RESEARCH ARTICLE

3,5-Bis[4-(diethoxymethyl)benzylidene]-1-methyl-piperidin-4-one, a Novel Curcumin Analogue, Inhibits Cellular and Humoral Immune Responses in Male Balb/c Mice

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Abstract: ***Background***: 3,5-Bis[4-(diethoxymethyl)benzylidene]-1-methyl-piperidin-4-one (BBP), a novel synthetic curcumin analogue has previously been shown to manifest potent immunosuppressive effects on the *in vitro* phagocytosis process of human neutrophils.

Objective: In the present study, BBP was investigated for it's *in vivo* innate and adaptive immune responses mediated by different humoral and cellular immune factors.

Methods: Male Balb/c mice were orally fed with BBP (5, 10 and 20 mg/kg) for a period of 14 days and immunized with sheep red blood cells (sRBC) on day 0 for the determination of adaptive responses. The effects of BBP on phagocytosis process of neutrophils isolated from blood of treated/untreated animals were determined. The ceruloplasmin and lysozyme serum levels and myeloperoxidase (MPO) plasma level were also monitored. The mechanism was further explored by assessing its effects on the proliferation of T and B lymphocytes, T-lymphocytes subsets $CD4^+$ and $CD8^+$ and on the secretion of Th1/Th2 cytokines as well as serum immunoglobulins (IgG, IgM) and delayed type hypersensitivity (DTH) reaction.

Results: BBP showed a significant dose-dependent reduction on the migration of neutrophils, Mac-1 expression, phagocytic activity and reactive oxygen species (ROS) production. In comparison to the sensitized control group, a dose-dependent inhibition was observed on lymphocyte proliferation along with the downregulation of effector cells expression and release of cytokines. Moreover, a statistically significant decrease was perceived in serum levels of ceruloplasmin, lysozyme and immunoglobulins and MPO plasma level of BBP-treated mice. BBP also dose-dependently inhibited sheep red blood cells (sRBC)-induced swelling rate of mice paw in DTH.

Conclusion: These findings suggest the potential of BBP as a potent immunosuppressive agent.

1. INTRODUCTION

The immune system is the body's diverse and prevalent defense systems which keep the integrity as well as defend human body from pathogenic assault. This system incorporates specific cells and molecules (producing an immune response), aimed to provide protection against infections [1]. Among the two major subdivisions of the immune system, the adaptive immune system is antigen-specific and upholds the specific feature of memory cells. This system involves the production of antigen-specific antibodies, which act by eliminating pathogens in the late phase of infection. On the contrary, a number of responses mediated by the innate immune system are constitutively present and ready to attack the invading pathogen [2]. These two systems are further composed of different cellular and humoral components, the combined

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action of which produces the strong immune response against infections. Phagocytes like neutrophils and macrophages are among the major cellular components of the innate immune system while the humoral barriers to infections include different enzymes and complement system [3, 4]. In a similar context, the adaptive immune system produces cellular immunity mediated by T cells while the humoral immunity is mediated by B cells. Although these two arms of defense system have independent functions, yet components of one system influence the function of other and vice versa [5, 6]. The homeostasis within the body of a healthy organism is maintained by the requisite balance of the immune system and its cells. An abnormal immune system results upon failing to sustain the balance, leading to impaired immune response. This explains the reason that most of the immune system disorders cause abnormally low activity (immune deficiency diseases) or overactivity of the immune cells (autoimmune diseases) [1].

Curcumin is the main ingredient of *Curcuma longa* and being multi-therapeutic agent has gained extensive economic value and pharmacological application as an antioxidant, anti-inflammatory, antitumor and chemoprotective agent [7]. Notwithstanding, curcumin's shortcoming *i.e.* low aqueous solubility, chemical instability, extreme biotransformation upon oral administration and limited systemic distribution hampers it's *in vivo* application and results in reduced biological activity [8]. Consequently, efforts have been directed to synthesize analogues of curcumin with similar safety profile but increased biological activity and solubility.

Ensuing the strategy of structural modification, previously we synthesized different novel series of α, β-unsaturated carbonyl-based compounds including curcumin and chalcone analogues. Among these compounds, a synthetic curcumin analogue namely 3,5-bis[4-(diethoxymethyl)benzylidene]-1-methyl-piperidin-4-one (BBP) significantly inhibited acetylcholinesterase and butyrylcholinesterase enzymes along with the repression of enzymes of arachidonic acid metabolism pathway [9, 10]. Recently, we have reported that BBP was a potent immunosuppressive agent as it has remarkably inhibited the *in vitro* phagocytosis process by granulocytes [11]. In continuing the efforts, the ongoing work was designed to investigate the immunosuppressive potential of the aforesaid potent novel synthetic compound on the eclectic parameters of non-specific and specific cellular and humoral immunity in male Balb/c mice.

2. MATERIALS AND METHOD

2.1. Chemicals and Reagents

Innovative Research, Inc. (Sarasota, FL, USA) supplied Lymphoprep™ gradient (1,077 mg/mL), concanavalin A (Con A), fluorescein isothiocyanate (FITC)-labeled opsonized *Escherichia coli*, phosphate-buffered saline (PBS) tablet, Hank's balance salt solution (HBSS), Roswell Park Memorial Institute (RPMI)-1640, penicillin, streptomycin, lipopolysaccharide (LPS), trypan blue reagent and sheep red blood cells (sRBC). FBS was purchased from PAA Laboratories (GE Healthcare, Little Chalfont, UK). Different types of conjugated antibodies like allophycocyanin (APC)-conjugated anti-mouse CD8 (0.2 mg/mL), APC-H7-conjugated CD4 (0.2 mg/mL), FITC-A conjugated CD11b

(0.5 mg/mL), PE-A conjugated CD18 (0.2 mg/mL), IgG-FITC (0.5 mg/mL) and Pharm Lyse™ lysing solution was acquired from BD Biosciences (Franklin Lakes, NJ, USA). Cyclosporin A (Cys A) (purity > 98%) was taken from Sigma (St. Louis, MO, USA). ^{3}H-thymidine, scintillation cocktail (Ultima Gold MV) were acquired from Perkin Elmer, Inc. (Waltham, MA, MA, USA). Hematoxylin and xylene were purchased from BDH (Merck KGaA, Darmstadt, Germany).

2.2. 3,5-Bis[4-(diethoxymethyl)benzylidene]-1-methyl-piperidin-4-one (BBP)

BBP has been synthesized previously by using Claisen-Schmidt condensation reaction [12]. The chemical structure of BBP is shown in Fig. (**1**). BPP was > 98% purity based on its physiochemical properties, HRESI-MS and NMR data.
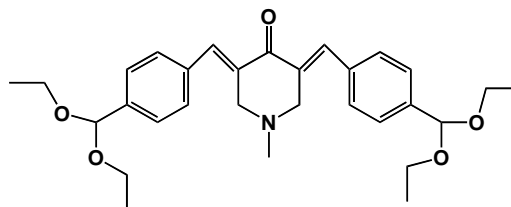
3,5-Bis[4-(diethoxymethyl)benzylidene]-1-methyl-piperidin-4-one (BBP)

Fig. (1). Chemical structure of 3,5-bis[4-(diethoxymethyl)-benzylidene]-1-methyl-piperidin-4-one.

Light yellow solid. mp: 142-144°C; ^{1}H NMR (500 MHz, CDCl$_3$) d: 7.63 (s, 2H), 7.32 (d, J ¼ 8 Hz, 4H), 7.15 (d,J ¼ 8 Hz, 4H), 5.59 (s, 2H), 3.29 (q, J ¼ 7.5, 8H), 2.71 (s, 4H), 2.12 (s, 3H), 1.20 (t, J ¼ 7.0, 12H); ^{13}C NMR (500 MHz, CDCl$_3$) d: 189.7, 148.2, 147.5, 139.8, 135.1, 128.4, 127.1, 101.4, 56.6, 51.2, 40.1, 16.7; HRESI-MS m/z: 495.2408 [M + 2H]$^+$, Microanalysis calculated for C$_{30}$H$_{39}$NO$_5$ (493.63), C: 72.99%, H: 7.96%, N: 2.84%. Found C: 72.95%, H: 7.99%, N: 2.87%.

2.3. Animals

The experimental animals used in this study (male Balb/c mice weighing 22-28 g) were supplied by the Laboratory Animal Resource Unit, Faculty of Medicine, Universiti Kebangsaan Malaysia (UKM). All methods used for animal studies were directed following a procedure permitted by the UKM Animal Ethics Committee (No. FF/2016/IBRAHIM/ 28-SEPT./780-SEPT.-2016-MAC.-2018). During the experiment, animals were housed in the aerated and temperature controlled room (22°C ± 3°C, 40 - 60 % humidity) with 12 h light/dark schedule. Standard food pellets along with water *ad libitum* were provided to the experimental animals during the whole study. The dosing volumes were changed according to the body weights monitored on regular basis. Before starting the experimental designs, animals were acclimatized for a period of one week.

2.4. Subchronic Oral Toxicity Study

The subchronic oral toxicity of BBP was investigated in male Balb/c mice (age; 10 to 12 weeks, body weight; 22-28 g) using the recommendations issued by Organization of Eco-

nomic Co-Operation and Development (OECD)(Guideline 423). Six groups, each containing six mice were randomly divided. BBP suspension was prepared by mixing BBP with sodium carboxymethyl cellulose in distilled water (1 %w/w) in a mortar. Four groups were orally fed with BBP suspension at 5, 10, 20 and 30 mg/kg body weight (BW) daily for 14 days, while the fifth group (vehicle control group) received 1% sodium carboxymethyl cellulose (NaCMC) and sixth group did not receive any treatment (normal control group). The mice were weighed frequently and observed for changes in their general behaviour, and appearance of any toxic symptoms *i.e.,* shivering, salivation, diarrhea, lacrimation, hair erection and convulsion as well as mortality and morbidity. At the end of study, animals were killed through cervical dislocation and weight of internal organs, including liver, kidney and heart were measured. Afterwards, these organs were collected for histological analysis.

2.5. Histological Analysis

The target organs collected from mice were prepared for histological analysis. The kidney, liver and heart tissue were fixed in10% formalin solution for overnight. After tissue processing, (paraffin)-embedding, and sectioning into 5 μm-thickness slices by using microtome, the sections were then stained with hematoxylin and eosin. The histological sections of aforementioned tissues were examined under Olympus light microscopy and morphological changes in all treatment groups were assessed.

2.6. Experimental Design

The study was conducted in three different experimental designs to determine eclectic parameters of non-specific and specific cellular and humoral immunity in Balb/c mice. After acclimatization of one week, the mice were separated into six groups arbitrarily with six animals per group. The first group received a regular diet (normal control group). The animals in vehicle control group were fed with 1% NaCMC in distilled water whereas third group received a known immunosuppressant, cyclosporin A (Cys A) which was given by oral gavage at a dose of 20 mg/kg BW (positive control group). For the first experimental design, the animals of group 4, 5 and 6 received BBP suspended in 1% w/w NaCMC in distilled water at doses of 5, 10 and 20 mg/kg BW, respectively, for 14 days. On day 15, the animals were sacrificed to collect blood samples in order to appraise the effects of BBP on the phagocytosis process by neutrophils and to determine serum levels of lysozyme and ceruloplasmin and plasma MPO (myeloperoxidase) levels. In the second experimental design, on day 0 of treatment, the mice were immunized intraperitoneally with sheep red blood cells, 5.0×10^9/mL (a T-cell-dependent antigen) as an antigenic stimulus. Prior to immunization, the sRBCs were resuspended in PBS after washing with cold PBS. The animals in group 4, 5 and 6 received BBP at aforementioned doses over a period of 14 days. At the end of the study, the mice were humanly killed, and spleen was collected for the analysis of lymphocytes proliferation and T lymphocyte phenotyping. The blood samples were also taken for the assessment of cytokines serum levels by activated T-cells. In the third experimental study, the animals were also immunized with sRBCs on day 0 of treatment and were continuously fed orally with

BBP for 14 days. On day 15, the treated and untreated mice were investigated for delayed type hypersensitivity (DTH) and samples were collected for measuring serum immuno-globulins. The dose of positive control used in this study was decided based on literature evidence [13, 14] while the safe doses for the novel synthetic analogue were selected based on toxicity study assessment.

2.7. Neutrophil's Isolation from Whole Mice Blood

The neutrophils were separated from the whole blood of treated mice by ensuing the method of Kumar et al., (2010) with minor modification [15]. Briefly, an equal volume (5 mL) of whole mice blood was carefully layered on Lympho-prep gradient layer previously pipetted into a falcon tube. This two-step gradient of Lymphoprep and blood inside the falcon tube was centrifuged at a speed of $400 \times g$, 4°C for 45 min. After centrifugation, the supernatant was cast off and the pellet was mixed with a pre-warmed lysing solution for the lysis of erythrocytes. After 2-3 minute incubation, the tube was again centrifuged at $200 \times g$ 4°C for 5 min. The supernatant formed was discarded and the pellet was resuspended in PBS. Finally, the cells were viewed under an inverted light microscope (Olympus ck 30, Tokyo, Japan) and calculated by using standard trypan blue method.

2.8. Cell Migration Assay

The inhibitory effect of BBP on chemotactic movement of neutrophils was measured by using a modified method of Ilangkovan *et al*. (2015) [16]. The neutrophils isolated from the non-immunized whole mice blood were calculated and to1.5 x 10^6 cells/ mL (final concentration). Cytoselect 24-well cell migration assay kit (Cell Biolabs, Inc. (San Diego, CA, USA) was used to perform the experiment. Initially, a total of 500 μL RPMI media comprising of chemotactic stimulant, fetal bovine serum (10%) was pipetted into the lower chambers while 300 μL of cell suspension in serum-free media was pipetted into the upper chamber of migration assay plate. Afterwards, the plate was kept for 2.5 h at 37°C in a humidified atmosphere (5% CO_2 incubator) to allow the cells to migrate towards the chemoattractant. After the specified time interval, the inserts were shifted to the new wells containing cell detachment solution to remove the adhered cells from the bottom side and again incubated at 37°C (5% CO_2) for 30 min to remove cells from the bottom of inserts. Finally, the inserts were discarded and dislodged cells were stained by adding the lysis buffer/CyQuant® GR dye solution. A fluorescence plate reader was used to analyse the results.

2.9. CD11b and CD18 Integrin Expression Assay

The Mac-1 expression assay was conducted as previously described by Ahmad et al. (2015) with minor changes [17]. LPS (3.5 μg/mL) was added to 100 μL of neutrophils cell suspension and incubated in CO_2 incubator (Shel Lab; Sheldon Manufacturing, Inc., Cornelius, OR, USA) at 37°C for 30 min. Thereafter, the tubes were simultaneously placed on ice and 10 μL of CD11b-FITC-A, CD18-PE-A were added to the mixture. Meanwhile, IgG- FITC was added as a negative control and tubes were incubated on ice for 1 hr. Afterwards, the tubes were centrifuged at a speed of 250 $\times g$

for 10 min and the supernatant was castoff while retaining the cell pellet. Finally, the cells within the pellet were washed twice with PBS at the same speed. The inhibition of adhesion molecule expression was analyzed by flow cytometer.

2.10. Phagocytic Assay

The phagocytic activity assay was determined by using the Phagotest assay kit (Glycotope Technology, Berlin, Germany). The isolated neutrophils from the whole blood collected from mice (100 μL) was mixed with 20 μL of FITC *Escherichia coli* and kept in water bath at (37°C) for 10 min. Thereafter, the samples were immediately transferred on ice to stop the process of phagocytosis. Subsequently, 100 μL of quenching solution was added which suppressed fluorescence of the bacteria to bind to the outside of the cell. The sample tubes were vortexed well and centrifuged at 250 ×g for 5 min after adding 3 mL of washing solution. The samples were then washed again and centrifuged at 250 ×g for 5 min (4°C). Finally, 200 μL of DNA staining solution was pipetted into each tube and vortexed. The phagocytic activity was examined by flow cytometer (BD FACS Canto, BD Biosciences) and indicated as (%) phagocytizing neutrophils.

2.11. Phagoburst Assay

The quantitative determination of oxidative burst of leukocytes isolated from the whole blood of Balb c/mice was investigated by using Phagoburst kit (Glycotope Technology, Germany). The assay was performed using manufacturer's guidelines. In brief, to trigger the cells, the neutrophils suspension (100 μL) was mixed with 20 μL of precooled *E. coli* and kept in a water bath at 37°C. After 10 min of incubation, 20 μL of substrate solution, dihydrorhodamine (DHR 123) was pipetted into respective tubes to determine the percentage of phagocytic cells producing reactive oxidants (conversion of DHR 123 to R 123 and their enzymatic activity amount of R 123 per cell). and incubated for another 10 min at 37°C. Lastly, 200 μL of DNA staining solution was added and cells were analysed by flow cytometry (BD FACS Canto, BD Biosciences).

2.12. Plasma Level of Myeloperoxidase Enzyme

Myeloperoxidase (MPO) level in the plasma was measured by using Mouse MPO ELISA kit (Abcam Biotechnology Company, Cambridge, UK) and the experiment was performed by using the manual guidelines. The whole blood collected from euthanized mice was centrifuged at $2000 \times g$ for 5 min at 4°C and plasma was separated. Briefly, 100 μL of each standard and sample was pipetted into the wells and incubated for 2.5 h at 37°C with gentle shaking. After the specified period, the wells were washed and pipetted with 100 μL of 1X prepared biotinylated antibody. After one hr, the process of washing was repeated. Finally, after adding 100 μL of prepared HRP- Streptavidin solution, 100 μL of TMB substrate reagent was put into each well and kept at 37°C for 30 min protected from light. In the end, 50 μL of Stop Solution was added to each well and a microplate reader was used to measure the plasma level of MPO.

2.13. Analysis of Serum Levels of Lysozyme and Ceruloplasmin

The effect of BBP on serum level of lysozyme and ceruloplasmin was determined by using a commercially available kit (Wuhan Biotech Co., Ltd, Wuhan, China). The whole mice blood was centrifuged at 5000 rpm for 20 min at 4°C to separate the serum. The collected serum was kept at 20°C till the experiment was done. The assay was conducted using manufacturer's guidelines.

2.14. Spleen Cell Suspension Preparation

To investigate the effects of synthetic analogue on lymphocyte proliferation and expression of effector cells, spleen cell suspension was prepared. From the animals immunized with 5.0×10^9 sRBCs/mL intraperitoneally, the spleen was removed aseptically and the splenocyte suspension was prepared using the method of Wang *et al.* (2013) with minor modification [18]. Briefly, after being humanly killed, the abdominal cavity of animals was cleaned with 70% alcohol swabs and an incision was made to remove the spleen. The spleen was immediately kept into 50 mL conical tube previously filled with cold PBS. Afterwards, before cutting the spleen into small pieces, it was positioned onto a cell strainer (70 μm). By using the plunger at the end of a syringe, the small pieces of spleen were pushed through the strainer and the cells were then washed with enough PBS. After centrifugation at a speed of $300 \times g$ for 5 min, the upper layer was removed and the cell pellet was resuspended in 2 mL pre-warmed Pharm Lyse lysing solution and kept for 3 min at 37°C. The washing step was again repeated at $250 \times g$ for 5 min. The top layer was aspirated and the cells were resuspended in 1 mL of complete medium (RPMI-1640). By using the standard trypan blue reagent, the viability of cells was assessed.

2.15. T and B Lymphocyte Proliferation Assay

The proliferative response of lymphocytes was assessed by the method of Varalakshmi *et al.* (2008) [19]. LPS and Con A at different concentrations (5 and 10 μg/mL, respectively) were used to stimulate the splenocytes. Concisely, 4×10^5 cells/mL of spleen cell suspension in RPMI-1640 complete medium were incubated with or without aforesaid mitogens in 96-well plates (200 μL/well) in 5% CO_2 (Shel Lab; Sheldon Manufacturing, Inc., Cornelius, OR, USA) at 37°C for a duration of 48 h. After 48 h, ^{3}H-thymidine at a concentration of 0.5 μCi/well was incorporated in all wells and incubated for 24 h. Upon completion of the incubation period, by using a Nunc cell harvester, the cells were harvested. Afterwards, 5 mL of scintillation fluid was added to determine the thymidine incorporation by a liquid scintillation counter (Perkin Elmer, Inc. Waltham, MA, USA). The data was represented as the stimulation index (SI), by applying the following formula:

SI = Average cpm with stimulation × 100

Average cpm without stimulation

2.16. T Lymphocyte Phenotyping Assay

To differentiate the T lymphocytes subset population, specific antibodies *i.e.* APC-conjugated CD8 for identifying CD8+ T-cells and APC-H7-conjugated CD4 for identifying

CD4+ T-cells were used (San Jose, CA, USA). With slight modification, the method previously explained by Gupta et al. (2006) was employed [20]. Briefly, after the lysis of RBCs by Pharm Lyse lysing solution, splenocytes cell suspension (1×10^6 cells/mL) was prepared. Thereafter, 50 μL of prepared cell suspension was added into the labeled 15 mL falcon tubes. Then 10 μL of APC-conjugated anti-mouse CD8 and APC-H7-conjugated anti-mouse $CD4^+$ antibodies were added into the respective tubes. The tubes were then incubated on ice for 30 min. After the specified time interval, the cell suspension was washed by using cold PBS (2 mL) and centrifuged at $250\times g$ for 5 min. Lastly, the supernatant was removed and the pellet was resuspended in 300 μL of PBS. The analysis was performed with multicolor flow cytometry using Cell Quest Pro Software and the results were expressed in percentage of $CD4^+$ and $CD8^+$ expression.

2.17. Th1/Th2 Cytokines Production

To evaluate the effect of synthetic analogue on Th1/Th2 cytokines in sensitized Balb/c mice, the serum was separated from whole blood collected from treated/untreated mice by centrifuging it at $2,000\times g$ for 20 min. The commercially available ELISA kits (R&D Systems Abingdon Science Park, UK) were used to determine sera levels of Th1 (IL-2, IFN-γ) and Th2 (IL-4) cytokines. Briefly, 50μL of standard, control and serum samples were added in a microplate precoated with the antibodies specific for particular Th1/Th2 cytokines and incubated for 2 h at 37°C. Subsequently, following the washing steps, 100 μL of cytokine specific conjugate was added into the wells. After 2 h of incubation, substrate solution (100 μL) was added and lastly, stop solution was pipetted into the wells. A microplate reader supplied by Thermos Fisher Scientific was used to measure the absorbance at 450 nm within 30 min of last step performed.

2.18. Delayed-type Hypersensitivity Reaction (DTHR)

The footpad reaction was performed using a modified method illustrated by George *et al.* (2011) [21]. On day 0 of treatment, all experimental animals were injected intraperitoneally with 200 μL sRBC (5×10^9 cells/mL). On day 14, the thickness of hind footpad of all experimental animals was recorded using digital Plethysmometer (Ugo Basile, USA). The right hind footpad's thickness was considered as control whereas, the left hind footpads of the animals were challenged by injecting 20 μL of sRBC (5×10^9) intradermally. The thickness of foot pad was again recorded after 24 h, and the changes between the paw thickness (right and left paw) were indicated as footpad reaction.

2.19. Measurement of Serum IgG and IgM Levels in Response to Sheep Red Blood Cells

Anti-sRBC IgG and IgM ELISA kit (Life Diagnostics, West Chester, US) were used to detect the serum level of anti-sRBC IgG and IgM. The experiment was conducted using the guidelines provided in the manual. Experimental animals (six groups with six animals each) were immunized by administering intraperitoneally 5×10^9 sRBC/mL in 200 μL on day 0 of the experiment (Ray and Dittel, 2010) [22]. Mice were orally fed with 5, 10 and 20 mg/kg of BBP for 14 days. The blood samples were withdrawn from all mice on day 8 to estimate the level of IgM antibody, and for IgG antibody levels, the blood samples were again taken from all animals on day 14. Serum was rapidly separated by centrifugation at $3500 \times g$ for 10 min at 4°C and stored at -20°C until used.

2.20. Statistical Analysis

All the results were analysed by using Graph Pad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). One-way analysis of variance (ANOVA) with post-Dunnett's test was employed to compare treated samples with a single control. All the values were expressed as mean ± standard deviation (SD) of six values. $p < 0.001$, $p < 0.01$, and $p < 0.05$ were considered to be statistically significant.

Fig. (2). Body weight of control and BBP (5, 10 and 20 and 30mg/kg) treated Balb/c mice during observation period. Results are presented as mean ± SD, with n=6 in each group. ***$P<0.001$; **$P<0.01$; *$P<0.05$, significantly different from the control group.

3. RESULTS

3.1. Subchronic Oral Toxicity

The alterations in body and organ weights along with the gross necropsy of body tissues is generally used to indicate the toxic effects of substances on animal and human bodies. In this study, male Balb/c mice were used to perform subchronic oral toxicity study of the novel synthetic compound, BBP. The compound at first three doses (5, 10, and 20 mg/kg) was well tolerated by all animals with no mortality or morbidity during the observation period. The color of hair, skin, oral activity, teeth, and eyes of treated mice and control were usual and no eye secretion, edema or salivation was observed. Similarly, the light reaction and behaviour activities of the mice were normal. As shown in Fig. (**2**), after treatment, the body weight as well as organ's weight of the treated mice among three groups (5, 10, and 20 mg/kg) increased steadily but were not significantly different as compared to the control mice. Contrary to this, the animals in the fourth group (30

Fig. (3). Histopathological examination (H&E, 10X) of heart, liver and kidney tissues of experimental animals. Normal control **(a, g, m)**, vehicle control **(b, h, n)**, 30 mg/kg **(c, i, o)**, 20 mg/kg **(d, j, p)**, 10 mg.kg **(e, k, q)**, 5 mg/kg **(f, l, r)**. (*); presence of tissue debris, (◄►); diameter of Bowman's capsule, (→); spotty necrosis.

mg/kg) witnessed significant signs of toxicity. Fig. (**2**) clearly showed the remarkable loss of weight in animal's body from 24.4 ± 2.0 g on 0 days to 15.2 ± 2.1 g on day 14. In addition, the animal's behaviour in this group was found to be abnormal throughout the study and feces were full of pus or mucus. Therefore, the presented results clearly indicated that BBP at concentrations of 5, 10 and 20 mg/kg was entirely safe for use in this study.

3.2. Effect of BBP on Histology of Heart, Liver and Kidney Tissues

Histological evaluation was performed to demonstrate the differences of treated tissue compared to normal tissue. Fig. (**3**) demonstrated that the gross necropsy findings of heart tissue treated by 30 mg/kg displayed extensive vacuolization and major myofibrils loss compared to normal heart tissue. It has been clearly observed by the presence of tissue debris (*). Besides, for other treatment groups, no major changes on vacuolization and no myofibrils loss was observed compared to normal heart tissue. In addition, in control group, the histopathology of the liver revealed a normal distribution of hepatocytes with clear visible nuclei, a portal triad and central vein. For the 30 mg/kg treatment, massive necrosis of centrolobular was clearly visualized inside and surrounding the terminal hepatic venule (THV) of the affected liver. Besides, the dilatation of central vein was significantly observed in 30 mg/kg group when compared with control and other groups. The nuclei of hepatocyte and hepatocytes sheets were also not clearly visualized at many areas of the liver. A sign of spotty necrosis (black arrow) in the liver explain the progression of liver tissue toxicity. However, the liver section of other treatment groups demonstrated similar aforementioned characteristics but gradually deficit when reaching a higher concentration of treatment (5-20 mg/kg). Correspondingly, prominent changes were observed on the Bowman capsule as well as convoluted tubules

in the kidney of 30 mg/kg treatment group. As shown in Fig. (**3**), the diameter of convoluted tubules was greatly increased compared with control and other treatment groups. Besides, the image suggesting the patchy necrosis of tubules or diffuse revelation of the renal tubular cells with flattening of cells because of tubular dilation (orange arrows); the cast formation within the tubules (yellow arrows); and sloughing of cells resulting in granular casts formation (red arrow). Finally, the blockage within the tubules due to the uncovered epithelium and cellular debris is visible (green arrow); and the reorganization of intercellular adhesion molecules cause the denuded tubular epithelial cells to lump together.

3.3. Effect of BBP on Neutrophil Migration

Cell migration assay was performed to explore the potential of BBP on leukocytes migration. 10% FBS served as a chemotactic stimulus. As illustrated in Fig. (**4**), a dose-dependent suppression of directional movement of neutrophils was seen i.e. by increasing the dose; reduced number of cells migrated. However, the *ex vivo* chemotactic migration inhibition of neutrophils was found to be significantly high ($p < 0.01$) at the highest dose of 20 mg/kg which was comparable to the positive control, cys A.

3.4. Effect of BBP on Mac-1 Expression

The regulation of CD11b/CD18 (Mac-1) surface expression is recognized imperative for adhesion and extravasation of leukocytes [23]. Fig. (**5**) depicted that the neutrophils purified from the blood of BBP treated mice followed a dose-dependent manner of inhibition of percentage expression of CD11b/CD18. However, in comparison to the untreated group, the inhibitory effects were found to be significant at the highest dose (20 mg/kg). Further, no substantial effects were reported at a dose of 5 mg/kg when compared with control (Table **1**).

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Fig. (4). Effect of BBP (5, 10 and 20 mg/kg) on the chemotaxis of neutrophils isolated from experimental animals. Results are represented as mean ± SD, with n=6 in each group. ***$P<0.001$; **$P<0.01$; *$P<0.05$, significantly different from the control group. **Abbreviations:** NSC; non sensitized control, VHC; vehicle treated control.

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Fig. (5). Flow cytometric evaluation of the effect of BBP (5, 10 and 20 mg/kg) on Mac-1 expression in neutrophils isolated from experimental animals. **Abbreviations:** NSC; non sensitized control, VHC; vehicle treated control, FITC-A; Fluorescein isothiocyanate-A, PE; phyco-erythrin.

3.5. Effect of BBP on Phagocytic Activity of Neutrophils

The capacity of isolated neutrophils from the treated and untreated groups of animals to engulf opsonized bacteria was investigated by using flow cytometer. The phagocytic activity was measured by comparing the percentage of phagocytosis with the reduction in the percentage of *E. coli* engulfment by phagocytic cells. The results obtained showed that the administration of BBP at high doses of 10 and 20 mg/kg caused the significant inhibition of bacteria engulfment by neutrophils in comparison to the control group ($p < 0.01$). Notably, mild phagocytic inhibitory activity was observed at a dose of 5 mg/kg. The potential effects of BBP on the neutrophil's phagocytic activity are shown in Table **1** and Fig. (**6**).

Table. 1. **Effect of various concentration of BBP on PMN Mac-1 expression activity (%), Phagocytic activity (%) and Phagoburst activity (%).**

Group	Dose (mg/kg)	% Activity		
		% Mac-1 Expression	**% Phagocytic Activity**	**% Phagoburst Activity**
NSC		83.49 ± 4.2	74.48 ± 3.6	83.14 ± 3.7
VHC		82.59 ± 3.4	74.20 ± 4.1	84.75 ± 4.6
BBP	5	81.55 ± 3.9	$70.25 \pm 3.7*$	80.69 ± 3.5
BBP	10	78.52 ± 5.8	$67.53 \pm 4.4**$	$77.45 \pm 4.4*$
BBP	20	$76.45 \pm 3.2*$	$63.13 \pm 3.3**$	$72.21 \pm 4.0**$
Cys A	20	$64.52 \pm 4.5***$	$48.29 \pm 5.6***$	$66.23 \pm 3.2***$

NSC, non-sensitized control; VHC, vehicle treated control; Cys A, cyclosporin A.

The values are expressed as the mean $\pm$ SD, with n=6 in each group. *** $P < 0.001$; **$P < 0.01$; *$P < 0.05$, significantly different from control group.

Fig. (6). Flow cytometric evaluation of the effect of BBP (5, 10 and 20 mg/kg) on phagocytic ability of neutrophils isolated from experimental animals. **Abbreviations:** NSC; non sensitized control, VHC; vehicle treated control, FITC-A; Fluorescein isothiocyanate-A.

3.6. Effect of BBP on Phagoburst Activity of Neutrophils

To appraise the effects of BBP on oxidative burst, the separated neutrophils from untreated and treated mice were incubated with bacteria. The phagoburst activity is expressed by the percentage of cells producing reactive oxygen radicals. The results obtained from flow cytometry (Table **1**, Fig. **7**) revealed that mice treated at doses of 5, 10 and 20 mg/kg exhibited a decrease in the percentage of leukocyte oxidative burst in a dose-dependent manner. Similar to Mac-1 expression, the optimal percentage of phagoburst activity was found at the highest dose of BBP and it was found comparative to the positive control, Cys A-treated group.

3.7. Effect of BBP on Plasma Level of Myeloperoxidase Enzyme

The myeloperoxidase enzyme level was determined by separating the plasma from the whole blood of control and treated mice and it was seen that all treated groups exhibited significant dose-dependent suppression of plasma MPO levels *i.e.* increase in the doses of BBP led to an increase in the inhibition of MPO levels in the plasma (Fig. **8**). Although a significant level (p < 0.001) of inhibition was seen at the highest doses of 20 and 10 mg/kg.

3.8. Effect of BBP on Serum Levels of Ceruloplasmin and Lysozyme

The *in vivo* immunomodulatory effects of BBP on serum level of lysozyme and ceruloplasmin was investigated in this study and the results are shown in Fig. (**9**). The obtained data depicted that the treatment of Balb/c mice with BBP (5-20 mg/kg) resulted in a significant (p< 0.001) inhibition of serum lysozyme in contrast to control. As shown in the figure, among all the tested doses, the best effects were noted at a dose of 20 mg/kg and comparable to that of the positive

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Fig. (7). Flow cytometric evaluation of the effect of BBP (5, 10 and 20 mg/kg) on oxidative burst by neutrophils isolated from experimental animals. **Abbreviations:** NSC; non sensitized control, VHC; vehicle treated control, FITC-A; Fluorescein isothiocyanate-A.

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decompressor
are needed to see this picture.

Fig. (8). Effect of BBP (5, 10 and 20 mg/kg) on plasma MPO level in treated Balb/c mice. Results are presented as mean ± SD, with n=6 in each group. ***$P<0.001$; **$P<0.01$; *$P<0.05$, significantly different from the control group. **Abbreviations:** NSC; non sensitized control, VHC; vehicle treated control.

control. For both lysozyme and ceruloplasmin, it can be clearly seen that the serum level was suppressed in a dose-dependent manner. Though for serum level of ceruloplasmin, compared to the control, mild inhibitory effects were seen at doses of 5 and 10mg/kg and more pronounced suppression was observed in the animals with 20 mg/kg treatment group.

3.9. Effect of BBP on Splenocyte Proliferation

Fig. (**10**) depicts the anti-proliferative activity of BBP. The obtained results revealed that the synthetic analogue potentially inhibited mitogen-induced splenic lymphocyte proliferation and this inhibition was found to follow a dose-

dependent manner. The reduced proliferation of lymphocytes from mice spleen treated with the synthetic compound was significant at doses of 10 and 20 mg/kg for both T and B-cells and it was comparable with the control group.

3.10. Effect of BBP on Lymphocyte Phenotyping

Based on the expression of surface markers, the splenic lymphocytes of treated mice were differentiated into T helper cells (CD4$^+$) and T suppressor cells (CD8$^+$), using flow cytometry. Splenocytes isolated from treated (BBP) and untreated mice (control group), immunized with sheep red blood cells were stained with specific conjugated antibodies and analyzed by flow cytometry. The results obtained are shown in Table **2**. The data revealed that in comparison to sensitized control (immunized with sRBC), the percentage expression of CD4+ and CD8$^+$ was significantly and dose-dependently suppressed at graded doses. However, the maximum inhibition by BBP was seen on the percentage of CD4 expression at 20 mg/kg dose and it was also found comparable to that of the Cys A, the immunosuppressant drug at a dose of 20 mg/kg. The immunosuppressive potential of our compound was strengthened by the inhibition of CD4$^+$, CD8$^+$ expression which was found more noticeable for CD4$^+$ T cell subset at the highest dose.

3.11. Effect of BBP on Serum Level of IL-2, IL-4 and IFN-γ

The effects of BBP at specified doses were investigated on Th1 and Th2 cytokines production by commercially available ELISA assay kit. The obtained results revealed that in comparison to the sensitized control group (immunized with sRBC), the sera level of IL-2 were significantly reduced and comparable to the Cys A-treated group at first two doses i.e. 20 and 10 mg/kg while for the third dose (5 mg/kg), less inhibition of serum level IL-2 was observed as shown in Fig. (**11**). On the contrary, the figure presented that the sera

Table 2.   Effect of various concentration of BBP on CD4$^+$ and CD8$^+$ expression (%) in spleen cells.

Group Dose (mg/kg)		T-cell Subset Detection (%)	
		CD4$^+$	CD8$^+$
SC	-	21.03 ± 2.6	30.62 ± 4.1
VHC	-	20.67 ± 3.2	30.03 ± 2.8
BBP	5	18.45 ± 3.9	28.08 ± 3.0
BBP	10	17.27 ± 4.3*	26.40 ± 3.7*
BBP	20	14.33 ± 4.6***	25.47 ± 4.7***
Cys A	20	10.63 ± 4.5***	16.13 ± 4.2***

SC, sensitized control; VHC, vehicle treated control; Cys A, cyclosporin A.

The values are expressed as the mean ± SD, with n=6 in each group. *** $P < 0.001$; **$P < 0.01$; *$P < 0.05$, significantly different from the control group.

Fig. (9). Effect of BBP (5, 10 and 20 mg/kg) on the serum lysozyme and ceruloplasmin level in treated Balb/c mice. Results are presented as mean ± SD, with n=6 in each group. *** $P<0.001$; **$P<0.01$; *$P<0.05$, significantly different from the control group. **Abbreviations:** NSC; non sensitized control, VHC; vehicle treated control.

Fig. (10). Effect of BBP (5, 10 and 20 mg/kg) on proliferation of T and B lymphocytes. Results are presented as mean ± SD, with n=6 in each group. *P* value; ***$P<0.001$; **$P<0.01$; *$P<0.05$, significantly different from the control group. **Abbreviations:** SC; sensitized control, VHC; vehicle treated control.

Fig. (11). Effect of BBP (5, 10 and 20 mg/kg) on the serum level of IL-2, IL-4 and IFN-γ from the experimental animals. Results are presented as mean ± SD, with n=6 in each group. ***$P<0.001$; **$P<0.01$; *$P<0.05$, significantly different from the control group. **Abbreviations:** SC; sensitized control, VHC; vehicle treated control.

Fig. (12). Effect of BBP (5, 10 and 20 mg/kg) on the serum level of IgG and IgM from the experimental animals. Results are presented as mean ± SD, with n=6 in each group. ***$P<0.001$; **$P<0.01$; *$P<0.05$, significantly different from the control group. **Abbreviations:** SC; sensitized control, VHC; vehicle treated control.

level of Th1 cytokine, INF-γ were found to be significantly suppressed at all the doses when compared to the sensitized control group. At the highest dose, 20 mg/kg, the inhibition of serum level of IL-4 was significantly higher than in the sensitized control group whereas, reduced inhibition activity was measured at the remaining two doses.

3.12. Effect of BBP-treated Mice on Delayed-type Hypersensitivity (DTH) Response

DTH response to sRBC was calculated as the measure of paw edema thickness (mm) for 5, 10 and 20 mg/kg BW of

each animal after treatment with BBP and compared with the sensitized control (immunized with sRBC). To evaluate the DTH reaction, the peak oedema at 24 h was taken as a parameter. Previously it was reported that sRBC caused an increase in the paw thickness within 18-24 h [24]. Correspondingly, in this study, an enhanced paw thickness was examined in the untreated group while the immunized treated groups that were administered with BBP at graded doses were found to elicit substantial inhibition in paw oedema as illustrated in Table **3**. Furthermore, among the three doses, significant suppression ($p > 0.01$) in paw volume was observed at 20 and 10 mg/kg groups.

Table 3.　Effect of BBP on Delayed Type Hypersensitivity response in Balb/c mice.

Paw Thickness (mm) Group 24 h Oedema		% Inhibition in Paw Oedema
SC	0.64 ± 0.25	-
VHC	0.68 ± 0.60	-
BBP (5mg/kg)	0.60 ± 0.73	6.25*
BBP (10mg/kg)	0.56 ± 0.97	12.50**
BBP (20mg/kg)	0.54 ± 0.86	15.62**
Cys A (20mg/kg)	0.37 ± 0.64	42.18***

SC, sensitized control; VHC, vehicle treated control; Cys A, cyclosporin A.

The values are expressed as the mean ± SD, with n=6 in each group; *p* value; *** $P < 0.001$; **$P < 0.01$; *$P < 0.05$, significantly different from the control group.

3.13. Effect of BBP on Serum Level of IgG and IgM Antibodies

In response to a T-cell dependent antigen (sRBC), the serum of treated mice was detected for IgG and IgM levels. The experiment was performed using the technique of ELISA. Data is shown in Fig. (**12**). The levels of immunoglobulins IgM and IgG were appreciably reduced dose-dependently in the sera of mice treated with BBP when compared to control. However, mild to weak inhibitory activity was detected for IgG antibody at a dose of 10 and 5 mg/kg, respectively, while a significant decrease ($p < 0.01$) in anti-SRBC IgM and IgG levels was noticed in the sera of mice treated with high dose as compared to the antibody levels in the sera of sensitized control group.

5. DISCUSSION

The defense system of humans is multifarious, the function of which involves an appropriate interaction among the immune cells to maintain the normal homeostasis. The modulation of immune responses is required upon the imbalance of the defense system [25]. It is well established that α, β-unsaturated carbonyl moieties are recognized as the reactive structures of synthetic molecules as well as natural products and hence exhibit miscellaneous pharmacological activities i.e., a potent antioxidant, anti-inflammatory, antibacterial, antitubercular and immunomodulatory properties [9, 12]. In our previous study, we investigated a series of α, β unsaturated carbonyl based compounds on the phagocytic function of neutrophils as an important indicator of innate immunity. Our results revealed that among 41 novel compounds, BBP showed potent inhibitory activity on the migration of neutrophils, *E. coli* engulfment, Mac-1 expression, and ROS production by human leukocytes [11]. The existing work was designed to explore the effects of BBP on the specific and nonspecific immunity parameters using male Balb/c mice. Our data indicated that the novel synthetic α, β unsaturated carbonyl based compound possessed great potential as a promising immunosuppressive agent. Indeed, the data acquired from the present *in vivo* work was found analogous with the data gathered from *in vitro* study. In this study, the oral administration of the synthetic curcumin analogue in

male Balb/c mice at three doses (5, 10 and 20 mg/kg) for 14 days was found safe since no toxic symptoms and abnormal behavior was witnessed. However, evident by the gross necropsy of the tissues (liver, kidney and heart) and remarkable loss of body weight manifested by treated animals, the dose of 30 mg/kg was not continued further in this *in vivo* study.

In the first experimental design, we investigated the nonspecific immune responses mediated by different cellular and humoral components of innate immune system. CD11b/ CD18 (Mac-1) is one of the most copious integrins on neutrophils and the adhesion of leukocytes to the vascular endothelium along with the subsequent steps involved in the resistance to microbes requires the adhesion of β2 integrin to the ligands. The downregulation of β2 integrin expression has also been associated with the progression of deadly systemic inflammatory disorders resulting in organ damage [26]. For the significant reduction of Mac-1 expression percentage compared with the control group, it is assumed that BBP may cause changes in the confirmation of Mac-1 complex because, upon the stimulation of neutrophils, CD11b/ CD18 move to the cell surface probably through conformational alterations [27]. Also, the inhibition may be due to altered protein kinase C (PKC), which plays a pivotal role in β2 integrin regulation and cell migration [28]. Correspondingly, this study demonstrated significant inhibitory effects of BBP on the movement of neutrophils towards the chemotactic stimuli as compared to control. The downregulation of Mac-1 could be one of the possible mechanism for the inhibition of FBS-induced chemotaxis (*ex vivo*) of neutrophils isolated from mice pretreated with BBP as chemoattractant triggered neutrophil's chemotaxis is mediated by Mac-1 [29, 30].

Neutrophils are multifunctional cells of innate immunity and after being accumulated at the site of infection, phagocytose the attacking microorganisms followed by the release of superoxide radicals and other secondarily derived ROS for the prompt killing of bacteria [31]. The immunomodulatory effects of BBP on the phagocytic capacity as well as subsequent oxidative burst by neutrophils in our ex vivo study may be due to the inhibition of Mac-1 expression that causes the antibodies against the complement surface receptors, CR3 on neutrophils to repress pathogens binding to phagocytic cells [32]. Another study described that suppression of Mac-1 results in inadequate ROS release degranulation in peritonitis models, as well as reduced binding, also causes abnormal phagocytosis of complement-opsonized particles [33].

Myeloperoxidase enzyme (MPO) being part of innate immunity plays an important role in cellular homeostasis and impaired levels cause instigation and development of serious and long-lasting inflammatory disorders. This enzyme is amply expressed in neutrophils and serves as a microbicidal agent by producing ROS that subsidizes the demolition and extermination of ingested microbes [34]. MPO bind with integrin exposed on neutrophil's surface which effectively causes the neutrophil's activation by boosting p38 mitogen-activated protein kinase (MAPK) phosphorylation [35]. The inhibition of MPO could be attributed to the inhibition of Mac-1, as the downregulation of Mac-1 suppress the sequential phagocytosis process and release of toxic radicals and enzymes.

Lysozyme has a central part in the nonspecific humoral immune mechanism which is produced by different phagocytic cells [36] and has the ability to stimulate phagocytes and trigger the complement system through its bactericidal and opsonin effects to prevent different infections. The potential inhibitory effects of BBP at the indicated doses on the serum level of lysozyme could possibly be due to inhibition of phagocytosis process as the aforesaid enzyme is released during the process of phagocytosis [37]. Furthermore, TNF-α stimulated raised level of lysozyme by phagocytic cells could also be suggested as one of the possible mechanism exerted by BBP [36]. Ceruloplasmin has been expressed as an imperative element of cell-mediated immunity and known to engage in the antioxidant activity. The repression of ceruloplasmin sera levels of experimental animals' dose-dependently could be attributed to the suppression of certain proinflammatory cytokines like TNF-α, IL-1, and IL-6 which cause the release of ceruloplasmin [38].

In the second experimental study, all the experimental animals were immunized with sRBC and the inhibitory potential of BBP at varying doses was assessed on the immune specific cellular and humoral responses. The suppression of T cell-mediated immune functions occupies a key position in the pathological process of different chronic inflammatory disorders. In our study, the antiproliferative action of BBP may be due to the inhibition of enzymes (ribonucleotide reductase and DNA polymerase) involved in process of DNA synthesis or it may have repressed the growth of cells during the S phase of cell cycle [39].

The immunosuppressive potential of BBP was strengthened by the inhibition of CD4, a CD8 expression which was found more noticeable for CD4+ T-cell subset at the highest dose (20mg/kg) and our results corroborated previously reported work [40]. CD4+ and CD8+ lymphocytes, owing to their specific membrane molecule are different functionally as well as phenotypically and have the ability to modify the immune responses by regulating the release of different cytokines [41].

T cell-mediated immunological responses were further explored by cytokines production; the altered levels of these cytokines occupy central part in the pathogenesis of many chronic inflammatory disorders and allergic reactions [42]. TNF-α is usually associated with Th1 subset of lymphocytes and involves in cell-mediated inflammatory and other immune processes. Whereas, Th2 lymphocytes secrete IL-4 and IL-5 cytokines particularly linked with humoral immunity as they cause B cells to proliferate and differentiate [43]. In our study, the potential reduction of cytokines production may be one of the promising mechanisms for inhibition of lymphocytes as mentioned in section 3.9. In addition, it has been documented that production of IL-12 is one of the major mechanism that affects the cytokine release in CD4+ cells [44].

DTH reaction particularly appears after the second contact with the antigen where T –helper cells cause the release of different immune cells and cytokines including IFN-γ, IL-2 responsible for inflammation [45]. The possible mechanism for significant immunosuppressive activity at the cellular immunity level could be due to the inhibition of T lymphocytes activation and the resultant release of cytokines. However, it could also be assumed that the compound may have affected the release of histamine and arachidonic acid metabolism pathway involved in the progression of DTH reaction [46].

The inhibition of the humoral response against sRBCs by BBP was signified by the substantial and dose-dependent decrease in serum antibodies level in mice. Lymphocytes along with the macrophages are involved in the production of immunoglobulins. Initially, IgM antibody is produced as a primary response while IgG antibody produced few days after immunization with sRBC. Numerous studies reported that the reduction in humoral immune response could be due to suppression of antigen processing and presentation, or the inhibition of proliferation of T and B lymphocytes or might be influenced by the release of different cytokines produced upon T cell and B cell stimulation [47].

CONCLUSION

The outcome of the current study corroborated that BBP, the synthetic curcumin analogue possessing α, β-unsaturated carbonyl moiety, exhibited strong suppressive effects on the cellular and humoral components of the innate and adaptive immune system of male Balb/c mice, and highlighted its significant immunomodulatory potential. The results obtained in this *in vivo* work are incongruent with our previous *in vitro* findings which showed that BBP markedly suppressed the innate immune defence by inhibiting chemotactic migration, engulfment activity of phagocytes and suppressing the ROS production by leukocytes [11]. These results suggest that BBP has the potential of developing as an effective therapeutic agent for the treatment of immune system disorders especially autoimmune diseases like arthritis psoriasis, lupus and eczema. However, further mechanistic studies should be accomplished as well as its pharmacokinetic and pharmacodynamics studies to confirm its mechanisms of action as a potent immunosuppressive drug candidate.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethics approval has been obtained from UKM Animal Ethics Committee (No. FF/2016/IBRAHIM/28-SEPT./780-SEPT.-2016-MAC.-2018).

HUMAN AND ANIMAL RIGHTS

The Balb/c mice used in this study were supplied by the Laboratory Animal Resource Unit, Faculty of Medicine, Universiti Kebangsaan Malaysia (UKM).

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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REFERENCES

[1] Arshad, L.; Jantan, I.; Bukhari, S.N.A.; Haque, M.A. Immunosuppressive effects of natural α, β-unsaturated carbonyl-based compounds, and their analogs and derivatives, on immune cells: A review. *Front. Pharmacol.,* **2017**, 8, 22.

[2] Lee, J.S.; Bukhari, S.N.A.; Fauzi, N.M. Effects of chalcone derivatives on players of the immune system. *Drug Des. Dev. Ther.,* **2015**, 9, 4761.

[3] Haque, M.A.; Jantan, I.; Bukhari, S.N.A. Tinospora species: An overview of their modulating effects on the immune system. *J. Ethnopharmacol.*, **2017**, 20, 67-85.

[4] Yuandani, I.J.; Ilangkovan, M.; Husain, K.; Chan, K.M. Inhibitory effects of compounds from *Phyllanthus amarus* on nitric oxide production, lymphocyte proliferation, and cytokine release from phagocytes. *Drug Des. Devel. Ther.*, **2016**, 10, 1935.

[5] Wallis, R.; Mitchell, D.A.; Schmid, R.; Schwaeble, W.J.; Keeble, A.H. Paths reunited: Initiation of the classical and lectin pathways of complement activation. *Immunobiology*, **2010**, 215, 1-11.

[6] Jantan, I.; Ahmad, W.; Bukhari, S.N.A.; Plant-derived immunomodulators: An insight on their preclinical evaluation and clinical trials. *Front. Plant Sci.,* **2015**, 6, 655.

[7] Mehrotra, S.; Agnihotri, G.; Singh, S.; Jamal, F. Immunomodulatory potential of *Curcuma longa*: A review. *South Asian J. Exp. Biol.,* **2013**, 3, 299-307.

[8] Vyas, A.; Dandawate, P.; Padhye, S.; Ahmad, A.; Sarkar, F. Perspectives on new synthetic curcumin analogs and their potential anticancer properties. *Curr. Pharm. Des.*, **2013**, 19, 2047-2069.

[9] Bukhari, S.N.A.; Jantan, I.; Masand, V.H.; Mahajan, D.T.; Sher, M.; Naeem-Ul-Hassan, M.; Amjad, M.W. Synthesis of α, β-unsaturated carbonyl based compounds as acetylcholinesterase and butyrylcholinesterase inhibitors: Characterization, molecular modeling, QSAR studies and effect against amyloid β-induced cytotoxicity. *Eur. J. Med. Chem.*, **2014a**, 83, 355-365.

[10] Bukhari, S.N.A.; Lauro, G.; Jantan, I.; Bifulco, G.; Amjad, M.W.; Pharmacological evaluation and docking studies of α, β-unsaturated carbonyl based synthetic compounds as inhibitors of secretory phospholipase A 2, cyclooxygenases, lipoxygenase and proinflammatory cytokines. *Bio Org. Med. Chem.*, **2014b**, 22, 4151-4161.

[11] Arshad, L.; Jantan, I.; Bukhari, S.N.A.; Jamil, S. Inhibitory effects of α, β-unsaturated carbonyl-based compounds and their pyrazoline derivatives, on phagocytosis of human neutrophils. Med.Chem. Res, 2018, 1-12.

[12] Bukhari, S.N.A.; Tajuddin, Y.; Benedict, V.J.; Lam, K.W.; Jantan, I.; Jalil, J.; Jasamai, M. Synthesis and evaluation of chalcone derivatives as inhibitors of neutrophils' chemotaxis, phagocytosis and production of reactive oxygen species. *Chem. Biol. Drug. Des.*, **2014c**, 83, 198-206.

[13] Borel, J.F.; Feurer, C.; Magnee, C.; Stähelin, H. Effects of the new anti-lymphocytic peptide cyclosporin A in animals. *Immunology*, **1977**, 32, 1017-1025.

[14] Thomson, A.; Whiting, P.; Simpson, J. Cyclosporine: immunology, toxicity and pharmacology in experimental animals. *Agents Actions*, **1984**, 15, 306-327, 6395677

[15] Kumar, S.; Jyoti, A.; Keshari, R.S.; Singh, M.; Barthwal, M.K.; Dikshit, M. Functional and molecular characterization of NOS isoforms in rat neutrophil precursor cells. *Cytometry* A, **2010**, 77, 467-477.

[16] Ilangkovan, M.; Jantan, I.; Mesaik, M.A.; Bukhari, S.N.A. Immunosuppressive effects of the standardized extract of Phyllanthus amarus on cellular immune responses in Wistar-Kyoto rats. Drug. Des. Devel. Ther., **2015**, 9, 4917-4930.

[17] Ahmad, W.; Jantan, I.; Kumolosasi, E.; Bukhari, S.N.A. Immunostimulatory effects of the standardized extract of Tinospora crispa on innate immune responses in Wistar Kyoto rats. Drug. Des. Devel. Ther., **2015**, 9, 2961-2973.

[18] Wang, C.; Yu, X.; Cao, Q.; Wang, Y.; Zheng, G.; Tan, T.K.; Zhao, H.; Zhao, Y.; Wang, Y.; Harris, D.C. Characterization of murine macrophages from bone marrow, spleen and peritoneum. *BMC Immunol.*, **2013**, 14, 6.

[19] Varalakshmi, C.; Ali, A.M.; Pardhasaradhi, B.; Srivastava, R.M.; Singh, S.; Khar, A. Immunomodulatory effects of curcumin: In-vivo. Int. Immunopharmacol., **2008**, 8, 688-700.

[20] Gupta, A.; Khajuria, A.; Singh, J.; Bedi, K.; Satti, N.; Dutt, P.; Suri, K.; Suri, O.; Qazi, G. Immunomodulatory activity of biopolymeric fraction RLJ-NE-205 from *Picrorhiza kurroa*. *Int. Immunopharmacol.*, **2006**, 6, 1543-1549.

[21] George, A.K.; Patrick, A.; Terrick, A. Immunomodulatory and erythropoietic effects of aqueous extract of the fruits of *Solanum torvum Swartz* (Solanaceae). *Pharmacogn. Res.*, **2011**, 3, 130-134.

[22] Ray, A.; Dittel, B.N. Isolation of mouse peritoneal cavity cells. *J. Vis. Exp.*, **2010**, 35, 1488.

[23] Hofman, P.; Piche, M.; Far, D.F.; Le Negrate, G.; Selva, E.; Landraud, L.; Alliana-Schmid, A.; Boquet, P.; Rossi, B. Increased *Escherichia coli* phagocytosis in neutrophils that have transmigrated across a cultured intestinal epithelium. *Infect. Immun.*, **2000**, 8, 449-455.

[24] Rondinone, S.N.; Giovanniello, O.A.; Barrios, H.A.; Nota, N.R. Effect of fractional cyclophosphamide dosage on sheep red blood cell-delayed-type hypersensitivity response in mice. *J. Immunol.*, **1983**, 4, 1600-1603.

[25] Haque, M.A.; Jantan, I.; Arshad, L.; Bukhari, S.N.A. Exploring the immunomodulatory and anticancer properties of zerumbone. *Food Funct.*, **2017**, 83410-83431.

[26] Koenderman, L.; Van Der Linden, J.A.; Honing, H.; Ulfman, L.H. Integrins on neutrophils are dispensable for migration into three-dimensional fibrin gels. *Throm. Haemost.*, **2010**, 104, 599-608.

[27] Yoshida, N.; Yoshikawa, T.; Tanaka, Y.; Fujita, N.; Kassai, K.; Naito, Y.; Kondo, M. A new mechanism for anti- inflammatory actions of proton pump inhibitors-inhibitory effects on neutrophil-endothelial cell interactions. *Aliment. Pharmacol. Ther.*, **2000**, 14, 74-81.

[28] Fogh, B.S.; Multhaupt, H.A.; Couchman, J.R. Protein kinase C, focal adhesions and the regulation of cell migration. *J. Histochem. Cytochem.*, **2014**, 62, 172-184.

[29] Coxon, A.; Rieu, P.; Barkalow, F.J.; Askari, S.; Sharpe, A.H.; Von Andrian, U.H.; Arnaout, M.A.; Mayadas, T.N. A novel role for the β2 integrin CD11b/CD18 in neutrophil apoptosis: A homeostatic mechanism in inflammation. *Immunity*, **1996**, 5, 653-666.

[30] Ley, K.; Laudanna, C.; Cybulsky, M.I.; Nourshargh, S. Getting to the site of inflammation: The leukocyte adhesion cascade updated. *Nat. Rev. Immunol.*, **2007**, 7, 678-689.

[31] Haugland, G.T.; Jakobsen, R.A.; Vestvik, N.; Ulven, K.; Stokka, L.; Wergeland, H.I. Phagocytosis and respiratory burst activity in lumpsucker (*Cyclopterus lumpus* L.) leucocytes analysed by flow cytometry. *PloS One*, **2012**, 7, e47909.

[32] Burg, N.D.; Pillinger, M.H. The neutrophil: Function and regulation in innate and humoral immunity. *Clin. Immunol.*, **2001**, 99, 7-17.

[33] Olza, J.; Aguilera, C.M.; Gil-Campos, M.; Leis, R.; Bueno, G.; Martínez-Jiménez, M.D.; Valle, M.; Cañete, R.; Tojo, R.; Moreno, L.A. Myeloperoxidase is an early biomarker of inflammation and cardiovascular risk in prepubertal obese children. *Diabetes Care*, **2012**, 35, 2373-2376.

[34] Kim, E.K.; Choi, E.-J. Pathological roles of MAPK signaling pathways in human diseases. *BBA Mol. Basis Dis.*, **2010**, 1802, 396-405.

[35] Yamamoto, Y., Gaynor, R.B. Therapeutic potential of inhibition of the NF-κB pathway in the treatment of inflammation and cancer. *J. Clin. Investig.*, **2001**, 107, 135-142.

[36] Rodríguez, M.C.; Rivas, G.A. Label-free electrochemical aptasensor for the detection of lysozyme. *Talanta*, **2009**, 78, 212-216.

[37] Ragland, S.A.; Criss, A.K. From bacterial killing to immune modulation: Recent insights into the functions of lysozyme. *PLoS pathogens*, **2017**, 13, e1006512.

[38] Wang, H.; Wang, M.; Chen, J.; Tang, Y.; Dou, J.; Yu, J.; Xi, T.; Zhou, C. A polysaccharide from *Strongylocentrotus nudus* eggs protects against myelosuppression and immunosuppression in cyclophosphamide-treated mice. *Int. Immunopharmacol.*, **2011**, 11, 1946-1953.

[39] De La Fuente, M.; Victor, V. Anti- oxidants as modulators of immune function. *Immunol. Cell Biol.*, **2000**, 78, 49-54.

[40] Luckheerman, R.V.; Zhou, R.; Verma, A.D.; Xia, B. CD+ T cells: Differentiation and functions. *Clin. Dev. Immunol.*, **2012**, *2012*, 925135.

[41] Kang, B.; Song, Y.; Kim, K.M.; Choe, Y.; Hwang, S.; Kim, T.S. Curcumin inhibits Th1 cytokine profile in CD4+ T cells by suppressing interleukin- 12 production in macrophages. *Br. J. Pharmacol.*, **1999**, *128*, 380-384.

[42] Stamm, C.; Barthelmann, J.; Kunz, N.; Toellner, K.M.; Westermann, J.; Kalies, K. Dose-dependent induction of murine Th1/Th2 responses to sheep red blood cells occurs in two steps: Antigen presentation during second encounter is decisive. *PloS One*, **2013**, *8*, e67746.

[43] Marshall, J.D.; Secrist, H.; Dekruyff, R.H.; Wolf, S.F.; Umetsu, D.T. IL-12 inhibits the production of IL-4 and IL-10 in allergen-specific human CD4+ T lymphocytes. *J. Immunol.*, **1995**, *155*, 111-117.

[44] Farrar, J.D.; Asnagli, H.; Murphy, K.M. T helper subset development: roles of instruction, selection, and transcription. *J. Clin. Investig.*, **2002**, *109*, 431-435.

[45] Von Bubnoff, D.; Geiger, E.; Bieber, T. Antigen-presenting cells in allergy. *J. Allergy Clin. Iimmunol.*, **2001**, *108*, 329-339.

[46] Benacerraf, B. Opinion: A hypothesis to relate the specificity of T lymphocytes and the activity of I region-specific Ir genes in macrophages and B lymphocytes. *J. Immunol.*, **1978**, *120*, 1809-1812.

[47] Amir, G.Z.; Azad, M.A.; Javidniac, K. Haussknechtia elymatica: A plant with immunomodulatory effects. *Iran J. Immunol.*, **2007**, *4*, 26-31.