

## ***In Silico Analysis of Phytoestrogens' Neuroprotective Effect on N-methyl-D-aspartate (NMDA) Receptors***

**Faisal Akhmal Muslikh<sup>1</sup>, Rizki Rahmadi Pratama<sup>2</sup>, Burhan Ma'arif<sup>3</sup>, Marisca Evalina Gondokesumo<sup>4\*</sup>**

<sup>1</sup> Faculty of Pharmacy, Bhakti Wiyata Health Sciences Institute, Kediri, East Java, Indonesia

<sup>2</sup> Faculty of Pharmacy, Islamic University of Kalimantan Muhammad Arsyad Al Banjari Banjarmasin, South Kalimantan, Indonesia

<sup>3</sup> Department of Pharmacy, Universitas Islam Negeri Maulana Malik Ibrahim, Malang, East Java, Indonesia

<sup>4</sup> Faculty of Pharmacy, University of Surabaya, Surabaya, East Java, Indonesia

\*Corresponding author

Email: [marisca@staff.ubaya.ac.id](mailto:marisca@staff.ubaya.ac.id)

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### ***A b s t r a c t***

**Keyword :**  
ADMET,  
In silico,  
Neurodegenerative,  
NMDA,  
Phytoestrogens

**Background:** Neurodegenerative diseases are neurological diseases that are a significant cause of death and disability globally. One of the causes is associated with cell signaling disorders, neuronal apoptosis, inflammation, and the deposition of aggregate proteins. The N-methyl-D-aspartate (NMDA) receptor is a glutamate receptor that, when activated, causes synaptic dysfunction and leads to neuronal death. Phytoestrogen compounds are able to replace the role of estrogen in maintaining body homeostasis, including in the CNS. **Objektif:** This study aims to determine the role of phytoestrogen compounds in inhibiting NMDA activation (IPBQ), which causes neurodegenerative diseases. **Methods:** The method used is molecular docking with the AutoDock 4.2.6. The prediction of physicochemical and pharmacokinetic properties used SwissADME, while the toxicity used pkCSM and ProTox II. **Results:** The results of docking using the IPBQ protein showed that the compounds  $\alpha$ -amyrin,  $\beta$ -amyrin and eudesmin had the best binding potential compared to 17 $\beta$ -estradiol which was the positive control. Physicochemical and pharmacokinetic tests showed that the three compounds had good permeability and strong lipophilicity, so they could penetrate cell membranes, and were not toxic, except for eudesmin, which was included in class IV in the toxicity test using ProTox II. **Conclusion:**  $\alpha$ -amyrin and  $\beta$ -amyrin compounds have the potential to treat neurodegenerative diseases against NMDA receptors (IPBQ).

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## INTRODUCTION

Neurodegenerative diseases are a heterogeneous group of neurological disorders that have a devastating impact on the lives of millions of people worldwide. This condition involves gradual damage to neurons in either the central nervous system (CNS) or peripheral nervous system (PNS).<sup>1,2</sup> Damage to the structure and function of the nervous tissue, together with the irreversible loss of neurons, results in disruption of vital communication pathways. This, in turn, causes disturbances in memory, cognition, behavior, sensory, and/or motor functions.<sup>2</sup>

Besides causing death worldwide, neurodegenerative diseases are also a cause of disability globally.<sup>1,3</sup> There are various factors associated with the emergence of neurodegenerative diseases, including genetics, cell signaling disorders, neuronal apoptosis, inflammation, deposition of aggregate proteins, mitochondrial dysfunction, oxidative stress, aging, gender, mutation, ethnicity, environmental exposure, and climate.<sup>1</sup> In neurodegenerative diseases, especially in the process of learning and memory formation, glutamate plays an important role in the ability or potential of something to develop and improve quality gradually over a long period of time, which is called the phenomenon of long-term potentiation (LTP).<sup>4,5</sup> N-methyl-D-aspartate (NMDA) receptors, which are ion channel receptors that respond to the neurotransmitter glutamate, are found in many excitatory synapses.<sup>1</sup> This activation of NMDA receptors can lead to synaptic dysfunction, which in turn causes neuronal damage and death.<sup>6</sup>

Phytoestrogen compounds are polyphenolic compounds derived from plants. These compounds have a structure similar to human endogenous hormones, especially 17 $\beta$ -estradiol.<sup>7-12</sup> Because of this similarity in structure, phytoestrogen compounds can replace estrogen in maintaining body balance.<sup>13,14</sup> In addition,

phytoestrogen compounds have many health benefits, including maintaining reproductive, heart, bone, skin, and central nervous system health, and they can help you lose weight.<sup>15</sup> In general, phytoestrogen compounds can be divided into two groups, namely the flavonoid and non-flavonoid groups.<sup>16</sup> Examples of phytoestrogen compounds from the flavonoid group include Genistein, Daidzein, Glycitein, Formononetin, Biochanin A, Equol, Quercetin, and Kaempferol. Meanwhile, from the non-flavonoid group, examples are Pinorexinol, Eudesmin,  $\alpha$ -amyrin, and  $\beta$ -amyrin.<sup>16,17</sup> This study aims to investigate the role of both phytoestrogen compounds from the flavonoid and non-flavonoid groups in inhibiting the activation of NMDA receptors (PDB ID 1PBQ), which can cause neurodegenerative diseases.

## METHODS

### Instruments

The tools used are in the form of a Legion 5 Pro 16AC6H laptop, which has 16.0 gigabytes of RAM specifications, an AMD Ryzen 7 5800H processor, Radeon graphics at 3.20 GHz, an NVIDIA GeForce RTX 3060 graphics card, and the Microsoft® Windows® 10 Pro operating system. Some of the software used in this study includes Avogadro, which is used to minimize energy, and the Swiss PDB viewer to optimize proteins. For molecular docking, Autodock 4.2.6 was used, while Discovery Studio visualizer and PyMOL were used to visualize the interaction between protein and ligand. SwissADME was used to predict pharmacokinetic and pharmacodynamic properties, while pkCSM and ProTox II were used to predict the toxicity properties of the tested compounds.

### Protein and Ligand Preparation

The NMDA receptor protein, obtained from the Protein Data Bank under the PDB code 1PBQ, was subjected to preparation for research purposes. This

involved the removal of water molecules and the addition of hydrogen atoms using the Discovery Studio Visualizer. Subsequently, the protein structure underwent optimization utilizing PDB Swiss, and the force field was established using GROMOS96. The resulting structure was then saved in the PDB file format. This study employed 11 phytoestrogen compounds sourced from PubChem, along with a reference compound that utilized native ligand molecules, specifically 5,7-dichloro-4-hydroxyquinoline-2-carboxylic acid and 17 $\beta$ -estradiol, for comparative purposes. The synthesis of these compounds involved the addition of hydrogen and the optimization of energy using the Avogadro software, utilizing a force field configuration based on MMFF94.<sup>18</sup>

### Molecular Docking

In this study, molecular docking was carried out between 11 phytoestrogen compounds that had been prepared against NMDA receptors using the ID PDB protein 1PBQ with the help of Autodock 4.2.6 software. The method starts by uploading proteins and ligands by Autodock. Subsequently, Gasteiger and Kollman partial charges are automatically incorporated into the test compound through detection and torque determination. The selection of the grid box is determined by the technique validation findings. Specifically, the grid box has dimensions of 40 x 40 x 40 and is positioned at coordinates (X = 5.083, Y = 37.494, Z = -16.921). The distance between grid points is 0.375 Å. The Lamarckian Genetic Algorithm is employed for conducting docking simulations, utilizing a population size of 150 individuals. The maximum number of evaluations is set at 2,500,000 for every 100 conformations. The evaluation of docking results involved identifying the optimal conformation with the highest energy binding score ( $\Delta G$ ) and the lowest inhibition constant ( $K_i$ ). Additionally, functional essential amino acid interactions

that contribute to docking interactions were detected using the Discovery Studio Visualizer.

### ADMET Prediction

For the purpose of conducting physicochemical and pharmacokinetic study, every substance was converted into a simplified molecular input line entry system (SMILES) format using ChemDraw Ultra 12.0. SMILES is employed to streamline the evaluation of pharmacokinetic and pharmacodynamic characteristics by comparing substances using IUPAC nomenclature.<sup>19,20</sup> Subsequently, the SMILES format that had been created was individually subjected to analysis on the SwissADME webtool (<http://www.swissadme.ch>) by clicking the run button. This analysis aimed to evaluate the pharmacokinetic and pharmacodynamic features of each chemical. The analysis yielded results for many parameters, including molecular weight, topological polar surface area (TPSA), hydrogen bond donor (HBD), hydrogen bond acceptor (HBA), log P, and Lipinski's five law parameters. These parameters were assessed using binary "Yes" or "No" responses.<sup>20</sup>

Meanwhile, to perform toxicity analysis, the SMILES format is used to predict the LD<sub>50</sub> value based on the globally harmonized system (GHS) using the online tool ProTox II ([http://tox.charite.de/protox\\_II/](http://tox.charite.de/protox_II/)), to predict the value of hepatotoxicity, skin sensitization, and toxicity of Ames, the pkCSM webtool (<http://biosig.unimelb.edu.au/pkcsm/prediction>) was used. The use of the SMILES format is done by writing SMILES in Canonical Smiles and then executing it by clicking the Start Tox-Prediction button.<sup>21</sup>

### RESULT

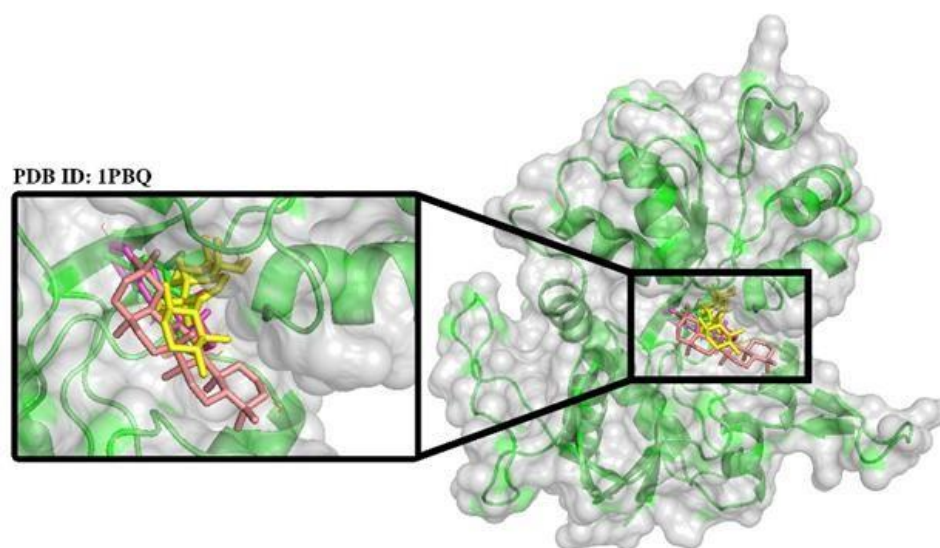
Molecular docking is a popular in silico method in drug discovery.<sup>22</sup> This approach facilitates the discovery of novel

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compounds with therapeutic properties, the anticipation of interactions between ligands and targets at the molecular scale, and the clarification of structure-activity correlations (SAR). The in silico approach is a method of predicting reactions between proteins target and ligands in computational drug design and has several advantages, such as lower application costs, reduced time and effort, and the ability to minimize the isolation of inactive compounds.<sup>23-25</sup> In addition, research conducted by Pinto et al. (2019)<sup>26</sup> and Muslikh et al. (2022)<sup>17</sup> showed

that an in silico approach can quickly determine the estrogenic activity of a compound.

Method validation by tethering receptors and native ligands was carried out using Autodock 4.2.6. From the results of method validation in the study, the RMSD value was 0.683 Å. RMSD results with values less than 2 Å indicate that the application is suitable for molecular docking processes that give results close to experimental results.<sup>27,28</sup>



**Figure 1.** Visualisasi ligand native dan protein target

The efficacy of molecular docking can be assessed by examining the values of binding energy ( $\Delta G$ ) and inhibition constant ( $K_i$ ). The energy binding value quantifies the potency of the biomolecular interaction between the ligand and the receptor.<sup>29</sup> A better affinity of the ligand for the receptor is shown by a lower energy binding value, which indicates that the contact between the ligand and the receptor is more stable.<sup>30</sup> In addition to energy binding values, the results of molecular docking also include poses, which can determine the interaction of ligands with proteins.<sup>30,31</sup>

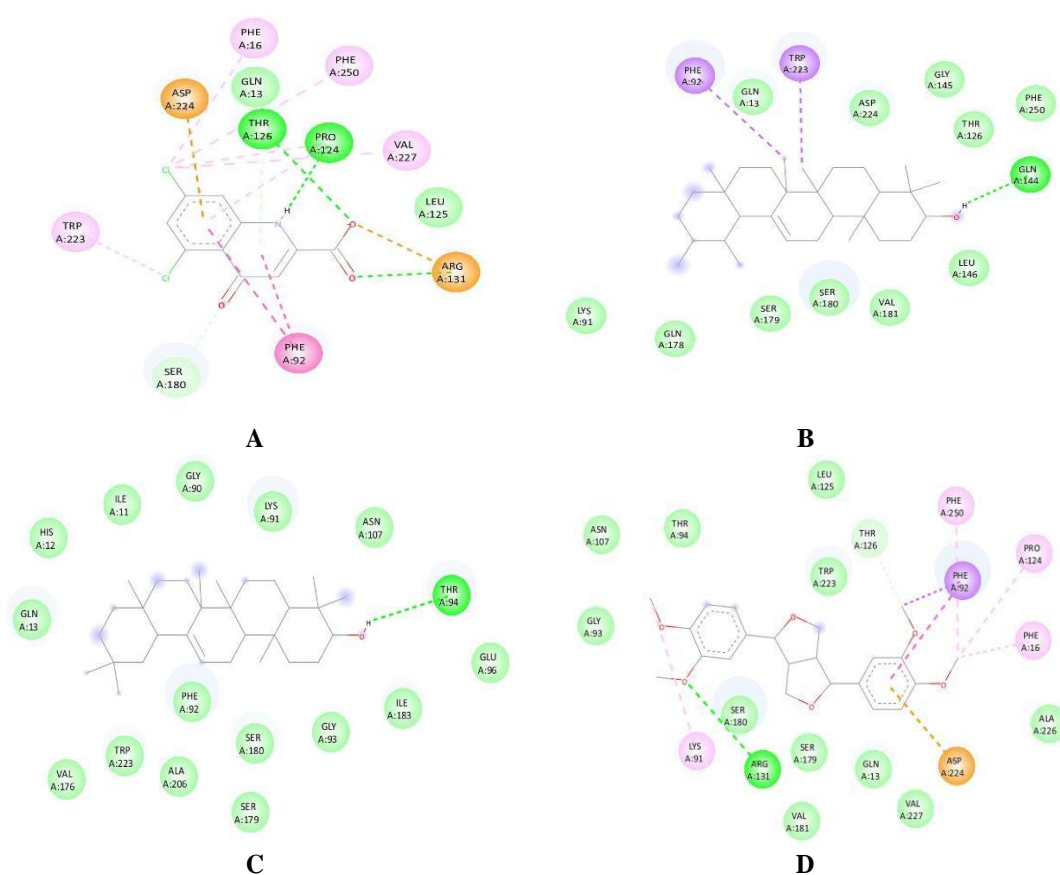
The results of the energy binding values in this study (table 1) show that the compounds  $\alpha$ -Amyrin,  $\beta$ -Amyrin,

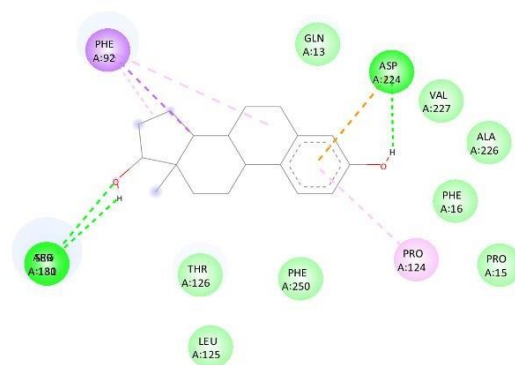
eudesmin, and  $17\beta$ -estradiol (comparison compounds) have low values compared to native ligands. This indicates that the compounds  $\alpha$ -Amyrin,  $\beta$ -Amyrin, and eudesmin have a higher potential than  $17\beta$ -estradiol in interacting with NMDA receptors with PDB ID 1PBQ.

The  $K_i$  value is the concentration needed to achieve 50% inhibition, and a lower  $K_i$  value implies a higher affinity of the ligand for macromolecules.<sup>32,33</sup> In Table 1, it can be seen that  $\alpha$ -Amyrin,  $\beta$ -Amyrin and Eudesmin show a strong affinity for the NMDA receptor with PDB ID 1PBQ compared to  $17\beta$ -estradiol because these three compounds have a lower  $K_i$  value than the comparator compound  $17\beta$ -estradiol.

**Table 1.** Energy binding and inhibition constant values of molecular docking for 1PBQ

Compounds	Energy binding (Kcal/mol)	Inhibition constant
$\alpha$ -Amyrin	-9.02	245.19 nM
$\beta$ -Amyrin	-8.66	446.13 nM
Eudesmin	-8.53	559.40 nM
17 $\beta$ -Estradiol	-8.66	446.91 nM
Ligand Native	-7.63	2.57 $\mu$ M
Pinosterol	-6.44	3.53 $\mu$ M
Kaemferol	-7.11	6.11 $\mu$ M
Genistein	-7.07	6.58 $\mu$ M
Glycitin	-6.83	9.86 $\mu$ M
Equol	-6.81	10.17 $\mu$ M
Biochanin A	-6.8	10.43 $\mu$ M
Formonetin	-6.76	11.16 $\mu$ M
Daidzein	-6.73	11.57 $\mu$ M
Quercetin	-6.72	11.90 $\mu$ M





## E

**Figure 2.** Visualization of 2D Docking Results, (A) ligand native, (B)  $\alpha$ -Amyrin, (C)  $\beta$ -Amyrin, (D) Eudesmin, (E) 17 $\beta$ -Estradiol.

**Table 2.** Similarity of Amino Acid Residues to native Ligand

Compounds	Amino acid residues	Number of amino acid residue bonds	Percentage of similarity of Amino Acid Residue to Native Ligand
Ligand Native	Van der wals: <b>GLN 13, LEU 125</b> Attractive charge: <b>ASP 224, ARG 131</b> Conventional Hydrogen bond: <b>THR 126, PRO 124</b> Carbon hydrogoen bond: <b>SER 180</b> Pi-Anion: <b>ASP 224, ARG 131</b> Pi-Donor hydrogen bond: <b>THR 126</b> Pi-Pi Stacked: <b>PHE 92</b> Alkyl: <b>TRP 223, PHE 16, PHE 250, VAL 227</b> Pi-Alkyl: <b>TRP 223, PHE 16, PHE 250, VAL 227, PRO 124</b>	20	100%
$\alpha$ -Amyrin	Van der wals: <b>GLN 13, ASP 224, GLY 145, THR 126, PHE 250, LEU 146, VAL 181, SER 180, SER 179, GLN 178, LYS 91</b> Conventional hydrogen bond: <b>GLN 144</b> Pi-sigma: <b>PHE 92, TRP 223</b>	14	35%
$\beta$ -Amyrin	Van der wals: <b>GLN 13, GLN 12, ILE 11, GLY 90, LYS 91, ASN 107, GLU 96, ILE 183, GLY 93, SER 180, SER 179, PHE 92, ALA 206, TRP 223, VAL 176</b> Conventional hydrogen bond: <b>THR 94</b>	16	20%
Eudesmin	Van der wals: <b>GLY 93, ASN 107, THR 94, LEU 125, TRP 223, ALA 226, VAL 227, GLN 13, SER 179, VAL 181, SER 180</b> Conventional Hydrogen Bond: <b>ARG 131</b> Carbon hydrogen bond: <b>THR 126</b> Pi-Anion: <b>ASP 224</b> Pi-Sigma: <b>PHE 92</b> Pi-Pi Stacked: <b>PHE 92</b> Alkyl: <b>PHE 250, PRO 124, PHE 16</b> Pi-Alkyl: <b>LYS 92</b>	20	65%
17 $\beta$ -Estradiol	Van der wals: <b>GLN 13, VAL 227, ALA 226, PHE 16, PRO 15, PHE 250, LEU 125, THR 126</b> Conventional hydrogen bond: <b>ARG 180, ASP 224</b> Pi-Anion: <b>ASP 224</b> Pi-sigma: <b>PHE 92</b> Pi-Alkyl: <b>PRO 124</b>	13	50%

The objective of examining amino acid residues in the interaction between the test drug and the target protein is to discern the specific interactions that take place and comprehend the significance of these interactions in the pharmacological impact of the test compound as an NMDA inhibitor. The bond interactions encompass hydrogen bonds, hydrophobic interactions, Van der Waals contacts, electrostatic interactions, and halogen interactions. Hydrogen bonds are the most robust form of non-covalent bonds, yet weaker than ionic or covalent ones. Hydrogen bonding is crucial in generating pharmacological activity and is commonly observed in this interaction.<sup>34</sup>

Amino acid residues refer to specific amino acids inside a protein that form a binding interaction with a ligand or chemical. The active site binding of the protein varies for each individual amino acid residue. The degree of amino acid residue similarity between the chemical and the control (native ligand) serves as an indicator of the possible similarity in the active site, which in turn suggests the likelihood of a strong binding and equivalent biological activity to that of the control.<sup>21,35,36</sup>

The SwissADME webtool was utilized to predict the pharmacokinetic and pharmacodynamic characteristics of the

compounds. The outcomes of molecular docking indicated that  $\alpha$ -Amyrin,  $\beta$ -Amyrin, and eudesmin were the most favorable compounds. Additionally, these compounds exhibited properties that are compatible with the human body, as shown in Table 3.<sup>37</sup> The assessment of this property is determined by evaluating the parameters outlined in Lipinski's five laws. Several Lipinski parameters were found to be below certain thresholds: the number of hydrogen bond donors (HBD) was less than 5, the number of hydrogen bond acceptors (HBA) was less than 10, the logarithm of the partition coefficient (log P) was less than 5, and the molecular weight was less than 500 g/mol. Compounds of a molecular weight less than 500 g/mol are deemed capable of traversing biological membranes. The H-acceptor and H-donor values represent the quantity of hydrogen bonds in the chemical, with larger values indicating a greater energy need for the absorption process. The log P value is a measure of the compound's solubility in the membrane fluid and is indicative of the compound's polarity.<sup>38</sup> The "boiled egg" graph in SWISSADME shows that the chemicals  $\alpha$ -Amyrin,  $\beta$ -Amyrin, and eudesmin can cross the blood-brain barrier and exert their effects, as indicated by their TPSA value of 79 Å<sup>2</sup>.<sup>18,39</sup>

**Table 3.** Analysis of the physicochemical and pharmacokinetic properties of the tested compounds

Compounds	BM ≤500 g/mol	Log P ≤ 5	HBA ≤ 10	HBD ≤ 5	Lipinski's Rule of Five	TPSA (Å <sup>2</sup> )
<b>Comparison compound</b>						
17 $\beta$ - estradiol	272.38	3.40	2	2	Yes	40.46
<b>Phytoestrogen test compounds</b>						
$\alpha$ -Amyrin	426.72	7.06	1	1	Yes	20.23
$\beta$ -Amyrin	426.72	7.20	1	1	Yes	20.23
Eudesmin	386.44	3.06	6	0	Yes	55.38

The toxicity assessment of the test compounds was conducted utilizing the online webtool ProTox II, which

categorizes substances according to the globally harmonized system (GHS) and classifies them into six LD50 toxicity

groups. Class I ( $LD50 \leq 5$  mg/kg) signifies a substance that is lethal if ingested. Class II ( $5 < LD50 \leq 50$  mg/kg) indicates that the substance is lethal if swallowed. Class III ( $50 < LD50 \leq 300$  mg/kg) denotes that the substance is toxic if eaten. Class IV ( $300 < LD50 \leq 2000$  mg/kg) signifies that the substance is harmful if swallowed. Class V ( $2000 < LD50 \leq 5000$  mg/kg) indicates that the substance is harmful if ingested. Lastly, class VI ( $LD50 > 5000$  mg/kg) indicates the absence of toxicity. The number is.<sup>40</sup> A substance with a higher LD50 value is considered to be less harmful or safer for the body, whereas a substance with a lower LD50 value is considered to be more toxic.<sup>42</sup> Forty-one The findings indicated that  $\alpha$ -Amyrin and  $\beta$ -Amyrin exhibited no hazardous characteristics and were classified as class VI, signifying the absence of toxicity.

The pkCSM webtool was utilized to forecast the hepatotoxicity, cutaneous

sensitization, and toxicity of Ames. Subsequently, the online program ProTox II was employed to assess the toxicity classification of substances, utilizing LD50 data. Hepatotoxicity is a significant form of toxicity that is crucial for identifying drugs that are detrimental to the liver.<sup>42,43</sup> Skin sensitization is the term used to describe hypersensitive reactions that are caused by chemicals capable of penetrating the outermost layer of the skin, known as the stratum corneum.<sup>43,44</sup> Ames toxicity is an approach used to evaluate the mutagenic and carcinogenic activities of various chemicals.<sup>43,44</sup> Based on Table 4, the compounds  $\alpha$ -Amyrin,  $\beta$ -Amyrin and eudesmin did not show toxicity to hepatotoxicity, skin sensitization, or Ames toxicity. The results of the toxicity test showed that the compounds  $\alpha$ -Amyrin,  $\beta$ -Amyrin and eudesmin were not toxic.

**Table 4.** Analysis of the toxicity of the test compounds

Compounds	Hepatotoxicity*	Skin Sensitization*	Ames Toxicity*	Toxicity Class LD <sub>50</sub> **
<b>Comparison compound</b>				
17 $\beta$ -estradiol	No	No	No	IV
<b>Phytoestrogen test compounds</b>				
$\alpha$ -Amyrin	No	No	No	VI
$\beta$ -Amyrin	No	No	No	VI
Eudesmin	No	No	No	IV

NB:

\* : using the online tool pkCSM

\*\* : using the online tool ProTox II

## DISCUSSION

The N-methyl-D-aspartate receptor is a glutamate receptor that is crucial for synaptic transmission and synaptic plasticity, which are fundamental processes involved in learning and memory. This receptor is not only essential for the development and functioning of the nervous system, but it also plays a role in neurotoxicity.<sup>45</sup> Recent research has linked NMDA activation to synaptic failure and neurodegenerative disorders, most notably

Alzheimer's disease (AD).<sup>46,47</sup> This toxicity is mediated by excessive Ca<sup>2+</sup> influx.<sup>45</sup>

Elevated levels of Ca<sup>2+</sup> signaling result in a steady decline in synaptic function and the degeneration of nerve endings. This is clinically associated with the progressive deterioration of cognitive abilities and memory, as well as the neurodevelopmental abnormalities observed in individuals with Alzheimer's disease. Activation of NMDAR leads to an elevation in the concentration of calcium ions in the cytosol, known as cytosolic free intracellular calcium ([Ca<sup>2+</sup>]<sub>i</sub>). This rise is



necessary for the occurrence of long-term potentiation (LTP) and long-term depression (LTD), as well as for synaptic plasticity in general.<sup>45</sup>

N-methyl-D-aspartate receptors are also present in other non-neural cells, such as central and peripheral glial cells, endothelium, bone, kidney, pancreas, and other cell types.<sup>48</sup> Astrocytes possess functioning NMDA receptors that are capable of reacting to glutamatergic input from neurons,<sup>49</sup> and neuroinflammatory processes.<sup>50,51</sup> Endothelial NMDA receptors may have a role in regulating the function of the blood-brain barrier (BBB). Elevated levels of glutamate in the brain have detrimental effects on neurons, hinder endothelial function, and compromise the integrity of the blood-brain barrier (BBB).<sup>52,53</sup>

This mechanism supports the fact that the compounds  $\alpha$ -Amyrin,  $\beta$ -Amyrin and eudesmin are able to bind NMDA with PDB ID 1PBQ, so that NMDA activation can be inhibited. However, the eudesmin compound has dangerous properties if ingested because it is included in class IV. Therefore, the compounds  $\alpha$ -Amyrin and  $\beta$ -Amyrin have the potential to be further developed as antineurodegenerative agents,  $\alpha$ -Amyrin and  $\beta$ -Amyrin also have activity in inhibiting the TLR2 receptor in neurodegenerative diseases.<sup>21</sup>

## CONCLUSION

$\alpha$ -Amyrin and  $\beta$ -Amyrin are phytoestrogen compounds that provide the best results in molecular docking, are acceptable to the body, and are not toxic. So that the compounds  $\alpha$ -Amyrin and  $\beta$ -Amyrin have the potential to treat neurodegenerative diseases against NMDA receptors (1PBQ).

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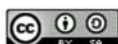
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