

THE POTENTIAL COMBINATION OF *CENTELLA ASIATICA*, *CURCUMA LONGA*, AND *PIPER NIGRUM* EXTRACTS IN TREATING BRAIN INJURY: *IN VITRO*, *IN VIVO* AND SILICO STUDIES

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ABSTRACT

Objective: Traumatic brain injury is a head injury that causes brain dysfunction. This disorder can have a bad effect if not treated quickly and appropriately. This study aims to examine the extract of Gotukola (*Centella asiatica*), turmeric (*Curcuma longa*), and black pepper (*Piper nigrum*) as natural medicines that can treat brain injury problems.

Methods: The parameters tested in this study included testing the memory of experimental animals using the Y-Maze method, *in vitro* inhibition of glutaminase, and in silico research through molecular docking and molecular dynamics on the compounds of each extract that have been previously reported.

Results: Each extract had activity in increasing memory, but a combination formula of the three extracts showed a significant increase in memory ($p < 0.05$, $n = 5$). The combination extract of gotu kola, turmeric, and black pepper in a ratio of 50:50:50 (combination 1), 25:50:50 (combination 5), and 25:12.5:50 (combination 8) continued its activity in inhibiting glutaminase. The results showed a significant decrease in glutaminase activity when applied to the three combination extract formulas. This study is also supported by in silico results showing that the asiaticoside compound identified in gotu kola extract, 1,5-bis (4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one compound from turmeric extract and Bacitritinib from black pepper extract have an important role in interacting with the target protein glutaminase with protein data bank 407D in molecular docking studies and interacting stably in molecular dynamics.

Conclusion: This study has supported the development of a combination extract formula of gotu kola, turmeric, and black pepper as a candidate for treating brain injury.

Keywords: Brain injury, Glutaminase, Natural resources, Molecular docking, Molecular dynamic

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INTRODUCTION

Brain injury is a force trauma (trauma) that affects the structure of the head, resulting in structural abnormalities and/or functional disorders of brain tissue [1]. Based on Indonesian Health data since 2018, the percentage of head injury cases was 11.9%, with the highest percentage in Gorontalo province at 17.9% [2]. Epidemiological data on brain injuries in Makassar City, Sout Sulawesi Province, Indonesia, especially at Dr Wahidin Sudirohusodo in 2005, numbered 861 cases; in 2006, there were 817 cases, and in 2007, there were 1078 cases [3].

Based on the effects on the head, injuries are classified into two mechanisms: primary injuries (Primary insult) and secondary injuries (secondary insult). Primary injury directly results from trauma that causes primary or mechanical damage. Meanwhile, secondary brain injury is described as a consequence of physiological disorders, such as ischemia, reperfusion, and hypoxia in areas of the brain at risk, sometime after the initial injury (primary brain injury) [4]. Secondary brain injury is sensitive to therapy, and its occurrence can be prevented. One of the causes of secondary brain injury is the excitotoxicity process mediated by the glutamine enzyme [5].

Glutamate is the main excitatory neurotransmitter in the central nervous system. It is important to keep glutamate concentrations low in the extracellular space to ensure adequate synaptic transmission and prevent excitotoxicity. Therefore, glutamate released from the presynaptic terminal must be removed from the synaptic cleft. In a healthy brain, toxic concentrations of glutamate can be prevented by the presence of excitatory amino acid transporters that take up glutamate [6, 7]. In astrocytes, glutamate is converted to glutamine by glutamine synthetase. Glutamine is released into the

extracellular space taken up by adjacent neurons and used to synthesize glutamate with the help of the enzyme glutaminase. Some glutamine is also oxidized and completely degraded as an energy substrate. This glutamate-recycling process is known as the glutamate-glutamine cycle, and this cycle results in changes in neuronal metabolism between periods of activity and rest and the energetic relationship between astrocytes and neurons [8, 9].

Gotu Kola (*Centella asiatica* L.) is a plant that contains antioxidants and can provide antihyperglycemic and antihypertension effects [10, 11]. This shows that Gotu Kola has potential as an alternative therapy for traumatic brain injury [12]. Another plant that can be used is turmeric. The curcumin compound from turmeric (*Curcuma longa* L.) has the potential as an analgesic, anti-inflammatory, and antioxidant [13, 14]. Administration of curcumin at a dose of 500 ppm orally for four weeks reduced oxidative stress and increased Brain-derived neurotrophic factor (BDNF) and C-AMP response element binding protein (CREB) levels in mice with a head injury model. These results illustrate that turmeric can reduce the negative impact of head injury, increasing synapse plasticity [15].

Black pepper (*Piper nigrum* L.) is also a candidate plant widely used in treating epilepsy. Piperine, a piperidine alkaloid, is a major component of many pepper plant families [16]. Modern pharmacological studies show that piperine has a variety of effects, including anti-oxidant, immune regulation, anti-tumor, effects on increasing drug metabolism, as well as mood and cognitive disorders [17–19]. Piperine can affect many brain diseases due to its efficient absorption and high membrane permeability [16]. For example, it was found that piperine can block transient receptor potential cation channel subfamily V member 1 (TRPV1) channels

instead of Ca^{2+} channels and is expected to become a new type of broad-spectrum antiepileptic drug. Black pepper contains the compound piperine. The piperin compound acts as an antioxidant, can inhibit oxidative stress, protects against free radicals and Radical Oxygen Species (ROS), and inhibits peroxidation [18, 20].

There is information on the chemical content of Gotu Kola, turmeric, and black piper plants and a history of their clinical use in treating brain injuries, making it possible to use a combination of these plants in treating and recovering from brain injuries. In this research, an assessment was carried out regarding the combined effects of these three plants as potential natural medicine candidates in treating brain injuries. The effects of combined dosage settings on the three plants were evaluated *in vitro* and *in silico*. This study was carried out to develop natural ingredient extracts into standardized herbal products and become phytopharmaceutical preparations for treating brain injuries in the future.

MATERIALS AND METHODS

Materials

The materials used in this research include distilled water, acetic acid (CH_3COOH), dimethyl sulfoxide (DMSO) (Merck-Germany), ethanol 70% v/v ($\text{C}_2\text{H}_5\text{OH}$), Enzyme-linked immunosorbent assay (ELISA) kit for Glutamate Receptor Ionotropic-AMPA 1 (GRIA1) was purchased from Cloud-Clone Corp (USA), sulfuric acid (H_2SO_4) (Merck-Germany), filter paper, L-glutamine (Sigma-Aldrich), sodium acetate (Merck-Germany), sodium Carboxyl Methyl Cellulose (Merck-Germany), animal *Rattus norvegicus* was purchased from animal laboratory Medical Faculty, Airlangga University-Indonesia. Sodium acetic (CH_3COONa) (Merck-Germany) and Nessler's reagent (Merck-Germany), buscopan® (hyoscine), Tebogan forte®, Gotu Kola, black pepper, and turmeric plant samples were obtained in Biringkanaya District, Makassar City, South Sulawesi, Indonesia ($5^{\circ}06'47.8''\text{S}$ $119^{\circ}30'29.0''\text{E}$). The samples of gotu kola herb, turmeric rhizome, and black pepper have been determined the classification of the plants in the Pharmaceutical Biology laboratory, Faculty of Health Sciences, Almarisah Madani University by Dr. Marwati, M. Sc. with the respective specimen codes, namely H-086/III/2023 (gotu kola), R-1161/III/2023 (turmeric), and B-0026/III/2023 (black pepper).

Extraction method

Dry samples of Gotu Kola (*Centella asiatica*), turmeric (*Curcuma domestica*), and black pepper (*Piper nigrum* L.) powder each weighed 100 g. Then, each sample was extracted by maceration using 500 ml of 70% v/v ethanol solvent. Extraction was carried out for 3×24 h while stirring occasionally, after which it was filtered. Each filtrate obtained was then macerated for 1×24 h, then filtered and concentrated using a rotary evaporator until a thick extract was obtained. Gotu kola, black pepper, and turmeric extracts were each stored in the refrigerator at a temperature of 2-8 °C and then used in each treatment.

Animal handling procedure

The procedures for handling experimental animals (*Rattus norvegicus*) in this study have received approval from the Research Ethics Code Institute of the Faculty of Medicine, Hasanuddin University, Makassar, South Sulawesi, Indonesia, with ID number 40611N4.6.4.5.31/PP36/2024. The test animal was a white rat (*Rattus norvegicus*) weighing 120 and 200 g. It was not sick, not registered and had never experienced drug treatment. The 30 test animals used in this research were divided into 6 groups, each consisting of 5 animals. Before being treated, they were acclimatized to the cage environment for 14 d and given daily food and drink.

Test the memory of experimental animals by the Y-Maze method

The group rats, consisting of the healthy control group, were given treatment and were without treatment for 25 d. Memory testing was then carried out using the Y-maze method [21] with slight modification. For the negative control, positive control, and treatment groups, mice were induced with 1.5 mg/KgBW of scopolamine for 10 d intraperitoneally. The memory testing was carried out using the Y-maze method. After the maze test, each treatment groups were given turmeric extract at a dose of 200

mg/KgBW, gotu kola extract at a dose of 200 mg/, and black pepper at a dose of 15 mg/KgBW. Variations in dose combinations were also carried out in this study, namely combining gotu kola, turmeric and black pepper extracts in various ratios of 50:50:50 (1), 50:25:25 (2), 25:50:25 (3), 25:25:25 (4), 25:50:50 (5), 50:50:25 (6), 25:25:50 (7), 25:12.5:50 (8), and 12.5:50:25 (9). Each extract and combination extracts were suspended with sodium CMC administered once a day orally using a cannula. The administration was carried out until the 15th day. After giving each group to the test animals, memory was tested using the Y maze method. The three-armed Y-maze method was 50 cm long, 12 cm wide, and 25 cm high. The arms were separated symmetrically at 120°. The rat was placed at the end of one arm of the maze and allowed to freely explore the Y-maze throughout the experiment, which usually lasted 5-8 min. The behavioral testing was performed in a closed and quiet room. A stopwatch recorded the mouse's activity during the experiment. In this procedure, a healthy control group was used without treatment, a group used ginkgo biloba 120 mg/Kg BW as a positive control, and a group of mice used CMC sodium suspension as a negative control, which was treated for 15 d.

Glutaminase inhibitor activity

The inhibitory activity of the glutaminase enzyme was tested using a modification of the method of [22, 23] with slight modification. In this test, a combination of gotu kola extract, turmeric, and black pepper was used in a ratio of 2:4:1. The combination of extracts was then weighed as much as 100 mg and dissolved in 10 ml DMSO-buffer phosphate solvent (5% v/v). The sample solution was then made into a concentration series (10-1000 ug/ml) for further analysis. The reaction mixture (0.5 ml) consisted of 0.1 sample solution in 5% DMSO, 0.1 ml 0.1 M acetate buffer (pH 4.9), 0.1 ml enzyme solution (2.0 U/ml glutaminase in the same container, and 0.2 ml of substrate solution (0.04 mmol L-glutamine in the same buffer) were incubated at 37° for 30 min. The reaction was stopped by adding 0.5 ml of H_2SO_4 (0.5 M) solution. The precipitated protein was removed by centrifugation (3000 rpm, 10 min), and 0.2 ml of supernatant was added to 3.8 ml of distilled water. After that, 0.5 ml of Nessler's reagent was added, and absorbance was measured at 420 nm for 10 min.

The percent inhibition of the glutaminase enzyme was calculated using the formula:

$$\text{Inhibition activity (\%)} = \frac{\text{Abs.blank} - \text{abs.sample}}{\text{abs.blank}} \times 100\% \dots\dots\dots [1]$$

Molecular docking simulation

The *in silico* activity of each extract of gotu kola, turmeric, and black pepper as candidates for treating brain injury was evaluated using molecular docking [24,25]. One of the target proteins in this research is glutaminase. Glutaminase protein was downloaded at the Protein Data Bank (<https://www.rcsb.org/>) with PDB ID 407D. Glutaminase is one of the enzymes involved in the mechanism of brain injury, so in this study, it was used as a target or receptor for searching for candidate chemical medicinal molecules from natural ingredients. There were 182 compounds consisting of 64 *Curcuma longa* L. compounds, 53 *Piper nigrum* L. compounds, and 65 *Centella asiatica* (L.) compounds. These compounds were obtained from several references that published chemical contents and were successfully identified in these three plants. The selected compounds are then confirmed for suitability for selected medicinal plants at <https://pubchem.ncbi.nlm.nih.gov/>. The among of 182 compounds were evaluated for their biological activity by molecular docking using Autodock Tools software (Autodock vina_1_1_2). Using the Autodock vina_1_1_2 program, a grid was formed with dimensions (28.23×25.62×31.95) Å with grid box coordinates (x, y, z) 15.7 Å, 33.33 Å and 42.81 Å on the enzyme glutaminase. The parameters evaluated in this study include binding affinity energy, hydrogen interactions, and inhibition constants for each compound.

Molecular dynamic simulation

To evaluate the ligand-target interactions for the compounds with minimal binding energy (stronger binding energy) further, molecular dynamics (MD) was utilized. YASARA (YASARA Bioscience GmbH, Vienna, Austria) (Land et al., 2018) software was used to simulate molecular dynamics. Amber14 is applied in conjunction

with periodic boundary conditions as a force field. The pH was kept at 7.4, and the temperature was adjusted to 310 K. To rebalance the system, TIP3P solvent and counterions (Na⁺, Cl⁻) were added. Ultimately, the simulation ran with a timestep of 0.25 fs for 100 ns. Every 25 ps, the following metrics are recorded and examined: radius of gyration, root mean square fluctuation (RMSF), and root mean square deviation (RMSD) [26].

Data analysis

Data analysis on *in vitro* testing from research results was analyzed using Microsoft Excel and statistically tested for normality, homogeneity, one-way ANOVA, and Kruskal-Wallis H tests using SPSS version 29.0. In silico evaluation, parameters include the orientation of the ligand structure, hydrophobic interactions, hydrogen bonds formed, and free energy values for each molecule's docking and molecular dynamic simulation.

RESULTS

Extraction and phytochemical screening

Gotu kola, turmeric, and black pepper are plant parts that can potentially have biological activity. In the study, gotu kola was used in herbaceous form (roots, stems, and leaves); in the turmeric plant, the rhizome was used, and the seeds were used in black pepper. Each gotu kola, turmeric, and black pepper powder were extracted by maceration using 70% ethanol as a solvent. Several references state that the maceration method for extracting gotu kola plants, turmeric rhizomes, and black pepper seeds effectively extracts the essential compounds [21, 22, 27]. The extraction solvent in 70% ethanol has also been proven to extract the compounds contained in these three plants. In general, triterpenoid compounds in the form of asiaticoside from the gotu kola plant are markers that have biological activity [28]. Likewise, turmeric rhizomes in curcuminoid

compounds [14] and black pepper seeds in the form of piperine compounds also have the same potential to provide biological activity [29]. The maceration method and the selected extracting solvent attracted these potential compound candidates.

Based on the phytochemical screening carried out using colorimetric method, information was obtained that gotu kola extract was positive for containing triterpenoid compounds, turmeric extract was positive for containing curcuminoid compounds, and black pepper seed extract showed positive results for containing alkaloids (table 1). This identification is still suspected of asiaticoside in gotu kola extract, curcumin in turmeric extract, and piperine in black pepper extract. These three metabolite compounds are the major compounds contained in each extract and are used as markers in future research development.

The memorial test by Y-maze test

Memory testing was carried out using the Y-maze method. This test aims to measure the memory of experimental animals to find food quickly and efficiently. The mouse's memory was calculated from entering the Y-maze until it found the food in its right arm of the Y-maze. Memory testing was carried out on rat test animals consisting of 4 treatment groups. Group 1 (healthy control) rat test animals were only given food and drink, group 2 (negative control) rat test animals were given 0.5% NaCMC, group 3 (positive control) rat test animals were given ginkgo biloba, and group 4 (gotu kola leaf extract treatment), Group 5 (turmeric extract treatment), group 6 (black pepper extract treatment) and group 7 until group 15 (extract combination). Before testing to obtain initial data, an acclimatization process was carried out for seven days. After that, initial data was obtained from the rat's introduction stage in the maze, which aimed at learning and forming spatial memory. This stage was carried out and observed for 1×24 h.

Table 1: Yield (%) of extracts and their phytochemical profile

Extract	Dry sample weight (g)	Extract weight (g)	Yield (%)	Organoleptic	Group compounds
Gotu Kola	50	7	14	The thick extract is blackish-green in colour, has a slightly bitter taste, and has a non-specific odour.	Terpenoid (+)
Turmeric rhizome	50	11	22	The thick extract is yellowish brown in colour, bitter in taste, and aromatic in smell	Curcuminoid (+)
Black pepper seed	50	9.88	19.76	Dry extract, black in color, spicy taste, and distinctive odor	Alkaloid (+)

Note: (+) is a positive result.

Table 2: Profile of memory before and after treatment in experimental mice (seconds)

Groups	Treatment	Observation (second)		
		Before treatment	Induction with scopolamine 1.5 mg/Kg BW	After treatment
1	Healthy control	140.7±40.28	-	-
2	Negative control (Na CMC)	144.0±77.86	167.4±63.82	169.1±68.72*
3	Positive Control (ginkgo biloba)	153.3±23.43	280.3±64.26	146.9±19.24*
4	Gotu kola extract (200 mg/KgBW)	142.0±6.44	191.3±7.31	137.8±7.18*
5	Turmeric extract (200 mg/KgBW)	145.2±88.27	198.3±34.84	133.0±23.70*
6	Black Pepper Extract (15 mg/KGBW)	120.6±25.27	317.2±137.60	150.9±13.69*
7	Combination 1 (50:50:50)	178.7±37.31	203.9±51.27	142.3±23.19*
8	Combination 2 (50:25:25)	153.9±30.63	192.2±59.16	149.8±44.08*
9	Combination 3 (25:50:25)	169.9±78.36	189.8±83.63	144.8±9.03*
10	Combination 4 (25:25:25)	196.6±64.46	217.3±19.43	181.3±72.78*
11	Combination 5 (25:50:50)	157.7±51.34	182.0±76.98	133.9±29.51*
12	Combination 6 (50:50:25)	167.6±10.17	213.4±60.91	155.9±19.54*
13	Combination 7 (25:25:50)	179.2±22.95	268.7±62.76	148.7±45.64*
14	Combination 8 (25:12.5:50)	155.7±29.50	254.9±69.28	143.1±40.95*
15	Combination 9 (12.5:50:25)	163.3±23.14	248.4±81.42	153.9±14.11*

Note: *The statistical analysis of samples were shows that the significant different among of samples ($p < 0.05$, $n = 3$, mean±SD).

The experimental data in table 2 shows changes in mice's memory before and after treatment, induced by Scopolamine and by treatment with the extract. Induction with Scopolamine showed a decrease in memory, observed in the longer it took for mice to leave the Y-maze, namely 169.1±68.72 seconds, compared to before induction, namely 144.0±77.86 sec. Similar things were experienced in all groups after being induced with Scopolamine compared with experimental data before induction. The decrease in memory of mice

using the Y-maze method after scopolamine induction was caused by Scopolamine quickly crossing the blood-brain barrier, causing the induction of muscarinic activity by depleting acetylcholine, which caused memory loss, oxidative stress, and causing a decrease in catalase activity in the hippocampus compared to initial data [30].

Data from the treatment of gotu kola extract, turmeric, and black pepper showed an increase in the memory of the experimental

animals, which was observed by decreasing the travel time (in minutes) for the mice to reach their food in the Y-maze. Administration of gotu kola extract suspension at a dose of 200 mg/Kg BW, turmeric extract at a dose of 200 mg/Kg BW, and black pepper extract at a dose of 15 mg/Kg BW were able to improve memory as evaluated using the Y-maze method (fig. 2). The improvement in memory after administration of the extract increased significantly $P < 0.05$ from the negative control group after scopolamine induction. Improved memory from each extract showed results that were not significantly different ($p > 0.05$) from

the positive control treatment group, ginkgo biloba (200 mg/Kg BW). This means that each extract can improve memory in mice after administration for 15 d. In this study, ginkgo biloba was used as a positive control because ginkgo biloba is one of the main compounds isolated from natural plants and works by activating the signaling pathway connected to erythroid 2 factor 2 (Nrf2), which is known as the primary molecular mechanism in protection against oxidative stress and deletion of its gene expression inhibits neuron regeneration by stimulating phase II genes through Kelch-like ECH associated protein-1 [31].

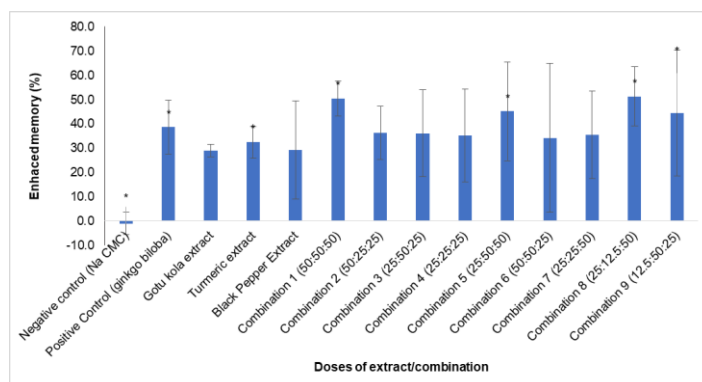


Fig. 1: Memory profile of test animals (mice) from each treatment by Y-maze method. Note: *The statistical analysis of samples were shows that the significant different among of samples ($p < 0.05$, $n = 3$, mean \pm SD)

In this study, an evaluation of memory enhancement activity was also conducted using several comparisons of gotu kola, turmeric, and black pepper extract combinations. Dose variations were carried out with different comparisons according to table 2. The evaluation results showed a significant increase in memory from the combination used and compared with the results of memory enhancement using a single extract and positive control. The combination of extracts 1 and 8 increased memory by $> 50\%$ (fig. 1) and was followed by a combination dose of extract 5 with a percentage increase of 46.8%. The combination of extracts at other doses did not show significant results in increasing memory, namely $< 40\%$ (post-hoc test, $p > 0.05$, $n = 3$). When observed from the increase in memory ability in test animals, the combination of doses 1 and 8 did not show a significant increase in memory (Post-hoc test, $p > 0.05$, $n = 3$). However, when compared with the percentage of memory increase between the combination doses 1 and 8 ($> 50\%$) and combination 5 ($> 45\%$) against the positive control, ginkgo biloba ($< 40\%$), significantly different results were obtained (Post-hoc test, $p > 0.05$, $n = 3$). Therefore, the administration of extracts with combinations of 1, 5, and 8, namely gotu kola, turmeric, and black pepper extracts, can be selected for further research.

Glutaminase inhibitor profile

Based on preliminary tests of the activity of gotu kola extract, skin, and black pepper as a memory enhancer, it was shown that combining the three natural extracts was proven effective in improving memory. The best combination uses a ratio of gotu kola, turmeric, and black pepper extract, namely 50:50:50 (combination 1), 25:50:50 (combination 5), and 25, 12.5, and 50 (combination 8). These combinations of the extracts were then applied to the *in vitro* glutaminase inhibition test.

Determination of inhibitory bioactivity of the combination extract of gotu kola, turmeric, and black pepper against glutaminase showed potent activity. Combinations with a ratio of 50:50:50 (combination 1), 25:50:50 (combination 5), and 25, 12.5, and 50 (combination 8) had IC_{50} values of 44.63 ± 11.84 , 57.94 ± 4.01 , and 56.29 ± 6.18 , respectively (fig. 2). According to Batabara *et al.* (2010), an IC_{50} value < 100 $\mu\text{g/ml}$ indicates strong inhibitory potential, an IC_{50} value of 100-450 $\mu\text{g/ml}$ indicates moderate inhibitory potential, and an IC_{50} value of 450-700 $\mu\text{g/ml}$ indicates weak inhibitory potential [32]. From the results of the tests, an IC_{50} value of < 100 $\mu\text{g/ml}$ was obtained, indicating potent activity. The statistical analysis results of the combination of extracts applied to the glutaminase enzyme showed significantly different IC_{50} values between samples. The combination of extract 1, namely at a ratio of 50:50:50, gave the most potent and most significant inhibitory effect with combinations 5 and 8 (post-hoc test, $p < 0.05$, $n = 3$).

In silico activity of combination extract by molecular docking

The *in vitro* activity of the combination of Gotu kola, turmeric, and black pepper extracts as a memory enhancer correlates with its bioactivity in inhibiting glutaminase. The prediction of active compounds from each extract that provide pharmacological activity for developing brain injury treatment needs to be made. This study also evaluated in silico activity through molecular docking to predict the interaction of active compounds identified in each extract against amino acid residues in the target protein. The target protein used was glutaminase protein with PDB id 407D. Each identified compound from gotu kola, turmeric, and black pepper extracts obtained from secondary data from searches in several sources was then monitored for interaction. Citric acid is one of the compounds used as a native ligand that has the potential to interact with the target protein. The native ligand pocket was reduced to obtain the pocket where the selected compounds interacted from each extract so that the visualization of the interaction between the identified compounds and the native ligand could be compared. The results of the native ligand redocking analysis against the target protein obtained an RMSD value of 1.5 Å. The RMSD value is stated to have good validity, so the ligand copy interaction pocket size can be used to dock compounds from each extract. The smaller RMSD value indicates that the interaction between the compound and the target protein is getting closer to the interaction between the native ligand and the target protein. Visualization of the docking method validation is shown in fig. 3.

The test compounds used in the study were 64 *Curcuma longa* L. compounds, 53 *Piper nigrum* L. compounds, and 65 *Centella asiatica* (L.) Urb. Compounds obtained from the results of literature studies. The observed free binding energy is the interaction energy of the ligand bond to the glutaminase enzyme. The results of the docking of the test compound molecules generally show interactions with the active site of the receptor, which are characterized by negative free binding energy values, but only a few compounds from *Curcuma longa* L. and *Centella asiatica* (L.) Urb. This showed the best results in inhibiting the target protein (table 3-5).

Based on the results of the in silico activity evaluation of identified compounds from gotu kola extract, it is shown that several potential compounds have the best interaction with the target protein of the glutaminase enzyme. Centellasaponin C, asiaticoside, and its derivatives, including asiaticoside B, D, E, and G, and Quercetin-3-O-rhamnoside compounds. The interaction of these compounds has a more negative binding affinity value (range -8 to -10 kcal/mol) compared to the native ligand 5-oxo-L-norleucine (-5.4 kcal/mol).

Table 3: Free energy value of binding, ligand and receptor interaction, and residues bound to glutaminase enzyme in gotu kola (*Centella asiatica* (L.) Urb) compound

No.	Compounds	Free binding affinity (kcal/mol)	Amino acid residue (H-Bond)
NL	5-OXO-L-NORLEUCINE	-5,4	ASN170, ASN117, GLN67, VAL266
1	Asiaticoside	-8,7	GLU163, ASN170, TYR248, LYS289
2	Asiaticin	-6,8	ARG496
3	Madekassosida	-8,6	ASN101, ARG99, ASP249, LYS289
4	Asam Asiatic	-9,3	-
5	Isoasiatic acid	-9,2	-
6	2 α ,3 β ,20,23-Tetrahydroxy-urs-28-ioc acid	-8,8	-
7	Centella-sapogenol A	-9,6	-
8	Asam madasiatic	-9,7	-
9	Centellin	-5,4	ASN170, SER166
10	1,3,7,9-Tetrahydroxy-6H-dibenzo[<i>b,d</i>]pyran-6-one	-8,0	TYR248, LYS71
11	Meso-inositol	-5,9	GLN67, SER68, TYR196, ASN170
12	Amygdalin	-8,5	SER166, ASN170, GLU163, ASN117
13	Brahmic acid	-9,6	-
14	Centellasaponin B	-9,3	-
15	Centellasaponin C	-9,1*	GLN67, ASN170, GLU168, TYR248
16	Asiaticoside C	-8,8	PHE100, GLU163, ASN117, ARG169, ASP249, ARG99
17	Asiaticoside D	-9,2*	ASP249, ASN117, ASN101
18	Asiaticoside E	-8,9*	VAL266, ASN170, GLU163, TYR31
19	Asiaticoside F	-8,2	VAL266, ASN170, GLU163, TYR31
20	Isoasiaticoside	-7,8	LEU287, MET290
21	Asiaticoside G8	-8,5*	ASP30, TYR31, LYS289, GLN67, ASN170
22	Centelloside E	-8,8	ARG99, ASP249, GLU163
23	Asiaticoside B	-8,3*	MET290, VAL266, SER68, LYS71, ASN117, GLU163, ASP288, LYS289
24	Centellasaponin A	-7,7	LYS289, ASN117, ASO249, LYS27
25	Centellasaponin D	-8,6	PHE100, GLU163, TYR196, ASN117
26	Chebuloide II	-9,0	GLU163, LYS27
27	Centelloside D	-8,4	ASP249, MET290, ASP288, ASN101
28	Castasterone	-7,8	ASN117, LYS27
29	Castillicetin	-9,1	GLU163, ASP249, SER68
30	Castilliferol	-9,2	SER68, ASP249
31	Polyacetylene I	-5,2	ASN170, ARG169
32	Polyacetylene II	-6,1	VAL266, SER68, ASN170
33	Polyacetylene III	-5,5	TYR248
34	Polyacetylene IV	-5,8	SER68
35	Polyacetylene V	-5,3	MET290
36	Centellicin	-5,3	TYR248
37	Glyceryl 2-phospho-1caprate	-5,8	TYR196, ASN117
38	Cadiyenol	-6,3	LYS27, ASN101, VAL266, SER68
39	Irbic acid (3,5-O-diceffeoyl-4-O-malonil quinic acid)	-8,0	LYS289, ASP288, LYS71, TYR196, TYR248, ASN101, ASN117, SER166
40	11-Oxoheneicosanyl cyclohexane	-5,1	SER68, VAL266
41	Apigenin	-7,8	ASN117, MET290, GLN67
42	Luteolin	-7,9	GLU163, ASN170, ARG169, ASN101, SER68
43	Myristicin	-5,7	ASN117
44	7-methoxycoumarin	-5,8	ASN117, SER68
45	Umbelliferone	-5,9	LYS71, ASN117
46	p-coumaric acid	-5,7	SER68, GLN67, MET290, TYR31
47	Ferulic acid	-5,9	TYR31, MET290, SER68
48	Caffeic acid	-5,7	ASN101, MET290, TYR31, GLN67
49	Kaempferol	-7,8	GLN67, SER68, MET290
50	Quercetin	-7,9	ASN101, GLN67
51	Chlorogenic acid	-8,0	MET290, ASN117, GLN67, SER68, ASN170
52	Kaempferol-3-arabinside	-8,4	GLU163, GLN67, TYR31, SER166
53	β -sitosterol	-7,8	-
54	Kaempferol-7-rhamnoside	-8,6	ASN117
55	Kaempferol-3-O-rhamnoside	-8,6	SER68, ASN101, ASN117, SER166
56	Quercetin-3-O-rhamnoside*	-9,0*	SER166, ASN170, GLU163, ASN117, SER68, ARG169
57	Kaempferol-5-glucoside	-8,2	ASN117, PHE100, GLY291, LYS289
58	Kaempferol-3-O-glucoside	-8,1	PHE100
59	Betulinic acid	-9,3	ASN117
60	Methyl asiatate	-8,9	-
61	Isothankunic acid	-9,0	-
62	Terminolic acid	-9,1	LYS289, ASP249, SER68
63	β -carotene	-5,9	-
64	Rutin	-8,5	MET290, PHE100, ASN117, ASN170, LYS27
65	Scheffurosia B	-8,8	PHE100, LYS289, ASP249, ARG99, ASN117, ASN170, GLU163

Note: *The selected compounds that have the best interaction in inhibiting the target protein based on evaluation of binding affinity and hydrogen interactions with amino acid residues.

Table 4: Free energy value of binding, ligand and receptor interaction, and residues bound to glutaminase enzyme in Turmeric extract (*Curcuma longa*) compounds

No.	Compounds	Free binding affinity (kcal/mol)	Amino acid residue (H-Bond)
NL	5-OXO-L-NORLEUCINE	-5,4	ASN170, ASN117, GLN67, VAL266
1	Curcumin	-7,6*	ASN101, LYS71, ASN170
2	Dimethoxy curcumin	-7,7	ASP30, TYR31
3	Bisdimeoxy curcumin	-7,6*	LYS27, GLN67, ASP30
4	Calebin A	-6,0	ASP30, LYS27, GLN67
5	1,7-bis(4-hydroxy-3-methoxyphenyl)-1,4,6-heptatriena-3-on	-7,9	LYS27
6	1-hydroxy-1,7-bis(4-hydroxyphenyl)-3-methoxyphenyl)-6-heptena-3,5-dion	-7,8*	LVS27, TYR31, GLN67
7	1,7-bis(4-hydroxyphenyl)-1-heptena-3,5-dion	-7,7	ASP30, LYS27, MET290
8	1,7-bis(4-hydroxyphenyl)-1,4,6-heptatrien-3-on	-7,6	TYR31, LYS27
9	1,5-bis(4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-on	-7,5*	ASN170, ASN117, LYS71, SER68, GLN67, ASN101, TYR248, VAL266
10	Curcumenon	-6,8	ASN101, LYS71
11	Dehydrocurdion	-6,7	SER68, ASN117
12	Germacrone 4,5-Epoxyde	-6,5	-
13	Bisabola-3,10-diena-2-on	-6,2	-
14	A-turmeron	-7,9*	ASN117, ASN170
15	Bisacumol	-6,4	ASP492
16	Bisacuron	-6,4	LYS71, GLU163
17	Curcumenol	-6,4	ASN117
18	Isoprocurcumenol	-6,9	TYR248, GLU163
19	Zedoaronediol	-7,0	ASN117
20	Procurcumenol	-7,0	ASN117, TYR196
21	Epiprocurcumenol	-7,0	ASN117, LYS71
22	Germacrone-13-al	-6,6	SER68
23	4-hydroxy-bisabola-2,10-diena-9-on	-6,3	-
24	4,5-di hydroxy bisabola-2,10-diena	-6,7	ASN117, TYR196
25	4-methoxy-5-hydroxy bisabola-2,10-diena-9-on	-6,5	ASN117
26	2,5-di hydroxy ibisabola-3,10-diena	-6,3	-
27	Procurcumadiol	-7,4	LYS71, ASN117
28	1,3,5,11-Bisabolatetraene	-5,9	-
29	3-Hydroxy-1,10-bisaboladien-9-one	-6,2	ASN117, LYS71
30	ar-Turmerone	-6,4	SER68
31	Curlone	-6,4	SER68
32	Bisacurone A	-6,6	TYR196, LYS71
33	Bisacurone B	-6,4	ASN117
34	Bisacurone C	-6,7	SER68
35	Bisacurone epoxide	-6,8	TYR31, LYS71
36	Turmeronol B	-6,2	ASN117
37	Dihydrocurcumenone	-6,7	ASN101, SER68
38	Isospathulenol	-7,1	VAL266
39	Isozedoaronediol	-7,3	TYR196
40	α -Phellandrene	-4,5	-
41	Sabinene	-4,8	-
42	Geraniol	-4,9	ASN117
43	Bergamotene	-5,7	-
44	p-Cymene	-5,1	-
45	Eucalyptol	-4,8	ASN117
46	Terpinolene	-5,0	-
47	β Thujene	-4,5	-
48	Terpineol	-5,3	GLU163, TYR31, GLN67
49	Camphene	-4,5	-
50	O-Cymene	-5,1	-
51	Cis- α -Bisabolene	-6,2	-
52	α -Curcumene	-6,2	-
53	γ -Himachelene	-6,5	-
54	α -Fernesene	-4,8	-
55	Alpha-terpinene	-4,9	-
56	β -Pinene	-4,6	-
57	β -Myrcene	-4,5	-
58	2-Carene	-4,6	-
59	3-Carene	-5,0	-
60	4-Carene	-4,6	-
61	Zingiberene	-5,4	-
62	β -Sesquiphellandrene	-6,1	-
63	p-Menthatriene	-5,0	-
64	Bisabolene	-6,1	-

Note: "The selected compounds that have the best interaction in inhibiting the target protein based on evaluation of binding affinity and hydrogen interactions with amino acid residues.

Table 5: Free energy value of binding, ligand and receptor interaction, and residues bound to glutaminase enzyme in black pepper (*Piper nigrum*) compounds

No.	Compounds	Free binding affinity (kcal/mol)	Amino acid residue (H-Bond)
NL	5-OXO-L-NORLEUCINE	-5,4	ASN170, ASN117, GLN67, VAL266
1	Piperin	-7,2	ASP249
2	Alpha-Thujene	-4,6	-
3	α -Pinene	-4,6	-
4	Camphene	-4,5	-
5	Sabinene	-4,8	-
6	β -Pinene	-4,6	-
7	β -Myrcene	-4,5	-
8	α -Phellandrene	-4,5	-
9	Delta-3-carene	-5,0	-
10	Alpha-terpinene	-4,9	-
11	p-Cymene	-5,1	-
12	Limonene	-5,0	-
13	1,3,6-Octatriene, 3,7-dimethyl-, (E)-(CAS) Beta Ocimene	-4,7	-
14	Gamma-terpinene	-5,1	-
15	Alpha-terpinolene	-5,0	-
16	Linalool	-4,6	TYR231, ARG215
17	Delta-elemene	-5,8	-
18	Alpha-cubenene	-5,9	-
19	Alpha-copaene	-5,9	-
20	Beta-elemene	-6,0	-
21	Alpha-gurjunene	-6,0	-
22	Trans-caryophyllene	-6,1	-
23	Alpha-humulene	-6,4	-
24	Germacrene-D	-6,3	-
25	Beta-selinene	-6,2	-
26	Alpha-selinene	-6,3	-
27	Delta-cadinene	-6,1	-
28	(-)-Caryophyllene oxide	-6,2	ASN117
29	Spathulenol	-6,5	LYS71
30	Eugenol	-5,4*	ASN117, ASN170
31	Gingerol	-6,7	TYR512, ARG496, LYS477
32	Zingerol	-5,6	SER166, SER68, LYS71
33	Carvacrol	-5,4	ASN101
34	Thymoquinone	-5,7	TYR248, ASN101
35	Cinnamaldehyde	-5,1	SER68
36	Cinnamic acid	-5,5*	GLN67, VAL266
37	β -Caryophyllene	-6,1	-
38	Calcone	-6,8	TYR248
39	Chloroquine	-6,1	-
40	Hydroxychloroquine	-6,2	TYR196
41	Anethole	-5,0	-
42	Fenchone	-5,0	ASN170
43	Metyl chavicol	-4,7	-
44	Cuminaldehyde	-5,3	VAL266
45	Baricitinib	-8,0*	SER166, ASN101, SER68, ASN117, ASN170, LYS71
46	Chavicine	-7,2	LYS71
47	Isochavicine	-7,1	-
48	Isopiperine	-7,1	VAL266
49	Piperidine	-3,2	-
50	Piperic acid	-6,4*	GLN67, ASN170
51	CMPD-1	-6,4*	ASN170, SER68, GLN67
52	CMPD-8	-5,8	TSER68, GLN67, VAL266
53	Adenosine 5'-monophosphate	-7,2	ASN117, GLN67, TYR196, ASN170

Note: *The selected compounds that have the best interaction in inhibiting the target protein based on evaluation of binding affinity and hydrogen interactions with amino acid residues.

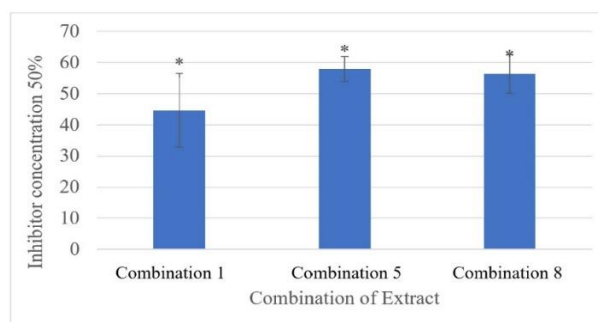


Fig. 2: The anti-glutaminase activity of combination extract. Test data showed that there was a significant difference in the inhibitory activity against glutaminase of each extract combination ($p < 0.05$, $n = 3$, $mean \pm SD$)

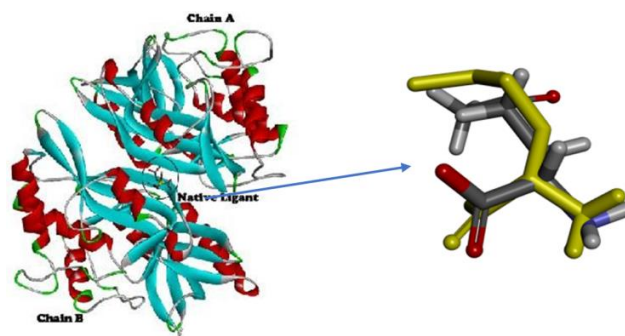


Fig. 3: Docking visualization of native ligand. (a) Yellow represents the copy ligand, and (b) gray represents the native ligand

The hydroxyl group located on the aromatic ring of Centellasaponin and asiaticoside derivatives shows hydrogen interactions at the amino

acid residues ASN170, ASN117, GLN67, and VAL266, depending on the position of each compound (fig. 4).

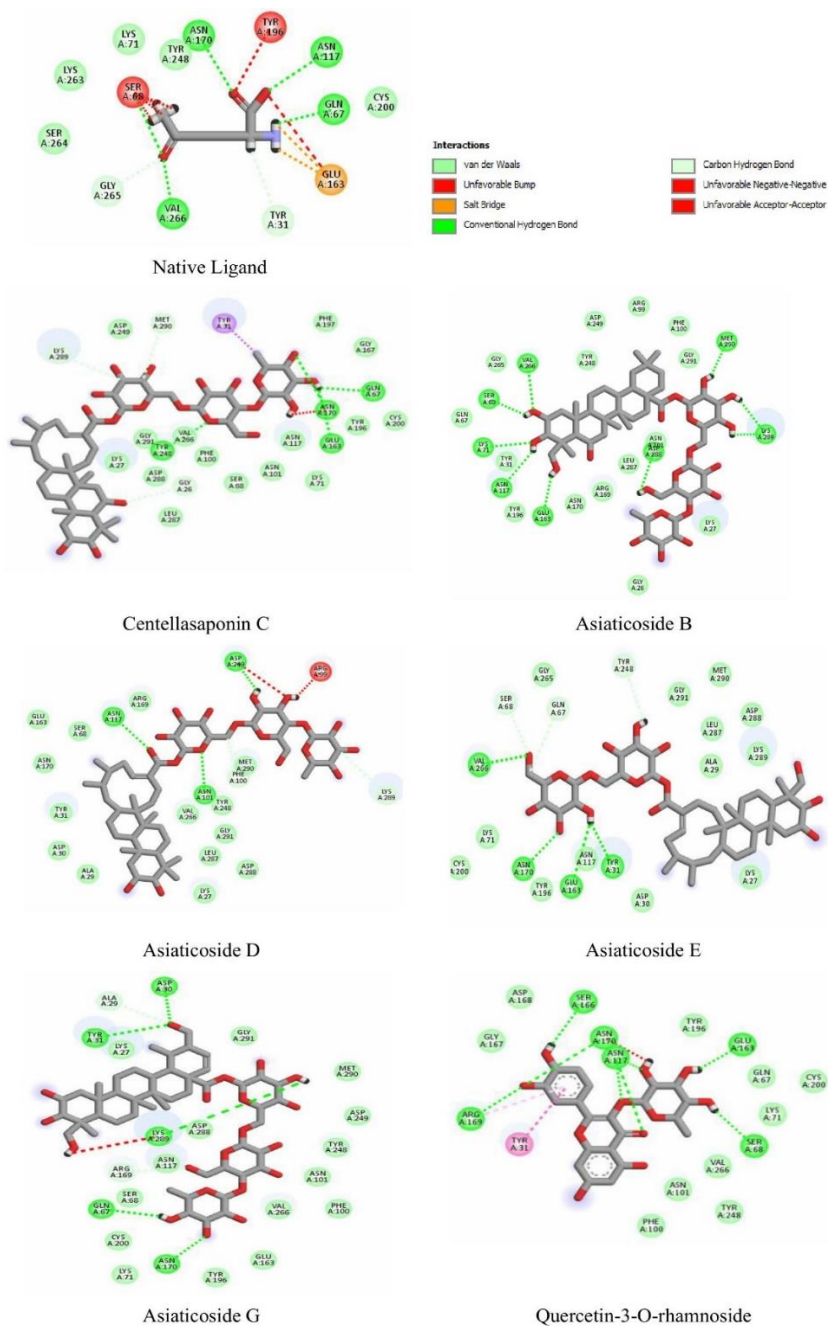
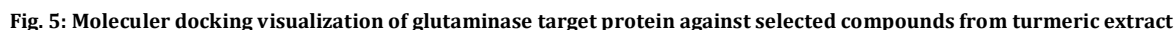


Fig. 4: Molecular docking visualization of glutaminase target protein against selected compounds from gotu kola extract

the most negative binding affinity values and interactions with key amino acids that were similar to the native ligand. The five compounds were curcumin, Bisdimethoxy curcumin, alpha-turmeron, 1-hydroxy-1,7-bis (4-hydroxyphenyl)-3-methoxyphenyl)-6-heptene-3,5-dione, and 1,5-bis (4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one with binding affinity values of each compound >7 kcal/mol (table 4). The binding affinity of each compound was evaluated to be more negative than the native ligand on the glutaminase protein.



ASN170, ASN117, and GLN67, and the hydroxyl group (OH) on the aromatic ring of the compound interacts with the amino acid VAL266. The compound's interaction with the key amino acids ASN170, ASN117, GLN67, and VAL266 is predicted to inhibit the target protein glutaminase. Meanwhile, black pepper extract showed 6 compounds inhibiting glutaminase *in silico*. Compounds like Baricitinib, Eugenol, Cinnamic acid, piperic acid, and CMPD-1 have negative binding affinity values (>6 kcal/mol) and even exceed native ligands (fig. 6 and table 5).

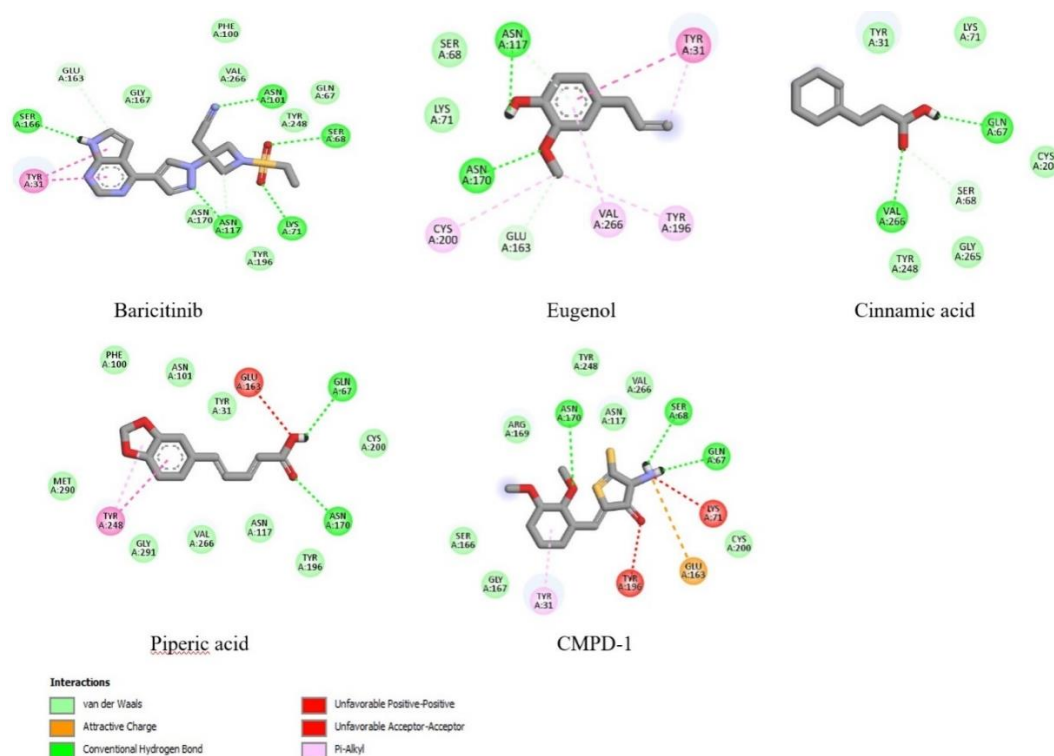


Fig. 6: Molecular docking visualization of glutaminase target protein against selected compounds from black pepper extract

Molecular dynamic simulation of extract compounds

In this study, one of the most active compounds was selected in silico from each extract. In the gotu kola extract, the molecular dynamic evaluation used the compound Asiaticoside B, and the compound 1,5-bis (4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-

one in the turmeric compound and Baricitinib in the black pepper extract compound. The three compounds were evaluated using RMSD, the Radius of gyration, and RMSF. The results of the molecular dynamic evaluation of each compound against the target protein glutaminase compared to the native ligand can be seen in fig. 7-9.

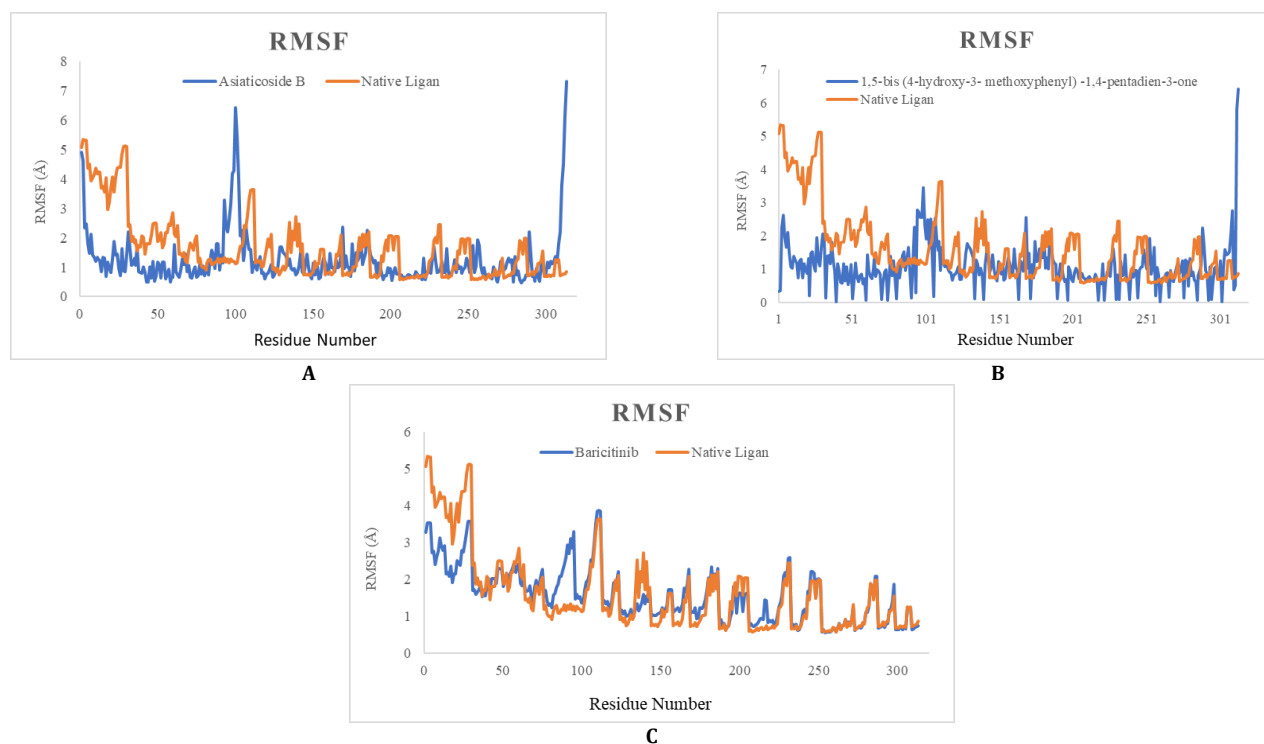


Fig. 7: Graph of RMSF profile during molecular dynamics simulation of 407D-Native ligand (orange line) and 407D-5-OXO-L-NORLEUCINE (blue line) complexes during 100 NM sampling time of the molecular dynamics simulation. A) Asiaticoside B, (B) 1,5-bis (4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one, and (C) Baricitinib

Of the three compounds, the 407D-Asiaticoside B complex showed the most stable binding complex when compared to the 1,5-bis (4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one and Baricitinib compounds. The 407D-Asiaticoside B binding complex showed an RMSD value of 2Å ranging from 0 to 60 NM (fig. 7). It began to increase in the range of 3Å to 90 NM and decreased until 100 NM.

The average RMSD value of the 407D-Asiaticoside B complex was <3 Å. While the 1,5-bis (4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one and Baricitinib compounds showed RMSD values >3 Å starting at 60 NM and started to decrease until 100 NM. RMSD value <3Å indicates that the protein is stable and does not experience significant conformational changes.

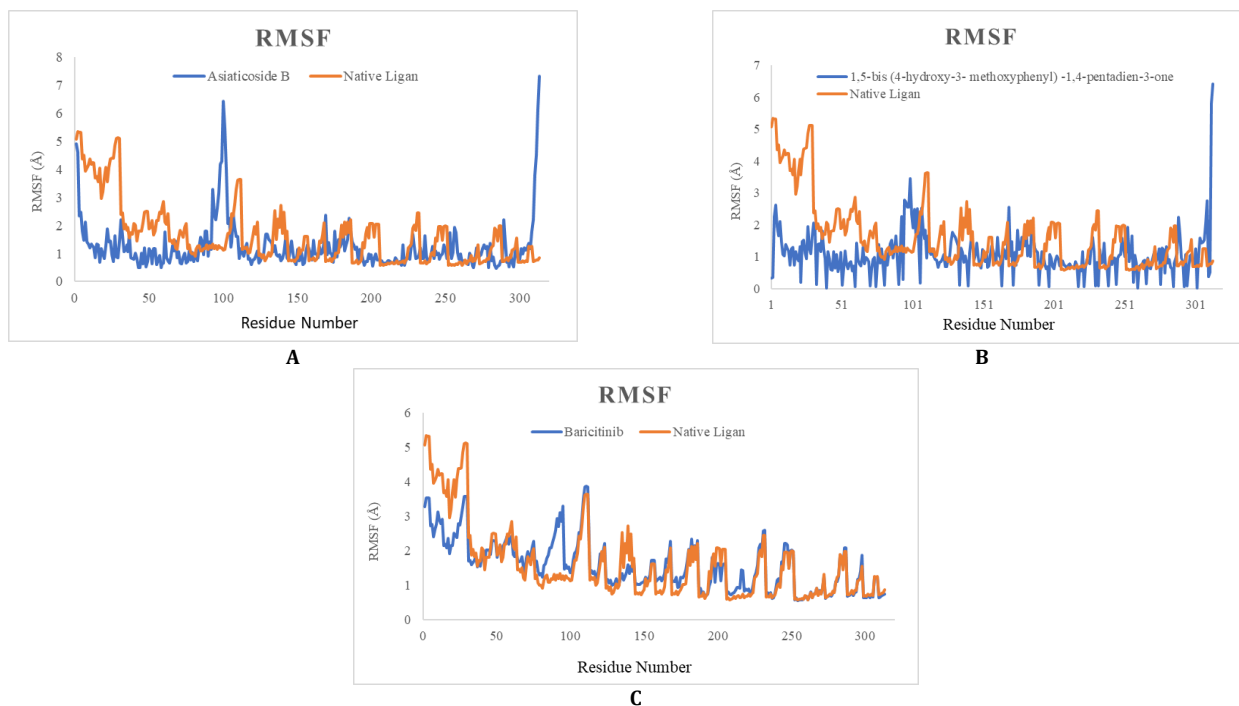


Fig. 8: Graph of RMSF profile during molecular dynamics simulation of 407D-Native ligand (orange line) and 407D-5-Oxo-L-Norleucine (blue line) complexes during 100 NM sampling time of the molecular dynamics simulation. A) Asiaticoside B, (B) 1,5-bis (4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one, and (C) Baricitinib

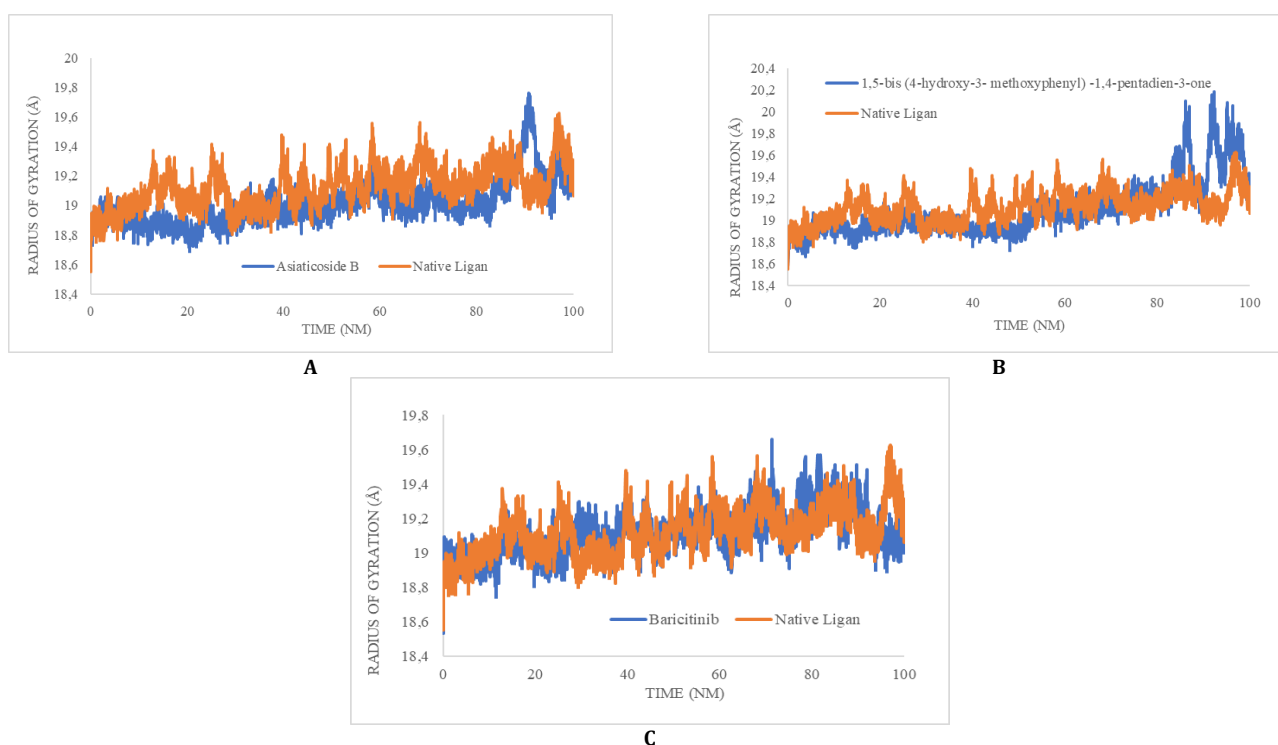


Fig. 9: The graph of Radius of Gyration profile during molecular dynamics simulation of 407D-Native ligand (orange line) and 407D-5-Oxo-L-Norleucine (blue line) complexes during 100 NM sampling time of the molecular dynamics simulation. A) Asiaticoside B, (B) 1,5-bis (4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one, and (C) Baricitinib

The visualization results of the RMSF value in the protein-ligand complex in each compound (fig. 8) tend not to experience significant fluctuations. Although there are fluctuations in the number of amino acids in sequence 100, they stabilize again until molecular dynamics occur. The average on the active site residue of the protein tends not to experience a significant increase in RMSF during the simulation process. The active site can maintain the structure because it has hydrogen bonds between residues ASN117, ASN170, GLN56, and VAL266, which can stabilize the position of the structure. Based on the visualization results of the RMSF value in fig. 10, it can be concluded that the active site residue is in a stable position.

Fig. 9 shows the profile of changes in the protein radius of gyration during molecular dynamics simulations of complexes with compound and native ligands. Changes in the size of 5A46 with ligands during molecular dynamics simulations did not experience significant changes in either the protein-ligand complex Asiaticoside B, 1,5-bis (4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one, and Baricitinib. Although at 80 NM, there was an increase in the radius of gyration, it returned to the appropriate size at 100 NM. This increase did not occur significantly, showing that the protein-ligand complex resulting from docking is quite stable. This can also be adjusted to the evaluation results of RMSD and RMSF values that did not experience significant changes.

DISCUSSION

This research examines the effects of gotu kola, turmeric, and black pepper extracts as candidates in treating brain injury, which is carried out in several stages, starting from the extraction process, phytochemical screening, and *in vitro* and *silico* tests. Secondary metabolite compounds such as asiaticoside derivatives from gotu kola, curcumin derivatives from turmeric, and piperine derivatives from black pepper can potentially treat brain injury. Several parameters used include the effects of extracts and combinations of extracts on improving memory and inhibiting glutaminase, which triggers an increase in neurotransmitters in the form of glutamate after brain injury.

Gotu Kola extract can stimulate memory, which is thought to be caused by the triterpenoid saponin compound (asiaticoside) contained therein. This compound is known to repair damaged blood vessels, thereby improving blood circulation to the brain. It is also able to regenerate cells and heal wounds. This process is initiated by inhibiting Na⁺/K⁺-ATPase in the brain, which depolarises calcium in the endoplasmic reticulum (ER). Depolarization of the RE causes continuous secretion of acetylcholine, resulting in stable central cholinergic neurotransmission and increased memory [12, 33, 34]. Turmeric extract is thought to contain curcuminoids essential in improving memory. Curcuminoids can work by reducing oxidative stress in the hippocampus, and curcumin shows its antioxidant potential by reducing oxidative stress [14, 35].

Meanwhile, in black pepper extract, the presence of a significant compound in the form of piperine is strongly suspected to have a role in improving memory. The antioxidant in piperine itself has a mechanism of action by donating one or more electrons to free radicals so that reactions caused by free radicals do not occur, which can cause oxidative stress [36, 37]. According to research conducted, piperine also stated there was a significant reduction in the increase in acetylcholinesterase that was induced by hyoscine because it has a high affinity for binding to acetylcholinesterase so that it cannot act as a catalytic enzyme against acetylcholine [38]. This follows the results obtained with the positive control, which showed that black pepper extract provided the same memory-enhancing activity as ginkgo biloba because it had a similar mechanism of action in inhibiting acetylcholinesterase and acting as an antioxidant. Combining these three natural ingredients is a novelty in searching for active ingredients with neuroprotective effects. To date, there have been no reports on the ability of individual extracts or combinations of extracts to inhibit glutaminase activity to assess their effects as neuroprotective.

Another consequence of brain injury is an increase in the neurotransmitter glutamate due to the excretion of glutaminase. Excess glutamate can cause excitotoxicity, which is a common

central pathological factor in many neurological diseases and injuries, especially those showing neuroinflammatory components, such as multiple sclerosis (MS), traumatic brain injury, acute brain anoxia/ischemia, epilepsy, glaucoma, meningitis, amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Huntington's disease, Parkinson's disease, and various other diseases. Glutamate itself becomes very toxic in multiple pathological conditions [39–41]. The existence of over-expression of glutamate is a parameter in evaluating the ability of a material to treat brain injury [42].

Glutamate plays a vital role in the normal development and function of the central nervous system (CNS). The benefits of glutamate are that it plays a crucial role in brain activity, including cognition. Glutamate also significantly contributes to the development of the CNS by contributing to the formation and elimination of synaptic nerve contacts, as well as cell migration, differentiation, and regulation of cell death. Given the strong and vital role of glutamate in many body functions and its rapid and robust effects on many target cells, glutamate must be present at the right time, in the right concentration, and in the right amount. Excess glutamate will cause excitotoxicity, a central pathological factor common to many neurological diseases and injuries, especially those showing a neuroinflammatory component, such as multiple sclerosis (MS), traumatic brain injury, acute brain anoxia/ischemia, epilepsy, glaucoma, meningitis, amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Huntington's disease, Parkinson's disease, and various other diseases. Glutamate becomes very toxic in multiple pathological conditions when its concentration exceeds its concentration. Meanwhile, glutamate deficiency can cause several problems, including neurological, mood, central nervous system, and metabolic disorders [39–41].

Several studies have reported that gotu kola, turmeric and black pepper extracts have neuroprotective effects. Asiaticoside is one of the asiatic acid derivatives in gotu kola that plays a role in inhibiting glutaminase through its antioxidant properties, so it can reduce the formation of ROS and inhibit the formation of lipid peroxidation [43]. The same thing also happens to the curcumin compound in turmeric which works by inhibiting glutaminase against glutamine metabolism through its antioxidant mechanism. Curcumin is also able to prevent the death of neuron cells after brain injury, one of the consequences of which comes from excessive glutaminase expression [44]. Meanwhile, piperine from black pepper works by improving cognitive disorders and restoring hippocampal neurotransmission, such as excessive glutamate expression due to increased glutaminase activity [45]. These studies support the results of our study and with the combination at certain doses used in this study, it can increase the effect as a neuroprotectant due to brain injury.

This study also observed the inhibitory interactions between chemical molecules by molecular docking of each extract against glutaminase protein to support the *in vitro* results. The parameters analyzed in this docking study were amino acid residues, hydrogen bonds, and free binding energy (ΔG) [46]. Observation of amino acid residue interactions aims to identify the interactions that occur between the ligand and the receptor. Hydrogen bonds are interactions that can stabilize the ligand bond with the receptor. Other interactions between ligands that can increase conformational stability are electrostatic interactions and van der Waals interactions [25, 47, 48].

The selected and active compounds in inhibiting glutaminase in *silico* from gotu kola extracts, such as centellasaponin and asiatica derivative, which are compounds of the triterpenoid saponin group and the Quercetin-3-O-rhamnoside compounds are phenolic compounds which are thought to play an essential role in the process of inhibiting over-expression of glutaminase. These compounds interact through hydrogen bonds with vital amino acids of the target protein, namely at residues ASN170, ASN117, GLN67, and VAL266 (fig. 4). For the curcumin derivatives specifically interacts through hydrogen bonds at the amino acid residue ASN170, while other compounds, namely bis dimethoxy curcumin, alpha-turmerone, and 1-hydroxy-1,7-bis (4-hydroxyphenyl)-3-methoxyphenyl)-6-heptene-3,5-dione specifically bond hydrogen with the key amino acid GLN67. However, for the compound 1,5-bis

(4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one, there is a hydrogen interaction at the key amino acids ASN170, ASN 117, GLN67, and VAL266 and is very similar to the hydrogen interaction of the native ligand compounds, namely ASN170, ASN117, GLN67, and VAL266 (fig. 5). The similarity of interactions with key amino acids of the target protein glutaminase allows competitive inhibition.

Similar things were also found in the *in silico* activity of black pepper extract compounds. There is a hydrogen interaction between the active site of the compound with specific amino acid residues, namely ASN117, ASN170, VAL266 and GLN67, so black pepper extract can inhibit excessive glutaminase activity, which triggers brain haemorrhage. The active site in the form of hydroxyl groups and methoxy groups of active compounds generally interact with hydrogen with amino acid residues ASN117, ASN170 and GLN67 and the critical amino acid VAL266 tend to interact with hydrogen with the carbonyl group (C = O) of the compound, especially in cinnamic acid compounds. Meanwhile, in the baricitinib compound, hydrogen interactions on the vital amino acids ASN117 and ASN170

occur in the nitrogen group of the pentacyclic structure of the baricitinib compound (fig. 6). The baricitinib compound reported in black pepper is an alkaloid with the best activity in inhibiting glutaminase, with a binding affinity value of -8.0 kcal/mol. This compound must be studied further regarding its mechanism and pharmacological action. However, based on the chemical structure, the nitrogen group in the alkaloid core certainly interacts with hydrogen with critical amino acids in the glutaminase target protein.

The selected compounds from each gotu kola, turmeric, and black pepper extract showed the best interaction with the target protein glutaminase, with a more negative binding affinity value than the native ligand. However, one of the candidate markers among the identified active compounds from each extract it was shown that the compound Asiaticoside B from gotu kola, 1,5-bis (4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one from turmeric, and Baricitinib from black pepper *in silico* gave strong activity. This is because the three compounds have the same active site that interacts with the amino acid residues of the target protein. Fig. 10 is a visualization of the synergistic effect of the three compounds on the target protein.

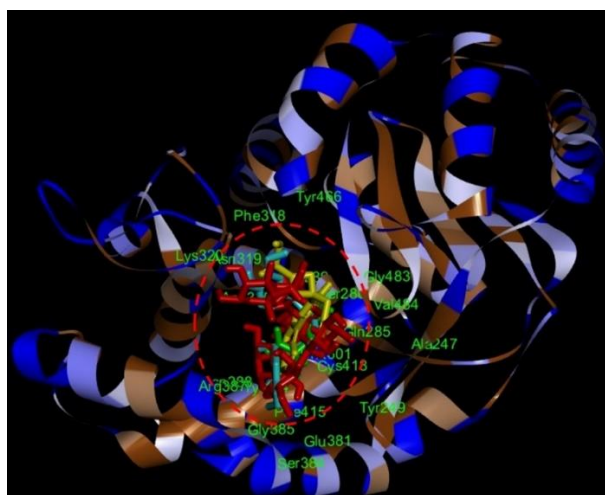


Fig. 10: Visualization of the interaction of Asiaticoside B (blue color), 1,5-bis (4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one (red color), and Baricitinib (green color) compounds against the target protein glutaminase. The red circle indicates that the three active compounds interact at the same active site compared to the native ligand (yellow color)

These results support the *in vitro* study on each extract and the combination extract of the three. *In vitro*, with parameters of increasing memory and inhibition of glutaminase, shows that the combination formula of the extract effectively increases memory and inhibits over-expression of glutaminase. The potential chemical content influences the synergistic effect of each extract in providing bioactivity and pharmacological effects. The chemical interaction of each compound with the receptor in providing a pharmacological effect is not convincing enough if it is not accompanied by the stability of the bond or interaction through molecular dynamics.

Molecular Dynamics is one of the best methods to investigate the dynamic behaviour of macromolecules at the molecular and atomic levels [49]. Molecular dynamics simulation aims to determine the stability of protein interactions with ligands in conditions similar to human physiology over a certain period. Several parameters evaluated are the RMSD (Root Means Standard Deviation) value, Radius of gyration, and RMSF (root means square fluctuation). These three parameters can evaluate the stability of the interaction of compounds with target proteins [50, 51].

Suppose the RMSD value ≥ 3 Å indicates that the protein has undergone conformational changes that are very different from its native condition. An increase in the RMSD value indicates that the protein structure is starting to open. The ligand seeks the appropriate binding site or coordinates on the protein. In contrast, a stable RMSD value indicates that the maximum conformation of the protein bound to the ligand is starting to be achieved so that the

protein can maintain its position [52, 53]. Similar things were also found in the 5A46-native ligand complex, which experienced an increase in RMSD values in the range of >3 Å. This indicates that the protein-native ligand complex tends to be less stable when compared to the protein-compound complex. However, observing the profile of the increase in RMSD values in the compounds 1,5-bis (4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one and Baricitinib did not provide a significant picture because at 100 NM time there was a decrease in RMSD values. Therefore, the RMSD observations on the three compounds showed fairly stable results.

The next parameter is the RMSF value in the 5A46 complex and the compound/native ligand. RMSF (Root mean Square Fluctuation) measures the deviation between the particle position and several reference positions. RMSF can be calculated for each amino acid residue that makes up the protein by looking at the extent of the fluctuation of the movement of each amino acid residue during the simulation [54, 55]. The purpose of RMSF analysis in molecular dynamics simulations is to obtain information about flexible and rigid amino acid residues during the simulation process [56]. Fig. 9 is a visualization of molecular dynamics on the RMSF parameters of the Protein-ligand complex. Low flexibility describes the stability of the interaction of the protein complex with the ligand. The maximum value of amino acid residues is 2.5 Å. RMSF values <2.5 Å indicate low flexibility. The flexibility of amino acid residues describes the stability of the interaction at the active site that binds to the test compound because the atoms that make up the amino

acid residues tend not to change many positions during the molecular dynamics simulation [57].

The protein radius of gyration is also analyzed in addition to RMSD and RMSF. The protein radius of gyration is the distance of the distribution of protein atoms from its main axis. The radius of gyration in molecular dynamics simulations provides information about changes in protein size during dynamic simulations. Therefore, the larger the radius of gyration, the larger the protein [52]. The profile of changes in the protein radius of gyration during molecular dynamics simulations of complexes with compound and native ligands. Changes in the size of 5A46 with ligands during molecular dynamics simulations did not experience significant changes in either the protein-ligand complex Asiaticosid B, 1,5-bis (4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one, and Baricitinib. Although at 80 NM, there was an increase in the radius of gyration, it returned to the appropriate size at 100 NM. This increase did not occur significantly, showing that the protein-ligand complex resulting from docking is quite stable. This can also be adjusted to the evaluation results of RMSD and RMSF values that did not experience significant changes. The study of molecular dynamic analysis of protein complexes with the most active compound ligands and native ligands showed quite good results and showed a stable protein-ligand complex.

Exploration in this study allows the development of a combination of gotu kola, turmeric, and black pepper extracts as an alternative treatment for brain injury. This study is still limited to exploring the bioactivity of single extracts and combination extracts used as candidates for active ingredients as neuroprotective. This discovery will be developed to obtain standardized natural product ingredients. Quantitative analysis of the levels of active compounds in the extract also needs to be done to regulate the dosage regimen as a neuroprotective. The form of the dosage formula is also essential to be developed to provide a better effect as a neuroprotective. In future developments, a combination dose of gotu kola, turmeric, and black pepper extracts can be applied as an alternative neuroprotective treatment. A combination dose regimen with a ratio of 50:50:50 of each extract can be formulated into a microencapsulation form and intended for oral administration. This approach can be done to produce phytopharmaceutical drug candidates that can be tested clinically.

CONCLUSION

Bioactivity studies of gotu kola, turmeric and black pepper extracts in treating brain injury through memory enhancement parameters in the Y-Maze, *in vitro* studies in inhibiting glutaminase expression and molecular docking approaches that have been carried out show a good correlation of activity. The bioactivity of the extracts individually has shown a good effect. In silico testing through molecular docking identified several compounds with glutaminase 5A46 inhibitory activity. Still, in each extract, only the most active compound was selected. It showed asiaticoside B, 1,5-bis (4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one, and Baricitinib compounds with more negative binding affinity values and have a hydrogen interaction profile to amino acid residues similar to the native ligand. The three compounds were also evaluated for their stability through molecular dynamics, and the evaluation results showed that they had stability when interacting with the target protein. After being evaluated, the combination of the extracts is very supportive of being developed as one of the standardized herbal products for treating brain injury.

ETHIC APPROVALS

This method has been equipped with a code of research ethics in the use of experimental animals as subjects for in vivo research obtained from the Research Ethics Code of Medical Faculty, Hasanuddin University, Makassar, South Sulawesi, Indonesia, with ID number 40611IN4.6.4.5.31/PP36/2024.

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AUTHORS CONTRIBUTIONS

AR, MA, AA, AAI: Concept or ideas, design, Clinical trial, Experimental Studies, Data Analysis, manuscript preparation. KP, SS, and HL: Literature search, collected the data, data acquisition, manuscript editing. NS, BY, SN: in silico design, *In vitro* experimental studies, statistical analysis, manuscript review, manuscript revision.

CONFLICT OF INTERESTS

The authors declare that is there not conflict of interest with the data contained in the manuscript.

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