

Inhibitory effect of Ginkgo biloba extract on human platelet aggregation

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The effect of pure flavonoids and Ginkgo biloba extract (GBE) on human platelet aggregation was investigated. Most of the flavonoids and vitamin E did not affect platelet aggregation in platelet-rich plasma (PRP); however some of these flavonoids inhibited platelet aggregation in gel-filtered platelets (GFP). GBE inhibited both ADP- and collagen-induced platelet aggregation in PRP, GFP and in whole blood in a dose-dependent manner. GBE at very low concentrations inhibited whole blood aggregation induced by ADP compared with those used for PRP or GFP. Flavonoids and GBE decreased the production of TxA₂ induced by collagen and ADP in PRP. However, no correlation was observed between the inhibition of platelet aggregation and the decrease of TxA₂ synthesis. GBE and flavonoids did not affect platelet membrane fluidity. However, the incubation of PRP with GBE increased cAMP levels in platelets, which is known to inhibit platelet activation by lowering intracellular Ca²⁺ levels. GBE is a mixture of many compounds, including flavonoids and ginkgolides, which affect metabolism of cAMP, TxA₂ and Ca²⁺ in platelets. It is effective in the inhibition of platelet aggregation, both in PRP and whole blood, and thus may be potentially used as an effective oral anti-platelet therapeutic agent.

Key words: flavonoids, Ginkgo biloba extract, human platelet aggregation, TxA₂ synthesis

Introduction

Platelet aggregation is fundamental to a wide range of physiological and pathological processes, including thrombosis and arteriosclerosis.^{1–3} Platelet aggregation is induced by various agents such as ADP, collagen, epinephrine, Von Willebrand factor, platelet-activating factor (PAF) and thrombin.^{2,4,5} The aggregation of platelets by these agonists is mediated, in part, through the intracellular formation of prostaglandin G₂, prostaglandin H₂ and thromboxane A₂ (TxA₂) from arachidonic acid, 20:4n–6 (AA).^{1–3} The activation of platelets leads ultimately to Ca²⁺ release through the inositol triphosphate (IP₃) path-

way, resulting in AA release, secretion of many factors and expression of several surface receptors, e.g., fibrinogen receptor (GPIIb/GPIIa).^{2,6} Conversely, inhibition of platelet aggregation is mediated by several anti-aggregating agents, such as prostacyclin (PGI₂), prostaglandin (PG) E₁, PGD₂, adenosine, and nitric oxide (NO).^{1–3,7} The intravascular release of PGI₂ and NO under normal arterial flow conditions inhibit platelet adhesion and aggregation by increasing cyclic AMP (cAMP) and cyclic GMP levels, respectively.³ PGI₂, an AA metabolite of endothelial cells, is the most potent inhibitor of platelet aggregation.³ PG-induced inhibition of platelet aggregation is mediated through an increase of cAMP synthesis due to the activation of adenylate cyclase.^{3,7,8} Activation of adenylate cyclase is initiated by binding of PGI₂ or PGE₁ through specific platelet surface receptors.^{6,8–10} cAMP level in platelets may also be increased by inhibiting cAMP phosphodiesterase activity.³

Hyperactivity of platelets, and their adhesion and aggregation at the site of the injury in atherosclerotic vessel walls, is critically important in the pathogenesis of

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cardiovascular disease.¹⁻⁴ In support of the pathophysiological role of platelets, it has been found that platelet inhibitory drugs, such as aspirin, reduce the incidence of myocardial infarction, stroke and death from cardiovascular disease in secondary prevention trials.¹¹ Given the high incidence of cardiovascular disease in developed countries, there is a great need for other anti-platelet drugs which do not increase bleeding time (a problem associated with glycoprotein IIb/IIIa antagonists^{8,12}), and circumvent the gastric problems associated with aspirin. This concept has stimulated research into the prevention of platelet hyperactivity by dietary supplements. Natural compounds were the first historical source of anti-thrombotic compounds (heparin, vitamin K antagonists, streptokinase, urokinase). Flavonoids are inhibitors of cyclic nucleotide phosphodiesterase and TxA₂ synthesis, two of the main mechanisms for the inhibition of aggregation of blood platelets. Some of these compounds could represent templates for the development of new inhibitors of platelet activation.

The extract of *Ginkgo biloba*, a plant used in Chinese traditional medicine for several thousands years, is thought to be beneficial in the treatment of peripheral and cerebral vascular insufficiency, age-associated cerebral impairment, hypoxic or ischaemic syndromes.^{13,14} Although complete analyses have not been conducted, laboratory studies have found that the standardised alcoholic extract of the *Ginkgo biloba* leaves (EGb 761) contains a great variety of apolar and polar compounds, such as long chain hydrocarbons and derivatives, acyclic acids and cyclic compounds, carbohydrates and derivatives, isoprenoids (sterols, terpenoids) and various compounds including (Z,Z)-4,4'-(1,4-pentadiene-1,5-diyl)diphenol, 6-hydroxykynurenic acid, cytokinins, β -lectins, carotenoids and number of flavonol and flavonol glycosides.¹³ The extract also contains a group of unique, closely related, bitter, 20-carbon diterpene lactone derivatives known as ginkgoglycides A, B, C and M. In addition, a similar 15-carbon sesquiterpene, designated bilobalide, is also present together with 6-hydroxybenzoic acid.¹³ Much research has focused on the free radical scavenging activity of the *Ginkgo biloba* extract, EGb 761, the potential benefits of which could be numerous.¹⁵⁻¹⁹ A very small body of conflicting data has revealed an inhibitory effect of EGb 761 on platelet aggregation.^{13,20,21}

The aim of this study, therefore, was to investigate the effect of *Ginkgo biloba* extract (GBE) on human platelet aggregation. Since GBE also contains flavonoids, it was also important to compare the anti-platelet properties of GBE with pure flavonoids such as catechin, myricetin, quercetin, apigenin, and morin. Flavonoids have been reported to exhibit a wide range of biological effects, including anti-inflammatory, anti-allergic, vasodilatory and anti-platelet actions.^{22,23} Although exact mechanisms are not yet established, more evidence is emerging to suggest that flavonoids exert these effects as antioxidants, free radical scavengers and chelators of divalent cations,^{22,23} but their role in platelet function is

not well characterised. We report in this paper that GBE is an effective anti-platelet regime as the extract contains various compounds which affect different activation pathways in platelets.

Materials and methods

Subjects

Healthy volunteers ($n=35$) took part in the study. All volunteers were non-smokers and abstained from medications for at least 14 days before the study. Volunteers fasted overnight before blood donation. The study was approved by the Joint Ethical Committee of the Grampian Health Board and the University of Aberdeen.

Materials

The TxB₂ assay kit and Amprep C-2 columns were obtained from Amersham (UK). Collagen, ADP and arachidonic acid were obtained from Helena Lab. (USA). Serum albumin (fat free), isobutyl-methylxanthine (IBMX), charcoal, and dextran T70 were obtained from Sigma (Poole, UK). Catechin, morin, myricetin, apigenin, and quercetin were obtained from Aldrich Chemical Co. α -Tocopherol was kindly supplied by Henkel (USA). GBE was kindly supplied by Indena SpA (Italy). All other reagents used were of analytical-grade quality.

Preparation of flavonoids

GBE was used as a suspension in phosphate-buffered saline (PBS), whereas pure catechin, morin, and myricetin were dissolved in ethanol (100% or 25%, as appropriate). In all experiments they were prepared fresh each day. Final concentrations of each flavonoid remained the same in all cases. Final concentrations of ethanol or DMSO did not exceed 0.13% in the platelet suspension.

Preparation of platelets and aggregation studies

Venous blood was collected through siliconised needles into plastic syringes: coagulation was prevented by mixing 9 vol of blood with 1 vol sodium citrate (final concentration, 13 mM). Platelet-rich plasma (PRP) was obtained by centrifugation of blood for 10 min at 180 xg. Platelet counts were performed in a Coulter cell Counter and the platelet numbers in PRP was adjusted to $2-3 \times 10^8$ cells/ml. Gel-filtered platelets (GFP) were prepared from PRP using a Sepharose 4B column as previously described.⁷ Platelet aggregation was monitored on a Packs-4 aggregometer (Helena Lab.) at a constant stirring speed of 1000 rpm at 37°C. PRP or GFP were incubated with flavonoids or GBE for different times, and then platelet aggregation was induced by adding ADP, collagen or AA. Control samples were incubated with vehicle alone. Platelet aggregation was performed within 1.5 h after the blood was taken.

Whole blood aggregation

Blood was taken from healthy subjects and mixed with sodium citrate, as described in the previous section. The blood was then incubated at 37°C in a water bath during the course of the experiment. A Chronolog whole blood aggregometer was used for these experiments. Typically, 500 µl of blood were pipetted into plastic tubes. To achieve a final volume of 1 ml, 400 µl of PBS and 100 µl of saline or GBE in solution were added and incubated for 1 min before addition of 5 µl of 1 mM ADP. Concentrations of GBE used were 0.5, 1.0, 2.0 and 4.0 mg/ml (final concentration). The average of several control aggregations and of the inhibition of aggregation by the different concentrations of GBE was determined so that the percentage inhibition could be calculated.

Thromboxane B₂ (TxB₂) assay

TxB₂, the breakdown product of TxA₂, was estimated using a TxB₂ assay kit, as described before.²⁴ Briefly, at the end of the platelet aggregation experiment, 400 µl plasma were acidified to pH 3 and then applied to an Amprep C-2 column. The TxB₂ was eluted using methylformate and then assayed according to the manufacturer's instructions.

Cyclic nucleotide assay

cAMP and cGMP were measured in PRP as previously described.²⁵ PRP aliquots (890 µl) were incubated with GBE (0, 0.125, 0.25, 1.0 and 2.0 mg/ml) for 4.5 min in the presence and absence of IBMX (a phosphodiesterase inhibitor). At 4.5 min, ethanol was added to the plasma at a ratio of 2:1, and after vortexing for approximately 10 s, it was kept at 4°C for 15 min. Subsequently, the samples were centrifuged at 1500 xg for 15 min at 4°C, the supernatant was dried down under air at 55°C, reconstituted in assay buffer (0.55 M acetate, pH 4.8 containing 0.1% gelatin), and frozen at -20°C until assayed.

Determination of platelet membrane microviscosity

Platelet membrane fluorescence polarisation was determined according to Dutta-Roy *et al.*²⁶ Typically, 50 µg platelet membrane protein in 50 mM Tris-HCl buffer, pH 7.4, containing 5 mM MgCl₂, were labelled with a fluorescence probe by incubating an equal volume of a 2 µM 1,6-diphenyl-1,3,5-hexatriene (DPH) dispersion in the same buffer for 1 h at 23°C. The steady-state fluorescence polarisation was measured in a Perkin-Elmer luminescence spectrophotometer (LS-SB) fitted with a polariser accessory. Excitation and emission wavelengths were 358 and 430 nm, respectively. Excitation and emission slits were 5 nm. The steady-state fluorescence polarisation, *P*, was calculated from Eq. (1):

$$P = \frac{I_{VV} - G.I_{VH}}{I_{VV} + G.I_{VH}} \quad G = \frac{I_{HV}}{I_{HH}} \quad (1)$$

where *I*_{VV} and *I*_{VH} are the fluorescence intensities recorded with the analysing polariser oriented, respectively, parallel and normal to the vertically oriented of the polarised excitation beam. *G* is the grating correction factor. *I*_{HV} and *I*_{HH} are the fluorescence intensities determined with the emission polariser vertically and horizontally when the excitation polariser was set in the horizontal position. Light scattering errors were minimised by assuring that measured anisotropies were concentration independent. The steady-state fluorescence anisotropy (*r*_s) was obtained from Eq. (2).

$$r_s = \frac{I_{VV} - G.I_{VH}}{I_{VV} + 2G.I_{VH}} = \frac{2P}{3 - P} \quad (2)$$

The effect of various flavonoids on the steady-state fluorescence anisotropy was determined by incubating DPH-labelled platelet membranes with the corresponding flavonoids or GBE for 30 min at 37°C. The fluorescence background of a blank sample (unlabelled membranes) never exceeded 15% of the fluorescence signal of membranes labelled with DPH.

Determination of plasma levels of vitamins C and E

Plasma levels of vitamin E were determined according to Belizzi *et al.*²⁷ and plasma vitamin C levels were determined according to Ross.²⁸

Statistical analysis

Results are presented as the mean ± SEM. Results were analysed by the Student's *t*-test. Values for amplitude of platelet aggregation in the presence of different concentrations of flavonoids and *Ginkgo biloba* extracts were plotted and regression analyses were evaluated by a computer assisted programme. Other statistical analyses were performed using ANOVA where appropriate, values were considered to be significantly different when *P* < 0.05.

Results

Effects of flavonoids on platelet aggregation

Firstly, we examined the effects of pure flavonoids on human platelet aggregation in PRP and GFP. Maximum amplitude of aggregation of platelets was obtained with the following concentrations of aggregating agents, 10 µM ADP and 1 µg/ml of collagen. The inhibitory effects of myricetin, morin and catechin on platelet aggregation were evaluated using different incubation times (10–30 min). Though the effects of each of the flavonoids did not appear to be time dependent, i.e., there was no significant difference between pre-incubations of 10 or 30 min, maximal inhibition of platelet aggregation was produced within 15 min. As it was difficult to prepare suspensions of flavonoids being investigated in buffer, experiments were carried out using ethanol or

Table 1. Percentage inhibition of platelet aggregation in PRP by various flavonoids

Flavonoids	Final concentrations (μM)	% Inhibition (mean \pm SEM)		
		Collagen (1 $\mu\text{g/ml}$)	ADP (10 μM)	Arachidonic acid (500 $\mu\text{g/ml}$)
Catechin	420	4.3 \pm 2.6	0	5.1 \pm 2.3
Morin	420	0	0	3.2 \pm 1.2
Quercetin	40	0	0	0
Myricetin	420	5.0 \pm 2.0	20.8 \pm 13	75 \pm 15*
Apigenin	420	0	0	0
α -Tocopherol	420	1.5 \pm 1.0	6.0 \pm 1.2	0

Experiments were performed as described in the Methods section. Each experiment was done in triplicate ($n=12$ individuals). Results are expressed as Mean \pm SEM.

* $P<0.05$ as compared with the solvent-treated controls.

DMSO as the solvent (final concentration $<0.13\%$). DMSO or ethanol at 0.13% concentration did not affect platelet aggregation in response to ADP or collagen compared with the untreated platelets. Table 1 shows the percentage inhibition of platelet aggregation by various flavonoids on PRP. Apigenin, α -tocopherol, quercetin and morin did not affect platelet aggregation induced by collagen, ADP or arachidonic acid. These three agonists are known to activate platelets via different mechanisms.² Conversely, catechin (4.3 \pm 2.6%) and myricetin (20.8 \pm 13.2%) had marginal effects on aggregation induced by collagen and ADP, respectively. Table 2 shows the percentage inhibition of four flavonoids, catechin, myricetin, morin and quercetin, on washed platelets. In contrast to PRP, aggregation of GFP was significantly inhibited by these flavonoids, except for apigenin and α -tocopherol. The concentrations of these flavonoids required to inhibit platelet aggregation by 50% (IC_{50}) in GFP induced by collagen were determined. The IC_{50} value for myricetin was $57\pm 6\mu\text{M}$, whereas for morin and catechin the values were considerably higher, 323 ± 78 and $591\pm 65\mu\text{M}$, respectively.

Table 2. Percentage inhibition of platelet aggregation in washed platelets by various flavonoids

Flavonoids	Final concentration (μM)	% Inhibition (mean \pm SEM)	
		Collagen (1 $\mu\text{g/ml}$)	Thrombin (1.5 U/ml)
Catechin	420	63.9 \pm 7.2*	0
Morin	420	85.1 \pm 3.0*	0
Myricetin	100	79.5 \pm 5.1*	6.5 \pm 2.5
Quercetin	40	32.5 \pm 12.5**	
Apigenin	420	0	0
α -Tocopherol	420	0	0

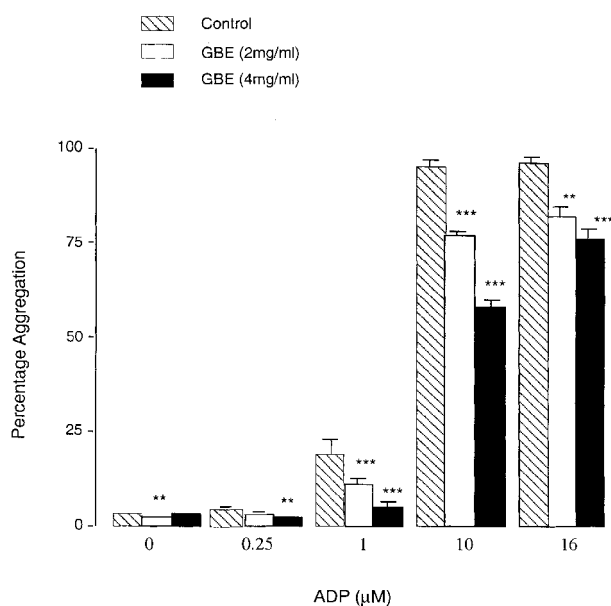
Experiments were performed as described in the Methods section. GFP were prepared as described.⁷ Each experiment was done in triplicate ($n=15$ individuals). Results are expressed as Mean \pm SEM. * $P<0.02$; ** $P<0.05$ compared with the solvent-treated controls.

Addition of platelet-poor plasma (PPP) to GFP decreased the inhibitory effects of flavonoids considerably (data not shown), indicating that plasma proteins probably reduced the inhibitory effects of flavonoids, possibly through binding to these molecules. Though the flavonoids tested were capable of inhibiting aggregation with collagen as the agonist, they were inactive when thrombin was used as the agonist.

It is possible that the plasma levels of vitamins E and C could affect the anti-platelet action of flavonoids. Therefore, the plasma levels of vitamins C and E of these subjects were determined, and in all cases they were found to be within the normal range (for vitamin C, $29.2\pm 2\mu\text{M}$, and for vitamin E, $8.5\pm 1.2\mu\text{g/ml}$).

Effect of GBE on platelet aggregation in PRP

Figure 1 shows the effect of incubation with GBE for 15 min at 2 and 4 mg/ml on platelet aggregation responses to ADP at concentrations of 0, 0.25, 1, 10 and 16 μM . The greatest inhibition (78.9 \pm 3.9%), was seen at the higher dose of GBE, 4 mg/ml, when platelets were stimulated with 1 μM ADP. The lower dose of ADP did not significantly stimulate the primary aggregation wave; however, significant inhibition with GBE can be observed at the higher concentrations of ADP. At 10 μM ADP concentration, which stimulated secondary aggregation, GBE at 4 mg/ml inhibited platelet aggregation by almost 42% compared with the control. At the highest dose of ADP (16 μM), the inhibition by GBE was less marked but was still significant (15.1 \pm 3.9% at 2 mg/ml GBE, $P=0.002$, and 19.9 \pm 3.8% inhibition at 4 mg/ml GBE, $P=0.0003$). GBE at 4 mg/ml concentrations under identical conditions inhibited collagen (1 $\mu\text{g/ml}$)-induced

**Figure 1.** Effect of GBE on ADP-induced platelet aggregation in PRP. PRP was prepared as described in the Methods section. PRP was then incubated with GBE for 15 min before ADP (0, 0.25, 1, 10, and 16 μM)-induced aggregation was initiated. ** $P<0.01$, *** $P<0.001$, paired t -test ($n=9$).

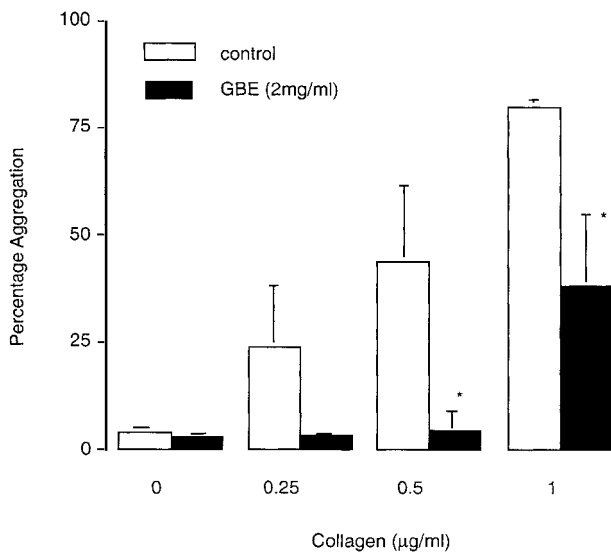


Figure 2. Effect of GBE on collagen induced-platelet aggregation. PRP was incubated with 2 mg/ml GBE for 4.5 min before collagen (0, 0.25, 0.5 and 1 µg/ml)-induced aggregation was initiated. * $P < 0.05$, paired t -test ($n = 4$).

platelet aggregation by 82% compared with control ($n = 4$, $P = 0.001$). Although 15 min incubation of GBE at 4 mg/ml with PRP was found to induce maximum inhibition of platelet aggregation induced by ADP or collagen, 4.5 min incubation with GBE at 2 mg/ml was found to be optimal. Figure 2 shows that GBE at 2 mg/ml inhibited collagen (0, 0.25, 0.5 and 1 µg/ml)-induced platelet aggregation. Inhibition was seen throughout the whole dose-response curve, the most significant inhibition of aggregation being at 0.5 µg/ml collagen ($91.1 \pm 2.4\%$, $P = 0.044$). The percentage inhibition of collagen induced-platelet aggregation appears higher than the inhibition of ADP-induced platelet aggregation throughout the dose-response curve. Like flavonoids, GBE did not inhibit AA-induced platelet aggregation; however, GBE was also equally effective in inhibiting platelet aggregation in GFP (data not shown).

Whole blood aggregation

A representative trace showing the effect of GBE on ADP-induced platelet aggregation in whole blood is shown in Figure 3. GBE inhibited ADP-induced aggregation in a concentration-dependent manner, reaching 100% inhibition with a 4 mg/ml dose. At 2 mg/ml of GBE, the inhibition of aggregation was much higher ($82.6 \pm 8.5\%$) in the whole blood than that seen in PRP with ADP (5 µM) at a similar concentration ($36.7 \pm 9.3\%$, $P < 0.002$). Maximal aggregation of whole blood in response to 5 µM of ADP was reached within 6 min of incubation (data not shown).

Effect of GBE on cyclic nucleotide synthesis

Cyclic AMP levels in platelets were determined after treating these cells with GBE at 0, 0.125, 0.25, 0.5, 1 and 2 mg/ml in PRP (in the absence and presence of IBMX)

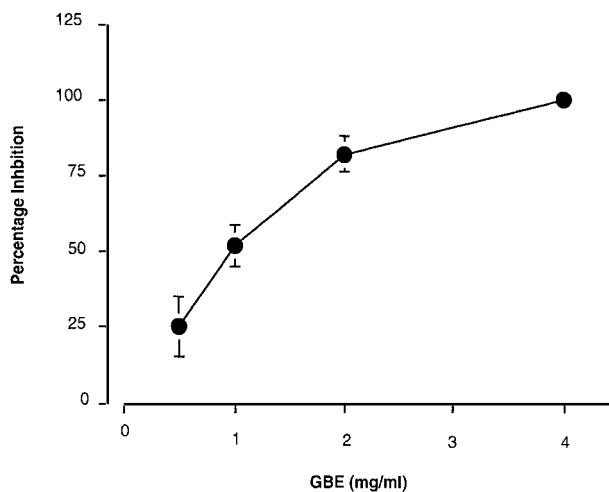


Figure 3. Effect of different of GBE on ADP-induced platelet aggregation in whole blood. Whole blood was incubated with GBE (0.5, 1, 2 and 4 mg/ml) for 4.5 min. before ADP (5 µM) was added to initiate aggregation ($n = 4$).

for 4.5 min (Figure 4). A dose-dependent increase in cAMP levels in response to GBE was observed; the cAMP level, which was 6.1 nM in the presence of IBMX, and in the absence of GBE, increased to 12.5 nM in the presence of 0.5 mg/ml GBE with IBMX ($P < 0.002$, $n = 4$). However the cGMP level which was 4.95 ± 0.25 nM remained essentially the same (5.0 ± 0.36 nM, $P > 0.5$, $n = 4$) in the presence of GBE (up to 2 mg/ml).

Effect of flavonoids and GBE on TxA_2 synthesis

To determine whether the inhibitory effect of GBE on platelet aggregation was due to the reduced synthesis of TxA_2 , levels of TxB_2 , the stable breakdown product of

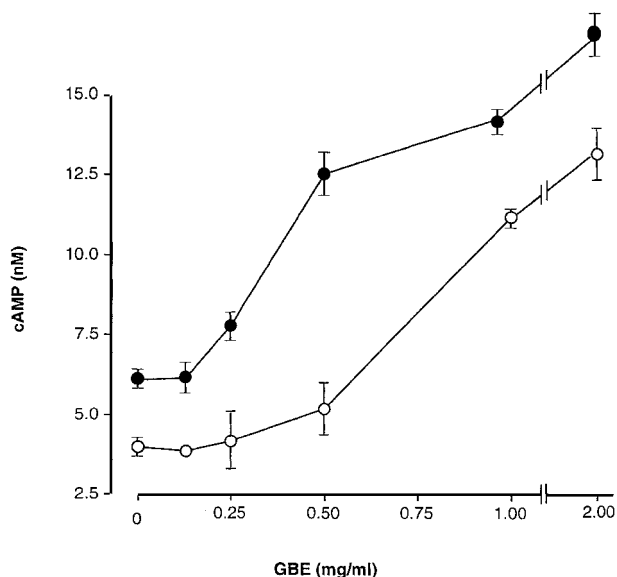


Figure 4. Effect of GBE on platelet cAMP levels in the presence and absence of IBMX. PRP was incubated with in the presence of IBMX with GBE at different concentrations (0.125, 0.25, 1, 2 mg/ml) for 4.5 min. cyclic AMP levels were then measured as described in the Methods section ($n = 4$). -IBMX (○-), +IBMX (●-).

TxA₂, were measured in washed platelets in the presence and absence of pure flavonoids. TxB₂ levels in collagen-stimulated washed platelets were markedly elevated above basal levels. Incubation of washed platelets with these flavonoids inhibited TxB₂ production induced by collagen to different degrees. Catechin was the least effective in inhibiting TxB₂ production by 48%, whereas morin and myricetin inhibited TxB₂ production by 65 and 95%, respectively, compared with controls (Table 3). The inhibition of platelet aggregation in washed platelets by the flavonoids was associated with reduced TxA₂ production. Incubation of PRP with GBE inhibited TxA₂ synthesis in the presence of both collagen and ADP, whereas it did not significantly affect AA-induced TxA₂ synthesis. At 4 mg/ml, GBE inhibited 26 and 40% of TxA₂ synthesis induced by ADP and collagen, respectively, whereas at 2 mg/ml GBE the reduction in TxA₂ was 18% with collagen and 17% with ADP. AA-induced TxA₂ synthesis was inhibited by only 5–10% even at the highest concentrations of GBE.

Membrane fluidity

To determine whether the modification of platelet membrane properties by the flavonoids or GBE was associated with the inhibition of platelet aggregation, membrane anisotropy of platelets was measured in the presence of flavonoids or GBE using 1,6-diphenyl-1,3,5-hexatriene as a fluorescence probe. There were no significant changes observed in the fluorescence anisotropy of membranes treated either with flavonoids, α -tocopherol or GBE compared with that of control membranes (0.225 \pm 0.005) (Table 4).

Discussion

Flavonoids have been reported to exert their anti-platelet action through cAMP phosphodiesterase activity.^{13,22,23,29} However, α -tocopherol, the major lipid soluble antioxidant with vitamin properties present in

Table 3. Inhibition of TxB₂ production by flavonoids

Flavonoid	Final concentration (μ M)	TxB ₂ formation (ng/ml)	% Platelet aggregation (in GFP)
Control	0	125.9	99
Catechin	210	48.7	91
	320	50.2	77.7
Morin	210	25.7	22.7
	320	25.2	14.5
	420	22.1	7.7
Myricetin	50	25	12.7
	75	28	9.5
	100	12.2	5.5

Experiments were performed as described in the Methods section. TxB₂ was measured after GFP were aggregated by collagen as described. Each experiment was done in triplicate ($n=15$ individuals).

Table 4. Effect of various flavonoids, α -tocopherol and GBE on

Treatment	Fluorescence anisotropy (r_s)
Control membranes	0.225 \pm 0.005
Catechin (420 μ M)-treated membranes	0.224 \pm 0.004
Apigenin (420 μ M)-treated membranes	0.223 \pm 0.007
Myricetin (100 μ M)-treated membranes	0.226 \pm 0.004
Morin (420 μ M)-treated membranes	0.227 \pm 0.005
α -Tocopherol (420 μ M)-treated membranes	0.228 \pm 0.009
GBE (2 mg/ml)-treated membrane	0.225 \pm 0.004
GBE (4 mg/ml)-treated membrane	0.226 \pm 0.005

The steady-state anisotropies of the platelet membranes were determined as described in the Methods section. The effects of various flavonoids, α -tocopherol and GBE on anisotropy of DPH-labelled membranes was determined by incubating these with the DPH-labelled membranes for 30 min at 23 °C ($n=9$). Results are expressed as mean \pm SEM. There was no statistical significant difference of the treated membranes from control.

tissues, affects eicosanoid synthesis by platelets only marginally.³⁰ Our data show that some of the flavonoids used in this study inhibited platelet aggregation in washed platelets but had very little effect in PRP. Moore *et al.*,³¹ also reported a minimum effect of various flavonoids on platelet aggregation in PRP, which could be the result of the proteins present in PRP reducing the inhibitory effects of these flavonoids, possibly by binding these compounds. Among the flavonoids, myricetin was the most potent inhibitor of platelet aggregation in GFP, whereas apigenin and α -tocopherol had no effect. The minimum concentration of flavonoid required to inhibit platelet aggregation by 50% (IC₅₀) was different for each flavonoid. In contrast to studies by Beretz *et al.*,³² in our experiments the concentration of catechin required to inhibit aggregation by 50% was approximately three times higher (591 \pm 65 vs. 187 \pm 82 μ M) and the concentration of morin was approximately one and a half times higher (323 \pm 78 vs. 193 \pm 38 μ M). Although these flavonoids inhibited platelet aggregation in GFP, no correlation was observed between the extent of inhibition of TxA₂ synthesis and platelet aggregation, indicating that other mechanisms may also be involved. Mower *et al.*³³ also demonstrated large variability in the platelet aggregation response to flavonoids in PRP. They reported that the platelets of one donor were completely resistant to the anti-aggregatory activity of flavonoids and showed no effect on cyclooxygenase activity.

Although the plasma level of vitamin E is not necessarily a good indicator of its levels in platelets or red blood cells,³⁰ no correlation was observed between the individual's plasma vitamin E level and percent of inhibition of platelet aggregation induced by flavonoids. This observation also holds true for vitamin C. This suggests that there was no association between plasma antioxidant levels and the ability of flavonoids to inhibit platelet aggregation.

The results show clearly that GBE produced a significant inhibitory effect on the aggregatory response

of platelets to ADP and collagen. GBE exerted a greater inhibition of platelet aggregation than the flavonoids examined both in PRP and GFP, whereas pure flavonoids were only effective in GFP. Of greater interest is that the inhibition by GBE appeared much greater in whole blood than in PRP, perhaps suggesting that GBE could affect other blood cells apart from platelets. A contributing factor may possibly be an effect of GBE on neutrophils. Salvemini *et al.*³⁴ proposed an EDRF-like factor, which is released from human neutrophils and acts synergistically with PGI₂ to inhibit platelet aggregation.³ This inhibitory activity was potentiated by superoxide dismutase, an enzyme which has been purified from leaves of *Ginkgo biloba*.³⁵ Furthermore, the strong free radical scavenging activities of *Ginkgo biloba* may inhibit the activation processes.¹⁵⁻¹⁹

The inhibition of ADP-induced platelet aggregation by GBE was dependent on the concentration of ADP used; 42% inhibition was observed at 10 μM ADP, whereas inhibition was lower at 16 μM ADP (8.0±2.7%). This indicates that 16 μM ADP is probably a supra-maximal dose and thus, although GBE may be having an inhibitory effect, it is less easily seen. A significant inhibition of aggregation by GBE was also seen at the 1 μM dose of ADP, which only stimulates the primary aggregation wave (63.6±7.3%). Therefore, we may conclude that GBE inhibits both primary and secondary aggregation induced by ADP.

The inhibition of collagen-induced aggregation by GBE was very marked at all doses of aggregant, indicating that GBE is a particularly good inhibitor of collagen-induced platelet aggregation. Given that GBE inhibited both ADP- and collagen-induced aggregation, it is clear that GBE does not act as a specific antagonist to platelet receptors because if this were the case, we would have expected specific antagonism. Considering the effect of incubation time with GBE in the PRP experiments, it appears that the shorter incubation time showed a trend for increasing the percentage inhibition of both the primary aggregation (from 56.0±7.5 to 63.6±7.2%) and the secondary aggregation with 4 μM ADP (data not shown). This implies that GBE was acting on the platelets to produce a fast inhibitory response further supporting the rationale for testing the cyclic nucleotide response to GBE. The results of the cyclic nucleotide assays show a dose-dependent effect of GBE on the levels of cyclic AMP in the platelets, but no effect on the levels of cGMP. This suggests that GBE may exert its inhibitory effect on platelet aggregation by increasing the levels of cyclic AMP within the platelets either by stimulating adenylate cyclase or inhibiting cAMP phosphodiesterase activity. Although not fully understood, a variety of molecular mechanisms for the role of cAMP in the inhibition of platelet activation have been proposed.² These include inhibition of the phospholipase C-mediated diacylglycerol (DAG) and IP₃ formation, alteration of the DAG signal for protein kinase C activation, as well as the protein kinase C signal, inhibition of GPIIb/GPIIIa-mediated platelet-platelet

contacts, indirect inhibition of phospholipase A₂, which releases AA (intra-platelet source of TxA₂) and, most importantly, antagonism of the Ca²⁺-mediated responses.² Previous work has shown that the incubation of platelets with stimulators of adenylate cyclase (prostacyclin), inhibits the subsequent Ca²⁺ response to agonists.² Thus, in future it would be interesting to monitor the direct effect of GBE on the intracellular levels of Ca²⁺ during agonist stimulation.

To determine whether there may be other mechanisms apart from the cyclic nucleotide response involved in the inhibitory effect of GBE on platelet aggregation, we also determined platelet TxA₂ synthesis and platelet membrane fluidity.³ Although GBE inhibited TxA₂ synthesis induced by collagen or ADP, it had very little effect on AA-induced TxA₂ synthesis. Similar to flavonoids, GBE did not affect platelet membrane fluidity. Since GBE inhibited both ADP- and collagen-induced platelet aggregation, the anti-aggregation action of GBE may be explained on the basis of its multiple constituents. Ginkgolides which are also present in GBE are known to inhibit mobilisation of Ca²⁺ in platelets, and thus inhibit PAF-induced platelet aggregation.³⁶ Since GBE is a mixture of various compounds, including quercetin as a glucorhamnoside ester, and ginkgolides, which are known to affect different cellular activation processes, GBE may be a superior anti-platelet therapy to individual flavonoids.

With regard to the clinical potential of GBE as a future anti-platelet drug, one must consider its actions not only upon platelets but also on the vessel wall, as these are closely related. With regard to the latter, Stucker *et al.*³⁷ have found that EGb 761 opposes vasospasm induced by thrombin serotonin and the thromboxane analogue (U46619). Comparison of the *in vitro* effects observed with GBE against *in vitro* studies with aspirin, thrombin inhibitors and GPIIb/GPIIIa antagonists should be entertained as crucial experiments as a follow-up to this paper. *In vitro* experiments comparing the effects of aspirin and the extract of *Ginkgo biloba*, EGb 761, on an experimental model of thrombosis induced by lasers in rats, concluded that aspirin and EGb 761 had a similar anti-thrombotic effect.³⁸

Aspirin is for the time being the gold standard anti-platelet agent for prevention of arterial thrombosis; being a cyclooxygenase inhibitor, it exerts effects through inhibition of the thromboxane-dependent amplification mechanism of activation of the fibrinogen receptor.³⁹ However, aspirin at low doses may not inhibit collagen-induced platelet aggregation in certain conditions,⁴⁰ and it becomes less effective if shear stress or ADP become relevant stimuli of platelet activation.³⁹ Furthermore, a ruptured plaque has the potential for promoting ADP, collagen and epinephrine-induced platelet aggregation.

In conclusion, GBE is equally effective in inhibiting platelet aggregation, both in PRP and whole blood, induced by collagen and ADP via increasing cAMP levels and lowering TxA₂ synthesis. Our data thus provide evidence that GBE has great potential for increasing the effectiveness of thrombosis prophylaxis

following an oral administration. Thrombosis and atherosclerosis are the results of complex pathological interactions involving platelets, leukocytes, the coagulation system, components of the vessel wall and haemodynamic parameters.³ GBE represents an interesting therapeutic approach because, in addition to its anti-platelet properties, it can interfere with other steps implicated in the development of vascular diseases.¹³ These claims, however, require further research to elucidate their effects *in vivo* bioavailability, stability and metabolism of the active compounds present in GBE responsible for inhibition of platelet function. The use of GBE in its present form as an oral therapeutic agent may be limited, as it may require large quantities (grams) to inhibit platelet activity *in vivo*. Studies are being carried out to address these issues in order to develop GBE as a successful anti-platelet agent.

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References

- Hamet P, Hidektasu S, Umeda F, Franks, DJ. Platelets and vascular smooth muscle: abnormalities of phosphodiesterase, aggregation, and cell growth in experimental and human diabetes. *Metabolism* 1983; **32**: 124–30.
- Kroll MH, Schafer AI. Biochemical mechanism of platelet activation. *Blood* 1989; **74**: 1181–95.
- Dutta-Roy AK. Insulin mediated processes in platelets, erythrocytes and monocytes/macrophages: effect of essential fatty acid metabolism. *Prostaglandins Leukotrienes Essent Fatty Acids* 1994; **51**: 385–99.
- Hamberg M, Svensson J, Samuelsson B. A new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc Natl Acad Sci USA* 1975; **74**: 2994–8.
- Dutta-Roy AK, Kahn NN, Sinha AK. Prostaglandin E₁: the endogenous physiological regulator of platelet mediated blood coagulation. *Prostaglandins Leukotrienes Essent Fatty Acids* 1989; **35**: 189–95.
- Furman MI, Grigoryev D, Bray PF, Dise KR, Goldschmidt-Clermont PJ. Platelet tyrosine kinase and fibrinogen receptor activation. *Circ Res* 1994; **75**: 172–80.
- Dutta-Roy AK, Ray TK, Sinha AK. Prostacyclin stimulation of the activation of blood coagulation factor X by platelets. *Science* 1986; **231**: 385–9.
- Harker, LA, Fuster V. Pharmacology of platelet inhibitors. *J Am Coll Cardiol* 1986; **8**: B21–32.
- Dutta-Roy AK, Sinha AK. Purification and properties of prostaglandin E₁/prostacyclin receptor of human blood platelets. *J Biol Chem* 1987; **262**: 12685–91.
- Dutta-Roy AK, Kahn NN, Sinha AK. Interaction of receptors for prostaglandin E₁/prostacyclin and insulin in human erythrocytes and platelets. *Life Sci* 1991; **49**: 1129–39.
- Yusuf S, Wittes J, Friedman I. Overview of results of randomized clinical trials in heart disease, unstable angina, heart failure, primary prevention with aspirin, and risk factor modification. *J Am Med Assoc* 1988; **260**: 2259–63.
- Coller BS, Anderson K, Weisman HF. New anti-platelet agents: platelet GPIIb/GPIIIa antagonists. *Thromb Haemost* 1995; **74**: 302–8.
- DeFeudis FV. *Ginkgo biloba* extract (Egb761) Pharmacological Activities and Clinical Applications. Paris: Elsevier, 1991.
- Kleijnen J, Knipschild P. *Ginkgo biloba*. *Lancet* 1992; **340**: 1136–9.
- Marcocci L, Packer L, Droylefaix MT, Sekaki A, Gardesalbert M. Antioxidant action of *Ginkgo biloba* extract EGB-761. *Methods Enzymol* 1994; **234**: 462–75.
- Yan, LJ, Droylefaix MT, Packer L. *Ginkgo biloba* extract (EGB761) protects human low density lipoproteins against oxidative modification mediated by copper. *Biochem Biophys Res Commun* 1995; **212**: 360–6.
- Khalil A, Fortun A, Bonnefontaousselot D, Gardesalbert M, Lepage S, Delattre J, Droylefaix MT. Antioxidant effect of EGB761 towards superoxide free radical action on LDL. *J Chim Phys Phys-Chim Biol* 1996; **93**: 143–50.
- White, HL, Scates PW, Cooper BR. Extracts of *Ginkgo biloba* leaves inhibit monoamine oxidase. *Life Sci* 1996; **58**: 1315–21.
- Pietri S, Sequin JR, d'Arbigny P, Drieu K, Culcasi M. *Ginkgo biloba* extract Egb 761 pretreatment limits free radical-induced oxidative stress in patients undergoing coronary by pass surgery. *Cardiovasc Drugs Ther* 1997; **11**: 121–31.
- Braquet P, Etienne A, Toupee C, Burgeon RH, Left J, Varagafitg BB. BN52021 and related compounds; a new series of highly specific PAF-acether inhibitor BN 52021. *Lancet* 1985; **1**: 1501.
- Stucker O, Pons C, Duverger JP, d'Arbigny P, Direu K. Effect of *Ginkgo biloba* extract (EGB 761) on in vitro induced, white-platelet, arterial-thrombosis formation. *Adv Ginkgo biloba Extract Res* 1994; **3**: 27–30.
- Landolfi R, Mower RL, Steiner M. Modification of platelet function and arachidonic acid metabolism by bioflavonoids. *Biochem Pharmacol* 1984; **33**: 1525–30.
- Cook NC, Samman S. Flavonoids: chemistry, metabolism, cardioprotective effects, and dietary sources. *J Nutr Biochem* 1996; **7**: 66–76.
- Dutta-Roy AK, Gordon MJ, Campbell FM, Crosbie L. Arachidonic acid uptake by human platelets is mediated by CD36. *Platelets* 1996; **7**: 291–5.
- Harper JS, Brooker G. Femtomole sensitive radioimmunoassay for cyclic AMP and cyclic GMP after acetylation by acetic anhydride. *J Nucleotide Res* 1975; **1**: 207–18.
- Dutta-Roy AK, Hoque L, Patterson BJ. Prostaglandin E₁ binding sites in rabbit erythrocyte membranes. *Eur J Biochem* 1993; **213**: 1167–73.
- Bellizzi MC, Dutta-Roy AK, Duthie GG, James WPT. Vitamin E binding activity of red blood cells in smokers. *Free Radic Res* 1996; **27**: 105–12.
- Ross M. Determination of ascorbic acid and uric acid in plasma by high performance liquid chromatography. *J Chromatogr B*. 1994; **657**: 197–200.
- Beretz A, Cazenave JP. Old and new natural products as the source of modern anti-thrombotic drugs. *Planta Med* 1991; **57**: S68–72.
- Dutta-Roy AK. α -Tocopherol-binding proteins: purification and characterization. *Methods Enzymol* 1997; **282**: 278–97.
- Moore PK, Griffiths RJ, Lofts FJ. The effect of some flavone drugs on the conversion of prostacyclin to 6-oxoprostaglandin E₁. *Biochem Pharmacol* 1983; **32**: 2813–7.
- Beretz A, Cazenave J-P, Anton R. Inhibition of aggregation and secretion of human platelets by quercetin and other flavonoids: structure-activity relationships. *Agents Actions* 1982; **12**: 382–7.
- Mower R, Landolfi R, Steiner M. Inhibition in vitro platelet aggregation and arachidonic acid metabolism by flavone. *Biochem Pharmacol* 1984; **33**: 357–63.
- Salvemini D, Denucci G, Gyglewski RJ, Vane JR. Human neutrophils and mononuclear cells inhibit platelet aggregation by releasing a nitric oxide-like factor. *Proc Natl Acad Sci USA* 1989; **86**: 6328–32.
- Duke MV, Salin, ML. Purification and characterization of an iron-containing superoxide dismutase from eucaryote, *Ginkgo biloba*. *Arch Biochem Biophys* 1985; **243**: 305–14.
- Etienne A, Baroggi N. Effects of ginkgolides on PAF-induced calcium mobilization in platelets. in: Braquet P, ed. *Ginkgolides, Chemistry, Biology, Pharmacology and Clinical perspectives*. S.A., J.R. Prous Science Publishers, 1988; 115–25.
- Stucker O, Pons C, Duverger JP, Drieu K. Effects of *Ginkgo biloba* extract (EGB-761) on arteriolar spasm in a rat cremaster muscle preparation. *Int J Microcirc Clin Exp* 1996; **16**: 98–104.
- Beloung E, Agvejouf O, Imault P, Ovalaane FA, Doutremepuich F. Experimental thrombosis model induced by laser beam: application of aspirin and an extract of *Ginkgo biloba*, EGB 761. *Thromb Res* 1996; **82**: 453–58.
- Schorr K. Anti-platelet drugs: a comparative review. *Drugs* 1995; **50**: 7–28.
- Roehm E. Low dose of aspirin inhibits arachidonic acid-precipitated platelet aggregation but not collagen-precipitated platelet aggregation. *Am J Cardiol* 1995; **76**: 637–8.