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Biochemical and biological assessment of the inhibitory potency of extracts from vinification byproducts of *Vitis vinifera* extracts against glycogen phosphorylase

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ABSTRACT

The inhibitory potency of thirteen polyphenolic extracts obtained from vinification byproducts of Greek varieties of *Vitis vinifera* against glycogen phosphorylase (GP) has been studied by kinetic experiments. GP is an enzyme involved in glucose homeostasis and a molecular target for the discovery of new hypoglycemic agents. Studies have shown that all extracts display significant inhibitory potency for GP *in vitro* with IC_{50} values in the range of low μg/mL. X-ray crystallographic analysis of GP crystals soaked with two of these extracts revealed that the most active ingredient is quercetin which binds at novel binding site, distinct from the other known sites of the enzyme. One of the most potent of the studied extracts had also a moderate effect on glycogenolysis in the cellular lever with an IC_{50} value of 17.35 μg/mL. These results highlight the importance of natural resources in the quest for the discovery of new hypoglycemic agents, while at the same time they can serve as the starting point for their exploitation for antidiabetic usage and the development of novel biofunctional foods.

1. Introduction

Type 2 diabetes mellitus (non-insulin dependent diabetes) is a metabolic disorder, characterized by hyperglycemia. Currently, it affects approximately 285 million people, and this number is expected to increase significantly during the following years (Smyth and Heron, 2006). This disease is associated with long-term complications including heart disease, strokes, kidney failure and limp blood circulation which can lead to amputation. At the moment an efficient therapy for the type 2 diabetes mellitus does not exist although several types of hypoglycemic drugs, aiming to reduce blood glucose levels, are in use (Cheng and Fantus, 2005; Krentz and Bailey, 2005; Padwal et al., 2005; van de Laar et al., 2005). However, these treatments have several adverse side effects with the main danger of causing hypoglycemia (Murata et al., 2004) and they are inadequate for 30–40% of the patients (Wagman and Nuss, 2001). Therefore, other therapeutic concepts are intensively investigated (Agius, 2007; Staehr et al., 2002; Treadway et al., 2001) while a nutritional therapy has been also suggested (McCarty, 2000).

Grapes, grape juice, and grape extracts are known to possess beneficial effects on a broad variety of pathological conditions, including cancer, cardiovascular diseases, ischemic stroke, neurodegenerative disorders, aging, hypertension, and hyperlipidemia (Guilford and Pezzuto, 2011; Haroutounian et al., 2013). Moreover, grape extracts display strong antioxidant and anticancer activities (Anastasiadi et al., 2010; Apostolou et al., 2013; Stagos et al., 2005, 2006). On the other hand, literature reports concerning the effects of extracts from *Vitis Vinifera* (common grapevine, European grape) on glucose/glycogen homeostasis are scarce (Orhan et al., 2006; Park et al., 2008; Zunino, 2009).

Glucose homeostasis is a complex process relying mainly on glycogen metabolism. Glycogen phosphorylase (GP; E.C. 2.4.1.1)

Abbreviations: GPb, Rabbit muscle glycogen phosphorylase b; PLP, pyridoxal-5′-phosphate; T2D, type-2 diabetes; Glc-1-P, α-D-glucose 1-phosphate; rmsd, root-mean-square deviation.

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catalyzes the first step of intracellular degradation of glycogen to Glc-1-P (Oikonomakos, 2002). As such, over the last decade, GP has been targeted for the discovery of new hypoglycemic agents (Hayes and Leonidas, 2010; Oikonomakos, 2002; Oikonomakos and Somsak, 2008; Somsak, 2011; Somsak et al., 2008). The efficacy of GP inhibitors on blood glucose control and hepatic glycogen balance has been confirmed from biological studies (Agius, 2007, 2010; Kurukulasuriya et al., 2003a,b; Treadway et al., 2001). The catalytic site of the enzyme has been probed with glucose and glucose analog inhibitors, designed on the basis of information derived from the crystal structure of the T-state GPb-α-D-glucose complex (Martin et al., 1991). GP is an allosteric enzyme and exists in two interconvertible structural conformations, denoted T (inactive) and R (active) according to the MWC model (Monod et al., 1965). Inhibitors like glucose, that stabilize the T conformation, block the entrance to the active site by stabilizing a closed position of a six residues long loop (280s loop). Thus, most inhibitors were designed to mimic the contacts of glucose that localize the closed position of the 280s loop. Apart from the active site, crystallographic studies (Oikonomakos, 2002) have identified four other binding sites, the allosteric, the inhibitor, the glycogen storage, and the indole-binding site that bind various modulators of the GP activity.

In the last decade research efforts for the discovery of novel GP inhibitors have diverged towards compounds from natural products. Thus, flavonoid derivatives (Kaiser et al., 2001; Kato et al., 2010; Oikonomakos et al., 2000; Tsitsanou et al., 2013), triterpenes (Wen et al., 2008) and benzoic acid derivatives (Li et al., 2008, 2011) have been found to display significant inhibitory potency against GP. *V. vinifera* extracts are rich in polyphenols like flavonoids, stilbenes, and phenolic acids (Anastasiadi et al., 2009) which have emerged as potent inhibitors of GP activity displaying as well hypoglycemic activity (Oikonomakos and Somsak, 2008; Somsak et al., 2008). In this work we present the biochemical assessment of a series of polyphenolic extracts obtained from the vinification byproducts (grape pomace, seeds and stems) of Greek *V. vinifera* varieties. We have also identified by X-ray crystallography screening the molecule of quercetin as the most potent bioactive ingredient of these extracts and assessed *ex vivo* their potential to inhibit glycogenolysis. This is the first endeavour reporting that *V. vinifera* extracts display potent inhibitory activity against GP, both *in vitro* and *ex vivo* and may greatly contribute towards the exploitation of these byproducts of the winemaking process, as a source of cheap raw material for the development of novel biofunctional foods for type 2 diabetes patients.

2. Materials and methods

2.1. Plant material

The samples studied constitute the vinification process byproducts obtained from red (Voidomato, Mavrotragano, Mandilaria, Moschomavro and Muscat Hamburg) and white (Assyrtiko) varieties of *V. vinifera* of Greece, Vinsanto constitutes the sun-dried Assyrtiko grapes. All samples were directly obtained by manual separation after the vinification process and were air-dried, millpowdered and stored at room temperature. Their cultivation sites and year of harvest are provided in Table 1.

2.2. Extracts preparation

Fifty grams of dried, powdered material (pomace, seeds or stems) were poured into a 200 mL mixture of methanol (MeOH)/H_2O/1.0 N HCl (90:9.5:0.5 v/v) and sonicated in an ultrasonic bath for 10 min. The liquid phase was separated by filtration and the remaining solid was re-extracted three additional times, using the same solvent system and procedure. The combined extracts were evaporated under vacuum to provide a slurry, which was dissolved in 30 mL of MeOH/H_2O (1:1) and centrifuged for 10 min (7000 rpm). The supernatant liquid was extracted with petroleum ether (3 × 30 mL) in order to remove the contained lipids and concentrated under vacuum. The remaining residue was poured into 30 mL of brine and extracted repetitively with ethyl acetate (EtOAc, 4 × 30 mL). Thus, all sugars contained were separated in the aqueous layer and discarded. The combined organic layers were dried over anhydrous magnesium sulfate and evaporated under vacuum. The remaining solid was weighed and dissolved in MeOH to 1 mg/mL, membrane filtered (0.45 μm) and subjected to HPLC analysis. In order to avoid the polyphenols degradation, all the aforementioned activities were performed in the absence of direct sunlight and at temperatures below 35 °C.

2.3. Determination of extracts chemical composition

2.3.1. Chemicals and reagents

Gallic acid, (+)-catechin, (−)-epicatechin, p-coumaric acid, ferulic acid, caffeic acid, syringic acid, kaempferol, quercetin, rutin and *trans*-resveratrol were purchased from Sigma–Aldrich (Steinheim, Germany). The Folin–Ciocalteu reagent was purchased from Fluka (Steinheim, Germany). All solvents used for the qualitative and quantitative determination of polyphenols were purchased from J.T. Baker (Griesheim, Germany) as analytical (polyphenol extraction) or HPLC (chromatographic analyses) grades. All remaining chemicals were of analytical grade and obtained from Sigma–Aldrich.

2.3.2. HPLC analyses

The chemical composition of extracts was determined using HPLC analysis which was carried out on a Hewlett Packard HP1100 system equipped with a quaternary pump and degasser. The column used was a Kromasil C18 column (250 mm × 4.6 mm, particle size 5 μm) connected with a guard column of the same material (8 × 4 mm). Injection was by means of a Rheodyne injection valve (model 7725I) with a 20 μL fixed loop. For the chromatographic analyses HPLC-grade water was prepared using a Milli-Q system, whereas all HPLC solvents (except acetonitrile) were filtered prior to use through cellulose acetate membranes of 0.45 μm pore size. Chromatographic data were acquired and processed using Chemstation software. The HPLC method used is a modified version of the method developed by Tsao and Yang (Tsao and Yang, 2003). More specifically, the analysis was carried out at 30 °C (maintained by a column thermostat) using samples of 20 μL, which were directly injected by means of a Rheodyne injection valve (model 7725I). The gradient eluted consisted of solvent A (obtained by the addition of 3% acetic acid in 2 mM sodium acetate aqueous solution) and solvent B (acetonitrile, CH_3CN). Run time was set at 70 min with a constant flow rate at 1.0 mL/min in accordance with the following gradient time table: at zero time, 95% A and 5% B; after 45 min, the pumps were adjusted to 85% A and 15% B; at 60 min, 65% A and 35% B; at 65 min, 50% A and 50% B; and finally at 70 min, 100% B. This routine was followed by a 30 min equilibration period with the zero time mixture prior to injection of the next sample. The column effluent was monitored at 280, 320, and 360 nm simultaneously. Peaks were identified by comparing their retention time and UV–vis spectra with the reference compounds, and data were quantitated using the corresponding curves of the reference compounds as standards. All standards were dissolved in methanol. An indicative HPLC chromatogram for the quantization of red grape stem extracts of Mavrotragano variety of Santorini 2009 is shown in Fig. 1.

2.3.3. Assessment of the Total Phenolic Content (TPC)

The TPC of the extracts was determined in accordance with a modified version of the Folin–Ciocalteu method (Singleton et al., 1999), A 100 μL sample of extract was added to a 10 mL flask containing 6 mL of deionized water. One milliliter of Folin–Ciocalteu reagent was added to the mixture, and the flask was stoppered and allowed to stand at room temperature for 3 min. A 1.5 mL portion of 20% Na_2CO_3 was added and the solution was diluted to the desire volume (10 mL) with deionized water. Absorbance was measured at 725 nm versus a blank after 2 h at room temperature. The results are expressed as gallic acid equivalents using the standard curve (absorbance versus concentration) prepared from authentic gallic acid.

2.3.4. Determination of total flavonoids

The total flavonoid content of the grapes was determined using a modified colorimetric method developed by Jia et al. (Jia et al., 1999). In particular, 1 mL of grape extract was added into a 10 mL flask containing 4 mL of deionized water. A 0.3 mL portion of 5% $NaNO_2$ was added to this mixture and allowed to stand for 5 min at room temperature. Then, 0.3 mL of 10% $AlCl_36H_2O$ was added, the mixture was allowed to stand for 1 min at room temperature and 2 mL of 1 M NaOH was added. The solution was diluted to 10 mL with the addition of deionized water and the absorbance of the solution versus a blank at 510 nm was measured immediately. The results are expressed as catechin equivalents using a standard curve (absorbance versus concentration) prepared from authentic catechin samples (Table 1). There is a great variation in the extracts compositions of the same vine production byproducts but acquired in different years, e.g. Assyrtiko 2006 and 2011. It is well established that the polyphenolic content of grapes and their products depends strongly on the specific climatic-soil conditions of each year (e.g. rainfall, cold-warm spring and summer etc.). Additionally, the biosynthesis of certain polyphenols (e.g. resveratrol) occurs either during normal development and/or as response to stress conditions, such as infection, wounding and UV irradiation (Naczk and Shahidi, 2004).