SHORT COMMUNICATION

Influence of *Piper sarmentosum* Aqueous Extract on the Expression of Osteocalcin in Glucocorticoid-induced Osteoporotic Rats

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ABSTRACT

Secondary osteoporosis is mainly caused by prolong used of glucocorticoid treatment. The *Piper sarmentosum* leaf aqueous extract was found to exhibit bone formatting osteocalcin activity against Dexamethasone induced osteoporotic rats. Thirty-two Sprague-Dawley rats were divided equally into four groups - G1: Sham-operated control group given intramuscular (IM) olive oil as vehicle and normal saline orally as vehicle; G2: Adrenalectomized (Adrx) control group given IM dexamethasone (DEX) (120 µg/kg/day) and normal saline orally as vehicle; G3: Adrx group given IM DEX (120 µg/kg/day) and aqueous extract of *Piper sarmentosum* leaves (125 mg/kg/day) orally; and G4: Adrx group given IM DEX (120 µg/kg/day) and glycyrrhizic acid (GCA) (120 mg/kg/day) orally. Immunohistochemical method with gold labelling was used to label the osteocalcin protein. Silver brightener was used, sprinkled on gold with a size of 5 nm so that the resulting image can be seen more clearly using a light microscope. The osteocalcin protein was measured quantitatively based on nomenclature report of the ASBMR Histomorphometry Committee (American Society for Bone Mineral Research). The activity shown by immunohisto-gold expression and localization of osteocalcin was comparable with the reference, glycyrrhizic acid, a potent inhibitor of 11β-hydroxysteroid dehydrogenase enzyme in RANKL-OPG pathway. As a conclusion, *Piper sarmentosum* may one day be utilized as an alternate treatment for individuals receiving long-term glucocorticoid therapy to prevent osteoporosis, therefore osteoporotic fractures.
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INTRODUCTION

Piper sarmentosum (Ps) is a creeping continental herb, locally known as "daun kadok" used mainly for flavouring of local cuisine [1]. It is used to alleviate headaches, toothaches, cough, asthma, and fever, according to Malay tradition [2]. More than 100 phytochemicals, including steroids, flavonoids, alkaloids, and essential oils, have been discovered in Ps. Plants naturally contain phenolic chemicals, including flavonoids (rutin and vitexin in aqueous extract), which have pharmacology effect such as antioxidant, antihypertensive, antibacterial, and antifungal properties based on previous studies [3]. The ferric reducing antioxidant test (FRAP), DDPH free radical scavenging assay, and β-carotene bleaching assay all showed that different portions of Ps active chemicals had antioxidative capabilities. [4]. It has been reported that the plant contains pharmacological characteristics [5] such as anti-tuberculous [6], anti-cancer [3], hypoglycaemic [7], larvicidal activity [8], antioxidant [9,3], anti-atherosclerotic [10], anti-inflammatory and anti-pyretic activities [11], and promotion of fracture healing [12]. A study by Ramli et al. [13] on the relationship of Ps aqueous extract with 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) stress enzyme in osteoporotic-induced rats revealed an increment in 11β-HSD1 dehydrogenase activity and reduction in its expression. In our previous studies, we

revealed the anti-osteoporotic effect of the Ps aqueous extract by observing the changes in gene expression, histomorphometry and biomechanical strength, by modulating the 11β-HSD1 enzyme activity in RANKL-OPG (osteoprotegerin) pathway, as comparable to the enzyme inhibitor, glycyrrhizic acid (GCA) [14,15,16]. In this study, we evaluated the Ps aqueous extract action by osteocalcin immunohisto-gold labelling expression.

Dexamethasone is a very potent synthetic glucocorticoid that can bind to glucocorticoid receptors [17] and promote human osteoclast formation [18,19]. Dexamethasone use for a long time can cause secondary osteoporosis. Few studies have shown that glucocorticoids increased the osteoclasts' life span directly by decreasing the apoptosis of mature osteoclasts [20]. In vivo study showed that glucocorticoids prevent osteoblast cells from proliferating and differentiating [21], reduced the activity and maturation of osteoblast [22], and induced the apoptosis osteoblast and osteocyte [20].

MATERIALS AND METHODS

Preparation of *Piper sarmentosum* aqueous extract

Plants of the *Piper sarmentosum* (Ps) were collected from its wild environment near a river stream. A voucher specimen (UKMB 29851) was deposited in the Herbarium Unit, Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM). The leaves were dried in a continuous airflow oven, crushed, and then boiled with distilled water to make an aqueous extract. The aqueous solution was transferred to the Herbal Technology Centre of the Malaysian Forest Research Institute (FRIM) to be freeze dried into powder. In this study, 125 mg/kg *Piper sarmentosum* powder was dissolved in normal saline to be used as a preventive therapy.

Animals and treatment

The UKM Animal Breeding Centre provided Sprague-Dawley rats (200-250 g) of either sex with ethical approval from the UKM Research and Animal Ethics Committee (PP/ANAT/2010/ELVY/14-JULY/313-JULY-2010-MAY-2012). Adrenalectomy and sham procedures were done, and treatment began two weeks after the surgery. Dexamethasone was mixed in olive oil and given intramuscularly at a rate of 120 g/kg/day for eight weeks, six days a week. Glycyrrhizic acid (GCA) (120 mg/kg/day) and *Piper sarmentosum* (125 mg/kg/day) were dissolved in normal saline and given orally once daily, six days a week, for eight weeks. The animals were kept on a regular diet. The adrenalectomized rats were given normal saline to replace the salt loss due to mineralocorticoid deficit post-adrenalectomy, while the sham-operated rats were given tap water ad libitum.

The rats were placed into four groups, each with eight rats, and were given the following therapy: the adrenalectomized (Adrx) rats were given dexamethasone (120 µg/kg/day) intramuscularly, and sham-operated rats (Sham) were given intramuscular olive oil. Oral administration of vehicle normal saline was given to the Sham group, whereas the adrenalectomized group was divided into two groups and given either *Piper sarmentosum* (125 mg/kg/day) or GCA (120 mg/kg/day) (Adrx + Ps and Adrx + GCA groups respectively). The rats were sacrificed humanely after 8 weeks of treatment. The surrounding tissues were removed from the right femoral bones, which were then wrapped in gauze and coated in aluminium foil after being soaked in PBS 7.4 pH. The bones were stored at a temperature of -80°C until they were analyzed.

Osteocalcin expression and localization

Gold labelling method was used during the immunohistochemical process to label the osteocalcin protein. Silver brightener was used, sprinkled on gold with a size of 5 nm so that the resulting image can be seen more clearly using a light microscope. The osteocalcin protein was measured quantitatively using Weibel 2 lines. The nomenclature was based on a recent report of the ASBMR Histomorphometry Committee (American Society for Bone Mineral Research) [23] which was modified according to computational compatibility. Sample images are taken using a digital camera connected with a microscope. The image was enlarged at a magnification of 200 times. Osteocalcin labelled with gold appears to be blackish after being covered with silver enlightenment coloring (argentum nitrate). Osteocalcin calculation and localization involved mineralizing bone cells found on the surface of trabecular bone. From each sample, eight slides were chosen. Three areas for each slide are chosen for evaluation. The chosen metaphyseal areas were located 1 mm from the lateral cortex and 3 to 7 mm from the lowest point of the growth plate line. These areas were chosen because, during the process of bone remodelling, trabecular bone retardation would cause the bone mass of this part to decrease [24]. One sample is given a value based on the average of the three locations. For each group of parameters tested, the average of all eight samples was used as a value. Video-Test Master 4.0 computer software is used for calculation of osteocalcin expression (in percentage) as volume and surface area.

Statistical analysis

Statistical Package for the Social Sciences (SPSS) software programme version 26 was used to analyse the data. The distribution of the data was assessed using the Shapiro-Wilk normality test. The comparison between treatment groups was done using parametric statistics (ANOVA test) and Tukey's post-hoc test. The

mean and standard error of the mean (SEM) were used to express the data.

RESULTS AND DISCUSSION

After eight weeks of treatment, the volume and surface of osteocalcin in the Adrx + Dexa group did not differ substantially from the Sham group. Dexamethasone treatment significantly reduced the osteocalcin volume and surface of Adrx + Dexa group compared to Adrx + Ps group. Adrx + Ps and Adrx + GCA groups were not significantly differ from the Sham group (Figure 1).

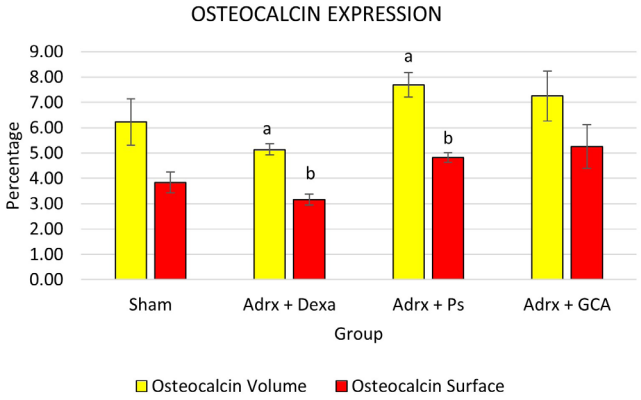

Figure 1 : Osteocalcin volume and surface after 8 weeks treatment. Sham: sham-operated group given normal saline as a treatment; Adrx + Dexa: adrx-control group given IM DEX 120 µg/kg/day; Adrx + Ps: Adrx group given IM DEX 120 µg/kg/day and *Piper sarmentosum* aqueous extract 125 mg/kg/day; and Adrx + GCA: Adrx group given IM DEX 120 µg/kg/day and GCA 120 mg/kg/day. The data is presented as a mean with SEM. At $p < 0.05$, the same alphabet indicates a significant difference between groups.

During osteocalcin immunohisto-gold localization, the trabecular bones of rats that received dexamethasone treatment in Adrx + Dexa group showed few silver-stained osteocalcin on the surface area compared to the other groups, as shown by arrow (Figure 2).

According to phytochemical study, *Piper sarmentosum* (Ps) leaf extract contains phenolic components such as naringenin, a natural antioxidant that scavenges superoxide [20]. Ps had a high phenolic content in both aqueous and boiled aqueous extractions [4]. Our previous study showed that Ps aqueous extract assist in osteoporotic fracture healing [12], strengthen the bone biomechanical properties [14], increased the osteoprotegerin (OPG) gene expression that modulate the RANKL-OPG pathway of bone remodelling [16] and improved the histomorphometry parameters [25].

Our present study showed that Ps aqueous extract significantly increased the osteocalcin protein expression in adrenalectomized rats given dexamethasone. In our prior study, we found a significant increament in

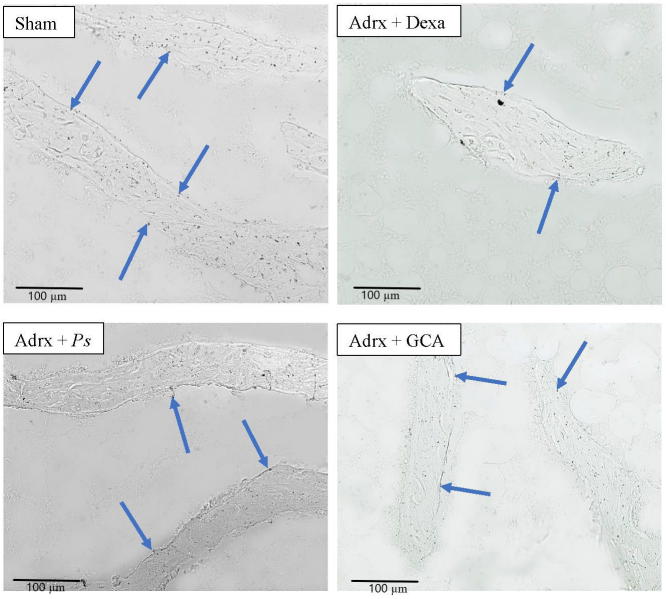

Figure 2 : Osteocalcin immunohisto-gold localization (blue arrow) after 8 weeks treatment (magnification 200x). Sham: sham-operated group given normal saline as a treatment; Adrx + Dexa: adrx-control group given IM DEX 120 µg/kg/day; Adrx + Ps: Adrx group given IM DEX 120 µg/kg/day and *Piper sarmentosum* aqueous extract 125 mg/kg/day; and Adrx + GCA: Adrx group given IM DEX 120 µg/kg/day and GCA 120 mg/kg/day.

the osteocalcin mRNA expression, a well-known bone formation indicator, in the glycyrrhizic acid (GCA) supplemented group, although no changes were seen in the Ps supplemented group. We also discovered that GCA influenced the RANKL/OPG mRNA expression ratio by stimulating OPG gene expression while inhibiting RANKL gene expression [16]. Thus, by reducing the activity of 11β -HSD1 reductase, GCA was able to limit bone resorption. 11β-HSD isoenzymes are known to be inhibited by glycyrrhizic acid by totally inhibiting the reductase activity of 11β-HSD1 [20]. This was associated with glucocorticoid-induced inhibition of bone-specific osteocalcin mRNA expression, which hinders the formation of the osteoblast phenotype [20]. In glucocorticoid-induced osteoporotic rats, Ps may play a role in regulating bone remodelling, but a higher dose or a longer treatment period may be required.

Piper sarmentosum has previously been shown to protect the bone against glucocorticoid-induced osteoporosis by modulating 11β-HSD1 expression and activity, resulting in bone resorption reduction [27,13]. *Piper sarmentosum* exhibited enhanced bone strength with better trabecular thickness, volume, and number, according to the biomechanical strength test and histomorphometry analysis [14,26]. *Piper sarmentosum* extract may potentially help with osteoporosis-related fractures [12]. As a result, *Piper sarmentosum* extract provided new promise for osteoporosis treatment [28].

CONCLUSION

Aqueous extract from *Piper sarmentosum* (Ps) leaves was able to improve the microstructure and cellular elements of glucocorticoid-induced osteoporotic bones in rats, by having a significant positive effect in reducing bone resorption in dexamethasone-treated rats through boosting osteocalcin expression. As a result, *Piper sarmentosum* extract could be used as a bone protective agent for patients on prolonged glucocorticoid therapy. However, further study should be done involving more specific flavonoids of aqueous extract particularly rutin and vitexin, using in-vitro and in-vivo studies.

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