CHAPTER IV

RESULTS

1. Effects of *P. mirifica* on bone loss in ovariectomized rats

1.1 Changes in body weight

The body weights of female rats on day 0 and day 90 during the three-month experiment are shown in Table 4.1. None of the groups of female rats had a significant difference in mean initial body weights (day 0). Although all rats were fed the same amount of soybean-free rat diet as did for SH rats, the body weight on day 90 of P0 rats, was significantly higher by 7.29% than that of the SH rats ($p = 0.03$). The body weights of P10 and P100 rats were significantly lower by 6.14 ($p = 0.006$), and 8.99 % ($p = 0.001$), respectively, than that of P0 rats and non-significant differences from that of SH rats. The P1000 rats did not gain weight during experimental period and their body weights at the end of experimental period were significantly lower by 15.15% ($p = 0.001$) than that of P0 rats. The EE rats gained weight and significant increase during experiment period ($p < 0.001$), but their body weights at the end of experimental period were significantly lower by 11.29% than that of P0 rats ($p = 0.001$).

Table 4.1 Changes of body weights, absolute and relative uterine weights and serum estradiol levels of initial control (IC) rats, sham control (SH) rats, and ovariectomized (OVX) rats treated with 0.1 mg/kg BW/day of 17 α-ethinylestradiol (EE), 0, 10, 100 and 1,000 mg/kg BW/day of *Pueraria mirifica* (P0, P10, P100 and P1000, respectively) for three months.

	Treatment						
	IC n=9	**SH** n=9	**EE** n=9	**P0** n=9	**P10** n=9	**P100** n=9	**P1000** n=9
Body weight on day 0 (g)	311±4	309±5	305±4	311±4	313±6	307±5	308±6
Body weight on day 90 (g)	-	340±6 [ac]	324±4 [abc]	365±6 [abc]	343±6 [ac]	332±6 [ac]	310±3 [bc]
Absolute uterine weight (mg)	-	496±29 [c]	545±21 [c]	148±5 [b]	257±20 [bc]	539±19 [c]	695±23 [bc]
Relative uterine weight (mg/100 g BW)	-	143±8 [c]	166±3 [bc]	40±2 [b]	76±7 [bc]	160±6 [c]	223±6 [bc]
Estradiol level on day 0 (pg/ml) n = 8/group	-	30.2±3.1	37.3±3.8	31.1±2.0	33.8±3.8	30.7±3.2	30.8±1.7
Estradiol level on day 90 (pg/ml) n=8/group	-	40.2±4.4 [c]	17.1±1.1 [ab]	17.5±1.2 [ab]	15.7±1.6 [ab]	20.2±1.4 [ab]	21.5±0.7 [ab]

Data are presented as means ± SEM.

[a] $p < 0.05$ day 0 *vs.* day 90 (within group)

[b] $p < 0.05$ *vs.* SH (between group)

[c] $p < 0.05$ *vs.* P0 (between group)

1.2 Changes in uterine weight

The absolute and relative uterine weights are shown in Table 4.1 and Figure 4.1. Three months after OVX, absolute uterine weights of P0 rats were greatly decreased by 70.16% in comparison with the SH rats ($p < 0.001$). Feeding of *P. mirifica* for P10, P100 and P1000 significantly increased absolute uterine weights, dose-dependently, by 73.65, 264.19 and 369.59% over that of the P0 rats ($p < 0.001$). The EE treatment highly increased uterine weights by 268.24% over the P0 rats ($p < 0.001$) and lower by 21.58% compared with the P1000 rats ($p < 0.001$). In addition, the absolute uterine weight of P1000 rats was higher by 40.12% ($p < 0.001$) than that of the SH rats. The difference among groups of relative uterine weights were statistically similar to the absolute uterine weights, except that the relative uterine weight in EE rats was higher than that of SH rats ($p < 0.04$).

1.3 Changes in serum estradiol levels

Serum estradiol levels are shown in Table 4.1. At the beginning of the experiment (day 0), no differences in serum estradiol levels were observed among SH, EE, P0, P10, P100 and P1000 rats. Serum estradiol levels were not significantly changed from day 0 to day 90 of experiment period in SH rats ($p = 0.095$). Three months after OVX, serum estradiol levels were decreased in *P. mirifica* and EE-treated rats. Three-month administration of *P. mirifica* at any doses did not increase serum estradiol levels and they were kept significantly lower than that of SH rats ($p < 0.001$).

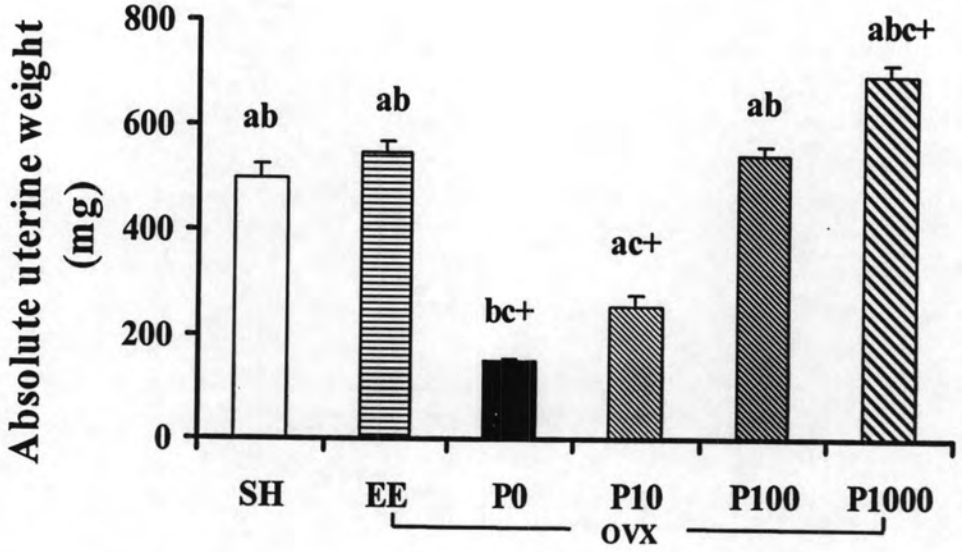

Figure 4.1 Absolute uterine weights in sham control (SH) rats and in ovariectomized (OVX) rats treated with 0.1 mg/kg BW/day of 17 α-ethinylestradiol (EE), and 0, 10, 100 and 1,000 mg/kg BW/day of *Pueraria mirifica* (P0, P10, P100 and P1000, respectively) for three months. Data are expressed as the mean ± SEM. $^{a}p < 0.001$ *vs.* P0; $^{b}p < 0.001$ *vs.* P10; $^{c}p < 0.001$ *vs.* P100; $^{+}p < 0.001$ *vs.* SH.

1.4 Changes in bone mineral density (BMD)

The trabecular bone mineral densities (TbBMDs) in the proximal tibial metaphysis (TM), the distal femoral metaphysis (FM) and the fourth lumbar vertebral body (L4) for all treatment groups are shown in Figure 4.2. The difference of TbBMDs between IC and SH groups was regarded as the age change from 7 to 10 months. The TbBMDs in TM and FM of SH group significantly decreased by 8.50 and 8.02%, respectively (p = 0.03) as the age change. The TbBMD in L4 of SH group non-significantly decreased by 6.26% (p = 0.053).

The influence of OVX on BMD was expressed by comparison between P0 and SH groups. The TbBMDs in TM, FM and L4 of P0 group were significantly deceased by 40.91, 33.28 and 15.15%, respectively, of the SH group (p = 0.001).

Feeding of P10 significantly prevented the decrease of TbBMDs in TM and FM by 28.98 and 30.98%, respectively (p = 0.01), but did not in L4 (p = 0.26). P100 treatment absolutely prevented the decrease of TbBMDs in TM, FM and L4, as shown by the percent prevention of 87.37, 85.79 and 81.89%, respectively (p = 0.001). The greater preventions were obtained by P1000 treatment in TbBMDs of TM, FM and L4 by 119.81, 110.86 and 130.06%, respectively (p = 0.001). Thus, the P1000 treatment substantially increased TbBMD in TM, FM and L4 compared with the SH group. The preventive effect of EE was significant and comparable to that of the P100, in TM, FM and L4 by 76.47, 77.86 and 74.28%, respectively (p = 0.001).

The cortical BMDs (CtBMDs) in all treatment groups for TM, FM, L4, TD and FD are shown in Figure 4.3. The CtBMDs slightly increased with age, and significantly in TM, FM, TD and FD by 2.38 (p = 0.001), 1.39 (p = 0.02), 1.18 (p = 0.001) and 2.02% (p = 0.001), respectively. On the other hand, changes in CtBMD was slightly and significantly decreased in L4 by 1.59% (p = 0.003).

The OVX for three months in P0 group did not obviously affect the CtBMDs of TM, FM, L4 and TD, excepting for the FD, where the CtBMD was slightly decreased and significantly by 0.68% (p = 0.02) over that of the SH rats.

Effects of P10 treatment were not found in CtBMDs of TM, FM, TD and FD compared with those of P0 group. On the other hand, significant increase was found in L4 by 1.12% ($p = 0.04$). Similar effects were exerted by P100 treatment, CtBMDs in TM, FM, TD and FD were comparable with those of P0, but it was significantly increased in the CtBMD of L4 by 1.51% ($p = 0.008$). The P1000 treatment significantly increased CtBMDs of TM, L4 and TD by 1.85, 2.03 and 0.68% ($0.001 \leq p \leq 0.009$), respectively, though CtBMD of FM was increased by 0.24% ($p = 0.67$) but it was not significant. Also, the P1000 treatment prevented the decrease of CtBMD of FD as shown by the percent prevention of 83.67% ($p = 0.04$). The effect of EE treatment on CtBMDs was comparable to that of P1000 treatment; it increased the CtBMDs in TM, L4 and TD by 1.74 ($p = 0.001$), 1.82 ($p = 0.002$) and 0.51% ($p = 0.05$), respectively, and did not affect FM and FD.

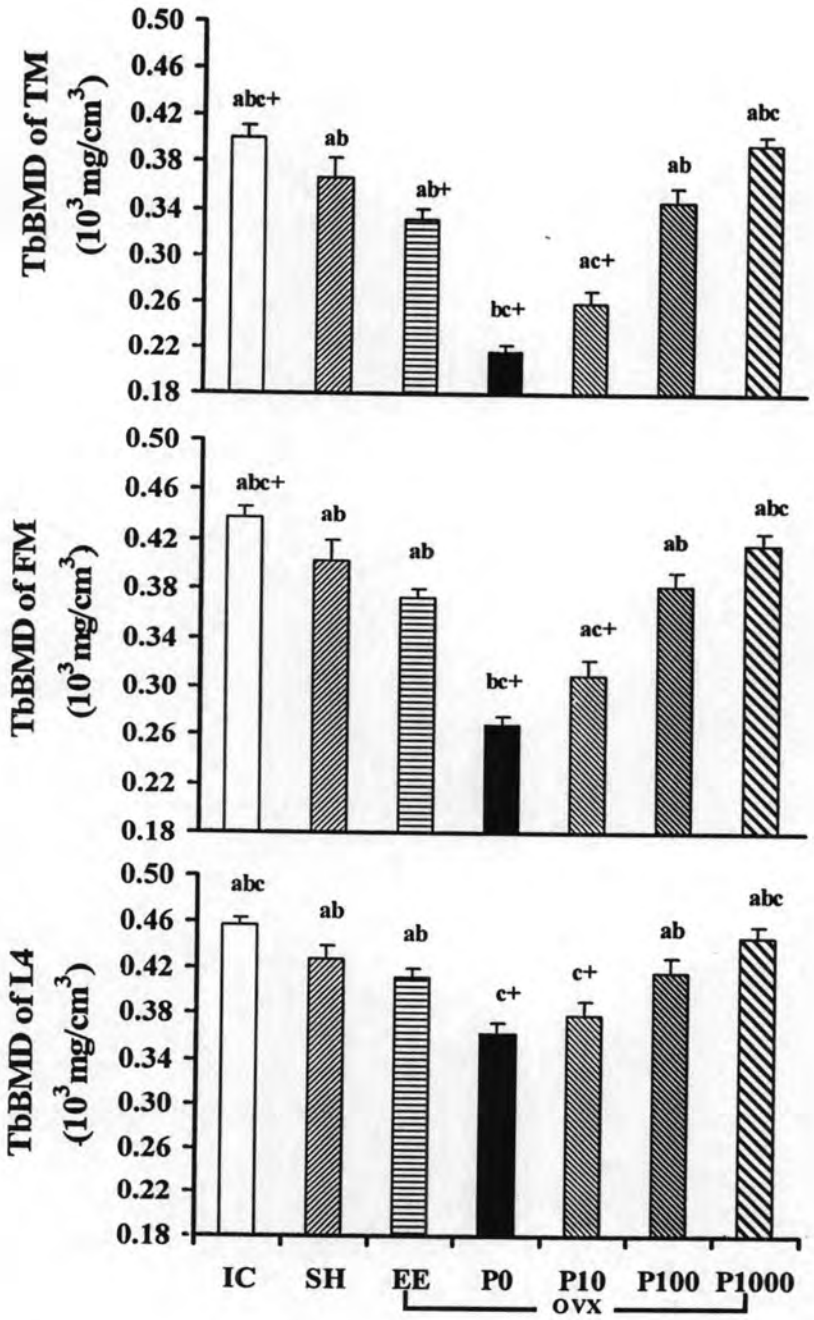

Figure 4.2 Trabecular bone mineral densities (TbBMDs) of proximal tibial metaphysis (TM), distal femoral metaphysis (FM) and the fourth lumbar vertebral body (L4) in initial control (IC), sham control (SH), and ovariectomized (OVX) female rats treated with EE, P0, P10, P100 and P1000 (abbreviations are the same as given in Figure 4.1). TbBMDs were determined on day 0 in IC group and day 90 in other 6 groups. Data are mean ± SEM. $^{a}p < 0.05$ *vs.* P0; $^{b}p < 0.05$ *vs.* P10; $^{c}p < 0.05$ *vs.* P100; $^{+}p < 0.05$ *vs.* SH.

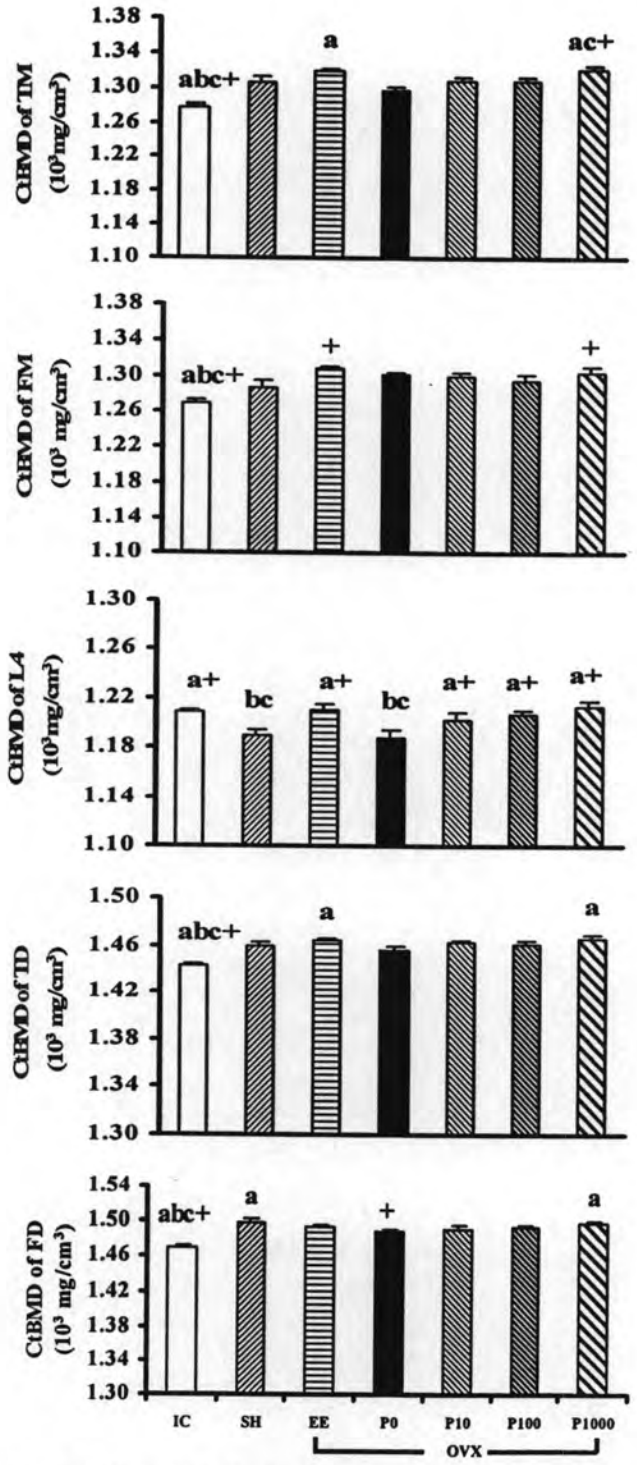

Figure 4.3 Cortical bone mineral densities (CtBMDs) of TM, FM and L4, tibial diaphysis (TD) and femoral diaphysis (FD) in IC, SH and OVX female rats treated with EE, P0, P10, P100 and P1000 (see Figure 4.1 and Figure 4.2 for abbreviations). CtBMDs were determined on day 0 in IC group and day 90 in other 6 groups. Data are mean ± SEM. $^{a}p < 0.05$ *vs.* P0; $^{b}p < 0.05$ *vs.* P10; $^{c}p < 0.05$ *vs.* P100; $^{+}p < 0.05$ *vs.* SH.

1.5 Changes in bone mineral content (BMC)

The effects of *P. mirifica* on bone mineral content (BMC) or bone mass in OVX rats were examined. The results for trabecular BMCs (TbBMCs) in TM, FM and L4 are shown in Figure 4.4. TbBMCs of TM, FM and L4 were not changed with age (comparison between SH and IC groups, $p>0.05$).

Three months after OVX, rats in P0 group significantly decreased TbBMCs in TM, FM and L4 by 35.72, 24.50 and 11.99% ($0.001 \leq p \leq 0.002$), respectively, of the SH group.

The P10 treatment significantly prevented the decrease of TbBMCs in TM, FM and L4 by 33.39, 45.67 and 70.75% ($0.02 \leq p \leq 0.04$), respectively. The greater prevention was found in P100, that is, the decreases of TbBMCs in TM, FM and L4 were prevented by 112.75, 104.92 and 95.75% ($0.001 \leq p \leq 0.002$), respectively. The effect of P1000 treatment was comparable to those of P100, the decreases of TbBMCs in TM, FM and L4 were prevented by 111.49, 77.36 and 106.91% ($0.001 \leq p \leq 0.002$), respectively. The effect of the EE treatment was intermediary between those of P10 and P100 treatments, that is, the decreases of TbBMCs in TM, FM and L4 were prevented by 82.41, 69.69 and 80.32% ($0.001 \leq p \leq 0.009$), respectively.

The cortical BMCs (CtBMCs) in all treatment groups are shown in Figure 4.5. Changes in CtBMCs with advancing age were either increase or decrease in relation to bone types and bone sites: no changes in TM and FM, significant decrease in L4 by 14.49% ($p = 0.004$), and significant increase in TD and FD by 5.60 and 7.09% ($0.007 \leq p \leq 0.04$), respectively.

Three months after OVX, rats in P0 group exhibited no changes of CtBMCs in all bone sites in comparison with those of the SH group.

The P10 treatment showed no effects on CtBMCs in all bone sites. The P100 treatment significantly increased CtBMCs in FM, L4 and FD by 5.76, 12.43 and 4.75% ($0.04 \leq p \leq 0.05$), respectively, but not in TM and TD. Significant increase by

the P1000 treatment were found in the CtBMCs in TM, FM and L4 by 4.57, 11.44 and 16.49% ($0.001 \leq p \leq 0.05$), respectively. However, non-significant increases in the CtBMCs were found in TD and FD by P1000 treatment. The effects of EE were similar to those of P100: with significant increase of CtBMCs in FM by 7.84% ($p = 0.009$), L4 by 14.49% ($p = 0.02$), and insignificant increases in TM, TD and FD.

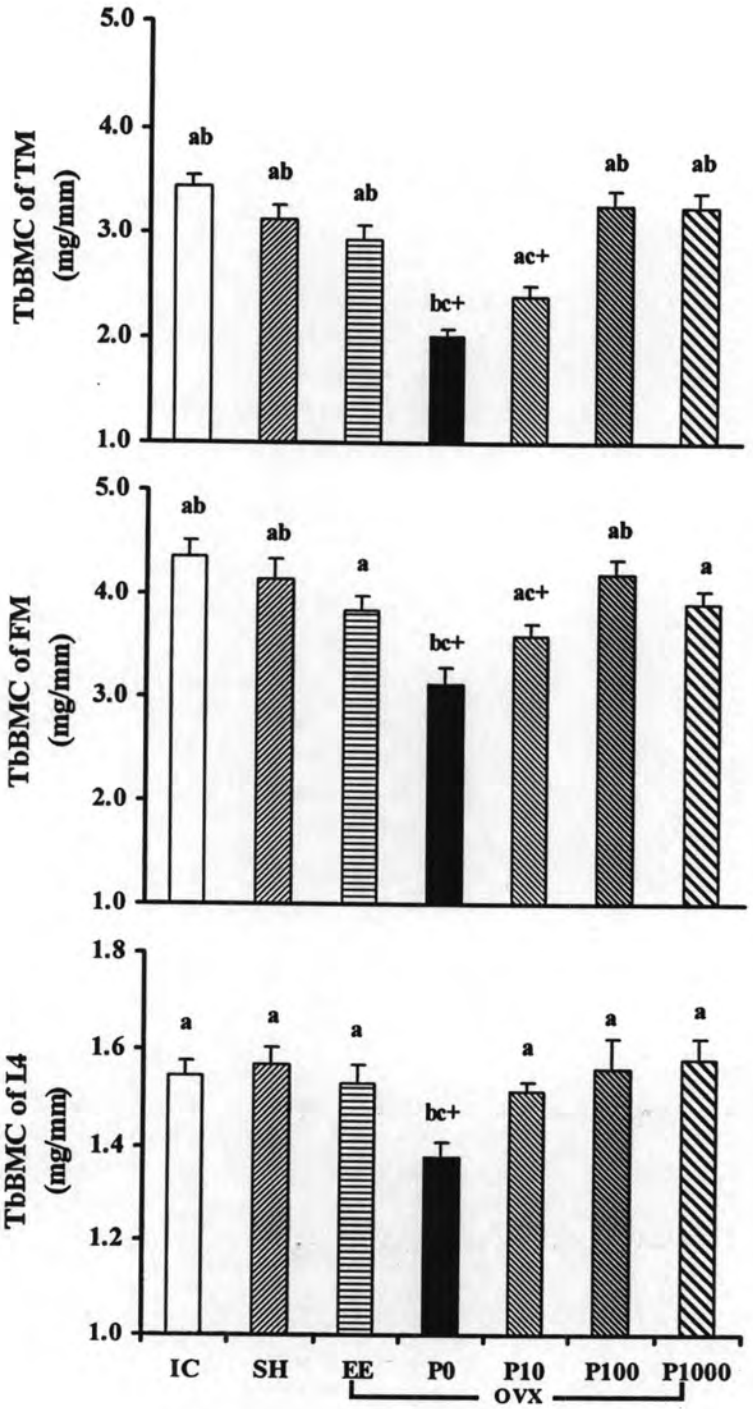

Figure 4.4 Trabecular bone mineral contents (TbBMCs) of TM, FM and L4 in IC, SH and OVX female rats treated with EE, P0, P10, P100 and P1000 (see Figure 4.1 and Figure 4.2 for abbreviations). TbBMCs were determined on day 0 in IC group and day 90 in other 6 groups. Data are mean ± SEM. $^{a}p < 0.05$ *vs.* P0; $^{b}p < 0.05$ *vs.* P10; $^{c}p < 0.05$ *vs.* P100; $^{+}p < 0.05$ *vs.* SH.

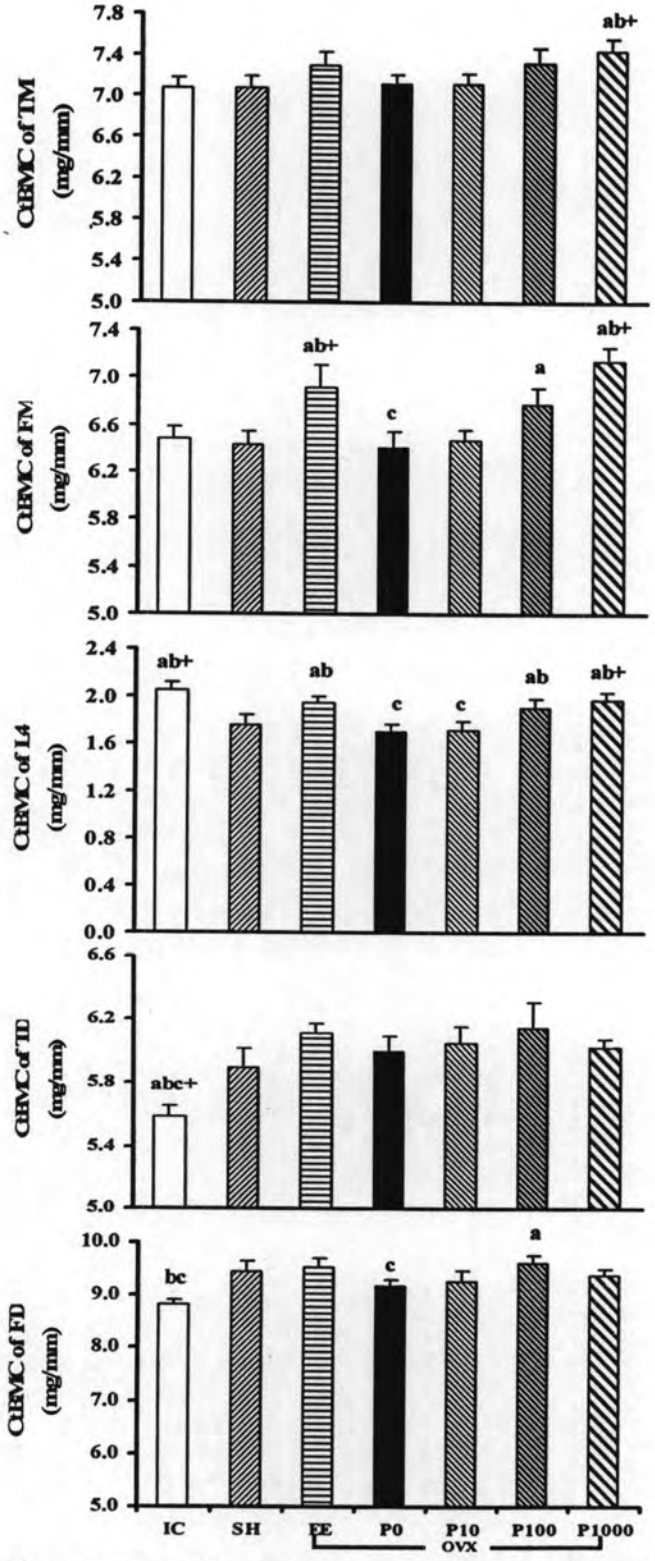

Figure 4.5 Cortical bone mineral contents (CtBMCs) of TM, FM, L4, TD and FD in IC, SH and OVX female rats treated with EE, P0, P10, P100 and P1000 (see Figure 4.1-4.3 for abbreviations). CtBMCs were determined on day 0 in IC group and day 90 in other 6 groups. Data are mean ± SEM. $^{a}p < 0.05$ *vs.* P0; $^{b}p < 0.05$ *vs.* P10; $^{c}p < 0.05$ *vs.* P100; $^{+}p < 0.05$ *vs.* SH.

1.6 Changes in bone histology

Histological sections revealed a normal trabecular conformation in SH rats. Below epiphyseal plate, metaphyseal region was fully filled with trabecular bones and its connectivity was intervened by small inter-trabecular spaces (Figure 4.6A). Three months after OVX, P0 rats showed sparse and thinner trabeculae which is resulted in greater inter-trabecular spaces (Figure 4.6C). The thicker trabeculae with high connectivity and narrowed inter-trabecular spaces were observed in P1000 rats (Figure 4.6D). Three months of EE treatment, bone deterioration caused by OVX was restored and became comparable with that of the SH rats (Figure 4.6B).

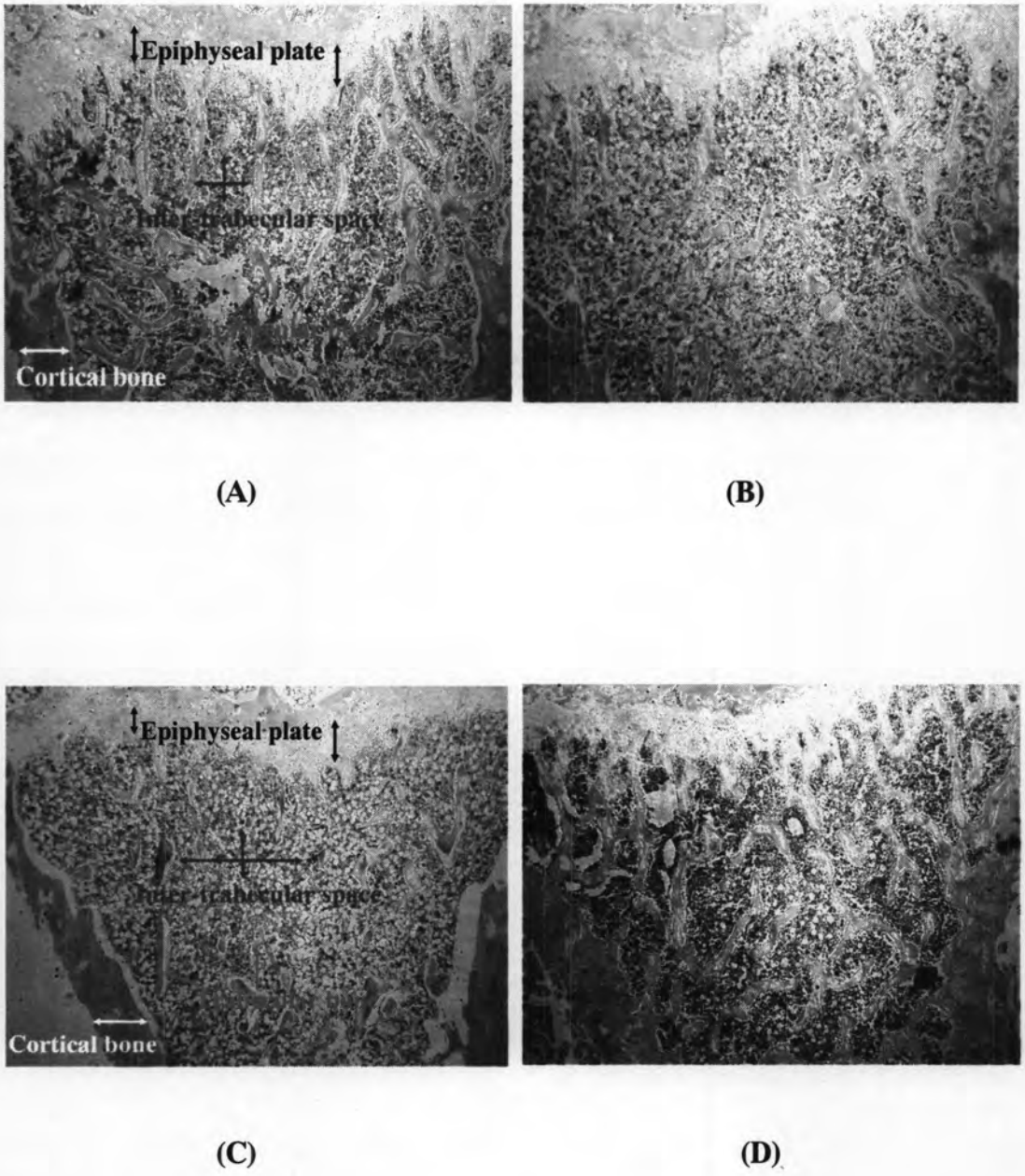

Figure 4.6 Histological section [stained with H & E (50x)] in longitudinal and medio-lateral plane of the proximal tibia at the epiphyseal growth plate and metaphyseal area of SH rats (A) and OVX rats treated with EE (B), P0 (C) and P1000 (D).

2. Effects of *P. mirifica* on bone loss in orchidectomized rats

2.1 Changes in body weight

Body weights of male rats at day 0 and day 90 of three-month experiment are shown in Table 4.2. The initial body weight between groups including IC group, were not significantly different. At day 90, body weight of SH rats significantly increased and were heavier than the other groups. ORX for three months in P0 rats significantly inhibited the increase of body weight compared with the SH rats (p = 0.04). Feeding of *P. mirifica* for three months dose-dependently suppressed the increase of the body weight for P10 (p = 0.09), P100 (p = 0.003) and P1000 (p = 0.008), respectively, in comparison with the day 0 body weight. Treatment of EE for three months also decreased the body weight from the day 0 weight (p = 0.01), and the day 90 body weight was comparable with that of P100 rats.

2.2 Changes in weights of seminal vesicles and ventral prostate glands

Changes in absolute and relative weights of seminal vesicles and ventral prostate glands are shown in Table 4.2. After three months of ORX in P0 rats, the absolute and relative weights of seminal vesicles and ventral prostate glands were highly significantly lower than that of SH rats (p = 0.001). Feeding of EE and *P. mirifica* at three different doses to ORX rats did not affect weights of these organs which were not significantly different between groups and comparable with the P0 rats.

Table 4.2 Body weights and absolute and relative weights of seminal vesicles and prostate glands of IC rats, SH rats, and ORX rats treated with EE, P0, P10, P100 and P1000 for three months.

	Treatment						
	IC n=9	**SH** n=9	**EE** n=9	**P0** n=9	**P10** n=9	**P100** n=9	**P1000** n=7**
Body weight on day 0 (g)	515±11	506±12	512±10	502±11	511±7	507±10	496±9
Body weight on day 90 (g)	-	572±17 [ac]	484±4 [abc]	542±8 [ab]	511±4 [bc]	479±7 [abc]	456±13 [abc]
Absolute seminal vesicle weight (mg)	-	2047±119 [c]	198±4 [b]	194±5 [b]	228±5 [b]	173±7 [b]	190±13 [b]
Relative seminal vesicle weight (mg/100 g BW)	-	360±24 [c]	41±1 [b]	38±2 [b]	45±1 [b]	35±1 [b]	42±3 [b]
Absolute prostate gland weight (mg)	-	549±36 [c]	76±8 [b]	92±12 [b]	76±8 [b]	92±12 [b]	94±14 [b]
Relative prostate gland weight (mg/100 g BW)	-	97±7 [c]	16±2 [b]	16±2 [b]	15±2 [b]	19±3 [b]	21±3 [b]

Data are presented as means ± SEM.

[a] $p < 0.01$ day 0 *vs.* day 90 (within group)

[b] $p < 0.05$ *vs.* SH (between group)

[c] $p < 0.05$ *vs.* P0 (between group)

** Two out of nine rats in P1000 group died from intra-tracheal feeding.

2.3 Changes in serum estradiol and testosterone levels

Serum estradiol and testosterone levels are shown in Table 4.3. At day 0, no differences among six groups of rats, SH, EE, P0, P10, P100 and P1000, were observed in serum estradiol levels. However, three months later (day 90), serum estradiol levels were significantly decreased in both SH and ORX rats treated with EE, P0, P10, P100 and P1000 ($0.001 \leq p \leq 0.02$). Serum estradiol level at day 90 in P0 group was the lowest among other five groups. Excluding the P0 group, serum estradiol levels at day 90 were not different between groups.

At day 0, no differences in serum testosterone levels were observed among six groups. Three month later, serum testosterone levels in SH rats were significantly decreased ($p = 0.03$) in comparison with day 0, however, this low level was still significantly higher than those of the other five groups of ORX rats ($p < 0.001$). Serum testosterone levels were very low and unable to be detected by the RIA technique.

Table 4.3 Changes in serum estradiol and testosterone levels in SH rats, and ORX male rats treated with EE, P0, P10, P100 and P1000, for three months.

Serum hormonal level	Treatments					
	SH n=8	**EE** n=8	**P0** n=8	**P10** n=8	**P100** n=8	**P1000** n=7
Estradiol level (pg/ml)						
-Day 0	23.4±1.0	26.1±1.1	24.9±0.5	25.4±1.0	24.6±1.1	23.5±1.2
-Day 90	16.8±1.0[ac]	14.8±1.9[ac]	10.6±1.8[ab]	17.1±1.2[ac]	16.0±1.1[ac]	17.1±1.4[ac]
Testosterone level (ng/ml)						
-Day 0	3.8±1.0	3.0±1.2	4.2±1.4	2.0±0.3	2.5±0.9	4.9±0.8
-Day 90	0.8±0.2[ac]	nd[ab]	nd[ab]	nd[ab]	nd[ab]	nd[ab]

Data are presented as means ± SEM.

[a] $p < 0.05$ day 0 *vs.* day 90 (within group)

[b] $p < 0.05$ *vs.* SH (between group)

[c] $p < 0.05$ *vs.* P0 (between group)

nd = non-detectable

2.4 Changes in bone mineral density (BMD)

Trabecular bone mineral densities (TbBMDs) at TM, FM and L4 in all treatment groups are shown in Figure 4.7. The difference of BMD between IC and SH groups was regarded as the age change from 7 to 10 months. Age changes of TbBMDs were not statistically significant in TM and L4 ($p > 0.05$), but it was significantly decreased in FM by 8.48% ($p = 0.008$).

The influence of ORX on TbBMDs was shown by the difference between TbBMDs in P0 and SH groups. The ORX significantly decreased the TbBMDs in TM, FM and L4 by 28.32, 34.38 and 27.90%, respectively ($p < 0.001$).

Treatment of P10 non-significantly prevented the decrease of TbBMDs in TM and FM ($p > 0.05$), but it significantly prevented the decrease of TbBMD in L4 by 50.81% ($p < 0.001$). Treatment of P100 highly prevented the decrease in TbBMDs in TM and L4 by 100.12 and 93.91%, respectively ($p < 0.001$). However, it prevented the trabecular bone loss only by 72.52% in FM ($p < 0.001$). Treatment of P1000 completely prevented the decrease in TbBMDs in TM, FM and L4 by 134.46, 124.17 and 137.37%, respectively ($p < 0.001$). As expected, EE administration highly prevented the decreases in the TbBMDs in TM, FM and L4 by 92.45, 86.91 and 94.28%, respectively ($p < 0.001$) in comparable with those of P100 administration.

Cortical bone mineral densities (CtBMDs) measured for all treatment groups in TM, FM, L4, TD and FD are shown in Figure 4.8. After age increasing for three months, the CtBMDs in TM, FM, TD and FD significantly increased in SH group over the IC group by 4.00, 3.07, 1.82 and 2.03%, respectively ($p < 0.001$). The increase in CtBMD by age was only 0.79% in L4 and non-significant ($p > 0.05$).

The ORX in P0 group did not affect the CtBMDs in TM and FM compared with those of the SH group. On the other hand, the CtBMDs in L4, TD and FD of P0 group were significantly lower than those of the SH group by 2.80 ($p < 0.001$), 0.82 ($p = 0.02$) and 0.90% ($p < 0.001$), respectively.

The treatment of P10 did not prevent the CtBMDs in TM, FM, L4, TD and FD from the ORX influence, compared with the P0 group. However, treatment of P100, prevented the decrease in CtBMDs in L4 and FD by 63.09 ($p = 0.001$) and 67.56% ($p = 0.01$), but not that in TD. Treatment of P100 significantly increased the CtBMDs in TM and FM compared with those in P0 group by 1.87 and 1.60%, respectively ($p < 0.01$). Treatment of P1000 also prevented the decrease in CtBMDs in L4 and FD by 103.24 ($p < 0.001$) and 80.81% ($p < 0.001$), respectively, but not that in TD. Treatment of P1000 increased the CtBMDs in TM and FM by 2.31 and 1.78% from those in P0 group ($p < 0.001$). The EE treatment also prevented the decrease in CtBMDs in L4 and FD by 66.36 ($p = 0.002$) and 55.33% ($p = 0.04$), respectively, but not that in TD. The EE administration significantly increased the CtBMD in TM by 2.20% ($p = 0.001$), but not that in FM.

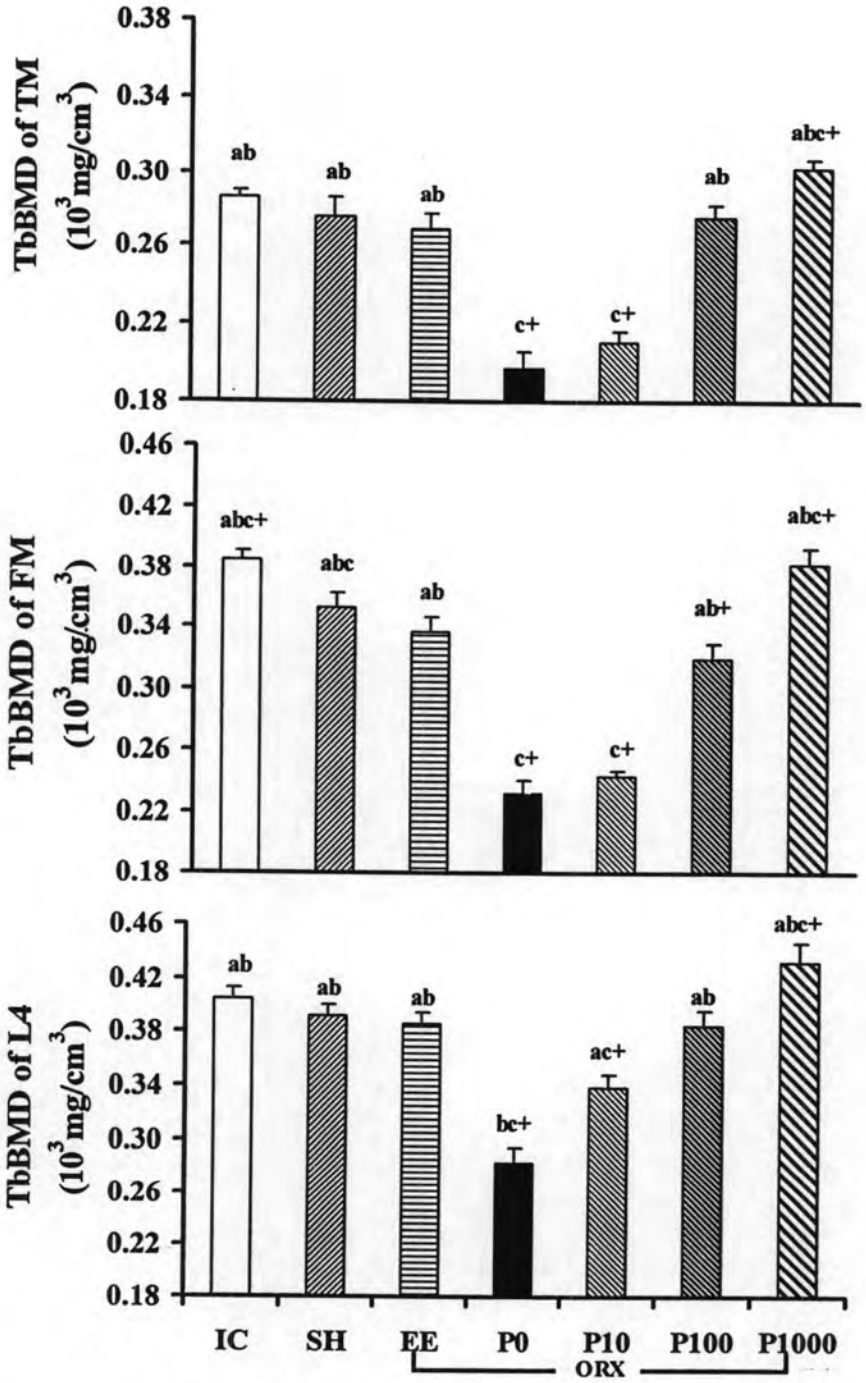

Figure 4.7 Trabecular bone mineral densities (TbBMDs) at the proximal tibial metaphysis (TM), distal femoral metaphysis (FM) and the fourth lumbar vertebral body (L4) in IC, SH, and ORX male rats treated with EE, P0, P10, P100 and P1000. TbBMDs were determined on day 0 in IC group and day 90 in other 6 groups. Data are mean ± SEM. $^{a}p < 0.05$ *vs.* P0; $^{b}p < 0.05$ *vs.* P10; $^{c}p < 0.05$ *vs.* P100; $^{+}p < 0.05$ *vs.* SH.

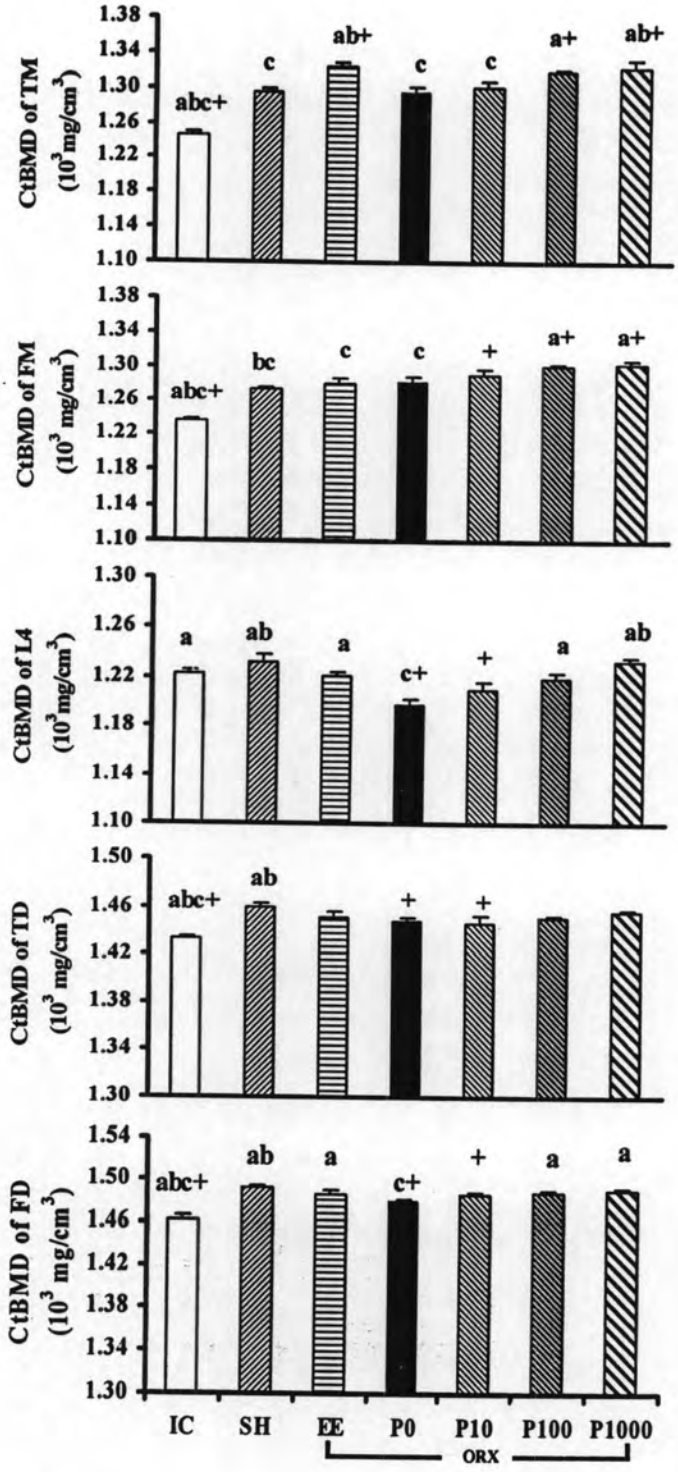

Figure 4.8 Cortical bone mineral densities (CtBMDs) at TM, FM, L4, TD and FD in IC, SH, EE, P0, P10, P100 and P1000 groups in male rats. CtBMDs were determined on day 0 in IC group and day 90 in other 6 groups. Data are mean ± SEM. $^{a}p < 0.05$ *vs.* P0; $^{b}p < 0.05$ *vs.* P10; $^{c}p < 0.05$ *vs.* P100; $^{+}p < 0.05$ *vs.* SH.

2.5 Changes in bone mineral content (BMC)

The effects of *P. mirifica* on bone mass in ORX rats were examined changes in BMC. The results for trabecular bone mineral contents (TbBMCs) in TM, FM and L4 are shown in Figure 4.9.

The three-month increase of age, from 7 to 10 months, between IC and SH group in TbBMCs were observed in TM, FM and L4 ($p > 0.05$).

The ORX for three months in P0 group obviously decreased TbBMCs in TM, FM and L4 by 34.13, 35.31 and 21.59%, respectively, from those of the SH group ($p < 0.001$).

The treatment of P10 significantly prevented the decrease in TbBMC in L4 by 55.26% ($p = 0.004$), but did not significantly prevent in the TM and FM. The treatment of P100 significantly prevented the decrease in TbBMCs in TM, FM and L4 by 67.44, 41.12 and 94.74% ($0.001 \leq p \leq 0.002$), respectively. The treatment of P1000 highly prevented the decrease in TbBMCs in TM, FM and L4 by 85.27, 76.17 and 110.53%, respectively ($p < 0.001$). Administration of EE highly prevented the decrease in TbBMCs in TM, FM and L4 by 82.17, 74.30 and 107.89%, respectively ($p < 0.001$) as effectively as those of P1000.

The cortical bone mineral contents (CtBMCs) in all treatment groups are shown in Figure 4.10. At day 90, the CtBMCs in SH group significantly increased in TM, FM, TD and FD by 11.77, 17.84, 11.72 and 11.91%, respectively ($p < 0.01$), while that of L4 did neither increase nor decrease from those of IC group.

The ORX for three months in P0 group significantly decreased CtBMCs in L4, TD and FD by 25.90, 11.20 and 11.53%, respectively ($p < 0.001$) over the SH group, however, the CtBMCs in TM and FM were not significantly changed.

The treatment of P10 slightly increased CtBMCs in all bone sites in comparison with P0 group but not significant ($p > 0.05$). The treatment of P100

significantly increased the CtBMCs in TM and FM by 8.09 ($p = 0.02$) and 13.32% ($p < 0.001$), respectively, from those in P0 group. However, P100 did not significantly prevent the decrease in CtBMCs in TD (28.72%; $p = 0.39$) and FD (36.13%; $p = 0.09$). Interestingly, the P100 highly significantly prevented the decrease in CtBMC in L4 by 59.72% ($p = 0.004$). The P1000 increased the CtBMCs in FM by 11.55% ($p = 0.008$), but did not in TM from those of P0 group. The P1000 also highly prevented the decrease in CtBMCs in L4, TD and FD by 83.33, 69.09 and 70.97% ($0.001 \leq p \leq 0.006$), respectively. Administration of EE significantly increased the CtBMCs in TM and FM by 13.4 and 12.56%, respectively, compared with those of P0 group ($p < 0.001$). The EE also significantly prevented the decrease in CtBMCs in L4, TD and FD by 55.56, 79.79 and 45.81% ($0.001 \leq p \leq 0.04$), respectively.

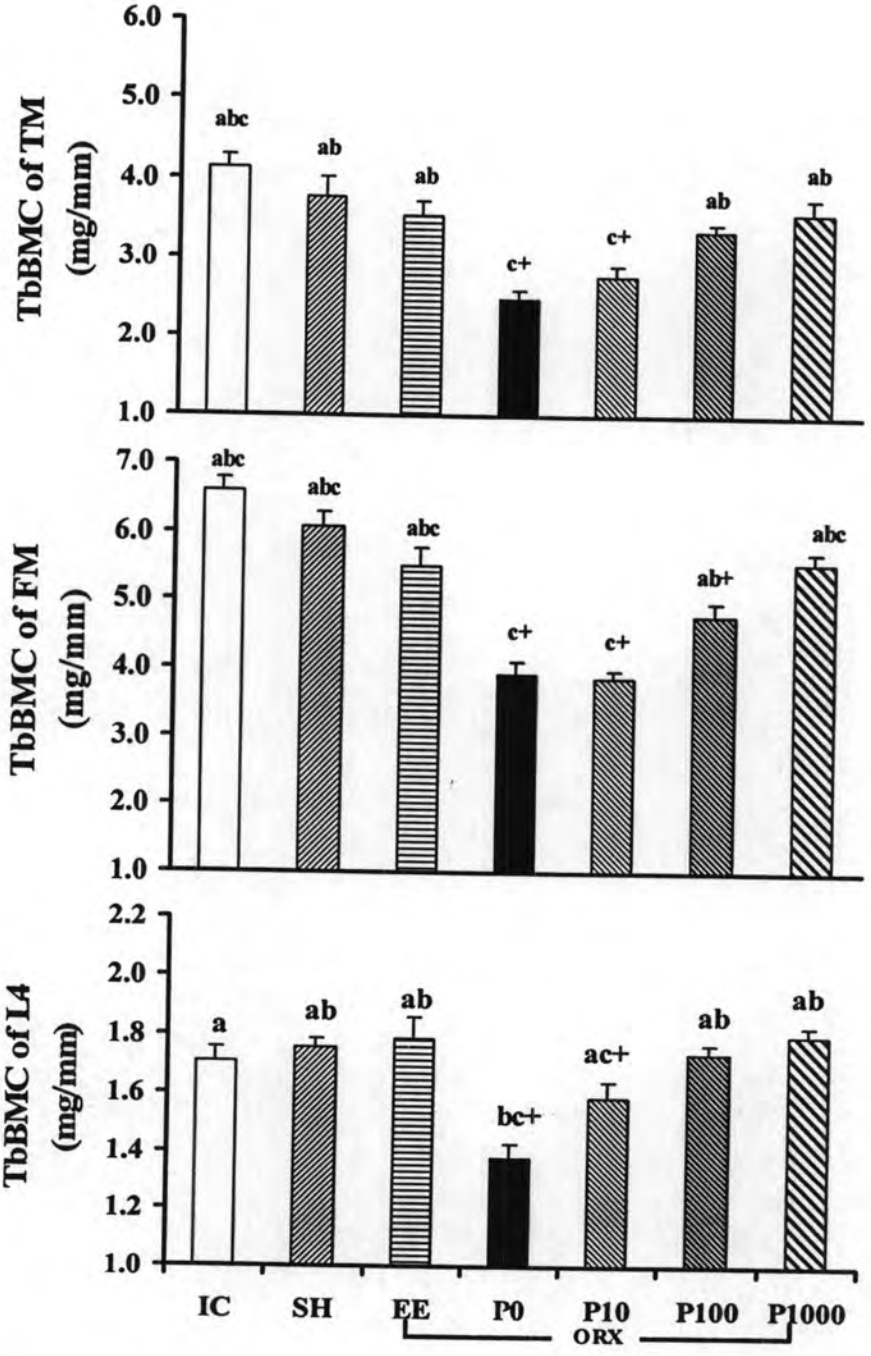

Figure 4.9 Trabecular bone mineral contents (TbBMCs) at the proximal tibial metaphysis (TM), distal femoral metaphysis (FM) and the fourth lumbar vertebral body (L4) in IC, SH, EE, P0, P10, P100 and P1000 groups in male rats. TbBMCs were determined on day 0 in IC group and day 90 in other 6 groups. Data are mean ± SEM. $^{a}p < 0.05$ *vs.* P0; $^{b}p < 0.05$ *vs.* P10; $^{c}p < 0.05$ *vs.* P100; $^{+}p < 0.05$ *vs.* SH.

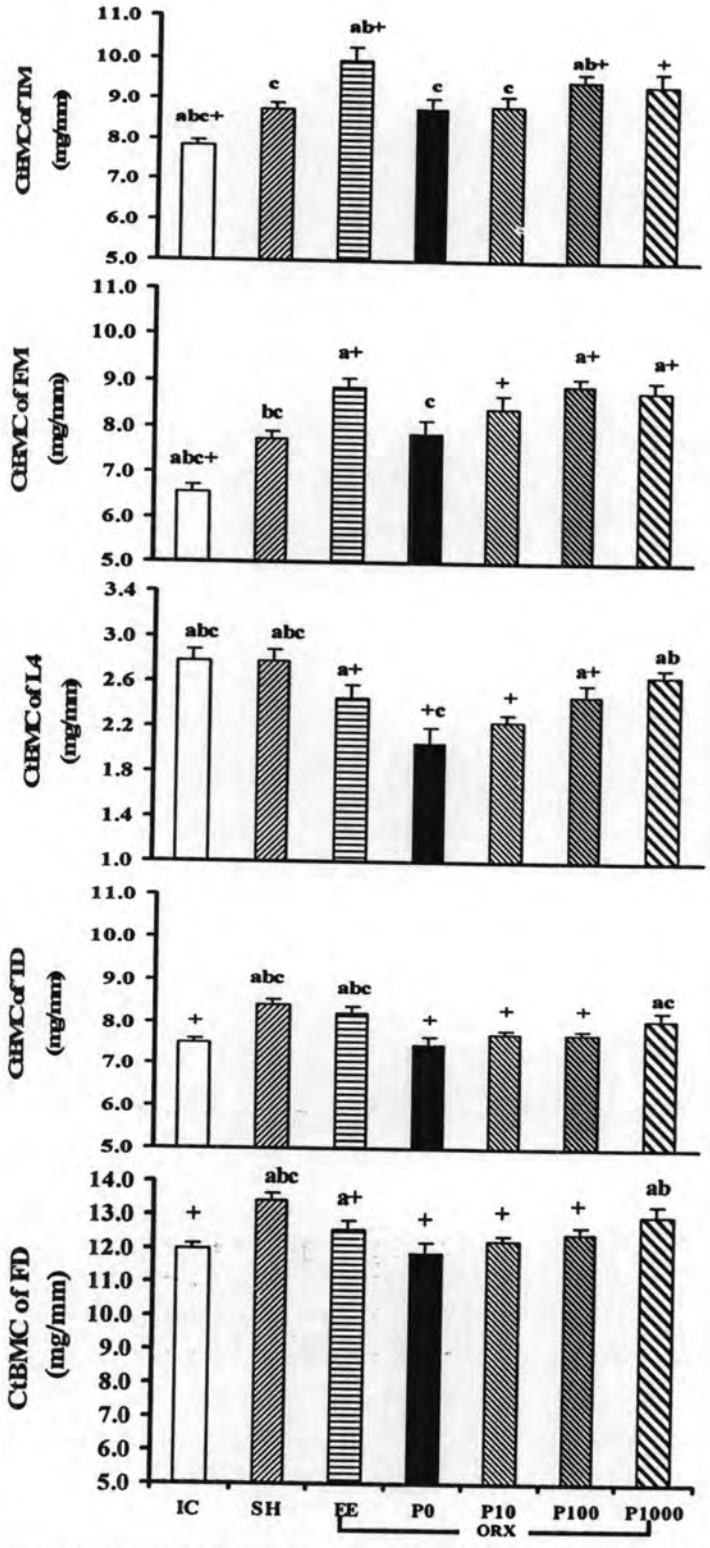

Figure 4.10 Cortical bone mineral contents (CtBMCs) at TM, FM, L4, TD and FD in IC, SH, EE, P0, P10, P100 and P1000 groups in male rats. CtBMCs were determined on day 0 in IC group and on day 90 in other 6 groups. Data are mean ± SEM. $^{a}p < 0.05$ *vs.* P0; $^{b}p < 0.05$ *vs.* P10; $^{c}p < 0.05$ *vs.* P100; $^{+}p < 0.05$ *vs.* SH.

2.6 Changes in bone histology

Histological sections in SH group revealed a normal trabecular conformation. Inside metaphyseal region (below epiphyseal plate) was completely filled with trabecular bones and its connectivity was intervened small inter-trabecular spaces (Figure 4.11A). Three months after ORX, P0 rats showed sparse and thinner trabeculae which is resulted in greater inter-trabecular spaces (Figure 4.11C). However, treatment of P1000 exhibited the thicker trabeculae with high connectivity and narrower inter-trabecular spaces than that of the SH treatment (Figure 4.11D). Three months of EE treatment, bone deterioration caused by ORX was restored and became comparable with the SH rats (Figure 4.11B).

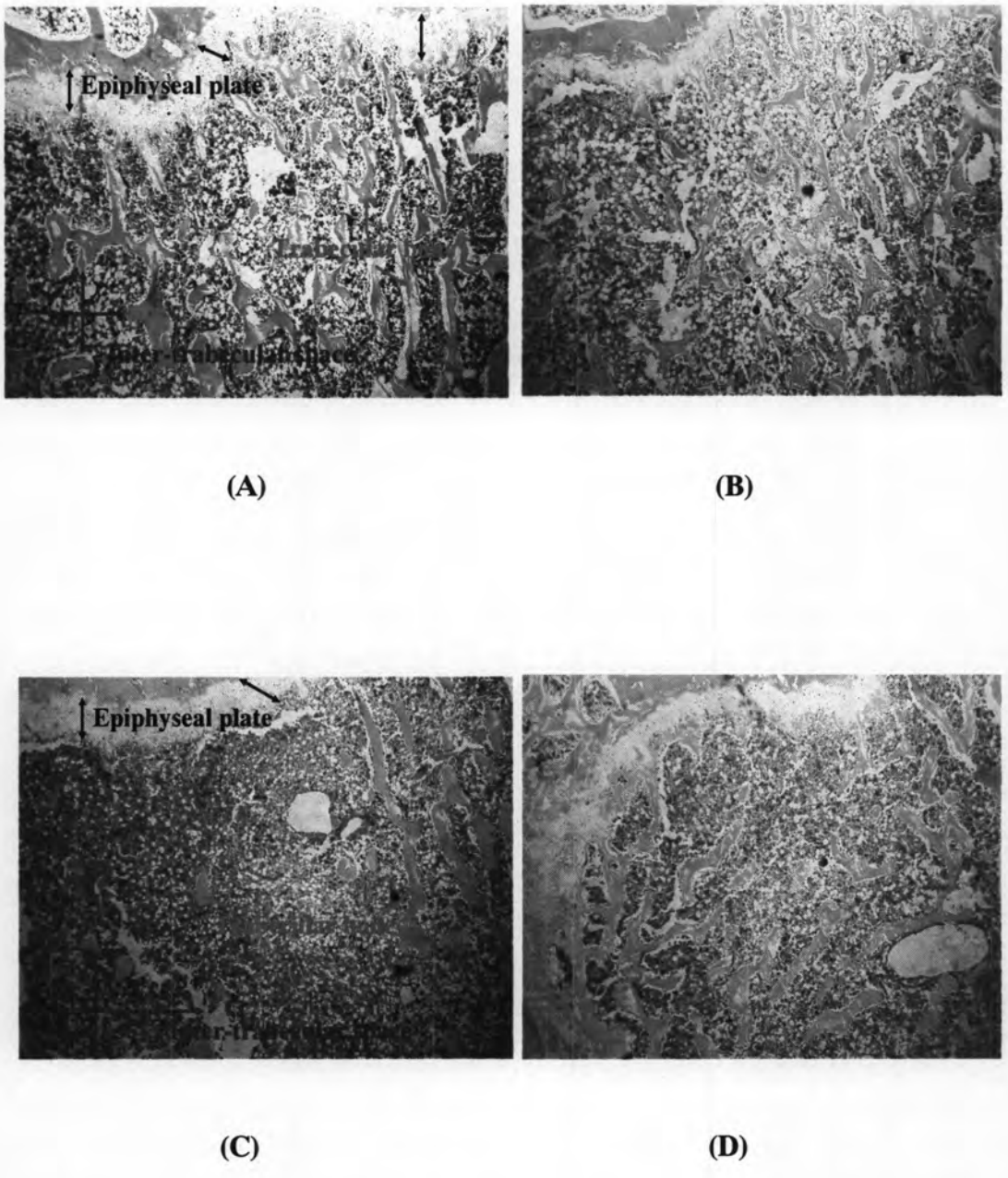

Figure 4.11 Histological section [stained with H & E (50x)] in longitudinal and medio-lateral plane of the proximal tibia at the epiphyseal growth plate and metaphyseal area of SH rats (A) and ORX rats treated with EE (B), P0 (C) and P1000 (D).

3. The effect of *P. mirifica* on TbBMD reduction comparing between OVX and ORX rats

Sex differences in response to *P. mirifica* treatment were seen only in the P10 group. The differences also depended on bone sites. In female, P10 treatment significantly prevented the decrease in TbBMDs in tibial metaphysis (TM) and femoral metaphysis (FM), but it was not in L4 ($p > 0.05$). In male, P10 treatment significantly prevented the decrease in TbBMD only in L4. In P100 and P1000 groups, sex differences in responses to the treatment could not be observed (Table 4.4).

Table 4.4 Comparison of the percentages of prevention on the TbBMD reduction after treated with *P. mirifica* between OVX and ORX rats.

		%Prevention		
Group	**Sex**	**TM**	**FM**	**L4**
P10	Female	28.18 ± 6.96*	30.98 ± 8.49*	ns
	Male	ns	ns	50.81 ± 10.01*
P100	Female	87.37 ± 8.25	85.79 ± 7.98	81.89 ± 8.38
	Male	100.12 ± 8.77	72.52 ± 7.64	93.91 ± 9.16
P1000	Female	119.81 ± 4.91	110.86 ± 7.05	130.06 ± 14.34
	Male	134.46 ± 6.43	124.17 ± 9.44	137.37 ± 12.26

Data are presented as means ± SEM.

* $p < 0.05$ female *vs.* male rats

ns = non-significant prevention *vs.* P0

4. Isoflavone contents in rodent diets and *P. mirifica*

4.1 Calibration curves of standard isoflavones and the percent recovery of isoflavone extraction

Highly linear calibration curves of standard isoflavones by HPLC technique were obtained, R^2 = 0.9881 – 0.9997 (Figure 4.12). Comparing between three consecutive extractions for the five isoflavones in rodent diets and *P. mirifica* samples, the percent recovery of the first extraction is the highest, ranging over 72.0 - 74.4%. Most of remaining isoflavones could be recovered by the second extraction, ranging over 19.6-22.0%, and only some of isoflavones remained for the third extraction, ranging over 5.8-6.8% (Figure 4.13).

4.2 Isoflavone contents in rodent diets and *P. mirifica*

Isoflavone contents in rodent diets and *P. mirifica* Cultivar Wichai-III are listed in Table 4.5, and their HPLC fingerprints are shown in Figure 3.4. The puerarin, a specific isoflavone found in *Pueraria spp.*, was not detected either in the standard rodent diets (C.P. 082) or in the soybean-free diet (C.P. 082/SBF). The total amount of four isoflavones, including daidzin, genistin, daidzein and genistein, in the standard soybean-based rodent diet ranged from 38.6 to 72.4 mg/100 g of diet. The variation of total isoflavones was considerably high, 1.10 – 1.88 times, between lots. In contrast, the soybean-free diet (C.P. 082/SBF) contained much lower total isoflavones (6.1 mg/100 g of diet), only 8-16% of the standard rodent diet.

High amounts of five isoflavones were found in *P. mirifica*, especially puerarin which accounts for more than half of the total isoflavones. However, the concentration of genistein and its glycoside form (genistin) in *P. mirifica* samples were very low compared with those of the standard rodent diets (C.P. 082): 0.3 $\pm$ 0.1 mg/100 g of *P. mirifica vs.* 0.9 $\pm$ 0.4 mg/100 g of diet for genistein, and 7.7 $\pm$ 0.2 mg/100 g of *P. mirifica vs.* 29.4 $\pm$ 7.9 mg/100 g of diet for genistin. The genistein was not found in the soybean-free diet (C.P. 082/SBF). The amounts of isoflavones

in aglycoside form (daidzein and genistein) were lower than those of respective glycoside forms (daidzin and genistin) in standard rodent diets and *P. mirifica*. The daidzein content was 0.34-0.57 times that of daidzin, and genistein was 0.01-0.06 times that of genistin.

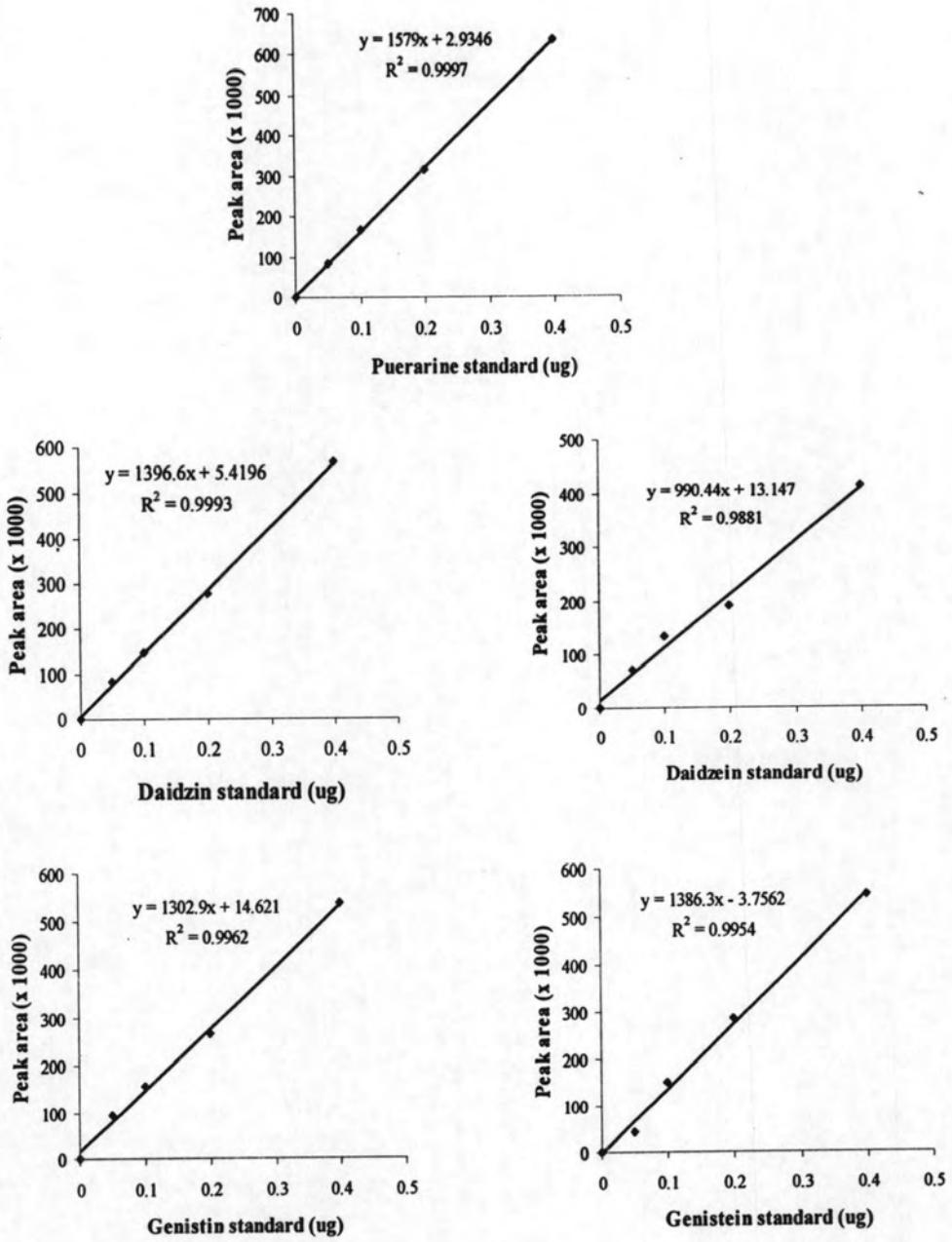

Figure 4.12 Calibration lines for the five standard isoflavones studied. Peak areas for the five amounts (0, 0.05, 0.10, 0.20, and 0.40 μg) of each isoflavone were determined by the high performance liquid chromatography (HPLC) technique.

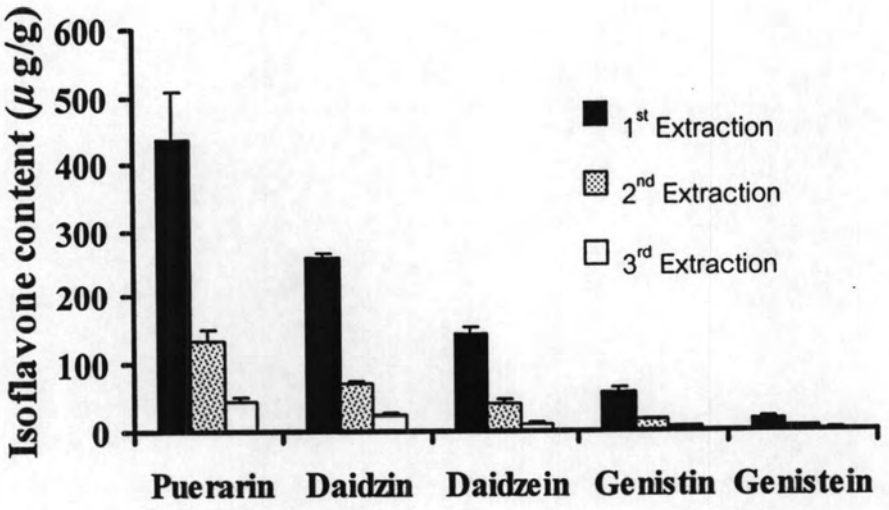

Figure 4.13 The concentration of three consecutive extractions of isoflavones, puerarin, daidzin, genistin, daidzein and genistein, in *Pueraria mirifica* Lot No. 990611 by high performance liquid chromatography (HPLC). Isoflavones were extracted by 70% ethanol.

Table 4.5 Isoflavone contents (mg/100 g sample) in standard and soybean-free rodent diets and in *P. mirifica* determined by the high performance liquid chromatography (HPLC) technique.

Samples	Isoflavones (mg/100 g sample)					
	Puerarin	Daidzin	Daidzein	Genistin	Genistein	Total
Standard rodent diet (C.P. 082)						
Lot No. 2	nd*	20.7 ± 0.6	10.2 ± 3.4	38.6 ± 2.6	1.4 ± 0.7	70.9 ± 3.0
Lot No. 10	nd	12.2 ± 0.2	4.5 ± 0.6	20.7 ± 0.9	1.2 ± 0.3	38.6 ± 2.9
Lot No. 18	nd	26.2 ± 0.9	9.1 ± 1.0	36.2 ± 1.1	0.9 ± 0.6	72.4 ± 3.7
Lot No. 21	nd	13.7 ± 0.8	5.4 ± 0.3	22.7 ± 0.8	0.8 ± 0.5	42.5 ± 0.8
Lot No. 24	nd	18.8 ± 1.8	10.0 ± 0.2	28.8 ± 3.5	0.4 ± 0.1	58.0 ± 5.6
Soybean-free rodent diet (C.P. 082/SBF) Lot No. 050119	nd	0.9 ± 0.1	3.0 ± 0.1	2.2 ± 0.1	nd	6.1 ± 0.2
Pueraria mirifica						
Lot No. 990609	86.5 ± 5.4	39.9 ± 2.4	22.8 ± 0.5	7.9 ± 0.4	0.3 ± 0.1	157.3 ± 8.7
Lot No. 990611	61.0 ± 4.8	34.9 ± 0.6	19.4 ± 0.8	7.6 ± 0.3	0.3 ± 0.1	123.2 ± 6.6

* nd = non-detectable