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Original Article

Evaluation of Kinetic Parameters of α-Amylase in the Presence of the Flavonoid Rich-Extract of *Ficus carica* L., an *In-vitro* Study

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Abstract

Plants contain different chemical constituents with the potential for insulin-mimetic action. Reduction of insulin resistance and inhibition of α-amylase have been used as therapeutic strategies in treating diabetes. Considering the possible role of normal flora bacteria in the human body's metabolism, the bacterial enzyme α-amylase may also be of interest from this point of view. This study investigated the effect of flavonoid-rich extracts of the fig plant (*Ficus carica* L.) on bacterial α-amylase. The flavonoid-rich extracts were obtained from fig leaves and fruits by solvent extraction. Subsequently, the kinetic parameters of the α-amylase enzyme in the presence of these extracts were studied using the Bernfeld method. The evaluated IC_{50} values for acarbose, leaf and fruit extracts, and quercetin were equal to 0.112, 2.029, 0.195, and 0.071 mg/mL, respectively. The separately determined kinetic parameters Km and Vmax of the α-amylase in the presence of the fruit extract were 551.488 mM and 0.018 mM/min, and in the presence of the leaf extract, were 991.518 mM and 0.016 mM/min, respectively. The mode of inhibition was determined to be competitive by both extracts. According to our investigations, fig plant flavonoid-rich extract can be suggested as a suitable alternative to chemical compounds for therapeutic use in diabetes mellitus.

Keywords: α-Amylase; *Ficus carica* L.; Flavonoids; Medicinal plant; Moraceae.

1. Introduction

The prevalence of diabetes as a chronic metabolic disease that occurs due to the lack of insulin hormone or impaired response of organelles to insulin has been increasing in recent decades. On the other hand, the prevalence of obesity and overweight as dangerous factors that can lead to various diseases, including diabetes, is increasing daily. People with diabetes develop secondary problems such as neuropathy, retinopathy, diabetic cataracts, and nephropathy by creating sorbitol in the body [1]. Despite the variety of effective antidiabetic drugs on the market, they can also have irreversible side effects. Due to the side effects of chemical and synthetic drugs, much research is now being done on the effects of compounds derived from medicinal plants in

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treating various diseases [2]. Pancreatic α-amylase (E.C. 3.2.1.1) is a critical enzyme that breaks down dietary carbohydrates such as starch into simple monosaccharides. Many plants have antidiabetic effects due to their ability to restore pancreatic tissue function by increasing insulin secretion or lowering postprandial glucose by delaying glucose uptake. They play this critical role by inhibiting carbohydrate-hydrolyzing enzymes such as α-amylase and α-glucosidase. Herbal medicines have fewer risks and side effects than chemical medicines due to their proximity and adaptation to the physiology of the human body. Human α-amylase is commonly considered the ultimate goal of these inhibitors. Due to the presence of natural flora bacteria in the human body that prevent the establishment of pathogenic bacteria in the body and involve in the body's metabolism, bacterial α-amylase can also be of interest from this point of view. Among different bacterial species, most studies have been performed on α-amylase of different strains of Bacillus [3].

In addition, many chronic diseases, such as diabetes, are associated with oxidative stress, which involves the production of reactive oxygen species (ROS) such as superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-). The role of free radicals in the pathogenesis and progression of diabetes is confirmed by an increase in the concentration of malondialdehyde (MDA) through lipid peroxidation in the pancreatic tissue of diabetic animal models [4]. Therefore, compounds without severe side effects with antidiabetic and antioxidant activities would be more valuable.

More than 400 species of plants with hypoglycemic ability have been identified. However, the search for new antidiabetic drugs from new plants is still interesting because they may contain substances that have a safer effect on diabetes [5]. The medicinal properties of these plants are attributed to secondary metabolites, including alkaloids, anthocyanins, flavonoids, quinones, steroids, and terpenoids [6]. Flavonoids are a group of natural substances with variable phenolic structures found in fruits, vegetables, cereals, tea, and wine. They have different favorable biological activities, especially antioxidant activity. Flavonoids protect plants from biotic and biological stresses and serve as a unique U.V. filter [7]. They act as signal molecules, allopathic compounds, phytotoxins, detoxifiers, and defensive antimicrobial compounds [8]. Quercetin is a powerful antioxidant flavonoid that is approximately six times more potent than vitamin C and is found in abundance in vegetables, fruit, onions, apples, red grapes, citrus fruits, broccoli, tomatoes, dark chocolate, and green and black tea [2].

Fig (*Ficus carica* L.) (Moraceae) is one of the edible plants in the Mediterranean and countries with dry and temperate climates, such as the Caspian Sea coast in Iran, southern Saudi Arabia, and Turkey. Although humans have been eating figs since immemorial, their nutritional and medicinal value have recently been extensively studied. Figs also appear to help prevent clogged arteries. The high fiber content of fig has laxative properties, and fig leachate inhibits the growth of cancer cells. Dried figs are a dietary supplement for people with diabetes. It has been reported that fig skin

can be used to treat diabetes [9]. Previous studies have shown that the decoction of fig leaves can effectively reduce cholesterol levels in diabetic rats. These medicinal properties have been reported due to this plant's high content of phenolic compounds [10, 11]. Gallic acid, chlorogenic acid, rutin, quercetin-3-O-rutinoside, and epicatechin are the most predominant phenolic acids and flavonoids in dried and fresh fig varieties. It is not known which of the compounds in fig leaves lowers blood glucose levels and what is the mechanism of action [12]. To better understand the subject, in the present study, we focused on the α-amylase inhibitory activity of the flavonoid-rich extracts of fig by determining their kinetic parameters in vitro.

2. Materials and Methods

Bacterial α-amylase with a specific activity of 130 (E.U./mg) of Merck, 3,5-dinitrosalicylic acid (DNSA), starch, sodium potassium tartrate, maltose, and quercetin were purchased from Fluka, and acarbose was from Sigma.

2.1. Plant material

Fresh leaves and fruits of the fig plant were collected in August 2019 from Chalus City in Mazandaran province. The voucher specimen (HUMZ-3162) was deposited at the Plant Biology Department, Faculty of Basic Sciences, University of Mazandaran, after identification by Dr. Alireza Naqinezhad as plant taxonomist. The leaves of the fig plant were dried at room temperature and away from sunlight and completely ground by a home mill (20 g). The fruits were washed and cut into slices to facilitate the drying process. Then it is completely dried with an ordinary fruit dryer, powdered with a home grinder (20 g), and stored in the refrigerator (4°C) for later use.

2.2. Preparation of flavonoid-rich extract

The flavonoid-rich extract was prepared by the Bharchura method with slight modification [12]. The 20 g of powdered leaves and fruits of the plant were separately extracted with 200 ml of 70% ethanol for 24 hours using an orbital shaker. The resulting plant extract was filtered through Whatman filter paper No.1, and the plant tissue was discarded. The obtained extract was concentrated in a vacuum and then defatted three times with 50 ml of chloroform in a separatory funnel. Then the resulting extract was washed triplicate with 50 ml of ethyl acetate. The solvent was removed at 45 °C using a rotary evaporator. The obtained flavonoid-rich fig leaves and fruit extract were stored in the refrigerator until use.

2.3. Measurement of total flavonoid content

The content of total flavonoids in the extracts was evaluated by the aluminum chloride colorimetric method [Malviya et al. 2010]. Quercetin was used as a reference to establish the calibration equation ($y = 0.5474 x + 0.1205$; $R^2=0.9917$), and the flavonoid content was expressed as mgQE/g of each extract.

2.4. Evaluation of α-amylase activity

The activity of the α-amylase was measured by Bernfeld's method with slight modification. 250 µL of α-amylase (1 mg/ml) was added to the mixture of 250 µL phosphate buffer pH 6.9 and

500 µL of starch solution (1%), then thoroughly mixed with a shaker. The reaction mixture was incubated at 40 °C for 15 min. Then the reaction was stopped with 1 ml of 96 mM 3,5-dinitrosalicylic acid (DNSA) at 100 °C in a boiling water bath for 7 min. The resulting complex of DNSA with α-amylase reaction product shows absorption at 540 nm on the spectrophotometer. As blank, the enzyme solution was replaced by 250 µL buffer solution. To express the activity of the α-amylase enzyme, a standard graph was prepared in the concentration range of 0.5-1.5 mM of maltose solution. The standard equation ($y = 1.931x + 0.0875$; $R^2 = 0.9829$) was used to calculate the enzyme activity. All experiments were performed in triplicate.

2.5. Amylase inhibition assay in the presence of flavonoid-rich extracts

Flavonoid-rich extracts from 10 to 50 µL were incubated with 250 µL of the enzyme (1 mg/mL) for 5 minutes. Then 500 µL of 1% starch solution in 0.02 M phosphate buffer was added, and the volume was adjusted to 1 mL with the same buffer. All samples were incubated for 15 minutes at 40 °C. Then, one ml of DNSA reagent was added to all tubes and heated in a 100°C water bath for 7 minutes. The samples were cooled, and 500 µL of distilled water was added to each tube. The absorbance of all samples was read at 540 nm. The inhibitory activity (%) was calculated using the formula: $(1 - A/B) \times 100$, where A was the enzyme activity in the presence of each extract, and B was the enzyme activity without the extracts. A solution of acarbose (1 mg/mL) was prepared in DMSO using an ultrasonic bath. Different volumes of this solution were poured into five test tubes, and 250 µL of the enzyme solution was added and incubated for 5 min. Then, 500 µl of 1% starch was added to all tubes, and after adjusting the volume to 1 ml, the enzyme activity was measured.

2.6. Effect of quercetin on α-amylase activity

The effect of quercetin as a standard flavonoid on enzyme activity was investigated. Five different volumes of quercetin solution (0.01g/mL) were added to 250 µL of the enzyme and incubated for 5 min. Then, 500 µL of 1% starch solution was added, and after adjusting the volume to 1 ml, the enzyme activity was measured.

2.7. Determination of kinetic parameters of the α-amylase

The Michaelis-Menten constant (Km) and maximal velocity (Vmax) in the presence of the flavonoid-rich extracts of *Ficus carica* were determined from the Michaelis-Menten and Lineweaver-Burk equations. For this purpose, the α-amylase (1 mg/mL) was preincubated with 40 µL of each extract for 5 min. After adding 25-125 µL of 1% starch solution, the total volume was adjusted to 700 µL by 0.02 M phosphate buffer and incubated for 5 min. Then, one mL of DNSA solution was added, and the test tubes were heated in a hot water bath at 100 °C for 7 minutes. Finally, 300 µL of distilled water was added to all tubes, and their absorbance read at 540 nm. A control sample was prepared for each experiment using phosphate buffer instead of enzyme solution.

2.8. Statistical Analysis

All data were expressed as Mean±SD at least triplicate and were analyzed by Prism software and Excel. One-way ANOVA was used to compare groups at a significant level of $p < 0.05$.

3. Results and Discussion

3.1 Measurement of the total flavonoid content of the extracts

Total flavonoids were measured by the aluminum chloride colorimetric method. As shown in **Figure 1**, the total flavonoid content of the leaf extract was 2.5 µg Q.E./mL and that of the fruit extract 53.5 µg Q.E./mL (micrograms of quercetin equivalent per mL of the extract). The standard diagram was drawn based on the measured absorbance against different concentrations of quercetin.

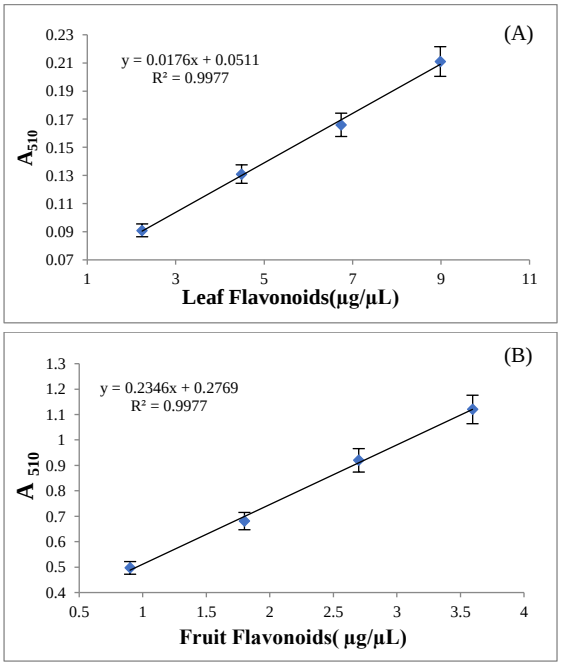

Figure 1. The resulting graphs for determination of flavonoid contents of (A): Fig leaf, (B): Fig fruit (values are shown as mean ± S.D. from triplicate measurements).

3.2. Results of α-amylase activity assay

According to Bernfeld's method and using the standard graph of maltose, the activity of the α-amylase enzyme was determined as 0.31 U/mL (**Figure 2**).

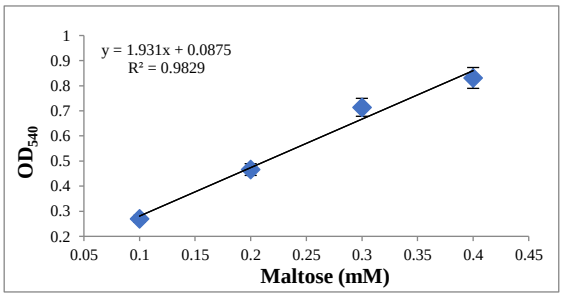

Figure 2. Standard graph of maltose at 540 nm (values are shown as mean ± S.D. from triplicate measurements).

3.3. Effect of flavonoid-rich extract of fig leaves and fruits on α-amylase activity

The enzyme activity was measured separately in the presence of fig leaves and fruit flavonoid-rich extracts. The results were calculated as the percentage of enzyme inhibition and shown in **Figure 3 C-D**. As a positive control, the experiment was performed with different concentrations of acarbose and quercetin (as a standard flavonoid). The results of these measurements are presented as a percentage of enzyme inhibition by increased concentration of both compounds (**Figure 3 A-B**).

According to the equations in **Figures 3A and B**, IC_{50} values for acarbose (as a drug) and quercetin (as a standard flavonoid) were determined to be 0.112 and 0.071 mg/mL, respectively. As shown in **Figures 3C and D**, the IC50 values as a measure of the inhibitory potential of fig leaf and fruit flavonoids on the α-amylase enzyme were found to be 2.029 mg/mL and 0.195 mg/mL, respectively.

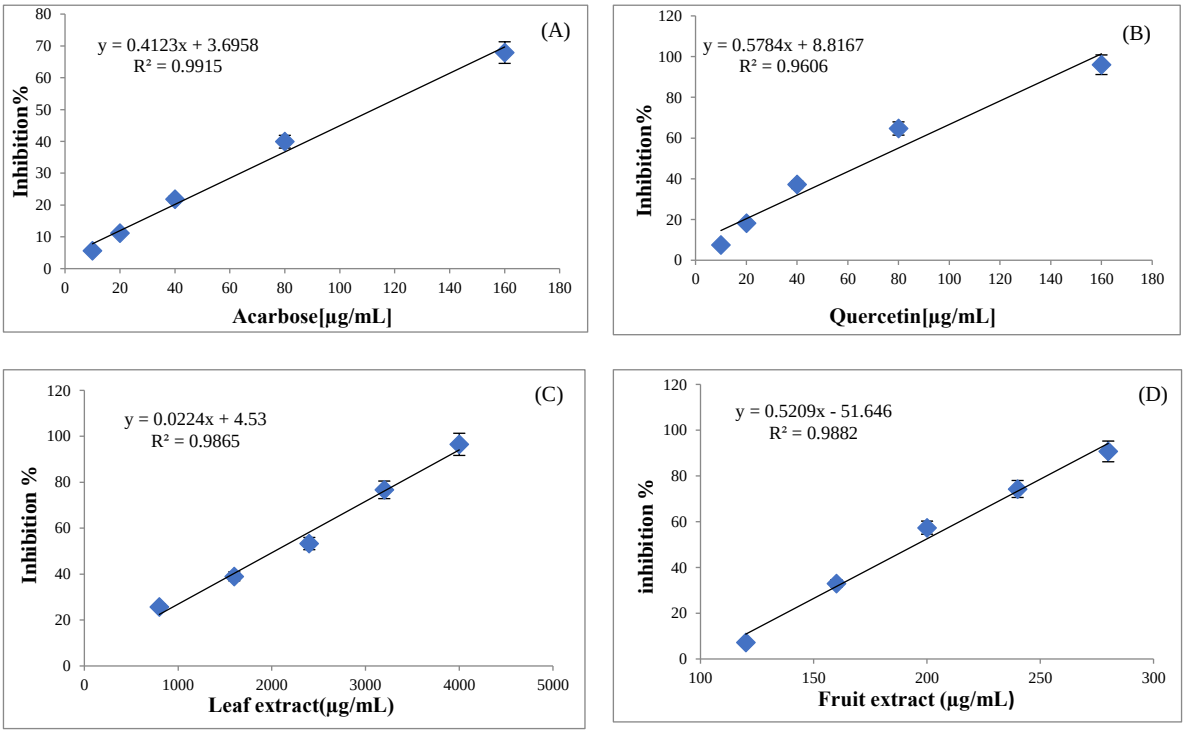

Figure 3. Inhibition percent of the α-amylase by various concentrations of A) acarbose, B) quercetin, C) flavonoid-rich extract of fig leaf, D) flavonoid-rich extract of fig fruit (values are shown as mean ± S.D. from triplicate measurements).

3.4. Investigation of the α-amylase kinetics in the presence of the extracts

In order to investigate the kinetics of the enzyme in the presence of different concentrations of starch as substrate at a specific incubation time, the reaction rate was measured. Then, the Lineweaver-Burk diagrams were drawn separately in the presence of the flavonoid extracts of fruits and leaves of fig. As shown in **Figure 4**, the slopes of the lines in the presence of the extracts were higher than that without extracts. In addition, it can also be seen that in the presence of the flavonoid extract of fig leaf, the slope of the line is greater than that of the fruit.

The kinetic parameters, the values of Km and Vmax of the α-amylase in the presence of the fruit and leaf flavonoid extracts of fig were shown in **Table 1.**

Table 1: The amounts of Km and Vmax for the amylase in the presence and absence of the flavonoids of Fig leaves and fruits.

	α-Amylase and the leaf flavonoids	**α-Amylase and the fruit flavonoids**	**α-Amylase without extract**
Km (mM)	991.518	551.488	321.800
Vmax (mM/min)	0.0165	0.0181	0.0193
Type of inhibition	Competitive	Competitive	--

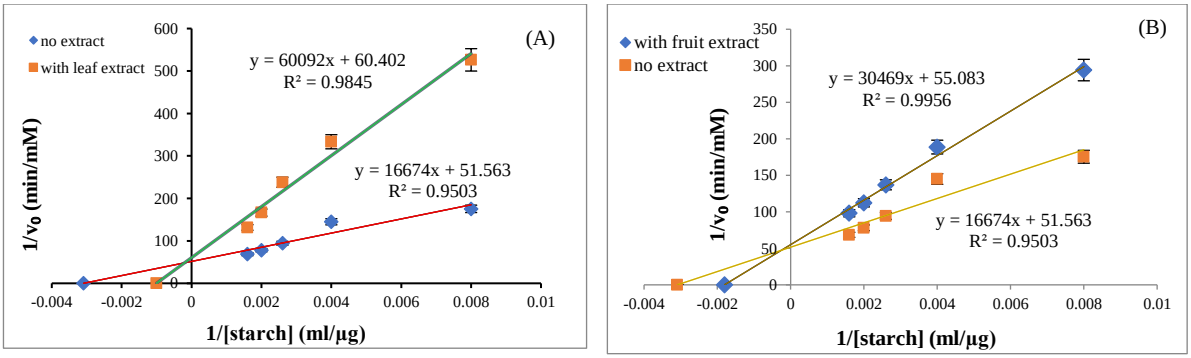

Figure 4. The Lineweaver-Burk plott of the α-amylase in the presence of the flavonoids of fig leaves (A) and the fruit (B) (values are shown as mean ± S.D. from triplicate measurements).

Six commercial varieties with different colors (black, red, yellow, and green) of *Ficus carica* were evaluated for total phenols, total flavonoids, antioxidant capacity, and profile of anthocyanins, which correlated well with the color appearance of the fig extract [14]. Phytochemical studies on different parts of *F. carica* revealed the presence of numerous bioactive compounds such as phenolic compounds, phytosterols, organic acids, anthocyanin composition, triterpenoids, coumarins, and volatile compounds such as hydrocarbons, aliphatic alcohols, and few other classes of secondary metabolites. Phenolic acids such as 3-O- and 5-O-caffeoylquinic acids, ferulic acid, quercetin-3-O-glucoside, quercetin-3-o-rutinoside, psoralen, bergapten, and organic acids (oxalic, citric, malic, quinic, shikimic, and fumaric acids) have been isolated from the water extract of the leaves of *F. carica*. The leaf extract induced a significant hypoglycemic effect in oral or intraperitoneal administration in streptozotocin-diabetic rats. In treated diabetic rats, weight loss was prevented, and plasma insulin levels significantly altered the survival index. Results indicated that the aqueous extract of *F. carica* has an evident hypoglycemic activity [14].

Further phytochemical studies on plants of the genus Ficus revealed the presence of ceramides, cerebrosides, steroids, pentacyclic triterpenes, flavonoids, and phenolic compounds (15-17). However, a preliminary phytochemical analysis of the bark of *F. vallis-choudae* D. indicated the presence of flavonoids, glycosides, alkaloids, tannins, and saponins [18]. The effect of the aromatic water of *Ficus carica* leaves was also investigated on streptozotocin-induced diabetic rats by Rashidi [9]. The results showed that oral consumption of the aromatic water of *Ficus carica* leaves reduced blood glucose levels, and the effect on blood glucose levels was dose-dependent. Wadood showed that fig fruit extract of *Relegiosa* species lowered blood glucose and total lipid levels in healthy rabbits but did not affect blood glucose concentration in alloxan diabetic rabbits [3]. Perez showed that blood glucose levels decreased significantly in the rats given basic and chloroform extracts from fig leaves compared to the control rat group [19]. Fig leaf decoction lowered blood glucose levels in diabetic patients. As reported, some studied cases indicated that fig skin, fruit, and leaves could reduce blood glucose levels. Differences in this field between studied cases can be due to

the source and method of preparation of the extracts or tested animals. It is not entirely clear which of the compounds found in fig leaves lowers blood glucose levels and what exactly is the mechanism of action.

Recently, the possible mechanisms of fig fruit extract in suppressing hepatic gluconeogenesis in diabetic rats were studied. To conduct this study, diabetic mice received fig fruit extract at a dose of 1 g/kg body weight twice daily for six weeks. Fasting blood sugar levels and two-hour oral glucose tolerance were measured. The results showed that fig fruit extract inhibited the expression of phosphoenol pyruvate carboxy kinase and glucose 6-phosphatase in the liver of diabetic rats. As a result, fig extract inhibits hepatic gluconeogenesis by activating protein kinase and regulating the levels of gluconeogenic enzymes [20]. Other studies on ethanolic, ethyl acetate, hexane, and butanol extracts of fig branches have shown many medicinal properties and can be very useful in increasing the total antioxidant capacity. Four-week consumption of dried figs can act as a suitable antioxidant barrier against free radicals and prevent many oxidative stress disorders, including cardiovascular disease, cancer, and preeclampsia in women [21]. A kinetic study of α-amylase from wheat *Eurygaster integriceps* was performed, similar to the idea implemented in this work [22]. This paper investigated the effect of Triticale extract with different concentrations on the α-amylase, and the extract displayed mixed inhibition of the enzyme activity. In a study by Subratty *et al*., the leaf of the Jack tree (*Artocarpus heterophyllus*), which is related to figs, significantly inhibited the α-amylase activity *in vitro*. It was found that the aqueous leaf extract significantly inhibited α-amylase activity in rat plasma. They observed the highest inhibitory activity (27%) at 1000 μg/mL [23].

Kinetic studies on α-amylase enzyme were performed using the Michaelis-Menten and Lineweaver-Burk equations to determine the type of inhibition. In the presence of the plant extract, the maximum velocity of the enzyme remained unchanged while the Km increased by 5.79 g/L, indicating it was a competitive inhibitor. They concluded that this extract acts as a starch blocker and reduces glucose after eating [23]. *Morinda lucida* (Rubiaceae) aqueous leaf extract has been reported to have the highest potency in inhibiting the enzymes α-amylase and α-glucosidase. The mode of inhibition of the aqueous extract on *α*-amylase was near competitive (IC_{50} = 2.3 mg/mL), but on *α*-glucosidase was a mixed non-competitive inhibition [24]. One of the strategies and methods of curing diabetes mellitus involves inhibiting carbohydrate-digesting enzymes in gastrointestinal glucose absorption, thereby lowering postprandial glucose levels. For example, the hydroxyl group on the flavon and benzene ring of catechins as natural polyphenolic phytochemicals interact with active enzyme sites forming a phenol-protein complex, eliminating enzyme access to the substrate [25]. The above studies clearly show increasing attention for alternative *α*-amylase inhibitors derived from plant extracts with higher potency and fewer side effects than existing therapeutic agents. Our study showed that the flavonoid-rich extracts from leaves and fruits of figs inhibited the activity of the α-amylase enzyme. According to our kinetic measurements, the values of Km and Vmax for

the inhibitor-free enzyme were 321.800 mM and 0.0193 mM/min, respectively. In the presence of fruit and leaf extracts, Km increased to 551.488 and 991.518 mM, and Vmax decreased slightly to 0.0181 and 0.0165 mM/min, respectively. Therefore, the enzyme inhibition by both plant extracts was a competitive type. The half maximum inhibitory concentration (IC_{50}) measured for the fruit and leaf extracts of fig, acarbose, and quercetin (**Figure 5**) demonstrates the usefulness of Ficus carica in ethnomedicine against diabetes.

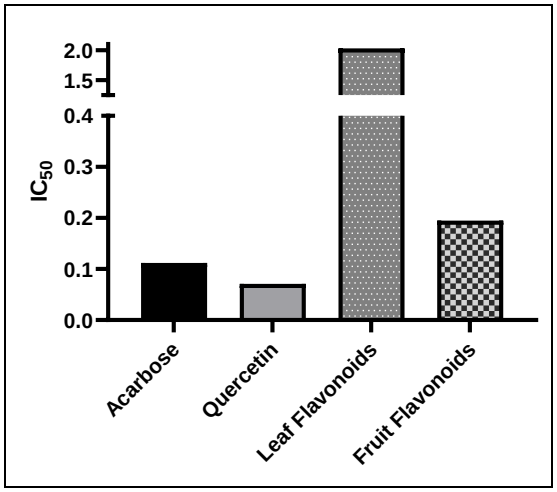

Figure 5: The determined IC_{50} values in α-amylase inhibition experiments for acarbose, quercetin, leaves flavonoids, and fruit flavonoids of *Ficus carica* L.

4. Conclusion

α-Amylase is a well-known molecular target for the treatment of diabetes mellitus. Despite being one of the most extensively studied enzymes, the search for new inhibitors with unique properties is still a current scientific goal. In this work, the flavonoids-rich extract from fig proved to be an effective inhibitor of the amylase enzyme. These results could support future use of the flavonoid-rich extract as a safe and inexpensive source.

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Conflict of interest

The authors declare to have no conflict of interest.

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