

T.R.
GEBZE TECHNICAL UNIVERSITY
INSTITUTE OF BIOTECHNOLOGY

**THE INVESTIGATION OF THE ANTI-CANCER
POTENTIAL OF WATERCRESS AS AFFECTED BY
SELENIUM NUTRITION**

EZGİ GÜNGER
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
DEPARTMENT OF BIOTECHNOLOGY

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GEBZE
2023

T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
BİYOTEKNOLOJİ ENSTİTÜSÜ

SELENYUM TAKVİYESİNİN ETKİLEDİĞİ
SU TERESİNİN ANTI-KANSER
POTANSİYELİNİN İNCELENMESİ

EZGİ GÜNGER
YÜKSEK LİSANS TEZİ
BİYOTEKNOLOJİ ANABİLİM DALI

DANIŞMANI
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ASST. PROF. ÜMİT BARIŞ KUTMAN

GEBZE
2023

	YÜKSEK LİSANS JÜRİ ONAY FORMU
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ABSTRACT

Functional foods can improve health and reduce the risk of diseases by offering specific physiological benefits in addition to essential nutrients. Watercress, a member of the Brassicaceae family, has health-promoting properties akin to cruciferous vegetables like broccoli and cabbage.

In this study, biofortification of watercress with selenium, which is one of the most commonly deficient micronutrients in humans, was achieved. Selenium at concentrations of 0.25 mg/L and 0.50 mg/L was administered to watercress plants. The results showed that selenium biofortification had no significant impact on watercress fresh biomass ($p>0.05$). A linear relationship between selenium accumulation and dosage was observed as expected. While the selenium level (0.50 mg Se/L) in the nutrient solution culture influenced the accumulation of S and Mo, it had no significant effect on other elements. Moreover, the study indicated that selenium treatments did not have a statistically significant impact on total phenolic analysis, antioxidant capacity analysis, chlorophyll and carotenoid levels, as well as nitrate capacity analysis in watercress. Additionally, experiments conducted on pancreatic cancer cells with the aim of assessing the anti-cancer effects of watercress revealed a significant decrease in cell proliferation and colony formation compared to the control group. Moreover, cell cycle arrest analysis demonstrated that watercress extracts induced apoptotic cell death in these cells. In conclusion, watercress shows potential as an anti-cancer candidate for pancreatic cancer cells, and selenium application enriches its contents.

Key Words: Watercress, Selenium, Biofortification, Cancer

ÖZET

Fonksiyonel gıdalar, temel besin maddelerinin yanı sıra belirli fizyolojik faydalar sunarak sağlığı iyileştirebilir ve hastalık riskini azaltabilir. Su teresi, Brokoli ve lahana gibi lahanagiller ailesine mensup olup, sağlığı destekleyen özelliklere sahiptir.

Bu çalışmada, insanlarda eksikliği sık raslanan mikrobesein maddelerinden biri olan selenyum ile su teresi bitkisinin biyofortifikasyonu gerçekleştirilmiştir. Su teresine 0.25 mg/L ve 0.50 mg/L konsantrasyonlarındaki selenyum uygulanmıştır. Sonuçlar, selenyum biyofortifikasyonunun su teresinin yaş ağırlığına istatistiksel olarak anlamlı bir etkisi olmadığını gösterdi ($p>0.05$). Beklendiği gibi, selenyum birikimi ile dozlar arasında doğrusal bir ilişki gözlemlendi. Topraksız besi kültüründeki 0.50 mg/L Se seviyesi, S ve Mo birikimini etkiledi, ancak diğer elementler üzerinde anlamlı bir etkisi olmadı. Ayrıca, çalışma, selenyum uygulamalarının su teresinde toplam fenolik analiz, antioksidan kapasite analizi, toplam klorofil ve karotenoid seviyeleri ve nitrat kapasite analizi üzerinde istatistiksel olarak anlamlı bir etkisi olmadığını ortaya koydu. Ek olarak, su teresinin pankreas kanseri hücreleri üzerindeki anti-kanser etkilerini değerlendirmek amacıyla yapılan deneyler, kontrol grubuna kıyasla hücre çoğalmasında ve koloni oluşturmada önemli bir azalma olduğunu ortaya koydu. Ayrıca, hücre döngüsü analizi, su teresi ekstraktlarının bu hücrelerde apoptotik hücre ölümünü tetiklediğini gösterdi. Sonuç olarak, su teresi, pankreas kanseri hücreleri için bir anti-kanser adayı olarak potansiyel göstermektedir ve selenyum uygulaması içeriğini zenginleştirmektedir.

Anahtar Kelimeler: Su teresi, Selenyum, Biyofortifikasyon, Kanser

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations and Acronyms</u>	<u>Explanations</u>
°C	: Celsius
h	: Hour
%	: Percentage
g	: Gram
w	: Weight
v	: Volume
mm	: Millimeter
mL	: Milliliter
mg	: Milligram
cm	: Centimeter
M	: Molar
μM	: Micromolar
μg	: Microgram
μm	: Micrometer
μL	: Microliter
kg	: Kilogram
L	: Liter
<i>p</i>	: P value
t	: Time
ANOVA	: Analysis of variance
CO ₂	: Carbon dioxide
e.g	: For example
et. al.	: And others
DW	: Dry weight
min	: Minute
NaCl	: Sodium Chloride
rpm	: Revolutions per minute
TPC	: Total phenolic content

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1. INTRODUCTION

Functional foods, including fruits and vegetables enriched with essential nutrients, play a crucial role in promoting overall health and preventing nutritional deficiencies. Furthermore, the production and promotion of these functional foods are of great importance in addressing widespread public health problems, especially in regions characterized by a high prevalence of nutritional deficiencies. Also, these fortified foods play a pivotal role in reducing nutritional disparities across communities, ultimately leading to improved community health and well-being. Selenium stands as a crucial micronutrient profoundly affecting human health. However, the global challenge of selenium deficiency has spurred investigations into biofortification as a potential solution to this problem. It can also enhance the nutritional quality of crops and have positive environmental impacts [1], [2].

Watercress, scientifically known as *Nasturtium officinale*, is a cruciferous vegetable that has garnered interest due to its potential as a functional food. Watercress is known for its high content of bioactive phytochemicals, including glucosinolates, phenolic compounds, carotenoids, and vitamin C [3], [4]. These substances have been linked to a range of health-enhancing characteristics, including antioxidative, anti-inflammatory, and anticancer effects. Among the notable bioactive compounds present in watercress is phenethyl isothiocyanate (PEITC), which has been the subject of extensive research for its potential in cancer prevention [5].

Selenium is a vital trace element with a fundamental role in human well-being. It participates in numerous physiological functions, such as supporting the immune system, converting thyroid hormones, and safeguarding the body against the detrimental effects of heavy metals and foreign substances. Inadequate selenium levels can result in diminished performance and, in severe cases, even fatalities [6]. Hence, it is crucial to maintain an appropriate selenium intake for the sake of one's overall health and welfare.

The process of biofortification, holds the potential to enhance the selenium levels in crops, rendering them more nutritious and advantageous for human consumption. Moreover, the biofortification of crops with selenium can yield favorable outcomes for human nutrition. Selenium is engaged in the synthesis of selenoproteins, which possess antioxidative characteristics and contribute to a range of physiological functions [7].

Pancreatic cancer is a highly aggressive and deadly disease, with a low survival rate. It is estimated that pancreatic cancer may soon become the second-leading cause of cancer death in the United States [8]. Therefore, there is an urgent need to develop better therapeutic options to improve patient outcomes. Considering watercress's potent antioxidant properties, particularly due to its high content of PEITC, it may present itself as a potential option for pancreatic cancer treatment.

1.1. Aim and Contribution of Thesis

In this thesis, selenium biofortification of watercress was conducted using two different concentrations, 0.25 mg/L and 0.50 mg/L, to explore its effects on various aspects of the plant. The study encompassed an examination of the accumulation of both micro and macro elements in the plant, as well as an assessment of chlorophyll and carotenoid levels. Furthermore, it investigated the impact of biofortification on antioxidant capacity, nitrate accumulation, and the total phenolic compound content. Additionally, the study delved into the evaluation of crude extracts obtained from the plant, analyzing their influence on pancreatic cancer cells in terms of cell proliferation, colony formation potential, and cell cycle arrest effects. This comprehensive investigation sheds light on the multifaceted implications of selenium biofortification in the context of watercress and its potential benefits for both plant and human health.

2. LITERATURE REVIEW

2.1. Watercress

Watercress, also known as *Nasturtium officinale* R. Br., is a resilient plant that belongs to the Brassicaceae family. It is a perennial species that is commonly found in wet regions. Despite having its origins in western Asia, Europe, India, and Africa, watercress can grow anywhere in the world, so its cultivation is not limited to these areas [9], [10]. Watercress is recognized as a safe edible plant by the European Food Safety Authority and has been included in their monograph "leaves, vegetables, herbs and edible flowers" [11]. Watercress is a popular ingredient in Mediterranean cuisine, enjoyed both raw in salads and cooked, particularly in soups. It's known for its sharp, peppery flavor and is recognized for its health-enhancing properties, including antioxidant, anti-inflammatory, and anticancer effects [12].

2.1.1. Chemical Composition of Watercress

Watercress contains a range of beneficial nutrients and compounds, including folic acid, carotenoids, glucosinolates, vitamins B, C, and E, pro-vitamin A, calcium, iron, iodine, and sulfur, which are associated with numerous health benefits [13]. This leafy green's high-water content, approximately 93%, results in its classification as a low-calorie food. Its carbohydrate and fat content are minimal, yet it offers a noteworthy protein content. Beyond these essential nutrients, watercress contains an array of bioactive compounds, including flavonols, hydroxycinnamic acids, and glucosinolates, with phenethyl isothiocyanate (PEITC) being the predominant compound. This comprehensive nutrient profile underscores watercress as a remarkable dietary choice with the potential for various health benefits.

2.1.2. Glucosinolates

Watercress, a member of the cruciferous vegetable family, is rich in glucosinolates, sulfur-containing compounds known for their potential anti-cancer properties [14]. The endogenous myrosinase enzyme is found in cruciferous

vegetables and catalyzes the hydrolysis of glucosinolates in the human body after consumption. When the plant tissue is chopped, chewed, or cut, the myrosinase enzyme shows its activity. This enzyme hydrolyzes thioglucosidic bonds by contacting glucosinolates and converts glucosinolates into hydrolysis products [15]. Although the products released vary depending on the reaction conditions, isothiocyanates and indoles formed because of enzymatic activity are the biologically active forms of glucosinolates. PEITC, obtained from glukonasturtiin, a form of glucosinolate that is converted into isothiocyanates through the action of myrosinase enzyme, is the most widely studied aromatic isothiocyanate [16].

PEITC (phenethyl isothiocyanate) has attracted interest in the field of anticancer research because of its potential as a powerful anti-cancer agent. Numerous studies have explored the mechanisms through which PEITC demonstrates its anticancer properties. One mechanism that has been discovered involves the triggering of cell-cycle arrest and apoptosis. PEITC has been demonstrated to result in cell-cycle arrest and the initiation of apoptosis in diverse cancer cell lines [17]. PEITC has been identified as an inhibitor of oxidative phosphorylation, resulting in the production of reactive oxygen species (ROS) and subsequent cell death in human prostate cancer cells [18]. Another study has shown that PEITC regulates intracellular ROS levels, playing a role in its anticancer activity [19].

2.2. History and Properties of Selenium

The crucial discovery of selenium was made in 1817 by the Swedish chemist Berzelius. It belongs to the VIA group of the periodic table, alongside elements like oxygen, sulfur, and tellurium. With an atomic weight of 78.96 and an atomic number of 34, selenium is a nonmetal [20], [21]. Selenium has six naturally occurring isotopes with the mass numbers 74, 76, 77, 78, 80, and 82. The two most prevalent isotopes are ^{80}Se and ^{78}Se , which have natural abundances of about 50 and 24%, respectively. Selenium in the environment is found in elemental state (Se^0), in the form of selenides (Se^{2-}), selenates (SeO_4^{2-}), or selenites (SeO_3^{2-}). Many factors, such as humidity level, pH, amount of free oxygen available, affect changes in selenium compounds. When the environment lacks oxygen and is acidic, the formation of selenium occurs more commonly if the environment does not have enough oxygen and is alkaline [22], [23]. Chemical characteristics like atomic size, ionization potential, and binding energy are

similar with those of sulfur. As a result, selenic acid, selenide selenite, and diselenide are selenium analogs of numerous sulfur compounds. The reaction between selenium and sulfur can result in the formation of selenyl sulfide bonds. Despite these similarities, the two elements are very different from one another, and different compounds with various chemical properties result from their replacement [20].

2.3. Selenium and Health

Selenium is an essential micronutrient necessary for the proper functioning of all organisms. However, it was long considered a toxic element due to symptoms such as poisoning, severe anemia, bone stiffness, hair loss, and blindness observed in humans and animals consuming or exposed to high doses of selenium [24].

As selenium can enter the body through inhalation, its concentration in the air should not exceed 0.2 mg/m³ to prevent harm. It is important to be cautious because the difference between a beneficial and a dangerous dose is very small [25].

Consumption in high doses is harmful, as well as insufficient supplementation causes an increased risk of developing many chronic degenerative diseases. Moreover, selenium deficiency can be associated with health problems such as muscle pain, proximal weakness, and skeletal muscle disorders [26]. It has also been observed that it may increase the risk of complications during pregnancy [27]. When the effects of selenium on the immune system are evaluated, it is seen that dietary selenium is important for a healthy immune system. In the deficiency of selenium, decrease in T cells and deterioration in lymphocytes can be seen. It has been observed that the immune response of T-lymphocytes is increased when a dietary supplement of 200 µg with sodium selenite is taken [28].

2.4. The Role of Selenium in Cancer Prevention and Treatment

Selenium is a vital trace element that plays a crucial role in maintaining human health. Research suggests that selenium may possess anti-cancer properties. The antioxidant effects of selenium in humans protect cells from the harmful effects of free radicals and have been shown to reduce the risk of cancer [29]. A study was conducted

in the USA to investigate the effect of selenium on cancer formation by comparing cancer rates in regions with high and low selenium levels. The study found that regions with high selenium had a lower rate of cancer, indicating that selenium at levels found in foods is not carcinogenic and has a protective function against cancer. This hypothesis was later supported by additional studies including breast cancer [30], colorectal cancer [31], prostate cancer [32], as well as overall cancer risk [33].

The effect of selenium on cancer development and progression varies according to the concentration or dose of the Se compound and its chemical form. The three most important Se compounds in cancer prevention; sodium selenite, l-selenomethionine and Se-methylselenocysteine. These compounds have different metabolic pathways and differ in reducing the risk of cancer. For example, l-selenomethionine is better absorbed than sodium selenite, but selenite is more toxic than Se-methylselenocysteine and selenomethionine. Selenium, essentially, has both beneficial and harmful effects for health. At nutritional levels, it functions as an antioxidant through selenoproteins, while at higher doses it acts as a pro-oxidant [34]. Studies show that high selenium exposure has a protective effect on cancer risk but still the relationship between selenium and cancer is complex and requires further investigation.

2.5. Selenium Biofortification

Biofortification is a promising functional agricultural strategy to increase the uptake and accumulation of certain nutrients by plant breeding, genetic engineering or fertilization of plants or agricultural products [35]. This strategy is especially important in order to meet the necessary micronutrients such as selenium, the deficiency of which causes various health problems for human health [36]. Studies on selenium transport and metabolism in plants have shown that selenium biofortification is the best strategy to increase selenium uptake in areas with low selenium levels, and sodium selenate and sodium selenite are the most common forms of selenium used for this [37].

Selenium metabolism in plants involves the uptake, translocation, assimilation, and metabolic fate of selenium within plant cells. Selenium has chemical and physical similarities to sulfur, and therefore, it follows similar metabolic routes as sulfur does in plants. Plants can absorb selenium from the soil through their roots and distribute it to various plant parts via the xylem and phloem [38]. Within the plant, selenium can become integrated into organic compounds like selenocysteine and selenomethionine.

These organic selenium compounds can act as building blocks for the production of selenoproteins, which serve crucial functions in a variety of biological processes [39]. Additionally, plants possess biochemical mechanisms capable of eliminating surplus selenium (Se) through the conversion of this element into volatile selenium compounds such as dimethyl-selenide (DMSe) or dimethyl-diselenide (DMDSe). This transformation process is commonly referred to as volatilization and is recognized as a means of detoxification [40].

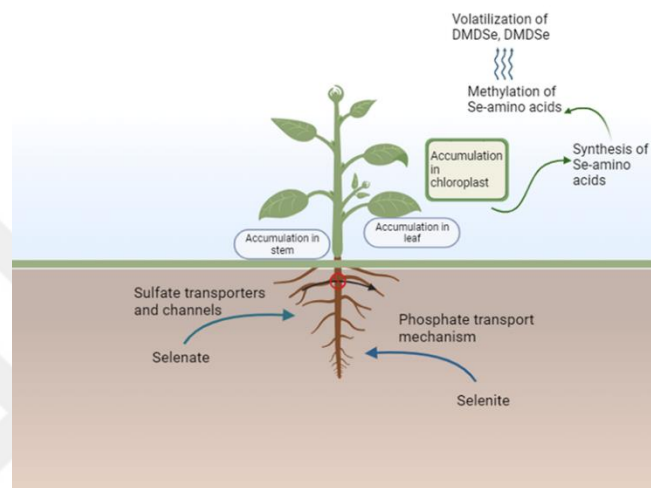


Figure 2.1: The schematic of Se metabolism in plants. Abbreviations: Se-amino acids, seleno-amino acids; DMSe, dimethyl-selenide.

A schematic of Se metabolism in plants is illustrated in Figure 2.1.[40].

2.6. Pancreatic Cancer

Pancreatic cancer is a highly lethal disease with limited treatment options and a high mortality rate [41]. Pancreatic cancer has a low 5-year survival rate of 12%. It ranks 10th among cancers diagnosed and approximately 64,050 new cases are expected in the United States in 2023 [42]. During the development and progression of pancreatic cancer, most patients do not develop obvious symptoms, making early diagnosis difficult. As the disease progresses to advanced metastatic stages, tumor cells become highly invasive. As a result, pancreatic cancer has become one of the deadliest malignancies [43].

Risk factors for pancreatic cancer can be divided into two groups: non-modifiable and modifiable factors. Non-modifiable risk factors include age, gender,

geographic location, blood type, family history, genetic predisposition, and diabetes. On the other hand, modifiable risk factors include factors such as gut microflora, smoking, alcohol consumption, chronic pancreatitis (CP), obesity, dietary habits, and infections [44].

Methods such as histopathological examination, tumor markers, computed tomography/positron emission tomography (CT/PET), endoscopic ultrasonography (EUS), magnetic resonance imaging (MRI), and endoscopic retrograde cholangiopancreatography (ERCP) are used in the diagnosis of pancreatic cancer. Methods such as histopathological examination, histopathology analysis and cytology are the methods considered as the "gold standard" in the diagnosis of pancreatic cancer. A clear diagnosis is critical before a treatment plan is created. Methods such as endoscopic ultrasonography (EUS) or computed tomography (CT) guided biopsy, acid cytology or exploratory biopsy during laparoscopy or open surgery are used to take samples of cancerous tissue [45], [46].

The most effective treatment for pancreatic cancer (PC) is known as surgical intervention. It can significantly prolong survival [47]. The indication for surgical resection depends on the stage of the tumor and its surgical removal. As neoadjuvant therapy, methods such as chemotherapy are often used to shrink the tumor and make it more suitable for surgery [48]. Radiotherapy, which uses high-energy radiation to kill cancer cells, is mainly used in patients with locally advanced PC [49]. Targeted therapies, on the other hand, give successful results in many types of cancer thanks to developing technology and scientific methods. Recently, new targets for targeted therapy for PC have been identified, such as PEGPH20 and CKAP4 [50]. More research is needed to prevent and treat pancreatic cancer, which still maintains high mortality rates despite many treatment modalities developed.

3. MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. Chemicals

The list of chemicals used in the experiments, along with their respective brands, is presented in Table 3.1.

Table 3. 1: Chemicals that were used in the thesis.

Chemicals	Company
Folin-Ciocalteu's Phenol Reagent	Merck
Methanol	Sigma Aldrich
Sodium Carbonate Anhydrous	Isolab
Gallic Acid	Merck
Sulfuric Acid	Sigma Aldrich
Standard Fetal Bovine Serum (FBS)	PanBiotech
DMEM Dulbecco's Modified Eagle Media	Gibco
Dimethyl Sulfoxide (DMSO)	BioFroxx
NutriCulture (1X) PBS Phosphate Buffered Saline	EcoTech Biotechnology
Thiazolyl Blue Tetrazolium Bromide (MTT) M: 414.33g/mol (1 g)	BioFroxx

3.2. Laboratory Devices

All laboratory devices used throughout the experiments were shown in Table 3.2.

Table 3.2: Laboratory devices used in this study.

LABORATORY DEVICES	BRANDS
CO2 incubator	Nüve
Laminar Flow Cabinet	Grafen Scientific
Spectrophotometer	PEAK Instruments
ZOE Fluorescent Cell Imager	Biorad
Light microscop	Soptop Optical Instruments
Water Bath	Elektro-mag
-80°C freezer	Nüve
Spin	DLAB
Smart garden system	Arzum Vahaa
The Mixer Mill MM 40	RETSCH MM 400
Closed-vessel microwave system	MARS
Water Bath	Electromag M24K

3.2. METHODS

3.2.1. Soilless Culture

The cultivation of watercress was carried out using the Arzum Vahaa smart garden system, which is suitable for soilless agriculture. 20 ml of solution A and 20 ml of solution B (the exact composition of which is undisclosed by the manufacturer) provided by Arzum Vahaa were added to 4.5 liters of water to provide a suitable environment for plant growth.

3.2.2. Experimental Design

In this experimental design Arzum Vahaa smart garden (3 replication $\times$ 3 selenium doses) were used. For the determination of selenium effect, 3 different selenium solutions (0, 0.25, 0.50 mg/L) were used. In all experiments selenium conditions were created by using sodium selenite (Na_2SeO_4) with a molecular weight of 188,94 g/mol and obtained from Acros Organics.

3.2.3. Harvest and Sample Storage

The leaves and stems of the plants were harvested to obtain both dry and fresh samples for the necessary analyses. Some of the fresh samples, in the quantities specified in Table 3.3. were stored at -80°C . Additionally, the samples, as indicated in Table 3.3. were dried at 65°C for two days to obtain dry samples, which were subsequently used for mineral element analysis and nitrate analysis.

Table 3.3: Sample Weights and Storage/Drying Conditions

	Control	0.25 mg/L Selenium	0.50 mg/L Selenium
The amount of samples stored at 80°C .	321 g $\pm$ 26	330 g $\pm$ 1	315 g $\pm$ 3
The amount of sample dried at 65°C .	59 g $\pm$ 7	56 g $\pm$ 12	65 g $\pm$ 4 g

3.2.4. Element Analysis

Dried plant materials were ground into a powder using a grinder. The ground samples were weighed one by one and placed in microwave digestion tubes. 2 ml of 30% hydrogen peroxide (H_2O_2) was then poured on top of it. Then 5 ml of 65% nitric acid (HNO_3) was added and in a closed-vessel microwave system was performed for the samples about 3 hours. Following digestion, the final volume was brought to 20

ml by filling each tube with 13 ml of double-distilled water. The digested samples were filtered using filter paper. All necessary elements concentrations including essential micro-, and macronutrients (except N) of both drainage and digested samples were analyzed by using Inductively coupled plasma optical emission spectrometry (ICP-OES). Control of accuracy of element analysis was performed by using standard reference materials certified by the National Institute of Standards and Technology.

3.2.5. Chlorophyll and Carotenoid Analysis

The packed frozen samples (-80°C) were placed into liquid nitrogen. Two 12 mm balls were then placed into each of the small jars (25 ml). The unpacked samples were taken to the ball mill jar. The jars were held with crucible tongs and immersed in liquid nitrogen for 20 seconds. They were then held by tongs and placed in the ball mill. The jars were compressed and locked securely. The ball mill was started at a frequency of 30 Hz for 30 seconds. After 30 seconds, the jars were held by tongs and opened. 5 ml of 80% Acetone was added to the jars while working on ice during the extraction. The jars were held by tongs and placed back in the ball mill. The jars were compressed and locked again, and the ball mill was started at 30 Hz frequency for 30 seconds. After 30 seconds, the jars were held by tongs and opened. The extracts were transferred to 15 ml falcons, which were put directly on ice. The falcons were vortexed and centrifuged at 7500 rpm for 15 minutes at 4°C. 2 ml of the supernatant was then transferred into 2 ml Eppendorf tubes, which were centrifuged again at 12000 rpm for 15 minutes at 4°C. After the preparation of the samples was completed, spectrophotometric measurements were conducted. The blank was set with acetone 80%. For each sample, 100 µl of the sample was taken, put into a quartz cuvette and diluted with acetone 80% of 1900 µl. Then, Chlorophyll levels of samples were measured with a spectrometer at appropriate wavelengths, 470 nm, 646.8 nm and 663.2 nm [51].

Calculations were made using the formula given below.

$$C_a = 12.25 * A_{663.2} - 2.79 * A_{646.8}$$

$$C_b = 21.5 * A_{646.8} - 5.1 * A_{663.2}$$

$$C_{x+c} = (1000A_{470} - 1.82 * C_a - 85.02 * C_b) / 198$$

3.2.6. Measurement of Total Phenolic Content (TPC)

Samples were analyzed for their total phenolic content (TPC) using the Folin-Ciocalteu's reagent method, following the procedure outlined by [52]. The total phenolic content was determined using a spectrophotometric method. Gallic acid standards at concentrations of 0.1, 0.2, 0.4, 0.8, and 1.2 mg/ml were prepared by dissolving Gallic acid in a solvent matching the background of plant extractions (70%). These standards, along with the samples and blanks, were pipetted in a specific order, including deionized water, standards, or plant samples, 0.5 N Folin Reagent, and 10% Na_2CO_3 . The tubes were thoroughly mixed by vortexing and kept in the dark for 30 minutes. Subsequently, the absorbance readings at 760 nm were taken using a quartz cuvette. It is important to note that standards were read first after blanking the spectrophotometer to ensure accurate results [52].

3.2.7. Determination of Trolox Equivalent Antioxidant Capacity (TEAC)

14 mM ABTS solution: 14 mM ABTS was prepared by dissolving 0.192 g of ABTS [(2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt)] in 25 ml of distilled water.

4.9 mM Potassium persulfate ($\text{K}_2\text{S}_2\text{O}_8$) solution: 4.9 mM potassium persulfate ($\text{K}_2\text{S}_2\text{O}_8$) was prepared by dissolving 0.033 g of potassium persulfate in 25 ml of distilled water.

ABTS^{•+} reagent: - The ABTS^{•+} reagent was prepared by mixing the solutions (14 mM ABTS and 4.9 mM Potassium persulfate ($\text{K}_2\text{S}_2\text{O}_8$) solution) in a 1:1 ratio (total 50 ml).

The mixture was stored in the dark at room temperature for 12-16 hours (overnight) to allow the formation of ABTS^{•+} radical cations.

First, a blank was set using absolute ethanol at 734 nm. Next, an ABTS solution was prepared by diluting ABTS-reagent with absolute ethanol to an absorbance of 0.70 (± 0.02) at 734 nm. Then, 1.0 ml of the diluted ABTS solution was added to 10 μl of Trolox standards (ranging from 0–1.50 mM), and absorbance readings were taken at 6 min for both the standards and plant samples. Finally, the Trolox standard curve equation was used to calculate the TEAC value of the samples. Initial reading was

taken during the initial 30 minutes to determine the time required for the watercress samples. The percentage inhibition of absorbance at 734 nm is calculated and plotted as a function of the concentration of Trolox [53].

3.2.8. Determination of Nitrate in Plant Tissue

0.1 g of dried watercress was weighed into 15 ml Falcon tubes. Then, 10 ml of dH₂O was added to each tube and vortexed. Next, the tubes were incubated for 1 hour in a water bath at 45°C. After incubation, the tubes were vortexed again. The tubes were then centrifuged at 4600 rpm for 15 minutes. 2 ml amount of the supernatant was carefully transferred to 2 ml Eppendorf tubes for storage. The samples were diluted 1/3. In addition, 0.1 ml of extract was transferred to falcon tubes and added 0.4 ml of reagent A. Simultaneously, 0.1 ml of each nitrate standard (0, 6.25, 12.5, 25, 50, 100, 250 and 500 ppm NO₃) prepared from KNO₃ was transferred to separate containers and 0.4 ml It was mixed with reagent A. The reaction mixture was left to stand for 20 minutes. Then, 9.5 ml of reagent B were added to the reaction mixture, causing the red color to change to green as the mixture became hot. The mixture was allowed to cool to room temperature for at least 3 hours. The spectrophotometer was calibrated using nitrate standards and absorbance was measured at 410 nm [54].

Reagent A: Salicylic acid, 5% (w/v), was dissolved in concentrated H₂SO₄ inside the fume hood.

Reagent B: 2M NaOH was prepared in dH₂O.

3.2.9. MTT Cell Viability Assay

The MTT assay was employed for assessing cellular viability and growth by quantifying metabolic activity. In this colorimetric assay, metabolically active cells reduce a yellow tetrazolium salt (MTT) to form purple formazan crystals. The intensity of the resulting solution's color is directly proportional to the quantity of viable and metabolically active cells. Panc-1 and MIA PaCa-2 cells were seeded at a density of 10,000 cells per well in 96-well plates and treated with watercress extracts at concentrations of 5, 10, 20 µl/ml, and 50 µl/ml for 24 hours. After the extract treatment, MTT was introduced to the cells. Afterward, the cells were incubated at 37°C for 4

hours. Following this incubation period, the culture medium was removed, and 100 μ l of dimethyl sulfoxide (DMSO) was added to the cells. The 96-well plate was then wrapped in aluminum foil and placed on a shaker for 10 minutes. Subsequently, the absorbance at 570 nm was measured using a plate reader [55].

3.2.10. Survival Assay

Panc-1 and MIA PaCa-2 cells were seeded at 50,000 density per well in 6-well plates and treated with watercress extracts 20 μ l/ml and 50 μ l/ml for 24, 48, 72 and 96 h. According to the time points, the cells were washed with 1X PBS and then 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 μ l was withdrawn from this mixture, and duplicate cell counts were performed for both cell lines using a hemocytometer [56].

3.2.11. Colony Formation Assay

The cells were incubated for 14 days at 37°C in an environment with 5% CO₂ and 95% humidity in a cell culture incubator, and their growth was monitored. After 14 days, Panc-1 and MIA PaCa-2 cells were first washed with a 1X PBS solution, and then 1 ml of Acetic acid/methanol mixture was added to each cell culture dish at a 1:3 ratio to fix the cells for 15 minutes. After 15 minutes, the fixation solution was removed from the culture dishes, and 1 ml of crystal violet dye was added to each well and left at room temperature for 20 minutes. After treating the cells with the dye, their images were captured [57].

3.2.12. Fluorescent Staining

The pancreatic cancer cells Panc-1 and MIA PaCa-2 were seeded at 100,000 cells/well into the 24 wells plate, and then incubated 24 h in a 37°C and 5% CO₂ incubator for adherence. Watercress extracts (50 μ l/ml) were applied to adherent cells. After 24h, the culture cabinet's lighting was turned off due to the dye's light sensitivity then the existing media in the 24-well plate was aspirated, and 250 μ l of the dye (Mito Tracker, DAPI and Lyso Tracker) and serum-free DMEM mixture was added to the

wells. The plate was then placed in an incubator at 37°C for a 15-minute incubation period. Subsequently, the plate was covered with foil, and cells were visualized by ZOE fluorescent microscope.

3.2.13. Cell Cycle Analysis with PI

Pancreatic cancer cells Panc-1 and MIA PaCa-2 were seeded at 500,000 cells per well into a 6-well plate. Watercress extracts (50 µl/ml) were applied to the adherent cells. The cells were collected and fixed by adding 1 ml of 70% cold ethanol, followed by a 15-minute incubation at room temperature. Subsequently, the samples were stored at -4 °C. The cells were separated from ethanol by centrifugation at 13200 rpm for 3 minutes. Each sample was incubated in the dark for further analysis. at 37°C for 30 minutes with a mixture of 990 µl of PBS and 10 µl of PI. Subsequently, the analysis of 10,000 cells per sample was performed using flow cytometry [58].

3.2.14. Statistical Analysis

All the obtained data were subjected to a one-way analysis of variance (ANOVA) at 95 % confidence. All analysis was performed using GraphPad software.

4. RESULTS

4.1. Soilless Culture Experiments

The application of selenium at doses of 0, 0.25 mg/L and 0.50 mg/L, after 27 days of planting watercress seeds, did not have any observable toxic or enhancing effects on the development of stems and leaves (Figure 4.1).

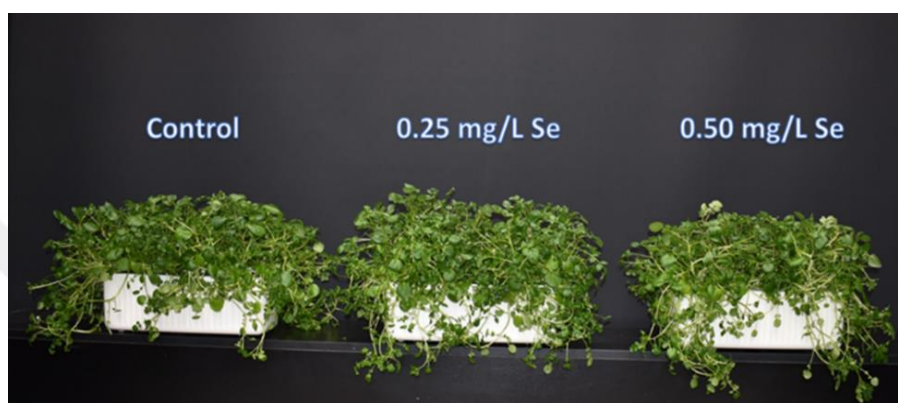


Figure 4.1: Shoot growth and general appearance of 48-day-old watercress grown in soilless culture supplied with three levels of Selenium (0, 0.25 mg/L, 0.50 mg/L).

Watercress harvested on the 27th day following selenium application was weighed, and the total weight of the harvested plants was recorded. The roots of the plants were counted and divided by the total weight to calculate the grams per plant. Statistical analysis was conducted to compare the weights of the treatment groups with the control group, which did not receive selenium treatment. The results indicated that there was no statistically significant difference in weight among these groups (Figure 4.2). Consequently, the application of selenium did not produce any discernible positive or negative impact on the overall fresh weight of the watercress plants.

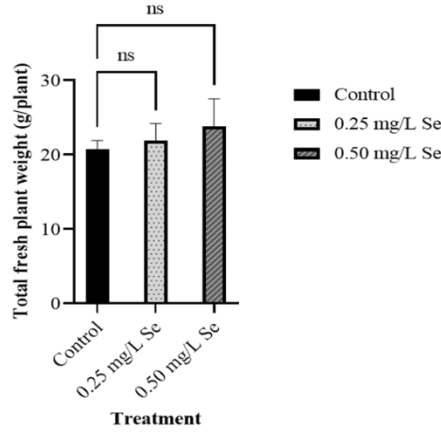


Figure 4. 2: The effect of different doses of selenium application on the total fresh biomass of watercress. Data are means $\pm$ standard deviation ($n = 3$) ($P > 0.05$) One-way ANOVA analysis was performed with a Sidak Post Hoc Test.

4.2. Effect of Selenium Biofortification on the Accumulation of Macro- and Microelements in Watercress

The concentration of selenium in watercress was analyzed using ICP analysis. Consistent with expectations, a linear relationship was observed between selenium accumulation and the dosage of selenium in the applied groups (Figure 4.3).

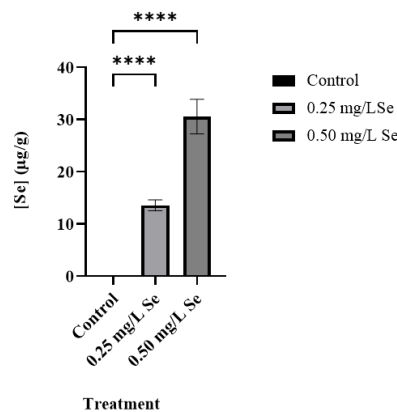


Figure 4. 3: Accumulation of selenium expressed on a dry weight basis in the total biomass of watercress grown under increasing levels of selenium in soilless culture ($p < 0.005$). Data are means $\pm$ standard deviation ($n = 3$) One-way ANOVA analysis was performed with a Sidak Post Hoc Test.

In the group where no selenium was applied (control group), the selenium levels were found to be 0 $\mu\text{g/g}$. In contrast, the group treated with 0.25 mg/L selenium

exhibited a substantial increase in selenium accumulation, with levels reaching 13.60 µg/g. The highest selenium accumulation was observed in the group treated with 0.50 mg/L selenium, with levels as high as 30 µg/g.

Table 4. 1: Mean values of micronutrient accumulation in watercress, expressed on a dry weight basis. The watercress was cultivated under varying selenium (Se) levels in a nutrient solution culture (n=3).

Treatments	Elemental Composition						
	B	Ca	Cu	Fe	Mn	Mo	Zn
Control	39.2±1 ^a	17±1 ^b	5.8±0.3 ^c	55.5±2 ^d	59.20±5 ^e	2.8±2 ^f	81.5±3 ^g
0.25 mg/L Se	46.1±2 ^a	19±1 ^b	6±0.1 ^c	65±3 ^d	59.8±3 ^e	3.4±0.2 ^f	69.5±7 ^g
0.50 mg/L Se	48.8±1 ^a	19±0.7 ^b	6.7±4 ^c	70±4 ^d	77±6 ^e	3.7±0.1 ^{fe}	93±3.9 ^g

Different letters at each column indicate significant difference between means according to one-way ANOVA ($p < 0.01$).

Table 4.2: Mean values of macronutrient accumulation expressed on watercress grown under increasing selenium in nutrient solution culture (n=3).

Treatments	Elemental composition (% dry mass)			
	K	Mg	P	S
Control	5.6±0.5 ^a	2.4±0.1 ^b	6.9±0.4 ^c	6.1±0.3 ^d
0.25 mg/L Se	5.2±0.2 ^a	3±0.1 ^b	8±0.6 ^c	7.5±0.4 ^d
0.50 mg/L Se	6.5±0.2 ^a	2.9±0.4 ^b	8.5±0.2 ^c	8.5±0.5 ^e

Different letters at each column indicate significant difference between means according to one-way ANOVA ($p < 0.01$).

The macro and microelement levels of watercress for both the control group and the selenium-treated watercress groups are presented in Table 4.1 and Table 4.2. The level of selenium (0.50 mg Se/L) in the solution culture influenced the accumulation of S and Mo but had no significant effect on B, Cu, Fe, Ca, Zn, P, Na, Mg, K, Cu, or B ($p > 0.005$) (Table 4.1).

4.3. Examining the Effects of Selenium Application on the Total Phenolic Compounds in Watercress

The gallic acid calibration curve has a concentration range of 0.1–0.16 mg/mL with the equation $y = 6.9269x + 0.0439$ (Appendix A). The total phenolic content of watercress in the control, 0.25 mg/L Se, and 0.50 mg/L Se-treated groups was 0.80 ± 0.1 ; 0.70 ± 0.1 ; 0.89 ± 0.14 mg GAE/g FW, respectively (Figure 4.4).

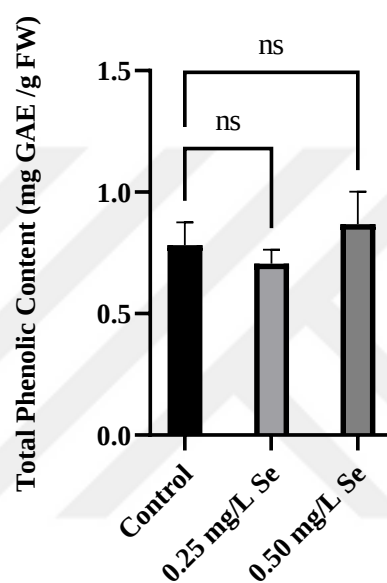


Figure 4.4: Evaluation of total phenolic contents (TPC) in methanolic extracts of watercress control group and groups treated with 0.25 mg/L Se and 0.50 mg/L Se. Data are means $\pm$ standard deviation ($n = 3$) ($p > 0.05$). One-way ANOVA analysis was performed with Sidak Post Hoc Test.

The effects of selenium application on the total phenolic compounds in watercress were examined using one-way ANOVA. The results indicated that there were no statistically significant changes observed when comparing the selenium-treated groups to the control group (Figure 4.4).

4.4. Determination of TEAC (Trolox Equivalent Antioxidant Capacity) in Selenium Biofortified Watercress

The Trolox Equivalent Antioxidant Capacity (TEAC) test or ABTS test is a widely used method to determine the total antioxidant capacity (TAC) of plant extracts, food, clinical fluids, and other samples. It is a test based on the reduction of antioxidant compounds in the sample by a metastable radical (ABTS•+). The reduction of ABTS is a color change, which can be measured spectrophotometrically [59]. The standard curve graph was drawn with the absorbance value obtained from the prepared standards (Appendix B).

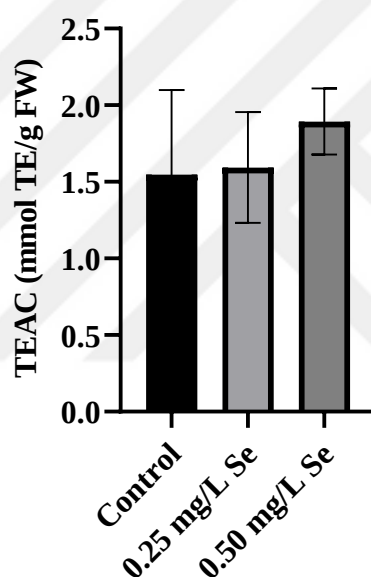


Figure 4.5: Antioxidant capacities of watercress extracts methanol: water (70:30, v: v) evaluated using the ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) assay. Data are means $\pm$ standard deviation ($n = 3$) ($p > 0.05$). One-way ANOVA analysis was with Sidak Post Hoc Test.

The effect of selenium application on the antioxidant capacity of watercress was assessed using the ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) test. The antioxidant capacity, measured as mm TE (Trolox Equivalent) per g fresh weight (FW), was determined as follows: Control group - 1.55 ± 0.55 , 0.25 mg/L Se group - 1.60 ± 0.4 , and 0.50 mg/L Se group - 1.90 ± 0.20 . Although antioxidant

capacity was found to be slightly increased, no statistically significant increase in antioxidant capacity was observed compared to the control group (Figure 4.5).

4.5. Influence of Selenium on Chlorophyll and Carotenoid Concentrations of Watercress

The effects of Se supplementation on the total chlorophyll and total carotenoid content in watercress are summarized in Figure 4.6.

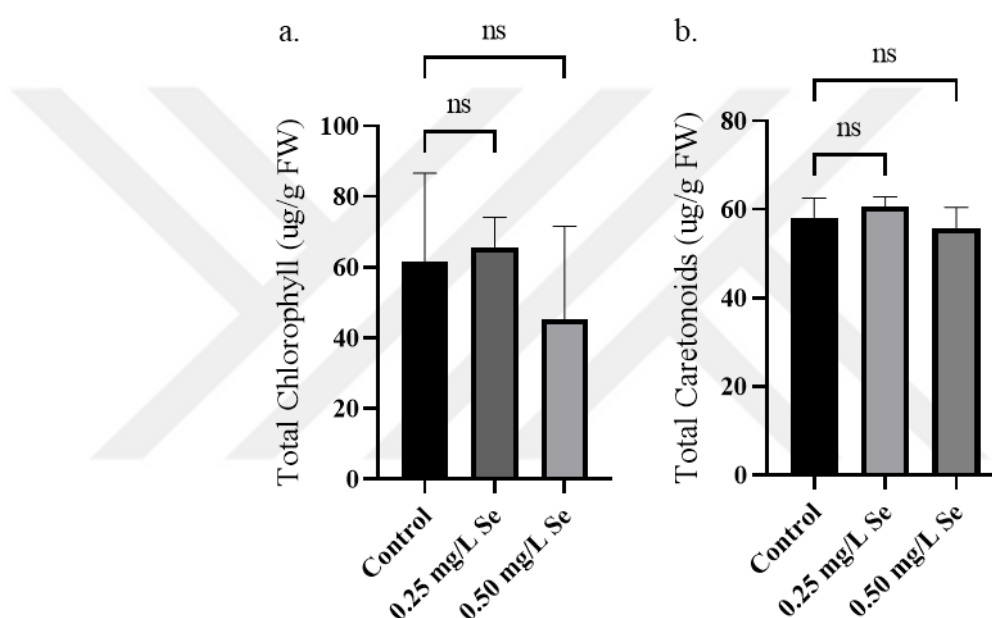


Figure 4.6: Effects of Selenium (Se) application rate on total chlorophyll and total carotenoids content in watercress. Data are means $\pm$ standard deviation ($n = 3$) ($p > 0.05$). One-way ANOVA analysis was performed with Sidak Post Hoc Test.

The total chlorophyll content control was measured as $61.47 \pm 25.11 \mu\text{g/g FW}$. In the group administered with a concentration of 0.25 mg/L Se, the total chlorophyll content was determined as $65.70 \pm 8.4 \mu\text{g/g FW}$. Similarly, in the group administered with a concentration of 0.50 mg/L Se, the total chlorophyll content was found to be $45.30 \pm 26.30 \mu\text{g/g FW}$ (Figure 4.6). Compared to the control group, the group administered with a concentration of 0.25 mg/L Se exhibited an increase of $2.30 \mu\text{g/g FW}$, while the group administered with a concentration of 0.50 mg/L Se showed a decrease of $4.75 \mu\text{g/g FW}$.

In this study, the total carotenoids content in the control group was measured as $58.20 \pm 18 \mu\text{g/g FW}$. When a concentration of 0.25 mg/L selenium (Se) was administered, the total carotenoids content in this group increased to $60.50 \pm 7.6 \mu\text{g/g FW}$. Conversely, in the group administered with a concentration of 0.50 mg/L Se , the total carotenoids content decreased to $55.80 \pm 6 \mu\text{g/g FW}$. Based on the results of the ANOVA statistical test, no statistically significant increase or decrease was observed compared to the control group in the total chlorophyll content and carotenoids content.

4.6. The Impact of Different Selenium Levels on Nitrate Content in Watercress

In the nitrate concentration results of watercress, the control group displayed a mean value of $44,250 \pm 5 \text{ mg/kg}$, while in the group treated with 0.25 mg/L of Se, it was measured at $33,190 \pm 7 \text{ mg/kg}$, and in the group exposed to 0.50 mg/L of Se, a value of $49,590 \pm 14 \text{ mg/kg}$ was recorded.

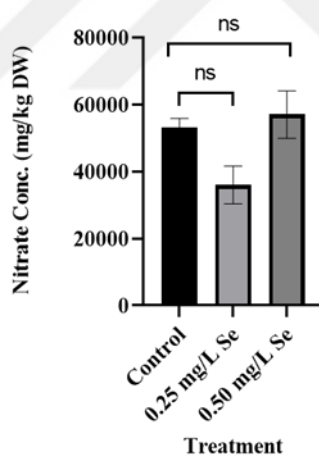


Figure 4. 7: The total nitrate levels of watercress were measured under different selenium biofortification levels. Data are means $\pm$ standard error ($n = 3$) ($p > 0.05$) One-way ANOVA analysis was performed with Sidak Post Hoc Test.

The total nitrate levels obtained from the calculations were examined using a one-way ANOVA statistical test in GraphPad Prism 9.5 software to investigate the potential effect of Selenium applications on total nitrate levels. The analysis revealed that the selenium application did not show any significant effect on the nitrate content (Figure 4.7).

4.7. False Positives in the MTT Test of Watercress Crude Extract on Pancreas Cancer Cells

To evaluate the impact of watercress extracts on the viability of pancreas cancer cells, an MTT assay was conducted. Despite applying the extracts at the recommended doses in the literature [60], surprisingly, particularly at high concentrations (0.20 $\mu\text{L/mL}$ and 0.50 $\mu\text{L/mL}$), the results showed significantly high viability levels, even though the cells were mostly nonviable (Figure 4.8). These results suggested that watercress extract might interact with the MTT compound, potentially leading to false-positive results for viability.

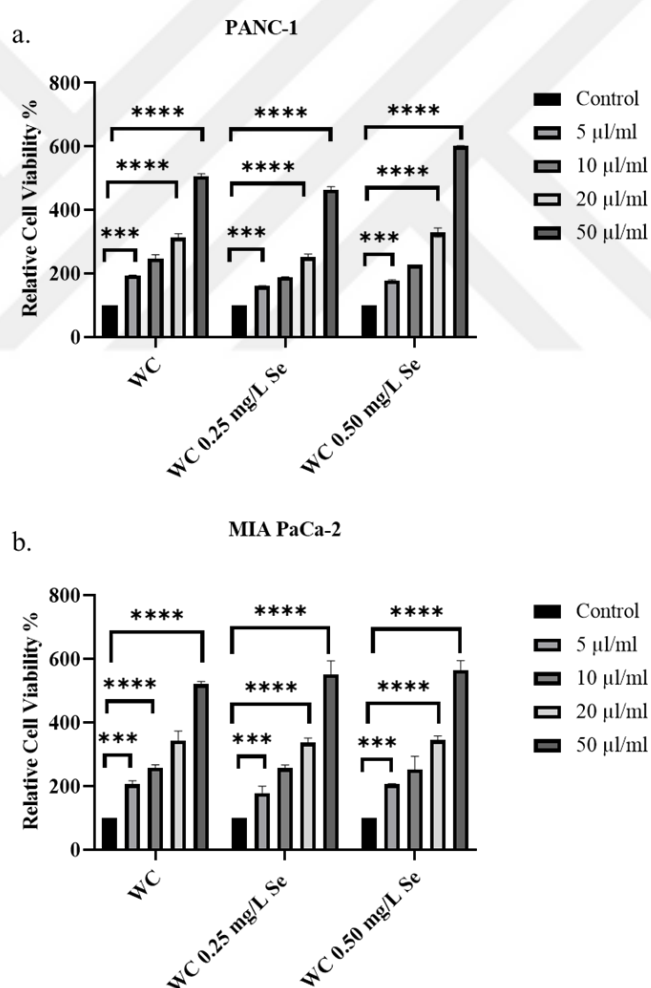


Figure 4. 8: MTT cell viability assay of MIA PaCa-2 and Panc-1 cells were performed to observe the effect of watercress extracts on cell viability (n=3). Each data point represents average $\pm$ SD of three separate experiments.

4.8. Watercress Extract Suppress Pancreas Cancer Cell Proliferation

The effects of the administered watercress extracts on cell viability were evaluated using a growth assay in MIA PaCa-2 and Panc-1 a cancer cell line. In all extracts (Watercress; Watercress 0.25 mg/L Se; Watercress 0.50 mg/L Se) applied at doses of 20 and 50 $\mu\text{L/mL}$ at the 24th, 48th, and 72nd hours, a significant decrease was observed compared to the control group in both cell line ($p < 0.0001$).

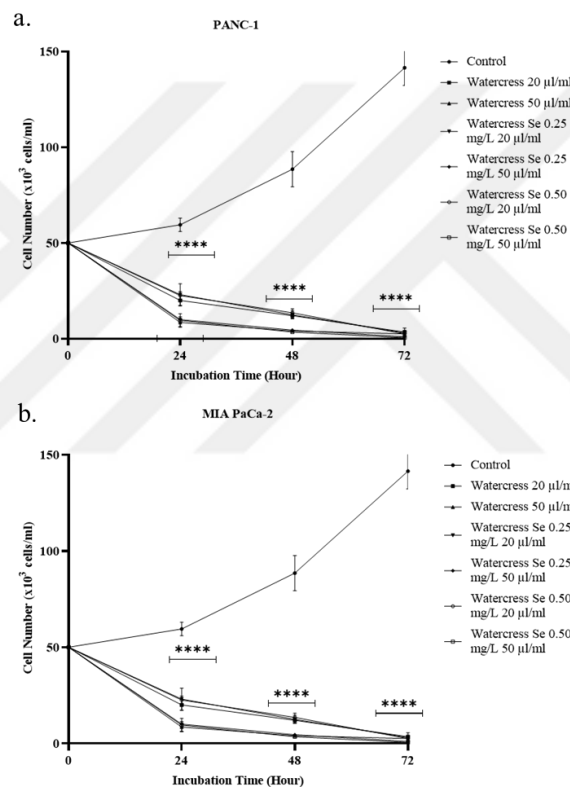


Figure 4.9: Cell viability was evaluated using a growth assay in MIA PaCa-2 and Panc-1 a cancer cell line. Data are means $\pm$ standard error ($n = 3$) ($P > 0.05$) One-way ANOVA analysis was performed with Sidak Post Hoc Test.

We observed a significant decrease when we compared the 0.20 $\mu\text{L/mL}$ dose to the 0.50 $\mu\text{L/mL}$ dose in all extracts (Watercress; Watercress 0.25 mg/L Se; Watercress 0.50 mg/L Se) applied at the 24th hour in both cell line ($p < 0.01$). However, in the counts made at 48 and 72 hours, there was no significant difference in cell viability when comparing the 0.20 $\mu\text{L/mL}$ dose to the 0.50 $\mu\text{L/mL}$ treatments ($p > 0.005$).

4.9. Colony Formation Potential of MIA PaCa-2 and Panc-1 Cells Treated Watercress Extracts

To assess the impact of watercress extracts (Watercress; Watercress 0.25 mg/L Se; Watercress 0.50 mg/L Se) on colony formation in MIA PaCa-2 and Panc-1 cancer cell lines, a colony formation assay was conducted.

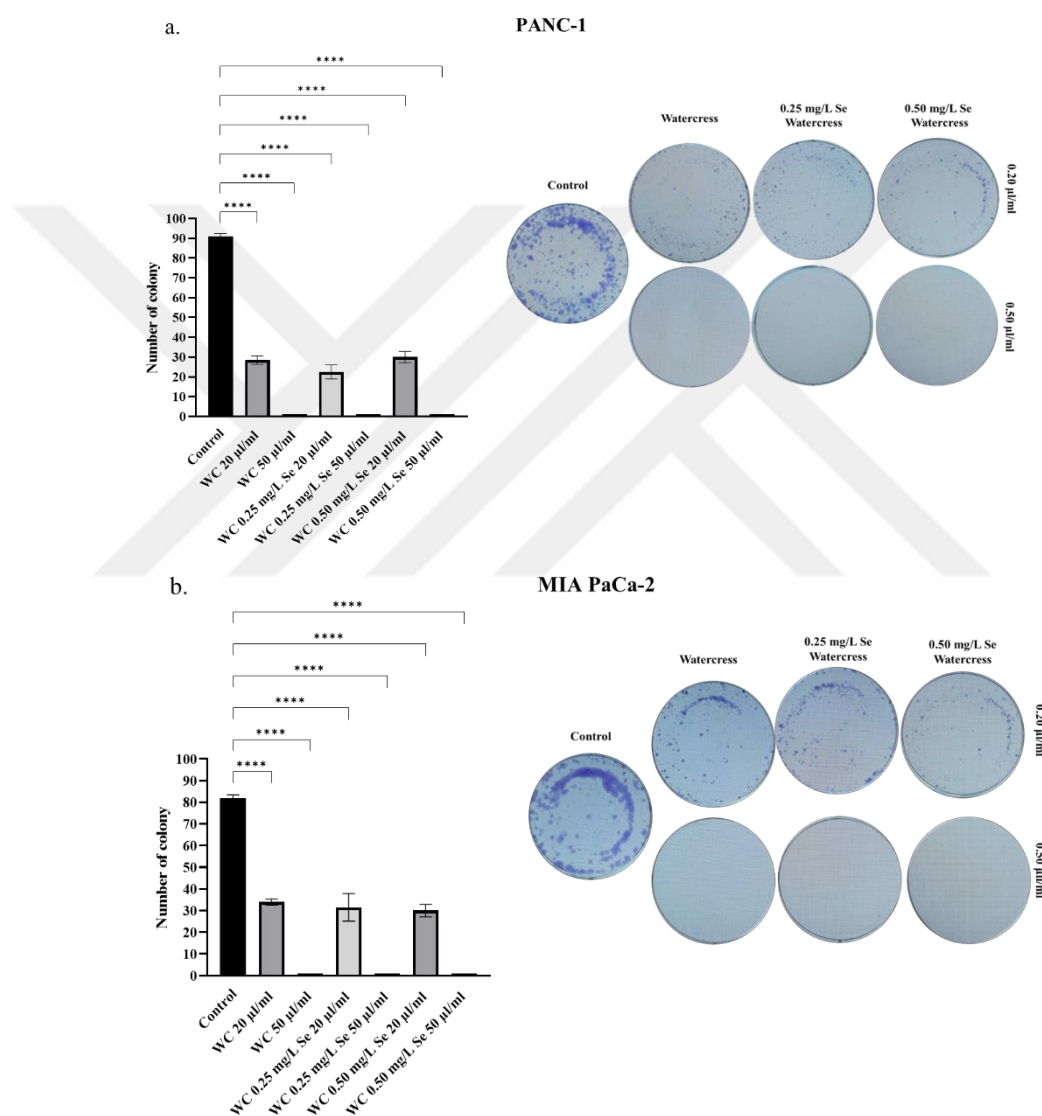


Figure 4.10: PANC-1, MIA PaCa-2 cells were exposed to 0.20 and 0.50 µl/ml of crude watercress extracts for 24 h. Then, the colony formation of cells was counted after 14 days of treatment.

In all extracts (Watercress; Watercress 0.25 mg/L Se; Watercress 0.50 mg/L Se applied at doses of 20 μ L/mL and 50 μ L/mL in both cell lines), a statistically significant decrease in colony count was observed compared to the control group (Figure 4.10).

4.10. Exploring the Impact of Watercress Extract Mitochondrial Membrane Potential, Nuclear Quantitation Using Fluorescent Staining

Panc-1 and MIA PaCa-2 cells were stained with MitoTracker and DAPI dyes to investigate the alterations in mitochondrial membrane potential, nuclear quantitation in the pancreatic cancer cell population following treatment with watercress extract (Figure 4.11). It was shown that for both Panc-1 and MIA PaCa-2 cells, watercress extracts increase the death rate.

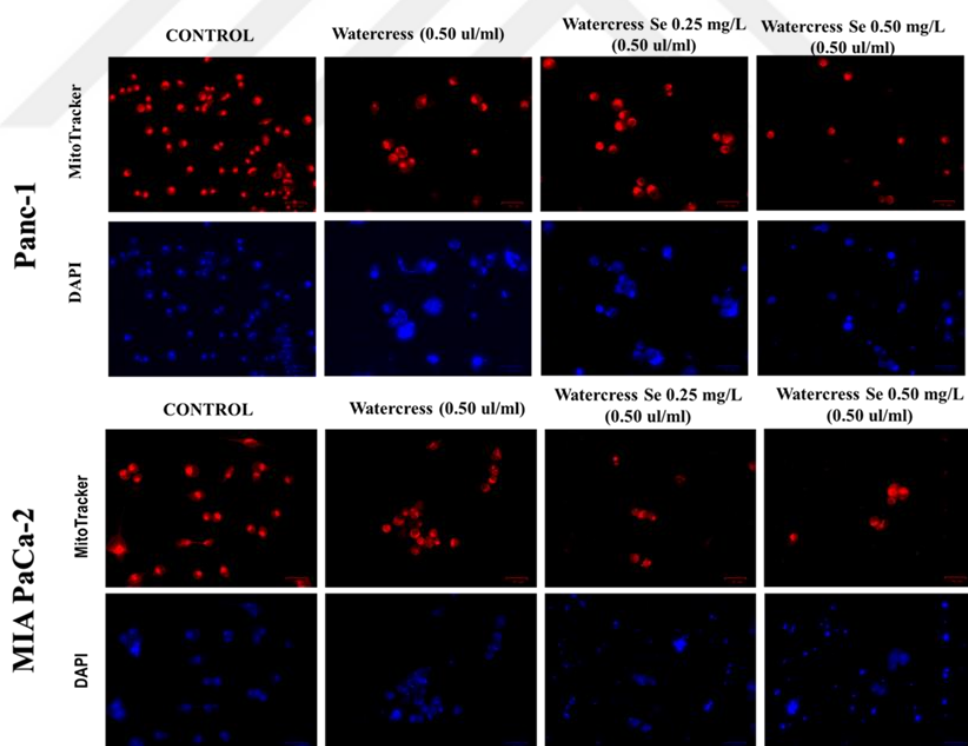


Figure 4. 11: 300,000 of Panc-1 and MIA PaCa-2 cells were seeded into 24 wells plate and treated with watercress extracts (50 μ l/ml) for 24 Cells were stained with MitoTracker and DAPI, dyes Scale bars represent 50 μ m.

4.11. The effects of Watercress Extract on the Cell Cycle on MIA PACA-2 and PANC-1 Cells

Flow cytometry was employed to assess various stages of the cell cycle at distinct time intervals. In this process, cells were treated with propidium iodide (PI) to enable the differentiation of cell cycle phases, and subsequently analyzed for their distribution across these phases.

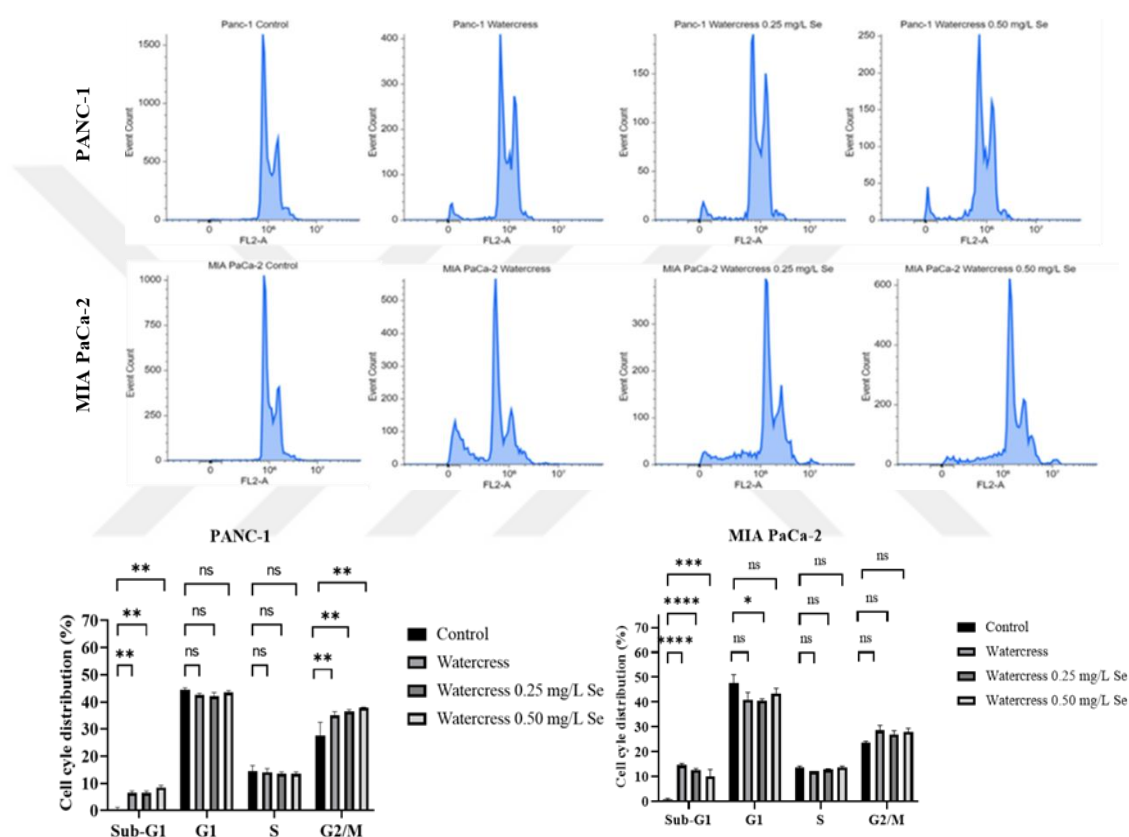


Figure 4.12: Cell cycle analysis using propidium iodide (PI) staining and flow cytometry. MIA PaCa-2 and PANC-1 cells were treated with watercress extracts at a dosage of 50 μ l/ml for 24 h (n=2).

The percentages of both cell lines in the sub-G1 phase (indicating apoptotic cells) exhibited a significant increase following treatment with extracts (Watercress; Watercress 0.25 mg/L Se; Watercress 0.50 mg/L Se) at a concentration of 50 μ l/ml for 24 hours. Specifically, in MIA PaCa-2 cells the percentages rose to 14%, 12% and 11 %, respectively, in contrast to the 0.8% observed in the control group. In Panc-1 cells the percentage rose to 6%, 8%, and 8, % respectively, in contrast to the 0.9% observed

in the control group (Figure 4.12). The population of cells in the G2/M phase increased in Panc-1 cells compared to the control group, whereas there was no significant change in MIA PaCa-2 cells.

5. DISCUSSION and CONCLUSION

In recent years, the cultivation of agricultural products rich in essential minerals and vitamins that positively impact human health and fulfill nutritional needs through appropriate fertilization types and programs has gained significant importance. Nowadays, maintaining a healthy and balanced diet has become more challenging than ever. Globally, microelement deficiency affects over three billion people, and this number continues to rise. In humans, the most observed micronutrient deficiencies are in zinc, iron, iodine, selenium, and cobalt [61].

Biofortification is a process aimed at increasing the nutritional value of crops by enhancing their content of essential vitamins, minerals, and other beneficial compounds. In this study, selenium, one of the most commonly observed micronutrient deficiencies, was employed for biofortification. The selection of plant species for selenium biofortification is a crucial decision. Different plant species have varying capacities to accumulate selenium and may accumulate different selenium forms. For instance, Brassica and Allium species like broccoli, watercress and garlic are naturally inclined to have higher selenium levels, making them potential favorites for biofortification initiatives. In this study, watercress (*Nasturtium officinale*) was chosen as the target plant species for selenium biofortification. The findings revealed notable fluctuations in selenium levels among the treatments in watercress. As the selenium concentration in the soil increased, there was a notable increase in the total selenium accumulation. Therefore, biofortification of watercress could be seen as a viable approach for boosting selenium levels in areas characterized by selenium deficiency. The advised daily selenium intake for individuals aged 14 years and older is 55 µg, with a maximum safe intake limit of 400 µg per day [62]. In this study, watercress treated with 0.25 mg/L selenium reached 13.60 µg/g DW, while watercress treated with 0.50 mg/L selenium reached 30 µg/g DW (Figure 4.3). These results provide valuable insight into the efficacy of selenium biofortification in increasing the selenium content of watercress, a potential step toward addressing dietary selenium deficiency.

Furthermore, it's worth noting that similar studies, such as the one conducted by [63] on watercress, have reported encouraging results. In their study, the application of 0.25 mg/L Se resulted in 44.59 $\mu\text{g g}^{-1}$ DW Se, while 0.50 mg/L Se yielded an impressive 248.43 $\mu\text{g g}^{-1}$ DW Se. These findings align with our research and provide further support for the feasibility and potential benefits of selenium biofortification in watercress.

The chemical structure of selenium is similar to the element sulfur. Plants take up selenium via sulfur carriers in the root plasma membrane. This indicates that the plant can compete with sulfur for selenium intake [64]. Selenium biofortification (0.50 mg/L Se) of watercress conducted in this thesis, a significant increase was observed in the sulfur level in the plant. Similarly, it has been shown that there is a significant increase in the sulfur level in the plant in the selenium biofortification of watercress by [63].

A statistically significant increase was observed in the concentration of the essential micronutrient molybdenum (Mo) in watercress at a dose of 0.50 mg/L selenium. Molybdenum is a critical nutrient for sulfur (S) metabolism, as it binds to Mo-requiring enzymes and facilitates the conversion of sulfite to sulfate, particularly through the action of enzymes such as sulfur oxidase. The observed significant increase in molybdenum concentration may be attributed to the enhanced uptake of sulfur by the watercress plant.

To understand the effects of selenium application on plant biomass, postharvest weighing was performed. The results showed that no significant effect on plant mass was observed at different selenium levels. Also, in another selenium biofortification study conducted with watercress, no significant effect on plant biomass was observed [63]. These results indicate that the application of selenium did not result in noticeable changes in plant biomass.

Watercress is renowned for its abundant phenolic content, which significantly contributes to its antioxidant properties [65]. The total phenolic content of watercress has been reported to range from 14.00 to 50.40 mg of or gallic acid equivalents per gram of dry weight [66], [67]. In this study, we determined that the total phenolic content of watercress in the control group, the 0.25 mg/L Se-treated group, and the 0.50 mg/L Se-treated group was 0.80 ± 0.1 mg GAE/g FW, 0.70 ± 0.2 mg GAE/g FW, and 0.89 ± 0.10 mg GAE/g FW, respectively. Saffaryazdi and her colleagues conducted a study where selenium biofortification was carried out. They observed an increase in

the total phenolic compound content in spinach, with the highest level being recorded at the highest applied selenium concentration (10 mg/L). However, it is worth noting that they also found that applying more than 1 mg/L of selenium negatively affected the growth parameters [68]. On the contrary, in this thesis, it was observed that selenium biofortification applied to watercress had no effect on the total phenolic content. In the light of this information, it is important to carry out more detailed studies in terms of having more information on the effect of selenium biofortification on the total phenolic content.

Antioxidants help protect cells from oxidative damage by stabilizing or neutralizing free radicals. The amount of antioxidants in plants is important for human health. By neutralizing free radicals in the body, antioxidants can reduce DNA damage to cells and reduce the risk of many diseases, including cancer [69]. The presence of phenolic compounds in watercress contributes to its antioxidant capacity [66]. We found that selenium biofortification didn't significantly affect the total phenolic compounds in watercress. As phenolic content is linked to antioxidant capacity in plants, we investigated how this might affect watercress's antioxidant capacity. Our results have supported this relationship. However, [70] reported that selenium biofortification has been shown to boost the antioxidant capacity of broccoli at different stages of its growth. In another study, which identified enhanced antioxidant capacity, conducted by [71] selenium-enriched basil leaves were observed to exhibit increased antioxidant capacity. Additional research is needed to examine the effects of harvesting at different times and analyzing specific plant regions. These studies can provide valuable insights into the plant's antioxidant capacity. It's also important to recognize that the effectiveness of selenium biofortification can vary depending on the plant species and the form of selenium used.

Carotenoids and chlorophyll play vital roles in plants and have significant implications for the well-being of both humans and plants. Carotenoids are actively engaged in various physiological functions within plants, including safeguarding against light damage, supporting growth, development, and responding to stress[72]. On the other hand, chlorophyll is accountable for the green pigment in plants and is indispensable for photosynthesis, the process through which plants transform sunlight into energy. Furthermore, chlorophyll contributes to human health by serving as a source of important nutrients and antioxidants[73]. Given the critical roles played by these pigments, we sought to explore the effects of selenium biofortification on them.

In the conducted studies, the impact of selenium biofortification on chlorophyll and carotenoids has been observed to vary. In their research, [74] reported an increase in chlorophyll and carotenoid levels in microgreen extracts biofortified with selenium at concentrations of 0.125, 0.25, and 0.50 mg/L Se. This observation suggests that selenium has a positive impact on these photosynthetic pigments, likely due to an increased rate of photosynthesis. In a study conducted by [75] it was observed that some levels of selenium application (1.30 and 6.57 mg/kg) had a favorable impact on maize plants in terms of growth, chlorophyll, and carotenoid content. In contrast, some levels of selenium application (13 and 26 mg/kg) had a detrimental effect on these aspects. This suggests that the dosage of selenium plays a critical role in determining its impact on plant growth and the levels of chlorophyll and carotenoids. In our study on selenium biofortification of watercress, we did not observe any statistically significant differences in chlorophyll and carotenoid levels. This outcome may be attributed to the inherent characteristics of watercress, selenium levels used in the study, or the timing of harvest.

Overall, the available literature suggests that the effect of selenium biofortification on total chlorophyll and carotenoid levels in plants is complex and may depend on various factors such as plant species, concentration of selenium applied. Further research is needed to fully understand the relationship between selenium biofortification and the levels of these pigments in plants.

Nitrates are naturally occurring components that humans typically obtain through the consumption of vegetables. They have been considered highly undesirable in the diet due to their potential carcinogenic effects [76]. The Acceptable Daily Intake (ADI) for nitrate, as established by the Food and Agriculture Organization (FAO) and the World Health Organization (WHO), is set at 3.7 mg of nitrate per kilogram of body weight per day [77]. Considering the potential health risks associated with excessive nitrate intake, we conducted an analysis within our research to investigate the effects of selenium biofortification on nitrate levels in watercress. While there is non-existing literature on the impact of selenium biofortification on nitrate levels in plants, in their study, [77] demonstrated that nitrate levels in broccoli and cauliflower were affected by fertilization. For instance, the nitrate level in broccoli treated with fertilizer (323 mg/kg) was observed to be higher than that in unfertilized broccoli (221 mg/kg). In the nitrate analysis conducted in this thesis, we observed that selenium biofortification did not lead to a significant change in watercress nitrate levels. The relationship between

nitrate levels in vegetables and various factors is complex, indicating the need for further extensive research in this area.

The MTT assay is one of the commonly utilized methods for assessing cell viability and cytotoxicity. In our study, we also employed the MTT assay to investigate the impact of crude watercress extracts on a pancreatic cancer cell line. However, when plant extracts are used to assess their cytotoxic potential, it is important to acknowledge that interference can occur, potentially leading to false-positive results in terms of cell viability. As observed in Figure 4.8, it is evident that watercress extract in the MTT assay resulted in false-positive outcomes. This condition has been similarly demonstrated by [78], where the MTT test also exhibited false-positive results when used in conjunction with certain plant extracts. Due to the presence of certain plant ingredients with reducing activity that transforms the MTT compound into the colored formazan, which serves as the basis of the assay, the MTT assay may not be appropriate for certain plant extracts.

We conducted a cell proliferation assay on Panc-1 and MIA PaCa-2 cells treated with watercress extracts, utilizing two dosages (20 $\mu\text{L/mL}$ and 50 $\mu\text{L/mL}$) previously recommended in the literature [60], [79]. The results revealed a significant reduction in cell numbers when compared to the control group. This indicates the potential anti-proliferative effects of the Watercress extracts on pancreatic cancer cells, which is a promising discovery. However, it's important to note that there was no significant difference observed between the watercress selenium treated groups extracts and watercress control group extracts. This suggests that the addition of selenium did not significantly alter the results.

We conducted a colony formation assay to investigate how the effects observed at the 24-hour mark in the proliferation assay would translate into colony formation outcomes. The results of the colony formation assay demonstrate a clear inhibitory effect of the extract treatments on cell proliferation, with treated groups exhibiting a significant reduction in colony formation compared to the control group. Moreover, a dose-response relationship was evident, indicating that higher treatment concentrations (50 $\mu\text{L/mL}$) resulted in more pronounced suppression of colony formation. These results are important for medical applications and encourage further investigation into new cancer treatments, though we still need to understand how they work at a deeper level.

To evaluate the effect of the watercress extracts on pancreatic cancer cells, cell imaging analyses were conducted using Mito Tracker, and DAPI staining. These analyses provided insights into the extract's impact on intracellular organelles and DNA. Mito Tracker staining was used to assess mitochondrial activity. A significant decrease in mitochondrial fluorescence intensity and deterioration in distribution patterns were observed following administration of the extracts.

The cell cycle analysis conducted on Panc-1 and MIA PaCa-2 cells revealed an increase in the sub-G1 phase in cells treated with the extract compared to the control group cells. This indicates that watercress extracts trigger apoptotic cell death in these cells.

In conclusion, the findings of this study suggest that watercress holds promise as a potential candidate for harnessing its anti-cancer potential against pancreatic cancer cells. Furthermore, the results indicate that selenium application to watercress contributes to the production of watercress with enriched contents. However, further research is required to elucidate the observed effects and investigate the underlying mechanisms of action.

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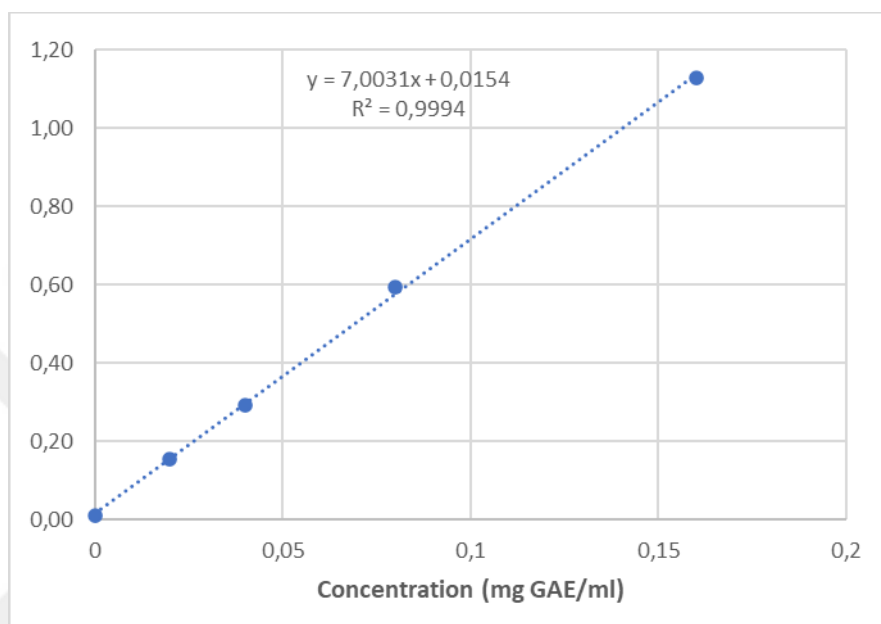
BIOGRAPHY

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APPENDICES

Appendix A: The gallic acid calibration curve has a concentration range of 0.1–0.16 mg/ml.



Appendix B: Trolox standards (ranging from 0–1.50 mM).

