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Unripe Fruit's Extract of Quince (*Cydonia oblonga* Miller) as a Potent Alpha-amylase Inhibitor

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Abstract: The use of alpha-amylase inhibitors has recently gained in popularity with the success and growth of carbohydrate restricted diets. In this study, two different stages from the unripe fruits of quince (*Cydonia oblonga* Miller) have been tested for their potentiality in alpha-amylase inhibition as a key enzyme in carbohydrates assimilation. Our results revealed that addition of different concentrations from extracts (0, 2, 4, 6, 8mg) of dry mass of each stage of unripe fruits resulted in drastically decrease in the enzymatic activity of alpha-amylase by the percent of (0%, 42.6%, 21%, 26.3%, and 16.9%) for the stage 1. Extracts from the stage 2 were more effective in enzymatic inhibition (0%, 26.9%, 3.8%, 0.2%, and 0.4%). The GC/MS analysis revealed that quince extract contains (sorbitol, quinic acid, p-vinylphenol and cyclopropane carboxylic acid). To explore which components are involved in the inhibition process, two pure components of the quince extract (sorbitol and quinic acid) were used in inhibition assay. Neither sorbitol nor quinic acid shows any significant inhibition; therefore, these two components could be excluded from the inhibition process. Our current study suggested that p-vinylphenol and cyclopropane carboxylic acid might act as α-amylase inhibitors *in vitro* separately or synergistically. The possible explanation for the presence of cyclopropane carboxylic acid (CPCA) in this critical phase of the unripe fruit will be discussed. This study suggests that the unripe fruits of quince can be used as a natural starch blocker containing alpha-amylase inhibitors which would be of interest for people requiring carbohydrate restricted diets.

Keywords: Quince Unripe Fruit, Alpha-amylase Inhibitor, P-vinylphenol, Cyclopropane Carboxylic Acid (CPCA).

1. Introduction

Diabetes mellitus is a metabolic disorder characterized by a congenital [type I insulin dependent diabetes mellitus/IDDM] or acquired [type II non-insulin-dependent diabetes mellitus/NIDDM] inability to transport glucose from the bloodstream into cells [1]. Several pharmacological strategies are used to improve diabetes *via* different modes of action such as stimulation of insulin release, increase the number of glucose transporters, inhibition of gluconeogenesis and reduction of absorption of glucose from the intestine [2]. The most effective therapeutic approach to decrease postprandial hyperglycemia is to retard absorption of glucose through inhibition of carbohydrate hydrolyzing enzymes in the digestive organs [3]. Alpha-amylase is one of the enzymes that catalyse the breakdown of starch to maltose and finally to glucose, which is the only sugar that can be utilized by the body [4].

The inhibition of these enzymes leads to a decrease in blood glucose level since monosaccharides are a form of carbohydrates, which are absorbed in the small intestine [5]. Recently, plants and their components have received much attention in the treatment of diabetes for various reasons and many researchers have focused on hypoglycemic agents from medicinal plants [2].

During the past years, several studies have revealed that quince tree (*Cydonia oblonga* Miller) is considered to be a good and cheap natural source for potent antioxidants including phenolic acids and flavonoids [6a, b, 7]. These antioxidants *per se* act as cell saviours

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via their capabilities as reducing agents, hydrogen donators, free radicals scavengers and singlet oxygen quenchers [8]. The scientific information supporting the health benefits claimed for quince leaf and fruit in folk medicine, namely cardiovascular disease, haemorrhoids, bronchial asthma, and cough is lacking in the literature [7, 9, 10 and 11]. The available data classify quince fruit as a source of health promoting compounds, due to its antioxidant, antimicrobial and anti-ulcerative properties [12-19]. For quince leaf, the free radical scavenging and anti-hemolytic activities have been confirmed [10]. It has been reported that quince posses anti-diabetic properties [20-22]. Moreover, Aslan *et al.*, (2010) recommended long-term use of quince in type II diabetic patients to protect against the complications of diabetes mellitus.

Recently, we have found out that the application of quince extract resulted in significant decrease in the glucose level in the blood of normal and UV exposed African catfish [23]. In this investigation, we attempted to understand the possible mode of quince extract to lower glucose level. Therefore, extracts of two different stages from the unripe fruits of quince (*Cydonia oblonga* Miller) have been tested for their potentiality in alpha-amylase inhibition as a key enzyme in carbohydrates assimilation.

2. Materials and Methods

2.1 Sample preparation and extraction

Healthy quince fruits were collected from quince trees cultivated in Faculty of Agriculture farm, Assiut University with two different ages (stage 1 and stage 2) before fruit ripening (See Fig. 1).

Fig. 1

The harvested fruits have been washed by tap water, dried by tissue papers subsequently cut into small pieces after removing seeds and finally crushed in liquid nitrogen. Twenty grams of fruit powder were thoroughly mixed with (3 X 300ml) of methanol at 40°C. Extract was filtered, concentrated vacuum (40°C) and redissolved in water.

2.2 GC/MS analysis for quince fruit methanolic extract

Methanolic leaf extract was analyzed using GC/MS, Agilent Model 6890N/5975B [Column DB 5ms, Agilent from [30, 0.25mm and 0.25mm] in the Analytical Chemistry Unit, Chemistry Department, Faculty of Science, Assiut University.

2.3 Chemicals and enzyme used

Sorbitol, quinic acid and pure alpha-amylase have been purchased from Sigma-Aldrich, Germany.

2.4 Alpha amylase inhibition assay

Amylase and amylase inhibitor activity assays were based on Bernfeld's method for amylase assay [24]. Amylase inhibitor extracts were mixed with amylase and incubated for 30 min. at 37°C. The reaction was started by adding extract-enzyme mixture to test tubes containing buffered starch solution (2mg starch in 20mM phosphate buffer of pH 6.9 containing 0.4mM NaCl) and was incubated for 20 min. This reaction was terminated by adding 3,5-dinitrosalicylic acid (DNS) reagent to the assay mixture. The assay tubes were kept in a boiling water bath for 5 min, cooled under tap water and the color formed by maltose oxidation was measured at 530nm. Controls without inhibitor were run simultaneously. One amylase unit is defined as the amount of enzyme that will liberate 1µmol of maltose from starch under the assay conditions (pH 6.9, 37°C, 5 min). Inhibitory activity is expressed as the percentage of inhibited enzyme activity out of the total enzyme activity used in the assay.

2.5 Statistical analysis

All experiments were carried out in a completely randomized design. The results were subjected to analysis of variance (one-way ANOVA) using PCSTAT program (Version 1A). The treatment means were compared using the least significant difference (LSD) values at a significance level of $P < 0.01$.

3. Results and Discussion

3.1 Rationale behind unripe fruit materials

Fruit ripening is a complex process, with several physiological changes taking place in a short time. These changes affect the flavor, color and texture of the fruit [25]. The involved enzymes in these metabolic activities are usually under cellular control. Alpha-amylase is a key enzyme plays a crucial role in starch hydrolysis during ripening. Cellular control of such enzyme could be achieved via plant hormones directly or indirectly by putative compounds found in the fruit and act as alpha-amylase inhibitors. This programme control ensures fruit ripening at the right time. Therefore, we collected unripe quince fruit from the trees at two different stages Fig. 1.

3.2 Alpha-amylase inhibition assay by crude extract

Generally, application of the crude extract of stage 1 in the reaction resulted in a sharp reduction in enzyme activity. The concentration 2mg of dry mass fruit caused remarkable decrease in the enzymatic activity by around 60%. Both second and third concentrations (4 and 6mg) reduce the alpha-amylase activity to around 80%. Addition of 8mg to the reaction tube resulted in drastic decrease in enzyme activity by approximately 100% (see Fig. 2). Interestingly, the utilization of crude extract from stage 2 was more effective than extract from stage 1.

3.3 GC/MS analysis

In an attempt to determine, which components have been involved in inhibition of alpha-amylase methanolic extract of more effective stage 2 was analyzed by GC mass spectrometry. The present results of GC/MS (Table 1) revealed that sorbitol is the major constituent in the quince fruit with the percent of 30.12%. Quinic acid has come in the second rank and represented by 23.49%. p-vinylphenol and cyclopropane carboxylic acid were also present with the percents of 11.24 and 10.18% respectively. Quince is one of the Rosaceae family and many species within this family can synthesize and accumulate sorbitol. This sugar alcohol synthesized in leaves and translocated to sink organs as the main sugar together with sucrose, and therefore its metabolism in sink organs has an important role in the growth of organs and plants [26]. Moreover, sorbitol (30% of the total) has been identified as a nonvolatile flavor component in the dwarf quince *Chaenomeles japonica* [27]. These findings are in the consistency of our current results the unripe fruit extract contains around 30 percent of sorbitol. To find out which component within those four detected, we have studied the possible effect of pure sorbitol and quinic acid on alpha-amylase activity.

In this experiment, pure sorbitol and quinic acid as major components of quince extracts were applied to the alpha-amylase assay by the same concentrations of the crude extract (2, 4, 6, 8mg dry mass). Our results revealed that neither sorbitol nor quinic acid by their concentrations did not affect the overall activity of alpha-amylase (data not shown).

The detected cyclopropane carboxylic acid in the unripe fruit of quince it might play a crucial role in alpha-amylase inhibition process. Cyclopropane and cyclopropene fatty acids are found in the phospholipids of many plants, bacteria, and parasitic protozoa [28]. As far as we know, this is the first report concerning the detection of cyclopropane carboxylic acid in this fruit, particularly in the stages before ripening.

Several hypotheses have been proposed for the possible biological role of cyclopropane fatty acids (CFAs) including temporal energy storage, membrane fluidity modification and retain rather than alter the membrane properties during growth phase transition [29]. This property for CFA represents a plausible explanation for its presence in the fruit during this growth phase in order to enable fruit tissues those undergoing fast enlargement. Moreover, the effects of cyclopropane carboxylic acid, on gluconeogenesis in rat and guinea pig kidney slices has been investigated and resulted in inhibition of glucose production from pyruvate by 86 percent in guinea pig but by only 22 percent in rat [30].

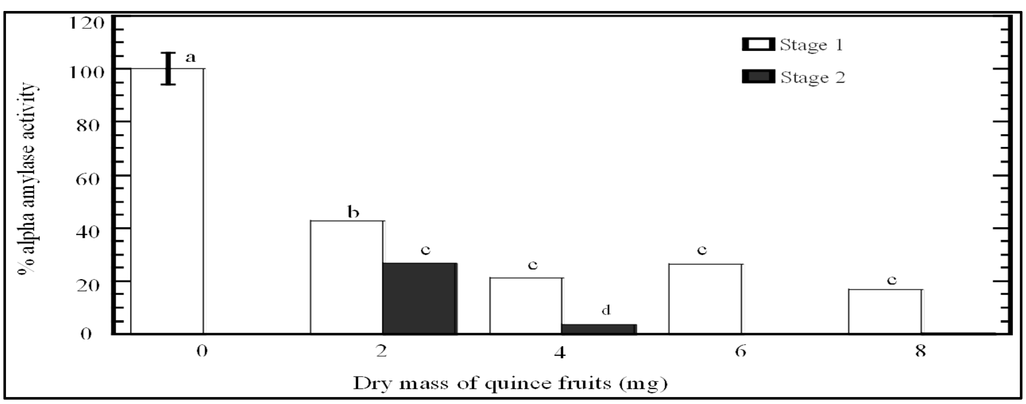

Fig. 2. Alpha-amylase inhibition by crude extracts of stage 1 and stage 2. The bar in first column shows the LSD ($P < 0.1$). Columns followed by the same letter are not significantly different at LSD ($P < 0.1$).

Table 1. Percentages of four major components of methanolic extract of stage 2 unripe quince (*Cydonia oblonga* Miller) fruit and their molecular weights as indicated by gas chromatography-mass spectrometry analysis.

Components	Molecular weight	Percent of total
Sorbitol	182	30.12
Quinic acid	192	23.49
p-vinylphenol	120	11.24
Cyclopropane carboxylic acid	86	10.18

The most promising finding in our study is the presence of p-vinylphenol with 11 percent in the quince extract. Based on the existing studies, it is found that polyphenols and flavonoids are among the natural active antidiabetic agents [31]. These compounds have been reported to exert various biological effects, including carbohydrate hydrolyzing enzyme inhibition and antioxidant activity. In agreement of our finding, it has been reported that polyphenolic compounds are able to inhibit the activities of digestive enzymes due to their ability to bind with proteins [32]. Inconsistency of our finding, recently it has been reported that quince posses anti-diabetic properties [20-22]. Moreover, Aslan *et al*., (2010) recommended long-term use of quince in type II diabetic patients to protect against the complications of diabetes mellitus. Based on our study it could be suggested that p-vinylphenol and cyclopropane carboxylic acid might act as potent alpha-amylase inhibitors separately or synergistically.

4. Conclusion

Extract of the unripe fruit of quince (*Cydonia oblonga* Miller) posses several biologically active components including sorbitol, quinic acid, p-vinylphenol and cyclopropane carboxylic acid. The last two components might be implicated in alpha-amylase inhibition. This privilege for this extract herein reflects a great potentiality for application of such extract in food and drug products, with remarkable benefits for human health.

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