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The Effect of *Nigella sativa* Extract on Repair of Osteoporosis through Suppression of TRAF 6 and NFATc 1 in Ovariectomized Rat

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ABSTRACT

Osteoporosis is a disease characterized by low bone mass and structural bone damage which results bone fragility and increases the risk of fracture. Increasing life expectancy makes women more vulnerable to osteoporosis due to hypo-estrogen in menopause. The research aimed to determine the effect of *Nigella sativa* extract in improving osteoporosis through suppression of Tumor Necrosis Factor receptor associated factor 6 (TRAF6) and Nuclear factor of activated T-cells cytoplasmic 1 (NFATc1) in ovariectomized (OVX) rats. This study was divided into 5 groups. There were OVX group, OVX + conjugated estrogen 20 mg / day and 3 groups treated with OVX +extract of *Nigella sativa* orally for 8 weeks at doses of 5, 10, and 20 mg /kg BW/day. The bone matrix thickness was stained by H&E, also number of TRAF6 and NFATc1 analyzed using flow cytometry. The results shown that the highest bone matrix thickness was obtained in the OVX + NS group 10 mg / kg BW (p=0,000), the lowest TRAF6 concentration in the OVX + group given NS 5 mg / kg BW (p= 0.001), and the concentration of NFATc1 was lowest in the OVX + CEE group (p = 0.009). The administration of *Nigella sativa* extract successfully improved osteoporosis through suppression of TRAF6 and NFATc1.

Keywords
Nigella sativa extract; osteoporosis; TRAF6; NFATc1

Introduction

The National Osteoporosis Foundation's (NOF) defines osteoporosis as a disease characterized by low bone mass, changes micro architecture of bone and structural bone damage which results bone fragility and increases the risk of fracture especially hip, spine and wrist and other [1,2]. Osteoporosis is referred to as "the silent disease" because the sufferer does not feel being attacked by osteoporosis, until experiencing a fracture with minimal trauma[3].One of the pathogenesis of postmenopausal osteoporosis is a decrease in estrogen concentration(hypoestrogen). Hypoestrogens cause an increase in the induction of various cytokines, such as interleukin (IL) 1, IL 6 and tumor necroting factor α (TNFα). These cytokines stimulate osteoclast differentiation, thereby triggering the process of osteoclastogenesis. The most important cytokine associated with estrogen deficiency-induced bone loss is TNFα produced by bone marrow T lymphocytes[4].Osteoclast differentiation is triggered by a bond between RANKL (receptor activator of nuclear factor κB ligand) and RANK. The RANKL-RANK interaction activates the nuclear factor of activated T-cells, cytoplasmic 1(NFATc1)-which is the main transcription factor for osteoclastogenesis and involves TRAF-6, c-Jun, c-Fos and p38. The nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) is the main transcription factor in osteoclast precursors NFATc1 will induce osteoclast specific genes such as tartrate-resistant acid

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phosphatase, calcitonin receptor and capthesin K, resulting in osteoclastogenesis [5-8]. Hormone replacement therapy (HRT)-by administering estrogen with or without progesterone - for postmenopausal osteoporosis therapy, is effective for fracture prevention in women aged less than 60 years and menopausal age less than 10 years. Giving HRT after the age of60 years is not recommended by the Women Health Initiative (WHI) because long-term administration will increase the risk of breast cancer [9].

This study aims to investigate exogenous estrogen as a phytoestrogens. Phytoestrogens are active ingredients derived from plants that have activities such as estrogen, which can be given to overcome estrogen deficiency in humans[10] such as *Nigella sativa* (NS) [11].

Ansari and Batra (2013) stated NS can prevent osteoporosis, but the mechanism of NS in preventing osteoporosis is not yet clear [12]. Thymoquinone (TQ) can inhibit RANKL which induces osteoclastogenesis in RAW 264.7 cells and BMMs cells and also bone resoption in male rats that are induced by lipo poly saccharide (LPS) for bone destruction [13].

Material and Method

Animals and experimental design

Fifteen Wistar female rat (Rattus norvegicus) weighting8-10 months-old. The rat were housed at a room temperature 29-320C, with 12 hours dark/like cycle and 50-60% relative humidity. They were fed with standard diet, with food and water ad libitum. The rats were ovariectomized under ketamine (40 mg/kg i.m respectively) according to Ingle DJ and Griffith JQ 1971 modified method. The OVX mice were acclimatized for four weeks prior to supplementation. After second acclimatization, they were randomly assigned to five groups such as control group (OVX group) was given distilled water, positive control-OVX+CEE) was treated with Conjugated Equine Estrogen (CEE) 20 mg/day (gavage). The third, fourth and fifth groups were supplemented with NS (5, 10, and 20 mg/kg BW/day) respectively. All groups were continued for 8 consecutive weeks. This study was approved by the Medical Research Ethics Commission of the Faculty of Medicine, University of Brawijaya Malang with 76/EC/KEPK-S3/03/2019 reference number.

Nigella sativa extraction

Nigella sativa seeds were identified and extracted with ethanol at UPT Laboratorium Herbal Materia Medica Batu-Indonesia. 50g of NS powder added with 95% ethanol. Furthermore, the extract was evaporated to obtain solid crystals. The product of 1000mg was then dissolved in DMSO. The extract was further diluted to obtain a dilution fraction of 1 mg/ml and 10 mg/ml.

Measurement of bone matrix thickness

Rat femurs were fixed in 10% neutral-buffered formaldehyde and then decalcified with decalcification solution for two weeks.The bones were embedded in paraffin, fixed and performed 4 μm histological section in border area of the cortex and bone trabeculae.

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Levels of TRAF6 and NFATc1

Serum TRAF6 and NFATc1 were perform at Biomedical laboratory, Faculty of Medicine, University of Brawijaya. Blood was collected and put in an EDTA tube for PBMC isolation. The pellets formed were stained with CD11b/GR1 antibodies, washed with staining buffer cells (2% Fetal Bovine Serum (FBS) in PBS). The cells were mixed with TRAF6 antibody (D-10) PE cat SC-8409PE and NFATc1 antibody (7A6) PE cat SC-7294 PE by incubation for 20 minutes in the dark at room temperature and then continue for flow cytometry analysis.

Results

Effect of NS on increasing the thickness of the bone matrix

The highest mean bone matrix thickness was obtained in the OVX + NS 10 mg/kg BW group (Figure 1). There was significant difference of mean bone matrix thickness in groups using one way ANOVA test (p= 0.000). Tukey's post hoc test was showed that the OVX group was significantly different from all other groups. The NS of 5 and 10 mg/kg BW produced a higher bone matrix thickness than OVX+CEE group ($p<0.05$), while OVX and NS 20 mg/kg BW groups compare with OVX+CEE (Figure 2).

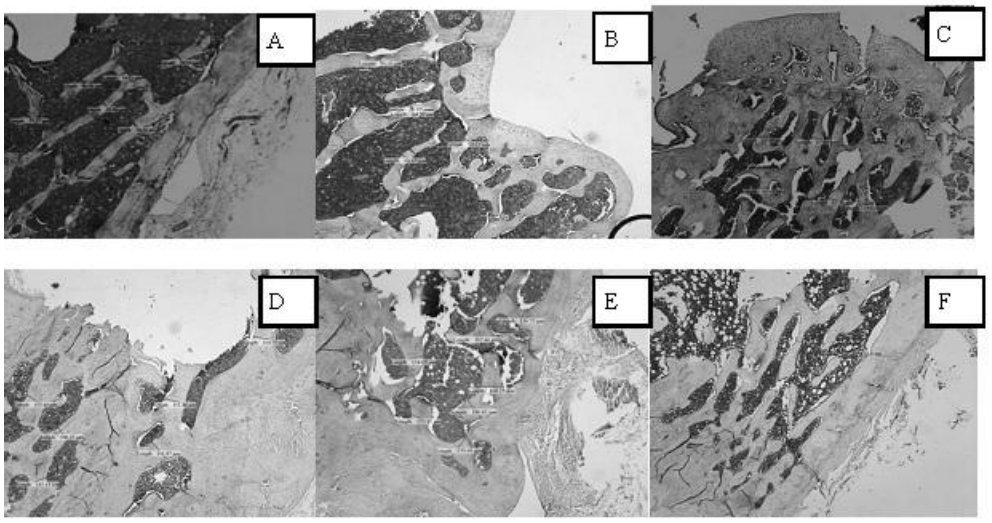

Figure 1. Photomicrograph of the femoral bone rat from each group showing thin matrices at OVX 4 weeks (A) and 12 weeks (B) and showing thick matrices at OVX+CEE (C), OVX + NS 5 mg/kg BW (D), OVX+NS 10 mg /kgBW (E) and OVX+ NS 20 mg/kg BW (F).

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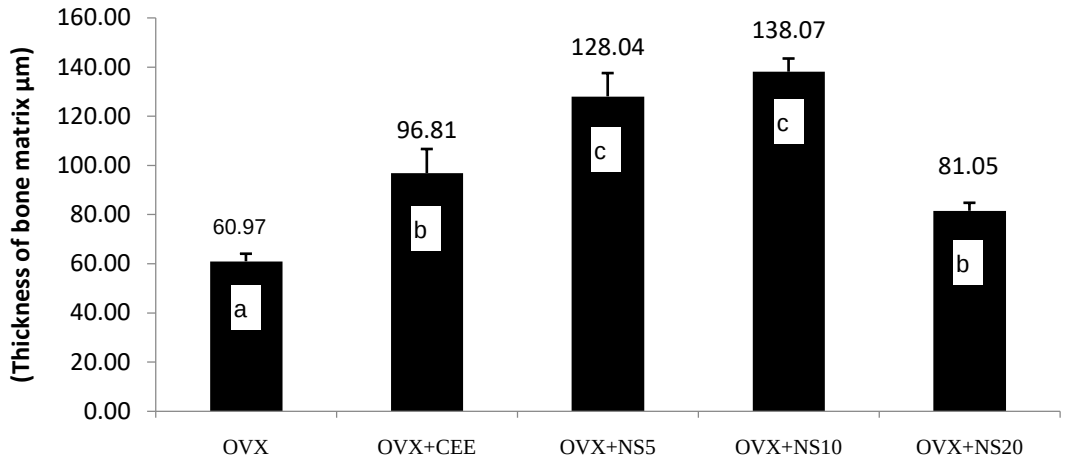

Figure 2. The mean thickness of the femoral bone matrix in each group (p= 0.000). The different letter notation indicates that there was significant difference (p<0.05).

Effect of NS on decreasing TRAF6

The comparison of the TRAF6 average in the group was tested using one way ANOVA and a significant difference was obtained p= 0.001<α. Tukey post hoc test was resulted obtained significant differences between the OVX group with OVX + CEE, OVX + NS 5 mg/kg BW and OVX + NS 10 mg/kg BW. There was no significant difference between OVX and OVX + NS 20 mg/kg. The lowest TRAF6 concentration was obtained in the OVX + NS 5 mg/kg BW group, even lower than OVX + CEE.

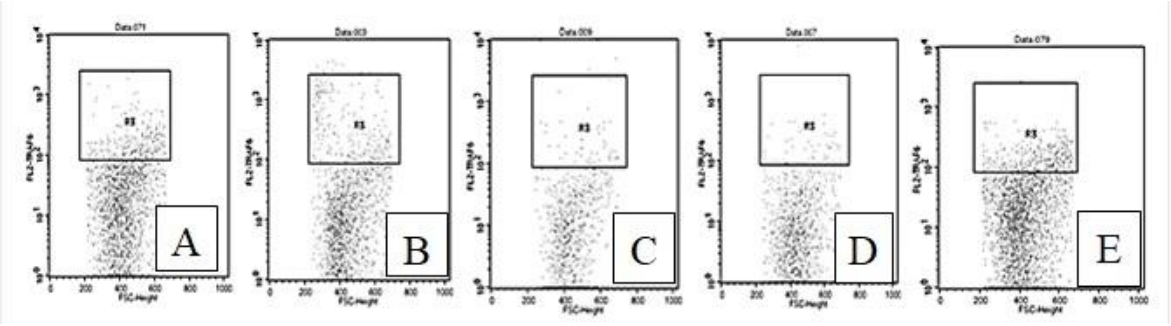

Figure 3. The flowcytometry of TRAF6 percentage in the OVX (A), OVX+CEE (B) and OVX+NS groups 5 mg/kg (C) OVX+NS groups 10 mg/kg (D), and OVX+NS groups 20 mg/kg (E) groups.

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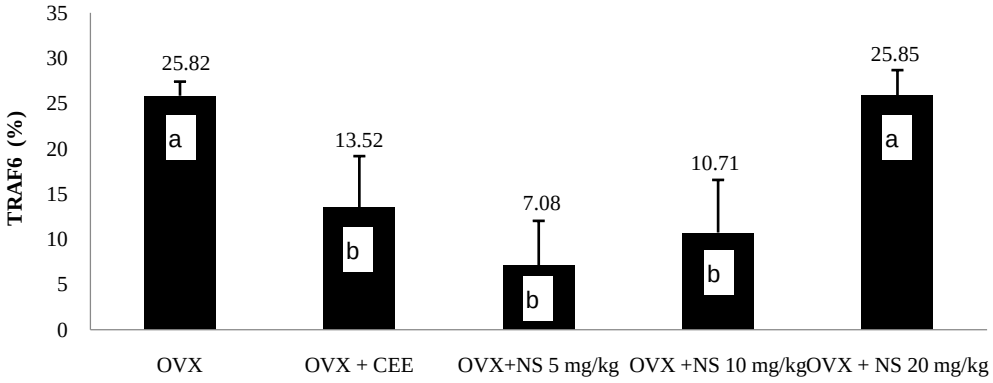

Figure 4. The average of TRAF6 percentage in each group (p=0.001).The different letter notation indicates that there was significant difference (p<0.05).

Effect of NS on decreasing NFATc1

The lowest NFATc1 concentration was obtained in the OVX + NS 5 mg / kg BW group. A comparison of NFATc1 in the group was tested using the one-way ANOVA test and a significant difference was obtained p= 0.009 (p<α). Tukey's post hoc test results showed that the OVX group was significantly different from all treatment group, while OVX+CEE group was not (Figure 6).

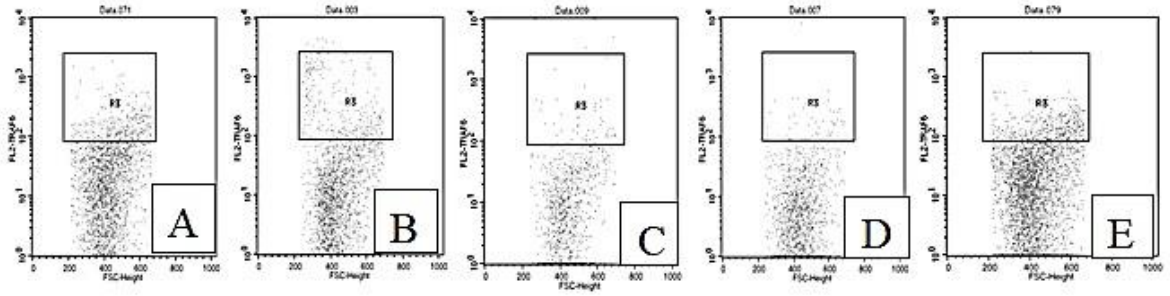

Figure 5. The flowcytometry of NFATc1 percentage of in the the OVX (A), OVX+CEE (B) and OVX+NS groups 5 mg/kg (C) OVX+NS groups 10 mg/kg (D), and OVX+NS groups 20 mg/kg (E) groups.

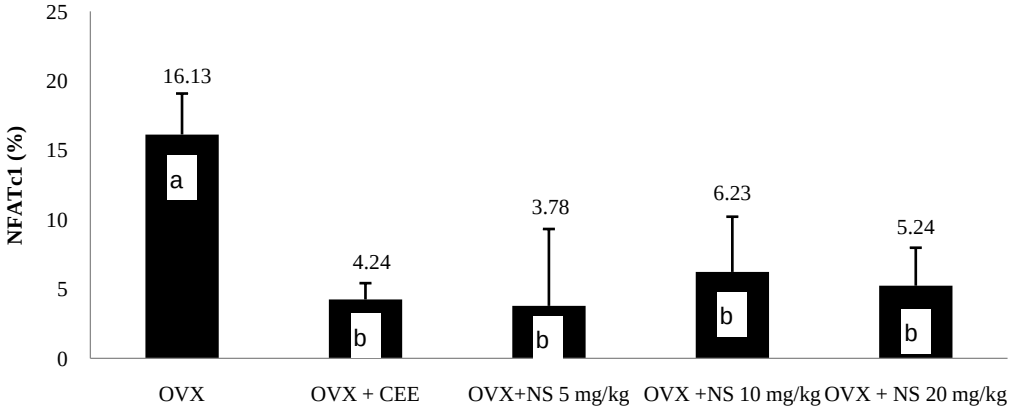

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Figure 6. The average of NFATc1 percentage in each group (p=0.009). The different letter notation indicates that there was significant difference (p<0.05)

Discussion

NS was significantly increased the thickness of the bone matrix maybe the presence of thymol, quercetin and thymoquinone in NS. Thymol was the main component of thyme oil whose structure was similar to Carvacrol. The effect of thyme (*Thymus vulgaris* L) containing thymol in male Sprague-Dawley rats could increase bone matrix thickness and prevented bone loss and calcium deficiency[14]. Thymol could bone matrix thickness and prevented bone loss and effectively prevented calcium deficiency[14-15]. Thymolinhibits osteoclast activity, suppress bone resorption, and protect bone loss, thereby increasing the thickness of the bone matrix [15].The quercetin (NS active compound) has an effect on increasing the thickness of the bone matrix[16,17]. Wong, et al., (2008) was shown that quercetin on collagen matrices can increase the formation of new bone locally, and could be used as bone graft material[16]. Orsolic, et al., (2018) confirmed the role of quercetin (quercetindyhidrate 98%) on increasing BMD, osteocalcin and calcium levels, phosphorus and triggering bone formation in osteoporosis rat (induced using retinoic acid), through their antioxidant mechanisms and estrogenic activity[17].

This was consistent with the results of the insilico test, that quercetin can bind to calcitonin receptors, which produce effects such as calcitonin[18].Thummuri, et al., (2015) used TQ at a dose of 2.5 μM, 5 μM and 7.5 μM at RAW 264.7 (mouse macrophage cell) and MC-3T3-E1 (murine pre-osteoblast). The results indicated that TQ could inhibit RANKL which induced osteoclastogenesis through suppression of phosphorylation from IKKα/β, p-65 (ser536) and NFKβ transcription activity. Thymoquinone could also inhibit NFATc1 and capthesin-K which were specific gene expression of osteoclasts. The ratswere treated with 5 μg / g BW LPS on days 1 and 4 (TQ 5 mg / kg orally 1 day before LPS administration)and continued until the 8th day. Data proven that TQ had a protective effect on inflammatory bone erosion by decreasing the number of osteoclasts. Thummuri et al. (2015) reportedtheincreasing of bone mineral content (BMC), bone mineral density (BMD) and bone architecture[13]. It was concluded that NS showed potential as an anti-osteoporosis might be due to the high content of unsaturated fatty acids, the presence of anti-inflammatory and anti-oxidant effect [19].In contrast,Seif (2014)treated 800 mg / kgBW NS powder mixed with aquadest suspension preparations. This causes a difference in the dose of NS extraction [19].This result consistent with Wong, *et al*., (2008) Seif (2014), Thummuri *et al*.,(2015), Orsolic, et al (2018), Sapkopta., *et al*. (2018) and Elbahnasawy *et al*. (2019). Increased thickness of the bone matrix in this study is likely to be the effect of thymol, quercetin and thymoquinone activities contained in NS [13-17,19].Tumor necrosis factor receptor-associated factor 6 (TRAF 6) has an important role in the process of osteoclastogenesis[20].TRAF 6 caused NFkB activation then interacts with NFATc 1.

TRAF 6 was involved in the downstream activation of several signaling pathways such as NF-κB [21].Decrease in TRAF 6 could be a marker of decreased osteoclastogenesis. Then there was a decrease in the process of osteoclast gene transcription carried out by NFATc [7].Tan et al. (2017) reported that TRAF6/c-Src/PI3K would inhibit the activation of NADPH oxidase-1 via intracellular ROS production and calcineurin phosphatase activity [7].The effect of NS on TRAF6 reduction might be due to quercetin and longifolene compounds. Quercetin was capable

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of binding to RE α and β and could quickly activate ERK in osteoblasts through the dependent RE pathway [22,23]. In silico analysis were obtained the ability of the bond between the active compound longifolene (bioactive NS) with ER has a binding affinity of -8.2 kcal/mol (unpublished). Quercetin and longifolene have estrogen-like effectsto decrease in TRAF 6[18].The administration of NS significantlyreducedNFATc 1 compared OVX group. The dose of 5 mg / kg BW had the lowest NFATc1 (fig 3b). This study indicatedNS 5 mg / kg BW gave a better effect to reduce NFATc 1 than giving conjugated estrogens.Hypoestrogens due to menopause will cause an increase in RANKL and a decrease in OPG production. The RANKL-RANK interaction activated NFATc1 (which is a major transcription factor for osteoclastogenesis) [6].The decrease in NFATc1 in this study was probably causes of active compounds in NS such as TQ which suppresses c-Fos and NFATc1 gene expression in osteoclast precursors [13]. Quercetin and longifolene bind to estrogen receptors and have estrogen-like effects [18].The mechanism of TQ blocking RANKL from inducing NF-KB was to weaken the phosphorylation of IkB kinase (IKKα/β)[13].Thus the process of osteoclastogenesis will be inhibited [20].NFATc1 is the main transcription factor that regulates osteoclast differentiation. In subsequent developments it was found that RANK signaling in inducing NFAT group expression, NFATc1 is important for osteoclast development because NFATc1-deficient precursor cells show an absolute failure to differentiate into osteoclasts [24]

Conclusion

In conclusion, our results show that the administration of the *Nigella sativa* extract successfully improved osteoporosis through suppression of TRAF6 and NFATc1. Therefore, our result suggests that *Nigella sativa* is a promoting candidate for prevention and therapy of osteoporosis.

Author Contributions: K and AR have played a role in designing the concept and experimental design. MKSH, K and AR have analyzed the results of histopathology, ELISA and flowcytometer. MKSH and SBS have conducted statistical analysis.

Limitation and future studied: Biomarker of osteoporosis and BMD in mice were not examined as a definite diagnosis of osteoporosis in live samples. It is necessary to check the estrogen receptors in the bones to see the impact of festrogen like from *Nigella sativa.*

Conflict of Interest
The authors declare no conflict of interest

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