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Chemical Interaction Analysis of L-Theanine Compounds from *Camellia sinensis* L. with Kainate Glutamate Receptors and Their Toxicity Effect as Anti Autism Candidates Based on In Silico

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Abstract. Autism is a neuropsychiatric disease, one of the causes of autism is damage to neurons. L-Theanine is a bioactive compound in *Camellia sinensis* L.which is analogous to L-Glutamate Acid structure and its neuroprotective effect. This study aimed to analyze the binding side of L-Theanine and L-Glutamate Acid to the kainate of glutamate receptor protein to determine and the effectiveness of its inhibitor function. Toxicity analysis is also used to determine the suitability of compounds as bioactive components to be consumed orally. The method used to analyze the interaction of compounds with target proteins is reverse docking. Toxicity analysis using the Toxtree 2.6.13 and collection of information from the Human Metabolome Database. The docking shows that L-Glutamate Acid and L-Theanine have the same site in the ionotropic Glutamate receptor protein, kainate1. The residual groups of the two compounds when binding to the similar glutamate receptor protein are THR (A: 91), GLU (A: 191), and ARG (A: 96). The binding affinity of the two compounds is almost the same, namely -5.0 kcal/mol for L-Glutamate Acid and -4.9 kcal/mol for L-Theanine. This allows L-Theanine to act as an inhibitor that blocks L-Glutamate Acid from binding to glutamate receptors on prostsynap membranes. The compound docking results show that L-Theanine has four bond side residues that are the same as the same L-Glutamate Acid and binding affinity of -5.0 kcal/mol. Analysis with the principle of RO5 Lipinski is known that L-Theanine compounds have the potential if taken orally. Therefore, the *C. sinensis* L. has potential as an anti-autism.

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INTRODUCTION

Autism Spectrum Disorder (ASD) or autism disorder is a neurodevelopmental disorder caused by various factors [1]. Autism is a neuropsychiatric disorder that is common in early childhood and will continue throughout one's life. The characteristic of autism disorder is their inability to communicate both verbally and non-verbally, not interested in the social environment, having a tendency to repeat an activity or even imitating certain activities with a form of unnatural attraction. This causes autism sufferers are often excluded from the community because social cognitive and language are the main things in human life [1, 2]. In addition to influencing the ability of social interaction, autism also has several symptoms related to neurologic namely movement problems, anxiety, convulsions, abnormal sleep patterns and hyperactivity problems (low attention), compulsive obsessive disorders, emotional instability, bipolar disorder or schizophrenia [3]. There are no definite data regarding cases of autism in Indonesia. However, in 2013 there were 100,000 million men with autism. This number is greater when compared to women with autism who only have a prevalence of 70.1 out of 100,000 women. In general, cases of the emergence of autism symptoms occur in individuals aged 15-19 years [4].

The factors that cause autism are quite numerous and varied by involving various metabolic processes. Genetic factors are factors that play a role in the emergence of the autism symptom phenotype [5]. In addition to internal factors, external factors can also lead to the emergence of autism. One of the external factors that cause autism is heavy metal mercury. Mercury is a zinc inhibitor to bind to metallothionein proteins, thus disrupting the level of metal homeostasis in the blood. Failure of homeostasis of metal levels in the blood affects the slowness of cognitive processes [6-8]. Disorders of the digestive system also become one of the causes of autism. Inflammation in the digestive tract and disruption of the digestive natural flora can also cause nutritional digestive disorders that have an impact on how the brain works [8]. Of the several factors that cause autism, neuronal ischemia is a direct factor that causes neuron degeneration due to glutamate poisoning [9].

To the best of our knowledge, there are no drugs specifically for autism sufferers. The use of drugs is usually only to reduce neurologic symptoms only. Selective serotonin reuptake inhibitors including citalopram, fluoxetine, and sertraline are drugs commonly used for depression, anxiety, and obsessive behavior [10]. However, the use of this drug has not been able to effectively cure because symptoms may reappear if the drug is stopped [11]. The use of medicinal plants can be used as a trusted source for finding new drugs for this purpose. Plants can be referred to as medicinal plants if there are plant parts containing active ingredients that have the potential for disease treatment media [12].

Camellia sinensis L. belongs to the family of Theaceae, the plant that is often consumed as a drink often used as a medicinal ingredient because of its bioactive content [13]. One of the bioactive compounds in the *C. Sinensis* which has the potential as a drug is L-Theanine. Previous research shown that L-Theanine has a neuropsychiatric effect, such as increasing attention when consumed together with caffeine [14], sharpens memory [15], increasing concentration when consumed together caffeine [15, 16], and reduce stress [17]. The results of other studies on experimental animals showed that L-Theanine can reduce high blood pressure so that it can prevent neuronal ischemia [18].

Thorne [13] explains that L-Theanine is a bioactive component in tea (*Camellia sinensis*) which is analogous to the glutamate structure. Neuroprotective produced by L-Theanine because it has a structure analogous to glutamic acid, allowing L-Theanine to prevent glutamic acid bonds with glutamate receptors (NMDA, AMPA and Kainate) at postsynaptic. Kakuda *et al.* [9] have revealed the neuroprotective effects of mice in vitro. There are no studies that reveal how the comparison of L-Theanine and L-Glutamate Acid in glutamate receptors in humans. This type of research can be done virtually without having to use tried subjects. Reverse docking is one of the analytical techniques to determine the binding site of a compound to macromolecules [19, 20]. The protein used in this study is ionotropic glutamate receptor, kainate 1. This protein is one type of glutamate receptor on the postsynaptic membrane of kainate type neurons [21]. In addition to determining compound interactions, toxicity tests are also needed to determine the feasibility of candidate compounds as certain anti-diseases.

EXPERIMENTAL

Extraction from Tea Leaf Samples Using The Maceration Technique Using 96% Ethanol.

The tea leaves samples were obtained from packaged in green tea leaves. The extraction process of *C. Sinensis* used a maceration method referring to the method developed by Maulina et al. [22]. Firstly, *C. Sinensis* leaves washed and dried without exposure to the light for three weeks. Furthermore, dried leaves were mashed to become a powder. Next, the leaf powder was macerated using 96% ethanol. This process was carried out for three days. We replaced the

maceration solution of *C. Sinensis* leaves every 24 h. The maceration solution was collected for further separation of solvent and dissolved compounds derived from *C. Sinensis* by using an evaporator.

Liquid Chromatography-Mass Spectrum (LCMS) Analysis to Determine The Content of Compounds Contained in Tea Plants.

The LC-MS analysis of *C. Sinensis* extract used the LC-MS 2010 EV, Shimadzu with a reverse-phase LC system, the ratio of a mobile phase of acetonitrile distillation water is 7: 3 and 10 mmol ammonium acetate. Retrieval of the sample from the *C. Sinensis* compounds was obtained from the highest content in the LCMS results. Selecting compounds with the highest composition based on LCMS results. L-Theanine is the most abundant compound, therefore, L-Theanine was selected as a specific sample for analysis.

Molecular Docking Process

Moreover, an in silico analysis is performed to detect interactions between selected bioactive compounds with target proteins. In silico screening in this research was conducted following the previous methods [23-25]. The analysis technique used is reverse docking, which is a technique for determining the binding side of bioactive compounds in the form of amino acid residues in proteins that are targeted for docking. The compound used as a control compound in this study is L-Glutamate Acid which is a natural neurotransmitter that has the ability to bind to the target protein in the form of glutamate receptor (Glutamate ionotropic receptor, kainate 1). While the test compounds in this study are L-Theanine which is a natural compound in tea which is considered to have a structure analogous to L-Glutamate Acid.

The initial stage in this silico analysis is collecting 3D structures of active compounds (ligands) in the PubChem database [26]. The file extension collected from the PubChem database is an SDF file. After collecting compounds, the next step is to determine the prediction target protein via the webserver. In this study, the web server used is SwissTargetPrediction and SuperPred. Because it has been determined that the protein taken is a glutamate receptor protein so that the results determined are selected for the type of glutamate receptor protein which is found in the results of the analysis of two web servers. The prediction results of protein are chosen for glutamate type Kainate protein (Glutamate ionotropic receptor, kainate 1). The next step is to search for protein ID in the UniProt database [27], then the obtained ID is used to download the 3D protein structure in the Protein Data Bank database [28]. The structure of 3D proteins downloaded in the GDP extension. The next step is to do compound docking using PyRx software. Before docking, the downloaded ligand compounds need to be changed in the PDB file extension through the Discovery Studio program because docking can only be done using the PDB file extension. The result of compound docking from the PyRx application is a list of ligand affinity bonds and proteins and docking compounds.

The next step is to visualize the results of the docking. Visualizing compounds with docking results using the PyMol application and Discovery Studio. The structure of the docking and protein compounds is opened simultaneously with the PyMol application simultaneously and the visualization is arranged in such a way as to facilitate observation. Next, the two files are saved into one file. The stored file from the combination of proteins and docking compounds is opened using the Discovery Studio application to determine compound bond amino acid residues with 2D diagram structure features. If the two compounds analyzed have the same amino acid residues, it can be said that the two compounds are located in the same site on the target protein.

The next step is the analysis of toxicity tests. Toxicity tests were carried out using the toxtree 2.6.13 and collection of information from the Human Metabolome Database (HMDB). HMDB is a human metabolite database consist of information about biological roles, chemical reactions, physiological concentrations, metabolic pathways, disease associations, and references [29]. Use of the toxtree 2.6.13 aims to determine the structure of the compound and its possibility to be potentially toxic or not. The way to use the application is to input the compound smiles to be predicted and then select the "estimate" command, then automatically the properties of the compound will appear along with information related to the possibility of the predicted toxicity. Analysis of the nature of the compound for safety in use must also follow the Lipinski RO5 (Rules of 5) principle [30]. Descriptions of the properties of compounds for fulfilling the Lipinski principle can be found in the Human Metabolome Database (HMDB).

RESULTS AND DISCUSSION

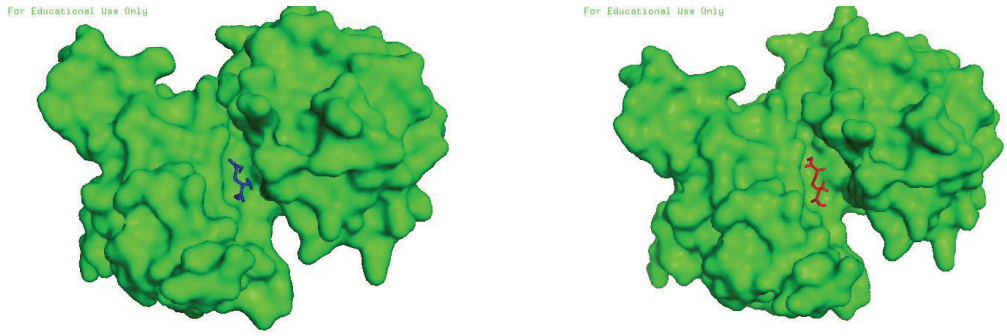
Analysis of LCMS

To analyze the bioactive compounds found in tea plants (*Camelia sinensis*), the LCMS technique was performed. Tea samples used for the analysis of compound contents are packaged green teas that are often found on the market with the "Tong Tji" brand. The results of LCMS analysis of tea leaf extracts showed that the dried tea leaves (green tea) contained as many as 89 compounds. One of the compounds with the highest content in tea extract is theanine with a concentration of 2.1%.

Of the several compounds above, their potential is then traced to overcome autism. Autism symptoms are closely related to neuropsychiatric, which are disorders that are closely related to nerves [1,2]. Compounds that have the potential to treat symptoms of autism must be related to neurologic metabolism. From some of the information above, the target protein for each bioactive compound is then searched. The target protein web server is used to find target proteins from bioactive compounds, one of which is the Swiss Target Prediction, Super Pred, and Stitch. The results of the prediction of target proteins indicate that the potential compound in neurologic processes is Theanine.

Docking Results of L-Glutamate Acid and Theanine Compounds to Ionotropic Glutamate Receptors, Kainate 1.

L-Theanine is predicted to have neuroprotective properties because it has a structure analogous to L-Glutamate Acid, which is a neurotransmitter compound. This is further proved by using an analysis of the interaction of L-Theanine compounds with ionotropic Glutamate receptor protein, kainate 1 which is a neurotransmitter receptor protein on neuron cell membranes using reverse docking techniques. Analysis of the interaction of L-Theanine against glutamate receptor protein compared with the interaction of L-Glutamate Acid compounds as control compounds against the same protein. The visualization of the results of L-Theanine and L-Glutamate Acid docking on Glutamate ionotropic receptors, kainate 1 can be seen in Fig. 1.



(a) Docking result of L-Glutamate Acid (b) Docking result of L-Theanine

FIGURE 1. visualization of L-Glutamate Acid and L-Theanine compounds using *PyMol*

When the two compounds are compared on the same side and visualized, it will appear the two compounds namely L-Theanine and L-Glutamate Acid have sufficient similar sites on the ionotropic Glutamate receptor protein, kainate 1. The visualization results can be seen in Fig. 2.

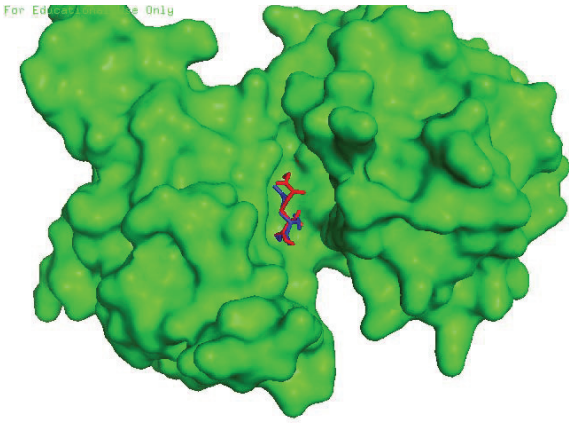
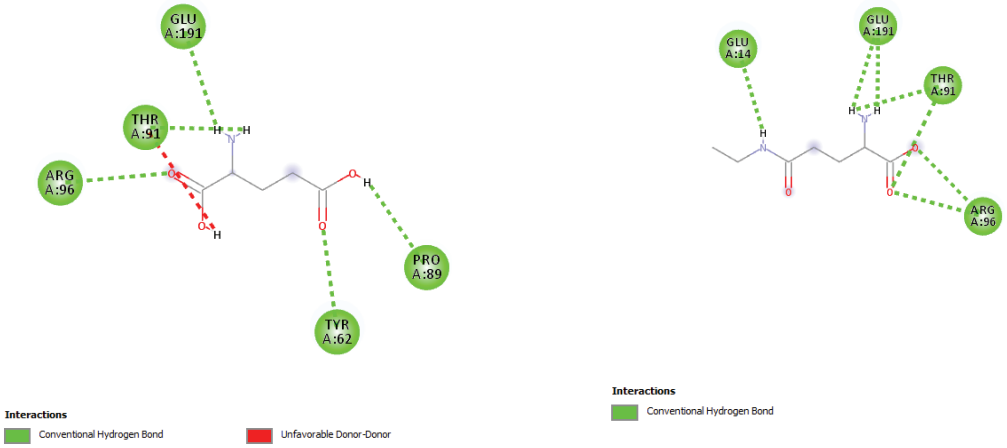


FIGURE 2. The Comparison of the results of docking L-Theanine and L-Glutamate Acid compounds to Protein Glutamate ionotropic receptors, kainate 1 based on visualization using *PyMol*
(Note: the red color shows L-Theanine compound and blue color L-Glutamate-Acid compound)

Ensuring the site of the two compounds in the protein is carried out by analyzing the interaction of ligand (compounds) on proteins. Visualization of ligand interaction is done using the Discovery Studio application. The results of the ligand interaction analysis can be seen in Fig. 3.



(a) Docking result of L-Glutamate Acid (b) Docking result L-Theanine

FIGURE 3. Ligand L-Glutamate Acid and L-Theanine interactions using Discovery Studio

Based on the docking results in Fig. 1 and 2, it is known that L-Glutamate Acid and L-Theanine bind to the same protein residue in the ionotropic Glutamate receptor protein, kainate 1 which is at the following residues: THR (A: 91), GLU (A: 191), and ARGs (A: 96). Whereas bond affinity data are presented in the following Table 1.

TABLE 1. Binding Affinity Ligand (L-Glutamate Acid and L-Theanine to Protein Glutamate receptor ionotropic, kainate 1

Ligand	Binding Affinity (kcal/mol)
GluK1_L-GlutamateAcid	-5,0
GluK1_L-GlutamateAcid	-4,7
GluK1_L-GlutamateAcid	-4,3
GluK1_L-GlutamateAcid	-4,1
GluK1_L-GlutamateAcid	-4,0
GluK1_L-Theanine	-4,9
GluK1_L-Theanine	-4,8
GluK1_L-Theanine	-4,5
GluK1_L-Theanine	-4,4

Note: the blue column has the same binding affinity in the same range of value

The results of the docking of the two compounds indicate that the binding affinity of the two compounds has almost the same value at RMSD O (the highest binding affinity value). L-Glutamate Acid has a binding affinity value of -5.0 kcal/mol while L-Theanine has almost the same value of -4.9 kcal/mol.

Toxicity Analysis of L-Theanine Compounds

Data obtained based on the analysis of the application of toxtree v2. 6.13 obtained that L-Theanine compounds do not contain functional groups which can increase the risk of toxicity because they do not have functional groups in the form of aliphatic secondary amines or their salts, cyano, N-nitroso, diazo, triazine or quaternary nitrogen, except in the following forms: > C = N + R2,> C = N + H2 or primary or tertiary hydrochloride or sulfuric salt. While the results of the analysis of Theanine compounds based on the RO5 Lipinski principle are described in Table 2 below.

TABLE 2. Potential Analysis of L-Theanine Compounds Based on Lipinski Principles

Indicator	Compound Characteristics	Acceptance
MWT (Molecular Weight) ≤ 500 dalton	174,2 Dalton	+
Bonding Donor H ≤ 5	3	+
Bonding Acceptor H ≤ 10	5	+
log*P* ≤ 5	-2,5	+
Rotatable Bond < 10	5	+

Notation: (+) accepted; (-) not accepted

Based on the analysis with the principle of RO5 Lipinski it is known that L-Theanine compounds have the potential to be consumed orally.

RESULTS AND DISCUSSION

Results of LCM and Docking Analysis of L-Theanine Compounds

Tea leaf extracts analyzed by LCMS showed that dried green tea leaves contained 89 compounds. Among all these compounds contains compounds that are neuroprotective namely theanine. Based on the results of docking it is known that L-Glutamate Acid and L-Theanine have the same site on Glutamate receptor ionotropic, kainate protein 1. The residual groups of the two compounds when binding to the similar glutamate receptor protein are THR (A: 91), GLU (A: 191), and ARGs (A: 96). The binding affinity of the two compounds is also almost the same, namely -5.0 kcal/mol for L-Glutamate Acid and -4.9 kcal / Mol for L-Theanine. This allows L-Theanine to act as an inhibitor that blocks L-Glutamate Acid from binding to glutamate receptors on prostsynap membranes. This inhibitory activity can prevent neuron degeneration due to excessive influx Ca^{2+} because L-Glutamate Acid remains bound to glutamate receptors and cannot be returned to previous presynaptic neurons [9].

The structure of L-Theanine which is analogous to L-Glutamate Acid [13] also allows L-Theanine to have antagonist properties in glutamate receptors, causing neuroprotective effects that can relieve neuropsychiatric symptoms that are symptomatic of autism. Kakuda [13] states that the binding side of L-Theanine to glutamate receptors is much lower than that of L-Glutamate Acid in the results of experiments in mice using ligand labeling techniques. However, the results of the compound analysis with docking visualization showed that binding affinity of L-Theanine and L-Glutamate Acid in Glutamate protein ionotropic receptor, kainate 1 (GluK 1) in humans has the power of binding affinity which is almost the same as similar bond residue. With the in silico approach shows that L-Theanine to humans has far more potential.

Toxicity Analysis of Theanine Compounds

Analysis of compound properties using the toxtree v2 application. 6.13 obtained that L-Theanine compounds do not contain functional groups which can increase the risk of toxicity so that it is safe for consumption. The compound analysis was also carried out using the principle of RO5 (Rule of Five) to determine the potential of compounds as oral bioactive. The data obtained show that L-Theanine fulfills the five principles of RO5 so that it has the potential

to be used as an oral bioactive. Lipinski [30] states that a compound has an oral bioavailability potential when it has a molecular weight of ≤ 500 daltons, an H ≤ 5 bond donor, and an H ≤ 10 acceptor, this requirement is related to the solubility of the compound. The L-Theanine compound has a molecular weight of 174.2 daltons, donor H bonds as much as 3 and H bond acceptors as much as 3. In solubility, the L-Theanine compound has the potential for oral bioavailability. Another requirement that must be met is the logP value must be ≤ 5. LogP is a measure of lipophilicity of a compound, namely the ability of chemical compounds to dissolve in fat, oil, lipids and non-polar solvents such as hexane or toluene [31]. Lipopolisity is related to the ability to absorb compounds in the intestine. A compound can be absorbed by the intestine if it has logP ≤ 5 [30]. L-Theanine has a logP value of -2.5, this indicates that L-Theanine has the ability to be absorbed by the intestine. The last requirement of the principle of RO5 is a rotatable bond. Rotatable bonds are the number of bonds that allow free rotation of the bond itself [32]. A compound is said to have the potential of bioactive (drug-like) if it has a rotatable bond of less than 10 [30]. L-Theanine has as many as 5 rotatable bonds that allow potential as bioactive (drug-like).

CONCLUSIONS

The results of the interaction of L-Theanine compounds against Glutamate proteins ionotropic receptors, kainate 1 (GluK 1) through reverse docking show that L-Theanine has the potential as an L-Glutamate Acid inhibitor because it has similar bond residues namely THR (A: 91), GLU (A: 191), and ARGs (A: 96). Both binding affinities are also almost the same, namely -5.0 kcal/mol for L-Glutamate Acid and -4.9 kcal/mol for L-Theanine. Theanine compounds do not have toxic potential based on the results of structural analysis using the application of toxtree 2.6.13. The principle analysis of RO5 also shows that L-Theanine also has oral bioactive potential. However, this study just analyzed the interaction of L-Theanine with one type of glutamate receptor namely kainate, so it needs a similar study that compares the interaction of L-Theanine compounds with other glutamate receptors, AMPA and NMDA. In addition, wet lab research also needs to be done to support the potential supporting data of L-Theanine as a neuroprotective bioactive specifically for autism.

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