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Cytotoxic Tolerance of Healthy and Cancerous Bone Cells to Anti-microbial Phenolic Compounds Depend on Culture Conditions

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Abstract

Carnosol and carnosic acid are polyphenolic compounds found in rosemary and sage with known anti-oxidant, anti-inflammatory, and anti-microbial properties. Here, we addressed the potential use of carnosol and carnosic acid for in vitro bone tissue engineering applications, specifically depending on their cytotoxic effects on bone marrow stromal and stem cells, and osteosarcoma cells in monolayer and 3D cultures. Carnosol and carnosic acid displayed a bacteriostatic effect on Gram-positive bacteria, especially on *S. aureus*. The viability results indicated that bone marrow stromal cells and bone marrow stem cells were more tolerant to the presence of carnosol compared to osteosarcoma cells. 3D culture conditions increased this tolerance further for healthy cells, while not affecting the cytotoxic potential of carnosol for osteosarcoma cells. Carnosic acid was found to be more cytotoxic for all cell types used in the study. Results suggest that phenolic compounds might have potential use as anti-microbial and anti-carcinogenic agents for bone tissue engineering with further optimization for controlled release.

Keywords Carnosol · Carnosic acid · Bone tissue engineering · Filter paper · 3D culture

Introduction

Osteosarcoma is a type of cancer that commonly develops in the metaphyseal regions of long bones. Though osteosarcoma may occur at any age, incidences during childhood and adolescence are more prevalent [28, 54]. Osteosarcoma has the capacity to metastasize to lungs and sometimes to other distant tissues such as the pancreas and skin [1, 15]. Though most of the

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osteosarcoma treatments were based on amputation, recent advances in therapeutic agents and the improved prosthetics made limb salvage surgeries more common [45]. Even though chemotherapy and proper surgical procedures decrease the risk of a relapse, still 15–25% of the patients have the risk of developing metastases [44, 53]. Recurrence occurs approximately in 10% of the patients who have undergone limb salvage surgery [43], and prognosis of the recurrence is poor, and unfortunately, chemotherapy with more than one agent provides a minimal improvement to the outcomes of the therapy [19, 26]. This is the reason why therapeutics that target primary and metastatic tumor cells with minimal damage to normal cells are actively studied for osteosarcoma [34].

Regeneration of a healthy bone tissue is regulated by chemical and mechanical cues as well as spatial factors and occurs free of scar formation [17, 32, 35]. However, if the defect size is large, it is considered as "critical size bone defect," and it cannot be healed naturally [14]. This factor also limits the success of salvage surgeries, and therefore tumor prosthesis, bone prosthesis composite, or osteochondral auto/allografts are used upon limb salvage operations to ensure healing [50]. Critical size bone defects can be filled with biological material and anti-carcinogenic compounds to suppress any potential cancer cells that remain after the surgery [33]. Another limit for the success of bone surgeries is microbial infections [41]. For the treatment of open fractures, internal fixation devices are used, and these operations carry a high risk of bone infections [39]. Similarly, limb salvage surgeries are also known to cause wound infections which may then result in loss of functionality, morbidity, and mortality [25]. Bone infections are usually treated by intravenous administration of antibiotics which are effective on Gram (+) bacteria, especially *Staphylococcus aureus*, and resistance to antibiotics caused the need of introducing alternative anti-microbial agents for clinical treatment [40]. Therefore, an agent that possesses anti-carcinogenic and anti-microbial properties would be beneficial to functionalize scaffolds with the aim of healing critical size bone defects after limb salvage surgeries.

Carnosol is a polyphenolic compound found in rosemary (*Rosmarinus officinalis*) and sage (*Salvia officinalis*) with anti-oxidant, anti-inflammatory, and anti-microbial properties [7, 24]. Furthermore, it is reported that carnosol has anti-carcinogenic properties in prostate, breast, skin, ovarian, colon, and intestinal cancers and leukemia [22, 48]. Carnosol is also shown to act as a potential anti-proliferative agent based on its increased efficacy on inhibiting cancer cell growth in suspension compared to cancer cells that are growing on monolayer [48]. Carnosic acid is another polyphenolic compound found in rosemary and sage with a similar chemical structure to carnosol. Carnosic acid may also undergo an oxidative degradation and rearrangement cascade, which generates other rosemary anti-oxidant compounds such as carnosol, rosmanol, galdosol, and rosmariquinone [11]. Similar to carnosol, carnosic acid also has chemo-preventive, anti-oxidant, anti-microbial, anti-obesity, anti-platelet, and anti-tumor properties [8, 11]. However, the effect of carnosol and carnosic acid on the viability of osteosarcoma cells is not known.

Here, we hypothesized that the cytotoxic effect of carnosol and carnosic acid would be used for the prevention of bone cancer. To test this, we cultured osteosarcoma, bone marrow stromal cells, and bone marrow stem cells with growth media containing different concentrations of carnosol or carnosic acid in 2D and 3D cell culture models. Further, the same cells were also cultured with the same conditions following 1-week osteogenic induction. Our results indicated that carnosol, but not carnosic acid, is a potential natural agent that can be used in bone tissue engineering as an anti-microbial and cancer preventive agent depending on its cytotoxic effect of osteosarcoma cells.

Materials and Methods

Determination of Anti-microbial Properties

Escherichia coli (ATCC® 25922™), *Staphylococcus aureus* (RSKK 1009; Refik Saydam National Type Culture Collection, Turkey), *Klebsiella pneumoniae* (FOR, DHA-2), and *Staphylococcus epidermidis* (NRRL B-4268) were used for anti-microbial activity determination test. Briefly, all the bacteria were streaked on tryptic soy agar (TSA) plates. For all bacterial types, one of the middle-sized colonies were transferred to tryptic soy broth for suspension culture. Liquid cultures were incubated at 37 °C for 24 h. Bacteria that were grown from one single colony were streaked on TSA plates again. The turbidity of the cultures was adjusted to 0.5 McFarland by diluting in Pepton water. Then, 10^5 dilution was made for all bacteria types, and 100-μL suspension was spread on TSA for calculation of cfu per milliliter.

Three different carnosol (Sigma-Aldrich, Germany) and carnosic acid (Sigma-Aldrich, Germany) concentrations (18, 30, and 60 μg/mL) were chosen according to cell culture test results for determination of anti-microbial activity. Carnosol and carnosic acid stocks were dissolved in DMSO, with 120, 60, and 36 μg/mL concentrations by diluting the main stock with broth. The test was performed in a 96-well plate, with 10^6 bacteria for each bacterial type. Eighty-microliter broth, 100 μL of each carnosol concentration, and 20 μL of bacteria were added to each well. Bacteria grown in tryptic soy broth were used as the positive control and 100 IU/mL of penicillin and 100 μg/mL of streptomycin was used as the negative control (Pen/Strep). The assay plate was incubated at 37 °C and 120 rpm for 24 h, by measuring the absorbance at 600 nm every 2 h.

Cell Culture

D1 ORL UVA mouse bone marrow stem cells [3, 6, 16], SaOs-2 human osteosarcoma cells, and HS-5 human bone marrow stromal cells were used for dose determination studies. Cells were seeded at a density of 10^4 cells/well in 96-well plates and cultured in DMEM high glucose medium (Gibco, USA) supplemented with 10% fetal bovine serum (Biological Industries, USA) and 1% Pen/Strep (Biological Industries, USA).

For the osteogenic induction, cells were seeded at a density of 10^3 cells/well in 96-well plates. After 24 h, growth medium of cells was replaced with osteogenic induction medium (10 mM β-glycerophosphate (Sigma-Aldrich, Germany), 50 μg/mL ascorbic acid (Sigma-Aldrich, Germany), and DMEM high glucose complete medium with 10% FBS and 1% Pen/Strep) and the cells were incubated with this medium for 1 week.

Determination of Effective Carnosol and Carnosic Acid Concentration

The determination of effective dose for carnosol (Sigma-Aldrich, Germany) and carnosic acid (Sigma-Aldrich, Germany) was conducted by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay for cells in normal or osteogenic conditions. Twenty-four hours after cell seeding, the media were discarded and the media containing varying carnosol and carnosic acid concentrations (0, 6, 18, 30, 42, and 60 μg/mL) were added to cell culture. Lyophilized carnosol was solubilized with DMSO (Amresco, USA) to prepare the 5 mg/mL stock solution, and all concentrations were prepared by diluting this stock with culture medium with an equal volume of DMSO in each concentration. MTT (Amresco, USA) test for cell

viability was applied after 24, 48, and 72 h treatment with carnosol or carnosic acid, and the outcome was assumed as directly proportional to the population of live cells [13, 36]. MTT tests were conducted by adding 10% MTT containing medium at 100 μL final volume in 96-well plates and incubating 4 h at 37 °C and 5% CO_2. Tetrazolium salt crystals were dissolved in DMSO, and absorbance of the samples was read at 570- and 650-nm wavelengths [56]. IC50 values were calculated as assuming the viability of control groups as 100%, and taking the phenolic compound concentrations that yield 50% cell survival according to the MTT absorbance values for data pertaining to 72 h [4, 11].

3D Cell Culture

In an effort to determine the effects of carnosol and carnosic acid on three-dimensional cell growth, Whatman paper (Grade 114) was selected as the scaffold material. Before, cell seeding papers were cut with a puncher for 96-well plates. Then, papers were sterilized by soaking in 90% ethanol for 30 min, aspirating the ethanol and leaving in the laminar flow hood overnight for drying [12]. Collagen is diluted with culture medium to 2.5 mg/mL from 3.7 mg/mL starting concentration [12]. For cytotoxicity tests, cells were seeded in a total of 2-μL volume. For cytotoxicity analysis, cells were resuspended in a mixture of collagen and growth medium and seeded on paper scaffolds by pipetting 2 μL of this mixture on each paper at a concentration of 10^4 cells/paper in 96-well plates and MTT assay was performed as described above. Cell-seeded paper scaffolds were moved to a new plate before MTT assay to eliminate the false signals that could come from the cells attached to the plate surface. The absorbance value of a similarly treated blank Whatman paper was used as background control.

Statistical Analyses

All the experiments were performed in triple repeats, and all results are displayed as the mean ± standard deviation. Within-group statistical analyses were performed using ANOVA. For cell growth, ANOVA was followed by Tukey post hoc test to detect significant growth or to illustrate the differences between cell types. For carnosol and carnosic acid applications, ANOVA followed by Dunnett's multiple comparison post hoc tests was applied, where the dose is compared to negative controls. Levels of significance were reported for 5%, 1%, and 0.1%.

Results

In order to determine the anti-microbial effect of carnosol and carnosic acid, *S. aureus* (Gram-positive), *S. epidermidis* (Gram-positive), *E. coli* (Gram-negative), and *K. pneumoniae* (Gram-negative) were treated with 18, 30, and 60 μg/mL of concentration for 24 h. Carnosol and carnosic acid were found to suppress the growth of *S. aureus* and to a limited extend the growth of *S. epidermidis* (Fig. 1). For *S. aureus* and *S. epidermidis*, stationary phase was observed, but the expected dose-dependent decrease in growth was only detected for *S. aureus* (Fig. 1). According to the curves of anti-microbial activity assay for carnosol and carnosic acid treatment, Pen/Strep stopped the growth of all bacterial types. On the other hand, when the samples were treated with carnosic acid, *S. aureus* stationary phase was observed for the control group, but for carnosic acid-treated groups (18 and 30 μg/mL), the bacteria were still in

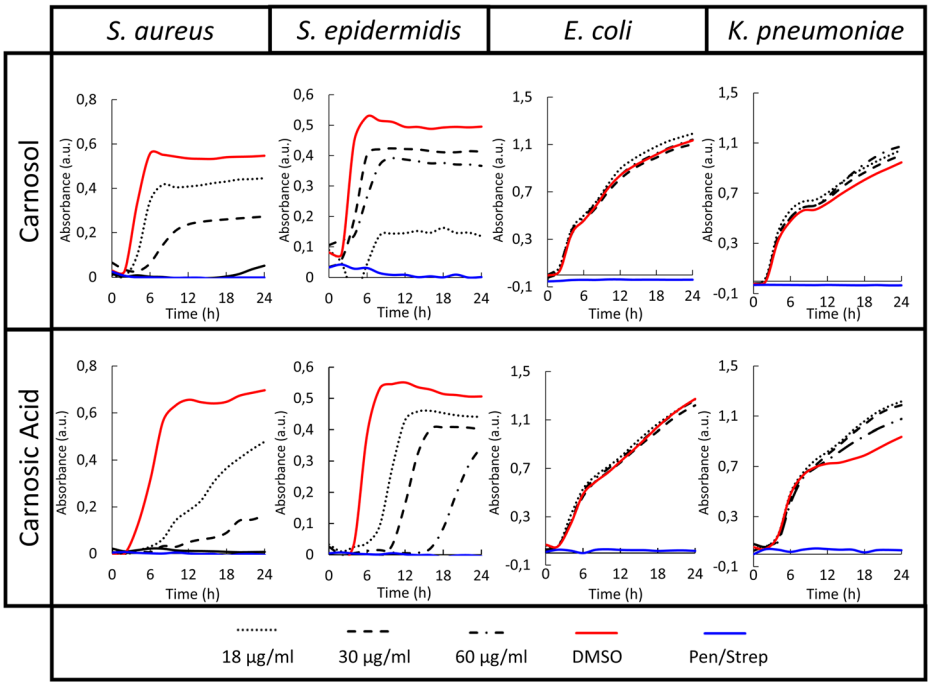

Fig. 1 Anti-microbial activity of carnosol and carnosic acid on *S. aureus*, *S. epidermidis*, *E. coli*, and *K. pneumoniae*. Increasing concentrations of both carnosol and carnosic acid decreased the growth of *S. aureus*. Both phenolic compounds decreased the growth of *S. epidermidis*. *Growth inhibition of S. epidermidis* was not concentration dependent for carnosol, but carnosic acid effected the same organism in a concentration-dependent manner. Both carnosol and carnosic acid did not have any inhibitory effect on growth of *E. coli* and *K. pneumoniae*

the logarithmic phase with a prolonged lag phase at the end of 24 h when compared to the control group (Fig. 1). Sixty micrograms per milliliter carnosol or carnosic acid, however, decreased the growth of *S. aureus* as much as Pen/Strep. Thus, both phenolic compounds, carnosol, and carnosic acid showed a bacteriostatic effect for the Gram-positive bacteria used in this study.

Documented over 3 days, all cell types used in this study showed a significant increase in cell viability on 2D monolayer cultures compared to their first day: D1 ORL UVA 162%, Saos-2 65%, and HS-5 47% (all $p < 0.001$) (Fig. 2a). At day 3, growth in D1 ORL UVA cells was 58% ($p < 0.01$) and 79% ($p < 0.001$) larger compared to Saos-2 and HS-5 cells. Application of osteogenic induction changed the growth pattern of cells (Fig. 2b); the viability of HS-5 cells increased 31% ($p = 0.02$), and Saos-2 cells decreased 63% ($p < 0.001$), while D1 ORL UVA cells remained similar to quiescent conditions. 3D scaffold-based culture also promoted significant growth for D1 ORL UVA (73%, $p = 0.02$) and HS-5 (88%, $p = 0.02$) cells, where cell growth was similar between cell types (Fig. 2c).

Gradual increase in the carnosol concentration in monolayer culture of D1 ORL UVA bone marrow stem cells caused 16%, 57%, 73%, and 97% decrease (all $p < 0.05$) in cell viability for 18, 30, 42, and 60 µg/mL concentrations, at the end of 24-h incubation (Fig. 3a). Extended to 72 h of exposure, D1 ORL UVA viability was 42%, 67%, 91%, 97%, and 97% lower (all $p < 0.001$) compared to negative control at 6, 18, 30, 42, and 60 µg/mL concentrations. HS-5 bone

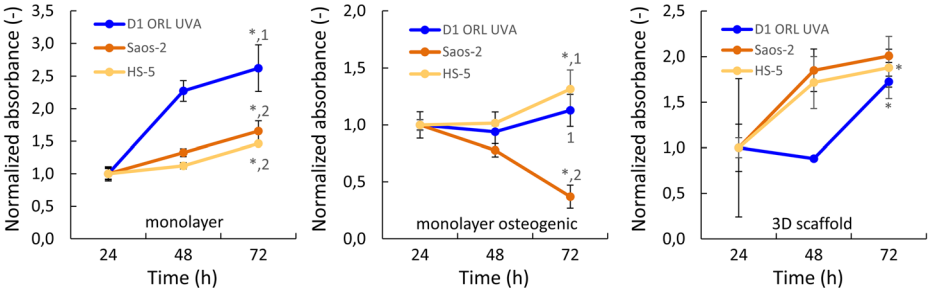

Fig. 2 Changes in cell viability of D1 ORL UVA bone marrow stem cells, HS-5 bone marrow cells, and Saos-2 osteosarcoma cells in **a** monolayer culture for 3 days, **b** monolayer culture for 3 days with 7 days a priori conditioning in osteogenic, and **c** culture for 3 days in 3D paper based scaffold. *$p \leq 0.05$ for viability at 72 h compared to 24 h calculated by ANOVA followed by Tukey's post hoc test. 1,2: differences in viability of cell type at 72 h calculated by ANOVA followed by Tukey's post hoc test

marrow cells similarly showed 31%, 62%, 65%, and 89% decrease (all $p < 0.05$) upon treatment with 18, 30, 42, and 60 μg/mL carnosol after 24 h (Fig. 3b). At 72 h of exposure, HS-5 viability was 19%, 68%, 92%, and 96% lower (all $p < 0.05$) compared to negative control at 18, 30, 42, and 60 μg/mL concentrations. A more detrimental effect was recorded for Saos-2 cells at 24 h where 17%, 63%, 96%, 99%, and 99% lower growth (all $p < 0.001$) for 6, 18, 30, 42, and 60 μg/mL carnosol concentrations compared to negative controls (Fig. 3c). Carnosol concentration of 30 μg/mL appeared to be extremely cytotoxic for osteosarcoma cells. Application of carnosic acid induced a significant dose-dependent reduction in the viability of all cell types (Fig. 3d–f). D1 ORL UVA cells responded to carnosic acid similar to their response to carnosol, but HS-5 cells appeared to have lower viability in high doses and Saos-2 cells were able to tolerate carnosic acid better until 60 μg/mL dose.

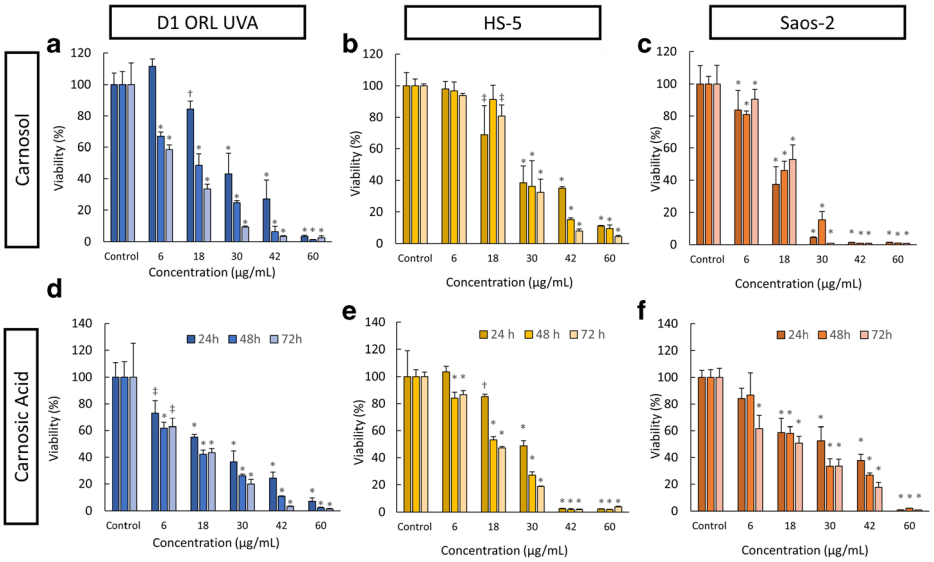

Fig. 3 Effect of carnosol treatment on monolayer cultured cell viability for 24, 48 and 72 h for **a** D1 ORL UVA, **b** HS-5, and **c** Saos-2 cells. Effect of carnosic acid treatment on monolayer cultured cell viability for 24, 48, and 72 h for **d** D1 ORL UVA, **e** HS-5, and **f** Saos-2 cells. †$p \leq 0.05$; ‡$p \leq 0.01$; *$p \leq 0.001$ for each time point compared to negative control calculated by ANOVA followed by Dunnett's post hoc test

To simulate the potential osteogenic behavior of bone marrow cells, an osteogenic induction cocktail was applied for 1 week prior to the 72-h application of carnosol or carnosic acid. Carnosol amount in osteogenic monolayer culture of D1 ORL UVA bone marrow stem cells caused 22%, 28%, 33%, and 73% decrease (all $p < 0.05$) in cell viability for 18, 30, 42, and 60 μg/mL concentrations at the end of 24-h incubation (Fig. 4a). Extended to 72 h of exposure, D1 ORL UVA viability was 9% larger and 42%, 67%, 91%, 97%, and 97% lower (all $p < 0.001$) compared to negative control at 6, 18, 30, 42, and 60 μg/mL concentrations, respectively. HS-5 bone marrow cells showed 30%, 41%, and 77% decrease (all $p < 0.001$) upon treatment with 30, 42, and 60 μg/mL carnosol after 24 h (Fig. 4b). Extended to 72 h of exposure, HS-5 viability was 20%, 51%, 70%, and 97% lower (all $p < 0.001$) compared to negative control at 18, 30, 42, and 60 μg/mL concentrations, respectively. Similar to monolayer culture, a more detrimental effect was recorded for Saos-2 cells at 72 h where 74%, 76%, 95%, 86%, and 98% lower (all $p < 0.001$) viability was recorded for 6, 18, 30, 42, and 60 μg/mL carnosol concentrations compared to negative controls (Fig. 4c). Application of carnosic acid on all cell types induced significant cytotoxicity; for D1 ORL UVA cells, the cytotoxic effect was seen at concentrations higher than 30 μg/mL, and for HS-5 and Saos-2 cells at 18 μg/mL concentration (Figs. 4d–f).

In discordance with monolayer results of the carnosol application, D1 ORL UVA bone marrow stem cells were able to tolerate carnosol application when cultured on the 3D scaffold (Fig. 5a). HS-5 cells were also able to tolerate low doses in 3D culture but showed 74%, 87%, and 93% decrease in viability (all $p < 0.001$) at 30, 42, and 60 μg/mL carnosol concentrations on 72 h (Fig. 5b). Saos-2 cells were more sensitive showing 18%, 35%, 88%, 96%, and 86% decrease in viability (all $p < 0.001$) at 6, 18, 30, 42, and 60 μg/mL carnosol concentrations compared to negative controls (Fig. 5c). Similar to carnosol treatment in the 3D culture system, carnosic acid treatment appeared to be tolerated well for D1 ORL UVA cells (Fig. 5d). For HS-5

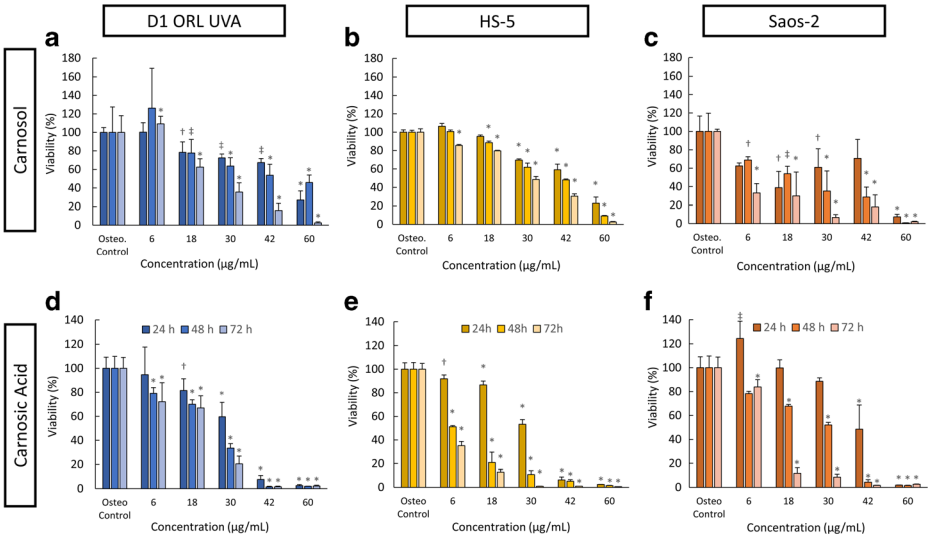

Fig. 4 Effect of carnosol treatment on osteogenic conditioned monolayer cultured cell viability for 24, 48, and 72 h for **a** D1 ORL UVA, **b** HS-5, and **c** Saos-2 cells. Effect of carnosic acid treatment on osteogenic conditioned monolayer cultured cell viability for 24, 48, and 72 h for **d** D1 ORL UVA, **e** HS-5, and **f** Saos-2 cells. †$p \leq 0.05$; ‡$p \leq 0.01$; *$p \leq 0.001$ for each time point compared to negative control calculated by ANOVA followed by Dunnett's post hoc test

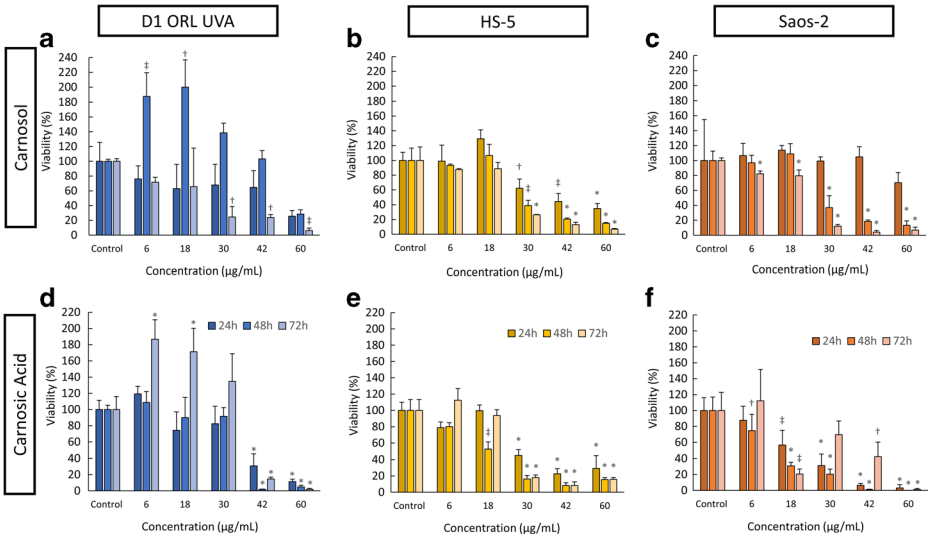

Fig. 5 Effect of carnosol treatment on 3D cultured cell viability for 24, 48, and 72 h for **a** D1 ORL UVA, **b** HS-5, and **c** Saos-2 cells. Effect of carnosic acid treatment on 3D cultured cell viability for 24, 48, and 72 h for **d** D1 ORL UVA, **e** HS-5, and **f** Saos-2 cells. †$p \leq 0.05$; ‡$p \leq 0.01$; *$p \leq 0.001$ for each time point compared to negative control calculated by ANOVA followed by Dunnett's post hoc test

and Saos-2 cells, carnosic acid was cytotoxic at concentrations higher than 30 μg/mL (Fig. 5e, f). Interestingly, unlike other cell types, D1 ORL UVA cells showed increased viability for 6 and 18 μg/mL carnosol concentrations at 48 h (271% and 230%, $p < 0.05$) and for the same carnosic acid concentrations at 72 h (186% and 171%, $p < 0.05$).

Discussion

Plant extracts are widely studied for use as anti-cancer and anti-microbial agents and nutrition supplements. Carnosol and carnosic acid are phenolic compounds with plant origin and approved by the European Union, Japan, and China as food additives, and they are the most commonly used anti-oxidants in the food industry (E392) [8]. Here, we investigated the anti-microbial and cytotoxic effects of both phenolic compounds for bone tissue engineering applications. Based on our 2D and 3D cell culture models, we identified carnosol but not carnosic acid, as a potential chemopreventive and anti-microbial agent for bone tissue engineering applications especially those that involve salvage surgeries to treat osteosarcoma.

Phenolic diterpenes carnosol and carnosic acid both have anti-microbial and nutraceutical properties alone and in combination with commercial antibiotics [29]. Consistent with our results, anti-microbial properties of carnosic acid were reported with minimal inhibitory concentration (MIC) values for both *S. aureus* (ATCC 25923) and *S. epidermidis* (DSM 1798) as 64 μg/mL and the compound was not as effective on *E.coli* and *K. pneumoniae* [52]. In vivo studies performed with mice also showed that carnosic acid diminished the growth of *S. aureus* that is internalized by macrophages with no harm to macrophages [30]. Because of their anti-microbial properties, these phenolic compounds can be utilized as potential candidates for preventing infections during bone defect treatments. In addition to their anti-microbial properties, the toxicity of these phenolic compounds on normal and cancer

cells was also studied and carnosol treatment apparently decreased the viability of bone cancer cells more than marrow stromal and bone marrow stem cells. The response of the same cells to carnosic acid was similar for the same conditions, with slightly higher cytotoxicity of carnosic acid on normal cells. The cytotoxicity of both phenolic compounds was diminished for stem cells but remained similar for normal and cancer cells in 3D culture.

Variety of cells and culture conditions we addressed in the study yield IC50 values, which is defined as the concentration at which the cell viability decreased to the half of the population compared to the control group, and were comparable to previous studies (Table 1). IC50 values of carnosol for MCF7 breast cancer cells were reported as 25.6 μM [23] and 82 μM [22]. For healthy cells, the IC50 values of carnosol were reported as 50 μM and 35.2 μM for BAEC and HUVEC cells, respectively, while these values were reported as 5.3 μM and 6.6 μM for HL60 (leukemia) and HT1080 (fibrosarcoma) cancer cell lines [11]. In another study, carnosol was reported to have no effect on breast, ovarian, and colon cancer models at concentrations lower than 25 μM and had dose- and time-dependent inhibitory effects for concentrations higher than 50 μM, while for healthy cells, viability reduction was only observed at concentrations higher than 200 μM [42]. Inhibitory effect of carnosol on the proliferation of adenocarcinomas was shown to be mediated by increasing intracellular cyclin B1, which regulates the progression from G_2 to M phase [49]. Carnosic acid that is extracted especially from rosemary, on the other hand, is reported to have anti-proliferative effects on various cancer cell lines such as HL-60 (myeloid leukemia), M14 and A375 (human melanoma), CaCo-2 (human colon carcinoma), HepG2 (hepatoma), and HCT-116 (colon cancer) and estrogen receptor negative human breast cancer cells by induction of G_1 cell cycle arrest [5, 18, 27]. Also, carnosic acid was reported to decrease the cell viability through apoptosis in RINm5F rat beta cells [27] and in human neuroblastoma IMR-32 cells [46]. In this research, we studied the response of normal and cancer cells that are derived from bone tissue to carnosol and carnosic acid treatment in 2D and 3D cell cultures. According to our results, the behavior of normal and cancer cells is different, and concentration and time dependent upon treatment. We also observed that the response of the same cell type to the same concentration of carnosol or carnosic acid differs in 2D and in 3D. Exposure to carnosol in 3D cell culture conditions affected osteosarcoma cells in lower doses, while healthy cells appeared to tolerate the compound in concentrations closer to antimicrobial levels. Furthermore, carnosol and carnosic acid acted as proliferative agents for healthy stem cells when applied in low doses for 3D culture, a trend that was not previously reported in related studies [55]. We believe that improved biomimicry in 3D culture may facilitate information on previously unknown molecular functions of phenolic compounds in osteogenesis.

Table 1 IC50 values (μg/mL) of carnosol and carnosic acid calculated after 72 h treatment of D1 ORL UVA, HS-5, and Saos-2 cells for different culture conditions

	Carnosol	Carnosic acid
D1 ORL UVA (monolayer)	12	40
HS-5 (monolayer)	25	18
Saos-2 (monolayer)	18	18
D1 ORL UVA (osteo)	23	22
HS-5 (osteo)	64	6
Saos-2 (osteo)	24	9
D1 ORL UVA (3D)	64	35
HS-5 (3D)	25	23
Saos-2 (3D)	20	64

In addition to their anti-microbial, anti-carcinogenic, and anti-inflammatory properties, herbal extracts can also be utilized as osteogenic inducers for in vitro differentiation of stem cells in cell culture and in tissue engineering applications [47]. Phenolic diterpenes found in herbs are gaining much interest because of their anti-inflammatory, anti-microbial, anti-cancer, and anti-oxidant properties. In addition to carnosol and carnosic acid, rosemary plants contain other phenolic diterpenes such as rosmanol and its isomers epi-rosmanol and epi-isorosmanol [9]. Rosmanol and epi-rosmanol were also reported to have anti-tumor effects especially on neuroblastoma cells [42]. Carnosic acid and carnosol are reported to be the most abundant and biologically most active components of rosemary plant [20], but the anti-oxidant effect of rosmanol is much higher than carnosol [31]. Because of this, in order to combine both anti-microbial, anti-carcinogenic, and anti-oxidant activities together at the highest level, the total extract of the plant or a mixture of all these phenolic compounds could be used simultaneously.

Encapsulation of the molecules is an alternative method to prevent the toxicity of the compound and to obtain a controlled and prolonged release system. It was previously reported that when *Calendula officinalis* extract was released from polymeric microspheres in collagen scaffolds, the toxicity of the extract on L929 fibroblasts was largely decreased and an extended release of the compound was achieved [21]. Another study showed that chlorophene-loaded nanospheres decreased the toxic effect of chlorophene on human red blood cells, while keeping its anti-microbial activity on *S. aureus* and *C. albicans* [37]. Previous studies also showed that molecules with varying molecular weights were entrapped within polymeric films, and by changing the crosslinking degree of the films, the release kinetics of the compounds were changed with no significant decrease in the anti-microbial properties [10]. In our study, the most effective carnosol and carnosic acid concentration which has the only anti-microbial effect was highly toxic on normal bone cells, so the release properties of these phenolic compounds might be improved and the toxic effect might be decreased by incorporation of a controlled release system.

Bone infections are very difficult to treat. Especially implant-related infections increase the duration and the cost of treatment and sometimes may result in morbidity or mortality [2]. It is crucial to prevent the adhesion of bacteria to implant surface in order to prohibit the biofilm formation which complicates the recognition of the bacteria by the immune system and the antibiotics [38]. If the implant or the scaffold is aimed to be used after tumor resection surgeries, incorporation of an anti-carcinogenic compound that does not damage the normal tissues as much as chemotherapeutic agents will have advantages over the commercial products. It is reported previously that such a scaffold was produced by doping of hydroxyapatite nanoparticles with selenium that can be used after tumor resection [51]. Similarly, the phenolic compounds carnosol and carnosic acid used in this study could be incorporated in a tissue engineering scaffold with a more complex release system and be utilized as an internal fixation system after tumor resection operations.

In conclusion, our study showed that phenolic diterpenes carnosol and carnosic acid both have an anti-microbial effect on *S. aureus,* which is the most commonly observed microorganisms in bone infections. The concentration that inhibits the growth of this bacteria was cytotoxic for monolayer cultures in this study, but in more accurate 3D conditions normal cells were able to better tolerate higher carnosol concentrations which are close to concentrations that have anti-microbial activity. We also suggest that carnosol could be encapsulated in controlled release systems to engineer its capabilities for bone tissue engineering in the future. Together with their anti-microbial and chemopreventive properties, these phenolic diterpenes are promising compounds for use in the treatment of bone defects especially formed after tumor resections.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflicts of interest.

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