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Anti-osteoporotic effects of syringic acid and vanilic acid in the extracts of waste beds after mushroom cultivation

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In recent years, the number of patients with osteoporosis has increased as population grows older. Therefore, the chemoprevention of osteoporosis by better nutrition is important. White-rot fungi degrades milled wood lignin for growth and development. This degradation results in the formation of phenolic compounds such as syringic acid (SA) and vanillic acid (VA). In the artificial culture of edible mushrooms using a mushroom bed, the disposal of waste beds after mushroom cultivation is an important issue. The present study investigated the presence and amount of both SA and VA in the discarded waste beds after mushroom cultivation. The extracts from waste beds after cultivation of shiitake mushrooms, *Lentinula edodes*; buna shimeji, *Hypsizygus marmoreus*; maitake, *Grifola frondosa*; king trumpet mushrooms, *Pleurotus eryngii*; and butterscotch mushrooms, *Pholiota microspora* were analyzed using high performance liquid chromatography. Although the content of SA and VA was considerably different among the mushrooms, SA and VA were present in extracts obtained from all the waste beds. We also demonstrated that SA and VA exert their anti-osteoporotic effect independently of the estrogen receptor-mediated pathway using murine monocytic RAW264.7 cells, ovariectomized mice, and human breast cancer MCF-7 cells. Thus, these results suggest that the extracts are effective sources of SA and VA, which are effective in preventing osteoporosis.

[**Key words:** Syringic acid; Vanillic acid; Waste beds after mushroom cultivation; Edible mushroom; White-rot fungi; Osteoclast; Osteoporosis; Estrogen receptor]

Osteoporosis is characterized by bone loss and micro-architectural deterioration of the skeletal tissue, which causes bone fragility and increases susceptibility to fracture. Osteoporotic bone loss is the result of high bone turnover, where bone resorption outpaces bone deposition (1–4). This imbalance in bone turnover is induced by estrogen (E2) deficiency in menopausal women. In recent years, as a result of an aging population, the number of osteoporosis patients worldwide has increased and bone health is becoming a serious problem. Bone health is profoundly affected by fitness and eating habits. Therefore, part of the solution to this serious problem is likely to include better nutrition.

The imbalance in bone turnover can be ameliorated with bioavailable estrogens including selective estrogen receptor (ER) modulators (5,6). Most estrogen activities are mediated by ERα and β, which are ligand-dependent transcriptional regulators. Several isoflavones are known to interact weakly with ERs due to their biophenolic structure, which is essential for the ligand–receptor association (7,8). Isoflavones can mimic or modulate endogenous estrogen activity by acting like partial ER agonists or antagonists, depending on the endogenous estrogen status. However, these compounds can also cause the proliferation of ER-positive human breast cancer cells (MCF-7) (9–11) and promote endometrial hyperplasia (12,13). Hence, excessive isoflavone intake can have adverse effects on breast and uterine cancer via ERs. Therefore, it is extremely important to identify compounds that exhibit anti-osteoporotic activity independent of ER-mediated pathways.

Mushrooms are the fruiting bodies of fungi. The artificial culture of edible mushrooms enables commercial production throughout the year. Many edible mushrooms have been cultivated in a sawdust-based culture system using a mushroom bed on a range of lignocellulosic materials (14,15). In the artificial culture of edible mushrooms using a mushroom bed, the disposal of waste beds after mushroom cultivation is an important issue. Various uses for waste bed material have been examined, including use as compost or pelleted fuel, recycling for re-use for mushroom culture, or conversion into bioethanol, but none of these has developed appreciably (16–18). Thus, many waste beds are incinerated after cultivation as recycling of these beds is difficult. Because of weak mushroom prices on Japanese markets, mushroom farmers in Japan need to reduce culture costs. Escalating energy bills, packaging material costs, and expenses for raw materials including sawdust affect mushroom farmers, particularly in small and medium-sized enterprise farms. Therefore, the development of systems to

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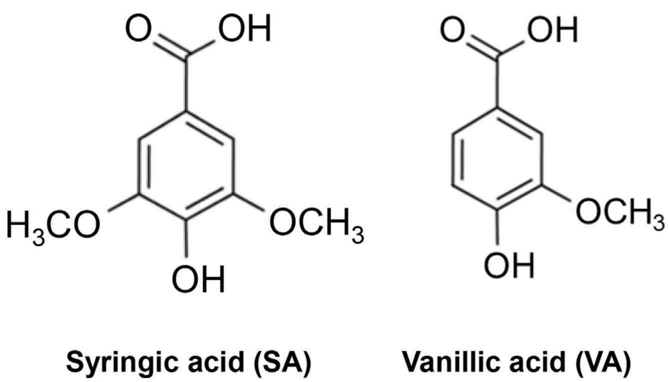

FIG. 1. Chemical structure of syringic acid (SA) and vanillic acid (VA).

recycle the discarded culture medium would lead to a reduction in labor and cost for mushroom farmers.

Degradation of milled wood lignins results in the formation of phenolic compounds such as syringic acid (SA) and vanillic acid (VA) (19–21). The structural formulae of SA and VA are shown in Fig. 1. Itoh et al. (22) reported that SA and VA are naturally present in the mycelium of the *Lentinula edodes*. However, little is known about whether and how much SA and VA exist in waste beds after mushroom cultivation. These compounds have no affinity for ERs (23) and have radical scavenging (24,25), anti-oxidative (24,25), and anti-inflammatory activities (22,26,27). Recently, the anti-osteoporotic activity of an SA-enriched diet was demonstrated in ovariectomized (OVX) postmenopausal mice (28). To our knowledge, the effect of VA on OVX mice has not been investigated, and neither SA nor VA has been studied for its effects on murine monocytic (preosteoclastic) RAW264.7 cells.

The present study aimed to investigate the presence and amount of both SA and VA in waste beds after mushroom cultivation and their anti-osteoporotic effects in RAW264.7 cells. It was established that SA and VA had anti-osteoporotic activity independent of the ER-mediated pathway, thus inhibiting osteoclast differentiation.

MATERIALS AND METHODS

Materials SA (>97% purity) and VA (>97% purity) were purchased from Tokyo Chemical Industry (Tokyo, Japan) and Sigma Aldrich (St. Louis, MO, USA), respectively. All other chemicals used in this study were of the highest purity available.

Preparation of waste beds after mushroom cultivation extracts Waste beds after cultivation of shiitake mushrooms *L.edodes* (KX-S055 and XR-1), buna shimeji *Hypsizygus marmoreus* (KX-BS023), maitake *Grifola frondosa* (MA52), king trumpet mushrooms *Pleurotus eryngii* (KX-EG071), or butterscotch mushrooms *Pholiota microspora* (KX-N008) were obtained from Kinokkusu Corporation, Sendai, Miyagi, Japan. The condition of mushroom culture and development and mushroom bed components are shown in Tables 1 and 2. Extracts of waste beds after mushroom cultivation were prepared as followed: Briefly, thirty volumes of hot water was added to the lyophilized waste bed including the mycelia (1 g) and boiled for 20 min. The extract was then filtered through 0.45 μm membrane filters and stored at −80°C until high performance liquid chromatography (HPLC) analysis. For later addition to RAW264.7 cells, the extract (KX-S055) was frozen in liquid nitrogen and dried after filtration.

Determination of SA and VA by HPLC An aliquot of sample was loaded on HPLC system comprising an L-2100 pump (Hitachi, Tokyo, Japan), a packed column (Inersil ODS-HL 250 × 4.6 mm, GL Science, Tokyo, Japan), and a L-2420 UV detector (Hitachi). In gradient elution, solution A: 10% acetonitrile aqueous solution (0.1% formic acid) and solution B: 30% acetonitrile aqueous solution (0.1% formic acid) were used and the following gradient program was applied: 0–90 min, 0–100% solution B. The flow-rate was 1.0 ml/min and absorbance was monitored at 260 nm. SA and VA levels in each sample were determined from a calibration curve using authentic samples.

Cell culture Murine monocytic RAW264.7 cells were purchased from American Type Culture Collection (Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2. For osteoclast differentiation, cells were plated in a 96-well plate and maintained in minimal essential medium-alpha (MEMα) supplemented with 10% charcoal-stripped FBS with antibiotics. Charcoal-stripped FBS was used to eliminate the effects of estrogenic compounds in FBS.

The human breast adenocarcinoma cell line (MCF-7) was obtained from Osaka Bioscience Institute, Japan. MCF-7 cells were maintained in phenol red-free DMEM, containing 10% (v/v) FBS, penicillin G and streptomycin, and incubated at 37°C in 5% CO_2. When the effects of SA, VA, and E2 were examined, the culture medium was prepared with phenol red-free DMEM containing 10% (v/v) charcoal-stripped FBS, penicillin G, and streptomycin.

Tartrate-resistant acid phosphatase staining of RAW264.7 cells RAW264.7 cells (3×10^3 cells/well) were cultivated in 96-well plates. After 24 h, 50 ng/mL receptor activator of nuclear factor kappa B (NF-κB) ligand (RANKL) or RANKL plus 17β-estradiol (E2) or RANKL plus SA or RANKL plus VA was added to the plate and cultured for 5 day. After fixation, the cells were stained by using a tartrate-resistant acid phosphatase (TRAP) staining kit (Cosmo Bio, Tokyo, Japan) according to the manufacture's protocol.

Measurement of TRAP activity in RAW264.7 RAW264.7 cells (3×10^3 cells/well) were cultivated in 96-well plates. After 24 h, RANKL or RANKL plus each sample was added to the plate and cultured for 3 day. The cells were fixed in 10% formalin and 90% ethanol and then dried. The dried cells were incubated for 20 min in 100 μL of citric acid buffer (pH 4.6, 5 mM *p*-nitrophenylphosphoric acid disodium salt) containing 0.1% Triton X-100. After incubation, the enzyme reaction mixture was removed and added to 100 μL of 0.1N NaOH in a 96-well plate. Absorption was measured at 415 nm using a microplate reader (Varioskan Flash, Thermo Fisher Scientific, Waltham, MA, USA).

Cell viability RAW264.7 cells (3×10^3 cells/well) were seeded into 96-well plates 1 day before treatment and were cultured with or without RANKL and each sample. For MCF-7 cell growth study, the cells were seeded in 96-well plates at a concentration of 2×10^3 cells/well in the medium. Next day, the cells were treated with E2 (10 nM), SA, and VA. The RAW264.7 and MCF-7 cells were cultured for 3 days and combined with MTT solution [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (50 ng/well)] and incubated for 4 h. Acid-isopropanol (0.04 N HCl in isopropanol) and 3% sodium lauryl sulfate were added to dissolve the reduced MTT crystals (formazan) present in the cells. After mixing, the absorbance was measured at 595 nm, with 655 nm as the reference, using a microplate reader.

Animals and administration All animal experiments were approved by the Institutional Animal Care and Use Committee and carried out according to the Kindai University Animal Experimentation Regulations (approval ID: KAAG-19-003).

For the kinetic analyses of SA and VA, female mice (Slc:ddY strain) were purchased from Japan SLC (Shizuoka, Japan). The animals were housed until 13-weeks-old under a 12-h/12-h light/dark cycle at 20 ± 2°C. Diet and tap water were supplied ad libitum. Mice, at a body weight of 38.4–43.9 g, were orally administered 100 mg/kg of SA (n = 5) or VA (n = 5) suspended in 0.1% carboxymethyl cellulose sodium salt solution. Vehicle solution was administered to control mice. Blood was collected from the heart 0.5 h after single oral administration of SA or VA. Serum samples were stored at −80°C until analysis. Serum samples (20 μl) were pipetted into

TABLE 1. Condition of mushroom culture and fruiting body development.

	Strain	Cultivation conditions		Fruiting body development conditions		Total time (days)
		Temperature (°C)	Time (days)	Temperature (°C)	Time (days)	
Shiitake (*L. edodes*)	KX-S055	20	120	19 ± 1	6	126
	XR-1	23	90	19 ± 1	5	95
Buna shimeji (*H. marmoreus*)	KX-BS023	23	70	13 ± 1	22	92
Maitake (*G. frondosa*)	MA52	23	35	18 ± 1	12	47
King trumpet mushroom (*P. eryngii*)	KX-EG071	23	23	15 ± 1	12	35
Butterscotch mushroom (*P. microspora*)	KX-N008	16 and 23	50 (30+20)	15 ± 1	16	66

TABLE 2. Mushroom bed components [weight (g) per block or bottle].

	Culture medium (sawdust)	Energy source	Fill ration (g/block or g/bottle)	Moisture content (%)
Shiitake (*L. edodes*)	*Quercus serrata*, 655 (360)	Rice bran, 36 (31.7) Flour containing wheat bran, 84 (73) Lime hydrat, 2.4 (2.4)	1200	61
Buna shimeji (*H. marmoreus*)	*Cryptomeria japonica*, 250 (62.5)	Rice bran, 60 (52.8) Dried okara, 20 (18) Wheat bran, 20 (17.4) Corncob meal, 30 (26) Supplement[a], 2 (1.7)	510	65
Maitake (*G. frondosa*)	*Quercus crispula*, 1225 (673)	Dried okara, 120 (108) Hominy feed, 96 (83.5) Supplement[b], 24 (23)	2400	63
King trumpet mushroom (*P. eryngii*)	*Cryptomeria japonica*, 233 (58.3)	Dried okara, 42 (15.1) Fresh bran, 21 (7.3) Supplement[b], 7 (2.7) Corncob meal, 44.7 (38.9)	500	68
Butterscotch mushroom (*P. microspora*)	*Quercus crispula*, 144.3 (79.4) and *Fagus crenata*, 144.3 (79.4)	Fresh bran, 39.9 (34.7) Supplement[c], 17.1 (16.3) Lime hydrat, 1.1 (1.1)	570	63

The number in parenthesis indicates the dry weight (g) per block or bottle. Shiitake and maitake: block cultivation. Buna shimeji, king trumpet mushroom, and butterscotch mushroom: bottle cultivation.

[a] Takaraclean (Yukiguni Maitake, Niigata, Japan).

[b] Neovitas HM (Nikka Whisky Distilling, Tokyo, Japan).

[c] Neovitas N (Nikka Whisky Distilling).

microtubes, and 20 μl of 100 mM acetylsalicylic acid (an internal standard) was added. Then, 80 μl of methanol was added to precipitate proteins. After vigorously mixed, the samples were centrifuged (3000 ×*g*, 10 min). Supernatants were diluted and filtered with syringe filter (0.45 μm). An aliquot of the final samples were injected into high performance liquid chromatography (HPLC) system.

To examine the effect of SA and VA on OVX mice, Slc:ddY female mice were purchased from Japan SLC. Mice were either OVX (n = 18) or sham-operated (Sham, n = 6) at 9 weeks of age. OVX mice were divided into OVX-control (n = 6), OVX-SA (n = 6), and OVX-VA (n = 6). The control group was fed a normal diet (Table 3) only, and the SA and VA group received SA- and VA-containing diets (100 mg/kg body weight/day) for 4 weeks. The mice were sacrificed under anesthesia after 4 weeks of each diet intake, and the humeri, femurs, and blood samples were collected. Other experimental conditions were as mentioned above identical to those described in the kinetic analyses.

To compare the effect of SA, VA, and E2 on uterine weight in OVX mice, Slc:ddY female mice were purchased from Japan SLC. After 1 week, the animals were either sham-operated (Sham group, n = 5) or ovariectomized (OVX, n = 20) at 9 weeks of age. Ovariectomised mice divided into 4 groups equally; OVX-control (n = 5), OVX-SA (n = 5), OVX-VA (n = 5), and OVX+E2 group (n = 5, 40 μg/kg b.w./day). After 1 week of operation, mice of OVX+E2 group were injected with E2 from abdominal cavity every day. The composition of control, SA, and VA diets were as mentioned above. After administering the diet for 4 weeks, the mice were sacrificed, and the uteri were harvested and weighed. Animals of other groups were injected with vehicle. Other experimental conditions were identical to those described in the kinetic analyses.

Extraction of total RNA from humeri and quantitative RT-PCR Total RNA was isolated from humeri by using the RNAqueous kit (Thermo Fisher Scientific). The RNA was subsequently treated with TURBO DNase-free kit (Thermo Fisher Scientific) to remove genomic DNA contamination. Two μg of each sample were reverse transcribed into cDNA with the high capacity cDNA reverse transcription kit (Thermo Fisher Scientific). For a quantitative analysis of each gene expression, the amplification of cDNAs was performed using SYBR Premix Ex Taq (Takara Bio Inc., Shiga, Japan), primers [TRAP: forward primer, 5′-GGAAATGGCCAATGCCAAAG-3′, reverse primer, 5′-ATCATGGTTTCCAGCCAGCAC-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward primer, 5′- ATGGTGAAGGTCGGTGTGAA; -3′, reverse primer, 5′- GAGTGGAGTCATACTGGAAC -3′] with a Thermal Cycler Dice (Takara Bio Inc.) according to the manufacturer's protocol. The expression data of TRAP gene was normalized to GAPDH expression level of the same individual sample.

TRAP activity in serum TRAP activity in the serum was measured by the colorimetric method. Briefly, 10 μL of serum was added to 40 μL of 50 mM citric acid buffer described above and incubated for 30 min at 37°C. The reaction was stopped by adding 80 μL of 0.1 N NaOH, and the absorbance at 405 nm was measured with a microplate reader (model 680; Bio-Rad Laboratories, Hercules, CA, USA). One unit is defined as the activity that hydrolyzes 1 μmol of substrate per min at 37°C.

Stereoscopic observation of femoral trabeculae The distal portions of the isolated right femur were ground with a whetstone. The produced pulverized preparation was observed with a stereoscopic microscope (SZ61, Olympus, Tokyo, Japan).

Computed tomography analysis Both the right and left femurs were scanned with an X-ray computed tomography system (LCT-100; ALOCA, Tokyo, Japan). Tomograms were taken at 0.1-mm intervals, and 60 slices from the end of the distal femoral area were analyzed. The bone mineral densities (cortical bone density, cancellous bone density, and total bone density) of each femur were measured. The

TABLE 3. Experimental diet components.

Component	Weight (%)
α-Cornstratch	50.58
Casein	22.22
Sucrose	10.00
Safflower oil	5.00
Cellulose	7.50
Mineral mix (AIN93)	3.50
Vitamin mix (AIN93)	1.00
Cholesterol	0.20

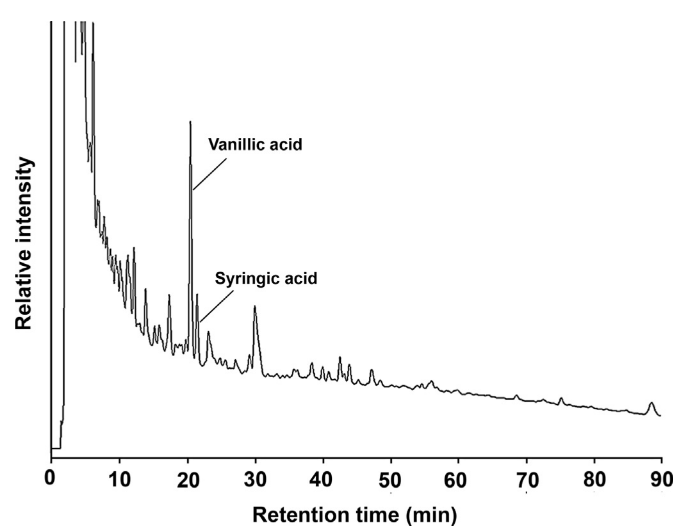

FIG. 2. HPLC chromatogram of extract from shiitake mushroom (KX-BS023) cultivation waste at UV 260 nm. The compound with retention time of 21.6 and 20.5 was identified as SA and VA with authentic samples, respectively.

TABLE 4. SA and VA contents (mg/100 g dry weight) in waste beds after mushroom cultivation.

	Strain	SA	VA
Shiitake (*L. edodes*)	KX-S055	28.1 ± 1.0	44.5 ± 1.0
	XR-1	31.2 ± 4.0	27.2 ± 1.9
Buna shimeji (*H. marmoreus*)	KX-BS023	8.7 ± 0.5	37.3 ± 2.5
Maitake (*G. frondosa*)	MA52	11.4 ± 0.4	5.7 ± 0.4
King trumpet mushroom (*P. eryngii*)	KX-EG071	7.3 ± 0.6	5.1 ± 0.2
Butterscotch mushroom (*P. microspora*)	KX-N008	16.5 ± 1.2	9.1 ± 0.7

Values are presented as means ± SD for at least three replicates.

results of bone mineral densities are expressed as the mean of values of the right and left bones.

Statistical analysis Data are presented as the mean ± standard deviation (SD). One-way analysis of variance followed by post hoc Tukey test was used to examine differences between the different groups. $P < 0.05$ was considered statistically significant.

RESULTS

SA and VA contents in waste beds after mushroom cultivation extracts Fig. 2 shows an HPLC chromatogram produced from a 50 µl sample of extract from shiitake mushroom (KX-BS023) cultivation waste. Two peaks, corresponding to the authentic SA and VA, were observed in all mushroom cultivation bed waste extracts in this study. The retention times of the two peaks were 21.6 and 20.5 min. The signal ion from these two fraction were corresponding to authentic SA and VA [M-H]- of *m/z* 197.05 and 167.03 detected by MS analysis (data not shown). Therefore, the two peaks, retention time is 21.6 and 20.5 min, were identified as SA and VA, respectively. SA and VA contents in waste bed were quantitated, based on the authentic SA and VA standard as shown in Table 4. The waste beds after shiitake mushroom (KX-S055) cultivation contained the most abundant total amount of SA and VA. Other strain of shiitake mushroom (XR-1) also has large amount of SA and VA, but the contents were slightly lower than that of KX-S055. Although the SA content was low, the VA content in buna shimeji waste was as high as that in shiitake mushroom waste. Although the waste beds after maitake, king trumpet mushrooms, and butterscotch mushroom cultivation also contained SA and VA, the content was lower than that of shiitake and buna shimeji.

Identification of SA and VA in mouse blood Next, we examined the kinetics of SA and VA in the serum of mice after oral administration. A representative HPLC chromatogram of serum 0.5 h after SA and VA administration is shown in Fig. 3A and B. Some peaks, not detected in control mice, were observed in blood of mice given SA and VA. The peak of retention time 21.6 in Fig. 3A and 20.5 in Fig. 3B was identified as SA and VA with authentic samples, respectively. These data suggest that SA and VA are partly absorbed without being significantly metabolized.

The serum levels of SA and VA 0.5 h after administration were 0.3 ± 0.04 mg/mL (1.4 ± 0.2 mM) and 0.3 ± 0.02 mg/mL (1.6 ± 0.1 mM), respectively. Although the C_{max} was not estimated in the present study, 20% or more of SA and VA administered was detected in intact form 0.5 h after administration, suggesting a high absorption ratio of SA and VA in mice. Thus, in this study, authentic SA and VA were used at the dose of 100 µM (below levels detected in serum) or less in subsequent *in vitro* assays.

Effect on osteoclast differentiation of SA and VA in the extracts of waste beds after mushroom cultivation The effect on osteoclast differentiation of SA and VA in the extracts of waste beds after mushroom cultivation was examined. Treatment with 0.1–1 mg/ml of the extracts (KX-S055) of waste beds after mushroom cultivation had no cytotoxic effects on RAW264.7 cells under our assay conditions (Fig. 4A). Although TRAP activity was increased in RANKL treated cells compared to that in untreated

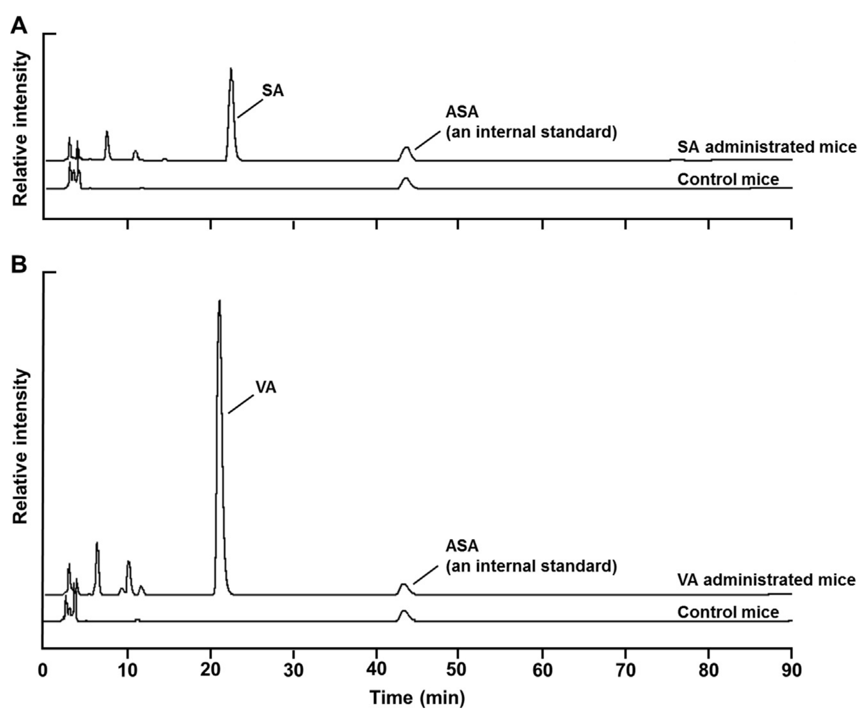

FIG. 3. Kinetics of SA and VA in the serum of mice after oral administration. (A) Representative HPLC chromatograms of serum 0.5 h after administration in 100 mg/kg of SA administered mouse (upper chromatogram) and control mouse (lower chromatogram). The peak of retention time 21.6 and 43.2 was identified as SA and acetylsalicylic acid (ASA) with authentic samples, respectively. (B) Representative HPLC chromatograms of serum 0.5 h after administration in 100 mg/kg of VA administered mouse (upper chromatogram) and control mouse (lower chromatogram). The peak of retention time 20.5 and 43.2 was identified as VA and ASA with authentic samples, respectively.

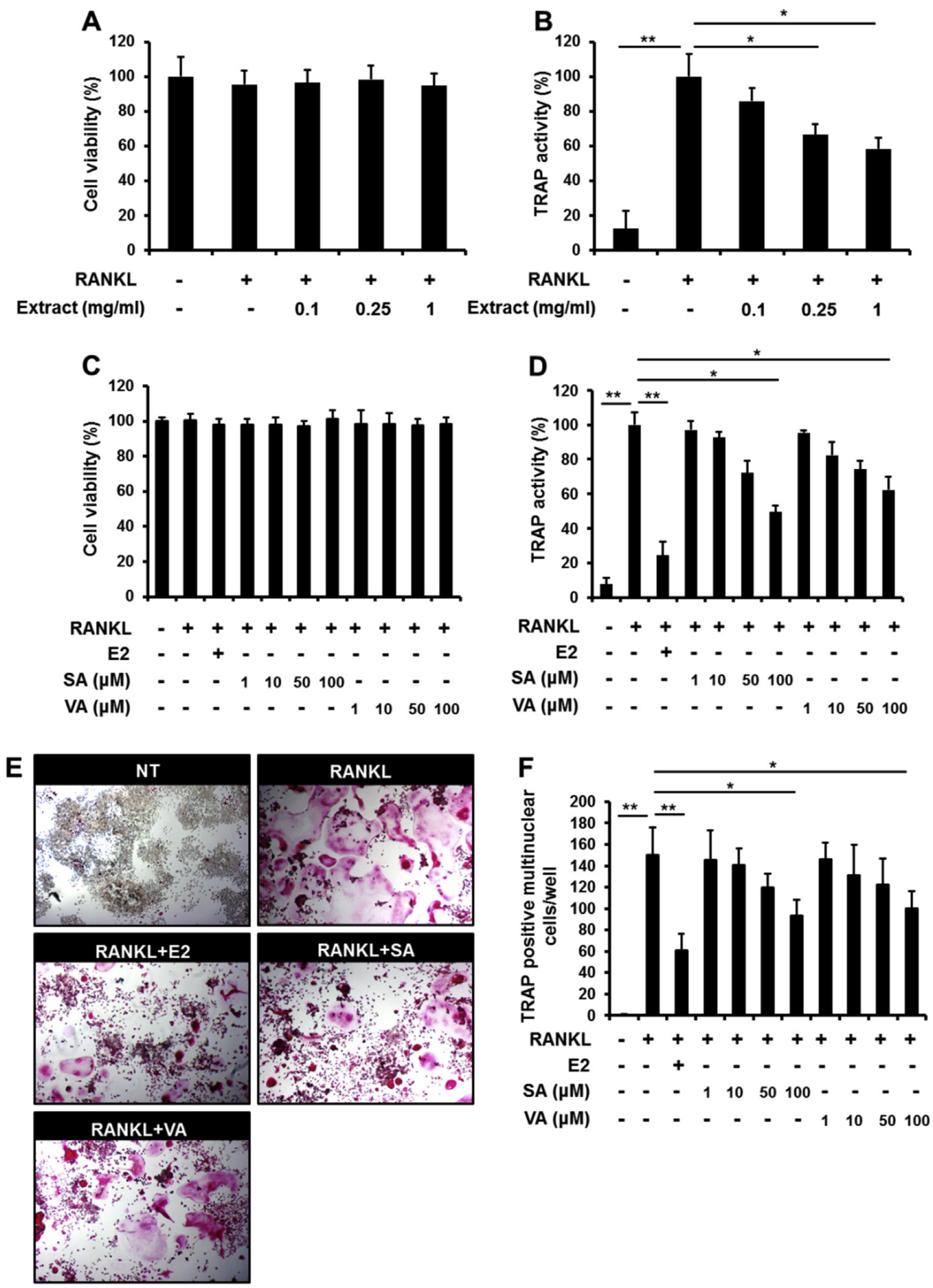

FIG. 4. Effect on osteoclast differentiation of SA and VA in the extracts of waste beds after mushroom cultivation. (A) Effect on cell viability of the extracts of waste beds after mushroom cultivation treatment; RAW264.7 cells were treated with RANKL and/or the extracts for 3 days, and proliferation was investigated using MTT assays. (B) Effect on TRAP activity of the extracts of waste beds after mushroom cultivation treatment; RAW264.7 cells were treated with RANKL and/or the extracts for 3 days. (C) Effect of SA and VA treatment on cell viability; RAW264.7 cells were treated with RANKL and/or E2, SA, and VA for 3 days, and proliferation was investigated using MTT assays. (D) Effect of SA and VA treatment on TRAP activity; RAW264.7 cells were treated with RANKL and/or E2, SA, and VA for 3 days. (E, D) Effect of SA and VA on osteoclastogenesis; cells were treated with RANKL and/or E2, SA, and VA for 5 days and TRAP stained. (E) Representative photograph of RAW264.7 cells cultured for 5 days. (F) The numbers of TRAP-positive cells of RAW 264.7 cells cultured for 5 days. Values are presented as means ± SD for at least three replicates. *$P < 0.05$; **$P < 0.01$.

cells, this RANKL-mediated activation was inhibited by the extracts in a dose-dependent manner (Fig. 4A). These results suggest that the SA and VA containing extracts of waste beds after mushroom cultivation suppress osteoclast differentiation.

Next, we examined whether authentic SA and VA suppresses the differentiation of osteoclasts. A range of 1–100 μM of SA and VA treatment showed no cytotoxic effects on RAW264.7 cells in our condition (Fig. 4C). As shown in Fig 4D, the TRAP activity in cells with RANKL treatment increased 13.1-fold compared to that without RANKL treatment. This activation by RANKL was inhibited by SA and VA in a dose-dependent manner. One hundred micro molar of SA and VA could inhibit over 50% and 37% of RANKL-induced TRAP activity, respectively. RAW264.7 cells differentiated into multinuclear TRAP-positive cells in the presence of RANKL, while monocytes were observed in non-treatment group (Fig. 4E). SA and VA effectively suppressed RANKL-induced differentiation into multinuclear TRAP-positive cells as wells as E2, a positive control (Fig. 4E). SA and VA treatment dose-dependently

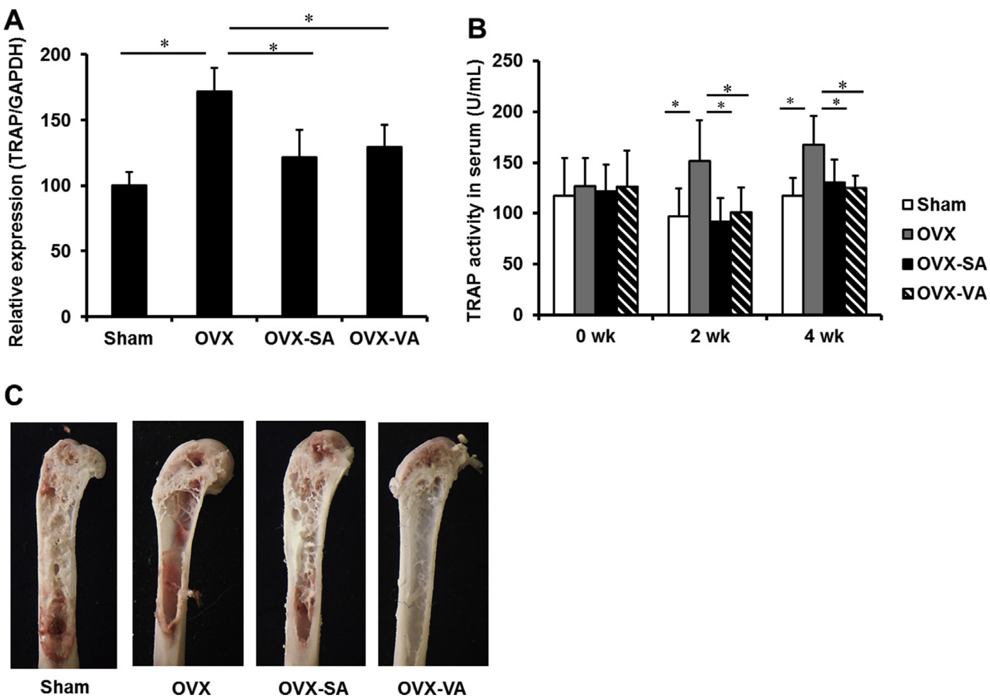

FIG. 5. Effect of the SA and VA diet on bone resorption in ovariectomized (OVX) mice. (A) Effects of SA and VA diet on gene expression of TRAP in humeri in the Sham-control, OVX-control, OVX-SA, and OVX-VA group. (B) Effects of SA and VA diet on serum TRAP activity in the Sham-control group (open bars), OVX-control group (shaded bars), OVX-SA group (closed bars), and OVX-VA group (hatched bars). (C) Effects of SA and VA diet on the femur trabeculae in the Sham-control, OVX-control, OVX-SA, and OVX-VA group. Representative images are shown. Values are presented as means ± SD. $^{*}P < 0.05$.

suppressed RANKL-induced increase in the number of TRAP-positive cells at 10–100 μM (Fig. 4F). The number of TRAP-positive cells in group treated with 100 μM SA and VA was significantly lower than that in controls (150.3 ± 26.0 vs. 93.2 ± 15.2, $P < 0.05$ and 150.3 ± 26.0 vs. 100.2 ± 16.4, respectively, $P < 0.05$). These results suggest that SA and VA suppress osteoclast differentiation.

Effect of SA and VA on bone resorption in OVX mice Next, we quantitated the gene expression levels of TRAP, a specific marker of mature osteoclasts, to investigate the effects of SA and VA on osteoclast differentiation *in vivo*. Fig. 5A shows the TRAP gene expression level in mice fed SA- or VA-containing diets. The expression of TRAP in humeri in the OVX-control group markedly increased compared to the Sham-control group. Dietary SA and VA each significantly suppressed the elevation of TRAP activity seen in the OXV-control mice.

The serum TRAP activity was determined as a bone resorption marker. When OVX mice were fed SA- or VA-containing diets for 2 and 4 weeks, serum TRAP activity significantly declined (Fig. 5B). These results suggest a positive effect on bone resorption *in vivo* as well as *in vitro*.

To confirm the bone loss-preventing effect of dietary SA and VA, the femoral trabeculae microstructure after the 4-wk feeding period was examined with a stereoscopic microscope. Isolated femurs were ground with a whetstone. The longitudinal cross-sections of the ground femur samples are shown in Fig. 5C. The number of femur trabeculae was well maintained in Sham mice. However, the number of trabeculae in OVX-control group significantly decreased compared with those in the Sham group. This drastic trabecular destruction in the OVX-control group was improved, partially but not perfectly, by the consumption of SA or VA. These observations indicate that an SA- or VA-containing diet protects the trabecular structure from degradation by osteoclasts. In addition, the positive effect of SA was stronger than that of VA.

Effect of SA and VA diet on uterine weight We next measured uterine weight changes caused by OVX treatment, SA, and VA diet. Uterine weight in Sham, OVX-control, OVX-SA, OVX-VA, and OVX-E2 group was 0.24 ± 0.04, 0.03 ± 0.02, 0.03 ± 0.02, 0.03 ± 0.01, and 0.08 ± 0.02 g, respectively. E2 treatment significantly restored the weights of uteri as well as the reported studies (29,30). However, SA and VA diet at this dose did not affect uterine weight in mice.

Effect of SA and VA on proliferation of MCF-7 cells To examine at the cellular level whether SA or VA exert any-estrogen-like effect, the proliferative effect of SA and VA on the human breast cancer cell line, MCF-7, was explored. Treatment of MCF-7 cells

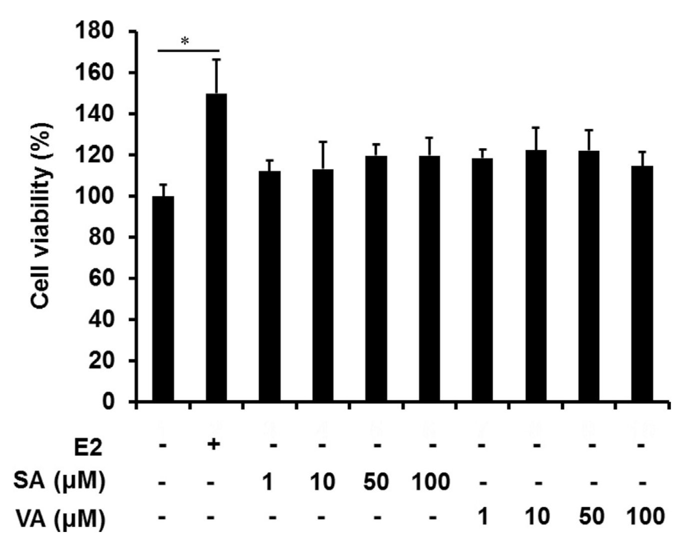

FIG. 6. Effect of SA and VA on MCF-7 cell proliferation; cells were treated with E2, SA, and VA for 3 days, and proliferation was investigated using MTT assays. Values are presented as means ± SD for at least three replicates. $^{*}P < 0.05$.

with SA or VA up to 100 μM for 4 days resulted in no enhanced proliferation without cell death, but 10 nM E2 stimulated twice the growth of MCF-7 cells (Fig. 6). This finding indicates that SA and VA does not support proliferation of MCF-7 cells, which express ERs and show E2-dependent growth.

DISCUSSION

In the present study, we demonstrated that SA and VA decreased the enzymatic TRAP activity and the number of TRAP-positive multinuclear cells in RANKL-induced osteoclasts in RAW264.7 cells. Therefore, SA and VA likely suppress the differentiation of RAW264.7 preosteoclasts to osteoclasts. Consistent with these results, the bone resorption marker (the gene expression levels of TRAP in bone marrow cells and TRAP activity in the serum) in the OVX-SA and OVX-VA groups were significantly lower than those in the OVX-control group after 4 weeks. This result is consistent with the hypothesis that SA- and VA-containing diets reduce bone resorption, thus, suppressing the degradation of femoral trabeculae in OVX mice. These findings indicate that SA- and VA-containing diets can partially but not completely protect the bones from degradation by osteoclasts in OVX mice.

We also determined the effect of SA and VA diets on the bone mineral density (BMD) using X-ray computed tomography. However, SA and VA diet feeding for 4 weeks did not alter the BMD in OVX mice in this study (data not shown). In our previous study, we reported that the BMD of OVX mice fed with an SA diet for 10 weeks improved significantly (28). The potential effect of VA on BMD is a limitation of this study. The improved effect of SA on the trabecular structure was stronger than that of VA. Therefore, a study with an administration period of 10 weeks or longer might be needed to clarify the suppressive effect of VA on reduced BMD in OVX mice. The difference between the effect of SA and VA on the trabecular structure may be due to the effect of these compounds on osteoblasts and/or osteocytes but not osteoclasts.

A better understanding of the kinetics of orally administered SA and VA in mice may contribute to the elucidation of the mechanism by which SA and VA suppress bone resorption in OVX mice. Large amounts of intact SA and VA were detected in serum, 0.5 h after SA and VA were orally administered to the mice. However, there were additional peaks observed in HPLC analysis of serum obtained from the mice fed with SA and VA, suggesting that some SA and VA enters the blood circulation as metabolites. The identification of these minor peaks, which are probably metabolites of SA and VA, is a limitation of this study. These metabolites of SA and VA may contribute to the bone loss preventing effects of the extracts in OVX mice.

As isoflavones, which suppress osteoclastogenesis, reportedly act as estrogen mimetics (7,31,32), the protective effects of SA and VA may reflect estrogen-like activities. The finding that SA and VA did not restore the weight of the uterus, which dramatically diminished after the OVX operation, suggested that SA and VA exhibit negligible estrogenic activity. The hypothesis that SA and VA exhibit negligible estrogenic activity was supported by the lack of enhanced cell proliferation in the presence of SA and VA. Furthermore, Simoncini et al. (23) showed that these compounds have no affinity for ERs. Thus, the bone loss-preventing activity of SA and VA might be not exerted through an ER-mediated pathway.

The mechanism through which SA and VA diets improve bone status in OVX mice is of great interest. SA inhibits the DNA-binding activity of nuclear factor-κB (NFκB), an oxidative stress responsive factor, in human colorectal cells (33). Calixto-Campos et al. (34) demonstrated that VA suppresses pro-inflammatory cytokine production and NFκB activation in mice model of inflammatory. Many reports have also suggested that the suppression of NFκB activation contributes to suppress osteoclastogenesis (35–37). Therefore, we propose that the effect of SA and VA is, at least in part, a result of the inhibition of NFkB transcriptional activity through antioxidant mechanisms.

SA and VA were present in extracts obtained from all waste beds after mushroom cultivation; however, the contents of SA and VA were considerably different among the mushrooms. No abnormal behaviors or adverse effects were observed in the mice on being fed with SA and VA-containing diets in this study. Thus, we expect that the extracts are likely to be safe nutritional materials that prevent osteoporosis. As culinary experience with the extracts is not available, the examination of safety of the extracts and/or SA and VA is important before applying to nutritional materials. Therefore, further acute and chronic toxicity evaluation and mutagenesis testing in mice is required.

Although both SA and VA were marginally present in extracts obtained from mushroom beds before seeding (data not shown), they were present in extracts obtained from all waste beds after mushroom cultivation. White-rot fungi degrades milled wood lignin, which is shield for acquisition of their energy source, for growth and development. As a result of lignin degradation, phenolic compounds, including SA and VA, are produced (19–21). Accordingly, SA and VA are likely produced as a result of milled wood lignin degradation during mushroom development.

In this study, the waste beds after shiitake mushroom (KX-S055) cultivation contained the highest amount of SA and VA. To investigate the difference in SA and VA content depending on the strain even in the same mushroom, the content of the waste beds after shiitake mushroom (XR-1) cultivation was examined. As observed in KX-S055, the total SA and VA content was higher in XR-1 than in other mushroom types. Shiitake mushrooms have stronger lignin degradation activity than other mushrooms. The difference of lignin degradation activity may contribute to the high SA and VA content. On the other hand, the SA and VA contents almost corresponded to the total culture period from seeding of inoculum to harvest. Moreover, the influence of the different types of sawdust, energy sources, and supplements for mushroom cultivation on lignin degradation should be taken into consideration. Therefore, it remains an open question why the SA and VA content are different between mushroom species, as the underlying biosynthetic pathways for SA and VA remain unclear.

In conclusion, based on the findings of our study, we propose that SA and VA are promising nutritional substances for the prevention of postmenopausal osteoporosis. However, further studies are needed to clarify the molecular mechanisms underlying the anti-osteoporotic activity of SA and VA.

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