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Veronika Leláková, Sophie Béraud-Dufour, Jan Hošek, Karel Šmejkal, Vilailak Prachyawarakorn, Phanruethai Pailee, Catherine Widmann, Jiří Václavík, Thierry Coppola, Jean Mazella, et al.

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1        **Therapeutic potential of prenylated stilbenoid macasiamenene F through its anti-**  
2        **inflammatory and cytoprotective effects on LPS-challenged monocytes and microglia**

3  
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27 **ABSTRACT**

28 ***Ethnopharmacological relevance***— *Macaranga* Thou. (Euphorbiaceae) is a large genus that  
29 comprises over 300 species distributed between Western Africa and the islands of the South  
30 Pacific. Plants of this genus have a long-standing history of use in traditional medicine for  
31 different purposes, including the treatment of inflammation. Fresh and dried leaves of certain  
32 *Macaranga* species (e.g. *M. tanarius* (L.) Müll.Arg.), have been used to treat cuts, bruises,  
33 boils, swellings, sores and covering of wounds in general. Several reports described  
34 *Macaranga* spp. being a rich source of polyphenols, such as prenylated stilbenes and  
35 flavonoids, mostly responsible for its biological activity. Similarly, an abundant content of  
36 prenylated stilbenes was also described in *M. siamensis* S. J. Davies, species recently  
37 identified (2001) in Thailand. While the respective biological activity of the prenylated  
38 stilbenes from *M. siamensis* was poorly investigated to date, our recent study pointed out the  
39 interest as the natural source of several novel anti-inflammatory stilbenoids isolated from this  
40 species.

41 ***Aim of the study***—This work investigated the potential anti-inflammatory effects of the  
42 stilbenoid macasiamenene F (MF) isolated from *M. siamensis* S.J. Davies (Euphorbiaceae) on  
43 the lipopolysaccharide (LPS)-induced inflammation of monocytes and microglia, major cells  
44 involved in the peripheral and central inflammatory response, respectively.

45 ***Materials and methods***—LPS-induced stimulation of TLR4 signaling led to the activation of  
46 inflammatory pathways in *in vitro* models of THP-1 and THP-1-XBlue™-MD2-CD14 human  
47 monocytes, BV-2 mouse microglia, and an *ex vivo* model of brain-sorted mouse microglia.  
48 The ability of the stilbenoid MF to intervene in the I $\kappa$ B/NF- $\kappa$ B and MAPKs/AP-1  
49 inflammatory cascade was investigated. The gene and protein expressions of the pro-  
50 inflammatory cytokines IL-1 $\beta$  and TNF- $\alpha$  were evaluated at the transcription and translation

51 levels. The protective effect of MF against LPS-triggered microglial loss was assessed by cell  
52 counting and an LDH assay.

53 **Results**—MF demonstrated beneficial effects, reducing both monocyte and microglial  
54 inflammation as assessed *in vitro*. It efficiently inhibited the degradation of I $\kappa$ B $\alpha$ , thereby  
55 reducing the NF- $\kappa$ B activity and TNF- $\alpha$  expression in human monocytes. Furthermore, the  
56 LPS-induced expression of IL-1 $\beta$  and TNF- $\alpha$  in microglia was dampened by pre-, co-, or  
57 post-treatment with MF. In addition to its anti-inflammatory effect, MF demonstrated a  
58 cytoprotective effect against the LPS-induced death of BV-2 microglia.

59 **Conclusion**—Our research into anti-inflammatory and protective effects of MF has shown  
60 that it is a promising candidate for further *in vitro* and *in vivo* investigations of MF  
61 intervention with respect to acute and chronic inflammation, including potentially beneficial  
62 effects on the inflammatory component of brain diseases such as stroke and Alzheimer's  
63 disease.

64

65 **Keywords:** natural stilbenoids, neuroinflammation, microglia, monocytes, prenyl

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66

67 **Abbreviations:**

68 A $\beta$ , amyloid beta; AD, Alzheimer's disease; AP-1, activator protein 1; BBB, blood-brain  
69 barrier; COXs, cyclooxygenases; DAMPs, damage-associated molecular pattern molecules;  
70 HD, Huntington's disease; I $\kappa$ B, inhibitor of NF-kappa B transcription factor; IL-1 $\beta$ ,  
71 interleukine 1 beta; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MAPKs, mitogen  
72 activated protein kinases; NF- $\kappa$ B, nuclear factor of kappa light polypeptide gene enhancer in  
73 B-cells; NSAIDs, non-steroidal anti-inflammatory drugs; PAMPs, pathogen associated  
74 molecular patterns; PD, Parkinson's disease; RA, rheumatoid arthritis; SEAP, secreted  
75 embryonic alkaline phosphatase; TLR4, toll-like receptor 4; TNF- $\alpha$ , tumor necrosis factor  
76 alpha.

## 77 **1. Introduction**

78 Inflammation represents an adaptive reaction of body tissues to invading pathogens,  
79 injuries, or endogenous signals from stressed, damaged, or dead cells. It is a physiological  
80 process that is beneficial, well-coordinated, and self-regulated. However, when the response  
81 to inflammatory stimuli becomes uncontrolled and continuous, chronic inflammatory  
82 conditions may develop and lead to severe pathologies (Medzhitov, 2008). Non-resolving  
83 inflammation contributes to the pathogenesis of both peripheral (neuropathic pain) and central  
84 nervous system diseases, such as stroke, traumatic brain injury, multiple sclerosis,  
85 amyotrophic lateral sclerosis, Alzheimer's disease, Huntington's disease, Parkinson's disease,  
86 or depression (Degan et al., 2018; Miller et al., 2009; Skaper et al., 2018). Moreover, a  
87 prolonged exposure to pathogens or an ongoing systemic inflammation of the kind observed  
88 in metabolic diseases such as diabetes or obesity can lead to a chronic state of  
89 neuroinflammation and ultimately result in neuronal cell death (Nicolas et al., 2017; Skaper et  
90 al., 2018).

91 Unrecovered and repeated acute inflammation, as well as long-term moderate  
92 exposure to noxious stimuli at the central or systemic level may result in a chronic state of  
93 disease. Nowadays, chronic low-grade inflammation is often related to diseases of modern  
94 civilization, such as metabolic syndrome, which is often characterized as a cluster of  
95 conditions such as hypertension, hyperglycemia, insulin resistance, obesity, and high levels of  
96 serum triglycerides, that increase the risk of diabetes mellitus type 2, cerebrovascular disease,  
97 and stroke (Lopez-Candales et al., 2017; Minihane et al., 2015). Some inflammatory  
98 autoimmune disorders, such as rheumatoid arthritis, are also based on chronic inflammation  
99 (Smolen et al., 2018). Systemic and CNS immune system crosstalk during inflammatory  
100 response is a critical consideration not to be overlooked (Minihane et al., 2015). Systemic  
101 macrophages and brain microglia together with CNS-associated macrophages play a crucial

102 role in surveillance of the internal environment and control of homeostasis (Ginhoux and  
103 Jung, 2014; Yin et al., 2017). Through toll-like receptors (TLRs), they transmit a signal  
104 during bacterial infection, injury, or brain damage, including that triggered by  
105 hypoxia/ischemia (Lehnardt et al., 2003).

106 Acute inflammation is often associated with a microbial infection that exemplifies  
107 itself in a form such as meningitis (Hoffman and Weber, 2009), bronchitis (Wark, 2008),  
108 pneumonia (Percopo et al., 2019), myocarditis (Blauwet and Cooper, 2010), or cystitis  
109 (Flores-Mireles et al., 2015). Moreover, Gram-negative bacteria such as *Klebsiella*  
110 *pneumoniae*, *Escherichia coli*, or *Pseudomonas* sp., possess in their outer membrane the  
111 endotoxin lipopolysaccharide (LPS), responsible for their pathogenic potential and massive  
112 clinical manifestations in humans (Sperandio et al., 2013). High peripheral concentrations of  
113 LPS can disrupt the blood-brain barrier (BBB) and promote neuroinflammation (Banks et al.,  
114 2015). LPS triggers an innate immune system response through TLR4 receptors linked with  
115 several inflammatory signaling pathways leading to the production of cytokines and the onset  
116 of inflammation (Pålsson-McDermott and O'Neill, 2004). We therefore used LPS from *E. coli*  
117 to induce TLR4-mediated inflammatory cascades in our experimental set up.

118 Stilbenoids are natural phenolic compounds found in foods (grapes, peanuts, berries,  
119 passion fruit, rhubarb), beverages (wine, white tea), and medicinal plants, including *Morus*  
120 spp., *Artocarpus* spp., and the less well known *Macaranga* spp. (Dvorakova and Landa, 2017;  
121 Hošek et al., 2019). Structurally, they share a common diarylethylene core and differ in its  
122 substitution, which may include prenyl group. Stilbenoids serve the plant as phytoalexins,  
123 protecting it against infection or harm. The strong anti-oxidant and anti-inflammatory  
124 properties of stilbenoids have multiple health benefits. Cardioprotective, neuroprotective,  
125 anti-diabetic and cancer preventive properties have been reported (Akinwumi et al., 2018;  
126 Dvorakova and Landa, 2017; Eräsalo et al., 2018; Hornedo-Ortega et al., 2018). The

127 neuroprotective potential of stilbenoids has been tested mostly on neurodegenerative  
128 disorders, such as AD. For instance, veraphenol, *cis*-scirpusin A and oxyresveratrol, stilbenes  
129 obtained from *Smilax* spp., showed significant inhibitory effects *in vitro* against  $\beta$ -secretase,  
130 the enzyme crucially responsible for the amyloid beta (A $\beta$ ) formation in AD (Jeon et al.,  
131 2007). Other than the most widely known and structurally simple stilbene *trans*-resveratrol,  
132 and its hydroxyderivates oxyresveratrol and piceatannol (found, *e.g.*, in grapes), whose  
133 antioxidant properties and therapeutic potential in cardiovascular diseases arouse great  
134 interest, have been studied very little to date. Little or no information is available about the  
135 biological activities of less-known stilbenoids, including those of the genus *Macaranga*  
136 (Hošek et al., 2019).

137         The genus of *Macaranga* comprises around 300 species and is native to Africa,  
138 Australia, Asia, and various islands of Indian and Pacific Oceans. Some species, such as *M.*  
139 *tanarius* (L.) Muell. Arg., in Thailand known as "Mek", have been used in traditional Thai  
140 medicine as anti-inflammatory treatment. Whereas the fresh leaves were used to cover  
141 wounds, a decoction from the dried root was administered against cough and fever  
142 (Kamarozaman et al., 2018; Phommart et al., 2005, Phupattanapong and Wongprasert, 1987).  
143 *M. sampsonii* Hance has been used for the treatment of swellings, cuts and sores (Quynh et  
144 al., 2018). The European expeditioners surveying the traditional medicinal plants of Papua  
145 New Guinea islands recorded the use of *M. aleuritoides* F. Muell. fruits and seeds by  
146 aborigine people to relieve abdominal pain. Various species of *Macaranga* were used for the  
147 treatment of diarrhea, constipation and stomach complaints in several islands, such as Tonga,  
148 Fiji, Java, Philippines, Bougainville Island and Malaysia (Holdsworth et al., 1983; Nick et al.,  
149 1995). *M. harveyana* (Muell. Arg.) Muell. Arg. known as "Loupata" was used in Tonga folk  
150 medicine for treatment of obstetric and gynecological disorders, such as secondary  
151 amenorrhea, post-partum hemorrhage and abdominal pain (Singh et al., 1984).

152 Several studies trying to describe the phytochemical profile of *Macaranga* species  
153 identified this genus as a rich source of polyphenols such as prenylated and geranylated  
154 stilbenes and flavonoids, terpenes, tannins, steroids and coumarins. Most of the described  
155 biological activities are attributed to stilbenes and flavonoids found in high amounts  
156 (Magadula, 2014; Ngoumfo et al., 2008).

157

158 *M. siamensis* S. J. Davies is a large-leaved plant, common in central and northern  
159 Thailand, firstly identified in 2001. The first phytochemical investigation provided by Pailee  
160 et al., 2015 demonstrated the high content of novel stilbenes, including macasiamenenes A, B,  
161 F, K, L, and P that have been isolated from dichloromethane extract of leaves and twigs. But,  
162 little information about their biological effects has been reported. Based on our previous study  
163 suggesting the anti-inflammatory properties of several prenylated stilbenoids contained in *M.*  
164 *siamensis* (Hošek et al., 2019), by the present work we undertook to compare the anti-  
165 inflammatory effects of seven compounds obtained from *Macaranga* to identify the  
166 therapeutic potential value of the most promising candidate, macasiamenene F (MF), for  
167 treatment of both systemic and central inflammation. The characterization of MF led us to  
168 describe for the first time its anti-inflammatory effects on microglial inflammation.

169

## 170 **2. Materials and methods**

171

### 172 **2.1. Animals**

173 Seven week-old male C57BL/6JRj mice (Janvier, France) were housed at 22°C with a  
174 12-h light-dark cycle and free access to water and food. All animal care and use were  
175 performed in accordance with the policies of European Community Directive 86/609/EEC.

176 The *ex vivo* experiments were approved by the local committee. Every effort was made to  
177 minimize the number of animals used and their suffering.

178

## 179 **2.2. Isolation of compounds and treatments**

180 Pailee et al. (2015) isolated the macasiamenene F (MF) along with L (**1**), K (**2**), P (**3**),  
181 A (**5**), B (**6**) and 2,6-diprenyl-resveratrol (**4**) from leaves and twigs of *M. siamensis* S. J.  
182 Davies (Euphorbiaceae) by chromatographic methods and identified them by spectroscopic  
183 methods, including NMR analysis. The voucher specimen (No. CRI 466) was deposited in the  
184 Laboratory of Natural Products, Chulabhorn Research Institute, Thailand (Pailee et al., 2015).  
185 The plant name has been checked with <http://www.theplantlist.org>. Compound **4**, 2,6-  
186 diprenyl-3,5,4'-trihydroxystilbene (**4**) was already known (Verotta et al., 2009), but  
187 compounds MF, **1–3**, **5**, and **6** were isolated for the first time. The purity of compounds was  
188 confirmed to be more than 98% using HPLC (Figure S1). DMSO was used as a vehicle; its  
189 concentration in cell culture never exceeded 0.1%. Apart from the dose-response studies,  
190 treatments were performed using appropriate serum-free medium at a concentration of 1  
191  $\mu\text{mol/L}$  (0.1  $\mu\text{mol/L}$  for compound **1**), which would not affect cell viability. At these  
192 concentrations, the viability measured by WST-1 was 100 % compared to non-treated cells  
193 (Fig. 1B). LPS from *E. coli* 0111:B4 (Sigma-Aldrich) freshly dissolved in PBS was used at a  
194 concentration of 1  $\mu\text{g/mL}$  (Taka et al., 2015; Triantafilou et al., 2001).

195

## 196 **2.3. Cell culture and differentiation**

197 The THP-1 human monocytic leukemia cell line was purchased from the European  
198 Collection of Authenticated Cell Cultures (Salisbury, UK). The BV-2 murine microglial cells  
199 were generated from primary microglial culture by infection with *v-raf/v-myc* oncogene  
200 carrying J2 retrovirus (Blasi et al., 1990). The THP-1 and THP-1-XBlue<sup>TM</sup>-MD2-CD14

201 (Invivogen, San Diego, CA, USA) cells were cultured in RPMI 1640 medium containing  
202 stabilized 2 mmol/L L-glutamine (Biosera, France), whereas the BV-2 cells were cultured in  
203 DMEM (4.5 g/L glucose, 4 mmol/L L-alanyl-glutamine, and w/o sodium pyruvate (Sigma).  
204 All of the cultures were supplemented with 10 % fetal bovine serum (FBS, Gibco) and  
205 antibiotics [100 U/mL penicillin and 100 µg/mL streptomycin (Gibco)]. Cells were grown in a  
206 water-saturated atmosphere of 5% CO<sub>2</sub> at a temperature 37°C. The experiments were  
207 performed on passages ranging from 3 to 16 for all types of cell lines. The THP-1 monocytes  
208 were differentiated into macrophages by adding 50 ng/mL of phorbolmyristateacetate (PMA)  
209 stimulation (Leláková et al., 2019). All subsequent experiments with all cell lines were  
210 performed after 2 h of incubation in serum-free medium.

211

#### 212 **2.4. Sorting of brain immune cells**

213 Immune cells were isolated from the brains of adult mice using an Adult Brain  
214 Dissociation Kit (Miltenyi Biotec, USA) according to the manufacturer's instructions. Brain  
215 homogenates were filtered using 70 µm cell strainers (BD Biosciences) and centrifuged (10  
216 min, 2,000 rpm). The resulting cell pellets were then re-suspended and washed with PBS at  
217 pH 7.2 containing 0.5 % BSA and 2.5 mM EDTA. They were then labeled with CD11b+  
218 MicroBeads (Miltenyi Biotec, USA) and incubated for 15 min at 4°C. CD11b+ mouse  
219 microglia and CNS associated macrophages were isolated using LS columns (Miltenyi Biotec,  
220 USA) in a magnetic field according to the manufacturer's instructions.

221

#### 222 **2.5. Ex vivo culture of microglia**

223 Brain-sorted microglia were seeded into 96-well tissue culture plate in DMEM  
224 (Gibco) containing 10 % fetal bovine serum (FBS, Gibco) and antibiotics [100 U/mL  
225 penicillin and 100 µg/mL streptomycin (Gibco)] at a density of  $7 \times 10^4$  cells/well. Microglial

226 cells were cultured on poly-L-lysine-covered plates at 37°C in an atmosphere containing 5 %  
227 CO<sub>2</sub> for 18 h. Thirty minutes after cell plating, MF at a concentration of 1 µmol/L, which has  
228 been determined to be non-toxic for both monocytes (Fig. 1) and microglia (Fig. 2), was  
229 added together with LPS (1 µg/ml) as a co-treatment. The supernatants and cell contents were  
230 collected and used subsequently to measure the levels of cytokines after 18 h.

231

## 232 **2.6. Cell viability: Dose-response relationships**

233 Undifferentiated floating THP-1 cells in serum-free RPMI 1640 medium were seeded  
234 into 96-well plates ( $5 \times 10^4$  cells/well). After 2 h, compounds MF, and **1–6** were added at  
235 concentrations ranging from 0.125 to 20 µmol/L. The cell viability was assessed 24 h later  
236 using Cell Proliferation Reagent WST-1 kit (Roche Diagnostics, Basel, Switzerland). Based  
237 on the resulting viability curves, the IC<sub>50</sub> values were calculated according to four parameters  
238 logistic (4PL) analysis. In the case of adherent BV-2 cells, the dose-response assays were  
239 carried out in exponential phase of growth established by a pilot study in 24-well plates.  
240 Briefly, BV-2 cells were seeded in the evening in complete DMEM medium at a density  $1.5 \times$   
241  $10^5$  cells/well, the next day washed by PBS, and the medium was replaced by serum-free  
242 medium. Cells were treated after 2 h with MF at concentrations ranging from 1 to 15 µmol/L.  
243 The mitochondrial activity and cell proliferation were evaluated after 24 h of incubation using  
244 a Cell Titer 96® Aqueous One Solution Cell Proliferation Assay (MTS; Promega, France)  
245 and manual counting of the living cells excluded from Trypan Blue (Sigma) staining,  
246 respectively. Cell mortality was assessed after 24 h using a Cytotoxicity Detection Kit (LDH;  
247 Roche) and represented as the percentage of lactate dehydrogenase (LDH) released by  
248 damaged cells as compared to cells treated with only the vehicle.

249

250

## 2.7. Determination of NF- $\kappa$ B/AP-1 activity

The activity of nuclear factor  $\kappa$ B (NF- $\kappa$ B) and activator protein-1 (AP-1) was measured on THP-1-XBlue<sup>TM</sup>-MD2-CD14 cells (Invivogen, CA, USA) derived from THP-1 human monocytes. This cell line stably expresses the NF- $\kappa$ B and AP-1 inducible secreted embryonic alkaline phosphatase (SEAP) reporter gene, the activity of which was detected using QUANTI-Blue<sup>TM</sup> reagent (Invivogen, San Diego, USA). Cells in serum-free medium RPMI 1640 medium were seeded ( $5 \times 10^4$  cells/well, 96-well plate) and incubated for 2 h at 37°C. Compounds MF, 2–6, and prednisone (P) used as a positive control, were added at the non-toxic concentration of 1  $\mu$ mol/L (0.1  $\mu$ mol/L was used for compound 1), together with vehicle DMSO (0.1 % (v/v)). After 1 h of incubation, the cells were treated with LPS (1  $\mu$ g/mL) to activate NF- $\kappa$ B/AP-1 and produce SEAP. After 24 h, 20  $\mu$ L of the supernatant was withdrawn, mixed with 175  $\mu$ L of QUANTI-Blue<sup>TM</sup> reagent and incubated for 30 min at 37°C. Spectrophotometric measurements at 655 nm were carried out in a Fluostar Omega Microplate Reader (BMG Labtech, Ortenberg, Germany).

## 2.8. Molecular docking

All of the simulations of ligand-protein docking on NF- $\kappa$ B were performed with AutoDockVina (Trott and Olson, 2010) using a PyRx virtual screening tool. Molecular dynamics were performed with a CUDA version of NAMD (Phillips et al., 2005) and VMD was used to prepare the molecular visualization and protein preparation. Docking simulations were performed using system with Core I7 and NVIDIA GTX 850 in the same manner we described previously (Leláková et al., 2019). The crystallographic coordinates of NF- $\kappa$ B bound to DNA were obtained from the RCSB Protein Data Bank with ID 3GUT. Only chain A (p65) and B (50) were used for simulations. All tested compounds meet the conditions of Lipinski rule of five, suggesting that tested compounds can penetrate cell membranes, pass

276 into cell compartments, and have several chemical groups forming hydrogen bonds. All  
277 compounds formed complexes with target protein structure with high binding energy ( $\Delta G$ )  
278 around -7.0 kcal/mol.

## 279 **2.9. Western blot analysis of TLR4-mediated signaling pathways**

280 THP-1-XBlue™-MD2-CD14 cells in 6-well plates at a density of  $3 \times 10^6$  cells/well  
281 were treated with MF or the vehicle (Veh). After 1 h, LPS (1  $\mu\text{g}/\text{mL}$ ) was added to the cells.  
282 Cells not stimulated with LPS represented the control (Ctrl). After 30 min, the cells were  
283 collected, washed in PBS, and homogenized in cold lysis buffer (50 mmol/L Tris-HCl, pH  
284 7.5; 1 mmol/L EDTA; 1mmol/L EGTA; 1 mmol/L sodium orthovanadate; 5 mmol/L sodium  
285 pyrophosphate; 50 mmol/L sodium fluoride; 270 mmol/L sucrose) with protease inhibitors  
286 (Roche Diagnostics, Basel, Switzerland). Equal amounts of proteins (9  $\mu\text{g}$ ) were separated in  
287 12 % polyacrylamide gel and transferred to a PVDF (polyvinylidene fluoride) membrane.  
288 This membrane was then incubated overnight at 4°C with the following monoclonal primary  
289 antibodies: anti-I $\kappa$ B- $\alpha$  (1:500; Cell Signaling, USA, product No. 4814), anti- $\beta$ -actin (1:5000;  
290 Abcam, Cambridge, UK, No. 8226), anti-SAPK/JNK (1:1000; Sigma-Aldrich, USA,  
291 SAB4200176), antiphospho-p44/42 MAPK (p-ERK1/2, 1:2000, No. 4370), anti-p44/42  
292 MAPK (ERK1/2, No. 4695), anti-phospho-p38 MAPK, and anti-phospho-SAPK/JNK  
293 (1:1000; Cell Signaling, MA, USA, No. 4511 and 4668) and polyclonal antibody: anti-p38  
294 MAPK (1:1000; Cell Signaling, MA, USA, 9212). Each membrane was then probed with the  
295 appropriate anti-mouse or anti-rabbit IgG secondary peroxidase-conjugated antibodies  
296 (1:2000, from Sigma-Aldrich, USA, No. A0168 and A0545). The ECL signal (Bio-Rad,  
297 USA) was detected using a Syngene PXi4 chemiluminescence imaging system  
298 (Cambridge,UK). Densitometric analysis was performed using AlphaEaseFC 4.0.0 software  
299 (Alpha Innotech, USA). The relative effects of MF and vehicle (Veh) on I $\kappa$ B- $\alpha$  were  
300 compared after normalization to  $\beta$ -actin.

301

## 302 **2.10. Isolation of RNA and qRT-PCR**

303 Total RNA was extracted using TRI Reagent<sup>®</sup> (Sigma), followed by treatment with  
304 TURBO DNase (Invitrogen). First-strand cDNAs were synthesized from 0.5 µg of total RNA  
305 using 200U/µL Superscript<sup>™</sup> IV Reverse transcriptase (Invitrogen) in reaction buffer in the  
306 presence of 2.5 µmol/L oligo d(T)<sub>20</sub> primers (Eurogentec, France), 0.5 mmol/L  
307 desoxyribonucleotide triphosphate (dNTP) mix, and 5 mmol/L dithiothreitol (DTT). The  
308 reaction was incubated for 5 min at 65°C, then for 60 min at 50°C, and it was finally  
309 inactivated by raising the temperature to 70°C for 15 min. Quantitative RT-PCR was  
310 performed using a LightCycler<sup>®</sup> 480 SYBR Green I Master with a LightCycler<sup>®</sup> 480 sequence  
311 detector (Roche Diagnostics). RPLO and GAPDH were used as reference genes for  
312 normalization. The following sequences of primers were used: mIL-1β (forward 5'-  
313 TGGTGTGTGACGTTCCCAT-3', reverse 5'-CAGCACGAGGCTTTTTTGTG-3'),  
314 mTNF-α (forward 5'-CATCTTCTCAA AATTCGAGTGACAA-3', reverse 5'-  
315 TGGGAGTAGACAAGGTACAACCC-3'), mRPLO (forward 5'-  
316 ACTGGTCTAGGACCCGAGAAG-3', reverse 5'-TCCCACCTTGTCTCCAGTCT-3'), and  
317 mGAPDH (forward 5'-CCAGTGAGCTTCCCGTTCA-3', reverse  
318 GAACATCATCCCTGCATCC-3') (Eurogentec, France).

319

## 320 **2.11. Immunodetection of inflammatory cytokines**

321 Differentiated THP-1 macrophages were pretreated for 1 h with the test compounds or  
322 prednisone 1 µmol/L (in the case of compound 1 0.1 µmol/L) dispersed in 0.1 % DMSO or  
323 with a 0.1 % DMSO solution alone (the vehicle). The release of TNF-α and IL-1β was  
324 evaluated by testing the supernatant after 24 h, using Human TNFα and IL-1β ELISA kits  
325 (Diacclone, France). For BV-2 cells, the expression of TNF-α and IL-1β was determined by

326 testing cell contents and supernatants, using AlphaLISA mIL1 $\beta$  (AL503C) and mTNF $\alpha$   
327 (AL505C) kits (PerkinElmer, MA, USA). The Amicon® Ultra 0.5mL filters (10K) for protein  
328 concentration (Merck Millipore, France) were used to concentrate supernatants before  
329 detection of IL-1 $\beta$  released from BV-2 cells. The total quantities of cytokines produced were  
330 calculated and normalized to the amounts of total proteins determined by Bradford protein  
331 assay (Biorad, France).

332

### 333 **2.12. Statistical analysis**

334 Statistical analysis was carried out using GraphPad Prism 6.00 software. Data were  
335 expressed as the mean $\pm$ SEM. Statistical analyses of differences between groups were  
336 performed using the parametric (n>30) Student's t-test (for comparisons of 2 groups) and  
337 non-parametric (n<30) Mann-Whitney (for 2 groups), and Kruskal-Wallis analysis, together  
338 with Dunn's post-hoc test (for more than 2 groups). Values of p less than 0.05 were  
339 considered statistically significant.

340

## 341 **3. Results**

342

### 343 ***3.1. Effects of Macaranga stilbenoids on the viability of THP-1 cells***

344 The prenylated stilbenoid MF has been found in the plant *M. siamensis* S. J. Davies,  
345 isolated by chromatographic methods, and identified by NMR analysis and other spectral  
346 methods (Pailee et al., 2015). We are the first to evaluate its effects on the viability of THP-1  
347 human monocytes. Figure 1B shows that MF displays one of the safest profiles. Its IC<sub>50</sub> was  
348 calculated at 5.7 $\pm$ 1.1  $\mu$ mol/L. The IC<sub>50</sub> values of compounds **1–6** were 1.8 $\pm$ 1.1, 3.3 $\pm$ 1.1,  
349 4.9 $\pm$ 1.1, 12.8 $\pm$ 1.1, 2.7 $\pm$ 1.1, and 3.9 $\pm$  1.1  $\mu$ mol/L, respectively (Hošek et al., 2019).

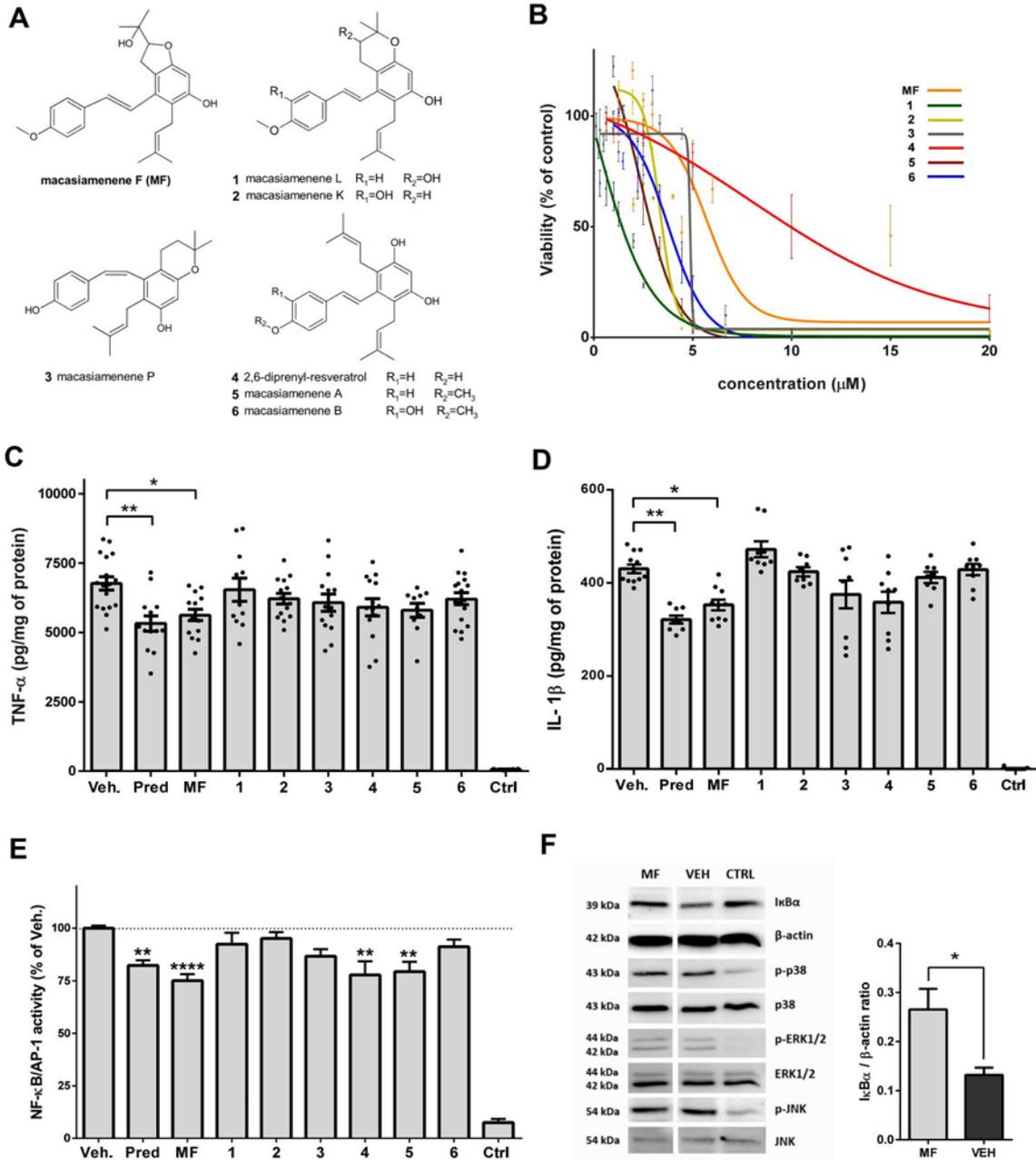
350 According to previous studies on humans evaluating the impact of oral supplementation of  
351 stilbenoids on their circulating concentration in plasma, targeting the concentration of 1  
352  $\mu\text{mol/L}$  that is accepted for the most studied stilbene resveratrol as lowest dose with a  
353 significant anti-inflammatory effect on peripheral cells (Chen et al., 2018) is a  
354 pharmacologically and clinically relevant (Boocock et al., 2007; Smoliga et al., 2011).  
355 Therefore to identify the most interesting anti-inflammatory candidate at this dose, we  
356 performed a non-exhaustive screening of the anti-inflammatory activities on stimulated  
357 macrophages of the seven compounds isolated from the plant *M. siamensis*.

358

### 359 ***3.2. MF reduces the secretion of inflammatory cytokines by acting on the $I\kappa\text{B}\alpha/\text{NF-}\kappa\text{B}$*** 360 ***pathway***

361 We first evaluated and compared to prednisone, a corticosteroid used in the  
362 management of inflammatory conditions or diseases, the effect of the seven compounds  
363 isolated from the plant *M. siamensis* at the concentration of 1  $\mu\text{mol/L}$  on the release of the  
364 pro-inflammatory cytokine  $\text{TNF-}\alpha$  (Fig. 1C) and  $\text{IL-1}\beta$  (Fig. 1D) in PMA-differentiated THP-  
365 1 macrophages. Interestingly, MF reduced the secretion of  $\text{TNF-}\alpha$  from macrophages  
366 stimulated by LPS by 22%, which was more than any of the other prenylated *Macaranga*  
367 stilbenoids (range of 3-16%) tested by Hošek et al. (2019). This anti-inflammatory effect of  
368 MF was comparable to that of prednisone (24 %), a routinely used corticoid anti-  
369 inflammatory drug (Fig. 1C). Moreover, a similar anti-inflammatory effect of MF was also  
370 observed on the secretion of  $\text{IL-1}\beta$  from macrophages stimulated by LPS (Fig. 1D).

371



372

373

374 **Figure 1: Effects of prenylated *M. siamensis* stilbenoids on NF- $\kappa$ B signaling of LPS-stimulated**

375 **THP-1 macrophages and THP-1 XBlue<sup>TM</sup>-MD2-CD14 monocytes. A, Test stilbenoids MF, 1-6; B,**

376 **Dose-response curves showing viability of THP-1 after a 24 h incubation with compounds MF, 1-6;**

377 **C, Effect on release of TNF- $\alpha$  protein; D, Effect on release of IL-1 $\beta$  protein; E, Activation of NF-**

378  **$\kappa$ B/AP-1 after 24 h; F, MF upstream regulation of I $\kappa$ B $\alpha$ . Data are presented as the mean $\pm$ SEM, n=3,**

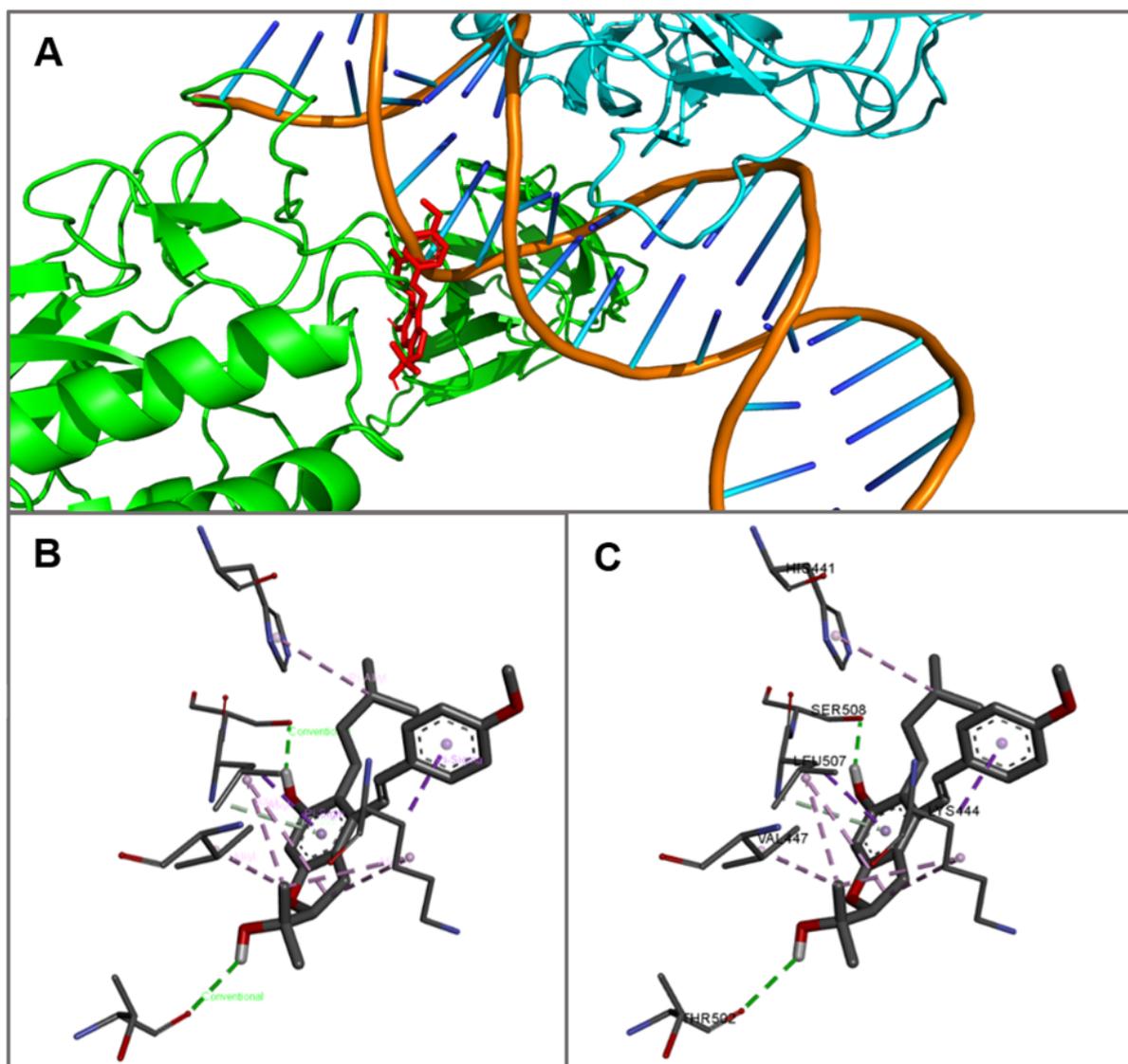
379 **\*indicates a significant difference of  $P < 0.05$ ,  $P < 0.01$ ,  $P < 0.0001$  versus only with the vehicle**

380 **treated cells (Veh) analyzed by the non-parametric tests Kruskal-Wallis in the case of C and D, and the**

381 **Mann and Whitney for E.**

382 We next focused on the regulation of TNF- $\alpha$  and IL-1 $\beta$  upstream, particularly on the  
383 transcription factors NF- $\kappa$ B and AP-1, which concomitantly control the expression of several  
384 pro-inflammatory cytokines, including TNF- $\alpha$  and IL-1 $\beta$ . The canonical inflammatory  
385 pathway that was triggered by TLR4 and led to overexpression of TNF- $\alpha$  involved NF-  
386  $\kappa$ B/AP-1 activation (Lawrence, 2009; MacIntyre et al., 2014). Again in this case MF showed  
387 one of the strongest inhibitory effects of the tested prenylated *M. siamensis* stilbenoids (Fig.  
388 1E), with a therapeutic potential greater than that of prednisone. Overall at the clinically  
389 transferable dose of 1  $\mu$ mol/L, the anti-inflammatory activities of compounds 4 and 5 are  
390 weaker than MF justifying it to be the most promising candidate for further evaluation of anti-  
391 inflammatory and protective effects. Then, to gain some mechanistic insights we investigated  
392 MF effect on the upstream activation of NF- $\kappa$ B/AP-1 which includes I $\kappa$ B $\alpha$  (Lawrence, 2009)  
393 and MAPKs (MacIntyre et al., 2014). Interestingly, MF reduced the degradation of I $\kappa$ B $\alpha$ , a  
394 direct inhibitor of NF- $\kappa$ B, but did not inhibit the activation (phosphorylation) of MAPKs  
395 known for leading to the activation of AP-1 (Fig. 1F). Therefore it is tempting to believe that  
396 MF could inhibit the inflammation in LPS-stimulated macrophages primarily through  
397 I $\kappa$ B $\alpha$ /NF- $\kappa$ B signaling, rather than by acting *via* the MAPK/AP-1 pathway.

398 Since inflammatory response of NF- $\kappa$ B is driven by its nuclear translocation and its  
399 binding activity, interfering with each of these steps or with both may account for MF anti-  
400 inflammatory effect. Since I $\kappa$ B $\alpha$  primary role is to bind to NF- $\kappa$ B for preventing its  
401 translocation to the nucleus, our results showing MF potential to inhibit I $\kappa$ B $\alpha$  degradation  
402 suggests that it could interfere with NF- $\kappa$ B nuclear translocation. Therefore we further  
403 investigated whether MF could also directly interfere with NF- $\kappa$ B to dampen its binding  
404 activity.



405

406

407 **Figure 2: Molecular docking pose of MF interactions with NF-κB (PDB 3GUT).** A, The bond of  
 408 MF to p50 subunit of NF-κB DNA binding pocket. Red - MF, green ribbon – p50 subunit, blue ribbon  
 409 – p65 subunit, orange double helix with colored sticks - DNA fragment; B, Interactions of MF with  
 410 p50 NF-κB depicting hydrogen bonds (green–classical, grey–non-classical) and hydrophobic  
 411 interactions (dark and light purple); C, MF forms interactions with p50 residues Thr502, Ser508  
 412 (classical hydrogen bonds), Leu507 (non-classical hydrogen bond and hydrophobic interactions),  
 413 His441, Lys444, and Val447 (hydrophobic interactions).

414

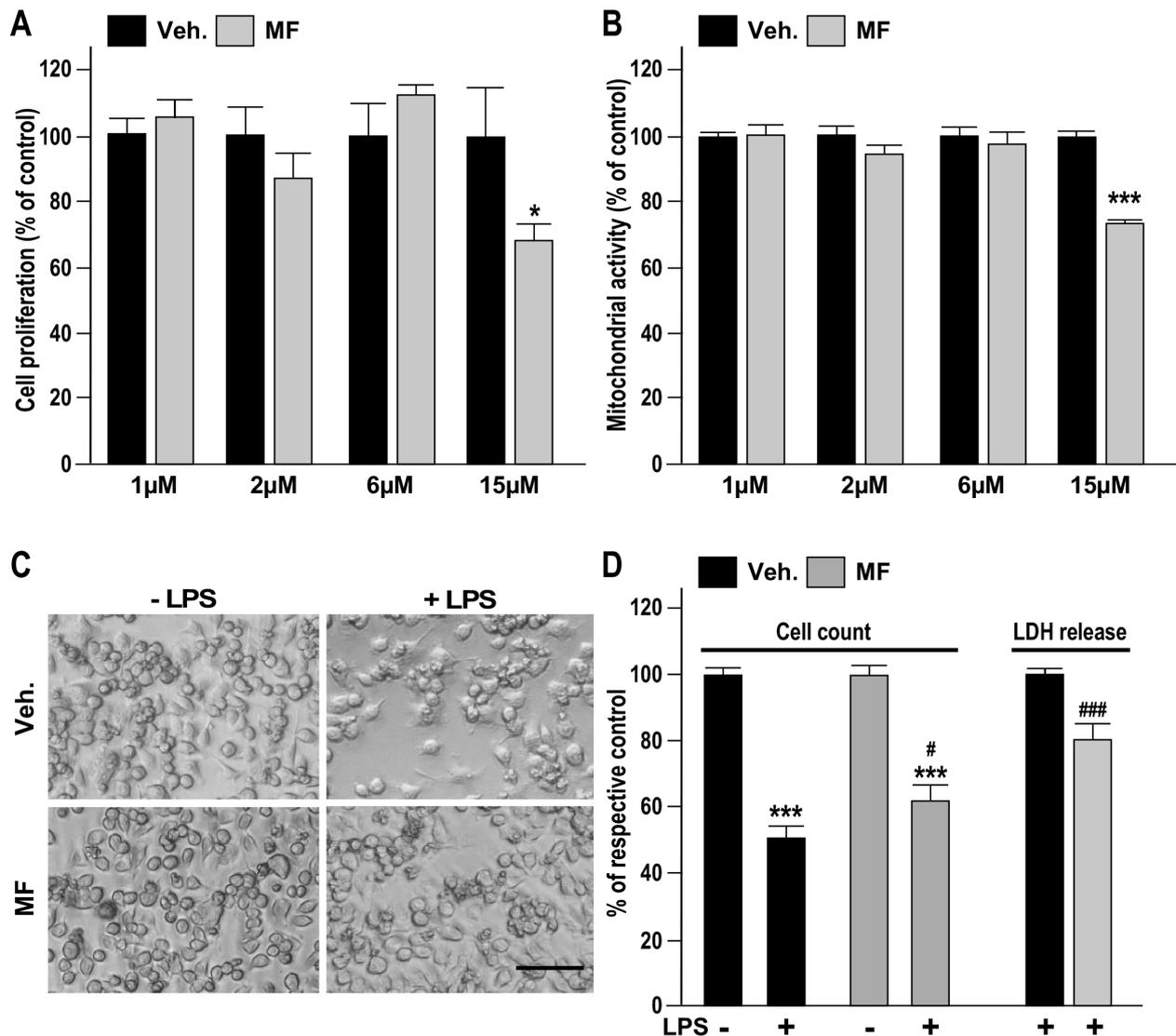
415 Thus, we evaluated the interaction of MF with NF-κB DNA binding site by simulating a  
 416 ligand-protein docking into DNA binding pocket of the NF-κB heterodimer p65/p50  
 417 according the method by (Trott and Olson, 2010) (Figure 1A). The hydroxyl groups of MF

418 tend to form conventional hydrogen bonds (O-H...O) with Thr502 and Ser508 residues. Non-  
419 classical hydrogen bond (N-H... $\pi$ ) was observed with Leu507. The alkyl hydrophobic  
420 interactions were detected with residues His441, Lys444, Val447, and Leu507. Two phenyl  
421 rings of MF typical for stilbene core tend to form  $\pi$ -alkyl hydrophobic bonds with Lys444 and  
422 Leu507 (Figure 2B and 2C). Overall MF displayed a high affinity ( $\Delta G = -6.7$  kcal/mol) for  
423 NF- $\kappa$ B DNA binding site similarly to other biologically active stilbenes inhibiting the NF-  
424  $\kappa$ B/AP-1 pathway (Leláková et al., 2019). Altogether our results suggest that MF may not  
425 only inhibit NF- $\kappa$ B activity by indirectly targeting its translocation preventing I $\kappa$ B $\alpha$   
426 degradation but also directly by interfering with the DNA binding site of NF- $\kappa$ B.

427

### 428 ***3.3. Anti-inflammatory dose of MF does not alter the proliferation and viability of BV-2*** 429 ***microglial cells***

430 We then tried to determine if this anti-inflammatory effect observed on monocytes  
431 could be extended to microglia, the immune cells of the brain. To evaluate whether the anti-  
432 inflammatory effect of MF could be obtained on the safe side within a concentration range  
433 similar to the active one used for monocytes, we first performed a dose-response study to  
434 assess whether MF had an effect on the proliferation of BV-2 microglia (Fig. 2A) and their  
435 corresponding mitochondrial activity (Fig. 2B), that are accepted indicators of BV-2 vital  
436 cellular functioning. Not only changes in mitochondrial activity reflect an alteration of the  
437 energy homeostasis of the cell physiology often associated with a switch from physiological  
438 to pathological states, but mitochondria alteration is known for triggering inflammation and to  
439 play a central role in pro-inflammatory signaling and inflammatory response (Meyer et al.,  
440 2018). Therefore it was crucial to know whether and at which dose MF may alter the  
441 physiological functioning of BV-2. The high dose of 15  $\mu$ mol/L altered the proliferation and  
442 activity of BV-2, but doses below 6  $\mu$ mol/L had no negative effect (Fig. 2A-B).



443

444

445 **Figure 2: Determination of the dose-limiting toxicity of MF and its protective effect against**  
 446 **inflammatory challenge in BV-2 cells.** **A**, Effect of a 24 h-application of MF at concentrations of 1,  
 447 2, 6, and 15 μmol/L on the proliferation of cells assessed by cell counting, \* indicates  $P < 0.05$ ,  
 448 significant difference in comparison with BV-2 cells treated only with the vehicle (Veh.); **B**, Effect of  
 449 a 24 h-application of MF on the mitochondrial activity, \*\*\* indicates  $P < 0.001$  compared to Veh.; **C**,  
 450 Representative snapshots (Scale bar = 150 μm) showing the MF-induced improved survival of BV-2  
 451 cultures injured by a 24 h-LPS challenge; **D**, Quantitative assessment of the beneficial effect of MF on  
 452 BV-2 cultures injured by a 24 h-LPS challenge, as determined by cell counting and the extent of LDH  
 453 release, \*\*\* indicates  $P < 0.001$  compared to the respective LPS non-stimulated control, # $P < 0.05$  and  
 454 ### $P < 0.001$  indicate significant differences between MF+LPS vs. Veh.+LPS. Data are presented as  
 455 the mean±SEM; n=6 measured in sextuplicates, and analyzed by Student's t-test.

456

457 We therefore chose the concentration 1  $\mu\text{mol/L}$ , previously identified and used to reduce  
458 the LPS-induced inflammatory response in monocytes, and used it for all subsequent  
459 experiments to evaluate the protective and anti-inflammatory potential of MF on microglia  
460 challenged by LPS (Kacimi et al., 2011) At this concentration, pretreatment with MF  
461 decreased the LPS-stimulated loss of microglia by 20% relative to the vehicle after 24 h (Fig.  
462 2C and D). This cellular protection was accompanied by a 20% reduction in the release of  
463 LDH by cells pretreated with MF as compared to cells treated only with the vehicle (Fig. 2D).

464

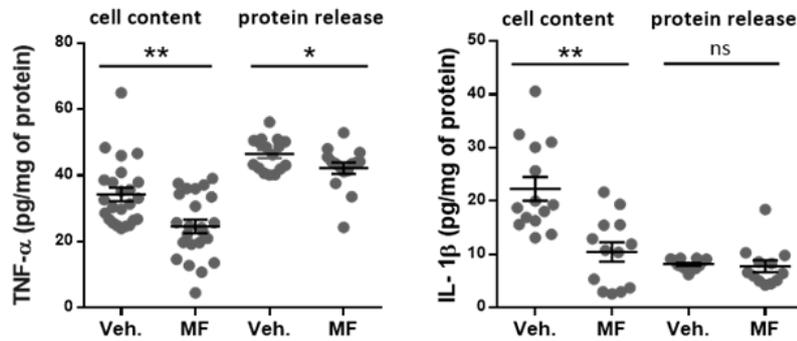
#### 465 ***3.4. Pretreatment with MF dampens the LPS-induced pro-inflammatory response of*** 466 ***BV-2 microglial cells***

467 The important anti-inflammatory properties of MF at 1  $\mu\text{mol/L}$ , established on LPS-  
468 stimulated monocytes, along with its protective action for BV-2 brain immune cells, led us to  
469 investigate its anti-inflammatory action in reducing the production of IL-1 $\beta$  and TNF- $\alpha$ . MF  
470 reduced the TNF- $\alpha$  and IL-1 $\beta$  protein expression in both intracellular content and release of  
471 proteins at the basal level, with no harmful stimuli (Fig. 3A).

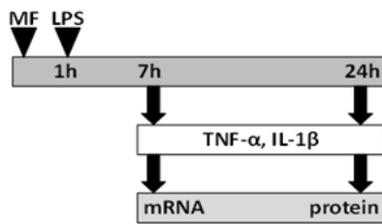
472 Moreover, BV-2 cells pretreated with MF showed an anti-inflammatory effect in the  
473 form of dampened BV-2 response to LPS-induced inflammation (Fig. 3D). Indeed, the  
474 intracellular content and release of both cytokines were also substantially reduced in cells  
475 pretreated with MF as compared to those treated only with the vehicle (Fig. 3D). This effect  
476 was correlated with a reduced level of gene expression for both pro-inflammatory cytokines:  
477 1 h of pretreatment of BV-2 cells with MF significantly reduced the expression of LPS-  
478 stimulated TNF- $\alpha$  and IL-1 $\beta$  mRNA by 26 % and 20 %, respectively (Fig. 3C).

479

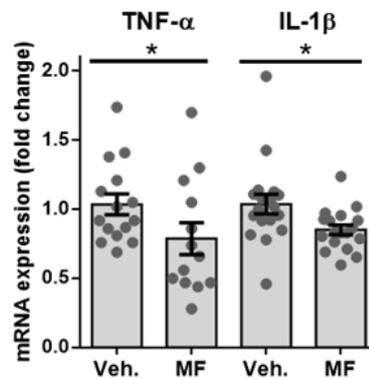
**A. Basal level 24h**



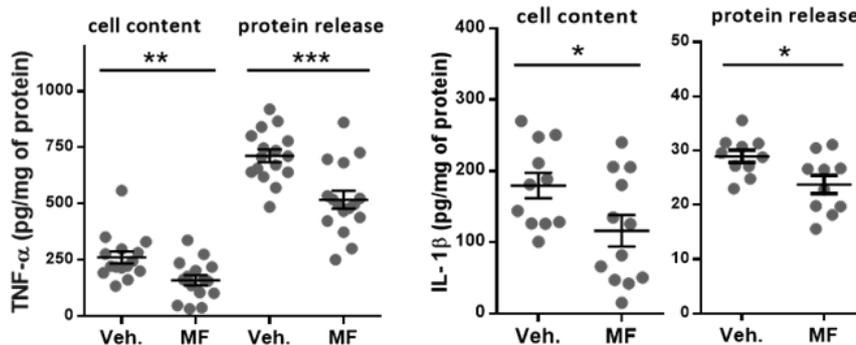
**B. PRE-treatment model**



**C. mRNA expression, 7h**



**D. LPS-activated 24h**



480

481 **Figure 3: Anti-inflammatory effects of MF pre-treatment on the expression of TNF- $\alpha$  and IL-1 $\beta$ .**

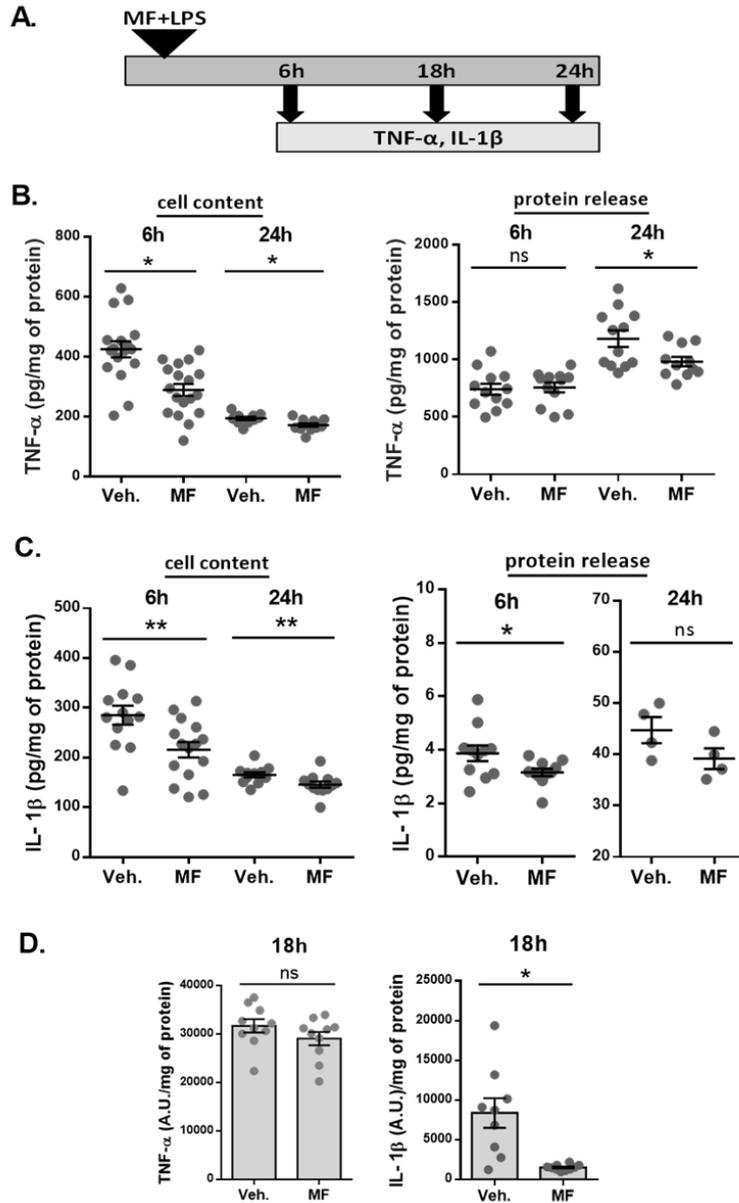
482 A, MF reduced the basal expression and release of TNF- $\alpha$  and IL-1 $\beta$  by BV-2 cells; B, Schema  
 483 illustrating the protocol of pre-treatment of BV-2 cells with MF (1  $\mu$ mol/L) and the timing of  
 484 evaluation of its effect on the mRNA and protein expression; C, MF reduced the LPS-stimulated  
 485 expression of TNF- $\alpha$  and IL-1 $\beta$  mRNA; D, MF reduced the LPS-induced expression and release of  
 486 TNF- $\alpha$  and IL-1 $\beta$  by BV-2 cells. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 vs. Veh. group. Data are expressed  
 487 as the mean $\pm$ SEM,  $n$ =3, measured in sextuplicates, analyzed by the non-parametric Mann and  
 488 Whitney test for two independent groups.

489

490 ***3.5. MF dampened the early stage of inflammation in a co-treatment with LPS***

491 The anti-inflammatory effect of pretreatment with MF is of great interest for  
492 nutritional intervention and the development of functional foods. But the fact that  
493 pretreatment with MF would need to be applied pre-emptively limits its clinical usefulness,  
494 particularly for the situations arising without warning. Therefore, we investigated the possible  
495 therapeutic use of MF as an anti-inflammatory drug during (a cotreatment protocol) or after (a  
496 posttreatment protocol) the establishment of inflammation.

497 To study the effect of MF in the early phase of inflammation, we assessed the cellular  
498 content and protein release of the inflammatory cytokines TNF- $\alpha$  and IL-1 $\beta$  in a culture  
499 medium of BV-2 cells after 6 h and 24 h of cotreatment with MF and LPS. Co-application of  
500 MF and LPS reduced both the intracellular level and the release of TNF- $\alpha$  which are normally  
501 induced by the application of LPS (Fig. 4). Marked inhibition (33 %) of the LPS-induced  
502 TNF- $\alpha$  up-regulation was already observed after 6 hours of cotreatment. This anti-  
503 inflammatory effect clearly influenced the level of TNF- $\alpha$  release, which was decreased by  
504 26 % after 24 h (Fig. 4B). Similarly, severe inhibition of the IL-1 $\beta$  response to LPS was  
505 reflected in both the cell content and the culture medium after 6 and 24 h of cotreatment (Fig.  
506 4C). We then determined the extent to which the beneficial effects observed in the BV-2 cell  
507 line could be extended to brain-sorted primary microglia (Fig. 4D). Whereas under the applied  
508 experimental conditions cotreatment with MF seemed not to be efficiently reduce the level of  
509 TNF- $\alpha$  in a statistically significant manner. Cotreatment with MF lowered the level of IL-1 $\beta$   
510 in primary microglia by factor of five, as compared to the vehicle (Fig. 4D).



511

512 **Figure 4: Anti-inflammatory effects of MF cotreatment with LPS in the BV-2 cell line and the**  
 513 ***ex vivo* culture of microglia cells.** A, Schema illustrating the cotreatment of BV-2 cells and primary  
 514 microglia with MF (1  $\mu$ mol/L) co-treatment with LPS (1  $\mu$ g/mL) together with the times at  
 515 which the cytokines were measured: 6 h and 24 h for the BV-2 cell line, and 18 h for the *ex vivo*  
 516 culture of microglia cells; B, TNF- $\alpha$  expressed in the cell content and protein release from the BV-2  
 517 cells at 6 and 24 h; C, IL-1 $\beta$  expression in cell content and protein release from BV-2 cells at 6 and  
 518 24 h; D, TNF- $\alpha$  and IL-1 $\beta$  protein contents in brain-sorted mouse microglia after 18 h of cotreatment  
 519 of MF with LPS or only the vehicle. Data are expressed as the mean $\pm$ SEM, n=3, measured in  
 520 sextuplicates, analyzed by the Mann-Whitney test. \* $P$ <0.05, \*\* $P$ <0.01 vs. Veh. group.

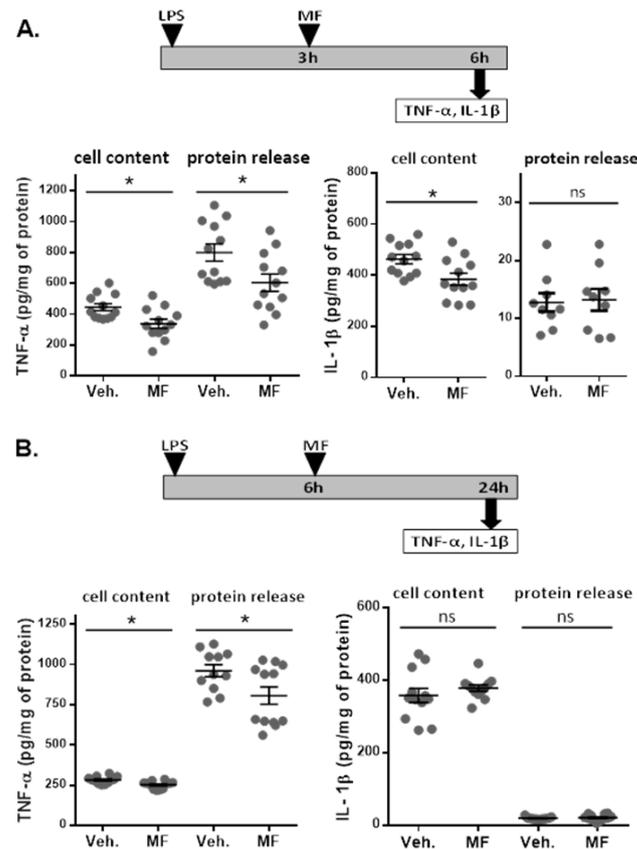
521

### 3.6. *Post-treatment with MF for resolving LPS-induced inflammation*

522  
523 Establishing the proof of the anti-inflammatory MF efficiency of MF as post-treatment  
524 is a mandatory steep toward clinical translation. Given as post-treatment, anti-inflammatory  
525 drugs are expected to resolve an ongoing inflammatory state by lowering the levels of  
526 cytokines such as TNF- $\alpha$  and IL-1 $\beta$ . After LPS challenge, the expression of such pro-  
527 inflammatory molecules is been initiated within a 3-6h time window, that also correlates with  
528 their in vivo expression, observed in severe acute CNS pathologies with massive  
529 inflammatory reaction, like stroke (Le Thuc et al., 2016; Nilupul Perera et al., 2006). As  
530 inhibition of inflammation is of interest to change the course of the pathology and improve  
531 stroke outcome, especially if starting the treatment after the recommended therapeutic  
532 window for thrombolysis with tPA (after 3-4.5 hours after the onset of stroke), we therefore  
533 investigated the effects of administering MF as a post-treatment, 3 or 6 h after the onset of  
534 LPS-induced inflammation (Figs. 5A and 5B, respectively) on TNF- $\alpha$  and IL-1 $\beta$   
535 inflammatory response, observed after 6 h or 24 h, respectively.

536 The amount of TNF- $\alpha$  were found to be reduced by approximately 20 % in both the  
537 cellular content and external medium, whether MF was applied 3 or 6 h after LPS (Fig. 5A).  
538 Treating the inflammation with MF 3 h after its induction reduced the IL-1 $\beta$  response to LPS  
539 found after 6 h, but treatment delayed by 6 h did not influence IL-1 $\beta$  levels 24 h after the  
540 onset of inflammation (Fig. 5B). This result may be explained by the fact that as IL-1 $\beta$  is  
541 known to be expressed rapidly during the first hours after LPS stimulation (Kwon et al., 2010)  
542 the association of late post-treatment and a low dose of MF would be too stringent conditions  
543 to show any anti-inflammatory effect. Therefore, increasing the dose and/or shorten the  
544 timing of administration may be anticipated option to further investigate for revealing and  
545 improving the MF anti-inflammatory effect on this particular cytokine. Moreover while  
546 several group have shown that BV-2 cells produce detectable quantity of interleukin, in our

547 settings, IL-1 $\beta$  was hardly induced and secreted as compared to TNF- $\alpha$  at the selected  
 548 measurement times (6 and 24 h). In these stringent settings, it is most likely that the difference  
 549 in MF effects at the dose of 1  $\mu$ mol/L on TNF- $\alpha$  and IL-1 $\beta$  could be explained by the timing  
 550 and level of the protein release in response to LPS challenge.  
 551



552

553 **Figure 5: Anti-inflammatory effects of posttreatment with MF.** Schema illustrating the protocol of  
 554 inducing inflammation with LPS (1  $\mu$ g/mL) in BV-2 cells and treating them 3 h (A) or 6 h later (B) by  
 555 applying MF post-treatment (1  $\mu$ mol/L). A, Effect of MF applied 3 h after the induction of  
 556 inflammation by LPS. MF post-treatment reduced both the protein content and release of the TNF- $\alpha$   
 557 and the IL-1 $\beta$  as evaluated 6 h after the application of LPS. B, When the MF posttreatment was  
 558 effectuated at 6 h after the induction of inflammation by LPS, the anti-inflammatory effect of reduced  
 559 TNF- $\alpha$  protein content was still found 24 h after the application of LPS, but that of the IL-1 $\beta$  protein  
 560 was not. That transitory expression of IL-1 $\beta$  apparently occurred entirely before the post-treatment  
 561 was applied. Data are expressed as the mean $\pm$ SEM, n=3, measured in sextuplicates, analyzed by the  
 562 Mann-Whitney test. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 vs. Veh. group.

563

## 564 **1. Discussion**

565 Inflammation accompanies many acute and chronic pathologies at both the peripheral  
566 and CNS levels. Acute inflammatory diseases are mostly infectious, but inflammation may  
567 also be a part of non-infectious pathologies, such as stroke, traumatic brain injury (Skaper et  
568 al., 2018), and hemorrhage (Goerge et al., 2008). Chronic inflammation is usually  
569 characterized as a slowly progressing mild inflammatory reaction that is linked with several  
570 long-term disorders, including atherosclerosis (Libby et al., 2002), inflammatory bowel  
571 diseases (Vochyánová et al., 2015), chronic obstructive pulmonary disease (Barnes et al.,  
572 2015), osteoarthritis (Robinson et al., 2016), or autoimmune disorders, such as allergic asthma  
573 (Barnes et al., 2015) or rheumatoid arthritis (Smolen et al., 2018).

574 Monocytes and microglia are major phagocytes of the innate immune system,  
575 providing the first line of defense. The presence of Toll-like receptors (TLRs) enables them to  
576 respond to different noxious stimuli, exogenous pathogen-associated molecular patterns  
577 (PAMPs) and some endogenous damage-associated molecular pattern molecules (DAMPs).  
578 TLR4 is a major receptor implicated in the inflammatory response recognizing bacterial LPS  
579 (Zhu and Mohan, 2010). Activation of TLR4 signaling leads to the expression of several pro-  
580 inflammatory cytokines, including TNF- $\alpha$  and IL-1 $\beta$ , mediated by the activation of NF- $\kappa$ B  
581 signaling pathway (Pålsson-McDermott and O'Neill, 2004). Both TNF- $\alpha$  and IL-1 $\beta$  are potent  
582 regulators of the innate immune system important for host defense. Especially, there is an  
583 increasing evidence pointing out a prominent role of IL-1 $\beta$  in acute neuronal injuries and  
584 many chronic brain diseases (Allan and Rothwell, 2003). Targeting the TLR4/NF- $\kappa$ B pathway  
585 therefore represents a potential therapeutic strategy for both acute and chronic inflammation-  
586 based diseases that are linked with abnormal secretion of cytokines (Kuzmich et al., 2017).

587 Plant-derived stilbenoids have demonstrated a promising ability to attenuate peripheral  
588 (Dvorakova and Landa, 2017; Eräsalo et al., 2018; Leláková et al., 2019) and CNS  
589 inflammation (Akinwumi et al., 2018; Hornedo-Ortega et al., 2018) *in vitro* and *in vivo*.  
590 However, clinical trials to investigate their potential in humans are still needed. Pterostilbene,  
591 a natural derivative of *trans*-resveratrol, that has frequently been investigated, has shown the  
592 ability to inhibit platelet aggregation *ex vivo* (Messina et al., 2015), improve high fat-induced  
593 atherosclerosis inflammation *via* NF- $\kappa$ B signaling *in vivo* (Zhang and Zhang, 2016), protect  
594 against myocardial ischemia-reperfusion (I/R) injury by reducing oxidative/nitrosative stress  
595 and the inflammatory response to it (Yu et al., 2017), and reduce the size of a myocardial  
596 infarction (Wu et al., 2017). At the CNS level, pterostilbene protects against hyperglycemia-  
597 (Yang et al., 2017) and glutamate-induced neuronal oxidative injury (Wang et al., 2016). In  
598 contrast, the stilbenoids of *Macaranga* spp. have not yet been thoroughly investigated.  
599 Among these compounds, MF displays a safe profile for therapeutic use, with low cytotoxic  
600 effects on THP-1 cells and on the BV-2, MOLT-3, HepG2, HuCCA-1, and A549 cell lines  
601 (Pailee et al., 2015). We focused on the activity of the transcriptional factors NF- $\kappa$ B and AP-  
602 1, both of which have been shown to concomitantly control the expression of various  
603 inflammatory cytokines during inflammatory response (MacIntyre et al., 2014). Whereas NF-  
604  $\kappa$ B is mostly responsible for the gene and protein expressions of TNF- $\alpha$  and IL-1 $\beta$  and is up-  
605 regulated by its direct cytoplasmic inhibitor I $\kappa$ B $\alpha$  (Lawrence, 2009), AP-1 is controlled  
606 predominantly by ERK1/2, p38, and JNK, members of the family of mitogen-activated  
607 protein kinases (MAPKs) (Zhou et al., 2018). In the present work, we reveal the promising  
608 potential of MF against peripheral inflammation by demonstrating its ability to: 1) intervene  
609 in the I $\kappa$ B $\alpha$ /NF- $\kappa$ B pathway and inhibit the degradation of I $\kappa$ B $\alpha$ , which leads to decreased  
610 activation of the transcriptional factor NF- $\kappa$ B, and 2) reduce secretion of inflammatory  
611 cytokine TNF- $\alpha$ , which is under the transcription control of NF- $\kappa$ B. This result is in

612 agreement with the ability of non-prenylated stilbenoids to reduce the phosphorylation of  
613 MAPKs (Leláková et al., 2019), whereas prenylated stilbenoids have been shown to affect the  
614 degradation of I $\kappa$ B $\alpha$  (Hošek et al., 2019).

615 Like monocytes at the peripheral level, microglia and CNS infiltrated monocytes are  
616 responsive to neuronal damage. Their activation is an alarming sign of the brain pathology  
617 that can occur in conditions such as microbial infection, traumatic brain injury, or stroke, and  
618 also degenerative disorders of the CNS (Dheen et al., 2007). In this work, we demonstrate the  
619 ability of MF to improve the *in vitro* native state of microglia by reducing the basal release of  
620 cytokines. This beneficial effect is associated with protection against LPS-induced loss of  
621 microglia.

622 Moreover, under inflammatory conditions, MF dramatically inhibits the LPS-  
623 stimulated gene and protein expressions of both TNF- $\alpha$  and IL-1 $\beta$ , whatever the stage of  
624 treatment (pre-, co-, and post-treatment). The greatest reduction of cytokines (up to 48 %) is  
625 achieved when the microglia are pretreated with MF before inflammation is induced.  
626 Although the up-stream signalization, such as level of NF- $\kappa$ B and I $\kappa$ B was not investigated in  
627 BV-2 cells, due to the phenotypic similarity of monocytes and microglia (Orihuela et al.,  
628 2016), it is tempting to anticipate that the response in mouse microglia may be similar. These  
629 results rivet attention on the potential for developing MF as a nutraceutical or dietary  
630 supplement, aimed at preventing any type of systemic and/or CNS inflammatory disorder, and  
631 thereby maintaining or improving organ function, preventing chronic disorders, promoting  
632 good health, or even delaying the process of aging (Nasri et al., 2014; Tauskela et al., 2016).

633 In the acute and chronic states of inflammatory disease, the ability of MF to reduce the  
634 secretion of the cytokines TNF- $\alpha$  and IL-1 $\beta$  may be of particular interest, because these  
635 cytokines are known to be involved in the activation of resident microglia and astrocytes  
636 during infection, inflammation, and the development of brain injury (Norden et al., 2016).

637 Their dysregulation and sustained activation can create a toxic circle, elevating the release of  
638 inflammatory factors that endanger the functioning and survival of neurons (Ramesh et al.,  
639 2013).

640 Our results show, that MF could be interesting as a supplementary compound used  
641 against chronic inflammatory diseases that would correspond to the so called “early phase of  
642 inflammation” in the present work. Low-grade systemic inflammation is like CNS  
643 inflammation (neuroinflammation), a pathological process that accompanies many persistent  
644 diseases and also the normal aging process nicknamed “inflammaging”. Inflammaging is  
645 possibly related to chronic activation of the innate immune system and changes in the  
646 functions of monocytes (Benedetto et al., 2017). Altered activation of microglia, characterized  
647 by elevated levels of TNF- $\alpha$  and IL-1 $\beta$ , is associated with neurodegenerative disorders, such  
648 as AD, Parkinson’s disease (PD), and Huntington’s disease (HD), and is a reaction to the  
649 presence of cellular aggregates and the misfolded proteins  $\beta$ -amyloid,  $\alpha$ -synuclein, and  
650 huntingtin, respectively. Reducing neuroinflammation could be a way to slow the progress of  
651 these diseases (Sastre et al., 2014). Inflammation underlying neurodegeneration is also present  
652 in multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), systemic lupus erythematosus  
653 (SLE), neuromyelitis optica (NMO), diabetes mellitus (DM), chronic bacterial meningitis,  
654 Lyme neuroborreliosis, and HIV-1 encephalitis (Amor et al., 2010; Ramesh et al., 2013).  
655 Some stilbenoids, such as *trans*-resveratrol, isorhapontigenin, gnetol, and pinosylvin, have  
656 shown significant potential to inhibit butyrylcholine esterase (BChE) enzyme, the increased  
657 activity of which is associated with the formation of A $\beta$  plaque in patients with AD  
658 (Sermboonpaisarn and Sawasdee, 2012). Oxyresveratrol exerts neuroprotective effects against  
659 Parkinsonian mimetic 6-hydroxydopamine (6-OHDA)-induced neurotoxicity in  
660 neuroblastoma cells (Chao et al., 2008). Studies of the “Mediterranean diet”, which is based  
661 on vegetables, fruits, whole grains, nuts, fish, and seafood, healthy fats, and olive oil, with

662 moderate consumption of red wine, and is rich in polyphenols, including stilbenoids, have  
663 reported beneficial effects, mitigating age-related and other neurodegenerative pathologies  
664 (Hornedo-Ortega et al., 2018).

665         The anti-inflammatory effects of MF may also be of interest for acute conditions such  
666 as cerebral ischemia, in which reduced blood flow and oxygen supply (hypoxia) lead to  
667 cerebral infarction. This condition triggers an intravascular inflammatory cascade that extends  
668 into the surrounding tissues of the injured brain, and is characterized by elevated levels of  
669 inflammatory cytokines, chemokines, adhesion molecules, reactive oxygen species, nitric  
670 oxide, extracellular proteases, and other substances flowing into and accumulating in immune  
671 cells, such as microglia (Le Thuc et al., 2015). A therapeutic strategy that intervened in the  
672 inflammatory process of cerebral ischemia might slow down and limit the brain damage  
673 caused by ischemia. The potential of stilbenoids to act against cerebral ischemia has been,  
674 however not thoroughly, investigated. It is noteworthy that a study using a transient rat middle  
675 cerebral artery occlusion model has shown that oxyresveratrol reduces the size of volume of  
676 an infarction and thus prevents neurological deficits induced by the ischemia/reperfusion (I/R)  
677 injury, inhibits the release of cytochrome c, and prevents the activation of caspase-3 (Andrabi  
678 et al., 2004). This indicates that as strong anti-oxidant and anti-inflammatory agents,  
679 stilbenoids could be effective in the treatment of I/R-injury, a devastating pathology without  
680 therapeutic opportunity.

681         Despite their relatively wide range, most of the anti-inflammatory drugs available on  
682 the market are linked with many side effects when used in the long term. The therapeutic and  
683 adverse effects of the most commonly prescribed non-steroidal anti-inflammatory drugs  
684 (NSAIDs) are related to the inhibition of cyclooxygenases (COXs). Their gastrointestinal,  
685 renal, and cardiovascular toxicity, together with numerous drug interactions, represent a risk  
686 especially for elderly patients who take multiple medications (Wongrakpanich et al., 2018).

687 Steroidal drugs can have pleiotropic and severe adverse effects (Aljebab et al., 2017).  
688 Biological treatment in the form of anti-TNF- $\alpha$  and anti-IL-1 $\beta$  antibodies has been shown to  
689 be highly effective for chronic disorders such as RA, especially when treatment is started in  
690 the early phase of the disease. However, this treatment represents an enormous financial  
691 burden for the health care system and is not accessible to all patients (Dinarello et al., 2012;  
692 Monaco et al., 2015). Thus, nutritional intervention and the development of functional food  
693 based on natural molecules that are not known to have profound side effects alter promise of  
694 preventing inflammation caused by several chronic and acute diseases. Many good candidates  
695 may be hidden in natural sources. MF would be among the most promising thanks to its  
696 ability to counteract both peripheral and CNS inflammation. MF is structurally a small  
697 molecule, effective at a pharmacologically relevant concentration. Synthesizing it in the  
698 laboratory could lower its production cost as compared to existing commercial treatments.

699 MF and its derivatives could fulfill the goal of finding a novel anti-inflammatory  
700 substance that is potent in prevention and/or supplementary treatment of inflammation-based  
701 pathologies of both systemic and central character and safe for long-term use.

702

## 703 **2. Conclusion**

704 Our current study shows that stilbenoid macasiamenene F isolated from a plant *M.*  
705 *siamensis* has significant anti-inflammatory effects. It is therefore an interesting candidate for  
706 further investigation using *in vivo* models where inflammation in the brain plays a major role,  
707 *e.g.*, cerebrovascular diseases. The increasing prevalence of chronic inflammatory disorders  
708 and more frequent occurrence of acute inflammatory diseases make the need for safe and  
709 effective anti-inflammatory therapeutic strategies. Other promising candidates may yet be  
710 found in natural sources, but with respect to the current state-of-the-art in nutraceuticals, MF

711 is the most likely to prove being effective for the prevention and treatment of inflammation-  
712 related pathologies, including secondary brain injury following a stroke, for which a  
713 therapeutic opportunity is severely lacking.

714

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725

726 **Author Contributions**

727 VP and PP isolated the studied molecules. VL, CH, NB, JH and KŠ designed the research  
728 project and interpreted the study data; VL, JV performed the experiments and collected the  
729 data; SBD, CW, TC and JM assisted the animal experiments. CH and NB share the position  
730 of last author. All authors critically revised the manuscript and approved the final version of  
731 the article, including the authorship list.

732

733 **Conflict of interest**

734 The authors declare to have no conflict of interest related to this publication and there has  
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736

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