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Quercetin and Kaempferol as Multi-Targeting Antidiabetic Agents against Mouse Model of Chemically Induced Type 2 Diabetes

Mrs. N DEEPA RAMANI, Mr. SHAIK RABBANI BASHA , Dr. SYED MOHAMMED AssociateProfessor1 , Asst Prof2,3 B.PHARMACY1,2, PHARM-D 3 Nimra College of Pharmacy, Jupudi, Krishna District, Andhra Pradesh-52145[1]

A. Article Info

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Abstract: Finding effective, safe medications that address several aspects of the metabolic condition known as diabetes is an urgent medical need. The potential of quercetin and kaempferol as multi-targeting antidiabetic agents was examined in this research. Using pharmacokinetic and docking software (AutoDock Vina 1.1.2), the druggability and binding affinities towards several antidiabetic targets were investigated for both drugs. According to our research, quercetin and kaempferol have good ADMET (absorption, distribution, metabolism, excretion, and toxicity) profiles and follow Lipinski's rule of five. In comparison to metformin, the positive control, both compounds exhibited stronger binding affinities for C-reactive protein (CRP), interleukin-1 (IL-1), dipeptidyl peptidase-4 (DPP-IV), peroxisome proliferator-activated receptor gamma (PPARG), protein tyrosine phosphatase (PTP), and sodium-glucose co-transporter-1 (SGLT-1). Kaempferol reduced α-amylase activity (in vitro) to a degree of 37.43 ± 0.42% and quercetin to a degree of 20.30 ± 0.49. In diabetic mice caused by streptozotocin-nicotinamide (STZ-NA), their oral supplementation led to a substantial decrease in blood glucose levels ($p < 0.001$), an improvement in lipid profile ($p < 0.001$), and an enhancement in total antioxidant status ($p < 0.01$). In addition, the growth of cancer cells Huh-7 and HepG2 was considerably reduced by both compounds ($p < 0.0001$), but the viability of the non-cancer Vero cell line was unaffected. Finally, compared to metformin, quercetin and kaempferol had stronger binding affinities to a variety of targets. Both compounds show promise for future research in diabetes treatment due to their antidiabetic capabilities in vitro and in vivo, as well as their anticancer activity. Maybe since they both target the same receptors, the two medications did not work together synergistically.

Keywords: type 2 diabetes; molecular docking; anticancer activity; kaempferol; quercetin; multi- target compounds

1. Introduction

A complicated metabolic non-communicable condition, diabetes mellitus (DM) may be caused by an abnormal rise in blood glucose level due to insulin secretion defects (type 1 DM), insulin action defects (type 2 DM), or both [1]. Diabetic epidemic levels have been achieved on a worldwide scale. Diabetes affects 415 million people worldwide (or almost 9% of the population) and is predicted to reach 642 million by 2040 [2]. Possible causes of the recent increase in diabetes incidence include reactive oxygen species (ROS) and impaired antioxidant defense systems [3]. There is a robust association between body mass index and the onset of type 2 diabetes [4]. Lipid and glucose homeostasis issues

individuals with diabetes often have metabolic abnormalities [5]. Serious issues affecting important organs such the eyes, liver, kidneys, and heart are common outcomes of chronic hyperglycemia [6]. Cancers of the pancreas, liver, and breast are all closely associated with type 2 diabetes, also known as non-insulin-dependent diabetic mellitus (NIDDM) [7].

In order to treat type 2 diabetes, one must make changes to their lifestyle and stick to a healthy weight, in addition to taking medication. Insulin analogs, sulfonylureas, and other release stimulators; thiazolidinediones, an insulin sensitizer; and acarbose, and biguanides, inhibitors of glucose absorption and synthesis in the liver, round out the list of these interventions [8]. Although gliptins, gli-tazones, sodium-glucose co-transporter (SGLT) inhibitors, and glucagon-like peptide 1 (GLP-1) agonists are also often recommended, metformin is still the predominant treatment choice [9,10]. Problems with the current state of diabetes care include a large and growing diabetic population, exorbitant healthcare costs, and the risk of side effects from pharmaceutical medications [9,11].

The "multi-target approach" is replacing the long-established "one drug" for "one target" paradigm in drug discovery as our knowledge of complicated illnesses grows [12]. When several variables contribute to the development of a disease, the traditional single-target treatment strategy often fails to address the complex pathophysiology [1,13]. Even at lower dosages, multi-targeting medicines may have a synergistic impact that increases their therapeutic potential [14]. An effective method to treat multifactorial disorders like diabetes might be the creation of cost-efficient multi-targeting medication candidates with safety profiles [13]. Computational and in vivo methods complement and enhance one another in the drug development process [15]. The use of bioinformatics tools and in silico computer-aided drug designs has drastically cut the time and money needed for drug discovery [16]. Drug development ideally involves in silico screening and optimization of druggability-based drug candidates while excluding chemicals/ligands with ADMET (absorption, distribution, metabolism, excretion, and toxicity) profiles that are undesirable [15,17,18]. As a tool for atomic-level prediction, molecular docking is widely utilized in the drug development process for the purpose of discovering promising new medicinal compounds.

of interactions between ligands and receptors [19].

Because of their reduced toxicity and antioxidant capabilities, medicines produced from plants have recently attracted a lot of interest [20,21]. In addition to oral antidiabetic medications such as sulfonylurea and metformin, several plant components are used for the management of diabetes on a global scale [22]. As a first-line oral therapy for diabetes, metformin (also known as dimethylbiguanide) is routinely used. The European herbal medicine Galega officinalis, which was found in 1918 to treat hyperglycemia, is its ancestor [23]. Because of their potential health advantages in treating a wide range of diseases, flavonoids—which are abundant in food plants—have attracted a lot of attention as secondary metabolites [24]. Plants containing polyphenols and polyflavonoids have shown promising results in treating a range of illnesses [24]. These compounds have antioxidant, anti-inflammatory, and neuroprotective properties. Flavonoids quercetin and kaempferol were studied for their possible antidiabetic effects on many targets involved in the development of diabetes. Fruits, vegetables, herbs, and grains are typical food sources of quercetin [25]. With its antioxidant, antiviral, anti-inflammatory, anti-obesity, and anti-allergic capabilities, quercetin has a wide range of biological activities [25-30]. The antioxidant, anticancer, antibacterial, neuroprotective, and hepatoprotective properties of kaempferol have made it a popular ingredient in a variety of foods, including barberries, onions, apples, citrus fruits, strawberries, and citrus fruits.

2. Results

2.1. In Silico Studies

2.1.1. Drug Scan and ADMET Profiling

The druggability of potential drug candidates can be evaluated using the Lipinski's Rule of Five (Ro5) [34]. This rule, also known as Pfizer's rule, sets certain criteria such as that the molecular weight (Mol. WT) should be less than 500 Daltons, the number of hydrogen bond donors (HBDs) should be no more than 5, the number of hydrogen bond acceptors (HBAs) should be less than 10, the number of rotatable bonds (RBNs) should be 10 or less, the octanal water partition coefficient (LogP) should no more than 5, and the molar refractivity (A) should be between 40 and 130. This rule evaluates the drug-likeness of a compound, indicating its potential pharmacological effectiveness and oral activity in the human body based on its pharmacokinetics and physicochemical parameters. Usually, compounds with no violation or minimal violation (not more than one) ensure potential activity, while two or more violations suggest limited oral effectiveness for drug candidates [17]. Both quercetin and kaempferol in our study followed Lipinski's Ro5, showing no violation as shown in Table 1. Metformin, the widely used antidiabetic drug, served as a reference. The chemical structures of both compounds are shown in Figure 1.

Table 1. Drug-likeness parameters of quercetin, kaempferol, and metformin.

Compounds	Mol. WT (g/mol)	HBD	HBA	RBN	LogP	A	Violation
Quercetin	302.24	5	7	1	1.23	78.03	0
Kaempferol	286.24	4	6	1	1.58	76.01	0
Metformin	126.12	3	6	0	−1.38	37	0

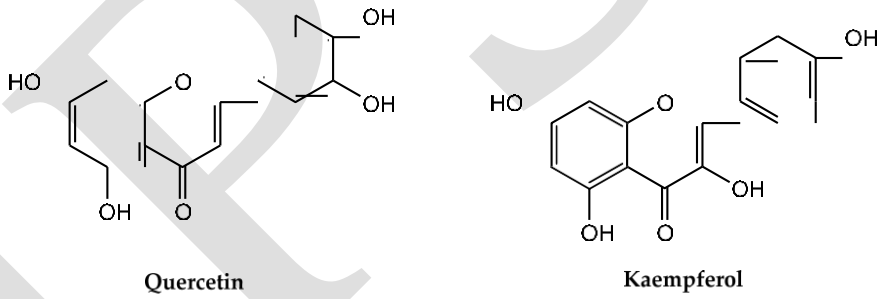

Figure 1. Chemical structure of quercetin and kaempferol.

In addition, quercetin, kaempferol, and metformin (the reference medication) were subjected to ADMET analysis. The discipline of medicinal chemistry relies on ADMET profiling to forecast the toxicity, distribution, metabolism, excretion, and absorption of potential therapeutic compounds, which in turn helps with drug development [35,36]. During the drug development phase, clinical trials are not conducted on therapeutic candidates that have negative ADMET profiles [18]. Results concerning the BBB and absorption in humans were consistent for both quercetin and kaempferol. Various other models, including the renal organic cation transporter, P-glycoprotein substrate, and human intestinal absorption (HIA), were also useful in determining if these compounds had sufficient medicinal candidate potential. Clusters of the cytochrome P50 isoenzyme are another key example; these enzymes are involved in a large portion of drug metabolism (around 75 percent), suggesting that quercetin and kaempferol might be useful in this context. The CYPEC9 and CYP2D6 enzymes belonged to this family, and none of these drugs inhibited their activity. And for all four of these chemicals, we found a decreased probability of P-glycoprotein substrate and inhibitor. Potential benefits, such as decreased efflux, renal clearance, and better bio-availability, which contribute to the desired pharmacokinetic profile, were also anticipated with regard to the renal organic cation transporter (ROCT) substrate. Additionally, the admetSAR and Swiss ADME research shown that neither chemical was carcinogenic nor harmful.

Table 2 lists all of the ADMET parameters for quercetin, kaempferol, and metformin (the typical antidiabetic medication).

Table 2. ADMET-related parameters of quercetin, kaempferol, and metformin.

	Quercetin	Kaempferol	Metformin
Absorption			
BBB	∓	∓	−
HIA			+
CaCo2 permeability	−	−	−
PGS	−	−	−
PGI	−	−	−
ROCT	−	−	−
Metabolism			
CYP3A4 Substrate	+	+	−
CYP2C9 Substrate	−	−	−
CYP2D6 Substrate	∓	∓	−
CYP3A4 Inhibition			−
CYP2C9 Inhibition	−	∓	−
CYP2C19 Inhibition	−		−
CYP2D6 Inhibition	∓	∓	−
CYP1A2 Inhibition			−
Toxicity			
Ames toxicity	NAT	NAT	NAT
Carcinogens	Non-carcinogenic	Non-carginogenic	Non-carginogenic

BBB; blood–brain barrier, HIA; human intestinal absorption, PGS; P-glycoprotein substrate, PGI; P-glycoprotein inhibitor ROCT; renal organic cation transporter, NAT; non-Ames toxic. + and ∓ represent presence and ab- sence respectively.

2.1.2. Molecular Docking

Using the AutoDock Vina 1.1.2 program, molecular docking was performed to investigate the binding affinities of the two chemicals towards various antidiabetic targets [37]. The following targets were identified: CRP, IL-1, DPP-IV, PPRG, PTP, and SGLT-1 [38-44]. In order to control inflammation, lipid metabolism, insulin signaling, and blood glucose levels in diabetes, it is essential to modulate these targets. In addition to inhibiting insulin-mediated glucose transport, CRP and IL-1 contribute to inflammation. Insulin sensitivity and lipid metabolism are enhanced by activating PPARG, while the activity of the hormones GLP-1 and GIP, which are essential for glucose homeostasis, is restored by inhibiting DPP-IV. When compared to metformin, quercetin and kaempferol exhibited superior binding affinities for all targets (Table 3). Both quercetin and kaempferol had binding affinities that varied from 5.8 to -8.4 (Kcal/mol) and 4.2 to -8.4 (Kcal/mol), respectively. While metformin (the positive control) had a binding affinity ranging from 4.2 to -5.3 (Kcal/mol), this was in contrast to other drugs. Table 3 further shows that quercetin and kaempferol had similar binding affinities to all of the targets.
Structure optimization of potential medications requires thorough examination of the ligand-receptor interaction [15]. Traditional hydrogen bonds, pi-alkyl, pi-sigma, amide pi-stacked, and hydrophobic contacts were among the many types of interactions uncovered by a thorough analysis of the ligand-receptor complex for quercetin, kaempferol, and metformin. Both compounds and the conventional medicine interact with each antidiabetic target in two dimensions, as shown in Figures 2 and 3. Figure 2 shows that among the quercetin compounds, CRP had the most hydrogen bonds, with five. Kaempferol, on the other hand, formed three hydrogen bonds with CRP and SGLT-1.

Table 3. Predicted binding energies and detailed docking interaction of quercetin, kaempferol, and metformin with selected antidiabetic targets.

Target Receptor	Ligand		Binding H-Bond Residues	No. of H-Bonds
	Name	Binding Energy (Kcal/mol)		
CRP	Quercetin	−5.8	Thr126, Arg58, Lys57, Val127, Glu62, Gly128, Glu130, Pro29	5
	Kaempferol	−5.6	Arg58, Val127, Thr126, Lys57, Thr56, Gly128, Glu62, Pro29, Arg58	3
	Metformin	−4.2	Glu293, Gln145, Ser21, Ile134, Ser132 Gly143, Phe142	1
IL-1	Quercetin	−7.4	Leu62, Lys63, Glu64, Ser153, Asn7, Ser5, Ser43, Gly61, Lys65 Typ90, Pro91,Lys63	3
	Kaempferol	6.8	Leu62, Ala1, Arg4, Val3, Ser45, Ser,43 Gly61, Lys65, Asn66, Glu64, Ser5, Thre68	1
	Metformin	−5	Tyr68, Lu62, Lys65, Glu64, Lys63, Ser43, Pro91, Ser5, Ser54, Val486	1
DDPIV	Quercetin	−6.6	Thr516, Glu464, Lys 433, Tyr414, Ser484, Thr411, Ala465 Glu464, Val486	2
	Kaempferol	−6.5	Tyr416, Tyr414, Ser485, Lys433, Thr411, Ala465, Asp65, Lys566	1
	Metformin	−4.3	Ser485, Glu464, Lys433, Tyr416, Tyr414, Thr411, Ala465, Lys466, Val486	2
PPRG	Quercetin	−8.4	Ile326, Cys285, Glue291, Glu295, Pro227, Phe226, Met329, Lue333, Met334, Val339, Phe363, Tyr321, Ser289, Arg288, Leu330, Ala292	3
	Kaempferol	−8.3	Phe363, Ile326, Lue333, Met329, Phe226, Glu295, Glu291, Ser284, Tyr327, Arg288, Cys285, Leu330, Ala292	--
	Metformin	−5.3	Gln410, Asp441, Asp396, Val390, Met439, Gln437	3
PTP	Quercetin	8.2	Arg221, Lys120, Ser216, Asp181, Gln266, Gly220, Gln262, Gly183, Ile219, Val64, Asp48, Phe182, Tyr46, Ala217	2
	Kaempferol	−8.3	Arg221, Val49, Ile219, Ser216, Asp181, Gly183, Gln226, Gly220, Gln261, Asp48	2
	Metformin	4.8	Ser80, Leu204, Gly209, Ser205, His208, Val221, pro210, Ser203, Arg79, Pro206, Gln78	6
SGLT-1	Quercetin	−8.4	Asp161, Tyr290, Ser81, Val160, Ile456, Thr156, Phe101, Gln457, Leu286, Trp289, Ala287, Ser72, Ile397, Ser393	2
	Kaempferol	−8.4	Asp161, Ser71, Tyr290, Ser81, Val160, Ile456, Thr156, Phe101, Ser393, Asn78, Trp289, Gln457, Leu286, Ser460	3
	Metformin	−5.3	Ser77, Ser81, Ser393, Val160, Lys157, Ile456, Thr156, Gly82, Asn78, Ile79	2

CRP (PDB ID: 1gnh), DPP-IV (PDB ID: 1j2e), IL1 (PDB ID: 9ilb), PRARG (PDB ID: 1prg), PTP (PDB ID: 2nt7), and SLGT1 (PDB ID: 7sla). Quercetin (PubChem CID: 5280343), Kaempferol (PubChem CID: 5280863) and Metformin (PubChem CID: 4091).

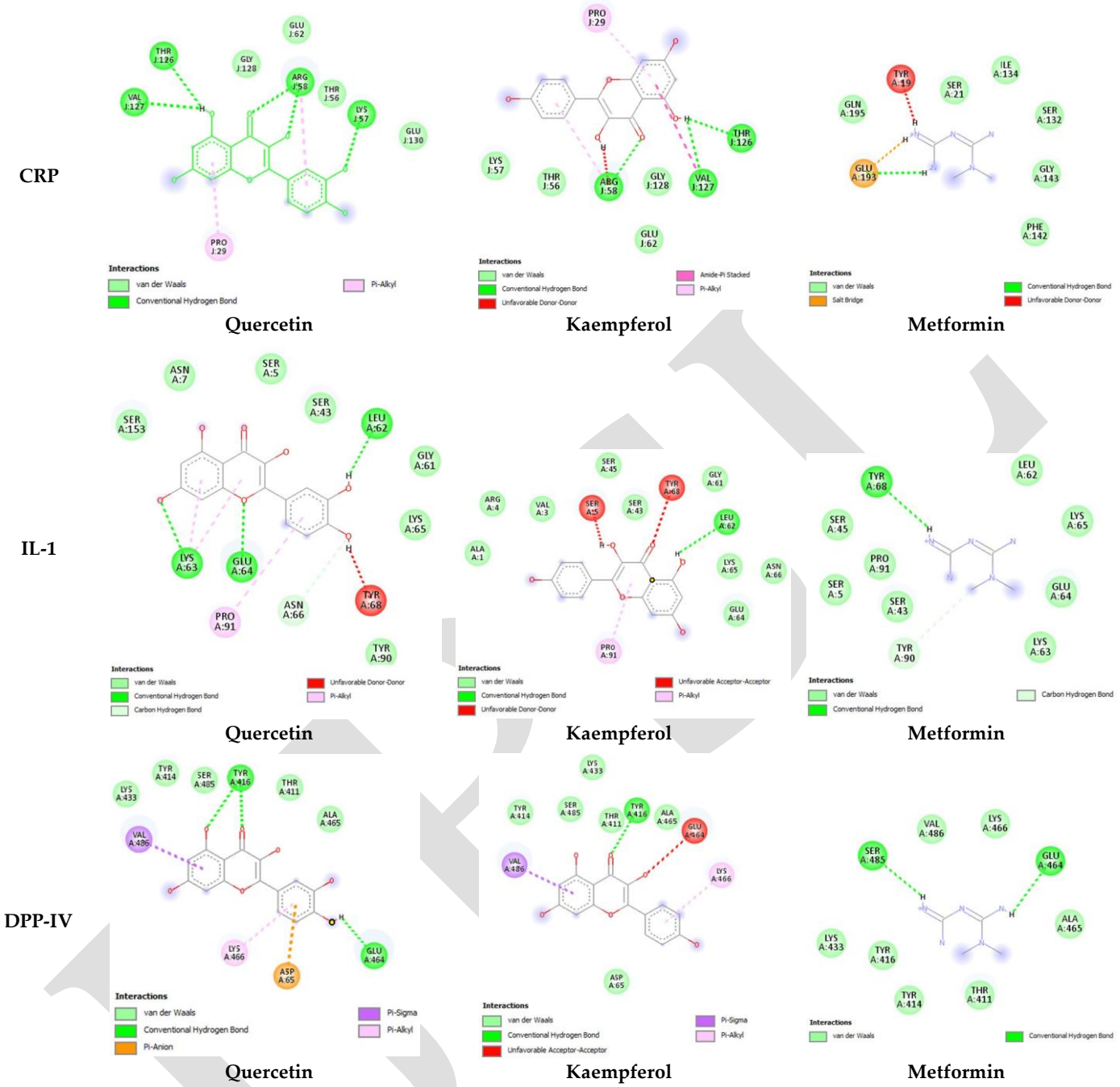

Figure 2. Two-dimensional representation of the interaction between quercetin, kaempferol, and metformin within the binding sites of CRP, IL-1, and the active site of DPP-IV. CRP (PDB ID: 1gnh), DPP-IV (PDB ID: 1j2e), and IL1 (PDB ID: 9ilb). Interaction analysis was carried out using BIOVIA Discovery Studio 2021 and the best pose was selected for each ligand.

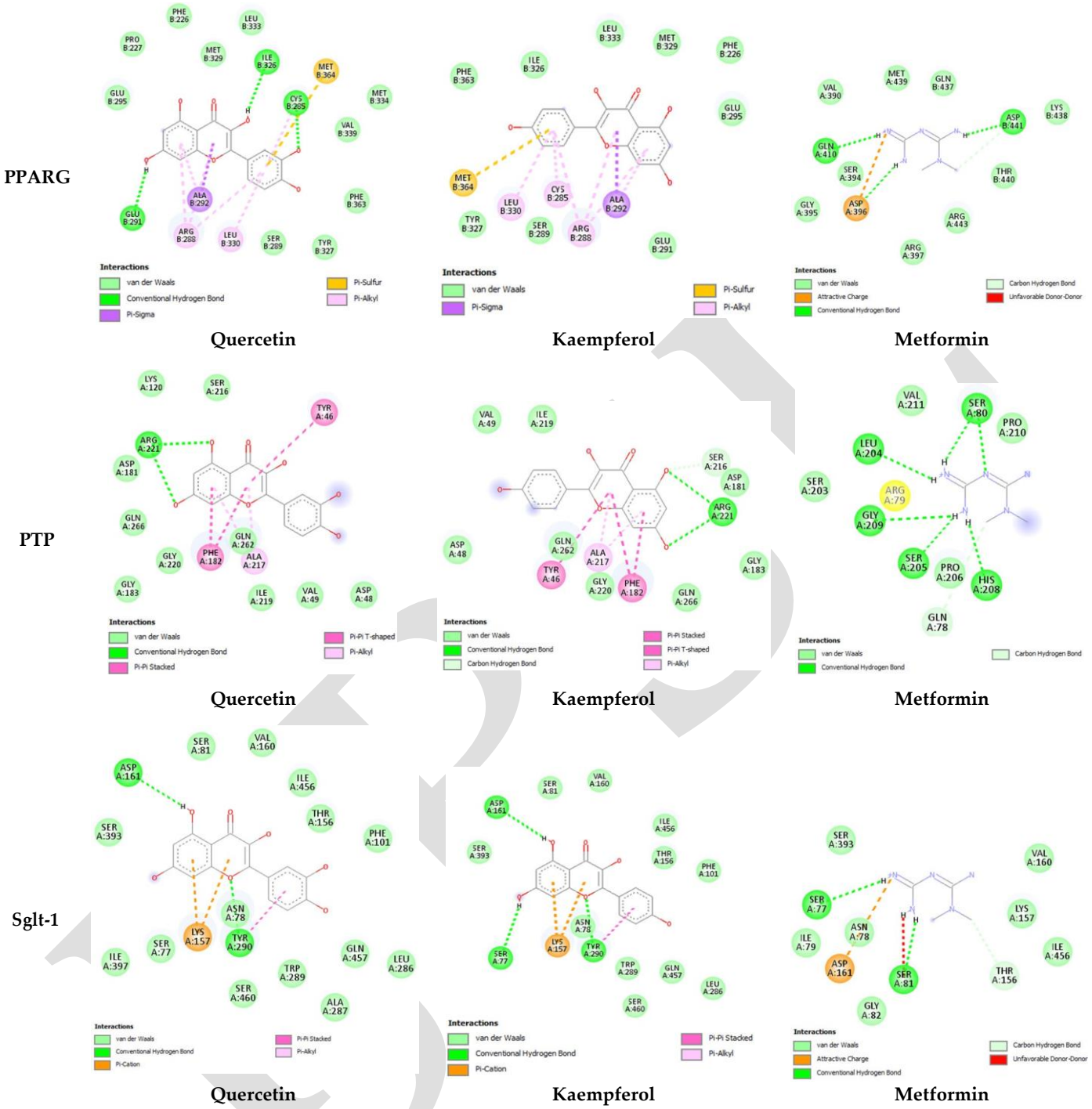

Figure 3. Two-dimensional representation of quercetin, kaempferol, and metformin interaction within canonical thiazolidinedione (TZD) binding sites of PPARG, active site of PTP, and binding site of SGLT-1. Quercetin (PubChem CID: 5280343), Kaempferol (PubChem CID: 5280863), and Metformin (PubChem CID: 4091). PRARG (PDB ID: 1prg), PTP (PDB ID: 2nt7), and SLGT-1 (PDB ID: 7sla). Interaction analysis was carried out using BIOVIA Discovery Studio 2021 and the best pose was selected for each ligand.

The α-Amylase Inhibition Assay for 2.2. In Vitro Anti-Hyperglycemic Activity
To decrease the rise in blood sugar levels after eating, one of the primary goals is to block pancreatic alpha-amylase enzymes [45]. When digested, this enzyme breaks down complex carbs into their component sugars. Glucose is released into the circulation less rapidly after eating when this enzyme is inhibited. There was an inhibition of alpha-amylase by 37.43 ± 0.42 and 20.30 ± 0.49% by kaempferol and quercetin, respectively.
2.3. Activities In Vivo That Reverse Diabetes

The results of the in silico studies prompted the use of a streptozotocin-nicotinamide (STZ-NA)-induced diabetic mouse model to evaluate the in vivo antidiabetic effects of quercetin and kaempferol. Blood glucose levels, serum lipid profiles, and liver antioxidant status were assessed in our in vivo investigation, which examined the effects of oral supplementation.

2.3.1. Quercetin, Kaempferol, and Their Combination's Impact on Diabetic Mice's Blood Glucose Levels
The optimum biomarker for monitoring and tracking patients' diabetes condition is blood glucose level since it meets all the required parameters for this purpose [46]. All of the animals showed a rise in blood glucose levels compared to the control group after injection of STZ-NA, indicating that the induction of diabetes was effective. In comparison to the normal control (Ctrl) group, the diabetic control (STZ-NA-treated) group had a considerably higher blood glucose level ($p < 0.001$). Blood glucose levels were considerably ($p < 0.001$) lower after oral administration of quercetin (20 mg/Kg), kaempferol (5 mg/Kg), and their combination compared to metformin (50 mg/Kg), as seen in Figure 4. In the combination group, quercetin (10 mg/Kg) and kaempferol (2.5 mg/Kg) were administered.

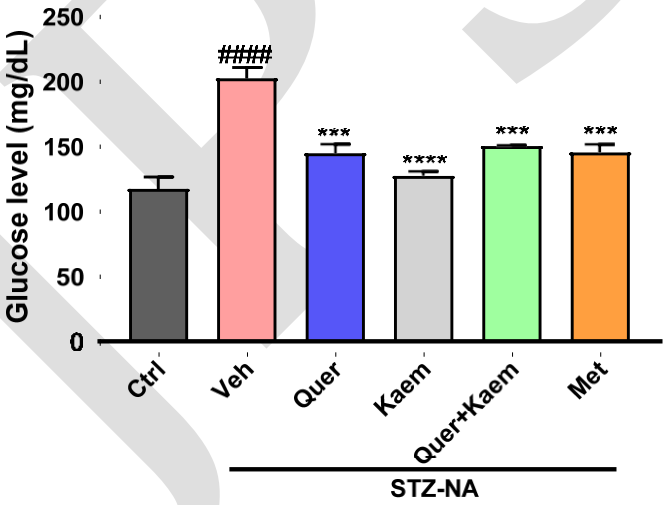

Figure 4. Graph showing the effect of quercetin, kaempferol, their combination, and metformin on blood glucose level. Data are expressed as mean ± SEM, where n = 3 (number of mice ineach group). One-way ANOVA (analysis of variance) and Tukey's multiple comparison test was applied to find the significance level and represented as p-value. #### $p < 0.0001$ shows comparison of diabetic control with normal control, *** $p < 0.001$ and **** $p < 0.0001$ show the comparison of other groups with diabetic control. Ctrl received 20% ethanol, representing normal control; Veh (vehicle) received normal saline, served as diabetic control; Quer received quercetin 20 mg/Kg; Kaem received kaempferol 5 mg/Kg; Quer + Kaem group received 10 mg/Kg of quercetin and 2.5 mg/Kg of kaempferol; and Met received metformin 50 mg/Kg served as positive control.

2.1.3. Effect of Quercetin, Kaempferol, and Their Combination on Lipid Profile of Diabetic Mice

Dyslipidemia is often observed in diabetic patients and is associated with an increase in cardiovascular diseases [47]. The induction of diabetes caused a significant increase in serum triglyceride (TG) and total cholesterol (TC) levels in diabetic mice, which are key factors for the development of cardiovascular diseases. However, in the current study, quercetin, kaempferol, and their combination significantly regulated both serum TG and TC levels, respectively, as shown in Figure 5.

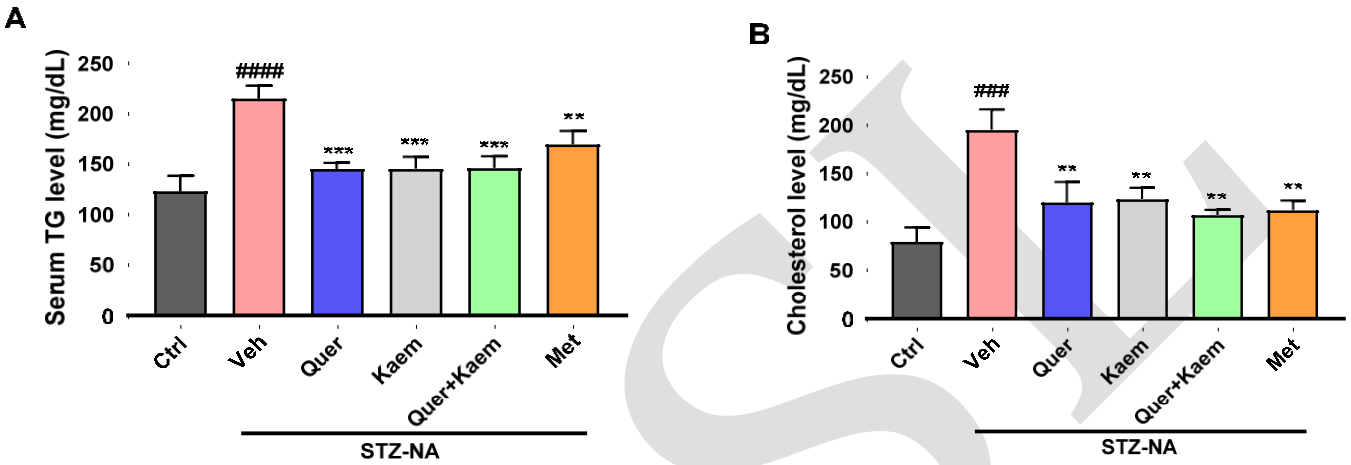

Figure 5. Effect of quercetin, kaempferol, and their combination on lipid profile; (**A**) serum triglyceride level and (**B**) cholesterol level of experimental mice. Data are presented as mean ± SEM, where $n = 3$. One-way ANOVA (analysis of variance) and Tukey's multiple comparison test were applied to find the significance level and represented as p-value. $^{\#\#\#\#}$ $p < 0.0001$ and $^{\#\#\#}$ $p < 0.001$ show comparison of diabetic control with normal control, *** $p < 0.001$ and ** $p < 0.01$ show the comparison of other groups with diabetic control.

2.1.4. Effect of Quercetin, Kaempferol, and Their Combination on Hepatic Markers of Diabetic Mice

The elevated levels of alkaline phosphatase (ALT), alanine aminotransferase (ALP), and bilirubin are associated with liver cell damage, leading to increased permeability and bringing these enzymes into the blood [48]. However, in this study, the induction of diabetes and the supplement of quercetin, kaempferol, and their combination showed no significant change in the serum ALT, ALP, and bilirubin levels, as shown in Table 4.

Table 4. Effect of quercetin, kaempferol, and their combination on level of serum ALT, ALP, and bilirubin.

Treatments	ALT (IU/L)	ALP (IU/L)	Bilirubin (mg/dL)
Control	69.0 ± 11	87.3 ± 6.3	0.55 ± 0.03
STZ-NA + Veh	80.5 ± 17	89 ± 07	0.64 ± 0.04
STZ-NA + Quer	68.0 ± 07	79.5 ± 2	0.605 ± 0.02
STZ-NA + Kaem	78.5 ± 08	78.5 ± 12	0.565 ± 0.11
STZ-NA + Quer + Kaem	85 ± 6.8	93 ± 15	0.625 ± 0.01
STZ-NA + Met		80.5 ± 18.9	92 ± 14	0.47 ± 0.03

Values are expressed as means ± SEM, where $n = 3$. Statistical analysis was applied to find level of significance as in Figure 4. Ctrl, normal control; Veh, vehicle (diabetic control); Quer, quercetin; Kaem, kaempferol; and Met, metformin (positive control).

2.1.5. Effect of Quercetin, Kaempferol, and Their Combination on Total Antioxidant Status (TAC) of Hepatic Tissues

Reactive oxygen species reduce overall antioxidant status in diabetic circumstances by oxidizing lipids and causing disturbances [49]. Mice treated with quercetin, kaempferol, or a combination of the two showed a substantial improvement ($p < 0.05$) in the antioxidant state of their liver tissue, in contrast to the diabetic control group. Figure 6 shows that compared to metformin, quercetin, kaempferol, and their combination all demonstrated an enhanced overall antioxidant status.

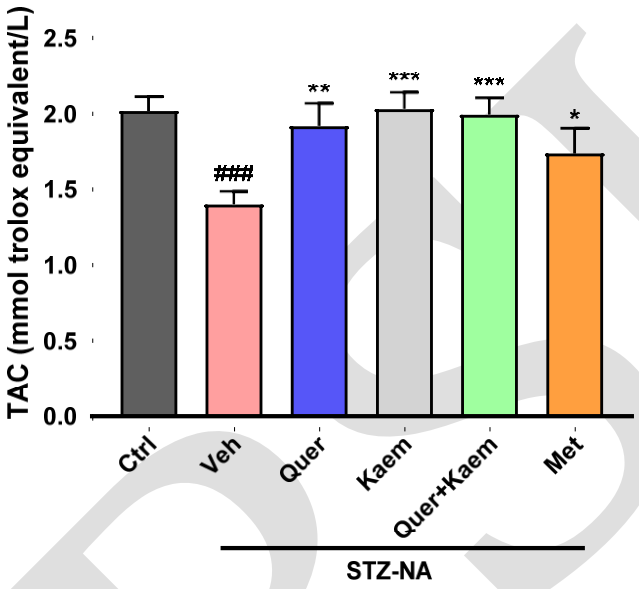

Figure 6. Effect of quercetin, kaempferol, and their combination on TAC status of liver tissue; values are means (n = 3) ± SEM, where n = 3. One-way ANOVA (analysis of variance) and Tukey's multiple comparison test were applied to find the significance level and represented as p-value. $^{###}$ $p < 0.001$ shows comparison of diabetic control with normal control, *** $p < 0.001$, ** $p < 0.01$, and * $p < 0.05$ show the comparison of other groups with diabetic control. Ctrl, normal control; Veh, vehicle (diabetic control); Quer, quercetin; Kaem, kaempferol; and Met, metformin (positive control).

2.2. Cell Viability Assay

Hyperinsulinemia is directly linked with the development of various types of cancer [50]. In this study, the cytotoxic effect of quercetin (36 µg/mL), kaempferol (15 µg/mL), and their combination (quercetin 18 µg/mL + 7.5 µg/mL) was explored against both cancer and non-cancer cell lines using an MTT assay. Quercetin, kaempferol, and their combination all significantly ($p < 0.0001$) inhibited the growth of Huh-7 and HepG2 cancer cell lines. However, no effect was observed on the viability of the Vero cell line (non-cancer). The cytotoxic activity of kaempferol was comparatively higher than quercetin in HepG2. It is evident from the MTT assay that the anticancer activity of both candidate drugs was comparable to doxorubicin (1.8 µg/mL), the standard anticancer drug (positive control), as shown in Figure 7.

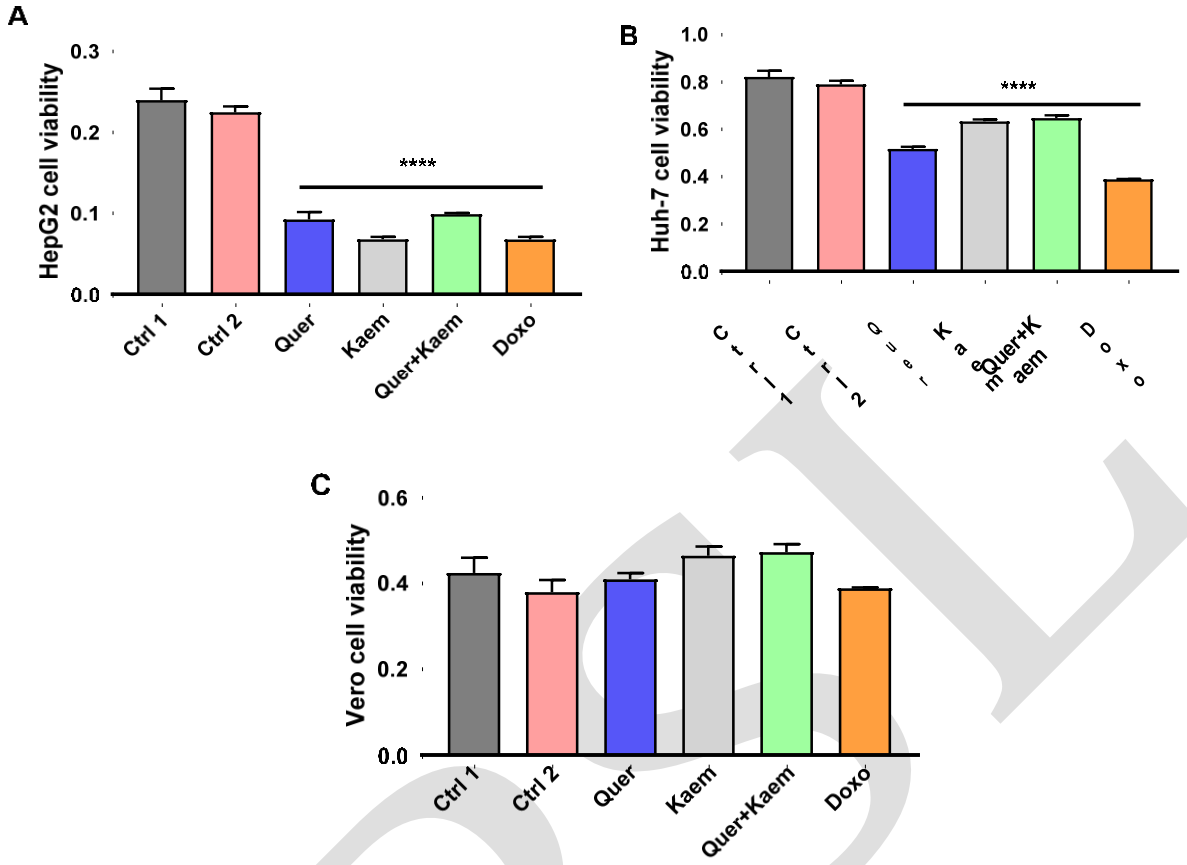

Figure 7. Effect of quercetin, kaempferol, and their combination on the viability of cancer and non-cancer cell lines. (**A–C**) Shows the effect of quercetin (36 µg/mL), kaempferol (15 µg/mL), their combination (18 µg/mL quercetin + 7.5 µg/mL kaempferol), and doxorubicin (1.8 µg/mL) on the viability of HepG2, Huh-7 (cancer), and Vero (non-cancer) cell lines, respectively. One-way ANOVA (analysis of variance) and Tukey's multiple comparison test were applied to find the significance level and represented as *p*-value. **** $p < 0.0001$ shows comparison of all treatment groups with both controls. Ctrl1, control with distilled water; Ctrl2, control with 0.1–0.2% dimethyl sulfoxide (DMSO); Quer, quercetin; Kaem, kaempferol; and Doxo, doxorubicin as standard anticancer drug. The absorbance was measured at 570 nm.

3. Discussion

There are several elements involved in the development of diabetes, which makes its pathophysiology complicated [1]. Finding safer and more effective alternatives is urgently needed due to the worldwide diabetes epidemics and the related worries about current drugs [9,11]. Researchers are captivated by the potential of multi-target medications to tackle complex illnesses like diabetes. Such medications have been steadily gaining approval from the FDA since 2015, a sign of their rising profile in the pharmaceutical industry [51]. A potentially useful alternative for the less invasive treatment of several diseases is the use of bioactive chemicals extracted from medicinal plants. Quercetin and kaempferol were investigated as possible ligands against several antidiabetic targets, such as CRP, IL-1, DPP-IV, PPARG, PTP, and SGLT-1, using in silico molecular docking in the present study. Using an in vitro alpha-amylase inhibition assay, as well as monitoring blood glucose level, lipid profile, and total antioxidant status in STZ-NA-induced mice model of type 2 diabetes, these compounds were further evaluated for their potential to treat diabetes. In addition, the MTT test was used to determine the cytotoxic effects of the two compounds against cancer and non-cancer cell lines.

We used computational methods to investigate these compounds' druggability and ADMET characteristics. We do not include medication candidates in our clinical studies if their ADMET profiles are negative.

pharmacological research [18]. Table 1 shows that quercetin and kaempferol are drug-like, and Table 2 shows that none of them has any possible hazardous or carcinogenic effects, and both have good ADMET profiles. These drugs' ADMET profiles are quite similar to metformin's. Benefits such as decreased efflux, renal elimination, and enhanced bio-availability are indicated by their HIA, bio-availability, and ROCT substrate status, and these factors lead to a favorable pharmacokinetic profile. Both compounds were shown to be suitable as therapeutic candidates according to an alternate model of P-glycoprotein substrate, the P50 isoenzyme model, which involves clusters of the cytochrome linked to around 75% of drug metabolism. Additionally, none of the drugs inhibited the activity of the CYPEC9 or CYP2D6 enzymes that belong to this family. The results show that these bioactive molecules, which have druggable properties and a good ADMET profile, may be better than synthetic chemicals like acarbose when used in drug formulation. Metabolically unstable states have been seen in acarbose [52].

In Figures 2 and 3, the molecular docking data provide light on the binding residues and interaction mechanisms of the two drugs at the binding sites of CRP, IL-1, DPP-IV, PPARG, PTP, and SGLT-1. When it comes to controlling hyperglycemia and its consequences, these objectives are crucial. Elevated C-reactive protein (CRP) levels inhibit insulin-mediated glucose transporter type 4 (GLUT4) translocation and contribute to inflammation, both of which are common in people with diabetes [39]. Among the cytokines linked to inflammation is interleukin (IL-I) [53]. The levels of nuclear factor kappa b (NF-kb) and nitric oxide are increased by inflammatory markers such as IL-6, IL-1, tumor necrosis factor alpha (TNF-α), and CRP. This activation of serine/threonine kinase leads to poor insulin signaling and the loss of pancreatic β-cells [38,39]. Cleavage of GIP and GLP-1 renders them inactive, and an increase in DPP-IV is a hallmark of diabetes. High glucose levels trigger the production of both GLP-1 and GIP, which are essential for maintaining glucose homeostasis via the stimulation of insulin secretion from pancreatic β-cells [40]. Treatment of type 2 diabetes often involves the use of dipeptidyl peptidase IV inhibitors, including vildagliptin, sitagliptin, and saxagliptin. These inhibitors prolong GLP's half-life [54]. Overexpression of PTP in skeletal muscles and other organs in type 2 diabetes inhibits the routes that insulin and leptin communicate [41]. Activated insulin receptors (IRs) reportedly undergo de-phosphorylation via PTP. The PI3K/AKT/GLUT4 signaling pathway is eventually downregulated once IRS-1 and IRS-2 are dephosphorylated [42]. Glucose absorption in the intestines and kidneys is caused by SGLT-1 upregulation in diabetes [43]. PPARG, or the glitazone receptor, plays a significant role in enhancing insulin sensitivity, lowering lipo-toxicity, and promoting fatty acid storage in fat cells [44]. It has been shown that PPARG agonists inhibit TNF-alpha, a protein that increases insulin resistance and promotes the expression of genes related to glucose and lipid metabolism [55]. Both compounds' interactions with the chosen targets revealed the presence of ligand-receptor complex formation mechanisms such as conventional hydrogen bonds, pi-alky, pi-sigma, and van der Waals contact. It seems that hydrophobic interactions, in addition to hydrogen bonding and van der Waals contact, promoted and stabilized the connection between ligands and receptors [52]. In the development of the ligand-receptor complex, both polar (Thr, Arg, Lys, Glu, Gln, Ser, Asn) and non-polar (Val, Gly, Leu, Ala) amino acids were involved (Figures 2 and 3). Table 3 shows that compared to the gold standard medicine metformin, an oral antidiabetic medication licensed by the FDA, quercetin and kaempferol had far greater binding affinities. A larger number of interacting amino acids at the target's binding sites may be related with their higher binding affinities. Further, the majority of interactions between the two drugs occurred via target residues that were identical to one another. This data points to the importance of certain amino acids in the development of ligand-receptor complexes. Further evidence of comparable routes for therapeutic benefits is provided by the shared binding residues, which point to similar binding styles and a common mechanism of action.

Figures 2 and 3 display the results of an investigation that reveals quercetin and kaempferol to be in inhibition mode when it comes to binding to CRP, IL-1, DPP-IV, PTP, and SGLT-1. It will need further research, however, to verify the

exists a complete inhibitory action for these substances. Both compounds have partial agonistic activity, as shown in Figure 3, when they bind to three known residues (Cys285, Arg288, Ser289) at the conventional thiazolidinedione (TZDs) binding sites of PPARG. It has been observed that compounds that create hydrophobic interactions with these PPARG residues affect co-activator recruitment and lead to transactivation by stabilizing the H3 and β-sheet regions [11,56].

Blood glucose levels rise because the alpha-amylase enzyme breaks down carbs into glucose. In order to keep postprandial hyperglycemia under control, it is crucial to inhibit this enzyme [45]. The alpha-amylase enzyme was inhibited to varying degrees by quercetin (20.30 0.49%) and kaempferol (37.43 0.42%). On the other hand, compared to acarbose (the positive control), their percentage inhibition was lower. This is corroborated by the results of Yang et al. (2023), who found that quercetin and kaempferol had less α-amylase inhibitory action than acarbose [57]. In addition, these compounds were tested for their in vivo antidiabetic potentials utilizing the STZ-NA-induced mice model of type 2 diabetes. All of the animals showed a rise in blood glucose levels compared to the control group after the STZ-NA injection, indicating that diabetes was successfully induced. When compared to the diabetic control group, the groups that were given quercetin, kaempferol, or a combination of the two orally demonstrated significantly lower blood glucose levels ($p < 0.001$), improved lipid profiles (Figure 5), and an improved total antioxidant status (Figure 6). Groups treated with STZ-NA had higher levels of triglycerides and serum cholesterol, both of which are risk factors for cardiovascular disease [58,59]. In order to treat diabetes-related problems, it is vital for a diabetic person to maintain their total antioxidant status [60]. It has been shown that insulin resistance and lipid peroxidation, caused by a decreased antioxidant state in diabetes persons, might harm the pancreas and other important organs [61]. The capacity of quercetin and kaempferol to control many targets, as proposed in silico, may explain the overall improvement in blood glucose, lipid profile, and antioxidant status that was found in the experimental animals. There was no synergistic impact seen in recent in vivo experiments when quercetin and kaempferol were administered together; rather, they had an additive effect. The same target for both drugs could explain why they don't work together. Nevertheless, quercetin's capacity to increase kaempferol's bio-availability is a possible benefit of mixing the two [31]. In addition, compared to conventional medications, kaempferol's antidiabetic impact was much stronger. Damage to liver cells may increase their permeability, which in turn can cause these enzymes to be released into the bloodstream, resulting in elevated levels of ALT and ALP [62]. Serum bilirubin, ALP, and ALT levels did not alter significantly in the experimental animals. Specifically, we are examining these drugs' potential in glycemic control, insulin sensitivity, and dyslipidemia control as part of our ongoing in vivo diabetes management research. Cardiovascular problems, diabetic nephropathy, and neuropathy are just a few examples of diabetes-related illnesses that need more research into these chemicals. Based on our research, these molecules may have therapeutic promise as multi-targeting antidiabetic drugs for the treatment of diabetes.

Several forms of cancer are more common in people with diabetes than in those without the disease. Insulin glargine is one of the antidiabetic drugs that has been linked to an increased risk of cancer [50]. So, it's certain that medications with anticancer and antidiabetic properties will be developed. Researchers found that quercetin, kaempferol, and a mix of the two greatly reduced the growth of cancer cell lines Huh-7 and HepG2. Even the normal Vero cell line did not show any obvious signs of cytotoxicity. Kaempferol and quercetin's capacity to induce apoptosis and cell cycle arrest in G1 and G2/M, respectively, in cancer cell lines, may explain their anticancer action. One possible action of quercetin is to promote cell death. According to references [31,63], quercetin and kaempferol reduce the proliferation of cancer cells by lowering the levels of miR-21, PKC, COX-2, and PI3K/AKT/mTOR signaling, while increasing the levels of p53 and caspase 3.

4. Materials and Methods

4.1. Reagents and Chemicals

From Gibco (Thermo Fisher Scientific Inc., Waltham, MA, USA), we procured Dulbecco's Modified Eagle medium (cat. number 41965-047), nicotinamide (cat. number AC12827100), penicillin-streptomycin (GibcoTM, cat. number 115140-122), and fetal bovine serum (FBS) (GibcoTM, cat. number 10500-064). Cais-son Labs of North Logan, UT, USA, was contacted to get trypsin-EDTA (TLR01). We acquired streptozotocin (sc-200719A) from Santa Cruz Biotechnology in Dallas, TX, USA. Sigma-Aldrich (St. Louis, MO, USA) supplied all of the chemicals used in the experiment, whereas Merck Co. (Darmstadt, Germany) supplied the ethanol (cat. number 459828). The quercetin and kaempferol were generously supplied by Dr. Nosheen Aslam, an associate professor at GCUF in Pakistan.

Cell Lines (4.2)

In accordance with earlier reports [64], the HepG2, Huh-7, and Vero cell lines were obtained from the National Institute for Biotechnology and Genetic Engineering (NIBGE) in Faisalabad, Pakistan. The cells were subsequently subcultured and kept at the Animal Cell Culture Lab (ACCL). The cells were trypsinized and subcultured on 10 cm cell culture plates after they reached 70-80% confluency. The following was utilized: penicillin (100 µg/mL), streptomycin (100 µg/mL), and Dulbecco's modified Eagle's medium (high glucose with 10% FBS). In a carbon dioxide incubator, cultured cell lines were maintained at a temperature of 37 ◦C, humidity of 95%, and CO2 levels of 5%.

4.3 In-Situ Investigations

4.3.1. Analyzing Drug Metabolism and Activity

The "Lipinski rule of five" (http://www. scfbio-iitd.res.in/software/drugdesign/lipinski.jsp, accessed on 12 January 2022) was used to perform the druggability test. The number 65. The ADMET-based characteristics of both substances and metformin were evaluated using the admetSAR and Swiss ADME online analyzer tools, respectively, as of 12 January 2022 and http://lmmd.ecust.edu.cn/admetsar2/, and http://www.swissadme.ch/, respectively.

4.3.2. Preparation of Proteins and Ligands

A complete set of receptor targets, including CRP, DPP-IV, IL1, PRARG, PTP, and SLGT1, were obtained in PDB format from the RCSB PDB (https://www.rcsb.org/structure, accessed on 30 January 2022). With the help of BIOVIA Discovery Studio 2021, we were able to extract all of the ligands and water molecules from the 3D structures. After including hydrogen and Kollman charges, the data was stored in PDBQT format using MGL Tool 1.5.7. From PubChem (https://pubchem.ncbi.nlm.nih.gov, retrieved on 30 January 2022). The ligand structures were obtained in SDF format. Using PyMol 2.5.4, the SDF format was transformed into PDB, and then stored in PDBQT format [52].

Section 4.3.3.Analysis of Docking

Using AutoDock Vina 1.1.2, the docking analysis was conducted. The parameters of the grid were determined with the help of auto dock 2.5.7. The target, ligand, grid size, and geometry-related data was stored in a *.txt* file. In order to optimize the docking coordinates and grid size, docking analysis was performed independently using the system command prompt. In order to see the results, the PDBQT file was converted to PDB format. BIOVIA Discovery Studio 2021 was used to examine the binding modes [52].

4.2. In Vitro Alpha-Amylase Inhibition Assay

The alpha-amylase enzyme inhibition assay was performed using the DNS method with minor modifications, as described in [45,66]. Forty microliters (40 µL) of the testing sample was added to 160 µL distilled water and 400 µL of 1% (w/v) starch solution.

Subsequently, 200 µL of α-amylase (0.5 mg/mL) was added, and the reaction mixtures were incubated for 3 min at 25 ˚C. After incubation, 200 µL from the reaction tubes was transferred to other tubes containing 100 µL of dinitrosalicylic acid (DNS) reagent. The reaction was stopped by incubating it at 90 ˚C for 15 min. Finally, after adding 900 µL of distilled water, absorbance was measured at 540 nm using a double-beam spectrophotometer, A & E Labs; AE-S90-2D (Guangzhou, China). Similarly, the blank incubation included all reaction components except the testing sample, which was replaced by distilled water. Acarbose was used as positive control. The percentage inhibition of testing samples and acarbose was calculated using the formula below.

$$\% \text{ Inhibition} = 1 - \frac{A_{unknown}}{A_{blank}} \times 100 \quad (1)$$

4.3. In Vivo Studies

4.3.1. Experimental Animals

The male Balb/c mice used in this experiment were purchased from the Laboratory Animals and Research Centre (LARC) in Faisalabad, Pakistan. They were housed in transparent plastic cages and given a standard diet along with an unbroken supply of clean, autoclaved water. The mice had an average body weight of 25-30 g. The conditions were maintained at a consistent 22-25 ◦C temperature and 50-60% humidity, with a 12-hour light-dark cycle. The Ethical Review Committee (Ref: AKT.FST/Misc./2022-31) and the Institutional Review Board (Ref: AKT.FST/Misc./2022-32) have given their stamp of approval for the investigational use of animals in this study. All animals were handled with the utmost care during the studies.

4.3.2. Diabetes Initiation

As previously mentioned, balb/c mice were induced with diabetes after acclimatization using a slightly modified method [67]. Following a 15-minute intraperitoneal administration of nicotinamide at a concentration of 240 mg/kg BW, all mice that had fasted overnight were given a single dose of STZ at 150 mg/kg BW, dissolved in a citrate buffer of 0.1 M. Hyperglycemia that did not occur while fasting was verified after 98 hours of taking STZ-NA. For 28 days, on alternating days, each group of mice received a different dosage of metformin, quercetin, kaempferol, or a combination of the three. Serum samples were taken by centrifuging at 3500 rpm for 10 minutes after the mice were killed at the conclusion of the experiment. The use of a 10% formaldehyde solution was also used for the preservation of organs. Additional organs were preserved at a temperature of -80 ◦C for future examination.

4.3.3. Classifying Animals

The animals were divided up at random. Group 1 received normal saline as control, group 2 received a single dose of STZ i.p + NA i.p (150 mg/kg B.W + 240 mg/kg B.W) served as diabetic control, group 3 containing STZ-NA diabetic mice received an oral dose of quercetin (20 mg/kg B.W), group 4 consisting of STZ-NA diabetic mice received an oral dose of kaempferol (5 mg/kg B.W), group 5 consisting of STZ-NA diabetic mice received the combination of quercetin and kaempferol (10 mg/Kg and (2.5 mg/Kg BW, respectively), and group 6 consisting of STZ-NA diabetic mice received an oral dose of metformin 50 mg/kg served as positive control.

4.3.4. Analysis of Blood Glucose Levels and Sampling

A glucometer (ON CALL Glucometer EZ II) was used to determine the blood glucose level from a drop of blood collected around the neck during sacrifice and blood collection. The strips used were On-Call blood glucose strip LOT 395375, which are routinely used in clinical applications.

(4.3.5) The Lipid Profile

Cholesterol Biomed Lot No. 150621 CHO-104400 and Triglyceride Biomed Lot No. 1701020 TG-1191000, two kits from Ebra in Miami, FL, USA, were used to assess total cholesterol and triglycerides, respectively.

4.3.6. Bilirubin, ALT, and ALP Levels

Ebra Mannheim of Miami, FL, USA, sold all of the diagnostic kits that were used. Bilirubin Ebra Lot No. 2002114 blt-001 was the kit used to test serum bilirubin levels. In order to prove liver damage, the levels of serum ALT and ALP were measured according to the manufacturer's guidelines. The relevant lots were ALT Ebra Lot No. BLT-00052 and ALK.PHOS Human Lot No. 0121 ETI-10700501.

4.3.7 Trans-antioxidant Capacity

The procedure outlined by Erel et al. [68] was used to determine the TAC capacity. The ABTS (2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) molecule is oxidized to ABTS+ in an acidic solution by hydrogen peroxide in this test, which produces a green hue. The number of antioxidants in the sample is closely correlated with the rate of bleaching, which causes the sample to become this hue. In short, acetate buffer (0.4 mol/L pH 5.8) and 5 µL of the sample were combined with 200 µL of reagent 1. In addition, 20 microliters of reagent 2 (ABTS+ in acetate buffer 30 mmol/L at pH 3.6) was added and left to incubate at room temperature for 5 minutes after addition. Following incubation, the samples' TACs were calibrated with Trolox and absorbance was measured at 660 nm. The absorbance of the blank was measured prior to combining the first two reagents. Expressed in mmole of Trolox equivalent per liter, the final results are presented.

4.4. Evaluate Cell Viability

An MTT test is a popular colorimetric method for determining cell viability and proliferation. It stands for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Aiming to identify the impact of quercetin, kaempferol, and their synergistic effect on cancer and non-cancer cell viability, the experiment was conducted. The cell concentration on an ELISA plate was 5 103 cells/well. Cells were treated with several solutions for 72 hours after 24 hours of sub-culturing, including quercetin (36 µg/mL), kaempferol (15 µg/mL) in 1% dimethyl sulfoxide (DMSO), a combination of the two (18 µg/mL of quercetin and 7.5 µg/mL of kaempferol), and doxorubicin (1.8 µg/mL) in water. Triplicates of each therapy were examined. The next step was to remove the media, wash the cells with PBS, and then treat each cell with 25 µL of MTT reagent, which is a 5 mg/mL solution. Using a microplate reader from LTEK Co., Ltd. (Seongnam, Republic of Korea), the formazan crystals were dissolved in 125 µL of DMSO after 4 hours of incubation, and the absorbance was measured at 570 nm [69].

4.5. Analyzing Statistics

For data analysis, GraphPad Prism (GraphPad, 7.07) was used. Unless otherwise indicated, the data are presented as the mean plus or minus the standard error of the mean. Using one-way ANOVA, we were able to identify all of the group differences. The results were deemed statistically significant when the p-values were * < 0.05, ** < 0.01, *** < 0.001, and **** < 0.0001 [70].

5. Conclusions

Based on the results of this research, we can say that quercetin and kaempferol are like antidiabetic superheroes: they help keep blood sugar levels stable, boost lipid profiles, and increase antioxidant status in general. Both chemicals demonstrated inhibitory and regulatory activity against several key targets in the development of diabetes. Molecular docking in silico studies showed that both drugs had stronger binding affinities to CRP, IL-1, DPP-IV, PPARG, PTP, and SGLT-1 than the widely used antidiabetic medicine metformin. Not only that, none of these chemicals has any harmful carcinogenic effects, and they both have good ADMET profiles. Good antitumor activity was also shown by these chemicals.

against cancer cell lines HepG2 and Huh-7, while having no impact on the viability of the non-cancer Vero cell line. The results of this research provide credence to the idea that quercetin and kaempferol might be useful antidiabetic drugs for diabetes treatment that target many targets at once.

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Institutional Review Board Statement: Ethical approval was issued by the Ethical Review Committee (Ref: AKT.FST/Misc./2022-31) and Institutional Review Board (Ref: AKT.FST/Misc./2022-32) on ethical standards in animal experimentation. During experiments, all animals were treated humanely.

Informed Consent Statement: Not applicable.

Data Availability Statement: All the data obtained from experiments are included in the manuscript.

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