ThymoDream™

Unlocking Nature for Deeper, More Restorative Nights

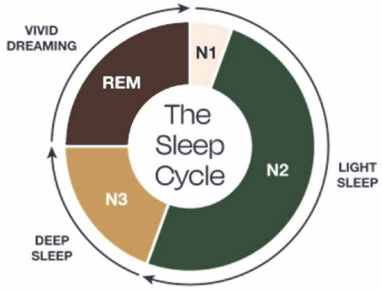
ThymoDream™

The connection between stress, sleep, and immunity is undeniable: stress disrupts sleep, and poor sleep weakens immunity. Traditional medications often cause unwanted side effects and dependency. ThymoDream™ offers a holistic, non-habit-forming, plant-based solution for enhanced overall wellness. Developed by Akay Bioactives and clinically substantiated, ThymoDream promotes deeper, more restorative sleep, effective stress relief, and balanced immunity—all without compromising memory.

36.8%

A staggering 36.8% of adults in the U.S. sleep less than seven hours per night, meaning that approximately 1 in 3 adults are not getting enough sleep (*CDC Behavioral Risk Factor Surveillance System (BRFSS), 2013–2022*).

Understanding the Significance of Sleep – Taking a Closer Look



Quality sleep is essential for our safety and well-being, affecting our attention, cognition, mood, and immune function (*Worley*, 2018). Chronic sleep deprivation can lead to serious health issues. In fact, in 2007, the International Agency for Research on Cancer classified inadequate sleep due to night shift work as a "probable" carcinogen because of circadian disruption. Thus, ensuring quality sleep each night is crucial for maintaining overall health.

Sleep goes through five stages: wake, N1, N2, N3, and REM. Non-rapid eye movement (NREM) sleep covers stages N1 to N3, with N3 being vital for repairing tissues, building muscles, and boosting the immune system. REM sleep helps with memory, emotions, brain development, and dreaming (*Patel et al., 2024*). To feel our best, we need to cycle through these various stages multiple times each night, which requires getting approximately 7-9 hours of sleep. However, 62% of adults worldwide report not getting enough sleep (*Philips Global Sleep Survey, 2019*). Many rely on melatonin or prescription medications, but these can cause grogginess and dependency. Due to these challenges, many people are now turning to plant-based, scientifically substantiated solutions like ThymoDream to relieve stress and support healthy sleep without unwanted side effects.

Introducing ThymoDream™

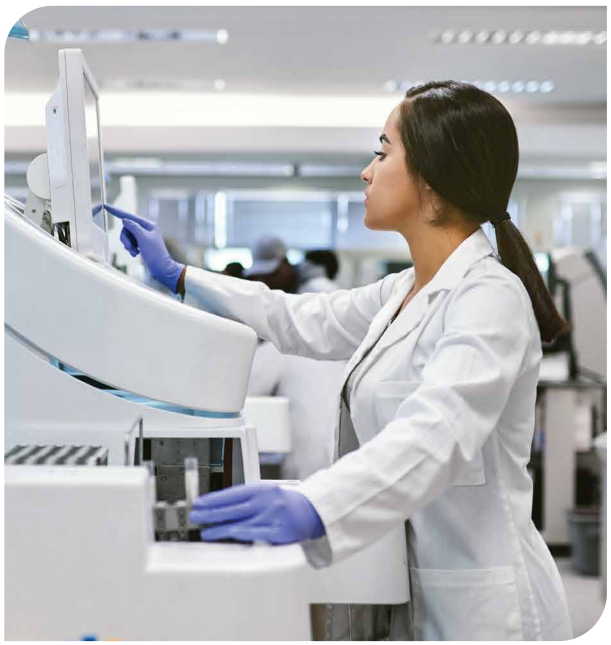
A perfect synergy of tradition and innovation, ThymoDream taps into the rich medicinal history of *Nigella sativa* (black cumin), that has been used for centuries in traditional remedies. Blending this heritage with modern science and advanced cold-press technology, ThymoDream is crafted to precision and delivers a unique composition of thymoquinone and carvacrol. Moreover, this plant-based, non-habit-forming solution is clinically substantiated to support the sleep-stress-immune axis, promoting deeper, restorative health.

Scientific Foundation of ThymoDream™

ThymoDream is supported by extensive scientific research, with over eight published studies encompassing both clinical and preclinical trials. These studies demonstrate its safety and efficacy in improving sleep quality, reducing stress, and enhancing overall wellness at a low dosage of 200 mg once before bedtime. This robust body of research positions ThymoDream as a premier, scientifically substantiated ingredient for occasional sleeplessness, stress, and immune health in otherwise healthy adults. Derived from responsibly sourced black cumin seeds, ThymoDream aligns with the body's natural processes without synthetic substitutes, offering a holistic, plant-based solution for better health with every use.

Experience the Difference:

- Patented black cumin seed extract that standardized for thymoquinone and carvacrol in a 10:1 ratio
- Clinically substantiated (4 RCTs)
- Proprietary cold-press technology
- Unique mechanism of action
- Clean label, oil soluble formulation
- Responsibly sourced with full scale traceability
- Suitable for different delivery formats (softgels, liquid-filled capsules, gummies)



ThymoDream™: Leading Innovation in the Sleep, Stress, and Immune Health Category

In an era where scientifically supported ingredients for holistic wellness are in high demand, ThymoDream stands out. ThymoDream offers a distinctive, plant-based solution that promotes restful sleep, reduces stress, and boosts immune function. Unlike synthetic and bioidentical alternatives, ThymoDream is melatonin-free and non-habit forming, plus it is available in an oil-soluble format that easily adapts to various applications like gummies, softgels, and liquid-filled capsules. With clean-label credentials (Organic, Halal, Kosher, Non-GMO, Allergen-free, Gluten-free, Vegan) and a commitment to ethical sourcing and sustainability, ThymoDream answers the call for natural, science-backed wellness solutions geared towards better sleep, improved stress management, and year-round wellness.

Clinically substantiated for sleep, stress, and immunity

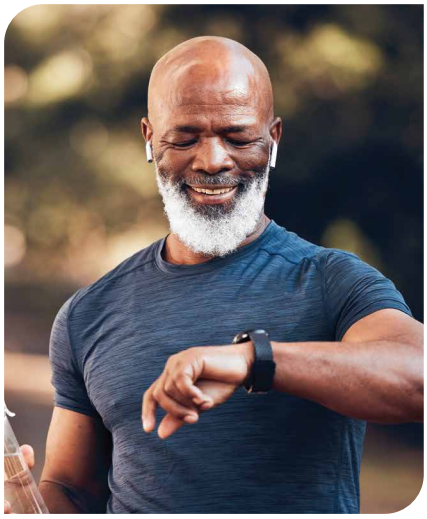
Proprietary cold-press technology

Unique mechanism of action

Clean label formulation

Responsibly sourced with full traceability

Low dosage of 200 mg per day

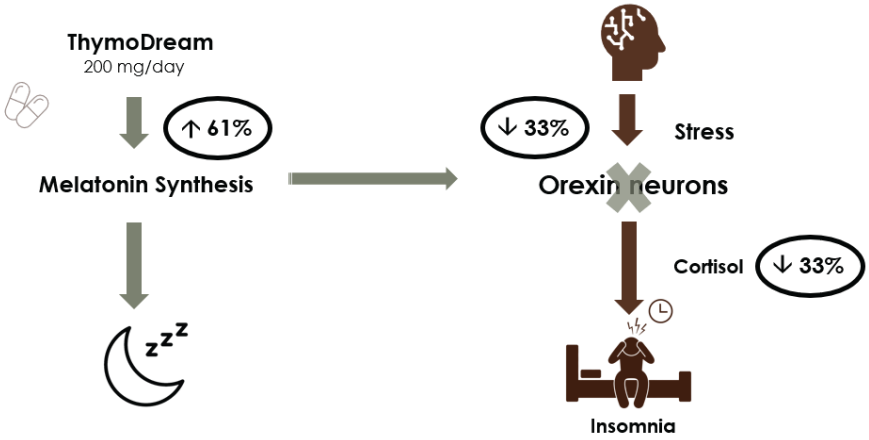


ThymoDream™: Deeper, Restorative Sleep for Healthier, Calmer Days

Backed by four human clinical studies, ThymoDream is confirmed for its safety, efficacy, and unique mechanism of action. These studies use validated questionnaires like the Pittsburgh Sleep Quality Index (PSQI), Depression Anxiety Stress Scale-21 (DASS-21), and Perceived Stress Scale-14 (PSS-14), along with biochemical assessments of melatonin, cortisol, and orexin level outcomes. Advanced evaluations, including polysomnography and actigraphy are also used to assess sleep quality, efficiency, latency, and wake after sleep onset, covering key NREM and REM stages. The research demonstrates that ThymoDream offers a comprehensive solution for sleep, stress, and immune health when used as a supplement.

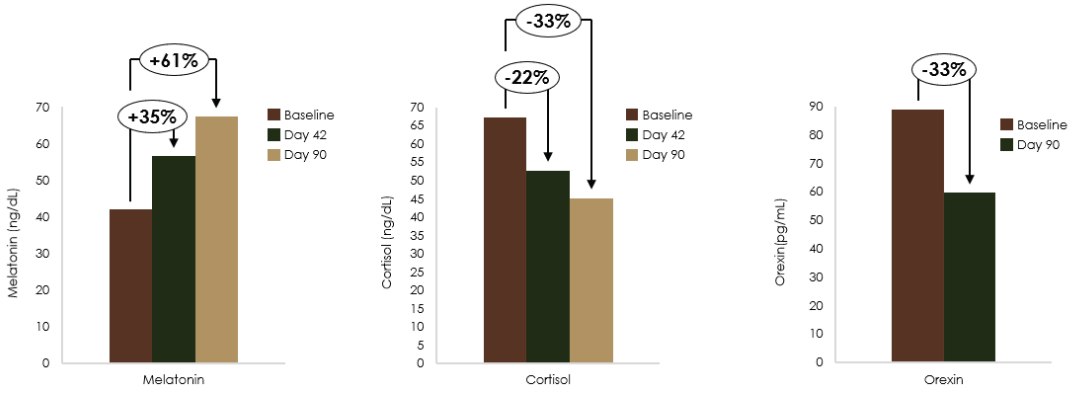
Mechanism of Action

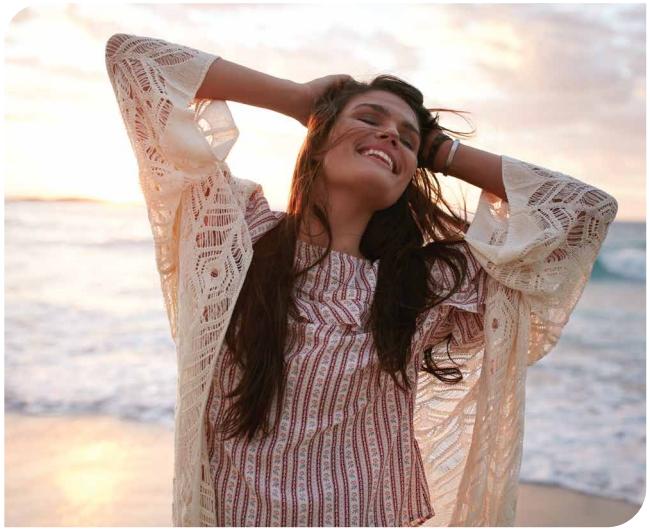
ThymoDream is standardized to thymoquinones, which act as selective dual orexin receptor antagonists (DORA). These bioactives modulate the hypothalamic-pituitary-adrenal axis, enhancing melatonin production by up to 61% and suppressing orexins—neuropeptides involved in cortisol release—by 33%. This mechanism of action improves sleep satisfaction, promotes stress relief, and boosts immune health. By managing orexin activity, ThymoDream fosters a balanced internal environment, calming both body and mind, and enhancing sleep quality and stress resilience for holistic wellness.



Melatonin, Cortisol and Orexin Level Outcomes

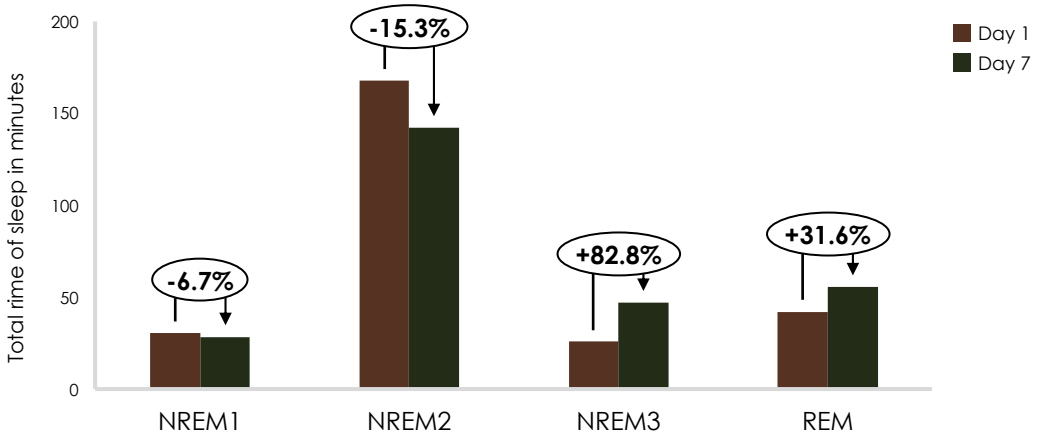
Modulating melatonin, orexin, and cortisol levels can help reduce stress and anxiety, promoting a calmer, more balanced mood and deeper, more restorative sleep. In a randomized, double-blind, placebo-controlled study, daily supplementation of 200 mg ThymoDream for 90 days increased melatonin levels by 61% and decreased orexin and cortisol levels by 33% in participants, demonstrating its ability to modulate the hypothalamic-pituitary-adrenal axis for better health outcomes (*Mohan et al., 2023*).





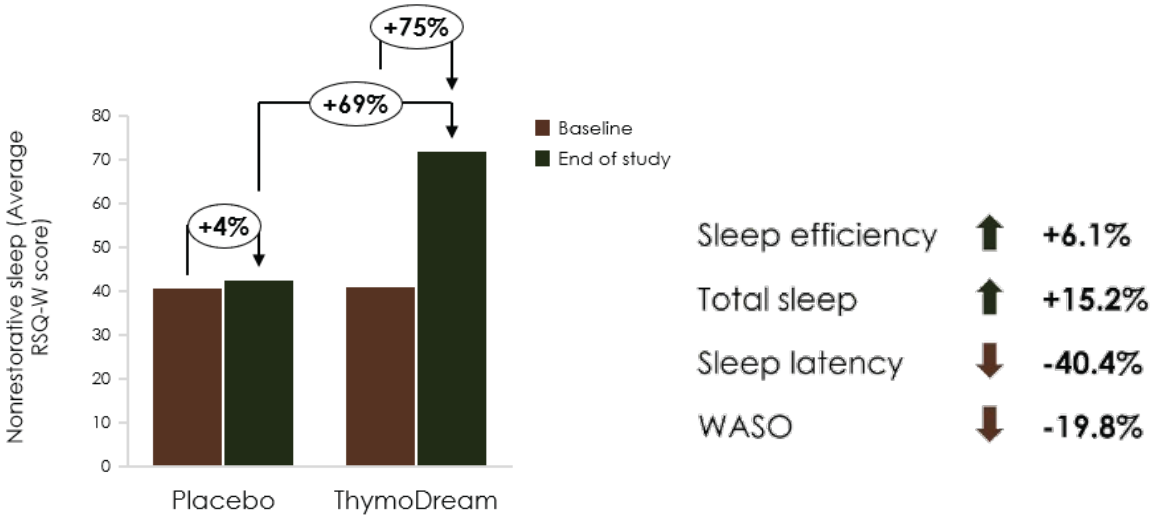
Sleep Cycle Duration – Improvements in Deepest and REM Sleep

Sleep progresses through various stages, but achieving NREM3 and REM sleep is crucial for restorative rest and essential for physical, emotional, and mental recovery. Participants who supplemented with ThymoDream (200 mg/day) for just 7 days experienced significant increases in NREM3 and REM sleep durations by 82.8% and 31.6%, respectively, as shown by polysomnography analysis (*Das et al., 2022*). These findings translate to nearly doubling NREM3 sleep time from 25 minutes to 47 minutes and increasing REM sleep from an average of 42 minutes to over 55 minutes after just one week of use.



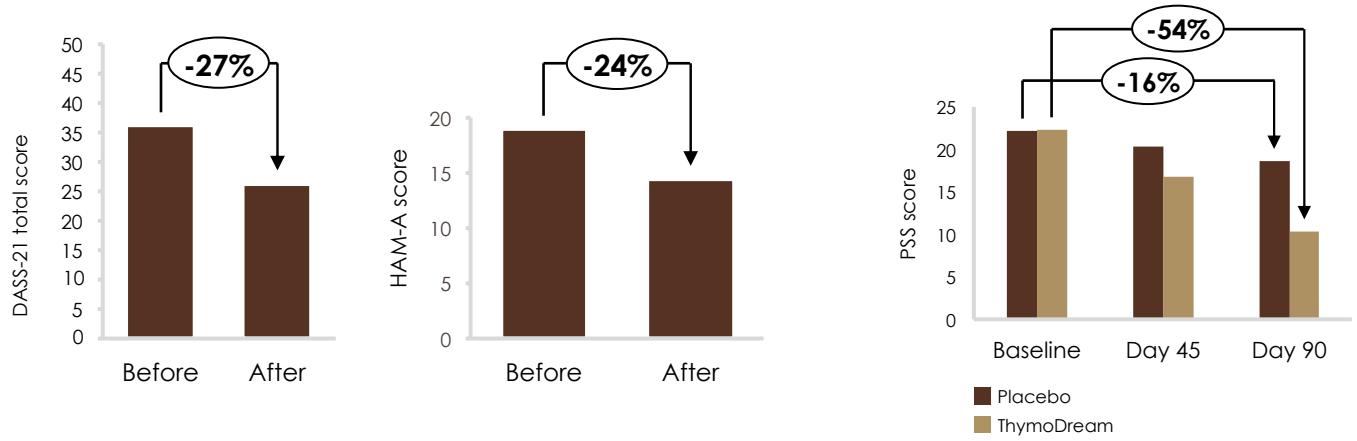
Sleep Satisfaction and Quality

Sleep satisfaction and quality can be measured using objective tools like actigraphy and subjective questionnaires like the Restorative Sleep Questionnaire (RSQ-W), that when combined provide a comprehensive sleep assessment. In a study with participants experiencing non-restorative sleep issues, those using ThymoDream (200 mg/night) showed a 75.3% improvement in sleep quality from baseline and a 68.9% enhancement over placebo in just 7 days. Other significant improvements included a 6.1% increase in sleep efficiency, 15.2% longer total sleep time (nearly an extra hour), a 40.4% reduction in sleep onset latency (falling asleep in just 15 minutes), and 19.8% less wakefulness after sleep onset (an average reduction of 10 minutes) (*Mohan et al., 2024*).



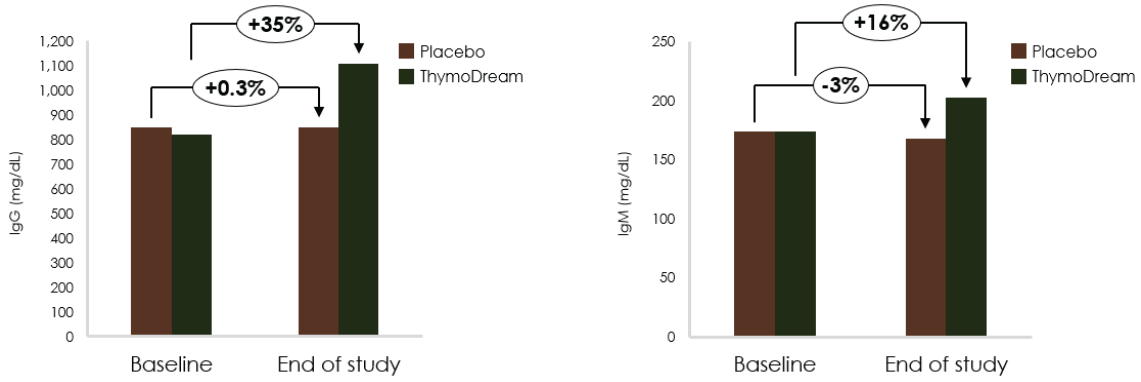
Stress Management

As a dual orexin receptor antagonist, ThymoDream effectively reduces perceived stress and anxiety levels. In both short-term and long-term studies, a daily intake of 200 mg of ThymoDream led to significant improvements in cortisol levels and validated metrics such as DASS-21, HAM-A, and PSS-14. Within just 28 days, DASS-21 scores dropped by 27.3%, with significant reductions in anxiety (-32.6%) and stress (-22.0%) scores. HAM-A scores decreased by 23.9%, while cortisol levels fell by 29.7% (*Das et al., 2022*). Long-term use, up to 90 days at the same dosage, showed sustained benefits, including further reductions in cortisol levels (-33%) and perceived stress scores (-54%) (*Mohan et al., 2023*).



Immune Support

High cortisol levels and lack of sleep can exacerbate stress and negatively impact immune health. However, supplementing with ThymoDream at a daily dosage of 200 mg significantly enhanced immune health in healthy participants within 90 days (*Mohan et al., 2023*). Key markers IgG and IgM increased by 35.6% and 16.4% from baseline, respectively. IgG, the most abundant antibody, makes up 75% of serum antibodies, while IgM, the largest antibody, constitutes about 5%. This increase in antibodies supports the body's ability to neutralize pathogens and clear antigens, thereby enhancing overall immune function.



Summary

ThymoDream is the key to unlocking nature's potential for deeper, more restorative sleep. It seamlessly blends traditional wisdom with modern science, offering a plant-based, melatonin-free solution that is scientifically backed to enhance sleep, manage stress, and boost immune health. By combining the benefits of black cumin seed oil with cutting-edge technology ThymoDream provides a revolutionary option for achieving deep, restorative sleep and overall well-being.

AKAY BIOACTIVES

To connect with a specialist or learn more about our ingredients, visit akaybioactives.com.

These statements have not been evaluated by the Food and Drug Administration.
This product is not intended to diagnose, treat, cure, or prevent any disease.

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A proprietary black cumin oil extract (*Nigella sativa*) (BlaQmax®) modulates stress-sleep-immunity axis safely: Randomized double-blind placebo-controlled study

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Objective: Stress, sleep, and immunity are important interdependent factors that play critical roles in the maintenance of health. It has been established that stress can affect sleep, and the quality and duration of sleep significantly impact immunity. However, single drugs capable of targeting these factors are limited because of their multi-targeting mechanisms. The present study investigated the influence of a proprietary thymoquinone-rich black cumin oil extract (BCO-5) in modulating stress, sleep, and immunity.

Methods: A randomized double-blinded placebo-controlled study was carried out on healthy volunteers with self-reported non-refreshing sleep issues ($n=72$), followed by supplementation with BCO-5/placebo at 200 mg/day for 90 days. Validated questionnaires, PSQI and PSS, were employed for monitoring sleep and stress respectively, along with the measurement of cortisol and melatonin levels. Immunity markers were analyzed at the end of the study.

Results: In the BCO-5 group, 70% of the participants reported satisfaction with their sleep pattern on day 7 and 79% on day 14. Additionally, both inter- and intra- group analyses of the total PSQI scores and component scores (sleep latency, duration, efficiency, quality, and daytime dysfunction) on days 45 and 90 showed the effectiveness of BCO-5 in the improvement of sleep ($p<0.05$). PSS-14 analysis revealed a significant reduction in stress, upon both intra ($p<0.001$) and inter-group ($p<0.001$) comparisons. The observed reduction in stress among the BCO-5 group, with respect to the placebo, was significant with an effect size of 1.19 by the end of the study ($p<0.001$). A significant correlation was also observed between improved sleep and reduced stress as evident from PSQI and PSS. Furthermore, there was a significant modulation in melatonin, cortisol, and orexin levels. Hematological/immunological parameters further revealed the immunomodulatory effects of BCO-5.

Conclusion: BCO-5 significantly modulated the stress-sleep-immunity axis with no side effects and restored restful sleep.

KEYWORDS

black cumin, Pittsburgh Sleep Quality Index, Perceived Stress Scale, stress, non-refreshing sleep, sleep quality, immunity

1. Introduction

Stress, insomnia, and immunity are the three major interconnected factors, that play key roles in the maintenance of health. It has been established that stress can negatively affect the quality and duration of sleep, which in turn impacts immunity (1). Stress, a mental condition generated by either extrinsic or intrinsic factors, may lead to various psychological, biological, and/or social issues. It has been identified as a major factor associated with sleep disorders. Since sleep is essential for energy, cell tissue repair, metabolic regulation, thermoregulation, cognition, motor actions, and immune functions (1, 2), it has been proposed that the 'stress-sleep-immunity' axis is critical for health (2).

Sleep and stress share multiple pathways and affect the central nervous system and circadian rhythm, leading to dysfunctions in metabolism, brain function and immunity (3). The hypothalamus-pituitary–adrenal axis (HPA) is the central system responsible for the neuroendocrine adaptation of the stress response. Orexins or hypocretins (Orexin A and Orexin B) are excitatory neuropeptides produced in the hypothalamus in response to stress stimuli (4). It modulates the activity of the HPA axis and autonomic nervous system to regulate the sleep–wake cycle, cognitive functioning, stress processing, and the metabolic and inflammatory responses (4). Orexins are also responsible for the release of cortisol, a major hormone released in response to stress (4).

Sleep and immune functions are interconnected. The sleep–wake cycle is one of the most important manifestations of the circadian rhythm, and its changes affect physical and mental activities, major organ functions, temperature regulation and immunomodulatory effects such as leukocytes and cytokine production and proliferation (1). It has been reported that one to three nights of sleep deprivation can lead to a significant elevation of inflammatory cytokines and can significantly reduce leukocyte, lymphocyte, and neutrophil counts (5).

Despite the importance of stress, sleep, and immunity, drugs/supplements capable of safely modulating the stress-sleep-immunity axis are limited because they act *via* multi-targeted mechanisms and side effects, including dependency, are common. *Nigella sativa*, commonly known as black cumin or black seed, is a culinary spice and an age-old medicinal herb with a wide range of health-beneficial pharmacological effects to attenuate oxidative stress, inflammation, immunity, energy metabolism, and cell survival (6, 7). Black cumin oil composed of both volatile and fixed oil fractions was identified as the major bioactive component of black cumin, in which thymoquinone (TQ) is considered the most active molecule (8). Black cumin has been reported to have antioxidant, anti-inflammatory, and neuroprotective effects as a function of its TQ content (6, 9–11). Recently, it was also reported that a novel formulation of black cumin oil extract containing TQ and carvacrol in a 10:1 (w/w) ratio (BCO-5) significantly alleviated stress and improved sleep quality in human volunteers when supplemented at 200 mg/day for 28 days (12). Jestin et al. conducted a 90-days safety assessment of BCO-5 among healthy human volunteers and established its safety for human consumption (13).

Based on previous studies, we hypothesized that BCO-5 would modulate the stress-sleep-immunity axis by reducing stress, improving sleep quality, and hence, the immunity. Thus, the present randomized, double-blinded, placebo-controlled study investigated the efficacy of BCO-5 in healthy subjects (25–65 years old) with significant stress and non-restorative sleep. Validated questionnaires were employed to analyze sleep quality [Pittsburgh Sleep Quality Index (PSQI)] and stress [Perceived Stress Scale (PSS)], along with changes in sleep and immune biomarkers.

2. Materials and methods

The proprietary formulation of black cumin oil used in this study (BCO-5; Patented and Registered as BlaQmax®) was manufactured by Akay Natural Ingredients, Cochin, India, following good manufacturing practices (Batch no: BCOQ 32/21 dated 12/04/2021). The dried black cumin seeds used for the manufacture of BCO-5 were identified and authenticated by a botanist, and the specimens were deposited at the Herbarium of Akay Natural Ingredients, Cochin, India (Voucher no: AK-NS-018). High-performance thin-layer liquid chromatography (HPTLC) (CAMAG HPTLC system, Switzerland) was employed to identify black cumin, as previously reported (13). The thymoquinone content was determined by high-performance liquid chromatography (HPLC) (Shimadzu Analytical India Private Limited, Mumbai, India) analysis, as reported previously (13). Analytical standard for TQ (CAS No: 490–91-5) was obtained from Sigma-Aldrich (Bangalore, India).

2.1. Subjects and design

In this randomized, double-blinded, placebo-controlled clinical trial, 150 volunteers (aged 25 to 65 years; healthy subjects) who were reported to experience significant stress and self-reported sleep issues such as non-restorative sleep, waking up at night multiple times, or having difficulty in a sound sleep for the past 4 weeks were selected. Volunteers were identified from the database of the contract research organization and the hospital where the study was conducted. After the initial description of the study, 96 participants were willing to participate and were further screened according to the inclusion and exclusion criteria (Table 1). Twenty-four subjects were eliminated after screening, and 72 volunteers were randomized into BCO-5 and placebo

TABLE 1 Inclusion and exclusion criteria.

Inclusion criteria
1. Healthy participants aged 25–65 years (both inclusive)
2. Participants with PSQI scores ≥5
3. Participants with PSS score between 14 and 26
4. Participants with body weight ≥ 50 kg
5. Approved birth control measures should be perceived by female participants of childbearing age and should have negative urine pregnancy test at the screening
6. Participants should refrain from smoking, caffeinated beverages, and alcohol consumption
7. Signed and informed consent should be provided by the participant and the participant should understand the study protocol
Exclusion criteria
1. Participants requiring medical treatment and suffering from health conditions like hypertension, diabetes, chronic renal failure, heart, thyroid and liver disease
2. Participants with hepatic or renal impairment (Alanine transaminase/Aspartate transaminase levels >3 upper limit of normal) (serum creatinine ≥2.0 mg/dl)
3. Subjects with history of conditions such as endocrine abnormalities including thyroid disease, psychiatric illness, drug abuse, smoking, addiction to alcohol, psychiatric illness
4. Participants who have went cardiovascular surgery or any other major surgery
5. Immuno-compromised state participants and those with immunodeficiency diseases like, HIV or Hepatitis B
6. Participants allergic to the composition of investigational product
7. Pregnant and lactating women
8. Participants with significant illness history or any medical derangements that can interfere with subject treatment, assessment, or compliance with the protocol
9. Participants currently participating or have participated in any other clinical trial, 1 month prior to start of this study
Any additional condition(s) that in the Investigators opinion would warrant exclusion from the study or prevent the subject from completing the study.

groups ($n = 36$/group). The study was conducted at the BGS Global Institute of Medical Sciences, Bangalore, India, under the guidance of a registered medical practitioner, according to the guidelines of the Clinical Trial Registry of India (CTRI/2021/05/033780 dated 25/05/2021). The protocol was reviewed and approved by the institutional ethics committee. Written informed consent was obtained from all subjects prior to the study. A cohort diagram representing the study design is shown in Figure 1.

The participants were requested to visit the study site on four different occasions; Visit I (day 0), Visit II (day 1), Visit III (day 45) and Visit IV (day 90/ end of study). During visit I, screening was performed against the inclusion/exclusion criteria, which included a structured medical interview and diagnosis as well as demographic and anthropometric measurements. The primary selection criterion was a PSQI score greater than 5. During visit II (day 1), the participants were asked to report at the study site with 10 h of fasting and were randomized, based on a computer-generated randomization code, into two groups to receive either a placebo or intervention. Blood (10 mL) was withdrawn for routine laboratory clinical parameters (biochemical and hematological) and markers of immunity. The baseline PSQI and PSS-14 scores were also recorded. Similar visits for blood and data collection were requested on day 45 (Visit III) and at the end of the study period, day 90 (Visit IV). Telephonic interviews were also performed on days 7 and 14 to inquire about adverse events, side effects, tolerance, and efficacy.

2.2. Sample size and randomization

Sample size calculation was performed using the G Power Statistical Software (3.1.9.7 Version, Franz Faul, University of Kiel, Kiel, Germany) (14). It was estimated that a total of 35 participants per group would be required, with an anticipated non-compliance or dropout rate of 20%, yielding 80% power, and 5% significance level. Participants were randomly allocated by permuted-block randomization (block size = 4) using a computer-generated allocation table.[1]

2.3. Intervention and dosage

Airtight high density polyethylene containers comprising 95 soft gel capsules (200 ± 10 mg extract/capsule) with either BCO-5 (intervention) or placebo were sequentially coded and provided to the participants. They were requested to consume one capsule daily after dinner, 20–30 min prior to bedtime. The total study duration was 90 ± 2 days. At the end of the study period (Visit IV), the remaining capsules were recorded. The effectiveness of participant blinding was evaluated by asking participants to predict the allocation (placebo/ intervention).

2.4. Pittsburgh Sleep Quality Index

The PSQI is a 19- item self-reported questionnaire designed to determine the overall sleep quality and disturbances over a period of one-month tenure. Four-point Likert scale is used to rate the severity/ frequency of the problems in such a way that '0' = not during the past month, '1' = less than once in a week, '2' = Once or twice a week, '3' = Three or more times a week. Each component yields a score ranging from 0 to 3, and the component scores are summed to yield a global PSQI score (from 0 to 21). A higher score indicates lower sleep quality (15). A score greater than 5indicates poor sleep quality or insomnia (16).

1 www.randomization.com

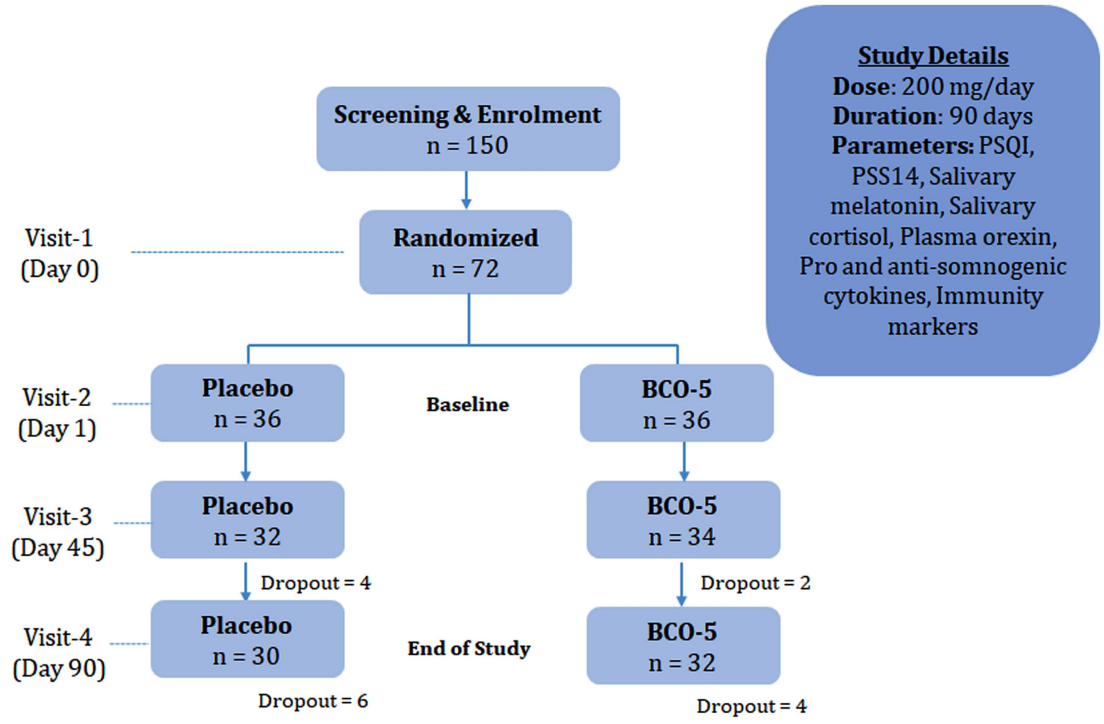

FIGURE 1
Cohort diagram depicting study design.

2.5. Perceived Stress Scale- 14 (PSS-14)

The PSS questionnaire was developed by Cohen et al. in 1983 to evaluate the perception of stressful conditions in a person's life based on their last one-month experience. It is the degree to which situations in life are appraised using a 14-item scale. A 5-point Likert scale, ranging from 0 (never) to 4 (very often), was used to evaluate the stress experienced. The PSS scores ranges from 0 to 56; with higher scores indicating higher perceived stress (17). Scores ranging to 0–18 indicate low stress; to 19–37 implies moderate, and to 38–56 as high stress.

2.6. Sleep and stress biomarkers

2.6.1. Estimation of salivary cortisol and melatonin

Cortisol and melatonin levels from the salivary samples were estimated using ELISA Kit methods of Neogen Corporation, KY, United States (Catalog No: 402710) and IBL-International, Germany (Catalog No: RE54041), respectively. Micro ELISA 96 well plates were used for the analysis. Absorbance was measured at 450 ± 2 nm using a Varioskan™ LUX multimode microplate reader (Thermo Scientific™, Waltham, MA, United States).

2.6.2. Orexin A assay

Plasma Orexin A levels were estimated using an ELISA kit method (Catalog No: E-EL-H1015) (Elabscience, Biotechnology Co., Limited Bethesda, United States). Measurements were conducted in Micro ELISA 96 well plates using a Varioskan™ LUX multimode microplate reader (Thermo Scientific™, Waltham, MA, United States) at 450 ± 2 nm.

2.7. Immunity biomarkers

2.7.1. Analysis of immunoglobulins

Serum immunoglobulin concentrations of IgG (Catalog no: E-EL-H0169) and IgM (Catalog no: E-EL-H1814) were analyzed using the ELISA kit method following the manufacturer's instructions at 450 ± 2 nm, using a Varioskan™ LUX multimode microplate reader (Thermo Scientific™, Waltham, MA, United States).

2.7.2. CD4+, CD8+ absolute count

Whole blood samples were collected in EDTA tubes, and 50 µL of the sample was added to a test tube containing a pre-dispensed, stabilized monoclonal antibody. The antibody mixture containing antibodies against CD4 and CD8 was provided by Becton Dickinson (San Diego, CA, United States) and conjugated with allophycocyanin (APC) and fluorescein isothiocyanate (FITC). The blood sample was diluted (1:10) using phosphate buffer after 15 min of incubation. The samples were analyzed using flow cytometry (FACS Calibur, Becton Dickinson, CA, United States). The sample analysis was performed in comparison with a specific fluorescence signal attributed to the presence of CD4 and CD8 antigens at the cell surface, with a side scatter signal for discriminating cells based on their shape and structure. The method of determination of absolute count was determined as described by Arneth (18).

2.7.3. Differential count

Whole blood sample were collected from the antecubital vein. The total and differential WBC counts were analyzed using automated cell cytometry (Quest Diagnostics, Inc., Madison, New Jersey, United States). Another 3 ml of whole blood was collected in EDTA vials and kept at room temperature until analysis within 24 h of sampling.

2.7.4. Analysis of cytokines

Serum concentrations of IL-1β (catalog no: E-EL-H0149), IL-2 (catalog no: E-EL-H0099), and IL-10 (catalog no: E-EL-H6154) were analyzed using ELISA kits, according to the manufacturer's instructions. Kits were purchased from Elabscience Biotechnology Co., Limited. Bethesda, United States. Absorbance was read at 450 ± 2 nm using a Varioskan™ LUX multimode microplate reader (Thermo Scientific™, Waltham, MA, United States).

2.8. Statistical analysis

Statistical analyses were performed using SPSS version 27.0. A 2 × 2 repeated measures ANOVA was employed to analyze statistical significance (treatment vs. time). Bonferroni test was used to adjust for multiple comparisons. The significance of the difference is represented as a '*p*' value. $p < 0.05$ was considered statistically significant. The reported values are arithmetic means with standard deviations (SD) or standard errors of the mean (SEM) as indicated. Pearson's correlation test was used to evaluate the significance of correlation between sleep quality (PSQI) and stress (PSS-14).

3. Results

3.1. Materials, subjects, and study design

HPTLC analysis confirmed that raw material used for preparing BCO-5 was *Nigella sativa* seed. It was in an oil form with 5.12% TQ content and 0.54% carvacrol in such a way that the TQ to carvacrol content ratio was 1:10. BCO-5 was food-grade and free from synthetic emulsifiers and food contaminants such as pesticides, heavy metals, mycotoxins, polyaromatic hydrocarbons, ethylene oxide, and microbial pathogens, as evident from their certificate of analysis.

The baseline anthropometric, haemodynamic, and other vital characteristics of the placebo and BCO-5 treated groups are provided in Supplementary Table S1. The average age and BMI of the participants in the placebo group were 25.76 ± 15.51 and 23.75 ± 0.79, respectively, while those of the BCO-5 group were 24.5 ± 16.62 and 24.29 ± 0.72, respectively. At the end of the study period, there were no significant differences between these factors.

No major side effects or adverse events were reported during the telephonic interviews on days 7 and 14. Three participants from the BCO-5 group and one from the placebo group reported bloating and borborygmus with a taste of oil in the mouth at different instances. However, all of them continued since they were satisfied with the improvement in their sleep quality. By day 14, no adverse events had been reported, and none of the participants showed signs of sleepiness or daytime drowsiness with fatigue. During the study period, 92% of participants in the BCO-5 group and 78% in the placebo group were found to be using supplements, although the consistency of use over 90 days could not be ascertained.

3.2. Influence of BCO-5 on sleep

Approximately 62% of the participants reported the beneficial effect of a single dosage. Upon telephonic interview on day 7, about 70% of participants reported satisfaction, which increased to 79% by day 14 (Figure 2). Reduction in sleep disturbances and better sleep were the two main types of feedback that were received.

3.2.1. PSQI on day 45

Intra-group comparison (baseline versus day 45) of the PSQI total score revealed a significant reduction ($p < 0.001$) in the BCO-5 group and a non-significant reduction in the placebo (Figure 3). Detailed analysis further revealed relative changes in various component scores corresponding to sleep quality ($p = 0.043$), sleep latency ($p = 0.003$), sleep duration ($p = 0.025$), overall sleep efficiency ($p < 0.001$), sleep disturbance ($p = 0.050$) and daytime dysfunction ($p < 0.001$) among BCO-5 participants. However, changes in sleep parameters were not significant in the placebo group (Figures 4A–F).

Inter-group comparison (Placebo versus BCO-5) of the PSQI component scores at baseline showed no significant difference ($p > 0.05$) between the BCO-5 and placebo. However, supplementation with BCO-5 resulted in a significant reduction in the PSQI total score (large difference effect size of 0.68; 95% CI: 7.55–8.59; $p < 0.001$). The overall effect size observed for various parameters were: sleep quality- 0.31 (95% CI: 1.14–1.61; $p = 0.024$), sleep latency- 0.31 (95% CI: 1.82–2.31; $p = 0.030$), sleep duration- 0.24 (95% CI: 1.59–1.92; $p = 0.002$), sleep efficiency- 0.52 (95% CI: 0.51–0.86; $p < 0.001$), sleep disturbance- 0.19 (95% CI: 1.00–1.00; $p = 0.030$), and daytime dysfunction- 0.29 (95% CI: 0.99–1.35; $p = 0.011$) compared to placebo (Figures 4A–F; Table 2). All component scores exhibited a moderate to large difference effect on the 45th day when compared to the placebo.

3.2.2. PSQI on day 90

By the end of the study period (day 90), BCO-5 supplementation revealed a continuous improvement in sleep parameters with a significant reduction in the PSQI total score and component scores on both intra and inter-group comparison. The total PSQI score of BCO-5 was significantly lower than that at baseline ($p < 0.001$) (Figure 3). The relative improvement in component scores for BCO-5 in comparison with baseline was sleep quality ($p = 0.003$), sleep latency ($p < 0.001$), sleep duration ($p = 0.008$), sleep efficiency ($p < 0.001$), sleep disturbance ($p = 0.009$) and daytime dysfunction ($p < 0.001$) (Figures 4A–F). However, the relative changes in the placebo group were not significant ($p > 0.05$).

Overall effect size observed for various component scores were: sleep quality– 0.57 (95% CI: 0.73–1.26; $p = 0.002$), latency– 0.82 (95% CI: 0.96–1.19; $p < 0.001$), duration– 0.33 (95% CI: 1.43–1.84; $p = 0.001$), efficiency– 0.47 (95% CI: 0.15–0.56; $p < 0.001$), disturbance– 0.20 (95% CI: 0.75–1.01; $p = 0.043$), daytime dysfunction– 0.56 (95% CI: 0.74–1.01; $p < 0.001$) and total PSQI– 0.86 (95% CI: 5.32–6.35; $p < 0.001$) (Figures 4A–F and Table 2).

Intra- and inter-group comparisons of the PSQI data corresponding to days 45 and 90 was also compared using paired and independent t-tests and were found to be significant (Supplementary Table S2).

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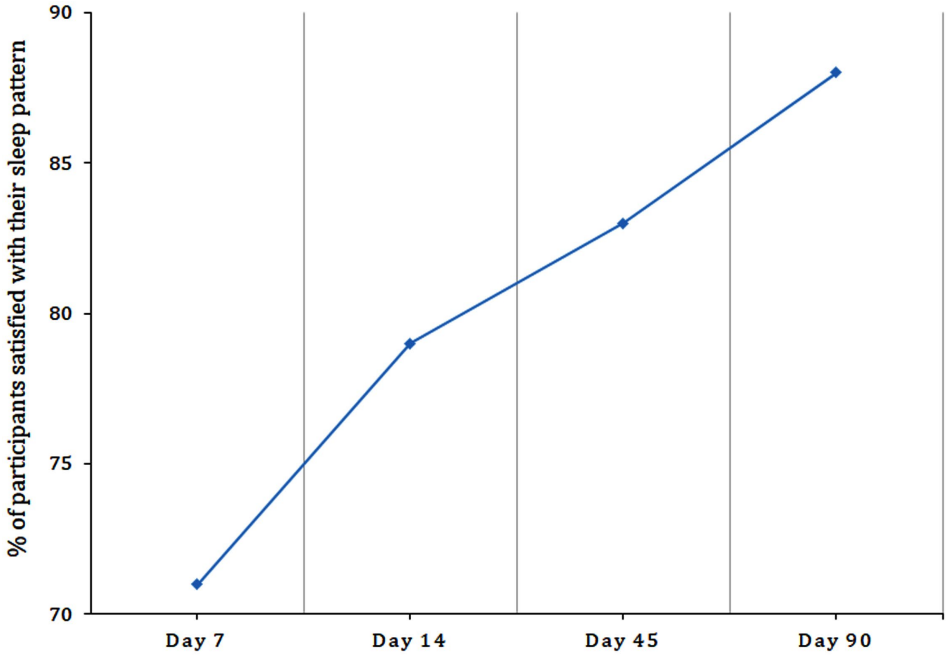

FIGURE 2
Graphical representation of the percentage of participants satisfied with their sleep pattern upon supplementation with BCO-5 as observed from sleep diary.

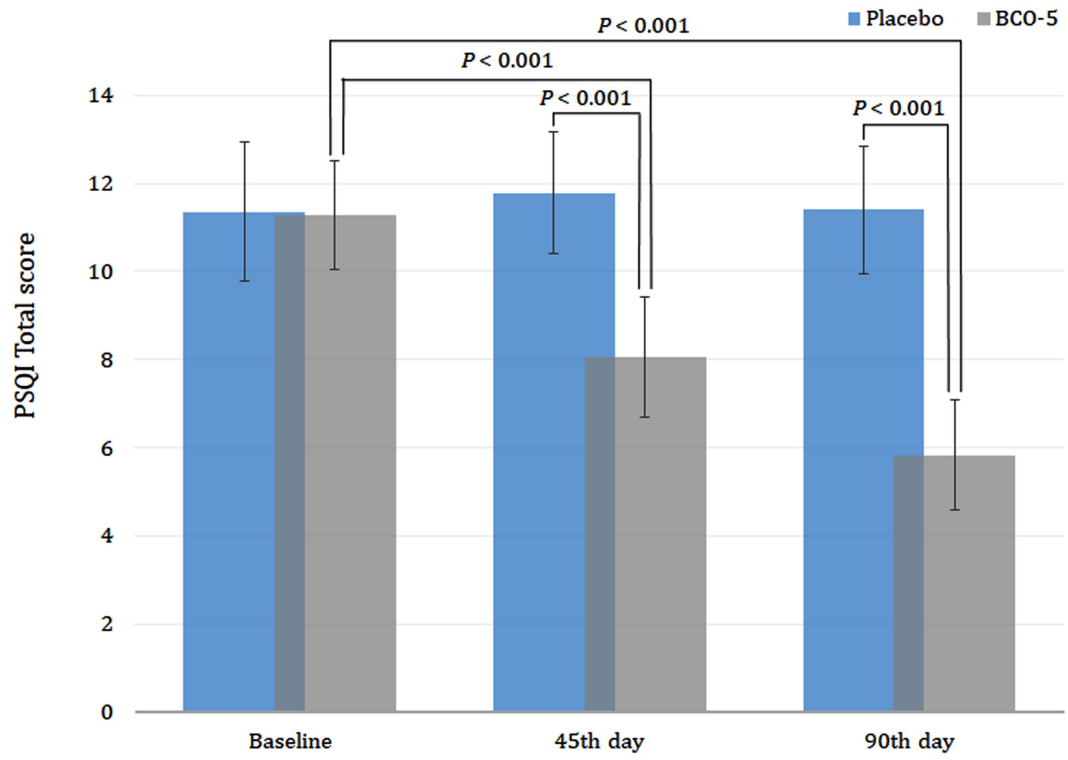

FIGURE 3
Effect of BCO-5 on PSQI total scores on 45th day 90th day compared to Placebo. The values are expressed as mean±SD.

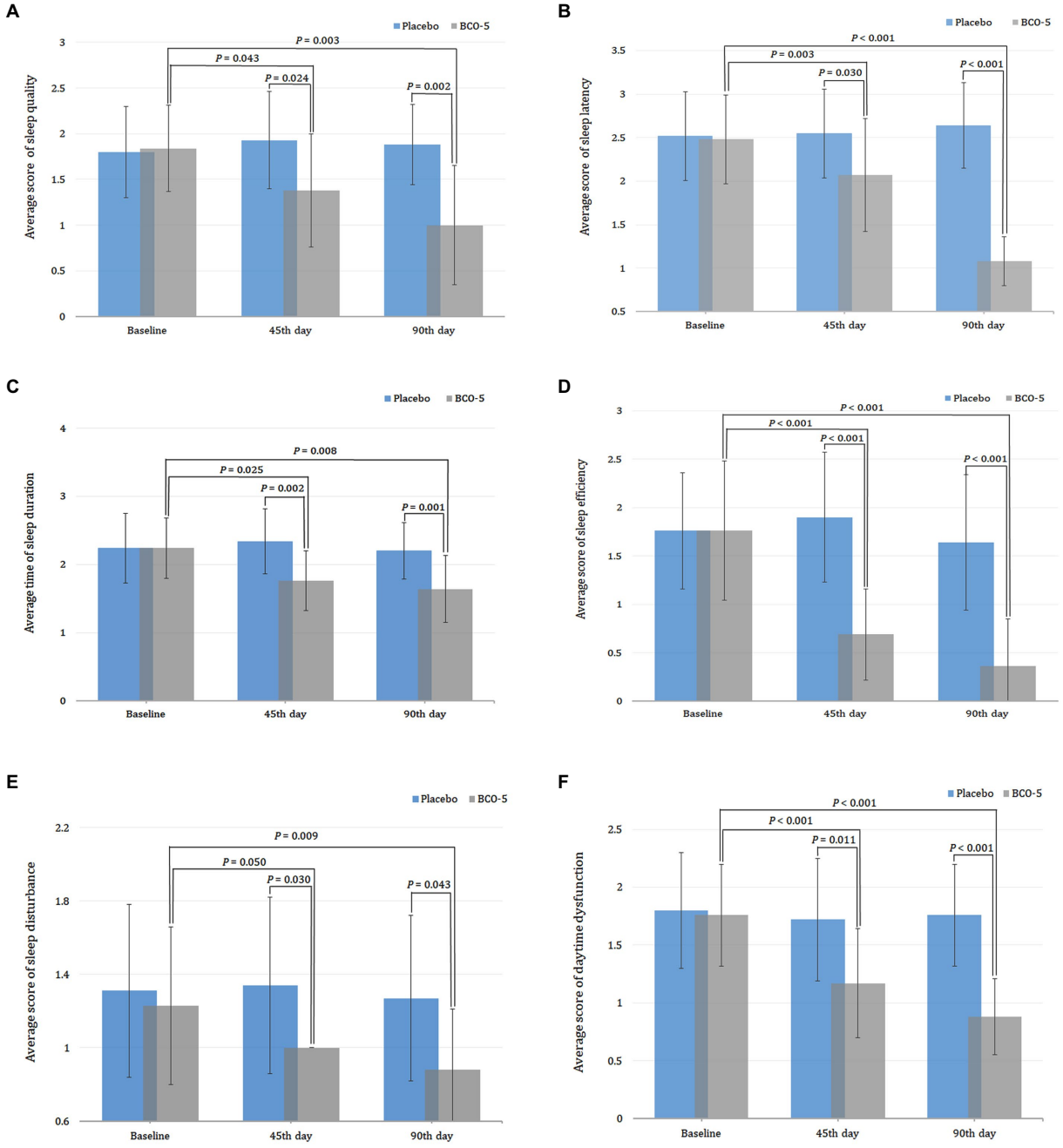

FIGURE 4
Relative changes in PSQI component scores. **(A)** Sleep quality. **(B)** Sleep latency. **(C)** Sleep duration. **(D)** Sleep efficiency. **(E)** Sleep disturbance. **(F)** Daytime dysfunction, when supplemented with BCO-5 on 45th day and 90th day compared to placebo. The values in **(B,C,E,F)** are provided as mean±SD.

3.3. Influence of BCO-5 on stress

Intra-group comparison of PSS-14 scores on the 45th day revealed a significant reduction ($p < 0.001$) in the BCO-5 group, whereas the placebo showed no significant change. Inter-group comparison also showed a significant reduction in stress in the BCO-5 group compared to the placebo. The effect size observed was 0.17 (95% CI: 15.51–18.06; $p = 0.045$) (Figure 5).

Both intra- and inter-group comparisons showed a significant effect of BCO-5 at the end of the study (day 90). The effect size observed was moderate effect of 0.43 (95% CI: 9.76–10.87; $p < 0.001$) (Figure 5). The data were also analyzed using paired and independent *t*-tests and were found to be significant (Supplementary Table S3).

3.4. Pearson's correlation between sleep and stress

BCO-5 administration showed a negative correlation between the PSQI and PSS-14 measures, with a coefficient of −0.314. This

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correlation was significant at the end of the study ($p < 0.05$). In contrast, placebo administered group exhibited a positive correlation between PSQI and PSS-14 parameters at the end of the study (0.178; $p = 0.357$).

3.5. Effect of BCO-5 on melatonin level

Both intra and inter-group analyses of salivary melatonin on day 45 showed no significant increase in placebo ($p > 0.05$), whereas a significant increase in the BCO-5 group was observed in both intra- ($p < 0.001$) and inter-group analyses ($p = 0.009$). The overall effect size was 0.84 (95% CI: 53.96–58.93) (Figure 6A). When extended to day 90, the placebo group exhibited no significant increase in melatonin levels while BCO-5 had a significant effect on both intra- ($p < 0.001$) and inter-group comparisons ($p < 0.001$). A large effect size [0.85 was observed at the end of the study (95% CI: 63.32–69.66) (Figure 6A)].

TABLE 2 Effect size and *p*-values of PSQI parameter (BCO-5 vs. Placebo) on day 45 and 90.

PSQI parameter	Time	Effect size	*P*-value
Sleep quality	45th day	0.31	0.024
	90th day	0.57	0.002
Sleep latency	45th day	0.31	0.030
	90th day	0.82	<0.001
Sleep duration	45th day	0.24	0.002
	90th day	0.33	0.001
Sleep efficiency	45th day	0.52	<0.001
	90th day	0.47	<0.001
Sleep disturbance	45th day	0.19	0.030
	90th day	0.20	0.043
Daytime dysfunction	45th day	0.29	0.011
	90th day	0.56	<0.001
Total PSQI	45th day	0.68	<0.001
	90th day	0.86	<0.001

A significance level $p < 0.05$ is considered as statistically different.

3.6. Effect of BCO-5 on cortisol level

The intra- and inter-group analysis of cortisol at the end of the 45th day exhibited a significant decrease ($p < 0.001$) for BCO-5, while the placebo showed no significant change ($p > 0.05$). The effect size observed upon analysis was 0.72 (95% CI: 48.73–55.70; $p = 0.044$).

At the end of the study (day 90), BCO-5 participants showed a significant decrease ($p < 0.001$), while the placebo exhibited no significance ($p > 0.05$) with respect to the baseline. Inter-group comparison at the end of the study also revealed a significant decrease in cortisol levels among BCO-5 participants compared to placebo. A large effect of 0.72 (95%CI: 40.99–48.51; $p = 0.013$) was observed (Figure 6B).

3.7. Effect of BCO-5 on orexin level

At the end of the study, the plasma concentration of orexin in the BCO-5 group, decreased significantly in upon both intra- and inter-group comparisons ($p < 0.001$), while the same for the placebo exhibited no significant change. The observed effect size had a large difference effect of 0.92 (95% CI: 57.07–61.99) (Figure 6C).

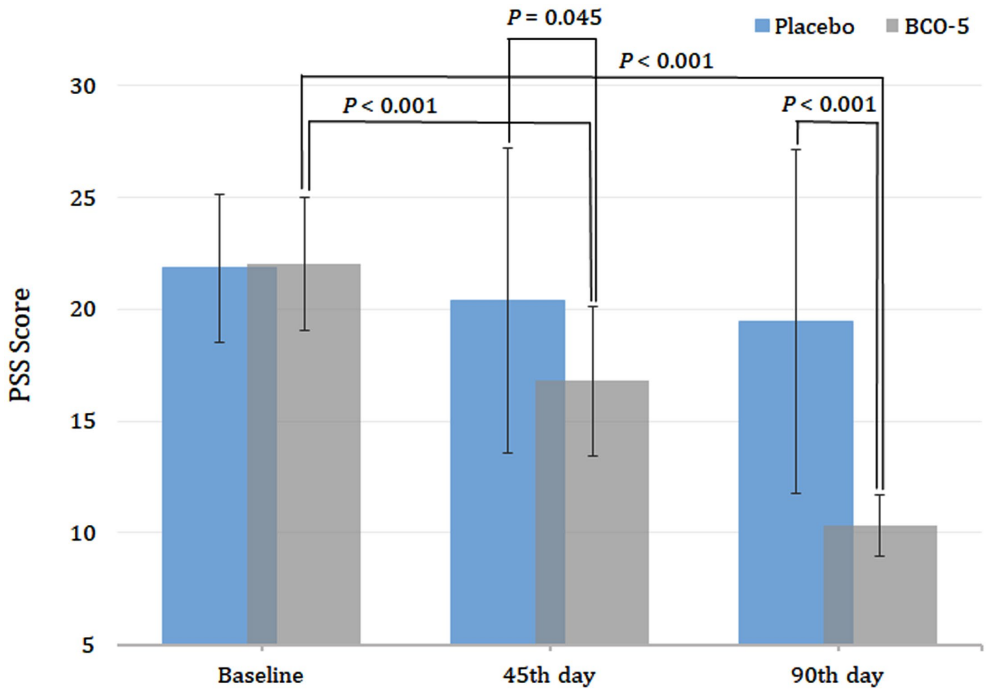

FIGURE 5
Relative changes in PSS-14 score upon supplementation with BCO-5 on day 45 and 90 compared to placebo. The values are expressed as mean±SD.

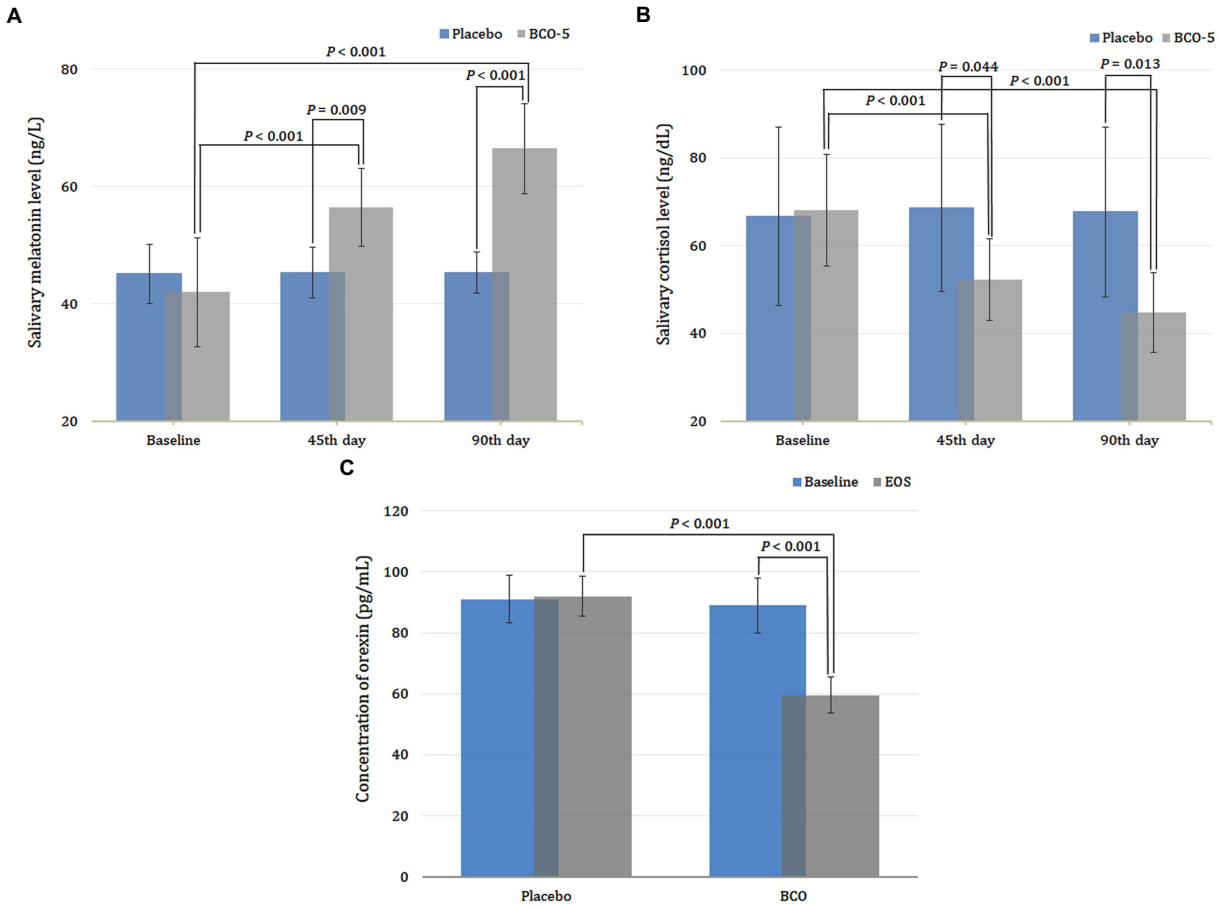

FIGURE 6
Changes in sleep biomarkers **(A)** melatonin and **(B)** cortisol and **(C)** orexin A on 45th day and 90th when supplemented with BCO-5 when compared to placebo. The values are expressed as mean±SD.

3.8. Effect of BCO-5 on immune markers

3.8.1. Influence on immunoglobulins

Intra-group analysis of IgM and IgG at the end of the study period revealed a significant increase compared to baseline ($p < 0.008$ and $p < 0.001$, respectively), whereas the placebo showed no significant effect. Inter-group analysis of IgM and IgG levels also showed a significant increase compared to the placebo. A moderate effect size of 0.41 (95% CI: 191.06–214.24; $p = 0.050$) was noted for IgM, and a large effect size of 0.87 (95% CI: 1091.09–1125.94; $p < 0.001$) for IgG (Table 3).

3.9. Influence on CD4+, CD8+ and CD4/CD8 ratio

Intra-group comparison of CD4+ and CD4/CD8 ratio exhibited a significant increase ($p = 0.015$ and $p < 0.001$respectively) in BCO-5 treated participants, whereas the placebo-supplemented group showed no significant effect ($p > 0.05$). CD8+ cells, on the other hand, showed a significant decrease ($p = 0.043$) compared to that at baseline.

Inter-group comparison revealed a significant increase in the CD4+ and CD4/CD8 ratio at the end of the study in BCO-5 treated participants. The effect size observed were: CD4+: 0.29 (95% CI: 765.07–819.02; $p < 0.001$) and CD4+/CD8+: 0.52 (95% CI: 1.85–2.08; $p = 0.032$) respectively. At the same time, CD8+ exhibited a much more significant decrease at the end of the study compared to the placebo, with a moderate effect size of 0.46 (95% CI: 387.90–425.91; $p = 0.042$) (Table 3).

3.9.1. Influence on differential count

At the end of the study, supplementation with BCO-5 increased the number of leukocytes, lymphocytes, and monocytes in both intra- and inter-group analyses. The relative effect size observed were, respectively, 0.34 (95% CI: 6342.14–6921.85; $p = 0.050$), 0.57 (95% CI: 35.80–40.26; $p = 0.009$), and 0.10 (95% CI: 5.57–6.97; $p < 0.001$). Neutrophils and eosinophils, on the other hand, showed a significant decrease, with an effect size of 0.57 (95% CI: 51.51–55.45; $p < 0.001$) and 0.31 (95% CI: 1.61–2.33; $p < 0.001$) respectively. The basophil count showed no significant difference (0.001; 95% CI: 0.17–0.27; $p = 0.294$) (Table 4).

3.9.2. Influence on cytokines- IL-1β, IL-2, and IL-10

Intra-group comparison of IL-1β, IL-2, and IL-10 showed no significant effect in the placebo. However, BCO-5 showed a significant

TABLE 3 Changes in immunity markers in participants from baseline to end of study in placebo and BCO-5 treated group and the *p*-values observed upon inter-group and intra-group comparison.

Parameters	Group	Baseline	End of study	*P*-values	
				Inter	Intra
IgG (mg/dL)	Placebo	848.04 ± 56.63	850.72 ± 52.53	<0.001	<0.001
	BCO-5	818.12 ± 63.65	1108.52 ± 42.22		
IgM (mg/dL)	Placebo	173.50 ± 24.55	168.15 ± 36.08	0.050	0.008
	BCO-5	174.15 ± 45.03	202.65 ± 28.69		
CD4 + cells	Placebo	655.91 ± 81.51	639.27 ± 91.90	<0.001	0.015
	BCO-5	703.18 ± 102.93	792.05 ± 60.84		
CD8 + cells	Placebo	398.32 ± 55.27	413.45 ± 57.10	0.042	0.043
	BCO-5	446.05 ± 34.18	406.91 ± 42.87		
CD4/CD8 ratio	Placebo	1.63 ± 0.33	1.57 ± 0.29	0.032	<0.001
	BCO-5	1.60 ± 0.23	1.97 ± 0.26		

Serum levels of immunoglobulin M, Serum levels of immunoglobulin G and the CD4/CD8 ratio of study participants at baseline and at the end of the study in the placebo and BCO-5 treated groups. Values are expressed as mean ± SD. Differences were considered statistically significant at $p < 0.05$.

TABLE 4 Changes in differential count in participants from baseline to end of study in placebo and BCO-5 treated group and their respective effect size.

Parameters	Group	Baseline	End of study	Effect size
TLC (cells/cmm)	Placebo	6,072 ± 777.56	5,792 ± 776.17	0.34
	BCO-5	5,992 ± 707	6632 ± 702.21	
Neutrophils (%)	Placebo	66.24 ± 3.44	63.32 ± 3.59	0.57
	BCO-5	64.29 ± 3.99	53.48 ± 4.77	
Lymphocytes (%)	Placebo	28.68 ± 3.90	31.29 ± 3.66	0.57
	BCO-5	27.30 ± 4.83	38.04 ± 5.40	
Eosinophils (%)	Placebo	1.38 ± 0.71	1.44 ± 0.78	0.31
	BCO-5	2.75 ± 1.00	1.98 ± 0.87	
Monocyte (%)	Placebo	3.44 ± 1.83	3.68 ± 1.58	0.10
	BCO-5	5.42 ± 1.65	6.28 ± 1.70	
Basophils (%)	Placebo	0.26 ± 0.11	0.26 ± 0.09	0.001
	BCO-5	0.23 ± 0.12	0.23 ± 0.12	

A significance level $p < 0.05$ is considered as statistically different.

increase in IL-1β ($p < 0.001$) and IL-2 ($p < 0.001$) and a significant decrease in IL-10 ($p < 0.001$) compared to their respective baseline values (Table 5).

Inter-group comparisons at the end of the study revealed a significant increase in the levels of IL-1β ($p < 0.001$) and IL-2 ($p < 0.001$). The effect sizes of 0.75 (95% CI: 6.31–7.18) and 0.85 (95% CI: 4.69–5.73) were observed in IL-1β and IL-2, respectively. The decrease in IL-10 was also found to be significant, with an effect size of 0.88 (95% CI: 13.08–14.42; $p < 0.001$) (Table 5).

3.10. Safety and adverse events

The results of the hematological and biochemical analyses are given in Supplementary Table S1. All clinical laboratory parameters were within the normal range before and after the study, and there was no significant difference between the groups or within the groups ($p > 0.05$). There were six dropouts from the placebo group and four dropouts from the BCO-5 group. However, none of the dropouts were due to any adverse side effects due to personal reasons. Confirmation of acceptability/tolerability and safety was provided by the satisfaction ratings at the end of the study. It was found that 2% of the participants in the intervention group (17% in the placebo group) were discontented with capsule intake.

4. Discussion

The present study investigated the ability of a novel formulation of black cumin oil extract (BCO-5) to modulate stress, sleep, and immunity when supplemented at a dose of 200 mg/day for 90 days. The interconnection between stress, sleep, and immunity are well established (19). Its mechanisms of action and pathogenesis are also known (20). The novelty of the present study lies in the fact that this is the first report on the positive influence of a botanical extract,

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TABLE 5 Changes in cytokines in participants from baseline to end of study in placebo and BCO-5 treated group and the *p*-values observed upon inter-group and intra-group comparison.

Parameters	Group	Baseline	End of study	*P*-values	
				Inter	Intra
IL-1β (pg/dL)	Placebo	2.41 ± 1.26	2.33 ± 1.79	<0.001	<0.001
	BCO-5	2.53 ± 1.20	6.75 ± 1.03		
IL-2 (pg/dL)	Placebo	1.91 ± 0.48	1.99 ± 0.75	<0.001	<0.001
	BCO-5	1.95 ± 0.57	5.21 ± 1.25		
IL-10 (pg/dL)	Placebo	25.59 ± 2.40	26.52 ± 4.44	<0.001	<0.001
	BCO-5	26.22 ± 3.40	13.76 ± 1.62		

A significance level $p < 0.05$ is considered as statistically different.

especially from a food component, on the safe modulation and alleviation of the stress-sleep-immunity axis. The rationale for the use of BCO-5 in the present study is a previous clinical study that employed polysomnography in healthy subjects with stress and sleep issues (12). Moreover, BCO-5 has also been shown to exhibit enhanced anti-inflammatory, anti-arthritic, acetylcholine esterase inhibitory, and neuroprotective effects in various preclinical studies (9, 10).

The study was conducted on healthy volunteers following a double-blind, placebo-controlled design. The total number of subjects ($n = 72$) enrolled and who completed the study was found to be statistically significant at the 80% power and 5% significance level. The baseline clinical laboratory tests and self-reported stress/sleep scores of the participants indicated that they were healthy but experienced significant sleep issues such as a non-restorative sleep (NRS) pattern due to stress/anxiety. The NRS is characterized by sleep disturbances and unsatisfactory sleep when awakening (21). Globally, more than 40% of the general population suffers from this sleep condition (22).

The primary objective of this study was to monitor the effect of BCO-5 on stress and sleep. To monitor stress, we used the well-validated short form of the Perceived Stress Scale (PSS-14), in which a higher score indicates a higher perception of stress (23). It provides a measure of the degree to which one's life is affected by stress and anxiety. The baseline scores revealed that the participants experienced significant stress and sleep issues due to various reasons on their personal and professional fronts. The stress level (higher score) in the placebo and treated groups was not significantly different at baseline. Supplementation with BCO-5 significantly reduced ($p < 0.001$) the PSS scores on days 45 and 90 indicating its primary efficacy. Moreover, the decrease on day 90 was significantly higher than that on day 45 ($p < 0.001$). At the end of the study, the participants felt more relaxed and placid compared to the placebo group.

The PSQI, one of the most commonly used and well-validated self-reported rating scale, was employed to monitor the seven-component scores, that is sleep quality, sleep latency, sleep duration, sleep disturbance, sleep efficiency, medication use and daytime dysfunction as a measure of the overall sleep quality (15). We observed a significant increase with an effect size of 0.31 ($p < 0.024$) and 0.57 ($p < 0.002$) in sleep quality on days 45 and 90 which indicates peaceful and restorative sleep. Better sleep quality has been shown to improve health, reduce daytime sleepiness, and improve well-being, and psychological functioning (24). Sleep latency is defined as the time that a person falls asleep after turning off lights. Inter-group comparison on the 45th and 90th days revealed a significant decrease in latency scores, with an effect size of 0.31 ($p = 0.03$) and 0.82 ($p < 0.001$), indicating the positive influence of BCO-5. Sleep disturbance is a major issue affecting a high percentage of the population and is a disorder/difficulty in initiating and maintaining sleep. The observation that sleeps disturbance scores were significantly reduced upon supplementation with BCO-5, with an effect size of 0.19 ($p = 0.030$) and 0.20 ($p = 0.043$), respectively on days 45 and 90, further shows its positive impact on sleep. BCO-5 also improved sleep duration, as evidenced by the results with an overall effect size of 0.24 ($p = 0.02$) and 0.33 ($p = 0.001$) respectively on days 45 and 90. Sleep duration refers to sleep obtained, either nocturnal or within 24-h time (25), and it was correlated with an increased risk of developing hypertension, diabetes, obesity, depression, heart attack, and stroke (26).

Sleep efficiency, which is the ratio of total sleep time to the time spent in bed, is another important component of the PSQI that captures the core problem for those suffering from insomnia. Poor sleep efficiency can increase daytime sleepiness and contribute to sleep debt (27). Supplementation with BCO-5 significantly improved sleep efficacy with an effect size of 0.56 and 0.47 on days 45 and 90, respectively. Moreover, BCO-5 has also shown a significant decrease in daytime dysfunction and inability to perform daily functions. The correlation between PSQI and PSS-14 was further evaluated using Pearson's correlation test and was found to be significant indicating the potential role of BCO-5 in reducing stress and improving sleep quality (-0.314; $p < 0.05$). In contrast, the placebo group showed a positive correlation at the end of the study (0.178; $p = 0.357$). Our findings were also in agreement with a previous report by Shelar et al. (28), who found a negative correlation between PSQI and PSS-14, reinforcing the relationship between reduced stress and increased sleep.

Furthermore, the measurement of salivary melatonin, cortisol, plasma orexin A, and cytokines provided an insight into the molecular basis of the action of BCO-5 on stress and sleep. Recently, there has been great interest in the development of Dual Orexin Receptor Antagonists (DORA) for treating chronic insomnia because orexin agonism during the day promotes wakefulness, and orexin receptor antagonists can promote sleep signals by enhancing melatonin levels (29, 30). Stress stimuli can generate orexins (orexin A and B) which express the corticotropin-releasing hormone (CRH). CRH binds to the anterior pituitary gland to release adrenocorticotropic hormone (ACTH), which further binds to its receptor in the adrenal cortex and releases cortisol, the major hormone produced in response to stress (31). Therefore, orexins act as molecular switches for the release of

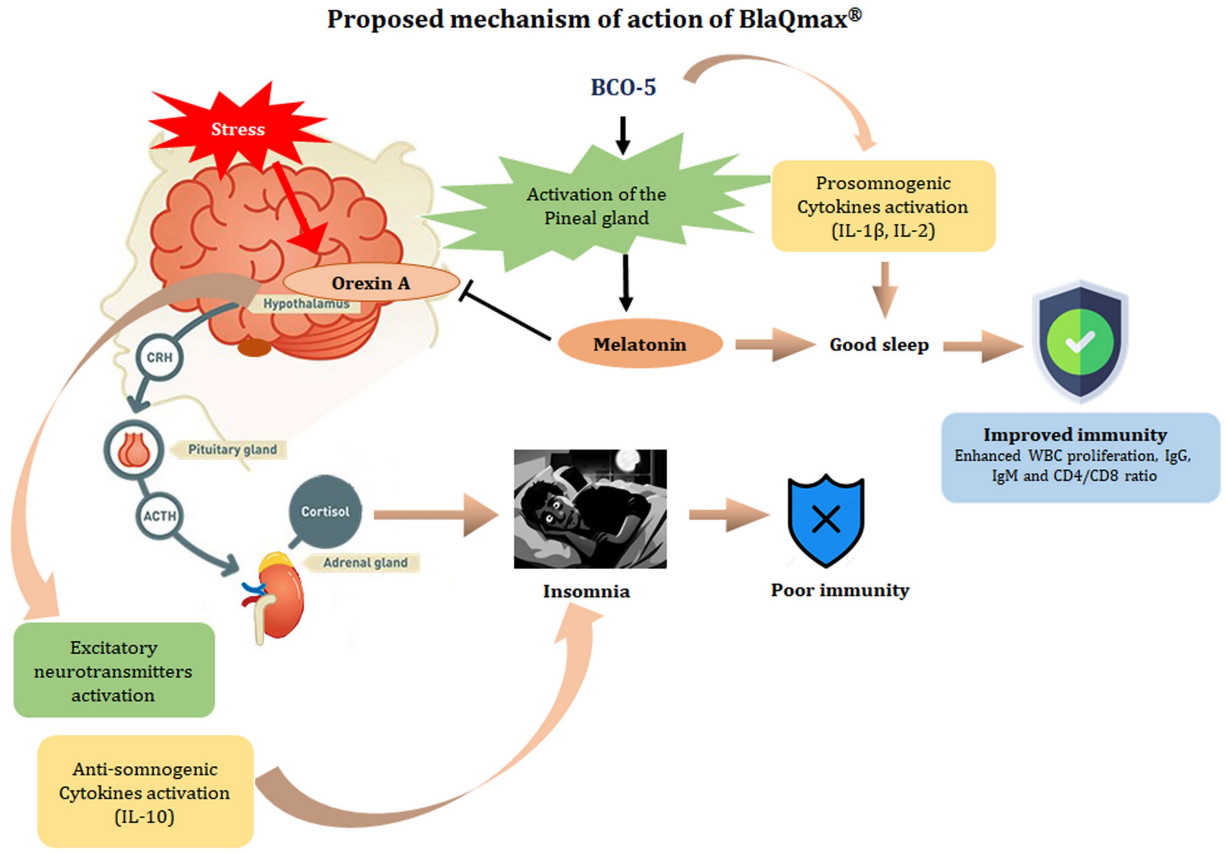

FIGURE 7
Proposed mechanism of action of BCO-5. Orexins (Orexin A and Orexin B) are the key neuropeptides produced in the hypothalamus and interact with the hypothalamic–pituitary–adrenal axis (HPA) to regulate sleep–wake cycle and neuroendocrine activation associated with stress. Orexins cause the release of corticotropin-releasing hormone (CRH) in the hypothalamus, which triggers the anterior pituitary to secrete adrenocortical-tropic hormone (ACTH), stimulating the release of cortisol – the major stress hormone, and promote wakefulness. In the nervous system, orexins result in the release of excitatory neurotransmitters such as dopamine, epinephrine, glutamate, and acetylcholine which further result in alertness/wakefulness. BCO-5 acts on the pineal gland and releases melatonin which acts as a natural inhibitor of orexins and thereby regulate HPA axis to reduce stress and promote sleep. In other words, BCO-5 act as a dual orexin receptor antagonist (DORA).

cortisol in response to a stress stimulus and regulate the sleep/wake cycle (Figure 7) (4). Melatonin, a hormone referred to as "the light of night," has been established as a natural inhibitor of orexins; hence, it can reduce stress by reducing cortisol levels and improve sleep quality (32). In agreement with this molecular mechanism, our results showed a significant increase in the salivary melatonin levels among the BCO-5 group, with a large effect size of 0.85 ($p = 0.009$) and a decrease in cortisol level with a large effect size of 0.72 ($p = 0.013$). The ability of BCO-5 to increase melatonin levels may be explained on the basis of previous studies showing that black cumin increases the concentration of 5-hydroxy tryptophan and tryptophan in the rat brain and plasma (33). Tryptophan and 5-hydroxy tryptophan can increase the concentration of serotonin, the biosynthetic precursor of melatonin (34, 35). We also observed a significant reduction in the plasma concentration of orexin A among the BCO-5 participants (0.92; $p < 0.001$), indicating that BCO-5 acts as a natural DORA. The molecular mechanism of BCO-5 in stress and sleep are depicted in Figure 7.

Sleep loss has been reported to trigger stress and elevate the plasma levels of various cytokines (36). Cytokines that alter sleep in humans and animals have been identified as IL-1β, IL-2, IL-4, IL-10, IL-13, IL-15, TNF-α, IFN-γ, and macrophage inhibitory protein-1β (MIP-1β), in which IL-1β, IFN-γ, IL-2, and TNF-α are considered pro-somnogenic cytokines (supporting sleep) and IL-4, IL-15, IL-10, and IL-13 were observed with sleep deprivation (anti-somnogenic) (5); IL-1β, IL-2, and IL-10 are the mainly studied cytokines that are known to be involved in sleep regulation (37). Our findings showed an elevated level of pro-somnogenic cytokines IL-1β and IL-2 and a decrease in anti-somnogenic IL-10, indicating the positive effect of BCO-5 on sleep modulation. Previous studies have also reported elevated levels of IL-2 and IL-1β upon improved sleep and an elevation in IL-10 levels upon sleep disorders (19, 38, 39).

Sleep and immunity are also interconnected. Epidemiological studies have shown that poor sleep increases susceptibility to diseases (1, 5, 40). Various biochemical studies have demonstrated that sleep loss can alter immune responses in both animals and humans (41–43). Leukocyte proliferation, humoral immunity, cell-mediated immunity, and immunoglobulin levels are reduced in participants with prolonged sleep disorders (44). Two or three nights of sleep deprivation can significantly decrease the neutrophil, lymphocyte and leukocyte counts (40). Recently, it has also been shown that every 60-min increase in sleep duration was associated with a significant increase in total leukocytes, lymphocytes, neutrophils, and monocyte count ($p < 0.001$) (45). Our results for the differential count at baseline agree

with this observation. We found that BCO-5 supplementation significantly enhanced leukocyte proliferation, as evident from the increase in the total leukocyte, lymphocytes, and monocytes counts.

Immunoglobulins (Ig) or antibodies are glycoproteins produced by plasma B cells which are critical for immune response and hence important markers of immunomodulatory effects. IgA, IgG, IgM, IgE, and IgD are immunoglobulins found in humans. IgG accounts for the most abundant immunoglobulin (75%) (46), whereas IgM is the largest antibody, accounting for approximately 5% of all antibodies in the serum. Immunoglobulins quickly recognize and initiate an immune response by directly neutralizing pathogens or clearing novel antigens (47, 48). Although various studies have attempted to correlate immunoglobulin levels with sleep disorders, the results are controversial and require further clarity. Everson reported a significant increase in IgG, IgM, and IgA levels after 3–5 days of sleep deprivation (49). However, other studies were unable to replicate this result and reported either no change or reduction in immunoglobulin levels upon sleep deprivation (40, 50). Our results indicated a significant increase in IgG and IgM levels in BCO-5 participants; however, the increase was within the normal range (48).

Cell-mediated immunity plays a critical role in antiviral responses (51). The absolute count of CD4+ T lymphocytes was significantly increased. This correlates with the increased levels of IL-2, a pro-somnogenic cytokine released by CD4+ cells. CD4+ cells are also known to promote immunoglobulins. However, a significant reduction was observed in the number of CD8 cells. The CD4/CD8 ratio is a direct marker of immune status, showing a decreasing trend in the placebo group and a significant increase in the BCO-5 group. In a previous study, Salem et al. reported a significant immunomodulatory effect of black cumin seed powder, with a significant increase in immunoglobulin, CD4+ cell absolute count, and lymphocyte counts (52). Certain animal studies have also reported immunomodulatory effects of black cumin (53–55).

The absence of significant side effects, adverse events, or toxic deviations in clinical laboratory parameters (biochemical/hematological) indicated its safety and suitability for supplementation. A detailed safety assessment of BCO-5 has recently been published, which also revealed no significant toxic effects (13). Acute and sub-chronic toxicity studies have also established the safety of BCO-5 and its optimized, safe dosage for human consumption of 900 mg/kg/day (56). The fact that more than 70% of the participants reported significant improvement in their sleep pattern by day 7 indicates the relatively fast action of BCO-5 as a natural stress/sleep aid.

The lack of an objective assessment of sleep parameters and the measurement of neurotransmitters and markers of innate immunity may be considered as limitation of the present study. Similarly, investigations of the bioavailability of the key bioactive molecules in BCO-5 would provide better insights into its mechanism of action.

5. Conclusion

In summary, the present randomized double-blinded placebo-controlled trial demonstrated the efficacy of a proprietary black cumin extract (BCO-5) to safely modulate the stress-sleep-immunity axis and further alleviate stress, establish restorative and restful sleep, and improve immunity among healthy subjects characterized by non-restorative sleep conditions. While PSS-14 demonstrated a significant reduction in stress, PSQI analysis established a positive effect of BCO-5 on sleep quality, sleep latency, sleep duration, and overall sleep efficiency when supplemented at a single dose of 200 mg/day. The percentage of participants who were satisfied with a single dose confirmed the rapid action of BCO-5. The improvements in stress and sleep reported by participants in the PSS-14 and PSQI questionnaires were in agreement with the changes in salivary melatonin and cortisol levels, plasma orexin, and cytokine levels. The influence of BCO-5 on immunity status was also clear from the differential counts, immunoglobulins, cytokines, and CD4+, CD8+, and CD4/CD8 ratios. The therapeutic potential of BCO-5 may be attributed to the pleiotropic mechanism of action that regulates the HPA axis and thereby modulates stress, sleep, and immunity functions, the three interconnected factors of optimized health, but with different pathways. Future studies should investigate the short-term effects of BCO-5 on various sleep problems by employing techniques such as actigraphy and its influence on anxiety, depression, and mood. Detailed investigation of the molecular mechanism of action is also recommended.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the Balagangadharanatha Swami Global Institute of Medical Sciences Institutional Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

Author contributions

MEM: recruitment and investigation of the trial. JT: conduct of the trial, data collection, and biochemical analysis. PP: drafting the original article. SD and PP: data analysis. MCM: review and editing of the original article. BP: principal investigator who supervised the study and was involved in the protocol development, data interpretation, review, and editing of the original article. All authors contributed to the article and approved the submitted version.

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Conflict of interest

BCO-5 used in the present study is a patented black cumin extract developed by Akay Natural Ingredients, Cochin, India and registered as BlaQmax®. PP and SD were employed by Akay Natural Ingredients. JT was employed by Leads Clinical Research and Bio Services Private Limited.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1152680/full#supplementary-material

References

1. Besedovsky L, Lange T, Haack M. The sleep-immune crosstalk in health and disease. *Physiol Rev*. (2019) 99:1325–80. doi: 10.1152/physrev.00010.2018

2. Tsigos C, Kyrou I, Kassi E, Chrousos GP. Stress: endocrine physiology and pathophysiology. *Endotext*. (2020)

3. Phan T, Malkani R. Sleep and circadian rhythm disruption and stress intersect in Alzheimer's disease. *Neurobiol Stress*. (2019):100133:10. doi: 10.1016/j.ynstr.2018.10.001

4. Akça ÖF, Uzun N, Kılınç İ. Orexin A in adolescents with anxiety disorders. *Int J Psychiatry Clin Pract*. (2020) 24:127–34. doi: 10.1080/13651501.2019.1711425

5. Garbarino S, Lanteri P, Bragazzi NL, Magnavita N, Scoditti E. Role of sleep deprivation in immune-related disease risk and outcomes. *Commun Biol*. (2021) 4:1–17. doi: 10.1038/s42003-021-02825-4

6. Hannan MA, Rahman MA, Sohag AAM, Uddin MJ, Dash R, Sikder MH, et al. Black cumin (*Nigella sativa* L.): A comprehensive review on Phytochemistry, health benefits, molecular pharmacology, and safety. *Nutrients*. (2021) 13:1784. doi: 10.3390/nu13061784

7. Ambati RR, Ramadan MF. *Nigella sativa* seed extracts in functional foods and nutraceutical applications In: M Fawzy Ramadan, editor. *Black cumin (Nigella sativa) seeds: Chemistry, Technology, Functionality, and Applications. Food Bioactive Ingredients*. Cham: Springer (2021)

8. Fawzy M, Hassanien R. *Black cumin (Nigella sativa) seeds: Chemistry, technology, functionality, and applications. Food bioactive ingredients*. Cham: Springer (2021).

9. Ratheesh M, Svenia JP, Sangeeth S, Sheethal S, Sony R, Sandya S, et al. Antioxidant, anti-inflammatory, and anti-arthritic effect of thymoquinone-rich black cumin (*Nigella sativa*) oil (BlaQmax®) on adjuvant-induced arthritis. *J Food Res*. (2021) 10:52. doi: 10.5539/jfr.v10n1p52

10. Ramalingam K, Ittiyavirah SP, Jyoti H. Acetylcholinesterase and growth inhibitory effects - various grades of *N. Sativa* oils. *Inter J Pharm Sci*. (2018) 10:245–50. doi: 10.13040/IJPSR.0975-8232.10(1).245-50

11. Ittiyavirah SP, Ramalingam K, Sathyan A, Rajasree RS, Kuruniyan MS, Quadri SA, et al. Thymoquinone-rich black cumin oil attenuates ibotenic acid-induced excitotoxicity through glutamate receptors in Wistar rats. *Saudi Pharma J*. (2022) 30:1781–90. doi: 10.1016/j.jsps.2022.10.007

12. Das SS, Ramalingam K, George S, Chakrapani BPS, Maliakel B, Ittiyavirah S, et al. Thymoquinone-rich black cumin oil improves sleep quality, alleviates anxiety/stress on healthy subjects with sleep disturbances– A pilot polysomnography study. *J Herb Med*. (2022) 32:100507. doi: 10.1016/j.hermed.2021.100507

13. Thomas JV, Mohan ME, Prabhakaran P, Das SS, Maliakel B, Krishnakumar IM. A phase I clinical trial to evaluate the safety of thymoquinone-rich black cumin oil (BlaQmax®) on healthy subjects: randomized, double-blinded, placebo-controlled prospective study. *Toxicol Rep*. (2022) 9:999–1007. doi: 10.1016/j.toxrep.2022.04.020

14. Thiagarajah K, Chee HP, Sit NW. Effect of alpha-S1-casein tryptic hydrolysate and L-Theanine on poor sleep quality: A double blind, randomized placebo-controlled crossover trial. *Nutrients*. (2022) 14:652. doi: 10.3390/nu14030652

15. Zhang C, Zhang H, Zhao M, Li Z, Cook CE, Buysse DJ, et al. Reliability, validity, and factor structure of Pittsburgh sleep quality index in community-based centenarians. *Front Psychiatry*. (2020):573530:11. doi: 10.3389/fpsyt.2020.573530

16. Zhong QY, Gelaye B, Sánchez SE, Williams MA. Psychometric properties of the Pittsburgh sleep quality index (PSQI) in a cohort of Peruvian pregnant women. *J Clin Sleep Med*. (2015) 11:869–77. doi: 10.5664/jcsm.4936

17. Barutcu Atas D, Aydin Sunbul E, Velioglu A, Tuglular S. The association between perceived stress with sleep quality, insomnia, anxiety, and depression in kidney transplant recipients during COVID-19 pandemic. *PLoS One*. (2021) 16:e0248117. doi: 10.1371/journal.pone.0248117

18. Arneth BM. Activation of CD4 and CD8 T cell receptors and regulatory T cells in response to human proteins. *Peer J*. (2018) 6:e4462. doi: 10.7717/peerj.4462

19. Lange T, Dimitrov S, Born J. Effects of sleep and circadian rhythm on the human immune system. *Ann N Y Acad Sci*. (2010) 1193:48–59. doi: 10.1111/j.1749-6632.2009.05300.x

20. Asif N, Iqbal R, Nazir CF. Human immune system during sleep. *Am J Clin Exp Immunol*. (2017) 6:92–6.

21. Chen N, Fong DYT, Li S, Wong JYH. Association between non-restorative sleep and quality of life in Chinese adolescents. *Int J Environ Res Public Health*. (2020) 17:1–10. doi: 10.3390/ijerph17197249

22. Tinajero R, Williams PG, Cribbet MR, Rau HK, Bride DL, Suchy Y. Nonrestorative sleep in healthy, young adults without insomnia: associations with executive functioning, fatigue, and pre-sleep arousal. *Sleep Health*. (2018) 4:284–91. doi: 10.1016/j.sleh.2018.02.006

23. Vallejo MA, Vallejo-Slocker L, Fernández-Abascal EG, Mañanes G. Determining factors for stress perception assessed with the perceived stress scale (PSS-4) in Spanish and other European samples. *Front Psychol*. (2018):37:9. doi: 10.3389/fpsyg.2018.00037

24. Harvey AG, Stinson K, Whitaker KL, Moskovitz D, Virk H. The subjective meaning of sleep quality: A comparison of individuals with and without insomnia. *Sleep*. (2008) 31:383–93. doi: 10.1093/sleep/31.3.383

25. Kline C. Sleep duration In: MD Gellman and JR Turner, editors. *Encyclopedia of Behavioral Medicine*. New York, NY: Springer (2013). 1808–10.

26. Institute of Medicine (US) Committee on Sleep Medicine and Research In: H Colten and B Altevogt, editors. *Sleep Disorders and Sleep Deprivation: An Unmet Public Health Problem*. Washington (DC): US National Academies Press (2006)

27. Reed DL, Sacco WP. Measuring sleep efficiency: what should the denominator be? *J Clin Sleep Med*. (2016) 12:263–6. doi: 10.5664/jcsm.5498

28. Tejas S, Krutika G, Mukta B, Waqas A. A study of perceived stress and sleep deprivation in an urban Indian population. *J Pharm Negat Results*. (2022) 13:7273–9. doi: 10.47750/pnr.2022.13.S07.875

29. Steiner MA, Michel A, Yanagisawa M, Clozel MM. The orexin system: basic science and role in sleep pathology. *Front Neurol Neurosci*. (2021) 45:22–37. doi: 10.1159/isbn.978-3-318-06844-3

30. Robinson CL, Supra R, Downs E, Kataria S, Parker K, Kaye AD, et al. Daridorexant for the treatment of insomnia. *Health Psychol Res*. (2022) 10:37400. doi: 10.52965/001c.37400

31. Veldhuis JD, Sharma A, Roelfsema F. Age-dependent and gender-dependent regulation of hypothalamic-adrenocorticotropic-adrenal axis. *Endocrinol Metab Clin N Am*. (2013) 42:201–25. doi: 10.1016/j.ecl.2013.02.002

32. Campino C, Valenzuela FJ, Torres-Farfan C, Reynolds HE, Abarzua-Catalan L, Arteaga E, et al. Melatonin exerts direct inhibitory actions on ACTH responses in the human adrenal gland. *Horm Metab Res*. (2011) 43:337–42. doi: 10.1055/s-0031-1271693

33. Tas U, Ayan M, Sogut E, Kuloglu T, Uysal M, Tanriverdi H, et al. Protective effects of thymoquinone and melatonin on intestinal ischemia-reperfusion injury. *Saudi J Gastroenterol*. (2015) 21:284–9. doi: 10.4103/1319-3767.166203

34. Perveen T, Haider S, Zuberi NA, Saleem S, Sadaf S, Batool Z. Increased 5-HT levels following repeated administration of *Nigella sativa* L. (Black Seed) oil produce

antidepressant effects in rats. *Sci Pharm*. (2014) 82:161–70. doi: 10.3797/scipharm.1304-19

35. Erland LAE, Saxena PK. Melatonin natural health products and supplements: presence of serotonin and significant variability of melatonin content. *J Clin Sleep Med*. (2017) 13:275–81. doi: 10.5664/jcsm.6462

36. Irwin MR, Carrillo C, Olmstead R. Sleep loss activates cellular markers of inflammation: sex differences. *Brain Behav Immun*. (2010) 24:54–7. doi: 10.1016/j.bbi.2009.06.001

37. Krueger JM, Majde JA, Rector DM. Cytokines in immune function and sleep regulation. *Handb Clin Neurol*. (2011) 98:229–40. doi: 10.1016/B978-0-444-52006-7.00015-0

38. Besedovsky L, Lange T, Born J. Sleep, and immune function. *Pflugers Arch*. (2012) 463:121–37. doi: 10.1007/s00424-011-1044-0

39. Rethorst CD, Greer TL, Toups MSP, Bernstein I, Carmody TJ, Trivedi MH. IL-1β and BDNF are associated with improvement in hypersomnia but not insomnia following exercise in major depressive disorder. *Transl Psychiatry*. (2015) 5:e611. doi: 10.1038/tp.2015.104

40. Ruiz FS, Andersen ML, Martins RCS, Zager A, Lopes JD, Tufik S. Immune alterations after selective rapid eye movement or total sleep deprivation in healthy male volunteers. *Innate Immun*. (2012) 18:44–54. doi: 10.1177/1753425910385962

41. Heiser P, Dickhaus B, Schreiber W, Clement HW, Hasse C, Hennig J, et al. White blood cells and cortisol after sleep deprivation and recovery sleep in humans. *Eur Arch Psychiatry Clin Neurosci*. (2000) 250:16–23. doi: 10.1007/PL00007534

42. Ruiz FS, Andersen ML, Zager A, Martins RCS, Tufik S. Sleep deprivation reduces the lymphocyte count in a non-obese mouse model of type 1 diabetes mellitus. *Braz J Med Biol Res*. (2007) 40:633–7. doi: 10.1590/S0100-879X2007000500005

43. Zager A, Andersen ML, Ruiz FS, Antunes IB, Tufik S. Effects of acute and chronic sleep loss on immune modulation of rats. *Am J Physiol Regul Integr Comp Physiol*. (2007) 293:R504–9. doi: 10.1152/ajpregu.00105.2007

44. Nie L, Pan XL, Zhang XB, Zhang S, Rao JX, Su ZF. Research on the correlation of immunity in patients with chronic insomnia. *Front Psychiatry*. (2022):1034405:13. doi: 10.3389/fpsyt.2022.1034405

45. Hoopes EK, D'Agata MN, Berube FR, Ranadive SM, Patterson F, Farquhar WB, et al. Consistency where it counts: sleep regularity is associated with circulating white blood cell count in young adults. *Brain Behav Immun Health*. (2021) 13:100233. doi: 10.1016/j.bbih.2021.100233

46. Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: from structure to effector functions. *Front Immunol*. (2014) 5:520. doi: 10.3389/fimmu.2014.00520

47. Sathe A, Cusick J. *Biochemistry, immunoglobulin M [Internet]*. Treasure Island (FL): StatPearls Publishing (2021).

48. Justiz VA, Jamal Z, Patel P, Kamleshun R. *Immunoglobulin*. Treasure Island (FL): StatPearls Publishing (2022).

49. Everson CA. Clinical assessment of blood leukocytes, serum cytokines, and serum immunoglobulins as responses to sleep deprivation in laboratory rats. *Am J Physiol Regul Integr Comp Physiol*. (2005) 289:R1054–63. doi: 10.1152/ajpregu.00021.2005

50. Hui L, Hua F, Diandong H, Hong Y. Effects of sleep and sleep deprivation on immunoglobulins and complement in humans. *Brain Behav Immun*. (2007) 21:308–10. doi: 10.1016/j.bbi.2006.09.005

51. Ganji A, Farahani I, Khansarinejad B, Ghazavi A, Mosayebi G. Increased expression of CD8 marker on T-cells in COVID-19 patients. *Blood Cells Mol Dis*. (2020) 83:102437. doi: 10.1016/j.bcmd.2020.102437

52. Salem A, Bamosa A, Alam M, Alshuraim S, Alyalak H, Alagga A, et al. Effect of *Nigella sativa* on general health and immune system in young healthy volunteers; a randomized, placebo-controlled, double-blinded clinical trial. *F1000 Res*. (2021) 10:1199. doi: 10.12688/f1000research.73524.1

53. Rosidah , Pratiwy FM. A mini-review: potential of *Nigella sativa* L. for immune fish against pathogen bacteria attack. *Int J Fish Aquat Stud*. (2022) 10:01–6. doi: 10.22271/fish.2022.v10.i4b.2695

54. Mahmoud HS, Almallah AA, Gad EL-Hak HN, Aldayel TS, Abdelrazek HMA, Khaled HE. The effect of dietary supplementation with *Nigella sativa* (black seeds) mediates immunological function in male Wistar rats. *Sci Rep*. (2021) 11:7542. doi: 10.1038/s41598-021-86721-1

55. Ghonime M, Eldomany R, Abdelaziz A, Soliman H. Evaluation of immunomodulatory effect of three herbal plants growing in Egypt. *Immunopharmacol Immunotoxicol*. (2011) 33:141–5. doi: 10.3109/08923973.2010.487490

56. Ramalingam K, Kuttan R, Sivadasan S, Ittiyavirah S, Madhavamenon KI. Safety assessment of thymoquinone-rich black cumin (*Nigella sativa*) oil (BlaQmax): acute and sub-chronic toxicity studies. *J Nutr Food Sci*. (2021) 11:811.

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A proprietary black cumin oil extract (*Nigella sativa*) (BlaQmax®) modulates stress-sleep-immunity axis safely: Randomized double-blind placebo-controlled study

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Objective: Stress, sleep, and immunity are important interdependent factors that play critical roles in the maintenance of health. It has been established that stress can affect sleep, and the quality and duration of sleep significantly impact immunity. However, single drugs capable of targeting these factors are limited because of their multi-targeting mechanisms. The present study investigated the influence of a proprietary thymoquinone-rich black cumin oil extract (BCO-5) in modulating stress, sleep, and immunity.

Methods: A randomized double-blinded placebo-controlled study was carried out on healthy volunteers with self-reported non-refreshing sleep issues ($n=72$), followed by supplementation with BCO-5/placebo at 200 mg/day for 90 days. Validated questionnaires, PSQI and PSS, were employed for monitoring sleep and stress respectively, along with the measurement of cortisol and melatonin levels. Immunity markers were analyzed at the end of the study.

Results: In the BCO-5 group, 70% of the participants reported satisfaction with their sleep pattern on day 7 and 79% on day 14. Additionally, both inter- and intra- group analyses of the total PSQI scores and component scores (sleep latency, duration, efficiency, quality, and daytime dysfunction) on days 45 and 90 showed the effectiveness of BCO-5 in the improvement of sleep ($p<0.05$). PSS-14 analysis revealed a significant reduction in stress, upon both intra ($p<0.001$) and inter-group ($p<0.001$) comparisons. The observed reduction in stress among the BCO-5 group, with respect to the placebo, was significant with an effect size of 1.19 by the end of the study ($p<0.001$). A significant correlation was also observed between improved sleep and reduced stress as evident from PSQI and PSS. Furthermore, there was a significant modulation in melatonin, cortisol, and orexin levels. Hematological/immunological parameters further revealed the immunomodulatory effects of BCO-5.

Conclusion: BCO-5 significantly modulated the stress-sleep-immunity axis with no side effects and restored restful sleep.

KEYWORDS

black cumin, Pittsburgh Sleep Quality Index, Perceived Stress Scale, stress, non-refreshing sleep, sleep quality, immunity

1. Introduction

Stress, insomnia, and immunity are the three major interconnected factors, that play key roles in the maintenance of health. It has been established that stress can negatively affect the quality and duration of sleep, which in turn impacts immunity (1). Stress, a mental condition generated by either extrinsic or intrinsic factors, may lead to various psychological, biological, and/or social issues. It has been identified as a major factor associated with sleep disorders. Since sleep is essential for energy, cell tissue repair, metabolic regulation, thermoregulation, cognition, motor actions, and immune functions (1, 2), it has been proposed that the 'stress-sleep-immunity' axis is critical for health (2).

Sleep and stress share multiple pathways and affect the central nervous system and circadian rhythm, leading to dysfunctions in metabolism, brain function and immunity (3). The hypothalamus-pituitary–adrenal axis (HPA) is the central system responsible for the neuroendocrine adaptation of the stress response. Orexins or hypocretins (Orexin A and Orexin B) are excitatory neuropeptides produced in the hypothalamus in response to stress stimuli (4). It modulates the activity of the HPA axis and autonomic nervous system to regulate the sleep–wake cycle, cognitive functioning, stress processing, and the metabolic and inflammatory responses (4). Orexins are also responsible for the release of cortisol, a major hormone released in response to stress (4).

Sleep and immune functions are interconnected. The sleep–wake cycle is one of the most important manifestations of the circadian rhythm, and its changes affect physical and mental activities, major organ functions, temperature regulation and immunomodulatory effects such as leukocytes and cytokine production and proliferation (1). It has been reported that one to three nights of sleep deprivation can lead to a significant elevation of inflammatory cytokines and can significantly reduce leukocyte, lymphocyte, and neutrophil counts (5).

Despite the importance of stress, sleep, and immunity, drugs/supplements capable of safely modulating the stress-sleep-immunity axis are limited because they act *via* multi-targeted mechanisms and side effects, including dependency, are common. *Nigella sativa*, commonly known as black cumin or black seed, is a culinary spice and an age-old medicinal herb with a wide range of health-beneficial pharmacological effects to attenuate oxidative stress, inflammation, immunity, energy metabolism, and cell survival (6, 7). Black cumin oil composed of both volatile and fixed oil fractions was identified as the major bioactive component of black cumin, in which thymoquinone (TQ) is considered the most active molecule (8). Black cumin has been reported to have antioxidant, anti-inflammatory, and neuroprotective effects as a function of its TQ content (6, 9–11). Recently, it was also reported that a novel formulation of black cumin oil extract containing TQ and carvacrol in a 10:1 (w/w) ratio (BCO-5) significantly alleviated stress and improved sleep quality in human volunteers when supplemented at 200 mg/day for 28 days (12). Jestin et al. conducted a 90-days safety assessment of BCO-5 among healthy human volunteers and established its safety for human consumption (13).

Based on previous studies, we hypothesized that BCO-5 would modulate the stress-sleep-immunity axis by reducing stress, improving sleep quality, and hence, the immunity. Thus, the present randomized, double-blinded, placebo-controlled study investigated the efficacy of BCO-5 in healthy subjects (25–65 years old) with significant stress and non-restorative sleep. Validated questionnaires were employed to analyze sleep quality [Pittsburgh Sleep Quality Index (PSQI)] and stress [Perceived Stress Scale (PSS)], along with changes in sleep and immune biomarkers.

2. Materials and methods

The proprietary formulation of black cumin oil used in this study (BCO-5; Patented and Registered as BlaQmax®) was manufactured by Akay Natural Ingredients, Cochin, India, following good manufacturing practices (Batch no: BCOQ 32/21 dated 12/04/2021). The dried black cumin seeds used for the manufacture of BCO-5 were identified and authenticated by a botanist, and the specimens were deposited at the Herbarium of Akay Natural Ingredients, Cochin, India (Voucher no: AK-NS-018). High-performance thin-layer liquid chromatography (HPTLC) (CAMAG HPTLC system, Switzerland) was employed to identify black cumin, as previously reported (13). The thymoquinone content was determined by high-performance liquid chromatography (HPLC) (Shimadzu Analytical India Private Limited, Mumbai, India) analysis, as reported previously (13). Analytical standard for TQ (CAS No: 490–91-5) was obtained from Sigma-Aldrich (Bangalore, India).

2.1. Subjects and design

In this randomized, double-blinded, placebo-controlled clinical trial, 150 volunteers (aged 25 to 65 years; healthy subjects) who were reported to experience significant stress and self-reported sleep issues such as non-restorative sleep, waking up at night multiple times, or having difficulty in a sound sleep for the past 4 weeks were selected. Volunteers were identified from the database of the contract research organization and the hospital where the study was conducted. After the initial description of the study, 96 participants were willing to participate and were further screened according to the inclusion and exclusion criteria (Table 1). Twenty-four subjects were eliminated after screening, and 72 volunteers were randomized into BCO-5 and placebo

TABLE 1 Inclusion and exclusion criteria.

Inclusion criteria
1. Healthy participants aged 25–65 years (both inclusive)
2. Participants with PSQI scores ≥5
3. Participants with PSS score between 14 and 26
4. Participants with body weight ≥ 50 kg
5. Approved birth control measures should be perceived by female participants of childbearing age and should have negative urine pregnancy test at the screening
6. Participants should refrain from smoking, caffeinated beverages, and alcohol consumption
7. Signed and informed consent should be provided by the participant and the participant should understand the study protocol
Exclusion criteria
1. Participants requiring medical treatment and suffering from health conditions like hypertension, diabetes, chronic renal failure, heart, thyroid and liver disease
2. Participants with hepatic or renal impairment (Alanine transaminase/Aspartate transaminase levels >3 upper limit of normal) (serum creatinine ≥2.0 mg/dl)
3. Subjects with history of conditions such as endocrine abnormalities including thyroid disease, psychiatric illness, drug abuse, smoking, addiction to alcohol, psychiatric illness
4. Participants who have went cardiovascular surgery or any other major surgery
5. Immuno-compromised state participants and those with immunodeficiency diseases like, HIV or Hepatitis B
6. Participants allergic to the composition of investigational product
7. Pregnant and lactating women
8. Participants with significant illness history or any medical derangements that can interfere with subject treatment, assessment, or compliance with the protocol
9. Participants currently participating or have participated in any other clinical trial, 1 month prior to start of this study
Any additional condition(s) that in the Investigators opinion would warrant exclusion from the study or prevent the subject from completing the study.

groups ($n = 36$/group). The study was conducted at the BGS Global Institute of Medical Sciences, Bangalore, India, under the guidance of a registered medical practitioner, according to the guidelines of the Clinical Trial Registry of India (CTRI/2021/05/033780 dated 25/05/2021). The protocol was reviewed and approved by the institutional ethics committee. Written informed consent was obtained from all subjects prior to the study. A cohort diagram representing the study design is shown in Figure 1.

The participants were requested to visit the study site on four different occasions; Visit I (day 0), Visit II (day 1), Visit III (day 45) and Visit IV (day 90/ end of study). During visit I, screening was performed against the inclusion/exclusion criteria, which included a structured medical interview and diagnosis as well as demographic and anthropometric measurements. The primary selection criterion was a PSQI score greater than 5. During visit II (day 1), the participants were asked to report at the study site with 10 h of fasting and were randomized, based on a computer-generated randomization code, into two groups to receive either a placebo or intervention. Blood (10 mL) was withdrawn for routine laboratory clinical parameters (biochemical and hematological) and markers of immunity. The baseline PSQI and PSS-14 scores were also recorded. Similar visits for blood and data collection were requested on day 45 (Visit III) and at the end of the study period, day 90 (Visit IV). Telephonic interviews were also performed on days 7 and 14 to inquire about adverse events, side effects, tolerance, and efficacy.

2.2. Sample size and randomization

Sample size calculation was performed using the G Power Statistical Software (3.1.9.7 Version, Franz Faul, University of Kiel, Kiel, Germany) (14). It was estimated that a total of 35 participants per group would be required, with an anticipated non-compliance or dropout rate of 20%, yielding 80% power, and 5% significance level. Participants were randomly allocated by permuted-block randomization (block size = 4) using a computer-generated allocation table.[1]

2.3. Intervention and dosage

Airtight high density polyethylene containers comprising 95 soft gel capsules (200 ± 10 mg extract/capsule) with either BCO-5 (intervention) or placebo were sequentially coded and provided to the participants. They were requested to consume one capsule daily after dinner, 20–30 min prior to bedtime. The total study duration was 90 ± 2 days. At the end of the study period (Visit IV), the remaining capsules were recorded. The effectiveness of participant blinding was evaluated by asking participants to predict the allocation (placebo/ intervention).

2.4. Pittsburgh Sleep Quality Index

The PSQI is a 19- item self-reported questionnaire designed to determine the overall sleep quality and disturbances over a period of one-month tenure. Four-point Likert scale is used to rate the severity/ frequency of the problems in such a way that '0' = not during the past month, '1' = less than once in a week, '2' = Once or twice a week, '3' = Three or more times a week. Each component yields a score ranging from 0 to 3, and the component scores are summed to yield a global PSQI score (from 0 to 21). A higher score indicates lower sleep quality (15). A score greater than 5indicates poor sleep quality or insomnia (16).

1 www.randomization.com

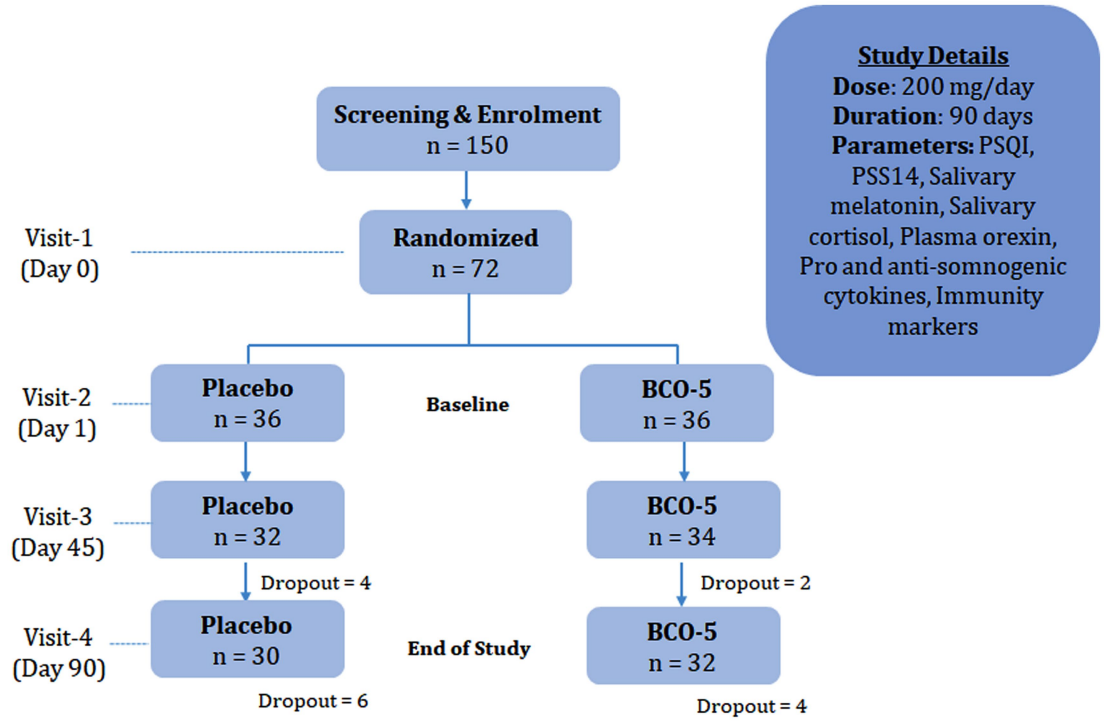

FIGURE 1
Cohort diagram depicting study design.

2.5. Perceived Stress Scale- 14 (PSS-14)

The PSS questionnaire was developed by Cohen et al. in 1983 to evaluate the perception of stressful conditions in a person's life based on their last one-month experience. It is the degree to which situations in life are appraised using a 14-item scale. A 5-point Likert scale, ranging from 0 (never) to 4 (very often), was used to evaluate the stress experienced. The PSS scores ranges from 0 to 56; with higher scores indicating higher perceived stress (17). Scores ranging to 0–18 indicate low stress; to 19–37 implies moderate, and to 38–56 as high stress.

2.6. Sleep and stress biomarkers

2.6.1. Estimation of salivary cortisol and melatonin

Cortisol and melatonin levels from the salivary samples were estimated using ELISA Kit methods of Neogen Corporation, KY, United States (Catalog No: 402710) and IBL-International, Germany (Catalog No: RE54041), respectively. Micro ELISA 96 well plates were used for the analysis. Absorbance was measured at 450 ± 2 nm using a Varioskan™ LUX multimode microplate reader (Thermo Scientific™, Waltham, MA, United States).

2.6.2. Orexin A assay

Plasma Orexin A levels were estimated using an ELISA kit method (Catalog No: E-EL-H1015) (Elabscience, Biotechnology Co., Limited Bethesda, United States). Measurements were conducted in Micro ELISA 96 well plates using a Varioskan™ LUX multimode microplate reader (Thermo Scientific™, Waltham, MA, United States) at 450 ± 2 nm.

2.7. Immunity biomarkers

2.7.1. Analysis of immunoglobulins

Serum immunoglobulin concentrations of IgG (Catalog no: E-EL-H0169) and IgM (Catalog no: E-EL-H1814) were analyzed using the ELISA kit method following the manufacturer's instructions at 450 ± 2 nm, using a Varioskan™ LUX multimode microplate reader (Thermo Scientific™, Waltham, MA, United States).

2.7.2. CD4+, CD8+ absolute count

Whole blood samples were collected in EDTA tubes, and 50 µL of the sample was added to a test tube containing a pre-dispensed, stabilized monoclonal antibody. The antibody mixture containing antibodies against CD4 and CD8 was provided by Becton Dickinson (San Diego, CA, United States) and conjugated with allophycocyanin (APC) and fluorescein isothiocyanate (FITC). The blood sample was diluted (1:10) using phosphate buffer after 15 min of incubation. The samples were analyzed using flow cytometry (FACS Calibur, Becton Dickinson, CA, United States). The sample analysis was performed in comparison with a specific fluorescence signal attributed to the presence of CD4 and CD8 antigens at the cell surface, with a side scatter signal for discriminating cells based on their shape and structure. The method of determination of absolute count was determined as described by Arneth (18).

2.7.3. Differential count

Whole blood sample were collected from the antecubital vein. The total and differential WBC counts were analyzed using automated cell cytometry (Quest Diagnostics, Inc., Madison, New Jersey, United States). Another 3 ml of whole blood was collected in EDTA vials and kept at room temperature until analysis within 24 h of sampling.

2.7.4. Analysis of cytokines

Serum concentrations of IL-1β (catalog no: E-EL-H0149), IL-2 (catalog no: E-EL-H0099), and IL-10 (catalog no: E-EL-H6154) were analyzed using ELISA kits, according to the manufacturer's instructions. Kits were purchased from Elabscience Biotechnology Co., Limited. Bethesda, United States. Absorbance was read at 450 ± 2 nm using a Varioskan™ LUX multimode microplate reader (Thermo Scientific™, Waltham, MA, United States).

2.8. Statistical analysis

Statistical analyses were performed using SPSS version 27.0. A 2 × 2 repeated measures ANOVA was employed to analyze statistical significance (treatment vs. time). Bonferroni test was used to adjust for multiple comparisons. The significance of the difference is represented as a '*p*' value. $p < 0.05$ was considered statistically significant. The reported values are arithmetic means with standard deviations (SD) or standard errors of the mean (SEM) as indicated. Pearson's correlation test was used to evaluate the significance of correlation between sleep quality (PSQI) and stress (PSS-14).

3. Results

3.1. Materials, subjects, and study design

HPTLC analysis confirmed that raw material used for preparing BCO-5 was *Nigella sativa* seed. It was in an oil form with 5.12% TQ content and 0.54% carvacrol in such a way that the TQ to carvacrol content ratio was 1:10. BCO-5 was food-grade and free from synthetic emulsifiers and food contaminants such as pesticides, heavy metals, mycotoxins, polyaromatic hydrocarbons, ethylene oxide, and microbial pathogens, as evident from their certificate of analysis.

The baseline anthropometric, haemodynamic, and other vital characteristics of the placebo and BCO-5 treated groups are provided in Supplementary Table S1. The average age and BMI of the participants in the placebo group were 25.76 ± 15.51 and 23.75 ± 0.79, respectively, while those of the BCO-5 group were 24.5 ± 16.62 and 24.29 ± 0.72, respectively. At the end of the study period, there were no significant differences between these factors.

No major side effects or adverse events were reported during the telephonic interviews on days 7 and 14. Three participants from the BCO-5 group and one from the placebo group reported bloating and borborygmus with a taste of oil in the mouth at different instances. However, all of them continued since they were satisfied with the improvement in their sleep quality. By day 14, no adverse events had been reported, and none of the participants showed signs of sleepiness or daytime drowsiness with fatigue. During the study period, 92% of participants in the BCO-5 group and 78% in the placebo group were found to be using supplements, although the consistency of use over 90 days could not be ascertained.

3.2. Influence of BCO-5 on sleep

Approximately 62% of the participants reported the beneficial effect of a single dosage. Upon telephonic interview on day 7, about 70% of participants reported satisfaction, which increased to 79% by day 14 (Figure 2). Reduction in sleep disturbances and better sleep were the two main types of feedback that were received.

3.2.1. PSQI on day 45

Intra-group comparison (baseline versus day 45) of the PSQI total score revealed a significant reduction ($p < 0.001$) in the BCO-5 group and a non-significant reduction in the placebo (Figure 3). Detailed analysis further revealed relative changes in various component scores corresponding to sleep quality ($p = 0.043$), sleep latency ($p = 0.003$), sleep duration ($p = 0.025$), overall sleep efficiency ($p < 0.001$), sleep disturbance ($p = 0.050$) and daytime dysfunction ($p < 0.001$) among BCO-5 participants. However, changes in sleep parameters were not significant in the placebo group (Figures 4A–F).

Inter-group comparison (Placebo versus BCO-5) of the PSQI component scores at baseline showed no significant difference ($p > 0.05$) between the BCO-5 and placebo. However, supplementation with BCO-5 resulted in a significant reduction in the PSQI total score (large difference effect size of 0.68; 95% CI: 7.55–8.59; $p < 0.001$). The overall effect size observed for various parameters were: sleep quality- 0.31 (95% CI: 1.14–1.61; $p = 0.024$), sleep latency- 0.31 (95% CI: 1.82–2.31; $p = 0.030$), sleep duration- 0.24 (95% CI: 1.59–1.92; $p = 0.002$), sleep efficiency- 0.52 (95% CI: 0.51–0.86; $p < 0.001$), sleep disturbance- 0.19 (95% CI: 1.00–1.00; $p = 0.030$), and daytime dysfunction- 0.29 (95% CI: 0.99–1.35; $p = 0.011$) compared to placebo (Figures 4A–F; Table 2). All component scores exhibited a moderate to large difference effect on the 45th day when compared to the placebo.

3.2.2. PSQI on day 90

By the end of the study period (day 90), BCO-5 supplementation revealed a continuous improvement in sleep parameters with a significant reduction in the PSQI total score and component scores on both intra and inter-group comparison. The total PSQI score of BCO-5 was significantly lower than that at baseline ($p < 0.001$) (Figure 3). The relative improvement in component scores for BCO-5 in comparison with baseline was sleep quality ($p = 0.003$), sleep latency ($p < 0.001$), sleep duration ($p = 0.008$), sleep efficiency ($p < 0.001$), sleep disturbance ($p = 0.009$) and daytime dysfunction ($p < 0.001$) (Figures 4A–F). However, the relative changes in the placebo group were not significant ($p > 0.05$).

Overall effect size observed for various component scores were: sleep quality– 0.57 (95% CI: 0.73–1.26; $p = 0.002$), latency– 0.82 (95% CI: 0.96–1.19; $p < 0.001$), duration– 0.33 (95% CI: 1.43–1.84; $p = 0.001$), efficiency– 0.47 (95% CI: 0.15–0.56; $p < 0.001$), disturbance– 0.20 (95% CI: 0.75–1.01; $p = 0.043$), daytime dysfunction– 0.56 (95% CI: 0.74–1.01; $p < 0.001$) and total PSQI– 0.86 (95% CI: 5.32–6.35; $p < 0.001$) (Figures 4A–F and Table 2).

Intra- and inter-group comparisons of the PSQI data corresponding to days 45 and 90 was also compared using paired and independent t-tests and were found to be significant (Supplementary Table S2).

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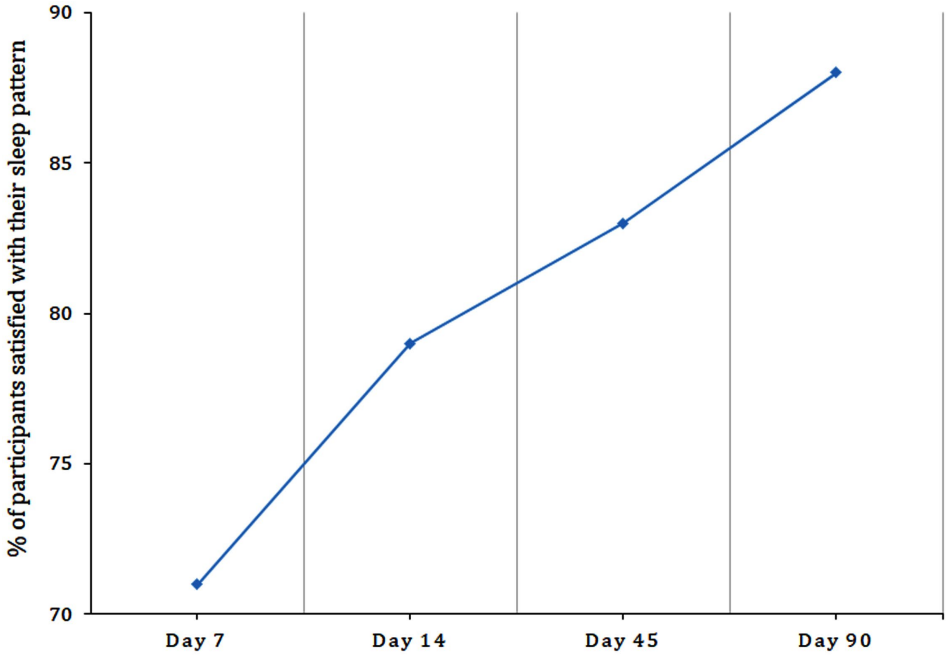

FIGURE 2
Graphical representation of the percentage of participants satisfied with their sleep pattern upon supplementation with BCO-5 as observed from sleep diary.

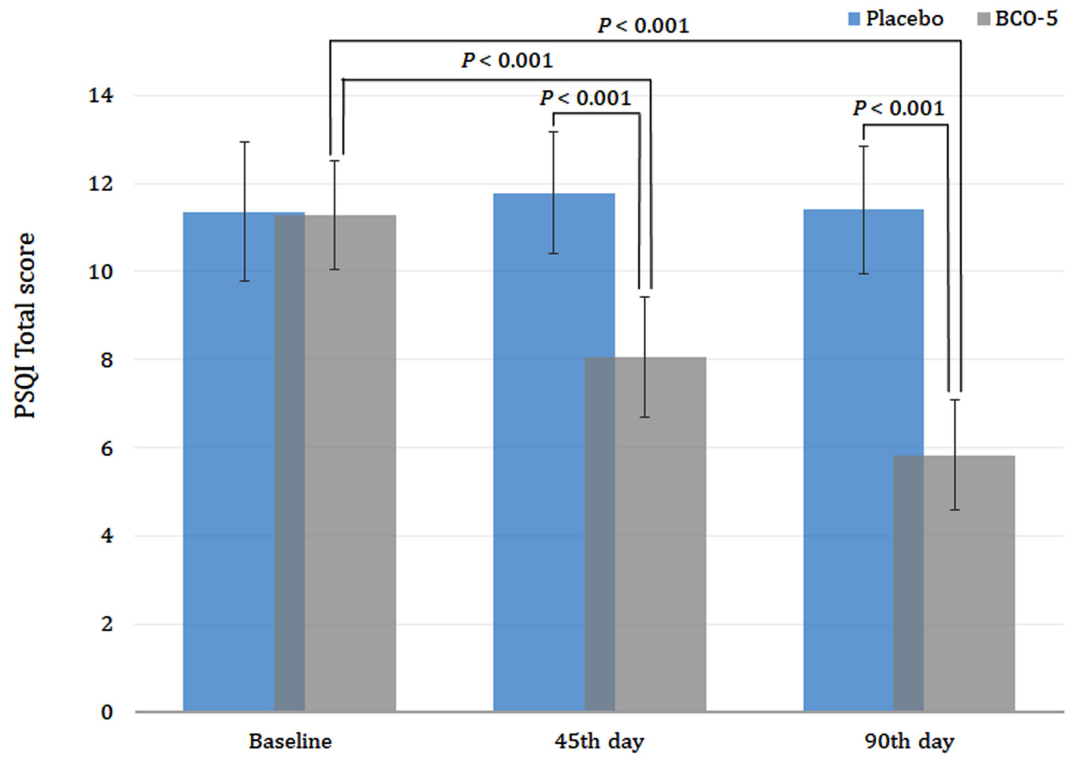

FIGURE 3
Effect of BCO-5 on PSQI total scores on 45th day 90th day compared to Placebo. The values are expressed as mean±SD.

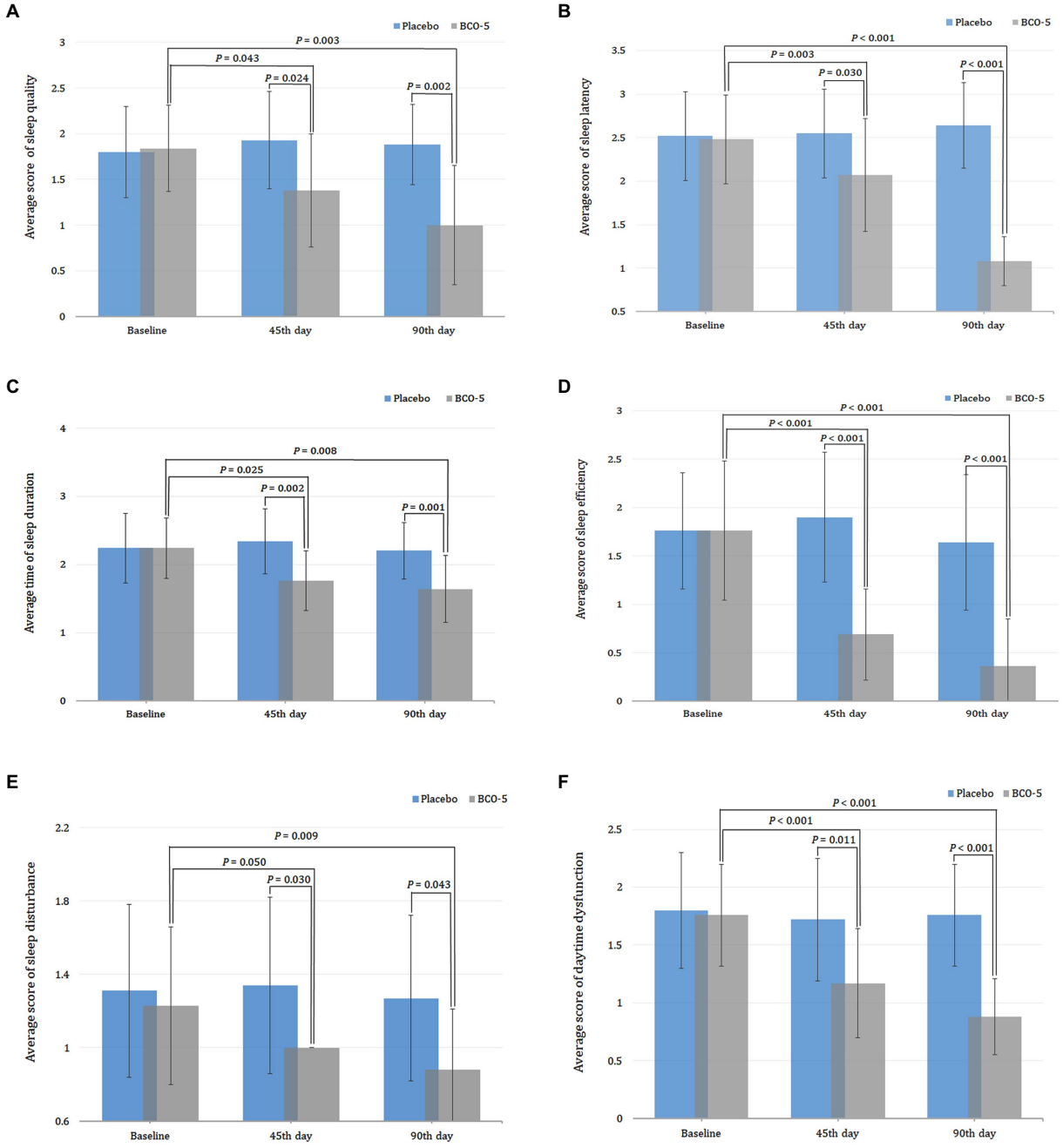

FIGURE 4
Relative changes in PSQI component scores. **(A)** Sleep quality. **(B)** Sleep latency. **(C)** Sleep duration. **(D)** Sleep efficiency. **(E)** Sleep disturbance. **(F)** Daytime dysfunction, when supplemented with BCO-5 on 45th day and 90th day compared to placebo. The values in **(B,C,E,F)** are provided as mean±SD.

3.3. Influence of BCO-5 on stress

Intra-group comparison of PSS-14 scores on the 45th day revealed a significant reduction ($p < 0.001$) in the BCO-5 group, whereas the placebo showed no significant change. Inter-group comparison also showed a significant reduction in stress in the BCO-5 group compared to the placebo. The effect size observed was 0.17 (95% CI: 15.51–18.06; $p = 0.045$) (Figure 5).

Both intra- and inter-group comparisons showed a significant effect of BCO-5 at the end of the study (day 90). The effect size observed was moderate effect of 0.43 (95% CI: 9.76–10.87; $p < 0.001$) (Figure 5). The data were also analyzed using paired and independent *t*-tests and were found to be significant (Supplementary Table S3).

3.4. Pearson's correlation between sleep and stress

BCO-5 administration showed a negative correlation between the PSQI and PSS-14 measures, with a coefficient of −0.314. This

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correlation was significant at the end of the study ($p < 0.05$). In contrast, placebo administered group exhibited a positive correlation between PSQI and PSS-14 parameters at the end of the study (0.178; $p = 0.357$).

3.5. Effect of BCO-5 on melatonin level

Both intra and inter-group analyses of salivary melatonin on day 45 showed no significant increase in placebo ($p > 0.05$), whereas a significant increase in the BCO-5 group was observed in both intra- ($p < 0.001$) and inter-group analyses ($p = 0.009$). The overall effect size was 0.84 (95% CI: 53.96–58.93) (Figure 6A). When extended to day 90, the placebo group exhibited no significant increase in melatonin levels while BCO-5 had a significant effect on both intra- ($p < 0.001$) and inter-group comparisons ($p < 0.001$). A large effect size [0.85 was observed at the end of the study (95% CI: 63.32–69.66) (Figure 6A)].

TABLE 2 Effect size and *p*-values of PSQI parameter (BCO-5 vs. Placebo) on day 45 and 90.

PSQI parameter	Time	Effect size	*P*-value
Sleep quality	45th day	0.31	0.024
	90th day	0.57	0.002
Sleep latency	45th day	0.31	0.030
	90th day	0.82	<0.001
Sleep duration	45th day	0.24	0.002
	90th day	0.33	0.001
Sleep efficiency	45th day	0.52	<0.001
	90th day	0.47	<0.001
Sleep disturbance	45th day	0.19	0.030
	90th day	0.20	0.043
Daytime dysfunction	45th day	0.29	0.011
	90th day	0.56	<0.001
Total PSQI	45th day	0.68	<0.001
	90th day	0.86	<0.001

A significance level $p < 0.05$ is considered as statistically different.

3.6. Effect of BCO-5 on cortisol level

The intra- and inter-group analysis of cortisol at the end of the 45th day exhibited a significant decrease ($p < 0.001$) for BCO-5, while the placebo showed no significant change ($p > 0.05$). The effect size observed upon analysis was 0.72 (95% CI: 48.73–55.70; $p = 0.044$).

At the end of the study (day 90), BCO-5 participants showed a significant decrease ($p < 0.001$), while the placebo exhibited no significance ($p > 0.05$) with respect to the baseline. Inter-group comparison at the end of the study also revealed a significant decrease in cortisol levels among BCO-5 participants compared to placebo. A large effect of 0.72 (95%CI: 40.99–48.51; $p = 0.013$) was observed (Figure 6B).

3.7. Effect of BCO-5 on orexin level

At the end of the study, the plasma concentration of orexin in the BCO-5 group, decreased significantly in upon both intra- and inter-group comparisons ($p < 0.001$), while the same for the placebo exhibited no significant change. The observed effect size had a large difference effect of 0.92 (95% CI: 57.07–61.99) (Figure 6C).

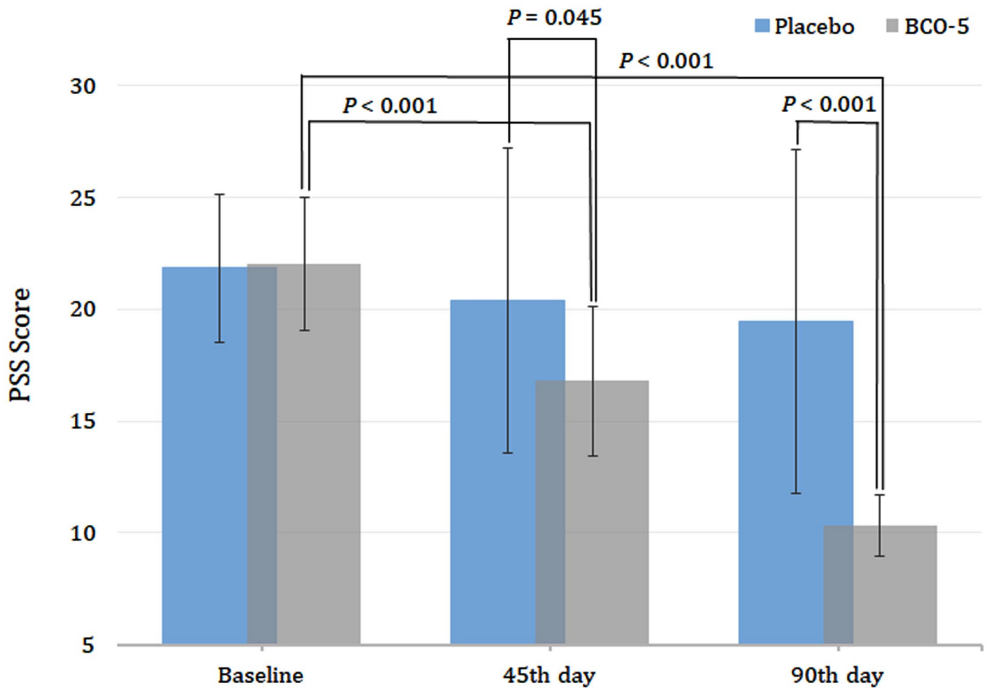

FIGURE 5
Relative changes in PSS-14 score upon supplementation with BCO-5 on day 45 and 90 compared to placebo. The values are expressed as mean±SD.

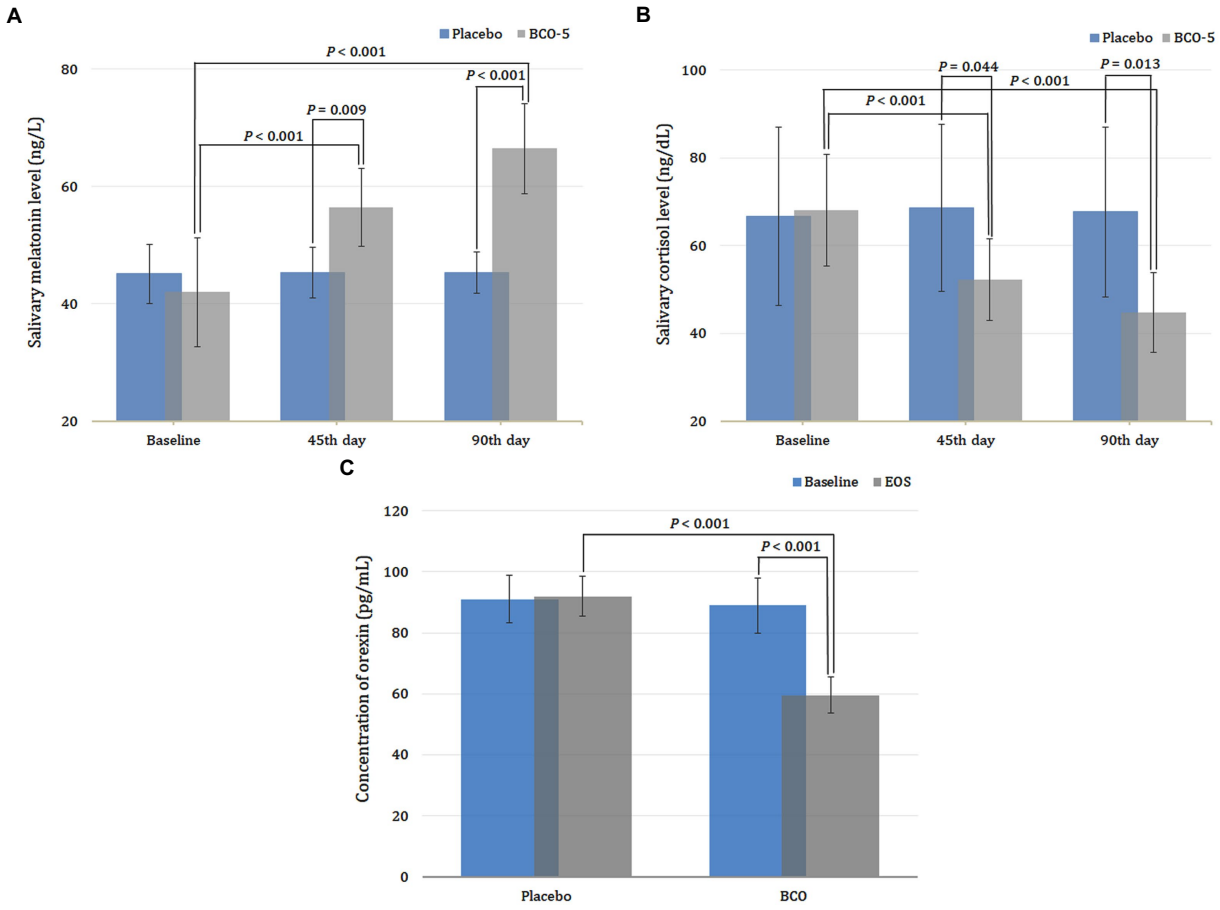

FIGURE 6
Changes in sleep biomarkers **(A)** melatonin and **(B)** cortisol and **(C)** orexin A on 45th day and 90th when supplemented with BCO-5 when compared to placebo. The values are expressed as mean±SD.

3.8. Effect of BCO-5 on immune markers

3.8.1. Influence on immunoglobulins

Intra-group analysis of IgM and IgG at the end of the study period revealed a significant increase compared to baseline ($p < 0.008$ and $p < 0.001$, respectively), whereas the placebo showed no significant effect. Inter-group analysis of IgM and IgG levels also showed a significant increase compared to the placebo. A moderate effect size of 0.41 (95% CI: 191.06–214.24; $p = 0.050$) was noted for IgM, and a large effect size of 0.87 (95% CI: 1091.09–1125.94; $p < 0.001$) for IgG (Table 3).

3.9. Influence on CD4+, CD8+ and CD4/CD8 ratio

Intra-group comparison of CD4+ and CD4/CD8 ratio exhibited a significant increase ($p = 0.015$ and $p < 0.001$respectively) in BCO-5 treated participants, whereas the placebo-supplemented group showed no significant effect ($p > 0.05$). CD8+ cells, on the other hand, showed a significant decrease ($p = 0.043$) compared to that at baseline.

Inter-group comparison revealed a significant increase in the CD4+ and CD4/CD8 ratio at the end of the study in BCO-5 treated participants. The effect size observed were: CD4+: 0.29 (95% CI: 765.07–819.02; $p < 0.001$) and CD4+/CD8+: 0.52 (95% CI: 1.85–2.08; $p = 0.032$) respectively. At the same time, CD8+ exhibited a much more significant decrease at the end of the study compared to the placebo, with a moderate effect size of 0.46 (95% CI: 387.90–425.91; $p = 0.042$) (Table 3).

3.9.1. Influence on differential count

At the end of the study, supplementation with BCO-5 increased the number of leukocytes, lymphocytes, and monocytes in both intra- and inter-group analyses. The relative effect size observed were, respectively, 0.34 (95% CI: 6342.14–6921.85; $p = 0.050$), 0.57 (95% CI: 35.80–40.26; $p = 0.009$), and 0.10 (95% CI: 5.57–6.97; $p < 0.001$). Neutrophils and eosinophils, on the other hand, showed a significant decrease, with an effect size of 0.57 (95% CI: 51.51–55.45; $p < 0.001$) and 0.31 (95% CI: 1.61–2.33; $p < 0.001$) respectively. The basophil count showed no significant difference (0.001; 95% CI: 0.17–0.27; $p = 0.294$) (Table 4).

3.9.2. Influence on cytokines- IL-1β, IL-2, and IL-10

Intra-group comparison of IL-1β, IL-2, and IL-10 showed no significant effect in the placebo. However, BCO-5 showed a significant

TABLE 3 Changes in immunity markers in participants from baseline to end of study in placebo and BCO-5 treated group and the *p*-values observed upon inter-group and intra-group comparison.

Parameters	Group	Baseline	End of study	*P*-values	
				Inter	Intra
IgG (mg/dL)	Placebo	848.04 ± 56.63	850.72 ± 52.53	<0.001	<0.001
	BCO-5	818.12 ± 63.65	1108.52 ± 42.22		
IgM (mg/dL)	Placebo	173.50 ± 24.55	168.15 ± 36.08	0.050	0.008
	BCO-5	174.15 ± 45.03	202.65 ± 28.69		
CD4 + cells	Placebo	655.91 ± 81.51	639.27 ± 91.90	<0.001	0.015
	BCO-5	703.18 ± 102.93	792.05 ± 60.84		
CD8 + cells	Placebo	398.32 ± 55.27	413.45 ± 57.10	0.042	0.043
	BCO-5	446.05 ± 34.18	406.91 ± 42.87		
CD4/CD8 ratio	Placebo	1.63 ± 0.33	1.57 ± 0.29	0.032	<0.001
	BCO-5	1.60 ± 0.23	1.97 ± 0.26		

Serum levels of immunoglobulin M, Serum levels of immunoglobulin G and the CD4/CD8 ratio of study participants at baseline and at the end of the study in the placebo and BCO-5 treated groups. Values are expressed as mean ± SD. Differences were considered statistically significant at $p < 0.05$.

TABLE 4 Changes in differential count in participants from baseline to end of study in placebo and BCO-5 treated group and their respective effect size.

Parameters	Group	Baseline	End of study	Effect size
TLC (cells/cmm)	Placebo	6,072 ± 777.56	5,792 ± 776.17	0.34
	BCO-5	5,992 ± 707	6632 ± 702.21	
Neutrophils (%)	Placebo	66.24 ± 3.44	63.32 ± 3.59	0.57
	BCO-5	64.29 ± 3.99	53.48 ± 4.77	
Lymphocytes (%)	Placebo	28.68 ± 3.90	31.29 ± 3.66	0.57
	BCO-5	27.30 ± 4.83	38.04 ± 5.40	
Eosinophils (%)	Placebo	1.38 ± 0.71	1.44 ± 0.78	0.31
	BCO-5	2.75 ± 1.00	1.98 ± 0.87	
Monocyte (%)	Placebo	3.44 ± 1.83	3.68 ± 1.58	0.10
	BCO-5	5.42 ± 1.65	6.28 ± 1.70	
Basophils (%)	Placebo	0.26 ± 0.11	0.26 ± 0.09	0.001
	BCO-5	0.23 ± 0.12	0.23 ± 0.12	

A significance level $p < 0.05$ is considered as statistically different.

increase in IL-1β ($p < 0.001$) and IL-2 ($p < 0.001$) and a significant decrease in IL-10 ($p < 0.001$) compared to their respective baseline values (Table 5).

Inter-group comparisons at the end of the study revealed a significant increase in the levels of IL-1β ($p < 0.001$) and IL-2 ($p < 0.001$). The effect sizes of 0.75 (95% CI: 6.31–7.18) and 0.85 (95% CI: 4.69–5.73) were observed in IL-1β and IL-2, respectively. The decrease in IL-10 was also found to be significant, with an effect size of 0.88 (95% CI: 13.08–14.42; $p < 0.001$) (Table 5).

3.10. Safety and adverse events

The results of the hematological and biochemical analyses are given in Supplementary Table S1. All clinical laboratory parameters were within the normal range before and after the study, and there was no significant difference between the groups or within the groups ($p > 0.05$). There were six dropouts from the placebo group and four dropouts from the BCO-5 group. However, none of the dropouts were due to any adverse side effects due to personal reasons. Confirmation of acceptability/tolerability and safety was provided by the satisfaction ratings at the end of the study. It was found that 2% of the participants in the intervention group (17% in the placebo group) were discontented with capsule intake.

4. Discussion

The present study investigated the ability of a novel formulation of black cumin oil extract (BCO-5) to modulate stress, sleep, and immunity when supplemented at a dose of 200 mg/day for 90 days. The interconnection between stress, sleep, and immunity are well established (19). Its mechanisms of action and pathogenesis are also known (20). The novelty of the present study lies in the fact that this is the first report on the positive influence of a botanical extract,

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TABLE 5 Changes in cytokines in participants from baseline to end of study in placebo and BCO-5 treated group and the *p*-values observed upon inter-group and intra-group comparison.

Parameters	Group	Baseline	End of study	*P*-values	
				Inter	Intra
IL-1β (pg/dL)	Placebo	2.41 ± 1.26	2.33 ± 1.79	<0.001	<0.001
	BCO-5	2.53 ± 1.20	6.75 ± 1.03		
IL-2 (pg/dL)	Placebo	1.91 ± 0.48	1.99 ± 0.75	<0.001	<0.001
	BCO-5	1.95 ± 0.57	5.21 ± 1.25		
IL-10 (pg/dL)	Placebo	25.59 ± 2.40	26.52 ± 4.44	<0.001	<0.001
	BCO-5	26.22 ± 3.40	13.76 ± 1.62		

A significance level $p < 0.05$ is considered as statistically different.

especially from a food component, on the safe modulation and alleviation of the stress-sleep-immunity axis. The rationale for the use of BCO-5 in the present study is a previous clinical study that employed polysomnography in healthy subjects with stress and sleep issues (12). Moreover, BCO-5 has also been shown to exhibit enhanced anti-inflammatory, anti-arthritic, acetylcholine esterase inhibitory, and neuroprotective effects in various preclinical studies (9, 10).

The study was conducted on healthy volunteers following a double-blind, placebo-controlled design. The total number of subjects ($n = 72$) enrolled and who completed the study was found to be statistically significant at the 80% power and 5% significance level. The baseline clinical laboratory tests and self-reported stress/sleep scores of the participants indicated that they were healthy but experienced significant sleep issues such as a non-restorative sleep (NRS) pattern due to stress/anxiety. The NRS is characterized by sleep disturbances and unsatisfactory sleep when awakening (21). Globally, more than 40% of the general population suffers from this sleep condition (22).

The primary objective of this study was to monitor the effect of BCO-5 on stress and sleep. To monitor stress, we used the well-validated short form of the Perceived Stress Scale (PSS-14), in which a higher score indicates a higher perception of stress (23). It provides a measure of the degree to which one's life is affected by stress and anxiety. The baseline scores revealed that the participants experienced significant stress and sleep issues due to various reasons on their personal and professional fronts. The stress level (higher score) in the placebo and treated groups was not significantly different at baseline. Supplementation with BCO-5 significantly reduced ($p < 0.001$) the PSS scores on days 45 and 90 indicating its primary efficacy. Moreover, the decrease on day 90 was significantly higher than that on day 45 ($p < 0.001$). At the end of the study, the participants felt more relaxed and placid compared to the placebo group.

The PSQI, one of the most commonly used and well-validated self-reported rating scale, was employed to monitor the seven-component scores, that is sleep quality, sleep latency, sleep duration, sleep disturbance, sleep efficiency, medication use and daytime dysfunction as a measure of the overall sleep quality (15). We observed a significant increase with an effect size of 0.31 ($p < 0.024$) and 0.57 ($p < 0.002$) in sleep quality on days 45 and 90 which indicates peaceful and restorative sleep. Better sleep quality has been shown to improve health, reduce daytime sleepiness, and improve well-being, and psychological functioning (24). Sleep latency is defined as the time that a person falls asleep after turning off lights. Inter-group comparison on the 45th and 90th days revealed a significant decrease in latency scores, with an effect size of 0.31 ($p = 0.03$) and 0.82 ($p < 0.001$), indicating the positive influence of BCO-5. Sleep disturbance is a major issue affecting a high percentage of the population and is a disorder/difficulty in initiating and maintaining sleep. The observation that sleeps disturbance scores were significantly reduced upon supplementation with BCO-5, with an effect size of 0.19 ($p = 0.030$) and 0.20 ($p = 0.043$), respectively on days 45 and 90, further shows its positive impact on sleep. BCO-5 also improved sleep duration, as evidenced by the results with an overall effect size of 0.24 ($p = 0.02$) and 0.33 ($p = 0.001$) respectively on days 45 and 90. Sleep duration refers to sleep obtained, either nocturnal or within 24-h time (25), and it was correlated with an increased risk of developing hypertension, diabetes, obesity, depression, heart attack, and stroke (26).

Sleep efficiency, which is the ratio of total sleep time to the time spent in bed, is another important component of the PSQI that captures the core problem for those suffering from insomnia. Poor sleep efficiency can increase daytime sleepiness and contribute to sleep debt (27). Supplementation with BCO-5 significantly improved sleep efficacy with an effect size of 0.56 and 0.47 on days 45 and 90, respectively. Moreover, BCO-5 has also shown a significant decrease in daytime dysfunction and inability to perform daily functions. The correlation between PSQI and PSS-14 was further evaluated using Pearson's correlation test and was found to be significant indicating the potential role of BCO-5 in reducing stress and improving sleep quality (-0.314; $p < 0.05$). In contrast, the placebo group showed a positive correlation at the end of the study (0.178; $p = 0.357$). Our findings were also in agreement with a previous report by Shelar et al. (28), who found a negative correlation between PSQI and PSS-14, reinforcing the relationship between reduced stress and increased sleep.

Furthermore, the measurement of salivary melatonin, cortisol, plasma orexin A, and cytokines provided an insight into the molecular basis of the action of BCO-5 on stress and sleep. Recently, there has been great interest in the development of Dual Orexin Receptor Antagonists (DORA) for treating chronic insomnia because orexin agonism during the day promotes wakefulness, and orexin receptor antagonists can promote sleep signals by enhancing melatonin levels (29, 30). Stress stimuli can generate orexins (orexin A and B) which express the corticotropin-releasing hormone (CRH). CRH binds to the anterior pituitary gland to release adrenocorticotropic hormone (ACTH), which further binds to its receptor in the adrenal cortex and releases cortisol, the major hormone produced in response to stress (31). Therefore, orexins act as molecular switches for the release of

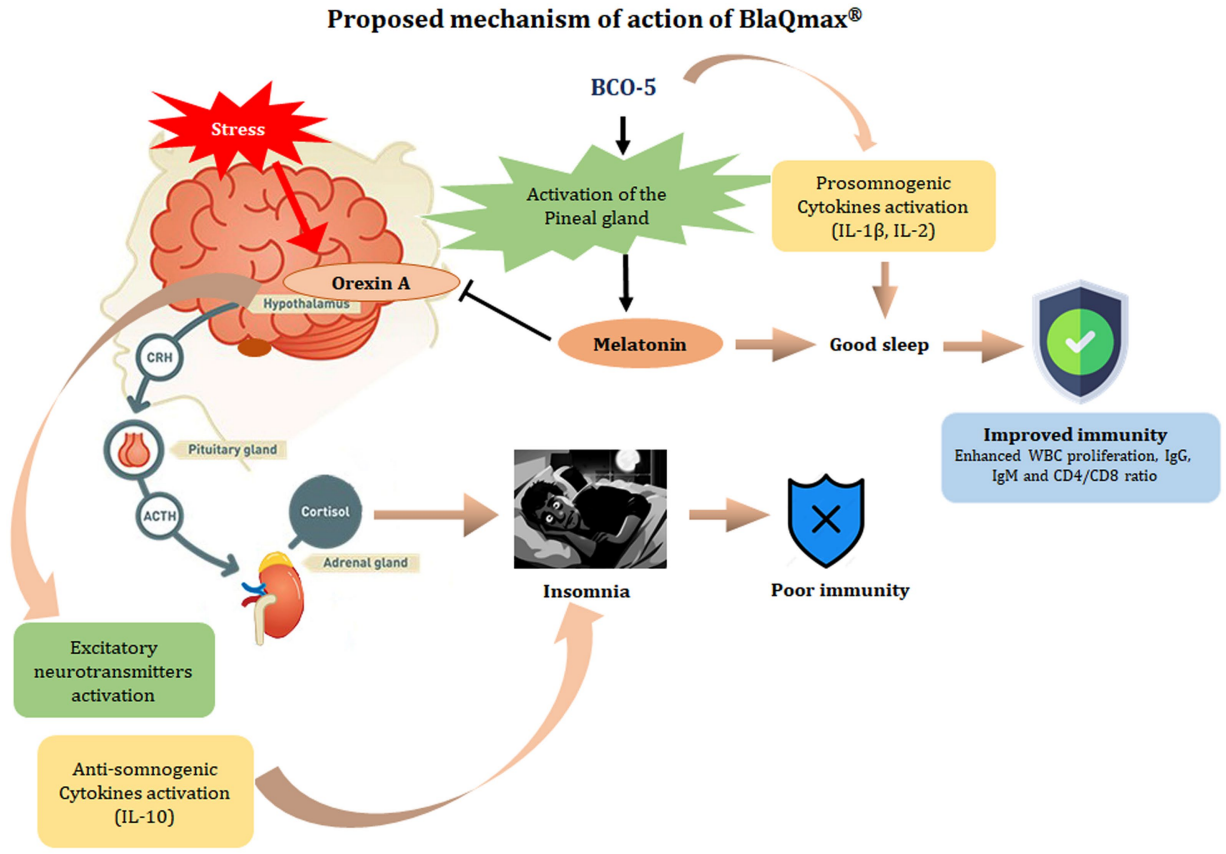

FIGURE 7
Proposed mechanism of action of BCO-5. Orexins (Orexin A and Orexin B) are the key neuropeptides produced in the hypothalamus and interact with the hypothalamic–pituitary–adrenal axis (HPA) to regulate sleep–wake cycle and neuroendocrine activation associated with stress. Orexins cause the release of corticotropin-releasing hormone (CRH) in the hypothalamus, which triggers the anterior pituitary to secrete adrenocortical-tropic hormone (ACTH), stimulating the release of cortisol – the major stress hormone, and promote wakefulness. In the nervous system, orexins result in the release of excitatory neurotransmitters such as dopamine, epinephrine, glutamate, and acetylcholine which further result in alertness/wakefulness. BCO-5 acts on the pineal gland and releases melatonin which acts as a natural inhibitor of orexins and thereby regulate HPA axis to reduce stress and promote sleep. In other words, BCO-5 act as a dual orexin receptor antagonist (DORA).

cortisol in response to a stress stimulus and regulate the sleep/wake cycle (Figure 7) (4). Melatonin, a hormone referred to as "the light of night," has been established as a natural inhibitor of orexins; hence, it can reduce stress by reducing cortisol levels and improve sleep quality (32). In agreement with this molecular mechanism, our results showed a significant increase in the salivary melatonin levels among the BCO-5 group, with a large effect size of 0.85 ($p = 0.009$) and a decrease in cortisol level with a large effect size of 0.72 ($p = 0.013$). The ability of BCO-5 to increase melatonin levels may be explained on the basis of previous studies showing that black cumin increases the concentration of 5-hydroxy tryptophan and tryptophan in the rat brain and plasma (33). Tryptophan and 5-hydroxy tryptophan can increase the concentration of serotonin, the biosynthetic precursor of melatonin (34, 35). We also observed a significant reduction in the plasma concentration of orexin A among the BCO-5 participants (0.92; $p < 0.001$), indicating that BCO-5 acts as a natural DORA. The molecular mechanism of BCO-5 in stress and sleep are depicted in Figure 7.

Sleep loss has been reported to trigger stress and elevate the plasma levels of various cytokines (36). Cytokines that alter sleep in humans and animals have been identified as IL-1β, IL-2, IL-4, IL-10, IL-13, IL-15, TNF-α, IFN-γ, and macrophage inhibitory protein-1β (MIP-1β), in which IL-1β, IFN-γ, IL-2, and TNF-α are considered pro-somnogenic cytokines (supporting sleep) and IL-4, IL-15, IL-10, and IL-13 were observed with sleep deprivation (anti-somnogenic) (5); IL-1β, IL-2, and IL-10 are the mainly studied cytokines that are known to be involved in sleep regulation (37). Our findings showed an elevated level of pro-somnogenic cytokines IL-1β and IL-2 and a decrease in anti-somnogenic IL-10, indicating the positive effect of BCO-5 on sleep modulation. Previous studies have also reported elevated levels of IL-2 and IL-1β upon improved sleep and an elevation in IL-10 levels upon sleep disorders (19, 38, 39).

Sleep and immunity are also interconnected. Epidemiological studies have shown that poor sleep increases susceptibility to diseases (1, 5, 40). Various biochemical studies have demonstrated that sleep loss can alter immune responses in both animals and humans (41–43). Leukocyte proliferation, humoral immunity, cell-mediated immunity, and immunoglobulin levels are reduced in participants with prolonged sleep disorders (44). Two or three nights of sleep deprivation can significantly decrease the neutrophil, lymphocyte and leukocyte counts (40). Recently, it has also been shown that every 60-min increase in sleep duration was associated with a significant increase in total leukocytes, lymphocytes, neutrophils, and monocyte count ($p < 0.001$) (45). Our results for the differential count at baseline agree

with this observation. We found that BCO-5 supplementation significantly enhanced leukocyte proliferation, as evident from the increase in the total leukocyte, lymphocytes, and monocytes counts.

Immunoglobulins (Ig) or antibodies are glycoproteins produced by plasma B cells which are critical for immune response and hence important markers of immunomodulatory effects. IgA, IgG, IgM, IgE, and IgD are immunoglobulins found in humans. IgG accounts for the most abundant immunoglobulin (75%) (46), whereas IgM is the largest antibody, accounting for approximately 5% of all antibodies in the serum. Immunoglobulins quickly recognize and initiate an immune response by directly neutralizing pathogens or clearing novel antigens (47, 48). Although various studies have attempted to correlate immunoglobulin levels with sleep disorders, the results are controversial and require further clarity. Everson reported a significant increase in IgG, IgM, and IgA levels after 3–5 days of sleep deprivation (49). However, other studies were unable to replicate this result and reported either no change or reduction in immunoglobulin levels upon sleep deprivation (40, 50). Our results indicated a significant increase in IgG and IgM levels in BCO-5 participants; however, the increase was within the normal range (48).

Cell-mediated immunity plays a critical role in antiviral responses (51). The absolute count of CD4+ T lymphocytes was significantly increased. This correlates with the increased levels of IL-2, a pro-somnogenic cytokine released by CD4+ cells. CD4+ cells are also known to promote immunoglobulins. However, a significant reduction was observed in the number of CD8 cells. The CD4/CD8 ratio is a direct marker of immune status, showing a decreasing trend in the placebo group and a significant increase in the BCO-5 group. In a previous study, Salem et al. reported a significant immunomodulatory effect of black cumin seed powder, with a significant increase in immunoglobulin, CD4+ cell absolute count, and lymphocyte counts (52). Certain animal studies have also reported immunomodulatory effects of black cumin (53–55).

The absence of significant side effects, adverse events, or toxic deviations in clinical laboratory parameters (biochemical/hematological) indicated its safety and suitability for supplementation. A detailed safety assessment of BCO-5 has recently been published, which also revealed no significant toxic effects (13). Acute and sub-chronic toxicity studies have also established the safety of BCO-5 and its optimized, safe dosage for human consumption of 900 mg/kg/day (56). The fact that more than 70% of the participants reported significant improvement in their sleep pattern by day 7 indicates the relatively fast action of BCO-5 as a natural stress/sleep aid.

The lack of an objective assessment of sleep parameters and the measurement of neurotransmitters and markers of innate immunity may be considered as limitation of the present study. Similarly, investigations of the bioavailability of the key bioactive molecules in BCO-5 would provide better insights into its mechanism of action.

5. Conclusion

In summary, the present randomized double-blinded placebo-controlled trial demonstrated the efficacy of a proprietary black cumin extract (BCO-5) to safely modulate the stress-sleep-immunity axis and further alleviate stress, establish restorative and restful sleep, and improve immunity among healthy subjects characterized by non-restorative sleep conditions. While PSS-14 demonstrated a significant reduction in stress, PSQI analysis established a positive effect of BCO-5 on sleep quality, sleep latency, sleep duration, and overall sleep efficiency when supplemented at a single dose of 200 mg/day. The percentage of participants who were satisfied with a single dose confirmed the rapid action of BCO-5. The improvements in stress and sleep reported by participants in the PSS-14 and PSQI questionnaires were in agreement with the changes in salivary melatonin and cortisol levels, plasma orexin, and cytokine levels. The influence of BCO-5 on immunity status was also clear from the differential counts, immunoglobulins, cytokines, and CD4+, CD8+, and CD4/CD8 ratios. The therapeutic potential of BCO-5 may be attributed to the pleiotropic mechanism of action that regulates the HPA axis and thereby modulates stress, sleep, and immunity functions, the three interconnected factors of optimized health, but with different pathways. Future studies should investigate the short-term effects of BCO-5 on various sleep problems by employing techniques such as actigraphy and its influence on anxiety, depression, and mood. Detailed investigation of the molecular mechanism of action is also recommended.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by the Balagangadharanatha Swami Global Institute of Medical Sciences Institutional Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

Author contributions

MEM: recruitment and investigation of the trial. JT: conduct of the trial, data collection, and biochemical analysis. PP: drafting the original article. SD and PP: data analysis. MCM: review and editing of the original article. BP: principal investigator who supervised the study and was involved in the protocol development, data interpretation, review, and editing of the original article. All authors contributed to the article and approved the submitted version.

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Conflict of interest

BCO-5 used in the present study is a patented black cumin extract developed by Akay Natural Ingredients, Cochin, India and registered as BlaQmax®. PP and SD were employed by Akay Natural Ingredients. JT was employed by Leads Clinical Research and Bio Services Private Limited.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2023.1152680/full#supplementary-material

References

1. Besedovsky L, Lange T, Haack M. The sleep-immune crosstalk in health and disease. *Physiol Rev*. (2019) 99:1325–80. doi: 10.1152/physrev.00010.2018

2. Tsigos C, Kyrou I, Kassi E, Chrousos GP. Stress: endocrine physiology and pathophysiology. *Endotext*. (2020)

3. Phan T, Malkani R. Sleep and circadian rhythm disruption and stress intersect in Alzheimer's disease. *Neurobiol Stress*. (2019):100133:10. doi: 10.1016/j.ynstr.2018.10.001

4. Akça ÖF, Uzun N, Kılınç İ. Orexin A in adolescents with anxiety disorders. *Int J Psychiatry Clin Pract*. (2020) 24:127–34. doi: 10.1080/13651501.2019.1711425

5. Garbarino S, Lanteri P, Bragazzi NL, Magnavita N, Scoditti E. Role of sleep deprivation in immune-related disease risk and outcomes. *Commun Biol*. (2021) 4:1–17. doi: 10.1038/s42003-021-02825-4

6. Hannan MA, Rahman MA, Sohag AAM, Uddin MJ, Dash R, Sikder MH, et al. Black cumin (*Nigella sativa* L.): A comprehensive review on Phytochemistry, health benefits, molecular pharmacology, and safety. *Nutrients*. (2021) 13:1784. doi: 10.3390/nu13061784

7. Ambati RR, Ramadan MF. *Nigella sativa* seed extracts in functional foods and nutraceutical applications In: M Fawzy Ramadan, editor. *Black cumin (Nigella sativa) seeds: Chemistry, Technology, Functionality, and Applications. Food Bioactive Ingredients*. Cham: Springer (2021)

8. Fawzy M, Hassanien R. *Black cumin (Nigella sativa) seeds: Chemistry, technology, functionality, and applications. Food bioactive ingredients*. Cham: Springer (2021).

9. Ratheesh M, Svenia JP, Sangeeth S, Sheethal S, Sony R, Sandya S, et al. Antioxidant, anti-inflammatory, and anti-arthritic effect of thymoquinone-rich black cumin (*Nigella sativa*) oil (BlaQmax®) on adjuvant-induced arthritis. *J Food Res*. (2021) 10:52. doi: 10.5539/jfr.v10n1p52

10. Ramalingam K, Ittiyavirah SP, Jyoti H. Acetylcholinesterase and growth inhibitory effects - various grades of *N. Sativa* oils. *Inter J Pharm Sci*. (2018) 10:245–50. doi: 10.13040/IJPSR.0975-8232.10(1).245-50

11. Ittiyavirah SP, Ramalingam K, Sathyan A, Rajasree RS, Kuruniyan MS, Quadri SA, et al. Thymoquinone-rich black cumin oil attenuates ibotenic acid-induced excitotoxicity through glutamate receptors in Wistar rats. *Saudi Pharma J*. (2022) 30:1781–90. doi: 10.1016/j.jsps.2022.10.007

12. Das SS, Ramalingam K, George S, Chakrapani BPS, Maliakel B, Ittiyavirah S, et al. Thymoquinone-rich black cumin oil improves sleep quality, alleviates anxiety/stress on healthy subjects with sleep disturbances– A pilot polysomnography study. *J Herb Med*. (2022) 32:100507. doi: 10.1016/j.hermed.2021.100507

13. Thomas JV, Mohan ME, Prabhakaran P, Das SS, Maliakel B, Krishnakumar IM. A phase I clinical trial to evaluate the safety of thymoquinone-rich black cumin oil (BlaQmax®) on healthy subjects: randomized, double-blinded, placebo-controlled prospective study. *Toxicol Rep*. (2022) 9:999–1007. doi: 10.1016/j.toxrep.2022.04.020

14. Thiagarajah K, Chee HP, Sit NW. Effect of alpha-S1-casein tryptic hydrolysate and L-Theanine on poor sleep quality: A double blind, randomized placebo-controlled crossover trial. *Nutrients*. (2022) 14:652. doi: 10.3390/nu14030652

15. Zhang C, Zhang H, Zhao M, Li Z, Cook CE, Buysse DJ, et al. Reliability, validity, and factor structure of Pittsburgh sleep quality index in community-based centenarians. *Front Psychiatry*. (2020):573530:11. doi: 10.3389/fpsyt.2020.573530

16. Zhong QY, Gelaye B, Sánchez SE, Williams MA. Psychometric properties of the Pittsburgh sleep quality index (PSQI) in a cohort of Peruvian pregnant women. *J Clin Sleep Med*. (2015) 11:869–77. doi: 10.5664/jcsm.4936

17. Barutcu Atas D, Aydin Sunbul E, Velioglu A, Tuglular S. The association between perceived stress with sleep quality, insomnia, anxiety, and depression in kidney transplant recipients during COVID-19 pandemic. *PLoS One*. (2021) 16:e0248117. doi: 10.1371/journal.pone.0248117

18. Arneth BM. Activation of CD4 and CD8 T cell receptors and regulatory T cells in response to human proteins. *Peer J*. (2018) 6:e4462. doi: 10.7717/peerj.4462

19. Lange T, Dimitrov S, Born J. Effects of sleep and circadian rhythm on the human immune system. *Ann N Y Acad Sci*. (2010) 1193:48–59. doi: 10.1111/j.1749-6632.2009.05300.x

20. Asif N, Iqbal R, Nazir CF. Human immune system during sleep. *Am J Clin Exp Immunol*. (2017) 6:92–6.

21. Chen N, Fong DYT, Li S, Wong JYH. Association between non-restorative sleep and quality of life in Chinese adolescents. *Int J Environ Res Public Health*. (2020) 17:1–10. doi: 10.3390/ijerph17197249

22. Tinajero R, Williams PG, Cribbet MR, Rau HK, Bride DL, Suchy Y. Nonrestorative sleep in healthy, young adults without insomnia: associations with executive functioning, fatigue, and pre-sleep arousal. *Sleep Health*. (2018) 4:284–91. doi: 10.1016/j.sleh.2018.02.006

23. Vallejo MA, Vallejo-Slocker L, Fernández-Abascal EG, Mañanes G. Determining factors for stress perception assessed with the perceived stress scale (PSS-4) in Spanish and other European samples. *Front Psychol*. (2018):37:9. doi: 10.3389/fpsyg.2018.00037

24. Harvey AG, Stinson K, Whitaker KL, Moskovitz D, Virk H. The subjective meaning of sleep quality: A comparison of individuals with and without insomnia. *Sleep*. (2008) 31:383–93. doi: 10.1093/sleep/31.3.383

25. Kline C. Sleep duration In: MD Gellman and JR Turner, editors. *Encyclopedia of Behavioral Medicine*. New York, NY: Springer (2013). 1808–10.

26. Institute of Medicine (US) Committee on Sleep Medicine and Research In: H Colten and B Altevogt, editors. *Sleep Disorders and Sleep Deprivation: An Unmet Public Health Problem*. Washington (DC): US National Academies Press (2006)

27. Reed DL, Sacco WP. Measuring sleep efficiency: what should the denominator be? *J Clin Sleep Med*. (2016) 12:263–6. doi: 10.5664/jcsm.5498

28. Tejas S, Krutika G, Mukta B, Waqas A. A study of perceived stress and sleep deprivation in an urban Indian population. *J Pharm Negat Results*. (2022) 13:7273–9. doi: 10.47750/pnr.2022.13.S07.875

29. Steiner MA, Michel A, Yanagisawa M, Clozel MM. The orexin system: basic science and role in sleep pathology. *Front Neurol Neurosci*. (2021) 45:22–37. doi: 10.1159/isbn.978-3-318-06844-3

30. Robinson CL, Supra R, Downs E, Kataria S, Parker K, Kaye AD, et al. Daridorexant for the treatment of insomnia. *Health Psychol Res*. (2022) 10:37400. doi: 10.52965/001c.37400

31. Veldhuis JD, Sharma A, Roelfsema F. Age-dependent and gender-dependent regulation of hypothalamic-adrenocorticotropic-adrenal axis. *Endocrinol Metab Clin N Am*. (2013) 42:201–25. doi: 10.1016/j.ecl.2013.02.002

32. Campino C, Valenzuela FJ, Torres-Farfan C, Reynolds HE, Abarzua-Catalan L, Arteaga E, et al. Melatonin exerts direct inhibitory actions on ACTH responses in the human adrenal gland. *Horm Metab Res*. (2011) 43:337–42. doi: 10.1055/s-0031-1271693

33. Tas U, Ayan M, Sogut E, Kuloglu T, Uysal M, Tanriverdi H, et al. Protective effects of thymoquinone and melatonin on intestinal ischemia-reperfusion injury. *Saudi J Gastroenterol*. (2015) 21:284–9. doi: 10.4103/1319-3767.166203

34. Perveen T, Haider S, Zuberi NA, Saleem S, Sadaf S, Batool Z. Increased 5-HT levels following repeated administration of *Nigella sativa* L. (Black Seed) oil produce

antidepressant effects in rats. *Sci Pharm*. (2014) 82:161–70. doi: 10.3797/scipharm.1304-19

35. Erland LAE, Saxena PK. Melatonin natural health products and supplements: presence of serotonin and significant variability of melatonin content. *J Clin Sleep Med*. (2017) 13:275–81. doi: 10.5664/jcsm.6462

36. Irwin MR, Carrillo C, Olmstead R. Sleep loss activates cellular markers of inflammation: sex differences. *Brain Behav Immun*. (2010) 24:54–7. doi: 10.1016/j.bbi.2009.06.001

37. Krueger JM, Majde JA, Rector DM. Cytokines in immune function and sleep regulation. *Handb Clin Neurol*. (2011) 98:229–40. doi: 10.1016/B978-0-444-52006-7.00015-0

38. Besedovsky L, Lange T, Born J. Sleep, and immune function. *Pflugers Arch*. (2012) 463:121–37. doi: 10.1007/s00424-011-1044-0

39. Rethorst CD, Greer TL, Toups MSP, Bernstein I, Carmody TJ, Trivedi MH. IL-1β and BDNF are associated with improvement in hypersomnia but not insomnia following exercise in major depressive disorder. *Transl Psychiatry*. (2015) 5:e611. doi: 10.1038/tp.2015.104

40. Ruiz FS, Andersen ML, Martins RCS, Zager A, Lopes JD, Tufik S. Immune alterations after selective rapid eye movement or total sleep deprivation in healthy male volunteers. *Innate Immun*. (2012) 18:44–54. doi: 10.1177/1753425910385962

41. Heiser P, Dickhaus B, Schreiber W, Clement HW, Hasse C, Hennig J, et al. White blood cells and cortisol after sleep deprivation and recovery sleep in humans. *Eur Arch Psychiatry Clin Neurosci*. (2000) 250:16–23. doi: 10.1007/PL00007534

42. Ruiz FS, Andersen ML, Zager A, Martins RCS, Tufik S. Sleep deprivation reduces the lymphocyte count in a non-obese mouse model of type 1 diabetes mellitus. *Braz J Med Biol Res*. (2007) 40:633–7. doi: 10.1590/S0100-879X2007000500005

43. Zager A, Andersen ML, Ruiz FS, Antunes IB, Tufik S. Effects of acute and chronic sleep loss on immune modulation of rats. *Am J Physiol Regul Integr Comp Physiol*. (2007) 293:R504–9. doi: 10.1152/ajpregu.00105.2007

44. Nie L, Pan XL, Zhang XB, Zhang S, Rao JX, Su ZF. Research on the correlation of immunity in patients with chronic insomnia. *Front Psychiatry*. (2022):1034405:13. doi: 10.3389/fpsyt.2022.1034405

45. Hoopes EK, D'Agata MN, Berube FR, Ranadive SM, Patterson F, Farquhar WB, et al. Consistency where it counts: sleep regularity is associated with circulating white blood cell count in young adults. *Brain Behav Immun Health*. (2021) 13:100233. doi: 10.1016/j.bbih.2021.100233

46. Vidarsson G, Dekkers G, Rispens T. IgG subclasses and allotypes: from structure to effector functions. *Front Immunol*. (2014) 5:520. doi: 10.3389/fimmu.2014.00520

47. Sathe A, Cusick J. *Biochemistry, immunoglobulin M [Internet]*. Treasure Island (FL): StatPearls Publishing (2021).

48. Justiz VA, Jamal Z, Patel P, Kamleshun R. *Immunoglobulin*. Treasure Island (FL): StatPearls Publishing (2022).

49. Everson CA. Clinical assessment of blood leukocytes, serum cytokines, and serum immunoglobulins as responses to sleep deprivation in laboratory rats. *Am J Physiol Regul Integr Comp Physiol*. (2005) 289:R1054–63. doi: 10.1152/ajpregu.00021.2005

50. Hui L, Hua F, Diandong H, Hong Y. Effects of sleep and sleep deprivation on immunoglobulins and complement in humans. *Brain Behav Immun*. (2007) 21:308–10. doi: 10.1016/j.bbi.2006.09.005

51. Ganji A, Farahani I, Khansarinejad B, Ghazavi A, Mosayebi G. Increased expression of CD8 marker on T-cells in COVID-19 patients. *Blood Cells Mol Dis*. (2020) 83:102437. doi: 10.1016/j.bcmd.2020.102437

52. Salem A, Bamosa A, Alam M, Alshuraim S, Alyalak H, Alagga A, et al. Effect of *Nigella sativa* on general health and immune system in young healthy volunteers; a randomized, placebo-controlled, double-blinded clinical trial. *F1000 Res*. (2021) 10:1199. doi: 10.12688/f1000research.73524.1

53. Rosidah , Pratiwy FM. A mini-review: potential of *Nigella sativa* L. for immune fish against pathogen bacteria attack. *Int J Fish Aquat Stud*. (2022) 10:01–6. doi: 10.22271/fish.2022.v10.i4b.2695

54. Mahmoud HS, Almallah AA, Gad EL-Hak HN, Aldayel TS, Abdelrazek HMA, Khaled HE. The effect of dietary supplementation with *Nigella sativa* (black seeds) mediates immunological function in male Wistar rats. *Sci Rep*. (2021) 11:7542. doi: 10.1038/s41598-021-86721-1

55. Ghonime M, Eldomany R, Abdelaziz A, Soliman H. Evaluation of immunomodulatory effect of three herbal plants growing in Egypt. *Immunopharmacol Immunotoxicol*. (2011) 33:141–5. doi: 10.3109/08923973.2010.487490

56. Ramalingam K, Kuttan R, Sivadasan S, Ittiyavirah S, Madhavamenon KI. Safety assessment of thymoquinone-rich black cumin (*Nigella sativa*) oil (BlaQmax): acute and sub-chronic toxicity studies. *J Nutr Food Sci*. (2021) 11:811.

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Original article

Thymoquinone-rich black cumin oil attenuates ibotenic acid-induced excitotoxicity through glutamate receptors in Wistar rats

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ABSTRACT

Inflammation-mediated alterations in glutamate neurotransmission constitute the most important pathway in the pathophysiology of various brain disorders. The excessive signalling of glutamate results in excitotoxicity, neuronal degeneration, and neuronal cell death. In the present study, we investigated the relative efficacy of black cumin (*Nigella sativa*) oil with high (5 % w/w) and low (2 % w/w) thymoquinone content (BCO-5 and BCO-2, respectively) in alleviating ibotenic acid-induced excitotoxicity and neuroinflammation in Wistar rats. It was found that BCO-5 reversed the abnormal behavioural patterns and the key inflammatory mediators (TNF-α and NF-κB) when treated at 5 mg/kg body weight. Immunohistochemical studies showed the potential of BCO-5 to attenuate the glutamate receptor subunits NMDA and GluR-2 along with increased glutamate decarboxylase levels in the brain tissues. Histopathological studies revealed the neuroprotection of BCO-5 against the inflammatory lesions, as evidenced by the normal cerebellum, astrocytes, and glial cells. BCO-2 on the other hand showed either a poor protective effect or no effect even at a 4-fold higher concentration of 20 mg/kg body weight indicating a very significant role of thymoquinone content on the neuroprotective effect of black cumin oil and its plausible clinical efficacy in counteracting the anxiety and stress-related neurological disorders under conditions such as depression and Alzheimer's disease.

1. Introduction

Neuroinflammation, an inflammatory response within the brain due to factors such as stress, infection, toxins, autoimmunity or injury, is interceded by the generation of cytokines, reactive oxygen species and secondary messengers produced by the glial cells in the central nervous system (CNS) (Di Sabato et al., 2016). Inflammation-mediated alteration in the glutamate neurotransmission constitutes the most critical pathway in the pathophysiology of various brain disorders since glutamate is the principal excitatory neurotransmitter responsible for the execution of primary physiological functions like the acquisition of memory, perception, and motor activity (Benussi et al., 2019). The glutamatergic neurotransmission occurs through the activation of *N*-methyl-D-aspartate receptors (NMDAR) (Zhou et al., 2020). However, excessive glutamatergic signalling may result in excitotoxicity, and neuronal degeneration, and even neuronal death (Malik et al., 2019, Cabeza et al., 2021). A balanced level of extracellular glutamate is thus needed for normal synaptic function (Halder et al., 2021).

Neuroinflammation causes increased production of proinflammatory cytokines by glial cells (Yang and Zhou, 2019). These proinflammatory cytokines, especially TNF-α, increase glutamate production by enhancing glutaminase and preventing glutamine

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uptake by inhibiting glutamate reuptake proteins (Iovino et al., 2020). As a result, the extracellular glutamate accumulates in the synapse leading to excitotoxicity and neuronal death (Kang et al., 2021). Therefore, the control of neuroinflammation through balancing the glutamate level in the brain was considered as an ideal therapy for various neurodegenerative diseases (Cunnane et al., 2020). Glutamate regulators like 6-diazo-5-oxo-norleucine (DON) (a glutaminase inhibitor) and ceftriaxone and riluzole (glutamate reuptake protein enhancers) are found to be effective, but are burdened with unknown target specific actions and thus with varying side effects (Clark and Vissel, 2016). Memantine, a medicine currently in practice for the treatment of various neurodegenerative diseases, is an NMDA receptor antagonist drug that has also been found to exhibit some side effects like hypertension, dizziness, headaches, constipation, and somnolence (Blanco-Silvente et al., 2018). Yet another approach in practice is the use of a combination of non-steroidal anti-inflammatory drugs (NSAID) (Ozben and Ozben, 2019). The use of specific anti-TNF biologicals like etanercept has also been shown to be effective, but is not cost-effective (Clark and Vissel, 2016). More importantly, most of these drugs are effective in the correction of only one pathogenic pathway, since they are target specific (Vezzani et al., 2019). Thus, there is tremendous interest in the development of multi-targeted therapeutic agents, which are safe (Zieba et al., 2022). Various natural agents, including the phytonutrients derived from food components, have been shown to possess significant multi-targeted anti-neuroinflammatory and neuroprotective effects without significant side effects Chen et al., 2021; Mohd Sairazi and Sirajudeen, 2020).

Nigella sativa L. belonging to the family of *Ranunculaceae*, generally referred to as black cumin or black seed is a popular kitchen spice and is generally recognized as safe (GRAS)-listed food component that has been widely used to treat a range of diseases and disorders worldwide (Tavakkoli et al., 2017). The oil fraction of black cumin, comprising both the essential and fixed oils, has been identified as its primary bioactive component (Mazaheri et al., 2019). While linoleic acid forms the major part of the fixed oil, thymoquinone (TQ) was identified as the most abundant molecule in the essential oil fraction (Gawron et al., 2021).Various studies have demonstrated the beneficial effects of natural compounds (Thajudeen et al., 2022, Rajasree et al., 2022, Ilyas et al., 2022). Several studies have linked the pharmacological effects of black cumin oil (antimicrobial, anti-inflammatory, antihypertensive, anti-obese, hypoglycemic, hypolipidemic, hepatoprotective, and neuroprotective) to its TQ content Kooti et al., 2016; Mukhtar et al., 2019).

Ibotenic acid is a potent NMDA receptor agonist resembling the chemical structure of glutamate (an excitatory neurotransmitter). An excitotoxin preferentially binds to NMDA receptors causing prolonged activation and excitotoxicity similar to glutamate. As a result, there occurs an excess influx of chloride and calcium ions and increased water entry in the neurons due to osmotic lysis. Ibotenic acid also induces neuronal loss throughout the nucleus basalis of the Meynert (nbM) complex and destroys the cholinergic cells in the ventral pallidum and substantia innominate complex (Van Dam and De Deyn, 2011). It was demonstrated that the intracerebroventricular administration (ICV) of IBO to rats could induce cortical cholinergic dysfunction with significant neuroinflammation and neurodegeneration (Karthick et al., 2016). Neuroinflammation, glutamate excitotoxicity, and cholinergic dysfunction are known to be the major pathological process that play key role in Alzheimer's disease.

The present study investigated the pathogenesis of neuroinflammation and receptor modulation by black cumin oil as a function of its TQ content employing ibotenic acid (IBO)-induced neurotoxicity model of rats.

2. Materials and methods

2.1. Animals

Colony inbred strains of adult Wistar rats weighing 200 – 250 g were used in the study. The animals were purchased from M/s Nagarjuna Herbal Concentrates Limited, Cochin, India and were housed at the animal house facility of the Department of Pharmaceutical Sciences, Centre for Professional and Advanced Studies, Kottayam, India, in a properly ventilated polypropylene cage under controlled temperature (22 – 25 ℃), relative humidity (60 – 80 %) and the light–dark cycle of 12 h. The animals were provided with standard rat feed (M/s VRK Nutritional Solutions, Pune, India) and water *ad libitum*. The animals were acclimatized to the laboratory conditions for a week prior to the experiments. The study was conducted in compliance with the Institutional Animal Ethics Committee guidelines (IAEC) of the Centre for Professional and Advanced Studies, Kottayam, India, with approval no: MGU/DPS/IAEC/2016/PD-5.

2.2. Materials

Ibotenic acid (Cat. No: I2765) was purchased from Sigma-Aldrich, Inc., MA, USA. GAD-65/67 polyclonal antibody (ITT1830), GluR-2 polyclonal antibody (ITT1923) and NMDAε1/2 polyclonal antibody (ITT3149) were purchased from Geno Technology Inc. St Louis MO, USA. A standard black cumin oil containing 2 % of TQ (BCO-2) and a TQ-rich black cumin oil containing 5 % thymoquinone (BCO-5) were obtained from M/s Akay Natural Ingredients, Kerala, India. Sunflower oil was employed as diluent and vehicle control.

2.3. Experimental design

2.3.1. Toxicity study

Detailed toxicity studies [single dose acute (14-day), and sub chronic (90-day) repeated-dose toxicity studies of black cumin oil containing 5 % (w/w) of TQ (BCO-5) were conducted as per OECD 423, 407 & 408 guidelines and reported previously (Kannan et al., 2022).

2.3.2. Ibotenic acid-induced model

Wistar rats of both sex weighing 200 – 250 g were randomly assigned into eight groups, with eight animals per group (Table 1). The dosage was selected based on toxicity studies (Kannan et al., 2022). Ibotenic acid was administered intracerebroventricular

Table 1
Grouping of animals, dose of drugs and route of administration followed in the study.

Groups	Drug	Dose	Route of administration
Normal control (C1)	Sunflower oil	–	Oral
IBO control (C2)	Ibotenic acid (IBO)	1 μL*	Intracerebroventricular (ICV)
Standard (S)	IBO + memantine	30 mg/kg	Oral
TQF1	IBO + BCO-5	10 mg/kg	Oral
TQF2	IBO + BCO-5	5 mg/kg	Oral
TQF3	IBO + BCO-5	2.5 mg/kg	Oral
TQN1	IBO + BCO-2	20 mg/kg	Oral
TQN2	IBO + BCO-2	10 mg/kg	Oral

* Ibotenic acid (IBO) was dissolved in sunflower oil (pH 7.2) at a concentration of 5 μg/μL.

(ICV) using the surgical procedures as described by Mahdi *et al*., and the treatment with TQF1, TQF2, TQF3, TQN1, TQN2 and memantine was continued for 30 days (Mahdi et al., 2019). Behavioral and cognitive parameters were assessed after 24 h of the last dose of treatment. The animals were sacrificed by cervical dislocation under anaesthetic conditions followed by decapitation, and the brains were removed for biochemical assays.

The rats were anaesthetized by injecting xylazine (20 mg/kg *i.p.*) and ketamine hydrochloride (80 mg/kg *s.c*) and were placed on a warm mat to maintain their body temperature. Sterile PBS drops were applied to both eyes, which prevented the dryness of the cornea during surgical procedures. About 70 % of ethanol was sprayed to the middle forehead of the rat and rubbed with dry cotton swabs. The same area on the forehead was wiped with 2 % chlorhexidine solution and the scrubbings with alcohol and chlorhexidine were repeated thrice. The hair on the forehead was shaved, and chlorhexidine antiseptic solution was applied to avoid contamination. The bregma in the brain was located using the thumb and index finger and the injection point was located using a measuring tape (1.0 ± 0.06 mm posterior to bregma, 1.8 ± 0.1 m m lateral to the sagittal suture and 2.4 mm in depth). The syringe was filled with 1 μL of IBO in sunflower oil (5 μg/μL) and placed at the injection point, perpendicular to the plane of injection. The reflected images of the syringe were aligned in both mirrors, with the lines drawn from a fixed perspective. The needle was inserted with the utmost care until the para film wrapping touches the skin and slowly injected the IBO solution or vehicle for five seconds at the rate of 0.2 μL/s. The syringe was kept steady and perpendicular throughout the procedure and an adequate time of 3 to 5 s were spent before the syringe was removed to avoid diffusion. The IBO injected rat was placed on a warm pad for recovery and its sternal recumbence was maintained. Adequate post-surgical care was maintained until it regained consciousness and were placed into separate cages to avoid contamination and any possible infections. The mobility of the rat in the cage was monitored post-operatively and checked for signs of infection or illness for 5 – 7 days.

Sunflower oil was injected instead of Ibotenic acid (C1) as a sham group and IBO (5 μg/μL) was injected as a control group (*C*2). Administration of the vehicle without the drug was given to both sham and control groups. Sham and control groups were used as positive and negative control groups, respectively (Glascock et al., 2011).

2.4. Behavioral analysis

Elevated plus maze: The elevated plus maze is a widely used method for evaluating anxiety and memory-related parameters. It consisted of two open arms measuring 50 × 10 cm and two closed arms measuring 50 × 10 × 40 cm with one roof. The two arms were arranged opposite to each other. The experimental animals (rats) were placed in the centre square of the maze facing towards the closed arm and allowed to explore the elevated plus-maze for five minutes freely. Before the next rat was released into the maze, the maze was washed with 20 % ethanol and dried. The number of open and closed arm entries and the amount of time spent on open and closed arms were measured and tabulated. The data from the above-said parameters were analyzed for the total number of entries (open arm) and the percentage of time spent on open arms (Pellow et al., 1985).

Open field test: The behavioral changes were evaluated using the open-field apparatus, a wooden box with dimensions of 60 × 60 × 35 cm. It consisted of four holes of 3.8 cm in diameter equally placed on the floor of the apparatus. The apparatus itself consists of lines; each animal was positioned individually at the centre of the open field and permitted for five minutes to explore freely in the apparatus. The total number of rearing (vertical activity), duration of rearing, grooming (protracted of the coat) and locomotion (number of line crossings) were measured (Pellow et al., 1985).

2.5. Acetylcholinesterase activity

Following the sacrifice of the animals, the brain was carefully removed without damage. The adhering blood particles were removed by washing with an ice-cold buffer solution and the tissues were weighed and homogenized in 0.1 M Phosphate buffer (pH 8). The activity was measured by determining the yellow anion formation from the reaction of the thiocholine generated by enzymatic hydrolysis of ATCh and Ellman's reagent. The change in absorbance was measured at 412 nm for 3 min at regular intervals of 30 s using a UV–visible spectrophotometer immediately after the enzyme was obtained. Acetylcholinesterase (AChE) inhibition was then evaluated by Ellman's method (Kannan et al., 2018).

2.6. Quantification of NF-κB and TNFα by ELISA

The quantification of cytokines was performed by ELISA kit method following the kit instructions (Cat No: E-EL-R0674; E-EL-R0019; Elabscience, Texas, USA). Optical density was read at 415 nm in an ELISA reader (Erba, Germany, Amable et al., 2013; Kim et al., 2016).

2.7. Immunohistochemical analysis

Four animals per group were processed for immunohistochemical analysis as per Kim et al., 2016. The collected samples were washed with ice-cold normal saline, post-fixed with 4 % paraformaldehyde, were cryoprotected in 30 % sucrose – PBS (0.1 M), and stored at −80 ℃ until processed. The sections were deparaffinised, hydrated and incubated with 3 % hydrogen peroxide for immunohistochemistry. Antigen retrieval was carried out by heating for 10 min in a 10 mM sodium citrate buffer (pH 6.0). Specimens were blocked for 30 min at room temperature using a protein block solution (BSA) and incubated with primary antibodies (*N*-methyl-D-aspartate receptor (NMDAε1/2) (dilution – 1:250); MPA – Selective glutamate receptor (GluR2) (dilution – 1:200) and glutamic acid decarboxylase 65/67 (GAD 65/67) (dilution – 1:250) at 4 ℃ overnight. Polymer-horseradish peroxidase anti-rabbit was used as a secondary antibody and 3, 3′-diaminobenzidine as the chromogen. The Immune-stained hippocampal coronal sections were scanned with a confocal laser microscope and the immune-stained cells were counted in the cornu Ammonis (CA1) area and dentate gyrus region (AP: Bregma 4.3 to 4.5 mm). Images of five replicate sections were analyzed (Yim et al., 2016).

2.8. Histopathology

For histopathology studies, the brain samples fixed in 10 % formal saline (10 mL of formaldehyde in 90 mL of physiological saline) were used. The paraffin-embedded sections were taken (100 μm thickness) and processed in alcohol - xylene series. The sections were stained with Haematoxylin-Eosin (H&E) dye and examined microscopically.

2.9. Statistical analysis

The statistical study was conducted using GraphPad Prism (Version 7.00) by one-way ANOVA accompanied by Tukey's multiple post-hoc test comparisons. All the values were expressed as Mean ± SEM with $P < 0.05$ considered as significantly different (a, b, c represents $P < 0.001$, $P < 0.01$ and $P < 0.05$ respectively when

compared with control; x, y, z represents $P < 0.001$, $P < 0.01$ and $P < 0.05$ respectively when compared with IBO control; ns – non-significant).

3. Results

3.1. Behavior studies

In the present study, the IVC administration of IBO induced behavioral changes, as evident from the significant decrease ($P < 0.001$) in the number of open arm entries and the time spent by the animals in the open arm (Group *C*2; IBO-treated) as compared to vehicle control animals (Group C1). BCO-5 treated group of animals showed significant protective activity at 5 mg/kg b. wt., while BCO-2 treated group showed lesser or no activity even at 20 mg/kg b. wt. ($P > 0.05$) (Fig. 1a and 1b). Significant improvement in both open arm entries and time spent by the animals in the open arm were observed in both TQF1 and TQF2 groups and were comparable to the memantine treated group (Group S). However, the effect was more pronounced in TQF2. Moreover, this was similar to the normal control group of animals indicating the reversal of the behavior in both TQF2 and memantine treated groups.

Ibotenic acid administration also produced a significant decrease ($P < 0.001$) in the ambulation and rearing behavior in disease control animals (IBO-treated) when compared to that of vehicle control (C1). Both memantine and BCO-5 at concentrations of 10 and 5 mg/kg body weight (TQF1 and TQF2 respectively) produced a significant increase ($P < 0.001$) in both the ambulation and rearing behavior and were similar to the behavior of the control group of animals ($P > 0.05$) (Fig. 1c and 1d). However, BCO-2 at both the tested doses of 20 and 10 mg/kg body weight produced only a minimum response, which was not statistically significant to the IBO control group *C*2.

3.2. Acetylcholinesterase activity

The acetylcholinesterase activity was significantly increased ($P < 0.01$) in the IBO- treated group (*C*2) compared with vehicle control demonstrating the neurotoxic effect of IBO. It was observed that both memantine and BCO-2 (10 and 20 mg/kg body weight) produced no significant change ($P > 0.05$) when compared with the treatment group, *C*2. BCO-5 on the other hand produced maximum effect at 5 mg/kg body weight itself, as evidenced from the significant decrease ($P < 0.01$) in AChE activity when compared to IBO control (Fig. 2). However, the AChE activity of BCO-5 at 10 mg/kg was slightly greater than TQF2, but has significantly decreased ($P < 0.05$) than *C*2, indicating the effect on AChE (Fig. 2).

3.3. Effect of BCO on brain tissue levels of NF-κB and TNF-α

The inflammatory marker TNF-α was significantly increased ($P < 0.001$) in the IBO- treated group compared with the control group. The treatment with memantine and BCO-5 at all the dosage produced a dose-dependent and significant decrease ($P < 0.001$) in the activity of TNF-α when compared to that of control. Meanwhile, BCO-2 showed only moderate activity (Fig. 4a).

The development of excitotoxic lesions in the brain by the administration of IBO was evident from the significant increase ($P < 0.001$) in the expression of NF-κB activity in IBO treated, compared to the vehicle control group of animals. However, treatment with memantine, BCO-5 (2.5, 5 and 10 mg/kg body weight) and BCO-2 (10 and 20 mg/kg body weight) significantly decreased ($P < 0.001$) the activity of NF-κB (Fig. 3b). However, BCO-5 at

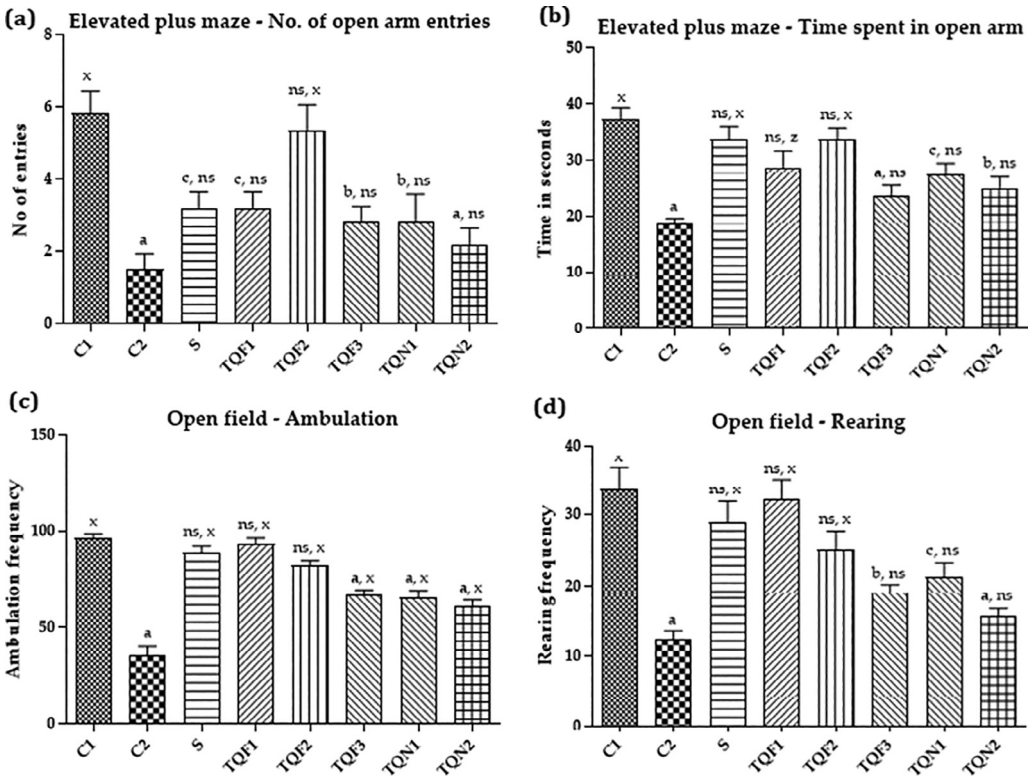

Fig. 1. Behavioural changes after IVC administration of IBO. Fig. 1a and 1b- BCO-5 treated group of animals showed significant protective activity at 5 mg/kg b. wt., while BCO-2 treated group showed lesser or no activity even at 20 mg/kg body weight. Fig. 1c and 1d- Both memantine and BCO-5 at concentrations of 10 and 5 mg/kg body weight (TQF1 and TQF2 respectively) produced a significant increase ($P < 0.001$) in both the ambulation and rearing behavior.

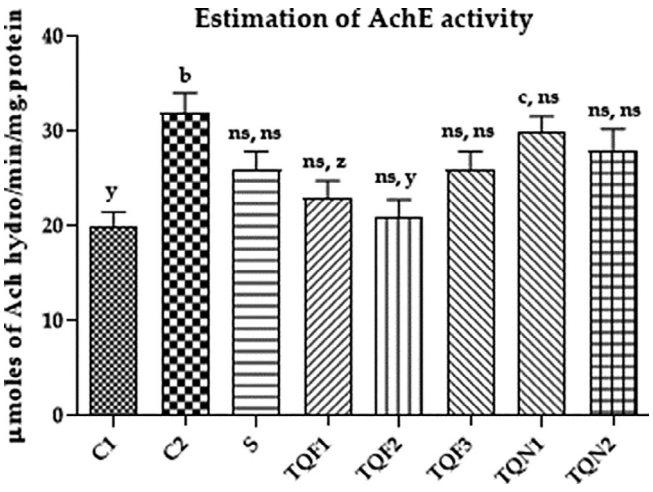

Fig. 2. The acetylcholinesterase activity in treatment and control group. The acetylcholinesterase activity was significantly increased ($P < 0.01$) in the IBO-treated group (C2) compared with vehicle control demonstrating the neurotoxic effect of IBO.

5 mg/Kg body weight itself showed a more significant effect compared to BCO-2 at 20 mg/kg b. wt.

3.4. Estimation of NMDA reactive cells by immunohistochemical analysis

Compared with the vehicle control group of animals, there was an exponential increase in the levels of NMDA positive cells ($P < 0.001$) in the IBO group (C2). Meanwhile, treatment with BCO-5 (2.5 and 10 mg/kg body weight) and BCO-2 (10 and 20 mg/kg body weight) produced significant alterations ($P < 0.001$) when compared to both IBO control and vehicle control (Fig. 4a and 4d). In contrast to this, BCO-5 at 5 mg/kg body weight produced a substantial decrease ($P < 0.001$) in reactive cells, which is comparable to the memantine treated group (S).

3.5. Estimation of GluR-2 reactive cells by immunohistochemical analysis

The quantity of GluR-2 reactive cells was significantly increased ($P < 0.001$) in IBO group compared to normal control. The results showed (Fig. 4b and 4e) that BCO-5 and BCO-2 had significantly reduced GluR-2 reactive cells ($P < 0.001$). However, the number of GluR-2 reactive cells in BCO-5 (5 mg/kg) treated group is comparable to normal (C1) rather than between BCO and C1 group.

3.6. Estimation of GAD 65/67 reactive cells by immunohistochemical analysis

The GAD levels in the brains of rats were decreased when administered with IBO as evidenced by the significant reduction ($P < 0.001$) in the number of GAD reactive cells in IBO treated group compared to that of control. Meanwhile, treatment with memantine and BCO-5 (10 and 5 mg/kg) significantly increased ($P < 0.001$) the number of GAD positive cells when compared to IBO control. BCO-5 at 2.5 mg/kg and BCO-2 at 20 mg/kg produced lesser activity ($P < 0.05$ and $P < 0.01$, respectively). BCO-2 at 10 mg/kg body weight showed no activity ($P > 0.05$) when compared to IBO control (Fig. 4c and 4f). On the whole, the order of activity of the black cumin oil with varying levels of TQ content was found to be in the order BCO-5 at 5 mg/kg body weight (TQF2) > BCO-5 at 10 mg/kg body weight (TQF1) > BCO-5 at 2.5 mg/kg body weight (TQF3) > BCO-2 at 20 mg/kg body weight (TQN1) > BCO-2 at 10 mg/kg body weight (TQN2).

3.7. Histopathology

The intracerebroventricular injection of IBO produced lesions in the brain of animals, as evidenced by the presence of necrosis and hyperplastic astrocytes in the IBO treated group. The treatment with memantine and BCO-5 at concentrations of 10 and 5 mg/kg body weight showed normal astrocytes and glial cells in all groups, except BCO-5 at 2.5 mg/kg body weight and BCO-2 at 20 mg/kg body weight where hyperplastic astrocytes were observed. The cerebellum of memantine, BCO-5 (10, 5 and 2.5 mg/kg body weight) and BCO- 2 at 20 mg/kg body weight treated animals were found to be normal. However, BCO-2 at 10 mg/kg body weight showed necrosis (Fig. 5).

4. Discussion

Over the past two decades, there has been a significant rise in the use of herbal medicines (Naseef et al., 2021, Ilyas et al., 2021). A number of botanical extracts and phytochemicals have also found scientific research-based applications as phytonutrients and are widely available as 'nutraceuticals' or 'dietary supplements' or as 'functional foods' (Rajasree et al., 2021, Laj et al., 2022). Black cumin seed is one such culinary spice possessing wide range of medicinal effects, especially on brain functions (Oskouei et al., 2018). Both the black cumin oil and its major component TQ have shown to exhibit beneficial neuropharmacological effects suitable for the management and treatment of Alzheimer's disease, Parkinson's disease, anxiety, depression, encephalomyelitis, epilepsy, traumatic brain injury, ischemia and other neurodegenera-

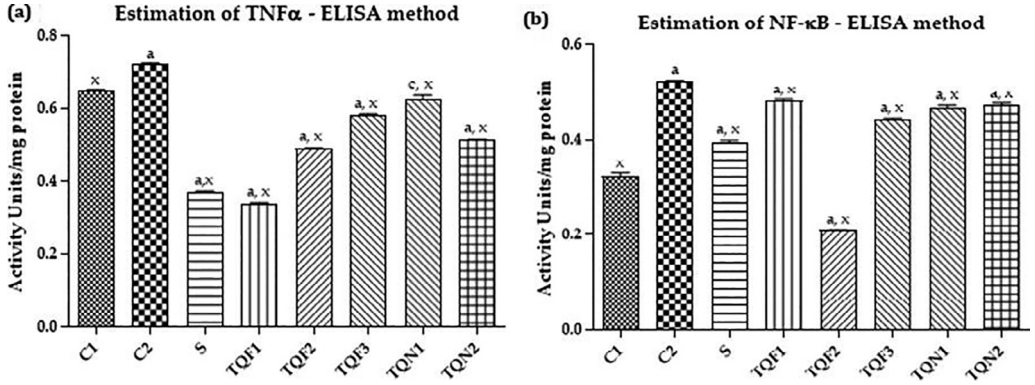

Fig. 3. Effect of BCO on brain tissue levels of NF-κB (3a) and TNF-α (3b).

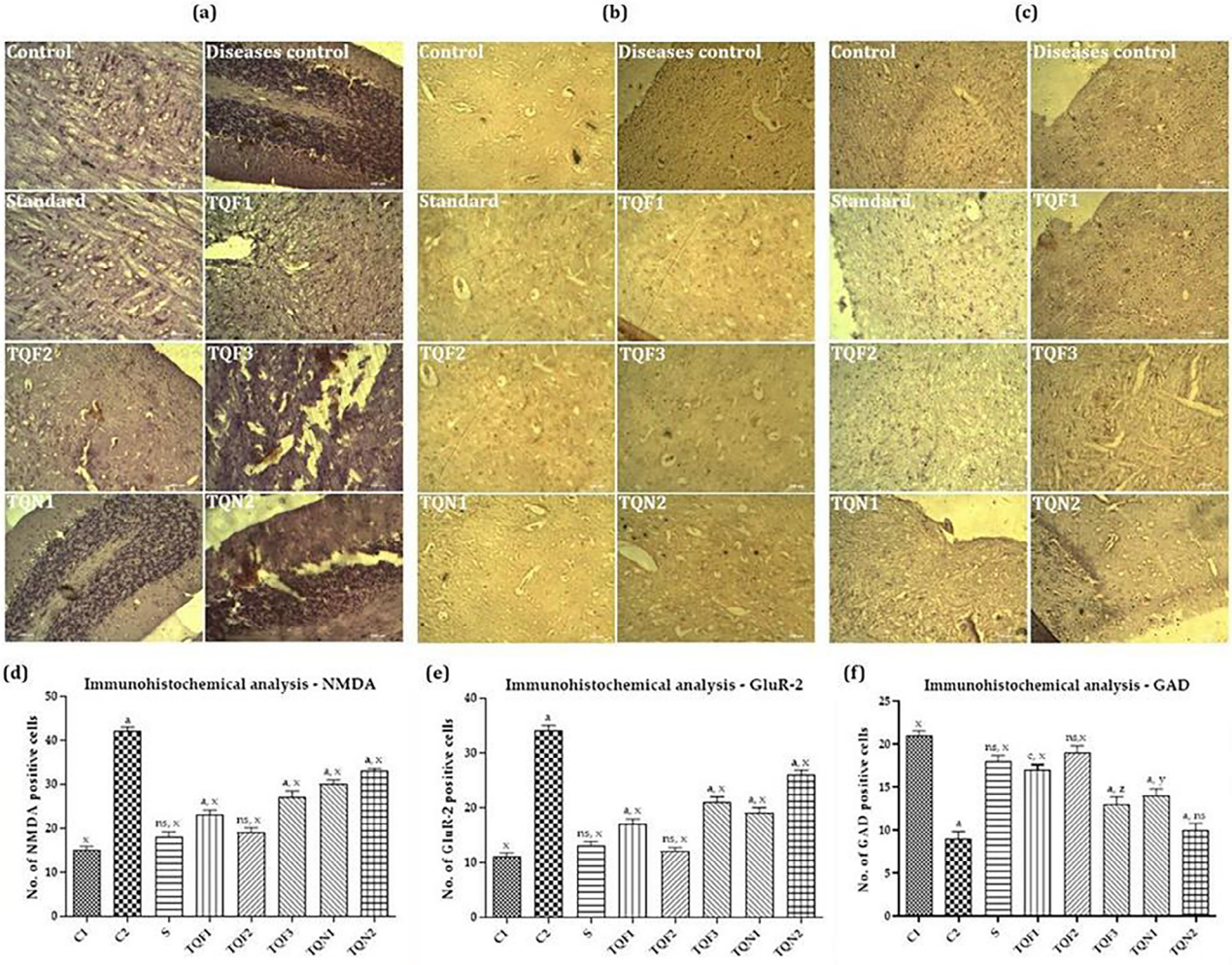

Fig. 4. Estimation of NMDA reactive cells, GluR-2 reactive cells and GAD 65/67 reactive cells by immunohistochemical analysis.

tive conditions (Samarghandian et al., 2018). However, although TQ has been regarded as the main bioactive molecule of black cumin oil, systematic investigations on the role of TQ content on neuroprotective effect has not been investigated yet (Tavakkoli et al., 2017). Thus, the present study attempted to evaluate the anti-neuroinflammatory effect and hence the neuroprotective activity of standardized black cumin oil as a function of its TQ content, using ICV administered IBO-induced neuroinflammatory model of rats.

Ibotenic acid has been shown to affect significant loss of nerve cells at various parts of the brain, including the striatum, hippocampus, substantia nigra, and cortex and produces vigorous gliosis in the region of neuronal loss (Zong et al., 2016). The non-neuronal inflammatory cells thus produced have the phenotype of macrophages and can damage the healthy axons and contribute to the increase in vascular permeability at the site of the lesion (Gaudet and Fonken 2018). Thus, ICV-administration of IBO has been shown to affect both the resident microglia and the protoplasmic astrocytes leading to inflammation and neuronal cell death. It is also validated as a model for anxiety-related behavioral changes and excitotoxic lesions resembling Alzheimer's disease (Tan and Kuner, 2021, Yang et al., 2020).

Significant behavioral changes, as observed in elevated plus-maze and open field study, clearly indicate the development of significant neurotoxicity upon administration of IBO as observed by Afees et al. (2022). The elevated plus-maze open field is a widely used behavioral experiment to assess the anxiolytic effects of pharmacologically active compounds (Walf and Frye 2007; Sestakova et al., 2013). In the elevated plus-maze experiment, both the number of entries and the time spent in the open arms were significantly increased upon treatment with BCO-5 compared to the IBO-treated disease control group, indicating the anxiolytic effects of BCO-5. The behavioral patterns were also reversed among BCO-5 treated animals, as evident from the significant increase in the ambulation and rearing frequencies compared to IBO group. However, the fact that BCO-2 showed no significant improvement in the behaviour indicates a definite role of TQ in the anxiolytic effect of black cumin oil. In a separate clinical study involving 15 healthy persons, we have shown that BCO-5 alleviated anxiety and stress levels. The anxiolytic effect of TQ due to its effects on the GABAergic pathways and on the NO-cGMP levels have already been reported (Gilhotra and Dhingra, 2011). The anxiolytic effect has great importance to reduce the psychiatric burden among the Alzheimer's patients (Becker et al., 2018).

Obesity is one of the major causes of the occurrence and development of cardiovascular events, thus the body weight gain was considered as one factor for cardiovascular risk (Powell-Wiley et al., 2021). It is noted that hyperlipidemia is closely associated with cardiovascular disease and high blood pressure is an important risk factor for CVD occurrence (Powell-Wiley et al., 2021). It has been demonstrated that the acetylcholine and cholinergic neurotransmission pathway play a vital role in learning, memory and

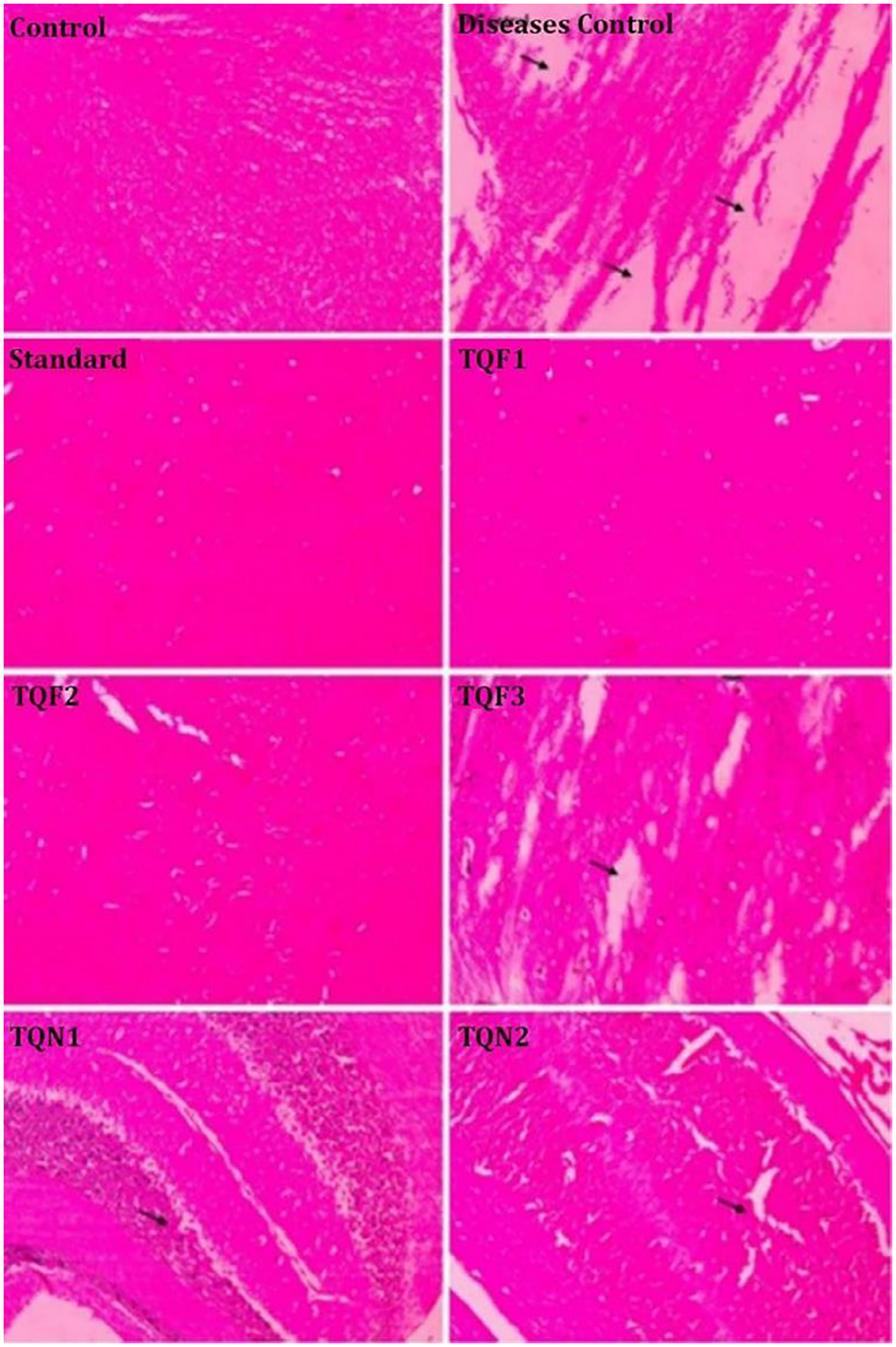

Fig. 5. Histopathological leisions in the brain of animals in response to different treatments.

spontaneous alertness, cognition, emotional behavior, wakefulness and attention (Lee et al., 2021, Luchicchi et al., 2014). Acetylcholinesterase (AChE) is an enzyme that catalyzes the breakdown and hence increased AChE causes the depletion of acetylcholine (Colovic et al., 2013). So, one of the therapeutic strategies for may ailments including Alzheimer's disease is the use of AChE inhibitors (Khazdair, 2015). In the present study, BCO-5 at 10 and 5 mg/kg body weight dosage produced significant inhibition of AChE activity and produced significantly high levels of acetylcholine in the brain tissues of animals as compared to IBO-treated disease control groups, which caused a significant reduction in acetylcholine levels compared to the vehicle control group. However, the BCO-2 group produced no significant effect, indicating the positive role of TQ in AChE inhibition and hence in the neuroprotective effect of black cumin oil. Previous research has shown the AChE inhibitory effects for other molecules found in black cumin oil, such as thymol, carvacrol, and thymohydroquinone (Silva et al., 2018).

Tumor necrosis factor (TNF-α) is an important pro-inflammatory cytokine and NF-κB is a transcription factor found in excessive levels in the brain tissues of Alzheimer's patients (Mohan et al., 2021, McCaulley and Grush, 2015). The TNF-α/NF-κB signalling pathway closely links neuroinflammation and neurodegeneration in the Alzheimer's pathogenesis (Zhou et al., 2014). NF- κB is a crucial mediator of neuronal inflammation and can regulate pro-inflammatory markers such as cytokines (Mohan et al., 2021). We observed a significant decrease in both TNF-α and NF-κB levels when treated with both BCO-5 and BCO-2, with a more significant effect for BCO-5 in a dose-dependent manner, further supporting the role of TQ in ameliorating the neuroinflammation. Various other phytochemicals, including curcumin, resveratrol, and green tea catechins have also been reported to modulate the brain tissue levels of TNF-α and NF-κB; but at higher doses (Jit et al., 2021). The significant activity of BCO-5 at relatively lower doses of 5 to 10 mg/kg body weight indicate the strong anti- neuroinflammatory effect, probably due to the better bioavailability of TQ. Previous reports have shown that TQ inhibits NF-kB mediated neural inflammation by activating Nrf2/ARE signalling pathway and prevents PI3k and Akt phosphorylation in BV2 microglia (Wang et al., 2015).

We have also studied the activity of black cumin oil on various receptors and their subtypes by using immunohistochemical analysis to gather better information on their pathway or mode of action. The NMDA receptor-mediated glutamatergic neurotransmission has been shown to play a crucial role in the synaptic functions and the plasticity of neurons. Excessive glutamatergic neurotransmission and neurotransmitter glutamate play a vital role in the pathogenesis of anxiety and neurodegenerative disorders, specifically in Alzheimer's (Schwartz et al., 2012, Celli et al., 2019). Strong activation of NMDA receptors leads to enhanced synaptic strength, leading to neuroinflammation and nerve cell death (Wang and Reddy, 2017). Besides, previous studies have shown that continuous activation of NMDA receptors can increase β amyloid (Aβ) levels in the brain (Fouad et al., 2018). The significant decrease in the NMDA-positive immunoreactive cells observed upon the immunohistochemical analysis in the present study may be due to the NMDAR antagonistic property of the BCO-5 in a dose-dependent manner.

We also studied the activity of BCO-5 on AMPA receptor GluR-2 subunits since they mediate fast excitatory neurotransmission along with specific synaptic responses related to cognition. The GluR-2 receptors present in the hippocampal cornu Ammonis (CA1) region of the brain play a significant role in dementia and behavioral changes (Gao et al., 2021, Li et al., 2020). Administration of IBO leads to the up-regulation of GluR-2 expression and the GluR- 2 positive immunoreactive cells were found to be significantly increased as reported previously (Karthick et al., 2016). However, the treatment with BCO-5 significantly reduced the level of GluR-2 immunoreactive cells, indicating its specific affinity on AMPA receptor subtypes. Thus, it could be concluded that BCO-5 acts by both NMDA and AMPA subtypes of glutamate receptors.

Apart from glutamate receptor studies, we also studied the effect of BCO-5 on GABA-ergic system by immunohistochemical analysis of glutamic acid decarboxylase (GAD), which is an enzyme involved in the biosynthesis of GABA. GAD 65 and 67 subtypes are present in the brain and had effects on cognitive performances, autism, Alzheimer's and neuropathic pain (Rozycka and Liguz-Lecznar, 2017). Ibotenic acid is proved to be an inhibitor of GAD (Maruca et al., 2020). The present study showed a dose-dependent increase in GAD when treated with BCO-5 as evidenced by the increased number of immunoreactive cells. The increase in the production of GAD can result in the conversion of the excess extracellular glutamate to GABA thereby decreasing the glutamate excitotoxicity (Gilhotra and Dhingra, 2011),. The observed results are also in line with the previou study of Gilhotra and Dhingra (2011), where TQ modulated the level of GABA in stressed mouse.

It is known that ICV injection of IBO leads to the direct exposure of the toxin to the nerve cells resulting in severe neuronal lesions in rats. The histopathological investigation from the study indicated the occurrence of inflammatory lesions as evident by hyperplastic astrocytes and necrosis in the cerebellum of the IBO-treated animals. The present study showed that the protection offered by BCO-5 at 5 mg/kg body weight was at par with memantine treatment. Both BCO-5 group and memantine treated group had normal cerebellum with normal astrocytes and glial cells. However, in the BCO-2 treated group, even at a higher dose, there were necrotic lesions. Thus, BCO-5 treatment has been shown to reverse the brain damage caused by IBO as compared to BCO-2.

Thus, the present study has shown that black cumin oil containing 5 % TQ (BCO-5) was highly effective in ameliorating neurotoxic effects induced by IBO, as compared to the common black cumin oil with around 2 % TQ (BCO-2). The low TQ containing BCO-2 even at a 4-fold higher dosage failed to show significant changes in majority of the parameters examined in the current study as compared to BCO-5. The anxiolytic effects of BCO-5 were evident from the increase in ambulation and rearing behavior. BCO-5 has shown protective effects on the various important pathways involved in the pathogenesis of neurodegenerative disease such as Alzheimer's disease. BCO-5 acted on the acetylcholine pathway (via AChE inhibition), glutamate pathway (via NMDA and AMPA subtypes of glutamate receptors, and GAD) and neuroinflammation (via NF-κB signalling pathway), improving associated anxiety- related behavior and protected the brain cells from necrosis. Thus, BCO-5 may be considered as a potent lead as compared to normal BCO-2 for improvement of various neurodegenerative conditions, especially to manage stress and anxiety, which very often makes significant psychological burden to the Alzheimer's disease patients.

5. Conclusions

In the present study, it was found that black cumin oil exhibits significant anti-neuroinflammatory and neuroprotective effect as a function of its TQ content. Black cumin oil with 5 % TQ (BCO-5) was found to offer a significant neuroprotective effect at 5 mg/kg body weight and even reversed the behavioral changes induced by the ICV-administration of IBO. Black cumin oil with 2 % TQ (BCO-2) on the other hand showed either poor protective effect or no effect even at 4-fold higher dosage. Moreover, BCO-5 significantly reduced the key mediators of neuroinflammation-TNF-α and NF-κB. Immunohistochemical analysis further revealed the significant ability of BCO-5 to decrease the NMDA and GluR-2 positive immune-reactive cells and increase the number of GAD. Histopathological results showed that treatment with BCO-5 could effectively protect from the neuronal damage caused by ICV-administration of IBO. Significant neuroinflammation and damage to the neuronal cells with the presence of hyperplastic astrocytes and necrosis were observed among IBO-treated rats. These changes in the biochemical markers together with the improvement in behavioral changes indicate the potential therapeutic effect of BCO-5 in counteracting the anxiety/stress-related neurological disorders, especially for Alzheimer's disease.

6. Institutional review board statement

The animal study protocol was approved by the institutional review board of Centre for Professional and Advanced Studies, Kottayam, India (approval number MGU/DPS/IAEC/2016PhD-05 dated 15–05-2016).

CRediT authorship contribution statement

Sibi P Ittiyavirah: Funding acquisition, Project administration, Validation, Conceptualization, Methodology, Software, Formal analysis, Investigation, Resources, Data curation, Writing – review & editing. **Kannan Ramalingam:** Funding acquisition, Project administration, Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – review & editing. **Arathy Sathyan:** Project administration, Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – review & editing. **R.S. Rajasree:** Project administration, Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Visualization, Supervision. **Mohamed Saheer Kuruniyan:** Funding acquisition, Project administration, Data curation, Writing – review & editing, Visualization. **Muhammed Elayadeth-Meethal:** Funding acquisition, Project administration, Data curation, Writing – review & editing, Visualization. **Punnoth Poonkuzhi Naseef:** Funding acquisition, Data curation, Writing – review & editing, Visualization, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Afees, O.J., Ayodele, B.R., Gbenga, A., Taiye, A.S., Yinka, O.S., Dayo, O., Sunday, F.O., Leviticus, A., Esther, O.O., Igbo, E.J., Dorcas, T.O., 2022. Localised streptozotocin-induced structural and cognitive changes in the hippocampal cornu ammonis 1 (CA-1) neurons and mitigating effects of Zingiber officinale. Phytomed. Plus 2, 100162.

Amable, P.R., Carias, R.B.V., Teixeira, M.V.T., da Cruz Pacheco, Í., Corrêa do Amaral, R. J.F., Granjeiro, J.M., Borojevic, R., 2013. Platelet-rich plasma preparation for regenerative medicine: optimization and quantification of cytokines and growth factors. Stem Cell. Res. Ther. 4, 1-13.

Becker, E., Rios, C.L.O., Lahmann, C., Ruecker, G., Bauer, J., Boeker, M., 2018. Anxiety as a risk factor of Alzheimer's disease and vascular dementia. Br. J. Psychiatry 213, 654–660.

Benussi, A., Alberici, A., Buratti, E., Ghidoni, R., Gardoni, F., Di Luca, M., Padovani, A., Borroni, B., 2019. Toward a glutamate hypothesis of frontotemporal dementia. Front. Neurosci. 13, 1–9. https://doi.org/10.3389/fnins.2019.00304.

Blanco-Silvente, L., Capella, D., Garre-Olmo, J., Vilalta-Franch, J., Castells, X., 2018. Predictors of discontinuation, efficacy, and safety of memantine treatment for Alzheimer's disease: meta-analysis and meta-regression of 18 randomized clinical trials involving 5004 patients. BMC Geriatr. 18, 160–168.

Cabeza, O.A., Zhang, Z., Khoury, E.S., Sheldon, R.A., Sharma, A., Zhang, F., Slusher, B.S., Kannan, R.M., Kannan, S., Ferriero, D.M., 2021. Neuroprotective effects of a dendrimer-based glutamate carboxypeptidase inhibitor on superoxide dismutase transgenic mice after neonatal hypoxic-ischemic brain injury. Neurobiol. Dis. 148, 105201.

Celli, R., Santolini, I., Van Luijtelaar, G., Ngomba, R.T., Bruno, V., Nicoletti, F., 2019. Targeting metabotropic glutamate receptors in the treatment of epilepsy: rationale and current status. Expert Opin. Ther. Targets 23, 341–351.

Chen, X., Drew, J., Berney, W., Lei, W., 2021. Neuroprotective natural products for Alzheimer's disease. Cells 10, 6–7.

Clark, I., Vissel, B., 2016. Excess cerebral TNF causing glutamate excitotoxicity rationalizes treatment of neurodegenerative diseases and neurogenic pain by anti-TNF agents. J. Neuroinflamm. 13, 1–16.

Colovic, M., Krstic, D., Lazarevic-Pasti, T., Bondzic, A., Vasic, V., 2013. Acetylcholinesterase inhibitors: pharmacology and toxicology. Curr. Neuropharmacol. 11, 315.

Cunnane, S.C., Trushina, E., Morland, C., Prigione, A., Casadesus, G., Andrews, Z.B., Beal, M.F., Bergersen, L.H., Brinton, R.D., de la Monte, S., Eckert, A., 2020. Brain energy rescue: an emerging therapeutic concept for neurodegenerative disorders of ageing. Nat. Rev. Drug Discov. 19, 609–633.

Di Sabato, D., Quan, N., Godbout, J., 2016. Neuroinflammation: the devil is in the details. J. Neurochem. 139, 136–153.

Fouad, I., Sharaf, N., Abdelghany, R., El Sayed, N., 2018. Neuromodulatory effect of thymoquinone in attenuating glutamate-mediated neurotoxicity targeting the amyloidogenic and apoptotic pathways. Front. Neurol. 236, 1–12.

Gao, T.H., Ni, R.J., Liu, S., Tian, Y., Wei, J., Zhao, L., Wang, Q., Ni, P., Ma, X., Li, T., 2021. Chronic lithium exposure attenuates ketamine-induced mania-like behavior and c-Fos expression in the forebrain of mice. Pharmacol. Biochem. Behavior 202, 173108.

Gaudet, A.D., Fonken, L.K., 2018. Glial cells shape pathology and repair after spinal cord injury. Neurotherapeutics 15, 554–577.

Gawron, G., Krzyczkowski, W., Lyzen, R., Kadzinski, L., Banecki, B., 2021. Influence of supercritical carbon dioxide extraction conditions on extraction yield and composition of *Nigella sativa* L. seed oil—modelling, optimization and extraction kinetics regarding fatty acid and thymoquinone content. Molecules 26, 6419.

Gilhotra, N., Dhingra, D., 2011. Thymoquinone produced antianxiety-like effects in mice through modulation of GABA and NO levels. Pharmacol Rep. 63, 660–669.

Glascock, J., Osman, E., Coady, T., Rose, F., Shababi, M., Lorson, C., 2011. Delivery of therapeutic agents through intracerebroventricular (ICV) and intravenous (IV) injection in mice. J. Vis. Exp. 56, 2968.

Halder, S., Anand, U., Nandy, S., Oleksak, P., Qusti, S., Alshammari, E.M., Batiha, G.E. S., Koshy, E.P., Dey, A., 2021. Herbal drugs and natural bioactive products as potential therapeutics: A review on pro-cognitives and brain boosters perspectives. Saudi Pharm J. 29, 879–907.

Ilyas, U.K., Elayadeth-Meethal, M., Kuruniyan, M.S., Quadri, S.A., Rajasree, R.S., Naseef, P.P., 2022. Densitometric quantification and optimization of polyphenols in Phyllanthus maderaspatensis by HPTLC. Saudi J. Biol. Sci. 29 (3), 1521–1529.

Ilyas, U., Katare, D.P., Naseef, P.P., Kuruniyan, M.S., Elayadeth-Meethal, M., Aeri, V., 2021. Immunomodulatory activity of Phyllanthus maderaspatensis in LPS-stimulated mouse macrophage RAW 264.7 cells. Separations 8 (9), 129.

Iovino, L., Tremblay, M.E., Civiero, L., 2020. Glutamate-induced excitotoxicity in Parkinson's disease: the role of glial cells. J. Pharmacol. Sci. 144, 151–164.

Jit, B.P., Pradhan, B., Dash, R., Bhuyan, P.P., Behera, C., Behera, R.K., Sharma, A., Alcaraz, M., Jena, M., 2021. Phytochemicals: potential therapeutic modulators of radiation induced signaling pathways. Antioxidants 11, 49.

Kang, Y.P., Mockabee-Macias, A., Jiang, C., Falzone, A., Prieto-Farigua, N., Stone, E., Harris, I.S., DeNicola, G.M., 2021. Non-canonical glutamate-cysteine ligase activity protects against ferroptosis. Cell Metab. 33, 174–189.

Kannan, R., George, S., PS, B.C., Maliakel, B., Ittiyavirah, S. and Krishnakumar, I.M., 2022. Thymoquinone-rich black cumin oil improves sleep quality, alleviates anxiety/stress on healthy subjects with sleep disturbances–A pilot polysomnography study. J. Herb. Med. 32, 100507.

Kannan, R., Ittiyavirah, S., Harindran, J., 2018. Acetylcholinesterase and growth inhibitory effects – various grades of *N. Sativa* oils. Int. J. Pharm. Sci. Res. 10, 245–250.

Karthick, C., Periyasamy, S., Jayachandran, K., Anusuyadevi, M., 2016. Intrahippocampal administration of ibotenic acid induced cholinergic dysfunction via NR2A/NR2B expression: implications of resveratrol against Alzheimer disease pathophysiology. Front. Mol. Neurosci. 9, 28.

Khazdair, M.R., 2015. The protective effects of *Nigella sativa* and its constituents on induced neurotoxicity. J. Toxicol. 15, 1–7.

Kim, S., Roh, J., Park, C., 2016. Immunohistochemistry for pathologists: protocols, pitfalls, and tips. J. Pathol. Transl. Med. 50, 411.

Kooti, W., Hasanzadeh-Noohi, Z., Sharafi-Ahvazi, N., Asadi-Samani, M., Ashtary-Larky, D., 2016. Phytochemistry, pharmacology, and therapeutic uses of black seed (*Nigella sativa*). Chin. J. Nat. Med. 14, 732–745.

Laj, N., Elayadeth-Meethal, M., Huxley, V.A.J., Hussain, R.R., Kuruniyan, M.S., Naseef, P.P., 2022. Quorum-sensing molecules: sampling, identification and characterization of N-acyl-homoserine lactone in Vibrio sp. Saudi J. Biol. Sci. 29 (4), 2733–2737.

Lee, H.J., Choi, T.I., Kim, Y.M., Lee, S., Han, B., Bak, I.S., Moon, S., Yu, D.Y., Shin, K.S., Kwon, Y.K., Moon, C., 2021. Regulation of habenular G-protein gamma 8 on learning and memory via modulation of the central acetylcholine system. Mol. Psychiatry 26, 3737–3750.

Li, W., Kutas, M., Gray, J.A., Hagerman, R.H., Olichney, J.M., 2020. The role of glutamate in language and language disorders-evidence from ERP and pharmacologic studies. Neurosci. Biobehav. Rev. 119, 217–241.

Luchicchi, A., Bloem, B., Viaña, J., Mansvelder, H., Role, L., 2014. Illuminating the role of cholinergic signalling in circuits of attention and emotionally salient behaviors. Front. Synaptic Neurosci. 6, 24.

Mahdi, O., Baharuldin, M.T.H., Nor, N.H.M., Chiroma, S.M., Jagadeesan, S., Moklas, M. A.M., 2019. Chemicals used for the induction of Alzheimer's disease-like cognitive dysfunctions in rodents. Biomed. Res. Ther. 6, 3460–3484.

Malik, A.R., Willnow, T.E., 2019. Excitatory amino acid transporters in physiology and disorders of the central nervous system. Int. J. Mol. Sci. 20, 5671.

Maruca, A., Rocca, R., Catalano, R., Mesiti, F., Costa, G., Lanzillotta, D., Salatino, A., Ortuso, F., Trapasso, F., Alcaro, S., Artese, A., 2020. Natural products extracted from fungal species as new potential anti-cancer drugs: a structure-based drug repurposing approach targeting HDAC7. Molecules 25, 5524.

Mazaheri, Y., Torbati, M., Azadmard-Damirchi, S., Savage, G.P., 2019. A comprehensive review of the physicochemical, quality and nutritional properties of Nigella sativa oil. Food Rev. Int. 35, 342–362.

McCaulley, M., Grush, K., 2015. Alzheimer's Disease: Exploring the role of inflammation and implications for treatment. Int. J. Alzheimers Dis. 15, 1–10.

Mohan, M., Abhimannue, A., Kumar, B., 2021. Modulation of proinflammatory cytokines and enzymes by polyherbal formulation *Guggulutiktaka ghritam*. J. Ayurveda Integr. Med. 12, 13–19.

Mohd Sairazi, N., Sirajudeen, K., 2020. Natural products and their bioactive compounds: neuroprotective potentials against neurodegenerative diseases. Evid. Based Complem. Altern. Med. 1–30. https://doi.org/10.1155/2020/6565396.

Mukhtar, H., Qureshi, A.S., Anwar, F., Mumtaz, M.W., Marcu, M., 2019. *Nigella sativa* L. seed and seed oil: Potential sources of high-value components for development of functional foods and nutraceuticals/pharmaceuticals. J. Essent. Oil Res. 31, 171–183.

Naseef, P.P., Elayadeth-Meethal, M., Anjana, A., Muhas, C., Kuruniyan, M.S., 2021. Therapeutic potential of induced iron depletion using iron chelators in COVID-19. Saudi J. Biol. Sci.

Oskouei, Z., Akaberi, M., Hosseinzadeh, H., 2018. A glance at black cumin (*Nigella sativa*) and its active constituent, thymoquinone, in ischemia: a review. Iran. J. Basic Med. Sci. 21, 1200–1206.

Ozben, T., Ozben, S., 2019. Neuro-inflammation and anti-inflammatory treatment options for Alzheimer's disease. Clin. Biochem. 72, 87–89.

Pellow, S., Chopin, P., File, S., Briley, M., 1985. Validation of open: closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J. Neurosci. Methods 14, 149–167.

Powell-Wiley, T.M., Poirier, P., Burke, L.E., Despres, J.P., Gordon-Larsen, P., Lavie, C.J., Lear, S.A., Ndumele, C.E., Neeland, I.J., Sanders, P., St-Onge, M.P., 2021. Obesity and cardiovascular disease: a scientific statement from the American Heart Association. Circulation 143 (21), e984–e1010.

Rajasree, R.S., Ittiyavirah, S.P., Naseef, P.P., Kuruniyan, M.S., Anisree, G.S., Elayadeth-Meethal, M., 2021. An evaluation of the antioxidant activity of a methanolic extract of Cucumis melo L. Fruit (F1 hybrid). Separations 8 (8), 123.

Rajasree, R.S., Ittiyavirah, S.P., Naseef, P.P., Kuruniyan, M.S., Elayadeth-Meethal, M., Sankar, S., 2022. The anti-inflammatory properties of the methanolic extract of Cucumis melo Linn. against prostate enlargement in Wistar rats. Saudi J. Biol. Sci. 29, (9) 103396.

Rozycka, A., Liguz-Lecznar, M., 2017. The space where aging acts: Focus on the GABA ergic synapse. Aging Cell 16 (4), 634–643.

Samarghandian, S., Farkhondeh, T., Samini, F., 2018. A review on possible therapeutic effect of *Nigella sativa* and thymoquinone in neurodegenerative diseases. CNS Neurol. Disord. Drug Targets 17, 412–420.

Schwartz, T., Siddiqui, U., Raza, S., 2012. Memantine as an augmentation therapy for anxiety disorders. Case Rep. Psychiatry 12, 1–3.

Sestakova, N., Puzserova, A., Kluknavsky, M., Bernatova, I., 2013. Determination of motor activity and anxiety-related behavior in rodents: methodological aspects and role of nitric oxide. Interdiscip. Toxicol. 6, 126–135.

Silva, E.R., de Carvalho, F.O., Teixeira, L.G., Santos, N.G.L., Felipe, F.A., Santana, H.S.R., Shanmugam, S., Quintans Júnior, L.J., de Souza Araújo, A.A., Nunes, P.S., 2018. Pharmacological effects of carvacrol in in vitro studies: a review. Curr. Pharm. Des. 24, 3454–3465.

Tan, L.L., Kuner, R., 2021. Neocortical circuits in pain and pain relief. Nat. Rev. Neurosci. 22, 458–471.

Tavakkoli, A., Ahmadi, A., Razavi, B., Hosseinzadeh, H., 2017. Black seed (*Nigella Sativa*) and its constituent thymoquinone as an antidote or a protective agent against natural or chemical toxicities. Iran. J. Pharm. Res. 16, 2–23.

Thajudeen, K.Y., Alsayari, A., Najib Ullah, S.N.M., Salam, S., Elayadeth-Meethal, M., Uoorakkottil, I., 2022. Validation, Optimization and Hepatoprotective Effects of Boeravinone B and Caffeic Acid Compounds from Boerhavia diffusa Linn. Separations 9 (7), 177.

Van Dam, D., De Deyn, P.P., 2011. Animal models in the drug discovery pipeline for Alzheimer's disease. Br. J. Pharmacol. 2011 (164), 1285–1300.

Vezzani, A., Balosso, S., Ravizza, T., 2019. Neuroinflammatory pathways as treatment targets and biomarkers in epilepsy. Nat. Rev. Neurol. 15, 459–472.

Walf, A., Frye, C., 2007. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. Nat. Protoc. 2, 322–328.

Wang, Y., Gao, H., Zhang, W., Zhang, W., Fang, L., 2015. Thymoquinone inhibits lipopolysaccharide-induced inflammatory mediators in BV2 microglial cells. Int. Immunopharmacol. 26, 169–173.

Wang, R., Reddy, P., 2017. Role of glutamate and NMDA receptors in Alzheimer's disease. J. Alzheimer's Dis. 57, 1041–1048.

Yang, X., Ji, J., Liu, C., Zhou, M., Li, H., Ye, S., Hu, Q., 2020. HJ22, a Novel derivative of piperine, Attenuates ibotenic acid-induced cognitive impairment, oxidativestress, apoptosis and inflammation via inhibiting the protein-protein interaction of Keap1-Nrf2. Int. Immunopharmacol. 83, 106383.

Yang, Q.Q., Zhou, J.W., 2019. Neuroinflammation in the central nervous system: symphony of glial cells. Glia 67, 1017–1035.

Yim, N., Ryu, S.W., Choi, K., Lee, K.R., Lee, S., Choi, H., Kim, J., Shaker, M.R., Sun, W., Park, J.H., Kim, D., 2016. Exosome engineering for efficient intracellular delivery of soluble proteins using optically reversible protein–protein interaction module. Nat. Commun. 7 (1), 1–9.

Zhou, X., Liang, J., Wang, J., Fei, Z., Qin, G., Zhang, D., Zhou, J., Chen, L., 2020. Up-regulation of astrocyte excitatory amino acid transporter 2 alleviates central sensitization in a rat model of chronic migraine. J. Neurochem. 155, 370–389.

Zhou, M., Yuan, M., Zhao, M., Hou, M., Med, C., Ma, M., Yu, M., 2014. Genistein antagonizes inflammatory damage induced by β-amyloid peptide in microglia through TLR4 and NF-κB. Nutrition 30, 90–95.

Zieba, A., Stępnicki, P., Matosiuk, D., Kaczor, A.A., 2022. What are the challenges with multi-targeted drug design for complex diseases? Expert. Opin. Drug Discov., 1–11

Zong, N., Li, F., Deng, Y., Shi, J., Jin, F., Gong, Q., 2016. Icariin, a major constituent from Epimedium brevicornum, attenuates ibotenic acid-induced excitotoxicity in rat hippocampus. Behav. Brain Res. 313, 111–119.

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A phase I clinical trial to evaluate the safety of thymoquinone-rich black cumin oil (BlaQmax®) on healthy subjects: Randomized, double-blinded, placebo-controlled prospective study

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ABSTRACT

Black cumin or black seed (*Nigella sativa L.*) is a popular medicinal herb and culinary spice belonging to *Ranunculacea* family. Thymoquinone (TQ) is the major active phytoconstituent in black cumin and is abundant in the volatile oil fraction. Though black cumin oil containing low TQ content (less than 1%) has been clinically investigated, clinical efficacy and safety data of TQ-rich oil is limited. A recent study with black cumin oil formulation containing 5% TQ (BCO-5) exhibited significant clinical efficacy to alleviate sleep disorders and stress. So, the present phase 1 randomized, double-blinded, placebo-controlled trial evaluated the safety of BCO-5 at a dose of 200 mg/adult/day for 90 days on healthy subjects (n = 70). Both the biochemical and hematological parameters were analysed along with the adverse events or side effects to establish the clinical safety of BCO-5. The study reported neither serious adverse side effects nor any significant alterations in the hematological parameters. The absence of significant changes in the biochemical parameters related to liver function (ALT, AST, ALP), renal function (serum creatinine and urea) were also observed. However, analysis of lipid profile showed a significant ($P < 0.05$) reduction in total cholesterol, LDL, VLDL and triglycerides, but within the normal range. In conclusion, BCO-5 is safe at 200 mg/adult/day for human consumption and may be clinically evaluated for various health beneficial pharmacological activities where black cumin oil has been shown to have positive effects.

1. Introduction

Black cumin or black seed (*Nigella sativa L.*) is an annual flowering herb belonging to the family *Ranunculaceae*. The herb possesses a wide range of medicinal properties in addition to its application as a culinary spice. It has been known in commerce for the past 2000 years and very often regarded as a "miracle cure" and "seed of blessing" owing to its long history of usage to treat various diseases [1]. Both the seeds and its oil have been used extensively in Indian and Arab traditional systems of medicine such as Ayurveda and Unani [2]. Black cumin is considered to be rich in proteins, mucilage, dietary fibre, and non-volatile phytochemicals such as alkaloids, saponins, tannins, minerals, and vitamins [3,4]. Despite its high phytochemical constituents, most of the therapeutic properties of black cumin has been attributed to its oil part obtained by cold-pressing or supercritical extraction methods [5]. Black cumin oil (BCO) contains both the fixed and essential oil fractions with thymoquinone (TQ) being the major molecule in the essential oil part along with other molecules including *p*-cymene, carvacrol, thymohydroquinone (THQ), dihydrothymoquinone (DHTQ), α-thujene, thymol, *t*-anethole, α-pinene, β-pinene and γ- terpinene [6]. The fixed oil fraction accounts for 32–40% (w/w) and contains linolenic acid as the major essential fatty acid along with arachidonic, eicosadienoic, oleic, palmitic, stearic and myristic acids. It was also found to contain β-sitosterol, cycloeucalenol, cycloartenol, and sterol esters in relatively low amounts [7–9]. The use of black cumin seeds and its oil in food is Generally Recognized as Safe (GRAS) by United States Food and Drug Administration (USFDA) (Code of federal regulation: 21CFR182.10) [10].

A number of extraction methods such as solvent extraction, cold-

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pressing, microwave-assisted extraction, Soxhlet extraction and supercritical fluid extraction techniques have been described for the black cumin oil preparation [11,12]. Though cold pressing was one of the oldest methods for the commercial scale production, supercritical extraction is now gaining importance as the green technology of preference [12]. Cold-pressed oil is widely available as black cumin oil and was found to contain only less than 0.5% TQ, when a validated high performance liquid chromatography (HPLC) method was used for quantification [13]. As per the current understanding, TQ has been identified as the major bioactive molecule responsible for the wide range of therapeutic effects and safety [1,14–18]. Hossen et al., and Ratheesh et al., demonstrated that the anti-inflammatory effect of black cumin oil was influenced by its TQ content [16,19]. Ramalingam et al., established the influence of TQ content on the acetylcholine esterase inhibition activity and also reported the dependence of TQ content on the acute toxicity of black cumin oil [17,18].

A number of pharmacological effects have been reported for black cumin and its oil including antioxidant, anti-inflammatory, anti-bacterial, anti-fungal, anti-viral, anti-cancer, hypoglycaemic, anti-hypertensive, hypolipidemic, cardioprotective, hepatoprotective, nephroprotective, neuroprotective, gastroprotective, immunomodulatory, anti-allergic and anti-obesity effects in various animals and human models (Fig. 1) [20–27]. An internet search using PubMed-Medline, Science Direct and Scopus search engines revealed about 76 clinical trials on either black cumin seed or oil; out of which, 31 were randomized double-blinded placebo-controlled trials and 17 were randomized control trials. Most of the clinical trials have used high dosage (3–10 mL/day) of cold-pressed oil due to the low TQ content. Therefore, a validated HPLC-method for TQ quantification and optimum dosage of TQ are important for both safety and efficacy.

In this regard, we developed a black cumin oil formulation containing 5% TQ (BCO-5; Patented and registered as BlaQmax®) (Patent No: US 10485837 B2; dated 26 November 2019). Preclinical toxicity studies as per Organization for Economic Co-operation and Development (OECD) guidelines could establish the possible safe dosage of BCO-5 in humans as not more than 900 mg/adult/day or not more than 50 mg of TQ/adult/day [17]. When supplemented at 200 mg/day, BCO-5 has been shown to improve the sleep quality by reducing sleep latency, sleep disturbance and deep sleep (REM sleep) duration with a significant reduction in anxiety and stress [28]. Thus, the present study was aimed to investigate the long-time safety of BCO-5 on healthy subjects at 200 mg/day for 90 days. Haematological and biochemical parameters were examined along with anthropometric measurements to monitor safety. All adverse events and side effects including food/water consumption were also recorded.

2. Materials and methods

2.1. Chemicals and Raw materials

Pharmacognosist identified dried seeds of black cumin were collected directly from the farmers and a voucher specimen (AK-NS-018) was stored at the herbarium of Akay Natural Ingredients, Cochin, India. The oil was produced at the Good Manufacturing Practices (GMP)-certified manufacturing plant of Akay Natural Ingredients, Cochin, India, (BlaQmax® Batch No: BCOQ 32/21 dated 12/04/2021). All solvents used for the analysis was of HPLC-grade and were purchased from M/s Sigma Aldrich, Bangalore, India. Analytical standard of TQ (CAS No: 490–91–5) was also obtained from M/s Sigma Aldrich, Bangalore, India.

High performance thin layer chromatography (HPTLC) in comparison with the botanical reference material was performed to confirm the identity of the raw material used for producing BCO-5. HPTLC densitometric analysis was performed on 10 cm × 20 cm aluminium-backed

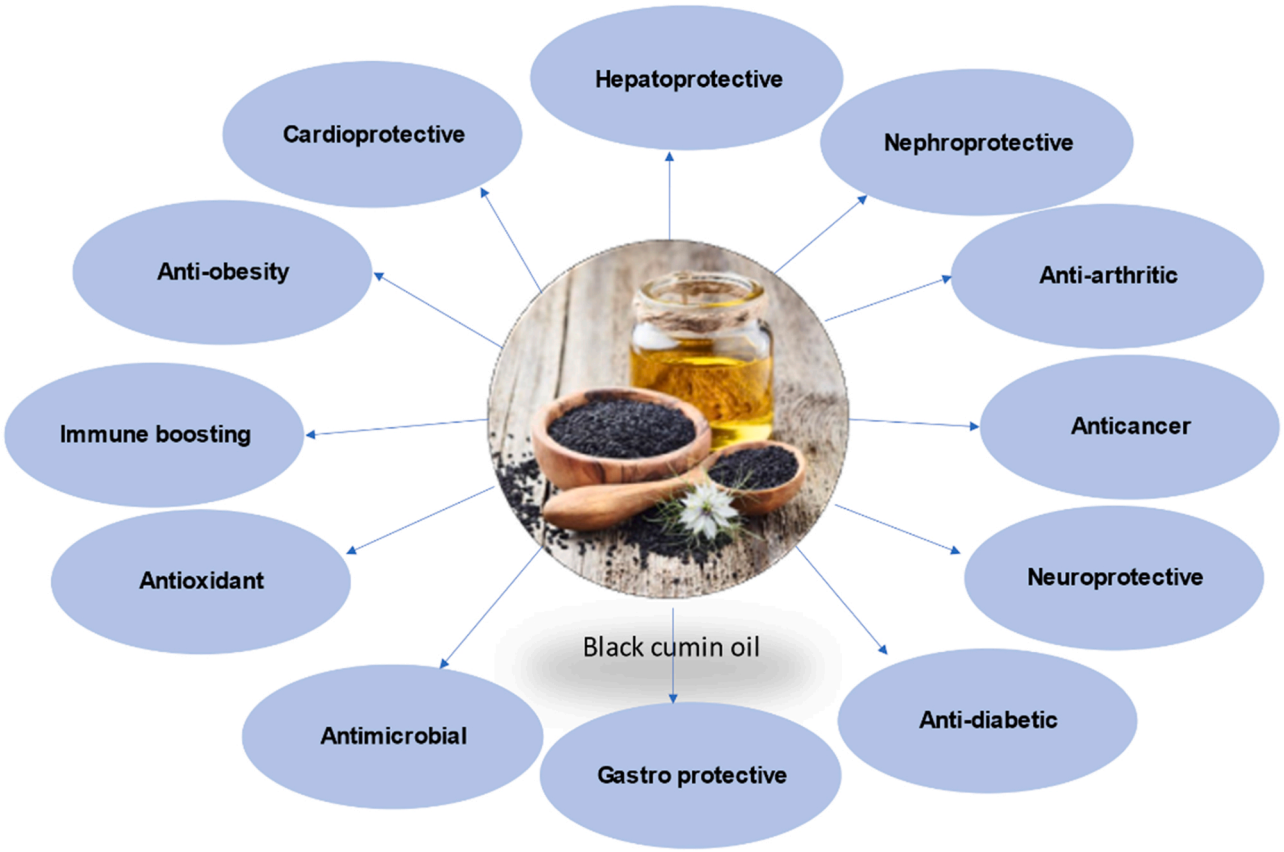

Fig. 1. Reported pharmacological effects of *Nigella sativa* and its oil.

plates coated with 0.2 mm layers of silica gel 60 F254 (E-Merck, Germany) employing CAMAG HPTLC system, Switzerland. Samples were applied to the TLC plates as 6 mm bands using an automatic TLC sampler fitted with a microlitre syringe. A constant application rate of 150 nL/s was used. Linear ascending development of the plates to a distance of 80 mm was performed with Toluene: ethyl acetate (7:3 v/v) as mobile phase in an automatic developing chamber previously saturated with mobile phase vapour for 30 min at 22 ℃. After development, the plates were treated with Vanillin/Sulphuric acid solution, heated to 110 °C and was scanned at 366 nm.

Thymoquinone content was estimated using a standardized HPLC method [29], employing SPD Shimadzu model instrument fitted with M20A photo-diode array detector (Shimadzu Analytical India Private Limited, Mumbai, India) and a reverse-phase C18 column (150 × 4.6 mm, 5 μm) (Phenomenex, Hyderabad, India). BCO-5 oil used in the study was found to contain, 5.2% (w/w) of TQ content.

2.2. Study materials

Identical soft gelatin capsules containing either BCO-5 or placebo (200 mg/capsule) were obtained from Akay Natural Ingredients, Cochin, India in tightly closed and sealed HDPE bottles (95 capsules/bottle). Detailed certificate of analysis and declaration of its suitability for human consumption was also received from the manufacturer. Identical capsules of flax seed oil were used as the placebo.

2.3. Participants and study design

The study was designed as a randomized, double-blinded, placebo-controlled trial to evaluate the safety of BCO-5 in healthy volunteers. During visit 1, 120 participants were screened as per the eligibility criteria and prior medical history. The selected seventy participants (both males and females) were randomized using computer generated randomization technique into two groups (n = 35/group) so as to receive either placebo (Group I) or BCO-5 (Group II) at a dose of 200 mg/day, 10–20 min before bed time.

The study duration was 90 days. Inclusion and exclusion criteria followed in the study are given in Table 1. A written informed consent was obtained from all study participants, prior to the study. Demographic and haematological parameters as well as vitals were recorded on Day 1 (Visit 2) and Day 90 (Visit 3) along with safety parameters. A cohort diagram displaying the study design is depicted in Fig. 2. The study was conducted at the Department of General Medicine, Balagangadharanatha Swami Global Institute of Medical sciences, BGS Health and Education city, Bangalore, India under the guidance of a qualified medical practitioner. The study was in strict compliance with the clinical research guidelines established by the Government of India and the declaration of Helsinki. The study was registered in clinical trial registry of India at http://ctri.nic.in/ [CTRI/2021/05/033780].

2.4. Safety outcomes

The adverse reactions were monitored using a questionnaire describing the earlier adverse effects reported upon exposure to black cumin, its oil or the active phytochemical thymoquinone. To judge the tolerance, the participants were informed to record any changes from the regular pattern of food or water intake, changes in sleep pattern, gastrointestinal disturbances, nausea or headache. Safety was further evaluated by recording the changes in anthropometric parameters, vital signs, haematological and biochemical parameters. The primary safety endpoints included the changes in liver and kidney function markers from the baseline till the end of study. Abnormality in physical examination, changes in vital signs, significant changes in clinical laboratory parameters and any other incidence of adverse events were considered for overall safety evaluation.

Table 1
Inclusion and exclusion criteria.

Inclusion criteria
1. Healthy male and females aged 18 – 50 years (both inclusive).
2. Participants having PSQI scores ≥ 5.
3. Healthy subjects with body weight ≥ 50 kg
4. Female participants of childbearing age agreeing to use approved birth control methods during the study and should have negative urine pregnancy test at the screening.
5. Participants ready to abstain from alcohol consumption, smoking and caffeinated beverages.
6. Participants who understand the study procedure and willing to provide signed informed consent to participate in the study.
Exclusion criteria
1. Subjects suffering from any chronic health conditions like diabetes, hypertension, chronic renal failure, heart, thyroid and liver disease and requiring medical treatment.
2. Participants with hepatic impairment (Alanine transaminase/Aspartate transaminase levels >3 upper limit of normal) or renal impairment (serum creatinine ≥2.0 mg/dl).
3. Participants with history of chronic metabolic disease, psychiatric illness, drug abuse, smoking, addiction to alcohol, endocrine abnormalities including thyroid disease.
4. Participants who have went cardiovascular surgery or any other major surgery.
5. Subjects with immunodeficiency disease, like, HIV or Hepatitis B / any other immuno-compromised state participants.
6. Subjects who may be allergic to any of the natural constituents of the investigational product.
7. Pregnant and lactating women.
8. History of clinically significant illness or any other medical disorder that may interfere with subject treatment, assessment or compliance with the protocol.
9. Currently participating or having participated in another clinical trial during the last 1 months prior to the beginning of this study.
Any additional condition(s) that in the Investigators opinion would warrant exclusion from the study or prevent the subject from completing the study.

2.5. Blood collection

Blood samples for the analysis of haematological and biochemical parameters were obtained from antecubital vein, after an overnight fasting (10 h). Blood samples were collected (10 mL) into plain vacutainer™ tubes for various analysis. Serum was separated after centrifugation (3500 rpm for 10 min at 4 ℃) of the clotted blood samples and stored at − 80 °C for biochemical analysis [30].

2.6. Analysis of serum biochemical parameters

The activities of liver function parameters like alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were estimated by the method of Huang et al., and Schumann et al., the lipid profile - Total cholesterol (TC), Low density lipoprotein (LDL), High density lipoprotein (HDL), Very low-density lipoprotein (VLDL) and Triglycerides (TG) were estimated by the method described by Rader and Hobbs; renal function markers, creatinine was estimated by the method of Peake and Whiting and urea by Rock et al., [31–35]. All tests were measured using an automated biochemical analyzer (Cobas 501, Roche diagnostics India Private Limited, Maharashtra, India).

2.7. Estimation of haematological parameters

Haematological parameters were measured employing Mindray auto haematology analyzer, BC-6000 (Abomed Biosystems Private Limited, Odisha, India).

2.8. Statistical analysis

IBM SPSS version 28 software was used for statistical analysis. Mean and standard deviation for continuous variables and percentages for categorical variables were reported accordingly. A 2 × 2 Repeated Measures ANOVA was employed to check the significance. '*P*' values

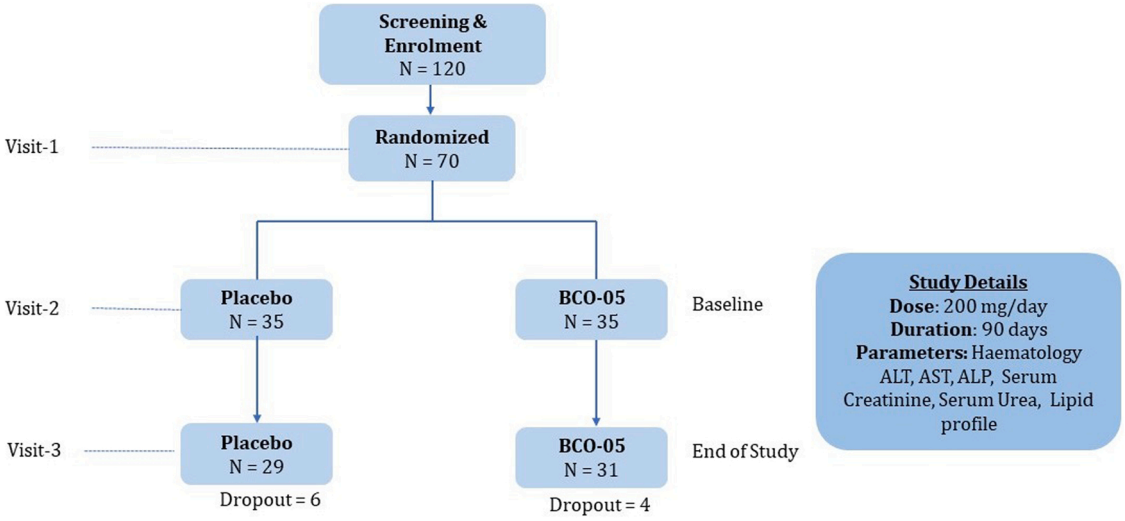

Fig. 2. Cohort diagram showing study design.

< 0.05 were considered as statistically significant [36].

3. Results

3.1. Analysis of study material

Physical, chemical and microbial analysis of the black cumin oil formulation (BCO-5) used in the current study, in comparison with the widely used cold-pressed oil, is depicted in Table 2. HPTLC analysis was employed to authenticate BCO-5 as *Nigella sativa* oil (Fig. 3). HPLC analysis showed 5.12% of TQ content which is almost 10 times higher than the TQ content in the normal cold-pressed oil.

3.2. Vital signs, anthropometric and demographic characteristics

Seventy participants including both males and females (n = 70) were enrolled in the study. BMI of males were 23.96 ± 0.78 and females were 23.44 ± 0.73, which was maintained throughout the study duration. It was observed that none of the subjects exhibited significant difference in pulse, systolic and diastolic blood pressure from the baseline until the end of study. The vital signs, anthropometric and demographic parameters at baseline and end of study of both males and females are listed in Table 3.

Table 2
Certificate of analysis of oil used in current study.

Parameters	Results
Physical characteristics	
Colour	Dark brown
Appearance	Free flowing liquid
Odour	Characteristic
Chemical characteristics	
Thymoquinone content	5.12%
Moisture content	0.32%
Solubility	Soluble in alcohol
Heavy Metals	
Lead	< 1 ppm
Mercury	< 0.1 ppm
Cadmium	< 0.5 ppm
Arsenic	< 1 ppm
Microbiology	
Total plate count	< 3000 cfu/g
Yeast & mold	< 100 cfu/g
E. coli	Absent/g
Salmonella	Absent/25 g
Coliforms	< 3 MPN/g

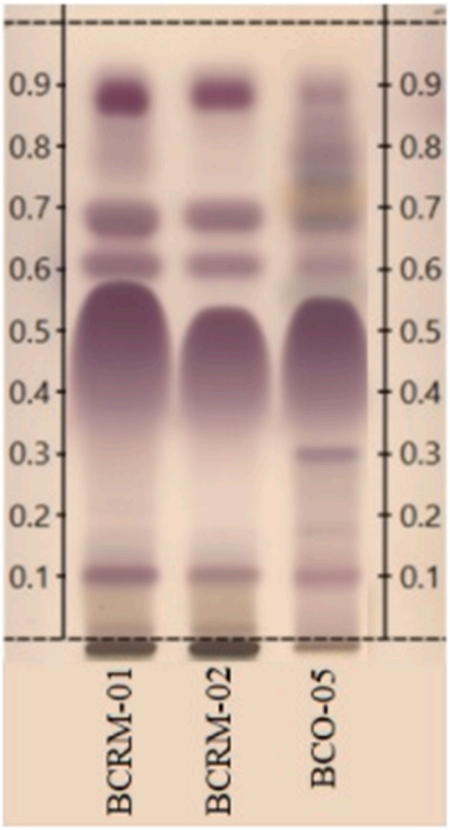

Fig. 3. HPTLC analysis of Black cumin seed and BCO-5; BCRM-01 - authentic reference of black cumin ; BCRM-02 - raw material used for the extraction of BCO-5.

3.3. Safety parameters

The recruited participants did not exhibit any signs of clinical toxicity, serious adverse or side effects. The details of haematological and biochemical assays in males and females are listed in Tables (4a, 4b). The hematological parameters were within normal range and revealed no significant difference ($P > 0.05$) at baseline verses end of study. Activities of liver toxicity markers (ALT, AST, ALP) and the markers of kidney function (serum creatinine, serum urea) exhibited no

Table 3
Changes in vital signs, demographic and anthropometric parameters from baseline to end of study of males and females in placebo and BCO-5 treated groups.

Parameters	Group	Male		Female	
		Baseline	End of study	Baseline	End of study
Age (yr)	Placebo	28.76 ± 8.19		30.42 ± 7.01	
	BCO-5	33.7 ± 9.85		35.55 ± 7.712	
Body weight (Kg)	Placebo	72.41 ± 5.84	73.17 ± 5.09	70.50 ± 6.29	71.25 ± 5.40
	BCO-5	72.60 ± 6.86	73.00 ± 6.34	72.72 ± 5.53	73.00 ± 3.82
BMI (Kg/m^2)	Placebo	23.96 ± 0.78	24.25 ± 0.67	23.44 ± 0.73	23.74 ± 1.00
	BCO-5	24.30 ± 0.69	24.43 ± 0.95	24.28 ± 0.79	24.38 ± 0.55
Temperature (°F)	Placebo	97.28 ± 0.41	97.48 ± 0.49	97.02 ± 0.46	97.64 ± 0.33
	BCO-5	97.52 ± 0.65	97.52 ± 0.60	97.22 ± 0.82	97.50 ± 0.39
Systolic BP (mmHg)	Placebo	119.35 ± 6.72	117.65 ± 5.86	116.33 ± 8.32	116.33 ± 7.63
	BCO-5	119.40 ± 7.81	116.30 ± 8.59	118.00 ± 7.39	113.45 ± 7.84
Diastolic BP (mmHg)	Placebo	73.94 ± 4.83	75.41 ± 5.80	75.50 ± 7.33	74.75 ± 6.90
	BCO-5	77.10 ± 7.31	74.15 ± 6.98	75.27 ± 6.08	73.00 ± 7.31
Pulse	Placebo	73.35 ± 3.33	73.76 ± 2.73	73.83 ± 4.65	76.00 ± 3.33
	BCO-5	74.25 ± 2.94	71.90 ± 3.80	74.18 ± 3.82	73.91 ± 5.94

BMI-body mass index, BP- blood pressure

significant difference ($P > 0.05$) from the baseline to the end of study. Intergroup comparison also showed no significant difference ($P > 0.05$) in BCO-5 group compared to placebo. However, a significant decrease in the lipid profile was observed from the baseline ($P < 0.05$) in BCO-5 group compared to placebo. Lipid profile of the individuals were also within the safe limit. The significant percentage change observed in male participants in TC, TG, LDL, VLDL and HDL were 12.1%, 19.66%, 16.33%, 12.76%, 8.21% and 15.27% respectively in BCO-5 group compared to the baseline which was statistically significant ($P < 0.05$), whereas in placebo the percent change noted were 5.23%, 8.88%, 3.64%, 1.56% and 0.21% respectively (Table 4a). The percentage difference observed in female participants were 9.78%, 12.82%, 14.52%, 11.93% and 9.59% in TC, TG, LDL, VLDL and HDL respectively in BCO-5 treated group which was statistically significant ($P < 0.05$) whereas placebo group showed 2.41%, 2.86%, 3.31%, 5.93% and 1.61% in TC, TG, LDL, VLDL and HDL respectively (Table 4b). Intergroup comparison also exhibited a significant decrease in lipid concentration from the baseline ($P < 0.05$). There were no reports regarding changes in food-water intake, gastrointestinal irritation due to prolonged intake of BCO-5 for 90-days duration.

3.4. Adverse events

No severe adverse effects were reported during the study. Three subjects from BCO-5 group and one from placebo reported bloating at the initial five days. Other two participants from BCO-5 group developed mild diarrhea during the study. A total of six subjects reported the borbhorigum and burping with a taste of black cumin oil in mouth at different instances during the 90-day trial period. Majority of the reported adverse effects were mild or moderate in black cumin oil treated group. Incidences of adverse effects are represented in Table 5. Further, all the biochemical parameters were analysed for adverse reactions and no significant variations from the normal serum levels were observed. There were no significant differences in the vital signs (pulse rate, systolic and diastolic blood pressure) of the participants at the two study points.

Table 4a
Changes in haematological and biochemical parameters in male participants from baseline to end of study in placebo and BCO-5 treated groups.

Parameters	Group	Baseline	End of study	Normal range
Hb (g/dL)	Placebo	13.24 ± 0.44	13.12 ± 0.22	13.2 – 16.6
	BCO-5	13.46 ± 0.69	13.28 ± 0.46	
RBC count (million/μL)	Placebo	4.88 ± 0.28	4.89 ± 0.27	4.7 – 6.1
	BCO-5	5.18 ± 0.47	4.94 ± 0.47	
MCV (fL/red cell)	Placebo	82.40 ± 4.51	83.54 ± 4.02	80 – 100
	BCO-5	78.67 ± 6.17	82.57 ± 2.65	
MCH (pg/cell)	Placebo	41.16 ± 55.38	27.05 ± 1.28	27.5 – 33.2
	BCO-5	25.63 ± 1.77	26.96 ± 1.06	
ALT (U/L)	Placebo	30.21 ± 6.10	26.84 ± 6.03	29 – 33
	BCO-5	26.48 ± 5.03	23.90 ± 5.79	
AST (U/L)	Placebo	28.64 ± 4.40	25.20 ± 6.36	10 – 40
	BCO-5	28.12 ± 4.10	24.31 ± 4.50	
ALP (U/L)	Placebo	84.94 ± 13.04	82.21 ± 11.88	44 – 147
	BCO-5	88.22 ± 14.13	89.07 ± 13.83	
Creatinine (mg/dL)	Placebo	1.02 ± 0.12	1.05 ± 0.14	0.74 – 1.35
	BCO-5	0.91 ± 0.11	0.88 ± 0.10	
Urea (mg/dL)	Placebo	19.90 ± 9.82	14.24 ± 1.84	7 – 20
	BCO-5	16.27 ± 1.55	18.77 ± 1.09	
TC (mg/dL)	Placebo	158.30 ± 23.59	166.58 ± 10.12	125 – 200
	BCO-5	184.33 ± 24.74	162.01 ± 20.19[a]	
TG (mg/dL)	Placebo	113.49 ± 23.30	123.57 ± 19.22	40 – 160
	BCO-5	134.40 ± 19.47	107.97 ± 18.41[a]	
LDL cholesterol (mg/dL)	Placebo	89.14 ± 16.86	92.39 ± 14.19	< 100
	BCO-5	113.79 ± 23.77	95.20 ± 18.89[a]	
HDL cholesterol (mg/dL)	Placebo	43.09 ± 2.38	43.18 ± 1.57	> 40
	BCO-5	42.23 ± 2.11	48.68 ± 1.95 [a]	
VLDL cholesterol (mg/dL)	Placebo	21.69 ± 5.02	22.03 ± 4.53	2 – 30
	BCO-5	25.93 ± 3.50	23.80 ± 4.57	

[a] $P < 0.05$ for 90th day versus baseline performed using paired sample t test; AST-aspartate aminotransferase; ALT-alanine aminotransferase; ALP-alkaline phosphatase; TC-total cholesterol; TG-triglycerides; LDL-low-density lipoprotein; HDL-high density lipoprotein; VLDL-very low-density lipoprotein; TLC-total leucocyte count; Hb-haemoglobin; MCV-mean corpuscular volume; MCH-mean corpuscular haemoglobin; RBC-red blood cell

4. Discussion

The study demonstrated the safety and tolerance of the proprietary formulation of thymoquinone-rich black cumin oil (BCO-5) when supplemented at a dosage of 200 mg/day for 90 days in healthy volunteers. Use of herbal medicines and supplements to maintain health and well-being has witnessed a great demand in the past couple of decades, and today it is reported that more than 80% of American population rely over them for their primary healthcare [37,38]. Poor quality of sleep and moderate stress has been reported as a major cause for the reduced quality of life, especially among the post-COVID patients around the world. While there are no specific medicines for such treatments in a safe manner, natural remedies, especially those derived from edible plants is of great significance. The previous data that BCO-5 helps to improve the sleep quality and modulate stress is a significant property which required to be exploited further [28]. In this regard, the present randomized controlled toxicity study is of great importance.

Biological activity of black cumin is mainly attributed to its essential oil part, particularly to TQ and carvacrol, the major components in the

Table 4b
Changes in haematological and biochemical parameters in female participants from baseline to end of study in placebo and BCO-5 treated groups.

Parameters	Group	Baseline	End of study	Normal range
Hb (g/dL)	Placebo	13.41 ± 0.49	13.39 ± 0.43	11.6 – 15
	BCO-5	13.45 ± 0.69	13.28 ± 0.46	
RBC count (million/ μL)	Placebo	4.87 ± 0.13	4.90 ± 0.19	4.2 – 5.4
	BCO-5	5.18 ± 0.47	4.94 ± 0.46	
MCV (fL/red cell)	Placebo	81.24 ± 3.15	81.93 ± 3.43	80 – 100
	BCO-5	78.67 ± 6.17	82.57 ± 2.65	
MCH (pg/cell)	Placebo	27.47 ± 0.77	27.06 ± 1.22	27.5 – 33.2
	BCO-5	25.63 ± 1.77	26.96 ± 1.06	
ALT (U/L)	Placebo	22.50 ± 2.46	22.23 ± 2.43	19 – 25
	BCO-5	22.73 ± 2.70	22.10 ± 3.04	
AST (U/L)	Placebo	20.87 ± 2.33	22.25 ± 3.99	9 – 32
	BCO-5	25.15 ± 3.12	23.55 ± 4.48	
ALP (U/L)	Placebo	77.88 ± 16.61	85.09 ± 15.58	44 – 147
	BCO-5	93.45 ± 15.56	78.45 ± 11.30	
Creatinine (mg/dL)	Placebo	0.97 ± 0.11	1.00 ± 0.18	0.59 – 1.04
	BCO-5	0.97 ± 0.12	0.98 ± 0.11	
Urea (mg/dL)	Placebo	11.83 ± 1.28	13.57 ± 1.70	9.8 – 20
	BCO-5	14.32 ± 2.56	18.75 ± 1.18	
TC (mg/dL)	Placebo	158.84 ± 17.61	162.68 ± 23.57	125 – 200
	BCO-5	165.35 ± 18.90	149.17 ± 18.67[a]	
TG (mg/dL)	Placebo	110.48 ± 30.62	107.31 ± 24.84	35 – 135
	BCO-5	126.05 ± 28.65	109.88 ± 26.16[a]	
LDL cholesterol (mg/ dL)	Placebo	99.15 ± 20.20	102.43 ± 20.25	< 100
	BCO-5	106.70 ± 23.55	91.20 ± 21.27[a]	
HDL cholesterol (mg/ dL)	Placebo	44.08 ± 2.80	43.37 ± 1.46	> 50
	BCO-5	41.98 ± 0.97	46.01 ± 1.96 [a]	
VLDL cholesterol (mg/dL)	Placebo	25.43 ± 5.46	23.92 ± 4.71	2 – 30
	BCO-5	24.14 ± 4.74	21.26 ± 5.21	

[a] $P < 0.05$ for 90th day versus baseline performed using paired sample t test; AST-aspartate aminotransferase; ALT-alanine aminotransferase; ALP-alkaline phosphatase; TC-total cholesterol; TG-triglycerides; LDL-low-density lipoprotein; HDL-high density lipoprotein; VLDL-very low-density lipoprotein; TLC-total leucocyte count; Hb-haemoglobin; MCV-mean corpuscular volume; MCH-mean corpuscular haemoglobin; RBC-red blood cell

Table 5
Adverse events reported.

Parameter	Placebo (n = 29)	BCO-5 (n = 31)
Number of participants with AEs	1(29)	4(31)
Number of AEs reported/participant (mean ± SD)	0.65 ± 0.23	0.85 ± 0.74
Number of participants with serious AEs (%)	0	0
Number of participants with severe AEs (%)	0	0
Number of participants with mild to moderate AEs (%)	3.44	12.90

AEs- Adverse events; BCO-5- Black cumin oil

essential oil part [39]. Strong antioxidant, free radical scavenging and anti-inflammatory effects of TQ have been believed to be the reason for the bioactivity of black cumin [39,40]. It has been found to elicit strong free radical scavenging effects and has been shown to upregulate the transcription genes responsible for the production of endogenous anti-oxidant defenses, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) and shown to upregulate the cytoprotective genes [41,42]. Preclinical studies have also confirmed the anti-convulsant, anti-microbial, anti-viral, anti-histaminic, immune-modulatory, anti-diabetic anti-hypertensive, anti-lipidemic and neuroprotective activities of TQ [1]. TQ also exhibited anti-carcinogenic, anti-neoplastic, anti-mutagenic and anti-proliferative activities [43,44].

Despite the natural status and favourable pharmacological effects, safety is an important element to be considered for the herbal remedies. Our previous studies have shown that TQ content is important for the safety and efficacy of black cumin oil [17,28]. In the single dose acute toxicity study on rats as per OECD guidelines, black cumin oil with 0.6% TQ showed an LD_{50} in the range of 300–2000 mg/kg b. wt., while the LD_{50} range for the one with 5% TQ was 50–300 mg/kg b. wt. [17]. Further subchronic studies suggested 5 mg TQ/kg b. wt. as a NOAEL of BCO-5, which translated to about 900 mg/day safe dosage for humans. In the current study, only 200 mg/day was used for 3 months since BCO-5 at this dosage was found to significantly improve the quality of sleep and reduce the stress [28].

The current study did not demonstrate any significant toxic effects, adverse effects or deviations in clinical parameters. Inter and intra-group comparison at the end of the study period did not reveal any significant deviation in the hematology and biochemical parameters of liver and kidney. However, the observed decrease in lipid profile and hematology were not in the toxic level, but in a positive manner indicating the modulation of lipid profile and immunity. The absence of significant changes on the relative activities of liver and kidney toxicity markers observed in the present study were in agreement with the previous findings. In a randomized, double-blinded, placebo controlled trial involving healthy volunteers, supplementation of black cumin oil at 5 mL/day for eight weeks was demonstrated to cause no adverse/toxic effects [45]. No deviations were noted in the activities of serum ALT, AST, ALP and in the levels of kidney markers (creatinine, urea) from the baseline till the end of study [45]. These findings were further in accordance with the randomized, double-blinded, placebo-controlled clinical trial of Amini et al., who employed the oil at 5 mL/day for 8 weeks [46]. In another study, supplementation of black cumin oil at different doses of 1.5, 3 and 4.5 mL/day for 20 days did not cause any adverse impacts or any significant deviations in the activities of serum ALT, AST, ALP and in the levels of renal function markers [47]. However, the supplementation of BCO-5 was found to offer a significant reduction in the levels of TC, TG and LDL cholesterol, with an enhancement in HDL. Our findings were supported by Amini et al., who observed a significant reduction in LDL when supplemented with 5 mL of black cumin oil per day for 8 weeks [46]. Bamosa et al., also reported that the consumption of black cumin powder at 2000 mg/day reduces the cholesterol in healthy subjects [48]. The possible mechanism for the hypolipidemic effect of black cumin may be via the activation of peroxisome proliferator-activated receptor gamma (PPAR-γ) gene, which once activated leads to elevated expression of CD-36 (a receptor for atherogenic LDL and ATP binding transporter A1). Moreover, TQ up-regulates hepatic LDL-receptor, inhibits 3-hydroxy 3-methyl glutaryl CoA reductase gene and downregulates Apo B100 gene leading to the reduced synthesis and increased clearance of LDL-cholesterol [49].

A brief report on the important clinical trials of black cumin oil and its beneficial effects are summarized in Table 6. In a recent randomized, double-blinded, placebo- controlled study, supplementation of cold-pressed oil at 500 mg twice daily for 8 weeks was effective in reducing clinical parameters associated with cardiac health viz, fasting blood sugar, total cholesterol, triglycerides, BMI, and blood pressure indicating its effectiveness in maintaining the cardiometabolic risk factors in Type 2 diabetes [30]. Kooshki et al., reported that the oil was found to provide the protective effect against inflammation [50], oxidative stress, fasting blood glucose and lipid profiles in type II diabetes mellitus participants, when supplemented at 500 mg × 2/day for 8 weeks [50]. In another randomized study using a TQ-rich oil (14.5%, evaluated using GC-MS), a dosage of 3 g/day for 12 weeks was found to offer significant reduction in body weight, BMI, fasting blood glucose, glycated hemoglobin, triglycerides, LDL-cholesterol and insulin resistance [51]. However, the analysis of TQ content in that study seems to

Table 6
Summary of clinical studies with BCO and thymoquinone.

Sl. No.	Intervention	Dose & Duration[a]	No. of subjects	Design	Conditions	Outcomes	Reference
1.	BCO	500 mg × 2/day 8 weeks	43	RCT, DB, PCCT	T2D	Decreased FBS, TC, TG, LDL-C, BMI, waist circumference, maintained BP	[30]
2.	BCO	500 mg × 2/day 8 weeks	50	DB, RCT	T2DM	Decreased serum FBG, TC, LDL, TG, MDA and increased HDL, offered cardio protection	[50]
3.	BCO Cold pressed (TQ-14.5%)	3 g × 3/day 12 weeks	72	DB, CCT	T2DM	Decreased body weight and BMI, FBS, glycated haemoglobin, triglycerides, LDL-cholesterol, Insulin and Insulin resistance, increased HDL	[51]
4.	BCO	2.5 mL × 2/day 6 weeks	60	CT	Participants with metabolic syndrome	Significantly reduced total cholesterol, LDL and FBS. Effective in subjects with insulin resistance when supplemented as an add on therapy	[52]
5.	BCO Cold pressed (TQ-0.7%)	500 mg × 2/day 8 weeks	80	RCT, DB, PCT	Asthma	Improves asthma with pulmonary function improvement	[53]
6.	NS boiled extract (TQ- 2%)	15 mL 12 weeks	29	DB	Asthmatic adults	Improvements in pulmonary function test and alleviations of the symptoms of asthma	[54]
7.	BCO	2.5 mL/day 12 weeks	70	Prospective, comparative, OL	CKD	Reduced blood urea, serum creatine, 24 h urine protein, improved clinical features and biochemical parameters	[55]
8.	BCO	2.5 mL/day 12 weeks	68	Prospective, comparative, OL	CKD	Decreased blood glucose, urea, serum creatine, urine protein and improved glomerular filtration	[56]
9.	Saturated BCO Cold pressed (TQ-0.98 mg/mL)	2.5 mL × 2/day 12 weeks	60	RCT, DB, PCCT	NAFLD	Decreased fatty liver and injury, TG, LDL-C and increased HDL-C	[57]
10.	BCO Cold pressed (TQ-12.5%)	3 g/day 8 weeks	50	DB, PC, RCT	Obese women	Decreased weight and increased SOD	[58]
11.	BCO Cold pressed	3 g/day 8 weeks	90	DB, RCCT	Obese women	Decreased weight, TG, VLDL, waist circumference,	[59]
12.	BCO (TQ- 0.01 mg)	1000 mg × 2/day 8 weeks	40	CO, DB, PCRCT	Obese & overweight women	Reduced obesity	[60]
13.	Thymoquinone	1 mg/ kg/ day	23	DB, PC, RCT, CO	Subjects with intractable epilepsy	Reduction in the frequency of seizures	[61]
14.	BCO Cold pressed	500 mg × 2/day 4 weeks	40	PC	Rheumatoid arthritis	Decreased swollen joints, morning stiffness	[62]
15.	(*Nigella sativa* Hydrogel) Standardised based on Thymoquinone	Topical application twice daily 4 weeks	60	RCT, DB, CCT	Acne vulgaris	Reduced comedones, papules and pustules	[63]
16.	BCO Baraka®	40–80 mg/Kg 10 weeks	30	RCT, CCO	Intractable epileptic children	No significant effect in seizure frequency and oxidative stress	[64]

[a] High dosage (1–10 mL/day) was used mainly. COVID-19-corona virus disease-19, T2DM-Type II diabetes mellitus, NAFLD-non-alcoholic fatty liver disease, CKD-chronic kidney disease, DB-double blind, PC-placebo controlled, RCT-randomized clinical trial, CCT-controlled clinical trial, CO-crossover, PCRCT-placebo controlled randomized clinical trial, OL-open label, PG-parallel group, CCO-controlled crossover

have issues since the recent safety studies did not support such a high dosage of an oil having 14.5% TQ. Further, Najmi et al., have also indicated the beneficial effect of steam distilled black cumin oil against insulin resistance, when supplemented at a dose of 5 mL/day for 6 weeks along with atorvastatin and metformin [52].

A couple of human studies have indicated the beneficial effect of cold-pressed BCO containing 0.7% TQ in asthma patients. A randomized, controlled trial reported significant improvement in mean asthma control score by normalization of the blood eosinophilia, and thus improving pulmonary function, when supplemented at 500 mg twice daily for 8 weeks [53]. In another study employing 15 mL/kg of 0.1% *Nigella sativa* boiled extract with 2% thymoquinone improved pulmonary function test and alleviated the symptoms of asthma [54].

Nephroprotection is another health function studied clinically. Alam et al., reported that black cumin oil at a dose of 2.5 mL/day for 12 weeks when supplemented along with alpha keto analogue of essential amino acid was found to offer significant improvement in blood urea, serum creatinine and total urine protein on patients with chronic kidney disease [55]. These findings were further supported by Ansari et al., who reported that the same dosage supplementation for 12 weeks to diabetic nephropathy patients with chronic kidney disease (stage 3 and 4) effectively reduced the respective hematological and biochemical parameters with improved glomerular filtration rate compared to control group [56].

Another area where black cumin oil has shown promising effect was in the management of non-alcoholic fatty liver disease (NAFLD). Khonche et al., reported that the supplementation of cold-pressed BCO with a thymoquinone fraction (0.98 mg/mL) at 2.5 mL when provided every 12 h for 3 months, could effectively decrease liver steatosis and injury, levels of triglycerides, LDL and increased HDL cholesterol among NAFLD patients [57].

Yet another area of pharmacological relevance is the overweight or obesity management with black cumin oil. Cold pressed BCO with 12.5% TQ by GC-MS analysis at 3 g/day was shown to significantly decrease the body weight with a significant elevation in SOD when subjected to obese population [58]. Another study has also reported a similar effect in reducing body weight, waist circumference, and other biochemical parameters [59]. Recently, Razmpoosh et al., reported that BCO with 0.01 mg TQ content/1000 mg was effective in managing obesity [60].

In addition to the above studies, black cumin oil has also shown some promising results in various other end points. In a double-blinded crossover clinical trial in children with refractory epilepsy, supplementation of TQ (1 mg/kg for four weeks) significantly reduced the frequency of seizure (anti-epileptic) [61]. Supplementation of cold-pressed BCO at 500 mg twice daily for one month was effective in managing rheumatoid arthritis by decreasing the disease activity score, swollen joints and morning stiffness [62]. The efficacy of topical application of BCO hydrogel on sixty participants with acne vulgaris was also reported. It was observed that the application of hydrogel twice

daily for sixty days effectively reduced comedones, papules and pustules [63].

However, some studies have also reported no significant beneficial effect for black cumin oil. For instance, Shawki et al., reported that the administration of BCO at 40–80 mg/kg/day for 10 weeks did not exhibit any significant improvement in the episodes of seizure or on the oxidative stress markers [64]. But, no information on the type of oil or its TQ content was provided. It has to be noted that none of the fore-mentioned clinical trials have not reported any serious adverse events or toxicity; even with the study which claimed 3 g/day supplementation of an oil having 14.5% TQ for 12 weeks [51]. Nausea, bloating, gastritis, fatigue, nasal dryness, and burning sensation were some of the side effects reported in various studies [40,65,66]. However, some of these patients were also diagnosed with other diseases and were under polypharmacy. Supplementation of BCO at 5 g/day has been shown to inhibit the activities of drug metabolizing enzymes like CYP2D6 and CYPA4 [67]. In summary, black cumin seed powder and its oil has not shown significant side effects or adverse events in various studies reported so far. Some of the side effects reported can be correlated to prior medical conditions and/or high dose and extended duration of the drug. In the present study, BCO-5 was also found to be safe when supplemented at a dose of 200 mg daily for 3 months.

5. Conclusion

Black cumin oil possesses significant pharmacological and therapeutic potential. The positive effects of black cumin and its oil have been delineated with about 76 clinical trials so far. High TQ content in BCO-5 was shown to support the small dosage of 100–200 mg/day contrary to the commonly available cold-pressed oil containing around 0.5% TQ and its high dosage (1–5 mL/day). It was also found to be safe without any major side effects or adverse events. Therefore, the present formulation of TQ-rich BCO-5 would be a potential nutraceutical ingredient for a number of health issues.

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CRediT authorship contribution statement

J.V.T - principal investigator who supervised the study and was involved in the protocol designing, data collection; **M.M.E** - recruitment of study participants, conduct of clinical trial; **P.P** - data analysis and drafting of original article; **S.D.S** - review & editing of original article; **B.M** - Resources and approval of study; **K.I.M**- Conceptualisation, approval of protocol, review & editing of original article.

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Declaration of competing interest

The authors disclose the following conflict of interests. BlaQmax® is a patented and registered product of Akay Natural Ingredients, Cochin, India. J.V.T & M.M.E belongs to non-profit research organizations who conducted the study and critically evaluated the data. P.P, S.D.S, B.M and K.I.M belong to the company Akay Natural Ingredients (Cochin, India), who prepared the study drugs, conceived the idea, and approved the protocol.

References

[1] M. Hannan, M. Rahman, A. Sohag, M. Uddin, R. Dash, M. Sikder, M. Rahman, B. Timalsina, Y. Munni, P. Sarker, M. Alam, M. Mohibbullah, M. Haque, I. Jahan, M. Hossain, T. Afrin, M. Rahman, M. Tahjib-Ul-arif, S. Mitra, D. Oktaviani, M. Khan, H. Choi, I. Moon, B. Kim, Black Cumin (*Nigella sativa* L.): a comprehensive review on phytochemistry, health benefits, molecular pharmacology, and safety, Nutrients 13 (2021) 1784, https://doi.org/10.3390/NU13061784.

[2] P. Sharma, M. Yelne, T. Dennis, Database on Medicinal Plants Used in Ayurveda, Central Council for Research in Ayurveda & Siddha, Deptt. of ISM & H, Min. of Health & Family Welfare, Government of India, New Delhi, 2005.

[3] Y. Kabir, H. Shirakawa, M. Komai, Nutritional composition of indigenous cultivar of black cumin seeds from Bangladesh, Prog. Nutr. 21 (2019) 428–434, https://doi.org/10.23751/PN.V21I1-S.6556.

[4] M. Ramadan, Nutritional value, functional properties and nutraceutical applications of black cumin (*Nigella sativa* L.): an overview, Int. J. Food Sci. Technol. 42 (2007) 1208–1218, https://doi.org/10.1111/J.1365-2621.2006.01417.X.

[5] O. Ketenoglu, S. Kiralan, M. Kiralan, G. Ozkan, M. Ramadan, Cold pressed black cumin (*Nigella sativa* L.) seed oil, Cold Presse Oils (2020) 53–64, https://doi.org/10.1016/B978-0-12-818188-1.00006-2.

[6] F. Forouzanfar, B. Bazzaz, H. Hosseinzadeh, Black cumin (*Nigella sativa*) and its constituent (thymoquinone): a review on antimicrobial effects, Iran. J. Basic Med. Sci. 17 (2014) 929–938.

[7] M. Sahak, N. Kabir, G. Abbas, S. Draman, N. Hashim, D. Adli, The role of *Nigella sativa* and its active constituents in learning and memory, Evid. -Based Complement. Altern. Med. 2016 (2016) 1–6, https://doi.org/10.1155/2016/6075679.

[8] S. Tembhurne, S. Feroz, B. More, D.M. Sakarkar, A review on therapeutic potential of *Nigella sativa* (kalonji) seeds, J. Med. Plants Res. 8 (2014) 167–177, https://doi.org/10.5897/JMPR10.737.

[9] I. Norsharina, I. Maznah, A. Aied, A. Ghanya, Thymoquinone rich fraction from *Nigella sativa* and thymoquinone are cytotoxic towards colon and leukemic carcinoma cell lines, J. Med. Plants Res. 5 (2011) 3359–3366.

[10] G.A. Burdock, Assessment of black cumin (*Nigella sativa* L.) as a food ingredient and putative therapeutic agent, Regul. Toxicol. Pharmacol. 128 (2022), https://doi.org/10.1016/J.YRTPH.2021.105088.

[11] N. Mohammed, M. Abd Manap, C. Tan, B. Muhialdin, A. Alhelli, A. Hussin, The effects of different extraction methods on antioxidant properties, chemical composition, and thermal behavior of black seed (*Nigella sativa* L.) oil, Evid. -Based Complement. Altern. Med. 2016 (2016), https://doi.org/10.1155/2016/6273817.

[12] M. Kiralan, G. Özkan, A. Bayrak, M. Ramadan, Physicochemical properties and stability of black cumin (*Nigella sativa*) seed oil as affected by different extraction methods, Ind. Crops Prod. 57 (2014) 52–58, https://doi.org/10.1016/J.INDCROP.2014.03.026.

[13] N. Barkah, K. Wiryawan, Y. Retnani, W. Wibawan, E. Wina, Physicochemical properties of products and waste of black seed produced by cold press method, IOP Conf. Ser. Earth Environ. Sci. 756 (2021) 1–8, https://doi.org/10.1088/1755-1315/756/1/012025.

[14] S. Haseena, M. Aithal, K. Das, S. Saheb, Phytochemical analysis of *Nigella sativa* and its effect on reproductive system, J. Pharm. Sci. Res. 7 (2015) 514–517.

[15] E. Yimer, K. Tuem, A. Karim, N. Ur-Rehman, F. Anwar, *Nigella sativa* L. (Black Cumin): a promising natural remedy for wide range of illnesses, Evid. Based Complement. Altern. Med. 2019 (2019), https://doi.org/10.1155/2019/1528635.

[16] M. Hossen, W. Yang, D. Kim, A. Aravinthan, J. Kim, J. Cho, Thymoquinone: an IRAK1 inhibitor with in vivo and in vitro anti-inflammatory activities, Sci. Rep. 7 (2017) 42995, https://doi.org/10.1038/SREP42995.

[17] K. Ramalingam, R. Kuttan, S. Sivadasan, S. Ittiyavirah, I.M. Krishnakumar, Safety assessment of thymoquinone-rich black cumin (*Nigella sativa*) oil (BlaQmax®): acute and sub-chronic toxicity studies, J. Nutr. Food Sci. 0 (2021) 1–4.

[18] K. Ramalingam, S. Ittiyavirah, J. Harindran, Acetylcholinesterase and growth inhibitory effects - various grades of *Nigella sativa* oils, Int. J. Pharm. Sci. 10 (2019) 245–250.

[19] M. Ratheesh, J.P. Svenia, S. Sangeeth, S. Sheethal, R. Sony, S. Sandya, I. M. Krishnakumar, Antioxidant, anti-inflammatory, and anti-arthritic effect of thymoquinone-rich black cumin (*Nigella sativa*) oil (BlaQmax®) on adjuvant-induced arthritis, J. Food Res. 10 (2021) p52, https://doi.org/10.5539/JFR.V10N1P52.

[20] E. Datau, Wardhana, E. Surachmanto, K. Pandelaki, J. Langi, Fias, Efficacy of *Nigella sativa* on serum free testosterone and metabolic disturbances in central obese male, Acta Med. Indones. 42 (2010) 130–134.

[21] M. Khan, H. Chen, M. Tania, D. Zhang, Anticancer activities of *Nigella sativa* (black cumin), African, J. Tradit., Complement., Altern. Med. 8 (2011) 226–232, https://doi.org/10.4314/AJTCAM.V8I5S.10.

[22] H. Hosseinzadeh, S. Taiari, M. Nassiri-Asl, Effect of thymoquinone, a constituent of *Nigella sativa* L., on ischemia-reperfusion in rat skeletal muscle, Naunyn Schmiede Arch. Pharm. 385 (2012) 503–508, https://doi.org/10.1007/S00210-012-0726-2.

[23] B. Amin, H. Hosseinzadeh, Black cumin (*Nigella sativa*) and its active constituent, thymoquinone: an overview on the analgesic and anti-inflammatory effects, Planta Med. 82 (2016) 8–16, https://doi.org/10.1055/S-0035-1557838.

[24] A. Hamdan, R. Idrus, M. Mokhtar, Effects of *Nigella sativa* on type-2 diabetes mellitus: a systematic review, Int. J. Environ. Res. Public Health 16 (2019) 4911, https://doi.org/10.3390/IJERPH16244911.

[25] I. Bocsan, R. Pop, O. Sabin, E. Sarkandy, P. Boarescu, Ş. Roşian, P. Leru, V. Chedea, S. Socaci, A. Buzoianu, Comparative protective effect of *Nigella sativa* oil and *Vitis vinifera* seed oil in an experimental model of isoproterenol-induced acute

myocardial ischemia in rats, Molecules 26 (2021) 3221, https://doi.org/10.3390/MOLECULES26113221.
[26] A. El-Far, M. Korshom, A. Mandour, A. El-Bessoumy, Y. El-Sayed, Hepatoprotective efficacy of *Nigella sativa* seeds dietary supplementation against lead acetate-induced oxidative damage in rabbit – Purification and characterization of glutathione peroxidase, Biomed. Pharmacother. 89 (2017) 711–718, https://doi.org/10.1016/J.BIOPHA.2017.02.044.
[27] U. Saleem, B. Ahmad, K. Rehman, S. Mahmood, M. Alam, A. Erum, Nephro-protective effect of vitamin C and *Nigella sativa* oil on gentamicin associated nephrotoxicity in rabbits, Pak. J. Pharm. Sci. 25 (2012) 727–730.
[28] S. Syam, R. Kannan, S. George, P. Chakrapani, B. Maliakel, S. Ittiyavirah, I. M. Krishnakumar, Thymoquinone-rich black cumin oil improves sleep quality, alleviates anxiety/stress on healthy subjects with sleep disturbances – a pilot polysomnography study, J. Herb. Med. 32 (2022), 100507, https://doi.org/10.1016/J.HERMED.2021.100507.
[29] N. Ahmad, R. Ahmad, A. Al-Layly, H. Al-Shawi, A. Al-Ali, M. Amir, A. Mostafa, Ultra-high-performance liquid chromatography-based identification and quantification of thymoquinone in *Nigella sativa* extract from different geographical regions, Pharmacogn. Mag. 14 (2018) 480, https://doi.org/10.4103/PM.PM_119_18.
[30] S. Hadi, R. Daryabeygi-Khotbehsara, P. Mirmiran, J. McVicar, V. Hadi, D. Soleimani, G. Askari, Effect of *Nigella sativa* oil extract on cardiometabolic risk factors in type 2 diabetes: a randomized, double-blind, placebo-controlled clinical trial, Phytother. Res. 35 (2021) 3747–3755, https://doi.org/10.1002/PTR.6990.
[31] X.-J. Huang, Y.-K. Choi, H.-S. Im, O. Yarimaga, E. Yoon, H.-S. Kim, Aspartate aminotransferase (AST/GOT) and alanine aminotransferase (ALT/GPT) detection techniques, Sensors 6 (2006) 756–782.
[32] G. Schumann, R. Klauke, F. Canalias, S. Bossert-Reuther, P.F.H. Franck, F.J. Gella, P.J. Jørgensen, D. Kang, J.M. Lessinger, M. Panteghini, F. Ceriotti, IFCC primary reference procedures for the measurement of catalytic activity concentrations of enzymes at 37 °C. Part 9: reference procedure for the measurement of catalytic concentration of alkaline phosphatase International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) Scientific Division, Committee on Reference Systems of Enzymes (C-RSE) (1)), Clin. Chem. Lab. Med. 49 (2011) 1439–1446, https://doi.org/10.1515/CCLM.2011.621.
[33] D. Rader, H. Hobbs, Disorders of lipoprotein metabolism, in: E. Braunwald, D. Kasper (Eds.), Harrison's Principles of Internal Medicine, Mc Graw Hill, New York, 2008, p. 2424.
[34] M. Peake, M. Whiting, Measurement of serum creatinine–current status and future goals, Clin. Biochem. Rev. (2006) 173–184. ⟨https://pubmed.ncbi.nlm.nih.gov/17581641/⟩.
[35] R. Rock, W. Walk, D. Jennings, Nitrogen metabolites and renal function, in: N. W. Tietz (Ed.), Fundamentals of Clinical Chemistry, 3rd ed..,, W.B. Saunders Company, Philadelphia, 1987, pp. 684–686.
[36] V. Singh, R.K. Rana, R. Singhal, Analysis of repeated measurement data in the clinical trials, J. Ayurveda Integr. Med. 4 (2013) 77–81, https://doi.org/10.4103/0975-9476.113872.
[37] M. Ekor, The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety, Front. Pharmacol. 4 (2014) 177, https://doi.org/10.3389/FPHAR.2013.00177.
[38] S. Thakkar, E. Anklam, A. Xu, F. Ulberth, J. Li, B. Li, Hugas Sarma, S. Crerar, S. Swift, T. Hakamatsuka, V. Curtui, W. Yan, X. Geng, W. Slikker, W. Tong, Regulatory landscape of dietary supplements and herbal medicines from a global perspective, Regul. Toxicol. Pharmacol. 114 (2020), 104647, https://doi.org/10.1016/J.YRTPH.2020.104647.
[39] O. Badary, M. Hamza, R. Tikamdas, Thymoquinone: a promising natural compound with potential benefits for COVID-19 prevention and cure, Drug Des., Dev. Ther. 15 (2021) 1833, https://doi.org/10.2147/DDDT.S308863.
[40] A. Tavakkoli, A. Ahmadi, B. Razavi, H. Hosseinzadeh, Black Seed (*Nigella sativa*) and its constituent thymoquinone as an Antidote or a protective agent against natural or chemical toxicities, Iran. J. Pharm. Res. 16 (Suppl) (2017) 2–23. ⟨https://pubmed.ncbi.nlm.nih.gov/29844772/⟩.
[41] S. Goyal, C. Prajapati, P. Gore, C. Patil, U. Mahajan, C. Sharma, S. Talla, S. Ojha, Therapeutic potential and pharmaceutical development of thymoquinone: a multitargeted molecule of natural origin, Front. Pharmacol. 8 (2017) 656, https://doi.org/10.3389/FPHAR.2017.00656/BIBTEX.
[42] Y. Mahmoud, H. Abdelrazek, Cancer: thymoquinone antioxidant/pro-oxidant effect as potential anticancer remedy, Biomed. Pharmacother. = Biomed. Pharmacother. 115 (2019), 108783, https://doi.org/10.1016/J.BIOPHA.2019.108783.
[43] M. Almoyad, Synergism from combination of targeted therapy and phytochemicals in colorectal cancer, (2018).
[44] M. Ahmad, F. Ahmad, S. Ashraf, H. Saad, S. Wahab, M. Khan, M. Ali, S. Mohan, K. Hakeem, M. Athar, An updated knowledge of Black seed (*Nigella sativa* Linn.): Review of phytochemical constituents and pharmacological properties, J. Herb. Med. 25 (2021), 100404, https://doi.org/10.1016/J.HERMED.2020.100404.
[45] H. Huseini, M. Amini, R. Mohtashami, M. Ghamarchehre, Z. Sadeqhi, S. Kianbakht, A. Fallah Huseini, Blood pressure lowering effect of *Nigella sativa* L. seed oil in healthy volunteers: a randomized, double-blind, placebo-controlled clinical trial, Phytother. Res. 27 (2013) 1849–1853, https://doi.org/10.1002/PTR.4944.
[46] M. Amini, H. Huseini, R. Mohtashami, Z. Sadeqhi, M. Ghamarchehre, Hypolipidemic effects of *Nigella sativa* L. seeds oil in healthy volunteers: a randomized, double-blind, placebo-controlled clinical trial, J. Med. Plants 10 (2011) 133–138.
[47] A. Akrom, E. Darmawan, Tolerability and safety of black cumin seed oil (Bcso) administration for 20 days in healthy subjects, Biomed. Res. 28 (2017) 4196–4201.
[48] A. Bamosa, A. Basil, A. Sowayan, S. Sowayan, Effect of oral ingestion of *Nigella sativa* seeds on some blood parameters, Saudi Pharm. J. 5 (1997) 126–129.
[49] A. Sahebkar, G. Beccuti, L.E. Simental-Mendía, V. Nobili, S. Bo, *Nigella sativa* (black seed) effects on plasma lipid concentrations in humans: a systematic review and meta-analysis of randomized placebo-controlled trials, Pharmacol. Res. 106 (2016) 37–50, https://doi.org/10.1016/J.PHRS.2016.02.008.
[50] A. Kooshki, T. Tofighiyan, N. Rastgoo, M. Rakhshani, M. Miri, Effect of *Nigella sativa* oil supplement on risk factors for cardiovascular diseases in patients with type 2 diabetes mellitus, Phytother. Res. 34 (2020) 2706–2711, https://doi.org/10.1002/PTR.6707.
[51] J. Heshmati, N. Namazi, M. Memarzadeh, M. Taghizadeh, F. Kolahdooz, *Nigella sativa* oil affects glucose metabolism and lipid concentrations in patients with type 2 diabetes: A randomized, double-blind, placebo-controlled trial, Food Res. Int. 70 (2015) 87–93, https://doi.org/10.1016/J.FOODRES.2015.01.030.
[52] A. Najmi, M. Nasiruddin, R. Khan, S. Haque, Effect of *Nigella sativa* oil on various clinical and biochemical parameters of insulin resistance syndrome, Int. J. Diabetes Dev. Ctries. 28 (2008) 11–14, https://doi.org/10.4103/0973-3930.41980.
[53] A. Koshak, L. Wei, E. Koshak, S. Wali, O. Alamoudi, A. Demerdash, M. Qutub, P. Pushparaj, M. Heinrich, Nigella sativa supplementation improves asthma control and biomarkers: a randomized, double-blind, placebo-controlled trial, Phytother. Res. 31 (2017) 403–409, https://doi.org/10.1002/PTR.5761.
[54] M. Boskabady, H. Javan, M. Sajady, H. Rakhshandeh, The possible prophylactic effect of Nigella sativa seed extract in asthmatic patients, Fundam. Clin. Pharmacol. 21 (2007) 559–566, https://doi.org/10.1111/J.1472-8206.2007.00509.X.
[55] M. Alam, M. Nasiruddin, S. Haque, R. Khan, Evaluation of safety and efficacy profile of *Nigella sativa* oil as an add-on therapy, in addition to alpha-keto analogue of essential amino acids in patients with chronic kidney disease, Saudi J. Kidney Dis. Transplant. Off. Publ. Saudi Cent. Organ Transplant. 31 (2020) 21–31, https://doi.org/10.4103/1319-2442.279943.
[56] Z. Ansari, M. Nasiruddin, R. Khan, S. Haque, Protective role of *Nigella sativa* in diabetic nephropathy: a randomized clinical trial, Saudi J. Kidney Dis. Transplant.: Off. Publ. Saudi Cent. Organ Transplant. 28 (2017) 9–14, https://doi.org/10.4103/1319-2442.198093.
[57] A. Khonche, H. Huseini, M. Gholamian, R. Mohtashami, F. Nabati, S. Kianbakht, Standardized *Nigella sativa* seed oil ameliorates hepatic steatosis, aminotransferase and lipid levels in non-alcoholic fatty liver disease: a randomized, double-blind and placebo-controlled clinical trial, J. Ethnopharmacol. 234 (2019) 106–111, https://doi.org/10.1016/J.JEP.2019.01.009.
[58] N. Namazi, R. Mahdavi, M. Alizadeh, S. Farajnia, Oxidative stress responses to nigella sativa oil concurrent with a low-calorie diet in obese women: a randomized, double-blind controlled clinical trial, Phytother. Res. 29 (2015) 1722–1728, https://doi.org/10.1002/PTR.5417.
[59] R. Mahdavi, N. Namazi, M. Alizadeh, S. Farajnia, Effects of *Nigella sativa* oil with a low-calorie diet on cardiometabolic risk factors in obese women: a randomized controlled clinical trial, Food Funct. 6 (2015) 2041–2048, https://doi.org/10.1039/C5FO00316D.
[60] E. Razmpoosh, S. Safi, M. Mazaheri, A. Salehi-Abargouei, N. Abdollahi, M. Nazari, H. Fallahzadeh, A. Nadjarzadeh, Effects of oral *Nigella sativa* oil on the expression levels and serum concentrations of adiponectin, PPAR-γ, and TNF-α in overweight and obese women: a study protocol for a crossover-designed, double-blind, placebo-controlled randomized clinical trial, Trials 20 (2019) 512, https://doi.org/10.1186/S13063-019-3568-0.
[61] J. Akhondian, H. Kianifar, M. Raoofziaee, A. Moayedpour, M.B. Toosi, M. Khajedaluee, The effect of thymoquinone on intractable paediatric seizures (pilot study), Epilepsy Res. 93 (2011) 39–43, https://doi.org/10.1016/J.EPLEPSYRES.2010.10.010.
[62] T. Gheita, S. Kenawy, Effectiveness of *Nigella sativa* oil in the management of rheumatoid arthritis patients: a placebo-controlled study, Phytother. Res. 26 (2012) 1246–1248, https://doi.org/10.1002/PTR.3679.
[63] S. Soleymani, A. Zargaran, M. Farzaei, A. Iranpanah, F. Heydarpour, F. Najafi, R. Rahimi, The effect of a hydrogel made by Nigella sativa L. on acne vulgaris: a randomized double-blind clinical trial, Phytother. Res. 34 (2020) 3052–3062, https://doi.org/10.1002/PTR.6739.
[64] M. Shawki, L. el Wakeel, R. Shatla, G. El-Saeed, S. Ibrahim, O. Badary, The clinical outcome of adjuvant therapy with black seed oil on intractable paediatric seizures: a pilot study, Epileptic Disord. 15 (2013) 295–301, https://doi.org/10.1684/EPD.2013.0602.
[65] R. Mohtashami, H. Fallah Huseini, M. Heydari, M. Amini, Z. Sadeqhi, H. Ghaznavi, S. Mehrzadi, Efficacy and safety of honey-based formulation of *Nigella sativa* seed oil in functional dyspepsia: a double blind randomized controlled clinical trial, J. Ethnopharmacol. 175 (2015) 147–152, https://doi.org/10.1016/J.JEP.2015.09.022.
[66] A. Alsamarai, M. Abdulsatar, A. Alobaidi, Evaluation of topical black seed oil in the treatment of allergic rhinitis, Anti Inflamm. Anti Allergy Agents Med. Chem. 13 (2014) 75–82, https://doi.org/10.2174/18715230113129990014.
[67] F. Al-Jenoobi, A. Al-Thukair, F. Abbas, M. Ansari, K. Alkharfy, A. Al-Mohizea, S. Al-Suwayeh, S. Jamil, Effect of black seed on dextromethorphan O- and N-demethylation in human liver microsomes and healthy human subjects, Drug Metab. Lett. 4 (2010) 51–55, https://doi.org/10.2174/187231210790980435.

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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 & Hosseinzadeh, 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-arthritic 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-arthritic 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-arthritic potential of *N. sativa.* The placebo-controlled anti-arthritic 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-arthritic effects of *N. sativa* with a significant decrease in DAS 28, percentages of CD8+, swollen joints, WBC count, duration of morning stiffness, and VAS (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 & Hosseinzadeh, 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 ℃), 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 0^{th} hour (Vo: before edematogenic agent injection) and 1^{st}, 2^{nd}, 3^{rd}, $4^{th,}$ and 5^{th} 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:

$$\% \text{ inhibition} = [(Vt - Vo)_{control} - (Vt - Vo)_{treated} / (Vt - Vo)_{control}] \times 100 \quad (1)$$

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 installed 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 phenzinemethosulphate, 0.3 mL of nitrobluetetrazolium 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 ℃ 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 H_2O_2 was added to initiate the test reaction. H_2O_2 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 NaN_3, 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 H_2O_2 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 ℃ 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 H_2O 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 $NaNO_2$ 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 1. List of Primers used in the RT-PCR analysis

Inflammatory markers	Forward primer	Reverse primer
IL-6	5'CCACTGCCTTCCCTACTTCA3'	5'TGGTCCTTAGCCACTCCTTC3'
COX-2	5'-ATCTGCCTGCTCTGGTCAATG-3'	5'-CAATCTGGCTGAGGGAACACA-3'
iNOS	5'-CAGCACAGAGGGCTCAAAGC-3'	5'-TCGTCGGCCAGCTCTTTCT-3'
TNF-α	5'GTCGTAAACCACCAAGC3'	5'GACTCCAAAGTAGACCTGCCC3'
GAPDH	5'CCTGCTTCACCACCTTCTTG3'	5'ATCCCATCACCATCTTCCAG3'

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 ℃. The reaction was initiated by the addition of 200 μM arachidonic acid to the reaction mixture followed by incubation of samples at 37 ℃ 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^{o}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.

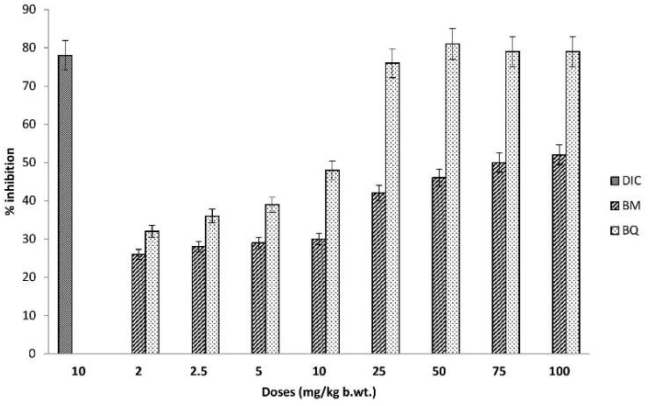

Figure 1. Effect of BM and BQ on carrageenan-induced paw edema rats (dose-response study)

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)

Groups	% Inhibition of paw volume		
	7th day	14th day	21st day
Adjuvant induced	0	0	0
BQ treated (50 mg/kg b.wt.)	69[a]	75[a]	80[a]
INDO (3mg/kg b.wt.)	37[a]	53[a]	72[a]

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

Groups	WBC (Cells×10^3/mL)	CRP (mg/mL)
Normal	3.6±0.02	51±0.32
AA	8.8±0.04[a]	77±0.65[a]
AA+BQ	3.9±0.01[a,b]	63±0.44[a,b]
AA+INDO	3.8±0.02[a,b]	57±0.46[a,b]

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).

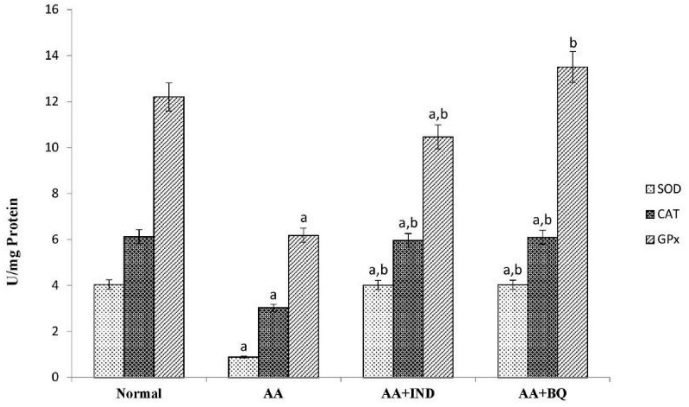

Figure 2. Effect of BQ on the activity of SOD, CAT, and GPx levels in adjuvant-induced arthritic rats

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 H_2O_2 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)].

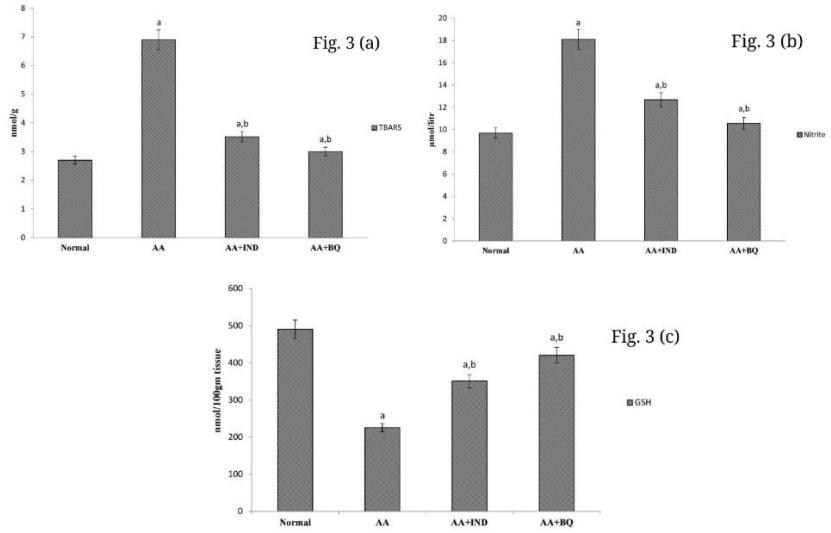

Figure 3. (a,b,c) Effect of BQ on the concentration of TBARS, nitrite, and GSH in experimental rats

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)].

Fig. 4 (a)

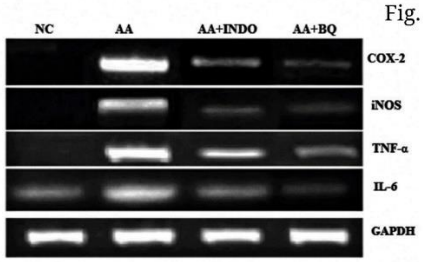

Fig. 4 (b)

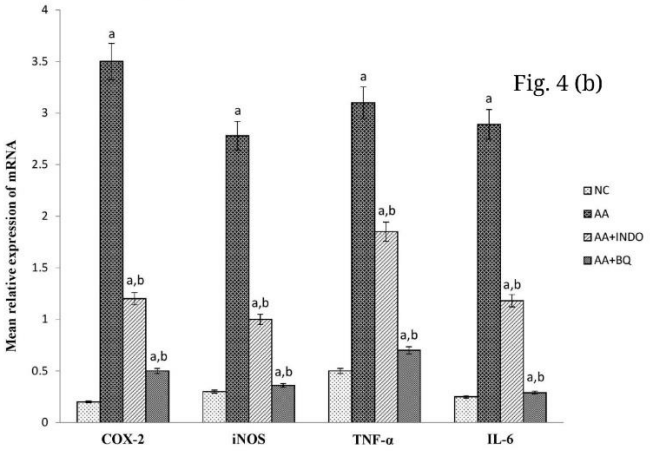

Figure 4. (a,b) Inhibitory effect of BQ on the gene expression of the pro-inflammatory markers

(a)-COX-2, iNOS, TNF-α & IL-6 determined by reverse transcriptase-PCR. GAPDH was used as a control. (b)-Mean relative expression of mRNA levels of COX-2, iNOS, TNF-α & IL-6 was represented by densitometry. *The values were expressed as mean ± SD, m=3 with six rats in each group.* a -Statistical difference with the control group P< 0.05, b – adjuvant-induced rats at *P*< 0.05.

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).

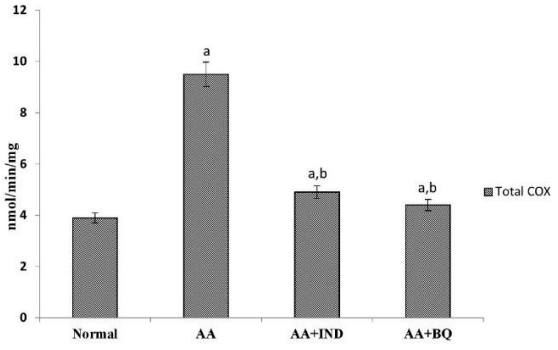

Figure 5. Effect of BQ on total COX activity

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).

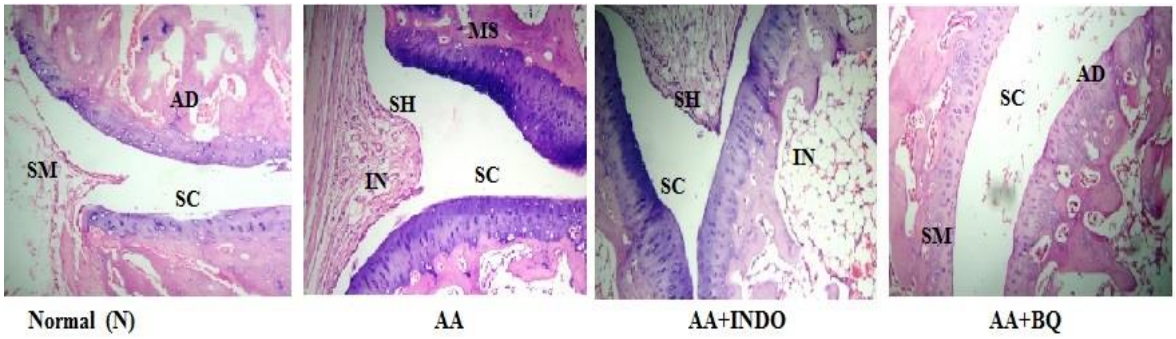

Figure 6. Histology of paw tissue (H&E stain 40×)

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 (Safiri 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-arthritic 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 after1 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 8^{th} 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-arthritic 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 PGE_2. 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 be remarkably 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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References

Abdulkhaleq, L. A., Assi, M. A., Abdullah, R., Zamri-Saad, M., Taufiq-Yap, Y. H., & Hezmee, M. (2018). The crucial roles of inflammatory mediators in inflammation: A review. *Veterinary World*, *11*(5), 627-635. https://doi.org/10.14202/vetworld.2018.627-635

Aebi, H. (1984). Catalase in vitro. *Methods in enzymology*, *105*, 121-126. https://doi.org/10.1016/S0076-6879(84)05016-3

Agergaard, N., & Jensen, P. T. (1982). Procedure for blood glutathione peroxidase determination in cattle and swine. *Acta veterinaria Scandinavica*, *23*(4), 515-527.

Ahmad, A., Husain, A., Mujeeb, M., Khan, S. A., Najmi, A. K., Siddique, N. A., Damanhouri, Z. A., & Anwar, F. (2013). A review on therapeutic potential of Nigella sativa: A miracle herb. *Asian Pacific journal of tropical biomedicine*, *3*(5), 337-352. https://dx.doi.org/10.1016%2FS2221-1691(13)60075-1

Al-Lawati, H., Vakili, M. R., Lavasanifar, A., Ahmed, S., & Jamali, F. (2020). Reduced Heart Exposure of

Diclofenac by Its Polymeric Micellar Formulation Normalizes CYP-Mediated Metabolism of Arachidonic Acid Imbalance in an Adjuvant Arthritis Rat Model: Implications in Reduced Cardiovascular Side Effects of Diclofenac by Nanodrug Delivery. *Molecular pharmaceutics*, *17*(4), 1377-1386. https://doi.org/10.1021/acs.molpharmaceut.0c00069

Alunno, A., Carubbi, F., Giacomelli, R., & Gerli, R. (2017). Cytokines in the pathogenesis of rheumatoid arthritis: new players and therapeutic targets. *BMC Rheumatol*, *1*, 3. https://doi.org/10.1186/s41927-017-0001-8

Amin, B., & Hosseinzadeh, H. (2016). Black Cumin (Nigella sativa) and Its Active Constituent, Thymoquinone: An Overview on the Analgesic and Anti-inflammatory Effects. *Planta medica*, *82*(1-2), 8-16. https://doi.org/10.1055/s-0035-1557838

Arjumand, S., Shahzad, M., Shabbir, A., & Yousaf, M. Z. (2019). Thymoquinone attenuates rheumatoid arthritis by downregulating TLR2, TLR4, TNF-α, IL-1, and NFκB expression levels. *Biomedicine & pharmacotherapy*, *111*, 958-963. https://doi.org/10.1016/j.biopha.2019.01.006

Benke, G. M., & Murphy, S. D. (1974). Effect of TOTP pretreatment on paraoxon and methyl paraoxon detoxification in rats. *Research communications in chemical pathology and pharmacology*, *8*(4), 665-672.

Bhattacharya, S., Mandal, S. K., Akhtar, M. S., Dastider, D., Sarkar, S., Bose, S., … Pramanick, A. (2020). Phytochemicals in the treatment of arthritis: current knowledge. *International Journal of Current Pharmaceutical Research*, *12*(4), 1-6. https://doi.org/10.22159/ijcpr.2020v12i4.39050

Biswas, S., Das, R., & Banerjee, E. R. (2017). Role of free radicals in human inflammatory diseases. *AIMS Biophysics*, *4*(4), 596-614. http://dx.doi.org/10.3934/biophy.2017.4.596

Brennan-Olsen, S. L., Cook, S., Leech, M. T., Bowe, S. J., Kowal, P., Naidoo, N., … Mohebbi, M. (2017). Prevalence of arthritis according to age, sex and socioeconomic status in six low and middle income countries: analysis of data from the World Health Organization study on global AGEing and adult health (SAGE) Wave 1. *BMC musculoskeletal disorders*, *18*(1), 271. https://doi.org/10.1186/s12891-017-1624-z

Briggs, A. M., Cross, M. J., Hoy, D. G., Sànchez-Riera, L., Blyth, F. M., Woolf, A. D., & March, L. (2016). Musculoskeletal Health Conditions Represent a Global Threat to Healthy Aging: A Report for the 2015 World Health Organization World Report on Ageing and Health. *The Gerontologist*, *56*(Suppl 2), S243-S255. https://doi.org/10.1093/geront/gnw002

Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., Li, Y., Wang, X., & Zhao, L. (2017). Inflammatory responses and inflammation-associated diseases in organs. *Oncotarget*, *9*(6), 7204-7218. https://doi.org/10.18632/oncotarget.23208

de Molon, R. S., Hsu, C., Bezouglaia, O., Dry, S. M., Pirih, F. Q., Soundia, A., Cunha, F. Q., Cirelli, J. A., Aghaloo, T. L., & Tetradis, S. (2016). Rheumatoid Arthritis Exacerbates the Severity of Osteonecrosis of the Jaws (ONJ) in Mice. A Randomized, Prospective, Controlled Animal Study. *Journal of bone and mineral research: the official journal of the American Society for Bone and Mineral Research*, *31*(8), 1596-1607. https://doi.org/10.1002/jbmr.2827

Faisal, R., Shinwari, L., & Jehangir, T. (2015). Comparison of the Therapeutic Effects of Thymoquinone and Methotrexate on Renal Injury in Pristane Induced Arthritis in Rats. *Journal of the College of Physicians and Surgeons-pakistan: JCPSP*, *25*(8), 597-601. https://doi.org/08.2015/jcpsp.597601

Faisal, R., & Imran, U. (2015). Comparative evaluation of thymoquinone and methotrexate in lung inflammation in murine model of rheumatiod arthritis. *J Postgrad Med Inst*, *29*, 88-92. Retrieved from https://jpmi.org.pk/index.php/jpmi/article/view/1686/0

Faisal, R., Ahmad, N., Fahad, Y. S., & Chiragh, S. (2018). Anti-Arthritic Effect of Thymoquinone in Comparison with Methotrexate On Pristane Induced Arthritis in Female Sprague Dawley Rats. *Journal of Ayub Medical College, Abbottabad: JAMC*, *30*(1), 3-7.

Fischer, E. A., Hetland, M. L., & Krabbe, S. (2020). Folinic acid alleviates side effects of methotrexate in arthritis patients with side effects despite folic acid supplementation: an observational cohort study. *Rheumatology (Oxford, England)*, *59*(11), 3566-3568. https://doi.org/10.1093/rheumatology/keaa277

Forouzanfar, F., Bazzaz, B. S., & Hosseinzadeh, H. (2014). Black cumin (Nigella sativa) and its constituent (thymoquinone): a review on antimicrobial effects. *Iranian journal of basic medical sciences*, *17*(12), 929-938.

Gheita, T. A., & Kenawy, S. A. (2012). Effectiveness of Nigella sativa oil in the management of rheumatoid arthritis patients: a placebo controlled study. *Phytotherapy research: PTR, 26*(8), 1246-1248. https://doi.org/10.1002/ptr.3679

Gholamnezhad, Z., Havakhah, S., & Boskabady, M. H. (2016). Preclinical and clinical effects of Nigella sativa and its constituent, thymoquinone: A review. *Journal of ethnopharmacology, 190*, 372-386. https://doi.org/10.1016/j.jep.2016.06.061

Grisham, M. B., Johnson, G. G., & Lancaster, J. R., Jr (1996). Quantitation of nitrate and nitrite in extracellular fluids. *Methods in enzymology, 268*, 237-246. https://doi.org/10.1016/s0076-6879(96)68026-4

Guo, Q., Wang, Y., Xu, D., Nossent, J., Pavlos, N. J., & Xu, J. (2018). Rheumatoid arthritis: pathological mechanisms and modern pharmacologic therapies. *Bone research, 6*, 15. https://doi.org/10.1038/s41413-018-0016-9

Hadi, V., Kheirouri, S., Alizadeh, M., Khabbazi, A., & Hosseini, H. (2016). Effects of Nigella sativa oil extract on inflammatory cytokine response and oxidative stress status in patients with rheumatoid arthritis: a randomized, double-blind, placebo-controlled clinical trial. *Avicenna journal of phytomedicine, 6*(1), 34-43.

Kakkar, P., Das, B., & Viswanathan, P. N. (1984). A modified spectrophotometric assay of superoxide dismutase. *Indian journal of biochemistry & biophysics, 21*(2), 130-132.

Kheirouri, S., Hadi, V., & Alizadeh, M. (2016). Immunomodulatory Effect of Nigella sativa Oil on T Lymphocytes in Patients with Rheumatoid Arthritis. *Immunological investigations, 45*(4), 271-283. https://doi.org/10.3109/08820139.2016.1153649

Li, Y., Kakkar, R., & Wang, J. (2018). In vivo and in vitro Approach to Anti-arthritic and Anti-inflammatory Effect of Crocetin by Alteration of Nuclear Factor-E2-Related Factor 2/hem Oxygenase (HO)-1 and NF-κB Expression. *Frontiers in pharmacology, 9*, 1341. https://dx.doi.org/10.3389%2Ffphar.2018.01341

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *The Journal of biological chemistry, 193*(1), 265-275.

Man, G. S., & Mologhianu, G. (2014). Osteoarthritis pathogenesis - a complex process that involves the entire joint. *Journal of medicine and life, 7*(1), 37-41.

Mittal, M., Siddiqui, M. R., Tran, K., Reddy, S. P., & Malik, A. B. (2014). Reactive oxygen species in inflammation and tissue injury. *Antioxidants & redox signaling, 20*(7), 1126-1167. https://dx.doi.org/10.1089%2Fars.2012.5149

Mollazadeh, H., & Hosseinzadeh, H. (2014). The protective effect of Nigella sativa against liver injury: a review. *Iranian journal of basic medical sciences, 17*(12), 958-966.

Nasuti, C., Fedeli, D., Bordoni, L., Piangerelli, M., Servili, M., Selvaggini, R., & Gabbianelli, R. (2019). Anti-Inflammatory, Anti-Arthritic and Anti-Nociceptive Activities of Nigella sativa Oil in a Rat Model of Arthritis. *Antioxidants (Basel, Switzerland), 8*(9), 342. https://dx.doi.org/10.3390%2Fantiox8090342

Noris, M., & Remuzzi, G. (2013). Overview of complement activation and regulation. *Seminars in nephrology, 33*(6), 479-492. https://doi.org/10.1016/j.semnephrol.2013.08.001

Ogawa, M., Matsuda, T., Ogata, A., Hamasaki, T., Kumanogoh, A., Toyofuku, T., & Tanaka, T. (2013). DNA damage in rheumatoid arthritis: an age-dependent increase in the lipid peroxidation-derived DNA adduct, heptanone-etheno-2'-deoxycytidine. *Autoimmune diseases, 2013*, 183487. https://dx.doi.org/10.1155%2F2013%2F183487

Ohkawa, H., Ohishi, N., & Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical biochemistry, 9*5(2), 351-358. https://doi.org/10.1016/0003-2697(79)90738-3

Rose, B. J., & Kooyman, D. L. (2016). A Tale of Two Joints: The Role of Matrix Metalloproteases in Cartilage Biology. *Disease markers, 2016*, 4895050. https://dx.doi.org/10.1155%2F2016%2F4895050

Safiri, S., Kolahi, A. A., Hoy, D., Smith, E., Bettampadi, D., Mansournia, M. A., … Cross, M. (2019). Global, regional and national burden of rheumatoid arthritis 1990-2017: a systematic analysis of the Global Burden of Disease study 2017. *Annals of the rheumatic diseases, 78*(11), 1463-1471. https://doi.org/10.1136/bmj.m791

Shimizu, T., Kondo, K., & Hayaishi, O. (1981). Role of prostaglandin endoperoxides in the serum thiobarbituric acid reaction. *Archives of biochemistry and biophysics, 206*(2), 271-276.

https://doi.org/10.1016/0003-9861(81)90091-6

Sokolove, J., & Lepus, C. M. (2013). Role of inflammation in the pathogenesis of osteoarthritis: latest findings and interpretations. *Therapeutic advances in musculoskeletal disease*, *5*(2), 77-94. https://doi.org/10.1177/1759720x12467868

Sproston, N. R., & Ashworth, J. J. (2018). Role of C-Reactive Protein at Sites of Inflammation and Infection. *Frontiers in immunology*, *9*, 754. https://dx.doi.org/10.3389%2Ffimmu.2018.00754

Tan, B. L., Norhaizan, M. E., Liew, W. P., & Sulaiman Rahman, H. (2018). Antioxidant and Oxidative Stress: A Mutual Interplay in Age-Related Diseases. *Frontiers in pharmacology*, *9*, 1162. https://dx.doi.org/10.3389%2Ffphar.2018.01162

van den Berg, R., Stanislawska-Biernat, E., & van der Heijde, D. M. (2011). Comparison of recommendations for the use of anti-tumor necrosis factor therapy in ankylosing spondylitis in 23 countries worldwide. *Rheumatology (Oxford, England)*, *50*(12), 2270-2277. https://doi.org/10.1093/rheumatology/ker270

Winter, C. A., Risley, E. A., & Nuss, G. W. (1962). Carrageenin-induced edema in hind paw of the rat as an assay for antiiflammatory drugs. Proceedings of the Society for Experimental Biology and Medicine. *Society for Experimental Biology and Medicine (New York, N.Y.)*, *111*, 544-547. https://doi.org/10.3181/00379727-111-27849

Yap, H. Y., Tee, S. Z., Wong, M. M., Chow, S. K., Peh, S. C., & Teow, S. Y. (2018). Pathogenic Role of Immune Cells in Rheumatoid Arthritis: Implications in Clinical Treatment and Biomarker Development. *Cells*, *7*(10), 161. https://dx.doi.org/10.3390%2Fcells7100161

Yimer, E. M., Tuem, K. B., Karim, A., Ur-Rehman, N., & Anwar, F. (2019). Nigella sativa L. (Black Cumin): A Promising Natural Remedy for Wide Range of Illnesses. *Evidence-based complementary and alternative medicine: eCAM*, *2019*, 1528635. https://doi.org/10.1155/2019/1528635

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Thymoquinone-rich black cumin oil improves sleep quality, alleviates anxiety/stress on healthy subjects with sleep disturbances– A pilot polysomnography study

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ABSTRACT

Introduction: Insomnia, the inability to get sleep or sleep well at night, is a universal health problem. In the present single-arm, open-label, pilot study, the safety and efficacy of a thymoquinone-rich (5% w/w) black cumin oil (*Nigella sativa*) (BCO) was investigated for the first time, among healthy subjects characterized with significant sleep problems.

Methods: Fifteen subjects were selected for the study and were provided with softgel capsules of BCO (200 mg × 1/day, after dinner) for 28 days. Polysomnographic analysis was performed to assess sleep quality, sleep efficiency and sleep latency (primary outcomes) at the beginning (visit 2) and after 7 days of supplementation (visit 3). Sleep quality index, anxiety and stress (secondary outcomes) were also measured at the beginning and at the end of the study period (28 days) using the validated questionnaires Pittsburgh Sleep Quality Index (PSQI), Depression Anxiety Stress Scale -21 (DASS-21) and Hamilton Anxiety Rating Scale-A (HAM-A), along with the measurements of cortisol and safety parameters.

Results: The results suggested a significant improvement in primary outcomes such as total sleep time, sleep latency and sleep efficiency with an increase of 82.49 % in Non-Rapid Eye Movement (NREM 3) and 29.38 % increase in Rapid Eye Movement sleep (REM sleep). The secondary outcome measurements also showed a significant reduction ($P < 0.05$) in anxiety and stress as evident from DASS-21 and HAM-A scales, with significant reduction in Cortisol. Haematological and biochemical analysis demonstrated the safety of BCO during the study period.

Conclusion: Supplementation of BCO was safe and found to significantly improve the sleep quality and ameliorate stress and anxiety.

[Clinical Trial Reg No: CTRI/2018/03/012,391 dated 07/03/2018]

1. Introduction

Sleep is a biological need of all higher life forms and sleep disorders can cause various health problems including chronic diseases (Koyanagi et al., 2014; Colten et al., 2006). According to the International Classification of Sleep Disorders – Version 3, sleep disorders are classified into six major classes in which insomnia accounts for approximately 9–15 % of the global population (Sateia, 2014). Nearly 25 % of adults in the United States are reported to have insufficient sleep, and the sleep-related problems are estimated to cost $15.9 billion every year, in addition to the cost due to related health issues, such as accidents, workplace absenteeism and worker productivity (Parthasarathy et al.,

Abbreviation: ACTH, Adrenocorticotropic hormone; BCO, Black Cumin Oil; CRH, Corticotropin-Releasing Hormone; DASS-21, Depression Anxiety Stress Scale -21; EEG, Electroencephalography; EMG, Electromyography; EOG, Electrooculography; HAM-A, Hamilton Anxiety Rating Scale-A; MRs, Mineralocorticoid Receptors; NREM, Non-Rapid Eye Movement; PSQI, Pittsburgh Sleep Quality Index; REM, Rapid Eye Movement; TQ, Thymoquinone; WASO, Wake After Sleep Onset.

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2016; Equihua-Benítez et al., 2017).

The molecular mechanism of sleep is generally controlled by the biomolecules melatonin, Ɣ-aminobutyric acid (GABA), glutamate and cortisol (Scammell et al., 2017). Melatonin is a hormone primarily produced by the pineal gland and has an inverse relationship with the amount of environmental light in such a way that its production spikes at night and gets inhibited during the day (Bartness et al., 1993). It binds to both melatonin receptor type 1A and melatonin receptor type 1B and induces sleep. So, these receptors are the targets for the melatonin agonists that are aimed to treat sleep disorders and circadian rhythm disorders (Cajochen et al., 2003).

Cortisol is another hormone produced by the adrenal cortex and its release is mediated by the hypothalamic-pituitary-adrenal (HPA) axis and follows a circadian rhythm characterized by a morning peak [cortisol awakening response (CAR)] followed by a slow decline throughout the day with either very low or undetectable amount at midnight (Martin and Crump, 2003; Pistollato et al., 2016). Dysregulated cortisol concentrations were shown to be correlated with diminished sleep quality (Backhaus et al., 2004). It is also known that GABA is associated with sleep, stress, and anxiety and the activation of GABA receptors favors sleep (Gottesmann, 2002).

A melatonergic drug called Ramelteon, was reported to be effective in treating sleep disorders and approved by the U.S. Food and Drug Administration (FDA) (Uchiyama et al., 2011). Tasimelteon (for the treatment of non-24 h sleep-wake disorder), Agomelatine (a melatonergic antidepressant) and Circadin® (a prolonged-release melatonin preparation) are other major drugs currently in use for the treatment of insomnia (Equihua-Benítez et al., 2017). However, melatonin based medicines have reported certain side effects such as drowsiness, headache, dizziness, or nausea (Wong et al., 2018).

During 2011, FDA warned a company that sells melatonin based brownies stating that melatonin has not been deemed as a safe food additive (Melatonin, 2021). Thus, there exists a great interest in natural and alternative agents for improving sleep quality. Valerian, L-theanine, St. John's wort (*Hypericum perforatum*), Aswagandha, Chammomile and Passionflower (*Passiflora incarnata*) are some of the natural agents currently in use (Melatonin, 2021; Hu et al., 2018). But most of these natural sleep aids need heavy dosage (> 600 mg/day) to produce a significant effect and hence the efficacy is in controversy (Hu et al., 2018).

Nigella sativa (NS), commonly known as black cumin or black seed is a medicinal herb widely used in the Indian and Arab systems of traditional medicine. It has been shown to possess a wide range of pharmacological effects, such as anti-inflammatory, anti-hypertensive, anti-ulcer, anti-diabetic, anti-microbial, spasmolytic, bronchodilator, hepatoprotective, renal protective, gastroprotective properties (Ahmad et al., 2013). Most of the therapeutic properties of this plant are dedicated to the seed oil in which thymoquinone (TQ) is identified as the most abundant bioactive principle (Ahmad et al., 2013). A number of preclinical studies on the effect of TQ in neurological problems such as epilepsy, Parkinson's disease, anxiety and memory have been reported (Farkhondeh et al., 2018). Thymoquinone treatment in mice has revealed significant anti-anxiety activity by modulating GABA and nitric oxide (NO) levels in the brain and plasma (Krishnakumar et al., 2018; Gilhotra and Dhingra, 2011). Hosseini et al., reported that intravenous injection of NS extract (400 mg/kg b. wt.) prevented lipopolysaccharide induced depression like behaviour in rats (Hosseini et al., 2012). Depression, anxiety and stress can have a significant role in the development of insomnia (Fernandez-Mendoza and Vgontzas, 2013). Another study reported that 250 mg/kg b.wt. dose of NS extract for 14 days significantly increased sleep time in rats (Guha et al., 2005). Thus, it was hypothesised that BCO would have significant effect in the amelioration of sleep disorders.

Moreover, earlier studies in the authors lab established the relaxing and sleepy effect of a black cumin oil containing 5% (w/w) of TQ and >45 % α-Linoleic acid its effects on various neurotransmitters and receptors (Krishnakumar et al., 2018). The neuro transmitters such as GABA, acetyl choline, NO, and glutamate levels were reported to have effects on sleep and wakefulness (Watson et al., 2010). Thus, the present pilot study investigated the efficacy of BCO on healthy subjects (35–65 years old) having significant stress, anxiety and sleep disturbances characterized by either waking up in the night for multiple times or having difficulty in initiating sleep for a minimum of 3 nights per week for the past 3 months (Sateia, 2014). While Polysomnography was employed to measure the sleep pattern, validated questionnaires were employed to analyze sleep quality (Pittsburgh Sleep Quality Index, PSQI), anxiety (Hamilton Anxiety Rating Scale, HAM-A) and stress (Depression Anxiety Stress Scales-21, DASS-21).

2. Materials & methods

2.1. General

Dried seeds of black cumin (black seed) were obtained from a selected region in India where it was cultivated with good agricultural practices. An authenticated botanist identified the sample and a voucher specimen (AK-NS-018) was deposited at the Herbarium of Akay Natural Ingredients, Cochin, India. Black seed oil used in the present study was produced at the manufacturing plant of Akay Natural Ingredients following good manufacturing practices (BlaQmax- batch no: BCOQ 02/17 date 20/08/2017). A proprietary cold-pressing extraction process was employed for the production. It was found to contain 5% (w/w) of thymoquinone upon high performance liquid chromatography (HPLC) analysis employing Shimadzu model LC 20AT, with an M20A photodiode array (PDA) detector (Shimadzu Analytical India Private Limited, Mumbai, India), fitted with a reverse phase C18 column (250 × 4.6 mm, 3 μm) (Phenomenex, Hyderabad, India). The detailed composition of BCO are provided in Table 1.

The present pilot study was designed as an open-label, single-arm study to evaluate the improvements in sleep quality, stress, and anxiety upon the supplementation of BCO. The study was approved by the Institutional Ethics Committee of M/s Aman Hospital and Research Centre, Gujarat, India [Reg No: CTRI/2018/03/012,391]. The inclusion and exclusion criteria are detailed in Table 2. The primary objectives were the sleep parameters such as the change in sleep latency, sleep duration, wake-after-sleep-onset (WASO) time, and sleep efficiency. Secondary outcomes were sleeping score, anxiety score, stress score, and safety.

Table 1
Composition of BCO used in the study.

Chemical Name	Percentage (w/w)	Method of estimation
Thymoquinone	5.89	HPLC*
Linoleic acid	48.92	
Palmitic acid	15.8	
Oleic acid	19.2	
Stearic acid	1.6	GC/MS**
Myristic acid	0.65	
Palmitoleic acid	0.51	
α-Linolenic acid	0.25	
p-cymene	3.86	
Trans 4- methoxy thujane	0.65	
Longifolene	1.13	
α-thujene	0.57	
Thymol	0.24	GC/MS MS***
β-pinene	0.18	
α- longipinene	0.15	
Lemonene	0.15	
Terpinene-4-ol	0.09	
α-pinene	0.16	

* Hadad et al., 2012.
** AOAC Official Method 969.33 Fatty Acids in Oils and Fats Preparation of Methyl Esters Boron Trifluoride Method First Action 1969.
*** Khalid and Shedeed, 2016.

Table 2
Inclusion and exclusion criteria for the study.

Inclusion criteria
a) Presence of at least one sleep complaint, such as difficulty in initiating sleep, which is present at least for 3 nights per week for at least 3 months
b) Subjects with sleep irregularities and having the Pittsburgh Sleep Quality Index (PSQI) Score greater than 5
c) Literate, agreeing to participate in the study
d) Having nearest health care centre in the residential area
Exclusion criteria
a) Having an allergy to black cumin or low blood pressure
b) Unpleasant occurrences such as death or hospitalization of a family member or divorce during the last three months
c) Prior history of depression or any other mental disorder
d) Lack of phone number or cell phone number
e) Possibility of changing the address during study

2.2. Subjects and design

Eighteen subjects (Age: 35–65 years) with sleep irregularities within the PSQI scores greater than 5 were selected for the study. The overall study period was 28 days and the study design was illustrated in the cohort diagram (Fig. 1). On Visit 1, screening of subjects was performed as per the inclusion criteria and three subjects who were not interested to participate in the study were excluded. The remaining fifteen subjects were enrolled for the study with a written informed consent for the participation of the study. The selected participants were requested to appear in two consecutive nights for Polysomnography analysis including their visit days to exclude the adaptation effects ('first night effects') and the values obtained in the first night were not used (Toussaint et al., 1995). The participants were requested to appear for Visit 2 (Day 1) to measure the baseline parameters such as blood collection for biochemical and hematological analysis, questionnaires (PSQI, DASS-21, HAM-A) and Polysomnography analysis. Following this, all subjects were directed to consume a single dose of BCO softgel capsules (200 mg × 1/day after dinner) for 28 days. During Visit 3, (after 7 days of BCO supplementation), the Polysomnography analysis was conducted again. The subjects were further asked to continue BCO intake at the same dose for the rest of the study period of 28 days. On Visit 4 (Day 28), all the participants completed the blood analysis and the questionnaires (PSQI, DASS-21, and HAM-A).

2.3. Polysomnography analysis

Sleepwear G3 Philips Respironics, USA instrument was used for polysomnography analysis. On visit 2 (Day 1) and visit 3 (Day 7), participants were directed to arrive in the Sleep Research Laboratory approximately two hours before their scheduled bedtime and allowed to sleep in temperature-controlled, sound-attenuated rooms. Bedtimes and rise times for each participant were determined from their sleep log for the past two weeks and maintained the same throughout all laboratory nights. Participants were asked to maintain their usual daytime activities, food habits and regular sleep-awake timings. Subjects were also asked to avoid napping if they routinely did not nap. No instructions were given to the participants to avoid a nap if they routinely napped. Participants were permitted to have caffeine in usual amounts in the morning but were asked to abstain from consuming caffeine or alcohol in the afternoon of the sleep recording nights. The BCO capsule was taken 20 min before bedtime. A small battery-operated clock was given for recording the sleep-awake timing. Scalp electrodes were placed for PSG recording and then allowed to sleep.

2.4. Pittsburgh sleep quality index (PSQI)

It is a self-rated questionnaire which measures sleep quality and disturbances over one month time interval (Buysse et al., 1989). Subjects were requested to fill the questionnaire on visit 2 (Day 1 of treatment; baseline) and visit 4 (Day 28 of treatment; End of study). Maximum time allowed to complete the questionnaire was 10 min.

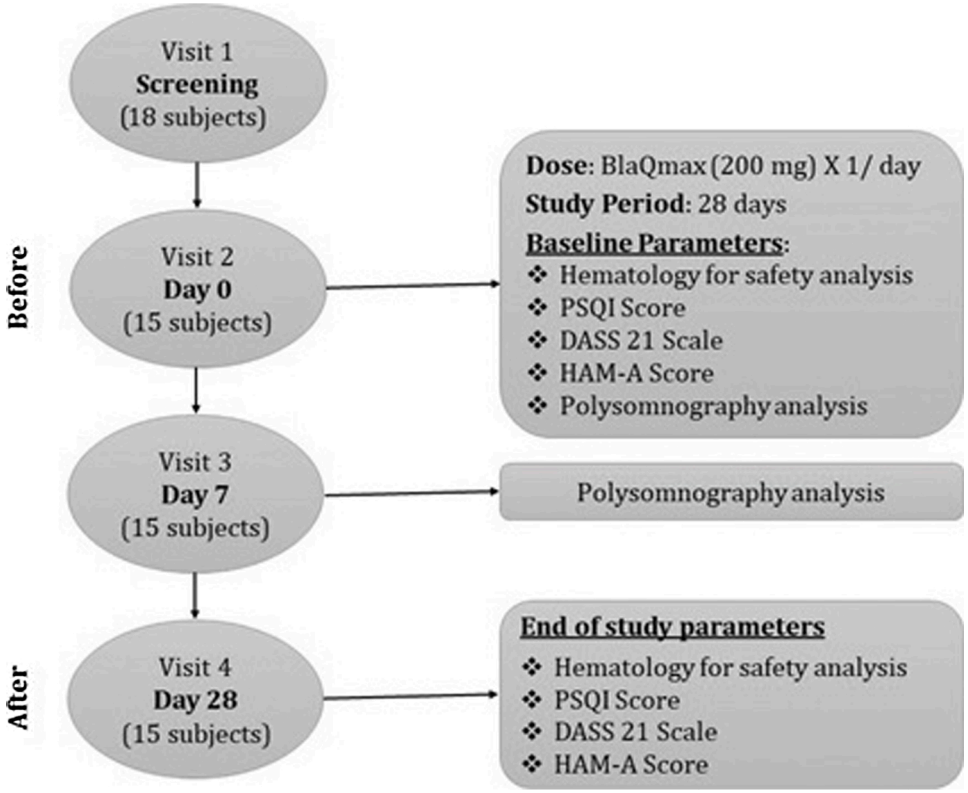

Fig. 1. Cohort diagram of a study plan.

2.5. Depression anxiety stress Scales-21 (DASS-21)

Validated form of the Depression Anxiety Stress Scales-21 (DASS-21), consisting of the 21-item self-report questionnaire, was employed to monitor the relaxation effect of BCO (Sinclair et al., 2012). The subjects were asked to rate all the items in the questionnaire in terms of how often they experienced depression, anxiety and stress in the past week using a 4-point scale, ranging from 0 (did not apply to me at all) to 3 (applied to me very much or most of the time) during the visit 2 (Day 1) and visit 4 (Day 28) (Lovibond and Lovibond, 1996).

2.6. Hamilton anxiety rating scale (HAM-A)

The Hamilton Anxiety Rating Scale (HAM-A) consists of 14 items to measure both psychic anxiety (mental agitation and psychological distress) and somatic anxiety (physical complaints related to anxiety) was employed in the study (Findikli et al., 2017; Bradley et al., 2018). The subjects were asked to fill the questionnaire on visit 2 (Day 1) and visit 4 (Day 28).

2.7. Estimation of cortisol level

The blood samples were collected on visit 2 (Day 1) and visit 4 (Day 28) between 9–10 am, and the serum cortisol levels were analysed using immunoassay kits (Rohini et al., 2015).

2.8. Safety parameters

The primary safety and tolerability of BCO at the present dosage of (200 mg × 1/day) was evaluated by specifically collecting the individual data regarding the adverse events, discomforts, and clinical changes. Blood collection was done both pre and post- treatment to measure hematological and biochemical parameters related to safety. Hematological parameters included hemoglobin content, RBC, hematocrit, platelet count, mean corpuscular volume, mean corpuscular hemoglobin, basophils, and eosinophils; in addition to neutrophils, lymphocytes, monocytes, and WBC. Liver and kidney function markers were also analysed.

2.9. Statistical analysis

Statistical analyses were carried out using the Statistical Package for Social Science (IBM SPSS Statistics. Version 25.0). The total sample size at the end of the study was 15. The efficacy endpoints included the comparison of data at the baseline with the end of the study (within-group comparison) using paired sample *t*-test and $P \leq 0.05$ was considered to be significant. The results were presented as mean ± SD. 95 % CI values are also provided.

3. Results

3.1. Effect of BCO in Polysomnography

The present study estimated the total sleep time, sleep continuity, sleep efficiency, sleep quality and latency using polysomnography. Non-rapid eye movement (NREM) and rapid eye movement (REM) periods were used to calculate the sleep efficiency. The relative periods of various sleep stages during the total sleep duration was divided into 4 stages as NREM1, NREM2, NREM 3 and REM. The results showed that the percentage of various sleep stages during the total sleep time was 11.4 % (NREM1), 63.1 % (NREM2), 9.7 % (NREM3) and 15.8 % (REM) respectively at the baseline (day 1, at the beginning of the study period) (Fig. 2). On Visit 3 (day 7), the percentage of total sleep time was significantly increased ($P \leq 0.05$) in NREM 3 stage by 82.77 % and REM stage by 31.58 %. Stage 1 (NREM1) and stage 2 (NREM2) on the other hand showed a significant reduction ($P \leq 0.05$) in percentage sleep time

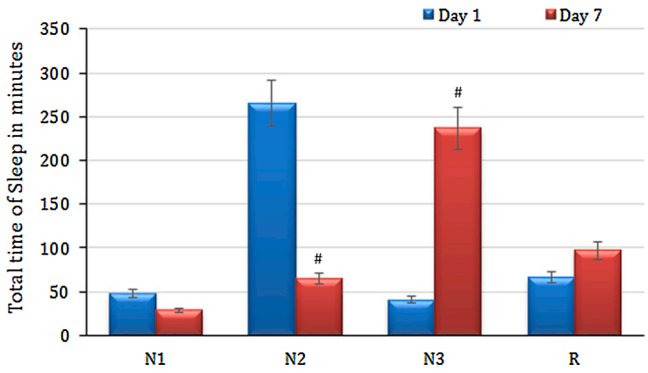

Fig. 2. Polysomnography analysis. The values are given as mean of total sleep time in minutes ± SD for different stages of sleep. N1-Non-random eye movement sleep phase 1 (NREM 1); N2- Non-random eye movement sleep phase 2 (NREM 2); N3- Non-random eye movement sleep phase 3 (NREM 3) and R-Random eye movement sleep phase (REM).

(6.7 % and 15.3 % respectively). Other parameters such as Total sleep time, sleep latency, WASO and Sleep efficiency were also improved after BCO treatment (Table 3).

3.2. Effect of BCO in PSQI score

In the present study, the baseline PSQI score was 10.75 ± 0.47 and was changed to 6.48 ± 0.86 by the end of the study period (day 28). Upon paired sample T-test, the difference was found to be statistically significant ($P \leq 0.001$; 95 % CI (3.71, 4.82); 39.72 %) in comparison with the baseline (Fig. 3a). Further analysis of the percentage improvement in component scores of PSQI indicated 30.36 % improvement in sleep quality, 27.32 % reduction in sleep latency and 25 % improvement in sleep duration with an average reduction of 50.66 % overall sleep disturbances (Fig. 3b).

3.3. Effect of BCO in DASS-21 score

The baseline DASS-21 total scores of the participants were in the range of 32–43 with an average score of 36.16 ± 5.22, which was changed to 26.29 ± 2.34 with a 27.29 % reduction by the end of the study period ($P \leq 0.05$; 95 % CI (6.75, 13.23)) (Fig. 4). Among the three sub-dimensions of DASS-21 Scale (anxiety, stress and depression), anxiety and stress showed significant reduction ($P \leq 0.05$; 95 % CI (4.23, 5.88); (3.86, 6.33) respectively)), with a percentage reduction of 32.58 and 21.96 respectively, at the end of study period when compared to baseline scores (Table 4). No significant change was observed in the depression sub-scale. The baseline sub-scale showed that the subjects were reported with normal depression (4.00 ± 1.41), but with severe anxiety (14.06 ± 1.38) and moderate stress (18.10 ± 2.21) scores. Upon supplementation with BCO, anxiety (9.00 ± 0.61) and stress (13.00 ± 0.45) were changed to mild states with no significant change in depression from its baseline scores (4.00 ± 1.41 verses 4.29 ± 0.78).

Table 3
Polysomnography analysis parameter changes.

Sleep parameters	Baseline	End of Study
Time in bed (min)	447.6 ± 53	437.8 ± 59
Total sleep time (min)	266 ± 48	312.5 ± 37*
Sleep latency (min)	27 ± 16	20 ± 7*
WASO (min)	154.6 ± 22	125.1 ± 15*
Sleep efficiency (%)	59.4 ± 28	71.4 ± 33*

The values are expressed as mean ± SD. * $P < 0.05$ vs baseline values.

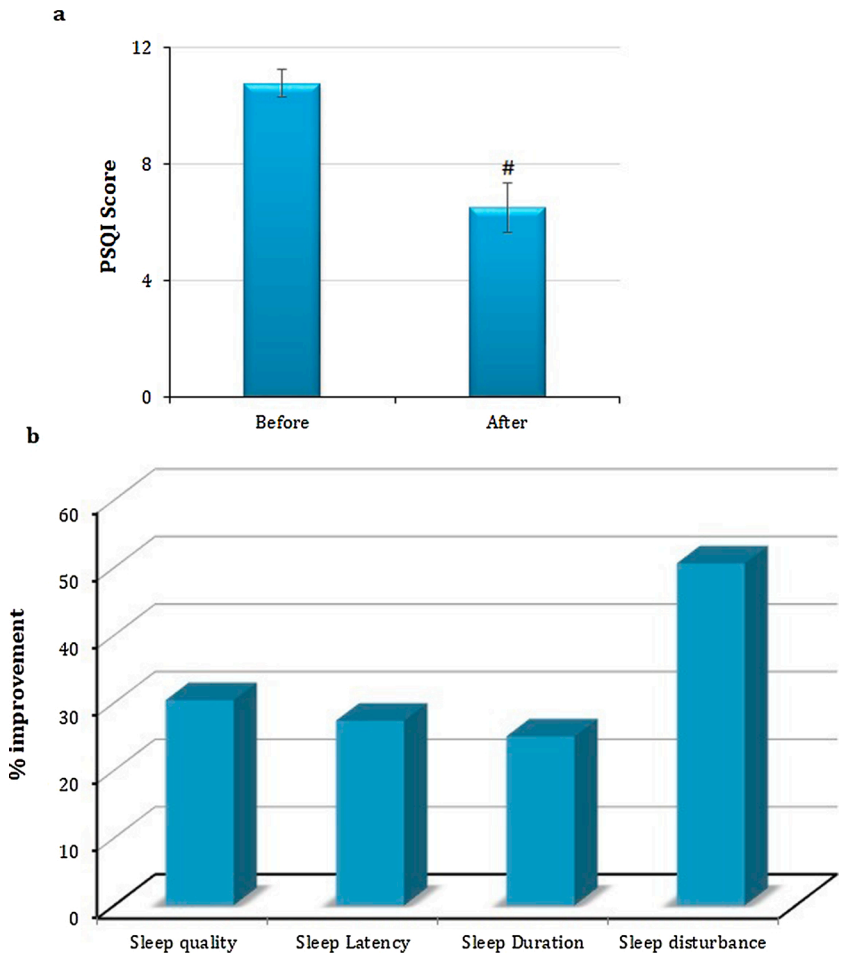

Fig. 3. Pittsburgh Sleep Quality Index (PSQI) analysis. (**a**) PSQI score - Values are expressed as mean ± SD. #$P \leq 0.001$ Vs baseline; (**b**) percentage improvement in sleep quality - percentage improvement in sleep quality, sleep latency, sleep duration and sleep disturbance after BCO treatment.

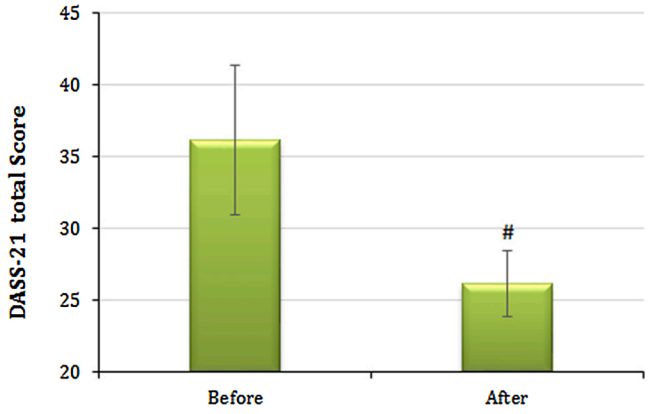

Fig. 4. Depression Anxiety Stress Scales-21 (DASS-21). Values are expressed as mean ± SD. $^{\#}P \leq 0.05$ Vs baseline.

3.4. Effect of BCO in HAM-A score

Upon HAM-A analysis, a significant reduction of 23.94 % ($P < 0.05$;) was observed in HAM-A scores during the study period (baseline 18.96 ± 0.35 to end of the study 14.42 ± 0.32, 95 % CI (4.29, 4.78 points)) (Fig. 5).

Table 4
Depression Anxiety Stress Scales-21 (DASS-21) sub dimensional scores*.

Period	Depression	Anxiety	Stress	Total
Baseline	4.00 ± 1.41 (normal)	14.06 ± 1.38 (severe)	18.10 ± 2.21 (moderate)	36.16 ± 5.22
End of study	4.29 ± 0.78 (normal)	9.00 ± 0.61$^{\#}$ (mild)	13.00 ± 0.45$^{\#}$ (mild)	± 2.34$^{\#}$

Values are expressed as mean ± SD. $^{\#}P \leq 0.05$ Vs baseline *As per DASS-21 scale, 0–9 score represents normal depression. In the anxiety sub-scale, 7–9 is considered as mild and 15–19 as severe. For the sub-class stress, score of 11–18 corresponds to mild and 19–26 as moderate.

3.5. Effect of BCO in cortisol level

The cortisol level showed a significant reduction at the end of the study in comparison with the baseline values. The statistical analysis using the Paired sample T-test gave the baseline value 12.26 ± 3.96 µg/dL which was changed to 8.51 ± 2.27 µg/dL ($P < 0.001$; 95 % CI (2.59, 4.96)), with a percentage difference of 29.72 % (Table 5).

3.6. Effect of BCO on safety parameters

The results of hematological and biochemical analyses are given in Table 6. It was observed that the treatment with BCO did not produce any significant ($P > 0.05$) change on hemoglobin content, RBC count, PCV, MCV, and MCH concentrations as compared to the baseline values. Neutrophils, lymphocytes, monocytes and WBC count were significantly

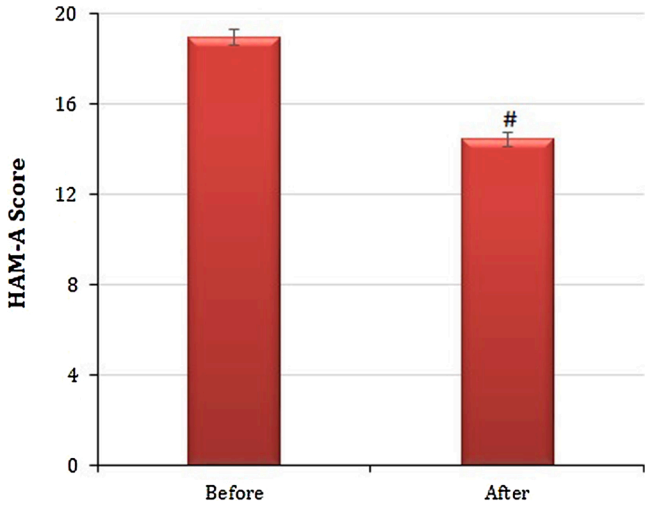

Fig. 5. Hamilton Anxiety Rating Scale (HAM-A). Values are expressed as mean ± SD. $^{\#}P \leq 0.05$ Vs baseline.

Table 5
Level of cortisol (μg/dL) in serum.

Time	Cortisol level
Baseline	12.26 ± 3.96
End of study	8.51 ± 2.27$^{\#}$

Levels of cortisol before and after treatment with BCO. Values are expressed as mean ± SD. $^{\#}P \leq 0.001$ Vs.

Table 6
Safety parameters – Haematological & Biochemical.

Parameters	Baseline	End of Study
Haemoglobin (g/dl)	13.08 ± 0.75	13.20 ± 0.76
RBC Count (million/μL)	5.38 ± 0.50	5.41 ± 0.45
WBC Count (No/μL)	7206.67 ± 914.54	6853.33 ± 1297.73
Packed cell volume (%)	39.22 ± 4.50	39.88 ± 3.47
Mean corpuscular volume (fl/red cell)	77.06 ± 12.09	75.95 ± 11.77
Mean corpuscular haemoglobin (pg/cell)	24.50 ± 5.20	24.52 ± 4.54
Neutrophils (%)	67.93 ± 3.86	65.33 ± 3.27
Lymphocytes (%)	30.60 ± 4.53	28.80 ± 3.61
Monocytes (%)	3.60 ± 1.45	3.27 ± 1.16
Eosinophil's (%)	1.67 ± 0.72	1.93 ± 0.59
Basophils (%)	Nil	Nil
SGOT (IU/L)	18.63 ± 5.24	19.16 ± 6.48
SGPT (IU/L)	21.68 ± 7.47	21.38 ± 8.29
S. Creatinine (mg/dl)	0.88 ± 0.14	0.90 ± 0.16

Results of safety parameters. Values are expressed as mean ± SD. A within-group comparison was done using a paired sample *t*-test.

reduced to normal range. Serum levels of aspartate aminotransferase (AST or SGOT) and alanine aminotransferase (ALT or SGPT), and creatinine have remained in the normal range with no significant change ($P > 0.05$) from the base values.

4. Discussion

The present study reports, for the first time, the safety and efficacy of a thymoquinone-rich (5% w/w) black cumin oil in improving sleep quality and reducing anxiety and stress among healthy sleep-deprived subjects. Polysomnography analysis followed by the oral administration of softgel capsules of 200 mg × 1/day for 7 days showed significant improvements in sleep latency, duration of deep sleep and total sleep time with a significant reduction in sleep disturbances. Further administration for 28 days revealed a significant reduction in anxiety and stress severity scores with an overall improvement in sleep quality without causing any adverse events or deviation in hematological and biochemical parameters related to safety.

Polysomnography is a comprehensive recording of the biophysiological changes during sleep (Kayabekir, 2019). It measures the sleep architecture in terms of two major phases, namely the non-rapid eye movement (NREM) and rapid eye movement (REM) phases (Kayabekir, 2019). The relative time periods of NREM during the total sleep duration was further divided into 4 stages as NREM 1, NREM 2, NREM 3 and NREM 4. The first two stages, NREM 1 and NREM 2, represent the initial time period taken by someone to fell asleep and is referred to as sleep latency. The later stages, NREM 3 and NREM 4, were recently combined and referred to as slow-wave sleep (SWS) stage because of the inherent difficulties in distinguishing these stages of deep sleep (Kayabekir, 2019). To monitor the various phases of sleep architecture while a subject is sleeping or trying to fall asleep, three concurrent physiological signal changes are collected using the non-invasive surface electrodes: electroencephalography (EEG), electrooculography (EOG) and electromyography (EMG). Thus, polysomnography requires whole night recording in a sleep laboratory. Additional signals related to breathing - nasal/oral airflow, thoracic effort, and abdominal effort may also be recorded simultaneously (Tripathi, 2008).

The present study estimated the total sleep time, sleep continuity, sleep efficiency, sleep quality and latency using polysomnography. Normally, NREM sleep constitutes about 75–85 % of total sleep time and REM sleep phase constitutes the remaining 20–25 %. The REM sleep increases as the night progress and is the longest in the last one-third of the sleep episode (Carskadon and Dement, 2021). Slow-wave sleep is identified by a preponderance of high-amplitude, low-frequency components as measured by EEG. The REM sleep is the deep sleep stage which was defined by a low-voltage EEG, similar to the activity of the brain during waking hours, with rapid and random movement of the eyes and low muscle tone (Irwin, 2015).

Supplementation of BCO for 7 days showed significant improvement in sleep latency and sleep efficiency as evident from the changes in the duration of various phases in the sleep architecture (Table 3). The significant reduction ($P \leq 0.05$) observed in NREM stages 1 and 2 (69 %) indicated improvement in sleep latency (reduction in the time taken to fall asleep) and the increase in SWS (NREM 3 and REM; 82.77 % and 23.8 % respectively) revealed enhancement in the duration of the deep sleep period. Wake time after sleep onset (WASO) was also found to be significantly reduced after treatment (Table 3), indicating a reduction in sleep disturbances. Similar variations in sleep stages have already been reported in the synthetic drugs used for the treatment of sleep disorders (Mathias et al., 2021).

An important factor in sleep disorders has been identified as hypothalamic-pituitary-adrenal axis (HPA-axis) dysregulation, particularly the alterations in circadian cortisol rhythmicity which is further related to stress (Dumbell et al., 2016). Corticotropin-releasing hormone (CRH) is released from the hypothalamus during stress and it triggers the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary. The ACTH can stimulate the adrenal cortex, which produces and secretes glucocorticoids (i.e., cortisol in humans), the end-product of HPA-axis activation. Cortisol initially binds to high-affinity mineralocorticoid receptors (MRs) and once the MRs have been saturated, cortisol binds to glucocorticoid receptors (GRs) and initiate a self-regulatory feedback system to shut down the HPA-axis and reduces blood cortisol concentration (Tsigos and Chrousos, 2002; Gunnar and Quevedo, 2007). Reduced cortisol level is correlated with enhanced sleep quality (Born et al., 1986; Vargas et al., 2018). It has also been demonstrated that increased cortisol concentration at night is also associated with enhanced NREM 1 and NREM 2 stages and reduced NREM 3 and REM (SWS) stages of sleep (Born et al., 1986; Follenius et al., 1992). Thus, the significant reduction of cortisol observed in the present study explains the reduction in NREM 1 & 2 and enhancement in

NREM 3 & REM stages (SWS) upon supplementation with BCO (Table 3).

The Pittsburgh Sleep Quality Index, a 19-item self-reported questionnaire that evaluates seven clinically derived domains of sleep difficulties (i.e., quality, latency, duration, habitual efficiency, sleep disturbances, use of sleeping medications, and daytime dysfunction), is a validated questionnaire widely used to identify clinically significant sleep impairments (Cole et al., 2006). Component scores of PSQI range from 0 to 3 and are summed to obtain a global score, which ranges from 0 to 21. The lower the PSQI score, better the sleep quality and a reduction in score indicates improvement in sleep quality (Buysse et al., 1989). A global PSQI score of more than 5 suggests a significant sleep disturbance (Buysse et al., 1989). The observed reduction in PSQI score (39.72 %) associated with the BCO treatment indicates improvement in sleep quality and further correlates with the polysomnography results (Figs. 3a & b).

DASS-21 has been a well-validated tool to measure stress, anxiety, and depression (Teo et al., 2019). According to DASS-21, a score ranging from 43 to 59 is generally considered to represent moderate depression, anxiety, and/or stress state. A higher score indicates severe to extremely severe states and lower scores denote normal state (Lovibond and Lovibond, 1996). The significant reduction ($P < 0.05$) in the DASS-21 score observed in the present study points out the relaxing or calming effect associated with BCO. The reduction was also significant in sub-dimensions of the scale, mainly in the anxiety and stress scores (Table 4). The observed reduction in DASS-21 was also supported by the results of HAM-A, which showed almost 23.94 % reduction in anxiety. The HAM-A scale is one of the first rating scale developed to measure the severity of anxiety symptoms and has been using widely in both clinical research settings (Findikli et al., 2017; Bradley et al., 2018). In HAM-A, each item is scored on a scale of 0 (not present) to 4 (severe), with a total score range of 0–56; where a score < 17 indicates mild severity, 18–24 mild to moderate severity and 25–30 for moderate to severe anxiety levels (Hamilton, 1959). Anxiety, depression or stress and are indirectly correlated with the development of insomnia (Fernandez-Mendoza and Vgontzas, 2013) and insomnia can also induce depression / anxiety. A two-fold enhancement in depression has been observed among individuals with insomnia (Li et al., 2016).

Supplementation of BCO did not show any adverse effects or clinically significant changes in either hematological or biochemical parameters, indicating the safety and tolerance at the investigational dosage 200 mg × 1/day for 28 days (Table 6). However, it is important to note the trend in respect of neutrophils, lymphocytes, monocytes, and WBC counts which showed a decrease and reversal to the normal levels from the initial elevated levels. These are well known immune function markers associated with sleep deprivation (Mullington et al., 2010). The subjects did not show any side effects or adverse events such as nausea, vomiting, stomach pain, dizziness or headache, indicating the tolerability of BCO. No morning sickness or tiredness was also reported. Thus, the present study reported the safety, tolerability, and efficacy of BCO as a safe natural sleep-aid with a significant influence on reducing stress and anxiety. Earlier clinical studies have revealed the hypolipidemic effect (5 mL/day for 56 days), anti-diabetic effect (700 mg/day for 40 days), anti-hypertensive effect (400 mg/day for 60 days) of cold-pressed black seed oil/seed/seed extract and no adverse events were reported with various dosages (Tavakkoli et al., 2017). The significant efficacy of BCO at a relatively low dose of 200 mg/day observed in the present study may be attributed to its relatively high levels of thymoquinone content (5% w/w) verses the normal cold-pressed oils with less than 0.4 % (w/w/) only. Thymoquinone has already been shown to be neuroprotective (Farkhondeh et al., 2018) with significant anti-inflammatory effects (Shaterzadeh-Yazdi et al., 2018). Anti-inflammatory and neuroprotective effects of black cumin seeds and oil have also been reported separately (Yimer et al., 2019; Khazdair, 2015).

Despite the fact that the study could implement the design with a successful gathering of information related to polysomnographic analysis and correlate the results with questionnaires and blood cortisol levels, major limitations include the small sample size and lack of a double-blinded and placebo-controlled design. The small sample size can be rationalized on the basis of the fact that this is a first time reported pilot clinical study to investigate the efficacy and safety of a TQ-rich black cumin oil in sleep disorder. According to Julious et al., a minimum sample size of 12 subjects per treatment arm is considered to be effective for a pilot clinical study (Julious, 2005). Moreover, Aickin suggested that early-phase studies should use small sample sizes and do not always require a placebo group in pilot studies aiming to check the efficacy and safety (Aickin, 2007). However, further randomized, controlled studies employing a larger sample size is warranted which should be addressed in future studies.

5. Conclusion

In conclusion, BCO (black cumin oil with 5 % w/w TQ) showed significant efficacy in subjects with sleep deprivation, both in primary and secondary outcomes. Significant improvement in sleep quality with a reduction in sleep latency and enhancement in deep sleep duration with an overall reduction in sleep disturbances pointed towards the promising role of BCO in sleep-disorders. Further analysis at the end of the study period (28 days) also indicated the significant benefits of BCO as a relaxant with a significant reduction in stress and anxiety severity scores. The hematological and biochemical analysis demonstrated the primary safety of BCO and its capacity to reduce cortisol levels. The beneficial effects of BCO observed in the present study can be mainly attributed to its volatile oil composition, mainly the TQ content. However, future studies with black cumin oils containing low levels of TQ (< 0.5 % w/w) are recommended to confirm the role of TQ in the sleep inducing effect of BCO.

CRediT authorship contribution statement

Kannan R: Methodology, Formal analysis, Data Curation and Manuscript drafting; **Syam Das S:** Manuscript drafting; **Sanju George and Baby Chakrapany PS:** Review & Editing; **Sibi Ittiyavirah**: Animal Experiments-Supervision; **Balu Maliakel:** Conceptualization, Review; **Krishnakumar IM;** Conceptualization, Visualization, Review, Editing & Resource

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Ethics approval and consent to participate

The study was approved by the Institutional Ethics Committee of M/s Aman Hospital and Research Centre, Gujarat, India [Reg No: CTRI/2018/03/012,391].

Consent for publication

Not applicable

Declaration of Competing Interest

The authors declare a conflict of interest. Kannan R Kannan, Sibi Ittiyavirah, Sanju George, Baby Chakrapany PS belongs to the non-profitable, and Government funded University and Syam Das S, Balu Maliakel and Krishnakumar IM belong to the organization that produces the test substance.

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References

Ahmad, A., Husain, A., Mujeeb, M., Khan, S.A., Najmi, A.K., Siddique, N.A., Damanhouri, Z.A., Anwar, F., 2013. A review on therapeutic potential of *Nigella sativa*: a miracle herb. Asian Pac. J. Trop. Biomed. 3, 337–352. https://doi.org/10.1016/S2221-1691(13)60075-1.

Aickin, M., 2007. The importance of early phase research. J. Altern. Complement. Med. 13, 447–450. https://doi.org/10.1089/acm.2007.6370.

AOAC, 1969. Official Method 969.33 Fatty Acids in Oils and Fats Preparation of Methyl Esters Boron Trifluoride Method First Action.

Backhaus, J., Junghanns, K., Hohagen, F., 2004. Sleep disturbances are correlated with decreased morning awakening salivary cortisol. Psychoneuroendocrinology 29, 1184–1191. https://doi.org/10.1016/j.psyneuen.2004.01.010.

Bartness, T.J., Powers, J.B., Hastings, M.H., Bittman, E.L., Goldman, B.D., 1993. The timed infusion paradigm for melatonin delivery: what has it taught us about the melatonin signal, its reception, and the photoperiodic control of seasonal responses? J. Pineal Res. 15, 161–190.

Born, J., Kern, W., Bieber, K., Fehm-Wolfsdorf, G., Schiebe, M., Fehm, H.L., 1986. Night-time plasma cortisol secretion is associated with specific sleep stages. Biol. Psychiatry 21, 1415–1424.

Bradley, P., Shiekh, M., Mehra, V., Vrbicky, K., Layle, S., Olson, M.C., Maciel, A., Cullors, A., Garces, J.A., Lukowiak, A.A., 2018. Improved efficacy with targeted pharmacogenetic-guided treatment of patients with depression and anxiety: a randomized clinical trial demonstrating clinical utility. J. Psychiatr. Res. 96, 100–107. https://doi.org/10.1016/j.jpsychires.2017.09.024.

Buysse, D.J., Reynolds, C.F., Monk, T.H., Berman, S.R., Kupfer, D.J., 1989. The Pittsburgh Sleep Quality Index: a new instrument for psychiatric practice and research. Psychiatry Res. 28, 193–213.

Cajochen, C., Kräuchi, K., Wirz-Justice, A., 2003. Role of melatonin in the regulation of human circadian rhythms and sleep. J. Neuroendocrinol. 15, 432–437.

Carskadon, M.A., Dement, W.C., n.d. Chapter 2-Normal Human Sleep: An Overview.

Cole, J.C., Motivala, S.J., Buysse, D.J., Oxman, M.N., Levin, M.J., Irwin, M.R., 2006. Validation of a 3-Factor scoring model for the Pittsburgh sleep quality index in older adults. Sleep 29, 112–116. https://doi.org/10.1093/sleep/29.1.112.

Colten, H.R., Altevogt, B.M., Research, I. of M. (US) C. on S.M, 2006. Functional and Economic Impact of Sleep Loss and Sleep-Related Disorders.

Dumbell, R., Matveeva, O., Oster, H., 2016. Circadian clocks, stress, and immunity. Front. Endocrinol. (Lausanne). 7, 37. https://doi.org/10.3389/fendo.2016.00037.

Equihua-Benítez, A.C., Guzmán-Vásquez, K., Drucker-Colín, R., 2017. Understanding sleep-wake mechanisms and drug discovery. Expert Opin. Drug Discov. 12, 643–657. https://doi.org/10.1080/17460441.2017.1329818.

Farkhondeh, T., Samarghandian, S., Shahri, A.M.P., Samini, F., 2018. The neuroprotective effects of thymoquinone: a review. DoseResponse 16 (15593). https://doi.org/10.1177/1559325818761455, 2581876145.

Fernandez-Mendoza, J., Vgontzas, A.N., 2013. Insomnia and its impact on physical and mental health. Curr. Psychiatry Rep. 15, 418. https://doi.org/10.1007/s11920-013-0418-8.

Findikli, E., Kurutas, E.B., Camkurt, M.A., Karaaslan, M.F., Izci, F., Fındıklı, H.A., Kardaş, S., Dag, B., Altun, H., 2017. Increased serum g protein-coupled estrogen receptor 1 levels and its diagnostic value in drug Naïve patients with major depressive disorder. Clin. Psychopharmacol. Neurosci. 15, 337–342. https://doi.org/10.9758/cpn.2017.15.4.337.

Follenius, M., Brandenberger, G., Bandesapt, J.J., Libert, J.P., Ehrhart, J., 1992. Nocturnal cortisol release in relation to sleep structure. Sleep 15, 21–27. https://doi.org/10.1093/sleep/15.1.21.

Gilhotra, N., Dhingra, D., 2011. Thymoquinone produced antianxiety-like effects in mice through modulation of GABA and NO levels. Pharmacol. Rep. 63, 660–669.

Gottesmann, C., 2002. GABA mechanisms and sleep. Neuroscience 111, 231–239.

Guha, D., Biswas, D., Purkayastha, S., 2005. Suppression of penicillin-induced epileptiform activity by Nigella sativa: possible mediation by neurotransmitters. Biog. Amin. 19, 309–321. https://doi.org/10.1163/156939105774647367.

Gunnar, M., Quevedo, K., 2007. The neurobiology of stress and development. Annu. Rev. Psychol. 58, 145–173. https://doi.org/10.1146/annurev.psych.58.110405.085605.

Hadad, G.M., Salam, R.A.A., Soliman, R.M., Mesbah, M.K., 2012. High-Performance liquid chromatography quantification of principal antioxidants in black seed (*Nigella sativa* l.) phytopharmaceuticals. J. AOAC Int. 95, 1043–1047. https://doi.org/10.5740/jaoacint.11-207.

Hamilton, M., 1959. The assessment of anxiety states by rating. Br. J. Med. Psychol. 32, 50–55.

Hosseini, M., Zakeri, S., Khoshdast, S., Yousefian, F.T., Rastegar, M., Vafaee, F., Kahdouee, S., Ghorbani, F., Rakhshandeh, H., Kazemi, S.A., 2012. The effects of *Nigella sativa* hydro-alcoholic extract and thymoquinone on lipopolysaccharide - induced depression like behavior in rats. J. Pharm. Bioallied Sci. 4, 219–225. https://doi.org/10.4103/0975-7406.99052.

Hu, Z., Oh, S., Ha, T.-W., Hong, J.-T., Oh, K.-W., 2018. Sleep-aids derived from natural products. Biomol. Ther. (Seoul). 26, 343–349. https://doi.org/10.4062/biomolther.2018.099.

Irwin, M.R., 2015. Why sleep is important for health: a psychoneuroimmunology perspective. Annu. Rev. Psychol. 66, 143–172. https://doi.org/10.1146/annurev-psych-010213-115205.

Julious, S.A., 2005. Sample size of 12 per group rule of thumb for a pilot study. Pharm. Stat. 4, 287–291. https://doi.org/10.1002/pst.185.

Kayabekir, M., 2019. Sleep physiology and Polysomnogram, physiopathology and symptomatology in sleep medicine, in: sleep medicine in clinical neurology [Working title]. IntechOpen. https://doi.org/10.5772/intechopen.82754.

Khalid, A.K., Shedeed, M.R., 2016. GC-MS analyses of black cumin essential oil produces with sodium chloride. Int. Food Res. J. 23, 832–836.

Khazdair, M.R., 2015. The protective effects of *Nigella sativa* and its constituents on induced neurotoxicity. J. Toxicol. 2015, 1–7. https://doi.org/10.1155/2015/841823.

Koyanagi, A., Garin, N., Olaya, B., Ayuso-Mateos, J.L., Chatterji, S., Leonardi, M., Koskinen, S., Tobiasz-Adamczyk, B., Haro, J.M., 2014. Chronic conditions and sleep problems among adults aged 50 years or over in nine countries: a multi-country study. PLoS One 9, e114742. https://doi.org/10.1371/journal.pone.0114742.

Krishnakumar, I.M., Balu, P., Maliakel Sibi, P., Ittiyavirah Kannan, R., 2018. US10485837B2. Novel Composition of Nigella sativa Seeds to Treat Anxiety, Stress and Sleep Disorders With Significant Memory Enhancement Properties and a Process for Producing the Same.

Li, L., Wu, C., Gan, Y., Qu, X., Lu, Z., 2016. Insomnia and the risk of depression: a meta-analysis of prospective cohort studies. BMC Psychiatry 16. https://doi.org/10.1186/s12888-016-1075-3, 375.

Lovibond, S.H., Lovibond, P.F., 1996. Manual for the Depression Anxiety Stress Scales. Psychology Foundation of Australia.

Martin, P.A., Crump, M.H., 2003. McDonald's Veterinary Endocrinology and Reproduction, 5th ed. Iowa State Press, Ames, Iowa.

Mathias, S., Wetter, T.C., Steiger, A., Lancel, M., n.d. The GABA uptake inhibitor tiagabine promotes slow wave sleep in normal elderly subjects. Neurobiol. Aging 22, 247–253.

Melatonin: In Depth | NCCIH, n.d. URL. https://nccih.nih.gov/health/melatonin.

Mullington, J.M., Simpson, N.S., Meier-Ewert, H.K., Haack, M., 2010. Sleep loss and inflammation. Best Pract. Res. Clin. Endocrinol. Metab. 24, 775–784. https://doi.org/10.1016/j.beem.2010.08.014.

Parthasarathy, S., Carskadon, M.A., Jean-Louis, G., Owens, J., Bramoweth, A., Combs, D., Hale, L., Harrison, E., Hart, C.N., Hasler, B.P., Honaker, S.M., Hertenstein, E., Kuna, S., Kushida, C., Levenson, J.C., Murray, C., Pack, A.I., Pillai, V., Pruiksma, K., Seixas, A., Strollo, P., Thosar, S.S., Williams, N., Buysse, D., 2016. Implementation of sleep and circadian science: recommendations from the sleep research society and national institutes of health workshop. Sleep 39, 2061–2075. https://doi.org/10.5665/sleep.6300.

Pistollato, F., Sumalla Cano, S., Elio, I., Masias Vergara, M., Giampieri, F., Battino, M., 2016. Associations between sleep, cortisol regulation, and diet: possible implications for the risk of alzheimer disease. Adv. Nutr. An Int. Rev. J. 7, 679–689. https://doi.org/10.3945/an.115.011775.

Rohini, G., Kalaivani, S., Kumar, V., Rajasekar, S., Tuckaram, J., Pandey, V., 2015. Estimation and comparison of serum cortisol levels in periodontally diseased patients and periodontally healthy individuals: a clinical-biochemical study. J. Pharm. Bioallied Sci. 7, 457. https://doi.org/10.4103/0975-7406.163501.

Sateia, M.J., 2014. International classification of sleep disorders-third edition: highlights and modifications. Chest 146, 14–0970. https://doi.org/10.1378/chest, 1387–1394.

Scammell, T.E., Arrigoni, E., Lipton, J.O., 2017. Neural circuitry of wakefulness and sleep. Neuron 93, 747–765. https://doi.org/10.1016/j.neuron.2017.01.014.

Shaterzadeh-Yazdi, H., Noorbakhsh, M.-F., Hayati, F., Samarghandian, S., Farkhondeh, T., 2018. Immunomodulatory and anti-inflammatory effects of thymoquinone. Cardiovasc. Hematol. Disord. Targets 18, 52–60. https://doi.org/10.2174/1871529X18666180212114816.

Sinclair, S.J., Siefert, C.J., Slavin-Mulford, J.M., Stein, M.B., Renna, M., Blais, M.A., 2012. Psychometric evaluation and normative data for the depression, anxiety, and stress Scales-21 (DASS-21) in a nonclinical sample of U.S. Adults. Eval. Health Prof. 35, 259–279. https://doi.org/10.1177/0163278711424282.

Tavakkoli, A., Mahdian, V., Razavi, B.M., Hosseinzadeh, H., 2017. Review on clinical trials of black seed (*Nigella sativa*) and its active constituent. Thymoquinone. J. pharmacopuncture 20, 179–193. https://doi.org/10.3831/KPI.2017.20.021.

Teo, Y.C., Hj Yusuf, A., Alice Lim, W.P., Ghazali, N.B., Abd Rahman, H., Lin, N., Koh, D., 2019. Validation of DASS-21 among nursing and midwifery students in Brunei. J. Public Health (Bangkok). 27, 387–391. https://doi.org/10.1007/s10389-018-0947-z.

Toussaint, M., Luthringer, R., Schaltenbrand, N., Carelli, G., Lainey, E., Jacqmin, A., Muzet, A., Macher, J.P., 1995. First-night effect in normal subjects and psychiatric inpatients. Sleep 18 (6), 463–469. https://doi.org/10.1093/sleep/18.6.463.

Tripathi, M., 2008. Technical notes for digital polysomnography recording in sleep medicine practice. Ann. Indian Acad. Neurol. 11, 129–138. https://doi.org/10.4103/0972-2327.41887.

Tsigos, C., Chrousos, G.P., 2002. Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress. J. Psychosom. Res. 53, 865–871.

Uchiyama, M., Hamamura, M., Kuwano, T., Nagata, H., Hashimoto, T., Ogawa, A., Uchimura, N., 2011. Long-term safety and efficacy of ramelteon in Japanese patients with chronic insomnia. Sleep Med. 12, 127–133. https://doi.org/10.1016/j.sleep.2010.10.006.

Vargas, I., Vgontzas, A.N., Abelson, J.L., Faghih, R.T., Morales, K.H., Perlis, M.L., 2018. Altered ultradian cortisol rhythmicity as a potential neurobiologic substrate for chronic insomnia. Sleep Med. Rev. 41, 234–243. https://doi.org/10.1016/j.smrv.2018.03.003.

Watson, C.J., Baghdoyan, H.A., Lydic, R., 2010. Neuropharmacology of sleep and wakefulness. Sleep Med. Clin. https://doi.org/10.1016/j.jsmc.2010.08.003.
Wong, A., Lee, C., Lee, J., 2018. Agomelatine overdose and related toxicity. Toxicol. Commun. 2, 62–65. https://doi.org/10.1080/24734306.2018.1503850.
Yimer, E.M., Tuem, K.B., Karim, A., Ur-Rehman, N., Anwar, F., 2019. Review Article *Nigella sativa* L. (Black Cumin): a Promising Natural Remedy for Wide Range of Illnesses. https://doi.org/10.1155/2019/1528635.

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Research Article

Safety Assessment of a Thymoquinone-rich Black Cumin Oil (BlaQmax®): Acute and Sub-chronic Toxicity Studies

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ABSTRACT

Background: Despite the increasing usage of black cumin (*Nigella sativa*) oil (BCO) in Dietary supplements/ Nutraceuticals, systematic investigations on the role of its major bioactive component, thymoquinone (TQ) on its safety aspects have not been reported. **Method:** Herein, we investigated the safety of BCO containing 0.6 and 5% (w/w) of TQ (BCO-0.6 and BCO-5) by single-dose acute and subchronic (90-day) repeated dose toxicity studies in Wistar rats, as per OECD guidelines. **Results:** While BCO-0.6 was safe at 300 - 2000 mg/kg b. wt. upon single-dose oral acute toxicity study, LD_{50} cut off dose for BCO-5 was in the range >50-300 mg/kg b. wt. Further repeated-dose (90-day) study at 0.1, 0.05 and 0.01 mL/kg b.wt. (94, 47, 9.4 mg/kg b.wt) of BCO-5 (i.e., at 5, 2.5 and 1 mg/kg b. wt. of TQ) established its no-observed-adverse-effect-level (NOAEL) as 0.1 mL/kg or 94 mg/kg b. wt. (5 mg of TQ/kg b. wt.). **Conclusion:** Black cumin oil containing 5% (w/w) of TQ content was found to have a NOAEL of 0.1 mL/kg or 94 mg/kg b. wt. in rodents; which also corresponds to a dose of 5 mg of TQ/kg b. wt. From this study, the safe human dosage may be derived as not more than 900 mg/kg b. wt. of BCO-5/day or 50 mg of TQ/adult/day.

Keywords: Black seed; Black cumin oil; Dietary supplement; *Nigella Sativa*; Nutraceutical; Repeated-dose; Safety; Thymoquinone; Toxicity

INTRODUCTION

Nigella sativa, commonly known as 'black cumin' or 'black seed' has received a recent interest owing to its health beneficial pharmacological activities as evident from the preclinical and clinical studies. In the field of Nutraceuticals, black cumin oil (BCO) is emerging as a miracle herbal agent for health and wellness. According to the American Botanical Council's herbal supplement market research, the sales of black cumin containing products in the USA increased by 200% in 2017 [1]. Black cumin is mainly growing in the Mediterranean region, Middle East, India and Pakistan [2,3]. The plant is mainly cultivated for its seed, and is an approved food flavouring agent and kitchen spice in USA and Europe (FDA 182.20; EFSA 8014-13-9). Seeds and oil of black cumin have been established to possess medicinal effects [2-5]. In the traditional systems of medicine such as Ayurveda and Unani, it is widely using in the treatment of various diseases [5]. Black cumin is known as the 'seed of blessings'. In the Arab systems of medicine, it is also regarded as a 'cure' for all forms of diseases [2-5].

Black cumin and its oil have been extensively researched for their therapeutic potential and has been shown to possess broad range of pharmacological activities, including antioxidant, anti-inflammatory, antimicrobial, hepatoprotective, anticancer, immunomodulatory, diuretic, antihypertensive, antidiabetic, anthelmintic, gastroprotective, analgesic, spasmolytic, bronchodilator and skin protective effects [6]. Several studies on black cumin and its oil have suggested that the biological activity of BCO could be specifically due to the essential oil components, particularly the thymoquinone (TQ). Several other active compounds, including p-cymene (7-15%), carvacrol (6-12%), 4-terpineol (2-7%), t-anethole (1-4%) and longifene (1-8%) have also been identified as volatile constituents of BCO in addition to the fatty acids such as linoleic acid, oleic acid, palmitic acid and linolenic acid [7].

Several clinical trials of black cumin oil at different dosage ranging from 1 to 12 mL/day have been reported [8]. While most of these studies have not provided an estimate of TQ levels in the oil used in the studies, it has been found that higher oil doses (5 to 10 mL/day) is generally required to provide significant health benefits, suggesting the need for a higher dose of TQ per day. Thus, black cumin oil with higher levels of TQ has recently been widely using as Dietary supplements and Nutraceuticals. However, most of the currently available supplements do not have standardised TQ levels on the label. Furthermore, no systematic studies have been performed on the role TQ content on the safety of these oils. Al-Ali et al. have reported the oral LD_{50} of synthetic thymoquinone

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as 794.3 mg/kg b. wt. in mice; however no details of this study are available in the public domain, except an abstract [9]. Dollah et al. stated that the supplementation of black cumin seed powder at 1 g/kg b. wt. did not produce major changes in liver and kidney function markers [10]. Yet another study by Zaoui et al. reported the oral LD_{50} of the fatty oil of black cumin as 28 mL/kg b. wt., with no indication of the TQ content in the tested oil [11].

The present study, therefore, attempted to investigate the safety of standardised black cumin oils containing varying TQ levels [0.6 and 5% (w/w)] prepared by a proprietary process of cold pressed extraction technique. In the typical protocol, oils containing varying levels of TQ were first subjected to single-dose acute toxicity study (14 days) to select their safe dose. The selected dosage of highest TQ containing oil was then employed for subchronic (90-day) repeated oral toxicity study to establish the 'no-observed-adverse-effect-level' (NOAEL) for future clinical interventions.

MATERIALS AND METHODS

Acquisition of materials

Black cumin oil containing 0.6% (BCO-0.6) and 5% TQ (BCO-5) were prepared by a proprietary process of cold-pressed extraction from Indian black seeds and were provided by M/s Akay Natural Ingredients, Cochin, India. Thymoquinone (TQ) content was measured by high-performance liquid chromatography (HPLC) method employing a photodiode array (PDA) detector at 254 nm [12].

Experimental animals

Colony inbred strains of adult Wistar rats of both sex weighing 200 to 250 g were purchased from M/s Nagarjuna Herbal Concentrates Limited, Kerala, India. The animals were housed at the animal house facility of Department of Pharmaceutical Sciences, Mahatma Gandhi University, Kottayam, India in properly ventilated polypropylene cages under controlled temperature (22 to 25°C), relative humidity (60 to 70%) and the light-dark cycle of 12 h. Standard rat feed and water were given ad libitum. One week before testing, the animals were acclimatised to the laboratory conditions and the study was performed as per the protocol accepted by the Institutional Animal Ethics Committee (IAEC). Approval No: MGU/DPS/IAEC/ 2016/PD-5.

Toxicity studies

Schematic representation of the protocol used in the present toxicity studies is depicted in Figure 1. The study was based on the guidelines set by the Organization of Economic Co-operation and Development (OECD). OECD 423 guidelines are employed for single dose acute and OECD 408 protocol was employed for subchronic (90-day) repeated dose toxicity studies [13,14].

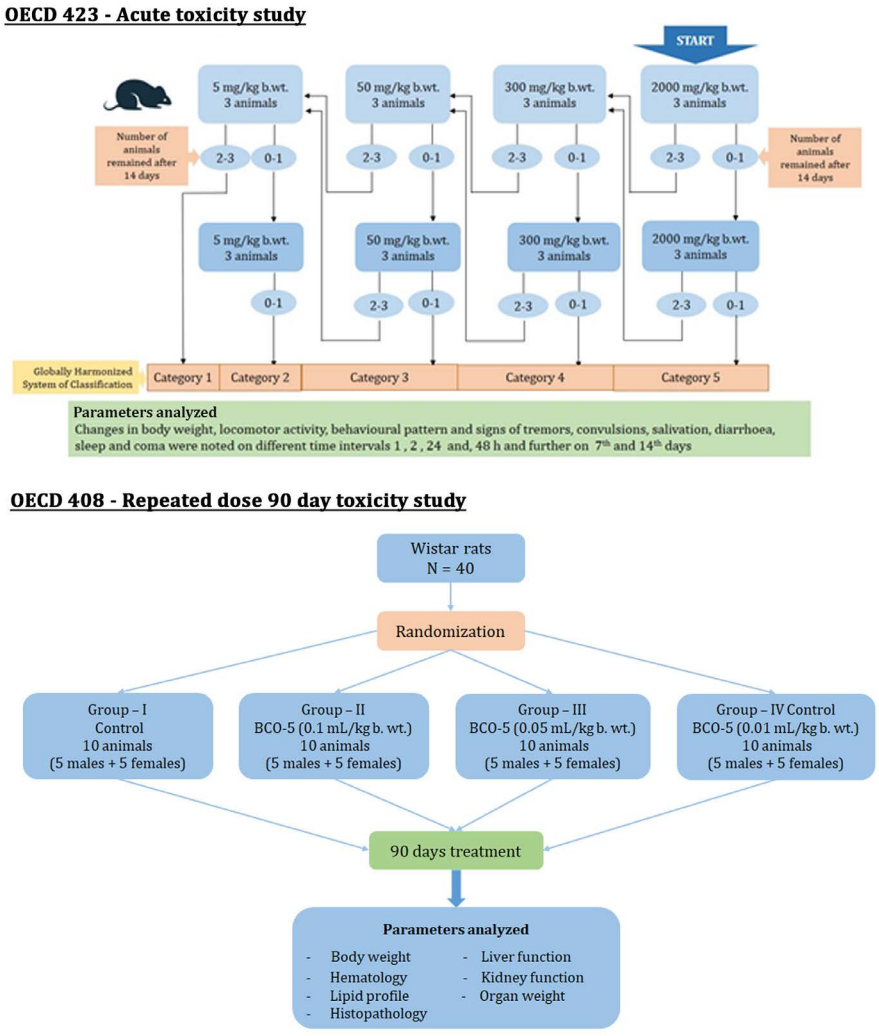


Figure 1: Schematic representation of protocols used in the study.

Acute toxicity study: The method involved a stepwise procedure with 3 animals of single-sex per group with defined doses of 5, 50, 300 and 2000 mg/kg of b. wt. [13]. Adult female Wistar rats weighing 200 to 250 g were used in the present study with a starting dose of 2000 mg/kg b. wt. Changes in body weight, locomotor activity, behavioural pattern and signs of tremors, convulsions, salivation, diarrhoea, sleep, and coma were noted on different time intervals 1, 2, 24 and, 48 h and further on 7th and 14th days. Any signs of adverse effects or toxicity were also noted during the same period.

Subchronic (90-day) repeated dose toxicity study: In the typical protocol, Wistar rats were randomised into three groups containing 5 males and 5 females per each group. Experimental groups G II, G III and G IV were fed with BCO-5 at three dose levels as shown in Table 1 and GI-the control group- was provided with the vehicle. The dosage of the drug was based on the outcomes of acute toxicity trials, and the quantity of drugs administered was decided on the basis of their body weight. Drugs were administered p.o. at the prescribed dose once daily using an oral catheter and continued for 90 days. During this period, the rats were monitored for any adverse reactions and mortality. Body weight and the intake of food/water were recorded. Blood samples were obtained for biochemical experiments on day 91 and the animals were sacrificed under anaesthesia by cervical dislocation. The organs were examined visibly for any type of abnormalities and were excised and weighed. The vital organs like liver, kidney, brain, and spleen were stored in formalin solution for histopathological studies.

Table 1. Dose levels of BCO-5 on animal groups.

Groups	Dose of oil in mL/kg b.wt.	Dose of oil in mg/kg b.wt.	Dose based on Thymoquinone content (mg/kg b.wt.)
Group I	Control	Control	Control
Group II	0.1	94	5
Group III	0.05	47	2.5
Group IV	0.01	9.4	0.5

Note: The dose in mg/kg was calculated based on the density of black cumin oil containing 5% thymoquinone.

Blood biochemistry

The blood was collected by retro-orbital puncture method and was stored in EDTA and non-EDTA vials for assaying haematological parameters and serum biochemistry. Investigated haematological parameters included Haemoglobin, Red blood cell (RBC) count, White blood cell (WBC) count and Platelet, and the serum biochemical parameters included Serum glutamic oxaloacetic transaminase (SGOT), Serum glutamic pyruvic transaminase (SGPT), Alkaline phosphatase (ALP), Albumin, Globulins, Total Protein, Total Bilirubin, Serum Urea, Creatinine, Cholesterol, High-density lipoproteins (HDL), Triglycerides, Low-density lipoproteins (LDL), Very low-density lipoprotein (VLDL) and Serum electrolytes. All the above analysis was conducted as per the standard methods [15].

Histopathology

The animals from the control and higher dose group were sacrificed by cervical dislocation under anaesthesia followed by decapitation and the organs like liver, kidney, spleen and brain were carefully removed. The collected samples were washed with ice-cold normal saline and fixed in 10% formal saline (10 mL of formaldehyde in 90 mL of physiological saline). Paraffin-embedded sections were taken (100-µm thickness), processed in alcohol-xylene series, and stained with Haematoxylin-Eosin (H&E) dye. The sections were examined microscopically for histopathological changes.

RESULTS

Single-dose toxicity

During the study period of 14 days, single-dose oral administration of BCO-0.6 at 2000 mg/kg b. wt. did not cause mortality or any adverse reactions. However, BCO-5 at the same dose caused a reduction in body weight. As per the OECD 423 guidelines, the study was then repeated with a starting dose of 300 mg/kg b. wt. of BCO-5. At this dose, the animals showed neither mortality nor changes in body weight, or any clinical signs of toxicity (Table 2). Repetition of the experiment at the same dose of BCO-5 provided similar results. Thus, LD_{50} cut off mg/kg b. wt. dose for BCO-5 was categorized as Category 3 (>50-300 mg/kg b. wt.), as per OECD.

Table 2: Observations for single-dose toxicity study (OECD 423) of BCO-5 at 300 mg/kg b.wt.

Observations (time)	1 h	2 h	24 h	48 h	Day 7	Day 14
Skin and Fur	N	N	N	N	N	N
Eyes	N	N	N	N	N	N
Mucous membrane	N	N	N	N	N	N
Salivation	N	N	N	N	N	N
Lethargy	2/3	2/3	2/3	N	N	N
Sleep	N	N	N	N	N	N
Coma	Nil	Nil	Nil	Nil	Nil	Nil
Convulsions	Nil	Nil	Nil	Nil	Nil	Nil
Tremors	Nil	Nil	Nil	Nil	Nil	Nil
Diarrhoea	Nil	Nil	Nil	Nil	Nil	Nil
Morbidity	Nil	Nil	Nil	Nil	Nil	Nil
Mortality	Nil	Nil	Nil	Nil	Nil	Nil

Note: N-Normal, 2/3, 1/3 -Observation ratio.

Subchronic (90-day) repeated dose toxicity study

Administration of BCO-5 at 0.1, 0.05 and 0.01 mL/kg b. wt. (i.e., at 5, 2.5 and 0.5 mg of TQ/kg b. wt.) for 90 days showed no significant ($P>0.05$) decrease in the body weight or in the food and water consumption among both adult male and female Wistar rats (Figure 2). Similarly, none of the animals produced mortality or major adverse events and clinical signs of toxicity. Necropsy revealed no morphological changes or any gross pathological anomalies in the organs. The organ weight such as liver, kidney,

spleen, and brain was also remained normal during the study period (Table 3). The assessment of haematological parameters did not show any significant variations (Table 4). Biochemical parameters of liver function, including the marker enzymes, bilirubin, total protein, albumin, and globulin were found unchanged during the study period (ALP levels showed significant change, but was within the safe limit) (Figure 3). In renal function tests, a dose-dependent change in urea levels was noted within the safe limit (Figure 4). However, no change in the creatinine levels was observed (Figure 4). The examination of lipid profile also showed no changes for total cholesterol and HDL, but a decreasing trend for LDL and VLDL (Figure 5). The triglyceride levels were found to be significantly decreased (Figure 5). The histopathological examination of liver, kidney, brain and spleen also showed no specific abnormal patterns (Figure 6), indicating the safety of BCO-5 at the optimized doses of up to 0.1 mL/kg (94 mg/kg b. wt.) or up to 5 mg/kg b. wt. of TQ, for long-term supplementation.

DISCUSSION

It has been shown that black cumin oil commonly produced by cold-pressing or supercritical extraction methods possess a wide range of pharmacological effects and therapeutic potential [6,16]. Many Nutraceuticals/Dietary supplements of black cumin oil produced by cold-pressing techniques and supercritical extraction are already available in the global marketplace. Though dosage ranging from 0.5 to 6 mL/day of black cumin oil was usually suggested by the nutraceutical companies, many of them are not standardized and no information is available on its TQ content. Moreover, no correlation between the TQ content and safety of black cumin oil has been reported to date. Therefore, the objective of the present study was to investigate the safety of black cumin oil as a function of TQ content.

International organizations like OECD and ICH have postulated several guidelines for the safety assessment of drugs and chemicals of both natural and chemical origin. Primarily, OECD 423

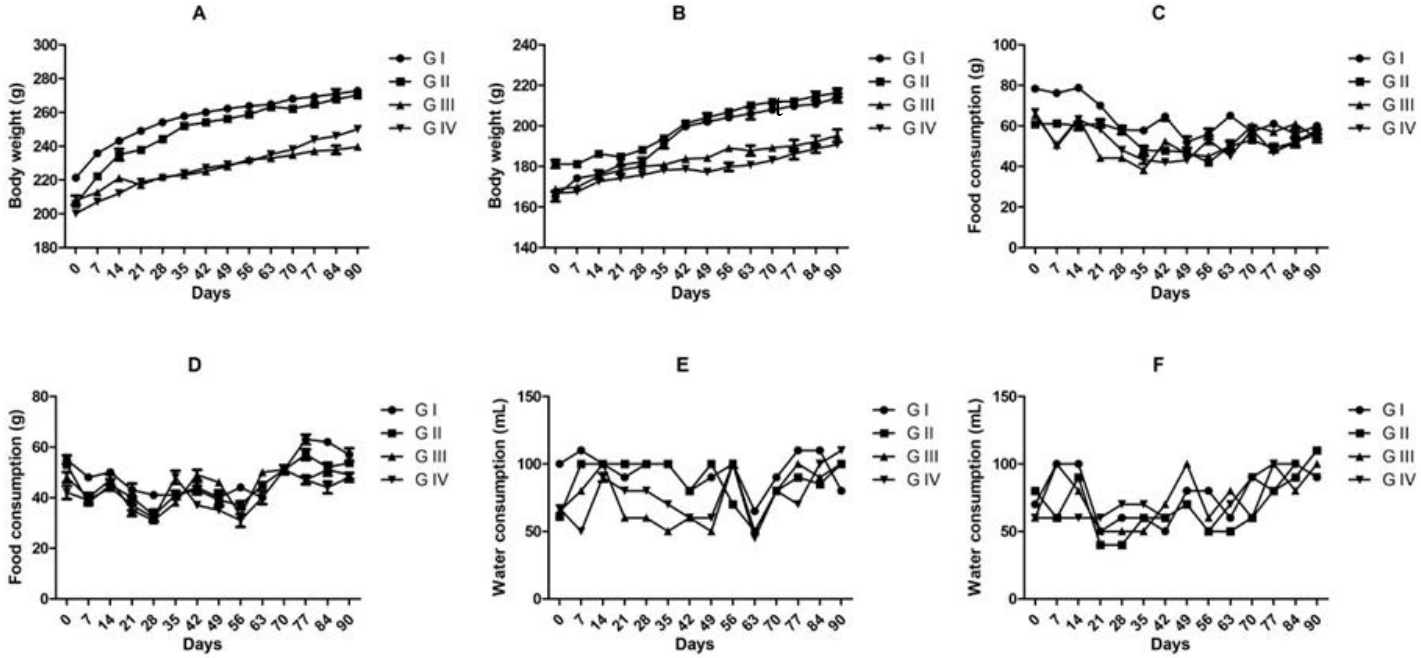
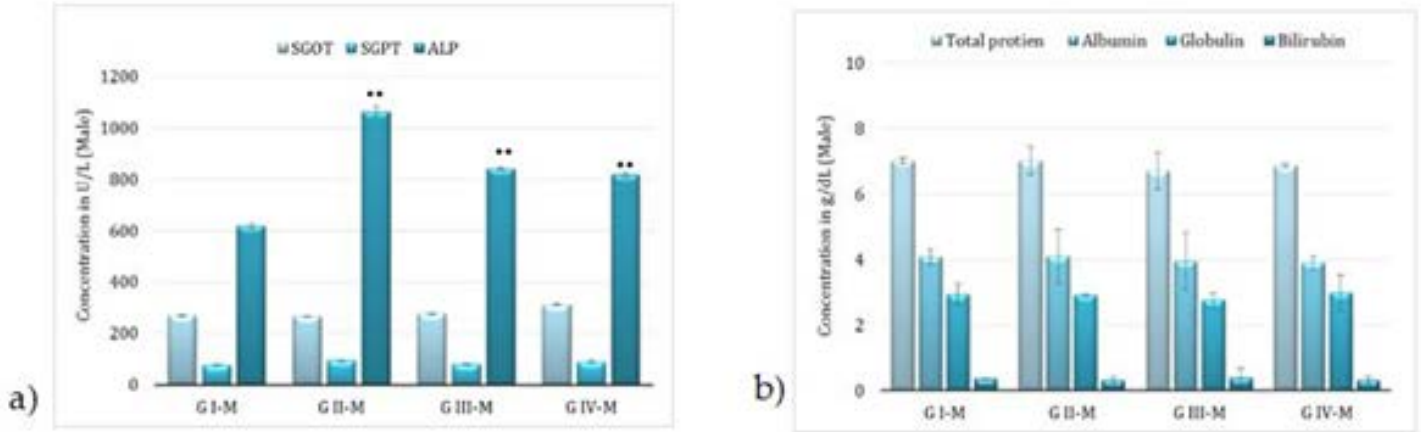


Figure 2: Body weight, food, and wate r consumption on 90-days toxicity studies of BCO-5. (A) Average body weight of male rats (n=5), (B) Average body weight of female rats (n=5), (C) Average daily food consumption of male rats (n=5), (D) Average daily food consumption of female rats (n=5), (E) Average daily water consumption of male rats (n=5), (F) Average daily water consumption of female rats (n=5).



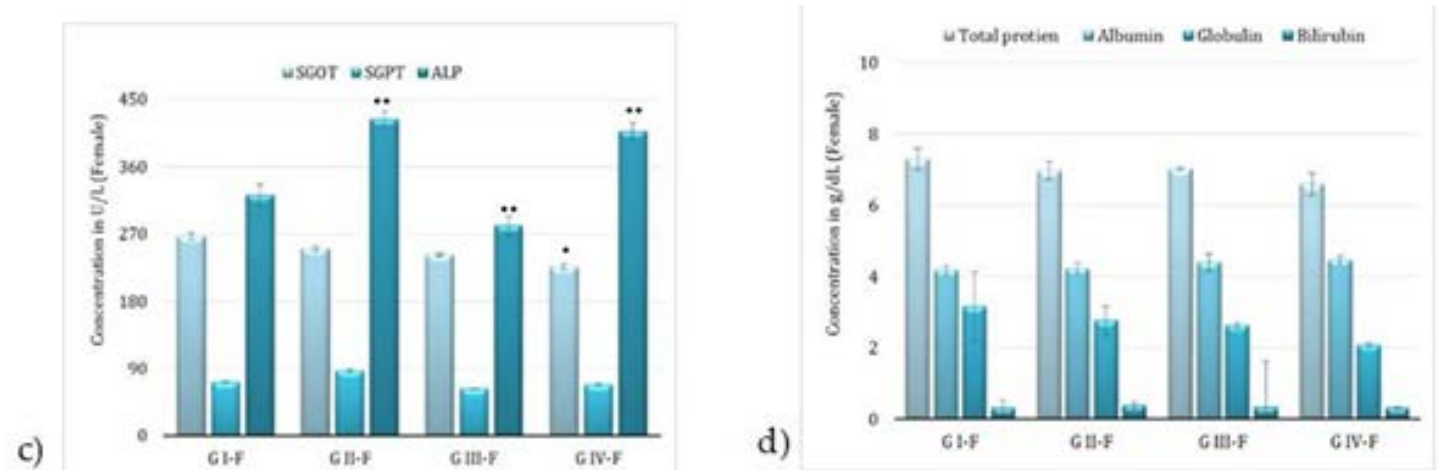


Figure 3: Liver function markers of animals, following 90-days of toxicity studies of BCO-5 – (a) & (b)–male rats and (c) & (d) – female rats (Mean ± SD, n=5, *- P<0.05, **-P<0.001).

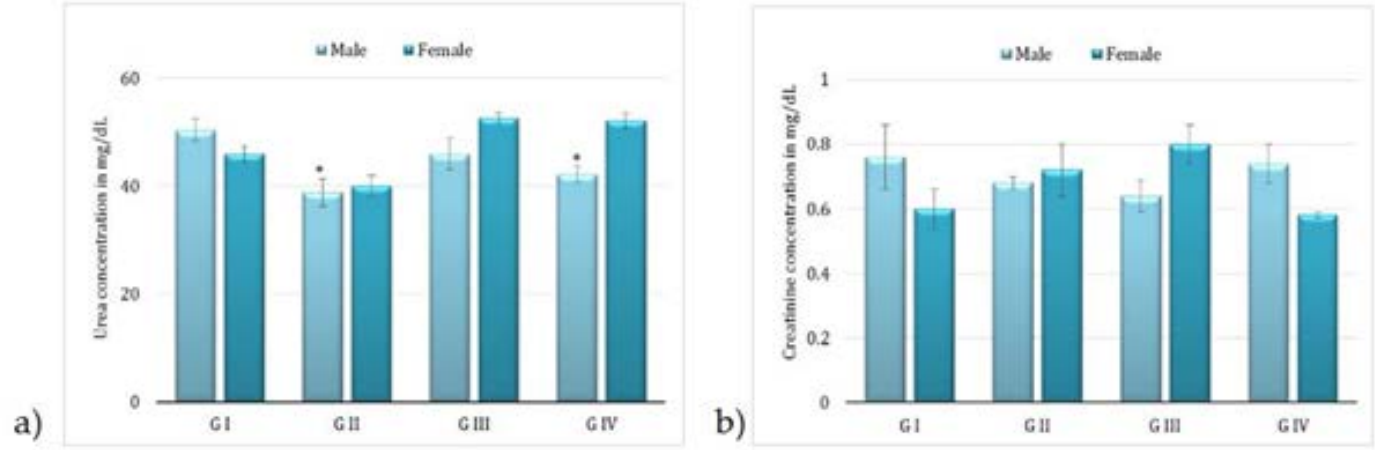


Figure 4: Kidney function tests following 90-days studies of BCO-5. a) Serum urea and b) serum creatinine (Mean ± SD, n=5, * - P<0.01).

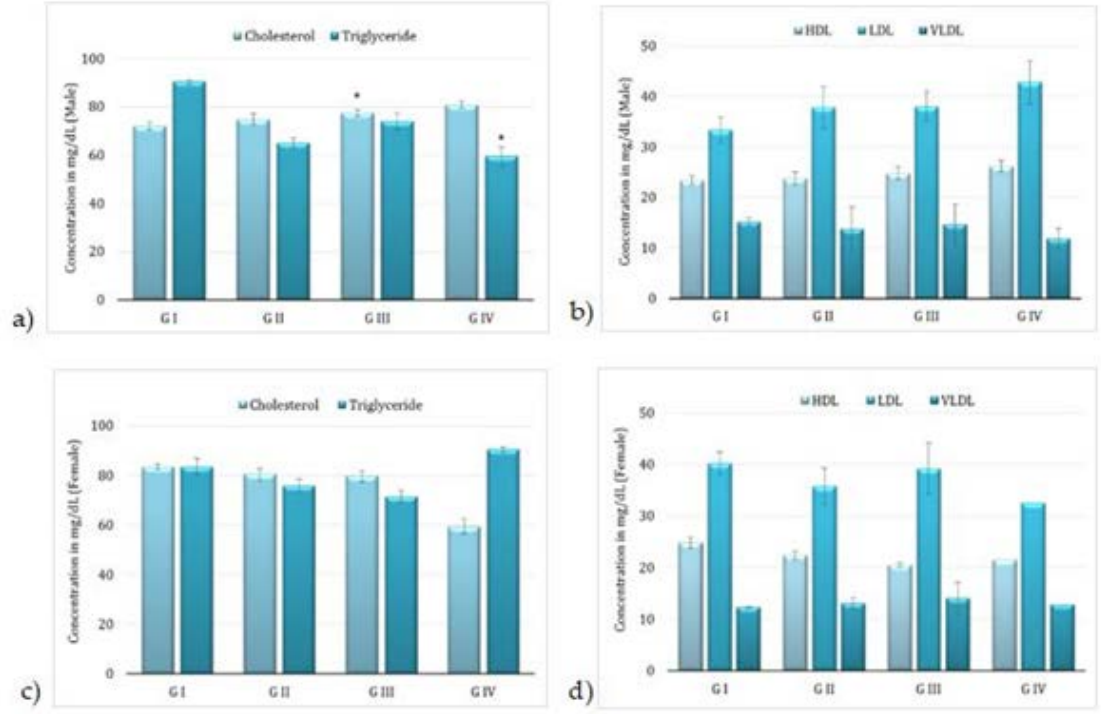


Figure 5: Lipid profile of following 90-days of toxicity studies of BCO-5 – (a) & (b)–male rats and (c) & (d)–female rats (Mean ± SD, n=5, *-P<0.001) Cholesterol (mg/dL), Triglyceride (mg/dL), HDL (mg/dL), LDL (mg/dL) and VLDL (mg/dL)

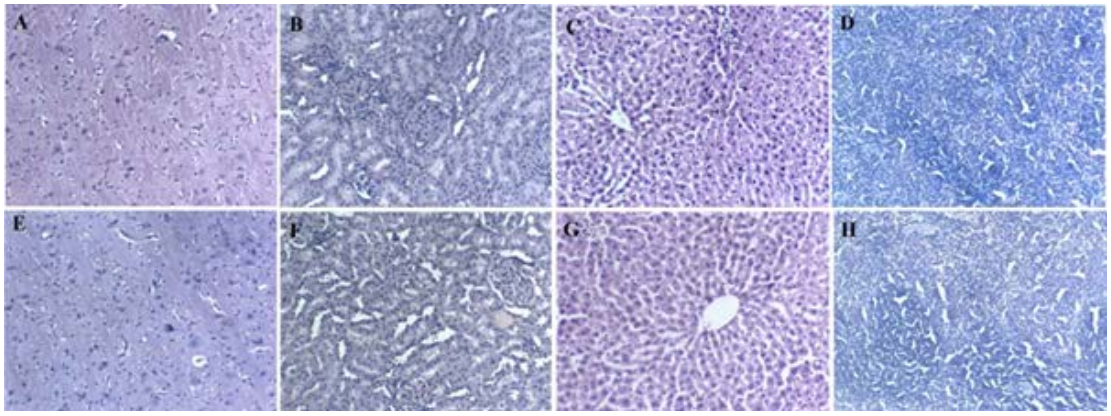


Figure 6: Histopathological results of 90 days of repeated-dose toxicity study (0.1 mL/kg) of BCO-5 by H&E staining. (A) Brain control (B) Kidney control (C) Liver control (D) Spleen control (E) Brain treated (F) Kidney treated (G) Liver treated (H) Spleen treated.

Table 3: Organ weight in g of male and female rats following 90-days repeated dose toxicity study of BCO-5.

-	Group I	Group II	Group III	Group IV
Male				
Liver	3.08 ± 0.20	3.63 ± 0.37ns	3.38 ± 0.21ns	3.36 ± 0.25ns
Spleen	0.32 ± 0.18	0.39 ± 0.22ns	0.32 ± 0.01ns	0.33 ± 0.08ns
Brain	0.58 ± 0.05	0.64 ± 0.17ns	0.64 ± 0.01ns	0.68 ± 0.09ns
Kidney	0.76 ± 0.08	0.79 ± 0.07ns	0.65 ± 0.13ns	0.68 ± 0.01ns
Female				
Liver	3.31 ± 0.24	3.78 ± 0.42ns	3.49 ± 0.37ns	3.27 ± 0.38ns
Spleen	0.34 ± 0.04	0.38 ± 0.06ns	0.36 ± 0.17ns	0.35 ± 0.32ns
Brain	0.75 ± 0.16	0.78 ± 0.05ns	0.75 ± 0.10ns	0.74 ± 0.13ns
Kidney	0.77 ± 0.12	0.80 ± 0.05ns	0.68 ± 0.05ns	0.66 ± 0.06ns

Note: Mean ± SD, n=5, ns-non-significant

Table 4: Haematological parameters of animals, following 90-day repeated dose toxicity study of BCO-5

-	Group I	Group II	Group III	Group IV
Male groups				
Hb (g/dL)	15.7 ± 0.51	15.08 ± 1.34^{ns}	15.08 ± 1.34^{ns}	14.78 ± 1.02^{ns}
RBC (10^6/cmm)	6.38 ± 0.02	6.14 ± 0.03^{ns}	6.16 ± 0.41^{ns}	5.96 ± 0.25^{ns}
Platelet (10^5/cmm)	4.32 ± 0.34	4.86 ± 0.13^{ns}	4.30 ± 0.14^{ns}	4.74 ± 0.18^{ns}
Total count	5400 ± 378.00	5260 ± 1003.30^{ns}	5220 ± 1003.30.30^{ns}	5660 ± 851.46^{ns}
Lymphocyte (%)	53 ± 6.04	57.0 ± 6.18^{ns}	57.0 ± 6.18^{ns}	54.6 ± 3.21^{ns}
Eosinophils (%)	8.0 ± 2.03	5.8 ± 1.02^{ns}	6.2 ± 2.06^{ns}	6.4 ± 2.85^{ns}
Female groups				
Hb (g/dL)	13.6 ± 0.24	14.34 ± 0.12^{ns}	13.94 ± 0.32^{ns}	14.20 ± 0.21^{ns}
RBC (10^6/cmm)	5.62 ± 0.21	5.86 ± 0.10^{ns}	5.68 ± 0.01^{ns}	5.80 ± 0.04^{ns}
Platelet (10^5/cmm)	4.54 ± 0.06	4.18 ± 0.22^{ns}	4.72 ± 0.42^{ns}	4.32 ± 0.09^{ns}
Total count	6400 ± 752.86	6500 ± 957.49^{ns}	7900 ± 724.44**	6540 ± 237.95^{ns}
Lymphocyte (%)	56.6 ± 2.52	59.0 ± 1.23^{ns}	56.01 ± 4.16^{ns}	58.2 ± 0.49^{ns}
Eosinophils (%)	5.4 ± 4.07	6.2 ± 2.54^{ns}	6.0 ± 0.09^{ns}	5.4 ± 1.53^{ns}

Note: Mean ± SD, N=5, ns-non-significant, **P<0.01

guidelines are most used for preliminary testing of drugs, which is a single-dose study. In the present study, black cumin oil with 0.6 and 5% TQ content (BCO-0.6 and BCO-5) were screened at various doses as per OECD guidelines, and found that both BCO-0.6 and BCO-5 are safe and produced no adverse events or mortality at 300-2000 mg/kg b. wt. of oil. However, BCO-5 was found to cause a significant body weight loss. Therefore, further studies at >50-300 mg/kg b. wt. dosage were carried out with BCO-5 and was found to be extremely safe with no abnormalities. In other words, the preliminary test as per OECD 423 showed a dependency of safety on TQ content of BCO in rodents. Previous acute toxicity studies on TQ also had reported a dose-dependent decrease in GSH levels, hypoactivity, respiratory difficulty and even mortality [17].

Subchronic (90-day) repeated dose study was then performed as per OECD 408 guidelines. At dosage of 0.1, 0.05 and 0.01 mL of BCO-5 per kg b. wt., none of the animals showed any specific change in any of the tested parameters including body weight, food/water consumption, haematology, biochemical, organ weight or histopathology, indicating its safety at the optimised dosage of 0.1 mL/kg b. wt. in rats. In terms of milligram weight of oil, the tested dosage is equivalent to 94, 47 and 9.4 mg of oil per kg b. wt. In terms of TQ content, the dosage can be considered as 5, 2.5 and 0.5 mg of TQ per kg b. wt. of animals (Table 1). Since 0.1 mL/kg b. wt. of BCO-5 corresponds to 5 mg/kg b. wt. of TQ, the corresponding safest human dose for clinical interventions or supplementation BCO-5 can be considered at 0.81 mg/kg b. wt./day of TQ according to the animal dose to human dose conversion [18]. In other words, the safest human dose of BCO-5 for a 60 Kg person may be considered as not more than 900 mg/day.

CONCLUSION

In summary, the present study demonstrated that the TQ content has a direct influence on the safety of black cumin oil and therefore recommends the standardisation and labelling of TQ content in nutraceuticals containing black cumin oil. While BCO with 0.6 % (w/w) of TQ content (BCO-0.6) was safe at 300-2000 mg/kg b. wt. upon single-dose acute toxicity study, the LD_{50} cut off dosage for BCO with 5% (w/w) TQ (BCO-5) was found to be in the Category 3 range (>50-300 mg/kg b. wt.) as per OECD guidelines. Further, repeated dose oral toxicity study on BCO-5 established its NOAEL as 0.1 mL/kg b. wt. or 94 mg/kg b. wt. In terms of TQ content, the safe dosage can be deduced as 5 mg of TQ per kg b. wt. in rodents. Upon conversion to human dosage, the safe dosage may be a maximum of 900 mg of BCO-5/adult/day or 48.6 mg of TQ/adult/day.

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CONFLICT OF INTEREST

The author(s) declared the following conflicts of interest concerning the authorship and/or publication of the article. KR, SI & RK belongs to the non-profitable institution and has no conflict of interest. SDS & KIM are employees at Akay who manufactured BCO.

CREDIT AUTHOR STATEMENT

Kannan Ramalingam: Investigation, Data curation and Writing-original draft preparation. Ramadassan Kuttan: Reviewing and Editing of Manuscript. Syam Das S: Data curation, Writing-Original draft preparation. Krishnakumar IM: Conceptualization, Methodology and Reviewing. Sibi Ittiyavirah: Conceptualization, Methodology, Supervision and Writing- Reviewing and Editing.

REFERENCES

1. Tyler S, Kimberly K, Veronica E, Claire M, Ryan S. Herbal Gram: Herbal Supplement Sales in US Increased 8.5% in 2017, Topping $ 8 Billion. Herb gram J Am Bot Counc. 2018;119:62-71.
2. Khan MA, Chen HC, Tania M, Zhang DZ. Anticancer activities of *Nigella sativa* (Black Cumin). African J Tradit Complement Altern Med. 2011;8(5 SUPPL):226-232.
3. Leong X, Mustafa MR, Jaarin K. *Nigella sativa* and Its Protective Role in Oxidative Stress and Hypertension. Evidence-Based Complement Altern Med. 2013;pp:1-9.
4. Bakathir HA, Abbas NA. Detection of the antibacterial effect of *Nigella sativa* ground seeds with water. Afr J Tradit Complement Altern Med. 2011;8(2):159-164.
5. Ahmad A, Husain A, Mujeeb M, Khan SA, Najmi AK, Siddique NA, et al. A review on therapeutic potential of *Nigella sativa*: A miracle herb. Asian Pac J Trop Biomed. 2013;3(5):337-352.
6. Randhawa MA, Alghamdi MS. Anticancer Activity of *Nigella sativa* (Black Seed)-A Review. Am J Chin Med. 2011;39(6):1075-1091.
7. Salim LZA, Mohan S, Othman R, Abdelwahab SI, Kamalidehghan B, Sheikh BY, et al. Thymoquinone induces mitochondria-mediated apoptosis in acute lymphoblastic leukaemia in vitro. Molec. 2013;18(9):11219-11240.
8. Tavakkoli A, Mahdian V, Razavi BM, Hosseinzadeh H. Review on clinical trials of black seed (*Nigella sativa*) and Its Active Constituent, Thymoquinone. J Pharmacopunct. 2017;20(3):179-193.
9. Amein Al-Ali, Abdul AA, Mohammad A R, Nisar AS. Oral and intraperitoneal LD_{50} of thymoquinone, an active principle of *Nigella sativa*, in mice and rats. J Ayub Med Coll. 2008;20(2):25-27.
10. Dollah MA, Parhizkar S, Latiff LA, Hassan MH. Toxicity effect of *Nigella sativa* on the liver function of rats. Adv Pharm Bull. 2013;3(1):97-102.
11. Zaoui A, Cherrah Y, Mahassini N, Alaoui K, Amarouch H, Hassar M. Acute and chronic toxicity of *Nigella sativa* fixed oil. Phytomed. 2002;9(1):69-74.
12. Hadad GM, Salam RAA, Soliman RM, Mesbah MK. High-Performance liquid chromatography quantification of principal antioxidants in black seed (*Nigella sativa* L.) phytopharmaceuticals. J AOAC Int. 2012;95(4):1043-1047.
13. Test No. 423: Acute Oral toxicity-Acute Toxic Class Method. OECD Publishing; 2002. (OECD Guidelines for the Testing of Chemicals, Section 4).
14. Test No. 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents. OECD Publishing; 1998. (OECD Guidelines for the Testing of Chemicals, Section 4).
15. Olaoye O, Onilude A, Idowu O. Quality characteristics of bread produced from composite flours of wheat, plantain and soybeans. Afric J Biotech. 2006;5(11).
16. Singh S, Das SS, Singh G, Schuff C, De-Lampasona MP, Catalán CAN. Composition, in vitro antioxidant and antimicrobial activities of essential oil and oleoresins obtained from black cumin seeds (*Nigella sativa* L.). Biomed Res Int. 2014;pp:918209.

17. Badary OA, Al-Shabanah OA, Nagi MN, Al-Bekairi AM, Elmazar MMA. Acute and subchronic toxicity of thymoquinone in mice. Drug Dev Res. 1998;44(2-3):56-61.

18. Nair AB, Jacob S. A simple practice guide for dose conversion between animals and human. J Basic Clin Pharm. 2016;7(2):27-31.

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ACETYLCHOLINESTERASE AND GROWTH INHIBITORY EFFECTS - VARIOUS GRADES OF *N. SATIVA* OILS

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Keywords:

Acetylcholinesterase, Sulforhodamine B assay, Black cumin oil, Neuroprotective

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ABSTRACT: Neuroprotection is utmost necessary due to the devastating neuronal injuries, including neurodegenerative disorders. *N. sativa* L. is called the Miracle herb due to its wide usage in traditional systems of medicine, especially Arabic. This plant has been studied for its therapeutic potential and found to possess a wide spectrum of activities, including anticancer and antioxidant properties. The present work is designed to investigate the acetylcholinesterase inhibitory and cytotoxic potential of various grades of black cumin oil for its neuroprotective activity. Thymoquinone is the most pharmacologically active ingredient found in black cumin oil. The various grades of black cumin containing 5%, 2% and 0.6% thymoquinone at different dose levels is used throughout this work. Acetylcholinesterase inhibitory is performed by using Ellman's method, and the cytotoxic potential is evaluated by using Sulforhodamine B assay employing IMR-32, U373-MG, and SK-N-SH cell lines. Black cumin oil containing 5% thymoquinone showed a dose-dependent increase in the acetylcholinesterase inhibition (64.47% inhibition at the dose of 20 µg/ml) which is similar to that of the standard acetylcholinesterase inhibitor donepezil (65.03% inhibition at the dose of 10 µg/ml). The cytotoxic potential evaluated by SRB assay indicated the black cumin oil containing 5% thymoquinone exhibiting the comparable growth inhibition with that of Adriamycin. Whereas, other grades of black cumin oil showed limited or no activity. It is concluded that *N. sativa* oil exhibits a neuroprotective activity in both the assays in a dose-dependent manner which indicates the variable effects of the concentration of thymoquinone present in the black cumin oil.

INTRODUCTION: Neuroprotection refers to the strategies and relative mechanisms which can defend the central nervous system (CNS) against neuronal injury due to both acute (*e.g.*, stroke or trauma) and chronic neurodegenerative disorders (*e.g.* Alzheimer's disease and Parkinson's disease) [1].

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Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by impairment of memory and cognitive function. Initially, mild cognitive impairment and deficits in short-term and spatial memory appear, but the symptoms become more severe with disease progression [2]. In traditional practices of medicine, plants have been used to enhance cognitive function and to alleviate other symptoms associated with AD [3].

Nigella sativa L. (Black cumin) is an annual herb belonging to the Ranunculaceae family. It is naturally distributed in countries bordering the

Mediterranean Sea and India. The black cumin seeds and the oil are being widely used over centuries for the treatment of various ailments throughout the world. It is one of the important drugs in traditional Indian systems like Ayurveda and Unani. Many studies on *N. sativa* suggest that its biological activity is attributed specifically to the components in the essential oil. So far, many active compounds have been identified and isolated from black cumin seeds. The major active constituents present in black cumin seeds are thymoquinone (30-48%), p-cymene (7-15%), carvacrol (6-12%), 4-terpineol (2-7%), t-anethole (1-4%), sesquiterpene longifene (1-8%) to name a few [4, 5].

Thymoquinone (TQ) which is chemically called as 2-isopropyl -5- methyl-1, 4- benzoquinone is the most pharmacologically active ingredient found in black cumin oil. It was reported that thymoquinone possessed various bioactivities, including anti-cancer and anti-oxidant properties [6]. Acute and chronic toxicity studies of black cumin oil and thymoquinone on laboratory animals were reported and stated to be safe, particularly when administered orally. In black cumin oil, the amount of TQ present is about 0.6%. In this study, we investigated the various grades of black cumin oil for its Acetylcholinesterase activity and also its cytotoxic potential by employing *in-vitro* methods.

MATERIALS AND METHODS:

Materials: Black cumin oils containing 2% TQ (TQT), 5% TQ (TQF) and normal black cumin oil containing 0.6% TQ (TQN) was obtained from Akay Flavours & Aromatics Pvt. Ltd., Kerala, India. Ellman's reagent (DTNB - 5,5'-dithiobis-(2-nitrobenzoic acid) (D8130), Acetylthiocholine iodide (01480), Acetylcholinesterase Type VI-S, (C3389) from electric eel, 200-1,000 units/mg protein were purchased from Sigma-Aldrich, Inc.

Methods:

Determination of Acetylcholinesterase Inhibitory Activity: Acetylcholinesterase (AChE) activity was determined according to the method of Ellman *et al*., (1961). Inhibition of AChE activity was measured using a UV-Spectrophotometer based on Ellman's method. The enzyme hydrolyzes the substrate Acetylthiocholine Iodide to thiocholine and acetic acid. Thiocholine is allowed to react with DTNB, and this reaction resulted in the development of a yellow color. The color intensity of the product is measured at 405 nm, and it is proportional to the enzyme activity. 500 µl of DTNB 3 mM, 100 µl of Acetylthiocholine iodide (AChI) 15 mM, 275 µl of Tris–HCl buffer 50 mM, pH 8 and 100 µl of each sample was dissolved in ethanol, water or DMSO, respectively, and were added to a 1 ml cuvette. This cuvette was used as a blank. In the reaction cuvette, 25 µl of buffer was replaced by the same volume of an enzyme solution 0.28 Uml^{-1}. The reaction was monitored for 5 min at 412 nm. The values were taken in triplicate [8, 9].

The percentage inhibition for each test solution was then calculated using the following equation:

Inhibition (%) = 1- (Absorbance of the sample /Absorbance of the blank) × 100

Evaluation of Cytotoxic Potential by Sulforhodamine B Assay: The cell lines were grown in RPMI 1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine. For present screening experiment, cells were inoculated into 96 well microtiter plates in 100 µL at plating densities as shown in the study details above, depending on the doubling time of individual cell lines. After cell inoculation, the microtiter plates were incubated at 37 °C, 5% CO_2, 95% air and 100% relative humidity for 24 h before addition of experimental drugs.

Experimental drugs were initially solubilized in dimethyl sulfoxide at 100 mg/ml and diluted to 1mg/ml using water and stored frozen before use. At the time of drug addition, an aliquot of frozen concentrates (1 mg/ml) was thawed and diluted to 100 g/ml, 200 g/ml, 400 g/ml and 800 g/ml with complete medium containing test articles. Aliquots of 10 µl of these different drug dilutions were added to the appropriate microtiter wells already containing 90 µl of the medium, resulting in the required final drug concentrations, i.*e.,* 10 g/ml, 20 g/ml, 40 g/ml, 80 g/ml.

After compound addition, plates were incubated under standard conditions for 48 h, and the assay was terminated by the addition of cold TCA. Cells were fixed in situ by the gentle addition of 50 µl of cold 30% (w/v) TCA (final concentration, 10% TCA) and incubated for 60 min at 4 °C.

The supernatant was discarded; the plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (50 µl) at 0.4% (w/v) in 1% acetic acid was added to each of the wells, and plates were incubated for 20 min at room temperature. After staining, the unbound dye was recovered, and the residual dyes were removed by washing five times with 1% acetic acid. The plates were air dried. The bound stain was subsequently eluted with 10 mM trizma base, and the absorbance was read on a plate reader at a wavelength of 540 nm with 690 nm reference wavelength.

Percent growth was calculated on a plate-by-plate basis for test wells relative to control wells. Percent growth was expressed in the ratio of average absorbance of the test well to the mean absorbance of the control wells × 100.

Using the six absorbance measurements [time zero (Tz), control growth (C), and test growth in the presence of drug at the four concentration levels (Ti)], the percentage growth was calculated at each of the drug concentration levels.

Percentage growth inhibition was calculated as:

$$[Ti/C] \times 100\%$$

A growth curve was constructed using percentage control growth versus drug concentration using the growth curve the concentration of drug causing 50% inhibition of cell growth (GI_{50}) was calculated. It was stated that the GI_{50} value of 10 or less than 10^{-6} is considered to demonstrate activity [10, 11].

RESULTS AND DISCUSSION: Acetylcholine, a neurotransmitter is essential for processing memory and learning. Pharmacological inhibitors of AChE are important in controlling diseases that involve impaired acetylcholine-mediated neurotransmission. For example, Alzheimer's disease (AD) involves selective loss of cholinergic neurons in the brain. In myasthenia gravis, auto-antibodies reduce the number of nicotinic acetylcholine receptors at the neuromuscular junction. AChE inhibition increases the synaptic concentration of acetylcholine and allows a higher occupancy rate and longer duration at its receptor – donepezil hydrochloride; an AChE inhibitor is used for the treatment of AD and other neurological disorders [12].

In our study AChE inhibitor activity of black cumin oil is estimated by using Ellman's method. The black cumin oils at the concentrations of 5%, 2% and normal black cumin oil at different doses were evaluated employing Donepezil (DPL) as the standard.

Jukic *et al*., 2007 studied the *in-vitro* AChE inhibitory potential on thymoquinone and their derivatives and reported the repressive potential of them as thymohydroquinone > carvacrol > thymoquinone > total essential oil > thymol > linalool. In the present work, we confirmed the AChE inhibitory activity of thymoquinone rich black cumin oil, which produced dose-dependent activity. The AChE inhibitory potential decreased in the following order: DPL > TQF > TQT > TQN. The inhibitory effect of the 20µg/ml concentration of TQF corresponds to the 10µg/ml concentration of DPL. Inhibitory activity of TQT was very weak and TQN even produced negative results. **Fig. 1** shows the AChE inhibitory activity of black cumin oil samples and the standard.

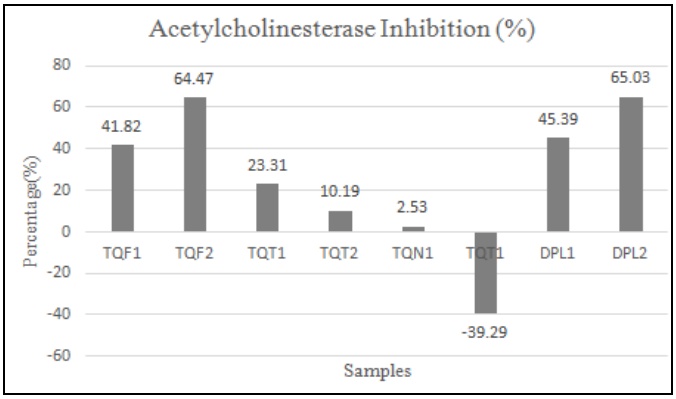

FIG. 1: AChE INHIBITORY ACTIVITY OF DIFFERENT GRADES OF BLACK CUMIN OIL USING ELLMAN'S METHOD. Where TQF1 - Black cumin oil containing 5% TQ (10µg/ml), TQF2 - Black cumin oil containing 5% TQ (20µg/ml), TQT1 - Black cumin oil containing 2% TQ (10µg/ml), TQT2 - Black cumin oil containing 2% TQ (20µg/ml), TQN1 - Black cumin oil containing 0.6% TQ (10µg/ml), TQN2 - Black cumin oil containing 0.6% TQ (20µg/ml), DPL1 – Donepezil (5µg/ml), DPL2 – Donepezil (10µg/ml).

Certain data suggest that cancer survivors have a decreased risk of Alzheimer's disease. A link between cancer and neurodegeneration is plausible as they share several genes and biological pathways, including inappropriate activation and deregulation of the cell cycle. Signaling along these pathways results in opposite endpoints: in the case

of cancer, uncontrolled cell proliferation, and in the case of neurodegeneration, apoptotic cell death. Proteins such as p53, a major regulator of apoptosis, and Pin1, which has a dual role in cell cycle control and protein folding, play a key part in the pathophysiology of both Alzheimer's disease and cancer [14].

Sulforhodamine B assay provides a rapid and sensitive method for measuring the drug-induced effects in both attached and suspension cultures. Sulforhodamine B is a bright pink aminoxanthone dye with two sulfonic groups, which bind to protein basic amino acid residues under mildly acidic conditions and the color development in the assay is rapid, stable and visible. The optical density of sulforhodamine B assay can be measured over a broad range of visible wavelengths in either a spectrophotometer or a 96-well plate reader. The major advantage of this assay over others is that this dye would not stain cell debris. Therefore, the sensitivity of sulforhodamine B assay is not affected by the presence of cell debris. So, it has been widely used for the cell growth studies [11, 15].

Three different cell lines IMR-32; U373-MG and SK-N-SH were used here in this study to evaluate the efficacy of the drug samples which contain three various concentrations of thymoquinone. The IMR-32 cell lines are a continuous hyperdiploid human neuroblastoma cell line, and this cell line is proved to be an excellent source for the isolation of human neuronal nAChR subunit cDNAs. The acetylcholine neurotransmitters play a vital role in both memory and behavior. Furthermore, it is also stated that wild-type human neuroblastoma IMR-32 cells can secrete long amyloid -protein [16, 17]. Amyloid beta acts as a neurotoxin when gets accumulated in the brain whereas the other cell line U373-MG which is human astrocytoma cell lines.

This cell line is proved to provoke inflammatory markers like IL-6 when triggered with certain inducers, and it is as well found that serotonin receptors can be expressed/ unexpressed to modify the release of IL-6. [18] SK-N-SH is a continuously cultured human neuroblastoma cell line, which is well known for studies related to neurodegenerative diseases. It is also proved that these cell lines are capable of synthesizing multiple neurotransmitters which includes dopamine, norepinephrine, acetylcholine, GABA, *etc*. The black cumin oil with different concentrations of thymoquinone; TQT, TQN, and TQF were used. Adriamycin (ADR) is used as a positive control whereas DPL is used as a standard. All the drug samples were dissolved in DMSO for the assay. The drug samples at the concentrations of 10 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml were used. The assay is done in triplicate, and the average values were used to determine the percentage control growth.

From the growth curve of IMR-32 cell lines, the GI_{50} of TQF was found to be 4.3, which showed maximum activity among all the drug samples followed by TQN at 24.1 and TQT at 30.9, which prove the dose-dependent activity of TQ in black cumin oil. The DPL is found to produce no activity whereas the positive control produced the GI_{50} value of less than 10. The growth curve of the IMR-32 cell line was shown in **Fig. 2A** and GI_{50} values in **Table 1**. When compared to neuroblastoma cell lines, the activity of black cumin oil is found to produce less action on glioblastoma cell line U373-MG. The GI_{50} value of TQF was 18.3 whereas of TQN, and TQT was 38.1 and 43.3 respectively. Here too donepezil has not produced any significant activity whereas Adriamycin produced GI_{50} of less than 10. The growth curve of the U373-MG cell line was shown in **Fig. 2B** and GI_{50} values in **Table 1**.

In SK-N-SH cell line, the drug samples TQF, TQT, and ADR were found to produce activity whereas TQN had shown the GI_{50} value of 35.4 and Donepezil with greater than 80. The growth curve of the SK-N-SH cell line was shown in **Fig. 2C** and GI_{50} values in **Table 1**. The SRB assay in the present study implicit that TQF produces activity, especially on neuronal cells (IMR-32 & SK-N-SH) than glial cells (U373-MG). Whereas TQT produced activity only on SK-N-SH cell line, and TQN produced limited or no activity on all the three cell lines, which replicates the action of black cumin oil based on TQ concentration.

CONCLUSION: In summary, DPL, the standard drug, and TQF exhibited a dose-dependent increase in AChE inhibitory activity. While TQT and TQN exhibited a dose-dependent decrease in AChE inhibition. This might be due to the variation in the active content namely TQ present in the oil of *N.*

sativa. These results are also correlating with GI_{50} values of various grades of black cumin oil as observed from the growth curve. Adriamycin is producing a consistent inhibition in all three cell lines. TQF is also producing a comparable effect while other samples TQT, TQN, and DPL failed to produce such growth inhibition. This work confirms the effect of various grades of black cumin oil for its neuroprotective activity. This study gives an insight into a thorough safety pharmacological studies for the oil of *N. sativa*.

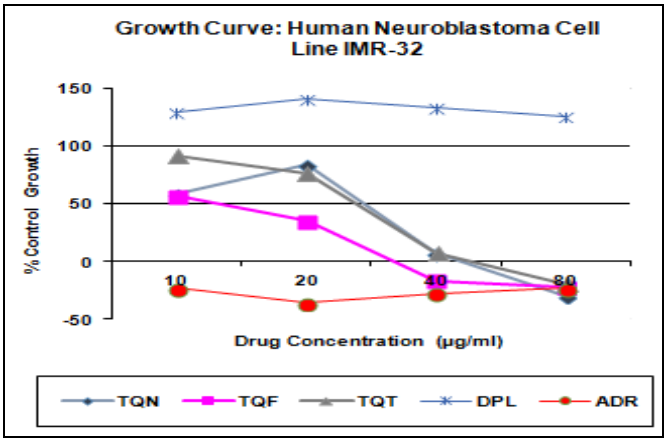

FIG. 2A: GROWTH CURVE: HUMAN NEUROBLASTOMA CELL LINE IMR-32 USING SRB ASSAY. Where TQF- Black cumin oil containing 5% TQ, TQT - Black cumin oil containing 2%, TQN - Black cumin oil containing 0.6% TQ, DPL – Donepezil, ADR - Adriamycin

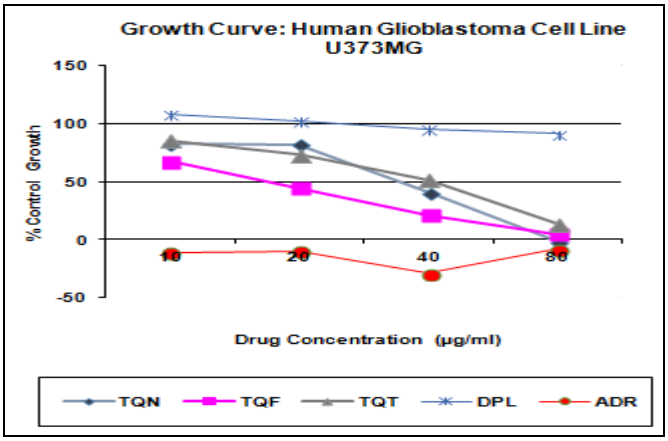

FIG. 2B: GROWTH CURVE: HUMAN GLIOBLASTOMA CELL LINE U373MG USING SRB ASSAY. Where TQF- Black cumin oil containing 5% TQ, TQT - Black cumin oil containing 2%, TQN - Black cumin oil containing 0.6% TQ, DPL - Donepezil, ADR - Adriamycin

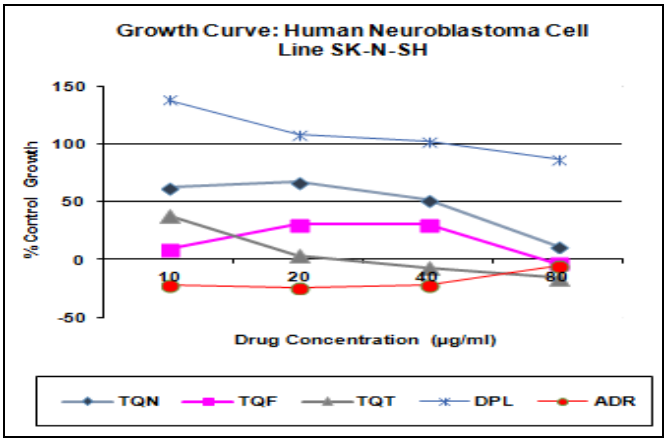

FIG. 2C: GROWTH CURVE: HUMAN NEUROBLASTOMA CELL LINE SK-N-SH USING SRB ASSAY. Where TQF- Black cumin oil containing 5% TQ, TQT - Black cumin oil containing 2%, TQN - Black cumin oil containing 0.6% TQ, DPL - Donepezil, ADR - Adriamycin

TABLE 1: GI_{50} VALUES OF DIFFERENT GRADES OF BLACK CUMIN OIL ALONGSIDE WITH DONEPEZIL AND ADRIAMYCIN

Samples	Drug concentrations (µg/ml) calculated from the graph		
	IMR-32	U373MG	SK-S-NH
TQN	24.1	38.1	35.4
TQF	4.3	18.3	<10
TQT	30.9	43.3	<10
DPL	NE	NE	>80
ADR	<10	<10	<10

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CONFLICT OF INTEREST: The authors declare no conflict of interest

REFERENCES:

1. Kumar GP and Khanum F: Neuroprotective potential of phytochemicals. Pharmacogn Rev 2012; 6(12): 81-90.
2. Merlo S, Spampinato S, Canonico PL, Copani A and Sortino MA: Alzheimer's disease: Brain expression of a

metabolic disorder? Trends Endocrinol Metab 2010; 21(9): 537-44.
3. Howes MJR and Houghton PJ: Plants used in Chinese and Indian traditional medicine for improvement of memory and cognitive function. Pharmacol Biochem Behav 2003; 75(3): 513-27.
4. Ahmad A, Husain A, Mujeeb M, Khan SA, Najmi AK and Siddique NA: A review on the therapeutic potential of Nigella sativa: A miracle herb. Asian Pac J Trop Biomed [Internet]. 2013; 3(5): 337-52. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23646296
5. Leong XF, Mustafa RM and Jaarin K: *Nigella sativa* and its protective role in oxidative stress and hypertension. Evidence-Based Complement Altern Med [Internet]. 2013 2013; 1-9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/23533459
6. Salim LZA, Mohan S, Othman R, Abdelwahab SI, Kamalidehghan B and Sheikh BY: Thymoquinone induces mitochondria-mediated apoptosis in acute lymphoblastic leukaemia in vitro. Molecules 2013; 18(9): 11219-40.
7. Nickavarm B, Mojab F, Javidnia K and Amoli MA: Chemical composition of the fixed and volatile oils of Nigella sativa L. from Iran. 2003; 58(9-10): 629-631tle. Z Naturforsch C 2003; 58(9-10): 629-31.
8. Adewusi EA and Steenkamp V: *In-vitro* screening for acetylcholinesterase inhibition and antioxidant activity of medicinal plants from Southern Africa. Asian Pac J Trop Med [Internet]. 2011; 4(10): 829-35. Available from: http://www.ncbi.nlm.nih.gov/pubmed/22014742
9. Ferreira A, Proença C, Serralheiro MLM and Araújo MEM: The *in-vitro* screening for acetylcholinesterase inhibition and antioxidant activity of medicinal plants from Portugal. J Ethnopharmacol 2006; 108(1): 31-7.
10. Orellana E and Kasinski A: Sulforhodamine B (SRB) assay in cell culture to investigate cell proliferation. BIO-PROTOCOL 2016; 6(21): e1984. Available from: http://www.bio-protocol.org/e1984
11. Vichai V and Kirtikara K: Sulforhodamine B colorimetric assay for cytotoxicity screening. Nat Protoc 2006; 1(3): 1112-6.
12. Soreq H: Acetylcholinesterase - new roles for an old actor. Nat Rev Neurosci 2001; 2(4): 294-302.
13. Jukic M, Politeo O, Maksimovic M, Milos M and Milos M: *In-vitro* acetylcholinesterase inhibitory properties of thymol, carvacrol and their derivatives thymoquinone and thymohydroquinone. Phyther Res 2007; 21(3): 259-61.
14. Driver JA, Beiser A, Au R, Kreger BE, Splansky GL and Kurth T: Inverse association between cancer and Alzheimer's disease: results from the Framingham Heart Study. BMJ 2012; 344(12): 1442.
15. Voigt W: Sulforhodamine B Assay and Chemosensitivity. Chemosensitivity [Internet]. 2005; 110: 039-48. Available from:http://link.springer.com/10.1385/1-59259-869-2:039.
16. Asami-Odaka A, Ishibashi Y, Kikuchi T, Kitada C and Suzuki N: Long amyloid beta-protein secreted from wild-type human neuroblastoma IMR-32 cells. Biochemistry 1995; 34(32): 10272-8.
17. Kormelink GPJ and Luyten WH: Cloning and sequence of full-length cDNAs encoding the human neuronal nicotinic acetylcholine receptor (nAChR) subunits beta3 and beta4 and expression of seven nAChR subunits in the human neuroblastoma cell line SH-SY5Y and/or IMR-32. FEBS Lett 1997; 400(3): 309-14.
18. Lieb K, Biersack L, Waschbisch A, Orlikowski S, Akundi RS and Candelario-Jalil E: Serotonin *via* 5-HT7 receptors activates p38 mitogen-activated protein kinase and protein kinase C ε resulting in interleukin-6 synthesis in human U373 MG astrocytoma cells. J Neurochem 2005; 93(3): 549-59.

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