

The Effects of Increasing Doses of *Nigella Sativa* and Conjugated Estrogen on Bone-Specific Alkaline Phosphatase (B-ALP), Procollagen Type 1 N-Terminal Propeptide (P1NP), Carboxy Terminal Crosslinked Telopeptide of Type 1 Collagen (CTX-1), and Osteoprotegerin (OPG) in an Osteoporosis Rat Model

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ABSTRACT

Introduction: Osteoporosis is characterized by decreased bone density, increasing the risk of fractures. Hormone therapy for post-menopausal osteoporosis has side effects, prompting the development of *Nigella sativa* (black cumin) as an alternative treatment. **Objective:** This study aims to determine the effect of administering *Nigella sativa* and conjugated estrogen as osteoporosis therapy in rats treated with bilateral oophorectomy, which have been verified as having osteoporosis based on bone biomarkers.

Materials and Methods: This study involved 72 female Wistar rats divided into six groups: K+ (positive control), K- (negative control), P1 (receiving conjugated estrogen), P2 (receiving 100 mg/kg BW of *Nigella sativa*), P3 (receiving 200 mg/kg BW of *Nigella sativa*), and P4 (receiving 400 mg/kg BW of *Nigella sativa*). After inducing post-menopausal osteoporosis through bilateral oophorectomy, the treatment groups received therapy for 28 days. Bone biomarkers such as bone-specific alkaline phosphatase (bALP), procollagen type 1 n-terminal propeptide (P1NP), carboxy-terminal crosslinked telopeptide of type 1 collagen (CTX-1), and osteoprotegerin (OPG) were measured using ELISA. **Results:** The 400 mg/kg BW administration of *Nigella sativa* significantly increased bALP and P1NP levels, indicating higher osteoblast activity. At the same dose, *Nigella sativa* also increased OPG levels, suppressing osteoclastogenesis in bone resorption. However, 100-400 mg/kg BW/day of *Nigella sativa* did not reduce CTX-1 levels, nor did it inhibit osteoclasts in bone resorption. Estrogen conjugation also increased OPG levels but did not significantly affect bALP and P1NP levels. **Conclusion:** *Nigella sativa* at a dose of 400 mg/kg BW significantly increases osteoblast activity and OPG levels. Estrogen conjugation increases OPG levels but does not affect bALP and P1NP. *Nigella sativa* can potentially serve as an effective alternative therapy for osteoporosis through a mechanism different from conjugated estrogen.

Keywords: *Nigella sativa*, Estrogen conjugation, B-ALP, Bone biomarkers, Osteoporosis rats.

INTRODUCTION

Osteoporosis is a condition characterized by decreased bone density, leading to an elevated risk of bone fragility and fractures.^{1,2} This condition is primarily caused by an imbalance between bone production and resorption, resulting in millions of fractures annually.³ Osteoporotic fractures, such as those in the hip, vertebrae, and wrist, are the most severe clinical consequences of osteoporosis and occur more frequently in women than in men. The incidence of osteoporosis rises sharply with age and affects areas with a high percentage of trabecular bone.⁴

Several studies have indicated that hormone replacement therapy (HRT) can address post-menopausal issues in women through the administration of estrogen and progesterone.⁵ However, during conjugated estrogen therapy, liver metabolism is approximately 4-5 times higher than peripheral metabolism. Long-term use of estrogen can affect hepatocyte cells, leading to coagulation disorders due to metabolism in the hepato-portal system. This impacts the production

of proteins involved in clotting and fibrinolysis, such as increasing factor VII, prothrombin fragments I and II, MMP II, and IX, raising the risk of congestive heart disease and thrombosis. Additionally, prolonged estrogen use increases the risk of venous thromboembolism due to resistant C-reactive protein and elevated production of fats and liver enzymes.⁶ Consequently, recent years have seen intensive development of alternative therapies for osteoporosis caused by menopause, one of which is the use of *Nigella sativa*.

The natural plant *Nigella sativa*, a member of the *Ranunculaceae* family, has long been used as a remedy for various acute and chronic diseases. It is commonly known as *habbatus sauda* or black cumin. The active component in this plant, found in its seeds, is known as thymoquinone (TQ).⁷⁻⁹

Nigella sativa is an alternative source of essential fatty acids in addition to vegetable oils and contributes significantly to the diet. Additionally, this seed is a source of fat, protein, and several essential minerals that positively impact human health in quantity and quality. Research indicates that *Nigella sativa* offers

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numerous therapeutic benefits, including anti-cancer, antioxidant, antibacterial, antifungal, antiparasitic, and anti-asthma properties. Furthermore, previous studies on *Nigella sativa* and its main active component, thymoquinone, have demonstrated effectiveness in treating bone and joint disorders.¹⁰

In this study, the dosage of *Nigella sativa* was determined based on in vivo toxicity studies, which was 470 mg/kg body weight (BW). Thus, we used 400 mg/kg BW and, for comparative purposes, 25% of this dose (100 mg/kg BW) and 50% of this dose (200 mg/kg BW).¹¹

Using bone biomarkers for early detection of osteoporosis provides a highly sensitive and specific indicator of the osteoporosis process.¹² In this study, several biomarkers were used to assess bone condition, including serum calcium, bone-specific alkaline phosphatase (bALP), procollagen type 1 N-terminal propeptide (P1NP), carboxy-terminal crosslinked teloepptide of type 1 collagen (CTX-1), and osteoprotegerin (OPG). This study aims to determine the effect of administering *Nigella sativa* (black cumin) and conjugated estrogen as osteoporosis therapy in rats treated with bilateral oophorectomy, which have been verified as having osteoporosis based on bone biomarkers.

MATERIALS AND METHODS

Study Design and Subject Recruitment

This experimental study was conducted in the laboratory of the Faculty of Veterinary Medicine, Universitas Airlangga. The test subjects were 3-month-old female Wistar white rats (*Rattus norvegicus*) weighing approximately 150 grams. The rats were provided with animal feed according to standard operating procedures. *Nigella sativa* (black cumin) was sourced from PT. Asimas Agriculus Sido Makmur Sentosa (Malang).

A total of 72 rats were acclimatized for seven days before undergoing the ovariectomy procedure to standardize hormonal cycle conditions. Subsequently, bilateral ovariectomy was performed on the test animals to induce post-menopausal osteoporosis. After 21 days, the hormonal status was re-examined to confirm the success of the ovariectomy through vaginal swabs and estrogen level measurements. Following the procedure, the rats were divided into six groups: K- (rats that underwent bilateral ovariectomy 21 days prior and received no treatment), K+ (rats that underwent bilateral ovariectomy 49 days prior and received no treatment), P1 (rats that underwent bilateral ovariectomy and received 10 µg/kg BW of conjugated estrogen, specifically 0.625 mg of Esthero preparation dissolved in MDA), P2 (rats that underwent bilateral ovariectomy and received 100 mg/kg BW of *Nigella sativa*), P3 (rats that underwent bilateral ovariectomy and received 200 mg/kg BW of *Nigella sativa*), and P4 (rats that underwent bilateral ovariectomy and received 400 mg/kg BW of *Nigella sativa*). For a total of 28 days, treatments were given once a day to the treatment groups.

Protein Quantification (ELISA)

Blood samples were collected 24 hours after the last treatment. Protein levels were analyzed using ELISA kits: bone-specific alkaline phosphatase (bALP) (Reed Biotech, RE3152R), carboxy-terminal crosslinked teloepptide of type 1 collagen (CTX-1) (Reed Biotech, RE1463R), Procollagen type 1 N-Terminal propeptide (P1NP) (Reed Biotech, RE2875R), and osteoprotegerin (OPG) (Reed Biotech, RE1765R), according to the manufacturer's protocol.

Statistical Analysis

Data were analyzed using the SPSS version 25 program. A descriptive analysis of the average research parameters in each group of experimental animals was conducted, followed by tests for normality and homogeneity of the data. Data normality was assessed using

the Shapiro-Wilk Test (data were considered normally distributed if p-value > 0.05), while homogeneity was evaluated using Levene's Test (data were considered homogeneous if p-value > 0.05). One-way ANOVA was used to analyze normally distributed and homogeneous data. For data that did not meet normality criteria, the Kruskal-Wallis test was employed. Statistical significance was indicated as * p-value < 0.05, ** p-value < 0.005, *** p-value < 0.001.

RESULTS

In this study, we measured the levels of several bone biomarkers, including bALP, P1NP, CTX-1, and OPG, to detect early osteoporosis.

bALP Level Test

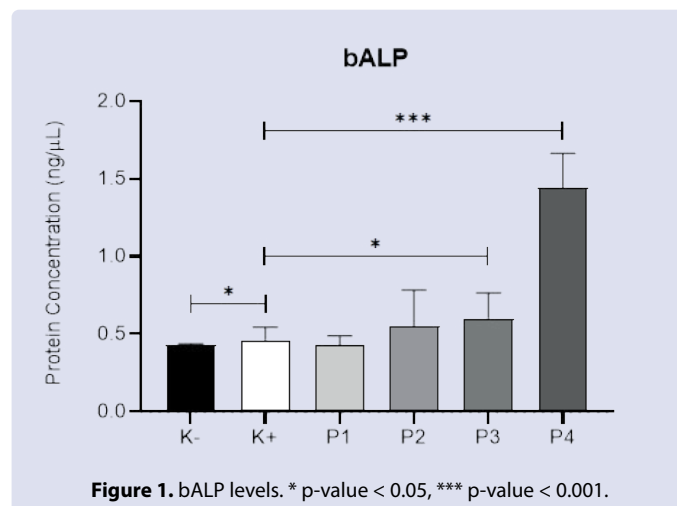
The levels of bALP, an osteoblast enzyme involved in bone formation, were measured using ELISA (Figure 1). Our results indicated that bALP levels in the K+ group (0.465 ± 0.080) significantly increased compared to the K- group (0.429 ± 0.009). The bALP levels in groups P1 (0.427 ± 0.061) and P2 (0.553 ± 0.229), which received 100 mg/kg BW of conjugated estrogen and *Nigella sativa*, respectively, did not demonstrate significant differences compared to the K+ group. In contrast, groups P3 and P4, which were given 200 mg/kg BW and 400 mg/kg BW *Nigella sativa*, respectively, demonstrated a significant increase in bALP levels (0.603 ± 0.163 and 1.444 ± 0.217) compared to the K+ group.

P1NP Level Test

The levels of P1NP, a bone remodeling biomarker, were measured using ELISA in serum (Figure 2). P1NP levels significantly increased in the K+ group (2623.00 ± 439.753) compared to the K- group (2316.08 ± 305.506). A significant increase in P1NP levels was also observed in the P4 group (5480.08 ± 1890.159), which received 400 mg/kg BW of *Nigella sativa* in comparison to the K+ group. No substantial differences in P1NP levels were found in groups P1 (2243.58 ± 441.895), P2 (2778.93 ± 515.461), and P3 (3045.11 ± 422.640), which received conjugated estrogen treatment, 100 mg/kg BW of *Nigella sativa*, and 200 mg/kg BW of *Nigella sativa*, respectively, compared to the K+ group.

CTX1 Level Test

The levels of CTX1, a bone resorption biomarker, were assessed using ELISA in serum (Figure 3). Our measurements indicated no substantial differences in CTX1 levels between the K- (0.1705 ± 0.0021) and K+ (0.1709 ± 0.0034) groups. Similarly, no significant differences were found between the P1 (0.1714 ± 0.0027), P2 (0.1721 ± 0.0031), P3 (0.1723 ± 0.0033), and P4 (0.1711 ± 0.0008) groups compared to the



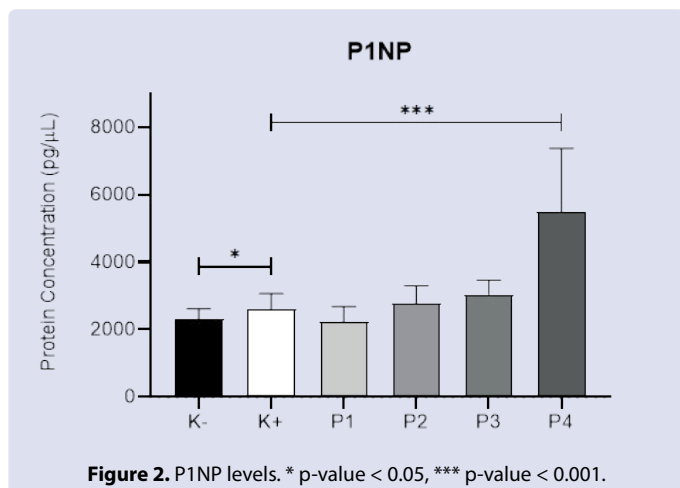


Figure 2. P1NP levels. * p-value < 0.05, *** p-value < 0.001.

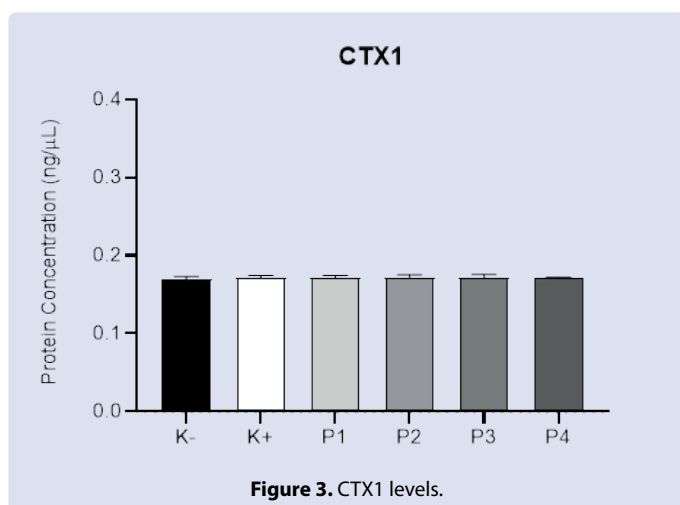


Figure 3. CTX1 levels.

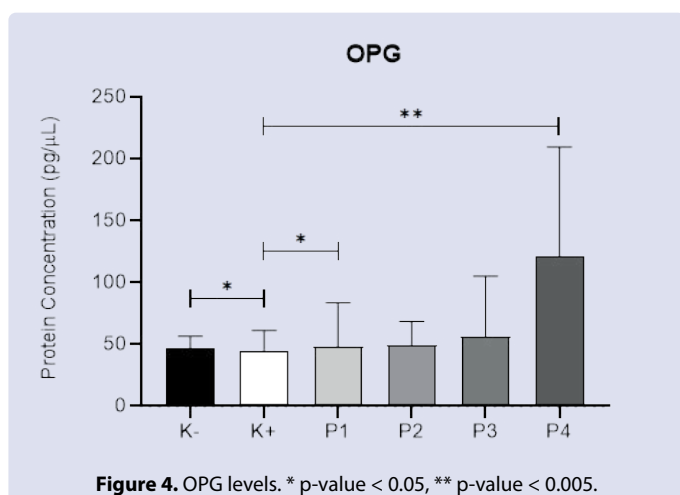


Figure 4. OPG levels. * p-value < 0.05, ** p-value < 0.005.

K+ group. Thus, neither the administration of conjugated estrogen nor 100, 200, and 400 mg/kg BW of *Nigella sativa* significantly affected CTX1 levels.

OPG Level Test

The levels of OPG, an inhibitor of osteoclast activation, were measured via ELISA in blood serum (Figure 4). The ELISA results revealed that OPG levels in the K+ group ($45,091 \pm 15,913$) significantly decreased compared to the K- group ($46,577 \pm 9,723$). Conjugated estrogen

treatment in the P1 group ($47,593 \pm 35,830$) significantly increased OPG levels in comparison to the K+ group. A substantial elevation in OPG levels was also observed in the P4 group ($120,812 \pm 88,673$), which received 400 mg/kg BW of *Nigella sativa* in comparison to the K+ group. In contrast, groups P2 ($48,878 \pm 19,419$) and P3 ($56,627 \pm 48,374$), which were given 100 mg/kg BW and 200 mg/kg BW of *Nigella sativa*, respectively, had no discernible impact on OPG levels when compared with the K+ group.

DISCUSSION

This study utilized 2-month-old female Wistar white rats (*Rattus norvegicus*) as a model for osteoporosis due to their bone structure's resemblance to that of humans. The study evaluated the effects of various doses of *Nigella sativa* compared with conjugated estrogen on direct markers of bone resorption and formation.

Bone-Specific Alkaline Phosphatase Levels

Bone-specific alkaline phosphatase (bALP) is a tetrameric enzyme attached to the osteoblast cell membrane via a glycosyl-phosphatidylinositol group.¹³ Although its specific function has not been fully understood, bALP contributes to the mineralization and formation of osteoid.¹⁴ This enzyme is found in various tissues, including the liver, bone, and placenta, with bone and liver isoforms being the most dominant (90%). bALP level measurement is used to assess osteoblastic activity, particularly in managing osteoporosis in premenopausal and post-menopausal women.

The study demonstrated that administering 10 μg/kg BW/day of conjugated estrogen to the P1 group reduced bALP levels in comparison to the control group, although this decline lacked statistical significance ($p > 0.05$). This decrease reflects a reduction in osteoblast activity in response to a decrease in osteoclast activity in osteoporosis.¹⁵

In contrast, the group receiving *Nigella sativa* therapy demonstrated increased bALP levels, indicating increased osteoblast activity. *Nigella sativa* does not function through a feedback mechanism involving bone resorption by osteoclasts but by enhancing osteoblast proliferation and survival.¹⁶ In vitro studies indicate that thymoquinone, a component of *Nigella sativa*, stimulates osteoblast proliferation, differentiation, and mineralization through the ERK pathway.¹⁷

Research on osteoporosis rat models found more osteoblasts in rats given *Nigella sativa* compared to controls.¹⁸ A dose of 400 mg/kg BW/day provided the most significant results in increasing bALP levels ($p < 0.005$). Thus, conjugated estrogen and *Nigella sativa* work through different mechanisms to inhibit the osteoporosis process.

Procollagen Type 1 N-Terminal Propeptide (P1NP) Levels

P1NP is an important parameter in examining osteoblast activity, especially because type I collagen is the main product of osteoblast synthesis. The amino-terminal propeptide (P1NP) is expressed from type I collagen deposits in the bone formation matrix during the bone remodeling process, making it an ideal bone formation marker.¹⁹

P1NP levels increased considerably in the K+ group relative to the K-group ($p < 0.005$), indicating osteoporosis. P1NP levels were lower in the treatment groups after conjugated estrogen therapy at a dose of 10 μg/kg BW in comparison to the K- and K+ groups, suggesting a decrease in osteoclast activity in osteoporosis, although this decline lacked statistical significance. In contrast, the group receiving *Nigella sativa* treatment demonstrated increased P1NP levels across various doses, indicating higher osteoblast activity. *Nigella sativa* induces osteoblast proliferation and osteogenic differentiation of stem cells.¹⁶ Additionally, *Nigella sativa* increases osteoblast activity through the ERK pathway.¹⁷ From these results, we can conclude that P1NP cannot

be used as a direct comparison between the groups receiving *Nigella sativa* and conjugated estrogen due to their different mechanisms of action in suppressing the occurrence of osteoporosis.

Carboxy-Terminal Crosslinked Telopeptide of Type 1 Collagen (CTX1) Levels

The telopeptides of type 1 collagen, including CTX-1 and NTX-1, are widely used biomarkers of bone resorption. CTX-1 is released during collagen degradation and measured by ELISA using a monoclonal antibody that targets the octapeptide sequence in the α -1(I) chain of the β -isoform. CTX-1 is a sensitive and specific bone resorption biomarker and demonstrates a rapid response to bisphosphonate therapy in post-menopausal osteoporosis.²¹ Nevertheless, blood must be collected in a fasting state since food consumption affects serum CTX-1 levels.²²

CTX-1 levels in this study were found to be greater in the K+ group than in the K-group, although the difference was not statistically significant ($p > 0.05$). There were no statistically significant differences in all treatment groups (P1 to P4), indicating that neither conjugated estrogen nor *Nigella sativa* was strong enough to suppress osteoclast activity. Groups P1 and P4 had almost the same mean CTX-1 levels, indicating that 400 mg/kg BW of *Nigella sativa* had similar effectiveness to conjugated estrogen in reducing bone matrix degradation by osteoclasts. The slight increase in CTX-1 in P2 and P3 may be due to the stimulation of osteoblasts in the formation of osteoclasts, but this is still controlled by *Nigella sativa* as a phytoestrogen through the formation of OPG by osteoblasts.^{23, 24} *Nigella sativa* extract contains various active components, such as thymoquinone, dithymoquinone, thymohydroquinone, and thymol, which are proven to be powerful antioxidants.¹⁰

Osteoprotegerin (OPG) Levels

Among various tissues and cell types, including osteoblasts, OPG is known for its secretion and production as a soluble receptor. It serves to inhibit osteoclastogenesis by acting as a decoy receptor for RANKL.²⁵ According to studies on rats, OPG overexpression in transgenic rat models and OPG therapy in normal rats promotes osteopetrosis, whereas OPG knockout rats have severe osteoporosis.²⁶ Recent studies have found that increased plasma OPG levels in post-menopausal women strengthen bone mass.

This study revealed an increase in OPG levels in the groups receiving 10 μ g/kg BW/day of conjugated estrogen and various doses of *Nigella sativa* compared to the K+ and K- groups. Administering 100-200 mg/kg BW of *Nigella sativa* increased OPG levels, although the difference was insignificant ($p > 0.005$). A substantial elevation in OPG levels occurred in the groups receiving 400 mg/kg BW of conjugated estrogen and *Nigella sativa*. This increase is attributed to the phenolic and flavonoid content of *Nigella sativa*, which are phytoestrogens.^{27, 28} The estrogenic effect of *Nigella sativa* was confirmed through uterine histopathology, examination of vaginal cells, and measurement of blood estrogen levels.²⁹ The rise in OPG levels is considered to be primarily caused by the phytoestrogen component of *Nigella sativa*.³⁰

CONCLUSION

Nigella sativa at a dose of 400 mg/kg BW substantially increases osteoblast activity and OPG levels, demonstrating its potential as an effective alternative therapy for osteoporosis. Low and medium doses of *Nigella sativa* did not show significant effects. Conjugated estrogen increased OPG levels but did not significantly affect bALP and P1NP levels. *Nigella sativa* inhibits osteoporosis differently from conjugated estrogen, potentially offering a safer and more effective therapeutic option.

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ETHICAL CONSIDERATION

The protocol of the study was approved by the Research Ethics Committee of Airlangga Academic Hospital (No. 2/KEH/140.09.2023).

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This research received no external funding.

CONFLICTS OF INTEREST

There is no conflict of interest in this study.

AUTHOR CONTRIBUTIONS

All authors contributed to article preparation and paper revision and have collectively assumed responsibility for all aspects of this study.

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