Enhancement of Vitamin D$_2$ Levels in *Pleurotus ostreatus* Using Ultraviolet Irradiation and Assessing Its Effect on Dexamethasone-Induced Osteoporosis in Mice

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**ABSTRACT**

Mushrooms are a dietary source of vitamin D$_2$ due to their high ergosterol content, which can be converted into vitamin D$_2$ after exposure to ultraviolet (UV) irradiation. Several reports have shown that regular consumption of UVB-irradiated mushrooms is effective in increasing bone calcification and modulation of host immunity. Therefore, this study aimed to enhance vitamin D$_2$ formation in oyster mushrooms (*Pleurotus ostreatus*) using UVB irradiation and assess its effect on dexamethasone-induced osteoporosis in mice. Oyster mushrooms were irradiated with a UVB lamp at doses ranging from 8.01 J/cm$^2$ - 24.03 J/cm$^2$. Vitamin D$_2$ levels were then measured using high-performance liquid chromatography (HPLC) with calciferol as the standard. The powder of vitamin D$_2$-enriched oyster mushrooms powder was then referred to as Oyster-D. Osteoporosis in BALB/c mice was induced using Dexamethasone at a dose of 0.0029 mg/20 g BW, orally for 30 days. The number of osteoclasts, osteoblasts, and osteocytes in the femur was assessed using hematoxylin-eosin staining, while the serum calcium levels were measured with spectrophotometry. The results showed that UVB irradiation with a dose of 48.06 J/cm$^2$ yielded the highest vitamin D$_2$ concentration at 11.333 µg/g. Furthermore, there was a significant increase in the number of osteocytes, osteoblasts, and serum calcium levels, while a reduction was observed in the number of osteoclasts. Based on these findings, UVB irradiation increased vitamin D$_2$ levels, and vitamin D$_2$-enriched oyster mushrooms had potential anti-osteoporosis effects.

**Keywords:** Vitamin D; *Pleurotus ostreatus*; UV irradiation; Osteoporosis.

**INTRODUCTION**

Vitamin D deficiency is a significant global health concern (Holick, 1996), with prevalence rates of 24%, 37%, and 40% in the United States, Canada, and Europe, respectively. Several studies have identified various factors contributing to its occurrence, including insufficient sun exposure due to the use of sunscreens that inhibit the nutrient’s synthesis in the skin. Furthermore, apart from this condition, prolonged or excessive consumption of dexamethasone, a glucocorticoid, can lead to decreased bone density. The expression of insulin-like growth factor-I has been reported to be inhibited by glucocorticoids, directly and indirectly reducing the activity of osteoblasts (Cashman, 2020; Cashman *et al*., 2016; Sarafin *et al*., 2015; Schleicher *et al*., 2016). This inhibition stimulates bone resorption, initiating bone loss in response to exposure to these compounds. The impediment of bone formation can disrupt bone remodeling, leading to an increased risk of fractures and decreased bone density (Canalis, 2003). Osteoporosis is a common metabolic disease characterized by decreased bone density, thereby necessitating careful attention (Giustina *et al*., 2018).

Mushrooms are edible macrofungi containing several vitamins and are often used in various traditional medicines (Valverde *et al*., 2015; Hasanah *et al*., 2023). These microfungi contain ergosterol, which can be converted into vitamin D$_2$ when exposed to ultraviolet (UV)
radiation (Sánchez, 2017). UVB irradiation has been proven to be effective in augmenting vitamin D2 levels in Agaricus bisporus and other cultivated mushrooms, including the white oyster variant. The use of UVB-irradiated mushrooms is an essential and cost-effective method for preventing vitamin D deficiency (Roberts et al., 2008). Exposure to UVB breaks the bonds in ergosterol, specifically in C9-C10, leading to the formation of an unstable intermediate "pre-vitamin D2". Subsequently, a thermally catalyzed mechanism converts pre-vitamin D2 to vitamin D2 (Chauhan et al., 2023). Alshammaa reported that white oyster mushrooms contained up to 48.27% more ergosterol per gram than button mushrooms, with only 27.6% (Alshammaa, 2017). This study suggested that white oyster mushrooms (Pleurotus ostreatus) could produce more vitamin D2 after exposure to UV radiation. Ergosterol conversion is also determined by the UV exposure, the UV spectrum, the UV position of fungi, and the moisture content. When exposed to UVB, the gills of the fungus exhibits the highest conversion to vitamin D2 (Jasinghe & Perera, 2005; Roberts et al., 2008).

The duration of UVB exposure is a major factor influencing vitamin D2 formation in mushrooms. When a fungus is exposed to UV radiation for an extended period, it increases the amounts of vitamin D2 (Chauhan et al., 2023). Vitamin D levels in the body have been reported to have a significant effect on calcium metabolism (Holick, 1996). Calcium, as the main component of bones, is essential for maintaining bone strength and density (Giustina et al., 2018). Furthermore, consuming oyster mushrooms enriched with vitamin D2 and calcium can be an effective strategy for minimizing the risk of osteoporosis (Valverde et al., 2015). Vitamin D plays a key role in the absorption of calcium from the digestive tract into the bloodstream. Several studies have shown that inadequate levels can impair calcium absorption from the food calcium irrespective of the adequacy of Ca (Chauhan et al., 2023). According to a previous study, vitamin D also helps to regulate Ca balance in the body. When calcium levels in the blood are too low, it stimulates Ca absorption from the small intestine, and vice versa (Cashman, 2020).

Calcium is the main component of bones and teeth (Giustina et al., 2018), and adequate levels in the blood are crucial for maintaining bone strength and density. When calcium levels in the blood are too low, the body resorts to drawing Ca from the bones to meet its physiological needs. This can lead to decreased bone density and an increased risk of osteoporosis (Cashman et al., 2016). Previous studies have also shown that blood Ca levels affect the production and release of parathyroid hormone. Furthermore, parathyroid hormone plays a crucial role in regulating blood Ca levels, and vitamin D influences the body's response to the hormone (Canalis, 2003).

Based on these findings, there is a close link between vitamin D and calcium levels in the blood, underscoring their concerted role in maintaining healthy bones, muscle function, and a well-functioning nervous system (Roberts et al., 2008). A deficiency in either vitamin D or calcium can lead to bone health problems and affect overall bodily function. This shows that it is important to evaluate blood calcium levels in relation to the anti-osteoporosis effect of vitamin D-enriched oyster mushroom powder. Therefore, this study aimed to enhance the vitamin D level of oyster mushrooms (Pleurotus ostreatus) using UVB irradiation and examine its effect on osteoclasts, osteoblasts, osteocytes, and serum calcium levels in dexamethasone-induced BALB/c mice.

**MATERIALS AND METHODS**

**UVB Irradiation of Oyster Mushrooms**

Fresh oyster mushrooms were purchased from a local farm located in Semarang, Indonesia. The cleaned oyster mushrooms were chopped into small pieces and placed on a tray (flat container). The samples were divided into four groups and exposed to UVB radiation (Narrowband UVB lamp TL-F72, 100W/12, length 106 cm). Furthermore, groups 1, 2, 3, and 4 were exposed to doses of 8.01, 16.02, 24.03, and 48.06 J/cm2, respectively. Mushrooms that were treated with UVB radiation were dried in a drying cupboard at a temperature of 40°C for 24 hours. The dried samples were ground with a grinder into a powder and sieved with a 120 mesh sieve.

**HPLC analysis of vitamin D2 levels**

The vitamin D2 content of UVB-irradiated oyster mushrooms was determined using a method described by Koyyalamudi, et al. (2009) with a few modifications. The sample was then saponified and the residue was dissolved in 10 ml ethanol (95%), followed by filtration through a 0.45 nm filter. A total of 20 microliters of the filtered sample with a concentration of 100 µg /mL was injected into the HPLC system (Waters 2690; Waters Corp., USA).
Furthermore, the HPLC system used the following components, including a UV-486 detector, a C18 column (250 × 2.00 mm, Waters Corp., United States), a mobile phase of acetonitrile/methanol (75:25, v/v) at a flow rate of 1.0 mL/minute, UV detection performed at 264 nm, a 10 μl injection, and a temperature of 30°C. The vitamin D₂ content was determined by comparing the retention times with those of calciferol standards (abcam, ab143588, UK) and quantified based on a calibration curve with analyte concentrations ranging from 5 to 40 μg/mL of vitamin D₂ (Koyyalamudi et al., 2009). Oyster mushroom powder with the highest vitamin D content was then referred to as Oyster-D.

**Animals and treatment**

Male BALB/c mice (2-3-month-old, 30 g) were obtained from the Integrated Research and Testing Laboratory (LPPT) (Yogyakarta, Indonesia). All mice were housed in cages at a room temperature of 23°C ± 1°C.°C, with 55% ± 5% relative humidity, 12 h illumination period, and alternating light and dark periods. Furthermore, the animal samples received water and a standard diet with libitum. The sample size was determined using the Federer formula \((t - 1) (n - 1) \geq 15\) with group numbers \(t\) of 6, leading to a minimum sample size of ≥4 for each group \(n\). The mice underwent 2 weeks of acclimatization before diet-based grouping was carried out. A total of 25 mice were randomly divided into 5 groups, namely a) Normal, b) Negative control induced with dexamethasone and treated with aquades as a placebo, c) induced with dexamethasone, and treated with Oyster-D powder (19.5 IU/30g BW), d) induced with dexamethasone, and treated with Oyster-D powder (31.2 IU / 30 g BW), and e) induced with dexamethasone, and treated with Oyster-D powder (39 IU/30g BW). All groups were fed with standard diet ad libitum, and dexamethasone induction was given at a dose of 0.0029 mg/20 g BW, orally for 30 days.

The study protocol was approved by the bioethical committee of Universitas Islam Sultan Agung (No. 428/XII/2021/bioethics committee).

**Serum Calcium Analysis**

Whole blood (approximately 1 ml) was collected in a collection tube and the serum was separated by centrifugation at 15,000 rpm for 10 min at 4 °C. All collected samples were subjected to a spectrophotometry analysis, and serum calcium levels were analyzed using a Microlab 300 spectrophotometer at a wavelength of 587 nm.

**Histological analysis of the femur**

The separated femur of each mouse was fixed in 10% neutral buffered formalin, femur trochlea head regions were longitudinally trimmed, embedded in paraffin, sectioned in 3–4 μm, and stained with hematoxylin and eosin (HE). In each prepared histological sample, the histological profiles were interpreted. Furthermore, the osteoporosis parameters, including osteoclast, osteoblrate, and osteocyte were estimated for bone.

**Statistical Analysis**

The statistical tests were performed using statistical software (SPSS, version 25.0, IBM Corporation, Armonk, NY, USA). Furthermore, the qualitative data were expressed as mean ± standard deviation (SD), and one-way ANOVA was used for multiple groups’ comparison. The least significant difference (LSD) test was used for comparison of the mean value of each group, and a \(P< 0.05\) was considered statistically significant.

**RESULTS AND DISCUSSION**

**Vitamin D₂ level Enhancement of UVB-irradiated oyster mushrooms**

The determination of vitamin D₂ levels in oyster mushrooms was carried out using High-Performance Liquid Chromatography (HPLC) with standard calciferol (vitamin D2) from ABCAM. The standard curve equation \(Y = 29463.0142x - 12708.6676\) was obtained, and the value of \(R\) was 0.9999. Furthermore, the results showed that UVB treatment led to an increased content of vitamin D₂ in the samples. Level observed in the 4 groups treated with different doses of UVB radiation was significantly higher compared to that of the normal (Table I). The highest level of 11.333 μg/g was found in the samples treated with 48.060 J/cm² of UVB-radiation.

**Serum Calcium Levels**

The highest serum Ca was found in the group treated with 39.0 IU of Oyster-D. Furthermore, the higher the dose of Oyster D, the higher the serum Ca level (Table II).

**Histological analysis of the femur**

The microscopic appearance of the femoral biopsy of dexamethasone-induced mice after treatment with Oyster-D (Figure 1).
The mean number of osteoclasts, osteoblasts, and osteocytes (Table III). According to the data, the dexamethasone-induced mice group without Oyster-D treatment had the largest number of osteoclasts compared to others. Furthermore, the Oyster-D treatment group receiving 39 IU had the highest number of osteoblasts compared to the untreated group (Table IV).

Table I. Mean of Vitamin D$_2$ levels of oyster mushroom at various doses of UVB-radiation.

<table>
<thead>
<tr>
<th>Without UVB-radiation</th>
<th>Dose of UVB-radiation (J/cm$^2$)</th>
<th>8.01</th>
<th>16.02</th>
<th>24.03</th>
<th>48.06</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin D$_2$ levels ± SD (µg/g) n=3</td>
<td>1.666±0.901</td>
<td>6.099±1.731</td>
<td>8.144±0.619</td>
<td>9.107±1.953</td>
<td>11.333±0.830</td>
</tr>
</tbody>
</table>

Table II. LSD Post Hoc of mean differences of Vitamin D$_2$ levels of oyster mushroom between various doses of UVB-radiation.

<table>
<thead>
<tr>
<th>Between group</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>UVB-radiation 8.01 J/cm$^2$</td>
<td>0.007*</td>
</tr>
<tr>
<td>UVB-radiation 16.02 J/cm$^2$</td>
<td>0.001*</td>
</tr>
<tr>
<td>UVB-radiation 24.03 J/cm$^2$</td>
<td>0.000*</td>
</tr>
<tr>
<td>UVB-radiation 48.06 J/cm$^2$</td>
<td>0.000*</td>
</tr>
<tr>
<td>UVB-radiation 16.02 J/cm$^2$</td>
<td>0.110</td>
</tr>
<tr>
<td>UVB-radiation 24.03 J/cm$^2$</td>
<td>0.031*</td>
</tr>
<tr>
<td>UVB-radiation 48.06 J/cm$^2$</td>
<td>0.001*</td>
</tr>
<tr>
<td>UVB-radiation 16.02 J/cm$^2$</td>
<td>0.444</td>
</tr>
<tr>
<td>UVB-radiation 24.03 J/cm$^2$</td>
<td>0.024*</td>
</tr>
<tr>
<td>UVB-radiation 48.06 J/cm$^2$</td>
<td>0.031*</td>
</tr>
</tbody>
</table>

Figure 1. Microscopic image of dexamethasone-induced mouse femoral biopsy with Hematoxylin-Eosin staining at 400x magnification. (A) osteoblasts; (B) osteocytes; (C) osteoclasts. (1) normal control, (2) without Oyster-D treatment, (3) 19.5 IU/30g BW Oyster-D treatment, (4) 31.2 IU/30g BW Oyster-D treatment, and (5) 39 IU/30g BW Oyster-D treatment.
This study enhanced vitamin D$_2$ formation in edible mushrooms using UV irradiation. Furthermore, the assessment of the effect of enhanced vitamin D$_2$ mushrooms in dexamethasone-induced osteoporosis in mice was also carried out.

HPLC was used to determine the level of the nutrient in white oyster mushrooms (Pleurotus ostreatus) with calciferol (vitamin D$_2$) as the standard. The findings showed a linear association between the UVB exposure period and vitamin D$_2$ levels. UVB irradiation at a high rate significantly increased the levels of the nutrient (Wu & Ahn, 2014). The levels in Pleurotus ostreatus increased because its ergosterol was converted into vitamin D$_2$ when exposed to ultraviolet (UV) radiation (Sánchez, 2017; Phillips et al., 2011). The observed differences in vitamin D$_2$ levels were due to the distinction of cultivars, moisture contents, UVB exposure period, temperature, mushroom preparation, and operating conditions.

According to Heo et al. (2020), mushroom powder irradiated by UVB rays contained up to 8.19 µg/g of vitamin D$_2$. The results showed that group 4 had the highest levels at 9.1074 µg/g, with extended irradiation of 45 minutes. Based on these findings, UVB irradiation affected the reaction of ergosterol related to the formation of vitamin D$_2$. Ergosterol was the most abundant content, and when irradiated with UVB, it underwent photolysis to produce pro-vitamin, which slowly isomerized to vitamin D$_2$ (Phillips et al., 2011; Villares et al., 2014).

Furthermore, before becoming vitamin D$_2$, system heating was performed using a reflux (Ahlborn et al., 2018; Zhang et al., 2018). Vitamin D$_2$ levels in white oyster mushrooms increased because ergosterol was exposed to UV radiation. When the compound was exposed to UV radiation, it underwent a bond breaking at C$_9$-C$_10$ accompanied by an isomerization reaction to form pro-vitamin D$_2$. The steric interaction between the methyl group’s C6 and C19 rings made pro-vitamin D$_2$ unstable, leading to single and s-trans bonds. The more the white oyster mushroom was exposed to UVB, the more the amount of cis and trans isomers produced, leading to an increase in vitamin D$_2$ (Chauhan et al., 2023).

The longer the UVB irradiation exposure time, the greater the energy exerted on oyster mushrooms, thereby affecting vitamin D$_2$ levels. A
high energy of 48.06 J/cm² caused a vitamin D₂ level of 11.33 ± 0.830 µg/g. After UVB irradiation, high levels caused the amount of ergosterol in fungal cell walls to decrease, impairing their function. Several studies had shown that ergosterol played a role in membrane fluidity and permeability (Iwaki et al., 2008). Vitamin D₂ levels in the present study were higher compared to the values reported by Heo et al. (2020) using superfine white button mushroom powder (7.1 ± 0.3 µg/g) (Bikle, 2014). In 94.28 minutes of UVB exposure, Keflie et al., 2019 obtained 315.5±8.2 µg/g of vitamin D₂ from white oyster mushroom (Pleurotus ostreatus).

According to the analysis of the number of osteocytes, osteoblasts, osteoclasts, and serum calcium levels, the Oyster-D treatment of 39.0 IU had a significant effect on the growth of osteoblasts compared to the untreated group. Glucocorticoids or corticosteroids could reduce osteoblasts and osteocytes through apoptosis, which was caused by caspase-3 activation. The anti-anabolic effect of glucocorticoids reduced IGF2, which functioned as a local regulator of osteoblast function. Glucocorticoids reduced the expression of OPG, causing the osteoclast lifespan to be elongated compared to osteoblasts and increasing the resorption of the bone matrix (Briot & Roux, 2015).

The presence of ergocalciferol (vitamin D₂) in white oyster mushrooms stimulated the growth of osteoblasts. Ergocalciferol was metabolized and transformed to 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), which bound to receptors in the bone to regulate mineralization (Pike et al., 2017). 1,25(OH)₂D₃ bound to VDR (Vitamin D Receptors) near each tissue through different genome genes, leading to various effects (Bikle, 2014; Pike et al., 2017). Previous studies had reported the effect of vitamin D₂-fortified mushrooms on circulating levels of 25(OH)D and parathyroid hormone (PTH). Distal tomographic microcomputer (micro-CT) analysis of the femur was used to assess the ability of the mushrooms to support bone growth, and histological evidence of soft tissue calcification was assessed to ensure the safety of high vitamin D₂ levels of white button mushrooms given to mice daily for ten weeks (Calvo et al., 2013).

Osteoblasts differentiated when transcription factors, such as osterix (Osx), runt-related transcription factor 2 (RUNX-2), and the WNT pathway were activated (Berardi et al., 2021; Vega et al., 2017). When 1,25-dihydroxy vitamin D bound to VDR at the RUNX-2 binding site on bone cells, it induced osteoblast transcription and differentiation (Bikle, 2014; Corrado et al., 2017). Osteoblasts produced OPG (osteoprotegerin), which served as a competitive inhibitor of RANK, preventing RANKL from binding to RANK. Osteoclastogenesis was a process that often led to a reduction in the number of osteoclasts. Vitamin D, calcitonin, estrogen, parathyroid hormone (PTH), serotonin, and leptin played essential role in osteoblast OPG expression. Furthermore, decreased osteoclastogenesis caused the production of osteoclast cells to reduce (Christakos et al., 2015).

Increasing the dosage of Oyster-D to 19.5-39.0 IU, led to an increase in the number of osteoblasts, osteocytes, and calcium serum levels, as well as a decrease in osteoclasts. The findings showed that exposing white oyster mushrooms to UVB radiation led to osteoporosis repair. This result had the potential to be expanded to prevent osteoporosis and continued into clinical studies to determine effectiveness, tolerability, and quality of life in osteoporosis patients.

In this study, there was evidence that osteocyte cells affected not only bone remodeling but could also improve bone structure. Osteocytes were located in areas close to the micro-damage where these processes occurred through apoptosis or were caused by other pathological factors. When increased bone remodeling due to pathological or physiological conditions that affected the bone structure occurred, osteocytes often increased the release of RANKL by the sclerotin protein, leading to the formation of osteoclasts. Several studies had shown that both substances had an essential role in bone remodeling (Kular et al., 2012; Prideaux et al., 2016). In this study, dexamethasone was used to induce osteoporosis in mice, which could be recognized in normal mice by transverse bending lumbar vertebrae. This was because mice had already developed osteoporosis or bone loss (Kim et al., 2021). Decreased blood calcium in osteoporosis was associated with the regulation of parathyroid and thyroid hormones that affected bone production and resorption. Calcium and vitamin D deficiency often affected the hormones regulating calcium. Uncontrolled calcium levels promoted parathyroid hormone secretion, leading to excessive bone remodeling that caused a loss in bone density and an increased risk of fracture (Felsenfeld et al., 2014).

The limitation of this study was the lack of bone density quantification, which led to inaccuracy in describing osteoporosis repair. However, measurements of the number of
Vitamin D Levels in Pleurotus ostreatus

osteoblasts, osteocytes, osteoclasts, and serum calcium levels were sufficient to represent a repair in the symptoms of osteoporosis.

CONCLUSION

In conclusion, this study showed the effect of 4 different doses of UVB irradiation on the vitamin D content of oyster mushrooms. The UVB irradiation significantly increased D$_2$ levels of oyster mushrooms up to 11.333 µg/g. Furthermore, the administration of vitamin D$_2$ 19.5 – 39.0 IU significantly increased serum calcium, the number of osteocytes and osteoblasts, as well as decreased the number of osteoclasts. Further studies were needed to ascertain the effects of UVB-irradiated mushrooms on bone density.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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