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ORIGINAL RESEARCH

Gingerols and Shogaols of *Zingiber officinale var. sunti Valeton* as Potential Allosteric Agonists of Human $GABA_A$ Receptor by in silico Pharmacology Approach

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Background: Gingerols and shogaols are the main active constituents in *Zingiber officinale* var. *sunti* Valeton has been reported for its anti-anxiety activity. Anti-anxiety drugs, such as benzodiazepines, alleviate anxiety disorders by enhancing the activity of gamma-aminobutyric acid type A ($GABA_A$) receptors through positive allosteric regulation at the α1/γ2 interface extracellular domain.

Purpose: To elucidate the binding energy and stability of gingerols and shogaols toward human $GABA_A$ receptor ($hGABA_AR$), compared to their known allosteric agonist (diazepam), and further phytochemical analysis in the ethanol extract of *Z. officinale* var. *sunti* Valeton rhizome (EEZO).

Methods: The ligands, namely gingerols (6-, 8-, and 10-gingerol) and shogaols (6-, 8-, and 10-shogaol), were evaluated by pre-ADMET screening tools and molecular docking simulation towards $hGABA_AR$ α1/γ2 subtype (PDB ID: 6X3X). Compounds with the best pre-ADMET profile and affinity were subjected to a 200 ns molecular dynamics (MD) simulation. The UPLC analysis was performed to detect and quantify gingerol and shogaol in EEZO.

Results: The best pre-ADMET prediction was shown by 6-gingerol, whereas the molecular docking simulations revealed that the best binding affinity and stability were shown by 6-gingerol (−7.41 kcal/mol) and 10-shogaol (−8.24 kcal/mol), which are comparable to that of diazepam. They build hydrogen bonds with α1 Ser206 and pi interaction with γ2 Phe77. The MD simulation confirmed that the stability of the 10-shogaol/$hGABA_AR$ and 6-gingerol/$hGABA_AR$ complexes is equal to that of diazepam/$hGABA_AR$. The UPLC analysis resulted in a level of 44.98 μg/mL for 6-gingerol and 2.52 μg/mL for 10-shogaol.

Conclusion: 6-Gingerol and 10-shogaol of EEZO may have the potential to be developed as novel allosteric agonists of human $GABA_A$ receptors, thus explaining their anti-anxiety activity. However, the activity towards the human $GABA_A$ receptor is lower than diazepam, its known allosteric agonist.

Keywords: anxiety, gamma-aminobutyric acid, GABA receptor agonist, red ginger, *Zingiberaceae*

Introduction

Human gamma-aminobutyric acid type A receptors ($GABA_ARs$) are pentameric ligand-gated ion channels that mediate fast inhibitory transmission in the brain.[1] $GABA_ARs$ activation by the endogenous agonist, GABA, opens chloride ion

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channels, hyperpolarizes the cells, and prevents potential action transmission.[2,3] The excitatory and inhibitory signaling balance is essential for information processing, cognition, and behavior. The GABAergic signaling impairment is associated with the pathophysiology of several neuropsychiatric disorders, including anxiety.[4,5] $GABA_A$Rs are the primary therapeutic target for anti-anxiety drugs.[6]

Benzodiazepines such as diazepam are one of the most commonly prescribed drugs for anxiety by targeting $GABA_A$Rs allosteric binding sites, located at the interface between the α-γ subunits. The allosteric binding sites for benzodiazepines and barbiturates are different from the orthosteric binding sites for the receptors' endogenous agonists, which are located at the interface between the α-β subunits.[7] The binding of diazepam to the allosteric site of $GABA_A$Rs potentiates GABA-related activation and enhances the inhibitory effects of endogenous GABA on the CNS.[8] A recent study by Kim et al described that diazepam binds to specific amino acid residues of $GABA_A$ receptors such as Phe77 and Tyr58 in the extracellular domain (ECD) of γ subunit and Tyr210, Ser205, His102, Phe100, and Tyr160 in the ECD of α subunit, which has important role for ligand affinity and drug efficacy.[9] However, the quick drug dependence and tolerance, the risk of abuse, memory impairment, and cognitive decline should be a concern.[8,10] Therefore, alternative medications with fewer side effects are required, and research in psychopharmaceuticals is a critical area of development.

It is perceived that plants have significant potential for development as neuroprotective agents by modulating multiple molecular and cellular pathways. Their bioactive compounds have been shown to influence the expression of $GABA_A$Rs in the amygdala and hippocampus.[11–13] Previous studies have demonstrated that ginger (*Zingiber officinale* Roscoe) of the family Zingiberaceae, exerts anti-anxiety activity in animal models, as evidenced by the elevated plus maze test and the light–dark box test, both are tests to measure anxiety in laboratory rodents.[14,15] The primary bioactive constituents of the rhizome of this plant, namely gingerols and shogaols, are thought to be responsible for its pharmacological activities.[16,17] Another species, namely *Zingiber officinale* var. *sunti* Valeton, commonly known as red ginger, is recognized for its high medicinal value, containing approximately 169 chemical constituents. However, its potential as an anti-anxiety has never been reported. Phytochemical screening has confirmed that gingerols and shogaols are also present abundantly in red ginger.[18] Considering everything, this study aims to elucidate the binding mode and stability of gingerols and shogaols toward the $GABA_A$Rs, and the best compounds in terms of binding energy and stability were determined for their presence and levels in the ethanol extract of *Zingiber officinale var. sunti Valeton* rhizome (EEZO).

Methods

Hardware and Software

The hardware is a computer with an Intel (R) Core i9-12th processor, 32GB DDR4 RAM, 1TB hard disk drive, 1TB solid-state drive, and Intel HD Graphics NVIDIA GeForce RTX 3070 8GB. The computer is equipped with Linux Ubuntu 23.10 operating systems equipped with Gaussian 09, Gauss View 5.0.8, AutoDock 4.2 (downloaded from The Scripps Research Institute official website http://autodock.scripps.edu/) equipped with MGLTools 1.5.6 (https://ccsb.scripps.edu/mgltools/downloads/) to simulate the process of docking of the ligands with human $GABA_A$ receptor, BIOVIA Discovery Studio Visualizer 2024 (https://discover.3ds.com/discovery-studio-visualizer-download), GROMACS 2020.1 (https://manual.gromacs.org/2016.3/index.html), ACPYPE (AnteChamber PYthon Parser interface), ForceGen 0.4, VMD 1.9.2, and MMPBSA.py.

Macromolecule Preparation

The 3D cryo-electron microscopic structure of human $GABA_A$ receptor ($hGABA_AR$) alpha1-beta2-gamma2 subtype in complex with GABA plus diazepam (PDB ID: 6X3X; resolution 2.92 Å; aggregation state: particle; PDB DOI https://doi.org/10.2210/pdb6X3X/pdb; deposited and released by Kim et al in 2020), was prepared by removing their ligand and the solvent using Biovia Discovery Studio 2024, analyzed for its active site and ligand interaction and prepared for the molecular docking simulation by adding polar hydrogen atoms and Kollman charges using AutoDockTools.

Ligand Preparation

The ligands, 6-gingerol (molecular formula of $C_{17}H_{26}O_4$; molecular weight of 294.4 g/mol), 8-gingerol (molecular formula of $C_{19}H_{30}O_4$; molecular weight of 322.4 g/mol), 10-gingerol (molecular formula of $C_{21}H_{34}O_4$; molecular weight of 350.5 g/mol), 6-shogaol (molecular formula of $C_{17}H_{24}O_3$; molecular weight of 276.4 g/mol), 8-shogaol (molecular formula of $C_{19}H_{28}O_3$; molecular weight of 304.4 g/mol), and 10-shogaol (molecular formula of $C_{21}H_{32}O_3$; molecular weight of 332.5 g/mol), were sourced from the PubChem database (https://pubchem.ncbi.nlm.nih.gov), and the Chem3D Ultra 12.0 was employed to build the ligands. Diazepam (PubChem CID 3016; molecular formula of $C_{16}H_{13}ClN_2O$; molecular weight of 284.74 g/mol) is used as the standard reference drug. All the ligands were subjected to the molecular mechanics force field 94 (MMFF94) in the AutoDockTools to produce accurate geometric structures and added the Gasteiger charges, partial atomic charges, and hydrogen, respectively, as described by Khumpirapang et al.[19]

Pre-ADMET and Drug-Likeness Predictions

The gingerols and shogaols were predicted for the pharmacokinetics parameters using the online ADMETlab 3.0 platform (https://admetlab3.scbdd.com/) for the in silico tool. The absorption properties of gingerols and shogaols were measured by three parameters, ie, F50% (bioavailability 50%), human intestinal absorption (HIA), and Caco-2 permeability. The distribution property of gingerols and shogaols was measured by two parameters, ie, the blood–brain barrier (BBB), plasma protein binding (PPB), and VDss (distribution volume at steady state). The metabolism property was predicted by CYP2D6 (cytochrome P450 subfamily 2D6, an enzyme that metabolizes antidepressants, including the serotonin-reuptake inhibitors paroxetine, fluvoxamine, and fluoxetine) and CYP3A4 (cytochrome P450 subfamily 3A4, an enzyme that metabolizes opioids, benzodiazepines, local anesthetics, immunosuppressants, calcium channel antagonists, and antihistamines). The excretion parameter was predicted by CLplasma penetration (Plasma Clearance; >15 mL/min/kg is categorized as high clearance; 5–15 mL/min/kg is categorized as moderate clearance; and < 5 mL/min/kg is categorized as low clearance) and T1/2 (Predicted Half-life; ultra-short half-life drugs: 1/2 < 1 h; short half-life drugs: T1/2 between 1–4 h; intermediate short half-life drugs: T1/2 between 4–8 h; long half-life drugs: T1/2 > 8 h). The toxicity parameter was calculated by the Ames toxicity and carcinogenicity in rats. In addition, the physicochemical properties were usually assessed according to the Lipinski Rule of Five (Ro5). The requirements for drug-like compounds to be orally effective should fulfill at least four criteria of the Lipinski Ro5, including a molecular mass < 500, cLogP, hydrogen bond acceptors (HBAs), hydrogen bond donors (HBDs), and molar refractivity. However, due to the limitations of the Lipinski Ro5 drug-likeness rules, a scoring method called Quantitative Estimate of Drug-likeness (QED) has been developed and equipped in the online ADMETlab 3.0 platform. QED combines eight physicochemical properties, which are molecular mass, cLogP, HBDs, HBAs, charge, aromaticity, stereochemistry, and solubility, fostering a score between 0 (not drug-like molecule) and 1 (the best drug-like molecule).[20]

The ligands with the best Pre-ADMET scores were further subjected to molecular docking and molecular dynamics (MD) simulation.[21]

Molecular Docking Simulation

Initially, a re-docking process of DZP404 (diazepam co-crystallized in $hGABA_AR$) into its original position within the allosteric site of the $hGABA_AR$ was performed and succeeded with an RMSD value of < 2 Å to evaluate the validity of docking. A grid box was settled at a dimension of 89.59 × 125.888 × 105.542 $Å^3$ spaced at 0.375 Å, based on the original position of diazepam, at the interface between α-γ subunits. Eventually, all the ligands (6-gingerol, 8-gingerol, 10-gingerol, 6-shogaol, 8-shogaol, and 10-shogaol) were docked in the active allosteric site of the $hGABA_AR$ (PDB ID: 6X3X) to obtain the best ligand, in terms of binding energy, by employing the Lamarckian genetic algorithm (LGA) set at 200 runs. The molecular docking simulation results were visualized using BIOVIA Discovery Studio Visualizer 2024. The binding affinity in terms of the docking score (kcal/mol), the inhibitory constant (Ki in μM), the hydrogen bond and the hydrophobic interactions, and the close contact residues were recorded and compared to that of diazepam. The best ligands that showed the lowest docking score/binding energy (kcal/mol) were further subjected to an MD simulation to evaluate the conformational stability of the ligand/receptor complex.[19]

Molecular Dynamics Simulation

The GROMACS (Groningen Machine for Chemical Simulation) 2020.1 with the AMBER99SB-ILDN force field was utilized for MD simulations. In this study, MD simulation was performed on a 200 ns time scale (3 fs time step). The GROMACS software generated the receptor and ligand architecture and parameters, which were followed by solvation and ion addition. The drug–receptor interactions decrease the energy up to a tolerance level of 1000 kJ/mol.nm, and the equilibration with the role of position restraint at the protein molecules for 0.1 ns, utilizing constant number of particles, volume, and temperature, abbreviated to NVT (to establish temperature equilibration) and constant number of particles, pressure, and temperature, abbreviated to NPT (to establish pressure equilibration), was adjusted. Analysis of MD simulation data included root-mean-square deviation (RMSD), root-mean-square fluctuation of residues (RMSF), radius of gyration (Rg), and the solvent accessible surface area (SASA).[13]

The best gingerols and shogaols of the 6-, 8-, and 10-gingerols, and 6-, 8-, and 10-shogaols, in terms of binding energy and stability, were further analyzed by laboratory experiments to confirm their presence and levels in the ethanol extract of *Z. officinale* var. sunti *Valeton* rhizome (EEZO).

Plant Materials and Extract Preparation

The fresh rhizomes of red ginger were harvested in the rainy season at Buniayu Village, Subang, West Bandung Regency, West Java, Indonesia. The plants were taxonomically authenticated by Arifin Surya Dwipa Irsyam (https://www.scopus.com/authid/detail.uri?authorId=57211286941; https://herbarium.sith.itb.ac.id/profil-kurator/), a certified botanist at the School of Life Sciences and Technology, Bandung Institute of Technology (Bandung, West Java, Indonesia), (https://herbarium.sith.itb.ac.id/koleksi/), and was confirmed as *Zingiber officinale var. sunti Valeton* (document number 2131/IT1.C11.2/TA.00/2024) with characteristics that matched those described in the references.

Fresh rhizomes were sorted (length of 3–5 cm, fresh in appearance, 9–10 months planted time), washed under tap water to separate from dirt and soil, peeled off, thinly sliced to 0.2–0.5 cm, and dried for 14 days at room temperature without direct sunlight. The dried rhizomes were ground to a medium degree of coarseness using a miller (Philips, Cucina 1741/1791) and sieved with mesh No. 40.[15] The coarse powder of the dried rhizomes (445 g) was cold-extracted with a technical grade ethanol 70% solvent (BrataChem, Indonesia), with a ratio of 1:10 for 3 × 24 h at 25 ± 2°C. The extracts were collected and filtered using Whatman qualitative filter paper (Merck, WHA1001090), and the solvent was evaporated in a vacuum rotary evaporator (Buchi, R-220) at 60 ± 2°C and 65 rpm to a thick consistency,[15] and resulted in a yield of 69 g of EEZO or 15.5%.

Determination of Nutritional Composition of the Ethanol Extract of *Zingiber officinale var. sunti Valeton* Rhizomes (EEZO)

The nutritional composition of EEZO was determined by following the Indonesian National Standard SNI 01–2891-1992 protocol to determine moisture, ash, protein, fat, carbohydrate, anthocyanin, and vitamin C.[22]

Analysis of Gingerols and Shogaols in Ethanol Extract of *Zingiber officinale var. sunti Valeton* Rhizomes (EEZO)

EEZO was analyzed using the ultra-performance liquid chromatography (UPLC) system (ACQUITY UPLC H-Class PLUS System, Waters) using a standard-addition technique with a mixture of standard 6-gingerol (MarkHerb Cat. No. PHE-10-5; https://markherb.odoo.com/en) and 10-shogaol (MarkHerb Cat. No. PHE-49-2; https://markherb.odoo.com/en). The standard solutions were prepared in increased concentrations for 6-gingerol (0, 0.02, 0.04, 0.06, 0.08, 0.10 mg/mL) and 10-shogaol (0, 0.001, 0.002, 0.003, 0.004, 0.005 mg/mL). Briefly, 100 mg of EEZO was dissolved in 10 mL of high-performance liquid chromatography-grade ethanol (Merck; CAS No. 64–17-5) by sonication using a FALC sonicator equipped with a digital timer for 1 min. The solution was diluted to 1.0 mg/mL and filtered through a 0.2 µm syringe filter. Approximately 1 mL of EEZO solution was spiked to each of the mixtures of standard 6-gingerol and 10-shogaol solutions, and 10 µL of the samples were injected into the C18 column, with a length of 250 mm, internal diameter of 4.6 mm, and particle size of 5 µm (Thermo Fisher Scientific, Waltham, MA, USA) as the stationary phase. The UPLC system used was

a gradient elution with a mobile phase A of water in 0.1% phosphoric acid and mobile phase B of acetonitrile in 0.1% phosphoric acid. The gradient mode was set as follows: 0 min 45% B, 8 min 50% B, 18 min 60% B, 35 min 80% B, and 40 min 45% B. Detection was set at 282 nm; flow rate at 1.0 mL/min, temperature at 25°C. The chromatographic run time was 45 min. Identification of the compounds was performed by comparing the retention times with those of 6-gingerol and 10-shogaol standards, the UV spectra, and UV absorbance ratios after co-injection of standards and samples. The measurement results (areas under the curve) were plotted against the standard concentrations on the x- and y-axis graphs, to obtain the linear regression equation and coefficient of correlation (R).[23,24]

Results

In silico Pharmacology in Terms of Pre-ADMET Properties and Drug-Likeness

The Pre-ADMET properties and drug-likeness estimation of gingerols and shogaols of *Z. officinale* var. *sunti* Valeton is presented in Table 1.

To evaluate the absorption properties of 6-gingerol, 8-gingerol, 10-gingerol, 6-shogaol, 8-shogaol, and 10-shogaol, Caco-2 permeability, HIA, and F50% (50% bioavailability) was estimated (Table 1). In this study, all gingerols and shogaols indicate good absorption as shown by the Caco-2 permeability value (Log Papp) higher than −5.15 cm/s. The Log Papp ranges between the best absorption of 6-shogaol (Log Papp −4.815 cm/s) to 8-gingerol (Log Papp −5.107 cm/s). The HIA estimation also confirms the good absorption properties of all gingerols and shogaols, with values ranging between 6-gingerol (the best absorption with an HIA of 0.001) to 10-shogaol (the lowest absorption with an HIA of 0.082); however, the predicted 50% bioavailability of all gingerols and shogaols results in <50%.

The distribution properties of 6-gingerol, 8-gingerol, 10-gingerol, 6-shogaol, 8-shogaol, and 10-shogaol were estimated by BBB, PPB, and VDss (Table 1). All gingerols and shogaols are categorized as BBB+, meaning they may have the ability to penetrate the BBB; however, only 6-gingerol exhibits the optimal PPB with a value of 80.039%. Moreover, all gingerols, but not shogaols, show an optimal VDss value.

The metabolism properties of gingerols and shogaols were evaluated by their probability to inhibit cytochrome P450 enzymes, in this study we chose CYP2D6 (cytochrome P450 subfamily 2D6, an enzyme that metabolizes antidepressants including the serotonin-reuptake inhibitors paroxetine, fluvoxamine, and fluoxetine) and CYP3A4 (cytochrome P450 subfamily 3A4, an enzyme that metabolizes opioids, benzodiazepines, local anesthetics, immunosuppressants, calcium channel antagonists, and antihistamines). When CYP2D6 is inhibited, antidepressants, including the serotonin-reuptake inhibitors, are not metabolized. Additionally, when CYP3A4 is inhibited, certain drugs such as benzodiazepines are not metabolized. Our study revealed that 6-gingerol, 8-gingerol, and 6-shogaol may weakly inhibit CYP2D6, while 10-gingerol, 8-shogaol, and 10-shogaol may strongly inhibit this enzyme. Furthermore, all gingerols and shogaols strongly inhibit CYP3A4 (Table 1).

The excretion properties of gingerols and shogaols were predicted by calculating CL Plasma Penetration and T1/2 (Table 1), which resulted in all compounds exhibiting moderate plasma clearance and ultra-short half-life (T1/2 of < 1 h), except 6-gingerol with a short half-life (T1/2 of 1.044 h). The estimation of toxicity revealed that gingerols and shogaols are safe as proven by their negative Ames values and non-carcinogenicity properties (Table 1).

The drug-likeness of gingerols and shogaols was estimated by QED (Quantitative Estimate of Drug-Likeness), which indicated that all compounds have moderate drug-likeness (QED values ranging from 0.471 to 0.65), except for 10-shogaol which has low drug-likeness with a QED value of 0.378. The QED estimation confirmed that the compound with the best drug-likeness properties is 6-gingerol (Table 1).

In silico Pharmacology in Terms of Binding Mode and Binding Affinity of the Ligands Towards $GABA_AR$

The binding mode and binding affinity of gingerols and shogaols of *Z. officinale* var. *sunti* Valeton was compared to those of diazepam, the known allosteric agonist of human $GABA_AR$ (Table 2). In this study, the re-docking of DZP404 (diazepam co-crystallized in protein) into its original position resulted in an RMSD value of 1.06 Å, demonstrating that both DZP404 molecules (the original and the re-docked) occupied the allosteric site (located between the α-γ subunits in

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Table 1 The Pre-ADMET Prediction and Drug-Likeness Properties of Gingerols and Shogaols

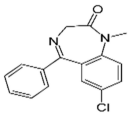
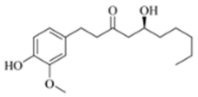
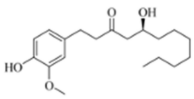
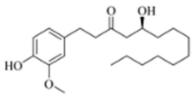
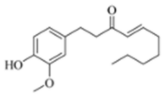
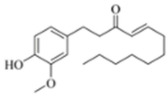
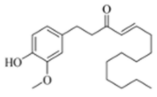
Name of Compound	Absorption			Distribution			Metabolism		Excretion		Toxicity		Drug-Likeness
	F50% (Bioavailability 50%)	HIA	Caco-2 (Log Papp in cm/s)	BBB	PPB (%)	VDss (L/kg)	CYP2D6 Inhibitor	CYP3A4 Inhibitor	CL Plasma Penetration (mL/min/kg)	T1/2	Ames Toxicity	Carcinogenicity	QED (Quantitative Estimate of Drug-Likeness)
6-gingerol	0.972 (< 50%)	0.001 (≥ 30%)	−5.104 (Optimal)	0.00 (BBB+)	80.039 (Optimal)	0.057 (Optimal)	0.258 (Low)	0.999 (High)	7.929 (Moderate)	1.044 h (Short)	0.2 (Negative)	0.323 (Non-carcinogen)	0.65 (Moderate)
8-gingerol	0.952 (< 50%)	0.003 (≥ 30%)	−5.107 (Optimal)	0.00 (BBB+)	95.168	0.127 (Optimal)	0.293 (Low)	0.999 (High)	6.454 (Moderate)	0.81 h (Ultra Short)	0.138 (Negative)	0.334 (Non-carcinogen)	0.571 (Moderate)
10-gingerol	0.983 (< 50%)	0.004 (≥ 30%)	−5.086 (Optimal)	0.00 (BBB+)	97.389	0.305 (Optimal)	0.909 (High)	1.0 (High)	5.891 (Moderate)	0.584 (Ultra Short)	0.109 (Negative)	0.322 (Non-carcinogen)	0.473 (Moderate)
6-shogaol	0.964 (< 50%)	0.014 (≥ 30%)	−4.815 (Optimal)	0.001 (BBB+)	98.474	−0.306	0.362 (Low)	0.997 (High)	6.271 (Moderate)	0.663 (Ultra Short)	0.346 (Negative)	0.009 (Non-carcinogen)	0.547 (Moderate)
8-shogaol	0.986 (< 50%)	0.043 (≥ 30%)	−4.854 (Optimal)	0.00 (BBB+)	98.795	−0.133	0.826 (High)	0.996 (High)	5.973 (Moderate)	0.571 (Ultra Short)	0.272 (Negative)	0.12 (Non-carcinogen)	0.471 (Moderate)
10-shogaol	0.989 (< 50%)	0.082 (≥ 30%)	−4.934 (Optimal)	0.00 (BBB+)	99.064	−0.008	0.936 (High)	0.999 (High)	5.72 (Moderate)	0.509 (Ultra Short)	0.239 (Negative)	0.129 (Non-carcinogen)	0.378 (Low)

Notes: Ames Toxicity, Category 1: Ames positive, category 0: Ames negative; Caco2, Cancer-Coli 2; optimal if > −5.15 Log Papp; CL, plasma Penetration Plasma Clearance; >15 mL/min/kg is categorized as high clearance; 5–15 mL/min/kg is categorized as moderate clearance; and < 5 mL/min/kg is categorized as low clearance; CYP2D6, Cytochrome P450 subfamily 2D6, an enzyme that metabolizes antidepressants including the serotonin-reuptake inhibitors paroxetine, fluvoxamine, and fluoxetine; CYP3A4, Cytochrome P450 subfamily 3A4, an enzyme that metabolizes opioids, benzodiazepines, local anesthetics, immunosuppressants, calcium channel antagonists, and antihistamines; F50%, 50% bioavailability; Category 1: F 50%+ or bioavailability < 50%, Category 0: F 50%− or bioavailability ≥ 50%; HIA, Human Intestinal Absorption; optimum if HIA close to 0; PPB, Protein Plasma Binding; optimal if PPB < 90%; QED, Quantitative Estimate of Drug-likeness; optimal drug-likeness if QED close to 1; T1/2, Predicted Half-life; ultra-short half-life drugs: T1/2 < 1 h; short half-life drugs: T1/2 between 1–4 h; intermediate short half-life drugs: T1/2 between 4–8 h; long half-life drugs: T1/2 > 8 h; VDss, Volume of Distribution at Steady State; optimal if VDss 0.04–20 L/kg.

the receptor extracellular domain) in almost the same geometrical conformation, and indicating that the method is valid (Figure 1). The binding energy of DZP404 in its allosteric binding site is −8.30 kcal/mol and this drug builds interactions with α: Ser206 (conventional hydrogen bond), with α: Gln204 (van der Waals), and with α: Phe100, His102, Tyr160, Tyr210 and γ: Tyr58, Phe77 (pi–pi interaction) as tabulated in Table 2.

More interestingly, all gingerols and shogaols are confirmed to occupy the allosteric site of human $GABA_AR$ by building interactions with critical residues in the sequence of binding energy from the highest to the lowest as follows,

Table 2 The Binding Mode and Affinity of Gingerols and Shogaols to Human $GABA_AR$ Compared to Diazepam (the Allosteric Agonist)

Ligand	2D structure	Molecule Description	Binding Affinity (kcal/mol)	Type of Interaction		
				Hydrogen Bond	Van der Waals	Pi Interaction
Diazepam (the allosteric agonist)		Molecular formula: $C_{16}H_{13}ClN_2O$; molecular weight: 284.74 g/mol	−8.30	α: **Ser206**	α: Gln204	α: Phe100, His102, Tyr160, **Tyr210** γ: Tyr58, **Phe77**
6-gingerol		Molecular formula: $C_{17}H_{26}O_4$; molecular weight: 294.4 g/mol	−7.41	α: Ala161, **Ser206, Tyr210**	α: **Ser205**	α: Tyr160 γ: Tyr79, Met130
8-gingerol		Molecular formula: $C_{19}H_{30}O_4$; molecular weight: 322.4 g/mol	−7.59	α: **His 102**, Ala161, **Tyr210** γ: Asn60	α: Tyr160	γ: **Phe77**
10-gingerol		Molecular formula: $C_{21}H_{34}O_4$; molecular weight: 350.5 g/mol	−7,68	α: Ala161, **Tyr210** γ: Asn60	α: **His102**, Tyr160	γ: Tyr58, **Phe77**, Ala79
6-shogaol		Molecular formula: $C_{17}H_{24}O_3$; molecular weight: 276.4 g/mol	−7.83	α: **His102**, Ala161, **Ser205, Tyr210**		α: Tyr160 γ: **Phe77**, Tyr58
8-shogaol		Molecular formula: $C_{19}H_{28}O_3$; molecular weight: 304.4 g/mol	−7,94	α: **His102**, Ala161, **Ser205, Tyr210**		α: Tyr160 γ: Tyr58
10-shogaol		Molecular formula: $C_{21}H_{32}O_3$; molecular weight: 332.5 g/mol	−8.17	α: **His102, Ser206**		α: Phe100, **Val203**, **Tyr210** γ: Tyr58, **Phe77**

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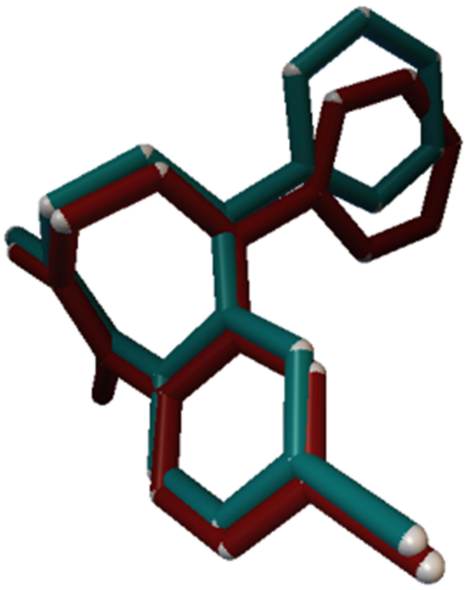

Figure 1 The superimposition of two DZP404 molecules (the green color molecule represents the re-docked DZP404; the red color molecule represents the original co-crystallized DZP404) in the allosteric of the human $GABA_A$ receptor. The RMSD value obtained is 1.06 Å (an RMSD value less than 2.0 Å indicates the validity of the method).

6-gingerol (−7.41 kcal/mol), 8-gingerol (−7.59 kcal/mol), 10-gingerol (−7.68 kcal/mol), 6-shogaol (−7.83 kcal/mol), 8-shogaol (−7.94 kcal/mol), and 10-shogaol (−8.17 kcal/mol) (Table 2), with the best affinity is shown by 10-shogaol. Gingerols and shogaols bind to human $GABA_AR$ by building conventional hydrogen bonds, van der Waals interaction or other hydrophobic interactions similar to those of diazepam, such as Tyr58, Phe77, Phe100, His102, Tyr160, Ser206, Tyr210 (Table 2 and Figure 2).

The Dynamics Stability of the Ligand-$GABA_ARs$ Complex (in Terms of RMSD and RMSF)

The dynamic stability of 6-gingerol/$GABA_AR$ and 10-shogaol/ $GABA_AR$ within 200 ns was determined using MD simulation, and compared to that of diazepam/$GABA_AR$. The resulting RMSD and RMSF graphs are shown in Figure 3.

The RMSD simulation shows that after 7 ns, the diazepam/$GABA_AR$ complex and 10-shogaol/$GABA_AR$ complex achieved equilibrium, and the system displayed steady fluctuation between 0.17 and 0.34 Å in the first 200 ns. Meanwhile, the 6-gingerol/$GABA_AR$ complex achieved equilibrium after 44 ns, and the system displayed steady fluctuation between 0.25 and 0.35 Å in the first 200 ns. The RMSD of the 6-gingerol/$GABA_AR$ complex showed a slightly higher value compared to the diazepam/$GABA_AR$ and 10-shogaol/$GABA_AR$ complexes. The RSMD simulation delineates that the ligand/receptor complexes remained stable during the simulation (Figure 3a). In addition, the RMSF simulation shows that the fluctuations were similar for diazepam/$GABA_AR$, 6-gingerol/$GABA_AR$, and 10-shogaol/$GABA_AR$ complexes (Figure 3b). However, residues 102, 203, 205, 206, 207, and 210 in 10-shogaol revealed a lower pattern than the other complexes.

The radius of gyration (Rg) and solvent-accessible surface area (SASA) analysis are depicted in Figure 4. The Rg serves as a quantitative metric to assess the structural stability of a protein structure during MD simulations. Rg provides insights into the spatial compactness and conformational flexibility of the protein within a biological environment. Lower Rg values are indicative of a more compact and rigid protein structure, reflecting reduced conformational fluctuations during the simulation.[25] In this study, the Rg value for the diazepam/$GABA_AR$ complex and 10-shogaol/$GABA_A$ R complex exhibited similar patterns after 1125000 ps, whereas the 6-gingerol/$GABA_AR$ complex revealed lower values compared to the diazepam/$GABA_ARs$ complex after 75000 ps (Figure 4a). The SASA analysis showed that diazepam, 6-gingerol, and 10-shogaol showed stable values between 112 nm^2 to 122 nm^2. During the simulation, 6-gingerol

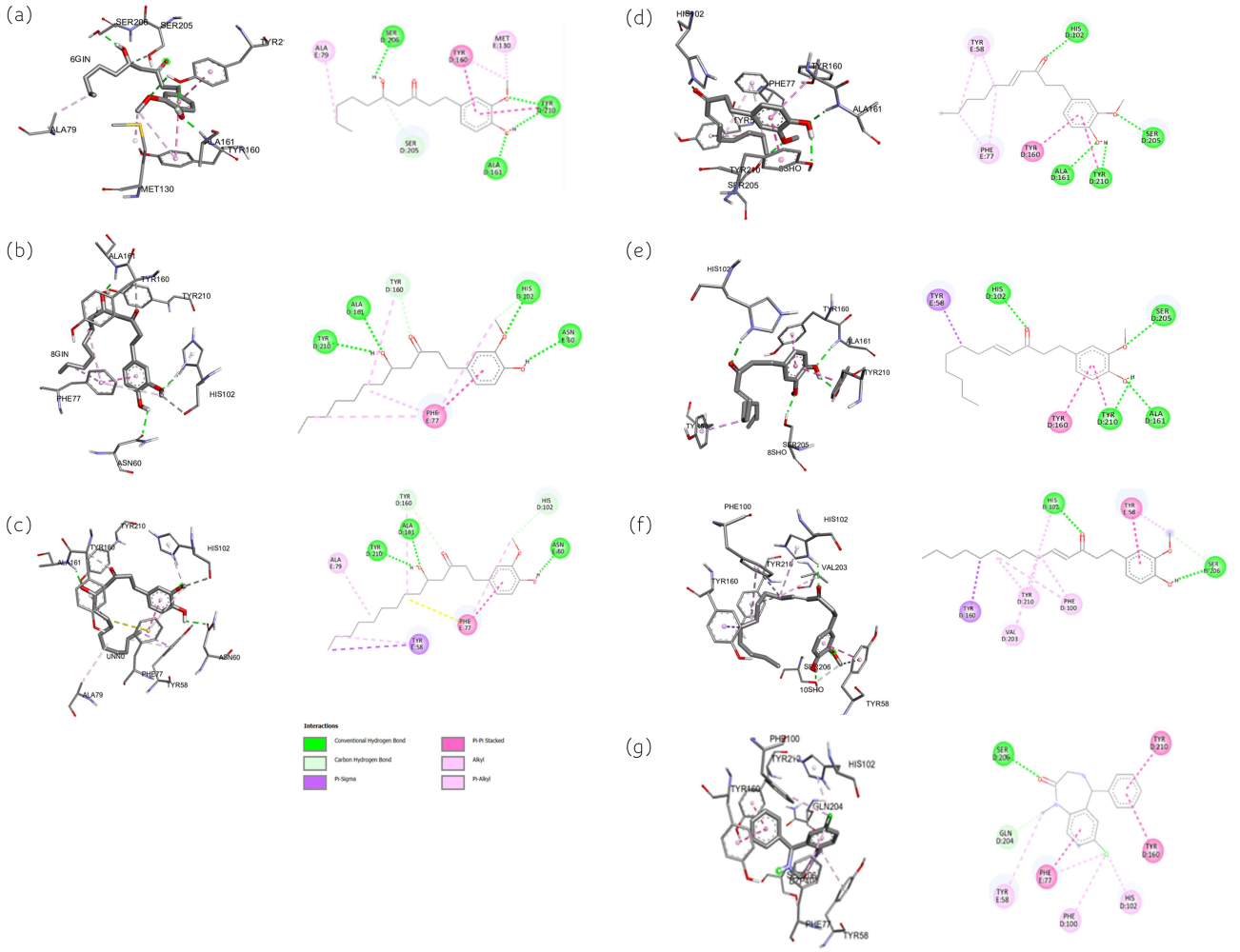

Figure 2 The binding mode of (**a**) 6-gingerol, (**b**) 8-gingerol, (**c**) 10-gingerol, (**d**) 6-shogaol, (**e**) 8-shogaol, (**f**) 10-shogaol, and (**g**) diazepam with the human $GABA_AR$. Figures on the left depict the amino acid residues involved in the interaction. Figures on the right illustrate the position of the ligands in the catalytic pocket of human $GABA_AR$.

fluctuated after 90 ns and 10-shogaol showed slight fluctuations after 120 ns (Figure 4b). A lower SASA value implies greater compactness and greater protein stability.[25]

The Nutritional Composition and the Levels of 6-Gingerol and 10-Shogaol in *Zingiber officinale var. sunti Valeton* Rhizome Extract (EEZO)

Determination of nutritional composition revealed that EEZO contains protein 3.16%, fat 11.52%, carbohydrate 73.43%, water/moisture 3.59%, and ash 8.30%. The total anthocyanins and vitamin C levels were 22.50 mg/100 g and 658.84 mg/100 g, respectively.

In this study, 6-gingerol and 10-shogaol were determined using UPLC analysis and showed that the 6-gingerol standard was eluted at 8.023 min and the 10-shogaol standard at 31.485 min (Figure 5a). EEZO was confirmed to contain 6-gingerol, eluted at 8.059 min, and 10-shogaol at 31.698 min, similar to the standards (Figure 5b). The levels of 6-gingerol and 10-shogaol in EEZO were quantitated using the linear regression equations of the standard addition method ($y = 4221.9x + 189885$ for 6-gingerol and $y = 3221.7x + 8112$ for 10-shogaol) and resulted in 44.98 µg/mL for 6-gingerol and 2.52 µg/mL for 10-shogaol.

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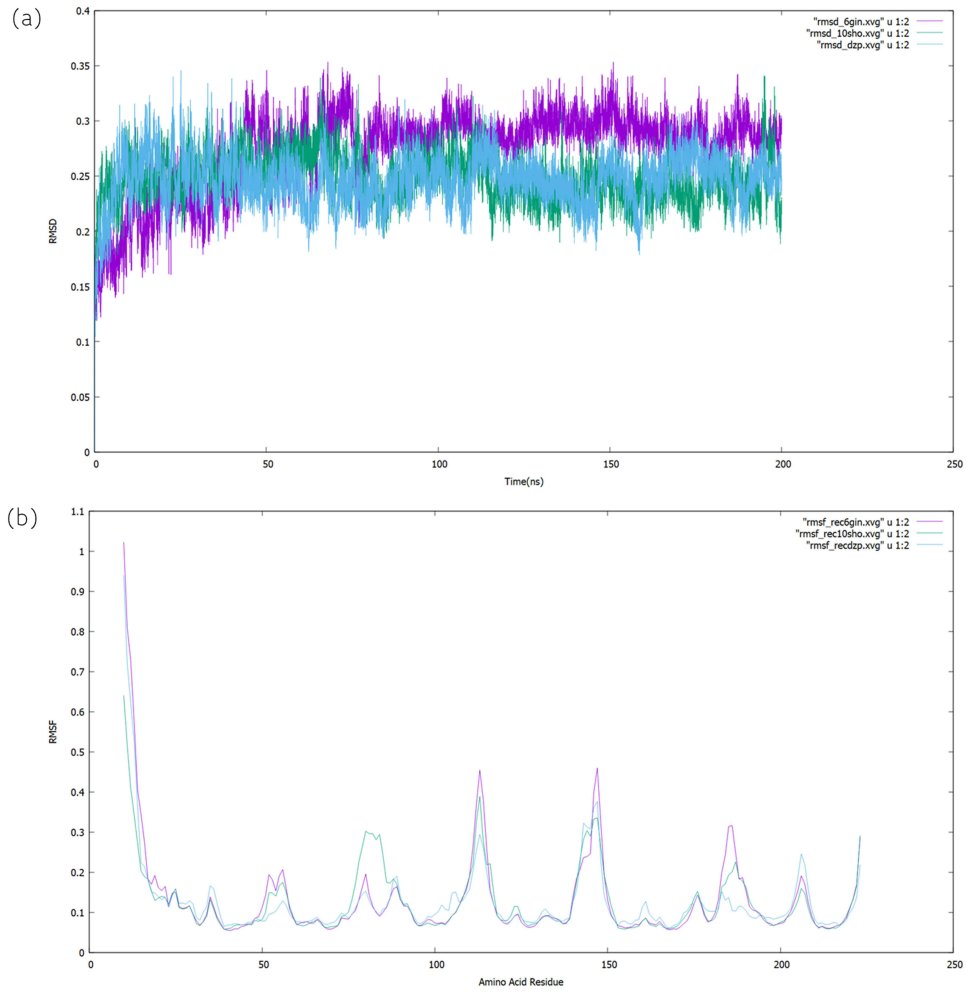

Figure 3 The RMSD (**a**) and RMSF (**b**) graphs of diazepam (blue), 6-gingerol (purple), and 10-shogaol (green) in complex with the human $GABA_AR$ within a 200-ns MD simulation.

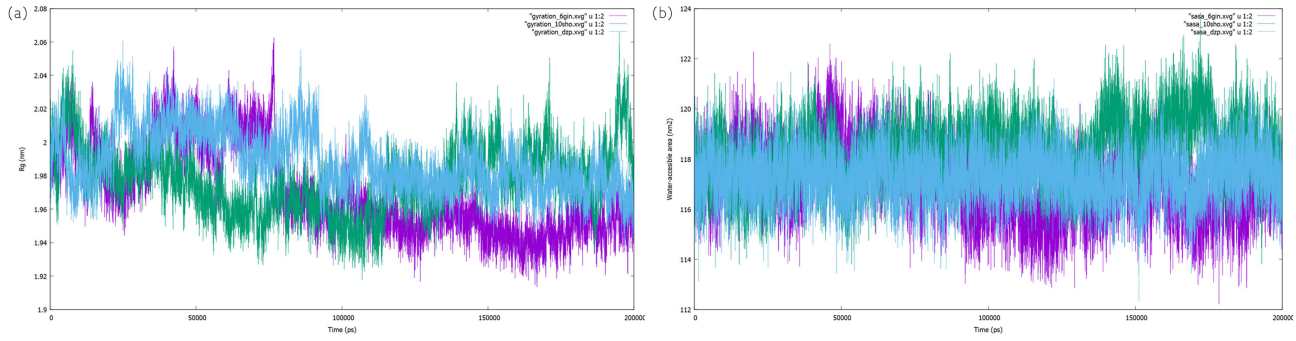

Figure 4 The radius of gyration (Rg) (**a**) and solvent accessible surface area (SASA) (**b**) plot of diazepam (blue), 6-gingerol (purple), and 10-shogaol (green) in complex with the human $GABA_AR$ within a 200 ns MD simulation.

Discussion

In our study, the pre-ADMET analysis revealed that all gingerols and shogaols indicate good absorption as proven by the Caco-2 permeability value (Log Papp) higher than −5.15 cm/s and the human intestinal absorption (HIA) estimation close to 0, with the best absorption is shown by 6-shogaol (Log Papp of −4.815 cm/s; HIA of 0.001). Caco-2 cells are human colon epithelial cancer cell lines that are utilized as a model of human intestinal absorption of drugs because they

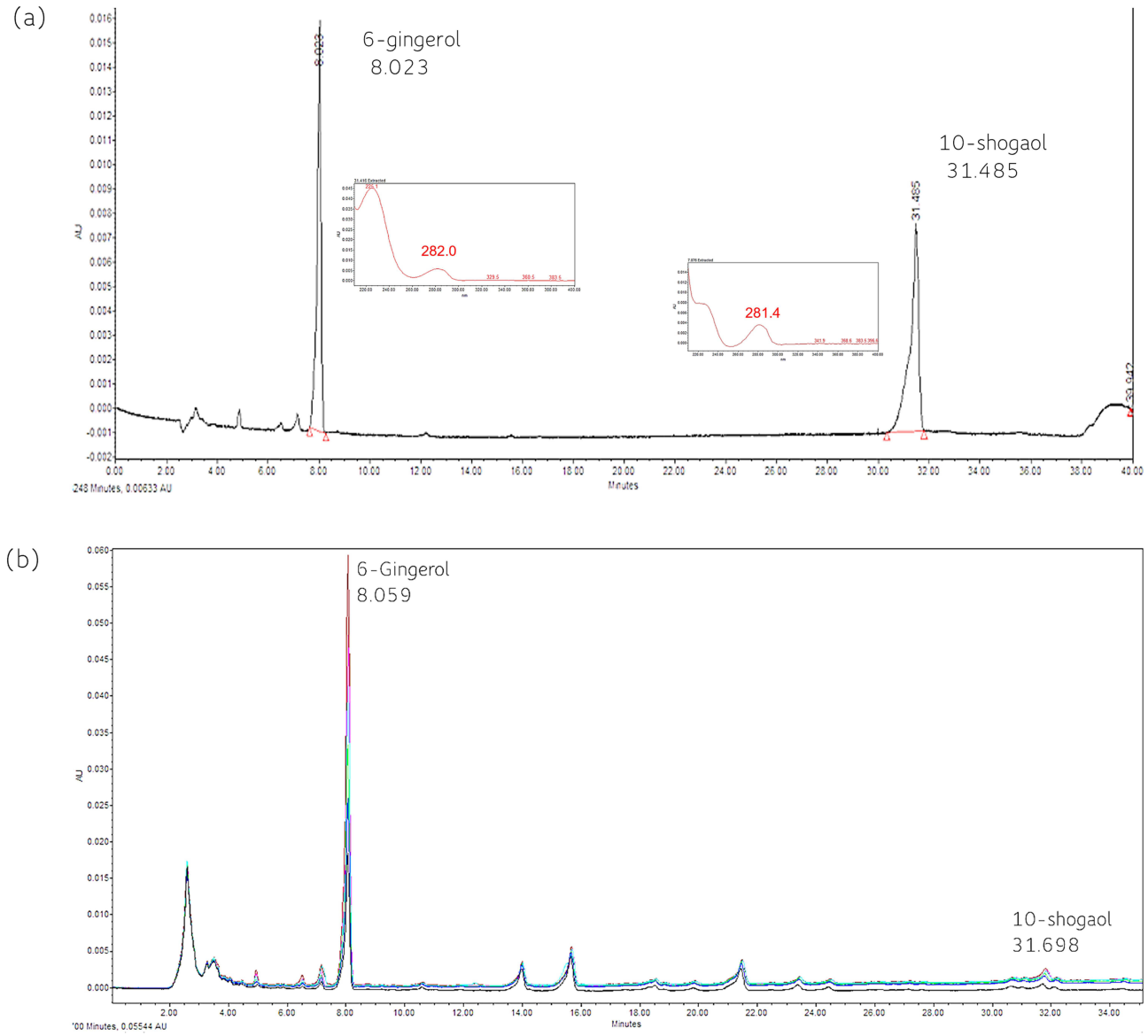

Figure 5 The chromatograms of standard 6-gingerol and 10-shogaol (**a**); and EEZO (**b**), were analyzed using ultra-performance liquid chromatography, stationary phase: a C18 column, and a gradient elution with water in 0.1% phosphoric acid (mobile phase (A) and acetonitrile in 0.1% phosphoric acid (mobile phase B). The gradient mode was set as follows: 0 min 45% B 55% A, 8 min 50% B 50% A, 18 min 60% B, 35 min 80% B, and 40 min 45% B. Detection was set at 282 nm; flow rate at 1.0 mL/min, temperature at 25 °C, and chromatographic run time for 45 min.

bear a resemblance to the human intestinal epithelium. The apparent permeability coefficient (Papp) was calculated from the permeation rate and compound concentration at time 0 h and time 2 h.[26] HIA is the most essential ADME property, indicating one of the key steps during the transport of drugs to their site of action.[27] In addition, these compounds satisfy the Lipinski Rules of Five,[28] and the Quantitative Estimate of Druglikeness (QED), thus confirming their good oral bioavailability. QED values range between 0 (all properties are unfavorable or not having drug-likeness) and 1 (all properties are favorable or comply with drug-likeness).[29] Our study is in agreement with a previous study, which also described that 6-gingerol, 8-gingerol, 10-gingerol, and 10-shogaol were able to cross the Caco-2 monolayer by passive diffusion.[30] Therefore, gingerols and shogaols may optimally be absorbed in the gastrointestinal tract, thus confirming their suitability for oral administration.

The estimation of distribution property delineated that gingerols and shogaols can penetrate the blood–brain barrier (BBB); moreover, 6-gingerol exhibits the optimal plasma protein binding (PPB of 80.039%). Only gingerols show an

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optimal volume of distribution at steady state (VDss) value, which should range between 0.04 and 0.7 L/kg.[31,32] PPB prediction is important in the ADME of drugs because it causes significant changes in the VD, clearance, and half-life of a drug.[33] It is the reversible binding of a compound with the plasma proteins caused by hydrophobic and electrostatic interactions. The fraction of bound compounds exists in equilibrium with the fraction of free compounds; only a fraction of free compounds can diffuse across cell membranes.[34] With its optimal PPB value and the ability to penetrate across the BBB, 6-gingerol, in particular, will be able to reach the central nervous system (CNS) and exert its biological activity.

Once entering the body, drugs will be biotransformed by hepatic enzymes, the subfamilies of cytochrome P450. These enzymes metabolize and convert drugs to more polar metabolites to be readily eliminated. CYP2D6 and CYP3A4 contribute to 2% of the overall hepatic CYP450 enzymes and are responsible for the metabolism of 25% of commonly prescribed drugs.[35] In this study, 6-gingerol may weakly inhibit CYP2D6, while 8-gingerol, 10-gingerol, 8-shogaol, and 10-shogaol may strongly inhibit this enzyme, while all gingerols and shogaols strongly inhibit CYP3A4. It should be considered that this type of inhibition may alter the metabolism of other drugs, such as antidepressants, including the serotonin-reuptake inhibitors (metabolized by CYP2D6) and benzodiazepines (metabolized by CYP3A4).[36,37]

Benzodiazepines (BZDs) are a class of anxiolytic compounds that act through positive allosteric modulation of $GABA_A$Rs at the α-γ subunits in the receptor's extracellular domain. BZD/$GABA_A$R complex enhances the influx of GABA-induced chloride ions, resulting in neuronal hyperpolarization and allosteric modulation of these receptors.[9,16] BZDs such as diazepam stabilize the affinity of the receptor's substrate, namely GABA, to bind at its orthosteric sites, and enhance the receptor stability.[9] Diazepam's action on $GABA_A$Rs activity could be influenced by specific binding with the target subunit. In this study, we confirmed that diazepam binds to allosteric pockets at the α-γ subunits and interacts with several amino acid residues, including α1: Phe100, α1: His102, α1: Tyr160, α1: Gln204, α1: Ser206, α1: Tyr210, γ2: Tyr58, and γ2: Phe77. The binding mode of diazepam shown here is in agreement with previous experiments in which diazepam interacts with α1: Phe100, α1: His102, α1: Ser206, α1: Tyr210, γ2: Tyr58, and γ2: Phe77.[9,38] Interestingly, gingerols and shogaols could enter and occupy the allosteric sites of the human $GABA_A$R at the α-γ subunits' extracellular domain. These compounds of interest can interact with essential amino acid residues that function for ligand affinity and efficacy, similarly to diazepam, such as Phe77 on the γ-subunit and Tyr210 on the α-subunit. These amino acid residues are important for stabilizing ligand/receptor binding. Mutating these residues to alanine and leucine will decrease ligand affinity, while substituting with tyrosine slightly improves it.[39] Diazepam forms a pi–pi stacking interaction with α1: His102, which is identified as an important amino acid residue in the BZD binding site. The mutation of His102 decreased the affinity of BZDs.[40] The formation of H-bonds is important in protein/ligand interaction to maintain the stability of the complex.[41] In our study, diazepam, gingerols, and shogaols have built several hydrogen bonds (H-bonds) with h$GABA_A$R. Shogaols α,β-unsaturated ketone side chain builds H-bonds to α1 his102, which indicates that the side chain was crucial for the formation of H-bonds and may contribute to the stability of the ligand-protein complex.

The inhibition constant (Ki) values of gingerols and shogaols with $GABA_A$Rs were found to be between −7.41 and −8.14 mm, which binding appears to correlate with the length of the aliphatic tail, with longer chains leading to higher affinity. Furthermore, the character of the side chain influences the binding affinity and effectiveness, as shogaols have an α,β-unsaturated ketone side chain, whereas gingerols have a β-hydroxy ketone side chain, thus highlighting 10-shogaol being the strongest ligand.[42,43] The presence of an α,β-unsaturated ketone in shogaols is responsible for their greater pharmacological activity than gingerols. The absence of this functional group or the addition of a β-hydroxyl group to the α,β-unsaturated ketone to afford [*n*]-gingerols may significantly reduce its bioactive properties.[44,45] The binding free energy score is a quantitative estimation of the most stable binding pose between the receptor and the ligand. The lower the binding energy, the more stable the bond between the ligand and the receptor. The stability of this bond can affect the activity of the ligand against the receptor, where the more stable the bond formed, the greater the possibility of ligand activity against the receptor.[25]

Our MD simulation revealed that both complexes of diazepam/$GABA_A$R and 10-shogaol/$GABA_A$R exhibit a similar RMSD pattern, thus confirming the stability of these complexes from their initial to final conformations. Meanwhile, the 6-gingerol-$GABA_A$R complex achieved equilibrium after 44 ns, and the system displayed steady fluctuation between 0.25 and 0.35 Å during 200 ns. The RSMD pattern of 6-gingerol was slightly higher than diazepam and 10-shogaol,

which indicates less stability. The lowest average RMSD value indicates the complex exhibits greater stability.[28] Moreover, the RMSF simulation shows similar patterns for diazepam/$GABA_AR$, 6-gingerol/$GABA_AR$, and 10-shogaol/$GABA_AR$ complexes, suggesting the stability of these complexes.[28] Considering all MD simulation results, 6-gingerol and 10-shogaol may work as the allosteric agonists of $GABA_AR$, similar to diazepam, because these compounds have shown moderate binding and stability with the receptor.

Due to variations in the ginger types and their growing locations, there may be slight differences in the composition and levels of bioactive constituents of gingers, as well as the nutritional quality and quantity of the extract are influenced by the extraction process. High-yield, high-quality bioactive chemical extraction is crucial, as is nutritional composition.[46] In the present study, the nutritional composition of EEZO is protein 3.16%, fat 11.52%, carbohydrate 73.43%, water/moisture 3.59%, ash 8.30%, total anthocyanins 22.50 mg/100 g, and vitamin C 658.84 mg/100 g. Our study is aligned with the previous reports, which described that gingers contained abundant levels of carbohydrates. Interestingly, protein and fat composition in our EEZO was higher compared to dried red ginger harvested in Bogor, West Java, Indonesia, and water extract-crystallization red ginger collected in Bekasi, West Java, Indonesia.[47,48] Vitamin C and anthocyanin levels in EEZO are also higher than those reported in red ginger planted in Lembang, West Java, Indonesia.[49] The high concentration of vitamin C in EEZO may contribute to its antioxidant activity. Previous studies described that red ginger exhibited better antioxidant activity compared to white ginger. Additionally, red ginger could dramatically increase its potential to inhibit Fe2+-induced lipid peroxidation in the rat brain. High concentrations of Fe have been linked to oxidative stress and play an important role in neurodegenerative disease.[50] The antioxidant activity of red ginger is enhanced by the presence of anthocyanidin, a class of flavonoids that exert antioxidant activity by inducing superoxide, nitric oxide, and peroxynitrite.[51]

Zingiber officinale var. *sunti Valeton* exhibits a broad spectrum of pharmacological activities, specifically antimicrobial, analgetic, antidiabetic, anti-inflammatory, antioxidant, anticancer, antihyperlipidemic, anti-hypercholesterolemia, and antihypertensive.[18,52,53] Its primary bioactive constituents, gingerols and shogaols, are considered responsible for this pharmacological effect. The presence of gingerols and shogaols in EEZO was confirmed using UPLC. Consistent with the previous studies,[43–45] our work identified 6-gingerol at 44.98 µg/mL as the most abundant compound in ginger, whereas 10-shogaol at 2.52 µg/mL was minor. The content of gingerols and shogaols is strongly correlated with the antioxidant activity of ginger rhizomes. Notably, a higher concentration of shogaols is associated with enhanced antioxidant, antibacterial, and antifungal activity.[52] Previous studies had described that the Zingiberaceae family plants, including *Z. officinale* Roscoe, exhibited anti-anxiety activity in animal models. Gingerols and shogaols were identified as the main bioactive compounds of these plants.[14,52,54] Interestingly, unlike benzodiazepines, this plant did not exhibit an amnestic effect (a decrease or loss of memory).[54] Our study predicted the potential of EEZO and its bioactive constituents as a promising alternative anti-anxiety drug. However, further preclinical and clinical studies, along with investigation into the pharmacological mechanism of EEZO, particularly 6-gingerol and 10-shogaol, are necessary to validate these findings.

Conclusion

Medicinal aromatic plants such as red ginger (*Zingiber officinale* var. *sunti Valeton*) have always become the topic of interest because of their numerous pharmacological activities. *Z. officinale* var. *sunti* Valeton contains major bioactive constituents, namely gingerols and shogaols, with hydrophobic properties in their structure, and thus may be able to penetrate the blood-brain barrier. These compounds possess pharmacophoric features required for occupying the allosteric sites at the interface of α-γ subunits of the human $GABA_AR$ extracellular domain, by interacting with important amino acid residues, such as Tyr210 on the α-subunit and Phe77 on the γ-subunit. In this study, 6-gingerol and 10-shogaol may have the potential to activate the human $GABA_AR$ through allosteric modulation that increases the affinity of $GABA_A$, the receptor's endogenous agonist, thus explaining their anti-anxiety activity. The activity towards the human $GABA_A$ receptor is comparable to that of diazepam, its known allosteric agonist. Moreover, the UPLC analysis of EEZO resulted in a level of 44.98 µg/mL for 6-gingerol and 2.52 µg/mL for 10-shogaol. Considering that there are more gingerols (the 8- and 10-gingerols) and shogaols (the 6- and 8-shogaols) in EEZO, making the plant in abundance with these compounds, *Z. officinale* var. *sunti* Valeton rhizomes may be developed as a plant-based anti-anxiety adjuvant agent

whose activity is attributed to all the gingerols and shogaols. However, further studies in animals and humans are needed to validate the in silico pharmacology approach and verify the efficacy and safety of this plant.

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Disclosure

The authors report no potential conflicts of interest in the research, authorship, or publication of this article.

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