

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 (2025) Haloxylon salicornicum Phytochemicals Suppress NF-kB, iNOS and Pro-inflammatory Cytokines in Lipopolysaccharide-Induced Macrophages. Chemistry and Biodiversity, 22 (2). 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: (cc) BY

Creative Commons: Attribution 4.0

Additional Information: This is an author-produced version of the published paper. Uploaded in accordance with the University's Research Publications Policy.

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) **Title:**

2	Haloxylon salicornicum Phytochemicals Suppress NF-KB, iNOS and Pro-inflammatory
3	Cytokines in Lipopolysaccharide-Induced Macrophages
4	Authors and affiliations:
5	Reem S. Alruhaimi ¹ , Omnia E Hussein ² , Sulaiman M. Alnasser ³ , Ibrahim Elbagory ⁴ ,
6	Mohammed A. Alzoghaibi ⁵ , Emadeldin M. Kamel ⁶ , Mohamed El Mohtadi ⁷ , Ayman M.
7	Mahmoud ⁸ *
8	¹ Department of Biology, College of Science, Princess Nourah bint Abdulrahman University,
9	Riyadh 11671, Saudi Arabia.
10	² Higher Technological Institute for Applied Health Sciences, Beni-Suef, Egypt.
11	³ Department of Pharmacology and Toxicology, College of Pharmacy, Qassim University,
12	Qassim 51452, Saudi Arabia.
13	⁴ Department of Pharmaceutics, Faculty of Pharmacy, Northern Border University, Rafha
14	76321, Saudi Arabia.
15	⁵ Physiology Department, College of Medicine, King Saud University, Riyadh, 11461, Saudi
16	Arabia.
17	⁶ Organic Chemistry Department, Faculty of Science, Beni-Suef University, Beni-Suef 62514,
18	Egypt.
19	⁷ Department of Biology, Edge Hill University, Ormskirk L39 4QP, UK.
20	⁸ Department 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: <u>a.mahmoud@mmu.ac.uk</u>

31 Abstract:

Haloxylon salicornicum is traditionally used for the treatment of several disorders associated 32 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 investigated the effect of *H. salicornicum* phytochemicals nuclear factor-kappaB (NF-κB), 35 inducible nitric oxide synthase (iNOS) and cytokines release by lipopolysaccharide (LPS)-36 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*. Treatment of LPS-challenged macrophages with the compounds resulted in remarkable 40 decrease in NF-KB p65 and iNOS mRNA abundance. All compounds suppressed the 41 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 revealed the ability of the isolated phytochemicals to bind NF-kB p65 and iNOS. In conclusion, 44 45 H. salicornicum is a rich source of phytochemicals with anti-inflammatory properties. The antiinflammatory efficacy of H. salicornicum phytoconstituents is mediated via their ability to 46 47 modulate NF-kB and iNOS, and suppress the release of NO, TNF-a, and IL-6 from macrophages. 48

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 cell proliferation at the injury site ^[1]. Inflammation can become a chronic condition in the case 57 of persistent cause or failure of the control mechanisms. This chronic condition can promote 58 cell proliferation and mutations and may finally result in cancer^[2]. In addition, chronic low-59 grade inflammation, known as inflammaging, has recently been acknowledged as a key 60 contributor to several disorders associated with aging ^[3]. Inflammaging is a mild inflammation 61 62 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. 63 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 organisms and foreign elements prior to the migration of leukocytes ^[4]. Macrophages display 66 67 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]. 68 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 the pro-inflammatory cytokines IL-6 and tumor necrosis factor (TNF)-α, and nitric oxide (NO) 73 generated by inducible NO synthase (iNOS)^[1]. Lipopolysaccharide (LPS) triggers the 74 75 expression of iNOS and inflammation induced by LPS is a central defense mechanism against bacterial infection ^[6]. Activation of nuclear factor- κ B (NF- κ B) is essential for the release of 76 inflammatory mediators and upregulation of iNOS^[1]. NF-kB regulates the expression of IL-6, 77 TNF- α , iNOS, and other mediators involved in inflammation ^[1]. NF- κ B could be activated by 78 several factors, including infections, tissue injury and reactive oxygen species (ROS), resulting 79 80 in the release of different mediators. However, the prolonged and excessive production of proinflammatory 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 85 inflammation in different disorders ^[7]. In this context, we have previously elucidated the 86 87 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* 88 and grows in Egypt and other countries ^[9]. The use of this species in folk medicine has been 89 acknowledged as it has been used for the treatment of sepsis, tuberculosis, diabetes mellitus 90 and other disorders associated with inflammation ^[10]. In an *in vitro* study, Bibi et al ^[10b] 91 92 demonstrated the inhibitory efficacies of H. salicornicum fractions on the growth of Mycobecterium tuberculosis H37Rv. In addition, the antibacterial activity of its alcoholic 93 extract against different strains has been recently reported [11]. The hepatoprotective efficacy of 94 95 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 96 97 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 98 our studies ^[8]. The effect of *H. salicornicum* on LPS-induced inflammatory response has not 99 been studied. This study investigated the effect of ten phytochemicals isolated previously ^[8a] 100 from the ethanolic extract of *H. salicornicum* on NF-KB, iNOS, and cytokine release by LPS-101 102 challenged macrophages.

- 103 **2. Materials and methods**
- 104 **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.: BSUCH2019-089) was archived in the Herbarium of the Faculty of Science (Registration code:
BSU- HERB19089).

110 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 111 112 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 organic layer was evaporated to produce corresponding extracts. The petroleum ether, ethyl 115 116 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 117 118 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'tetramethoxyflavone (10) (Fig. 1)^[8a]. 121

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 µg/ml of compounds 1-10 for 1 h and 127 then challenged with 1 µg/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 OD260/280 \geq 1.8 were used for the synthesis of cDNA. Amplification of cDNA was achieved using SYBR 132 133 Green Master Mix (ThermoFisher Scientific, USA) and the following primers: NF-kB p65 F:5'CTATGTGTGCAGACGAAGCC3' and R:5' AGACCGAGGACTAGGCAGAC3' 134 (NM_001402548.1; amplicon size: 91 bp); iNOS F:5'GCCCAGCCAGCCAAC3' and 135 136 R:5'GCAGCTTGTCCAGGGATTCT3' (NM_001313922.1; amplicon size: 108 bp), and βactin F:5'GTGCTATGTTGCTCTAGACTTCG3' and R:5'ATGCCACAGGATTCCATACC3' 137 (NM_007393.5; amplicon size: 174 bp). The obtained data were analyzed using the $2^{-\Delta\Delta Ct}$ 138 method $^{[13]}$. 139

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**

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.

154 **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.

158 **3. Results**

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

161 Treatment of macrophages with 1 µg/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-KB mRNA (fold 164 change 2.17 \pm 0.19 and 1.73 \pm 0.17 for 5 and 10 μ g/ml, respectively) (Fig. 2B). Compounds 2, 3, and 4 downregulated NF-KB mRNA significantly at 5 and 10 µg/ml (P<0.001, P<0.01 and 165 166 P<0.001, and fold changes at 5 μ g/ml; 1.66 \pm 0.37, 2.08 \pm 0.26 and 1.76 \pm 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 decreased following treatment of the LPS-induced cells with 5 μ g/ml (1.69 \pm 0.15, 1.62 \pm 0.22, 168 169 and 1.82 ± 0.23) and $10 \,\mu$ 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 \pm 0.19, 2.05 \pm 0.28, and 2.13 \pm 0.20) and 10 μ g/ml (1.44 \pm 0.23, 1.75 ± 0.20 , and 1.99 ± 0.17) (Fig. 2I-K). All compounds showed a trend decrease in NF- κ B 172 173 mRNA abundance; however, the dose-dependent effect was non-significant. NF-kB mRNA 174 abundance at the 5 µg/ml concentration of compounds 1, 3, 8, 9, and 10 was significantly higher 175 than the control (P < 0.05).

3.2. *H. salicornicum* phytochemicals downregulate iNOS and nitrite in LPS-challenged 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 μ g/ml of compounds 1-10 remarkably decreased iNOS mRNA (fold change: 2.26 ±

181 $0.37, 1.96 \pm 0.22, 2.59 \pm 0.47, 1.90 \pm 0.23, 2.03 \pm 0.29, 2.55 \pm 0.34, 2.01 \pm 0.11, 2.14 \pm 0.67,$

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 ± 0.10
- 186 $0.17, 1.99 \pm 0.24, 1.87 \pm 0.26$, and 1.85 ± 0.19 , respectively) (Fig. 3B-K).

187 NO levels produced by LPS challenged cells ($40.62 \pm 7.59 \,\mu$ 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 μ g/ml 189 of compounds 1-10 significantly reduced NO release by LPS-challenged cells (27.07 ± 4.53 , 190 $19.32 \pm 2.98, 24.07 \pm 2.28, 19.34 \pm 2.31, 21.05 \pm 3.61, 26.47 \pm 4.60, 15.02 \pm 2.65, 15.63 \pm$ 191 2.37, 21.48 \pm 1.98, and 25.03 \pm 2.47 μ M, respectively) (Fig. 4B-K). The 10 μ 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 $13.83 \pm 2.15 \mu$ 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 LPS198 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**.

211 Similar to TNF- α , IL-6 release from LPS-challenged cells (55.04 \pm 7.93 pg/ml) was significantly higher than the control cells $(10.50 \pm 1.81 \text{ pg/ml})$ as shown in Figure 6A 212 213 (P<0.001). Compounds 1-10 significantly reduced IL-6 levels when supplemented at 5 µg/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 , 18.83 ± 2.52 , 21.23 ± 2.57 , 18.73 ± 2.45 , 30.03 ± 5.08 , and 24.43 ± 2.87 pg/ml, respectively 215 216 (Fig. 5B-K). Despite non-significant as compared to the lower concentration, 10 µg/ml of 217 compounds 1-10 remarkably (P<0.001) reduced IL-6 release $(20.07 \pm 3.28, 13.63 \pm 1.79, 23.59)$ 218 \pm 3.68, 13.41 \pm 1.92, 14.17 \pm 1.53, 14.54 \pm 2.40, 17.98 \pm 1.94, 13.60 \pm 1.81, 22.17 \pm 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-κB and iNOS

221 Molecular docking simulation data represented in Figures 7-10 and Table 1 show the binding affinity of compounds 1-10 with NF-kB RelA. Compounds 1, 2, and 3 (Fig. 7) exhibited lowest 222 223 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). Compounds 1, 2, and 3 exhibited -7.5, -7.2 and -8.0 kcal/mol binding energy with iNOS and 231 232 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-kB RelA, compounds 4, 5, and 6 233 234 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 kcal/mol binding energy and polar binding and hyrdrophobic interactions with 2 and 8 amino 236 237 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-kB and iNOS are 240 represented in Figures 15A and 15B, respectively.

241 **4. Discussion**

H. salicornicum has been traditionally used in the treatment of disorders associated with 242 inflammation such as sepsis, diabetes and tuberculosis ^[10]. The antibacterial efficacy of its 243 ethanolic extract was reported ^[10b] and our recent work revealed the suppressing effects of its 244 245 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. 246 247 salicornicum on NF-kB 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-KB 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 other cellular processes ^[1]. The ability of NF-*κ*B to alter the biology of cells is attributed to the 256 numerous genes it controls ^[1]. In resting cells, NF-κB is localized in the cytoplasm bound to 257 IkB and the first step in its activation is the post-translational modification of IkBs ^[17]. 258 Following activation, NF-KB induces the expression of several genes encoding inflammatory 259 260 mediators and it also plays central roles in the survival and differentiation of innate and inflammatory immune cells ^[18]. Hence, dysregulation of NF-KB activation promotes 261 inflammatory disorders^[18] and its modulation is of valuable therapeutic benefit. In response to 262 various stimuli, including bacterial infection, IkB is ubiquitinated and degraded by proteasome 263 and NF-kB subunit dimers translocate into the nucleus to bind DNA and promote gene 264 expression ^[19]. Macrophages express pattern recognition receptors (PRRs) that detect several 265 microbial components such as LPS and activate NF-kB, resulting in the release of 266 inflammatory mediators ^[17]. 267

268 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 269 270 the expression of NF-κB p65 and exhibited binding affinity with RelA dimer, demonstrating their NF-kB inhibitory activities. All compounds showed a concentration-dependent trend in 271 272 downregulating NF-kB 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 p65 in TNF- α /IFN- γ -treated HaCaT cells *in vitro* ^[20]. Compound **2** (aesculetin) exhibited anti-275 276 inflammatory activities mediated via suppression of NF-kB 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 277 lung injury ^[22] and neuroinflammation ^[23] induced by LPS. Our study introduced new 278

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 compound 4 (fucosterol) was supported by previous studies showing its potent anti-283 inflammatory efficacy. For instance, in a mouse model of acute liver injury (ALI), fucosterol 284 suppressed NF-κB p65 immunostaining ^[24]. It downregulated NF-κB p65 gene expression and 285 inhibited matrix metalloproteinase in UVB-induced HaCaT cells ^[25]. Our *in vitro* findings 286 287 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 interactions. Compounds 5 (β -sitosterol) and 6 (β -sitosterol-3-O- β -D-glucoside) showed 289 290 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 LPS-induced lung epithelial cells ^[26] and reduced neuroinflammation by inhibiting NF-KB in 293 microglial cells ^[27]. Compound 7 (scopolin) remarkably downregulated NF-*k*B p65 in LPS-294 295 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 296 297 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-κB was not investigated ^[28]. Our study introduced new information on the involvement of NF-kB suppression in the anti-299 inflammatory efficacy of scopolin. Compounds 8 (5,7,2'-trihydroxyflavone), 9 (5,7,2'-300 301 trihydroxy-6-methoxyflavone), and 10 (5-hydroxy-6,7,3',4'-tetramethoxyflavone) decreased 302 NF-κB 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-κB p65 and the controlling effect of NF-kB on the expression of iNOS and pro-inflammatory cytokines, all compounds 308 decreased iNOS mRNA and suppressed the release of NO, TNF-α, and IL-6. The activation of 309 310 macrophages with LPS and other stimulants leads to the release of NO and pro-inflammatory cytokines. This is a direct consequence of NF-KB activation ^[18]. NO is one of the pro-311 inflammatory mediators produced via iNOS activation in macrophages and other cells ^[18]. In 312 313 inflammation, the expression of iNOS is upregulated in different cells, including macrophages, microglia, and neutrophils, resulting in the generation of excess NO^[30]. This surplus NO leads 314 315 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 damage ^[30]. TNF- α is pleiotropic cytokine with various effects on the body cells and a key 317 318 regulator of pro-inflammatory responses implicated in several inflammatory and autoimmune disorders ^[31]. TNF- α is involved in the regulation of both acute and chronic inflammation and 319 may cause cell death via apoptosis or necroptosis ^[32]. Likewise, IL-6 is produced in response 320 to tissue damage, infections and other conditions to contribute to the defense mechanism. 321 322 Despite the controlled mechanism of production, prolonged and uncontrolled release of IL-6 is implicated in inflammation and autoimmune disorders ^[33]. Isoscopoletin, aesculetin, and 323 altechromone A (compounds 1-3) significantly downregulated iNOS and suppressed NO, 324 TNF- α , and IL-6 production from LPS-induced macrophages. All three compounds exhibited 325 326 binding towards iNOS with many amino acid residues were observed in the binding mode of all compounds. These findings are directly related to the ability of these compounds to 327 downregulate NF-κB^[20-23]. In a study on colitis, aesculetin decreased NO, TNF-α, and IL-6 328

^[21], suppressed TNF-a and IL-6 in LPS-induced lung injury ^[22] and downregulated iNOS and 329 cytokines in LPS-induced neuroinflammation^[23]. The current study showed for the first time 330 the efficacy of altechromone A to bind with iNOS (in silico) and downregulate (in vitro) iNOS, 331 332 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. 333 334 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 has been reported to downregulate TNF-a, and IL-6 gene expression in a mouse model of ALI 336 ^[24] and β -sitosterol suppressed the generation of these cytokines in LPS-induced lung epithelial 337 cells ^[26] and microglial cells ^[27]. Undaria pinnatifida-derived fucosterol was effective in 338 339 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 hydrophobic interactions with 8 residues of iNOS. The effects of scopolin on these mediators 342 343 is scarcely reported. In a rat model of arthritis, Ervcibe obtusifolia-derived scopolin reduced IL-6^[28]. The isolated flavonoids (compounds 8, 9, and 10) showed inhibitory activities on the 344 345 expression of iNOS and the production of NO, TNF-α, and IL-6, effects that could be explained by their NF-kB inhibition efficacy. All flavonoids showed hydrophobic interactions with ten 346 347 amino acid residues of iNOS. The lack of data showing the protein expression levels of NF-kB 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**

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- κ B 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- κ B 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.

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.
- aM. S. Abduh, R. S. Alruhaimi, H. A. Alghtani, O. E. Hussein, M. H. Abukhalil, E. M. 381 [5] 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. M. Hassanein, M. N. Bin-Jumah, A. M. Mahmoud, Food and Chemical Toxicology 2023, 384 180, 114055; dS. A. Antar, W. Abdo, R. S. Taha, A. E. Farage, L. E. El-Moselhy, M. E. Amer, 385 A. S. Abdel Monsef, A. M. Abdel Hamid, E. M. Kamel, A. F. Ahmeda, A. M. Mahmoud, Life 386 Sci 2022, 291, 120260; eA. M. Mahmoud, O. A. M. Abd El-Ghafar, M. A. Alzoghaibi, E. H. 387 388 M. Hassanein, Life Sci 2021, 278, 119600.

389	[6]	A. Płóciennikowska, A. Hromada-Judycka, K. Borzęcka, K. Kwiatkowska, <i>Cell Mol Life Sci</i>
390	[-]]	2015 , $/2$, 557-581.
391	[/]	am. S. Addun, M. A. Alzognaidi, A. M. Alzognaidi, A. Bin-Ammar, M. F. Alotaidi, E. M.
392		Kamel, A. M. Manmoud, <i>Life Sci</i> 2023 , 121612; bR. S. Alrunaimi, G. Mostata-Hedeab, M.
393		S. Abduh, A. Bin-Ammar, E. H. M. Hassanein, E. M. Kamel, A. M. Mahmoud, Front
394		<i>Pharmacol</i> 2023 , <i>14</i> , 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, <i>Chemistry Africa</i> 2023 , <i>6</i> , 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, <i>Saudi Pharm J</i> 2023 , <i>31</i> , 101766; bS. A.
404		Ramadan, E. M. Kamel, M. A. Ewais, A. A. Khowailed, E. H. M. Hassanein, A. M.
405		Mahmoud, <i>Environ Sci Pollut Res Int</i> 2023 , <i>30</i> , 49197-49214.
406	[9]	L. Boulos, <i>Flora of egypt, Vol. 4</i> , 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. Siddigi, Y. Zhang, S. U. Kazmi, A. J. B. Malik, m. c. letters,
409		2010 , <i>20</i> , 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-Regaiev, K. A. Qureshi,
412		Antibiotics (Basel) 2023. 12.
413	[12]	M. Ahmad. S. Fram. <i>Pak J Pharm Sci</i> 2011 , <i>24</i> , 377-382.
414	[13]	K. J. Livak, T. D. Schmittgen, <i>Methods (San Diego, Calif.</i>) 2001 , <i>25</i> , 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 , <i>126</i> , 131-138.
417	[15]	S Dallakvan A Olson Methods Mol Biol 2015 , 1263, 243-250
418	[16]	A. C. Wallace, R. A. Laskowski, J. M. Thornton, <i>Protein Eng</i> 1995 , <i>8</i> , 127-134.
419	[17]	M. S. Havden, S. Ghosh, <i>Cell</i> 2008 , <i>132</i> , 344-362.
420	[18]	T. Liu, L. Zhang, D. Joo, SC. Sun, Signal Transduction and Targeted Therapy 2017 , 2
421	[.0]	17023
422	[19]	M Karin M Delhase SeminImmunol 2000 12 85-98
423	[20]	D-Y Seo I-W Park S-H Kim S-R Oh S-B Han O-K Kwon K-S Ahn in
424	[20]	International Journal of Molecular Sciences Vol. 25 2024
425	[21]	S-K Wang T-X Chen W Wang L-L Xu Y-O 7hang 7 lin Y-B Liu Y-7 Tang Journal
426	[2]	of Ethnonbarmacology 2022 296 115489
427	[22]	T Chen O Guo H Wang H Zhang C Wang P Zhang S Meng Y Li H li T Yan Free
128	[22]	Radical Research 2015 /0 1/50-1/68
420	[23]	I 7bu C Nang E Luo H Pan K 7bang I Liu P 7bou I Cao X Chang H He V Oiu L
427	[23]	Wang H Long V Liu T Van <i>Physiol Behav</i> 2016 163 184-192
430	[24]	W Mo C Wang LLi K Chen V Yia S Li L Yu X Lu W Wang C Guo Castroenterol
431	[24]	Doc Dract 2019 2018 282/120
43Z 122	[25]	MS Vim C H Oh M I Vim I V Hwang Distochamistry and Distochagy 2012 90
433	[25]	мз. кип, Gп. Он, мз. кип, Jк. пwang, <i>Photochemistry and Photobiology</i> 2013 , <i>0</i> 9, 011 010
434	[24]	911-910. V Chan I Chan V Dan M Wang 7 Yang W 7hang O Li C Liu 7 Sun S Nia Dissour
430	[20]	A. CHEH, J. CHEH, T. KEH, W. Wahy, Z. Yany, W. Zhany, Q. LI, C. LIU, Z. SUH, S. NIE, DISCOV
430 127	[77]	WEU ZUZJ, 30, 740-700. av Thang I Than S Chang T Thuang S Waimai V Li T Chan D Jing D Thang C
43/ 120	[27]	at. Liteny, J. Litao, S. Onany, Z. Lituany, S. Wainer, A. Li, Z. Onen, B. Jiny, D. Litany, G. Zhao, Journal of Neuroimmune Dharmacelegy 2022 , 19, 400, 702; by Sup. L. Cas, M.
438 420		LINAU, JUUITIALUI INVEULUITIITIUHE PHALMACUUUYY 2023 , 18, 690-703; DY. SUN, L. GAO, W. Hou J. Wu, <i>RieMed Decearch International</i> 2020 , 2020, 7522207
437		$\Box \cup u$, J. vvu, <i>Diviveu Research IIItel Hatiohai</i> 2020 , 2020, 7532300.

- 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.
- Ahmeda, A. S. Alanazi, A. M. Lamsabhi, A. M. Mahmoud, *RSC Advances* 2023, *13*,
 12361-12374; bA. M. Sayed, E. H. M. Hassanein, S. H. Salem, O. E. Hussein, A. M.
 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

453 Tables:

Compound	Lowest binding energy (kcal/mol)	Polar interacting residues	Hydrophobic interacting residues		
1 -5.9 Thr60, His58 T		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		Asn186	Arg187, Val248, Arg246, Lys218		
5	5 -7.6 Asn186		Val248, Lys218, Arg246, Arg187		
6	-8.2 Asn186, Arg33, Asp217		Ala192, Val248, Arg187, Lys218, Asp217		
7	7 -7.1 Arg278, Ser11 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		

	T-1.1. 1 D'-1	· · · · · · · · · · · · · · · · · · ·	- C II 1º	• 1		
454	Table I. Bind	ing aminuties	OI H. Salice	o <i>rnicum</i> pny	toconstituents	towards NF-KB.

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		

457	Table 2. Binding affinities	of <i>H</i> .	salicornicum	phytoconstituents	towards iNOS.
-----	-----------------------------	---------------	--------------	-------------------	---------------

461 Figures:



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 \pm SD, (N = 3). *P<0.05 and ***P<0.001 vs Control. ##P<0.01 and 467 ###P<0.001 vs LPS.



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.



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



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.



- 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.



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.



498 Fig. 11. Molecular docking of compounds 1, 2, and 3 with iNOS showing the crystal structure499 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.



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



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



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