Erzincan Üniversitesi
Fen Bilimleri Enstitüsü Dergisi
2024,17(1), 164-173
ISSN: 1307-9085, e-ISSN: 2149-4584
Araştırma Makalesi

Erzincan University
Journal of Science and Technology
2024,17(1), 164-173
DOI: 10.18185/erzifbed.1376859
Research Article

The Combined Effect of Vulpinic Acid and Doxorubicin in Breast Cancer MCF-7 Cells

Esma Kübra KAĞAN YENİÇERİ [1], Ahmet ALTAY [2*]

[1] Institute of Science and Technology, Erzincan Binali Yıldırım University, 24030, Erzincan, Turkey
[2]Department of Chemistry, Faculty of Arts and Science, Erzincan Binali Yıldırım University, 24100, Erzincan, Turkey

Received: 16/10/2023, **Revised:** 11/12/2023, **Accepted:** 11/12/2023, **Published:** 28/03/2024

Abstract

Breast cancer is a major global health problem that imposes a significant social and economic burden on individuals and societies. Chemotherapy, a common treatment approach, often leads to resistance and unwanted side effects, especially in the advanced stages of the disease. This has led to a search for more effective and less toxic anticancer agents. Lichens, associations of fungi and algae, are gaining attention for their potential in cancer therapy. Lichens are rich sources of secondary metabolites with diverse biological effects, including anti-tumor properties. In this study, we summarise the combined effects of vulpinic acid a lichen acid, with doxorubicin, a chemotherapeutic drug, on breast cancer MCF-7 cells. The results of the XTT assay and subsequent Compusyn analysis showed that VA and doxorubicin, a chemotherapeutic drug, alone exhibited potent anti-proliferative effects in a dose- and time-dependent manner, and interestingly, when used in combination, they produced an antagonistic effect in the same cancer line. These results provide the first example of a study to show what effect the combination of VA and Dox will have on other breast cancer cell lines.
Keywords: breast cancer, vulpinic acid, doxorubicin, combination effect.

Vulpinik Asit ve Doksorubisinin Meme Kanseri MCF-7 Hücrelerinde Kombine Etkisi

Öz

Meme kanseri bireylere ve toplumlara önemli sosyal ve ekonomik yük getiren önemli bir küresel sağlık sorunudur. Yaygın bir tedavi yaklaşımı olan kemoterapi, özellikle hastalığın ileri evrelerinde sıklıkla direnç ve istenmeyen yan etkilere yol açmaktadır. Bu durum daha etkili ve daha az toksik antikanser ajanlarının araştırılmasına yol açmıştır. Mantar ve alglerin birleşimi olan likenler, kanser tedavisindeki potansiyelleri nedeniyle dikkat çekmektedir. Likenler, anti-tümör özellikleri de dahil olmak üzere çeşitli biyolojik etkilere sahip, zengin ikincil metabolit kaynaklarıdır. Bu çalışmada, bir liken asit olan vulpinik asidin kemoterapötik bir ilaç olan doksorubisin ile meme kanseri MCF-7 hücreleri üzerindeki kombine etkileri araştırıldı. XTT testinin sonuçları ve ardından Compusyn analizleri, VA ve kemoterapötik bir ilaç olan doksorubisinin, yalnız hallerinde doz ve zaman bağımlı olarak güçlü anti-proliferatif etkiler sergilediğini, öte yandan ilginç bir şekilde, kombine halinde kullanıldıklarında aynı kanser hattında antagonistik bir etki sergiledikleri belirlendi. Bu sonuçlar, VA ve Dox kombinasyonunun diğer meme kanseri hücre hatları üzerinde nasıl bir etki yaratacağını gösteren bir çalışmanın ilk örneğini sağlamıştır.
Anahtar Kelimeler: meme kanseri, vulpinik asit, doksorubisin, kombine etki

*Corresponding Author: aaltay2013@gmail.com
Esma Kübra KAĞAN YENİÇERİ, https://orcid.org/0000-0001-8120-8900
Ahmet ALTAY, https://orcid.org/0000-0001-8120-8900

1. Introduction

Cancers are malignant tumors that result from the uncontrolled growth of cells in tissues or organs [1]. Cancer is not only a growing public health problem but also a social and economic burden for societies. Breast cancer is the most commonly diagnosed cancer and the second most common cause of cancer-related death in women after lung cancer [2, 3]. Today, the most common treatments for breast cancer are surgery, radiotherapy, and chemotherapy. Depending on the type and stage of cancer, chemotherapy is used to treat the disease, prevent the spread of the tumor, slow its growth, and improve some symptoms of cancer, and in some cases, chemotherapy is the only treatment option. Some of the chemotherapeutic drugs commonly used for breast cancer treatment are cisplatin, docetaxel, and doxorubicin [4]. The mechanism of action of each chemotherapy drug may be the same and/or different. Doxorubicin (DOX), for example, interacts with DNA to provide its most effective mechanism of action against cancer. Doxorubicin stabilizes the topoisomerase-DNA complex that forms after topoisomerase II cuts the DNA during DNA transcription. This prevents the DNA double helix from recombining, thus preventing DNA from pairing [5]. Although these synthetic drugs, including Dox, are used in the treatment of cancer, they have many undesirable side effects in patients undergoing treatment [6]. For instance, nausea, vomiting, irregular heartbeat, neutropenia, baldness, heart failure, and cardiomyopathy are some of the side effects of doxorubicin [4]. As cancer is one of the most common diseases of our time, and the number of cancer diagnoses is increasing every day, scientists are working hard to discover and develop new drugs with fewer side effects [7]. More than 60% of cancer drugs in use today are developed from natural sources such as plants, bacteria, fungi, and marine organisms [8]. Medically important plants with pharmaceutical properties have been used for centuries by the general public in all developing and developed countries for the treatment of a wide range of diseases [9].

Many of the therapeutic properties of these medicinal plants are due to the secondary metabolites that they contain. Secondary metabolites have many biological effects including antiviral, antibacterial, antifungal, antiprotozoal, anti-herbicidal, mutagenic, antioxidative, antiulcer, antipyretic, anti-inflammatory, and anti-tumor [10]. Besides medicinal plants, there are still many natural resources that have not been adequately explored, including lichens formed by the symbiotic association of fungi and algae [11]. Similar to medicinal plants, lichens have many biological properties, including anti-cancer effects, thanks to the lichen secondary metabolites. Vulpinic acid (VA), one of the lichens' secondary metabolites, is known to have many biological properties including antimicrobial [12], antidiabetic [13], and anticancer activities [14]. Even, our previous study found that VA has significant anti-cancer potential in many cancers, including breast cancer [11]. The drugs used in chemotherapy either become less effective during treatment or cancer cells can develop resistance to these drugs. For this reason, several drugs may be given in combination to make the treatment more effective [15, 16]. Indeed, studies of the combined effects of currently used chemotherapeutic drugs and secondary metabolites with anticancer potential have gained momentum over recent years. While the literature contains studies on the anti-cancer effects of the chemotherapy agents DOX and VA

on breast cancer alone, no data exist on the effect of these two compounds on cancer cells when used together [11]. In this regard, the dose- and time-dependent anticancer effects of the two aforementioned compounds, both alone and in combination at different doses, were comparatively investigated on the breast cancer cell line MCF-7.

2. Material and Methods

2.1. Cell lines and culture

The breast cancer MCF-7 cell line was purchased from the ATCC (American Type Culture Collection, LGC Promochem, UK). The MCF-7 cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich). Basal mediums were supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS) (HyClone), 1% l-glutamine (Thermo Fisher Scientific), 1% penicillin, and streptomycin (Sigma-Aldrich). Cells were maintained in a 5% CO_2 incubator at 37 °C. All the studies were carried out in the biosafety -II cabine (Nuve, Turkey).

2.2. Preparation of chemicals doxorubicin and vulpinic acid.

Vulpinic Acid (VA) was purchased from Cayman Chemicals Company. Doxorubicin (DOX) was purchased from Cell Signaling Technology. The chemicals were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich) as a stock solution and stored at −20 °C until usage. The various concentrations of VA ranging from 5 to 150 µM were performed with the culture mediums. Similarly, the different concentrations of DOX were prepared ranging from 0.05-100 µM with the complete culture medium.

2.3. Cell proliferation assay

The dose- and time-dependent antiproliferative effects of vulpinic acid (VA) and doxorubicin (DOX), alone and in combination, on breast cancer cell lines were investigated using 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay. The principle underlying the assay is that only living cells have an active metabolism and can convert XTT to purple formazan, which has a maximum absorbance of 490 nm [17, 18]. Firstly, the dose- and time-dependent antiproliferative effects of DOX and VA alone on MCF-7 cell was investigated. For this purpose, both cell lines were plated in 96-well plates with 7500 cells per well in 150 µL of medium and kept overnight in a 5% CO_2 incubator at 37°C. This process was performed separately for each time point (24, 48, and 72h). The following day, different doses (0.1, 1, 10, 50, and 100 µM) obtained from the stock solution of Dox were applied to the cell line, and the plates were kept in the 5% CO_2 incubator for each period. The same procedure was performed for VA at varying concentrations depending on the cell type (10, 25, 50, 75, 100, and 150 µM). At the end of each incubation period, the old medium in the wells was removed and the wells were washed with PBS. Subsequently, 50 µL of XTT solution was added to the wells together with 100 µL of fresh medium, and after incubation in the 5% CO_2 incubator for 5 hours, the absorbances were measured at 490 nm. The cells containing only fresh medium were used as vehicle control. The cell proliferation was calculated with the help of the following equation;

Proliferation % = [Abs $_{(extract\text{-}treated\ cells)}$ - Abs $_{(extract\ in\ cell\text{-}free\ medium)}$] / [Abs $_{(control\ group\ cells)}$ - Abs $_{(cell\text{-}free\ medium)}$].

Considering the data obtained from XTT assays, different concentrations of DOX and VA were combined (VA+DOX), and XTT experiments were performed similarly. The absorbances obtained here and in the previous step were entered into the Compusyn program and CI Combinational Index (CI) values were calculated. CI is the value indicating the effect on the cell of combining two or more drugs. The CI value obtained from combination studies between 1 and 10 means that the effect is antagonistic, equal to 1 means that the effect is additive, and between 0.9 and 0 means that the effect is synergistic [19].

2.4. Statistical analysis

The results of the XTT assay were analyzed using the unpaired t-test with the GraphPad Prism software version 6.0. The COMPUSYN program was used to calculate the Combination Index (CI) results. Three independent experiments were conducted in every group, and all measurements were performed three times. $p < 0.05$ was considered statistically significant.

3. Results and Discussion

To determine the antiproliferative effects of DOX and VA alone against MCF-7 cancer cells, different concentrations of DOX ranging from 0.1 to 100 µM and various concentrations of VA ranging from 10 to 150 µM were applied to the MCF-7 cells for three different periods (24, 48, and 72 h). The results of the XTT experiments were presented in Table 1.

Table 1. Dose- and time-dependent inhibitory effects of DOX and VA on MCF-7 cells.

24 h			
Doxorubicin (µM)	**%inhibition**	**Vulpinic Acid (µM)**	**%inhibition**
0.1	46.24	10	10.29
1	57.74	25	25.82
10	86.31	50	30.21
50	≥100	75	37.60
100	≥100	100	56.25
48 h			
Doxorubicin (µM)	**%inhibition**	**Vulpinic Acid (µM)**	**%inhibition**
0.1	83.01	10	52.48
1	85.90	25	58.69
10	97.25	50	59.86
50	≥100	75	71.88
100	≥100	100	73.03
72 h			
Doxorubicin (µM)	**%inhibition**	**Vulpinic Acid (µM)**	**%inhibition**
0.1	94.81	10	78.72
1	94.55	25	88.35
10	97.51	50	89.79
50	≥100	75	≥100
100	≥100	100	≥100

Examining the dose-dependent inhibitory effects of DOX on MCF-7 cells at the end of each period individually from Table 1, it can be seen that DOX inhibited 46.24% cell proliferation at 0.1 µM, and this inhibition increased to 86 % at 10 µM after 24 h. At a concentration of 50 µM, DOX almost completely stopped cell proliferation. The 48 h results showed that DOX inhibited cell proliferation by 86.3 % at a concentration of 0.1 µM, and by increasing the concentration to 10 µM, inhibition increased to 97.2 %. At the end of 72 h, even the lowest dose of DOX increased cell proliferation inhibition to around 95%. These data reveal that DOX has a strong anticancer effect on MCF-7 cells in a dose- and time-dependent manner.

Similarly, when the antiproliferative activity of VA, one of the lichen metabolites, was tested against MCF-7 cells after 24 h, it was found to have a significant dose-dependent antiproliferative effect between 10 and 100 µM. While VA showed 10.29 % inhibition at the lowest dose of 10 µM, the inhibition rate reached 50 % at the highest dose of 100 µM. A dose-dependent inhibition was observed with increasing incubation time. VA, for instance, was found to inhibit cell proliferation by 52.4 % and 78.7 % at the lowest concentration of 10 µM after 48 and 72 h, respectively.

Detailed information on the anti-cancer mechanism of DOX, which is commercially used as a chemotherapeutic agent, is available in the literature. The number of studies on the biological activity of VA, a natural product, including its anti-cancer activity, is increasing day by day. In addition, it has been reported in several studies on the mechanism of action of VA in cancer. Kilic et al. (2018) reported the antiproliferative and apoptotic effects of VA on human breast cancer MCF-7 and non-cancerous MCF-12A cell lines. The results showed that VA significantly inhibited cell viability and induced apoptosis of human breast cancer cells while While it has no significant cytotoxic or apoptotic effects on non-cancerous cells [14]. Cansaran-Duman et al. (2021) investigated the role of vulpinic acid (VA) on the progression of breast cancer and reported that VA could downregulate the expression of 12 miRNAs by silencing the FOXO-3 gene [20]. Another study reported that VA exhibited anti-cancer activity in the breast cancer cell lines MCF-7 and MDA-MB-453 by inhibiting Thioredoxin reductases-1 (TRXR1) enzyme, which is recognized as a cancer marker, preventing migration and inducing apoptosis [11].

Recently, as cancer tumors develop resistance to the drugs used in chemotherapy over time, and the effects of these drugs are not sufficient, there has been an increase in the use of combinations of different drugs to treat patients. For example, Jin et al (2021) applied the combination of platycodin and docetaxel to prostate cancer DU-145 cells and reported that it synergistically inhibited cell growth, upregulated the Bax/Bcl-2 ratio, increased apoptosis, and ROS production, and suppressed the AKT/mTOR and ERK signaling pathways. [15]. Shokrzadeh et al (2021) applied the combination of Dox with lutein to the MCF-7 cells and reported that the result was a significant decrease in antioxidant enzymes while a significant increase in ROS levels, with a synergistic anticancer mechanism. [16].

In this regard, at this stage of the study, the aim was to apply different doses of combinations of VA, which is a natural compound and may have much fewer side effects in living organisms compared to synthetic drugs, and DOX, a drug widely used in chemotherapy, to MCF-7 cancer cells and then determine whether this situation is synergistic or antagonistic in the cell. The period over which the results of VA and DOX would be used to decide on combination studies was evaluated by looking at Table 1. For each dose of DOX, from lowest to highest, similar doses of VA were combined and applied to the cells, taking into account the data at 48 hours, which is the most effective period. For this, each combined dose of DOX + VA was prepared and applied to MCF-7 cells for 48 h and subjected to antiproliferative activity tests using XTT assay. The absorbance values obtained from the XTT tests were processed in the Compusyn program and CI values were calculated (Figure. 1).

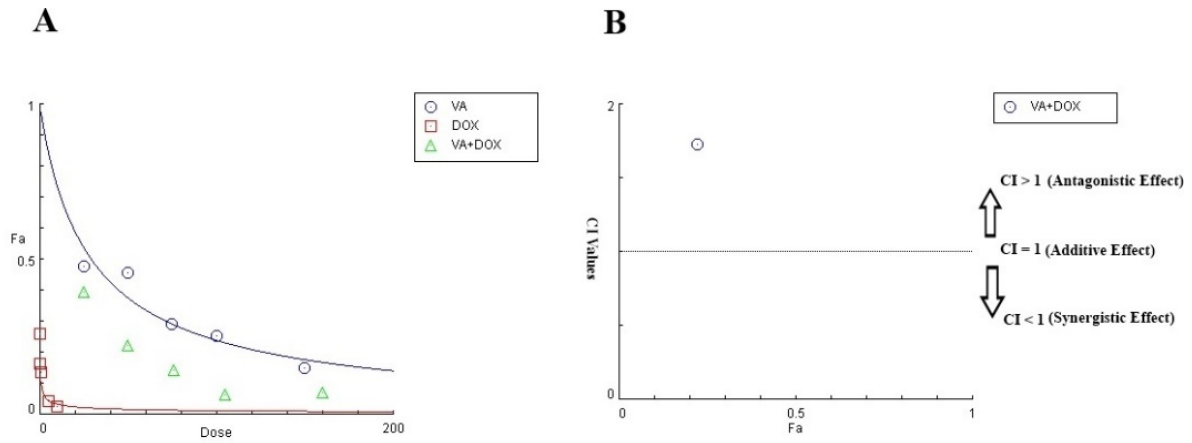

Figure 1. Compusyn results of vulpinic acid (VA) and Doxorubicin (DOX) in MCF-7 cells. **A)** Dose effect curves for VA, DOX, and VA+ DOX combination, **B)** Combination Index Value – Fa (Fraction Affected Level) for VA+ DOX. The value obtained in the combination index indicates a mild antagonism when 1.1 - 1.2, a moderate antagonism when 1.2 - 1.45, 1.45 - 3.3 antagonism, 3.3 - 10 strong antagonism, > 10 very strong antagonism, 0.85 - 0.9 mild synergy, 0.7 - 0.85 moderate synergy, 0.3 - 0.7 synergy, 0.1 - 0.3 strong synergy, < 0.1 very strong synergy.

Table 2. Combined effects and CI values of VA+DOX in MCF-7 cells

VA+DOX (µM)	Absorbance	CI
25-0.05	0.394	4.73265
50-0.1	0.222	1.72574
75-1	0.144	4.19017
100-5	0.064	2.62389
150-10	0.070	6.37395

CI: Combination Index

Looking at the data in Table 2, although each combined version of VA and DOX reduces the proliferation of MCF-7 cancer cells, each of the CI values is greater than 1. This indicates that the two compounds used in combination produced an antagonistic effect in this cell line, weakening each other to some extent, rather than having a synergistic effect. MCF-7 cells are known to be estrogen receptor-positive (ER+). Therefore, anti-cancer agents that act on this cell line bind to these receptors and exert their effects on the cell. While both compounds studied here showed a strong antiproliferative effect alone, presumably by binding to these receptors, when combined they showed an antagonistic situation, reducing the rate or ability to bind to this receptor and some extent preventing each other from binding. Although the combined use

of VA+ DOX has an antagonistic effect on MCF-7 cells in this study, further studies are needed to determine the effect of the combination on MDA-MB-453, MDA-MB-231, and other similar breast cancer cell lines.

4. Conclusion

As a result, this study showed that VA and DOX alone exerted a dose- and time-dependent anticancer effect on MCF-7 cells, whereas it was expected that they would exert a stronger anticancer effect when combined, but they weakened each other's effect by showing an antagonistic effect.

Ethics in Publishing

There are no ethical issues regarding the publication of this study.

Author Contributions

Conceived and designed the experiments: Dr. Ahmet Altay (AA), and Esma Kübra KAĞAN YENİÇERİ (EKKY). Performed the experiments: AA, and EKKY. Analyzed the data: AA, and EKKY. Contributed reagents/materials/analysis tools: AA, and EKKY. Wrote the paper: AA, and EKKY. The final version of the manuscript was read and approved by all authors.

Acknowledgments

I would like to thank the 100/2000 YÖK Doctoral Project, which financially supported my doctoral thesis in the field of "Natural and Herbal Products/Cosmetics". This study is part of the doctoral thesis. I would also like to thank the Erzincan Binali Yıldırım University Basic Sciences Application and Research Center and, Medicinal Aromatic Plants Application and Research Center for all the experiments that were carried out.

References

[1] Hausman, D. M. (2019). What is cancer?. Perspectives in biology and medicine, 62(4), 778-784.

[2] DeSantis, C. E., Ma, J., Gaudet, M. M., Newman, L. A., Miller, K. D., Goding Sauer, A., Siegel, R. L. (2019). Breast cancer statistics, 2019. CA: a cancer journal for clinicians, 69(6), 438-451.

[3] Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A., & Bray, F. (2021). Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: a cancer journal for clinicians, 71(3), 209-249.

[4] Nicoletto, R. E., & Ofner III, C. M. (2022). Cytotoxic mechanisms of doxorubicin at clinically relevant concentrations in breast cancer cells. Cancer Chemotherapy and Pharmacology, 89(3), 285-311.

[5] Thorn, C. F., Oshiro, C., Marsh, S., Hernandez-Boussard, T., McLeod, H., Klein, T. E., & Altman, R. B. (2011). Doxorubicin pathways: pharmacodynamics and adverse effects. Pharmacogenetics and genomics, 21(7), 440.

[6] Epstein, J. B. (2007). Mucositis in the cancer patient and immunosuppressed host. Infectious disease clinics of North America, 21(2), 503-522.

[7] Temel, Y., Kucukler, S., Yıldırım, S., Caglayan, C., & Kandemir, F. M. (2020). Protective effect of chrysin on cyclophosphamide-induced hepatotoxicity and nephrotoxicity via the inhibition of oxidative stress, inflammation, and apoptosis. Naunyn-Schmiedeberg's archives of pharmacology, 393, 325-337.

[8] Mondal, S., Bandyopadhyay, S., K Ghosh, M., Mukhopadhyay, S., Roy, S., & Mandal, C. (2012). Natural products: promising resources for cancer drug discovery. Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents), 12(1), 49-75.

[9] Baytop, T. (1999). Therapy with medicinal plants in Turkey (past and present). Publication of the istanbul University, 312, 2-3.

[10] Mitrović, T., Stamenković, S., Cvetković, V., Nikolić, M., Tošić, S., & Stojičić, D. (2011). Lichens as source of versatile bioactive compounds. Biologica Nyssana, 2(1), 1-6.

[11] Kalın, Ş. N., Altay, A., & Budak, H. (2022). Inhibition of thioredoxin reductase 1 by vulpinic acid suppresses the proliferation and migration of human breast carcinoma. Life Sciences, 310, 121093.

[12] Lauterwein, M., Oethinger, M., Belsner, K., Peters, T., & Marre, R. (1995). In vitro activities of the lichen secondary metabolites vulpinic acid,(+)-usnic acid, and (-)-usnic acid against aerobic and anaerobic microorganisms. Antimicrobial agents and chemotherapy, 39(11), 2541-2543.

[13] Demir, Y., Ceylan, H., Türkeş, C., & Beydemir, Ş. (2022). Molecular docking and inhibition studies of vulpinic, carnosic and usnic acids on polyol pathway enzymes. Journal of Biomolecular Structure and Dynamics, 40(22), 12008-12021.

[14] Kılıç, N., Aras, S., & Cansaran-Duman, D. (2018). Determination of vulpinic acid effect on apoptosis and mRNA expression levels in breast cancer cell lines. Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents), 18(14), 2032-2041.

[15] Jin, Y. Y., Li, Y. J., Ge, D. S., Zhu, L. L., Wang, Y. Y., Luo, J., ... & Chen, Y. Q. (2021). Combination of Platycodin D with docetaxel synergistically suppressed cell growth in DU-145 by enhancing apoptosis and alleviating autophagy. European Journal of Integrative Medicine, 42, 101302.

[16] Shokrzadeh, M., Mortazavi, P., Moghadami, A., Khayambashi, B., & Motafeghi, F. (2021). Synergistic antiproliferative and anticancer activity of carotenoid lutein or coenzyme Q10 in combination with doxorubicin on the MCF7 cell line. Applied In Vitro Toxicology, 7(4), 167-174.

[17] De Logu, A., Pellerano, M. L., Sanna, A., Pusceddu, M. C., Uda, P., & Saddi, B. (2003). Comparison of the susceptibility testing of clinical isolates of Mycobacterium tuberculosis by the XTT colorimetric method and the NCCLS standards method. International journal of antimicrobial agents, 21(3), 244-250.

[18] Altay, A., & Budak, H. (2023). Diffractaic acid exhibits thioredoxin reductase 1 inhibition in lung cancer A549 cells.

[19] Sever, A. (2018). Mcf-7 hücrelerinde oksidatif hasara karşı cisplatin ile kombinasyonda resveratrolün sinerjik etkisi ve oksidatif stres indeksi (Doctoral dissertation, Anadolu University (Turkey)).

[20] Cansaran-Duman, D., Yangin, S., & Çolak, B. (2021). The role of vulpinic acid as a natural compound in the regulation of breast cancer-associated miRNAs. Biological Research, 54.