Antioxidant, Anti-inflammatory, and Anti-arthritic Effect of Thymoquinone-rich Black Cumin (*Nigella sativa*) oil (BlaQmax®) on Adjuvant-induced Arthritis

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Abstract

Rheumatoid Arthritis (RA) is a complex autoimmune disorder involving chronic and persistent inflammation, principally influencing the synovial joints which further prompting the obliteration of articular cartilage. Although black cumin (*Nigella sativa*) oil has already studied for its anti-arthritic properties, the current study was focused on the comparative evaluation of the antioxidant and anti-inflammatory properties of a thymoquinone (TQ)-rich (5% w/v) black cumin oil (BQ) with the commonly available standard black cumin oil (BM) containing 0.4% (w/v) TQ, and subsequent investigation on the potential application of BQ in the management of RA. Adjuvant-induced arthritis (AA) was instigated by a single intradermal infusion of 0.1 mL of Complete Freund's adjuvant (CFA) on the paw of adult Wistar rats. Based on the primary dose-response study using the carrageenan-induced paw edema model, 50 mg/kg b.wt. of BQ was employed for the treatment. The endogenous antioxidants (SOD, Catalase, GPx, and GSH), pro-inflammatory cytokines (COX-2, Nitrate, iNOS, TNF-α, IL-6), lipid peroxidation, and histopathology were evaluated to monitor the influence of BQ in AA rats. Adjuvant-induced animals showed a critical downregulation in antioxidant status with elevated levels of pro-inflammatory cytokines and lipid peroxidation. But, the treatment with BQ significantly reversed the antioxidant and inflammatory markers with downregulation of the pro-inflammatory gene expressions. Histopathology showed a significant reduction in the massive cell infiltration and epidermal edema of the paw tissue in AA rats when administered with BQ and indicated its potential effect to alleviate RA conditions in experimental rats.

Keywords: adjuvant-induced arthritis, antioxidants, black cumin, black seed, *Nigella sativa*, thymoquinone

1. Introduction

Inflammation is a complex pathological and physiological process that occurs with numerous specific and varied molecular signals produced by the macrophages, leukocytes, mast cells, and by the activation of complement factors (Noris & Remuzzi, 2013). Rheumatoid Arthritis (RA) is a complex autoimmune disorder that predominantly affects skeletal joints and cartilage tissues. Approximately 1% of world population has been diagnosed with arthritic conditions with a male/female ratio of 3:1 (Brennan-Olsen et al., 2017; Briggs et al., 2017). Chronic synovial inflammation is a prominent indication of RA, mainly characterized by joint swelling and narrowing of joint space, causing severe pain (Sokolove & Lepus, 2013). The pathogenesis of arthritis has shown to involve extensive proliferation of the cells of the synovial tissue and lesions in the articular cartilage with irregular surface erosions, decreased thickness, leukocyte infiltration, and pannus formation along with the involvement of free radicals (Man & Mologhianu, 2014). The inflammatory mechanism has been identified as the main pathway leading to the progression and destruction of the joints in RA (Guo et al., 2018).

Like in every other inflammatory or autoimmune disorder, the oxidative stress generated by the ROS (Reactive Oxygen Species) has its critical role in the progression of RA (Tan et al., 2018). The prominent biomolecules of the body such as proteins, lipids, and DNA were found to encounter devastating damages by ROS (Ogawa et al., 2013). As the system encounters this drastic attack, the endogenous antioxidants such as SOD, CAT, GPx, and...
GSH were drastically downregulated with significant elevation of key inflammatory markers such as TNF-α (tumor necrosis factor-α), IL-1 and IL-6 (interleukins), inflammatory enzymes like iNOS (inducible nitric oxide synthase) and COX-2 (cyclooxygenase-2) produced by the T cells (Li et al., 2018). A significant elevation in the enzymes of the tissues such as matrix metalloproteinase (MMPs) has also been observed due to cartilage damage (Rose & Kooyman, 2016).

NSAIDs (Non-Steroidal Anti-Inflammatory Drugs) and DMARDs (Disease-Modifying Anti-Rheumatoid Drugs) have been prominently using in the treatment of RA, albeit a specific treatment protocol for its cure is yet to be defined (van den Berg et al., 2011). On the other hand, the side effects of these drugs cause major limitations when considering their usage for a longer duration. Recently, Nutraceuticals have been proven to be a more reliable alternative, primarily due to their safety and cost-efficiency. *Nigella sativa*, a medicinal herb and culinary spice commonly known as black cumin or black seed has been extensively used in the traditional systems of treatment of several diseases including the common cold, headache, asthma, rheumatic diseases, and cancer (Mollazadeh & Hosseinazadeh, 2014). The oil of black cumin, mainly produced by cold-pressing process, has been shown to possess antioxidant, anti-inflammatory, analgesic, antipyretic, anti-asthmatic, anti-hypertensive, antimicrobial, and anti-neoplastic effects (Yimer et al., 2019; Ahmad et al., 2013; Forouzanfar et al., 2014). Nasuti et al., 2019 have reported the anti-artheritic potential of black cumin oil in Complete Freund’s adjuvant (CFA)-induced arthritic rats when treated with a high dosage of 1596 and 798 mg/kg b.wt. Arjumand et al., (2019) showed significant attenuation of inflammatory markers, along with the downregulation of mRNA expression levels of TLR's, interleukins, and NF-kB on CFA-induced RA rats when treated with black cumin oil. Moreover, Faisal et al. have reported the anti-artheritic effect of thymoquinone on pristine-induced arthritic rats at 2 mg/kg b.wt. with a significant reduction of paw histopathology, synovial cytology, hyperplasia, paw volume, paw weight, and TLC reduction (Faisal et al., 2015; Faisal et al., 2018).

Human clinical studies have also revealed the anti-artheritic potential of *N. sativa*. The placebo-controlled anti-artheritic study conducted by Hadi et al., 2016 in female patients suffering from RA reported significant decrease in DAS28 (Disease Activity Score-28) when supplemented with *N. sativa* oil capsules at a dose of 1g/day for 8 weeks. Similar studies by Kheirouri et al., 2016 and Gheita et al., 2011 also reported potential anti-artheritic effects of *N. sativa* with a significant decrease in DAS 28, percentages of CD8+, swollen joints, WBC count, duration of morning stiffness, andVAS (Visual Analog scale) score compared to placebo when supplemented at a dose of 1 g/day. Their studies have also pointed out a considerable increase in levels of regulatory T cell percentage, CD4+/CD25+, and CD4+/CD8+ ratio. However, the dosage as high as 3 g/day for powder and 10 mL/day for oil has been reported as tolerated and free of other side effects (Z. Gholamnezhad et al., 2016).

The majority of pharmacological properties of black cumin seed and extract have been ascribed to its most abundant constituent, thymoquinone (2-isopropyl-5-methyl-1,4-benzoquinone)’ (Amin & Hosseinazadeh, 2016). Thymoquinone (TQ) levels in commonly available cold-press extract may range from 0.1 to 0.5% (w/v) depending on the quality and geographical location of black cumin. BlaQmax® (BQ) utilized in the current investigation is a proprietary black cumin extract containing 5% (w/v) of TQ produced from Indian black cumin seeds by a cold-pressing method. Since BQ contains around 10-fold TQ concentration than the common cold-pressed extract, we hypothesized that BQ may provide better efficacy at a relatively lower dosage. Subsequently, the current investigation was focused on the relative examination of a thymoquinone (TQ)-rich (5% w/v) black cumin extract (BQ) with the commonly available standard black cumin extract (BM) containing 0.4% (w/v) TQ to investigate the role of TQ in the anti-inflammatory potential and antioxidant properties of black cumin extract and its conceivable viability in the regulation of RA utilizing CFA-induced arthritis model of rats.

2. Materials and Methods

2.1 Materials and Reagents

Standard black cumin extract containing 0.4% (w/v) of thymoquinone (BM) and BQ containing 5% (w/v) of thymoquinone prepared by the proprietary process of cold-pressing process were obtained as a gift from Akay Natural Ingredients, Cochin, India. Analytical grade chemicals and biochemical agents were obtained from Merck, Mumbai, India, and Sigma-Aldrich, MO, USA. All the antibodies, ELISA, RNA isolation, and RT-PCR kits were purchased from Sigma-Aldrich, MO, USA. Kit for measuring plasma CRP (C-reactive proteins) level was purchased from M/s Diasys Diagnostics GmbH, Germany.

2.2 Animals

Wistar rats (Male, weighing 150 ± 10 g) were allocated into different groups (Carrageenan and Adjuvant induced) with 6 animals per group. All animals utilized in the assessment were raised in the host animal facility and
maintained with constant temperature (24–26°C), humidity (55–60%), and 12:12 h light-dark cycle- photoperiod. Commercial balanced laboratory diet (Amrut Laboratory Animal Feeds, Maharashtra, India) and regular water were given as often as necessary. The animals received compassionate care, consistent according to institutional animal ethics guidelines. All assessments were driven by the standard procedure and guidelines of the Animal Ethics Committee CPCSEA (Registration No. CAF/Ethics/446) Government of India.

2.3 Experimental Design

2.3.1 Carrageenan-induced Acute Inflammatory Model

The mitigating anti-inflammatory action of black cumin extracts was estimated using the carrageenan-induced acute inflammatory model (Winter et al., 1962). Sixteen groups of rats were subjected to intraperitoneal administration of various doses (2, 2.5, 5, 10, 25, 50, 75, 100 mg/kg b.wt.) of BM and BQ and standard medication diclofenac (DIC) (3 mg/kg b.wt.). Following 60 minutes, 0.1 mL of 1% carrageenan (an edematogenic agent) suspension in 0.9% NaCl solution was infused into the sub-plantar tissue of the right hind paw. The paw volume was estimated by a Plethysmometer. The paw measurements were analyzed at the 0th hour (Vo: before edematogenic agent injection) and 1st, 2nd, 3rd, 4th and 5th hour post-administration time points (Vt). The contrast between Vt and Vo was taken as the edema value. The level of percentage of inhibition was then dictated by the equation:

\[
\% \text{ inhibition} = \left( \frac{V_t - V_o}{V_t} \right)_{\text{control}} \times 100
\]

2.3.2 Adjuvant-induced Chronic Arthritic Model

The experimental animals were segregated into 4 groups as follows: Normal control rats (NC), Adjuvant-induced arthritic control rats (AA), AA+ BQ, and AA+ Indomethacin (INDO). Right hind paw of all the animals except normal control rats (NC) were immunized with 0.1 mL CFA consisting of heat eliminated Mycobacterium tuberculosis in paraffin emulsion. Two of these groups were administrated orally with BQ (50 mg/kg b.wt.) and INDO (3 mg/kg b.wt.) in normal saline. The experiment was conducted within a tenure of 21 days. Animals were kept fasted overnight and euthanized to collect tissues. On the following day, tissue collected from treated paws were analyzed, fixed in 10% formalin buffer, and subjected to decalcification for 7 days in 20% EDTA solution. Later the tissues were instilled in paraffin for histopathological analysis. Blood parameters and biochemical analysis were also assessed.

2.4 Analytical Procedures

2.4.1 Assay of Superoxide Dismutase (SOD)

Superoxide dismutase (SOD) was analyzed according to the SOD activity determination protocol by Kakkar et al., (1984). Tissue samples were homogenized in 0.25M sucrose solution and the homogenate was subjected to centrifugation. The reaction blend containing of 1.2 mL sodium pyrophosphate, 0.1 mL phenazinemethosulphate, 0.3 mL of nitroblue tetrazolium and 0.2 mL NADH was diluted with 3 mL of distilled water and 0.2 mL NADH was added to trigger the reaction cascade. Samples were incubated at 30°C for 90 sec. and immediately after incubation, the reaction was stopped by adding 1 ml glacial acetic acid. 4 mL n-butanol was added to the reaction blend and vigorously mixed. This blended mixture was permitted to settle for about 10 min and afterward subjected to centrifugation. The butanol layer was collected cautiously and the color intensity of the chromogen in butanol fraction was estimated at 560 nm against blank butanol.

2.4.2 Assay of Catalase Activity (CAT)

Catalase activity (CAT) was estimated following the standardized protocol of Aebi, (1984). Homogenized paw tissue was subjected to centrifugation and the supernatant was collected for further analysis. About 1.995 mL of 50 mM phosphate buffer (pH 7.0) was taken into a tube and added to 5 μL of tissue supernatant. 1.0 mL freshly prepared 30 mM H₂O₂ was added to initiate the test reaction H₂O₂ deterioration in the samples was estimated by spectrophotometry at 240 nm absorbance.

2.4.3 Assay of Glutathione Peroxidase (GPx)

The activity of glutathione peroxidase (GPx) was estimated by the protocol of Agergaard et al., (1982). To a 1-mL cuvette, 0.890 mL of 100 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM NaN₃, 0.2 mM NADPH, 1 U/mL glutathione reductase and 1 mM glutathione were added. Total volume was made up to 0.9 mL with paw tissue homogenate. 100 μL of 2.5 mM H₂O₂ was added to initiate the test reaction, and NADPH transformation to NADP⁺ was estimated by measuring the absorbance for 3 min spectrophotometrically. The activity of GPx was expressed as n/moles of NADPH oxidized to NADP+/min/mg protein, utilizing the molar
extinction coefficient of 6.22×10^6 (cm^-1 M^-1) for NADPH.

2.4.4 Estimation of Glutathione (GSH)

Glutathione content (GSH) was assessed according to the strategy depicted by Benke & Murphy (1974). Sample tissues were homogenized in 5 mL of precipitating solution and subjected for 5 minutes’ incubation at room temperature. Incubated homogenate was filtered through course grade filter paper and the collected filtrate was used in further analysis. Test reaction system contained 3 mL of 0.3 M phosphate solution and 1 mL of 0.04% (5,5-dithio-bis-(2-nitrobenzoic acid) DTNB in 0.2 mL filtrate. The reaction mixture in tubes was capped and blended carefully by inversion. GSH content was estimated by spectrophotometric analysis, rapidly within 4 minutes at 412 nm.

2.4.5 Estimation of Lipid Peroxidation- Malondialdehyde (MDA)

The extent of tissue lipid peroxidation was estimated by measuring malondialdehyde (MDA). Paw tissue samples were homogenized at 1:9 ratio (1g tissue in 9 ml 1.15% KCl) and the homogenate was subjected to centrifugation at 3000 rpm for 5 min. The supernatant was carefully collected and the MDA was estimated by the protocol of Ohkawa et al., (1979). The test solution was prepared by the addition of 0.2 mL of tissue homogenate, 0.2 mL of 8.1% SDS, 1.5 mL of 20% acetic acid (pH 3.5), and 1.5 mL of the aqueous solution of TBA and made up to 4 mL and samples are kept in a boiling water bath at 90°C for 1 hour. The reaction mixture was then cooled under tap water, followed by the addition of 1 mL of distilled water and 5 mL of n-butanol: pyridine reagent. Tube contents were vigorously mixed well and subjected to 10 min centrifugation at 4000 rpm. The supernatant was collected and the pink color was read at 532 nm against blank butanol.

2.4.6 Determination of Nitrite

Nitric oxide (NO) levels were estimated by the Griess reaction method (Grisham et al., 1996). The rapid conversion of NO to nitrite and nitrate in the presence of H2O2 was measured. Equivalent volumes of paw tissue supernatant and Griess reagent (1% sulphanilamide and 0.1% N-[naphthyl]ethylenediamine dihydrochloride: 1:1) was blended to initiate the reaction. The absorbance of the product formed was estimated by spectrophotometric analysis at 550 nm. The measure of nitrite was determined from a NaNO2 standard curve.

2.5 RT-PCR Analysis of Inflammatory Markers

Total RNA was isolated from paw tissues using a total RNA isolation kit (Sigma Aldrich, MO, USA) according to the manufacturer's directions. Oligonucleotides for rat IL-6, TNF-α, iNOS, COX-2, and GAPDH were designed using primer designing tool Primer-BLAST and as follows:+

<table>
<thead>
<tr>
<th>Inflammatory markers</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>5’CCACCTGCTTCCCTACTTCA3’</td>
<td>5’TGTCCTCTAGCCACACTTCTC3’</td>
</tr>
<tr>
<td>COX-2</td>
<td>5’-ATCTGCGCTGCTTGGTCAATG-3’</td>
<td>5’-CAATCTGCTGAGGGAACACA-3’</td>
</tr>
<tr>
<td>iNOS</td>
<td>5’-CAGCACAGAGGGCTCAAGC-3’</td>
<td>5’-TGTCGCCAGCTTTCTTCT-3’</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5’GTCGTAAACCACCAAGC3’</td>
<td>5’GACTCCAAAGTAGAAGCTTGGC3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5’CCTGCTTACCACCTTCTTGG3’</td>
<td>5’ATCCACCATCACCATCTCCAG3’</td>
</tr>
</tbody>
</table>

Reverse transcription and DNA amplification steps were performed independently in two-step RT-PCR using an Eppendorf thermocycler. In the initial reaction, about 5μL of Total RNA was utilized for cDNA synthesis as a template. Along with template, dNTPs, oligo (dT) and reverse transcriptase enzyme were added to an RNase free tube. The subsequent steps included the addition of appropriate forward and reverse primers, RT-PCR premix with the enzyme, synthesized cDNA, and dNTPs. Amplified PCR products were isolated and separated by agarose gel electrophoresis on 1.5% agarose gel containing ethidium bromide at ~50V, and the gel was then visualized by an E-gel imager. Band intensities were estimated from the obtained gel images through densitometry. GAPDH is used as a standard against the PCR products of corresponding samples.

2.6 Activity of Total-COX in Paw Tissue

Total-Cyclooxygenase activity (COX) was measured by the technique portrayed by Shimizu et al., (1981). Paw tissue samples incubated with Tris–HCl buffer (pH 8), 5 mM glutathione, and 5 mM hemoglobin for 1 min at 25°C. The reaction was initiated by the addition of 200 μM arachidonic acid to the reaction mixture followed by incubation of samples at 37°C for 20 min. After incubation, the reaction was terminated by adding 10% trichloroacetic acid in 1N hydrochloric acid to the reaction mixture. All the samples were subjected to centrifugation and separation followed by the addition of 1% thiobarbiturate. The activity of COX was dictated
by spectrophotometric analysis at 530 nm absorbance.

2.7 Blood Parameters – WBC Count and CRP Level

The whole blood collected from the animal was immediately transferred into EDTA tubes at room temperature and centrifuged for 10 min at 2000 rpm. Serum was kept at -80°C for further analysis. Leukocytes (WBCs) count was determined by a hemocytometer. C-reactive protein (CRP) was estimated with standard assay kit (Diasys Diagnostic Systems GmbH, Germany).

2.8 Histopathological Analysis of Paw Tissue

Whole paw tissue was collected from the rats after 21 days of CFA immunization and treatment with BQ. Specimen were quickly sectioned out at a thickness of 5 µm and were fixed in a 10% buffered solution of formalin. Later the paraffin-embedded tissue segments were deparaffinized and stained with hematoxylin-eosin (H&E) for histopathology. Samples were then analyzed and captured under a light microscope (Labomed LX300) for evaluating basic anomaly, morphological changes, and inflammatory cell infiltration. Histopathologic assessment of paw tissue aggravation and inflammation was determined in a protocol blinded experiment by two different spectators.

2.9 Assay of Protein

The concentration of total protein in each sample was determined by the methodology of Lowry et al., (1951). The color variance exhibited by the samples in proportion to the protein concentration was estimated by spectrophotometric analysis at 660 nm.

2.10 Statistical Analysis

All the experimental data were investigated utilizing the analytical statistical program SPSS/PC+, variant 11.0 (SPSS Inc., Chicago, IL, USA). One-way ANOVA was utilized for the examination trial of critical contrasts among groups. Pair-fed correlations between the groups were evaluated by Duncan’s multiple range tests and the differences at p < 0.05 was considered statistically significant.

3. Results

3.1 Effect of BM and BQ on Carrageenan-induced Paw Edema Rats (Dose-response Study)

Percentage inhibition of edema following the different doses (2, 2.5, 5, 10, 25, 50, 75, 100 mg/kg b.wt.) of oral administration of BM and BQ in carrageenan-induced acute paw edema model of rats is given in Figure 1. At 50 mg/kg b.wt., BQ exhibited a potent inhibitory effect as compared to BM. BQ even exhibited a comparatively higher anti-inflammatory effect than the i.p. administrated standard drug diclofenac at 10 mg/kg b.wt.

![Figure 1. Effect of BM and BQ on carrageenan-induced paw edema rats (dose-response study)](image)

Values expressed as mean ± SD, m=3 with six rats in each group.

3.2 Effect of BQ on Adjuvant-induced Arthritic Rats

Paw volume of all the CFA-induced arthritic control rats (AA) was remarkably increased as compared to the normal untreated control rats. The paw volume was maximum on day 7 in all AA rats and the treatment with BQ notably (p< 0.05) reduced the paw volume, corresponding to inhibition of 69, 75, and 80% respectively on the 7th, 14th, and 21st day. The percentage of inhibition by BQ was higher than the standard drug indomethacin treated rats on day 21 (Table 2).
Table 2. The percentage inhibition of paw volume by BQ (adjuvant model)

<table>
<thead>
<tr>
<th>Groups</th>
<th>% Inhibition of paw volume</th>
<th>7th day</th>
<th>14th day</th>
<th>21st day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adjuvant induced</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BQ treated (50 mg/kg b.wt.)</td>
<td></td>
<td>69±a</td>
<td>75±a</td>
<td>80±a</td>
</tr>
<tr>
<td>INDO (3mg/kg b.wt.)</td>
<td></td>
<td>37±a</td>
<td>53±a</td>
<td>72±a</td>
</tr>
</tbody>
</table>

The values expressed as mean ± SD, m=3 with six rats in each group. a-Statistical difference with the adjuvant-induced group at P<0.05

3.3 Effect of BQ on Hematological Parameters

The effects of BQ in hematological parameters of the AA rats are as shown in Table 3. The results reveal that the levels of WBC and CRP were remarkably increased (p< 0.05) in AA rats and were significantly decreased upon treatment with BQ.

Table 3. The Inhibitory Effect of BQ on the concentration of plasma CRP, WBC count

<table>
<thead>
<tr>
<th>Groups</th>
<th>WBC (Cells×10³/mL)</th>
<th>CRP (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>3.6±0.02</td>
<td>51±0.32</td>
</tr>
<tr>
<td>AA</td>
<td>8.8±0.04a</td>
<td>77±0.65a</td>
</tr>
<tr>
<td>AA+BQ</td>
<td>3.9±0.01a,b</td>
<td>63±0.44a,b</td>
</tr>
<tr>
<td>AA+INDO</td>
<td>3.8±0.02a,b</td>
<td>57±0.46a,b</td>
</tr>
</tbody>
</table>

The Values expressed as mean ± SD, m=3 with six rats in each group. a-Statistical difference with the control group at P < 0.05. b- Statistical difference with adjuvant rats at P < 0.05.

3.4 Effect of BQ on Antioxidant Enzymes and Oxidative Stress

CFA-induced arthritis created significant oxidative stress as shown by the remarkable (p<0.05) reduction in the activities of SOD, GPx, and CAT levels in comparison with the normal control group (p<0.05) when the paw tissue homogenates were analyzed. However, the upregulated oxidative stress and detrimental effects caused by CFA induced arthritis were improved by treatment with BQ. The ameliorating effect of BQ (p<0.05) increased antioxidant levels; which were even higher than observed in indomethacin-induced rats (Figure 2).

![Figure 2. Effect of BQ on the activity of SOD, CAT, and GPx levels in adjuvant-induced arthritic rats](image)

The values are expressed as mean± SD, m=3 with six rats in each group. a - Statistical difference with the normal control group at P< 0.05. b – Statistical difference with adjuvant-induced rats at P< 0.05. SOD: U/mg-enzyme concentration required to inhibit chromogen production by 50% in 1 min. Catalase: U-μmol H₂O₂ decomposed/min. GPx: U-μmol NADPH oxidized/min. AA- Adjuvant-induced arthritis, INDO-Indomethacin, BQ- BlaQmax.

3.5 Effect of BQ on the Concentration of TBARS, Nitrite, and GSH

CFA-induced arthritic (AA) control rats shown to have remarkably higher (p<0.05) levels of TBARS, nitrite
concentration, and also the concentration levels of GSH was notably declined. Administration of BQ to the AA-induced group significantly reduced (p<0.05) TBARS and nitrite concentration and increased GSH levels in comparison with indomethacin-induced rats [Figure 3. (a, b, c)].

![Figure 3](http://example.com/figure3.png)

Figure 3. (a,b,c) Effect of BQ on the concentration of TBARS, nitrite, and GSH in experimental rats

The values are expressed as mean± SD, m=3 with six rats in each group. a - Statistical difference with the normal control group at P< 0.05. b – Statistical difference with adjuvant-induced rats at P< 0.05. AA-Adjuvant induced arthritis, INDO-Indomethacin, BQ- BlaQmax.

### 3.6 Effect of BQ on Pro-inflammatory Markers and Cytokines by RT-PCR.

CFA induced arthritic rats exhibited upregulated gene expression of COX-2 and iNOS. The levels of IL-6 and TNF-α were also significantly inclined in AA rats. In comparison with indomethacin-induced rats, treatment with BQ down-regulated the expressions of these pro-inflammatory markers and cytokines [Figure 4. (a, b)].
3.7 Effect of BQ on Total-COX Activity

The activity of total-COX was remarkably elevated (p<0.05) CFA induced rats in comparison with the normal control group (p<0.05). However, the elevation of COX was remarkably mitigated (p<0.05) upon treatment with BQ, as compared to rats treated with indomethacin (Figure 5).

![Figure 5. Effect of BQ on total COX activity](image)

The values are expressed as mean±SD m=3 with six rats in each group. a - Statistical difference with the normal control group at P< 0.05. b – Statistical difference with adjuvant-induced rats at P< 0.05. AA-Adjuvant induced arthritis, INDO-Indomethacin, BQ- BlaQmax.
3.8 Effect of BQ on Paw Histopathology

The indications obtained from the histopathological studies are that the inflammatory cell infiltration, changes in the smooth contour of articular cartilage with irregular surface erosions, decreased thickness and destruction of the articular cartilage, cartilage loss of basophilia, and fainting or absence of tidemark lines of calcified cartilage were markedly suppressed in AA rats administered with BQ (Figure 6).

![Histology of paw tissue](image)

Figure 6. Histology of paw tissue (H&E stain 40x)

SM- Normal thin synovial membrane, AD- Normal subintimal membrane with supporting adipose tissue, SC- Synovial cavity, MS- Mild synovitis, SH- Thickening of the subintimal membrane with synovial hyperplasia, IN- Inflammatory cell infiltration.

4. Discussion

Inflammation is a natural immune response of the body towards harmful irritants or stimuli and is determined by the type of cells involved in its pathways. It is considered acute or chronic depending on the duration of persistence (Chen et al., 2018). RA is a systemic autoimmune disorder causing synovial joint deformities, functional loss, and cartilage degradation (Yap et al., 2018). Around 20 million people around the world are estimated to be prone to RA conditions (Safari et al., 2019). The currently available medications are shown to cause a lot of adversities such as heartburn, anemia, abdominal pain, peptic ulcer, gastrointestinal bleeding, cataracts, high/low blood pressure, insomnia, muscle atrophy, osteoporosis, body weight gain, and immunity loss upon continuous usage (Bhattacharya et al., 2020; Al-Lawati et al., 2020, Fischer et al., 2020). So, safe and efficient natural agents having the ability to manage or to inhibit the progress of the RA conditions are of great significance. A significant volume of research towards the development of nutraceuticals and phytopharmaceuticals is in progress in this regard (Guo et al., 2018).

The current investigation was focused on the anti-inflammatory, antioxidant, and anti-arthritis activity of black cumin extract as a function of TQ content. The injection of 0.1 mL of a 1% carrageenan-induced acute inflammation caused significant paw edema, which showed a dose-dependent decrease when treated with both BM and BQ. This model has two distinct phases; in the initial phase immediately after 1 h of post-injection, the mast cells released serotonin and enzymes, along with an increase in the prostaglandins at the site of inflammation. Whereas in the latter part, the accumulation of polymorphic nuclear cells (PMNs) and IL-6 by the macrophages occur, eventually leading to the generation of ROS and lysosomal enzymes causing severe tissue damage (Mittal et al., 2014). Treatment with BQ administrated orally from the 8th day of CFA-injection onwards showed a remarkable suppression of paw edema (80% of inhibition) in comparison with the untreated control group and was even significantly higher than the standard drug indomethacin-induced group, by day 21. The increase of paw thickness, with swelling and redness leading to prolonged joint destruction, disability, and functional loss is an important clinical feature of RA (de Molon et al., 2016). The fact that BQ significantly inhibited the paw edema at 50 mg/kg b.wt. as compared to BM, indicates its potent anti-inflammatory effect and plausible efficiency in RA management.

The arthritis induction mechanism using CFA (Complete Freund's adjuvant) in which animals were immunized with heat-killed Mycobacterium tuberculosis (MT) particles is regarded as a highly reproducible model of RA. Further studies on Complete Adjuvant-induced Arthritis (AA) revealed a significant anti-arthritis effect of BQ, indicating its effect on chronic inflammatory RA condition. Pathophysiology of RA is characterized by the synthesized free radicals from neutrophils, NO, ROS, cytokines, and prostaglandins (Biswas et al., 2017). Biosynthesis of prostaglandins is catalyzed by the enzyme COX (cyclooxygenase) and the inhibition of COX
was found to provide relief from the inflammation and pain. Lipids of the cell membrane are oxidized by free radicals, causes the formation of lipid peroxidation (Abdulkhaleq et al., 2018). In AA rats, high levels of COX and lipid peroxidation was observed as a result of elevated oxidative stress and inflammation. But, the administration of BQ significantly impeded the deleterious effects of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8) delivered during the development of inflammation and hence to reduce PGE2. Serum levels of pro-inflammatory cytokines were also observed in RA condition, whereas the levels of anti-inflammatory cytokines (IL-4 and IL-10) are comparatively declined (Alunno et al., 2017). Moreover, the primary endogenous antioxidants SOD, Catalase, Glutathione peroxidase, and GSH are shown to beRemarkably enhanced in BQ-treated animals, leading to the obstruction of lipid peroxidation as apparent from the reduction in TBARS and nitrite levels.

Earlier studies on arthritic patients have shown that serum CRP levels and WBC are useful biomarkers for monitoring the development of inflammatory processes during the pathogenesis of RA (Sproston & Ashworth, 2018; Yap et al., 2018). This was found to agree in the present study since CRP and WBC went significantly high upon adjuvant injection and subsequently reduced to the normal range when treated with BQ indicating its bioavailability and anti-inflammatory potential. A recent investigation by Nasuti et al., 2019, has also summarized an attenuating effect of black cumin extract in arthritic-model of rats, but at a comparatively higher dose (1596 mg/kg b.wt.) of oral administration.

Major pathological markers of inflammation in RA such as inflammatory cell infiltration, the altered contour of articular cartilage with irregular surface erosions, decreased thickness and destruction of the articular cartilage, mononuclear cellular aggregations, the proliferation of collagen fibers, and fainting or absence of tidemark lines of calcified cartilage and cartilage loss of basophilia were found to be markedly higher in the histopathology analysis of paw tissues of AA rats. Treatment with BQ was found to markedly suppress these pathological markers of inflammation as evident from the suppression of reactive mesothelial cells and subsequent reduction in the swelling in hind paws and knee joints of rats; suggesting the significant attenuation of the paw pathology. Taken together, thymoquinone-rich black cumin extract (5%) (BQ) possesses a significant anti-inflammatory effect than the conventional black cumin extract oil with low levels of TQ (< 0.5%) (BM) indicating a role of thymoquinone in the anti-inflammatory effect. Further studies on CFA-induced arthritic animals have also justified the plausible efficacy of BQ in RA management, as evident from its inhibitory effect on pro-inflammatory mediators, nitrite, and lipid peroxidation and also its capacity to modulate WBC and the endogenous antioxidant systems by alleviating the oxidative stress. Further histopathology analysis also correlated the effect of BQ in RA conditions. Thus, the present results justify the use of black cumin oil in traditional medicine for decades, especially against various inflammatory diseases and BQ offers a way to make use of its potential therapeutic effects at a relatively low dosage.

Conflict of Interest

Authors disclose the conflict of interest. BQ is a thymoquinone-rich black cumin extract patented by M/s Akay Natural Ingredients, Cochin, India, and registered as BlaQmax®. M. Ratheesh, Jose P. Svenia, S. Sheethal, Rajan Sony, and S. Sandya belongs to a non-profitable academic research organization and have no conflict of interest.

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