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Identification of chalcone-based antileishmanial agents targeting trypanothione reductase

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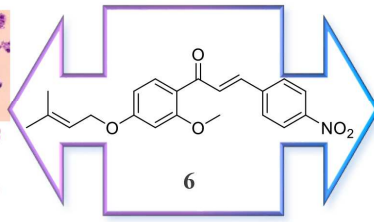
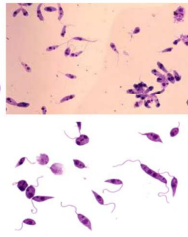
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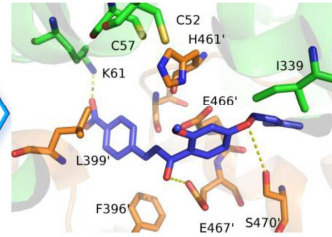
L. Donovanii

$IC_{50} = 3.0 \mu M$
(promastigote)

$IC_{50} = 14.0 \mu M$
(amastigote)



Trypanothione Reductase



$K_D = 0.6 \mu M$
 $K_i = 0.45 \mu M$

27 use of natural derived products, i.e. chalcones, as potential source of antileishmanial agents. Thirty-
28 one novel chalcone compounds have been synthesized and their activity has been evaluated against
29 promastigotes of *Leishmania donovani*; 16 compounds resulted active against *L. donovani* in a
30 range from 3.0 to 21.5 μM , showing low toxicity against mammalian cells. Among these molecules,
31 **6** and **16** showed good inhibitory activity on both promastigotes and intracellular amastigotes,
32 coupled with an high selectivity index. Furthermore, compounds **6** and **16** inhibited the
33 promastigote growth of other leishmanial species, including *L. tropica*, *L. major* and *L. infantum*.
34 Finally, **6** and **16** interacted with high affinity with trypanothione reductase (TR), an essential
35 enzyme for the leishmanial parasite and compound **6** inhibited TR with sub-micromolar potency.
36 Thus, the effective inhibitory activity against *Leishmania*, the lack of toxicity on mammalian cells
37 and the ability to block a crucial parasite's enzyme, highlight the potential for compound **6** to be
38 optimized as novel drug candidate against leishmaniasis.

39

40

41 **Keywords**

42 Chalcone, drug discovery, leishmaniasis, natural products, neglected tropical disease, trypanothione
43 reductase

44

45 **1. INTRODUCTION**

46 Leishmaniasis are vector-borne infections caused by protozoan parasites belonging to the genus
47 *Leishmania* that are transmitted through the bite of phlebotomine sand flies of the genus
48 *Phlebotomus* in the Old World [1]. Most forms of the diseases are zoonotic and only 21 of the 30
49 *Leishmania* species that infect mammals may cause human infection.

50 Most infections caused by *Leishmania* parasites are asymptomatic, but in symptomatic patients
51 clinical manifestations range from cutaneous leishmaniasis (CL), which may result in disfiguring

52 scars if left untreated, to the potentially fatal visceral leishmaniasis (VL), which is characterized by
53 fever, splenomegaly, pancytopenia and weight loss [2].

54 Leishmaniasis are distributed in Asia, Africa, Latin America and Southern Europe, with an
55 estimation of 1.5-2.0 million new cases per year of CL and 0.5 million cases of VL [3]. Despite this,
56 leishmaniasis are classified among the most neglected diseases, based on their strong association
57 with poverty and on the limited resources invested in their diagnosis, treatment and control [2].
58 Chemotherapy is the only method for protection against leishmaniasis, since there is currently no
59 approved vaccine for humans [4]. Treatment of leishmaniasis comprises liposomal amphotericin B,
60 pentavalent antimonials, paromomycin and miltefosine; these drugs are plagued by several
61 limitations including high costs, toxicity, route of administration and poor efficacy [5]. Moreover,
62 the increasing emergence of *Leishmania* parasites that are resistant to antimonial drugs is a serious
63 problem in several endemic regions [6]. This scenario emphasizes the need of developing novel
64 effective, safe and economically feasible antileishmanial agents.

65 The implementation of the genome project for many trypanosomatid species lead to the
66 identification of several drug targets suitable for gaining parasite selective inhibition [7]. Indeed,
67 targeting a unique and essential parasite metabolic pathway, which is absent in mammals is
68 generally considered a successful therapeutic strategy.

69 In this context, the thiol-dependent redox polyamine metabolism represents an essential
70 detoxifying system by means of which the parasite eliminates its toxic endogenous metabolites [8].
71 While the mammalian redox defense machinery is based on glutathione (GSH), the protozoan
72 parasites from the Trypanosomatidae family, including *Trypanosoma* and *Leishmania*, strictly
73 depend on a different pathway for supporting their intracellular redox homeostasis; they employ
74 trypanothione (*N1,N8*-bis-glutathionyl-spermidine) in its reduced thiol form T(SH)₂ [9].
75 Trypanothione disulfide (TS₂) is obtained by means of two consecutive steps, each involving the
76 conjugation of GSH to N₁ and N₈ amino groups of spermidine by the ATP-dependent C-N ligase
77 trypanothione synthetase (TryS). Trypanothione reductase (TR), a NADPH-dependent flavoprotein,

78 reduces TS₂ to T(SH)₂, thus ensuring an intracellular reducing environment. The inhibition of TryS
79 and/or TR is known to disrupt the parasite redox balance [10, 11]; these two enzymes can be
80 regarded as validated molecular targets for the development of effective and selective
81 antileishmanial drugs [9, 12].

82 TR inhibition may be achieved by competing with trypanothione binding to the active site; for
83 example, trivalent antimony Sb(III), the active form of the antimonial drug sodium stibogluconate
84 (SSG) [13], a number of metals, such as Ag(I) and Au(I) [14-16] and some TR inhibitors, such as
85 azole and diaryl compounds [17, 18] have been reported to directly bind the trypanothione binding
86 site.

87 Natural products (NPs) including flavonoids, isoflavonoids, saponins, alkaloids, tannins and
88 indoles have been shown to exert antileishmanial effects [19, 20]. Among them, chalcones (1,3-
89 diaryl-2-propen-1-ones), prominent secondary metabolites and precursors of flavonoids, can be
90 considered “privileged structures”, i.e. evolutionary-chosen molecules that have evolved to achieve
91 an inherent affinity for diverse biological macromolecules in the natural selection process [21].
92 Indeed, chalcones display a wide range of pharmacological effects, including antioxidant,
93 antimutagenic, antimitotic, antimetastatic and antiinflammatory activity [22, 23]. The
94 antileishmanial potential of chalcones has also been demonstrated [20]; naturally occurring
95 chalcones such as licochalcone A and isocordoin (Figure 1), isolated from *Glycyrrhiza glabra* and
96 *Lonchocarpus xuul*, respectively, were able to efficiently inhibit the proliferation of different
97 *Leishmania* species [24-26]. Unfortunately, the intrinsic cytotoxicity of these molecules may
98 represent an undesired aspect. In this study, the chalcone framework was selected as main scaffold
99 to develop a new series of effective and safe antileishmanial agents and targeting a key enzyme in
100 the polyamine-trypanothione pathway, ie TR.

101 Therefore, we have synthesized 31 novel chalcones, evaluated their activity against *Leishmania*
102 vs. mammalian cells and tested both interaction and ability to inhibit *L. donovani* TR.

103

104 **2. RESULTS**

105 *2.1 Design strategy*

106 A small library of 31 chalcone-based analogues was designed and synthesized (Figure 1). In
107 particular, the A-ring of the main scaffold was properly functionalized at the positions 2 and 4: the
108 C-4 position was occupied by a suitable alkoxy function (O-R), namely 3,3-dimethylallyloxy (or
109 prenyloxy) and propargyloxy affording Series 1 and 2, respectively. The C-2 position was
110 differently functionalized by introduction of hydroxy, methoxy, prenyloxy and propargyloxy groups
111 (O-R₁). In order to perform a Structure Activity Relationship (SAR) study, different moieties were
112 introduced as B-ring, namely pyridine or aryl functions bearing methoxy, bromo, nitro, and fluorine
113 substituents.

114

115 *2.2. Synthesis*

116 All the tested chalcones were readily synthesized through the classic base-catalyzed Claisen-
117 Schmidt procedure (as shown in Scheme 1). In details, the selected acetophenone was reacted at
118 room temperature with the appropriate aldehyde in ethyl alcohol and in the presence of a 50%
119 KOH/H₂O solution, to give the desired final compounds (**1-31**, Table 1). The acetophenone
120 intermediates were obtained by reaction of 2,4-dihydroxyacetophenone with the appropriate alkyl
121 halide to obtain the 2-OH,4-alkoxyacetophenones (**32, 33**) and 2,4-bi-functionalized-acetophenones
122 (**34-37**).

123

124 *2.3. Biological and Enzymatic Assays*

125 First, 31 chalcone-based derivatives were investigated for their antileishmanial effect on the
126 promastigotes of *L. donovani*. Then, for 16 analogues that inhibited parasite growth at micromolar
127 level, cytotoxicity against mammalian kidney epithelial cells and affinity for TR enzyme (SPR-
128 based assay) were also evaluated. This allowed us to identify two promising molecules in terms of
129 activity and selectivity that were then further investigated for their ability to inhibit promastigote

130 growth of different parasitic species, including *L.tropica*, *L.major* and *L.infantum* and to affect the
131 growth *L.donovani* amastigotes. Finally, the mechanism of TR inhibition was also studied.

132

133 2.3.1. *In vitro* inhibition of *Leishmania* promastigote growth

134 To assess the antileishmanial potential of the synthesized compounds, a reference strain of *L.*
135 *donovani* (MHOM/NP/02/BPK282/0cl4) was employed in two different stages of the parasitic life
136 cycle; the extracellular promastigote form is found in the sandfly vector, while intracellular
137 amastigote form is specifically found in the host cell.

138 In a first experiment, 31 chalcone-based derivatives were investigated for their antileishmanial
139 efficacy on the promastigote forms of *L. donovani* (Table 1). Amphotericin B was employed as
140 reference compound. Data were expressed as IC₅₀, i.e. the concentration of compound that is
141 required to inhibit growth by 50%.

142 Among the tested chalcones, compounds **1-16** turned out to effectively inhibit the promastigote
143 growth with micromolar potency and IC₅₀/72h values ranged from 21.5 μM (compound **12**) to 3.0
144 μM (compound **6**). Concerning analogues **17-31**, no antileishmanial effect was observed at the
145 maximal dose of 40.0 μM and were then discarded from further evaluation. Among derivatives with
146 a simple phenyl function as B-ring included in Series 1 (**7, 19-21**), the substituent at the 2-position
147 of the A-ring markedly affected the inhibitory behaviour against *Leishmania*. In details, compound
148 **7**, bearing a methoxy function, showed an IC₅₀/72h of 15.0 μM, while the presence of hydroxy,
149 acethoxy, and prenyloxy (**19, 20** and **21**, respectively) rendered the derivatives almost inactive. By
150 keeping the most favourable 2-methoxy-4-prenyloxy substitution pattern in the A-ring, further
151 modifications were applied to the B-aryl ring. The presence of electron-donating moieties, such as
152 methoxy groups, on several positions of the B-ring (compounds **8, 23, 24**) gave different results, as
153 only the bulky 3,4,5-trimethoxylated analogue **8** proved to inhibit parasite growth with an IC₅₀/72h
154 of 11.0 μM. The effect of different electron-withdrawing groups, namely bromo, nitro, and fluoro,
155 on the *para* position of the B-ring (compounds **5, 6**, and **22**, respectively) was also investigated:

156 compound **6**, with the nitro substituent, resulted to be the most active among the series, showing an
157 $IC_{50/72h}$ of 3.0 μM , followed by the bromo-derivative **5** ($IC_{50/72h} = 16.0 \mu M$). The introduction of
158 a heterocyclic furyl group (**18**) led to a loss of activity, while a 4- and 3-pyridyl moiety (**3** and **4**,
159 respectively) allowed to retain good activities ($IC_{50/72h} = 10.5 \mu M$). The corresponding pyridyl-
160 based analogues (**1** and **2**), characterized by a 2-hydroxyl function, retained leishmanicidal activity
161 ($IC_{50/72h} = 5.0 \mu M$ and $8.5 \mu M$, respectively).

162 A different trend of potency was observed in the 4-propargyloxy Series 2 upon applying
163 different B-ring functionalization. The insertion on B-ring of methoxy groups (**27**, **28** and **29-31**)
164 and *para*-NO₂ functions (analogues **25** and **26**) led to inactive compounds. Interestingly, the
165 presence of a *para*-F phenyl B-ring and of a 2-propargyloxy A-ring (compound **16**) conferred
166 an effective ability to inhibit *Leishmania* growth ($IC_{50/72h} = 12.5 \mu M$). Moreover, the *para*-Br
167 derivatives **12** and **15** retained a moderate antileishmanial activity ($IC_{50/72h} = 21.5 \mu M$ and 15.0
168 μM , respectively) that was comparable to the corresponding Br-derivative **5** of Series 1. The
169 pyridine-based analogues **10**, **11**, and **13**, **14** with a 2-methoxy-4-propargyloxy and 2,4-bis-
170 propargyloxy A-ring, respectively, showed low micromolar potencies ($IC_{50/72h}$ values ranging
171 from 4.0 μM to 9.5 μM), similar to that of the corresponding Series 1 analogues (**1-4**). A reduction
172 of activity was observed for compound **9**, designed as the 2-hydroxy congener of **11** and **14**
173 derivatives.

174 Compound **6** and **16** were further investigated for their antileishmanial efficacy on the promastigote
175 forms of three other parasitic species, ie *L. tropica*, *L. major* and *L. infantum*. Interestingly, the
176 results obtained highlight diverse susceptibilities of different parasitic species to the compounds., In
177 detail, compound **6** exhibited an $IC_{50/72h}$ of 5.2 μM , 3.3 μM and 1.6 μM on *L. tropica* , *L. major*
178 and *L. infantum*, respectively, while compound **16** showed an $IC_{50/72h}$ of 13 μM , 10 μM and 1.6
179 μM on *L. tropica*, *L. major* and *L. infantum*, respectively. Thus, both compound **6** and **16** exhibited
180 the highest inhibitory activity on *L. infantum*, revealing *L. infantum* as the most susceptible species
181 to the examined chalcones.

182 2.3.2 *In vitro* mammalian cell toxicity

183 The cytotoxic effect against mammalian cells was evaluated for the most active chalcones (**1-16**)
184 by using Vero cell line (mammalian kidney epithelial cells) (Table 1). Data were expressed as 50%
185 cytotoxic concentration (CC_{50}). The tested compounds generally displayed moderate to low
186 cytotoxicity, with $CC_{50}/72h$ values above 30 μM . In particular, analogues **5**, **6** and **16** were
187 characterized by low toxicity, with cytotoxic effect detected only at 600 μM . On the contrary, the
188 pyridine-based chalcones showed an unfavorable SI due to their moderate cytotoxic effects. It is
189 noteworthy that $CC_{50}/72h$ value of the reference compound amphotericin B was 200 μM , lower
190 than those of compound **6** and **16**. The cytotoxic effect against human acute monocytic leukemia
191 cell line (THP-1) was also evaluated (Table 1) and the selectivity index (SI) was calculated as
192 CC_{50}/IC_{50} ratio. Analogue **5** showed a higher cytotoxic effect on THP-1 than on the Vero cell line.
193 On the other hand, compound **6** and **16**, the most promising of the series, showed the same low
194 cytotoxic effect on the Vero cell line and on THP-1. The cytotoxicity assays allowed to identify
195 compounds **6** and **16** as the most promising of the series. Indeed they showed a remarkable
196 antileishmanial potency against the extracellular form of the parasite ($IC_{50}/72h = 3.0 \mu M$ and 12.5
197 μM , for compound **6** and **16**, respectively) that was coupled with a good SI on mammalian cells
198 (200 and 48, for compound **6** and **16**, respectively).

199

200 2.3.3 *Inhibition of L. donovani amastigote growth*

201 Considering the promising results obtained for **6** and **16**, these compounds were selected to be
202 tested for their efficacy against the amastigote stage of *L. donovani*. The amastigote assay was
203 performed by using metacyclic promastigotes to infect differentiated THP-1 macrophagic cells;
204 amastigotes transformed from metacyclic promastigotes proliferated inside host macrophages. An
205 inversion of potencies was observed when focusing on inhibitory activity of **6** and **16** on
206 amastigotes growth, with respect to activities recorded on promastigotes (Figure 2a-

207 2b). Indeed, compound **16** exhibited a higher inhibitory effect on amastigotes than on
208 promastigotes (4.5 μM vs 12.5 μM , $\text{IC}_{50}/72\text{h}$ calculated on amastigote and promastigote cultures,
209 respectively), while compound **6** showed a lower potency on amastigotes than on promastigotes
210 (14.0 μM vs 3.0 μM , $\text{IC}_{50}/72\text{h}$ detected on amastigote and promastigote cultures, respectively).

211

212 2.3.4. Evaluation of activity toward trypanothione reductase

213 2.3.4.1. Surface Plasmon Resonance screening

214 For Surface Plasmon Resonance (SPR) experiments, TR from *Leishmania* spp. has been
215 immobilized by amine coupling on COOH5 sensorchips, while chalcones were analytes; FastStep
216 SPR experiments were performed by stepped analyte gradient injections (ranging between 1.5 and
217 100 μM). Screening of the 16 most active chalcones (compounds **1-16**) demonstrated that two
218 molecules, compounds **6** and **16**, interacted directly and with high affinity with TR. K_D values
219 calculated for these compounds were K_D (compound **6**) = $0.6 \pm 0.2 \mu\text{M}$ and K_D (compound **16**) = 2.4
220 $\pm 0.5 \mu\text{M}$ (Figure 3).

221

222 2.3.4.2. Enzymatic assays

223 Kinetic studies were performed on compound **6**, endowed with the highest activity against the
224 promastigote forms of *L. donovani* and affinity toward TR. Steady state kinetic experiments were
225 carried out at various concentrations of TS_2 and compound **6**, while fixed TR and NADPH
226 concentrations (10 nM and 40 μM , respectively) were maintained. After starting the reaction by the
227 addition of NADPH, the absorbance decrease at 340 nm, indicative of NADPH oxidation, was
228 measured. As shown in Figure 4, compound **6** competitively inhibited the binding of TS_2 to TR.
229 Each line in the Dixon plot represents linear regression analysis of reciprocal of average fitted rates
230 of TS_2 reduction for different substrate concentrations, as a function of inhibitor concentration. The
231 K_M and k_{cat} of TR used for the K_i calculation were $23.0 \pm 1.0 \mu\text{M}$ and $11.4 \pm 0.3 \text{ s}^{-1}$ respectively

232 [17]. The value of K_i calculated from the Dixon plot analysis was $0.45 \pm 0.11 \mu\text{M}$, about three times
233 lower than that of Sb(III) ($1.5 \mu\text{M}$) [13].

234

235 2.4. Docking studies

236 The crystal structure of TR from *L. infantum* revealed two crucial cysteine residues (Cys52 and
237 Cys57) in the active site, involved in a concerted nucleophilic attack to the TS_2 disulfide bridge to
238 produce the reduced substrate $\text{T}(\text{SH})_2$. In order to gain functional and structural insight into the
239 mechanism of inhibition, molecular docking simulations of compound **6** to TR were performed
240 using the x-ray structures of the enzyme in both reduced and oxidized states. Figure 5a illustrates
241 the most probable and energetically favourable binding modes of compound **6** at the active site of
242 TR in oxidized state (PDB code: 2JK6) and Figure 5b shows the conformation with the lowest
243 energy in the most populated cluster. As shown in TableS1, the most populated cluster contains
244 32/100 poses and the lowest energy pose in this cluster displays a binding energy of -7.63 kcal/mol
245 corresponding to a $K_i=2.56 \mu\text{M}$. Figure 5c displays the conformation with the lowest energy in the
246 most populated cluster, resulting from the docking procedure performed using TR in reduced state
247 (PDB code: 4ADW); the clusters and the energies of the poses are reported in TableS2. In this case,
248 the most populated cluster contains 16/100 poses and the lowest energy pose in this cluster displays
249 a binding energy of -6.66 kcal/mol , corresponding to a $K_i=13.19 \mu\text{M}$.

250 As shown in Figure 5, both the docking procedures performed using the TR structures in the
251 oxidized and reduced state gave similar results. Interestingly, in both the procedures the most
252 populated clusters occupy the same portion of the trypanothione cavity volume. Compound **6** binds
253 to TR in both oxidized and reduced states at the same hydrophobic pocket close to the two catalytic
254 cysteines lined by the following residues: Ile339, Ile458', His461', E466', E467', S470', F396',
255 L399'. Compound **6** establishes electrostatic interactions with and K61, E467' and S470'.
256 Interestingly, compound **6** appears to have a higher affinity for the oxidized form of TR.

257

258 3. DISCUSSION

259 To support the urgent need of safe and effective agents against leishmaniasis, a plethora of
260 bioactive compounds with antileishmanial activity and acting through different mechanisms have
261 been synthesized [27, 28]. Evidence indicates that a number of natural and synthetic chalcones
262 exhibit antileishmanial activity [25, 29-32]. In this study, we evaluated the antileishmanial effect of
263 a small library of synthetic chalcones and we investigated the mechanism of action of selected
264 compounds. Among the 31 tested compounds, 16 (**1-16**) turned out to inhibit the promastigote form
265 of *L.donovani* with micromolar potency, and among them, two (**6** and **16**) showed good potency
266 ($IC_{50/72h}$ values of 3.0 and 12.5 μM , respectively), even if lower with respect to the reference drug
267 amphotericin B. Furthermore compounds **6** and **16** maintained a good inhibitory activity when
268 tested on amastigotes of *L.donovani* (IC_{50} values of 14.0 and 4.5 μM , respectively). Interestingly,
269 these derivatives were characterized by low toxicity when tested on Vero cells and THP-1 cells,
270 being three times less toxic than amphotericin B and thus exhibiting a very favorable SI.

271 Moreover, compounds **6** and **16** efficiently inhibited the promastigote growth of other leishmanial
272 species, including *L.tropica*, *L.major* and *L.infantum*, being particularly active on *L.infantum*.

273 In an effort to assess the mechanism by which **6** and **16** inhibit *Leishmania* growth, we evaluated
274 their affinity and activity against TR, a pivotal enzyme involved in the parasite detoxification. The
275 enzymes of the trypanothione pathway are not present in mammals, and are often considered among
276 the most promising antileishmanial targets. However, it was previously demonstrated that at least
277 90% of TR inactivation needs to be obtained by inhibitor compounds to kill the parasite; therefore,
278 effective TR inhibitors should have submicromolar inhibition activity [12]. Here, we observed that
279 compound **6** showed a submicromolar K_i value vs. TR ($0.45 \pm 0.11 \mu M$) that is about 6 times lower
280 than the $IC_{50/72h}$ value vs. promastigotes and 30 times lower than the $IC_{50/72h}$ vs. the amastigotes.
281 This result is in agreement with numerous results present in literature showing that specific and
282 efficient trypanosomatid TR inhibitors have been found to be less active on the parasites growth.
283 This apparent paradox was shown by Krieger and coworkers who produced conditioned TR

284 knockout in *T. brucei* [33], demonstrating that the redox metabolism of the parasite was affected
285 only when TR was titrated down to less than 5% of normal. Thus, compound **6** is a good TR
286 inhibitor, with a K_i value remarkably lower than Sb(III) and azole-based compounds and in the
287 same order of magnitude of RDS 777, a diaryl sulphide derivative [17, 18].

288 The docking experiments furnished a possible binding mode of compound **6** to the catalytic site.
289 Indeed, compound **6** binds to TR in both the oxidized and reduced states to a hydrophobic pocket
290 close to the catalytic site, which was already shown to be part of RDS 777 binding site in the TR
291 trypanothione cleft. Interestingly, F396' and E467' lining the compound **6** binding site have been
292 already identified as important residues to establish interaction with other TR inhibitors [18].

293 This study has some limitations, including the employment of a small library of chalcones for the
294 screening of antileishmanial activity and the lack of *in vivo* pharmacokinetic and pharmacodynamic
295 testing of the selected compounds.

296

297

298 **4. CONCLUSIONS**

299 A small library of 31 chalcone-based analogues were synthesized and tested for their
300 antileishmanial activity. Among tested compounds, **6** and **16** were found to be significantly active in
301 *in vitro* evaluation against *L. donovani* promastigotes and amastigotes without eliciting cytotoxic
302 effects towards human cells, thus showing optimal performance in terms of potency and selectivity.
303 Furthermore, compounds **6** and **16** inhibited the promastigote growth of other leishmanial species,
304 including *L. tropica*, *L. major* and *L. infantum*. Finally, compounds **6** and **16** interacted with TR and
305 compound **6** effectively inhibited TR activity, providing evidence that TR inhibition could represent
306 one of the possible mechanisms of action of this molecule. In conclusion, the effective inhibitory
307 activity against different *Leishmania* species, the lack of toxicity on mammalian cells and the ability
308 to block a crucial parasite enzyme target, highlight the potential for the chalcone **6** to be further
309 optimized and to develop novel drug candidates against leishmaniasis.

310

311 **5. EXPERIMENTAL SECTION**

312 *5.1. Chemistry.*

313 Starting materials, unless otherwise specified, were used as high grade commercial products.
314 Solvents were of analytical grade. Reaction progress was followed by thin layer chromatography
315 (TLC) on precoated silica gel plates (Merck Silica Gel 60 F254) and then visualized with a UV254
316 lamplight. Chromatographic separations were performed on Merck silica gel columns by flash
317 method (Kieselgel 40, 0.040-0.063 mm, 240 – 400 mesh). Melting points were determined in open
318 glass capillaries, using a Büchi apparatus and are uncorrected. ¹H NMR and ¹³C NMR spectra were
319 recorded on a Varian Gemini spectrometer 400 MHz and 101 MHz, respectively, and chemical
320 shifts (δ) are reported as parts per million (ppm) values relative to tetramethylsilane (TMS) as
321 internal standard; standard abbreviations indicating spin multiplicities are given as follows: s
322 (singlet), d (doublet), t (triplet), br (broad), q (quartet) or m (multiplet); coupling constants (*J*) are
323 reported in Hertz (Hz). Mass spectra were recorded on a Waters ZQ 4000 apparatus operating in
324 electrospray mode (ES). Analyses indicated by the symbols of the elements were within ± 0.4 % of
325 the theoretical values. Compounds were named relying on the naming algorithm developed by
326 CambridgeSoft Corporation and used in Chem-BioDraw Ultra 14.0.

327

328 *5.2. Williamson Reaction: General Procedure*

329 A mixture of selected hydroxylated acetophenone (1.0 eq), alkyl halide (1.1-1.5 eq), K₂CO₃ (1.1
330 eq) in acetone, was heated for 6-10 h at 80 °C; reaction progress was monitored by TLC. Upon
331 reaction completion, the mixture was hot filtered and the solvent was evaporated under reduced
332 pressure. The resulting crude product was purified by column chromatography over a silica gel
333 using a mixture of petroleum ether/EtOAc as the eluent to give the desired pure product.

334 5.2.1. *1-(2-hydroxy-4-((3-methylbut-2-en-1-yl)oxy)phenyl)ethan-1-one (32)* Reaction of 2,4-di-
335 hydroxyacetophenone (5.0 mmol, 0.75 g) and 3,3-dimethylallyl bromide (5.5 mmol, 0.82 g) gave
336 the crude **32** that was purified by flash chromatography (petroleum ether/EtOAc 9:1), 97% yield,
337 mp 42-44 °C. ¹H-NMR (CDCl₃) δ 1.88 (s, 3H, CH₃), 1.91 (s, 3H, CH₃), 2.55 (s, 3H, COCH₃), 4.65
338 (d, 2 H, *J* = 6.6 Hz, OCH₂), 5.55 (t, 1H, *J* = 6.6 Hz, CH), 6.44 (d, *J* = 1.8 Hz, 1H, H-3), 6.54 (dd, *J*
339 = 1.8 and 8.6 Hz, 1H, H-5), 7.78 (d, *J* = 8.6 Hz, 1H, H-6).

340 5.2.2. *1-(2-hydroxy-4-(prop-2-yn-1-yloxy)phenyl)-1-ethanone (33)*. Reaction of 2,4-
341 dihydroxyacetophenone (5.0 mmol, 0.75 g) and propargyl bromide solution 80 wt. % in toluene (5.5
342 mmol, 0.80 g) gave the crude final product **33** that was purified by flash chromatography
343 (petroleum ether/EtOAc 7:3), 93% yield, mp 64-66 °C. ¹H-NMR (CDCl₃) δ 2.55 (s, 3H, CH₃), 2.60
344 (s, 1H, CH), 4.72 (s, 2H, OCH₂), 6.44 (d, *J* = 1.8 Hz, 1H, H-3), 6.55 (dd, *J* = 1.8 and 8.6 Hz, 1H, H-
345 5), 7.70 (d, *J* = 8.6 Hz, 1H, H-6).

346 5.2.3. *1-(2-methoxy-4-((3-methylbut-2-en-1-yl)oxy)phenyl)ethan-1-one (34)*. Reaction of **32** (4.2 g,
347 20.1 mmol) with methyl iodide (4.18 g, 30.25 mmol) gave the crude final product **34** that was
348 purified by flash chromatography (petroleum ether/EtOAc 9.75:0.25), 93% yield, mp 74-76 °C. ¹H-
349 NMR (CDCl₃) δ 1.77 (s, 3H, CH₃), 1.81 (s, 3H, CH₃), 2.57 (s, 3H, COCH₃), 3.00 (s, 3H, OCH₃),
350 4.58 (d, 2 H, *J* = 6.6 Hz, OCH₂), 5.48 (t, 1H, *J* = 6.6 Hz, CH), 6.42 (d, *J* = 1.8 Hz, 1H, H-3), 6.53
351 (dd, *J* = 1.8 and 8.6 Hz, 1H, H-5), 7.83 (d, *J* = 8.6 Hz, 1H, H-6).

352 5.2.4. *1-(2,4-bis((3-methylbut-2-en-1-yl)oxy)phenyl)ethan-1-one (35)*. Reaction of **32** (4.2 g, 20.1
353 mmol) with 3,3-dimethylallyl bromide (4.41 g, 30.25 mmol) gave the crude final product **35** that
354 was purified by flash chromatography (petroleum ether/EtOAc 9.75:0.25) as transparent oil, 56 %
355 yield. ¹H NMR (CDCl₃) δ 1.75 (s, 3H, CH₃), 1.80 (s, 3H, CH₃), 1.84 (s, 3H, CH₃), 1.88 (s, 3H,
356 CH₃), 2.55 (s, 3H, COCH₃), 4.50 (d, 2H, *J* = 6.6 Hz, OCH₂), 4.57 (d, 2H, *J* = 6.6 Hz, OCH₂), 5.44

357 (t, 1H, $J = 6.6$ Hz, CH), 5.60 (t, 1H, $J = 6.6$ Hz, CH), 6.45 (d, $J = 1.8$ Hz, 1H, H-3), 6.58 (dd, $J =$
358 1.8 and 8.6 Hz, 1H, H-5), 7.81 (d, $J = 8.6$ Hz, 1H, H-6).

359 5.2.5. *1-(2-methoxy-4-(prop-2-yn-1-yloxy)phenyl)ethan-1-one (36)*. Reaction of **33** (3.16 g, 17.7
360 mmol) with methyl iodide (3.75 g, 26.55 mmol) gave the crude final product **36** that was purified by
361 flash chromatography (petroleum ether/EtOAc 9.5:0.5), 92% yield, mp 93-95 °C. ^1H NMR
362 (CDCl_3) δ 2.59-2.61 (m, 4H, COCH_3 and CH), 3.95 (s, 3H, OCH_3), 4.78 (s, 2H, OCH_2), 6.57 (d, $J =$
363 1.8 Hz, 1H, H-3), 6.59 (dd, $J = 1.8$ and 8.6 Hz, 1H, H-5), 7.82 (d, $J = 8.6$ Hz, 1H, H-6).

364 5.2.6. *1-(2,4-bis(prop-2-yn-1-yloxy)phenyl)ethan-1-one (37)*. Reaction of **33** (3.16 g, 17.7 mmol)
365 with propargyl bromide solution 80 wt. % in toluene (4.97 g, 26.55 mmol) gave the crude final
366 product **37** that was purified by flash chromatography (petroleum ether/EtOAc 9.5/0.5), 75 % yield,
367 mp 101-103 °C. ^1H NMR (CDCl_3) δ 2.59-2.61 (m, 5H, COCH_3 and CH_2), 4.78 (s, 2H, OCH_2), 4.81
368 (s, 2H, OCH_2), 6.57 (d, $J = 1.8$ Hz, 1H, H-3), 6.57 (dd, $J = 1.8$ and 8.6 Hz, 1H, H-5), 7.80 (d, $J =$
369 8.6 Hz, 1H, H-6).

370 5.3. Claisen–Schmidt reaction: General Procedure

371 To an ethanol solution of acetophenone (**32-37**, 1.0 eq) and the selected benzaldehyde (1.1 eq), a
372 KOH aqueous solution (50 % p/v, 6 eq) was added dropwise. The reaction mixture was stirred at
373 room temperature overnight, then diluted with water and acidified with 6N HCl. The separated solid
374 was collected by *vacuum* filtration and was purified by flash chromatography using petroleum
375 ether/EtOAc as eluent. The final products were crystallized from suitable solvent.

376 5.3.1. *(E)-1-(2-hydroxy-4-((3-methylbut-2-en-1-yl)oxy)phenyl)-3-(pyridin-4-yl)prop-2-en-1-one (1)*.
377 Starting from **32** (0.22 g, 1.0 mmol) and 4-pyridinecarboxaldehyde (0.19 g, 1.1 mmol) the crude
378 final product **1** was obtained and was purified by crystallization from EtOH to obtain a red-orange
379 solid (0.14 g), 45% yield, mp 102-103 °C. ^1H NMR (CDCl_3) δ 1.77 (s, 3H, CH_3), 1.82 (s, 3H, CH_3),
380 4.59 (d, $J = 6.4$ Hz, 2H, OCH_2), 5.49-5.53 (m, 1H, $\text{CH}=\text{C}$), 6.50 (d, $J = 1.8$ Hz, 1H, H-3), 6.52 (dd,

381 $J = 1.8$ and $J = 8.4$ Hz, 1H, H-5), 7.49 (d, $J = 4.4$ Hz, 2H, H-2' and H-6'), 7.71 (d, $J = 15.6$ Hz, 1H,
382 =CH), 7.73 (d, $J = 15.6$ Hz, 1H, CH=), 7.81 (d, $J = 8.8$ Hz, 1H, H-6), 8.70 (d, $J = 4.4$ Hz, 2H, H-3'
383 and H-5'). ^{13}C NMR (CDCl_3) δ 18.2, 25.7, 65.3, 101.7, 108.6, 113.8, 118.5, 122.0, 124.7, 131.2,
384 138.5, 141.0, 142.0, 148.8, 149.5, 166.0, 166.9, 192.1. ESI-MS (m/z): 310 (M + H); Anal.
385 $\text{C}_{19}\text{H}_{19}\text{NO}_3$ (C, H, N).

386 5.3.2. (*E*)-1-(2-hydroxy-4-((3-methylbut-2-en-1-yl)oxy)phenyl)-3-(pyridin-3-yl)prop-2-en-1-one (**2**).

387 Starting from **32** (0.22 g, 1.0 mmol) and 3-pyridinecarboxaldehyde (0.19 g, 1.1 mmol) gave the
388 crude final product **2** that was purified by crystallization from EtOH to obtain a orange solid (0.21
389 g), 67% yield, mp 108-109 °C. ^1H NMR (CDCl_3) δ 1.77 (s, 3H, CH_3), 1.82 (s, 3H, CH_3), 4.59 (d, J
390 = 6.4 Hz, 2H, OCH_2), 5.49-5.53 (m, 1H, $\text{CH}=\text{C}$), 6.51 (d, $J = 2.0$ Hz 1H, H-3), 6.53 (dd, $J = 8.8$ and
391 2.0 Hz, 1H, H-5), 7.32-7.36 (m, 1H, H-5'), 7.62 (d, $J = 15.6$ Hz, 1H, =CH), 7.82 (d, $J = 16.0$ Hz, 1H,
392 CH=), 7.84 (d, $J = 8.8$ Hz, 1H, H-6'), 7.79 (d, $J = 7.6$ Hz, 1H, H-6), 8.61 (d, $J = 4.8$ Hz, 1H, H-4'),
393 8.94 (s, 1H, H-2'). ^{13}C NMR (CDCl_3) δ 18.66, 25.6, 65.2, 102.7, 107.6, 113.8, 119.2, 123.5, 125.7,
394 131.2, 138.4, 144.0, 144.1, 149.8, 149.5, 166.0, 166.7, 192.8. ESI-MS (m/z): 310 (M + H); Anal.
395 $\text{C}_{19}\text{H}_{19}\text{NO}_3$ (C, H, N).

396 5.3.3. (*E*)-1-(2-methoxy-4-((3-methylbut-2-en-1-yl)oxy)phenyl)-3-(pyridin-4-yl)prop-2-en-1-one (**3**).

397 Starting from **34** (0.23 g, 1.0 mmol) and 4-pyridinecarboxaldehyde (0.19 g, 1.1 mmol) gave the
398 crude final product **3** that was purified by flash chromatography (DCM/MeOH 9.75:0.25) and then
399 crystallized from AcOEt/n-hexane to obtain a solid(0.09 g), 30% yield, mp 102-103 °C. ^1H NMR
400 (CDCl_3) δ 1.79 (s, 3H, CH_3), 1.82 (s, 3H, CH_3), 3.96 (s, 3H, OCH_3), 4.51 (d, $J = 6.4$ Hz, 2H,
401 OCH_2), 5.44-5.51 (m, 1H, $\text{CH}=\text{C}$), 6.30 (d, $J = 1.8$ Hz, 1H, H-3), 6.42 (dd, $J = 1.8$ and 8.4 Hz, 1H,
402 H-5), 7.49 (d, $J = 4.4$ Hz, 2H, H-2' and H-6'), 7.70 (d, $J = 15.6$ Hz, 1H, =CH), 7.72 (d, $J = 15.6$ Hz,
403 1H, CH=), 7.80 (d, $J = 8.8$ Hz, 1H, H-6), 8.65 (d, $J = 4.4$ Hz, 2H, H-3' and H-5'). ^{13}C NMR
404 (CDCl_3) δ 18.5, 24.5, 55.2, 64.3, 101.9, 107.6, 113.8, 119.1, 123.0, 125.7, 131.2, 138.5, 144.1,
405 144.6, 148.8, 149.3, 162.1, 166.5, 192.5. ESI-MS (m/z): 324 (M + H); Anal. $\text{C}_{20}\text{H}_{21}\text{NO}_3$ (C, H, N).

406 5.3.4. (*E*)-1-(2-methoxy-4-((3-methylbut-2-en-1-yl)oxy)phenyl)-3-(pyridin-3-yl)prop-2-en-1-one (**4**).
407 Starting from **34** (0.23 g, 1.0 mmol) and 3-pyridinecarboxaldehyde (0.19 g, 1.1 mmol) gave the
408 crude final product **4** that was purified by flash chromatography (DCM/MeOH 9.75:0.25) and then
409 crystallized from AcOEt/n-hexane to obtain an orange solid (0.14 g), 45% yield, mp 98-100 °C. ¹H
410 NMR (CDCl₃) δ 1.78 (s, 3H, CH₃), 1.83 (s, 3H, CH₃), 3.93 (s, 3H, OCH₃), 4.78 (d, *J* = 4 Hz, 2H,
411 OCH₂) 5.49-5.53 (m, 1H, CH=C), 6.61 (s, 1H, H-3), 6.66 (d, *J* = 8.8 Hz, 1H, H-5) 7.34-7.37 (m,
412 1H, H-5'), 7.56 (d, *J* = 15.6 Hz, 1H, =CH), 7.62 (d, *J* = 16 Hz, 1H, CH=), 7.80 (d, *J* = 8.8 Hz, 1H,
413 H-6'), 7.89 (d, *J* = 7.6 Hz, 1H, H-6), 8.60 (d, *J* = 4.8 Hz, 1H, H-4'), 8.84 (s, 1H, H-2'). ¹³C NMR
414 (CDCl₃) δ 18.4, 24.7, 55.8, 64.9, 101.5, 107.6, 119.3, 123.5, 127.1, 129.7, 131.3, 132.7, 138.4,
415 141.1, 148.0, 148.6, 149.3, 163.3, 168.3, 192.2. ESI-MS (*m/z*): 324 (M + H); Anal. C₂₀H₂₁NO₃ (C,
416 H, N).

417 5.3.5. (*E*)-3-(4-bromophenyl)-1-(2-methoxy-4-((3-methylbut-2-en-1-yl)oxy)phenyl)prop-2-en-1-one
418 (**5**). Starting from **34** (0.23 g, 1.0 mmol) and 4-bromobenzaldehyde (0.20 g, 1.1 mmol) gave the
419 crude final product **5** that was purified by flash chromatography (petroleum ether/AcOEt 4:1) and
420 then crystallized from AcOEt/n-hexane to obtain a yellow solid (0.32 g), 81% yield, mp 96-98 °C.
421 ¹H NMR (CDCl₃) δ 1.78 (s, 3H, CH₃), 1.83 (s, 3H, CH₃), 3.91 (s, 3H, OCH₃), 4.59 (d, *J* = 6.4 Hz,
422 2H, OCH₂), 5.49-5.53 (m, 1H, CH=C), 6.64 (d, *J* = 2.0 Hz, 1H, H-3), 6.72 (dd, *J* = 2.0 and 8.8 Hz,
423 1H, H-5), 7.62 (d, *J* = 15.6 Hz, 1H, =CH), 7.66 (d, *J* = 15.8 Hz, 1H, CH=), 7.76 (d, *J* = 8.8 Hz, 2H,
424 H-2' and H-6'), 7.80 (d, *J* = 8.4 Hz, 1H, H-6), 8.24 (d, *J* = 8.8 Hz, 2H, H-3' and H-5'). ¹³C NMR
425 (CDCl₃) δ 18.3, 24.9, 54.2, 65.1, 100.3, 106.6, 118.7, 119.9, 123.4, 128.2, 128.4, 131.8, 133.5,
426 138.9, 145.4, 162.4, 168.8, 191.2. ESI-MS (*m/z*): 402 (M + H); Anal. C₂₁H₂₁BrO₃ (C, H,).

427 5.3.6. (*E*)-1-(2-methoxy-4-((3-methylbut-2-en-1-yl)oxy)phenyl)-3-(4-nitrophenyl)prop-2-en-1-one
428 (**6**). Starting from **34** (0.23 g, 1.0 mmol) and 4-nitrobenzaldehyde (0.17 g, 1.1 mmol) gave the crude
429 final product **6** that was purified by crystallization from EtOH to obtain a yellow solid (0.28 g),
430 77% yield, mp 147-147 °C. ¹H NMR (CDCl₃) δ 1.77 (s, 3H, CH₃), 1.82 (s, 3H, CH₃), 3.93 (s, 3H,

431 OCH₃), 4.57 (d, *J* = 6.4 Hz, 2H, OCH₂), 5.49-5.53 (m, 1H, CH=C), 6.52 (d, *J* = 1.6 Hz, 1H, H-3),
432 6.58 (dd, *J* = 1.6 and 8.0 Hz, 1H, H-5), 7.68 (d, *J* = 16.0 Hz, 1H, =CH), 7.59 (d, *J* = 16.0 Hz, 1H,
433 CH=), 7.72 (d, *J* = 8.4 Hz, 1H, H-2' and H-6'), 7.81 (d, *J* = 8.4 Hz, 1H, H-6), 8.26 (d, *J* = 8.4 Hz,
434 1H, H-3' and H-5'). ¹³C NMR (CDCl₃) δ 18.2, 25.8, 55.7, 65.1, 105.8, 118.9, 121.3, 124.1, 128.6,
435 131.0, 133.2, 138.2, 141.9, 160.7, 164.2, 183.2. ESI-MS (*m/z*): 368 (M + H); Anal. C₂₁H₂₁NO₅ (C,
436 H, N).

437 5.3.7. (*E*)-1-(2-methoxy-4-((3-methylbut-2-en-1-yl)oxy)phenyl)-3-phenylprop-2-en-1-one (7).

438 Starting from **34** (0.23 g, 1.0 mmol) and benzaldehyde (0.11 g, 1.1 mmol) gave the crude final
439 product **7** that was purified by flash chromatography (petroleum ether/AcOEt 9.5:0.5) to obtain a
440 yellow solid (0.14 g), 42% yield, mp 147-149 °C. ¹H NMR (CDCl₃) δ 1.78 (s, 3H, CH₃), 1.82 (s,
441 3H, CH₃), 3.90 (s, 3H, OCH₃), 4.58 (d, *J* = 6.4 Hz, 2H, OCH₂), 5.49-5.53 (m, 1H, CH=C), 6.53 (d, *J*
442 = 1.6 Hz, 1H, H-3), 6.57 (dd, *J* = 1.6 and 8.0 Hz, 1H, H-5), 7.30-7.43 (m, 3H, H-3'-H-5'), 7.53 (d, *J*
443 = 15.6 Hz, 1H, CH=), 7.61-7.67 (m, 2H, H-2' and H-6'), 7.68 (d, *J* = 15.6 Hz, 1H, CH=), 7.77 (d, *J*
444 = 8.4 Hz, 1H, H-6). ¹³C NMR (CDCl₃) δ 18.2, 25.8, 55.7, 65.0, 99.3, 105.8, 118.9, 122.0, 127.2,
445 128.2, 128.8, 129.9, 132.8, 135.5, 141.9, 160.4, 163.5, 190.6. ESI-MS (*m/z*): 323 (M + H); Anal.
446 C₂₀H₂₂O₃ (C, H).

447 5.3.8. (*E*)-1-(2-methoxy-4-((3-methylbut-2-en-1-yl)oxy)phenyl)-3-(3,4,5-trimethoxyphenyl)prop-2-

448 *en*-1-one (**8**). Starting from **34** (0.23 g, 1.0 mmol) and 3,4,5-trimethoxybenzaldehyde (0.21 g, 1.1
449 mmol) gave the crude final product **8** that was purified by crystallization from EtOH to obtain a
450 yellow solid (0.29 g), 71% yield, mp 62-64 °C. ¹H NMR (CDCl₃) δ 1.78 (s, 3H, CH₃), 1.82 (s, 3H,
451 CH₃), 3.90 (s, 3H, OCH₃), 3.89 (s, 9H, OCH₃), 4.57 (d, *J* = 6.4 Hz, 2H, OCH₂), 5.49-5.53 (m, 1H,
452 CH), 6.52 (d, *J* = 1.6 Hz, 1H, H-3), 6.57 (dd, *J* = 1.6 and 8.0 Hz, 1H, H-5), 6.81 (s, 2H, H-2' and H-
453 6'), 7.34 (d, *J* = 15.6 Hz, 1H, CH=), 7.54 (d, *J* = 15.6 Hz, 1H, CH=), 7.70 (d, *J* = 8.4 Hz, 1H, H-6).
454 ¹³C NMR (CDCl₃) δ 18.7, 25.7, 55.6, 56.7, 56.9, 60.5, 63.8, 99.9, 103.8, 107.4, 118.5, 118.9, 123.0,

455 126.6, 131.3, 132.8, 138.5, 138.7, 145.9, 153.1, 153.8, 162.2, 168.8, 190.6. ESI-MS (m/z): 413 (M +
456 H); Anal. C₂₄H₂₈O₆ (C, H).

457 5.3.9. (*E*)-1-(2-hydroxy-4-(prop-2-yn-1-yloxy)phenyl)-3-(pyridin-3-yl)prop-2-en-1-one (**9**). Starting
458 from **33** (0.19 g, 1.0 mmol) and 3-pyridinecarboxaldehyde (0.19 g, 1.1 mmol) gave the crude final
459 product **9** that was purified by crystallization from EtOH to obtain an orange solid (0.22 g), 79%
460 yield, mp 122-124 °C. ¹H NMR (CDCl₃) δ 2.58 (s, 1H, C≡CH), 4.77 (d, J = 1.6 Hz, 2H, OCH₂),
461 6.61 (d, J = 1.8 Hz, 1H, H-3), 6.66 (dd, J = 8.8 and 1.8 Hz, 1H, H-5), 7.34-7.37 (m, 1H, H-5'), 7.56
462 (d, J = 15.6 Hz, 1H, =CH), 7.64 (d, J = 15.6 Hz, 1H, CH=), 7.81 (d, J = 8.4 Hz, 1H, H-6'), 7.89 (d,
463 J = 4.8 Hz, 1H, H-6), 8.60 (d, J = 4.8 Hz, 1H, H-4'), 8.84 (s, 1H, H-2'). ¹³C NMR (CDCl₃) δ 55.8,
464 76.3, 76.8, 101.6, 106.7, 127.6, 129.6, 131.3, 132.6, 133.8, 134.9, 145.6, 147.9, 149.8, 160.3, 162.4,
465 190.1. ESI-MS (m/z): 280 (M + H); Anal. C₁₇H₁₃NO₃ (C, H, N).

466 5.3.10. (*E*)-1-(2-methoxy-4-(prop-2-yn-1-yloxy)phenyl)-3-(pyridin-4-yl)prop-2-en-1-one (**10**).
467 Starting from **36** (0.20 g, 1.0 mmol) and 4-pyridinecarboxaldehyde (0.19 g, 1.1 mmol) gave the
468 crude final product **10** that was purified by flash chromatography (DCM/MeOH 9.75:0.25) and then
469 crystallized from ethanol to obtain a brown solid (0.11 g), 36% yield, mp 98-100 °C. ¹H NMR
470 (CDCl₃): δ 2.56 (s, 1H, C≡CH), 3.91 (s, 3H, OCH₃), 4.75 (d, J = 2.0 Hz, 2H, OCH₂), 6.58 (d, J =
471 1.6 Hz, 1H, H-3), 6.64 (dd, J = 2 and 8.4 Hz, 1H, H-5), 7.40 (d, J = 5.6 Hz, 2H, H-2' and H-6'),
472 7.54 (d, J = 16.0 Hz, 1H, =CH), 7.65 (d, J = 16.0 Hz, 1H, CH=), 7.79 (d, J = 8.4 Hz, 1H, H-6), 8.63
473 (d, J = 5.6 Hz, 2H, H-3' and H-5'). ¹³C NMR (CDCl₃) δ 55.7, 56.6, 76.3, 77.7, 102.5, 107.7, 113.8,
474 118.2, 123.5, 125.7, 130.2, 138.7, 144.5, 146.3, 149.9, 150.5, 166.0, 166.7, 192.6. ESI-MS (m/z):
475 294 (M + H); Anal. C₁₈H₁₅NO₃ (C, H, N).

476 5.3.11. (*E*)-1-(2-methoxy-4-(prop-2-yn-1-yloxy)phenyl)-3-(pyridin-3-yl)prop-2-en-1-one (**11**).
477 Starting from **36** (0.20 g, 1.0 mmol) and 3-pyridinecarboxaldehyde (0.19 g, 1.1 mmol) gave the
478 crude final product **11** that was purified by flash chromatography (DCM/MeOH 9.75:0.25) and then

479 crystallized from ethanol to obtain a yellow solid (0.21 g), 72% yield, mp 120-122 °C. ¹H NMR
480 (CDCl₃): δ 2.58 (s, 1H, C≡CH), 3.93 (s, 3H, OCH₃), 4.78 (d, *J* = 1.6 Hz, 2H, OCH₂), 6.62 (s, 1H,
481 H-3), 6.66 (d, *J* = 8.8 Hz, 1H, H-5), 7.32-7.36 (m, 1H, H-5'), 7.56 (d, *J* = 15.6 Hz, 1H, =CH), 7.62
482 (d, *J* = 16 Hz, 1H, CH=), 7.81 (d, *J* = 8.8 Hz, 1H, H-6'), 7.89 (d, *J* = 8 Hz, 1H, H-6), 8.61 (d, *J* =
483 4.4 Hz, 1H, H-4'), 8.84 (s, 1H, H-2'). ¹³C NMR (CDCl₃) δ 55.7, 56.6, 76.3, 77.7, 101.7, 108.5,
484 113.3, 122.3, 125.7, 131.2, 136.5, 141.0, 142.0, 148.8, 149.5, 151.2, 166.5, 166.9, 192.0. ESI-MS
485 (*m/z*): 294 (M + H); Anal. C₁₈H₁₅NO₃ (C, H, N).

486 5.3.12. (*E*)-3-(4-bromophenyl)-1-(2-methoxy-4-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one (**12**).
487 Starting from **36** (0.20 g, 1.0 mmol) and 4-bromobenzaldehyde (0.20 g, 1.1 mmol) gave the crude
488 final product **12** that was purified by flash chromatography (petroleum ether/EtOAc 9:1) and then
489 crystallized from ethanol to obtain a yellow solid (0.35 g), 95% yield, mp 131-133 °C. ¹H NMR
490 (CDCl₃) δ 2.59 (t, *J* = 2.4 Hz, 1H, C≡CH), 3.92 (s, 3H, OCH₃), 4.77 (d, *J* = 1.6 Hz, 2H, OCH₂),
491 6.61 (s, 1H, H-3), 6.65 (d, *J* = 8.4 Hz, 1H, H-5), 7.49 (d, *J* = 17.2 Hz, 1H, =CH), 7.53 (d, *J* = 8.4
492 Hz, 2H, H-2' and H-6') 7.61 (d, *J* = 15.6 Hz, 1H, CH=), 7.77 (d, *J* = 8.4 Hz, 1H, H-6) 8.26 (d, *J* =
493 8.8 Hz, 2H, H-3' and H-5'). ¹³C NMR (CDCl₃) δ 55.9, 56.6, 76.2, 77.9, 99.7, 106.2, 122.3, 124.9,
494 127.1, 129.6, 130.0, 132.1, 132.0, 134.9, 140.6, 160.8, 162.0, 190.2. ESI-MS (*m/z*): 372 (M + H);
495 Anal. C₁₉H₁₅BrO₃ (C, H).

496 5.3.13. (*E*)-1-(2,4-bis(prop-2-yn-1-yloxy)phenyl)-3-(pyridin-4-yl)prop-2-en-1-one (**13**). Starting
497 from **37** (0.23 g, 1.0 mmol) and 4-pyridinecarboxaldehyde (0.19 g, 1.1 mmol) gave the crude final
498 product **13** that was purified by crystallization from EtOH to obtain a yellow solid (0.08 g), 27%
499 yield, mp 156-158 °C. ¹H NMR (CDCl₃) δ 2.58-2.60 (m, 2H, C≡CH), 4.78 (d, *J* = 2.0 Hz, 2H,
500 OCH₂) 4.80 (d, *J* = 2.4 Hz, 2H, OCH₂), 6.69 (d, *J* = 1.6 Hz, 1H, H-3), 6.72 (dd, *J* = 2.0 and 8.4 Hz,
501 1H, H-5), 7.46 (d, *J* = 5.2 Hz, 2H, H-2' and H-6'), 7.57 (d, *J* = 15.6 Hz, 1H, =CH), 7.73 (d, *J* = 15.6
502 Hz, 1H, CH=), 7.84 (d, *J* = 8.8 Hz, 1H, H-6), 8.66 (d, *J* = 4.4 Hz, 2H, H-3' and H-5'). ¹³C NMR

503 (CDCl₃) δ 55.9, 56.0, 76.2, 77.9, 100.7, 107.6, 113.8, 118.4, 122.4, 124.7, 131.2, 138.5, 140.9,
504 142.0, 150.5, 166.7, 167.9, 192.1. ESI-MS (*m/z*): 318 (M + H); Anal. C₂₀H₁₅NO₃ (C, H, N).

505 5.3.14. (*E*)-1-(2,4-bis(prop-2-yn-1-yloxy)phenyl)-3-(pyridin-3-yl)prop-2-en-1-one (**14**). Starting
506 from **37** (0.23 g, 1.0 mmol) and 3-pyridinecarboxaldehyde (0.19 g, 1.1 mmol) gave the crude final
507 product **14** that was purified by crystallization from ethanol to obtain a yellow solid (0.23 g), 72%
508 yield, mp 156-158 °C. ¹H NMR (CDCl₃): δ 2.58-2.60 (m, 2H, C≡CH), 4.78 (d, *J* = 2.0 Hz, 2H,
509 OCH₂), 4.80 (d, *J* = 2.4 Hz, 2H, OCH₂), 6.51 (s, 1H, H-3), 6.53 (d, *J* = 8.8 Hz, 1H, H-5), 7.32-7.36
510 (m, 1H, H-5'), 7.62 (d, *J* = 15.6 Hz, 1H, =CH), 7.82 (d, *J* = 16 Hz, 1H, CH=), 7.84 (d, *J* = 8.8 Hz,
511 1H, H-6'), 7.79 (d, *J* = 7.6 Hz, 1H, H-6), 8.61 (d, *J* = 4.8 Hz, 1H, H-4'), 8.94 (s, 1H, H-2'). ¹³C
512 NMR (CDCl₃) δ 56.3, 56.9, 76.4, 76.7, 77.9, 101.1, 107.2, 123.4, 127.6, 128.5, 130.5, 131.6, 131.7,
513 132.9, 141.0, 158.0, 159.2, 160.7, 165.0, 190.0. ESI-MS (*m/z*):318 (M + H); Anal. C₂₀H₁₅NO₃ (C,
514 H, N).

515 5.3.15. (*E*)-1-(2,4-bis(prop-2-yn-1-yloxy)phenyl)-3-(4-bromophenyl)prop-2-en-1-one (**15**)
516 Starting from **37** (0.23 g, 1.0 mmol) and 4-bromobenzaldehyde (0.20 g, 1.1 mmol) gave the crude
517 final product **15** that was purified by flash chromatography (petroleum ether/EtOAc 9:1) and then
518 crystallized from EtOH to obtain a yellow solid (0.37 g), 95% yield, mp 123-125 °C. ¹H-NMR
519 (CDCl₃) δ 2.57-2.59 (m, 2H, 2 C≡CH), 4.78 (d, *J* = 8.8 Hz, 4H, 2 OCH₂), 6.70 (s, 1H, H-3), 6.73 (d,
520 *J* = 8.4 Hz, 1H, H-5), 7.46-7.54 (m, 4H, H-2'-H-6'), 7.56 (d, *J* = 15.6 Hz, 1H,=CH), 7.62 (d, *J* = 16
521 Hz, 1H, CH=), 7.80 (d, *J* = 8.4 Hz, 1H, H-6). ¹³C NMR (CDCl₃) δ 55.8, 56.0, 76.1, 76.3, 99.6,
522 106.1, 122.8, 124.2, 127.6, 129.6, 131.0, 132.1, 132.8, 134.9, 140.6, 160.3, 162.1, 190.1. ESI-MS
523 (*m/z*): 396 (M + H); Anal. C₂₁H₁₅BrO₃ (C, H).

524 5.3.16. (*E*)-1-(2,4-bis(prop-2-yn-1-yloxy)phenyl)-3-(4-fluorophenyl)prop-2-en-1-one (**16**). Starting
525 from **37** (0.23 g, 1.0 mmol) and 4-fluorobenzaldehyde (0.14 g, 1.1 mmol) gave the crude final
526 product **16** that was purified by flash chromatography (petroleum ether/EtOAc 4:1) and then

527 crystallized from EtOH to obtain a yellow solid (0.30 g), 91% yield, mp 159-161 °C. ¹H NMR
528 (CDCl₃) δ 2.57-2.59 (m, 2H, 2 C≡CH), 4.77 (d, *J* = 2.4 Hz, 2H, OCH₂), 4.79 (d, *J* = 2.4 Hz, 2H,
529 OCH₂) 6.71 (s, 1H, H-3), 6.72 (d, *J* = 2.4 Hz, 1H, H-5), 7.06-7.11 (m, 2H, H-2' and H-6'), 7.48 (d,
530 *J* = 15.6 Hz, 1H, =CH), 7.59-7.62 (m, 2H, H-3' and H-5'), 7.65 (d, *J* = 16 Hz, 1H, CH=), 7.79 (d, *J*
531 = 9.6 Hz, 1H, H-6). ¹³C NMR (CDCl₃) δ 56.0, 56.6, 76.2, 76.3, 77.7, 77.8, 101.1, 107.2, 115.8 (d, *J*
532 = 22 Hz), 123.4, 126.8, 126.9, 130.1 (d, *J* = 8.5 Hz), 131.6, 131.7, 132.9, 141.0, 158.1, 161.7, 165.0,
533 190.0. ESI-MS (*m/z*): 335 (M + H); Anal. C₂₁H₁₅FO₃ (C, H, N).

534 5.3.17. (E)-N-(4-(3-(2-methoxy-4-((3-methylbut-2-en-1-yl)oxy)phenyl)-3-oxoprop-1-en-1-
535 yl)phenyl)acetamide (**17**). Starting from **34** (0.23 g, 1.0 mmol) and 4-acetamidobenzaldehyde (0.18
536 g, 1.1 mmol) gave the crude final product **17** that was purified by crystallization from EtOH to
537 obtain a yellow solid (0.12 g), 58% yield, mp 131-133 °C. ¹H NMR (CDCl₃) δ 1.78 (s, 3H, CH₃),
538 1.82 (s, 3H, CH₃), 2.21 (s, 3H, COCH₃), 3.90 (s, 3H, OCH₃), 4.57 (d, *J* = 6.4 Hz, 2H, OCH₂), 5.49-
539 5.54 (m, 1H, CH), 6.52 (d, *J* = 2.0 Hz, 1H, H-3), 6.57 (dd, *J* = 8.0 and 2.0 Hz, 1H, H-5), 7.23 (br,
540 1H, NH), 7.46 (d, *J* = 16.0 Hz, 1H, =CH), 7.53 (d, *J* = 8.0 Hz, 2H, H-2' and H-6'), 7.57 (d, *J* = 8.0
541 Hz, 2H, H-3' and H-5'), 7.64 (d, *J* = 16.0 Hz, 1H, CH=), 7.75 (d, *J* = 8.2 Hz, 1H, H-6). ¹³C NMR
542 (CDCl₃) δ 18.2, 23.3, 25.8, 55.7, 65.1, 105.8, 118.9, 121.3, 124.5, 129.6, 131.0, 134.2, 138.6, 145.9,
543 161.5, 163.2, 169.9, 188.2. ESI-MS (*m/z*): 380 (M + H); Anal. C₁₉H₂₀O₄ (C, H).

544 5.3.18. (E)-3-(furan-2-yl)-1-(2-methoxy-4-((3-methylbut-2-en-1-yl)oxy)phenyl)prop-2-en-1-one
545 (**18**). Starting from **34** (0.23 g, 1.0 mmol) and 2-furaldehyde (0.11 g, 1.1 mmol) gave the crude final
546 product **18** that was purified by purified by flash chromatography (petroleum ether/EtOAc 4:1) to
547 obtain a brown oil (0.16 g), 51% yield. ¹H NMR (CDCl₃) δ 1.77 (s, 3H, CH₃), 1.82 (s, 3H, CH₃),
548 3.90 (s, 3H, OCH₃), 4.58 (d, *J* = 6.4 Hz, 2H, OCH₂), 5.47-5.50 (m, 1H, CH), 6.48-6.51(m, 2H,
549 furfuryl), 6.56 (d, *J* = 1.8 Hz, 1H, H-3), 6.64 (dd, *J* = 1.8 and 8.4 Hz, 1H, H-5), 7.26-7.27 (m, 1H,
550 furfuryl), 7.42 (d, *J* = 15.6 Hz, 1H, =CH), 7.48 (d, *J* = 15.6 Hz, 1H, CH=), 7.76 (d, *J* = 8.2 Hz, 1H,
551 H-6). ¹³C NMR (CDCl₃) δ 18.2, 24.3, 55.7, 64.2, 101.2, 107.9, 112.5, 113.9, 119.5, 120.8, 123.4,

552 131.6, 138.9, 143.2, 152.5, 163.4, 168.8, 191.5. ESI-MS (m/z): 313 (M + H); Anal. C₂₀H₁₅NO₃ (C,
553 H, N).

554 5.3.19. (*E*)-1-(2-hydroxy-4-((3-methylbut-2-en-1-yl)oxy)phenyl)-3-phenylprop-2-en-1-one (**19**) [34].

555 Reaction of **32** (1.0 mmol, 0.17 g) and benzaldehyde (1.1 mmol, 0.12 g) gave the crude **19** that was
556 purified by crystallization from EtOH to obtain a yellow solid (0.2 g), 64% yield, mp 92-95 °C. ¹H
557 NMR (CDCl₃) δ 1.76 (s, 3H, CH₃), 1.81 (s, 3H, CH₃), 4.56 (d, $J = 6.0$ Hz, 2H, OCH₂), 5.47 (t, $J =$
558 6.0 Hz, 1H, CH), 6.49 (dd, $J = 2.0$ and 8.4 Hz, 1H, H-5), 6.52 (d, $J = 1.8$ Hz, 1H, H-3), 7.43-7.55
559 (m, 3H, H-3'-H-5'), 7.60 (d, $J = 15.6$ Hz, 1H, =CH), 7.63-7.65 (m, 2H, H-2' and H-6'), 7.82 (d, $J =$
560 8.4 Hz, 1H, H-6), 7.89 (d, $J = 15.3$ Hz, 1H, CH=), 10.45 (br, 1H, OH). ¹³C NMR (CDCl₃) δ 18.6,
561 19.5, 65.0, 101.8, 108.2, 113.9, 118.6, 120.5, 128.4, 128.8, 130.7, 130.5, 130.0, 134.2, 138.6, 143.7,
562 165.0, 166.1, 191.7, (In accordance with previously published spectroscopic data). MS (ESI⁺) m/z :
563 309 (M + H); Anal. C₂₀H₂₀O₃ (C, H).

564 5.3.20. (*E*)-1-(2,4-bis((3-methylbut-2-en-1-yl)oxy)phenyl)-3-phenylprop-2-en-1-one (**21**). Reaction

565 of **35** (1.0 mmol, 0.29 g) and benzaldehyde (1.1 mmol, 0.12 g) gave the crude **21** that was purified
566 by flash chromatography (petroleum ether/EtOAc 9.75:0.25) to obtain a white oil (0.04 g), 10%
567 yield. ¹H NMR (CDCl₃) δ 1.76 (s, 3H, CH₃), 1.82 (s, 3H, CH₃), 1.85 (s, 3H, CH₃), 1.89 (s, 3H,
568 CH₃), 4.51 (d, $J = 6.0$ Hz, 2H, OCH₂), 4.58 (d, $J = 6.0$ Hz, 2H, OCH₂), 5.47-5.61 (m, 2H, CH), 6.47
569 (d, $J = 2.0$, 1H, H-3), 6.61 (dd, $J = 2.0$ and 8.4 Hz, 1H, H-5), 7.38-7.42 (m, 3H, H-3'-H-5'), 7.63-
570 7.67 (m, 2H, H-2' and H-6'), 7.69 (d, $J = 15.6$ Hz, 1H, =C), 7.70 (d, $J = 8.4$ Hz, 1H, H-6), 7.81 (d, J
571 = 15.3 Hz, 1H, C=). ¹³C NMR (CDCl₃) δ 18.6, 18.7, 20.5, 20.7, 24.5, 24.8, 65.1, 65.4, 101.8, 107.3,
572 118.6, 119.6, 123.5, 128.1, 128.4, 128.6, 135.5, 138.0, 145.2, 162.4, 168.7, 191.9. ESI-MS (m/z):
573 378 (M + H); Anal. C₂₅H₂₈O₃ (C, H).

574 5.3.21. (*E*)-3-(4-fluorophenyl)-1-(2-methoxy-4-((3-methylbut-2-en-1-yl)oxy)phenyl)prop-2-en-1-one

575 (**22**). Starting from **34** (0.23 g, 1.0 mmol) and 4-fluorobenzaldehyde (0.14 g, 1.1 mmol) gave the
576 crude final product **22** that was purified by flash chromatography (petroleum ether/EtOAc 9:1) and

577 then crystallized from EtOH to obtain a white solid (0.12 g), 35% yield, mp 81-83 °C. ¹H NMR
578 (CDCl₃) δ 1.78 (s, 3H, CH₃), 1.83 (s, 3H, CH₃), 3.91 (s, 3H, OCH₃), 4.59 (d, *J* = 6.4 Hz, 2H, OCH₂),
579 5.49-5.53 (m, 1H, CH), 6.53 (d, *J* = 1.6 Hz, 1H, H-3), 6.58 (dd, *J* = 1.6 Hz and 8.0 Hz, 1H, H-5),
580 7.06-7.11 (m, 2H, H-2' and H-6'), 7.46 (d, *J* = 16.0 Hz, 1H, =CH), 7.57-7.60 (m, 2H, H-3' and H-
581 5'), 7.65 (d, *J* = 16.0 Hz, 1H, CH=), 7.76 (d, *J* = 8.8 Hz, 1H, H-6). ¹³C NMR (CDCl₃) δ 18.2, 25.8,
582 55.7, 65.1, 103.8, 107.4, 118.9, 119.3 (d, *J* = 22 Hz), 124.6, 130.0 (d, *J* = 8.5 Hz), 131.2, 138.4,
583 145.9, 162.7, 168.2, 193.4. ESI-MS (*m/z*): 341 (M + H); Anal. C₂₁H₂₁FO₃ (C, H).

584 5.3.22. (*E*)-1-(2-methoxy-4-((3-methylbut-2-en-1-yl)oxy)phenyl)-3-(4-methoxyphenyl)prop-2-en-1-
585 one (**23**). Starting from **34** (0.23 g, 1.0 mmol) 4-methoxybenzaldehyde (0.14 g, 1.1 mmol) gave the
586 crude final product **23** that was purified by flash chromatography (petroleum ether/EtOAc 4:1) and
587 then crystallized from EtOH to obtain a yellow solid (0.23 g), 66% yield, mp 62-64 °C. ¹H NMR
588 (CDCl₃) δ 1.78 (s, 3H, CH₃), 1.83 (s, 3H, CH₃), 3.86 (s, 3H, OCH₃), 3.90 (s, 3H, OCH₃), 4.58 (d, *J*
589 = 7.2 Hz, 2H, OCH₂), 5.49-5.53 (m, 1H, CH), 6.53 (d, *J* = 2.0 Hz, 1H, H-3), 6.57 (dd, *J* = 2.0 Hz
590 and 8.8 Hz, 1H, H-5), 6.90-6.95 (m, 2H, H-3' and H-5'), 7.40 (d, *J* = 16.0 Hz, 1H, =CH), 7.54-7.57
591 (m, 2H, H-2' and H-6'), 7.66 (d, *J* = 15.6 Hz, 1H, CH=), 7.74 (d, *J* = 8.8 Hz, 1H, H-6). ¹³C NMR
592 (CDCl₃) δ 18.2, 24.8, 55.7, 64.8, 64.0, 100.9, 107.8, 118.9, 122.5, 126.2, 127.8, 128.7, 132.8, 135.5,
593 141.9, 159.4, 160.4, 162.5, 190.1. ESI-MS (*m/z*): 353 (M + H); Anal. C₂₂H₂₄O₄ (C, H).

594 5.3.23. (*E*)-3-(3,4-dimethoxyphenyl)-1-(2-methoxy-4-((3-methylbut-2-en-1-yl)oxy)phenyl)prop-2-en-
595 1-one (**24**). Starting from **34** (0.23 g, 1.0 mmol) and 3,4-dimethoxybenzaldehyde (0.18 g, 1.1 mmol)
596 gave the crude final product **24** that was purified by flash chromatography (petroleum ether/EtOAc
597 4:1) and then crystallized from DCM/petroleum ether to obtain a yellow solid (0.29 g), 77% yield,
598 mp 83-85 °C. ¹H NMR (CDCl₃) δ 1.78 (s, 3H, CH₃), 1.83 (s, 3H, CH₃), 3.90 (s, 6H, OCH₃), 3.93 (s,
599 3H, OCH₃), 4.59 (d, *J* = 7.4 Hz, 2H, OCH₂), 5.48-5.52 (m, 1H, CH), 6.54 (s, 1H, H-3), 6.58 (d, *J* =
600 8.4 Hz, 1H, H-5), 6.89 (d, *J* = 8.4 Hz, 1H, H-5'), 7.13 (d, *J* = 2.1, 1H, H-2'), 7.20 (dd, *J* = 8.4 and
601 2.1 Hz, 1H, H-6'), 7.37 (d, *J* = 15.6 Hz, 1H, =CH), 7.63 (d, *J* = 15.6 Hz, 1H, CH=), 7.73 (d, *J* = 8.8

602 Hz, 1H, H-6). ¹³C NMR (CDCl₃) δ 18.2, 24.8, 55.7, 56.2, 64.8, 64.0, 100.9, 107.8, 118.5, 122.5,
603 125.3, 127.8, 132.1, 134.4, 141.9, 159.4, 160.4, 161.4, 162.5, 190.4. ESI-MS (*m/z*): 383 (M + H),
604 Anal. C₂₃H₂₆O₅ (C, H).

605 5.3.24. (*E*)-1-(2-methoxy-4-(prop-2-yn-1-yloxy)phenyl)-3-(4-nitrophenyl)prop-2-en-1-one (**25**).
606 Starting from **36** (0.20 g, 1.0 mmol) and 4-nitrobenzaldehyde (0.17 g, 1.1 mmol) gave the crude
607 final product **25** that was purified by crystallization from ethanol to obtain a white solid (0.22 g),
608 67% yield, mp 178-180°C. ¹H NMR (CDCl₃) δ 2.59 (t, *J* = 2.4 Hz, 1H, C≡CH), 3.94 (s, 3H, OCH₃),
609 4.78 (d, *J* = 2.4 Hz, 2H, OCH₂), 6.61 (d, *J* = 2.0 Hz, 1H, H-3), 6.67 (dd, *J* = 2.0 and 8.4 Hz, 1H, H-
610 5), 7.64 (d, *J* = 16.0 Hz, 1H, =CH), 7.70 (d, *J* = 16.0 Hz, 1H, CH=), 7.73 (d, *J* = 8.4 Hz, 2H, H-2'
611 and H-6'), 7.82 (d, *J* = 8.4 Hz, 1H, H-6), 8.26 (d, *J* = 8.8 Hz, 2H, H-3' and H-5'). ¹³C NMR
612 (CDCl₃) δ 55.7, 56.5, 77.7, 105.8, 121.3, 124.1, 128.6, 131.0, 133.2, 138.2, 160.7, 164.2, 183.2.
613 ESI-MS (*m/z*): 338 (M + H); Anal. C₁₉H₁₅NO₅ (C, H, N).

614 5.3.25. (*E*)-1-(2,4-bis(prop-2-yn-1-yloxy)phenyl)-3-(4-nitrophenyl)prop-2-en-1-one (**26**). Starting
615 from **37** (0.23 g, 1.0 mmol) and 4-nitrobenzaldehyde (0.17 g, 1.1 mmol) gave the crude final
616 product **26** that was purified by flash chromatography (petroleum ether/EtOAc 9:1) and then
617 crystallized from ethanol to obtain a yellow solid (0.37 g), 95% yield, mp 186-188 °C. ¹H NMR
618 (CDCl₃) δ 2.58-2.60 (m, 2H, C≡CH), 4.75 (d, *J* = 2.4 Hz, 2H, OCH₂), 4.78 (d, *J* = 2.4 Hz, 2H,
619 OCH₂) 6.66 (d, *J* = 2.0 Hz, 1H, H-3), 6.70 (dd, *J* = 2.0 and 8.8 Hz, 1H, H-5), 7.60 (d, *J* = 15.6 Hz,
620 1H, =CH), 7.66 (d, *J* = 16 Hz, 1H, CH=), 7.74 (d, *J* = 8.8 Hz, 2H, H-2' and H-6'), 7.83 (d, *J* = 8.4
621 Hz, 1H, H-6), 8.23 (d, *J* = 8.8 Hz, 2H, H-3' and H-5'). ¹³C NMR (CDCl₃) δ 55.7, 55.9, 76.2, 76.6,
622 77.7, 77.8, 101.8, 107.3, 123.1, 127.7, 130.5, 133.2, 138.2, 162.8, 164.4, 187.2. ESI-MS (*m/z*): 362
623 (M + H) Anal. C₂₁H₁₅NO₅ (C, H, N).

624 5.3.26. (*E*)-1-(2-methoxy-4-(prop-2-yn-1-yloxy)phenyl)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one
625 (**27**) Starting from **36** (0.20 g, 1.0 mmol) and 3,4,5-trimethoxybenzaldehyde (0.21 g, 1.1 mmol)

626 gave the crude final product **27** that was purified by crystallization from EtOH to obtain a yellow
627 solid (0.27 g), 73% yield, mp 106-108 °C. ¹H NMR (CDCl₃) δ 2.58 (s, 1H, C≡CH), 3.86 (s, 3H,
628 OCH₃), 3.94 (s, 3H, OCH₃), 4.75 (d, *J* = 2.0 Hz, 2H, OCH₂), 6.65 (s, 1H, H-3), 6.67 (d, *J* = 8.4 Hz,
629 1H, H-5), 6.81 (s, 2H, H-3' e H-5'), 7.37 (d, *J* = 15.6 Hz, 1H, =CH), 7.58 (d, *J* = 8.8 Hz, 2H, H-2'
630 and H-6'), 7.64 (d, *J* = 15.6 Hz, 1H, CH=), 7.78 (d, *J* = 8.8 Hz, 1H, H-6). ¹³C NMR (CDCl₃) δ 55.8,
631 55.9, 56.0, 56.2, 56.3, 76.2, 76.6, 77.8, 77.9, 100.9, 107.8, 110.4, 118.5, 123.0, 123.6, 125.8, 131.0,
632 132.8, 142.5, 149.2, 157.8, 162.2, 190.5. ESI-MS (*m/z*): 382 (M + H); Anal. C₂₂H₂₂O₆ (C, H, N).

633 5.3.27. (*E*)-3-(3,4-dimethoxyphenyl)-1-(2-methoxy-4-(prop-2-yn-1-yloxy)phenyl)prop-2-en-1-one
634 (**28**). Starting from **37** (0.23 g, 1.0 mmol) and 3,4-dimethoxybenzaldehyde (0.18 g, 1.1 mmol) gave
635 the crude final product **28** that was purified by flash chromatography (petroleum ether/EtOAc 9:1)
636 and then crystallized from EtOH to obtain a yellow solid (0.22 g), 69% yield, mp 130-132 °C. ¹H
637 NMR (CDCl₃) δ 2.58 (s, 1H, C≡CH), 3.90 (s, 6H, OCH₃), 3.93 (s, 3H, OCH₃), 4.76 (s, 2H, OCH₂),
638 6.60 (s, 1H, H-3) 6.64 (d, *J* = 8.4 Hz, 1H, H-5), 6.88 (d, *J* = 8.4 Hz, 1H, H-5'), 7.12 (d, *J* = 2.2 Hz,
639 1H, H-2'), 7.19 (dd, *J* = 8.0 and 2.2 Hz, 1H, H-6'), 7.33 (d, *J* = 15.6 Hz, 1H, =CH), 7.62 (d, *J* =
640 15.6 Hz, 1H, CH=), 7.72 (d, *J* = 8.0 Hz, 1H, H-6). ¹³C NMR (CDCl₃) δ 55.7, 55.8, 56.2, 56.5, 76.7,
641 77.9, 101.1, 107.1, 110.0, 111.4, 123.6, 123.9, 125.8, 127.3, 131.8, 142.7, 147.1, 154.5, 157.8,
642 161.4, 190.4. ESI-MS (*m/z*): 353 (M + H); Anal. C₂₁H₂₀O₅ (C, H).

643 5.3.28. (*E*)-1-(2,4-bis(prop-2-yn-1-yloxy)phenyl)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (**29**).
644 Starting from **37** (0.23 g, 1 mmol) and 3,4,5-trimethoxybenzaldehyde (0.21 g, 1.1 mmol) gave the
645 crude final product **29** that was purified by crystallization from ethanol to obtain a yellow solid
646 (0.27 g), 67% yield, mp 147-138 °C. ¹H NMR (CDCl₃) δ 2.55-2.58 (m, 2H, C≡CH), 3.94 (s, 6H,
647 OCH₃), 3.97 (s, 3H, OCH₃), 4.77-4.79 (m, 4H, OCH₂), 6.71 (d, *J* = 2.2 Hz, 1H, H-3) 6.88 (dd, *J* =
648 8.4 and 2.0 Hz, 1H, H-5), 6.81 (s, 2H, H-2' and H-6'), 7.42 (d, *J* = 15.6 Hz, 1H, =CH), 7.63 (d, *J* =
649 16.0 Hz, 1H, CH=), 7.81 (d, *J* = 8.8 Hz, 1H, H-6). ¹³C NMR (CDCl₃) δ 55.8, 55.9, 56.0, 56.4, 56.5,

650 76.2, 76.7, 77.8, 77.9, 101.1, 107.1, 110.2, 111.3, 123.7, 125.4, 128.3, 132.5, 142.7, 149.1, 150.8,
651 151.1, 157.8, 161.4, 190.3. ESI-MS (m/z): 407 (M + H); Anal. C₂₄H₂₂O₆ (C, H).

652 5.3.29. (*E*)-1-(2,4-bis(prop-2-yn-1-yloxy)phenyl)-3-(3,4-dimethoxyphenyl)prop-2-en-1-one (**30**).

653 Starting from **37** (0.23 g, 1.0 mmol) and 3,4-dimethoxybenzaldehyde (0.18 g, 1.1 mmol) gave the
654 crude final product **30** that was purified by flash chromatography (petroleum ether/EtOAc 4:1) and
655 then crystallized from EtOH to obtain a yellow solid (0.24 g), 65% yield, mp 137-139 °C. ¹H NMR
656 (CDCl₃) δ 2.55-2.58 (m, 2H, C≡CH), 3.94 (s, 6H, OCH₃), 4.77-4.79 (m, 4H, OCH₂), 6.71 (d, J =
657 2.2 Hz, 1H, H-3) 6.88 (d, J = 8.4 and 2.2 Hz, 1H, H-5), 7.16 (d, J = 8.2 Hz, 1H, H-5'), 7.20 (d, J =
658 2.0 Hz, 1H, H-2'), 7.22 (dd, J = 8.0 and 2.2 Hz, 1H, H-6'), 7.40 (d, J = 15.6 Hz, 1H, =CH), 7.64 (d,
659 J = 16.0 Hz, 1H, CH=), 7.75 (d, J = 8.8 Hz, 1H, H-6). ¹³C NMR (CDCl₃) δ 55.8, 55.9, 56.0, 56.5,
660 76.2, 76.7, 77.8, 77.9, 101.1, 107.1, 110.0, 111.1, 122.9, 123.8, 125.1, 128.3, 132.6, 142.7, 149.1,
661 151.1, 157.8, 161.4, 190.4. ESI-MS (m/z): 377 (M + H); Anal. C₂₃H₂₀O₅ (C, H).

662 5.3.30. (*E*)-1-(2,4-bis(prop-2-yn-1-yloxy)phenyl)-3-(4-methoxyphenyl)prop-2-en-1-one (**31**).

663 Starting from **37** (0.23 g, 1.0 mmol) and 4-methoxybenzaldehyde (0.14 g, 1.1 mmol) gave the crude
664 final product **31** that was purified by flash chromatography (petroleum ether/EtOAc 9:1) and then
665 crystallized from EtOH to obtain an orange solid (0.20 g), 59% yield; mp 120-122 °C. ¹H NMR
666 (CDCl₃) δ 2.57-2.60 (m, 2H, C≡CH), 3.86 (s, 3H, OCH₃), 4.76 (d, J = 1.6 Hz, 2H, OCH₂), 4.78 (d,
667 J = 2.0 Hz, 2H, OCH₂) 6.70 (d, J = 2.2 Hz, 1H, H-3), 6.72 (d, J = 8.2 Hz, 1H, H-5), 6.92 (d, J = 8.4
668 Hz, 2H, H-3' and H-5'), 7.42 (d, J = 15.6 Hz, 1H, =CH), 7.58 (d, J = 8.4 Hz, 2H, H-2' and H-6'),
669 7.66 (d, J = 15.6 Hz, 1H, CH=), 7.76 (d, J = 8.8 Hz, 1H, H-6). ¹³C NMR (CDCl₃) δ 55.9, 56.0,
670 56.5, 76.2, 76.7, 77.7, 77.9, 101.1, 107.1, 110.0, 111.1, 122.4, 123.7, 125.2, 128.9, 132.7, 132.8,
671 142.7, 149.8, 157.8, 161.2, 190.5. ESI-MS (m/z): 345 (M + H); Anal. C₂₀H₁₅NO₃ (C, H, N).

672 5.3.32. 2-cinnamoyl-5-((3-methylbut-2-en-1-yl)oxy)phenyl acetate (**20**). Compound **19** (0.31 g, 1.0
673 mmol) was reacted with acetic anhydride (10 mL) and the mixture was heated under reflux for 4 hr

674 and then poured into ice/water. The solid was collected and crystallized from EtOH to give **19** as
675 white solid (0.23 g), 66 % yield, mp 121-122 °C. ¹H NMR (CDCl₃) δ 1.79 (s, 3H, CH₃), 1.82 (s,
676 3H, CH₃), 2.35 (s, 3H, COCH₃), 4.56 (d, *J* = 6.0 Hz, 2H, OCH₂), 5.47-5.50 (m, 1H, =CH), 6.41 (d, *J*
677 = 2.0, 1H, H-5), 6.60 (dd, *J* = 1.8 and 8.4 Hz, 1H, H-3), 7.27 (d, *J* = 15.6 Hz, 1H, =CH), 7.33-7.45
678 (m, 3H, H-3', H-4', H-5'), 7.53-7.61 (m, 2H, H-2' and H-6'), 7.65 (d, *J* = 15.3 Hz, 1H, =CH), 7.78
679 (d, *J* = 8.4 Hz, 1H, H-6). ¹³C NMR (CDCl₃) δ 18.61, 20.5, 24.5, 65.1, 104.8, 118.6, 113.6, 118.6,
680 118.9, 120.5, 127.7, 128.2, 139.7, 135.9, 138.0, 145.2, 154.4, 154.7, 166.0, 169.11, 191.7. ESI-MS
681 (*m/z*): 351 (M + H); Anal. C₂₀H₂₀O₃ (C, H).

682 5.4. Parasitology.

683 5.4.1. *Parasites.* Promastigote forms of a *L. donovani* reference strain
684 (MHOM/NP/02/BPK282/0cl4), *L. major* reference strain (MHOM/SU/73/5-ASKH), *L. tropica*
685 reference strain (MHOM/SU/74/K27), *L. infantum* reference strain (MHOM/ TN/ 80/ IPT1) were
686 cultured at 26°C in HOMEM (Gibco Thermo Fisher Scientific Inc., Waltham, USA), a liquid
687 custom made medium supplemented with 20% foetal bovine serum (FBS, EuroClone SpA, Milan,
688 Italy) and 1% penicillin-streptomycin (EuroClone SpA).

689 5.4.2. *Cell cultures.* THP-1 cells (human leukemia monocytic cell line) were cultured at 37 °C in
690 RPMI-1640 (EuroClone SpA) liquid medium supplemented with 10% FBS (EuroClone SpA), 1%
691 levoglutamine (EuroClone SpA), Mercaptoethanol (Gibco) 50 μM , 1% penicillin-streptomycin.
692 Vero cells (kidney of African green monkey epithelial cell line) were cultured at 37 °C in MEM
693 liquid medium supplemented with 10% FBS (EuroClone SpA), 1% levoglutamine (EuroClone
694 SpA), 1% penicillin-streptomycin (EuroClone SpA).

695 5.4.3. *Promastigote growth inhibition assay.* The late log/stationary phase of promastigotes were
696 seeded with complete HOMEM medium at 10⁶/mL in 96-well plates and incubated with tested
697 compounds at a range concentration of 40 μM – 1.6 μM in a 26 °C incubator for 72 h. The
698 antileishmanial drug amphotericin B was used as standard drug (positive control). Each experiment

699 was performed in duplicate. Stock solution of the compounds was 8 mM in DMSO. To estimate the
700 concentration at which the compounds caused 50% inhibition of growth (IC₅₀), the AlamarBlue
701 assay was employed (Life Technologies, Thermo Fisher Scientific Inc., Waltham, USA). The
702 AlamarBlue assay includes a colorimetric growth indicator based on detection of metabolic activity.
703 Specifically, the system incorporates an oxidation-reduction (REDOX) indicator that fluoresces and
704 changes color in response to chemical reduction of growth medium resulting from cell growth: the
705 method monitors the reducing environment of proliferating cells; the cell permeable resazurin is
706 added (nonfluorescent form, blue color) and, upon entering cells, is reduced to resorufin
707 (fluorescent form, red color) as result of cellular metabolic activity. Evaluation was performed by
708 adding 20 µL of AlamarBlue and incubating at 26 °C for 24 h. The reducing environment was
709 evaluated after 24 hours by absorbance measurement at the Multiskan Ascent Plate Reader (Thermo
710 Fisher Scientific Inc.) at 550 nm and 630 nm.

711 *5.4.4. Antiamastigote assay.* Human acute monocytic leukemia cell line (THP1) were infected with
712 *L. donovani* promastigotes for the assessment of the activity of compounds against the amastigote
713 form of *Leishmania* parasite. Cells were seeded in a 96-well plate (10⁵ cells/mL) in complete
714 RPMI-1640 medium and PMA (0.1 µM, Cayman Chemical Company, Ann Arbor, Michigan, USA)
715 was added for the cells adherence. Cells were incubated at 37 °C in a 5% CO₂ incubator. After 48 h,
716 the medium was replaced with fresh medium containing stationary phase promastigotes that were
717 then phagocytized by monocytic cells and transformed into intracellular amastigotes. After 24 h of
718 incubation, chalcone compounds were added and the plates were incubated at 37°C in a 5% CO₂
719 incubator for 72 h. After incubation, wells were washed, fixed, and stained with Giemsa. Staining
720 was detected using a Nikon Eclipse E200 light microscope (Nikon, Tokyo, Japan). The infectivity
721 index (% of infected macrophages x average number of amastigotes per macrophage) was
722 determined by counting at least 100 cells in duplicate cultures.

723 *5.4.5. Citotoxicity test.* Mammalian kidney epithelial cells (Vero cell line) were seeded (10⁵/mL)
724 with complete MEM medium in 96-well plates and incubated with test compounds up to a

725 concentration of 600 μ M at 37 °C in a 5% CO₂ incubator. Similarly, THP1 were seeded in a 96-well
726 plate (10⁵ cells/mL) in complete RPMI-1640 medium and PMA (0.1 μ M) was added for the cells
727 adherence. After 72 h of incubation, 20 μ L of AlamarBlue reagent was added to each well and
728 incubated at 37 °C for 24 h. Reduction of resazurin to resorufin was evaluated after 24 h by
729 absorbance measurement at the Multiskan Ascent Plate Reader (Thermo Fisher Scientific Inc.,) at
730 550 nm and 630 nm. DMSO was also tested on *Leishmania* promastigotes and no toxicity was
731 detected. Thus, DMSO did not influence the toxicity of the compounds. Each experiment was
732 performed in duplicate. The selectivity index (SI) for each compound was calculated as the ratio
733 between cytotoxicity (CC₅₀/72h) in Vero cells and activity (IC₅₀/72h) against *Leishmania*
734 promastigotes.

735 5.5. Surface Plasmon Resonance (SPR) measurements

736 SPR experiments were carried out using a SensiQ Pioneer system (SensiQ, ICxNomadics Inc.).
737 The sensor chips (COOH5 SensiQ) were chemically activated by injection of 250 μ l of a 1:1
738 mixture of N-hydroxysuccinimide (50 mM) and N-ethyl-N-(3-dimethylaminopropyl)carbodiimide
739 (200 mM) at a flow rate of 25 μ l/min. Recombinant *Li*TR was immobilized on the activated sensor
740 chip via amine coupling. The reaction was carried out at a rate of 10 mL/min in 20 mM sodium
741 acetate at pH 4.5; the remaining N-hydroxysuccinimide esters were blocked by injecting 100 μ L of
742 1 M ethanolamine hydrochloride. Recombinant *Li*TR was captured to approximately 2000 RU. The
743 chalcone compounds (analytes) were dissolved at a concentration of 10 mM or 20 mM in
744 dimethylsulfoxide (DMSO), and diluted 1:100 in HEPES-buffered saline (HBS: 10 mM HEPES,
745 pH 7.4; 150 mM NaCl; 0.005 % surfactant P20).

746 FastStep injections of samples (100 μ M analytes in HBS + 1% DMSO), and reference buffer
747 (HBS + 1% DMSO) were performed: either the inhibitor and reference buffer were automatically
748 diluted in HBS and injected by 7 serial doubling steps (step contact time = 15 s, nominal flow rate =
749 200 μ l/min). The following analytes were injected: 0-17 s: analyte concentration=1.56 μ M; 17-33 s:
750 3.12 μ M; 33-48 s: 6.25 μ M; 48-62 s: 12.5 μ M; 63-78 s: 25 μ M; 78-93 s: 50 μ M; 94-100 s: 100 μ M;

751 for each injection, a maximal RU value was obtained. In control experiments, the sensor chip was
752 treated as described above in the absence of immobilized protein. The interaction of the
753 immobilized protein with the analytes was detected by mass concentration dependent changes of the
754 refractive index on the sensor chip surface. The changes in the observed SPR signal are expressed
755 as Resonance Units (RU). Typically, a response change of 1000 RU corresponds to a change in the
756 surface concentration on the sensor chip of about 1 ng of protein per mm². The increase in RU
757 relative to baseline indicates complex formation between the immobilized protein and the analytes.
758 For each concentration, the plateau region represents the steady-state phase of the interaction. The
759 decrease in RU after 100 s indicates analyte dissociation from the immobilized ligand after buffer
760 injection.

761 Each sensorgram is the average of three different experiments. Sensorgrams were subjected to
762 global analysis using QDat software 2.2.0.23; for each analyte concentration a % Response was
763 calculated, allowing a local Rmax fit (according to the molecular weight of each compound) and
764 displaying as a response relative to the Rmax. % Response vs. analyte concentration was plotted,
765 and K_D values were calculated for each analyte both from Scatchard plots and from global analysis
766 using the QDat software, by fitting a simple 1:1 binding model to the data.

767 *5.6. Enzymatic assay*

768 *LiTR* was cloned and purified as previously described by Baiocco et al. [13] [35]. Enzyme
769 inhibition assays were carried out at 25 °C using a diode array Hewlett–Packard HP8452A
770 spectrophotometer. The solution containing TR 40 nM, TS₂ (75 μM, 100 μM, 200 μM, 400 μM)
771 and chalcone compound **6** (30 nM, 50 nM, 70 nM, 1 μM) were allowed to equilibrate for 2 min in
772 a quartz cuvette. Assays were initiated by addition of NADPH 40 μM and the absorbance decrease
773 at 340 nm, which indicates the oxidation of NADPH, was followed. The concentrations of NADPH
774 was calculated using the molar extinction coefficient $\epsilon = 6,222 \text{ M}^{-1} \text{ cm}^{-1}$ at 340 nm. Trypanothione
775 disulfide (Bachem) and NADPH (Sigma) were used for the experiments.

776 *5.7. Docking experiments*

777 The pdb coordinates of Compound **6** were designed using the WebGL server [36]. Docking
778 calculations were performed by the Autodock 4.0 software [37]. Docking procedures were
779 performed using the structures of TR in both the oxidized form (PDB code: 2JK6) and the reduced
780 form (PDB code 4ADW) downloaded from the protein data bank (PDB code: 2JK6). The TR
781 structure was edited using the software from the ADT package to remove all water molecules and
782 add hydrogen atoms. Non-polar hydrogens and lone pairs were then merged and each atom within
783 the macromolecule was assigned a Gasteiger partial charge. A grid box of $80 \times 80 \times 80$ points, with
784 a spacing of 0.375 \AA , was positioned at the active-site gorge. The Lamarckian genetic algorithm
785 (LGA) was employed with the maximum number of generations and energy evaluations of 631 and
786 1000334, respectively.

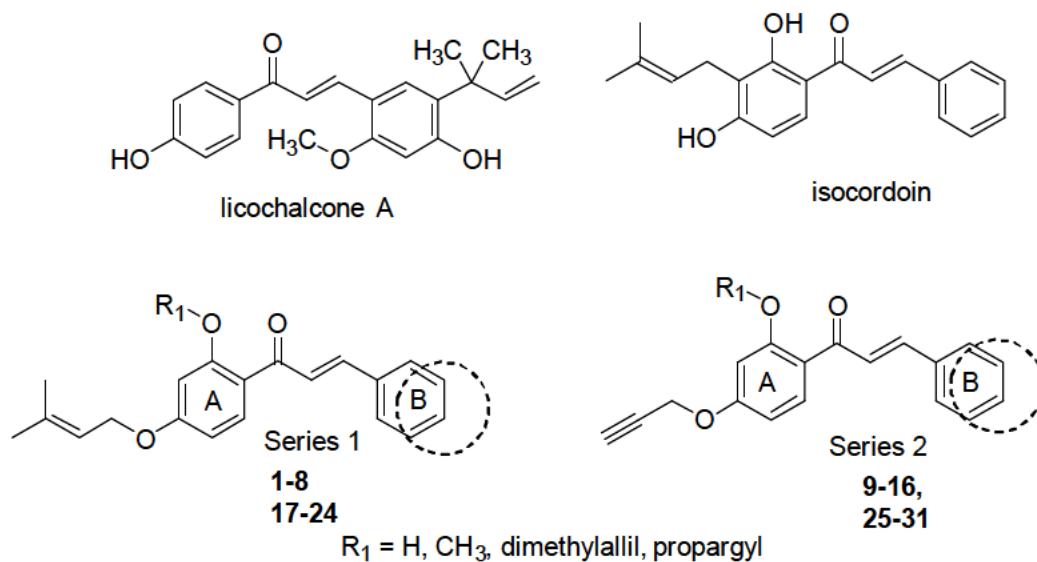
787 **Acknowledgments**

788 This work was supported by Fondazione Del Monte di Bologna e Ravenna (Prot. Nr. 349
789 bis/2016), by the University of Bologna (RFO funds) and by the Ministry of Education (PRIN 2015,
790 Prot. 20154JRJPP). We would like to thank Dr. David Staid for his help to conceive the docking
791 experiments.

792

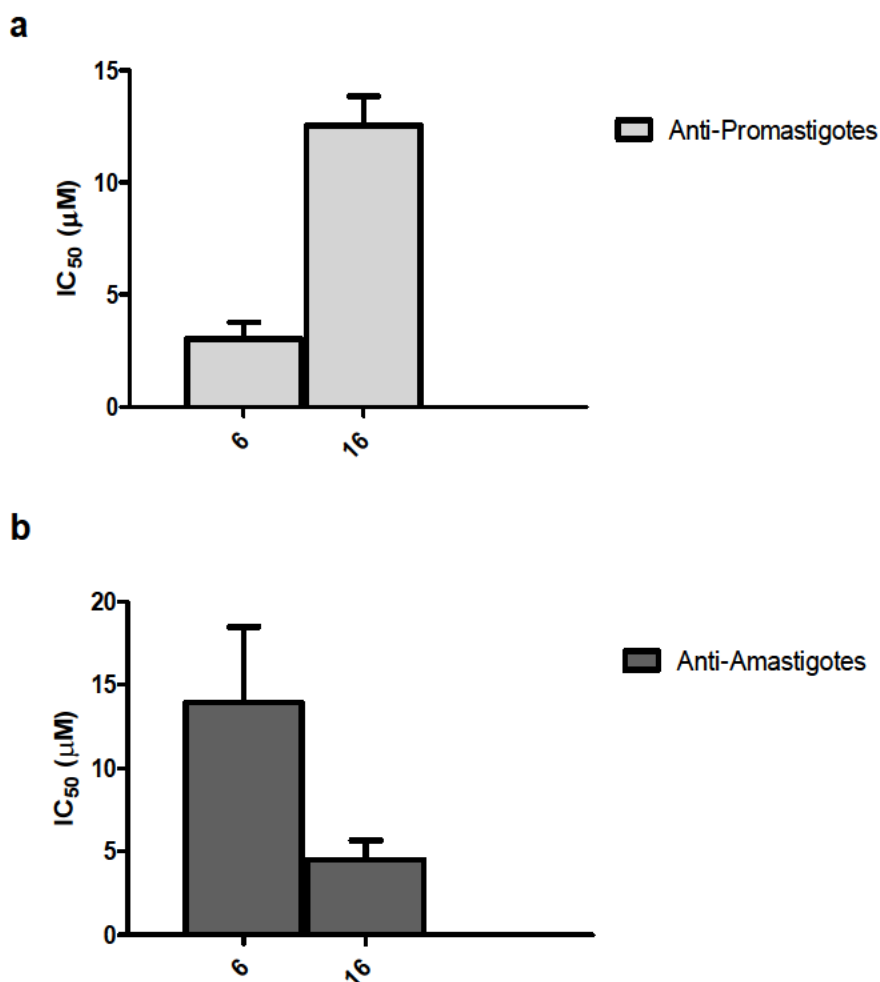
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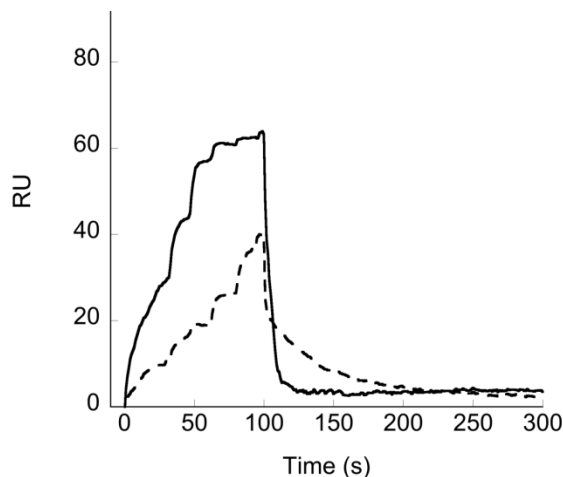
796 **Figure 1.** Structures of licochalcone A, isocordoin and general structure of the newly synthesized
797 compounds (Series 1 and 2).



799

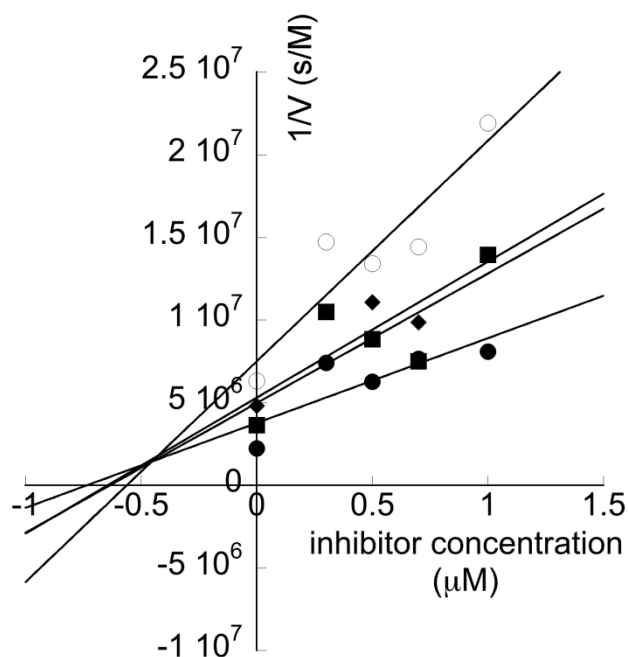
800 **Figure 2.** Antipromastigote (2a) and antiamastigote (2b) activity of compound 6 and compound 16.
801 *L. donovani* promastigotes were treated with tested compounds at a concentration range of 40 - 1.5
802 µM for 72 h, then the effect of the chalcones was evaluated by the AlamarBlue® assay. For the
803 amastigote assay, *Leishmania*-infected THP-1 cells were treated with tested compounds at a

804 concentration range of 40-1.5 μM for 72h, then fixed and stained with Giemsa. Results from three
805 independent experiments performed in duplicates are shown. $\text{IC}_{50/72\text{h}}$, as concentration of
806 compound required to inhibit growth by 50%, is plotted in y axis. Bars represent mean values \pm
807 standard errors.
808



809

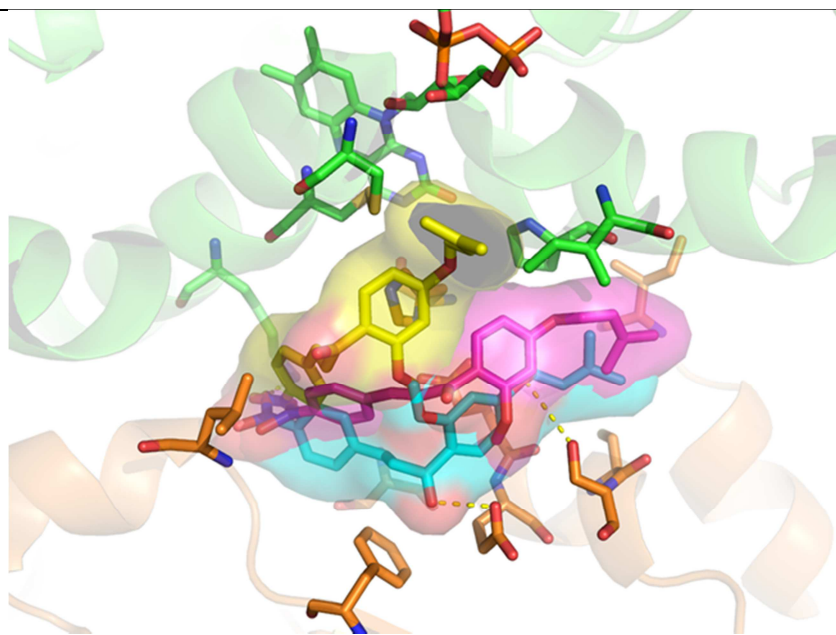
810 **Figure 3.** SPR binding curves (sensorgrams) obtained by injecting different concentrations (range
811 1.5-100 μM) of compounds **6** (full line) and **16** (dashed line) on a surface of covalently immobilized
812 TR; dissociations phases are also shown. RU; response units.
813



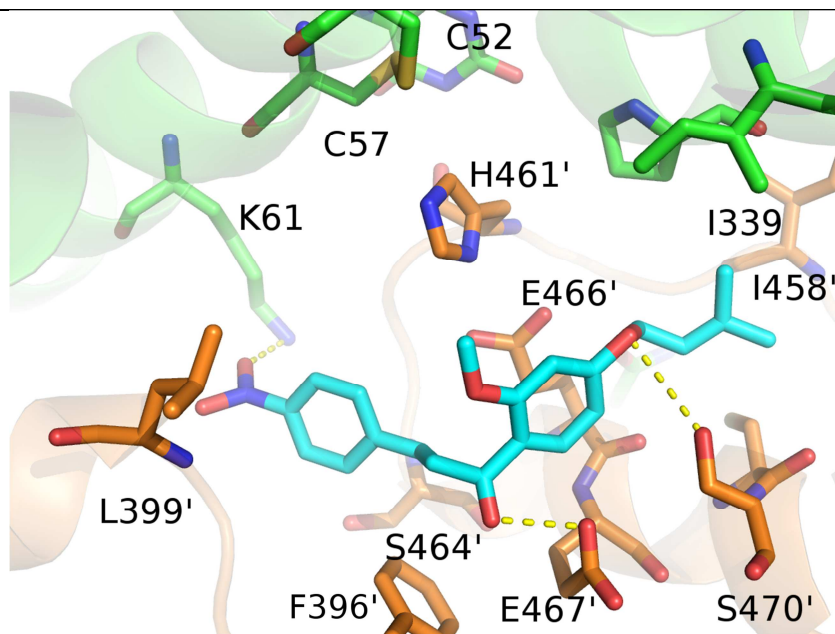
814

815 **Figure 4** Dixon plot of TR inhibition by compound **6** (concentration range: 0-1.0 μM). Open
816 circles $[\text{TS}_2] = 75 \mu\text{M}$; filled squares $[\text{TS}_2] = 100 \mu\text{M}$; filled diamonds $[\text{TS}_2] = 200 \mu\text{M}$ and filled
817 circles $[\text{TS}_2] = 400 \mu\text{M}$.
818

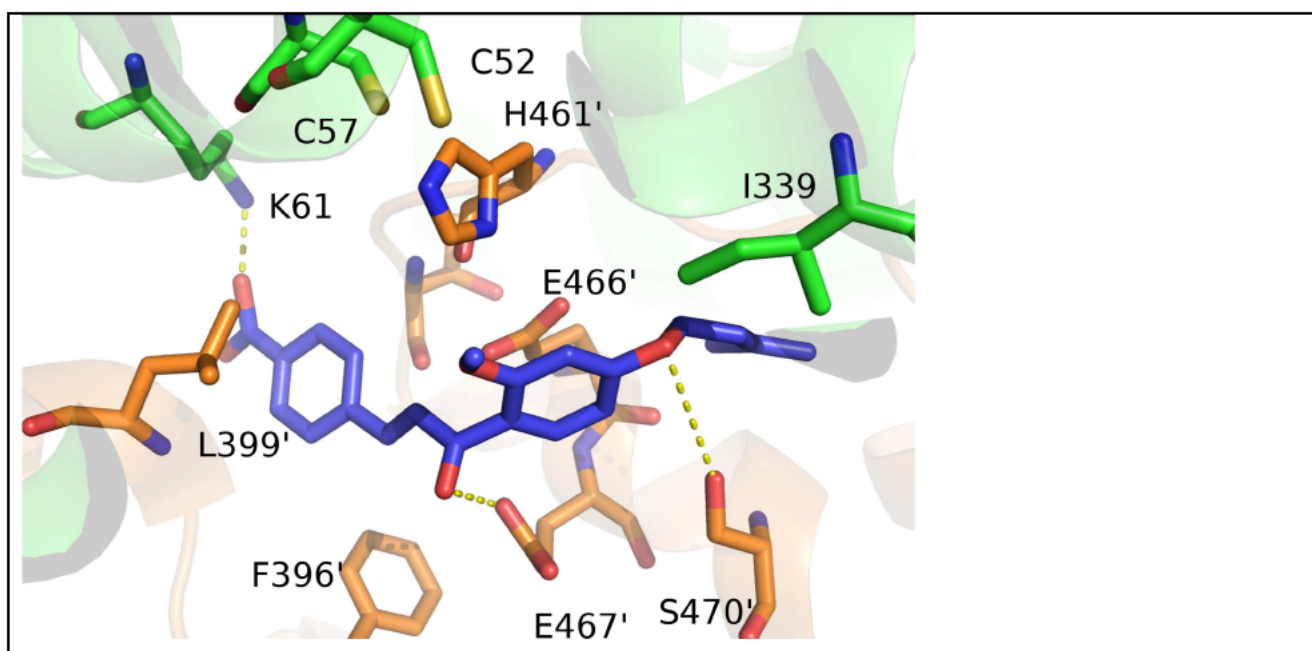
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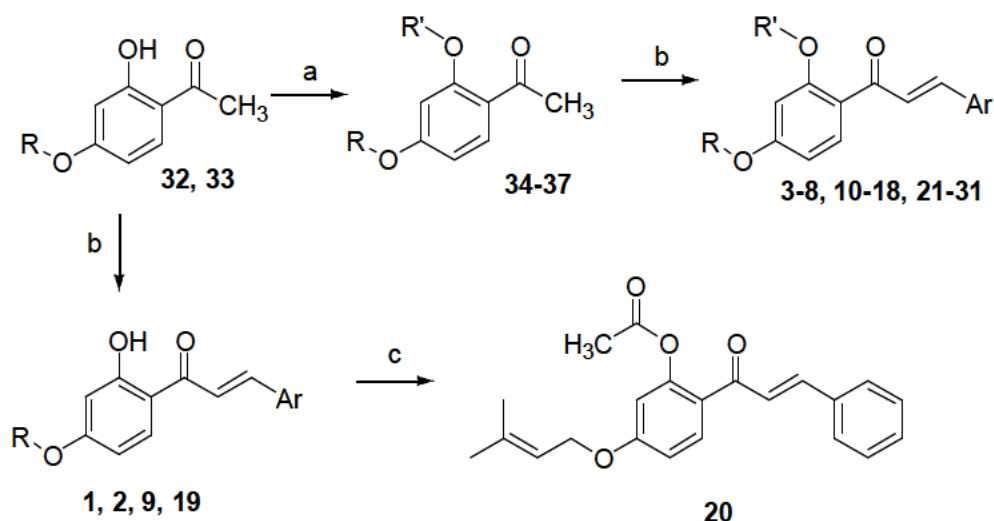
b



c



819 **Figure 5.** Blow up of the complex between compound **6** and TR obtained by docking experiments
 820 using both the oxidized form (PDB code: 2JK6) (a and b) and the reduced form (PDB code 4ADW)
 821 (c) of the protein. **a.** In A are represented the lowest energy poses belonging to the most populated
 822 clusters (reported in TableS1). The pose 3 belonging to cluster 1 is colored pink; the pose 60
 823 belonging to cluster 2 is colored cyan; the pose 94 belonging to cluster 3 is colored yellow. **b.** In b,
 824 the pose of compound **6** docked in the oxidized form of TR belonging to the most populated cluster
 825 (pose 60 belonging to cluster 2) is represented. **c.** In c, the pose of compound **6** docked in the
 826 reduced form of TR belonging to the most populated cluster (pose 61 belonging to cluster 3) is
 827 represented. Compound **6** and the residues interacting with it are indicated and represented as sticks.
 828 The two TR subunits are colored in green and orange whereas compound **6** docked in the oxidized
 829 TR is colored cyan and compound **6** docked in reduced TR is colored blue. The picture was
 830 obtained using PyMOL (The PyMOL Molecular Graphics System, Version 2.0 Schrödinger, LLC.)
 831
 832



833

834 **Scheme 1.** Synthetic route of compounds **1-37**^a

835 ^aReagents and conditions: a) selected alkyl bromide, K₂CO₃, acetone, reflux; b) KOH 50%, EtOH,
 836 rt, 18 h; c) acetic anhydride, reflux.

837

838
839
840

Table 1. Inhibitory activity of chalcones **1-31** against promastigotes of *L. donovani* growth, cytotoxicity in mammalian kidney epithelial cells and in a human monocytic cell line and selectivity indexes.

Comp	Structure	<i>L. donov</i> IC ₅₀ (μ M) ^a	Vero CC ₅₀ (μ M) ^b	SI ^{c,d}	THP-1 CC ₅₀ (μ M)	SI ^{c,e}
1		5.0	40.0	8	16.0	3.2
2		8.5	210.0	24.7	100.0	11.8
3		10.5	16.0	1.5	16.0	1.5
4		10.5	22.0	2	40.0	3.8
5		16.0	600.0	37.5	100.0	6.3
6		3.0	600.0	200	600.0	200
7		15.0	50.0	3.3	50.0	3.3
8		11.0	40.0	3.6	25.0	2.3
9		17.5	40.0	2.3	25.0	1.4
10		4.0	20.0	5	16.0	4
11		9.5	15.0	1.6	16.0	1.7

12		21.5	100.0	4.6	100.0	4.6
13		7.0	10.0	1.4	11.0	1.6
14		4.0	6.0	1.5	15	3.8
15		15.0	420.0	28	25.0	1.6
16		12.5	600.0	48	600.0	48
17		n.i.	n.d.	n.d.	n.d.	n.d.
18		n.i.	n.d.	n.d.	n.d.	n.d.
19		n.i.	n.d.	n.d.	n.d.	n.d.
20		n.i.	n.d.	n.d.	n.d.	n.d.
21		n.i.	n.d.	n.d.	n.d.	n.d.
22		n.i.	n.d.	n.d.	n.d.	n.d.
23		n.i.	n.d.	n.d.	n.d.	n.d.

24		n.i.	n.d.	n.d.	n.d.	n.d.
25		n.i.	n.d.	n.d.	n.d.	n.d.
26		n.i.	n.d.	n.d.	n.d.	n.d.
27		n.i.	n.d.	n.d.	n.d.	n.d.
28		n.i.	n.d.	n.d.	n.d.	n.d.
29		n.i.	n.d.	n.d.	n.d.	n.d.
30		n.i.	n.d.	n.d.	n.d.	n.d.
31		n.i.	n.d.	n.d.	n.d.	n.d.
Amph B		0.3	200.0	666	200.0	666

841

842 ^aIC₅₀/72h represents concentration of a compound that causes 50% growth inhibition and is the
843 mean of two independent determinations. The experimental error was within 50%. ^bCC₅₀/72h
844 represents 50% cytotoxic concentration. ^cSI; Selectivity index (SI = CC₅₀/IC₅₀). ^dSI was calculated
845 considering CC₅₀ on Vero cells. ^eSI was calculated considering CC₅₀ on THP1 cells. Comp;
846 compounds. n.i.; not inhibiting parasite growth up to 40 μM. n.d.; not determined due to the low
847 antileishmanial potency. Amph B; amphotericin B.

848

- 850 [1] D. Pace, *Leishmaniasis*, *J. Infect.*, 69 Suppl 1 (2014) S10-18.
- 851 [2] WHO, Control of the leishmaniasis: report of a meeting of the WHO Expert Committee on the
852 Control of Leishmaniasis, Geneva, 22-26 March 2010, in: WHO technical report series, 2010.
- 853 [3] D. Savoia, Recent updates and perspectives on leishmaniasis, *J. Infect. Dev. Ctries*, 9 (2015)
854 588-596.
- 855 [4] P.J. Hotez, B. Pecoul, "Manifesto" for advancing the control and elimination of neglected
856 tropical diseases, *PLoS Negl. Trop. Dis.*, 4 (2010) e718.
- 857 [5] J.F. Barbosa, S.M. de Figueiredo, F.M. Monteiro, F. Rocha-Silva, C. Gaciele-Melo, S.S.
858 Coelho, S. Lyon, R.B. Caligiorne, *New Approaches on Leishmaniasis Treatment and Prevention: A
859 Review of Recent Patents*, *Recent Pat. Endocr. Metab. Immune Drug Discov.*, 9 (2015) 90-102.
- 860 [6] R.K. Jha, A.K. Sah, D.K. Shah, P. Sah, The treatment of visceral leishmaniasis: safety and
861 efficacy, *JNMA J. Nepal Med. Assoc.*, 52 (2013) 645-651.
- 862 [7] D. Smirlis, M.B. Soares, Selection of molecular targets for drug development against
863 trypanosomatids, *Sub-cellular biochemistry*, 74 (2014) 43-76.
- 864 [8] S. Muller, E. Liebau, R.D. Walter, R.L. Krauth-Siegel, Thiol-based redox metabolism of
865 protozoan parasites, *Trends in parasitology*, 19 (2003) 320-328.
- 866 [9] K. Augustyns, K. Amssoms, A. Yamani, P.K. Rajan, A. Haemers, Trypanothione as a target in
867 the design of antitrypanosomal and antileishmanial agents, *Curr. Pharm. Des.*, 7 (2001) 1117-1141.
- 868 [10] A. Ilari, A. Fiorillo, P. Baiocco, E. Poser, G. Angiulli, G. Colotti, Targeting polyamine
869 metabolism for finding new drugs against leishmaniasis: a review, *Mini Rev. Med. Chem.*, 15
870 (2015) 243-252.
- 871 [11] L.R. Krauth-Siegel, M.A. Comini, T. Schlecker, The trypanothione system, *Sub-cellular
872 biochemistry*, 44 (2007) 231-251.
- 873 [12] A. Ilari, A. Fiorillo, I. Genovese, G. Colotti, Polyamine-trypanothione pathway: an update,
874 *Future Med. Chem.*, 9 (2017) 61-77.
- 875 [13] P. Baiocco, G. Colotti, S. Franceschini, A. Ilari, Molecular Basis of Antimony Treatment in
876 Leishmaniasis, *J. Med. Chem.*, 52 (2009) 2603-2612.
- 877 [14] P. Baiocco, A. Ilari, P. Ceci, S. Orsini, M. Gramiccia, T. Di Muccio, G. Colotti, Inhibitory
878 Effect of Silver Nanoparticles on Trypanothione Reductase Activity and *Leishmania infantum*
879 Proliferation, *ACS Med. Chem. Lett.*, 2 (2011) 230-233.
- 880 [15] A. Ilari, P. Baiocco, L. Messori, A. Fiorillo, A. Boffi, M. Gramiccia, T. Di Muccio, G. Colotti,
881 A gold-containing drug against parasitic polyamine metabolism: the X-ray structure of
882 trypanothione reductase from *Leishmania infantum* in complex with auranofin reveals a dual
883 mechanism of enzyme inhibition, *Amino acids*, 42 (2012) 803-811.
- 884 [16] G. Colotti, A. Ilari, A. Fiorillo, P. Baiocco, M.A. Cinellu, L. Maiore, F. Scaletti, C. Gabbiani,
885 L. Messori, Metal-based compounds as prospective antileishmanial agents: inhibition of
886 trypanothione reductase by selected gold complexes, *ChemMedChem*, 8 (2013) 1634-1637.
- 887 [17] P. Baiocco, G. Poce, S. Alfonso, M. Coccozza, G.C. Porretta, G. Colotti, M. Biava, F. Moraca,
888 M. Botta, V. Yardley, A. Fiorillo, A. Lantella, F. Malatesta, A. Ilari, Inhibition of *Leishmania*
889 *infantum* trypanothione reductase by azole-based compounds: a comparative analysis with its
890 physiological substrate by X-ray crystallography, *ChemMedChem*, 8 (2013) 1175-1183.
- 891 [18] F. Saccoliti, G. Angiulli, G. Pupo, L. Pescatori, V.N. Madia, A. Messori, G. Colotti, A.
892 Fiorillo, L. Scipione, M. Gramiccia, T. Di Muccio, R. Di Santo, R. Costi, A. Ilari, Inhibition of
893 *Leishmania infantum* trypanothione reductase by diaryl sulfide derivatives, *J. Enzyme Inhib. Med.
894 Chem.*, 32 (2017) 304-310.
- 895 [19] I.A. Rodrigues, A.M. Mazotto, V. Cardoso, R.L. Alves, A.C. Amaral, J.R. Silva, A.S. Pinheiro,
896 A.B. Vermelho, *Natural Products: Insights into Leishmaniasis Inflammatory Response*, *Mediators
897 Inflamm.*, 2015 (2015) 835910.

898 [20] A. Oryan, Plant-derived compounds in treatment of leishmaniasis, *Iran J. Vet. Res.*, 16 (2015)
899 1-19.

900 [21] B.E. Evans, K.E. Rittle, M.G. Bock, R.M. DiPardo, R.M. Freidinger, W.L. Whitter, G.F.
901 Lundell, D.F. Veber, P.S. Anderson, R.S. Chang, et al., Methods for drug discovery: development
902 of potent, selective, orally effective cholecystokinin antagonists, *J. Med. Chem.*, 31 (1988) 2235-
903 2246.

904 [22] N.K. Sahu, S.S. Balbhadra, J. Choudhary, D.V. Kohli, Exploring pharmacological significance
905 of chalcone scaffold: a review, *Curr. Med. Chem.*, 19 (2012) 209-225.

906 [23] J.R. Dimmock, D.W. Elias, M.A. Beazely, N.M. Kandepu, Bioactivities of chalcones, *Curr.*
907 *Med. Chem.*, 6 (1999) 1125-1149.

908 [24] M. Liu, P. Wilairat, S.L. Croft, A.L. Tan, M.L. Go, Structure-activity relationships of
909 antileishmanial and antimalarial chalcones, *Bioorg. Med. Chem.*, 11 (2003) 2729-2738.

910 [25] R. Shivahare, V. Korthikunta, H. Chandasana, M.K. Suthar, P. Agnihotri, P. Vishwakarma,
911 T.K. Chaitanya, P. Kancharla, T. Khaliq, S. Gupta, R.S. Bhatta, J.V. Pratap, J.K. Saxena, S. Gupta,
912 N. Tadigoppula, Synthesis, structure-activity relationships, and biological studies of
913 chromenochalcones as potential antileishmanial agents, *J. Med. Chem.*, 57 (2014) 3342-3357.

914 [26] R. Borges-Argaez, T. Vela-Catzin, A. Yam-Puc, M.J. Chan-Bacab, R.E. Moo-Puc, M.
915 Caceres-Farfan, Antiprotozoal and Cytotoxic Studies on Some Isocordoin Derivatives, *Planta Med.*,
916 75 (2009) 1336-1338.

917 [27] H. Hussain, A. Al-Harrasi, A. Al-Rawahi, I.R. Green, S. Gibbons, Fruitful decade for
918 antileishmanial compounds from 2002 to late 2011, *Chem. Rev.*, 114 (2014) 10369-10428.

919 [28] N. Singh, B.B. Mishra, S. Bajpai, R.K. Singh, V.K. Tiwari, Natural product based leads to
920 fight against leishmaniasis, *Bioorg. Med. Chem.*, 22 (2014) 18-45.

921 [29] S. Gupta, R. Shivahare, V. Korthikunta, R. Singh, N. Tadigoppula, Synthesis and biological
922 evaluation of chalcones as potential antileishmanial agents, *Eur. J. Med. Chem.*, 81 (2014) 359-366.

923 [30] P. Boeck, C.A. Bandeira Falcão, P.C. Leal, R.A. Yunes, V.C. Filho, E.C. Torres-Santos, B.
924 Rossi-Bergmann, Synthesis of chalcone analogues with increased antileishmanial activity, *Bioorg.*
925 *Med. Chem.*, 14 (2006) 1538-1545.

926 [31] E.C. Torres-Santos, M.I. Sampaio-Santos, F.S. Buckner, K. Yokoyama, M. Gelb, J.A. Urbina,
927 B. Rossi-Bergmann, Altered sterol profile induced in *Leishmania amazonensis* by a natural
928 dihydroxymethoxylated chalcone, *J. Antimicrob. Chemother.*, 63 (2009) 469-472.

929 [32] M. Chen, S.B. Christensen, T.G. Theander, A. Kharazmi, Antileishmanial activity of
930 licochalcone A in mice infected with *Leishmania major* and in hamsters infected with *Leishmania*
931 *donovani*, *Antimicrob. Agents Chemother.*, 38 (1994) 1339-1344.

932 [33] S. Krieger, W. Schwarz, M.R. Ariyanayagam, A.H. Fairlamb, R.L. Krauth-Siegel, C. Clayton,
933 Trypanosomes lacking trypanothione reductase are avirulent and show increased sensitivity to
934 oxidative stress, *Mol. Microbiol.*, 35 (2000) 542-552.

935 [34] M.C. do Nascimento, W.B. Mors, Chalcones of the root bark of *Derris sericea*,
936 *Phytochemistry*, 11 (1972) 3023-3028.

937 [35] P. Baiocco, S. Franceschini, A. Ilari, G. Colotti, Trypanothione reductase from *Leishmania*
938 *infantum*: cloning, expression, purification, crystallization and preliminary X-ray data analysis,
939 *Protein Pept. Lett.*, 16 (2009) 196-200.

940 [36] S. Yuan, H.C.S. Chan, Z. Hu, Implementing WebGL and HTML5 in Macromolecular
941 Visualization and Modern Computer-Aided Drug Design, *Trends in biotechnology*, 35 (2017) 559-
942 571.

943 [37] G.M. Morris, R. Huey, W. Lindstrom, M.F. Sanner, R.K. Belew, D.S. Goodsell, A.J. Olson,
944 AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility, *Journal of*
945 *computational chemistry*, 30 (2009) 2785-2791.

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- The development of new, effective and safe antileishmanial drugs is urgently needed.
- The enzyme trypanothione reductase, by disrupting *Leishmania* parasite redox balance, represents a validated molecular target for the development of antiparasitic agents.
- Chalcone as useful template for the design of novel antileishmanial compounds.
- 16 of the newly synthesized chalcones were active against *L.donovani in vitro*.
- Chalcone **6** potently inhibits leishmanial trypanothione reductase.