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In-vitro effect of phenolic compounds on platelet activation and Monocytes-Platelets aggregates by flow Cytometry

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ABSTRACT

Phenols are natural, high-added value molecules, abundant in many fruits and vegetables, known for their significant biological activities, and beneficial effect to human health. Platelets play a central role in clot formation, resulting in haemostasis, after endothelium injury. Platelet activation and aggregation are common factors in cardiovascular diseases and generally responsible for atherothrombotic events. A series of flavonoids, namely; kaempferol, quercetin, myricetin catechin and epicatechin, three phenolic acids (caffeic, ferulic and coumaric) and major phenolic compounds obtained from pomegranate peels and sea buckthorn berries were examined for their effect on human platelet activation induced by collagen using flow cytometry. Surface biomarker P-selectin levels were estimated at different concentrations of phenolic compounds and certain agonist concentration. All compounds were found to inhibit platelet activation induced by collagen to a wide range. Epicatechin, with IC_{50}value 0.08 mM, was the most potent one followed by myricetin, coumaric acid, ferulic acid and the phenols from the two plants with IC_{50}values ranging from 0.2-0.39 mM. Based on the fact that monocytes adhere to platelets and form aggregates during the activation procedure, the monocytes-platelet aggregates were measured. Platelet activation levels and monocytes-platelet aggregates, in the presence of various concentrations of phenolic compounds, decrease upon time, with monocytes remaining though at high levels 70-80 % of initial value. The study gave further insight to the potential of phenolic compounds as inhibitors of platelet activation and therefore their use as medication for thrombosis and cardiovascular diseases. Monocytes-platelet aggregates in combination with platelet-surface biomarkers or separately may give valuable information on targeting atherothrombotic events.

Keywords: Phenolic compounds; Platelet activation; Flow Cytometry.

1. INTRODUCTION

Phenolic compounds (phenolic acids and flavonoids) are first known for their antioxidant properties and are widely distributed in fruits and vegetables [1-10]. Antioxidants protect cells from the damage caused by free radicals. Free radicals are constantly generated in the human body resulting in extensive damage to tissues leading to various diseases such as cancer, Alzheimer's, renal diseases, cardiac abnormalities, etc. [2]. Studies have shown that ingestion of flavonoids reduces the risk of cardiovascular diseases, metabolic disorders, and other related diseases by reducing oxidative stress, inhibiting platelet aggregation,acting as vasodilators in blood vessels [11,12]. Thus, plants with antioxidant properties play vital functions in exhibiting beneficial effects and are used as an alternative source of medicine to mitigate diseases associated with oxidative stress and cancer [13-14]. On the other, platelets play a central role in clot formation, resulting in haemostasis, after endothelium injury. Platelet activation and aggregation are common factors in atherothrombotic events and process. Activated plateletsrelease several pro-inflammatory molecules such as P-selectin and CD40 ligand, convert GPIb/IIIa complex (a central platelet

receptor mediating platelet aggregation) into an active form, which allows platelet aggregation. Platelet activation is stimulated by bound platelet secretion products and local prothrombotic factors resulting in haemostasis [15,16]. These platelet products, called platelet biomarkers are of great importance, as they may predict thrombotic situations. Among these, P-selectin CD62P, CD63and CD40L GPIIb/IIIA are well studied [17,18]. CD62P is found in the α-granules of platelets. Presence of CD62-P on the surface of the platelets indicates platelets in activated state while their absence implies resting state of the platelets [17,18]. Flow cytometry can give valuable information on platelet function and surface biomarkers including, P-selectin [19,20]. Furthermore, monocytes are central mediators in the development of atherosclerotic plaques.They circulate in blood and eventually migrate into tissues including the vessel wall where they give rise to macrophages and dendritic cells.

Monocyte-platelet aggregates (MPAs) are formed in responce to platelet activation and are complexes detectable in the peripheral blood [21]. Monocyte population comprises three distinct functional subsets delineated by the cell surface expression of CD14 and CD16: the most abundant classical $CD14^{+}CD16^{-}$ ($CD14^{+}$), intermediate $CD14^{+}CD16^{+}$ and non-classical $CD14^{dim}CD16^{+}$ ($CD16^{+}$) monocytes [22]. The existence of monocyte subsets with distinct roles in haemostasis and inflammation indicates their specializedfunction [22].

As it was reported, circulating MPA level increases in patients with acute thrombotic events, such as myocardial infarction or stroke as well as in subjects with underlying atherothrombotic risk factors including hypertension and diabetes [23-25]. Circulating MPAs are also increased in patients with a variety of autoimmune disorders.The level of MPAs reflects the degree of platelet hyperactivity and may be used as an indicator of blood thrombogenicity. Cross-talk between platelets and monocytes is now regarded as a crucial pathophysiological mechanism linking thrombosis and inflammation and is believed to mediate, at least in part, the pro-inflammatory action of activated platelets. The different subsets of MPAs can be identified based on expression of the CD14 and CD16 markers. In patients with stable coronary artery disease (CAD), CD14monocytes were decreased, whereas CD16monocytes were increased by 90% compared with healthy subjects with angiographically normal coronary arteries. I seems that stable CAD is associated with expansion of the nonclassical monocyte subset and increased expression of inflammatory markers on monocytes. Flow cytometric analysis of monocyte subsets and marker expression may provide valuable information on vascular inflammation [23-26]. In other words, identification of monocyte subsetsalone or in combination with the platelet-surface biomarkers can contribute to selective therapeutic targets.

Endothelial dysfunction and inflammation are key mechanisms of vascular disease. As it was reported [21], heterogeneity of monocyte subpopulations may be related to the development of vascular dysfunction in CAD.

We sought to investigate and compare a series of natural phenolic compounds and phenolics obtained from two plants for their effect on platelet reactivity using flow cytometry. In parallel, MPA levels were estimated under the same conditions as a potential contributor marker for diagnosis of atherothrombotic events.

2. MATERIALS AND METHODS

2.1. Materials

Phenolic compounds used, were obtained from Merck. Monoclonal antibodies CD 45 anti HU APC-Cy7 were purchased from EXBIO, CD 61 PE from Beckman Coulter, CD 42b FITC from Diaclone, CD 14 FITC from Biolegend, CD 16 PE/Cy7 from Biolegend and CD62P from PE ORIGENE. CD62P-P Selectin (Origene Tech), CD61 FITC (GPIIb/IIIa) (Exbio) and CD45 Krome (Beckman Coulter).

Pomegranates and sea buckthorn (*Hippophaes ramnoides*) fruits were obtained from the local market, Thessaloniki, Greece.

The extraction and recovery of phenolic compounds from pomegranate peels and sea buckthorn berries was processed as previously reported [27]. Total phenolic compounds obtained from the two plants were estimated as mg of gallic acid using the Folin-Ceucalteu reagent [28].

2.2.1. **Preparation of Human platelets**

Whole blood collected in citrate buffer (3,8% sodium citrate) slow centrifuged for 10 minutes at 150 x g and the Platelet Rich Plasma (PRP) was collected. Platelets were re suspended to a final concentration to 25,000plts/μl. Sodium citrate was selected as the anticoagulant instead of heparin and ethylene diamine tetra acetic acid (EDTA) given its lesser impact on complement activation pathways.

2.2.2. Platelet labeling

The monoclonal antibodies CD62P-P Selectin, CD61 FITC (GPIIb/IIIa) and CD45 were used to labeling platelets for detection of membrane activation markers in circulating platelets before and after the incubation of the agonist and

polyphenols. Briefly, in 40μl of cold PBS buffer (to prevent activation of platelets), 2μl of the PRP was added, followed by addition of the polyphenols in various concentrations. After incubation of 10min collagen was added and incubated 5 min at room temperature. Subsequently, the antibody (CD) was added, followed by a final incubation of 10 min at room temperature in the dark. In a final step, 300μl of cold PBS buffer was added to stop the reaction and the samples was measured directly on the cytometer. To minimize in vitro artifacts, fixation or washing procedures were omitted. The concentration of the agonist used is 3.45mg/ml collagen

2.2.3. Monocytes labeling

The same, as above, procedure was followed for monocytes' labeling, using CD14 και CD16 monoclonal antibodies and 5μl whole blood instead of PRP.

In both cases, after incubation with monoclonal antibodies lytic solution Versalyse (Beckman Coulter) was added, the sample incubated for 10minand subsequently measured for monocytes-platelet aggregates in the presence of phenolic compounds.

2.3. Flow Cytometer

For the platelet aggregation experiments the multiple analyzer (Beckman Coulter NaviosEX Flow Cytometer) was used. Activated platelets were analyzed by quantifying the expression of the markers CD62P-P Selectin, CD61 (GPIIb/IIIa) and CD45. Data on 5,000 to 20,000 platelets per sample were collected and analyzed. Platelets were detected by light scattering (Forward & Side scatter-FS /SS) and their population was electronically selected (gated) and analyzed for activation as detected by the binding of the specific probes [29-31].

2.4. Statistical analysis

Statistical analysis was carried out using the SPSS, version 25, program. All experiments were run in triplicate and the results expressed as mean ± standard deviation (SD) values. All data were considered statistically significant at $p < 0.05$.

3. RESULTS AND DISCUSSION

Flavonoids consist of a fifteen-carbon skeleton with two benzene rings (A and B) linked via a heterocyclic pyrane ring (C). They contain several subcategories including, flavonols, flavanols, flavones and others. Here, three flavonols (quercetin, kaempferol, myricetin),two flavanols (catechin and epicatechin) and three phenolic acids (ferulic, caffeic, coumaric) were examined for their effect on human platelet reactivity in *in vitro* experiments. Figure 1a shows the chemical structures of the flavonols and flavanols and 1b the structures of the phenolic acids.It was found that upon increase of concentrationof phenolic compound, the percentage of platelet activation significantly decreases and thus the corresponding % inhibition of platelet activation increases accordingly. Table 1 gives the IC_{50} values in mM of all the examined compounds. As it can be seen from Table 1 epicatechen and myricetin are the most potent among the flavonoids examined. Figure 2 depicts the dot plot of P-selectin levels on collagen-activated platelets at three different concentrations of epicatechin. The percentage of P-selectin levels decreases going from lower to higher concentrations of epicatechin (from left to the right, Figure 2) thus giving high levels of inhibition of platelet reactivity, with IC_{50} value at 0.08 mM. Catechin and epicatechin are isomers withtwo chiral centers on the molecule (on C2 and C3 carbon atoms , Figure 1a). Catechin is with trans configuration and two stereo isomers (+)-catechin, (-)-catechin. Similarly, epicatechin is cis configuration and two stero isomers, (+)-epicatechin and (-)-epicatechin (Figure 1a).

Interestingly enough, catechin with IC_{50} at 4.4 mM is considered the less potent one, emphasizing the importance of stereo configuration of specific functional group (Trans and Cis isomerism) in the molecule for appearance of biological activity (structure-activity relationship). There have been numerous studies on cardioprotective effects of flavanols consumption suggesting that catechins may be one of the key mediators of cardiovascular health in previously established heart-healthy diets, such as the DASH diet or the Mediterranean diet. Catechins are also reported to have antibacterial properties [32-34]. From the three flavonols examined, myricetin showed higher inhibition of platelet activation than quercetin and kaempferol with IC_{50} at 0.21 mM. Myricetin is also known for several activities [35]. Besides antioxidant, anticancer and anti-inflammatory activities, it may also protect against diseases such as Parkinson's and Alzheimer's [35]. Kaempferol and quercetin are well studied and their biological and positive effect to human health have been reported in many published articles [36,37]. Phenolic acids are also well distributed in plants(fruits and vegetables) showing antioxidant and antiplatelet activities [6,38,39]. Inhibition of platelet activation was also observed with the three phenolic acids with coumaric acid as the most potent one (Table 1). It was found that all compounds presented a dose dependent effect with satisfactory linearityin most cases. The effect of coumaric acid on inhibition of platelet activation, indicatively, is shown in Figure 3, with a satisfactory linear correlation between concentration and % inhibition of platelet

aggregation and $R^2$0.975. Caffeic acidshowed also a dose dependent effect with the lowest linearity. Specifically, at low concentrations the linearity is sufficiently good, while higher concentrations add less to linearity. These results are in accordance with our previous [unpublished data] experiments on inhibition of platelet aggregation induced by caffeic acid, using conventional aggregometer. Ferulic acid with IC_{50} value at 0.39 mM is considered equipotent to phenols from pomegranate peels. Major phenolic compounds isolated from pomegranate peels and sea buckthorn berries as previously reported [27]are also included in the study, based on the fact that they contain most of the phenolic compounds examined here. As it can be seen in Table 1 sea buckthorn phenolics presented higher inhibition of platelet activation than those of pomegranate with comparable values to myricetin and coumaric acid. In all cases examined, inhibition of platelet activation was dependent on concentration in a dose manner.

In the frame of a previous report [25] that degranulated, P-selectin-positive platelets, aggregate with leukocytes in vitro and rapidly lose surface P-selectin in vivo, we also attempted to examine the levels of MPAsin the presence of the phenolic compounds at different concentrations. As it has been reported [26], in vivo tracking of activated platelets in baboons, and humans(with coronary intervention, and acute myocardial infraction), showed that circulating MPAsare a more sensitive marker of in vivo platelet activation than platelet surface-P selectin.Therefore, MPAs % was also estimated in all cases based on $CD14^+$ measurements. Figure 4 shows dot plots of MPAs % on collagen-activated platelets at three differen concentrations of epicatechin, by measuring CD14+ levels. It is evident that MPAs % decreases, sililarly to platelet activation % (figure 2) going from lower to higher concentrations. Figure 5 gives the percentage of platelet activation (a) and MPAs (b) at different concentations of ferulic acid. It is evident that both, platelet activation % and MPAs % decrease upon increasement of concentration of ferulic in a linear manner.Furthermore, in order to compare the servival of P-selectin levels and MPAs versus time, we conducted measurements, at different time intervals (15-180 min) after their first evaluation (zero time). It was found that in all cases, after 3 hours the P-selectin levels decrease and accordingly do so MPAs, with the latter still remaining at high levels at 70 to 83% , compared to P-selectin levels which recorded a little lower values (data obtained but not shown). Indicatively, Figure 6 gives the MPAs% on collagen-activated platelets incubated with 1.6 mM ferulic acid,recorded at differernt time intervals (15-120 min) indicating the existence of MPAs after 3 hrs and in the presence of a platelet-activation inhibitor at the highest concentration.

Table - 1: IC_{50}values (concentration in mM) of inhibition of platelet activation % recorded by phenolic compounds

Compound	IC_{50}(mM)
Kaempferol	0.85
Quercetin	2.36
Catechin	4.44
Epicatechen	0.08
Myricetin	0.21
Ferulic acid	0.39
Caffeic acid	2.6
Coumaric acid	0.23
Phenols from Pomegranate peels	0.37
Phenols from sea buckthorn berries	0.22

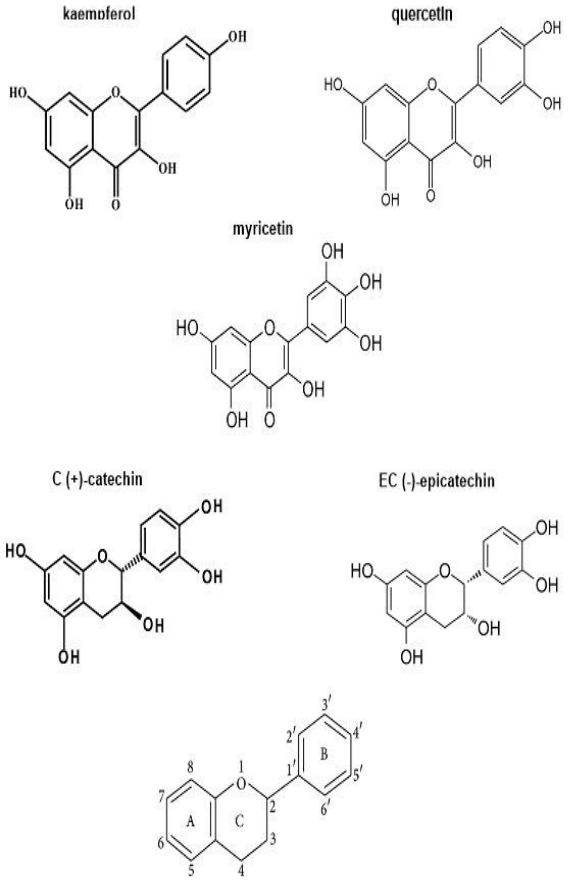

Figure -1a

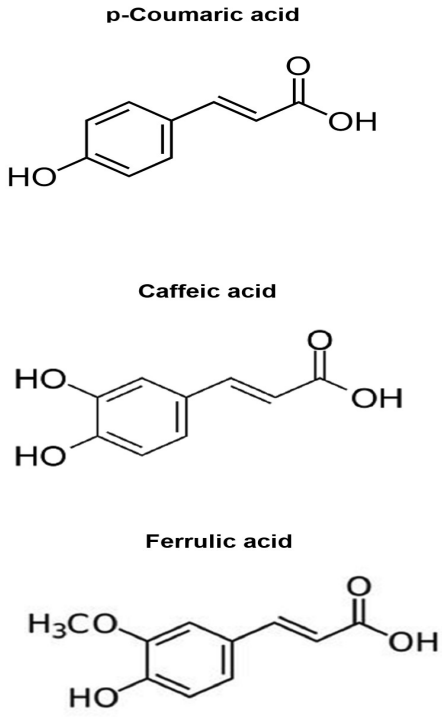

Figure -1b

Figure - 1: Chemical structures of flavonoids (a) and phenolic acids (b) examined .

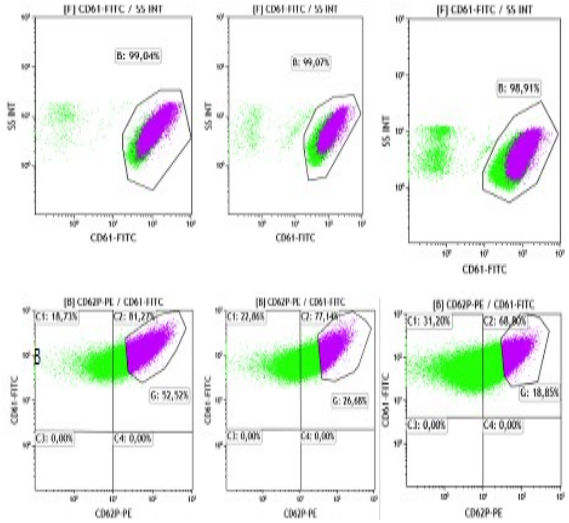

Figure - 2: Representative dot plots of the effect of epicatechin on collagen-activated platelets by Flow Cytometry in three –color abeling experiments. The percentage of activated platelets are compared to control in platelets incubated with epicatechin at 0.05, 0.1 and 0.5 mM respectively, from left to the right. The expression of CD-61P and CD62-P are shown on a two-dimension dot-plot (A and B).

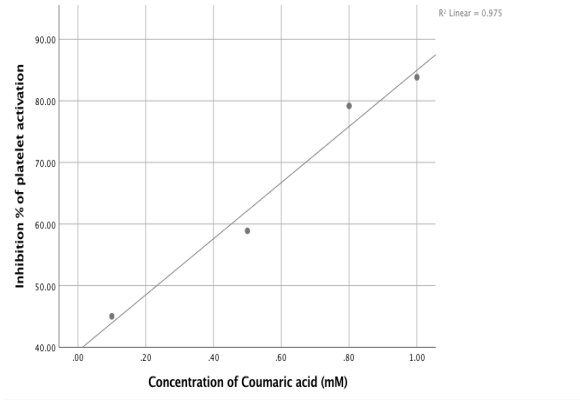

Figure - 3: Dose dependent effect of coumaric acid at different concentrations on inhibition of platelet activation (%) induced by collagen.

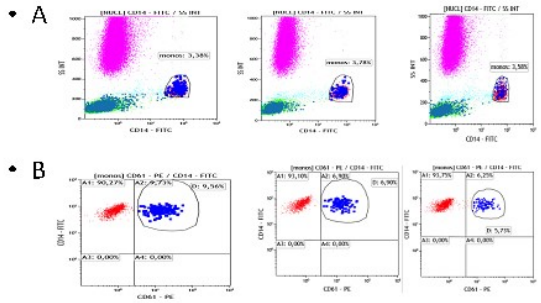

Figure – 4: Representative dot plots of MPAs recorded on collagen-activated platelets at three concentrations of epicatechin (0.05, 0.1and 2 mM respectively, left to the right) in whole blood analyzed by flow cytometry in three color labeling experiments The expression of CD14+ is shown on a two dimensional dot-plot(A and B).

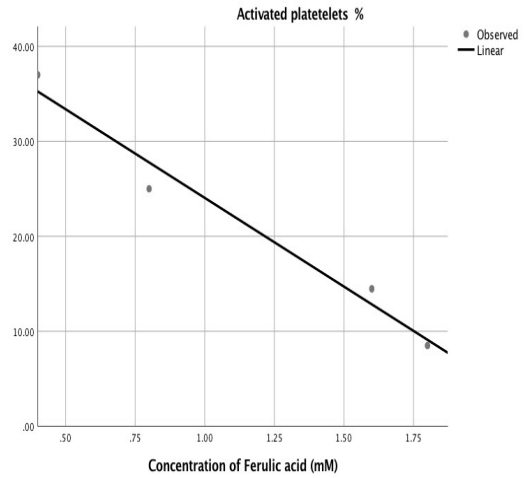

Figure – 5a

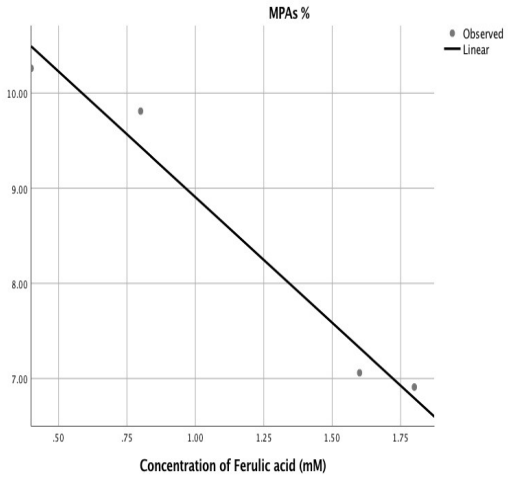

Figure – 5a

Figure – 5: Effect on platelet activation (a) and MPAs (b) percentage levels of ferulic acid at three different concentrations.

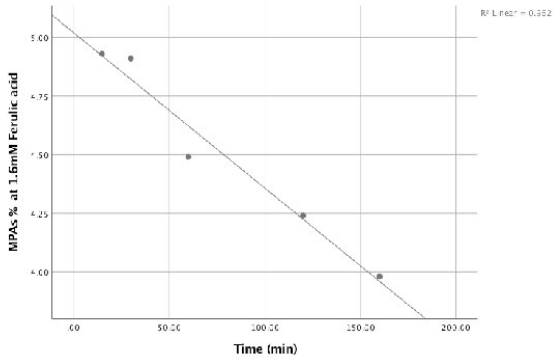

Figure - 6: Change of MPAs % level versus time in the presence of 1.6 mM ferulic acid.

4. CONCLUSION

All compounds tested, found to exert inhibitory effect on human platelet activation induced by collagen, to a varying degree, supporting previous reports for their high impact to human health, when consumed. The study highlighted the importance of epicatechin as the most potent one and its possible applications.

These in vitro experiments showed that P-selectin levels decrease upon time, but don't seem to reach zero levels after some time (three hrs) in the presence of phenolic compounds. Therefore,flow cytometric determination of MPAs levels together with surface P-selectin biomarker, may give valuable information on human platelet activation and contribute to diagnosis of cardiovascular events.

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Conflicts of Interest: The authors declare no conflict of interest.

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