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Salvia clandestina L. is a wild perennial species present in the Salento area of Italy. Here, we examined the in vitro effects of an aqueous extract of S. clandestina L. on the MG-63 osteosarcoma cell line. The extract reduced osteosarcoma cell viability mainly by way of apoptosis, as we observed (1) upregulation of gene and protein expression of p53, cyclin-dependent kinase inhibitors p21WAF1 and p27Kip1, and proapoptotic BAX; (2) activation of caspases; and (3) induction of a sub-G1 peak in the cell cycle. The mitogen-activated protein kinases (MAPKs) JNK1/2 and p38 are activated and involved in the intracellular effects of the S. clandestina extract, as preincubation with the JNK1/2 inhibitor SP600125 or the p38 inhibitor SB203580 significantly decreased S. clandestina extract–induced cytotoxicity and inhibited increase in p53, p21WAF1, p27Kip1, and BAX. SP600125 also inhibited mRNA levels for all the aforementioned proteins, while SB203580 only affected p53 mRNA. Furthermore, S. clandestina extract treatment counteracted epithelial-to-mesenchymal transition, inhibited cell migration, and decreased the expression and activity of matrix metalloproteinase MMP2. In addition, S. clandestina extract enhanced the cytotoxic activity of cisplatin on MG-63 cells through downregulation of the Akt/PI3K protein kinase. We conclude that S. clandestina extract may be a novel agent for osteosarcoma treatment.

Keywords: Salvia clandestina L.; MG-63 osteosarcoma; apoptosis; MAPKs; epithelial-to-mesenchymal transition; MMPs; phenolic compounds; danshensu

Introduction

Osteosarcoma is the most frequent primary solid malignancy of the bone in pediatric and adolescent patients since there exists a direct relationship between rapid bone growth and osteosarcoma. Osteosarcoma is associated with resistance to chemotherapy and the tendency to metastasize to the lungs. Lung metastases are associated with particularly poor prognoses among patients with osteosarcoma. It is estimated that approximately 20% of the sarcoma patients have detectable metastasis at diagnosis. During metastasis, many regulatory events occur that include the involvement of several specific molecules. The main metastatic processes include migration, invasion, circulation, adhesion, and colonization. In the early stages, the alteration of the extracellular matrix (ECM), which is responsible for cell–cell contact, induces metastasis and migration. Thus, matrix metalloproteinases (MMPs) contribute to the ability of osteosarcoma cells to invade and metastasize. For these reasons, MMPs have been considered as an attractive therapeutic target for the treatment of inflammation-related diseases, such as cancer. Even when combined treatments are used, the prognosis for osteosarcoma metastatic disease remains poor, and more than half of the cases relapse; at present, the best
treatment strategy to manage osteosarcoma patients with lung metastasis remains unclear. In addition, despite advances, chemotherapy often is difficult for patients due to the systemic toxicities of these agents. Thus, there is a need to identify new and effective treatment strategies in order to overcome the limitations of current approaches. It has been demonstrated that diet has an impact on specific types of tumor; in addition, dietary natural compounds, such as phytochemicals, can influence cancer risk and tumor behavior, preventing invasion and metastatic phase.

Fruit, vegetables, and medicinal plants represent the main dietary sources of polyphenols, which exert many biological activities, including antioxidant, anti-inflammatory, antimicrobial, antiproliferative, and proapoptotic activity. Emerging evidence suggests that long-term intake of polyphenols has positive effects on the incidence of several cancers, type II diabetes, cardiovascular disease, and neurodegenerative diseases.

We previously examined the composition and antioxidant activity of phenols from S. clandestina, a wild perennial species present in the Salento (Apulia, Italy) area. Among the 29 compounds detected, a high content of danshensu (3-(3,4-dihydroxy-phenyl) 2-hydroxy-propinic acid), a powerful antioxidant and a cardioprotective agent, was observed; the danshensu content in S. clandestina shoots (4.96 mg g⁻¹ DW) was more than fourfold the level reported for leaf extracts of S. miltiorrhiza.

Starting from recent studies demonstrating antitumor properties of bioactive molecules contained in S. clandestina extracts, in the present study we tested for antitumor and antimigration effects of the extracts in an in vitro model of osteosarcoma using MG-63 cancer cells.

**Materials and methods**

**Materials**

Eagle’s minimum essential medium, antibiotics, glutamine, and fetal bovine serum (FBS) were purchased from Celbio (Milan, Italy). Antibodies to caspase-9 and caspase-3, Bax, Bid, Bcl-2, poly (ADP-ribose) polymerase (PARP), MMP2, MMP9, phospho-Akt/PKB, phospho-JNK1/2, and phospho-p38 were obtained from Cell Signaling Technology (Celbio). Antibodies to p21<sup>WAF1/CIP1</sup>, p53, p27<sup>Kip1</sup>, CDKs, cyclin E, E-cadherin, vimentin, JNK1/2, p38, and goat anti-rabbit antibody conjugated with peroxidase and control antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Cell-permeable broad-spectrum caspase inhibitor z-VAD-fmk, JNK1/2 inhibitor SP600125, p38 inhibitor SB203580, and MMP inhibitor GM-6001 were obtained from Calbiochem (Darmstadt, Germany). All other reagents were purchased from Sigma (Milan, Italy).

**Plant material and phenolic extract preparation**

S. clandestina L. wild plants were randomly collected from a fallow field in the town of Arnesano, near Lecce, Salento, Italy in April 2017. The plant material was harvested at the floral budding stage. Plants were identified by the Systematic Botanical Laboratory and voucher specimens deposited in the Herbarium of the Department of Biological and Environmental Sciences and Technologies, University of Salento, Italy (voucher no. Sc 24978). After collection from the field, shoots were oven dried at 35 °C until reaching a constant weight. A dried sample (1 g) was mixed with 100 °C H₂O at a ratio of 1:20 (w/v) in a flask, and the suspensions were brought to boil with refluxing for 20 min, according to Zhou et al., and as indicated in Nicoli et al. After centrifugation, the resulting solutions were filtered using a 0.2-μm PFTE membrane and analyzed as described below.

**HPLC ESI/MS-TOF analysis**

Phenolic characterization (Fig. S1 and Table S1, online only) was carried out using an Agilent 1200 liquid chromatography system (Agilent Technologies, Palo Alto, CA) equipped with a standard autosampler. The HPLC column was an Agilent Extended C18 (1.8 μm, 2.1 × 50 mm). Separation was carried out at 40 °C with a gradient elution program at a flowrate of 0.5 mL/min. The mobile phases consisted of water plus 0.1% formic acid (A) and acetonitrile (B). The following multistep linear gradient was applied: 0 min, 5% B; 13 min, 25% B; and 19 min, 40% B. The injection volume in the HPLC system was 5 μL, and the extract was diluted 1:2 by phase A. The HPLC system was coupled to an Agilent 6320 TOF mass spectrometer equipped with a dual ESI interface (Agilent Technologies) operating in negative ion mode. Detection was carried out within a mass range of 50–1700 m/z. Accurate mass measurements of each peak from the total ion current chromatogram were obtained.
by means of an ISO pump (Agilent G1310B) using a dual nebulizer ESI source that introduces a low flow (20 μL/min) of a calibration solution that contains the internal reference masses at m/z 112.9856, 301.9981, 601.9790, and 1033.9881, in negative ion mode. The accurate mass data of the molecular ions were processed through the MassHunter (Agilent Technologies) software.

**Quantitative analysis and total phenolics quantification**

In order to establish the relationship between peak area and concentration, linear regression models were carried out using the following standards: danshensu, caffeic acid, rosmarinic acid, and salvianolic acid B. Phenolic compound contents were expressed in microgram per gram of dry matter (μg/g DW) and microgram per milliliter of extract (μg/mL) (Table S2, online only).

The total phenolic content (TPC) of the *S. clandestina* aqueous extract was determined according to the Folin-Ciocalteu method by reading the absorbance at 765 nm with a JASCO V-550 UV/VIS spectrophotometer (JASCO Corporation, Tokyo, Japan); results were expressed in terms of caffeic acid equivalent (CAE) as mg/g DW and mg/mL of extract by using a caffeic acid standard curve (equation: \( y = 4.3039x \)) (Table S2, online only). All measurements were performed in triplicate.

**Cell lines**

The human osteosarcoma cell line MG-63 was cultured in Eagle’s minimum essential medium supplemented with 10% FBS and maintained at 37 °C in a humidified incubator with 5% CO₂. Cells were grown to 70–80% confluence and then treated with extracellular nucleotides at various concentrations and for different incubation periods.

**Cell viability assay and cell cycle analysis**

The SRB (sulforhodamine B) assay and the conversion of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenol tetrazolium bromide) by osteosarcoma cells were used as indicators of cell number. Viable cells were also counted by the trypan blue exclusion assay and light microscopy. The data presented are means ± standard deviation (SD) from eight replicate wells per microtiter plate.

MG-63 cells were fixed in 70% cold ethanol and kept overnight at 4 °C. Cells were then resuspended in 20 μg/mL propidium iodide (PI) and 100 μg/mL RNase (final concentrations). Cell cycle was evaluated with the FL3 detector in the linear mode using FloMax software (Partec, Cornaredo, Milano, Italy).

**Cell apoptosis assay**

Cell apoptosis was determined by the annexin V-FITC/PI assay. After treatment, cells were collected and resuspended with 500 mL binding buffer at a concentration of 10⁶ cells/mL. After adding 5 mL annexin V-FITC and 10 mL of propidium iodide solution, cells were mixed and incubated at room temperature in the dark for 10 minutes. Then, the samples were analyzed using a CyFlow® Space cytometer (Partec) and FloMax software (Partec), as described previously.

**Preparation of subcellular fractions and western blotting**

Subcellular fractions were obtained as previously reported. Western blotting analysis, immunodetection, and densitometric analysis were performed as previously described.

**Reverse transcriptase-polymerase chain reaction**

Total RNA from portions of tissue or cells was extracted using the SV Total RNA Isolation System (Promega, Madison, WI) according to the manufacturer’s instructions. The procedures for the reverse transcriptase-polymerase chain reaction and/or real-time PCR were performed as described previously in the presence of 2 μM of specific primers for different genes and for β-actin (Celbio).

**Cell wounding assay**

The cells were seeded in 12-well culture dishes and cultured until they reached confluence. The cells were then scraped with a 20-μL micropipette tip, denuding a strip of the monolayer approximately 500 μm in width, as described previously.

**MMP gelatin zymography**

After treatment, the culture medium was collected and centrifuged at 14,000 rpm for 5 min at 4 °C to remove cells and debris as described previously. MMP gelatin zymography assay was performed as previously indicated.

**Statistical analysis**

The analysis was performed by a different person than the experimenters. Each experiment was repeated at least four times. Data points reported in
Figure 1. *S. clandestina* aqueous extract decreases MG-63 cells viability. (A) Osteosarcoma cells treated or not with increasing concentrations of *S. clandestina* aqueous extract; cell viability was determined by the MTT assay and data are presented as percent of control (means ± SD of six independent experiments with eight replicates each); *P* < 0.05 compared with untreated cells. (B) Top: The cell cycle was investigated by flow cytometry in propidium iodide–stained cells after treatment with 60 μg/mL of *S. clandestina* extract. Bottom: The percentage of cells in different cell cycle phases. (C) Cells treated or not for 12 and 24 h with 60 μg/mL of *S. clandestina* extract; apoptosis was determined by the annexin V-FITC/PI assay.

the figures are given as means ± standard deviation (SD). The data were analyzed using GraphPad® Prism 5.0 Software (GraphPad Software, San Diego, CA). Either an unpaired Student's *t*-test or one-way ANOVA was performed. A *P* value of less than 0.05 was considered statistically significant. When the results were at least *P* < 0.05, we performed a post hoc analysis using the Bonferroni–Dunn test.
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Results

Salvia clandestina *L.* extracts inhibit cell proliferation

In vitro proliferation data were obtained by the MTT assay and confirmed by the SRB assay to rule out potential effects of *S. clandestina* extracts on mitochondrial enzymes. Furthermore, comparable results were obtained when cell number was directly determined by cell counting (data not shown); consequently, we used the MTT assay in the combined experiments reported hereafter. Exposure of serum-free medium starved MG-63 cells to increasing doses of *S. clandestina* aqueous extracts (2.5–200 μg/mL) induced, starting from about 25 μg/mL TPC, a significant dose-dependent reduction in cell viability (Fig. 1A). It should be noted that low doses of *S. clandestina* extract increased cell viability due to the polyphenol paradox effect. After 24-h incubation with *S. clandestina* extract, the IC_{50} value was 60 μg/mL (Fig. 1A); therefore, this concentration was used for all subsequent experiments. Exposure of the MG-63 cells to *S. clandestina* extract at concentrations ranging from 2.5 to 200 μg/mL for 24 h resulted in a dose-dependent activation of apoptosis (Fig. S2, online only).

Cell cycle arrest and induction of apoptosis

The effect of the extract on the cell cycle was investigated by flow cytometry in PI (propidium iodide)-stained cells after treatment for 6, 12, and 24 hours. Notably, 60 μg/mL *S. clandestina* extract causes a significant and time-dependent increase in the percentage of cells in the sub-G_{1} phase (Fig. 1B). After 24 h of incubation, most of the cells treated have undergone apoptosis (Fig. 1C).

*S. clandestina* extract (60 μg/mL) caused the activation of caspase-9, -7, and -3, with the generation of activated heterodimers of low molecular mass in a time-dependent manner (Fig. 2A). Activation of caspases leads to the cleavage of several target proteins, such as PARP. Indeed, as shown in Figure 2A, the extract significantly increased the amounts of cleaved PARP after 12-h treatment, thus confirming the induction of apoptosis. Caspase-9 has been linked to the mitochondrial death pathway, and Bcl-2 proteins regulate this pathway. Thus, we assessed the effects of 60 μg/mL *S. clandestina* extract on the expression of BAX and Bcl-2 proteins by western blot analysis of mitochondrial fractions. *S. clandestina* extract increased the expression of BAX and Bcl-2 proteins by western blot analysis of mitochondrial fractions. *S. clandestina* extract increased the expression of BAX and Bcl-2 proteins by western blot analysis of mitochondrial fractions.

Figure 2. *S. clandestina* extract induces apoptosis in MG-63 cells. Cytosolic nuclear and mitochondrial fractions were separated by SDS-PAGE and analyzed by western blotting using (A) anti-PARP-1, anti-caspase-3, -7, and -9 and (B) anti-BAX, anti-Bcl2, anti-cyt-c, and anti-BID antibodies. The purity of fractions was assessed by immunoblotting with anti-β-actin, A, or with anti-porin, B, monoclonal antibodies. (C) Cells treated with and without *S. clandestina* extract and also pretreated with the cell-permeable broad-spectrum caspase inhibitor z-VAD-fmk (100 μM; left: quantification of the percentage of apoptotic nuclei was obtained by fluorescent staining with 4′,6-diamidino-2-phenylindole (DAPI) (means ± SD; n = 6); P < 0.001 by one-way ANOVA; values with shared letters are not significantly different according to Bonferroni/Dunn post hoc tests; right: viable cell number evaluation after 24-h treatment. Statistically significant differences between cells treated with an aqueous extract plus z-VAD-fmk and with *S. clandestina* extract alone (*P < 0.01) or with untreated cells (§P > 0.05) by Student's *t*-test.
In order to prove that the activation of caspases is an essential step in the apoptotic pathway induced by the *S. clandestina* extract, cells were pretreated with the cell-permeable broad-spectrum caspase inhibitor z-VAD-fmk (100 μM) for 1 h, and then treated with 60 μg/mL *S. clandestina* extract for 24 hours. Blockage of caspase activity markedly inhibited the cell death and apoptosis process induced by *S. clandestina* extract (Fig. 2C). These results suggest that the mechanism of cytotoxic effects induced by *S. clandestina* extract on MG-63 cells is related to its apoptosis-inducing activity.

**Signal transduction pathways involved in S. clandestina extract-mediated effects**

*S. clandestina* extract induced a time-dependent phosphorylation of the mitogen-activated protein kinases (MAPKs) JNK1/2 and p38, without affecting their basal levels (Fig. 3A). Preincubation with the JNK1/2 inhibitor SP600125 (1 and 10 μM) or with the p38 inhibitor SB203580 (1 and 10 μM) significantly decreased extract-induced cytotoxicity (Fig. 3B). Thus, *S. clandestina* extract activated MAPKs, thereby forcing apoptosis.

*S. clandestina* extract (60 μg/mL) caused a significant decrease in the expression of cyclin D1 and the cyclin-dependent kinases CDK4 and CDK6 but no detectable changes in CDK2 protein expression (Fig. 4A). *S. clandestina* extract also modulated the expression of the CDK inhibitors p21\textsuperscript{WAF1} and p27\textsuperscript{Kip1} inasmuch as extract treatment produced a significant increase in both inhibitors (Fig. 4A). Since p21\textsuperscript{WAF1} expression may be regulated by p53, we checked the effect of the extract on p53 levels. Indeed, p53 levels were significantly increased (Fig. 4A). Therefore, we analyzed p53, p21\textsuperscript{WAF1}, and p27\textsuperscript{Kip1} transcripts by real-time-PCR using specific primers, with β-actin as an internal control. As shown in Figure 4B, *S. clandestina* extract induced the upregulation of these three transcripts in a time-dependent manner.

The expression of p53, p21\textsuperscript{WAF1}, and p27\textsuperscript{Kip1} was examined by both western blot and real-time PCR analysis in MG-63 cells pretreated with p38 or JNK1/2 inhibitors before *S. clandestina* extract incubation. Both SB203580 and SP600125 inhibited the effects of the extract on increasing p53, p21\textsuperscript{WAF1}, and p27 protein expression (Fig. 4C). Furthermore, SP600125 also inhibited the levels of the three
Figure 4. *S. clandestina* extract induces p53. (A) Cell lysates were separated on an SDS gel and immunoblotting was performed using antibodies to the indicated proteins. Sequential incubation with anti-β-actin antibody confirmed equal protein loading. These results are representative of six independent experiments. (B) Real-time PCR; mRNA levels are presented as fold change values relative to control. Data are expressed as the mean ± SD of six different experiments. For p27^Kip1, p21^WAF1, and p53, P < 0.0001 by one-way ANOVA (n = 6); values with shared lowercase (for p27^Kip1), uppercase (for p21^WAF1), or bold (for p53) letters are not significantly different according to Bonferroni/Dunn post hoc tests. (C) Cells were pretreated with the JNK1/2 inhibitor SP600125 or the p38 inhibitor SB203580, and then incubated with *S. clandestina* extract. Immunoblotting was done against the indicated proteins after 48-h treatment. Sequential incubation with anti-β-actin antibody confirmed equal protein loading. (D) Cells were pretreated with the JNK1/2 inhibitor SP600125 or the p38 inhibitor SB203580 and then incubated with *S. clandestina* extract for different times (5 min for p53; 10 min for p21^WAF1; and 20 min for p27^Kip1). Real-time PCR was performed with specific primers for p53, p21^WAF1, p27^Kip1, and the housekeeping gene β-actin. mRNA levels are presented as fold change values relative to control. Data are expressed as the mean ± SD of six different experiments. For p27^Kip1, p21^WAF1, and p53, P < 0.0001 by one-way ANOVA (n = 6); values with shared lowercase (for p27^Kip1), uppercase (for p21^WAF1), or bold (for p53) letters are not significantly different according to Bonferroni/Dunn post hoc tests. (E) MG-63 cells were treated or not with *S. clandestina* extract for the indicated times or were pretreated with the JNK1/2 inhibitor SP600125 or the p38 inhibitor SB203580 and then incubated with *S. clandestina* extract for 20 minutes. Real-time PCR was performed with specific primers for mRNAs for BAX and the housekeeping gene β-actin. mRNA levels are presented as fold change values relative to control. Data are expressed as the mean ± SD of six different experiments. P < 0.0001 by one-way ANOVA (n = 6); values with shared letters are not significantly different according to Bonferroni/Dunn post hoc tests.

transcriptional regulation of proapoptotic members of the Bcl-2 family is involved in the initiation of apoptosis that is central to the activity of p53. The increased expression of the proapoptotic protein BAX, a Bcl-2 family member, was observed following *S. clandestina* extract incubation (Fig. 2B), suggesting that p53-mediated induction of Bcl-2 proapoptotic family members may contribute to *S. clandestina* extract–induced apoptosis. Consistently, the upregulation of BAX mRNA (Fig. 4E) and protein (Fig. 4C) was inhibited by both SP600125...
S. clandestina extract inhibits cell migration. Differential cell migration rate was examined using a wound closure assay. Cells were treated or not with 20 μg/mL of S. clandestina extract and monitored by microscopy at the indicated times. (A) Migration rate (average ± SD) and degree of wound closure were assessed by measuring the distance between wound edges at the indicated time intervals in at least eight randomly chosen regions of four different experiments normalized to 100% wound closure for control cells. \( P < 0.001 \) by one-way ANOVA \((n = 4)\); values with shared letters are not significantly different according to Bonferroni/Dunn post hoc test. (B) Cells were treated with the JNK1/2 inhibitor SP600125 or the p38 inhibitor SB203580 for 30 min before stimulation with or without S. clandestina extract. After 24 h, migration was evaluated. Results (means ± SD) from four independent experiments are presented. Migration was expressed as the percentage of unstimulated cells at 24 hours. \( P < 0.001 \) by one-way ANOVA \((n = 4)\); values with shared letters are not significantly different according to Bonferroni/Dunn post hoc tests.

and SB203580, indicating that S. clandestina extract increased p53 transcriptional activity in an MAPK-dependent manner.

S. clandestina extract inhibits the migration of osteosarcoma cells

Osteosarcoma cell migration is associated with its metastatic potential;\(^5,6\) thus, we used in vitro cell culture wound closure assays to further study the effects of S. clandestina extract. Wounds were created in confluent cell cultures, and repopulation of the wound was evaluated by measuring the width of the wound over time. The width of the wound was plotted as the percentage of the control in order to quantify the effect of 20 μg/mL S. clandestina extract on cell migration (this concentration was used since it did not change cell viability, as shown in Fig. 1A). The 20 μg/mL extract significantly decreased cell migration (Fig. 5A and B), apparently not due to cytotoxicity (Fig. 1A). This effect was blocked by the JNK1/2 inhibitor SP600125 (Fig. 5B).

S. clandestina extract induces downregulation of MMP2

MMPs play a central role in ECM degradation during osteosarcoma metastasis;\(^5\) thus, to check whether S. clandestina extract affects the activity of MMP2 and MMP9, the main MMPs expressed by MG-63 cells, we evaluated their activities in cell culture–conditioned medium using zymographic analysis. Treatment of cells with
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**Figure 6.** *S. clandestina* aqueous extract inhibits MMP2 mRNA and protein. (A) Real-time PCR for MMP2 and for the housekeeping gene β-actin. mRNA levels are presented as fold change values relative to control. Data were expressed as the mean ± SD of six different experiments. (B) Conditioned media and cell lysates were subjected to gelatin zymography and western blot analysis with anti-MMP antibodies. (C) Cells were pretreated for 30 min with 10 nM of the MMP inhibitor GM-6001 and then incubated with *S. clandestina* extract, in the presence or absence of 15 nM human recombinant active MMP2. After 24 h, migration was evaluated. Results (means ± SD) from four independent experiments are presented. Migration was expressed as the percentage of that of unstimulated cells. Statistically significant differences were observed between unstimulated cells and cells treated with GM-6001 (*P < 0.0001), and between cells treated with *S. clandestina* extract alone and cells treated with *S. clandestina* extract plus human recombinant active MMP2 ($P < 0.0001$) by Student’s *t*-test. (D) Western blotting of total lysates was performed with anti-E-cadherin (E-cadh) and anti-vimentin (Vimen) monoclonal antibodies. Sequential incubation with anti-β-actin antibody confirmed equal protein loading. The figures are representative of four independent experiments.

*S. clandestina* extract caused a time-dependent decrease in MMP2 activity but no change in MMP9 activity (Fig. 6B). Consistently, *S. clandestina* extract also induced downregulation of MMP2 mRNA and protein levels (Fig. 6A and B).

In order to understand whether decreased MMP2 was involved in the decrease in cell migration induced by the extract, we used the MMP inhibitor GM-6001 to inhibit MG-63 cell mobility. As shown in Figure 6C, preincubation of cells with 10 nM GM-6001 markedly decreased cell migration, mimicking the inhibitory effect of *S. clandestina* extract. In addition, the use of human recombinant active MMP2 at the physiological concentration of 15 nM$^{17}$ reversed the effect of *S. clandestina* extract on cell migration (Fig. 6C).

**S. clandestina** extract reverses epithelial-to-mesenchymal transition in osteosarcoma cells

Epithelial-to-mesenchymal transition (EMT) is a crucial step for the invasion and metastasis of osteosarcoma cells. It was found that *S. clandestina* extract inhibited EMT, and this is accompanied by the upregulation of the epithelial marker E-cadherin and downregulation of the protein levels of the mesenchymal marker vimentin, as shown by western blot (Fig. 6D).

**Effects of *S. clandestina* extract and cisplatin on MG-63 cells**

Cisplatin is a well-known chemotherapeutic agent, but tumor cell resistance to it often compromises its efficacy. We investigated the hypothesis that *S. clandestina* extract may be able to potentiate the cytotoxic activity of cisplatin. The viability of MG-63 cells treated with increasing concentrations of cisplatin (from 0.1 to 200 μM) or with the combination of both cisplatin and *S. clandestina* extract (60 μg/mL). That concentration of the extract was chosen on the basis of the MTT assay after 24-h incubation. Cisplatin caused dose-dependent cytotoxicity (Fig. 7A), and the simultaneous addition of 60 μg/mL *S. clandestina* extract enhanced its antiproliferative potency (Fig. 7A; $P < 0.001$). We studied the effect on the cell cycle of *S. clandestina* extract in combination with cisplatin by using flow cytometry of PI-stained cells after treatment for 24 hours. Addition of *S. clandestina* extract (60 μg/mL) caused a further significant increase in the percentage of apoptotic cells beyond that caused by cisplatin alone (Table 1).

Activation of caspase-9 and caspase-3 together with an increase in cleaved PARP was detected, thus revealing that *S. clandestina* extract could potentiate cisplatin-induced apoptosis through the activation of the intrinsic apoptosis pathway (Fig. 7B). Cells exposed to 60 μg/mL extract for 24 h showed a significant decrease in the protein level of Akt/PKB, a kinase promoting survival and growth in response to many extracellular signals. Such
downregulation of Akt/PKB was higher in cells treated with a combination of 60 μg/mL S. clandestina extract and 100 μM cisplatin compared with either agent alone (Fig. 7B).

**Discussion**

Osteosarcoma is the most common primary bone tumor in children and young adults. The currently used treatment for osteosarcoma is a combination of doxorubicin, cisplatin, and methotrexate before and after surgery, with an overall 5-year survival rate of less than 40%. This therapy helps in the survival of patients with nonmetastatic conditions, but, since osteosarcoma metastasizes to the lungs in 80% of cases, the survival rate is very low. In addition, chemotherapeutic drugs often cause various side effects. Therefore, the identification of efficient alternative drugs with less toxicity is crucial. The use of medicinal plants to improve health is an ancient practice and, more recently, many kinds of bioactive phytochemicals have been identified to exhibit antitumor and cytotoxic activities. The screening and the evaluation of the antitumor features of phytochemicals could lead to the development of new pharmacologically active molecules for cancer therapy. In recent years, the potential use of *Salvia* as a new anticancer agent has been recognized. The genus *Salvia*, consisting of nearly 1000 species, is among the important aromatic and medicinal plants worldwide. Among these species, we previously reported the phenolic characterization of *S. clandestina* L., a wild species present in the Salento (Apulia, Italy) area. The main phytochemical components of *S. clandestina* extract are presented in the table in Figure 8. In this *in vitro* study, we have demonstrated that an extract obtained from this plant has antitumor effects in osteosarcoma cells.

### Apoptotic death of cancer cells occurs in response to the administration of many natural compounds with antitumor activities, and we show here that *S. clandestina* extract also exerts its antitumor effects through apoptosis. Specifically, it induces the characteristic features of apoptosis, including activation of caspase-9, caspase-3, and caspase-7, and the cleavage of PARP. Caspase activation is regulated by various proteins, including the Bcl-2 family, and after *S. clandestina* extract treatment, Bcl-2 and BAX expression levels decreased and increased, respectively. It is noteworthy that many agents that inhibit cell cycle progression help to eliminate cancer cells via apoptotic or nonapoptotic mechanisms.

### Cell cycle checkpoints are the most important cell cycle regulators, which act when DNA damage or other cell damage has occurred. If the damage cannot be properly repaired, cells will either continue to divide with aberrant DNA or undergo apoptosis. The cyclin-dependent serine/threonine kinases and their regulatory cyclin subunits are responsible for governing the checkpoints in a cell division cycle. Normal progression through the G1 phase of the cell cycle is dependent upon the activities of the CDK4/cyclin D and CDK2/cyclin E complexes. In our study, we showed that *S. clandestina* extract decreased the expression of CDK4 and cyclin E and, conversely, increased p21<sup>WAF1/CIP1</sup> and p27<sup>Kip1</sup> expression, thus indicating that the activity of the CDK4/cyclin D and CDK2/cyclin E complexes was inhibited.

The p53 tumor suppressor is involved in cell cycle regulation, DNA repair, and apoptosis. We show here that *S. clandestina* extract induces apoptosis by upregulating p53 mRNA and protein. Many pathways mediate the apoptosis caused by p53, including BAX and Bcl-2 proteins. The BAX gene promoter contains several consensus sequences for p53

### Table 1. Cell cycle investigated by flow cytometry in propidium iodide

| S. clandestina (60 μg/mL) | Cisplatin (100 μM) | Sub G<sub>0</sub>/G<sub>1</sub> | G<sub>0</sub>/G<sub>1</sub> | S | G<sub>2</sub>/M |
|---------------------------|-------------------|-----------------|---------------|---|-----------|
| -                         | -                 | 1.39 ± 0.11     | 66.95 ± 2.57  | 14.49 ± 1.7 | 17.06 ± 0.61 |
| +                         | -                 | 24.97 ± 3.34*   | 52.75 ± 0.48* | 9.56 ± 0.21* | 12.71 ± 0.47* |
| -                         | +                 | 6.65 ± 0.57†    | 80.37 ± 2.23* | 7.67 ± 0.57† | 5.31 ± 0.37† |
| +                         | +                 | 37.48 ± 1.93†   | 45.36 ± 2.23† | 14.05 ± 0.57† | 3.11 ± 0.37† |

*P < 0.05 compared with untreated cells.
†P < 0.05 compared with cells treated with *S. clandestina* extract alone.
Indeed, polyphenols clandestina 0.0001 compared with cells BAXisible to fostercytochrome We show that transcripts. It is not fully known why standard deviation of five inde-

extract induces the activation of JNK1/2 In osteosarcoma, Akt/PKB hyperacti-

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Figure 7. S. clandestina aqueous extract sensitizes osteosarcoma cells to cisplatin. Increasing concentrations of cisplatin in the presence or absence of 60 μg/mL of S. clandestina extract were added to MG-63 cells. (A) Cell viability was assessed after 24 hours. Values are means ± standard deviation of five independent experiments with eight replicates in each and are presented as percent of control. *P < 0.0001 compared with cells treated with cisplatin alone, based on an unpaired Student's t-test. (B) Cells were treated or not with cisplatin, with S. clandestina extract, or with cisplatin plus S. clandestina extract. Cell lysates were then subjected to western blot analysis. Sequential incubation with anti-β-actin antibody confirmed equal protein loading. The figures are representative of four independent experiments.

bind.21 BAX is able to foster cytochrome c release in the cytosol, thus activating caspase-9 and leading to apoptosis. In addition, BAX may bind to Bcl-2, thus inhibiting its apoptosis suppression function. In MG-63 cells, p53 upregulates BAX and p21WAF1/CIP1 transcripts. It is not fully known why some cells undergo apoptosis in response to p53 activation, whereas others are simply arrested in the cell cycle. It may depend on p53 level, extracellular survival factors, or intrinsic activation of intracellular survival pathways. Multiple signaling pathways have been shown to influence p53 activation, including the MAPKs ERK1/2, JNK1/2, and p38.22 Much evidence shows that activation of p38 and JNK1/2 by a variety of cell stimuli, including natural products from Salvia miltiorrhiza Bunge, is correlated with apoptosis.23 We show that S. clandestina extract induces the activation of JNK1/2 and p38 and that their inhibition prevents apoptosis. Furthermore, p53 mRNA blockage after JNK1/2 inhibition suggests that the transcriptional induction of p53 signaling occurs downstream of JNK1/2. Consistent with this, various studies have demonstrated that JNK1/2 can, directly or indirectly, modulate p53 and its targets.24 Phosphorylation of p53 can regulate its activity by altering protein stabil-

In order to improve the overall effectiveness of cancer treatment and minimize toxicity and side effects, the use of natural compounds is promising because they have lower toxicities than conventional chemotherapy agents.27 In addition, it has been shown that by combining phytochemicals with chemotherapy, the effectiveness of the cancer treatment can be improved.27 Indeed, polyphenols are able to increase the sensitivity of different cancer cells to chemotherapy drugs.27 Accordingly, a significant inhibition of cell viability and an increase in apoptosis were observed in MG-63 cells treated with the combination of S. clandestina extract and cisplatin. The bioactive components modulate other cell signaling pathways in addition to MAPKs, among them the PI3K/Akt pathway, which is constitutively activated in various types of cancers, including osteosarcomas.28 Akt/protein kinase B (PKB) is a key signaling kinase downstream of PI3K that regulates cell survival; it inhibits apoptosis through its ability to phosphorylate and inactivate several components of the intrinsic apoptosis machinery, including BAX and caspase-9.29 In osteosarcoma, Akt/PKB hyperactivi-

treatment can be improved.

Tumor metastasis is a complex process that depends upon the ability of tumor cells to undergo
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Effects of *S. clandestina* L. extract on osteosarcoma

Figure 8. Anticancer effects and mechanisms of *S. clandestina* extract. The extract can directly inhibit cancer cell proliferation and kill cancer cells by induction of cell cycle arrest and apoptosis, respectively. It also overcomes drug resistance by increasing sensitivity to cisplatin, and, therefore, might be used in combination chemotherapy. In addition, *S. clandestina* extract decreases the metastatic potential of osteosarcoma cells.

The EMT, to dissociate from the primary tumor in order to enter the blood or lymphatic vessels, and to reattach at distant sites. Since invasion and migration contribute to the recurrence and metastasis of cancer cells, the suppression of migration might be important for successful cancer therapy. Some *in vivo* and *in vitro* studies have demonstrated that phytochemicals extracted from *Salvia miltiorrhiza* inhibit the invasion and migration of various cancer cells and also in osteosarcoma cells. Here, we show that the migration of MG-63 cells is decreased by low concentrations of *S. clandestina* extract through the down-regulation of MMP2 and the inhibition of the EMT. MMP-induced degradation of the ECM regulates the metastatic ability of cancer cells, and, among them, MMP2 plays an important role in osteosarcoma invasion and metastasis. In addition, the expression of MMP2 is associated with pulmonary metastasis and related to the prognosis of osteosarcoma. Consequently, MMP2 is considered a drug target in cancer treatment, inasmuch as its inhibition could limit cancer progression and metastasis.

EMT is a critical step in cancer metastasis. It consists of epithelial cells temporarily losing their epithelial characteristics and acquiring the characteristics of mesenchymal cells. The regulation of the activities of the inducers of EMT is an obvious strategy to inhibit osteosarcoma progression. Recent evidence indicates that natural plant compounds can modulate pathological EMT or its deleterious effects through acting on different cellular signal transduction pathways. In the present paper, we show that *S. clandestina* extract can effectively reverse the EMT, thus inhibiting osteosarcoma metastasis, by regulating the expression of E-cadherin and vimentin.

The effects of the main components of *S. clandestina* extracts were also evaluated (Fig. S3, online).
only) on osteosarcoma cells, but the most important anticancer effects are due to a synergistic action of the aqueous extract components of S. clandestina, as demonstrated also for S. officinalis extracts. These data support the administration of the extract as it is, as that would be less expensive and simpler compared with the isolation of single components.

The results presented here demonstrate that S. clandestina extract can counteract osteosarcoma (MG-63 cells) by (1) apoptosis induction, (2) inhibition of metastatic potential, and (3) potentiation of cisplatin effects (Fig. 8). Although further investigation is required, including in vivo studies, our study supports our hypothesis that S. clandestina extract may be a novel and effective agent for the treatment of osteosarcoma. An appropriate method of administration of S. clandestina L. extract remains to be elucidated in order to overcome the limitation of the poor bioavailability of polyphenol compounds, as well as to develop appropriate formulations in order to use S. clandestina in human therapy.

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Author contributions
A.M., S.M., and E.N. presented the concept. E.S. and C.N. performed methodology. E.S. carried out the investigation. A.M., L.D.B., and C.N. wrote the original draft. S.M. and L.D.B. helped in writing—reviewing and editing. S.M. and E.N. were involved in supervision. All authors read and agreed to the published version of the manuscript.

Supporting information
Additional supporting information may be found in the online version of this article.

Figure S1. The total ion current chromatogram of HPLC/MS-TOF of the S. clandestina aqueous extract.

Figure S2. S. clandestina extract induces apoptosis in MG-63 cells.

Figure S3. Effects of phenolic compounds of S. clandestina aqueous extract on MG-63 cell viability.

Table S1. HPLC/ESI-TOF-MS accurate masses of [M–H]− ions of constituents of S. clandestina aqueous extract and tentative identification.

Table S2. Total phenolic content (TPC) and phenolic compounds quantification of the S. clandestina aqueous extract.

Competing interests
The authors declare no competing interests.

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