

ANTICANCER ACTIVITY OF *Ornithogalum umbellatum* L. IN COLORECTAL CARCINOMA CELLS MEDIATED BY CHANGES IN CELL REDOX STATUS

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ABSTRACT. Colorectal cancer is the third most common malignancy in the world. This paper aims to examine the anticancer activity of the plant *Ornithogalum umbellatum* (Linnaeus, 1758), through its influence on the cells' redox status. Experimental research was performed on human colorectal cancer cells, HCT-116, which were treated with an aqueous extract of the plant. After a 24-hour treatment, the inhibitory concentration was determined using a standard cytotoxic test. The inhibitory concentration was used to examine the effects of the treatment on the concentrations of intracellular components of the redox system. The obtained results show that the concentration of reactive oxygen species, nitric oxide, and reduced glutathione decreased, while the concentration of oxidized glutathione increased. This research concludes that the aqueous extract of the plant *Ornithogalum umbellatum* has an anticancer effect on the human colorectal cancer cell line HCT-116, mediated by changes in redox status.

Keywords: oxidative stress, nitric oxide, glutathione, cancer cell proliferation

INTRODUCTION

Colorectal cancer (CRC), a malignancy of the colon and rectum, has been steadily increasing over the past decades. In 2022, more than 1.9 million new cases were diagnosed, and approximately 850,000 patients died from this disease (CHHIKARA and PARANG, 2022). CRC currently ranks as the third most common malignancy in men and the second in women, representing the second leading cause of cancer-related mortality worldwide (BRAY *et al.*, 2018; SUNG *et al.*, 2021). Notably, more than 65% of patients are diagnosed in high-income countries, highlighting the strong association between CRC incidence and modern lifestyle factors (<https://www.iarc.who.int/featured-news/colorectal-cancer-awareness-month-2022/>).

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Despite the remarkable advances in cancer therapeutic approaches, including surgery, radiotherapy, and chemotherapy, no optimal and universally effective therapy has yet been achieved. Conventional treatments are often limited by severe side effects and high recurrence rates, while the emergence of resistance to anticancer drugs remains a major barrier to long-term success (HOLOHAN *et al.*, 2013; DAS *et al.*, 2020). Consequently, over the past years, research has increasingly focused on antioxidant natural products, exploring them as both potential anticancer agents and as supplement strategies to reduce the adverse effects of conventional therapies (SAMMAR *et al.*, 2019).

Reactive oxygen species (ROS) are free radicals that are highly reactive molecules due to their unpaired electrons. ROS, including superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^{\cdot}$), are mainly produced under physiological conditions through mitochondrial respiratory processes by an incomplete reduction of molecular oxygen (DROGE, 2002). Physiological concentrations of ROS serve as signaling molecules, but when produced in excessive amounts, they can oxidize biomolecules within the cell, including nucleic acids, proteins, lipids, and carbohydrates, leading to structural and functional cell damage (HALLIWEL and GUTTERIDGE, 2015). In cancer, ROS regulate pathways that promote malignant transformation, cell survival, proliferation, invasion, and migration (PRASAD *et al.*, 2017). To sustain this oncogenic phenotype, cancer cells deliberately maintain elevated ROS levels (TRACHOOTHAM *et al.*, 2009; PRASAD *et al.*, 2017). In contrast, excessive ROS accumulation can also trigger apoptosis or ferroptosis signaling pathways in cancer cells (DI MEO and VENDITTI, 2020; VALKO *et al.*, 2006). Another important effect of ROS accumulation, particularly $O_2^{\cdot-}$, is the reduction of nitric oxide (NO). NO is a short-lived signaling molecule synthesized from L-arginine by nitric oxide synthase (constitutive – cNOS and inducible – iNOS) (MONCADA and HIGGS, 1993). $O_2^{\cdot-}$ rapidly reacts with NO to form highly toxic peroxynitrite ($ONOO^-$) (WINK and MITCHELL, 1998).

Given that ROS are essential for maintaining the oncogenic phenotype, redox homeostasis is of critical importance for cancer cell survival. Redox homeostasis refers to processes maintaining balance between ROS production and the activity of the antioxidant defense system, which includes both nonenzymatic and enzymatic components (HALLIWEL and GUTTERIDGE, 2015). In contrast to normal cells, cancer cells generally exhibit lower enzymatic antioxidant protection, which allows them to sustain elevated ROS levels that support cancer progression (TRACHOOTHAM *et al.*, 2009; JELIC *et al.*, 2021). In addition to antioxidants naturally present in human cells, many antioxidants are derived from other organisms, particularly plants. Plants are rich sources of antioxidant compounds, which they continuously produce as secondary metabolites (PETRUK *et al.*, 2018; EL-HARAKEH *et al.*, 2021). These metabolites include diverse classes such as flavonoids and polyphenols, which can be extracted from various plant tissues and have the potential to modulate redox homeostasis and support anticancer therapy (GUPTA *et al.*, 2020).

The genus *Ornithogalum* L., which belongs to the family Hyacinthaceae, is broadly distributed throughout Europe, Africa, and Asia (MARTINEZ-AZORIN *et al.*, 2010). Among its species is *Ornithogalum umbellatum* (Linnaeus, 1758), a herbaceous plant that commonly inhabits crops, grasslands, gardens, and meadows (COROMINAS *et al.*, 2017). Its fresh leaves and bulbs are edible, and both the bulbs and aerial parts of the plant are particularly rich in antioxidants, as demonstrated by the high antioxidant capacity measured in this plant (ADAWIA *et al.*, 2016; AYDIN, 2020; KAVANOZ, 2022). In addition, species of the genus *Ornithogalum* have been used in traditional medicine, especially in the treatment of cancer and inflammatory conditions (ANDRIĆ, 2015).

This study aims to investigate the anticancer activity of the aqueous extract of the plant *Ornithogalum umbellatum*, through its effect on the redox status of human colorectal cancer cell line HCT-116.

MATERIAL AND METHODS

The results presented in this paper were obtained during experimental research conducted in the Laboratory for Biomedical Research, Department of Biomedical Sciences at the State University of Novi Pazar. All chemicals used during this experiment were of standard pro analysis of degree of purity and were procured from the manufacturer SIGMA (St. Luis, USA). DMEM (Dulbecco's Modified Eagle Medium), FBS (fetal bovine serum), and antibiotics/antifungals were procured from QIAGEN (Hilden, Germany).

Preparation of *Ornithogalum umbellatum* for the experiment.

For this procedure, 50 g of chopped and dry plant material was transferred to an Erlenmeyer. 250 ml of water was added to the plant material. The plant material was macerated three times at room temperature using fresh solvent every 24 hours. The obtained filtrate was evaporated to dryness in a vacuum evaporator for three days. The plant extract was then transferred into sterile bottles and stored in the refrigerator at +4 °C.

Cell cultures and treatment

Experimental research was performed on the human colorectal cancer cell line HCT-116, procured from the Curie Institute / CNRS UMR 144. Cells were grown in complete medium: DMEM + 10% FBS + antibiotic/antifungal, at a temperature of 37 °C, in the presence of 5% CO₂ and in a humid atmosphere. At a cell confluence of about 90%, separation of cells adherent to the flask substrate was performed.

The cells were treated with different concentrations (100 µg/mL; 50 µg/mL; 25 µg/mL; 12.5 µg/mL and 6.25 µg/mL) of the aqueous extract of plant *O. umbellatum*. After the treatment, the cells were incubated for 24 hours at a temperature of 37 °C in a humid atmosphere in the presence of 5% CO₂. Then, cell viability and concentrations of oxidation/antioxidation system parameters were determined.

MTT – cell viability assay

The MTT assay is used to determine the viability of cells treated with different concentrations of the aqueous extract of plant *O. umbellatum*. Mitochondrial dehydrogenase from living cells reacts with the yellow tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), resulting in a soluble purple formazan, which is what the MTT test is based on (MOSMANN, 1983; KISS *et al.*, 2021).

For the MTT assay, a 96-well plate was seeded with cell suspension. The plate was incubated for 24 hours at a temperature of 37 °C in a humid atmosphere in the presence of 5% CO₂. After 24 hours, the cells were treated with different concentrations of the aqueous extract of the plant *O. umbellatum* (100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, 6.25 µg/mL). After a 24-hour incubation of control (untreated) and treated cells, the medium was aspirated, then MTT solution at a concentration of 5 µg/mL was added to each well. The plates were incubated for 3 hours at a temperature of 37 °C in a humid atmosphere in the presence of 5% CO₂. After incubation, the medium was aspirated, and then DMSO was added to each well. The absorbance was read at 550 nm on an ELISA reader. Based on the absorbance readings for control cells (untreated cells, 100% viability) and cells treated with different concentrations of *O. umbellatum* aqueous extract, the percentage of viable cells was calculated.

Cell preparation to determine concentrations of oxidation/antioxidation system parameters

The inhibitory concentration (IC_{50}) of the aqueous extract of the plant *O. umbellatum* was 29 $\mu\text{g/mL}$, which was determined based on the results of the MTT test and using the *CalcuSyn* program. This effective concentration was used to examine the effects of the aqueous extract of the plant *O. umbellatum* on the parameters of the oxidation/antioxidation system in HCT-116 colorectal cancer cells.

Cell cultures in two flasks, at a confluency of 80%, were treated with an aqueous extract of the plant *O. umbellatum* at a concentration of 29 $\mu\text{g/mL}$. The concentrations of hydrogen peroxide and nitrogen monoxide were determined in the cells from the first flask. The concentrations of reduced and oxidized glutathione were determined in the cells from the second flask. While the cells from the third and fourth flasks served as controls for determining the concentrations of the parameters of the oxidation/antioxidation system. After the treatment, the cells were incubated for 24 hours at a temperature of 37 $^{\circ}\text{C}$ in a humid atmosphere in the presence of 5% CO_2 . Then the medium was aspirated, and 2 mL of trypsin was added per flask. The detached cells were transferred to a clean test tube and counted under a microscope. After counting, the cells were centrifuged for 5 minutes at 4000 rpm. The supernatant was discarded, and the cells were washed three times in PBS, so the resuspended cells were ready for further analysis.

Determination of $\text{O}_2^{\cdot-}$, H_2O_2 , NO concentration

Nitroblue-tetrazolium assay is used for the determination of the concentration of $\text{O}_2^{\cdot-}$. Nitroblue-tetrazolium is reduced to nitroblue-formazan in the presence of $\text{O}_2^{\cdot-}$ (AUCLAIR and VOISIN, 1985). The resuspended cells were added to a plate with 96 wells, and the absorbance was read at 630 nm on an ELISA reader. The concentration of formazan indicates the concentration of $\text{O}_2^{\cdot-}$.

To determine the concentrations of hydrogen peroxide and nitrogen monoxide, cell contents were extracted by disrupting cell membrane stability by the freeze-thaw method. After washing, the cells were resuspended in PBS, and their number was adjusted to $10^6/\text{mL}$. Then the cells were frozen at -20 $^{\circ}\text{C}$ and thawed at 37 $^{\circ}\text{C}$ in three cycles lasting 15 minutes each. After lysis, the cells were centrifuged for 20 minutes at 4500 rpm. The supernatant was separated, and the concentrations of hydrogen peroxide and nitrogen monoxide were determined in it. The same extraction procedure was performed with control cells. The determination of the concentration of H_2O_2 relies on the reaction mixture, which contains phenol red and peroxidase. Hydrogen peroxide oxidizes phenol red in the presence of peroxidase as a catalyst (PICK and KEISARI, 1980). Color intensity (absorbance) is detected on an ELISA reader at a wavelength of 600 nm.

Sulfanilamic acid and N-1-naphthylethylenediamine react with NO_2^- to give a stable purple nitrogen compound whose color intensity is directly proportional to the nitrite concentration. This test is based on the diazotization reaction known as the Griess method (BEUTLER, 1984). The absorbance is read on an ELISA reader at a wavelength of 550 nm.

Determination of reduced (GSH) and oxidized glutathione (GSSG) concentration

To determine the concentration of reduced and oxidized glutathione, after washing in PBS, the cells were adjusted to a number of $10^6/\text{mL}$, centrifuged for 5 minutes at 4000 revolutions/minute, and then resuspended in 1 mL of 2.25% sulfosalicylic acid (+4 $^{\circ}\text{C}$). Cell membrane lysis was performed by the freeze-thaw method. After lysis, the samples were centrifuged for 20 minutes at 4500 rpm. The supernatant was separated, and the concentrations of oxidized and reduced glutathione were determined in it. The same extraction procedure was performed with control cells. The concentration of GSH determination test is based on the oxidation of GSH by 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB) (BEUTLER, 1975a). GSSG is reduced to GSH in the presence of the enzyme

glutathione reductase and NADPH as a cofactor, which is what this method is based on (BEUTLER, 1975b).

Statistical analysis

The data obtained were analyzed using the SPSS 13.0 software package for Windows. Results are presented as arithmetic mean $\pm$ standard error (S.E.M). Data between the groups studied were analyzed using an independent t-test. The criterion for statistical significance is $p < 0.05$. The results are presented graphically.

RESULTS AND DISCUSSION

The anticancer activity of *Ornithogalum umbellatum* has been documented in the literature, with reports demonstrating cytotoxic effects against lung, endometrial, and colon cancer cell lines (ALTIPARMAK *et al.*, 2019; PHING *et al.*, 2021). In addition, the plant's high content of phenols and flavonoids has been associated with remarkable antioxidant potential (AKGEYIK *et al.*, 2023). This suggests a possible connection between its cytotoxic effect and disruption of cellular redox status. Despite these promising findings, no study to date has specifically investigated the effects of *O. umbellatum* on components of cellular redox status. The present work opens a novel perspective by linking its anticancer activity to the disruption of cellular redox status.

In our study, 24-hour treatment with *O. umbellatum* significantly reduced the viability of CRC cells. The extract exhibited a concentration-dependent cytotoxic effect, with an IC_{50} value of 29 $\mu\text{g/mL}$ (Tab. 1). Moreover, the treatment with a determined IC_{50} induced a disruption of redox status, which reflected in altered ROS and NO levels and glutathione balance, which contribute to oxidative stress and ultimately lead to cell death.

Table 1. Effects of different concentrations of the aqueous extract of the plant *Ornithogalum umbellatum* (Linnaeus, 1758) on the viability of human colorectal cancer cell line HCT-116. Data represent mean $\pm$ S.E.M; * $p \leq 0.05$ treatment versus control.

Treatment ($\mu\text{g/mL}$)	6.25	12.5	25	50	100
Cell viability (%)	33.67 $\pm$ 2.53	41.85 $\pm$ 3.39	51.58 $\pm$ 4.29	53.56 $\pm$ 4.62	62.51 $\pm$ 5.43
IC_{50}	29 $\mu\text{g/mL}$				

Oxidative stress is a condition in the cell caused by an excess production of free radicals or by reduced capacity of the antioxidant protection system, leading to various pathophysiological changes in cells (ALKADI, 2020). In cancer cells, ROS and NO act as secondary messengers that maintain oncogenic signaling and promote their progression (VALKO *et al.*, 2006). Because of this, cancer cells are more resistant to oxidative stress compared with normal cells (PRASAD *et al.*, 2017). By maintaining elevated ROS levels, cancer cells promote proliferation, invasiveness, and metastatic potential through the modulation of gene expression and the activation of mitogenic transcription factors (REUTER *et al.*, 2010; MOLONEY and COTTER, 2018; FIOCCHETTI *et al.*, 2019). Conversely, suboptimal ROS levels can suppress these processes (MOLONEY and COTTER, 2018).

In colorectal cancer cells, the intracellular levels of ROS and NO are tightly regulated to sustain their viability. The results obtained in this study show that 24-hour treatment with

aqueous extract of *O. umbellatum* induces a decrease in O_2^- , H_2O_2 , and NO levels in colorectal cancer cells (Fig. 1).

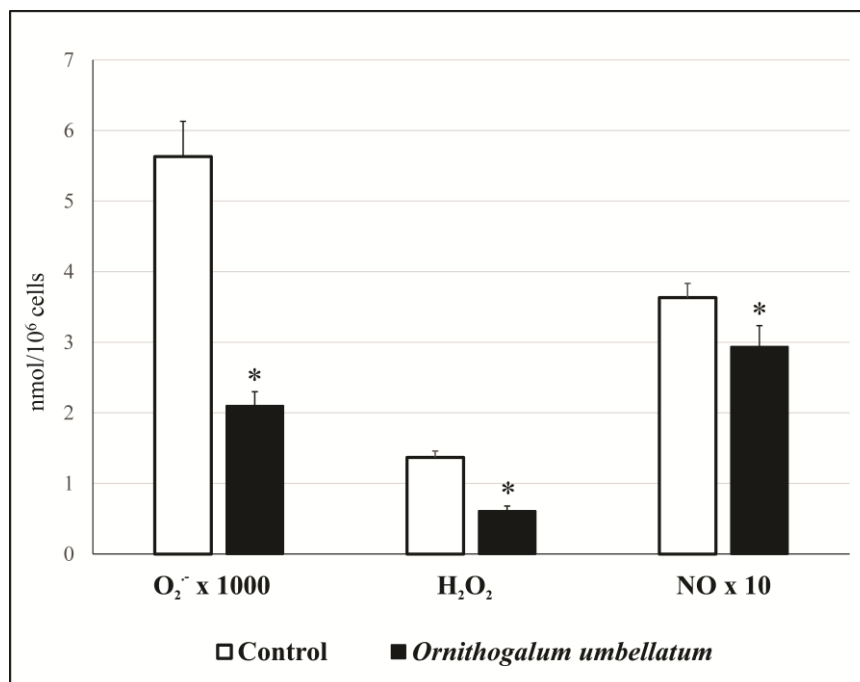


Figure 1. Effects of the aqueous extract of the plant *Ornithogalum umbellatum* (Linnaeus, 1758) on the concentration of O_2^- , H_2O_2 , and NO in human colorectal cancer cell line HCT-116. Data represent mean $\pm$ S.E.M; * $p \leq 0.05$ treatment versus control.

Gallic acid is a low-molecular-weight trihydroxybenzoic acid, which is one of the main phenolic constituents of *O. umbellatum* (AKGEYIK *et al.*, 2023). Gallic acid has been shown to inhibit the activation and nuclear accumulation of specific transcription factors responsible for the regulation of NOS expression (PANDURANGAN *et al.*, 2015; SERRELI and DEIANA, 2023). This mechanism prevents NOS synthesis and leads to a decrease in NO levels, consistent with results obtained in our study. In addition, the aqueous extract of this plant contains other phenolic compounds, particularly high concentrations of procatechuic and caffeic acids (AKGEYIK *et al.*, 2023). These phenolic acids possess multiple hydroxyl groups, which provide them with strong antioxidant capacity. Due to their antioxidant activity, procatechuic and caffeic acids contribute to the reduction of O_2^- levels. Such low levels of O_2^- subsequently result in decreased H_2O_2 formation. Furthermore, the activity of the GSH/GSSG system also contributes to the decrease of O_2^- levels. This eventually disrupted redox signaling pathways that are crucial for maintaining the oncogenic phenotype of cancer cells (VALKO *et al.*, 2006).

The GSH/GSSG system is one of the primary mechanisms of non-enzymatic antioxidant protection that is continuously active in the cells. Reduced glutathione (GSH) directly neutralizes ROS and RNS and serves as an electron donor in the detoxification of H_2O_2 catalyzed by glutathione peroxidase, which is subsequently oxidized to glutathione disulfide (GSSG) (STADTMAN, 1991; MEISTER, 1992). GSSG is a highly reactive molecule that, under physiological conditions, is rapidly reduced back to GSH by the enzyme glutathione reductase (GR). This process protects the cell from oxidative damage and enables the maintenance of redox homeostasis. The results obtained in this study show that a 24-hour treatment with an aqueous extract of *O. umbellatum* resulted in a significant decrease in GSH levels accompanied by a significant accumulation of GSSG (Fig. 2). This imbalance can be attributed to the presence of quercetin. The aforementioned extract contains quercetin, a

flavonoid known to inhibit GR activity (RAT *et al.*, 2016; LEE *et al.*, 2023). The inhibitory effect of quercetin on GR activity causes excessive accumulation of GSSG. The low concentration of GSH is the result of its oxidation and the inability of GR to reduce GSSG (LEE *et al.*, 2023). Elevated GSSG levels trigger a loss in mitochondrial membrane integrity, leading to disturbances in permeability and mitochondrial-cytoplasmic exchange (CIRCU and Aw, 2008). This disruption initiates apoptotic signaling cascades of cancer cells (CIRCU and Aw, 2008). Consequently, the main cause of the observed cytotoxicity appears to be the excessive accumulation of GSSG.

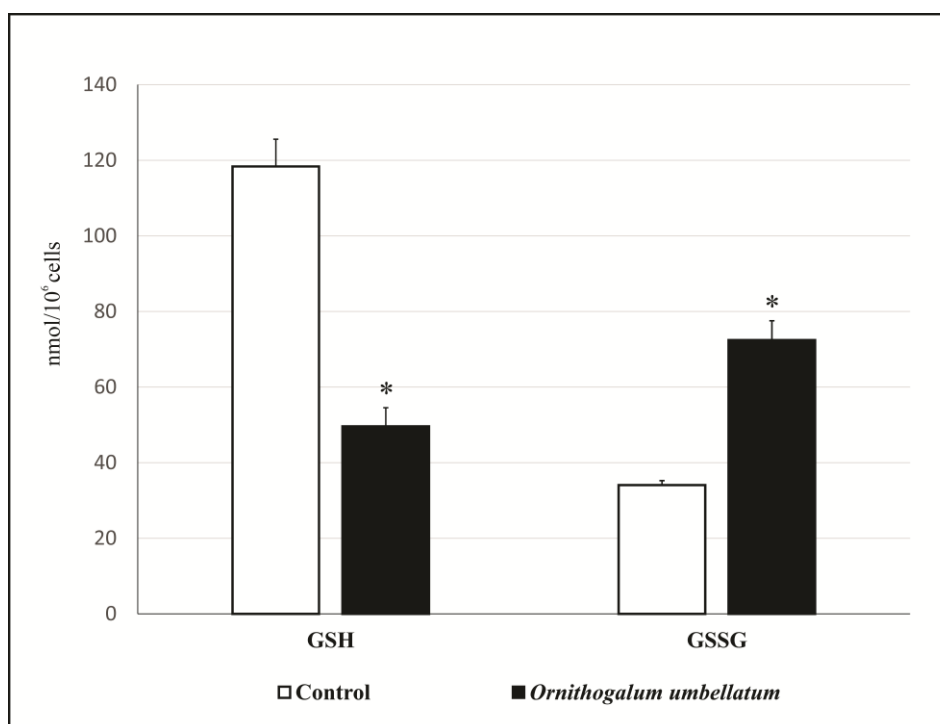


Figure 2. Effects of the aqueous extract of the plant *Ornithogalum umbellatum* (Linnaeus, 1758) on the concentration of GSH and GSSG in human colorectal cancer cell line HCT-116. Data represent mean $\pm$ S.E.M; * $p \leq 0.05$ treatment versus control.

Treatment with the aqueous extract of *O. umbellatum* plant resulted in a disruption of redox status in colorectal cancer cells. Although it led to a reduction in O_2^- , H_2O_2 , NO, and GSH levels, this effect was most likely not the main cause of decreased cell viability. Additionally, recent research has demonstrated that elevated concentration of any free radical is generally more effective in causing cancer cell death (GORRINI *et al.*, 2013). Taken together, these findings suggest that the decline in the colorectal cancer cells' viability is primarily caused by the excessive accumulation of GSSG.

CONCLUSION

This research concludes that the aqueous extract of *O. umbellatum* plant has an anticancer effect on the human colorectal cancer cell line HCT-116, mediated by changes in redox status. The disruption of redox balance in the cells is caused by flavonoids and phenols from the aqueous extract of the plant. The main cause of the decrease in cell viability is the excessive accumulation of GSSG, which induces programmed cell death. Future research will examine the effect of the aqueous extract of *Ornithogalum umbellatum* on the migration of colorectal cancer cells.

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