RASĀYAN *J. Chem.*
Vol. 16 | No. 4 |2370-2377| October - December | 2023
ISSN: 0974-1496 | e-ISSN: 0976-0083 | CODEN: RJCABP
http://www.rasayanjournal.com
http://www.rasayanjournal.co.in

IDENTIFICATION OF POTENTIAL ALPHA-AMYLASE INHIBITORS FROM ZIZIPHUS TRINERVIA USING GC-MS AND COMPUTATIONAL APPROACH

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ABSTRACT

The goal of this study is to identify and separate the *Ziziphus Trinervia* Fruit's secondary metabolites using GC-MS methods. The GC-MS results revealed that 3,6,9,12-Tetraoxatetradecan-1-ol (**1**), Methyl salicylate (**2**), Diethyl Phthalate **(3)** and 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl hexasiloxane (**4**) are the most abundant secondary metabolites in the ethanolic extract. The identified compounds (**1-4**) were screened for drug-likeness using SWISS-ADME and their pharmacokinetics properties were evaluated by pkCSM. The target protein, Human pancreatic alpha-amylase (4W93), is docked with the identified compounds (1-4) using MOE software. The docking results indicate that compound **4** exhibits a comparable binding affinity for the target protein as standard α-amylase inhibitors. This suggests that it has the potential to interact effectively with the target enzyme. The *in-silico* study reports that compound **4** could be a promising drug candidate against inhibiting the function of human pancreatic α-amylase. These findings could contribute to the development of new therapeutic options for managing diabetes. Researchers and healthcare professionals will likely be interested in exploring this potential avenue for treatment.

Keywords: *Ziziphus Trinervia*; GC-Ms Analysis; Phytoconstituents; Docking; α-Amylase Inhibitors; Diabetes.
RASĀYAN *J. Chem.*, Vol. 16, No. 4, 2024

INTRODUCTION

Amylase inhibitors are perceived as starch blockers since they shut down dietary starches from being processed and consumed by the body. This has the option of treating obesity and diabetes mellitus a metabolic issue described by chronic hyperglycemia coming about because of defects in insulin discharge.[1,2] α-amylase inhibitors are drug-design focuses on the improvement of compounds for the action of diabetes Obesity and Hyperlipaemia.[3] Protein inhibitors of α-amylase happen generally in plants and fruits.[4,5] Several fruits contain secondary metabolites that have been studied for their potential benefits in managing diabetes. It's important to note that while certain compounds in fruits may show promise in preclinical studies, individual responses can vary, and more research is needed to establish their efficacy and safety in human populations. Additionally, moderation is key, as fruits also contain natural sugars. Fang Hua and coworkers evaluated α-amylase inhibitory effects of the phytoconstituents isolated from *Camellia sinensis* L.O. Kuntze. In 2020, Analy Aispuro-Pérez *et al.*[7], prepared a series of aryl imines under the microwave, and in-vivo analysis was carried out against the α-amylase enzyme. The outcomes displayed that the maximum of the compounds presented decent inhibitory activity. The α-amylase inhibition was carried out in contradiction of *Justicia carnea* leaf extract. The overall result indicates the potential to prevent postprandial hyperglycemia by retardation down carbohydrate hydrolysis.[8] *Ziziphus* family Rhamnaceae have fruits like drupes or dry is related to an Arabic term while the ancient Greeks used the name ziziphon for the jujube. The broadly tamed jujubes are *Z. mauritiana* Lam which is also known as the Indian jujube, while the wild diversity of *Ziziphus* is *Z. nummularia*. *Z. Mauritius* or *Z. jujube* species are cultivated in vast parts of the world. According to Ayurveda,

http://doi.org/10.31788/RJC.2023.1648554

Z. nummularia root treats cough, bile, and headache. Its bark extract has been used to treat dysentery and diarrhea. The leaves help reduce obesity and antipyretic. The fruits are used to improve digestion, and laxatives, remove biliousness and aphrodisiac, burning sensations, and vomiting. In addition, it is widely used for illnesses like tuberculosis and blood diseases. The seeds are also used in the treatment of leukemia and eye diseases.[9-11] Drug discovery activity is a significant matter in the pharmaceutical industry since it is a cost-effective and time-consuming job for evolving new drugs.[12] In the course of the screening process, several key steps are taken to eliminate compounds with side effects. In-silico drug design programs show an important character in the drug design process.[13] Therefore, the purpose of this research is to evaluate the inhibitory activity of α-amylase by extracting biochemicals from the fruit extract of *Ziziphus Trinervia* with the help of GC-Ms and a computational approach.

EXPERIMENTAL

Collection and Preparation of Plant Materials

The *Ziziphus trinervia* fruits were collected in Courtallam, Tamilnadu, India. The newly picked fruits were washed under running tap water and followed by rinsed in distilled water. After being chopped into little pieces, the fruit bulbs were allowed to be shade-dried at room temperature. The dried fruits were extracted with ethanol solvent in a Soxhlet apparatus for 8 hours. The isolated samples were filtered and the solvents were removed.

Gas Chromatography-Mass Spectrometry (GC–MS) Analysis

Ziziphus trinervia fruit extracts were subjected to GC–MS studies utilizing the MassHunter GC/MS (Agilent Technologies, Inc.). The isolated samples were determined by comparing the phytochemicals retention time (min), peak area, peak height, and mass spectral patterns with the compound spectrum database kept in the National Institute of Standards and Technology (NIST) library.[14]

Molecular Docking Analysis

Bioactive structures (**1-4**) were obtained from the PubChem database. The α-amylase target protein (PDB code: 4w93) was obtained from the Protein Data Bank(https://pubchem.ncbi.nlm.nih.gov/). The protein structure was prepared by structure correction and 3D protonation. Energy minimization of the protein structure was performed using Amber12: EHT Forcefield. Bioactive structures obtained from PubChem were used as ligands. Ligand structures were energy minimized using Amber12: EHT Forcefield. Molecular docking studies were performed using MOE 09 docking software. Preset parameters included 5 docked positions, London dG scoring, and the induce-fit model. The MOE 09 docking program[15] was used to screen for protein-ligand interactions. Discovery Studio Visualizer was employed to extract probable binding conformations of bioactive ligands. Molecular docking is a computational technique used to predict the binding mode and affinity of molecules. The London dG scoring function is commonly used to estimate the free energy of binding.

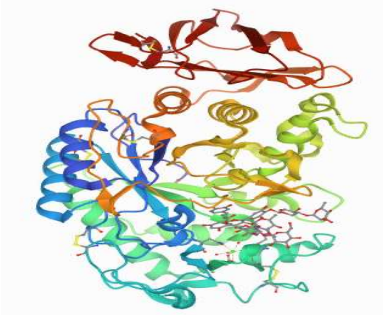


Fig.-1: Crystal Structure of α-amylase Protein

Drug-likeness and ADMET Properties Analysis

SwissADME, an online server[16], was utilized to analyze the drug-likeness properties of the selected bioactive compounds (ligands **1-4**). Lipinski's rule[17], a widely used rule of thumb in drug design, was applied to filter the bioactive compounds based on their physicochemical properties. The requirements were as follows: partition coefficient (log P) not to exceed 5, total polar surface area not to exceed 140, molecular

mass not to exceed 500 Da, and hydrogen bond donors and acceptors not to exceed 5. The pkCSM server[18] was employed for the analysis of Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) properties of ligands **1-4**. The NCBI PubChem database was used to obtain the SMILES data. The retrieved SMILES data was used as the input for both the SwissADME and pkCSM online servers.

RESULTS AND DISCUSSION

Gas Chromatography-Mass Spectroscopy (GC-MS) Analysis

The GC-MS analysis of *Ziziphus Trinervia* Fruit extract has four compounds having retention times of between 0.939 and 7.708 min. The compound's prediction and identification were done based on the NIST reference library. The compounds are 3,6,9,12-Tetraoxatetradecan-1-ol (**1**), Methyl salicylate (**2**), Diethyl Phthalate (**3**), and 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl hexasiloxane (**4**). The collected *Ziziphus Trinervia* Fruit phytoconstituents are recorded in Table-1 and the chromatogram is in Fig.-2.

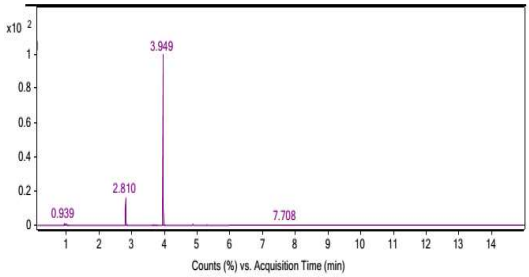


Fig.-2: GC–MS/MS Chromatogram of Ethanolic Extract of *Ziziphus Trinervia* Fruit

Table-1: Quantitative Phytoconstituents Identified from *Ziziphus Trinervia* Fruit Through the GC-MS Analysis

No	Retention Time (min.)	Relative content (%)	Formula	Metabolites	Molecular Weight
1	0.939	8.82	C10H22O5	3,6,9,12-Tetraoxatetradecan-1-ol	222.15
2	2.810	89.0	C8H8O3	Methyl salicylate	152.05
3	3.949	84.0	C12H14O4	Diethyl Phthalate	222.09
4	7.708	24.8	C12H38O5Si6	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl	430.13

Docking Analysis

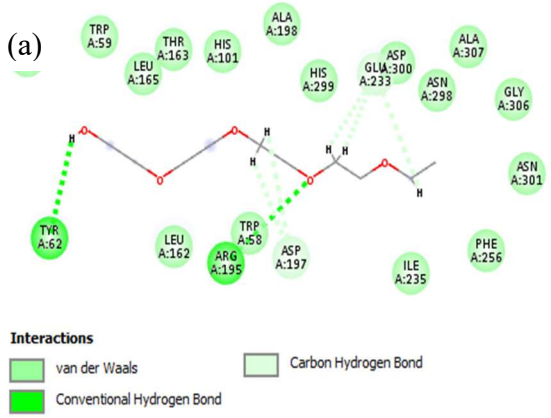
To reveal the binding modes of bioactive phytoconstituents (**1-4**), a docking simulation was performed with a crystal structure of α-amylase. Figs-3a and b show 2D and 3D interactions of inhibitor and phytoconstituents of *Ziziphus Trinervia* Fruit in the active site pocket of α-amylase protein. As seen from the 2D binding interactions of ligand **1**, the oxygen forms a hydrogen bond with ARG 195 and hydrogen in the hydroxyl groups forms an H-bond with TYR 62 in the active pocket. Further, the ligand interacts with amino acids namely ASP 300, GLU 233, and ASP 197 through a covalent hydrogen bond. Likewise, in the case of compound **2**, the hydrogen bond is observed between hydroxyl hydrogen and ASP197, and pi-alkyl interaction with LEU 162. The binding mode orientation of ligand **3** shows the formation of hydrogen bonds with ASN 298 and HIS 299 amino acids in the active pocket. Besides, the alkyl group in the compound forms pi-alkyl interaction with HIS 201 and LEU 162. The bioactive ligand **4** is the most active compound (-8.40 kcal/mol) in this series. The binding mode shows a good number of pi-alkyl interactions with amino acids such as HIS 305, TRP 58, TRP 59, THR 163, LEU 165, ALA 307, ILE 235, and HIS 299. Compound **4** showed Vander Waals interactions near amino acids in the protein structure. The binding interaction of standard Acarbose showed hydrogen bonds with ALA 307, ASN 298, GLU 233, and HIS 299 in the active pocket of the α-amylase protein. Further, it forms pi-alkyl interaction with LEU 165 and TRP 59 amino acid residue. When we compare the binding energy results of the ligand series with the standard drug, phytoconstituent **4** showed the closest value to the standard drug.

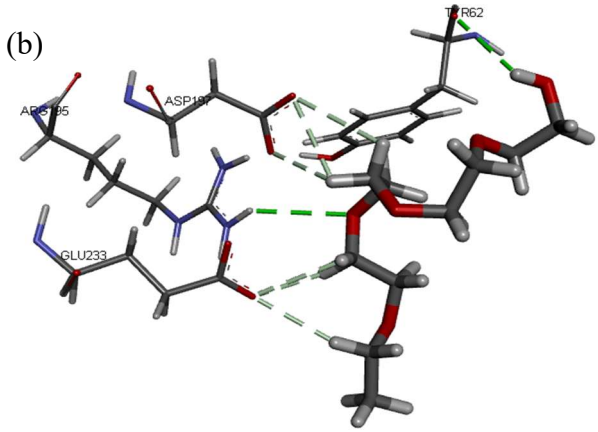
Drug-Likeness Properties Analysis

The drug-likeness properties of bioactive compounds **1-4** were assessed by Pfier's rule (or Lipinski's rule). This investigation is based on the structure of the drug material and it is an initial standard to measure the structural resemblance of a perfect drug.[19]

Table-2: Docking Results of Phytoconstituents and Acarbose onto the Active Site α-amylase Protein

Ligand No	Binding energy (kcal/mol)	Interactions	H-bond
1	-6.08	ASP 300; GLU 233; ASP 197	TYR 62; ARG 195
2	-4.61	TYR 62; LEU 165; HIS 299	ASP 197
3	-5.47	HIS 201; LEU 162; ASP 197	ASN 298; HIS 299
4	-8.4	HIS 305; TRP 58; TRP 59; THR 163; LEU 165; ALA 307; ILE 235; HIS 299	
Acarbose	-8.65	GLY 104; THR 163; LEU 165; TRP 59	ALA 307; ASN 298; GLU 233; HIS 299





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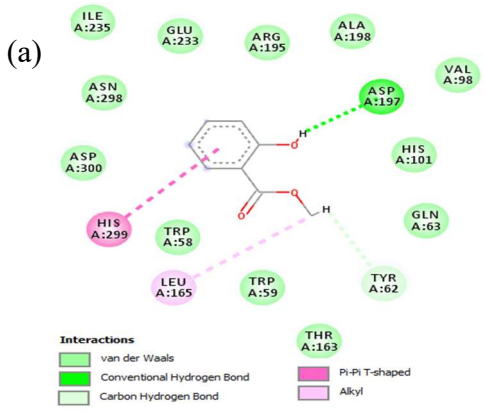

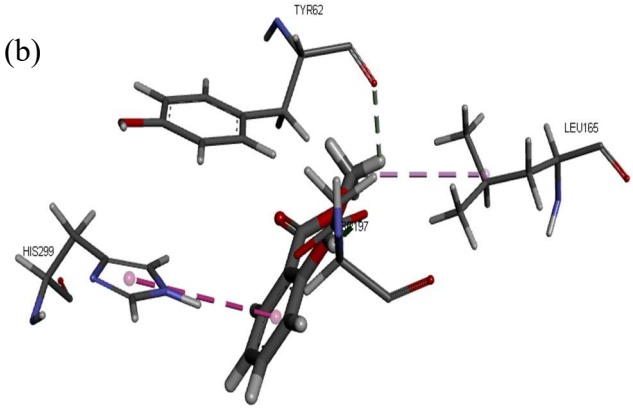

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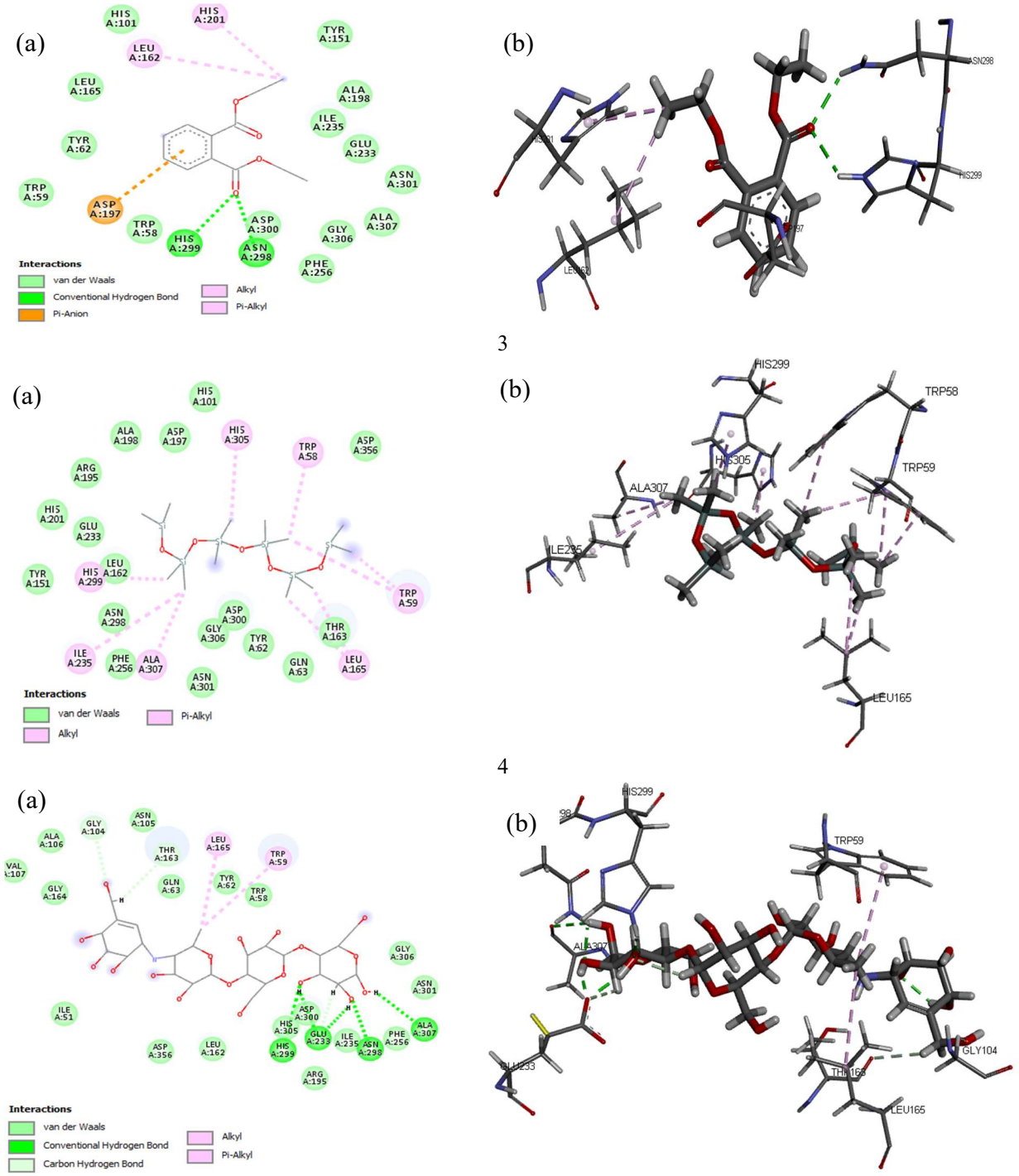

Acarbose

Fig.-3: Binding Pose, (a) 2D and (b) 3D Interactions of Inhibitor and Phytoconstituents of *Ziziphus trinervia* Fruit in the Active Site Pocket of the α-amylase Protein

However, it is not compulsory for a drug to follow every rule to be a potential drug applicant. It appears that your study aligns with the proposition by Bickerton *et al.*[20], suggesting that oral bioavailability may not necessarily impact the bioactivity or pharmacological potencies of drug candidates. This observation implies that the selected ligands (**1-4**) exhibited favorable structural properties, making them suitable candidates for drug development. These structural properties likely contribute to their potential as ideal drugs.

Table-3: Drug-Likeness Properties of Phytoconstituents

Ligand	MM	Log p	HBA	HBD	Molar refractivity	TPSA
1	222.28	0.61	5	1	55.69	57.15
2	152.15	1.66	3	1	39.74	46.53
3	222.24	2.3	4	0	58.61	52.6
4	430.94	1.82	5	0	113.11	46.15

Pharmacokinetic Profile Analysis

The pharmacokinetic properties inspection of a drug compound is defined based on its ADMET properties. ADMET study is remarkably beneficial in the initial phase of drug discovery to enable a significant reduction of clinical trial disappointments.[18] Water solubility, GI absorption, skin permeability, and $CaCo_2$ permeability were assessed. Compounds **1, 3**, and **4** exhibited good absorbance rates, with compound **3** showing the highest absorption percentage (96.25%). Compound **2** showed a moderate absorption percentage (79.19%). VDss, CNS permeability, and BBB membrane permeability were examined.[21] The bioactive compounds showed distribution volumes lower than expected values, indicating relatively low distribution (Table-4). For BBB membrane penetration, all compounds had log BB values > 0.3, suggesting potential passage through the BBB membrane. For CNS penetration, the log PS values were in the range of > -2 to < -3, indicating impenetrability. CYP450 metabolism scores were analyzed, reflecting the role of CYP450 in drug metabolism in the liver system.[22] Total drug clearance, a combination of hepatic and renal clearance, ranged from -0.63 to 1.11 mL/min/kg.[23] The absence of skin allergic reactions and hepatotoxic effects was noted for all analyzed compounds. hERG inhibition (I and II) was examined for cardiotoxicity, and none of the compounds exhibited inhibitory actions. Compound **2** did not show AMES toxicity, while others exhibited AMES toxicity. Predicted scores for LD50, LOAEL, and maximum tolerated dosage indicated no significant toxicity concerns. Based on the comprehensive ADMET analysis, it was concluded that the identified phytoconstituents (compounds **1-4**) could be potential α-amylase inhibitor drugs.

Table-4: Pharmacokinetics, Toxicities, and Receptor Binding Properties of Phytoconstituents using pkCSM Web server

	Ligand No	**1**	**2**	**3**	**4**	Unit
Absorption	Water solubility	-0.74	-0.32	-1.75	-3.95	Numeric (log mol/L)
	Caco2 permeability	1.35	1.24	1.31	1.08	Numeric (log mol/L)
	Intestinal absorption (Human)	95.19	79.19	96.25	91.11	Numeric (% Absorbed)
	Skin Permeability	-3.60	-2.48	-2.63	-2.18	Numeric (log Kp)
	P-glycoprotein substrate	No	No	No	No	Categorical (Yes/No)
	P-glycoprotein I inhibitor	No	No	No	Yes	Categorical (Yes/No)
	P-glycoprotein II inhibitor	No	No	No	No	Categorical (Yes/No)
Distribution	VDss (human)	-0.30	-0.25	-0.24	-0.48	Numeric (log L/kg)
	Fraction unbound (human)	0.79	0.45	0.34	0.47	Numeric (Fu)
	BBB permeability	-0.092	-0.19	0.025	-0.97	Numeric (log BB)
	CNS Permeability	-3.18	-2.16	-2.43	-3.97	Numeric (log PS)
Metabolism	CYP2D6 Substrate	No	No	No	No	Categorical (Yes/No)
	CYP3A4 Substrate	No	No	No	No	Categorical (Yes/No)
	CYP1A2 inhibitor	No	No	Yes	No	Categorical (Yes/No)
	CYP2C19 inhibitor	No	No	No	Yes	Categorical (Yes/No)
	CYP2C9 inhibitior	No	No	No	No	Categorical (Yes/No)
	CYP2D6 inhibitior	No	No	No	No	Categorical (Yes/No)
	CYP3A4 inhibitior	No	No	No	No	Categorical (Yes/No)
Excretion	Total Clearance	1.11	0.63	0.81	0.94	Numeric (log ml/min/kg)
	Renal OCT2 substrate	No	No	No	No	Categorical (Yes/No)
Toxicity	Max. tolerated dose (human)	0.79	1.03	1.07	0.19	Numeric (log mg/kg/day)

	hERG I inhibitor	No	No	No	No	Categorical (Yes/No)
	hERG II inhibitor	No	No	No	No	Categorical (Yes/No)
	Oral Rat Acute Toxicity (LD50)	1.64	1.86	2.28	3.65	Numeric (mol/kg)
	Oral Rat Chronic Toxicity (LOAEL)	0.80	2.64	2.52	0.55	Numeric (log mg/kg_bw/day)
	Hepatotoxicity	No	No	No	No	Categorical (Yes/No)
	Skin Sensitisation	No	No	No	No	Categorical (Yes/No)

CONCLUSION

GC-MS analysis in ethanol extract of *Ziziphus Trinervia* Fruit identified peaks indicating the presence of phytochemical constituents such as 3,6,9,12-Tetraoxatetradecan-1-ol (**1**), Methyl salicylate (**2**), Diethyl Phthalate (**3**) and 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl hexasiloxane (**4**) Also, a docking investigation was carried out to find the binding affinities and the possible interactions of the ligand-protein complexes. The assumed α-amylase inhibitory metabolites displayed moderate (-4.61 kcal/mol) to high affinities (-8.4 kcal/mol) toward the active Pocket. Finally, this study demonstrated that metabolite **4** showed comparable binding energies with standard drugs. Drug-likeness and ADEMT score of bioactive compounds were evaluated by SWISS-ADME, and pkCSM online servers, respectively. Overall, the *in-silico* results confirmed that they could be used as s promising α-amylase inhibitors. We believe that the insights gained from the *in-silico* study may be of great value for the discovery and development of the novel α-amylase inhibitor drug.

ACKNOWLEDGMENTS

The authors are very much thankful to the management authorities of Sri Parasakthi College for Women (Autonomous), Courtallam, Tamilnadu for their cooperation in providing essential research facilities.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing, and approved the final draft for publication. The research profile of the authors can be verified from their ORCID IDs, given below:

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