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Synthesis and pharmacological activity of chalcones derived from 2,4,6-trimethoxyacetophenone in RAW 264.7 cells stimulated by LPS: Quantitative structure–activity relationships

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Abstract—Inhibition of nitric oxide (NO) production by altering the expression of induced enzymes involved is potentially an important strategy for obtaining antiinflammatory agents. In the search for hits to obtain lead compounds for new drugs of this class, 14 synthetic chalcones derived from 2,4,6-trimethoxyacetophenone were evaluated in terms of their inhibitory action, in vitro, in relation to NO production in murine macrophages of the line RAW 264.7 induced by bacterial lipopolysaccharides (LPS). All the compounds were obtained by aldolic condensation between the acetophenone and corresponding aldehydes, under basic conditions. The mean IC_{50} values, calculated through dose versus inhibitory effect curves, in four independent experiments, varied between 1.34 and 27.60 μM, and were compared with the positive control, compound 1400W (IC_{50} = 3.78 μM), a highly selective inhibitor of iNOS (induced nitric oxide synthase). Eight chalcones gave mean IC_{50} values less than or equal to those obtained for 1400W, which suggests that these molecules may act as inhibitors of inflammatory process. The QSAR study reveals that electron-withdrawing groups in the B-ring seem to increase the inhibition of nitrite production, mainly when in position 2. A substitution in the ortho position of the A-ring seems to be necessary for the activity.

1. Introduction

In the complex reaction systems involved in the response of a host to pathogens, chemical mediators, such as nitric oxide (NO), play a pivotal role. NO is a free radical gas produced from L-arginine and molecular oxygen by catalytic reaction of nitric oxide synthase (NOS),[1] which exists in three different isoforms: endothelial NOS (eNOS) and neuronal NOS (nNOS), which are constitutively expressed and play an important role in physiological processes including neurotransmission and vascular homeostasis, and inducible NOS (iNOS), which can be expressed by many cells, such as macrophages, Kupffer cells, neutrophils, fibroblasts, vascular smooth muscle cells, and neuronal and endothelial cells in response to pathological stimuli.[2]

Most interest has been focused on the mechanism of iNOS expression in macrophages and consequently the role of NO production in their immune responses. NO can react with superoxide derived from macrophages to generate highly cytotoxic peroxynitrite which destroys the invading microorganisms.[3,4] iNOS is highly expressed by lipopolysaccharide (LPS)-activated macrophages and this contributes to the pathogenesis of septic shock.[5] Thus, the inhibition of NO production by iNOS is an important target in the treatment of inflammatory diseases.

Chalcones are essential intermediate compounds in flavonoid biosynthesis, and they are easily found in arboreal or smaller plants. They can be obtained by several chemical methods and Claisen–Schimidt's condensation (aldolic condensation) is the most used one.[6] Many studies have shown that they are compounds of great chem-

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ical and pharmacological interest because they exhibit many biological activities,[7,8] such as antifungal,[9,10] antiviral,[11] antibacterial,[12] antimalarial,[13] anti-leishmania,[14,15] antinociceptive,[16] antiinflammatory,[17–19] and anti-tumoral,[18,20,21] among others. Substitutions in the A- and B-rings of chalcones can result in compounds with different biological activities.

Hydroxylated and chlorated chalcones have been reported to inhibit NO synthesis and also iNOS protein expression in RAW 264.7 cells.[18,19] Other chalcones derived from 4-dimethylaminochalcone have also been found to inhibit nitrite production and iNOS protein expression in the LPS-stimulated RAW 264.7 macrophage.[17,22] 2′-substituted chalcones, including those derived from 2,4,6-trimethoxyacetophenone, have been found to be effective in inhibiting the biosynthesis of interleukin (IL)-1 from human peripheral blood monocytes stimulated with LPS.[23]

In this study, we evaluated the inhibition of nitrite production in RAW 264.7 cells by some chalcones derived from 2,4,6-trimethoxyacetophenone. A quantitative structure–activity analysis was carried out to determine a relationship with the pharmacological effects.

2. Results and discussion

2.1. Chalcones

2,4,6-Trimethoxyacetophenone (**TMA**) and 14 synthetic chalcones derived from it were prepared as shown in Figure 1,[6] with yields varying between 43% and 97%. All reagents used were obtained commercially (Merck, Sigma–Aldrich), except xanthoxyline, which was prepared as previously described.[24] This method permitted the obtainment of substituted compounds in the B-ring. The lowest yields were obtained with compounds **2** and **13**. In the case of **13**, the low yield can be explained by the presence of a dimethylamine group, which can stabilize the carbonyl group in the aldehyde, decreasing its reactivity. Regarding compound **2**, the presence of a nitro group in position 3 in the aldehyde must increase the electropositivity of the carbonyl group, increasing its reactivity and consequently its yield; however, this was not observed and the low yield may be due to losses during the purification process. Compounds **4** and **5** also possess a nitro group in positions 2 and 4, and, as the other compounds, presented good yields.

Compound **11** exhibited all the spectroscopic data, including the elementary analysis, confirming the proposed structure; however, its experimental melting points was different from those reported in the literature. Five new compounds (**1**, **3**, **4**, **6** and **14**) were confirmed by chemical identification data: ^{1}H NMR, ^{13}C NMR, IR, and elementary analysis. ^{1}H NMR spectra revealed that all the structures were geometrically pure and configured E ($J_{H\alpha-H\beta}$ = 14–17 Hz).

2.2. Evaluation of chalcone's ability to inhibit NO production

Chalcones were evaluated for their ability to inhibit NO synthesis by the RAW 264.7 macrophage stimulated with LPS. Their IC_{50} values were calculated for concentrations from 0.1 to 30 μM and compared with those of the positive drug 1400W (*N*-(3-(aminomethyl)benzyl)acetamidine), a highly selective inhibitor of iNOS, which is 5000 times more selective to this isoform than eNOS, and 200 times more than nNOS.[25,26] All compounds were able to inhibit the nitrite production in a concentration-dependent manner with mean IC_{50} values varying from 1.34 to 27.60 μM. Table 1 shows the yields, IC_{50} values, and percentage (%) of cellular viability at the IC_{50} level.

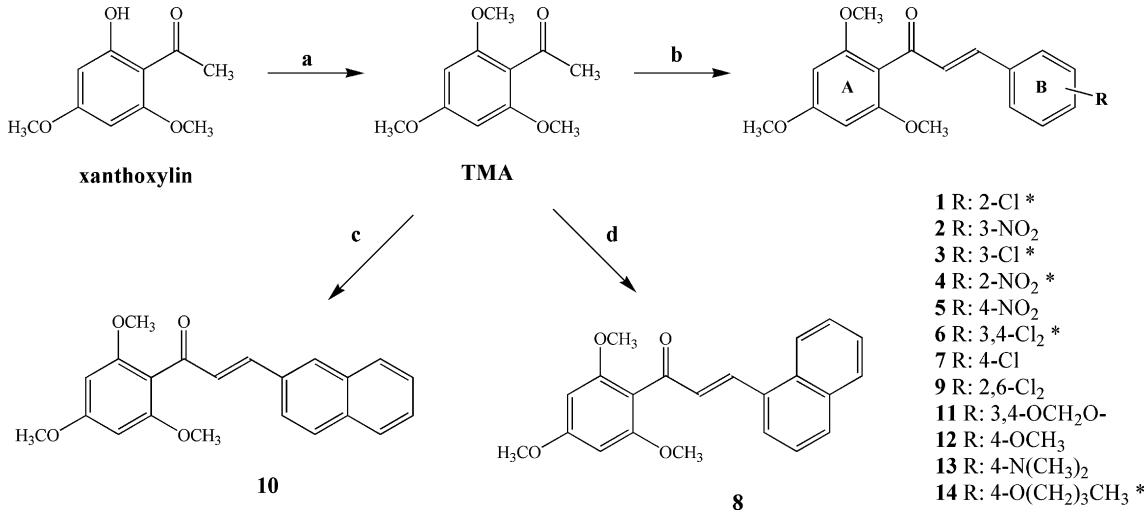

Figure 1. Synthesis of 2,4,6-trimethoxyacetophenone (**TMA**) and corresponding chalcones. (a) Me_2SO_4, NaOH/Me_2CO, reflux, 24 h. (b) Corresponding aldehyde, KOH 50% v/v, methanol, rt, 24 h. (c) 2-Naphthaldehyde, KOH 50% v/v, methanol, rt, 24 h. (d) 1-Naphthaldehyde, KOH 50% v/v, methanol, rt, 24 h. *New compounds.

Table 1. Yield (%), IC_{50} values ± confidence intervals of inhibitory effect, and cellular viability (at IC_{50} level) for some selected chalcones

Chalcone	R	Yield (%)	IC_{50} ± confidence intervals (μM)	Cell viability at IC_{50} (%)
1	2-Cl	80	1.70 (1.44–1.97)	100
2	3-NO_2	43	1.78 (0.97–3.27)	75
3	3-Cl	91	2.21 (0.38–12.9)	100
4	2-NO_2	60	2.43 (2.06–2.79)	100
5	4-NO_2	79	2.45 (0.46–13.20)	70
6	3,4-Cl_2	89	2.46 (2.12–2.79)	100
7	4-Cl	94	4.24 (2.02–8.93)	75
8	1-Naphthyl	97	4.62 (2.75–6.49)	100
9	2,6-Cl_2	63	7.26 (2.01–26.30)	100
10	2-Naphthyl	88	11.00 (8.08–14.90)	100
11	3,4-OCH_2–O–	78	13.10 (9.11–18.70)	100
12	4-OCH_3	84	15.20 (10.10–22.80)	100
13	4-$N(CH_3)_2$	53	24.90 (1.77–351.00)	90
14	4-$O(CH_2)_3$–CH_3	92	27.60 (5.59–136.00)	100
1400W			3.78 (1.87–7.63)[a]	82

[a] Under our experimental conditions.

The structure–activity analysis demonstrated that all chalcones with substituents that reduce the electronic density of the B-ring, such as chlorine atoms (**1**, **3**, **6**, **7**, and **9**) or nitro groups (**2**, **4**, and **5**), showed the greatest inhibition activity and selectivity in the inhibition of nitrite production. It was possible to observe also that the position of the chlorine atoms in the B-ring influences the inhibition of the nitrite production. In the molecules with only one chlorine atom, a proximity of this to the carbonyl group (position 2, **1**) gave the lowest IC_{50} value, followed by the molecule with the chlorine atom in position 3 (**3**) and then in position 4 (**7**). Considering the standard deviation of the experimental error, we can observe the same effect for the molecules that have a nitro group as the substituent, with better activity being observed when it is in position 2 (**4**), followed by the positions 3 (**2**) and then 4 (**5**). The decreases in the activity follow the decrease in the inductive effect of the chlorine atom and nitro group. However, the molecules with nitro groups showed, in general, greater cytotoxicity than the molecules with chlorine atoms.

Batt et al. showed that chalcones obtained from 2,4,6-trimethoxyacetophenone (**TMA**), like **2**, **5**, **7**, **8**, **9**, **10**, **12**, **13** in this study, among others, inhibited the biosynthesis of IL-1 from human peripheral blood monocytes stimulated by LPS, and a qualitative study of the structure–activity relationship revealed that the electron-withdrawing groups in the B-ring, regardless of the position, give enhanced potency.[23] This is in accordance with our results, except regarding the activity being independent of the position of the substituents. Furthermore, the same authors reported that an ortho substituent in A-ring is absolutely required for activity, because this substitution presumably induces a conformational change in the molecule, forcing the A-ring to be orthogonal to the enone.[23] Our results show that 2′,4′,6′-trimethoxylated chalcones in the A-ring, where this conformational change in the molecule probably occurs, also are active.

Ko et al. observed that, substitutions in chalcones by chlorine atoms in positions 3 and 4 of the B-ring and di-substituted at A-ring induced the inhibition of NO production and the iNOS protein expression in RAW 264.7 cells stimulated by LPS.[19] In our case, of the molecules that had two chlorine atoms in the B-ring, **6** (with chlorine atoms in positions 3 and 4) was more active than **9** (with chlorine atoms in positions 2 and 6) and thus, the conformation also seems to be important to the activity.

Chalcones with substituents that increase the electronic density of the B-ring, such as methoxy (**12**), butoxy (**14**) or dimethylamine (**13**) groups, did not show significant activity in the inhibition of the nitrite production. Won et al. demonstrated that a di-methoxylated chalcone in positions 2′ and 5′ in the A-ring and 4-hydroxylated in the B-ring presented inhibitory activity in relation to NO production in RAW 264.7 cells.[18] Herencia et al.[22] and Rojas et al.[17] showed that 4-dimethylaminochalcone derivatives have a high level of inhibitory activity in relation to the induction of iNOS in the same cells used in our study. It is interesting to observe that in Rojas et al.[17] the more active chalcones are those which are di-methoxylated in positions 2′ and 5′, and 2′ and 6′. In our case, were tested 2′,4′,6′-trimethoxylated chalcones, and **13**, which has as substituent a dimethylamine group as the ring B, did not show satisfactory activity.

Varying the structure of the B-ring, the presence of the methylenedioxyl group (**11**) makes its inhibitory activity as poor as that of **12** (with a methoxyl group). The 1-naphthyl group as the B-ring (**8**) exhibited good activity, better than the 2-naphthyl group (**10**), probably due to its spatial conformation.

Five compounds (chalcones **1**, **3**, **4**, **6**, and **8**) presented IC_{50} and cytotoxicity values less than or equal to those obtained for the 1400W, 3.78 (1.87–7.63) μM, under our experimental conditions. Figure 2 shows the concentration–response curves for nitrite production inhibition for the four most potent chalcones (**1**, **4**, **6**, and **8**) and their respective cell viability.

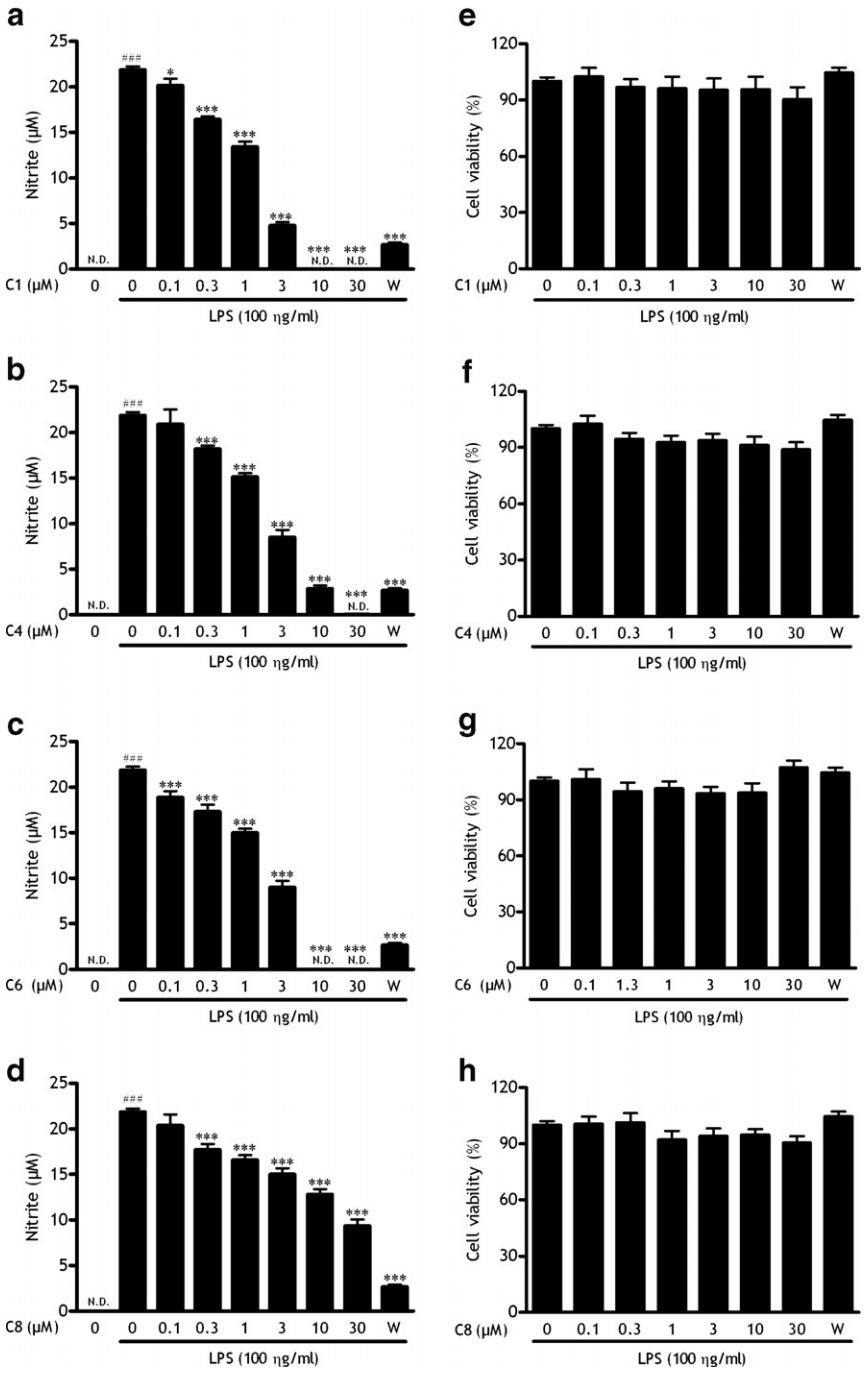

Figure 2. Effect of chalcones **1**, **4**, **6**, and **8** on nitrite production (a–d) and cell viability (e–h) in vitro. RAW 264.7 macrophages were plated at 1×10^{-6} cells/ml for 2 h. The non-adherent cells were removed by washing the plates twice with PBS. Chalcones were then added to the plates at various concentrations, as indicated in the graphs, for 24 h. Nitrite concentration was measured by the Gries method, while cell viability was measured by MTT assay. The graphs represent means (±SEM) of four independent experiments. $^{\#\#\#}P < 0.001$ compared with non-stimulated cells (first column); $^{*}P < 0.05$, $^{***}P < 0.001$ compared with cells stimulated with LPS (second column). One-way ANOVA followed by Bonferroni's test.

2.3. Theoretical quantitative structure–activity relationship

Preliminary calculations of the HOMO and LUMO surfaces for the chalcones **1**–**14** of Table 2 suggested some relationship between the activity and the location of these orbitals in distinct parts of the molecules. In the case of chalcones, higher potency seemed to be related to a higher contribution of HOMO in the **TMA** moiety and LUMO in the **R** moiety. A similar trend with other classes of compounds has been noted in a theoretical study on the antispasmodic activity of xanthoxyline derivatives; Santos et al.[27] found a relationship between this activity and the reactivity indices of HOMO and LUMO, as well as their sums over two distinct parts of the molecules. Also, Galeazzi et al.[28,29] found the same trend in recent studies on the herbicide activity of sulfonylureas and imidazolinones. These observations prompted us to try a similar study on the chalcones.

Table 2. Biological activity [Log(1/*C*), $C = IC_{50}$], activity calculated from equation *N* [EQ(*N*)] and quantum chemical descriptors found to correlate with biological activity of the chalcones

No.	R groups	Log(1/*C*)	EQ(1)	EQ(2)	$\sum R_H$(TMA)	$\sum R_L$(R)
1	R_1, X = 2-Cl	5.77	5.391	5.668	9.108	9.109
2	R_1, X = 3-NO_2	5.75	5.658	5.773	10.206	11.964
3	R_1, X = 3-Cl	5.65	5.521	5.564	8.018	9.197
4	R_1, X = 2-NO_2	5.61	5.632	5.794	10.430	12.076
5	R_1, X = 4-NO_2	5.61	5.651	5.766	10.132	11.835
6	R_1, X = 3,4-Cl_2	5.61	5.728	5.370	5.986	10.124
7	R_1, X = 4-Cl	5.37	5.352	5.075	2.892	9.552
8	R_2, X = H	5.34	4.986	4.816	0.178	11.276
9	R_1, X = 2,6-Cl_2	5.14	5.204	5.804	10.535	9.445
10	R_3	4.96	4.972	4.825	0.272	11.010
11	R_1, X = 3,4-OCH_2O–	4.88	4.789	4.833	0.353	9.673
12	R_1, X = 4-OCH_3	4.82	4.710	4.851	0.539	8.822
13	R_1, X = 4-$N(CH_3)_2$	4.60	4.648	4.823	0.248	8.960
14	R_1, X = 4-$O(CH_2)_3CH_3$	4.56	4.690	4.843	0.461	8.829
15	R_1, X = 3-CH_2NH_2		5.473	5.315	5.406	8.595
16	R_1, X = 2,3-$(OH)_2$		4.229	4.799	0.001	6.737
17	R_1, X = 4-CH_3		4.849	4.906	1.125	8.704
18	R_1, X = 3-OCH_3		4.659	4.848	0.511	8.543
19	R_1, X = 2,6-$(OCH_3)_2$		4.918	4.896	1.014	9.334
20	R_1, X = 2,5-$(OCH_3)_2$		4.745	4.819	0.206	9.672
21	R_1, X = 2,4,6-$(OCH_3)_3$		4.702	4.852	0.552	8.745
22	R_1, X = 3-OCH_3-4-OH		4.698	4.829	0.310	9.168
23	R_1, X = 4-F		5.159	4.972	1.808	9.659
24	R_1, X = 2-F		5.650	5.393	6.224	9.597
25	R_1, X = 4-Br		5.568	5.205	4.259	9.752
26	R_1, X = 2-OH		4.177	4.815	0.165	6.080
27	R_1, X = 2-OH-4-OCH_3		4.186	4.818	0.198	6.074
28	R_1, X = 3,4,5-$(OCH_3)_3$		4.768	4.843	0.458	9.340
29	R_1, X = 2,4,5-$(OCH_3)_3$		4.832	4.832	0.346	9.961
30	R_1, X = 2-COOH		5.500	5.767	10.148	10.873
31	R_1, X = 4-COOH		5.574	5.727	9.730	10.888
32	R_1, X = 4-NH_2		4.691	4.830	0.319	9.107
33	R_1, X = 3-CH_3-4-$N(CH_3)_2$		4.690	4.828	0.298	9.141
34	R_1, X = 3-$N(CH_3)_2$		4.536	4.808	0.095	8.532
35	R_1, X = 3-CF_3		5.369	5.771	10.190	10.079
36	R_1, X = 3-CF_3-4-Cl		5.517	5.758	10.050	10.872
37	R_1, X = 3-CF_3-4-NO_2		5.757	5.776	10.240	12.642
38	R_1, X = 4-CF_3		5.453	5.755	10.025	10.427
39	R_1, X = 3,5-Cl_2		5.421	5.721	9.667	9.831
40	R_1, X = 2,4-Cl_2		5.600	5.627	8.682	10.120
41	R_2, X = OH		5.087	4.803	0.041	12.198
42	R_1, X = 3-OCH_3-4-$OCH_2C_6H_6$		4.640	4.828	0.302	8.809
43	R_4, Y = S, X = H		5.104	4.932	1.391	9.926
44	R_4, Y = S, X = NO_2		5.767	5.749	9.958	12.379
45	R_4, Y = O, X = CH_3		4.650	4.842	0.454	8.588
46	R_5, X = H		4.551	4.849	0.526	7.818
47	R_5, X = CH_3		4.550	4.840	0.431	7.988
48	R_6		5.100	4.827	0.290	11.802

The R groups are those shown in Figure 3.

In order to evaluate the effect of various substituents on the activity, QSARs were calculated based on chalcones **1**–**14** of Table 2, and the predictions for another 34 non-synthesized chalcones were carried out. Attempts to correlate the activity with Log *P*, surface area, molecular volume, the energies of the HOMO and LUMO, E_{HOMO} and E_{LUMO}, and their difference, $\Delta E (= E_{LUMO} - E_{HOMO})$, were not successful. The best equations were found to correlate the activity with the sums of the reactivity indices of HOMO and LUMO. These descriptors encode information that quantifies the magnitude of the frontier orbitals in distinct parts of the molecules. Concerning the drug–receptor interactions, it can be stated that regions of the molecules that contain a greater HOMO contribution tend to be electron-donor 'anchors' to the receptor site, and the regions that contain a greater LUMO contribution tend to be electron-acceptor 'anchors'. In the equations below, $\sum R_H$(TMA) is the sum of the reactivity indices of the HOMO of the **TMA** moiety (Fig. 3), $\sum R_L$(R) is the sum of reactivity indices of the LUMO of the R moiety, the number in parentheses is the 95% confidence interval of the coefficient, *n* is the number of compounds taken into account in the regression, *r* is the multiple correlation coefficient, *s* is the standard deviation, and *F* is the ratio of regression to residual. In the construction of each equation, the two most discrepant

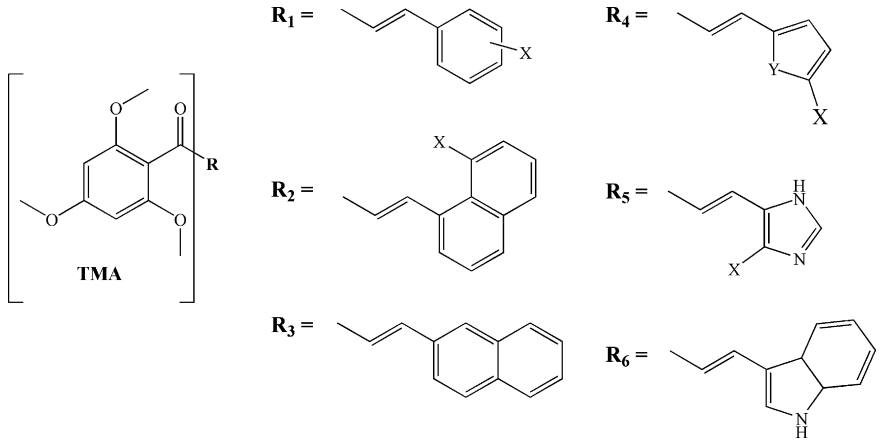

Figure 3. The 2,4,6-trimethoxyacetophenone moiety (**TMA**) and **R** groups of the chalcones.

compounds were removed from the data set in order to achieve optimal statistical quality.

Eq. 1 best quantifies the appearance of HOMO and LUMO in the series of chalcones experimentally tested. In this case, the two most discrepant compounds were chalcones **1** and **8** of Table 2. This equation indicates that the electronic properties play an important role in the interactions of chalcones with the receptor site. According to this model, $\sum R_H(\mathrm{TMA})$ should have an optimal value for maximum activity, and $\sum R_L(\mathrm{R})$ should be as high as possible. Apparently, this effect can be achieved by electron-withdrawing substituents in the R moiety (Table 2). The calculated correlation coefficient for these variables is 0.154.

$$\begin{aligned}\mathrm{Log}(1/C) = {} & -0.024(\pm 0.007)\left[\sum R_H(\mathrm{TMA})\right]^2 \\ & + 0.307(\pm 0.081)\sum R_H(\mathrm{TMA}) \\ & + 0.154(\pm 0.073)\sum R_L(\mathrm{R}) \\ & + 3.185(\pm 0.711) \qquad (1)\end{aligned}$$

$$n = 12;\ \ r = 0.980;\ \ s = 0.103;\ \ F_{3,8} = 63.55;$$

$$\sum R_H(\mathrm{TMA})_{\mathrm{OPTIMAL}} = 6.356$$

Attempts were also made to construct parabolic and linear models with one variable. Eq. 2 was the best equation with only one variable. This equation was obtained by removing compounds **1** and **9** (Table 2) from the regression. Despite its good statistical quality, the absence of a descriptor that counterpoises $\sum R_H(\mathrm{TMA})$ in this equation may give predictions that are too optimistic. This trend can be observed in Table 2.

$$\begin{aligned}\mathrm{Log}(1/C) = {} & 0.095(\pm 0.028)\sum R_H(\mathrm{TMA}) \\ & + 4.799(\pm 0.185) \qquad (2)\end{aligned}$$

$$n = 12;\ \ r = 0.919;\ \ s = 0.193;\ \ F_{1,10} = 54.08$$

The antiinflammatory potency of the chalcones can be assessed by comparing the values of Table 2 with the activity value of the standard inhibitor, 1400W, for which $\mathrm{Log}(1/\mathrm{IC}_{50}) = 5.42$. All compounds with activity values higher than 5.42 can be considered more active than 1400W. Thus, as can be seen in Table 2, chalcones **1**–**6** are more active than 1400W, and chalcones **7**–**14** are less active. For the non-synthesized chalcones, it is possible to predict that the presence of electron-withdrawing substituents (like CF_3, NO_2, and COOH) in the R moiety must increase the activity.

3. Conclusions

In summary, of the 14 synthesized and tested molecules, eight (**1**–**8**) gave a mean IC_{50} value less than or equal to that obtained for the positive control drug, compound 1400W, a highly selective inhibitor of iNOS. The structure–activity analysis demonstrates that chalcones with substituents that reduce the electronic density in the B-ring, such as chlorine atoms or nitro groups, show the greatest activity and selectivity in the inhibition of nitrite production. However, molecules with nitro groups show, in general, a slightly higher cytotoxicity than molecules with chlorine atoms. The inhibitory activity is also dependent on the position of the electron-withdrawing group in the B-ring, and position 2 seems to be the more important, with chalcone **1** being the most active.

According to the QSAR studies, the presence of electron-withdrawing substituents (like CF_3, NO_2, and COOH) in the R moiety of chalcones would increase their antiinflammatory activity. Such substituents exert an influence on the location of HOMO and LUMO in the molecules and a correlation with the activity is suggested. These results, together with the results of earlier studies,[27] demonstrate that the reactivity indices of HOMO and LUMO encode important information about the compounds, in relation to their activity.

Thus, the results showed that these chalcones are probably active in inflammatory processes, since NO production is an important step of the inflammation. The inhibition of the production of NO and PGE_2, mainly

in macrophages, which affects the expression of the induced enzymes involved, may be an important strategy for obtaining new drugs.[17,18]

4. Experimental

4.1. Synthesis

The 2,4,6-trimethoxyacetophenone (**TMA**) and the chalcones were synthesized as shown in Figure 1. All reagents used were obtained commercially (Merck, Sigma–Aldrich), except xanthoxyline, which was prepared as previously described.[24] The 2,4,6-trimethoxyacetophenone (**TMA**) was obtained by magnetic agitation of a solution of xanthoxyline (7.50 g; 38.25 mmol), NaOH (2.62 g; 65.5 mmol in 50 ml of water), acetone (300 ml), and Me_2SO_4 (5.24 ml; 38.25 mmol), under reflux for 24 h. After evaporation of the solvent, the solid formed was recrystallized in hexane, obtaining the 2,4,6-trimethoxyacetophenone (**TMA**) crystals. This methodology of synthesis was used for the obtainment of 3-bromine-2,4,6-trimethoxyacetophenone from monobromide xanthoxyline in a previous study,[24] however, the synthesis of 2,4,6-trimethoxyacetophenone (**TMA**) from xanthoxyline has not been previously described. The substituted chalcones (**1**–**14**) were prepared by magnetic agitation of **TMA** (0.25 g; 1.2 mmol), methanol (20 ml), KOH 50% w/v (5 ml), and the corresponding aldehyde (1.2 mmol), at room temperature for 24 h. Distilled water and hydrochloric acid (10%) were added to the reaction for total precipitation of the compounds. The compounds were then obtained by vacuum filtration and later recrystallized in dichloromethane and hexane. Chalcones **11** and **13** were previously described, respectively, by Phrutivorapongkul et al.[11] and by Edwards et al.[20] Compounds **2**, **5**, **7**, **8**, **9**, **10**, **12**, and **13** were cited by Batt et al.,[23] and structures **1**, **3**, **4**, **6**, and **14** are new chalcones.

4.2. Physico-chemical data on synthesized compounds

The purified chalcones were obtained in yields between 43% and 97%. The structures were identified using melting points (mp), infrared spectroscopy (IR), ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy, and, for the unpublished ones, also elementary analysis. Melting points were determined with a Microquímica MGAPF-301 apparatus and are uncorrected. IR spectra were recorded with an Abb Bomen FTLA 2000 spectrometer on KBr disks. NMR (^{1}H and ^{13}C NMR) spectra were recorded on a Brucker Ac-200F (200 MHz) or Varian Oxford AS-400 (400 MHz) instrument, using tetramethylsilane as an internal standard. Elementary analysis was carried out using a CHNS EA 1110. Percentages of C and H were in agreement with the product formula (within ±0.4% of theoretical values for C). The purity of the synthesized chalcones was analyzed by thin-layer chromatography (TLC) using Merck silica pre-coated aluminum plates of 200 μm thickness, with several solvent systems of different polarities. Compounds were visualized with ultraviolet light (λ = 254 and 360 nm) and using sulfuric anisaldehyde solution followed by heat application as the developing agent and purified by recrystallization from hexane and dichloromethane. ^{1}H NMR spectra revealed that all the structures were geometrically pure and configured E ($J_{H\alpha-H\beta}$ = 14–17 Hz).

4.2.1. TMA: 2,4,6-trimethoxyacetophenone. $C_{11}H_{14}O_4$, creamy solid; mp = 100–102 °C. ^{1}H NMR ($CDCl_3$) δ 2.45 (s, 3H, CH_3), 3.81 (s, 3H, *p*-OCH_3), 3.78 (s, 6H, *o*-OCH_3), 6.09 (s, 2H, H3, H5). RMN ^{13}C ($CDCl_3$) δ 32.41 (CH_3), 55.31 (*m*-OCH_3), 55.71 (*p*-OCH_3), 90.49 (C3, C5), 113.60 (C1), 158.25 (C2, C6), 162.25 (C4), 201.61 (C=O). Yield = 84%.

4.2.2. 1: (2*E*)-3-(2-Chloro-phenyl)-1-(2′,4′,6′-trimethoxyphenyl)-2-propen-1-one. $C_{18}H_{17}ClO_4$, light yellow solid; mp = 112–114 °C; IR ν_{max}/cm^{-1} 1666, 1206 (C=O), 1585 (C=C), 1231, 1019 (C–O), 3015, 2971, 2942, 2844, 1602, 1458, 1440, 1416, 1081, 972, 821, 766 (Ar) (KBr). ^{1}H NMR ($CDCl_3$) δ 3.78 (s, 6H, OCH_3), 3.86 (s, 3H, OCH_3), 6.16 (s, 2H, H3′, H5′), 6.91 (d, 1H, J = 16.0 Hz, Hα), 7.27–7.28 (m, 2H, H4, H5), 7.38 (d, 1H, J = 8.0 Hz, H3), 7.66 (d, 1H, J = 8.0 Hz, H6), 7.77 (d, 1H, J = 16.0 Hz, Hβ). ^{13}C NMR ($CDCl_3$) δ 55.69 (*p*′-OCH_3), 56.13 (*o*′-OCH_3), 90.97 (C3′, C5′), 111.66 (C1′), 127.27 (Cα), 128.04 (C5), 130.28 (C6), 131.08 (C4), 131.48 (C3), 133.52 (C2), 135.28 (C1), 140.17 (Cβ), 159.21 (C2′, C6′), 162.82 (C4′), 194.30 (C=O). Anal. Calcd for $C_{18}H_{17}ClO_4$: C, 64.97; H, 5.15. Found: C, 64.20; H, 5.52. Yield = 80%.

4.2.3. 2: (2*E*)-3-(3-Nitro-phenyl)-1-(2′,4′,6′-trimethoxyphenyl)-2-propen-1-one. $C_{18}H_{17}NO_6$, dark yellow solid; mp: 144–146 °C (lit. p.f.: 146–148 °C); IR ν_{max}/cm^{-1} 1675, 1206 (C=O), 1588 (C=C), 1528, 1352, 855 (N=O), 1231, 1017 (C–O), 2965, 2943, 2842, 1608, 1467, 1434, 1413, 1081, 972, 917, 817 (Ar) (KBr). ^{1}H NMR ($CDCl_3$) δ 3.79 (s, 6H, OCH_3), 3.87 (s, 3H, OCH_3), 6.18 (s, 2H, H3′, H5′), 7.06 (d, 1H, J = 16.0 Hz, Hα), 7.43 (d, 1H, J = 16.0 Hz, Hβ), 7.56 (dd, 1H, J = 7.9 Hz, H5), 7.84 (d, 1H, J = 7.7 Hz, H6), 8.20 (d, 1H, J = 7.9 Hz, H4), 8.35 (s, 1H, H2). ^{13}C NMR ($CDCl_3$) δ 51.80 (*p*′-OCH_3), 52.26 (*o*′-OCH_3), 87.02 (C3′, C5′), 107.62 (C1′), 119.00 (C2), 120.58 (C4), 126.15 (Cα), 127.69 (C5), 130.11 (C6), 133.23 (C1), 136.48 (Cβ), 144.90 (C3), 155.42 (C2′, C6′), 159.18 (C4′), 189.35 (C=O). Yield = 43%.

4.2.4. 3: (2*E*)-3-(3-Chloro-phenyl)-1-(2′,4′,6′-trimethoxyphenyl)-2-propen-1-one. $C_{18}H_{17}ClO_4$, yellow solid; mp = 133–135 °C; IR ν_{max}/cm^{-1} 1644, 1202 (C=O), 1602 (C=C), 1231, 1022 (C–O), 3014, 2940, 2841, 1458, 1412, 969, 888, 825, 792 (Ar) (KBr). ^{1}H NMR ($CDCl_3$) δ 3.75 (s, 6H, OCH_3), 3.83 (s, 3H, OCH_3), 6.13 (s, 2H, H3′, H5′), 6.92 (d, 1H, J = 16.0 Hz, Hα), 7.25–7.30 (m, 2H, H5, H6), 7.27 (d, 1H, J = 16.0 Hz, Hβ), 7.37 (d, 1H, H4), 7.46 (s, 1H, H2). ^{13}C NMR ($CDCl_3$) δ 55.69 (*p*′-OCH_3), 56.14 (*o*′-OCH_3), 90.95 (C3′, C5′), 111.77 (C1′), 126.65 (Cα), 128.29 (C6), 130.14 (C2), 130.27 (C4), 130.35 (C5), 134.99 (C3), 137.16 (C1), 142.18 (Cβ), 159.17 (C2′, C6′), 162.87 (C4′), 193.89 (C=O). Anal. Calcd for $C_{18}H_{17}ClO_4$: C, 64.97; H, 5.15. Found: C, 64.67; H, 5.63. Yield = 91%.

4.2.5. 4: (2*E*)-3-(2-Nitro-phenyl)-1-(2′,4′,6′-trimethoxy-phenyl)-2-propen-1-one. $C_{18}H_{17}NO_6$, yellow solid; mp = 153–155 °C; IR ν_{max}/cm^{-1} 1674, 1207 (C=O), 1603 (C=C), 1233, 1018 (C–O), 1522, 1337, 855 (N=O), 2968, 2943, 2844, 1470, 1439, 1415, 970, 949, 814 (Ar) (KBr). ^1H NMR ($CDCl_3$) δ 3.80 (s, 6H, OCH_3), 3.85 (s, 3H, OCH_3), 6.15 (s, 2H, H3′, H5′), 6.81 (d, 1H, J = 16.0 Hz, Hα), 7.52 (d, 1H, J = 8.0 Hz, H6), 7.65 (d, 1H, J = 16.0 Hz, Hβ), 7.65–7.72 (m, 2H, H4, H5), 8.01 (d, 1H, J = 8.0 Hz, H3). ^{13}C NMR ($CDCl_3$) δ 55.68 (p'-OCH_3), 56.08 (o'-OCH_3), 90.81 (C3′, C5′), 110.62 (C1′), 125.12 (C3), 129.53 (Cα), 130.30 (C6), 131.58 (C4), 133.73 (C1), 133.77 (C5), 140.14 (Cβ), 148.51 (C2), 159.18 (C2′, C6′), 162.98 (C4′), 194.69 (C=O). Anal. Calcd for $C_{18}H_{17}NO_6$: C, 62.97; H, 4.99; N, 4.08. Found: C, 62.94; H, 5.65; N, 3.94. Yield = 59%.

4.2.6. 5: (2*E*)-3-(4-Nitro-phenyl)-1-(2′,4′,6′-trimethoxy-phenyl)-2-propen-1-one. $C_{18}H_{17}NO_6$, light yellow solid; mp = 173–175 °C (lit. p.f.: 176–178 °C); IR ν_{max}/cm^{-1} 1683, 1209 (C=O), 1591 (C=C), 1223, 1019 (C–O), 1513, 1341, 847 (N=O), 2938, 2836, 1613, 1465, 1411, 980, 950, 806 (Ar) (KBr). ^1H NMR ($CDCl_3$) δ 3.79 (s, 6H, OCH_3), 3.87 (s, 3H, OCH_3), 6.16 (s, 2H, H3′, H5′), 7.06 (d, 1H, J = 16.4 Hz, Hα), 7.43 (d, 1H, J = 16.4 Hz, Hβ), 7.66 (d, 2H, H2, H6), 8.23 (d, 2H, H3, H5). ^{13}C NMR ($CDCl_3$) δ 55.75 (p'-OCH_3), 56.21 (o'-OCH_3), 90.97 (C3′, C5′), 110.99 (C1′), 124.31 (C3, C5), 129.05 (C2, C6), 132.65 (Cα), 140.05 (C1), 141.71 (Cβ), 148.51 (C4), 159.47 (C2′, C6′), 163.21 (C4′), 193.01 (C=O). Yield = 79%.

4.2.7. 6: (2*E*)-3-(3,4-Dichloro-phenyl)-1-(2′,4′,6′-trimethoxy-phenyl)-2-propen-1-one. $C_{18}H_{16}Cl_2O_4$, light yellow solid; mp = 125–127 °C; IR ν_{max}/cm^{-1} 1672, 1205 (C=O), 1587 (C=C), 1229, 1018 (C–O), 2940, 2800, 1603, 1467, 1412, 969, 948, 839, 808 (Ar) (KBr). ^1H NMR ($CDCl_3$) δ 3.77 (s, 6H, OCH_3), 3.86 (s, 3H, OCH_3), 6.15 (s, 2H, H3′, H5′), 6.92 (d, 1H, J = 16.0 Hz, Hα), 7.27 (d, 1H, J = 16.0 Hz, Hβ), 7.35 (d, 1H, H5), 7.43 (d, 1H, H6), 7.58 (s, 1H, H2). ^{13}C NMR ($CDCl_3$) δ 55.71 (p'-OCH_3), 56.18 (o'-OCH_3), 90.98 (C3′, C5′), 111.76 (C1′), 127.44 (Cα), 130.12 (C6), 130.65 (C2), 131.01 (C5), 133.32 (C4), 134.11 (C3), 135.46 (C1), 140.84 (Cβ), 159.27 (C2′, C6′), 162.97 (C4′), 193.54 (C=O). Anal. Calcd for $C_{18}H_{16}Cl_2O_4$: C, 58.87; H, 4.39. Found: C, 59.51; H, 5.04. Yield = 89%.

4.2.8. 7: (2*E*)-3-(4-Chloro-phenyl)-1-(2′,4′,6′-trimethoxy-phenyl)-2-propen-1-one. $C_{18}H_{17}ClO_4$, light yellow solid; mp = 128–130 °C (lit. p.f.: 132–133 °C); IR ν_{max}/cm^{-1} 1672, 1208 (C=O), 1606 (C=C), 1229, 1017 (C–O), 3005, 2965, 2961, 2835, 1568, 1472, 1451, 1402, 1088, 972, 952, 809 (Ar) (KBr). ^1H NMR ($CDCl_3$) δ 3.77 (s, 6H, OCH_3), 3.85 (s, 3H, OCH_3), 6.16 (s, 2H, H3′, H5′), 6.92 (d, 1H, J = 16.0 Hz, Hα), 7.32 (d, 1H, J = 16.0 Hz, Hβ), 7.33 (d, 2H, H2, H6), 7.44 (d, 2H, H3, H5). ^{13}C NMR ($CDCl_3$) δ 55.70 (p'-OCH_3), 56.18 (o'-OCH_3), 90.98 (C3′, C5′), 111.94 (C1′), 129.31 (Cα), 129.66 (C3, C5), 129.74 (C2, C6), 133.82 (C4), 136.23 (C1), 142.54 (Cβ), 159.18 (C2′, C6′), 162.81 (C4′), 194.05 (C=O). Yield = 94%.

4.2.9. 8: (2*E*)-3-(1-Naphthyl)-1-(2′,4′,6′-trimethoxy-phenyl)-2-propen-1-one. $C_{22}H_{20}O_4$, creamy solid; mp = 172–174 °C (lit. p.f.: 176–178 °C); IR ν_{max}/cm^{-1} 1640, 1205 (C=O), 1586 (C=C), 1227, 1025 (C–O), 3003, 2942, 2840, 1605, 1466, 1454, 1412, 1082, 974, 818, 798, 771 (Ar) (KBr). ^1H NMR ($CDCl_3$) δ 3.81 (s, 6H, OCH_3), 3.88 (s, 3H, OCH_3), 6.20 (s, 2H, H3′, H5′), 7.05 (d, 1H, J = 16.0 Hz, Hα), 7.47–7.55 (m, 3H, H3, H6, H7), 7.81 (d, 1H, J = 6.8 Hz, H2), 7.86–7.89 (m, 2H, H5, H8), 8.07 (d, 1H, J = 8.0 Hz, H4), 8.25 (d, 1H, J = 16.0 Hz, Hβ). ^{13}C NMR ($CDCl_3$) δ 55.73 (p'-OCH_3), 56.19 (o'-OCH_3), 91.02 (C3′, C5′), 112.04 (C1′), 123.64 (Cα), 125.43 (C2, C8), 125.75 (C3), 126.35 (C6), 126.94 (C7), 128.96 (C4), 130.59 (C5), 131.78 (C9), 132.63 (C10), 133.93 (C1), 140.94 (Cβ), 159.32 (C2′, C6′), 162.83 (C4′), 194.08 (C=O). Yield = 97%.

4.2.10. 9: (2*E*)-3-(2,6-Dichloro-phenyl)-1-(2′,4′,6′-trimethoxy-phenyl)-2-propen-1-one. $C_{18}H_{16}Cl_2O_4$, beige solid; mp = 115–117 °C (lit. p.f.: 119–121 °C); IR ν_{max}/cm^{-1} 1649, 1206 (C=O), 1590 (C=C), 1231, 1028 (C–O), 3009, 2943, 2842, 1604, 1556, 1458, 1414, 1080, 975, 820, 776 (Ar) (KBr). ^1H NMR ($CDCl_3$) δ 3.79 (s, 6H, OCH_3), 3.85 (s, 3H, OCH_3), 6.15 (s, 2H, H3′, H5′), 7.08 (d, 1H, J = 16.4 Hz, Hα), 7.15 (dd, 1H, J = 8.0 Hz, H4), 7.33 (d, 2H, J = 8.0 Hz, H3, H5), 7.49 (d, 1H, J = 16.4 Hz, Hβ). ^{13}C NMR ($CDCl_3$) δ 55.69 (p'-OCH_3), 56.12 (o'-OCH_3), 90.88 (C3′, C5′), 111.44 (C1′), 128.97 (C3, C5), 129.77 (Cα), 132.94 (C4), 135.28 (C2, C6), 136.91 (C1), 137.13 (Cβ), 159.53 (C2′, C6′), 163.02 (C4′), 193.80 (C=O). Yield = 63%.

4.2.11. 10: (2*E*)-3-(2-Naphthyl)-1-(2′,4′,6′-trimethoxy-phenyl)-2-propen-1-one. $C_{22}H_{20}O_4$, light yellow solid; mp = 139–141 °C (lit. p.f.: 145–146 °C); IR ν_{max}/cm^{-1} 1639, 1206 (C=O), 1588 (C=C), 1231, 1027 (C–O), 3002, 2945, 2845, 1622, 1605, 1459, 1415, 1083, 980, 859, 826 (Ar) (KBr). ^1H NMR ($CDCl_3$) δ 3.78 (s, 6H, OCH_3), 3.87 (s, 3H, OCH_3), 6.18 (s, 2H, H3′, H5′), 7.07 (d, 1H, J = 16.4 Hz, Hα), 7.48–7.55 (m, 4H, H3, H6, H7, Hβ), 7.70 (d, 1H, J = 8.4 Hz, H4), 7.81–7.83 (m, 2H, H5, H8), 7.90 (s, 1H, H1). ^{13}C NMR ($CDCl_3$) δ 55.71 (p'-OCH_3), 56.19 (o'-OCH_3), 91.01 (C3′, C5′), 112.11 (C1′), 124.09 (Cα), 126.86 (C3), 127.39 (C6), 128.00 (C7), 128.75 (C1), 128.80 (C5), 129.49 (C8), 130.49 (C4), 132.83 (C10), 133.55 (C9), 134.45 (C2), 144.49 (Cβ), 159.14 (C2′, C6′), 162.68 (C4′), 194.53 (C=O). Yield = 88%.

4.2.12. 11: (2*E*)-3-(1,3-Benzodioxol-5-yl)-1-(2′,4′,6′-trimethoxy- phenyl)-2-propen-1-one. $C_{19}H_{18}O_6$, creamy solid; mp = 124–126 °C (lit. p.f.: 143–144 °C); IR ν_{max}/cm^{-1} 1639, 1206 (C=O), 1599 (C=C), 1264, 1034 (C–O), 3009, 2938, 2839, 1503, 1467, 1451, 1417, 976, 925, 824, 807 (Ar) (KBr). ^1H NMR ($CDCl_3$) δ 3.79 (s, 6H, OCH_3), 3.86 (s, 3H, OCH_3), 5.99 (s, 2H, –OCH_2O–), 6.10 (s, 1H, H2), 6.15 (s, 2H, H3′, H5′), 6.80 (d, 1H, J = 15.4 Hz, Hα), 6.98 (d, 2H, J = 8.0 Hz, H5), 7.05 (d, 2H, H6), 7.28 (d, 1H, J = 14.8 Hz, Hβ). ^{13}C NMR

($CDCl_3$) δ 55.70 (p'-OCH_3), 56.16 (o'-OCH_3), 90.91 (C3′, C5′), 101.76 (–O*C*H_2O–), 107.02 (C2), 108.74 (C5), 112.12 (C1′), 125.08 (C6), 127.51 (Cα), 129.68 (C1), 144.33 (Cβ), 148.51–149.81 (C3, C4), 159.01 (C2′, C6′), 162.56 (C4′), 194.44 (C=O). Anal. Calcd for $C_{19}H_{18}O_6$: C, 66.66; H, 5.30. Found: C, 65.62; H, 5.97. Yield = 78%.

4.2.13. 12: (2*E*)-3-(4-Methoxy-phenyl)-1-(2′,4′,6′-trimethoxy-phenyl)-2-propen-1-one. $C_{19}H_{20}O_5$, creamy solid; mp = 112–114 °C (lit. p.f.: 116–118 °C); IR ν_{max}/cm^{-1} 1673, 1203 (C=O), 1598 (C=C), 1256, 1026 (C–O), 2961, 2938, 2837, 1635, 1512, 1467, 1420, 1081, 979, 820, 804 (Ar) (KBr). ^{1}H NMR ($CDCl_3$) δ 3.76 (s, 6H, OCH_3), 3.84 (s, 3H, OCH_3), 3.86 (s, 3H, OCH_3), 6.16 (s, 2H, H3′, H5′), 6.84 (d, 1H, J = 14.8 Hz, Hα), 6.89 (d, 2H, H3, H5), 7.31 (d, 1H, J = 14.8 Hz, Hβ), 7.47 (d, 2H, H2, H6). ^{13}C NMR ($CDCl_3$) δ 55.62 (p-OCH_3), 55.69 (p'-OCH_3), 56.16 (o'-OCH_3), 90.92 (C3′, C5′), 112.15 (C1′), 114.50 (C3, C5), 127.21 (Cα), 127.91 (C1), 130.35 (C2, C6), 144.56 (Cβ), 158.96 (C4), 161.62 (C2′, C6′), 162.48 (C4′), 194.73 (C=O). Yield = 84%.

4.2.14. 13: (2*E*)-3-[4-(Dimethylamino)phenyl]-1-(2′,4′,6′-trimethoxy-phenyl)-2-propen-1-one. $C_{20}H_{23}NO_4$, yellow solid; mp = 148–150 °C (lit. p.f.: 153–155, 149–150 °C); IR ν_{max}/cm^{-1} 2994, 2937, 2839, 1628, 1210 (C=O), 1597 (C=C), 1230, 1029 (C–O), 1304 (C–N), 1529, 1453, 1442, 1413, 1084, 968, 945, 813 (Ar) (KBr). ^{1}H NMR ($CDCl_3$) δ 3.01 (s, 6H, N–CH_3), 3.76 (s, 6H, OCH_3), 3.86 (s, 3H, OCH_3), 6.16 (s, 2H, H3′, H5′), 6.65 (d, 2H, H3, H5), 6.78 (d, 1H, J = 16.0 Hz, Hα), 7.27 (d, 1H, J = 16.0 Hz, Hβ), 7.41 (d, 2H, H2, H6). ^{13}C NMR ($CDCl_3$) δ 40.38 (N–*C*H_3), 55.68 (p'-OCH_3), 56.15 (o'-OCH_3), 90.91 (C3′, C5′), 111.99 (C3, C5), 112.49 (C1′), 122.84 (Cα), 124.67 (C1), 130.47 (C2, C6), 146.20 (Cβ), 152.05 (C4), 158.80 (C2′, C6′), 162.19 (C4′), 194.95 (C=O). Yield = 53%.

4.2.15. 14: (2*E*)-3-(4-Butoxy-phenyl)-1-(2′,4′,6′-trimethoxy-phenyl)-2-propen-1-one. $C_{22}H_{26}O_5$, creamy solid; mp = 110–112 °C; IR ν_{max}/cm^{-1} 1667, 1204 (C=O), 1587 (C=C), 1228, 1021 (C–O), 2946, 2871, 2840, 1562, 1508, 1457, 1413, 1062, 980, 947, 824 (Ar) (KBr). ^{1}H NMR ($CDCl_3$) δ 0.93–1.02 (m, 3H, –CH_3), 1.43–1.57 (m, 2H, –C*H*$_2$–CH_3), 1.63–1.83 (m, 2H, –C*H*$_2$–CH_2–CH_3), 3.76 (s, 6H, OCH_3), 3.85 (s, 3H, OCH_3), 3.98 (t, 2H, –O–CH_2–), 6.16 (s, 2H, H3′, H5′), 6.83 (d, 1H, J = 16.7 Hz, Hα), 6.87 (d, 2H, J = 8.7 Hz, H3, H5), 7.30 (d, 1H, J = 16.7 Hz, Hβ), 7.45 (d, 2H,J = 8.7 Hz, H2, H6). ^{13}C NMR ($CDCl_3$) δ 14.05 (–CH_3), 19.43 (–*C*H_2–CH_3), 31.42 (–*C*H_2–CH_2–CH_3), 55.68 (p'-OCH_3), 56.15 (o'-OCH_3), 68.06 (–O–*C*H_2–), 90.97 (C3′, C5′), 112.23 (C1′), 115.01 (C3, C5), 127.07 (Cα), 127.67 (C1), 130.33 (C2, C6), 144.68 (Cβ), 158.96 (C2′, C6′), 161.28 (C4), 162.48 (C4′), 194.72 (C=O). Anal. Calcd for $C_{22}H_{26}O_5$: C, 71.33; H, 7.07. Found: C, 67.54; H, 7.36. Yield = 92%.

4.3. Biological assays

4.3.1. Cell culture. RAW 264.7 cells (ATCC TIB71) purchased from Banco de Células do Rio de Janeiro (Rio de Janeiro, RJ, Brazil) were maintained at 37 °C in DMEM (GIBCO BRL, USA) supplemented with 10% heat-inactivated calf serum (GIBCO BRL, USA), glucose (4.5 g/l), sodium bicarbonate (1.5 g/l), L-glutamine (2 mM), streptomycin (100 μg/ml), and penicillin (100 U/ml) in a humidified atmosphere of 5% CO_2.

4.3.2. Nitrite quantification. Cells were plated at 1×10^6 cells/ml in 96-well culture plates. After 2 h of incubation at 37 °C non-adherent cells were removed by washing the plates repeatedly. Compounds in various concentrations (as indicated in the graphs) were then added to the plates. Thirty minutes later cells were stimulated with LPS (100 ηg/ml) for 24 h. The isolated supernatants (100 μl) were mixed with 100 μl of Gries reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride in 2% phosphoric acid) and incubated at room temperature for 5 min. The concentration of nitrite was measured by OD reading at 550 nm using a $NaNO_2$ standard curve. CI_{50} was calculated using Graphpad Prism Software.

4.3.3. Cell viability. MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl tetrazolium bromide) is a substrate which produces a dark blue formazan product when incubated with living cells by cleavage of the MTT ring in active mitochondria. An MTT assay was used to measure cell viability following treatment with indicated compounds. After removal of the supernatants, the cells were incubated with MTT (0.05 mg/ml) at 37 °C for at least 4 h. Supernatants were then gently removed and 100 μl of dimethylsulfoxide (DMSO) was added to the wells. After 15 min of gently mixing the absorbance was determined at 550 nm. Naive cells were considered as 100% of viability.

4.3.4. Statistical analysis. All data are expressed as means ± SEM. Statistical significance of differences between the groups was determined by One-way ANOVA followed by the Bonferroni test. Statistical analysis and calculation of IC_{50} values were performed using GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA).

4.4. Quantitative structure–activity relationships—computational methods

Initially, a conformational search was performed for all chalcones by means of the 'conformer distribution' resource implemented in the Spartan'04 package[30] with a semi-empirical AM1 level of theory.[31] Chalcones **1**–**13** were submitted to the systematic method. For chalcone **14**, the Monte Carlo method had to be employed, since the systematic method has been shown to be more time-consuming. The default settings were used for chalcones **15**–**48** (in this case, the choice of the systematic or Monte Carlo method depended on the complexity of the molecule). After optimization of all conformers, the lowest energy conformers of each chalcone were optimized using the AM1 method and a gradient tolerance of 8.4×10^{-6} Hartrees $Bohr^{-1}$. The eigenvalues and eigenvectors, printed in log files, were used in the calculation of the reactivity indices of HOMO and LUMO

over each atom '*i*', $R_H(i)$ and $R_L(i)$, respectively. These are calculated as follows

$$R_H(i) = \frac{f_r^E(i)}{-E_{HOMO}} \times 100 \quad f_r^E(i) = \sum (C_{HOMO,n})^2$$

$$R_L(i) = \frac{f_r^N(i)}{E_{LUMO} - E_{HOMO}} \times 100 \quad f_r^N(i) = \sum (C_{LUMO,n})^2$$

where f_r^E and f_r^N are the electrophilic and nucleophilic atomic frontier electron densities, respectively, and $C_{HOMO,n}$ *and* $C_{LUMO,n}$ are the coefficients of the atomic orbital X_n in the HOMO and LUMO, respectively. In order to better represent quantitatively the contributions of these orbitals in two distinct parts of the molecules, the sums of the indices R_H and R_L, $\sum R_H$ and $\sum R_L$, were calculated over the 2,4,6-trimethoxyacetophenone moiety (**TMA**, Fig. 3) and the variable radical moiety (**R**, Fig. 3), which gave $\sum R_H(TMA)$, $\sum R_L(TMA)$, $\sum R_H(R)$, and $\sum R_L(R)$. These summations were used as QSAR descriptors, since high correlations between R_H and R_L arise for various atoms of the same moiety. Other descriptors taken into account were Log *P*, surface area, molecular volume, the energies of the HOMO and LUMO in electron volt, E_{HOMO} and E_{LUMO}, and their difference, ΔE ($=E_{LUMO} - E_{HOMO}$). The QSAR equations and statistics were calculated with the BuildQSAR program.[32]

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