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INHIBITION OF α-AMYLASE AND α-GLUCOSIDASE ACTIVITY BY EPIGALLOCATECHIN-3-GALLATE (EGCG) A POLYPHENOL COMPONENT OF GREEN TEA, *CAMELLIA SINENSIS* AND A KNOWN HSP90 INHIBITOR

Jyoti Patki*, Madhuresh Raj, Thankamani Marar, Suchismita Mazumdar

School of Biotechnology and Bioinformatics, D Y Patil deemed to be University, Navi Mumbai, India

**Corresponding author: jyoti.tope@dypatil.edu*

ABSTRACT

Current diabetic therapy suffers from the adverse effects and limited pharmacologic spectrum of synthetic drugs. Starch digestive enzyme inhibitors have emerged as attractive targets in the management of hyperglycaemia but the discovery of target modulators has not been as promising. Plant derived inhibitory compounds especially polyphenols are gaining increasing scientific attention for their potential to control obesity and achieve glycaemic control while being consumed as a component of normal diet. The present study is an attempt to validate the anti-diabetic prospects of a natural source Heat shock protein 90 (Hsp90) inhibitor Epigallocatechin-3-gallate (EGCG) (a polyphenolic constituent of *Camellia sinensis* or green tea). Multiple modulatory effects beneficial in diabetes, cardiovascular diseases and cancer have been attributed to EGCG. However, the exact molecular mechanisms underlying these effects are still unclear. Here we have employed *in vitro* assays to test the direct interaction of EGCG with α-amylase and α-glucosidase that are established hyperglycaemic targets. The activity of these enzymes from different sources (pancreatic, intestinal and liver) was assayed in the presence of EGCG. Acarbose a standard anti diabetic drug was used as control. Our study showed that EGCG significantly inhibited both enzymes with greater potential for α-glucosidase inhibition. Kinetics studies predicted non-competitive inhibition. This study lays strong evidence to anti-diabetic property of EGCG by confirming one of the many proposed mechanisms underlying its biological effects.

Keywords: EGCG, diabetes, hyperglycaemia, α-amylase, α-glucosidase, Hsp90.

1. INTRODUCTION

Hyperglycaemia is the major cause of diabetic complications and long-term therapeutic regimen benefit from glycaemic control [1]. Glucosidases are enzymes of the digestive tract strategically situated tobreak down the dietary carbohydrates into simple absorbable mono-saccharides. Inhibition of these enzymes retards the rate of carbohydrate digestion and delays the carbohydrate absorption thereby reducing post-prandial hyper-glycaemia [2, 3]. Liver glucosidases inhibitors reduce the rate of glycogenolysis thereby decreasing blood glucose levels as a short-term effect and marginal reduction in haemoglobin A1c levels as a long-term effect [4].

Glucosidase inhibitors therefore constitute an important part of the consortium of anti-diabetic agents. However, adverse side effects including diarrhoea, gastro-intestinal distress, meteorism and pronounced hypoglycaemia at high doses have been reported for several leading glucosidase inhibitors like acarbose, miglitol and voglibose [1, 2, 5]. Plant derived compounds should be principally more acceptable sources of safe and effective enzyme inhibitors due to their natural origin, rich chemical diversity and low cost of preparation. Moreover, plants that are resistant to insect and microbial pathogens are important sources of glucosidase inhibitors [6, 7]. Polyphenolic components of plants like guava, shirazi thyme, neem and Malabar plum have been studied for their effectiveness against carbohydrate digesting enzymes [8, 9].

Green tea (*Camellia sinensis*) is known to have several health benefits. The leaves of green tea contain a wide spectrum of polyphenols known to show anti-diabetic effect through α amylase inhibition. Epigallocatechin-3-gallate (EGCG) that accounts for 80% of catechin content of green tea has been reported to inhibit α glucosidase (EC 3.2.1.20) and α amylase (EC 3.2.1) by direct interaction with these enzymes [10, 11]. EGCG is also shown to possess anti-tumour activity through

association with the molecular chaperones, heat shock protein 90 (Hsp90) and hsp70 and modulates the activity of several cancer associated cell signaling protein kinases and receptors [12, 13]. Although there have been reports of glucosidase inhibition by plant polyphenolic extracts containing EGCG, the effect of EGCG in isolation is not very clear. Greater interest in EGCG effect also stems from the possible interaction of this catechin with other anti-diabetic targets given its multiple targeting potential. In the present study we tried to address the question of whether the EGCG as a major component of green tea extract could result in pharmacologically significant inhibition of glucosidase enzymes. Alpha amylase and alpha glucosidase enzymes of chicken origin were used and inhibition was evaluated through *in vitro* assays. The results were compared with the standard glucosidases inhibitor, Acarbose. The mechanism of inhibition was elucidated through enzyme kinetic studies.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Pancreas, liver and intestinetissues of chicken were obtained from the local poultrycentre and were immediately transferred to the laboratory packed in ice-box maintained at 4°C. All the chemicals and reagents used in the present work were of analytical grade or of ultrapure grade. Sodium chloride,starch, acetic acid, 3,5-dinitrosalicylic acid (DNS), p-nitrophenyl d-gluco-pyranoside (p-NPG), sodium carbonate, monosodium and disodium phosphate were purchased from Sisco Research Laboratories Pvt Ltd (SRL). EGCG was purchased from Now Foods as green tea extract capsules (200 mg/capsule). Dimethyl sulfoxide (DMSO) was purchased from HiMedia Laboratories (Mumbai, India) Acarbose (Glucobay®) was purchased from Bayer Pharmaceuticals Pvt Ltd (Mumbai, India). Spectroscopic readings were taken on UV visible Spectrophotometer (Biochrom Libra S70 double beam spectrophotometer, Biochrom Pvt Ltd, Cambridge).

2.2. Preparation of crude enzyme extract from pancreatic, liver and intestinal tissues

For preparation of crude α-amylase and α-glucosidase extract the chicken tissue (pancreas and liver for amylase and intestine for glucosidase) were washed free of blood with 0.9% NaCl, cleared of other tissues and 200 mg was weighed and homogenized in 10 ml of ice-cold phosphate buffer (0.2M, pH 7.4). The tissue homogenate was then centrifuged at 8000 rpm for 10 min at 4°C. The supernatant collected was used as source of crude enzyme [8, 14].

2.3. Enzyme inhibition screening starch-agar plate assay

Stock solutions of the compounds Acarbose (standard) and EGCG (test) were prepared in dimethylsulfoxide (DMSO). Various concentrations were made from the stock to be used in inhibition assays (10, 20 and 50µM).

Clear supernatant obtained after tissue homogenization and centrifugation was screened for enzyme activity by starch agar plate method. Plates with agar containing 2% starch were prepared. For control, each enzyme-extract 50 µl and 50 µl of phosphate buffer (20 mM pH 6.9) was added in the wells and incubated at 37°C for 30 min. Plate was stained with iodine solution and plates were inspected for appearance of zone of clearance.

For inhibition studies, 50µl of inhibitor (different concentrations of Acarbose or EGCG) was placed in the well and incubated at 37°C for 20 minutes and then 50 µl of enzyme solution was added. The plates were incubated overnight and then stained with the iodine. The area of zone of clearance was compared [15]. Each incubation was conducted in triplicate.

2.4. α-Amylase inhibitory assay:

The α-amylase inhibition assay was performed using the method described by Miller [16] with some modifications. Every concentration of the standard and test compounds was incubated with the enzyme extracts (pancreas and liver) of fixed protein concentration at 37°C for 30min. Preparations with enzyme extracts and buffer without the inhibitor were treated similarly and used as control. After pre-incubation, 1% starch solution was added as substrate and further incubated at 37°C for 30min. The reaction was then stopped by adding dinitro-salicylic acid (DNS, colour reagent) and heating the reaction mixture in a boiling water bath for 10min. All the tubes were cooled and the absorbance was measured at 540 nm. One unit of enzyme was defined as the amount of enzyme needed to produce 1µM of maltose under assay conditions.

The percentage inhibition of enzyme was calculated using the following formula,

$$(\%)inhibition = \left(\frac{Abs(Control) - Abs(Sample)}{Abs(Control)}\right) \times 100$$

The 50% inhibitory concentration (IC_{50}) for pancreatic and liver α-amylase was determined from plots of

percent inhibition versus inhibitor concentration. Each incubation was conducted in triplicate.

2.5. α-Glucosidase inhibitory assay:

Supernatant of the intestinal tissue homogenate was used as α-glucosidase enzyme extract. For each concentration of standard and test compound, pre-incubation with enzyme extract at 37°C for 15 min was done. Enzyme extracts with 0.1 M phosphate buffer (pH 6.5) and no inhibitor were considered as control. The reaction was initiated by adding p-nitrophenyl-d-glucopyranoside (pNPG) (30 mM) as substrate. Following incubation at 37°C for 15 min, the reaction was terminated with addition of 1 M Na_2CO_3. The absorbance was measured at 410 nm. Enzyme activity was determined based on the amount of p-nitrophenol produced.Percentage inhibition was determined as described for α-amylase. The 50% inhibitory concentration (IC_{50}) for intestinal α-glucosidase was determined from plots of percent inhibition versus inhibitor concentration.Each incubation was conducted in triplicate.

2.6. Determination of Km and Vmax

The kinetics of α-amylase and α-glucosidase enzymes were studied by using a fixed enzyme concentration (in this case the whole un-diluted enzyme extract), fixed standard and test compound concentrations in the reaction mixtures and fixed incubation time. The substrate concentrations ranged from 5 to 30mg/mL in the final assay volume. The absorbance was recorded at 540 nm and the amount of substrate produced was determined from the maltose/p-nitro phenol standard curve (0-1 mM). The mode of inhibition was defined on the basis of kinetic parameters Km and Vmax by plotting Lineweaver-Burk plot [17] Dixon plots were constructed to determine inhibition constant Ki for Acarbose and EGCG [18].

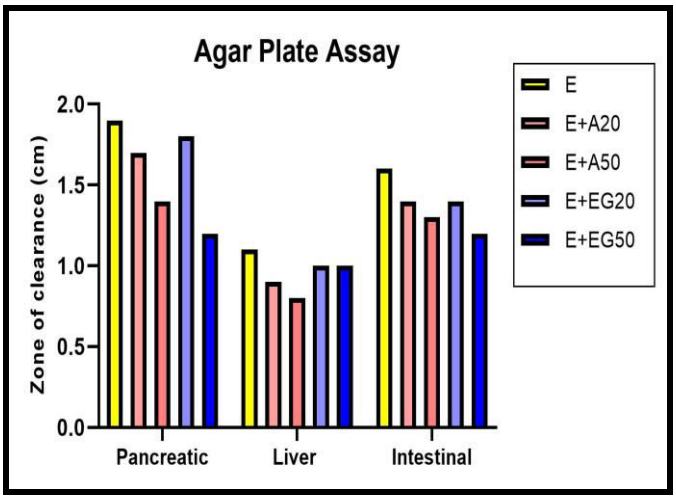
2.7. Statistical Analysis

Most data were expressed as the mean ± SD of three replicates. Graphpad Prism 8 software was used for analysis of data. Unpaired t test was used to evaluate the possible differences among the means and p values <0.05 were considered as significant.

3. RESULTS AND DISCUSSION

This study intended to throw light on the presence and nature of glucosidase enzyme inhibitory potential of the major catechin-3-gallate of green tea, EGCG. Being a dietary component EGCG is an attractive candidate for inhibition of digestive enzymes. At the same time being an Hsp90 inhibitor of natural origin there is a barrage of scientific speculation supporting its anti-diabetic potency. In one of our own studies, we have demonstrated that Gedunin, another natural-product Hsp90 inhibitor strongly interacts and modulates six different molecular targets of diabetes [19]. Encouraged by both these cause-effect theories the present study was outlined to verify the effect part of the hypothesis.

Pancreatic and intestinal glucosidases are the primary targets of modern diabetic therapy as their inhibition causes effective retardation of glucose absorption and reduction in post-prandial hyperglycaemia (PPHG). Additionally, inhibition of liver glucosidases reduces hepatic glycogenolysis further contributing to reduction in blood glucose [8]. The controlled hyperglycaemia leads to reduced haemoglobin glycation as a long-term effect [20]. In this study we used pancreatic, intestinal and liver glucosidases of avian origin which were used in the form of respective tissue homogenates. Acarbose which is an established glucosidase inhibitor was used as positive control to enable comparison of inhibitory activity of EGCG.



E- only enzyme, E+A20/ 50- enzyme with Acarbose (20 μM)/(50 μM), E+EG20/ 50- enzyme with EGCG (20 μM)/(50 μM)

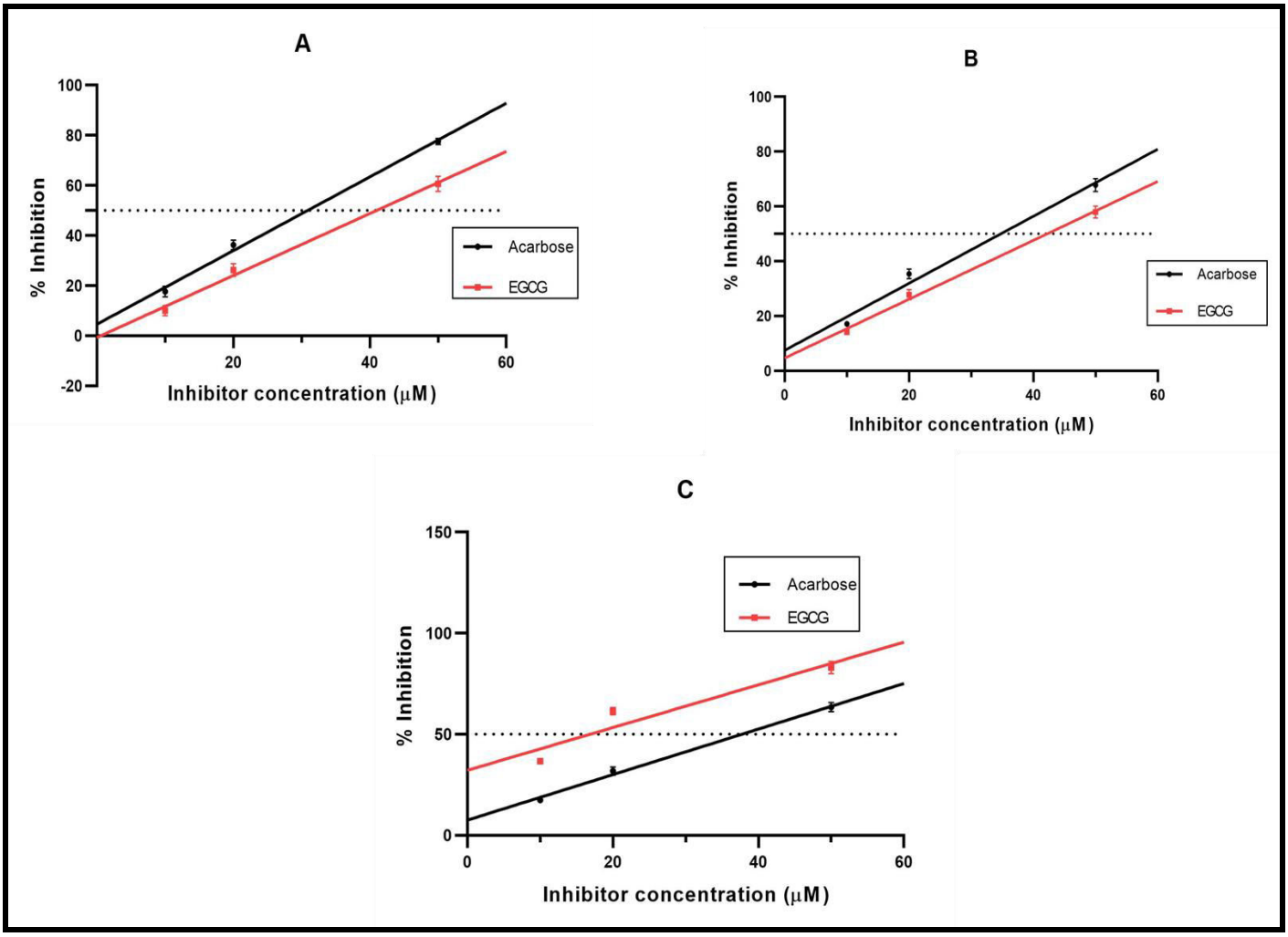
Fig. 1: Agar plate screening assay for enzyme activity of the tissue extracts and enzyme inhibition of inhibitor compounds

The preliminary agar-plate analysis results indicate reasonable activity for the three crude enzyme extracts making them passable to be used in the quantitative inhibitory assays (**Fig. 1**) Distinct inhibition of all three

enzymes by both Acarbose and EGCG was evident from the depletion in zone of clearance diameters. The agar plate analysis was treated only as a qualitative screening step, the results of which would be substantiated by quantitative inhibitory assays. Nevertheless, a dose dependent effect was clearly observed.

3.1. α-Amylase and α-Glucosidase inhibitory assay

The inhibition by EGCG and Acarbose was studied from the dose-reponse curve (Fig. 2). IC_{50} of EGCG for pancreatic and liver α-amylase was 40 µM and 42 µM while that for α-glucosidase was found to be 16 µM. This was significantly lower in comparison to amylase enzymes ($p<0.01$) as well that of Acarbose for the same enzyme ($p<0.05$). The decrement in enzymatic velocity of the enzymes was found to be inhibitor concentration dependent. Nearly 83% inhibition of intestinal α-glucosidase was seen at the highest tested dose of 50 µM by EGCG. In comparison the pancreatic and liver amylase inhibtion was only 60.64% (pancreas) and 57.94% respectively for EGCG (50 µM). The pancreatic and liver enzyme inhibition was comparable to that of the standard inhibitor, Acarbose (IC50; 35 µM).



(A) pancreatic α-amylase (B) liver α-amylase (C) intestinal α-glucosidase by Acarbose (10, 20 and 50 µM) and EGCG (10, 20 and 50 µM). Inhibitor concentration values corresponding to 50% inhibition indicate IC_{50}. Data points are expressed as mean ±SE, n=3

Fig. 2: Dose-response curve showing dose-dependent inhibition

The findings indicate that EGCG is a strong intestinal α-glucosidase inhibitor while pesenting moderate to weak α-amylase inhibition. Similar observations were reported by Yilmazer-Musa (2017) [1] where isolated

catechins including EGCG exhibited less than 50% enzyme inhibition at the highest dose tested for α-amylase. On the other hand with IC_{50} values lower than Acarbose, EGCG was documented to be the most potent inhibitor of α-glucosidase among all catechins studied and resulted in complete inhibtion of the enzyme. The relative potency of EGCG against α-glucosidase is reasoned to be stucture based such that the presence of esterified gallate group explains the good interaction with α-glucosidase while lack of correct stereospicifc orientations of C, A and B rings in flavan-3-ols limits α-amylase catalytic site interaction [21-23].

Besides inhibition of alpha amylase and glucosidase EGCG has also been reported to cause inhibtion of the enzyme catalysing gluconeogenesis, Pepck through the activation of AMP-activated potein kinase [10].

A study on the carbohydrate digesting enzyme inhibition of aqueous extracts of *S oblonga* also documents more inhibitory effect towards alpha glucosidase than alpha amylase. The inhibitory activity is credited to the secondary metabolite components of the extract like tannins, flavonoids and terpenoids [24]. It has also been pointed out that as alpha glucosidase inhibition would affect not only carbohydate digestion but also the rate of monosaccharide absorption, its inhibition is more critical in diabetic control [25]. A single dose of green tea extract taken with test meal was reported to decrease starch digestion and absorption in humans by a study published in scientific reports [26].

Interestingly, Acarbose itself was slightly less potent in its effect to inhibit α-glucosidase as compared to α-amylase. The IC_{50} of Acarbose for α-glucosidase was 37μM (Fig. 2) compared to the 35 μM for α-amylase. Similar finding was repoted by Griffith T (2015) [27]. This study attributed the difference in the affinity for the two enzymes to the difference in the binding propeties of the large saccharide chain of Acarbose. In spite of being specifically prescibed for α-glucosidase inhibition of disaccharide digestion, weaker inhibitory effect of Acarbose on α-glucosidase is reported by many studies.

The α-glucosidase inhibition of tea extracts was attributed to the synergistic effect of polyphenols and other constituents in a study evaluating the potential of green, black and oolong tea [2]. Significant inhibitory effects of catechins have been reported and the galloyl moiety is considered critical for the effect [28] An IC_{50} value of 246 μg/ ml for α-glucosidase was reported for lipophilic EGCG derivative (L-EGCGd) which was also shown to significantly reduce plasma glucose levels [29]. Kamiyama O et al. (2010) have documented good rat intestinal maltase inhibition by EGCG with an IC_{50} of 16 μM and rabbit glycogen phosphorylase with IC_{50} 34 μM. The study proposes dietary supplementation of gallated catechin in diabetic control [30].

There is a great variation in the IC_{50} values documented by different studies with respect to both EGCG as well as Acarbose. An extensive study of alpha amylase inhibition by dietary polyphenols has shown that changes in the type of substrate and concentration affect the apparent potency of an inhibitor. Even when Acarbose was used as inhibitor, the reported differences in the IC_{50} values ranged from 0.9 to 23,100 μM [31]. The mechanism of inhibition also contributes to the differences and therefore, IC_{50} values of EGCG for amylose and amylopectin were observed to be 5 μM and 60μM respectively in the same study. These factors should account for the variation in the calculated IC_{50} values in different reports. The assay reagents, source of enzyme, enzyme purity as well as purity of the inhibitor are all factors that influence the results of any study. Apart from temperature and pH there appears to be no consensus regarding other parameters of enzyme inhibition assay in published literature. In the present study the usage of enzymes of avian origin and and crude nature of the extracts strongly influence the ranges of percentage enzyme inhibition and IC_{50} values of both the compounds tested.

3.2. Kinetic studies

To analyse the mode of inhibition of enzymes, the effect of various inhibitor concentrations on kinetics of enzymes was studied. The Km and Vmax values determined from the Lineweaver-Burk (LB) plots of each of the enzymes are listed in the table 1.

EGCG inhibitory effect on all three enzymes (Fig. 3) was characterized by a decrease in Vmax values with increasing inhibitor concentration while the Km remained reasonably constant with the uninhibited-enzyme Km value. On the other hand, Acarbose typically exhibited increase in Km at higher concentrations while no change in maximum velocity of enzymes was observed at all studied doses (Fig.3). These results are clearly indicative of non-competitive inhibition on the part of EGCG. As substantially documented earlier, in this study also Acarbose showed competitive inhibition of glucosidases. The competitive

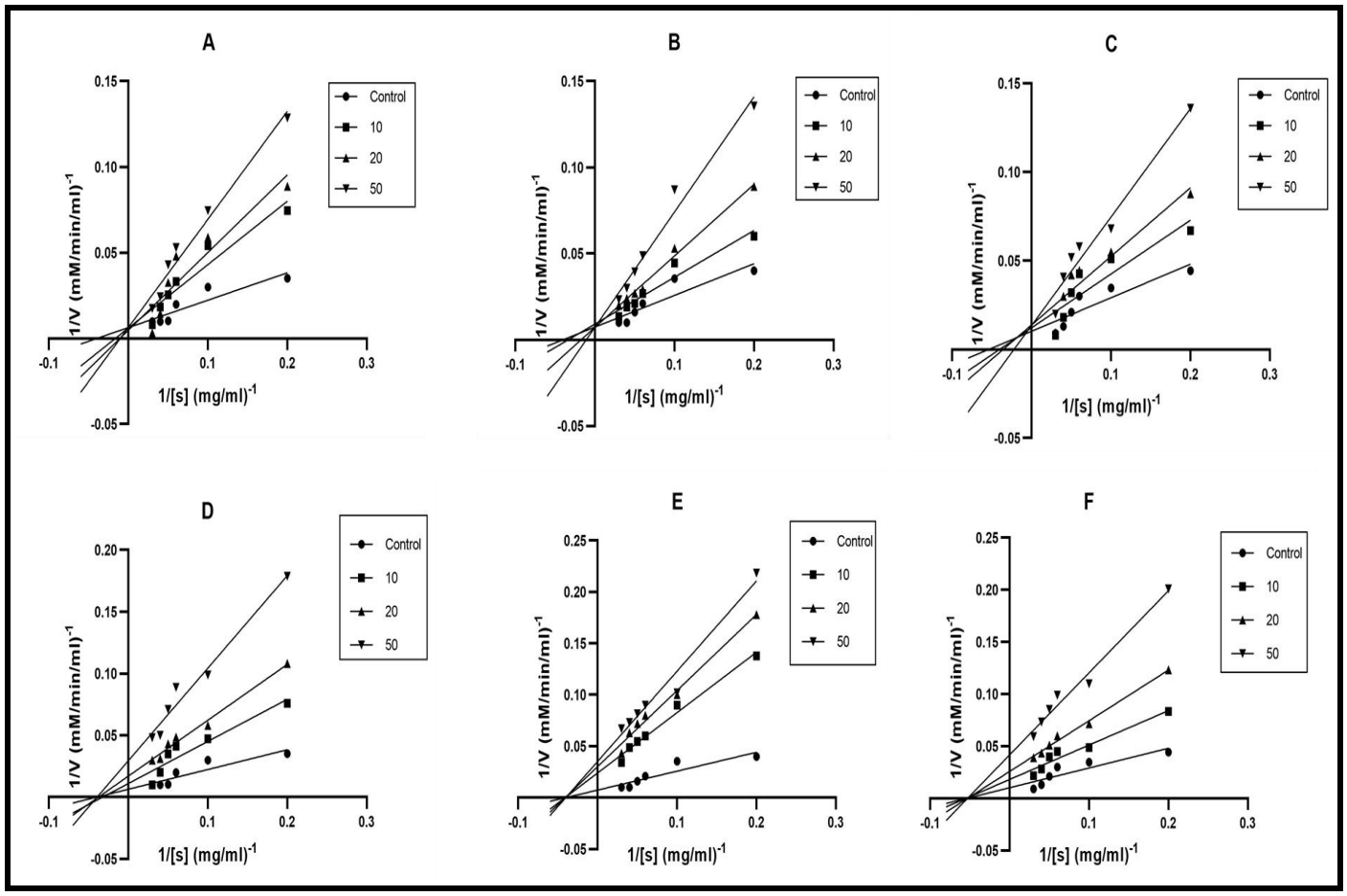
nature of Acarbose is attributed to the pseudo-sugar ring and glycosidic nitrogen linkage that mimics the glycosidic bond of carbohydrates [32].

Non-competitive inhibition for tea polyphenols has been reported Yang X etal (2014) [2]. Zhang W et al (2016) have documented non-competitive inhibition of alpha amylase by EGCG with decrease in Vmax and unchanged Km in enzyme kinetic studies [33].

Table 1: Kinetic parameters of α-Amylase and α-glucosidase

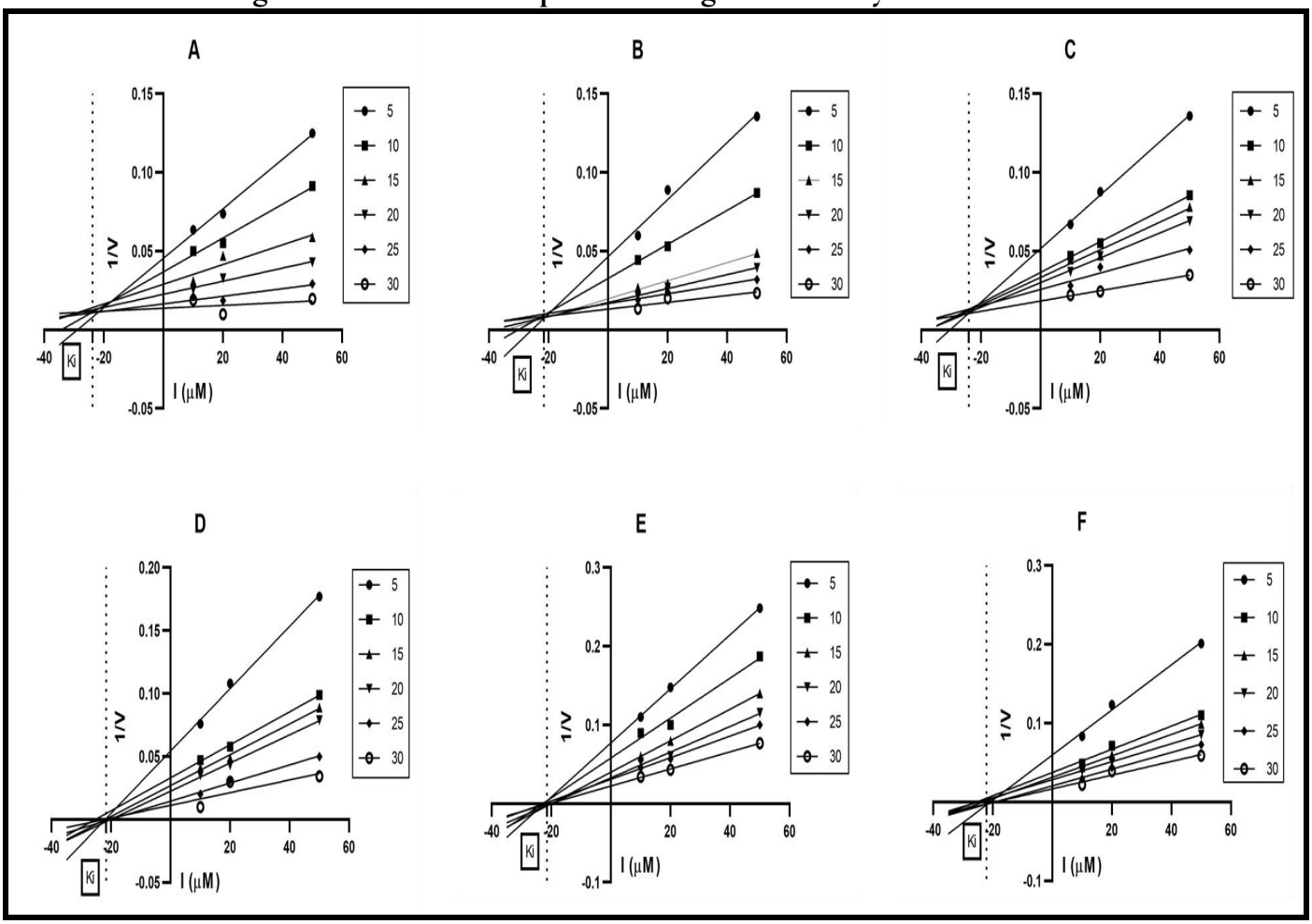
Enzyme and Inhibitor	Km (mg/mL)	Vmax ($mMmin^{-1}mL^{-1}$)	Ki(μM)
PE (no inhibitor)	25.64	0.1587	-
PE+A50	100.00	0.1567	23.84±1.64
PE+EGCG50	25.60	0.034	21.64±1.97
LE (no inhibitor)	25.00	0.1369	-
LE+A50	100.00	0.1369	21.66±1.64
LE+EGCG50	25.00	0.0285	21.48±1.31
IE (no inhibitor)	18.51	0.0978	-
IE+A50	45.24	0.0739	24.16±1.15
IE+EGCG50	18.86	0.0243	22.16±2.73

PE-Pancreatic enzyme, LE-Liver enzyme, IE-Intestinal enzyme, A50-Acarbose 50μM, EGCG50-EGCG 50μM, Km-Michaelis constant, Vmax-maximum velocity of enzyme activity, Ki-Inhibition constant determined from Dixon plots. Standard deviation of Ki values is calculated from the intersection points of extrapolated trendlines in Dixon plots. Km and Vmax values unchanged with respect to control enzyme are highlighted with same colour



(A) *pancreatic α-amylase by Acarbose* ***(B)*** *liver α-amylase by Acarbose* ***(C)*** *intestinal α-glucosidase by Acarbose (10, 20 and 50 μM) and* ***(D)*** *pancreatic α-amylase by EGCG* ***(E)*** *liver α-amylase by EGCG* ***(F)*** *intestinal α-glucosidase by EGCG (10, 20 and 50μM). The X intercepts represent -1/Km and Y intercepts represent 1/V. Data points are expressed as mean ± SE, n=3*

Fig. 3: Lineweaver-Burk plots showing kinetic analysis of inhibition



(A) *pancreatic α-amylase by Acarbose* ***(B)*** *liver α-amylase by Acarbose* ***(C)*** *intestinal α-glucosidase by Acarbose (10, 20 and 50 μM) and* ***(D)*** *pancreatic α-amylase by EGCG* ***(E)*** *liver α-amylase by EGCG* ***(F)*** *intestinal α-glucosidase by EGCG (10, 20 and 50 μM). The mean of intercept values represents -Ki. Data points are expressed as mean ±SE, n=3*

Fig. 4: Dixon plots showing kinetic analysis of inhibition

In silico docking studies of EGCG with alpha amylase and alpha glucosidase have also demonstrated non-competitive inhibition where EGCG is shown to block the entry of the deeply buried substrate binding site. This study also demonstrated that the blocking effect of EGCG is stronger for alpha glucosidase as compared to alpha amylase, substantiating our results [10].

An advantage of non-competitive or mixed inhibitors over the competitive ones is that they may not be overwhelmed by high substrate concentration that ensues with increased carbohydrate food intake and are effective at lower dosages [34].

On the basis of non-competitive mode of EGCG observed in this study, it can be interpreted that out of the various mechanisms of inhibition described for α-glucosidase inhibitors, the potency to make ionic or hydrophobic interactions with sites other than the active site, should be most likely mode of action [35].

The inhibitor constants (Ki) were determined from Dixon plots (Fig. 4) constructed by plotting reciprocal of enzyme velocity (1/V) against inhibitor concentrations at different values of [S]. For each [S] value points lie on a straight line. Extrapolated lines for different [S] values intersect at a single point for which i

= -Ki. Ki is the dissociation constant of EI complex. This method can distinguish between competitive and non-competitive inhibitors [18].

The Ki determined for EGCG and Acarbose for the three enzymes are listed in Table 1. The Ki is the concentration required to produce half maximum inhibition and is a quantitative index of inhibitor potency. It is a better estimate of inhibitory capacity compared to IC_{50}which is dependent on enzyme concentration used and its value is always greater than Ki [36].

4. CONCLUSION

This study showed that EGCG, the most abundant catechin found in green tea as well as several plant extracts, is a potent inhibitor of carbohydrate digesting enzymes alpha amylase and alpha glucosidase. The greater inhibitory potency for alpha glucosidase is attributed to smaller size and structural features of EGCG. The mode of inhibition appears to be non-competitive and therefore warrants greater pharmacologic interest due to advantages over competitive inhibitors. Considering the complex range of modulating effects credited to EGCG as a Hsp90 inhibitor the present finding is encouraging in terms of search of a multi-target agent in diabetic therapy. Dietary supplementation or preparation of nutraceuticals or functional foods using catechin gallates would help potentiate and at the same time simplify diabetic therapy and minimize side effects. Further research to address questions like optimal dosage, stability in the gastrointestinal tract, in vivo efficacy and potential for inhibition of other diabetic targets is desirable.

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Conflict of Interest:

The authors declare that they have no conflict of interest

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