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In vitro anti-inflammatory effects of naturally-occurring compounds from two Lauraceae plants

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ABSTRACT

The *in vitro* anti-inflammatory effects of seven known lignans and one dihydrochalcone isolated from the leaves of two Lauraceae species (*Pleurothyrium cinereum* and *Ocotea macrophylla*), were evaluated through the inhibition of COX-1, COX-2, 5-LOX and the aggregation of rabbit platelets induced by PAF, AA and ADP. (+)-de-4″-*O*-methylmagnolin **4** was found to be a potent COX-2/5-LOX dual inhibitor and PAF-antagonist (COX-2 IC_{50} 2.27 μM; 5-LOX IC_{50} 5.05 μM; PAF IC_{50} 2.51 μM). However, all compounds exhibited an activity at different levels, indicating good anti-inflammatory properties to be considered in further structural optimization studies.

Key words: COX, furofuran lignans, 5-LOX, *Ocotea macrophylla*, platelet aggregation, *Pleurothyrium cinereum*.

INTRODUCTION

Prostaglandin H_2 synthase (also named cyclooxygenase, COX), which has three isoforms, COX-1, COX-2 and COX-3 (Simmons et al. 2004), and lipoxygenase (LOX) catalyze the rate-limiting step in the formation of prostaglandins (PGs) and leukotrienes (LTs), respectively. The delineation of their distinct functions in physiological and pathological processes has promoted plenty of studies that are focused on the search for potential anti-inflammatory agents. In this way, COX-2 inhibitors were developed as a safer alternative to traditional non-steroidal anti-inflammatory drugs (NSAIDs) because there was less risk of gastrointestinal ulceration (Charlier and Michaux 2003).

Platelets, being the smallest cellular component in the blood, change shape from disc-shaped into spiny spheres, in the presence of stimuli such as arachidonic acid (AA). Activated platelets bind to fibrinogen, aggregate and release adenosine diphosphate (ADP) and serotonin, among others, contained in their intracellular granules (Rao 1993). Thromboxane A_2 (TxA_2), an AA metabolite, ADP and platelet activating factor (PAF) intensify the extent of platelet aggregation as an autocatalytic process rather than promote an equilibrium response (Armstrong 1996). Under pathophysiological conditions, platelet activation may result in peripheral, cardiovascular or cerebrovascular thrombosis with severe consequences. The relationship between platelet aggregation and acute inflammation had been previously discussed (Prescott et al. 2002), as well as the possible risk of suffering cardiovascular problems (myocardial infarction and thromboembolic events) by using COX-2-specific inhibitors, since the healthy balance between COX-2-mediated prostacyclin and COX-1-dependent thromboxane is disturbed (Konstam et al. 2001). Therefore, the search for COX inhibitory compounds with antiplatelet effects, which possess a different mechanism from that of NSAIDS, appeared to be a relevant way to provide protection against harmful cardiovascular events in treating inflammatory problems (Bombardier et al. 2000).

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Several Lauraceae plants have shown anti-inflammatory (Lin et al. 2007, da Silva Filho et al. 2004) and anti-platelet (Ballabeni et al. 2007) properties, as well as furofuran and aryltetralin lignans isolated from other families (Kim et al. 2009, Kim and Yun-Choi 2007, Chen et al. 1996). Furthermore, relevant results were obtained in pharmacological studies for the furofuran lignan yangambin, isolated from the leaves of *Ocotea duckei* (Lauraceae) (Morais et al. 1999), which exhibited excellent cardiovascular properties (Tibiriçá 2001), and was found to differentiate putative PAF receptor subtypes in the gastrointestinal tract of rats (Jesus-Morais et al. 2000). Thus, as part of our research on the biological activity of Lauraceae compounds, this paper reports the in vitro evaluation of the anti-inflammatory and anti-platelet effects of three 1-aryltetralin lignans [(+)-otobaphenol **1**, 4-hydroxy-3-methoxy-3′,4′-methylenedioxy-$\Delta^{7,8,7',8'}$-6.7′,8.8′-lignan **2**, (8*R*)-3,4-dimethoxy-3′,4′-methylenedioxy-$\Delta^{7',8'}$-6.7′,8.8′-lignan **3**], two furofuran lignans ((+)-de-4″-*O*-methylmagnolin **4**, (+)-demethylpiperitol **5**), and a dihydrochalcone (dihydroflavokawin B **6**) previously isolated from *Pleurothyrium cinereum* van der Werff (Coy and Cuca 2008a, b). These properties were assessed through inhibition of COX-1, COX-2, 5-LOX and aggregation of rabbit platelets induced by PAF, AA and ADP, respectively. In addition, two diastereomeric dibenzylbutane lignans (*meso*-3,4,5,3′,4′,5′-hexamethoxy-8.8′-lignan **7** and *threo*-3,4,5,3′,4′,5′-hexamethoxy-8.8′-lignan **8**) were isolated from the leaves of *Ocotea macrophylla* Kunth, which were identified on the basis of spectroscopic analyses (^{1}H, ^{13}C NMR, 2D NMR, HRMS) (Fig. 1).

MATERIALS AND METHODS

General Experimental Procedures

Melting points were determined on a Fisher-Johns melting point apparatus without correction. Ultraviolet (UV) spectra and colorimetric measurements were performed on a Cary 50 Scan (Varian instruments) spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer, using deuterated chloroform ($CDCl_3$) as solvent, and tetramethylsilane (TMS) as internal shift reference. High resolution mass spectra (HRMS) were determined on a micromass quadrupole-time of flight (qToF) (Waters Inc.) [with an electrospray ionization (ESI) source and in the positive ion mode] mass spectrometer. Platelet aggregation was measured on a Chrono-Log (Chrono-Log Corporation) optical aggregometer.

Plant Material, Extraction and Isolation

Whole plants of *P. cinereum* van der Werff and *O. macrophylla* Kunth were collected in November 2005 in the Awá indigenous reservation in Alto Albí, Tumaco county, department of Nariño, Colombia, and in June 2006 by the side of Nocaima-Vergara road in Nocaima county, department of Cundinamarca, Colombia, respectively. Voucher specimens (numbers COL518334 for *P. cinereum* and COL517191 for *O. macrophylla*, which were identified by the botanist Adolfo Jara Muñoz) were deposited at Herbario Nacional Colombiano, Universidad Nacional de Colombia. Detailed extraction and isolation processes for compounds **1-8** have been previously reported (Coy and Cuca, 2008a, b, Coy et al. 2010).

Cells and Reagents

COX-(ovine) inhibitor screening and 5-LOX-(potato) inhibitor screening kits were purchased from Cayman Chemical Company, Ann Arbor, MI, USA (Catalogs No. 560101 and No. 760700, respectively). AA, PAF, ADP and ginkgolide B were acquired from Sigma-Aldrich. Platelet-rich plasma (PRP) was obtained from blood collected from the central ear artery of female White New Zealand rabbits with a body weight between 3 and 4 kg.

COX-1, COX-2, and 5-LOX Inhibition Assays

The ability of the test compounds listed in Table I to inhibit ovine COX-1, COX-2, and potato 5-LOX were determined using enzyme immunoassay (EIA) kits, according to the reported methods (Rao et al. 2003, Chowdhury et al. 2008).

Platelet Aggregation Induced by PAF, AA and ADP

Anti-platelet activity was carried out according to the reported method (Koch 2005), which was approved by

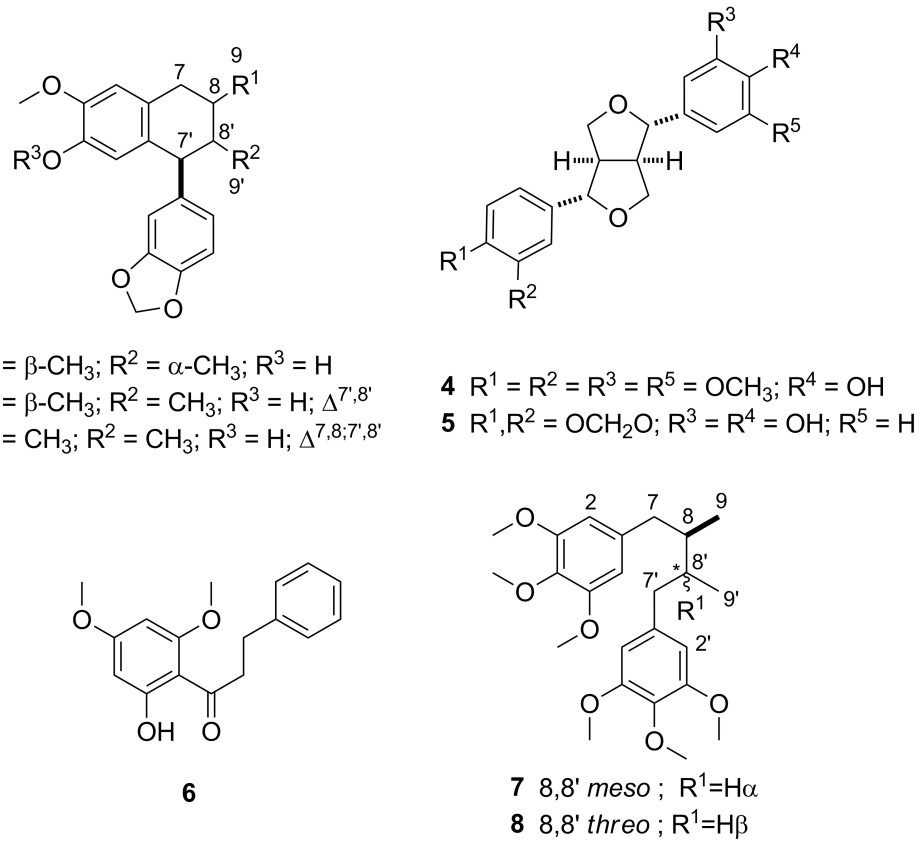

Fig. 1 – Structures of compounds **1-8**.

TABLE I
COX-1, COX-2 and 5-LOX enzyme inhibition results of compounds *1-8*.

Compound	IC_{50} (μM)[a]		
	COX-1	COX-2	5-LOX
1	479	49.6	8.39
2	368	65.2	9.45
3	388	80.1	116
4	35.6	2.27	5.05
5	24.6	22.2	4.22
6	1.22	457	127
7	322	16.3	46.4
8	432	15.6	78.2
celecoxib	8.32	0.0512	11.3
aspirin	0.411	2.53	–
caffeic acid	–	–	3.73

[a]Values are means of two experiments, standard deviation from the mean is <10% of the mean value.

the ethics committee at Universidad Nacional de Colombia. Briefly, convenient PRP was stirred at 800 rpm and maintained at 37°C. Samples of PRP were preincubated for 5 min at 37°C with test compounds (selected concentrations according to the previous work of Koch 2005) in dimethyl sulfoxide (DMSO). Aggregation was induced by the addition of 10 μL diluted PAF, AA or ADP, according to the test. The final concentration was 7.20 nM for PAF, 100 μM for AA, and 4.00 μM for ADP on PRP. In order to eliminate the effect of the solvent on the aggregation, the final concentration of DMSO was fixed at 0.5%, which did not affect the aggregation measurement. The inhibition of platelet aggregation *versus* a solvent control was calculated as percentage.

STATISTICS

Half-maximal inhibition concentrations (IC_{50}, μM) were determined by a non-linear regression analysis using GraphPad prism 5.00 (GraphPad software, San Diego, CA, USA).

TABLE II
Inhibitory effects of compounds *1-8* on the aggregation of rabbit platelets induced by PAF, AA and ADP.

Compound	IC_{50} (μM)[a]		
	PAF (7.20 nM)	AA (100 μM)	ADP (4.00 μM)
1	846 ± 67	645 ± 33	>999
2	140 ± 13	156 ± 21	>999
3	24.8 ± 2.3	28.6 ± 2.5	77.6 ± 4.3
4	2.51 ± 0.16	73.6 ± 3.1	10.3 ± 1.8
5	3.84 ± 0.45	74.7 ± 2.8	15.6 ± 1.4
6	767 ± 35	43.2 ± 2.6	678 ± 44
7	38.9 ± 3.2	147 ± 23	565 ± 37
8	50.3 ± 3.7	168 ± 34	478 ± 41
aspirin	10.3 ± 1.8	5.33 ± 0.45	545 ± 36
ginkgolide B	0.925 ± 0.097	75.6 ± 1.0	>999

[a]The data were expressed as means 95% confidence intervals of 4 rabbits.

RESULTS AND DISCUSSION

The *in vitro* abilities of the compounds **1-8** to inhibit the isozymes COX-1 and COX-2 (which appeared to be dose-dependent) were determined in the COX-catalyzed transformation of AA into PGH_2, which was then reduced to $PGF_{2\alpha}$ and detected by the enzyme immunoassay (EIA), following the reported methodology (Rao et al. 2003), whose results are shown in Table I. Whereas lignans **1**, **4** and **7-8** showed an activity against COX-2 (IC_{50} 7.21-49.6 μM range), the dihydrochalcone 6 displayed a very low activity. However, **6** was the most potent COX-1 inhibitor (IC_{50} 1.22 μM) among test compounds, being three times less active than the control. Aryltetralin (**1-3**) and dibenzylbutane (**7-8**) lignans exhibited selective inhibition against COX-2, and the dibenzylbutane lignans were significantly more active than the aryltetralins, and *threo*-isomer **8** was a better inhibitor than the *meso*-isomer **7**. The furofuran lignans (**3-5**) inhibited the action of both COX isozymes with lower selectivity, although **4** was almost fifteen times more selective than **5**.

The *in vitro* inhibitory effect of compounds **1-8** against 5-LOX was determined in the lipoxygenation reaction using a purified lipoxygenase to transform AA into hydroperoxides, which are detected and measured by the addition of a chromogen, following the reported methodology (Chowdhury et al. 2008). As expected, compounds having a free phenolic OH group (**1-2**, **4-5**) inhibited 5-LOX (IC_{50} 4.22-18.2 μM range), **5** being the most potent 5-LOX inhibitor that had a comparable activity (IC_{50} 4.22 μM) to that of the positive control that was used (caffeic acid, IC_{50} 3.73 μM). Compounds **3** and **6-8** exhibited weak 5-LOX inhibitory effect (IC_{50} 46.4 to 127 μM). Regarding the diastereomeric lignans **7-8**, the *meso*-isomer **7** was displayed a lower IC_{50} value than its *threo*-isomer **8**, in contrast to their COX-inhibition. By comparing the COX and 5-LOX inhibition results, **4** appeared to be the best candidate as a COX-2/5-LOX dual inhibitor, which is a current matter of interest in the development of anti-inflammatory agents (Chowdhury et al. 2008, Charlier and Michaux 2003).

In addition, the capability to inhibit the aggregation of rabbit platelets induced by PAF, AA and ADP was evaluated *in vitro*, according to the reported method (Koch 2005), whose results are shown in Table II. Compounds **1-3** showed no significant antiplatelet effect in the test. Compound 6 had the highest IC_{50} value for AA-induced platelet aggregation, indicating that the antiplatelet activity is likely a consequence of COX-1 inhibition. Lignans **4-5** were found to be the most active compounds, since they inhibited the platelet aggregation induced by the three agonists, showing important selectivity for PAF-induced aggregation. The diastereomeric dibenzylbutane lignans **7-8** also displayed selectivity towards PAF-inhibition, the *threo*-isomer being more active than the *meso*-isomer. All compounds showed lower activity than the positive controls; how-

ever, compound **5** presented a valuable pharmacological profile, because of its COX-2/5-LOX dual inhibition and antiplatelet activities. Recently, a very closely related compound isolated from *Magnolia fargesii* (Magnoliaceae) was found to be a good inhibitor of the production of nitric oxide (NO) and PGE_2, as well as the expression of inducible nitric oxide synthase (iNOS) and COX-2, respectively, through the suppression of IκBα degradation and NFκB activation (Kim et al. 2009).

In conclusion, these results imply that aryltetralin and furofuran lignans might be excellent candidates for further studies aiming for structural optimization to improve their activity, and delineate their anti-inflammatory effects. Our study clearly demonstrated that these lignans have good anti-inflammatory properties that are in agreement with the literature (Lin et al. 2007, da Silva Filho et al. 2004, Ballabeni et al. 2007, Kim et al. 2009, Kim and Yun-Choi 2007, Chen et al. 1996, Morais et al. 1999, Jesus-Morais et al. 2000). However, further structure-activity studies and biological analyses are required to clarify the underlying mechanism and to draw unambiguous conclusions regarding the antiinflammatory potential of aryltetralin and furofuran lignans.

LIST OF ABBREVIATIONS

^{13}C NMR	carbon nuclear magnetic resonance
^{1}H NMR	hydrogen nuclear magnetic resonance
2D NMR	two-dimensional nuclear magnetic resonance
AA	arachidonic acid
ADP	adenosine diphosphate
CC	column chromatography
$CDCl_3$	deuterated chloroform
$CHCl_3$	chloroform
COX	cyclooxigenase
DMSO	dimethyl sulfoxide
EIA	enzyme immunoassay
ESI	electrospray ionization
Et_2O	diethyl ether
EtOAc	ethyl acetate
HRMS	high resolution mass spectrometry
IC_{50}	half-maximal inhibition concentrations (in μM)
iNOS	inducible nitric oxide synthase
LOX	lipoxygenase
LTs	leukotrienes
m/z	mass/charge relationship
MeCN	acetonitrile
MeOH	methanol
NSAIDs	nonsteroidal anti-inflammatory drugs (NSAIDs)
PAF	platelet activating factor
PGHS	prostaglandin H_2 synthase
PGs	prostaglandins
PRP	platelet-rich plasma
qToF	quadrupole time of flight
TMS	tetramethylsilane
TxA_2	thromboxane
UV	ultraviolet

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RESUMO

Os efeitos anti-inflamatórios *in vitro* de sete conhecidos lignanos e uma dihidrocalcona isolados das folhas de duas espécies da família Lauraceae (*Pleurothyrium cinereum* e *Ocotea macrophylla*) foram avaliados por meio da inibição da COX1, COX-2, 5-LOX e agregação de plaquetas de coelhos induzida por PAF, AA e ADP. A (+)-4″-*O*-metilmagnolina-4 foi encontrada como mais potente inibidora tanto da COX-2 quanto de 5-LOX e antagonista de PAF (COX-2 IC_{50} 2,27 μM; 5-LOX IC_{50} 5,05 μM; PAF IC_{50} 2,51 μM). Entretanto, todos compostos mostram uma atividade em intensidades diferentes, indicando boas propriedades anti-inflamátorias a serem consideradas para futuros estudos de modificações e otimização estruturais.

Palavras-chave: COX, lignanos furofurânicos, 5-LOX, *Ocotea macrophylla*, agregação plaquetária, *Pleurothyrium cinereum*.

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