JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Plum and Soy Aglycon Extracts Superior at Increasing Bone Calcium Retention in Ovariectomized Sprague Dawley Rats

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ABSTRACT: Plant-derived polyphenols have been shown to influence bone turnover and bone properties in the estrogen-depleted state. We used a crossover design in ovariectomized rats (n = 16 rats for each diet) to investigate the effect of supplementation of two doses each of blueberry, plum, grape, grape seed extract, and resveratrol on bone. We tested the aglycon and glucoside forms of genistein to quantify differences in efficacy on bone calcium retention. Rats were given an intravenous dose of ^{45}Ca to prelabel bone, and bone calcium retention was assessed by urinary excretion of ^{45}Ca:Ca ratio during an intervention period compared with nonintervention. Genistein aglycon increased bone calcium retention significantly ($p < 0.05$) more than the glucoside (22% vs 13%, respectively). Plum extract (0.45% w/w total dietary polyphenols) and resveratrol (0.2% w/w total dietary polyphenols) were also effective, increasing bone calcium retention by 20% ($p = 0.0153$) and 14% ($p = 0.0012$), respectively. Several polyphenolic-rich diets improved bone calcium retention.

KEYWORDS: *genistein aglycon, genistein glucoside, bone calcium retention, bone resorption, plant-derived polyphenols*

INTRODUCTION

Various plant sources of polyphenolic compounds have been studied for their ability to prevent postmenopausal bone loss associated with estrogen deficiency. The link between soy consumption in the Asian diet and reduced fracture risk[1] has sparked many studies examining soy isoflavones and postmenopausal bone health. More recently, plum, blueberry, and grape products have also been implicated in preventing postmenopausal bone loss due to their polyphenolic components.[2−6]

In Western countries, soy is not consumed as often or in sufficient quantities to match the soy consumed in Asia, where beneficial effects to bone have been observed. In addition, soy in Western countries is typically consumed as an ingredient or in supplemental form that contains extracted isoflavones from soy rather than in whole soy foods.[7] The ability of soy isoflavones to attenuate bone resorption in an estrogen deficient state remains controversial.[8] Discrepancies have been attributed to a number of factors including an individual's ability to convert daidzein to equol, the effect of food matrix on bioavailability of isoflavones compared with extracted isoflavones, and specific isoflavone composition. In foods, isoflavones exist as glycosides and require cleavage of the sugar moiety in the gut to be absorbed in the intestine.[9] Although there have been contrary reports of bioavailability of glycoside compared with aglycon forms of genistein,[10,11] there is interest in comparing glycoside to aglycon forms for bone health as the aglycon form was used in various studies that have demonstrated a strong bone preserving effect in postmenopausal women.[12−15] The presence of the sugar moiety may have an influence on where the isoflavone is absorbed in the gut and how the isoflavone is subsequently conjugated inside the body.

Isoflavones and other phenolics have demonstrated estrogenic activity[16,17] and are being investigated for their biological effect on the estrogen-depleted state. Depletion of estrogen results in a marked increase in osteoclastogenesis, causing bone resorption. During this postmenopausal period, bone formation lags behind resorption, and net bone loss ensues.[18]

There is emerging evidence to suggest that certain fruits, vegetables, and other dietary compounds found in the Western diet may be protective to bone, especially from prune, grape products, and blueberries.[19−21] Grape seed extract has also shown promise in maintaining bone health as supplementation with grape seed extract was able to recover bone lost while on a calcium deficient diet in rats.[22] Dried plum is one of the most extensively studied botanicals for its role in bone health because of a potentially anabolic effect in bone. Dried plum was very effective in protecting bone in ovariectomized (OVX) rats when compared with the effect of intermittent teriparatide (parathyroid hormone 1−34) administration, which is the most effective anabolic drug therapy currently available for osteoporosis treatment.[3] Plum, as a daily supplement of 100 g/d dried plum for one year, was able to increase bone mineral density (BMD) in the spine and ulna of postmenopausal women compared with those who were supplemented with an equal amount of dried apple.[23] While these extracts appear to be diverse, they are rich sources of specific polyphenol forms

Received: August 8, 2013
Revised: May 9, 2014
Accepted: June 4, 2014
Published: June 4, 2014

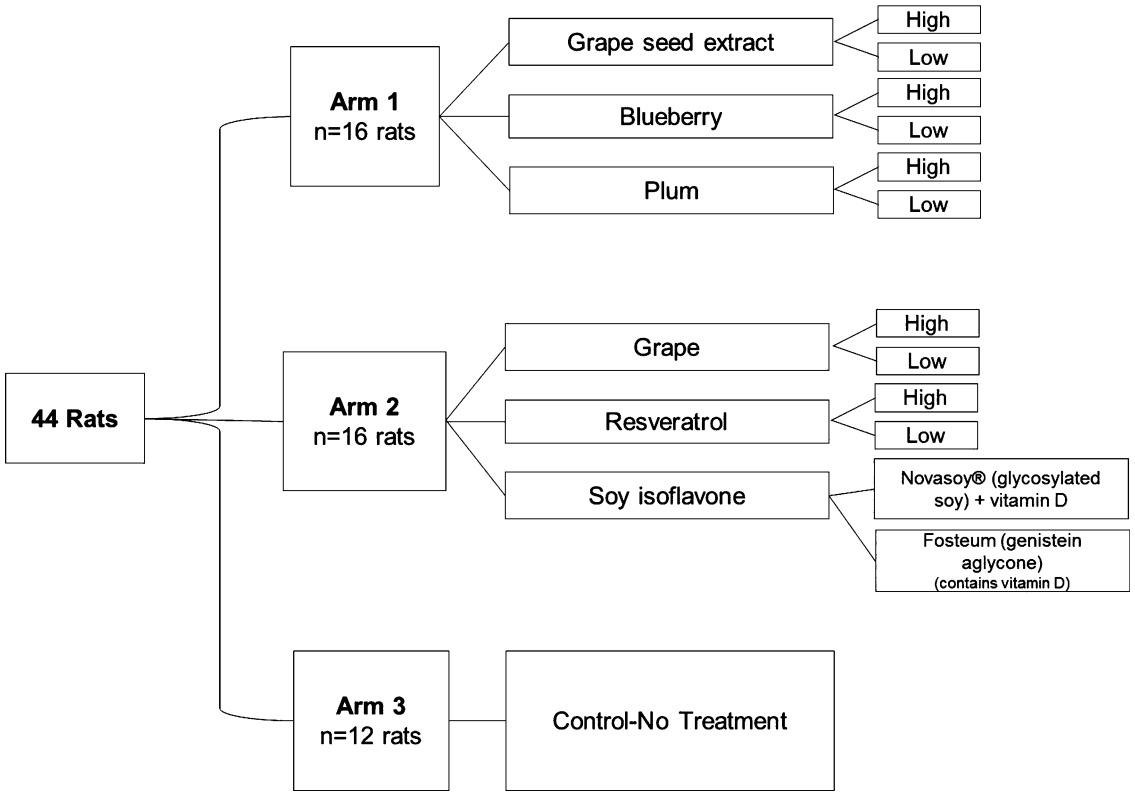

Figure 1. Study design. Forty-four rats were designated into one of three study arms. Rats in arm 1 (n = 16) received blueberry and plum fruit powder and grape seed extract to compare the effect of polyphenols in the whole fruit. Rats in arm 2 (n = 16) received whole fruit polyphenols from grape to compare against the isolated resveratrol polyphenols. Rats in arm 2 were also given genistein in the aglycon form and glycosylated soy to determine the effect of conjugation. Rats in arm 3 (n = 12) were not given a dietary botanical to establish an unperturbed excretion of ^{45}Ca excretion.

including quercetin, anthocyanins, proanthocyanidins, hydroxycinnamates, and resveratrol, which can be classified as possible bioactive agents responsible for observed bone health benefits associated with these foods. For example, resveratrol, found in grape products, extracts, and supplements, increased bone formation markers and decreased expression of receptor activator of nuclear factor kappa-B (RANK) in human primary monocytes,[21] showing potential to alter osteoclastogenesis.

Although the bioactive foods and ingredients described above have been shown to benefit bone by traditional measures of bone density and material properties, these approaches require chronic feeding and terminal measures. This has largely prevented comparisons among multiple diets and dose response effects. The aims of this study were to directly compare botanical sources and to study the effective dose of extracts to increase bone calcium retention in OVX rats using a novel technology that does not require sacrifice and evaluates responses quickly so that multiple comparisons can be efficiently tested. This involves prelabeling bones with ^{45}Ca as we have done previously with ^{41}Ca in human studies[24,25] as a means of screening botanical extracts for their ability to improve bone calcium retention. By injecting rats with ^{45}Ca, allowing the isotope to incorporate into bone, and quantifying the amount of ^{45}Ca in urine, a direct measure of net calcium tracer lost from bone is obtained. The long half-life of ^{45}Ca (163 days) allows multiple diets to be compared using a crossover design in the same rat, providing greater power to detect differences among diets. This method allows for screening of numerous diets on a relatively small number of rats during a short time period.

The method for determining bone calcium retention was derived from a well-established protocol using another bone seeking tracer, ^{3}H-tetracycline,[19,26−28] and adapted by us for calcium tracers in rats[29−31] and humans.[32] ^{3}H-tetracycline was the tracer of choice initially because it was assumed that it assessed only bone resorption from prelabeled bones in rats because it attaches tightly to hydroxyapatite and was slow to release.[33−35] However, we showed through kinetic modeling of both ^{3}H-tetracycline and calcium tracers that both isotopes leave bone during bone resorption and can re-enter bone during bone formation. Our findings suggest that these two tracers give comparable values and that net bone calcium retention is a more accurate description of what is being measured with bone seeking tracers.[30] Mühlbauer and Fleish[36] evolved the method to use daily measures of urinary ^{3}H-tetracycline from prelabeled bone of young rats given multiple injections of the tracer in order to continuously monitor bone resorption through 10-day diet periods and 10-day washout periods using a crossover design. We established that older OVX rats and a single isotope administration could be used in a study that evaluated effects of time of stabilization to OVX and time from dose on tracer behavior.[31] We further validated urinary tracer excretion against bone disappearance of the tracer[31] and adequacy of 10-day diet and washout periods.[29] Quantifying the urinary tracer appearance of a bone label enables rapid screening and multiple comparisons of efficacy of diets designed to improve bone calcium retention.

We hypothesized that both source and dose effects would be observed through testing high and low doses of various extracts from grape seed, grape, and blueberry, as well as plum powder,

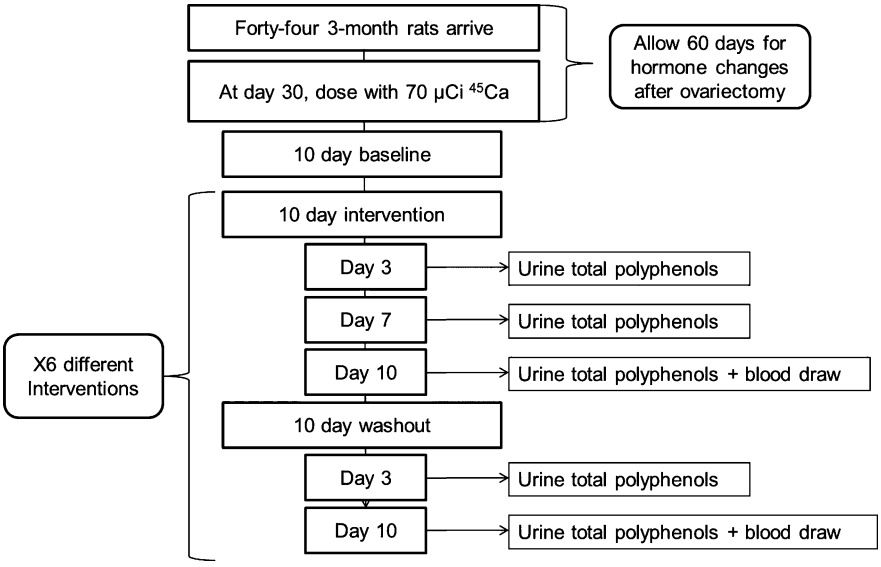

Figure 2. Study timeline for arrival of ovariectomized (OVX) rats, dosing with ^{45}Ca, and urine and blood collection pattern.

and resveratrol. We also used this screening method to determine whether genistein in an aglycon form is more effective at increasing bone calcium retention than its glucoside counterpart.

MATERIALS AND METHODS

Chemicals. A retrospective analysis of phenolic compounds from grape, blueberry, and plum extract was conducted after the conclusion of the study. Extracts were stored at −80 °C, reconstituted in 2% formic acid in ddH_2O at ∼1 mg/mL, and analyzed for key phenolic components by LC−MS as described by Song et al.[37]

Rats. Forty-four OVX Sprague Dawley rats (3 month old) were shipped from Harlan (Indianapolis, IN) and given approximately 30 days to acclimate to their environment, and to stabilize hormone changes post-OVX, while being fed AIN93-M polyphenol-free diet (soybean oil was replaced with corn oil) and distilled water ad libitum. The rats were housed individually in a humidity- and temperature-controlled room with a 12 h light and 12 h dark cycle. Food intake was monitored twice weekly, and body weight was recorded weekly. All procedures performed on rats were approved by and in compliance with the standards set by Purdue University's Animal Care and Use Committee.

Study Design. The study design (Figure 1) was a randomized, crossover intervention trial to evaluate 12 different polyphenolic-containing diets on bone turnover. The rats were randomized into one of three groups: (1) 16 rats received six diets of plum, blueberry, and grape seed extract at two doses, (2) 16 rats received six diets of grape extract and resveratrol at two doses each, and Novasoy (glycosylated soy) and Fosteum (genistein aglycon) at a single dose, and (3) 12 rats received no treatment to establish unperturbed ^{45}Ca excretion over time to verify that the regression lines computed from the nondiet periods were not confounded by diets for both calcium retention and the biomarker of bone turnover. Within each arm, the diets were randomized by polyphenolic source and then by dose.

The rats were injected with 70 μCi of ^{45}Ca in saline via a tail vein catheter approximately 30 days after arrival, moved to metabolic cages, and allowed 29 days for the isotope to be eliminated from soft tissues and fully incorporated into bone. Urine was collected for 24 h twice weekly during this equilibration period. The metabolic cages contained screens that prevented food debris and feces from contaminating the urine; the cages were cleaned once every 3 days throughout the duration of the experiment. Following the equilibration period, a baseline level of urinary tracer excretion was assessed during an initial 10 day baseline period (Figure 2), which was followed by a 10 day intervention and 10 day washout period. For each diet, there was n = 16 rats with 10 days of 24 h urine samples for each rat. The intervention and washout collections were repeated for 6 total intervention/washout cycles, and the total duration of the study was 189 days. Throughout the baseline, intervention, and washout, urine was collected for 24 h for 10 days during the intervention and 6 days during the washout. Urine was analyzed as a 24 h sample and was not pooled over multiple days. Food intake and body weight were monitored every 5 days. Blood was taken at the end of each diet and washout via the saphenous vein, and serum was stored at −80 °C to assay for bone-specific alkaline phosphatase. The urine collected at the end of each intervention and washout period was stabilized with 1% ascorbic acid at a 4:1 ratio immediately after the 24 h collection period, stored at −80 °C, and used to assay for total polyphenols and cross-linked N-telopeptides of type I collagen (NTx). Rats were killed using an excess of CO_2.

Diets. All diets were formulated to be isocaloric and to have approximately equal macronutrient, vitamin, and mineral content. The AIN93-M polyphenol-free diet[38] (Table 1) was used as a basal diet with the replacement of soybean oil with corn oil to prevent confounding from residual isoflavones. A higher amount of corn starch than in the original AIN93-M diet was used to facilitate the incorporation of up to 25% fruit powder, while maintaining the ability to pellet the diets. Research Diets, Inc. (New Brunswick, NJ),

Table 1. Modified AIN93-M Diet

ingredient	gm/kg
casein	140
L-cystine	1.8
corn starch[a]	495.692
maltodextrin[a]	125
sucrose	100
cellulose	50
corn oil[b]	40
tert-butylhydroquinone	0.008
mineral mix[c]	35
vitamin mix	10
choline bitartrate	2.5

[a]Modified from AIN93-M diet to enable pelleting while replacing up to 25% of corn starch with fruit powders. [b]Soybean oil in AIN93-M diet replaced with corn oil to avoid residual soy isoflavone interaction. [c]Mineral mix contains phosphorus at 3000 mg/kg diet and calcium at 5000 mg/kg diet.

dry blended the extracts and fruit powders into the basal diet prior to pelleting the diets. Each extract or fruit powder was added to the diet to create two doses of varying polyphenolic content and stored at −20 °C. The diets were formulated based on the goal of delivering 0.2% and 1% w/w total polyphenols for each extract where possible (Table 2). The total polyphenol content in the extracts was determined using the Folin−Ciocalteu method;[39] however, levels of resveratrol and soy isoflavones were added based on the manufacturers' specified levels of resveratrol and total isoflavones, respectively.

Table 2. Botanical and Total Polyphenol Composition in Modified AIN93-M Diet

botanical (% total polyphenols in extract)	exptl dose	% w/w diet	
		product	total polyphenols
grape seed extract (82.7)	high	1.2	1
	low	0.25	0.2
plum[a] (2.22)	high	20	0.45
	low	9	0.2
blueberry[b] (1.65)	high	25	0.4
	low	9	0.15
grape (32.8)	high	3	1
	low	0.6	0.2
resveratrol (99.9)	high	0.2	0.2
	low	0.1	0.1
genistein aglycon (6.67)	aglycon	0.6	0.04
glycosylated soy[c] (40.0)	glucoside	0.1	0.04

[a]Corn starch and cellulose in the AIN93-M diet were replaced by carbohydrates and fiber from plum powder. [b]Corn starch in the modified AIN93-M diet was replaced with maltodextrin from blueberry powder. [c]Additional 2960 IU of vitamin D/kg diet to match Fosteum (genistein aglycon).

We could achieve this goal with grape seed extract (Sensient, Indianapolis, IN) and grape skin extract (Sensient, Indianapolis, IN), which were both formulated to deliver 0.2% and 1% w/w total dietary polyphenols in the low and high doses, respectively. The blueberry and plum powder had lower phenolic content; therefore, these were added to the diet at levels that have been shown to be effective in bone.[3−6] The blueberry powder (Sensient, Indianapolis, IN) was added at levels of 9% and 25% w/w in the diet (delivering 0.15% and 0.40% w/w total dietary polyphenols, respectively) and plum powder (donated by the California Dried Plum Board, Los Altos, CA) was added at 9% and 20% w/w diet to deliver 0.20% and 0.45% w/w total dietary polyphenols, respectively. The added carbohydrates from the blueberry and plum powders were balanced by removing corn starch from the diet formula. Additionally, cellulose was removed to balance the added fiber in the plum powder. Resveratrol and isoflavones were added at lower levels than the fruit powders and extracts because they are isolated components rather than a complex botanical matrix. Resveratrol (Chromadex, Inc., Irvine, CA) was added at 0.1% and 0.2% w/w total dietary polyphenols. Novasoy (Archer Daniels Midland Company, Decatur, IL), which contains genistein glycoside plus other isoflavones, and Fosteum (Primus Pharmaceuticals, Inc., Scottsdale, AZ), which is genistein aglycon, were added to the diet to deliver an equivalent amount of total isoflavone content (0.04% w/w total dietary polyphenols) and to be consistent with the range of doses published in similar studies.[40,41] The diets delivered 5000 mg/kg diet calcium and 5000 mg/kg diet total phosphorus. Vitamin D was added to the Novasoy (glycosylated soy) diet at 2960 IU/kg diet to match the vitamin D present in the Fosteum supplement.

^{45}Ca and Total Calcium in Urine. Urine was centrifuged at 3500 rpm for 10 min at 4 °C, decanted, and diluted to the nearest 0.5 mL with ultrapure water; the total volume was recorded. Scintillation vials were prepared with 15 mL of Ecolite(+) (MP Biomedicals LLC., Solon, OH) and 1 mL of urine, and ^{45}Ca was measured on a Beckman LS 6500 scintillation counter (Beckman Instruments, Inc., Fullerton, CA). The ^{45}Ca values were corrected for decay and were reported as a percentage of original dose. Urinary total calcium was determined by vortexing, diluting, and analyzing urine samples on the atomic absorption spectrometer, AAnalyst 300 (PerkinElmer Instruments, Waltham, MA). For each 24 h urine sample of each rat, the ratio of percent dose ^{45}Ca to milligrams of total calcium was determined as the ^{45}Ca:Ca ratio. ^{45}Ca:Ca excretion during intervention was compared with ^{45}Ca:Ca excretion during nonintervention periods to determine percent bone calcium retention due to dietary polyphenols.

Biochemical Markers of Bone Turnover. Urine from day 10 of each intervention and washout period was stored at −80 °C for cross-linked N-telopeptides of type I collagen (NTx) analysis. Urine NTx was assayed in triplicate using an enzyme-linked immunosorbent assay (ELISA) from Wampole Laboratories R, Inc. (Princeton, NJ). The microtiter plate in the kit was precoated with an antibody specific to NTx. Samples and standards were added, followed by a series of reagents designed to produce a color change in the wells containing NTx. Bound NTx was analyzed spectrophotometrically at a wavelength of 450 nm, and concentration of NTx was determined by comparison to a standard curve.

Blood was taken at the end of each botanical diet period and washout period, and serum bone alkaline phosphatase was assayed using an ELISA from MyBioSource (San Diego, Ca). Serum samples were diluted 2-fold with saline and added to the wells of a microtiter plate that were precoated with an antibody specific to bone alkaline phosphatase. A series of reagents were added to produce a color change in the wells that contained bone alkaline phosphatase. Bone alkaline phosphatase was analyzed spectrophotometrically at 450 nm, and the concentration of bone alkaline phosphatase was determined by comparison to a standard curve.

Total Polyphenol Analysis. To confirm the dose dependent increase in polyphenol absorption from diets, urinary total polyphenols were quantified following solid phase extraction based on a modification of methods as described by Medina-Remón et al.[42] Briefly, urine samples were diluted to approximately 1 mL using a 50 mM sodium phosphate buffer (pH = 3.0). Strata-X polymeric reversed phase extraction tubes (Phenomenex Inc., Torrance, CA) were used for solid phase extraction. The tubes were initially washed with 3 mL of methanol, followed by 6 mL of phosphate buffer. The entire urine sample was applied to the column and washed with 6 mL of phosphate buffer, followed by 6 mL of 5% methanol. The samples were eluted with 6 mL of methanol, dried, and resolubilized with ethanol. Samples were then pipetted in triplicate onto a 96-well plate, along with gallic acid (Sigma-Aldrich, St. Louis, MO) in ethanol to create a standard curve, and an ethanol blank. The Folin−Ciocalteu reagent (2 N, Sigma-Aldrich, St. Louis, MO) was diluted to 0.2 N and added to the plate, and then samples were incubated for 7 min at room temperature. An equal amount of 7% sodium bicarbonate solution was added to the reaction, and the plate was incubated for 30 min prior to being analyzed by a spectrophotometer at 750 nm. Total polyphenols of the urine samples were calculated from the gallic acid standard curve and were reported as gallic acid equivalents.

Statistical Analysis. The ^{45}Ca:Ca ratio was transformed using the natural logarithm to correct for skewedness. For each rat, a simple linear regression model was fit through all of the nonintervention periods (baseline and washout data points) creating a regression line as illustrated in Figure 3A and Figure 3B. During the intervention period, a predicted value from the regression line for each observation of each rat was determined using the model described. The predicted value for each observation was subtracted from the experimental value measured during the botanical diet period, and the mean of differences was taken for that botanical diet of that rat. The means from each dietary intervention were averaged across all rats, and 95% confidence intervals were calculated. The values were exponentiated and reported in the original scale as percent improvement in calcium retention compared with baseline.

The data for food intake, weight change, feeding efficiency ratio, body weight, and total polyphenols were normal and were analyzed without transformation. Means and standard deviations were calculated for these variables, a repeated measures analysis of variance

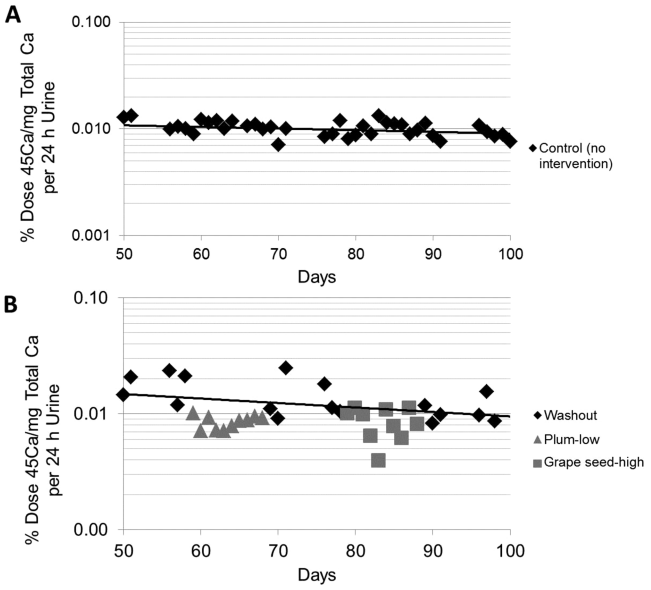

Figure 3. Plot of days vs ^{45}Ca:Ca ratio for (A) a control rat that did not receive a botanical diet and (B) two botanical diet periods and washout periods in one rat. Each data point represents a single 24 h urine collection. Different diets are indicated by different symbols. A regression line was fit through all of the nonintervention periods, which include baseline and washout data points. During the botanical diet periods, the residual was determined by finding the difference between the predicted value from the regression line and the experimental value.

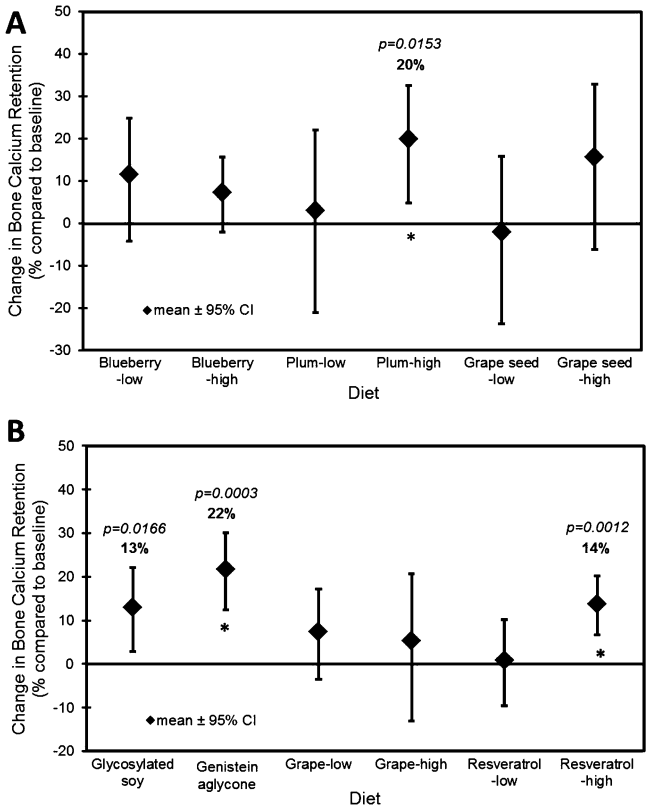

Figure 4. Effect of dietary botanicals on bone calcium retention in ovariectomized (OVX) rats (n = 16 rats per diet) was reported as mean ± 95% confidence intervals for (A) arm 1 and (B) arm 2. A value of "0" represents no change from the regression line computed from nonintervention periods. A confidence interval that excludes "0" indicates a significant change from the regression line at α = 0.05. The * indicates significant difference (p < 0.05) between high and corresponding low dose of the same dietary botanical as determined by a t test. For soy, the * indicates significant difference (p < 0.05) between genistein aglycon and glycosylated soy.

(ANOVA) was performed, and differences were determined by contrasts between high and low doses within each extract, and between genistein aglycon and glycosylated soy. t tests were performed for food intake, body weight, weight change, and feeding efficiency ratio to determine differences between control and botanical diets with a Bonferroni correction to adjust for multiple comparisons of means.

For each rat, NTx values were transformed to correct for skewedness using the natural logarithm. Subsequently, the difference in NTx between intervention and washout was determined for each botanical diet as change in NTx. The means and 95% confidence intervals of the differences were taken for each diet. A repeated measures ANOVA was performed followed by contrasts to determine differences between high and low doses of each diet, and between genistein aglycon and glycosylated soy. The means for each diet were transformed back to the original scale and reported as a ratio with 95% confidence interval. SAS software (version 9.3 SAS Institute, Cary, NC) was used for all analyses.

RESULTS

Bone Calcium Retention. Percent change in bone calcium retention was determined by finding the difference between the ^{45}Ca:Ca ratio excreted during the botanical diet compared with the residual line of all control diet periods. The urinary ^{45}Ca:Ca ratio of the control rats in arm 3 (Figure 1) verified that the regression line was not altered by diets in similarly aging rats (Figure 3A). The ratio of percent dose of ^{45}Ca to milligrams of Ca in the 24 h urine samples and residuals for two diets for one rat in arm 1 is shown in Figure 3B. Using this method, bone calcium retention was significantly improved due to dietary intervention with glycosylated soy (13%; p = 0.0166), genistein aglycon (22%; p = 0.0003), resveratrol-high (14%; p = 0.0012), and plum-high (20%; p = 0.0153) compared with baseline (Figures 4A and 4B). The genistein aglycon supplement, Fosteum, increased bone calcium retention 2-fold compared to glycosidic mixed isoflavone supplement, Novasoy (p < 0.05). The high dose of plum (0.45%) and resveratrol (0.2%) improved bone calcium retention compared with the lower doses (p < 0.05).

Food Intake and Body Weight. There was no significant difference in body weight between high and low doses of any botanical diet intervention despite some differences in the contributing factors (Table 3). In arm 1, plum-low increased the food intake, feeding efficiency ratio, and weight change compared with the plum-high diet (p < 0.0001). Blueberry-low increased the feeding efficiency ratio and weight change compared with the blueberry-high diet (p = 0.05). In arm 2, grape-low and resveratrol-high decreased the feeding efficiency ratio compared with control (p < 0.05), and glycosylated soy, grape-low, and resveratrol-high diets decreased the weight change of rats compared to those on the control diet (p < 0.05). There was a weak, but significant, positive correlation between change in body weight and net bone calcium retention (r = 0.21; p = 0.002); however, change in body weight was not correlated with NTx (p = 0.69).

Analysis of Extracts. Analysis of key phenolic compounds in grape, blueberry, and plum extracts highlighted some compositional differences between the different extracts (Table 4). The most prominent constituents in the plum

dx.doi.org/10.1021/jf403310q | J. Agric. Food Chem. 2014, 62, 6108−6117

Table 3. Food Intake, Body Weight, Weight Change, and Feeding Efficiency Ratio[a] for Ovariectomized Rats Expressed As Mean ± Standard Deviation

variable	feeding efficiency ratio	food intake, g/day	body wt, g	wt change, g/day
arm 1 rats				
blueberry-low	0.07 ± 0.05*	15.7 ± 1.3	354.2 ± 26.1	1.06 ± 0.70*
blueberry-high	0.03 ± 0.03	14.9 ± 1.44	351.4 ± 29.2	0.45 ± 0.56
plum-low	0.06 ± 0.05**	15.3 ± 2.2**	352.9 ± 22.1	0.92 ± 0.83**
plum-high	−0.02 ± 0.07	12.7 ± 2.3	346.1 ± 21.6	−0.20 ± 0.80
grape seed extract-low	0.03 ± 0.04	14.5 ± 1.0	351.1 ± 24.2	0.48 ± 0.64
grape seed extract-high	0.05 ± 0.03	15.1 ± 1.3	352.7 ± 25.0	0.78 ± 0.57
arm 2 rats				
glycosylated soy	0.03 ± 0.03	14.9 ± 1.1	377.0 ± 24.7	0.50 ± 0.40†
genstein aglycon	0.05 ± 0.04	15.0 ± 1.5	376.1 ± 28.2	0.74 ± 0.57
grape-low	0.06 ± 0.02†	16.0 ± 1.5	362.4 ± 22.5	1.01 ± 0.43†
grape-high	0.07 ± 0.03	17.0 ± 1.5	363.6 ± 20.3	1.14 ± 0.63
resveratrol-low	0.05 ± 0.03†	15.2 ± 1.5	360.1 ± 22.2	0.83 ± 0.60†
resveratrol-high	0.07 ± 0.03	15.2 ± 1.5	362.6 ± 22.0	1.05 ± 0.48
arm 3 control rats	0.16 ± 0.06	15.2 ± 1.2	370.2 ± 20.1	2.78 ± 1.24

[a]Feeding efficiency ratio as change in body weight/food intake. The * and ** denote significant differences with $p = 0.05$ and $p < 0.0001$, respectively, between low and corresponding high groups of the same extract diet as determined by contrasts after ANOVA. The † denotes significant difference from control with $p < 0.05$ as determined by t tests performed using a Bonferroni correction to adjust for multiple comparisons.

Table 4. Key Phenolic Compounds Identified in Extracts[a]

	mg/g extract[b]		
analytes	plum	grape	blueberry
phenolic acids			
gallic	0.15	11.85	0.45
ferulic		3.16	2.22
caffeic	2.82	0.37	0.45
stilbenoids			
resveratrol			
other stilbene derivatives		9.16	2.10
chlorogenic acids			
3-0-caffeoylquinic	11.62		4.19
caffeoylquinic (other forms)	21.75	3.28	1.85
flavonoids			
catechin		2.84	1.25
epicatechin		0.81	
quercetin- glucosides	0.09	0.14	0.23
genistein			
daidzein			
cyanidin-glycosides		0.94	1.85
acetylated cyanidins	0.27	0.71	0.19
peonidin-glycosides		3.21	1.21
acetylated peonidins		1.78	
delphinidin-glycosides		5.51	9.77
acetylated delphinidins	0.23	2.12	2.60
petunidin-glycosides		7.66	7.10
acetylated petunidins		2.61	
malvidin-glycosides		22.44	21.69
acetylated malvidins		7.72	
total polyphenols[c]	22.2	328	16.5

[a]Phenolic compounds were tested in duplicate using LC−MS[41] to assay for key compounds. [b]Resveratrol contained 295.12 mg resveratrol/g sample and grape seed extract contained 827 mg/g total polyphenols as determined using the Folin−Ciocalteu[38] method. Grape seed extract was unavailable for analysis using LC−MS. [c]Total polyphenols were determined through the Folin−Ciocalteu method.[38]

extract were chlorogenic acids (11.62 mg/g extract 3-0-caffeoylquinic and 21.75 mg/g extract other forms of caffeoylquinic), which were also notably higher than the content found in grape (3.28 mg/g extract chlorogenic acids) and blueberry (5.04 mg/g extract total chlorogenic acids). Grape and blueberry were both characterized as being higher in anthocyanins (54.7 mg/g extract and 44.41 mg/g extract total anthocyanins, respectively) compared with plum (0.5 mg/g extract total anthocyanins); however, grape had a greater amount of gallic acid compared with blueberry (11.85 mg/g extract compared with 0.45 mg/g extract, respectively), and blueberry contained a higher level of chlorogenic acids than grape (5.04 mg/g extract compared with 3.28 mg/g extract, respectively). Grape extract did not contain resveratrol, but did have other stilbene derivatives (9.16 mg/g extract).

Biochemical Markers of Bone Turnover. Grape seed extract-high ($p = 0.0056$) and grape-high ($p = 0.004$) dietary interventions reduced NTx compared with each corresponding baseline period (Figure 5A and Figure 5B). Resveratrol-low significantly increased NTx compared with the corresponding baseline period ($p = 0.0039$). Blueberry, plum, soy, grape seed extract, grape-low, and resveratrol-high diets did not produce a significant change in NTx.

The aglycon soy diet significantly increased serum bone alkaline phosphatase compared with baseline ($p = 0.03$, data not shown); however, none of the other diets impacted bone alkaline phosphatase.

Urine Total Polyphenols. There was a significant dose response of urinary total polyphenols in blueberry ($p = 0.005$) and grape seed extract ($p < 0.0001$) interventions of arm 1 and grape ($p = 0.0002$) dietary intervention of arm 2 rats (Figure 6A and Figure 6B). There was no difference in total polyphenols between glycosylated soy and genistein aglycon ($p = 0.27$).

DISCUSSION

In this crossover intervention trial in OVX rats, we identified that although both isoflavone-containing diets were effective at increasing bone calcium retention, the aglycon form of genistein was significantly more effective than the glucoside. In addition, we demonstrated that higher levels of resveratrol and plum (0.20% and 0.45% total polyphenols, respectively) increased bone calcium retention.

dx.doi.org/10.1021/jf403310q | J. Agric. Food Chem. 2014, 62, 6108−6117

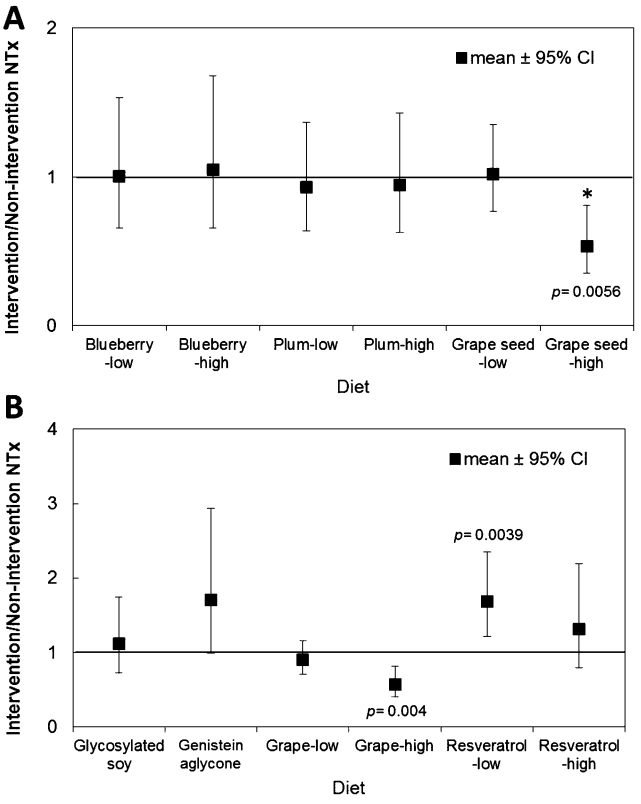

Figure 5. Effect of dietary botanicals on urinary NTx in ovariectomized (OVX) rats was reported as mean ± 95% confidence interval. A value of "1" represents no change from the regression line computed from nonintervention periods. A confidence interval that excludes "1" indicates a significant change from the regression line at α = 0.05. Contrasts were performed after ANOVA. For blueberry (n = 15 for low; n = 16 for high), plum (n = 15 for low; n = 14 for high), and grape seed extract (n = 14 for low; n = 15 for high) diets (A) * indicates a significant ($p < 0.05$) difference between NTx values during the low and corresponding high doses of the same diet. For soy (n = 14 for genistein aglycon; n = 15 for glycosylated soy), grape extract (n = 16 for low and high), and resveratrol (n = 16 for low and high) (B) there was a trend for NTx to be lower during the grape-high diet compared with the grape-low diet (p = 0.078). NTx, urinary collagen type 1 cross-linked N-telopeptide.

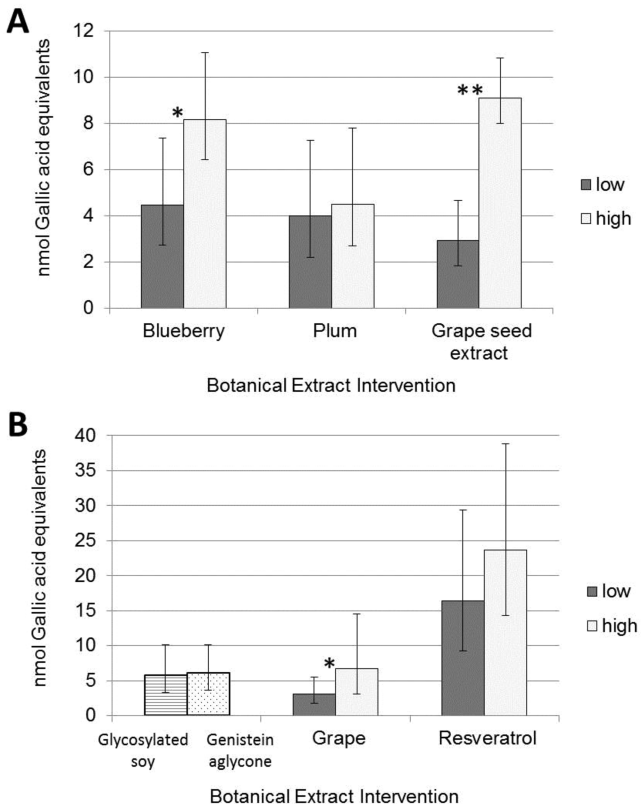

Figure 6. Effect of dietary botanicals on urinary total polyphenols in 24 h urine of ovariectomized (OVX) rats (n = 16 rats per diet). Values are reported as mean ± standard deviation with contrasts performed to detect differences between means. For blueberry, plum, and grape seed extract diets (A) * and ** indicate low is significantly different from high at p = 0.005 and $p < 0.0001$, respectively. All arm 1 diets were significantly different from baseline at p = 0.0015. For soy, grape, and resveratrol diets (B) * indicates that low is significantly different from high at p = 0.0002. There was a trend for total polyphenols during low to be different from high during the resveratrol-high diet with p = 0.06. All arm 2 diets were significantly different from baseline at $p < 0.0001$.

Dietary aglycon soy elicited an improvement to bone calcium retention that was approximately double the response from a diet with glycosylated soy (22% for aglycon soy compared with 13% for glycosylated soy), which supported our hypothesis that the aglycon form of genistein is more effective than the glucoside when tested on an equivalent mass basis of total polyphenols. We did not observe a difference in urinary total polyphenols between genistein aglycon and glycosylated soy, which supports that the glycosylated soy and genistein aglycon diets were formulated to have equivalent isoflavone content. We are first to directly compare soy aglycon to glucoside for effect on bone in an estrogen-depleted rodent model. Through this direct comparison, we were able to demonstrate that the aglycon form of genistein was more effective than glycosylated soy in increasing bone calcium retention.

Cleavage of the sugar moiety is believed to be required for genistin (glycosylated) to be effectively absorbed in the intestine,[9] but absorption of genistein (aglycon) occurs readily[11] and as early in the digestive process as in the stomach.[43] After the glucoside is hydrolyzed and absorbed in the gut, it is conjugated to mostly glucuronic acid by both intestinal and hepatic enzymes. Consumption of the aglycon form of genistein has the potential to lead to a higher presence of unconjugated genistein in the blood due to enhanced uptake in the gut and some absorption in the stomach. This is biologically important because unconjugated genistein has been shown to bind more strongly to estrogen receptor-β than its glucuronide metabolites,[16] demonstrating the potential for a more estrogenic effect on bone. We were able to demonstrate the enhanced efficacy of the aglycon form of genistein on bone in an estrogen-depleted rodent model. Future research should directly compare the metabolites produced from consumption of aglycon and glycoside soy to more closely link this mechanism to the increase in bone calcium retention from the aglycon form of soy.

Estrogen in bone works primarily by decreasing the production of inflammatory cytokines (interleukin (IL)-1, IL-6, and RANKL and M-CSF (macrophage colony stimulating factor)) in osteoblasts and precursor cells to regulate osteoclast activity.[44,45] A drop in estrogen leads to a prominent increase in osteoclast activity, which greatly increases bone resorption with a lag in subsequent bone formation.[18] Although the estrogenic activity of phytoestrogens such as isoflavones has been shown to elicit an antiresorptive response on bone,[24] we observed that

dx.doi.org/10.1021/jf403310q | J. Agric. Food Chem. 2014, 62, 6108−6117

genistein aglycon increased bone calcium retention, consistent with bone formation. This is evidenced by an increase in BAP, which is a marker of bone turnover and often an indicator of an anabolic effect to bone. Our finding suggests that the ability of isoflavones to reduce net bone loss works by stimulating the anabolic bone building effect to counter the increase in resorption caused by loss of estrogen, rather than by mitigating bone resorption only. Urinary calcium tracer excretion is specific to bone mineral balance and more precise than biochemical markers of bone turnover.

Our finding that short-term supplementation of dried plum at 20% w/w diet, delivering 0.45% w/w total dietary polyphenols (plum-high), to OVX rats resulted in a 20% increase in bone calcium retention was similar to the 15% increase in bone tracer retention observed by Mühlbauer et al. using a similar method in OVX rats with a lower percentage (8% w/w diet) of dried prune extract for 10 days.[19] The effects we observed on bone calcium retention from plum is also consistent with doses reported in the literature to be protective to bone in OVX rats, i.e., 15% and 25%.[3−5,46,47] In postmenopausal women, plum supplemented at 100 g/d prevented bone loss and increased BMD;[23,47] the control diet of 100 g/d dried apples also protected bone from loss, but to a lesser degree than dried plum supplementation.[23] This level of dried plum intake would be equivalent to the 25% w/w diet given to rodents and similar to the 20% w/w diet given in this study, assuming that food intake in women totals approximately 400 g/d on a dry weight basis.

We did not find a significant effect on bone with diets of blueberry, grape seed extract, or grape extract in OVX rats. Evaluation of the phenolic profiles of these extracts reveals that chlorogenic acids are more prominent in plum compared to the other extracts. Both chlorogenic acid[48] and dried plum powder[49] have been found to have an inhibitory effect on the RANKL pathway involved in osteoclastogenesis. However, we did not observe a decrease in NTx, a marker of bone resorption, with dietary supplementation with dried plum.

Previous work demonstrated that blueberry at 5% w/w diet prevented the loss of whole-body BMD in OVX rats.[50] Additionally, blueberry at 10% w/w diet fed to prepubertal[6,51] rats prevented bone loss later in life; the suggested mechanism was that phenolic acids found in blueberry promoted osteoblast differentiation. Although sera phenolic acid derivatives were described,[6] the phenolic profile of the blueberry extract was not reported. The lack of effect observed in our study could potentially be attributed to differences in blueberry phenolic profile and concentration of phenolic compounds in the extract. While abundant in anthocyanins, blueberry had lower levels of chlorogenic acids compared with the plum powder, which may have impacted the efficacy of the blueberry diet.

Grape seed extract when given with calcium was shown to have a protective effect on bone in adult and growing intact rats when given immediately following a low calcium diet.[2,22] We tested grape seed extract in an estrogen-depleted rodent model and did not find an effect on bone. Catechins, the most abundant polyphenols present in grape seed extract,[52] have been shown to have estrogenic activity,[53,54] and there is evidence to suggest that catechins from green tea have a role in preventing postmenopausal bone loss.[55,56] however, the binding affinity of different catechins to estrogen receptor-β should be investigated further as this receptor more directly impacts bone.

We did not see an effect of grape extract (0.2−1% w/w total polyphenols in diet) on bone calcium retention; however, we did find that resveratrol-high, which delivered 0.2% dry matter as total polyphenols in the diet, increased bone calcium retention by 14%. Mühlbauer et al.[19] fed rats 8% w/w diet red wine residue and observed a reduction in bone resorption; however, the measured total polyphenols and resveratrol content were not reported. Despite anthocyanins and gallic acid being more prominent in grape than the other extracts, our analysis of the phenolic profile of grape extract showed an absence of resveratrol, a compound which has been proven to have estrogenic activity[17] and was effective as an isolated compound in our study. Further analysis comparing grape extracts with varying levels of resveratrol should be tested to determine if efficacy is due primarily to resveratrol.

Total polyphenols were significantly higher during the botanical interventions than during baseline and washout periods, suggesting that polyphenols were absorbed and cleared into urine from test diets. Total polyphenols were not significantly different during the washout compared with baseline, which suggests clearance of prior diet in the system and no carryover effect.

Biochemical markers of bone turnover were not always consistent with bone calcium retention response to diets. Grape seed extract-high and grape-high supplementation decreased NTx without a change in bone calcium retention. Resveratrol-low induced an increase in NTx, but had no significant effect on bone calcium retention. The increase in bone calcium retention for glycosylated soy, aglycon soy, plum-high, and resveratrol-high failed to lower NTx or increase bone alkaline phosphatase.

In our crossover study with OVX rats, dietary plum, resveratrol, genistein glucoside, and genistein aglycon increased bone calcium retention by 13−22%. To our knowledge we are the first to directly compare and report the enhanced effect of genistein aglycon on bone. Additionally, we confirmed efficacy of dried plum on bone and highlighted chlorogenic acids as the group of phenolic compounds contributing to increased bone calcium retention. Although we did not observe a bone protective effect with a resveratrol-devoid grape extract, we did observe a strong bone protective effect with resveratrol, which suggests that resveratrol may be the active component in other grape products that have been shown to have bone protective effects. Future studies could extend this work by evaluating the effect of specific classes of botanical polyphenolics on bone strength measures.

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Funding

This research was supported in part by the United States Department of Agriculture National Needs Fellowship for Foods for Health and by Grant P50-AT00477 from the National Institutes of Health.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

P. J. Lachcik is thanked for helping with handling and performing procedures on rats. J. R. Burgess is thanked for helping with designing and performing the plan for total polyphenol analysis.

■ ABBREVIATIONS USED

OVX, ovariectomized; BMD, bone mineral density; RANK, receptor activator of nuclear factor kappa-B; RANKL, receptor activator of nuclear factor kappa-B ligand; M-CSF, macrophage colony stimulating factor; IL, interleukin; NTx, cross-linked N-telopeptides of type I collagen; ANOVA, analysis of variance

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