Title:

Haloxylon salicornicum Phytochemicals Suppress NF-κB, iNOS and Pro-inflammatory Cytokines in Lipopolysaccharide-Induced Macrophages

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Abstract:

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

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

1. Introduction

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

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

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

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

2. Materials and methods

2.1. Plant collection and isolation of phytochemicals

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

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

2.2. Cells and treatments

RAW 264.7 murine macrophages (VACSERA, Egypt) were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (Gibco/BRL) at 37°C and under 5% CO_2. The cells were seeded in a 96-well plate (1×10^5 cells/mL) in DMEM and allowed to adhere for 5 hours. The cells were treated with 5 and 10 µg/ml of compounds 1-10 for 1 h and then challenged with 1 µg/ml LPS from *Escherichia coli* (Sigma, USA) for 24 h. The medium and cells were collected for analysis.

2.3. qRT-PCR

The harvested cells were washed in cold PBS and Trizol reagent (ThermoFisher Scientific, USA) was used for the isolation of RNA. Following its quantification, RNA with OD260/280 ≥ 1.8 were used for the synthesis of cDNA. Amplification of cDNA was achieved using SYBR Green Master Mix (ThermoFisher Scientific, USA) and the following primers: NF-κB p65 F:5'CTATGTGTGCAGACGAAGCC3' and R:5' AGACCGAGGACTAGGCAGAC3' (NM_001402548.1; amplicon size: 91 bp); iNOS F:5'GCCCAGCCAGCCCAAC3' and R:5'GCAGCTTGTCCAGGGATTCT3' (NM_001313922.1; amplicon size: 108 bp), and β-actin F:5'GTGCTATGTTGCTCTAGACTTCG3' and R:5'ATGCCACAGGATTCCATACC3' (NM_007393.5; amplicon size: 174 bp). The obtained data were analyzed using the $2^{-\Delta\Delta Ct}$ method [13].

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

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

2.5. Molecular docking

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

2.6. Statistical analysis

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

3. Results

3.1. *H. salicornicum* phytochemicals downregulate NF-κB p65 in LPS-challenged macrophages

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

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

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

with 5 µg/ml of compounds **1-10** remarkably decreased iNOS mRNA (fold change: 2.26 ± 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, 2.59 ± 0.50, and 2.085 ± 0.54, respectively) (Fig. 3B-K). The effect of compounds **3**, **6**, **9**, and **10** on iNOS was significant ($P<0.05$) as compared to the control cells. The higher concentration of compounds **1-10** resulted in more potent suppression effect on iNOS mRNA abundance (fold 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 ± 0.17, 1.99 ± 0.24, 1.87 ± 0.26, and 1.85 ± 0.19, respectively) (Fig. 3B-K).

NO levels produced by LPS challenged cells (40.62 ± 7.59 µM) was significantly ($P<0.001$) higher than the control cells (4.47 ± 0.81 µM) as shown in Figure 4A. Treatment with 5 µg/ml of compounds 1-10 significantly reduced NO release by LPS-challenged cells (27.07 ± 4.53, 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 ± 2.37, 21.48 ± 1.98, and 25.03 ± 2.47 µM, respectively) (Fig. 4B-K). The 10 µg/ml of compounds **4**, **5**, and **6** showed a concentration dependent effect on NO levels (8.39 ± 1.70, 7.96 ± 1.05, and 13.83 ± 2.15 µM, respectively) (Fig. 4E-G). At the same concentration, compounds **1**, **2**, **3**, **7**, **8**, **9**, and **10** ameliorated NO significantly ($P<0.001$) and the observed 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, and 16.77 ± 1.20 µM, respectively (Fig. 4E-G).

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

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

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

Similar to TNF-α, IL-6 release from LPS-challenged cells (55.04 ± 7.93 pg/ml) was significantly higher than the control cells (10.50 ± 1.81 pg/ml) as shown in Figure 6A ($P<0.001$). Compounds **1**-**10** significantly reduced IL-6 levels when supplemented at 5 µg/ml with reported levels of 23.52 ± 3.02, 19.47 ± 1.55, 30.61 ± 5.44, 19.03 ± 1.27, 19.97 ± 1.86, 18.83 ± 2.52, 21.23 ± 2.57, 18.73 ± 2.45, 30.03 ± 5.08, and 24.43 ± 2.87 pg/ml, respectively (Fig. 5B-K). Despite non-significant as compared to the lower concentration, 10 µg/ml of compounds **1**-**10** remarkably ($P<0.001$) reduced IL-6 release (20.07 ± 3.28, 13.63 ± 1.79, 23.59 ± 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 21.13 ± 1.75 pg/ml, respectively) as depicted in Figure 5B-K.

3.4. *H. salicornicum* phytochemicals exhibit binding affinity toward NF-κB and iNOS

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

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

4. Discussion

H. salicornicum has been traditionally used in the treatment of disorders associated with inflammation such as sepsis, diabetes and tuberculosis [10]. The antibacterial efficacy of its ethanolic extract was reported [10b] and our recent work revealed the suppressing effects of its methanolic and ethanolic extracts on CIS-induced liver and kidney inflammation and toxicity [8]. In this study, the effects of ten phytochemicals isolated from the ethanolic extract of *H. salicornicum* on NF-κB and iNOS expression, and the release of NO and pro-inflammatory cytokines by LPS-induced macrophages were investigated. In addition, the binding affinity of the isolated phytochemicals with NF-κB and iNOS was explored using molecular docking.

The data revealed the inhibitory efficacy of the isolated compounds on NO, TNF-α, and IL-6 by LPS-challenged macrophages. These effects were associated with decreased NF-κB and iNOS mRNA abundance. Inhibition of NF-κB represents an effective strategy for the attenuation of dysregulated inflammation and its consequent disorders. NF-κB is a transcription

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

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

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

304 flavonoid compounds possess the ability to suppress LPS-induced NF-κB upregulation.
305 Flavonoids are known of their ability to suppress NF-κB and inflammatory responses
306 associated with different disorders [7b, 8b, 29].
307 Owing to the ability of compounds **1**-**10** to downregulate NF-κB p65 and the controlling effect
308 of NF-κB 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-κB 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-κB [20-23]. In a study on colitis, aesculetin decreased NO, TNF-α, and IL-6

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

5. Conclusion

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

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

Conflict of Interest

The authors declare no conflict of interest.

Availability of data and materials

The manuscript contains all data supporting the reported results.

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Authors' Contributions:

Conceptualization: A.M.M.; Methodology: R.S.A., O.E.H., S.M.A., I.E., M.A.A., M.E., 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: A.M.M., O.E.H., and M.E.; Formal analysis: A.M.M., and E.M.K.; Resources: S.M.A., I.E., M.A.A. and R.S.A.; Supervision: A.M.M.; Writing-Original draft: A.M.M. and O.E.H.; Writing-review and editing: A.M.M.

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Tables:

Table 1. Binding affinities of *H. salicornicum* phytoconstituents towards NF-κB.

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

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

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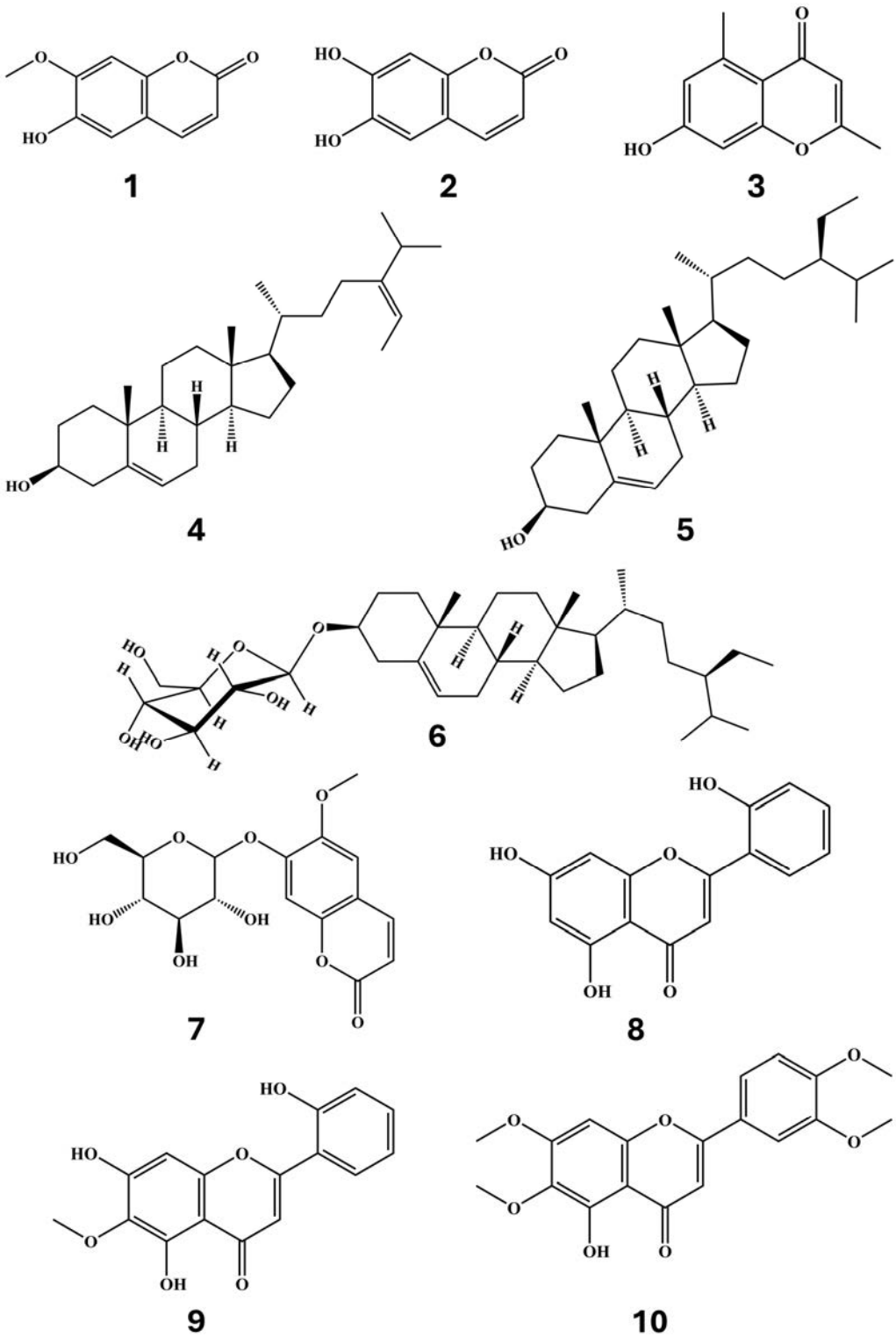
461 Figures:



462

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

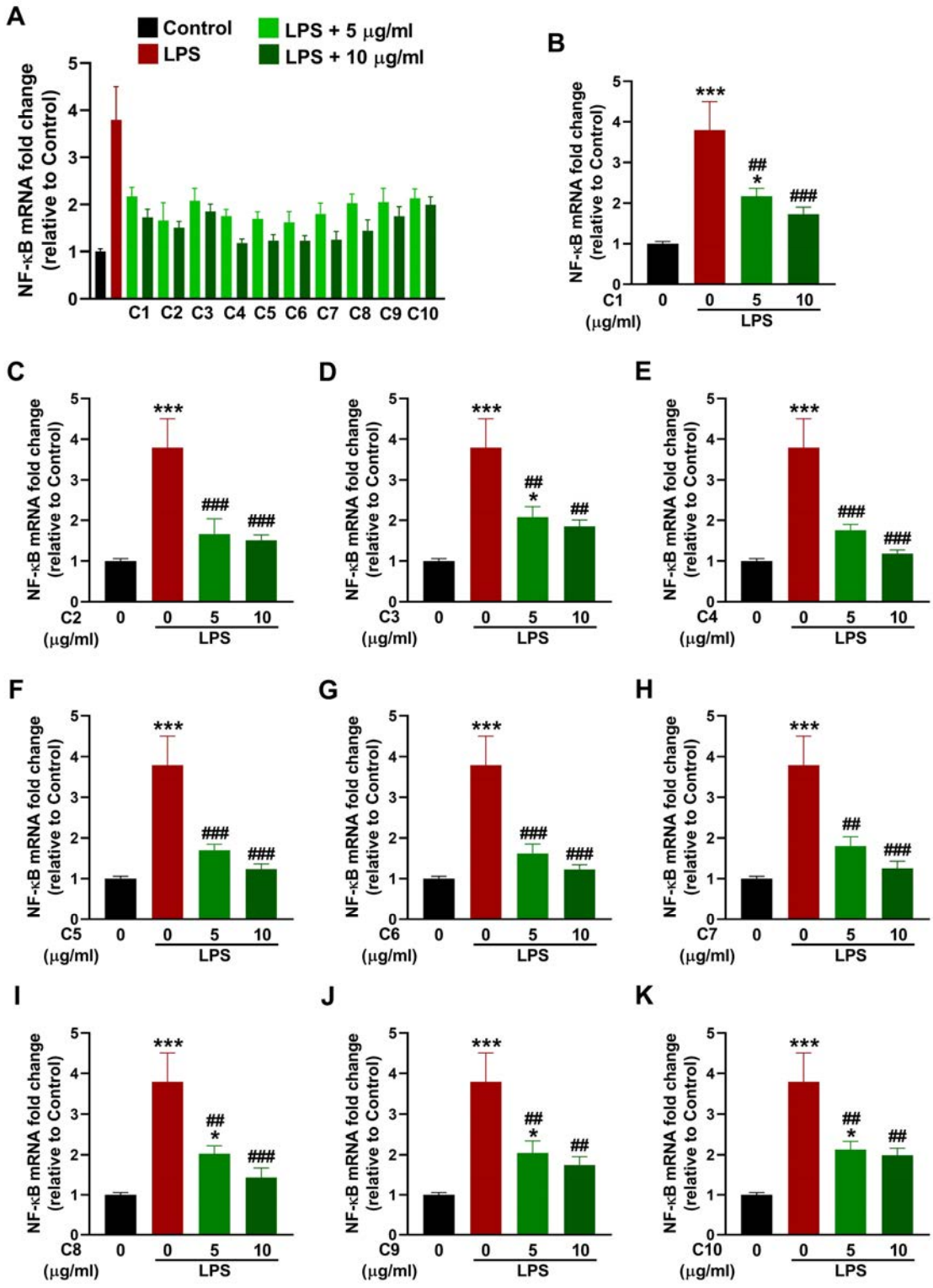


Fig. 2. Effect of compounds **1-10** on NF-κB p65 mRNA abundance in LPS-induced macrophages. Data are Mean ± SD, (N = 3). *P<0.05 and ***P<0.001 vs Control. $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs LPS.

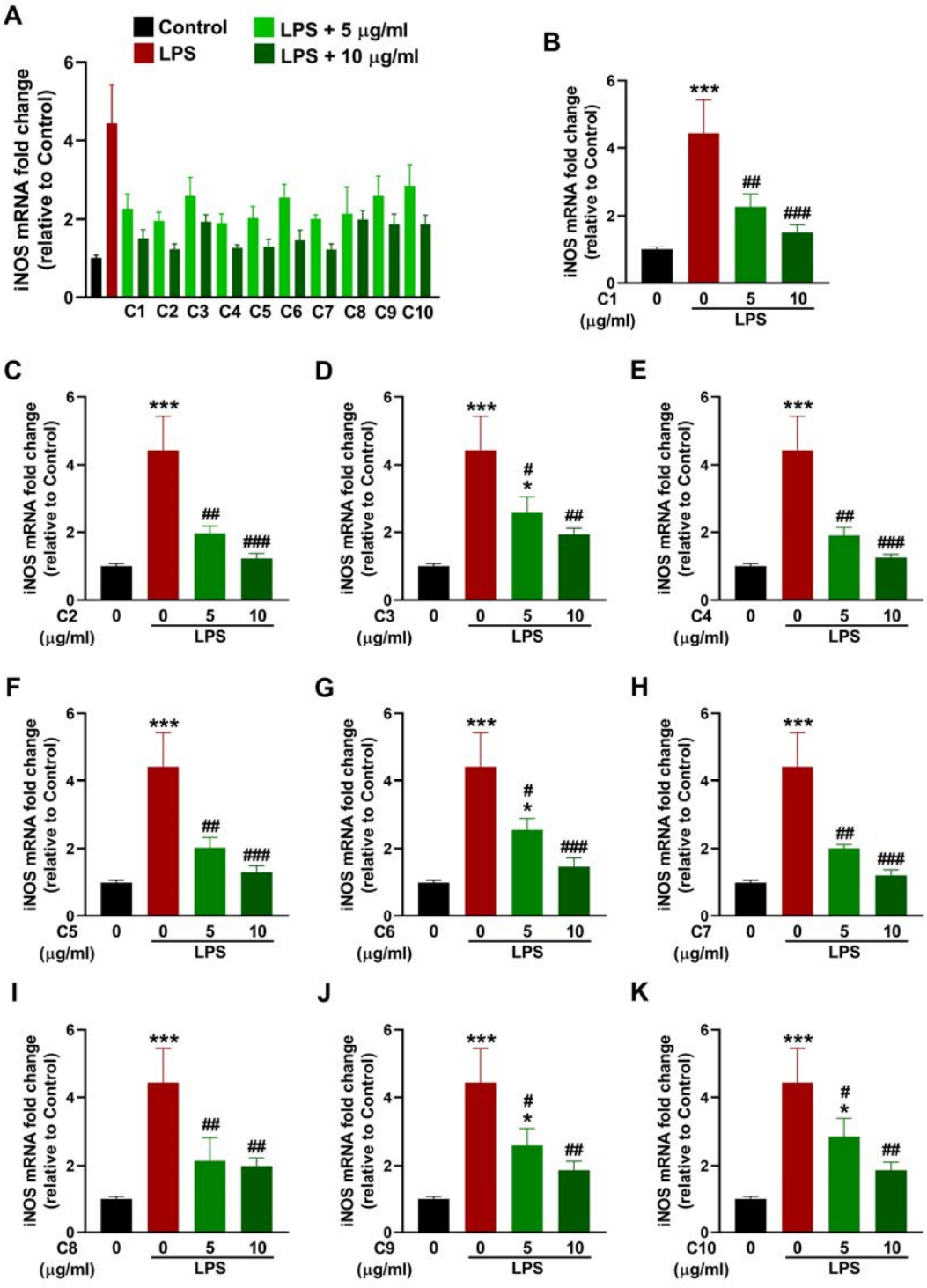
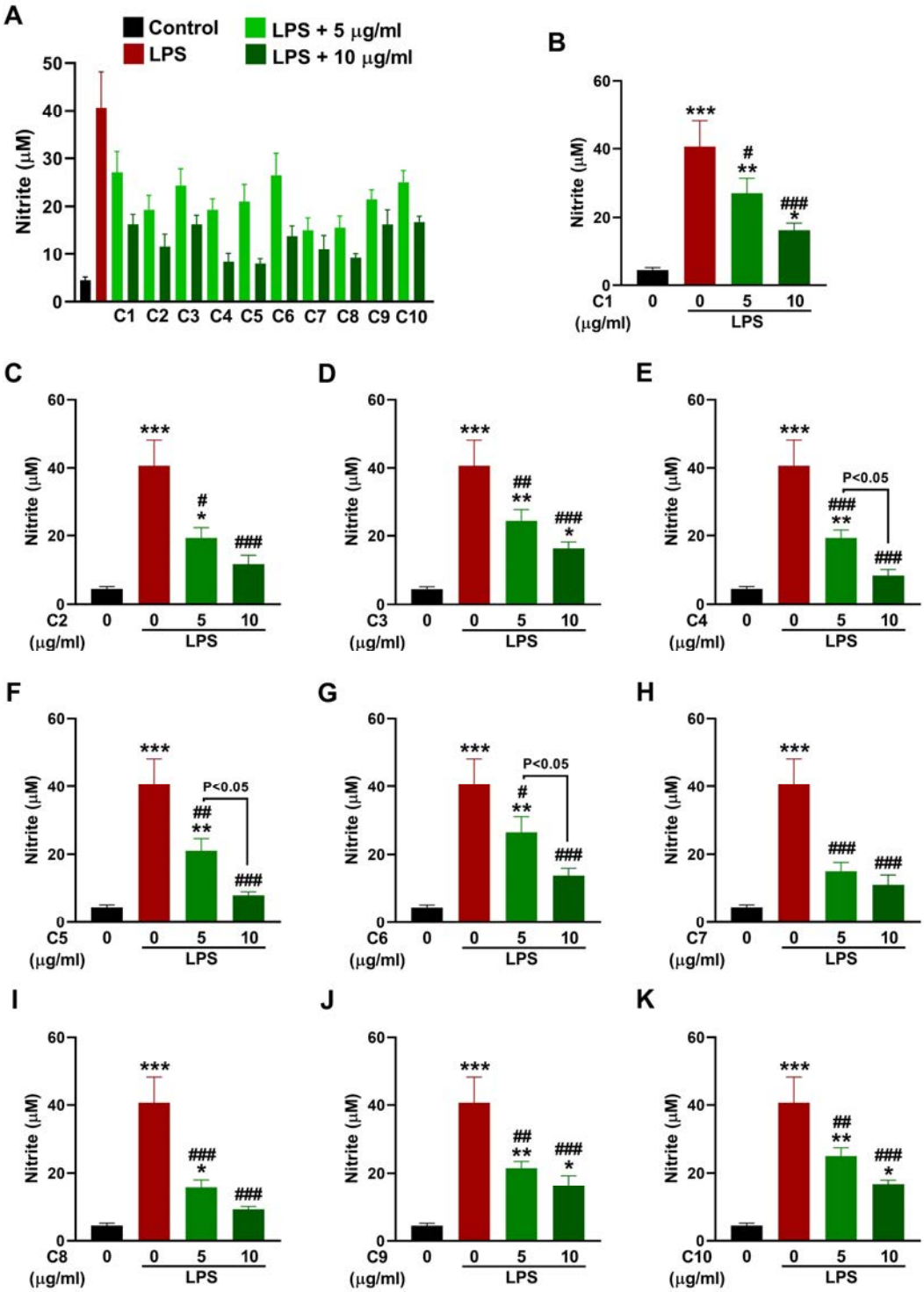


Fig. 3. Effect of compounds **1**-**10** on iNOS mRNA abundance in LPS-induced macrophages. Data are Mean ± SD, (N = 3). *P<0.05 and ***P<0.001 vs Control. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs LPS.



472

473 Fig. 4. Effect of compounds **1**-**10** on NO production by LPS-induced macrophages. Data are
474 Mean ± 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.

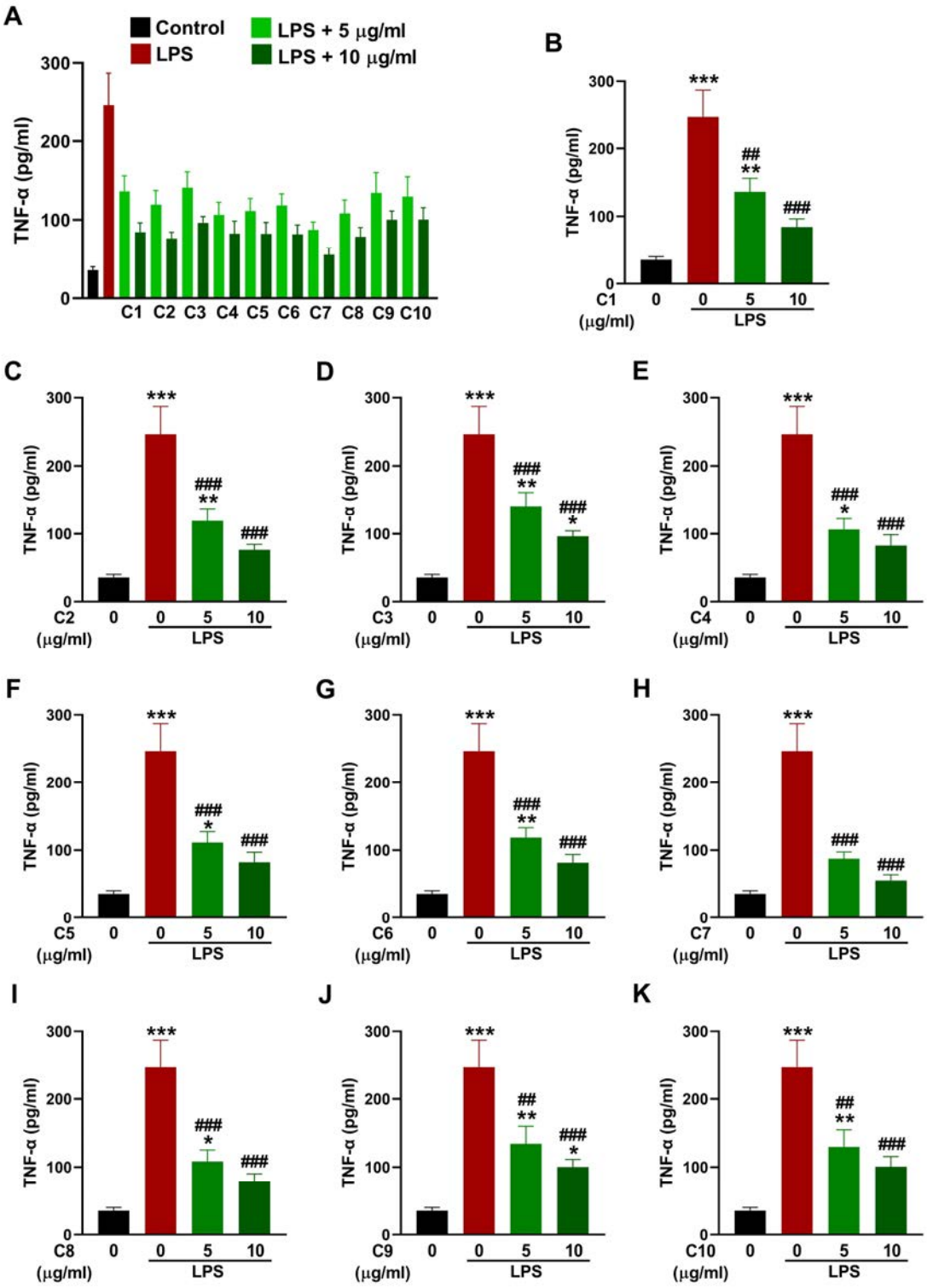
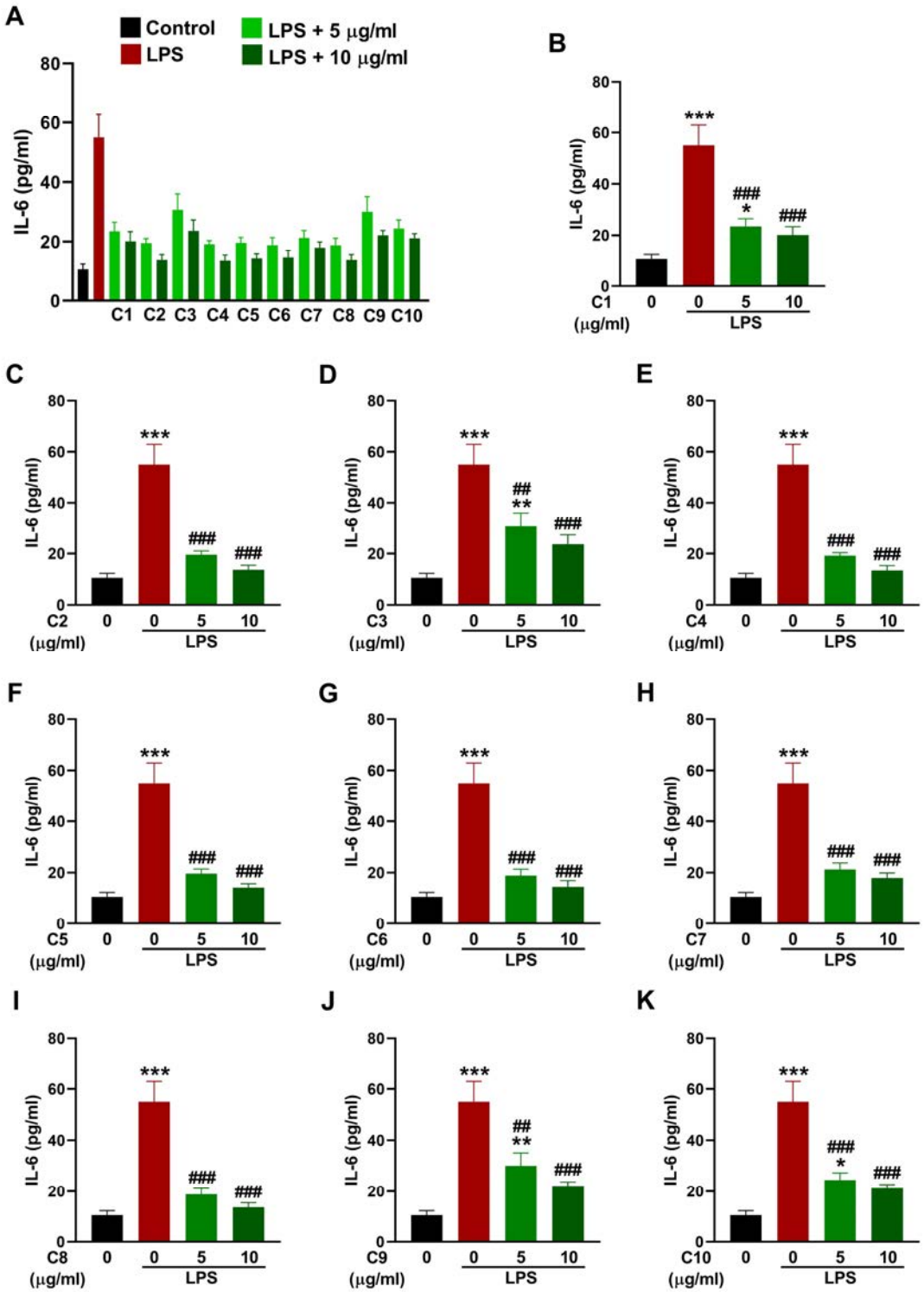


Fig. 5. Effect of compounds **1-10** on TNF-α release by LPS-induced macrophages. Data are Mean ± SD, (N = 3). *P<0.05, **P<0.01 and ***P<0.001 vs Control. $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs LPS.



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

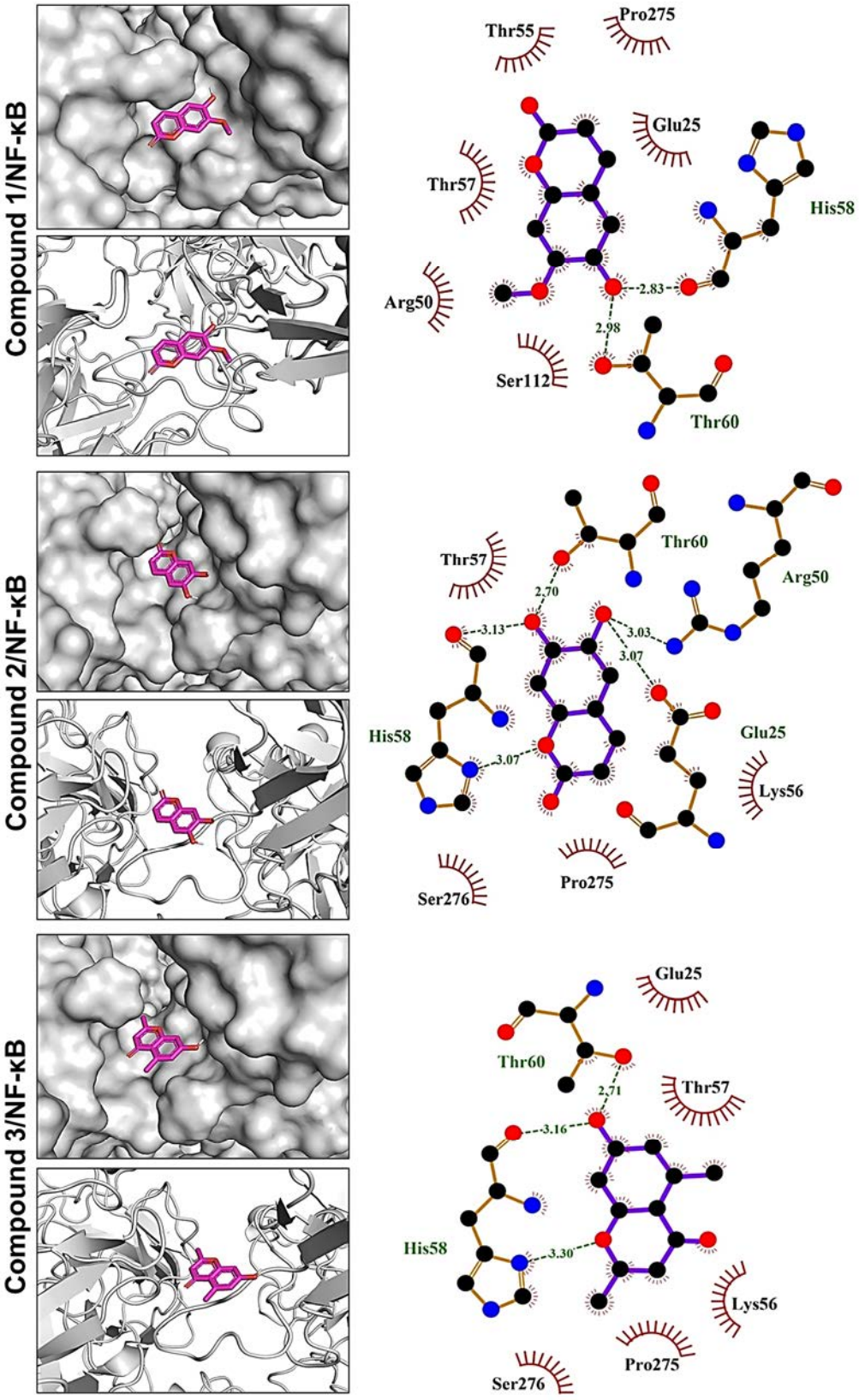


Fig. 7. Molecular docking of compounds **1**, **2**, and **3** with NF-κB RelA dimer showing the crystal structure and amino acid residues involved in polar bonding and hydrophobic interactions.

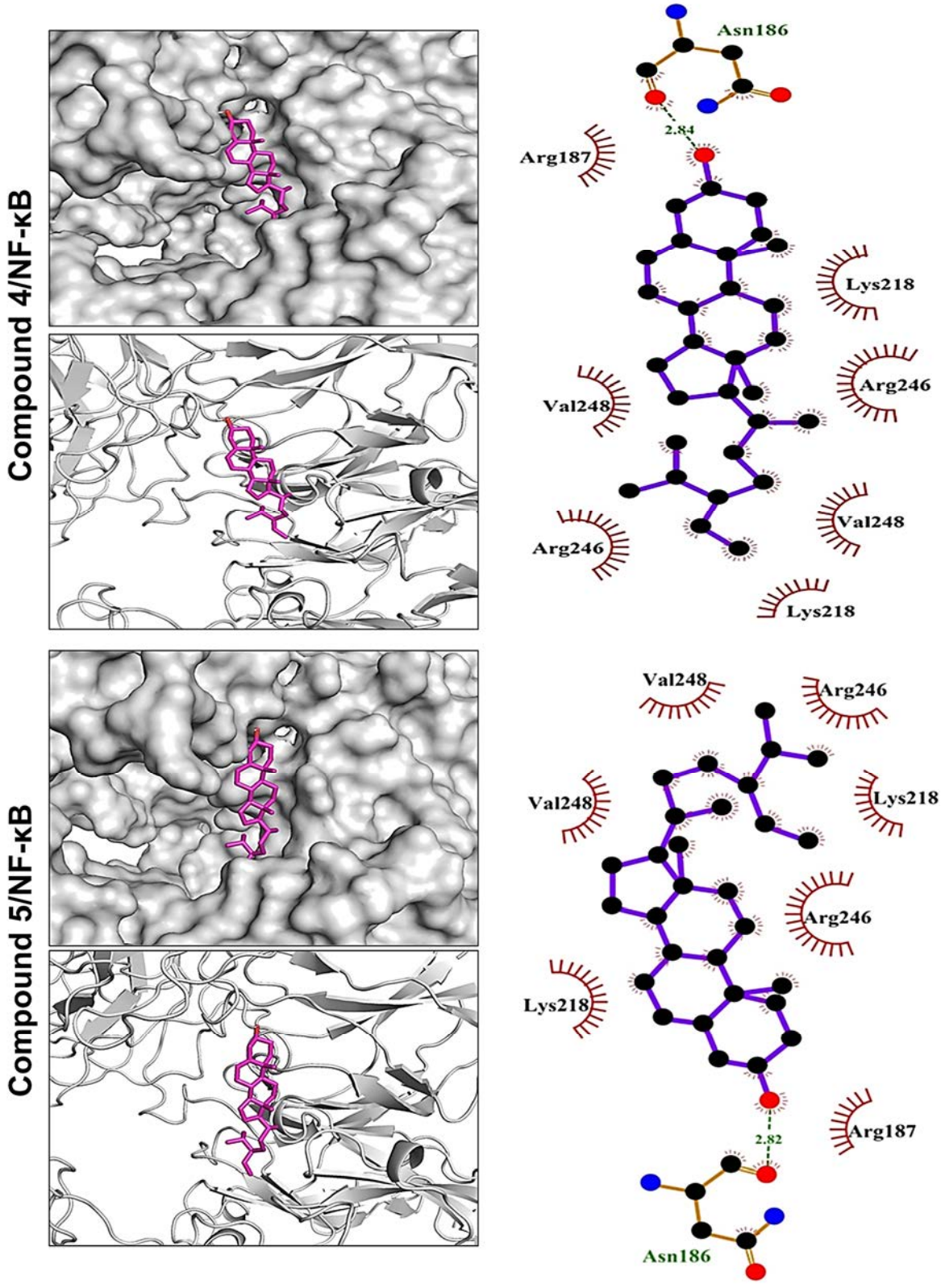


Fig. 8. Molecular docking of compounds **4** and **5** with NF-κB RelA dimer showing the crystal structure and amino acid residues involved in polar bonding and hydrophobic interactions.

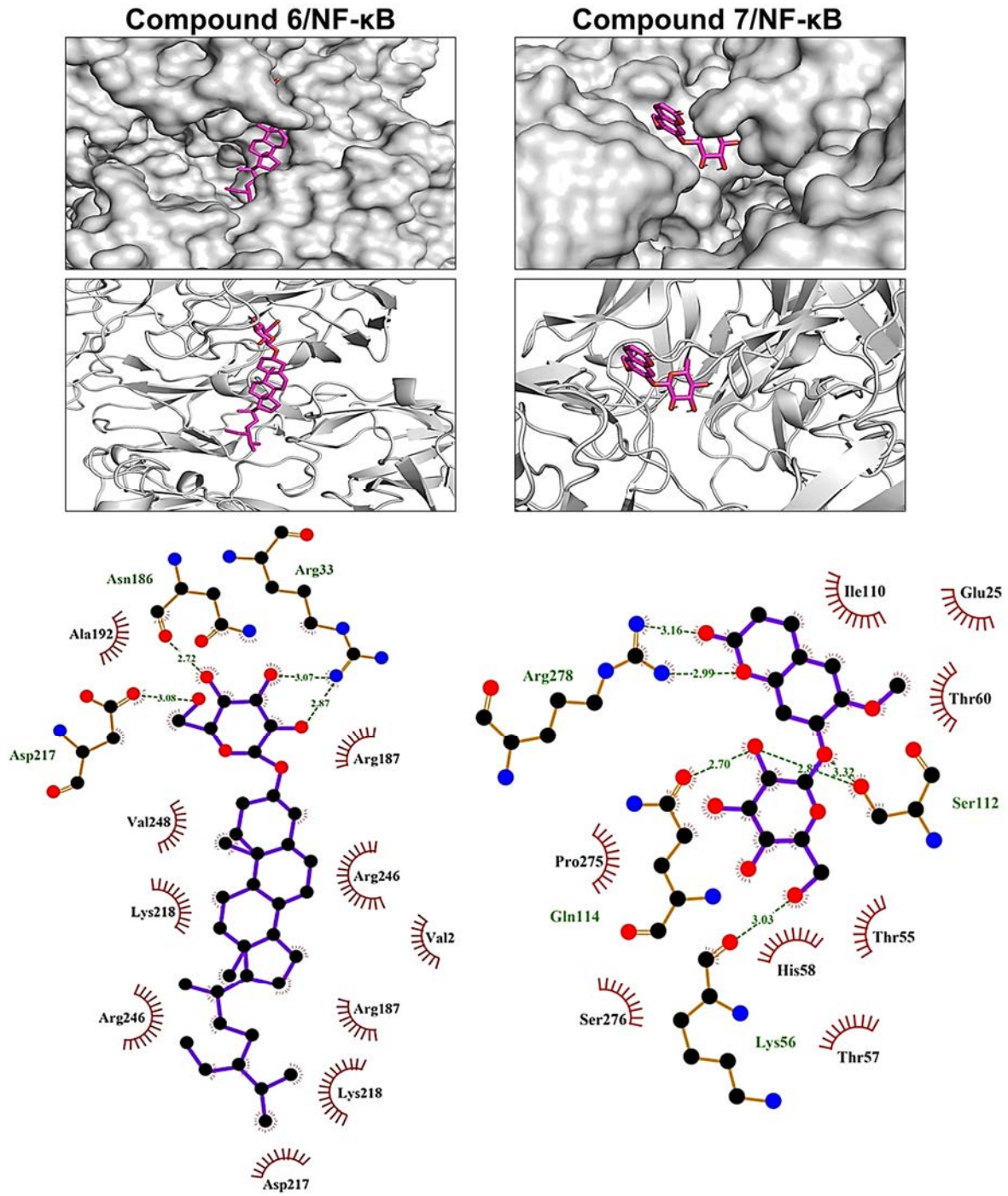


Fig. 9. Molecular docking of compounds **6** and **7** with NF-κB RelA dimer showing the crystal structure and amino acid residues involved in polar bonding and hydrophobic interactions.

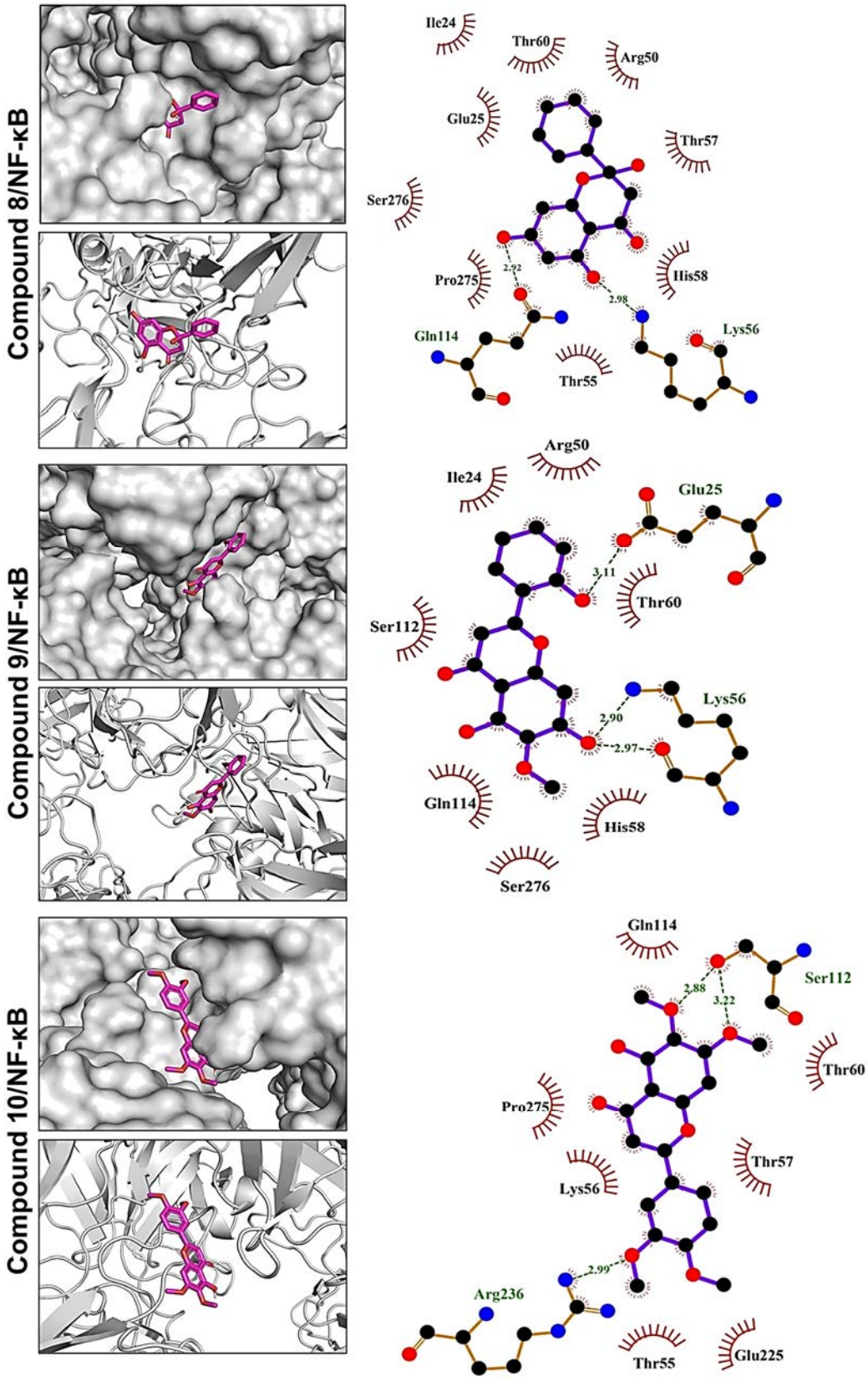


Fig. 10. Molecular docking of compounds **8**, **9**, and **10** with NF-κB RelA dimer showing the crystal structure and amino acid residues involved in polar bonding and hydrophobic interactions.

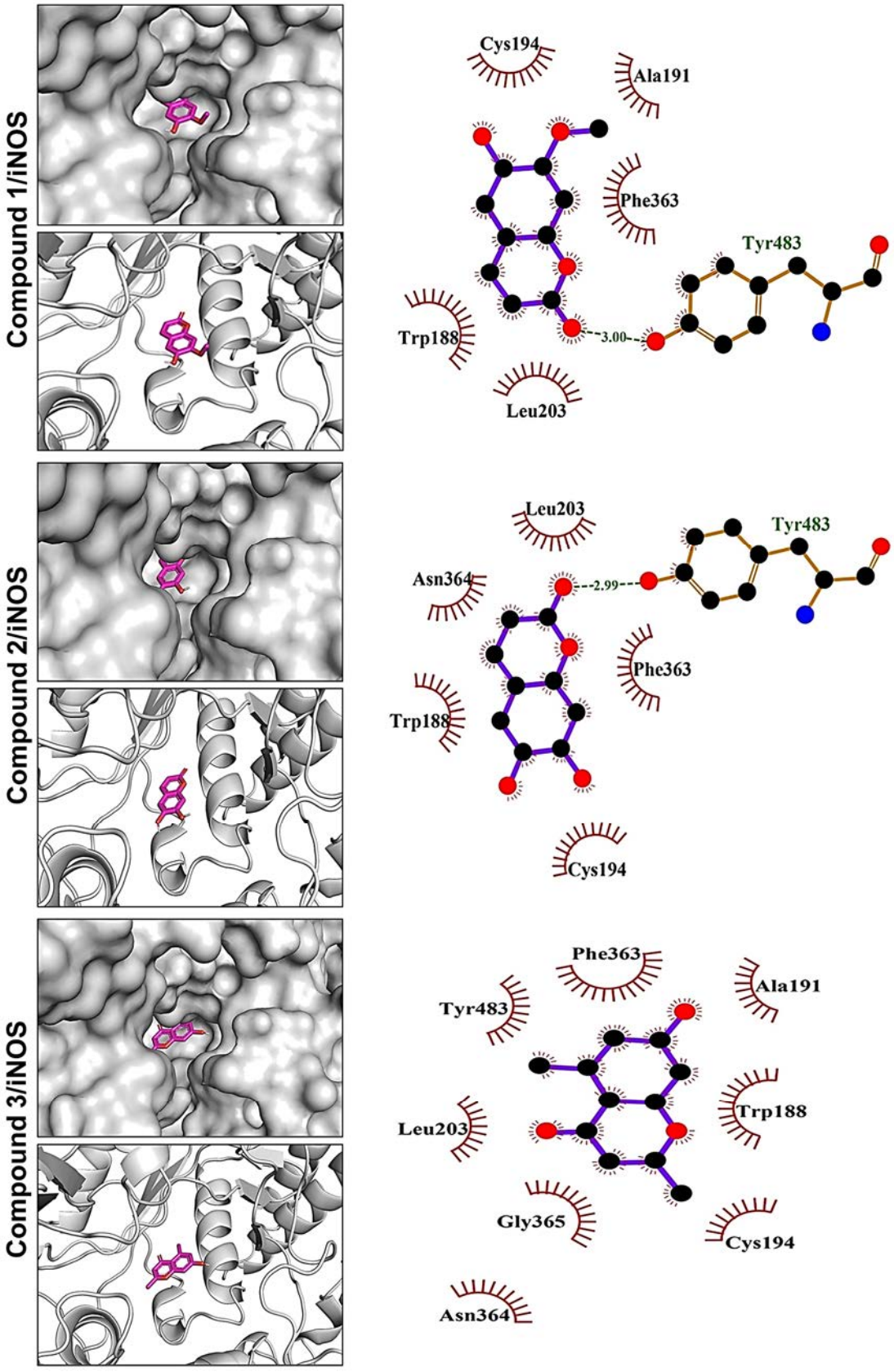


Fig. 11. Molecular docking of compounds **1**, **2**, and **3** with iNOS showing the crystal structure and amino acid residues involved in polar bonding and hydrophobic interactions.

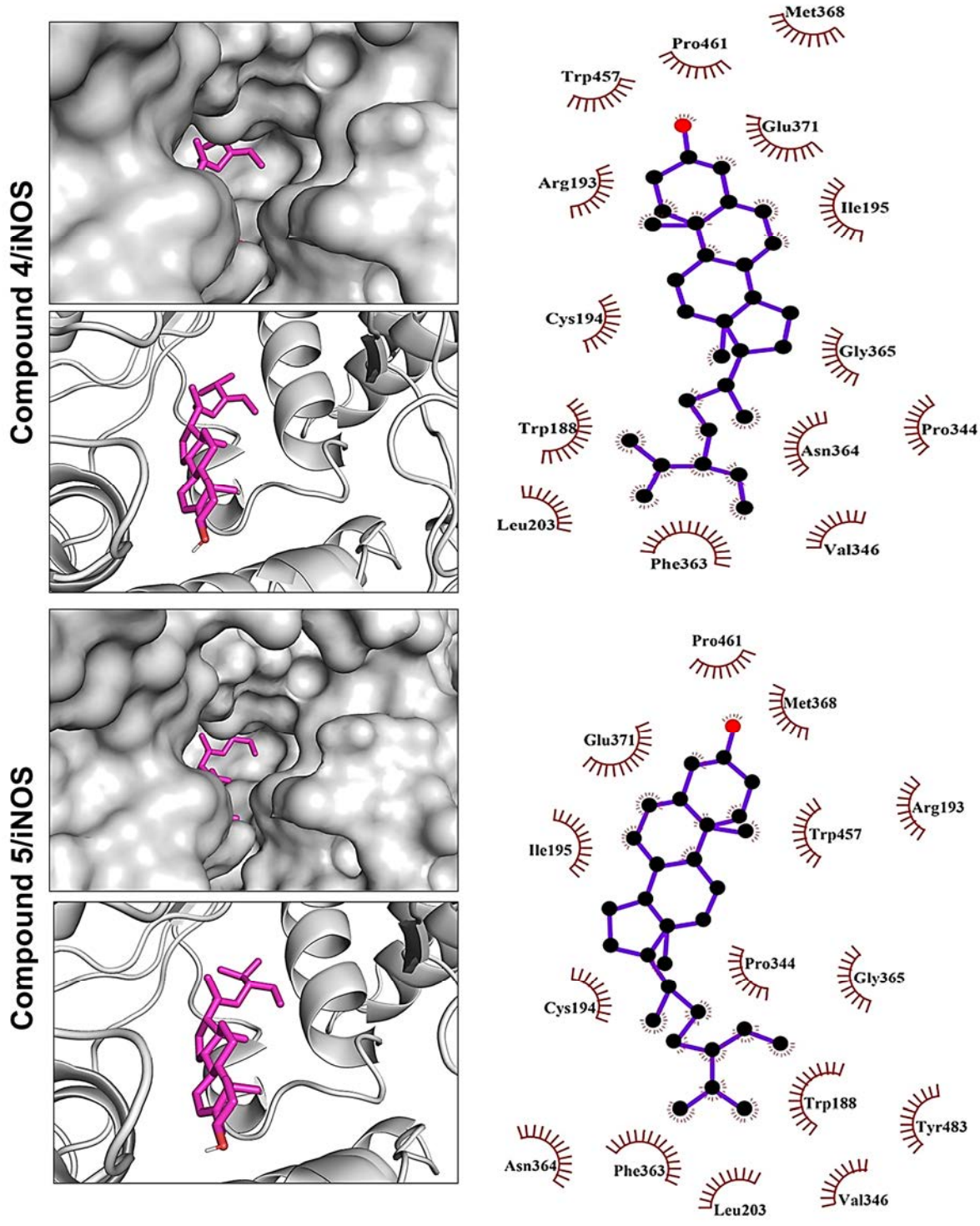
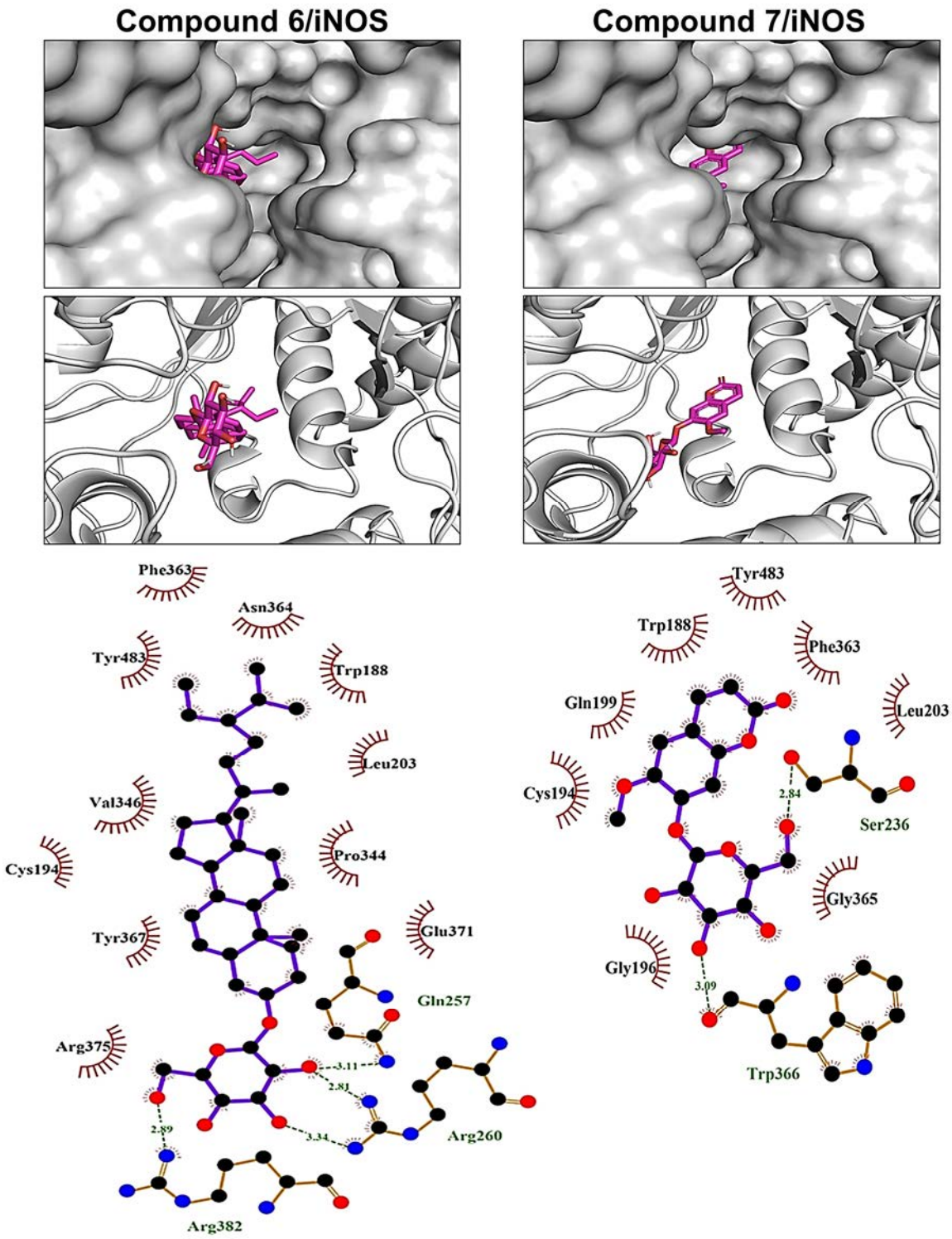
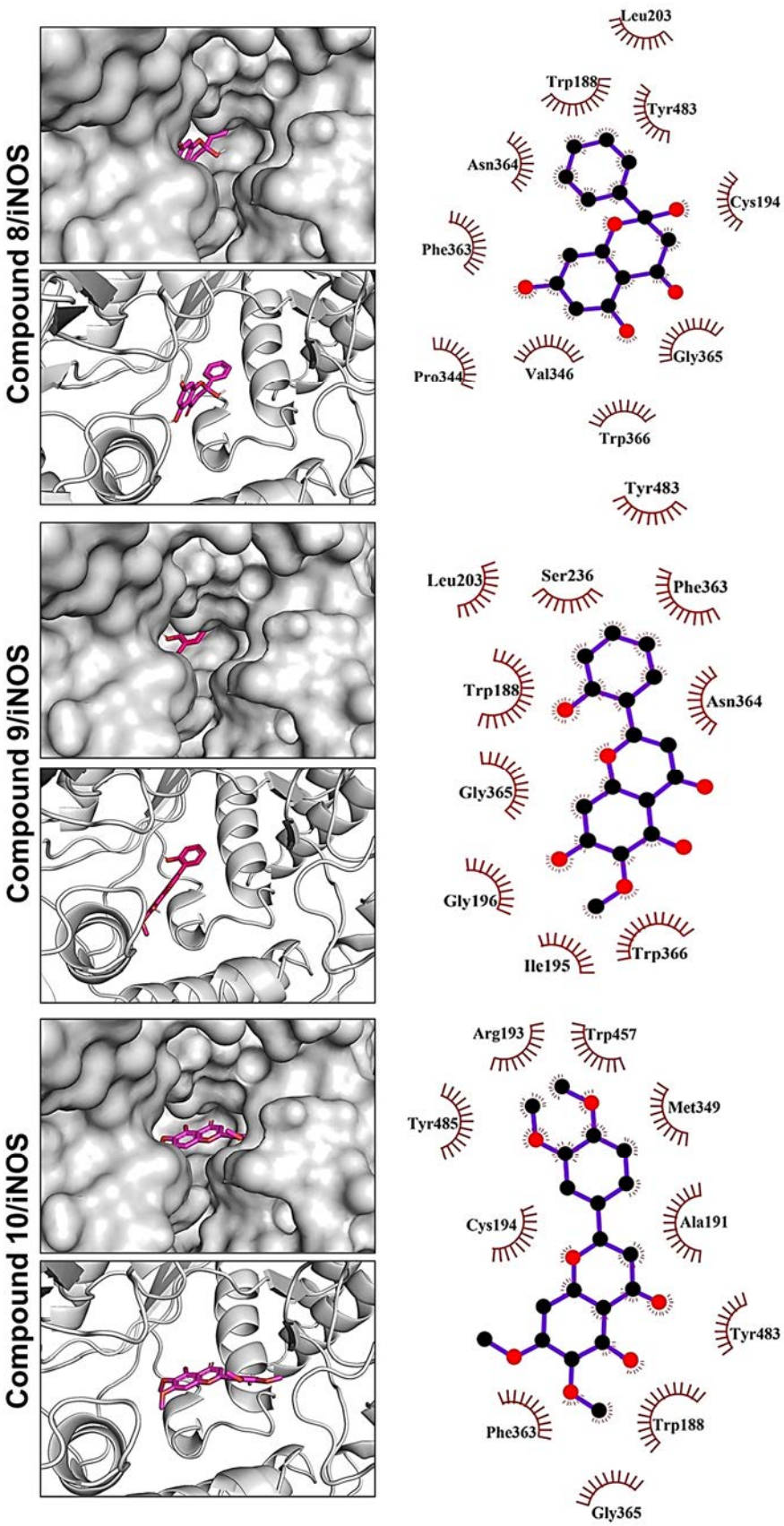


Fig. 12. Molecular docking of compounds **4** and **5** with iNOS showing the crystal structure and amino acid residues involved in polar bonding and hydrophobic interactions.



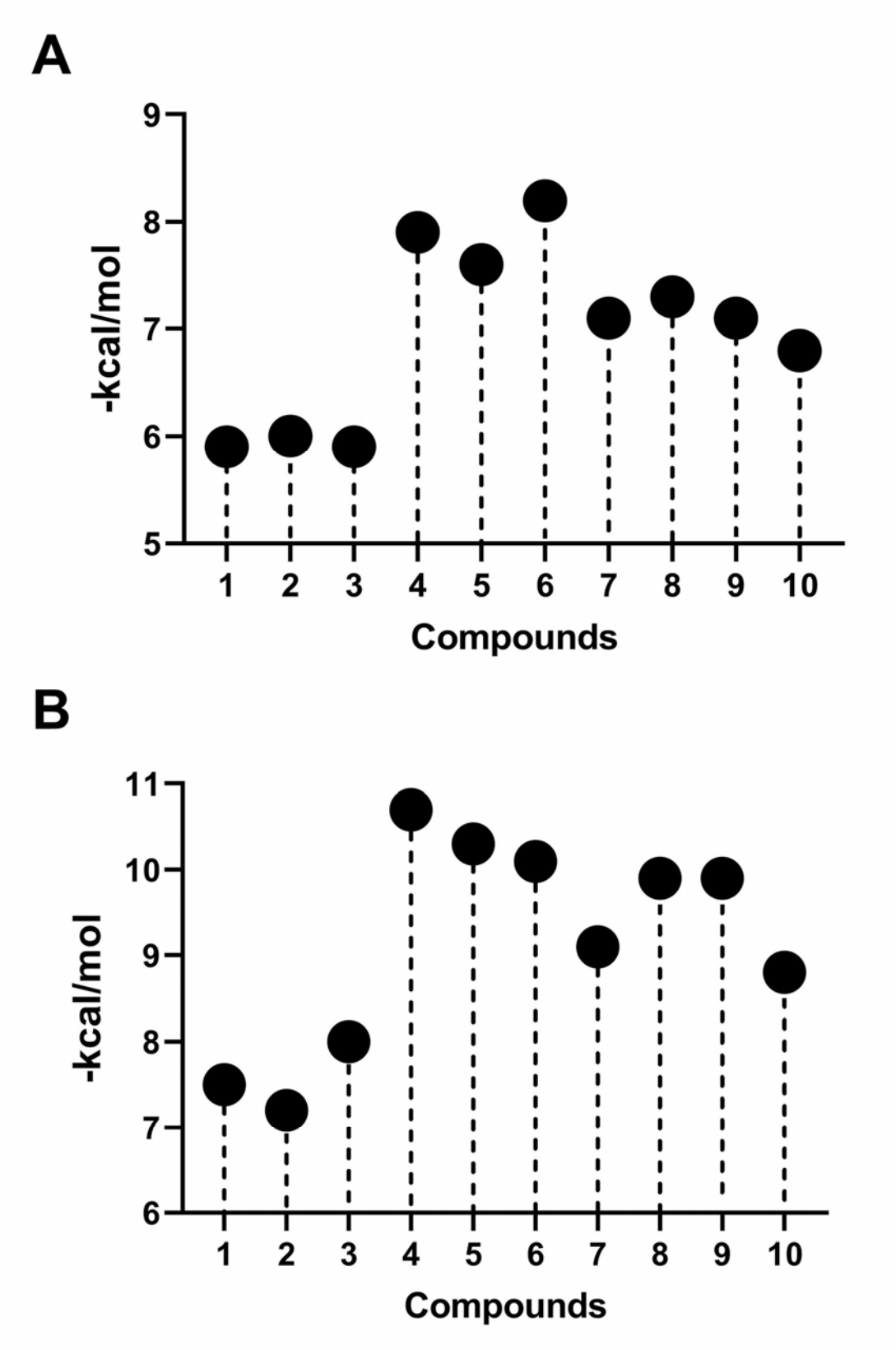
503

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-κB RelA and (B)
511 iNOS.