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Biological Evaluation of Several Coumarin Derivatives Designed as Possible Anti-inflammatory/Antioxidant Agents*

CHRISTOS KONTOGIORGIS and DIMITRA HADJIPAVLOU-LITINA†

Department of Pharmaceutical Chemistry, School of Pharmacy, Aristotelian University of Thessaloniki, Thessaloniki 54124, Greece

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Several linear and angular coumarins designed and synthesised as possible anti-inflammatory and antioxidant agents were evaluated for their biological activities, using the carrageenin-induced rat paw oedema model. In general, the compounds were found to be potent anti-inflammatory agents. Compound (4) was found to possess protective properties in adjuvant-induced arthritis in rats. The compounds were found to interact with 1,1-diphenyl-2-picryl-hydrazyl stable free radical (DPPH) whereas most of them were essentially inactive in other tests. The anti-inflammatory activity seemed to be connected with their reducing activity. R_M values were determined as an expression of their lipophilicity which was also calculated as clog *P*. Only a poor relationship existed between lipophilicity and anti-inflammatory activity.

Keywords: Carrageenin paw oedema; Anti-inflammatories; Antioxidants; Arthritis; Coumarins

INTRODUCTION

Coumarin is a naturally occuring constituent of many plants and essential oils. Pharmacologically it is classified as a flavonoid along with a range of other compounds.[1] Flavonoids have widespread biological activities, affecting many mammalian cell functions, including inhibition of mitochondrial enzyme systems.[2] They have a reputation for anti-inflammatory, antiallergic and antihemorrhagic activities.[3] Several medicinal plant derived coumarins or synthetic coumarins affect numerous enzymes and are able to modulate the functional activity of several types of cells. Their anti-inflammatory activity has been studied extensively in several *in vitro* and *in vivo* models. Various stages of inflammation are affected by the compounds including the formation of granulose tissue, chronic arthritis and, in the early stages, increased permeability of capillaries. Coumarins have also been found to inhibit lipid peroxidation, to scavenge hydroxyl radicals, superoxide radicals, hypochlorous acid[4] soybean lipoxygenase/or 5-lipoxygenase[5] and to possess anti-inflammatory and anticoagulant abilities.[6]

Recently the synthesis of several coumarin derivatives with significant anti-inflammatory and antioxidant activity have been reported.[7–9] As a part of an effort to design improved anti-inflammatory/antioxidants drugs, the biological evaluation of several synthesised novel coumarins has been undertaken. The structural modifications of the novel synthesised compounds involve the (a) angular or linear structure of the coumarins, (b) the reversal of functions in the structure, (c) the nature of the heterocyclic ring condensed in positions 7,8 and (d) the nature of the 6-, 7- and 8-substituents.

Non-steroidal anti-inflammatory drugs have a broad spectrum of effects and it has been suggested that the variations in both efficacy and their tolerability are partly due to differences in their physicochemical properties which determine their distribution in the body and their ability to pass through and to enter the interior of membranes.[10]

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†Corresponding author. Tel.: +30310-997627. Fax: +30310-997679. E-mail: hadjipav@pharm.auth.gr

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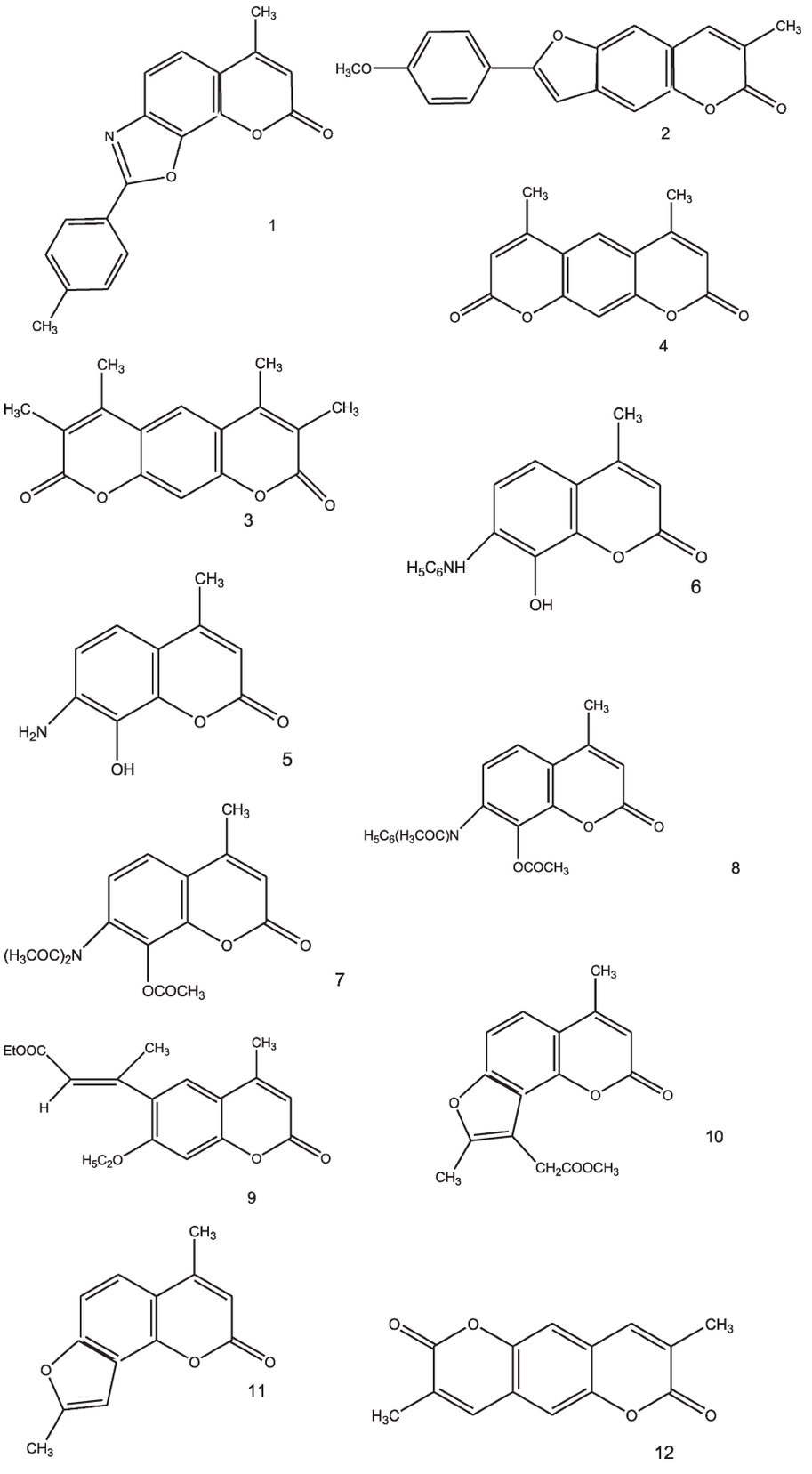

FIGURE 1 Structures of the tested compounds.

Thus, partition coefficients such R_M values were also performed here and compared with the corresponding theoretically calculated values for n-octanol-water.

MATERIALS AND METHODS

All reagents were obtained from commercial sources and were of analytical grade. The compounds to be tested were kindly provided by Professor Nicolaides and his colleagues[11–16] (Figure 1).

Spectra were recorded with a Perkin–Elmer UV–Vis 554 spectrophotometer (Perkin–Elmer Corporation Ltd., Lane Beaconsfield, Bucks, England).

All the chemicals used were of analytical grade and commercially available from Merck. Trypsin (pancreasprotease) 200Fip U/g, salicylic acid (SA), acetylsalicylic acid (ASA), *N*-tosyl-methyl-arginine ester (TAME) 1,1-diphenyl-2-picrylhydrazyl (DPPH), nordihydroguairetic acid (NDGA) were purchased from the Aldrich Chemical Co. (Milwaukee, WI, USA). Soybean Lipoxygenase, linoleic acid sodium salt and indomethacin were obtained from Sigma Chemical, Co. (St. Louis, MO, USA) and carrageenin, type K, was commercially available. Freund's adjuvant referred to 0.6 mg desiccated *Mycobacterium butyricum* suspended in 0.1 mL liquid parafin.

Physicochemical Studies

Reversed phase TLC (RPTLC) was performed on silica gel plates impregnated with 55% (v/v) liquid paraffin in light petroleum ether[17] with methanol/water mixture (70/30, v/v) as mobile phase. The plates were developed in closed chromatography tanks saturated with the mobile phase at 24°C. Spots were detected under UV light or by iodine vapour. R_M values (a lipophilicity index) were determined from the corresponding R_f values (from ten individual measurements) using the equation $R_M = \log[(1/R_f) - 1]$ (See Table I).

Biological Assays

In Vivo Studies

Inhibition of Carrageenin-induced Oedema[7]

Oedema was induced in the right hind paw of Fisher 344 rats (150–200 g) by the intradermal injection of 0.1 mL 2% (w/v) carrageenin in water. Both sexes were used but pregnant females were excluded. The animals, bred in our laboratory, were housed under standard conditions and received a diet of commercial food pellets and water *ad libitum*. During the experiment period food but not water was withheld. These studies were in accordance with recognized guidelines on animal experimentation (Guidelines for the Care and Use of Laboratory Animals published by the Greek Government 160/1991, based on EU regulations 86/609).

TABLE I % Inhibition of the carrageenin induced paw oedema (% CPE in 0.1 mmol/Kg). Experimentally determined (R_M) and theoretically calculated values (clog *P*) of lipophilicity

Compounds	% CPE	R_M	Clog *P* [30]
1	56.3	−0.136	3.86
2	36	−0.271	4.49
3	ND	0.0762	2.58
4	63.5	−0.0506	1.68
5	ND	ND	1.33
6	ND	ND	3.68
7	ND	ND	3.73
8	ND	ND	4.76
9	23.7	0.618	4.07
10	ND	ND	2.21
11	ND	0.588	2.97
12	48.5	ND	1.68
Coumarin	23.7	ND	1.41
Indomethacin	57	ND	4.18

ND = not determined.

The tested compounds, 0.1 mmol/Kg body weight, were suspended in water with a few drops of Tween 80 and ground in a mortar before use and were given intraperitoneally (i.p) at the same time as the carrageenin. The rats were euthanized 3.5 h after carrageenin injection. The experiment was repeated twice for each compound (two groups of 6 animals). The difference between the weight of the injected and uninjected paws was calculated for each animal. The change in paw weight was compared with that in control animals (injected with water) and expressed as a percentage inhibition of the oedema (CPE% values Table I). Indomethacin in 0.1 mmol/Kg (57% inhibition) was administered as a standard comparator drug. Values for CPE% are the mean from two different experiments with a standard error of the mean less than 10%. Statistical studies used student's *T*-test ($p < 0.01$ compared with control values).

Induction of Adjuvant Induced Disease (AID)

Groups of 6 animals were used. Rats were divided into 3 groups: groups 1 and 2 were injected[18] with Freund's adjuvant (FA) intradermally, into the base of the tails of the animals and were treated with compound (**4**), which was found to be the most active in the carrageenin paw oedema test. Compound (**4**) was injected i.p in a dose of 0.001 mmol/Kg from day zero once every other day for the following 24 days. Group 3 used as an absolute control, was injected with liquid vehicle only. Adjuvant arthritis was developed ca 14 days post FA administration. Arthritic score was measured every 2 days from the comencement (14th day). For quantification of arthritis (arthritic score), a single point was assigned for each

TABLE II Assessment of the preventive action of (**4**) on the Adjuvant Induced Disease (AID) manifestations (body weight change, inflammation-arthritic score, zoxazolamine paralysis)

Examined parameters (mean ± SD)	AID rats treated with (**4**)	AID rats-controls treated only with the liquid vehicle	Absolute-controls, normal animals, treated with the liquid vehicle only
Percent change of body weight (g ± SD)	−10.5 ± 0.2	−14.8 ± 4.5	18 ± 4.4
Inflammation-(arthritic scores)*	2.25	8.80	No arthritic scores are observed
Zoxazolamine paralysis (minutes ± SD)	232.5 ± 19.6	390.0 ± 8.6	127.5 ± 5.3

* Counted on the 24th day after FA injection.

inflammed wrist or ankle area and an additional point was given for each involved phalangeal joint, up to a maximum of 5 points per extremity (Table II). On the 24th day and at least 12 h after the last injection, animals were administered zoxazolamine i.p 10 mg/1mL/gr, as an aqueous suspension with a few drops of Tween 80 and the duration of the paralysis was recorded. The body weight, the duration of zoxazolamine paralysis and inflammation were assessed on the 24th day (Tables II and III). The experiment was conducted in duplicate.

In Vitro Studies

In the *in vitro* assays each experiment was performed at least in triplicate and the standard deviation of absorbance was less than 10% of the mean.

Effects of the Test Compounds on the Fe^{2+}-ion-stimulated Peroxidation of Linoleic Acid[19]

Linoleic acid sodium salt (90 mg) was dissolved and diluted to 50 mL with 0.2 M phosphate buffer pH 7.4 and oxygen (100%) bubbled through the solution. 2 mL aliquots of the linoleic acid sodium solution were incubated for 2 h at 37°C with 2.5 mM Fe^{2+} ($FeSO_4$) and 1 mM of the test compounds. The amount of peroxidation which occurred during this time was measured by the 2-thiobarbituric acid (TBA) method. The amount of TBA-reactive material in each sample was determined by measuring the absorbance of the aqueous layer at 535 nm. The inhibition of Fe^{2+}-stimulated oxidation of linoleic acid caused by each compound is the mean value for three to five experiments (Table IV).

TABLE III Effect of compound (**4**) on the onset (day of appearance after Adjuvant's injection, FA) and severity of arthritis (arthritic scores) in Adjuvant Induced Disease (AID) rats

Days after FA's injection	AID rats treated*	AID rats controls*
0	0	0
2	0	0
4	0	0
6	0	0
8	0	0
10	0	0
12	0	0
14	0.66	6.60
16	1.08	8.10
18	2.80	11.80
20	2.50	14.00
22	2.25	10.80
24	2.10	8.80

* Number of arthritic scores (SD less than 10%); phenylbutazone 80 mg/Kg *p.o* gave arthritic score of 0.3 (for controls the arthritic score was 9.0) on the 24th day.

Competition of the Test Compounds With Dimethyl Sulfoxide (DMSO) for Hydroxyl Radicals[20]

The hydroxyl radicals generated by the Fe^{3+}/ascorbic acid system, were detected according to the method of Nash (1953), by the determination of formaldehyde produced from the oxidation of dimethyl sulfoxide. The reaction mixture contained EDTA (0.1 mM), Fe^{3+} (167 μM), DMSO (33 mM) in phosphate buffer (50 mM, pH 7.4), the test compound and 150 μL of ascorbic acid (10 mM in phosphate buffer) added finally to start the reaction). After 30 min of incubation (37°C) the reaction was stopped with CCl_3COOH (17%, w/v) and the formaldehyde formed was detected spectrophotometrically at 412 nm (Table IV).

TABLE IV (1) Effects of the examined compounds and reference drugs on the mediated oxidation of dimethyl sulfoxide (33 mM) HO% *in vitro*; (2) % inhibition of soybean Lipoxygenase *in vitro* (% LOX); (3) % inhibition of Fe^{2+}-stimulated oxidation of linoleic acid (% Lin.); (4) % Interaction of the examined compounds and reference drugs with the stable free radical DPPH

Compound	% HO·	% LOX	% Lin	% DPPH
1	NR	No	NT	89.5
2	NR	22.6	NR	30
3	NR	ND	80.4	23
4	ND	ND	ND	94
5	ND	ND	ND	70.9
6	ND	ND	ND	77.4
7	ND	ND	ND	50.3
8	NR	16.1	ND	18.2
9	45	NR	36	42.2
10	18.7	NR	NR	11.4
11	53.6	NR	NR	22.4
12	46.4	17.3	NR	5.5
Coumarin	NR	36	1.2	ND
ASA	ND	ND	ND	80.6
NDGA	ND	ND	83.7	ND

ND = not determined; NR = no result under experimental conditions; ASA, acetylsalicylic acid; NDGA, nordihydroguaeretic acid.

Interaction of the Test Compounds With 1,1-diphenyl-2-picryl-hydrazyl (DPPH) Stable Free Radical[21]

To a solution of DPPH (0.1 mM) in absolute ethanol an equal volume of the test compound dissolved in ethanol was added. As control solution, ethanol was used. After 20 min at room temperature the absorbance was recorded at 517 nm. Acetylsalicylic acid was used as an appropriate standard possessed 80.6% activity at 0.1 mM (Table IV).

Scavenging Activity of Superoxide Anion Radical[8]

The superoxide anion was generated by the xanthine–xanthine oxidase system and measured by the nitroblue tetrazolium (NBT) method. To the reaction mixture in phosphate buffer 7.4 (0.1 mol/L) containing xanthine, NBT and test compound (1 mmol/L final concentration) xanthine oxidase (0.07 U/mL) was added. After incubating for 10 min at room temperature the absorbance was recorded at 560 nm (Table IV).

Inhibition of β-glucuronidase[22]

Compounds in acetate buffer (0.1 M, pH 7.4) were tested against β-glucuronidase (0.1 mL of 1 U/mL) using 2.5 mM *p*-nitrophenyl-β-D-glucopyranosiduronic acid. After incubation at 37°C for 30 min, 2 mL of 0.5 N NaOH solution was added to the mixture and the absorbance of the mixture was measured at 410 nm (Table IV).

Inhibition of Proteolysis[21]

Tosyl arginine methyl ester (TAME) was used as substrate for trypsin. The reaction mixture consisted of 1.5 mL buffer (0.1 M tris–HCl, pH 7.8 in 50% methanol, v/v) and 1.4 mL TAME (0.01 M in 50%, v/v methanol). The test compounds (0.1 mM) dissolved in 50% methanol were added. The reaction was started by addition of 0.1 mL trypsin (1 mg/mL 0.001 N HCl). The increase in the absorbance at 256 nm was determined over the next 4 min (Table IV).

Soybean Lipoxygenase Inhibition[21]

The test compounds dissolved in 60% aqueous ethanol (final concentration 0.1 mM), were incubated at room temperature with sodium linoleate (0.1 mM) and 0.15 ml of enzyme solution ($1/10^4$, w/v in saline). The conversion of sodium linoleate to 13-hydroperoxylinoleic acid at 234 nm was recorded and compared with an appropriate standard inhibitor (nordihydroguaretic acid 0.1 mM, 83.7% inhibition) (Table IV).

RESULTS AND DISCUSSION

Biological Studies

The *in vivo* anti-inflammatory effects of the tested coumarins, presented in Table I, were assessed by using the functional model of carrageenin-induced rat paw oedema as percentage of weight increase in the right hind paw in comparison to the uninjected left hind paw. Carrageenin-induced oedema is a non-specific inflammation resulting from a complex of diverse mediators.[23] Since oedemas of this type are highly sensitive to non-steroidal anti-inflammatory drugs (NSAIDs), carrageenin has been accepted as a useful agent for studying new anti-inflammatory drugs.[24] This model reliably predicts the anti-inflammatory efficacy of the NSAIDs and during the second phase it detects compounds that are anti-inflammatory agents as a result of inhibition of prostaglandin amplification.[25] As shown in Table I, all the investigated compounds induced protection against carrageenin-induced paw oedema. The protection ranged from 23.7–63.5% while the reference drug, indomethacin, induced 57% protection at an equivalent concentration. Compound (**4**) was the most potent (63.5%) whereas compound (**9**) had the lowest effect (23.7%).

Adjuvant induced disease (AID) is a good experimental model of rheumatoid arthritis and is often used for testing agents for anti-inflammatory activity. Rats treated with compound (**4**) did not develop arthritis indicating that this coumarin derivative exhibited possible immunomodulating activity, an activity to be further confirmed. The time course of adjuvant arthritis development expressed as arthritic score is shown in Table III. Arthritic score, body weight loss and *in vivo* metabolism impairment (expressed as the duration of the induced paralysis) and cachexia were significantly reduced in the experiment (Table II). Compound (**4**) has been found to restore liver disfunction of arthritic rats and to influence their body and liver weight (Table II). The percent change of body weight for compound (**4**) was −10.5%. In the same experimental protocol phenylbutazone (80 mg/Kg *p.o*) gave an arthritic score of 0.3 (for controls, the arthritic score was 9.0) on the 24th day after the administration of FA.[26] The duration of zoxazolamine-induced paralysis was reduced to normal levels. Although, *N*-alkyl-7-aminocoumarins are substrates of cytochrome P_{450}-isozymes,[27] the present data require further investigation regarding the mechanism of action, in order to delineate that the hepatoprotective effect of compound (**4**) during inflammation could be mediated via the ability of compound to induce drug metabolizing enzymes.

We determined the inhibition of Fe^{2+}-stimulated oxidation of linoleic acid by compounds (**1**–**12**) in

order to find out if the test compounds act as antioxidants in a non biological system (Table IV). Only compounds (**3**) and (**9**) as substrates inhibited this type of lipid peroxidation (36–80.4%). Compound (**3**) showed the highest inhibition whereas compound (**9**) showed inhibition equipotent to coumarin. No attempt was made to find the concentration of substrate-compound which produces maximal inhibition.

All compounds were found to interact with the stable free radical DPPH (Table IV). This interaction indicates their radical scavenging ability in an iron-free system. Compounds (**8**), (**10**) and (**12**) were found to have very low activity, whereas compounds (**11**) and (**4**) showed the highest interactions (89.5–94%). Compounds (**2**), (**3**) and (**11**) produced approximately 25% interaction whereas (**5**) and (**6**) an average of 79.1%. Compounds (**7**) and (**9**) were found to interact with DPPH satisfactorily (42.2–50.3%). In general, this interaction expresses the reducing activity of the test compounds and indicates their ability to scavenge free radicals. The inhibitory action on carrageenin induced rat paw oedema of coumarins (**1**), (**2**) and (**4**) is correlated to the reducing ability (as interaction with DPPH). For compound (**12**), a correlation exists between the $HO^{\bullet}$ scavenging ability and the carrageenin inhibitory activity.

During the inflammatory process, phagocytes generate the superoxide anion radical at the inflammed site and this is connected to other oxidizing species such as $HO^{\bullet}$. Hydroxyl radicals are produced by reactions which depend on transition metals, particularly iron.[28] Thus, superoxide anion radical production and the $HO^{\bullet}$ scavenging abilities of these coumarins, were tested. The superoxide anion radical was measured by the reduction of NBT to formazan. The assay was also adapted to assess the ability of antioxidants to react with $O_2^{\cdot-}$. However, none of the coumarins, tested at a final concentration of 1 mM, showed any significant ability to scavenge $O_2^{\cdot-}$. (data not shown).

The competition of coumarins with DMSO for $HO^{\bullet}$ generated by the Fe^{3+}/ascorbic acid system, expressed as the inhibition of formaldehyde production, was used for the evaluation of their hydroxyl radical scavenging activity. In these experiments (Table IV) compounds (**1**–**8**) did not show any inhibition, whereas compounds (**9**–**12**) (0.1 mM), inhibited significantly the oxidation of DMSO (33 mM). Compound (**11**) was found to be the most active. The order of decreasing $HO^{\bullet}$ scavenging activity was (**11**) > (**12**) > (**9**) > (**10**). There was not a clear distinction between the activity of comarins possessing the angular and linear structure. However, the nature of the heterocyclic ring (condensed to the C7,8 of the coumarin e.g. furyl-), seemed to be important.

No inhibition was observed on soybean lipoxygenase (LOX) under our experimental conditions, except for compounds (**2**), (**4**) and (**12**) (16.1–22.6%, Table IV). Compounds (**4**) and (**12**) showed similar inhibition values. A clear distinction between the reversed form of the pyranono-coumarin ring system (compounds **4** and **12**) and the two methyl-groups (**3**- and **4**-) concerning the biological response, could not be found. However, it could be assumed that a site specific scavenging may occur through an ability of the compounds to chelate Fe^{2+}.

In this investigation all compounds were studied in order to gain insight into the mechanism of their antiphlogistic action. These derivatives were tested with regard to their antioxidant ability as well as their ability to inhibit β-glucuronidase and to affect trypsin-induced proteolysis. The role played by proteases in the early stage of inflammatory process is well documented. Some anti-inflammatory agents have been reported to exhibit antiproteolytic activity.[29] It was found that the examined coumarins, under the reported experimental conditions, did not inhibit these enzymes *in vitro* (data not shown).

The value of the *in vitro* tests conducted here is that they enable the possibility of direct pro-oxidant or anti-oxidant effects of compounds to be investigated *in vivo*. The fact that such effects could be feasible, by *in vitro* testing, does not mean that they actually occur *in vivo* and further studies are underway to examine this question. The ability of lipophilic molecules to concentrate within hydrophobic regions, such as the interior of membranes, cannot be ignored.

Physicochemical Studies

Lipophilicity is an important physicochemical parameter for the kinetics of biologically active compounds. Antioxidants of hydrophilic or hydrophobic character are both needed to act as radical scavengers in the aqueous phase or as chain breaking antioxidants in biological membranes. The lipophilicity of most of these coumarins was determined from RPTLC and expressed as R_M values (Table I). This is considered to be a reliable fast and convenient method for expressing lipophilicity.[17] From our results it can be concluded that the R_M values could not be used as a successful relative measure of the overall lipophilic/hydrophilic balance of these molecules as indicated by the difference with the clog *P*-calculated values [Biobyte]- which express their theoretical lipophilicity in the standard octanol–water system. This difference could be attributed to the different nature of the hydrophilic and lipophilic phases in the two systems. Our attempt to correlate lipophilicity as clog *P* (theoretically calculated lipophilicity)[30] (Table I) or R_M (Table I) with the reducing ability of the tested

compounds was unsuccessful ($r < 0.31$). A poor relationship existed between anti-inflammatory activity (CPE) and lipophilicity.

From our results, it can be concluded, that lipophilicity is not the main property contributing to the anti-inflammatory/antioxidant activity of the investigated coumarins.

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