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ORIGINAL CONTRIBUTION

Antiplatelet effect of new lipophilic hydroxytyrosol alkyl ether derivatives in human blood

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Abstract
Purpose To investigate the in vitro antiplatelet and anti-inflammatory effects of five alkyl hydroxytyrosol (HT) ether derivatives in human whole blood and compare these effects with those of HT.
Methods Blood samples from healthy volunteers were incubated with HT and HT alkyl ether derivatives (ethyl, butyl, hexyl, octyl and dodecyl). Maximum intensity of platelet aggregation was induced with collagen, arachidonic acid or ADP. Calcium-induced thromboxane B_2 and nitric oxide production, LPS-induced prostaglandin E_2 and nitric oxide production and LPS-induced interleukin 1β production were measured.
Results All compounds inhibited platelet aggregation, thromboxane B_2 and inflammatory mediators in a concentration-dependent manner. The concentrations of each compound that inhibited the corresponding variable by 50 % compared to control samples (IC_{50}) were in the range of 10^{-7}–10^{-6} M for HT hexyl ether; for the other compounds, these values were in the range of 10^{-5} M. The IC_{50} for thromboxane B_2 production was in the range of 10^{-4} M. The effects of HT alkyl ether derivatives were greater than those of HT. These compounds increased nitric oxide production. There was no direct relationship between the effects of these compounds and alkyl chain length. Maximum effects were observed in the C4–C6 range.
Conclusions Alkyl ether derivatives of HT exert antiplatelet and anti-inflammatory effects that are greater than those of HT.

Keywords Hydroxytyrosol alkyl ether derivatives · Platelet aggregation · Prostanoids · Nitric oxide · Inflammatory mediators

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Introduction

The "Mediterranean diet" forms part of strategies to prevent cardiovascular disease and has been shown to have benefits in reducing coronary heart disease and all-cause mortality [1, 2]. Virgin olive oil, the main source of dietary fat in the Mediterranean diet [3], contains several components whose biological effects have been amply demonstrated. Specifically, its so-called minor components such as polyphenols [4], antioxidant compounds that help stabilize olive oil, have a number of biological effects that may explain the beneficial effect of olive oil in preventing cardiovascular disease [5, 6].

Hydroxytyrosol (3,4-dihydroxyphenylethanol) (HT) is an *o*-diphenolic compound present in virgin olive oil as either a secoiridoid derivative [7] or an acetate ester [8]. Several studies have demonstrated its ability to help prevent cardiovascular disease, mainly by protecting against low-density lipoprotein oxidation [9]. Hydroxytyrosol was also shown to inhibit platelet aggregation [10, 11] and to have anti-inflammatory activity [12] and protective effects

against induced oxidative stress through its ability to scavenge free radical species in different cell lines [13].

Platelet activation, the main cause of arterial thrombus formation, results from endothelial dysfunction caused in the early stages of atherogenesis, mainly due to the damage caused by the risk factors such as diabetes, hyperlipidemia and obesity. Accordingly, the pharmacological prevention of arterial thrombotic events with aspirin or clopidogrel (among other drugs) usually aims to inhibit platelet function [14]. Hydroxytyrosol inhibits platelet aggregation both in vitro and ex vivo, and modifies the synthesis of prostanoids, nitric oxide (NO) and some leukocyte inflammatory mediators [10, 11, 15, 16]. However, the low lipophilicity of this compound has led to efforts to synthesize HT derivatives with a better hydrophile/lipophile balance in order to improve their pharmacodynamic profile and increase bioavailability. Virgin olive oil intake supplies not only HT but also other antioxidant compounds that could exert a synergistic effect with HT itself. However, the studies with HT are due to the future development of a compound from a natural source with potential use in the prevention of cardiovascular disease in humans. This requires increasing the lipophilicity of HT in order to try to increase the amount of product to interact with its molecular target in the cells. In this connection, a series of hydrophobic derivatives (alkyl HT ethers) of HT have been synthesized [17] and found to be stable when digested in vitro, rapidly absorbed, and partially metabolized by Caco-2/TC7 cells [18]. Between 40 and 85 % of the compound transferred across the enterocyte monolayer is not metabolized and would be expected to reach the portal blood, and subsequently the liver, unmodified. The HT derivatives tested to date are absorbed well by human hepatoma cells [19].

The aims of the present study were to investigate the in vitro antiplatelet effect of five alkyl HT ether derivatives in human whole blood and to compare these effects with HT.

Materials and methods

Material

Thromboxane B_2, prostaglandin E_2 and interleukin 1β enzyme immunoassay kits were from GE Healthcare (Barcelona, Spain). The nitrite/nitrate ELISA kits were obtained from Cayman Chemical (Ann Arbor, MI, USA). Collagen and adenosine diphosphate (ADP) were obtained from Menarini Diagnóstica S.A. (Barcelona, Spain). All other reagents were from Sigma Chemical Corp. (St. Louis, MO, USA). Hydroxytyrosol alkyl ether derivatives were supplied by the Department of Organic Chemistry and Pharmaceutics, School of Pharmacy, University of Seville (Spain). Alkyl HT ethers were chemically synthesized from HT according to the methods of Madrona et al. [17] (Fig. 1).

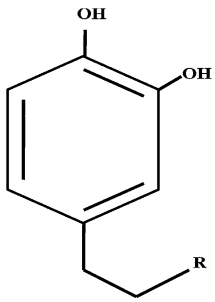
Study design

This in vitro study was carried out in blood samples from healthy volunteers who had not taken any medication during the 15 days before inclusion and who had not consumed virgin olive oil during the 24 h before inclusion. Sodium citrate 3.8 % at a proportion of 1:10 was used as the anticoagulant. In each group of experiments we used 6 samples from different volunteers (3 men and 3 women in each group) with a mean age of 33.6 ± 1.8 years. The study was approved by the Clinical Assays Committee of the Hospital Regional Universitario Carlos Haya in Malaga, Spain.

Different concentrations of HT and of HT alkyl ether derivatives (10^{-7}–10^{-3} M) were incubated at 37 °C before inducers were added. All drugs were diluted in a mixture of ethanol/PBS (100 μL ethanol plus 900 μL PBS) at a proportion of 1:10. Maximum concentration of ethanol in blood samples was 0.01 %.

Analytical techniques

All techniques were run in a single-blind manner, that is, the persons who did the assays were unaware of the origin and nature of the samples.



R	Name
OH	Hydroxytyrosol (HT)
OC_2H_5	HT ethyl ether
OC_4H_9	HT butyl ether
OC_6H_{13}	HT hexyl ether
OC_8H_{17}	HT octyl ether
$OC_{12}H_{25}$	HT dodecyl ether

Fig. 1 Chemical structures of hydroxytyrosol and hydroxytyrosyl alkyl ethers

Platelet aggregometry

Platelet aggregation was measured as electronic impedance in whole blood (500 μL of sample diluted with 500 μL PBS). We used a Chrono-Log 540 aggregometer (Chrono-Log Corp., Haverton, PA, USA) with collagen (1 μg/mL), adenosine diphosphate (ADP, 2.5 μM) and arachidonic acid (400 μM) to induce aggregation. Drugs were incubated at 37 °C for 10 min before the aggregation inducer was added, and the aggregation was recorded for 10 min. Maximum intensity of aggregation was quantified as the maximum change in electronic impedance in samples without the drug or a given concentration of each drug. The concentrations of aggregating agents were chosen according to the previous experiments that yielded EC_{50} values of 1.09 ± 0.1 μg mL^{-1} for collagen ($n = 10$), 2.5 ± 0.1 μM for ADP and 392 ± 19 μM for arachidonic acid ($n = 10$).

Type-1 and type-2 cyclooxygenase inhibitory activity [20, 21]

The production of thromboxane A_2 was determined by measuring its stable metabolite thromboxane B_2, as an index of type-1 cyclooxygenase (COX-1) activity. Samples of whole blood were incubated with the alkyl ether derivative and then stimulated with calcium ionophore A23187 (50 μM) at 37 °C during 30 min; then, they were centrifuged at $10{,}000 \times g$ for 3 min, and the amount of thromboxane B_2 (TxB_2) in the supernatant was determined with an enzyme immunoassay kit.

Cyclooxygease-2 (COX-2) activity was measured in blood samples incubated firstly with alkyl ether derivatives, then with bacterial membrane lipopolysaccharide (LPS) (1 μg/mL) for 18 h at 37 °C. The 96-well plate was then centrifuged, and the plasma was removed and frozen at

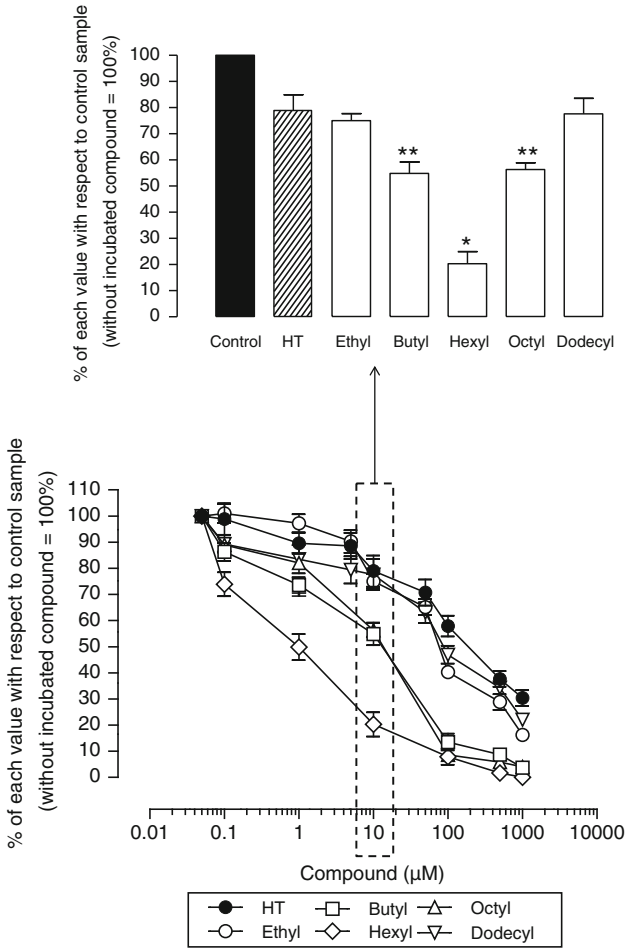


Fig. 2 Collagen-induced platelet aggregation in human whole blood after incubation with hydroxytyrosol (HT) and HT alkyl ether derivatives. The box shows data corresponding to the 10 μM concentration. Each point is the mean ± SEM of six independent experiments. *$p < 0.05$ with respect to all the groups. **$p < 0.05$ with respect to HT, ethyl and dodecyl derivatives

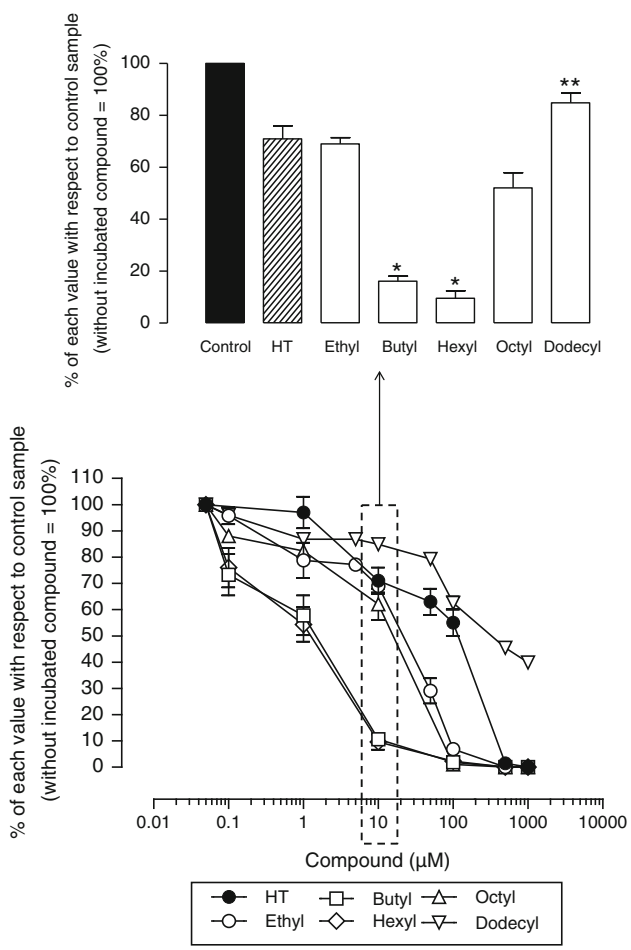


Fig. 3 Arachidonic acid-induced platelet aggregation in human whole blood after incubation with hydroxytyrosol (HT) and HT alkyl ether derivatives. The box shows data corresponding to the 10 μM concentration. Each point is the mean ± SEM of six independent experiments. *$p < 0.05$ with respect to HT, ethyl, octyl and dodecyl derivatives. **$p < 0.05$ with respect to octyl derivative

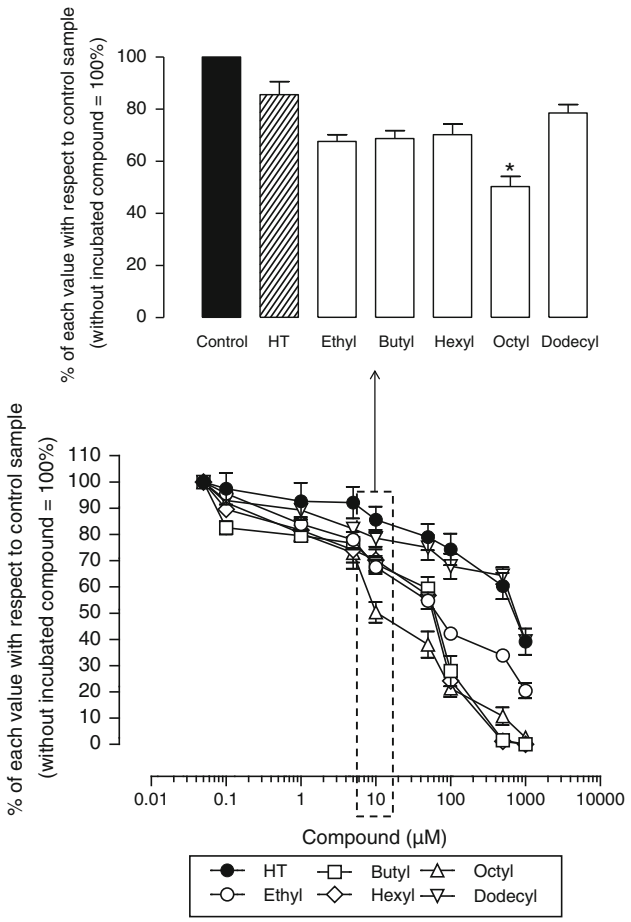


Fig. 4 Adenosine diphosphate (APD)-induced platelet aggregation in human whole blood after incubation with hydroxytyrosol (HT) and HT alkyl ether derivatives. The box shows data corresponding to the 10 μM concentration. Each point is the mean ± SEM of six independent experiments. *$p < 0.05$ with respect to all the groups

−80 °C. Prostaglandin E_2 was measured in the supernatant with a commercial enzyme immunoassay kit. In each experiment a sample of blood without LPS induction was incubated, and LPS-induced prostaglandin E_2 production was calculated as the difference between the LPS-induced samples and noninduced samples.

Nitric oxide production

Nitric oxide production was incubated with the alkyl ether derivative and then induced with the calcium ionophore A23187 (50 μM) and bacterial membrane LPS (1 μg/mL) for 18 h at 37 °C, and measured as nitrite + nitrate concentration in the supernatant of induced blood samples with an enzyme immunoassay kit. The experiments were designed to determine NO production with the drug under study or in ethanol/PBS solution (control sample) for 10 min at 37 °C. Briefly, leukocytes were incubated for 10 min at 37 °C with 200 μL buffer containing 10 mM HEPES, 1 mM EDTA, 1 mM DL-dithiothreitol (DTT), 10 μg mL^{-1} leupeptin, 4.8 mM DL-valine, 1 mM NADPH, 1 mM $MgCl_2$, 1 mM $CaCl_2$ and 20 μM L-arginine, and then with 10 μL saline or the corresponding concentration of the drug. Basal nitrite + nitrate production was first determined; after this step calcium A 23187 ionophore or LPS was added, and after 30 min (calcium-induced samples) or 18 h (LPS-induced samples) of incubation at 37 °C, the increase in nitrite + nitrate production was recorded.

Interleukin 1β production

Blood was incubated with the corresponding alkyl ether derivative and then with bacterial membrane LPS (1 μg/mL) for 18 h at 37 °C. The plates were then centrifuged, and the plasma was removed and frozen at −80 °C. Interleukin 1β was measured in the supernatant with a commercial enzyme immunoassay kit. In each experiment a sample of blood without LPS induction was incubated, and LPS-induced interleukin 1β production was calculated as the difference between the LPS-induced samples and noninduced samples.

Statistical analysis

The data in the text, tables and figures are expressed as the mean ± standard error of six experiments. The results were analyzed statistically with the SPSS personal computer program (Statistical Program for Social Sciences, v. 19.0, licensed by the Central Computer Services at the University of Malaga). Significance of the differences in each variable was determined with ANOVA analysis. For pairwise comparison of groups the Tukey's post hoc transformation was used. Differences were considered significant when $p < 0.05$.

Results

Hydroxytyrosol and HT ethers inhibited platelet aggregation induced by collagen or arachidonic acid and ADP in whole blood (Figs. 2, 3, 4). Maximum intensity of aggregation in samples induced with ADP was 22.4 ± 1.7 ohms. After induction with collagen, maximum aggregation was 20.0 ± 1.2 ohms; in blood samples induced with arachidonic acid, maximum intensity of aggregation was 24.3 ± 1.6 ohms.

There was a biphasic inhibitory effect depending on the length of the carbon chain (R in Fig. 1): an increase in the antiplatelet effect from two (ethyl) to six (hexyl) carbons, and a decrease in this effect for compounds with eight (octyl) or twelve (dodecyl) carbons. To highlight these differences, the results with the 10 μM concentration are boxed in Figs. 2, 3 and 4. Table 1 shows the concentrations of each compound that inhibited maximum platelet aggregation recorded in control samples by 50 % (IC_{50}).

Table 1 Mean values of the concentrations of each compound that inhibited 50 % (IC_{50}, μM) maximum intensity of platelet aggregation induced with 1 μg/mL collagen (Imax-COL), 400 μM arachidonic acid (Imax-AA) or 2.5 μM ADP (Imax-ADP), calcium-induced thromboxane B_2 (TxB_2) production, LPS-induced prostaglandin E_2 (PGE_2), LPS-induced interleukin 1β (IL-1β) production and LPS-induced nitric oxide (iNOS) production

HT alkyl ethers						
	HT	Ethyl	Butyl	Hexyl	Octyl	Dodecyl
Imax-COL	193 ± 25*	79.3 ± 4.1	17.5 ± 2.5	0.5 ± 0.04[a]	15.9 ± 0.2	47.7 ± 6.8
Imax-AA	190 ± 23*	30.2 ± 2.3	2.9 ± 0.5[b]	2.3 ± 0.4[b]	52.2 ± 0.5	85.0 ± 4.4
Imax-ADP	716 ± 65*	66.5 ± 9.6	70.2 ± 7.2	60.9 ± 2.5	125 ± 11[c]	317 ± 37[c]
TxB_2 (COX-1)	72.9 ± 5.2*	237 ± 48	551 ± 50	457 ± 36	342 ± 32	786 ± 93
PGE_2 (COX-2)	60.1 ± 3.2	11.4 ± 1.0[†]	6.9 ± 0.5[†]	1.9 ± 0.1[†a]	24.1 ± 3.2[†]	62.1 ± 7.1
IL-1β	77.5 ± 6.8	62.5 ± 6.4	3.1 ± 0.4[†d]	6.3 ± 0.8[†d]	>1,000	>1,000
iNOS	0.3 ± 0.02	0.3 ± 0.03	0.5 ± 0.03	1.0 ± 0.2[†a]	3.7 ± 0.5[†]	14.7 ± 0.8[†]

HT hydroxytyrosol

[a] $p < 0.05$ with respect to all the HT alkyl ether derivatives

[b] $p < 0.05$ with respect to ethyl, octyl and dodecyl derivatives

[c] $p < 0.05$ with respect to ethyl, butyl and hexyl derivatives

[d] $p < 0.05$ with respect to ethyl derivative

* $p < 0.05$ with respect to all the groups

[†] $p < 0.05$ with respect to HT group

For HT these values were significantly higher than with all the HT ethers. The greatest antiaggregation potency in whole blood was found with HT hexyl ether, except when ADP was used as the inducer. The effects on platelet aggregation were greater when collagen and arachidonic acid were used as the inducer compared to ADP.

All six compounds inhibited TxB_2 production in a concentration-dependent manner (Fig. 5; Table 1). However, the effects were most significant with concentrations up to 100 μM. Thromboxane B_2 production in control samples was 1.35 ± 0.11 nmol/10^9 platelets, and carbon chain length apparently had no influence on this effect.

Type 2 cyclooxygenase activity measured as LPS-induced prostaglandin E_2 production was inhibited after incubation with each of the HT derivatives (Fig. 6; Table 1). For a concentration of 10 μM, there was a biphasic inhibitory effect depending on carbon chain length (Fig. 6), as was also observed in the aggregometry assays.

Interleukin 1β production induced with LPS was reduced in a concentration-dependent manner after incubation with HT and HT ethyl, butyl and hexyl ethers (Fig. 7; Table 1). No significant effect was observed with HT octyl and dodecyl ethers.

The HT derivatives increased calcium-induced and reduced LPS-induced nitrite + nitrate production in whole blood in a concentration-dependent manner (Figs. 8, 9; Table 1). Nitrite + nitrate concentration in control samples was 1.3 ± 0.01 μM. There was a tendency toward a biphasic pattern in the effects of different inducers depending on carbon chain length.

Discussion

We report initial evidence that lipophilic HT ether derivatives have antiplatelet aggregating effects in human blood. Some compounds in virgin olive oil inhibit platelet function, for example, phenolic extracts from waste products of the olive oil production process [22], HT [10, 11], HT acetate [10, 11], oleuropein [23] and phenolic isochromans [24]. Feeding virgin olive oil to rats or rabbits was found to significantly reduce collagen-induced platelet aggregation [25, 26]. Our results confirm this effect of HT, but also show that HT alkyl ethers have a greater antiaggregating effect than HT in blocking platelet aggregation. It was recently reported that olive oil extract inhibited both the activation and aggregation of human platelets in vitro, and this finding led the authors to postulate a possible anti-inflammatory effect of extract concentrations equivalent to 50–250 μM of HT [22]. These data are consistent in quantitative terms with those we obtained for HT in the present study.

Some aspects of the antiplatelet effect of alkyl HT ether derivatives should be taken into consideration, that is, the potency of the antiplatelet effect, the possible mechanisms of their antiplatelet effect and the relationship between carbon chain length and potency of the antiplatelet effect. Firstly, alkyl HT ethers inhibit platelet aggregation induced with collagen, arachidonic acid and ADP. In this connection, and with the exception of HT ethyl ether (which showed a similar effect with all three aggregation inducers tested here), the effects of other HT compounds were

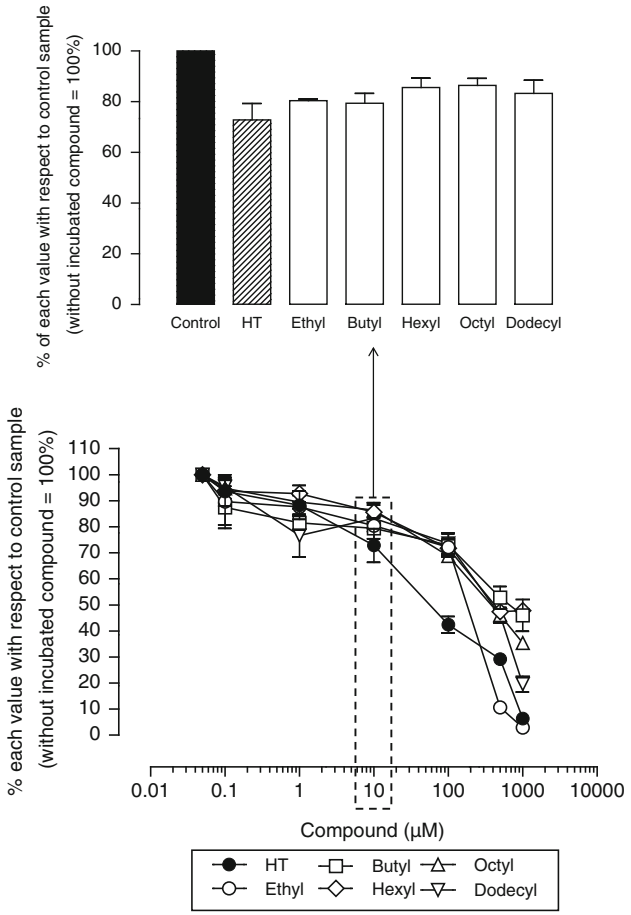


Fig. 5 Calcium-induced thromboxane B_2 production after incubation with hydroxytyrosol (HT) and HT alkyl ether derivatives. The box shows data corresponding to the 10 μM concentration. Each point is the mean ± SEM of six independent experiments

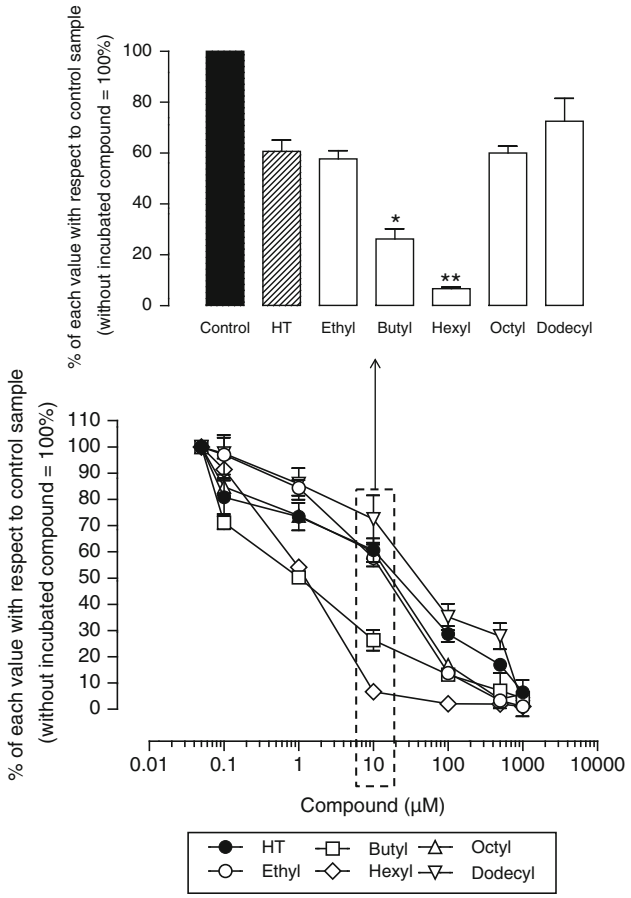


Fig. 6 Bacterial membrane LPS-induced prostaglandin E_2 production as an index of type-2 cyclooxygenase activity, after incubation with hydroxytyrosol (HT) and HT alkyl ether derivatives. The box shows data corresponding to the 10 μM concentration. Each point is the mean ± SEM of six independent experiments. $*p < 0.05$ with respect to HT, ethyl, octyl and dodecyl derivatives. $**p < 0.05$ with respect to all the groups

greater when platelet aggregation was induced with collagen or arachidonic acid. In addition, IC_{50} values were in the 10^{-7}–10^{-6} M range for HT hexyl ether, whereas for the other compounds these values were in the 10^{-5} M range. The inhibitory effects of HT on platelet aggregation are consistent with those published previously [10, 11]. Regarding other derivatives, HT acetate showed an inhibitory effect on platelet aggregation; in qualitative terms its behavior was similar to that of alkyl ether derivatives although in quantitative terms its effect was weaker, with an IC_{50} in the 10^{-5} M range [11]. These findings show that HT alkyl ether derivatives, especially the six-carbon compound, have a quantitatively greater antiplatelet effect than HT.

Secondly, the antiplatelet drugs commonly used in clinical practice to inhibit platelet activation act through two pathways: inhibiting platelet thromboxane synthesis (aspirin) or increasing cAMP levels by blocking membrane receptors for ADP (clopidogrel) [13]. The compounds analyzed in this study inhibited thromboxane synthesis, although this effect was observed with concentrations in the 10^{-4} M range, that is, a molar concentration range higher than the IC_{50} found for the antiplatelet effect. It was previously shown that HT at a concentration of 400 μM in whole blood reduced collagen- and thrombin-induced thromboxane production by approximately 75 % [23]. Interpolating the results plotted in Fig. 5 for a concentration of 400 μM of the phenolic compounds compared here shows that HT would reduce thromboxane production by 66 % and most HT alkyl ether derivatives would reduce production by 45–48 %, the exception being the 71 % reduction expected for the ethyl derivative. However, the main mechanism of action of these compounds appears not to be the inhibition of thromboxane synthesis. Instead, platelet aggregation may be inhibited by the joint actions of several mechanisms, one of which may be the stimulation of calcium-dependent (constitutive pathway) NO production, as occurs with aspirin [27]. The alkyl ether derivatives

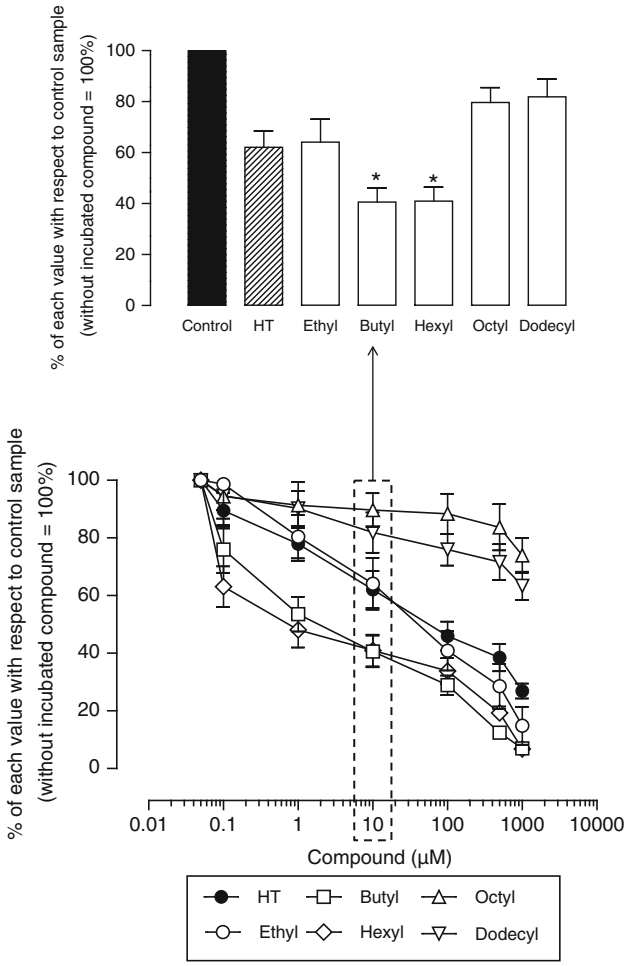


Fig. 7 Bacterial membrane LPS-induced interleukin 1β production after incubation with hydroxytyrosol (HT) and HT alkyl ether derivatives. The box shows data corresponding to the 10 μM concentration. Each point is the mean ± SEM of six independent experiments. *$p < 0.05$ with respect to HT, ethyl, octyl and dodecyl derivatives

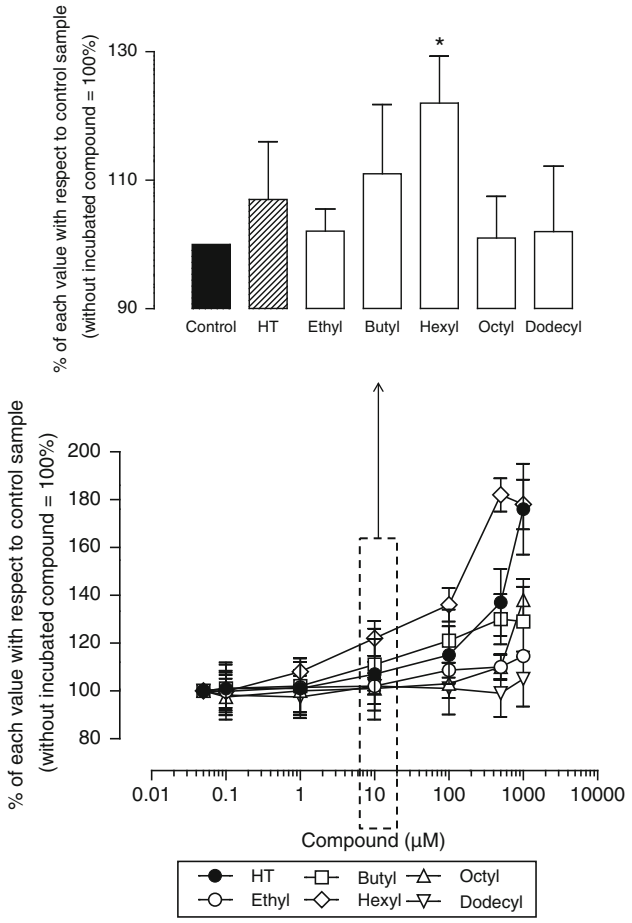


Fig. 8 Calcium-induced nitrite + nitrate production as an index of constitutive nitric oxide synthase activity, after incubation with hydroxytyrosol (HT) and HT alkyl ether derivatives. The box shows data corresponding to the 10 μM concentration. Each point is the mean ± SEM of six independent experiments. *$p < 0.05$ with respect to ethyl and octyl derivatives

increased NO production in a concentration-dependent manner, which may be one of the mechanisms of inhibition of platelet function. This behavior was previously reported for HT, HT acetate [10, 11] and other flavonoids [28].

Other targets to be taken into account in cardiovascular disease are inflammatory pathways. The inflammatory process is known to influence the genesis and evolution of vascular disease [29, 30], especially in the early stages. In addition, inflammatory mediators may facilitate the sequence of biochemical events in atherosclerosis and thrombogenesis [30]. The inhibition of these pathways by some compounds may thus be an important mechanism in preventing cardiovascular disease, since they may enhance a possible antiplatelet effect, as occurs with aspirin [29, 30]. In the present study we investigated three biochemical pathways of tissue inflammation: COX-2, inducible NO synthase (iNOS) and interleukin 1β production. These pathways were inhibited in a concentration-dependent manner by HT and HT alkyl ether derivatives (Figs. 6, 7, 8, 9; Table 1). In general, the ethyl, butyl and hexyl derivatives showed a greater effect than HT. Earlier research showed that in human whole blood samples, HT and HT acetate inhibited the production of an LPS-induced inflammatory mediator [11]. It has also been shown that HT exerts an anti-inflammatory effect by suppressing the expression of COX-2 and iNOS in human monocytes [31]. Our study provides evidence that HT alkyl ether derivatives (mainly the ethyl, butyl and hexyl derivatives) have an anti-inflammatory effect greater than that of HT. This effect may contribute significantly to their potential ability to prevent vascular disease, in addition to their documented antiplatelet effect. All these effects require that these derivatives of HT penetrate inside platelets and leukocytes, although this has not been studied a good penetration of

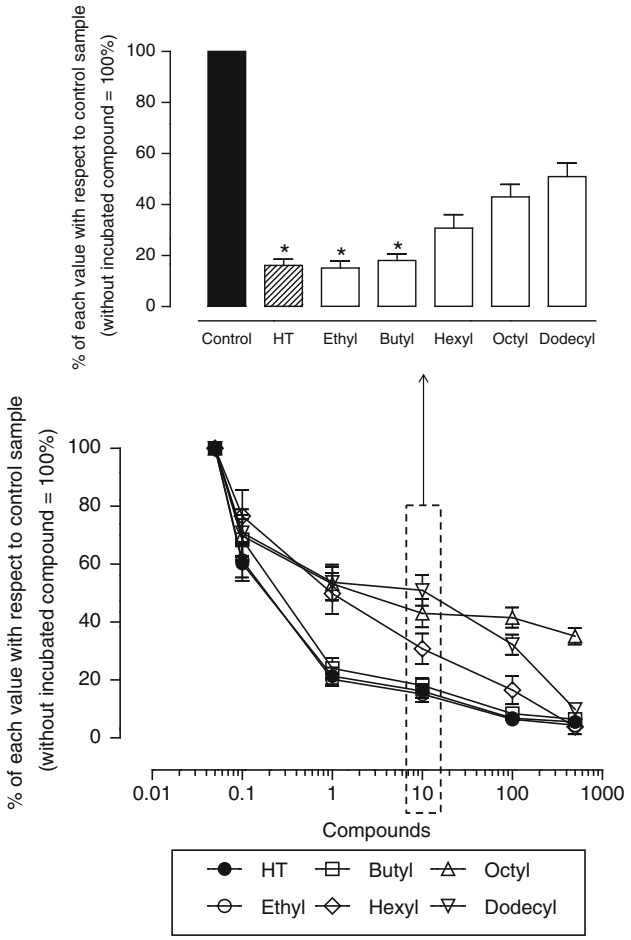


Fig. 9 Bacterial membrane LPS-induced nitrite + nitrate production as an index of inducible nitric oxide synthase activity, after incubation with hydroxytyrosol (HT) and HT alkyl ether derivatives. The box shows data corresponding to the 10 μM concentration. Each point is the mean ± SEM of six independent experiments. $*p < 0.05$ with respect to hexyl, octyl and dodecyl derivatives

these ethers through biological membranes has been demonstrated [18, 19], for that reason it could be assumed that also pass the cytoplasmic membranes of platelets and leukocytes, however, this statement requires further specific studies.

Finally, it is important to note the relevant role of the lipophilic nature of synthetic phenolic compounds in their antiplatelet and anti-inflammatory potential in human whole blood samples. Our results show that there is no direct relationship between the effects of HT alkyl ether derivatives and alkyl chain length (carbon atoms). A critical chain length for maximum lipophilicity and maximum antioxidant effect has been documented both for ether and ester derivatives [32, 33]. These earlier studies reported that medium-sized carbon chains in the range of C4–C10 were optimal for maximum effect. We found that the greatest antiplatelet and anti-inflammatory effects were obtained with the C4 and C6 derivatives. These results are consistent with those reported by Laguerre et al. [31] and Tofani et al. [32] concerning the lipophilicity and antioxidant potential of these derivatives. According to Tofani et al. [32], molecular dynamics data showed that the folded conformation and consequent shielding of the catechol moiety may partially account for the weaker effect of long-chain derivatives compared to medium-chain forms. Moreover, Laguerre et al. [31] postulated that the medium-sized derivatives might interact under more favorable conditions with target molecules in the cell to exert their effects, due to their higher lipophilicity and thus greater penetration into the cell.

Although our experiments document these in vitro effects in human whole blood samples, further studies are needed to demonstrate these benefits after oral administration of these compounds. Additional research should aim to characterize their mode of action in vivo and to determine the relationship between ether compounds and kinetic parameters that can provide evidence regarding the possible relationships between their potency of action and the carbon chain length.

In summary, our results show that HT alkyl ether derivatives exert antiplatelet and anti-inflammatory effects that are greater in quantitative terms than the effect of HT. This effect consists of a slight decrease in platelet thromboxane synthesis, an increase in constitutive NO production and inhibition of the inducible activities of cyclooxygenase and nitric oxide synthase.

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