Anti-inflammatory and antiplatelet activity of isolated phenolics from wine wastes

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Abstract— Interesting fractions or/and constituents from wine wastes (both red and white marc) were obtained by solvent extraction and sorption/desorption techniques. The specific fractions may have applicability in food and pharmaceutical industries, by presenting simultaneously antioxidant activity (DPPH method), antiplatelet activity (*in vitro*) and anti-inflammatory (inhibition of cycloogygenases 1 and 2) properties. Specific components, such as gallic acid, quercetin and kaempferol, which identified in the active fractions, were also tested for their contribution to the above mentioned activities.

***Keywords*—**Wine wastes, phenolics, antiplatelet activity, anti-inflammatory activity.

I. Introduction

Grapes are reported to exert favourable effects on human health. Their annual production is nearly 60.000.000 tn and mostly (80%) is used in winemaking industry **[1]**. Enological industries produce a large amount of wastes. These contain various valuable secondary by-products such as phenolics, sugars, organic acids etc., which are beneficial materials for neutraceutical and pharmaceutical applications, whereas their free disposal increases the chemical and biochemical oxygen demand **[2]**. Thus, isolation of some phenolics or their use for production of high added value products are very important both for economic and environmental aspects [**3]**.

Phenolic compounds can be considered as high added-value by-products and the use of low-cost industrial wastes could greatly reduce their production costs and increase the margin profit of the products [**4]**. The isolation of such chemical constituents from winery wastes is very important, since they can be used in food and pharmaceutical industries. Phenolics are potent antioxidants and as it has been reported, have beneficial effects against cardiovascular disease, cancer, allergy, anti-inflammatory and antiplatelet activity and anti-carcinogenic effects **[1, 4].**

Cardiovascular diseases are the leading cause of mortality and morbidity worldwide, accounting for nearly 30% of global deaths. Intravascular thrombosis, such as coronary thrombosis, deep venous thrombosis, and acute arterial thrombosis, is a major cardiovascular disease. The general pathogenesis of thrombosis includes platelet activation. Patients with coronary heart disease tend to have increased platelet reactivity. Therefore, antiplatelet therapy is a promising approach for preventing thrombosis **[5].** Some phenolics, including gallic acid, quercetin, kaempferol, are anti-platelet activity sources **[6, 7, 8].**

The objectives of our study were the isolation of phenolics from red and white wine wastes using solvent extractions and sorption/desorption techniques. Phenolic fractions analysed using HPLC and LC-MS/MS and exhibit significant antioxidant activity. Moreover, their *in vitro* antiplatelet activity was evaluated as well as their anti-inflammatory activity was tested measuring the inhibition of cyclooxygenases 1 and 2 (COX-1 and COX-2).

II. Materials and Methods

A. Wine wastes

Grape marc, white and red from malagouzia and syrah variety respectively, were kindly provided by Ktima Gerovassiliou, a wine-making factory in Epanomi (Thessaloniki, Greece) in the vintage 2013. Samples were dried at ambient temperature and milled in a commercial blender (i.d. ≤ 1 mm). 100 g of dry weight were extracted with a certain volume of ethanol in a sonicator bath (General sonic, 41 kHz, 320 W) at 35^{o}C, 20 min, pH 2.0, to increase the yield of extracted phenols and their antioxidant activity. The extracts thus obtained, here after called crude extracts, were centrifuged (4500 rpm, 10 min), stored in the refrigerator (-20 ^{o}C) and further analyzed.

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B. HPLC and ESI-MS analysis

Polyphenols analysis were carried out using a Thermo Finnigan Spectra HPLC system (San Jose, California) model UV 6000 LP, equipped with EZChromeElite software, Version 3.1.7., four Q-Grad pumps, a diode array detector (DAD; the wavelengths used were 280 and 360 nm) and injection valve (20μL loop). The separation was performed with a Grace Smart RP C-18 column (250x4.6 mm i.d.; 5 μm particle size).

The mobile phase consisted of 2% (v/v) acetic acid in milli-Q water (eluent A) and 100% acetonitrile (eluent B) using a gradient program as follows: from 0 min, 100% A; 4 min, 85% A/15% B; 20 min, 60% A/40% B; 40 min, 45% A/55% B. Total run time was 40 min and a flow rate of 1.0 mL/min.

Electrospray ionization mass spectrometry (ESI-MS) infusion experiments were carried out by using a Thermo Fisher Scientific (Bremen, Germany) model LTQ Orbitrap Discovery MS. The experiments were run using a standard ESI source operating in a positive ionization mode. Source operating conditions were a 3.7 kV spray voltage and a 300 °C heated capillary temperature.

C. In vitro platelet aggregation

Platelet aggregation experiments were performed by a conventional photometric technique in a four channel aggregometer, at 37°C, with continuous recording of light transmission, according to the method of Born **[9].** Platelets were obtained from venous blood of healthy donors. The blood was immediately mixed with 3.8% sodium citrate solution and was centrifuged at 1000 rpm for 10 min to yield platelet rich plasma (PRP). Collagen was used as aggregation agent. The aggregation was determined by recording the increase of light transmission. The aggregometer calibration was performed using platelet poor plasma (PPP; 100% T) obtained by centrifugation (1500 × g, 15 min) **[10].** The results are expressed as antiplatelet activity and calculated from **equation (1).**

Antiplatelet Activity = (maximum aggregation of collagen - maximum aggregation of sample)/maximum aggregation of collagen*100% **(1)**

D. Anti-inflammatory activity

The anti-inflammatory activity of crude extracts and phenolic fractions was tested using COX (cyclooxygenaces 1 and 2) activity assay kit (CAYMAN CHEMICAL, USA). The values were determined according to the manufacturer's instructions and the activity of samples on the enzymes, is expressed as percent inhibition (% inhibition).

E. Determination of antioxidant activity

Antioxidant activity was estimated using the 2,2-diphenyl-1-picrilhydrazyl (DPPH) method **[11].** The DPPH solution (0.1 g per L in ethanol) was prepared daily, stored in a flask covered with aluminium foil and kept in the dark at 4 °C between measurements. The percent decrease in absorbance was recorded for each concentration and percent quenching of DPPH radical was calculated on the basis of the observed decrease in absorbance of the radical **(equation 2)**.

% Inhibition = $[(A_{DPPH} - A_{Extr})/A_{DPPH})] * 100$ **(2)**

Where A_{DPPH} is the absorbance value of the DPPH blank sample and A_{Extr} is the absorbance value of the test solution.

F. Data analysis

Statistical analysis was carried out using the Microsoft Office Excel. All experiments were run in triplicate and the results expressed as mean ± standard deviation (SD) values. All data were considered statistical significant at $p < 0.05$.

III. RESULTS AND DISCUSSION

A. Wine wastes

In this study, crude extracts from white and red marc were tested for their antiplatelet and anti-inflammatory activity. Furthermore, an isolation of some of the most active phenolics in these extracts was carried out by solvent extraction and subsequent sorption/desorption experiments **(unpublished data) [12].** In **Figure 1(a)** the HPLC profile of the initial white marc extract (possessing antioxidant activity 87±1.92 %), before sorption, is given, whereas, in **Figure 1(b)**, the HPLC profile of the sample after sorption/ desorption (possessing antioxidant activity 85±2.62 %) using aluminum oxide is given.

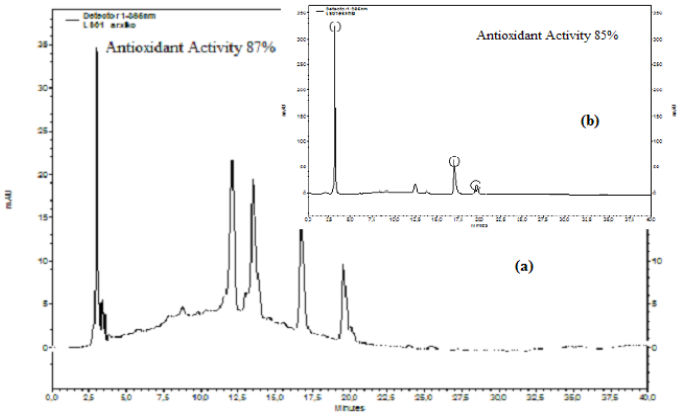

Fig 1. HPLC analysis of crude white marc extract (a) and white marc sample after sorption/desorption method (b), at 360 nm.

B. HPLC and ESI-MS analysis

According to HPLC and ESI-MS analysis, gallic acid, quercetin and kaempferol were identified in the initial and final extracts from red and white marc respectively. In **Figures 2, 3** and **4** the HPLC and ESI-MS profile of these phenolics identified, in white marc extract, is given.

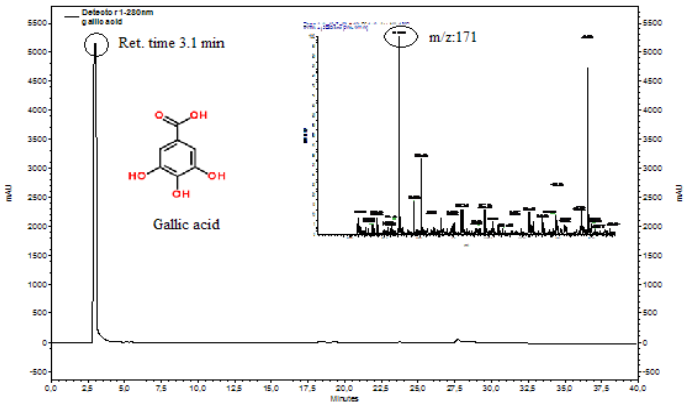

Fig 2. HPLC and ESI-MS profile of isolated gallic acid (retention time 3.1 min, m/z 171), at 280 nm from white marc extract.

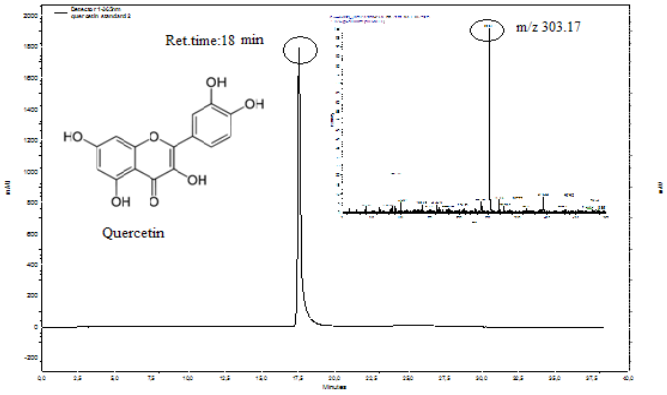

Fig 3. HPLC and ESI-MS profile of isolated quercetin (retention time 18 min, m/z 303.17), at 360 nm from white marc extract.

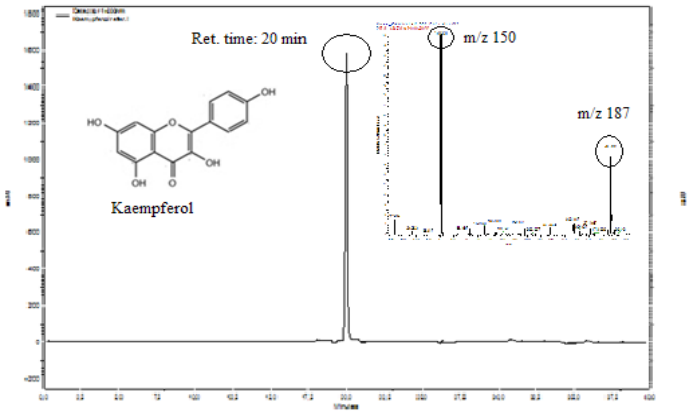

Fig 4. HPLC and ESI-MS profile of isolated kaempferol, (retention time 20.0 min, m/z 150,287) at 280nm from white marc extract.

C. In vitro platelet aggregation

Red and white marc samples, due to their high antioxidant activity (96 and 87% respectively, according to DPPH assay) were also tested for antiplatelet activity *in vitro*. Their inhibitory effect on platelet aggregation induced by collagen is shown in **Figure 5.**

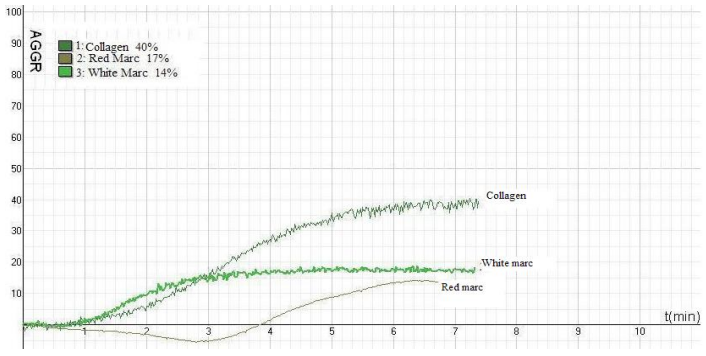

Fig 5. Effect of red and white marc on human platelet aggregation induced by collagen **[2].**

Furthermore, in another series of experiments, isolated phenolics from final fractions from sorption /desorption experiments, possessing high antioxidant activity **(Fig. 1)** were also examined for their antiplatelet activity. The isolated phenolics gallic acid, quercetin and kaempferol found to exert strong inhibition of platelet aggregation **(Fig. 6)** at the concentration of 1 ppm and the data are in aggrement with recent reports **[8]**. Dose/response experiments of all samples reported here are in progress and will give an insight of synergism or of the autonomous and independent action of each one of the compounds examined. Interesting, resveratrol has not been detected in the initial crude extracts neither of red nor of white marc samples. Thus our data suggest that not only resveratrol, a constituent of red grapes which eventually passes to the wine, but a number of other phenolic compounds that go to wine wastes may contribute to the health benefits to humans, by inhibiting platelet aggregation.

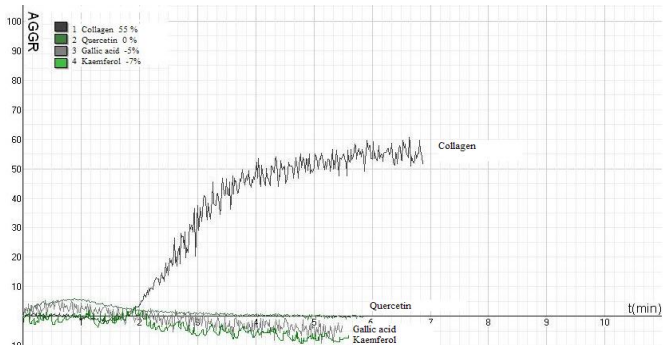

Fig 6. Effect of quercetin, gallic acid and kaempferol on human platelet aggregation induced by collagen.

D. Anti-inflammatory activity

In the present study, the anti-inflammatory activity of the initial white and red marc extracts and of isolated gallic acid, and quercetin is presented in **Figure 7**.

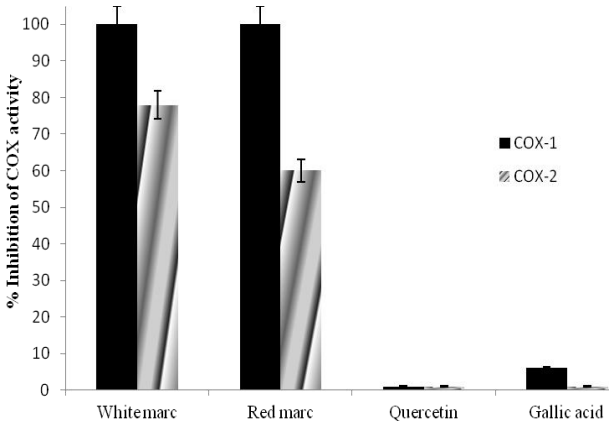

Fig 7. Effect of red and white marc extracts, quercetin and gallic acid on COX-1 and COX-2 activity.

The cyclooxygenase (COX-1 and COX-2) isoenzymes, catalyze the conversion of arachidonic acid to prostaglandin H_2 (PGH_2), a potent platelet aggregator of vascular smooth muscle contraction and inhibitor of platelet thromboxane synthase. Prostaglandins on the other hand play critical roles in several biological processes, including the regulation of immune function**,** kidney development and gastrointestinal integrity. COX-1 and COX-2 are isoforms, and differ mainly in their pattern of expression. COX-1 is expressed in most tissues, whereas COX-2 is induced by numerous physiologic stimuli **[13]**. Metabolites of arachidonic acid are critical for numerous biologic processes, including inflammation, ovulation, implantation, angiogenesis, platelet aggregation, and immunologic function. Eicosanoids are the products of arachidonic acid metabolism, and the cyclooxygenase enzymes play a key role in the production of eicosanoids. Since the first report that aspirin and indomethacin inhibited prostaglandin production by blocking cyclooxygenase activity **[14]** it has been found that nonsteroidal anti-inflammatory drugs (NSAIDs) directly affect cyclooxygenase activity, either by covalently modifying the enzyme (as in the case of aspirin) or by competing with the substrate for the active site.

Our results, give a first indication that the initial extracts have strong effects against COX-1 (~99% for both red and white marc extracts) and COX-2 (60% and 78% from red and white marc respectively) enzymes. Quercetin lack of effect on both COX-1 and COX-2 enzymes, however, gallic acid showing marginal inhibition only of COX-1 (~6%) **(Fig.7)**. Thus, the effect of all the other constituents in the final fraction, such as kaempferol etc., should further be investigated to clarify their contribution/ or not, to anti-inflammatory activity.

IV. Conclusion

Specific phenolic extracts were obtained from red and white marc samples and showed significant inhibition of platelet aggregation induced by collagen in a series of *in vitro* experiments, in addition to their high antioxidant activity.

Red and white marc extracts also showed significant anti-inflammatory activity (~99 % inhibition of COX-1 and ~78 % inhibition of COX-2 enzymes).

Considering the annual production of these wastes and the multifunctional properties of phenolic fractions obtained, their valorization seems very profitable in food, neutraceutical and pharmaceutical applications.

Acknowledgment

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