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Original article

Design, synthesis and pharmacobiological evaluation of novel acrylic acid derivatives acting as lipoxygenase and cyclooxygenase-1 inhibitors with antioxidant and anti-inflammatory activities

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ABSTRACT

A series of novel acrylic acid derivatives bearing at the 3 position thienyl, furfuryl and 3,5-ditert-butyl-4-hydroxyphenyl substituents have been designed, synthesized and tested as potential dual lipoxygenase/cyclooxygenase-1 (LOX/COX-1) inhibitors and as antioxidant and anti-inflammatory agents. Some compounds have shown moderate antioxidant and COX-1 inhibitory activities, very good anti-inflammatory activity and an inhibition of soybean lipoxygenase (LOX) higher than caffeic acid. In particular, compound **4I** disclosed a moderate *in vitro* LOX inhibition with an IC_{50} = 100 μM whereas compounds **1I** and **2II** exhibited the best, albeit poor, activity as COX-1 inhibition (75% inhibition at 100 μM). Good radical scavenging properties were shown by compounds **4I**, **3I** and **1II**. Docking simulations performed on LOX inhibitor **4I** and COX-1 inhibitor **1I** indicated that hydrophobic key interactions may govern the enzyme-inhibitor binding.

1. Introduction

Arachidonic acid (AA) is metabolised to eicosanoids by the cyclooxygenase (COX), lipoxygenase (LOX) and epoxygenase pathways [1].

In the COX pathway, the COX-1, COX-2, and COX-3 isoforms convert AA to the hydroxyendoperoxide PGH_2, which is further metabolized to prostaglandins (PGs), prostacyclin (PGI_2) and thromboxane A_2 (TXA_2). In contrast, AA is initially converted to hydroperoxyeicosatetraenoic acids (HPETE), subsequently to hydroxyeicosatetraenoic acids (HETE) and then to the leukotrienes (LTs) via the LOX pathway [2,3]. PGs and LTs produced in the COX and LOX pathways, respectively, have been recognized as pro-inflammatory mediators in numerous inflammatory diseases, allergic disorders [4–6] proliferation and neoangiogenesis [7,8].

Many non-steroidal anti-inflammatory drugs (NSAIDs) were found to inhibit these enzymes. Acetylsalicylic acid, a classical NSAID, is a selective COX-1 inhibitor whereas naproxen and indomethacin are non-selective COX inhibitors. In the late 1990s, selective COX-2 inhibitors (i.e, coxibes) have been introduced in clinical trials for the treatment of inflammatory conditions without causing gastric irritation. However, several coxibes (e.g., rofecoxib and valdecoxib) have been withdrawn because they have been associated with myocardial infraction and cardiovascular thrombotic events [9–11]. COX-2-specific inhibitors, such as celecoxib (Celebrex®) and rofecoxib (Vioxx®) lack antiplatelet activity because they do not inhibit the COX-1 isoform and therefore do not inhibit thromboxane synthesis [12]. These thrombotic side effects seem to be due to the decreased level of vasodilatory and anti-aggregatory prostacyclin (PGI_2) along with an increased level of the prothrombotic platelet aggregator TXA_2 [3,13–15]. In addition, it has been pointed out that inhibiting COX pathway could shunt the metabolism of AA toward the 5-LOX pathway [15], increasing the formation of leukotrienes leading to inflammation. Unfortunately, NSAIDs-induced important adverse effects e.g. asthma and gastrointestinal damages, that limit their use [16–18].

Compounds that combine COX and LOX inhibition may present multiple advantages because they synergistically block metabolic pathways of arachidonic acid cascade and thrombosis and possess

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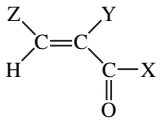


Y: -Ph, -H **Z:** different substituents **X:** -OH, -NHOH, -NHR

Fig. 1. Substituted acrylic acids and amides, inhibitors of soybean lipoxygenase with antioxidant and anti-inflammatory activities.

a wide range of anti-inflammatory activities [19]. It has been clearly shown that dual inhibitors of the COX/LOX enzymatic pathways offer a better alternative approach in designing new efficacious drugs with excellent safety profile and least side effects. During the last few years different groups have synthesized dual COX/LOX inhibitors [13–15,20–23]. Moreau et al. [14,15] have presented some aryl-acrylic acids as dual COX/LOX inhibitors while Abdellatif et al. [24,25] as COX (COX-1/2) inhibitors. Recently, we have reported a series of substituted acrylic acids and amides having potent inhibitory activity against soybean lipoxygenase as well as good antioxidant and anti-inflammatory activities (Fig. 1). These analogues have been used in the present design and synthesis. Some of the referred acrylic acids have also been tested as COX-1 inhibitors with promising results [26–29].

Starting from the observation that several pyrazoline, thiophene, di-*tert*-butylphenol, hydrazone, pyrrolidine and pyrazole derivatives were potent dual 5-LOX and COX inhibitors [30], we have designed a series of acrylic acid derivatives containing a 5-membered heterocyclic and the 3,5-ditert-butyl-4-hydroxyphenyl (DTBHP) substituents as potential dual COX/LOX inhibitor [31–48]. Indeed, DTBHP derivatives have been proved to be good oral anti-inflammatories devoid of ulcerogenic drawbacks. Some compounds (**I**–**IV**) showing dual COX/5-LOX activity are presented in Fig. 2 [43–48]. Compound S-2474 (**III**) in which the DTBHP moiety is linked with a vinyl group to the heterocyclic ring is undergoing clinical trials as an anti-arthritic drug [43,44]. Other studies have shown that compounds possessing a propenone moiety, represented by **V** in Fig. 2, exhibit also dual COX/LOX inhibition [49].

Our design supported from our previous findings [26–29] addressed new acrylic acid derivatives combining appropriate structural and physicochemical features in order to inhibit soybean lipoxygenase and cyclooxygenase-1 and to present both antioxidant and anti-inflammatory activities. The synthesized compounds were screened for free radical scavenging ability, *in vitro* soybean LOX and COX-1 inhibition and *in vivo* anti-inflammatory activity.

Finally, docking studies were undertaken to gain insight into the possible binding mode of the most active compounds in the LOX and COX-1 binding sites.

2. Results and discussion

2.1. Chemistry

The synthesis of the aryl-acrylic acids was accomplished according to the Knoevenagel condensation indicated in Scheme 1. The aryl-acrylic acids of series **I** were obtained by the condensation of a suitable aldehyde with phenylacetic acid and acetic anhydride in the presence of triethylamine, while the 3-substituted-acrylic acids of series **II** were obtained by the condensation of the suitable aldehyde with malonic acid in the presence of pyridine and piperidine.

All the reactions proceeded smoothly and in good yields (43–75%), with the only exception of compounds **1I** and **4II** which were obtained in lower yields (28 and 27%, respectively). All the compounds formed were recrystallized from ethanol/water. The structures of the synthesized compounds, given in Table 1, were confirmed by IR, ^{1}H NMR, ^{13}C NMR and elemental analysis. All the acids present the characteristic absorption in the IR (nujol) (nearly 3200 (O–H), 1720 (C=O), 1625 (C=C), cm^{-1}) and the expected ^{1}H NMR signals for the *E* configuration of the double bond. In some cases the H of the COOH group was not detectable. Spectral data are consistent with the proposed structures and are in agreement with previous findings (226–27, 29).

Compounds **1II** [50,51], **2II** [50] and **3II** [51] were previously reported, whereas compound **3I** has been used as an intermediate for the synthesis of naphtho[2,1-*b*]-furanecarboxylic acids by oxidative photochemical cyclization [52]. Compound **4II** was synthesized according to our procedure [28,29], differing from the Knoevenangel condensation reaction of 3,5-di-tert-butyl-benzaldehyde with malonic acid in dioxane reported by Munteanu et al. [53].

2.2. Physicochemical studies

Since lipophilicity is a significant physicochemical property for the absorption, distribution, metabolism and excretion of drugs (ADME properties), we measured it experimentally as R_M values by the RPTLC (reverse-phase thin-layer chromatography) method and compared it with the corresponding log *P* values in *n*-octanol-buffer estimated by Clog *P* [54]. RPTLC is considered to be a reliable, fast and convenient method for expressing lipophilicity [55]. Apart from the important role of lipophilicity for the ADME

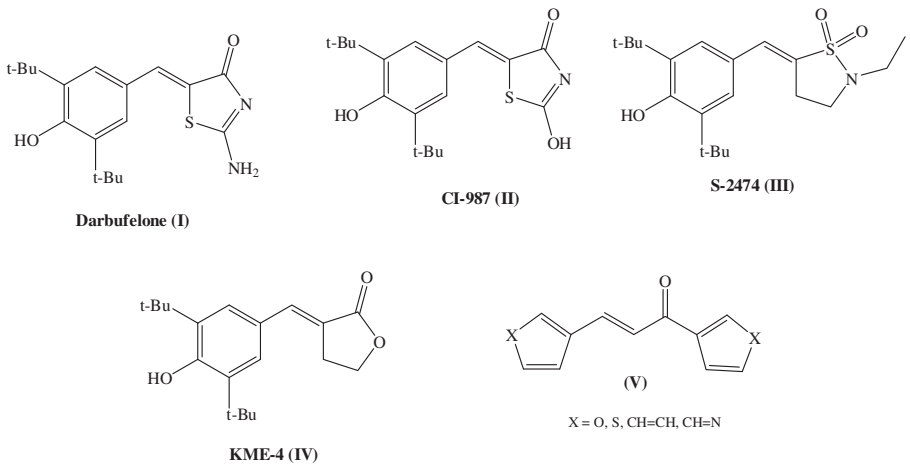
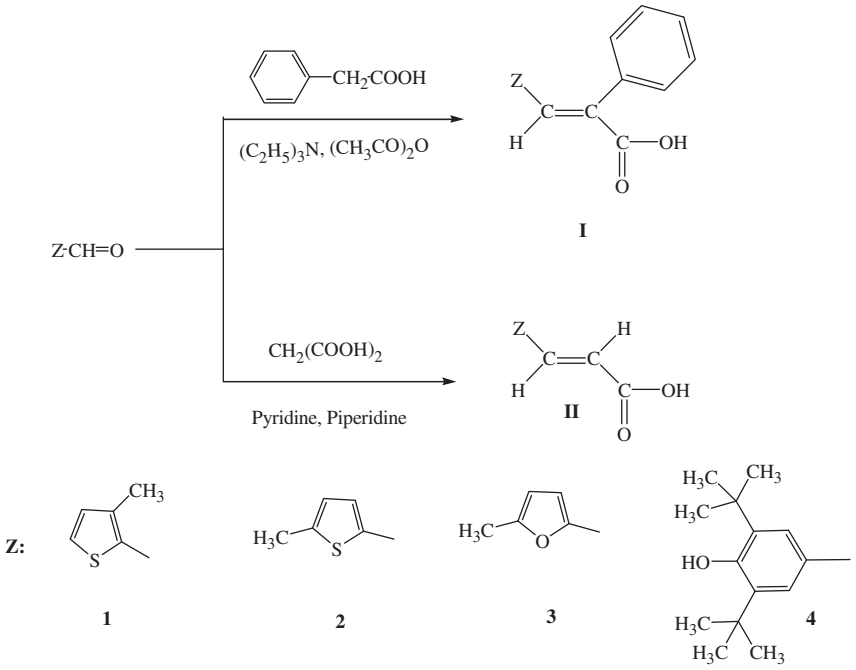


Fig. 2. Dual COX/LOX inhibitors.



Scheme 1. Synthesis of aryl-acrylic acid derivatives.

properties, also the radical scavenging property (i.e., in an aqueous phase) or the chain-breaking antioxidant activity (i.e., in biological membranes) both taken into account in the present study are influenced by lipophilicity.

Unfortunately, the results showed in Table 1 indicated that R_M values did not result any sound correlation with log *P* and therefore, in the present case, such parameters were unsuited for modelling lipophilicity. The lack of correlation between log *P* and R_M might be due to the different nature of the hydrophilic and lipophilic phases used in the two experimental systems.

2.3. Biological results and discussion

Protection against inflammation and radical attack, inhibition of LOX and COX-1, and toxicity were measured as reported in the Experimental Section.

In acute *in vivo* toxicity experiments, the examined compounds did not present toxic effects in doses up to 0.5 mmol/kg body weight.

Acute inflammation is due to the release of chemical mediators, which cause edema as a result of extravasations of fluid and proteins from the local microvasculature and accumulation of polymorphonuclear leukocytes at the inflammatory site. The *in vivo* anti-inflammatory effects of the tested acrylic acids were assessed by using the carrageenin-induced rat paw edema (CPE) model and are presented in Table 2 as percentage of weight decrease at the right hind paw. The induced edema is a non-specific inflammation highly sensitive to NSAIDs and it is largely accepted as a useful tool for studying new anti-inflammatory agents [56]. It reliably assesses the anti-inflammatory potency of the NSAIDs and detects during the second phase of inflammation process that they act as anti-inflammatory agents by inhibiting prostaglandin amplification [57]. All the tested acids at 100 μM concentration induced a significant protection against the CPE ranging from 10 to 75% edema weight decrease with the exception of **4II** (2%) while the reference drug indomethacin induced a 47% edema weight decrease at an equivalent dose. The presence of the phenyl group at position 2 in the 2,3-disubstituted derivatives **3I** and **4I** seems to favourably affect the biological response. In fact, compounds **3I** and **4I** were found to be more potent than the corresponding monosubstituted derivatives **3II** and **4II** (Table 2). A preliminary multiple regression analysis of anti-inflammatory activity versus several physicochemical parameters (lipophilicity, steric and electronic descriptors) of the examined compounds revealed no significant correlation.

Compounds were evaluated for the inhibition of soybean lipoxygenase LOX by the UV-based enzyme assay of Taraborewala et al. [58]. While one may not extrapolate the quantitative results of this assay to the inhibition of mammalian 5-LOX, it has been shown that inhibition of plant LOX activity by NSAIDs is qualitatively quite similar to the inhibition observed on the rat mast cells LOX and this assay may be used as a qualitative or semi-quantitative screen for such activity.

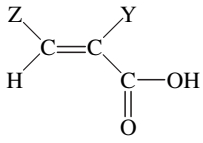
Examination of LOX IC_{50} inhibition values shows that compound **4I** is the most active inhibitor followed by compound **4II** (Table 2). Not surprisingly, the presence of the DTBHP group favours the inhibitory activity.

Most LOX inhibitors are antioxidants or free radical scavengers [59], and some studies suggested a relationship between LOX inhibition and the ability of the inhibitor to reduce the Fe^{3+} at the active site to the catalytically inactive Fe^{2+} [60,61] LOXs contain a "non-heme" iron per molecule in the enzyme active site as high-spin Fe^{2+} in the native state and the high spin in the activated state Fe^{3+}. Several LOX inhibitors proved to be excellent ligands for Fe^{3+}. It has been demonstrated that their mechanism of action is presumably related to their coordination with the catalytically crucial Fe^{3+}.

Although lipophilicity is referred to, as an important physicochemical property for LOX inhibition [25–28], no clear relationship between LOX inhibitory activity and log *P* emerged for our compounds. Nonetheless, it must be stressed that the highly lipophilic compounds **4I** and **4II** resulted the most active, while the less lipophilic one, that is compound **3II** was the less active inhibitor. The introduction of a phenyl ring at position 2 led to improved inhibitory activity with the exception of compound **1I** (Table 2).

Compounds **2II** and **1II** exhibited the highest *in vivo* anti-inflammatory activity in CPE model with a 77.5 and 63%, edema

Table 1
Chemical structures, physicochemical and reaction data of aryl-acrylic acid derivatives **1**–**4**



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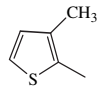
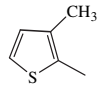
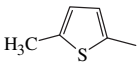
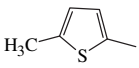
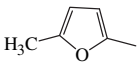
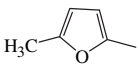
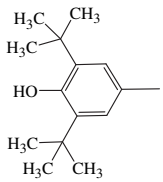
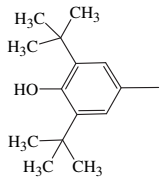
Compd.	Z	Y	Formula*	R_f	Clog P^{**}	$R_M^{\#}$ (±SD)	mp °C	yield%
1I			$C_{14}H_{12}O_2S$	0.51[a]	3.73	−0.563(0.040)[d]	198–200	28
1II		H	$C_8H_8O_2S$	0.87[b]	2.38	−0.461(0.038)[d]	174–175	59
2I			$C_{14}H_{12}O_2S$	0.76[c]	3.73	−0.553(0.022)[d]	154–155	43
2II		H	$C_8H_8O_2S$	0.87[b]	2.38	−0.458(0.027)[d]	163–164	64
3I			$C_{14}H_{12}O_3$	0.77[c]	3.26	−0.583(0.014)[d]	144–145	46
3II		H	$C_8H_8O_3$	0.83[c]	1.91	−0.482(0.013)[d]	154–155	75
4I			$C_{23}H_{28}O_3$	0.71[c]	6.37	−0.540(0.011)[d]	145–147	72
4II		H	$C_{17}H_{24}O_3$	0.86[c]	5.02	−0.133(0.005)[d]	95–97	27

*Elemental analyses for molecular formula (±0.4%).
**Theoretically calculated clog *P* values.
#R_M values are the average of at least 10 measurements.
[a] $CH_3COOC_2H_5$:$CHCl_3$:−petroleum ether 40–65 °C (2:1:1).
[b] $CHCl_3$.
[c] CH_2Cl_2:$CH_3COOC_2H_5$ (2:1).
[d] CH_3OH:H_2O:CH_3COOH, (77:23:0.1).

weight reduction respectively (Table 2). These values were even higher than that observed with an equimolar amount of the reference compound indomethacin (47%).

COX-1 plays a role as a "housekeeping enzyme", for example maintaining the lining of the stomach and in endothelial cells contributing to the normal function of the cardiovascular system via the release of prostacyclin. Thus, inhibition of COX-1 is involved in the appearance of the unwanted side effects. Based on the above we found interesting to examine inhibitory activity of compounds towards COX-1 at 100 μM (Table 2). They resulted moderately active and no correlation was observed between the anti-inflammatory activity and COX-1 inhibition. This is particularly evident for compound **1I** that exhibited the highest inhibition potency but a weak anti-inflammatory activity in the rat (21% edema weight reduction) and for compound **2I** which had a good anti-inflammatory activity (53%) but presented a low COX-1 (12.5%).

Table 2
In vivo anti-inflammatory activity and *in vitro* lipoxygenase (LOX) and cycloxygenase (COX-1) inhibitory activities of acrylic acid derivatives **1**–**4**.

Compd	CPE (%)[a]	LOX[b] IC_{50} (μM)	COX-1[c]
1I	21.0**	310	75
1II	63.0**	255	37.5
2I	53.0*	290	12.5
2II	77.5*	280	75
3I	34.0*	240	nt
3II	10.0*	315	nt
4I	43.0*	100	nt
4II	2.0*	220	nt
Caffeic acid		600	
SC-560			(IC_{50} = 7.8 nM) 100%
Indomethacin	47*		
NDGA		515	

*$p < 0.01$.
**$p < 0.05$.
[a] % of reduction of the rat paw edema (CPE %) induced by carrageenin at the dose of 0.01 mmol/Kg/body weight.
[b] Soybean lipoxygenase inhibition expressed as IC_{50} (μM).
[c] Cyclooxygenase inhibitory activity at 100 μM, expressed as % of inhibition. Statistical data: student's *t*-test.

It is well known that free radicals play an important role in inflammatory action [62]. Consequently, compounds with antioxidant properties could be expected to offer protection in inflammation and lead to potentially effective drugs. In fact, many non-steroidal anti-inflammatory drugs have been reported to act either as radical scavengers or as inhibitors of free radical production. Thus, we found interesting to test these derivatives for their antioxidant ability in comparison to well known antioxidants, i.e., 3,5-di-tert-butyl-4-hydroxy-toluene (DTBHP), nordihydroguaretic acid (NDGA), trolox and caffeic acid (Tables 2–5) [63].

A number of assays have been used for the measurement of the antioxidant activity [64–67]. Each method relates to the generation of different radical, acting through a variety of mechanisms and the measurement of a range of end points at a fixed time intervals. The reducing abilities of the examined compounds were determined by the use of the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) at 0.05 and 0.1 mM concentration [29] after 20 and 60 min (Table 3). The reducing abilities of the tested compounds ranged nearly 37% with compound **4II** and small differences were observed among the compounds with time and the concentration. As expected, the DTBHP derivatives (**4II** and **4I**) presented higher reducing ability.

It is well known that rates of reactive oxygen species (ROS) production are increased in most diseases [68,69]. The cytotoxicity of $O_2^{-\bullet}$ and H_2O_2 in living organisms is mainly due to their transformation into •OH reactive radical and $^1O_2$. During the inflammatory process, phagocytes generate the superoxide anion radical at the inflamed site and this is connected to other oxidizing species

Table 3
Reducing ability (RA %) of acrylic acid derivatives **1**–**4**.

Compd.	RA % 0.05 mM		RA % 0.1 mM		RA % 0.2 mM	
	20 min	60 min	20 min	60 min	20 min	60 min
1I	1.7	no	5.9	2.8	3.4	7.2
1II	3.2	no	3.2	2.5	9.7	7.2
2I	3.6	2.9	6.7	4.9	9.3	7.3
2II	3.5	1.0	4.4	4.3	6.4	2.6
3I	3.2	1.4	3.6	4.8	10.0	6.5
3II	4.8	3.0	8.6	7.6	9.6	6.7
4I	2.8	5.3	7.6	12.2	17.3	66.2
4II	28.1	26.9	31.6	36.9	47.7	26.5
NDGA	84.8	83.1	81.0	82.6	90.3	94.6

no: no activity under the used experimental conditions.

Table 4
Radical scavenging activity (%) of hydroxyl (HO,) and superoxide ($O_2^{-\bullet}$) radicals by acrylic acid derivatives **1**–**4**.

Compd.	HO• (%) 0.01 mM	HO• 0.1 mM	$O_2^{-\bullet}$ (%) 0.1 mM (1 mM)
1I	100	99.4	no (100.0)
1II	99.8	99.8	57.1
2I	98.9	97.9	no (100.0)
2II	99.3	97.8	no (66.7)
3I	99.8	100	50.0
3II	98.1	99.6	14.3
4I	99.8	99.5	85.7
4II	97.8	100	35.7
Trolox	88.2	73.4	–
Caffeic acid	–	–	46.5 (86.1)

no: no action under the used experimental conditions.

such as the radical •OH. Hydroxyl radicals are among the most reactive oxygen species and are considered to be in part responsible for tissue damage occurring in inflammation.

Therefore, a series of assays were performed to evaluate the radical scavenging properties of our compounds. Their competition with DMSO for •OH radicals generated by the Fe^{3+}/ascorbic acid system expressed as percent inhibition of formaldehyde production was used for the evaluation of their radical scavenging activity at 0.1 mM concentration (Table 4) [29]. All the compounds strongly inhibited the oxidation of DMSO (33 mM) at 0.1 mM with an activity higher than the reference compound Trolox.

An additional assay was performed to evaluate the radical scavenging property of our compounds, at 0.1 mM concentration, on non-enzymatically generated superoxide anion radicals (Table 4). Compound **4I** presented the highest activity, followed by compound **1II** (85.7 and 57.1%, respectively).

Mixing heme proteins with H_2O_2 generates powerful oxidizing activated heme species and radicals on amino-acids side chains that can cause lipid peroxidation. As a model of such reactions we used the peroxidation of arachidonic acid by a mixture of heme and H_2O_2 [29]. All the tested compounds significantly inhibited the lipid peroxidation at 0.1 mM concentration with the exception of compounds **1II**, **2II** and **3II** (Table 5). The highest activity was exhibited by compounds **4II** bearing the DTBHP group.

Generation of the ABTS (2,2′-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid)) radical cation, formed the basis for a further determination of the antioxidant activity of our compounds. All compounds showed remarkable activity with the exception of compound **1I** (18.8%) (Table 5).

Azo compounds generating free radicals through spontaneous thermal decomposition are useful for *in vitro* studies of free radical production. The water soluble 2,2′-azobis (2-amidinopropane) hydrochloride (AAPH) has been extensively used as a clean and controllable source of thermally produced alkylperoxyl free

Table 5
Antioxidant activities of acrylic acid derivatives **1**–**4** in the heme dependent lipid peroxidation (LP %), $ABTS^{+\bullet}$ - decolorization ($ABTS^{+\bullet}$ %) and inhibition of linoleic acid peroxidation (AAPH) assays.

Compd	LP% 1 mM	$ABTS^{+\bullet}$% 0.1 mM	AAPH% 0.1 mM
1I	35.5	18.8	20.1
1II	1.2	41.7	64.9
2I	55.8	41.1	37.5
2II	1.2	44.2	73.4
3I	49.2	65.6	32.6
3II	7.9	37.1	74.9
4I	39.3	60.3	60.1
4II	54.1	53.9	74.8
Trolox	nt	88.0	63.0
Ascorbic Acid	nt	95.8	nt

nt: not tested.

radicals. Also this assay was performed to better characterize the antioxidant activity of our compounds. All compounds presented a good activity at 0.1 mM concentration (20.1–74.9%).

2.4. Docking studies

Even if the observed LOX and COX-1 inhibitory affinities of the analyzed acrylic acids were quite low, we decided to perform some docking simulations on the most active compounds to get some clues on the nature and type of interactions governing the inhibitor binding [70,71].

Simulations were carried out with GOLD, a software based on a genetic algorithm to explore the ligand conformational space [72]. Docking poses were obtained by applying both Chemscore and Goldscore, fitness functions available for scoring. As easily interpretable results were obtained on the basis of a recently published work [73], all the results reported in the present paper are referred to the Chemscore fitness function. The coordinates for the reference protein were obtained from 1IK3 (soybean lipoxygenase) and 1Q4G (ovine cyclooxygenase-1) available from the Protein Data Bank (PDB) with a resolution of 2 Å [74]. These complexes were prepared for docking studies by adding hydrogen atoms, removing water and co-crystallized inhibitors and refined by using the Protein Preparation tool implemented in Maestro 7.5 [75]. Enzyme-inhibitor interactions within a radius equal to 12 Å centered on reported bound inhibitors were taken into account.

The docking simulation of the most active compound **4l** toward LOX showed that the enzyme-inhibitor complex was stabilized by hydrophobic interactions occurring between the aromatic moieties of the ligand and lipophilic residues of the binding site. In particular the 3,5-di-tert-butyl-4-hydroxy-phenyl group was oriented towards the hydrophobic region lined by Leu565, Ile572, Val566, Leu515 and Trp519 while the 2-phenyl moiety protruded into a hydrophobic cavity formed by residuesLeu277, Ile772, Thr274, Leu273, Ile857 and Ile557. In addition, the carboxylic functional group was supposed to coordinate the iron ion as it showed a similar structural arrangement compared to the peroxide group of the co-crystallized ligand (Fig. 3).

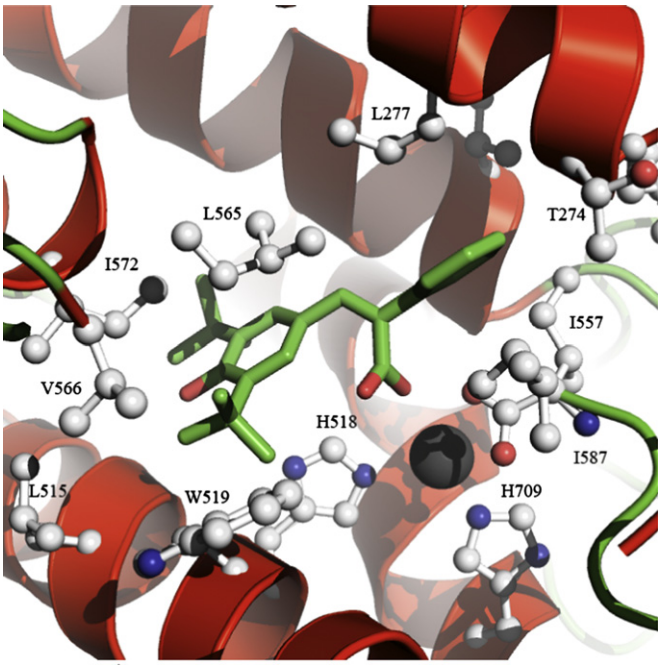


Fig. 3. Representation of the top-scored docking pose of compound **4l**, rendered as a stick model, into the soybean LOX binding site (PDB code 1IK3). Side-chains of relevant binding site residues are rendered as ball and stick models. The iron ion is rendered as a gray sphere.

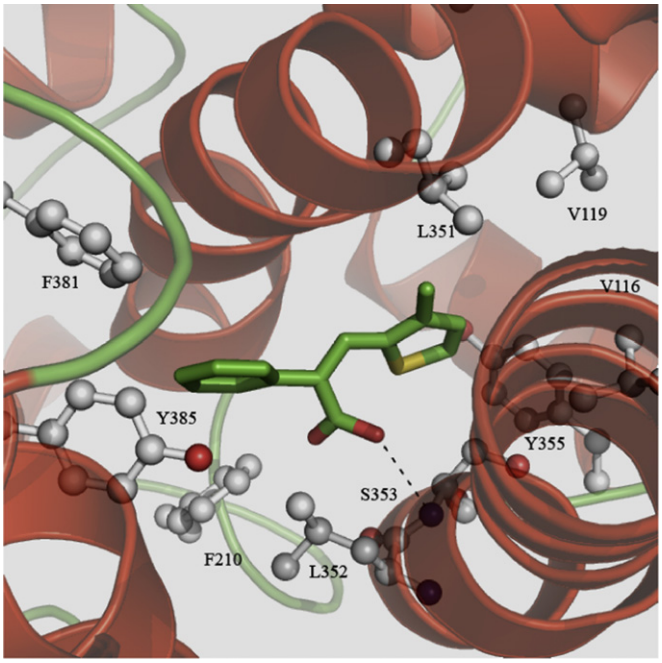


Fig. 4. Representation of the top-scored docking pose of compound 1l, rendered as a stick model, into the COX-1 binding site (PDB code 1Q4G). Side-chains of relevant binding site residues are rendered as ball and stick models. A possible HB is indicated with a dashed line.

The docking top-score binding pose indicated the availability of some room in the para position of the 3,5-di-tert-butyl-4-hydroxy-phenyl ring but the removal of the OH group might eliminate the good antioxidant activity of this compound. However, room for medium-sized substituents could be found at position 2 or on the 2-phenyl ring of the acrylic acid derivatives.

As far as the COX-1 inhibition is concerned, the most active inhibitor **1l** was docked into the enzymatic binding site. It could establish many hydrophobic interactions with its 2-phenyl ring leaned towards Phe381 and Tyr385 and the 3-methylthiophen ring pointing towards Leu531, Val116 and Leu359. Finally the carboxylic group could establish an H-bond interaction with the NH group of the backbone of Ser353. In this case, structural modifications aiming at the improvement of binding affinity could be made at position 3 where larger room may be available for hydrophobic substituents (Fig. 4).

3. Conclusions

The present study showed that certain novel acrylic acid derivatives possess a high anti-inflammatory activity. Moreover, most of them proved to be potent •OH scavengers, inhibited lipid peroxidation and *in vitro* soybean LOX, and exhibited a good antioxidant activities in a variety of tests. The high antiradical *in vitro* activity of the tested compounds supported, at least in part, the good *in vivo* anti-inflammatory activity.

The most active compounds docked successfully into the active site of the inhibited enzymes. Inhibitory activity of the most potent compounds was explained mostly by hydrophobic interactions.

Two compounds, that are **1II** and **2II,** were found to present a promising anti-inflammatory profile with a high antiedematous activity, significant inhibitory activity on LOX and COX-1 and good hydroxyl radical scavenging activity. However, the inhibition of

both COX-1 and LOX enzymes was generally very low. Since inhibition of COX-1 is involved in the appearance of the unwanted side effects, this dual inhibition might produce a protective effect as well as a reduction in inflammation. Some of the synthesized compounds might constitute initial leads for the design of new more potent therapeutics against inflammation. This design could be guided by the insights gained by our preliminary docking studies on LOX and COX active sites and by our previous results [26–29]. Starting from these data, further investigations will be carried out to gain insight into the mechanism of antiphlogistic activity of the examined compounds.

4. Experimental section

Materials: All chemicals, solvents and reagents were of analytical grade and purchased from commercial sources. Soybean Lipoxygenase and porcine heme were obtained from Sigma Chemical, Co. (St. Louis, MO, USA). The kit for COX activity assay was purchased by Cayman. For the *in vivo* experiments, male and female Fischer-344 rats (180–240 g) were used.

Synthesis: All starting materials were obtained from commercial sources and used without further purification. Melting Points (uncorrected) were determined on a MEL-Temp II (Lab. Devices, Holliston, MA, USA). UV–Vis spectra were obtained on a Perkin–Elmer 554 double beam spectrophotometer and on a Hitachi U-2001 spectrophotometer. Infrared spectra (film as Nujol mulls) were recorded with Perkin–Elmer 597 spectrophotometer (Perkin–Elmer Corporation Ltd., Lane Beaconsfield, Bucks, England) and a Shimadzu FTIR-8101M. The ^{1}H Nucleic Magnetic Resonance (NMR) spectra were recorded at 300 MHz on a Bruker AM 300 spectrometer (Bruker Analytische Messtechnik GmbH, Rheinstetten, Germany) in $CDCl_3$ or DMSO using tetramethylsilane as an internal standard unless otherwise stated ^{13}C NMR spectra were obtained at 75.5 MHz on a Bruker AM 300 spectrometer in $CDCl_3$ or DMSO solutions with tetramethylsilane as internal reference unless otherwise stated. Representative ^{13}C NMR spectra are given. Chemical shifts are expressed in δ (ppm) and coupling constants *J* in Hz. Mass spectra were determined on a VG-250 spectrometer (VG-Labs., Tritech, England) with ionization energy maintained at 70 eV. Elemental analyses for C and H gave values acceptably close to the theoretical values (±0.4%) in a Perkin–Elmer 240B CHN analyzer. Reactions were monitored by thin-layer chromatography (TLC), on aluminum cards precoated with 0.2 mm of silica gel and a fluorescent indicator.

4.1. General procedure

4.1.1. Synthesis of substituted 2-phenyl-3-heteroaryl-acrylic acids **1I–4I**

Title compounds were prepared by a Knoevenangel reaction, as illustrated in Scheme 1, according to literature methods [26–29]. A suitable aldehyde (0.015 mol) was condensed with phenylacrylic acid (0.015 mol) and acrylic acid anhydride (10 mL) in the presence of triethylamine (5 mL). The reaction mixture was refluxed for 5 h. The solution was poured into 2 N HCl, then on ice and the formed precipitate was collected by filtration and recrystallized from 50% aqueous ethanol. In case that no precipitate was formed an extraction with 3 × 100 mL $CHCl_3$ was made and the organic phase was collected, dried over $MgSO_4$ and evaporated to dryness affording a residue that was recrystallized from 50% aqueous ethanol.

*4.1.1.1. (E)-3-(3-Methyl-thiophen-2-yl)-2-phenylacrylic acid (**1I**).* IR (Nujol) (cm^{-1}): 3150, 2918–2705, 1720, 1666, 1606, 1384; ^{1}H NMR ($CDCl_3$): δ, (ppm) 2.40 (3H, s, CH_3), 6.80 (1H, d, $J = 6$ *Hz*), 7.16 (1H, d, $J = 6$ *Hz*), 7.28–7.47 (6H, ms), 11.00 (1H, s, COO*H*); ^{13}C NMR ($CDCl_3$): 12.4, 122.5, 125.9, 126.0, 128.6, 129.1, 129.6, 130.2, 133.5, 133.6, 134.8, 138.5, 139.0, 168.9; Anal. C, H, N. Calcd %: ($C_{14}H_{12}O_2S$) C: 68.83, H: 4.95; Found %: C: 68.98, H: 4.77.

*4.1.1.2. (E)-3-(5-Methyl-thiophen-2-yl)-2-phenylacrylic acid (**2I**).* IR (Nujol) (cm^{-1}): 3175, 2961–2876, 1730–1713, 1615, 1385; ^{1}H NMR ($CDCl_3$): δ, (ppm) 2.33 (3H, s, CH_3), 6.62–7.62 (8H, ms), 11.28 (1H, s, COO*H*); Anal. C, H, N. Calcd %: ($C_{14}H_{12}O_2S$) C: 68.83, H: 4.95; Found %: C: 68.82, H: 4.73.

*4.1.1.3. (E)-3-(5-Methyl-furan-2-yl)-2-phenylacrylic acid (**3I**).* IR (Nujol) (cm-1): 3175, 2961–2897, 1700–1679, 1662, 1388; ^{1}H NMR ($CDCl_3$): δ, (ppm) 2.21 (3H, s, CH_3), 5.77–5.89 (2H, m), 7.26–7.75 (6H, ms); ^{13}C NMR ($CDCl_3$): 13.7, 109.1, 117.3, 126.0, 127.9, 128.6, 129.2, 129.3, 130.0, 135.0, 139.0, 149.0, 155.0, 168.0; Anal. C, H, N. Expected %: ($C_{14}H_{12}O_3$) C: 73.67, H: 5.30; Found %: C: 73.94, H: 4.98.

*4.1.1.4. (E)-3-(3,5-Di-tert-butyl-4-hydroxyphenyl)-2-phenylacrylic acid (**4I**).* IR (Nujol) (cm-1): 3196–2940, 2870, 1730, 1653, 1380; ^{1}H NMR ($CDCl_3$): δ, (ppm) 1.08–1.37 (18H, m, 6xCH_3), 7.06–7.61 (8H, m), 7.93 (1H, s, O*H*); Anal. C, H, N. Calcd %: ($C_{23}H_{28}O_3$) C: 78.38, H: 8.01; Found %: C: 78.06, H: 8.25.

4.2. General procedure for the synthesis of 3-heteroaryl-acrylic acids **1–4II** [26–29]

Title compounds were prepared, as illustrated in Scheme 1, by a Knoevenangel condensation of the suitable heteroarylaldehyde with malonic acid. Malonic acid (0.01 mol) was dissolved in 1.12 mL of pyridine and the heteroaldehyde (0.01 mol) and piperidine (0.01 mol) were added. The mixture was refluxed until the emission of CO_2 stopped. Then the solution was poured into 2 N HCl and then on ice. The formed precipitate was collected by filtration and recrystallized from water or from 3:1 water/ethanol mixture. If no precipitate was formed an extraction with 3 × 100 mL $CHCl_3$ or CH_2Cl_2 was made and the organic phase was collected, dried over $MgSO_4$, and evaporated to dryness affording a residue that was recrystallized from aqueous ethanol.

4.2.1. (E)-3-(3-Methylthiophen-2-yl)acrylic acid (**1II**)

IR (Nujol) (cm^{-1}): 3100–2900, 1720, 1650, 1380; ^{1}H NMR ($CDCl_3$): δ, (ppm) 2.58 (3H, s, CH_3), 6.18 (1H, d, $J = 15$ *Hz*, C*H*=CH), 6.89 (1H, d, $J = 6$ *Hz*), 7.31 (1H, d, $J = 6$ *Hz*), 7.96 (1H, d, $J = 15$ *Hz*); ^{13}C NMR ($CDCl_3$): 14.4, 114.9, 128.0, 131.4, 133.6, 137.9, 142.3, 172.3; Anal. C, H, N. Calcd %: ($C_8H_8O_2S$) C: 57.12, H: 4.79; Found %: C: 57.31, H: 4.89.

4.2.2. (E)-3-(5-Methylthiophen-2-yl)acrylic acid (**2II**)

IR (Nujol) (cm^{-1}): 3100, 2961–2876, 1773–1753, 1655, 1387; ^{1}H NMR ($CDCl_3$): δ, (ppm) 2.58 (3H, s, CH_3), 6.09 (1H, d, $J = 15$ *Hz*, C*H*=CH), 6.74 (1H, d, $J = 3$ *Hz*), 7.09 (1H, d, $J = 3$ *Hz*), 7.80 (1H, d, $J = 15$ *Hz*, CH=C*H*); ^{13}C NMR ($CDCl_3$): 15.9, 119.9, 126.7, 127.9, 132.4, 139.7, 144.0, 171.0; Anal. C, H, N. Calcd %: ($C_8H_8O_2S$) C: 57.12, H: 4.79; Found %: C: 57.51, H: 4.89.

4.2.3. (E)-3-(5-Methylfuran-2-yl)acrylic acid (**3II**)

IR (Nujol) (cm^{-1}): 3100, 2900–2950, 1750–1700; ^{1}H NMR (DMSO-d_6, $CDCl_3$): δ, (ppm) 2.36 (3H, s, CH_3), 6.10 (1H, d, $J = 3$ *Hz*), 6.23 (1H, d, $J = 15$ *Hz*, C*H*=CH), 6.56 (1H, d, $J = 3$ *Hz*), 7.45 (1H, d, $J = 15$ *Hz*, CH=C*H*), 8.27 (1H, s, COO*H*); ^{13}C NMR ($CDCl_3$): 13.9, 109.0, 112.7, 117.5, 133.0, 141.0, 149.3, 171.6; Anal. C, H, N. Calcd %: ($C_8H_8O_3$) C: 63.15, H: 5.30; Found %: C: 63.14, H: 5.43.

4.2.4. (E)-3-(3,5-Di-tert-butyl-4-hydroxyphenyl)acrylic acid (**4II**)

IR (Nujol) (cm^{-1}): 3175, 2940–2854, 1740, 1687–1615; ^{1}H NMR ($CDCl_3$): δ, (ppm) 1.37–1.43 (18H, m, 6xCH_3), 6.51 (1H, s, O*H*),

7.09–7.15 (1H, d, $J = 18$ *Hz*, CH=CH), 7.22–7.84 (3H, m); Anal. C, H, N. Calcd %: ($C_{17}H_{24}O_3$) C: 73.88, H: 8.75; Found %: C: 73.85, H: 8.42.

4.3. Physicochemical studies

4.3.1. Determination of R_M values

Reversed phase TLC (RPTLC) was performed on silica gel plates impregnated with 55% (v/v) liquid paraffin in light petroleum ether. The mobile phase was a methanol/water mixture (77/23, v/v) containing 0.1% of acetic acid. 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 vapours. R_M values were determined from the corresponding R_f values (from ten individual measurements) using the equation $R_M = \log [(1/R_f)-1]$ [29].

4.3.2. Estimation of lipophilicity as Clog P

Lipophilicity was theoretically calculated as Clog *P* values in *n*-octanol-buffer by CLOGP Programme of Biobyte Corp [76].

4.4. Biological experiments

4.4.1. Experiments in vivo

4.4.1.1. Inhibition of the carrageenin-induced edema [27,29]. Edema was induced in the right hind paw of Fisher 344 rats (150–200 g) by the intradermal injection of 0.1 mL 2% carrageenin in water. Both sexes were used. Females pregnant were excluded. Each group was composed of 6–15 animals. The animals, which have been bred in our laboratory, were housed under standard conditions and received a diet of commercial food pellets and water *ad libitum* during the maintenance but they were entirely fasted during the experiment period. Our studies were carried out in accordance with recognised guidelines on animal experimentation.

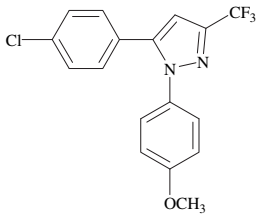
The tested compounds 0.01 mmol/kg body weight, were suspended in water, with few drops of Tween 80 and ground in a mortar before use and were given intraperitoneally simultaneously with the carrageenin injection. The rats were euthanized 3.5 h after carrageenin injection. The difference between the weight of the injected and not-injected paws was calculated for each animal. The change in paw weight was compared with that in control animals (treated with water) and expressed as a percent inhibition of the edema (CPE % values in Table 2). Indomethacin 0.01 mmol/kg was used as reference compound. CPE % are the mean values from two different experiments with a standard error of the mean less than 10%.

4.4.2. Experiments in vitro

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.

4.4.2.1. Soybean lipoxygenase inhibition study in vitro [27,29]. *In vitro* study was evaluated as reported previously. The tested compounds dissolved in ethanol were incubated at room temperature with sodium linoleate (0.1 mM) and 0.2 mL of enzyme solution ($1/9 \times 10^4$ w/v in saline). The conversion of sodium linoleate to 13-hydroperoxylinoleic acid at 234 nm was recorded and compared with the appropriate standard inhibitor (Table 2).

4.4.2.2. In vitro cyclooxygenase-1 (COX-1) inhibition study [27]. Cyclooxygenase (COX) activity was determined by using arachidonic acid (AA) as substrate and N,N,N,N-tetramethylphenylenediamine (TMPD) as co-substrate. The reaction mixture (1 mL) contained 0.75 μM heme, 128 μM TMPD, 80 μM AA and 1.5 μg enzyme, in 0.1 M Tris/HCl (pH 8.5). The oxidation of substrate was measured, at room temperature by monitoring the increase of absorbance at 611 nm. The absorption due to the spontaneous oxidation of TMPD was subtracted from the initial rate of oxidation observed in presence of AA. The inhibition of the compounds at 0.1 mM concentration was determined after preincubation for 6 min with the enzyme in the presence of heme and TMPD and the reaction was started by adding AA. SC-560 has been used as a reference COX-1 inhibitor (Table 2).



SC-560

4.4.2.3. Determination of the reducing activity of the stable radical 1,1-diphenyl-picrylhydrazyl (DPPH) [27,29]. To a solution of DPPH in absolute ethanol an equal volume of the compounds dissolved in DMSO was added. A stock solution (10 mM) for the compounds was used, the concentrations of the final solutions of the compounds were 0.05, 0.1 and 0.2 mM. After 20 and 60 min at room temperature the absorbance was recorded at 517 nm (Table 3).

4.4.2.4. Competition of the tested compounds with DMSO for hydroxyl radicals [27,29]. The hydroxyl radicals generated by the Fe^{3+}/ascorbic acid system, were detected by the determination of formaldehyde produced from the oxidation of DMSO. The reaction mixture contained EDTA (0.1 mM), Fe^{3+} (167 μM), DMSO (33 mM) in phosphate buffer (50 mM, pH 7.4), the tested compounds and ascorbic acid (10 mM). After 30 min of incubation at 37° C the reaction was stopped with CCl_3COOH (17% w/v) and the % competition of the tested compounds with DMSO for hydroxyl radicals was determined (Table 4).

4.4.2.5. Non enzymatic assay of superoxide radical scavenging activity [27,29]. The superoxide producing system was set up by mixing PMS, NADH and air–oxygen. The production of superoxide was estimated by the nitroblue tetrazolium method. The reaction mixture containing compounds, 3 μM PMS, 78 μM NADH, and 25 μM NBT in 19 μM phosphate buffer pH 7.4 was incubated for 2 min at room temperature and the absorption measured at 560 nm against a blank containing PMS. The tested compounds were preincubated for 2 min before adding NADH and the % superoxide radical scavenging activity was determined (Table 4).

4.4.2.6. Heme protein-dependent lipid degradation [27,29]. 50 μM heme, arachidonic acid (0.4 mM) the compounds at the various concentrations tested, H_2O_2 (0.5 mM) were incubated together for 10 min at 37 °C in KH_2PO_4–KOH buffer (50 mM, pH 7.4). The product of peroxidation was detected using the TBA test. The compounds were added in DMSO solution, which has no effect on the assay (Table 5) and % heme protein-dependent lipid degradation was determined.

4.4.2.7. $ABTS^{+\cdot}$ - decolorization assay for antioxidant activity [77]. ABTS was dissolved in water to a 7 mM concentration. ABTS radical cation ($ABTS^{+\cdot}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. For the present study, the $ABTS^{+\cdot}$ solution was diluted with ethanol to an absorbance of 0.70 at 734 nm and equilibrated at 30 °C. Stock

solutions of the tested compounds in DMSO were diluted so that, after introduction of a 10 mL aliquot of each dilution into the assay, they produced between 20% and 80% inhibition of the blank absorbance. After addition of 1.0 mL of diluted $ABTS^{+\cdot}$ solution ($\lambda = 734$ nm) to 10 mL of antioxidant compounds or Trolox standards (final concentration 0–0.1 mM) in ethanol the absorbance reading was taken at 30 °C exactly 1 min after the initial mixing (Table 5).

Ten microliters of the 16 mM linoleic acid dispersion were added to the UV cuvette containing 0.93 mL of 0.05 M phosphate buffer, pH 7.4, thermostatted at 37 °C. The oxidation reaction was initiated at 37 °C under air by the addition of 50 μL of 40 mM AAPH solution. Oxidation was carried out in the presence of aliquots (10 μL) of the tested compounds. In the assay without antioxidant, lipid oxidation was measured in the presence of the same level of DMSO. The rate of oxidation at 37 °C was monitored by recording the increase in absorption at 234 nm caused by conjugated diene hydroperoxide formation (Table 5).

4.4.2.8. Inhibition of linoleic acid peroxidation [78]. Production of conjugated diene hydroperoxide by oxidation of linoleic acid in an aqueous dispersion was monitored at 234 nm. 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) is used as a free radical initiator. This assay can be used to follow oxidative changes and to determine the inhibition of linoleic acid peroxidation induced by each tested compound.

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