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ANTIOSTEOPOROTIC EFFECT OF THE RHIZOME OF *DRYNARIA FORTUNEI* (KUNZE) (POLYPODIACEAE) WITH SPECIAL EMPHASIS ON ITS MODES OF ACTION

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Abstract: One of the elderly age health issues is a silent epidemic, the osteoporosis. Beside the extensive chemical investigations, the antiosteoporotic studies of various plants and their constituents have been carried out. The dried rhizome of *Drynaria fortunei* (Kunze) J. Sm. (termed as Rhizoma *Drynariae*) is generally adopted in traditional therapy systems to treat osteoporosis. In addition to stimulatory role in bone formation, the current studies have proposed inhibitory effect of Rhizoma *Drynariae* on bone resorption. The pharmacological data of the extracts and total flavonoid contents of Rhizoma *Drynariae* have been discussed in this review article to summarize various modes of action of Rhizoma *Drynariae* and its compounds against osteoporotic models of osteoblasts, osteoclasts and animals. Being a player in bone resorption, cathepsin k is an important target molecule for managing osteoporosis through the use of Rhizoma *Drynariae* and its total flavonoids. Conclusively, further investigations should be conducted to develop future drug of choice using Rhizoma *Drynariae* for clinical treatment of osteoporosis.

Keywords: Rhizoma *Drynariae*, tissue engineered cartilage, cartilage regeneration, flavonoid, osteoporosis

One of the serious systemic skeletal diseases is osteoporosis, which emerges owing to many etiologies including hypogonadism, hypersteroidism, and menopause (1, 2). In osteoporosis, mineral density of bone tissue becomes significantly low, which results in deteriorated and fragile bones. Such bones are extremely prone to breakage (3).

Bone physiology depends on the delicate balance between bone resorption and regeneration. Bone resorption depends on deterioration of osteoblasts and osteoclasts through the process of apoptosis. Reversibly, bone regeneration involves the uninterrupted supply of new osteoblasts and osteoclasts (4). The functions of osteoblasts and osteoclasts are principally related with increased consumption of glucocorticoids, decreased level of sex steroids and senescence. Additionally, the prolonged lifespan of osteoclasts and shortened lifespan of osteoblasts leads to disturbance in equilibrium between bone resorption and regeneration.

An upregulation in osteoclastogenesis and osteoblastogenesis in the marrow has been observed in people with decreased level of sex steroids under the effect of upregulated levels and influence of cytokines, such as IL-1 and IL-6 (5). In addition, there is diminished bone formation in both aged male and female during each remodeling cycle. Furthermore, there is significant increase in the rate of bone remodeling in menopausal women (6).

On excessive usage of glucocorticoids, there is reduction in the intestinal absorption of calcium. Moreover, glucocorticoid excess also induces defects in the metabolism of vitamin D. Both of these changes lead to hypercalciuria and various pathological changes, including increased resorption

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of bones as well as decreased proliferation of osteoblasts. As a result, reduction in the biosynthesis of various macromolecules occurs resulting in deficiency of sex steroids, this situation escorts to hyperparathyroidism (7). Additionally, there is increased apoptosis as well as reduced synthesis of osteoblasts and osteocytes, on excessive usage of glucocorticoids (8).

The factors responsible for osteoporosis development include modifiable and non-modifiable factors. The former category involves medication use, calcium and vitamin D usage, sex hormones, anorexia nervosa, alcohol consumption, and smoking, while age, gender, body size, family history, and ethnicity are named as non-modifiable factors (2). The osteoporosis can be prevented or treated through daily exercise, balance diet, and various drugs. Estrogen, bisphosphonates, calcitonin, and sodium fuoride are the drugs which are used to treat osteoporosis (9).

The usual therapeutic approaches for managing osteoporosis focus on the remodeling of bones through various modes involving the provision of estrogens, calcium and phosphorus in bones; the stimulation of parathyroid hormone (PTH) synthesis; the induction of OPG (osteoprotegerin) expression, osteoblast proliferation, and the osteoclast apoptosis; and reduction in the level of IL-1, 4, and 6 (10).

Therapies that play important role in reducing bone resorption include vitamin D, calcium, hormone, and bisphosphonate. Little therapeutic outcome has been noted in terms of improved bone mineral density when vitamin D or calcium alone is supplemented in deficient persons (10). Hormone therapy is another approach for managing the osteoporosis in the postmenopausal women, however it should be dealt as a short-term therapy since its long-term use may lead to development of breast and/or uterus cancer (11). Along with the preventive effect against osteoporosis, bisphosphonate intake produces some undesired effects in upper gastrointestine that may persist for many years after their discontinuation (12). For example, potential undesired effect of estrogen is an increased risk of venous thrombosis (13). Additionally, therapies that play important role in stimulating the bone regeneration include PTH, which is involved in bone anabolism. At a dose of 20 μg, PTH (1-34) effectively inhibited fracture risk in postmenopausal women. However, the discontinuity of PTH is followed by decline in bone mineral density (14). This drawback of PTH has been managed by using an antiosteoporotic compound, strontium ranelate after discontinuing PTH (15, 16).

The above given summary of synthesized drugs used for preventing and treating the osteoporosis elaborates their specific drawbacks. In comparison to these drugs, some natural therapeutic agents from various medicinal plants are found to possess promising antiosteoporotic activity with lesser side effects, even after long-term use. It is noteworthy that the plant medicines possess plenti-

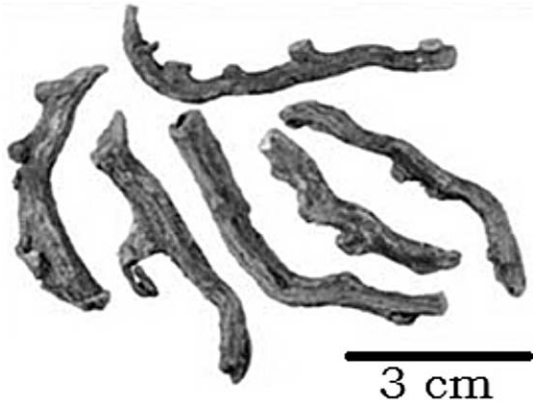
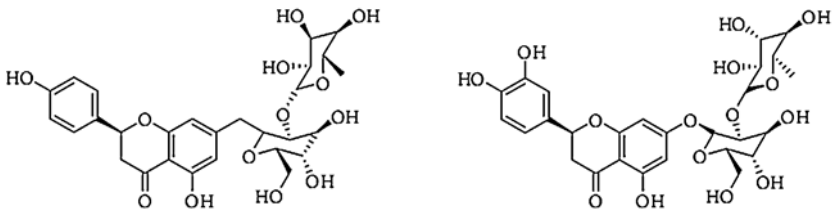


Figure 1. Rhizoma *Drynariae*



Naringin Neoeriocitrin

Figure 2. Chemical structure of naringin and neoeriocitrin, isolated from *Drynaria fortunei* (20)

ful active compounds. It is useful in treating osteoporosis, which is a disease with multipathways and multitargets of pathogenesis (16).

Drynaria fortunei is a well known medicinal plant whose dried rhizome is extensively used for treatment of bone diseases, inflammation, and hyperlipemia (17). It is especially used in Traditional Chinese Medicines (TCM). *Drynaria fortunei* (Kunze) J. Sm. is a perennial pteridophyte that belongs to family, Polypodiaceae (18). Due to use of dried rhizome of *Drynaria fortunei* in disease therapy, the term "Rhizoma *Drynariae*" is generally used for this herbal drug. In TCM, Rhizoma *Drynariae* is also called as "Yang-tonifying" herb; it means herb used to treat bone diseases (18, 19). Moreover, various flavonoids and triterpenoids are found in Rhizoma *Drynariae* extract, and these compounds are considered to be responsible for improving the bone cell viability (20). Naringin and neoeriocitrin are leading examples of flavonoids isolated from Rhizoma *Drynariae* extract (18). These compounds have antiosteoporotic activity, possibly due to its capability of activating the estrogen receptors as well as replacing estrogen (21). The important examples of triterpenoids isolated from Rhizoma *Drynariae* extract include 24-ethyl-9,19-cyclolanost-25-en-3; 3-ol, hop-22(29)-ene and fern-9(11)-ene, respectively (18). Naringin has been demonstrated to possess the capability of stimulating new bone formation (20). Figure 1 reveals the structure of Rhizoma *Drynariae*. In addition, Figure 2 describes the chemical structure of naringin and neoeriocitrin, isolated from *Drynaria fortunei* (20).

The pharmacological data of the extracts, serum, and Rhizoma *Drynariae* total flavonoids has been found from literature search about various modes of action of Rhizoma *Drynariae* and its compounds against osteoporotic models of osteoblasts, osteoclasts and animals (22-27). For example, significant increase in alkaline phosphatase activity in the cell lines, enhancement in proteoglycan synthesis, and improvement in calcification of the cultivated chick embryo bone primordium is observed after Rhizoma *Drynariae* injection (28). After treatment of rat osteoblasts with Rhizoma *Drynariae*, considerable bone recovery *via* its antioxidant action has been reported (29). A biochemical study has reported significant effect of Rhizoma *Drynariae* on osteclastic cell lines (23, 30).

This review article reports the summary of current studies about antiosteoporotic potential of the dried rhizome of *Drynaria fortunei* with special focus on its mode of action.

ANTIOSTEOPOROTIC POTENTIAL OF THE DRIED RHIZOME OF *DRYNARIA FORTUNEI*

The Rhizoma *Drynariae* has effectively been used for many years in the eastern Asia (especially in China and Korea) as an anti-inflammatory, hypolipemic, anti-arteriosclerotic, and anti-osteoporotic agent (31-33). Numerous current studies elaborate therapeutic effectiveness of *Drynariae* Rhizoma in osteoporosis and bone fracture in various models such as the ovariectomized rat model. The modes of action of Rhizoma *Drynariae* include stimulation of BMP-2 and ALP (alkaline phosphatase), aggregation of bone matrix proteins such as type I collagen, and increased expression of up-regulated Runx2 and osteocalcin (34). Beside Rhizoma *Drynariae* total flavonoids, Rhizoma *Drynariae* has been used for bone treatment in different forms including Rhizoma *Drynariae* medicated-extracts and serum.

Extracts

The mouse bone cells culture was exposed to various dilutions of *Drynariae* Rhizoma extract to investigate its anti-resorption potential. From colorimetric MTT assay conducted for determination of mitochondrial activity of these cells, significant anti-resorptive effect of *Drynariae* Rhizoma extract was observed. It is noteworthy that no cytotoxicity was observed in this experiment. This outcome is in agreement with another report (35). A dilution of 100 µg/mL of *Drynariae* Rhizoma extract produced maximum bone protective effect. Alternatively, osteoclasts contain cathepsin k, an endoproteinase that is found largely in the lysosomes, which is considered to be responsible for bone resorption *via* matrix degradation. Cathepsin k is present only in the osteoclasts. Cathepsin k expression can be inhibited by gene silencing, leading to inhibition of bone resorption as well as the collagen decomposition (36), while cancellous bone turnover is observed when cathepsin k overexpression. Thus, cathepsin k may be considered as a specific biomarker in bone resorption. These information suggest that cathepsin k may be a crucial target (37, 38).

Another experiment involving the treatment of mouse long bone cells such as osteoclasts and osteoblast with wortmannin (the PI3-kinase inhibitor) and calphostin C (a specific inhibitor of protein kinase C), was conducted. The results revealed the inhibitory effect on the osteoclast-reconciled intracellular dispensation of cathepsin k. Similar findings were obtained when *Drynariae* Rhizoma extract was used instead of wortmannin. On the other hand, mannose-6-phosphate receptor

is involved in the re-entrance of the secreted proenzymes into cells. Thus, one more experiment was carried out to study the inhibitory effect of wortmannin and *Drynariae* Rhizoma extract on the possibility of this re-entrance in the absence or presence of mannose-6-phosphate. The results revealed the dose-dependent inhibitory effect on the osteoclast-reconciled intracellular dispensation of cathepsin k. Additionally, an elevated level of wortmannin and *Drynariae* Rhizoma potency was observed in the presence of mannose-6-phosphate. Conclusively, dose-dependent inhibition of *in vitro* bone resorption and cathepsin k processing by *Drynariae* Rhizoma was noted. In this way, *Drynariae* Rhizoma extract can be taken as a prodrug with bone resorption inhibiting feature, since it possesses capability of ceasing the maturing process of cathepsin k in osteoclasts-containing long bone cells (39). Later on, Jeong et al. (34) studied the effect of *Drynariae* Rhizoma extract on bone tissue formation, ossification, using MC3T3-E1 (non-transformed osteoblasts) and rat bone marrow cells. As a result, *Drynariae* Rhizoma was found to be involved in stimulation of the ALP activity and mineralization in a dose-dependent in a concentration range of 50-150 µg/mL. Moreover, significantly elevated levels of bone morphogenetic protein-2 and ALP mRNA were noted at a concentration of 100 µg/mL of *Drynariae* Rhizoma. Additionally, non-significant elevated levels of type I collagen mRNA were noted at a concentration of 60 µg/mL of *Drynariae* Rhizoma, leading to the gene expression inhibition of collagenase-1 during 15-20 days of culture. Decisively, these anabolic effects of *Drynariae* Rhizoma results in an increase in proliferation and differentiation of osteoblasts *in vitro*, revealing the bone protective activity of *Drynariae* Rhizoma (40).

In order to verify the *in vitro* results that Rhizoma *Drynariae* extract promotes *in vitro* bone cell viability through an increase in intracellular total protein, alkaline phosphatase and acid phosphatase (23), the systemic effect of Rhizoma *Drynariae* extract on bone structure in normal mice has been examined using micro-CT scanning. Wong and Rabie used 8 week old male BALB/c mice to compare the bone structures of mice treated with and without Rhizoma *Drynariae* extract. The results of this *in vivo* study show that there is 0.25 mm enhancement in the proximal end of the left tibia of each mouse. The quantitative morphometric analysis of the bone structures revealed that there was an increase in the bone density as evident from augmented bone volume/tissue volume ratio and bone trabeculae by 6.45% and 10.00%, respectively, in Rhizoma *Drynariae* extract treated mice. It could be concluded from these results that the bone density can be improved by oral intake of Rhizoma *Drynariae* extract. This *in vivo* study supported the previous *in vitro* findings about the anabolic effect of Rhizoma *Drynariae* extract, i.e., this extract enhanced the bone cell activity (23, 41). In another study to test bone strengthening activity of Rhizoma *Drynariae*, Wong et al. investigated the systemic effect of Rhizoma *Drynariae* extract on bone formation in eight week old male BALB/c mice. The results showed that there is enhancement in the trabecular number and bone density by 10% and 6.45%, respectively, which reveals the change in bone histomorphology in Rhizoma *Drynariae* extract treated mice as compared to control group of these animals. As evident from the identification of osteoblasts and osteocytes in the newly formed bone, it was also noted that there was induction of new bone formation on the margins of the defects in Rhizoma *Drynariae* treated mice. It indicates the systemic effect of this extract on bone formation and healing. In addition, this activity of Rhizoma *Drynariae* could be due to its active constituent, naringin. Naringin has been demonstrated to be involved in the up-regulation of osteogenic factor expression, which results in angiogenesis and/or osteogenesis. As concluding remarks, the osteogenic effect of naringin and other active ingredients of Rhizoma *Drynariae* on fracture-prone bones including femur neck and lumbar spine may also be investigated (42).

Serum

The influence of serum medicated with different doses of RDTF (Rhizoma *Drynaria* total flavonoids) on the osteoblasts of newborn SD rats cultured by collagenase method, was investigated. These cells were then tested for MTT, PNPP, PI and Annexin V/PI analysis and it was observed the significantly increased proliferation and alkaline phosphatase activity in RDTF treated osteoblasts than that of the untreated osteoblasts, demonstrating the anti-osteoporosis activity in a time dependent manner (43).

Rhizoma Drynaria total flavonoid extract

RDTF are obtained through extraction of dry rhizomes of *Drynaria fortunei*, followed by isolation and purification (44). Naringin is the main active constituent of RDTF. The RDTF play an important role in treatment of the bones with lesions, low density and strength, increased blood viscosity, low

bone mineralization, and resorption especially postmenopausal osteoporosis (39, 45-49).

Kang et al. prepared water and ethanol extract of *Drynariae* Rhizoma to compare the extraction efficacy of total flavonoids. Moreover, both extracts were tested *in vitro* for comparing their antioxidant and anti-osteoporosis features. The ethanol extract showed better extraction efficacy and antioxidant activity in comparison to that of water extract. Additionally, the ethanol extract showed better proliferation and differentiation of cultured mouse (KP100 CD-1) osteoblastic cells *in vitro*. Based on these results, it can be concluded that the ethanol extract of *Drynariae* Rhizoma might be more effective for osteoporosis treatment (50).

In postmenopausal osteoporosis, Rhizoma *Drynariae* is capable of sustaining the normal trabecular structure as well as inhibiting rate of bone turnover through mimicking the estrogen. In addition, RDTF possess potent anti-oxidative and osteoprotective activity, possibly through its potential of restoring OVX-induced osteoporosis in rats. Moreover, the increased osteoblasts proliferation as well as the reduced osteoclasts activity in rat bones-treated with RDTF, was observed *in vitro* (51).

Smad is a protein that plays a role in intracellular signaling for silk threonine kinase receptors type I and II, which are involved in the generation of bones. In order to examine the influence of RDTF on expression of the Smad1 and Smad5 mRNA, Zhu et al. (52) administered RDTF to the ovariectomized rats (except normal group consisting of non-ovariectomized rats) in high, moderate, and low doses. The femur bone assays of the sacrificed rats showed that Smad1 and Smad5 were more expressed in RDTF treated rats, as compared to normal group rats. This effect was dose independent (52).

Pang et al. (53) conducted a study to explore the bone protection in young ovariectomized (C57/BL6J) mice by using RDTF based on its estrogen-like activity. The maximum increase in trabecular-rich bone mineral densities at distal femur and lumbar spine in the ovariectomized mice treated with 0.173 mg of RDTF per gram mice weight per day, was observed. The peripheral quantitative computed tomography approach was employed to study bone mineral densities. The co-incubation of RDTF with 17β-estradiol antagonist ICI 182, 780 in rat osteoblast-like UMR-106 cells did not show stimulation of osteoblasts by RDTF; it showed ER-dependent osteoblastic functions of RDTF. Moreover, transient transfection in UMR-106 cells was also studied. It revealed that ERE-dependent luciferase activity depended on dose of RDTF through ER-α and ER-β. Conclusively, RDTF not only activates ER leading to growth regulation of the osteoblasts, but also protects mice from ovariectomy-induced osteoporosis. Additionally, the postmenopausal women with osteoporosis should also be clinically treated to assess therapeutic efficacy of RDTF. Such results were already published by Liu et al., demonstrating that RDTF-3H-TdR mixture promotes the alkaline phosphatase activity in UMR-106 cells in a dose- and time-dependent manner in osteoblast culture *in vitro* (54).

In order to provide experimental confirmation about the mode of Rhizoma *Drynariae* for osteoporosis treatment, an interventional study was conducted to study the effect of RDTF on the osteoclasts through detection of bone mineral density, biomechanics, serum cathepsin k concentration, and cathepsin k mRNA expression in the proximal metaphysis of the tibia in 72 female Sprague-Dawley ovariectomized rat models of osteoporosis. Additionally, a three-point bending approach was used for measuring the maximum load of the tibia to assess the influence of RDTF on bone strength. In comparison to that in the estrogen and normal groups, there were significant differences in the bending load, serum cathepsin k concentrations, and cathepsin k mRNA expression in the RDTF rats. Moreover, RDTF showed a concentration-dependents effect on bone mineral density (55). In a study about interventional effect of RDTF on cathepsin k, bone density of the ovariectomized rat model with osteoporosis under treatment using RDTF was determined and found RDTF mediated-increase in bone density, in comparison to the control group (56-60).

Isolated compound - naringin

The leading example of flavonoid isolated from Rhizoma *Drynariae* extract is naringin that plays an important role in bone metabolism and osteogenesis (39, 61, 62). It is capable of suppressing the retinoic acid-provoked osteoporosis in rats; enhancing expression of BMP-2 resulting in bone formation; increasing the proliferation and osteogenic differentiation of bone mesenchymal stem cells in osteoporosis diseases in human (63-65). Due to double directional adjusting effect (i.e., estrogenic and anti-estrogenic functions), naringin is found to manage osteoporosis *via* selectively binding with estrogenic receptor (66, 67). In addition, current studies have proved the usefulness of naringin in ovariectomized-induced bone loss in mice/rats (68-71).

Table 1. Clinical trials using Rhizoma *Drynariae* for treating osteoporosis.

No.	Treatment	No. of subjects	Treatment group	Treatment duration (days)	Bone mass density change (g/cm^2)	Adverse effects (%)	Ref.
1	*Rhizoma Drynariae*	34	PT	180	+0.005 (lumbar); +0.025 (femur)	Not observed	73
	Tibolone	35	HT		+0.043 (lumbar); +0.051 (femur)	Uterine bleeding (8.6%)	
2	*Rhizoma Drynariae*	48	PT	180	+0.024 (femur)	Not observed	74
	Estradiol valerate, medroxyprogesterone	42	HT		+0.015 (femur)		
3	*Rhizoma Drynariae*	40	PT	180	+0.038 (femur)	Not observed	75
	Nilestriol	40	HT		+0.039 (femur)		
4	*Rhizoma Drynariae*	50	PT	90	+0.103 (forearm)	Not observed	76
	Nilestriol	30	HT		+0.056 (forearm)		

Clinical trials using Rhizoma *Drynariae* for treating osteoporosis

The fundamental remedies for osteoporosis include bisphosphonates and estrogen, but these agents have significant side effects. Therefore, natural remedies are currently being investigated, particularly from plants, since possibility of finding of natural ingredients is significantly higher in plants than indiscriminate prospect in conventional approaches, as evident from their folk use in osteoporosis. In contrast to the admired view "herbals are natural and harmless", herbal safety is of prime importance, since some herbals have been reported to have serious side effects including hepatotoxicity. Thus, clinical usage of herbal drugs should be recommended after thorough quality control testing and standardization. Table 1 reflects the clinical trials using Rhizoma *Drynariae* for treating osteoporosis. It is clearly evident from most of the studies that the anti-osteoporotic activity of Rhizoma *Drynariae* is comparable to various standards including tibolone, estradiol valerate, medroxyprogesterone, and nilestriol, in terms of bone mass density change during study for 180 days. Moreover, these studies reported no adverse effects of Rhizoma *Drynariae*, except for tibolone that resulted in uterine bleeding (8.6%) (72).

CONCLUSION

The literature study proposes various modes of anti-osteoporotic activity of RDTF as given here: Firstly, RDTF promoted the proliferation of MCF-7 (human breast cancer cell line) and ROS17/2.8 (osteoblast-like cell line), even more efficiently than estradiol and genistein showing the estrogen-like effect of RDTF. Secondly, RDTF inhibited the lacunae production in osteoclasts leading to inhibition of cellular expression of cathepsin k. Finally, RDTF inhibited transport and expression of the cathepsin k precursor in cells, with the same effectiveness as that of wortmannin. These studies verify that RDTF treats osteoporosis through inhibition of cathepsin k.

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