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Inhibitory Effects of Red Grape Seed Extracts on Pancreatic α-amylase and Lipase

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Abstract: The purpose of present study was to investigate the effect of water and ethanol extracts of red grape seeds on inhibition of pancreatic α-amylase and lipase *in vitro*. Total proanthocyanidins were determined in grape seed extracts (GSEs). Different concentrations (400, 800, 1300 and 1800 ppm) of each GSE were employed to evaluate their potentials on pancreatic α-amylase and lipase after pre-incubation with enzyme or substrate. The obtained results showed that ethanol grape seed extract contains higher concentration of proanthocyanidins (32.64 mg/g grape seeds) compared with water grape seed extract (26.72 mg/g grape seeds). Data also showed that the GSEs possessed inhibitory effects against some pancreatic enzymes (i.e. pancreatic α-amylase and lipase). Inhibitory activity of each GSE is occurred either pre-incubation with enzyme or substrate. There was a positive correlation between GSE concentration and inhibitory activity. The ethanol grape seed extract (EGSE) had higher inhibitory effect against pancreatic α-amylase and lipase in comparison with water grape seed extract (WGSE). In conclusion, the GSEs rich in compounds that inhibit α-amylase and lipase may provide a safe, natural and cost-effective in the treatment of diabetes and obesity.

Key words: Red grape seeds · Inhibitory effect · α-amylase · Lipase · *In vitro*

INTRODUCTION

Diabetes is a group of metabolic diseases characterized by chronic hyperglycemia resulting from deficiency in insulin secretion or action. One therapeutic approach for treating diabetes is to decrease the postprandial glycemia by the inhibition of enzymes responsible for the carbohydrate hydrolysis, such as α-amylase [1]. Pancreatic α-amylase (EC 3.2.1.1) is a key enzyme in the digestive system and catalyses the initial step in hydrolysis of starch to a mixture of smaller oligosaccharides consisting of maltose, maltotriose and a number of α-(l→6) and α-(1→4) oligoglucans. These are then acted on by α-glucosidases and further degraded to glucose which on absorption enters the blood-stream. Hence, retardation of starch digestion by inhibition of enzymes such as α-amylase plays a key role in the control of diabetes. Inhibitors of pancreatic α-amylase delay carbohydrate digestion causing a reduction in the rate of glucose absorption and lowering the post-prandial serum glucose levels [2]. Some inhibitors currently in clinical use are acarbose and miglitol which inhibit glycosidases such as α-amylase. However, many of these synthetic hypoglycemic agents have their limitations, are non-specific, produce serious side effects and fail to elevate diabetic complications. The main side effects of these inhibitors are gastrointestinal viz., bloating, abdominal discomfort, diarrhea and flatulence [3]. Herbal medicines or natural products are getting more importance in the treatment of diabetes as they are free from side effects and less expensive when compared to synthetic hypoglycemic agents [4]. Pancreatic lipase is a key enzyme for the digestion of dietary triglycerides. The inhibition of lipase activity retards the fat absorption and therefore ameliorates obesity [5]. It is well known that dietary fat is not directly absorbed from the intestine unless it has been subjected to the action of pancreatic lipase. Thereby, to suppress weight gain, it would be effective to reduce fat absorption by lipase inhibition. Orlistat, a specific pancreatic lipase inhibitor, is clinically used for preventing obesity and hyperlipidemia [6]. On the other hand, lipase inhibitory materials derived from natural products, such as chitosan [7] and the polyphenolic constituents of oolong tea extract [8] have been reported previously.

Grapes (*Vitis vinifera*) are considered as the world's largest fruit crops, with an approximate annual production of 58 million metric tones [9]. It has various biological functions, due to its rich polyphenol ingredients, most of

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which are contained in its seeds (60-70%) and skin (30%). However, large quantities of grape seed wastes are produced annually by the food processing industry-wine, juice etc. Grape seeds particularly contain two-thirds of the phenols of the grape, 5-8% by weight. Catechin, epicatechin and epicatechin gallate and gallic acid are the monomeric compounds identified in grape seeds. These, along with dimers, trimers and oligomers of catechin and epicatechin are referred to as procyanidins or proanthocyanidins [10]. Polyphenols in grape seeds have also been reported to have a variety of biological activities, including antioxidant, antithrombotic, antitumor, antibacterial, antiviral, anti-inflammatory, antiallergic, protective and antinutritional effects [11-15]. The antinutritional effects of GSE have been ascribed to the ability of procyanidins to interact with digestive enzymes [16]. The inhibition of digestive enzymes upon interaction with procyanidins has been proven for lipases [17], proteases [18], as well as α-amylase and α–glucosidases [12, 19]. Indeed, it is well known that phenolic compounds can have strong affinities with proteins and particularly with human salivary proline rich proteins (PRPs) and histatins [20] to form both non-covalent and covalent associations according to the phenolic compound size. Other studies, Laurent *et al*. [21] have demonstrated that insoluble complexes, which result from interactions between proteins and tannins, are stable throughout the digestive tract. Gonçalves *et al*. [22] studied the interactions between porcine pancreatic lipase (PL) and grape seed procyanidins by several methods. They found that an inhibitory effect of grape seed procyanidins on lipase hydrolytic activity. Recent study indicates that GSE inhibits pancreatic α-amylase and intestinal α–glucosidases related to delay postprandial hyperglycemia [23]. Yilmazer-Musa *et al*. [24] evaluated the inhibitory effect of grape seed extract and its constituent flavan-3-ol monomers (catechins) on α-amylase and α-glucosidase activity. The results showed that grape seed extract strongly inhibited both α-amylase and α-glucosidase activity, with equal and much higher potency, respectively, than acarbose.

The present study was aimed to investigate the inhibitory effects of grape seed extracts on pancreatic α-amylase and lipase activities *in vitro*.

MATERIALS AND METHODS

Materials: Plant Material: Grape (*Vitis vinifera* L., variety Red Roumy), as large clusters with red berries, was purchased from a local market at Giza, Egypt.

Chemicals: Pancreatin (from porcrine pancreas) was purchased from Sigma Chemical Co., USA. All other chemicals were of analytical reagent grade.

Methods

Preparation of Grape Seed Extracts (GSEs): Two different extracts were prepared from red grape seeds using the procedure described by Badavi *et al*. [25] with some modifications as follows: Grape seeds were separated from the grapes manually, air dried (in shade, 25-30°C) for one week and milled to fine powder. To prepare water grape seed extract (WGSE), 0.2 g of grape seed powder was macerated in 20 ml of distilled water (DW) for 24 h at 5°C and was stirred three times. The mixture filtered with cheese cloth and the resulting filtrate was used as WGSE. The same method was used for the extraction of grape seed with ethanol 80%. After extraction with ethanol, the solvent was removed from the obtained extract by evaporation. The residue was re-dissolved in the same volume of DW. The obtained solution was used as ethanol grape seed extract (EGSE).

Determination of Total Proanthocyanidins: Total proanthocyanidins were determined in grape seed extracts based on the procedure reported by Sun *et al*. [26] as follows: Grape seed extract (0.5 ml) was mixed with 3 ml of vanillin-methanol (4% v/v) and 1.5 ml of hydrochloric acid. The resulting mixture was allowed to stand for 15 min at room temperature. The developing color was measured at 500 nm using Jenway 6300 spectrophotometer. The concentration was calculated from the standard curve prepared using serial concentrations of standard catechin solution.

Assay of α-Amylase Inhibitory Activity: α-amylase (1-4-α-D-glucan glucanohydrolase, EC 3.2.1.1) inhibitory activity was assayed for grape seed extracts under investigation based on the method of Bernfeld [27] with a little modification of Karthic *et al*. [28] as follows: Known volumes (0.05-0.2 ml) of each grape seed extract were individually added to test tubes then 0.2 ml of pancreatic α-amylase enzyme solution (0.1 mg/ml of 2 mM phosphate buffer solution, pH 6.9) and 0.1 ml phosphate buffer solution (pH 6.9) were added. The solutions were mixed well and incubated for 20 min at 37ºC after that 0.1 ml of starch solution (1%) was added then mixed well and incubated for 5 min at room temperature. The enzyme reaction is interrupted by adding of 0.5 ml of dinitrosalicylic acid reagent (1% 3, 5-dinitrosalicylic acid

and 30% Rochelle salt in 0.4 M NaOH). The tubes were heated for 5 min in a boiling water bath and then cooled in running tap water. The absorbance (A) was measured at 540 nm using Jenway 6300 spectrophotometer. Control was prepared using the same procedure by replacing the extract with buffer. Blank was prepared using the same procedure by replacing the enzyme with buffer. The inhibition percent of α-amylase enzyme was calculated using the following equation:

$$\text{Inhibition (\%)} = \frac{\text{Acontrol - Asample}}{\text{Acontrol}} \times 100$$

Assay of Lipase Inhibitory Activity: The lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) inhibitory activity was assayed for grape seed extracts based on the method of Mia *et al*. [29] with the following modification:

Preparation of Substrate Emulsion: It was prepared by adding 10 ml of the alcoholic solution of olive oil (0.866 g purified olive oil was dissolved in 100 ml a mixture of 95% ethanol and 5% methanol) to 400 ml of the buffer solution (0.05 *M* Tris buffer and 0.008 *M* sodium deoxycholate, pH 9.3) and gently mixing by inverting the container 5 times. The solution was stable for 4 weeks if stored in the refrigerator.

Procedure: Three determinations were carried out to assay lipase inhibitory activity. In first determination, lipase solution (0.5%) was heated at 70ºC for 10 min (to lipase inactivation). Inactive lipase solution (0.1 ml) and grape seed extract (0.2 ml) were added to 0.5 ml of substrate emulsion in test tube. After mixing, the absorbance was measured at 440 nm against buffer solution as reagent blank using Jenway 6300 spectrophotometer. In second determination, active lipase solution (0.1 ml) was added to 0.5 ml of substrate emulsion, mixed well and then incubated for 3 min. In the end of the period, the grape seed extract (0.2 ml) was added, mixed well and the O.D was measured against buffer solution. Lipase activity in international units (IU) per 100 ml solution was estimated as follows:

$$\text{Lipase activity} = \frac{\text{Decrease of O.D in 3 min}}{\text{O.D of substrate emulsion}} \times 40$$

In third determination, a known volume (0.05-0.2 ml) of each grape seed extract was added to 0.1 ml of enzyme solution, mixed well and incubated at room temperature for 30 min, then 0.5 ml of substrate emulsion was added and mixed well. The absorbance was measured after 3 min at 440 nm against buffer solution and the remained lipase activity was calculated using the previous equation. Lipase inhibitory activity was calculated by the difference.

Statistical Analysis: The results were analysed by an analysis of variance ($P<0.05$) and the means separated by Duncan's multiple range test. The results were processed by CoStat computer program (1986).

RESULTS AND DISCUSSION

Total Proanthocyanidins Content: Proanthocyanidins are the most abundant phenolic compounds in grape seeds and are high-molecular-weight polymers comprised of dimmers or trimers of (+)-catechin and (-)-epicatechin [30]. Proanthocyanidins content was calorimetrically determined in both grape seed extracts by vanillin-hydrochloric acid assay. A vanillin-hydrochloric acid (HCl)) assay is specific for flavavn-3-ol. The obtained results showed that grape seed extracts contain a logical amount of proanthocyanidins (Fig. 1). Ethanol grape seed extract contains higher concentration of proanthocyanidins (32.64±0.25 mg/g grape seeds) compared with water grape seed extract (26.72±0.36 mg/g grape seeds). The difference in their proanthocyanidins content may be due to their respective polarities. The content of phenolics (catechin and procyanidins) in grape seeds is clearly affected by four agroecological factors: the cultivar, the year of production, the site of production and the degree of maturation [31]. The present results are in agreement with those obtained by Yamakoshi *et al*. [32], Shi *et al*. [33], Nakamura *et al*. [34], Liu and White [35] and Georgiev *et al.*[36], who reported that grape seeds contain proanthocyanidins. Prieur *et al*. [37] found that 55% of the procyanidins extracted from grape seeds consisted of more than five monomer units and determined that their mean degree of polymerization ranged from 2.3 to 15.1 (by thiolysis) and from 2.4 to 16.7 (by gel permeation chromatography). Gu *et al*. [38] reported that dry grape seeds contain an average of 35.3 mg of total proanthocyanidins per gram of sample. Silvan *et al*. [39] reported that the phenolic profile of GSE mainly consisted on flavonols, phenolic acids, catechins and proanthocyanidins and anthocyanins. Among them, catechins and proanthocyanidins were the major compounds, representing 77.6% of total phenolic compounds determined.

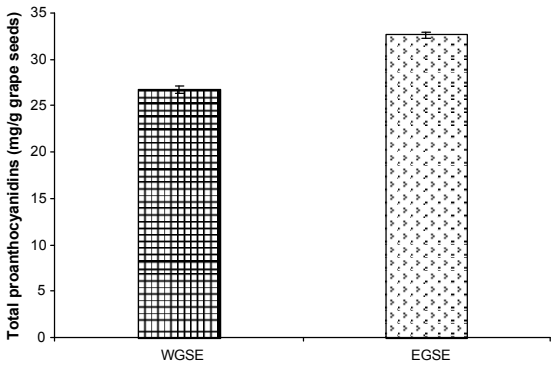

Fig. 1: Total proanthocyanidins content of water grape seed extract (WGSE) and ethanol grape seed extract (EGSE).

Inhibition of Digestive Enzymes by Grape Seed Extracts *in Vitro*: The inhibitory effects of grape seed extracts on some digestive enzymes associated with carbohydrate and lipid digestion were studied. Pancreatic α-amylase and lipase were used. In this study, the enzyme activities were assayed after pre-incubation of individual grape seed extract at various concentrations with enzyme for suitable period as well as after pre-incubation with substrate for suitable period.

α-amylase Inhibition: The effect of individual GSE on pancreatic α-amylase activity after pre-incubation with enzyme was evaluated and the data obtained are given in Table 1. Data illustrated that pre-incubation of pancreatic α-amylase with GSEs led to significant inhibition of enzyme activity, i.e. the pre-incubation of pancreatic α-amylase with each GSE led to decreasing the enzyme activity. Data also revealed that the inhibitory activity of both grape seed extracts was increased with increasing the concentration of each extract. In other words, there was positive correlation between GSE concentration and inhibitory activity. The maximum inhibition percentage (74.86%) was observed with EGSE at higher concentration (1800 ppm). However, the inhibition percentage of α-amylase reached 52.48% with WGSE at the same concentration. Noteworthy, it has been found that ethanol grape seed extract was also more effective than water grape seed extract. On the other hand, pre-incubation of α-amylase substrate with individual GSE at various concentrations led also to significant inhibition of pancreatic α-amylase. Data in Table 2 illustrate the inhibitory activity of GSEs after pre-incubation of each GSE at various concentrations with amylase substrate. As seen previously, there was also positive correlation between

Table 1: Inhibitory effect of water grape seed extract (WGSE) and ethanol grape seed extract (EGSE) on pancreatic α-amylase after pre-incubation of enzyme with extract

Treatment	Concentration (ppm)	Inhibition%
WGSE	400	7.53^{h}±0.07
	800	23.07^{g}±0.05
	1300	39.38^{f}±0.05
	1800	52.48^{d}±0.05
EGSE	400	39.94^{e}±0.10
	800	62.45^{c}±0.06
	1300	68.64^{b}±0.05
	1800	74.86^{a}±0.06
LSD 0.05	-	0.192

Values are means of three replicates ± SE. Numbers in the same column followed by the same letter are not significantly different at $P<0.05$.

Table 2: Inhibitory effect of water grape seed extract (WGSE) and ethanol grape seed extract (EGSE) on pancreatic α-amylase after pre-incubation of substrate with extract

Treatment	Concentration (ppm)	Inhibition%
WGSE	400	5.16^{g}±0.04
	800	12.13^{f}±0.09
	1300	21.60^{d}±0.03
	1800	30.37^{c}±0.37
EGSE	400	13.95^{e}±0.13
	800	30.11^{c}±0.14
	1300	48.16^{b}±0.14
	1800	61.66^{a}±0.13
LSD 0.05	-	0.495

Values are means of three replicates ± SE. Numbers in the same column followed by the same letter are not significantly different at $P<0.05$.

GSE concentration and inhibitory activity. The inhibition percentages of pancreatic α-amylase were 13.95, 30.11, 48.16 and 61.66% with EGSE whilst were 5.16, 12.13, 21.60 and 30.37% with WGSE at concentrations 400, 800, 1300 and 1800 ppm, respectively. The results obtained in Tables (1 and 2) showed generally that the inhibitory activity of GSEs was considerable higher when GSE pre-incubated with enzyme than that when GSE pre-incubated with enzyme substrate.

Lipase Inhibition: The effects of GSEs at different concentrations (400, 800, 1300 and 1800 ppm) on pancreatic lipase were studied either after pre-incubation of GSE with enzyme or with enzyme substrate. Inhibitory effects of GSEs after pre-incubation with enzyme are recorded in Table 3. The obtained results demonstrated that both WGSE and EGSE had lipase inhibitory activity. EGSE showed a strong inhibitory potential with inhibition percentage ranged from 52.66 to 17.05% for concentrations ranging from 1800–400 ppm. WGSE also possessed high inhibitory activity but less than that of EGSE at the same concentration, where maximum inhibition reached 45.44%.

Table 3: Inhibitory effect of water grape seed extract (WGSE) and ethanol grape seed extract (EGSE) on pancreatic lipase after pre-incubation of enzyme with extract

Treatment	Concentration (ppm)	Inhibition%
WGSE	400	$8.51^{g}\pm0.26$
	800	$22.05^{e}\pm0.59$
	1300	$37.55^{c}\pm0.42$
	1800	$45.44^{b}\pm0.28$
EGSE	400	$17.05^{f}\pm0.34$
	800	$26.05^{d}\pm0.26$
	1300	$38.15^{c}\pm0.37$
	1800	$52.66^{a}\pm0.44$
LSD 0.05	-	1.156

Values are means of three replicates ± SE. Numbers in the same column followed by the same letter are not significantly different at $P<0.05$.

Table 4: Inhibitory effect of water grape seed extract (WGSE) and ethanol grape seed extract (EGSE) on pancreatic lipase after pre-incubation of substrate with extract

Treatment	Concentration (ppm)	Inhibition%
WGSE	400	$0.79^{h}\pm0.10$
	800	$13.60^{f}\pm0.21$
	1300	$28.78^{d}\pm0.67$
	1800	$42.63^{c}\pm0.30$
EGSE	400	$2.88^{g}\pm0.16$
	800	$20.38^{e}\pm0.37$
	1300	$44.50^{b}\pm0.28$
	1800	$61.41^{a}\pm0.29$
LSD 0.05	-	1.021

Values are means of three replicates ± SE. Numbers in the same column followed by the same letter are not significantly different at $P<0.05$.

Similar trend of GSEs in inhibition of pancreatic lipase when pre-incubation with enzyme was found when enzyme substrate was pre-incubated with GSEs. Data in Table 4 illustrate the inhibitory activities of GSEs after pre-incubation with lipase substrate. Data revealed that EGSE at different concentrations (400-1800 ppm) had inhibitory activity more than that of WGSE. The inhibition percentages of EGSE and WGSE at higher concentration (1800 ppm) were 61.41 and 42.63%, respectively. A dose response relationship of GSEs on lipase inhibitory activity was observed. In other words, there was a positive correlation between inhibition percentage and concentration of grape seed extracts (WGSE and EGSE). The results obtained in Tables (3 and 4) showed generally that the inhibitory activity of GSEs was considerable higher when GSE pre-incubated with enzyme substrate than that when GSE pre-incubated with enzyme.

From previous results, generally, it can be concluded that the extracts of red grape seeds possess inhibitory effects against some pancreatic enzymes associated with digestion of starches (amylase) and lipids (lipase). Inhibitory activity of each GSE is occurred either pre-incubation with enzyme or substrate. There is a positive correlation between inhibitory activities of GSEs and their concentrations in enzyme assay medium. The ethanol grape seed extract had higher inhibitory effect against pancreatic amylase and lipase in comparison with water grape seed extract. On the other hand, the inhibitory effects of GSEs may be due to their contents of polyphenolic compounds especially proanthocyanidins. These polyphenolic compounds have ability to interact with digestive enzymes (as protein) or enzyme substrates [16, 21-23, 40]. The higher inhibitory effect of ethanol grape seed extract in comparison with water grape seed extract was associated with its content of polyphenolic compounds [11]. The obtained results were supported by Moreno *et al*. [17], who found that grape seed extract at a concentration of 1 mg/ml resulted in 80% inhibition against pancreatic lipase and they also suggested that the inhibitory effect may be caused by a synergistic action of several phenolic compounds including procyanidins within the extracts. Adisakwattana *et al*. [41] found that GSE significantly inhibited pancreatic lipase in dose-dependent manner and this inhibition may be caused by oligomeric procyanidins. Gonçalves *et al*. [12] studied the ability of grape seed procyanidins to inhibit α-amylase activity using a colorimetric method. They concluded that the increasing degree of polymerization of the procyanidin fractions is responsible for their increasing inhibition. This inhibition occurs through the formation of a stable interaction between procyanidin and enzyme as determined using fluorescence quenching. Wang *et al*. [42] investigated the interactions between proanthocyanidins (PC) and porcine pancreatic lipase (PL) was from variant aspects of lipase conformation, activity, kinetics and thermodynamics. The results showed that 34% inhibitory rate of PC on PL is achieved after about 30-min incubation and the inhibitory rate increases with the increase of PC concentration.

Finally, the present study indicates that red grape seed extracts are potent inhibitors of key enzymes in the digestion of carbohydrates and lipids *in vitro* and these inhibitory activities are related to polyphenol content in red grape seed extracts. More studies and *in vivo* experiments are needed to examine whether grape seed extracts can inhibit digestive enzymes *in vivo*.

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