



## The red wine phenolics *trans*-resveratrol and quercetin block human platelet aggregation and eicosanoid synthesis: Implications for protection against coronary heart disease

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### Abstract

A number of lines of evidence suggest that red wine may be more effective than other alcoholic beverages in decreasing the risk of coronary heart disease (CHD) mortality. This protection over and above that due to ethanol itself may be explained by phenolic components with which red wines are richly endowed. We have studied the effects of the trihydroxy stilbene *trans*-resveratrol on human platelet aggregation and on the synthesis of three eicosanoids from arachidonate by platelets, i.e. thromboxane B<sub>2</sub> (TxB<sub>2</sub>), hydroxyheptadecatrienoate (HHT) and 12-hydroxyeicosatetraenoate (12-HETE). These effects were compared with the actions of other wine phenolics (quercetin, catechin and epicatechin) and antioxidants ( $\alpha$ -tocopherol, hydroquinone and butylated hydroxytoluene). *trans*-Resveratrol and quercetin demonstrated a dose-dependent inhibition of both thrombin-induced and ADP-induced platelet aggregation, whereas ethanol inhibited only thrombin-induced aggregation. The other compounds tested were inactive. *trans*-Resveratrol also inhibited the synthesis of TxB<sub>2</sub>, HHT, and to a lesser extent 12-HETE, from arachidonate in a dose-dependent manner. Quercetin inhibited only 12-HETE synthesis, and hydroquinone caused slight inhibition of TxB<sub>2</sub>

*Abbreviations:* CHD, coronary heart disease; HHT, hydroxyheptadecatrienoate; 12-HETE, 12-hydroxyeicosatetraenoate; TxB<sub>2</sub>, thromboxane B<sub>2</sub>; TxA<sub>2</sub>, thromboxane A<sub>2</sub>; BHT, butylated hydroxytoluene; HQ, hydroquinone; PRP, platelet-rich plasma; PPP, platelet-poor plasma; DMSO, dimethyl sulfoxide; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

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synthesis, the remaining compounds being ineffective. De-alcoholized red wines inhibited platelet aggregation; their ability to inhibit the synthesis of  $\text{TxB}_2$  but not that of 12-HETE from labelled arachidonate by washed human platelets was proportional to their *trans*-resveratrol concentration. These results are consistent with the notion that *trans*-resveratrol may contribute to the presumed protective role of red wine against atherosclerosis and CHD.

**Keywords:** Atherosclerosis; Thromboxane  $\text{B}_2$ ; Hepoxilins; Anti-oxidants; Resveratrol; Quercetin

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## 1. Introduction

Epidemiological evidence from many studies involving hundreds of thousands of human subjects overwhelmingly supports the notion that moderate alcohol consumption is a negative risk factor for coronary heart disease (CHD) mortality [1,2]. Approximately half of this risk reduction may be attributed to increased concentrations of circulating high-density lipoprotein cholesterol (HDL-C) which is increased by ethanol in a dose-dependent manner [3,4]. Reduced platelet coagulability is another mechanism which is thought to play a beneficial role in this process [5,6]. However, other mechanisms such as inhibition of lipoprotein oxidation, free-radical scavenging, and modulation of eicosanoid metabolism [7–10] may reduce the likelihood of atherosclerosis and its sequelae, including CHD, in moderate drinkers. Ethanol itself is unlikely to account for all of these latter postulated effects, and interest has therefore focused upon other constituents of alcoholic beverages which may be the agents responsible.

A clue to the identity of these putative agents has been provided by a series of publications suggesting that, independent of the effects of ethanol consumption, wine consumption confers additional protection against CHD mortality. The evidence favouring this view has come largely from analyses utilizing World Health Organization statistics and data from the Organization for Economic Co-operation and Development on a country-by-country basis [11–14], but at least one experimental study in human subjects [15], as well as an earlier investigation in cholesterol-fed rabbits [16] have concurred in the conclusion that not only wine in general, but red wine specifically is the most potent alcoholic beverage conferring protection against atherosclerosis or favourably modifying established biochemical risk factors for this process. Since wines, and especially red wines differ most notably from all other forms of beverage alcohol in their content of phenolic (and particularly flavonoid) constituents [17], attention has been directed to these compounds to define their potential role as anti-atherogenic agents. Many, such as quercetin and catechin, are widely distributed among plant and vegetable products, but the tri-hydroxy stilbene, *trans*-resveratrol, has to date been reported in very few components of the human diet, red wine being the only significant source [18]. Moreover, its presence in herbal Japanese folk medication has led to a number of animal experiments suggesting that it may have anti-inflammatory and anti-coagulatory properties that could protect against atherosclerosis and CHD [19–22]. We have therefore studied the effects of

*trans*-resveratrol upon human platelet coagulation and on the synthesis of certain eicosanoids related to this process, and compared these effects with those of other phenolic constituents of red wine.

## 2. Materials and methods

### 2.1. Study design

Human platelet-rich plasma (PRP) was stimulated to coagulate in the presence of ADP or thrombin. Washed platelets were also incubated with  $^{14}\text{C}$ -labelled arachidonate, and the synthesis of the following eicosanoids was determined: thromboxane  $\text{B}_2$  ( $\text{TxB}_2$ ), a stable metabolite of the pro-coagulatory thromboxane  $\text{A}_2$  ( $\text{TxA}_2$ ); hydroxyheptadecatrienoate (HHT), an intermediate of the cyclo-oxygenase pathway associated with the synthesis of  $\text{TxA}_2$ , and 12-hydroxyeicosatetraenoate (12-HETE), a product of the 12-lipoxygenase pathway associated with synthesis of the heptoxilins. The ability of *trans*-resveratrol and of a number of anti-oxidants ( $\alpha$ -tocopherol, butylated hydroxytoluene (BHT) and hydroquinone (HQ)), and phenolics present in red wine [quercetin, catechin and epicatechin] to inhibit these processes was determined by adding these substances in varying concentrations and calculating the concentration required to cause 50% inhibition ( $\text{ID}_{50}$ ).

### 2.2. Chemicals

Thrombin (50 U/mg) and ADP were obtained from Sigma (St. Louis, Mo., USA).  $[1-^{14}\text{C}]$ arachidonic acid (55 mCi/mmol) was purchased from Amersham (Oakville, ON). Red wines (generously provided by the Liquor Control Board of Ontario) with *trans*-resveratrol concentrations of 5.8 and 78.5  $\mu\text{mol/l}$  measured by a direct gas chromatographic-mass spectrometric assay [23] were dealcoholized by rotary evaporation at 37°C and the total phenolic concentration was measured with the Folin reagent [24] using quercetin as standard. *trans*-Resveratrol, BHT, HQ, catechin, epicatechin and quercetin were all from Sigma;  $\alpha$ -tocopherol and Ecolite scintillation fluid were from ICN (Cleveland, OH, USA). Dimethyl sulfoxide (DMSO) and HPLC-grade solvents (hexane, diethyl ether, methanol and absolute ethanol) were from Caledon (Georgetown, ON, Canada). 12-HETE and arachidonic acid were from Cayman Chemical Co. Inc. (Ann Arbor, MI, USA). TLC plates were from Merck (EM Science, Gibbstown, NJ, USA). All glassware was siliconized by rinsing with a 1% (v/v) solution of Aquasil (Pierce, Rockford, IL, USA).

### 2.3. Preparation of plasma and platelets

Healthy volunteers who had not taken aspirin or non-steroidal anti-inflammatory drugs for at least 2 weeks were used for this study which was approved by the Human Experimentation Committee for our institution. Blood was obtained by venipuncture and collected in a syringe containing 0.13 M sodium citrate (1:9 (v/v) to blood). PRP was prepared by centrifugation of blood at  $140 \times g$  for 15 min. Most of the plasma was removed, and platelet-poor plasma [PPP] was then prepared by centrifuging the residual blood at  $900 \times g$  for 5 min. The concentration of platelets in the PRP was determined in a cell counter (Coulter Electronics, Burlington, ON, Canada).

#### 2.4. Platelet aggregation

Platelet aggregation was determined using a Payton aggregometer (Payton Associates, Buffalo, NY, USA). The absorbance of the PPP was measured, whereupon samples of PRP containing  $3 \times 10^5$  platelets were transferred to siliconized cuvettes, and the volume was adjusted to 500  $\mu\text{l}$  with Buffer A (137 mM NaCl, 1 mM KCl, 0.42 mM  $\text{NaH}_2\text{PO}_4$ , 0.5 M  $\text{MgCl}_2$ , 5.5 mM glucose and 20 mM Hepes [pH 7.4]). The platelet suspension was stirred at 800 rev./min at 37°C in the aggregometer for 1 min. Final concentrations of 1–1000  $\mu\text{M}$  of test compounds in DMSO (*trans*-resveratrol, BHT, HQ, quercetin, catechin and epicatechin) were added to the suspension; DMSO (0.2% (v/v) final concentration) was added to the control samples.  $\alpha$ -Tocopherol, which was not soluble in DMSO, was added in 1  $\mu\text{l}$  ethanol which in itself, did not affect aggregation. To some samples, 1–10  $\mu\text{l}$  of ethanol was added. After incubation for 2 min, aggregation was induced using thrombin (120 mU/ml final concentration) or ADP (2  $\mu\text{M}$  final concentration), dissolved in Buffer A. Light transmission was recorded until equilibrium was attained. The change in optical density due to aggregation was expressed as the percentage of the difference in optical density between PRP and PPP.

#### 2.5. Eicosanoid synthesis

Samples of PRP containing  $75 \times 10^6$  platelets were transferred to centrifuge tubes, and platelets were collected by centrifugation at  $900 \times g$  for 5 min. They were resuspended in 1 ml Buffer A, and incubated for 1 min in a shaking water bath at 37°C. Samples of test compounds (1–100  $\mu\text{mol/l}$ ) were added in DMSO; DMSO (0.2% (v/v) final concentration) was added to control samples. After 2 min, 5  $\mu\text{l}$  of ethanol containing 10  $\mu\text{g}$  arachidonic acid (100 000 counts/min) was added, followed after 15 s by thrombin (160 mU/ml). Platelet suspensions were shaken for 20 min at 37°C. The assay was stopped by placing the tubes on ice, and adding 3 ml ethyl acetate. The pH of the lower phase was adjusted to 3, the upper phase was collected, and the aqueous phase was re-extracted with a further 3 ml of ethyl acetate. The pooled ethyl acetate phases were extracted a final time with water, and then dried under  $\text{N}_2$ . All phase separations were accomplished by centrifugation at  $100 \times g$  for 5 min. The residue was dissolved in methanol, and separated into 2 portions. To measure  $\text{TxB}_2$ , one portion was separated by TLC using ethyl acetate/acetic acid (99:1 (v/v)). The other portion was separated by TLC using ethyl acetate/hexane/acetic acid (30:70:1 (v/v)), which gives better resolution of HHT and 12-HETE. Metabolites were visualized by autoradiography using Kodak XAR film. Bands were identified from autoradiographs, scraped, incubated for 1 h in methanol/water (1:1 (v/v)), and counted in 10 ml scintillation fluid.

#### 2.6. Data analysis

Data are presented as the mean  $\pm$  S.D. Statistical analyses were performed using Student's *t*-test.

### 3. Results

#### 3.1. Platelet coagulation

*trans*-Resveratrol inhibited both thrombin-induced and ADP-induced aggregation

of PRP (Fig. 1A). Standard anti-oxidants (HQ, BHT and  $\alpha$ -tocopherol) were ineffective. Of the wine phenolics tested, catechin and epicatechin had little effect, but quercetin inhibited both thrombin-induced and ADP-induced aggregation (Fig. 1B), whereas ethanol inhibited only thrombin-induced aggregation (not shown). The  $ID_{50}$  values for *trans*-resveratrol were a little higher than those of quercetin and (for thrombin-induced aggregation) 3 orders of magnitude lower than that of ethanol on a molar basis (Table 1). De-alcoholized red wine low in *trans*-resveratrol (5.8  $\mu\text{mol/l}$ ) also blocked both thrombin-induced and ADP-induced platelet aggregation (Fig. 1C), possibly due to its quercetin content, although other as yet unidentified anti-aggregatory agents may also have contributed.

### 3.2. Eicosanoid metabolism

*trans*-Resveratrol strongly inhibited the synthesis by platelets of  $\text{TxB}_2$  ( $ID_{50}$ ,  $7.12 \pm 0.85 \mu\text{mol/l}$ ) and HHT ( $ID_{50}$ ,  $6.09 \pm 2.25 \mu\text{mol/l}$ ) from arachidonate, and had a less pronounced inhibitory effect ( $ID_{50}$ ,  $76.9 \pm 26.4 \mu\text{mol/l}$ ) on 12-HETE synthesis (Fig. 2). In all instances the inhibition yielded a log-linear dose-response relationship with *trans*-resveratrol concentration (Fig. 2). Of the other wine phenolics tested, quercetin ( $ID_{50}$ ,  $3.29 \pm 1.38 \mu\text{mol/l}$ ) inhibited 12-HETE synthesis (Fig. 3) but not that of  $\text{TxB}_2$  or HHT (not shown). Of the standard anti-oxidants, HQ inhibited  $\text{TxB}_2$  synthesis, but much less than did *trans*-resveratrol at equimolar concentration (Fig. 4); ethanol, catechin and epicatechin in concentrations up to 10  $\mu\text{M}$  did not alter the synthesis of any of these three eicosanoids. The de-alcoholized red wine with high *trans*-resveratrol concentration (78.5  $\mu\text{mol/l}$ ) produced a much more profound inhibition of  $\text{TxB}_2$  (and also HHT) synthesis than the preparation with low resveratrol concentration (5.8  $\mu\text{mol/l}$ ), but both preparations were equivalent in their capacity to inhibit 12-HETE synthesis (Fig. 5).

## 4. Discussion

Atherosclerosis is a multifactorial disease to which a large number of distinct biochemical mechanisms have been postulated to contribute. One of the first to be identified was an increase in total plasma cholesterol concentration subsequently ascribed to an increase in the low-density lipoprotein (LDL) fraction [25]. Next came the realization that impairment of reverse cholesterol transport, best characterized by a decrease in HDL, could also promote this pathological process [26]. Recognition of pathways for cholesterol influx other than the classical Brown-Goldstein LDL receptor [27], including the scavenger receptor which mediates the unregulated endocytosis of modified LDL, focused attention on LDL oxidation as a mechanism promoting cholesterol deposition and foam-cell formation in the blood vessel wall [7,8]. Endothelial damage and impaired production of a smooth muscle relaxing factor now known to be nitric oxide or a related compound has also been recently elucidated [28,29], while inflammatory changes (mediated at least in part by leukotrienes) may play a role in the infiltration of the vascular media by cells, thereby disrupting the elastic lamina and other structures crucial to blood vessel integrity [30]. Finally, the importance of platelet coagulation in the formation and pro-

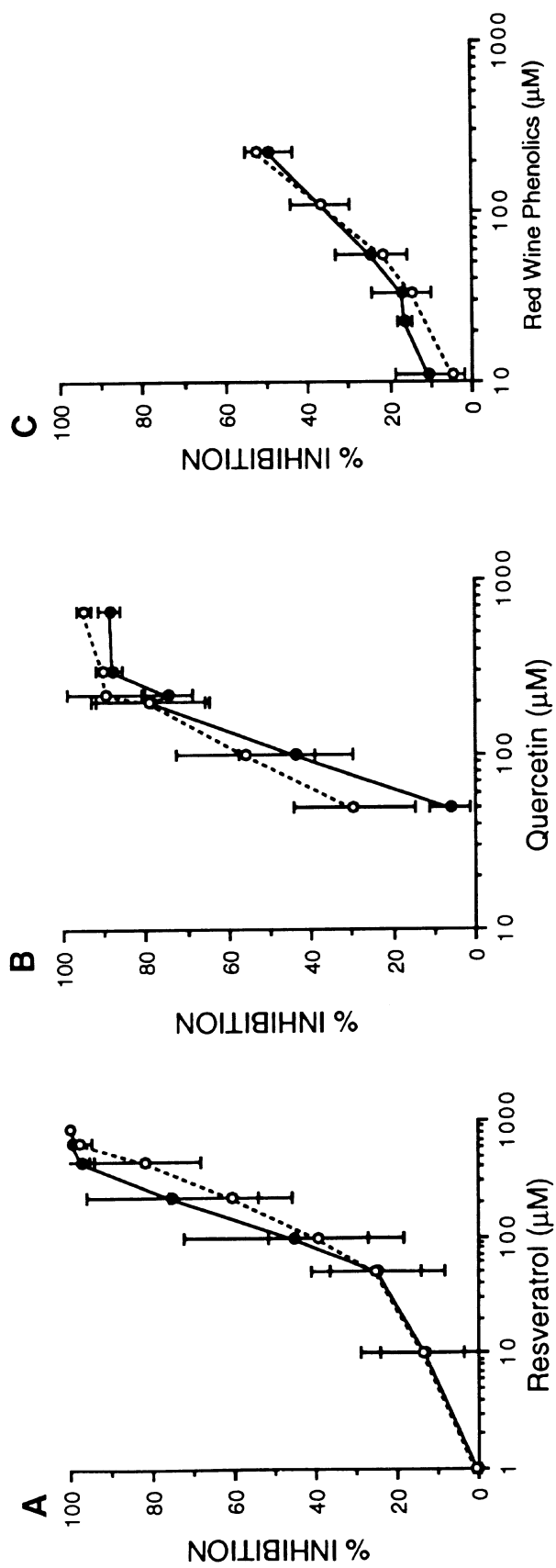


Fig. 1. Inhibition (means  $\pm$  S.D.) of platelet aggregation induced by ADP (solid line) and thrombin (broken line) by *trans*-resveratrol (A,  $n = 10$ ), quercetin (B,  $n = 3$ ) and red wine phenolics (C,  $n = 3$ ) with concentration (log scale). Each point was assayed in triplicate in each experiment which utilized a different platelet-rich plasma.

Table 1  
Concentration ( $\mu\text{mol/l}$ ) required to inhibit platelet aggregation by 50%

Compound	ADP	Thrombin
Resveratrol (11)	129.9 $\pm$ 64.4	164.7 $\pm$ 67.3
Quercetin (3)	101.7 $\pm$ 41.7	92.9 $\pm$ 22.1
Ethanol (3)	No effect	132.6 $\pm$ 40.2 <sup>a</sup>

Number of independent experiments in parentheses; each experiment was the mean of triplicates.

<sup>a</sup>Given in mmol/l.

gression of the atherosclerotic plaque and in precipitating vascular occlusion hardly requires emphasis [27,30].

The data presented in this report, supplemented by other published findings, suggest that each of these mechanisms is favourably influenced by one or more constituents present in red wine. Firstly, it increases the circulating concentration of HDL and of its major apolipoprotein, apo-AI, and favourably influences the LDL-cholesterol, HDL-cholesterol ratio [15]. These effects are shared with most other alcoholic beverages and are therefore likely to be alcohol-dependent. Secondly, it contains several phenolic compounds (including quercetin, catechin, epicatechin and

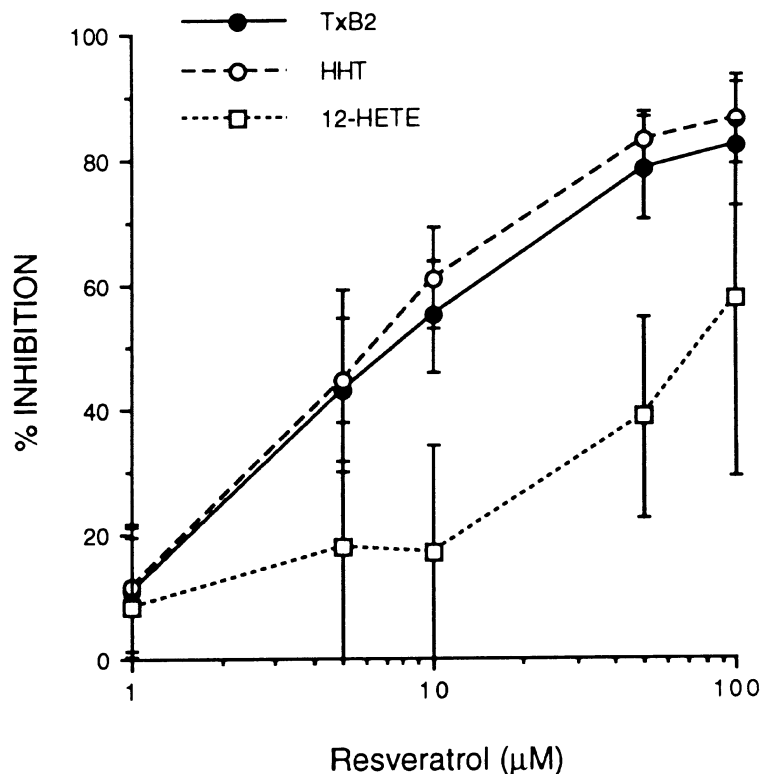


Fig. 2. Inhibition (mean  $\pm$  S.D.) of eicosanoid synthesis by washed human platelets from labelled arachidonate by *trans*-resveratrol (concentration on log scale). Each point is based on 7 independent experiments using a different platelet preparation.

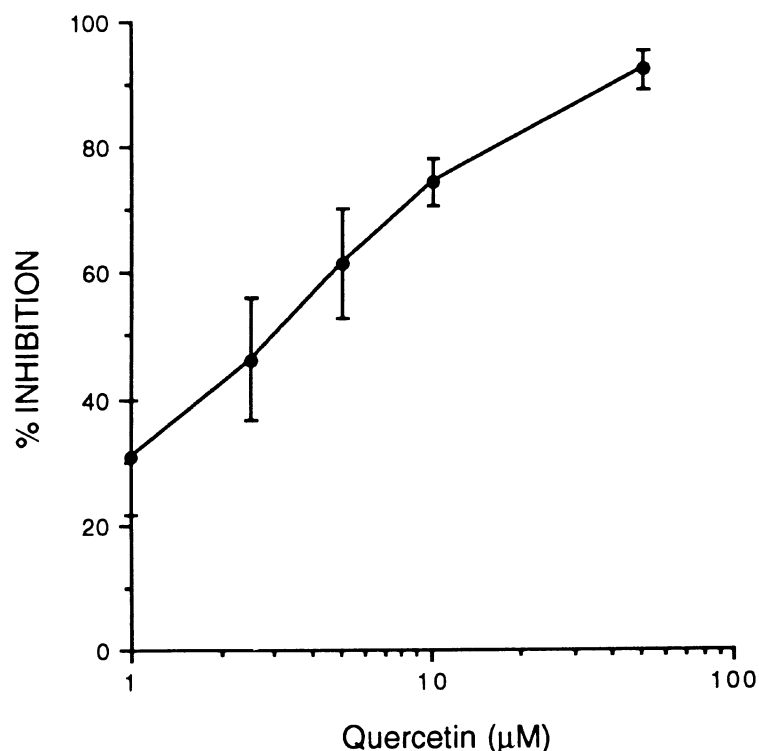


Fig. 3. Inhibition (mean  $\pm$  S.D.) of 12-HETE synthesis by washed human platelets from labelled arachidonate by quercetin (concentration on log scale). Each point is based on 3 independent experiments using a different platelet preparation.

*trans*-resveratrol) which have been shown to block LDL oxidation in vitro and to be more potent than  $\alpha$ -tocopherol in so doing [31,32]. Thirdly, it promotes nitric oxide formation by isolated vascular endothelium, an effect which is best accounted for by its polyphenolic content, with quercetin being the most potent of the relevant compounds tested [33]. Fourthly, alcohol itself has been convincingly established to have anti-coagulatory properties although its effects are mainly seen with aggregation induced by agents which stimulate phospholipase C, such as thrombin and collagen [34].

To these findings, we can now add the following in-vitro observations concerning red wine phenolics. Firstly, at least two of these (quercetin and *trans*-resveratrol) inhibit ADP-induced as well as thrombin-induced platelet coagulation, and they do so at ID<sub>50</sub> concentrations dramatically less than that of ethanol. Secondly, quercetin (and to a lesser extent *trans*-resveratrol) inhibits the 12-lipoxygenase pathway of arachidonate metabolism, blocking the synthesis of hepoxilins which are mediators of calcium mobilisation, vascular permeability and neutrophil activation [35]. Specifically, the synthesis of 12-HETE which has been postulated to be pro-atherogenic by virtue of impairing endothelial function and prostacyclin production [36] was markedly reduced. Finally, *trans*-resveratrol blocks the synthesis of TxA<sub>2</sub> from arachidonate as measured by the production of its stable metabolite TxB<sub>2</sub>. Of



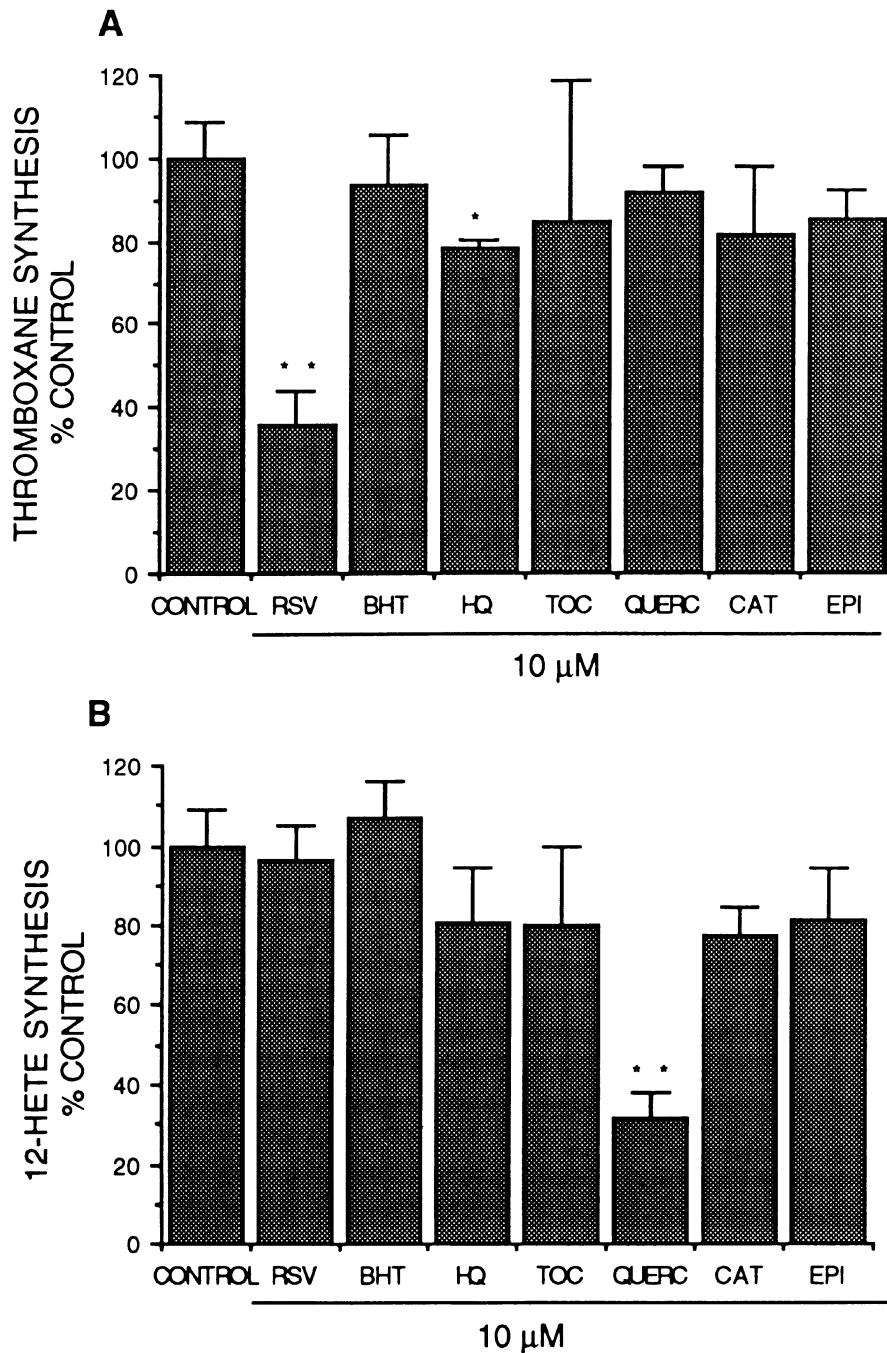


Fig. 4. Effect of *trans*-resveratrol, antioxidants and other wine phenolics on the synthesis of thromboxane B<sub>2</sub> (A, top) and 12-HETE (B, bottom) from labelled arachidonate by washed human platelets at a fixed concentration of 10 μmol/l as a percentage of the control (DMSO above). Data are mean ± S.D. of 4 experiments in each of which a different sample was used. Apart from *trans*-resveratrol, none of these agents altered HHT synthesis at the concentration tested. RSV, *trans*-resveratrol; TOC, α-tocopherol; QUERC, quercetin; CAT, catechin; EPI, epicatechin. \**P* < 0.05; \*\**P* < 0.001.

particular interest was the finding that de-alcoholized red wine low in *trans*-resveratrol (5.8 μmol/l) blocked thrombin-induced and ADP-induced platelet aggregation, and demonstrated moderate inhibition of 12-HETE but not TxB<sub>2</sub> or HHT synthesis. The pattern of inhibition thus mimicked that of quercetin which is present

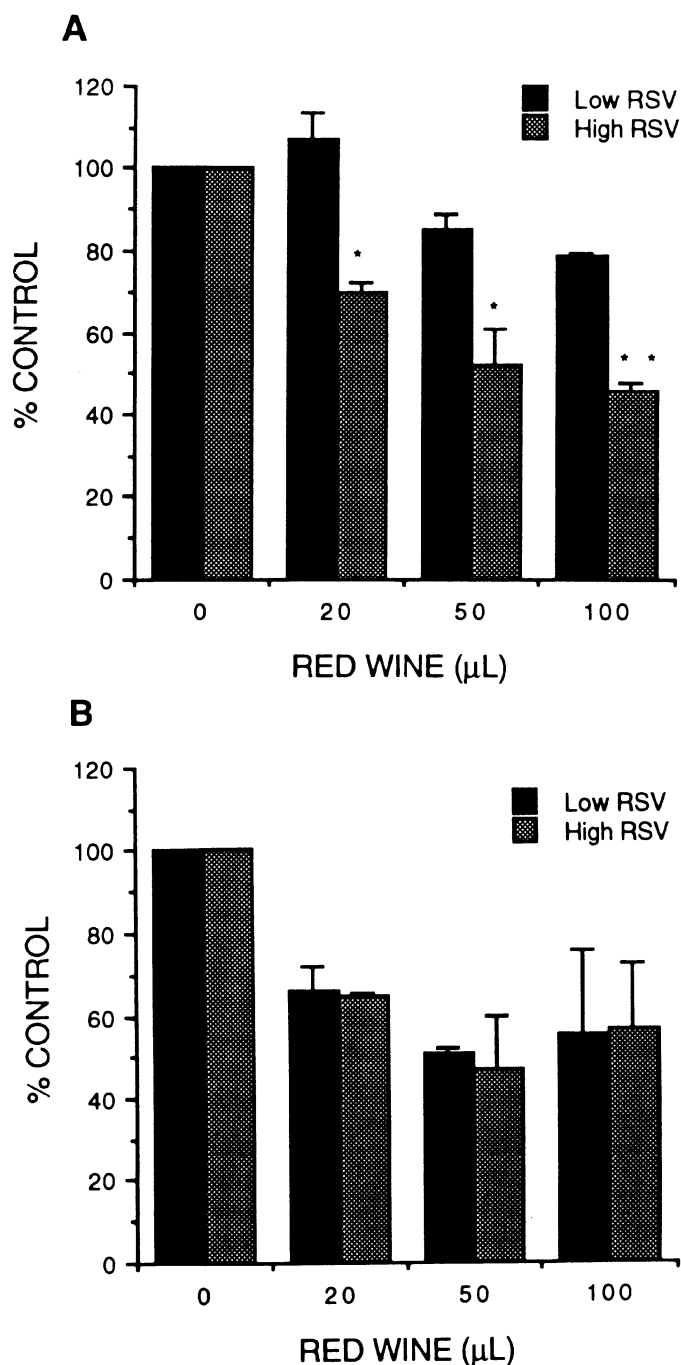


Fig. 5. Effects of de-alcoholized red wines of high (78.5  $\mu\text{mol/l}$ ) and low (5.8  $\mu\text{mol/l}$ ) *trans*-resveratrol concentration on the synthesis of  $\text{TxB}_2$  (A,  $n = 4$ ) and 12-HETE (B,  $n = 4$ ) from labelled arachidonate by washed human platelets. The total reaction volume was 1 ml of which the wine comprised 0–100  $\mu\text{l}$ . The total phenolics [24] of the high and low resveratrol wines were 4.56 and 5.94  $\mu\text{mol/l}$ , respectively. The bars and hatched lines represent the range of values in two independent experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ .

in most red wines in the range of 50–100  $\mu\text{mol/l}$  [17], and was not consistent with that of *trans*-resveratrol. On the other hand, the de-alcoholized red wine high in *trans*-resveratrol (78.5  $\mu\text{mol/l}$ ) inhibited both  $\text{TxB}_2$  and HHT synthesis. Since the wine comprised 10% of the final reaction mixture, the *trans*-resveratrol concentra-

tion (7.85  $\mu\text{mol/l}$ ) can fully account for the inhibition of  $\text{TxB}_2$  synthesis demonstrated in Fig. 5 on the basis of its  $\text{ID}_{50}$  ( $7.12 \pm 0.85 \mu\text{mol/l}$ ).

Although *trans*-resveratrol and quercetin inhibit both ADP-induced and thrombin-induced platelet aggregation at molar concentrations in the same order of magnitude, it is uncertain whether these effects are achieved by the same or different mechanisms. Thrombin, but not ADP, involves phosphoinositide-specific phospholipase C activation following binding of the agonist to G proteins and accompanied by a rise in cytosolic free  $\text{Ca}^{2+}$  and activation of protein kinase C, each of which signal may independently or synergistically induce platelet aggregation [37,38].  $\text{TxA}_2$ , synthesis of which from arachidonate is inhibited by *trans*-resveratrol but not by quercetin, also stimulates phospholipase C. Thus, it is conceivable that quercetin blocks thrombin-induced platelet aggregation by directly inhibiting phospholipase C activity whereas *trans*-resveratrol acts through inhibition of  $\text{TxA}_2$  production. Since increased levels of cyclic AMP suppress the response of platelets to pro-aggregatory agonists [34], stimulation of platelet adenyl cyclase activity is another possible site of action for these agents.

Whether these *in vitro* effects are reproduced *in vivo* during the consumption of red wine can only be determined by experiments on human subjects; these are currently under investigation by our group. However, it is worth noting that in enological studies of red wines from all parts of the globe, we have rarely encountered a *trans*-resveratrol concentration  $< 5 \mu\text{mol/l}$ , concentrations as high as  $100 \mu\text{mol/l}$  being recorded [37]. Moreover, we have also found quite high concentrations of *cis*-resveratrol in most red wines (in some cases exceeding those of the *trans*-isomer) although the biological effects of this compound have yet to be described. The moderate consumption of red wine (recommended doses are 2–5 glasses or a maximum of 375 ml/day) could therefore provide resveratrol in sufficient amounts to favourably influence eicosanoid metabolism and possibly also platelet aggregation depending upon its absorption, metabolism, and residence time within the blood circulation and relevant tissues. The present results provide a clear rationale for investigations into the bioavailability and pharmacokinetics of resveratrol which are under way in our laboratory.

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