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Influence of flavonoids' lipophilicity on platelet aggregation

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Flavonoids are natural polyphenolic compounds present in a wide spectrum of plants that have a beneficial effect on human health. In the context of cardiovascular diseases related to plaque and thrombus formation, flavonoids exhibit an antiaggregatory effect. Previously, it has been reported that all tested flavonoids exhibit an antiaggregatory effect on platelet aggregation when measured by impedance aggregometry on whole blood, in the test of aggregation induced by adenosine diphosphate (ADP). As not all flavonoids have the same targets within signaling pathways, an assumption of a common non-specific mechanism related to lipophilicity is to be considered. To test this hypothesis, reverse-phase thin layer chromatography was used to assess the lipophilicity of flavonoids; impedance aggregometry was used for testing of platelet aggregation and flow cytometry to monitor the influence of flavonoids on platelet activation. Lipophilicity analysis showed a highly negative correlation of log*P* and *MINaAC* for groups of flavones and flavanones. As determined by flow cytometry, the exposition of receptors necessary for the promotion of platelet activation and primary clot formation was diminished, *i.e.*, lowered expression of the activated form of integrin αIIbβ3 was observed in the presence of flavanone. Platelet membrane stabilization by flavonoids as a mechanism of antiaggregatory effect has been supported by impedance aggregometry experiments when specific inhibitors of platelet aggregation signaling pathways (U73122, indomethacin, verapamil) were used in the presence of a weak (ADP) and a strong (TRAP-6) agonist of aggregation. While individual flavonoids can have specific targets within aggregation signaling pathways, all flavonoids share a common non-specific mechanism of platelet aggregation inhibition related to their lipophilicity and membrane stabilization that, to some extent, contributes to their antiaggregatory effect.

Keywords: flavonoids, platelets, lipophilicity, flow cytometry, antiaggregatory activity

Flavonoids are natural polyphenolic compounds present in a wide spectrum of plants. One of the prominent and well documented beneficial effects on human health that has been confirmed in epidemiological studies is known as French paradox, *i.e.*, although

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French population consumes fat-rich diet, incidence of cardiovascular diseases is lower than expected as their diet is also rich in polyphenols (1, 2). Based on their accessibility to humans through the dietary intake of fruits, vegetables and some beverages, flavonoids have been a topic of research for many years including their possible use as lead compounds for novel pharmaceutical agents. Many *in vitro* studies on plant and fruit extracts or on pure flavonoid compounds confirm antithrombotic, especially antiplatelet, effect in dose depending manner although without revealing the mechanism of action (3–5). In regard to cardiovascular diseases related to plaque and thrombus formation, flavonoids exhibit the antiaggregatory effect. For some flavonoids, this is attributed to the inhibition of cyclooxygenase, lipoxygenase, tyrosine-kinase, phosphodiesterase or phospholipase which participate in platelet signaling pathway (6–8).

Lipophilicity of flavonoids has been a subject of studies exploring flavonoid-membrane interactions. According to the results presented in previous studies, flavonoids are positioned within the membrane depending on their polarity, with more hydrophobic ones which penetrate deeper in the core of membrane bilayer, interact with membrane transporter proteins, and decrease membrane fluidity (9, 10). Platelet membrane is the best example of membrane fluidity; it functions as a dynamic structure involved in processes of activation and aggregation. These processes depend strongly on phospholipid transverse distribution in resting and activated state, and processes in the inner leaflet of platelet membrane (11).

Work of Lipinski *et al.* (12) represents the milestone for determination of the relationship between pharmacokinetic and physicochemical parameters by establishing "rule-of-five" and uses log*P* as a measure of the compound's lipophilicity. Partition coefficient (*P*) represents a ratio between the concentration of the non-ionized form of the compound in the *n*-octanol-water system expressed in logarithmic scale (log*P*). While the shake-flask method is the gold standard for log*P* determination, lipophilicity is nowadays more often determined indirectly by liquid chromatography (13). The simple approach is the determination by planar chromatography that uses chromatographic parameter R_M (parameter calculated based on the retention factor of substance) as a surrogate measurement of lipophilicity. To bypass laborious and time-consuming experimental methods, several computational approaches for the prediction of lipophilicity (log*P*) have been developed based on pool of predefined atoms, fragments and their contribution factors (14–16).

In the previous work by Bojić *et al.* (17) it has been shown that all tested flavonoids, without exception, exhibit the antiaggregatory effect on platelet aggregation induced by adenosine diphosphate (ADP). As not all flavonoids have the same targets within signaling pathways, an assumption of a common non-specific mechanism related to lipophilicity is to be considered. Thus, the aim of this study was to examine the flavonoids antiaggregatory mechanism based on lipophilicity and influence of flavonoids on platelet membrane rigidity/fluidity.

EXPERIMENTAL

Subjects

Blood samples were collected at Croatian Institute of Transfusion Medicine (Zagreb, Croatia) in 4.5-mL vacutainer tubes with tri-sodium citrate (3.8 %) as an anticoagulant. Fifty blood donors who were not on any antiaggregatory therapy gave written, informed consents before participating in research. The study was conducted according to Helsinki declaration and with approval of Ethics Committee of the Croatian Institute of Transfusion

Medicine and the University of Zagreb Faculty of Pharmacy and Biochemistry. All measurements were conducted in two hours after the blood sample collection.

Reagents

Flavonoid aglycons used in this work are listed in Table I. Flavonoid solutions were prepared by dissolving standards in methanol (Kemig, Croatia) for the purpose of thin-layer chromatography analysis, while serial of doubling dilutions ($1/2^n$) in dimethyl sulfoxide (DMSO, Merck KgaA, Germany) in the concentration range of 500 mmol L^{-1} to 62 nmol L^{-1} was used for platelet aggregation analysis. ADP, collagen, TRAP-6, arachidonic acid (AA) and ristocetin were obtained from Roche Diagnostics (Germany), and saline (0.9 % NaCl) with and without calcium chloride from the Croatian Institute of Transfusion Medicine. Inhibitor of cyclooxygenase, indomethacin, and calcium channels blocker, verapamil hydrochloride, were purchased from Acros Organics (Thermo Fisher Scientific, Belgium) and inhibitor of phospholipase C (PLC), U73122 from Merck KgaA. Anti-integrin αIIbβ3 antibody (PAC-1) labelled with fluorescein isothiocyanate (FITC), anti-CD61 labelled with phycoerythrin (PE), FITC mouse IgM, κ isotype control and PE mouse IgG1, κ isotype control for flow cytometry experiments were purchased from Becton Dickinson (USA). Reverse-phase thin-layer chromatography was performed on Merck RP-18 F254S plates with a mobile phase consisting of water, acetonitrile and glacial acetic acid (last two solvents were obtained from Kemig, Croatia, while distilled water was prepared in house).

Reverse-phase thin-layer chromatography and computational determination of lipophilicity

Twenty flavonoid compounds were analyzed by reverse-phase thin-layer chromatography (RP-TLC) using acetonitrile/water/glacial acetic acid in a ratio 66:33:1 (*V*/*V*/*V*) as a mobile phase. After development, flavonoids were detected as dark spots on a chromatographic plate with the fluorescent indicator (wavelength 254 nm). Based on retention factor, R_F, chromatographic parameter R_M was calculated as follows $R_M = \log [(1/R_F) - 1]$.

Calculation of theoretical log*P* was performed using online applications Chemicalize (https://chemicalize.com), ChemSketch (http://www.chemspider.com), Molinspiration (https://www.molinspiration.com) and SwissADME (http://www.swissadme.ch). SwissADME is calculating log*P* in five different ways and expresses their consensus log*P* value. Values of experimentally determined chromatographic parameter R_M were correlated with calculated log*P* values using MS Office Excel (Microsoft, USA) and reported as correlation coefficient and adjusted squared correlation coefficient at a significance level of $p < 0.05$.

Impedance aggregometry

Impedance aggregometry on whole blood was monitored on Multiplate® instrument (Roche Diagnostics, Germany) and was used for determination of minimum antiaggregatory concentration (*MINaAC*) of flavanone in the presence of a set of agonists (TRAP-6, ADP, collagen, AA, ristocetin) by previously described method (17). Briefly, the influence of serial doubling concentrations of flavanone on platelet aggregation was measured by using original Multiplate procedure as follows: 300 μL of blood was incubated for 3 minutes at 37 °C with 20 μL flavonoid and 300 μL of saline-$CaCl_2$ preheated at 37 °C. Vehicle – DMSO (final concentration 3 %) was used as a negative control (untreated sample). Aggregation was induced by addition of 20 μL of the agonist with final concentrations: 6.3 μmol L^{-1} ADP, 3.1 μg mL^{-1} collagen, 31 μmol L^{-1} TRAP-6, 0.48 μmol L^{-1} AA, and 0.3 mg mL^{-1} ristocetin. *MINaAC* was

determined in three independent measurements for treated and untreated sample using the Student's *t*-test. The same pre-defined Multiplate protocol was followed for the assessment of the combined effect of inhibitory drugs (U73122 hydrate, verapamil hydrochloride, indomethacin) and flavanone. Measurements were conducted with TRAP-6 as a strong and ADP as a weak agonist using 15 and 60 x *MINaAC* of flavanone to ensure repeatable inhibition effect on platelet aggregation. U73122 hydrate, verapamil hydrochloride, and indomethacin were used in concentrations previously reported in the literature (18–20), *i.e.*, 4 μmol L^{-1} U73122, 82.5 μmol L^{-1} verapamil and 0.21 μmol L^{-1} indomethacin in platelet aggregation test induced with 30 μmol L^{-1} TRAP-6, while in platelet aggregation test induced with 6.3 μmol L^{-1} ADP concentrations were for U73122, verapamil and indomethacin 3.2 μmol L^{-1}, 52 μmol L^{-1} and 0.21 μmol L^{-1}, resp. Results of aggregation for untreated sample (DMSO, 0.8 %), flavanone treated, inhibitor drug-treated, and for the sample treated with the combination of flavanone and inhibitor drug were statistically assessed using ANOVA.

Flow cytometry

Platelet-rich plasma (PRP) was obtained by centrifugation of freshly drawn citrate blood samples at 150 *g* for 10 min. Platelet concentration was adjusted to 20.000 per μL with phosphate-buffered saline (pH 7.4). One mL of PRP was pre-incubated with three dilutions of flavanone (30, 244 and 488 μmol L^{-1}) or with the vehicle as a control (DMSO, 0.8 %) for 15 minutes at 37 °C. An aliquot of 72 μL treated sample (flavanone, DMSO) was mixed with agonist TRAP-6 (final concentration 21 μmol L^{-1}) and the excess amount of anti-CD61 (PE) to mark platelet population and PAC-1 FITC to monitor the activated form of integrin αIIbβ3. The sample was incubated in the dark at 37 °C, for 30 min. Flow cytometry runs were performed on FC500, Beckman Coulter (France). To distinguish platelet cell population based on cell size and granularity, forward *vs.* side scatter was used collecting 10.000 CD61 positive events. Isotype controls FITC labelled mouse IgM κ, and IgG1 PE were used to monitor the level of non-specific background signal. Activated platelets were assessed as the percentage of platelets with positive activation-dependent platelet surface marker PAC-1 FITC in CD61 positive cells gate. Results were obtained as a measurement in triplicate and tested with paired Student's *t*-test for significance. To confirm the concentration level of flavanone which caused statistically significant inhibition of platelet activation, measurements were repeated on five independent samples in triplicate.

Statistical analysis

The antiaggregatory effect of flavanone and inhibitor drugs were assessed with one-way ANOVA test with *post hoc* Tukey analysis for multiple comparison. For comparison of R_M and log*P* linear regression analysis was performed. Paired Student's *t*-test was used for the estimation of *MINaAC* and platelet activation inhibition measured by flow cytometry. If not otherwise stated, statistical analysis was performed within R v2.8.1 environment (R-project, Austria).

RESULTS AND DISCUSSION

Lipophilicity of flavonoids

To assess the lipophilicity of flavonoids reverse-phase thin-layer chromatography was conducted on a series of 20 compounds. Chromatographic parameter, R_M, the surrogate of

Table I. Experimental (chromatographic parameter R_M*), calculated parameters (log*P*) of flavonoid lipophilicity and MINaC*[a]

Flavonoid	R_M	*MINaAC* (μmol L^{-1})	log*P*_ ChemAxon	log*P*_ ChemSketch	log*P*_ Molinspiration	Swiss_ ilog*P*	Swiss_ xlog*P*3	Swiss_ wlog*P*	Swiss_ mlog*P*	Swiss_ silicos-it	Swiss_ consensuslog*P*
Flavones											
Flavone[b]	0.3892	3.815	2.97	3.56	3.74	2.55	3.56	3.46	2.27	4.04	3.18
6-Hydroxyflavone[c]	–0.0378	0.954	2.66	3.72	3.23	2.28	3.62	3.17	1.66	3.52	2.85
7-Hydroxyflavone[c]	–0.0884	15.259	2.66	3.32	3.23	2.22	3.62	3.17	1.66	3.52	2.84
Tangeretin[d]	0.4202	30.518	2.18	2.66	3.78	3.71	3.04	3.5	0.63	4.21	3.02
Tectchrysine[e]	0.5202	0.954	3.16	3.13	3.48	2.88	3.85	3.17	1.33	3.52	2.95
Chrysine dimethyl ether[e]	0.5202	1.907	3.01	3.27	3.75	2.95	3.62	3.48	1.57	4.04	3.13
Chrysine[b]	0.0126	3.815	3.01	2.88	2.94	2.27	3.52	2.87	1.08	3.02	2.55
Acacetin[d]	0.0378	3.815	2.85	3.15	3.00	2.56	3.35	2.88	0.77	3.03	2.52
Diosmetin[e]	–0.3590	7.629	2.55	3.10	2.28	2.47	3.1	2.59	0.22	2.55	2.19
Apigenin[b]	–0.3892	3.815	2.71	2.10	2.46	1.89	3.02	2.58	0.52	2.52	2.11
Luteolin[e]	–0.5942	7.629	2.40	2.40	1.97	1.86	2.53	2.28	-0.03	2.03	1.73
Flavonols											
3,6-Dihydroxyflavone[c]	–0.0378	0.119	2.42	3.64	2.94	1.97	3.05	2.87	1.08	3.02	2.4
3,7-Dihydroxyflavone[c]	–0.0631	1.907	2.42	3.27	2.94	2.01	3.05	2.87	1.08	3.02	2.4
Galangin[b]	0.0000	122.070	2.76	2.83	2.65	2.08	2.25	2.58	0.52	2.52	1.99
Rhamnetin[d]	–0.2083	0.954	2.30	2.58	2.22	2.23	1.87	2.29	–0.31	2.06	1.63
Isorhamnetin[d]	–0.3203	7.629	2.30	1.76	1.99	2.35	1.87	2.29	–0.31	2.06	1.65
Quercetin[b,f]	–0.5863	15.259	2.77	2.07	1.68	1.63	1.54	1.99	–0.56	1.54	1.23
Flavanons											
Pinocembrin-7-methylether[e]	0.3100	0.954	3.28	4.11	3.13	2.75	3.20	2.78	1.52	3.06	2.66
Pinocembrin[e]	–0.1076	15.259	3.14	3.93	2.60	2.11	2.88	2.48	1.27	2.55	2.26
Hesperetin[b]	–0.4536	1.907	2.68	2.90	1.94	2.24	2.60	2.19	0.41	2.08	1.91
Flavanone[b]	0.1811	0.062	3.10	3.62	3.18	2.41	3.14	3.07	2.47	3.57	2.93

[a] MINaAC represents minimal antiaggregatory concentration (taken from reference 17).

Flavonoids purchased from: [b] Merck KgaA, Germany, [c] ChromaDex, USA, [d] BioChemika, Switzerland, [e] Extrasynthese, France.

[f] In the form of quercetin dihydrate.

log*P*, was in the range from –0.59 to 0.52. The highest R_M values were obtained for tectochrysin and chrysin dimethyl ether, and the lowest for luteolin (Table I).

Table II. Results of regression analysis for chromatographic parameter R_M *and log*P *for flavonoids from Table I*

Application	Adjusted R^2	Multiple R	p-value
log*P*_ChemAxon	0.1556	0.4448	0.0434
log*P*_ChemSketch	0.2605	0.5454	0.009
log*P*_Molinspiration	0.8545	0.9283	<0.001
Swiss_ilog*P*	0.5649	0.7659	<0.001
Swiss_xlog*P*3	0.4011	0.6566	0.0012
Swiss_wlog*P*	0.7269	0.8605	<0.001
Swiss_mlog*P*	0.4678	0.7032	<0.001
Swiss_silicos-it	0.7530	0.8748	<0.001
Swiss_consensuslog*P*	0.7315	0.8631	<0.001

Computational values of log*P* determined in Chem Axon were in the range of 2.18 to 3.28 with the highest value for pinocembrin-7-methylether. The same compound had the highest value of ChemSketch calculated log*P* (4.11). In the case of Molinspiration calculated log*P*, the most lipophilic flavonoids were flavone (3.74), tangeretin (3.78) and techtochrysin (3.48). SwissADME calculates log*P* according to five different algorithms and expresses their consensus log*P* value. These values were the highest for flavone (3.18), tangeretin (3.02) and chrysin dimethyl ether (3.13) which is in accordance with experimentally determined R_M values.

Results of linear regression (Table II) for multiple log*P* and R_M showed strong positive correlation $R = 0.8631$ and $R = 0.9283$ ($p < 0.001$) for calculated SwissADME consensus log*P* and Molinspiration program, resp. ChemSketch and Chemicalize log*P* showed moderate positive correlation to chromatographic parameter R_M $R = 0.5454$ ($p = 0.009$) and $R = 0.4448$ ($p = 0.0434$).

Based on the strong positive correlation between R_M parameter and log*P* calculated by Molinspiration and SwissADME application and moderate positive correlation with ChemSketch and Chemicalize log*P*, it can be concluded that chromatographic parameter R_M is a good surrogate of log*P* and a good predictor of flavonoid lipophilicity.

This approach was also previously used as a part of QSAR study of flavonoids as bacteriostatic agents against *Escherichia coli*, with the conclusion that high correlation between calculated log*P* (Clog*P*) values and predicted MIC_{50} implies that hydrophobicity plays a major role in the antibacterial activity of flavonoids by their interaction with membranes (21). When inhibition of Syk protein tyrosine kinase activity and human mast cells degranulation was studied on a set of flavonoids, a significant relationship was observed between compound log*P* and the observed inhibitory effect (22). Log*P* was also included in global models for the evaluation of toxicity of flavonoid compounds in the metabolic environment of mammals (23). As a measure of lipophilicity, log*P* offers a very convenient way of predicting the possible extent of flavonoid action *in vivo* based on their interaction with biomembranes.

Relationship between inhibition of platelet aggregation and lipophilicity of flavonoids

For flavonoids listed in Table I previously tested in platelet aggregation assay induced by ADP (17), *MINaAC* values were correlated with calculated log*P* (Table III).

*Table III. Correlations for log*P *and minimal antiaggregatory concentration (MINaAC) of flavonoids*

Application	Flavone	Flavonols	Flavanons	Flavons and flavonols	Flavons and flavonons	Flavonols and flavonons
log*P*_ChemAxon	–0.7576[a]	0.6691	0.1599	–0.0103	–0.5922[a]	0.0724
log*P*_ChemSketch	–0.2667	0.0082	0.3013	–0.1214	–0.2005	–0.1545
log*P*_Molinspiration	0.1908	0.1341	–0.2218	–0.0597	0.1498	–0.0047
Swiss_ilog*P*	0.5384	0.0062	–0.6678	–0.0279	0.4078	–0.1949
Swiss_xlog*P*3	–0.3550	–0.1014	–0.2666	–0.3042	–0.2297	–0.2414
Swiss_wlog*P*	0.2406	0.0428	–0.3616	–0.1106	0.1785	–0.0545
Swiss_mlog*P*	–0.2522	0.0983	–0.2195	–0.1419	–0.2668	–0.1333
Swiss_silicos-it	0.2836	0.0303	–0.3728	–0.1070	0.1996	–0.1066
Swiss_consensuslog*P*	0.1157	0.0185	–0.3645	–0.1628	0.0542	–0.1611

[a] Statistically significant correlation ($p < 0.05$).

The relationship was tested on a whole set of flavonoids, as well as individual classes of flavonoids and their combinations (flavones, flavonols, flavanones, flavones and flavonols, flavones and flavanones, flavonols and flavanones). Statistically significant correlation was found between *MINaAC* and log*P* calculated by Chemicalize application: strong negative correlation $R = –0.7576$ ($p = 0.0069$) for flavone group and moderate negative correlation $R = –0.5922$ ($p = 0.0200$), for combined groups of flavone and flavanone. Van Dijk *et al.* (24) found that flavonols exhibited a substantially higher affinity for liposomes than flavanones attributing this result with a far more planar configuration of the flavonols in comparison with the tilted configuration of flavanones. Oteiza *et al.* (10) proposed lipophilicity of flavonoid molecule as a basis for flavonoid interactions with biomembranes as well as interactions of polyphenols at the surface of bilayers through hydrogen bonding that can act to reduce the access of deleterious molecules, thus protecting the structure and function of membranes. Results of the study by Arora *et al.* (25) on the modulation of membrane fluidity by flavonoids suggest that partition into the hydrophobic core causes a dramatic decrease in lipid fluidity of the membrane.

Our results are in concordance with the assumption that flavonoids have a tendency to localize in the membrane bilayer based on their lipophilicity. In that sense, flavonoids with higher lipophilicity have greater potency to interact with the membrane and consequently cause inhibition of platelet aggregation at a lower concentration, presumably by causing a decrease in lipid fluidity. Thus, the stabilized platelet membrane is less susceptible to the inducers of platelet aggregation. This is in accordance with high negative correlation observed between values of log*P* calculated by Chemicalize application with *MINaAC* values; flavonoids with higher values of log*P* have lower values of *MINaAC* and stronger antiplatelet effect.

When average values of log*P* (Chemaxon) for different classes of flavonoids were calculated (based on data in Table I), the following trend was observed: flavanones (2.50), flavonols (2.76), flavones (3.05). The same trend was observed when comparing antiaggregatory effect; the higher lipophilicity is, the more prone antiaggregatory effect is, *i.e.*, *MINaAC* are lower: flavanons (24.66 μmol L^{-1}), flavonols (7.75 μmol L^{-1}), flavones (4.55 μmol L^{-1}).

Although this observation confirms objective of our work, it should be noted that not all classes of flavonoids were equally represented in our study (flavanones, $n = 4$, flavonols, $n = 6$, flavones, $n = 11$).

Influence of flavonoids and antiplatelet drugs combinations on primary hemostasis

When compared to our previous study, an antiaggregatory activity which was evaluated on a set of 30 flavonoids in the platelet-aggregation assay induced by ADP (17), flavanone showed much higher potency with *MINaAC* concentration in the nanomolar range (63 nmol L^{-1}). As this was the lowest *MINaAC* that we have observed among all flavonoids present in our library of compounds, further studies were conducted using different commercially available inducers of platelet aggregation for impedance aggregometry, namely TRAP-6, collagen, ristocetin and arachidonic acid (Table IV).

Table IV. Effects of flavanone on platelet aggregation depending on the agonist used in impedance aggregation assay

Agonist	*MINaAC* (μmol L^{-1})	*p*-value
ADP	0.063	0.0366
TRAP-6	2.000	0.0310
Collagen	0.500	0.0175
Ristocetin	0.500	0.0270
AA	0.500	0.0037

AA – arachidonic acid, ADP – adenosine diphosphate, *MINaAC* – minimal antiaggregatory concentration, TRAP-6 – thrombin receptor-activating peptide (hexapeptide)

In the herein presented study, *MINaAC* of 0.063 μmol L^{-1} was observed when a weak agonist ADP was used to induce aggregation. When TRAP-6, hexapeptide surrogate of thrombin, was used as an inducer of platelet aggregation *MINaAC* of 2 μmol L^{-1} was obtained. Similarly, stronger inducers of platelet aggregation required more flavanone to observe a statistically significant decrease of platelet, *i.e.*, *MINaAC* was 0.5 μmol L^{-1} in platelet aggregation assays induced by collagen, ristocetin and AA.

Experiments on the combined inhibitory effect of flavanone and known inhibitors of platelet aggregation were designed on Multiplate® analyzer to assess their interactions and, to some extent, indicate a possible mechanism of flavanone action in the presence of strong or weak agonist. A PLC inhibitor U73122, calcium channels blocker verapamil and cyclooxygenase inhibitor indomethacin were used for this purpose. Each experiment included the determination of platelet aggregation of either flavanone or the inhibitor (known antiplatelet drug), or the combination of flavonoid and inhibitor. Platelet aggregation assays in this instance were induced by a strong (TRAP-6) or a weak (ADP) agonist of aggregation. To assess the statistical significance of different treatments one-way ANOVA with *post hoc* test was used for results assessment. The results of these analyses are shown in Fig. 1.

The statistically significant difference between treated samples was observed for the combination of U73122, an inhibitor of PLC, and flavanone, for both (TRAP-6, ADP) agonist applied (Fig. 1a). As expected, flavanone standard reduced platelet aggregation by 14 %,

U73122 reduced platelet aggregation by 25 % while the combination of the two reduced platelet aggregation by 35 % in TRAP-6 induced platelet assay. However, the combined effect of flavanone and U73122 on platelet aggregation induced by ADP was a reduction of 50 %. Significant combined inhibitory effect of flavanone and U73122 on the first and most important enzyme in platelet activation cascade, even in the presence of a strong agonist, could be the consequence of specific inhibition of PLC-mediated signaling pathways upstream of PLC. As membrane fluidity is involved in the regulation of platelet activation, *i.e.*, change of cell shape, pseudopodia formation and exposition of receptors that stimulate platelet aggregation (26, 27), our assumption is that flavonoids as lipophilic substances influence membrane fluidity by increasing membrane rigidity thus reducing the exposition of receptors at the membrane surface and subsequent activation of platelet aggregation signaling pathways. Experiments on G protein-coupled receptor show that increased membrane fluidity stimulates an active form of the receptor (28). Flavanone can interact in a non-specific way with platelet membrane, and, thus, interfere in signal transduction of G-coupled proteins to PLC.

When combined with verapamil, flavanone showed a reduction of platelet aggregation induced by TRAP-6 (Fig. 1b). However, this effect was not observed when ADP was used as platelet aggregation inducer. As both pathways induced by ADP and TRAP-6 include calcium liberation from dense tubules it could be assumed that this is not a common target (29). Rather, upstream signaling pathways unique to TRAP-6 such as Rho/Rho-kinase could be possible targets of flavanone (29). The absence of combined inhibitory influence of flavanone and indomethacin regardless of the used agonist (Fig. 1c), indicates that the antiaggregatory effect of flavanone is not achieved downstream of cyclooxygenase 1.

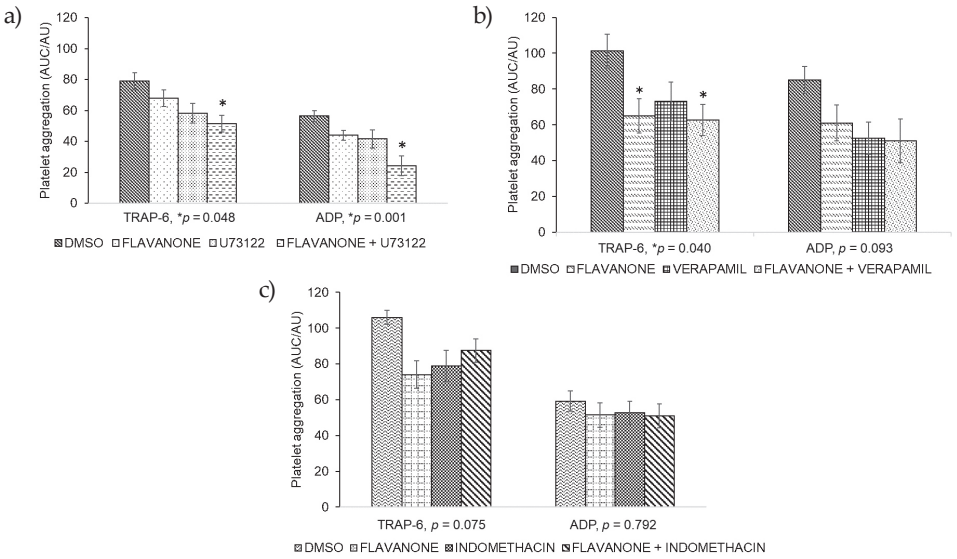

Fig. 1. Results of impedance aggregometry assays induced by a strong (TRAP-6) and a weak (ADP) agonist of aggregation for: a) flavanone and a phospholipase C inhibitor U73122, b) calcium channels blocker verapamil and c) cyclooxygenase inhibitor indomethacin, and flavanone and inhibitor drug combinations. Asterisks indicate statistical significance.

Although the methodology used in these experiments lacks sensitivity to pinpoint precise target of flavanone, results obtained are in accordance with the presumption of the non-specific mechanism of platelet aggregation through the interaction with the membrane rigidity.

Flow cytometry

If flavanone influences membrane rigidity, expression of receptors on platelet surface will be reduced, *e.g.*, by inhibiting transverse diffusion also known as flip-flop. To assess the expression of receptors in the presence and the absence of flavonoid, flow cytometry was used. Platelet activation trough modulation of inside-out signaling of integrin αIIbβ3 is significantly inhibited by flavanone (Figs. 2 and 3).

The observed effect is dose-dependent ($R = 0.9897$, $p < 0.05$, Fig. 2). An example of flow cytometry results is shown in Fig. 3: statistically significant reduction in the receptor exposition is achieved at higher concentrations of flavanone (488 μmol L^{-1}) when compared to the untreated samples of the same participant ($p = 0.0031$). This result was confirmed subsequently by the analysis of five independent blood samples ($p = 0.0018$).

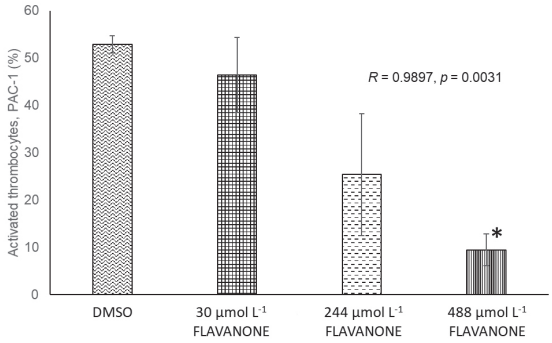

Fig. 2. Inhibitory effect of flavanone on platelet aggregation induced by TRAP-6 on platelet-rich plasma.

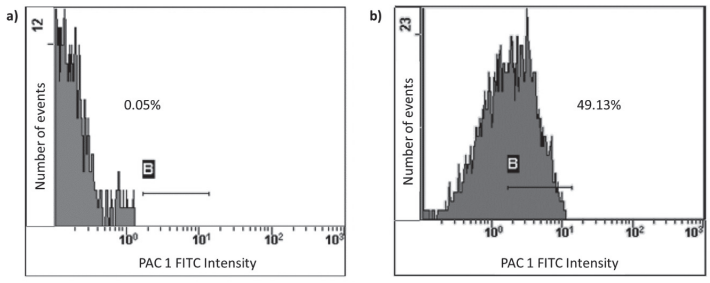

Fig. 3. Flow cytometry analysis of platelet activation induced by TRAP-6 in: a) absence and b) the presence of flavanone. Mean fluorescence intensity is measured using integrin αIIbβ3 antibody.

Despite the noticeable effect, when compared with *MINaAC* observed in impedance aggregometry, only a rather high concentration of flavanone achieved significant inhibition of platelet activation in flow cytometry experiments. This could be due to the difference in the type of samples (impedance aggregometry uses whole blood while platelet-rich plasma is used for flow cytometry experiments), as well as experimental set up (concentration, sample manipulation, time, *etc.*). This is in accordance with the results of Ostertag *et al.* (30) and Vaiyapuri *et al.* (31) who also used higher concentrations of phenolic compounds to see measurable results in flow cytometry experiments. Although they present complementary methods, flow cytometry and impedance aggregometry comprise different aspects of platelet function and are therefore not interchangeable when agonist-induced platelet reactivity is assessed (32). As for methodology, impedance aggregometry, by performing in whole blood, enables other blood elements to influence platelet aggregation, therewith, functions under more physiological conditions (33).

CONCLUSIONS

In platelet aggregation assays induced by ADP, all flavonoids showed antiaggregatory activity (17), suggesting a non-specific mechanism of action related to their lipophilicity and stabilization of platelet membrane. Lipophilicity analysis showed a highly negative correlation between log*P* and *MINaAC* for groups of flavones and flavanones confirming the objective of our study. If so, the exposition of receptors necessary for the promotion of platelet activation and primary clot formation should be diminished. This was confirmed by flow cytometry in which lowered expression of the activated form of integrin αIIbβ3 was observed in the presence of flavanone. Platelet membrane stabilization by flavonoids as a mechanism of antiaggregatory effect has been supported by impedance aggregometry experiments when specific inhibitors of platelet aggregation signaling pathways were used in the presence of a weak and strong agonist of aggregation. While individual flavonoid can have specific targets within aggregation signaling pathways, flavonoids share a common non-specific mechanism of platelet aggregation inhibition related to their lipophilicity and membrane stabilization that, to some extent, contributes to their antiaggregatory effect.

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Acronyms, abbreviations, symbols. – AA – arachidonic acid, ADP – adenosine diphosphate, CD – cluster of differentiation, Clog*P* – calculated log*P*, FITC – fluorescein isothiocyanate, Ig – immunoglobulin, log*P* – logarithm of partition coefficient, *MINaAC* – minimal antiaggregatory concentration, PAC-1 – anti-integrin αIIbβ3 antibody, PE – phycoerythrin, PLC – phospholipase C, PRP – platelet-rich plasma, R_M – chromatographic parameter, surrogate measurement of lipophilicity, TRAP-6 – thrombin receptor-activating peptide (hexapeptide).

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