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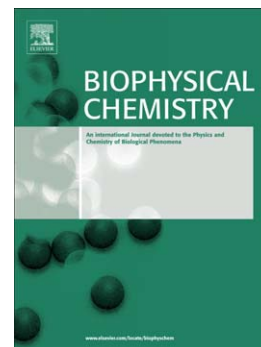
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Effect of Resveratrol on Platelet Aggregation by Fibrinogen Protection

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KEYWORDS: Resveratrol, fibrinogen, NMR, FTIR, platelet adhesion and aggregation

ABBREVIATIONS

RSV, 3,5,4'-trihydroxy-trans-stilbene; FBG, fibrinogen; EP, epinephrine; SEM, Scanning
Electron Microscopy; PS, polystyrene; PRP, platelet rich plasma.

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ABSTRACT

The effect of resveratrol (RSV) in inhibiting platelet adhesion and aggregation, as well as fibrinogen (FBG) conformational changes promoted by epinephrine (EP), were studied, by using complementary experimental techniques.

NMR and IR spectroscopies were used to investigate possible protective effects by RSV toward FBG, in presence of EP. The protective effect of RSV towards FBG was highlighted by spin nuclear relaxation experiments, that were interpreted for determining the thermodynamic equilibrium constants of FBG-EP interaction, and by infrared measurements, that showed EP-induced conformational changes of FBG.

The ability of RSV in inhibiting platelet adhesion and aggregation promoted by EP was evaluated by Scanning Electron Microscopy (SEM), measuring the platelet adhesion and aggregation degree, in comparison to data obtained for platelet aggregation in platelet rich plasma (PRP).

The experimental combined approach pointed out that RSV is able to protect both FBG and platelets from the denaturant and aggregating action of EP at stress level concentration.

INTRODUCTION

Blood platelet activation plays a crucial role in many important physiological and pathological processes, such as several arterial disorders, including strokes and myocardial infarction [1]. The activation of platelets is also considered to be an important step in inflammatory processes, i.e. synthesis and release of inflammatory substances like cytokines and prostanoids. It is well known, that blood platelets also participate in allergic and non allergic inflammation and tumor progression [2].

Natural products having antioxidant properties, are useful for understanding the mechanism of pathologies onset. Resveratrol (3,5,4'-trihydroxy-*trans*-stilbene, RSV) exhibits a wide range of biological activities, including anti-inflammatory and anti-tumoral effects [3]. RSV may be responsible for the protection against coronary heart diseases [4,5]. The mechanism of RSV action has not been totally elucidated, but it seems to be related to its antioxidant activity, its ability to inhibit ribonucleotide reductase, DNA polymerase and cyclooxygenase 2 (COX-2) transcription, and its action as agonist for the estrogen receptor [6]. RSV inhibits the biological activity of blood platelets [3,7], causing reduction of thrombin-induced aggregation, decreasing platelet adhesion to type I collagen [8] and fibrinogen (FBG) [9] and inhibiting platelet aggregation induced by ADP, collagen, and thrombin [7,9]. RSV also acts as an antioxidant against free radical generation in blood platelets [10] and may be responsible for modulating signaling pathway.

Several studies relevant to platelets morphology and ultrastructure of platelets interacting with other species, i.e. fibrin networks in animals and humans, via microscopy techniques (TEM and SEM) are reported [11]. The studies allow to verify the modification of animals platelets morphology (including humans, from which they are originated) and the aggregation stage induced by the presence of coagulant and/or anti-aggregation species like epinephrine (EP) and/or resveratrol (RSV), respectively.

FTIR studies on protein conformational changes induced by several substances, surfaces or physical parameters, have also been reported [12,13,14], shedding light on the relation between proteins structure and their bioactivity.

Thus, it is interesting to underline the power of an integrated characterization approach based on spectroscopic/microscopic studies and biological tests. On these reasoning, the RSV effect towards platelet adhesion and aggregation induced by EP was evaluated together with the antioxidant ability to preserve the FBG native conformation in the presence of EP with the aim of understanding the potential effects of antioxidant compounds in regulating physiological or pathological processes. Moreover, platelet aggregation and protein denaturation processes were studied by using relaxation and infrared spectroscopy measurements, compared with biological data also of exploiting chemical-physical methods in biological approaches.

Improving the safety and functioning of blood-contacting devices, often requires the development of strategies that allow to avoid adverse reactions, particularly platelet adhesion and aggregation at the implants surface. Although, the effect of RSV in reducing platelet aggregation in platelet rich plasma (PRP) was largely demonstrated [15,16], the ability of this molecule to influence platelet adhesion and aggregation at the interface with polymeric surfaces has never been proved. The results obtained and reported in this paper show that RSV is able to reduce platelet aggregation induced by epinephrine not only in PRP but also on polystyrene. The presence of the antioxidant compound really contributes to enhance the hemocompatibility of blood-contacting devices.

EXPERIMENTAL SECTION

Materials. Epinephrine (EP) and *trans*-resveratrol (3,5,4'-trihydroxy-*trans*-stilbene, RSV) were purchased from Sigma Aldrich Chemie GmbH (Buchs, Switzerland) and used without further

purification. Fibrinogen (FBG, Human Plasma, MW=341 kDa) was purchased from Calbiochem (San Diego, USA) and used without further purification.

All materials used in platelet adhesion and aggregation studies were supplied by Sigma Aldrich (Switzerland).

NMR Measurements. All the ^1H spectra were recorded on a Bruker AMX 400. The solutions were obtained by dissolving the appropriate amounts of epinephrine (EP) and resveratrol (RSV) in D_2O .

The spin-lattice relaxation rates were measured using the $(180^\circ-\tau-90^\circ-t)_n$ sequence. The τ values used for the selective and non-selective experiments were the following: 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.8, 1, 1.4, 2, 3, 4, 5, 8, 20 s respectively, and the delay time (t) was 20 s. The 180° selective inversion of the proton spin population was obtained by a selective soft gaussian perturbation pulse (width: 60 ms, attenuation level: 120dB) [17]. The FID (Free Induction Decay) was processed using an exponential window function with line broadening of 1 Hz. The selective and non-selective spin-lattice relaxation rates refer to the H-6 of epinephrine. Since in general the recovery of proton longitudinal magnetization after a 180° pulse is not a single exponential, due to the sum of different relaxation terms, the selective spin-lattice relaxation rates were calculated using the initial slope approximation and subsequent three parameter exponential regression analysis of the longitudinal recovery curves. The maximum experimental error in the relaxation rate measurements was 5%. The affinity index was calculated by linear regression analysis of the experimental data.

All the spectra were processed using the Bruker Software XWINNMR, version 2.5 on Silicon Graphics O₂ equipped with RISC R5000 processor, working under the IRIX 6.3 operating system.

Calculation of the Affinity Index. The analysis of non-selective R_1^{NS} and selective R_1^{SE} proton spin-lattice relaxation rates, allows to determine the *affinity index* for the ligand-macromolecule equilibrium, as previously reported in literature [18,19,20].

To remove the effects of different correlation times and different proton densities, and to isolate the effects of restricted motions, due to the interaction of the ligand with the macromolecule, it a *normalized affinity index* should be used [21]:

$$\Delta R_{1N}^{SE} = \frac{KR_{1b}^{SE}[M_0]}{(1 + K[L])R_{1f}^{SE}} \quad (1)$$

where K is the thermodynamic equilibrium constant, R_{1f}^{SE} and R_{1b}^{SE} are the selective spin-lattice relaxation rates for the free and bound ligand fractions, M_0 and L are the macromolecule and ligand concentrations, respectively.

The dependence of the normalized relaxation rate, enhancements ΔR_{1N}^{SE} by the macromolecule concentration $[M_0]$ is represented by a straight line passing through the origin of the axes with slope:

$$[A_I^N]_L^T = \frac{KR_{1b}^{SE}}{(1 + K[L])R_{1f}^{SE}} \quad (2)$$

$[A_I^N]_L^T$ is still a constant at constant temperature and ligand concentration and it is defined as *normalized affinity index* ($\text{dm}^3 \text{mol}^{-1}$).

Calculation of the Binding Constants of epinephrine-fibrinogen complexes. Plotting $1/\Delta R_1^{SE}$ as function of the ligand concentration $[L]$, a linear correlation is obtained, the slope (S) and the intercept (I) of the straight line being:

$$S = \frac{1}{R_{1b}^{SE}[M_0]} \quad (3)$$

and

$$I = \frac{1}{KR_{1b}^{SE}[M_0]} \quad (4)$$

Since the concentration of the macromolecule is known and the relaxation rate of the free ligand can be directly measured, the relaxation rate of the bound ligand R_{1b}^{SE} can be calculated by the equation 3 and this value can be used in order to obtain the equilibrium constant parameter, K, from equation 4.

FTIR-ATR Measurements. Solutions for the FTIR-ATR experiments were obtained by dissolving the appropriate amounts of ligands and protein in PBS, in order to obtain the following concentrations: FBG 7.33×10^{-6} M (2.0 mg/mL); EP 80-90 pg/mL (stress concentration); RSV 5.0×10^{-4} M.

The spectra were obtained with a Thermo FT-IR spectrometer Nicolet 5700, operating between 3000 and 900 cm^{-1} . An MCT detector was used, and the apparatus was purged with dry nitrogen. Typically, 300 scans at a resolution of 2.0 cm^{-1} were averaged. The frequency scale was internally calibrated with a reference He-Ne laser to an accuracy of 0.01 cm^{-1} . An attenuated total reflection (ATR) cell for liquid with germanium crystal was used to record the spectra in solution. The collected IR spectra of FBG-EP and FBG-EP-RSV do not contain the vibrations of the two small molecules (EP and RSV), since their concentrations used in the experiments were well below the IR detection limit. Because of its high surface activity, the FBG, once the protein solution is introduced into the ATR cell, rapidly adsorbs onto the surface of germanium crystal. Since it is well-known that surface adsorption may affect the protein conformation [22,23], spectra of both the protein and protein-hormone systems were collected at predetermined intervals during their residence times (0-10 min) into the cell. The recorded spectra did not differ from each other, suggesting that the surface of the ATR germanium crystal did not significantly affect the FBG secondary structure, at least within the experimental time interval.

Spectral Processing. All the spectra in solution were taken in a single beam mode. The sample spectrum was obtained by subtracting the solvent spectrum from the sample solution spectrum. The scale factor for solvent subtraction was chosen so that the spectral region between 2000 and 1700 cm^{-1} was flat.

Spectral Enhancement. In order to improve the observability of the overlapping bands, mathematical resolution enhancement was performed by a spectral deconvolution process that is similar to the Fourier self-deconvolution [24,25], except that mathematical operations are performed in the spectral domain, rather than in the Fourier domain. The spectral deconvolution process moves intensity from the outer wings of a band to the center of the band, therefore reducing its effective half-width and improving its observability [26]. The quality of the deconvolution procedure is controlled by two variables, namely, a half-band-width of the Lorentzian line used for deconvolution and the resolution enhancement achieved.

Blood collection

Selected donors were healthy people who had fasted for more than 8 h and had not received any medication for at least 14 days. Blood samples were collected by adding 3.8% (w/v) trisodium citrate water solution as anticoagulant, at 9:1 ratio blood/citrate (v/v). Platelet-rich plasma (PRP) was generated by spinning whole blood at 150 g for 15 min at room temperature; platelet-poor plasma (PPP) was prepared by spinning citrated blood at 2000 g for 15 minutes at room temperature. PRP was suspended in PPP up to a final platelet concentration of 200.000/ μL .

Platelet adhesion and aggregation on polystyrene

Incubation of platelets with samples. The ability of RSV to inhibit platelet adhesion and aggregation, induced by epinephrine (EP), on polystyrene (PS) surface, was evaluated. The response of the PRP to PS in presence of EP 15 mM, and different RSV concentrations solubilized in hydro-ethanolic solution (EtOH, 0.3 mM) was tested. Moreover, as it is well

known that high concentration of EtOH reduces platelet aggregation in vitro [27], the effect of EtOH 0.3 mM on the PRP and EP system (without RSV) was also evaluated.

Epinephrine hydrochloride was reconstituted with a filtered (0.2 μm) PBS solution and resveratrol solutions at different concentrations (0.5, 15, 60, 100 mM) were formulated by adding appropriate volumes of hydro-ethanolic solution.

An aliquot of 10 μL of each RSV solution, or 10 μL of ethanol 0.3 mM, was then added to 440 μL of PRP and incubated for 10 min at 37°C. Then, 50 μL of EP solution was added. Each of the above samples was then added to the bottom of each well of a 24-multiwell plate. The samples were then incubated for 1 h at 37°C without shaking. Each sample was tested in triplicate.

Evaluation of platelet adhesion and aggregation on PS by Scanning Electron Microscopy (SEM). After the incubation, the samples were treated for SEM analysis following the procedure already reported [28]. Briefly, the samples were rinsed carefully with phosphate buffered saline (PBS) to remove any non-adhering platelet and incubated in 2.5% (v/v) glutaraldehyde in 100 mM sodium cacodylate for 30 min. Then, the samples were washed in 100 mM cacodylate buffer for 30 s, rinsed with distilled water and left standing in primary dehydration solution (70% v/v EtOH in H₂O) for 15 min. They were then transferred to secondary dehydration solution (90% v/v EtOH in H₂O) for 15 min, and subsequently to absolute ethanol for 15 min for total platelet dehydration. Finally, the samples were desiccated overnight under vacuum and gold sputtered with an automatic sputter coater (BAL-TEC MED 010, Balzers, Germany). SEM at 15 kV accelerating voltage was used to analyse the degree of platelet adhesion and aggregation to PS. The shape of adhered platelets was described according the modified Cooper morphology [29] (Table 1).

Table 1. Classification of adherent platelets according to their morphology, as reported by Cooper et al. [29]

| Platelet shape | Morphology |
|-----------------------|---|
| Round (R) | round or disc-shaped morphology and absence of pseudopodia |
| Dendridic (D) | disc-shaped morphology, few pseudopodia and absence of spreading |
| Spread-Dendridic (SD) | disc-shaped morphology with several pseudopodia, some of them flattened onto the material surface and with hyaloplasma expanded among pseudopodia |
| Spreading (S) | disc-shaped morphology with hyaloplasma expanded among pseudopodia |
| Fully Spread (FS) | disc-shaped morphology with hyaloplasma completely spread and absence of distinct pseudopodia |

Platelet aggregation in Platelet-rich plasma (PRP)

Platelet aggregation induced by EP alone and in presence of RSV and ethanol, was quantified by measuring maximal aggregation (percent of maximal light transmittance). Briefly, 10 μ L of PBS or resveratrol solution or ethanol solution were added to 440 μ L of PRP at 37°C in the aggregometer cuvette, in order to achieve the following final concentrations of the tested compounds: (a) RSV 100 μ M, (b) EtOH 0.3 mM. After 10 min of incubation at 37°C, 50 μ L of EP solution were added in order to obtain a final concentration of the platelet agonist of 15 μ M. The aggregation curves were then registered.

Statistical analysis

Multiple comparison were performed by one-way ANOVA and individual differences tested by Fisher's test after the demonstration of significant intergroup differences by ANOVA. Differences with $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Nuclear Relaxation Measurements

The hypothesized protective effect exerted by resveratrol (RSV) toward fibrinogen (FBG) can be investigated by Nuclear Magnetic Resonance (NMR) spectroscopy, in particular by spin nuclear relaxation data. As reported in Material and Methods section, the behaviour of selective spin-lattice relaxation rate values of epinephrine (EP) in the presence of different amounts of FBG plays a key role in understanding whether the interaction occurs. In particular, the calculation of the *affinity index* from relaxation rates enhancement, allows to quantify the strength of such recognition processes. Moreover, the comparison of the affinity indexes calculated for EP-FBG systems in absence or in presence of RSV, allows to establish the differences in the extent of the interaction, i.e. the effect of such polyphenol in decreasing EP-FBG interaction. Figure 1 reports the enhancement of proton selective relaxation rates of epinephrine due to the presence of fibrinogen in the presence of resveratrol (line a) and without resveratrol (line b). Data highlighted a strong diversity in the affinity indexes for the two systems; in particular the strength of the interaction between epinephrine and FBG was strongly decreased by the presence of resveratrol. This protective effect can also be evaluated analysing the complex formation thermodynamics in the two systems, which can be deeply investigated by calculating the values of the equilibrium constants that describe the formation of epinephrine-fibrinogen complexes in the presence and absence of resveratrol.

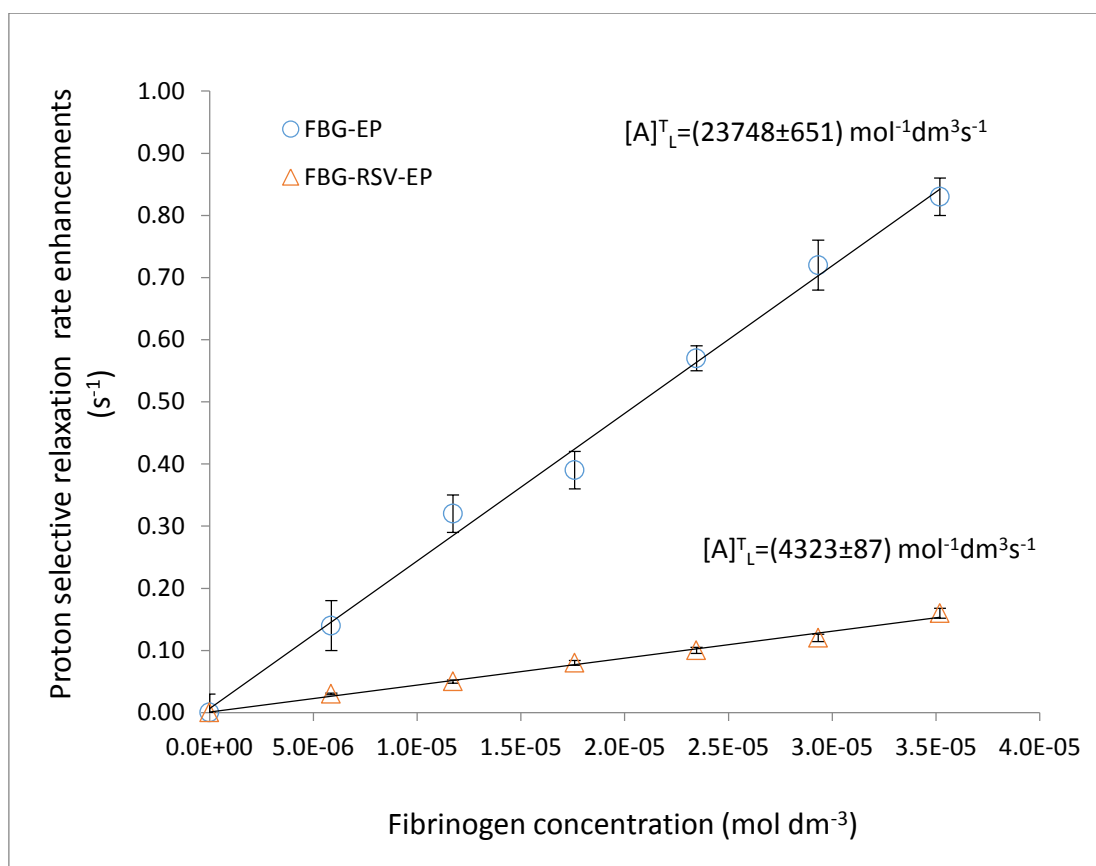


Figure 1. Proton selective relaxation enhancements of epinephrine versus fibrinogen concentration in the presence of 500 μM of resveratrol and without resveratrol with the affinity indexes calculated for the two systems.

The contribution to the proton selective relaxation rate due to the fraction of EP bound to FBG, may be dependent also on small ligand concentration. In particular, the reciprocal of relaxation rate enhancements measured varying epinephrine amounts at a fixed FBG concentration with and without resveratrol, which typically shows a linear trend in relation to ligand concentration, can be used to calculate the thermodynamic equilibrium constants (K) as well as the relaxation rate of the bound ligand in the two systems. Figure 2 shows the values of $1/\Delta R_1^{SE}$ versus epinephrine concentration in the presence of a fixed amount of fibrinogen (a) without resveratrol and (b) with 500 μM of resveratrol. From the slope and the intercept of the straight lines obtained by fitting the experimental data, the equilibrium constants which describe the formation

of epinephrine-fibrinogen complexes, in the absence and presence of resveratrol, have been calculated as (148.47 ± 6.47) and $(9.03 \pm 0.40) \text{ dm}^3 \text{ mol}^{-1}$, respectively. The dissociation constants K_D , obtained from equilibrium constants ($K_D = 1/K_{eq}$), which measure the tendency of the epinephrine-fibrinogen complexes in the absence and presence of resveratrol to release the single components, have been calculated as $(6.7 \times 10^{-3} \pm 3 \times 10^{-4})$ and $(1.1 \times 10^{-1} \pm 5 \times 10^{-3}) \text{ mol dm}^{-3}$, respectively. These results confirm that resveratrol is able to protect fibrinogen from the denaturant activity of epinephrine, i.e. avoiding coagulation processes when the plasma concentration of epinephrine reaches high levels due to stress conditions.

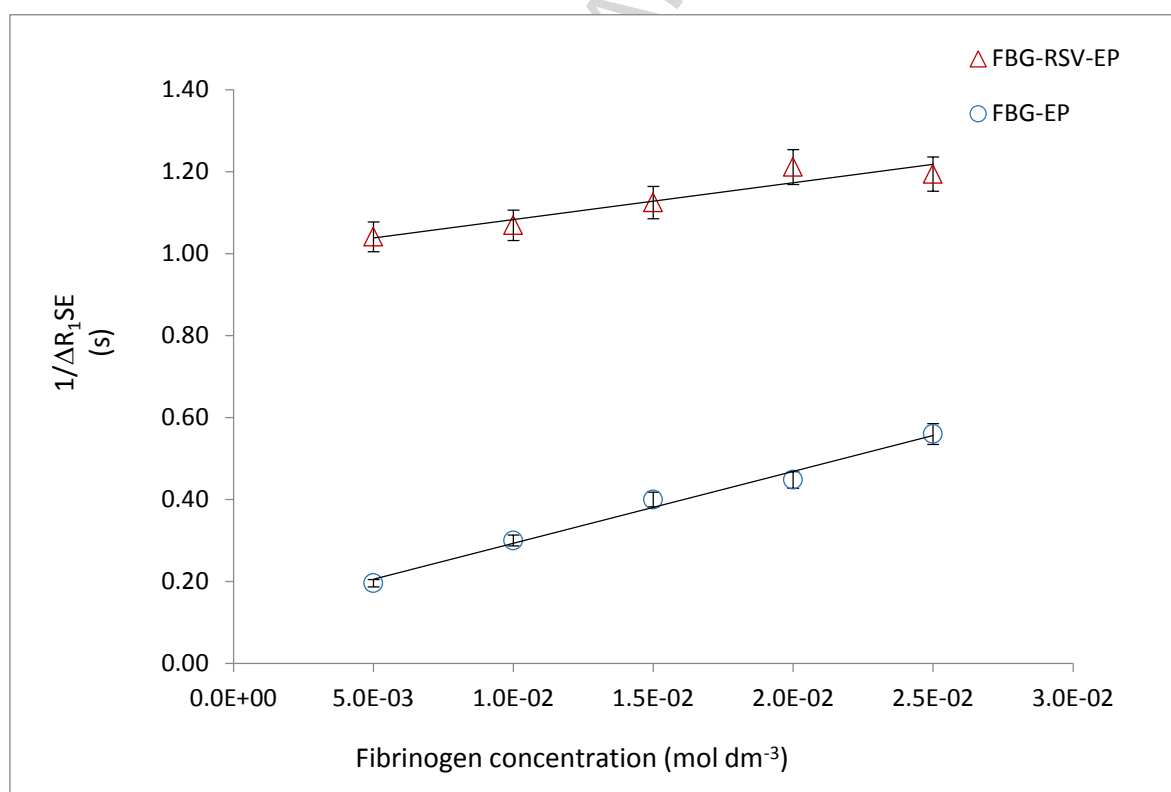


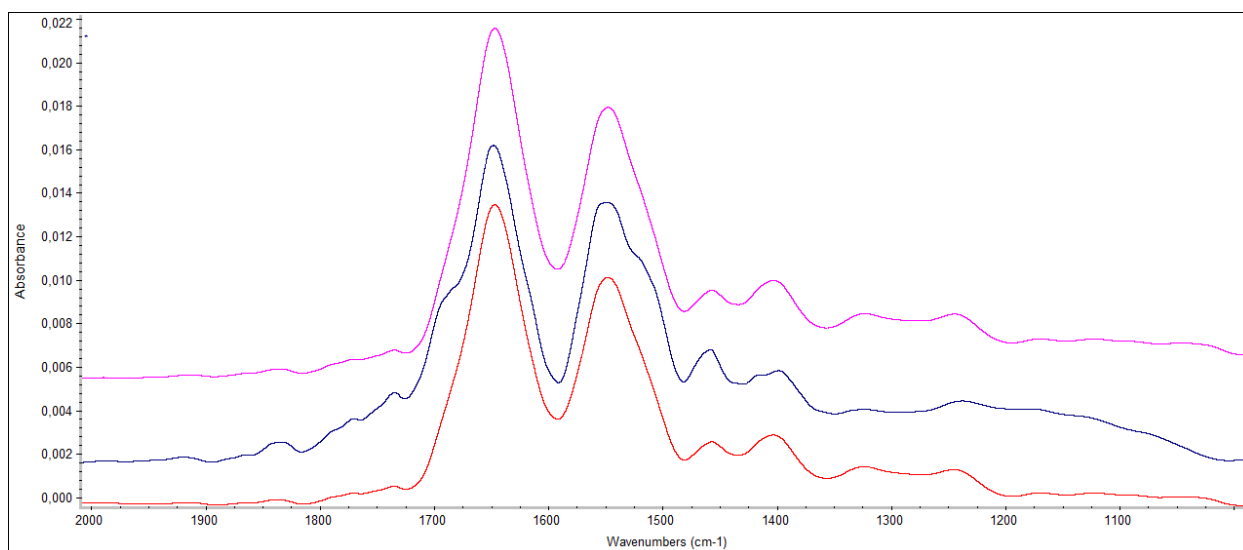
Figure 2. Reciprocal of selective relaxation rate enhancements in relation to epinephrine concentration without resveratrol and in the presence of 500 μM of resveratrol. The values of the calculated K for the two systems are (148.47 ± 6.47) and $(9.03 \pm 0.40) \text{ dm}^3 \text{ mol}^{-1}$, respectively.

Fourier Transform Attenuated Total Reflectance Infrared Spectroscopy

Infrared spectroscopy was applied to the study of both the protein-hormone (FBG-EP) and protein-hormone-antioxidant (FBG-EP-RSV) systems with the aim of identifying the protein secondary structural changes induced by EP and evaluating the ability of RSV to inhibit the protein-hormone interaction, thus preserving the FBG native conformation.

The structural changes were identified by the variations observed in the Amide I and Amide III regions of the protein infrared spectrum, which are assigned to the combination of the C=O stretching and N-H bending modes and to the C-N stretching and N-H bending modes respectively of the peptide group [30]. It is, in fact, well known that both the Amide I and Amide III regions are very sensitive to changes in protein secondary structure [31]. The Amide I and III regions in the IR spectra of protein, protein-hormone and protein-hormone-antioxidant systems were deconvoluted to better identify the vibrational modes associated with the single components of the protein secondary structure. They are reported in Figure 3a. Table 2 summarizes the main wavenumbers observed in the infrared spectra together with their assignments. The original IR spectra of the studied systems (obtained as the difference between the sample spectrum and that of the buffer) do not contain the contribution of neither the hormone nor resveratrol vibrations, since both the epinephrine and resveratrol concentrations used in the experiments were too low to allow their vibrational absorption of to be detected by IR.

a)



b)

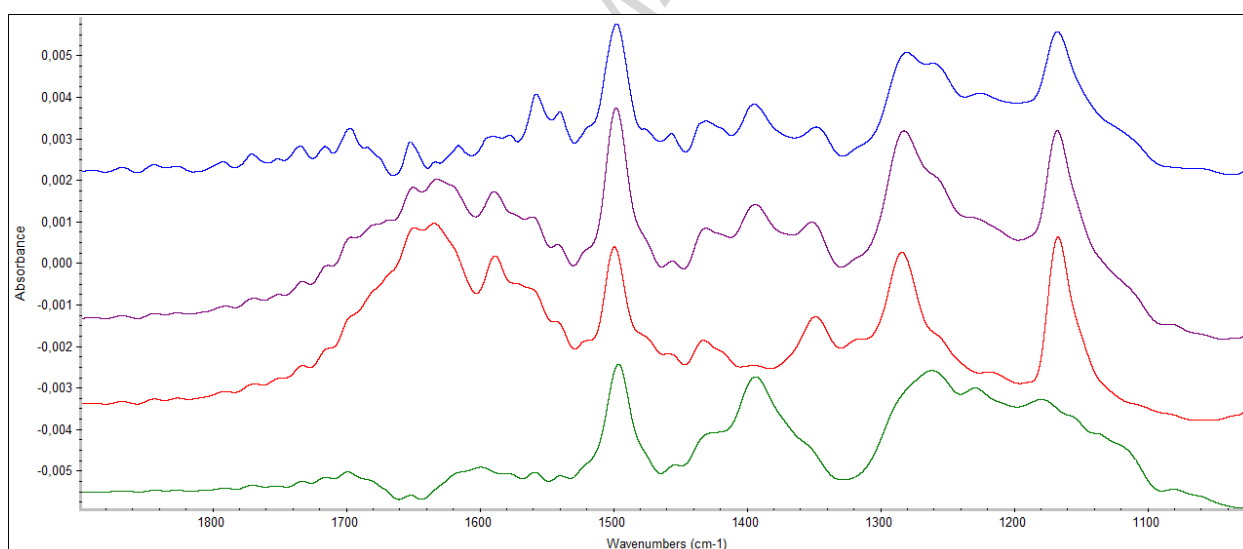


Figure 3. a) Amide I and Amide III deconvoluted difference FTIR-ATR spectra of: native FBG (red spectrum), FBG-EP system (blue spectrum), FBG-EP-RSV system (pink spectrum) in normal saline solution. b) Difference FTIR-ATR spectra of: EP (green spectrum), RSV (red spectrum), EP + RSV spectra (violet spectrum), EP-RSV complex (blue spectrum)

Table 2. Main wavenumbers observed in the FBG Amide I and Amide III spectral ranges and their assignments in terms of protein secondary structure.

| System | Amide I components | | | Amide III components | | |
|------------|--------------------|-----------------|----------------|----------------------|-----------------|----------------|
| | Random-coil | α -helix | β -sheet | Random-coil | α -helix | β -sheet |
| FBG | 1680 | 1650 | 1625 | n.d. | 1325 | 1245 |
| FBG-EP | 1680 | 1648 | 1615 | n.d. | 1325 | 1235 |
| FBG-EP-RSV | 1680 | 1650 | 1625 | n.d. | 1325 | 1245 |

Thus, the wavenumbers observed in the spectra of the protein-hormone and protein-hormone-antioxidant systems directly reflect the FBG secondary structure in the presence of the two molecules, and the variations eventually observed in the spectrum of FBG with respect to that of the native protein can only be assigned to the conformational changes of the protein induced by the presence of these molecules. This statement is also based on the fact that the effect of the ATR crystal surface on the protein conformation could be neglected. In fact, if significant protein conformational changes would be induced by the ATR crystal surface, they should be clearly observed in the spectra of all the analyzed systems. The FBG secondary structure revealed by the spectra of both FBG and FBG-EP-RSV corresponds to the conformation of the native protein [31], whereas the spectrum of FBG-EP (with epinephrine at the stress level concentration) reveals some variations in the Amide I and Amide III regions, supporting the hypothesis of an interaction between FBG and EP, responsible for the protein conformational changes. In Figure 3 can be clearly observed that the spectrum of FBG-EP shows an intensity increase of the vibrational modes of both random-coil and β -sheet components with respect to that of α -helix in the Amide I region and a wavenumber drop of the β -sheet component in the Amide III region when compared to the spectrum on native FBG. These findings are in good

agreement with the infrared data previously published [32] that strongly suggested the role of EP at the stress level concentration in inducing significant FBG conformational changes.

On the contrary, when resveratrol is added to fibrinogen solution, the subsequent addition of epinephrine does not induce any variations in the protein secondary structure, as revealed by the IR spectrum of FBG-EP-RSV. This spectrum shows, in fact, the same wavenumbers and the same band intensities for the different Amide I and Amide III components than those observed in the IR spectrum of native FBG. These data strongly support the hypothesis that the antioxidant compound inhibits the protein interaction with the hormone, thus preserving its native conformation.

A possible explanation of the ability of resveratrol to preserve the native conformation of fibrinogen in the presence of the stress level concentration of epinephrine may be based on the RSV interaction with EP in solution as revealed by the infrared analysis of this system. The infrared spectrum of the EP-RSV system appears in fact different from the “sum spectrum” obtained simply by the mathematical process of summing the RSV and EP IR spectra (Figure 3b). The spectrum of the EP-ESV complex shows sharp absorptions at 1558 and 1540 cm^{-1} together with a strong intensity decrease of the 1650 and 1620 cm^{-1} absorptions with respect to EP+RSV spectrum. When interaction between two molecules occurs the infrared spectrum of the complex, in fact, shows some wavenumber shifts together with some band intensity variations, due to changes in both the bond spring constants and bond angles [30,31].

Platelet adhesion and aggregation on polystyrene (PS)

The PS adherent platelets after contact with PRP are few and not aggregated, as shown by SEM images (Figure 4a). On the contrary, the presence of EP 15 mM induces a high degree of aggregation on PS surface as demonstrated in Figure 4b. In fact, numerous large aggregates are present. Platelets forming aggregates have lost their individuality and it is impossible to

distinguish single platelets each other. Anyway, there are also a few small clusters and single platelets. The presence of ethanol 0.3 mM is not able to reverse the pro-aggregating effect of epinephrine. In fact, as shown in Figure 4c, many large platelet aggregates are present on PS surface, together with few single adherent platelets. In the aggregates it was impossible to distinguish single platelets as well as without ethanol. The adding of RSV 0.5 mM completely inhibits platelet aggregation and decreases the degree of platelet adhesion as shown in Figure 4d. No aggregates are present on the PS surface but just a few single platelets. The degree of platelet adhesion decreases by increasing the resveratrol concentration. As reported in Figure 4e. The presence of RSV 15 mM involves a further reduction in the degree of platelet adhesion which further decreases at a resveratrol concentration of 60 mM (Figure 4f) and becomes negligible for the highest tested value of 100 mM (Figure 4g).

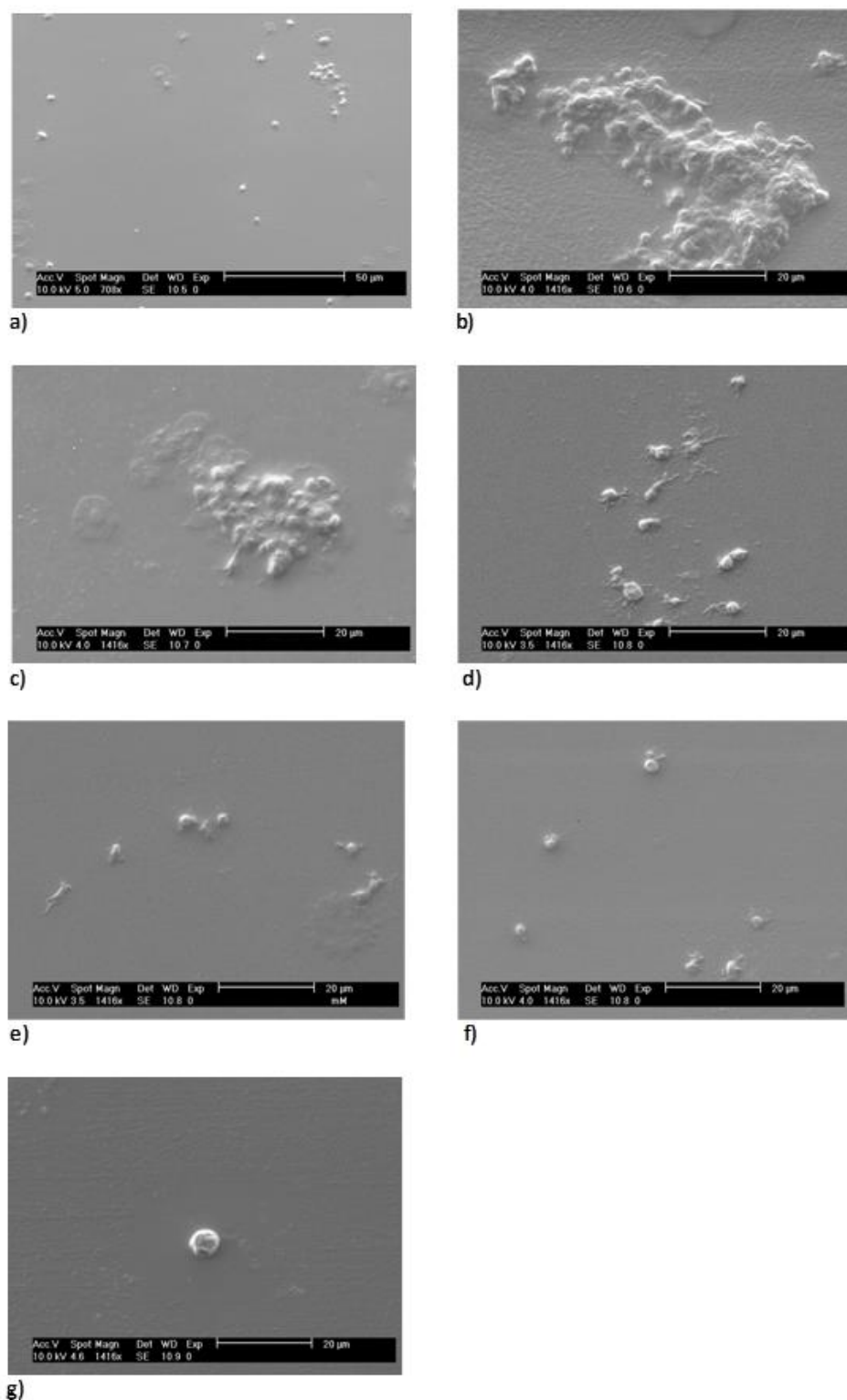


Figure 4. Scanning Electron Microscopy images of adhered and aggregated platelets on PS after contact with: **a)** PRP; **b)** PRP+EP 15 mM; **c)** PRP+ EP 15 mM+EtOH 0.3 mM; **d)** PRP+ EP 15 mM+RSV 0.5 mM; **e)** PRP+ EP 15 mM+RSV 15 mM; **f)** PRP+ EP 15 mM+RSV 60 mM; **g)** PRP+ EP 15 mM+RSV 100 mM.

SEM observations are confirmed by counting the number of platelets adhering to the PS (Figure 5). There is a very high degree of platelet adhesion and aggregation in presence of epinephrine 15 mM and epinephrine 15 mM+ethanol 0.3 mM. Platelets are aggregated, fused each other and it is impossible to assess their exact number. On the contrary, the presence of resveratrol decreases significantly the number of adherent platelets in comparison to the PRP and this effect results to be dose-dependent. At the maximum concentration of resveratrol tested there are a very few platelets adhering.

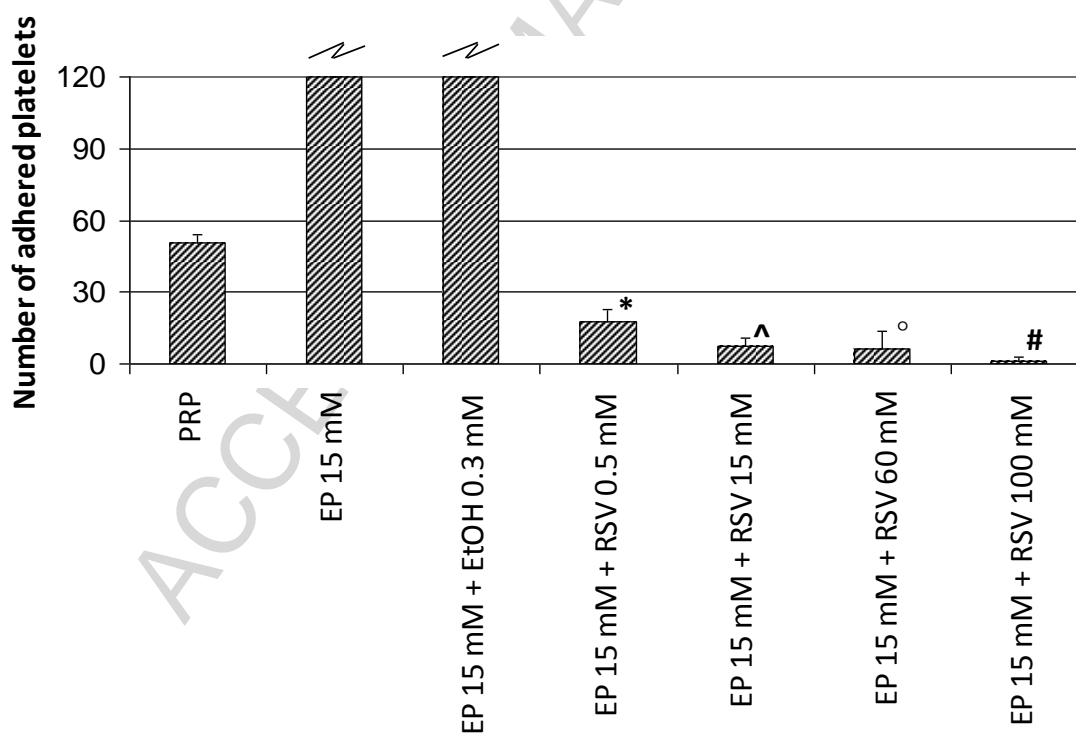


Figure 5. Number of adherent platelets on PS after contact with epinephrine (EP) 15 mM and resveratrol (RSV) 0.5, 15, 60 and 100 mM. The negative control is PRP; the positive controls are EP 15 mM and EP 15 mM + EtOH 0.3 mM. Data are means \pm SE of counts from five fields of three specimens. ⚡ It is not possible to assess the exact number of adherent platelets, since big aggregates are present on the PS surface. *, ^, °, # are statistically different from PRP (Control).

Because of the close correlation between platelet shape and functional state, the distribution of platelet shape has been analysed and reported in Figure 6. This analysis has been not performed for EP 15 mM and EP 15 mM + EtOH 0.3 mM because in the latter two cases the majority of platelets is in the form of aggregates. As shown in Figure 6, the presence of resveratrol induces an increase in the percentage of platelets in less reactive shapes (round and dendritic) in comparison to the control (PRP). At the drug concentration of 100 mM, the 100% of the adhering platelets have round or disc-shaped morphology with the absence of pseudopodia.

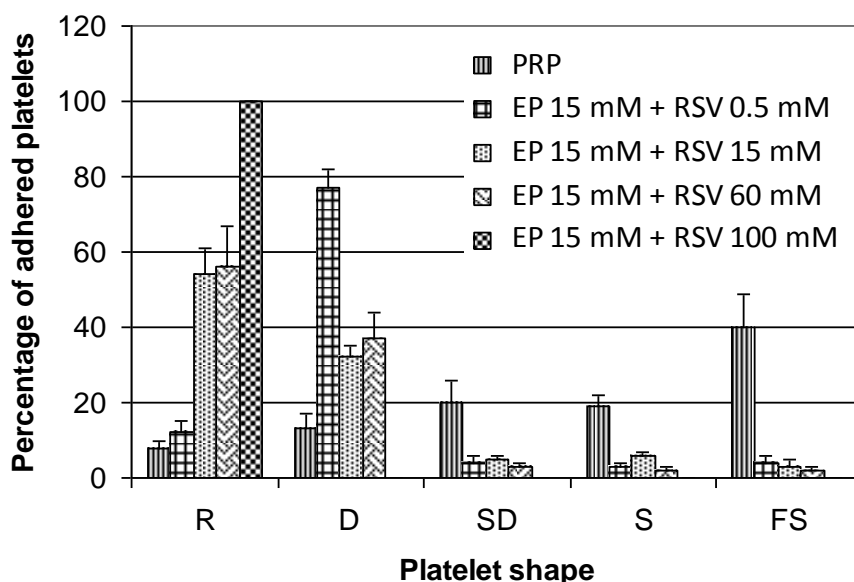


Figure 6. Percentage of platelet shape in contact with epinephrine (EP) 15 mM and different concentration of resveratrol (RSV). Data are mean \pm SE of counts from five fields on three specimens. Platelet shapes: R = round, D = dendritic, SD = spread dendritic, S = spreading, FS = fully spread.

Platelet aggregation in Platelet-rich plasma (PRP)

The ability of RSV to affect platelet aggregation in PRP has been also evaluated. In PRP, RSV 15 mM reduces significantly platelet aggregation stimulated by EP 15 mM (Table 3). This effect

is due mainly to the drug because the ethanol 0.3 mM induces a small decrease in the percentage of maximal aggregation in comparison to the control.

Table 3. Platelet maximal aggregation

| Samples | Maximal aggregation (%) |
|-----------------------------|-------------------------|
| Control (PRP + EP 15 mM) | 86 ± 2 |
| PRP + EP 15 mM + EtOH 15 mM | 73 ± 3 |
| PRP + EP 15 mM + RSV 15 mM | 13 ± 2 |

Values are mean±SE of two experiments each one performed in triplicate. $p < 0.05$ vs control

CONCLUSION

In this paper the ability of resveratrol in preserving fibrinogen from denaturation and platelets from aggregation was studied using combined spectroscopic and biological analyses. The spin nuclear relaxation showed that the strength of the interaction between epinephrine and fibrinogen was strongly decreased by the presence of resveratrol. The values of the equilibrium constants, which describe the formation of epinephrine-fibrinogen complex, significantly decreased in presence of resveratrol. These data were confirmed by IR spectroscopic measurements that emphasized the protective effect of the antioxidant compound towards fibrinogen. The infrared data, also, showed that fibrinogen was not denatured by the interaction with epinephrine in presence of resveratrol. Resveratrol inhibited protein/ hormone interaction, and thus reduced the degree of platelet adhesion and aggregation. In fact, SEM analysis revealed that the presence of resveratrol completely inhibited the platelet aggregation and the degree of platelet adhesion decreased by increasing the resveratrol concentration. Moreover, resveratrol resulted also able to reduce the percentage of platelet aggregation in PRP.

The protective properties of resveratrol towards fibrinogen from the denaturant activity of epinephrine can be explained on the basis of its ability to binding the catecholamine.

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DECLARATIONS OF INTEREST

The authors report no declarations of interest.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest

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CAPTIONS TO FIGURES

Figure 1. Proton selective relaxation enhancements of epinephrine versus fibrinogen concentration in the presence of 500 μM of resveratrol and without resveratrol with the affinity indexes calculated for the two systems.

Figure 2. Reciprocal of selective relaxation rate enhancements in relation to epinephrine concentration without resveratrol and in the presence of 500 μM of resveratrol. The values of the calculated K for the two systems are (148.47 ± 6.47) and $(9.03 \pm 0.40) \text{ dm}^3 \text{ mol}^{-1}$, respectively.

Figure 3. a) Amide I and Amide III deconvoluted difference FTIR-ATR spectra of: native FBG (red spectrum), FBG-EP system (blue spectrum), FBG-EP-RSV system (pink spectrum) in normal saline solution. b) Difference FTIR-ATR spectra of: EP (green spectrum), RSV (red spectrum), EP + RSV spectra (violet spectrum), EP-RSV complex (blue spectrum)

Figure 4. Scanning Electron Microscopy images of adhered and aggregated platelets on PS after contact with: **a)** PRP; **b)** PRP+EP 15 mM; **c)** PRP+ Ep 15 mM+EtOH 0.3 mM; **d)** PRP+ EP 15 mM+RSV 0.5 mM; **e)** PRP+ Ep 15 mM+RSV 15 mM; **f)** PRP+ EP 15 mM+RSV 60 mM; **g)** PRP+ EP 15 mM+RSV 100 mM.

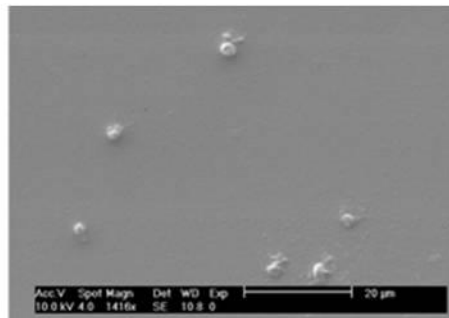
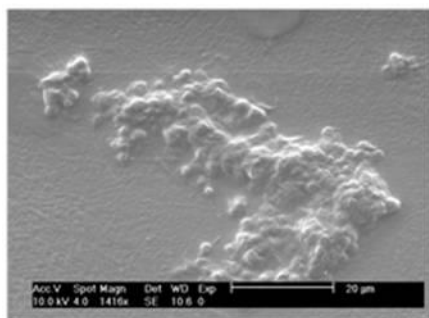
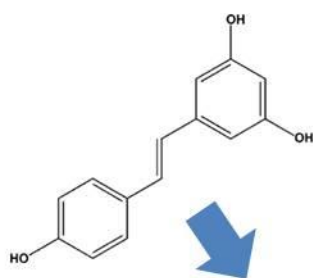
Figure 5. Number of adherent platelets on PS after contact with epinephrine (EP) 15 mM and resveratrol (RSV) 0.5, 15, 60 and 100 mM. The negative control is PRP; the positive controls are EP 15 mM and EP 15 mM-Ethanol 0.3 mM. Data are means \pm SE of counts from five fields of three specimens. \swarrow It is not possible to assess the exact number of adherent platelets, since big aggregates are present on the PS surface. *, ^, °, # are statistically different from PRP (Control).

Figure 6. Percentage of platelet shape in contact with epinephrine (EP) 15 mM and different concentration of resveratrol (RSV). Data are mean \pm SE of counts from five fields on three specimens. Platelet shapes: R =round, D = dendridic, SD = spread dendridic, S = spreading, FS = fully spread.

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Graphical abstract

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Highlights

- This study demonstrated the ability of resveratrol to preserve fibrinogen from denaturation and platelets from aggregation using combined spectroscopic and biological analyses. SEM analysis revealed that the presence of resveratrol completely inhibited the platelet aggregation and the degree of platelet adhesion decreased by increasing the resveratrol concentration.
- The protective properties of resveratrol towards fibrinogen from the denaturant activity of epinephrine can be explained on the basis its ability to bind the catecholamine

[1] A. D. Blann, Platelets: The universal killer?, *Biochimica et Biophysica Acta* 1772 (2007) 715-717.

[2] E. Damiani, S. E. Ullrich, Understanding the connection between platelet-activating factor, a UV-induced lipid mediator of inflammation, immune suppression and skin cancer, *Progress in Lipid Research* 63 (2016) 14–27.

[3] B. Olas, B. Wachowicz, Resveratrol, a phenolic antioxidant with effects on blood platelet functions, *Platelets* 16 (2005) 251-260.

[4] L. Fremont, Biological effects of resveratrol, *Life Sci.* 66 (2000) 663-673.

[5] B.N.M. Zordoky, I.M. Robertson, J. R. B. Dyck, Preclinical and clinical evidence for the role of resveratrol in the treatment of cardiovascular diseases, *Biochimica et Biophysica Acta* 1852 (2015) 1155–1177.

[6] O. Daniel, M.S. Meier, J. Schlatter, J. Schlahter, P. Frischhnecht, Selected phenolic compounds in cultivated plants: ecologic functions, health implications, and modulation by pesticides, *Environ. Health Perspect.* 107 (1999) 109-114.

[7] B. Olas, B. Wachowicz, J. Saluk-Juszczak, T. Zielinski, Effect of resveratrol, a natural polyphenolic compound, on platelet activation induced by endotoxin or thrombin, *Thrombosis Research* 107 (2002) 141-145.

[8] H. M.Zbikowska, B. Olas, B. Wachowicz, B. T.Krajewski, Response of blood platelets to resveratrol, *Platelets* 10 (1999) 251-256.

- [9] H. M. Zbikowska, B. Olas, Antioxidants with carcinostatic activity (resveratrol, vitamin E and selenium) in modulation of blood platelet adhesion, *J Physiol. Pharmacol.* 3 (2000) 513-520.
- [10] B. Olas, H. M. Zbikowska, B. Wachowicz, T. Krajewski, A. Buczynski, A. Magnuszewska, Inhibitory effect of resveratrol on free radical generation in blood platelets, *Acta Biochim. Pol.* 46 (1999) 961–966.
- [11] E. Pretorius, W. A. Vieira, H. M. Oberholzer, R. E. J. Auer, Comparative scanning electron microscopy of platelets and fibrin networks of human and different animals, *Int. J. Morphol.* 27(1) (2009) 69-76.
- [12] B.Y. Zaslavsky, V. N. Uversky, A. Chait, Analytical applications of partitioning in aqueous two-phase systems: Exploring protein structural changes and protein–partner interactions *in vitro* and *in vivo* by solvent interaction analysis method, *Biochimica et Biophysica Acta* 1864 (2016) 622-644.
- [13] G. Sekar, S. Sugumar, A. Mukherjee, N. Chandrasekaran, Multiple spectroscopic studies of the structural conformational changes of human serum albumin-Essential oil based nanoemulsions conjugates, *Journal of Luminescence* 161 (2015) 187-197.
- [14] L. Shang, Y. Wang, J. Jiang, S. Dong, pH-Dependent Protein Conformational Changes in Albumin: Gold Nanoparticle Bioconjugates: A Spectroscopic Study, *Langmuir* 23(5) (2007) 2714–2721.
- [15] K. L. Lannan, M. A. Refaai, S. K. Ture, C. N. Morrell, N. Blumberg, R. P. Phipps, S.L. Spinelli, Resveratrol preserves the function of human platelets stored for transfusion, *Br. J. Haematol.* 172(5) (2016) 794-806.
- [16] I. K. Toliopoulos, Y. V. Simos, S. Oikonomidis, S.C. Karkabounas, Resveratrol diminishes platelet aggregation and increases susceptibility of K562 tumor cells to natural killer cells. *Indian J Biochem. Biophys.* 50(1) (2013) 14-18.

- [17] C. Bauer, R. Freeman, T. Frenkiel, J. Keeler, A. J. Shaka, Gaussian pulses, *J. Magn. Reson.* 58 (1984) 442-457.
- [18] R. Freeman, H. D. W. Hill, B. L. Tomlinson, L. D. Hall, Dipolar contribution to NMR spin-lattice relaxation of protons, *J. Chem. Phys.* 61 (1974) 4466-4474.
- [19] C. Bonechi, S. Martini, C. Rossi, Interaction study of bioactive molecules with fibrinogen and human platelets determined by ^1H NMR relaxation experiments, *Bioorganic & Medicinal Chemistry* 17(4) (2009) 1630-1635.
- [20] S. Martini, C. Bonechi, G. Corbini, C. Rossi, Determination of the modified 'affinity index' of small ligands and macromolecular receptors from NMR spin-lattice relaxation data, *Chemical Physics Letters*, 447(1-3) (2007) 147-153.
- [21] S. Martini, C. Bonechi, M. Casolaro, G. Corbini, C. Rossi, Drug-protein recognition processes investigated by NMR relaxation data. A study on corticosteroid-albumin interactions. *Biochem. Pharmacol.* 71 (2006) 858-864.
- [22] T. J. Lenk, B. D. Ratner, R. M. Gendreau, K. K. Chittur, IR spectral changes of bovine serum albumin upon surface adsorption, *J. Biomed. Mater. Res.* 23 (1989) 549-569.
- [23] R. Barbucci, A. Magnani, Conformation of human plasma proteins at polymer surfaces: the effectiveness of surface heparinization, *Biomaterials* 15(12) (1994) 955-962.
- [24] W. E. Blas, G. W. Helsey, In *Deconvolution of Absorption Spectra*; Academic Press: New York, 1981.
- [25] J. K. Kauppinen, D. J. Moffat, H. H. Mantsch, D. G. Cameron, Fourier Self-Deconvolution: A Method for Resolving Intrinsically Overlapped Bands, *Appl. Spectrosc.* 35 (1981) 271-276.
- [26] *Instructions Infrared Data System (CDS-3 Application Program Rev. D)*; Perkin-Elmer Corporation: Waltham, MA, 1985.

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- [27] M. Miceli, L. Alberti, F. Bennardini, P. Di Simplicio, G. Seghieri, G. H. R. Rao, F. Franconi, Effect of low doses of ethanol on platelet function in long-life abstainers and moderate-wine drinkers, *Life Sciences* 73 (2003) 1557-1566.
- [28] S. Lamponi, A.M. Aloisi, R. Barbucci, The role of Fbg in platelet adhesion to polymeric materials in conditions of psychological stress, *Biomaterials* 20 (1999) 1791-1797.
- [29] T. M. Ko, L. J. C. Cooper, Surface characterization and platelet adhesion studies of plasma-sulphonated polyethylene, *Biomaterials* 14 (1993) 657-664.
- [30] L. J. Bellamy, *The Infrared Spectra of Complex Molecules*; Methuen: London, 1958.
- [31] R. M. Gendreau, Biomedical fourier transform infrared spectroscopy: Application to proteins. In *Spectroscopy in the Biomedical Sciences*; Gendreau, R. M., Ed.; CRC Press: Boca Raton, FL, 1986; pp. 21-52.
- [32] R. Barbucci, S. Lamponi, A. Magnani, Fibrinogen conformation and platelet reactivity in relation to material-blood interaction: effect of stress hormones, *Biomacromolecules* 4(6) (2003) 1506-1513.