Synthesis and Anti-Inflammatory Activity of Chalcones and Related Mannich Bases

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Abstract: Chalcones and Mannich bases have been reported to present antiinflammatory activities as well as inhibitory activities on several factors implicated in inflammation disorders.

A series of chalcones and some related Mannich bases were prepared by Claisen-Schmidt condensation of appropriate acetophenones with appropriate aromatic aldehyde. Mannich bases were derived from chalcones, with formaldehyde and the corresponding amine. The compounds were tested *in vitro* for their ability to inhibit various enzymes involved in the arachidonic acid cascade, for their antioxidant behaviour and *in vivo* for anti-inflammatory activity.

Some chalcones and Mannich bases present strong anti-inflammatory and antioxidant activities. Almost all the tested compounds present high inhibitory activity on lipid peroxidation. Some compounds showed potent inhibitory effect on superoxide anion formation. Among the tested compounds **5** and **6** showed the highest lipoxygenase (LO) inhibitory activity. All the tested compounds inhibit both the proteolytic and esteratic activities of trypsin and chymotrypsin.

The results indicated that the anti-inflammatory effects of the compounds were partially mediated, through their antioxidant activity. Attempts to correlate quantitatively structure with activity revealed that lipophilicity and molar refractivity influence the biological response.

Key Words: Chalcones, Mannich bases, anti-inflammatory, antioxidant.

INTRODUCTION

Chalcones are a chemical class that has shown promising therapeutic efficacy for the management of several diseases. Many papers have been presented in the literature with references to structural modifications of the chalcone template [1]. In fact, not many structural templates can claim association with such a diverse range of pharmacological activities, among, which cytotoxicity, antitumour, anti-inflammatory, antiplasmodial, immunosuppression and antioxidant, are widely cited [2]. They considered as the precursor of flavonoids and isoflavonoids. Chemically they consisted of open-chain flavonoid by a three carbon α,β-unsaturated carbonyl system. [3] Recently much attention has been paid on the synthesis of chalcones mainly from acetophenones and aromatic aldehydes by Claisen-Schmidt condensation.

The anti-inflammatory action of chalcone derivatives *per se* has been examined and appears to be associated with suppression of inflammatory mediators, such as nitric oxide (NO) and tumor necrosis factor- (TNF), which are generated by macrophages stimulated with lipopolysaccharide (LPS) [4]**.** This protective mechanism could derive from simultaneous inhibition of the production of various inflammatory mediators [5] and/or by a direct inhibitory action of the activation of transcription factors (NF-κB, AP-1) that regulate the inflammatory response [4, 6]. The beneficial activity of chalcones may also originate from their ability to induce endogenous cytoprotective pathways such as heme oxygenase-1 (HO-1) [7], a potent antioxidant enzyme that protects against a variety of stressful insults [8, 9].

Published results have revealed that conversion of various acyclic conjugated styryl ketones e.g. chalcones, into the corresponding Mannich bases was often accompanied by increased bioactivity both *in vitro* and *in vivo* [10]. Won *et al*., [11, 12] synthesized (*E*)-1(2hydroxyphenyl)-3(thiophen-2-yl)prop-2-en-1-one, a chalcone derivative (Fig. **1**) which was tested *in vitro* for its inhibitory activity on chemical mediators released from mast cells, neutrophils, macrophages and microglial cells with satisfactory results.

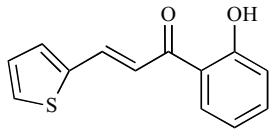

Fig. (1). (*E*)-1(2hydroxyphenyl)-3(thiophen-2-yl)prop-2-en-1-one.

Recently we have reported the synthesis and the biological evaluation of some new aryl-acetic acids (Fig. **2**) having potent antioxidant/anti-inflammatory activity and inhibiting high soybean lipoxygenase [13].

In an effort to continually develop potent anti-inflammatory agents, we tried to modify the above molecules keeping the main –CH(C_6H_5)=CH-CO- group and to synthesize a novel series of chalcones and relative Mannich bases. The

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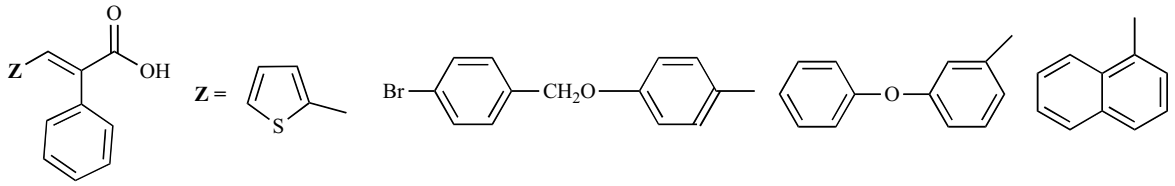


Fig. (2). General Structure of the aryl-acetic acids.

synthesized compounds were screened for detailed free radical scavenging ability, *in vitro* lipoxygenase inhibition, *in vivo* anti-inflammatory activity and they were studied in terms of structure activity relationships.

The suggested structural variations could affect both efficacy and their tolerability partly due to differences in their physicochemical properties, which determine their distribution in the body and their ability to pass through and to enter in the interior membranes [14, 15]. Today there is an increase interest in the development of non-acidic anti-inflammatory agents (NSAIDs), since the used acidic NSAIDs cause development of untoward side effects [16].

CHEMISTRY

The synthesis of the chalcones was accomplished according to the Claisen-Schmidt condensation of appropriate substituted acetophenones or hydroxyacetophenones (o-/p-) with appropriate aromatic aldehyde, as indicated to Scheme **1**. The corresponding reactions proceeded smoothly and in good yields (38-89 %). All the compounds formed were recrystallized by methanol/ether. (Table **1** and Fig. **3**) The synthesis of chalcones **1** [17], **2** [11, 12], **4** [18], **6** [19] and **7** [20] has been reported.

Appropriate hydroxychalcones reacted with formaldehyde and the corresponding amine in ethanol, to give Mannich bases **8**, **9**, **10** and **11** (Scheme **2**). Reactions were monitored by thin layer chromatography. The corresponding reactions proceeded smoothly and in good yields (55-86 %). All the compounds formed were recrystallized by ethanol absolute. (Table **1** and Scheme **2**)

The structures of all the synthesized compounds are confirmed by IR, ^{1}H-NMR, ^{13}C-NMR and elemental analysis. All the derivatives present the characteristic absorption in the IR (nujol) ((O-H), (C=O), (C=C)). The ^{1}H-NMR spectra spectroscopy revealed that they possessed the E configuration which was based on the J values of 9-15 Hz of the olefinic protons (Tables **2a**-**2c**). The ^{13}C-NMR spectra also confirmed the suggested structures. The results are consistent with the proposed structures and are in agreement with previous findings [21].

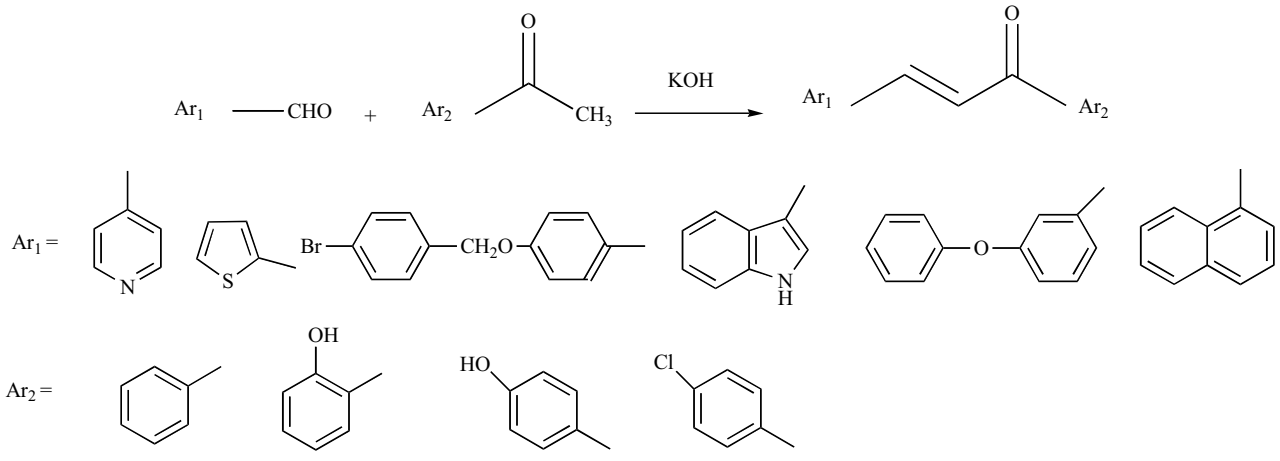
Physicochemical Studies

a) Determination of Lipophilicity as R_M Values

Since lipophilicity is a significant physicochemical property determining distribution, bioavailability, metabolic activity and elimination, we tried to determine experimentally their lipophilicity from RPTLC method as R_M values and to compare them with the corresponding theoretically calculated clog *P* values in n-octanol-buffer [22]. This is considered to be a reliable, fast and convenient method for expressing lipophilicity. Apart from the important role of lipophilicity for the kinetics of biologically active compounds, antioxidants of hydrophilic or lipophilic character are both needed to act as radical scavengers in the aqueous phase or as chain-breaking antioxidants in biological membranes.

From our results (Table **1**) it can be concluded that R_M values could be used as a successful relative measure of the overall lipophilic/hydrophilic balance of these molecules. We tried to linearly correlate the R_M values with the theoretically calculated clog P values.

The correlation coefficient r is low (r = 0.694). We could attribute this to the different nature of the hydrophilic and lipophilic phases in the two systems.



Scheme 1: Synthesis of chalcones.

Table 1. Synthesized Chalcones and Mannich Bases and their Physicochemical Properties. Lipophilicity Values: Experimentally Determined R_M and Theoretically Calculated clog *P* Values

Compound	ClogP*	R_M#	m.p. (ºC)	yield	Formula#
1	2.46	-0.694	a	70	
2	3.60	0.051	b	38	
3	6.51	0.954	113-118	59	$C_{22}H_{17}O_3Br$
4	6.06	0.303	c	62	$C_{21}H_{16}O_3$
5	6.70	0.489	115-118	89	$C_{22}H_{16}O_2BrCl$
6	4.57	0.053	d	87	
7	3.03	-0.513	e	54	
8	3.39	-0.044	66-69	86	$C_{16}H_{17}NO_2S$
9	6.29	-0.013	119-122	55	$C_{25}H_{24}NO_3Br$
10	5.84	-0.688	278-280	80	$C_{24}H_{23}NO_3$
11	2.60	-0.484	189-191	61	$C_{23}H_{27}N_3O_2$

*Theoretically calculated clog *P* values; #R_M values are the average of at least 10 measurements; #the given formulas correspond to the new synthesized compounds; a [17], b [11], c [18], d [19], e [20].

b) Determination of Lipophilicity as Clog P

Lipophilicity was theoretically calculated as Clog P values in n-octanol-buffer by CLOGP Programme of Biobyte Corp [23].

BIOLOGICAL RESULTS AND DISCUSSION

In this investigation, we synthesized some novel chalcones and Mannich bases that were expected to offer protection against inflammation and radical attack and inhibition of LO, by application of standard synthetic methods summarized in Schemes **1-2**.

The interaction of the examined compounds with the stable radical DPPH was studied at 0.1, 0.2 and 1mM after 20 and 60 min (Table **3**), indicating their scavenging/reducing ability in an iron free system.The reducing abilities are very low with the exception of chalcones **3** and **4**. No differ-

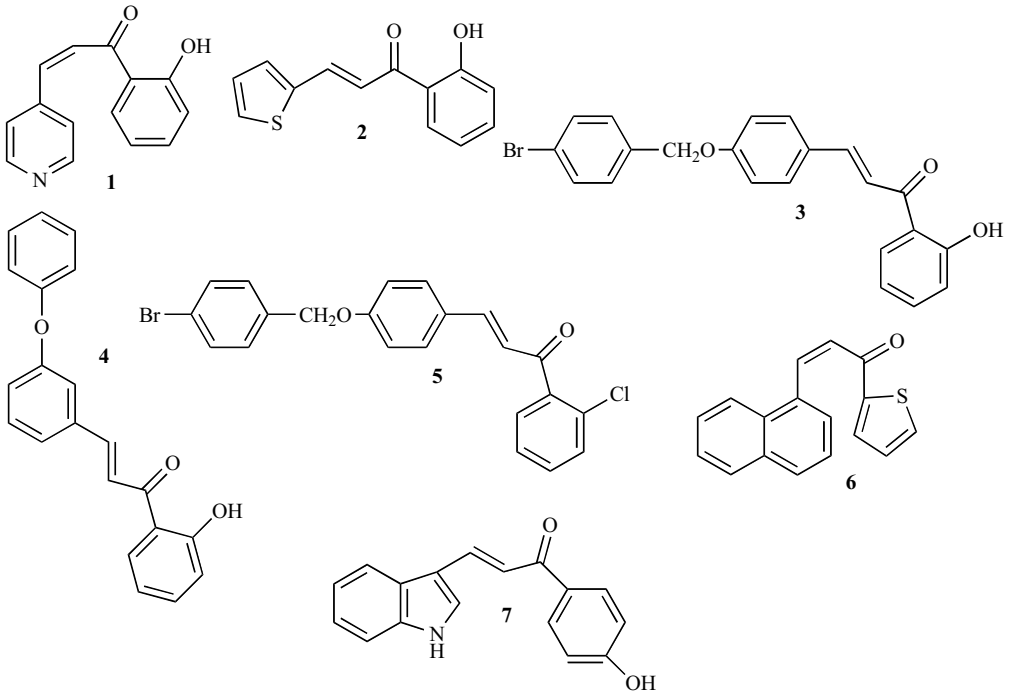
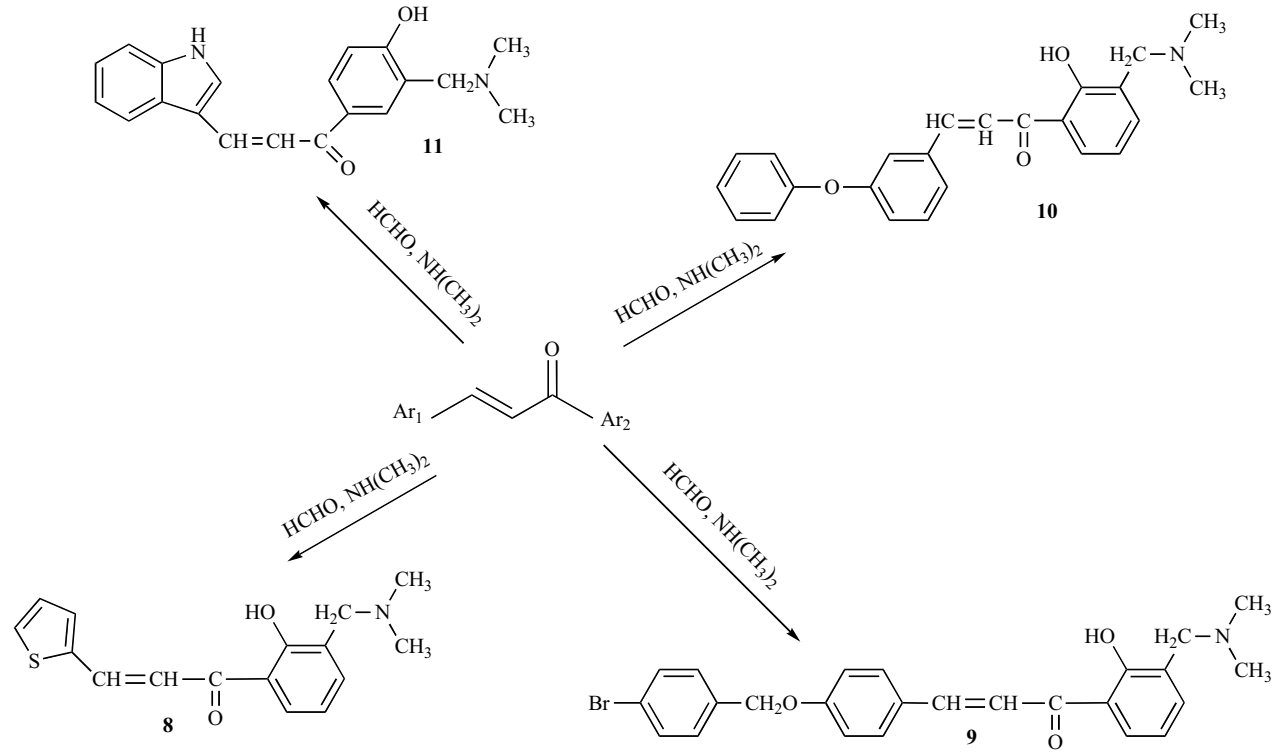


Fig. (3). Structures of the synthesized chalcones 1-7.



Scheme 2. Synthesis of Mannich bases.

Table 2a. [1]H-NMR Data for the New Chalcones and Mannich Bases

N	δ ppm
3*	5.17(2H, s), 6.91-792 (12H m aromatic and 2H db), 9.88 (OH, s)
5*	5.12 (2H, s, CH_2), 6.89-7.97 (12H, m 10 aromatic and 2H db)
8**	2.20 (6H, s –$N(CH_3)_2$), 4.20-4.48 (2H, br,-CH_2-), 6.92-7.01 (1H, m aromatic), 7.40-7.41 (4H, m 3H aromatic and 1H db),7.51-7.89 (2H, s, aromatic), 8.02-8.07 (1H, d –CH=, *J* = 15 Hz)
9**	1.60 (6H, s, -–$N(CH_3)_2$), 2.89 (2H, s, -CH_2), 5.10 (2H, s, -CH_2), 6.99-7.68 (12H, 11 aromatic and 1H db), 7.83-7.86 (1H, d –CH=, *J* = 9 Hz), 9.89 (1H, s OH)
10**	1.79-2.40 (6H, m–$N(CH_3)_2$), 3.40-3.83 (2H, br –CH_2-), 5.19 (1H, s –OH), 6.83 (1H, s aromatic), 7.04—7.60 (13H, 11 aromatic and 2H db)
11**	2.00-2.79 (6H, m–$N(CH_3)_2$), 4.54 (2H, s, -CH_2), 6.86-7.49 (6H, m aromatic), 7.90-7.95 (1H, d , -CH= , *J* = 15 Hz), 8.23-8.26 (2H, m 1H aromatic and 1H from the db) 10.00 (1H, s –OH), 11.40 (1H, s, NH)

*Measured in $CDCl_3$; ** Measured in $CDCl_3$-DMSO-d_6.

Table 2b. [13]C-NMR Data for the New Chalcones and Mannich Bases

N	δ ppm
3*	40.02, 69.40, 69.46, 76.57, 77.00, 77.42, 115.10, 115.36, 118.00, 118.60, 118.76, 120.10, 122.30, 127.82, 129.05, 130.31, 130.54, 131.86, 132.00, 135.40, 136, 145.09, 160.82, 163.37, 163.57 , 193.62
5*	76.58, 115.33, 119.50, 122.13, 128.00, 128.88, 129.00, 129.82, 130.31, 131.83, 135.40, 145.00, 160.63, 189.16
8**	76.10, 76.60, 77.00, 118.20, 118.40, 128.10, 129.00, 132.20, 132.30, 135.90, 137.00, 137.40, 177.20, 192.70
9**	44.50, 58.70, 61.10, 69.30, 69.50, 115.40, 127.80, 129.00, 131.00, 134.00, 139.00,140.00, 148.00, 154.00, 163.40, 190.70
11**	40.20, 40.50, 55.00, 102.70, 107.60, 112.00, 121.40, 122.30, 123.60, 136.50, 137.20, 150.70., 168.00, 185.00

*Measured in $CDCl_3$; ** Measured in $CDCl_3$-DMSO-d_6.

Table 2c. IR Data for the New Chalcones and Mannich Bases

N	cm^{-1}
3	3060, 3100,1680, 1580, 1100
5	1660, 1580, 1250, 650
8	3050, 1640, 1580, 1250
9	3450, 1680, 1570, 1250, 630
10	3500-3450 broad, 1710, 1580
11	3450, 1680, 1620, 1580, 1250

Table 3. Interaction % with the Stable Radical DPPH

Compounds	% 0.1 mM		% 0.2 mM		% 1 mM	
	20 min	60 min	20 min	60 min	20 min	60 min
1	4	4	1	1	no	no
2	17	20	20	23	17	23
3	46	46	nd	nd	nd	nd
4	45	44	nd	nd	nd	nd
5	3	2	1	1	no	2
6	4	5	2	2	no	2
7	3	4	10	8	1	4
8	no	no	1	6.4	no	1
9	2	5	10	9	no	no
10	3	4	2	1	5	2
11	no	4	10	9	7	13

no: no action under the experimental conditions; nd not determined under the reported conditions NDGA 81 % and 83 % at 0.1 mM (20 and 60 min), 80 % at 0.2 mM and 95.6 % at 1 mM.

ences were observed within the compounds with the time and the concentration. The presence of hydroxyl group is correlated with higher activity e.g. chalcone **3** is more potent than **5,** in which hydroxyl group has replaced by a chlorine atom. Lipophilicity of the whole molecule does not seem to influence the results. A decrease in the interaction values was observed when chalcones **3** and **4** were transformed to Mannich bases.

It is known that the rates of reactive oxygen species ROS production are increased in most diseases [24, 25]. The cytotoxicity in living systems is mainly due to the transformation of $O_2^{\cdot-}$ and the H_2O_2 into hydroxyl radicals, reactive metal and $^1O_2$. During the inflammatory process, phagocytes generate the superoxide anion radical at the inflammed site and this is connected to other oxidizing species.

Non enzymatic superoxide anion radicals were generated and the compounds were tested as possible scavengers. The majority of the compounds present medium scavenging activity (**2**, **5**, **6**, **9** and **11**) ranged from 19-44 %. Two chalcones **1** and **4** do not present any activity. Chalcone **3** is more potent compared to **5** in which a chlorine atom has replaced the OH group. (Table **4**) Lipophilicity of the whole molecule does not seem to increase the scavenging activity. The most potent chalcone **7** and the most potent Mannich base **8,** present low *clog P* values (3.03 and 3.39 respectively, Tables **1** and **4**). In some cases, Mannich bases scavenge superoxide anion stronger than the corresponding chalcones (e.g. **2** and **8**).On the contrary Mannich bases **9** and **11** present lower scavenging ability than the corresponding chalcones **3** and **6**. (Table **4**) In one case (compounds **4** and **10**), the results remain constant.

We tried to study the behaviour of chalcones and Mannich bases on lipid peroxidation. As a model of such reactions we used the peroxidation of arachidonic acid by a mixture of heme and H_2O_2. Mixing heme proteins with H_2O_2 generates powerfully oxidizing activated heme species and radicals on amino-acids side chains that can cause lipid peroxidation. Comparing the % inhibition values of lipid peroxidation among the tested o-OH substituted chalcones,

Table 4. % Superoxide Radical Scavenging Activity ($O_2^{-\cdot}$); % Inhibition of Heme Dependent Lipid Peroxidation (LP %); *In Vitro* Inhibition of Soybean Lipoxygenase (LO) (IC_{50}); Inhibition % of Induced Carrageenin Rat Paw Edema (CPE %)

Compounds	$O_2^{-\cdot}$ (%) 0.1mM	% LP- 0.1 mM	IC_{50} µM	CPE (%)[a] 0.01 mmoles/ kg body weight
1	No	83	680	52**
2	44	54	425	45**
3	60	35	450	22**
4	No	15	140	31**
5	33	85	80	55**
6	42	83	55	45**
7	77	85	515	80**
8	63	44	380	66**
9	19	36	350	38**
10	7	25	1000	8**
11	25	74	(77 % at 0.1mM)	77**
CA	86	6	600	
Indomethacin	47*			

no: no action under the experimental conditions; [a] statistical studies were done with student's T-test, * $p<0.01$, ** $p<0.05$.

compound **1** is the most effective. Between chalcones **3** and **5,** the o-Cl (**5**) analogue shows higher inhibition. (Table **4**) Lipophilicity of substituent influences the biological activity (π-*OH* = -0.67, π- *Cl* = 0.71 π-*OH* < π-*Cl*). Higher π value is correlated with potent inhibition Taking under consideration the transform of chalcones to Mannich bases the % inhibition values of lipid peroxidation remain almost the same for chalcone **2** and Mannich base **8**, chalcone **3** and Mannich base **9.** For Mannich base **10** the inhibition increases (chalcone **4**) and for base **11** it lowers (chalcone **7**). Lipophilicity does not affect inhibition.

We tried to linearly correlate the % of lipid peroxidation inhibitory activity (LP %).

$$\text{Log LP \%} = -0.061\ (0.048)\ \text{CMR} - 0.280(0.165)\ I_{2\text{-OH}} + 2.471(0.454) \qquad \textbf{Eq. 1}$$

$N = 9$, $r = 0.898$, $r^2 = 0.806$, $q^2 = 0.46$, $s = 0.100$, $F_{2,6} = 12.4$, $\alpha = 0.01$

The indicator variable $I_{2\text{-OH}}$ takes value 1/0 for the examples where a hydroxyl group exists at 2-position (or ortho-position) of the phenyl ring. The presence of 2-OH group has a negative effect. CMR refers to overall molar refractivity. Since MR is primarily a measure of bulk and of polarizability of the molecule the negative sign with CMR suggest steric hindrance and in a rough way the larger the molecule is, the lower the inhibition of lipid peroxidation. Lipophilicity does not contribute to the biological activity. *Clog P* cannot replace MR. Although, the above equation **1** is not sharp in terms of r, it clearly points out the significant role of steric effects.

Lipoxygenases are a class of non heme iron containing enzymes that contribute to the eicosanoid pathway. These enzymes are precursors for the inflammatory mediators, leukotrienes and lipoxins and thus we found interesting to test our compounds for their inhibitory activity against soybean LO. In this investigation all compounds were studied in order to gain insight into their LO-inhibition. We used the UV absorbance based enzyme assay [26]. While one may not extrapolate the quantitative results of this assay to the inhibition of mammalian 5-LO, it has been shown that inhibition of plant LO activity by NSAIDs is qualitatively similar to their inhibition of the rat mast cell LO and may be used as a simple qualitative screen for such activity.

Perusal of IC_{50} values shows that compound **6** is the most active, within the set followed by chalcone **5** (Table **4**). The most of the LO inhibitors are antioxidants or free radical scavengers [27], since lipoxygenation occurs *via* a carbon centred radical. Some studies suggest a relationship between LO inhibition and the ability of the inhibitors to reduce the Fe^{3+} at the active site to the catalytically inactive Fe^{2+}. LOs 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 LO inhibitors are excellent ligants for Fe^{3+}. It has been demonstrated that their mechanism of action is presumably related to its coordination with a catalytically crucial Fe^{3+}.

Among the o-OH substituted chalcones, compound **4** is the most potent. Comparing the nature of o-substituent on the substituted acetophenone, chalcone **5** is more potent than **3** (IC_{50} 80µM) (Table **4**). It seems that the lipophilicity, π-values of o-substituents influences the inhibitory activity (π-

OH = -0.67, π- *Cl* = 0.71). For Mannich base **11** it was not possible to determine the IC_{50} value (Table **4**).

Regression analyses of the IC_{50} LO values revealed that lipophilicity as experimental lipophilicity values-R_M values- is the main physicochemical parameter that influences the biological response (Eq. 2). Lipophilicity is referred [28-31] as an important physichochemical property for lipoxygenase inhibition and in this case is verified by our experimental data.

$$\text{Log } 1/\text{ IC}_{50} = 0.874(0.513)\ R_M + 3.691(0.258) \qquad \textbf{Eq. 2}$$

N= 8, r = 0.862, $r^2 = 0.743$, $q^2 = 0.622$, s = 0.27, $F_{1,6} = 17.37$, $\alpha = 0.01$

Considering the role played by proteases in the early stage of inflammatory process we evaluated the ability of compounds to inhibit these enzymes. All the tested compounds highly inhibit trypsin at 0.1mM. No differences are observed between chalcones **2**, **3**, **4** and their corresponding Mannich bases, which all significantly inhibit trypsin induced proteolysis. A decrease is observed in the case of chalcone **7** and its base **11** (Table **5**).

Regression analyses of % inhibition values of trypsin proteolysis (% TrPr) indicates that lipophilicity expressed in theoretically calculated values of lipophilicity *clog P*, mainly affects the biological response. It was not possible to correlate our experimental data with the experimentally determined lipophilicity values R_M.

$$\text{Log \% TrPr} = 0.097(0.056)\textit{clog P} + 1.363(0.273) \qquad \textbf{Eq. 3}$$

N =9, r = 0.845, $r^2 = 0.714$, $q^2 = 0.526$, s = 0.108, $F_{1,7} = 17.41$, $\alpha = 0.01$

Although Eq. **3** is not sharp in terms of it points out the role of lipohilic effects.

Mannich bases **9** and **11** inhibit strongly chymotrypsin induced proteolysis. In general, chalcones seems to be poor inhibitors (with the exception of chalcone **5**). This does not stand for their bases. The indolyl Mannich base is the most potent against chymotrypsin. No role for lipophilicity was found, although the most potent compound **5** has the higher *clog P* value (6.70) Table **5**.

The indolyl derivative **11** presents the lower IC_{50} value on the inhibition of trypsin acting as an esterase. Taking under consideration the nature of o- substituents, chalcone **5** (o-Cl) presents higher inhibition compared to chalcone **3** (o-OH). It seems that the positive hydrophobic contribution of o-Cl grou*p* *(π- o-Cl = 0.71)* increases the biological response. The same results have been taken from chymotrypsin. Again chalcone **5** was found the most potent (Table **5**).

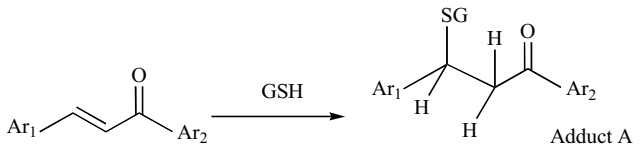
Chalcones **3** and **4** highly inhibit the enzymic activity of trypsine as esterase. In this case the o-OH substituted derivative is more potent than the corresponding o-Cl. Overall lipophilicity of the molecules-as *clog P*-does not seem to influence this type of inhibition. Mannich bases **8**, **9** and **11** present higher IC_{50} values compared to the corresponding chalcones. On the contrary chalcone **4** is more potent than Mannich base **10**.

No much difference is observed between the IC_{50} values of chalcones **3** and **5**. Mannich base **11** is the most potent among the other bases and equipotent to its chalcone. Chalcone **3** is less active than base **9** and chalcone **2**~**8**. The stereochemistry of the aldehydic part of the chalcone (Ar_1) seems to be important. Considering chalcones **1, 2**, **3** and **4**, compound **4** is more effective.

Table 5. ***In Vitro*** **Inhibition % of Trypsin Proteolytic Activity (Tr.Pr %);** ***In Vitro*** **Inhibition of Chymotrypsin Proteolytic Activity IC_{50};** ***In Vitro*** **Inhibition of Trypsin Esteratic Activity IC_{50};** ***In Vitro*** **Inhibition of Chymotrypsin Esteratic Activity IC_{50}**

Compounds	Tr. Pr % 0.1mM	Ch- pr. IC_{50} µM	Tr.- Est. IC_{50} µM	Est.-Ch- IC_{50} µM
1	50	650	Nd	Nd
2	96	(39% at 0.1mM)	420	300
3	100	(90% at 0.1mM)	135	450
4	100	(40% at 0.1mM)	115	140
5	79	20	470	500
6	55	(90% at 0.1mM)	460	370
7	39	530	185	60
8	100	100	350	315
9	100	35	420	330
10	90	300	Nd	Nd
11	33	10	70	61
Salicylic acid	54		100	> 100

Glutathione conjugation is an important pathway by which reactive electrophilic compounds are detoxified. It protects vital cellular constituents against chemical reactive species by virtue of its nucleophilic sulphydryl group and constitutes an *in vivo* antioxidant protective mechanism.The nucleophilic addition of GSH to electron-deficient carbon double bonds occurs mainly in compounds with α,β-unsaturated double bonds.[32] Alkylation with a cellular thiol such as glutathione GSH may occur with the α,β-unsaturated chalcones and Mannich bases, leading to the adducts A and we have studied this possibility. (Scheme **3**) From our experimental results it points out that α, β-unsaturated compounds **2**, **3**, **6**, **7**, **8** and **11** are conjugated with GSH and present significant alkylation rates. For these compounds an alkylation may to occur, leading to the adduct A (Scheme **3**).



Scheme 3.

For the compounds **3** and **7** the alkylation proceeds, higher when the concentration of GSH is high (10GSH). For compound **6** the alkylation proceeds higher when the concentration of GSH is low (0.2 mM) due to stereochemical reasons. All compounds

In acute toxicity experiments, the *in vivo* examined compounds did not present toxic effects in doses up to 0.5 mmol/kg body weight. Ulcerogenicity was not found. 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-inflammarory effects of the tested compounds were assessed by using the carrageenin-induced rat paw edema (CPE) model and are presented in Table **4**, percentage of weight increase at the right hind paw. The induced oedema is a non-specific inflammation highly sensitive to non-steroidal anti-inflammatory agents (NSAIDs). Thus it has been accepted as a useful tool for studying new anti-inflammatory agents [33]. It reliably predicts the anti-inflammatory potency of the NSAIDs and detects during the second phase that are anti-inflammatory agents as a result of inhibition of prostaglandin amplification [34]. All the tested compounds induced protection (ranged from 8-80 %) against to carrageenin induced paw edema while the reference drug indomethacin induced 47 % protection at an equivalent dose. Chalcone **7** was the most potent (80 %) followed by the corresponding Mannich base **11** (77 %). The presence of the o-Cl -group in chalcone **5** seems to highly increase the biological response. The lipophilic contribution π of this group is important ($\pi = 0.71$).

We tried to linearly correlate the expressions of anti-inflammatory, antioxidant, free radical scavenging activity and LO inhibition activity for all tested compounds. None of these correlations were satisfactory enough ($r < 0.6$). Presumably these activities proceed *via* at least partially different mechanisms.

In this investigation all compounds were studied in order to gain insight in the mechanism of their antiphlogistic action. It is well known that free radicals play an important role in inflammatory process. [35] Consequently, compounds with antioxidant properties could be expected to offer protection in rheumatoid arthritis and inflammation and to lead to potentially effective drugs. In fact, many non steroidal anti-inflammatory drugs have been reported to act either as inhibitors of free radical production or as radical scavengers. [36] Thus, we tested these derivatives with regard to their antioxidant ability and in comparison to well known antioxidant agents eg. nordihydroguaiaretic acid NDGA, caffeic acid CA

CONCLUSION

In conclusion, the broad spectrum of the observed antioxidant activity of the majority of the examined compounds allows us to propose them in treating human diseases that involves ROS**.**

The present study has shown that certain chalcones and the corresponding Mannich bases possess significant anti-inflammatory activity. Their synthesis is almost simple with satisfactory yields. Most of them are potent superoxide anion scavengers and inhibit *in vitro* soybean lipoxygenase and lipid peroxidation. Hydrophobicity, the presence of a free 2-OH group and steric requirements, are the most important factors in terms of SAR

Although the anti-inflammatory mechanism explaining the activity on CPE remains unclear, the *in vivo* antiinflammatory activity of the synthesized compounds seems to be related with their high scavenging and reducing activity, *in vitro*.

Three compounds **7**, **8** and **11** were found to present a promising anti-inflammatory profile, whereas compound **5** and **6** present high LO inhibitory activity.

MATERIALS AND METHODS

General

All the chemicals used were of analytical grade and commercially available by Merck, 1,1-diphenyl-2-picrylhydrazyl (DPPH), nordihydroguairetic acid (NDGA) are purchased from the Aldrich Chemical Co. Milwaukee, WI, (USA). Soybean Lipoxygenase, linoleic acid sodium salt. Arachidonic Acid (AA), NADH, Nitrotetrazolium Blue (NBT), porcine heme and indomethacin were obtained from Sigma Chemical, Co. (St. Louis, MO, USA) and carrageenin, type K, was commercially available. For the *in vivo* experiments, male and female Fischer-344 rats (180-240 g) were used. N-methylphenazonium-methyl sulfate was purchased by Fluka.

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 Hitachi U-2001 spectrophotometer. Infrared spectra (film as Nujol mulls) were recorded with a Shimadzu FTIR-8101M. The ^{1}H Nucleic Magnetic Resonance (NMR) spectra were recorded at 300MHz 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. Elemental analyses were obtained on an acceptable range (± 0.4 %) in a Perkin-Elmer 240B CHN analyzer (The Perkin-Elmer Corporation Ltd.). Reactions were monitored by thin-layer chromatography (TLC) by Fluka, on aluminum cards precoated with 0.2 mm of silica gel and fluorescent indicator.

General Procedure for the Preparation of Compounds 1-7 [37, 11, 17, 18,19, 20]

The mixture of the aromatic aldehyde (10 mmol) and the acetylated aromate (10 mmol) were dissolved in 30 mL of MeOH and stirred during addition of 5 mL of potassium hydroxide (15% m/V). After 24 hrs the product precipitated. About 2 mL of glacial acetic acid were added. Solids were then filtered with suction and washed twice with cold MeOH. The chalcones formed in this manner were used for the preparation of the corresponding Mannich bases after purification (crystallization from methanol/ether). Synthesis and physicochemical properties of compounds **1, 2**, **4**, **6** and **7** are cited in references [12, 17, 18, 19, 20].

General Procedure for the Preparation of the Mannich Bases 8-11 [38]

A mixture of chalcones **2, 3, 4** (0.01 mol), formaldehyde (36-38% w/v, 0.01 mol), and dimethylamine (0.01 mol) in ethanol (40 mL) was heated under reflux for 40 - 80 hrs. A mixture of chalcone **6** (0.005 mol), formaldehyde (36-38% w/v, 0.01 mol) and dimethylamine (0.01 mol) in ethanol (40 mL) was heated under reflux for 80 hrs. On cooling, the solvent was removed in vacuo, yielding an oil which was dissolved and recrystallized from absolute ethanol. Reactions are monitored by thin layer chromatography TLC.

Physicochemical properties of the new compounds are indicated in Table **1** whereas spectroscopical and elemental analyses are given in Tables **2a-c**.

Biological Experiments

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.

Determination of the Reducing Activity of the Stable Radical 1,1-diphenyl-picrylhydrazyl (DPPH) [32]

To a solution of DPPH in absolute ethanol an equal volume of the compounds dissolved in DMSO was added. As control solution ethanol was used. The concentrations of the solutions of the compounds were 0.1, 0.2 and 1 mM. After 20 and 60 min at room temperature the absorbance was recorded at 517 nm and compared with the appropriate standards NDGA (Table **3**).

Non Enzymatic Assay of Superoxide Radicals-Measurement of Superoxide Radical Scavenging Activity [32]

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. Caffeic acid was used as an appropriate standard (Table **4**).

Heme Protein-Dependent Lipid Degradation [32]

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. Caffeic acid was used as an appropriate standard (Table 4).

Soybean Lipoxygenase Inhibition Study *In Vitro [32]*

In vitro study was evaluated as reported previously. The tested compounds dissolved in DMSO were incubated at room temperature with sodium linoleate (0.1 mM) and 0.2 ml of enzyme solution (1/9 x 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 **4**). Caffeic acid was used as an appropriate standard.

Inhibition of Proteolysis of Trypsin and Chymotrypsin Activities [32]

0.2 ml of 100μg/ml trypsin or chymotrypsin solution was added to 0.8 ml of 0.1mol/phosphate buffer (pH 7.6) including the tested compounds and preincubated at 37° C for 30 min and then 1 ml of 1% albumin in 0.1 mol/l phosphate buffer was added and incubated at 37° C for 30 min. After the incubation 3ml of 5% trichloroacetic acid was added to the incubated solution and allowed to set at room temperature for 1 hr. The absorbance at 280 nm was recorded and the IC_{50} values of the tested compounds were determined

Inhibition of Trypsin and Chymotrypsin Activities as Esterases [39]

Tosyl arginine methyl ester (TAME) was used as substrate for trypsin whereas BTEE was used as substrate for Chymotrypsin. The reaction mixture consisted of 1.5mL buffer (0.1M tris–HCl, pH 7.8 in 50% methanol, v/v) and 1.4mL TAME or BTEE (0.01M in 50%, v/v methanol). The test compounds dissolved in DMSO were added and their IC_{50} values were determined. The reaction was started by addition of 0.1mL trypsin or chymotrypsin(1mg/mL 0.001N HCl). The increase in the absorbance at 256 nm was determined over the next 4 min (Table **5**).

Stability Studies and Incubation with Glutathione (GSH) [40]

Solutions of the compounds were prepared in water using phosphates buffer solution (PBS) pH 7.4 and in order to achieve dissolution the solvent contained approximately 10% v/v alcohol. The concentrations of the solutions were chosen so that the absorption maxima were between 0.5 and 1. The test compounds are incubated for 24h at 37°C and their UV

spectra were recorded. All determinations were carried out in duplicate. The error limits of the ε values were approximately 2%. The experiment was repeated in the presence of GSH using thiol/test compound, 2/1 and 10/1 and incubation at 37°C for 24h and their UV spectra were recorded. The results are given in Table **6**.

Table 6. Stability Studies and Incubation with Glutathione (GSH)

α/α	λ_{max}	ε_{max}
1*	260	2440
1**	260	2695
1***	260	5500
2*	320	3360
2**	320	3165
2***	320	4365
3*	360	280
3**	360	200
3***	360	80
4*	310	4090
4**	310	4110
4***	310	4360
5*	370	1335
5**	370	3035
5***	370	2320
6*	300	8905
6**	300	1430
6***	300	6770
7*	300	23225
7**	300	10425
7***	300	6755
8*	330	3295
8**	330	2905
8***	330	3435
9*	250	7720
9**	250	8775
9***	250	13690
10*	270	2170
10**	270	2390
10***	270	4605
11*	300	19670
11**	300	16490
11***	300	18165

*compound 0.1mM + 0 mM GSH.

** compound 0.1mM + 0.2 mM GSH.

***compound 0.1mM + 1mM GSH.

In VIVO ASSAYS

Inhibition of the Carrageenin-Induced Edema [32]

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 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 uninjected 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 Table **4**. Indomethacin in 0.01 mmol/kg (47 %). Values CPE % are the mean from two different experiments with a standard error of the mean less than 10 %.

Statistical Analysis

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. The statistical significance for the in vivo results was performed using the Student's T-test.

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