Original Research

The Effects of *Cosmos caudatus* (Ulam Raja) Supplementation on Serum and Bone Minerals Levels in Ovariectomized Rats

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**Summary.** Osteoporosis is a consequence of estrogen deficiency and has been associated with oxidative stress. *Cosmos caudatus* (ulam raja), a local plant, has been shown to improve bone histomorphometry in ovariectomized rats. This study further determined the effects of *Cosmos caudatus* on serum and bone minerals levels in ovariectomised rats. Female Sprague Dawley rats were divided into 4 groups, (I) sham operated (SO) (II) ovariectomised (OVX) (III) ovariectomised + 500mg/kg *Cosmos caudatus* extract (CC) and (IV) ovariectomised + estrogen 64.5 ug/kg of rat weight (E2). Rats were treated for 8 weeks. Ovariectomy reduced serum calcium and phosphate levels compared to SO group (*p* = 0.042 for both) but did not cause any changes in bone minerals levels. Groups treated with CC and E2 showed significant increase in serum calcium levels (*p* = 0.018 for both) and serum phosphate levels (*p* = 0.016, *p* = 0.002 respectively) compared to ovariectomized group. However, both *Cosmos caudatus* and E2 did not affect the minerals levels of femur and fifth lumbar bones (L5). In conclusion, *Cosmos caudatus* is comparable to estrogen in improving estrogen deficient-induced osteoporosis by maintaining serum minerals homeostasis.

**Industrial relevance.** Osteoporosis has grown to be a major concern especially among postmenopausal women. Studies have been done extensively in finding alternative treatment for osteoporosis. This is in view of the various side effects caused by the existing treatment agents such as estrogen. *Cosmos caudatus*, one of the local plants, has been explored and was observed to improve bone histomorphometric in ovariectomised rats. It has also been shown to enhance fracture healing in ovariectomized rats. This study further examined the effects of *Cosmos caudatus* on minerals homeostasis which further proved the effectiveness of this plant as an alternative treatment for osteoporosis.

**Keywords.** *Cosmos caudatus*; estrogen; bone minerals; serum minerals; ovariectomy

**INTRODUCTION**

Osteoporosis is a serious public health concern worldwide. The prevalence of osteoporosis cases occurring in Malaysia is 24.1% in 2005. In Hong Kong, it usually occurs in 10 per 1000 population in both genders above the age of 70 years old. It was reported that the prevalence of osteoporosis was 12.6% in Thailand, 16.1% in China and 10.08% in Taiwan. Asian population has higher prevalence of osteoporosis compared to the western countries. This may be due to the fact that Asians have lower body mass index and shorter height.

One of the causes of osteoporosis is oxidative stress. Oxidative stress is associated with the decline in estrogen hormone during menopause which leads to an increase in inflammatory cytokines such as tumor necrosis factor-α and interleukins. Protection against osteoporosis by statins is linked to a reduction of oxidative stress and restoration of nitric oxide formation in aged and ovariectomized rats. This shows how oxidative stress, namely free radicals, contributes to osteoporosis.

Studies have been done to explore new medicinal agents from amongst the various natural products available which possess several beneficial properties such as antioxidants and anti-inflammatory. One natural product of interest is *Cosmos caudatus* (Figure 1), a popular herb in Malaysia and known as ulam raja by the locals. The plant has several beneficial effects such as to enhance blood circulation, act as anti-aging agent, able to reduce body heat, strengthen bone marrow and treat infections associated with pathogenic microorganisms. *Cosmos caudatus* embodies hexane and dichloromethane extracts, which have an inhibitory potential towards few enzymes related to hyperglycemia and hypertension prevention. Its leaves have been shown to possess alpha-glucosidase inhibitory activity which may benefits type 2 diabetes mellitus. In a randomized controlled trial, subjects who were given *C. caudatus* for eight weeks showed significant improvement in insulin resistance and insulin sensitivity.
As many other edible vegetables in Malaysia, *C. caudatus* also has latent effect of anti-tumor promotion. *Cosmos caudatus* also contain polyphenols, carotenoids, ascorbic acid, and protein. Its ascorbic acid content is more than 100 mg ascorbic acid/100 g. In addition, *C. caudatus* has a number of proanthocyanidins that contributes to its major antioxidant effects.

Previous study has shown that *C. caudatus* improved bone histomorphometric parameters in ovariectomized rats. The present study further investigated the mechanism of *C. caudatus* in improving bone structure of ovariectomized rats by looking at the mineral levels in the serum and the bones.

### MATERIALS AND METHODS

**Animals and treatment.** Thirty-two female Sprague Dawley rats, aged three months old, weighing 200-250g, were randomly divided into four groups with eight rats in each group. The rats were obtained from Laboratory Animal Unit, Faculty of Medicine, Universiti Kebangsaan Malaysia. The treatment groups and protocol are shown in Table 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Group symbol</th>
<th>Oral administration</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Sham-operated control group</td>
<td>SO</td>
<td>Normal saline</td>
<td>8 weeks</td>
</tr>
<tr>
<td>II</td>
<td>Ovariectomized control group</td>
<td>OVX</td>
<td>Normal saline</td>
<td>8 weeks</td>
</tr>
<tr>
<td>III</td>
<td>Ovariectomized + <em>C. caudatus</em> group</td>
<td>CC</td>
<td>500 mg/kg <em>C. caudatus</em> extract</td>
<td>8 weeks</td>
</tr>
<tr>
<td>IV</td>
<td>Ovariectomized + estrogen</td>
<td>E2</td>
<td>64.5 μg/kg estrogen</td>
<td>8 weeks</td>
</tr>
</tbody>
</table>

The sham and ovariectomy procedures were done according to the previously described protocol. This study was approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (FP/FAR/2012/NORAZLINA/23-MAY/443-MAY-2012-MAY-2013).

**Diets and treatment.** During the experimental period, all the rats were fed with normal rat chow (Gold Coin, Malaysia) and deionised water ad libitum. An aqueous extract of *C. caudatus* with the concentration of 500g/300mL was prepared by School of Chemical Sciences & Food Technology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia using water extraction method which was previously described by Huda-Faujan et al. Briefly, the edible portions of the fresh samples were cleaned and washed. Then the samples were air-dried using a fan. Samples were then blended with distilled water in a ratio of 3:1 (water: herb) and filtered. The filtrates were then evaporated using a vacuum evaporator at 50°C to produce a viscous mass.

The 500mg/kg dose was prepared for each rat depending on the weight of the rat. For every 200 g of rat, 0.06ml of *C. caudatus* extract (500g/300mL) was given. Estrogen (conjugated equine estrogen, Premarin-Wyeth, Canada) was prepared by crushing the tablet containing 0.625mg of conjugated estrogen which was then dissolved in deionized water. It was then administered to the rats orally at a dose of 64.5 μg/kg /day. The doses of *C. caudatus* and estrogen were chosen based on previous studies.

**Blood collection.** Blood was taken from orbital sinus of rats under general anesthesia at week 0 and week 8. To collect the blood, a capillary tube was inserted at the angle of 30 degree to the nose, into medial canthus of the eyes. Plasma was separated from blood by centrifuging and was stored in -70°C until analysis.

**Serum minerals analysis.** Colorimetric calcium detection kit (product number ab102505) from Abcam (United Kingdom) was used to determine serum calcium. Serum phosphate was determined by using Phosphate Assay Kit (Colorimetric), product number ab65622 purchased from Abcam (United Kingdom). The ELISA machine was used to read the absorbance in the samples.

**Bone extraction.** For bone sample, the rats were euthanized by overdose of diethyl ether at the end of treatment period. Left femur and fifth lumbar vertebrae (L5) were dissected and cleaned from all soft tissues. The bones were dried in an oven at 100°C for 24 hours and then ashed in furnace at 800°C for 12 hours. The ash was weighed and then dissolved in 3 ml nitric acid for further analysis. Femur and lumbar vertebrae were chosen because these bones represent the different types of bone, i.e. cortical and trabecular. The proportion of trabecular bone is higher compared to cortical bone in lumbar vertebra while femur is one of the bones which has high cortical content.
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Bone minerals analysis. Before bone analysis was done, lanthanum chloride stock was prepared by mixing 58.64g lanthanum oxide (Sigma USA) with 250ml HCL. Distilled water was then added to make the solution become 1L. The solution was stored in a dark bottle and was kept refrigerated. On the day of analysis, 20ml from the stock was mixed with distilled water to make up to IL solution.

During bone analysis, the bones were separated into two groups; femur and L5. Dilution for each bone was done differently. For femur, it was diluted 12500x by mixing 200µl sample (femur) with 4.8ml lanthanum chloride (solution A). Next, 10µl sample from solution A was taken and mixed with 4.99ml lanthanum chloride (duplicate was done). For L5, 100µl sample (L5) was mixed with 4.9ml lanthanum chloride (solution B) to get the dilution factor of 5000x. This was followed by mixing 50µl from solution B with 4.95ml lanthanum chloride (duplicate was done).

The parameters that were measured were the calcium and magnesium levels in the bones. The aid of a Shimadzu atomic absorption spectrometer, model AA-680 (Japan) was used to evaluate the calcium and magnesium levels in the bone.

Statistical analysis. Data were analysed by One-Way analysis of variance (ANOVA) test using the Statistical Package for Social Sciences (19.0.1; SPSS, Inc., Chicago, IL) software. The Tukey’s honestly significant difference test was selected as the post-hoc test. Data with pre and post values were analysed using Paired t test. Values are presented as mean ± standard error of mean (SEM). Differences at 5% level were considered significant.

RESULTS AND DISCUSSION

Serum mineral levels. Serum calcium levels were lower in ovariectomized group after eight weeks of treatment (0.40 ± 0.05) compared to sham-operated group (0.48 ± 0.03) (p<0.05) (Figure 2). The ovariectomized rats also had lower serum calcium compared to CC and E2 groups (0.49 ± 0.02 and 0.49 ± 0.01 respectively) (p<0.05). For serum phosphate levels, ovariectomized rats had lower values while Cosmos caudatus and estrogen groups had higher values at week 8 compared to week 0 (p<0.05) (Figure 3). In addition, after eight weeks of treatment, serum phosphate levels were lower in ovariectomized group compared to groups treated with C. caudatus and estrogen (p<0.05).

The reduction in serum calcium and phosphate levels in ovariectomized rats were in accordance with a previous study17. Another previous study on ovariectomised mice also observed reduction in phosphorus levels in the plasma18. These findings in
animals echo the human in which serum calcium was found to be significantly low in postmenopausal women than in the premenopausal women\(^1\). It was found that the imbalance in serum calcium in ovariectomized rats was associated with downregulation of epithelial calcium transport protein mRNA expression in the intestine\(^2\). On the contrary, serum phosphorus was observed to decline with age in premenopausal women but increased between the age of 46 and 60 years old\(^3\).

In addition, estrogen-deficiency induced by ovariectomy triggered an increase in oxidative stress and DNA damage\(^4\). It has also been shown that oxidative stress may play a role in the reduction of intestinal calcium absorption\(^5\). Thus, the increase in oxidative stress after ovariectomy may also contributes to the reduction in serum calcium levels seen in this study.

The rats treated with estrogen were able to restore the serum mineral levels back to its normal values. The conjugated estrogens act as hormone replacement therapy that can mimic the function of endogenous estrogen. Estrogen administration was shown to upregulate calcium influx channel which plays a role in duodenal calcium absorption\(^6\). However, conflicting results were observed in previous studies. Ovariectomized rats treated with ethinylestradiol for 12 weeks showed increased serum levels of calcium and phosphorus\(^2\). While another study did not find significant changes in serum calcium and phosphorus levels in ovariectomized rats treated with estrogen\(^2\). This may be due to the different dose being used and different duration of treatment.

The serum calcium and phosphate levels were significantly increased in group treated with 500 mg/kg \textit{C. caudatus}. \textit{Cosmos caudatus} is rich in mineral content and contains 270 mg calcium, 37 mg phosphorus, 4.6 mg iron, 4mg sodium, 426 mg potassium per 100 g of the plant\(^7\). Furthermore, \textit{C. caudatus} has a number of proanthocyanidins and ascorbic acid that contribute to its major antioxidant effects\(^8\). These properties of \textit{C. caudatus} may be responsible in reversing the serum mineral effects induced by ovariectomy as seen in this study.

\textit{Bone mineral levels}. There were no significant findings in calcium levels in femur and L5 bones between all groups (Figures 4 and 5). Similar observations were seen in magnesium levels in both femur and L5 bones (Figures 6 and 7).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4}
\caption{Femoral bone calcium content in ovariectomized rats treated with \textit{Cosmos caudatus} and estrogen. [SO]: Sham-operated rats; [OVX]: Ovariectomized rats; [CC]: Ovariectomized rats treated with 500 mg/kg \textit{Cosmos caudatus}; [E2]: Ovariectomized rats treated with 64.5 μg/kg Estrogen. All values are expressed as mean ± SEM.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig5}
\caption{Fifth lumbar bone calcium content in ovariectomized rats treated with \textit{Cosmos caudatus} and estrogen. [SO]: Sham-operated rats; [OVX]: Ovariectomized rats; [CC]: Ovariectomized rats treated with 500 mg/kg \textit{Cosmos caudatus}; [E2]: Ovariectomized rats treated with 64.5 μg/kg Estrogen. All values are expressed as mean ± SEM.}
\end{figure}
It has been shown that bone calcium and magnesium levels were decreased in ovariecctomized rats\textsuperscript{17}. In another study, bone calcium content was reduced in rats sixteen weeks after ovariecctomy compared to the control group\textsuperscript{29}. However, in the present study, no significant findings were observed in bone mineral levels. Similar findings were seen in a study which reported that one month or two months post-ovariecctomy did not change bone mineral elements even though differences were seen in histomorphometric parameters\textsuperscript{30}. These findings suggest that measurement at different time points may yield different observations.

In the present study, treatment was started eight weeks after ovariecctomy which lasted for another eight weeks. Previous studies which used various treatment modalities have applied different time points to initiate treatment after the ovariecctomy. In one study, treatment of ovariecctomized rats was started 4 weeks after ovariecctomy\textsuperscript{31}. In another study, the rats were given only one week of recovery after ovariecctomy, before treatment started\textsuperscript{32}. The discrepancies in bone mineral levels seen in the present study compared to previous studies may be due to the different time point applied.

This study also did not observe any increment in bone mineral levels in group treated with estrogen. This contradicts with the findings of a previous study which observed improved bone calcium content upon treatment with estrogen in ovariecctomized rats\textsuperscript{33}. The previous study treated the rats for eight weeks which was similar to the present study. However, their study started the treatment two weeks after ovariecctomy as opposed to the present study, eight weeks after ovariecctomy. This may be the reason why the effects of estrogen on bone mineral levels were not apparent.

Previously, \textit{C. caudatus} was found to be able to reverse the adverse effects of ovariecctomy on structural bone histomorphometric parameters, such as trabecular bone volume (BV/TV), trabecular number (Tb.N), and trabecular separation (Tb.Sp)\textsuperscript{12}. \textit{Cosmos caudatus} has also been shown to improve dynamic bone histomorphometric parameters in ovariecctomised rats\textsuperscript{34}. In addition, \textit{C. caudatus} has shown potential in improving fracture healing by increasing bone biomechanical parameters in ovariecctomized rats\textsuperscript{35}. Despite the beneficial effects of \textit{C. caudatus} on bone seen in the previous studies, the present study failed to find any positive changes on the bone mineral levels. Factors other than bone mineral levels such as bone remodeling modulation may contribute to the improvements seen in the previous studies. It has been shown that \textit{C. caudatus} prevented the increment of bone resorption in ovariecctomized rats\textsuperscript{36}. In conclusion, \textit{C. caudatus} has the potential in protecting bone from bone loss by restoring the imbalance in serum mineral levels induced by ovariecctomy. The effects of \textit{C. Caudatus} was comparable to that of estrogen.
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REFERENCES

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