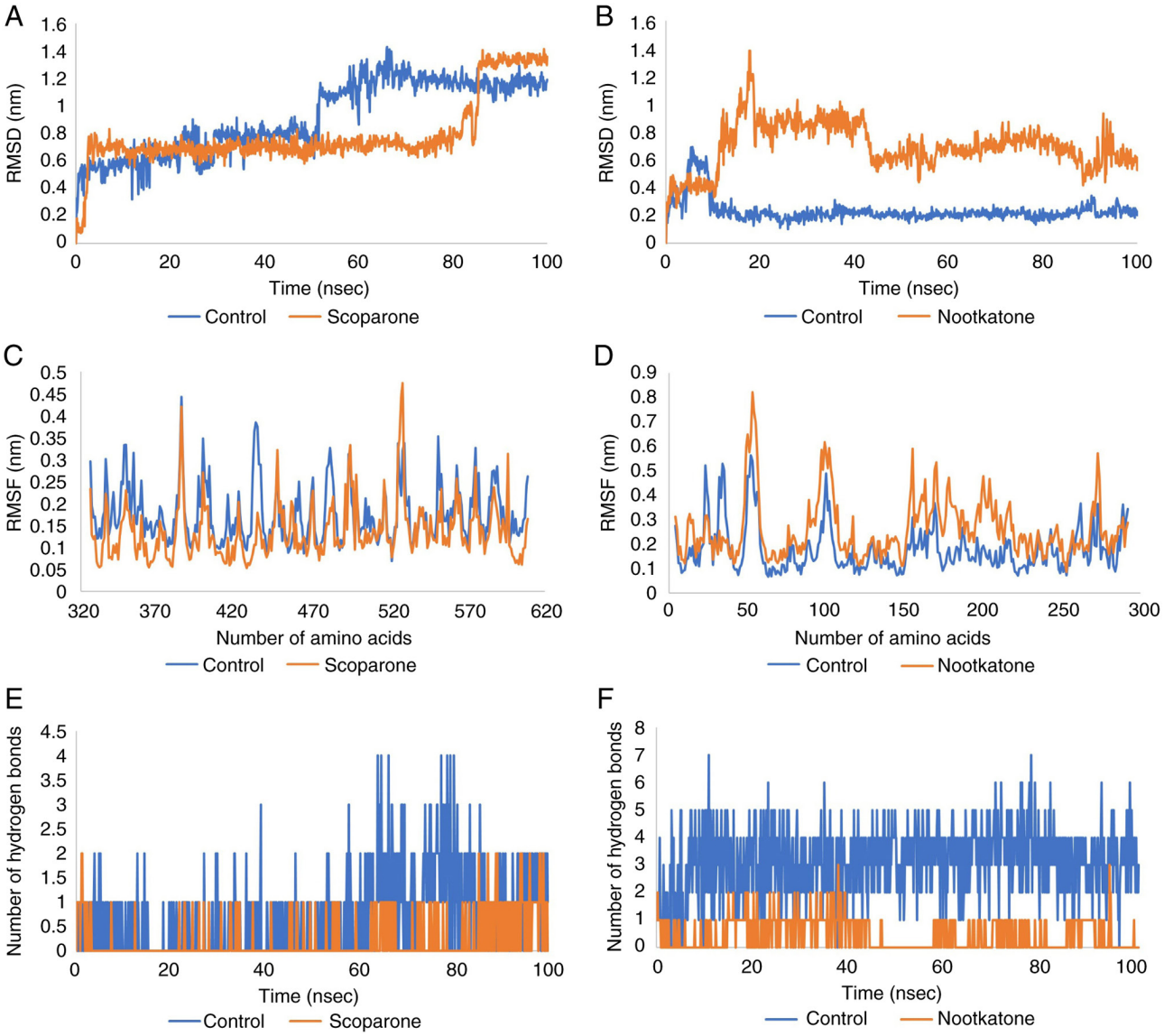

Figure 4. Results of molecular dynamics simulation. (A) RMSD scoparone-Keap 1 complex, (B) RMSD nootkatone-NMDA complex, (C) RMSF scoparone-Keap 1, (D) RMSF nootkatone-NMDA, (E) hydrogen bond number scoparone-Keap 1, (F) hydrogen bond number nootkatone-NMDA. RMSD, root-mean-square deviation; RMSF, root-mean-square fluctuation; Keap1, Kelch-like ECH associated protein 1; NMDA, N-methyl-D-aspartate.

bonds, the stability of the ligand in the active binding cavity of a protein is determined. The shorter the duration of engagement, the more effective it will be. The number of hydrogen bonds in the scoparone-Keap1 and nootkatone-NMDA complex was less than that of the control, as shown in Fig. 4E and F.

Discussion

The molecular docking method aimed to assign to the antioxidant herbal compounds found in the peels of *Citrus sinensis*. Through the use of various bioinformatics techniques, anti-inflammatory drugs can be identified and modified, which may lead to the discovery of novel aspects of anti-inflammatory disorders that are less well-known in society, thereby revealing human indifference to these issues. It is essential to be well informed and to conduct in-depth research on the proteins involved in anti-inflammation and the traditional herbal medications used to treat this condition. It is necessary to compile and discuss the vast amount of knowledge gained synergistically.

Keap1 protein and NMDA receptor play a crucial role in the process of increased oxidative stress in brain injury conditions. The presence of mechanical trauma to the brain causes damage to the BBB, leading to an increase in glutamate release and the induction of NMDA receptors (3,6,9). This induction causes an increase in extracellular Ca^{2+} ions, thus increasing oxidative stress conditions in the cell and disrupting the process of releasing the Keap1-Nrf2 protein, consequently inactivating the Nrf2 protein (20,28). In the present study, LC/HRMS revealed that the scoparone and nootkatone compounds in orange peel extract (*Citrus sinensis*) had good pharmacokinetic potential (the property of penetrating the BBB and meeting Lipinski criteria), molecular docking, and molecular dynamics.

The scoparone ligand binds to five amino acids in Keap1 protein: Arginine415, glycine530, serine508, tyrosine525

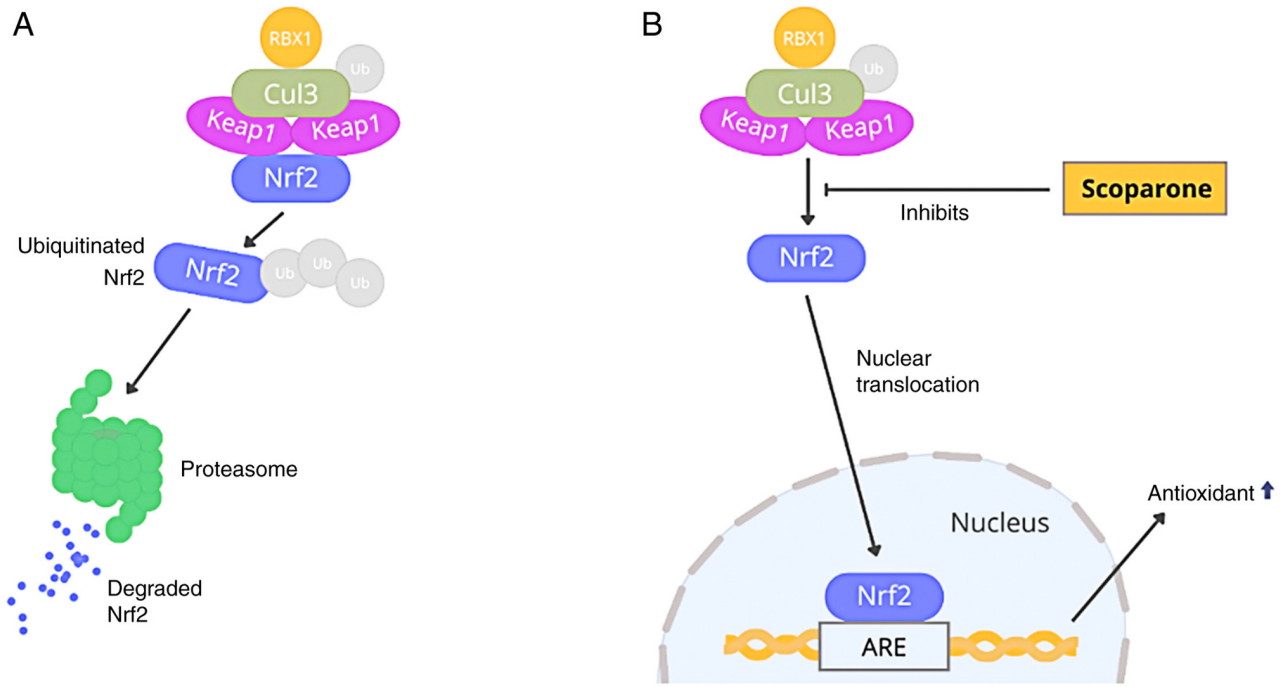

Figure 5. Keap1-Nrf2 antioxidant pathway. (A) Keap1 binding with Nrf2 ubiquitinates Nrf2, leading to the proteasomal degradation of Nrf2. (B) Inhibition of Keap1-Nrf2 binding by scoparone allows for the nuclear translocation of Nrf2, leading to the activation of ARE, increasing the cellular antioxidant product. Keap1, Kelch-like ECH associated protein 1; Nrf2, nuclear factor erythroid 2-related factor 2; ARE, antioxidant response element; RBX1, ring-box 1; Cul3, cullin3.

and alanine556, with a higher binding affinity value than the control ligand. The bonds between Keap1 protein and scoparone ligands are six hydrogen bonds and three hydrophobic bonds. The bond between the scoparone ligands on the amino acids arginine415 and serine508 is a polar area (an electrostatic interaction occurs), and the amino acid tyrosine525 is a non-polar area (a hydrophobic bond occurs) (20). However, in the control ligand there are only two hydrogen bonds in the amino acids arginine483 and serine508 and two hydrophobic bonds in the amino acid tyrosine525; thus, the scoparone ligand has a higher level of stability and binding affinity compared to the control ligand (20,27). The results of molecular dynamics simulation revealed that the scoparone ligands had a better conformational stability level (less fluctuation, RMSF parameters) and a greater docking fit rate (average of scoparone, 0.7 nm; and control average, 1 nm; RMSD parameters) than the control ligands (29). These results indicate that the scoparone ligand has a better and more stable inhibitory potential against Keap1 protein than the control ligand. Therefore, the *in silico* findings demonstrated that scoparone is a potential inhibitor of Keap1, which may lead to the enhanced release of Nrf2 and its subsequent activation of the antioxidative enzyme pathway.

Previous studies have specifically demonstrated the antioxidant effects of scoparone in various tissues, such as neuronal and myocardial tissues. The study by Wu *et al* (30) in 2019 demonstrated decreased levels of ROS and malondialdehyde (MDA), and increased levels of glutathione peroxidase and SOD in an *in vitro* model of oxygen glucose deprivation/reoxygenation injury using hippocampal neurons treated with 25, 50 and 100 μM scoparone. The study by Lyu *et al* (31) in 2021 demonstrated that mice with myocardial hypertrophy treated with scoparone at 60 mg/kg body weight daily via oral gavage exhibited an alleviation of cardiac hypertrophy and fibrosis, and ROS production via the inhibition of ras-related C3 botulinum toxin substrate 1 that stimulates oxidative stress (31).

It has been shown that the Nrf2 pathway is a critical pathway in the body's response against oxidative stress. Keap1 is known as a Nrf2 regulator by targeting Nrf2 in the ubiquitination process. Keap1 is also known to have a stress-related receptor. When activated under stress conditions such as oxidative stress, Nrf2 is released from the ubiquitination process, translocates to the cell nucleus, and stimulates transcription of various antioxidants and neurotrophins in the brain, as illustrated in Fig. 5 (20,28).

The increased extracellular glutamate and aspartate concentrations in the rat hippocampus following transient cerebral ischemia (stroke) have been the focus of intense investigation for over two decades. Considering this hypothesis, investigations using different animal models have found that NMDA receptor antagonists may improve outcomes after TBI and stroke, paving the way for large-scale placebo-controlled human trials in TBI and stroke (32).

The results of molecular docking of the nootkatone compound to the NMDA receptor protein revealed the presence of bonds in five amino acids, namely phenylalanin92, proline124, glysine144, tryptopan223 and phenylalanin250, with one hydrogen bond and nine hydrophobic bonds, as shown in Fig. 3. Binding to the amino acids phenylalanine92, proline124, glysine144 and phenylalanine250 are receptor residues of the NMDA protein on control ligands and have been shown to act as antagonists *in vitro* studies (33). These results indicate that nootkatone ligands have good potential as NMDA receptor inhibitors. The binding affinity results showed that the nootkatone and control ligands had the same value, namely

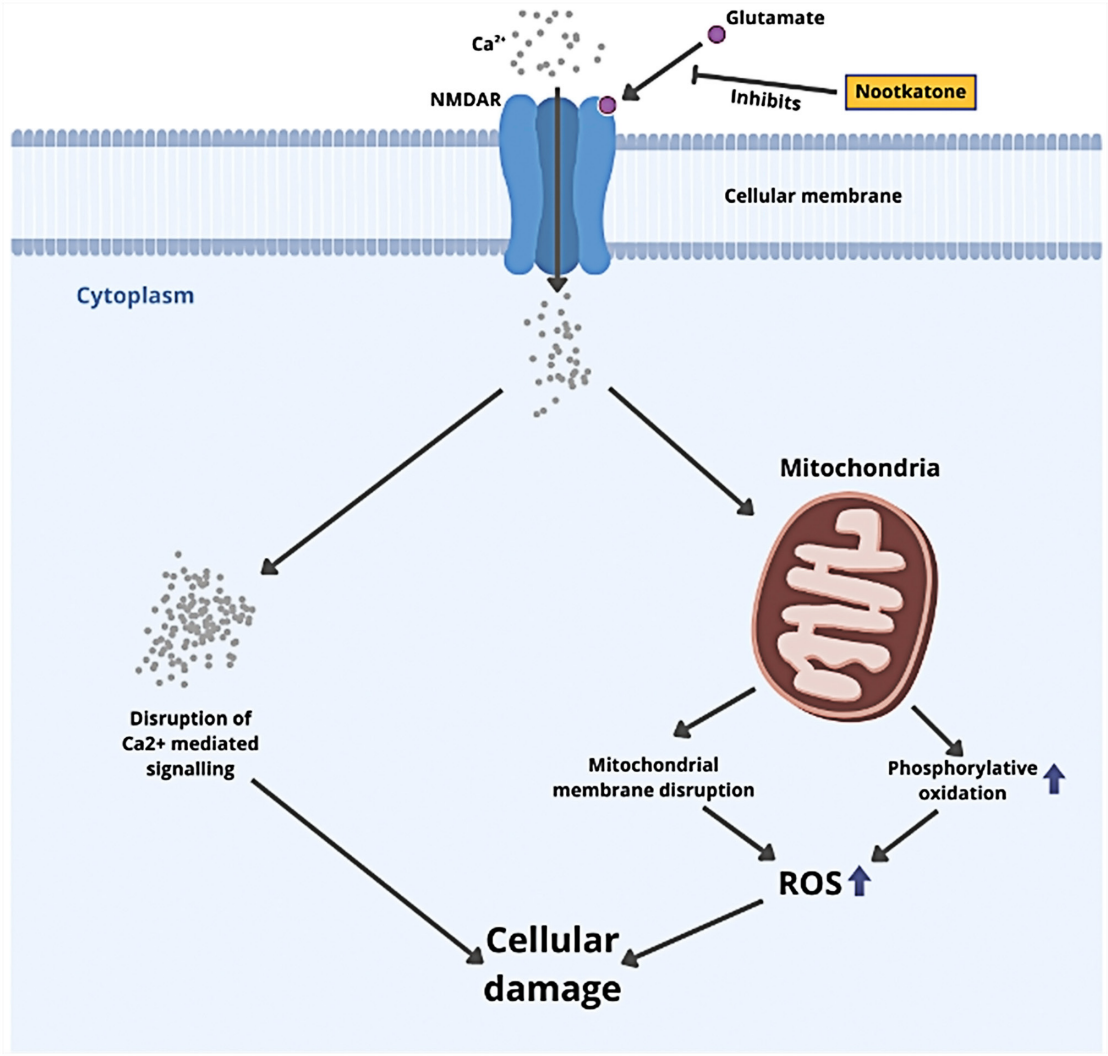

Figure 6. Glutamate binding with NMDAR leads to an influx of Ca^{2+}. Hyper influx of Ca^{2+} caused by excitotoxicity can disrupt Ca^{2+}-mediated cellular signaling. The influx of Ca^{2+} also increases the cellular energy demand, stimulating phosphorylative oxidation, and ROS as its byproducts. Ca^{2+} disrupts mPTP, disrupting mitochondrial membrane. Disruption of mitochondrial membrane and higher phosphorylative oxidation leads to an increase in the levels of cytoplasmic ROS. High level of ROS and the disruption of Ca^{2+} mediated signaling leads to cellular damage. Inhibition of glutamate-NMDAR binding by nootkatone can be beneficial to reduce excitotoxicity-mediated cellular damage. NMDAR: N-methyl-D-aspartate receptor, mPTP, mitochondrial permeability transition pore; ROS, reactive oxygen species.

-7.8 Kcal/mol. The results of molecular dynamics show that the control ligand has a better conformational stability level (less fluctuation, RMSF parameters) and has a greater docking fit (control ligand 0.2 nm average and nootkatone ligand 0.6 nm, RMSD parameter) than the nootkatone ligand. However, the RMSD results of the nootkatone ligand were still acceptable because the value was <3 nm (34). Control ligands have better potential to act as NMDA receptor inhibitors than nootkatone ligands.

NMDA receptor is required for neurodevelopment, central nervous system tissue remodeling and plasticity, and dendritic spine formation (memory formation). The primary mechanism underlying subsequent brain damage following TBI is the increased NMDA receptor activation. The ion channel activity of the NMDA receptor at the postsynaptic membrane regulates Ca^{2+} redistribution. The NMDA receptor-induced Ca^{2+} excess contributes to neuronal damage and synaptic plasticity disruption. NMDA receptor activation also causes nitrative stress and mitochondrial dysfunction following TBI. This is linked to NMDA receptor activity modulating neuronal nitric oxides synthase (35). This is in line with the findings of the present study that NMDA has a strong affinity for nootkatone, comparable to the control ligand. *Citrus sinensis* peel may lower glutamate excitotoxicity, oxidative stress and apoptosis, as shown in Fig. 6 (35,36).

Previous research has demonstrated the antioxidant effects of nootkatone in neuronal and renal tissues. The study by Yan *et al* (37) revealed a significant decrease in MDA levels in the hippocampal neurons of mice with D-galactosamine-induced liver injury treated with nootkatone at 5 and 10 mg/kg body weight intragastrically. Park *et al* (38) demonstrated a significant decrease in ROS production and an increase in the levels of antioxidant enzymes, such as HO-1 and NQO1 in mice exposed to lipopolysaccharide (LPS) and in LPS-stimulated microglia model treated with nootkatone. The study by Chen *et al* (39) revealed a significant increase in the levels of catalase and SOD in mice with unilateral ureteral obstruction treated orally with nootkatone at 10 mg/kg body weight.

The importance of Keap1 and Nrf2 in the antioxidative process and their subsequent anti-inflammatory and various beneficial effects can be applied in various pathological conditions, such as TBI. The pathological process of secondary brain injury caused by delayed neurochemical reaction following initial mechanical trauma involves neuronal cell death (40). Various clinical and preclinical studies have demonstrated that the type of neuronal cell death involved in various parts of the brain, such as the hippocampus, cortex and thalamus is mainly apoptotic (38-40).

The release of ROS, particularly mitochondrial ROS in ischemia-related brain pathologies, such as stroke and TBI, is one of the triggers for neuronal apoptosis (41-43). Previous studies have indicated that increased ROS generation in neuronal cytosol leads to the release of various pro-apoptotic proteins, such as cytochrome *c* (44,45). This increase in apoptosis suggests that oxidative stress plays a key role in apoptosis. The molecular docking analysis performed herein demonstrated that *Citrus sinensis* peel compounds with a high binding affinity to Keap1, such as scoparone, had a higher binding affinity than the control ligand. This finding suggests *Citrus sinensis* peel has potential for use as an oxidative stress inhibitor for the treatment of in brain injury via the inhibition of Keap1 binding to Nrf2.

In conclusion, some compounds, notably scoparone and nootkatone, in *Citrus sinensis* peel exhibit promising oxidative stress therapeutic potential by exerting inhibitory effects on the Keap1 and NMDA pathways in brain injury. However, the present study utilized the LC/HRMS and *in silico* computational methods, which were insufficient to analyze practical efficacy and various factors in clinical conditions. Hence, further *in vitro* and *in vivo* studies are warranted to validate the therapeutic potential of ethanol extract of *Citrus sinensis* peel in brain injury.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the Figshare repository (https://doi.org/10.6084/m9.figshare.20552073.v1) and on the MetaboLights Compound Database (https://www.ebi.ac.uk/metabolights/MTBLS5785). Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).

Authors' contributions

MFRS and WMS 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. MFRS, GFAP, RAV, AAAM, WMS, LDF, FLS and MH were involved in the conception and design of the study, and in the revising and reviewing of the manuscript. MFRS, GFAP, AAAM, and RAV confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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