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In Silico Study of Compounds Bioactive Guava (*Psidium guajava*) as Antidiarrheal Assisted Liquid Chromatography-Mass Spectroscopy

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ABSTRACT

Guava leaves (*Psidium guajava* L.) are widely used in traditional medicine to treat diarrhoea; however, the specific bioactive compounds and their molecular mechanisms remain insufficiently characterised. This study aimed to identify the secondary metabolites in guava leaf methanol extract using LC–MS and evaluate their antidiarrheal potential through molecular docking against three key human protein targets involved in diarrheal pathophysiology: CFTR (fluid secretion), M3 muscarinic receptor (intestinal motility), and TLR4 (bacterial toxin–induced inflammation). LC–MS profiling detected 11 major compounds, including quercetin, kaempferol, isorhamnetin, myristicin, gallic acid, and sinapic acid. The selected compounds were prepared and docked using AutoDock Vina, following proper protein optimisation, grid-box definition, and method validation through redocking. Kaempferol, quercetin, and isorhamnetin showed the most favourable binding affinities across the three targets, with binding energies ranging from −7.8 to −10.2 kcal/mol, indicating strong and stable interactions. Key hydrogen-bonding and hydrophobic interactions at the CFTR regulatory domain, M3 receptor-binding pocket, and TLR4–MD2 interface support their potential biological relevance. All major compounds met Lipinski's Rule of Five, suggesting good oral drug-like properties. These findings indicate that flavonoid-rich compounds in *Psidium guajava*, particularly kaempferol and quercetin, possess promising antidiarrheal potential via multimodal molecular mechanisms. Further in vitro and in vivo studies are required to validate these computational predictions in humans.

Keywords: *Psidium guajava,* LC–MS, Molecular Docking, Antidiarrheal

INTRODUCTION

Diarrhoea remains a primary global health concern, particularly among infants and young children, and is characterised by increased stool frequency and looseness due to alterations in intestinal motility, secretion, absorption, or inflammatory responses. According to the World Health Organisation (WHO), diarrhoea accounts for more than 1.7 billion cases annually. It remains one of the leading causes of mortality in children under five years of age, worldwide (WHO, 2024). In Indonesia, diarrhoea consistently ranks among the top causes of morbidity and mortality in children, reflecting the urgent need for effective and accessible therapeutic strategies (Ministry of Health RI 2020).

The underlying mechanisms of diarrhea vary by etiology and involve disruptions in ion transport, excessive intestinal motility, and inflammation, which are triggered by infectious agents. Three molecular pathways play central roles in diarrheal pathophysiology: (1) fluid hypersecretion mediated by the cystic fibrosis transmembrane conductance regulator (CFTR), which regulates chloride and water secretion in intestinal epithelial cells (Thiagarajah et al., 2015); (2) acetylcholine-driven hypermotility through the activation of the M3 muscarinic receptor on smooth muscle cells (Broad et al., 2016); and (3) immune activation via Toll-like receptor 4 (TLR4) in response to lipopolysaccharides (LPS) produced by gram-negative bacteria (Lu et al., 2008). These pathways collectively represent rational therapeutic targets for antidiarrheal drug discovery.

Psidium guajava L. (guava) is widely used in traditional medicine throughout Southeast Asia, including Indonesia, to treat diarrhea, dysentery, and gastrointestinal disturbances. Phytochemical studies have demonstrated that guava leaves contain abundant secondary metabolites, such as flavonoids, tannins, phenolic acids, terpenoids, and alkaloids (Arima & Danno, 2002; Díaz-de-Cerio et al., 2017). Flavonoids, such as quercetin and kaempferol, are particularly associated with antidiarrheal effects

through several mechanisms, including inhibition of intestinal smooth muscle contraction, modulation of ion transport, and anti-inflammatory activity (Gutiérrez et al., 2008; Ojewole et al., 2008). Tannins and phenolic acids also contribute to antimicrobial and spasmolytic properties, thereby reducing stool output in infectious diarrhea (Cowan 1999).

Despite substantial ethnopharmacological documentation, the specific molecular mechanisms through which individual guava leaf metabolites exert antidiarrheal activity remain unclear. Previous studies have primarily relied on broad phytochemical screening or in vivo antidiarrheal assays without integrating more advanced analytical techniques. Comprehensive LC–MS profiling of guava leaves from Indonesian cultivars remains limited, and studies combining LC–MS data with molecular docking approaches are rare. Furthermore, most available docking studies do not target proteins directly involved in diarrhea, such as CFTR, M3, and TLR4, thereby restricting mechanistic interpretation (Joseph et al., 2021).

Based on these limitations, this study aimed to: (1) identify and characterize secondary metabolites present in *Psidium guajava* leaf methanol extract using LC–MS, and (2) evaluate the antidiarrheal potential of selected metabolites through molecular docking against CFTR, M3 muscarinic receptor, and TLR4, three key targets representing fluid secretion, intestinal motility, and inflammation pathways. The integration of metabolite profiling with in silico docking is expected to provide a more mechanistic and systematic understanding of the antidiarrheal properties of *Psidium guajava (P. guajava*).

RESEARCH METHODS

Tool

The tools used were a computer with Windows 11 version 22H2 64 bit specifications and Chemdraw 3D and MarvinSketch programs version 24.1.0, AutodockTools-1.5.6., PyRx version 0.9.8, Molegro Molecular Viewer version 2012.2.5.0, Discovery Studio version 20.10, and helper programs others that are based on online servers such as Pubchem at https://pubchem.ncbi.nlm.nih.gov/, pkCSM at https://biosig.lab.uq.edu.au/pkcsm/, and Protein Data Bank (PDB) at https://www.rcsb.org/ .

Material

Guava leaves (*Psidium guajava* L.) were collected, cleaned, air-dried at room temperature for seven days, and ground into a fine powder (40-mesh). Methanol (analytical grade), distilled water, and all solvents used for LC–MS analysis were obtained from certified suppliers. The protein structures used in molecular docking were CFTR (PDB ID: *5UAK*), M3 Muscarinic Receptor (CHRM3) (PDB ID: *4U15*), and TLR4–MD2 complex (PDB ID: *3FXI*). All ligands identified by LC–MS were retrieved from PubChem.

Sample Extraction

The extract was obtained via maceration. A total of 500 g of powdered guava leaves was immersed in 2.5 L of 100% methanol for 72 h at room temperature with occasional stirring. The filtrate was collected, and the residue was re-macerated twice under identical conditions. The combined extracts were concentrated using a rotary evaporator at 45 °C to obtain a viscous methanol extract. The extract yield was recorded for analysis. (Raihan & Yunitasari, 2022).

Identification Profile of Phytochemicals with LC-MS

Compound identification was performed using LC–MS/MS equipped with a QToF detector (Xevo G2-S, Waters, USA). Chromatograms and mass spectra were analysed using MassLynx software to annotate compounds based on m/z values, fragmentation patterns, and comparison with reference databases (PubChem, METLIN, and MassBank).

Ligand Preparation

The identified LC–MS compounds were downloaded from PubChem in SDF format and energy-minimised using MarvinSketch 24.1. Optimisation was carried out at pH 7.4. A conformational search was performed, and the lowest-energy conformation was saved in PDBQT format using AutoDockTools 1.5.6.

Protein Preparation

Protein structures are first obtained from databases such as the Protein Data Bank (PDB). Docking is done using Autodock Tools. Docking results are then selected from results that have low binding affinity values and saved in pdb format.(Agu et al., 2023).

Validation of Docking Method

The docking method was validated using AutoDock Tools software by redocking (re-docking) on six receptors. The results of this process obtained data in the form of grid box parameters and RMSD values (Astuty & Komari, 2022).

Docking Results Analysis

Molecular docking data analysis was performed based on Gibbs free binding energy (ΔG) values, RMSD values, amino acid interactions, visualization of docking results, and the Lipinski rule (Rule of Five). To see amino acid interactions, Molegro Molecul Viewer (MMV) software was used in 2D and 3D forms.(Fadillah et al., 2023).

Ligand Based Drug Likeness Screening (drug scan)

Ligand Based Drugs Likeness Screening (Drug Scan) Observation of drug candidates is carried out on ligands by paying attention to Lipinski's Rule of Five drug rules. (http://www.scfbio-iitd.res.in/software/drugdesign/lipinski.jsp) which includes a molecular weight of <500 g/mol, hydrogen bond donor (<5), hydrogen bond acceptor (<10), molar refractory between 40-130 (Cavin et al., 2024).

RESULTS AND DISCUSSION

Guava leaves (*Psidium guajava* L.) contain various active compounds, such as flavonoids, tannins, alkaloids, and other phenolic compounds, with antibacterial, antioxidant, and anti-inflammatory properties. (Indraswara et al., 2024). Extraction methods are used to obtain bioactive compounds. The extraction method used in this study was maceration. The maceration method is generally used for the extraction of natural materials because it can prevent damage to components that are unstable at high temperatures or during heating. In addition, the maceration method is a simple procedure that requires minimal equipment. The process involves soaking the powder in a solvent with simple ingredients. (Asworo & Widwiastuti, 2023). In this study, methanol was chosen as the extraction solvent because it offers the most efficient and comprehensive extraction of the major bioactive compounds in Psidium guajava, especially flavonoids, phenolic acids, tannins, and other polar-to-semi-polar metabolites. (Ridhwan Anshor Alfauzi et al., 2022)

After extracting guava leaves with methanol, the next step is to identify the bioactive compounds in the extract using Liquid Chromatography-Mass Spectrometry (LC-MS). LC-MS was chosen because it combines the separation of physicochemical properties from liquid chromatography with mass spectrometry for specific detection. Liquid chromatography separates the components of a sample and charges the ions, which are then detected by a mass spectrometer. Profit from the use of LC-MS, namely, can analyse a wider variety of components, such as thermally unstable, polar, or high-molecular-weight molecules, even proteins (Sinurat et al., 2024.).

Figure 1. Total Ion Chromatogram of *Psidium guajava*

LC–MS profiling of the methanolic extract of *Psidium guajava* leaves revealed 11 major chromatographic peaks, representing the dominant secondary metabolites in the sample. The total ion chromatogram (TIC) displayed well-resolved peaks with retention times ranging from 0.45 to 19.22 min, indicating a diverse composition of polar to moderately non-polar compounds.

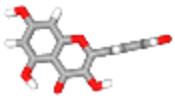
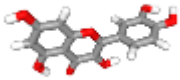
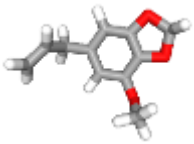
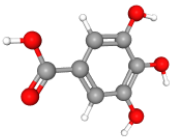
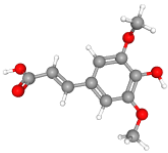
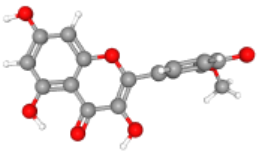
Compound annotation was performed by comparing the detected m/z values and fragmentation patterns with reference spectra available in the MassBank, METLIN, and PubChem databases. Based on this approach, six compounds were confidently identified, whereas five peaks remained unassigned owing to insufficient spectral matching. The identified metabolites were primarily phenolic acids, flavonoids, and aromatic ethers, consistent with previously reported phytochemical profiles of guava leaves. Five peaks were classified as unknown compounds, reflecting the chemical diversity of guava leaf metabolites and suggesting the presence of additional constituents not fully represented in major spectral libraries. The retention time distribution indicates that most guava leaf metabolites are moderately polar, consistent with the methanol extraction. These LC–MS results served as the basis for selecting representative compounds for subsequent molecular docking analysis. Table 1 summarises the retention times, putative compound names, molecular formulas, and molecular weights of the primary metabolites detected.

Table 1. Retention Time, Compound Name, Chemical Structure, and Molecular Weight Obtained from LC-MS Spectra

No.	Retention Time (min)	Predicted Compound	Molecular Formula	Molecular Weight (g/mol)
1	0.45	Gallic acid	$C_7H_6O_5$	170.12
2	2.65	Unknown compound	—	204.34
3	3.10	Unknown compound	—	178.22
4	5.76	Myristicin	$C_{11}H_{12}O_3$	192.21
5	7.70	Unknown compound	—	206.23
6	8.21	Isorhamnetin	$C_{16}H_{12}O_7$	316.26
7	9.03	Quercetin	$C_{15}H_{10}O_7$	302.23
8	9.30	Kaempferol	$C_{15}H_{10}O_6$	286.23
9	9.69	Sinapic acid	$C_{11}H_{12}O_5$	224.21
10	13.48	Unknown compound	—	285.33
11	19.22	Unknown compound	—	358.38

The next stage is the molecular docking process, namely, by preparing the protein and the test ligand. The ligands to be tested were prepared in MarvinSketch, and the compounds were prepared using clean 2D and protonation methods, selecting the lowest-energy value.

Table 2. Structure of Test Ligands from Guava Leaf (*Psidium guajava*)

No.	Compound	Structure	Compound	Structure
1	Kaempferol		Quercetin	
2	Myristicin		Gallic Acid	
3	Sinapic Acid		Isorhamnetin	

Afterwards, protein preparation was performed, and the protein structure was first obtained from a database such as the Protein Data Bank (PDB). The downloaded file was opened with the MOLEGO molecular viewer software to separate the native ligand from the protein. After that, preparation was carried out by removing water molecules using Discovery Studio. The removal of water molecules aims to reduce the computational burden, thereby increasing the simulation time. Therefore, the presence of water molecules will make the simulation impractical. After removing the water molecules, hydrogen atoms and charges were added. Hydrogen atoms are added to correctly define hydrogen-bonding sites and protonation states, while partial charges are applied to represent the electrostatic properties of amino acids. Both steps are essential for accurate calculation of ligand–receptor interactions during molecular docking. After the receptor was prepared, it was stored in a pdbqt file (Kalontong et al., 2022).

Removal of residue besides essential amino acids, reviewed, returned with review journal, macromolecules, selected ones attached by PDB. (Hasan et al., 2022). The receptors used in this study, namely as many as six receptors, including 3O96, 3B8E, 4DKL, 4DAJ, 5WB7, and 3ALQ.

Redocking of native ligands into their respective receptors was conducted to assess the reliability of the docking protocol. Four receptors—5WB7, 3O96, 4DKL, and 4DAJ—exhibited RMSD values ranging from 0.55 to 1.83 Å, falling within the acceptable threshold of ≤ 2.0 Å. These values indicate accurate reproduction of the crystallographic binding poses and confirm that the docking parameters were appropriate for these targets (Ramadhan & Musfiroh, 2021).

Receptor 3B8E yielded an RMSD of 3.25 Å, which is above the ideal limit but not excessively

high. This suggests that docking predictions for this receptor may still offer valuable qualitative insights, but should be interpreted with caution, given reduced pose accuracy. In contrast, the redocking of receptor 3ALQ produced an RMSD of 31.96 Å, indicating a complete failure to reproduce the native ligand conformation. Therefore, docking results associated with this receptor are not considered valid and were excluded from biological interpretation.

Table 2. List of Native Ligands for Each Receptor

No.	Native Ligand	Run	Binding Energy (kcal/mol)	RMSD (Å)
1	5WB7	77	−10.08	1.02
2	3O96	30	−9.57	0.60
3	3ALQ	11	−2.78	31.96
4	3B8E	35	−7.47	3.25
5	4DKL	—	−2.53	1.83
6	4DAJ	26	−9.01	0.55

Overall, the validation results support the reliability of docking predictions for four of the six receptors, allowing confident interpretation of ligand–receptor interactions for the validated targets (Sekolah et al., 2024).

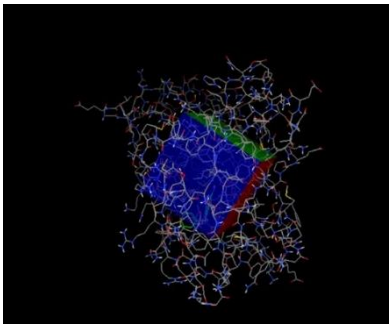


Figure 2. Protein Preparation Using Autodock Tools

The Lipinski test checks how a drug moves through the body. If a drug does not pass this test, it is better for injection than for taking by mouth. The size of a drug affects how it moves through cell walls. Drugs smaller than 500 g/mol move through cell walls more easily than larger ones. Hydrogen bonds help describe how a drug is absorbed. If a drug has 10 or more donor hydrogen bonds and 5 or more acceptor hydrogen bonds, it needs more energy to be absorbed. Another important factor is the log P value, which shows how a drug mixes with fats and oils. Table 4 shows that all six test drugs meet the Lipinski rules.

Table 3. Lipinski Results for Each Compound

No.	Compound	Molecular Weight (<500g/mol)	Hydrogen Donor (<5)	Hydrogen Acceptor (<10)	Log p (<5)
1	Gallic acid	286,239	4	6	2,2824
2	Isorhamnetin	302,238	5	7	1,988
3	Kaempferol	295,382	0	3	3,4559
4	Myristicin	322,269	7	8	0.7426
5	Quercetin	290,271	5	6	1,5461
6	Sinapic acid	121,161	3	3	-0.6719

The Lipinski analysis showed that all six compounds from Psidium guajava leaf extract meet the standards of Lipinski's Rule of Five. This means they have good properties for possible oral drugs. The compounds have molecular weights between 121.161 g/mol (sinapic acid) and 322.269 g/mol (myristicin), all below the 500 g/mol limit. Lower molecular weight helps them cross cell membranes more easily, suggesting they are the right size for intestinal absorption.

The number of hydrogen-bond donors (HBD) ranges from 0 in kaempferol to 7 in myristicin. Hydrogen-bond acceptors (HBAs) range from 3 to 8. These numbers fit within Lipinski's limits of 5 or fewer for HBD and 10 or fewer for HBA. This balance helps with both solubility and receptor binding. Too

much hydrogen bonding can make it hard for substances to pass through membranes. Flavonoids like quercetin (HBD = 5; HBA = 6) and isorhamnetin (HBD = 5; HBA = 7) have strong hydrogen-bonding abilities. This might help them interact well with amino acids in molecular docking.

The logP values of the compounds ranged from −0.67 (sinapic acid) to 3.46 (kaempferol), which is within the recommended range (≤5). This means the compounds can cross lipid membranes and still dissolve in water for distribution in the body. Kaempferol, with the highest logP (3.4559), can easily pass through membranes. Sinapic acid, with a low, negative logP, is highly water-soluble but still acceptable. A negative logP means the compound dissolves better in water than in fats, so it has low membrane permeability. In this study, sinapic acid's negative logP explains its moderate docking affinity and suggests it might need help with absorption, rather than just passing through on its own.

The next step is to analyse the docking results. We examine the binding energy values for the six test ligands. Binding energy is the energy needed for a compound to attach to a target receptor. A lower (negative) binding energy indicates the compound binds more strongly to the target protein, making the bond more stable (Prasiska Wulandari et al., 2023). Based on the molecular docking results, quercetin is the best test ligand because it binds to several receptors with the lowest binding energies.

Table 4. Molecular Docking Results of Native Ligands with Test Ligands

No.	Senyawa	Binding energi (kkal/mol)					
		5WB7	3O96	3ALQ	3B8E	4DKL	4DAJ
1	Kaempferol	-7,54	-7,18	Does not meet criteria		-4,88	95,38
2	Quercetin	-9,72	-6,92			-5,46	150,38
3	Isorhamnetin	-3,65	-6,58			-5,14	194,49
4	Gallic acid	-4,23	-3,50			-3,50	186,5
5	Sinapic acid	-2,7	-4.31			-2,49	-2,25
6	Myristicin	-9,4	-7,37			-5,39	199,33

The study looked at how six main compounds from Psidium guajava interact with specific antidiarrheal receptors (5WB7, 3O96, 3B8E, and 4DKL). The results showed that these compounds bind to the receptors differently. The flavonoids, especially quercetin, kaempferol, and isorhamnetin, had the best binding energies. This aligns with prior research indicating that polyphenolic compounds bind well to proteins because they can form many hydrogen bonds and stack with other molecules (Panche et al., 2016; Boots et al., 2008).

Quercetin had the strongest interaction with receptor 5WB7 (−9.72 kcal/mol), better than the other compounds. Its strong binding might be due to its five hydroxyl groups, which help it form more hydrogen bonds and fit well into the receptor. Other studies also found that quercetin can block gastrointestinal and inflammatory targets (Gutiérrez et al., 2008; D'Andrea, 2015).

Kaempferol showed strong binding to receptors 5WB7 and 3O96, with values of −7.54 kcal/mol and −7.18 kcal/mol, respectively. This means it interacts well with a range of targets. Its flat, ring-like structure and hydroxyl groups help it form strong bonds in receptor sites (Alam et al., 2022). Isorhamnetin, a modified form of quercetin, also showed strong binding. This is common for methylated flavonoids, which often keep their binding ability and can pass through cell membranes more easily (Walle, 2007).

Interaction Amino Acid Residues

After molecular docking, it is important to check how amino acids interact. These interactions can include hydrogen bonds, hydrophobic interactions, van der Waals interactions, and electrostatic interactions. Visualising docking results helps identify how compounds interact with amino acids on protein receptors. The antidiarrheal effect of Psidium guajava is due to its main constituents, such as quercetin, kaempferol, and isorhamnetin. These chemicals interact with key receptors that control gut movement, inflammation, the gut lining, and ion transport. These actions match known pathways that cause diarrhoea.

First, flavonoids can bind firmly to receptors involved in movement, such as 5WB7. This may help slow down fast intestinal movements. Flavonoids are known to inhibit acetylcholine-induced muscle contractions, thereby helping relax muscles (Ojewole, 2008; Gutiérrez et al., 2008). Second, flavonoids can interact with receptors linked to inflammation, such as 3O96. This means they might reduce inflammation signals. Quercetin and kaempferol are known to inhibit NF-κB activation and reduce cytokine release, thereby reducing inflammation and fluid secretion (Boots et al., 2008; D'Andrea, 2015).

Third, flavonoids can bind to receptors that regulate the epithelial barrier, such as 3B8E. This suggests they might strengthen intestinal barriers. Evidence shows quercetin can improve barrier strength and reduce leaks (Suzuki & Hara, 2011).

Guava compounds might help reduce chloride release or change how electrolytes move in the body. This is because they interact with specific receptors, such as 4DKL. Stopping chloride from leaving cells through CFTR-like pathways can help reduce secretory diarrhoea (Thiagarajah et al., 2015; Cui et al., 2019).

The docking results show that flavonoids from Psidium guajava work in several ways. They reduce movement, limit inflammation, strengthen the body's barriers, and regulate ion transport. These actions explain why guava leaves are traditionally used to treat diarrhoea.

Table 5. Results of Amino Acid Residue Interactions

No.	Compound	Bond Hydrogen	Bond Hydrophobic
1	5WB7	ASP: 313, ASN: 222	GLY:316, ALA:317, LEU:496, PHE:224, PHE:258, LUE:312, ILE:115
2	3O96	ALA:700, MET:696, GLU:694, ALA:645	ILE:621, LEU:747, ILE:677, LYS:647, ILE:691
3	3B8E	GLY :117, TYR:121	ILE:16, VAL:8, ALA:9, THR:56, SER:59, ILE:60, PHE:34, LEU:22
4	4DKL	VAL:209 (hydrogen)	-
5	4DAJ	LEU:449, ALA:416	ALA:415, VAL:386, CYS:395, ARG:414
6	Kaempferol	VAL:209	ALA: 230, TRP: 208

The analysis of amino acid interactions shows that each ligand makes hydrogen bonds and hydrophobic contacts. These help the ligand stay in place in the receptor pocket. Hydrogen bonds are important for positioning the ligand, while hydrophobic interactions make the binding stronger by holding the ligand in nonpolar parts of the protein (Hubbard & Haider, 2010). Some native ligands, such as 5WBJ and 3O96, interact with residues including ASP313, ASN222, ALA700, and GLU694. These interactions help keep the ligands stable in the active site. The key finding is that VAL209 is contacted by both the native ligand 4DKL and the strong test ligand kaempferol. This shows that VAL209 is important for ligand binding in this receptor. Kaempferol also interacts with ALA230 and TRP208, which are hydrophobic residues. These support π–π and van der Waals interactions, which are linked to strong binding in flavonoids (Panche et al., 2016). These interaction patterns suggest that compounds targeting VAL209 and nearby hydrophobic residues may be more stable and active.

CONCLUSION

This study found that kaempferol and quercetin are the most promising compounds in guava leaves for treating diarrhea. Kaempferol had the lowest binding energy of −9.72 kcal/mol and interacted with an important amino acid, VAL209, which is also targeted by the natural ligand. This suggests it might have a significant biological effect. Both kaempferol and quercetin meet Lipinski's criteria, meaning they are likely to be good drugs and can be taken by mouth.

The new part of this study is connecting compounds found in guava leaves with specific receptors related to diarrhea. This gives new ideas about how these compounds might work at the molecular level. However, these results are early predictions made using computer models. While kaempferol and quercetin show good potential, more lab and animal studies are needed to confirm their effectiveness against diarrhea.

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