Original Article

Reactive oxygen species (ROS) are a crucial factor in the anticancer activity of *Oliveria decumbens* extract against the A431 human skin cell line

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ABSTRACT

Globally, skin cancer is a main public health challenge whose incidence is continuously increasing. Given the limitations of conventional t herapies, new research and novel therapies may be promising for reducing skin cancer morbidity and mortality. Phytochemicals are attractive resources for new therapy design in cancer research due to their cost-effectiveness and lower side effects. In the present study, the anti-cancer activity of *Oliveria decumbens* (*O.decumbens*) extract was investigated on the human skin cancer A431 cell line A431. The aqueous extract of the O.*decumbens* plant was prepared using the traditional method. Then IC50 was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay under different concentrations of *O. decumbens*. Cell apoptosis was investigated by Annexin V-FITC/Propidium Iodide (PI) and flow cytometry. Cell cycle was investigated by PI staining and flow cytometry. Reactive oxygen species (ROS) production was analyzed by DCFH-DA (2', 7' -dichlorofluorescein-diacetate) staining and flowcytometry.IC50 for cell viability was determined 475 µg/ml. Cell cycle analyses showed G1 arrest in treated cells compared to control cell. Results also confirmed significant increase of apoptotic cells (8.2±%1, P<0.05) under IC50 concentration of the extract in comparison to the control group (2.5±0.99%). A significant increase in ROS level was observed in *O.ecumbens* treated cells compared to control cells (738± 170 vs 316±55 in the control group, P<0.05.).Overall, the present results indicate that *O.decumbens* extract can inhibit skin cancer cell proliferation via inhibition of cell cycle and apoptosis. It seems that ROS production plays a critical role in the anticancer effect of *O. decumbens* extract. Therefore, its potential option for future treatment of skin cancer should be considered.

Keywords: *Oliveriadecumbens*, Aqueous extract, ROS, Skin cancer, A431cell line

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1. Introduction

Skin cancer is the deadliest type of malignant cancer which is considered as major causes of cancer- related mortality worldwide (1,2). Skin cancer is generally classified into two major types including melanoma arising from melanocytes and non-melanoma skin cancer(NMSC) with epidermal derived cells origin (3). Reported global incidence of skin cancer is an underestimate due to lack of diagnostic criteria and underreporting(3). However, epidemiologic evidence suggests an increasing burden of both NMSC and melanoma (3,4). Despite the lower incidence of melanoma (1% of all skin cancers), its mortality rate is much higher than that of NMSC. Generally, non-melanoma skin cancers (NMSCs) are divided into two main subtypes; squamous cell carcinoma (SCC), and basal cell carcinoma (BCC) (5). Surgical excision, chemotherapy, photothermal therapy (PTT), immunotherapy, and biotherapy are the main methods of skin cancer treatment, which have been limited due to high toxicity, drug resistance, and poor selectivity(6). Therefore, the search for new approaches with high efficacy and low toxicity has been considered as a necessity for skin cancer treatment (6). In recent years, medicinal plants or drugs with natural resources have received more attention for treatment of a variety of cancers due to their cost-effectiveness and lower side effects (7). The protective effects of phytochemicals on skin cancer animal and cell lines models have been reported in several studies, and it seems that therapeutic potentials of the reported phytochemicals can be promising (7-10). *Oliveria decumbens* Vent. (*O. decumbent*), also known as Mashkourak or Den, is a single aromatic species in Iran belonging to the Apiaceae/Umbelliferae family,which wildly grows in the western and southern parts of Iran, especially in the western foothills of the Zagros Mountain range (11,12). Flavonoids such as kaempferol derivatives, monoterpene compounds such as thymol and carvacrol, and phenylpropanoids such as myristicin have been reported as as the major bioactive ingredients of *O. decumbens*, which is traditionally consumed for the treatment of human health problems such as fever, indigestion, abdominal pain, and diarrhea (11-13). Studies have been also shown that *O. decumbens* could be a multi-bioactive medicinal plant in other diseases such as cancer. Khodavirdipour et al. recently showed that ethanolic extract of *O.decumbens* can promote apoptosis and inhibit metastatic behavior in HT-29 colorectal cancer line (14). Cytotoxic effects of *O. decumbens* essential oil (OEO) on MCF-7 breast cancer cell line have also been reported (15). The anticancer effects of *O.decumbens* have been less investigated. In this study, for the first time, anti-skin cancer effects of *O.decumbens* on A431 cancer cell line as epidermoid squamous cell carcinoma were investigated.

2. Materials and Methods

2.1. Plant extract

For plant material, *O. decumbens* sample was purchased from the herbal shops (Attari). A voucher specimen was identified by a botanist. The plant materials were washed, dried in the shade, and crushed for extraction. Powdered plant materials (500 g) were successively extracted in a Soxhlet's apparatus.

2.2. Cell Culture

Human epidermoid squamous cell carcinoma, A431 cell line, was obtained from the National Cell Bank of Iran and grown in RPMI 1640 medium (Gibco RL, Grand Island, NY) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), 1% glutamine and 1% nonessential amino acids in a 5% humidified CO_2 atmosphere.

2.3. Cell viability by flow cytometry

A431 cells (1×10^5) were seeded into each well of a 12-well culture plate and incubated overnight at 37°C. Cells were treated with 1000 µg/ml of the plant extract for 48 h. The next day, cells were harvested and stained with propidium iodide (or PI) (50 µg/ml PI and 100 µg/ml RNase A in PBS), and PI fluorescence was determined using a FACScan instrument.

2.4. Cell viability by MTT assay

Cells were plated in 96-well plates (5×10^3 cells/well) and incubated overnight at 37°C. The next day, cells were treated with different concentrations of the extract (0, 15, 30, 75, 250,500, 1000, 1500 µg/ml) and incubated at 37°C for 48 hours. For viability assay, 10 µl MTT (stock solution (5 mg/ml) was added, and the plates were incubated for another 4 hours at 37°C. Formazan crystals were dissolved with 150 µl DMSO (150 µl/well). Absorbance was measured using a microplate reader (Thermo Fisher Scientific, Inc.) at 490 nm.

2.5. Apoptosis Assay

A431 cells (1×10^5) were seeded into each well of a 12-well culture plate and incubated overnight at 37°C. Cells were treated with IC50 concentrations of the plant extract for 48 h. After 48 h, treated cells were detached and apoptosis was detected using the Annexin V-FITC/Propidium Iodide (PI) apoptosis detection Kit (MiltenyiBiotec, Bergisch Gladbach, Germany). The percentage of apoptotic cells was calculated by flow cytometry (BD FACSCalibur flow cytometer, USA).

2.6. Cell cycle assay

A431 cells (1×10^5) were seeded into each well of a 12-well culture plate and incubated at 37°C overnight. Cells were treated with IC50 concentrations of the plant extract for 48 hours after which , treated cells were detached and fixed with ice-cold ethanol (70% w/w) at -20°C for 2 hours. After PBS washing, cells were stained with PI (50 µg/ml PI and 100 µg/ml RNase A in PBS) for 30 min at 37°C. The calculated percentage of cells in different phases of the cell cycle was analyzed by flow cytometry (BD FACS Calibur flow cytometer, USA).

2.7. ROS detection assay

A431 cells (1×10^5) were seeded into each well of a 12-well culture plate and incubated overnight at 37°C. Cells were treated with IC50 concentrations of the plant extract for 48 hours. Cells were then detached and treated with 10 µM DCFH-DA (2', 7' -dichlorofluoresceindiacetate) for 30

minutes. Then treated cells were then washed twice with PBS to remove the extracellular compound, and DCFH-DA fluorescence was detected by flow cytometry (BD FACSCalibur, USA).

2.8. Statistical Analysis

SPSS 27.0 software (SPSS, Inc., Chicago, IL, USA) was used to analyze the data which h are presented as the mean ± SD. Unpaired Student's t-test was used to compare control and treatment groups. $p<0.05$ was considered statistically significant.

3. Results

3.1. *decumbens* extract reduced A431 cell viability

The sensitivity of A431 cells to *O. decumbens* extract was first evaluated by PI staining.48 hours treatment of *O. decumbens* (1000 µg/ml) extract can inhibit the cell viability by more than 80% (Figure 1A). To evaluate the of 50% growth inhibition (IC_{50}), MTT assay was performed, and data analyses showed that O. *decumbens* treatment for 48 hours reduced the cell growth of A431 cells in a dose-dependent manner. The concentration to achieve 50% inhibition (IC_{50}) was 475 µg/ml (Figure 1B).

3.2. *decumbens* extract induced G1 arrest and apoptosis in A431 cells

Cell cycle arrest and apoptosis are two major causes of cell growth inhibition. The effect of *O. decumbens* extract on A431 cell cycle distribution was investigated under IC50 concentration of the extract for 48h by PI staining and flow cytometry. The results showed that G1proportion significantly increased in treated group (55.6± 3.9%) compared to control group (36.5±3.2%) ($P<0.01$). Treatment with the extract also showed a significant decrease in S phase (30.7±2.1 vs 38.1±2.8 in control group $P<0.05$).No significant change was observed in G2 phase (Figure 2A, B). The effect of *O. decumbens* extract on A431 cell apoptosis was evaluated by Annexin V-FITC/PI staining and flow cytometry. As shown in Figure 3, treatment with the extract significantly decreased viable cells (71.1±4.2% vs 92±4.8% in the control group, $P<0.05$). The results also confirmed a significant increase of apoptotic cells (8.2±%1, $P<0.05$) under IC50 concentration of the extract compared to control group (2.5±0.99%). Necrotic cells also significantly increased in treatment group in comparison with control group (18.5±5.5% vs 3.7±2.1%, $P<0.05$).

3.3. *decumbens* extract increased ROS generation of A431 cells

The production of intracellular ROS was investigated using DCFH-DA staining by flow cytometry. As shown in Figure 4, the level of ROS in *O.* decumbens treated cells was significantly higher than in control cells (738±170% vs 316±55% in control group, $P<0.05$).

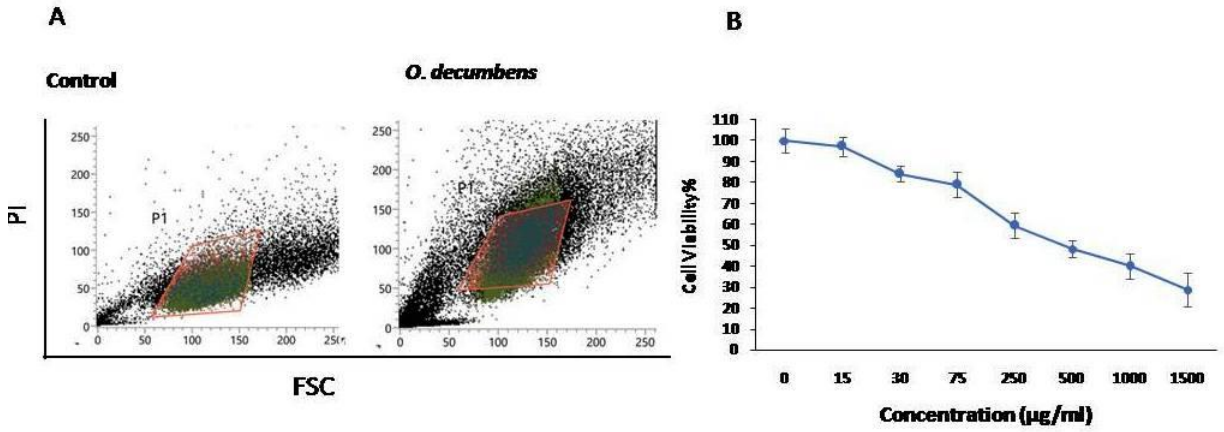

Figure 1.A) Cell viability was assessed by PI staining and flow cytometry after 48 hours of treatment with 1000µg/ml. B), MTT assay was performed to determine IC50 after 48 hours of extract treatment.

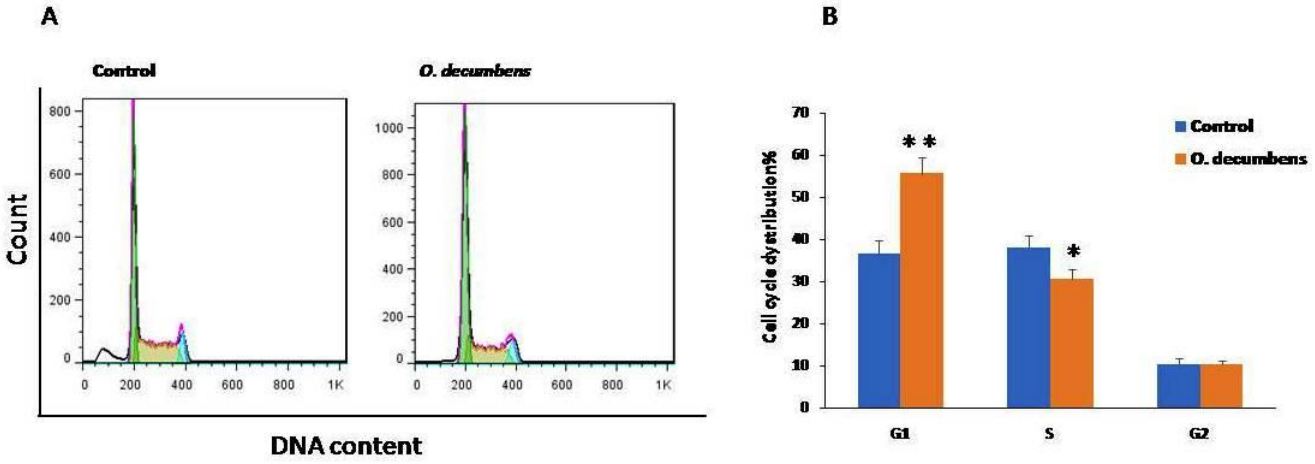

Figure 2A,B).Apoptotic cells were assessed by Annexin V-FITC/Propidium iodide (PI) staining using flow cytometry. Dot plots represent early apoptotic cells in the lower right quadrant, late apoptotic cells in the upper right quadrant, and viable cells in the lower left quadrant (LL). The Percentage of apoptotic cells from three biological replicates is expressed as the mean in the bar graph. Data are presented as mean±SD. *p<0.05.

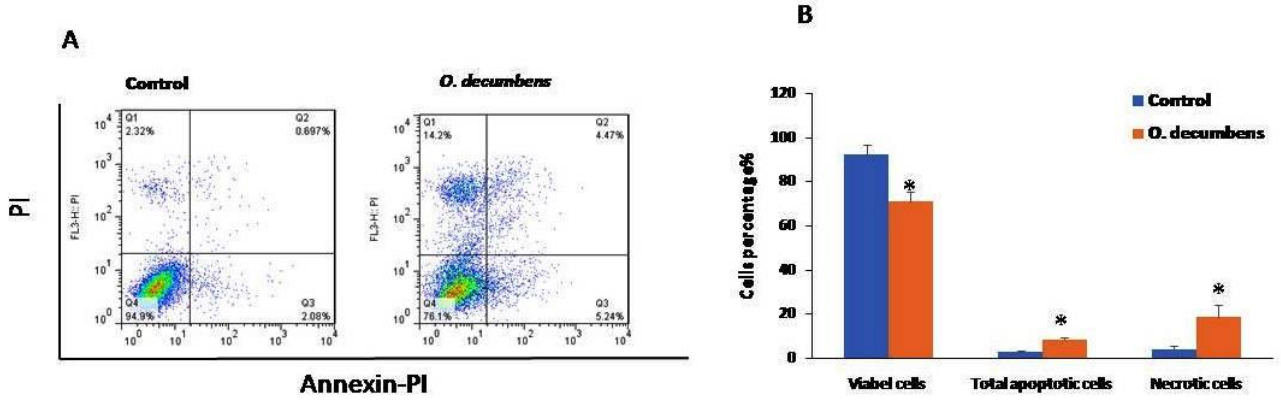

Figure 3A,B):After 48 hours of treatment, cells were labeled with PI and analyzed by DNA flow cytometry. The data show the percentage of cells in each phase of the cell cycle. G2/S and G1.The Percentage of apoptotic cells of three biological replicates is expressed as the mean in the bar graph. All experiments were performed in triplicate. Data are presented as mean±SD; **p<0.01,*p<0.05

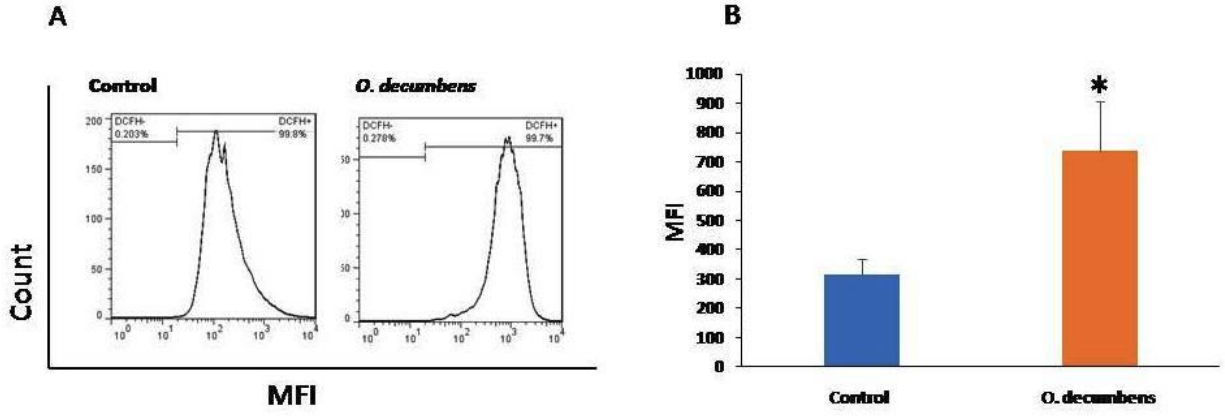

Figure4A, B): Detection of ROS in treated and control cells by flow cytometry using a DCFH-DA assay. A) Plots of the fluorescence intensities of the DCF dye. Cells are exposed to 900 µM H_2O_2 for 60 minutes before their incubation with the DCFH-DA probe for flow cytometry measurements. B) Quantitative histograms of the mean fluorescence intensity of the DCFH-DA probe with regard to the background level of untreated cells. Data are expressed as mean ± SD of three independent experiments (*p < 0.05)

4. Discussion

The incidence of skin cancer as a serious health problem is increasing especially in developed countries with higher prevalence of the cancer risk factors (4,16). Surgical excision, radio therapy, chemotherapy, or cryosurgery are the most widely used methods for the treatment of skin cancer (1,6,17). Despite all the drawbacks of current therapies, herbal remedies are attracting the attention of researchers to develop more selective and effective anticancer drugs with lower side effects (18-20). Studies have been shown that anticancer effects of natural compounds are as a result of potentiating apoptosis, inhibiting cell proliferation and inhibiting metastasis (18). *O. decumbens* Vent, endemic plant of Iran, the various pharmacological activities including antiviral, antidiabetic, and antifungal have been reported in different studies (12). Although anticancer effect of *O.decumbens* has been less studied, each of chemical compounds of *O.decumbens* such as *O.decumbens* such as thymol, carvacrol and gamma-terpinene, in individually has anticancer, anti-proliferative, and pro-apoptotic properties (21, 22). Khodavirdipour et al. recently showed that ethanolic extract of *O. decumbens* can promote apoptosis in HT-29 colorectal cancer line (14). The data in this study also confirmed the anti-apoptotic effect of aqueous extract of *O.decumbens* against A431 skin cancer cells. The induction of apoptosis in HepG2 hepatocellular carcinoma by carvacrol, a major medicinal compound in the Apiaceous family has been also been reported. Carvacrol can activate the mitochondrial pathway and mitogen-activated protein kinase, leading to induction of apoptosis (23). Based on our results, it seems that theincrease of ROS production is the main mechanism of apoptotic induction of *O. decumbens* aqueous extract. Jamali and et al. showed that *Oliveria decumbens* vent essential oil (OEO) can inhibit the proliferation of mouse models and human breast cancer cell lines. Their results showed that the increased level of ROS generation led to the disruption mitochondrial membrane potential ($\Delta\Psi m$), caspase 3 activation and apoptosis (24). Reactive oxygen species (ROS) are by-products of many cellular processes and act as a double-edged sword (25). Maintenance of ROS to a certain level is necessary for cellular proliferation and survival. An imbalance between oxidants and antioxidants leads to higher levels of ROS, which result in damage to biomolecules, cell membranes and organelles, resulting in cell death (25). Oxidative stress activates the DNA damage response (DDR) and the stressed cancer cell moves towards cell cycle arrest. It has been confirmed that manipulation of ROS levels in cancer cells can be promising and useful for the development of cancer therapeutic strategies (26, 27). Cell cycle analysis also confirmed G1 arrest under treatment of *O. decumbens* extract in A431 skin cancer cells. S-phase cell cycle arrest with OEO treatment has been reported elsewhere (24). According to GC/MS analysis, thymol is one of the main component of *O. decumbens* extract that causes DNA damage through ROS induction (28). Kang and colleagues also showed that thymol induced G2/M phase cell cycle arrest in gastric carcinoma cells (29). According to our data in this study which confirmed the previous study, *O. decumbens* extract has enough potential with many benefits in skin cancer therapy. However, more studies are needed to understand the molecular and biochemical mechanism by which limits cancer cell growth.

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Authors' Contribution

Study concept and design: F. SH, Z. J.
Data Acquisition: M. D., Z.J.
Analysis and interpretation of data: Z. J.
Drafting of the manuscript: M.D.
Critical revision of the manuscript: Z. J., F. SH.
Statistical analysis: M. D. and Z.H.

Ethics

This research was conducted with the approval of North Tehran Branch, Islamic Azad University, and Tehran, Iran (Ethical code: IR.IAU.TNB.REC.1401.064).

Conflict of Interest

The authors declare that they have no conflict of interests.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

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