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ANTITUMOR EFFECTS OF *TANACETUM BALSAMITA* ESSENTIAL OILS BY DOWNREGULATION NRF-2 AND MMP-9 IN BREAST CANCER

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Abstract: The aim of this study was to investigate antitumor capacity of different *Tanacetum balsamita* essential oils by measuring apoptosis rate, MMP-9 concetration and Nrf-2 expression level in human MBA-MB-468 and MDA-MB-231 cells. The tested EOs expressed proapoptotic effects, significantly inhibited the MMP-9 concentration and down-regulated the expression level of Nrf-2. The obtained data suggest that the tested EOs exert considerable antitumor activity elevating apoptosis level and inhibiting the motility of tested cancer cells. The reduced levels of Nrf-2 expression suggest decreased defense potential for oxidative disturbances, which could be the major antitumor mechanism detected in the study.

Keywords: *Tanacetum balsamita* essential oils, breast cancer cell lines, Nrf-2, MMP-9, apoptosis rate

Introduction

Cancer represents one of the most persistent groups of diseases with high mortality worldwide. Breast cancer is the second most common cancer of female worldwide. Despite the extensive use of multimodal chemotherapies, their efficiency is limited, emphasizing the need for novel therapeutic approaches with higher cytotoxicity against malignant cells and acceptable demaging outcomes at healthy tissues. Naturally occurring compounds such as terpenoids, phenolics, flavonoids, and alkaloids due to their significant

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therapeutic effects, find increasing medical application in cancer prevention and therapy (Anand et al., 2022). Natural compounds can be used alone or in combination with chemotherapeutics, which allows the application of lower doses of chemotherapeutics in order to overcome drug resistance and toxicity on healthy tissues (Raimondo et al., 2015). Various plant secondary metabolites used in traditional medicine, classified as alkaloids, saponins, terpenes, glycosides and polyphenols, have shown positive anticancer properties *in vitro* and *in vivo*. Accordingly, various products of plant origin, including essential oils isolated from aromatic plants, rise significant interest regarding their anticancer properties (Lin et al., 2019; Sauter, 2020).

Nrf-2 (Nuclear factor erythroid 2- related factor 2) is one of the main regulators of redox homeostasis with predominant antioxidative property and its up-regulation is indicated in breast cancer cell growth and progression. Induction of apoptosis, limits an excessive proliferation of breast cancer cells. Nrf-2 values are used as biomarkers for various specific types of cancer.

Matrix metalloproteinases (MMPs) are proteases with a fundamental role in the degradation of extracellular matrix (ECM) components. MMPs are involved as essential molecules in multiple and diverse physiological processes, such as reproduction, tissue repair, and regulation of inflammatory processes. Its activity is controlled at various levels such as at transcription level, pro-peptide activation level and by the activity of a family of tissue inhibitors of metalloproteinase - endogenous inhibitors of MMPs. Most cancer deaths are the result of tumor dissemination, a process that includes the activity of metalloproteinases. MMPs are used in precision medicine for they role as biomarkers (Mustafa et al., 2022).

The aim of this study was to investigate antitumor capacity of different *Tanacetum balsamita* essential oils by measuring apoptosis rate, MMP-9 concetration and Nrf-2 expression level in human MBA-MB-468 and MDA-MB-231 cell line.

Materials and methods

2.1. Cell culture and treatment

The human breast cancer cell line MDA-MB-468 and MDA-MB-231 were obtained from American Tissue Culture Collection. The cells were grown according to the procedure described in detail in the paper Obradovic et al. (2020). Two essential oils of *Tanacetum balsamita* from different organs (leaf (LEO) and stems (SEO)) were used in experiments. After short-term and long-term

treatments (24 h and 72 h), the evaluation of cell proliferation, concentration of MMP-9 and Nrf-2 were performed. The stock solution LEO and SEO was prepared in the concentration of 10 mg mL^{-1}, while the tested concentrations were 1 μg mL^{-1} and 10 μg mL^{-1}. All treatments were performed in triplicate for each assay. After the completed treatments, the concentrations of MMP-9 and Nrf-2 were measured as well as a percentage of apoptotic and necrotic cells.

2.2. Determination of invasive potential of MMP-9 concentration

Matrix metalloproteinases are endopeptidases that play a key role in cell invasion by degrading matrix components such as collagen type IV, native collagen, and others. With the help of a kit for the quantitative determination of the concentration of total MMP-9, it is possible to analyze the invasive potential of cells from cell lysates. This test uses a quantitative sandwich enzyme immunoassay technique. Samples and reagents from MMP-9 (Elabscience, ELISA) must first be prepared as described in the provided instructions. Untreated (control) and treated cells, were trypsinized, centrifuged, and washed three times in PBS. Then cells were sonificated and supernatant was used further in the protocol. Absorbance was measured by using ELISA reader at 450 nm. The concentrations of Human MMP-9 in the samples calculate by comparing the OD of the samples to the standard curve.

2.3. Determination the concentrations of Human NFE2L2 (Nuclear Factor, Erythroid Derived 2, Like 2)

This test uses a quantitative sandwich enzyme immunoassay technique. Samples and reagents from NFE2L2 (Elabscience, ELISA) must first be prepared as described in the provided instructions. Untreated (control) and cell with treatment were trypsinized and then centrifuged and washed three times in PBS. Then cells were sonificated and supernatant was used further in the protocol. Absorbance was measured by using ELISA reader at 450 nm. The concentrations of Human NFE2L2 in the samples calculate by comparing the OD of the samples to the standard curve.

2.4. Determination of type of cell death

Apoptosis and necrosis were analyzed by double staining with annexin V-FITC and 7-AAD. Annexin V binds to the cells with exposed phosphatidylserine, whereas 7-AAD labels the cells with membrane damage. Apoptotic cells were detected using the Annexin V-FITC/7-AAD Kit (Apoptosis Detection Kit, Beckman Coulter, USA). Staining was performed according to the manufacturer's

instructions and Shounan protocol (Shounan et al., 1998). After the treatment the cells were collected, washed in PBS and resuspended in ice cold binding buffer. Ten thousand events were analyzed on Flow cytometer Cytomics FC500 (Beckman Coulter, USA). The percent of viable (Annexin V-7-AAD-) cells, early apoptotic (Anexin V+7-AAD-) cells, late apoptotic (Annexin V+7-AAD+) cells, and necrotic cells (Anexin V-7-AAD+) cells were evaluated by Flowing Software (http://www.flowingsoftware.com/).

Results and discussion

The group of matrix metaloproteinases to which MMP-9 belongs, is considered particularly important for the invasiveness of cancer cells during metastasis. In addition to the examination of the migration potential of the cells, in our previus study (Vukic et al., 2022), an analysis of the parameters of the invasion abilities of these cells was carried out. The total concentration of MMP-9 is detected by an enzymatic, ELISA reagent kits method. The results presented on Figure 1. show that LEO and SEO exerted a statistically significant decrease in the concentration of MMP-9 after both treatments compared to the control group of cells. The strongest effect was exerted by LEO at a concentration of 10 μg mL^{-1} on the MDA-MB-231 cell line after long-term treatment (72 h).

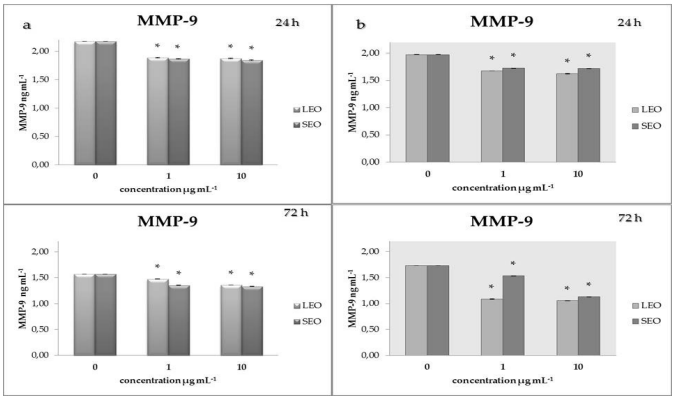

Figure 1. The effects of two concentrations of LEO and SEO on concentration of MMP-9 in a) MDA-MB-468 cells and b) MDA-MB-231 cells after 24 h and 72 h of treatment. Results are presented as the mean of three independent experiments ± standard error; * $p < 0.05$ relative to control.

Cancer biomarkers can play an essential role in fields such as cancer diagnosis and prognosis, monitoring disease progression, predicting disease recurrence, monitoring, and predicting treatment efficacy, as well as cancer screening. MMP-9 is involved as essential molecules in multiple and diverse physiological processes, such as reproduction and regulation of inflammatory processes, but also helps cancer cells to evade primary tumor tissue and migrate across ECM. Since MMP-9 plays an important role in cancer cell progression, it represents a convenient biomarker, already established as such for several cancers (Huang, 2018).

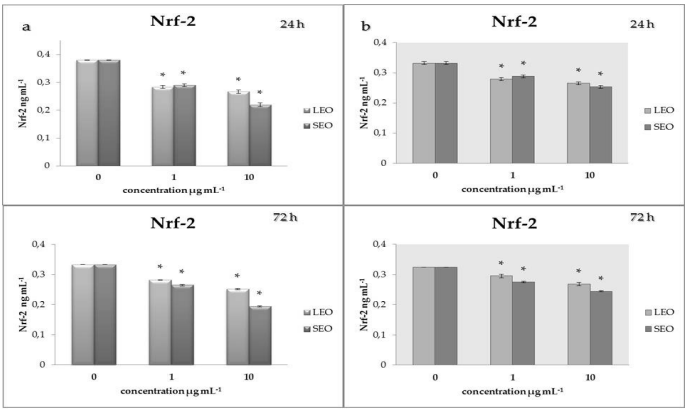

Figure 2. The effects of two concentrations of LEO and SEO on concentration of Nrf-2 in a) MDA-MB-468 cells and b) MDA-MB-231 cells after 24 h and 72 h of treatment. Results are presented as the mean of three independent experiments ± standard error; * $p < 0.05$ relative to control.

In healthy cells, Nrf-2 exerts basic levels of expression, which prevents antioxidative stress and maintaining physiological levels of reactive oxygen species; however, cancer cells overexpress Nrf-2, which is associated with various phenomena, such as the development of drug resistance, angiogenesis, development of cancer stem cells phenotype, and metastasis (Kumar et al., 2022). Redox mechanism-based therapy is known to play an important role in cancer treatment; however, Nrf-2 is responsible for the regulation of antioxidant and cytoprotective properties through the activation of several genes involved in glutathione (GSH) synthesis and chemoresistance. The concentration of total glutathione after 24 h and 72 h of incubation with various concentrations of LEO and SEO showed an increase in the levels of glutathione compared to the control in our previus study (Vukic et al., 2022).

In healthy tissues, Nfr-2 has been extensively considered as one of primary methods for cancer prevention. However, since it exerts cytoprotective action, chemopreventive drugs activate Nrf-2 in cancer cells, and its elevated activity may promote cancer cell survival and proliferation, implying that inhibition of Nrf-2 may be essential pharmacological target in antitumor therapies (Kumar et al., 2022). Nrf-2 targeting cancer progression can provide a new perspective in designing more effective drugs.

The results of our study show that LEO and SEO exerted a statistically significant decrease in the expression level of Nrf-2 after both treatments compared to the control group of cells and presented on Figure 2. The strongest effect was shown by SEO at a concentration of 10 μg mL^{-1} on the MDA-MB-468 cells line after long-term treatment (72 h).

Apoptosis is highly regulated process of cell death and is crucial in maintaining tissue homeostasis, regulating cell division ratio and preventing carcinogenesis. The components of EOs from various plants have been indicated to induce the apoptosis in numerous cancer cell types. Since our previous results suggest decreased viabilty of the tested EOs (Vukic et al., 2022), we have performed the measurments of apoptotic potential as one of the mechanisms of recorded antitumor activity in the study. Apoptosis inducing agents are expected to be sucessful antitumor drugs since apoptosis is protective mechanism against cancer development that acts to remove genetically dameged cells from tissue before they undergo clonal expansion (Goldar et al., 2015).

The type of cell death was determined by flow cytometric analysis of the treated cells stained with Annexin V FITC and 7-AAD. Both cell lines (MDA-MB-468 and MDA-MB-231) were treated for 24 h and 72 h, with concentration 1 μg mL^{-1} and 10 μg mL^{-1}. The effects of LEO and SEO showed statistically significant time- and dose- dependent proapoptotic effect in MDA-MB-468 and MDA-MB-231 cells, as shown on Figure 3. The strongest apoptosis level compared to non tretaed cells was recoreded for LEO essential oil in concentration of 10 μg mL^{-1} in MDA-MB-231 cell line.

The tested essential oils expressed antiproliferative activity, and proapoptotic effects. The investigated oils significantly inhibited the MMP-9 concentration and down-regulated the expression level of Nrf-2.

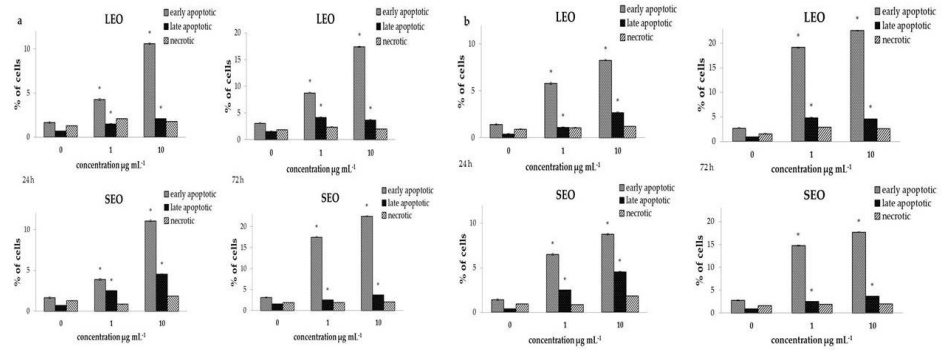

Figure 3. Flow cytometric analysis of Annexin V-FITC/7-AAD stained a) MDA-MB-468 cells and b) MDA-MB-231 cells for 24 h and 72 h with LEO and SEO at two different concentrations. The percentages of early apoptotic (Annexin V+7-AAD-, lower right quadrant), late apoptotic (Annexin V+7-AAD+, upper right quadrant) and necrotic cells (Annexin V−7-AAD+, upper left quadrant) in non-treated and treated cells are indicated on dot plots). Results are presented as the mean of three independent experiments ± standard error; * $p < 0.05$ relative to control.

Conclusion

The obtained data suggest that the tested oils exert considerable antitumor activity by down-regulating Nrf-2 level, elevating apoptosis rate and inhibiting the motility of tested breast cancer cell lines. The reduced levels of Nrf-2 expression suggest decreased defense potential for oxidative disturbances, which could be the major antitumor mechanism detected in the study.

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