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In Vitro Anti-Amylase Activity of Diadzein and Evaluation of its Synergistic Role with Acarbose in Combination

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ABSTRACT

Diabetes mellitus is a worldwide health issue and one of the leading causes of morbidity. The mortality creates a pandemic situation in developing nations. The changes in quality of life and negligence in health care statistics are responsible for increasing the chances of developing diabetes mellitus. Accordingly, there is an upward trend in its occurrence, particularly in industrialized urban zones. Type 2 diabetes mellitus (DM) is characterised by debilitated insulin hormone secretion and increased insulin obstruction. The utilization of therapeutic plants and their phytochemicals for curing diabetes isn't only a quest for more secure options in contrast to pharmaceuticals, which temporarily bring down the blood glucose, forestall coronary illness and hypertension, and enhance the antioxidant system, insulin activity, and discharge. Flavonoids are very useful for their multi-directional activities including therapeutic approaches for diabetes. Numerous studies have been carried out to explore their potential role in the treatment of diabetes. Flavonoids from the biggest family of polyphenolic herbal compounds have many functions to manage diabetes such as proliferating the *β* cell of the pancreas, regulating the insulin signaling pathway, progression of glucose metabolism, etc. With *in vitro* experiments, it has further been established that Daidzein and Eriodyctiol (Abbreviation) inhibit PPA with IC_{50} values of 26 and 22 uM respectively. Their interaction with PPA has also been established by docking studies. Diadzein exhibits noncompetitive kinetics during interaction with PPA with a K_M value of 0.49 mM in contrast to the competitive mode of inhibition of PPA by Eriodyctiol. Moreover, Daidzein augments the inhibitory potential of commonly used amylase inhibitor acarbose in a synergistic way. It inhibits lipid peroxidation, which remains unaffected by the presence of acarbose. So Eriodyctiol and Daidzein (Abbreviation) can be considered suitable alternatives to acarbose in the treatment of Diabetes mellitus.

Keywords: *Flavonoids; Hyperglycemia; Acarbose; Amylase; Combination Therapy; Hyperglycemia*

Introduction

Diabetes: A Global Concern

Diabetes is sure to be among the most crucial medical issues in the 21st century (Chakrabarti & Rajagopalan, 2002). Diabetes mellitus is a heterogeneous disease and has become a curse for the entire world, of which already 100 million people have become victims (Balaji *et al.,* 2019; American Diabetes Organization, 2003). This disease adversely affects other crucial

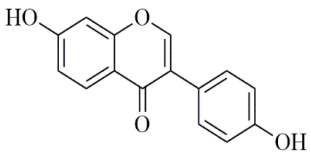
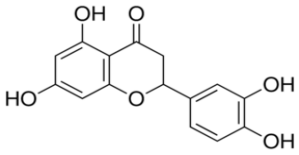
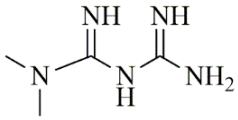
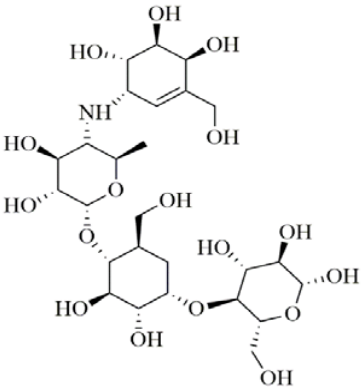
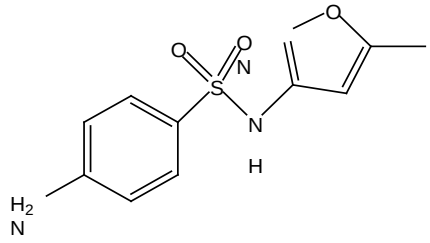
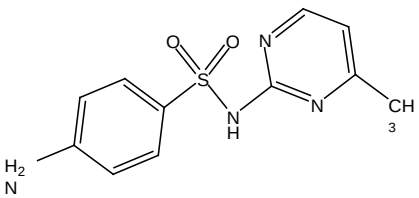
cellular functions namely the cardiovascular system, increasing the risk of heart attack, neuropathy, and retinopathy (Inzucchi, 2002; Boivin *et al.,* 1988; Eyre *et al.,* 2004; Schreiber *et al.,* 2015). Type 2 Diabetes comprises 90% of the diabetic population and is expanding rapidly. At the point when the way of life is gradually becoming too complex, due to poor control of blood glucose, the administration of accurate medicine and proper treatment choices are crucial for type 2 diabetes (Breuer, 2003; DiNicolantonio *et al.,* 2015). To prevent postprandial hyperglycemia which is an outcome of type II diabetes originates from insulin resistance and inadequate insulin secretion from the beta cells of the pancreas. Although several lines of therapies are already in progress, one effective therapy is to reduce the activity of starch degrading enzymes like α amylase to keep control over the amount of free glucose in the system, thereby reducing the glycemic index. Flavonoids have attracted much attention in the past few years for their amylase inhibition potential. Another significant treatment alternative is the use of alpha-glucosidase inhibitor like acarbose, an authorized drug entity for the treatment of prediabetes in numerous nations (Eyre *et al.,* 2004). It has been utilized worldwide for over 20 years for the treatment of type 2 diabetes (Schreiber *et al.,* 2015). Several studies have surveyed the role of acarbose in combating these conditions.

Mode of Function of Alpha Glucosidase Inhibitor

Alpha-glucosidase inhibitors act as competitive or noncompetitive inhibitors of enzymes needed to digest carbohydrates: specifically alpha-glucosidase enzymes in the brush border of the small intestines (Phan *et al.,* 2013; Hung *et al.,* 2012). This group of enzymes hydrolyzes oligosaccharides into simpler form of sugars in the small intestine.

Currently prescribed alpha-glucosidase inhibitor drugs like acarbose, miglitol (synthetic derivative of 1-deoxynojirimycin), metmorfin and voglibose (from microbial origin) are often associated with some undesirable adverse effects such as bloating, diarrhea and abdominal pain if the drugs are prescribed for long term administration to subdue DM (Anisimov *et al.,* 2003; Bolen *et al.,* 2007) (Table 1). These drugs are inhibitory against amylase and drive starch digestion into the colon, followed by fermentation using bacterial enzymes (Lankisch *et al.,* 1998). The production of gases and bloating resulting from the bacterial fermentation of starch causes the unpleasant gastrointestinal side effects typical for this class of anti-diabetic drugs. Metmorfin, another antidiabetic drug, interferes with renal dysfunction and cannot be administered to patients with renal impairment (Inzucchi *et al.,* 2014).

Table 1: Structure of Different Flavonoids and Antidiabetic Drugs

Name of the Drug	Structure of Flavonoids
Daidzein	
Eriodyctiol	
Metformin	
Acarbose	
Sulfamethoxazole (SMX)	
Sulfamerazine (SMZ)	

Drug-Target Relationships: From the Magic Bullet to the Multi-Target Paradigm

The idea of “magic bullets” proposed by Paul Erlich is applicable to the drugs that have been used as single disease target is reoriented to drug repurposing concept (Strebhardt & Ullrich, 2008). The earlier ‘one-drug one-target one disease” technique has driven drug discovery a lot in the late twentieth century and has been successfully established as a targeted therapeutic approach. Repurposing of drug entities bearing broad polypharmacologies, may contribute due to their clinical viability and provides knowledge into the development of new repositioning chances (Talevi & Bellera, 2020; Dudley *et al.,* 2011; Turner *et al.,* 2016). In this regard, the mention of sulfamethoxazole (SMX) (Abbreviation) and sulfamerazine (SMZ) (Abbreviation) with their amylase inhibitory potency is noteworthy in the context of amylase inhibition (Maity *et al.,* 2016; Malla *et al.,* 2021) (Table 1). SMX and SMZ repress porcine pancreatic amylase (PPA) (Abbreviation) in a non-competitive mode with a normal IC_{50} range of 0.94 mM and 0.96 mM individually. Interaction of SMX and SMZ with PPA showed progressive extinguishing of tryptophan fluorescence with shift in lambda max emission (λmax). The reaction of SMX and SMZ with PPA is accompanied by the entropy-driven factor (24.8 cal mol^{-1} K^{-1} and 22.8 cal mol^{-1} K^{-1} respectively) with negative commitment from enthalpy change factor. SMX and SMZ meddle with the action of acarbose in a synergistic mode to decrease the usually prescribed dosage of acarbose as obvious from the *in vitro* PPA hindrance study. In synopsis, loss of PPA action in the presence of SMX and SMZ is characteristic of auxiliary conformational changes of PPA. This is additionally sustained from the schematic model as well as from the docking study performed with the sulfur drug and PPA (Maity *et al.,* 2016; Malla *et al.,* 2021). Several classes of medicines for type 2 diabetes are available in the market including acarbose, metmorfin, voglibose etc. Each class of medicine targets to neutralize the blood glucose level. Additionally medicinal plants are also included as a useful resource of ingredients namely isoflavonoids that can be a boon for the arena of drug development in the cure of wide variety of diseases (Phan *et al.,* 2013; Hung *et al.,* 2012; Formica & Regelson, 1995). Diadzein, a plant estrogen, derived from soya products is effective against diseases like cardiovascular, breast cancer and diabetes. However whether diadzein targets amylase and can serve as an antidiabetic drug with amylase inhibitory potential is not known (Table 1). Earlier studies depicted that diadzein is neither associated with improved insulin sensitivity nor regulatory role on the control of glycemic (Gobert *et al.,* 2010; Ye *et al.,* 2015). At this point, it is crucial to check the role of diadzein as an amylase inhibitor, the type of inhibition by this molecule is required in order to use this molecule for therapeutic purpose. Flavonoids like diadzein and eriodyctiol either work exclusively or in combination with other conventional drug molecule, to produce satisfactory pharmacological effect. This might nullify the discomfort associated with the administration of antidiabtic drugs like acarbose. This may offer a new approach to the diabetic patient care.

Materials and method

Molecular Docking: The procedure of assessing binding energy and the chemistry of protein-ligand binding is carried out by docking ligands to proteins and analyzing the three-

dimensional structure of the molecule using different software. The various steps that need to be conducted are mentioned as follows:

Ligand Screening

The initial screening of the Ligands calls for the use of the SwissADME program available on the web (https: //www.swiss adme.ch/) that helps for the assessment of selected compounds (Daina *et al.,* 2017).

Protein Preparation and Active Site Determination

The protein of interest for the study is amylase (PDB ID- 2QMK). The protein structures are retrieved from the website rcsb.org, commonly known as the Protein Data Bank (Berman *et al.,* 2000) in .pdb format. To carry out protein-ligand docking, the protein needs to be prepared by conducting several edits of the structure. The modifications include the elimination of water molecules (since water molecules can interrupt the binding site of the protein) and attached ligands from the protein using Auto Dock Tools (Version 1.5.6) (Trott & Olson, 2009; Formica & Regelson, 1995) software. Further modifications include the addition of partial charges and polar hydrogen atoms. 3D conformations of the ligand molecules were downloaded from the web-based site called PubChem. The .sdf files of the ligands were converted using PyMOL (4.6.0) (Schrödinger, 2015; Balaji *et al.,* 2019). Ligand preparation was carried out with the help of AutoDock Tools (1.5.6) wherein Gasteiger charges were attached to the ligand and the hydrogens of non-polar nature were unified. The ligand molecule was then saved in the. pdbqt format.

Molecular Docking Using Auto Dock Vina (4.2.6)

The initial step that is to be followed to dock protein and ligand structures is grid preparation. It was done for the protein using AutoDock Tools (1.5.6) (Trott & Olson, 2009). The grid box is an indicator of the region where the docking will take place. The grid dimensions were obtained for the amylase molecule. It acts as a prerequisite for conducting docking in the software. Finally, in-silico docking was done using AutoDock Vina (4.2.6) (Trott & Olson, 2009). The docking results were calculated by Auto dock vina using its Scoring function and results were displayed in the form of Scores and RMSD values.

Residue Analysis

PyMOL (4.6.0) and Discovery Studio 2021 were used for visualization of interactions of the docked structure at the ligand sites and Discovery Studio was used to study the interactions between the enzyme and the ligands (Biovia, 2015).

Amylase Assay

All the reagents including Daidzein, Eriodictyol and Porcine pancreatic α-amylase were purchased from Sigma (St. Louis, MO, USA). Acarbose was procured from Bayer (Leverkusen, Germany). All are of analytical grade.

Porcine pancreatic α-amylase was taken in 50 mM phosphate buffer saline, pH 7.0. The flavonoids in different concentrations were dissolved in DMSO. The assay was conducted following standard protocol using the following formula (Maity *et al.,* 2016).

% Inhibition = [ΔAbs_{540} control - ΔAbs_{540} sample] x 100

The extent of PPA inhibition had been calculated as IC_{50} values (Inhibitory concentration of the flavonoid required for 50% inhibition of the test samples).

Determination of the PPA Inhibitory Mode of Action of Flavonoids

The kinetics of inhibition of flavonoids against PPA have been determined with diadzein and eriodictyol for a range of substrate concentrations in the absence or presence of different concentrations of ligands. The mode of inhibition had been determined from the nature of the curve using the Lineweaver-Burk plot. Respective Km (dissociation constant) and V_{max} (maximum reaction velocity) values for each of the flavonoid had been estimated from the slope and intercept of the curve by plotting the inverse of PPA reaction velocity (V) versus 1/ [substrate (starch) concentration] (Johnson, 2013).

Effect of Daidzein *in Vitro* PPA Inhibition in Combination with Acarbose

To judge the effect of Diadzein to inhibit PPA action, daidzein was applied alone at the respective half inhibitory concentrations (IC_{50}) in presence of acarbose (3 µM and 6 µM). % of PPA inhibition had been estimated by the usual DNS assay as described earlier.

In Vitro Anti-Lipid Peroxidation Assay in Presence of Flavonoids

Lipid peroxide formation has been measured by standard protocol (Malla *et al.,* 2021). The reaction is monitored after the addition of FeSO4 followed by an incubation of 20 minutes at 37°C temperature in the presence of diadzein. The intensity of the pink colour developed was measured at 535 nm. The percentage of inhibition by flavonoids had been calculated as stated above.

Results

Molecular Docking Study of Daidzein, Eriodyctiol and Metformin with Amylase

Screening of Ligands

The SwissADME online tool was used to assess the properties of different ligands namely Daidzein and Eriodyctiol in comparison to Metformin. The results have been summarized in Table 3. The analysis shows high bioavailability scores in the case of all the ligands used. Bioavailability can be defined as the drug or molecule that is available in the blood circulation and is responsible for carrying out the intended functions. Daidzein and Eriodyctiol show no violation of the Lipinski Rule which asserts their potential use as viable drugs Also, Metformin shows viable bioavailability scores coupled with zero violations of the Lipinski rules.

Table 2: Bioavailability Score and Radar Charts of Selected Ligands

Ligand	Bioavailability Score	Lipinski Rule	Radar
Eriodyctiol	0.55	Yes; 0 violation	

Daidzein	0.55	Yes; 0 violation	
Metformin	0.55	Yes; 0 violation	

Table 3: Average Docking Score of Ligands with the Confidence Interval (The Docking Scores Were Obtained Upon Running the Program)

Compound name	Average Binding Energy (Kcal/mol)	Std Deviation	Average Sample Size
Diadzein	-8.08	0.495	5
Eriodyctiol	-8.62	0.370	5
Metformin	-5.14	0.149	5

Interactions of the Ligands and Amylase Using Molecular Docking:

Daidzein shows mostly Pi-Pi stacked interaction with aromatic amino acid residues along with a single hydrogen bond with Aspartic acid. Eriodyctiol interaction with amylase is dominated by hydrogen bonds with polar amino acid residues. Metformin accounts for five hydrogen bonds, Vander Waals interaction, and unfavourable donor-donor interaction.

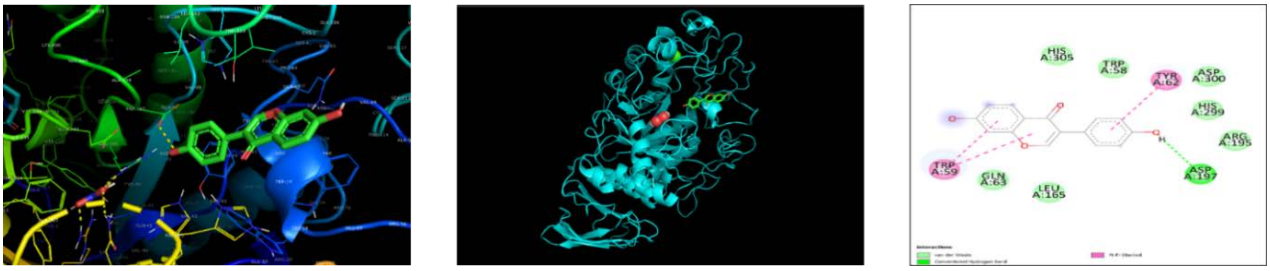


Figure 1A: Interaction between Daidzein and Porcine Pancreatic Amylase Amino Acid Residues B. Interaction between Daidzein and Amylase; C. 2-D Interaction between Daidzein and Amylase

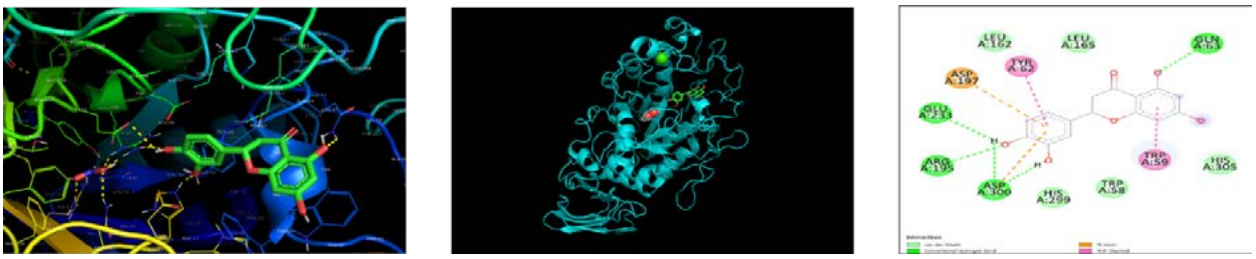


Figure 1B: Interaction between Eriodyctiol and Amylase Amino Acid Residues B. Interaction between Eriodictyol and Amylase; C. 2-D Interaction between Eriodyctiol and Amylase

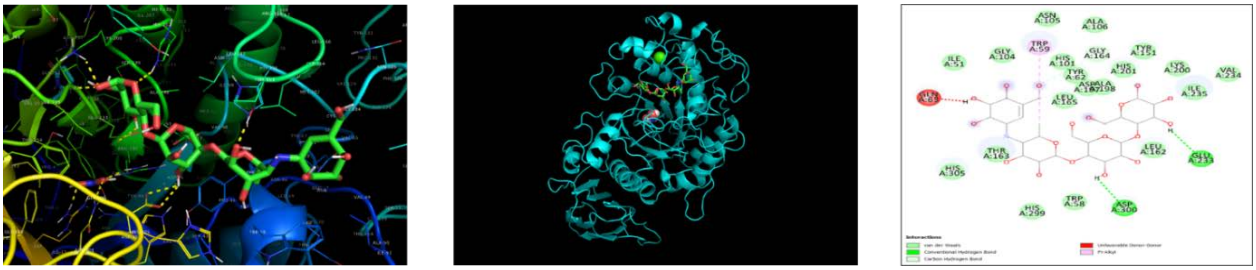


Figure 1C: Interaction between Metformin and Amylase Amino Acid Residues B. Interaction between Metformin and Amylase; C. 2-D Interaction between Metformin and Amylas

Inhibition of PPA Activity by Flavonoids:

Starch digestion by alpha-amylase is mediated through the formation of β-glycosyl enzyme intermediate involving acidic carboxylic acids namely, Asp197, Glu233, and Asp300 present in the catalytic cleft followed by its hydrolysis. From a recent in silico study, involvement of residues of the enzyme including Phe178, Phe303, His280, His351, Arg315, Arg442 and Tyr158 are evident during interaction with the inhibitors (Ernawati *et al.,* 2018). Polyphenolic compounds like flavonoids are implicated as one of the important groups of amylase inhibitors (Lo Piparo *et al.,* 2008; Islam *et al.,* 2020). Interaction between flavonoids and α amylase is facilitated by the presence of several hydroxyl groups on the B ring of the flavonoid skeleton. Hydrogen bonding between the hydroxyl groups in position R6 or R7 of the ring A and position R4/ or R5/ of the ring B of the flavonoids and the catalytic triad (Asp197, Glu233, and Asp300 present in active cleft) is further stabilized through conjugated π-system of the flavonoids (Lo Piparo *et al.,* 2008).

Daidzein is an isoflavone with a B ring attached at 3 positions of the pyrene C ring. The richest sources of this flavonoid are soybeans and soy products. Eriodictyol is a flavanone, bitter in taste, and extracted from a plant, Yerba Santa (Eriodictyon californicum) (Deng *et al.,* 2020). It is also abundant in citrus fruit. This flavanone is of immense health importance protecting against diabetes, cancer, neuro diseases and hepatic insult. Eriodictyol enhances insulin productivity thereby stimulating glucose-utilization aiding cAMP/PKA signaling pathway (Hameed *et al.,* 2018). However, their anti-amylase activity is not explored so far. In the present study, all the flavonoids suppress PPA activity in a dose dependent manner with respective IC_{50} values in the range of 20-26 µM Figure 2, Table 4.

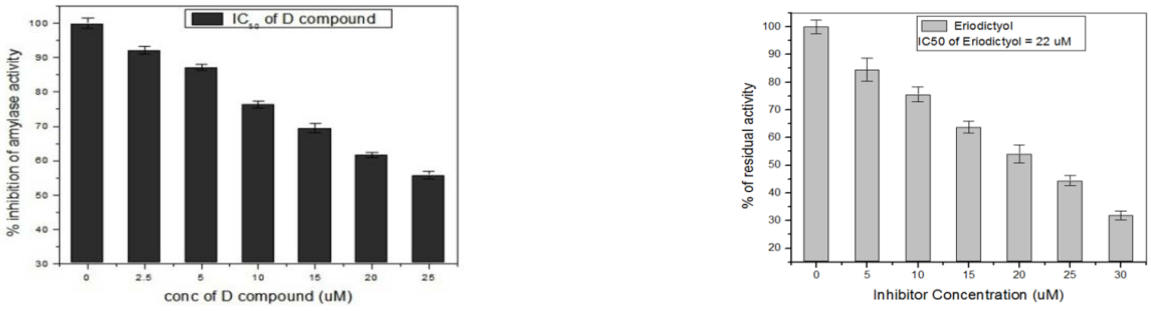


Figure 2: Inhibition of PPA Activities by Flavonoids (A. Diadzein; B. Eriodictoyl)

Estimation of PPA inhibitory action of flavonoids: To further explore the kinetics as well as the nature of inhibition of PPA by the flavonoids, a double reciprocal plot has been plotted for Diadzein and Eriodictyol. Km and V_{max} values derived from each of the kinetic studies have been presented in Table 4. From the result, it is evident that eriodyctiol competitively inhibits PPA whereas daidzein executes noncompetitive mode of inhibition on PPA action.

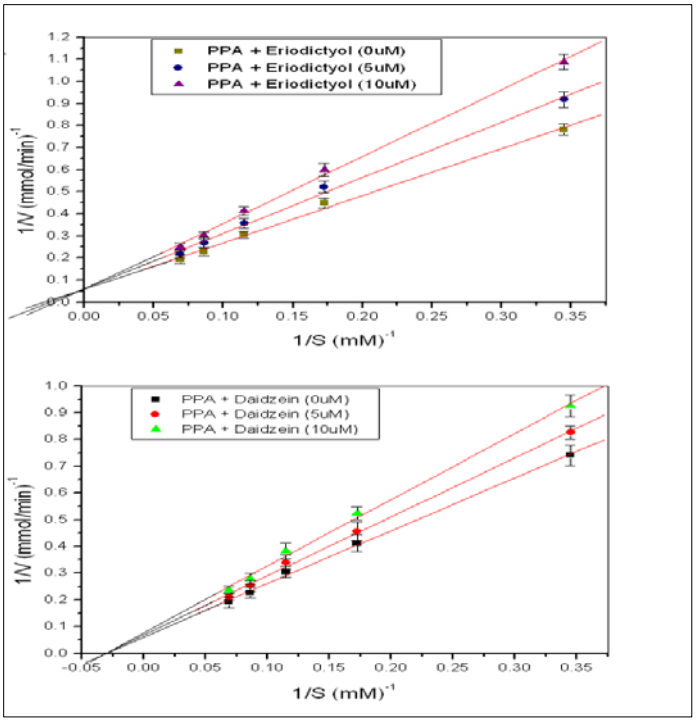


Figure 3: Kinetics of PPA Inhibitions by Flavonoids (A. Eriodictyol, B. Daidzein) by Lineweaver-Burk Plot

Table 4: Kinetic Parameters of PPA Inhibition by Flavonoids

Flavonoids	**K_M (mM)**	**Nature of inhibition**	**IC_{50}**
Diadzein	0.49	Diadzein	26 uM
Eriodictyol	0.41	Competitive	22 uM

Lipid Peroxidation Inhibition and Acarbose in Combination with Diadzein:

There are various reports that indicate reactive oxygen species (ROS) (Abbreviation) has played a crucial role in maintaining cellular homeostasis. Any alteration of the ROS level leads to cell death. ROS are very transient, reactive and unstable in nature. There are varieties of polyunsaturated fatty acids (PUFA) in the cell membrane that help to maintain membrane integrity. There are various reports states that reactive oxygen species (ROS) sometimes target these polyunsaturated fatty acids and peroxidises and as a result cell membrane loses its integrity. ROS is having short half-life, albeit their oxidation products are relatively stable. So they are good indicator of oxidative stress. When ROS attack polyunsaturated fatty acids (PUFAs) of the membranes, they give rise to highly reactive aldehydes. These lipid peroxidation products covalently bind to specific amino acids, commonly histidine, cysteine, or lysine following Michael addition. The resulting modification generates alteration in protein structure with functional impairment. The role of lipid peroxidation affects pathophysiology and aggravates chronic diseases namely Alzheimer's disease, diabetes mellitus, hypertension, and cancers in affected populations (Erejuwa *et al.,* 2012; Butterfield *et al.,* 2010; Zanini *et al.*, 2013). Plethora of information being available deciphering the protective role of diadzein to decline the oxidative stress, lowering of serum triglyceride level and reduced inflammation prompted to evaluate its anti-amylase inhibitory role (Zanini *et al.,* 2013). However, it is ineffective to reduce the glycemic index as reported by (Gobert *et al.,* 2010) mentioned earlier. In order to maintain the protective role of diadzein, any possible interference by acarbose is conducted on the anti-amylase activity of daidzein during lipid peroxidation.

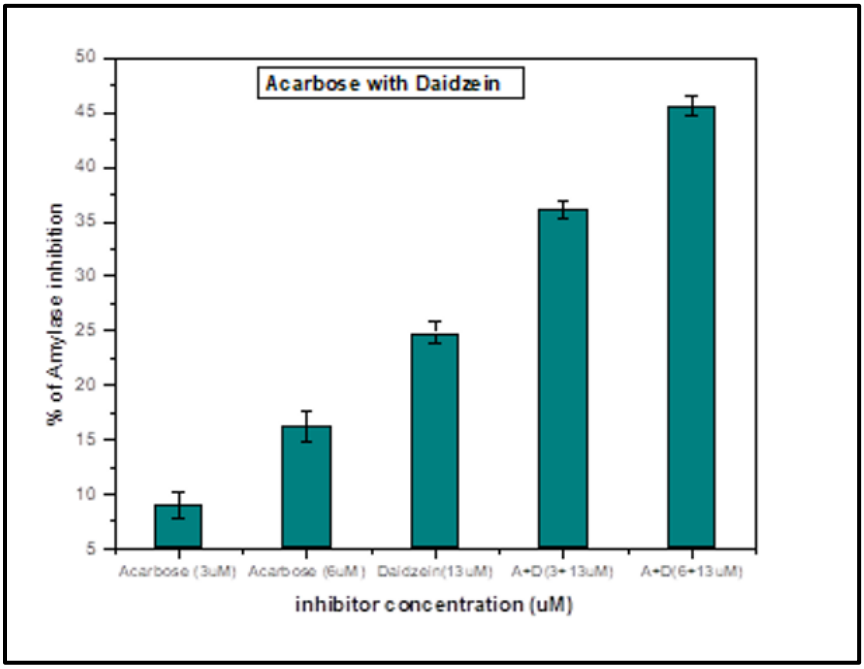


Figure 4: Synergistic Inhibitory Action of Diadzein towards PPA Activity in Presence of a Reduced Dose of Acarbose

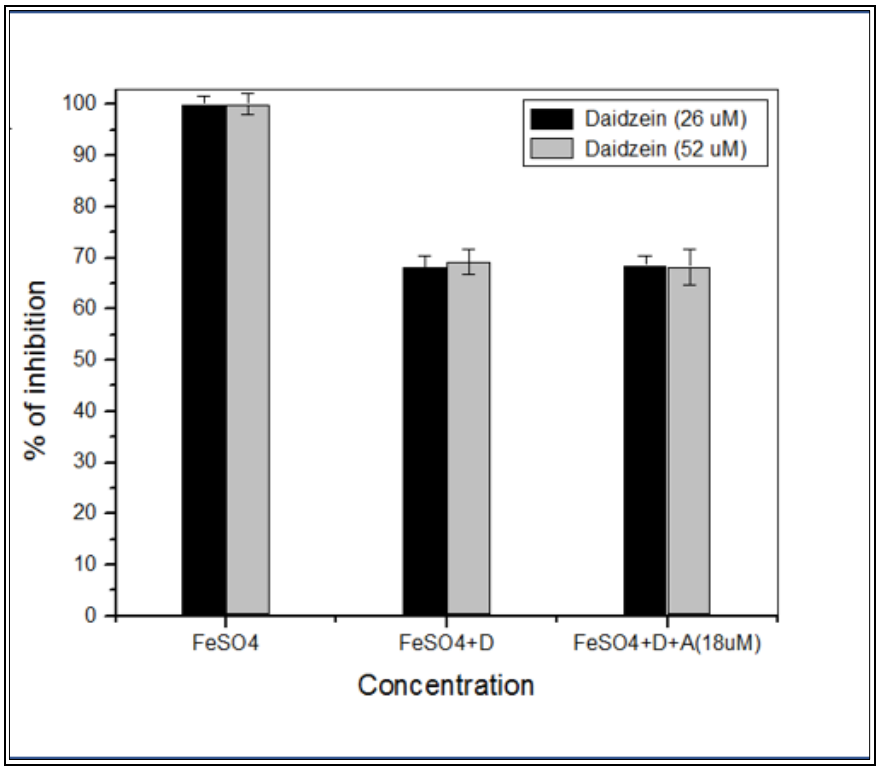


Figure 5: Inhibition of Lipid Peroxidation by Daidzein in Absence and Presence of Acarbose

Lipid peroxidation assay has been performed in the presence of diadzein at its respective IC_{50} values (26 µM respectively) as deduced from Table 4. No remarkable reduction in the inhibition of lipid peroxidation by D has been manifested when carried out in presence of acarbose (18 µM = 1/2 of the IC_{50} value of acarbose for PPA inhibition). The extent of lipid peroxidation induced by FeSO4 has been taken as 100% which has been diminished to 68.1% in the presence of 26 µM Diadzein (IC_{50} for PPA inhibition). As lipid peroxidation is not influenced by the presence of acarbose, so *in vitro* combination study with diadzein and acarbose can be considered together might produce encouraging results during PPA inhibition (Figure 5).

Conclusion

This *in vitro* report of PPA inhibition by deidzein is to be extrapolated to in vivo studies with several diabetic volunteers and should be judged in detail in various combinations of other flavonoids with well-known diabetic drugs like acarbose and metmorfin or with other amylase inhibitors. Flavonoids occupy a key position in the management of critical diseases like diabetes and cancer, with minimal side effects as referred to by several earlier reports. Since they are embedded with several health-promoting activities like antimicrobial, anticancer, antidiabetic, and anti-lipid peroxidation potential, their inclusion in day-to-day life proves to be very much beneficial. They can be considered as natural repurposed natural medicine for the treatment of Diabetes, the deadly one especially in post covid tough situation.

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