Improvement of calcium balance by *Fructus Ligustri Lucidi* extract in mature female rats was associated with the induction of serum parathyroid hormone levels

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**Abstract**

*Fructus Ligustri Lucidi* (FLL) is a commonly prescribed herb in many kidney-tonifying Traditional Chinese Medicinal formulae for the treatment of osteoporosis. The present study aimed to identify the active fractions in FLL and to characterise its effects on Ca balance, calcitriol hormone levels as well as bone properties in mature female rats fed diets containing different levels of Ca. In the present study, 4-month-old Sprague–Dawley female rats were treated with either FLL ethanol extract (EE), ethyl acetate-soluble fraction of EE (EAF), water-soluble fraction of EE (WF) or their vehicle for 12 weeks on a medium-Ca diet (MCD, 0·6 % Ca, 0·65 % P). Then, the Sprague–Dawley female rats treated with WF or its vehicle for 12 weeks were fed diets containing different levels of dietary Ca (low-Ca diet (LCD), 0·1 % Ca, 0·65 % P; MCD; high-Ca diet (HCD), 1·2 % Ca, 0·65 % P). The results demonstrated that WF from EE but not EAF exerted a prominent effect on Ca balance by inhibiting urinary and faecal Ca excretion. WF significantly increased Ca balance in rats fed MCD or HCD with an associated increase in serum parathyroid hormone (PTH) levels. WF did not alter bone mineral density or bone mineral content of the tibia in all the rats fed with different levels of dietary Ca. In conclusion, WF was responsible for the positive actions of FLL on Ca absorption and balance. The regulation of Ca balance by WF might involve its action in stimulating PTH production in the mature female rats.

**Key words:** *Fructus Ligustri Lucidi*: Calcium balance: Mature rats: Dietary calcium: Parathyroid hormone

*Fructus Ligustri Lucidi* (FLL), the fruit of *Ligustrum lucidum* Ait, is a commonly prescribed herb to nourish the endocrine and renal systems and to strengthen the bones in Traditional Chinese Medicine¹. In the clinical practice of Traditional Chinese Medicine, FLL is a component of many kidney-tonifying Chinese herbal formulae for the treatment of osteoporosis². Our previous study demonstrated that the ethanol extract (EE) of FLL could improve bone properties in the aged female rats, mainly by elevating Ca absorption and balance².³. FLL was proven to have improved efficiency in utilising Ca from diet, mainly via its actions on increasing serum 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) level and vitamin D-dependent Ca binding proteins (CaBP)². Our results demonstrated that FLL enhanced the transcellular Ca transport in aged female rats fed with sufficient dietary Ca². Our recent study reported that FLL increased the circulating

**Abbreviations:** 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; μCT, micro-computed tomography; BS, bone surface; BV, bone volume; C, control, CaBP, Ca binding proteins; Cr, creatinine; EAF, ethyl acetate-soluble fraction of ethanol extract; EE, ethanol extract; FLL, *Fructus Ligustri Lucidi*; HCD, high-Ca diet; LCD, low-Ca diet; MCD, medium-Ca diet; NDS, non-digestible saccharides; PTH, parathyroid hormone; TV, tissue volume; WF, water-soluble fraction of ethanol extract.

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Increased calcium balance by *Fructus Ligustri Lucidi*

**Methods**

**Preparation and fractionation of Fructus Ligustri Lucidi**

*Fructus Ligustri Lucidi* (FLL) was obtained from Jiangsu province of China. A voucher specimen was deposited in The Hong Kong Polytechnic University. The crude plant (40 kg) was extracted with 70% ethanol by the reflux method twice; and each lasted 1 h. The mixture was filtered to collect the filtrate, which was evaporated to almost dryness using a rotary evaporator under reduced pressure. The residue was finally lyophilised to dryness to obtain the EE (yield 11-42%). The EE was then suspended in hot water and partitioned between ethyl acetate and water to obtain the ethyl acetate-soluble fraction (EAF) and the water-soluble fraction (WF). The two fractions were made into dried powders by a process of evaporation and lyophilisation.

Oleanolic acid and ursolic acid are the two main active compounds in FLL; and oleanolic acid is a commonly used chemical marker for the authentication of FLL according to the Chinese Pharmacopoeia (Edition 2010). HPLC detection of the two compounds in the EE of FLL and its two fractions, EAF and WF are shown in the Appendix. A C18 HPLC column (4.6 × 250 mm, 5 μm) was used in the HPLC analysis. A mobile phase consisting of MeOH–0.1% acetic acid (87:13) was run at a flow rate of 1 ml/min, and the detection wavelength was set at UV 204 nm. Oleanolic acid and ursolic acid were adequately resolved from other unknown compounds and could be clearly identified by the retention time in the EE of FLL and its EAF (see Appendix (a)). WF was further analysed by HPLC under another condition (see Appendix (b)). A mobile phase consisting of acetonitrile and 0.1% trifluoroacetic acid was run at a flow rate of 0.8 ml/min, and the detection wavelength was set at UV 230 nm. The linear gradient elution was set as follows: from the beginning, acetonitrile increased from 8 to 40% in 40 min, and then increased to 70% in the next 20 min. The two most prominent peaks in WF were identified to be nuezhenide and salidroside (see Appendix (b)).

**Animal study design**

A total of thirty-two 4-month-old mature (average weight 238.5 (± 2.5) g) Sprague–Dawley female rats (Experimental Animal Center of the Hong Kong Chinese University, Hong Kong, China) were used in the first animal study. Upon acclimatisation with a medium-Ca diet (MCD; TD Teklad 98005, 0.6% Ca, 0.65% P) for 5 d, the mature rats were randomly divided into four groups: the control group (C, treated with distilled water) and FLL EE, FLL EAF or FLL WF as the treatment groups and pair-fed with MCD. The dosages of EE, EAF and WF were given according to their actual extraction ratio, namely 700, 126 and 57.4 mg/kg per d, respectively. Total treatment period was 12 weeks. In the second experiment, the effects of the active fraction on Ca balance and bone properties were studied. A total of sixty 4-month-old mature Sprague–Dawley female rats (220–250 g; Experimental Animal Center of the Hong Kong Chinese University, Hong Kong, China) were randomly divided into three groups with differing dietary Ca levels and treated with either FLL EE water (WF, 574 mg/kg per d) or its vehicle (distilled water) for 12 weeks. All rats were fed a MCD (TD 98005, 0.6% Ca, 0.65% P) for 5 d before the initiation of the treatment regimen. Diets containing different levels of Ca were low-Ca diet (LCD, TD 05004, 0-1% Ca, 0.65% P), MCD and high-Ca diet (HCD, TD 05005, 1.2% Ca, 0.65% P).
All diets were purchased from Harlan Teklad (Madison, WI, USA). All rats had free access to distilled water, and were fed 15 g/d per rat of the respective diet, the minimum average food intake of the rats during the accretion period. The body weight of the animals was recorded weekly. The rats were housed in a room that provided alternating 12 h of light and 12 h of darkness with the room temperature at 23 ± 1°C and humidity 55 ± 5%. Husbandry of the animals was based on the National Institutes of Health Guide for Care and Use of Laboratory Animals. The experimental protocol was approved by the Animal Ethics Committee of The Hong Kong Polytechnic University.

Sample collection

The animals, 2 d before being killed, were housed individually in metabolic cages for collection of urine and faeces. On the day of killing, blood was withdrawn from the abdominal aorta under light diethyl ether anaesthesia, and the serum was prepared. Rat tibias were collected, cleaned of all soft tissue, wrapped in saline-soaked towels and stored at −20°C for further analysis.

Table 1. Effects of the ethanol extract (EE) of Fructus Ligustri Lucidi (FLL) and its fractions on serum or urine chemistries in mature female rats fed a medium-calcium diet (MCD; 0·6 % calcium, 0·65 % phosphorus) for 12 weeks

(Mean values with their standard errors, n=6–8)

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<th>Weight gain (g)</th>
<th>Serum Cr (mg/l)</th>
<th>Serum 1,25(OH)2D3 (pg/ml)</th>
<th>Urinary Ca:Cr (mg/mg)</th>
<th>Urinary P:Cr (mg/mg)</th>
<th>Serum 1,25(OH)2D3 (pg/ml)</th>
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Cr, creatinine; 1,25(OH)2D3, 1,25-dihydroxyvitamin D3; PTH, parathyroid hormone; C, vehicle-treated group; EAF, ethyl acetate-soluble fraction in the EE of FLL; WF, water fraction in the EE of FLL.

* Mean values were significantly different from those of the vehicle-treated group (P<0.05).

Detection of calcitropic hormones

Serum levels of intact PTH (1–84) were detected using rat bioactive intact PTH ELISA assay (Immutopics, Inc., San Clemente, CA, USA). Serum 1,25(OH)2D3 was extracted with two separate extraction columns and measured by competitive enzyme immunoassay (Immundiagnostik AG, Bensheim, Germany).

Micro-computed tomography analysis of rat tibia

Left tibias were thawed at room temperature before testing. The bone properties of tibia were studied using cone-beam X-ray micro-computed tomography (μCT; vivaCT40; Scanco Medical AG, Basserdorf, Switzerland). The μCT images of the proximal metaphysis in the left tibia head were obtained by scanning at medium resolution with 21 μm increments at 70 kVp and 110 μA with a tube voltage of 50 kV, tube content of 0·1 mA, slice thickness of 13 μm and pixel size of 13 μm. The scanning process was controlled by an OpenVMS (Intel Itanium) workstation. The scanning positions of the tibia head were 2·5–7 mm from the knee joint. A total of 210 consecutive slices from metaphysis bone were obtained for evaluation. After evaluation, a μCT reconstruction model was generated and three dimensional bone parameters were calculated by software. Bone content parameters included apparent bone mineral density, bone mineral content and bone volume: tissue volume ratio (BV:TV). Bone structural parameters included trabecular number, trabecular thickness, trabecular separation, bone surface:bone volume ratio (BS:BV) and connectivity density.

Statistical analysis

Data from these experiments were reported as means with their standard errors. All statistical analyses were performed using PRISM version 4.0 (GraphPad Software Inc., La Jolla, CA, USA). Analysis of the effects of diet, herb and interaction of both factors as grouping variables was performed by two-way ANOVA. Inter-group differences were analysed by unpaired t-test as a post-test. Differences in P value of less than 0·05 were considered statistically significant.
Results

Effects of Fructus Ligustri Lucidi fractions on body weight, serum and urine chemistries

Body weight in the mature female rats increased gradually within the experimental period and the FLL treatment did not alter the weight gain (Table 1). Serum Ca and P were not altered by FLL treatment in mature female rats (Table 1). Urinary Ca:Cr was significantly decreased in rats by treatment with FLL EE and FLL WF (P<0·05 v. C, Table 1). Urinary P:Cr levels were not significantly altered in rats upon treatment with EE and its fractions (Table 1). EE appeared to increase serum 1,25(OH)2D3 levels; however, the increase was not statistically significant (Table 1). Similarly, serum PTH levels tended to increase in rats upon EE and WF treatment, but the increase did not reach statistical significance (Table 1).

Effects of Fructus Ligustri Lucidi fractions on calcium balance

Faecal Ca excretion in rats was significantly decreased by EE and its WF treatment (P<0·05 v. C, Fig. 1(a)). Both the calculated Ca net balance and Ca absorption rate in the mature rats were enhanced simultaneously by EE and WF treatment (P<0·05 and P<0·01 v. C, respectively; Fig. 1(b) and (c)).

Effects of Fructus Ligustri Lucidi water fraction on body weight, serum and urine calcium and phosphorus levels in rats fed different levels of dietary calcium

Body weight increased slowly in all six rat groups during the experimental period and weight gain in mature rats was not significantly altered in response to treatment with FLL WF (Table 2). However, two-way ANOVA analysis indicated that dietary Ca level altered the weight gain significantly (P<0·05, Table 2). In particular, LCD induced more weight gain in both vehicle- and WF-treated female rats (Table 2). Serum Ca did not alter in rats fed LCD or MCD in response to WF treatment (Table 2). However, HCD significantly increased serum Ca levels in the vehicle-treated rats (P<0·05 v. C; Fig. 1(a)). Both the faecal Ca excretion, Ca net balance and Ca absorption rate in each treatment group. Sprague–Dawley female rats (4 months old) were treated with vehicle (C; distilled water) and the ethanol extract (EE), ethyl acetate-soluble fraction (EAF) or water fraction (WF) of Fructus Ligustri Lucid (FLL), at the dosage of 700, 126 and 574 mg/kg per d, respectively. Total treatment period was 12 weeks. At the end of experiment, faeces and urine were collected for detection of Ca content. Ca net balance and Ca absorption rate were calculated according to their faecal and urinary Ca excretion. Values are means with their standard errors represented by vertical bars (n 6–8). Mean values were significantly different from those of vehicle-treated group: *P<0·05, **P<0·01.

Fig. 1. Contents of (a) faecal Ca, (b) Ca net balance and (c) Ca absorption rate in each treatment group. Sprague–Dawley female rats (4 months old) were treated with vehicle (C; distilled water) and the ethanol extract (EE), ethyl acetate-soluble fraction (EAF) or water fraction (WF) of Fructus Ligustri Lucid (FLL), at the dosage of 700, 126 and 574 mg/kg per d, respectively. Total treatment period was 12 weeks. At the end of experiment, faeces and urine were collected for detection of Ca content. Ca net balance and Ca absorption rate were calculated according to their faecal and urinary Ca excretion. Values are means with their standard errors represented by vertical bars (n 6–8). Mean values were significantly different from those of vehicle-treated group: *P<0·05, **P<0·01.
Table 2. Effects of water fraction of *Fructus Ligustri Lucidi* (FLL) on weight gain and chemistries levels of calcium, phosphorus in serum and urine of normal mature female rats fed a low-calcium diet (LCD; 0·1 % calcium, 0·65 % phosphorus), medium-calcium diet (MCD; 0·6 % calcium, 0·65 % phosphorus) or high-calcium diet (HCD; 1·2 % calcium, 0·65 % phosphorus) for 12 weeks (Mean values with their standard errors, n = 6–8)

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**P** (two-way ANOVA)

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Cr, creatinine; 1,25(OH)2D3, 1,25-dihydroxyvitamin D3; PTH, parathyroid hormone.

* Mean values were significantly different from those of the vehicle-treated group fed with a similar level of dietary Ca (P < 0·05).

Mean values were significantly different from those of the LCD-fed rats in vehicle-treated groups: † P < 0·05, ††† P < 0·001.

Effects of *Fructus Ligustri Lucidi* water fraction on bone properties of rat tibia in rats fed with different levels of dietary Ca (Table 3). Higher levels of dietary Ca significantly improved bone mineral density, trabecular number and structure in proximal metaphysis of the rat tibia. Our results showed that higher dietary Ca significantly increased trabecular bone microarchitecture in rats through increasing trabecular number (t < 0·05), thickness (t < 0·05), and decreasing trabecular separation (t < 0·05). Accordingly, the highest Ca absorption rate was found in the LCD-fed rats (Fig. 2(c)). Accordingly, the highest Ca absorption rate was found in the LCD-fed rats (Fig. 2(c)). Accordingly, the highest Ca absorption rate was found in the LCD-fed rats (Fig. 2(c)). Accordingly, the highest Ca absorption rate was found in the LCD-fed rats (Fig. 2(c)).
in response to MCD or HCD (P<0·01 v. LCD-fed rats, Table 3), suggesting a lower rate of bone resorption in these groups. Connectivity density in bone was improved by MCD and HCD treatment in rats (P<0·05 v. LCD-fed rats, Table 3). Similarly, WF treatment did not result in any significant changes of bone microarchitecture in rats (Table 3).

Discussion

Our previous studies reported that FLL EE exerted positive effects on Ca metabolism in aged female rats(2). The present study extended our previous findings to demonstrate that FLL (EE) could increase Ca balance and Ca absorption in mature female rats. Furthermore, WF from EE, but not EAF, exhibited a prominent effect on Ca balance, indicating that WF might be the active fraction of EE for Ca regulation. The subsequent experiment confirmed that WF could offer significant enhancing effects on Ca balance and absorption when the dietary Ca was adequate. The positive actions of WF on Ca balance were associated with the rise in serum PTH levels without altering serum 1,25(OH)2D3 levels in mature normal female rats. Furthermore, the increase in PTH levels by FLL treatment did not induce apparent bone loss in the mature-female-rat model. In addition, WF was found to help prevent surges of serum Ca levels with increasing dietary Ca in the mature female rats.

The mechanism of the actions of FLL on vitamin D metabolism in female rats appeared to alter with age. In contrast to our previous study(2) in which serum 1,25(OH)2D3 levels increased in response to FLL treatment in aged female rats, serum 1,25(OH)2D3 levels did not alter in response to treatment with FLL EE and its fractions in the mature female rats. Previous studies by others(16,17) showed that the levels of serum 1,25(OH)2D3 declined with age. Thus, it is possible that the lower background levels of serum 1,25(OH)2D3 in the aged rats allowed further stimulation of its biosynthesis by FLL in our previous study(2). In contrast, the regulation of 1,25(OH)2D3 biosynthesis in mature rats might be tightly regulated by its stimuli and further stimulation might be inhibited by the physiological feedback mechanisms(18), thereby preventing further increase of serum 1,25(OH)2D3 levels by FLL in mature rats in the present study.

Our results showed that WF treatment did not alter serum Ca levels in the rats fed LCD or MCD. Serum Ca levels were even down-regulated in the HCD-fed rats in response to WF treatment. Thus, the results indicated that WF treatment could prevent the surge of serum Ca in rats in response to HCD feeding. Moreover, WF treatment improved Ca net balance and Ca absorption rate in both the MCD- and HCD-fed rats through suppressing urinary and faecal Ca excretion. Most importantly, urinary Ca excretion was decreased greatly in the WF-treated HCD-fed rats. Thus, it was intriguing to find that serum Ca levels in WF-treated HCD fed rats were suppressed despite the induction of intestinal Ca absorption rate as well as the suppression of urinary Ca excretion by WF treatment in these animals.

Our results also showed that WF treatment significantly increased serum PTH levels in the MCD- and HCD-fed rats. It is well known that the hormonal regulation of PTH is complex and involves circulating Ca levels and 1,25(OH)2D3(19). Low levels of extracellular Ca stimulate PTH secretion within minutes; while elevated levels of Ca inhibit hormone release and favour the degradation within parathyroid cells(20). Another major regulator of PTH secretion is 1,25(OH)2D3 in which PTH mRNA expression in the parathyroid gland is
Table 3. Effects of water fraction of *Fructus Ligustri Lucidi* (FLL) on the bone structural and bone content parameters in tibia metaphysis of normal mature female rats fed a low-calcium diet (LCD; 0·1 % calcium, 0·65 % phosphorus), medium-calcium diet (MCD; 0·6 % calcium, 0·65 % phosphorus) or high-calcium diet (HCD; 1·2 % calcium, 0·65 % phosphorus) for 12 weeks (Mean values with their standard errors, *n* 6–8).

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* P (Two-way ANOVA)

BS, bone surface; BV, bone volume; Conn-D, connectivity density; aBMD, apparent bone mineral density; BMC, bone mineral content; TV, tissue volume.

* Mean values were significantly different from those of LCD-fed rats in vehicle-treated groups (*P* < 0·05).

The lack of negative effects of high serum PTH levels on bone could be related to the overall Ca status/balance of the rat. The lack of negative effects of high serum PTH levels on bone could be related to the overall Ca status/balance of the rat. The lack of negative effects of high serum PTH levels on bone could be related to the overall Ca status/balance of the rat. The lack of negative effects of high serum PTH levels on bone could be related to the overall Ca status/balance of the rat.
WT-treated animals as well as the protective effects of WT on bone. The present study clearly demonstrated that WT fraction could improve Ca balance in rats fed MCD or HCD, with a significant reduction of urinary Ca excretion as well as positive effects on intestinal Ca absorption. Our previous study showed that FLL extract could exert direct effects on the mineralisation process in osteoblast-like cells (22). Thus, the positive effects of WT on bone and Ca balance might counteract the negative effects of high PTH levels in rats in the present study; resulting in no obvious changes in bone properties in the WT-treated animals fed MCD or HCD. Future studies are needed to investigate the mechanism by which WT treatment exerts protective effects on bone in the midst of increased PTH levels in mature female rats.

Currently, the most well-studied non-vitamin D dietary factors that increase Ca balance in humans or animals are non-digestible saccharides (NDS), including monosaccharides (29–33) and fructo-oligosaccharides (34–38). Several mechanisms that contribute to the actions of NDS have been proposed: (1) NDS could be fermented by residing bacteria to produce byproducts, such as SCFA in the caecum and colon to result in a decrease in intestinal pH that eventually leads to an increase in Ca solubility (39); (2) in vitro studies suggested that NDS indirectly open tight junctions in the epithelial cells by increasing intracellular Ca ion concentration, which in turn activates paracellular Ca transport and benefit Ca absorption (40); (3) NDS could increase active Ca transport by the activation of calbindin 9k in large intestine only (41). Our previous results indicated that FLL increased 1,25(OH)2D3-dependent CaBP expression in duodenum (2) as well as renal 1-hydroxylase expression (4) in aged female rats. In addition, the present study clearly demonstrated that FLL increased serum PTH levels in mature female rats. Thus, the mechanism of actions involved in increasing Ca balance by FLL and by NDS appears to be different.

Although FLL also contains quantitative constituents of polysaccharides (12–13), the active constituents in FLL that are responsible for Ca regulation might not be NDS, as the reported mechanism of NDS is different from that of FLL. Moreover, it was found that oleanolic acid and ursolic acid (29–31), the commonly reported active ingredients in FLL, are not contained in the identified active fraction of WF in the present study. Further study is required to identify the active components in FLL that exert the reported positive effects on Ca balance in vitro. Nuzhenide and salidroside, two major compounds that can be isolated from WF, might be candidates for explorations in future studies.

In summary, our present study demonstrated that FLL EE could improve Ca absorption and Ca balance, independent of the ages of the female rats. WF might be responsible for the positive effects of EE in regulating Ca absorption and Ca balance. The beneficial effects of WF in the mature female rats when dietary Ca was sufficient might be mediated by its direct or indirect action on PTH production. Most importantly, the increase in PTH levels induced by WF treatment did not result in any bone loss and the surges of serum Ca levels associated with high Ca feeding can also be prevented by WF treatment in mature female rats. These results suggested that WF is a potential agent that can be administrated orally to improve Ca balance and might be useful for the prevention of osteoporosis.

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**References**

Increased calcium balance by *Fructus Ligustri Lucidi*

Appendix

(a). Overlapped HPLC chromatograms for quantitative analysis of oleanolic acid and ursolic acid in the 70% ethanol extract of *Fructus Ligustri Lucidi* (FLL), and its sub-fractions. (A) Ethyl acetate fraction of FLL (EAF); (B) 70% ethanol extract of FLL (EE); (C) water fraction of FLL (WF); (D) ursolic acid standard; (E) oleanolic acid standard; (1) oleanolic acid; (2) ursolic acid.

(b). HPLC profile of water-soluble fraction of *Fructus Ligustri Lucidi* run under a new condition. The two quantitative identified compounds in this fraction were salidroside and nuezhenide, which were shown in the figure, respectively.