EURASIAN JOURNAL of MOLECULAR & BIOCHEMICAL SCIENCES

(EURASIAN MOL BIOCHEM SCI)

e-ISSN: 2822-4019

Volume : 4 - Number: 1 - June/December 2025

https://dergipark.org.tr/en/pub/ejmbs

Eurasian Journal of Molecular and Biochemical Sciences

Official Journal of Erzurum Technical University Faculty of Science

Year	Volume	Issue / Number
2025	4	1

All articles in this journal are available free of charge from https://dergipark.org.tr/tr/pub/ejmbs

Eurasian Mol Biochem Sci is published twice a year

Indexing:

Eurasian Journal of Molecular and Biochemical Sciences (Eurasian Mol Biochem Sci) is indexed and abstracted by the following indexes and platforms (Alphabetical order); *Crossref, Cosmos Impact Faktor Google Scholar, Ideal Online, Index Copernicus.*

Eurasian Journal of Molecular and Biochemical Sciences

Year	Volume	Issue / Number
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Influence of ABA and CaO Nanoparticles on Antioxidative Potential of Calli Derived from Two Einkorn (*Triticum monococcum*) Ecotypes

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***Cite:**, Halis B, Ertüfekci O, Yazıcılar B, Geyik M.Ş, Başkut A.Ü, Nadaroğlu H. Influence of ABA and CaO Nanoparticles on Antioxidative Potential of Calli Derived from Two Einkorn (Triticum monococcum) Ecotypes. Eurasian Mol Biochem Sci 202;4(1): 1-10*

Received: 27 March 2025, Accepted: 1 July 2025 *DOI: 10.5281/zenodo.15876631*

Abstract

CaO nanoparticles (NPs) are important macromolecules that act as signal transducers in plants' growth and survival processe s. This study analyzed the responses of two different (Incedere and Kurekdere) *Triticum monococcum* landraces to various an tioxidant and physiological processes under tissue culture conditions. Two *T. monococcum* landraces were cultivated in 1-and 10-mM mannitol, consisting of 0.5 ppm and 1.5 ppm Cao NPs. CaO NPs significantly enhanced the activation of growth factor s in the two tested landraces. TOS, DPPH, CUPRAC, PPO, and GR were significant with Ca2+ NP application and demonstrat ed a high level associated with the tolerance degrees of the varieties. When the results of the total antioxidant test were analyz ed, it was detected that oxidant levels decreased significantly when CaO NPs were treated at increasing concentrations. While the antioxidant capacity of CaO NPs was limited at low concentrations (10-30 µg/mL) in the first 7 days, a high promotion wa s detected at higher concentrations (50 µg/mL). The activity increased in the second week, and the antioxidant effect continue d, especially in the 30 and 50 µg/mL groups. While a significant increase was observed in the results of wheat samples treated with CaO NPs in the 1st week, it was seen that the rise in copper ion-reducing activity became more balanced in the 2nd week. This trend shows that CaO NPs activate phenolic metabolism in short-term applications, but cellular regulation mechanisms c ome into play in the long term and balance the enzyme activity. The 2nd week's data show that GR activity reached a plateau l evel in certain dose groups. It is demonstrated that GR activity in the 50 µg/mL group did not change compared to the 1st wee k , but a slight decrease was observed in the 10 and 30 µg/mL applications. ABA and CaO NPs were observed to positively affe ct wheat development.

Keywords: *Triticum monococcum*, Callus, CaO NP, ABA, Callus

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Introduction

Einkorn (*Triticum monococcum* L. ssp. *monococcum*) is a diploid (2n = 2x = 14) primitive wheat and a close relative of durum (*Triticum turgidum* ssp. *durum*) and bread (*Triticum aestivum* ssp. *aestivum*) wheat. It originated in the Karacadag Mountains in Türkiye and was distributed to Europe during the Green Revolution (1). Due to its yields on poor soils, *T. monococcum* is still growing in cultivated lands as the essential cereal crop of many agricultural regions such as South Europe, Minor Asia, the Caucasus, and North Africa. Today, *T. monococcum* is one of the most critical grown food crops broadly cultivated in Türkiye, Balkan countries, southern Italy, southern France, Spain, and Morocco (2,3). *Triticum monococcum* is cultivated for its whole grain and enriched grain nutrition, folic acids, fiber, minerals, and vitamins. Various polymorphism structures, small genome sizes, and easy cultivation procedures are detected in *T. monococcum* (4). Extensive studies on modern wheat breeding programs have been started, followed by investigations on resistance to pests and diseases, tolerance to abiotic stresses, and functional foods. *Triticum monococcum* has been used extensively for the development of cultivars resistant to stress conditions (5). It exhibits high yield potential, grain quality, carotenoids, tocopherols, and phenolic acids (6). It is currently used in bread wheat production because einkorn possesses interesting nutritional traits, including low gluten content and minimal toxicity. Einkorn is known as a main source of micronutrients. The macro- and micronutrients and fibre contained in cereals and cereal-based products are important for the growth and development of plants; a deficiency of these elements can lead to reduced growth and development. Nanobiotechnology, as a biotechnology and agricultural field, has many novel applications, including nano-pesticide fertilizers, herbicides, or genes, improving seed sprouting, expansion, and plant conservation against environmental stresses. Nanotechnology has more comprehensive applications than biotechnology, such as gene transformation, genomics, proteomics, bioinformatics, and other technologies (7,8). Nanotechnologies can improve yield volume in less yielding crops, which contributes to sustainable agriculture. Furthermore, nanotechnology has assisted novel potential for improving the quality of foods, flavor, advanced protein content, and enhanced nutritional values. Nanoparticles have supported the development of yield productivity by introducing such qualities as biotic tolerance and increased environmental stress tolerance to the crops (9). Nanoparticles (NPs), including a class of metal-oxide (CaO, TiO2, and CuO—ZnO) with physicochemical properties, called nanomaterials (NM), have suggested new opportunities in developing plant growth to improve crop productivity. NPs have matchless physicochemical features and great potential as scaffolds for biomolecule interaction. With their high pertinence to in vitro applications, the use of NPs for maintaining and controlling callus development is a promising and worthy topic due to their increased effectiveness, resistance, progression, and, incredibly, their high specific surface area, which can induce interactions with living cells (10). Calcium (Ca^{2+}) is the primary plant nutrient, which is the central task of Ca^{2+} in plant expansion to ensure structural support to cell walls. Calcium is also well-known as a subsidiary precursor when plants are mechanically or biochemically injured. CaO is among the most favorable heterogeneous base catalysts due to its relatively high base sites and nontoxicity. CaO NPs can improve the plant's agronomic traits by eliminating oxidative stress (11). ABA is a significant phytohormone, and its functions and biosynthesis have been extensively studied at almost all enzymatic steps

through molecular-genetic, biochemical, and physiological approaches (12). Endogenous ABA levels significantly promote response to abiotic stresses, and they adjust some aspects of biochemical responses to a variety of biotic and abiotic stresses (13). Several researchers report that both the adaptation and survival of plant cells and tissues to various abiotic stress conditions may be improved by exogenous ABA (14). It is usually in vitro culture media to improve somatic embryogenesis and promote somatic embryo quality by improving dehydration tolerance and inhibiting precocious germination. ABA is also applied to induce somatic embryos to transform a stable phase in in vitro culture mediums and during synthetic seed studies (15). Recently, many studies have been published on the act of in vitro culture on enviromental e stress and the improvement of stress-resistance plants through in vitro selection (16,17). However, the effects of CaO NPs and ABA on the in vitro culture of einkorn wheat are currently not well known. This study aimed to determine the effects of ABA+CaO NPs treatments on oxidative stress in callus tissues of two landraces of einkorn.

Materials and Methods

Plant Material and Callus Induction: In our study, two ecotypes (İncedere and Kürekdere) of einkorn (Triticum monococcum) were used as the material for the response to CaO NPs and ABA applications. The mature seeds were disinfected with 1% sodium hypochlorite for 5 min, washed three times with ddH2O, and incubated with autoclaved water overnight at 4 °C. Mature embryos, aseptically obtained from swelling seeds and placed with the scutellum side up, were cultured in vitro on hormone-free MS medium (18). The explants were in total darkness for a total of one month at 25 ± 1 °C. After one month, callus induction appeared and was used for enzyme activity studies.

CaO NPs and ABA Treatments: The calli were transferred onto the callus development medium, including MS basal medium (pH 5.7), and 0.8 % agar was added with 2 mg/L of 2,4-D for 1 week in a growth cabinet at 28 °C, under a 16/8-h photoperiod condition. The calli were transferred to two distinct media containing CaO NPs and ABA, with treatments applied during the first and second weeks, in response to the CaO NPs+ABA treatments. In the first week, compact callus was transferred on hormone MS medium (18) 4 mg L–1 2,4-D (2,4-dichlorophenoxyacetic acid) in the presence of 1 and 10 mm ABA, consisting of 0.5 ppm and 1.5 ppm CaO NPs. For the second week, the callus was transferred to hormone-free MS medium (18) 4 mg L–1 2,4-D in the presence of 1 and 10 mm ABA, consisting of 0.5 ppm and 1.5 ppm CaO NPs for two weeks.

Determination of DPPH● Free Radical Removal Activity: DPPH● free radical removal activities of callus samples were performed according to a modification of the Blois method (19). 1 mM DPPH● solution was used as a free radical. Callus was transferred to the test tubes successively, and their total volume was complemented to 3 mL with distilled water. Then, 1 mL of DPPH● solution was added to each sample. After incubating for 30 minutes in the dark at room temperature, the absorbance changes at 517 nm were measured against the blank sample formed from ethanol. The control sample was prepared using 3 mL of ethanol and 1 mL of DPPH● solution. Reduced absorbance gives the remaining amount of DPPH● solution, i.e., free radical removal activity. BHA (Butylated Hydroxyanisole) and α-tocopherol were used as standard antioxidant compounds. DPPH● free radical calculations were made according to the following equation.

DPPH Free Radical Removal Activity (%) = (1- (A numune)/Akontrol)x 100

A sample refers to the absorbance value found after adding the sample to the DPPH● radical solution, and a control refers to the absorbance value of the control sample containing only the DPPH● radical solution. BHA and α-tocopherol were used as positive controls.

Determination of Cupric Reducing Antioxidant Capacity: Cupric Reducing Antioxidant Capacity (CUPRAC) analyses of callus samples were carried out by a modified procedure of the CUPRAC method, developed by (20). Callus samples were transferred to the test tubes, then 0.25 mL 0.01 M copper (II) chloride ($CuCl_2$) solution and 0.25 mL 7.5 x 10–3 M ethanolic neocuprin solution were added successively. Then, 0.25 mL of 1 M ammonium acetate buffer solution was added. Subsequently, after incubation for 30 minutes at room temperature, the absorbance change was read using the Epoch ™ Microplate UV-Vis Spectrophotometer at 450 nm against the blend of distilled water. For the control, distilled water was used instead of the sample. The increased absorbance level shows more copper reduction capacity.

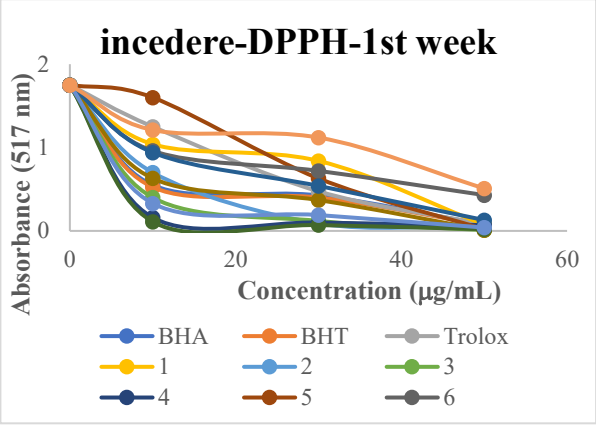
Determination of PPO Activity: To determine PPO activity, 0.5 g frozen leaves were ground to a powder under liquid nitrogen. The frozen plant powder was added to the extraction solution (0.05 M sodium phosphate containing 5 mM ascorbic acid and 1% (w/v) polyvinyl pyrrolidone at pH: 6.5). The suspension was centrifuged at 20000 x g for 15 min at 4 °C. The supernatants were filtered through Whatman No: 4 filter paper and assayed for the enzymatic activity. PPO activity was determined by measuring the increase in absorbance at 420 nm with a spectrophotometer (UV-1208 Shimadzu JAPAN). Then, 50 µl of crude extract was added to a 3 mL substrate mixture containing 0.20 M sodium phosphate buffer (pH: 6.5), 25 mM catechol. Enzyme activity was calculated from the linear portion of the curve. One unit of PPO activity was defined as the amount of enzyme that can cause an increase in absorbance of 0.001/minute (21,22).

Determination of Glutathione Reductase Activity: Glutathione reductase (GR) (EC 1.8.1.7)) activity determination is based on monitoring the oxidation of NADPH at 340 nm. Activity measurement was performed by measuring the change at 340 nm for 3 min in 1 mL of a mixture containing 50 mM potassium phosphate (pH=7) buffer, 2 mM Na2 EDTA, 0.15 mM NADPH, 0.5 mM GSSG, and 100 µL of enzyme extract (234,2).

Statistical Analysis: Each experiment was repeated three times. Analysis of variance was conducted using a one-way ANOVA test using SPSS 21.00, and means were compared by the Duncan test at the 0.05 confidence level.

Result

DPPH• Free Radical Scavenging Activity Findings: The DPPH radical scavenging activity of CaO NPs was compared with the standard antioxidants BHA (Butylated Hydroxyanisole), BHT (Butylated Hydroxytoluene), and Trolox. While the antioxidant capacity of CaO NPs was limited at low concentrations (10-30 µg/mL) in the first round, a significant increase was observed at higher concentrations (50 µg/mL). In the second round, the activity increased, and the antioxidant effect continued, especially in the 30 and 50 µg/mL groups (Figures 1,2).



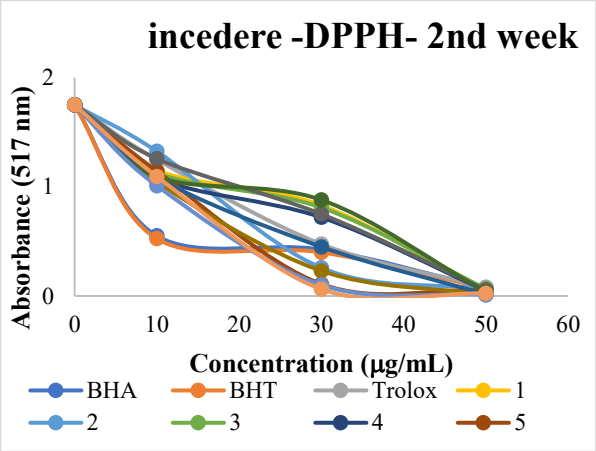
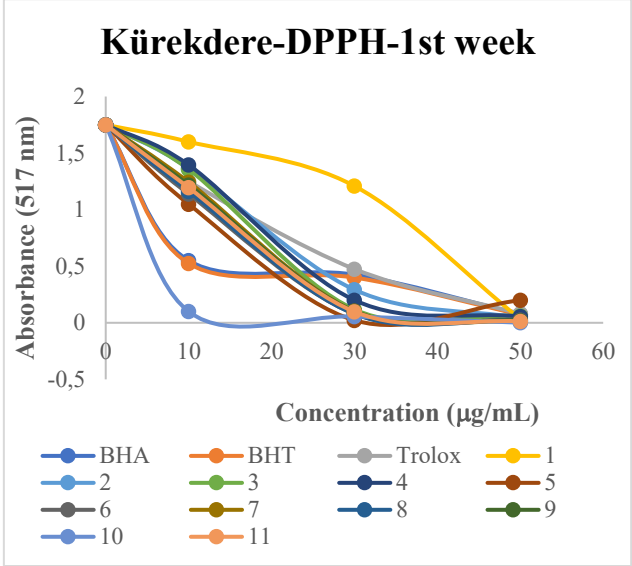


Figure 1. Comparison of DPPH radical scavenging activity of wheat samples treated with CaO NPs and ABA at various concentrations (10-50 µg/mL) with standard antioxidants BHA, BHT, and trolox1: Control, 2:Ca^{+}, 3:Ca^{-}, 4: 0.5 ppm CaO NPs, 5: 1 ppm CaO NPs, 6: 1 mM ABA, 7: 10 mM ABA, 8: 1 mM ABA + 0.5 ppm CaO NPs, 9:1 mM ABA+ 1.5 ppm CaO NPs, 10: 10 mM ABA + 0.5 ppm CaO NPs, 11: 10 mM ABA + 1.5 ppm CaO NPs



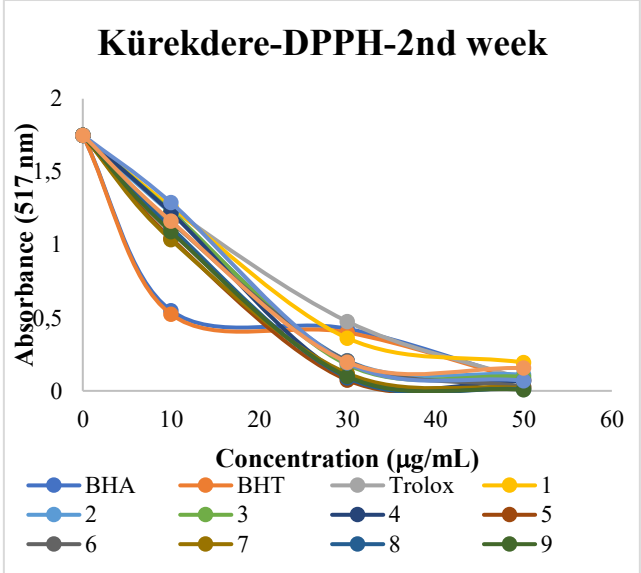
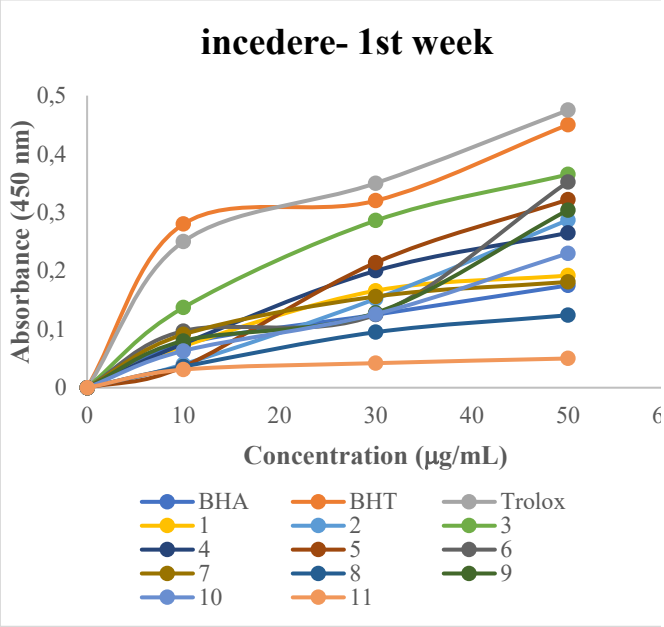


Figure 2. DPPH radical scavenging activity of wheat callus samples treated with CaO NPs at various concentrations (10-50 µg/mL). Trolox1: Control, 2:Ca^{+}, 3:Ca^{-}, 4: 0.5 ppm CaO NPs, 5: 1 ppm CaO NPs, 6: 1 mM ABA, 7: 10 mM ABA, 8: 1 mM ABA + 0.5 ppm CaO NPs, 9:1 mM ABA+ 1.5 ppm CaO NPs, 10: 10 mM ABA + 0.5 ppm CaO NPs, 11: 10 mM ABA + 1.5 ppm CaO NPs

CUPRAC findings: The copper ion (Cu^{2+}) reduction capacities of wheat callus samples treated with CaO NPs, determined by the CUPRAC method, were analyzed in comparison with standard antioxidants (BHA, BHT, Trolox). While a significant increase was observed in the results of wheat callus samples treated with CaO NPs during the first week, the enhancement in copper reduction activity appeared to stabilize and

become more balanced by the second week.This situation shows NPs can lead to different biochemical interactions in plant tissues over time (Figure 3, 4).



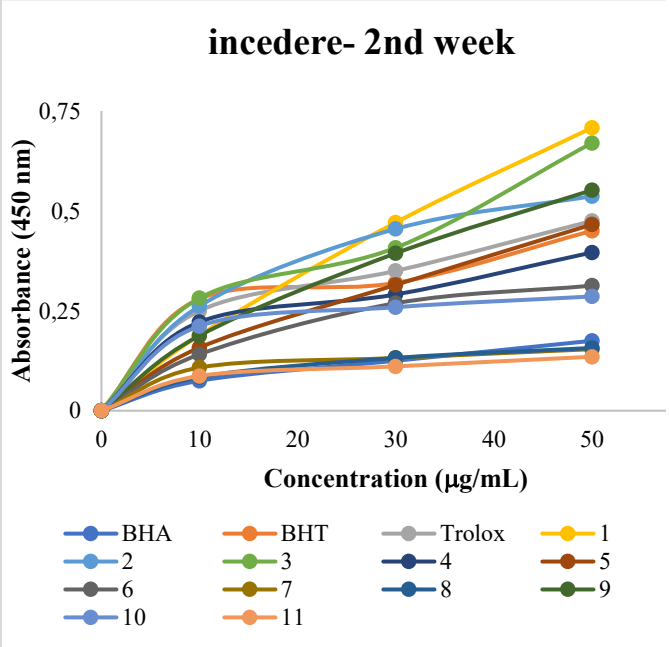
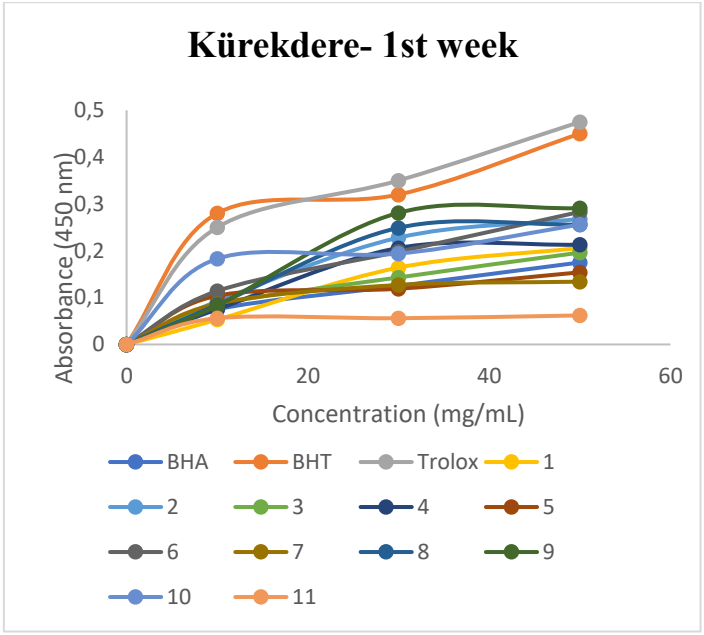


Figure 3 cupric (Cu^{2+}) reducing activities of wheat samples treated with CaO NPs at various concentrations (10-50 µg/mL). Trolox1: Control, 2:Ca^{+}, 3:Ca^{-}, 4: 0.5 ppm CaO NPs, 5: 1 ppm CaO NPs, 6: 1 mM ABA, 7: 10 mM ABA, 8: 1 mM ABA + 0.5 ppm CaO NPs, 9:1 mM ABA+ 1.5 ppm CaO NPs, 10: 10 mM ABA + 0.5 ppm CaO NPs, 11: 10 mM ABA + 1.5 ppm CaO NPs



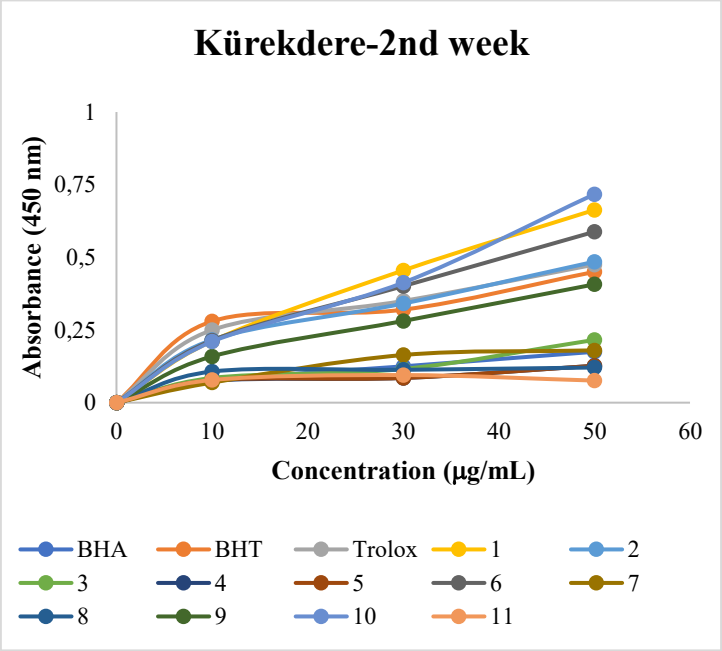


Figure 4. Cupric ion (Cu^{2+}) reducing activities of wheat samples treated with CaO NPs at various concentrations (10-50 µg/mL). Trolox1: Control, 2:Ca^{+}, 3:Ca^{-}, 4: 0.5 ppm CaO NPs, 5: 1 ppm CaO NPs, 6: 1 mM ABA, 7: 10 mM ABA, 8: 1 mM ABA + 0.5 ppm CaO NPs, 9:1 mM ABA+ 1.5 ppm CaO NPs, 10: 10 mM ABA + 0.5 ppm CaO NPs, 11: 10 mM ABA + 1.5 ppm CaO NPs

PPO Activity Findings: Polyphenol oxidase (PPO) is an enzyme that plays a role in plant defense mechanisms through the oxidation of phenolic compounds. Changes in PPO activity were analyzed to evaluate the effects of CaO NP applications on phenolic metabolism. The first-week results showed that CaO NPs treatment caused a dose-dependent increase in PPO activity ($p < 0.01$). It was determined that PPO activity increased by 42.8% in the 50 µg/mL CaO NPs applied group compared to the control group. The second week's data revealed that PPO activity remained stable in the high-concentration groups (30 and 50 µg/mL) compared to the first week ($p > 0.05$). Still, a slight decrease was experienced in the 10 µg/mL application ($p < 0.05$). This trend shows that CaO NPs activate phenolic metabolism in short-term applications, but cellular regulation mechanisms come into play in the long term and balance the enzyme activity (Figure 5).

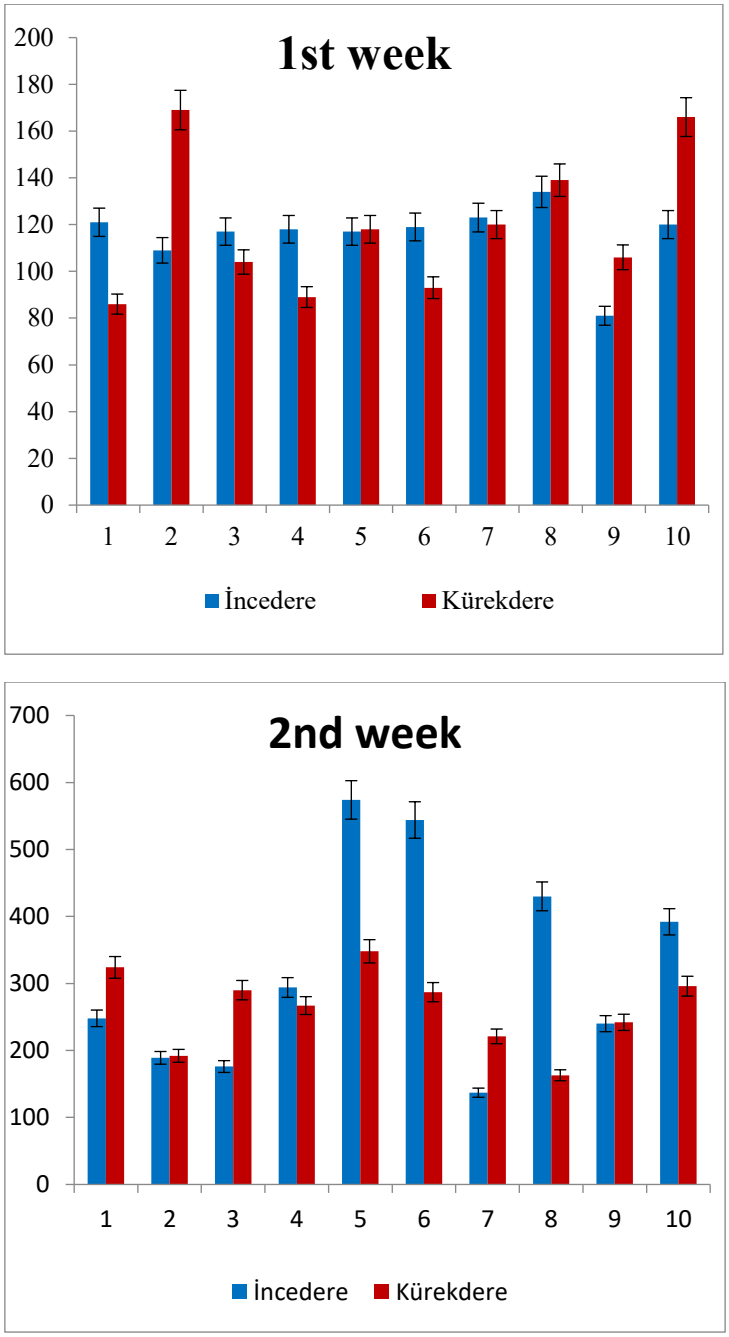


Figure 5. PPO enzyme activity in the 1st and 2nd weeks of Incedere and Kürekdere. 1: Control, 2:Ca^{+}, 3:Ca^{-}, 4: 0.5 ppm CaO NPs, 5: 1 ppm CaO NPs, 6: 1 mM ABA, 7: 10 mM ABA, 8: 1 mM ABA + 0.5 ppm CaO NPs, 9:1 mM ABA+ 1.5 ppm CaO NPs, 10: 10 mM ABA + 0.5 ppm CaO NPs, 11: 10 mM ABA + 1.5 ppm CaO NPs

Glutathione Reductase Activity Findings: Glutathione reductase (GR) is an antioxidant enzyme that is critical in maintaining cellular redox homeostasis. Our study determined that CaO NPs application in the 1st week caused a significant increase

in GR activity ($p < 0.05$). In particular, it was determined that GR activity increased by 24.6% and 38.2% in 30 and 50 µg/mL CaO NPs applications, respectively, compared to the control group. The 2nd-week data show that GR activity reached a plateau in certain dose groups; GR activity in the 50 µg/mL group did not change compared to the first week ($p > 0.05$), but a slight decrease was observed in 10 and 30 µg/mL applications ($p < 0.05$) (Figure 6).

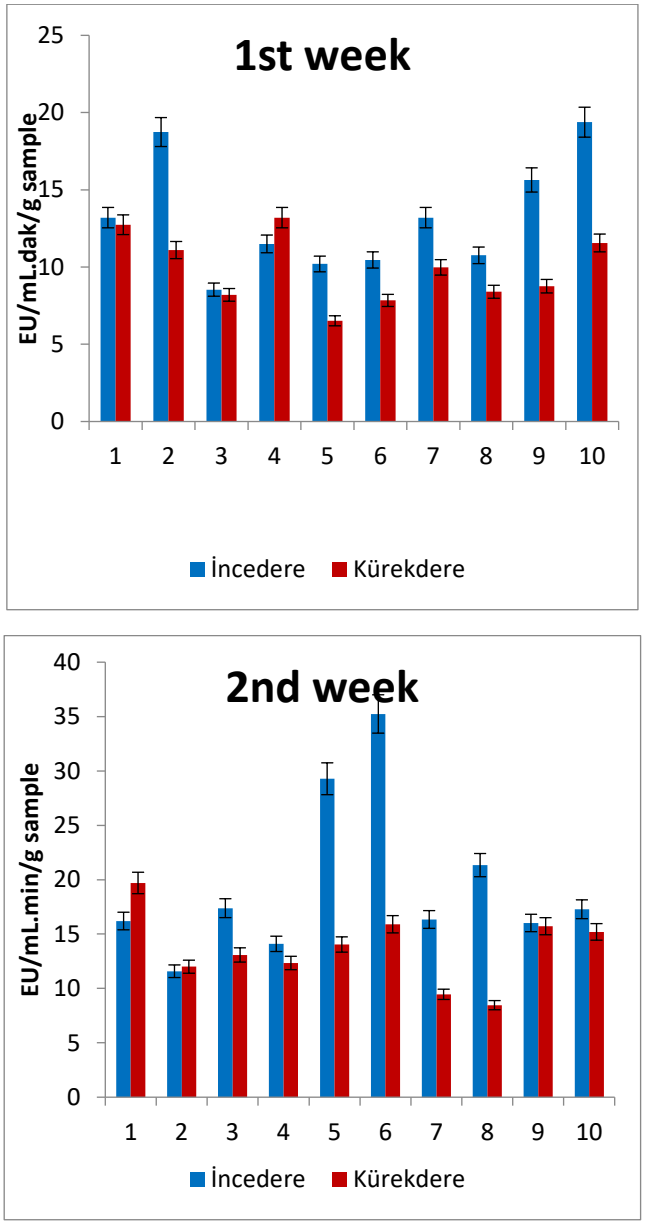


Figure 6. Effect of CaO NPs and ABA applications Glutathione reductase enzyme activity. 1: Control, 2:Ca^{+}, 3:Ca^{-}, 4: 0.5 ppm CaO NPs, 5: 1 ppm CaO NPs, 6: 1 mM ABA, 7: 10 mM ABA, 8: 1 mM ABA + 0.5 ppm CaO NPs, 9:1 mM ABA+ 1.5 ppm CaO NPs, 10: 10 mM ABA + 0.5 ppm CaO NPs, 11: 10 mM ABA + 1.5 ppm CaO NPs

Discussion

Nanoparticles (NPs), either alone or together with hormones, have been applied to several plant varieties and were detected in cells containing predominantly salicylic acid and ABA for improving growth and development in vitro cell culture. Upon the type and concentrations, the synergistic influence of NPs with plant tissues affects many physiological and biochemical changes during the cellular growth process (25). In this study, treatments of CaO NPs+ABA greatly influenced the callus improvement and the process of precious antioxidant compounds. CaO NPs+ABA at two doses was implemented in vitro on callus developed and maintained in the MS media in composition with 4 mg L–1 2,4-D (2,4-dichlorophenoxyacetic acid), including 0.5, 1.5 ppm CaO NPs nanoparticulate and 1 and 10 mM ABA. Based on the CUPRAC rates, samples of first-week cultured calli in the presence of CaO NPs enhanced hormone response and greatly promoted CaO NPs markers as compared to control callus, and there was an upwards in Cuprac amount that was collected in the presence of CaO NPs. Conversely, samples of second-week cultured callus cells in the presence of Ca2+ NPs have a more stable hormone response, without changing CUPRAC rate, as compared to the untreated callus (Figure 3, 4). This event was considered to be a positive feedback treatment through signal transduction. The feedback organizing results between CUPRAC and Ca2+ NPs have been reported on adaptation and developmental conditions in various plant species (12). These findings agree with those published by (26) without nanoparticle treatments in the study on Saponaria prostrata plant extract by two different in vitro bioanalytical methods, including CUPRAC and FRAP. Our study demonstrated that under CaO NPs conditions, the DPPH content declined by a larger level than in the untreated callus in both the İncedere and Kürekdere ecotypes of *T. monococcum* (Figure 1, 2).

The positive impacts of CaO NPs in ABA presence were deficient at 0.5 ppm than at 1.5 ppm concentration. Bursal et al. (2022) (27) found a parallel result for DPPH decreased in water extract compared to the control set of S. prostrata. However, their results indicated considerable free radicals scavenging activity when compared to the standards used. (28) found similar results in garlic extract with different solvents. PPO activity is closely associated with the synthesis and degradation of metabolites in plants. Callus types are essential factors affecting PPO activity (29). Both ecotypes were quickly observed for better PPO activities with Ca2+ NPs concentrations of 1 ppm and 10 ppm. Conversely, the same ecotypes responded with more stable PPO activity at 1.5 ppm CaO NPs 1- 10 ppm in a long time. Our results indicated that PPO activity varied remarkably depending on the CaO NPs+ABA degrees and period of treatments but independent of genotype (Figure 5). The first-week treatments showed a significant increase in GR activity, primarily due to the enhanced effect of CaO NPs, whereas in the second week, the activity slightly decreased (Figure 6). In the current study, the antioxidant enzyme GR activity is the lowest on the premier concentration of ABA 10 mM, presumably due to deactivation. These results suggest that the duration of treatment and GR activity may be regulated differently. These outcomes agree with those published by (30) in the study on Mung bean germinating seedlings fluoride stress at different concentrations for 5 days.

Conclusion

In this study, the synergistic effects of CaO NPs and ABA on callus development and antioxidant responses in *Triticum monococcum* ecotypes were evaluated under *in vitro* conditions. The combined application of CaO NPs and ABA significantly enhanced callus improvement and modulated key antioxidant parameters such as TOS, CUPRAC, DPPH, and PPO activities. Notably, CaO NPs treatments effectively reduced oxidative stress induced by ABA, especially at higher concentrations, suggesting their potential role in stress mitigation and cellular adaptation. The most pronounced changes were observed during the first week of treatment, where CaO NPs contributed to a heightened antioxidant enzyme response, particularly GR activity. However, this effect diminished over time, highlighting the importance of treatment duration. The observed decrease in DPPH content and modulation of PPO activity suggest that nanoparticle concentration and treatment period play critical roles in determining physiological responses, independent of genotype. Overall, these findings emphasize the promising role of CaO NPs-particularly in combination with ABA-as bioactive agents for enhancing stress resilience and promoting callus development in cereal crops.

Declaration of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Eurasian Journal of Molecular and Biochemical Sciences

Evaluating the Synergistic Effects of Oleuropein and Vitamin C on Head and Neck Cancer Cell Viability and Migration

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Cite: *Beyaz Z. F, Öztürk E. Kara A, Evaluating the Synergistic Effects of Oleuropein and Vitamin C on Head and Neck Cancer Cell Viability and Migration. Eurasian Mol Biochem Sci 2025;4(1): 11-17*

Received: 27 March 2025, Accepted: 1 July 2025 *DOI: 10.5281/zenodo.15876824*

Abstract

Cancer's standard treatment is generally based on cytotoxic drugs, radiotherapy, chemotherapy, and surgery. One treatment type in the study is phytotherapy, which uses plant components to treat diseases or improve health. Oleuropein, obtained from olive leaves, is active in olive leaves and fruit. It affects health, including skin protection, anti-aging properties, antiviral effects, and antioxidant benefits. Recent studies have demonstrated that it has a positive impact on humans, including its potential for cancer prevention and antimicrobial properties. The other substance to be combined with oleuropein is vitamin C, which plays an essential role in the body, inactivates carcinogenic substances, and is a well-known antioxidant that forms a protective shield against cancer. We investigated the therapeutic effects of combining oleuropein and vitamin C on head and neck cancer cells (FaDu). We conducted cell viability and scratch (migration) analyses on FaDu cells treated with oleuropein and/or vitamin C at different doses for 24 hours. The cell viability analyses revealed that treatments with oleuropein and vitamin C significantly reduced cell viability. At the same time, the combined applications of oleuropein and vitamin C also considerably reduced cell viability. In the cell migration test, we observed that oleuropein and vitamin C decreased the cell migration. The results indicate that applying oleuropein or vitamin C leads to decreased cell viability. However, the combined applications of oleuropein and vitamin C offer an effective treatment option by reducing cell viability and migration.

Keywords: Cancer, Oleuropein, Vitamin C, Head and neck cancer

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Introduction

Today and in the future, cancer is one of the most common diseases that threaten human health. Cancer is a multifaceted and complex disease that occurs when normal cells in the body grow and multiply uncontrollably (Alexander & Vidyasagar, 1993). While normal cells grow and divide within a particular cycle and die, cancer cells lose these control mechanisms when necessary and multiply uncontrollably. This is one of the main factors that make cancer treatment difficult. The development and spread of cancer are associated with many biological, genetic, and environmental factors (Ertan, 1967).

Cancer is classified into numerous subtypes that can originate from various tissues and organs. It can develop anywhere in the body and present itself with different symptoms. Common symptoms of cancer include abnormal growth or swelling, skin changes, chronic fatigue, excessive weight loss, and a long-term cough. Cancer is usually diagnosed with a series of tests and imaging methods. These include blood tests, magnetic resonance imaging (MRI), x-rays, ultrasound, and computerized tomography (CT). Early diagnosis is one of the most important factors that increase the success of treatment (İbrahim, 2020).

Cancer treatment varies depending on the type of disease, its stage, and the patient's general health status. Treatment options include surgical intervention, chemotherapy, immunotherapy, radiotherapy, and targeted treatments (Forasitiere et al., 2023). In recent years, the effects of natural compounds on cancer have also been investigated, and the effects of compounds such as oleuropein and vitamin C on cancer cells are the subject of scientific studies.

Head and neck cancer is a type of cancer that occurs with the uncontrolled growth of various tissues in the head and neck region. These cancers usually develop in regions such as the mouth, tongue, pharynx, nose, throat, ear, larynx, and thyroid. Head and neck cancer, which is a significant health problem worldwide, can lead to serious complications if not diagnosed early. The treatment process varies depending on the type and stage of the cancer, and treatment becomes more complex in advanced stages (Powell et al., 2011).

Head and neck cancer risk factors include tobacco use, excessive alcohol consumption, prolonged sun exposure, nutritional deficiencies, and genetic factors. In recent years, an increase in the incidence of head and neck cancer has been observed, and some of this increase is associated with human papillomavirus (HPV) (Waridel et al., 1997). Certain types of HPV can cause cancer in the head and neck region, so widespread use of HPV vaccines stands out as an important strategy in combating this type of cancer.

Oleuropein is one of the main components of the olive tree and has potent antioxidant and anticancer properties. It was first discovered by Bourquelot and Vintilesco in 1908, and its structure was fully described in 1960. Oleuropein is known for its antiatherogenic, antimicrobial, anti-inflammatory, and antiviral effects (Gikas et al., 2007). Olive fruits and leaves are rich in oleuropein. This compound, which is found in higher amounts, especially in unripe olives, decreases as the olive ripens (Omar, 2010). Oleuropein is a natural additive in many industries due to its ability to neutralize superoxide anions (Preedy & Watson, 2020). Studies on the effects of oleuropein in the fight against cancer have shown that this compound can stop the growth of cancer cells. However, further clinical studies are needed to establish the direct validity of this effect in humans (Boskou, 2008).

Vitamin C (ascorbic acid) is a water-soluble vitamin essential for the human body. It is naturally found in many fruits and vegetables. It has many vital functions, including supporting the immune system, enhancing iron absorption, providing antioxidant effects, and contributing to collagen production (Sözmen, 2002). Vitamin C can prevent cellular damage by neutralizing

free radicals as a powerful antioxidant. With this feature, it has the potential to slow down the aging process and reduce disease risks. It is thought to protect against colds and viral infections due to its immune system-strengthening effects (Morpa, 2005). Although studies on the impact of vitamin C on cancer have not reached a definitive conclusion, it is suggested that adequate vitamin C intake may help prevent the development of certain types of cancer. However, further scientific research is required to validate this effect. The study aims to investigate the therapeutic effects of oleuropein and/or vitamin C, which are essential compounds with antioxidant and anti-inflammatory properties. Laboratory studies aim to examine their impact on head and neck cancer cells.

Material and methods

FaDu cells are hypopharyngeal cancer cell lines and are used as models in laboratory head and neck cancer research. These cells, isolated by Dr. J.C. Moloney in 1956, are of the squamous cell carcinoma type and serve as an important model in research, including cancer biology, metastasis, tumor growth, and drug testing (Akao et al., 2007; Shen et al., 2017).

Frozen FaDu cell lines used in the study were stored in liquid nitrogen. The cells were seeded in T-25 flasks in RPMI-160 medium and incubated at 37°C with 5% CO2. When cells reached 80% confluency, the medium was aspirated, and the cells were washed with phosphate-buffered saline (PBS). They were then incubated with trypsin-EDTA (0.25%). The cells were then resuspended in fresh medium and reseeded in new flasks at the desired density.

Drug Preparations: Vitamin C (176 g/mol, Sigma-Aldrich) was dissolved in distilled water and then diluted with the culture medium. Oleuropein (Santa Cruz) was dissolved in ethanol and further diluted with the culture medium. The final ethanol concentration was reduced to a non-toxic level, maintaining a dilution ratio of 1:10,000. To evaluate the combined effect, cells were treated with 25 μM Vitamin C and concentrations of 50 μM and 80 μM of oleuropein for 24 hours. The treated concentrations were selected based on the effective dose ranges reported in the literature. Vitamin C doses were based on the concentrations found to be effective in human cell lines (Taşkın, 2023). Oleuropein doses were determined by considering the 50–80 μM range, which has been reported to have anticancer effects in a study (Öztürk et al., 2022).

Cell Viability Assay: FaDu cells were seeded in 96-well plates (2500 cells/well) and incubated for 24 hours. Following incubation, cells were treated with Vitamin C and /or oleuropein or their combination for 24 hours. After treatment, CCK-8 reagent was added to each well, and the plate was incubated for 3 hours at 37°C. Absorbance was measured at 450 nm using an ELISA plate reader (Epoch Bio-Tek, USA).

Wound Healing Assay (Scratch Assay): Cells were seeded in a 6-well plate and incubated until confluency. A scratch was created using a pipette tip, and detached cells were removed by washing with PBS. Cells were treated with Vit C and/or Oleuropein, then incubated for 24 hours. Wound closure was monitored under an inverted microscope at 0 and 24 hours. The disclosure area was measured using ImageJ software, and then the closer areas were estimated from the images of 0 and 24 hours of incubation.

Result

Cell Viability Assay Results: Figure 1 shows the cell viability values (%) of FaDu cells for 24 h. Compared to the control group, treatments with oleuropein (OLE) and Vitamin C (Vit-C) significantly decreased cell viability. In particular, oleuropein treatment at concentrations of 50 μM and 80 μM decreased viability rates. In addition, using Vitamin C and oleuropein together (Vit-C + OLE 50 μM and Vit-C + OLE 80 μM) had a more significant effect than using either alone.

This suggests that these two compounds may have a synergistic effect. The decrease in cell viability may indicate the anti-proliferative or toxic effects of these compounds. These results may require further mechanistic studies to understand the effects of oleuropein and/or Vitamin C on cells.

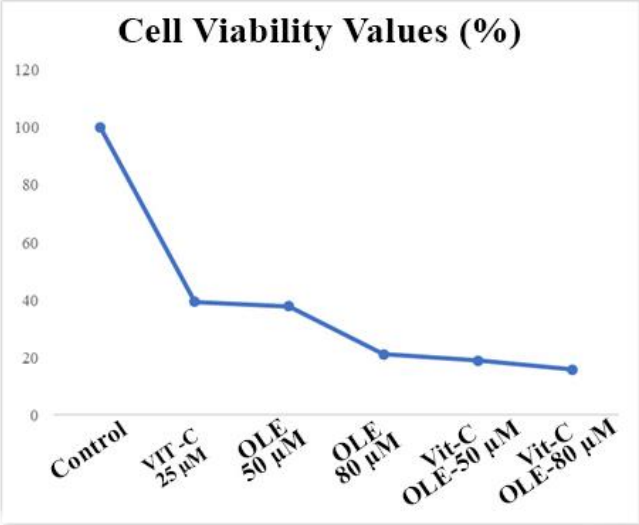

Figure 1. Effect of vitamin C and/or oleuropein application on the viability of FaDu cells after 24 h of incubation. Vitamin C (25 µM), oleuropein (50 µM and 80 µM), and combinations of these compounds were applied to FaDu cells for 24 h. Cell viability was measured using the CVDK-8 kit; results are presented as mean ± standard deviation (n = 5).

Wound Healing Assay: Wound healing analysis showed that oleuropein and Vitamin C individually reduced cell migration. The combination treatment significantly inhibited cell migration compared to individual treatments. These findings suggest that combining oleuropein and Vitamin C effectively reduces cell viability and migration, indicating its potential as a therapeutic strategy for head and neck cancer. The scratched cells and discolored areas seen in Figure 2, and the discolored distance measurements seen in Figure 3.

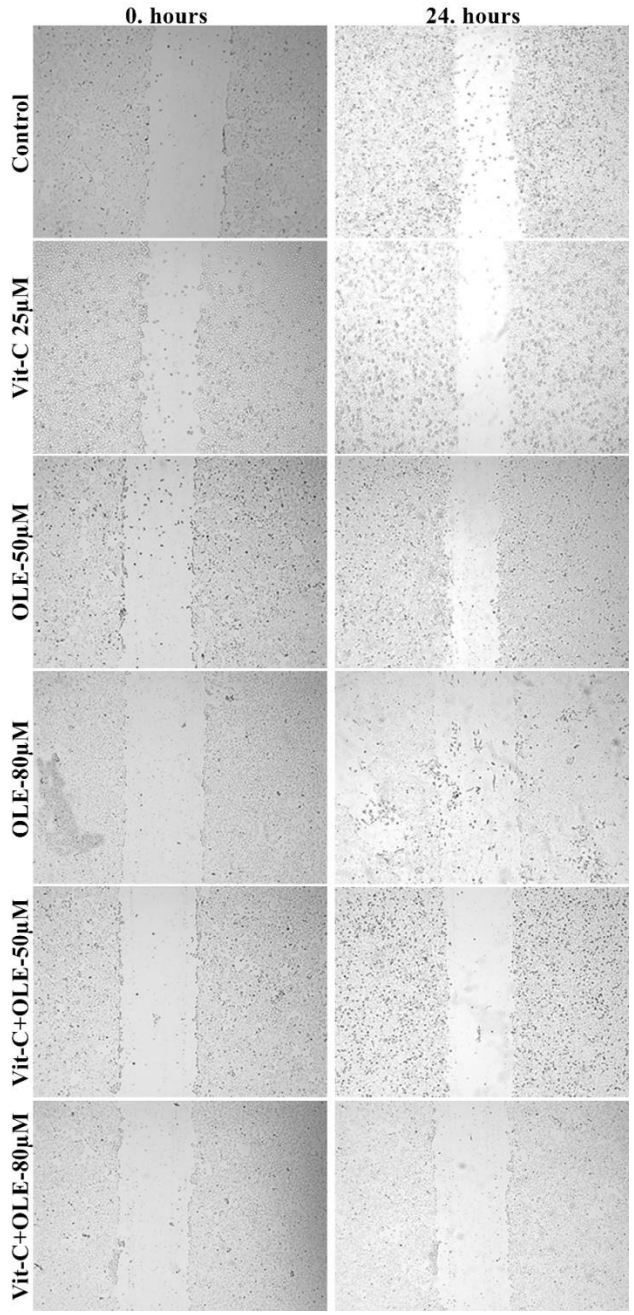

Figure 2. The illustration shows FaDu cells treated with Vit C and/or Oleuropein at 0 and 24 hours of the scratch test. At 0 hours, a scratch was created on the confluent layer of FaDu cells using a sterile pipette tip, and images were taken. At the end of the 24th hour, the migration of cells was evaluated along with the wound closure process. The images visually reflect the effects of different treatment groups on the wound area.

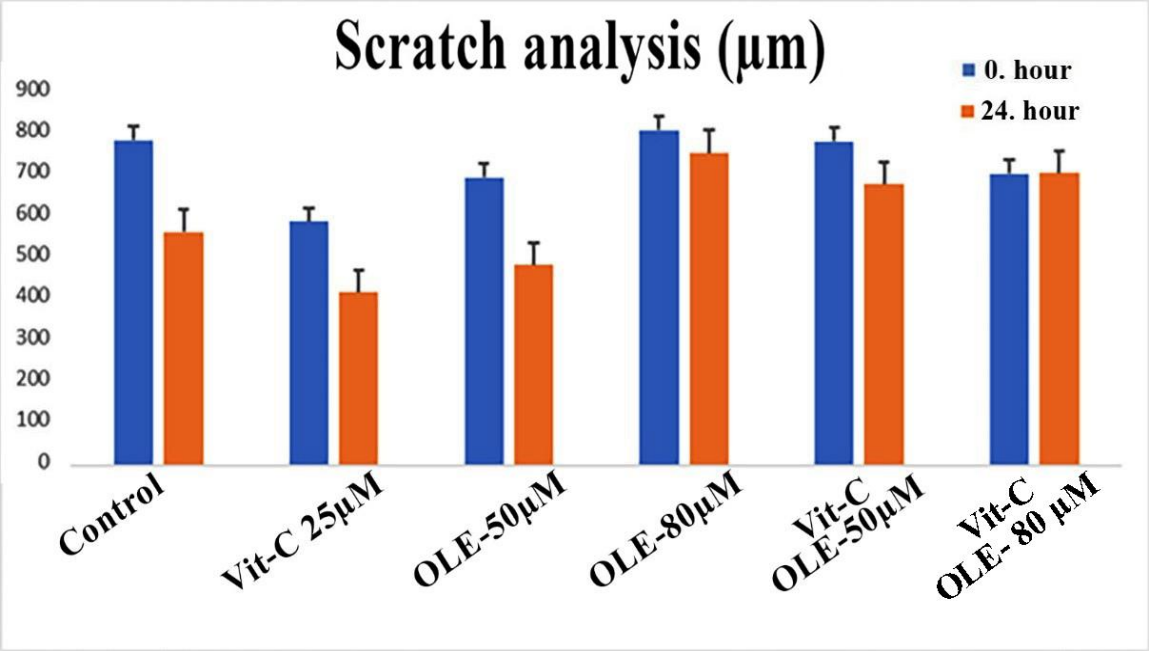

Figure 3. Graph of wound closure percentages analyzed from microscopic images obtained at 0 and 24 hours within the scope of the scratch (wound healing) analysis. Vitamin C (25 µM), oleuropein (50 µM and 80 µM), and combinations of these compounds were applied to FaDu cells; wound openings were measured with ImageJ software. The results are presented as mean ± standard deviation (n = 5), and *p < 0.05 indicates statistically significant differences compared to the control group.

Discussion

This study investigated the effects of oleuropein (OLE) and Vitamin C (Vit-C) treatments on the FaDu cell line. The findings demonstrate that treating these compounds alone and in combination has a significant impact on cell viability and migration. The results of our study reveal the effects of oleuropein and Vitamin C on cancer cells. These findings are parallel to similar studies in the literature. In particular, a significant decrease in cell viability was observed when oleuropein was applied at 50 µM and 80 µM concentrations. This finding is consistent with the effects reported in previous studies on the anticancer properties of oleuropein and the mechanisms of inhibiting cancer cells. Li et al. (2012) reported that oleuropein exhibited anti-proliferative effects on prostate cancer cells. Similarly, in this study, oleuropein treatment significantly decreased cell viability. Additionally, the cell viability-reducing effect of high doses of Vitamin C has been previously demonstrated. Jacob and Sotoudeh (2002) stated that high Vitamin C concentrations can create cytotoxic effects. This study observed that high-dose Vitamin C treatment reduced cell viability.

In our study, combining oleuropein and Vitamin C resulted in greater cell viability loss than using either compound alone. This result suggests that these two compounds have a synergistic effect. This synergy is also consistent with other studies in the literature. Lee et al. (2013) suggested that combining oleuropein and Vitamin C created synergistic effects in cancer cells. Similarly, in our study, the combination of these two compounds showed a more substantial anti-proliferative effect. The synergistic impact indicates that these compounds can yield more effective

treatment results when used in combination. This situation provides a crucial foundation for developing new treatment strategies for cancer.

The results of the wound healing test on cell migration also reveal that these compounds can affect cell motility. It was observed that cell migration decreased when both compounds were applied separately; however, the combination treatment provided a more potent inhibition of migration. This result shows that oleuropein and Vitamin C can inhibit cell migration. Similarly, Lee et al. (2013) reported that oleuropein inhibited cell migration and reduced cancer cell invasion. Consistent with this, Impellizzeri et al. (2011) also showed that oleuropein reduced oxidative stress and inhibited inflammatory signaling, which are key processes in cancer metastasis.

Vitamin C is a compound known for its properties that limit cell motility. Mandl et al. (2009) demonstrated that Vitamin C inhibits migration of various cancer cell types through its antioxidant and pro-oxidant mechanisms, depending on concentration. In addition, Cárcamo et al. (2002) highlighted the role of Vitamin C in regulating HIF-1α and collagen synthesis, further implicating it in the control of wound healing and tumor progression. These findings represent a crucial step in understanding the effects of these compounds on wound healing and metastasis processes, particularly in the context of cancer treatment and management. Öztürk et al. (2022) investigated the cytotoxic and genotoxic effects of olive leaf extract on colorectal cancer cell lines. In the study, olive leaf extract demonstrated significant cytotoxic effects against colorectal cancer cells, while exhibiting minimal toxicity in normal cell lines. This study supports the potential therapeutic effects of oleuropein on cancer cells. Furthermore, Han et al. (2016) confirmed the anti-metastatic potential of oleuropein in breast cancer cells by demonstrating its effect on matrix metalloproteinases (MMPs), which are critical enzymes in cancer cell invasion. Similarly, Du et al. (2010) found that oleuropein suppressed proliferation and induced apoptosis in prostate cancer cells. The findings obtained in our study indicate that olive leaf extract and oleuropein have a similar effect in preventing the proliferation and migration of cancer cells. This parallelism confirms the anticancer effects of oleuropein (Koyun et al., 2022).

In conclusion, this study investigated the effects of oleuropein and Vitamin C on the FaDu cell line, revealing that both compounds exhibited anti-proliferative and migration-inhibitory effects. The combination treatment further enhanced these effects, creating a synergistic effect. However, the results of this study require more detailed and comprehensive studies before they can be applied clinically. Future research should aim to understand better the molecular mechanisms and clinical effects of these compounds.

Author Contributions: FB, EÖ, and AK contributed equally to the conception, execution, and writing of this study. All authors have approved the final version of the manuscript.

Declaration of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Eurasian Journal of Molecular and Biochemical Sciences

Investigating The Factors Affecting Obesity Using Machine Learning Algorithms

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Cite: *Camli O, Sevim D. E,. Investigating the factors affecting obesity using machine learning algorithms. Eurasian Mol Biochem Sci 2025;4(1): 18-24*

Received: 14 April 2025, Accepted: 2 july 2025 *DOI: 10.5281/zenodo.15876882*

Abstract

Obesity is a global health concern driven by a complex interaction of behavioral, dietary, genetic, and lifestyle factors. This study investigates key predictors of obesity using four machine learning-based feature selection techniques: Boruta, Recursive Feature Elimination (RFE), Lasso Logistic Regression, and Genetic Algorithms. A dataset of 2,111 individuals from Mexico, Peru, and Colombia—comprising demographic, behavioral, dietary, and physical activity-related variables—was analyzed using R. All four methods consistently identified high-calorie food consumption, frequent snacking, low water intake, low physical activity, and family history of overweight as the most robust predictors. In contrast, variables such as gender, smoking, and transportation mode were excluded by all algorithms, indicating limited relevance in this context. The partial selection of features like alcohol intake and caloric monitoring by specific methods highlights the importance of algorithm-specific sensitivity to non-linear patterns. These findings emphasize the central role of lifestyle-related factors in obesity risk and support the use of machine learning to derive interpretable, data-driven insights for public health policy and targeted intervention strategies.

Keywords: Obesity, Machine Learning, Feature Selection, Boruta, Lasso, Genetic Algorithm, RFE, Public Health

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Introduction

Obesity has emerged as one of the most pressing globa l health issues, contributing to increased risk of chroni c conditions such as type 2 diabetes, cardiovascular dis eases, and certain cancers. According to the World Hea lth Organization (2023), more than one billion people worldwide are affected by obesity, and this number co ntinues to rise. Understanding the underlying factors c ontributing to obesity is essential for developing effecti ve prevention and intervention strategies.

The multifactorial nature of obesity—encompassing lif estyle, behavioral, environmental, and genetic compon ents—presents significant challenges to researchers. C

onventional statistical approaches often fall short in ha ndling high-dimensional data and detecting nonlinear relationships among variables. In contrast, machine le arning (ML) algorithms are increasingly recognized for their ability to analyze complex data structures and un cover hidden patterns (1).

Recent studies have highlighted the growing use of ML methods in obesity-related research. For instance, Kni ghts et al. (2024) employed a combination of experime ntal statistical design and ML algorithms—specifically Random Forest and Support Vector Machines (SVM)— to identify latent risk factors for obesity. Their results i ndicated that Random Forest provided better feature i mportance interpretability, while SVM excelled in clas sification performance.

Fernandes et al. (2023) conducted a comprehensive co mparison of ML algorithms including Decision Trees, k -Nearest Neighbors (k-NN), Naive Bayes, and Support Vector Machines on obesity prediction tasks. Among t hese, SVM achieved the highest accuracy, followed clos ely by Decision Trees.

Similarly, Iqbal et al. (2024) trained classification mod els such as Logistic Regression, Random Forest, and G radient Boosting using physical activity-based features , demonstrating the predictive value of behavioral vari ables. Random Forest outperformed the others in term s of accuracy and precision.

Lucena et al. (2024) further expanded the approach by combining physical activity and dietary features and co mparing algorithms such as XGBoost, Artificial Neural Networks (ANN), and Logistic Regression. Their result s showed that XGBoost yielded the most optimal result s, particularly in recall and AUC metrics.

From a national perspective, Kitiş and Goker (2023) a pplied Naive Bayes, SVM, and Random Forest to classi fy obesity stages among the Turkish population, report ing that Random Forest achieved the best balance betw een sensitivity and specificity, providing region-specifi c insights to the literature.

These studies illustrate that machine learning not only enhances predictive accuracy but also contributes to the interpretability and application of findings in public health and clinical decision-making. They also demonstrate the relevance of integrating diverse algorithmic strategies and varied feature sets for robust modeling.

In this study, we employ four distinct machine learning-based feature selection methods to identify the most influential variables associated with obesity. These include the Boruta algorithm, Recursive Feature Elimination (RFE), Lasso Logistic Regression, and a Genetic Algorithm-based approach. By comparing the results of these algorithms, we aim to obtain robust and consistent insights into the key determinants of obesity

Material and methods

This study employed four machine learning-based feature selection methods—Boruta, Recursive Feature Elimination (RFE), Lasso Logistic Regression, and Genetic Algorithms—to identify the most significant predictors of obesity. These methods were chosen for their complementary strengths in handling high-dimensional data, capturing nonlinear relationships, and offering interpretability. All methods were applied to a dataset comprising 2,111 records collected from individuals in Mexico, Peru, and Colombia. The dataset includes demographic, behavioral, dietary, and physical activity-related variables. All statistical analyses were conducted in R, a comprehensive environment for statistical computing and graphics (7).

Table 1. Variables selected by each feature selection method

Feature	Boruta	RFE	Lasso	Genetic Algorithm
Gender	✗	✗	✗	✗
Age	✗	✓	✗	✗
Family history of obesity	✓	✓	✓	✓
High-calorie food (FAVC)	✓	✓	✓	✓
Vegetable consumption (FCVC)	✗	✓	✗	✗
Number of meals (NCP)	✗	✓	✓	✓
Frequency of snacking (CAEC)	✓	✓	✓	✓
Smoking (SMOKE)	✗	✗	✗	✗
Water intake (CH2O)	✓	✓	✓	✓
Caloric monitoring (SCC)	✓	✗	✗	✗
Physical activity (FAF)	✓	✓	✓	✓
Technology use (TUE)	✗	✗	✗	✓
Alcohol consumption (CALC)	✗	✗	✗	✓
Public Transportation	✗	✗	✗	✗
Walking	✗	✗	✗	✗
Motorbike	✗	✗	✗	✗
Bike	✗	✗	✗	✗
Automobile	✗	✗	✗	✗
Weight	✗	✗	✗	✗

Data Set

The dataset used in this study comprises 2,111 records from individuals in Mexico, Peru, and Colombia. While 23% of the data were collected directly from survey responses, the remaining 77% were synthetically generated using the SMOTE (Synthetic Minority Over-sampling Technique) technique in the Weka environment. Given the class imbalance in the original dataset, the SMOTE method was used to synthetically generate additional samples for the minority class. While this technique helps to mitigate model bias towards the majority class, it may also introduce artificial correlations that could influence the feature selection process. Thus, findings based on synthetic data should be interpreted with caution. The dataset includes 19 features covering a range of demographic, behavioral, dietary, and physical activity-related factors.

Gender is encoded as a binary variable (1 for male, 0 for female). Age is a continuous variable representing each respondent's age in years. Variables such as family history of overweight, high-calorie food consumption (FAVC), vegetable consumption (FCVC), smoking (SMOKE), and caloric monitoring (SCC) are binary indicators (0 or 1). Other variables such as the number of daily main meals (NCP), food consumption between meals (CAEC), water intake (CH2O), physical activity (FAF), technology usage (TUE), and alcohol consumption (CALC) are encoded on ordinal scales.

In addition, the dataset includes five dummy variables

to indicate the participant's primary mode of transportation (Automobile, Bike, Motorbike, Public_Transportation, Walking). The target variable is binary: "NObeyesdad" (a term from the original dataset) takes the value 1 if the individual is classified as obese, and 0 otherwise.

Boruta Algorithm

Boruta is a wrapper-based feature selection algorithm that leverages the random forest classifier to assess the importance of each feature by comparing it with shadow (randomized) counterparts. It iteratively removes features that are statistically less important than the maximum importance of shadow features, aiming to identify all relevant variables rather than a minimal optimal subset. In this study, Boruta was used to determine which features consistently contribute to predicting obesity status. The analysis was performed in R using the Boruta package (8).

Recursive Feature Elimination (RFE)

Recursive Feature Elimination (RFE) is a widely used wrapper-based feature selection method that recursively eliminates the least important features based on the predictive power of a specified learning algorithm (9). In each iteration, a model is trained, feature importances are calculated, and the least relevant feature is removed from the dataset. This process is repeated until a predefined number of features is reached, allowing for the identification of a feature subset that maximizes model performance.

In this study, RFE was implemented using logistic regression as the base learner. To enhance the generalizability and stability of the selected feature subset, 10-fold cross-validation was applied during the selection process. The analysis was performed using the caret package in R, which provides an efficient and customizable framework for feature selection and model evaluation (10).

Lasso Logistic Regression

Lasso (Least Absolute Shrinkage and Selection Operator) Logistic Regression introduces an L1 penalty to the traditional logistic regression cost function (11). This penalty term shrinks the coefficients of less important variables to zero, effectively performing variable selection while preventing overfitting. In this study, Lasso regression was conducted using the glmnet package in R, with the regularization parameter λ optimized through cross-validation (12).

Genetic Algorithm

Genetic Algorithms (GA) are population-based metaheuristic optimization techniques inspired by natural evolution (13,14). Each candidate solution (i.e., subset of features) is encoded as a binary chromosome. Through iterative selection, crossover, and mutation operations, GA explores the feature space to identify subsets that optimize a chosen fitness function—in this case, classification accuracy via logistic regression. The analysis in this study was carried out using the GA package in R (15).

Result

A comparative summary of the variables selected by each method is presented in Table 1. The selection status of each variable was determined based on the internal evaluation criteria of the corresponding feature selection algorithm. For instance, Boruta selects features by comparing their importance against shadow attributes, while Lasso shrinks coefficients of less important variables to zero. The table uses check marks (✓) to indicate selected features and cross marks (✗) for features that were not selected. The features consistently identified across all algorithms included high-calorie food consumption (FAVC), frequency of snacking (CAEC), water intake (CH2O), physical activity level (FAF), and family history of overweight. These variables were considered robust predictors of obesity status.

These findings suggest a strong and consistent association between dietary patterns, physical activity, and family history with obesity status. While variables

like gender, smoking, transportation mode, and weight were not selected by any algorithm, others such as caloric monitoring, vegetable consumption, and alcohol intake were selected by at least one method. This variability indicates that some features may carry conditional or algorithm-specific predictive power. The consistency of key predictors across multiple models reinforces their relevance for future health policy and intervention design.

Discussion

The results of this study reinforce the growing consensus in the literature that obesity is strongly influenced by behavioral and lifestyle-related variables. Across all four machine learning algorithms applied high-calorie food consumption, frequent snacking, low water intake, low physical activity levels, and a family history of obesity were consistently identified as key predictors. These findings align with previous research demonstrating the centrality of these variables in determining obesity risk (3, 4).

Interestingly, variables such as gender, smoking status, and transportation preferences were not selected by any of the algorithms. This may indicate that their influence is either less pronounced in the current dataset or potentially mediated by stronger behavioral variables. The non-selection of gender, in particular, contrasts with earlier studies suggesting gender-specific obesity trends, highlighting the importance of data context, variable interactions, and feature representations (2). Moreover, one notable finding is the non-selection of weight as a predictor, despite its central role in defining obesity. This may be explained by the dataset's labeling strategy: individuals are already categorized as obese or non-obese based on their weight and related metrics. As such, weight does not serve as an independent predictor in this context but is instead embedded within the target variable. Similarly, gender—though commonly reported as a determinant of obesity—may not have demonstrated significant predictive power in this dataset due to potential mediation by behavioral factors such as dietary habits and physical activity, which are directly accounted for in the models.

Some variables, including vegetable consumption (FCVC), caloric monitoring (SCC), and alcohol intake (CALC), were selected by only one of the four methods. This suggests that such features may possess conditional or non-linear effects that are captured differently depending on the algorithm used. For instance, the Genetic Algorithm, being a heuristic optimizer, was the only method to capture the predictive value of alcohol consumption, indicating the possible contribution of hidden interactions or non-linear patterns.

The variability in feature selection across different algorithms may stem from their inherent methodological differences. For instance, Lasso tends to eliminate collinear features by selecting only one representative, while RFE considers feature combinations iteratively, potentially retaining correlated predictors. The Genetic Algorithm, by contrast, explores a broader solution space, making it more likely to detect nonlinear or interaction-based effects. Therefore, variables selected uniquely—such as alcohol consumption (CALC) and technology use (TUE)—might exhibit subtle predictive relationships that only some algorithms can capture depending on their optimization objectives.

These findings illustrate the strength of a multi-algorithmic approach to feature selection, particularly in complex health datasets. The consistency of core variables across multiple models strengthens their validity and relevance for real-world applications. This study highlights the potential of machine learning not only in prediction but also in variable prioritization for public health interventions.

Public health practitioners and clinicians may benefit from focusing on the consistently selected features when designing preventive interventions or personalized obesity risk assessments. Furthermore,

policy makers should consider emphasizing healthy eating habits, hydration, and physical activity in targeted health programs, especially for individuals with a family history of obesity.

It is important to acknowledge that the use of synthetically generated data through SMOTE may affect the stability and generalizability of the selected features. Although this approach helps to balance the dataset, it might also amplify patterns not representative of real-world variability. Future work using fully empirical or external validation datasets is necessary to confirm the robustness of these results.

Although our study provides valuable insights into obesity determinants in Latin American populations, caution should be exercised when generalizing these findings to other regions. Genetic, cultural, and environmental differences may influence the importance of certain features. Future research should incorporate data from diverse geographic and socio-economic contexts to validate the universality of our results.

Conclusion

This study demonstrated the effectiveness of machine learning-based feature selection methods in identifying key factors associated with obesity. By applying Boruta, Recursive Feature Elimination (RFE), Lasso Logistic Regression, and Genetic Algorithms to a dataset consisting of both real and synthetically generated health-related data, we found that high-calorie food consumption, frequent snacking, low water intake, low physical activity, and family history of obesity were consistently selected as important predictors across all models.

These findings highlight the significance of behavioral and lifestyle-related variables over demographic factors such as gender and transportation mode, which were not selected by any of the algorithms. The convergence of results across different models reinforces the reliability of the identified predictors and supports their consideration in targeted public health interventions.

Our results emphasize the value of employing multiple feature selection techniques for robust, interpretable modeling in health data analysis. Future studies may further benefit from incorporating longitudinal data and additional contextual variables to improve prediction accuracy and intervention design. Additionally, given that our data represent individuals from specific Latin American countries, future studies should explore whether the identified predictors hold across other populations with varying genetic and lifestyle backgrounds

Author Contributions

Conceptualization: Onur Camli Formal Analysis: Onur Camli Investigation: Onur Camli, Dilek Sevim Methodology: Onur Camli Project Administration: Onur Camli Writing – Original Draft: Onur Camli, Dilek Sevim

Writing – Review & Editing: Onur Camli, Dilek Sevim

Declaration of Interest: The author declares that there is no conflict of interest regarding the publication of this paper.

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Eurasian Journal of Molecular and Biochemical Sciences

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Synergistic Anticancer Effects of Low-Frequency Magnetic Field and Doxorubicin on Glioblastoma Cell Line

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Cite: *Ergene H, Aydemir, Arslan M. E, Berber G, Men D. E, Karataş H, Arslan E, Aksakal C, Türkez H. Synergistic Anticancer Effects of Low-Frequency Magnetic Field and Doxorubicin on Glioblastoma Cell Line. Eurasian Mol Biochem Sci 2025;4(1): 25-36*

Received: 25 March 2025, Accepted: 11 May 2025 *DOI: 10.5281/zenodo.15876928*

Abstract

Glioblastoma (GBM) is one of the most aggressive primary brain tumors of the central nervous system, with a limited median survival of approximately 15 months despite current treatment options. In this study, the effects of a low-frequency magnetic field (LF-MF) in combination with the chemotherapeutic agent doxorubicin (DOX) on the glioblastoma cell line (U87MG) were investigated. In the experimental design, U87MG cells were exposed to LF-MF at the intensity of 1 mT and treated with different concentrations of DOX (5 µg/ml and 10 µg/ml). Cell viability was assessed using the MTT assay, nuclear morphological changes were analyzed with Hoechst 33258 staining, and apoptotic and necrotic cell percentages were determined by flow cytometry. The results showed that DOX alone reduced cell viability in a dose-dependent manner (IC_{50}= 3.22 µg/ml). However, in the presence of LF-MF, DOX's cytotoxic effect increased, leading to a decrease in the IC_{50} value to 2.18 µg/ml. Flow cytometry analyses revealed that while 5 µg/ml and 10 µg/ml DOX alone increased apoptotic cell percentages to 23.4% and 38.7%, respectively, these rates increased to 37.2% and 52.5% when combined with LF-MF. Compared to the control group, LF-MF did not alter toxicity in the healthy fibroblast cell line (HDFa) and had no additional effect on DOX-induced cell death. However, in glioblastoma cells, LF-MF-supported DOX treatment significantly enhanced cell death, suggesting that LF-MF may improve DOX efficacy by increasing cell membrane permeability and reactive oxygen species (ROS) production. This study demonstrates that the combination of LF-MF and DOX exerts a synergistic effect on glioblastoma cells by enhancing cell death. LF-MF-assisted chemotherapy strategies could be a potential alternative in glioblastoma treatment, but further molecular and biochemical studies are needed to elucidate the underlying mechanisms.

Keywords: Glioblastoma, Low-Frequency Magnetic Field, Doxorubicin, Apoptosis, Chemotherapy, Cell Viability.

Introduction

GBM is the most common malignant primary brain tumor, accounting for approximately 57% of all gliomas and 48% of primary malignant central nervous system tumors. Patients diagnosed with this disease have a

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significantly limited prognosis, with a median survival of less than two years (1). Recent advancements in molecular profiling have led to a revised classification of glioblastoma, particularly emphasizing the analysis of isocitrate dehydrogenase (*IDH*) mutation status, which provides a deeper understanding of the disease's fundamental pathogenesis. Histologically, glioblastoma subtypes are characterized by high-grade astrocytic tumors, regions of microvascular proliferation, and/or areas of focal necrosis. In addition, distinct histological variations are observed in *IDH*-wild-type glioblastomas. Giant cell glioblastomas contain large, highly pleomorphic multinucleated giant cells, whereas gliosarcomas exhibit sarcomatous mesenchymal metaplasia along with malignant astrocytic features. At the molecular level, mutations in isocitrate dehydrogenase 1 (*IDH1*) and *IDH2*, along with O6-methylguanine-DNA methyltransferase (*MGMT*) promoter methylation, are considered prognostic indicators associated with better clinical outcomes (2). Current treatment strategies for glioblastoma include tumor-treating fields with low-intensity electric fields and adjuvant temozolomide administration. In cases of tumor recurrence, there is no standardized treatment protocol; however, therapeutic options such as surgical resection, radiotherapy, chemotherapy, or systemic treatments like bevacizumab may be considered based on the patient's condition. For patients who respond to treatment, median survival extends beyond 15 months. Epidemiological data from the United States between 2000 and 2014 indicate that the one-year relative survival rate for glioblastoma improved from 34.4% (2000–2004) to 44.6% (2005–2014), averaging 41.4%. Despite short-term survival rate improvements over time, the five-year survival rate has remained relatively constant, with only 5.8% of patients surviving beyond five years after diagnosis. Glioblastoma exhibits a slight male predominance, and its incidence increases with age. Among elderly patients receiving only supportive care, the median survival is less than four months. According to registry data from 2011 to 2015, the age-adjusted annual incidence of glioblastoma in the United States was 3.21 per 100,000 individuals. The incidence varies by age and gender, with a median age at diagnosis of 65 years, and the highest prevalence observed in the 75–84 age group. Given these statistics, there is an urgent need to develop novel therapeutic approaches that can shorten treatment duration and reduce the economic burden associated with glioblastoma management (3,4).

The incorporation of magnetic field (MF) applications alongside chemotherapy has emerged as a promising approach in cancer treatment, potentially enhancing therapeutic success and reducing treatment duration. However, the optimal intensity and strength of the applied magnetic field remain uncertain. The widespread use of static magnetic fields (SMF) in medicine has necessitated the exploration of their biological effects and mechanisms of interaction with biological systems (5). The presence of magnetic materials within the cells and organisms can influence various metabolic and biological processes, including stress responses, proliferation, and structural alignment. In general, SMF interactions have been deemed safe at the organ and organism levels. However, some adverse effects have been reported by subjects exposed to strong SMFs. Experimental studies conducted on C57BL/6 mice subjected to continuous SMFs ranging from 2 to 12 T for 28 days have indicated no significant safety concerns (6). The effects of MF on cancer progression have been tested in a metastatic human cancer model, in which mice were implanted with a human breast tumor and exposed to modulated MF at specific intervals for six weeks. Additionally, a control group was treated with the chemotherapeutic agent cyclophosphamide. The results showed that neither MF nor cyclophosphamide significantly reduced the number of metastases. However, both treatments suppressed the spread and growth of

metastases, with MF exhibiting a greater inhibitory effect than chemotherapy (7). Furthermore, previous studies have demonstrated that MF exposure at specific intensities and durations can inhibit tumor growth by 40% to 50%. This inhibition has been associated with a reduction in tumor cell proliferation, an increase in apoptotic cell death, and decreased expression of certain proteins. The effects of MF may also be linked to alterations in the recombination rate of free radicals, supporting the theory that its anti-tumor activity is mediated through free radical interactions. These findings confirm the potential anti-cancer effects of magnetic fields and suggest that this therapy is non-toxic, further reinforcing its viability as an adjunctive treatment in oncology (8).

GBM is the most prevalent and lethal form of central nervous system tumors. The limitations of current treatment methods and the prolonged treatment duration impose a significant economic burden on both patients and healthcare systems. In this context, reducing treatment duration and mitigating the disease burden is a fundamental objective to alleviate these economic challenges. Recent literature suggests that magnetic fields (MF) may exert beneficial biological effects against certain types of cancer (9). In particular, low-frequency modulated MF has been observed to inhibit tumor growth and limit metastatic spread. The underlying inhibition mechanism is believed to involve the dynamics of free radical recombination and redox signaling pathways (10). Building upon these scientific findings, our study aimed to investigate whether the combination of low-frequency magnetic field exposure and the anti-cancer drug DOX exhibits a synergistic effect. This investigation was conducted *in vitro* using the glioblastoma (U87MG) cell line (11).

Material and Method

Cell Cultures: The human fibroblast cell line (ATCC® PCS-201-012™) was cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% penicillin/streptomycin antibiotic mixture, 10% fetal bovine serum (FBS), and maintained at 37°C in a humidified incubator with 5% CO_2 until confluence was reached. To investigate the effects of low-frequency magnetic field (LF-MF) exposure, cells were subjected to 1 mT magnetic field intensity in the presence and absence of DOX (5 µg/ml) for 24 hours of application time. The experiments were conducted using 48-well culture plates and performed in triplicate to ensure reproducibility (12,13). The U87MG human glioblastoma cell line (ATCC® HTB-14™) was cultured in DMEM supplemented with 1% penicillin/streptomycin and 5% FBS under standard conditions at 37°C in a humidified atmosphere of 5% CO_2. Cells were allowed to proliferate until they reached 80% confluence before being subjected to experimental conditions.The effects of LF-MF were initially evaluated under non-toxic conditions to establish safe magnetic field intensities. Once the optimal parameters were identified, LF-MF exposure was applied in combination with DOX (5 µg/ml) at pre-determined concentrations for anti-cancer investigations (14).

MTT Cell Viability Assay: To assess cell viability, U87MG and fibroblast cells were seeded in 48-well plates at a density of 10^4 cells per well in 100 µL of culture medium. Cells were incubated for 24 hours at 37°C in a CO_2 incubator before treatment with DOX alone, LF-MF alone, or a combination of both. Following treatment, 10 µL (5 mg/ml) of MTT reagent (thiazolyl blue tetrazolium bromide) was added to each well, and the plates were gently agitated for 1 minute to ensure uniform mixing. As a positive control, 1 % Triton-X was added to the wells. Cells were incubated for an additional 3–4 hours at 37°C in the dark to allow MTT reduction by metabolically active cells. After incubation, the culture medium was carefully aspirated without disrupting the cell monolayer, and 100 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. Absorbance was

measured at 570 nm using a microplate reader. The results were analyzed to determine the percentage of viable cells in each treatment group (15).

The Device Generating Uniform ELF-MFs: The ELF-MF-generating system used in this study comprises a culture plate holder encircled by a copper coil. This coil, consisting of 150 turns of 1.5 mm copper wire, is capable of producing magnetic fields up to 30 mT without causing any noticeable heat buildup in the coil. Magnetic field intensity is highest at the edges of the culture plate holder and gradually decreases toward the center. Therefore, cell cultures were selectively grown in these peripheral regions to ensure more consistent and reliable experimental conditions. Magnetic field strength was monitored and adjusted using a Gauss meter (Eles, EE406-HP1, TR). To generate magnetic fields of 1 mT, electrical currents of 0.6 V/1.07 A were applied to the coil.

Nuclear Morphology Analysis Using Hoechst 33258 Fluorescent Staining: Hoechst 33258 fluorescent staining was used to evaluate nuclear morphology and detect apoptotic changes in the treated cells. Cells were cultured under the same experimental conditions and treated with DOX alone, LF-MF alone, or in combination (Magnetic fields and DOX applied simultaneously). A negative control group was included, in which no treatment was applied. After 24 hours of incubation, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for 30 minutes. Following fixation, cells were washed with PBS and stained with 1 µM Hoechst 33258 dye at room temperature for 5 minutes. Stained cells were then analyzed under a fluorescence microscope (Leica® DM IL LED), and images were captured to assess nuclear integrity and morphological changes associated with apoptosis (16,17).

Flow Cytometry Analysis: Apoptotic and necrotic cell populations were quantified using flow cytometry. Following treatment, cells were detached by incubation with trypsin for 5 minutes at room temperature and collected by centrifugation at 300 × g. A total of 5×10^4 cells were resuspended in 500 µL of 1× binding buffer. To detect apoptotic and necrotic cells, 5 µL of Annexin V-FITC and 5 µL of propidium iodide (PI, 50 µg/mL) were added to each sample, and cells were incubated in the dark at room temperature for 5 min. Samples were analyzed using a flow cytometer with dual filters for FITC and rhodamine detection. The percentages of apoptotic and necrotic cells were determined by distinguishing Annexin V-positive and PI-positive populations (18).

Statistical Analysis: All experiments were performed in triplicate, and data were analyzed using One-Way ANOVA to assess the statistical significance of differences between treatment groups. Multiple comparisons were conducted using Dunnett's test (against the control group) and Tukey's post hoc test to compare treatment groups. A significant threshold of $p < 0.05$ was applied. Statistical analyses were performed using GraphPad Prism version 7.0 software

Results

In this study, patients were divided into four groups according to the treatment protocol, and the sample number and average age of the groups were determined as follows: Group I (n=41; 70.73±10.69 years), Group II (n=16; 68.25±5.18 years), Group III (n=18; 73.00±8.00 years), Group IV (n=37; 71.86±8.42 years). The healthy control group (Group V, n=30; 69.18±12.25 years) without any systemic disease was formed. There was no statistically significant difference between the patient groups and the control group ($p>0.05$). Shapiro-Wilk tests were performed to determine whether there was a significant difference between the groups in our study parameters. As a result of these tests, the mean, standard error (SE), median, first and third quartile (Q1-Q3), minimum, maximum and p value results of the groups according to the parameters are shown in Table 1. According to this table, significant differences were found between the

groups in CAT, ARE, MPO, TAS, TOS and OSI values ($p<0.05$). No significant difference was found between the groups in the measurement of SOD, tGSH, GPx, MDA, and PON levels ($p>0.05$).

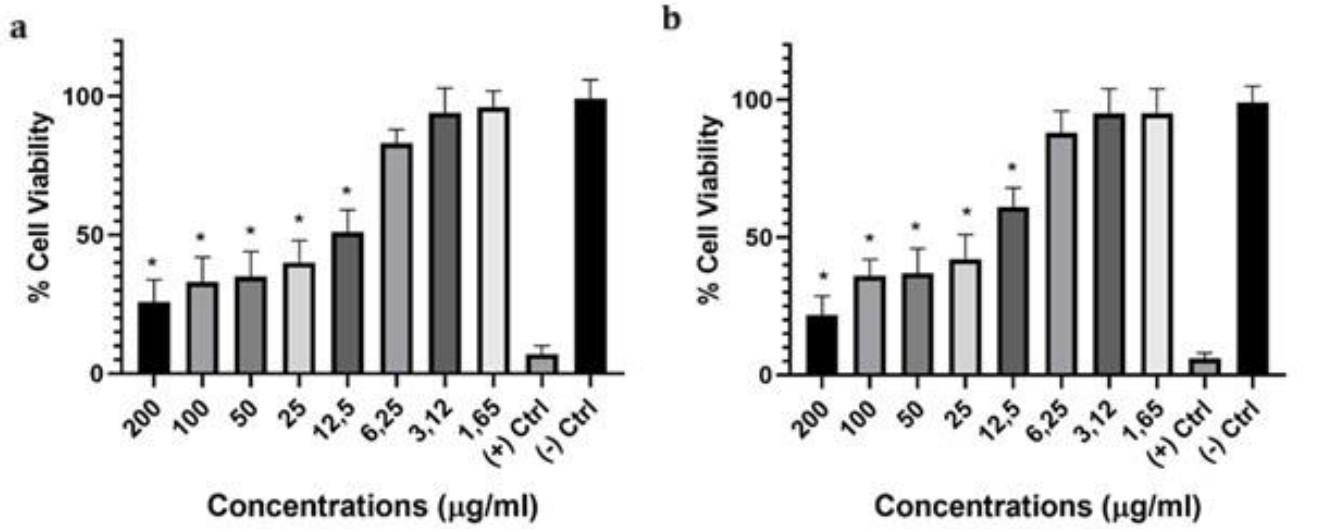
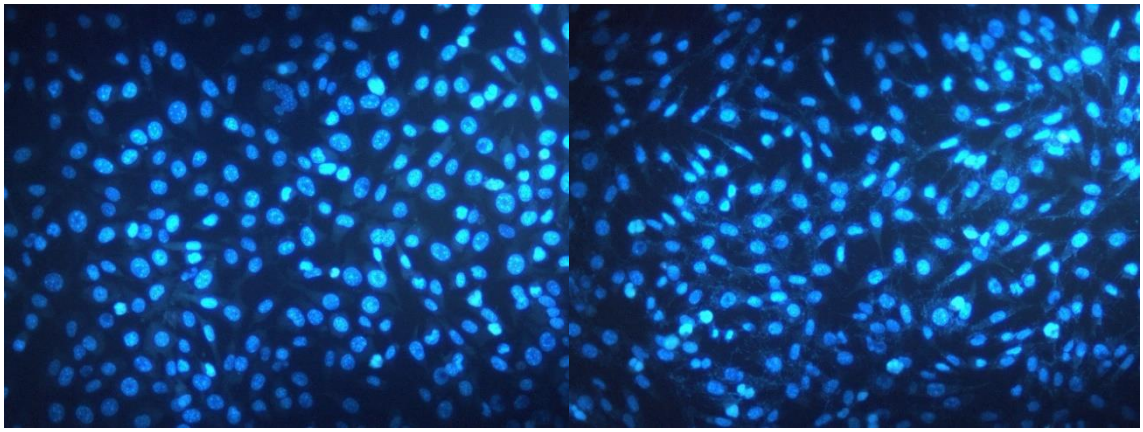


Figure 1. Presents the cytotoxicity analysis results of human dermal fibroblast adult (HDFa) cells following a 24-hour exposure to different doses of DOX. The results are categorized into two experimental conditions: a. Cytotoxicity without Magnetic Field. This group represents the viability of HDFa cells treated with DOX in the absence of a magnetic field. b. Cytotoxicity Under Magnetic Field Application: This group illustrates the viability of HDFa cells subjected to DOX treatment under the influence of an applied magnetic field.

The effects of different concentrations of DOX and magnetic field application on the nuclear morphology of human dermal fibroblast adult (HDFa) cells were investigated. Fluorescence microscopy analyses using Hoechst 33342 staining revealed no significant morphological alterations in the nuclei following DOX treatment. Similarly, no changes in nuclear integrity or morphology were observed when the magnetic field was applied alone or in combination with DOX (Figure 3). These results suggest that the magnetic field does not have a significant effect on DOX-induced nuclear alterations in healthy fibroblast cells and does not modify cellular morphology.

a- Negative Control

b- Magnetic field-applied cells



c- DOX (5 µg/ml)

d- DOX and Magnetic field (5 µg/ml)

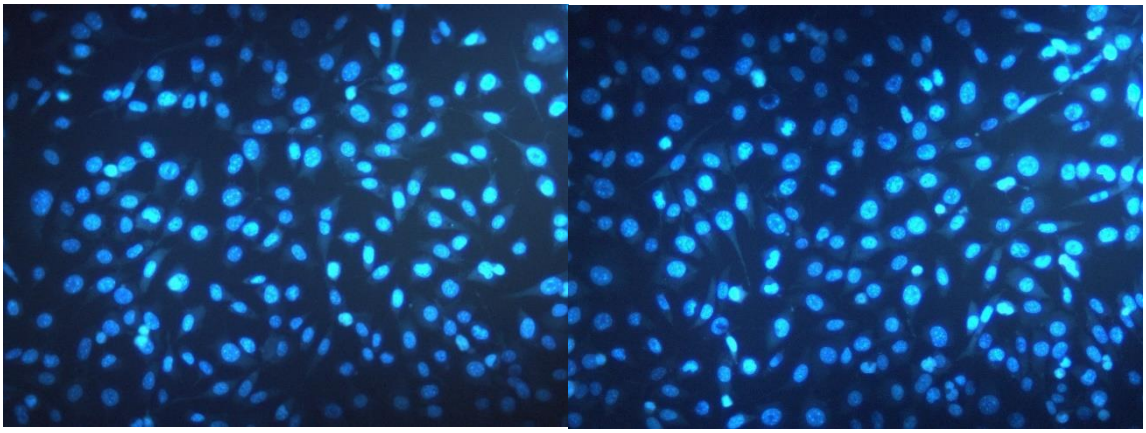


Figure 2: Nuclear Morphologies of HDFa Cells Treated with Different Concentrations of DOX, Visualized Using Hoechst 33258 Fluorescent Nuclear Staining. a- Negative Control (only cell culture), b- Magnetic field-applied (1 mT) cells, c- DOX (5 µg/ml) applied cell culture, d- DOX (5 µg/ml) and Magnetic field (1 mT) applied cell culture

Notably, when DOX was administered in conjunction with a magnetic field (MF), a more pronounced decrease in cell viability was observed. Under the same DOX concentrations, the viability of U87MG cells further declined to 65.2% ± 2.8% at 5 µg/ml DOX + MF, 42.8% ± 3.4% at 5 µg/ml DOX + MF, and 21.5% ± 2.9% at 10 µg/ml DOX + MF (Figure 3). These findings suggest that the presence of a magnetic field significantly enhances the cytotoxic effect of DOX on glioblastoma cells ($p < 0.05$). The observed increase in cytotoxicity in the presence of MF indicates that the magnetic field may enhance DOX efficacy through several potential mechanisms, including increased cell membrane permeability, enhanced drug uptake, or modulation of cellular stress responses. The data suggest that MF-assisted chemotherapy could be a promising strategy to potentiate the effects of chemotherapeutic agents like DOX in glioblastoma treatment. However, further molecular and biochemical analyses are required to elucidate the precise mechanisms underlying this effect. Investigating key apoptotic markers, oxidative stress indicators, and intracellular drug accumulation levels could provide deeper insights into how MF influences DOX-induced cytotoxicity in glioblastoma cells.

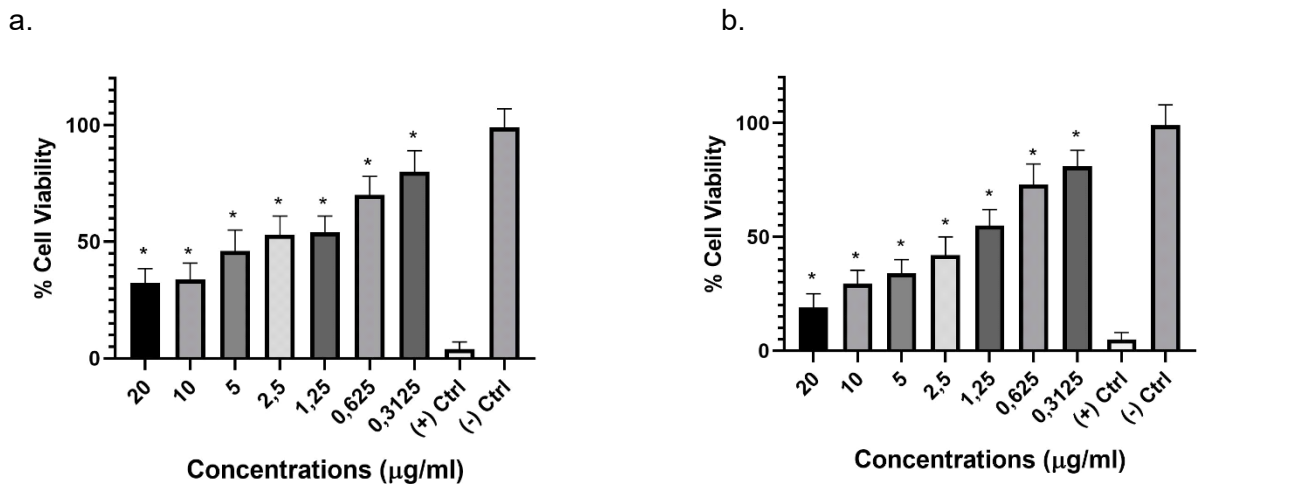


Figure 3. Cell viability analysis of U87MG cells treated with different concentrations of DOX for 24 hours. a- Cytotoxicity observed without a magnetic field showed a dose-dependent decrease in cell viability. b- In the presence of a magnetic field, DOX-induced cytotoxicity was significantly enhanced, suggesting that the magnetic field increases the drug's effectiveness in glioblastoma cells

In this study, the cytotoxic effects of DOX on human glioblastoma (U87MG) cells were evaluated using the MTT assay under two conditions: with and without a magnetic field (MF). The half-maximal inhibitory concentration (IC_{50}) values were calculated to quantify DOX's cytotoxic potential in both conditions. The results demonstrated that in the absence of a magnetic field, the IC_{50} value of DOX was determined to be 3.22 μg/mL, indicating the concentration required to reduce cell viability by 50%. However, when a magnetic field was applied, the IC_{50} value significantly decreased to 2.18 μg/mL (Figure 4). This reduction in IC_{50} suggests that the presence of a magnetic field enhances the cytotoxic efficacy of DOX, allowing for a similar level of cell death at lower drug concentrations. The observed increase in DOX efficacy under MF conditions suggests that the magnetic field may facilitate enhanced drug uptake, altered intracellular distribution, or modulation of cellular stress response mechanisms, thereby potentiating DOX's cytotoxic effects. These findings support the potential use of magnetic field-assisted chemotherapy as a strategy to enhance the effectiveness of chemotherapeutic agents while potentially reducing the required dosage and associated side effects. Further molecular and mechanistic studies are needed to fully elucidate the underlying interactions between MF exposure and DOX cytotoxicity in glioblastoma cells.

a. b.

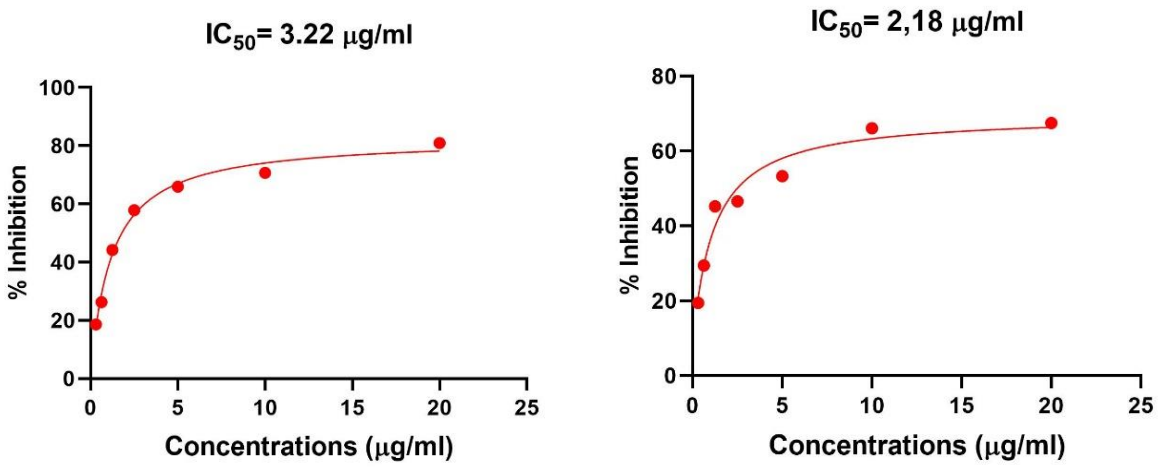


Figure 4. The IC50 value of DOX (DOX) in U87MG cells was a- 3.22 µg/mL without a magnetic field and decreased to b- 2.18 µg/mL with a magnetic field.

Flow cytometry analysis was conducted to evaluate the effects of DOX, applied at two different concentrations (5 µg/ml and 10 µg/ml), on the cell death mechanisms of the human glioblastoma cell line (U87MG), both in the presence and absence of a magnetic field. The results indicate that DOX induces cell death in a dose-dependent manner. In the absence of a magnetic field, treatment with 5 µg/ml DOX increased apoptotic and necrotic cell populations, with **early apoptotic cells increasing from 5.4% (control) to 18.7%,** and **late apoptotic cells rising from 7.2% to 22.4%.** Similarly, necrotic cell percentages showed a modest increase from **3.8% to 9.6%.** At a higher concentration of 10 µg/ml, apoptotic and necrotic cell death was more pronounced, with **early apoptosis reaching 29.1%, late apoptosis increasing to 35.8%,** and **necrosis rising to 15.2%.** When a magnetic field was applied in combination with DOX, a more significant increase in cell death was observed. Under these conditions, treatment with 5 µg/ml DOX resulted in **early apoptotic cell percentages of 25.3%, late apoptosis at 30.5%,** and **necrosis at 12.7%,** indicating a statistically significant enhancement compared to the non-magnetic condition. Similarly, at 10 µg/ml DOX, the presence of a magnetic field further amplified apoptotic cell death, with **early apoptotic cells reaching 38.4%, late apoptotic cells increasing to 44.2%,** and **necrotic cells rising to 19.6%**. These findings are illustrated in **Figure 5.** The observed enhancement in apoptotic cell percentages when DOX was combined with a magnetic field suggests that the magnetic field may potentiate cell death mechanisms and improve the therapeutic efficacy of the chemotherapeutic agent. This effect could be attributed to alterations in cell membrane permeability, increased production of reactive oxygen species (ROS), or modifications in drug uptake mechanisms.

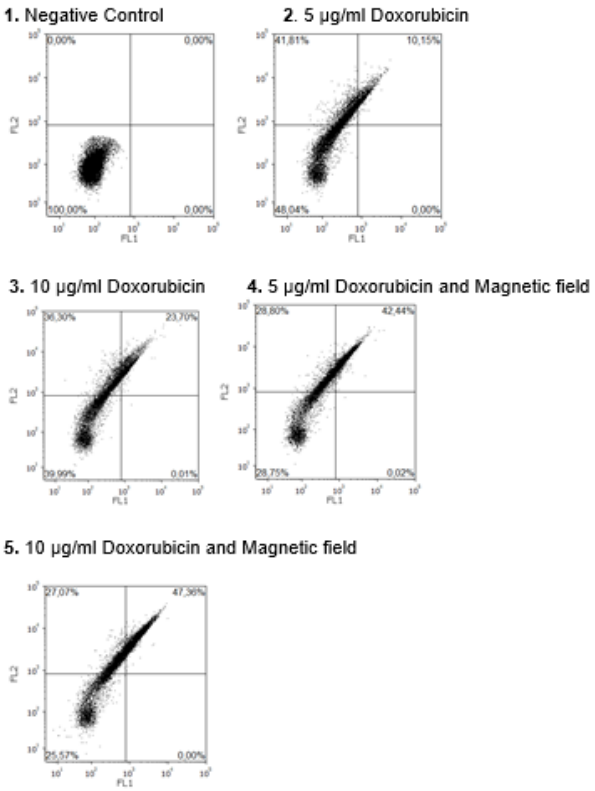


Figure 5: Flow Cytometry Analysis of Changes in Cell Death Mechanisms Following the Application of DOX (5 µg/ml and 10 µg/ml) and a Magnetic Field in the Human Glioblastoma (U87MG) Cell Line

Discussion

In the present study, it was demonstrated that the application of a low-frequency magnetic field (LF-MF) significantly enhanced the cytotoxic efficacy of DOX (DOX) in U87MG glioblastoma cells. While LF-MF alone did not induce marked cytotoxicity, its combination with DOX led to a significant decrease in cell viability, an increase in apoptotic cell populations, and a reduction in the IC_{50} value of DOX, indicating a synergistic interaction between the two modalities. These findings are consistent with a growing body of evidence suggesting that LF-MFs can act as adjuvants to chemotherapy, sensitizing cancer cells to drug-induced cytotoxicity. Previous studies have shown that LF-MFs can independently inhibit cancer cell proliferation by inducing oxidative stress and apoptosis. It was shown that exposure of breast cancer cells to a 1 mT LF-MF (50–275 Hz) caused G0/G1 cell cycle arrest and elevated ROS production, ultimately leading to suppressed proliferation (19). Similarly, it was demonstrated that spinning LF-MF exposure resulted in increased superoxide production and selective glioma cell death while sparing normal neurons and glial cells (20). Moreover, pulsed magnetic fields (20 mT) were reported to induce physical disruption of cancer cell membranes without affecting normal cells, suggesting a tumor-selective membrane destabilization effect (21).

It was shown in recent *in vitro* studies that exposure to LF-MF enhances the intracellular accumulation of chemotherapeutic agents, possibly by increasing membrane permeability or modulating ion channel activity. In particular, one study indicated that brief LF-MF exposure significantly increased DOX uptake in breast cancer cells by activating calcium-permeable *TRPC1* channels, ultimately leading to higher intracellular drug levels and greater cytotoxicity. The decrease in IC_{50} observed in our study (from 3.22 to 2.18 µg/mL) similarly supports the hypothesis that magnetic field exposure facilitates more efficient drug delivery or retention within glioblastoma cells. Our results also align with the literature reporting synergistic effects when LF-MF is combined with chemotherapy. In previous *in vitro* models, it was shown that LF-MF significantly enhanced DOX cytotoxicity in MCF-7 breast cancer cells, reducing the IC_{50} from ~2 µM to ~0.25 µM and increasing apoptotic cell fractions (22). In our study, a 1 mT LF-MF reduced the IC_{50} of DOX from 3.22 to 2.18 µg/mL (67% decrease), supporting the notion that LF-MF enhances drug sensitivity in glioblastoma. Likewise, it was demonstrated that static magnetic fields (10 mT) prolonged DOX-induced ROS in MCF-7 cells, overcoming GSH-mediated resistance and triggering oxidative damage beyond repair thresholds (23). Mechanistically, increased ROS generation appears to be a key contributor to the observed synergy. In our

study, as in earlier findings, the combination treatment led to greater oxidative stress and cell death compared to DOX alone. It was also previously shown that LF-MF can amplify DOX-induced oxidative damage, and quenching ROS with antioxidants mitigated this effect in glioma cells (20).

In addition to drug uptake, oxidative stress appears to be a critical component of the synergistic mechanism. It was previously demonstrated that magnetic fields can elevate reactive oxygen species (ROS) production, exacerbating oxidative damage induced by chemotherapeutic agents. Our results align with these observations, as the combination of LF-MF and DOX caused a marked increase in apoptosis compared to DOX treatment alone. This effect may be attributed to a ROS-mediated amplification of mitochondrial dysfunction and DNA damage pathways, both of which are known targets of DOX-induced cytotoxicity. Supporting this, earlier studies showed that ROS accumulation under combined LF-MF and drug exposure surpasses the threshold for cancer cell survival, resulting in enhanced apoptotic death. Another contributing factor is enhanced drug uptake. It was reported that magnetic fields can alter membrane permeability and transport, enabling more efficient DOX entry into cancer cells. For example, short-term LF-MF exposure was shown to stimulate TRPC1-mediated calcium influx and vesicular DOX uptake in breast cancer cells, doubling intracellular DOX levels (24). Although intracellular DOX accumulation was not directly quantified in our study, the enhanced cytotoxic response suggests that similar uptake mechanisms may be involved. Moreover, our study also confirms that LF-MF exerts minimal effects on healthy cells. HDFa fibroblasts did not exhibit increased cytotoxicity or morphological changes under LF-MF and DOX co-treatment. This is supported by other studies showing that normal cells, including endothelial and muscle cells, are relatively resistant to magnetic field-induced damage, which has been attributed to differences in membrane composition and basal ROS levels (21,24)

Importantly, the selective sensitivity of glioblastoma cells to LF-MF-assisted chemotherapy observed in our study is consistent with previous reports indicating that normal cells are less affected by magnetic field exposure. It was previously shown that healthy fibroblasts and neuronal cells maintained their viability under LF-MF, even in the presence of chemotherapeutic agents. Our findings confirm this selectivity, as HDFa cells showed no significant change in viability or morphology under LF-MF and DOX treatment. This suggests that LF-MF may offer a therapeutic advantage by preferentially sensitizing malignant cells while sparing healthy tissue, thereby potentially reducing off-target toxicity in clinical settings. While our findings corroborate much of the recent literature, it must be noted that certain studies have reported limited or no synergy, especially under conditions of weak field strength or short exposure duration. For instance, it was shown that 50 Hz exposure at 100 μT for 3 h failed to enhance DOX efficacy in MCF-7 cells, despite inducing redox changes (25). This highlights the importance of optimizing LF-MF parameters to achieve a synergistic effect. In light of the findings, in line with recent data showing that appropriately applied LF-MFs can selectively sensitize glioblastoma cells to DOX through a combination of ROS-mediated apoptosis and potentially increased drug uptake. These findings support the continued exploration of LF-MF as a non-invasive adjuvant strategy in glioblastoma therapy, with the potential to enhance therapeutic efficacy while minimizing systemic toxicity. The synergistic interaction observed in our study also appears to be frequency and intensity dependent. In earlier work, insufficient field strength or exposure duration was found to yield minimal enhancement of drug efficacy. In contrast, field

intensities ranging from 1 to 10 mT, as employed in our study, have consistently been associated with increased chemotherapy sensitivity and apoptosis in various tumor models, including glioblastoma, breast, and lung cancer cells.

All in all, our findings demonstrate that low-frequency magnetic field (LF-MF) exposure significantly enhances the cytotoxic efficacy of doxorubicin (DOX) in U87MG glioblastoma cells, primarily through mechanisms involving increased oxidative stress and potentially augmented drug uptake. While LF-MF alone exhibited minimal cytotoxicity, its combination with DOX markedly reduced cell viability, elevated apoptotic rates, and lowered the IC_{50} value of DOX, indicating a synergistic interaction. Importantly, this effect appeared to be selective for cancer cells, as healthy fibroblasts remained unaffected under the same treatment conditions. These results align with and expand upon existing literature suggesting that LF-MFs can serve as effective adjuvants in chemotherapy by sensitizing malignant cells while sparing normal tissue. The observed synergy was found to be dependent on field strength and exposure parameters, underscoring the importance of optimizing LF-MF application in future studies. Overall, this study supports the therapeutic potential of LF-MF-assisted chemotherapy as a non-invasive and selective strategy for enhancing treatment outcomes in glioblastoma. Existing literature supports the notion that LF-MFs can augment ROS levels and influence calcium signaling, thereby enhancing chemotherapeutic efficacy. For instance, Hajipour Verdom et al. demonstrated that a 10 mT static magnetic field increased ROS generation, amplifying DOX-induced cytotoxicity in MCF-7 breast cancer cells. Additionally, the interplay between calcium signaling and ROS has been implicated in cancer cell apoptosis, suggesting that LF-MFs may modulate these pathways to potentiate DOX effects (23). To strengthen the conclusions of the current study, future investigations should incorporate direct assessments of ROS levels, drug uptake, and calcium signaling. Such measurements would provide a clearer understanding of the mechanisms by which LF-MFs enhance DOX efficacy, aligning the study's findings with established literature and offering more definitive insights into potential therapeutic application.

Author Contributions: Conceptualization: Hilal Ergene, Murat Aydemir, Mehmet Enes Arslan Formal Analysis: Murat Aydemir, Cihat Aksakal Investigation: Gürkan Berber, Dilara Esra Men, Hatice Karataş Methodology: Gürkan Berber, Dilara Esra Men, Hatice Karataş, Elif Arslan Project Administration: Hasan Türkez, Cihat Aksakal Writing – Original Draft: Elif Arslan, Hilal Ergene Writing – Review & Editing: Mehmet Enes Arslan, Hasan Türkez

Declaration of Interest: The author declares that there is no conflict of interest regarding the publication of this paper.

Funding: This study was supported by Tubitak 2209-A with the project number of 1919B012317274.

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Eurasian Journal of Molecular and Biochemical Sciences

Antibiofilm Potential and Chemical Characterization of Crude Methanolic Extracts from *Astragalus gummifer*

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Cite: *Tosunoğlu R, Ertürk A, Coşkun M.K, Yazici A. Antibiofilm Potential and Chemical Characterization of Crude Methanolic Extracts from Astragalus gummifer. Eurasian Mol Biochem Sci 2025;4(1): 37-44*

Received: 7 April 2025, Accepted: 8 july 2025 *DOI: 10.5281/zenodo.15877017*

Abstract

Astragalus species are widely recognized for their potent bioactive properties; however, the antibiofilm potential of *Astragalus gummifer* remains unexplored. In the present study, we assessed the antimicrobial and antibiofilm activity of A. gumnifer metabolic extracts against referenced bacterial and Candida strains. Plant material was collected from the Palandöken Mountain region in Erzurum, Turkey. The crude extract was obtained by methanol extraction and concentrated at 62 °C, followed by dissolution in distilled water for microdilution and crystal violet assays. In addition to these assays, untargeted metabolite profiling was performed on the methanolic extract. Fourier Transform Infrared Spectroscopy (FT-IR) and Quadrupole Time-of-Flight Liquid Chromatography–Mass Spectrometry (Q-TOF-LC/MS) analyses were employed for compound identification. A total of nine known secondary metabolites were successfully identified in the crude extract of *A. gummifer*.

Keywords: *Astragalus gumnifer*, Biofilm, Qtof lcms, Kristal violet

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Introduction

Bacterial biofilms are complex microbial communities encased in a self-produced extracellular polymeric substance (EPS) that adhere to surfaces. Unlike planktonic (free-floating) bacteria, biofilm-forming bacteria exhibit enhanced resistance to antibiotics, disinfectants, and the host immune system, making them a major concern in medical, industrial, and environmental settings [1], [2], [3]. Biofilms are responsible for a wide range of persistent infections, such as chronic wounds, dental plaque, catheter-associated infections, and implant-related complications [4]. Additionally, they contribute to industrial biofouling and water contamination, leading to significant economic and health challenges [5]. Understanding biofilm formation and developing effective strategies to inhibit or disrupt these structures is crucial for improving infection control and industrial hygiene [6], [7], [8]. Plants produce many metabolites important for human health [8], [9], [10]. Some of these plants are the Astragalus species that have gained importance in recent years. The flora of Turkey includes more than 440 species of the Astragalus genus, 211 of which are known to be endemic [11]. Astragalus species are used in various ways. Because of their high nutritional properties, some astragalus species are used as animal feed. Astragalus species that are not eaten by animals play an important role in preventing soil erosion. In addition, some astragalus species are uprooted and used as fuel, while others are preferred as ornamental plants due to their aesthetic appearance and beautiful flowers [12]. Astragalus species attract attention not only with their industrial use but also with their immune system modulatory, antioxidant, anti-inflammatory, hepatoprotective and neuroprotective properties. Studies show that this plant supports the immune system, delays cellular aging and may positively affect cardiovascular health [13]. Many of the Astragalus species can also be consumed by humans due to their antiviral and liver protective properties. The Astragalus gummifer, which grows in the mountains of Eastern Anatolia, especially Turkey and Iran, in high altitude and arid regions is a remarkable medical plant. It is a perennial plant belonging to the Fabaceae (legume) family, distributed across the arid and semi-arid regions of Turkey. When the chemical composition of A. gummifer is examined, it is known that there are many secondary metabolites such as saponins, flavonoids, polysaccharides, phenolic compounds and alkaloids. However, studies investigating the effects of Astragalus species on biofilm formation are quite limited, and this indicates that more research is needed to evaluate the antibiofilm potential of A. gummifer [9], [14], [15]. In the light of this information, the antibiofilm activity of A. gumnifer extract against pathogenic microorganism was shown for the first time in the present study. Moreover, chemical structure was investigated.

Material and methods

Chemicals

All chemicals used in the study, such as ethanol, crystal violet dye, phosphate buffered saline (PBS), Mueller-Hinton Agar (MHA), broth (MHB) and potato dextrose agar (PDA) were of analytical grade and were purchased commercially. Extraction of Astragalus gummiferFresh Astragalus gummifer was collected from Palandöken Mount, Erzurum city in Turkey. The plant (leaves and petiole) was air dried. Then, 20 g of plant parts were added to 99% methanol for 72 hours for maceration. It was then filtered through whatman paper. The methanolic extract obtained was evaporated with the help of a rotary evaporator (Scilogex, RE100 Pro) at 62℃. The remaining extract was dissolved in 1 mL of sterile dH2O.

Bacterial and Yeast Culture Conditions

Four bacterial strains (Pseudomonas aeruginosa PAO1 and ATCC 27853 Escherichia coli (ATCC 25922), Staphylococcus aureus (ATCC 25923) and

Enterococcus faecalis (ATCC 29212)) and four yeast strains (Candida albicans ATCC 90028, C. tropicalis KUEN 1025, C. parapsilosis ATCC 22019 and C. dubliniensis CBS 7987) were used to test antimicrobial and antibiofilm activity assay. Bacteria and yeast strains were grown on MHA and PDA medium, respectively.

Microdilution Assay

The microdilution assay was conducted using modified EUCAST protocol [16]. In brief, an overnight culture was grown in medium at 37°C with continuous shaking at 150 rpm. After incubation, the final inoculum was adjusted to an optical density of 0.08–0.1 at 600 nm. In a 96-well plate, 100 µL of the extract at increasing concentrations (0.5–512 mg/L) was combined with 100 µL of the microbial culture, bringing the total volume to 200 µL. Medium served as the negative control. The plates were then incubated under static conditions at 37°C for 24 hours. The minimum inhibitory concentration (MIC) was determined as the lowest concentration at which no microbial growth was observed. The results were determined by taking the averages of three repeated experiments.

Crystal Violet Assay

The antibiofilm activity was assessed using a modified crystal violet (CV) assay [17]. Initially, microbial cultures were prepared in a 96-well plate following a similar procedure to the microdilution test. After 48 hours of incubation, non-adherent cells were discarded, and the wells were rinsed with sterile water. Subsequently, 0.1% crystal violet dye was added to each well and allowed to stain for 20 minutes. Excess dye was then removed, and the wells were washed thoroughly with tap water. Finally, 30% acetic acid was added to dissolve the bound dye, and absorbance was measured at 590 nm using a spectrophotometer (Thermo, Multiscan). All measurements were performed in triplicate, and the results are expressed as the mean ± standard deviation.

Fourier-Transform İnfrared Reflectance Spectroscopy (FT-IR)

FT-IR Attenuated total reflectance (ATR) spectroscopy (Bruker VERTEX 70V) was used to characterise the crude extract. The spectra were calculated in the frequency range of 500-4000 cm-1 [18].

Quadrupole Time-of-Flight Liquid Chromatography Mass Spectrometry (Q-TOF-LC/MS)

The crude extract was characterised using Q-TOF-LC/MS system (Agilent Technologies, CA, USA). The gradient system with the mobile phase consisted of solvent A: ultra-pure water/%1 formic Acid and solvent B: formic Acid/acetonitrile. The column temperature was set to 35 °C. The electrospray ionization (ESI) mode was selected using a fragmentation voltage of 90. The gradient profile used in the Q-TOF-LC/MS was taken in Table 1. Compounds of the unknown peaks were investigated using devices library, PubChem and Mass bank [19]

Table 1: The gradient profile used in the Q-TOF-LC/MS separation of crude extract of *Astragalus gummifer*.

Time (min)	Flow Rate (mL/min)	%A	%B
0-1	0,4	90	10
1-15	0,4	80	20
15-18	0,4	100	0
18-24	0,4	80	20

Result

In this study, the antimicrobial and antibiofilm effects of the methanolic extract of the A. gumnifer on bacterial and fungal culture was examined. Interestingly, in our study, the methanolic extract of A. gumnifer was not found to have any lethal or growth inhibitory properties against either bacteria or yeast. Therefore, in the microdilution test we performed, no results were obtained up to the value of 512 mg/L. Despite this, crystal violet staining revealed significant antibiofilm activity against S. aureus, E. coli, C. albicans, and C. parapsilosis. At a concentration of 8 µg/mL, the extract inhibited biofilm formation by 66% in S. aureus and 70% in E. coli. Similarly, at 64 µg/mL, biofilm inhibition rates reached 83% for C. albicans and 86% for C. parapsilosis. These results are illustrated in Figure 1. Notably, no antibiofilm activity was detected against two clinical strains of P. aeruginosa, or against C. dubliniensis and C. tropicalis.

FTIR-ATR spectra provide information about the chemical characteristic functional groups of crude extract [20]. The absorbance wavelength graph of the FTIR result is presented in figure 2. The peak at 1074-1112/cm was ascribed to C-N stretching amine. The peak at 1116,49/cm was attributed to C=C stretching. The peak at 1651/cm was attributed C=N stretching imine/oxime or C=O stretching conjugated ketone or alkenes. The peak at 2947/cm was ascribed to CH3 asymmetry stretching. The peak at 3338/cm was was attributed ıntermolecular bonded alcohol O-H stretching.

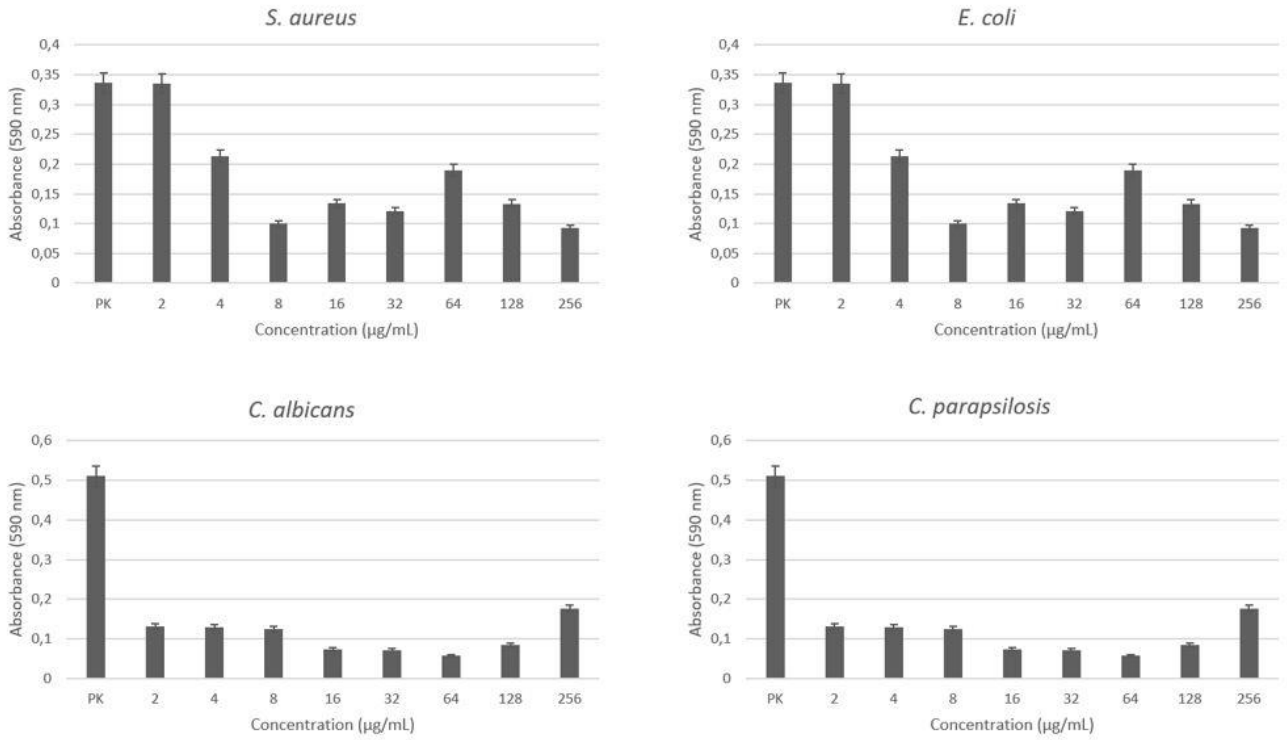

Figure 1. Antibiofilm activity of methanolic extract of A. gumnifer against S. aureus, E. coli, C. albicans and C. parapsilosis. Results obtained from the average of three replicates. Standard error was taken in the results. PK is the group that contains only cells, without astragalus extract.

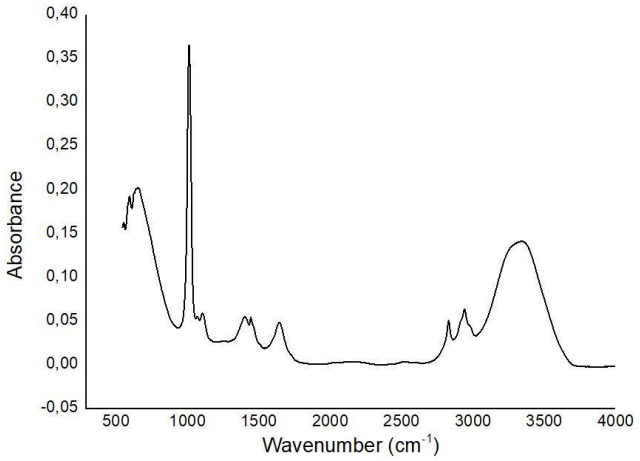

Figure 2. FTIR analysis of methanolic extract of *A. gumnifer*

Q-TOF-LC/MS analyses were carried out to determine the molecular weight and possible structure of the compounds in the crude extract [19], [21] As an outcome of the analyses, the most abundant peak was 871,57232 m/z. The library search revealed that this peak resembles Pheophytin a. Pheophytin a is a derivative of chlorophyll. It is found in plants, algae, and cyanobacteria and is formed when the magnesium (Mg) ion in chlorophyll a is replaced by hydrogen (H) ions. The other most abundant peak was m/z. 607.28967 m/z. The library search revealed that this peak resembles Euphornin. It is an alkaloid of plant origin. According to the library screening, the other most abundant peak, 326.22906 m/z, is understood to be a compound of aromatic origin. Figure 3 displays the m/z data acquired from the ESI ion scan of the crude extract analysis. Nonetheless, further studies are needed for comprehensive characterization.

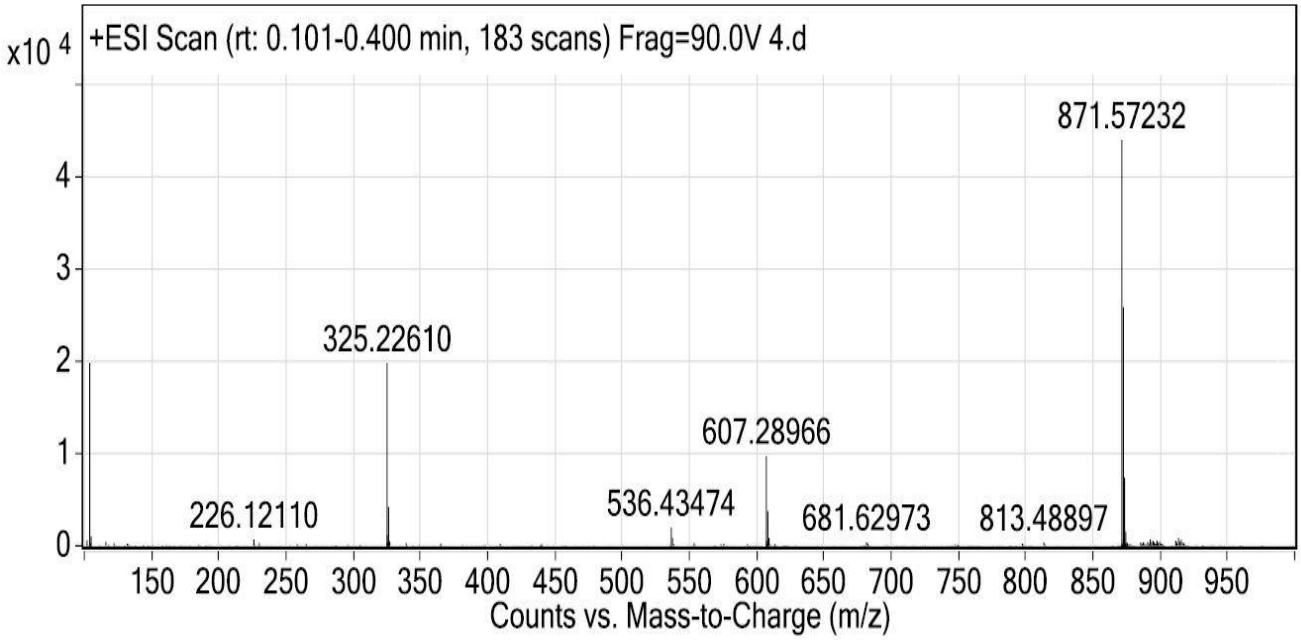

Figure 3: ESI-MS positive ionization for the major peaks in A. gummifer crude extract

Table 2: Chemical Compounds identified in the A. gumnifer extract through Q-TOF-LC/MS analysis.

Compounds	Molecular Formula	ions	m/z	Score
Pheophytin a	C55 H74 N4 O5	(M+H)+	871.57239	97,82
Glabrin C	C41 H64 N8 O9	(M+H)+	813.48901	62,45
Euphornin	C33 H44 O9	(M+Na)+	607.28967	94,1
Desmethylmaprotiline glucuronide	C25 H29 N O6	(M+H)+	440.20584	76,51
Histidyl-histidyl-phenylalanine	C21 H25 N7 O4	(M+H)+	440.20584	76,26
Linalyl anthranilate	C17 H23 N O2	(M+Na)+	296.16275	46,03
alpha-Terpinyl anthranilate	C17 H23 N O2	(M+Na)+	296.16275	46,03
1-(p-Methoxyphenyl)-5-piperidino-1-penten-3-one	C17 H23 N O2	(M+Na)+	296.16275	46,03
Alpha-Pyrrolidinopropiophenone	C13 H17 N O	(M+Na)+	226.12109	82,69

Discussion

Plants produce a wide variety of metabolites that enable their survival and adaptation to environmental stress factors. These compounds are generally divided into two groups: primary and secondary metabolites. Primary metabolites are directly involved in essential physiological processes such as growth, development, and reproduction, while secondary metabolites mostly function in environmental interactions [22], [23]. Secondary metabolites include various structural groups such as alkaloids, flavonoids, terpenoids, phenolic compounds, and glycosides. These compounds fulfill many vital roles in plants, such as defense against herbivores, resistance to pathogens, protection from ultraviolet radiation, competitive advantage, and signaling. Moreover, many secondary metabolites are of great importance to humans, as they are widely used in drug development, food additives, cosmetic products, and agricultural biotechnology Therefore, a detailed understanding of the structural diversity and biological functions of secondary metabolites is crucial for both basic science and applied research. In the present study, we initially investigated the antimicrobial and antibiofilm properties of A. gummifer collected from Erzurum Palandöken Mountain. Locally known as 'geven', A. [24], [25], [26]gummifer was first evaluated for antimicrobial activity using agar well diffusion (data not shown) and microdilution assays. No antimicrobial activity was observed against the reference microorganisms tested in this study. This could be attributed to the insolubility of the antimicrobial compounds in the methanolic extract. Another possible explanation is the degradation of compounds in the extract at 62°C. Additionally, the timing of plant collection might have influenced the results, as the plant materials used in this study were collected during September and October [27], [28].

Although the crude extract of A. gumnifer had no antimicrobial results, interestingly it showed antibiofilm activity against some bacterial and yeast strains. This situation suggests that antimicrobial and antibiofilm properties are independent of each other. In fact, there are many studies addressing this issue. The antibiofilm property is attributed to the prevention of microorganism adhesion to surfaces without killing them. In this context, A. gummifer extract may have

inhibited the quorum sensing mechanism or interfered with the motility mechanisms responsible for initial adhesion [28], [29], [30], [31]. Inhibition of biofilm is very valuable in drug-resistant strains such as C. albicans and S. aureus. Slightly different from this observation, methanol extracts of Astragalus membranaceus enriched with flavonoid fractions have been shown to exhibit potent antibacterial and antibiofilm activity against Bacillus cereus. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were reported as 1.56 mg/mL and 6.25 mg/mL, respectively, and biofilm formation was significantly suppressed as shown by crystal violet test [32].

Destroying the biofilms is as important as inhibiting pathogens. In fact, 80% of infectious diseases today are caused by biofilms. The fact that biofilms are so effective necessitates the discovery of compounds with antibiofilm properties. In the current study, upon analyzing the chemical composition of the crude extract of A. gumnifer, the presence of aromatic compounds and alkanes was identified. Library screening of the Q-TOF-LC/MS results led to the identification of a total of nine compounds. A list of these compounds is presented in Table 2. Upon examination, none of these compounds correspond to previously identified substances known to exhibit antibiofilm properties. This highlights the need for further investigation of the pure forms of these compounds for their potential antibiofilm activity in future studies.

Author Contributions

RT, AE, MKC and AY were made the experiments. AY designed and supervised the experiment. RT and AY contributed to the writing.

Acknowledgements

This study was supported by Tübitak (2209-A).

Declaration of Interest

There is no conflict of interest between the authors.
All authors have read and approved the manuscript.

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Eurasian Journal of Molecular and Biochemical Sciences

Densovirinae: An Eco-Friendly Alternative in Biological Control

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Cite: *Aş Y, Eroğlu G.B., Densovirinae: An Eco-Friendly Alternative in Biological Control. Eurasian Mol Biochem Sci 2025;4(1): 45-55*

Received: 28 March 2025, Accepted: 29 April 2025 *DOI: 10.5281/zenodo.15877070*

Abstract

Entomopathogenic viruses are among the most important biological control agents due to their narrow host spectrum, low *in vivo* production costs, and environmentally friendly properties. In particular, asymptomatic viruses with the potential to cause oral infection are seen as an environmentally sustainable alternative to chemical pesticides in the control of harmful insects. The fact that these viruses do not harm non-target organisms and their ability to protect the natural balance of the ecosystem offer significant advantages in the field of biological control. In addition, their minimal environmental impact contributes to the implementation of sustainable approaches to agricultural pest management. Densoviruses are non-enveloped, single-stranded, linear DNA genome viruses of very small size in the subfamily Densovirinae of the family Parvoviridae that infect only invertebrates. These viruses have been isolated from many insect orders including Blattodea, Diptera, Hemiptera, Hymenoptera, Coleoptera, Lepidoptera and Orthoptera. They have also been reported to infect decapod crustaceans and echinoderms. Although the discovery of densoviruses dates back some sixty years, their use as biological control agents was not seriously considered until it was demonstrated that these viruses do not infect vertebrates. However, *in vivo* and *in vitro* studies in mammals have shown that densoviruses do not infect vertebrates, and this has accelerated research into their potential use in biological control over the last two decades. In this review, the general characteristics of densoviruses and their potential use in biological control are discussed in detail.

Keywords: Entomopathogenic viruses, Parvoviridae, densovirinae, biocontrol agent

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Introduction

Biological control is a control method that has been developed using the natural pressure mechanisms of nature and has a very low probability of harming the environment (1). In this type of control, predators, parasitoids or pathogens are used to reduce the numbers of harmful insects, whose populations have

increased significantly in some years, below the economic damage threshold. While predators and parasitoids in biological control are methods based on the use of beneficial insects against the target organism, pathogens are micro-organisms that cause the target organism to become sick or die. These microorganisms include viruses, fungi, bacteria, protozoa and nematodes, and are bioagents that can reduce pest populations well below the economic damage threshold in a short period of time (2). The main reasons for the widespread use of microbial agents in biological control are that, unlike chemicals, they are host-specific, do not harm non-target organisms, do not leave residues in nature, are environmentally friendly and reliable (3). Microbial insecticides are used effectively to control many harmful insects. The fact that microbial insecticides are not toxic to non-target humans and other living organisms, and that the toxins they produce are generally specific at the species or genus level, makes biological control preferable to chemical control (4). Viruses are the most commonly used of these micro-organisms, and viruses usually enter their hosts orally through the digestive system and cause infection (1). The digestive system of the virus-infected insect is disrupted, many organs are damaged and as a result the insect becomes sick and dies. The main factors limiting the use of entomopathogenic viruses are their narrow host range, the need to replicate in the host and their slow action. One of the most important viruses in the field of microbial control are the members of the family Baculoviridae, which are more resistant to natural conditions (1). Most of the known hosts of baculoviruses belong to the order Lepidoptera, while others belong to the orders Diptera and Hymenoptera (5). Another group of viruses, densoviruses, have a wider distribution and have been isolated from many insect orders, including Blattodea, Diptera, Hemiptera, Hymenoptera, Lepidoptera, Odonata and Orthoptera (6). In addition, recent studies have reported the presence of densoviruses in some Coleoptera species that are storage pests (7-10). Densoviruses, also known as densonucleosis viruses (DNV), are so named because infected cells characteristically develop intensely stained nuclei (11, 12). These viruses, which belong to the subfamily Densovirinae of the family Parvoviridae in the order Piccovirales, are known to be among the smallest DNA viruses with dimensions of 18-28 nm (13-15). DNVs replicate in the nuclei of invertebrate hosts and form large circular inclusions. First identified in 1964 from Galleria mellonella larvae, DNVs have a non-enveloped single-stranded DNA genome, making them more resistant to environmental conditions and distinct from other viruses (8). DNVs offer significant advantages for biological control because they are specific to invertebrates, can be spread by oral infection, are stable, and show distinct morphological symptoms in the host (16).

Classification of densoviruses: The family Parvoviridae, introduced in 1975, was split into two subfamilies in 1993 as Parvovirinae, which infects vertebrates, and Densovirinae, which infects invertebrates (14). This two-subfamily classification has long been supported by phylogenetic analyses, as both subfamilies have a very narrow host spectrum. In 2012, a genus known as Chapparvovirus was introduced, but isolates belonging to this genus have been detected in some vertebrate tissues (kidney and liver), faeces, and even blood (17-23). At the same time, the identification of endogenous chapparvovirus sequences in arthropod genomes has highlighted the need to revise the classification of both the family Parvoviridae and the subfamily Densovirinae (24, 25). Densoviruses have been divided into genera based on the sequence similarity of the NS1 protein, which has been isolated from many insect species and is highly conserved in all densoviruses. If the NS1 proteins have more than 85% amino acid sequence similarity, densoviruses can be considered members of the same

species. In addition, members of the same genus should have at least 35% similarity between their NS1 proteins (26).

As new isolates are identified, revisions are made to the classification of both the Parvoviridae family and the Densovirinae subfamily. According to the latest update of the ICTV, the family Parvoviridae is now divided into three subfamilies (Densovirinae, Parvovirinae and Hamaparvovirinae) and one unclassified genus (Metalloincertoparvovirus) (26). The subfamily Densovirinae is divided into 11 genera in 2020 (Figure 2). According to data from the ICTV taxonomy browser, the 11 genera of the subfamily Densovirinae contain 64 species, and the distribution of these species within the genera is as follows Aquambidensovirus (24 species), Leafambidensovirus(6species),Diciambidensovirus (3species) ,Hemiambidensovirus (3 species), Iteradensovirus (7 species), Miniambidensovirus (1 species), Muscodensovirus(2 species), Pefuambidensovirus(2species),Protoambidensovirus(7species), Scindoambidensovirus(6species) and Tetuambidensovirus (3 species). All genera except Aquambidensovirus have been isolated from insects of these genera. Studies to isolate insects that are harmful to households, agriculture and forestry and to evaluate the usefulness of these isolates for biological control are very valuable.

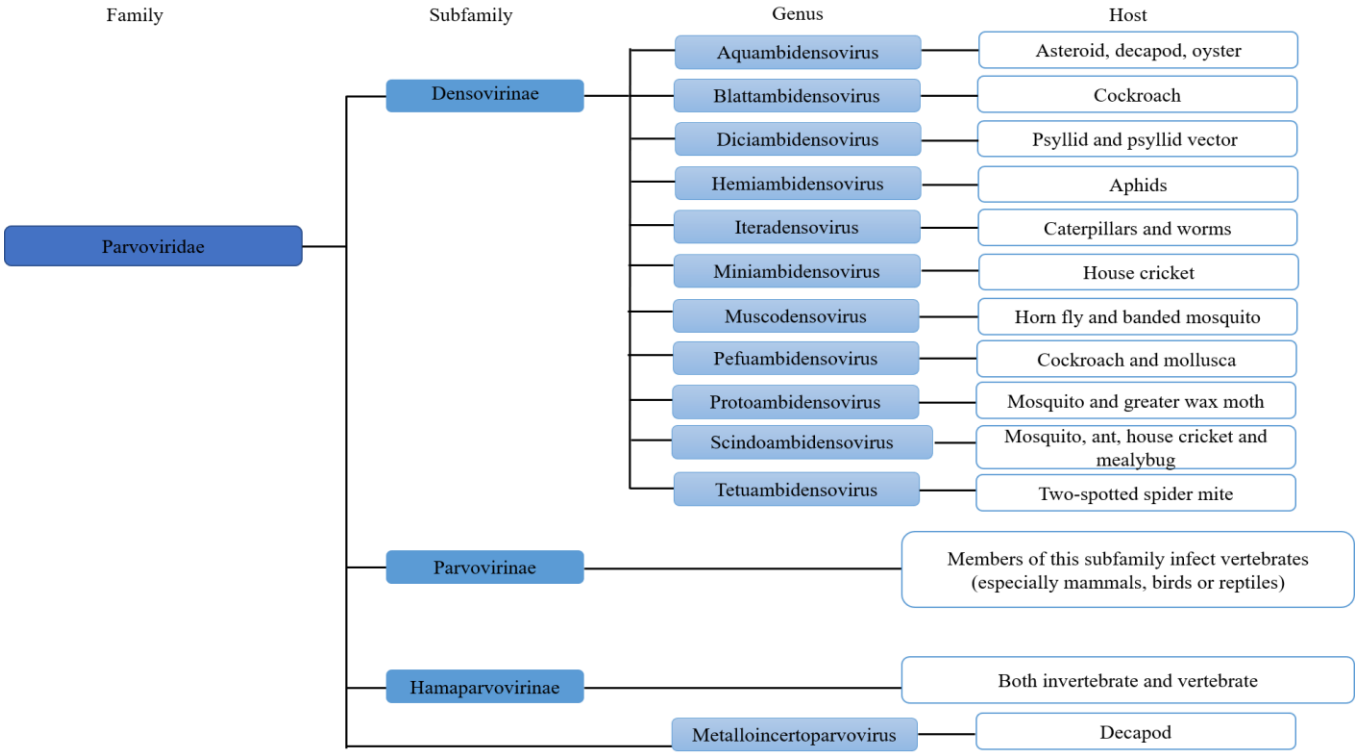

Figure 1: Classification of densoviruses (ICTV taxonomy browser)

Genome organization of densoviruses: DNVs are single-stranded, non-enveloped, single-stranded DNA viruses with icosahedral symmetry and a genome size of approximately 4000-6000 bp (27). All DNVs have a structure consisting of Y-shaped inverted terminal repeats (ITRs), which are required for replication and genome packaging (28-30) (Figure 2a). The molecular mass of DNVs is approximately 6 MDa, of which 20±30% is DNA and the remainder is mainly protein (12). Two types of proteins are encoded in the genome: structural (VP) and non-structural (NS). NS are responsible for the replication of the viral genome and

are considered an important parameter for virus classification, especially since the protein known as NS1 is highly conserved in all DNVs (14, 26) (Figure 2b). Structural proteins are responsible for the formation of the viral capsid (31). All DNV genomes have NS and VP genes equally distributed in the 5' half of each strand (12) (Figure 2c).

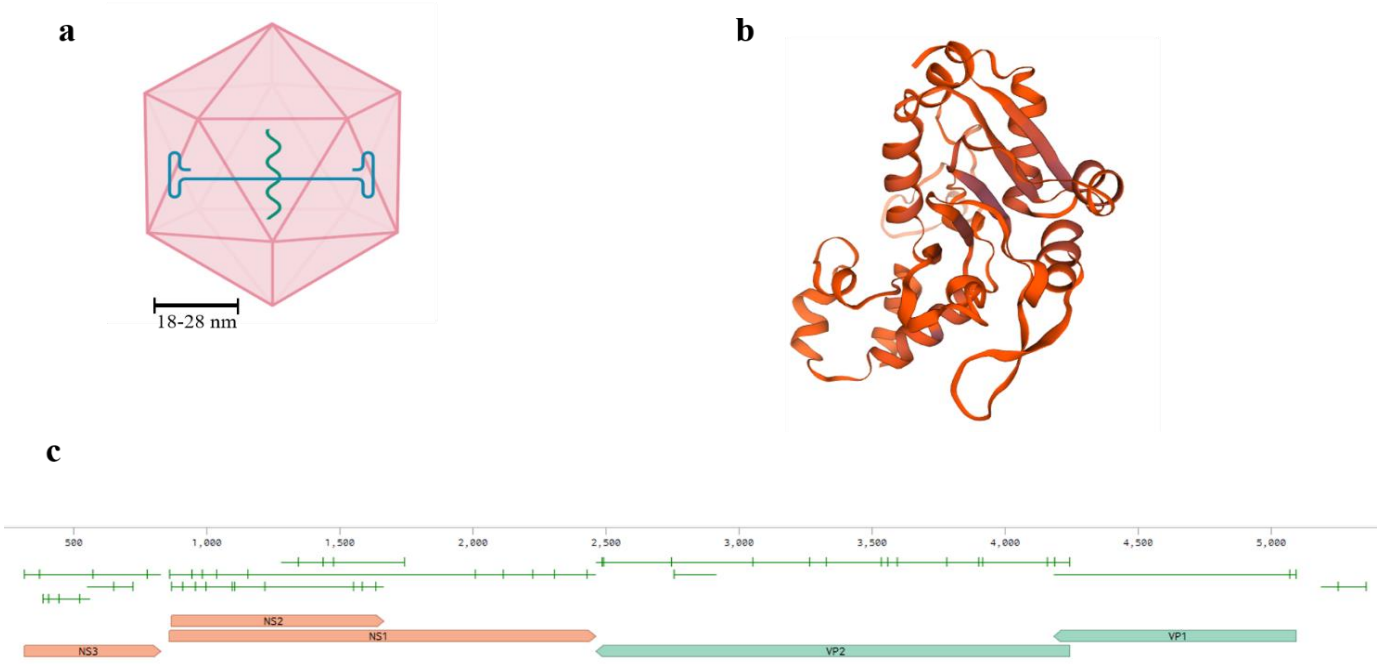

Figure 2: Structure of densoviruses **(a)** Morphological structure of the virus particle with icosahedral capsid symmetry [This figure was drawn using BioRender (https://www.biorender.com)], **(b)** Structure of the NS1 protein [This figure was drawn using SWISS-MODEL (https://swissmodel.expasy.org/)], **(c)** DNV genome map [Benchling online tool using the genome sequence of Tenebrio molitor densovirus- Türkiye (TmDNV-TR) isolate (10)].

The N-terminal extension of the minor capsid protein VP1, the largest VP in DNVs, encodes phospholipase A2 (PLA2), which is required for endosomal exit (2, 16). Other parvoviruses lacking PLA2 (notably members of the Hamaparvovirinae) use an alternative endosomal pathway (32).

Use of densoviruses as gene transfer vectors: Terminal sequences make up 8-18% of the densovirus genome. Since terminal sequences contain all the elements necessary for replication and packaging, the remaining part of the genome is suitable for foreign DNA insertion. In some cases, where viral functions are lacking, a helper plasmid (usually pUCA) containing VP and/or NS genes can be used. In this case, the transducer genome, helper plasmid and trans-producing cells must be incubated long enough to undergo at least one cell division to produce transducer particles. This is because fundamental processes such as gene expression and replication in DNVs are directly linked to host cell division (11, 33). Liu et al. (2017) developed a non-defective recombinant Aedes albopictus densovirus (AaeDV) microRNA (miRNA) expression system. Thus, it was reported that recombinant AaeDVs developed for use in mosquito control can be used to overexpress or reduce the expression of the target gene in larvae (34) (Figure 3).

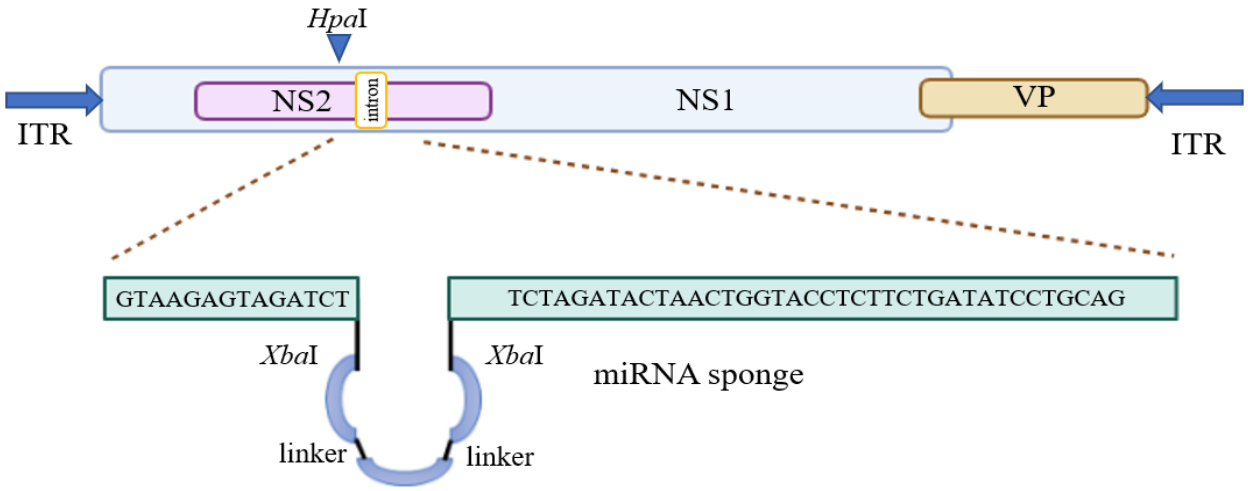

Figure 3. Schematic organization of recombinant AaeDV plasmids [This figure was drawn using BioRender (https://www.biorender.com) (modified from 34)]

Replication of densoviruses: DNVs enter the cell via clathrin-mediated endocytosis and replicate in the host nucleus (13, 35). They enter the cell using various receptors such as glycoproteins, glycans and glycolipids on the cell surface (36-38). After endocytosis, they pass through the endolysosomal system of the host cell and reach the nucleus. During this time, the viral particle is exposed to an acidic environment ranging from pH 7.4 to pH 4.0 (32, 39). Although this acidic environment is known to induce conformational changes in the viral capsid, the mechanism of viral escape from endosomes is not fully understood (35). Once the virus reaches the nucleus, mRNA is transcribed and NS1 initiates replication. It then begins to replicate using the host's S-phase replication mechanism (37). During replication, the hairpin structure is continuously opened and replicated. The rolling hairpin replication process then begins. In this process, it refolds to change the direction of replication to move along the genome. This produces a molecule containing many copies of the genome, and newly formed ssDNAs are removed from this concatemer and packaged into the capsid. Finally, mature virions exit the cell by exocytosis or lysis, terminating the replication process (Figure 4). Vendeville et al (2009) infected the Lymantria dispar 652 cell line with Junonia coenia DNV to determine the early endocytotic steps in DNV infection. According to the data obtained, DNV infection progressed with rapid clathrin-mediated uptake, slow traffic from low pH and late endosomal compartments, and integration of the cytoskeletal network (35).

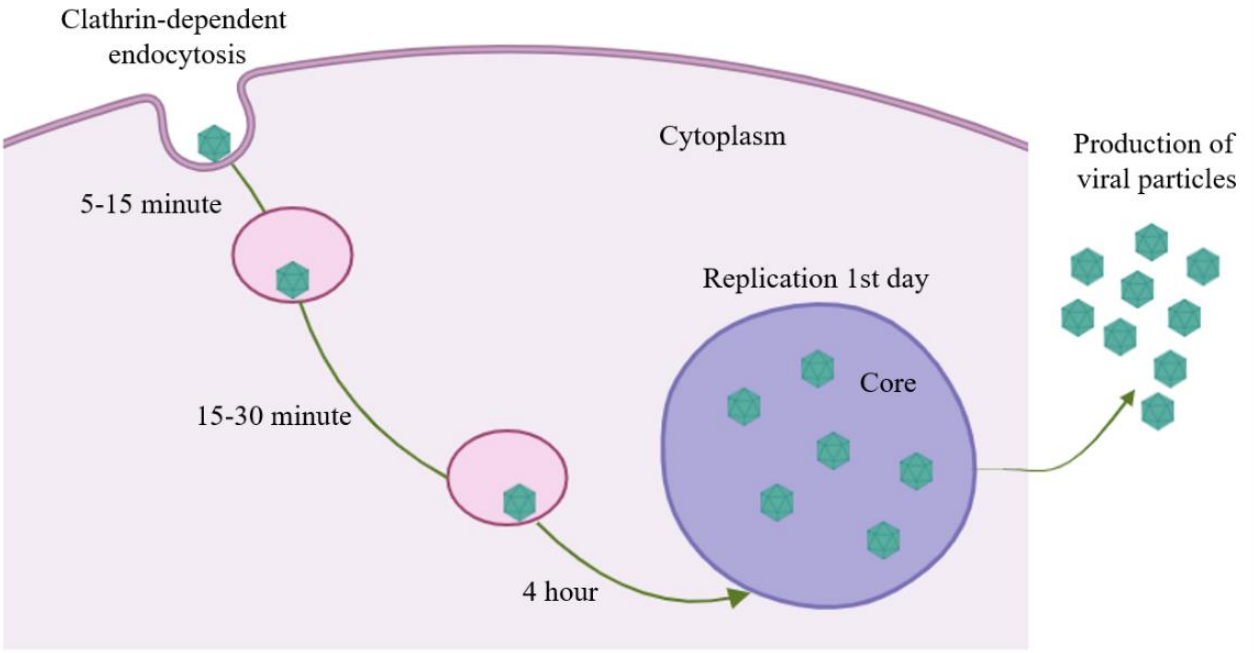

Figure 4. Densovirus replication process [This figure was drawn using BioRender (https://www.biorender.com) (modified from 31)].

Densovirus symptoms in insects: DNV infection is characterized by nuclear hypertrophy caused by the accumulation of large virion particles and cytoplasmic paracrystalline virion arrays in insect tissues (6). DNVs cause severe diseases in their host organisms and are widely used for the biological control of significant insect pests due to their high virulence and transmissibility. Additionally, densoviruses represent an advancement over classical biopesticides, offering a species-specific alternative to traditional insecticides (40). Symptoms of DNV infection typically begin with anorexia and lethargy, followed by limping and molting or inhibition of metamorphosis. Infected larvae gradually turn white and become paralyzed. In cockroaches and house crickets, common symptoms include paralysis of the hind legs and uncoordinated movements (41). DNV infect host larvae, causing a variety of symptoms such as changes in cuticle pigmentation, loss of movement, and death. The severity of parasitism and damage varies significantly depending on the type of infection. The most striking symptom observed in infected larvae is the complete blackening of the body. As a result, densovirus infections, especially in storage pests like *Zophobas morio*, are commonly referred to as "black wasting disease" (42). Prior to this blackening, dark discoloration is observed in the midgut of larvae (27). Some species of densoviruses also induce tumor-like lesions in the intestines of their hosts. Infected larvae exhibit severe deterioration of midgut epithelial cells, accompanied by thickening and opacification of the intestinal wall. These changes result in the clouding of intestinal contents. In larvae infected through feeding, the virus was found to replicate in midgut cells, leading to a pathogenic mechanism that damages the intestinal barrier (43) (Figure 5). The peritrophic membrane is a critical component in insects, serving as a protective lining in the midgut. It acts as a significant barrier, making it difficult and complex for orally ingested viruses to penetrate and initiate infection. A study to investigate how DNV capsids adhere to and cross this barrier to reach target cells used a combined

methodology including microscopy, biochemistry, proteomics and transcriptomics. The results showed that the peritrophic membrane, exposed mucins and non-mucin protein receptors serve as binding sites for DNV and other insect pathogens. This highlights the interaction between the virus and components such as chitin, glycans and proteins as key mechanisms underlying this process (16).

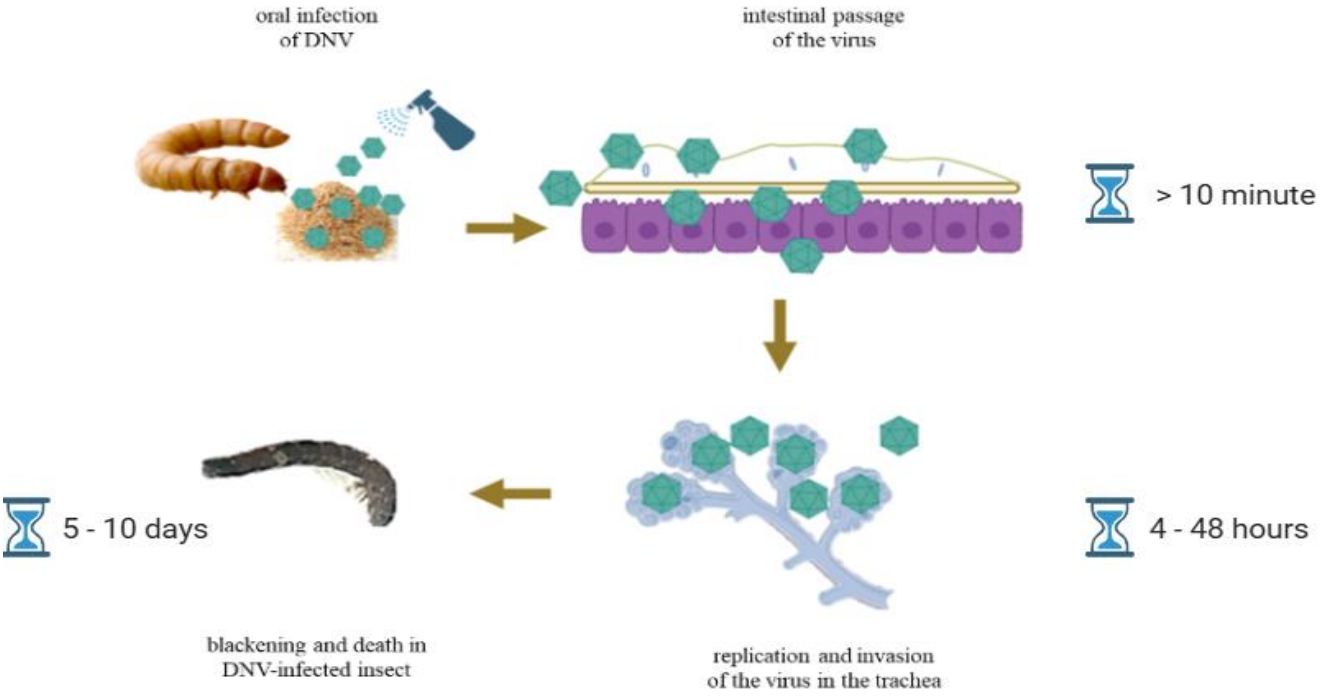

Figure 5: Schematic of densovirus pathogenesis in Tenebrio molitor. The virus is transferred to the larval food and the larva is able to ingest the virus orally. The virus quickly reaches the midgut and crosses the intestinal epithelium. It then targets the tissues, particularly the trachea, where it replicates and causes the larva to blacken and then die. [This figure was drawn using BioRender (https://www.biorender.com) (modified from 31)]

Usability of densoviruses in biological control and host range: The use of insect viruses in biological control has been known since 1911 (44). Isolates of the baculovirus group, particularly DNA viruses, have been commercially available since 1975 (45). To date, 60 baculovirus isolates have been registered and are used as an alternative to chemical pesticides in the control of insect pests (46, 47). Although densoviruses (DNV) were first discovered 60 years ago, they were not considered as candidates for biological control until it was shown that they do not infect vertebrates (12). However, since *in vivo* and *in vitro* studies over the last 20 years have shown that they cannot replicate in vertebrates at all, they are now emerging as an important biological control agent as an alternative to baculoviruses (27, 48, 49). Some characteristics of DNVs, such as high virulence and host specificity, inability to infect vertebrates, and high resistance to extreme environmental conditions, make them potentially effective biological control agents against populations of agriculturally and medically important pests (16, 42). In addition, because densoviruses are DNA viruses and therefore have a

DNA repair mechanism, they do not allow mutations to alter their host specificity (50, 51).

The first study to use densovirus as an insecticide for biological control purposes involved the use of *Galleria mellonella* cadavers infected with GmDNV to control beehives heavily infested with this pest. In this study, the formulation was applied in three doses and after 15 days of infection, 95% mortality was observed at the highest dose and 73% at the lowest dose. One month later, 100% mortality was observed for all three doses. In addition, pest parasites were not affected and contributed to the further spread of the virus (52). In another study, during the survey of arboviruses in China, a new densovirus (DNV) was isolated from adult female *Culex pipiens pallens* (CppDNV), and found to cause cytopathic effects in C6/36 cells (53). The first mosquito-specific densovirus (MDV) was found in *Aedes aegypti* larvae in a Russian laboratory colony in 1972, and to date MDVs have been isolated from many mosquito species, including important disease vectors such as *A. aegypti*, *Aedes albopictus*, *Anopheles gambiae*, *Anopheles sinensis*, *Culex pipiens* and *Culex pipiens* pallans (54). The reason for the large number of studies on densoviruses in the mosquito group is that they act as vectors and can transmit diseases from animal to animal. Another group of insects in which there has been a considerable amount of work on densoviruses are cockroaches (55). Many studies of densoviruses isolated from cockroaches have resulted in new densoviruses being isolated and entered into databases (56, 57). In addition, densoviruses isolated from species of the family Noctuidae (*Spodoptera littoralis*, *Spodoptera frugiperda*, *Pseudoplusia includens*, *Mythimna loreyi* and *Helicoverpa armigera*), an important family of agricultural pests, are also important for the biocontrol of these insects (58-61). In recent years, it has been reported that densovirus isolates have been obtained from these insects of the family Tenebrionidae, known as storage pests, which are reared in culture and sold commercially, but no biotest data are available (7-10). In future studies, biotest studies with these insects have potential importance for the biocontrol of storage pests. To date, there is only one commercial densovirus-based product (Biokiller, China). This product contains a densovirus (PfDNV) isolated from *Periplaneta fuliginosa* and has been developed and sold in gel form for cockroach control for twenty years (62).

As a result of studies on the host specificity of DNVs, it has been shown that GmDNV, CeDNV and AdDNV have a host range limited to their original hosts, whereas other DNVs isolated from Lepidoptera have a wider host range (63-65). This situation is a disadvantage, albeit a minor one, for certain species and limits studies. The host range of DNVs infecting other groups of mosquitoes extends to different species. *A. albopictus*, *Aedes cantans*, *Aedes caspius*, *Aedes geniculatus*, *Aedes vexans*, *Culex pipiens*, etc. among the mosquitoes with a very wide range of species, there are many studies on densoviruses (54). In terms of sensitivity, which is an interesting aspect of the host spectrum issue, *Bombyx mori* is at the top of the list of hosts with the highest sensitivity to densoviruses. Among the economically important silkworm species, a few are susceptible to BmDNV-2 and almost all strains susceptible to BmDNV-1 are resistant to BmDNV-2. Therefore, the mode of inheritance of resistance to BmDNV infection has been investigated and it has been found that insensitivity to each virus is genetically controlled by a recessive gene that is not sex-linked (66). As a result, the host range and specificity of densoviruses vary between insect species.

Mammalian cell toxixity: The Parvoviridae family is a large group of viruses that can infect both vertebrates and invertebrates (26). However, there are uncertainties regarding the host spectrum of the Densovirinae subfamily that infects invertebrates. Although it was reported in the 1960s that densoviruses

(DNV) could infect mouse L cells (67-69), it was later understood that these observations did not indicate viral replication. DNV infection was studied in terms of viral transcription, replication, integration, and production by El Far et al (2004). The findings showed that L cells and other vertebrate cells did not support densovirus replication or transcription, whereas viral DNA replication and transcription occurred in *L. dispar* cells (LD652). It has been reported that the viral genome was removed from the plasmid in LD652 cells and both NS and VP mRNAs were detected, but not in L cells. It has been stated that the viral genome was integrated into the host chromosome in L cells, but transcription did not occur after integration. As a result, it has been shown that densoviruses can perform efficient replication and transcription in insect cells, but cannot initiate infection in mammalian cells. This increases the potential of densoviruses as gene therapy vectors (27).

Result

In today's world, where crop pressure and inadvertent use of pesticides threaten the sustainability of ecosystems, the discovery of innovative biological alternatives has become an urgent necessity. Densoviruses, viruses of the Parvoviridae family, stand out as a promising solution due to their unique mechanisms of action and positive environmental profile in pest management. The use of these viruses in biological control not only allows selective targeting of pest populations, but also contributes to the protection of biodiversity by offering a less chemical-dependent and more sustainable control method. The fact that densoviruses are DNA viruses and have a DNA repair mechanism prevents the viruses from undergoing mutations that would change their host specificity (50, 51). This feature ensures ease of use and avoids any negative impact on the ecosystem. In addition, all densoviruses have a very narrow host spectrum, making them safe to use in biological control. Densoviruses have many advantages, such as being specific to invertebrates, being able to multiply by oral infection, being stable and showing clear morphological symptoms in the host (16). For all these reasons, the use of densoviruses as biological control agents is included in many studies in the current literature, and their use as an effective biological agent can be envisaged in the future.

Author Contributions: Conceptualization: Yasemin Aş, Gözde Büşra EROĞLU Investigation: Yasemin Aş, Gözde Büşra EROĞLU Writing – Original Draft: Yasemin Aş, Gözde Büşra EROĞLU Writing – Review & Editing: Gözde Büşra EROĞLU

Declaration of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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