Impact on cardioprotective effect of *Psidium guajava* leaves extract in streptozotocin-induced Wistar mice with molecular *in silico* analysis

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RESEARCH ARTICLE

Abstract

Cardiovascular disease (CVD) and its complications have been regarded as the leading cause of morbidity and mortality. The drugs available in the market are effective to treat CVD, but with many adverse reactions. Nowadays, herbal products are the attention of researchers because of their less adverse effects. In this study, the cardioprotective effects of ethanolic leaves extract of *Psidium guajava* Linn. (Guava) (*P. guajava*) were evaluated in streptozotocin (STZ)-treated animal models. Mice acquired for the study were divided into five groups, each consisting of six mice. The toxin-induced mice were treated with the ethanolic leaves extract of *P. guajava* (300 mg/kg body weight [b.w.]). The results were compared to the standard drug (glibenclamide)-treated mice (3 mg/kg b.w.). The following parameters were considered for further investigations: creatine kinase-muscle brain (CK-MB), creatine kinase (CK), troponin, lysosomal, and mitochondrial enzymes. Then the docking study was accomplished. The levels of cardiac marker enzymes and lysosomal enzymes increased significantly in the toxin-induced mice, while the level of mitochondrial enzyme decreased significantly. During treatment with the ethanolic leaves extract of *P. guajava,* the levels of all parameters were notably reversed to normal range ($P < 0.05$). Further, in docking analysis, the interaction of compounds, such as alpha-terpineol, cyclopentanecarboxamide, guaiol (a sesquiterpenoid alcohol), 1H-cyclopropanaphthalene, tetracyclotridecan-9-ol, dormin/abscisic acid, and epiglobulol, with the respective protein molecules, evidenced the cardioprotective effect of *P. guajava* leaves. Hence, it was concluded that the ethanolic leaves extract of *P. guajava* leaves have a cardioprotective effect.

Keywords: *Psidium guajava*; lysosomal enzymes; mitochondrial enzymes; cardiac markers

Introduction

India is known as the global capital of diabetes mellitus. Diabetes mellitus causes several secondary disorders; CVD is one of the diabetes-related disorders. The end products of glycation make for an important role in the cardiac disorder associated with diabetes (Buynes and Thorpe, 2000). According to the World Health Organization (WHO), an estimated 17.3 million individuals died due to CVD in 2008, and it may increase to 23 million by 2030. In India in 1990, nearly 2.6 million people died due to CVD (Kumar *et al*., 2011; Sivakumar and Dhanarajan, 2001). CVD is a substantial health burden with an ever-increasing prevalence, and has remained the foremost reason for morbidity and mortality worldwide. Some allopathic drugs, such as aspirin, anti-arrhythmic drugs, and receptor blocker, are available for myocardial infarction, but these drugs are quite expensive and have a number of adverse reactions (Vishal *et al*., 2010, Xie *et al.,* 2022).

Nowadays, scientists are exploring to develop drugs from plants because of their positive effects to treat diseases (Panche *et al.,* 2016). Medicinal plant *Psidium guajava* Linn (guava; *P. guajava*) belongs to family Myrtaceae. *P. guajava*, a tropical plant, is used globally as a food as well as medicine because of its various medicinal properties. Although *P. guajava* has a variety of medicinal benefits, it is the most common and classic therapy for gastrointestinal infections, including diarrhea, dysentery, stomach ache, and indigestion (Gutiérrez *et al.*, 2008).

P. guajava is cultivated throughout India, mostly in the states of Andhra Pradesh, Assam, Bihar, Maharashtra, Uttar Pradesh, and West Bengal (Daswani *et al.*, 2017). Leaves of the plant contain a large number of phytoconstituents, such as alkaloids, terpenoids, phenols, flavonoids, and ethanol extracts (Manikandan *et al*., 2013, 2016; Manikandan and Vijaya Anand, 2015). Zakaria and Mohd (1994) established that the leaves of *P. guajava* contained caryophyllene, alpha-pinene, quercetin, and limonene compounds. Its leaves are used to study medicinal properties in the treatment of malaria (Gessler *et al*., 1994), diarrhea (Lutterodt *et al*., 1992), diabetes (Manikandan *et al*., 2018), and bacterial diseases (Canceras *et al*., 1993). Therefore, the present investigation intended to find changes in cardiac marker, lysosomal, and mitochondrial enzymes in the STZ)-induced and *P. guajava* leaves' extract-treated mice with *in silico* analysis. The analysis was done to analyze the binding affinity of phytoconstituents of *P. guajava* leaves with marker enzymes.

Materials and Methods

Source of chemicals

All the chemicals and reagents used were of higher analytical grade required for the assays and were obtained from Sigma-Aldrich (Bangalore, India), unless otherwise noted specifically. The biochemical analysis was done with the help of the autoanalyzer Turbochem 100 and the reagents as well as calibrators were obtained from iChem™ 100.

Extract preparation

Fresh leaves of *P. guajava* were obtained from Tiruchirappalli, Tamil Nadu, India, and authenticated and deposited at Rapinat Herbarium, St. Joseph College, Tiruchirappalli, India. The leaves were shade-dried, powdered, and stored till further use. Extract of *P. guajava* leaves was obtained by the hot percolation method using Soxhlet apparatus, with ethanol used as a solvent.

Animals

In the present investigation, male albino mice, aged 6–8 weeks and weighing approximately 150–180 g, were used as the study model. The mice were kept in clean and dry plastic cages. All the animals were fed with a commercially available standard diet (Sai Durga Feeds and Foods, Bengaluru, India). The study was conducted at Srimad Andavan Arts and Science College (SAASC), Tiruchirappalli, Tamil Nadu, India, and ethical clearance to conduct the study with mice model was obtained from the Institutional Animal Ethics Committee, SAASC, Tiruchirappalli, India. (CPCSEA approval No. 790/03/ac/CPCSEA).

The animals were split into the following five groups, G1–G5, each with six mice: Control G1 group was given a standard diet and saline water; negative control G2 group: diabetic mice induced with STZ (60 mg/kg body weight [b.w.]); G3 group was treated with 300-mg/kg b.w. of ethanolic leaves extract of *P. guajava*; G4 group comprised G2 mice treated with 300-mg/kg b.w. of ethanolic leaves extract of *P. guajava*; and G5 group comprised G2 mice treated with glibenclamide, 3-mg/kg b.w., for 45 days. The animals were fasted overnight (for about 12–18 h) on the last day of the experiment prior to the induction of anaesthesia and collection of blood samples. The animals were sacrificed at the end of experimental period by cervical dislocation under diethyl ether anesthesia. Blood samples were collected from the heart of animals for different biochemical assays. All biochemical parameters were quantified and determined by using commercial kits.

Biochemical analysis

In this study, we evaluated cardiac marker, lysosomal, and mitochondrial enzymes. The cardiac marker enzymes

included were CK-MB (Apple *et al*., 1988), CK (Okinaka *et al*., 1961), and troponin (Burtis and Ashwood, 1996). The lysosomal enzymes included were acid phosphatase (ACP; King, 1965), β-D-glucuronidase (Kawai and Anno, 1971), β-N-acetyl glucosaminidase (Moore and Morris, 1982), cathepsin D (Sapolsky *et al*., 1973); and mitochondrial enzymes evaluated were isocitrate dehydrogenase (ICH; Bell and Baron, 1960), α-ketoglutarate dehydrogenase (KDH; Reed and Mukherjee, 1969), succinate dehydrogenase (SDH; Slater and Bonner, 1952), malate dehydrogenase (MDH; Mehler *et al*., 1948), nicotinamide adenine dinucleotide (NAD)+hydrogen (H) (NADH) dehydrogenase (Tsoo *et al*., 1967), and cytochrome-C-oxidase (Pearl *et al*., 1963).

Molecular computational analysis

Targets selection

The Protein Data Bank (PDB) was used for retrieving the three dimensional structure of cardiac markers were CK-MB (PDB ID 3B6R), CK (PDB ID 1I0E), and troponin (PDB ID 1J1D). Similarly, for evaluation of lysosomal enzymes were ACP (PDB ID 1RPA), β-D-glucuro-nidase (PDB ID 6LEJ), β-N-acetyl glucosaminidase (PDB ID4GVF), and cathepsin D (PDB ID 4OBZ). Same as for evaluation of mitochondrial enzymes were ICH (PDB ID 3LC6), KDH (PDB ID 7WGR), SDH (PDB ID 1NEK), MDH (PDB ID 2PWZ), NADH dehydrogenase (PDB ID 5XTC), and cytochrome-C-oxidase(P DB ID 5Z62).

Protein pre-processing

All the selected target proteins were imported by the maestro platform of the Schrodinger software, using appropriate PDB Id and the protein preparation wizard module. The retrieved three-dimensional (3D) structures were pre-processed by following addition of hydrogen, zero-order bonds to metals, converting selenomethionine (SeMet) to methionine (Met), creating disulfide bonds, and filling in missing side chains. In addition, inhibitors were removed, H-bonds were optimized, and energy minimization was applied by using the OPLS4 force field.

Ligand preparation

Previously we found GC-MS analysis was used to determine 21 phytoconstituents in ethanolic leaves extract of *P. guajava* (Manikandan *et al.*, 2018). The PubChem database 3D structures were obtained in the structure data file (SDF) format. In addition, the LigPrep module was applied to refine the structure. In all, 32 various states of stereoisomerism were obtained after applying the OPLS4 force field (Schrödinger software 2021-2: LigPrep, Schrödinger, LLC, New York, NY, 2021).

Phytoconstituents in ethanolic leaves extract of *P. guajava* were investigated for molecular docking analysis using the "extra precision" (XP) mode of Glide (a ligand docking program for predicting protein-ligand binding modes) module. A pose viewer was used to investigate the docked ligand interaction with protein for ideal conformation. Both protein and ligand complex interaction modules generated 2D interactions (Leslie *et al*., 2021).

Statistical analysis

The present investigation was conducted by using one-way analysis of variance (ANOVA). Statistical testing was done using Statistical Package of Social Science (SPSS) version 14.0 for Windows. The data were represented as mean ± standard deviation (SD), and $P < 0.05$ was considered statistically significant.

Results

Based on the previous results, the phytoconstituents from *P. guajava* were docked with targeted proteins. The top interacting molecules were highlighted among the docked 21 phytoconstituents. The levels of cardiac markers, such as CK, CK-MB, and troponin, are shown in Table 1. These markers were notably increased in the STZ-treated diabetic animals, compared to the control group. However, the increased markers reverted to normal levels following the treatment with ethanolic extract of *P. guajava* leaves, compared to the standard drug-treated and control groups. No notable changes were observed in the plant extract-treated mice group. Table 2 shows the molecular interaction of cardiac target proteins with *P. guajava* phytoconstituents. The top compounds were bound at the same location and their 2D interactive structures were observed (Figure 1). Further, the molecular interactions are presented in Table 2. Among cardiac target proteins, PG9, PG15, and PG19 were observed as being the top-ranked molecules.

The results of ACP, cathepsin D, β-D-glucuronidase, and β-N-acetyl glucosaminidase in control group, toxin-treated group, and ethanolic leaves extract of *P. guajava* group are shown in Table 3. Compared to the control group, the concentration of lysosomal enzymes was augmented in the toxin-induced diabetic mice but decreased in the ethanolic leaves extract of *P. guajava* group. No notable changes were observed in the plant alone-treated group.

Among 21 phytoconstituents of *P. guajava* docked with target proteins, the 3D interaction of five compounds is shown in Figure 2. Figure 3 shows the 3D interaction of compounds with specific ligands. We observed that all top five compounds were docked in the same region.

Table 1. Phytochemicals profiling of *P. guajava* using GC-MS analysis.

Compounds ID	Compound name	Pub Chem ID
PG1	Hydroquinone	785
PG2	Naphthalene	931
PG3	Eucalyptol	2758
PG4	Azulene	9231
PG5	Alpha-terpineol	17100
PG6	Alloaromadendrene	91354
PG7	Cyclopentanecarboxamide	226274
PG8	Guaiol	227829
PG9	1H-Cyclopropanaphthalene	318639
PG10	Farnesol	445070
PG11	Tetracyclotridecan-9-ol	585744
PG12	Caryophyllene	5281515
PG13	Humulene	5281520
PG14	Cis-alpha-bisabolene	5352653
PG15	Dormin/abscisic acid	5375199
PG16	Gamma-muurolene	6432308
PG17	1,6,10-Dodecatrien-3-ol	6436889
PG18	Beta-bisabolene	10104370
PG19	Epiglobulol	11858788
PG20	4-Isopropyl-1,6-dimethyl-1,2,3,4 tetrahydronaphthalene	12302242
PG21	Copane	12303908

Further, the molecular interaction was studied for top three compounds as shown in Table 4. Among lysosomal-targeted proteins, PG5, PG7, PG9, PG11, and PG15 were observed as the top-ranked molecules.

The levels of ICH, KDH, SDH, MDH, NADH dehydrogenase, and cytochrome-C-oxidase in the study groups are shown in Table 5. Compared to the control group, the enzymes levels of tricarboxylic acid cycle (TCA), including ICH, KDH, SDH, and MDH, as well as respiratory enzymes, including the levels of NADH-dehydrogenase and cytochrome-C-oxidase, were significantly decreased in the toxin-induced group. However, the levels were significantly increased to the normal range following treatment with ethanolic extract of leaves of *P. guajava*. No change was observed in the plant extract-treated group.

Among 21 phytoconstituents of *P. guajava* docked with target proteins, the top-ranked compounds for 3D interaction are shown in Figure 4. All compounds were docked in the same region. Further, molecular interaction was studied, which is given in Table 6. Among mitochondrial-targeted proteins, PG5, PG7, PG9, and PG8 were observed as the top-ranked molecules. Figure 5 presents the interaction of the binding of ligands to specific phytoconstituents determined in *P. guajava* leaves extract.

Table 2. Quantity of cardiac marker enzymes creatine kinase-myoglobin binding (CK-MB), creatine kinase (CK), and troponin in an animal model.

Parameters/groups	G1	G2	G3	G4	G5
CK-MB ((IU/L)	0.87 ± 0.01^{a}	3.63 ± 0.62^{b}	0.84 ± 0.02^{a}	0.93 ± 0.05^{c}	$0.90\pm0.03^{a,c}$
CK (U/L)	35.62 ± 0.87^{a}	98.42 ± 0.65^{b}	35.61 ± 0.79^{a}	40.25 ± 0.65^{c}	$37.12\pm0.32^{a,c}$
Troponin (ng/L)	0.58 ± 0.03^{a}	1.95 ± 0.08^{b}	0.57 ± 0.02^{a}	0.62 ± 0.03^{c}	0.61 ± 0.02^{c}

Values are given as mean ± SD of five experimental groups for each rat.
Values marked with superscript $^{a, b, c}$ differ significantly at $P \leq 0.05$ (Duncan's multiple range test [DMRT]).

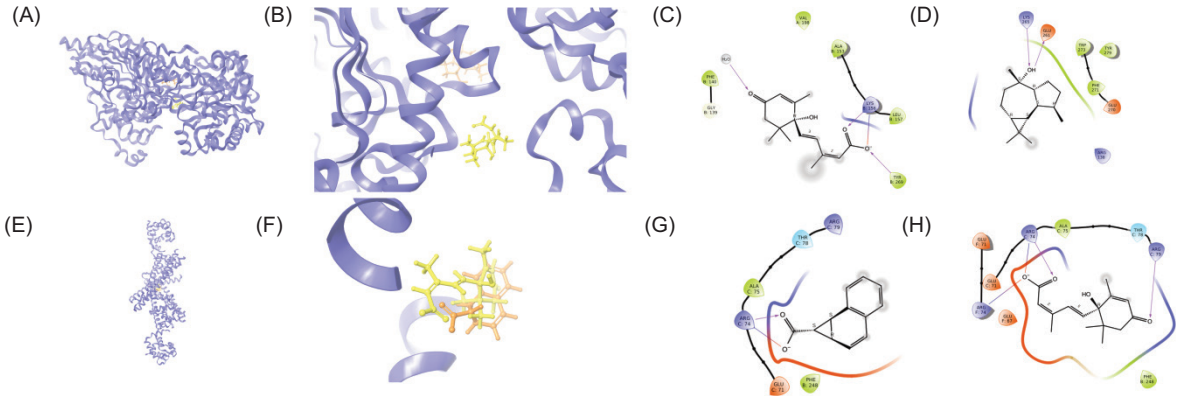

Figure 1. The 21 phytochemicals extracted from *P. guajava* leaves were docked with cardiac marker target proteins. The three-dimensional docked complex structure of (A,B) creatine kinase (CK) from the brain, and (E,F) troponin. The two-dimensional structure of CK from the brain with (C) PG15 and (D) PG19; and that of troponin with (G) PG9 and (H) PG15.

Table 3. Molecular interaction of cardiac marker enzymes with *P. guajava* phytochemicals.

Target/ Compounds ID	Compounds Pubchem ID	Amino acid interaction (3-letter code)	Bond length (Å)	Glide score (kcal/mol)
Creatine kinase from brain				
PG15	5375199	ARG74, ARG79	(2.17, 3.61, 1.84, 3.06, 2.26) (2.06, 2.16)	–2.609
PG9	318639	ARG74	(1.68, 1.80)	–2.46
Troponin				
PG15	5375199	TYR269, LYS156,	1.80 (1.85, 4.02)	–2.868
PG19	11858788	GLU261, LYS265	1.58, 1.91	–2.772

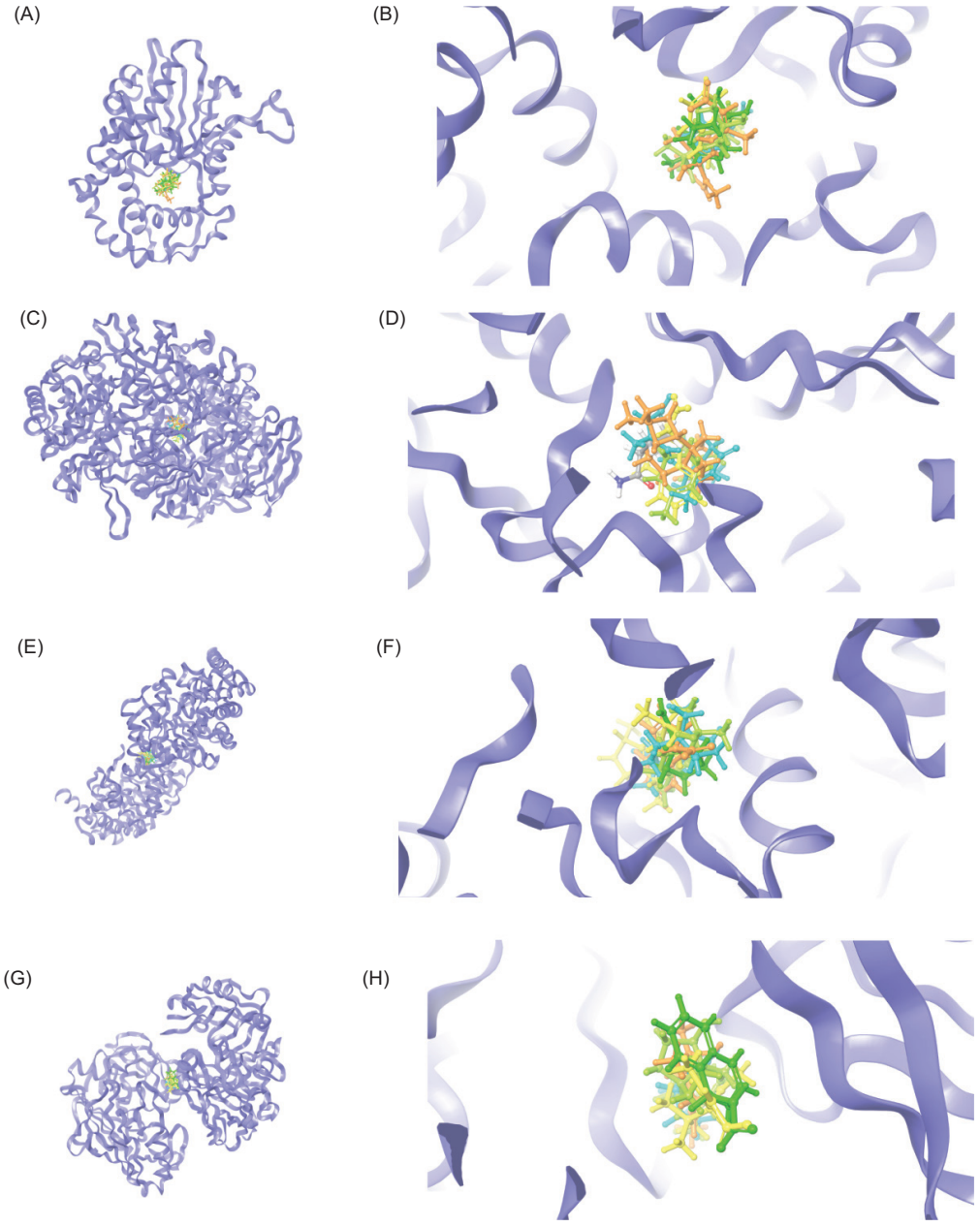

Figure 2. The 21 phytochemicals extracted from *P. guajava* leaves were docked with lysosomal target proteins. The three-dimensional docked complex structure of (A,B) acid phosphatase (ACP), (C,D) β-D-glucuronidase, (E,F) β-N-acetyl glucosaminidase, and (G,H) cathepsin D.

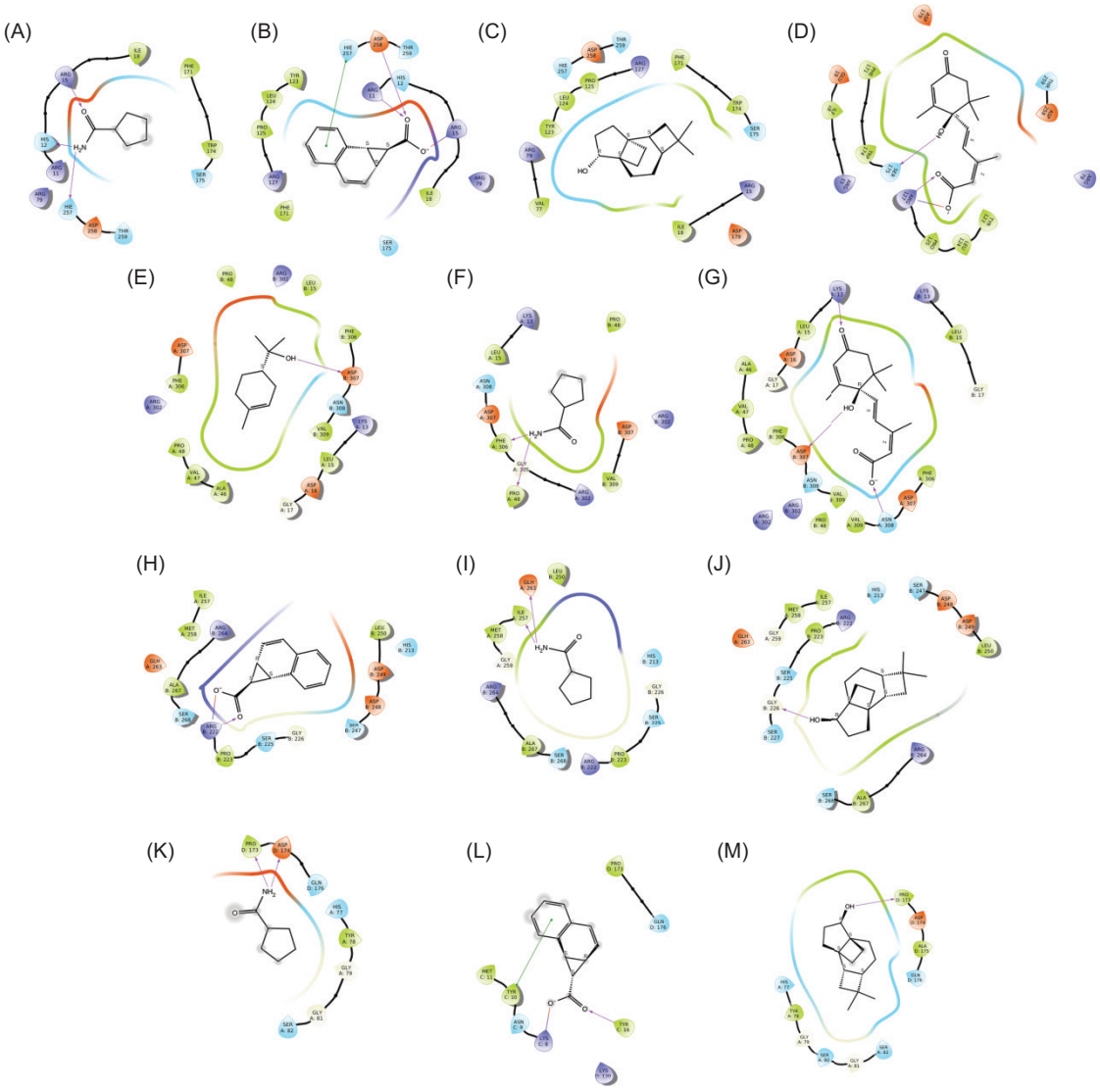

Figure 3. The two-dimensional structure of acid phosphatase (ACP) with (A) PG7, (B) PG9, (C) PG11, and (D) PG15; that of β-D-glucuronidase with (E) PG5, (F) PG7, and (G) PG15; that of β-N-acetyl glucosaminidase with (H) PG9, (I) PG7, and (J) PG11; and that of cathepsin D with (K) PG7, (L) PG9, and (M) PG11.

Table 4. Effect of *P. guajava* leaves extract on acid phosphatase (ACP), cathepsin D, β-D-glucoronidase, and β-N-acetyl glucosaminidase proteins in control and STZ-induced rat models.

Parameters/groups	G1	G2	G3	G4	G5
ACP	125.36±1.09^{a}	173.31±0.98^{b}	124.52±1.03^{a}	136.42±1.10^{c}	130.53±1.68^{a,c}
Cathepsin D	23.21±1.02^{a}	48.73±1.72^{b}	24.35±0.98^{a}	29.65±1.15^{c}	27.67±1.27^{c}
β-D-glucuronidase	22.36±0.54^{a}	36.23±0.73^{b}	22.72±0.65^{a}	26.45±0.92^{c}	25.32±0.75^{c}
β-N-acetyl glucosaminidase	48.81±1.16^{a}	75.64±1.92^{b}	48.12±1.65^{a}	52.65±0.68^{c}	51.43±0.79^{a,c}

The effect of *P. guajava* leaves extract on lysosomal enzymes ACP, cathepsin D, β-D-glucuronidase, and β-N-acetyl glucosaminidase are expressed in µmol/h/100 mg.
Values are given as mean ± SD of five experimental groups, each with six mice.
Values marked with superscript $^{a, b, c}$ differ significantly at P ≤ 0.05 (DMRT).

Table 5. Molecular interaction of lysosomal target proteins with *P. guajava* phytochemicals.

Target/ Compounds ID	Compounds Pubchem ID	Amino acid interaction (3-letter code)	Bond length (Å)	Glide score (kcal/mol)
Acid phosphatase (ACP)				
PG15	5375199	SER175, ARG127	1.88 (1.81, 1.85, 3.32)	–5.118
PG9	318639	LEU124, ARG11, ASP258, ARG15	2.71, 2.41, 2.05 (1.84, 2.09, 2.76)	–4.052
PG7	226274	ARG15, HIE257, HIS12	1.81, 2.01, 1.93	–3.403
β-D-glucuronidase				
PG15	5375199	ASN308, LYS13, ASP307	1.90, 1.93, 1.66	–4.884
PG7	226274	PHE306, PRO48	1.70, 2.08	–3.958
PG5	17100	ASP307	2.02	–3.851
β-N-acetyl glucosaminidase				
PG9	318639	ARG222, SER268	(1.61, 1.96, 4.78), 2.79	–5.639
PG11	585744	GLY226	2.20	–4.54
PG7	226274	GLH263, ILE257	1.95, 2.00	–4.337
Cathepsin D				
PG7	226274	PRO173, ASP174	2.02, 2.22	–3.187
PG9	318639	LYS8, TYR16	(1.89, 2.85), 1.84	–2.884
PG11	585744	PRO173	1.93	–2.713

Discussion

Extract of *Psidium guajava* leaves

The extract of *P. guajava* leaves was obtained with ethanol, as it has a polarity index of 5.2 and is safer and found to have advantages over other organic solvents (Hikmawanti *et al.,* 2021). Ethanol is considered as a suitable solvent for recovering most of phytoconstituents and extraction of antioxidant components and foods derived from plants (Sultana *et al.,* 2009). In our previous study, extraction done with ethanol demonstrated to have more phytoconstituents than other extraction solvents, such as aqueous chloroform, petroleum, ether, and hexane (Manikandan *et al.*, 2016).

Effect of cardiac markers

The CK-MB has a vital role in identifying patients with myocardial infarction and other cardiovascular complications. The CK-MB enzyme is present in the myocardial cells of the heart. In myocardial infarction, cells of the myocardium are damaged due to the toxin, which releases CK-MB in the bloodstream. The present study is focussed on the STZ-induced diabetes and its effect on the myocardial cells of the heart. A previous study conducted by Seager *et al*. (1984) proved that myocardial complications happen in STZ-treated chronic diabetic animals. Findings of the present study also demonstrate the same results. The administration of ethanolic extract of *P. guajava* leaves revealed a considerable reduction in glucose and lipid peroxidation levels. The treatment also increased significantly the levels of glutathione, glutathione peroxidase, superoxide dismutase, and catalase in the liver, compared to the levels in diabetic mice (Manikandan *et al.*, 2016; Sinha, 1972).

Following the treatment with ethanolic extract of *P. guajava* leaves, the increase in chemical compounds was reversed to normal levels. This happened because the leaves extract restored myocardial cell damage and lead to decrease in CK-MB release in the bloodstream.

A similar effect was observed in the doxorubicin-induced mice treated with *Grewia unbellifera* (family Malvaceae) and *Gmelina arborea* or gamhar (family Lamiaceae) (Arafa *et al*., 2014). In myocardial infarction, the level of CK enzyme is increased in circulation (Okinada *et al*., 1961). In the current study, STZ induced myocardial damage and favored the release of CK in the blood. Following the treatment with ethanolic extract of *P. guajava* leaves, the level of CK decreased in the blood, because the secondary metabolites found in the leaves regenerated myocardial cells and prevented the release of CK enzyme.

In the control group, no significant changes were observed in mice when treated with the plant extract. In recent years, death of cardiac cells is determined by using the serum troponin level. In animals, the increased concentration of troponin indicates myocardial infarction (O'Brien *et al*., 2006). This was also proved in the present investigation, wherein the level of troponin was increased in the blood due to the toxin STZ-induced myocardial infarction. However, treatment with *P. guajava* leaves extract reduced the level of troponin in the blood.

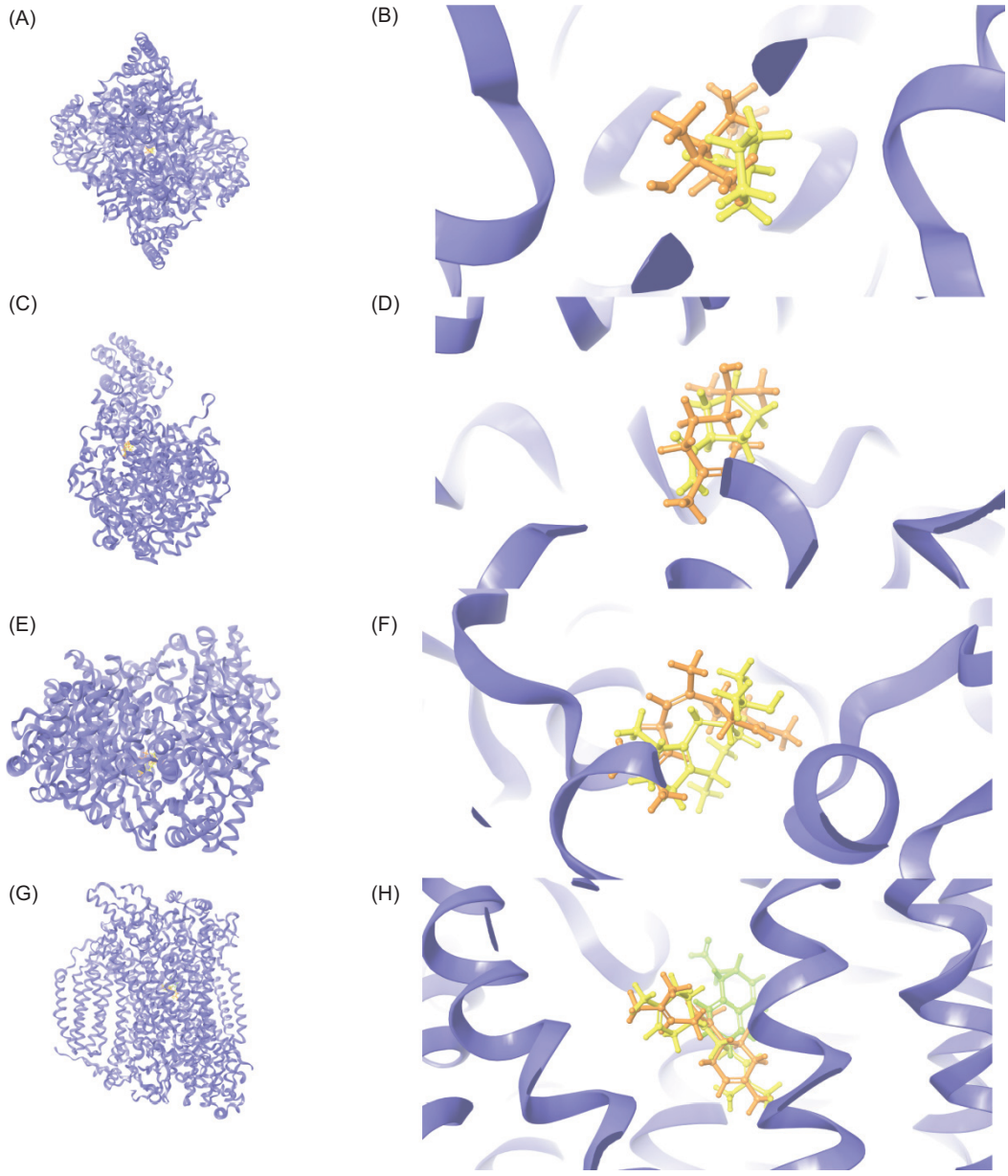

Figure 4. The 21 phytochemicals extracted from *P. guajava* leaves were docked with mitochondrial target proteins. The three-dimensional docked complex structure of (A,B) α-ketoglutarate dehydrogenase, (C,D) succinate dehydrogenase, (E,F) malate dehydrogenase, and (G,H) cytochrome-C-oxidase.

This effect was due to the prevention of leakage of troponin enzyme from myocardial cells. Significant changes were observed, compared to the negative control group. Vijayakumar *et al.* (2018) demonstrated that the leaves extract of *P. guajava* and its isolated quercetin fraction significantly controlled the lipid profile, which is one of the major risk factors for CVD pathogenesis in carbon tetrachloride-toxicated mice.

Phytoconstituents have been widely and effectively used in the treatment of various diseases, and the phytophysical properties of these compounds have been extensively studied and followed by *in silico* studies against diabetic targets. In the present investigation, phytoconstituents, such as abscisic acid, cyclopropanaphthalene, and epiglobulol, showed affinity toward CK and troponin. The abscisic acid and cyclopronaphthalene compounds adhered to CK in brain proteins and epiglobulol, and abscisic acid adhered to troponin. The target phytochemical compounds are evaluated with the binding affinity with the cardiac markers and further can be selected as hits.

Table 6. Effect of the ethanolic extract of *P. guajava* leaves on ICH, KDH, SDH, MDH, NADH, and cytochrome oxidase in a rat model.

Parameters/groups	G1	G2	G3	G4	G5
Isocitrate dehydrogenase (ICH)	612.31±16.72[a]	201.43±20.32[b]	613.46±21.32[a]	602.63±17.65[c]	604.89±20.23[c]
Alpha-keto dehydrogenase (KDH)	141.62±9.62[a]	90.43±8.82[b]	140.87±10.36[a]	134.78±9.61[c]	136.31±11.62[a,c]
Mitochondrial succinate dehydrogenase (SDH)	215.31±16.81[a]	107.61±15.4[b]	216.68±11.32[a]	200.68±10.56[c]	201.73±9.65[c]
Mitochondrial malate dehydrogenase (MDH)	299.61±11.83[a]	170.78±10.53[b]	300.60±10.82[a]	278.72±19.61[c]	288.63±10.57[c]
Nicotinamide adenine dinucleotide (NADH) dehydrogenase	32.36±1.16[a]	15.32±0.95[b]	32.01±1.29[a]	29.31±0.81[c]	30.37±1.57[c]
Cytochrome-C-oxidase	7.25±0.61[a]	3.82±0.35[b]	7.01±0.26[a]	6.71±0.54[c]	7.01±0.90[c]

The effect of *P. guajava* leaves extract on mitochondrial enzymes ICH, KDH, SDH, MDH, NADH, and cytochrome-C-oxidase are expressed in µmol/h/100 mg.
Values are in given as mean ± SD of five studied groups for each rat.
Values marked with superscript [a, b, c] differ significantly at P ≤ 0.05 (DMRT).

Effect on lysosomal enzymes

Acid phosphatise is one of the important lysosomal enzymes. Myocardial injury because of toxin causes the release of ACP enzyme from lysosome to cytosol (Decker and Wildentha, 1980). The release of ACP enzyme may cause cell injury and cell death because of the attacking of alternative pathway in cardiac cells (Hoffstein *et al.,* 1975). ACP level was increased in the present study because of necrosis in cardiac cells.

In diabetic condition, the renin-angiotensin-aldosterone system (RAAS) system leads to oxidative damage and activates necrosis (Frustaci *et al.,* 2000). This was observed in the present investigation. However, the level of ACP reverted to normal if treated with the plant leaves extract. The phytoconstituents present in the extract may protect cardiac cells from necrosis and reduces ACP level in the serum.

Karthikeyan *et al.* (2007) proved that the treatment with grape seeds reduced ACP level in the serum. This was proved in the present study too. The animal cells contain cathepsin D, a lysosomal enzyme (Sudharsan *et al.,* 2006). Cathepsin D may disturb the oxygen radical, which could lead to cardiac tissue damage. The level of cathepsin D is increased in STZ-induced mice, but treatment with the plant leaves extract may reverse the cathepsin D level to normal. The phytoconstituents may protect cellular membranes from damage and inhibit the release of lysosomal enzymes.

The free fatty acid level is increased in diabetes. These free fatty acids are deposited in the blood to reduce the cell membrane stability of cardiac cells. This damage caused to cellular membranes releases lysosomal enzymes, which include β-D-glucuronidase and β-N-acetyl glucosaminidase. In the STZ-induced mice, the levels of these two enzymes were significantly increased, compared to the control group. However, treatment with the plant leaves extract decreased the levels of these enzymes. This was due to the decreased concentration of free fatty acid by the plant leaves extract, which reduced injuries to the cellular membrane and subsequently decreased the levels of lysosomal enzymes.

The results of the present study concluded that compounds such as abscisic acid, cyclopropanaphthalene, and cyclopenanecarboxamide bind to ACP; compounds such as abscisic acid, cyclopentanecarboxamide, and alpha-terpineol bind to β-D-glucuronidase; compounds such as cyclopropanaphthalene, tetra cyclotridecan-9-ol, and cyclopentanecarboxamide bind to beta-N-acetyl glucosaminidase; and cathepsin-D binds to the compounds such as cyclopentanecarboxamide, cyclopropanaphthalene, and tetra cyclotridecan-9-ol. These compounds are found to have higher binding affinity and glide scores with respective lysosomal enzymes.

Effect on mitochondrial enzymes

Prolonged oxidative stress releases free radicals, which affect and alter the structure and functioning of mitochondrial membrane (Subashini and Sumathi, 2012). Normally, TCA cycle enzymes are found in the matrix of the mitochondria. Damage to the membrane may reduce ICH, KDH, SDH, and MDH levels. In this study, owing to increased oxidative stress in toxin-treated mice, the levels TCA cycle enzymes were reduced. Nevertheless, treatment with the plant leaves extract significantly increased the levels of TCA cycle enzymes because of the antioxidant disposition of *P. guajava*.

NADH dehydrogenase and cytochrome-C-oxidase enzymes are found in the inner membranes of

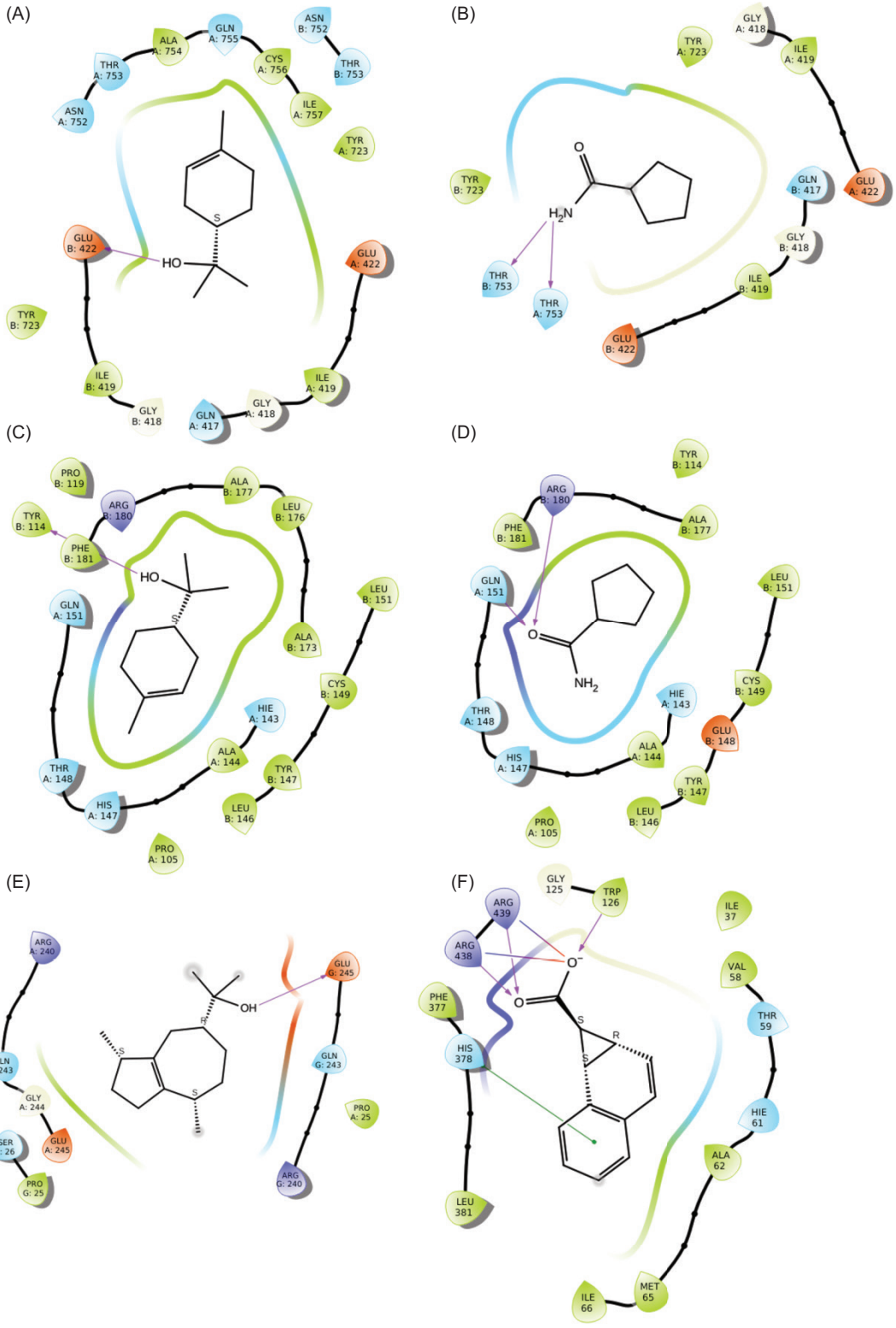

Figure 5. The two-dimensional docked complex structure of α-ketoglutarate dehydrogenase with (A) PG5 and (B) PG7; that of succinate dehydrogenase with (C) PG5 and (D) PG7; that of malate dehydrogenase with (E) PG8; and that of cytochrome-C-oxidase with (F) PG9.

Table 7. Molecular interaction of mitochondrial target proteins with *P. guajava* phytochemicals.

Target/ Compounds ID	Compounds Pubchem ID	Amino acid interaction (3-letter code)	Bond length (Å)	Glide score (kcal/mol)
α-Ketoglutarate dehydrogenase (KDH)				
PG7	226274	THR753 (A & B chain)	(2.08 & 2.12)	–4.067
PG5	17100	GLU422	1.97	–3.984
Succinate dehydrogenase (SDH)				
PG7	226274	ARG180, GLN151	2.07, 1.97	–4.422
PG5	17100	THR114	2.24	–2.714
Malate dehydrogenase (MDH)				
PG8	227829	GLU245	2.05	–3.327
Cytochrome-C-oxidase				
PG9	318639	ARG438, ARG439, TRP126	(2.32, 4.91) (1.72, 2.38, 3.77) 2.20	–8.629

mitochondria. The levels of these enzymes are directly proportional to the levels of phospholipids (Subashini and Sumathi, 2012). In the present investigation, the levels of phospholipids were reduced in the toxin-treated animals. This decreasing concentration of phospholipids may reduce levels of both enzymes. However, the levels of these enzymes were significantly increased when animals were treated with the plant leaves extract. This could be due to the inhibition of degradation of phospholipids. A similar effect was also observed in the study conducted by Subashini and Sumathi (2012). Vijayakumar *et al.* (2020) established that isolated quercetin fractions exhibited more reasonable activity than that of the ethanolic extract of *P. guajava* leaves.

In silico approach

Following the above experiments, an *in silico* approach was carried out on phytoconstituents against cardiac target myocardial enzymes, which include KDH, SDH, MDH, and cytochrome-C-oxidase. Virtual screening of phytoconstituents are found to have binding affinity towards the myocardial enzymes, such as cyclopentanecarboxamide, and alpha-terpineol which are able bound to the KDH enzyme, cyclopentanecarboxamide and alpha-terpineol able to tightly bound to the SDH, guaiol (a sesquiterpenoid alcohol) compounds are able to bind to the MDH and 1H-cyclopropanaphthalene to cytochrome-C-oxidase enzyme. The phytoconstituents were screened for their binding affinity and the selected compounds were investigated for further *in silico* cardioprotective effects by docking with selected target proteins.

Based on docking energy and good interaction with the active site, ligand molecules were selected for further investigation. The docking studies confirmed the inhibition of cardiac target proteins to show the cardioprotective activity of various phytoconstituents discovered in *P. guajava*.

Conclusion

The present study concluded the positive effects of ethanolic extract of *P. guajava* leaves on the cardiac marker and mitochondrial enzymes of STZ-induced diabetic mice. We studied the phytoconstituents of *P. guajava* leaves against multiple target proteins, and observed that alpha-terpineol, cyclopentanecarboxamide, guaiol, 1H-cyclopropanaphthalene, tetracyclotridecan-9-ol, dormin/abscisic acid, and epiglobulol were the top-ranked molecules of cardiac, lysosomal, and mitochondrial target proteins. The limitation of the present investigation was that it did not cover molecular-level parameters to confirm overall medicinal benefits of *P. guajava*, which in the future could be a potential drug for diabetes-related cardiac complications.

Author Contributions

Conceptualization of the study was accomplished by Ramasamy Manikandan, Balasubramanian Balamuralikrishnan, and Arumugam Vijaya Anand. Methodology, data curation, and formal analysis were done by Ramasamy Manikandan, Arthi Boro, Pushparaj Karthika, Arunkumar Malaisamy, Shanmugam Velayuthaprabhu, and Rengasamy Lakshminarayanan Rengarajan. Software and bioinformatics analysis was done by Pushparaj Karthika and Meyyazhagan Arun. Writing of original draft was done by Ramasamy Manikandan and Balasubramanian Balamuralikrishnan. Working group coordination was done by Balasubramanian Balamuralikrishnan. Reviewing and editing was done by Arumugam Vijaya Anand, Arunkumar Malaisamy, Shanmugam Velayuthaprabhu, and Rengasamy Lakshminarayanan Rengarajan. Reviewing and interpretation was done by Arumugam Vijaya Anand and Balasubramanian Balamuralikrishnan. All the authors read and finalized the published version of the manuscript.

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Conflicts of Interest

The authors declared to have no conflict of interest.

Data Availability Statement

All the authors confirmed that the data supporting this study are accessible upon request.

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