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The protective effects of β-caryophyllene on LPS-induced primary microglia M_1/M_2 imbalance: A mechanistic evaluation

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ABSTRACT

Aims: Neuroinflammation is observed as a routine characterization of neurodegenerative disorders such as dementia, multiple sclerosis (MS) and Alzheimer's diseases (AD). Scientific evidence propounds both of the neuromodulatory and immunomodulatory effects of CB_2 in the immune system. β-Caryophyllene (BCP) is a dietary selective CB_2 agonist, which deserves the anti-inflammatory and antioxidant effects at both low and high doses through activation of the CB_2 receptor.

Methods: In this study, we investigated the protective effects of a broad range concentration of BCP against LPS-induced primary microglia cells inflammation and M_1/M_2 imbalance and identifying the portion of the involvement of related signaling pathways on BCP effects using pharmacological antagonists of CB_2, PPAR-γ, and sphingomyelinase (SMase).

Key findings: The protective effects of BCP on LPS-induced microglia imbalance is provided by the M_2 healing phenotype of microglia, releasing the anti-inflammatory (IL-10, Arg-1, and urea) and anti-oxidant (GSH) parameters and reducing the inflammatory (IL-1β, TNF-α, PGE_2, iNOS and NO) and oxidative (ROS) biomarkers. Moreover, we showed that BCP exerts its effects through CB_2 receptors which overproduction of ceramides by SMase at middle to higher concentrations of BCP reduce the protective activity of BCP and results in the activation of the PPAR-γ pathway.

Significance: In conclusion, the low concentration of BCP has higher selective anti-inflammatory effects rather than high levels. On this occasion, BCP by modulating the microglia is able to have potential therapeutic effects in neuro-inflammation conditions and microglia cells such as MS and AD.

1. Introduction

Inflammation is generally considered as one of the earliest and complex biological responses of the immune system against infection and injuries. Inflammation may be initiated by the release of chemical factors by injured cells to construct a kind of physical barrier to the diffuse of infection and to promote healing of damaged tissue following the clearance of pathogen. In the context, innate immunity cells mainly macrophages initiate acute inflammation which in chronic conditions the prolonged immune reactions impairs healing processes, leading to cells or tissues damage and creating the pains [1,2]. In central nervous system (CNS), neuroinflammation is obviously observed as a routine characterization and core stone of neurodegenerative disorders such as dementia, multiple sclerosis (MS) and Alzheimer's disease (AD) [3].

Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; IBMX, 3-isobutyl-1-methylxanthine; AD, Alzheimer's disease; Arg-1, arginase-1; CNS, central nervous system; cAMP, cyclic adenosine monophosphate; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; DTNB, 2,2′-dinitro-5,5′-dithiodibenzoic acid; EAE, experimental autoimmune encephalomyelitis; FBS, fetal bovine serum; FSK, forskolin; GPCR, G protein-coupled receptors; HO-1, heme oxygenase-1; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HMGB1, high mobility group box 1; IMP, imipramine; iNOS, inducible nitric oxide synthase; IL, interleukin; LPS, lipopolysaccharide; MPP^+, 1-methyl-4-phenylpyridinium; MS, multiple sclerosis; NO, nitric oxide; Nrf2, nuclear factor erythroid 2-related factor 2; NFκB, nuclear factor κB; ANOVA, one-way analysis of variance; OGD/R, oxygen-glucose deprivation and re-oxygenation; PPAR-γ, peroxisome proliferator-activated receptor-gamma; PMSF, phenylmethane sulfonyl fluoride; PBS, phosphate-buffered saline; KPE, potassium phosphate buffer; PGE_2, prostaglandin E_2; *p*, P values; ROS, reactive oxygen species; T_{reg}, regulatory T-cells; SMase, sphingomyelinase; SIF, stock isotonic Ficoll; TLR-4, Toll-like receptor-4; GSH, total SH groups; TNF-α, tumor necrosis factor-α; CB_1, type 1 cannabinoid receptor; M_1, type 1 macrophage; CB_2, type 2 cannabinoid receptor; M_2, type 2 macrophage; BCP, β-caryophyllene; Aβ, β-amyloid

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Due to the inaccessibility of adaptive immune system, CNS resident macrophage cells, microglia, represent a crucial role to protect and defend against various injury and harmful stimuli including pathogens, physical and chemical irritants, and damages. Microglia as one of the professional phagocyte cells can be usually classified into two main groups of inflammatory and anti-inflammatory cells regarding to their metabolism and secretory mediators that named type 1 (M_1) and type 2 (M_2) macrophage, respectively [4–6]. M_1 cells are activated by lipopolysaccharide (LPS) or IFN-γ, which they encourage the inflammation circumstances by enhancing the expression of inflammatory mediators and genes including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6 and prostaglandin E_2 (PGE_2). In addition, M_1 cells introduce unique merit on metabolizing the arginine to nitric oxide (NO) by inducible nitric oxide synthase (iNOS) [4–6]. M_1 cells have several beneficial functions consisting of bactericidal, anti-cancer and phagocytic activities. Like a double-edged sword, aberrant and over-activation of M_1 phenotype microglia cells promote neuronal injury and destruction associated with different neurodegenerative diseases due to the liberation of cytotoxic and pro-inflammatory agents including NO, reactive oxygen species (ROS), TNF-α, IL-1β and IL-6 [7–9]. Beyond the inflammatory activity of macrophages, M_2 polarized microglia cells may play an important role in constructive processes such as wound healing and tissue repair. Furthermore, M_2 cells are able to abrogate the inflammation and immune reactions through producing the anti-inflammatory mediators such as IL-10 and IL-4 as well as increasing the expression and activity levels of arginase-1 (Arg-1) and subsequent anti-inflammatory and healing products like ornithine and urea [6,10]. Thereupon, long-term activation of M_1 phenotype microglial cells results in CNS injuries and cell damages, although M_2 cells by antagonizing the inflammation reveal the regeneration and healing states [6,10]. Therefore, the strategy of measuring the M_1/M_2 ratio could be considered as a new therapeutically immunotherapy method for M_1 phenotype dominant diseases such as MS. In this way, determination of M_2 anti-inflammatory mechanisms is necessary for pharmacological polarization of the M_1 imbalanced M_1/M_2 ratio towards M_2.

The endocannabinoids system as an emerged biological system is composed of endocannabinoids and cannabinoid receptors, which typically consist of type 1 and 2 (CB_1 and CB_2). Scientific evidence propounds that endocannabinoids may act as both neuromodulators and immunomodulators in the immune system especially through the CB_2 receptor [11–13]. Amazingly, the CB_2 receptors and its specific ligands have recently acquired more attention among other types of cannabinoid receptors, as a main therapeutic target for the regulation of neuroinflammation and alleviation of activated M_1 microglia due to firstly non-psycho activity unlike CB_1 receptor activation, and secondly the CB_2 receptors are broadly placed in an immune system cell [14–16]. Furthermore, several studies have indicated that the activation of CB_2 receptor reduces the pro-inflammatory mediators in response to detrimental stimuli, resulting in the augmentation of anti-inflammatory mediators and increase of neuronal survival [14,17,18]. In this regard, there are several studies which support the implication of the activation of the peroxisome proliferator-activated receptor-gamma (PPAR-γ) pathway, leastwise in part, is involved in anti-inflammatory effects of endocannabinoids by their feature to inhibit the activation of nuclear factor κB (NFκB) and the expression of the pro-inflammatory cytokines [17,19].

β-Caryophyllene (BCP) is a dietary phytocannabinoid classified as a selective CB_2 agonist (Ki = 155 ± 4 nM), which found in abundance in medicinal plants such as *Syzygium aromaticum*, *Origanum vulgare* L. and *Piper nigrum* L. [20,21]. In this context, several studies support the controversy that BCP at both low and high doses may exert the anti-inflammatory and anti-oxidant effects through activation of CB_2 receptors [11,22,23]. On this occasion, BCP at low doses (lower than 2 μM or 10 mg/kg) provides the healing and protection including analgesic [20], anti-inflammation [20], reducing the neuropathic pain [24], and anti-apoptogenic effects against noxious stimuli like MPP^+ (1-methyl-4-phenylpyridinium)-induced Parkinson model [23] and glutamate excitotoxicity [25] by completely acting on the CB_2 receptors because of the addition of CB_2 antagonist totally blocks the effects of BCP. Otherwise, several studies also demonstrated that BCP only at high doses (> 25 mg/kg and 10 μM) can introduce the protective activities, such as amelioration effects on animal models of multiple sclerosis [26], ulcerative colitis [27,28] and neuropathic pain [21,28] as well as in-vitro model of β-Amyloid (Aβ)-induced toxicity on BV-2 microglia cell line [22]. Similarly, it has been showed that selective CB_2 agonist JWH-133 at low doses affects the inflammatory and anti-inflammatory cytokines (IL-12p40 and IL-10, respectively) concentration-dependently (10 nM-1 μM), while this effect is disrupted at high dose of 5 μM, which was not discussed [29]. Nevertheless, there is not any study evaluating the possible immunomodulatory activity of BCP on primary microglia M_1/M_2 balances in relation to the CB_2 receptor.

Eventually, we aimed to investigate the protective effects of a broad range concentration of BCP against LPS-induced primary microglia cells inflammation and M_1/M_2 imbalance and identifying the portion of the involvement of related signaling pathways on BCP effects using pharmacological antagonists of CB_2, PPAR-γ, and sphingomyelinase (SMase).

2. Materials and methods

2.1. Chemicals and antibodies

β-Caryophyllene (BCP, C9653 SIGMA), JWH-133 as a selective CB_2 cannabinoid receptor agonist (J2753-25MG), DMEM culture media, penicillin plus streptomycin (pen/strep), amphotericin B, fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), LPS (L2880 SIGMA), Ficoll, DNAse I, Dispase II, and other cell culture materials were obtained from Sigma-Aldrich chemical Co. (St. Louis, MO, USA). Bradford reagent (B6916 SIGMA), 2′,7′-dichlorofluorescin diacetate (DCFH DA, code D6883), Griess reagent (G4410 SIGMA), sulfanilamide, n-(1-naphthyl) ethylenediamine, DTNB (2,2′-dinitro-5,5′-dithiodibenzoic acid), triton-X, sulfosalicylic acid, potassium phosphate buffer, EDTA disodium salt, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), KCl, dithiothreitol (DTT), Nonidet P40, phenylmethane sulfonyl fluoride (PMSF) and protease inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO). AM-630 a selective CB_2 cannabinoid antagonist (sc-200365A) and GW-9662 a selective PPAR-γ antagonist (sc-202641), as well as imipramine hydrochloride (IMP, sc-207753A) and AM-251 a selective CB_1 cannabinoid antagonist (sc-200366), were purchased from Santa Cruz Biotechnology Inc. Commercially available colorimetric urea kit, QuantiChrom™ urea assay kit (DIUR-100) was obtained from BioAssay Systems, Hayward, CA (Santa Cruz, CA, USA). ELISA kits (TNF-α, IL-1β, IL10, and PGE_2) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), were purchased from eBioscience (San Diego, CA, USA) and Roche Diagnostic (Mannheim, Germany), respectively. Cyclic AMP (cAMP) assay kit for biologics was HitHunter® DiscoveRx (90-0075LM10).

2.2. Primary microglia isolation

Primary microglia cells (C57BL/6, 8 weeks old, 28 ± 5 g, n = 8) were isolated using the method of Lee and Tansey was previously described [4,30] with minor modification. In review, mice brains after decapitation were detached from meninges and underwent mechanically and enzymatically dissociation by papain (final concentration 1 mg/mL), dispase II (final concentration 1.2 U/mL) and DNAse I (final concentration 20 U/mL). After that, the minced brain was transferred to 15 mL tube containing 3 mL of enzymes without serum FBS medium in cell culture incubator for 20 min. After neutralizing the enzymes with 5 mL of complete medium, the cells were centrifuged for 5 min at 250 × *g* in controlled room temperature and the supernatants were slowly removed. The pellets were re-suspended in 4 mL per brain of 37% stock isotonic Ficoll (SIF; nine parts of Ficoll with one part 10 ×

HBSS), and slowly poured on 4 mL of 70% SIF. Over the 37% SIF layer, 4 mL of 30% SIF was slowly pipetted, followed by 2 mL of HBSS. The mixture was centrifuged gradient 40 min at 300 ×*g* (18 °C) with no brake. The 70–37% interphase was collected into another clean 15-mL conical tube and added 6 mL of HBSS for each 2 mL of interphase volume collected to ensure the Ficoll containing the interphase was diluted about three times, and centrifuged 7 min at 500 ×*g* at 4 °C. Finally, the cells were counted using a hemocytometer and they were seeded on the culture plate for subsequent assays.

2.3. Cell culture

Isolated adult mice microglia cells were cultured in DMEM enriched with 1% v/v of pen/strep (100 ×), and 10% v/v of heat-inactivated FBS and also supplemented with 0.5 μg/mL amphotericin B and 2 mM L-glutamine. Cells were maintained at 37 °C and 5% v/v CO_2, in a humidified incubator.

2.4. Assessment of the level of cAMP

To study the functional CB_2 selectivity of BCP effects, a wide range of dose-response was carried out using forskolin (FSK)-induced cAMP production. For this meaning, the cells were cultured in 96-well plates (5×10^4 cells/mL) overnight. After discarding the supernatant, the cells were chilled for 10 min at room temperature in enriched phosphate-buffered saline (PBS) with 500 μM 3-isobutyl-1-methylxanthine (IBMX). Afterward, the cells were treated with different concentrations (10^{-11}–10^{-4} M) of BCP or JWH-133 in the presence or the absence of CB_1 (AM251, 50 nM) or CB_2 (AM630, 100 nM) antagonists, and then incubated for 30 min at 37 °C in a final volume of 100 μL. Following another 30 min of incubation with 10 μM FSK at 37 °C, intracellular cAMP levels were assessed by HitHunter® for adherent cells enzyme fragment complementation using chemiluminescent detection assay according to the manufacturer's instructions at an optical density of 450 nm in reference of 540 nm. The high-affinity CB_2 receptor ligand JWH-133 was used as positive control.

2.5. Experimental design

2.5.1. Protocol 1

For investigating the possible protective effects of BCP against LPS-induced inflammation on microglia cells, the cells were pre-incubated with either BCP (0.2–25 μM) or JWH-133 (1 μM, [25,27,31]) for 24 h. Next, these cells also were incubated with LPS (1 μg/mL) for another 24 h (Table 1).

2.5.2. Protocol 2

For evaluation of the possible role of CB_2 receptor in the protective effects of BCP against LPS-induced inflammation on microglia cells, the cells were first pre-incubated with pharmacological CB_2 receptor antagonist AM-630 (1 μM, [20]) for 30 min and then added either BCP (0.2–25 μM) or JWH-133 (1 μM, [21,25,27,31]) for 24 h. Next, these cells also were incubated with LPS (1 μg/mL) for another 24 h (Table 1).

2.5.3. Protocol 3

For evaluation of the possible role of PPAR-γ receptor in the protective effects of BCP against LPS-induced inflammation on microglia cells, the cells were first pre-incubated with pharmacological PPAR-γ receptor antagonist GW 9662 (1 μM, [25,27]) for 30 min and then added either BCP (0.2–25 μM) or JWH-133 (1 μM, [21,25,27,31]) for 24 h. Next, these cells also were incubated with LPS (1 μg/mL) for another 24 h (Table 1).

2.5.4. Protocol 4

For investigating the possible role of sphingomyelinase in the protective effects of BCP, SMase inhibitor IMP was used. The cells were initially exposed with IMP (1 μM, [32,33]) for 30 min, and then incubated using either BCP (0.2–25 μM) or JWH-133 (1 μM) for 24 h. Next, these cells also were incubated with LPS (1 μg/mL) for another 24 h (Table 1).

Table 1
Summary protocols of experimental design.

Protocols				
1	For 24 h: BCP (0.2-25 μM) Or JWH-133 (1 μM)	For 24 h: LPS (1 μg/ml)	Assay	
2	For 30 min: AM-630 (1 μM)	For 24 h: BCP (0.2-25 μM) Or JWH-133 (1 μM)	For 24 h: LPS (1 μg/ml)	Assay
3	For 30 min: GW 9662 (1 μM)	For 24 h: BCP (0.2-25 μM) Or JWH-133 (1 μM)	For 24 h: LPS (1 μg/ml)	Assay
4	For 30 min: IMP (1 μM)	For 24 h: BCP (0.2-25 μM) Or JWH-133 (1 μM)	For 24 h: LPS (1 μg/ml)	Assay

2.6. Cell proliferation assay

Briefly, about 7000 cells were seeded in 96-wells plate and treated according to protocols at 37 °C in 5% v/v CO_2 incubator. After the duration of the protocol treatment, 10 μL of MTT reagent (stock solution: 5 mg/mL) was added to each well which incubated for the next 3 h. formazan crystals were dissolved in 100 μL DMSO and the absorbance was read using StatFAX 2100 ELISA plate reader (Awareness Inc., USA) at 570 nm in referencing 620 nm.

2.7. Protein determination

Total protein contents were measured according to Bradford's method [34].

2.8. Assessment of the levels of anti/pro-inflammatory cytokines

Anti-inflammatory (IL-10) and pro-inflammatory cytokines (IL-1β, TNF-α and PGE_2) were assessed using the sandwich ELISA method according to the manufacturer's manual. The cells were cultured (10^6 cells/each 6-well plate) and incubated with compounds based on the experimental protocols at 37 °C in 5% v/v CO_2 incubator. Finally, the supernatants were collected and the levels of cytokines measured [35]. The levels of cytokines were reported as pg/mg protein.

2.9. Measurement of the intracellular reactive oxygen species (ROS) level

The level of intracellular ROS was examined using DCFH-DA as described previously [11,36]. Microglia cells (5×10^4 cells/well) were seeded in 96 well plates and treated according to the experimental

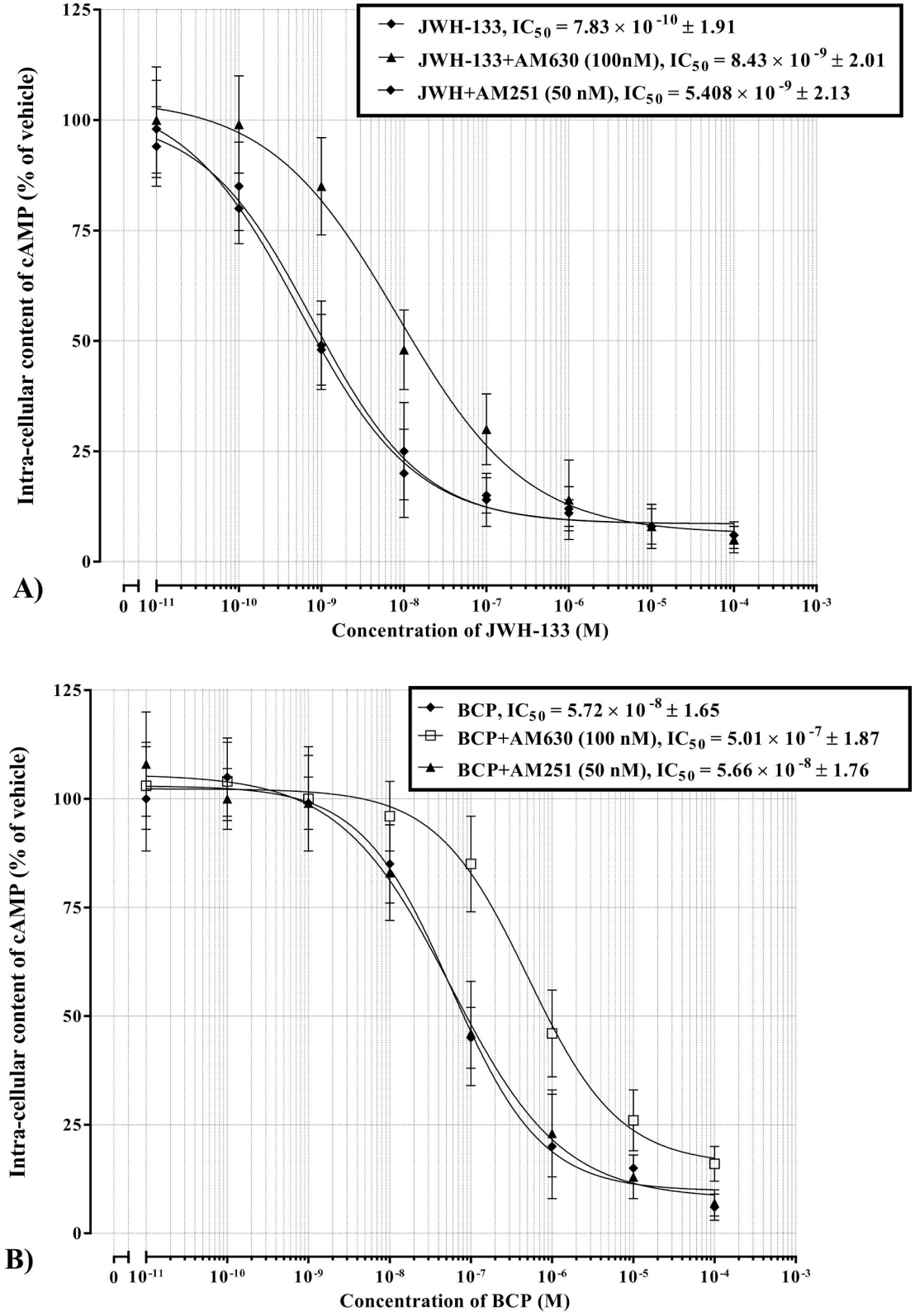


Fig. 1. The effect of A) JWH-133 or B) BCP on the level of FSK-induced intracellular cAMP production, in the presence or absence of CB_1 (AM251, 50 nM) or CB_2 (AM630, 100 nM) antagonists. Data were shown as mean ± SEM, n = 6.

protocols at 37 °C in 5% v/v CO_2 incubator. After that, DCFH-DA was added to the cells and incubated for 30 min later. The fluorescence intensity was read out using ELISA reader at excitation wavelength of 504 nm and the emission wavelength of 524 nm.

2.10. Assessment of the level of nitric oxide (NO) metabolites

The level of nitric oxide was investigated as a form of nitrite production using Griess method which described previously [35,37]. Microglia cells were cultured at a density of 10^6 cells per each 6 well-plate

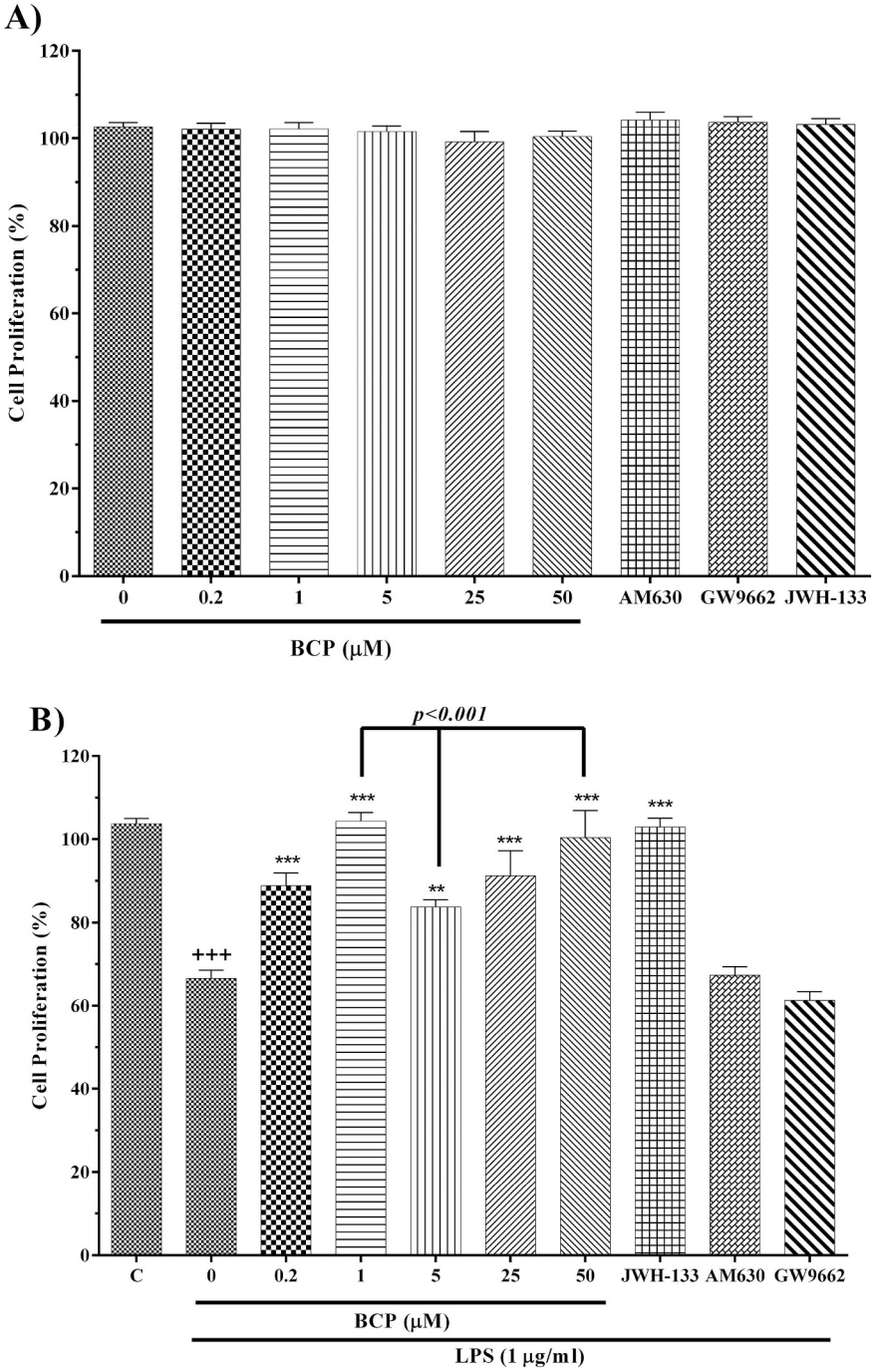


Fig. 2. The effect of different concentrations of BCP on the level of cell proliferation; data were shown as mean ± SEM, n = 8 for each protocol of experiment. After passing the Normality test, comparisons between groups were done using ANOVA with Tukey-Kramer's *post-hoc* multiple comparisons test. For comparing the respected concentrations of BCP to each combination, two-way ANOVA test was done with the following Holm-Sidak's *post-hoc* multiple comparisons test; A) the effect of BCP (0.2–50 μM), JWH-133 (1 μM), AM630 (1 μM) and GW9662 (1 μM) on cell proliferation of microglia during 48 h incubation; B) the effect of BCP (0.2–50 μM), JWH-133 (1 μM), AM630 (1 μM) and GW9662 (1 μM) on cell proliferation of microglia during 24 h incubation and then 24 h LPS exposure (1 μg/mL); C) the combination effects of BCP (0.2–50 μM) or JWH-133 (1 μM) with AM-630 (1 μM) on cell proliferation of microglia during 24 h incubation and then 24 h LPS exposure; D) the combination effects of BCP (0.2–50 μM) or JWH-133 (1 μM) with GW-9662 (1 μM) on cell proliferation of microglia during 24 h incubation and then 24 h LPS exposure; E) the combination effects of BCP (0.2–50 μM) or JWH-133 (1 μM) with IMP (1 μM) on cell proliferation of microglia during 24 h incubation and then 24 h LPS exposure. (*) shows a comparison between BCP and respected non-treated LPS group, **: $p < 0.01$ and ***: $p < 0.001$; ($^{+}$) compares non-treated LPS group to control group, $^{+++}$: $p < 0.001$; (^{x}) compares different combinations of BCP to the respected concentration of BCP alone in the presence of LPS, ^{x}: $p < 0.05$ and ^{xxx}: $p < 0.001$; lines show a comparison between 5 and 25 μM, and 1 μM of BCP. Abbreviations: BCP: β-caryophyllene, LPS: lipopolysaccharides, IMP: imipramine.

and treated according to the experimental protocols at 37 °C in 5% v/v CO_2 incubator. Then, the supernatants were gathered to examine the concentration of nitrite using Griess reagent in a spectrophotometer at 540 nm. In brief, 50 μL of supernatant was incubated with equal volumes of sulfanilamide and n-(1-naphthyl)-ethylenediamine in 2 N hydrochloric acid at controlled room temperature for 10 min. The concentration of nitrite was determined using sodium nitrite standard curve [4]. The level of NO was reported as nmol/mg protein.

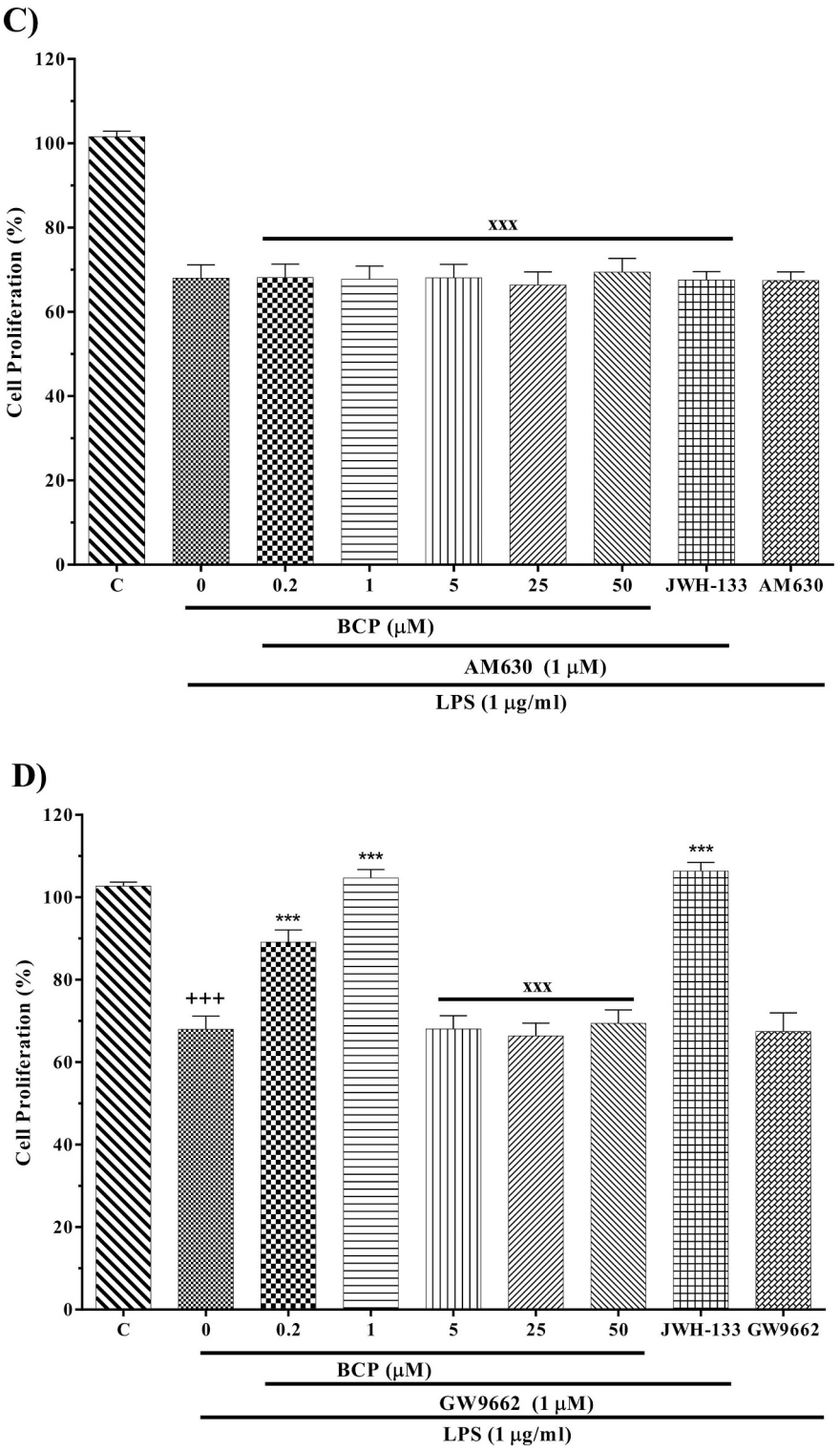


Fig. 2. (*continued*)

2.11. Assessment of the level of urea

The level of urea was determined using colorimetric urea assay kit according to the manufacturer's instructions. The cells were cultured at the density of 2×10^6 cells per each 6 well-plate and treated according to the experimental protocols at 37 °C in 5% v/v CO_2 incubator. Then, the supernatants were gathered to examine the concentration of urea at 430 nm. In brief, 50 μL of supernatant was mixed with equal volumes of medium (blank) and 50 μL urea-solution (5 mg/dL or 850 μM), and then incubated with 200 μL working reagent at controlled room temperature for 50 min. Urea concentration (nmol/mg protein) of the sample is calculated by the standard curve of urea.

2.12. Assessment of the intracellular level of total –SH groups (GSH)

The total level of –SH groups as an index of anti-oxidant defense was measured using DTNB which reacts with the –SH moieties and produces a yellow complex dye with a peak absorbance at 412 nm. In brief, the cells

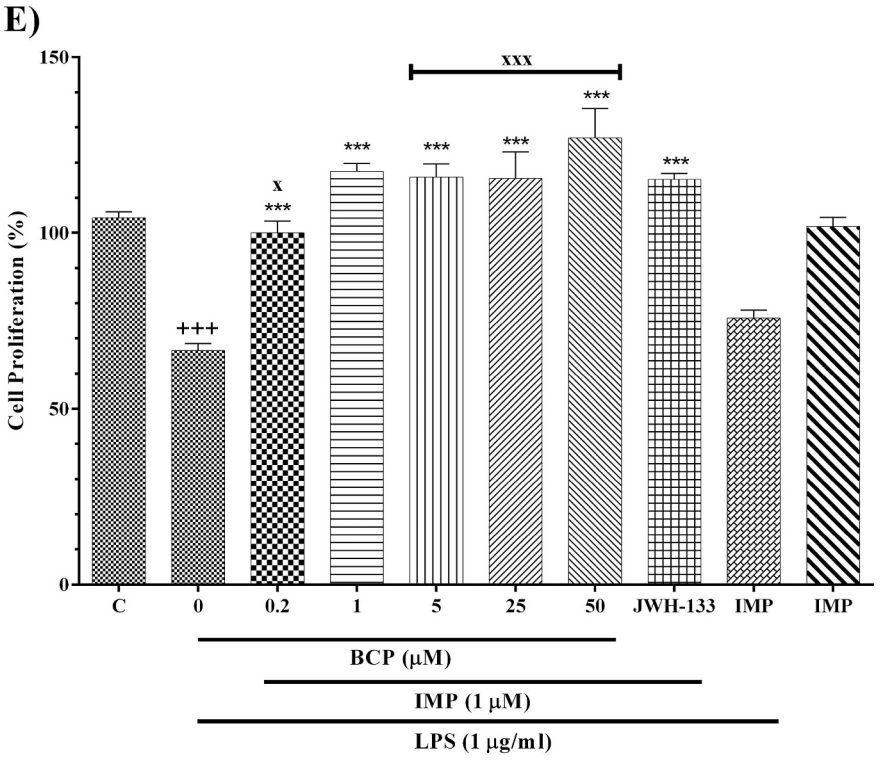


Fig. 2. (*continued*)

were cultured at the density of 10^6 cells per each 12-well plate and incubated with compounds based on the experimental protocols section at 37 °C in 5% v/v CO_2 incubator. After that, the cells were harvested to evaluate intra-cellular amount of GSH. After cell collection, the cells were lysed using lysis buffer (1 mL 0.1% Triton-X and 0.6% sulfo-salicylic acid in 0.1 M potassium phosphate buffer (KPE) with 5 mM EDTA disodium salt, pH 7.5), and then homogenized (DIAX 100, Heidolph, Schwabach, Germany) the suspension in cold water (0–4 °C) for 2–3 min along with vortexing every 30 s. 20 μL of samples were undergone of assessment. Sample absorbance was read at 412 nm against KPE buffer alone (A1). Then, 120 μL DTNB reagent (2 mg/3 mL KPE) was added to the mixture, and absorbance was read again after 2 min away from the light at controlled room temperature (22–25 °C) (A2). The absorbance of the DTNB reagent alone was also read as a blank (B). Total thiol concentration (nmol/mg protein) was calculated using the following equation [38,39]:

$$\text{Total thiol concentration (nmol/mg protein)} = (A_2 - A_1 - B) \times \frac{1.07}{0.05} \times 13.6.$$

2.13. Assessment of the level of iNOS

Intra-cellular level of iNOS was assessed using a commercially available sandwich ELISA kit according to the manufacturer's manual. The cells were cultured (2×10^6 cells/each 6-well plate) and incubated with compounds based on the experimental protocols at 37 °C in 5% v/v CO_2 incubator. After that, the cells were collected and subsequently lysed using lysis buffer, and then homogenized (DIAX 100, Heidolph, Schwabach, Germany) the suspension in cold water (0–4 °C) for 2–3 min along with vortexing every 30 s. The samples were centrifuged at 12,000*g* at 4 °C for 10 min and then 50 μL of supernatants were undergone of assessment. The level of iNOS was reported as ng/mg protein.

2.14. Assessment of the level of Arg-1

Intra-cellular level of Arg-1 was assessed using a commercially available sandwich ELISA kit according to the manufacturer's manual. The cells were cultured (2×10^6 cells/each 6-well plate) and incubated with compounds based on the experimental protocols at 37 °C in 5% v/v CO_2 incubator. After that, the cells were collected and subsequently lysed using lysis buffer, and then homogenized (DIAX 100, Heidolph, Schwabach, Germany) the suspension in cold water (0–4 °C) for 2–3 min along with vortexing every 30 s. The samples were centrifuged at 12,000*g* at 4 °C for 10 min and then 50 μL of supernatants were undergone of assessment. The level of Arg-1 was reported as ng/mg protein.

2.15. Statistical analysis

Data were analyzed using GraphPad Prism ® 6 (GraphPad Software, San Diego, CA) software and showed as means ± SEM. Normality test was carried out based on Kolmogorov–Smirnov and Bartlett's tests that assess the homogeneity of variances. After passing the tests, comparisons between groups were done using one-way analysis of variance (ANOVA) with Tukey-Kramer *post-hoc* multiple comparisons test. To compare respected concentrations of BCP to each combination, two-way ANOVA test was done with the following Holm-Sidak's *post-hoc* multiple comparisons test. P values (*p*) lower than 0.05, 0.01 and 0.001 were statistically considered significant differences.

3. Results

3.1. The effects of BCP and JWH-133 on the level of FSK-induced cAMP production

Our results indicated that JWH-133 (10^{-11}–10^{-4} M) concentration-dependently decreases the level of cAMP following FSK stimulation (Fig. 1A). Pre-incubation of JWH-133 with AM630, but not with AM251, made a right shift in a dose-response curve in which increased the level of IC_{50} of JWH-133 (Fig. 1A). We also found that BCP (10^{-11}–10^{-4} M) reduces the level of FSK-induced cAMP production in a concentration-dependent manner (Fig. 1B). Likewise, AM630, but not AM251, provided a right shift in the concentration-response curve of BCP (Fig. 1B).

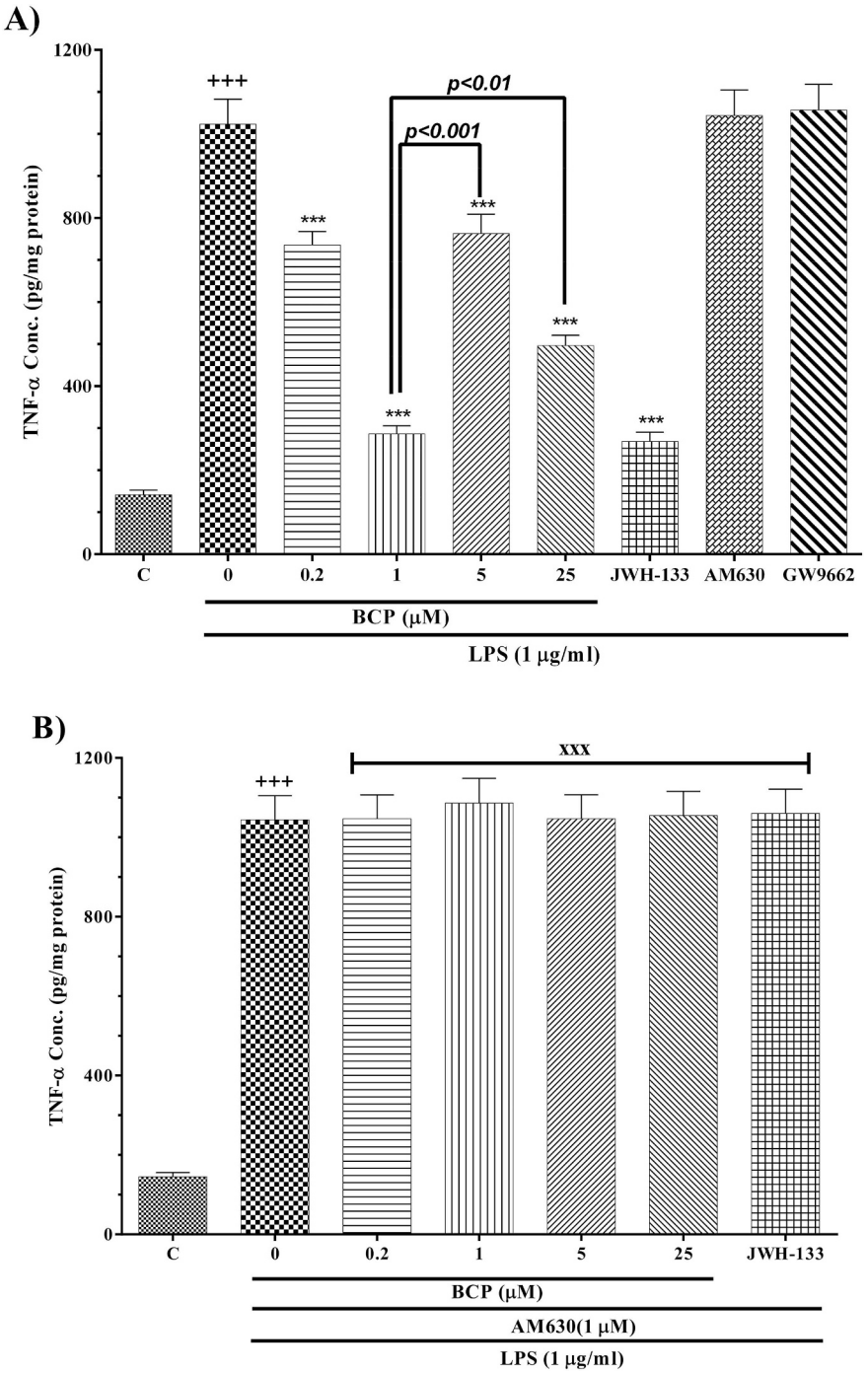


Fig. 3. The effect of different concentrations of BCP on the level of TNF-α production; Data were shown as mean ± SEM, n = 8 for each protocol of experiment. After passing the Normality test, comparisons between groups were done using ANOVA with Tukey-Kramer's *post-hoc* multiple comparisons test. For comparing the respected concentrations of BCP to each combination, two-way ANOVA test was done with the following Holm-Sidak's *post-hoc* multiple comparisons test; A) the effect of BCP (0.2–25 μM), JWH-133 (1 μM), AM630 (1 μM) and GW9662 (1 μM) on the level of TNF-α production of microglia during 24 h incubation and then 24 h LPS exposure (1 μg/mL); B) the combination effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with AM-630 (1 μM) on the level of TNF-α production of microglia during 24 h incubation and then 24 h LPS exposure; C) the combination effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with GW-9662 (1 μM) on the level of TNF-α production of microglia during 24 h incubation and then 24 h LPS exposure; D) the combination effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with IMP (1 μM) on the level of TNF-α production of microglia during 24 h incubation and then 24 h LPS exposure. (*) shows a comparison between BCP and respected non-treated LPS group, ***: $p < 0.001$; ($^{+}$) compares non-treated LPS group to control group, $^{+++}$: $p < 0.001$; (^{x}) compares different combinations of BCP to the respected concentration of BCP alone in the presence of LPS, ^{x}: $p < 0.05$ and ^{xxx}: $p < 0.001$; lines show a comparison between 5 and 25 μM, and 1 μM of BCP. Abbreviations: BCP: β-caryophyllene, LPS: lipopolysaccharides, IMP: imipramine.

3.2. The effects of LPS and BCP on cell proliferation

Preincubation with different concentrations of BCP (0.2–50 μM), JWH-133 (1 μM), AM-630 (1 μM) or GW9662 (1 μM) as well as IMP (1 μM) presented no significant effects on proliferation of microglial cells compared to control group (Fig. 2A and E). LPS (1 μg/mL) significantly decreased cell proliferation compared to the control group ($p < 0.001$, Fig. 2B). BCP (0.2–50 μM) and JWH-133 (1 μM)

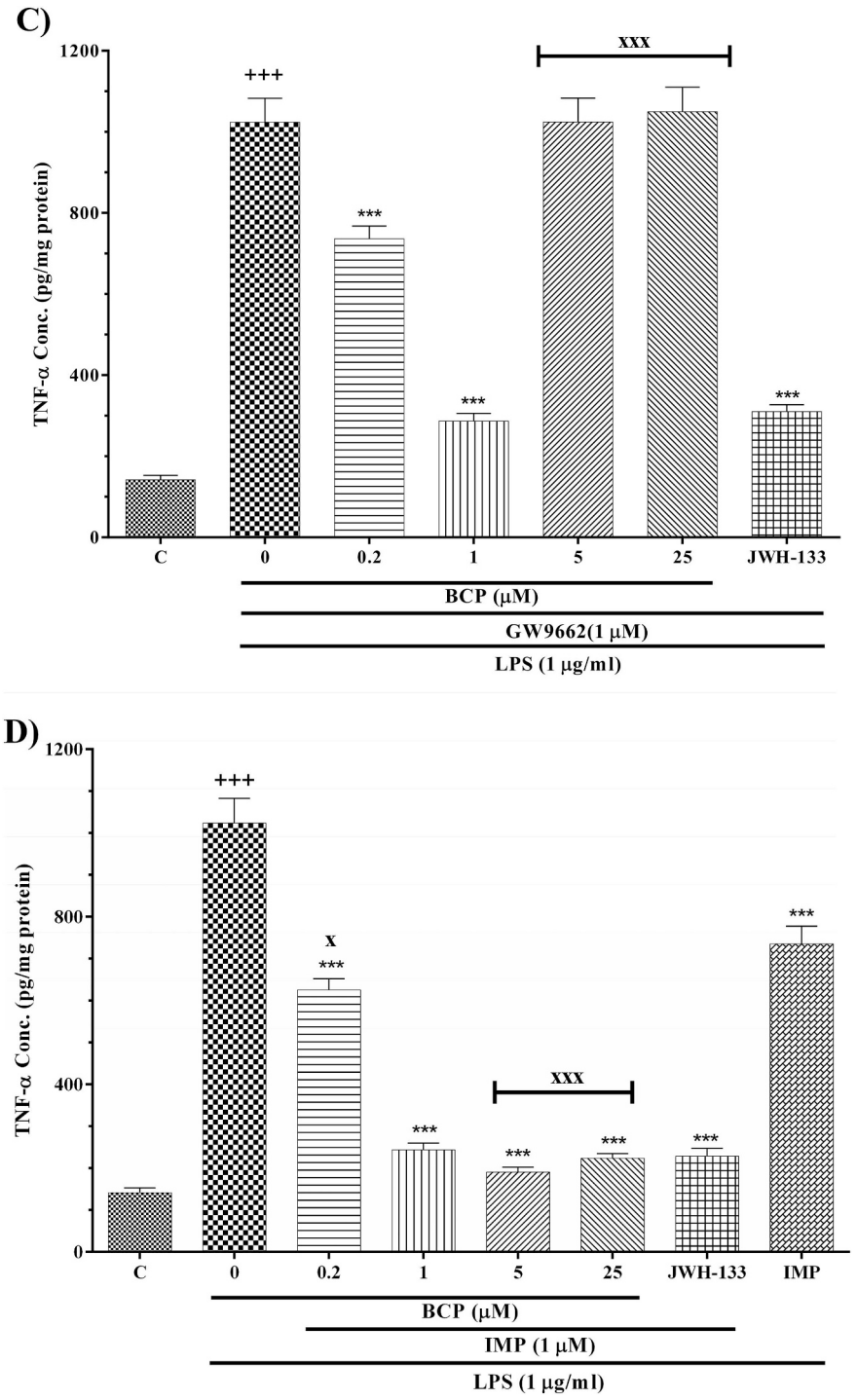


Fig. 3. (*continued*)

significantly increased cell proliferation in comparison to LPS treated group ($p < 0.001$–0.01 for all cases, Fig. 2B). Pretreatment along with AM-630 (1 μM) completely abolished the protective effects of all tested concentrations of BCP and JWH-133 against LPS-induced toxicity ($p < 0.001$ for all cases, Fig. 2C). Applying GW9662 before high concentrations of BCP (5–50 μM) significantly reduced the protective response against LPS-induced toxicity comparing to same concentrations that received no combinations ($p < 0.001$ for all cases, Fig. 2D). In comparison to BCP alone group, pre-incubation of BCP (0.2, 5, 25 and 50 μM) along with IMP (1 μM) significantly increased the cell proliferation ($p < 0.001$–0.05 for all cases, Fig. 2E).

3.3. The effects of LPS and BCP on inflammatory and anti-inflammatory cytokines, and PGE_2 levels

Twenty-four hours incubation with LPS (1 μg/mL) significantly increased the levels of TNF-α, IL-1β, PGE_2 and IL-10 production in comparison to control group ($p < 0.001$ for all cases, Fig. 3–6A). Pre-incubation of the cells with BCP (0.2–25 μM) or JWH-133 (1 μM) significantly decreased the levels of TNF-α, IL-1β and PGE_2 comparing to LPS group ($p < 0.001$ for all cases, Fig. 3–5B). BCP (0.2, 1 and 25 μM) or JWH-133 (1 μM) significantly increased the level of IL-10 compared with LPS group ($p < 0.001$ to 0.05 for all cases, Fig. 6A). In the presence of AM630, the effects of all concentrations of BCP or JWH-133 on the levels

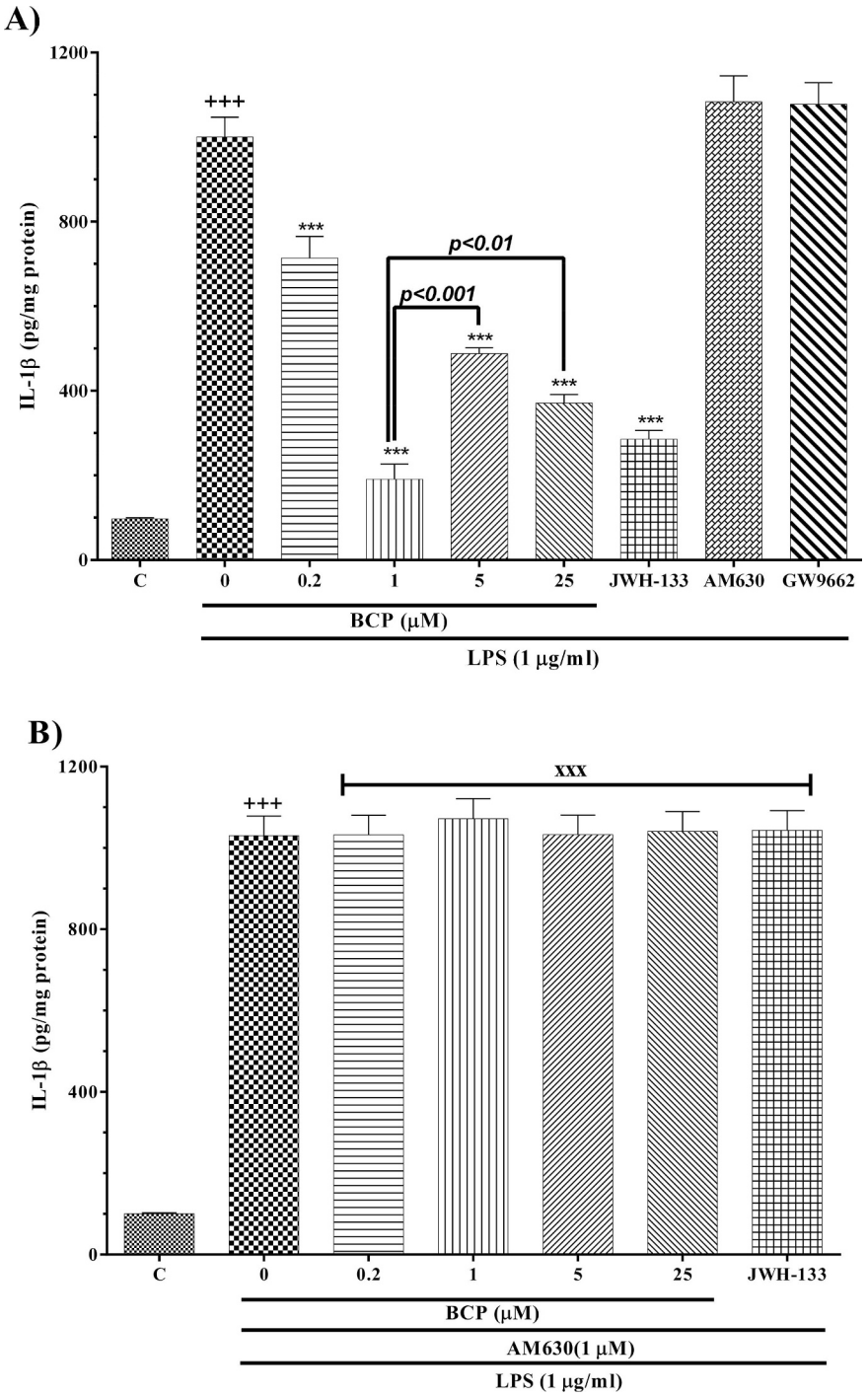


Fig. 4. The effect of different concentrations of BCP on the level of IL-1β production; data were shown as mean ± SEM, n = 8 for each protocol of experiment. After passing the Normality test, comparisons between groups were done using ANOVA with Tukey-Kramer's *post-hoc* multiple comparisons test. For comparing the respected concentrations of BCP to each combination, two-way ANOVA test was done with the following Holm-Sidak's *post-hoc* multiple comparisons test; A) the effect of BCP (0.2–25 μM), JWH-133 (1 μM), AM630 (1 μM) and GW9662 (1 μM) on the level of IL-1β production of microglia during 24 h incubation and then 24 h LPS exposure (1 μg/mL); B) the combination effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with AM-630 (1 μM) on the level of IL-1β production of microglia during 24 h incubation and then 24 h LPS exposure; C) the combination effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with GW-9662 (1 μM) on the level of IL-1β production of microglia during 24 h incubation and then 24 h LPS exposure; D) the combination effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with IMP (1 μM) on the level of IL-1β production of microglia during 24 h incubation and then 24 h LPS exposure. (*) shows a comparison between BCP and respected non-treated LPS group, ***: $p < 0.001$; ($^{+}$) compares non-treated LPS group to control group, $^{+++}$: $p < 0.001$; (^{x}) compares different combinations of BCP to the respected concentration of BCP alone in the presence of LPS, ^{xxx}: $p < 0.001$; lines show a comparison between 5 and 25 μM, and 1 μM of BCP. Abbreviations: BCP: β-caryophyllene, LPS: lipopolysaccharides, IMP: imipramine.

of TNF-α, IL-1β, PGE_2 and IL-10 following LPS stimulation were completely omitted compared to respected concentrations of BCP or JWH-133 alone ($p < 0.001$ for all cases, Fig. 3–6B). The combination of GW9662 with BCP also caused a complete omission of the effects of BCP (5 and 25 μM) on levels of TNF-α, IL-1β, PGE_2 and IL-10 comparing to respected concentrations of BCP alone ($p < 0.001$ for both cases, Fig. 3–6C). Pretreatment of the cells with IMP (1 μM) significantly attenuated the levels of TNF-α, IL-1β and PGE_2 in comparison to LPS group

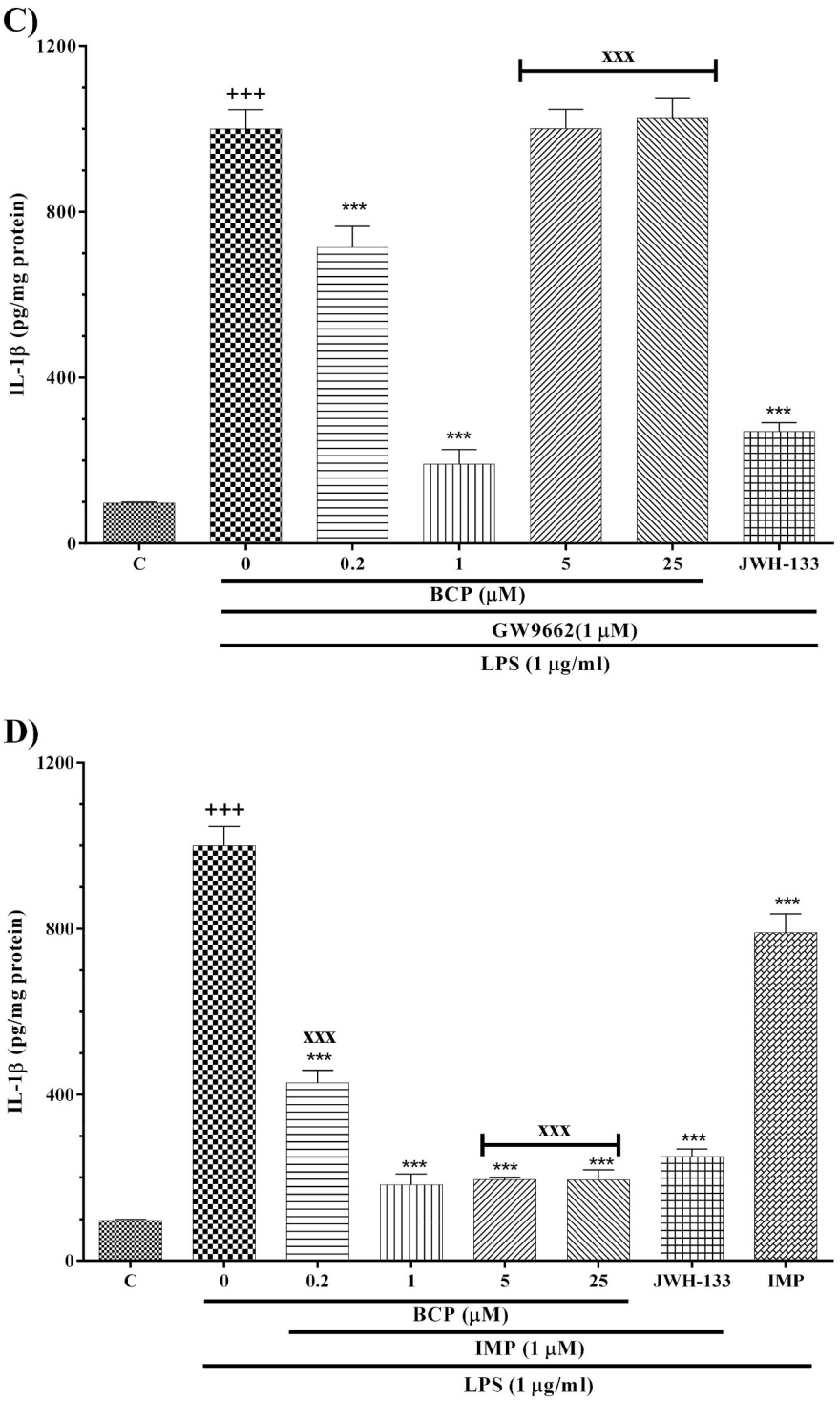


Fig. 4. (*continued*)

($p < 0.001$ for all cases, Fig. 3–5D). Premedication of BCP (0.2, 5 and 25 μM) with IMP (1 μM) significantly decreased the levels of TNF-α, IL-1β and PGE_2, whereas enhanced the level of IL-10 more than each concentration of BCP alone ($p < 0.001$–0.05 for all cases, Fig. 3–6D).

3.4. The effects of LPS and BCP on the levels of NO and iNOS

LPS (1 μg/mL) significantly increased the levels of NO and iNOS comparing to the control group ($p < 0.001$ for all cases, Fig. 7A, and E). Preincubation with either BCP (0.2–25 μM) or JWH-133 (1 μM) significantly reduced the amounts of NO and iNOS in comparison to LPS group ($p < 0.001$ for all cases, Fig. 7A, and E). Using the AM-630 (1 μM), the effects of BCP (0.2–25 μM) or JWH-133 (1 μM) were significantly abolished on the levels of NO and iNOS against LPS stimulation compared to respected concentrations of BCP alone ($p < 0.001$ for all cases, Fig. 7B and F). The reducing effects of BCP (5 and 25 μM) on the levels of NO and iNOS were also significantly reversed using pre-incubation with GW9662 ($p < 0.001$ for all cases, Fig. 7C and G). Premedication of the cells with IMP (1 μM) significantly attenuated the levels of NO and iNOS in comparison to LPS-treated group ($p < 0.001$ for all cases, Fig. 7D and H). Also, pre-incubation with IMP (1 μM) markedly augmented the protective

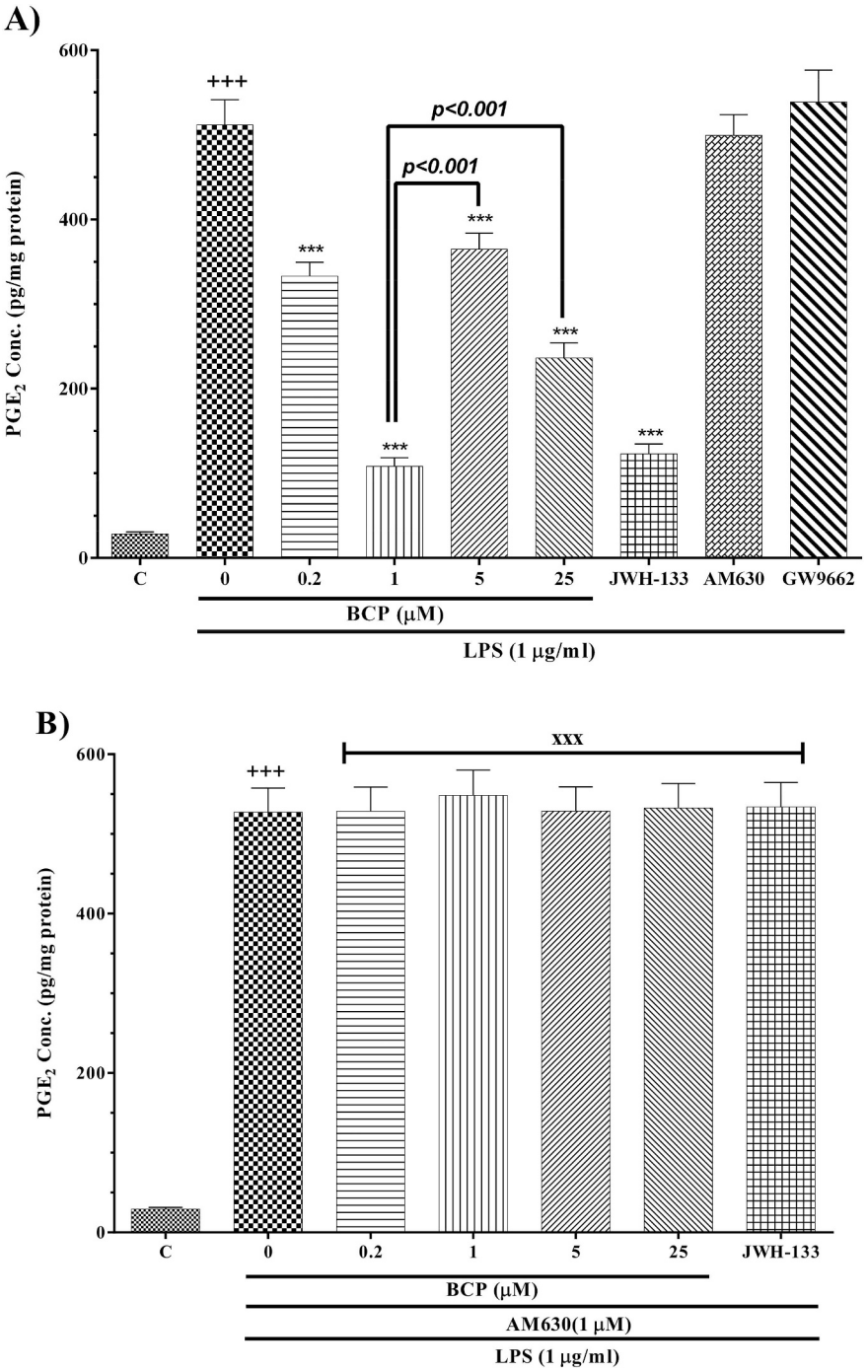


Fig. 5. The effect of different concentrations of BCP on the level of PGE_2 production; Data were shown as mean ± SEM, n = 8 for each protocol of experiment. After passing the Normality test, comparisons between groups were done using ANOVA with Tukey-Kramer's *post-hoc* multiple comparisons test. For comparing the respected concentrations of BCP to each combination, two-way ANOVA test was done with the following Holm-Sidak's *post-hoc* multiple comparisons test; A) the effect of BCP (0.2–25 μM), JWH-133 (1 μM), AM630 (1 μM) and GW9662 (1 μM) on the level of PGE_2 production of microglia during 24 h incubation and then 24 h LPS exposure (1 μg/mL); B) the combination effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with AM-630 (1 μM) on the level of PGE_2 production of microglia during 24 h incubation and then 24 h LPS exposure; C) the combination effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with GW-9662 (1 μM) on the level of PGE_2 production of microglia during 24 h incubation and then 24 h LPS exposure; D) the combination effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with IMP (1 μM) on the level of PGE_2 production of microglia during 24 h incubation and then 24 h LPS exposure. (*) shows a comparison between BCP and respected non-treated LPS group, ***: $p < 0.001$; ($^{+}$) compares non-treated LPS group to control group, $^{+++}$: $p < 0.001$; (^{x}) compares different combinations of BCP to the respected concentration of BCP alone in the presence of LPS, ^{xx}: $p < 0.01$ and ^{xxx}: $p < 0.001$; lines show a comparison between 5 and 25 μM, and 1 μM of BCP. Abbreviations: BCP: β-caryophyllene, LPS: lipopolysaccharides, IMP: imipramine.

effects of BCP (0.2, 5 and 25 μM) on levels of NO and iNOS comparing to respected concentrations of BCP alone ($p < 0.001$ for all cases, Fig. 7D and H).

3.5. The effects of LPS and BCP on the levels of urea and Arg-1

LPS (1 μg/mL) significantly decreased the levels of urea and Arg-1 in comparison to control group ($p < 0.001$ for all cases, Fig. 8A, and E). Pre-

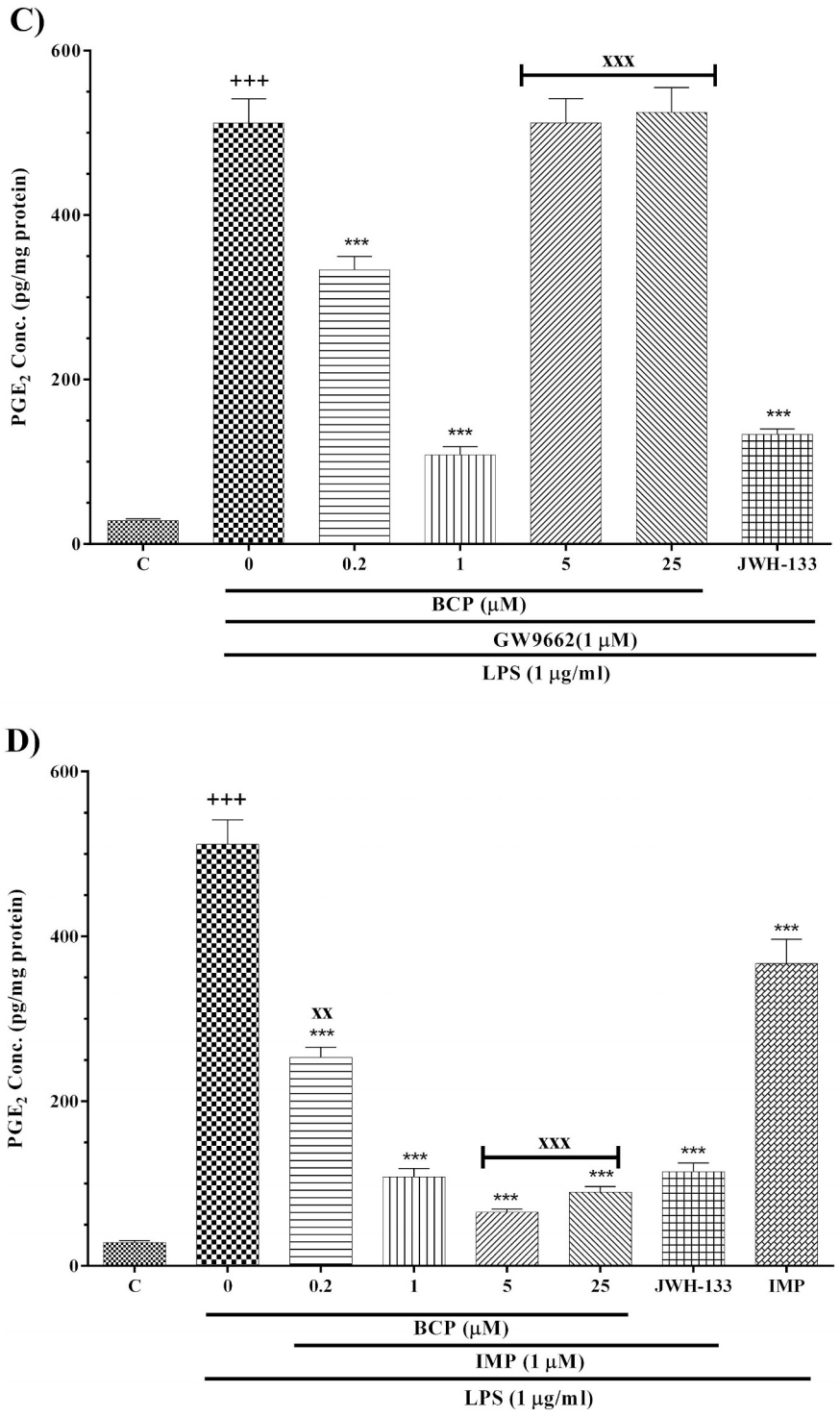


Fig. 5. (*continued*)

incubation of the cells with either BCP (0.2–25 μM) or JWH-133 (1 μM) significantly elevated the levels of urea and Arg-1 in comparison to LPS group ($p < 0.001$–0.05 for all cases, Fig. 8A, and E). AM-630 (1 μM) significantly abolished the effects of BCP (0.2–25 μM) on the levels of urea and Arg-1 against LPS stimulation comparing to respected concentrations of BCP alone ($p < 0.001$ for all cases, Fig. 8B and F). In the presence of GW9662 (1 μM), the effects of BCP (5 and 25 μM) were significantly declined on the levels of urea and Arg-1 ($p < 0.001$ for all cases, Fig. 8C and G). Premedication of the cells with IMP (1 μM) significantly increased the levels of urea and Arg-1 in comparison to LPS-treated group ($p < 0.001$ for all cases, Fig. 8D and H). Preincubation of IMP (1 μM) also notably improved the increasing effects of BCP (0.2, 5 and 25 μM) on the levels of urea and Arg-1 comparing to respected concentrations of BCP alone ($p < 0.001$–0.05 for all cases, Fig. 8D and H).

3.6. The effects of LPS and BCP on the levels of NO/urea and iNOS/Arg-1 ratios

LPS (1 μg/mL) significantly increased the levels of NO/urea and iNOS/Arg-1 ratios comparing to the control group ($p < 0.001$ for all cases, Fig. 9A, and E). In the presence of LPS, both of BCP (0.2–25 μM) and JWH-133 (1 μM) significantly reduced the levels of NO/urea and

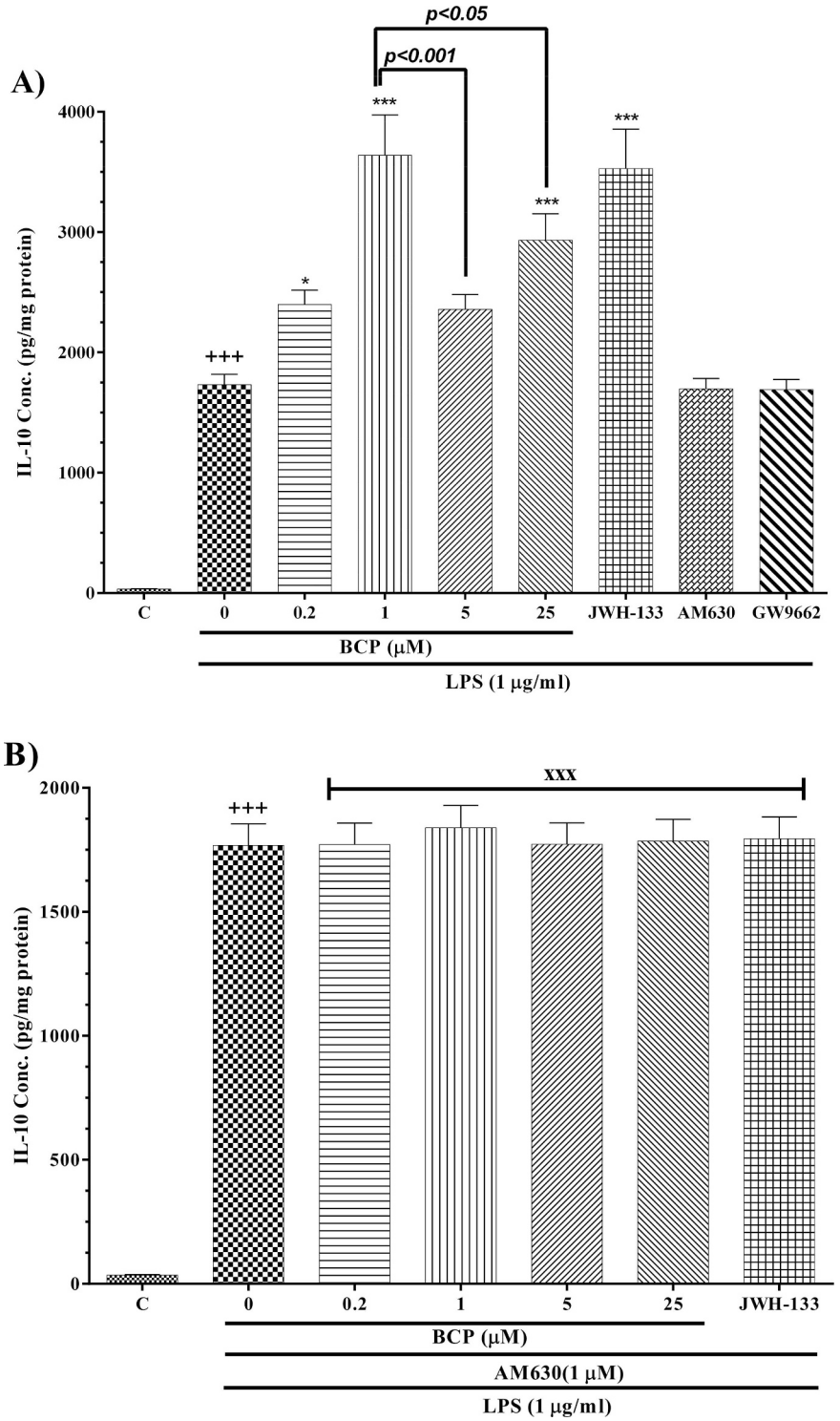


Fig. 6. The effect of different concentrations of BCP on the level of IL-10 production; Data were shown as mean ± SEM, n = 8 for each protocol of experiment. After passing the Normality test, comparisons between groups were done using ANOVA with Tukey-Kramer's *post-hoc* multiple comparisons test. For comparing the respected concentrations of BCP to each combination, two-way ANOVA test was done with the following Holm-Sidak's *post-hoc* multiple comparisons test; A) the effect of BCP (0.2–25 μM), JWH-133 (1 μM), AM630 (1 μM) and GW9662 (1 μM) on the level of IL-10 production of microglia during 24 h incubation and then 24 h LPS exposure (1 μg/mL); B) the combination effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with AM-630 (1 μM) on the level of IL-10 production of microglia during 24 h incubation and then 24 h LPS exposure; C) the combination effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with GW-9662 (1 μM) on the level of IL-10 production of microglia during 24 h incubation and then 24 h LPS exposure; D) the combination effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with IMP (1 μM) on the level of IL-10 production of microglia during 24 h incubation and then 24 h LPS exposure. (*) shows comparison between BCP and respected non-treated LPS group, *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$; ($^{+}$) compares non-treated LPS group to control group, $^{+++}$: $p < 0.001$; (^{x}) compares different combinations of BCP to the respected concentration of BCP alone in the presence of LPS, ^{xxx}: $p < 0.001$; lines show a comparison between 5 and 25 μM, and 1 μM of BCP. Abbreviations: BCP: β-caryophyllene, LPS: lipopolysaccharides, IMP: imipramine.

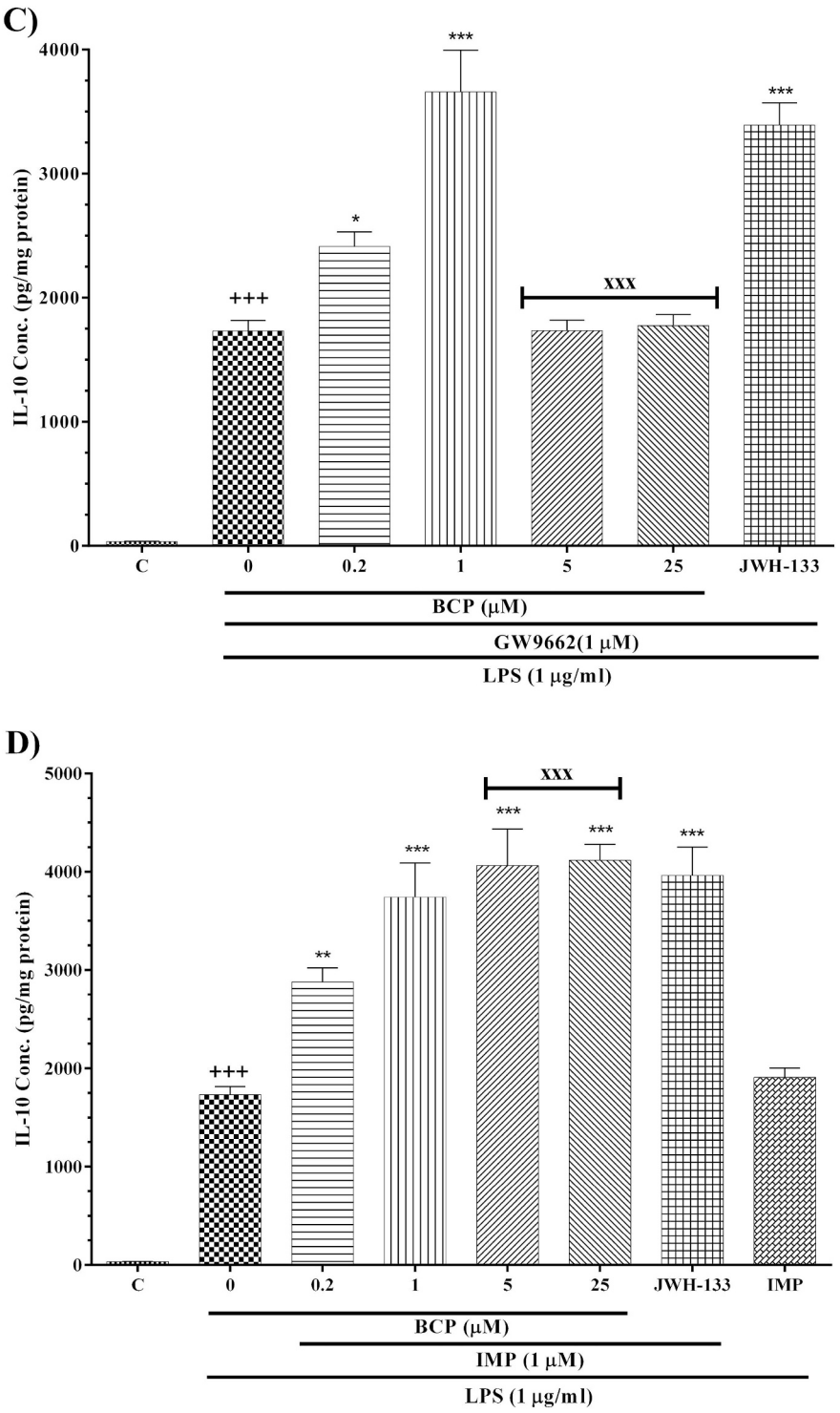


Fig. 6. (*continued*)

iNOS/Arg-1 ratios ($p < 0.001$ for all cases, Fig. 9A, and E). Using the AM-630 (1 μM) provided a significant reduction in the effects of BCP (0.2–25 μM) on the levels of NO/urea and iNOS/Arg-1 ratios against LPS stimulation compared to respected concentrations of BCP alone ($p < 0.001$ for all cases, Fig. 9B and F). By GW9662 (1 μM), there did not show significant protective effects of BCP (5 and 25 μM) on the levels of NO/urea and iNOS/Arg-1 ratios ($p < 0.001$ for all cases, Fig. 9C and G). Premedication of the cells with IMP (1 μM) significantly attenuated the levels of NO/urea and iNOS/Arg-1 ratios compared to LPS-treated group ($p < 0.001$ for all cases, Fig. 6D, H and L). IMP (1 μM) also significantly enhanced the reducing effects of BCP (0.2, 5 and 25 μM) on the levels of NO/urea and iNOS/Arg-1 ratios comparing to respected concentrations of BCP alone ($p < 0.001$ for all cases, Fig. 9D and H).

3.7. The effects of LPS and BCP on the level of intracellular ROS

Exposure to LPS (1 μg/mL) resulted in a significant increase in the level of ROS compared to the control group ($p < 0.001$, Fig. 10A). In the presence of LPS, BCP (0.2–25 μM) and JWH-133 (1 μM) significantly inhibited the level of ROS in comparison to LPS group ($p < 0.001$ for all cases, Fig. 10A). AM-630 (1 μM) completely

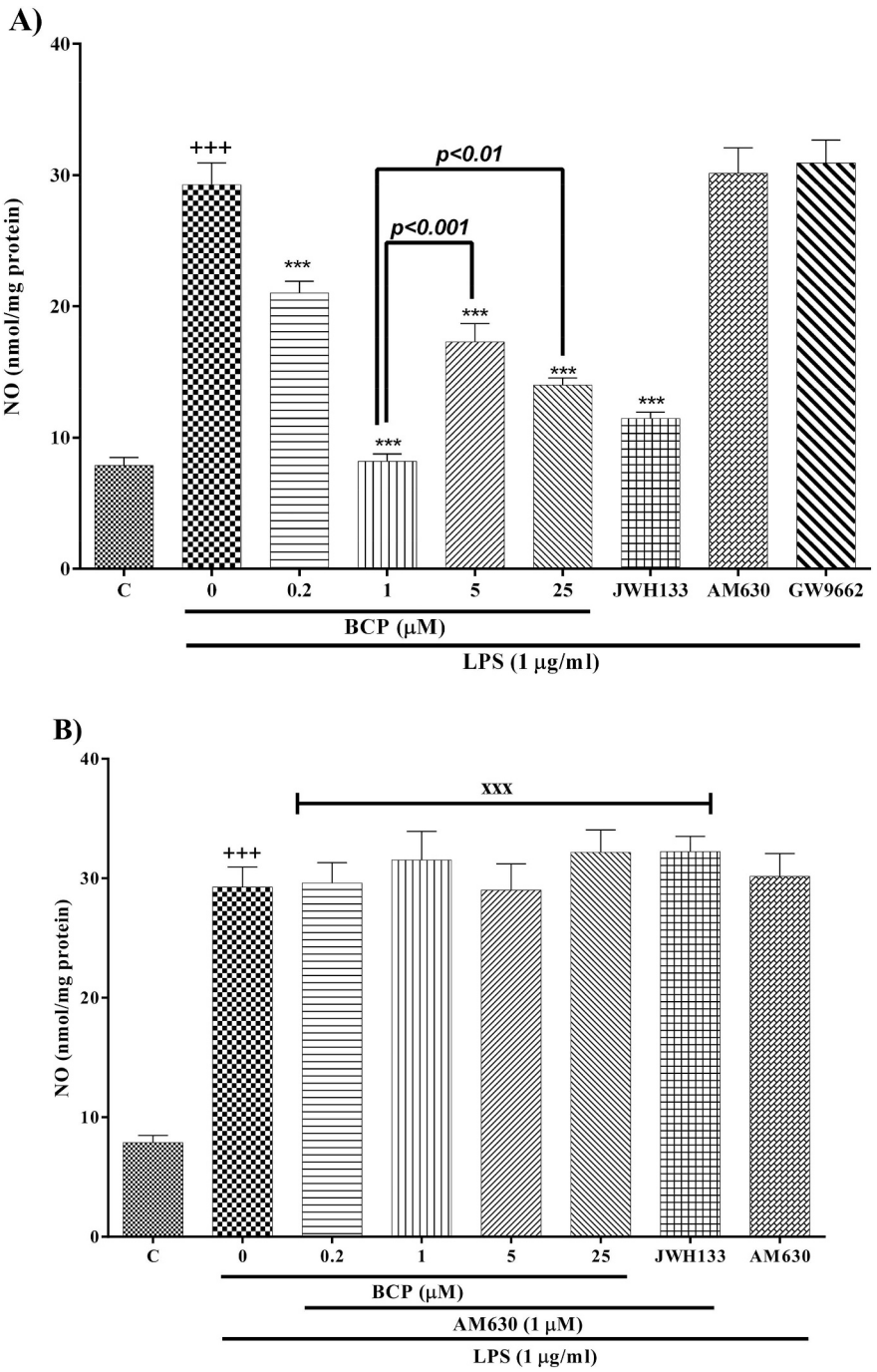


Fig. 7. The effect of different concentrations of BCP on the levels of NO production and iNOS expression; Data were shown as mean ± SEM, n = 8 for each protocol of experiment. After passing the Normality test, comparisons between groups were done using ANOVA with Tukey-Kramer's *post-hoc* multiple comparisons test. For comparing the respected concentrations of BCP to each combination, two-way ANOVA test was done with the following Holm-Sidak's *post-hoc* multiple comparisons test; A) the effect of BCP (0.2–25 μM), JWH-133 (1 μM), AM630 (1 μM) and GW9662 (1 μM) on the level of NO production of microglia during 24 h incubation and then 24 h LPS exposure (1 μg/mL); B) the combination effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with AM-630 (1 μM) on the level of NO production of microglia during 24 h incubation and then 24 h LPS exposure; C) the combination effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with GW-9662 (1 μM) on the level of NO production of microglia during 24 h incubation and then 24 h LPS exposure; D) the combination effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with IMP (1 μM) on the level of NO production of microglia during 24 h incubation and then 24 h LPS exposure; E) the effect of BCP (0.2–25 μM), JWH-133 (1 μM), AM630 (1 μM) and GW9662 (1 μM) on the level of iNOS expression of microglia during 24 h incubation and then 24 h LPS exposure (1 μg/mL); F) the combined effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with AM-630 (1 μM) on the level of iNOS expression of microglia during 24 h incubation and then 24 h LPS exposure; G) the combined effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with GW-9662 (1 μM) on the level of iNOS expression of microglia during 24 h incubation and then 24 h LPS exposure; H) the combined effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with IMP (1 μM) on the level of iNOS expression of microglia during 24 h incubation and then 24 h LPS exposure. (*) shows a comparison between BCP and respected non-treated LPS group, ***: $p < 0.001$; ($^{+}$) compares non-treated LPS group to control group, $^{+++}$: $p < 0.001$; (^{x}) compares different combinations of BCP to the respected concentration of BCP alone in the presence of LPS, ^{xxx}: $p < 0.001$; lines show a comparison between 5 and 25 μM, and 1 μM of BCP. Abbreviations: BCP: β-caryophyllene, LPS: lipopolysaccharides, IMP: imipramine.

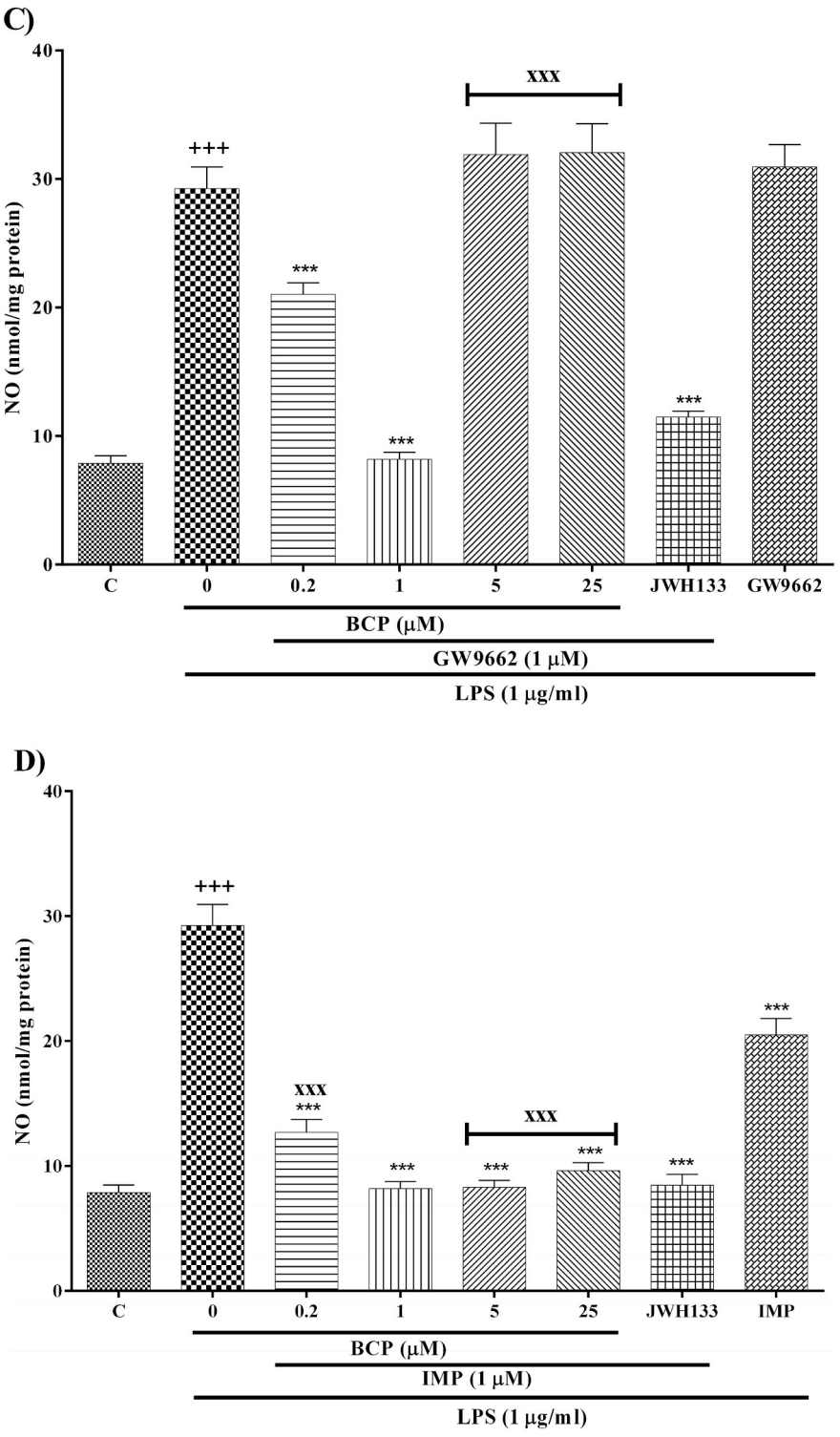


Fig. 7. (*continued*)

declined the effects of either BCP (0.2–25 μM) or JWH-133 (1 μM) on ROS level compared to respected concentrations of BCP alone ($p < 0.001$ for all cases, Fig. 10B), whereas GW9662 markedly removed the effect of BCP (5 and 25 μM) on the level of ROS ($p < 0.001$ for both cases, Fig. 10C). Notably, IMP (1 μM) attenuated ROS level comparing to LPS group ($p < 0.001$, Fig. 10D). IMP (1 μM) also markedly improved ROS reducing effects of BCP (0.2, 5 and 25 μM) comparing to respected concentrations of BCP alone ($p < 0.001$ for all cases, Fig. 10D).

3.8. The effect of LPS and BCP on intracellular GSH

In comparison to the control group, LPS (1 μg/mL) significantly decreased the level of GSH ($p < 0.001$, Fig. 11A). Pre-incubation with BCP (1 and 25 μM) or JWH-133 (1 μM) markedly increased the level of GSH compared to the LPS group ($p < 0.001$ for all cases, Fig. 11A). In the presence of AM-630 (1 μM), the effects of either BCP (1 and 25 μM) or JWH-133 (1 μM) were significantly omitted on GSH level ($p < 0.001$ for all cases, Fig. 11B). GW9662 (1 μM) significantly mitigated the effect of BCP (25 μM) on GSH level ($p < 0.001$, Fig. 11C).

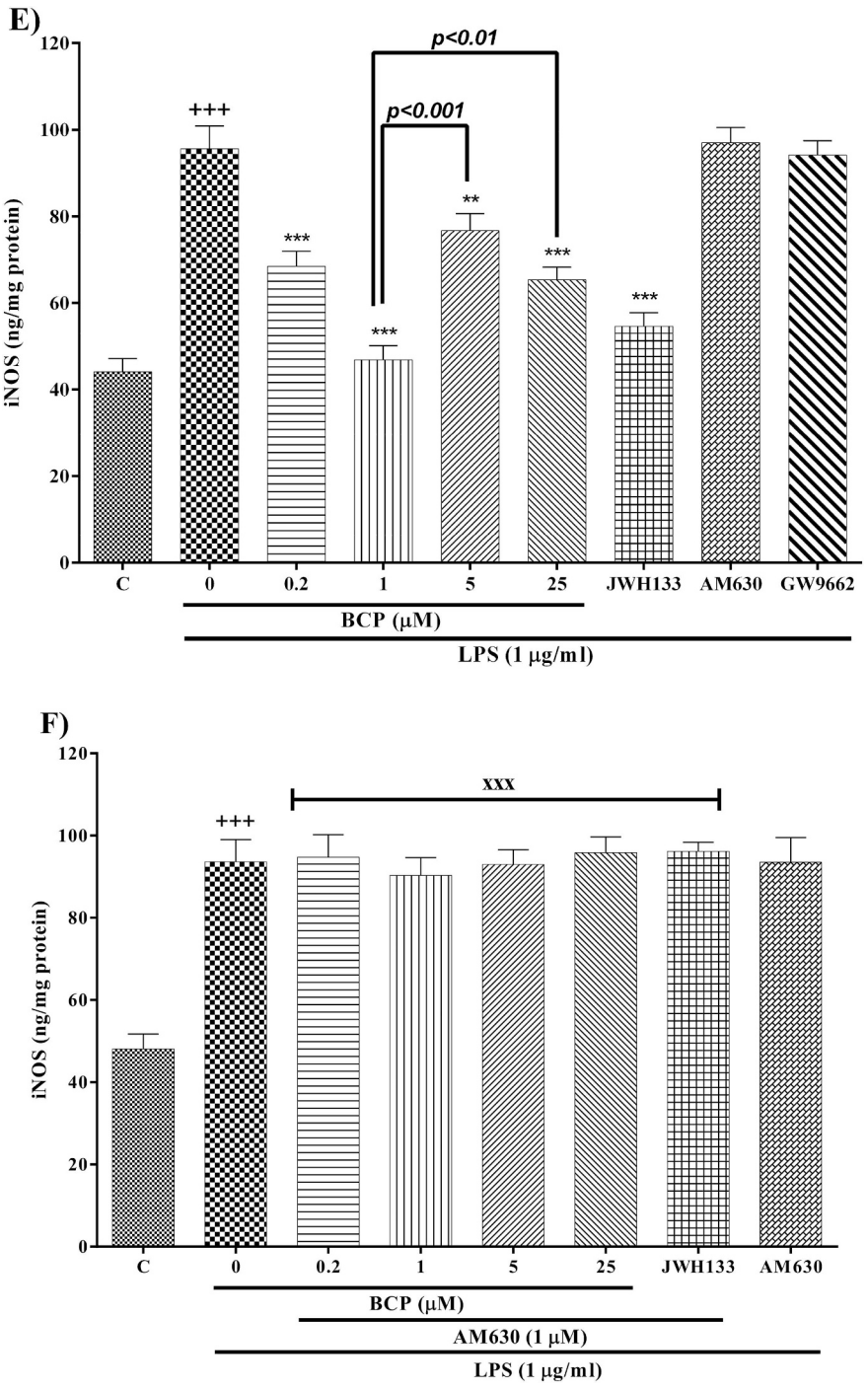


Fig. 7. (*continued*)

IMP (1 μM) significantly enhanced the effect of BCP (0.2, 5 and 25 μM) on the level of GSH comparing to LPS group ($p < 0.001$–0.05 for all cases, Fig. 11D).

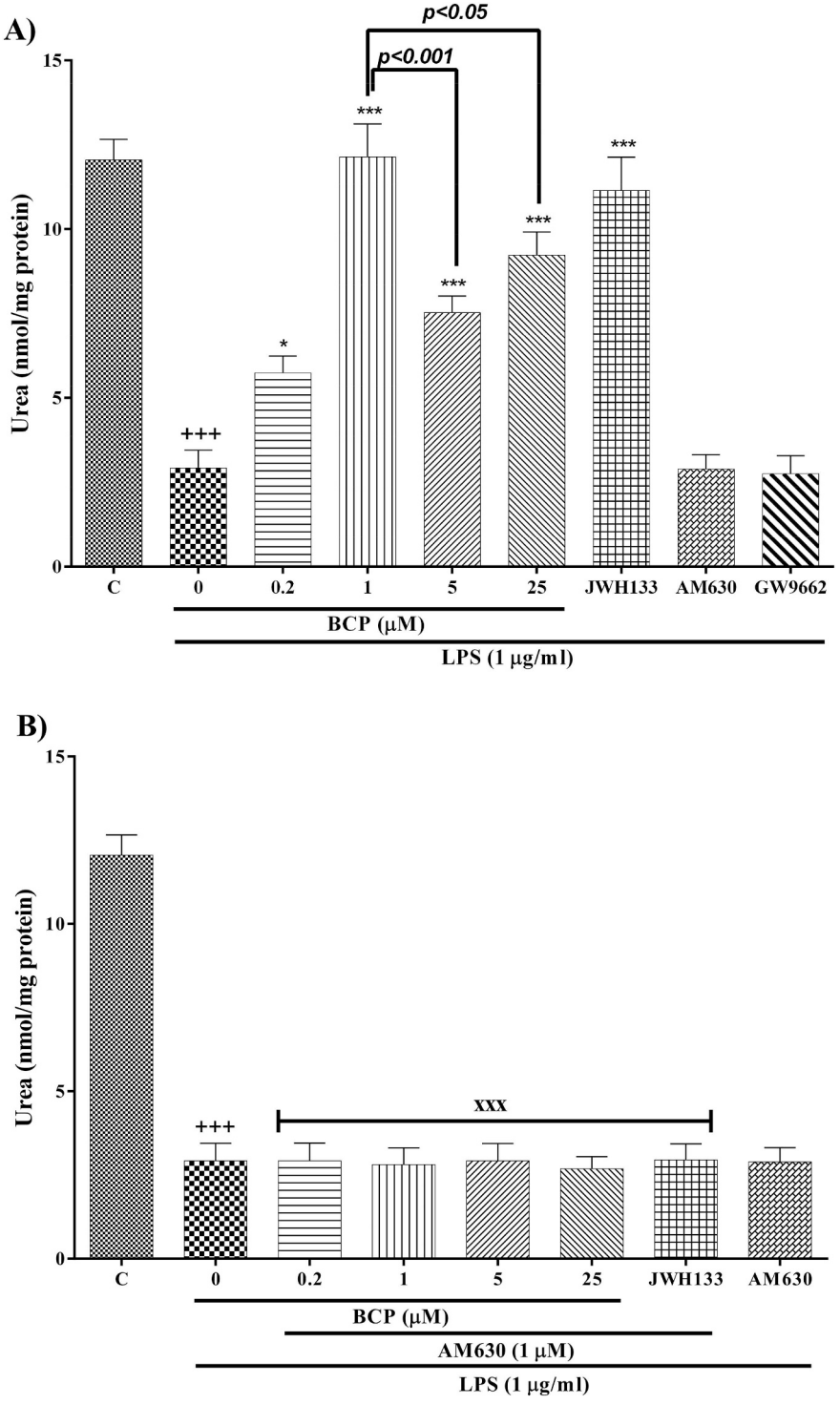
4. Discussion

To our knowledge, this is the first report on immunomodulatory properties of BCP on LPS-induced the inflammatory state of primary mice microglia and M_1/M_2 imbalance. As result, we indicated that BCP (0.2–25 μM) provides anti-inflammatory activities through the CB_2 receptor. Interestingly, we demonstrated that the anti-inflammatory and protective effects of BCP at high concentrations (5 and 25 μM) are also PPAR-γ receptor-mediated in addition to the CB_2 receptor. In addition, we found that the protective effects of low concentrations of BCP (0.2 and 1 μM) are more potent than high concentrations of BCP. In the presence of the SMase inhibitor IMP (1 μM), there did not show different anti-inflammatory effects between low and high concentrations of BCP in which 5 μM of BCP provides a protection as well as 1 μM.

Endotoxin LPS is considered as a potent inflammatory agent which provides an imbalance on microglia phenotypes towards M_1 state via Toll-like receptor-4 (TLR-4) [40–42]. In the present study, we indicated that 24 h exposure to LPS (1 μg/mL) causes a significant reduction in cell proliferation of primary microglia compared to control group. Our results also showed that LPS leads to a significant reduction in the level of GSH as an antioxidant defense of cells, and an increment in ROS formation. Similarly, it has been showed that stimulation of microglia

cells by LPS results in an elevation in the levels of ROS and NO which considered as one of M_1 phenotype characteristics [4,42,43]. Previous studies demonstrated that LPS produces its effect through an increase in the levels of pro-inflammatory cytokines as well as NO production and iNOS expression [4,42]. Likewise, it was illustrated that LPS also decreases the levels of Arg-1 expression and its product urea and subsequent reduction in anti-inflammatory phenotype M_2 macrophage population [4,42,44]. Therewith, several studies presented that the levels of NO/urea or iNOS/Arg-1 ratios could be considered as well marker to predict the rate of M_1/M_2 phenotypes. They also pointed out that assessment of inflammatory (TNF-α and IL-1β) and anti-inflammatory (IL-10) cytokines are necessary to have more accurate insight on the M_1/M_2 balances [4,42,44]. In agreement to previous studies, we showed that LPS induces inflammatory microglia M_1 phenotype by increasing the levels of TNF-α, IL-1β and PGE_2, NO and iNOS, and decreasing the urea and Arg-1 levels. In light of these results, LPS causes an increase in the levels of NO/urea (M_1/M_2) and iNOS/Arg-1 (M_1/M_2) ratios.

CB_2 receptors as a member of the G protein-coupled receptor superfamily (GPCR) are mainly expressed in immune system cells



(caption on next page)

Fig. 8. The effect of different concentrations of BCP on the levels of urea production and Arg-1 expression; Data were shown as mean ± SEM, n = 8 for each protocol of experiment. After passing the Normality test, comparisons between groups were done using ANOVA with Tukey-Kramer's *post-hoc* multiple comparisons test. For comparing the respected concentrations of BCP to each combination, two-way ANOVA test was done with the following Holm-Sidak's *post-hoc* multiple comparisons test; A) the effect of BCP (0.2–25 μM), JWH-133 (1 μM), AM630 (1 μM) and GW9662 (1 μM) on the level of urea production of microglia during 24 h incubation and then 24 h LPS exposure (1 μg/mL); B) the combination effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with AM-630 (1 μM) on the level of urea production of microglia during 24 h incubation and then 24 h LPS exposure; C) the combination effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with GW-9662 (1 μM) on the level of urea production of microglia during 24 h incubation and then 24 h LPS exposure; D) the combination effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with IMP (1 μM) on the level of urea production of microglia during 24 h incubation and then 24 h LPS exposure; E) the effect of BCP (0.2–25 μM), JWH-133 (1 μM), AM630 (1 μM) and GW9662 (1 μM) on the level of Arg-1 expression of microglia during 24 h incubation and then 24 h LPS exposure (1 μg/mL); F) the combined effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with AM-630 (1 μM) on the level of Arg-1 expression of microglia during 24 h incubation and then 24 h LPS exposure; G) the combined effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with GW-9662 (1 μM) on the level of Arg-1 expression of microglia during 24 h incubation and then 24 h LPS exposure; H) the combined effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with IMP (1 μM) on the level of Arg-1 expression of microglia during 24 h incubation and then 24 h LPS exposure. (*) shows comparison between BCP and respected non-treated LPS group, *: $p < 0.05$, **: $p < 0.01$ and ***: $p < 0.001$; ($^{+}$) compares non-treated LPS group to control group, $^{+++}$: $p < 0.001$; (^{x}) compares different combinations of BCP to the respected concentration of BCP alone in the presence of LPS, ^{x}: $p < 0.05$ and ^{xxx}: $p < 0.001$; lines show a comparison between 5 and 25 μM, and 1 μM of BCP. Abbreviations: BCP: β-caryophyllene, LPS: lipopolysaccharides, IMP: imipramine.

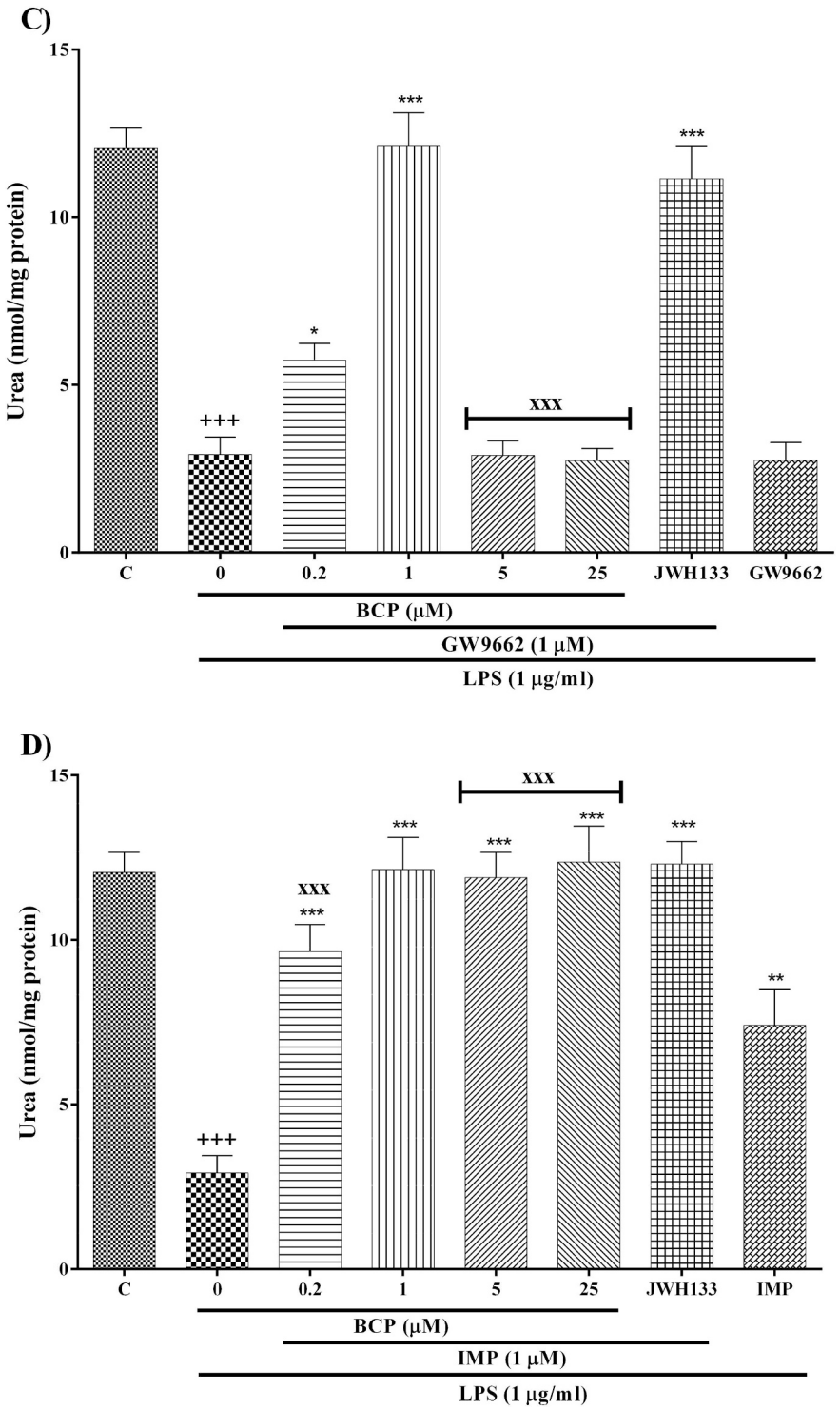
including microglia. In this context, many studies emphasize on the notion that the CB_2 receptor agonists are dose-dependently able to decrease the level of cAMP production in present of FSK stimulation [5,11,18]. For this aim, we initially evaluated the effects of either BCP or JWH-133, a known selective CB_2 receptor agonist, on the inhibition of FSK-induced cAMP levels in the presence or the absence of CB_1 or CB_2 antagonist in microglia cells. JWH-133 and selective antagonists were applied in this study to have better insight into the activity of BCP on CB_2 receptors. As results, both BCP and JWH-133 dose-dependently decreased the level of cAMP content, which in the present of a CB_2 antagonist, BCP dose-response curve was impressed to a right shift similar to JWH-133. Indeed, these findings confirm that first, the CB_2 receptor is expressed in our cells, and secondly, BCP acts through CB_2 receptors at used concentrations in this study, not the CB_1 receptor. In agreement with our results, Gertsch and coworkers also showed in CB_2 receptor-transfected CHO-K1 cells that BCP has a selective effect on the stimulation of CB_2 receptor [20].

Microglia as CNS resident macrophages play crucial and defensive roles on CNS and regeneration of it due to the absence of the adaptive immune cell in the brain such as T lymphocytes [4,42,45]. We showed that BCP (0.2–50 μM) not only has no toxicity effect on microglial cells but also protects the cells against LPS-induced cytotoxicity. There are many supporting studies that demonstrate the protective activity of BCP against chemical and natural toxins. It was demonstrated that BCP (10, 25 and 50 μM) prevents the Aβ-induced cytotoxicity in murine microglia (BV2) cell line [22]. Furthermore, it has been showed that BCP (0.5 and 1 μM) protects glial cells (C6 glioma cells) against glutamate excitotoxicity [25]. Recently, Wang et al. also indicated that BCP (1 and 2.5 μM) ameliorates MPP^{+}-induced cytotoxicity [23]. As expected, our observations showed that the protective effects of BCP are also CB_2 receptor-mediated on LPS-induced toxicity because of CB_2 antagonist completely reversed the effects.

Inflammation is considered as a complex process and condition which act as double-edged swords. Acute inflammation could be a protective and defensive mechanism against many internal and external damaging factors and stimuli; but in chronic conditions, it can cause a lot of damages [35,42,46]. Activation of microglia cell by LPS results in the overexpression and overproduction of inflammatory cytokines and biomarkers. In the present study, we indicated that mice isolated microglia cells produce an excessive amount of inflammatory biomarkers in the presence of LPS, which pre-treatment with BCP significantly declines them through the CB_2 receptor. Previous studies showed that BCP could effectively alleviate the inflammatory conditions. It was showed that BCP abrogates hypoxia-induced activation of BV-2 microglial cells through activation of CB_2 receptor and reduction in the levels of inflammatory cytokines TNF-α, IL-1β and IL-6 as well as inhibition of NF-κB [47]. Furthermore, Yawei Hu and co-workers showed that BCP inhibits Aβ oligomer-induced neuroinflammation in BV-2 microglial cells via the reduction in the levels of production and expression of TNF-α, IL-6, IL-1β, and PGE_2 as well as suppression the nuclear translocation of NF-κB and the activation of NF-κB transcriptional activity [22]. However, they did not investigate the relationship of these effects with CB2 receptor activity. Yang and colleagues also showed that BCP (0.2–25 μM) regulates neuronal death and inflammation in the in-vitro model of cerebral ischemia-reperfusion injury, oxygen-glucose deprivation, and re-oxygenation (OGD/R). They showed that BCP also inhibits TNF-α and IL-1β secretion through inhibiting high mobility group box-1 (HMGB-1)-TLR4 signaling pathway [48]. Recently, it has been indicated that BCP can decrease inflammatory cytokines as well as oxidative stress markers in stimulated lymphocytes [28].

Anti-inflammatory cytokine IL-10 diverts naïve microglia cells towards M_2 phenotype which plays an important role in blunting the destructive inflammatory cascades by down-regulating the secretion of pro-inflammatory cytokines, and reduction in M_1 population [49,50]. Moreover, IL-10 deficiency, particularly in the CNS, overexpresses the pro-inflammatory cytokines which consequently leads to neuronal dysfunction, and behavior and cognition impairment [51,52]. Herein, we additionally observed that BCP enhances the level of IL-10 which is mediated by the CB_2 receptor. In agreement with our results, several studies presented that CB_2 receptor ligands efficiently increase the level of IL-10 production and secretion. Fernando Correa and co-workers described the protective and anti-inflammatory effects of CB_2 receptor agonists are tightly attributed to IL-10 level. They also indicated that the exogenous IL-10 as like as CB_2 receptor agonist JWH-133 significantly enhances IL-10 release by LPS/IFN-γ-activated macrophages which addition of CB_2 receptor antagonist SR144558 completely prevents the effect of JWH-133 [29]. Similarly, Robinson et al. demonstrated that CB_2-selective cannabinoid ligands exert anti-inflammatory effects through the increase in the level of IL-10 release, CD_4 expression and regulatory T-cells (T_{reg}) population which CB_2 expression knockout totally blocks the effect of CB_2 agonist [53]. In addition, Alberti et al. explained that BCP dose-dependently increases the level of IL-10 in CB_2 dependent manner since CB2 antagonist completely declines BCP effects [26]. These studies support our findings of the stimulatory effect of BCP on IL-10 release which is mediated by the CB_2 receptor.

Excessive production and expression of NO and iNOS are involved in pathogenesis of many types of diseases as such inflammatory disease, cancer, MS and allergic reactions like asthma and atopic dermatitis [1,54–58]. Moreover, it has been demonstrated that a reduction in the levels of NO and iNOS lead to the recovery, improvement, and treatment of diseases with imbalanced M_1/M_2 ratio through skewing M_1 towards M_2 phenotype [1,55–58]. Hence, evaluation of the levels of NO production and iNOS protein expression in the presence of LPS stimulation is important for the assessment of inflammation [42,59,60]. Accordingly, we evaluated the effects of pre-medication of microglia cells along with BCP in the presence of LPS stimulation. As results, all concentrations of BCP notably mitigate both the levels of NO production and iNOS expression. Also, in this case, the protective effects of



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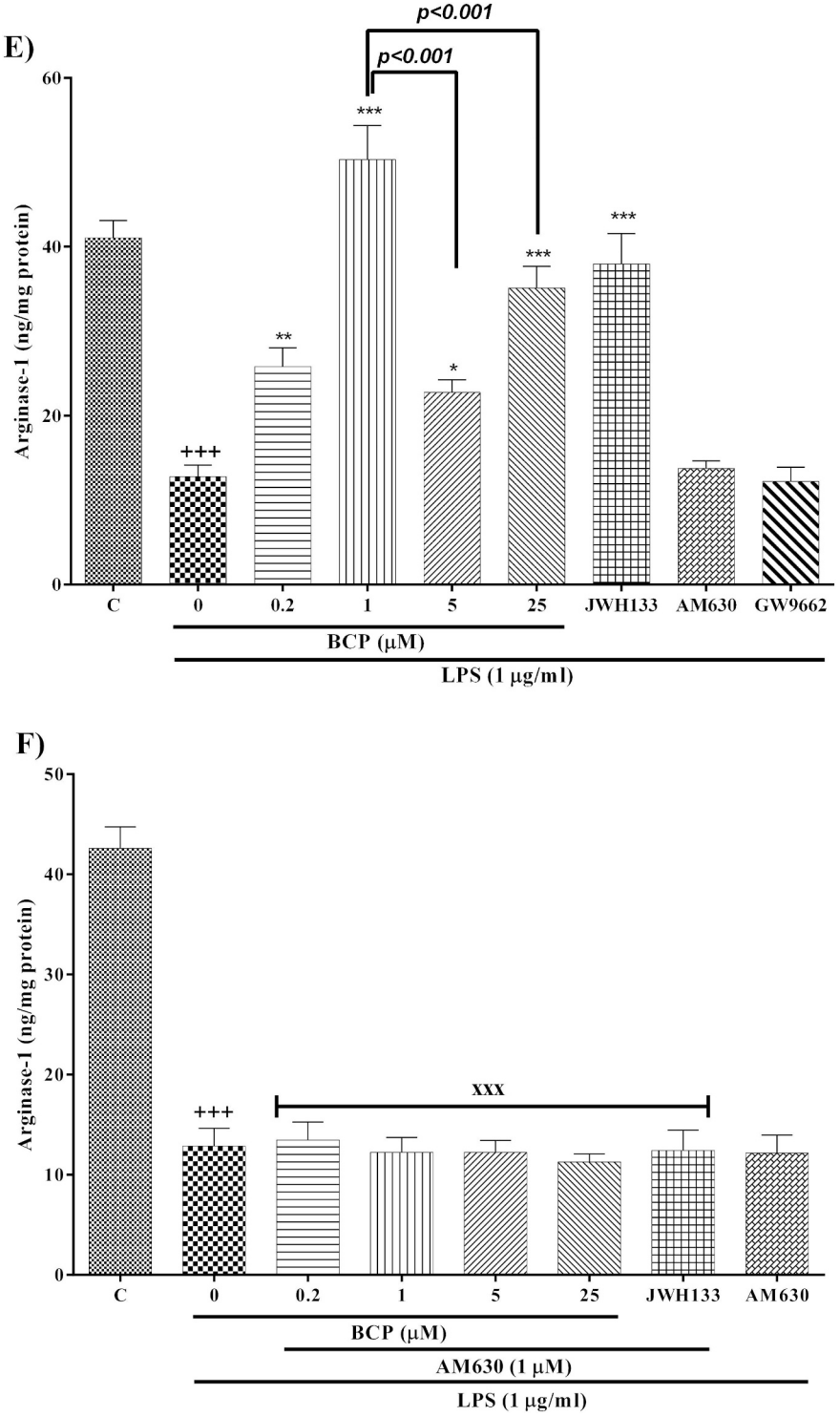
BCP are dependent on CB_2 receptors which the addition of CB_2 receptor antagonist AM630 totally reverses the effects of BCP. In line with our results, it has been showed that BCP suppresses the expression of NO, PGE_2, iNOS, and COX-2 induced by $A\beta_{1-42}$ in BV-2 cells [22]. Moreover, BCP (25 and 50 μM as well as 25 and 50 mg/kg, respectively) shows a significant decrease in the levels of NO and ROS comparing to untreated both of in-vitro (isolated macrophages and lymphocytes) and in-vivo experimental autoimmune encephalomyelitis (EAE) models [28]. Furthermore, Assis et al. illustrated that BCP suppresses the overproduction of ROS and NO induced by glutamate which these effects are totally blocked using the CB_2 antagonist [25].

One of the well-known features to investigate the anti-inflammatory macrophage pattern is the assessment of the levels of Arg-1 expression and its consequent product urea as well as the calculation of the ratios of Arg-1/iNOS or Urea/NO which presents the M_1/M_2 balances. Once the M_1/M_2 balance polarized to M_2, the protection is usually observed especially in the central nervous system [61,62]. Noteworthy, iNOS and Arg-1 compete for the same substrate L-arginine (L-Arg) which overexpression of Arg-1 can deplete the cells from L-Arg and hydrolyzes into ornithine and urea [63,64], there would be a little access to the substrate for iNOS and consequently decrease NO [64]. Therefore, in order to unveil the mechanisms involving in NO and iNOS reduction by BCP,



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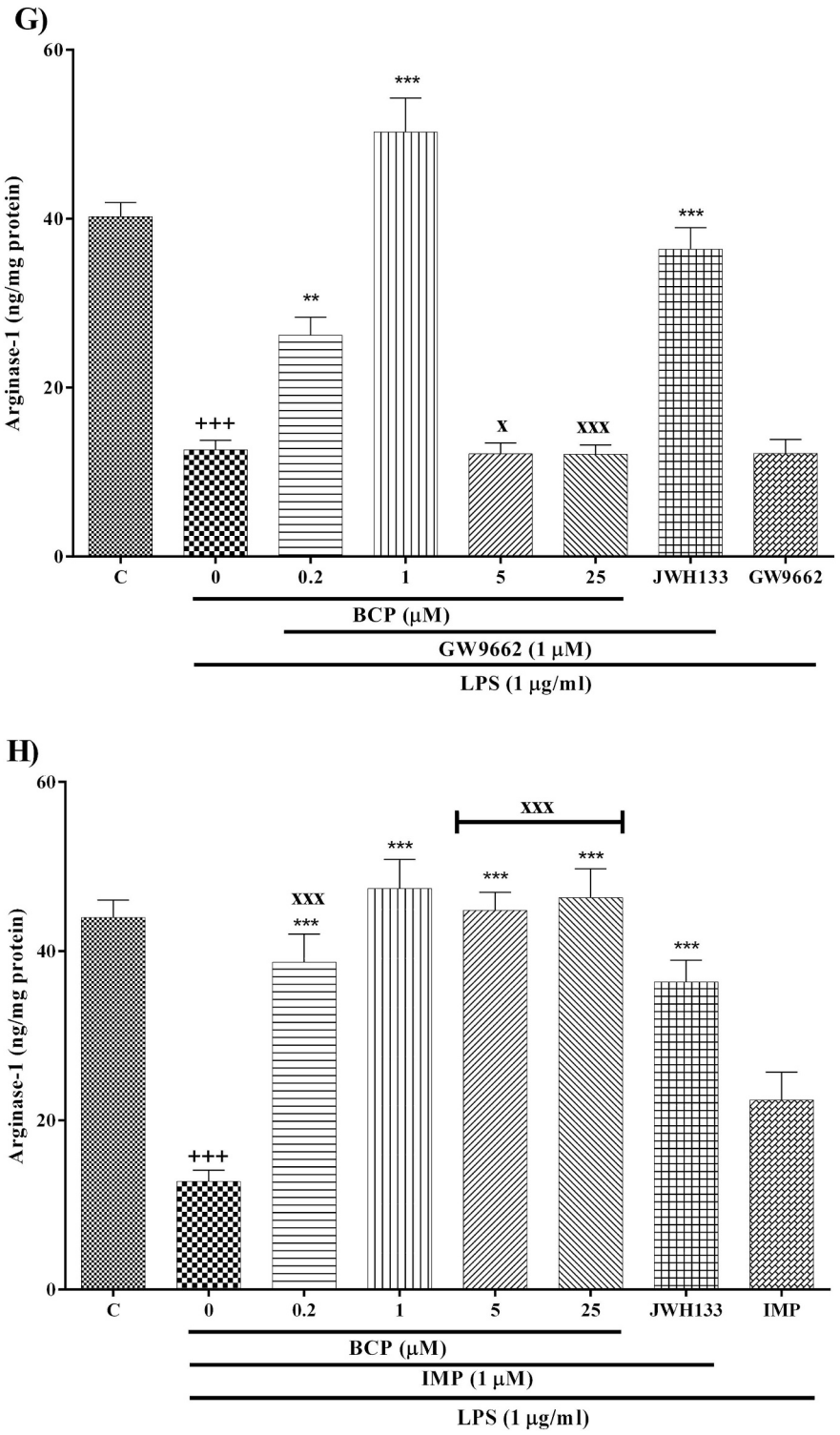
we examined the expression Arg-1 and production levels of urea. According to our results, we showed that all tested concentrations of BCP increase both of the levels of urea production and Arg-1 expression, which these effects are mediated through CB_2 receptor because the incubation of the cells with CB_2 receptor antagonist AM630 completely abolishes the effects of BCP. Moreover, we achieved that the imbalanced and increased levels of NO/urea (M_1/M_2) or iNOS/Arg-1 (M_1/M_2) by LPS are notably declined using BCP and skewed towards M_2 phenotype. In fact, BCP decreases the production and expression of NO and iNOS possibly throughout increases the levels of urea and Arg-1 as result of the diversion in M_1 dominant of M_1/M_2 balance towards anti-inflammatory and healing microglia M_2 phenotype. These findings are also supported throughout our previous information regarding the decrease in the levels of inflammatory cytokines and an increase in the level of IL-10 which collectively polarizes the anti-inflammatory M_2 phenotype. Likewise, these effects are depending on the CB_2 receptor. In accordance with these effects of BCP on CB_2 receptors, several studies also showed that CB_2 receptor ligands considerably polarize the resting or other types of macrophages (M_0 or M_1) into M_2 phenotype showing the reduction in the release of pro-inflammatory cytokines



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which their effects depend on the CB_2 receptor. We found no studies regarding the evaluation of the effects of BCP on microglia polarization, however, there are many supportive studies about protection of CB_2 receptor ligand and their effects on microglia polarization towards M_2 phenotype. In one study, Tao and co-workers determined the protective effects of CB_2 receptor-mediated on secondary brain injury during germinal matrix hemorrhage (GMH). They explained that CB_2 receptor ligand JWH-133 suppresses neuro-inflammation by regulating microglial M_1/M_2 polarization towards M_2 phenotype [65]. A CB_2 agonist 0-1966 also introduced the protective and healing effects against secondary traumatic brain injury (TBI) by reducing the neuroinflammation and providing the healing macrophage state [66]. Denaës et al. presented that JWH-133 protects against alcoholic liver disease throughout the anti-inflammatory and anti-steatogenic properties of CB_2 receptor and polarization of Kupffer cell from M_1 towards M_2 state [67]. These studies also support our conclusion regarding BCP and JWH-133 effects which they regulate M_1/M_2 balance towards M_2 phenotype.

Following the stimulation, an excess ROS production to a level

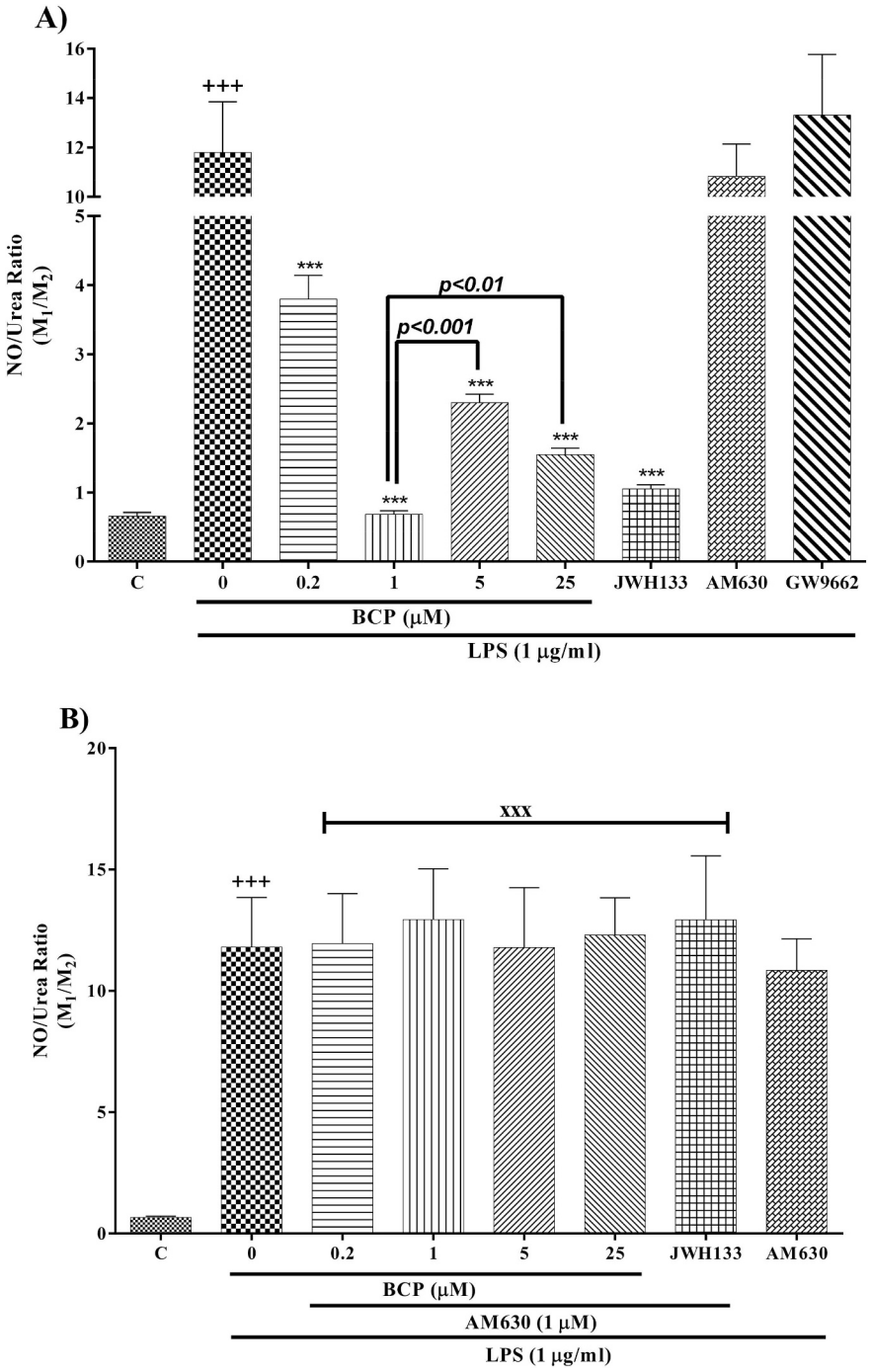
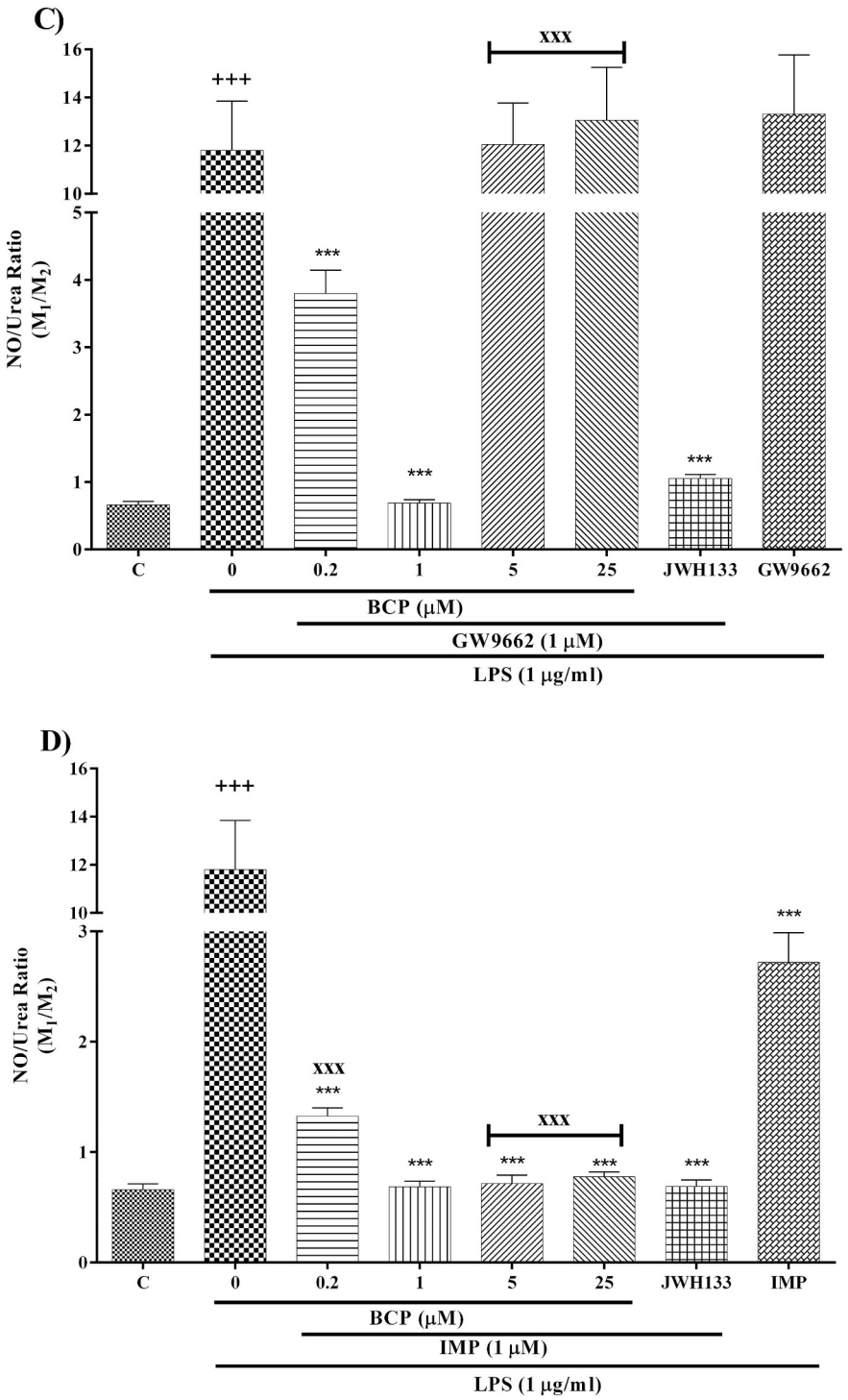


Fig. 9. The effect of different concentrations of BCP on the levels of NO/urea and iNOS/Arg-1 ratios (M_1/M_2); Data were shown as mean ± SEM, n = 8 for each protocol of experiment. After passing the Normality test, comparisons between groups were done using ANOVA with Tukey-Kramer's *post-hoc* multiple comparisons test. For comparing the respected concentrations of BCP to each combination, two-way ANOVA test was done with the following Holm-Sidak's *post-hoc* multiple comparisons test; A) the effect of BCP (0.2–25 μM), JWH-133 (1 μM), AM630 (1 μM) and GW9662 (1 μM) on the level of NO/urea (M_1/M_2) ratio of microglia during 24 h incubation and then 24 h LPS exposure (1 μg/mL); B) the combination effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with AM-630 (1 μM) on the level of NO/urea (M_1/M_2) ratio of microglia during 24 h incubation and then 24 h LPS exposure; C) the combination effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with GW-9662 (1 μM) on the level of NO/urea (M_1/M_2) ratio of microglia during 24 h incubation and then 24 h LPS exposure; D) the combination effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with IMP (1 μM) on the level of NO/urea (M_1/M_2) ratio of microglia during 24 h incubation and then 24 h LPS exposure; E) the effect of BCP (0.2–25 μM), JWH-133 (1 μM), AM630 (1 μM) and GW9662 (1 μM) on the level of iNOS/Arg-1 (M_1/M_2) ratio of microglia during 24 h incubation and then 24 h LPS exposure (1 μg/mL); F) the combined effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with AM-630 (1 μM) on the level of iNOS/Arg-1 (M_1/M_2) ratio of microglia during 24 h incubation and then 24 h LPS exposure; G) the combined effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with GW-9662 (1 μM) on the level of iNOS/Arg-1 (M_1/M_2) ratio of microglia during 24 h incubation and then 24 h LPS exposure; H) the combined effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with IMP (1 μM) on the level of iNOS/Arg-1 (M_1/M_2) ratio of microglia during 24 h incubation and then 24 h LPS exposure. (*) shows a comparison between BCP and respected non-treated LPS group, ***: $p < 0.001$; ($^{+}$) compares non-treated LPS group to control group, $^{+++}$: $p < 0.001$; (^{x}) compares different combinations of BCP to the respected concentration of BCP alone in the presence of LPS, ^{xxx}: $p < 0.001$; lines show a comparison between 5 and 25 μM, and 1 μM of BCP. Abbreviations: BCP: β-caryophyllene, LPS: lipopolysaccharides, IMP: imipramine.



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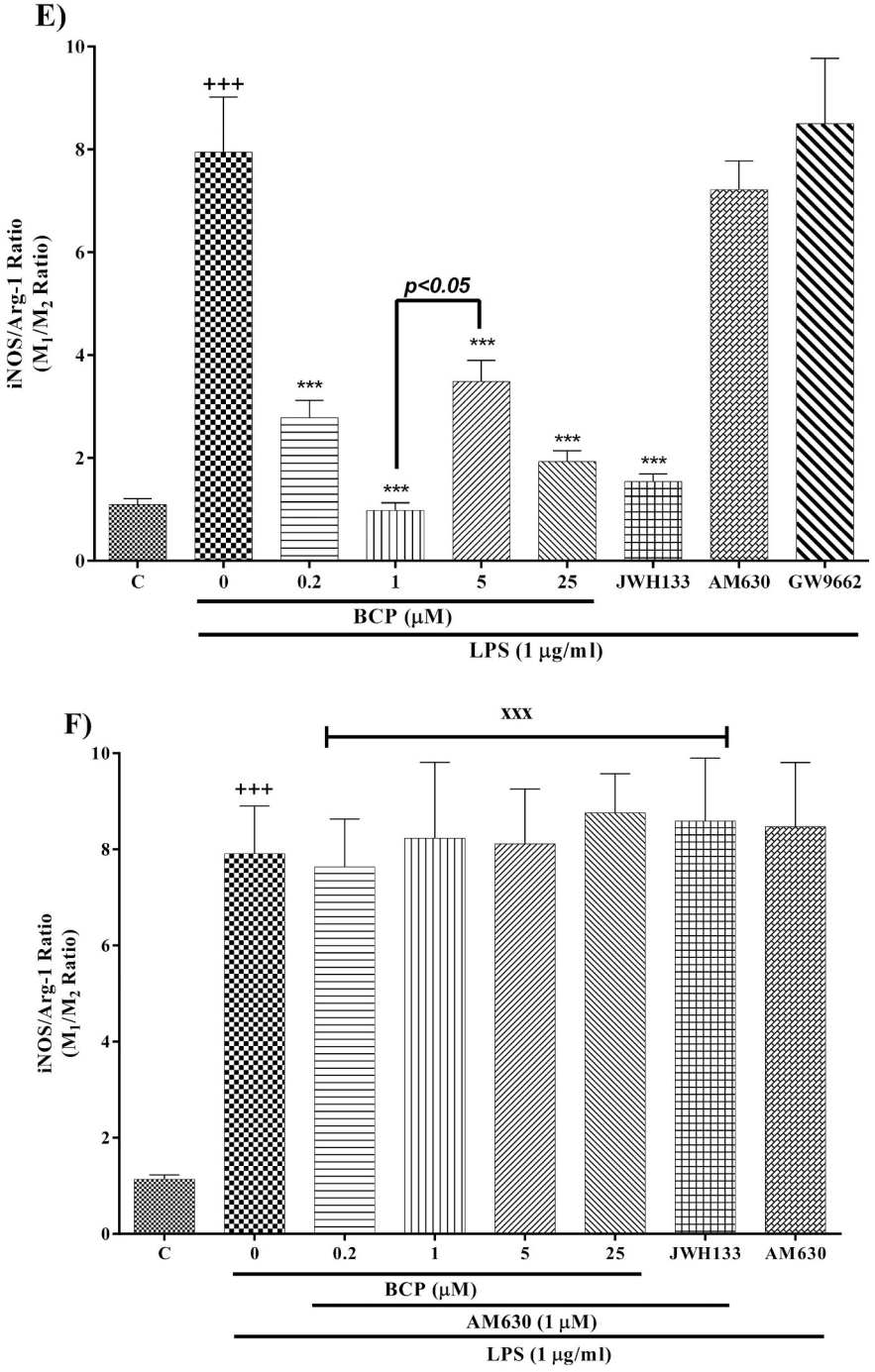
higher than physiological concentrations results in oxidative stress, although some essential roles and advantages of ROS cannot be ignored in normal cell functions including proliferation, differentiation, and cell death [68], long-term sustained high levels of ROS leads to the destructive effects on proteins and other macromolecules. Using generation of ROS, the M_1 phenotype of microglia cells has defensive and protective roles against injuries. Contextually, anti-oxidant systems such as thiol-based (GSH) components dampen over-activity of ROS signaling pathways [40,42,68]. In the present study, LPS exacerbated the level of ROS production, whereas pre-medication with all concentrations of BCP decreases ROS generation along with the increase in the cellular content of GSH as the anti-oxidant factor. Interestingly, we also found that the effects of BCP on ROS and GSH are CB2 receptor-mediated. In accordance with these findings, the effects of BCP were also evaluated on ROS generation level using both in-vitro and in-vivo models which BCP shows a decreasing effect in the level of ROS [28]. Also, Assis et al. demonstrated that BCP diminishes the levels of ROS and NO which the effects are completely blocked by using CB_2 antagonist [25]. Recently, it was also documented that BCP attenuates the level of ROS formation by MPP^+-induced toxicity in human SH-SY5Y cells, while the effects completely eliminated using CB_2 antagonist AM-630 [23]. It was also described that BCP increases the levels of GSH and



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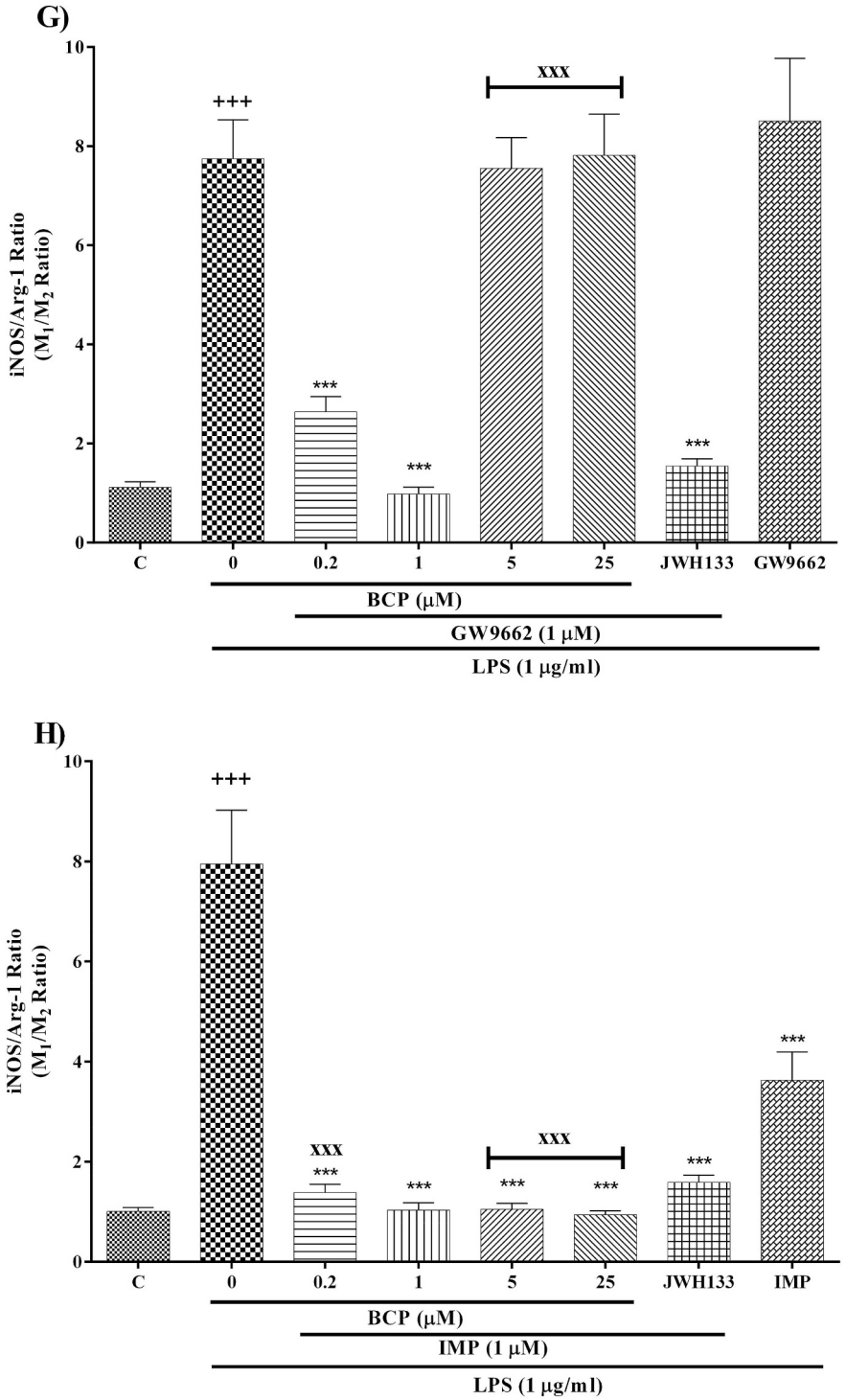
glutathione peroxidase activity against glutamate excitotoxicity or in-vitro model of Parkinson's disease (MPP^+) [23,25]. Furthermore, Jinghua Shan and colleagues presented the protective effects of BCP is depended on CB_2 receptor signaling pathway on maintaining osteoblast function, the prevention of ROS generation and an elevation in the intracellular level of GSH [24]. Additionally, it has been noticed that BCP activates the anti-oxidant signaling pathway nuclear factor erythroid 2–related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) which encourage the levels of GSH and glutathione peroxidase activity [25]. In this way, several studies expressed that Nrf2/HO-1 signaling pathways increase the levels of anti-inflammatory cytokines, and also abrogate inflammatory responses [69]. These studies support our findings on BCP effects on the prevention of ROS formation, and the increment of GSH level.

Intriguingly, we also revealed that BCP has not a consistency in a relationship between applied concentrations and achieved the response in all measured parameters, and there is an abrupt gap which the protective responses of BCP are divided into two phases. Indeed, the concentration-dependent effects of BCP are mediated throughout the CB_2 receptor and the addition of CB_2 antagonist completely abolishes the effects of BCP. To explain the fluctuated effects of the CB_2 agonist BCP, we should consider the involvement of multiple CB_2 post-receptor signaling pathways which are functionally important in different concentrations. However, the effects of each CB_2 agonist on a single signaling there should be concentration-dependent without any fluctuation. Actually, primary evaluation of wide range concentrations of BCP on the reduction in the level of FSK-induced cAMP production also confirmed that applied concentrations of BCP are in this range and act



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selectively through CB_2 receptor which the addition of CB_2 antagonist provides a right shift in a dose-response curve like JWH-133. In the light of previous studies, CB_2 receptor ligands may also show the protective activity through the PPAR-γ receptor [70–73]. Therefore, to have a better understanding and unveiling the involvement mechanisms, we also considered another set of experiment to investigate the effects of the same concentrations of BCP on LPS-induced microglia inflammation and polarization using a PPAR-γ antagonist GW9662. Unlike to low concentrations (0.2 and 1 μM) of BCP, the results of the current study showed that the effects of BCP at high concentrations (5 and 25 μM) are dependent to both PPAR-γ and CB_2 receptors. Certainly, it means that the second phase of the protective effects of BCP is one consequence following CB_2 receptor stimulation which can be seen only at high concentrations of BCP. In this regard, we can additionally conclude that the effects of BCP at high concentrations are possibly mediated through post-receptors either of signaling pathways or mediators acting on the PPAR-γ receptor as consequence of activation of CB_2 receptors since the application of CB_2 receptor antagonist similarly declines all achieved effects of BCP. In line with our findings, other studies also showed that BCP (48 mg/kg) mitigates Alzheimer-like disease model via both of CB_2 receptor and PPAR-γ signaling pathways [74]. In addition, Bento et al. showed that BCP (12.5–50 mg/kg) has

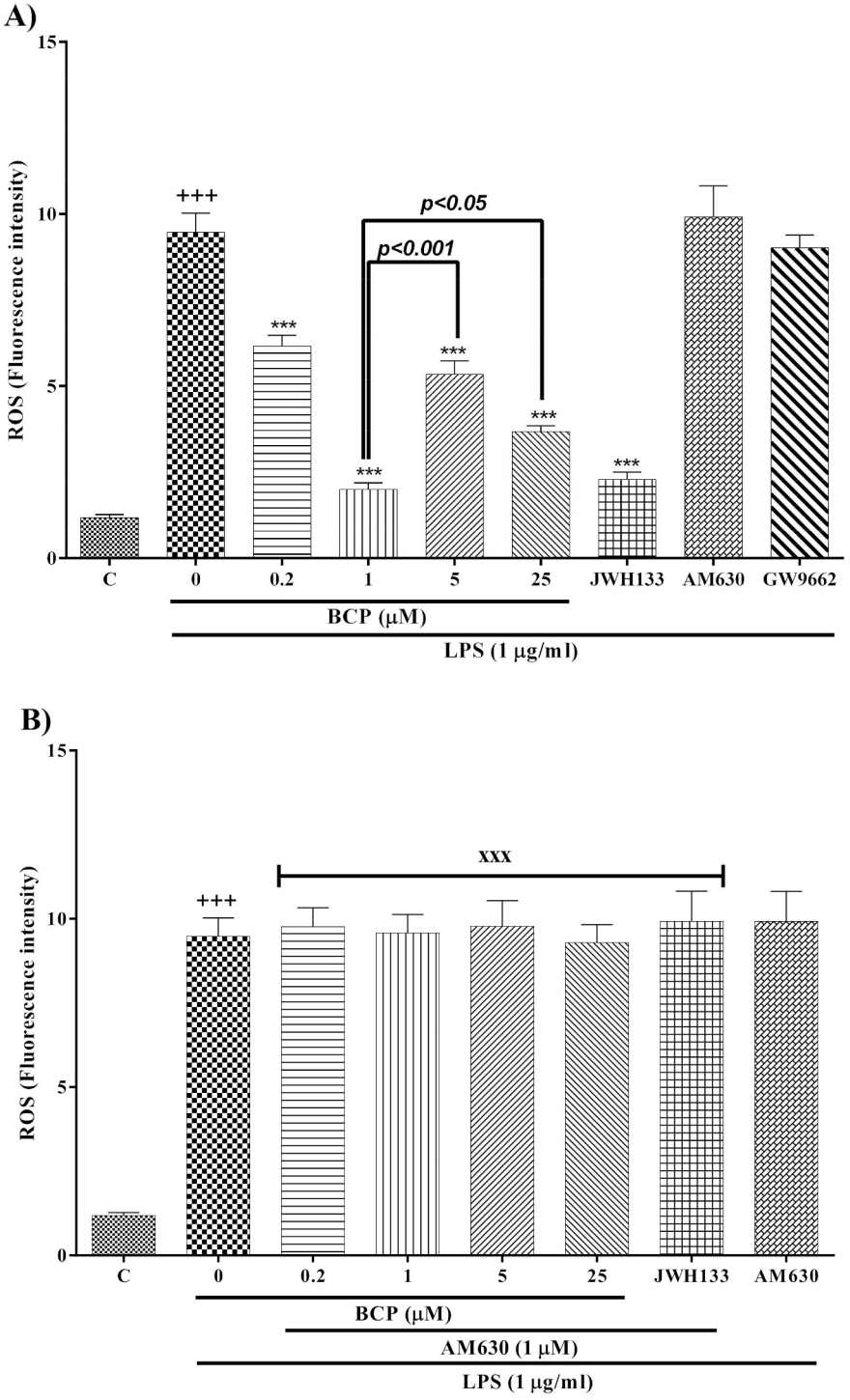


Fig. 10. The effect of different concentrations of BCP on the level of ROS production; data were shown as mean ± SEM, n = 8 for each protocol of experiment. After passing the Normality test, comparisons between groups were done using ANOVA with Tukey-Kramer's *post-hoc* multiple comparisons test. For comparing the respected concentrations of BCP to each combination, two-way ANOVA test was done with the following Holm-Sidak's *post-hoc* multiple comparisons test; A) the effect of BCP (0.2–25 μM), JWH-133 (1 μM), AM630 (1 μM) and GW9662 (1 μM) on the level of ROS production of microglia during 24 h incubation and then 24 h LPS exposure (1 μg/mL); B) the combination effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with AM-630 (1 μM) on the level of ROS production of microglia during 24 h incubation and then 24 h LPS exposure; C) the combination effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with GW-9662 (1 μM) on the level of ROS production of microglia during 24 h incubation and then 24 h LPS exposure; D) the combination effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with IMP (1 μM) on the level of ROS production of microglia during 24 h incubation and then 24 h LPS exposure. (*) shows a comparison between BCP and respected non-treated LPS group, ***: $p < 0.001$; ($^{+}$) compares non-treated LPS group to control group, $^{+++}$: $p < 0.001$; (^{x}) compares different combinations of BCP to the respected concentration of BCP alone in the presence of LPS, ^{xxx}: $p < 0.001$; lines show a comparison between 5 and 25 μM, and 1 μM of BCP. Abbreviations: BCP: β-caryophyllene, LPS: lipopolysaccharides, IMP: imipramine.

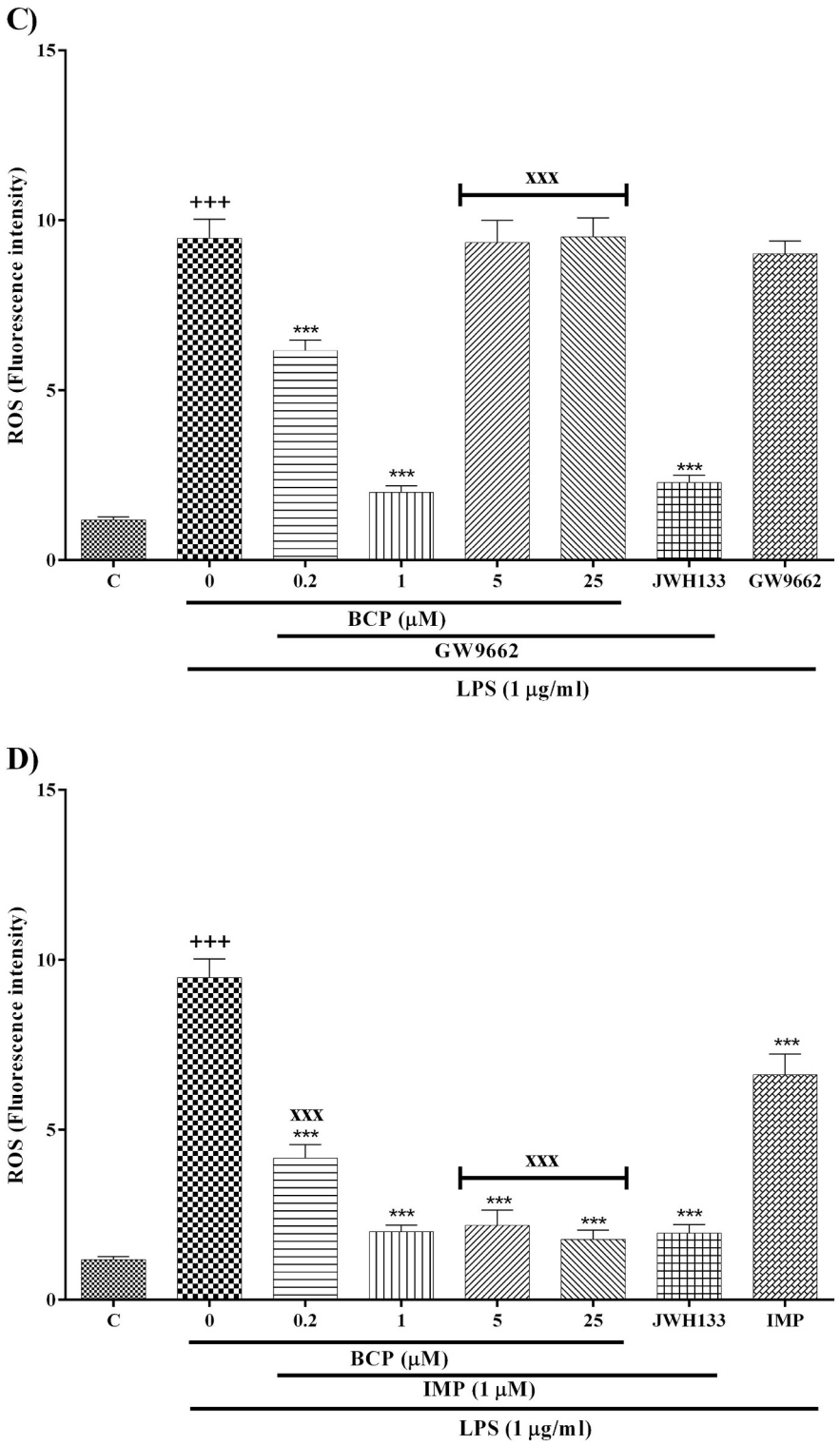


Fig. 10. (*continued*)

therapeutic and preventive effects on DSS (dextran sulfate sodium) model of colitis which are totally declined using either CB_2 or PPAR-γ antagonists [27]. To our knowledge so far, there is no study to explain the molecular mechanism to connect the activation of CB_2 to activation of PPAR-γ, although this behavior may occur for most of the CB_2 receptor ligands. Noteworthy, CB_2 receptor ligand JWH-015 introduced anti-cancer activity on hepatocellular carcinoma through CB_2 receptor which this effect is also PPAR-γ receptor-mediated since the administration of either PPAR-γ antagonist or specific PPAR-γ si-RNA totally blocked the effects of JWH-015 [72]. Fernando Correa et al. also described that activation of CB_2 receptor using JWH-133 leads to decrement in the level of pro-inflammatory cytokine IL-12 which was not concentration dependent without any explanation for possible mechanisms. [29].

Several studies showed that CB_2 receptor stimulation leads to the activation of $G_{i\alpha}$ which attenuates cell cAMP contents, while long-term exposure or high concentrations of selective CB_2 receptor ligands due to the nature of GPCR lead to initiation of other CB_2 receptor-dependent

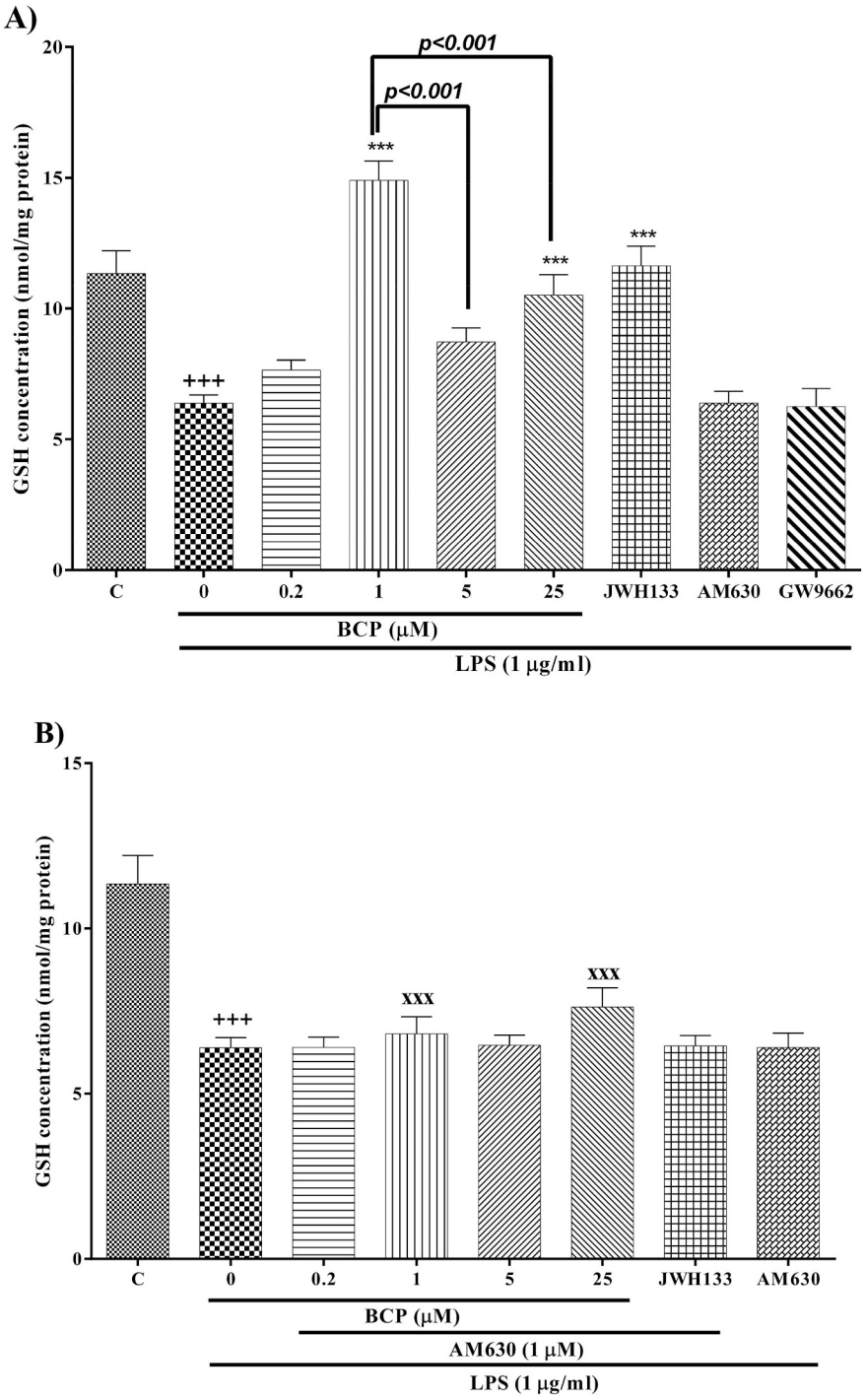
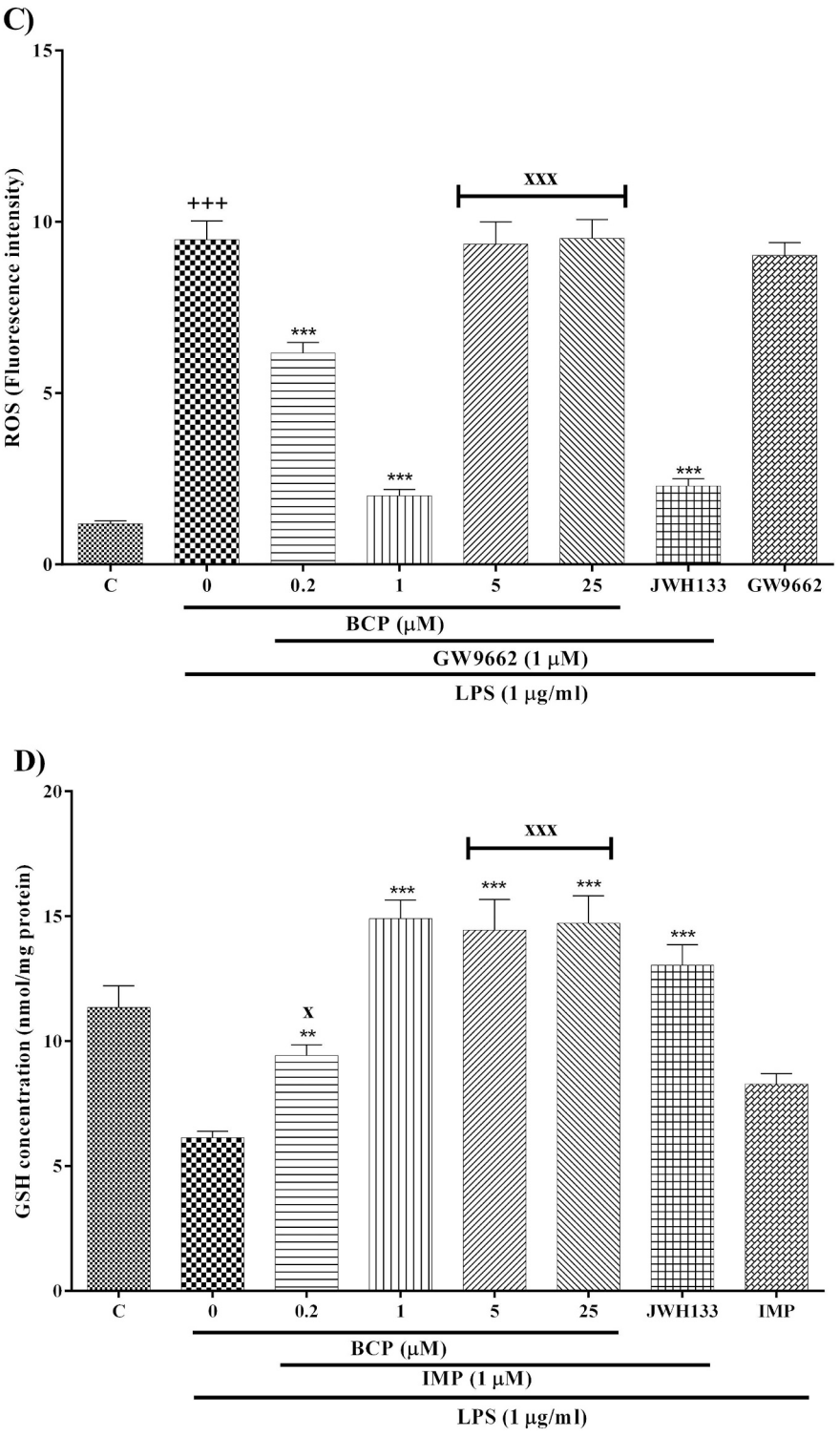


Fig. 11. The effect of different concentrations of BCP on the level of GSH; Data were shown as mean ± SEM, n = 8 for each protocol of experiment. After passing the Normality test, comparisons between groups were done using ANOVA with Tukey-Kramer's *post-hoc* multiple comparisons test. For comparing the respected concentrations of BCP to each combination, two-way ANOVA test was done with the following Holm-Sidak's *post-hoc* multiple comparisons test; A) the effect of BCP (0.2–25 μM), JWH-133 (1 μM), AM630 (1 μM) and GW9662 (1 μM) on the level of GSH of microglia during 24 h incubation and then 24 h LPS exposure (1 μg/mL); B) the combination effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with AM-630 (1 μM) on the level of GSH of microglia during 24 h incubation and then 24 h LPS exposure; C) the combination effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with GW-9662 (1 μM) on the level of GSH of microglia during 24 h incubation and then 24 h LPS exposure; D) the combination effects of BCP (0.2–25 μM) or JWH-133 (1 μM) with IMP (1 μM) on the level of GSH of microglia during 24 h incubation and then 24 h LPS exposure. (*) shows a comparison between BCP and respected non-treated LPS group, **: $p < 0.01$ and ***: $p < 0.001$; ($^{+}$) compares non-treated LPS group to control group, $^{+++}$: $p < 0.001$; (^{x}) compares different combinations of BCP to the respected concentration of BCP alone in the presence of LPS, ^{x}: $p < 0.001$ and ^{xxx}: $p < 0.001$; lines show a comparison between 5 and 25 μM, and 1 μM of BCP. Abbreviations: BCP: β-caryophyllene, LPS: lipopolysaccharides, IMP: imipramine.

signaling pathways including either βγ-subunit or β-arrestin internalization [75,76]. One of the important targets of related components is sphingomyelinase (SMase) activation by CB_2-activated βγ-subunit which results in the production of ceramides the activator of the PPAR-γ receptor [75,76]. On this basis, we investigated the possible interaction of sphingomyelinase activity using IMP, a known SMase inhibitor, to determine the causes of two phases of selective CB_2 agonist BCP responses [32,33]. In the present study, we revealed that the



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combination of IMP with BCP increases all the protective effects of BCP at all concentrations against LPS-induced inflammation with a single phase concentration-response curve. We may conclude that high concentrations of BCP over-stimulate βγ-subunit which it more activates SMase leading to excessive production of ceramides although we cannot exclude other mechanisms like internalized GPCR mediated signaling.

Some studies showed that ceramides are involved in the activation and expression of PPAR-γ which in the presence of its antagonist the effects are totally disappeared [70,73]. Additionally, it was showed that either CB_1 or CB_2 receptor activation induces apoptosis through ceramides de novo synthesis in colon cancer cells [77]. JWH-133 also showed anti-tumoral activity against gliomas through CB_2 receptor and related ceramides production because of using either the CB_2 antagonist or ceramides production inhibitors completely blocked the effects of JWH-133 [78]. Intriguingly, Vara et al. explained the participation of PPAR-γ receptor in the anti-tumoral activity of Δ9-tetrahydrocannabinol (THC) and JWH-015 on hepatocellular carcinoma [72]. They also demonstrated that these effects are PPAR-γ receptor-mediated which pharmacological inhibition of PPAR-γ caused a significant right shift of the dose-response curve. Furthermore, they

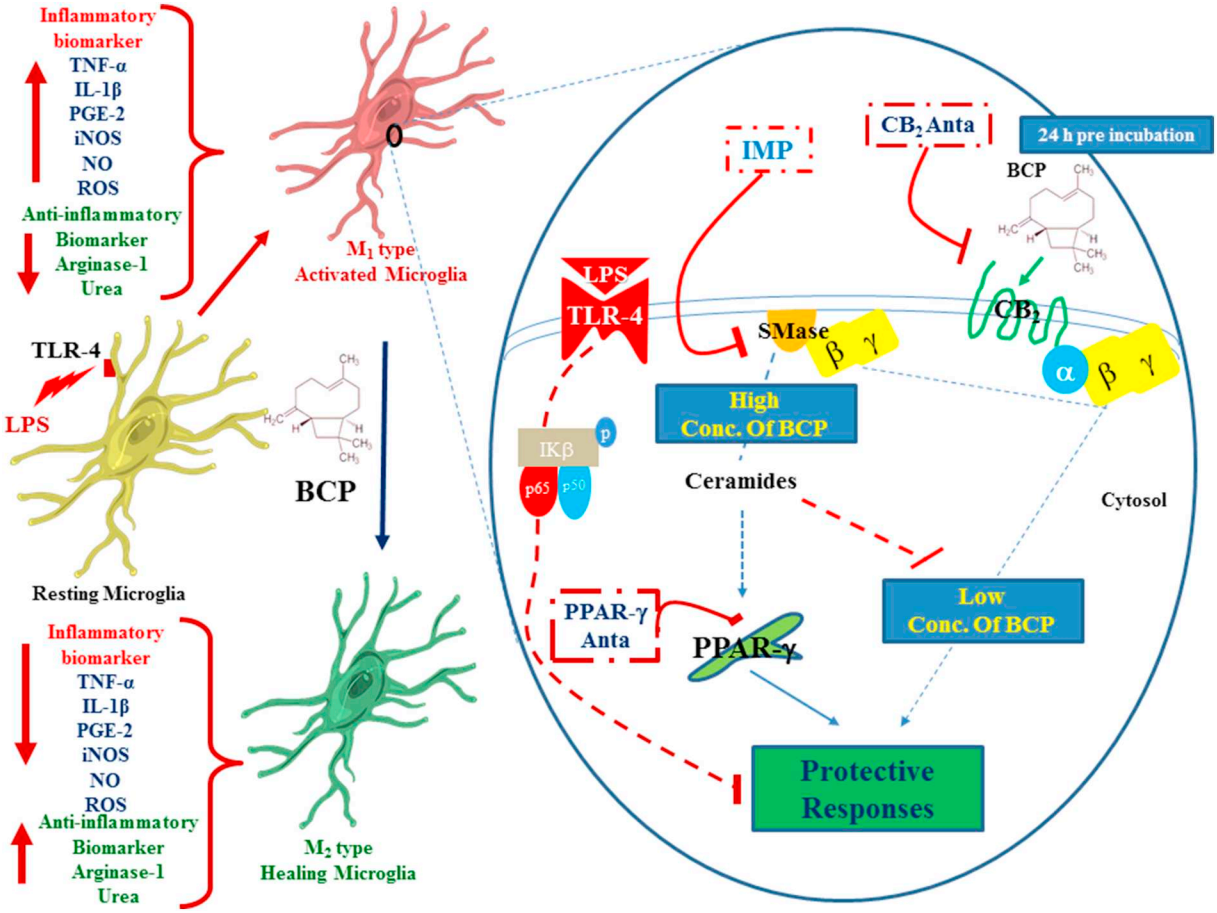

Fig. 12. The summarized of possible involved mechanisms in the protective and modulatory effects of BCP on M_1 polarized cells.

observed that cannabinoids induce ceramides accumulation in HepG2 cells which link CB_2 and PPAR-γ activation [72]. These studies may support our findings on the contribution of ceramides between CB_2 and its related PPAR-γ signaling. However, as limitation and suggestion of the present study, assessment of the levels of cytoplasmic and nuclear fractions of PPAR-γ and ceramides in the presence or absence of CB_2 antagonist may be supportive to evaluate the relation between CB_2 and PPAR-γ receptors.

5. Conclusion

Taken together, the results of the present study indicate the protective effects of BCP on LPS-induced microglia imbalance by providing the M_2 healing phenotype of microglia, releasing the anti-inflammatory (IL-10, Arg-1 and urea) and anti-oxidant (GSH) parameters and reducing the inflammatory (IL-1β, TNF-α, PGE_2, iNOS and NO) and oxidative (ROS) biomarkers (Fig. 12). Moreover, we showed that BCP exerts its effects through CB_2 receptors which over-production of ceramides by SMase at middle to higher concentrations of BCP reduce the protective activity of BCP and results in the activation of the PPAR-γ pathway. Collectively, we can conclude the low concentrations of BCP, and possibly other CB_2 receptor ligands, have higher selective anti-inflammatory effects rather than high levels of ligand which may activate other adverse effects. On this occasion, BCP by modulating the microglia is able to have therapeutic and protective effects in neuro-degenerative disorders by involving the neuro-inflammation conditions and microglia cells such as MS and Alzheimer's diseases.

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Compliance with ethical standards

Conflict of interest

The authors declare that there are no conflicts of interest.

Declaration of transparency and scientific rigor

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigor of preclinical research recommended by funding agencies, publishers and other organizations engaged with supporting research.

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