


Please cite the Published Version

Alruhaimi, Reem S, Hussein, Omnia E, Alnasser, Sulaiman M, Elbagory, Ibrahim, Alzoghaibi, Mohammed A, Kamel, Emadeldin M, El Mohtadi, Mohamed and Mahmoud, Ayman M  (2024) Haloxylon salicornicum Phytochemicals Suppress NFκB, iNOS and Proinflammatory Cytokines in LipopolysaccharideInduced Macrophages. Chemistry and Biodiversity. e202401623 ISSN 1612-1872

DOI: <https://doi.org/10.1002/cbdv.202401623>

Publisher: Wiley

Version: Accepted Version

Downloaded from: <https://e-space.mmu.ac.uk/636124/>

Usage rights:  [Creative Commons: Attribution 4.0](https://creativecommons.org/licenses/by/4.0/)

Additional Information: This is an accepted manuscript of an article which appeared in final form in Chemistry and Biodiversity, published by Wiley

Data Access Statement: The manuscript contains all data supporting the reported results.

Enquiries:

If you have questions about this document, contact openresearch@mmu.ac.uk. Please include the URL of the record in e-space. If you believe that your, or a third party's rights have been compromised through this document please see our Take Down policy (available from <https://www.mmu.ac.uk/library/using-the-library/policies-and-guidelines>)

1 **Title:**

2 ***Haloxylon salicornicum* Phytochemicals Suppress NF- κ B, iNOS and Pro-inflammatory**
3 **Cytokines in Lipopolysaccharide-Induced Macrophages**

4 **Authors and affiliations:**

5 Reem S. Alruhaimi¹, Omnia E Hussein², Sulaiman M. Alnasser³, Ibrahim Elbagory⁴,
6 Mohammed A. Alzoghaibi⁵, Emadeldin M. Kamel⁶, Mohamed El Mohtadi⁷, Ayman M.
7 Mahmoud^{8*}

8 ¹Department of Biology, College of Science, Princess Nourah bint Abdulrahman University,
9 Riyadh 11671, Saudi Arabia.

10 ²Higher Technological Institute for Applied Health Sciences, Beni-Suef, Egypt.

11 ³Department of Pharmacology and Toxicology, College of Pharmacy, Qassim University,
12 Qassim 51452, Saudi Arabia.

13 ⁴Department of Pharmaceutics, Faculty of Pharmacy, Northern Border University, Rafha
14 76321, Saudi Arabia.

15 ⁵Physiology Department, College of Medicine, King Saud University, Riyadh, 11461, Saudi
16 Arabia.

17 ⁶Organic Chemistry Department, Faculty of Science, Beni-Suef University, Beni-Suef 62514,
18 Egypt.

19 ⁷Department of Biology, Edge Hill University, Ormskirk L39 4QP, UK.

20 ⁸Department of Life Sciences, Faculty of Science and Engineering, Manchester Metropolitan
21 University, Manchester M1 5GD, UK.

22

23 *Corresponding author:

24 **Ayman M. Mahmoud**

25 Department of Life Sciences, Faculty of Science and Engineering, Manchester Metropolitan
26 University, Manchester M1 5GD, UK

27 ORCID ID: 0000-0003-0279-6500

28 E-mail: a.mahmoud@mmu.ac.uk

29

30

31 **Abstract:**

32 *Haloxylon salicornicum* is traditionally used for the treatment of several disorders associated
33 with inflammation. Despite it is a defense response against tissue injury and infections,
34 inflammation can become a chronic condition that can negatively impact the body. This study
35 investigated the effect of *H. salicornicum* phytochemicals nuclear factor-kappaB (NF-κB),
36 inducible nitric oxide synthase (iNOS) and cytokines release by lipopolysaccharide (LPS)-
37 challenged macrophages *in vitro*. The binding affinity of the tested phytochemical towards NF-
38 κB and iNOS was investigated using molecular docking. Ten compounds (four coumarins,
39 three sterols and three flavonoids) were isolated from the ethanolic extract of *H. salicornicum*.
40 Treatment of LPS-challenged macrophages with the compounds resulted in remarkable
41 decrease in NF-κB p65 and iNOS mRNA abundance. All compounds suppressed the
42 production of nitric oxide (NO) and the pro-inflammatory cytokines (tumor necrosis factor
43 (TNF)-α and interleukin (IL)-6) from macrophages challenged with LPS. Molecular docking
44 revealed the ability of the isolated phytochemicals to bind NF-κB p65 and iNOS. In conclusion,
45 *H. salicornicum* is a rich source of phytochemicals with anti-inflammatory properties. The anti-
46 inflammatory efficacy of *H. salicornicum* phytoconstituents is mediated via their ability to
47 modulate NF-κB and iNOS, and suppress the release of NO, TNF-α, and IL-6 from
48 macrophages.

49 **Keywords:** *Haloxylon*; Inflammation; Cytokines; Macrophages.

50 **1. Introduction**

51 Inflammation is a complex stereotypical response of the body to damage caused by different
52 factors. Infections, exposure to toxic chemicals, ischemic injury are among the causes of tissue
53 damage and inflammation [1]. The inflammatory response as a defense mechanism is controlled
54 by several mediators produced by different cells. These mediators include cytokines,
55 leukotrienes, prostaglandins, vasoactive molecules, complement components and others. The

56 inflammatory response triggers changes and immune response for tissue repair and enhancing
57 cell proliferation at the injury site ^[1]. Inflammation can become a chronic condition in the case
58 of persistent cause or failure of the control mechanisms. This chronic condition can promote
59 cell proliferation and mutations and may finally result in cancer ^[2]. In addition, chronic low-
60 grade inflammation, known as inflammaging, has recently been acknowledged as a key
61 contributor to several disorders associated with aging ^[3]. Inflammaging is a mild inflammation
62 that has been reported in aging tissues, including cardiovascular, nervous and other tissues ^[3].
63 Different cells are involved in the inflammatory and immune responses to tissue injury.
64 Macrophages represent a key player in inflammation and body adaptive immunity. Given their
65 wide tissue distribution, macrophages confer immediate response and defense against invading
66 organisms and foreign elements prior to the migration of leukocytes ^[4]. Macrophages display
67 a range of immune responses, including innate immunity against pathogens such as bacteria
68 and adaptive immunity through the release of interleukins (ILs) and antigen presentation ^[4].
69 However, abnormal immune responses, including inflammation are implicated in numerous
70 chronic disorders, including diabetes, atherosclerosis, and heart, liver and kidney diseases ^[5].
71 The immunomodulatory function of macrophages is mediated via the secretion of cytokines,
72 leukocyte recruitment and phagocytosis. During inflammatory responses, macrophages secrete
73 the pro-inflammatory cytokines IL-6 and tumor necrosis factor (TNF)- α , and nitric oxide (NO)
74 generated by inducible NO synthase (iNOS) ^[1]. Lipopolysaccharide (LPS) triggers the
75 expression of iNOS and inflammation induced by LPS is a central defense mechanism against
76 bacterial infection ^[6]. Activation of nuclear factor- κ B (NF- κ B) is essential for the release of
77 inflammatory mediators and upregulation of iNOS ^[1]. NF- κ B regulates the expression of IL-6,
78 TNF- α , iNOS, and other mediators involved in inflammation ^[1]. NF- κ B could be activated by
79 several factors, including infections, tissue injury and reactive oxygen species (ROS), resulting
80 in the release of different mediators. However, the prolonged and excessive production of pro-

81 inflammatory mediators in dysregulated inflammation provokes damage both local and
82 systemic [6]. Therefore, NF-κB and its regulated cytokines and iNOS represent key targets for
83 the development of drugs targeting dysregulated inflammation as well as disorders associated
84 with excessive ROS.

85 Plants and their derived phytochemicals have demonstrated efficacies against dysregulated
86 inflammation in different disorders [7]. In this context, we have previously elucidated the
87 beneficial effects of *Haloxylon salicornicum* against inflammation induced by cisplatin (CIS)
88 in rat liver and kidney [8]. This plant is a desert shrub that belongs to the family *Chenopodiaceae*
89 and grows in Egypt and other countries [9]. The use of this species in folk medicine has been
90 acknowledged as it has been used for the treatment of sepsis, tuberculosis, diabetes mellitus
91 and other disorders associated with inflammation [10]. In an *in vitro* study, Bibi et al [10b]
92 demonstrated the inhibitory efficacies of *H. salicornicum* fractions on the growth of
93 *Mycobacterium tuberculosis* H37Rv. In addition, the antibacterial activity of its alcoholic
94 extract against different strains has been recently reported [11]. The hepatoprotective efficacy of
95 the ethanolic extract of *H. salicornicum* was reported in rats challenged with carbon
96 tetrachloride [12]. However, these studies didn't investigate its effects on inflammation. The
97 effects of the methanolic and ethanolic extracts of *H. salicornicum* against inflammation
98 associated with kidney and liver injury, respectively, induced by CIS were recently reported in
99 our studies [8]. The effect of *H. salicornicum* on LPS-induced inflammatory response has not
100 been studied. This study investigated the effect of ten phytochemicals isolated previously [8a]
101 from the ethanolic extract of *H. salicornicum* on NF-κB, iNOS, and cytokine release by LPS-
102 challenged macrophages.

103 **2. Materials and methods**

104 **2.1. Plant collection and isolation of phytochemicals**

105 *H. salicornicum* was collected from Beni-Suef governorate (latitude - longitude: 29.370824 -
106 31.094077, Egypt) in June 2019. The plant was identified by taxonomists at the Botany and
107 Microbiology Department, Beni-Suef University (Egypt) and a voucher specimen (No.: BSU-
108 CH2019-089) was archived in the Herbarium of the Faculty of Science (Registration code:
109 BSU- HERB19089).

110 Ten compounds were isolated from the aerial parts of *H. salicornicum* as reported in our
111 previous study^[8a]. Briefly, the aerial parts of *H. salicornicum* (3 kg) were dried, powdered, and
112 extracted using ethanol by cold maceration. The resultant extract was concentrated under
113 reduced pressure, yielding a residue of 218 g. This residue was dissolved in warm water and
114 sequentially partitioned with petroleum ether, ethyl acetate, chloroform, and *n*-butanol. Each
115 organic layer was evaporated to produce corresponding extracts. The petroleum ether, ethyl
116 acetate, and *n*-butanol extracts underwent sequential chromatographic fractionation using
117 different stationary phases and eluents to afford the purified compounds (**1-10**)^[8a]. The isolated
118 compounds (**1-10**) were identified as isoscopoletin (**1**), aesculetin (**2**), altechromone A (**3**),
119 fucosterol (**4**), β -sitosterol (**5**), β -sitosterol-3-*O*- β -D-glucoside (**6**), scopolin (**7**), 5,7,2'-
120 trihydroxyflavone (**8**), 5,7,2'-trihydroxy-6-methoxyflavone (**9**), and 5-hydroxy-6,7,3',4'-
121 tetramethoxyflavone (**10**) (Fig. 1)^[8a].

122 **2.2. Cells and treatments**

123 RAW 264.7 murine macrophages (VACSERA, Egypt) were grown in DMEM supplemented
124 with 10% fetal bovine serum (FBS) and 1% antibiotics (Gibco/BRL) at 37°C and under 5%
125 CO₂. The cells were seeded in a 96-well plate (1×10^5 cells/mL) in DMEM and allowed to
126 adhere for 5 hours. The cells were treated with 5 and 10 μ g/ml of compounds 1-10 for 1 h and
127 then challenged with 1 μ g/ml LPS from *Escherichia coli* (Sigma, USA) for 24 h. The medium
128 and cells were collected for analysis.

129 **2.3. qRT-PCR**

130 The harvested cells were washed in cold PBS and Trizol reagent (ThermoFisher Scientific,
131 USA) was used for the isolation of RNA. Following its quantification, RNA with OD_{260/280}
132 ≥ 1.8 were used for the synthesis of cDNA. Amplification of cDNA was achieved using SYBR
133 Green Master Mix (ThermoFisher Scientific, USA) and the following primers: NF- κ B p65
134 F:5'CTATGTGTGCAGACGAAGCC3' and R:5' AGACCGAGGACTAGGCAGAC3'
135 (NM_001402548.1; amplicon size: 91 bp); iNOS F:5'GCCCAGCCAGCCCAAC3' and
136 R:5'GCAGCTTGTCCAGGGATTCT3' (NM_001313922.1; amplicon size: 108 bp), and β -
137 actin F:5'GTGCTATGTTGCTCTAGACTTCG3' and R:5'ATGCCACAGGATTCCATACC3'
138 (NM_007393.5; amplicon size: 174 bp). The obtained data were analyzed using the $2^{-\Delta\Delta C_t}$
139 method [13].

140 **2.4. Determination of NO, TNF- α and IL-6**

141 NO production was determined in the culture medium as nitrite using Griess reagent [14]. In a
142 96-well plate, 100 μ l of the medium was mixed with equal volume of Griess reagent and kept
143 at room temperature for 10 min. The absorbance was measured at 540 nm and nitrite content
144 was determined using a standard curve. TNF- α and IL-6 were assayed using ELISA kits
145 (ELabscience, China).

146 **2.5. Molecular docking**

147 The affinity of *H. salicornicum* phytochemicals towards NF- κ B RelA (PDB: 5u01), and iNOS
148 (PDB: 3EAI) was investigated using PyRx virtual screening software (version 0.8) [15].
149 Autodock Tools (ADT; v1.5.6) was employed for target protein preparation which included the
150 removal of water molecules, addition of polar hydrogens, and assignment of Gasteiger charges.
151 Ligands were prepared by optimizing their geometry and assigning appropriate torsional
152 degrees of freedom. PyMOL (v2.3.2) and LigPlot (v2.2.8) [16] were used for visualization of
153 binding mode and protein-ligand interactions, respectively.

154 **2.6. Statistical analysis**

155 The data are represented as mean \pm standard deviation (SD). Analysis of the statistical
156 differences was carried out using one-way ANOVA followed by Tukey's test on GraphPad 8.
157 A P value <0.05 was considered significant.

158 **3. Results**

159 **3.1. *H. salicornicum* phytochemicals downregulate NF- κ B p65 in LPS-challenged** 160 **macrophages**

161 Treatment of macrophages with 1 μ g/ml LPS resulted in significant ($P<0.001$) upregulation of
162 NF- κ B mRNA (fold change 3.79 ± 0.71) (Fig. 2A). Treatment of the LPS-induced macrophages
163 with two different concentrations of compound **1** remarkably suppressed NF- κ B mRNA (fold
164 change 2.17 ± 0.19 and 1.73 ± 0.17 for 5 and 10 μ g/ml, respectively) (Fig. 2B). Compounds **2**,
165 **3**, and **4** downregulated NF- κ B mRNA significantly at 5 and 10 μ g/ml ($P<0.001$, $P<0.01$ and
166 $P<0.001$, and fold changes at 5 μ g/ml; 1.66 ± 0.37 , 2.08 ± 0.26 and 1.76 ± 0.14 , and at 10
167 μ g/ml; 1.51 ± 0.13 , 1.85 ± 0.16 , and 1.18 ± 0.16 , respectively) (Fig. 2C-E). NF- κ B mRNA was
168 decreased following treatment of the LPS-induced cells with 5 μ g/ml (1.69 ± 0.15 , 1.62 ± 0.22 ,
169 and 1.82 ± 0.23) and 10 μ g/ml (1.23 ± 0.13 , 1.25 ± 0.11 , and 1.26 ± 0.17) of compounds **5**, **6**,
170 and **7**, respectively (Fig. 2F-H). Compounds **8**, **9**, and **10** exerted significant effect on NF- κ B
171 mRNA at both 5 μ g/ml (2.02 ± 0.19 , 2.05 ± 0.28 , and 2.13 ± 0.20) and 10 μ g/ml (1.44 ± 0.23 ,
172 1.75 ± 0.20 , and 1.99 ± 0.17) (Fig. 2I-K). All compounds showed a trend decrease in NF- κ B
173 mRNA abundance; however, the dose-dependent effect was non-significant. NF- κ B mRNA
174 abundance at the 5 μ g/ml concentration of compounds **1**, **3**, **8**, **9**, and **10** was significantly higher
175 than the control ($P<0.05$).

176 **3.2. *H. salicornicum* phytochemicals downregulate iNOS and nitrite in LPS-challenged** 177 **macrophages**

178 Macrophages challenged with LPS showed upregulated iNOS mRNA (fold change: $4.43 \pm$
179 1.01) significantly as compared to the control cells ($P<0.001$; Fig. 3A). Treatment of the cells

180 with 5 $\mu\text{g/ml}$ of compounds **1-10** remarkably decreased iNOS mRNA (fold change: $2.26 \pm$
181 0.37 , 1.96 ± 0.22 , 2.59 ± 0.47 , 1.90 ± 0.23 , 2.03 ± 0.29 , 2.55 ± 0.34 , 2.01 ± 0.11 , 2.14 ± 0.67 ,
182 2.59 ± 0.50 , and 2.085 ± 0.54 , respectively) (Fig. 3B-K). The effect of compounds **3**, **6**, **9**, and
183 **10** on iNOS was significant ($P < 0.05$) as compared to the control cells. The higher concentration
184 of compounds **1-10** resulted in more potent suppression effect on iNOS mRNA abundance (fold
185 change: 1.51 ± 0.23 , 1.22 ± 0.15 , 1.94 ± 0.18 , 1.25 ± 0.10 , 1.30 ± 0.19 , 1.47 ± 0.25 , $1.21 \pm$
186 0.17 , 1.99 ± 0.24 , 1.87 ± 0.26 , and 1.85 ± 0.19 , respectively) (Fig. 3B-K).

187 NO levels produced by LPS challenged cells ($40.62 \pm 7.59 \mu\text{M}$) was significantly ($P < 0.001$)
188 higher than the control cells ($4.47 \pm 0.81 \mu\text{M}$) as shown in Figure 4A. Treatment with 5 $\mu\text{g/ml}$
189 of compounds 1-10 significantly reduced NO release by LPS-challenged cells (27.07 ± 4.53 ,
190 19.32 ± 2.98 , 24.07 ± 2.28 , 19.34 ± 2.31 , 21.05 ± 3.61 , 26.47 ± 4.60 , 15.02 ± 2.65 , $15.63 \pm$
191 2.37 , 21.48 ± 1.98 , and $25.03 \pm 2.47 \mu\text{M}$, respectively) (Fig. 4B-K). The 10 $\mu\text{g/ml}$ of
192 compounds **4**, **5**, and **6** showed a concentration dependent effect on NO levels (8.39 ± 1.70 ,
193 7.96 ± 1.05 , and $13.83 \pm 2.15 \mu\text{M}$, respectively) (Fig. 4E-G). At the same concentration,
194 compounds **1**, **2**, **3**, **7**, **8**, **9**, and **10** ameliorated NO significantly ($P < 0.001$) and the observed
195 levels were 16.31 ± 2.06 , 11.69 ± 2.57 , 14.22 ± 1.68 , 11.07 ± 2.89 , 9.23 ± 0.84 , 16.27 ± 3.02 ,
196 and $16.77 \pm 1.20 \mu\text{M}$, respectively (Fig. 4E-G).

197 **3.3. *H. salicornicum* phytochemicals attenuate TNF- α and IL-6 release from LPS-** 198 **challenged macrophages**

199 LPS-challenged cells released significantly higher levels of TNF- α ($246.71 \pm 40.42 \text{ pg/ml}$) as
200 compared to the non-challenged cells ($35.67 \pm 4.51 \text{ pg/ml}$) ($P < 0.001$, Fig. 5A). Compounds **1**,
201 **2**, and **3** remarkably reduced TNF- α release when supplemented at 5 $\mu\text{g/ml}$ (136.02 ± 19.98 ,
202 119.31 ± 18.04 , $141.10 \pm 20.55 \text{ pg/ml}$, respectively) and 10 $\mu\text{g/ml}$ (84.27 ± 11.93 , 76.42 ± 8.08 ,
203 and $96.39 \pm 9.16 \text{ pg/ml}$, respectively) (Fig. 5B-D). Likewise, compounds **4-10** reduced TNF- α
204 release from LPS-challenged cells significantly at both 5 $\mu\text{g/ml}$ (106.27 ± 16.17 , $111.09 \pm$

205 17.38, 118.44 ± 14.84 , 87.59 ± 10.02 , 109.65 ± 17.01 , 134.81 ± 25.74 , and 129.70 ± 27.11
206 pg/ml, respectively) and 10 $\mu\text{g/ml}$ (83.19 ± 16.29 , 81.06 ± 14.81 , 79.67 ± 11.93 , 55.34 ± 8.50 ,
207 78.40 ± 12.14 , 100.32 ± 10.98 , and 97.96 ± 15.04 pg/ml, respectively) concentrations as shown
208 in Figure 5E-K. All compounds showed a trend decrease in TNF- α with increased
209 concentration, and the effect of the 5 $\mu\text{g/ml}$ was significant when compared to control cells
210 except for compound 7.

211 Similar to TNF- α , IL-6 release from LPS-challenged cells (55.04 ± 7.93 pg/ml) was
212 significantly higher than the control cells (10.50 ± 1.81 pg/ml) as shown in Figure 6A
213 ($P < 0.001$). Compounds **1-10** significantly reduced IL-6 levels when supplemented at 5 $\mu\text{g/ml}$
214 with reported levels of 23.52 ± 3.02 , 19.47 ± 1.55 , 30.61 ± 5.44 , 19.03 ± 1.27 , 19.97 ± 1.86 ,
215 18.83 ± 2.52 , 21.23 ± 2.57 , 18.73 ± 2.45 , 30.03 ± 5.08 , and 24.43 ± 2.87 pg/ml, respectively
216 (Fig. 5B-K). Despite non-significant as compared to the lower concentration, 10 $\mu\text{g/ml}$ of
217 compounds **1-10** remarkably ($P < 0.001$) reduced IL-6 release (20.07 ± 3.28 , 13.63 ± 1.79 , 23.59
218 ± 3.68 , 13.41 ± 1.92 , 14.17 ± 1.53 , 14.54 ± 2.40 , 17.98 ± 1.94 , 13.60 ± 1.81 , 22.17 ± 1.63 , and
219 21.13 ± 1.75 pg/ml, respectively) as depicted in Figure 5B-K.

220 **3.4. *H. salicornicum* phytochemicals exhibit binding affinity toward NF- κ B and iNOS**

221 Molecular docking simulation data represented in Figures 7-10 and Table 1 show the binding
222 affinity of compounds **1-10** with NF- κ B RelA. Compounds **1**, **2**, and **3** (Fig. 7) exhibited lowest
223 binding energies -5.9, -6.0 and -5.9 kcal/mol, respectively, and showed common amino acid
224 residues (Thr60, His58, Thr57, Arg50, Pro275, and Glu25) in their binding patterns.
225 Compounds **4**, **5**, and **6** exhibited the lowest binding energies (-7.9, -7.6, and -8.2 kcal/mol,
226 respectively) and all formed a polar bond with Asn186 and hydrophobic interactions with
227 Arg187, Val248, Arg246, and Lys218 (Fig. 8-9). Compound **7** showed -7.1 kcal/mol binding
228 energy, and hydrophobic interactions and polar bonding with 8 and 4 amino acid residues,

229 respectively (Fig. 9). Compounds **8**, **9**, and **10** showed -7.3, -7.1, and -6.8 kcal/mol binding
230 energy and 2 polar binding and 9, 7, and 7 hydrophobic interactions, respectively (Fig. 10).
231 Compounds **1**, **2**, and **3** exhibited -7.5, -7.2 and -8.0 kcal/mol binding energy with iNOS and
232 all bond to the amino acid residues Tyr483, Cys194, Trp188, Leu203, and Phe363 as shown in
233 Figure 11 and Table 2. Similar to their binding with NF- κ B RelA, compounds **4**, **5**, and **6**
234 showed the lowest binding energies (-10.7, -10.3, and -10.1 kcal/mol, respectively) and bound
235 to several amino acids as shown in Figures 12 and 13 and Table 2. Compound **7** exhibited -9.1
236 kcal/mol binding energy and polar binding and hydrophobic interactions with 2 and 8 amino
237 acid residues, respectively (Fig. 13). Compound **8**, **9**, and **10** (Fig. 14) exhibited hydrophobic
238 interactions only, each with 10 residues and showed binding energies of -9.9, -9.9 and -8.8,
239 respectively. The lowest binding energies of all compounds with NF- κ B and iNOS are
240 represented in Figures 15A and 15B, respectively.

241 **4. Discussion**

242 *H. salicornicum* has been traditionally used in the treatment of disorders associated with
243 inflammation such as sepsis, diabetes and tuberculosis [10]. The antibacterial efficacy of its
244 ethanolic extract was reported [10b] and our recent work revealed the suppressing effects of its
245 methanolic and ethanolic extracts on CIS-induced liver and kidney inflammation and toxicity
246 [8]. In this study, the effects of ten phytochemicals isolated from the ethanolic extract of *H.*
247 *salicornicum* on NF- κ B and iNOS expression, and the release of NO and pro-inflammatory
248 cytokines by LPS-induced macrophages were investigated. In addition, the binding affinity of
249 the isolated phytochemicals with NF- κ B and iNOS was explored using molecular docking.
250 The data revealed the inhibitory efficacy of the isolated compounds on NO, TNF- α , and IL-6
251 by LPS-challenged macrophages. These effects were associated with decreased NF- κ B and
252 iNOS mRNA abundance. Inhibition of NF- κ B represents an effective strategy for the
253 attenuation of dysregulated inflammation and its consequent disorders. NF- κ B is a transcription

254 factor that is activated in response to excess ROS, bacterial infection and tissue injury. It
255 regulates the expression of numerous mediators involved in inflammatory response as well as
256 other cellular processes ^[1]. The ability of NF- κ B to alter the biology of cells is attributed to the
257 numerous genes it controls ^[1]. In resting cells, NF- κ B is localized in the cytoplasm bound to
258 I κ B and the first step in its activation is the post-translational modification of I κ Bs ^[17].
259 Following activation, NF- κ B induces the expression of several genes encoding inflammatory
260 mediators and it also plays central roles in the survival and differentiation of innate and
261 inflammatory immune cells ^[18]. Hence, dysregulation of NF- κ B activation promotes
262 inflammatory disorders ^[18] and its modulation is of valuable therapeutic benefit. In response to
263 various stimuli, including bacterial infection, I κ B is ubiquitinated and degraded by proteasome
264 and NF- κ B subunit dimers translocate into the nucleus to bind DNA and promote gene
265 expression ^[19]. Macrophages express pattern recognition receptors (PRRs) that detect several
266 microbial components such as LPS and activate NF- κ B, resulting in the release of
267 inflammatory mediators ^[17].

268 Among the subunits, RelA (p65) has a central role in mediating the transcription of target genes
269 via direct contribution to DNA binding ^[18]. The tested phytochemicals in this study suppressed
270 the expression of NF- κ B p65 and exhibited binding affinity with RelA dimer, demonstrating
271 their NF- κ B inhibitory activities. All compounds showed a concentration-dependent trend in
272 downregulating NF- κ B p65 mRNA in LPS-challenged macrophages. The *in silico* findings
273 showed that compounds **4**, **5**, and **6** exhibited the lowest binding energy. These findings added
274 support to a recent study showed the ability of compound **1** (isoscopoletin) to suppress NF- κ B
275 p65 in TNF- α /IFN- γ -treated HaCaT cells *in vitro* ^[20]. Compound **2** (aesculetin) exhibited anti-
276 inflammatory activities mediated via suppression of NF- κ B both *in vitro* and in an *in vivo* model
277 of colitis ^[21]. Other studies demonstrated the inhibitory effect of aesculetin on NF- κ B in acute
278 lung injury ^[22] and neuroinflammation ^[23] induced by LPS. Our study introduced new

279 information that compound **3** (altechromone A) exhibits anti-inflammatory activity mediated
280 via downregulation of NF- κ B p65 in LPS-challenged macrophages. *In silico*, compounds **1**, **2**,
281 and **3** showed similar binding energies (-5.9, -6.0 and -5.9, respectively) and all bound to Thr60,
282 His58, Thr57, Pro275, and Glu25 amino acid residues of RelA. The suppressive effect of
283 compound **4** (fucosterol) was supported by previous studies showing its potent anti-
284 inflammatory efficacy. For instance, in a mouse model of acute liver injury (ALI), fucosterol
285 suppressed NF- κ B p65 immunostaining ^[24]. It downregulated NF- κ B p65 gene expression and
286 inhibited matrix metalloproteinase in UVB-induced HaCaT cells ^[25]. Our *in vitro* findings
287 supported the inhibitory effect of fucosterol and *in silico* exploration added further support by
288 showing its ability to bind to 8 amino acid residues with polar bonds and hydrophobic
289 interactions. Compounds **5** (β -sitosterol) and **6** (β -sitosterol-3-*O*- β -D-glucoside) showed
290 binding with 8 and 13 amino acids of RelA, respectively. Given the similar structure of
291 compounds **4**, **5**, and **6**, their binding with RelA included common amino acid residues
292 (Asn186, Arg187, Val248, Arg246, and Lys218). β -sitosterol effectively suppressed NF- κ B in
293 LPS-induced lung epithelial cells ^[26] and reduced neuroinflammation by inhibiting NF- κ B in
294 microglial cells ^[27]. Compound **7** (scopolin) remarkably downregulated NF- κ B p65 in LPS-
295 induced macrophages and exhibited *in silico* binding marked by polar bonding with 4 residues
296 and hydrophobic interactions with 8 residues of RelA. Scopolin's anti-inflammatory efficacy
297 was investigated in very few studies. In arthritis in rats, scopolin isolated from *Erycibe*
298 *obtusifolia* reduced IL-6 and inflammation, but its effect on NF- κ B was not investigated ^[28].
299 Our study introduced new information on the involvement of NF- κ B suppression in the anti-
300 inflammatory efficacy of scopolin. Compounds **8** (5,7,2'-trihydroxyflavone), **9** (5,7,2'-
301 trihydroxy-6-methoxyflavone), and **10** (5-hydroxy-6,7,3',4'-tetramethoxyflavone) decreased
302 NF- κ B p65 mRNA in LPS-induced macrophages and all exhibited 2 polar bonding and 9, 7,
303 and 7 hydrophobic interactions with RelA amino acid residues, respectively. Hence, these

304 flavonoid compounds possess the ability to suppress LPS-induced NF- κ B upregulation.
305 Flavonoids are known of their ability to suppress NF- κ B and inflammatory responses
306 associated with different disorders [7b, 8b, 29].

307 Owing to the ability of compounds **1-10** to downregulate NF- κ B p65 and the controlling effect
308 of NF- κ B on the expression of iNOS and pro-inflammatory cytokines, all compounds
309 decreased iNOS mRNA and suppressed the release of NO, TNF- α , and IL-6. The activation of
310 macrophages with LPS and other stimulants leads to the release of NO and pro-inflammatory
311 cytokines. This is a direct consequence of NF- κ B activation [18]. NO is one of the pro-
312 inflammatory mediators produced via iNOS activation in macrophages and other cells [18]. In
313 inflammation, the expression of iNOS is upregulated in different cells, including macrophages,
314 microglia, and neutrophils, resulting in the generation of excess NO [30]. This surplus NO leads
315 to a pro-inflammatory response in different organs and reacts with ROS to produce
316 peroxynitrite that further increases ROS and pro-inflammatory mediators and provokes DNA
317 damage [30]. TNF- α is pleiotropic cytokine with various effects on the body cells and a key
318 regulator of pro-inflammatory responses implicated in several inflammatory and autoimmune
319 disorders [31]. TNF- α is involved in the regulation of both acute and chronic inflammation and
320 may cause cell death via apoptosis or necroptosis [32]. Likewise, IL-6 is produced in response
321 to tissue damage, infections and other conditions to contribute to the defense mechanism.
322 Despite the controlled mechanism of production, prolonged and uncontrolled release of IL-6 is
323 implicated in inflammation and autoimmune disorders [33]. Isoscopoletin, aesculetin, and
324 altechromone A (compounds **1-3**) significantly downregulated iNOS and suppressed NO,
325 TNF- α , and IL-6 production from LPS-induced macrophages. All three compounds exhibited
326 binding towards iNOS with many amino acid residues were observed in the binding mode of
327 all compounds. These findings are directly related to the ability of these compounds to
328 downregulate NF- κ B [20-23]. In a study on colitis, aesculetin decreased NO, TNF- α , and IL-6

329 ^[21], suppressed TNF- α and IL-6 in LPS-induced lung injury ^[22] and downregulated iNOS and
330 cytokines in LPS-induced neuroinflammation ^[23]. The current study showed for the first time
331 the efficacy of altechromone A to bind with iNOS (*in silico*) and downregulate (*in vitro*) iNOS,
332 NO, TNF- α , and IL-6 in LPS-induced macrophages. Compounds **4**, **5**, and **6** markedly
333 decreased iNOS mRNA, TNF- α , and IL-6 and dose-dependently suppressed NO production.
334 Moreover, all three compounds exhibited the lowest binding energy with iNOS *in silico* where
335 all showed dense hydrophobic interactions and compound **6** showed polar bonding. Fucosterol
336 has been reported to downregulate TNF- α , and IL-6 gene expression in a mouse model of ALI
337 ^[24] and β -sitosterol suppressed the generation of these cytokines in LPS-induced lung epithelial
338 cells ^[26] and microglial cells ^[27]. *Undaria pinnatifida*-derived fucosterol was effective in
339 reducing NO and pro-inflammatory cytokines generation from macrophages ^[34]. Scopolin
340 remarkably decreased iNOS mRNA, NO, and pro-inflammatory cytokines in LPS-induced
341 macrophages and exhibited *in silico* binding marked by polar bonding with 2 residues and
342 hydrophobic interactions with 8 residues of iNOS. The effects of scopolin on these mediators
343 is scarcely reported. In a rat model of arthritis, *Erycibe obtusifolia*-derived scopolin reduced
344 IL-6 ^[28]. The isolated flavonoids (compounds **8**, **9**, and **10**) showed inhibitory activities on the
345 expression of iNOS and the production of NO, TNF- α , and IL-6, effects that could be explained
346 by their NF- κ B inhibition efficacy. All flavonoids showed hydrophobic interactions with ten
347 amino acid residues of iNOS. The lack of data showing the protein expression levels of NF- κ B
348 and iNOS could be considered as a limitation of this study. However, the results of mRNA
349 abundance and levels NO, TNF- α , and IL-6 confirm downregulation of NF- κ B and iNOS
350 following treatment of the LPS-challenged macrophages with the tested compounds.

351 **5. Conclusion**

352 This study introduced new information on the anti-inflammatory efficacy of *H. salicornicum*
353 phytochemicals. The isolated ten compounds showed potent inhibitory effects on the

354 expression of NF- κ B p65 and iNOS, and the release of NO, TNF- α , and IL-6 from LPS-induced
355 macrophages. In addition, all compounds exhibited affinities to bind to NF- κ B p65 and iNOS,
356 suggesting their potential as candidate inhibitors of these proteins. Therefore, *H. salicornicum*
357 is rich in anti-inflammatory phytochemicals and further studies are recommended to investigate
358 the exact molecular mechanisms underlying their effect on inflammatory mediators. *In vivo*
359 studies and clinical trials to investigate the anti-inflammatory and immunomodulatory
360 efficacies of the studied compounds are recommended.

361 **Conflict of Interest**

362 The authors declare no conflict of interest.

363 **Availability of data and materials**

364 The manuscript contains all data supporting the reported results.

365 **Acknowledgment**

366 Princess Nourah bint Abdulrahman University Researchers Supporting Project Number
367 (PNURSP2024R381), Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia.

368 **Authors' Contributions:**

369 Conceptualization: A.M.M.; Methodology: R.S.A., O.E.H., S.M.A., I.E., M.A.A., M.E.,
370 E.M.K., and A.M.M.; Investigation: R.S.A., O.E.H., M.E., E.M.K., and A.M.M.; Data curation:
371 A.M.M., O.E.H., and M.E.; Formal analysis: A.M.M., and E.M.K.; Resources: S.M.A., I.E.,
372 M.A.A. and R.S.A.; Supervision: A.M.M.; Writing-Original draft: A.M.M. and O.E.H.;
373 Writing-review and editing: A.M.M.

374 **References:**

- 375 [1] Q. Zhang, M. J. Lenardo, D. Baltimore, *Cell* **2017**, *168*, 37-57.
376 [2] N. Singh, D. Baby, J. P. Rajguru, P. B. Patil, S. S. Thakkannavar, V. B. Pujari, *Ann Afr Med*
377 **2019**, *18*, 121-126.
378 [3] aN. Watson, B. Ding, X. Zhu, R. D. Frisina, *Ageing Res Rev* **2017**, *40*, 142-148; bA. L.
379 Gruver, L. L. Hudson, G. D. Sempowski, *J Pathol* **2007**, *211*, 144-156.
380 [4] X. Zhang, D. M. Mosser, *J Pathol* **2008**, *214*, 161-178.
381 [5] aM. S. Abduh, R. S. Alruhaimi, H. A. Alqhtani, O. E. Hussein, M. H. Abukhalil, E. M.
382 Kamel, A. M. Mahmoud, *Life Sci* **2023**, *313*, 121281; bA. Alhusaini, L. Fadda, I. H. Hasan,
383 E. Zakaria, A. M. Alenazi, A. M. Mahmoud, *Biomolecules* **2019**, *9*; cR. S. Alruhaimi, E. H.
384 M. Hassanein, M. N. Bin-Jumah, A. M. Mahmoud, *Food and Chemical Toxicology* **2023**,
385 *180*, 114055; dS. A. Antar, W. Abdo, R. S. Taha, A. E. Farage, L. E. El-Moselhy, M. E. Amer,
386 A. S. Abdel Monsef, A. M. Abdel Hamid, E. M. Kamel, A. F. Ahmeda, A. M. Mahmoud, *Life*
387 *Sci* **2022**, *291*, 120260; eA. M. Mahmoud, O. A. M. Abd El-Ghafar, M. A. Alzoghbi, E. H.
388 M. Hassanein, *Life Sci* **2021**, *278*, 119600.

- 389 [6] A. Płóciennikowska, A. Hromada-Judycka, K. Borzęcka, K. Kwiatkowska, *Cell Mol Life Sci*
390 **2015**, *72*, 557-581.
- 391 [7] aM. S. Abduh, M. A. Alzoghaibi, A. M. Alzoghaibi, A. Bin-Ammar, M. F. Alotaibi, E. M.
392 Kamel, A. M. Mahmoud, *Life Sci* **2023**, 121612; bR. S. Alruhaimi, G. Mostafa-Hedeab, M.
393 S. Abduh, A. Bin-Ammar, E. H. M. Hassanein, E. M. Kamel, A. M. Mahmoud, *Front*
394 *Pharmacol* **2023**, *14*, 1204641; cA. Fadel, A. M. Mahmoud, J. J. Ashworth, W. Li, Y. L. Ng,
395 A. Plunkett, *International Journal of Biological Macromolecules* **2018**, *109*, 819-831; dA.
396 M. Mahmoud, M. Bin-Jumah, M. H. Abukhalil, in *Inflammation and Natural Products*
397 (Eds.: S. Gopi, A. Amalraj, A. Kunnumakkara, S. Thomas), Academic Press, **2021**, pp.
398 175-203; eF. C. Asogwa, C. G. Apebende, G. W. Ugodi, P. Ebo, H. Louis, A. I. Ikeuba, C. J.
399 Asogwa, T. E. Gber, I. J. Ikot, A. E. Owen, *Chemistry Africa* **2023**, *6*, 1349-1361; fF. C.
400 Asogwa, A. Ibezim, F. Ntie-Kang, C. J. Asogwa, C. O. B. Okoye, *Scientific African* **2020**, *7*,
401 e00229.
- 402 [8] aS. A. Ramadan, E. M. Kamel, R. S. Alruhaimi, A. Bin-Ammar, M. A. Ewais, A. A.
403 Khowailed, E. H. M. Hassanein, A. M. Mahmoud, *Saudi Pharm J* **2023**, *31*, 101766; bS. A.
404 Ramadan, E. M. Kamel, M. A. Ewais, A. A. Khowailed, E. H. M. Hassanein, A. M.
405 Mahmoud, *Environ Sci Pollut Res Int* **2023**, *30*, 49197-49214.
- 406 [9] L. Boulos, *Flora of egypt, Vol. 4*, Al Hadara Publishing Cairo, **2005**.
- 407 [10] aM. Ajabnoor, M. Al-Yahya, M. Tariq, A. J. F. Jayyab, **1984**, *55*, 107-109; bN. Bibi, S. A. K.
408 Tanoli, S. Farheen, N. Afza, S. Siddiqi, Y. Zhang, S. U. Kazmi, A. J. B. Malik, m. c. letters,
409 **2010**, *20*, 4173-4176.
- 410 [11] O. A. Rugaie, H. A. Mohammed, S. Alsamani, S. Messaoudi, L. M. Aroua, R. A. Khan, S. A.
411 Almahmoud, A. D. Altaleb, M. Alsharidah, M. Aldubaib, K. A. Al-Regaiey, K. A. Qureshi,
412 *Antibiotics (Basel)* **2023**, *12*.
- 413 [12] M. Ahmad, S. Eram, *Pak J Pharm Sci* **2011**, *24*, 377-382.
- 414 [13] K. J. Livak, T. D. Schmittgen, *Methods (San Diego, Calif.)* **2001**, *25*, 402-408.
- 415 [14] L. C. Green, D. A. Wagner, J. Glogowski, P. L. Skipper, J. S. Wishnok, S. R. Tannenbaum,
416 *Analytical Biochemistry* **1982**, *126*, 131-138.
- 417 [15] S. Dallakyan, A. J. Olson, *Methods Mol Biol* **2015**, *1263*, 243-250.
- 418 [16] A. C. Wallace, R. A. Laskowski, J. M. Thornton, *Protein Eng* **1995**, *8*, 127-134.
- 419 [17] M. S. Hayden, S. Ghosh, *Cell* **2008**, *132*, 344-362.
- 420 [18] T. Liu, L. Zhang, D. Joo, S.-C. Sun, *Signal Transduction and Targeted Therapy* **2017**, *2*,
421 17023.
- 422 [19] M. Karin, M. Delhase, *Semin Immunol* **2000**, *12*, 85-98.
- 423 [20] D.-Y. Seo, J.-W. Park, S.-H. Kim, S.-R. Oh, S.-B. Han, O.-K. Kwon, K.-S. Ahn, in
424 *International Journal of Molecular Sciences, Vol. 25*, **2024**.
- 425 [21] S.-K. Wang, T.-X. Chen, W. Wang, L.-L. Xu, Y.-Q. Zhang, Z. Jin, Y.-B. Liu, Y.-Z. Tang, *Journal*
426 *of Ethnopharmacology* **2022**, *296*, 115489.
- 427 [22] T. Chen, Q. Guo, H. Wang, H. Zhang, C. Wang, P. Zhang, S. Meng, Y. Li, H. Ji, T. Yan, *Free*
428 *Radical Research* **2015**, *49*, 1459-1468.
- 429 [23] L. Zhu, C. Nang, F. Luo, H. Pan, K. Zhang, J. Liu, R. Zhou, J. Gao, X. Chang, H. He, Y. Qiu, J.
430 Wang, H. Long, Y. Liu, T. Yan, *Physiol Behav* **2016**, *163*, 184-192.
- 431 [24] W. Mo, C. Wang, J. Li, K. Chen, Y. Xia, S. Li, L. Xu, X. Lu, W. Wang, C. Guo, *Gastroenterol*
432 *Res Pract* **2018**, *2018*, 2824139.
- 433 [25] M.-S. Kim, G.-H. Oh, M.-J. Kim, J.-K. Hwang, *Photochemistry and Photobiology* **2013**, *89*,
434 911-918.
- 435 [26] X. Chen, J. Chen, Y. Ren, M. Wang, Z. Yang, W. Zhang, Q. Li, C. Liu, Z. Sun, S. Nie, *Discov*
436 *Med* **2023**, *35*, 946-955.
- 437 [27] aY. Zheng, J. Zhao, S. Chang, Z. Zhuang, S. Waimei, X. Li, Z. Chen, B. Jing, D. Zhang, G.
438 Zhao, *Journal of Neuroimmune Pharmacology* **2023**, *18*, 690-703; bY. Sun, L. Gao, W.
439 Hou, J. Wu, *BioMed Research International* **2020**, *2020*, 7532306.

- 440 [28] R. Pan, Y. Dai, X. Gao, Y. Xia, *Int Immunopharmacol* **2009**, *9*, 859-869.
- 441 [29] aE. M. Kamel, A. Bin-Ammar, A. A. El-Bassuony, M. M. Alanazi, A. Altharawi, A. F.
- 442 Ahmeda, A. S. Alanazi, A. M. Lamsabhi, A. M. Mahmoud, *RSC Advances* **2023**, *13*,
- 443 12361-12374; bA. M. Sayed, E. H. M. Hassanein, S. H. Salem, O. E. Hussein, A. M.
- 444 Mahmoud, *Life Sci* **2020**, *259*, 118173.
- 445 [30] J. N. Sharma, A. Al-Omran, S. S. Parvathy, *Inflammopharmacology* **2007**, *15*, 252-259.
- 446 [31] J. R. Bradley, *The Journal of Pathology* **2008**, *214*, 149-160.
- 447 [32] G. D. Kalliolias, L. B. Ivashkiv, *Nat Rev Rheumatol* **2016**, *12*, 49-62.
- 448 [33] T. Tanaka, M. Narazaki, T. Kishimoto, *Cold Spring Harb Perspect Biol* **2014**, *6*, a016295.
- 449 [34] M. S. Yoo, J. S. Shin, H. E. Choi, Y. W. Cho, M. H. Bang, N. I. Baek, K. T. Lee, *Food Chem*
- 450 **2012**, *135*, 967-975.

451

452

453 Tables:

454 Table 1. Binding affinities of *H. salicornicum* phytoconstituents towards NF- κ B.

Compound	Lowest binding energy (kcal/mol)	Polar interacting residues	Hydrophobic interacting residues
1	-5.9	Thr60, His58	Thr57, Arg50, Thr55, Pro275, Ser112, Glu25
2	-6.0	Thr60, His58, Glu25, Arg50	Thr57, Pro275, Ser276, Lys56
3	-5.9	Thr60, His58	Thr57, Glu25, Ser276, Lys56, Pro275
4	-7.9	Asn186	Arg187, Val248, Arg246, Lys218
5	-7.6	Asn186	Val248, Lys218, Arg246, Arg187
6	-8.2	Asn186, Arg33, Asp217	Ala192, Val248, Arg187, Lys218, Asp217
7	-7.1	Arg278, Ser112, Lys56, Gln114	Pro275, Ile110, Glu25, Thr60, Thr55, His58, Ser276, Thr57
8	-7.3	Gln114, Lys56	Ser276, Ile24, Thr60, Arg50, Glu25, Pro275, Thr55, Thr57, His58
9	-7.1	Glu25, Lys56	Ser112, Ile24, Arg50, Gln114, Thr60, His58, Ser276
10	-6.8	Ser112, Arg236	Pro275, Gln114, Lys56, Thr57, Thr55, Glu225, Thr60

455

456

457 Table 2. Binding affinities of *H. salicornicum* phytoconstituents towards iNOS.

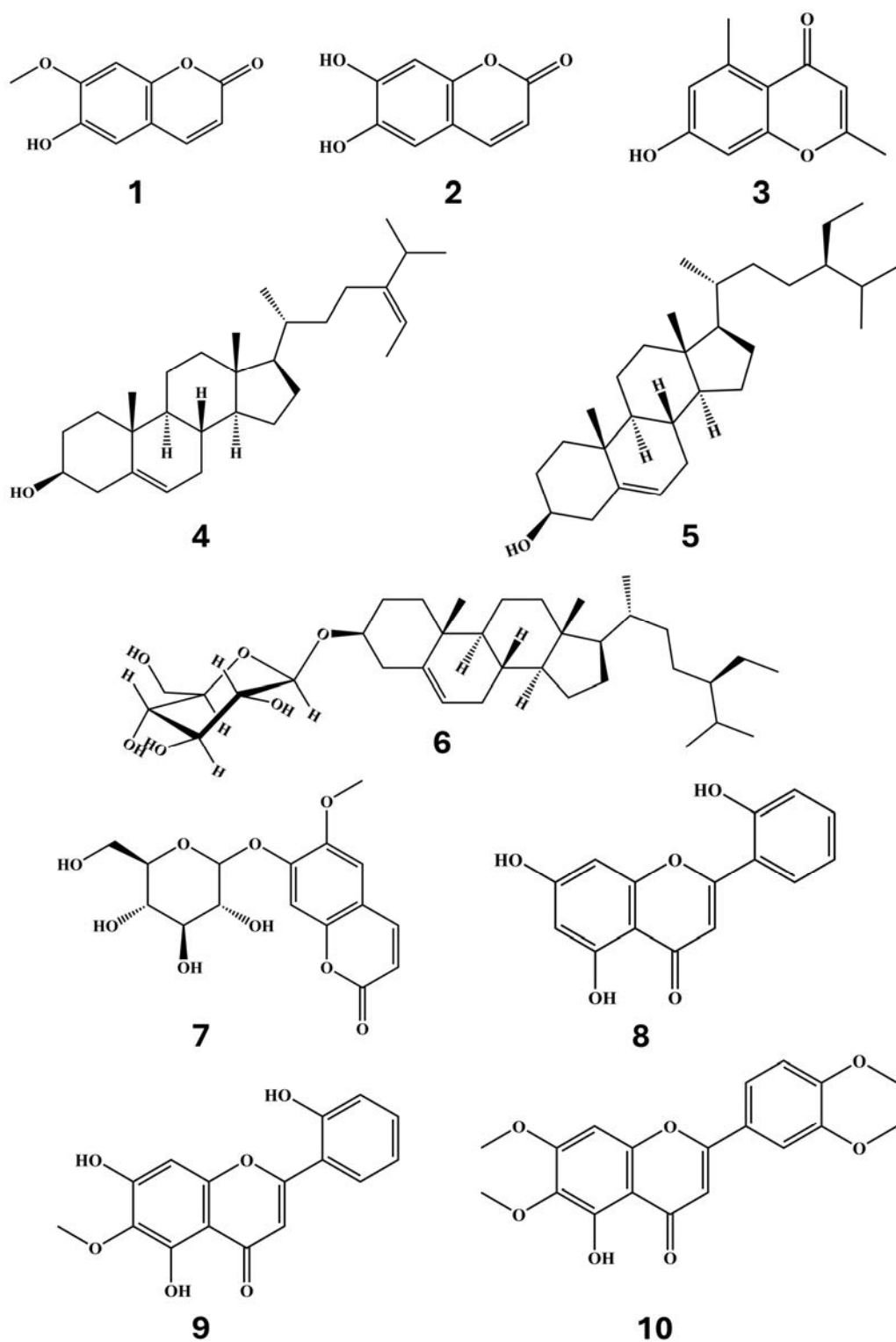
Compound	Lowest binding energy (kcal/mol)	Polar interacting residues	Hydrophobic interacting residues
1	-7.5	Tyr483	Cys194, Ala191, Trp188, Leu203, Phe363
2	-7.2	Tyr483	Cys194, Asn364, Trp188, Leu203, Phe363
3	-8		Cys194, Ala191, Asn364, Trp188, Tyr483, Leu203, Gly365, Phe363
4	-10.7		Cys194, Trp457, Pro461, Met368, Arg193, Trp188, Leu203, Phe363 Glu371, Ile195, Gly365, Pro344, Asn364, Val346
5	-10.3		Cys194, Ile195, Glu371, Pro461, Pro344, Asn364, Phe363, Leu203, Met368, Trp457, Gly365, Arg193, Trp188, Tyr483, Val346
6	-10.1	Gln257, Arg260, Arg382	Cys194, Tyr483, Phe363, Asn364, Trp188, Val346, Leu203, Tyr367, Arg375, Pro344, Glu371
7	-9.1	Ser236, Trp366	Cys194, Gln 199, Trp188, Tyr483, Gly196, Phe363, Leu203, Gly365
8	-9.9		Cys194, Phe363, Asn364, Leu203, Trp188, Tyr483, Gly365, Pro344, Val346, Trp366
9	-9.9		Tyr483, Leu203, Ser236, Phe363, Trp188, Gly365, Gly196, Trp366, Ile195, Asn364
10	-8.8		Cys194, Tyr485, Arg193, Trp457, Met349, Ala191, Phe363, Trp188, Gly365, Tyr483

458

459

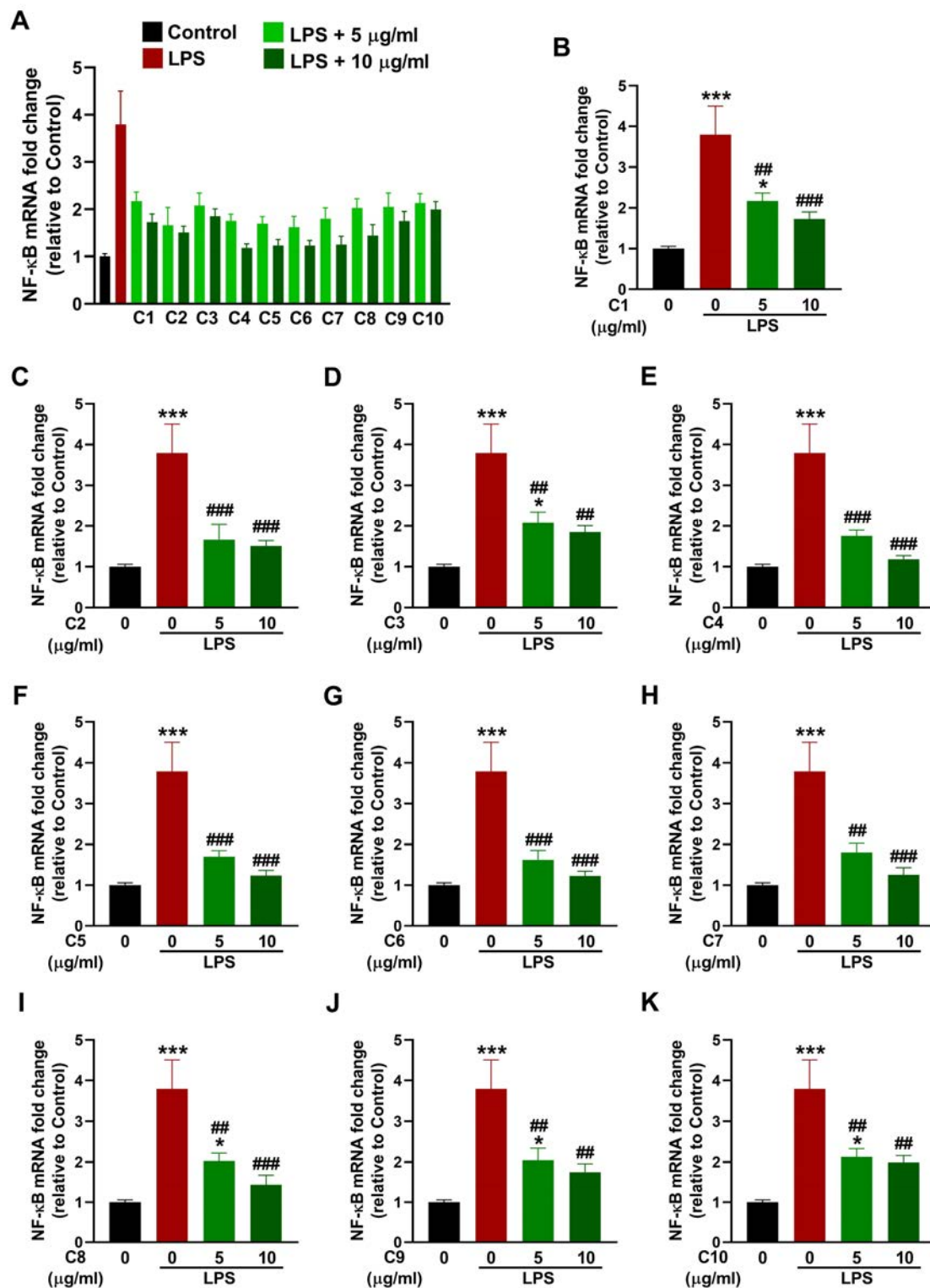
460

461 Figures:



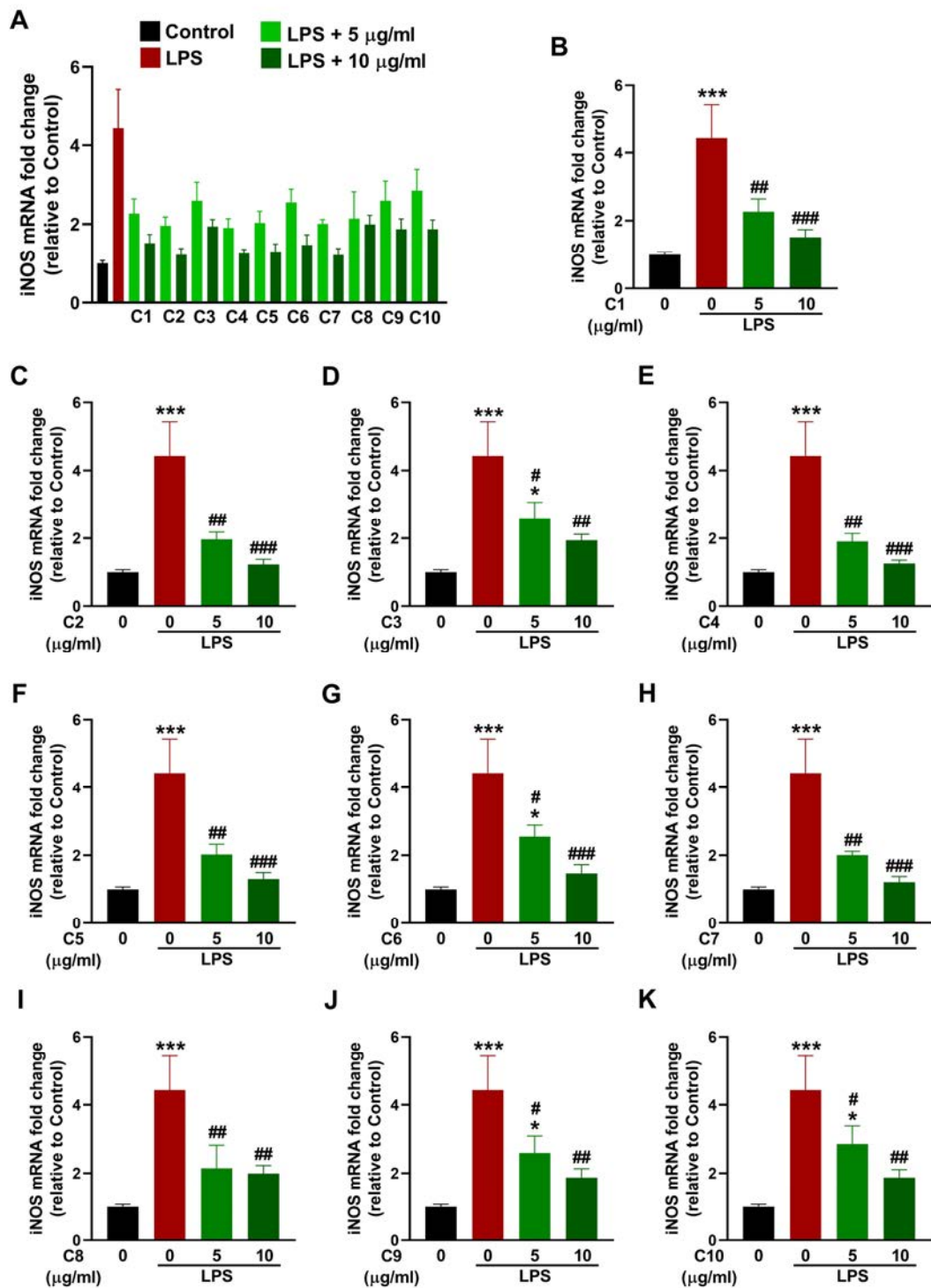
462

463 Fig. 1. Chemical structure of the isolated compounds (1-10).



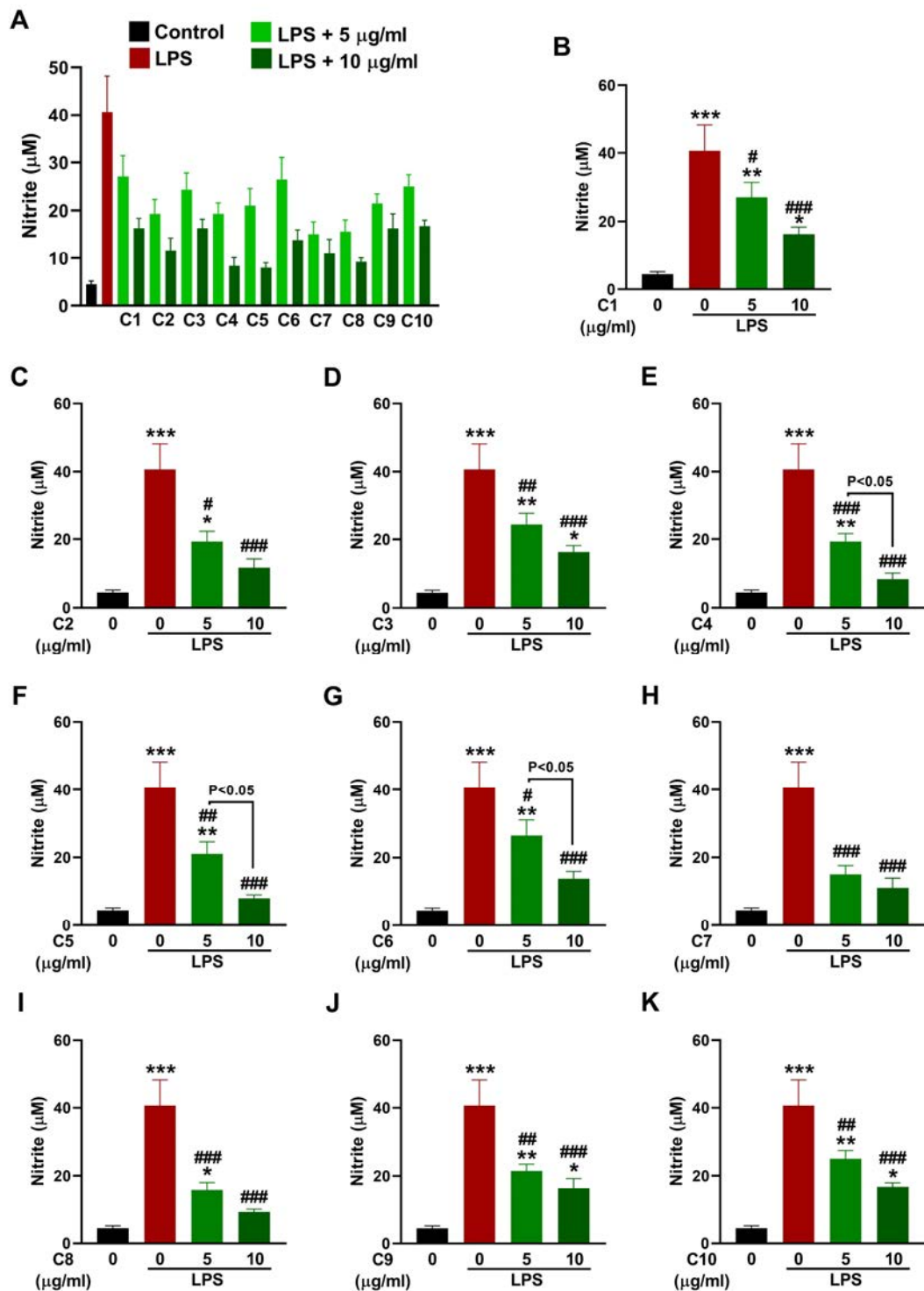
464

465 Fig. 2. Effect of compounds **1-10** on NF-κB p65 mRNA abundance in LPS-induced
 466 macrophages. Data are Mean ± SD, (N = 3). *P<0.05 and ***P<0.001 vs Control. ##P<0.01 and
 467 ###P<0.001 vs LPS.



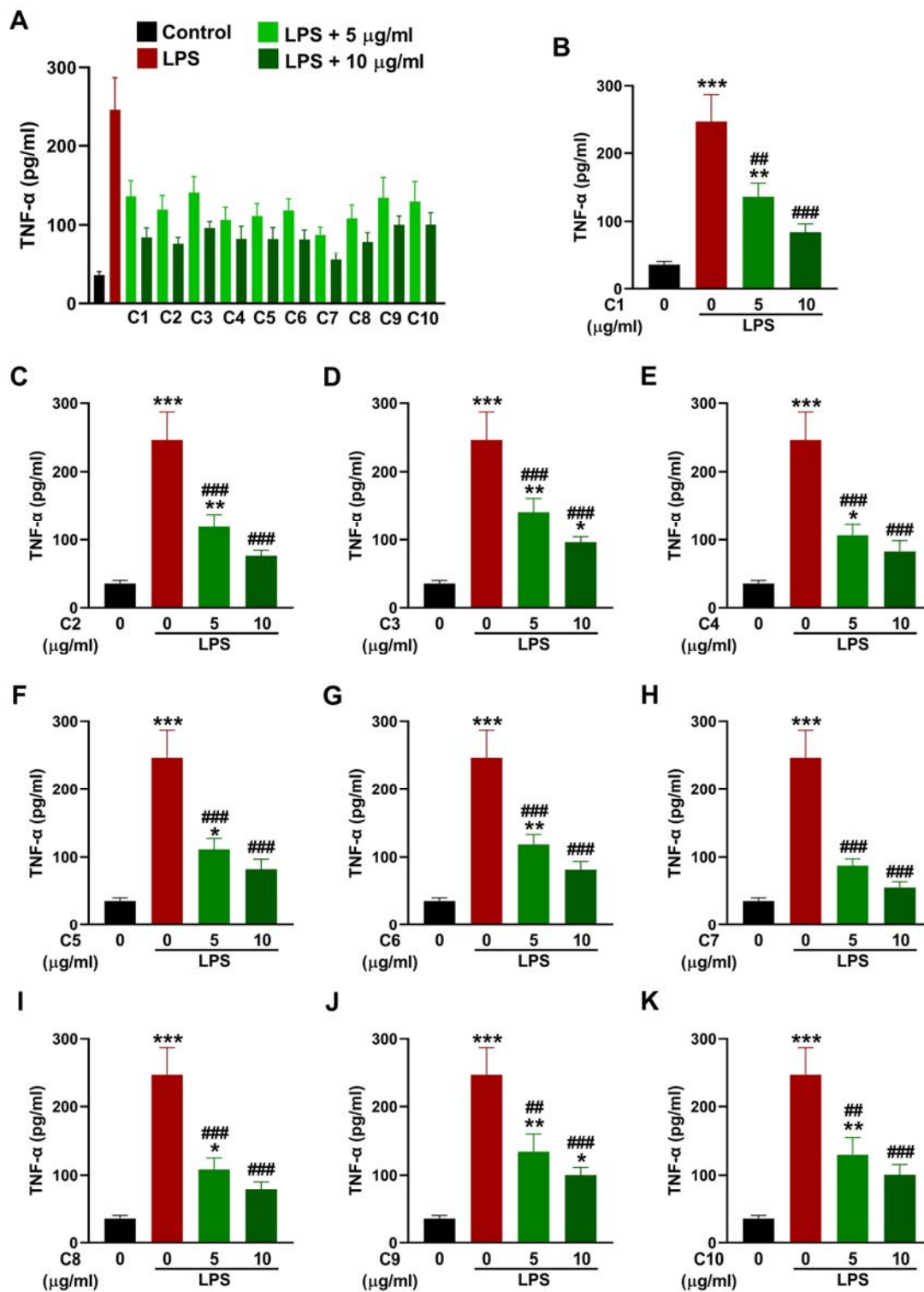
468

469 Fig. 3. Effect of compounds **1-10** on iNOS mRNA abundance in LPS-induced macrophages.
 470 Data are Mean \pm SD, ($N = 3$). * $P < 0.05$ and *** $P < 0.001$ vs Control. # $P < 0.05$, ## $P < 0.01$ and
 471 ### $P < 0.001$ vs LPS.



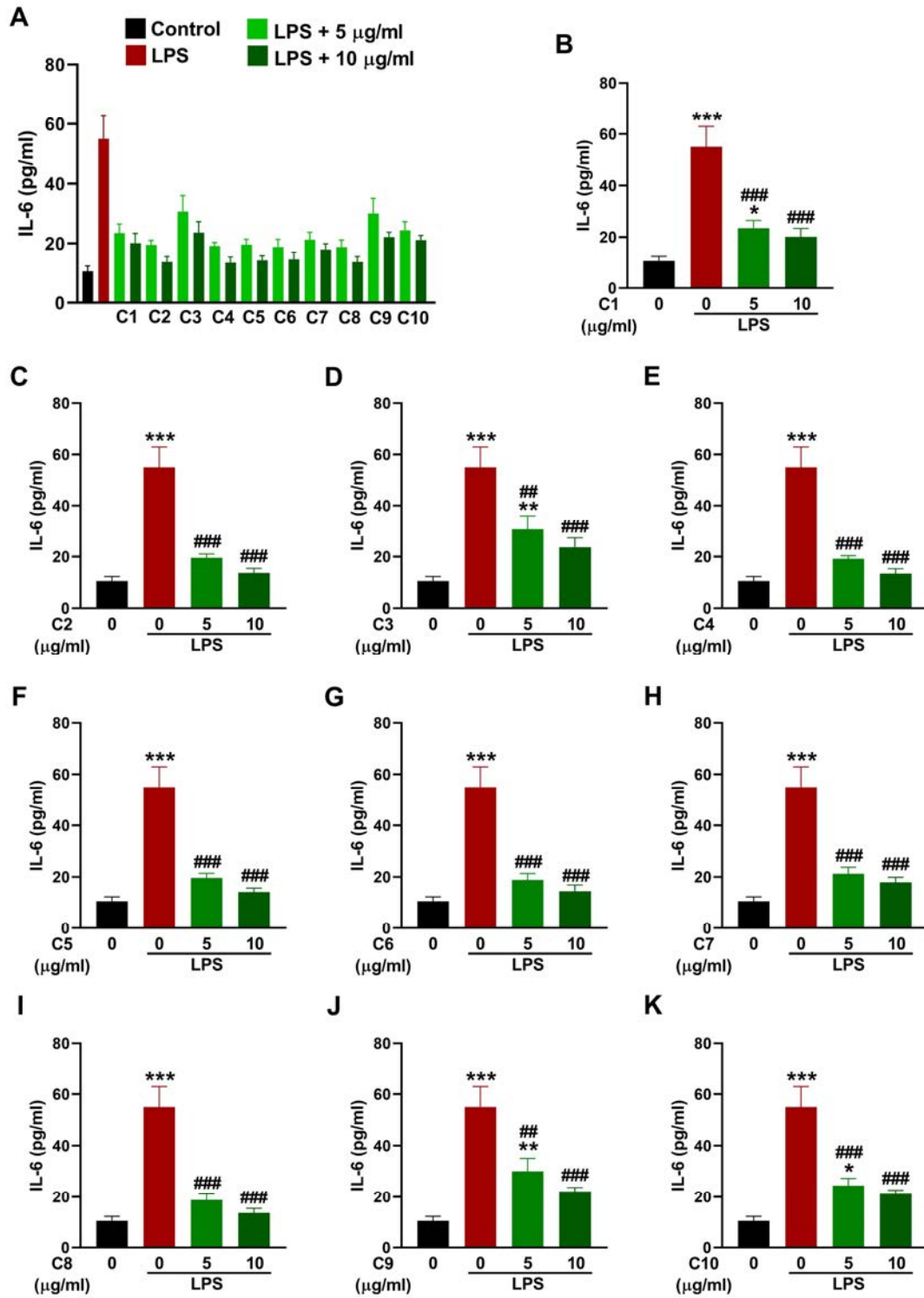
472

473 Fig. 4. Effect of compounds **1-10** on NO production by LPS-induced macrophages. Data are
 474 Mean \pm SD, ($N = 3$). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs Control. # $P < 0.05$, ## $P < 0.01$ and
 475 ### $P < 0.001$ vs LPS.



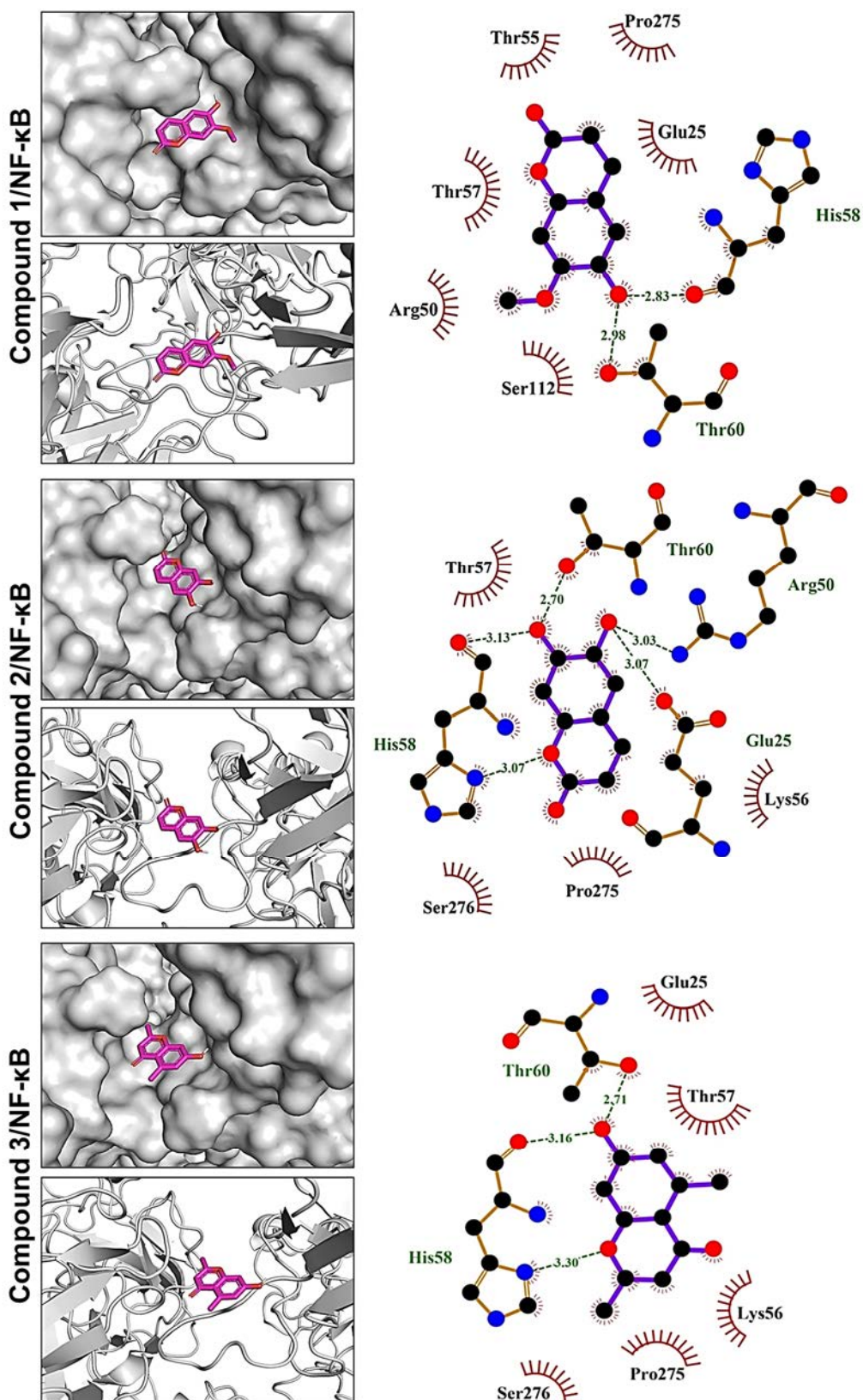
476

477 Fig. 5. Effect of compounds **1-10** on TNF- α release by LPS-induced macrophages. Data are
 478 Mean \pm SD, ($N = 3$). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs Control. ## $P < 0.01$ and ### $P < 0.001$ vs
 479 LPS.



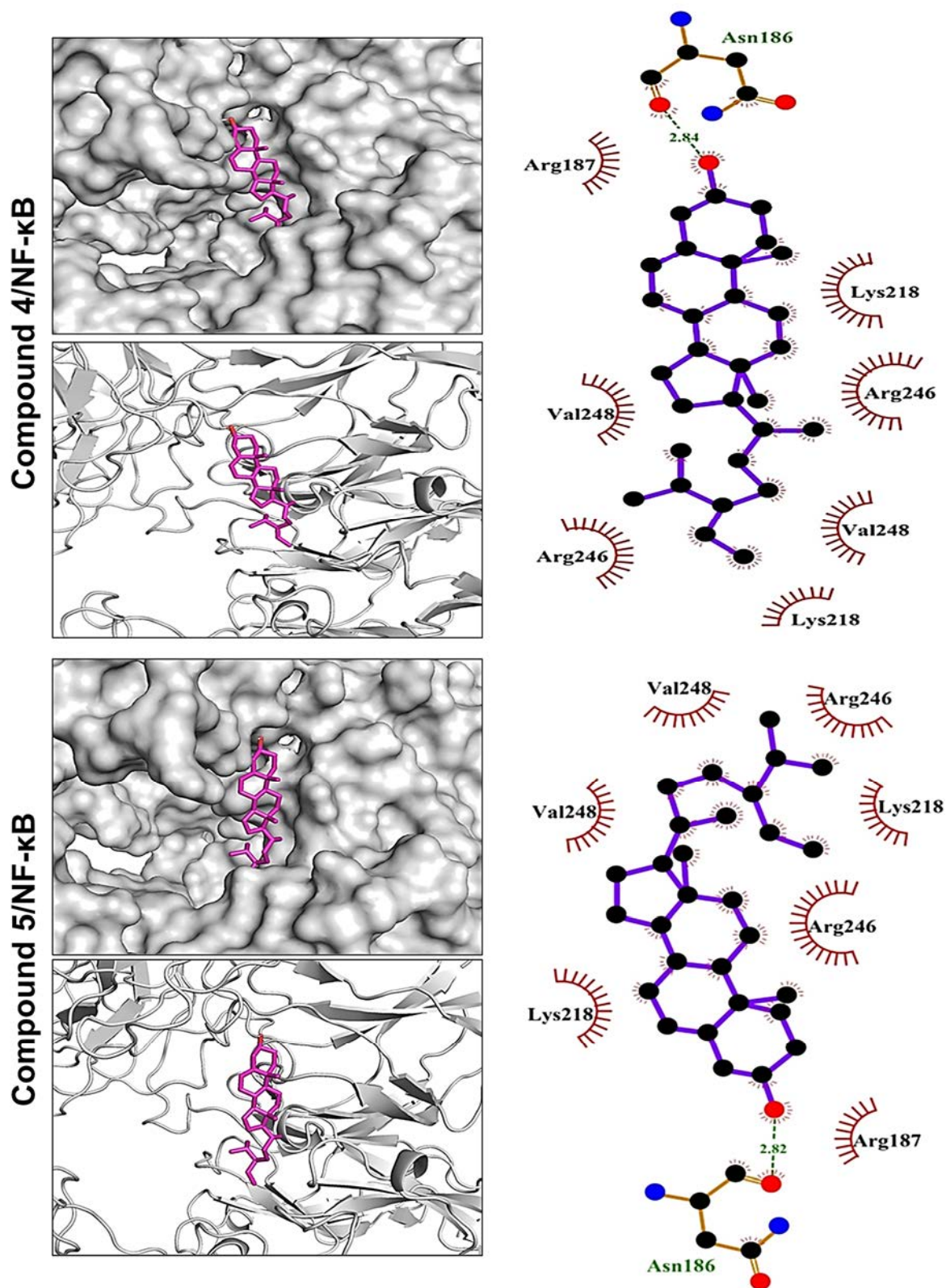
480

481 Fig. 6. Effect of compounds **1-10** on IL-6 release by LPS-induced macrophages. Data are Mean
 482 \pm SD, ($N = 3$). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs Control. ## $P < 0.01$ and ### $P < 0.001$ vs LPS.



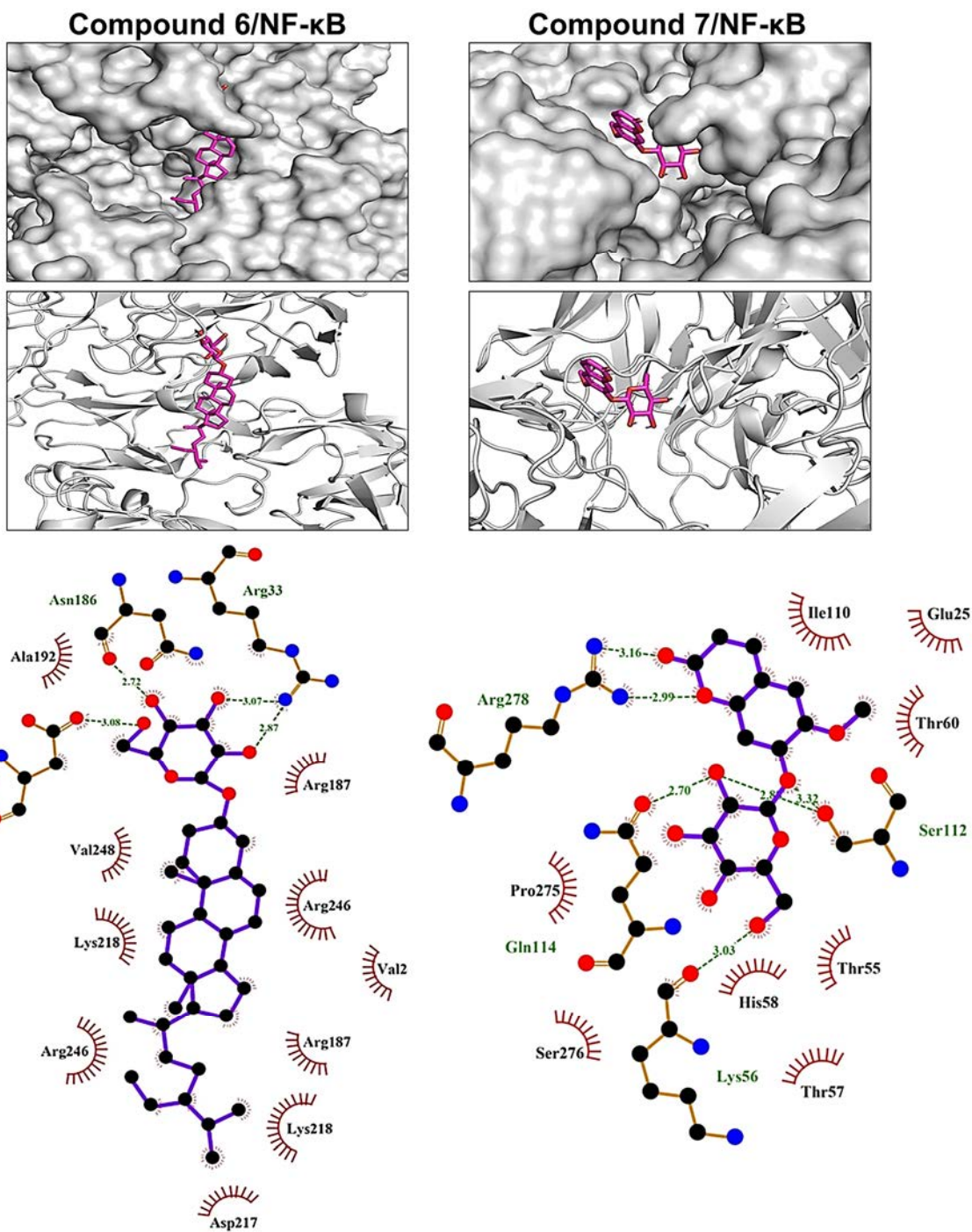
483

484 Fig. 7. Molecular docking of compounds 1, 2, and 3 with NF-κB RelA dimer showing the
 485 crystal structure and amino acid residues involved in polar bonding and hydrophobic
 486 interactions.



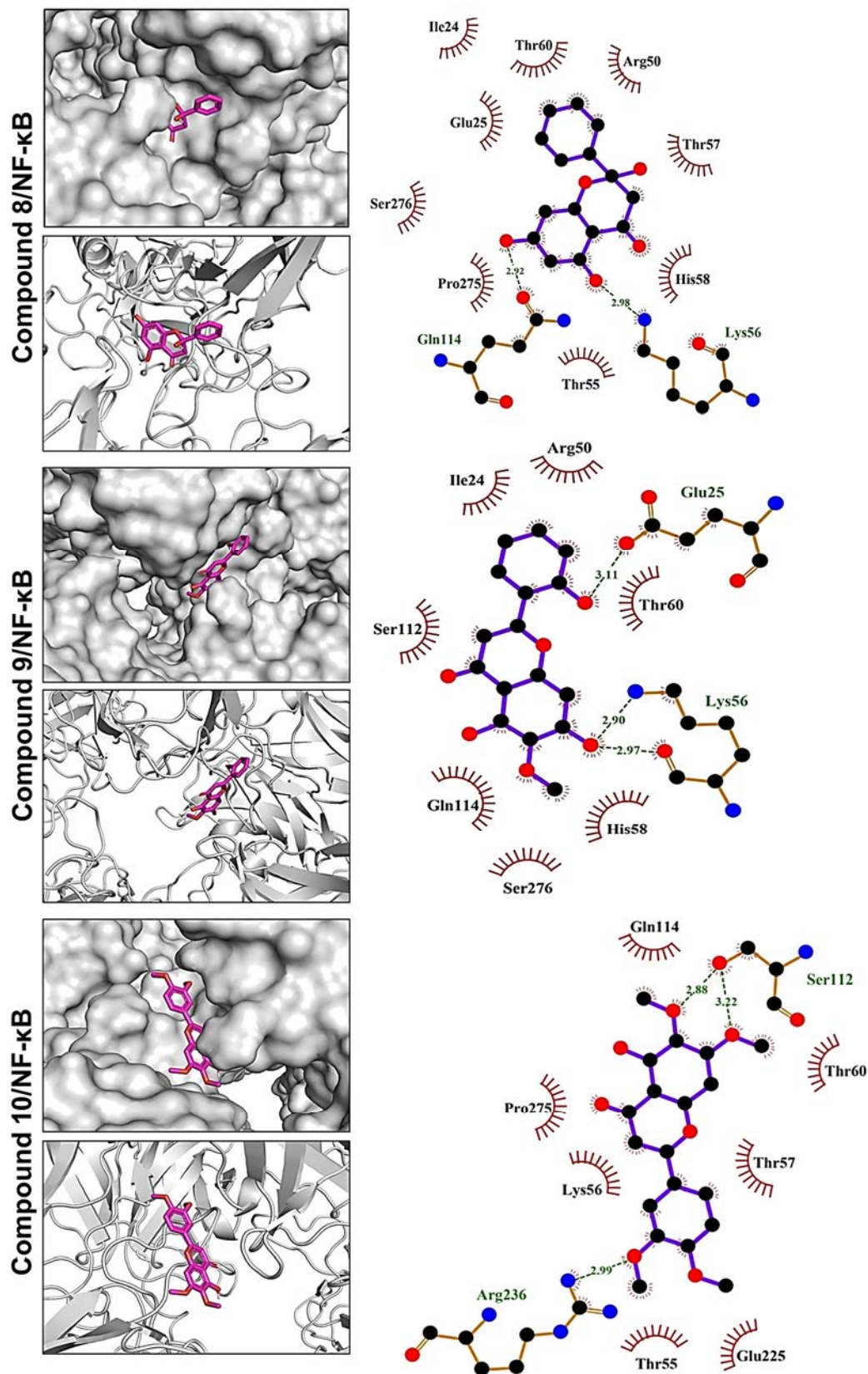
487

488 Fig. 8. Molecular docking of compounds **4** and **5** with NF-κB RelA dimer showing the crystal
 489 structure and amino acid residues involved in polar bonding and hydrophobic interactions.



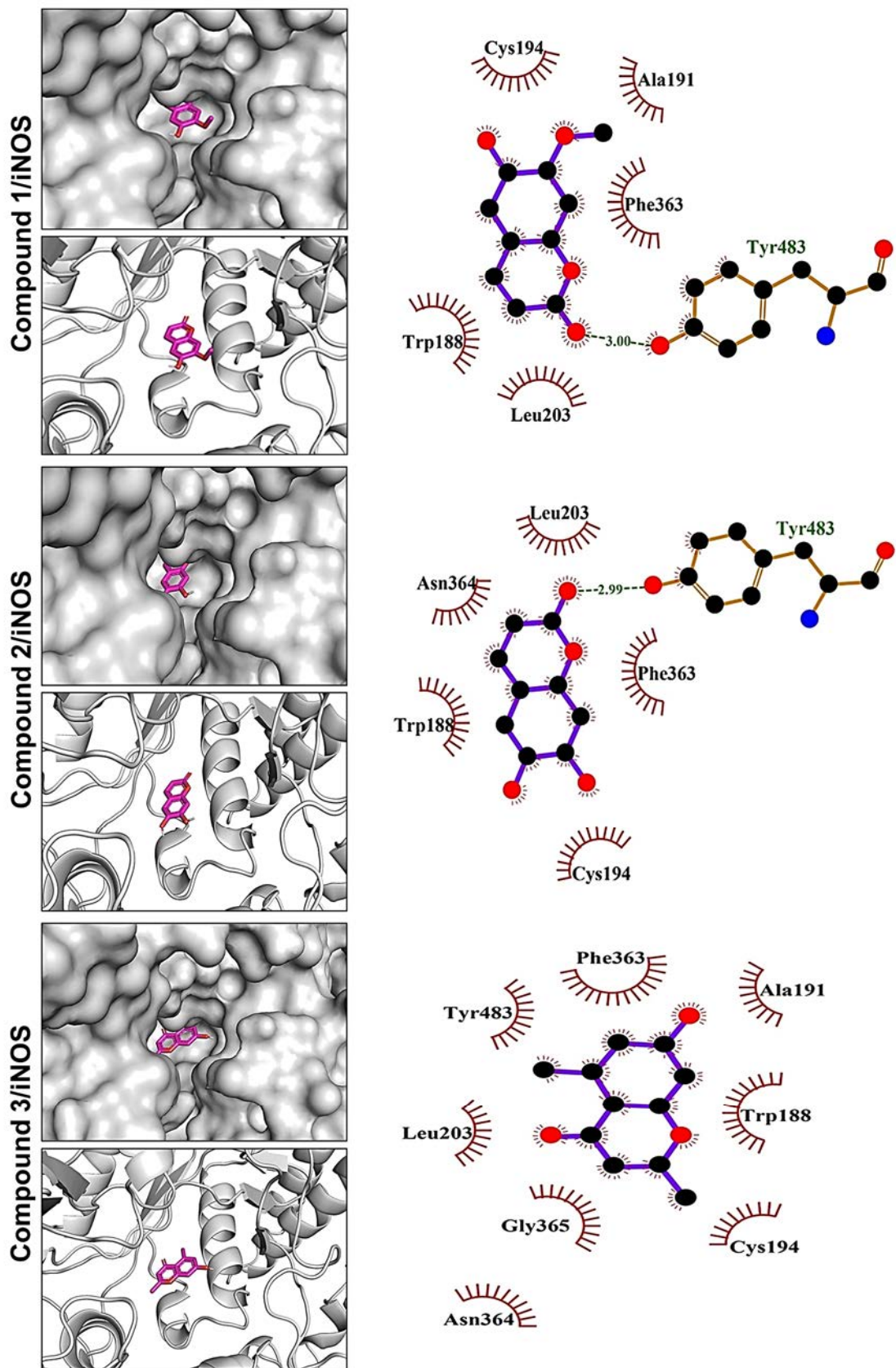
490

491 Fig. 9. Molecular docking of compounds **6** and **7** with NF-κB RelA dimer showing the crystal
 492 structure and amino acid residues involved in polar bonding and hydrophobic interactions.



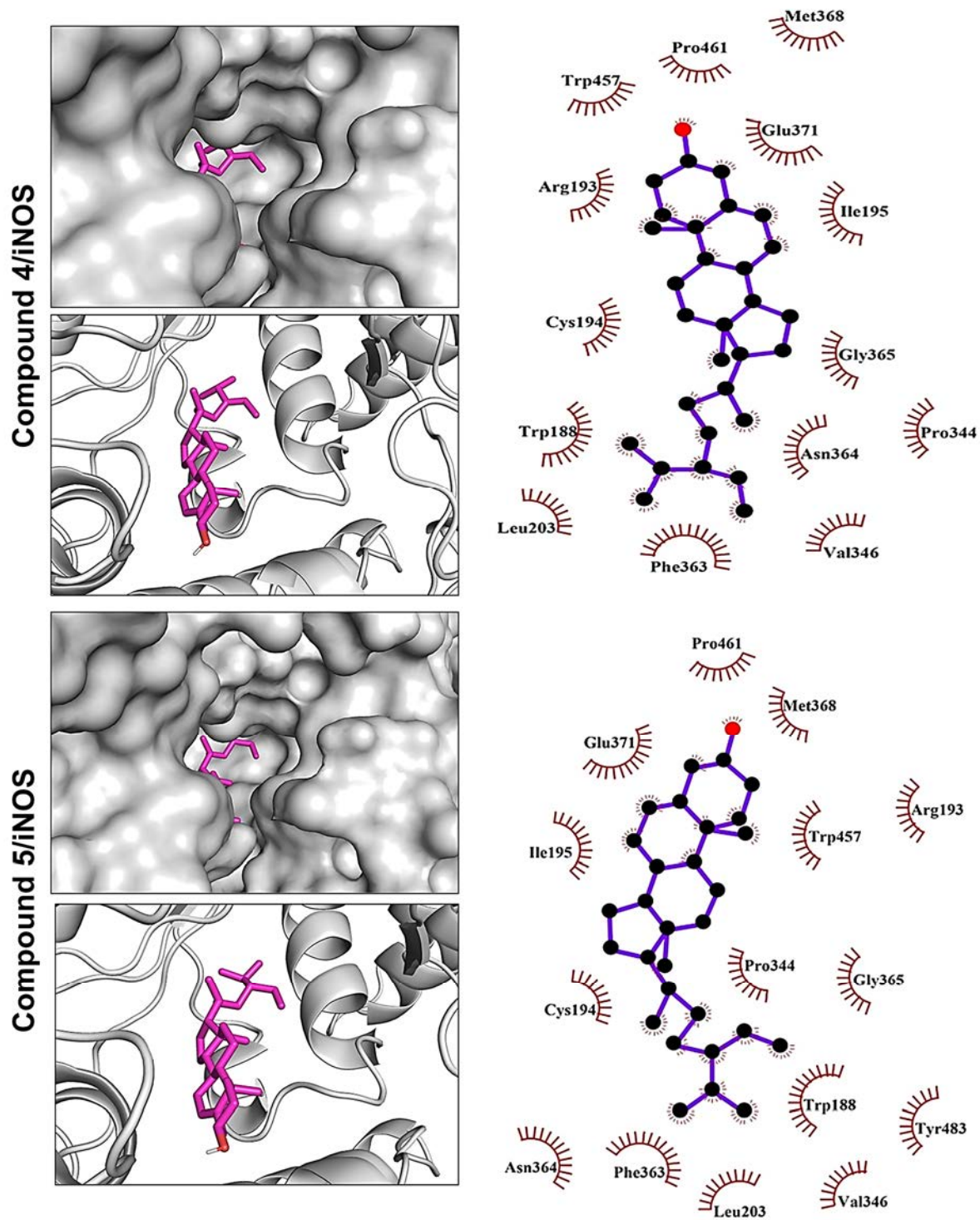
493

494 Fig. 10. Molecular docking of compounds 8, 9, and 10 with NF-κB RelA dimer showing the
 495 crystal structure and amino acid residues involved in polar bonding and hydrophobic
 496 interactions.



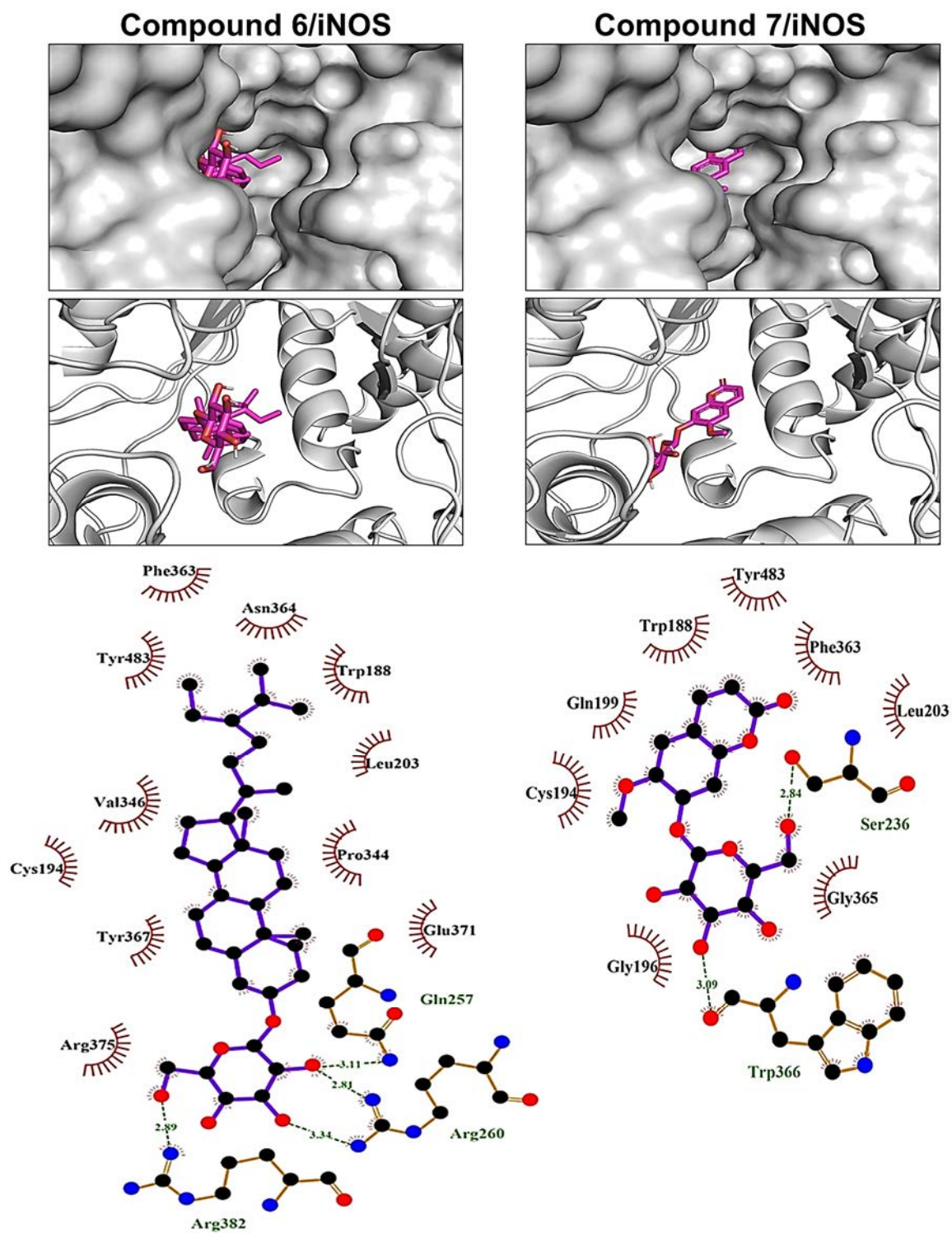
497

498 Fig. 11. Molecular docking of compounds 1, 2, and 3 with iNOS showing the crystal structure
 499 and amino acid residues involved in polar bonding and hydrophobic interactions.



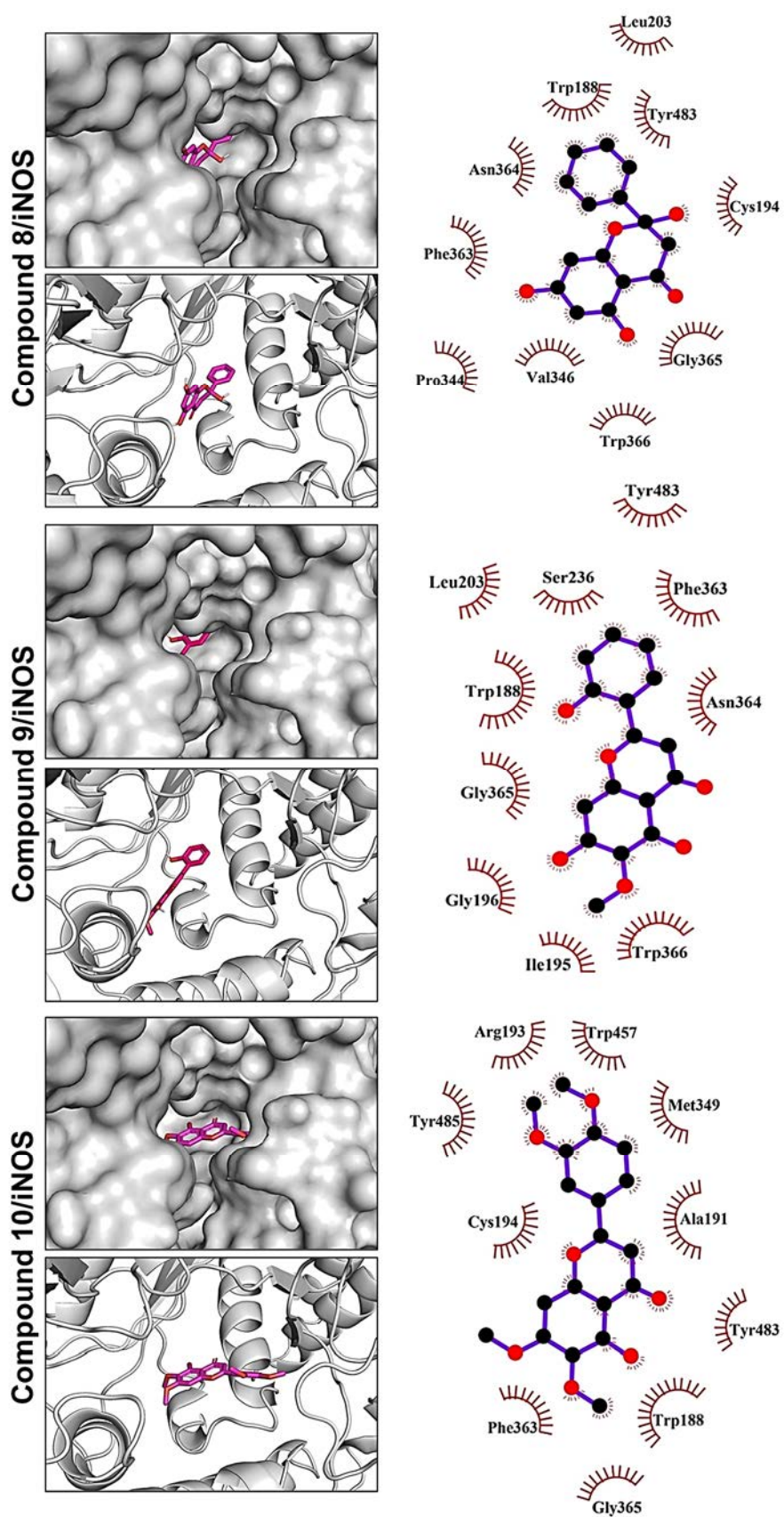
500

501 Fig. 12. Molecular docking of compounds **4** and **5** with iNOS showing the crystal structure and
 502 amino acid residues involved in polar bonding and hydrophobic interactions.



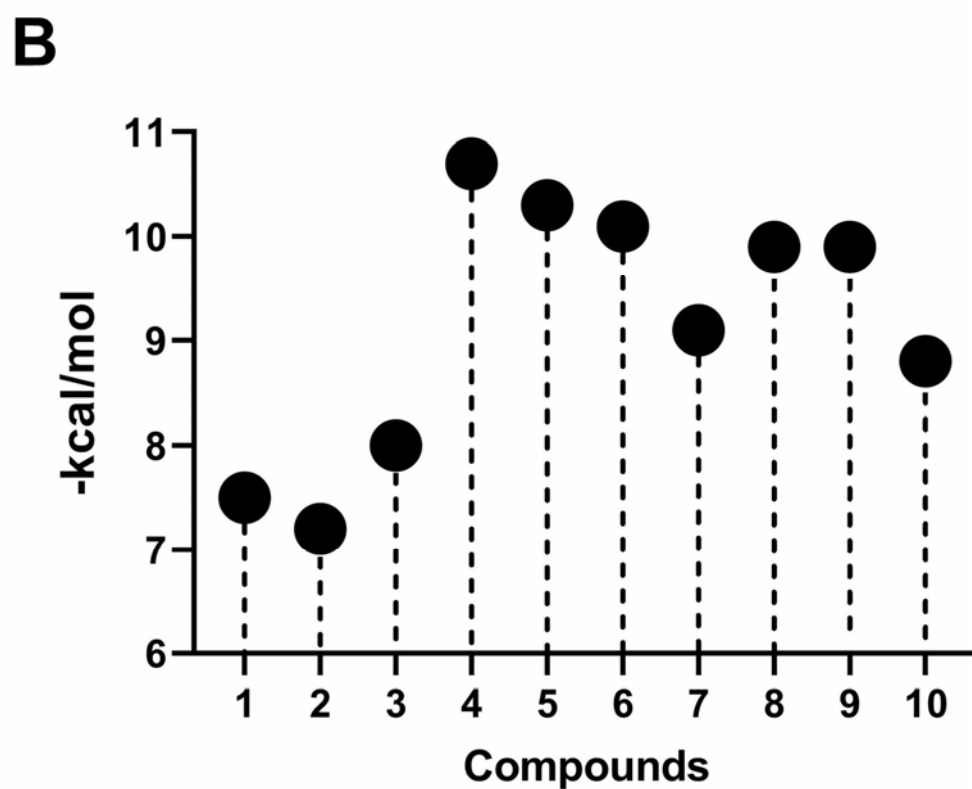
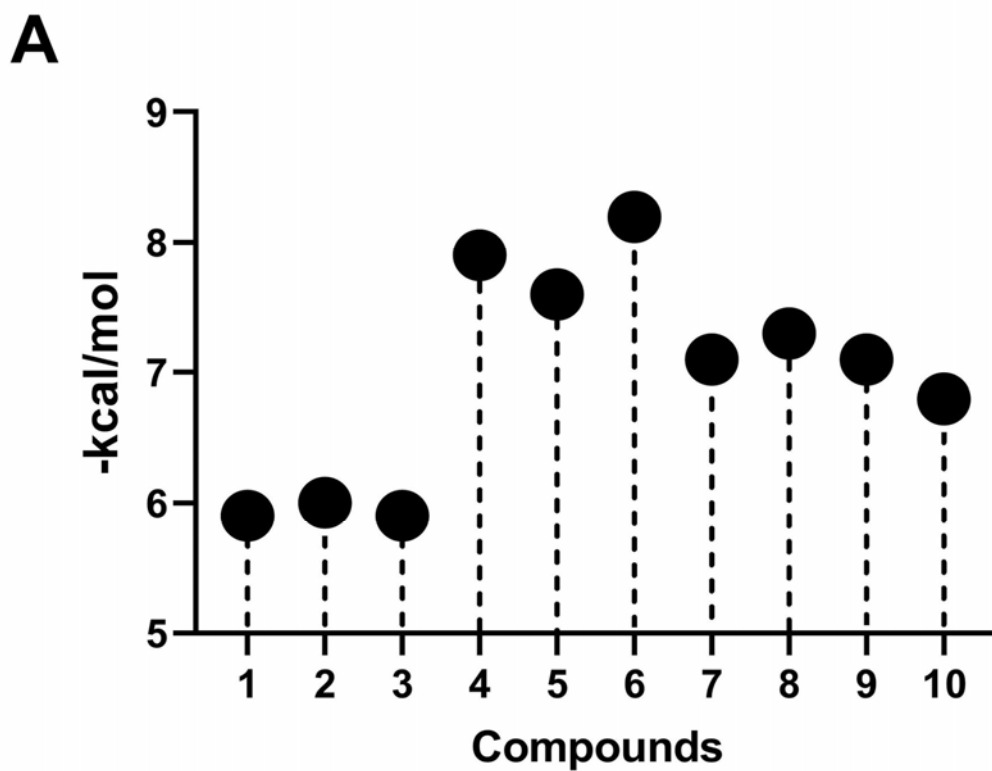
503

504 Fig. 13. Molecular docking of compounds **6** and **7** with iNOS dimer showing the crystal
 505 structure and amino acid residues involved in polar bonding and hydrophobic interactions.



506

507 Fig. 14. Molecular docking of compounds **8**, **9**, and **10** with iNOS showing the crystal structure
 508 and amino acid residues involved in polar bonding and hydrophobic interactions.



509

510 Fig. 15. Lowest binding energy (kcal/mol) of compounds 1-10 with (A) NF- κ B RelA and (B)
 511 iNOS.