



Berberis lycium Royle fruit extract mitigates oxi-inflammatory stress by suppressing NF- κ B/MAPK signalling cascade in activated macrophages and Treg proliferation in splenic lymphocytes

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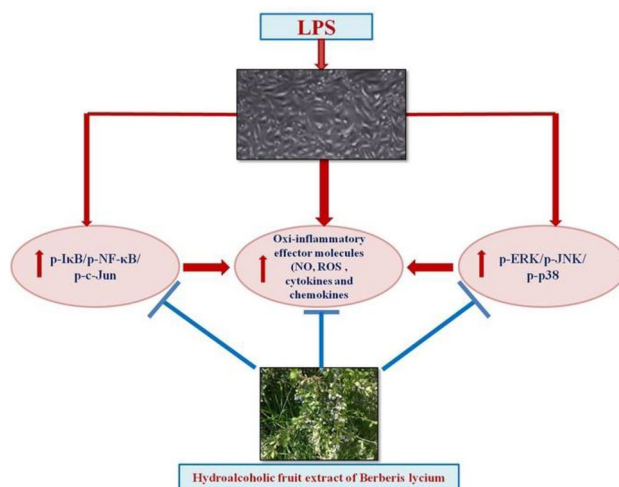
Abstract

Although *Berberis* plant species have been advocated as immune modulators, information regarding their mechanism(s) of action is limited. Therefore, in the present study we assessed the efficacy of *Berberis lycium* Royle fruit extract (BLFE) in the attenuation of lipopolysaccharide (LPS)-induced oxi-inflammatory aggravation and concanavalin A (Con-A)-induced proliferation in murine peritoneal macrophages and lymphocytes, respectively. BLFE strongly suppressed production of the oxidative and inflammatory effector molecules nitric oxide (NO), reactive oxygen species (ROS), inducible nitric oxide synthase (iNOS), inflammatory cytokines (TNF- α /IL-6/IL-1 β /IFN- γ) as well as chemokines (MCP-1 and RANTES), with a concomitant enhancement in heme oxygenase-1 (HO-1) and IL-10 levels. Subsequent mechanistic analysis revealed that BLFE strongly inhibited the phosphorylation of I κ B α as well as MAPKs such as extracellular signal-regulated kinase (ERK), p38 MAPK, and c-Jun NH2-terminal kinase (JNK), thereby directly resulting in the suppression of nuclear factor- κ B (NF- κ B) and c-Jun activation, ultimately culminating in the observed attenuation of inflammatory molecules. Additionally, BLFE appeared to mitigate Con-A-induced proliferation of Tregs (CD3+ CD4+ CD25+) thereby suggesting its modulatory effects on adaptive immune cells. UPLC–DAD–ESI–QTOF–MS/MS of BLFE revealed the presence of major bioactive phenolics and alkaloids including chlorogenic acid, rutin, catechin and quercetin 3-D-galactoside, berberine and magnoflorine, which could have synergistically contributed to the above findings. Overall, this work provides evidence that BLFE may be effective in the mitigation of inflammatory disorders, especially those associated with NF- κ B/MAPK activation.

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Extended author information available on the last page of the article

Graphical abstract



Keywords *Berberis lycium* · Fruit extract · Oxi-inflammation · NF- κ B · c-Jun · MAPKs · Tregs

Introduction

Inflammation is a natural immune response to invading pathogens and injury, but the process is also implicated in the pathogenesis of various chronic inflammatory disorders, such as rheumatoid arthritis, inflammatory bowel disease and neurodegenerative disorders (Jiang et al. 2017; Sharma et al. 2018). Inflammatory responses involve both innate and adaptive immune cells, as well as effector ROS molecules such as NO and various cytokines and chemokines including TNF- α , IL-1 β , IL-6, MCP-1 and RANTES. These responses are under tight transcriptional regulation involving both NF- κ B-dependent and -independent inflammatory pathways such as ERK, p38 MAPK, and JNK (Lee et al. 2017; Wang et al. 2017). Lipopolysaccharide-mediated activation of immune cells and initiation of inflammatory responses have been implicated in the pathological aggravation of various inflammatory disorders and considering this, inflammatory pathways are molecular targets of various steroidal and NSAID medications. However, due to known side effects and rising costs of traditional therapeutics, a global emphasis is focused on identification and development of much safer and affordable plant-based anti-inflammatory agents (Sharma et al. 2018). In this context, there are growing evidences that plant secondary metabolites and plant-based extracts can confer protection against inflammatory damage by suppressing the production of effector molecules as well as their mediatory pathways such as NF- κ B and MAPKs (Bajpai et al. 2018; Kim et al. 2018).

Berberis lycium Royle, also known Indian berberry, is a valuable plant that is wildy distributed in the Himalayan

region at 2000–2700 m altitude (Ali et al. 2015; Pradhan and Saha 2015). Traditionally, it has been utilized to treat various ailments such as diarrhoea, intestinal colic, jaundice, internal wounds, diabetes and rheumatism (Ali et al. 2015). Preliminary investigations have indicated that different parts of this plant (especially bark and roots) exhibit anti-microbial, anti-inflammatory, anti-carcinogenic and hepatoprotective activities which are thought to be due to the presence of various bioactive phytochemicals such as alkaloids, phenolics and anthocyanins (Andola et al. 2011; Chand et al. 2011; Ali et al. 2015). However, despite these emerging findings, there appears to be a distinct dearth of studies pertaining to understanding the molecular mechanism(s) governing the putative anti-inflammatory and anti-oxidant effect of this plant. Therefore, keeping the foregoing discussion in view, we hypothesized that *B. lycium* fruit extract (BLFE) could ameliorate inflammatory aggravation in innate immune cells by modulating NF- κ B- and MAPK-mediated signaling pathways. To test our hypothesis, we assessed the influence of BLFE on LPS-mediated inflammation in murine macrophages as well as Con-A-mediated proliferation in splenic lymphocytes. Further, the BLFE was characterized by UPLC–DAD–ESI–QTOF–MS/MS so as to correlate and ascertain the diversity of prevalent bioactive phytochemicals. Therefore, the present study attempts to provide a holistic understanding of molecular mechanisms governing the immunomodulatory and anti-inflammatory attributes of BLFE.

Materials and methods

Preparation of BLFE

Berberis lycium fruits at maturation stage were collected from Kangra valley (Himachal Pradesh) located in western Himalayan region of India. BLFE was prepared using hydroalcoholic (50:50 water and ethanol) solvent system with minor modifications as described by Bhatt et al. (2018). Briefly, after separation of seeds from fruits (1000 g), pulp was used for the extraction of BLFE for 24 h at room temperature by percolation and the process was repeated twice. After extraction, samples were filtered using Whatman filter paper and concentrated using a rotary evaporator (Buchi, India) followed by lyophilisation (Labconco, USA). The total phenolic content (TPC) and total flavonoid content (TFC) of BLFE were estimated using Folin–Ciocalteu spectrometric and aluminium chloride colorimetric methods, respectively (Joshi et al. 2015), and are reported as gallic acid (Sigma-Aldrich, US) equivalent of BLFE (GAE/mg) on fresh weight (FW) basis. The antioxidant activity (AA) of BLFE was assessed using DPPH and ABTS assay as described by Joshi et al. (2015).

Animal husbandry

Six-to-eight-week-old Balb/C mice were obtained from animal house facility, CSIR-IHBT, Palampur. Mice were maintained under normal 12-h dark:12-h light cycle at 25 ± 2 °C temperature and 40–70% relative humidity in individual ventilated caging system (Citizen, India). Animals were fed with commercially available basal diet and water ad libitum. All experimental procedures were approved by the Institutional animal ethics committee (Approval no. IAEC/IHBTP4/Jan 2016) and carried out in accordance with guidelines prescribed by the National Regulatory Authority, CPCSEA, Government of India.

Macrophage culture and assessment of cellular viability

Peritoneal macrophages from mice were isolated as described previously (Sharma et al. 2018). For cellular assays, lyophilized BLFE was reconstituted completely Roswell Park Memorial Institute (RPMI)-1640 (HiMedia laboratories, Mumbai, India) (supplemented with 10% FBS and 1% antibiotic–antimycotic solution) while control group cells were treated with RPMI-1640 alone. The toxicity of different concentrations of BLFE (25, 50, 100, 200, and 400 µg/ml) on viability of peritoneal macrophages was estimated by measuring the reduction of

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (HiMedia laboratories, Mumbai, India) to formazan. Briefly, 0.35×10^6 cells/well were seeded into 96-well culture plate in 200 µl media for 48 h in the presence or absence of BLFE. After visual confirmation of cell morphology, MTT assay was used to determine and calculate cell viability as reported by Sharma et al. (2018).

Inflammatory stimulation and assessment of nitric oxide production

Briefly, peritoneal macrophages (0.35×10^6) were seeded in 96-well plates for 6 h in RPMI-1640 supplemented with 10% FBS (HiMedia laboratories, Mumbai, India). The cells were treated with different concentrations of reconstituted BLFE (50, 100 and 200 µg/ml) for 12 h in a CO₂ incubator, followed by stimulation with LPS (Sigma-Aldrich, US) (1.5 µg/ml) for another 16 h. Finally, NO production was measured using the Griess reagent as per the manufacturer's instruction (Promega, USA). Dexamethasone (Dex, 2 µg/ml) was used as positive control for all experiments.

Assay for intracellular ROS determination in stimulated macrophage

Briefly, macrophages (1.5×10^6 /well) were seeded on 24-well plates and treated with various concentrations of BLFE. At the end of experiment, ROS accumulation was assessed using carboxy-2,7-dichloro-4-fluorescein diacetate (DCFDA); (Sigma-Aldrich, US) dye as described previously by Sharma et al. (2018). Finally, fluorescence images were captured with EVOS FL cell imaging system (Thermo Fisher Scientific, US) for comparative analysis.

Measurement of cytokines in culture supernatants

Macrophages (1.5×10^6) were treated with three concentrations (50, 100 and 200 µg/ml) of BLFE for 12 h, as described in the previous section. Estimation of TNF-α, IL-6, IL-1β, IFN-γ and IL-10 cytokines in cell culture supernatants was performed using commercially available sandwich ELISA kits following the manufacturer's protocol (eBioscience, San Diego, CA, USA). All the samples were assessed in triplicates.

Total RNA isolation and qRT-PCR

At the end of the experiment, total RNA was isolated and qRT-PCR for TNF-α, IL-1β, IL-6, IFN-γ, IL-10, MCP-1 and RANTES was performed as reported previously (Sharma et al. 2018). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) expression was used as an internal control to

normalize mRNA expression. The primer sequences used for mRNA analysis were as presented below:

Primers	Primer sequence (5' end–3' end)
GAPDH-F	TCA CTC AAG ATT GTC AGC AATGC
GADPH-R	TCA TGA GCC CTT CCA CAA TG
IL1B-F	CGT TCC CAT TAG ACA ACTGCA CTA
IL1B-R	TCC ATT GAG GTG GAG AGCTTTC
IL6-F	CTT CCA TCC AGT TGC CTT CTTG
IL6-R	GGG AGT GGT ATC CTC TGTGAA GTC
iNOS-F	AGC GAG GAG CAG GTG GAA
iNOS-R	CCT TTT TTG CCC CAT AGG AAA
TNF- α -F	GAA ACA CAA GAT GCT GGGACAGT
TNF- α -R	CAT TCG AGG CTC CAG TGA ATTC
IFN- γ -F	CTC CGG CCT AGC TCT GAGA
IFN- γ -R	TGA CTG TGC CGT GGC AGT AA
IL-10-F	ATT TGA ATT CCC TGG GTG AGAA
IL-10-R	TGC TCC ACT GCC CTT GCT CTT CTC
MCP-1-F	CTG AAG CCA GCT CTC TCT CTC TCT TCCT
MCP-1-R	GAG CCA ACA CGT GGA TGCT
RANTES-F	TCC CTG TCA TCG CTT GCT CTA
RANTES-R	CGG ATG GAG ATG CCG ATTT

Protein extraction and immunoblotting

Total proteins were isolated and quantified as described earlier (Sharma et al. 2018). The primary antibodies anti-iNOS, anti-I κ B α , anti-p-I κ B α , anti-NF- κ B, anti-p-NF- κ B, anti-Nrf-2, anti-p38, anti-p-p38, anti-c-Jun, anti-p-c-Jun, anti-ERK, anti-p-ERK, anti-JKN and anti-p-JNK were obtained from Cell Signalling Technologies, USA. Anti- β -tubulin antibodies were purchased from Santa Cruz Biotechnology, USA, and used as an internal control. Western blot images were quantified using ImageJ software.

Splenic immunophenotyping

Briefly, the mouse spleen was aseptically removed, washed in PBS and then dissociated into a single-cell suspension using syringe plunger, followed by centrifugation at 400 g for 10 min. The single-cell suspension was then treated with RBC lysis buffer for 10 min to rupture any erythrocytes present. The remaining splenocytes were then washed twice in PBS by centrifugation at 400 g for 5 min. This final pellet was then re-suspended in RPMI 1640 supplemented with 10% FBS and 1% antibiotic–antimycotic solution (i.e. Complete RPMI), and counted in a haemocytometer. The isolated splenocytes (1×10^6) were seeded in six-well plates and incubated with BLFE (50, 100, and 200 μ g/ml) for 12 h followed by stimulation with Con-A (7 μ g/ml) (Sigma-Aldrich, US) for 36 h. After incubation, phenotypic characterization

of splenocytes was performed by flow cytometer analysis using AMNIS ImageStream[®]X Mark II Imaging Flow Cytometer (Merck Millipore, Germany). Splenocytes were suspended in FACS buffer (PBS, pH 7.4, with 0.5% BSA and 0.1% sodium azide) and incubated at 4 °C for 15 min with FITC-anti-mouse CD3, PE-anti-mouse CD4, and APC-anti-mouse CD25 antibodies (eBioscience, San Diego, CA) as previously described by Sharma et al. (2017). The cells were washed twice with PBS and re-suspended in FACS buffer. CD3+, CD4+ and CD4+ CD25+ sub-populations were analysed by the IDEAS software, Germany.

UPLC–DAD–ESI-QTOF-MS/MS analysis

To assess, the individual phenolics and alkaloids of BLFE UPLC–ESI-QTOF-MS/MS (Waters, Milford, MA, USA) were performed. Briefly, a mixture of methanol:water (50:50 v/v) was used to prepare the sample (10 mg/ml). Prior to injection into UPLC system (BEH C18 column, 2.1 mm x 100 mm, 1.7 μ m particle size), the sample was filtered using a 0.22- μ m Millex GV syringe filter (Millipore, MA, USA). Briefly, for phenolic and alkaloid characterizations, 0.1% formic acid in water as solvent A and 0.1% formic acid in acetonitrile as solvent B were used as mobile phase, with 0.25 ml/min flow rate at 25 °C column temperature. For chromatographic separations of phenolics and quercetin 3-D-galactoside, the used mobile phase consisted of 0–0.3 min (10% B), 0.3–7 min (45% B), 7–8 min (95% B), 8–9 min (95% B), 9–10 min (10% B) and 10–12 min (10% B). For analysis of alkaloids, the used mobile phase (solvent A 0.05% formic acid in water and acetonitrile as solvent B with 0.3 ml/min flow rate) consisted of 0–0.2 min (13% B), 0.2–3.5 min (30% B), 3.5–4 min (13% B), and 4–5 min (13% B) (Bala et al. 2015). Additionally, the mobile phase used for the characterization of berberine consisted of 0–0.5 min (10% B), 0.5–5 min (35% B), 5–7 min (90% B), 7–8.5 min (90% B), 8.5–9 min (10% B) and 9–12 min (10% B). Standard stock solutions (1 mg/ml) of gallic acid, pro-catechuic acid, chlorogenic acid, catechin, caffeic acid, p-coumaric acid, naringin, cinnamic acid, kaempferol and quercetin 3-D-galactoside were prepared in HPLC-grade methanol (Sigma-Aldrich, USA). The quantity of the detected compounds was reported as mg/g of fruit extract. The MS/MS experiment was performed on a Q-TOF mass spectrometer equipped with an electron spray ionization source (Micro-mass, Manchester, UK) and software Masslynx v4.1. The MS parameters in positive mode were as follows: capillary voltage 3.2 kV, cone voltage 30 V, source block temperature 80 °C, scan range, m/z 100–1000, source block temperature 80 °C, RF lens1 45, aperture 0.5, RF lens 20.6, cone gas 50 l/h, desolvation temperature 220 °C, desolvation gas 400 l/h, argon collision gas pressure 3.2×10^{-3} mbar and ion energy 23.0 eV.

Bioactivity validation of phytomolecules characterized in BLFE

In an attempt to ascertain and validate the apparent biological effects of BLFE, two of the characterized compounds, berberine (Sigma-Aldrich, USA) and rutin (Sigma-Aldrich, USA) in BLFE were analysed for the inhibition of LPS-stimulated inflammatory responses in macrophages. Briefly, cells were pre-exposed to different concentrations (25, 50 and 100 $\mu\text{M}/\text{ml}$) of berberine and rutin, respectively, for 12 h followed by LPS induction for another 16 h and subsequent analysis of NO production and iNOS expression by RT-PCR and western blotting as detailed in previous sections.

Statistical analyses

Results are presented as the mean \pm SD. One-way ANOVA, followed by Tukey's test was used to perform the statistical analyses. Differences with p value ≤ 0.05 were considered as statistically significant.

Results

Characterization and cytocompatibility of BLFE

Analysis of TPC revealed that BLFE contained 46.7 ± 4.3 μg gallic acid equivalent per mg fresh weight, while 14.1 ± 1.72 $\mu\text{g}/\text{mg}$ gallic acid equivalent of TFC was also observed. Moreover, the anti-oxidant potential of BLFE assessed by DPPH and ABTS assay showed an IC_{50} of 101.7 ± 2.6 $\mu\text{g}/\text{ml}$ and 26.9 ± 1.2 $\mu\text{g}/\text{ml}$, respectively. These results suggest that BLFE could be useful in ameliorating oxi-inflammatory stress in a biological system. Pursuing this, we initially evaluated cytotoxicity of BLFE in macrophages. As shown in Fig. 1a, BLFE treatment even up to a range of 400 $\mu\text{g}/\text{ml}$ did not result in any apparent cytotoxic effects. Based on these results, BLFE concentrations of 50, 100 and 200 $\mu\text{g}/\text{ml}$ were selected for further experiments.

BLFE ameliorates NO production and iNOS expression in activated macrophages

LPS stimulation to macrophages increases NO production, which is regulated by iNOS. Therefore, to analyse the effect of BLFE on NO production, the cellular nitrite level (an indicator of NO production) of activated macrophages treated with different concentration of BLFE was measured. As presented in Fig. 1b, stimulation with LPS resulted in significant ($p \leq 0.05$) enhancement in NO levels (20.9 μM) as compared to control (5.7 μM ; vehicle alone) while BLFE treatment (50, 100 and 200 $\mu\text{g}/\text{ml}$) exerted a significant ($p \leq 0.05$)

yet concentration-independent inhibition in NO production (up to 15.5 μM) compared to LPS-stimulated macrophages (Fig. 1b). Subsequent analysis of iNOS mRNA expression revealed a significant ($p \leq 0.05$) increase in LPS-stimulated macrophages in comparison with control. On the other hand, BLFE at 200 $\mu\text{g}/\text{ml}$ significantly inhibited ($p \leq 0.05$) the expression of iNOS by 61% in comparison with LPS-stimulated cells (Fig. 1c). Further, protein analysis showed a strong accumulation in iNOS in LPS-treated cells which were significantly ($p \leq 0.05$) attenuated by BLFE treatment at 100 and 200 $\mu\text{g}/\text{ml}$ BLFE (Fig. 1d). Here, the results indicate that the attenuation of NO by BLFE may be associated with inhibition of the iNOS expression. The anti-inflammatory drug dexamethasone (Dex) also showed a strong attenuation in iNOS at both transcriptional and translational levels.

BLFE augments redox homeostasis by reducing ROS accumulation and Nrf-2 expression while enhancing HO-1 expression in LPS-stimulated macrophages

As illustrated in Fig. 2a, BLFE treatment to LPS-stimulated cells inhibited the accumulation of ROS, which was in accordance with NO production assay. Analysis of mRNA expression of the anti-oxidant enzyme HO-1 demonstrated a non-significant reduction of 44.2% in LPS-treated cells as compared to control cells, which was significantly ($p \leq 0.05$) increased in BLFE-treated cells (up to 84.8%) in comparison with LPS-stimulated cells (Fig. 2b). Immunoblotting for the analysis of Nrf-2 expression showed a significant ($p \leq 0.05$) increase (32.4%) in LPS-stimulated macrophages which were also significantly attenuated in the presence of BLFE (up to 53.1%) albeit in a dose-independent manner. Interestingly, dexamethasone treatment did not appear to significantly enhance HO-1 (as compared to BLFE) while both dexamethasone and BLFE showed similar effects in modulating Nrf-2 expression in LPS-stimulated cells (Fig. 2c).

BLFE modulates key cytokine and chemokine production in LPS-stimulated macrophages

To address the effect of BLFE on pro- and anti-inflammatory mediators, we next investigated the mRNA expression level of the inflammation regulatory effector cytokines TNF- α , IL-1 β , INF- γ , IL-6 and IL-10 in LPS-stimulated macrophages by qRT-PCR. The results indicated that LPS insult to macrophages significantly ($p \leq 0.05$) increased the expression of TNF- α , IL-1 β , INF- γ and IL-6 by 4.3-, 153.2-, 3.1- and 22.8-folds, respectively in comparison to control (Fig. 3a–d). In contrast, treatment with different concentrations (50, 100 and 200 $\mu\text{g}/\text{ml}$) of BLFE significantly ($p \leq 0.05$) inhibited the mRNA expression of TNF- α , IL-1 β , and IL-6 by 3.8-, 6.9- and 4-folds, respectively, in

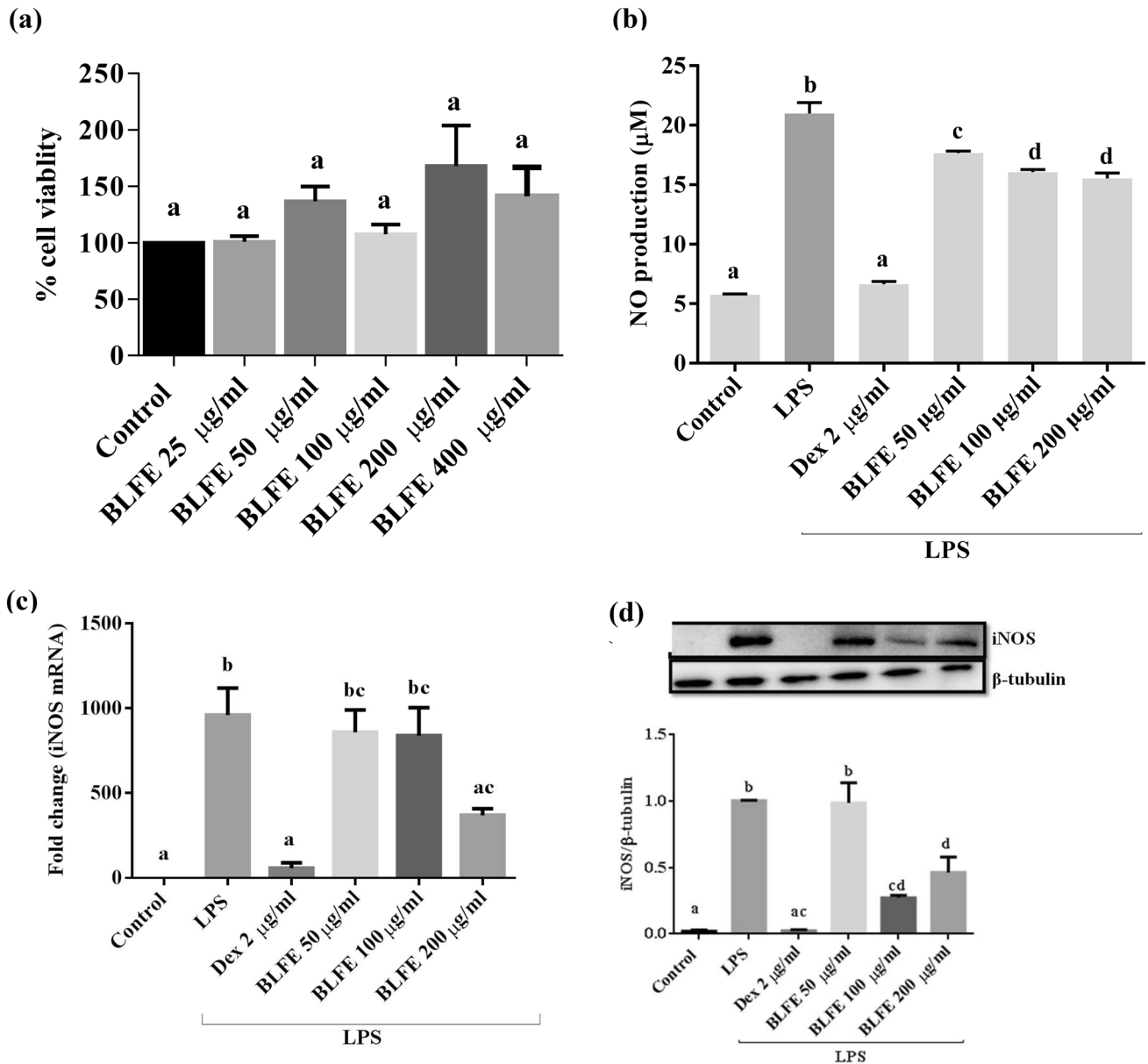


Fig. 1 BLFE attenuates NO production and iNOS expression in LPS-stimulated murine peritoneal macrophages. **a** Percentage cellular viability of macrophages treated at different concentrations of BLFE for 48 h. Cells pre-treated with BLFE (50, 100, 200 µg/ml) for 12 h

followed by stimulation with LPS (1.5 µg/ml) for another 16 h. **b** NO production. iNOS mRNA (**c**) and protein expression (**d**). Values are mean ± SD of three independent experiments; values with different letters are significantly different at $p < 0.05$

comparison to LPS-stimulated cells. However, BLFE-mediated suppression of $\text{INF-}\gamma$ could only be observed at the highest concentration of 200 µg/ml (Fig. 3d). No change in IL-10 levels could be observed in LPS-treated cells, whereas a significant ($p \leq 0.05$) increase of 10.1-fold was noted in BLFE-treated cells (Fig. 3e). Analysis of cytokine levels in culture supernatants is presented in Fig. 4a–e. On induction with LPS, a significant ($p \leq 0.05$) enhancement in the level of TNF- α , IL-1 β and IL-6 by 206.9-, 4.9- and 487.7-folds was observed, but had no significant effect on $\text{INF-}\gamma$

(1.2-fold) production. On the other hand, 100 and 200 µg/ml BLFE exposure to stimulated cells strongly inhibited the production of these cytokines. In particular, a significant decrease in all measured pro-inflammatory cytokines with a concomitant increase in IL-10 levels was observed specifically at 200 µg/ml of BLFE treatment (Fig. 4e). Furthermore, it was observed that the inhibition of pro-inflammatory cytokines at 200 µg/ml was either equal or greater than the inhibition by dexamethasone. Similarly, analysis of chemokines RANTES and MCP-1 revealed significant

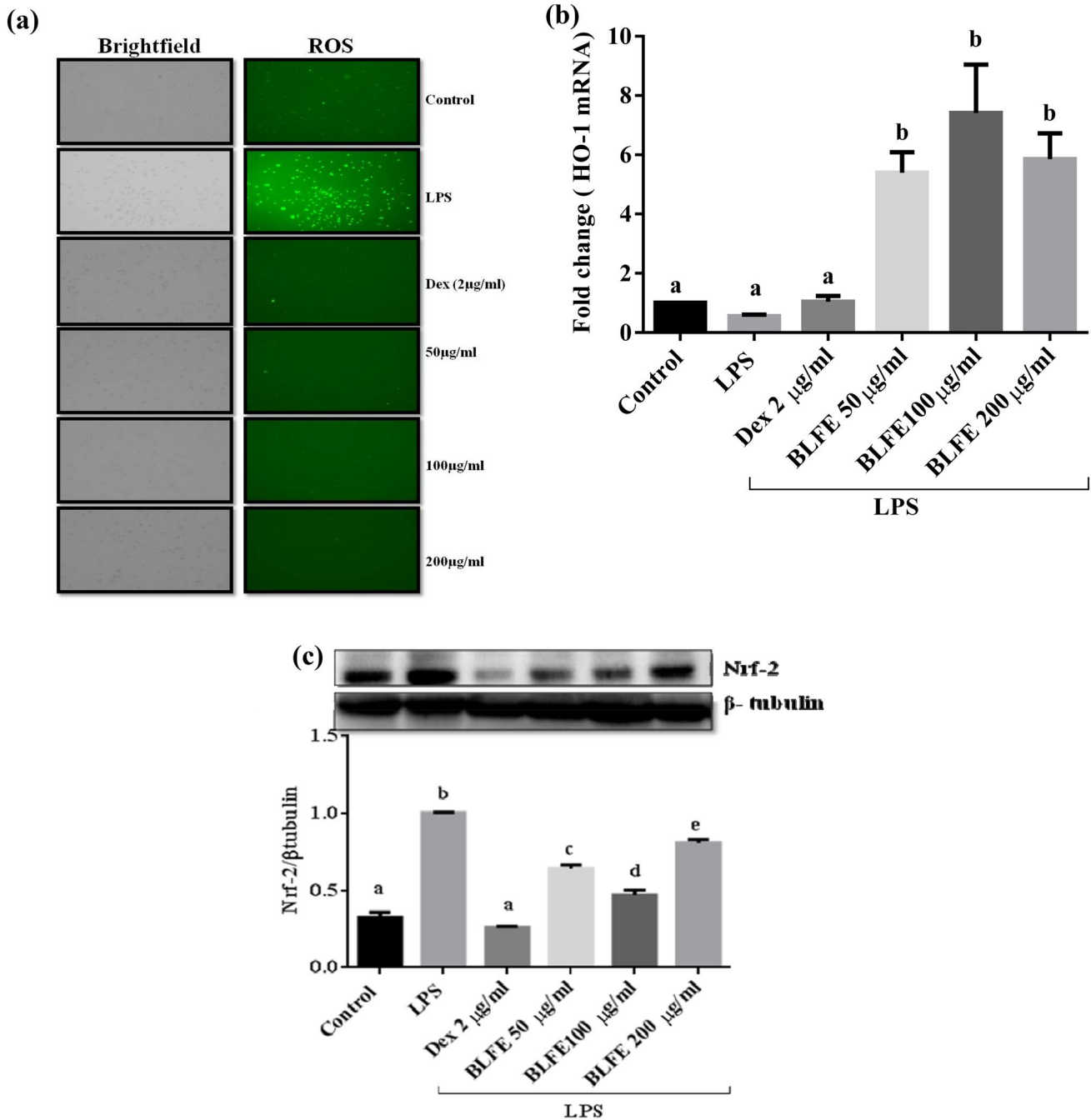


Fig. 2 BLFE ameliorates redox stress in peritoneal macrophages. Cells pre-treated with BLFE (50, 100, 200 µg/ml) for 12 h followed by stimulation with LPS (1.5 µg/ml) for another 16 h. **a** ROS accumulation by DCFDA assay. **b** mRNA expression of HO-1 and **c** Nrf2

expression by immunoblotting. Values are mean ± SD of three independent experiments; values with different letters are significantly different at $p < 0.05$

upregulation in LPS-stimulated cells, which was again significantly ($p \leq 0.05$) attenuated by the presence of BLFE at all tested concentrations (Fig. 5a, b).

BLFE suppresses the activation of IκBα, NF-κB and c-Jun transcription factors

To investigate whether attenuation of inflammatory responses by BLFE are mediated through the NF-κB pathway, we examined the effect of BLFE on LPS-induced

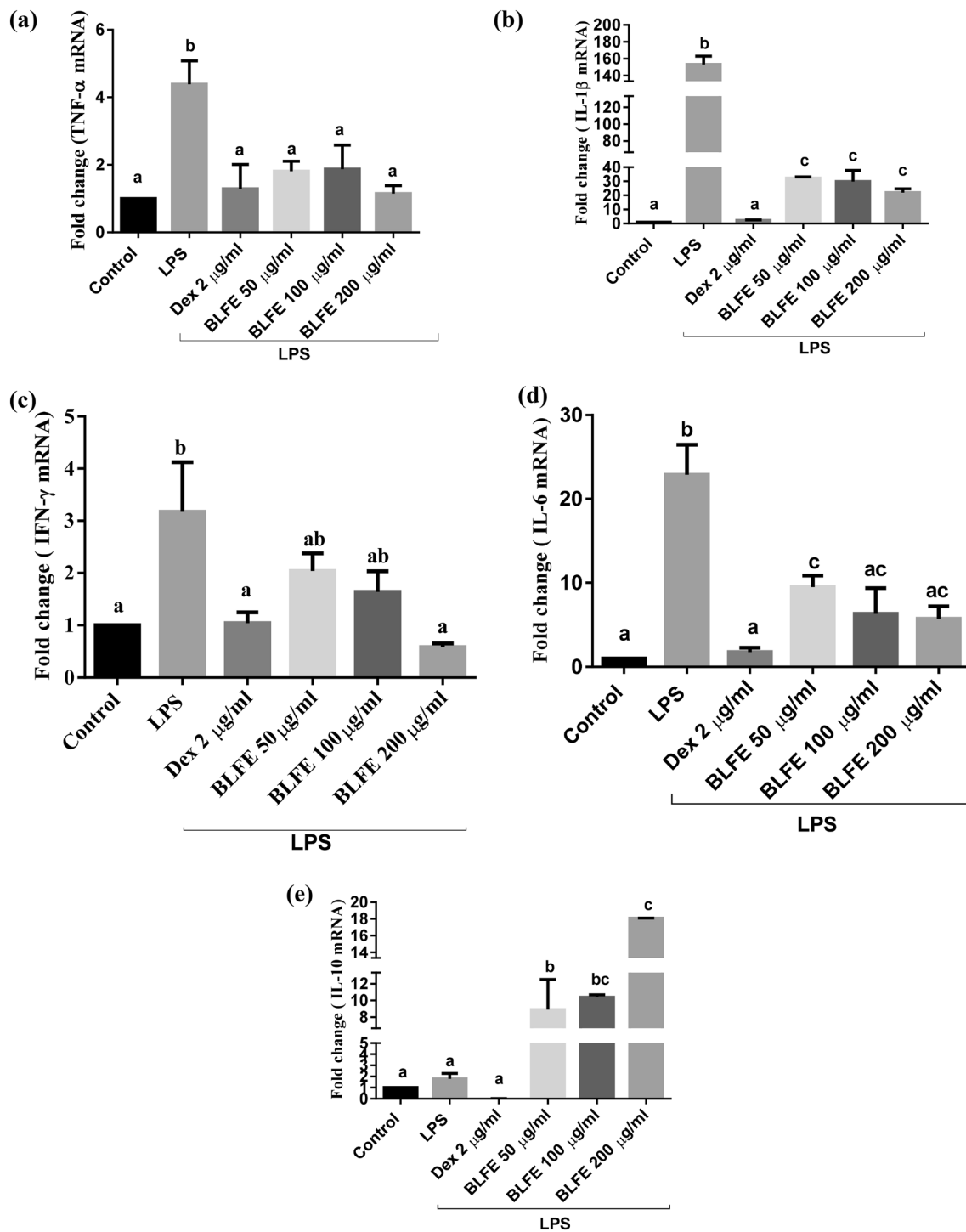


Fig. 3 BLFE augments inflammatory homeostasis in LPS-stimulated macrophages. Cells pre-treated with BLFE (50, 100, 200 μg/ml) for 12 h followed by stimulation with LPS (1.5 μg/ml) for another 16 h. **a** TNF-α, **b** IL-1β, **c** IFN-γ, **d** IL-6 and **e** IL-10 mRNA fold change was

measured using qRT-PCR. Values are mean ± SD of three independent experiments; values with different letters are significantly different at $p < 0.05$

phosphorylation of IκBα and NF-κB in stimulated cells (Fig. 6a, b). Compared with un-stimulated (control) cells, the phosphorylation of IκBα significantly ($p \leq 0.05$)

increased in LPS-stimulated cells (Fig. 6a). In contrast, phosphorylation in BLFE-treated cells was significantly ($p \leq 0.05$) decreased for all tested concentrations.

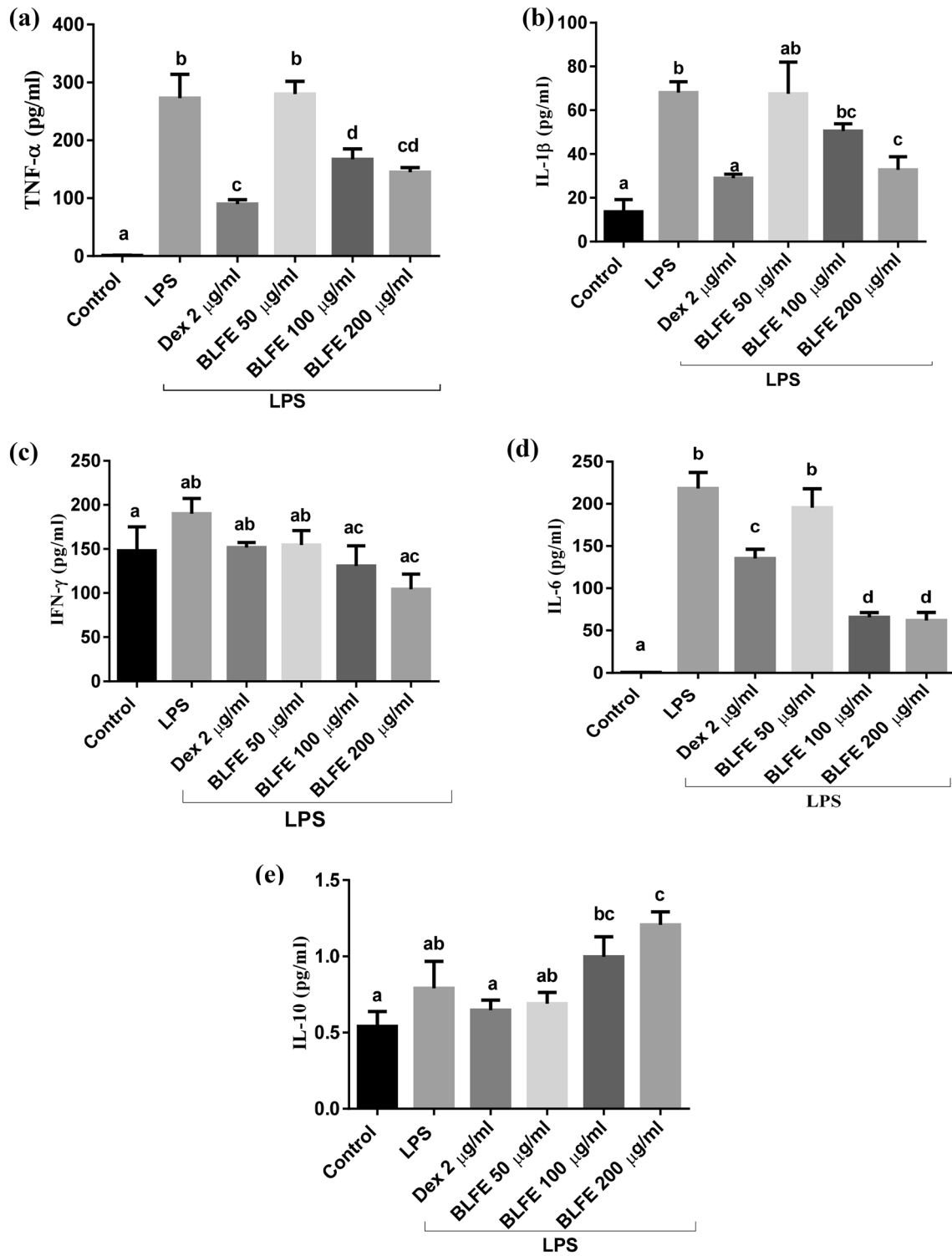


Fig. 4 BLFE modulates interleukin production in LPS-stimulated macrophages. Cells pre-treated with BLFE (50, 100, 200 μg/ml) for 12 h followed by stimulation with LPS (1.5 μg/ml) for another 16 h. **a** TNF-α, **b** IL-1β, **c** IFN-γ, **d** IL-6 and **e** IL-10 production was

measured using ELISA. Values are mean ± SD of three independent experiments; values with different letters are significantly different at $p < 0.05$

Fig. 5 BLFE alleviates chemokine production in LPS-stimulated murine peritoneal macrophages. Cells pre-treated with BLFE (50, 100, 200 $\mu\text{g/ml}$) for 12 h followed by stimulation with LPS (1.5 $\mu\text{g/ml}$) for another 16 h. **a** RANTES and **b** MCP-1 fold change was measured using qRT-PCR. Values are mean \pm SD of three independent experiments; values with different letters are significantly different at $p < 0.05$

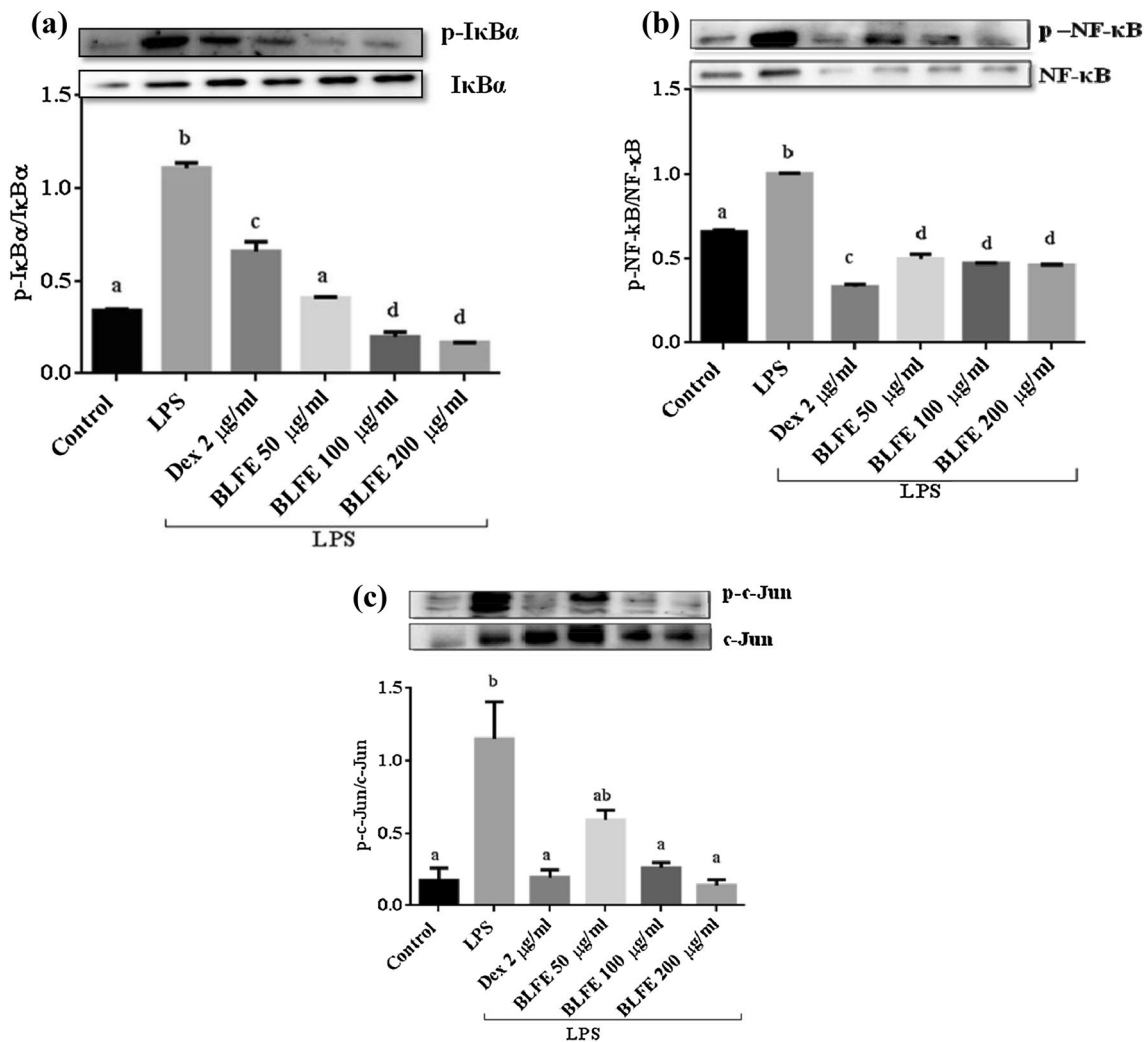
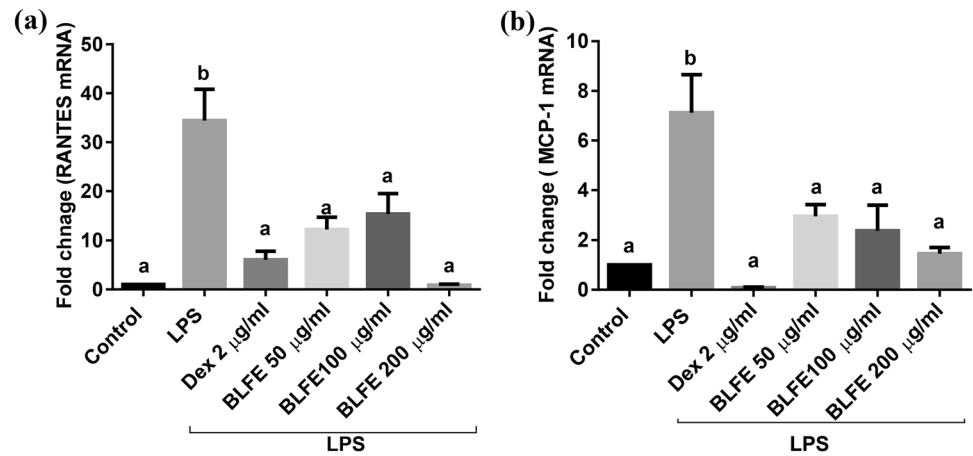


Fig. 6 BLFE inhibits activation of **a** p-IκBα/IκBα, **b** p-NF-κB/NF-κB and **c** p-c-Jun/c-Jun expression in LPS-stimulated murine peritoneal macrophages. Cells pre-treated with BLFE (50, 100, 200 $\mu\text{g/ml}$) for

12 h followed by stimulation with LPS (1.5 $\mu\text{g/ml}$) for another 16 h. Values are mean \pm SD of three independent experiments; values with different letters are significantly different at $p < 0.05$

Interestingly, the inhibitory effect of BLFE on $\text{I}\kappa\text{B}\alpha$ phosphorylation was significantly ($p \leq 0.05$) higher than dexamethasone. Furthermore, it was observed that all tested concentrations of BLFE significantly ($p \leq 0.05$) suppressed the activation of NF- κB in a concentration-independent manner (Fig. 6b). These results suggest that inhibition of inflammatory effector molecules by BLFE is mediated via attenuation of NF- κB phosphorylation. Next, we examined the potential role of BLFE on LPS-induced c-Jun expression in murine macrophages (Fig. 6c). The results revealed that in the presence of BLFE, LPS-induced phosphorylation of c-Jun was markedly suppressed by all the tested concentrations.

BLFE suppresses the activation of MAPKs (ERK, JNK and p38) in LPS-stimulated macrophages

The regulatory role of MAPKs in LPS-induced activation of NF- κB and other inflammatory markers was analysed through the phosphorylation of ERK, JNK and p38 in stimulated macrophages. As shown in Fig. 7a–c, LPS stimulation significantly ($p \leq 0.05$) enhanced the phosphorylation of ERK1/2, JNK and p38 in comparison to the control (un-stimulated) cells. Conversely, BLFE pre-treatment significantly ($p \leq 0.05$) downregulated the phosphorylation of ERK1/2, JNK and p38 (albeit at 100 and 200 $\mu\text{g}/\text{ml}$ only). These results confirmed that the suppressive effect of BLFE on NF- κB and inflammatory effector molecules is regulated by inhibition of MAPKs.

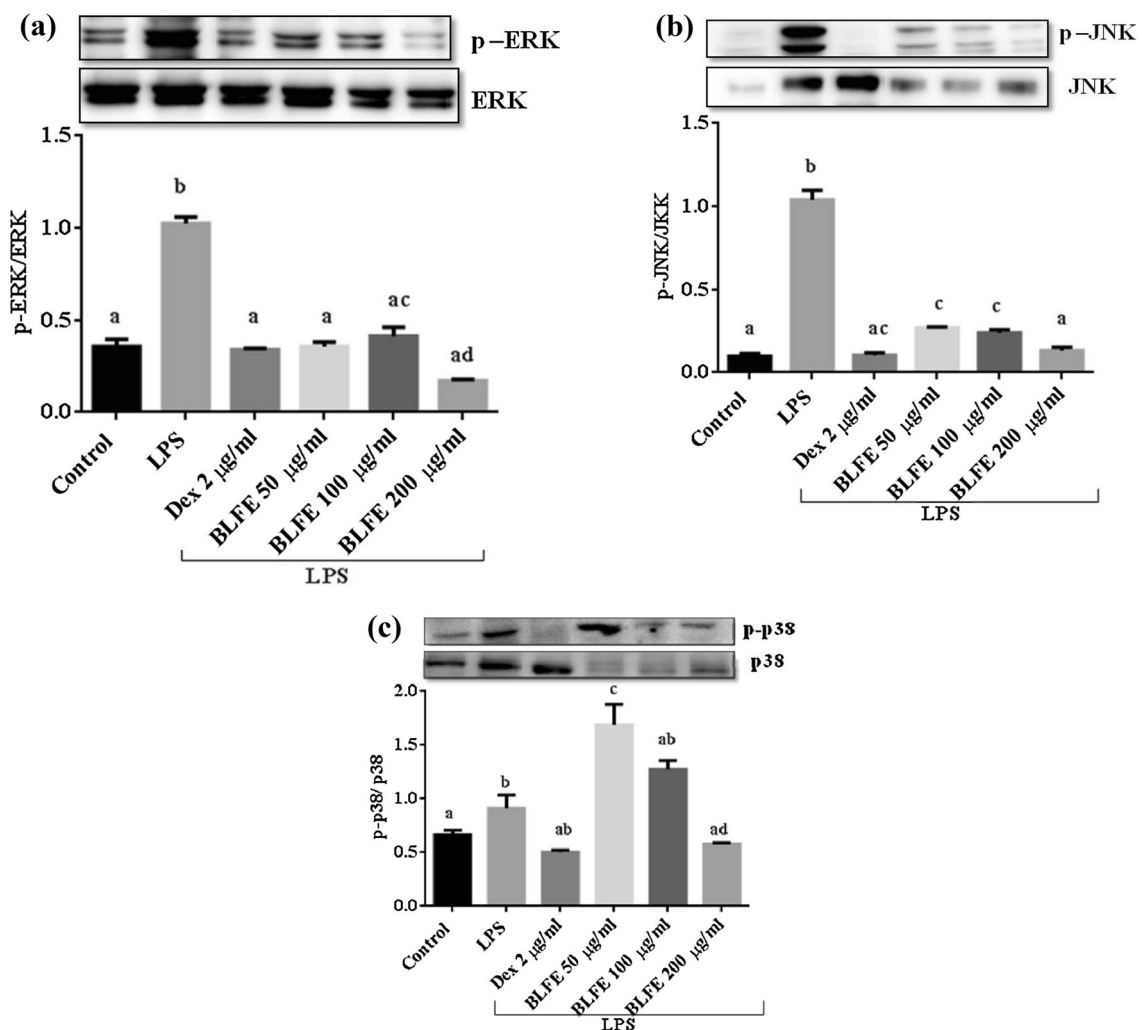


Fig. 7 BLFE decreases phosphorylation of **a** p-ERK/ERK **b** p-JNK/JNK and **c** p-p38/p38 expression in LPS-stimulated peritoneal macrophages. Cells pre-treated with BLFE (50, 100, 200 $\mu\text{g}/\text{ml}$) for 12 h

followed by stimulation with LPS (1.5 $\mu\text{g}/\text{ml}$) for another 16 h. Values are mean \pm SD of three independent experiments; values with different letters are significantly different at $p < 0.05$

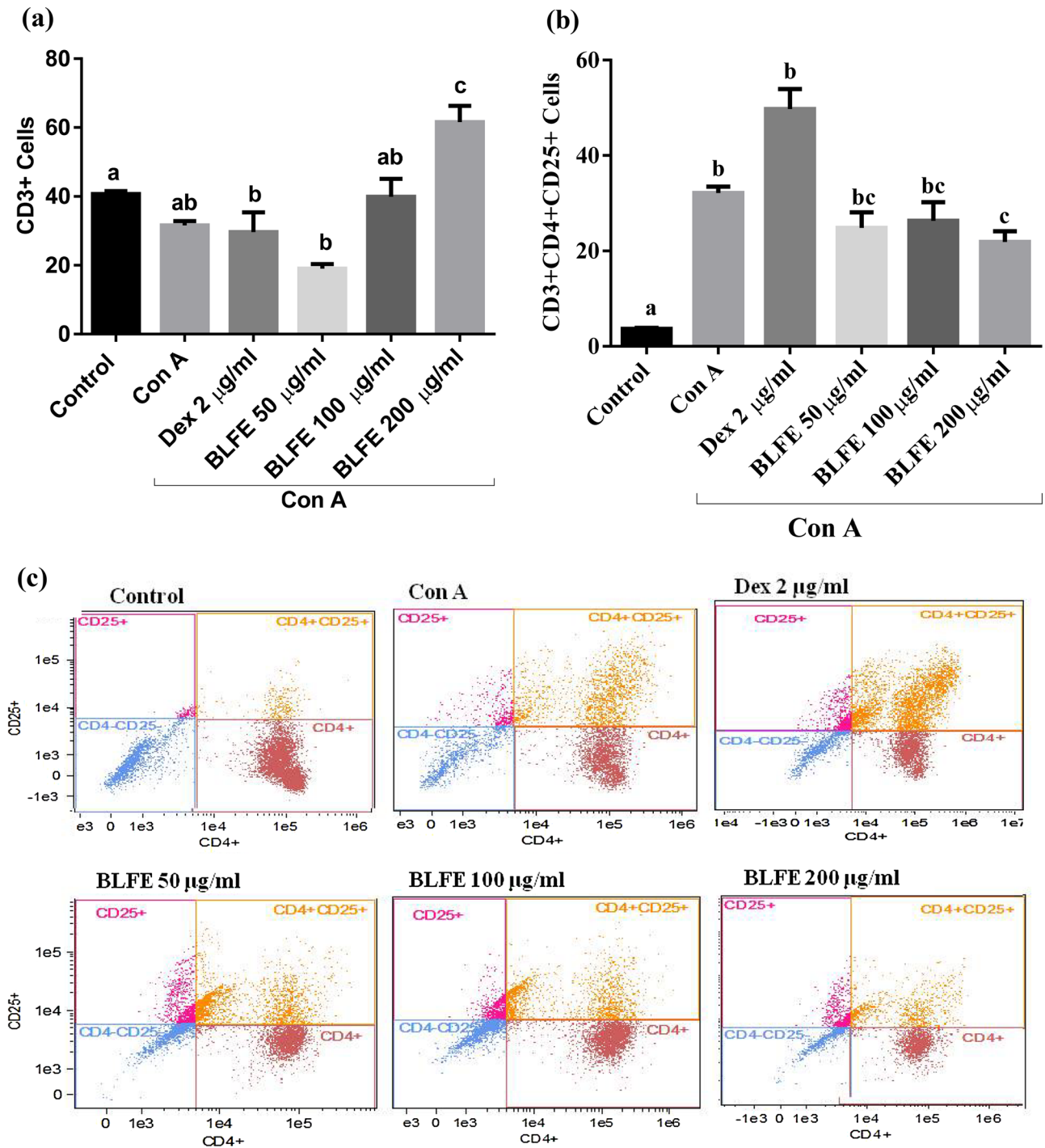


Fig. 8 Effect of BLFE on T-cell population in Con A-stimulated murine splenocytes. Cells pre-treated with BLFE (50, 100, 200 $\mu\text{g/ml}$) for 12 h followed by stimulation with Con A (7 $\mu\text{g/ml}$) for another 36 h. **a** CD3+ **b** CD3+ CD4+ CD25+ cell populations were

analyzed using flow cytometry and **c** CD4+ vs CD25+ scatterplots. Values are mean \pm SD of three independent experiments; values with different letters are significantly different at $p < 0.05$

BLFE treatment dysregulates CD3+ CD4+ CD25+ Treg homeostasis in Con-A-stimulated splenocytes

Con-A-treated splenocytes showed a non-significant 22.4% decrease in CD3+ cells as compared to control (Fig. 8a). On

the other hand, analysis of BLFE (200 $\mu\text{g/ml}$) treatment on splenocytes revealed a significant ($p \leq 0.05$) increase (95.2%) in CD3+ population in comparison to Con-A-stimulated cells (Fig. 8a). However, we noticed a significant decrease in CD3+ CD4+ cell population in Con-A-stimulated cells

as compared to the control although BLFE treatment did not result in any significant changes compared to Con-A-treated cells (data not shown). Additionally, compared with un-stimulated cells, a significant increase of 88.5% was observed in CD3+ CD4+ CD25+ cell population in Con-A-treated cells, while pre-treatment with BLFE (200 µg/ml) significantly decreased CD3+ CD4+ CD25+ numbers by 31.8% as compared with stimulated cells (Fig. 8b).

Characterization of BLFE by UPLC–DAD–ESI-QTOF-MS/MS

Table 1 shows the retention time and MS/MS spectral data of the bioactive compounds present in the BLFE characterized using UPLC–ESI-QTOF-MS/MS. A total of 16 compounds from different classes of phytochemicals including eight alkaloids and eight phenolics (seven flavonoids and one phenolic acid) were identified by comparing mass fragmentation patterns (Table 1). The LC/MS chromatogram (total ion chromatogram) in positive ion mode and mass spectrum of BLFE are presented in Fig. 9a and supplementary file S1, respectively. On the basis of retention time, UVλ_{max} and mass fragmentation patterns with respect to standards, peaks 3, 4, 6, 7, 8 and 16 were identified as chlorogenic acid, catechin, magnoflorine, rutin and quercetin 3-D-galactoside and berberine, respectively (Table 1; Supplementary files S1 and S2). Additionally, peaks 1, 2, 5 and 9–14 in Fig. 9a were identified as cyanidin-3-O-galactoside, cyanidin-3-O-glucoside, demethylated product of magnoflorine, berbamine, myricetin-3-glucoside, petunidin-3-O-glucoside,

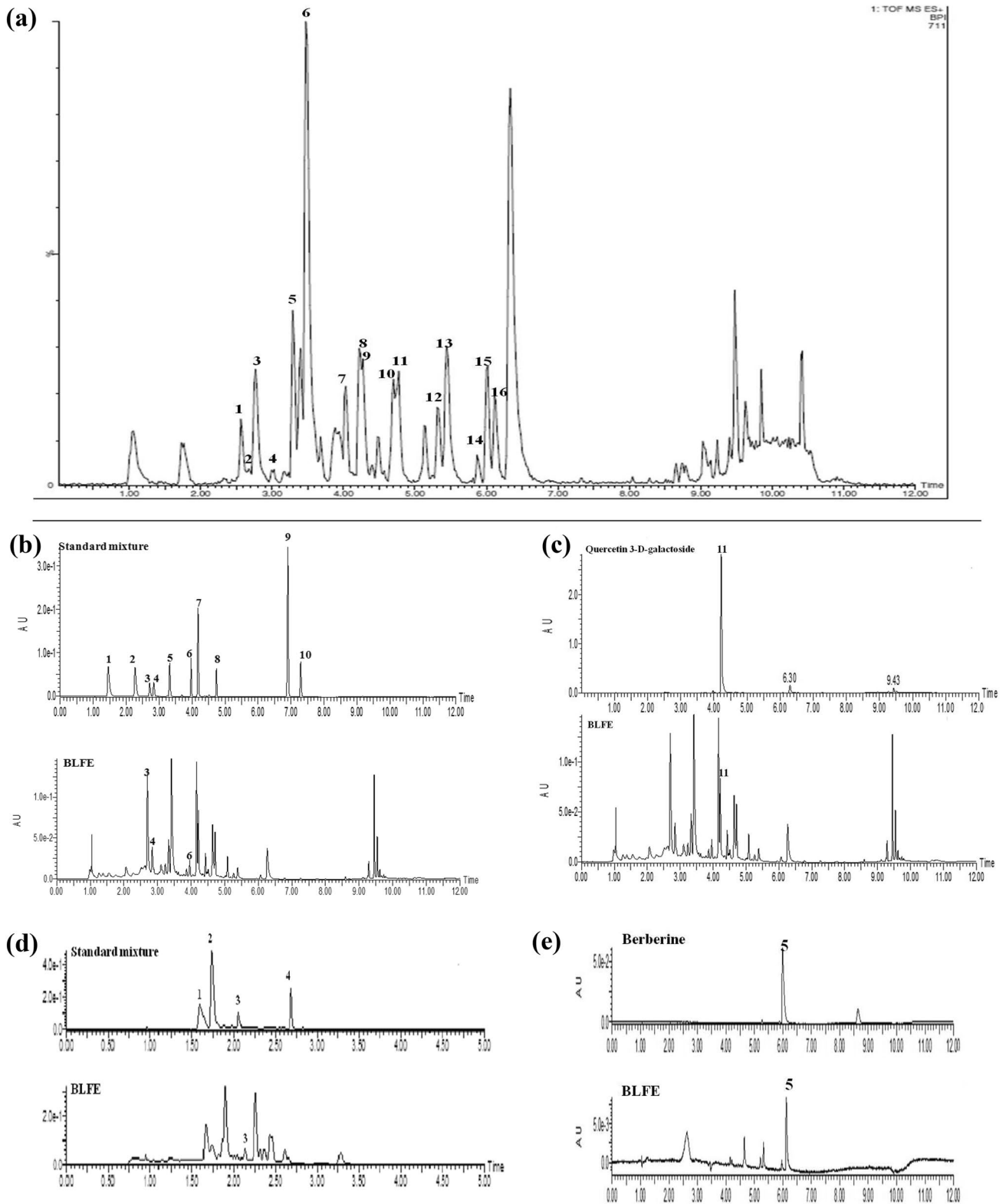
jatrorrhizine, dihydroxy derivative of jatrorrhizine, protopine and palmatine, respectively, by comparison of their mass fragmentation patterns (Table 1 and Supplementary file S1). Further, a total of four phenolics, namely chlorogenic acid (10.0 ± 0.9 mg/g), catechin (1.8 ± 3.2 mg/g), rutin (0.62 ± 0.7 mg/g) and quercetin 3-D-galactoside (2.51 ± 1.2 mg/g) as well as two alkaloids, namely magnoflorine (17.22 ± 3.8 mg/g) and berberine (0.58 ± 0.1 mg/g) in BLFE were quantified by UPLC–DAD method (Table 2 and Fig. 9b–e).

Berberine and rutin corroborate the anti-inflammatory effects of BLFE

The phytochemicals berberine and rutin were characterized in BLFE and preliminary assessment of their biological effects corroborated the apparent anti-inflammatory attributes of BLFE. We observed that treatment with berberine (at 50 and 100 µM/ml) and rutin (at 100 µM/ml) caused a significant ($p \leq 0.05$) decrease in NO production in LPS-induced macrophages (Fig. 10a). Further assessment of iNOS expression, both at transcriptional as well as translational levels, indicated its robust inhibition in the presence of berberine and rutin in LPS-stimulated macrophages (Fig. 10b, c). These observations suggest that the presence of these compounds could have been at least partially responsible for observed cellular effects of BLFE in the present study.

Table 1 Retention time, mass and mass fragmentation of bioactive compounds identified in BLFE using UPLC–ESI-QTOF-MS/MS

S. no.	RT	[M + H]	MS ²	Identification	References
1	2.565	449	354, 287, 192	Cyanidin-3-O-galactoside	(Junqueira-Gonçalves et al. 2015; Pradhan and Saha 2015)
2	2.658	449	354, 287, 192	Cyanidin-3-O-glucoside	(Junqueira-Gonçalves et al. 2015; Pradhan and Saha 2015)
3	2.750	355	163	Chlorogenic acid	(Pradhan and Saha 2015; Bhatt et al. 2018)
4	2.84	291	139	Catechin	(Bhatt et al. 2017)
5	3.401	328	297, 265	Demethylated product of magnoflorine	
6	3.475	342	297, 265	Magnoflorine	(Kukula-Koch 2017)
7	4.032	611	465, 303	Rutin	(Bhatt et al. 2018)
8	4.217	465	303	Quercetin 3-D-galactoside	(Plazonić et al. 2009; Ruiz et al. 2010)
9	4.268	609		Berberine	(Kukula-Koch 2017)
10	4.700	479	317	Myricetin-3-glucoside	(Ruiz et al. 2010)
11	4.756	479	317	Petunidin-3-O-glucoside	(Dou et al. 2007; Ruiz et al. 2010)
12	5.313	338	323	Jatrorrhizine	(Dou et al. 2007; Shuang-lai et al. 2011)
13	5.443	370	338, 323	Dihydroxy derivative of jatrorrhizine	(Zhang et al. 2008)
14	6.0	354	–	Protopine	(Zhang et al. 2008; Sun et al. 2014; Och et al. 2017)
15	6.112	352	337	Palmatine	(Dou et al. 2007; Shuang-lai et al. 2011)
16	6.335	336	321	Berberine	(Dou et al. 2007; Shuang-lai et al. 2011)



Discussion

Plant species and products enriched with bioactive secondary metabolites are considered potent cytoprotective

agents against various oxidative and inflammatory disorders. Indeed, in the present study, analysis of TPC, TFC and AA indicated a strong presence of bioactive entities in BLFE, which is consistent with previous

Fig. 9 Characterization of BLFE using **a** UPLC–ESI–QTOF–MS/MS (TIC spectra). 1—cyanidin-3-*O*-galactoside, 2—cyanidin-3-*O*-glucoside, 3—chlorogenic acid, 4—catechin, 5—demethylated product of magnoflorine, 6—magnoflorine, 7—rutin, 8—quercetin 3-*D*-galactoside, 9—berbamine, 10—myricetin-3-glucoside, 11—petunidin-3-*O*-glucoside, 12—jatrorrhizine, 13—dihydroxy derivative of jatrorrhizine, 14—protopine, 15—palmatine, 16—berberine. **b–e** UPLC–DAD chromatogram of **b** phenolic mixture: 1—gallic acid, 2—pro-catechuic acid, 3—chlorogenic acid, 4—catechin, 5—caffeic acid, 6—rutin, 7—*p*-coumaric acid, 8—naringin, 9—cinnamic acid and 10—kaempferol; **c** 11—quercetin 3-*D*-galactoside; **d** alkaloid mixture: 1—syringin, 2—cardifolioside 3—magnoflorine 4—tinocardiside and **e** 5—Berberine

studies using different parts of *Berberis* species (Campisi et al. 2014; Pradhan and Saha 2015). On subsequent UPLC–DAD–ESI–QTOF–MS/MS analysis, we observed that BLFE had a high content of secondary metabolites chlorogenic acid, rutin, catechin, quercetin 3-*D*-galactoside, berberine and magnoflorine, which are generally recognized for their anti-oxidant and anti-inflammatory potential (Kuo et al. 2004; Nakanishi et al. 2010; Hwang et al. 2013; Guo et al. 2018; Xianchu et al. 2018). It is thus evident that fruits of *Berberis* species are an important source of bioactive compounds that could be useful in developing an alternate therapeutic strategy for the alleviation of inflammatory and oxidative cellular stressors. To test this possibility, ex vivo anti-inflammatory efficacy of BLFE using LPS-stimulated macrophages and Con-A-activated splenic T cells was investigated. The unifying hallmark of LPS-induced inflammation is the abnormal and excessive production of various effector molecules through the activation of inflammatory pathways such as NF- κ B and MAPKs. Here, in the present study, we observed that pre-treatment with BLFE strongly suppressed the majority of inflammatory mediators through the inhibition of NF- κ B, c-Jun and MAPKs (ERK, JNK and p38) in stimulated macrophages and thus exhibited excellent anti-inflammatory activity. BLFE treatment attenuated NO production as well as suppressed iNOS expression compared to LPS-stimulated cells. Additionally, BLFE at both 100 and 200 μ g/ml inhibited protein expression of iNOS, while iNOS mRNA expression could only be significantly influenced at the concentration of 200 μ g/ml. These results suggest that interference with

iNOS protein expression may be the primary contributing factor for the apparent inhibitory effects of BLFE on NO production in activated macrophages. These results are in agreement with studies performed by Chiou et al. (2000) and Shin et al. (2016) using andrographolide and berberine, respectively, in stimulated Raw 264.7 cells. Analysis of characterized phytomolecules (berberine and rutin) in BLFE also validated these observations wherein a strong attenuation of NO production and iNOS expression was observed. This also suggests that the presence of a milieu of such characterized bioactive components in BLFE could be responsible for the diverse anti-inflammatory effects as observed in the present study.

To further substantiate the anti-inflammatory attributes of BLFE, various inflammatory cytokines and chemokines were assessed. The results revealed that LPS treatment significantly augmented the levels of TNF- α , IL-1 β , IL-6 and INF- γ , while pre-treatment with BLFE (100 and 200 μ g/ml) significantly reduced the levels of all the tested cytokines. In contrast, significantly increased IL-10 production in the presence of BLFE was also observed suggesting immunomodulatory attributes of BLFE. These results suggest that BLFE modulated the production of effector inflammatory molecules as well as regulatory molecules, thereby suggesting its efficacy in maintaining inflammatory homeostasis. The present findings are similar to previous studies using different plant extracts wherein secondary metabolite-enriched plant fractions exhibited anti-inflammatory effects (Sharma et al. 2018; Kumar et al. 2016; Kang et al. 2007). In addition, studies on isolated chlorogenic acid and catechin have also shown significant inhibitory effects on inflammation and cytokine levels (Noll et al. 2012; Hwang et al. 2013). The increased levels of MCP-1 and RANTES are also known to attract and recruit immune cells at the site of infection that may further augment inflammatory damage. We observed that BLFE treatment attenuated the production of these chemokines, thereby further supporting its potency in the amelioration of inflammation (Qin et al. 2016; Park et al. 2017).

To explore the molecular mechanism(s) governing the apparent anti-inflammatory attributes of BLFE, we assessed the activation of inflammatory regulators, NF- κ B and MAPKs (ERK, JNK and p38). It was observed that LPS

Table 2 Quantification (mg/g) of phenolics and alkaloids in BLFE using UPLC–DAD–ESI–QTOF–MS/MS

S. no.	RT (min)	Mass (g/mol)	UV λ max (nm)	Identification	Quantity (mg/g)
01	2.71	355	217.8, 325.8	Chlorogenic acid	10 \pm 0.9
02	2.70	291	205.8, 286.8	Catechin	1.8 \pm 3.2
03	3.96	611	210, 350.8	Rutin	0.62 \pm 0.75
04	4.22	465	208, 254.8, 354.8	Quercetin 3- <i>D</i> -galactoside	2.51 \pm 1.2
05	6.3	336	263.8, 346.8, 426.8	Berberine	0.58 \pm 0.1
06	2.04	342	219.8, 267.8	Magnoflorine	17.22 \pm 3.8

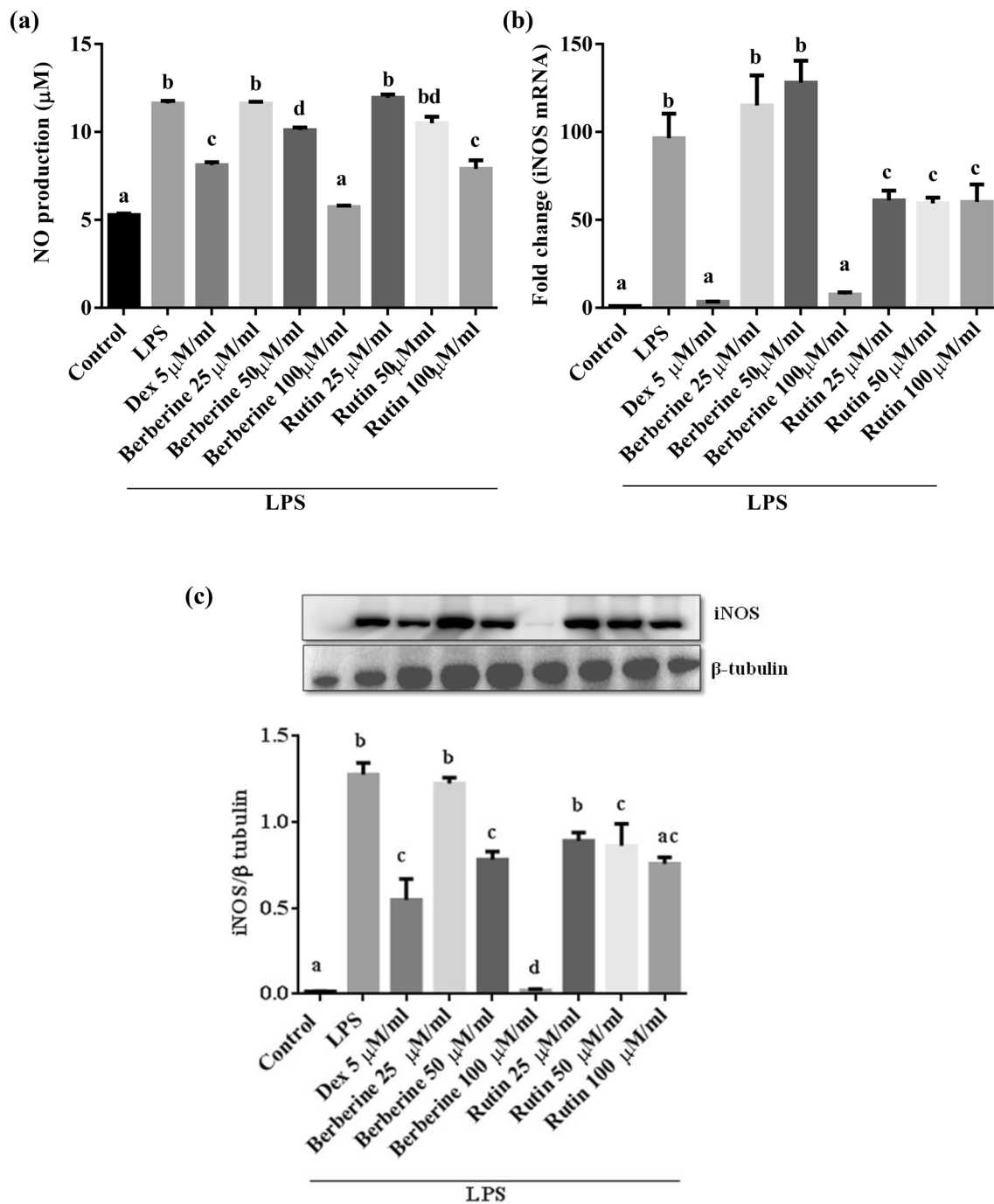


Fig. 10 BLFE constituents berberine and rutin ameliorate NO production and iNOS expression in stimulated peritoneal macrophages. Cells pre-treated with (25, 50 and 100 µM/ml) for 12 h followed by stimulation with LPS (1.5 µg/ml) for another 16 h. **a** NO production.

b mRNA expression of iNOS and **c** iNOS expression by immunoblotting. Values are mean ± SD of three independent experiments; values with different letters are significantly different at $p < 0.05$

treatment significantly upregulated NF-κB activation, which was strongly inhibited by pre-treatment with BLFE. Similarly, phosphorylation of IκBα, an upstream regulator of NF-κB, was also equally suppressed by BLFE suggesting that the apparent BLFE-mediated inhibitory effect on NF-κB

phosphorylation could be attributed to the downregulation of IκBα phosphorylation. Overall, it appears that suppression of NF-κB induced by IκBα inhibition on account of BLFE treatment would have contributed to the observed attenuation of inflammatory effector molecules in LPS-stimulated

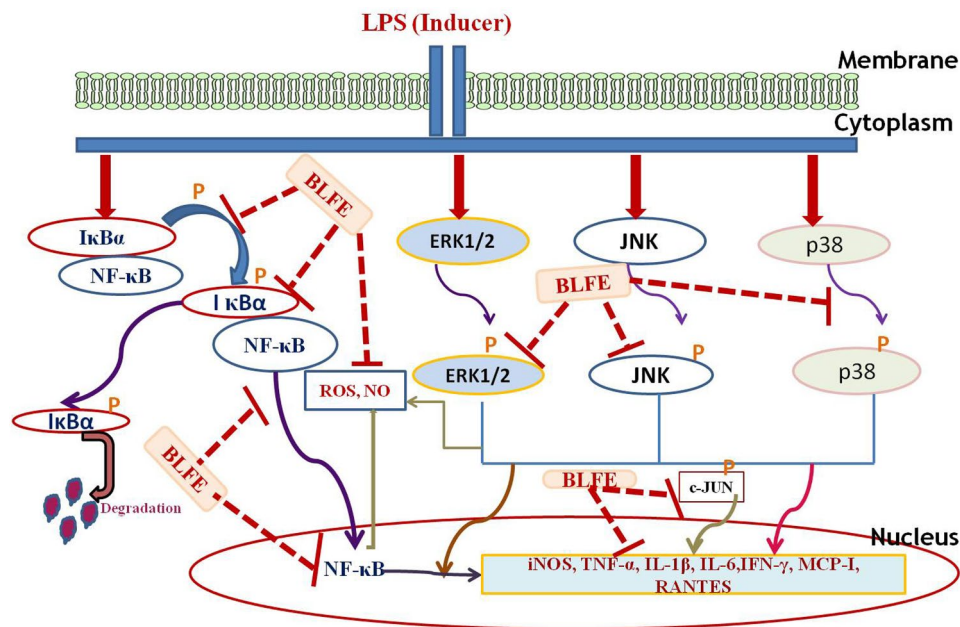


Fig. 11 Schematic diagram of probable signalling cascade mediating BLFE-induced anti-inflammatory effects. LPS stimulation augments inflammatory response by enhancing effector molecules such as NO, ROS, cytokines and chemokines through the activation of NF- κ B, c-Jun and MAPK (ERK, JNK and p38) signalling pathways. BLFE suppresses LPS-induced I κ B α as well as MAPK-dependent

NF- κ B activation, which culminates in the suppression of inflammatory effector molecules. Notwithstanding this, the inhibitory effect of BLFE on c-Jun, ERK, JNK and p38 activation could also directly result in inhibition of iNOS, cytokine and chemokine levels. Red “dotted inverted-T” symbols signify the possible inhibitory effects of BLFE

macrophages. In agreement with our results, a study on LPS-stimulated THP-1 cells showed I κ B α -dependent inhibition of NF- κ B activation with *Angelica dahuricae* Radix extract treatment (Kang et al. 2007). Similarly, another finding showed that Apo-9-fucoanthinone exposure reduced the activation of NF- κ B by inhibiting the I κ B phosphorylation in LPS-stimulated zebra fish model (Kim et al. 2018). In addition to plant extracts, it has been shown that pure magnoflorine and rutin are also potent NF- κ B modulators against extracellular stressors (Lee et al. 2012; Guo et al. 2018). LPS-induced activation of NF- κ B and c-Jun by MAPKs such as ERK, JNK and p38 pathways is also known to promote inflammatory responses (Wang et al. 2017; Bajpai et al. 2018). As such, in the present study, it was observed that phosphorylation of MAPKs (ERK, JNK and p38) was significantly increased with LPS treatment while BLFE exposure significantly decreased the expression of ERK and JNK at all the test concentrations. However, p38 as well as c-Jun phosphorylation could only be attenuated at 100 and 200 μ g/ml BLFE. Overall, inhibition of these MAPKs signalling pathways suggested multi-faceted molecular targets of BLFE-mediated inflammatory suppression. These findings are consistent with previous studies using resveratrol, magnoflorine, blueberries, and *Angelica dahuricae* Radix extract (Kang et al. 2007; Wang et al. 2017; Guo et al. 2018).

The inflammatory microenvironment can also disturb the cellular redox homeostasis, thereby causing enhanced ROS production and accumulation, which in turn can further stimulate NF- κ B-mediated inflammation resulting in a vicious oxi-inflammatory cycle. Considering the observed potent anti-inflammatory attributes of BLFE in the present study, we next sought to determine the influence of BLFE on ROS production and related mechanism. It was observed that LPS treatment increased ROS accumulation in macrophages which was strongly attenuated by BLFE treatment. On the contrary, a decrease in gene expression of HO-1 in LPS-stimulated macrophages was observed which was significantly enhanced by BLFE treatment. The anti-oxidant enzyme HO-1 plays a crucial role in mitigating inflammation and oxidative stress (Pae and Chung 2009; Och et al. 2014) and thus it appears that increased HO-1 production could have contributed to observed attenuation of ROS in BLFE-treated cells. These observations are consistent with other studies which illustrated that enhanced HO-1 expression plays a significant role in mediating the anti-inflammatory effects of various natural compounds in LPS-stimulated macrophages (Li et al. 2011; Och et al. 2014). When under significant oxidative stress, the Nrf-2 orchestrates and augments antioxidant response elements (such as SOD/catalase/HO-1) for mitigative response. In the present study, we observed that LPS-treated cells strongly

upregulated the protein expression of Nrf-2, possibly due to prevalent cellular oxidative stress, which the cells tried to counter (albeit unsuccessfully). In contrast, Nrf-2 expression in BLFE-treated cells was on par with the control. This suggests that BLFE treatment would have attenuated cellular ROS accumulation before it could reach a threshold so as to cause an activation of Nrf-2. HO-1 is also known to be activated by Nrf-2 independent of the mechanism involving NF- κ B and MAPKs (p38, ERK and JNK) that may have contributed to its observed activation in BLFE-treated cells (Wijayanti et al. 2004; Och et al. 2014). Overall, the apparent BLFE-mediated attenuation of the inflammatory pathways of NF- κ B, c-Jun with MAPKs could have directly contributed in the alleviation of ROS accumulation and subsequent inhibition of the oxi-inflammatory vicious cycle accompanied, in part, by the activation of HO-1 pathway. Assessment of T cell proliferation revealed that Con-A treatment induced the development of CD3+ CD4+ CD25+ Treg cells as compared to control which was significantly attenuated by BLFE treatment, albeit only at a higher concentration (200 μ g/ml). Interestingly, the same concentration of BLFE also appeared to upregulate total CD3+ cells compared to stimulated control. It has been shown that Con-A can encourage the maturation and activation of Treg cells which contributes to immune-related disorders such as during inflammation. Overall it appears that BLFE could exert beneficial effects on adaptive immune cells as well by controlling Con-A-mediated proliferation of Treg cells (Shinohara and Tsukimoto 2018).

Conclusion

In the present study, we observed that BLFE treatment exerted anti-oxidant and anti-inflammatory attributes by inhibiting LPS-mediated induction of pro-inflammatory effector molecules. Based on the observed results, we speculate that BLFE may suppress NF- κ B- and c-Jun-mediated inflammatory responses via I κ B α -dependent as well as MAPK (ERK, JNK and p38)-dependent pathways (Fig. 11). Overall, it is reasonable to conclude that the apparent inhibitory effects of BLFE on LPS-induced NF- κ B, c-Jun, ERK, JNK and p38 activation resulted in the attenuation of subsequent production of NO, pro-inflammatory cytokines and chemokines. The apparent diverse immunosuppressive attributes of BLFE could be ascribed to the presence of different bioactive molecules, such as berberine and rutin, as well as their mutual synergism in influencing the inflammatory pathways. Taken together, these findings provide compelling evidence that *B. lycium* fruits could be a new addition in the development of anti-inflammatory functional foods. Future research directions may include in vivo assessment of preventive as well as

therapeutic attributes of BLFE against various inflammatory disorders.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest.

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