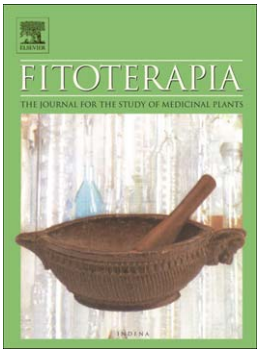


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# Anti-inflammatory and antioxidant effects of a combination of cannabidiol and moringin in LPS-stimulated macrophages

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## Abstract

Inflammatory response plays an important role in the activation and progress of many debilitating diseases. Natural products, like cannabidiol, a constituent of *Cannabis sativa*, and moringin, an isothiocyanate obtained from myrosinase-mediated hydrolysis of the glucosinolate precursor glucomoringin present in *Moringa oleifera* seeds, are well known antioxidants also endowed with anti-inflammatory activity. This is due to a covalent-based mechanism for ITC, while non-covalent interactions underlie the activity of CBD. Since these two mechanisms are distinct, and the molecular endpoints are potentially complementary, we investigated in a comparative way the protective effect of these compounds alone or in combination on lipopolysaccharide-stimulated murine macrophages. Our results show that the cannabidiol (5μM) and moringin (5μM) combination outperformed the single constituents that, at this dosage had only a moderate efficacy on inflammatory (Tumor necrosis factor-α, Interleukin-10) and oxidative markers (inducible nitric oxide synthase, nuclear factor erythroid 2-related factor 2, nitrotyrosine). Significant upregulation of Bcl-2 and downregulation of Bax and cleaved caspase-3 was observed in cells treated with cannabidiol-moringin combination. Treatment with the transient receptor potential vanilloid receptor 1 antagonist was detrimental for the efficacy of cannabidiol, while no effect was elicited by cannabinoid receptor 1 and cannabinoid receptor 2 antagonists. None of these receptors was involved in the activity of moringin. Taken together, our *in vitro* results testify the anti-inflammatory, antioxidative, and anti-apoptotic effects of the combination of cannabidiol and moringin.

**Abbreviations**

LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α, IL-1β, interleukin-1β; IFN-γ, interferon-γ IL-6, interleukin-6; NO, nitric oxide; iNOS, nitric oxide synthase; CBD, cannabidiol; THC, tetrahydrocannabinol; CB1, cannabinoid

receptor 1; CB2, cannabinoid receptor 2; TRPV1, transient receptor potential vanilloid receptor 1; GLs; glucosinolates; ITCs, isothiocyanates; GMG, glucomoringin; LPS, lipopolysaccharide; H&E, hematoxylin and eosin staining; IL-10, interleukin-10;SDS-PAGE, SDS-polyacrylamide gel electrophoresis; Nrf2, nuclear factor erythroid 2-related factor 2; TLR4, toll-like receptor 4.

***Keywords:*** cannabidiol, moringin, combination therapy, anti-inflammation, LPS, macrophages

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## 1. Introduction

Inflammation is a natural defense response that arises in any tissue against different harmful stimuli, such as chemicals, tissue damage trauma, infection or exposure to microbiological toxins, such as lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria. Inflammation can cause several diseases, including some cancers [1, 2]. Macrophages play a key role in the initiation, maintenance, and resolution of inflammatory process. During inflammation, activated macrophages secrete a number of different pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interferon-γ (IFN-γ), interleukin-6 (IL-6), and oxidative stress mediators, such as nitric oxide (NO), which is synthesized by inducible nitric oxide synthase (iNOS) [3].

*Cannabis sativa* L. (hemp) and *Moringa oleifera* are the most important folklore medicinal plants used by many cultures, particularly in South Asia, for centuries. The beneficial effects of natural compounds derived from these and other medicinal plants with anti-inflammatory and antioxidant activities in inflammatory diseases have been studied for many years [4, 5]. Cannabinoids, active compounds present in the plant *Cannabis sativa* L. (hemp), are potent anti-inflammatory agents belonging to a class of unique meroterpenoids obtained from the alkylation of an olivetollike alkyl resorcinol with a monoterpene unit [6]. Approximately 61 cannabinoids are known to exist and cannabidiol (CBD) and tetrahydrocannabinol (THC) are the two most abundant cannabinoids found in hemp. CBD exerts its anti-inflammatory function via endogenous receptors, such as cannabinoid receptor 1 (CB1), cannabinoid receptor 2 (CB2), and transient receptor potential vanilloid receptor 1 (TRPV1) [7]. Although THC has shown anti-inflammatory effects, it has been demonstrated that THC produces learning and memory impairment, hypothermia, aggressive behavior and analgesia, impairment of the prepulse inhibition of the startle reflex and catalepsy-like immobilization. Accordingly, application of THC in the clinical therapy has been strongly limited by their

psychotropic effects. On the other hand, CBD is a very promising compound, showing anti-inflammatory, immunosuppressive, analgesic, anxiolytic and anti-cancer effects without any psychotropic effects [8]. Moreover, both THC and CBD have been shown to modulate the function of immune cells, including repression of humoral response, immune cell proliferation, maturation, and migration, and antigen presentation [9].

Glucosinolates (GLs) are sulfur- and nitrogen-containing natural compounds largely present in the species of the order Brassicales. Upon hydrolysis by endogenous β-thioglucosidase called myrosinase (EC 3.2.1.147), GLs produce a number of important bioactive products, among which isothiocyanates (ITCs) are definitely the most striking [10]. ITCs are well documented for their antioxidant and neuroprotective effects [11, 12]. Glucomoringin (GMG) (4-(α-L-rhamnopyranosyloxy)benzyl is an uncommon member of the GL family present in vegetables belonging to the family Moringaceae: *Moringa oleifera* commonly called the "horse–radish tree", is the most widely distributed species in the genus Moringa [13]. The glycosylated compound 4-(α-L-rhamnopyranosyloxy)benzyl isothiocyanate (moringin), resulting from myrosinase-hydrolysis (>99%) of GMG at neutral condition, has been shown to exhibit many biological activities, including effective antioxidant, anti-inflammatory [14], and anti-tumor activities [15].

Since inflammation and oxidative stress are closely associated pathophysiological features involved in many incurable diseases, drugs with anti-inflammatory and antioxidative effects are in demand. Accordingly, the combined application of anti-inflammatory CBD and the antioxidant moringin may be a potential cocktail for therapeutic consideration. To this end, we have evaluated the anti-inflammatory, antioxidative, and anti-apoptotic effects of the combination of low doses of CBD (5μM) and moringin (5μM), in comparison with monotherapy by either drug alone, in *in vitro* lipopolysaccharide (LPS)-stimulated murine macrophage RAW cells. In addition, we investigated whether cannabinoid receptors CB1, CB2 and vanilloid TRPV1 are involved in CBD-mediated anti-inflammatory effects.

## 2. Materials and methods

### 2.1. Extraction and isolation of CBD

*Cannabis sativa* L, obtained from greenhouse cultivation at CREA-CIN, Rovigo (Italy), was collected in November 2013. The isolation and purification of cannabinoids was done in accordance with their legal status (Authorization SP/106 23/05/2013 of the Ministry of Health, Rome, Italy). Pure CBD (>99%) (**Fig. 1A**) was isolated from the tops of an Italian variety of industrial hemp (named Carmagnola) according to a standardized method of the cannabinoid purification [16] to avoid any trace of THC that could interfere in the trial or cause legal limitation.

### 2.2. Isolation and purification of GMG and myrosinase

GMG was purified from *M. oleifera* L. (fam. Moringaceae) seeds according to a method previously described [17]. In brief, GMG was purified by two sequential chromatographic steps: isolation through anion exchange chromatography, followed by gel filtration to attain purification to homogeneity. GMG was unambiguously characterized by $^{1}H$ and $^{13}C$ NMR spectrometry [18] and the purity was assessed by HPLC analysis of the desulfo-derivative according to the ISO 9167-1 method accepted by the European Union, Commission Regulation, EEC No. 1864/90 [19], yielding GMG with a purity of about 99% based on peak area value. The enzyme myrosinase was isolated from seeds of *Sinapis alba* L. as reported by Pessina et al. [20]] with some modification. The specific activity of the stock solution used in the current study was 60 U/mg of soluble protein. The enzymatic activity was 32 U/mL and the solution was kept at 4 °C in sterile saline solution at neutral pH until use. One myrosinase unit was defined as the amount of enzyme able to hydrolyze 1μmol/min of sinigrin at pH 6.5 and 37 °C [21].

### 2.3. Enzyme-catalyzed hydrolysis of GMG

GMG (1.64 mM) was dissolved in RPMI-1640 medium and cell treatment required the enzyme-catalyzed hydrolysis of the phytochemical. The action of myrosinase enzyme (0,64U/ mL) for 15 min allowed fast production of moringin, before the cell treatment (**Fig. 1B**). The total conversion of pure GMG into moringin was confirmed by HPLC analysis of the GMG desulfo-derivative, which allowed us to observe the reduction of GMG until its complete disappearance in the reaction mixture [22].

### 2.4. Cell culture conditions and drug treatment

The murine macrophage cell line RAW 264.7 was acquired from (Centro substrati cellulari, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia, Italy). Macrophage cells were cultured in monolayer using RPMI-1640 medium (CARLO ERBA, Italy) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich Co. Ltd, USA). The cells were grown in logarithmic phase at 37°C in a moisturized atmosphere of 5% $CO_2$ and 95% air. Experiments were performed with cells not surpassing 30 passages. For drug treatment, cells were grown until 70%-80% confluence followed by 2h pretreatment with either CBD (2.5 or 5μM) or moringin (2.5 or 5μM) or CBD (2.5 or 5μM)-MOR (2.5 or 5μM) cocktail (CBD-moringin). Then, the cells were stimulated with lipopolysaccharides from *Escherichia coli* 0111:B4 (LPS) (10μg/mL; Sigma-Aldrich Co Ltd, USA) for 24h by adding LPS directly into CBD-, moringin-, and CBD-moringin-treated cell culture medium. Untreated cells (CTR), moringin (2.5 or 5μM), CBD (2.5 or 5μM), GMG (10μM), and myrosinase treated cells without LPS activation were also included as controls. After LPS

stimulation, the cells were either fixed or harvested for further analyses. For antagonists study, cells were incubated for 2h with different combinations of SR141716A (CB1 antagonist; 1μM) or AM630 (CB2 antagonist; 100nM) or Capsazepine (TRPV1 antagonist; 1μM) before CBD or moringin or CBD-moringin cocktail administration. Cells treated with vehicle (0.1% DMSO) and antagonists with or without CBD, moringin, CBD-moringin, and LPS were also included as controls. All antagonists were purchased from Tocris Bioscience, UK. All the experiments were made in triplicates and repeated for three independent times.

**2.5. Immunocytochemistry**

Cells on coverslips (10 mm; Thermo SCIENTIFIC, Germany) were fixed with 4% paraformaldehyde at room temperature for 15 min followed by phosphate buffered saline (PBS, pH 7.5) washes. Morphological changes in the cells were assessed by hematoxylin and eosin staining (H&E) applying a standard protocol. For immunocytochemical staining, cells were 4% paraformaldehyde-fixed and administered with PBS-buffered 3% hydrogen peroxide (H2O2) at room temperature for 15 min to suppress the endogenous peroxidase activity. Then, cells were blocked with horse serum + 0.1% Triton X-100 for 20 min followed by incubation for overnight at 4°C with primary antibodies against examined proteins: anti-TLR-4 (1:100 Abcam), IκB-α (1:200 Cell Signaling Technology), NFκBp65 (1:200 Cell Signaling Technology), TNF-α (1:100 Cell Signaling Technology), Interleukin-10 (IL-10) (1:100 Santa Cruz Biotechnology Inc), Bax (1:100 Santa Cruz Biotechnology Inc) and Bcl-2 (1:100 Santa Cruz Biotechnology Inc) . After PBS wash, cells were incubated with biotinylated secondary antibody (1:200, Vector Laboratories, Burlingame, CA) and streptavidin ABComplex-HRP (ABC-kit from Dako, Glostrup, Denmark). The immunostaining was developed with peroxidase substrate kit DAB (Vector Laboratories, Burlingame, CA) (brown color; positive staining) and counterstaining with nuclear fast red (Vector Laboratories, Burlingame, CA) (pink background; negative staining). Microscopy was performed using light microscopy (LEICA DM 2000 combined with LEICA ICC50 HD camera). Immunocytochemistry images (n=3 photos from each group) were assessed for densitometry analysis using LEICA Application Suite V4.2.0 software to calculate the percentage of positive staining of the cells. All images are representative of three independent experiments.

**2.6. Protein extraction and western blot analysis**

Cells exposed to varied drug combination were harvested following 24h of incubation. After washing with ice-cold PBS, the cells were lysed using Buffer A [320mM Sucrose, 10mM, 1mM EGTA, 2mM EDTA, 5mM $NaN_3$, 50mM NaF, β-mercaptoethanol, and protease/phosphatase inhibitor cocktail (Roche, USA)] in ice for 15 min, followed by centrifugation at 1000g for 10 min

at 4°C. The supernatant was served as cytosolic extract. The pellet was further lysed using Buffer B [150mM NaCl, 10mM Tris-HCl (pH 7.4), 1mM EGTA, 1mM EDTA, Triton x-100, and protease/phosphatase inhibitor cocktail (Roche, USA)] in ice for 15 min, followed by centrifugation at 15,000g for 30 min at 4°C. The supernatant was collected and used as nuclear extract. Protein concentrations were calculated using the Bradford assay (Bio-Rad, USA). Twenty micrograms of proteins were heated for 5 min at 95°C, resolved by 8% or 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a PVDF membrane (Immobilon–P, Millipore, USA). Membranes were blocked in 5% skim milk in PBS for 1 h at room temperature followed by incubation for overnight at 4°C with particular primary antibodies . The following primary antibodies were used: Nuclear factor erythroid 2-related factor 2 (Nrf2; 1:200, Santa Cruz Biotechnology Inc, ), iNOS (1:500 Cell Signaling Technology), , and cleaved caspase-3 (1:500 Cell Signaling Technology) Then, membranes were washed in PBS 1X and incubated with HRP-conjugated anti-rabbit IgG secondary antibody (1:2000; Santa Cruz Biotechnology Inc) for 1 h at room temperature. To ascertain that blots were loaded with equal amounts of protein lysates, they were also incubated with antibody for GAPDH HRP Conjugated (1:1000; Cell Signaling Technology). Also, expression of cleaved-caspase 3 was normalized on expression of caspase 3 (1:500; Cell Signaling Technology). The relative expression of protein bands was visualized using an enhanced chemiluminescence system (Luminata Western HRP Substrates, Millipore) and protein bands were acquired and quantified with ChemiDoc™ MP System (Bio-Rad) and a computer program (ImageJ software) respectively. All blots are representative of three independent experiments.

### 2.7. Statistical data analysis

GraphPad Prism version 6.0 program (GraphPad Software, La Jolla, CA) was used for statistical analysis of the data. One-way ANOVA and Bonferroni's multiple comparison test were used to analyze the difference between the means of the treatment groups and the control group. Differences with a *p*-value of less than 0.05 were considered statistically significant. Results are expressed n ± SEM of n experiments.

## 3. Results and discussion

In our study, we have evaluated the protective effect of the combination of low doses of CBD and moringin, in comparison with monotherapy by either drug alone, in *in vitro* LPS-stimulated murine macrophage RAW cells. We found that a cocktail of CBD (5μM) and moringin (5μM) showed more protection than either CBD (5μM) or moringin (5μM) alone. No significant protection was

observed in LPS-activated macrophages treated with CBD (2.5µM), moringin (2.5µM) and CBD (2.5µM) and moringin (2.5µM) combination (data not shown). LPS-induced cell morphological changes, such as increase in cell size and production of lamellipodia and filopodia (**Fig.1D**), were reduced in CBD-moringin cocktail (**Fig.1G**), while CBD (**Fig.1E**) or moringin (**Fig.1F**) treatment alone produced only a medium level of protection. Interestingly, we found selective anti-inflammatory and antioxidative effects from CBD and moringin, respectively. Immunocytochemistry results showed negative staining for pro-inflammatory marker TNF-α expression in CBD (Fig. 2C) and CBD-moringin cocktail (Fig. 2E) treated LPS-activated macrophages, while moringin (Fig. 2D) showed relatively less positive staining for TNF-α than LPS control (**Fig.2B**). Dense positive staining of TNF-α was noticed in LPS control (Fig. 2B), while normal untreated cells (Fig. 2A) showed negative staining. Anti-inflammatory marker IL-10 showed positive staining in LPS-stimulated cells treated with CBD alone (Fig. 3C) and a combination of CBD-moringin (Fig. 3E), while moringin alone (Fig. 3D) showed partial positive staining for IL-10 upregulation. Normal untreated cells (Fig. 3A) and LPS control cells (Fig. 3B) showed negative staining for IL-10. Similar modulatory effect of CBD on inflammatory markers TNF-α and IL-10 has been demonstrated in different *in vitro* and *in vivo* models [24-26]. We suppose that the significant modulation of these inflammatory markers in CBD-moringin treated LPS-activated macrophages was prominently induced by CBD and not by moringin. However, our results showed an average anti-inflammatory effects of moringin at low dose of 5µM. On the contrary, LPS-induced oxidative stress markers, such as iNOS, Nrf2 and nitrotyrosine were markedly regulated by moringin alone and CBD-moringin combination. Negative immunostaining was observed for nitrotyrosine in moringin alone (Fig. 4D) and CBD-moringin treatment (Fig. 4E), while partial positive staining was noticed in CBD alone (Fig. 4C) treatment. LPS control (Fig. 4B) showed marked positive staining for nitrotyrosine.. Western blot results revealed that moringin alone and CBD-moringin in combination significantly enhanced the nuclear level of antioxidative marker Nrf2 in LPS-activated macrophages (;, while CBD alone treatment increase Nrf2 expression but less than CBD-moringin in combination (Fig. 5A). iNOS level (Fig. 5B) was elevated in LPS-activated macrophages, while moringin alone and a combination of CBD-moringin showed no expression for iNOS.. CBD alone treatment downregulated LPS-activated iNOS level. These results demonstrated the potential antioxidative effect of moringin over CBD, even at low doses. Our findings also suggest that the significant reduction of these oxidative stress markers in CBD-moringin treated LPS-activated macrophages might be resulted from the effect of moringin.

Then, we assessed the modulatory effect of CBD, moringin, and a combination of CBD-moringin in LPS-induced Toll-like receptor 4 (TLR4) pathway. Toll-like receptors (TLRs) are a family of cell

surface receptors consisting of at least 11 members (TLR1-9), differentially expressed on leukocyte subsets and nonimmune cells [27]. TLRs control key features of innate and adaptive immune responses by identifying endogenous cues of tissue damage as well as pathogen-associated molecular patterns. Upon stimulation, TLRs activate the expression of inflammatory cytokines via MyD88-dependent and MyD88-independent signaling pathways. TLR4 is the receptor for the endotoxin LPS of gram-negative bacteria. TLR4, upon binding with LPS, recruits MyD88 and TRIF proteins, which in turn mediate IκB-α degradation and NFκB activation [28]. LPS-stimulated activation of TLR4 and NFκB transcription factor has been reported in macrophages [29]. In our study, we found that both CBD (Fig. 6C) and moringin (Fig. 6D) downregulated the expression of pro-inflammatory markers TLR-4 and NFkBp65 (Fig. 8 C and D). Immunocytochemistry results showed dense immunolocalization for TLR-4 (**Fig. 6B**) and NFκBp65 (**Fig.8B**) in LPS-stimulated macrophages. Treatment with CBD (5μM) or moringin (5μM) monotherapy showed relatively less immunolocalization for TLR4 (Fig.6 C and D respectively) as well as NFκBp65 (Fig. 8 C and D respectively) than LPS control (Fig.6 B and Fig. 8 B) , while a combination of CBD (5μM)-moringin (5μM) showed negative immunolocalization for TLR-4 (Fig. 6 E) and NFκBp65 (Fig. 8 E) , which proposed the plausible additive anti-inflammatory effect of CBD and moringin in combination. On the contrary, significant dense immunolocalization of IκB-α was noticed in a combination of CBD-moringin (Fig. 7 E), although CBD (Fig. 7 C) and moringin alone (Fig. 7 D) treatment also partially increased IκB-α expression. LPS-induced macrophages (Fig. 7 B) showed negative immunolocalization for IκB-α. Moreover, we noticed that the additive anti-inflammatory effect produced by a combination of CBD-moringin was reflected together with apoptosis inhibition. LPS-induced apoptosis induction has been demonstrated in macrophages and other type of cells [30].

Our immunocytochemistry results showed positive staining for Bax in LPS-induced macrophages (Fig. 9B), downregulated by CBD-moringin combination (Fig.9E);. CBD (Fig. 9C) or moringin (Fig. 9D) monotherapy also reduced the expression of Bax, but less than that of CBD-moringin in combination. In addition, it was found a negative staining of Bcl-2 in LPS-induced macrophages (Fig. 10B), while CBD-moringin in combination (Fig. 10E) showed significant positive staining. CBD (Fig. 10C) or moringin (Fig. 10D) monotherapy also partly enhanced the expression of Bcl-2, but less than that of CBD-moringin in combination. Finally, western blot results showed that monotherapy treatment with moringin and a combination of CBD-moringin decreased cleaved caspase-3 expression in LPS-activated macrophages. CBD alone did not reduce cleaved caspase-3 expression level compared to LPS control (Fig. 11B). . These results indicated the additive anti-inflammatory and anti-apoptotic effects of low dose CBD-moringin drug combination in LPS-

stimulated macrophages. Similar approach of low dose CBD-moringin combination in *in vivo* models for inflammatory diseases may render more preclinical evidence.

In our study, we further investigated whether cannabinoid receptors CB1, CB2 and vanilloid TRPV1 are involved in the anti-inflammatory effect of CBD. Our results demonstrated that CBD might act preferably via TRPV1 receptors since TRPV1 antagonist pretreatment blocked the protective effect of CBD in LPS-stimulated macrophages, while CB2 and CB1 antagonists showed relatively less inhibition towards the protective role of CBD (**Fig.12A**). Furthermore, TRPV1 antagonist and cocktail of CB1/CB2/TRPV1 antagonists terminated the protection elicited by CBD-moringin in combination (**Fig.12C**), which suggested the regulatory role of TRPV1 receptor for CBD-moringin combination. Our results are in line with the previous reports, where it has been demonstrated that CBD does not exhibit high affinity for the CB1 or CB2 receptors [31], and that CBD may exert anti-inflammatory effects via TRPV1 receptors [32]. However, moringin-mediated protection was not affected by CB1, CB2, and TRPV1 antagonists (**Fig.12B**), which implied that the defensive antioxidative effect of moringin in LPS-activated macrophages might depend on other receptors, and not depend on CB1, CB2 and TRPV1 receptors.

## 4. Conclusions

Overall, our *in vitro* findings show additive anti-inflammatory effect of CBD and moringin mix in LPS-activated mouse macrophages. Moreover, our results suggest that combination of low dose CBD-moringin drug might be a promising therapeutical approach for inflammatory diseases.

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## Conflict of Interest

Authors declare no conflict of interest.

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**Figure legends**

**Figure 1**. Low dose CBD-moringin combination treatment inhibits LPS-stimulated morphological changes and cell death in murine macrophages RAW 264.7. A) Chemical structure of CBD. B) Production of moringin (4-(α-L-rhamnopyranosyloxy)benzyl isothiocyanate) from myrosinase-catalyzed hydrolysis of glucomoringin (GMG). C) Eosin/hematoxylin (E/H) staining of RAW 264.7 cells untreated (CTR) (C), incubated with LPS (LPS) (D), incubated with LPS and treated with 5 µM CBD (LPS + CBD) (E), incubated with LPS and treated with 5 µM moringin (LPS + moringin) (F), or incubated with LPS and treated with 5 µM CBD +5 µM moringin (LPS + CBD + moringin) (G). All images were acquired at 40x (size bar 50 µm). Arrows indicate the points of connections among the cells.

**Figure 2**. Immunohistochemical analysis for TNF-α. in RAW 264.7 cells untreated (CTR) (A), incubated with LPS (LPS) (B), incubated with LPS and treated with 5µM CBD (LPS + CBD) (C), incubated with LPS and treated with 5µM moringin (LPS + moringin) (D), and incubated with LPS and treated with 5 µM CBD + 5 µM moringin (LPS + CBD + moringin) (E). All images were acquired at 40x (size bar 50 µm). Arrows indicate the points of connections among the cells. Densitometric analysis for TNF-α (F), *p*****< 0.0001 *vs* LPS, *p*****< 0.001 *vs* LPS + CBD, *p*****< 0.001 *vs* LPS + moringin, *p*****< 0.001 *vs* LPS + CBD + moringin. ND not detectable.

**Figure 3.** Immunohistochemical analysis for IL-10 in RAW 264.7 cells untreated (CTR) (A), incubated with LPS (LPS) (B), incubated with LPS and treated with 5µM CBD (LPS + CBD) (C), incubated with LPS and treated with 5µM moringin (LPS + moringin) (D), and incubated with LPS and treated with 5 µM CBD + 5 µM moringin (LPS + CBD + moringin) (E). All images were acquired at 40x (size bar 50 µm). Arrows indicate the points of connections among the cells. Densitometric analysis for IL-10 (F), *p*****< 0.001 vs LPS + CBD, *p*****< 0.001 vs LPS + moringin, *p*****< 0.001 vs LPS + CBD + moringin.

**Figure 4.** Immunohistochemical analysis for nitrotyrosine in RAW 264.7 cells untreated (CTR) (A), incubated with LPS (LPS) (B), incubated with LPS and treated with 5µM CBD (LPS + CBD) (C), incubated with LPS and treated with 5µM moringin (LPS + moringin) (D), and incubated with LPS and treated with 5 µM CBD + 5 µM moringin (LPS + CBD + moringin) (E). All images were acquired at 40x (size bar 50 µm). Arrows indicate the points of connections among the cells. Densitometric analysis for nitrotyrosine (F), *p*****< 0.0001 vs LPS, *p*****< 0.001 vs LPS + CBD, *p*****< 0.001 vs LPS + moringin, *p*****< 0.001 vs LPS + CBD + moringin.

**Figure 5**. Western blot analysis for Nrf2 (A); *p*****< 0.0001 vs LPS, *p*****< 0.001 vs LPS + CBD, *p*****< 0.001 vs LPS + moringin, *p*****< 0.001 vs LPS + CBD + moringin. Western blot analysis

for iNOS (B); *p*****< 0.0001 vs LPS, *p****=0.0002 vs LPS + CBD, *p*****< 0.001 vs LPS + moringin, *p*****< 0.001 vs LPS + CBD + moringin. GAPDH was used to normalize the signal. ND not detectable.

**Figure 6.** Immunohistochemical analysis for TLR4 in RAW 264.7 cells untreated (CTR) (A), incubated with LPS (LPS) (B), incubated with LPS and treated with 5μM CBD (LPS + CBD) (C), incubated with LPS and treated with 5μM moringin (LPS + moringin) (D), and incubated with LPS and treated with 5 μM CBD + 5 μM moringin (LPS + CBD + moringin) (E). All images were acquired at 40x (size bar 50 μm). Arrows indicate the points of connections among the cells. Densitometric analysis for TLR4 (F) *p*****< 0.0001 vs LPS, *p*****< 0.001 vs LPS + CBD, *p*****< 0.001 vs LPS + moringin, *p*****< 0.001 vs LPS + CBD + moringin.

**Figure 7.** Immunohistochemical analysis for IkBα in RAW 264.7 cells untreated (CTR) (A), incubated with LPS (LPS) (B), incubated with LPS and treated with 5μM CBD (LPS + CBD) (C), incubated with LPS and treated with 5μM moringin (LPS + moringin) (D), and incubated with LPS and treated with 5 μM CBD + 5 μM moringin (LPS + CBD + moringin) (E). All images were acquired at 40x (size bar 50 μm). Arrows indicate the points of connections among the cells. Densitometric analysis for IkBα (F), *p*****< 0.0001 vs LPS, *p*****< 0.001 vs LPS + CBD, *p*****< 0.001 vs LPS + moringin, *p*****< 0.001 vs LPS + CBD + moringin. ND not detectable.

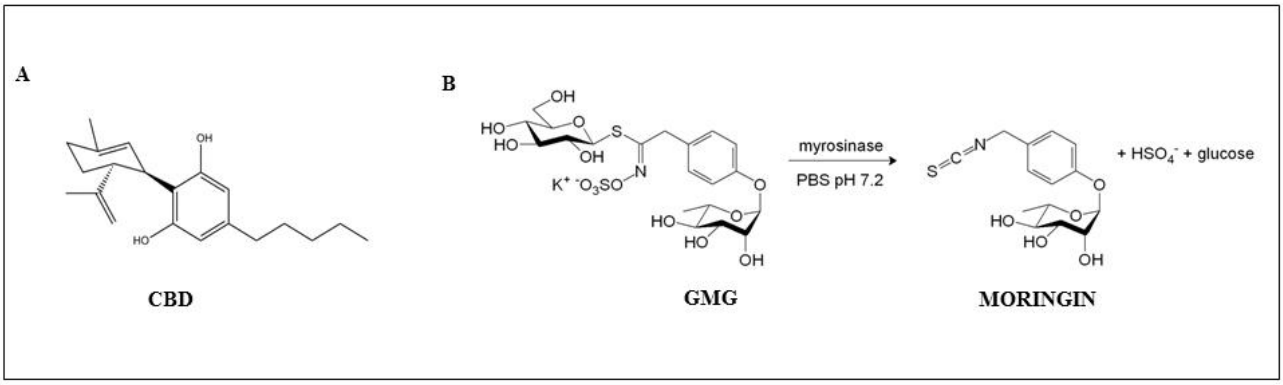
**Figure 8.** Immunohistochemical analysis for NFkB in RAW 264.7 cells untreated (CTR) (A), incubated with LPS (LPS) (B), incubated with LPS and treated with 5μM CBD (LPS + CBD) (C), incubated with LPS and treated with 5μM moringin (LPS + moringin) (D), and incubated with LPS and treated with 5 μM CBD + 5 μM moringin (LPS + CBD + moringin) (E). All images were acquired at 40x (size bar 50 μm). Densitometric analysis for NFkB (F), *p*****< 0.0001 vs LPS, *p*****< 0.001 vs LPS + CBD, *p*****< 0.001 vs LPS + moringin, *p*****< 0.001 vs LPS + CBD + moringin. ND not detectable.

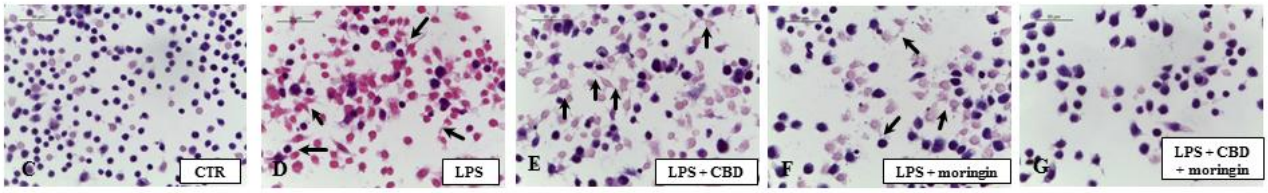
**Figure 9.** Immunohistochemical analysis for Bax in RAW 264.7 cells untreated (CTR) (A), incubated with LPS (LPS) (B), incubated with LPS and treated with 5μM CBD (LPS + CBD) (C), incubated with LPS and treated with 5μM moringin (LPS + moringin) (D), and incubated with LPS and treated with 5 μM CBD + 5 μM moringin (LPS + CBD + moringin) (E). All images were acquired at 40x (size bar 50 μm). Arrows indicate the points of connections among the cells.

**Figure 10.** Immunohistochemical analysis for Bcl-2 in RAW 264.7 cells untreated (CTR) (A), incubated with LPS (LPS) (B), incubated with LPS and treated with 5μM CBD (LPS + CBD) (C), incubated with LPS and treated with 5μM moringin (LPS + moringin) (D), and incubated with LPS and treated with 5 μM CBD + 5 μM moringin (LPS + CBD + moringin) (E). All images were acquired at 40x (size bar 50 μm). Arrows indicate the points of connections among the cells.

**Figure 11.** Densitometric analysis for Bax/Bcl-2 Ratio (A), *p***= 0.0039 vs LPS, *p*****< 0.001 vs LPS + CBD, *p*****< 0.001 vs LPS + moringin, *p*****< 0.001 vs LPS + CBD + moringin. ND not detectable. B) Western blot analysis for cleaved caspase-3. *p*****< 0.0001 vs LPS, *p*****< 0.001 vs LPS + CBD, *p*****< 0.001 vs LPS + moringin, *p*****< 0.001 vs LPS + CBD + moringin. Caspase 3 was used to normalize the signal.

**Figure 12.** CBD acts preferably via TRPV1 receptor in LPS-stimulated murine macrophages RAW 264.7. Macrophages were incubated with CB1 (1 μM), CB2 (100 nM) and TRPV1 (1 μM) antagonists before CBD and LPS administration. Eosin/hematoxylin (E/H) staining for RAW 264.7 incubated with LPS and treated with 5 μM CBD (LPS + CBD) (A), incubated with LPS and treated with 5 μM moringin (LPS + moringin) (B) and incubated with LPS and treated with 5 μM CBD + 5 μM moringin (LPS + CBD + moringin) (C). All images were acquired at 40x (size bar 50 μm).

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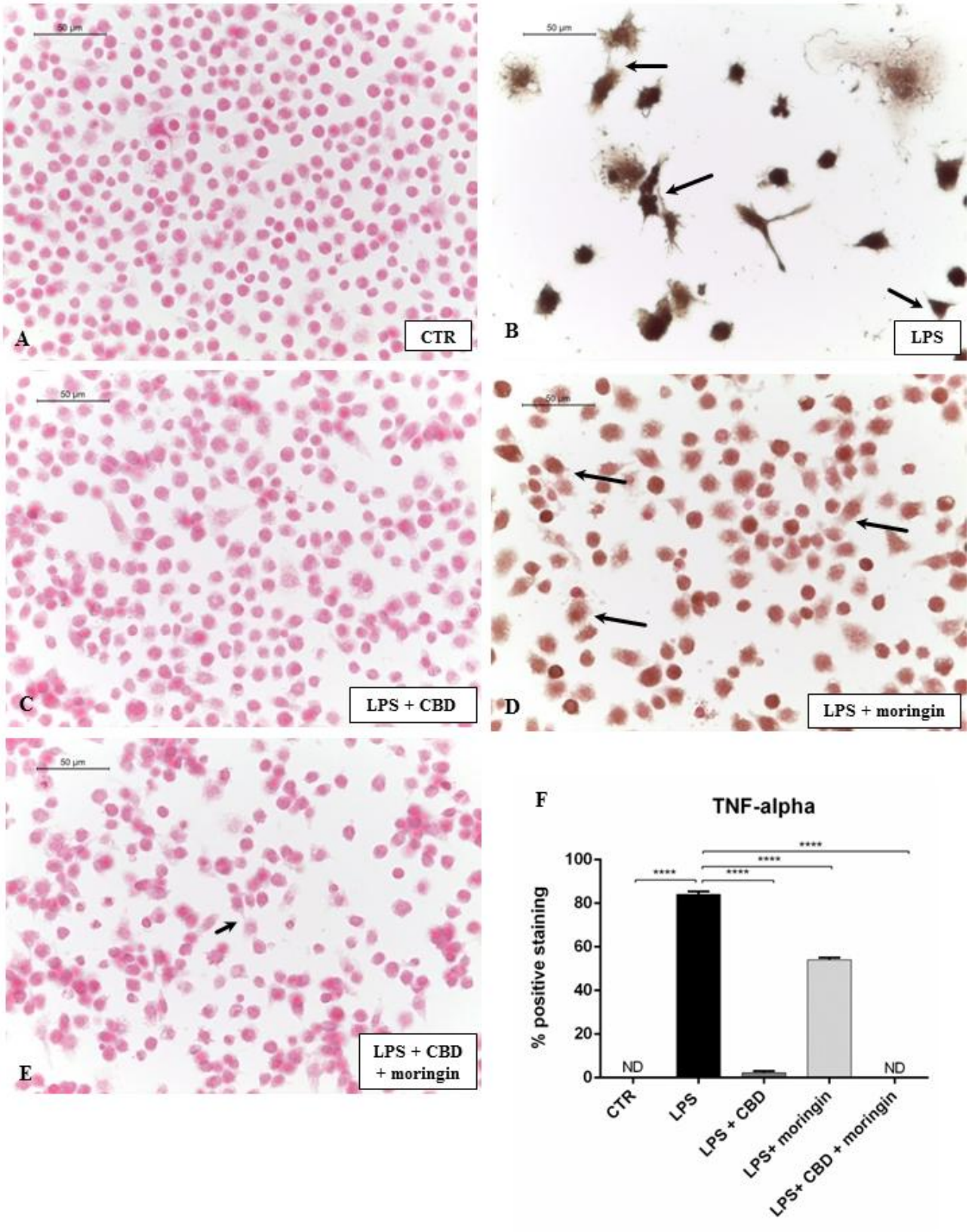

A
CTR
B
LPS
C
LPS + CBD
D
LPS + moringin
E
LPS + CBD
+ moringin
F
TNF-alpha
% positive staining
100
80
60
40
20
0
ND
ND
****
****
****
****
CTR
LPS
LPS + CBD
LPS+ moringin
LPS+ CBD + moringin

**Figure 2**

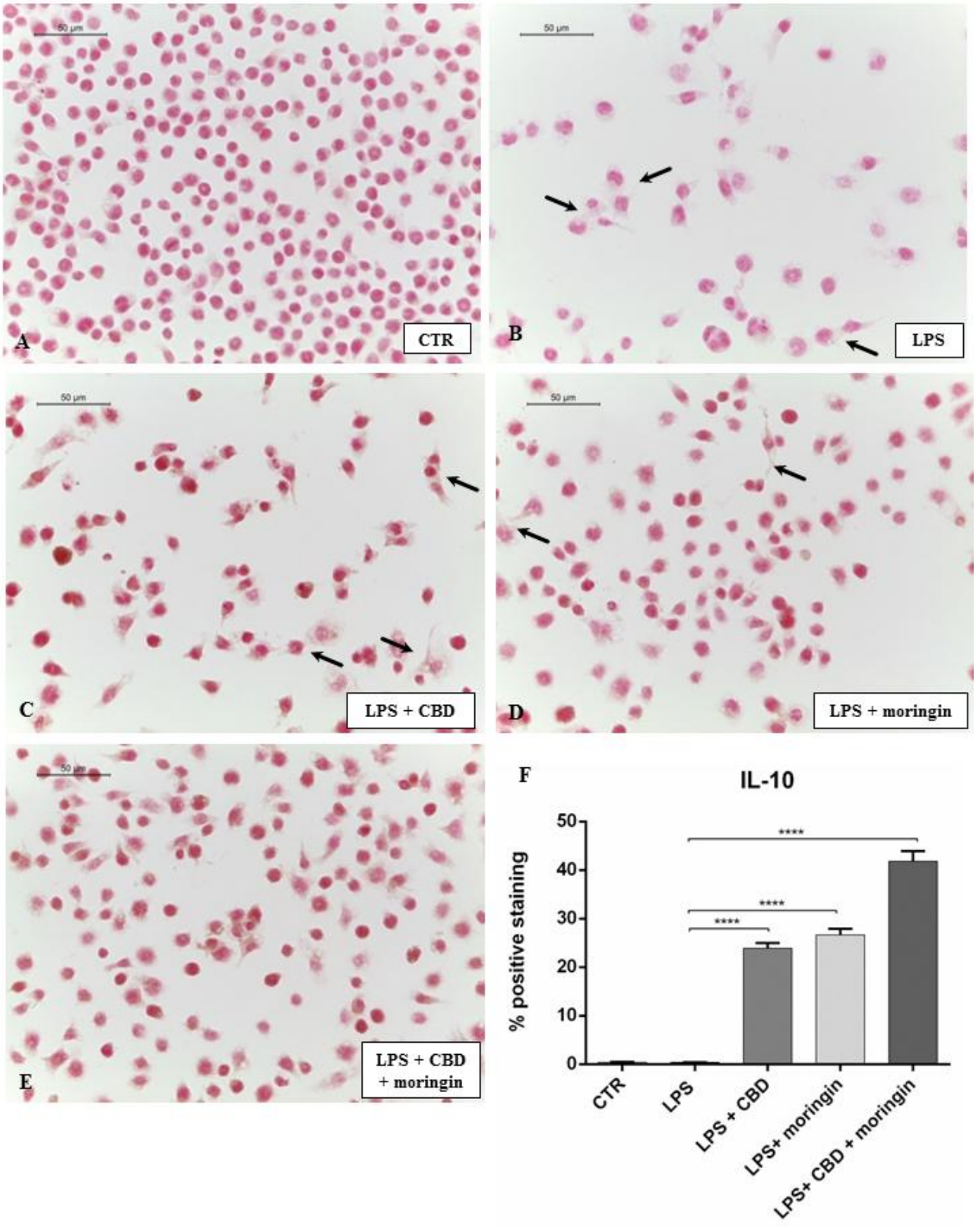

A
CTR
B
LPS
C
LPS + CBD
D
LPS + moringin
E
LPS + CBD
+ moringin
F
IL-10
% positive staining
50
40
30
20
10
0
****
****
****
CTR
LPS
LPS + CBD
LPS+ moringin
LPS+ CBD + moringin

**Figure 3**

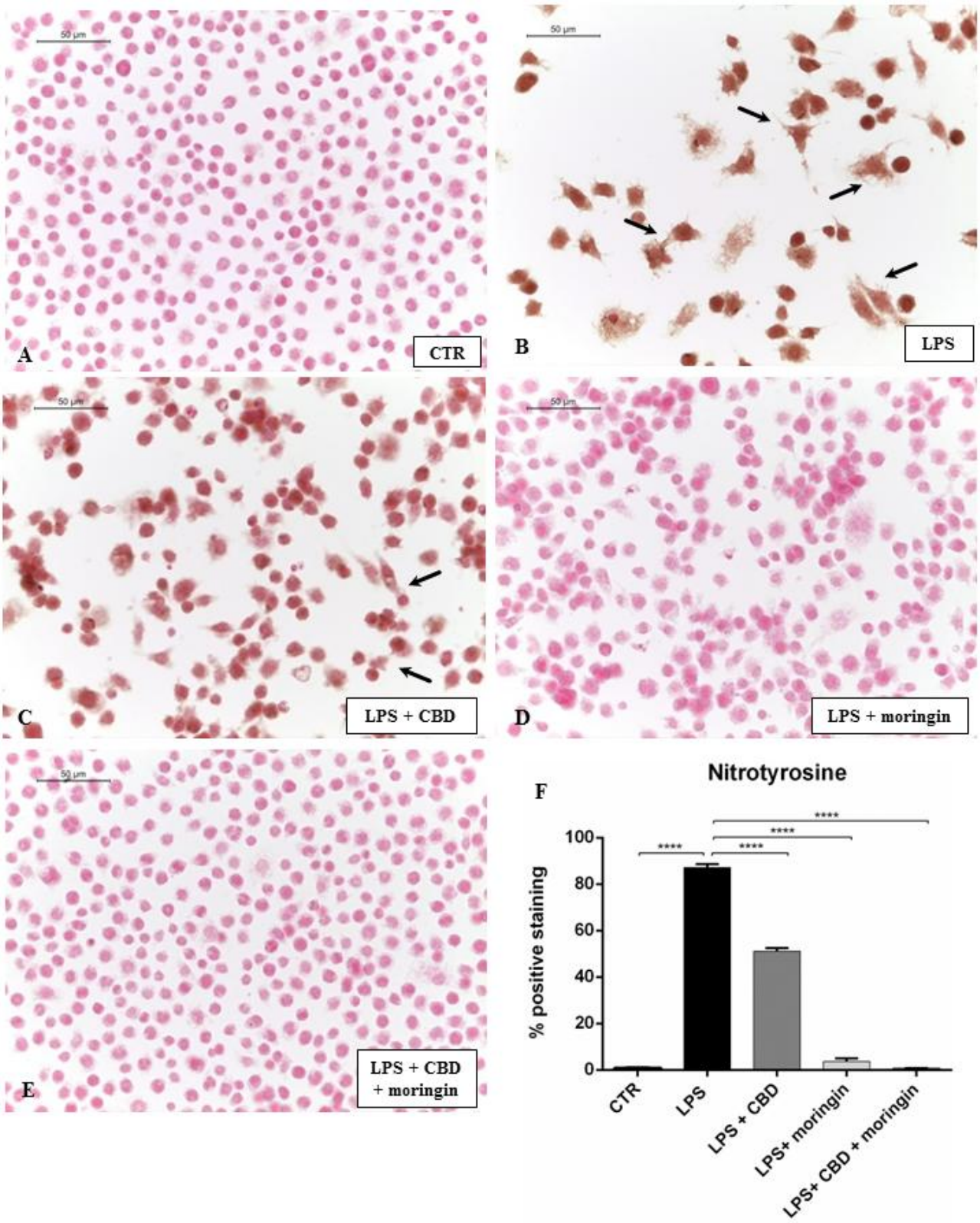
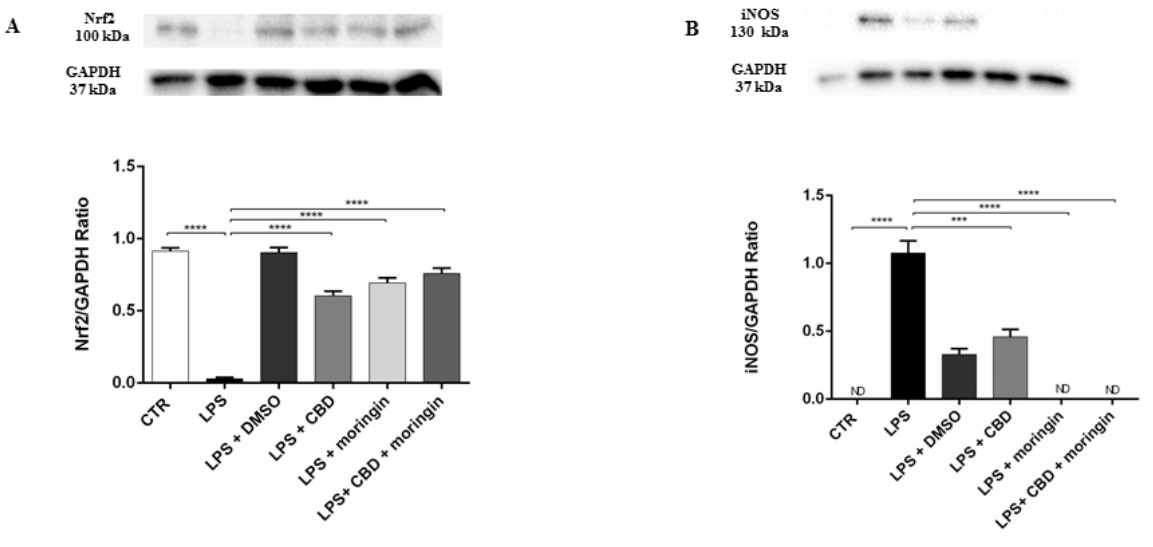

A
CTR
B
LPS
C
LPS + CBD
D
LPS + moringin
E
LPS + CBD
+ moringin
F
Nitrotyrosine
% positive staining
100
80
60
40
20
0
****
****
****
****
CTR
LPS
LPS + CBD
LPS+ moringin
LPS+ CBD + moringin

**Figure 4**

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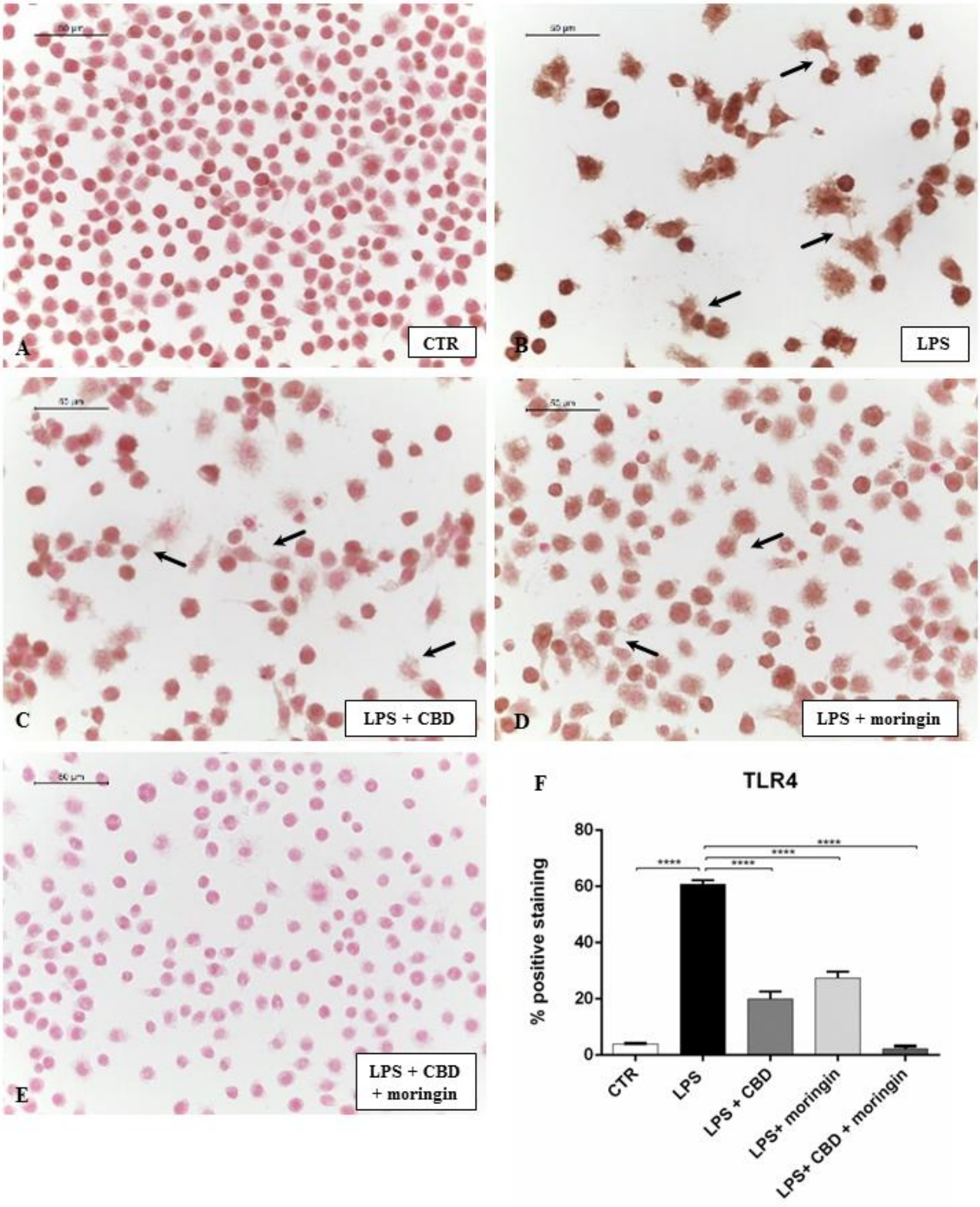

A
CTR
B
LPS
C
LPS + CBD
D
LPS + moringin
E
LPS + CBD
+ moringin
F
TLR4
% positive staining
80
60
40
20
0
****
****
****
****
CTR
LPS
LPS + CBD
LPS+ moringin
LPS+ CBD + moringin

**Figure 6**

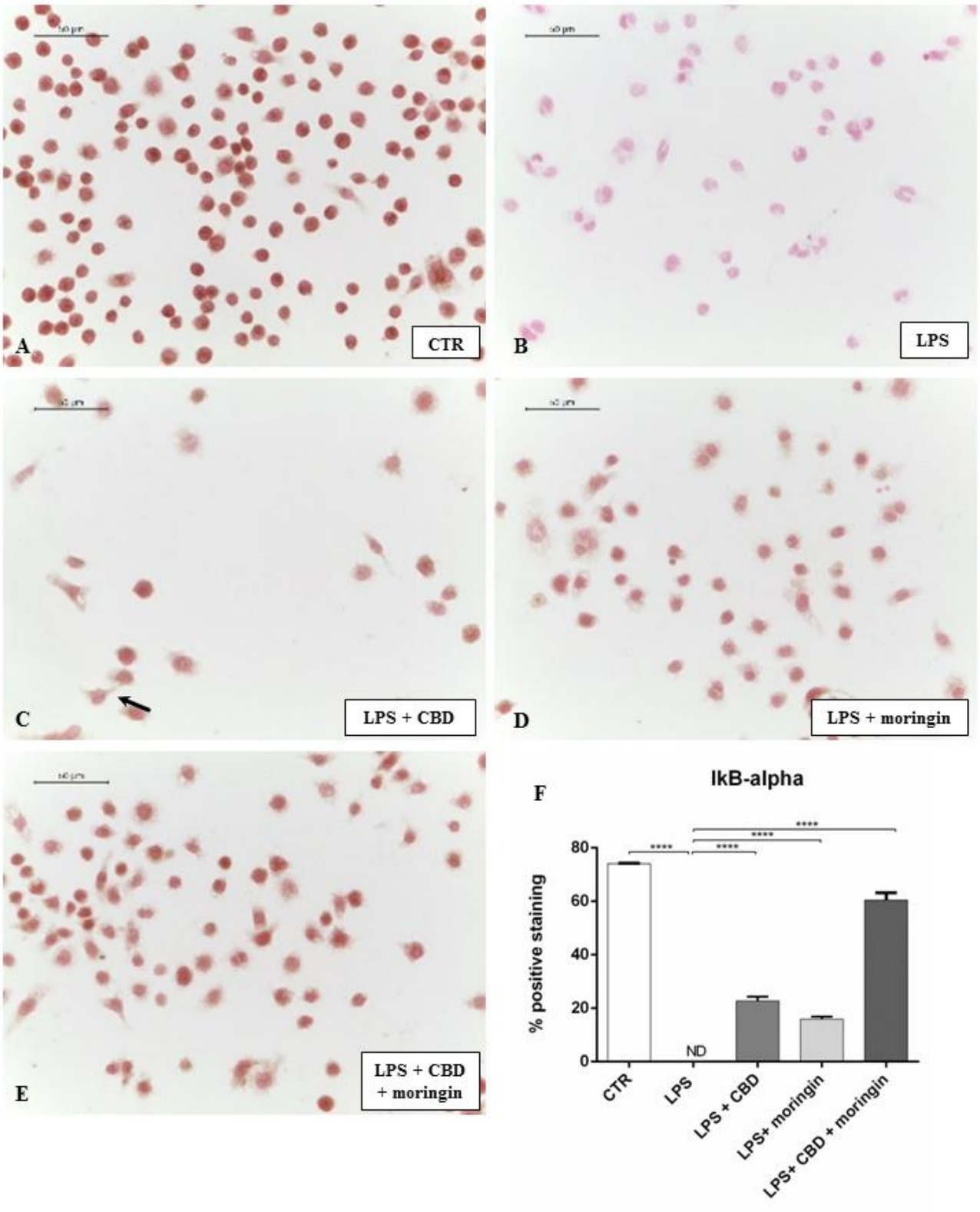



**Figure 7**

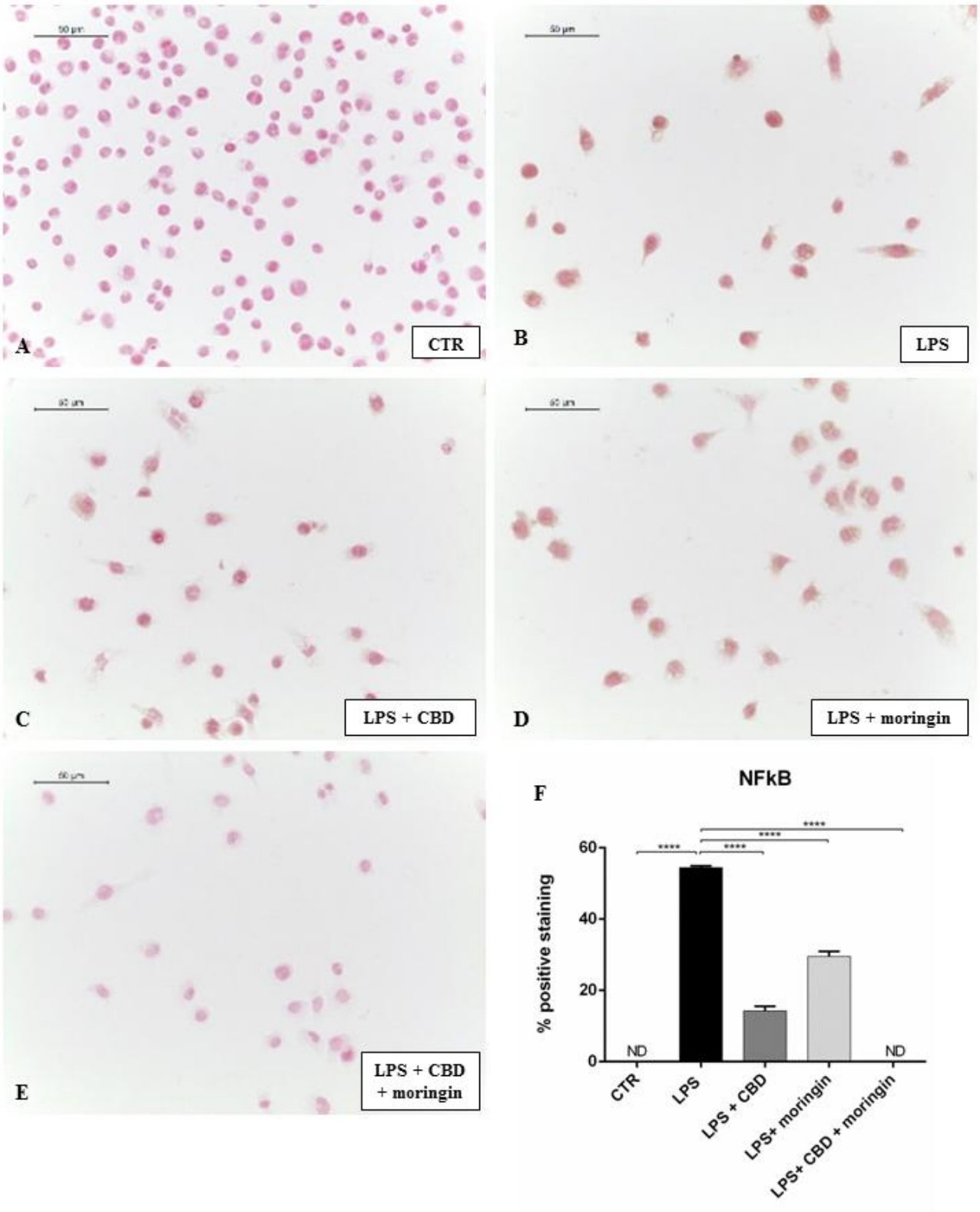

A
CTR
B
LPS
C
LPS + CBD
D
LPS + moringin
E
LPS + CBD
+ moringin
F
NFkB
% positive staining
60
40
20
0
ND
ND
****
****
****
****
CTR
LPS
LPS + CBD
LPS+ moringin
LPS+ CBD + moringin

**Figure 8**

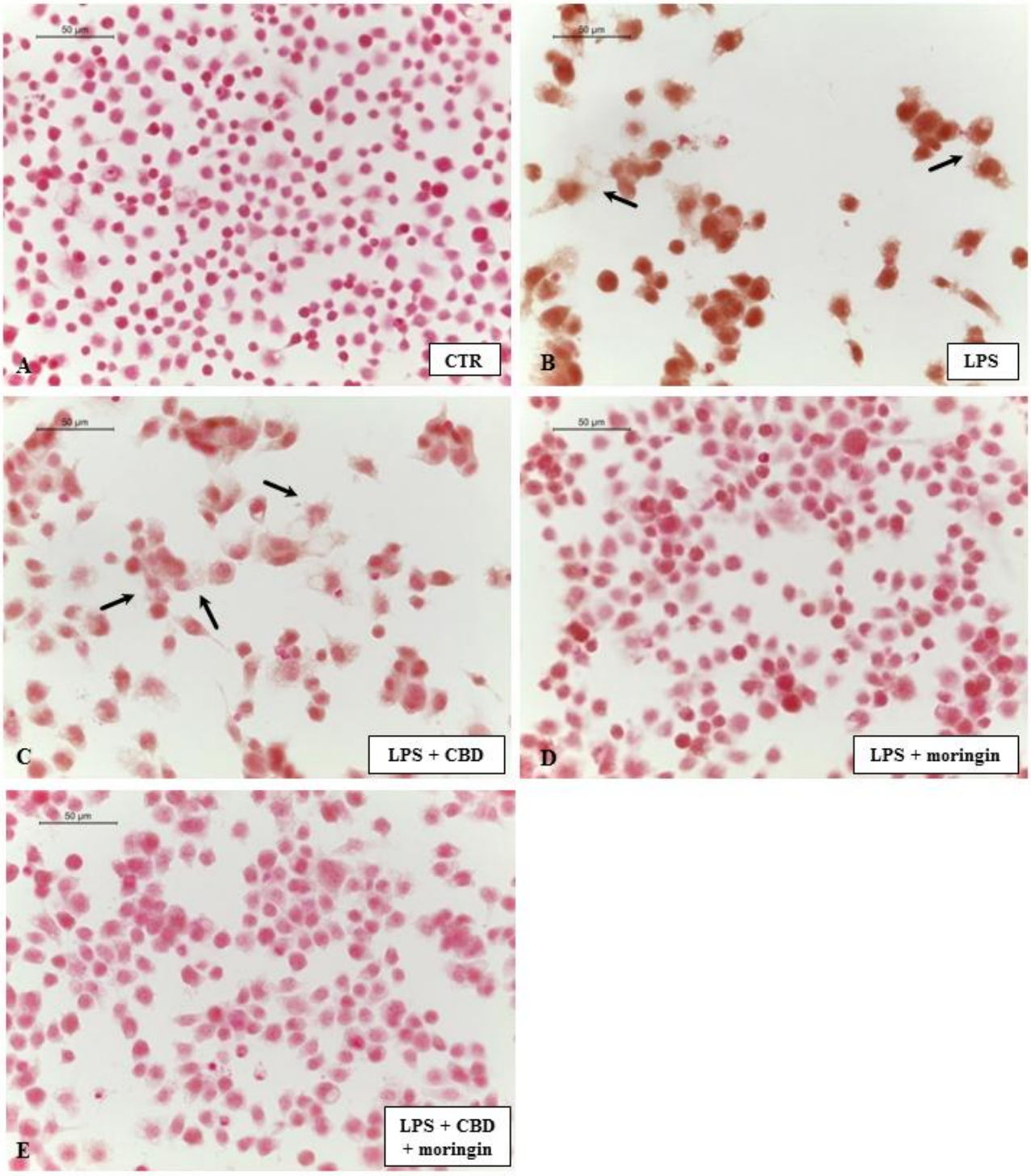
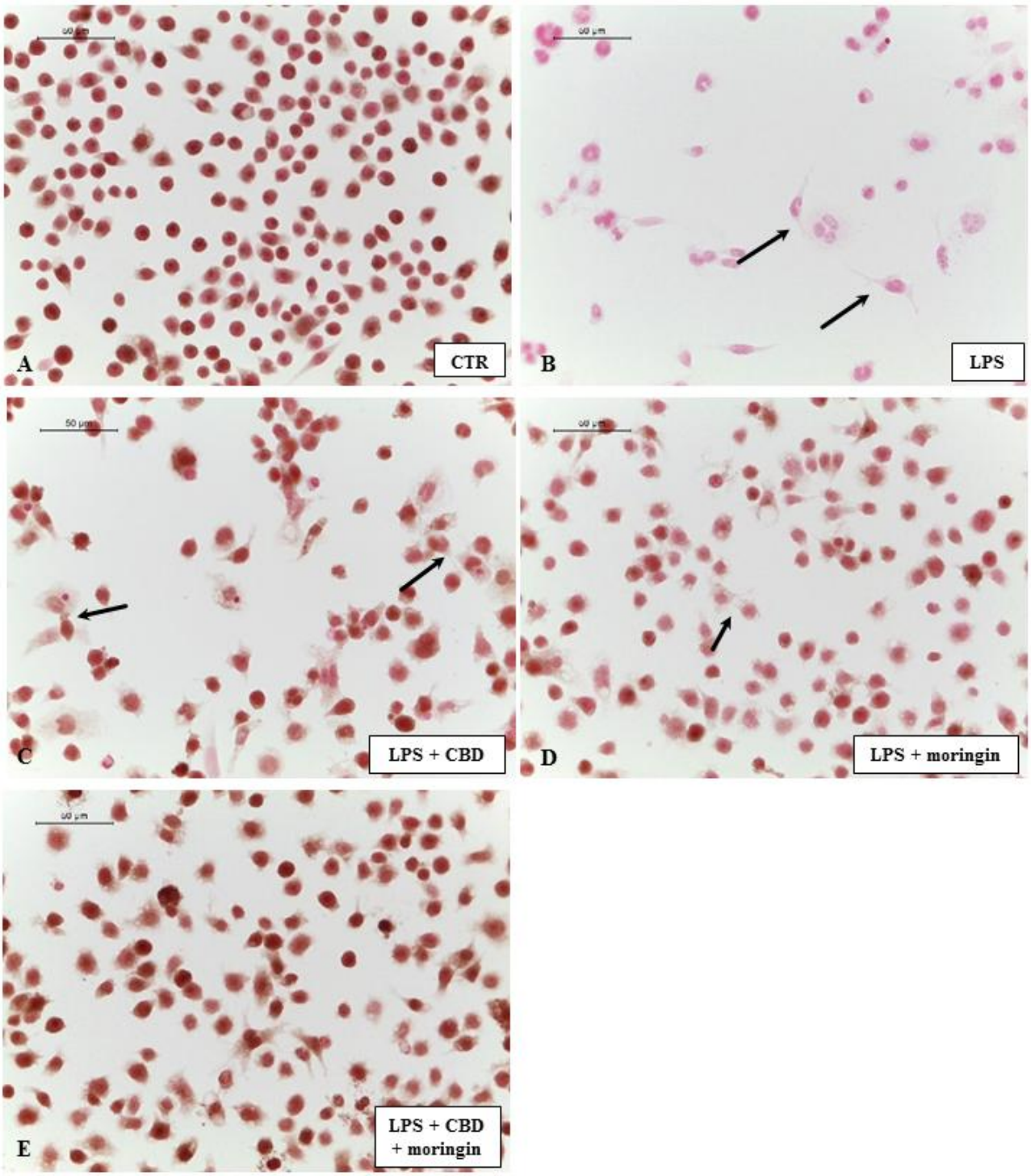



**Figure 9**

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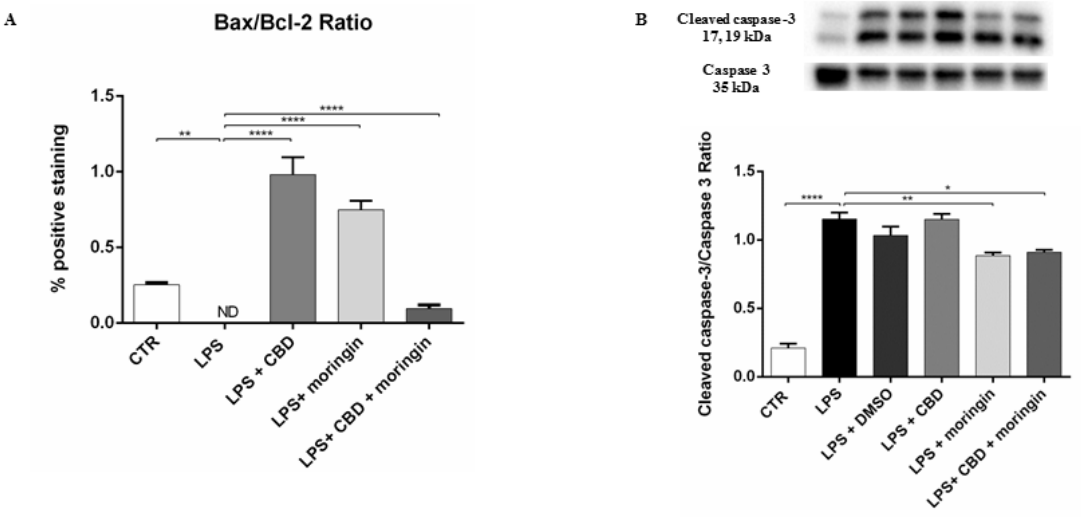


**Figure 11**

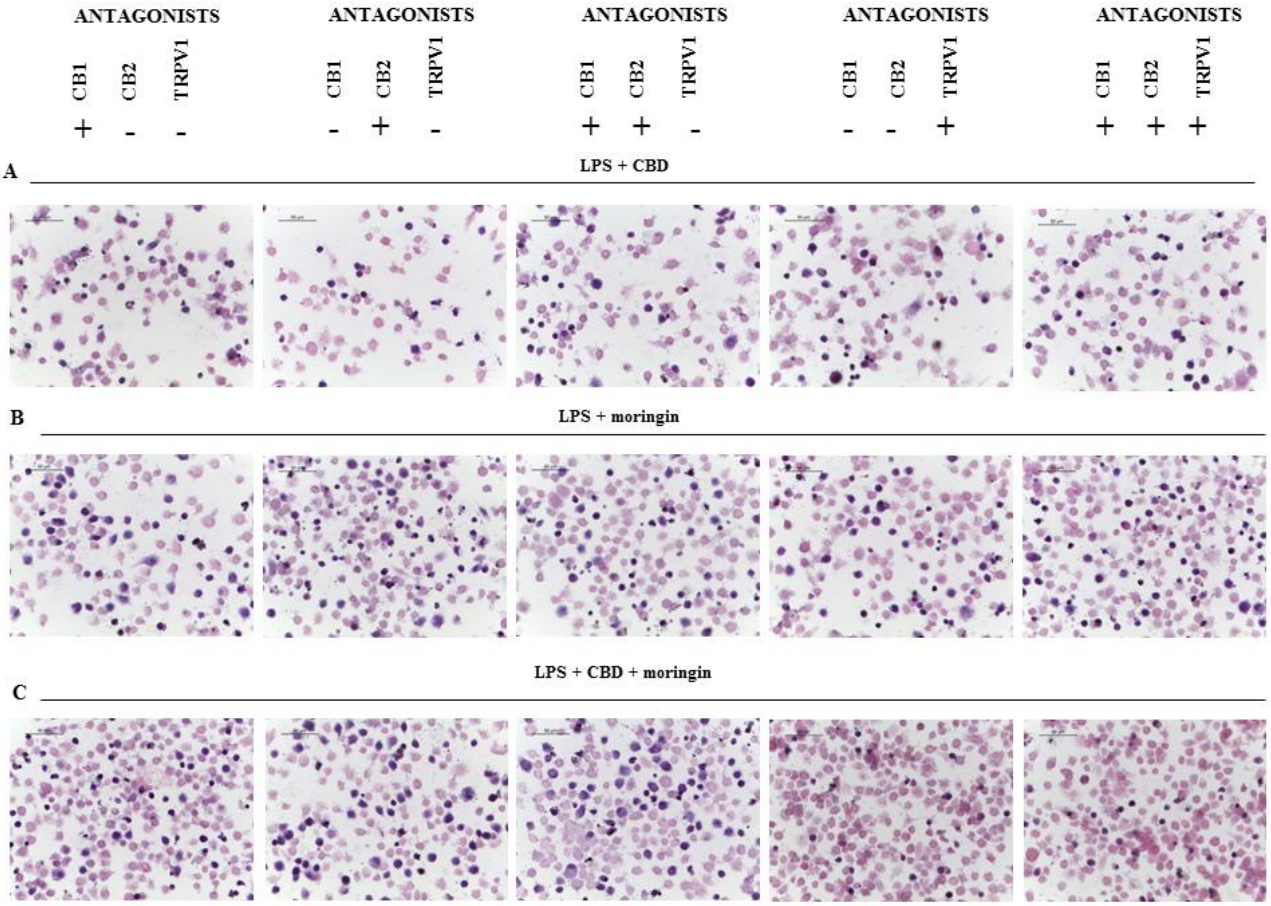


Figure 12

Graphical abstract