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THE BIOLOGICAL ACTIVITY OF *Ocimum minimum* L. FLOWERS ON REDOX STATUS PARAMETERS IN HCT-116 COLORECTAL CARCINOMA CELLS

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ABSTRACT. Medicinal plants have widely been used as a natural source of remedies for treating several diseases, including colorectal cancer. *Ocimum minimum* L. is a very important dietary plant used in traditional and modern medicine, due to its health beneficial effect realized by cytotoxic, proapoptotic, antioxidant/prooxidant, antiviral and antimicrobial activity. The biological activity of *O. minimum* flowers has been evaluated in HCT-116 colorectal carcinoma cells through antiproliferative activity by MTT assay, pro-apoptotic activity by AO/EB and concentrations of redox status parameters ($O_2^{\cdot-}$ and lipid peroxidation) by colorimetric methods. The protein expression of iNOS was analyzed by immunocytochemistry, while the antimigratory effect was measured by xCELLigence system. The treatment with *O. minimum* shows the antiproliferative, proapoptotic, impact on redox status parameters and antimigratory effect on HCT-116 cells. Based on obtained results, the pharmacological effect of *O. minimum* is evident against HCT-116 colorectal carcinoma cells, suggesting that this plant may be good start material for future anticancer therapy investigation.

Keywords: *Ocimum minimum* L., colorectal carcinoma, cytotoxic activity, redox status, antimigratory potential.

INTRODUCTION

Colorectal cancer is the third most common cancer in Serbia with 12.2% of newly diagnosed cases in 2020 (VEKIC *et al*., 2020). This is an aggressive type of cancer that develops slowly and usually starts with polypoid changes in the epithelial cells of the colon or rectum (RAWLA *et al*., 2019). The development of colorectal cancer may be associated with various risk factors due to the patient's lifestyle (physical inactivity, nutritional habit, obesity) or environmental conditions (HAGGAR and BOUSHEY, 2009). Chronic gastrointestinal tract

diseases, such as Crohn's disease and ulcerative colitis, express high levels of oxidative stress, so if left untreated, they could contribute to the development of colorectal cancer (PIECHOTA-POLANCZYK and FICHNA, 2014). Oxidative stress in combination with the inflammation process in the gastrointestinal tract may participate in ROS-dependent pathways thus inducing tissue damage and facilitating the development of malignancy (BHATTACHARYYA *et al*., 2014).

Oxidative stress is a disbalance between the production of reactive oxygen (ROS) and nitrogen species (RNS) and its elimination by antioxidants. The most important ROS species (superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$), and organic peroxides) are normal products during the biological reduction of molecular oxygen (COLLIN, 2019). Nitrogen reactive species (RNS) include relatively unreactive nitric oxide ($NO^{\bullet}$) and its derivative the peroxynitrite ($ONOO^-$) which is a powerful oxidant, able to damage many biological molecules (DI MEO *et al*., 2016). In normal conditions low ROS/RNS are controlling signal transduction, cell proliferation, and apoptosis, but in high concentrations causes oxidative damage to DNA, proteins, and lipids thus increasing the risk for mutagenesis and cancer development (DRÖGE, 2002; COLLIN, 2019). Lipid peroxidation (autooxidation) is a process of oxidation of polyunsaturated fatty acids and involves the production of peroxides, ROS, and other reactive organic free radicals. Markers for oxidative damage in cells are 8-hydroxy-2-deoxyguanosine (8-OHdG), as a marker of oxidative DNA damage; protein groups, a marker of protein oxidation; malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), as markers of lipid peroxidation. During cancer initiation ROS induces alterations in DNA, while in promotion and progression stages stimulate abnormal gene expression, cell proliferation, and decrease of apoptosis (AGGARWAL *et al*., 2019).

The treatment of colorectal cancer includes a combination of surgical resection, chemotherapy, radiotherapy, and immunomodulatory therapy, but almost 40% of patients relapse through late metastasis (MIYAMOTO *et al*., 2014). Many commercially used chemotherapeutics are plant-derived products. Considering that plants have huge potential to distribute new drugs, with confirmed health benefits and selective anticancer outcomes, research in this field is very significant (TALIB *et al*., 2020). Plant-derived agents are powerful drugs used in the pharmacological industry due to their efficiency in suppressing, reversing, or postponing the process of carcinogenesis (CRAGG and PEZZUTO, 2016). The plants from the *Ocimum* L. genus are widely used in traditional and modern medicine for treating various diseases because of their antitumor, anti/prooxidant, antibacterial, antimicrobial, antiviral, and immunomodulatory effects (SHAHRAJABIAN *et al*., 2020). The anticancer and anti-inflammatory activities were demonstrated in the case of *Ocimum basilicum* L. (BEHBAHANI, 2014), *O. sanctum* L. (LUKE *et al*., 2021), *O. gratissimum* L. (CHEN *et al*., 2011) and *O. kilimandscharicum* Gürke (DE LIMA *et al*., 2014). *Ocimum minimum* L., small leaved basil, is a well-known specie from the genus *Ocimum*, and it is widely used in nutrition and traditional medicine for treating various diseases and conditions.

Considering that *O. minimum* is a widely known medicinal herb and lack in data on its pharmacological action, the aim of this study is to evaluate its biological effect on redox status parameters in HCT-116 colorectal cancer cells. The anticancer effects were examined by cytotoxic activity followed by the investigation of *O. minimum* ability to induce apoptosis by AO/EB staining. Modulation of redox homeostasis by *O. minimum* was examined through monitoring the concentration of superoxide anion ($O_2^{\cdot-}$) and malondialdehyde (MDA), while protein expression of iNOS was detected by immunofluorescence quantification. The migratory potential of *O. minimum* on HCT-116 cells was investigated with the xCELLigence system.

MATERIAL AND METHODS

Chemicals

Phosphate buffered saline and Dulbecco's Modified Eagle Medium (DMEM) were obtained from GIBCO, Invitrogen, USA. Ethidium bromide (EB) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were bought from SERVA, Germany. Acridine Orange (AO) was obtained from Acros organics, New Jersey, USA. The primary antibody iNOS, secondary antibody Alexa 488 and diamidino-2-phenylindole (DAPI) were from Thermo Scientific, USA. The 2-thiobarbituric and hydrochloric acid were from Sigma-Aldrich, USA. Trichloroacetic acid and methanol were from VWR Prolabo, USA. Fetal bovine serum (FBS) was obtained from Capricorn Scientific GmbH, Germany. Polyvinyl alcohol mounting medium was bought from Fluka Analytical, Switzerland. All solvents and chemicals were of analytical grade.

Identification, collection, and preparation of methanol extract from O. minimum. flowers

The flowers of *O. minimum* were collected in Gornja Trešnjevica, Aranđelovac, Serbia (GPS 44º13′0″ N; 20º33′0″ E) in July 2019. A voucher of an identified plant by taxonomic and botanical characteristics was kept in the Herbarium of the Department of Biology and Ecology, Faculty of Science, University of Kragujevac, Serbia (No. 134/2020). The methanol extract of *O. minimum* flowers was prepared by dissolving 20 g dried plant material in 500 mL of pure methanol (99.8%) in an ultrasonic bath for 15 min and then left to dissolve another 24 h. The plant material was macerated three times at room temperature using a fresh solvent (methanol) every 24 hours. The samples were filtered through a filter paper (Whatman No.1) and the filtrates were collected and evaporated to dryness using a rotary evaporator (IKA, Germany) at 40ºC. Methanolic extract of *O. minimum* flowers were kept in sterile sample tubes and stored in a refrigerator at 4°C until use.

Cell culturing

The human colorectal cancer cell line (HCT-116) was obtained from American Type Culture Collection, USA. Cells were maintained in optimal conditions, according to the procedure described in MILUTINOVIĆ *et al*. (2015).

Determination of cell proliferation by MTT assay

The effect of *O. minimum* treatment on the viability of HCT-116 colorectal carcinoma cells was determined by MTT assay (MOSMANN, 1983), described in detail in NIKODIJEVIĆ *et al*. (2021). For cell viability assay, cells were seeded in 96 well plates (10^4 cells/well). After 24 h of incubation cells were treated with methanol extract of *O. minimum* concentrations (1–500 µg/mL), while the untreated cells served as control. The assay was performed 24, 48 and 72 h after the treatment. Percentages of cell viability were obtained as the ratio between the absorbance of treated and untreated cells. Half-maximal inhibitory concentration (IC_{50}) was calculated from the dose-response curves by the CalcuSyn program.

Fluorescence microscopic analysis of cell death

Acridine orange/ethidium bromide (AO/EB) double staining assay was performed to determine of cell death type on HCT-116 colorectal carcinoma cells (BASKIĆ *et al*., 2006. Cells were seeded in 96 well plates (10^4 cells/well) and after 24 h they were treated with *O. minimum*

in concentration of 10 and 100 µg/mL. Untreated cells served as control. The percentages of viable (V) and early apoptosis (EA) cells were proportionally calculated, concerning the total cell number per sample (a minimum of 300 cells was counted per sample). The assay was performed using an inverted fluorescent microscope (Nikon Ti-Eclipse) at 400x magnification, 24 h after treatment.

Detection of superoxide anion radical

Determination of superoxide anion radical ($O_2^{\cdot-}$) was measured by NBT assay (AUCLAIR and VOISIN, 1985), as described in NIKODIJEVIĆ *et al*. (2021). The cells were seeded in a 96-well plate (10^4 cells/well) and treated with 100 µL of *O. minimum* in the concentration range of 1-500 µg/mL. Untreated cells served as a control. The assay was performed 24, 48, and 72 h after treatment. The results for $O_2^{\cdot-}$ were firstly expressed in nmol/mL and then calculated concerning the number of viable cells based on results from the MTT assay.

TBARS – lipid peroxidation assay

The concentration of malondialdehyde, as a marker of oxidative stress, was determined and calculated with the TBARS assay (BUEGE and AUST, 1978), described in detail in NIKODIJEVIĆ *et al*. (2021). The assay was performed 24, 48, and 72 h after applied treatment. After 24 h the cells were treated with 100 µL of *O. minimum* methanol extract in concentration of 1–500 µg/mL, while the untreated cells served as control. The results were presented in pmol/mg of protein.

Immunocytochemistry quantification of iNOS protein expression

The effect of *O. minimum* on protein expression of iNOS was detected by the immunofluorescence method on HCT-116 colorectal carcinoma cells (HIGUCHI *et al*., 2001). The HCT-116 cells ($5x10^4$ cells/well) were seeded in 6 well plates on glass coverslips. After 24 h cells were treated with 2 mL of *O. minimum* extract in concentrations of 10 and 100 µg/mL. Untreated cells served as control. The further procedure of immunocytochemistry assay was described in NIKODIJEVIĆ *et al*. (2021). The cells were visualized using a fluorescent microscope Nikon Ti-Eclipse at 600x magnification. Micrograph processing and quantification of cell fluorescence were performed by ImageJ software. The results are presented as relative fluorescence per cell.

Migration assay with the xCELLigence system

The rate of cell migration was monitored in real-time with the xCELLigence system in CIM-plates (ACEA Biosciences, Inc.). The HCT-116 cells were grown in a 75 cm^2 flask with 15 mL of complete medium, in a CO_2 incubator (5% CO_2 and 37ºC). Before performing the assay, it is necessary to replace the complete medium with a MEM Eagle (medium with a minimum amount of food). In CIM-Plate 16 was added 30 µL MEM without FBS to the top plate, then incubate for 30 min at room temperature (22–24ºC). In each well of the bottom plate was added 160 µL DMEM with the appropriate chemoattractant (20% FBS). The plates were placed on the appliance and returned to the incubator for 30 minutes. Thereafter, the $40x10^4$ cells were resuspended in 100 µL MEM and seeded on top of CIM-Plate. The incubation period lasted 24 h for the cells to adhere to the bottom of the top plate. After that, the cells were treated with *O. minimum* in the concentration of 10 and 100 µg/mL, while untreated cells served as control. Readings were noted at 25 scans, at 5 min intervals, and then followed by scans at every 15 min intervals during 72 h of the experiment.

Statistical analysis

The data are expressed as mean ± standard error. Biological activity was examined in two individual experiments, performed in triplicate for each dose. The magnitude of the correlation between variables was done by SPSS (Chicago, IL) statistical software package (SPSS for Windows, ver. 17, 2008). The IC_{50} values were calculated from dose curves by the CalcuSyn program (BIOSOFT, Cambridge GB – United Kingdom). The relative intensity of fluorescence for iNOS protein expression in control and treated cells were measured by the ImageJ program (Wayne Rasband, ImageJ).

RESULTS AND DISCUSSION

Flavonoids and phenolic acids as bioactive secondary metabolites show a wide range of anticancer effects that include modulation of reactive oxygen species and nitrogen, inducing apoptosis, suppressing proliferation, effects on migration potential, and expression of genes and proteins involved in various stages of cancer progression (ANANTHARAJU *et al.*, 2016; KOPUSTINSKIENE *et al.*, 2020).

In our previous report (JOVANKIĆ *et al.*, 2022) the HPLC analysis indicated that methanol extract of *O. minimum* flowers contains high amounts of bioactive compounds. The most dominant flavonoids were rutin, quercetin-3O-glucopyranoside and catechin, while the most dominant phenolics were chlorogenic, caffeic and protocatechuic acid.

The results in this study are mainly discussed according to reported anticancer activities of the identified secondary metabolites by HPLC analysis in the extract of *O. minimum* flowers.

Determination of cell viability and apoptotic effect of Ocimum minimum on HCT-116 colorectal cancer cells

In vitro effect *of O. minimum* on the HCT-116 cells viability was investigated by MTT assay during 72 h. Our results showed a significant time and dose-dependent reduction of cell viability on HCT-116 colorectal carcinoma cells (Fig. 1).

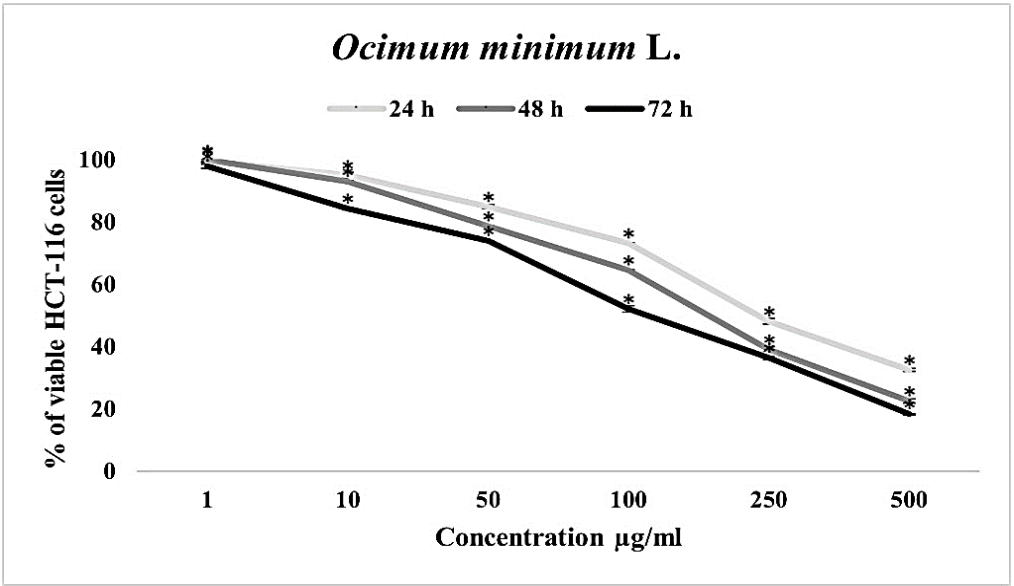

Figure 1. Antiproliferative effects of *O. minimum* on HCT-116 colorectal carcinoma cells. The results are presented as the mean of two independent experiments ± standard error. * Statistically significant difference ($p<0.05$) in relation to control values.

Half-maximal inhibitory concentration (IC_{50}) was used as an indicator for cytotoxicity. Based on IC_{50} values (Tab. 1) *O. minimum* induced significant time-dependent cytotoxicity on HCT-116 cells, especially after 72 h (IC_{50}=109.53 µg/mL).

Table 1. Cytotoxic effects of *O. minimum* expressed as IC_{50} value (µg/mL) on HCT-116 colorectal cancer cell line.

Cell line	Treatment	24 h	48 h	72 h
HCT-116	*Ocimum minimum*	252.31	161.53	109.53

Flavonoids display important anticancer properties by controlling ROS-scavenging enzyme activities, contributing to arresting the cell cycle, inducing apoptosis, autophagy, and suppressing cancer cell proliferation and invasiveness (KOPUSTINSKIENE *et al*., 2020). Our results are in correlation with other authors who confirmed cytotoxicity from other species in the genus *Ocimum* against different types of cancer cell lines. Essential oils and ethanol extract of *O. basilicum* showed an antiproliferative effect in HeLa, FemX, K562, and SKOV3 cells (ZARLAHA *et al*., 2014). *In vitro* cytotoxic activity of *O. viride* Willd essential oil was confirmed against HT-26 colon cancer cells with an IC_{50} value of ~0.034 ± 0.001µg/mL (BHAGAT *et al*., 2020). *In vitro* cytotoxic activity of *O. sanctum* ethanolic and aqueous extracts was demonstrated in HT-15 and HT-29 colon carcinoma, IMR-32 neuroblastoma, and A-549 lung cancer cells (SUNDARAM *et al*., 2011). Our previous study reported a significant reduction of cell viability in breast cancer model systems under *O. minimum* treatment (JOVANKIĆ *et al*., 2022).

The performed AO/EB analysis for determination of cell death type indicated that treatment with *O. minimum* induces early apoptosis in HCT-116 cells without necrotic changes. The percentage of early apoptosis slightly increased in a dose-dependent manner during 24 h, suggesting that cytotoxicity may be mediated by this apoptotic change (Tab. 2, Fig. 2).

Table 2. The effect of *O. minimum* on the percentage of viable and early apoptotic changes on HCT-116 colorectal carcinoma cells, after 24 h of applied treatment. The results are presented as the mean of two independent experiments ± standard error.

	Percentage of apoptotic changes in HCT-116 cells	
Concentration	**Viable cells**	**Early apoptosis**
Control	98.27±0.24	1.73±0.76
10 µg/mL	95.58±0.70	4.42±0.75*
100 µg/mL	94.52±0.68	5.48±0.79*

*Statistically significant difference ($p<0.05$) compared to control values.

The central regulator of tissue homeostasis is apoptosis, which regulates normal cell growth, proliferation, and cell death (XU *et al*., 2011). Numerous studies show a beneficial effect of flavonoids as prooxidants capable to promote apoptosis and suppress proliferation through the inhibition of important signal pathways in cancer cells such as EGFR/MAPK, PI3K, AKT and NF-κB (ABOTALEB *et al*., 2019). Our reported results about proapoptotic effects of *O. minimum* are correlated with other authors who confirmed this action in several cancer model systems. It has been demonstrated that flavonoids can induce apoptotic changes via down-regulation of PI3Kγ mediated PI3K/AKT/mTOR/p70S6K/ULK signaling pathway in human breast cancer cells (ZHANG *et al*., 2018). Quercetin induces apoptosis in CT-26, LNCaP,

MOLT-4, and Raji cell lines and reduces cell viability in a dose-dependent manner *in vitro* and *in vivo* assays (HASHEMZAEI *et al*., 2017). Rutin stimulates antiproliferative and pro-apoptotic effects against B164A5 murine melanoma cells (DANCIU *et al*., 2017).

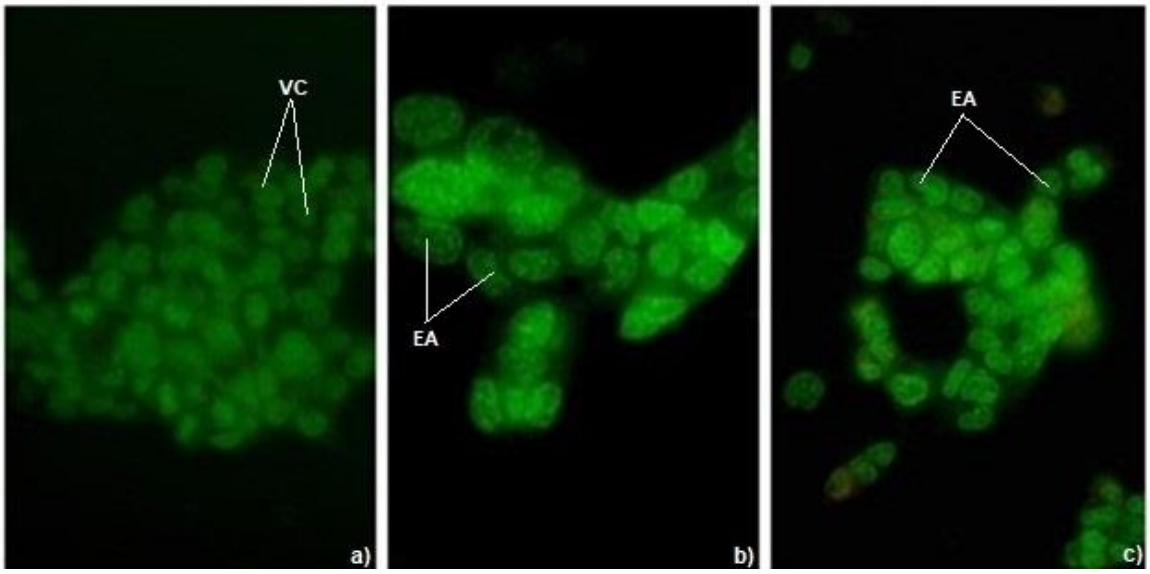

Figure 2. Typical morphological changes in HCT-116 colorectal carcinoma cells stained with AO/EB after treatment with *O. minimum* (a-control, b-10 µg/mL, c-100 µg/mL).

Mediation of redox homeostasis by Ocimum minimum

The concentration of $O_2^{\cdot-}$ was measured by NBT assay for 72 h. The obtained results presented in Fig. 3. indicate on strong prooxidant effect in a dose and time-dependent manner, suggesting the presence of oxidative stress in HCT-116 induced by *O. minimum* treatment. The increased concentration of ROS could be the reason for enhanced apoptotic morphological changes in the *O. minimum* extract-treated HCT-116 cells.

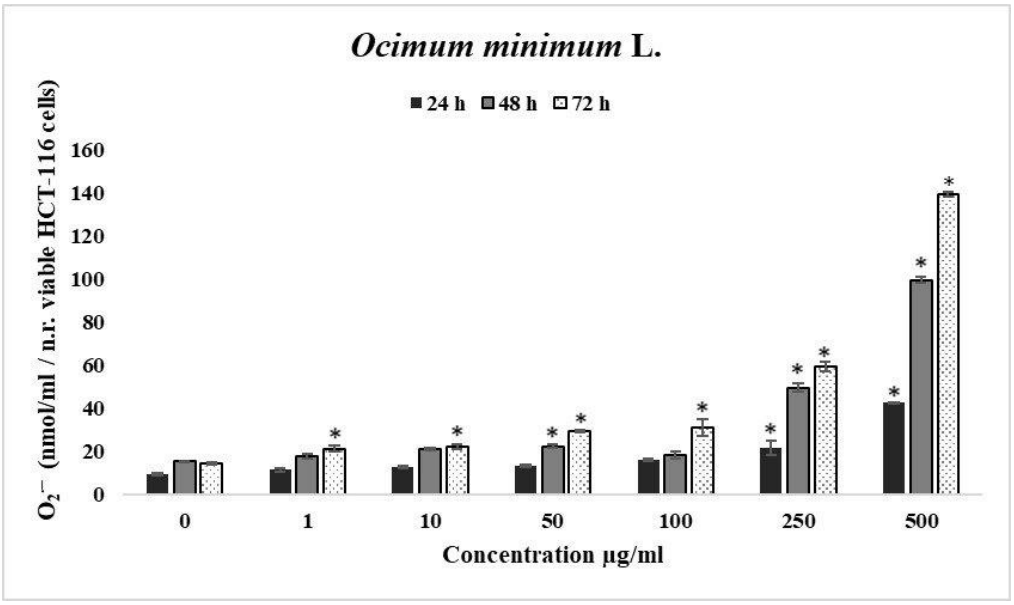

Figure 3. The concentration of superoxide anion radical ($O_2^{\cdot-}$) in HCT-116 colorectal carcinoma cells after 24, 48, and 72 h of *O. minimum* treatment. The results are presented as the mean of two independent experiments ± standard error. *Statistically significant difference ($p<0.05$) compared to control values.

SRIDEVI *et al*. (2016) have reported that ethanolic extract of *O. sanctum* significantly increases ROS levels in NCI-H460 cells. Our previous studies showed that specific phenolic components from plants could act like strong prooxidants, inducing pro-apoptotic and cytotoxic effects in HCT-116 and SW-480 colorectal carcinoma cells (MILUTINOVIĆ *et al*., 2015).

The concentration of malondialdehyde (MDA) was measured by TBARS assay for 72 h. Results showed that *O. minimum* pronounces significant prooxidant effect on HCT-116 cells after 24 h and 72 h of applied treatment. Antioxidant capacity was detected after 48 h of treatment, suggesting that cells had recovered from oxidative stress in meantime (Fig. 4).
It has been shown that elevated ROS concentration can cause apoptosis by increasing the level of cellular lipid peroxidation by disrupting the permeability of the mitochondrial membrane (PELICANO *et al*., 2004). Dysfunction of the mitochondrial membrane is an early hallmark of apoptosis (GREEN and LEEUWENBURGH, 2002). Dietary flavonoids are powerful anti-cancer agents, which can cause anti or prooxidant effects due to their polyphenolic structure (SAK, 2014). Ethanolic extract of *O. sanctum* increased lipid peroxidation in A549 human lung adenocarcinoma cells which prevents MDA enzymatic activity and cytoprotection of cancer cells (WIHADMADYATAMI *et al*., 2019). Quercetin caused a significant increase in MDA concentration in CK2 human laryngeal carcinoma cells (DURGO, 2007). The plant natural compounds which cause prooxidant effects via cytotoxicity-mediated apoptosis in cancer cells may be a good starting point for cancer-specific therapy.

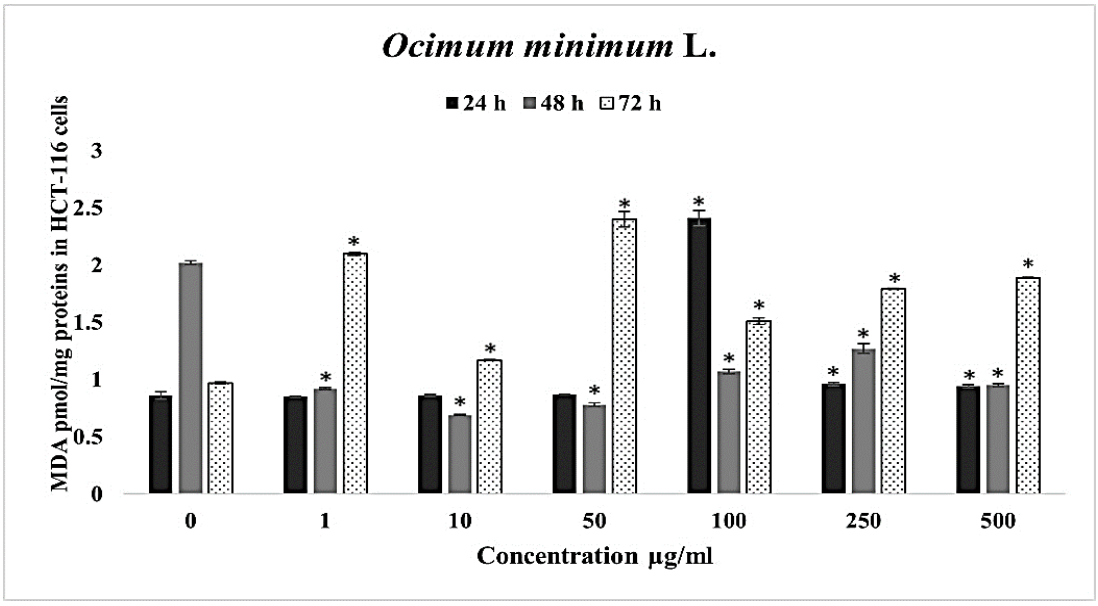

Figure 4. The concentration of MDA, presented in pmol/mg of protein in HCT-116 colorectal carcinoma cells after 24, 48, and 72 h of *O. minimum* treatment. The results are presented as the mean of two independent experiments ± standard error. *Statistically significant difference ($p<0.05$) compared to control values.

Inducible nitric oxide synthase (iNOS) is an enzyme that produces nitric oxide (NO). High levels of NO promote proliferation, angiogenesis, and metastasis in the cancer microenvironment (BASUDHAR *et al*. 2017). The protein expression of iNOS was reduced at a dose of 100 µg/mL, while at a dose of 10 µg/mL it was increased in HCT-116 cells treated with *O. minimum* compared to control values (Figs. 5 and 6).

Authors reported that pretreatment with quercetin produced an inhibitory effect of iNOS expression in RAW cells (MU *et al*., 2001). Ellagic acid reduces the expression of iNOS, TNF-

a, and IL-6 in colon cancer induced in rats by 1,2-dimethylhydrazine (UMESALMA and SUDHANDIRAN, 2010).

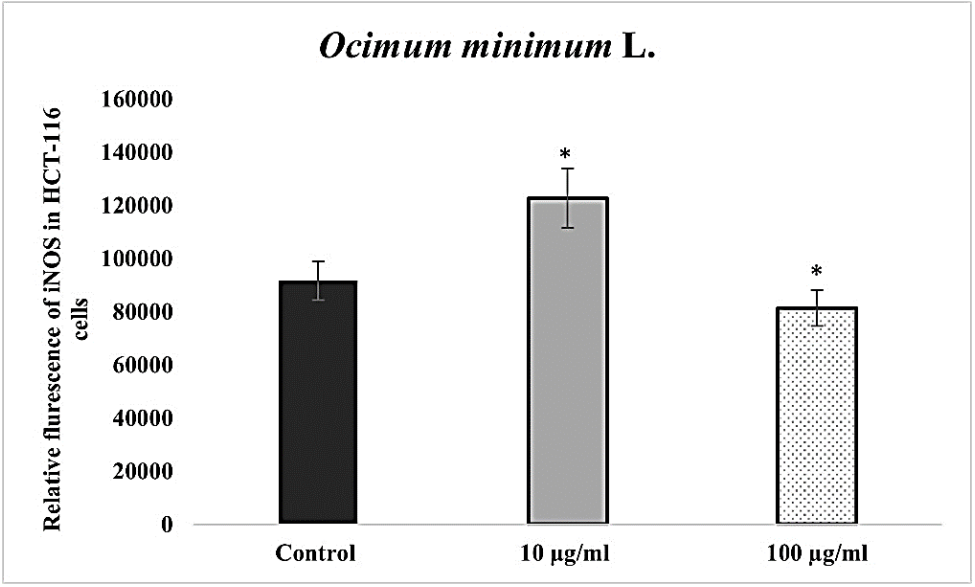

Figure 5. The protein level of iNOS in HCT-116 colorectal carcinoma control and cells treated with *O. minimum* (10 and 100 µg/mL), 24 h after treatments. The data are means ± SE of two independent experiments. *p<0.05 compared to untreated cells.

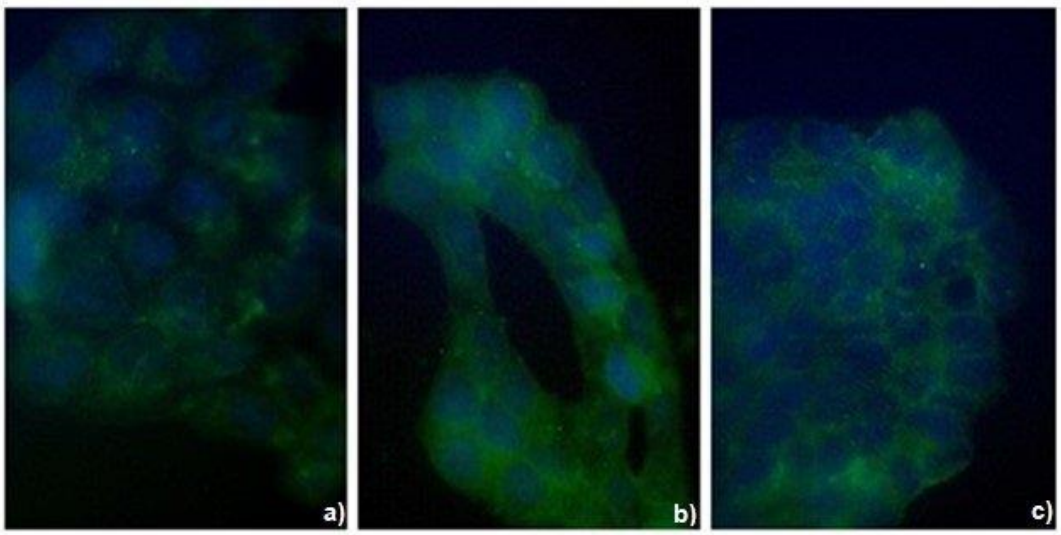

Figure 6. Protein expression of iNOS in HCT-116 control colorectal carcinoma cells a) and 24 h after treatments with 10 b) and 100 µg/mL c) *O. minimum*. The nuclei are colored blue (DAPI color), while iNOS is green (secondary antibody conjugated to Alexa 488).

The migratory potential of O. minimum against colorectal carcinoma HCT-116 cells

Antimigratory potential of *O. minimum* was evaluated with RTCA DP Instrument Flexible Real-time Cell Monitoring System for 72 h. Results presented in Table 3. indicated on significant antimigratory effect of *O. minimum* against HCT-116 cells generally in a dose and time-dependent manner. An exception was only in the concentration of 10 µg/mL 24 h after applied treatment, where is an evident promigratory effect (Tab. 3).

Table 3. Antimigratory effect of *O. minimum* on HCT-116 colorectal carcinoma cells compared with control. The results are presented as the mean of two independent experiments ± standard error.

Concentration	24 h	48 h	72 h
Control	100.00±0.05	100.00±0.09	100.00±0.023
10 µg/mL	133.47±1.07*	68.73±5.84*	48.29±4.04*
100 µg/mL	42.12±3.25*	13.00±2.37*	1.00±0.29*

* Statistically significant difference ($p<0.05$) in relation to control values.

Cancer metastasis is the main cause of death in patients since this process stimulates tumor cells to migrate and invade distinct organs in presence of oxidative stress (PISKOUNOVA *et al*., 2015; ZHUYAN *et al*., 2020). Phenol complex catechin-lysine induces a selective anti-migratory effect by inhibition of JAK2/STAT3 and WNT pathways in colorectal, breast, and pancreatic cancer cell lines (SILVA *et al*., 2019). Previous studies had suggested that rutin dominantly inhibits adhesion and migration in colorectal HT-29 and lung A549 cancer cells (BEN SGHAIER *et al*., 2016). The same effect was confirmed with apigenin against DLD-1 and SW-480 human colorectal adenocarcinoma cells (DAI, 2016).

In this study its shown that methanol extract of *O. minimum* flowers has significant antiproliferative, proapoptotic, prooxidant and antimigratory effects against HCT-116 colorectal carcinoma cells. The synergic action of phytochemicals identified in *O. minimum* flowers showed important pharmacological effect whit potential use as a dietary supplement against colorectal cancer in combination with another chemotherapeutics. Inhibition of crucial signal pathways involved in different stages of cancer progression by *O. minimum* treatment may be a promising therapeutic strategy against this disease.

Acknowledgments

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