MCBU SBED
MANİSA CELAL BAYAR ÜNİVERSİTESİ SAĞLIK BİLİMLERİ ENSTİTÜSÜ DERGİSİ
MANISA CELAL BAYAR UNIVERSITY JOURNAL OF INSTITUTE OF HEALTH SCIENCE
ISSN: 2147-9607

ARAŞTIRMA MAKALESİ
RESEARCH ARTICLE
CBU-SBED, 2023, 10 (4): 289-295

The Root Extracts of *Valeriana Officinalis* L. may Control Programmed Cell Death Pathways in Breast Cancer Cell Line, MCF-7

Valeriana Officinalis L. Kök Ekstraktları Meme Kanseri Hücre Hattı MCF-7'de Programlanmış Hücre Ölümü Yolaklarını Kontrol Edebilir

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Gönderim Tarihi / Received:27.02.2023
Kabul Tarihi / Accepted: 22.03.2023
DOI: 10.34087/cbusbed.1257111

Öz

Günümüzde doğal ürünlerden elde edilen fitokimyasallar kanserin tedavisi ve önlenmesi için yeni stratejiler geliştirmede umut verici ajanlar olarak kabul edilmektedir. Sirkadiyen ritme bağlı uyku bozukluklarını ve uykusuzluğu tedavi etmek için yaygın olarak kullanılan bir takviye olan kediotu (*Valeriana officinalis L.*) kök ekstreleri bu bağlamda iyi bir aday olabilir. Bu çalışmada, kediotu kökü ekstraktının bir insan meme kanseri modeli olan MCF-7 hücrelerinde programlanmış hücre ölüm mekanizmalarını indükleyip indüklemediği hipotezini ortaya koyduk. Hipotezi test etmek için MCF-7 hücrelerini 24 saat boyunca farklı konsantrasyonlarda ekstrakt ile muamele ettik. Apoptotik morfolojiyi ve apoptotik indeksi değerlendirmek için Giemsa boyamasını, otofajik akı ile ilişkili vakuolleri belirlemek için ise monodansilkadaverin testini kullandık. Bulgularımız, kediotu köklerinden elde edilen ekstraktların insan meme kanseri hücresi MCF-7 üzerinde doza bağlı bir şekilde apototik ve otofajik etkiye sahip olduğunu göstermiştir. Ayrıca, hücrelerin tipik parke taşı morfolojisi ekstrakt uygulamalarından sonra bozulmuş ve hücreler birbirleriyle temasını kaybetmiştir. Bu morfolojik değişiklik, ekstrasellüler matriks veya komşu hücrelerle hücre iletişiminin kaybı ile indüklenen programlanmış bir hücre ölümü tipi olan anoikise atfedilmiştir. Sonuç olarak, bu çalışmada birçok metodolojik eksiklik olmasına rağmen, bulgularımız *Valeriana officinalis* L.'nin meme kanseri tedavisinde potansiyel bir anti-kanser ajan olabileceğini düşündürmektedir.

Anahtar kelimeler: Fitoterapi, *Valeriana officinalis* L., Kediotu, Meme kanseri, Apoptosis, Otofaji, Anoikis

Abstract

Natural product-derived phytochemicals are now accepted as promising agents in developing new strategies for cancer treatment and prevention. The root extracts of valerian (*Valeriana officinalis* L.), which is a supplement widely used for improving circadian rhythm-dependent sleep disorders and insomnia, might be a good candidate in that context. In the present study we hypothesized whether extract of valerian root induce programmed cell death machineries in a human breast cancer model, MCF-7 cells. To test the hypothesis, we treated MCF-7 cells with the extract at different concentrations for 24 h. Giemsa staining was used to evaluate the apoptotic morphology and apoptotic index, and monodancylcadaverine assay was used to determine vacuoles that are associated with autophagic flux. Our results indicated that extracts of the roots of valerian have apototic and autophagic effect on human breast cancer cell MCF-7 in a dose dependent manner. Moreover, the typical

cobblestone morphology of the cells was disrupted after the extract treatments and the cells lost contact with each other. This morphological alteration was attributed to anoikis, is a programmed cell death type induced by loss of cell communication with extracellular matrix or neighboring cells. In conclusion, although this study has many methodological shortcomings, our findings suggest that *Valeriana officinalis* L. might be a potential anti-cancer agent for the treatment of breast cancer.

Keywords: Phytotherapy, *Valeriana officinalis* L., Valerian, Breast cancer, Apoptosis, Autophagy, Anoikis

1. Introduction

Breast cancer is one of the three most common cancers worldwide [1]. Although current conventional strategies seem to be hallmarks to treat or prevent of breast cancer, a series obstruction, such as side effects of anti-cancer agents and increased mortality, remains be an unresolved issue [2]. Recent advances in cancer chemoprevention with natural product-derived phytochemicals have encouraged researchers to develop novel and original strategies against various types of cancer [3-7]. Although their low toxicity and widespread availability make phytochemicals attractive in cancer treatment, cellular events underlying the mechanisms of action of phytochemicals and molecular targets have not yet been fully elucidated [8, 9]. However, evidence regarding this area have been increased with growing understanding the role of natural compounds such as capsaicin, catechins, lycopene, cucurbitacin B, curcumin, and resveratrol [9-11]. The root extracts of plant known as *Valeriana officinalis* L. or valerian has long been used as a sleep aid due to its sedative and hypnotic effect [12, 13]. In herbal therapy, valerian root is also known to be widely used against depression, cardiac arrhythmia [14], anxiety and epilepsy [15]. However, the potential effect of valerian on apoptotic and/or non-apoptotic pathways in cancer has not yet been resolved. Recently, it has been proposed that valeric acid, a product of valerian, may act as a histone deacetylase inhibitor and decrease proliferation of breast cancer cells, MCF-7 and MDA-MB-231 [16]. In the present study, our goal was to evaluate the potential effect of the extracts of valerian root on the apoptotic and non-apoptotic cell death machinery in a breast cancer cell line model MCF-7.

2. Materials and Methods

2.1. Maintenance of Cell Culture

Human breast cancer cell line MCF-7 were obtained from ATCC (HTB-22™) and grown in RPMI 1640 medium containing 10% fetal bovine serum, 1% L-glutamine and 1% penicillin-streptomycin at 37 °C containing 5% CO_2 atmospheric conditions.

2.2. Preparation of the Root Extracts

The root extracts of valerian were purchased commercially in capsule form (Shiffa Home, Turkey) and pulverized. To main stock solution, 625 mg of extract was diluted in 5 mL distilled water, heated to 100 °C in a water bath for 20 min and centrifuged at 1000 x g for 10 min. The supernatant was filtered through a hydrophilic membrane containing 0.22 µM pores and stored at +4 °C until used in the experiments. In the treatments, the main stock solution was diluted in culture medium with dimethyl sulfoxide (DMSO) to obtain the specified doses.

2.2. MTT Assay

To test cytotoxicity of the extracts of valerian root, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. Briefly, 100 µL cells at a density of 1 x 10^4 were grown in 96 well plates containing 100 µL medium at 37 °C and 5% CO_2 atmosphere for 24 hours. Subsequently, the increasing concentrations of the root extract (0,5 mg/mL, 1 mg/mL, 2 mg/mL, 4 mg/mL, 8 mg/mL, and 10 mg/mL) in 100 µL serum-free medium were added to wells in five replicates and incubated for 24 hours at 37 °C and 5% CO_2 atmosphere. At the end of incubation time, MTT solution (5 mg/mL) (M5655, Sigma) was added to each well at a volume of 20 µL and incubated for 4 hours at same temperature and atmospheric condition and 200 µL of solubilisation solution (DMSO) was added to each well. To determine background absorbance values, 200 µL of DMSO was added to 3 wells. After 5 minutes incubation in shaker, absorbance value of wells was measured in a microplate reader (Tecan, Switzerland) at 570 nm with a reference wavelength of 720 nm. The mean percentage of viability was calculated with following formula:

$$Cytotoxicity = 1 - \frac{Mean\ absorbance\ of\ treated\ cells - Background\ absorbance}{Absorbance\ of\ positive\ control - Background\ absorbance}\ x\ 100$$

IC_{50} values of valerian root extracts at 24 hours were determined as 3.67 mg/mL with commercial software (GraphPad Prism 9, ver.9.3.1, CA, USA).

2.3. Giemsa Staining

Morphological and apoptotic analyses were accomplished with conventional Giemsa staining. To stain MCF-7 cells with Giemsa, 500 µL cells at a density of 1 x 10^5 cell/well were seeded onto 12 mm ∅ sterile coverslips in 24-well plates for 24 hours under an atmosphere of 5% CO_2 at 37°C. At the end of incubation, cells were treated with medium including valerian root extracts at concentrations of 3,67 mg/mL-IC_{50}-, 5 mg/mL and 10 mg/mL for 24 hours and fixed with methanol for 10 minutes. After the washing periods with PBS, cells were stained with 4% Giemsa (48900, Sigma) for 15 minutes at room temperature. Morphological and apoptotic evaluation and collection of images were performed

with an Olympus BX43 microscope equipped with an Olympus DP74 camera. Five ROIs of 1.063 x 837 pixels for each experimental group were randomly selected and cells representing apoptotic morphology were counted. Mean apoptotic index was calculated with following equation:

$$AI = \frac{\sum Apoptotic\ cell}{\sum Apoptotic\ cell + \sum Non-apoptotic\ cell}\ x\ 100$$

2.4. Monodansylcadaverine (MDC) Staining for the Determination of Autophagic Vacuoles

To monitorize autophagic vacuoles, monodansylcadaverine (MDC) staining was used. Briefly, 500 µL cells were grown onto sterile coverslips (12 mm ∅) in 24-well plates including at a density of 1 x 10^5 cells/well for 24 hours at 37 °C in 5% CO_2. Three of wells were left blank to further evaluation as positive control and valerian root extract was added to the remaining wells at different concentrations (3,67 mg/mL-IC_{50}-, 5 mg/mL, and 10 mg/mL). After the incubation for 24 hours in same temperature and atmospheric conditions, cells were washed three times for 5 minutes each with phosphate buffered solution (PBS) and stained with 0.005 mM of MDC in serum-free medium for 10 minutes at 37 °C in 5% CO_2. At the end of staining period, cells were fixed with cold methanol at -20 °C for 10 minutes and washed with PBS. Cells were then analyzed in a fluorescence microscope (Olympus BX43, Japan) using a DAPI (4′,6-diamidino-2-fenilindol)- specific filter cube and the images containing regions of interest (ROIs) were acquired as jpg and tiff formats by using DP70 camera system (Olympus, Japan). To statistical quantification, images were transferred to ImageJ software (ver. 1.46c, NIH, Bethesda, MD, ABD, http://rsb.info.nih.gov/ij/) and "cell counter" plug-in of the software was used to count MDC-labeled autophagic vacuoles in randomly selected five cells for each experimental group.

2.5. Statistical Analysis

The number of autophagic vacuoles and apoptotic index between groups were determined by one-way analysis of variance (ANOVA) using GraphPad Prism 9 (ver.9.3.1, CA, USA). Tukey was used for post-hoc correction. All results were expressed as mean SEM and $p < 0.05$ was considered a significant difference between groups.

3. Results

3.1. MTT Assay

The half of the maximum inhibitory concentration (IC_{50}) is the most widely used method for determining the efficacy of a drug. This value indicates how much drug is needed to inhibit a biological process by half, thus providing a measure of the potency of an antagonist drug in pharmacological research. Therefore, in our study, we first determined the IC_{50} value of the root extracts of *V. officinalis* L. at 24 hours by MTT assay, which is a standard and cost-effective application. Our results showed that the 24-hour IC_{50} concentration of this extract was 3.67 mg/mL for MCF-7 cells (Figure 1).

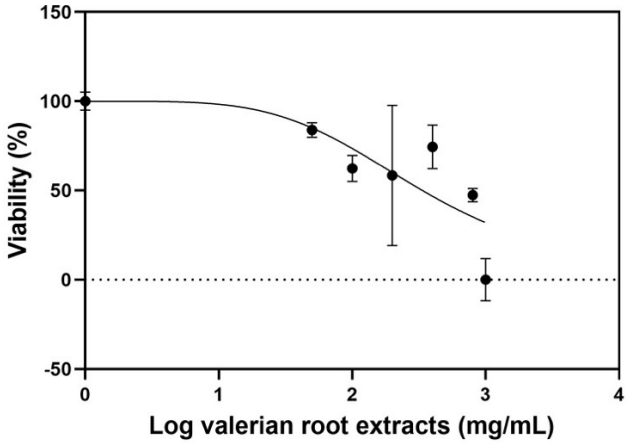

Figure 1. The effect of the valerian root extracts on human breast cancer cell line MCF-7 measured by MTT assay in the presence of the root extract concentrations (0,5 mg/mL, 1 mg/mL, 2 mg/mL, 4 mg/mL, 8 mg/mL, and 10 mg/mL) for 24 hours. The IC_{50} for MCF-7 was 3.67 mg/mL.

3.2. Giemsa Staining

Histological analysis of MCF-7 cells by Giemsa staining showed that the cells in the control group were bipolar and formed colonies in places, thus giving a "cobblestone appearance" typical for these cells in monolayer culture. At higher magnification (100 x), we found that the cells exhibited a diffuse cytoplasm with prominent Golgi areas. The nuclear membrane and nucleolus were clearly observed and regions of heterochromatin and euchromatin could be distinguished in control group (Figure 2a). After treatment with IC_{50}, 5 mg/mL and 10 mg/mL concentrations of the root extract we observed that colonization of cells is gradually reduced compared to control group, presumably due to disruption of epithelium-like morphology in the cells (Figure 2 a-d). In particular, the cobblestone appearance of the cells disappeared after the treatment of the root extracts at concentration of 10 mg/mL (Figure 2d). Furthermore, some cells showed typical features of apoptotic morphology such as loss of bipolar organization, increased cytosolic vacuolization, chromatin condensation and bleb formation at concentrations of IC_{50}, 5 mg/mL and 10 mg/mL (Figure 2b-d). Statistically, the apoptotic index (AI) for MCF-7 cells was gradually increased at all treated concentrations of the valerian root. The number of apoptotic cells was significantly increased especially at 10 mg/mL concentration of the extracts ($p < 0.05$; mean ± SEM: 17.10 ± 2.95) when compared to control group (Figure 2e). These results suggest that the root extract of valerian had a dose-dependent apoptotic effect on MCF-7 breast cancer cells.

3.3. MDC Staining

The autophagic effect of the extracts of valerian root was analyzed by identification of autophagic vacuoles using MDC. This compound is an autofluorescent substrate and widely used for probing autophagic vacuoles in cultured cells [17]. We found that the number of autophagic vacuoles, which was low in the control group, increased with the treatment of extracts, and this increase was statistically significant, especially at 5 mg/mL and 10 mg/mL concentrations as compared to control group ($p < 0.001$ and $p < 0.0001$, respectively; Figure 3). This finding suggests that increasing doses of the root extracts of valerian may have an autophagic effect in breast cancer cells.

4. Discussion

Cancer is one of the major health problems threatening the human health. The elucidation of some of the molecular mechanisms involved in the functioning of cancer with today's advancing technologies has led to the development of a large number and variety of new anti-cancer drugs. However, conventional chemotherapies using chemically synthesized drugs remain insufficient to control cancer. Strong adverse reactions due to chemotherapy, failure of improvement the survival rate and financial problems led to the emergence of alternative approaches for the treatment and prevention of cancer. Naturally occurring compounds or phytochemicals derived from plants, marine life of microorganisms are not only natural sources of drugs but also an important source for cancer treatment. Many phytochemicals and derivative compounds are known to have anti-tumor potential for the treatment of cancer patients and do not produce the adverse effects of synthetic chemotherapy. However, the underlying mechanisms by which natural products prevent cancer have not yet been fully elucidated. Therefore, *in vitro,* and *in vivo* evaluation of candidate plant extracts for potential anti-cancer activity is important to establishing effective and side-effect-free phytochemical-based anti-cancer treatment strategies. In this study, the potential effect of valerian root extracts on apoptotic and non-apoptotic cell death mechanisms in human breast cancer cell line MCF-7 was investigated by conventional methodology. Our finding showed that increasing doses valerian root extracts may cause both apoptotic and autophagic effect. Our ANOVA results indicated that while apoptotic activity was significantly increased only after 10 mg/mL concentration of the root extract, the autophagic activity increased after the 5 mg/mL as well as 10 mg/mL concentration of the root extract. This finding could be suggested as at the lower concentration of the valerian root extract, for instance 5 mg/mL, but not the IC_{50}, cells may undergo autophagy rather than apoptosis. Autophagy (type 2 cell death) is an important physiological event in eukaryotic cells and involves the degradation of cellular components in lysosomes after being surrounded by a membrane [18, 19]. As a quality control mechanism, autophagy is triggered by intracellular or extracellular signals including nutritional, metabolic, oxidative, or pathogenic imbalances [20]. Typically, autophagy flux proceeds with de novo formation of a double layered vesicular membrane. Vesicle elongation, maturation of autophagosome that surrounding cellular debris, autophagosome-lysosome fusion and degradation of cargo are subsequent stages of autophagic flux [21]. In early stages of tumorigenesis, autophagy as a survival pathway and quality control mechanism prevents tumor initiation and suppresses cancer progression. When tumors progress to later stages and are exposed to environmental stresses, autophagy contributes to the survival and growth of tumors and increases the aggressiveness of cancers by facilitating metastasis [22]. Apoptosis (type 1 cell death) is a genetically conserved physiological type of death regulated by intracellular and/or extracellular signals and characterized by morphological changes in the cell, including nuclear fragmentation and condensation, mitochondrial outer membrane permeabilization (MOMP), blebbing, cell shrinkage and apoptotic body formation [23]. Previous reports indicated that various plant-derived natural compounds may induce apoptosis [24, 25]. On the other hand, disrupted circadian rhythm is a potential risk factor for cancer prognosis [26, 27]. Most recently, Shi et al. (2021), based on this possible relationship between circadian rhythm and cancer development, investigated the anti-tumor activity of the valeric acid in breast cancer [16]. Although this compound may inhibit to cell proliferation by mediating epigenetic modification, there is currently insufficient information about its possible mechanism of action. In our study, classical cobblestone-like morphology of MCF-7 cells was disrupted upon the treatment of root extracts. We, therefore, considered that the root extracts of valerian alter the cellular morphology and disrupt intercellular communication thereby prevent colonization of MCF-7 cells in monolayer culture. In epithelial cells, the breakdown of cellular connections such as integrins results in the activation of an apoptotic pathway called anoikis [28]. Considering that MCF-7 is an epithelial cancer cell, the apoptotic morphology induced by the treatment of valerian extract in our study may be related to anoikis. Indisputably, to confirm this scenario, the expression levels of cell surface molecules involved in cellular adhesion, extracellular matrix components or molecules associated with the external apoptotic pathway need to be investigated.

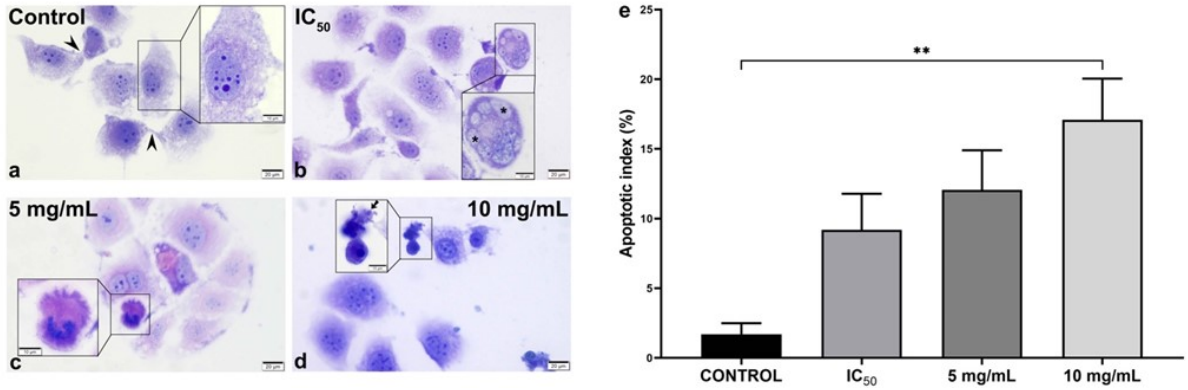

Figure 2. Morphological comparison of apoptotic and non-apoptotic cells at increasing concentrations (3.67 mg/mL-IC_{50}-, 5 mg/mL and 10 mg/mL) of valerian root extracts by Giemsa staining (a-d). Cellular contacts between non-apoptotic cells in control group were indicated with arrowheads. The cytosolic vacuolization (asterix), chromatin condensation and bleb formation (arrow) are hallmarks of apoptosis (a-d, insets). The number of apoptotic cells was significantly increased in the group treated with 10 mg/mL extract for 24 hours compared to the control group (**$p < 0.05$; mean ± SEM: 17.10 ± 2.95) (e).

For instance, it has been found that suppression of the protein tyrosine phosphatase 1B, an inhibitor of insulin signaling pathways and highly expressed in the mammary gland, may modulate cell-cell adhesion, and have an anoikis-like effect [29].

Only a few reports on the effect of compounds isolated "Valerianaceae" family members on autophagy have published in the literature. Iridoid glycosides of *Valeriana jatamansi*, a member of the Valerianaceae family, were found to cause autophagy-mediated cell death in human colorectal carcinoma cells [30]. Consistently, iridoid esters of *Valeriana jatamansi* have been found to induce apoptotic and autophagic cell death in breast cancer [31]. To the best of our knowledge, this is the first study to demonstrate the autophagic effect of *Valeriana officinalis* L. root extracts in breast cancer cell line MCF-7. However, the molecular mechanisms underlying this agent-mediated autophagy in breast cancer are not yet clear.

In conclusion, in this report, we have focused the hypothesis that valerian (Valeriana officinalis L.) root extract may have an inducing effect on cell death mechanisms in human breast cancer cell MCF-7. The most prominent result of this study is the finding that the root extracts of *Valeriana officinalis L.* increases cell death mechanisms in the human breast cancer cell line MCF-7 in a dose dependent manner. Possible death mechanisms in this process are autophagy, apoptosis and anoikis, an apoptotic type of death associated with the loss of cellular connections. To our knowledge, this is the first study in the literature to report that *Valeriana officinalis L.* root extracts have an autophagic effect on human breast cancer cells MCF-7. Based on histologic analysis, we concluded that the cells loss of contact and classical cobblestone-like morphology of the cells and their displacement from each other by treatment may lead to anoikis. However, this claim requires verification with detailed analyses.

Based on histologic analysis, we concluded that the loss of contact and the classic cobblestone-like morphology of the cells and their displacement from each other by treatment may lead to anoikis. In fact, if this hypothesis is confirmed by future studies testing in detail the expression levels of the players involved in the anoikis mechanism, the potential of *Valeriana officinalis L.* extracts as an important strategy in response to epithelium-derived tumor cells will increase.

This study has some limitations. Firstly, we suggest that early findings from this study be verified by molecular biological techniques such as immunocytochemistry, western blotting or flow cytometry targeting specific molecules that play a fundamental role in apoptosis, autophagy and anoikis pathways. In this study, due to funding constraints, the potential effects of *Valeriana officinalis L.* extracts were only investigated in a breast cancer cell line. The fact that the study was based on a single cell line can be considered as a research limitation. Therefore, there is a need to investigate whether the extracts would have a similar effect on normal control cells and cell lines modeling different types of cancer. Finally, in our study, a commercial product was used as *Valeriana officinalis* L. root extracts. Of the approximately 12000 plant taxa in our country, 3649 are endemic [32]. The use of extracts obtained from valerian plants growing in our country in future research and obtaining possible expected results may enable the production of a novel anti-cancer drug that will provide added value to our country.

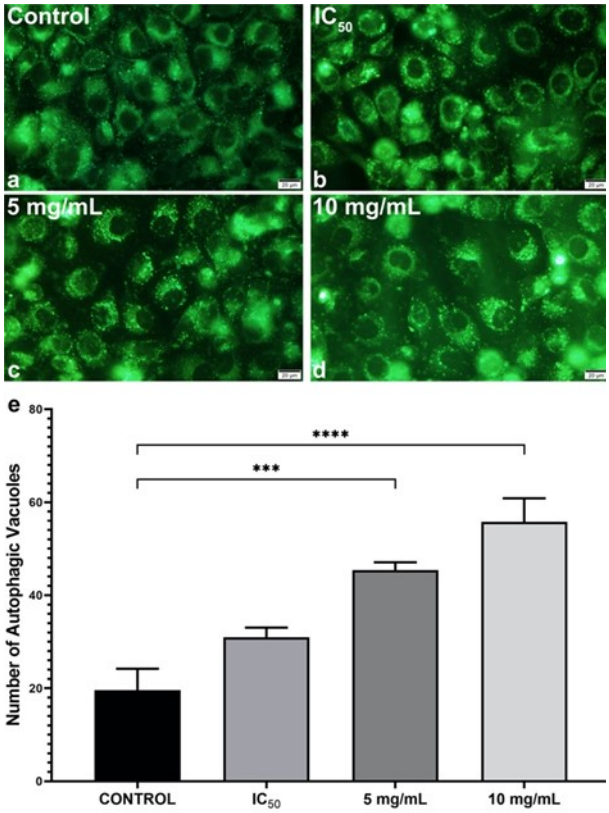

Figure 3. Representing images of MDC-positive autophagic vacuoles (a-d). ANOVA results showed that autophagic vacuoles were significantly increased after 24 hours treatments of 5 mg/mL and 10 mg/mL extracts as compared to control group (*** $p < 0.001$, mean ± SEM: 45.4 ± 1.7; ****$p < 0.0001$, mean ± SEM: 55.8 ± 5.03, respectively) (e).

5. Acknowledgements and Conflicts of Interest

This work was supported by the Manisa Celal Bayar University Scientific Research Projects Coordination Unit. Project Number: 2021-121. The authors declare no conflict of interest.

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