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Butrin, Isobutrin, and Butein from Medicinal Plant *Butea monosperma* Selectively Inhibit Nuclear Factor-κB in Activated Human Mast Cells: Suppression of Tumor Necrosis Factor-α, Interleukin (IL)-6, and IL-8

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ABSTRACT

Activation of mast cells in rheumatoid synovial tissue has often been associated with tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-8 production and disease pathogenesis by adjacent cell types. *Butea monosperma* (BM) is a well known medicinal plant in India and the tropics. The aim of this study was to examine whether a standardized extract of BM flower (BME) could inhibit inflammatory reactions in human mast cells (HMC) using activated HMC-1 cells as a model. Four previously characterized polyphenols—butrin, isobutrin, isocoreopsin, and butein—were isolated from BME by preparative thin layer chromatography, and their purity and molecular weights were determined by liquid chromatography/mass spectrometry analysis. Our results showed that butrin, isobutrin, and butein significantly reduced the phorbol 12-myristate 13-acetate and calcium ionophore A23187-induced inflammatory gene expression and production of TNF-α, IL-6, and IL-8 in HMC-1 cells by inhibiting the activation of NF-κB. In addition, isobutrin was most potent in suppressing the NF-κB p65 activation by inhibiting IκBα degradation, whereas butrin and butein were relatively less effective. In vitro kinase activity assay revealed that isobutrin was a potent inhibitor of IκB kinase complex activity. This is the first report identifying the molecular basis of the reported anti-inflammatory effects of BME and its constituents butrin, isobutrin, and butein. The novel pharmacological actions of these polyphenolic compounds indicate potential therapeutic value for the treatment of inflammatory and other diseases in which activated mast cells play a role.

Mast cells are emerging key players in the erosive and inflammatory events leading to joint destruction in inflammatory diseases (Maruotti et al., 2007). Accumulation of mast cells in rheumatoid synovial tissues and their activation and degradation associated with proinflammatory cytokines and matrix-degrading enzymes at cartilage erosion sites suggest that they could be usefully selected as a therapeutic target (Woolley and Tetlow et al., 2000). Activated mast cells produce a wide variety of inflammatory mediators, such as eicosanoids, proteoglycans, proteases, and several proinflammatory and chemotactic cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-8 (Blue et al., 1993). TNF-α reportedly plays a pivotal role in the pathogenesis of inflammatory arthritis, especially due to its ability to regulate IL-1β expression, this being important for the induction of prostanoid and matrix metalloproteinases production by synovial fibroblasts and chondrocytes (Arend and Dayer, 1995). Another proinflammatory cytokine, IL-6, is

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ABBREVIATIONS: TNF, tumor necrosis factor; IL, interleukin; RA, rheumatoid arthritis; NF-κB, nuclear factor-κB; BM, *Butea monosperma*; TLC, thin layer chromatography; BME, extract of *Butea monosperma* flowers; LC/MS, liquid chromatography and mass spectrometry; PMACI, phorbol 12-myristate 13-acetate and calcium ionophore A23187; IKK, IκB kinase complex; HMC, human mast cell(s); PMA, phorbol 12-myristate 13-acetate; A23187, calcimycin, $C_{29}H_{37}N_3O_6$; MTT, 3-(4,5-dimethylthiazol-2-yl)-diphenyl-tetrazolium bromide; ELISA, enzyme-linked immunosorbent assay; R_f, retention factor; HPLC, high-performance liquid chromatography; DPPH, 2,2-diphenyl-2-picrylhydrazyl hydrate; ORAC, oxygen radical absorbance capacity assay; RT-PCR, real-time polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; F, forward; R, reverse; ASA, ascorbic acid; ROS, reactive oxygen species.

found in high levels in arthritic joints and is also a potent mediator of inflammatory processes (Ershler and Keller, 2000). IL-8 is a neutrophil chemoattractant factor involved in inflammation (Möller et al., 1993). Several recent lines of evidence indicate that production of TNF-α, IL-6, and IL-8 by activated mast cells could drive synovitis in patients with inflammatory arthritis (Woolley and Tetlow, 2000). It has been shown that mice deficient in mast cell activation were resistant to the induction of arthritis in the K/BxN (KRN T-cell receptor transgenic mouse on the C57BL/6xNOD genetic background) model of rheumatoid arthritis (RA) (Lee et al., 2002). These data suggest that inhibition of inflammatory mediators produced by activated mast cells could provide benefit in RA and other inflammatory diseases.

Master transcription factor NF-κB plays an important role in the expression of inflammatory mediators such as TNF-α, IL-6, and IL-8 that play a crucial role in joint destruction and therefore are key molecular targets for therapeutic intervention in inflammatory diseases such as RA (DiDonato et al., 1997; Azzolina et al., 2003). For these reasons, NF-κB is an obvious target of anti-inflammatory therapeutics (DiDonato et al., 1997).

Butea monosperma (BM) is a commonly grown plant in India. Traditionally, most of the plant parts are used for the treatment of various inflammatory, metabolic, and infectious diseases. The plant constituents have been reported to possess hepatoprotective (Wagner et al., 1986), anti-inflammatory (Shahavi and Desai, 2008), antidiabetic (Somani et al., 2006), antihelmintic (Iqbal et al., 2006), antidiarrheal (Gunakkunru et al., 2005), antifungal (Yadava and Tiwari, 2007), and antimicrobial (Gurav et al., 2008) activities. In short, approximately 45 medicinal uses are associated with this plant, and half of the number of claims have been scientifically studied and reported in the literature (Burli and Khade, 2007). Phytochemical analysis of BM extract identified the presence of flavones and flavanols (Yadava and Tiwari, 2007), chalcones (Gupta et al., 1970), isoflavones (Bandara et al., 1990), pterocarpans (Bandara et al., 1990), leucocyanidin tetramer (Seshadri and Trikha, 1971), as well as triterpenes (Bandara et al., 1990) and sterols (Mishra et al., 2000). Chokchaisiri et al. (2009) carried out a reinvestigation of the BM flower constituents and showed the presence of seven flavonoid glucosides, with two of them (butrin and isobutrin) isolated previously (Wagner et al., 1986). Three glucosides—coreopsin, isocoreopsin, and sulfurein—were identified, and the remaining two were new and have been assigned the structures monospermoside and isomonospermoside (Gupta et al., 1970; Chokchaisiri et al., 2009). Extract of BM flowers shows anticonvulsive activity, due to the presence of a triterpene (Kasture et al., 2002). Alcoholic extract of BM flowers has also been reported to exhibit antifertility activity (Razdan et al., 1970), with butrin showing both male and female contraceptive properties (Bhargava, 1986).

In the present study, we used preparative thin layer chromatography (TLC) to isolate butrin, isobutrin, isocoreopsin, and butein from a hydroalcoholic extract of BM flowers (BME) and determined the purity of the isolated polyphenols by liquid chromatography/mass spectrometry (LC/MS). We then investigated the effect of butrin, isobutrin, isocoreopsin, and butein on the expression of proinflammatory cytokines and the activation of NF-κB by using phorbol 12-myristate 13-acetate and calcium ionophore A23187 (PMACI)-activated HMC-1 human mast cells. Our results showed that isobutrin was the most potent inhibitor of the inflammatory stimuli-induced production of TNF-α, IL-6, and IL-8 via modulation of NF-κB. Butrin and butein also significantly inhibited the PMACI-induced TNF-α, IL-6, and IL-8 expression ($p < 0.05$), but isocoreopsin had no affect on the expression of these inflammatory mediators in HMC-1 cells. Further studies revealed that isobutrin inhibits NF-κB activation by inhibiting IκBα degradation, and it was found to be a potent inhibitor of IκB kinase complex (IKK) enzyme activity in vitro.

Materials and Methods

Materials. Phorbol 12-myristate 13-acetate (PMA), calcium ionophore A23187, α-thioglycerol, 3-(4,5-dimethylthiazol-2-yl)-diphenyl-tetrazolium bromide (MTT), gallic acid, 2,2-diphenyl-2-picrylhydrazyl hydrate, naphthyl ethylene diamine dihydrochloride, sulfanillic acid, ascorbic acid, sodium acetate, ferric chloride, and Trolox were purchased from Sigma-Aldrich (St. Louis, MO). Iscove's modified Dulbecco's medium was from Invitrogen (Carlsbad, CA), and iron-supplemented calf serum was from HyClone Laboratories (Logan, UT). 2,2′-Azobis 2-amidinopropane dihydrochloride was purchased from Wako Bioproducts (Richmond, VA). Antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA) and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). NF-κB inhibitor (parthenolide) was purchased from Calbiochem (San Diego, CA). Kits for performing cytokine-specific enzyme-linked immunosorbent assays (ELISAs) were purchased from R&D Systems (St. Paul, MN). A kit for assaying the IKK activity was purchased from Cell Signaling Technology Inc. HMC-1 cells were kindly provided by Dr. J. H. Butterfield (Division of Allergic Diseases, Mayo Clinic, Rochester, MN).

Preparation of BM Extract. The *B. monosperma* flowers were collected from the medicinal plant garden of Ajmal Khan Tibbiya College (Aligarh, India), and a voucher specimen was deposited in the Department of Moelejat, Faculty of Unani Medicine, Aligarh Muslim University (Aligarh, India). The *B. monosperma* flowers were dried and ground to a fine powder in a coffee mill, and the extract (BME) was prepared as described previously (Wagner et al., 1986). In brief, the dried powder (500 g) of *B. monosperma* flower was extracted with methanol in a Soxhlet extractor for 20 h. Removal of the solvent was done under reduced pressure in a rotatory evaporator (Rotavapor; BÜCHI Labortechnik AG, Flawil, Switzerland) and freeze-dried (Labconco, Kansas City, MO). We obtained 100 g of orange-colored powder, which was stored at 4°C in an airtight container. Required quantity of this freeze-dried powder was dissolved in Ultrapure water (Cayman Chemical, Ann Arbor, MI), filter-sterilized (0.45-μm Millex filters; Millipore, Billerica, MA), and added to the culture medium.

Preparative TLC. To screen for the presence of known constituents, BME was chromatographed on silica gel as described previously (Wagner et al., 1986). In brief, flavonoids were isolated by preparative TLC on silica gel (0.5 mm; Merck, Darmstadt, Germany), with eight spots becoming visible using the solvent system ethyl acetate/formic acid/acetic acid/water (100:11:11:26). Spots with retention factor (R_f) value of 0.20, 0.32, 0.39, 0.45, 0.69, 0.79, 0.90, and 0.96 were collected for further analysis. In this solvent system, R_f values of 0.32 and 0.45 correspond to butrin and isobutrin, respectively (Wagner et al., 1986).

High-Performance LC/MS. LC/MS analysis of BME and TLC-purified BME fractions was performed on a 1100 capillary LC system equipped with a model 1100 diode array detector (Agilent Technologies, Wilmington, DE), and a quadrupole time-of-flight mass spectrometer (Micromass Q-TOF1; Waters, Milford, MA) equipped with an electrospray ionization source. The binary solvent system used was solvent A, 0.1% formic acid in water and solvent B, 0.1% formic

acid with acetonitrile. Whole BME and TLC-purified fractions were dissolved in 1 ml of a mixture containing solvent A/solvent B (1:1, v/v), filtered using 0.22-μm syringe filter (Millipore), and 10 μl of filtrate was injected onto a C18 reversed-phase column (Chromegabond WR C18 column, 5-μm particles, 120-Å pore size, 15 cm × 2.1 mm; ES Industries, West Berlin, NJ). A linear gradient was used: 0 min, 10% B to 26% B at 20 min; then to 65% B at 35 min; and finally to 100% B at 36 min. The flow rate was 0.2 ml/min, and the HPLC flow was directed into the mass spectrometer ion source after leaving the diode array detector. The mass spectrometer was operated in the negative ion mode and under the following conditions: capillary voltage, 2200 V; source temperature, 100°C; desolvation temperature, 350°C; and cone voltage, 30 V. UV-visible spectra were recorded from 220 to 500 nm. Spectra were scanned over a mass range of *m/z* 150 to *m/z* 1200 at 2.0 s per cycle.

DPPH Radical Scavenging Assay. The hydrogen atom or electron donation ability of phenolic compounds of BME was measured against stable 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH) radical as described previously (Blois, 1958). BME or its isolated polyphenols (1–10 μg/ml) were added with DPPH (0.1 mM) in methanol and allowed to stand at room temperature for 30 min, and then the absorbance was measured at 517 nm with a spectrophotometer (model UV-1800; Shimadzu, Kyoto, Japan). Radical scavenging activity of isolated phenolic compounds of BME was expressed as the percentage of inhibition of free radical and was calculated using the formula $[(A_B - A_A)/A_B] \times 100$, where A_B is absorbance of blank and A_A is absorbance of sample. Ascorbic acid, a known antioxidant, was used as positive control.

Nitric Oxide Scavenging Assay. NO scavenging activity was measured by using Griess reagent as described previously (Sreejayan and Rao, 1997), with slight modifications. In brief, Griess reagent was modified by using naphthyl ethylene diamine dihydrochloride (0.1%, w/v). Scavenging of NO was competed with oxygen leading to inhibited production of NO. Two milliliters of sodium nitroprusside (10 mM) with BME or its polyphenols (1–10 μg) at physiological pH was incubated at 25°C for 150 min. After incubation, 0.5 ml of the reaction mixture containing nitrite was pipetted and mixed with 1 ml of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for complete diazotization. Then, 1 ml of naphthyl ethylene diamine dihydrochloride was added, mixed, and allowed to stand at 25°C for 30 min, and the pink-colored chromophore was read at 540 nm. Percentage of scavenging activity was calculated by using the equation $[(A_B - A_A)/A_B] \times 100$, where A_B is absorbance of blank and A_A is absorbance of sample. Ascorbic acid was used as positive control.

Oxygen Radical Absorbance Capacity Assay. The ORAC assay was performed as described previously using fluorescein as the fluorescent probe (Huang et al., 2002). The fluorescein was used as a target of free radical attack with 2,2′-azobis 2-amidinopropane dihydrochloride as a peroxyl radical generator. Trolox was used as the control standard. The antioxidative activity of samples (1–10 μg) was determined from its ability to protect the fluorescence of the indicator in the presence of peroxyl radical. Results are expressed as micromoles of Trolox equivalence per milligram of dried BME or its phenolic compounds. Ascorbic acid was used as positive control. Each experiment was done in triplicates.

Treatment of HMC-1 Cells. HMC-1 cells were grown in Iscove's modified Dulbecco's medium supplemented with 10% iron-supplemented calf serum and 1.2 mM α-thioglycerol, 1% penicillin-streptomycin, and 2.5 μg/ml amphotericin B at 37°C, 5% CO_2 in a plug-sealed flask (BD Biosciences Discovery Labware, Bedford, MA). Serum-free HMC-1 cells were treated with different doses of isolated polyphenols of BME (1–10 μg/ml) for 2 h before stimulation with 40 nM PMA plus 1 μM A23187 (PMACI) for different times. HMC-1 cells cultured without PMACI or BME constituents served as controls. Cytotoxicity of phenolic compounds was determined using MTT assay (Cory et al., 1991).

Real-Time PCR. Quantitative real-time polymerase chain reaction (RT-PCR) was used to quantify the expression of mRNAs for TNF-α, IL-6, and IL-8 with expression of GAPDH was used as endogenous control. Total RNA was separated from HMC-1 cells by TRIzol reagent (Invitrogen) according to the manufacturer's instruction (Invitrogen). First-strand cDNA was synthesized using 1 μg of total RNA and the SuperScript First-Strand cDNA synthesis kit (Invitrogen). Primers used for PCR-assisted amplification were as follows: TNF-α (GenBank accession number NM_000595; F, 5′-AGG ACG AAC ATC CAA CCT TCC CAA-3′ and R, 5′-TTT GAG CCA GAA GAG GTT GAG GGT-3′), IL-6 (GenBank accession number NM_000600; F, 5′-AAA TTC GGT ACA TCC TCG ACG GCA-3′ and R, 5′-AGT GCC TCT TTG CTG CTT TCA CAC-3′), IL-8 (GenBank accession number NM_000584; F, 5′-AGA AAC CAC CGG AAG GAA CCA TCT-3′ and R, 5′-AGA GCT GCA GAA ATC AGG AAG GCT-3′), and GAPDH (GenBank accession number NM_002046.3; F, 5′-TCG ACA GTC AGC CGC ATC TTC TTT-3′ and R, 5′-ACC AAA TCC GTT GAC TCC GAC CTT-3′). PCR amplification was carried out using the core kit for SYBR Green (Quanta Biosciences, Gaithersburg, MD) and the Step One Real-Time PCR System (Applied Biosystems, Foster City, CA). Typical profile times used were initial step, 95°C for 10 min followed by a second step at 95°C for 15 s and 60°C for 30 s for 40 cycles with melting curve analysis. The level of target mRNA was normalized to the level of GAPDH and compared with control (untreated sample). Data were analyzed using ΔΔCT method (Pfaffl, 2001).

Enzyme-Linked Immunosorbent Assay. HMC-1 cells were stimulated with PMACI for 24 h with or without pretreatment with polyphenols of BME. TNF-α, IL-6, and IL-8 produced in the culture medium were quantified using the cytokine specific ELISA kit according to the instructions of the manufacturer (R&D Systems) using a Synergy HT microplate reader (BioTek Instruments, Winooski, VT).

Western Immunoblotting Analysis. Stimulated and control HMC-1 cells were washed with ice-cold phosphate-buffered saline and lysed using the cell lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM EGTA, and Complete protease and phosphatase inhibitors) as described previously (Rasheed et al., 2009b). Cytoplasmic and nuclear fractions were prepared as described previously (Rasheed et al., 2009a). Total lysate or nuclear/cytoplasmic fraction protein (35 μg/lane) was resolved by SDS-polyacrylamide gel electrophoresis (10% resolving gel with 4% stacking) and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked with blocking buffer containing nonfat dry milk powder in Tris-buffered saline containing 0.1% Tween 20, and probed with 1:200 to 1:1000 diluted primary antibodies (Cell Signaling Technology Inc. and Santa Cruz Biotechnology, Inc.) specific for the target protein. Immunoreactive proteins were visualized by using 1:1000 diluted horseradish peroxidase-linked secondary antibodies and enhanced chemiluminescence (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) (Rasheed et al., 2009b). Images were captured by using AFP-Imaging System (Minimedical Series, Elms Ford, NY).

NF-κB DNA Binding Activity Assay. Activation of NF-κB p65 in HMC-1 cells pretreated with BME or purified butrin, isobutrin, isocoreopsin, or butein and then stimulated with PMACI was determined using a highly sensitive transcription factor ELISA kit according to the instructions of the manufacturer (Assay Designs, Ann Arbor, MI). The assay uses streptavidin-coated plates with bound NF-κB-biotinylated consensus sequence to capture the active form of NF-κB. The captured active NF-κB is incubated with a specific antibody. The assay is developed with a chemiluminescent substrate and the signal is detected using a luminometer (Lumat LB 9507; Berthold Technologies, Bad Wildbad, Germany), and the values are expressed as relative light units.

In Vitro IKKβ Kinase Assay. Effect of BME or TLC-purified different fractions of BME on IKK activity was determined using HTScan IKKβ kinase assay kit according to the instructions of the manufacturer (Cell Signaling Technology). The kit provides a means of performing kinase activity assays with recombinant human IKKβ kinase. It includes active IKKβ kinase (supplied as glutathione transferase fusion protein), a biotinylated peptide substrate, and a phosphoserine antibody for detection of the phosphorylated form of the substrate peptide. Purified IKKβ kinase was pretreated with different doses of BME 5 min before the treatment of substrate peptide. Assay was performed on a 96-well high binding streptavidin-coated plate (Thermo Fisher Scientific, Waltham, MA) and the absorbance (A) of each well was read at 450 nm using a Synergy HT ELISA plate reader (BioTek Instruments). Each kinase assay was performed in triplicate. Percentage of inhibition of IKKβ kinase activity was calculated using the formula $1 - [(A_{\text{inhibited}} - A_{\text{blank}})/(A_{\text{uninhibited}} - A_{\text{blank}})] \times 100$.

Densitometric Analysis. Densitometric measurements of the scanned bands were performed using UN-SCAN-IT software (Silk Scientific Inc., Orem, UT). Each band was scanned five times, and the mean band intensity (pixels/band) was obtained. Data were normalized to suitable loading controls and expressed as mean ± S.D.

Statistical Analysis. All measurements were performed in duplicates and repeated at least three times. All statistical analyses were performed using Origin 6.1 software (OrginLab Corp., Northampton, MA; one-paired two-tailed t test with one-way analysis of variance and Tukey's post hoc analysis), and $p < 0.05$ was considered significant. Values shown are mean ± S.E.M. unless stated otherwise.

Results

Screening of Flavonoids Present in BME. BME was first chromatographed on silica gel, and eight different spots were identified, with R_f values of 0.20, 0.32, 0.39, 0.45, 0.69, 0.79, 0.90, and 0.96 (data not shown), and then analyzed by HPLC using a reversed phase C18 column (Fig. 1A). Preparative TLC was used to separate fractions of BME and fractions were analyzed by HPLC under identical experimental conditions. HPLC chromatogram of different fractions prepared by TLC showed that the four fractions with R_f values 0.32, 0.45, 0.69, and 0.96 were cleanly resolved by TLC as evident by single HPLC peak of each of these fractions (Fig. 1, B–E). These fractions were further analyzed by mass spectrometry (Fig. 1, B–E, insets) and fraction 2 (R_f = 0.32), fraction 4 (R_f = 0.45), fraction 5 (R_f = 0.69), and fraction 9 (R_f = 0.96) gave single sharp peak at 595, 595, 433, and 271 m/z, respectively (Fig. 1, insets). Previous studies have shown that R_f values 0.32 and 0.42 corresponds to butrin and isobutrin, respectively (Chokchaisiri et al., 2009), and it has also been reported that butrin, isobutrin, isocoreopsin, and butein having molecular weights of 596.53, 596.53, 434.39, and 596.53, respectively, are active constituents of BME (Gupta et al., 1970; Chokchaisiri et al., 2009). Based on the known R_f values, molecular weights, and use of commercially available butein (Extrasynthese, Genay, France) standard, we identified that fractions 2, 4, 5, and 9 contained purified butrin, isobutrin, isocoreopsin, and butein, respectively (Fig. 1). Chemical structures of these purified polyphenols were obtained from http://pubchem.ncbi.nih.gov and are shown in Fig. 1 (B–E, insets). Purified polyphenols concentrations were determined spectrophotometrically using commercially available butein (Extrasynthese) as standard.

Antioxidant Activity of BM Polyphenols. Antioxidant potential of the butrin, isobutrin, isocoreopsin, butein, or BME was determined by three different test systems, namely, DPPH radical scavenging assay, NO radical scavenging capacity assay, and ORAC assay. The reactivity toward the stable free radical DPPH was measured at 540 nm. A maximum of 42% inhibition of DPPH radical was observed by BME at 10 μg/ml. Butrin, isobutrin, isocoreopsin, and butein with the same concentration (10 μg/ml) inhibited DPPH radical activity by 35, 40, 27, and 28%, respectively (Fig. 2A). Ascorbic acid (ASA) was used as a positive control inhibited 56% DPPH radical activity. Effect of BM polyphenols on 2,2′-azobis 2-amidinopropane dihydrochloride-induced peroxyl radical was determined by ORAC assay. The maximum ORAC values (±S.E.M.) of three independent assays with 10 μg/ml butrin, isobutrin, isocoreopsin, butein, BME, or ASA were 146 ± 8.6, 132 ± 11.2, 143 ± 9.2, 149 ± 4.2, 153 ± 10.8, or 112 ± 9.6 Trolox equivalents, respectively (Fig. 2B). NO scavenging assay showed butrin, isobutrin, isocoreopsin, butein, and BME reduced 16, 29, 28, 23, and 39% of NO activity, respectively, at 10 μg/ml, whereas ASA with the same concentration inhibited 44% of NO activity (Fig. 2C).

Effect of Butrin, Isobutrin, Isocoreopsin, and Butein on Cell Viability and TNF-α Expression in Activated HMC-1 Cells. HMC-1 cells were pretreated with isolated polyphenols of BME (1–60 μg/ml) for 2 h and then stimulated with PMA (40 nM) plus A23187 (1 μM PMACI) for 24 h. We first examined the cytotoxicity of phenolics of BME alone or in combination with PMACI on HMC-1 cells after 24-h treatment using MTT assay. BME and its polyphenolic constituents up to 60 μg/ml and PMACI (40 nM PMA plus 1 μM A23187) were not toxic to the cells as determined by MTT assay (data not shown). Next, we examined whether the polyphenols isolated from BME could modulate the gene expression of potent proinflammatory mediator TNF-α in HMC-1 cells. As shown in Fig. 3A, pretreatment with isobutrin and butein (1–10 μg/ml) dose-dependently inhibited PMACI-induced mRNA expression of TNF-α ($p < 0.05$) compared with controls. Purified butrin inhibited the expression of TNF-α to a lesser extent, whereas isocoreopsin had no affect on the mRNA expression of TNF-α. Whole BME extract was used as a positive control and significantly inhibited the PMACI-induced TNF-α mRNA expression in HMC-1 cells ($p < 0.05$) (Fig. 3A). To determine whether this inhibition of gene expression also influenced the production of TNF-α, culture supernatants were assayed using a TNF-α-specific ELISA. As shown in Fig. 3B, pretreatment with 1 to 10 μg/ml isobutrin, butein, or whole BME significantly decreased the PMACI-induced TNF-α production in the culture supernatant of activated HMC-1 cells ($p < 0.05$). In agreement with studies on mRNA expression, isocoreopsin had no effect on TNF-α production (Fig. 3B).

Effect of Butrin, Isobutrin, Isocoreopsin, and Butein on PMACI-Induced Expression and Production of IL-6 in HMC-1 Cells. HMC-1 cells were pretreated with butrin, isobutrin, isocoreopsin, butein, or BME (1–10 μg/ml) for 2 h and then stimulated with PMACI for 8 h. The level of IL-6 mRNA was quantified by quantitative RT-PCR method. Our results showed that HMC-1 cells treated with PMACI had higher levels of IL-6 mRNA compared with the levels of IL-6 mRNA detected in unstimulated mast cells. However, IL-6

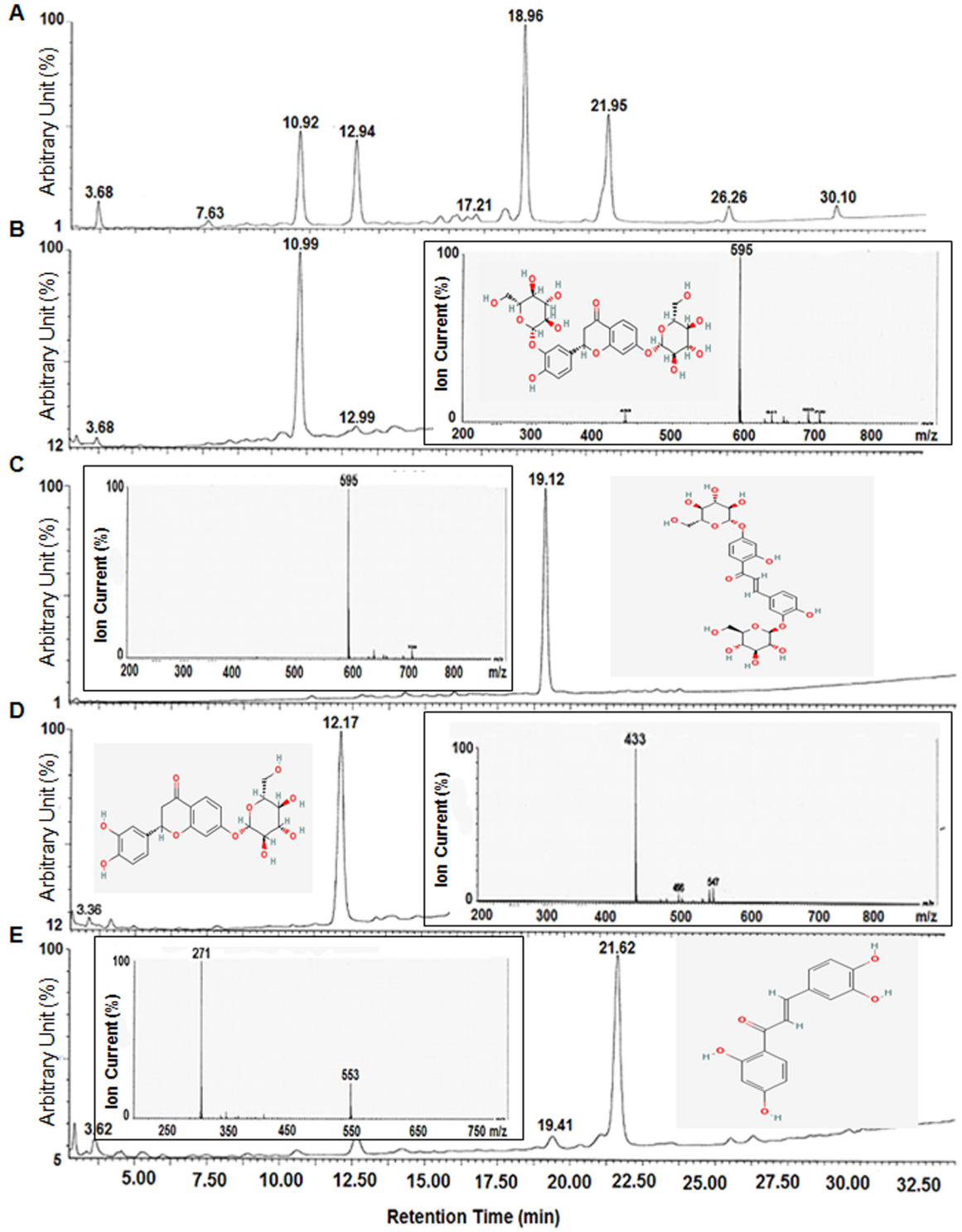

Fig. 1. Screening of BM polyphenols. HPLC chromatogram of the whole BME (A) and the preparative TLC-purified fractions of BME (B–E). Inset, mass spectrometry-chromatogram of TLC-purified fractions of BME and chemical structures of BM polyphenols used in this study. B, butrin: molecular weight 596, molecular formula $C_{27}H_{32}O_{15}$, and International Union of Pure and Applied Chemistry (IUPAC) name (2*S*)-2-[4-hydroxy-3-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxyphenyl]-7-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-hydroxymethyl)oxan-2-yl]oxy-2,3-dihydrochromen-4-one. C, isobutrin: molecular weight 596, molecular formula $C_{27}H_{32}O_{15}$, IUPAC name (*E*)-1-[2-hydroxy-4-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxyphenyl]-3-[4-hydroxy-3-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxyphenyl]prop-2-en-1-one isobutrin. D, isocoreopsin: molecular weight 434, molecular formula $C_{27}H_{32}O_{15}$, IUPAC name (2*S*)-2-(3,4-dihydroxyphenyl)-7-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]ox y-2,3-dihydrochromen-4-one. E, butein: molecular weight 272, molecular formula $C_{15}H_{12}O_5$, IUPAC name (*E*)-1-(2,4-dihydroxyphenyl)-3-(3,4-dihydroxyphenyl)prop-2-en-1-one.

mRNA levels showed a significant decline in the samples pretreated with butrin, isobutrin, butein or BME and then stimulated with PMACI ($p < 0.05$) (Fig. 3C). PMACI-induced IL-6 expression was inhibited by isocoreopsin as well, but only at high concentration (Fig. 3C). When culture supernatants were assayed for IL-6 protein levels results were sim-

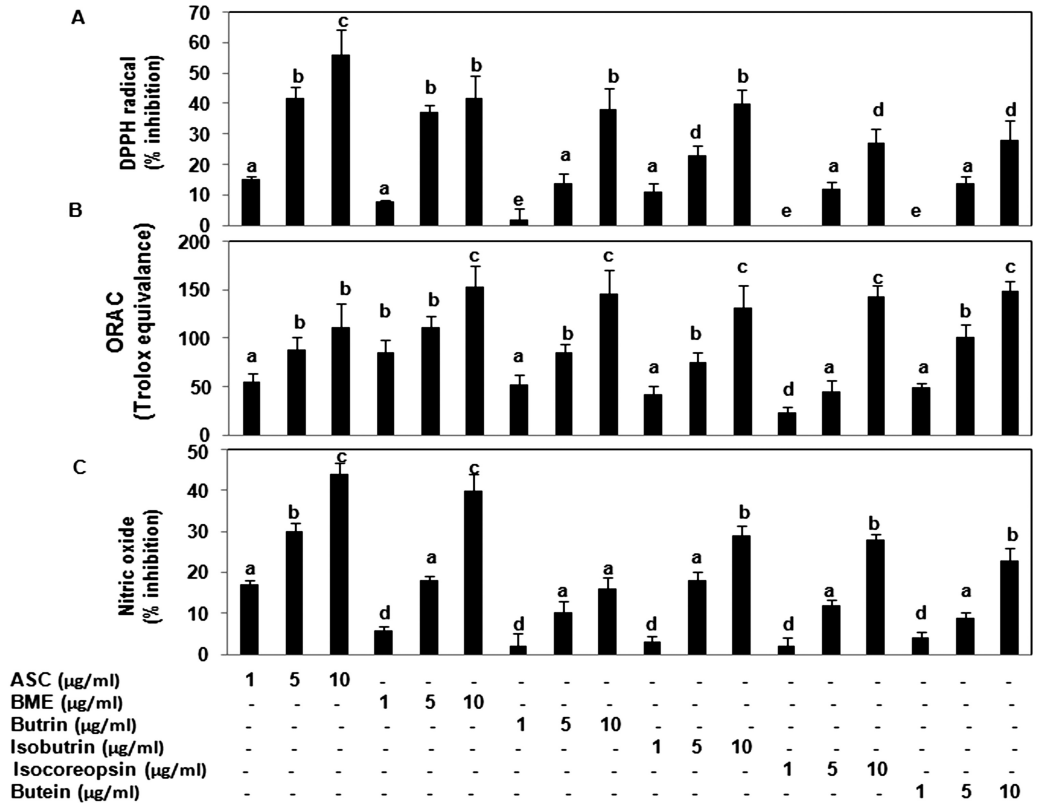

Fig. 2. Antioxidant potential of BM polyphenols. A, DPPH radical-scavenging capacity of BM polyphenols. The radical scavenging ability of BM polyphenols was analyzed by measuring their inhibitory effects on the absorbance of DPPH radicals at 517 nm. Ascorbic acid was used as a positive control. The reaction was performed in triplicates, and results were expressed as percentage of inhibition of the absorbance of the DPPH radical ± S.E.M. B, ORAC of BM polyphenols. The antioxidant activity of BM polyphenols by ORAC was conducted in microplates using fluorescein as the fluorescent probe. Ascorbic acid was used as positive control. The reaction was performed in triplicates and results were expressed as micromoles of Trolox equivalence per microgram of dried BME and its polyphenols. C, percentage of inhibition of NO radicals in the presence of BM polyphenols. The radical scavenging ability of BM polyphenols was analyzed by measuring their inhibitory effects on the absorbance of the NO reaction product at 540 nm. Ascorbic acid was used as a positive control. The reaction was performed in triplicates and results were expressed as percentage of inhibition of the absorbance of NO reaction product ± S.E.M. Bars with the same letter do not differ statistically ($p < 0.05$).

ilar to those obtained with IL-6 mRNA expression (Fig. 3C). As shown in Fig. 3D, pretreatment with purified butrin, isobutrin, butein or BME significantly inhibited the PMACI-induced IL-6 production at low doses ($p < 0.05$), isocoreopsin inhibited the IL-6 production only at a higher dose of 10 μg/ml (Fig. 3D). These results also point out that compared with other polyphenols used isobutrin showed the maximum inhibitory effect on IL-6 expression and production ($p < 0.001$) in activated HMC-1 cells.

Butrin, Isobutrin, and Butein Down-Regulated the Expression and Production of IL-8 in PMACI-Stimulated HMC-1 Cells. Pretreatment of HMC-1 cells with butrin, isobutrin, butein or BME inhibited PMACI-induced gene expression of IL-8 in a dose-dependent manner as determined by quantitative RT-PCR (Fig. 3E). The maximal inhibitory effect of BM polyphenols was found at 10 μg/ml concentration ($p < 0.001$). The results showed that HMC-1 cells stimulated with PMACI had enhanced levels of IL-8 compared with the levels detected in untreated HMC-1 cells. It is important to note that HMC-1 cells pretreated with different doses of BME or purified polyphenols and then stimulated with PMACI produced significantly reduced levels of IL-8 in the culture supernatant ($p < 0.05$) (Fig. 3F). Pretreatment with 10 μg/ml isocoreopsin had no effect on the expression and production of IL-8 in stimulated HMC-1 cells compared with controls stimulated with PMACI ($p > 0.05$) (Fig. 3, E and F).

Effect of BM Polyphenols on NF-κB Activation in PMACI-Stimulated HMC-1 Cells. To evaluate the mechanism of inhibition of TNF-α, IL-6, and IL-8 gene expression in HMC-1 cells, NF-κB activation was studied. Stimulation of HMC-1 cells with PMACI induced degradation of IκBα and nuclear translocation of NF-κB p65 (Fig. 4). Pretreatment with butrin, isobutrin, butein, or BME significantly inhibited the PMACI-induced degradation of IκBα ($p < 0.05$), but the inhibition was more pronounced by isobutrin pretreatment, whereas isocoreopsin failed to block the PMACI-induced IκBα degradation (Fig. 4, B and C). We also used a highly sensitive ELISA method to determine the activation of NF-κB by PMACI in HMC-1 cells (Fig. 4A). Butrin, isobutrin, butein, or BME significantly inhibited the activation and DNA binding activity of NF-κB p65 ($p < 0.05$), whereas pretreatment with isocoreopsin had no effect on PMACI-induced activation of NF-κB p65 in HMC-1 cells (Fig. 4A). Treatment of HMC-1 cells with parthenolide, a well known NF-κB inhibitor used as positive control, blocked the PMACI-induced TNF-α (Fig. 3, A and B), IL-6 (Fig. 3, C and D), and IL-8 (Fig. 3, E and F) expression and production in HMC-1 cells as determined by quantitative RT-PCR and by cytokine-specific ELISA. Together, these results suggest that butrin,

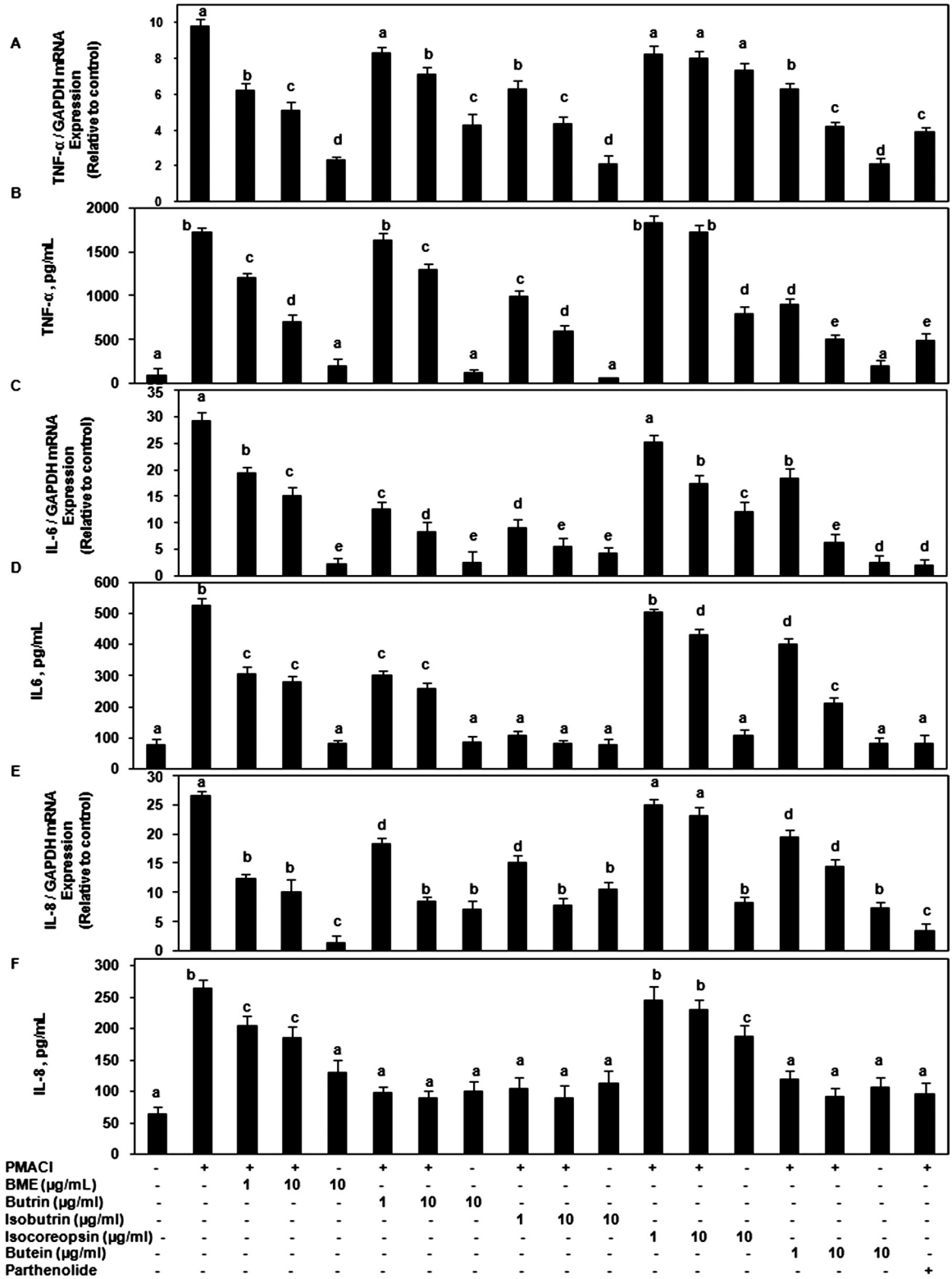

Fig. 3. Effect of BM polyphenols and specific inhibitor of NF-κB on TNF-α, IL-6, and IL-8 gene expression and production in PMACI-stimulated human mast cells. HMC-1 cells were pretreated with butrin, isobutrin, isocoreopsin, butein, or BME (1–10 μg/ml) for 2 h and stimulated by PMA (40 nM) plus A23187 (1 μM) for 8 h (A, C, and E) or 24 h (B, D, and F). Gene expression of TNF-α (A), IL-6 (C), and IL-8 (E) was determined by quantitative RT-PCR normalized to GAPDH and then compared with the levels present in untreated cells. Level of TNF-α (B), IL-6 (D), and IL-8 (F) in the culture medium was quantified by sandwich ELISA. Concentration of NF-κB inhibitor (parthenolide) used in these studies was 50 μM. Results are representative (mean ± S.E.M.) of four independent experiments and differ without a common letter ($p < 0.05$).

isobutrin, butein, or BME exert the inhibitory effect on TNF-α, IL-6, and IL-8 expression by inhibiting the activation of NF-κB.

Inhibition of IKKβ Kinase Activity by BM Polyphenols. Effect of purified BM polyphenols on the phosphorylating activity of IKKβ kinase was also determined using an

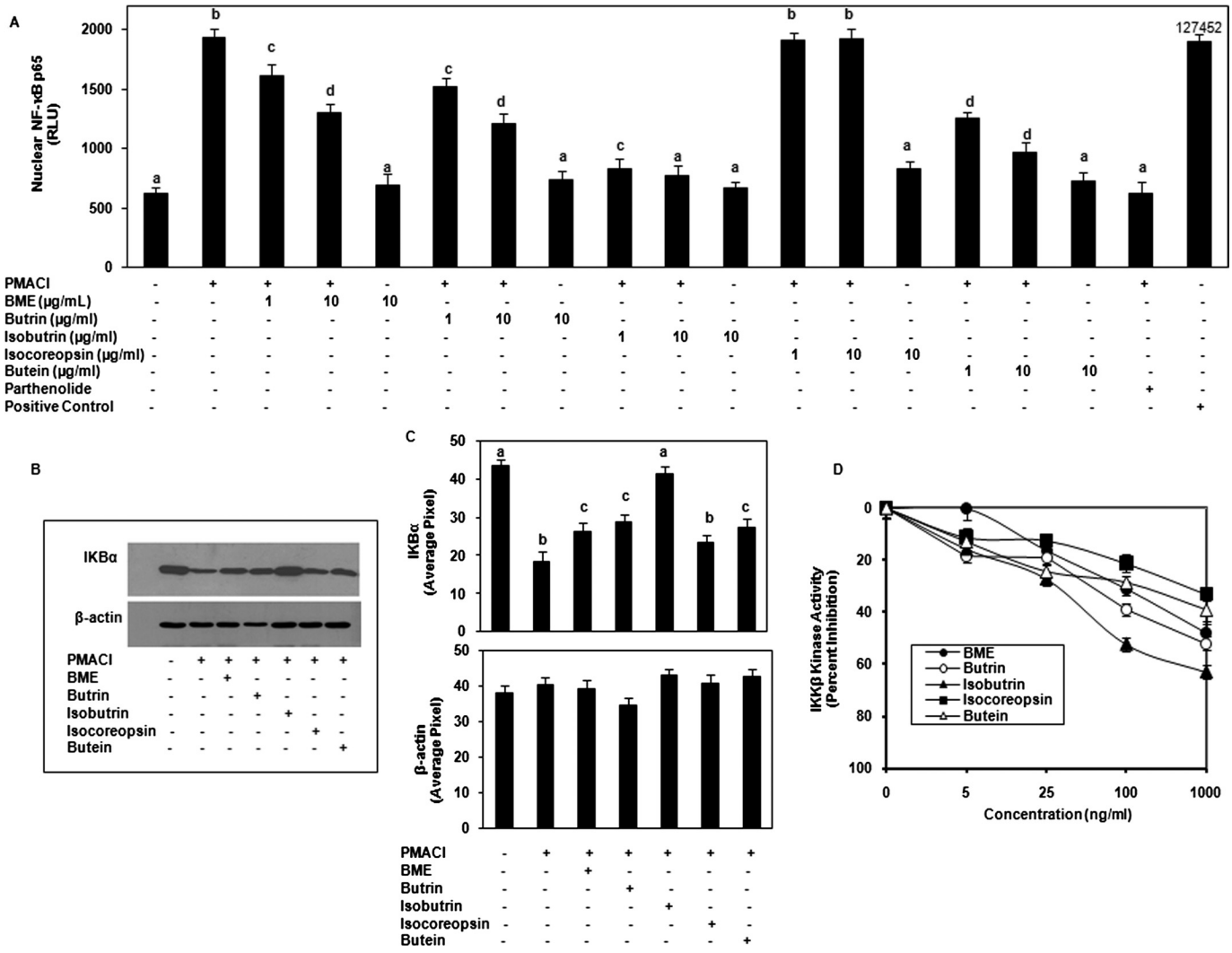

Fig. 4. Effect of BM polyphenols on the activation of NF-κB in PMACI-stimulated HMC-1 cells. Cells were pretreated with butrin, isobutrin, isocoreopsin, butein, or BME (1–10 μg/ml) for 2 h before PMA (40 nM) plus A23187 (1 μM) stimulation. A, NF-κB p65 was determined in nuclear extracts by highly sensitive and specific ELISA (Assay Designs). The potent inhibitor for IκB kinase, parthenolide (50 μM), was used as a positive control. TNF-α-treated HeLa cell extract (supplied with kit) was also used as a positive control. The assay is developed with a chemiluminescent substrate and the signal is detected using a luminometer (Lumat LB 9507; Berthold Technologies). Nuclear NF-κB p65 activity was expressed as relative light units (RLU). Results are representative (mean ± S.E.M.) of four independent experiments and differ without a common letter ($p < 0.05$). B, IκBα degradation was analyzed by Western immunoblotting using antibodies specific for the IκBα (Cell Signaling Technology Inc.). β-Actin was used as protein loading control. C, band images were digitally captured and the band intensities (pixels/band) were obtained using the Un-Scan-It software and are expressed in average pixels. Data shown is cumulative of three experiments and the optical density values are mean ± S.D. and differ without a common letter ($p < 0.05$). D, BM polyphenols inhibited the IKKβ kinase activity in vitro. IKKβ kinase activity was determined in the absence or presence of BM polyphenols (0–1000 ng/ml) by using the HTScan IKKβ kinase assay kit (Cell Signaling Technology Inc.). Each bar represents the mean ± S.E.M. of three independent experiments.

HTScan IKKβ kinase assay kit (Cell Signaling Technology Inc.). Purified IKKβ kinase was pretreated with different doses of butrin, isobutrin, isocoreopsin, butein, or BME (5–1000 ng/ml) 5 min before incubation with the substrate peptide. Figure 4D shows that IKKβ kinase activity was inhibited by all four purified BM polyphenols. A maximum of 63% inhibition of enzymatic activity was observed with 1 μg/ml isobutrin ($p > 0.001$). In contrast, IKK activity was inhibited 52 and 34% by butrin and butein, respectively, at the same dose, and isocoreopsin inhibited 33% IKK activity. Whole BME used as positive control inhibited 48% enzyme activity (Fig. 4D). These data suggest that butrin, isobutrin, and butein suppress the activation of NF-κB by inhibiting the enzyme activity of IKK complex. Although butein and isocoreopsin both inhibited the IKK enzyme activity to a similar degree, why isocoreopsin was not effective in suppressing NF-κB activation in HMC-1 cells needs further investigation.

Discussion

Both RA and osteoarthritis are characterized by variable degrees of chronic inflammation, cartilage destruction, and joint deformity. Histopathological observations of the "rheumatoid lesion"—a term used to describe the cartilage-pannus junction and cartilage erosion sites (Maruotti et al., 2007)—have identified several cell types, each of which may contribute different mediators to the inflammatory and degradative processes, with these processes usually being microenvironmental in nature. Although macrophages, fibroblastic synoviocytes, chondrocytes, lympho-

cytes, and neutrophils are recognized as important contributors to arthritic joint pathology, the mast cell has generally been neglected. Increased numbers of mast cells are found in the synovial tissue and fluid of patients with inflammatory arthritis (Woolley and Tetlow, 2000; Maruotti et al., 2007), and especially at sites of cartilage erosion (Woolley and Tetlow, 2000). Mast cells activation has been reported in these locations for a significant proportion of rheumatoid specimens (Tetlow and Woolley, 1995). Because these cells contain a variety of potent mediators, including histamine, heparin, proteinases, leukotrienes, and multifunctional cytokines (Woolley and Tetlow, 2000), its potential contributions to the processes of inflammation and matrix degradation have recently become evident (Maruotti et al., 2007). The human mast cell line HMC-1 is a useful tool for studying cytokine activation pathways (Shin et al., 2007). The spectrum of proteins and other molecules produced by HMC-1 cells with PMACI stimulation supports the well recognized role of mast cells in inflammation (Azzolina et al., 2003; Shin et al., 2007). Proinflammatory cytokines are important mediators of inflammation, immunity, proteolysis, cell recruitment, and proliferation, and these have received much attention over the past decade (Rasheed and Haqqi, 2008), and TNF-α, IL-6, and IL-8 have been demonstrated in the rheumatoid lesion by immunohistochemistry (Woolley and Tetlow, 2000). TNF-α, IL-6, and IL-8 play a major role in triggering and sustaining the inflammatory response in mast cells (Azzolina et al., 2003; Shin et al., 2007). These reports indicate that the inhibition of proinflammatory cytokines production by mast cells may be key to suppress the inflammatory symptoms.

Free radicals derived from oxygen, nitrogen, and sulfur molecules in the biological system are highly active to react with other molecules due to their unpaired electrons. These radicals are important groups of molecules called reactive oxygen species (ROS)/reactive nitrogen species, which are produced during cellular metabolism and functional activities and have important roles in cell signaling, apoptosis, gene expression, and ion transportation. However, excessive ROS attack on bases in nucleic acids, amino acid side chains in proteins, and double bonds in unsaturated fatty acids, causes oxidative stress, which can damage DNA, RNA, proteins, and lipids, resulting in an increased risk for cardiovascular disease, cancer, RA, and other diseases (Lü et al., 2009). Intracellular antioxidant enzymes and intake of dietary antioxidants may help to maintain an adequate antioxidant status in the body. In the past decades, new molecular techniques, cell cultures, and animal models have been established to study the effects and mechanisms of antioxidants on ROS/reactive nitrogen species.

In the Ayurvedic system of medicine, the flowers of BM commonly known as "flame of the forest" are used for relieving burning sensation, treatment of gout, leprosy, and other skin diseases (Burli and Khade, 2007). In Unani system of medicine, BM flower extract is used as an aphrodisiac, expectorant, tonic, and diuretic and in biliousness, and it is an important component of the Indian medicinal system (Burli and Khade, 2007). The flowers of this plant are rich in flavonoids (Gupta et al., 1970; Chokchaisiri et al., 2009) and are known to have many medicinal properties (Wagner et al., 1986; Kasture et al., 2002; Burli and Khade, 2007). In the present study, we prepared an extract from BM flowers (BME) and investigated the active principles. Preparative TLC, analytical HPLC, and mass spectrometry results showed that BME was rich in flavonoids butrin, isobutrin, isocoreopsin, and butein. Earlier investigations have shown that butrin and isobutrin from BM flowers possess antihepatotoxic activity (Wagner et al., 1986; Burli and Khade, 2007), whereas butein showed estrogenic and postcoital anticonceptive activities (Chokchaisiri et al., 2009). Studies have indicated that the BM plant has great potential in treating several ailments, where free radicals have been reported to be the major factors contributing to the disorders (Lavhale and Mishra, 2007). Results of this study showed that BME and its purified fractions possess potent free radical scavenging properties. The observed antioxidant activity of BME could be due to the higher phenolic content in the extract. It is well documented that the capacity of polyphenols to act as antioxidants depends upon the number and position of hydroxyl groups and other features in their chemical structures (Heijnen et al., 2001). Structural analysis of purified BM polyphenols (Fig. 1) has shown that butrin, isobutrin, isocoreopsin, and butein contain 9, 10, 6, and 4 hydroxyl groups, respectively, resulting in their high ability to donate hydrogen atoms and to support the unpaired electron. Isobutrin and butrin, with a high number of hydroxyl groups showed potent antioxidant activity even at 1 μg/ml, perhaps because of high hydrogen-donating ability, which may nullify the effect of unpaired electrons; and these results are in agreement with previously reported results (Lavhale and Mishra, 2007). Many of the biological actions of polyphenols have been attributed to their antioxidant properties, either through their reducing capacity per se or through their possible influences on intracellular redox status (Lü et al., 2009). Here, we show for the very first time that butrin, isobutrin, and butein inhibited the PMACI-induced gene expression and production of TNF-α, IL-6, and IL-8 in human mast cells (HMC-1). Our data demonstrated that, in addition to being a potent antioxidant, isobutrin was most effective in suppressing the expression of these inflammatory mediators in HMC-1 cells. This could form the basis of a new strategy for the treatment of mast cell-mediated inflammatory diseases.

NF-κB/Rel transcription factors are present in the cytosol in an inactive state, complexed with the inhibitory IκBα proteins (DiDonato et al., 1997). Most agents, including PMACI, activate NF-κB through a common pathway based on phosphorylation-induced, proteasome-mediated degradation of IκBα, and its degradation exposes the nuclear localization signal and allows the NF-κB to translocate to the nucleus and bind the promoter of the target genes (Finco et al., 1994; Azzolina et al., 2003). The key regulatory step in this pathway involves activation of a high-molecular-weight IIKK complex, whose catalysis is generally carried out by three tightly associated IKK subunits—IKKα and IKKβ that serve as catalytic subunits of the kinase, and IKKγ that serves as the regulatory subunit (Zandi et al., 1997). Activation of IKK depends on the phosphorylation of serines 177 and 181 in the activation loop of IKKβ (serines 176 and 180 in IKKα), which causes conformational changes resulting in kinase activation (DiDonato et al., 1997). It is well documented that NF-κB specifically regulates the production of

TNF-α, IL-6, and IL-8, and patients with inflammatory diseases have higher levels of these molecules (Shin et al., 2007). In this study, we show that pretreatment of human mast cells with butrin, isobutrin, and butein decreased the PMACI-induced increase in nuclear NF-κB/p65 levels and IκBα degradation in HMC-1 cells, whereas treatment with isocoreopsin failed to block the PMACI-induced NF-κB/p65 and IκBα degradation (Fig. 4, A and B). To gain further insight into the mechanism, we used an in vitro kinase activity assay. Our results showed that butrin, isobutrin, and butein inhibited the IKKβ kinase activity in vitro, indicating that the observed inhibition of NF-κB in human mast cells may be due to the inhibition of IKK activity causing IκBα to accumulate in the nucleus. Similar results were obtained with the IKK inhibitor parthenolide, which also resulted in the accumulation of IκBα, inhibition of NF-κB, and consequent reduced mRNA expression and production of TNF-α, IL-6, and IL-8 in activated HMC-1 cells. Taken together, these results indicate that butrin, isobutrin, and butein inhibited the PMACI-induced TNF-α, IL-6, and IL-8 production via repressing the NF-κB activation in HMC-1 mast cells.

This is the first study to demonstrate that butrin, isobutrin, and butein, isolated from *Butea monosperma* flower, inhibited the PMACI-induced activation in human mast cells. The results of the present study indicate that BM polyphenols down-regulate the production of TNF-α, IL-6 and IL-8 via inhibiting the NF-κB activation by suppressing IKK activity and inhibiting IκBα phosphorylation and degradation. Butrin, isobutrin, and butein or compounds derived from them could form the basis of new strategy for the treatment of mast cell-mediated inflammatory diseases.

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