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GEBZE TECHNICAL UNIVERSITY
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THE POTENTIAL AUTOPHAGY-RELATED MOLECULAR TARGETS OF WATERCRESS EXTRACTS IN PANCREAS CANCER CELLS

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A THESIS OF MASTER OF SCIENCE
DEPARTMENT OF BIOTECHNOLOGY

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GEBZE TEKNİK ÜNİVERSİTESİ
LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ

SU TERESİ ÖZÜTLERİNİN PANKREAS KANSERİ HÜCRELERİNDE OTOFAJİ İLE İLİŞKİLİ POTANSİYEL MOLEKÜLER HEDEFLERİ

NASİP ÜMRAN TURAN

YÜKSEK LİSANS TEZİ
BİYOTEKNOLOJİ ANABİLİM DALI

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EKİM 2024

MASTER of SCIENCE JURY APPROVAL FORM

A thesis submitted by Nasip Ümran TURAN defended on 18/10/2024 before the jury formed with the 16/07/2024 date and 2024/38 numbered decision of the GTU Graduate Administration Board, has been accepted as a MASTER of SCIENCE thesis in the Department of Biotechnology.

JURY

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To my

beloved family

ABSTRACT

Functional foods are dietary products that offer health benefits beyond basic nutrition, enhancing the nutritional profile of the diet, preventing chronic diseases, supporting immune function, and enabling personalized nutrition. This study investigates the potential therapeutic effects of selenium-enriched watercress, a promising functional food candidate, on pancreatic cancer. Watercress plants grown in new generation hydroponic systems at different selenium concentrations (0, 0.25, and 0.50 mg/L) were extracted using 70% ethanol. Sets of experiments were conducted to evaluate cytotoxicity, colony formation, cell survival, membrane potentials, and mTOR and autophagy-related protein expressions on PANC-1 and MIA PaCa-2 pancreatic cancer cells. Selenium-enriched watercress extracts at 0.25 mg/L and 0.50 mg/L concentrations significantly inhibited cancer cell proliferation and colony formation. Fluorescent staining analysis showed that watercress extracts had a significant effect on cell death and survival in pancreatic cancer cells through reactive oxygen species and cellular mitochondrial activity in a dose-dependent manner. These findings highlight the potential of selenium-enriched watercress to modulate key pathways involved in cancer progression and demonstrate its feasibility as a functional food in cancer treatment. Further research is necessary to fully elucidate the mechanisms through which selenium-enriched watercress exerts its effects and to optimize its therapeutic application as a functional food.

Keywords: Functional foods, Watercress, Selenium, Cancer

ÖZET

Fonksiyonel gıdalar, temel beslenmenin ötesinde sağlık yararları sunan, diyetin besin profilini geliştiren, kronik hastalıkları önleyen, bağışıklık fonksiyonunu destekleyen ve kişiselleştirilmiş beslenmeye olanak sağlayan diyet ürünleridir. Bu çalışma, umut verici bir fonksiyonel gıda adayı olan selenyumla zenginleştirilmiş su teresinin pankreas kanseri üzerindeki potansiyel terapötik etkilerini araştırıyor. Yeni nesil hidroponik tarım sistemlerinde selenyumca zengin ortamda (0, 0.25 and 0.50 mg/L) selenyumca zenginleştirilen watercress bitkisinin, deneylerde kullanılmak üzere %70 EtOH kullanılarak özütü elde edilmiştir. Sitotoksisite, koloni oluşumu, hücre sağkalımı, membran potansiyelleri, mTOR ve otofajiye bağlı protein ekspresyonlarını değerlendirmek amacıyla PANC-1 ve MIA PaCa-2 pankeras kanseri hüreleri üzerinde deneyler yapıldı. Elde edilen sonuçlarda, selenyumla 0,25 mg/L ve 0,50 mg/L konsantrasyonlarda zenginleştirilmiş su teresi, kanser hücresi çoğalmasını, koloni oluşumunu önemli oranda inhibe ettiği görüldü. Floresan boyama analizi, su teresi ekstraktlarının pankreas kanseri hücreleri üzerinde reaktif oksijen türleri ve hücresel mitokondriyal aktivite yoluyla doza bağlı bir şekilde hücre ölümü ve hücre sağkalımı üzerinde dikkate değer bir etkiye sahip olduğunu gösterdi. Bu bulgular, selenyumla zenginleştirilmiş su teresinin kanserin ilerlemesinde rol oynayan temel yolları modüle etme potansiyelini vurguluyor ve kanser tedavisinde fonksiyonel bir gıda olarak uygulanabilirliğini ortaya koyuyor. Selenyumla zenginleştirilmiş su teresinin etkilerini gösterdiği mekanizmaları tam olarak aydınlatmak ve fonksiyonel bir gıda olarak terapötik uygulamasını optimize etmek için daha fazla araştırma yapılması gerekmektedir.

Anahtar Kelimeler: Fonksiyonel gıda, su teresi, selenyum, kanser

ACKNOWLEDGEMENTS

First and foremost, I would like to extend my heartfelt gratitude to my esteemed advisor, Prof. Dr. Elif Damla ARISAN, whose unwavering support and invaluable contributions have been instrumental in my personal and professional development. Throughout my master's degree, I faced many challenges and setbacks, but her steadfast encouragement and exemplary role modeling strengthened my resolve and bolstered my self-confidence. Every day, I appreciate the profound wisdom in her guidance, for which I am deeply grateful.

I would also like to express my deepest gratitude and sincere thanks to my co-supervisor, Asst. Prof. Ümit Barış KUTMAN, for his invaluable collaboration and guidance in structuring my thesis. His profound contributions and unwavering support were crucial to successfully completing my master's thesis. I also extend my deep gratitude to the members of KUTMAN Lab for their support and shared experiences throughout this thesis.

ARISAN Lab is a unique place where joy and intense focus coexist, creating a nest of knowledge and goodwill. Thank you to all my labmates for providing a peaceful and motivating daily environment. Special thanks to Başak Dalbayrak for her patience and willingness to answer my questions in detail, time and time again. I am also grateful to my dear friend Rıdvan Aziz Ayaz, who has supported me from the very beginning and whose advice I have always admired.

I want to thank my bachelor's advisor, Assoc. Prof. Hasan Ufuk ÇELEBİOĞLU and my mentor, Ezgi SARI, and my best friends Gamze ÖZBAY and Aybike Yaren YILDIZ for making my academic journey more meaningful at every step. I am the first-gen highest educated in my family, and I am grateful to my beloved mother, Sabriye İNAN, and brother, Ümit TURAN, for their support. Also, the biggest thanks to my precious daughter, Şans, who never took her tiny paws off my laptop while writing this thesis.

Finally, I would like to thank all my professors and friends of the GTU Institute of Biotechnology for making this place a home for us.

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LIST OF SYMBOLS AND ABBREVIATIONS

°C	:	Celcius
h	:	Hour
%	:	Percentage
mL	:	Milliliter
mg	:	Milligram
µg	:	Microgram
µL	:	Microliter
p	:	P value
AMPK	:	AMP-activated protein kinase
ATG5	:	Autophagy Related 5
ATG7	:	Autophagy Related 7
ATG12	:	Autophagy Related 12
BODIPY	:	Fluorinated Boron-Dipyrromethene
CO_2	:	Carbon dioxide
dH_2O	:	Distilled water
DMEM	:	Dulbecco's Modified Eagle Medium
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic Acid
EtOH	:	Ethanol
GPxs	:	Glutathione peroxidases
HBBS	:	Hank’s Balanced Salt Solution
HSD	:	Honestly significant difference
kDa	:	Kilodaltons
LC-3 A/B	:	Microtubule-associated protein 1A/1B-light chain 3
Min	:	Minute
mTOR	:	Mammalian Target of Rapamycin
NaCl	:	Sodium Chloride
NF-κB	:	Nuclear factor kappa-light-chain-enhancer of activated B cells
PBS	:	Phosphate-Buffered Saline
PEITC	:	Phenethyl isothiocyanate
PI	:	Propidium Iodide
PI3K	:	Class III phosphatidylinositol 3-kinase
ROS	:	Reactive Oxygen Species
RPM	:	Revolutions per minute
TBS	:	Tris-Buffered Saline
TBS-T	:	Tris-Buffered Saline-Tween 20
TrxRs	:	Thioredoxin reductases
ULK1	:	Unc-51 Like Autophagy Activating Kinase 1
ROS	:	Reactive Oxygen Species
Se	:	Selenium

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1. INTRODUCTION

1.1. The Importance and Health Benefits of Functional Foods

Functional foods are dietary products that provide additional health benefits beyond their essential nutritional value. Functional food covers a wide range of natural and fortified foods, including fruits, vegetables, nuts, cereals, dairy products, and beverages. These foods are functionalized by enriching them with bioactive compounds, such as vitamins, minerals, and other health-promoting nutrients **[1].** The importance of functional foods in nutrition has grown as studies are increasingly highlighting their ability to address various health issues. Functional foods enhance the nutritional profile of the diet, prevent chronic diseases, support immune function, and enable personalized nutrition **[2]**. Dietary fibers from vegetables and cereals are crucial for maintaining digestive health and have been linked to reduced risks of certain cancers and cardiovascular diseases **[3-5]**. Cruciferous vegetables such as broccoli, cauliflower, and cabbage contain phytochemicals beneficial for cancer prevention. Additionally, foods rich in antioxidants, like berries, nuts, and green tea, help protect the body from oxidative stress and inflammation **[6,7]**.

Figure 1.1 Categorization of Functional Foods [8].

The production and distribution of functional foods are particularly vital for addressing public health issues in regions with limited access to nutritious food. Integrating functional ingredients into everyday foods can lead to significant health improvements. Moreover, the production and consumption of functional foods offer environmental and economic benefits. Sustainable farming practices and the use of locally sourced ingredients can reduce the carbon footprint of food production. The market for functional foods can also drive economic growth by creating new opportunities for farmers, food manufacturers, and retailers **[9,10]**. Interest in functional foods has increased due to emerging nutritional deficiencies and associated health issues. Thus, functional foods therefore play a vital role in improving overall health and well-being. By enhancing the diet's nutritional profile, preventing chronic diseases, supporting immune function, and enabling personalized nutrition, functional foods are essential in modern nutrition. Their integration into daily diets and sustainable production methods can significantly improve public health and contribute to economic growth **[11]**.

1.2. Watercress and Its Bioactive Properties

Nasturtium officinale R. Br., commonly known as watercress, is a semi-aquatic perennial herb belonging to the Brassicaceae family. Native to Western Asia, Europe, India, and Africa, watercress has the ability to adapt to many environments, allowing it to be grown outside its original habitat and thrive in other climates **[12,13]**. Watercress has been used as a medicinal and food crop for many decades, and now it has become a global phenomenon and is a crucial element in both culinary and health communities worldwide. The European Food Safety Authority has acknowledged watercress safety and nutritional significance due to its beneficial effects on human health **[14-17]**.

Figure 1.2 Image of *Nasturtium officinale* R. Br. (Watercress).

Watercress contains a wide variety of nutrients and chemicals, including folic acid, carotenoids, glucosinolates, vitamins B, C, and E, pro-vitamin A, calcium, iron, iodine, and sulfur. These elements are well known for their benefits for overall well-being. Watercress is considered a low-calorie food due to its high water content of about 93% **[18]**. Although it contains low amounts of carbohydrates and lipids, it also provides a significant amount of protein. Watercress contains a significant amount of bioactive components, including flavanols, hydroxycinnamic acids, and glucosinolates. Previous research showed that isothiocyanates exhibit potent antioxidant, anti-inflammatory, and anticancer activities, helping to detoxify carcinogens and protecting cells from oxidative damage. The most common of these is phenethyl isothiocyanate (PEITC) **[19,20]**. Phenethyl isothiocyanate (PEITC) has garnered significant attention in cancer research for its potential as a potent anti-cancer agent. Several studies have been conducted on the mechanisms by which PEITC exerts its anticancer effects **[21]**. In cancer research with phenethyl isothiocyanates, the link between cell cycle arrest and induction of apoptosis, one of the key mechanisms, comes to the fore **[22]**. Moreover, PEITC has been shown to cause cell cycle arrest and trigger apoptosis in various cancer cell lines. PEITC has been identified as a regulator of intracellular ROS levels and contributes to anticancer activity. It selectively induces oxidative stress in cancer cells by modulating ROS levels, leading to their apoptosis while sparing normal cells **[23]**. It has been shown to play a significant role in regulating multiple signaling pathways involved in cancer progression. PEITC modulates autophagy, a process critical for cellular homeostasis and survival, which can contribute to cancer cell death when dysregulated. Additionally, PEITC inhibits the NF-κB and Akt/mTOR pathways, both of which are crucial for cell survival, proliferation, and metastasis. NF-κB is a transcription factor involved in inflammatory responses and cell survival, while the Akt/mTOR pathway is key in regulating cell growth, metabolism, and survival **[24-26]**.

1.3. Selenium and Health

Selenium (Se) is an essential micronutrient required in trace amounts and plays a vital role in human metabolism, development, and hormonal equilibrium. It is a crucial component of selenoproteins, and its deficiency can lead to serious chronic diseases **[27]**. Humans possess a total of 25 selenoproteins, which may be categorized into six distinct functional groups. These groups include proteins responsible for selenium transport, selenocysteine synthesis, protein folding, hormone metabolism, redox signaling, and reductase/peroxidase activity. Selenoproteins are present in several cellular compartments, including mitochondria, endoplasmic reticulum, nucleus, cell membrane, and Golgi membrane. They serve numerous activities, such as acting as antioxidants, reducing inflammation, metabolizing hormones, and regulating endoplasmic reticulum stress **[28]**. Moreover, certain selenoproteins control inflammatory cytokines, modulate immune function, and inhibit pro-inflammatory pathways, all contributing to their role in reducing inflammation in the case of cancer. For example, glutathione peroxidases (GPxs) and thioredoxin reductases (TrxRs), both selenoproteins, are critical in mitigating oxidative stress and maintaining cellular redox balance, which is crucial for cancer prevention **[29-31]**. Recent research has suggested that selenium supplementation may improve the efficacy of some cancer therapies. Selenium compounds have been shown to increase the cytotoxicity of chemotherapeutic agents, thereby improving their efficacy **[32]**. In addition, selenium's involvement in DNA repair mechanisms highlights its potential in cancer prevention and therapeutic health **[33]**. However, balancing selenium intake is crucial, as excess selenium can lead to toxicity and adverse health effects. This delicate balance highlights the selenium-enriched functional foods, which aim to provide an optimal level of selenium through regular diets **[34]**. Ensuring proper selenium levels in these foods can optimize selenium supplementation in clinical settings, offering a safe and effective approach to enhancing public health.

1.4. Selenium Biofortification in Functional Foods

Biofortification is a promising agricultural application that increases the specific nutrient content in crops through effective agronomic practices, conventional plant breeding, or state-of-the-art biotechnological methods. The increasing practice of enriching functional foods with selenium is a crucial strategy to address selenium deficiency and improve public health. This methodology is particularly pertinent for cultivating selenium-enriched foods that ensure sufficient intake through daily diets, notably in regions characterized by low selenium soil levels **[35]**. Hydroponic cultivation, a soil-free agricultural method, offers numerous advantages for biofortification. This approach allows for precise control over nutrient delivery, ensuring optimal growth conditions and consistent enrichment levels. Hydroponics uses water more efficiently, reduces the need for pesticides, and minimizes environmental impact compared to traditional farming methods. Additionally, it allows for year-round cultivation, increasing the feasibility of producing nutrient-enriched crops regardless of soil conditions. The feasibility of using hydroponic cultivation for other nutrient enrichments is equally promising. For instance, iron-enriched spinach can be grown hydroponically to combat iron deficiency anemia, a common nutritional issue. Similarly, fortifying tomatoes with lycopene, an antioxidant, can be achieved through hydroponics to enhance their health benefits and potentially reduce the risk of chronic diseases **[36, 37]**

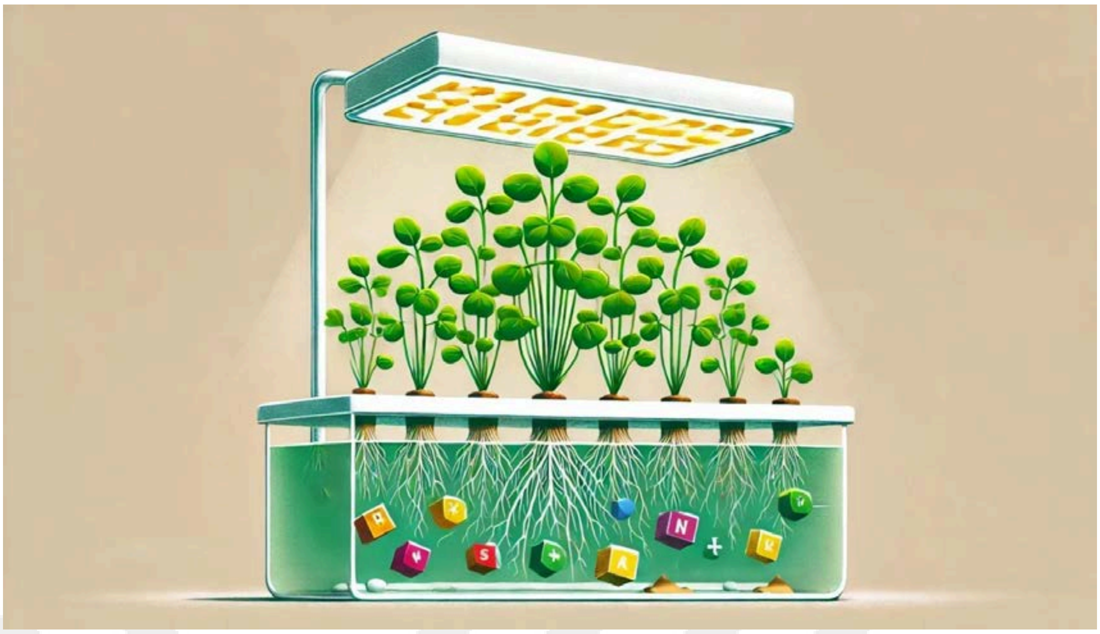

Figure 1.3. Illustration of hydroponic cultivation system **[38]**.

Enriching watercress with selenium through hydroponics exemplifies how this method can enhance the nutritional quality of functional foods. Selenium is vital for antioxidant defense, immune function, and thyroid hormone metabolism, making its inclusion in diets crucial for overall health. By cultivating selenium-enriched watercress hydroponically, we can ensure a reliable source of this essential nutrient, addressing deficiencies and promoting health benefits **[39,40]**. Agronomic biofortification techniques, notably the application of selenium fertilizers, have been efficaciously employed in the cultivation of staple crops such as wheat, rice, and broccoli **[41]**. These selenium-enriched crops are subsequently utilized in the production of an array of functional foods, encompassing bread, cereals, and vegetable-based products. Empirical studies have substantiated that biofortified crops substantially elevate selenium status among populations, thereby ameliorating the incidence of health complications associated with selenium deficiency **[42]**. In addition to increasing selenium intake, biofortified functional foods offer numerous health benefits. They are effective in enhancing antioxidant capacity, fortifying immune function, and diminishing the risk of chronic diseases, including malignancies. The evolution of selenium-enriched functional foods is congruent with the escalating consumer predilection for nutritionally fortified foods that proffer health benefits surpassing fundamental nutrition **[43]**. The future of selenium biofortification appears auspicious, underpinned by ongoing research dedicated to refining biofortification methodologies, elucidating the bioavailability of selenium from biofortified foods, and investigating the synergistic interactions of selenium with other nutrients. As global awareness of health intensifies, selenium biofortification in functional foods is set to play a crucial role in improving nutritional status and promoting overall well-being **[44]**.

1.5. Pancreatic Cancer

Pancreatic cancer is a highly lethal malignancy with limited diagnostic and treatment modalities and is expected to become the leading cause of cancer-related deaths in the United States over the next two to three decades. The five-year survival rate for individuals diagnosed with this cancer in the USA is a mere 13%, primarily due to diagnoses at advanced or metastatic stages. While the median age for pancreatic cancer diagnosis is 70 years, recent trends indicate an increasing incidence among younger individuals, particularly those assigned female at birth. Even for patients with localized, resectable tumors, the prognosis remains poor, with a five-year post-surgery survival rate of only 20% [45, 46]. The development of pancreatic cancer is influenced by both non-modifiable and modifiable risk factors. Non-modifiable risk factors that are beyond the control of the individual include age, gender, ethnicity, blood type, genetic predisposition, and family history. Modifiable risk factors associated with pancreatic cancer that can be modified through lifestyle modifications or medical interventions include gut microflora, alcohol consumption, smoking, obesity, diet, and infections [47, 48].

1.6. Role of Nutrition in Pancreatic Cancer

Nutrition is a significant determinant of cancer risk, influenced by metabolic and genetic variables. Diets rich in fruits, vegetables, and whole grains are linked to a reduced chance of developing cancer, while those heavy in processed meats and sweets are associated with an increased risk [49, 50]. The etiology of pancreatic cancer is multifactorial, and emerging evidence indicates a potential link between nutrition and pancreatic cancer risk. Nutritional factors play a pivotal role in pancreatic carcinogenesis through mechanisms such as oxidative stress, inflammation, and insulin resistance [51-53]. Epidemiological studies have shown a relationship between consuming fruits and vegetables and a reduced risk of pancreatic cancer. This inverse association has been corroborated by case-control studies highlighting significant benefits from consuming fruits and vegetables such as citrus fruits, melons, berries, dark green vegetables, tomatoes, beans, peas, deep yellow vegetables, fiber, and whole grains. Alongside findings that a plant-based diet may reduce the risk of pancreatic cancer, diets rich in red and processed meats have been associated with a higher risk [54]. These foods contain high saturated fats and heterocyclic amines, which can cause oxidative DNA damage and promote carcinogenesis. Additionally, cooking meats at high temperatures increases cancer risk by producing carcinogenic compounds such as polycyclic aromatic hydrocarbons [55-57]. The Western diet, characterized by high consumption of red and processed meat, refined grains, and sugary drinks, is associated with an increased risk of pancreatic cancer. Conversely, the Mediterranean diet, which emphasizes fruits, vegetables, whole grains, legumes, nuts, and olive oil, is linked to a reduced risk. The protective effects of the Mediterranean diet are attributed to its high content of anti-inflammatory and antioxidant compounds and its favorable impact on metabolic health [58-60]. Consuming several nutrient-rich foods, exceptionally functional foods that offer additional health benefits can significantly lower cancer risk. Functional foods like selenium-enriched products enhance antioxidant capacity and immune function, improving cancer prevention. Integrating these foods into a balanced diet supports metabolic health and reduces inflammation, playing a vital role in reducing the risk of pancreatic cancer [61, 62].

1.7. Molecular Targets of Autophagy in Pancreatic Cancer

Autophagy is a conserved cellular process that maintains homeostasis by degrading and recycling damaged organelles, misfolded proteins, and other intracellular debris. This process is crucial for cellular survival under stress conditions such as nutrient deprivation, hypoxia, and oxidative stress. Autophagy can act both as a tumor suppressor and a promoter, depending on the context and stage of cancer development. It is essential to identify the specific molecular targets that play a role in regulating autophagy in order to create feasible treatment approaches for pancreatic cancer **[63-66]**. Figure 1.4. illustrates the comprehensive pathway and machinery mechanism of stress-induced autophagy.

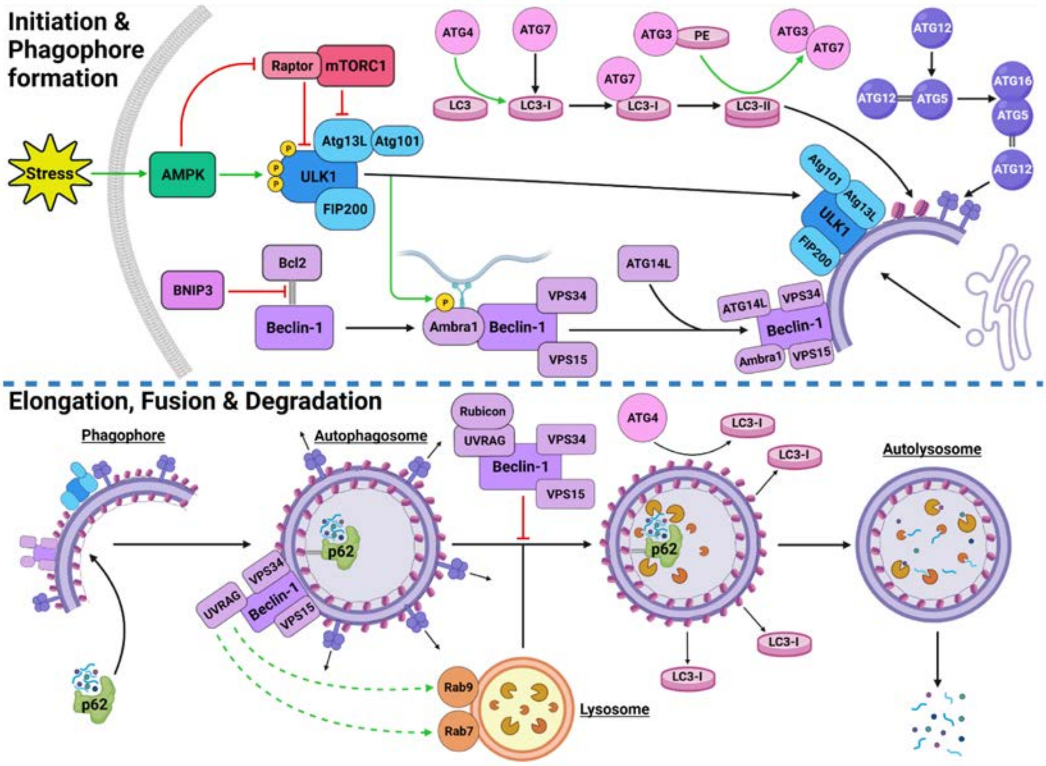

Figure 1.4 Stress-induced autophagy pathway and machinery **[67]**.

Major proteins and mechanisms that regulate autophagy include ATGs, Beclin-1, LC3, and the mammalian target of rapamycin (mTOR) pathway. Autophagy-related (ATG) proteins play a crucial role in the start and execution of autophagy. ATG5, ATG7, and ATG12 play a vital role in creating autophagosomes, which are vesicles with two membranes surrounding cellular components meant for destruction **[68]**. Beclin-1 is an essential component of the class III phosphatidylinositol 3-kinase (PI3K) complex that initiates autophagosome formation. Down-regulation of Beclin-1 has been associated with decreased autophagy and increased tumorigenesis. Microtubule-associated protein 1A/1B-light chain 3 (LC3) is involved in the elongation and closure of autophagosomes. The conversion of LC3-I to LC3-II is a marker of autophagosome formation and autophagic flux **[69-71]**. mTOR is a major cell growth, proliferation, and survival regulator. mTOR complex 1 (mTORC1) inhibits autophagy by phosphorylating and inactivating the ULK1 complex, which is required for the initiation of autophagy. In pancreatic cancer, the mTOR pathway is often hyperactivated, contributing to increased cell growth and resistance to therapy **[72, 73]**.

1.8. mTOR and Autophagy Relationship in Pancreatic Cancer

The link between mTOR and autophagy is particularly meaningful in pancreatic cancer. mTOR acts as a central negative regulator of autophagy. In the times when nutrients are abundant, mTORC1 is activated and inhibits autophagy by phosphorylating ULK1 (Unc-51 Like Autophagy Activating Kinase 1) and preventing its interaction with AMPK (AMP-activated protein kinase), a positive regulator of autophagy. Conversely, under nutrient deprivation or cellular stress conditions, mTORC1 activity is suppressed, activating autophagy to maintain cellular homeostasis by recycling damaged organelles and proteins **[74, 75]**. In Figure 1.5., the diagram displays the main key players in the mTOR pathway.

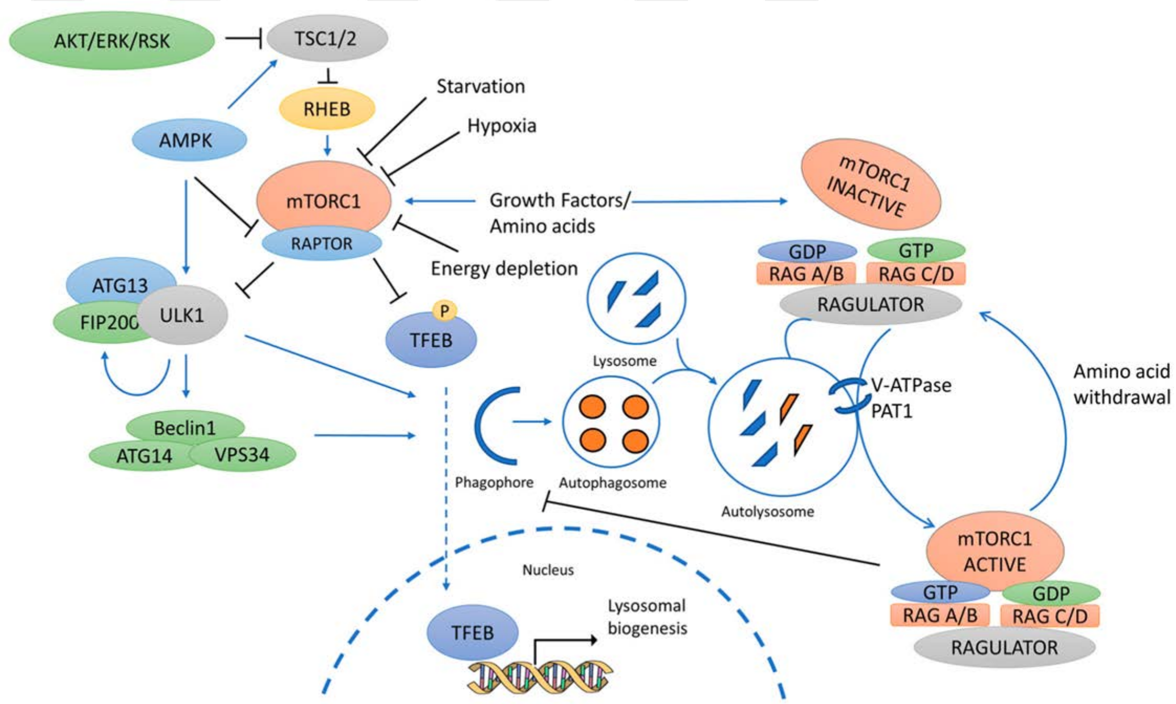

Figure 1.5. Leading key players of the mTOR signaling pathway **[76]**.

In pancreatic cancer, mTOR signaling is frequently upregulated due to mutations in upstream regulators such as PI3K (phosphoinositide 3-kinase), PTEN (phosphatase and tensin homolog), and KRAS **[77, 78]**. These mutations result in the hyperactivation of the mTOR pathway, promoting tumor growth and survival by enhancing protein synthesis, lipid biosynthesis, and glucose metabolism **[79]**. This pathway's activation also contributes to the cancer cells' ability to avoid apoptosis and sustain uncontrolled proliferation. Recent studies have discovered that excessive mTOR signaling in pancreatic cancer is linked to heightened resistance to chemotherapy and targeted treatments, making it a crucial target for therapeutic intervention. Inhibiting mTOR can induce autophagy and potentially sensitize cancer cells to therapeutic agents. For instance, mTOR inhibitors like rapamycin and its analogs (rapalogs) have shown promise in preclinical studies by inducing autophagy, which can lead to the degradation of pro-survival proteins and the inhibition of cell growth **[80, 81]**. Suppression of mTOR activity can trigger autophagy, potentially making cancer cells more sensitive to therapy. For example, mTOR inhibitors such as rapamycin and its analogs (rapalogs) have shown potential in preclinical studies by stimulating autophagy, which can result in the degradation of pro-survival proteins and suppression of cell proliferation **[82, 83]**. Furthermore, the combination of mTOR inhibitors with autophagy inhibitors (e.g., chloroquine) has shown enhanced anti-tumor effects by preventing the recycling of cellular components and leading to increased cancer cell death. This dual inhibition strategy disrupts the metabolic adaptability of cancer cells and helps overcome drug resistance [84-86]. Understanding the intricate relationship between mTOR and autophagy in pancreatic cancer provides a foundation for developing targeted therapies that effectively inhibit tumor growth and improve patient outcomes.

1.9. Aim of This Study

The aim of this thesis is to investigate the anti-cancer effects of selenium biofortified watercress ethanol extracts on pancreatic cancer cell lines, which have been studied on various diseases for their bioactive and therapeutic properties. In the scope of the thesis, it is aimed to comparatively investigate the therapeutic effect of Se-biofortified watercress extracts on cell survival, lipid metabolism, and autophagy in two different pancreatic cancer cell lines with different mutations.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Cell Lines and Reagents

PANC-1 (CRL-1469), MIA PaCa-2 (CRL-1420), and AsPC-1 (CRL-1682) cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO- Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (GIBCO- Life Technologies, Carlsbad, CA) and 1% penicillin/streptomycin (GIBCO, Invitrogen Co) and incubated in 37 °C with 5% CO_2 (NUVE, Istanbul, Turkiye).

2.2. Methods

2.2.1. Plant Growth and Ethanol Extraction of Watercress

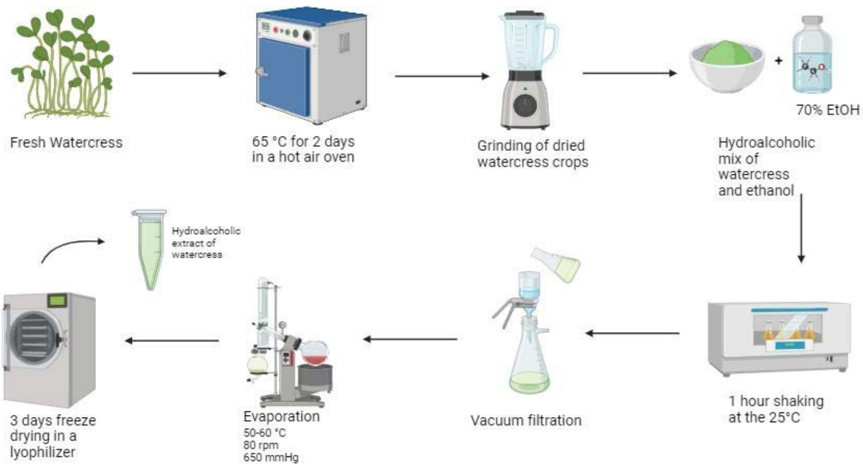

Figure 2.1. Schematic representation of watercress EtOH extraction.

Watercress crops are obtained from Ezgi Günger’s MSc thesis under the collaboration of Cellular Solutions Laboratory and Plant Nutrition and Physiology laboratory at Gebze Technical University, Institute of Biotechnology. Arzum Vahaa smart garden home type hydroponic farming systems was used in this experimental design, each condition repeated for 3 times. Three different selenium concentrations (0, 0.25, 0.50 mg/L) were used to determine the selenium effect. In all treatment groups, selenium conditions were created using sodium selenete (Na_2SeO_4) with a molecular weight of 188.94 g/mol obtained from Acros Organics. Harvested leaves and stems of the plants were stored in a -80 °C freezer samples for the necessary analyses. Fresh watercress crops were incubated in a 65 °C hot air oven for 2 days. The dried crops were ground until powdered. The powder obtained from watercress crops was incubated with 70% EtOH at 25 °C for 1 hour. The resulting watercress ethanol mixture was filtered by vacuum filtration, and alcohol was removed by evaporation technique. After that, the concentrated watercress extract was dried for 3 days by freeze dry method. The watercress extract was extracted with EtOH at a ratio of 1:10000 and aliquoted and stored in a -80 °C freezer for use throughout this study.

2.2.2. Cell Culture

PANC-1 (CRL-1469) and MIA PaCa-2 (CRL-1420) cell lines were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO- Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (GIBCO-Life Technologies, Carlsbad, CA) and 1% penicillin/streptomycin (GIBCO, Invitrogen Co) and incubated in 37 °C with 5% CO_2 (NUVE, Istanbul Türkiye). Passaging of cells was followed and observed by inverted microscope and cell growth media were changed in every 2-3 days intervals depending on their condition.

2.2.3. Cell Thawing

Frozen cell lines with freezing media containing (90% FBS – 10% DMSO) in 80 C cryotubes were quickly thawed and the cells were transferred to centrifuge tubes by adding medium at the1:5 ratio. Cells were centrifuged at 1500 rpm for 5 minutes. After centrifuge, the supernatant was removed, and the pellet was dissolved with the growth media. Cells were cultivated by placing 3 ml of complete DMEM in T25 flask and were place in 37 °C with 5% CO_2 incubator. The media was changed every 2 days until the cells reached the confluency.

2.2.4. Cell Freezing

The cells that had adhered and proliferated on the surface of a T25 cell culture flask were detached enzymatically using 500 µl of trypsin-EDTA. To ihnibite the trypsin-EDTA activity, 1 ml of complete medium was added. The cells were then transferred into sterile Falcon tubes and centrifuged at 1500 rpm for 5 minutes to collect them. Following centrifugation, the cells were resuspended in growth medium and counted using a hemocytometer. After counting, the cells were mixed with a freezing medium containing FBS and DMSO at a 9:1 ratio, and transferred into cryotubes. To preserve cells with low passage numbers, the tubes were stored at -80°C.

2.2.5. Dose-Dependent Cell Viability Assay (MTT Assay)

The effects of Se-biofortified watercress extracts on pancreatic cancer cell lines were investigated with colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide) assay. The method is based on the colorimetric analysis to detect a color change, which occurs when the dehydrogenase enzyme increases in living and intact mitochondrial cells. The color change results from the production of purple formazan salt after cleaving the yellow MTT spacer's tetrazolium ring. The pancreas cancer cells PANC-1 and MIA PaCa-2 were seeded at 3x103 cells/well into the 96-well plate and incubated overnight in a 37 °C and 5% CO2 incubator for adherence. Then, cells were treated with 0-500 µg/mL Se enriched watercress extract and incubated at 37 °C and 5% CO2 incubator for 24 h. After the incubation period, the medium in the wells was renewed. Then, 10µl of MTT reagent (5 mg/mL) was added to the medium on the cells. The samples were incubated at 37 °C and 5% CO2 for 4 hours. After 4 hours, all the medium was discarded, and 100 µl of dimethyl sulfoxide (DMSO) was added to each well and incubated in the dark on the shaker at room temperature for 15 minutes for the formazan crystals to dissolve. Absorbance was measured at two wavelengths at 570 nm in a microplate reader [87].

2.2.6. Colony Formation Assay

Pancreas cancer cells PANC-1 and MIA PaCa-2 were seeded in a 6-well plate at the density of $2.5x10^3$ cells and incubated overnight in a 37 °C and 5% CO_2 incubator for adherence. After 24 h of incubation, cells were treated with different dosages (0, 25, 50, 100, 250, and 500 µg/mL) of ethanol (EtOH) extracts of Se-biofortified (0.25 mg/L and 0.50 mg/L) watercress for 24 h. After 24 h of treatment application, the treated culture medium was discarded, and cells were allowed to form colonies in fresh, complete DMEM for ten days. Then, the media was discarded, and wells were washed with 1X PBS twice and incubated with fixation solution (3:1 (methanol:acetic acid)) for 15 min. The fixed cells were stained with 0.5% crystal violet dye and incubated at room temperature for 20 minutes. After incubation, crystal violet dye was discarded, cells were washed with dH_20 twice, and the colony formation potential was visualized. Further, all colonies were counted for numerical analysis using ImageJ Software **[88]**.

2.2.7. Cell Survival Assay

Panc-1 and MIA PaCa-2 cells were seeded at 10.000 density per well in 24-well plates and treated with non-biofortified and Se-biofortified watercress extracts 0, 25, 50 100, 250 and 500µl/ml for 24h. According to the time points, the cells were washed with 1X PBS and treated with trypsin-EDTA. After centrifugation at 13200 rpm for 2 minutes, the resulting cell pellet was homogenized with 1 ml of DMEM. Subsequently, 10 µl was withdrawn from this mixture, and duplicate cell counts were performed for both cell lines using a hemocytometer **[89]**.

2.2.8. Fluorescent Staining

PANC-1 and MIA PaCa-2 pancreatic cancer cells were placed in 24-well plates at a density of 3×10^4 cells per well. They were left overnight to attach to the surface. The cells were exposed to specific concentrations of 100 µg/ml, 250 µg/ml, and 500 µg/ml of three distinct selenium biofortified (0.25-0.50 mg/L) and non-enriched watercress extracts for a duration of 24 hours. After, the cells were rinsed with 1X phosphate-buffered saline (PBS) and then subjected to staining with MitoSpy™ Red CMXRos, BODIPY® 493/503, DAPI, DCFDA, and Propidium Iodide (PI) dye. The staining solutions were prepared by diluting them at a ratio of 1:1000 in 1X phosphate-buffered saline (PBS) for each well. Subsequently, the cells were placed in a 5% CO_2 incubator and incubated at 37 °C for a duration of 30 minutes. The subcellular mitochondrial mass and oxidative activity of living cells were observed, and cell pictures were captured using the Invitrogen EVOS™ M5000 Imaging System. Fluorescence images were analyzed for relative fluorescent intensity using ImageJ Software for quantitative analysis.

2.2.9. Total Protein Isolation.

Pancreatic cancer cells PANC-1 and MIA PaCa-2 were seeded in 6-well plates as 2.5 $\times 10^5$ cells per well. The plates were then incubated overnight in a 37 °C and 5% CO2 incubator to allow the cells to adhere. The cells were treated with certain concentrations (0.25 mg/L - 0.50 mg/L) of watercress ethanol (EtOH) extract. After the treatment, the culture medium was withdrawn from the cells, and the cells were washed twice with 1 mL of ice-cold 1X PBS. The cells were disrupted on ice using a suitable volume (30-80 µl) of protein lysis solution (M-PER Mammalian Protein Extraction Reagent) and subsequently agitated for 20 minutes at 4°C. Following the lysis step, the cells were gently removed from the surface and placed into little tubes with a volume of 1.5 mL. Next, the centrifugation process was used to eliminate any remaining cell debris. The centrifugation was performed at a speed of 13,200 revolutions per minute for a duration of 15 minutes at a temperature of +4°C. Following centrifugation, the liquid portion (supernatant) was transferred to fresh 1.5 mL microcentrifuge tubes and kept at -20 °C.

2.2.10. Determination of Protein Concentration with Bradford Assay

The protein content was quantified using the Bradford assay. The standard curve was generated by generating various bovine serum albumin (BSA) concentrations, starting from a stock concentration of 2 mg/mL. The concentrations used were 25 µg/mL, 125 µg/mL, 250 µg/mL, 500 µg/mL, 750 µg/mL, 1000 µg/mL, 1500 µg/mL, and 2000 µg/mL. 2 microliters of each BSA solution and blank were added to the 96-well plate and then mixed with 100 microliters of Bradford Reagent. The mixture was incubated at room temperature for 10 minutes. The absorbance measurement was conducted at a wavelength of 595 nm using a microplate reader. Next, a blank solution consisting of 2 µl of mammalian protein extraction reagent (M-PER) was prepared. Then, 2 µl of each extracted protein sample was put in 96-well plates on ice. After that, 100 µl of Bradford reagent was added to the protein samples and incubated for 10 minutes in the dark. Following the incubation period, the absorbance at 595 nm was determined using a microplate reader. Protein concentrations were then estimated based on the standard curve that was previously established **[90]**.

2.2.11. Immunoblotting

According to the Bradford assay result, 20 µg of protein samples from each protein lysate were combined with 4X LDS sample buffer at a ratio of 1:4. This mixture was then placed in 1.5 ml microcentrifuge tubes to denature and straighten out the proteins. The tubes were incubated in a heat block at a temperature of 95°C for a duration of 5 minutes. Next, 2 µl of protein ladder and samples were placed onto a 12%-15% polyacrylamide Bis-Tris micro gel. The gel was electrophoresed at a voltage range of 80-120 V using a running buffer with a concentration of 1X for a duration of 60-90 minutes. Following gel running, the polyvinyl fluoride (PVDF) membrane was subjected to methanol activation for a duration of 10 seconds and subsequently rinsed with distilled water for a period of 1 minute. A model for transferring wet sandwiches was developed, using a vertical transfer buffer that is 1X the size of the pre-wetted sponge pads and filter papers. The transfer module was positioned inside a compact gel tank, and the cassette was filled with a transfer buffer that was diluted to a concentration of 1X. The proteins were electrophoretically transferred to the polyvinylidene difluoride (PVDF) membrane at a constant voltage of 20V for a duration of 60 minutes. In order to minimize the presence of non-specific binding, the PVDF membrane was treated with a solution of 5% skim milk in 1X TBS-T (Tris-buffered saline with 0.1% Tween 20) for a duration of 1 hour at room temperature. Subsequently, the membrane was subjected to incubation with the chosen primary antibody (diluted 1:1000 in a solution of 5% skim milk and 1X TBS-T) for an extended period of time at a temperature of +4 °C. Following the incubation with the main antibody. The membrane underwent three rounds of washing with 1X TBS-T for a duration of 10 minutes each. The membrane was exposed to a horseradish peroxidase (HRP)-linked secondary antibody (diluted 1:1000 in a solution of 5% skim milk and 1X TBS-T) that is compatible with the primary antibody. This incubation took place for a duration of 1 hour at room temperature. Next, the membrane was rinsed three times with 1X TBS-T and once with 1XTBS for a duration of 5 minutes each. A solution of enhanced chemiluminescence (ECL) was developed for the purpose of detecting and quantifying proteins on the membrane. Following a 1-minute incubation of the membrane in a solution of ECL, and analyzed using the ChemiDoc™ XRS+ Imaging System.

2.2.12. Statistical Analysis

The significance of the effects of the treatments for each experiment was evaluated by using analysis of variance (ANOVA) using GraphPad Prism software. Western blot quantification and relative fluorescence intensity were analyzed with Image J software.

3. RESULTS

3.1. Selenium Accumulation Levels Analyzed by ICP-OES

Selenium concentration in watercress crops was analyzed by ICP-OES analysis. As anticipated, a linear correlation was determined between the amount of selenium accumulated and the dose in the treated groups **[91]**.

Table 1. ICP-OES analysis results of selenium biofortificated watercress crop adapted from Ezgi Günger's MSc Thesis **[91]**

Sodium Selenate Treatment (Na_2SeO_4) (mg/L)	**Tissue Selenium (µg/g)**
Control	*ns*
0.25	13.60
0.50	30.0

3.2. Selenium Biofortified Watercress Extracts Reduced Cell Viability and Proliferation in a Dose-dependent Manner

The effects of the selenium biofortified watercress extract on the relative cell viability of PANC-1 and MIA PaCa-2 pancreatic cancer cell lines were investigated using the colorimetric MTT assay. MTT cell viability assay of pancreatic cancer cells has proceeded to evaluate cytotoxic responses against five different concentrations (25 µg/ml, 50 µg/ml, 100 µg/ml, 250 µg/ml, and 500 µg/ml) of each of two different Se-biofortified (0.25 mg/L and 0.50 mg/L) and non-biofortified concentrations of watercress extracts through EtOH extraction method for 24 h (Figure 3.1). Considering the PANC-1 cells, it was shown that both selenium biofortified and non-biofortified watercress extracts inhibited cell viability significantly due to increasing doses. In particular, 500 µg/ml remarkably reduced cell viability by 40% compared to the control group. When the effects of selenium biofortified and non-biofortified watercress extracts were compared, the non-biofortified treatment group showed results very close to the 0.50 mg/L Se-biofortified group. Furthermore, it was observed that watercress extract enriched with 0.25 mg/L selenium increased cell proliferation by 5% at doses of 25 µg/ml and 50 µg/ml (Figure 3.1).

Considering MIA PaCa-2 cells, at a dose of 500 µg/ml, Se-biofortified and non-biofortified watercress extracts treatment groups showed approximately 40-50% decrease in cell viability. When the effect of selenium biofortified and non-biofortified groups on MIA PaCa-2 cells was compared, similar results were obtained in the non-biofortified group and 0.50 mg/L Se-biofortified groups in a dose-dependent manner. While non-biofortified and 0.50 mg/L Se-biofortified treatment groups significantly decreased cell viability, it was observed that cell proliferation increased in the 0.25 mg/L Se-biofortified treatment group at doses of 25 µg/ml and 50 µg/ml compared to the control group (Figure 3.1). Selenium biofortified and non-biofortified watercress extracts showed a dose-dependent cytotoxic effect on PANC-1 and MIA PaCa-2 cells. However, in groups treated with 0.25 mg/L selenium biofortified watercress, cell proliferation was observed in both cell lines at 25 µg/ml and 50 µg/ml.

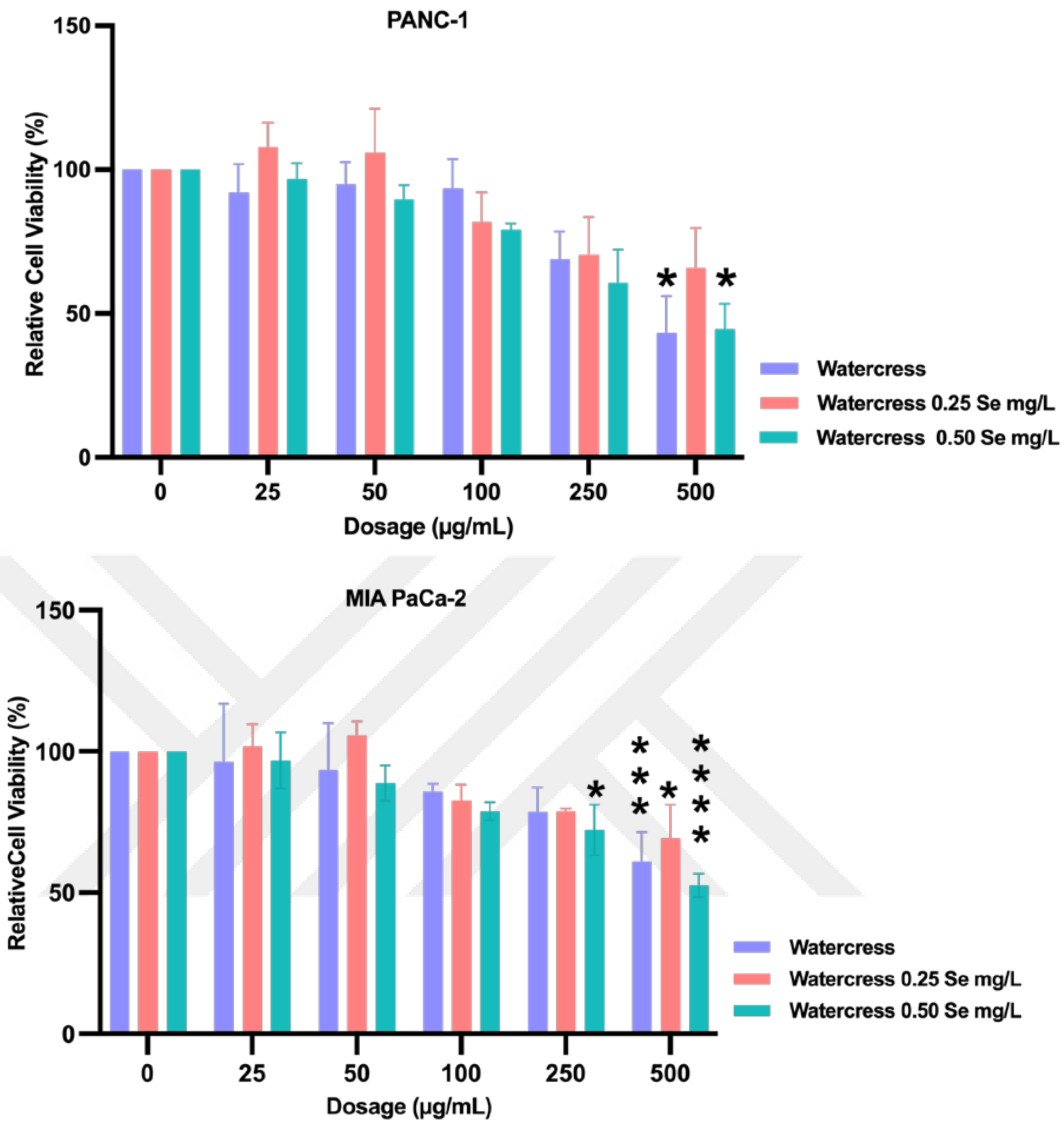

Figure 3.1. MTT cell viability assay. PANC-1 (top) and MIA PaCa-2 (bottom) pancreatic cell lines to evaluate cytotoxic responses against different concentrations (0, 25, 50, 100, 250, and 500 µg/ml) of selenium biofortified (0.25 - 0.50 mg/L) and non-biofortified watercress extracts for 24 h. Different letters indicate significant differences between means according to Tukey's HSD test. (p < 0.05) (ns: P>0.05, *: P≤0.05, **: P≤0.01, ***: P≤0.001, ****:P≤0.0001)

Colony formation assay was performed to observe the ability of pancreatic cancer cells to form colonies in the presence of Se-biofortified and non-biofortified watercress EtOH extracts. PANC-1 and MIA PaCa-2 pancreatic cancer cells were treated with non-biofortified, 0.25 mg/L and 0.50 mg/L selenium biofortified watercress EtOH extracts at doses of 25 µg/ml, 50µg/ml, 100 µg/ml, 250 µg/ml, and 500 mg/L for 24 hours (Figure 3.2). Colonies in each treatment group were counted using ImageJ Software (Figure 3.3).

In Se-biofortified treatment groups, both PANC-1 and MIA PaCa-2 cells showed a significant decrease in colony-forming ability at the dose of 500 μg/ml compared to control groups. In the non-biofortified treatment groups, the ability of pancreatic cancer cells to form colonies decreased dose-dependently at the dose of 100 μg/ml compared to the control groups. The colony-forming ability of PANC-1 and MIA PaCa-2 pancreatic cancer cells was significantly reduced in all non-biofortified and Se-biofortified (0.25-0.50 mg/L) treatment groups at a concentration of 500 μg/ml compared to the control group (Figure 3.2 – Figure 3.3).

As a result of the MTT cell viability assay and colony formation assay, 100 μg/ml, 250 μg/ml, and 500 μg/ml of non-biofortified and Se-biofortified watercress EtOH extracts were determined as selected doses for further applications of this study.

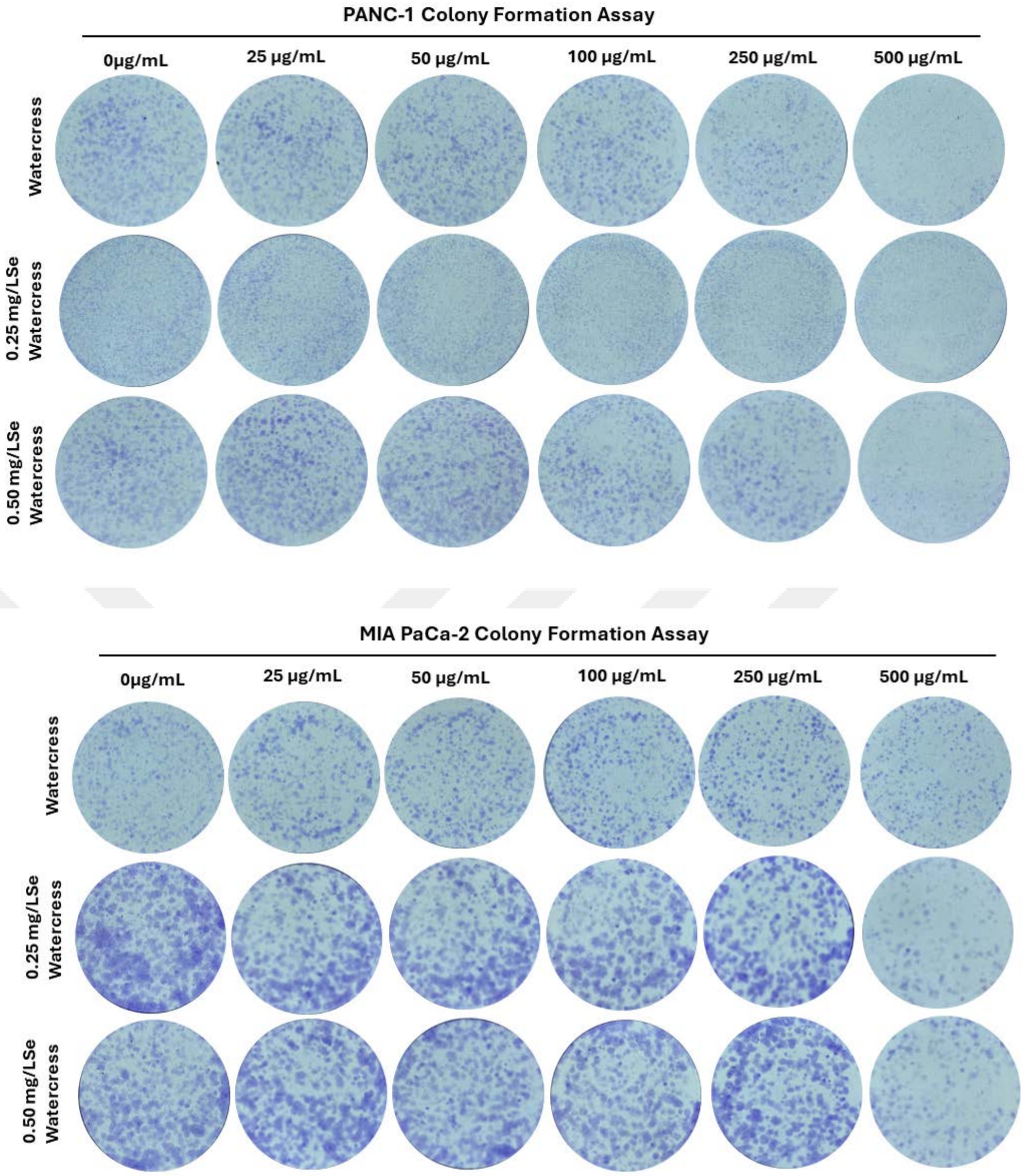

Figure 3.2. Colony Formation Assay. PANC-1 (top) and MIA PaCa-2 (bottom) cancer cells were exposed to different concentrations (0, 25, 50, 100, 250, and 500 µg/ml) of selenium biofortified (0.25 - 0.50 mg/L) and non-biofortified watercress extracts for 24 h. The colony formation of cells was counted after ten days of treatment with media replenishment every two days.

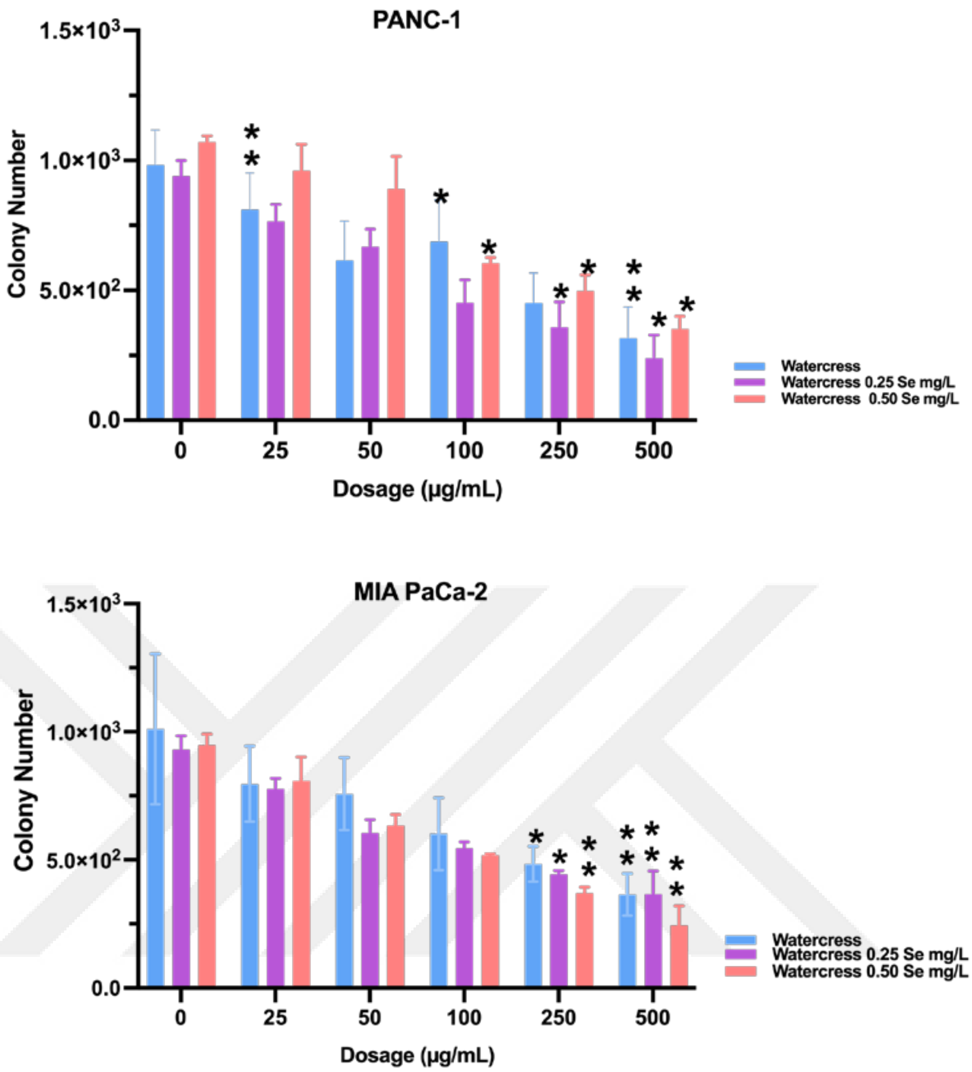

Figure 3.3. Numerical analysis of colony formation. Images obtained from three independent replicates treated with different concentrations (0, 25, 50, 100, 250, and 500 µg/ml) of selenium biofortified (0.25 - 0.50 mg/L) and non-biofortified watercress extracts and colony counting was performed with ImageJ software. Different letters indicate significant differences between means according to Tukey's HSD test. (n=3, p < 0.05) (p < 0.05) (ns: P>0.05, *: P≤0.05, **: P≤0.01, ***: P≤0.001, ****:P≤0.0001)

To investigate the long-term effects of Se-biofortified and non-biofortified watercress extracts on pancreatic cancer cells PANC-1 and MIA PaCa-2, cell survival assay was performed against five different concentrations (25 µg/ml, 50 µg/ml, 100 µg/ml, 250 µg/ml, and 500 µg/ml) of each of two different Se-biofortified (0.25 mg/L and 0.50 mg/L) and non-biofortified concentrations of watercress extracts through EtOH extraction method for 24, 48 and 72h (Figure 3.4). In PANC-1 cells, a decrease in cell survival was observed in the non-biofortified watercress extracts treatment group compared to the control group at 100 ug/ul ($3.3x10^4$ and $2.4x10^4$ cells, respectively).

All doses showed parallel development in the first 24 hours with the control group. Within the first 24 hours, there is a significant cell death with 100 μg/ml and 500 μg/ml doses compared to control ($1.6x10^4$ cells and $2.4x10^4$ cells, respectively). At 24 hours, the most significant cell death was detected at 250 μg/ml compared to the control group ($1.1x10^4$ and $3.3x10^4$ cells, respectively). Significant decreases in cell survival started at the 48th treatment hour at a dose of 100 ug/ul compared to the control group ($2.2x10^4$ and $4.2x10^4$ cells, respectively). At a dose of 500 μg/ml, a notable decrease in cell number was detected at 48th hours ($9x10^3$ cells) and 72 h hours ($8x10^3$ cells) of the treatment compared to the control group ($4.3x10^4$ cells). However, the doses of 25 μg/ml and 50 μg/ml did not show significant cytotoxic effects compared to the control group (Figure 3.4-A).

Considering the 0.25 mg/L Se-biofortified treatment group of the PANC-1 cells, in the 24 h, cell numbers were significantly decreased at the dose of 100 μg/ml ($1.5x10^4$ cells), 250 μg/ml ($1.2x10^4$ cells), and 500 μg/ml ($7x10^3$ cells) compared to the control group (3.6×10^4 cells). Also, in the treatment groups of 25 μg/ml ($2.9x10^4$ cells) and 50 μg/ml ($2x10^4$ cells), cell survival of PANC-1 cells decreased slightly compared to the control group. (Figure 3.4-B). At the 48th treatment hour, a significant decrease was observed in the cells treated with 50 μg/ml ($3x10^4$), 100 μg/ml ($1.9x10^3$ cells), 250 μg/ml ($1x10^3$) and 500 μg/ml (6.7x103 cells) doses compared to the control group ($5.2x10^4$) (Figure 3.4-B).

In the treatment group of 0.50 mg/L Se-biofortified watercress extract, cell numbers of PANC-1 cells significantly decreased compared to the control group ($3.3x10^4$ cells) by 24 h at the 500 μg/ml ($9.6x10^3$ cells) dose treatment, which continued notably at 48 and 72 h (Figure 3.4-C)

Considering MIA PaCa-2 cells treated with non-biofortified watercress extract, a cytotoxic effect was observed at doses of 100 μg/ml ($1x10^4$ cells), 250 μg/ml ($1x10^4$ cells), and 500 μg/ml ($7.8x10^3$ cells) compared to the control group ($2.6x10^4$ cells) at 24 h. At a dose of 250 μg/ml, the number of cells decreased dramatically at 72 hours compared to the control group. In the 500 μg/ml ($2.3x10^4$ cells) treated group, a decrease in cell survival of the MIA PaCa-2 cells was again observed compared to the control group ($6.4x10^4$) (Figure 3.4-D)

In the 0.25 mg/L Se-biofortified treatment group, the cell survival of MIA PaCa-2 cells decreased after the dose of 50 μg/ml. Compared to the control group ($3.4x10^4$ cells), at 24h 50 μg/ml. 50 μg/ml ($1.6x10^4$ cells), 100 μg/ml ($1.3x10^4$ cells), 250 μg/ml

($7.5x10^3$ cells), ve 500 μg/ml ($3.9x10^3$ cells) treatment doses of 0.25 mg/L Se-biofortified watercress extract decreased the cell survival of MIA PaCa-2 cells significantly. In the 72-hour treatment group, doses of 100 μg/ml, 250 μg/ml, and 500 μg/ml significantly decreased the cell survival of MIA PaCa-2 cells compared to the control group (6.4x104) (Figure 3.4-E).

In MIA PaCa-2 cells treated with watercress extract enriched with 0.50 mg/L Se, compared to the control group, cell survival was significantly decreased at doses of 100 μg/ml and higher. The doses of 100 μg/ml ($1.2x10^3$ cells), 250 μg/ml ($1x10^4$ cells), and 500 μg/ml ($5x10^3$ cells) at 24 hours compared to the control group ($2.8x10^4$ cells). Subsequently, the most radical decrease in cell survival in this treatment group, compared to the control group ($6.7x10^4$ cells), occurred at the dose of 500 μg/ml (5×10^3 cells) at 72 hours (Figure 3.4-F).

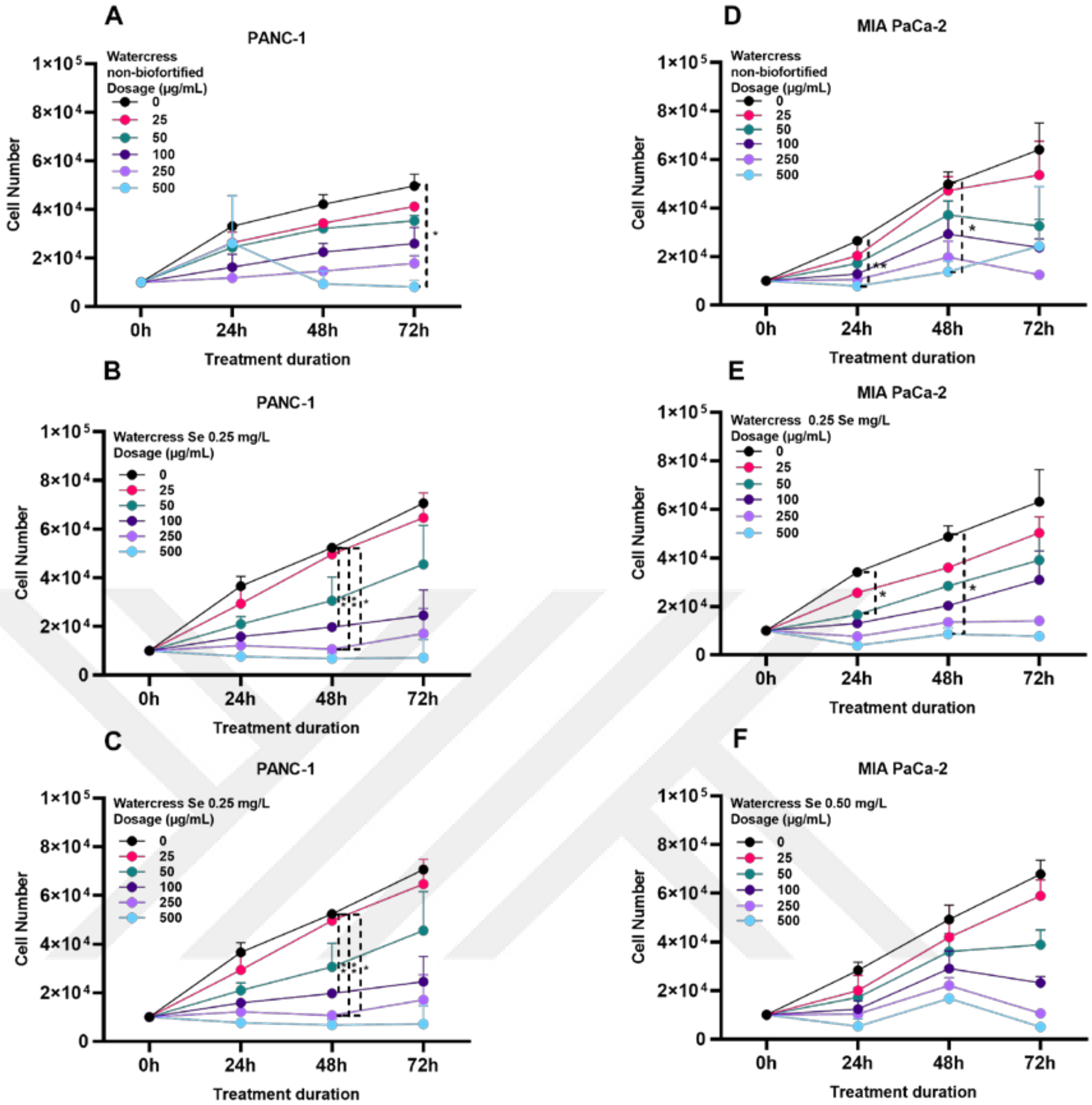

Figure 3.4. Cell survival assay. The effects of long-term (24, 48, and 72h) exposure of PANC-1 (A, B, C) and MIA PaCa-2 (D, E, F) pancreatic cancer cells to different concentrations (0, 25, 50, 100, 250, and 500 µg/ml) of non-biofortified and selenium biofortified (0.25 - 0.50 mg/L) and non-biofortified watercress extracts. Each data point represents the mean ± SD of two independent experiments with at least two repeats of each condition. Two-way ANOVA analysis was performed with Dunnett's test. (ns: P>0.05, *: P≤0.05, **: P≤0.01, ***: P≤0.001, ****:P≤0.0001)

3.3. Fluorescent Staining Analysis to Investigate the Cell Death and Mitochondrial Membrane Potential and Reactive Oxygen Species of PANC-1 and MIA PaCa-2 Cells Treated with Different Concentrations of Se Biofortified Watercress Extract

The dose-dependent effects of different concentrations of selenium biofortified watercress extracts on cell death, mitochondrial membrane potential, and reactive oxygen species were monitored by fluorescent staining. A significant dose-dependent decrease in MitoSpy fluorescence intensity was observed in the group treated with non-biofortified watercress extract. In Figure 3.5, a slight decrease was observed in the mitochondrial membrane potential of PANC-1 pancreatic cancer cells treated with non-selenium enriched watercress EtOH extract for 24 h at doses of 100 μg/ml and 250 μg/ml compared to the control group. However, a significant decrease was observed in the mitochondrial membrane potential at the 500 μg/ml dose compared to the control group. In order to quantify the signal ratio in the fluorescence images, fluorescence intensity analysis was performed. Figure 3.11-A shows the results of the fluorescence signal obtained using ImageJ software. A significant decrease in the relative fluorescence intensity of MitoSpy was detected in the control group and in cells treated with 500 μg/ml non-biofortified watercress extract.

In PANC-1 cells treated with 0.25 mg/L Se-biofortified watercress EtOH extract shown in Figure 3.6. In both, the doses of 100 μg/ml and 250 μg/ml showed a similar decrease in mitochondrial membrane potential on the PANC-1 pancreas cancer cells compared to the control group. In the 500 μg/ml dose treatment group, a significant decrease was observed in the mitochondrial membrane potential compared to the control group. The relative fluorescent intensity analysis showed that the MitopSpy fluorescence signals at the dose of 500 μg/ml significantly decreased compared to the control group (Figure 3.11-A).

In Figure 3.7, the treatment group of 0.50 mg/L Se-biofortified watercress EtOH extract on PANC-1 pancreatic cancer cells showed the most effective decrease in the mitochondrial membrane potential at the dose of 500 μg/ml compared to the control group. At a dose of 250 μg/ml, a slight decrease in the mitochondrial membrane potential of PANC-1 cells was observed compared to the control group. Finally, at the dose of 100 μg/ml, there was no significant decrease was observed in the mitochondrial membrane potential of the cells compared to the control group.

In the PANC-1 cells treated with non-biofortified watercress extract, DAPI signals were more intense in cells treated with the dose of 500 µg/ml compared to the control group. At the doses of 100 µg/ml and 250 µg/ml, DAPI signals showed slightly more intensity compared to the control group. Examination of the fluorescence intensity is shown in Figure 3.11-A. reveals a significant increase in fluorescence intensity at the 500 µg/ml dose compared to the control group.

In Figure 3.6, the PANC-1 cells treated with 0.25 mg/L Se-biofortified watercress EtOH extracts at the doses of 250 µg/ml and 500 µg/ml, an increase in DAPI fluorescence intensity was observed compared to the control group. At the dose of 100 µg/ml, an increase was also observed compared to the control group. According to the fluorescent intensity analysis results shown in Figure 3.11-A, the 0.25 mg/L Se-biofortified group was detected as the most effective treatment at the dose of 500 µg/ml.

In PANC-1 cells treated with 0.50 mg/L Se-biofortified watercress EtOH extract shown in Figure 3.7. In PANC-1 pancreatic cancer cells treated with 100 µg/ml and 250 µg/ml doses, there was a significant increase in DAPI fluorescence intensity compared to the control group. However, at the dose of 500, although intensity was observed, no significant increase was observed compared to the control group. It was determined that there was a decrease in the 500 dose compared to the control group shown in Figure 3.11-A.

In PANC-1, cells with non-biofortified watercress, EtOH extract showed a dose-dependent decrease in the fluorescence of the DCFDA dye, which indicates the reactive oxygen species responsiveness of the cells. The most significant decrease was observed at a dose of 500 µg/ml compared to the control group. Relative fluorescence analysis also confirmed the change in DCFDA fluorescence at the 500 µg/ml dose. At doses of 100 µg/ml and 250 µg/ml, DCFDA fluorescence in PANC-1 cells was also shown to decrease compared to the control group. (Figure 3.11-A).

In the 0.25 mg/L Se-biofortified watercress EtOH extract treatment group, a dose-dependent decrease in the fluorescence of DCFDA dye was observed (Figure 3.6). The most significant decrease was observed at a dose of 500 µg/ml compared to the control group. Relative fluorescence analysis also confirmed the change in DCFDA fluorescence at a dose of 500 µg/ml. At 100 µg/ml and 250 µg/ml doses, DCFDA fluorescence in PANC-1 cells also decreased compared to the control group (Figure 3.11-A).

Considering the 0.50 mg/L Se-biofortified watercress treatment group of PANC-1 cells, similarly to other groups, a dose-dependent decrease in the fluorescence signals of DCFDA dye was observed compared to the control groups (Figure 3.7). The most significant decrease was observed at the dose of 500 µg/ml compared to the control group. In the relative fluorescence analysis, a significant decrease was shown in DCFDA fluorescence at a dose of 500 µg/ml (Figure 3.11-A). At 100 µg/ml and 250 µg/ml doses, DCFDA fluorescence in PANC-1 cells decreased compared to the control group (Figure 3.7).

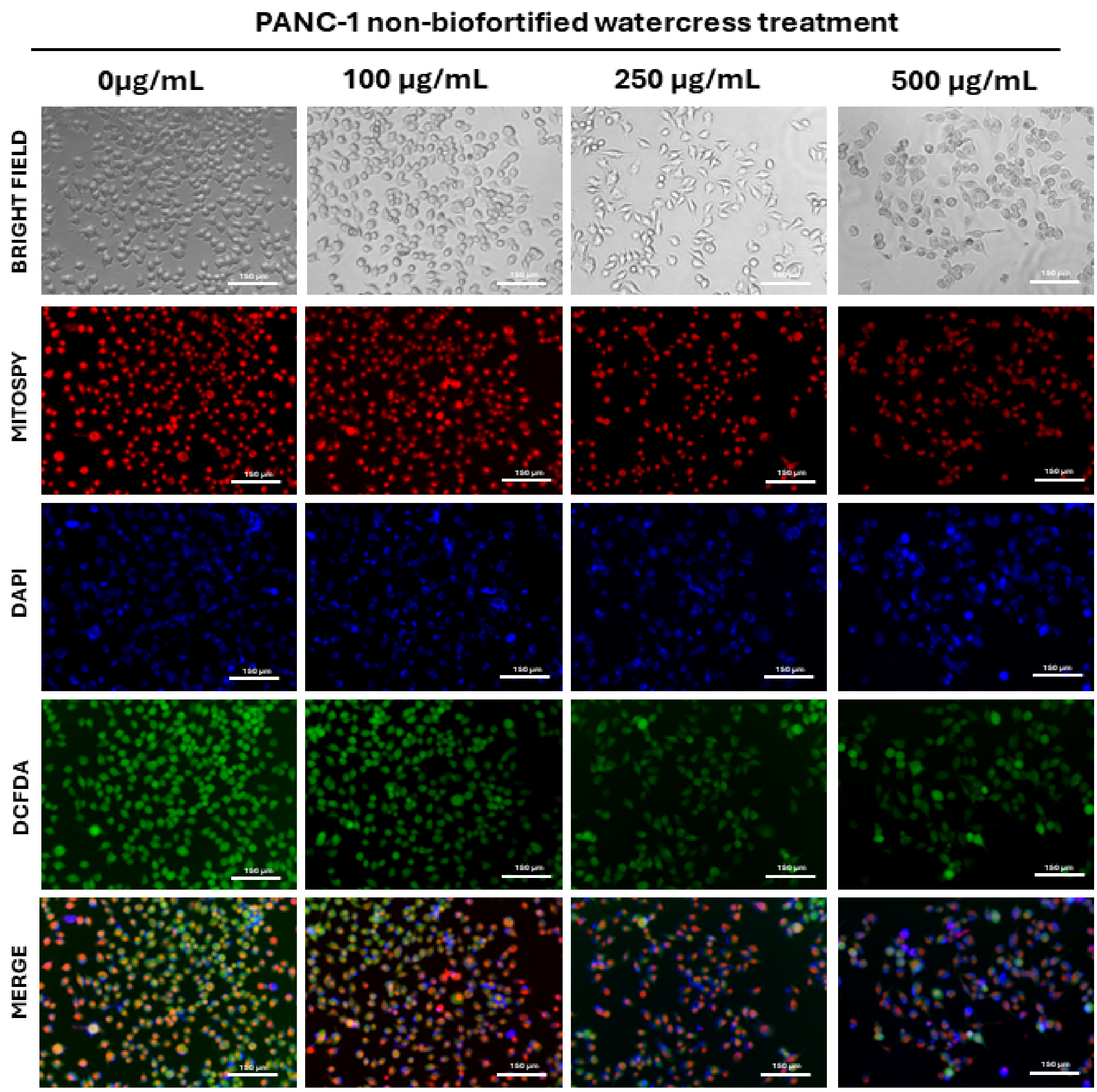

Figure 3.5 Fluorescent staining images of PANC-1 pancreatic cancer cells. Cells were treated with selected (100, 250, and 500 µg/mL) of non-biofortified watercress extracts for 24h. MitoSpy, DAPI, and DCFDA stains were used to analyze the cells' mitochondrial, cell nuclei, and reactive oxygen species potential. Images were taken with 20X objectives. (Scale=150µm)

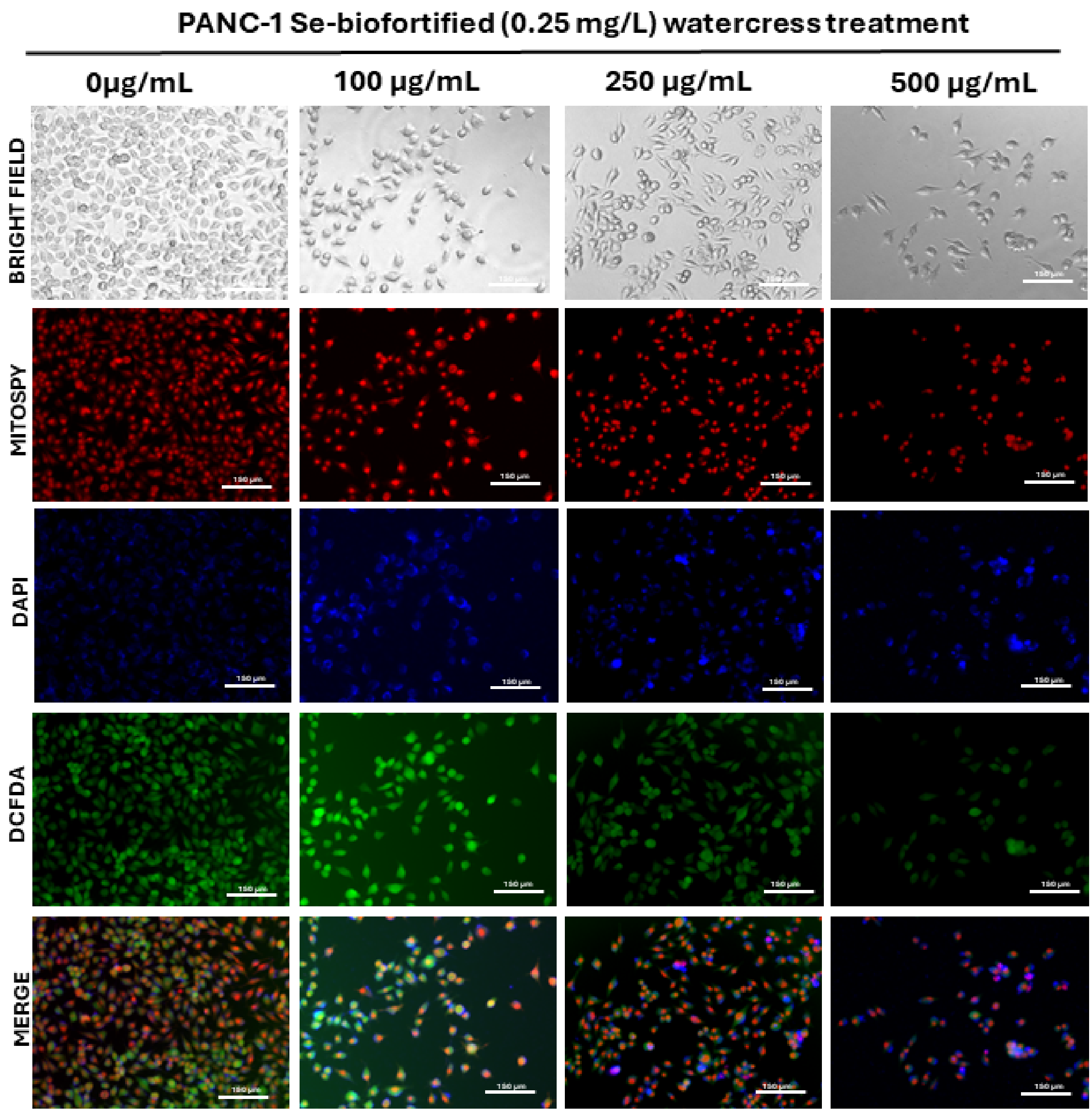

Figure 3.6 Fluorescent staining images of PANC-1 pancreatic cancer cells. Cells were treated with selected (100, 250, and 500 μg/mL) of Se-biofortified (0.25 mg/L) watercress extracts for 24h. MitoSpy, DAPI, and DCFDA stains were used to analyze the cells' mitochondrial, cell nuclei, and reactive oxygen species potential. Images were taken with 20X objectives. (Scale=150μm)

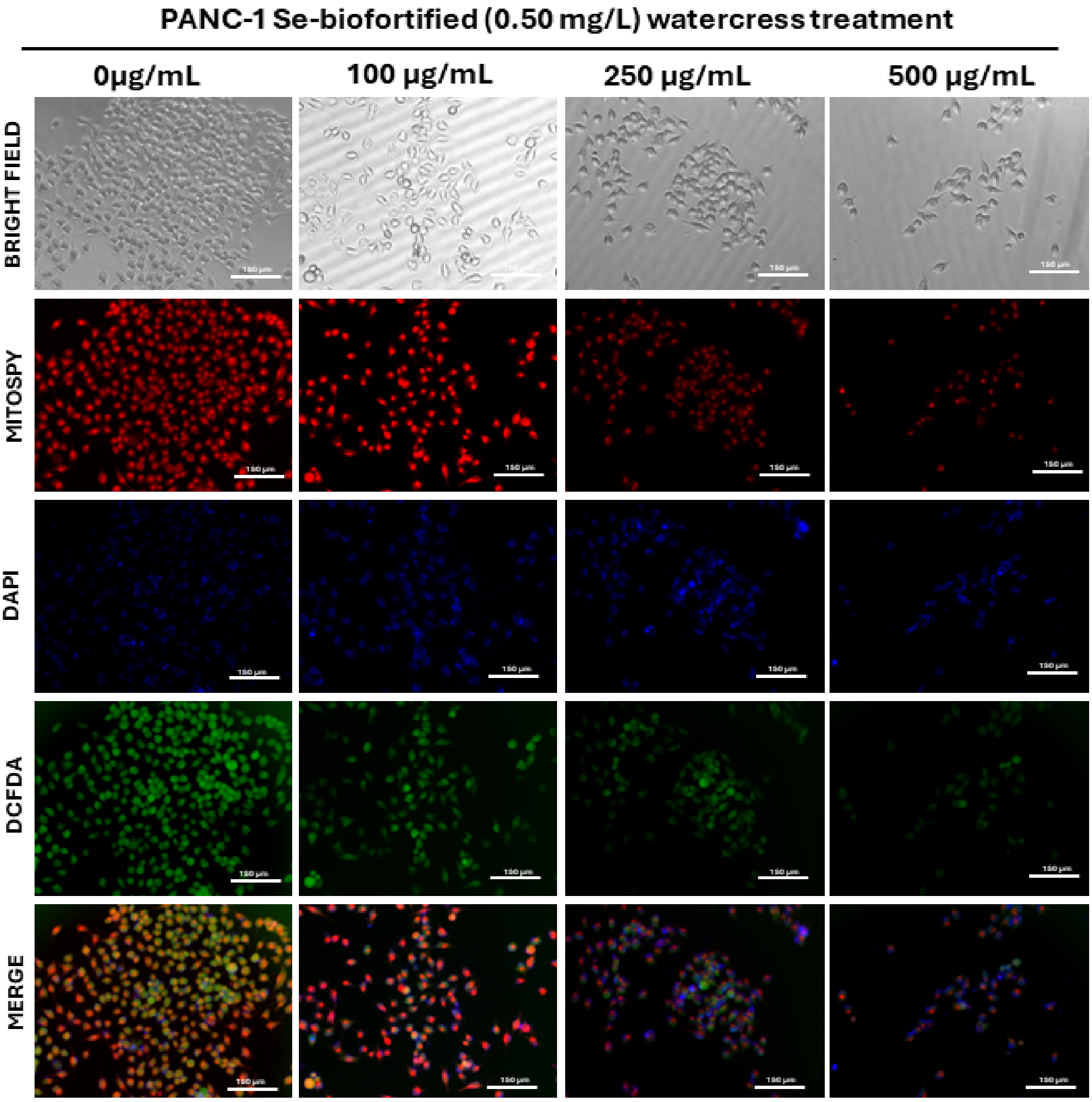

Figure 3.7 Fluorescent staining images of PANC-1 pancreatic cancer cells. Cells were treated with selected (100, 250, and 500 µg/mL) of Se-biofortified (0.25-0.50 mg/L) and non-biofortified watercress extracts for 24h. MitoSpy, DAPI, and DCFDA stains were used to analyze the cells' mitochondrial, cell nuclei, and reactive oxygen species potential. Images were taken with 20X objectives. (Scale=150µm)

Considering MIA PaCa-2 cells, a significant dose-dependent decrease was observed in MitoSpy fluorescence intensity in the non-biofortified watercress treatment group (Figure 3.8). According to the relative fluorescence intensity analysis shown in Figure 3.11-B, at a dose of 500 µg/ml, the mitochondrial membrane potential of MIA PaCa-2 cells was significantly decreased compared to the control group.

In MIA PaCa-2 cells treated with 0.25 mg/L Se-biofortified watercress, the mitochondrial membrane potential monitored by MitoSpy was slightly decreased at doses 100 μg/ml and 250 μg/ml, while there was an important decrease at dose 500 μg/ml compared to the control group (Figure 3.9). This significant decrease was also detected by the relative fluorescence analysis of the 500 μg/ml dose compared to the control group (Figure 3.11-B).

In Figure 3.10, the MIA PaCa-2 cells treated with 0.50 mg/L Se-biofortified watercress showed that MitoSpy fluorescence intensity was decreased dramatically at doses 250 μg/ml and 500 μg/ml, and there was also a considerable decrease observed at the dose 100 μg/ml compared to the control group. The relative fluorescence analysis (Figure 3.11-B) showed that 500 μg/ml doses have the most effective treatment, which decreases the mitochondrial membrane potential of MIA PaCa-2 cells compared to the control group. Figure 3.8 shows MIA PaCa-2 cells treated with non-biofortified watercress extract; DAPI fluorescence increased at the doses of 100 μg/ml, 250 μg/ml, and 500 μg/ml compared to the control group. The results of the relative fluorescence analysis showed that the dose of 500 μg/ml significantly increased the DAPI signals compared to the control group (Figure 3.11-B).

In experimental groups treated with 0.25 mg/L Se-biofortified watercress, DAPI fluorescence intensity was observed at doses of 100 μg/ml and 500 μg/ml, while a slight intensification was observed at dose 250 μg/ml compared to the control group. Relative fluorescence analysis showed a significant increase in DAPI fluorescence at a dose of 500 μg/ml compared to the control group (Figure 3.11-B). Figure 3.10 shows the MIA PaCa-2 cells treated with 0.50 mg/L Se-biofortified watercress EtOH extracts. In this group, 0.50 mg/L Se-biofortified treatments showed similar results with the control group. Relative fluorescence analysis showed that there was a slight increase in DAPI fluorescence at a dose of 500 μg/ml compared to the control group in Figure 11-B.

Regarding the non-biofortified watercress treatment group dyed with DCFDA, a decrease in DCFDA signals was observed at doses 250 μg/ml and 500 μg/ml compared to the control group (Figure 8). However, an increase was observed at 100 μg/ml doses compared to the control group of the non-biofortified watercress EtOH extract treatment group. According to the results of relative fluorescence analysis, the DCFDA fluorescence intensity in the treatment groups decreased dose-dependently compared to the control group of MIA PaCa-2 cells (Figure 3.11-B)

In MIA PaCa-2 cells treated with 0.25 mg/L Se-biofortified watercress, a significant decrease was observed in DCFDA at the dose of 500 μg/ml, and there was a considerable decrease at dose of 100 and 250 μg/ml compared to the control group. This significant decrease was also confirmed by the relative fluorescence analysis of the 500 μg/ml dose compared to the control group (Figure 3.11-B).

Figure 3.10 shows the MIA PaCa-2 cells treated with 0.50 mg/L Se-biofortified watercress, DCFDA decreased in a dose-dependent manner compared to the control group. The dose of 500 μg/ml showed a most significant impact on the MIA PaCa-2 cell line. The DCFDA signals of MIA PaCa-2 cells were significantly decreased by the doses of 100 μg/ml and 250 μg/ml. The analysis of relative fluorescence indicated a significant decrease in DCFDA relative fluorescence intensity at a dose of 500 μg/ml, as shown in Figure 3.11-B.

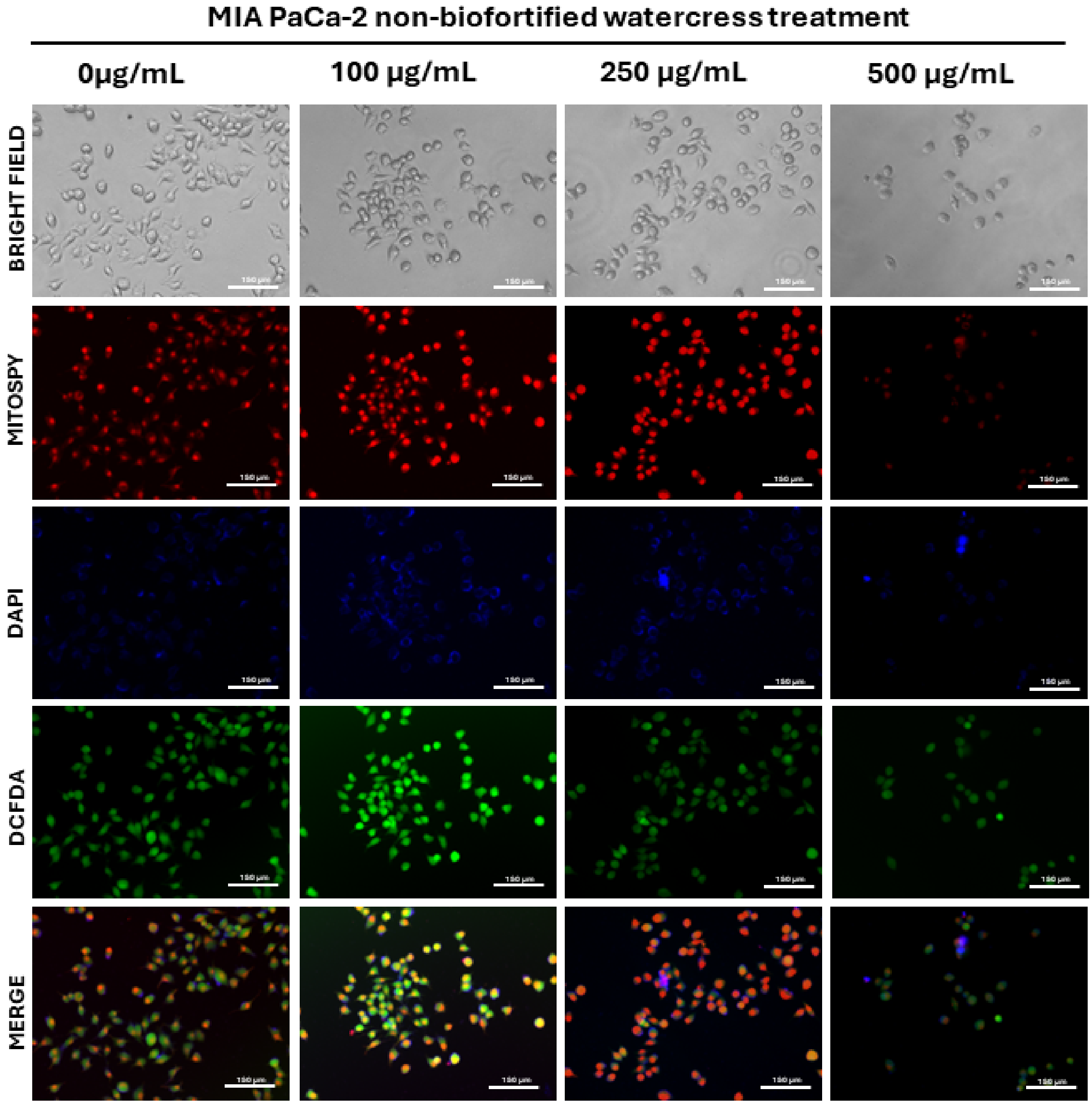

Figure 3.8 Fluorescent staining images of MIA PaCa-2 pancreatic cancer cells. Cells were treated with selected (100, 250, and 500 µg/mL) of non-biofortified watercress EtOH extracts for 24 h. MitoSpy, DAPI, and DCFDA stains were used to analyze the cells' mitochondrial, cell nuclei, and reactive oxygen species potential. Images were taken with 20X objectives. (Scale=150µm)

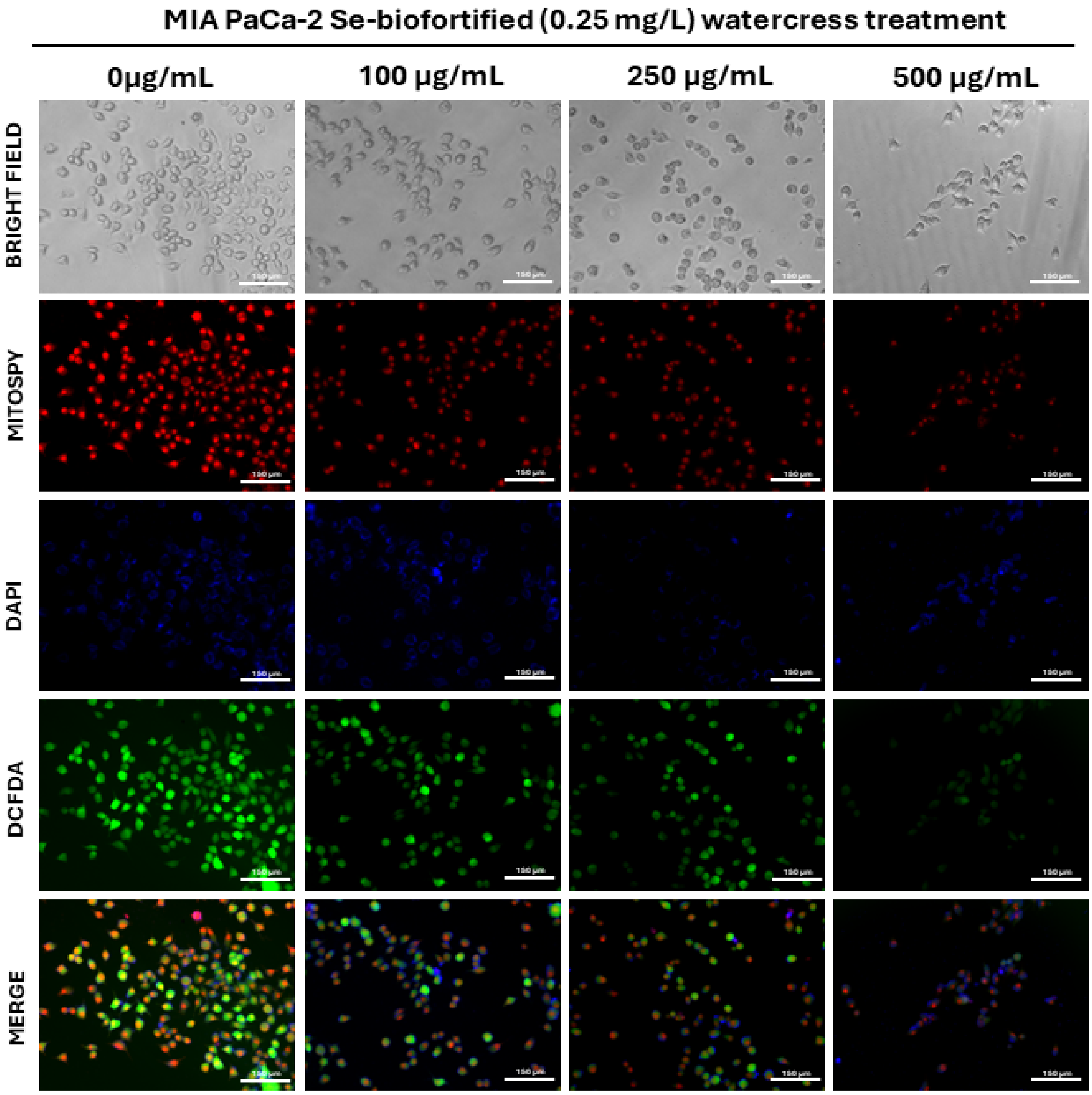

Figure 3.9 Fluorescent staining images of MIA PaCa-2 pancreatic cancer cells. Cells were treated with selected (100, 250, and 500 μg/mL) of 0.25 mg/L Se-biofortified watercress EtOH extracts for 24 h. MitoSpy, DAPI, and DCFDA stains were used to analyze the cells' mitochondrial, cell nuclei, and reactive oxygen species potential. Images were taken with 20X objectives. (Scale=150μm)

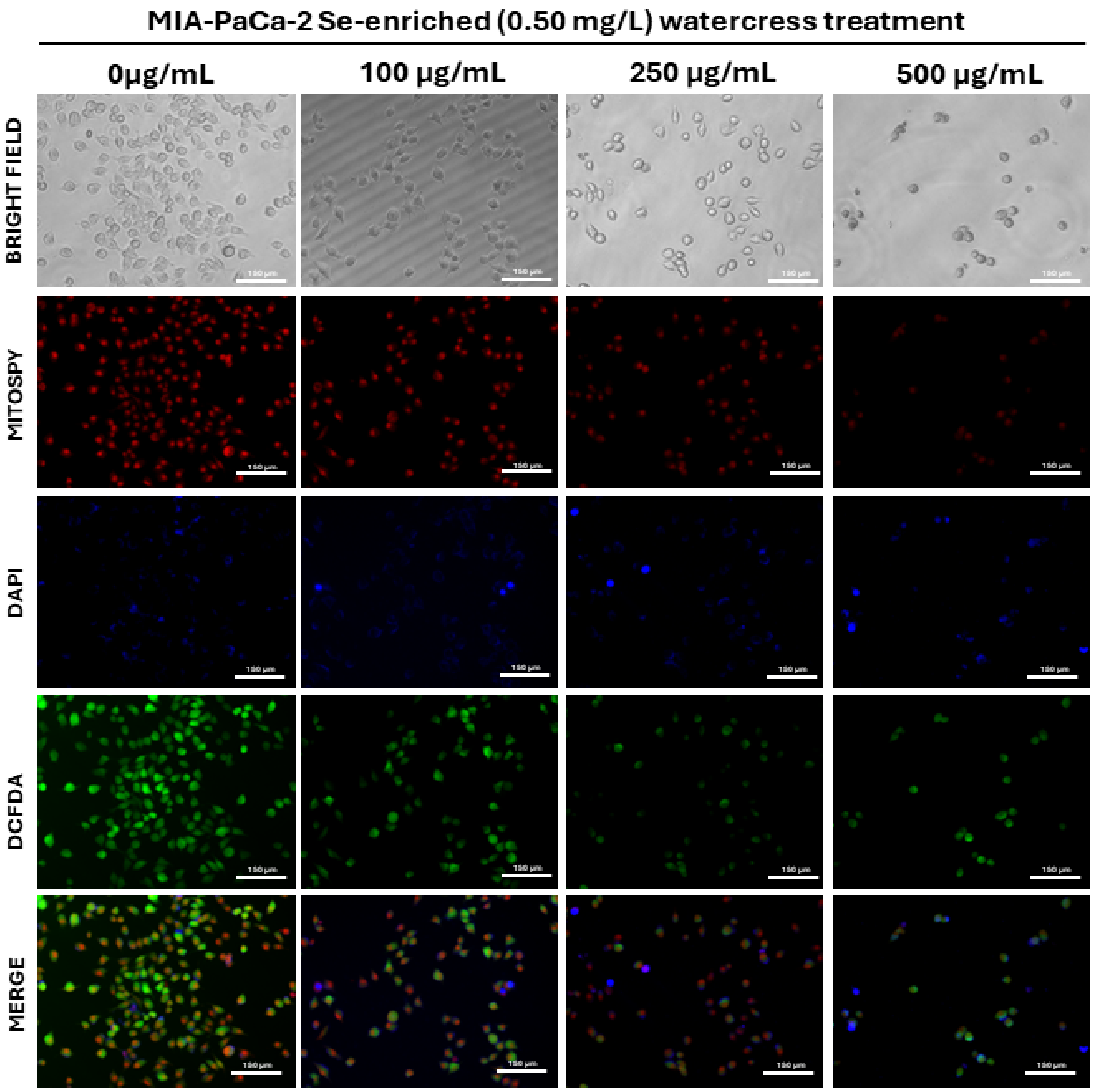

Figure 3.10 Fluorescent staining images of MIA PaCa-2 pancreatic cancer cells. Cells were treated with selected (100, 250, and 500 µg/mL) of 0.50 mg/L Se-biofortified watercress EtOH extracts for 24 h. MitoSpy, DAPI, and DCFDA stains were used to analyze the cells' mitochondrial, cell nuclei, and reactive oxygen species potential. Images were taken with 20X objectives. (Scale=150µm)

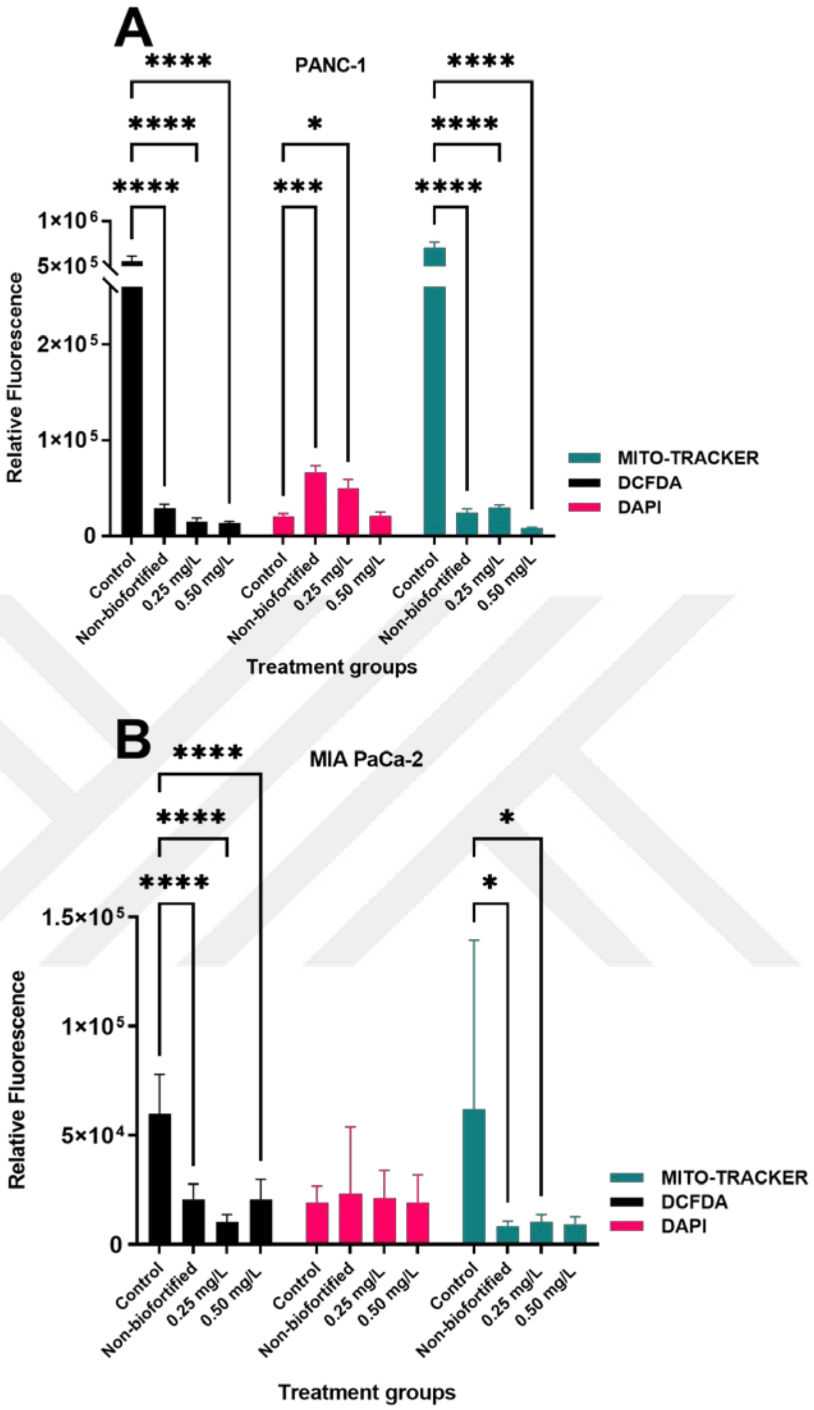

Figure 3.11 Relative fluorescence intensity analysis of PANC-1 (**A**) and MIA PaCa-2 (**B**) pancreatic cancer cells. Cells were treated with 500 µg/mL of Se-biofortified (0.25-0.50 mg/L) and non-biofortified watercress extracts for 24 h. MitoSpy, DAPI, and DCFDA stains were used to analyze the cells' mitochondrial, cell nuclei, and reactive oxygen species potential. Relative fluorescence intensity was investigated by measuring 15 cells for each stained treatment group. Each data point represents the mean ± SD. Two-way ANOVA analysis was performed with Dunnet's test. (ns: P>0.05, *: P≤0.05, **: P≤0.01, ***: P≤0.001, ****:P≤0.0001)

3.4. Lipid Droplet Formation in PANC-1 and MIA PaCa-2 Pancreatic Cancer Cells Exposed to Different Selenium Biofortified Watercress Extracts

Figure 3.12 shows the BODIPY and PI staining of PANC-1 cells treated with non-biofortified watercress EtOH extract. In this treatment group, lipid droplets were decreased at doses of 100 μg/ml and 250 μg/ml compared to the control group. However, an increase was observed at doses of 500 μg/ml according to the control group. In PANC-1 cells treated with 0.25 mg/L Se-biofortified watercress, shown in Figure 3.13. It was observed that the watercress EtOH extracts enriched with 0.25 mg/L of Se showed slightly increased lipid droplets at doses of 100 μg/ml, 250 μg/ml, and 500 μg/ml. Regarding the PANC-1 cells shown in Figure 3.14, the 0.50 mg/L Se-biofortified watercress EtOH extracts treatment did not change the lipid droplet accumulation significantly, but the dose of 500 showed a more significant. Additionally, it is important to note that no signal was observed in any treatment group for PANC-1 cells when stained with PI for all treatment groups.

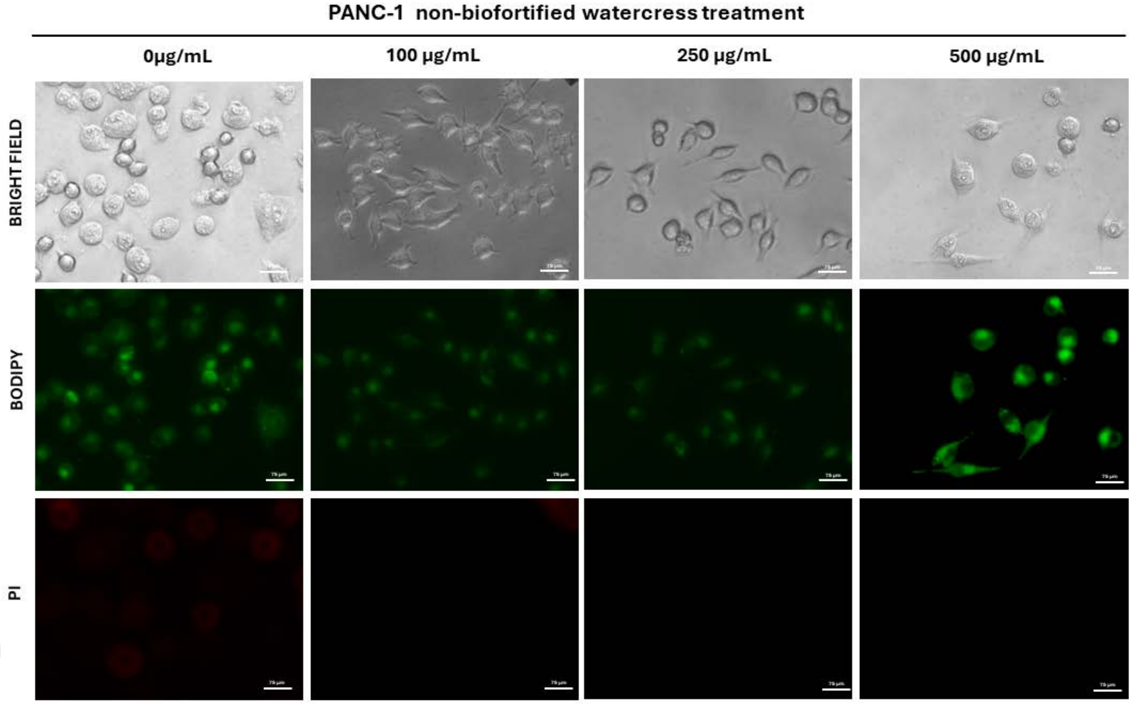

Figure 3.12 Lipid droplets and PI imaging of PANC-1 pancreatic cancer cells. Cells were treated with selected (100, 250, and 500 μg/ml) of non-biofortified watercress EtOH extracts for 24 h. BODIPY was used to analyze cells' lipid droplet formation ability, and PI was used to detect apoptotic cells. Images were taken with 40X objectives.

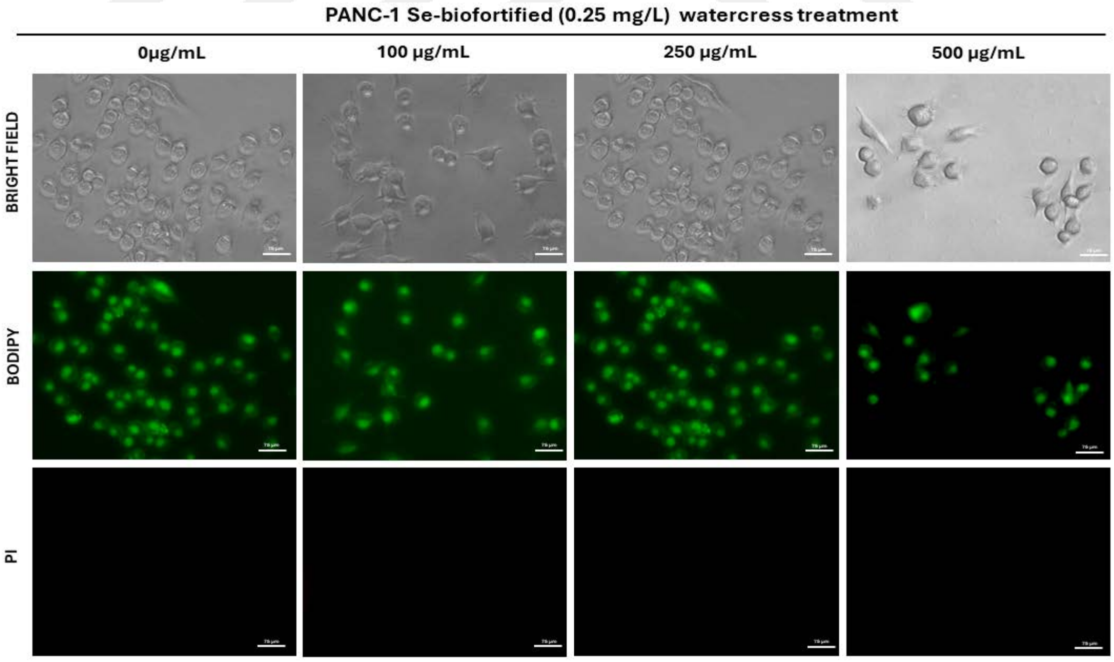

Figure 3.13 Lipid droplets and PI imaging of PANC-1 pancreatic cancer cells. Cells were treated with selected (100, 250, and 500 μg/ml) of 0.25 mg/L Se-biofortified watercress EtOH extracts for 24 h. BODIPY was used to analyze cells' lipid droplet formation ability, and PI was used to detect apoptotic cells. Images were taken with 40X objectives

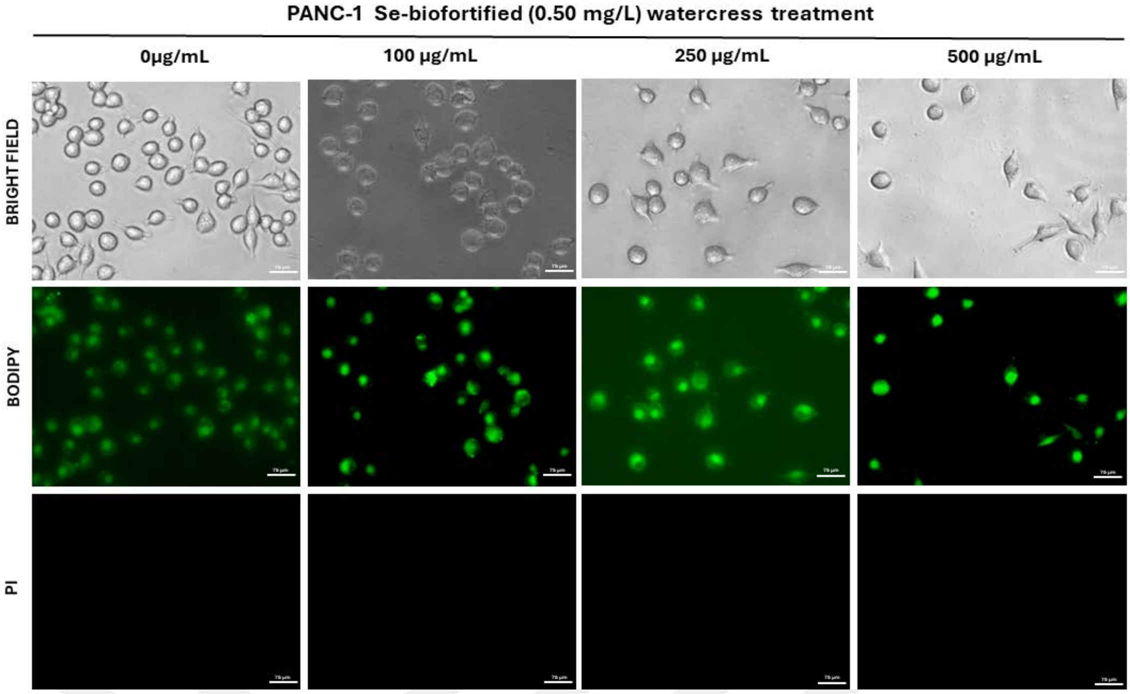

Figure 3.14 Lipid droplets and PI images of PANC-1 pancreatic cancer cells. Cells were treated with selected (100, 250, and 500 µg/ml) of 0.50 mg/L Se-biofortified watercress extracts for 24 h. BODIPY was used to analyze cells' lipid droplet formation ability, and PI was used to detect apoptotic cells. Images were taken with 40X objectives.

In MIA PaCa-2 cells treated with non-biofortified watercress are shown in Figure 3.15. Lipid droplets were reduced at doses of 100 µg/ml and 250 µg/ml. However, there is an increase observed in the lipid droplet formation of MIA PaCa-2 cells at the dose of 500 µg/ml compared to the control group. In PI staining, cell death was observed in non-biofortified watercress-treated MIA PaCa-2 cells at doses of 250 µg/ml and 500 µg/ml. Figure 3.16 shows the MIA PaCa-2 cells treated with 0.25 mg/L Se-biofortified watercress extract. The formation of lipid droplets was reduced at doses of 100 µg/ml, 250 µg/ml, and 500 µg/ml. Additionally, at the doses 250 µg/ml and 500 µg/ml, slight cell death was observed in PI-stained cells. Regarding MIA PaCa-2 cells treated with 0.50 mg/L watercress extract, which is shown in Figure 3.17, the formation of lipid droplets was decreased in a dose-dependent manner at doses of 100 µg/ml, 250 µg/ml, and 500 µg/ml, respectively. Finally, in PI-stained MIA PaCa-2 groups, cell death was observed only at a dose of 500 µg/ml.

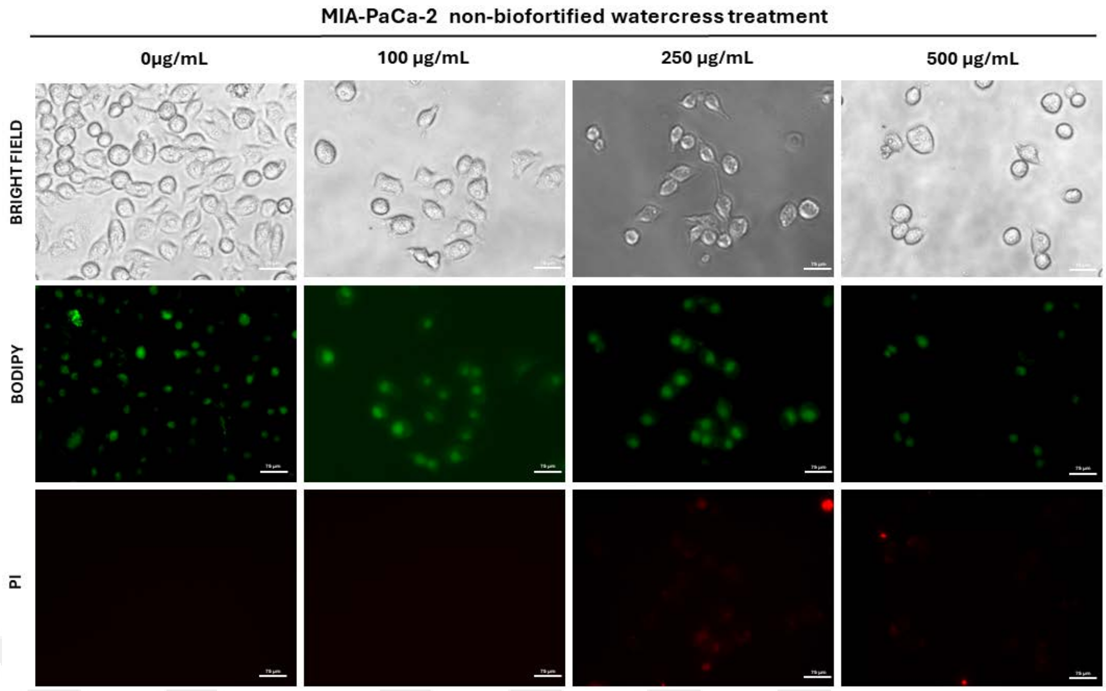

Figure 3.15 Lipid droplets and PI images of MIA PaCa-2 pancreatic cancer cells. Cells were treated with selected (100, 250, and 500 µg/ml) of non-biofortified watercress extracts for 24h. BODIPY was used to analyze cells' lipid droplet formation ability, and PI was used to detect apoptotic cells. Images were taken with 40X objectives.

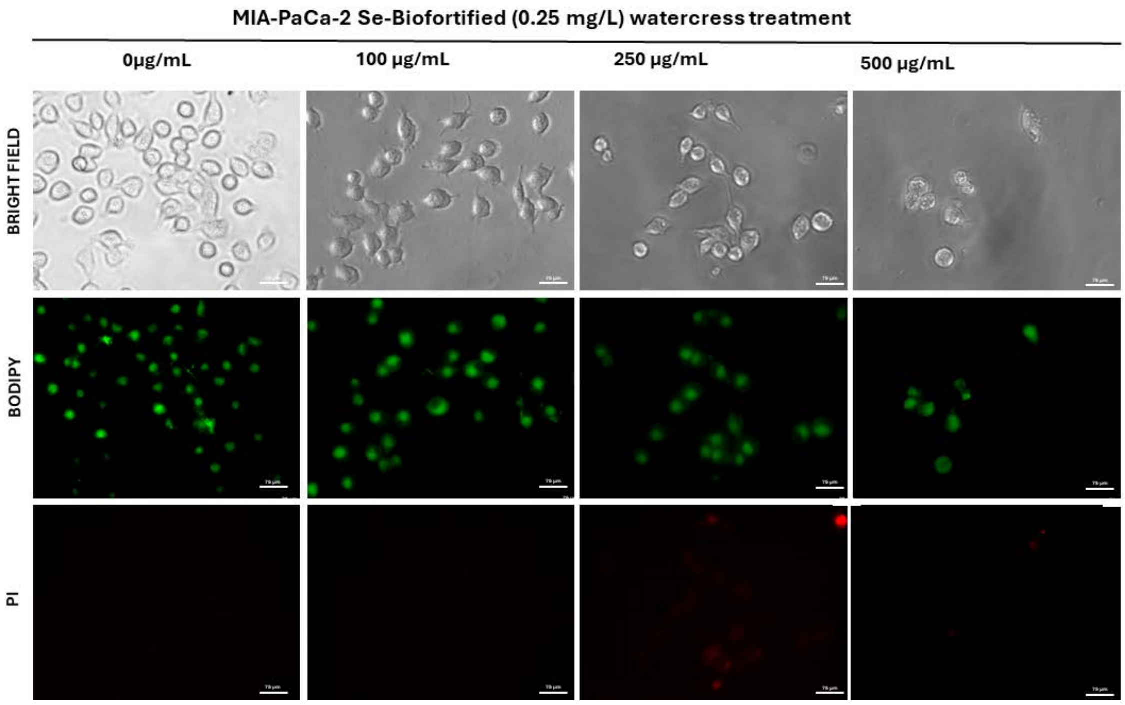

Figure 3.17 Lipid droplets and PI images of MIA PaCa-2 pancreatic cancer cells. Cells were treated with selected (100, 250, and 500 µg/ml) of 0.50 mg/L Se-biofortified watercress extracts for 24h. BODIPY was used to analyze cells' lipid droplet formation ability, and PI was used to detect apoptotic cells. Images were taken with 40X objectives.

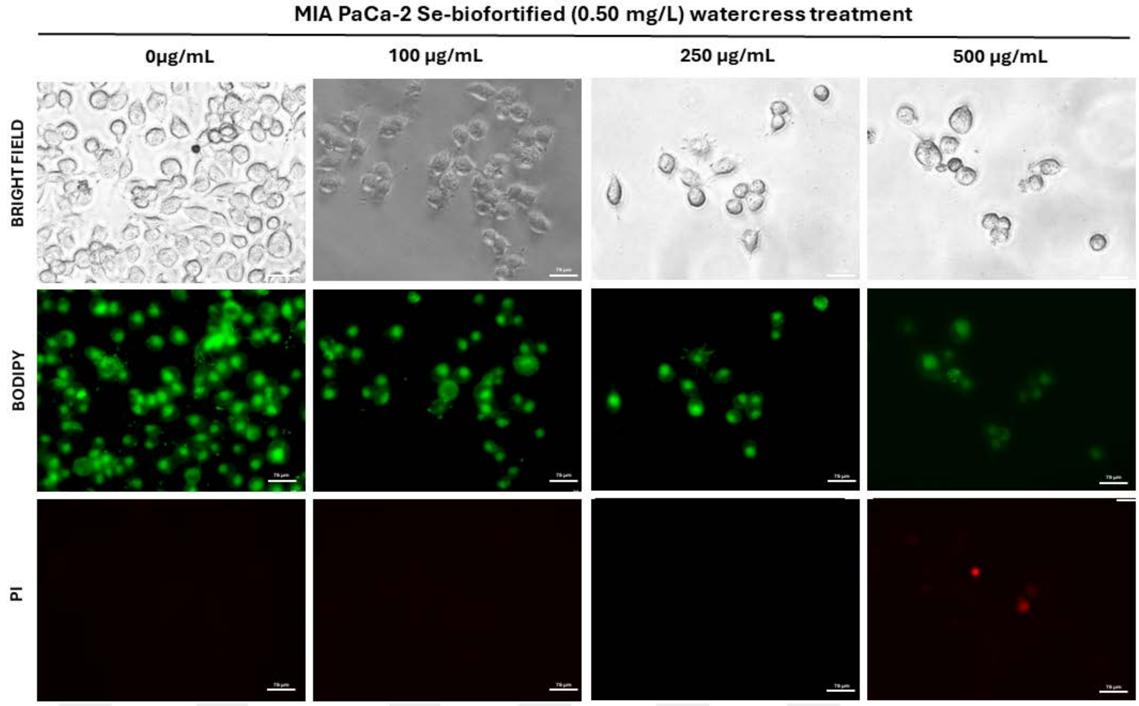

Figure 3.17 Lipid droplets and PI images of MIA PaCa-2 pancreatic cancer cells. Cells were treated with selected (100, 250, and 500 μg/ml) of 0.50 mg/L Se-biofortified watercress extracts for 24h. BODIPY was used to analyze cells' lipid droplet formation ability, and PI was used to detect apoptotic cells. Images were taken with 40X objectives.

3.5. mTOR and Autophagy-Related Protein Expression Analysis

mTOR and autophagy-related protein expressions of PANC-1 and MIA PaCa-2 cells treated with different concentrations (0, 100, 250, and 500 μg/ml) of Se-biofortified (0.50 mg/L) watercress extracts were analyzed by western blotting technique. In PANC-1 cells, mTOR was upregulated at doses of 100 μg/ml, 250 μg/ml, and 500 μg/ml of 0.50 mg/L Se-biofortified watercress extract compared to the control group, while mTOR was downregulated in the starvation-treated group with Hank's Balanced Salt Solution (HBBS) for 4 hours. ATG5 expression decreased dramatically at dose 100 compared to the control group, and this decrease was also seen slightly at doses 250 μg/ml and 500 μg/ml. ATG5 expression decreased in starved PANC-1 cells compared to the control group. In PANC-1 cells, LC-3 A/B A/B expression was not observed as a double band, as expected (Figure 3.6).

In MIA PaCa-2 cells, a decrease in mTOR levels was observed at doses of 100 μg/ml and 500 μg/ml, whereas other treatment groups showed similar expression levels compared to the control group. A decrease in ULK1 expression was observed at doses of 100 μg/ml and 500 μg/ml. The starvation group also showed expression levels similar to those of the control group. ATG5 expression increased dramatically in the starvation group, followed by decreases at doses 100 μg/ml, 250 μg/ml, and 500 μg/ml. Lipidation of LC-3 A/B is related to autophagic activity; therefore, Se-biofortified (0.50 mg/L) watercress extracts at doses of 100 μg/mL and 250 μg/mL induced the lipidation level of LC-3 A/B of MIA PaCa-2 cells, compared to the untreated negative control. Increased LC-3 A/B lipidation was observed in the HBBS-starved group compared to the control group. High-dose treatment led to increased lipidation of LC-3 A/B, whereas at a dose of 500 μg/mL, there was no increase compared to the control group (Figure 3.6).

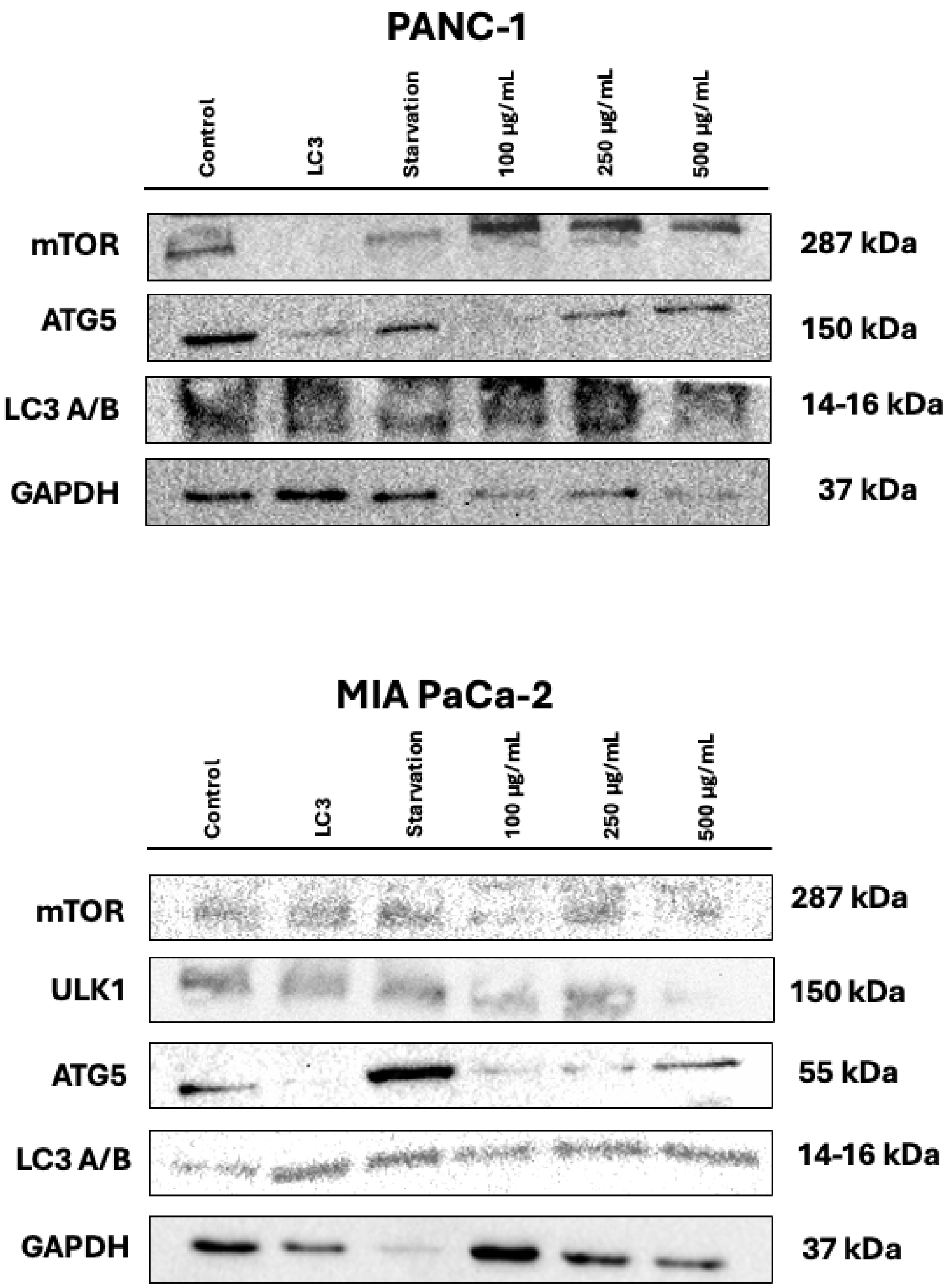

Figure 3.18 Effects of 0.50 mg/L Se-biofortified 500 µg/ml watercress EtOH extracts on pancreatic cancer cell lines for 24 h, and the protein expression levels of important molecular targets associated with mTOR and autophagy by western blotting. GAPDH was used as a loading control.

4. DISCUSSION AND CONCLUSION

In recent years, functional foods have gained significant attention for their potential in disease prevention and health promotion. Watercress stands out for its impressive health-promoting qualities, including antioxidant, anti-inflammatory, and potential anticancer effects **[61]**. Watercress contains several chemical constituents, such as glucosinolates, isothiocyanates, phenolic compounds, flavonoids, vitamins, and minerals, making it a powerful superfood **[14, 19]**. Besides the importance of bioactive compounds, essential elements are also vital for human health. Selenium is crucial for human health due to its role in antioxidant defense, immune function, and thyroid hormone metabolism. Selenium comprises several selenoproteins that help neutralize harmful free radicals and DNA damage, a significant factor in cancer development. **[32]**. The biological fortification of watercress with selenium, an essential trace element often deficient in society, further enhances its nutritional and therapeutic benefits, making it a completely functional food.

Selenium is a mineral that needs to be obtained from external sources by the human body. Promoting selenium intake in the population is essential, as well as reducing health problems caused by its deficiency and ensuring it is easy and accessible for everyone **[37]**. Additionally, while emphasizing selenium consumption, other health-promoting bioactive compounds should also be taken into consideration. Functional foods can be a natural and environmentally friendly solution to the increasing nutrient deficiency in society. The watercress utilized in this study was grown using a hydroponic technique and biofortified with selenium. Hydroponic systems are crucial due to their ability to facilitate the precise and effective cultivation of plants in nutrient-dense solutions, eliminating the requirement for soil. This approach not only enhances the nutritional value of the crops but also fosters ecological sustainability. Hydroponic farming necessitates less water and space in comparison to conventional agriculture, diminishes the likelihood of soil-borne illnesses, and permits meticulous control of nutrient levels to enhance plant growth and nutritional value. Hydroponic systems may utilize these benefits to create high-quality functional meals, such as selenium biofortified watercress, which can be made more readily available to a broader range of people **[34]**. Today, given the critical need for innovative approaches to combat diseases like, the potential of hydroponic systems to produce nutrient-enriched plants, such as selenium-biofortified watercress, becomes particularly relevant. The enhanced nutritional profile of these crops could offer additional therapeutic benefits, supporting existing treatments and contributing to overall patient health.

Pancreatic cancer is the fourth leading cause of cancer death in both men and women, with a poor prognosis and a five-year survival rate of less than 5%. Even for patients with localized, resectable tumors, the prognosis remains discouraging, with a five-year survival rate of only 20% **[41, 42, 58]**. The current medical treatments for pancreatic cancer primarily include surgical intervention, chemotherapy, and radiation therapy. Even so, chemotherapy encounters many obstacles that restrict its effectiveness **[47, 73]**. Gemcitabine, commonly used in conjunction with additional medicines such as nab-paclitaxel or FOLFIRINOX (a combination of fluorouracil, leucovorin, irinotecan, and oxaliplatin), is the established chemotherapy regimen. Although these combinations, the overall rates of response and advantages in terms of survival are still moderate. A significant obstacle in chemotherapy for pancreatic cancer is the compact stromal tissue that encompasses the tumor, restricting the transportation of drugs. The desmoplastic response restricts the infiltration of chemotherapeutic drugs into the tumor, diminishing their efficacy. Moreover, pancreatic cancer cells frequently acquire resistance to treatment through many pathways. These factors include the increase in drug efflux pumps, modifications in drug metabolism, and variations in apoptotic pathways. The presence of hypoxia and nutritional deprivation in the tumor microenvironment exacerbates treatment challenges by enhancing cancer cell survival and resistance to therapy. These problems emphasize the necessity for innovative therapy methods and the significance of incorporating techniques to overcome the physical and biological obstacles of pancreatic cancer [75-78]. Due to these challenges in the diagnosis and treatment of pancreatic cancer, there is growing interest in exploring complementary and alternative therapies, such as functional foods and nutraceuticals, to improve the efficacy of conventional treatments. Optimal nutrition and bioactive substances found in functional foods, such as selenium biofortified watercress, have the potential to enhance patient outcomes in cancer treatment. This can be achieved by targeting molecular pathways implicated in cancer growth and resistance. **[41- 43]**. Nutrition and dietary habits play an essential role in pancreatic cancer, and nutrient intake significantly influences the risk of developing pancreatic cancer and the prognosis of those who create the disease **[51]**. Specific dietary components, such as phenolic compounds and isothiocyanates, have shown potential protective effects and therapeutic benefits. These compounds can neutralize free radicals and reduce oxidative stress, lowering cancer risk. Consistently consuming foods high in phenolics has been linked to a reduced likelihood of developing various cancers, including pancreatic cancer **[52]**. Isothiocyanates in cruciferous vegetables like watercres11s, broccoli, Brussels sprouts, and kale exhibit significant anti-cancer properties. These sulfur-containing compounds prevent cancer by activating detoxification enzymes, promoting apoptosis, and halting the cancer cell cycle **[20]**. In a previous study on HT-29 cells, it has been demonstrated that PEITC induces G1 cell cycle arrest through activation of the p38 MAPK signaling pathway **[21]**. Enriching plants abundant in phenolic compounds with trace elements such as selenium provides a supplementary defense against cancer. Augmented consumption of phenolic compounds and trace elements directly from dietary sources, as opposed to supplements, may mitigate pancreatic cancer risk and ameliorate treatment-related side effects **[84, 87]**.

In this study, the anti-cancer and therapeutic effects of watercress extracts, which were enriched with 0.25 mg/L and 0.50 mg/L Se concentrations and turned into a functional food, were evaluated on PANC-1 and MIA PaCa-2 cell lines. The MTT assay was employed to assess the cellular metabolic activity of the treated cancer cells and provided a quantitative measure of cell viability and allowed us to compare the effects of different selenium concentrations and treatment doses on PANC-1 and MIA PaCa-2 cells. The cytotoxic responses of six different concentrations (0, 25, 50, 100, 250, and 500 μg/ml) of selenium biofortified watercress extracts (0.25 mg/L and 0.50 mg/L) were evaluated on PANC-1 and MIA PaCa-2 pancreatic cancer cell lines over 24 hours using the ethanol (EtOH) extraction method.

Our findings revealed that both selenium biofortified and non-biofortified watercress extracts significantly inhibited cell viability in a dose-dependent manner in PANC-1 and MIA PaCa-2 pancreatic cancer cell lines. In the PANC-1 cell line, the non-biofortified watercress extract exhibited a more pronounced cytotoxic effect across the tested concentrations. Conversely, in MIA PaCa-2 cells, the watercress extract enriched with 0.50 mg/L selenium showed a more significant cytotoxic effect. Both cell lines demonstrated relative resistance to the 0.25 mg/L selenium biofortified watercress extract, particularly at lower doses of 25 µg/ml and 50 µg/ml. This increased proliferation might be due to the activation of pro-survival pathways in response to mild oxidative stress, highlighting the need for precise dosing in therapeutic applications **[88]**. In 2006, Boyd et al. reported that crude watercress extract effectively inhibited DNA damage, reduced cell proliferation and impaired the invasive ability of colon cancer cells in vitro. Their findings highlight the extract's potential as a chemoprotective agent by targeting multiple stages of carcinogenesis, including initiation, proliferation and metastasis. Thus, it supports its role in the prevention of colorectal cancer, which, like pancreatic cancer, is a gastrointestinal cancer and shares the BRCA2 mutation **[95]**.

Interestingly, the highest cytotoxic effect in PANC-1 cells was observed at 500 µg/ml dose of non-biofortified watercress extract, followed by 0.50 mg/L selenium biofortified extract at the same concentration. This indicates that high doses of non-biofortified and selenium biofortified watercress extracts can effectively reduce cell viability in PANC-1 cells in a selenium-independent manner. The similar cytotoxic effects of the non-biofortified and 0.50 mg/L Se-biofortified watercress extracts suggest that selenium's addition does not significantly enhance the extract's efficacy against PANC-1 cells. This may be because watercress already has highly bioactive compounds, making it a great functional food candidate.

In MIA PaCa-2 Cells, the most effective cytotoxic effect of both non-biofortified and Se-biofortified watercress extracts was observed at the dose of 500 µg/ml. At the doses of 25, 50, 100, and 250 µg/ml, relative cell viability decreased compared to the control group, but this was still not enough to be considered a cytotoxic effect. Further, MIA PaCa-2 cells treated with 0.25 mg/L Se-biofortified watercress showed robust resistance. At the 50 µg/ml dose, relative cell viability was surprisingly increased compared to the control. The genetic profiles of MIA PaCa-2 and PANC-1 cell lines highlight their distinct molecular characteristics of pancreatic cancer. The MIA PaCa-2 cells have mutations that lead to aggressive behavior, invasiveness, and resistance to apoptosis. This makes them an ideal model for studying the metastatic and invasive properties of pancreatic cancer. Previous study showed that phenethyl isothiocyanate inhibited proliferation and induced apoptosis in pancreatic cancer cells *in vitro* in the MIA PaCa-2 xenograft animal model. Watercress is an excellent source of phenethyl isothiocyanate and our current findings overlap with the outcomes of previous studies on the MIA PaCa-2 line. On the other hand, the PANC-1 cells, despite carrying critical oncogenic mutations, maintain an epithelial phenotype and are less invasive **[89]**. The difference in the findings from the cell viability analysis is entirely related to the different genetic backgrounds of the two cell lines. In conclusion, watercress extract showed a significant dose-dependent effect on cell viability in both pancreatic cancer cell lines. Lastly, false positivities were observed in the MTT assay due to the phenolic contents in a previous study **[91]**. To prevent this, we changed the treatment medium before applying the MTT reagent during the experiment. It is important to note that we did not observe false positivities in the MTT assay, and we recommend that this be considered for further studies with watercress **[84]**.

Colony formation is a vital ability to evaluate the survival, proliferation, and form of a colony, which is essential for understanding the tumorigenic potential of cancer cells **[85]**. We conducted a colony formation assay to evaluate the colony-forming ability of pancreatic cancer cells from a single cell. Pancreatic cancer cells PANC-1 and MIA PaCa-2 were exposed to different concentrations of Se-biofortified (0.25 - 0.50 mg/L) watercress ethanol extracts for 24 h. In both cell lines, a dose-dependent decrease in colony-forming ability was observed. The colony formation assay results demonstrate a pronounced inhibitory effect of the extract treatments on cell proliferation, as evidenced by a significant reduction in colony formation in the treated groups compared to the control group. The observed decrease in the colony formation ability of MIA PaCa-2 cells correlates positively with the results of the previous MTT assay. However, in PANC-1 cells, 0.25 mg/ml Se-biofortified watercress extract showed an increase in relative cell viability in MTT results but a significant decrease in colony formation. Loss of the ability to form colonies indicates a reduced ability of cancer cells to sustain long-term growth and form new tumor masses. This can significantly reduce the likelihood of tumor recurrence and metastasis. Colony formation is directly linked to the proliferative capacity of cancer cells. When cells lose this ability, it indicates a reduced capacity to undergo multiple rounds of cell division. This can lead to slower tumor growth and potentially increase the effectiveness of therapies that rely on the rapid proliferation of cancer cells to exert their effects. Cancer cells that lose their ability to form colonies may become more sensitive to therapeutic interventions such as chemotherapy, radiation therapy, and targeted therapies. Colony-forming ability is also linked to the capability of cancer cells to invade other parts of the body.

Cells that can form colonies are more likely to survive and form new tumors in distant organs. Therefore, loss of the ability to form colonies may indicate a reduced capacity for cancer to spread, which is an important factor in cancer-related deaths **[85]**. Our findings revealed that the watercress extract significantly reduced the colony-forming ability of PANC-1 and MIA PaCa-2 pancreatic cancer cell lines in a dose-dependent manner, independently from selenium concentrations.

Cell survival assay was performed to analyze the proliferation of pancreatic cancer cells in different concentrations of Se-biofortified watercress extract for different increasing times. PANC-1 and MIA PaCa-2 cells were treated with non-biofortified and Se-biofortified (0.25 - 0.50 mg/L) watercress extract. The results revealed a significant reduction in cell numbers for both pancreatic cell lines compared to the control group. These results indicate the potential anti-proliferative effects of watercress extracts on pancreatic cancer cells. However, it is important to note that no significant differences were observed between the group treated with selenium biofortified watercress extracts and the non-biofortified treatment group. This suggests that selenium addition did not significantly alter the outcomes. Nevertheless, compared to control, a dose and time-dependent decrease in cell survival was investigated at all doses in the non-biofortified and Se-biofortified treatment groups. These results are significant for medical applications and encourage further investigation into novel cancer treatments, although a deeper understanding of the underlying mechanisms is still needed.

Fluorescent staining analyses were performed using MitoSpy and DAPI, PI, BODIPY, and DCFDA staining to evaluate pancreatic cancer cells of watercress extracts for mitochondrial membrane potential, cell death, lipid metabolism, and ROS. These analyses provided information on the effect of the extract on intracellular organelles and DNA. We also combined this with a relative fluorescence intensity analysis to evaluate the staining images quantitatively.

MitoSpy is a dye used to assess mitochondrial activity and health by selectively staining mitochondria in live cells. In our study, MitoSpy staining revealed significant changes in mitochondrial fluorescence intensity and distribution patterns in PANC-1 and MIA PaCa-2 pancreatic cancer cell lines treated with selenium biofortified watercress extracts. A dose-dependent decrease in mitochondrial fluorescence intensity was observed, indicating disrupted mitochondrial function and potential induction of mitochondrial-mediated apoptosis. For pancreatic cancer, mitochondrial dysfunction is crucial as it can lead to impaired energy production and increased oxidative stress, promoting cell death. The observed mitochondrial disruption suggests that selenium biofortified watercress extracts might help induce apoptosis in pancreatic cancer cells by disturbing mitochondrial potentials. This finding is promising because mitochondrial functioning is vital for cancer cell survival and proliferation, and its impairment can be a valuable therapeutic strategy **[58]**. Xiao et al. used MitoSpy staining to demonstrate that treatment with Phenethyl Isothiocyanate (PEITC) significantly increased mitochondrial superoxide production in human prostate cancer cells. This increase in mitochondrial reactive oxygen species (ROS) suggests that PEITC disrupts mitochondrial function, leading to oxidative stress. The study highlights the role of mitochondrial ROS as a key mediator in PEITC-induced apoptosis, as evidenced by the pronounced ROS accumulation in treated cells compared to controls **[96]**.

DAPI is a fluorescent stain that binds strongly to A-T rich regions in DNA, used to visualize nuclear morphology and assess cell viability. In our study, DAPI staining showed increased fluorescent signals in PANC-1 cells dose-dependently, indicating increased nuclear condensation and fragmentation, which are hallmarks of apoptosis. This effect was not as pronounced in MIA PaCa-2 cells, suggesting a different response between the two cell lines. MIA PaCa-2 cells are more invasive than PANC-1 cells, and if watercress did not sufficiently disrupt the cell membrane, this may explain why there was no significant change in DAPI signaling. However, the nuclear changes observed in PANC-1 cells indicate that selenium biofortified watercress extracts might induce apoptosis through DNA damage and chromatin condensation **[88]**. A previous study also investigated the effect of watercress and PEITC, an important bioactive compound of watercress, on MCF-7 breast cancer cells and suggested that these compounds are associated with cell cycle arrest, DNA damage, and compromised cell viability in cancer cells **[90]**. This finding may be necessary for pancreatic cancer therapy as it highlights the potential of selenium biofortified watercress extracts to trigger apoptotic pathways in cancer cells, leading to their effective elimination. In a study conducted on animal experiments, daily watercress intake was found to cause inhibition of experimental Ehrlich tumor growth. The complex bioactive compounds of watercress make it an undoubted functional food candidate **[23]**.

In our study, DCFDA staining revealed that selenium biofortified watercress extracts significantly reduced ROS levels in both pancreatic cancer cell lines. In they context of pancreatic can cer, controlling ROS levels is crucial as oxidative stress is known to cause cancer progression and resistance to treatment. However, it is important to emphasize that the reduction in ROS levels is not directly associated with increased selenium concentrations. Furthermore, Xiao et al. highlighted the role of mitochondrial ROS as a key mediator in PEITC-induced apoptosis, as evidenced by the marked accumulation of ROS in treated cells compared to controls in prostate cancer cells **[96]**.

BODIPY staining showed variations in lipid droplet formation in both PANC-1 and MIA PaCa-2 cells treated with selenium biofortified watercress extracts. The results indicate a dose-dependent effect on lipid accumulation, with higher doses leading to a more significant reduction in lipid droplets. The results indicate a dose-dependent effect on lipid accumulation, with higher doses leading to a more significant reduction in lipid droplets. Altered lipid metabolism is a hallmark of cancer cells, providing energy and building blocks for rapid proliferation **[94]**. The observed decrease in lipid droplets suggests that selenium-biofortified watercress extracts might disrupt lipid metabolism, depriving pancreatic cancer cells of the necessary resources for growth. Singh et al. demonstrated through BODIPY staining that Phenethyl Isothiocyanate (PEITC) treatment significantly reduced the number of neutral lipid droplets in prostate tumor cells. This decrease in lipid droplets suggests that PEITC inhibits lipid metabolism in these cells. The findings indicate that disrupting lipid storage could be a key mechanism through which PEITC exerts its anticancer effects, potentially impairing the energy supply needed for tumor growth and progression **[96]**.

We performed PI fluorescence staining to detect death cells with disrupted membranes. Although the number of cells decreased as the dose increased in the experimental groups, no significant result was observed in PI staining. This may be due to the medium change we made during the experimental phase to improve image quality. PANC-1 and MIA PaCa-2 are adherent cells; when they die, they lose their adhesion properties and start to float. Since the medium was changed, most of the dead cells may have floated away from the medium, and therefore, no signal from the PI stain could be detected.

We investigated the effects of selenium biofortified watercress extracts on mTOR and autophagy-related protein expressions in PANC-1 and MIA PaCa-2 pancreatic cancer cell lines. Our findings reveal different responses in these cell lines and emphasize the complex interaction between mTOR signaling and autophagy in the presence of watercress extracts. mTOR is a central regulator of cell growth, proliferation, and survival, and it plays a crucial role in cancer biology. Autophagy, a cellular degradation process that recycles damaged organelles and proteins, is intricately linked to mTOR signaling. Dysregulation of the mTOR-autophagy axis can significantly impact cancer cell survival and therapeutic resistance.

In PANC-1 cells, mTOR expression was upregulated with selenium biofortified watercress extract treatment compared to the control. This suggests that selenium biofortified extracts may activate mTOR signaling pathways in these cells, potentially promoting cell growth and proliferation. The downregulation of mTOR in the starvation-treated group (HBBS) aligns with known responses to nutrient deprivation, which typically inhibit mTOR activity to conserve cellular resources **[95,96]**. Cavell et al. demonstrated that PEITC inhibits mTORC1 activity through a mechanism that relies on TSC2, a key negative regulator of mTORC1 in mouse embryonic fibroblast (MEFs). Their findings revealed that PEITC treatment significantly reduces the phosphorylation of critical mTORC1 targets, such as p70 S6K and 4E-BP1, both of which play essential roles in regulating protein synthesis and cell growth **[98]**.

ATG5, a critical protein in autophagy, showed a marked decrease in expression with selenium biofortified watercress extract treatment in PANC-1 cells. This decrease indicates that selenium biofortified watercress extract may inhibit autophagy initiation in PANC-1 cells. Similarly, starvation conditions reduced ATG5 expression, which might reflect a cellular adaptation to stress by modulating autophagy pathways. The absence of an apparent double band for LC-3 A/B in PANC-1 cells complicates the interpretation of autophagy, suggesting that further investigation is required to understand the impact on autophagy comprehensively. Liu et al. demonstrated that Phenethyl Isothiocyanate (PEITC) induces autophagy in Cal-27 oral squamous cell carcinoma cells through upregulation of autophagic markers, such as ATG5 and LC3-II. Their study showed that PEITC treatment led to increased levels of ATG5 and the conversion of LC3-I to LC3-II, which are critical indicators of autophagy **[98]**.

In contrast, MIA PaCa-2 cells displayed a decrease in mTOR levels with selenium biofortified watercress extract treatment, indicating that selenium biofortified watercress extract might inhibit mTOR signaling in these cells, potentially reducing cell proliferation. ULK1, another autophagy-related protein, also showed decreased expression, aligning with the observed mTOR downregulation, as ULK1 is regulated by mTORC1. ATG5 expression in MIA PaCa-2 cells increased under starvation conditions as expected. Then it showed a significant decrease with selenium biofortified watercress extract treatment, suggesting a complex regulatory mechanism where selenium biofortified extracts might suppress autophagy initiation. A previous study indicated that ATG5 plays an essential role in the regulation of PEITC-induced autophagic and apoptotic cell death in prostate cancer cells **[23]**. Watercress is a rich source of PEITC, and its effects were similar to those observed in the ATG5 expression in pancreatic cancer cells. Lipidation of LC-3 A/B, a marker of autophagosome formation, increased with selenium biofortified watercress extract treatment, indicating enhanced autophagic activity. However, there was no increase in LC-3 A/B lipidation at higher concentrations, suggesting a possible threshold effect where higher concentrations might inhibit autophagy.

The differential expression patterns of mTOR, ATG5, and LC-3 A/B in PANC-1 and MIA PaCa-2 cells underline the cell-specific responses to selenium biofortified watercress extracts. In PANC-1 cells, the upregulation of mTOR and downregulation of ATG5 suggest promoting cell growth and inhibiting autophagy, potentially supporting tumor progression. Conversely, in MIA PaCa-2 cells, the downregulation of mTOR and increased LC-3 A/B lipidation imply that selenium biofortified extracts may induce autophagic cell death, offering a therapeutic potential to counteract cancer cell survival. Understanding these mechanisms could pave the way for developing targeted therapies that exploit the unique vulnerabilities of specific cancer cell types **[62,73,74,94,95]**.

While this study has promising results, further research must address its limitations. This study Metinler arasında satır boşluğu olmamalıinvestigated the effects of selenium-biofortified watercress extract on PANC-1 and MIA PaCa-2 pancreatic cancer cell lines as in vitro models. As an initial step for further research, it is required to investigate the effects on the healthy pancreas cell lines. Several factors must be considered when translating these findings from the laboratory to real-world applications. When consumed as part of a diet, the effectiveness of selenium-biofortified watercress depends on the digestion and absorption of its bioactive compounds. Once ingested, these compounds must navigate the digestive system and be efficiently absorbed in the intestines. Post-absorption, they undergo a metabolic transformation in the liver, which can alter their form and bioactivity. In the human body, selenium and other compounds from watercress interact with numerous dietary components. These interactions can enhance or inhibit their bioactivity, making it difficult to interpret in vitro results **[98]**. *In vivo* studies are essential to determine the therapeutic potential and safety of selenium-biofortified watercress extracts in a more complex biological system. Moreover, genetic differences among individuals can influence the metabolism and utilization of selenium and other bioactives, leading to variable responses. Thus, clinical trials are necessary for further studies in line with the results obtained from *in vivo* animal experiments to investigate the different effects depending on people.

Se-biofortified watercress as a functional food offers significant potential for sustainable health solutions and direct therapeutic effects. Functional foods like selenium-biofortified watercress can be crucial in disease prevention and health promotion. Integrating such foods into daily diets could inspire a shift towards improved overall nutritional quality and public health, potentially reducing the incidence of various diseases, including cancer. Evaluating tumor growth inhibition, metastasis, and overall survival in pancreatic cancer models will provide valuable insights into the clinical relevance of these findings. Further studies are needed to elucidate the precise molecular mechanisms involved. Investigating the role of upstream and downstream effectors of mTOR and autophagy pathways and other signaling pathways will deepen our understanding of how these extracts modulate cellular processes in pancreatic cancer cells.

In conclusion, our findings suggest that biofortified watercress as a functional food has preventive and therapeutic potential against pancreatic cancer cell lines. The different effects observed in targeted pancreatic cancer cell lines highlight the complexity of mTOR and autophagy signaling pathways when affected by bioactive compounds.

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BIOGRAPHY

Nasip Ümran TURAN enrolled in the Biotechnology Department at Bartin University in 2016. During her undergraduate studies, she conducted research on the relationships between phenolic compounds and gut microbiota. In 2019, she completed an Erasmus+ internship on carbohydrate-binding proteins at the Technical University of Denmark. After graduating in 2020, she joined the Biotechnology MSc program at the Institute of Biotechnology, Gebze Technical University in 2021. Since 2023, she has been working on the Local Food Trace ERASMUS+ (2022-KA220-VET-B4D7B161) and ARDEB (2023-A-113-05) projects.

PUBLICATIONS AND PRESENTATIONS FROM THE THESIS

Turan Ümran N., Arısan Damla E., Kutman Ümit B., (2024), "Effects Of Watercress (Nasturtium Officinale) Extract Grown at Different Selenium Levels On Pancreatic Cancer Cell Lines," 1st International Congress on Biotech Solutions for Sustainability-Kocaeli, Türkiye, 7-10 May.

APPENDICES

APPENDIX-A: COLONY FORMATION ASSAY REPLICATES OF PANC-1 CELL LINE

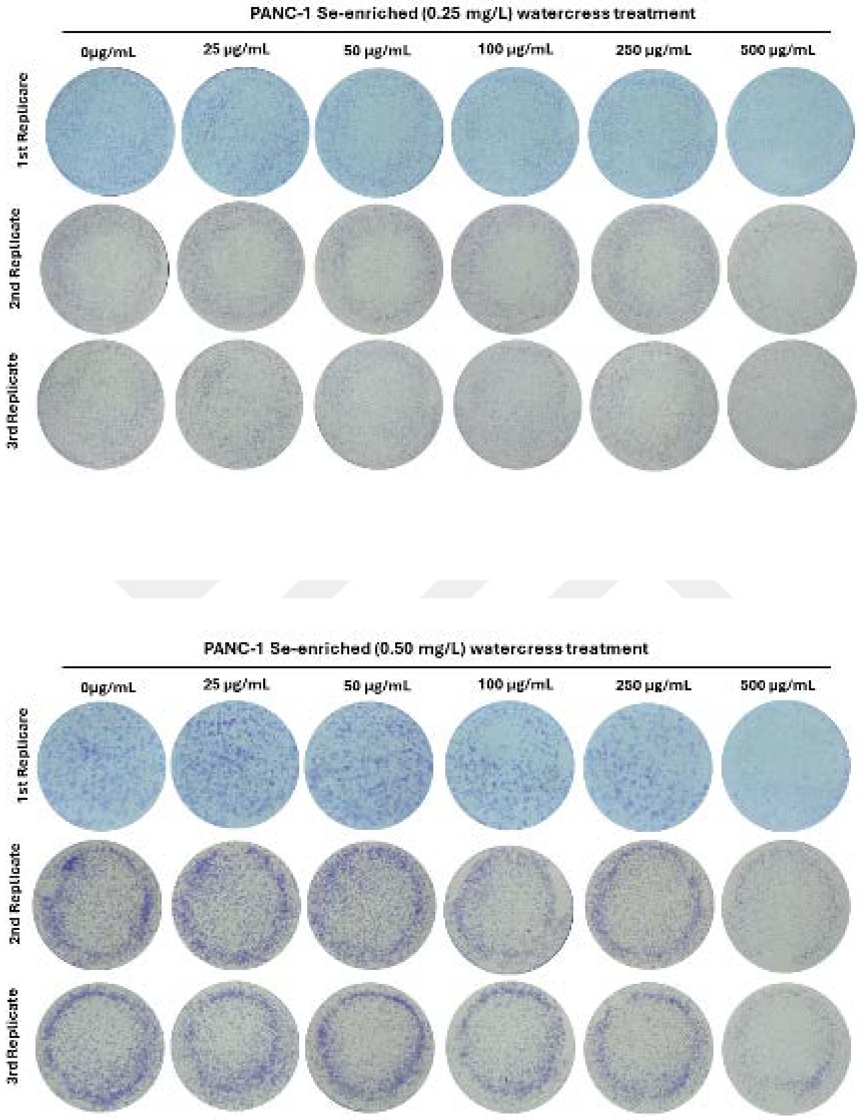

Figure A.1: 3 replicates of colony formation assay on PANC-1 cells. Pancreatic cancer cells were exposed to different concentrations (0, 25, 50, 100, 250, and 500 μg/ml) of selenium biofortified (0.25 - 0.50 mg/L) and non-biofortified watercress extracts for 24 h. The colony formation of cells was counted after ten days of treatment with media replenishment every two days.

APPENDIX-B: COLONY FORMATION ASSAY REPLICATES OF MIA PaCa-2 CELL LINE

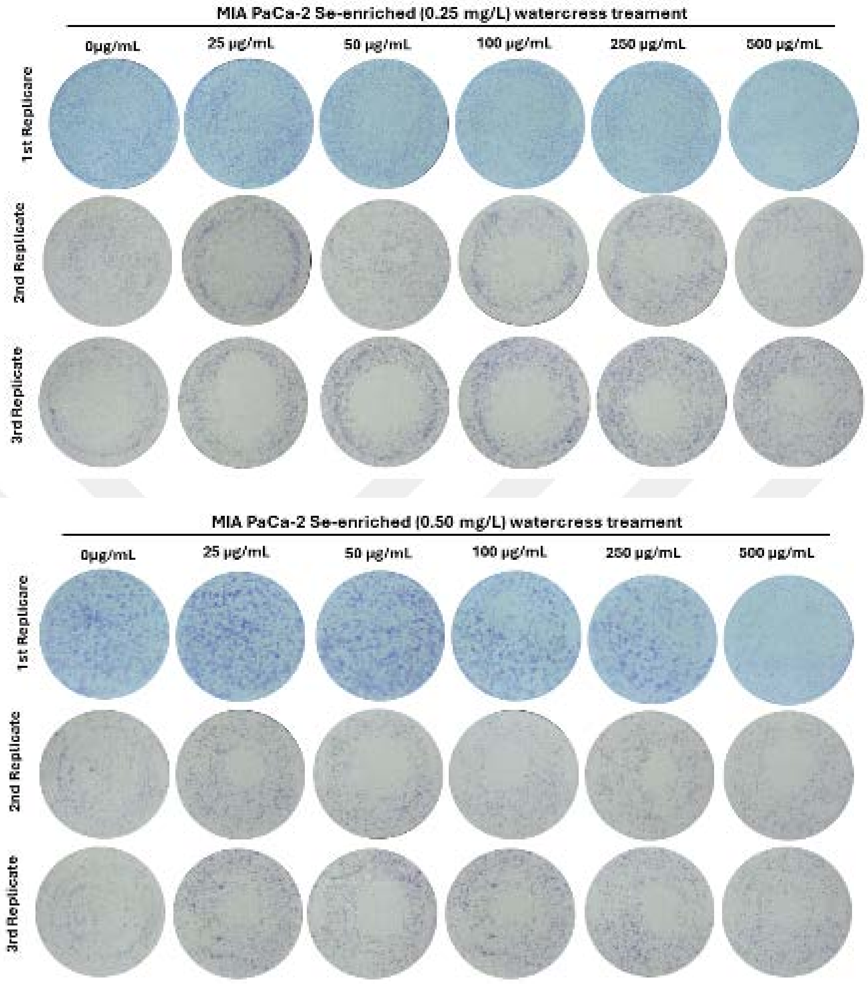

Fıgure A.2: 3 replicates of colony formation assay on PANC-1 cells. Pancreatic cancer cells were exposed to different concentrations (0, 25, 50, 100, 250, and 500 µg/ml) of selenium biofortified (0.25 - 0.50 mg/L) and non-biofortified watercress extracts for 24 h. The colony formation of cells was counted after ten days of treatment with media replenishment every two days.