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Review

Outer Membrane Vesicles (OMVs) as Antibiotic Carriers: A Promising Approach

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Abstract

The misuse and overuse of antibiotics have driven the emergence of antibiotic-resistant bacteria in recent decades. With the increasing incidence of resistant strains and the significant slowdown in new antibiotic discoveries, treating bacterial infections has become more challenging. Therefore, there is an urgent need to explore alternative treatments, such as using bacterial outer membrane vesicles (OMVs) for targeted delivery. OMVs are nanoscale, spherical structures originating from Gram-negative bacteria's outer membrane. These vesicles are naturally released by almost all types of Gram-negative bacteria into their environment during growth and play crucial roles in pathogenesis by transporting specific biomolecules, such as toxins and other virulence factors, to host cells. Due to their unique ability to encapsulate and transport various bioactive molecules across the Gram negative cell membrane, nanosized OMVs hold significant potential as a novel platform for antibiotic delivery. This review discusses biogenesis, biofunctions, and antibacterial applications of OMVs.

Keywords: Antibacterial therapy, antibiotic resistance, drug delivery, Gram-negative bacteria, OMVs

1. Introduction

Antibiotics are commonly used for the treatment of bacterial infections. The frequent or improper use of antibiotics has resulted in the widespread development of bacterial resistance to these drugs. The increasing incidence of multidrug-resistant pathogenic bacteria has made conventional antimicrobial treatment ineffective (1). Particularly, Gram-negative bacteria pose a significant challenge to antibiotic treatment due to their intricate, double-membrane cell structure (2). In an effort to combat infections caused by Gram-negative bacteria, research has focused on developing new types of drugs and discovering new therapeutic approaches to overcome the limitations of current medications.

Gram-negative bacteria, like most other cells, release membrane vesicles, known as outer membrane vesicles (OMVs), to mediate a range of cellular functions. OMVs are small, spherical, bilayered particles released during normal growth and stress conditions (3). They range in size from 20 to 300 nm in diameter and consist of components from the outer membrane and periplasmic space of the bacterium (3). OMVs play crucial roles in many bacterial

activities including intercellular communication, biofilm formation and pathogenesis (1). In recent years, it has been established that OMVs are involved intracellular communication, known as quorum sensing, both within bacterial communities and between bacteria and host cells. This communication is facilitated by the capability of OMVs to transport a diverse array of biomolecules including proteins, lipids, nucleic acids, and small signaling molecules across the Gram negative cell envelope.

OMVs can encapsulate and transport these molecules, thereby enhancing their stability and concentration in the extracellular environment (4). The unique property of OMVs to transport molecules suggests that OMVs can serve as promising vehicles for antibiotic delivery, offering a novel approach to combat bacterial infections caused by Gram negative bacteria. OMVs as antibiotic carriers could enhance drug delivery efficiency, reduce side effects, and potentially overcome mechanisms of resistance. This paper aims to provide a comprehensive overview of the potential of using OMVs as antibiotic carriers, including their biogenesis, biofunctions, isolation, and purification. In addition, the limitations and concerns regarding the clinical use of OMVs are discussed.

2. Formation and Composition of OMVs

OMVs were first identified in the 1960s, but it is only in recent decades that research on their formation, functions, and potential applications has significantly expanded (5). The formation of OMVs is a complex and highly regulated process influenced by various factors. For instance, conditions such as temperature fluctuations, ultraviolet radiation, nutrient availability, environmental stress, antibiotic exposure, and changes in bacterial growth status (6). OMVs are essentially formed through the outward budding of the outer membrane (OM) of Gram-negative bacteria. The budding occurs at sites where the lipoprotein connections between the peptidoglycan layer and the outer membrane are absent or disrupted, encapsulating biological molecules such as proteins, genetic material, and virulence factors derived from both the outer membrane and the periplasm (Fig 1) (1, 7).

Although various models have been proposed to explain the complex biogenesis process, such as the enrichment or depletion of specific local proteins, alterations in membrane curvature, and the accumulation of periplasmic proteins and peptidoglycan fragments, the exact mechanism of OMV formation is not fully understood (8).

3. Functions of OMVs

OMVs have the ability to naturally package and deliver a diverse array of molecular cargoes including proteins, lipids, nucleic acids, and small signaling molecules. Through their cargoes, OMVs carry out various physiological and pathological biofunctions**.** They have been shown to play crucial roles in bacterial communication (quorum sensing), biofilm formation, genetic exchange and pathogenesis.

3.1. Bacterial Communication and Biofilm Formation

OMVs are involved in quorum sensing, a bacterial communication process that relies on the production and detection of signaling molecules known as autoinducers. OMVs can encapsulate and transport these signaling molecules, thereby enhancing their stability and concentration in the extracellular environment. By facilitating the transfer of quorum-sensing signals, OMVs help bacteria monitor their population density and coordinate collective behaviors such as virulence factor production, antibiotic production, motility and biofilm formation (9).

Bacterial biofilms play a significant role in the development of chronic infections in humans. Biofilms are clusters of microorganisms that mainly consist of polysaccharides, secreted proteins, and extracellular DNA. Bacteria in biofilms can display antibiotic resistance up to 1,000 times higher than their planktonic counterparts. OMVs have been identified as crucial elements within the biofilm matrix (10). This discovery highlight that OMVs participate to biofilm formation

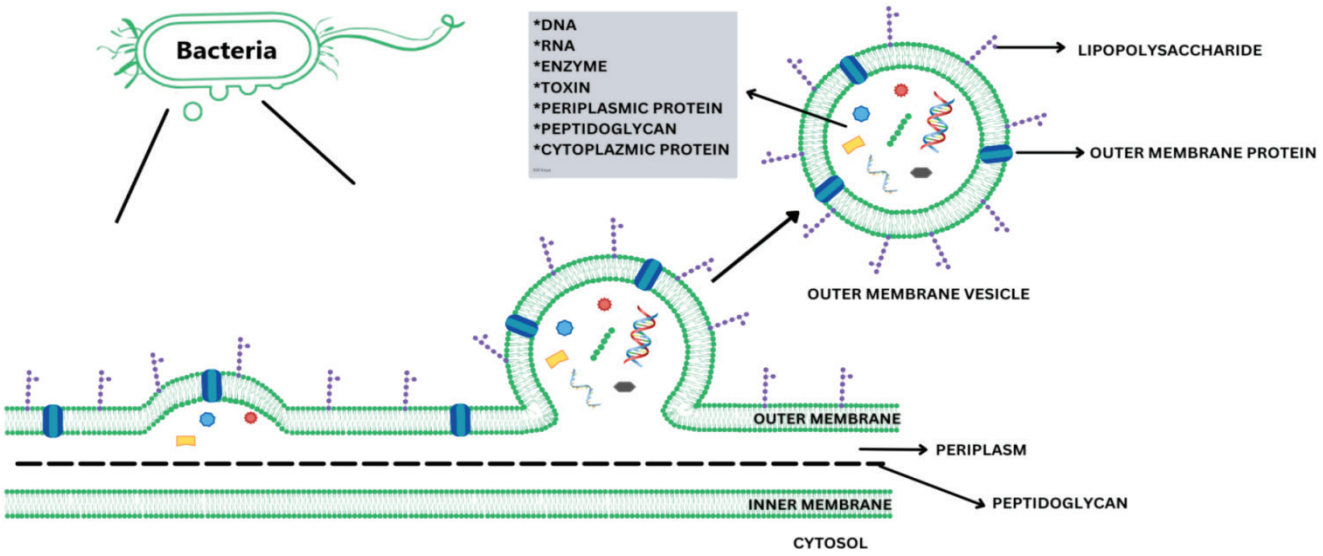

Figure 1. Representation of the formation, release and composition of OMVs. OMVs bud from outer membrane containing proteins and lipids from the outer membrane and material from the periplasm.

3.2. Horizontal Gene Transfer

OMVs can carry genetic material, such as plasmids or fragments of chromosomal DNA, facilitating horizontal gene transfer between bacterial cells. This property is especially important in the spread of antibiotic resistance genes, which can be delivered to neighboring cells through OMVs, contributing to the emergence of multidrug-resistant bacteria (1,11).

3.3. Pathogenesis

The role of OMVs in bacterial pathogenesis has been increasingly recognized. Many pathogenic bacteria release OMVs that help them establish infections by facilitating tissue invasion, immune evasion, and host cell interactions. OMVs contribute to bacterial pathogenesis by delivering microbial toxins and virulence factors directly into host cells during infection and weakening the immune response (12). For instance, *P. aeruginosa* produces OMVs that carry virulence factors, including toxins, that damage host cells and tissues (13). Similarly, *Porphyromonas gingivalis* OMVs carrying gingipain toxin can damage the supporting tissues of teeth, leading to severe periodontitis (1).

After mature OMVs formed, they gradually adhere to the host membrane, enter the cell, and release their cargo at a specific location. They can aid in immune evasion by modulating host immune responses or acting as decoys for antibodies (14). When OMVs interact with host cells, they can trigger immune responses such as the activation of macrophages, dendritic cells, or epithelial cells, either by inducing inflammation or immune tolerance. This immune modulation can be beneficial for bacteria, as it allows them to evade host defenses or establish chronic infections. In *Neisseria gonorrhoeae*, OMVs can trigger apoptosis of macrophages, affecting innate immun response and worsening the sexually transmitted disease gonorrhoea (15). OMVs from *Helicobacter pylori* (*H. pylori*) can reduce the secretion of interleukin 8 (IL-8) or lipopolysaccharide (LPS) and allow *H. pylori* to evade the immune response and establish a persistent infection (16).

4. Production, Isolation and Purification of OMVs

The process of isolating and purifying OMVs typically involves a series of steps, including

cultivation, low-speed centrifugation, sterile filtration, ultrafiltration, ultracentrifugation, density gradient separation, and gel filtration (17). The production of OMVs begins with culturing bacteria in an appropriate growth medium. Typically, OMVs are harvested from the culture after a sufficient cultivation period. After culturing, bacteria in the culture are removed by low-speed centrifugation (usually 2000×*g* ~ 10,000×*g*) (3). The supernatant is then passed through a sterile filter (0.22 μm or 0.45 μm) to eliminate any remaining bacteria. 100–500 kDa ultrafiltration membranes are used to to concentrate OMVs and remove non-OMV-associated proteins. Finally, the cell-free supernatant is subjected to ultracentrifugation at a speed range between 100,000 and 200,000 g at 4 ◦C for at least 2 h to collect OMVs (18). OMVs can be further purified by density gradient centrifugation with iodixanol, and ultracentrifuged at 100, 000 g for 18 h. After ultracentrifugation, the obtained OMV pellet is resuspended in PBS and stored at -20 or -80 ºC (17).

5. Characterization of OMVs

The determination of quantification and quality of purified OMVs is crucial for subsequent applications. The morphology and structure of OMVs are characterized by various optical microscopes such as transmission electron microscopy (TEM), scanning electron microscopy (SEM), cryogenic-transmission electron microscopy (cryo-TEM), and atomic force microscopy (AFM) (19, 20). TEM is the most commonly employed technique to visualize OMVs morphology and structure. SEM shows the three-dimensional structure of OMVs, but its resolution is lower than TEM. Cryo-TEM has also been utilized to visualize the morphology and structure of OMVs (21).

Cryo-TEM examines a frozen sample, effectively preventing morphological changes caused by dehydration and chemical fixatives. Atomic Force Microscopy (AFM) is another method used to analyze the structure of OMVs with high resolution. It enables real-time visualization of OMVs in air or liquid without the need for extensive sample preparation, allowing for detailed observation of surface properties. (22). Dynamic Light Scattering (DLS) is usually employed to measure the OMVs hydrodynamic diameter, size distribution and zeta potential (23).

Nanoparticle Tracking Analysis (NTA) is an alternative light scattering technique for determining the size distribution and concentration of nanoparticles. NTA works on principles similar to DLS but offers enhanced accuracy in assessing the size distribution of heterogeneous particles. Furthermore, NTA not only measures the size of OMVs but also counts single particles, allowing for simultaneous measurement of OMV yield (21, 24).

6. OMVs as antibiotic delivery vehicles

The rising prevalence of multidrug-resistant pathogenic bacteria has rendered conventional antimicrobial treatments ineffective, underscoring the critical need for novel strategies to combat bacterial infections. The capacity of OMVs to carry diverse biomolecules across the Gram-negative bacterial cell membrane highlights their potential as natural carriers for delivering antibiotics (25). This approach could offer a significant advantage in overcoming the challenges associated with effectively treating infections caused by these difficult-to-treat bacteria.

Kadurugamuwa and Beveridge (1996) were the first to discover that bacteria exposed to antibiotics release OMVs containing some of the drug. They cultured the *Pseudomonas aeruginosa* (*P. aeruginosa)* strain PAO1 in the presence of gentamicin and isolated gentamicin loaded OMVs (g-MVs) (26). Gentamicin containing OMVs (g-MVs) exhibited a significantly stronger inhibitory capacity against gentamicin-impermeable *P. aeruginosa* strains when compared to the effects of free antibiotics [10]. In another study, it was observed that g-MVs isolated from *Shigella flexneri* (*S. flexneri*) were able to attach to and penetrate human epithelial cells, effectively delivering the antibiotic to intracellular

S. flexneri, resulting in significant inhibition of bacterial growth (27). Similarly, gentamicin-containing *P. aeruginosa* OMVs were also reported to effectively kill some Gram-positive organisms such as *Bacillus subtilis* and *Staphylococcus aureus (*28). Study carried out by Huang et al demonstrated that quinolone antibiotics—such as levofloxacin, ciprofloxacin, and norfloxacin loaded OMVs isolated from *Acinetobacter baumannii* can effectively penetrate and kill pathogenic bacteria, including *Klebsiella pneumoniae* and *P. aeruginosa*, in vitro (20). Moreover, in a mouse model of intestinal bacterial infection, these antibiotic-loaded OMVs significantly reduced the bacterial load in the small intestine and feces of infected mice [20]. Tashiro et al reported that gentamicin-loaded OMVs from *Buttiauxella agrestis* (*B. agrestis*) demonstrated a potent bactericidal effect against *B. agrestis* as well as against *E. coli* and *P. aeruginosa (11)*.

The studies demonstrated that as nanoscale materials, OMVs can improve the efficiency of drug uptake and delivery. Additionally, OMVs offer excellent biostability, ensuring efficient drug transport. They protect bioactive molecules from degradation, enhancing cargo stability and enabling the efficient delivery of functional cargo to target cells. OMVs readily fuse with target cell membranes, exhibit excellent membrane stability and biocompatibility, and demonstrate prolonged circulation times within the bloodstream. These properties make them ideal for a variety of biomedical applications. Moreover, they can be genetically modified by molecular techniques. Compared to synthetic nanoparticles, OMVs generally exhibit lower toxicity, making them safer for therapeutic applications. Although OMVs have numerous advantages as antibiotic delivery platforms, several limitations hinder their widespread use as drug carriers such as pathogenicity, immunogenicity and controlled drug loading and release. One of the most significant drawbacks of OMV use in human is their potential to cause pathogenicity and immunogenicity in the host (1, 29). Derived from bacterial outer membranes, OMVs often contain LPS, which can provoke strong immune responses. High LPS levels may lead to systemic inflammation. In addition, OMVs derived from pathogenic bacteria can deliver virulence factors, leading to toxicity in host cells. Strategies such as LPS modification or genetic engineering of bacterial strains can help reduce this risk (30).

7. Conclusion

In today's of increasing antibiotic resistance, OMVs are attracting increasing attention as an alternative approach to the treatment of infections caused by Gram-negative bacteria due to their wide range of applications, especially in drug delivery. Compared to other drug delivery platforms, their lower toxicity, excellent biostability, and especially their ability to transport and protect the drugs they contain into targeted cells make OMVs an alternative to conventional antimicrobial therapies. This makes them a promising antibiotic delivery vehicle. However, the safety and efficacy of OMVs in clinical applications requires further study. Technical challenges such as the production, purity, stabilization and large-scale production of these structures should also be considered. Furthermore, further research on the effects of the biological materials carried by OMVs and their effects on the immune system is important. In conclusion, OMVs should be considered as a potential tool in the fight against antibiotic resistance and research in this field should be supported. Future studies will be critical for the safe and effective use of OMVs in clinical practice.

Conflicts of interest: The authors declare no conflicts of interest related to this work.

Ethics approval: Not applicable

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Original Article

Hepato(Geno)toxicity Assessment of Different Bromelain Food Supplements in HepG2 Cell Line Model by the Comet Assay

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Abstract

Introduction: Food supplements have become increasingly popular to support nutrition, improve overall health, and prevent diseases, yet their safety and efficacy remain under scrutiny due to limited regulation and inconsistent quality standards. Bromelain, a proteolytic enzyme mixture derived from pineapples, has gained attention due to its traditional medicinal uses and anti-inflammatory, antiedematous, and antithrombotic effects. This study aimed to evaluate the hepato(geno)toxicity potential of bromelain food supplement samples on human liver cell line, purchased from a pharmacy and an online retailer. In view of the increasing usage of bromelain, its promising pharmacological activities, and the limited toxicological data, the research highlights the potential risks associated with unregulated and widely accessible bromelain products, particularly those purchased online.

Methods: A colorimetric MTT (3-dimethylthiazol-2,5-diphenyltetrazolium bromide) assay was conducted to determine the IC_{50} concentrations, while the genotoxic potential of bromelain food supplement samples on human liver cell line, were evaluated using the alkaline single-cell gel electrophoresis assay (Comet Assay).

Results: No statistically significant differences in genotoxic potential were observed for either product in the HepG2 cell line when compared to the solvent control and the negative control at both tested concentrations (IC_{25} and IC_{50}).

Conclusion: Although no risks were identified for the tested products, the safety and authenticity of food supplements purchased online remain uncertain. Addressing regulatory and safety gaps requires harmonized vigilance systems, stricter marketing regulations, and increased public awareness to ensure their safe use.

Keywords: Bromelain food supplement, hepato(geno)toxicity, comet assay, MTT

1. Introduction

Food supplements are defined as “products with a determined daily intake dose prepared as capsule, tablet, lozenge, single-use powder package, liquid ampoule, dropper bottle and other similar liquid or powder forms which consist of nutrients such as vitamins, minerals, carbohydrates, fatty acids, fibers, amino acids or other physiological or nutritional effects of plant, plant and animal origin substances, bioactive substances and similar substances, alone or in mixtures, in order to supplement normal nutrition” by Turkish Food Codex Notification on food supplements (2013/49) (1).

Nowadays, due to factors such as population growth and increasing work intensity have led to disruptions in nutrition, resulting in changes of the eating habits. Consequently, food supplements have become increasingly common and popular. (2). The development of communication opportunities has raised interest in food supplements. Other factors rising this interest include people believing that supplements are natural and reliable, with no side effects, and that food supplements are easily accessible. Many people take food supplements to maintain their nutrition, stay healthy and active, and treat and prevent diseases often without consulting a health professional (3). A survey in the USA revealed that 61.2% of 376 randomly selected adults reported using food supplements. Some of these participants indicated that they used these products based on their physician's recommendation, while most stated that they obtained food supplements through friends, family, or social media (4).

The Ministry of Food, Agriculture, and Livestock licenses food supplements in Türkiye, and within the framework of the principles followed, manufacturers or importers offer the food supplements they produce and/or import to the market with the permits they receive in a very short period and through a very simple application process. There is no obligation to analyze food supplements inspected by the Ministry of Food, Agriculture and Livestock. In Türkiye, food supplements are frequently advertised across various media platforms. Their safety largely depends on the manufacturer's declaration, and the absence of rigorous safety assessments similar to those required for drugs poses a potential risk to public health. Furthermore, some products on the market are occasionally produced illegally (5). The safety of using food supplements should be questioned because they are taken in an discriminated and unregulated manner (3).

Bromelain is a mixture of proteolytic enzymes predominantly extracted from pineapples (*Ananas comosus L.*). It contains various closely related proteinases that exhibit antiedematous, anti-inflammatory, antithrombotic, and fibrinolytic activity both *in vitro* and *in vivo.* Traditionally, bromelain has been used to relieve edema, in digestive system disorders, and topically in wound and burn treatment (6). Since 1875, bromelain has been studied for its wide-ranging therapeutic benefits, including reducing blood clots, relieving conditions like angina and sinusitis, aiding recovery from injuries, and improving the absorption of medications, especially antibiotics (7, 8). Recent research also investigates bromelain's potential use in innovative treatments, suggesting it may serve as an effective drug against COVID-19 disease, as supportive and prophylactic therapy in *in vitro* or *in silico* investigations (9).

Bromelain products are available for purchase in pharmacies, markets, and through various online platforms. They are commercially available in tablet, capsule, gel, and sachet forms. Bromelain is absorbed in its active form through the gastrointestinal tract, with around 40% of the total being absorbed as its high molecular weight form. According to pharmacokinetics, the maximal blood concentration of bromelain was reached within one hour after oral dosing. Additionally, there are combined formulations of bromelain with active ingredients such as resveratrol, quercetin, curcumin, vitamin C, hyaluronic acid, and collagen. (6, 10).

Based on our observations, it is noteworthy that the edema-decreasing effect is often misinterpreted by users and bromelain food supplements are taken unintentionally to lose weight without being supported by proper diet or exercise. Additionally, consumers frequently purchase food supplements online due to their affordability. However, the safety

and authenticity of food supplements purchased through online platforms cannot be guaranteed.

The aim of this study is to evaluate the hepato(geno) toxicity of several bromelain food supplement samples on human liver cell lines, purchased from a pharmacy and an online retailer. The growing use of bromelain in recent years, the inadequacy of its toxicological studies, and its promising pharmacological activities proven by various studies served as the motivation for this work. The comet assay was employed to assess the hepato(geno) toxicity potential of bromelain food supplements in the HepG2 cell line**.**

2. Methods

2.1 Test samples

One of the bromelain food supplement samples was purchased from an online retailer (coded as e-BRM), while the other was obtained from a pharmacy (coded as p-BRM). Seven capsules (3.13 g) of the p-BRM sample and five tablets (4.9 g) of the e-BRM sample were weighed, and extracts were prepared using the maceration method with 20 mL of absolute ethanol as the solvent. The samples were mixed and left to stand overnight in the ethanol solution. The following day, they were filtered under vacuum, and this process was repeated three times. The combined filtrates were collected in a flask, and the solvent was evaporated under a vacuum at 50 °C using a rotary evaporator to obtain the extracts. A total of 0.66 g of extract (yield of 21.08%) was obtained from the p-BRM sample and 0.58 g (yield of 11.84%) was obtained from the e-BRM sample. The resulting extracts were stored at 4 °C until the day of the experiments. The extracts were solved in the culture medium with DMSO ratio fixed as 1% to reach the final concentrations in the day of the experiments.

2.2 Cell culture

The human HepG2 cells (liver cancer cell line, HB-8065; American Type Culture Collection) were incubated in 25 cm^2 flasks in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal calf serum (FCS) and 1% antibiotics (100 U/mL penicillin; 100 µg/mL streptomycin) under 5% CO_2 at 37 °C. The experiments were conducted using cells at passage numbers between 15 and 20.

2.3 Cell Viability Analysis and Dose Determination

A colorimetric MTT (3-dimethylthiazol-2,5-diphenyltetrazolium bromide) assay was performed to calculate the IC_{50} concentration, which represents the concentration at which 50% of cellular function is inhibited, indicating cell viability. A suspension of 1×10^4 cells was seeded into each well of a flat-bottom 96-well plate and incubated at 37 °C with 5% CO_2 for 24 hours to allow cell adhesion. Following the incubation period, the culture medium was removed, and the cells were exposed to the samples at final concentrations of 25, 50, 75, and 100 µg/mL (the extracts were solved in the culture medium with DMSO ratio fixed as 1%) for 24 hours to establish a dose-response curve and calculate the IC_{50} value. The culture medium was used as a negative control, while medium containing 1% dimethyl sulfoxide (DMSO) served as a solvent control. Each concentration was tested in triplicate, and controls were repeated six times. At the end of the incubation period, extraction solutions were removed, and cell viability was determined using the MTT assay, which relies on the reduction of tetrazolium salt to formazan dye by mitochondrial dehydrogenase enzymes in viable cells. Cells were incubated for 4 hours at 37 °C in 50 µL of MTT-containing medium (1 g/L), after which the resulting formazan crystals were dissolved in 160 µL of DMSO. The absorbance of the dissolved crystals was measured at 570 nm using an ELISA microplate reader, and relative cell viability (%) was calculated.

2.4 Genotoxicity

The cells were seeded into a flat-bottom 24-well plates at a density of 2×10^5 cells per well and incubated at 37 °C with 5% CO_2 for 24 hours to allow cell adhesion. Subsequently, both the e-BRM

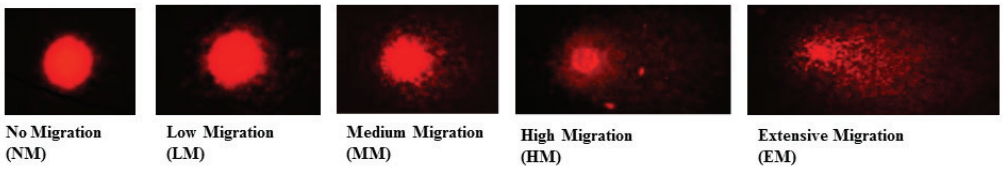

Figure 1. Comet pattern of undamaged cells to damaged cells (13).

and p-BRM samples were applied at two different final concentrations of 75 µg/mL (IC_{25}) and 100 µg/mL (IC_{50}), and the cells were incubated for an additional 24 hours. At the end of the incubation period, the extraction solutions were removed, the cells were washed with PBS, trypsinized, collected, and centrifuged 400 x g for 10 min. The supernatant was discharged, and the cell density was adjusted to $1x10^6$ cells/ml by using cold PBS.

The genotoxicity potential of e-BRM and p-BRM samples were examined by performing alkaline single cell gel electrophoresis assay (Comet Assay) according to Singh *et al*. with slight modification (11). The cells were suspended in 0.65% low melting agarose and spread onto slides pre-coated with 0.65% high melting agarose. The slides were covered with coverslips to allow the agar to solidify. After solidification, the coverslips were removed, and the slides were treated for one hour with cold lysis solution (stock lysis pH 10: 5 M NaCl, 100 mM EDTA, 10 mM Tris; freshly supplemented with 1% Triton X-100 and 10% dimethyl sulfoxide on the day of the experiment) to lyse the cells. Following the lysis stage, the experiments were carried out in a dark room. To allow the unwinding of the DNA double-strand structure in an alkaline environment, the slides were incubated in a solution containing 1 mM EDTA and 300 mM NaOH (pH > 13) for 30 minutes. Subsequently, electrophoresis was performed for 30 minutes at 15 volts and 300 mA. After this step, neutralization was carried out in three sets for a total of 15 minutes using 0.4 M Tris buffer (pH 7.5), followed by fixation of the slides with 50%, 75%, and 100% ethanol sequentially. The slides were then stained with 25 µg/mL ethidium bromide and visualized under an Olympus BX51 fluorescence microscope at 40x magnification. Damaged and undamaged cells were scored visually (Fig 1). A total of 100 cells were scored with two slides analyzed per condition. The total comet score was calculated according to following formula (12). Arbitrary Unit: 0 x No Migration (NM) + 1 x Low Migration (LM) + 2 x Medium Migration (MM) + 3 x High Migration (HM) + 4 x Extensive Migration. Figure 1 represents the microscope pattern of cells undamaged cells to damaged cells with appearance of comet. Comparisons were made with the negative control (culture medium), solvent control (1% DMSO) and positive control (100µM H_2O_2). All samples were tested in triplicate.

2.5 Statistics

Total comet scores (TCS) were expressed as the mean and standard deviation of the mean (n=3). Statistical analyses were conducted using the SPSS 20.0 software. The normality of the distribution continuous variables was tested by Shapiro–Wilk's test. Since the distribution was found to be normal, the means of the results obtained from three replicates (n=3) at different concentrations were compared using (ANOVA), with a Tukey multiple comparison, post hoc test for comparisons of different treatments versus the respective controls. Statistical significance was considered for $p<0.05$.

3. Results

The percentage of cell viability after 24 hours of exposure to increasing concentrations is shown in Figure 2. A proportional decrease in cell proliferation was observed with increasing concentrations of the e-BRM sample. However, no dose-dependent linear response was detected for the p-BRM sample. The IC_{25} and IC_{50} values for the e-BRM sample were calculated as 76.45 µg/mL and 103.48 µg/mL, respectively. Based on these results, the *in vitro* alkaline comet assay was performed at two different final concentrations (75 and 100 µg/mL) for both samples, with each condition tested in triplicate.

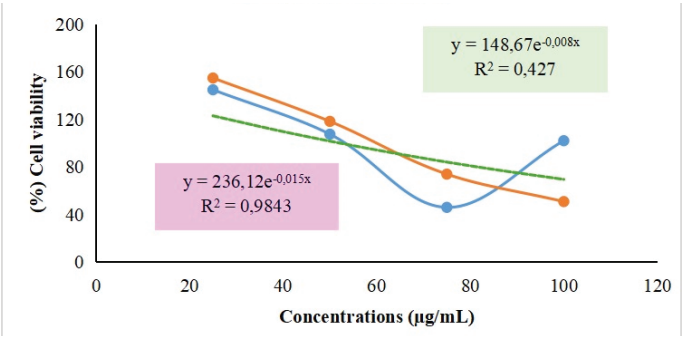

Figure 2. Percentage of the HepG2 cell viability after 24-hour incubation with different concentrations of bromelain food supplement extract samples

The mean TCS values of the samples are presented in Table 1. When comparing the genotoxic potential of both products in the HepG2 cell line with the solvent control and the negative control, no statistically significant differences were observed at either concentration (75 or 100 µg/mL) for both samples. However, the positive control showed a statistically significant difference in terms of its potential to cause genotoxic damage.

Table 1. Mean TCS Values After Sample Applications in the HepG2 Cell Line

Sample (n=3)	Final Concentrations	Total Comet Score (mean±SD) TCS±SD
Negative control	0	3,79 ± 0,96
Solvent control (DMSO)	%1	7,44 ± 0,94
Positive control (H_2O_2)	100 µM	182,71 ± 62,91***
e-BRM	75 µg/mL	6,49 ± 4,26
	100 µg/mL	4,73 ± 1,99
p-BRM	75 µg/mL	8,68 ± 2,89
	100 µg/mL	6,67± 2,12

Total comet score (TCS): 0 x No Migration (NM) + 1 x Low Migration (LM) + 2 x Medium Migration (MM) + 3 x High Migration (HM) + 4 x Extensive Migration (EM). a Significance of positive control compared with each sample at * p< 0.05; ** p<0.005; *** p<0.001.

4. Discussion

Food supplements (FSs) are increasingly popular but pose global regulatory and safety challenges due to inconsistent product quality, misleading marketing, and gaps in post-market surveillance (14). Furthermore, manufacturers are not obliged to disclose potential with other substances, alcohol, and medicinal products (15). Internet marketing exacerbates these issues by enabling cross-border distribution that bypasses regulations. In the U.S., over half of plant food supplements (PFSs) feature misleading labels, claiming to treat diseases like diabetes, heart disease, and cancer despite regulatory bans. Similarly, the FDA has recorded numerous instances of deceptive marketing. In Europe, unauthorized claims also mislead consumers, creating risks tied to unclear product efficacy (14). Regulatory systems for FSs differ between regions. The European Food Safety Authority (EFSA) provides guidelines, but the absence of centralized post-market vigilance leaves gaps. Only a few countries, such as France and Denmark, have dedicated nutrivigilance systems relying on voluntary reporting (16, 17). In Türkiye, although a centralized vigilance system monitors the medicinal products, there is no centralized vigilance system for food supplements, which may have a risk for monitoring the safety profile of food supplements (18). The complexity of assessing adverse drug reactions (ADRs) further complicates the evaluation of FSs safety. Challenges include the lack of diagnostic markers for herb- and drug-induced liver injuries (HILI/DILI) and inconclusive *in vitro* studies on toxicity and bioavailability. The interplay of these challenges emphasizes the necessity for more robust regulatory frameworks and mandatory pre-market safety studies for PFSs (14).

Numerous studies investigated the potential beneficial properties (9), while only few studies previously focused on the safety and toxicity of bromelain or bromelain supplements. The preclinical toxicity studies, especially LD50 studies, were published before the 1980s, and neither abstracts nor full texts of these studies are available online. Therefore, according to the review articles citing these older studies, generally describe bromelain as an acutely safe and well-tolerated phytochemical compound (19, 20).

According to the World Health Organization's (WHO) VigiAccess database, 1285 adverse drug

reactions (ADRs) have been reported for bromelain-containing products in the post-marketing period since they were first marketed, and a trend of increasing reported ADRs has been observed since 2018. Gastrointestinal disorders; general disorders such as oedema and pain; nervous system disorders such as dizziness, somnolence and headache; skin and subcutaneous tissue disorders such as pruritus, rash, and urticaria were the most frequently reported ADRs on bromelain-containing products. Eight of 1285 ADRs were related hepatobiliary disorders, including acute/chronic hepatic failure, cholecystitis, hepatitis, drug-induced liver injury (DILI), abnormal hepatic function, jaundice, and portal vein thrombosis. Also, elevated transaminase enzymes levels were recorded in the database. ADRs have been reported the most frequently in Asia, subsequently in Europe (21). To minimize adverse effects when taking food supplements, it is critical to consider the requirements of the user, diseases, regularly used medications, even medications prescribed for acute illnesses.

Drug interactions are often ignored in the use of bromelain food supplements. However, studies have shown that bromelain can increase the plasma concentration of antibiotics and has an anticoagulant effect (22). Also, four ADRs related to drug interactions for bromelain-containing products have been recorded in the VigiAccess database (21). On the other hand, exposure to bromelain via inhalation has been associated with asthma and IgE-mediated allergic reactions in a few case studies (23, 24).

Since the liver is the primary target organ for substances entering systemic circulation and plays a central role in metabolism, as well as the bioactivation and detoxification of compounds, the liver models are essential for toxicity testing. Primary human hepatocyte cultures are considered the gold standard for such studies; however, they have notable limitations, including donor variability, high culturing costs, and isolation challenges. As an alternative, the human hepatocellular carcinoma cell line HepG2 is widely used in toxicology and cancer research. HepG2 cells offer several advantages, such as being of human origin, ease of availability, the ability to produce highly reproducible results, and a limited but functional metabolic capacity (25). For this reason, HepG2 cells were selected in our study to determine hepato(geno)toxicity potential of the selected bromelain food supplements.

The cytotoxic and anticancer properties of bromelain were investigated in various carcinoma cell line (26, 27, 28, 29). Murthy et al. treated HepG2 cells with bromelain at concentrations of 25 µg/mL, 50 µg/mL, 100 µg/mL, and 125 µg/mL for 24, 48, and 72 hours, demonstrating a decrease in cell viability in a concentration-dependent manner. The IC_{50} value of bromelain for 24 hours was reported as 1.35µM (approximately 44.55 µg/mL) (29). In our study, although a proportional decrease in cell proliferation was observed with increasing concentrations of the e-BRM sample, the p-BRM sample did not exhibit a dose-dependent linear response. The IC_{25} and IC_{50} values for the e-BRM sample were determined to be 76.45 µg/mL and 103.48 µg/mL, respectively. The IC_{50} value in our study was higher than the result reported by Murthy *et al*. In fact, direct comparison between the two studies may not entirely appropriate, as our study focused on evaluating the effects of bromelain extracted from a food supplement on cell proliferation, whereas the other study utilized pure bromelain samples.

To the best of our knowledge, no studies have investigated the genotoxicity of bromelain food supplement products. In our study, no statistically significant differences in genotoxic potential were observed for either product in the HepG2 cell line when compared to the solvent control and the negative control at both tested concentrations (75 and 100 µg/mL).

5. Conclusion

No hepato(geno)toxic effects were observed for either product. While this study does not indicate a risk associated with the tested products, the safety and authenticity of food supplements purchased through online platforms cannot be guaranteed. In summary, although PFSs are widely marketed and consumed, significant regulatory and safety gaps are present. Addressing these challenges requires the

implementation of harmonized vigilance systems, stricter marketing regulations, and increased public awareness to ensure the safe use of FSs and mitigate potential health risks.

Conflicts of interest: The authors declare no conflicts of interest related to this work.

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Declarations: Not applicable

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Ethics approval: Not applicable

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Original Article

Investigations of Some Chlorocresol Hydrazones Against Tyrosinase Enzyme by Molecular Docking Method: In Silico Study

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Abstract

Introduction: Tyrosinase, found in various organisms, including plants and mammals, and is responsible for pigmentation as well as the undesirable browning of fruits and vegetables, is a multi-copper enzyme involved in the synthesis of melanin in human. As it is known, melanin provides protection against harmful ultraviolet radiation, which can lead to serious conditions like skin cancers. However, excessive melanin accumulation could result in hyperpigmented spots, creating aesthetic concerns. Tyrosinase inhibitors could potentially lead to the development of novel skin-whitening agents, anti-browning compounds for food preservation, and also for insect control substances. Recently, a wide spectrum of numerous moderate to potent tyrosinase inhibitors have been identified and reported.

Methods: In this study, by using the AutodockVina Virtual Screening Tool, some hydrazide-hydrazone compounds starting from p-chlorocresol were screened for interactions and binding mode of the tyrosinase active site. The enzyme-ligand interactions were analyzed using Biovia Discovery Studio software. Moreover, drug-likeness potential of the compounds was examined by using SwissADME online web tool.

Results: The results showed that compound S5, which did not violate the Lipinski and Veber rules and had a binding energy of -7.9 kcal/mol, could be a potential inhibitor of the tyrosinase enzyme.

Conclusion: Identifying the interactions between the tested ligands and the tyrosinase enzyme will contribute to the development of new hydrazide-hydrazone derivatives aiming the inhibition of tyrosinase.

Keywords: p-chlorocresol, docking, druglikeness, hydrazone, tyrosinase

1. Introduction

Melanin is a pigment produced by specialized cells in the deepest layer of the epidermis and is crucial for safeguarding the skin against the damaging effects of ultraviolet (UV) radiation (1,2). It also contributes to the color of our skin, eyes, and hair, influencing our overall appearance. While melanin primarily serves a protective role against UV rays, an excess proliferation in certain areas of the skin

can result in hyperpigmentation, which can pose aesthetic concerns (3).

Hyperpigmentation, a common skin condition, resulting from the excessive production or accumulation of melanin, the pigment responsible for skin color (4). This overproduction occurs during the process of melanogenesis, where the tyrosinase (TYR) enzyme catalyzes the oxidation of L-tyrosine to L-dopa, and subsequently to dopaquinone, the precursor of melanin. While melanin provides natural protection against UV radiation, its overproduction can lead to skin disorders like melasma and dark spots (5-7). As a result, TYR inhibitors have gained significant attention not only in the cosmetic but also food industries. In cosmetics, they are used to lighten skin and reduce the appearance of hyperpigmentation, whereas in the food industry, they can prevent browning in fruits and vegetables.

Although several TYR inhibitors are available, some, such as kojic acid and hydroquinone, have raised concerns about safety and long-term efficacy. On the other hand, newer compounds like thiamidol and deoxyarbutin promise safer alternatives (Fig 1). For instance, thiamidol, has demonstrated potent inhibitory effects on TYR, with a clinical trial confirming its efficacy in improving skin tone after topical application. Another compound, deoxyarbutin, has been reported to lighten skin effectively without causing irritation or damage to melanocytes (8).

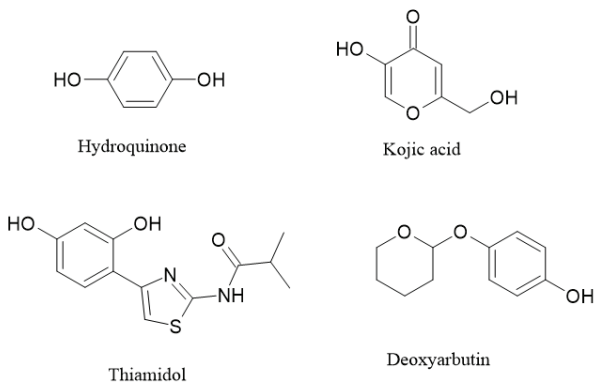


Figure 1. Chemical structures of some tyrosinase inhibitors

Hydrazones are organic compounds characterized by the -CH=N-NH_2 functional group, that are widely used in chemical synthesis as intermediates for the production of heterocyclic compounds, pharmaceuticals, and coordination complexes (9,10). Biologically, hydrazones exhibit diverse therapeutic effects, including antibacterial, antifungal, antiviral, anticancer, and anti-inflammatory activities (11-13). Their structural flexibility makes them valuable in medicinal chemistry for designing compounds with targeted biological properties.

In the current study, the potential inhibitory activity of 2-(4-chloro-3-methylphenoxy)-*N*'-[(aryl) methylidene]acetohydrazides (**S1-10**), which had been synthesized previously by our research group was investigated *in silico* by using molecular docking simulation method (14).Based on the docking studies, among the ten compounds studied, one (**S5**) appeared to have the highest inhibition on TYR activity.

2. Methods

2.1 Chemistry

As shown in Table 1, 2 - (4-Chloro-3-methylphenoxy)-*N*'-[(aryl)methylidene] acetohydrazides (**S1-10**) were successfully synthesized by using a well-known method and the synthetic protocol and spectral data of the molecules (**S1-10**) were reported previously by our research group (14).

2.2 Molecular modeling study

2.2.1 Protein preparation

The recently reported high-resolution X-ray structure of TYR (2.78 Å) (PDB ID 2Y9X: https://www.rcsb.org/structure/2Y9X) (15) was used in this study. After acquiring the protein crystal structure, all water molecules were deleted except those located in the ligand binding site. While co-crystallized ligand tropolone was deleted, Cu^{+2} ions in the active site were kept constant. The protein was saved in .pdb format and subsequently converted to pdbqt format using Autodocktools 1.5.6. (16). Then, the region of co-crystallized ligand were determined as the locations of the grid box (-9.923,

-26.885, -43.059) by using Autodocktools 1.5.6. and Discovery Studio 2021 (Accelrys Software Inc., Discovery Studio Modeling Environment, Release 4.0, San Diego, 2013.).

2.2.2 Preparation of the ligands

Compounds **S1-10** were drawn by using Spartan 4.0, and each molecule's energy was also minimized using Spartan 4.0 (17). The most stable isomer, *E,* was selected in the drawing of the all ligands. The conformations with the lowest energy were saved in .pdb format and then converted to .pdbqt format by using Autodocktools 1.5.6. (18,19).

2.2.3 Molecular docking

The determination of the grid box region (-9.923, -26.885, -43.059) and dimensions (40x40x40 Å) to include tropolone was performed using AutodockTools 1.5.6. and Discovery Studio 2021. Molecular docking was subsequently conducted by using Autodock Vina (19), with each docking process repeated a minimum of three times to ensure result accuracy. The docking scores and conformations of each molecule were then visualized by using Discovery Studio 2021, and the binding energies of all ligands for each molecule are summarized in Table 1.

2.3 Determination of druglikeness profile

The present study focuses on evaluating the drug-likeness of compounds **S1-10** according to Lipinski's 5 rules and Veber's rule. All of these compounds were assessed in terms of molecular weight, LogP, number of hydrogen bond donors/ acceptors, topological polar surface area, and the number of rotatable bonds (20). Additionally, these compounds were examined for gastrointestinal (GI) absorption and blood-brain barrier (BBB) permeability. All of these data were obtained from the online web server SwissADME (21).

3. Result and Discussion

The p-chlorocresol hydrazones listed in Table 1 and the co-crystallized ligand tropolone were drawn using Spartan. They were then prepared for docking studies by using AutodockTools 1.5.6. In the first step, docking studies were conducted with tropolone using the parameters reported in the material and method section. In the mentioned validation study, a similarity was observed between

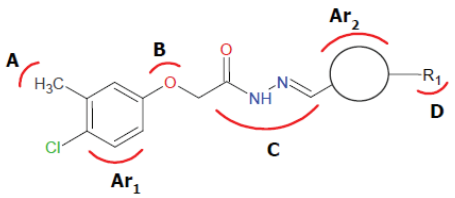


Table 1. Tested compounds **S1-10** and their binding energy against active site of TYR enzyme and their druggability

Compound	Ar_2	R_1	Binding Energy (ΔG=kcal/mol)	Lipinski rule n_{viol}	Veber rule n_{viol}
S1	Phenyl	2-Cl,3-OCH_3	-7.0	0	0
S2	Phenyl	2,5-$(OH)_2$	-7.1	0	0
S3	Phenyl	4-OCF_3	-6.9	0	0
S4	Phenyl	3-OCH_3, 4-F	-7.1	0	0
S5	Phenyl	2,6-$(CH_3)_2$	-7.9	0	0
S6	Phenyl	2,6-F_2	-7.3	0	0
S7	Thiophene	4-C_6H_5	-7.3	0	0
S8	Pyrazole	3-C_6H_5	-7.7	0	0
S9	Phenyl	2-OCF_3	-6.9	0	0
S10	Phenyl	3-NO_2, 4-CH_3	-7.1	0	0
Tropolone			-5.5		

the tropolone placed in the active site with the co-crystallized ligand tropolone, with a RMSD value of 1.029 Å. Subsequently, the binding potentials of compounds **S1-10** to the tyrosinase enzyme were investigated.

When the results of molecular modeling studies were examined, it was determined that the p-chlorocresol ring of compounds **S4**, **S6**, **S7**, **S9**, and **S10** was located in the tropolone binding site. In terms of hydrogen bond interactions, the hydrazone group (*C*) of molecules **S6** and **S7** forms H-bond interactions with the His244 amino acid at distances of 2.36 Å and 1.88 Å, respectively. Additionally, the hydrazone group (*C*) of the **S4** molecule interacted with the Val283 amino acid through an H-bond at a distance of 2.46 Å. Hydrogen bond interactions of all compounds with the active site, via substituents (*D*) on the hydrazone side chain, were identified and presented in Fig 2. Furthermore, compounds **S4**, **S6**, and **S9**, which contain -F substitution, exhibit halogen interactions with the active site (Fig 2).

Analysis of the hydrophobic interactions between the compounds in Fig 2 and the active site reveals that the p-chlorocresol ring, the starting material, engageed in pi-sigma interactions with the Val283 amino acid. Additionally, compound **S6** exhibited a pi-anion interaction with the Glu322 amino acid, while compound **S10** formed a pi-cation interaction with the His285 amino acid. Compounds **S4** and **S10** displayed pi-pi T-shaped interactions with the active site, whereas compound **S7** demonstrated a pi-pi stacked interaction. The hydrophobic interactions (pi-alkyl and alkyl) of the mentioned compounds with amino acids Leu63, Ala80, Phe192, His259, His263, Phe264, Val283, Pro284, and His285 enhanced their affinity for the active site.

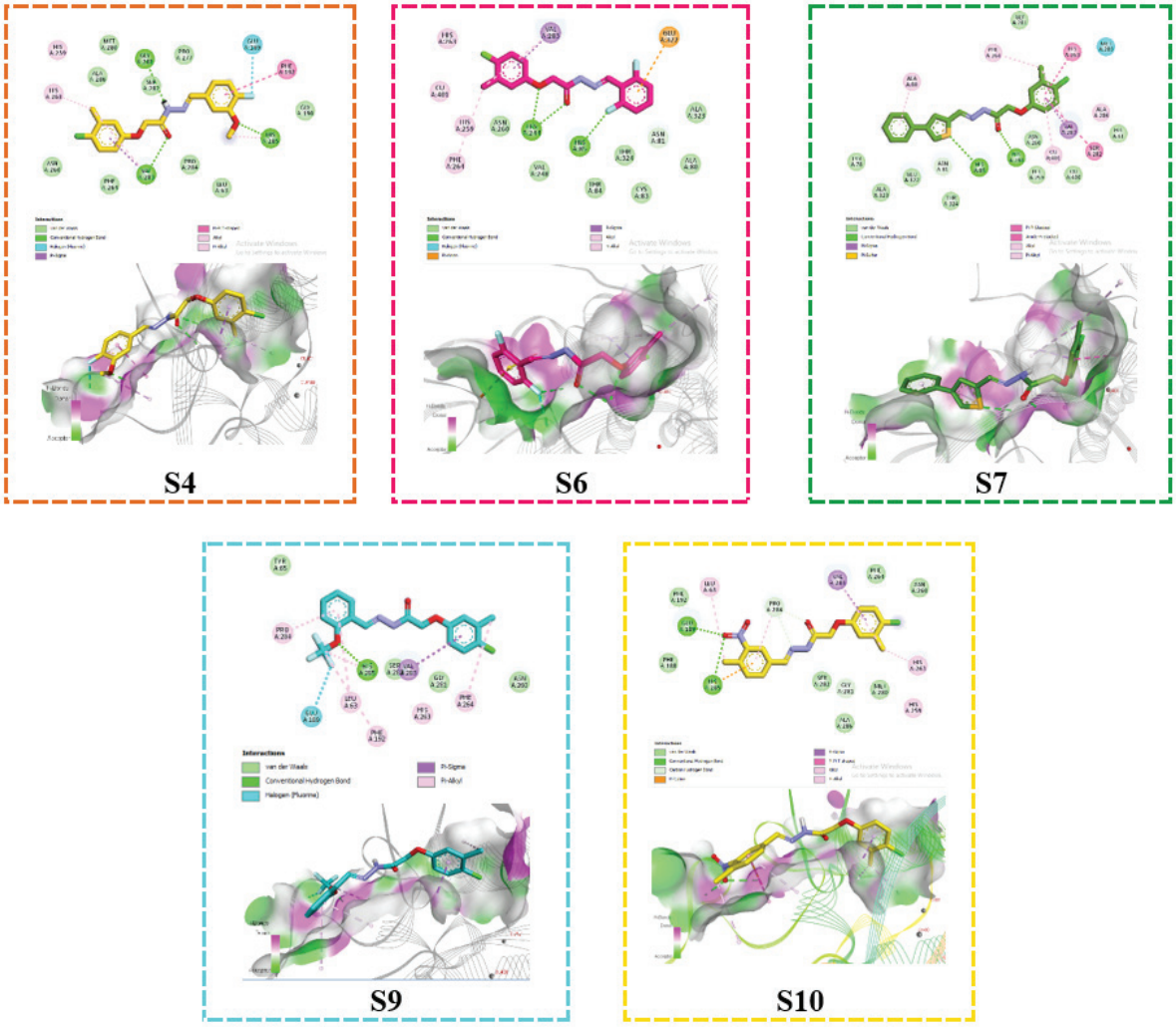
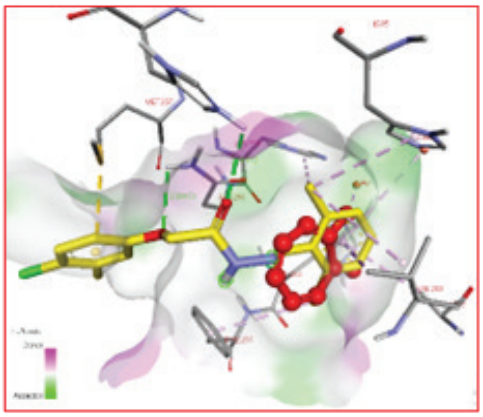


Figure 2. The interactions of compounds **S4, S6, S7, S9,** and **S10** with the active site of the TYR enzyme.

Table 2. Interactions of compound **S5** with the active site of tyrosinase enzyme (**S5** is yellow, tropolone is red).



Func. Group	Residue	Interaction	Distance (Å)
B	*Asn260*	*H-bond*	*2.83*
C	*His244*	*H-bond*	*2.88*
D	*Val283*	*Alkyl*	*4.86*
D	*His263*	*Pi-Alkyl*	*4.44*
D	*His85*	*Pi-Alkyl*	*4.61*
D	*Phe264*	*Pi-Alkyl*	*4.76*
D	*His259*	*Pi-Alkyl*	*4.97*
Ar_1	*Met257*	*Pi-Sulfur*	*3.75*
Ar_2	*Val283*	*Pi-Sigma*	*3.56*
Ar_2	*His263*	*Pi-Pi Stacked*	*4.00*
Ar_2	*Ala286*	*Pi-Alkyl*	*4.99*

When the results of molecular modeling studies for compounds **S1**, **S2**, **S3**, **S5**, and **S8 were** examined, it has been determined that the phenyl ring in the hydrazone side chain is positioned in the tropolone binding site. In terms of hydrogen bond interactions, the hydrazone group of the **S1** molecule formed H-bond interactions with the Val283 and Ser282 amino acids at distances of 2.10 Å and 2.36 Å,

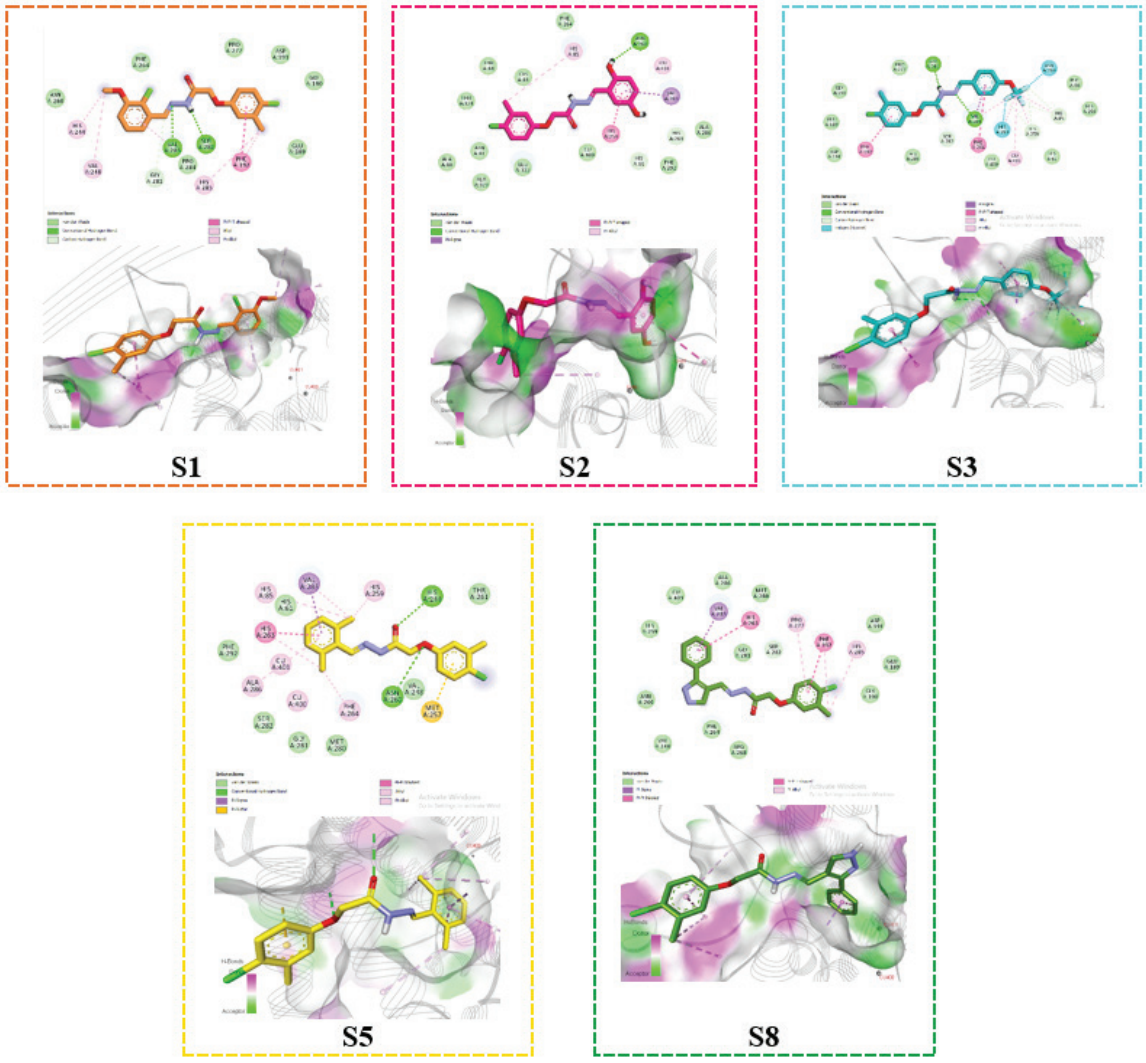


Figure 3. The interactions of compounds **S1, S2, S3, S5,** and **S8** with the active site of the TYR enzyme.

respectively. Similarly, the **S2** molecule formed H-bond interactions with the Asn260 amino acid at a distance of 2.52 Å through the phenol group in its hydrazone side chain, while no H-bond interaction with the active site was detected for the **S8** molecule. The hydrazone group of the **S3** molecule formed H-bond interactions with Val283 and Gly281 amino acids at distances of 2.70 Å and 2.74 Å, respectively. Additionally, the -OCF_3 group in the side chain of the hydrazone group interacted via halogen bonds with the His259, Asn260, and His263 amino acids (Fig 3).

Examining the hydrophobic interactions of the compounds depicted in Fig 3 with the active site has revealed that the p-chlorocresol ring, the starting material, engages in pi-pi T-shaped interactions with the amino acids Phe192, Phe264, and His259, as well as pi-sigma interactions with Val283. Additionally, hydrophobic interactions (pi-alkyl and alkyl) between these compounds and amino acids His85, Phe192, His244, Val248, His263, Phe264, Pro277, Val283, and His285 further has enhanced their affinity to the active site.

Among the compounds **S1-S10**, the compound predicted to have the highest in silico TYR inhibition has been **S5**, with a binding energy of -7.9 kcal/mol (Table 2). For compound **S5**, an H-bond interaction was identified between its hydrazone C=O group and the His244 amino acid at a distance of 2.88 Å, as well as between its ether (*B*) chain and the Asn260 amino acid at a distance of 2.83 Å. Lastly, it is thought that pi-sulfur interactions with the Met257 amino acid contributed to its binding energy.

The BOILED-Egg model is a tool used to predict the passive gastrointestinal (GI) absorption and brain access of small molecules, which is useful for drug discovery and development. It provides a simple graphical estimation of passive intestinal absorption and brain penetration as a function of WLOGP and TPSA. When the plotted molecule falls inside the white ellipse, good intestinal absorption is expected, while falling inside the yellow ellipse indicates a high probability of crossing the blood-brain barrier (BBB). Molecules located in the gray area are predicted to neither be absorbed by the GI tract nor cross the BBB (21). Based on this information, it is predicted that compounds, except for **S2**, **S7**, and **S10**, can cross the BBB and have high GI absorption. Additionally, none of the compounds, except **S8**, are Pgp substrates (Fig 4).

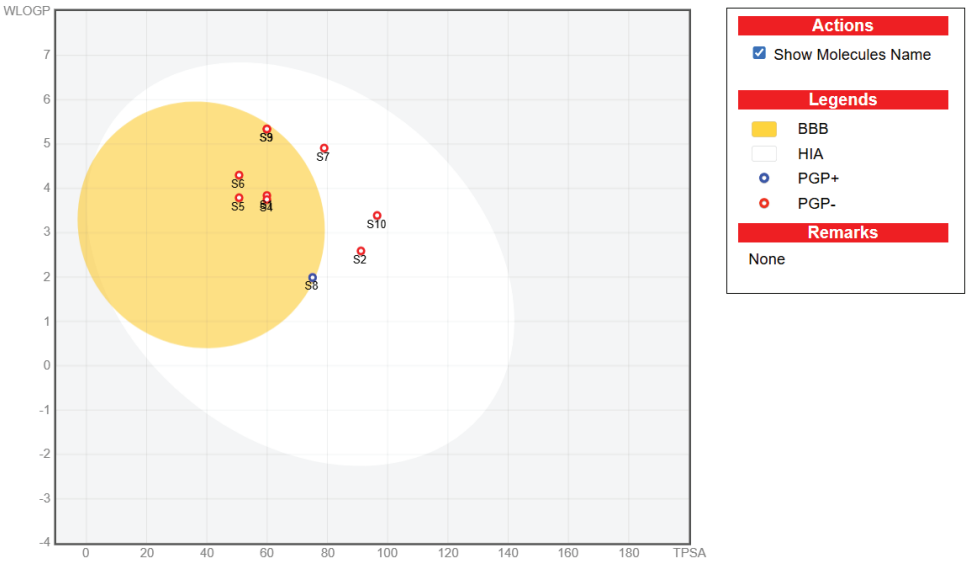


Figure 4. Graphical distribution of compounds **S1-S10** using the boiled egg predictive model.

4. Conclusion

In conclusion, the docking study conducted in this study clarifies the molecular interactions between the compounds **S1-S10**, previously synthesized by our research group, and the *Agaricus Bisporus Mushroom Tyrosinase* enzyme, sheds light on the TYR enzyme inhibition potential of the compounds.

The hydrazone group in the compounds was observed to interact with the enzyme binding site via hydrogen bonding, reinforced by hydrophobic interactions, and demonstrated a higher binding energy than the co-crystallized ligand, tropolane. When examining the drug-likeness potential of the compounds, it was determined that they do not violate either Lipinski's or Veber's rules, and their predicted GI absorption and BBB permeability were presented using the boiled-egg model. Among the tested compounds, **S5** (ΔG=-7.9 kcal/mol) showed the highest affinity, displaying a binding profile characterized by hydrogen bonds, hydrophobic interactions, and electrostatic contacts. The identification of hydrogen bond interactions between the hydrazone group of the tested ligands and TYR enzyme will contribute to the development of new hydrazide-hydrazone derivatives targeting enzyme inhibition.

Conflicts of interest: The authors declare no conflicts of interest related to this work.
Ethics approval: Not applicable

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Original Article

The Role of Critical Molecules in Proliferation and Apoptosis in Tumoral and Peri-tumoral Microenvironments of Gastric Cancer

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Abstract

Introduction: Gastric cancers (GC) are one of the main causes of cancer-related deaths worldwide. Despite the dizzying progress in diagnostic and therapeutic strategies, many GC cases are diagnosed at advanced stages. Various signaling pathways have been identified to play vital roles in GC. Although the importance of these signaling pathways in GC has been demonstrated, further clinical studies are needed. Therefore, the expression of some signaling proteins in the tumoral and peri-tumoral microenvironments of GC are examined in this study.

Methods: Protein expression levels were analyzed using Western blotting method in tumor and non-tumorous tissues from nine GC sufferers. The Kaplan-Meier method was used for the log-rank test to estimate survival curves and examine the survival distribution.

Results: It was found that overexpression of c-MYC, mTOR, and AKT alongside decreased degrees of AMPK and TRAIL in GC tissues. Kaplan-Meier survival analysis highlighted worse survival outcomes in patients with high mTOR and AKT expression, while high TRAIL levels were associated with improved survival. Interestingly, a poorer prognosis was associated with higher c-MYC expression, highlighting the oncogenic role of this protein in GC through the AKT/mTOR pathway. On the other hand, decreased AMPK expression implied a lack of tumor-suppressive properties, most likely due to mTOR activation.

Conclusion: These outcomes highlight the intricate interplay between GC's tumor-suppressive and carcinogenic mechanisms. Targeting these molecules by inhibiting mTOR or activating AMPK may provide new treatment approaches which can significantly increase TRAIL sensitivity. Moreover, further studies are necessary to confirm the present results and establish the integrated therapy approaches.

Keywords: AKT, AMPK, c-MYC, gastric cancer, mTOR, TRAIL

1. Introduction

Gastric cancers (GC) are among the leading reasons for cancer-associated mortality worldwide. The development and progression of GC involve complex interactions between various signaling pathways that modify cellular growth, proliferation, and apoptotic approaches. Molecules like c-MYC, mechanistic target of rapamycin (mTOR), AKT, AMP-activated protein kinase (AMPK), and TNF-related apoptosis-inducing ligand (TRAIL) are very important in this situation.

c-MYC is one of the carcinogenic transcription factors which is essential for cell division and cell growth. Most gastric malignancies can be cause carcinogenesis by activating the AKT/mTOR pathway through c-MYC overexpression. In particular, it has been demonstrated that stomach-specific overexpression of c-MYC activates the AKT/mTOR pathway and triggers gastric adenoma (1).

mTOR is an essential enzyme that controls metabolism and cell proliferation. In addition to c-MYC expression, activation of the mTOR pathway plays a crucial role in cellular growth and differentiation (2). Uncontrolled cell proliferation and treatment resistance in various malignancies, including leukemias, are related with the activation of the PI3K/AKT/mTOR pathway (3).

AMPK functions as the primary regulator of cellular energy homeostasis and inhibits cell proliferation through the suppression of the mTOR signaling pathway. By suppressing the mTOR pathway and lowering c-MYC expression in cancer cells, AMPK activation can impede the development of tumors. Compound C, which is an AMPK inhibitor, has been shown to cause autophagy of cancer cells (4,5).

TRAIL is one death ligand which might cause cancer cells to undergo apoptosis. It was shown that the efficacy of TRAIL is determined by the state of intracellular signaling pathways, and interactions with the AMPK/mTOR pathway can affect TRAIL sensitivity.

Variations in the expression levels and interactions of these molecules play important roles in the development of GC. Particularly, c-MYC overexpression through the AKT/mTOR pathway can promote tumorigenesis, at the same time as AMPK activation can suppress tumor growth through inhibiting the mTOR pathway (6). Investigating the expression of c-MYC, mTOR, AKT, AMPK, and TRAIL is crucial for understanding the molecular mechanisms of GC and identifying potential therapeutic targets. The present study investigates the functions of c-MYC, mTOR, AKT, AMPK, and TRAIL within the tumor and peri-tumor microenvironments of GC.

2. Methods

The study included the same patients' tumor and tumor around non-tumorous tissues of nine GC patients. The study was approved by the Medical Ethics Committee of the Istanbul Medical Faculty, Istanbul University (Approval No: 19.06.2019/119785) and was conducted according to the principles stated in the Declaration of Helsinki.

2.1. Western Blot analysis

The analysis determined specific protein expression in GC tissues, including tumor and adjacent non-tumorous tissues from the same patients. Tissue samples were subjected to lysis using a lysis buffer maintained at low temperatures, and the concentrations of proteins were determined by utilizing a protein assay kit. (DC kit; Bio-Rad, Hercules, CA, USA). Using sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with a 4–20% gradient, 40 µg of protein from each cell and group was separated and then transferred to polyvinylidene difluoride membranes. After transfer, the membranes were blocked with a blocking buffer (0.1 Triton X-100 with 5% dry milk in Tris-buffered saline-Tween 20 (TBS-T)) for 60 min. At the end of the blocking period, the membranes were washed with TBS-T and TRAIL (Invitrogen, Carlsbad, Germany, #MA1-41027), Caspase-3 (Invitrogen, Carlsbad, Germany #MA5-11516), c-MYC (Invitrogen, Carlsbad, Germany #PA5-85185), mTOR (Invitrogen, Carlsbad, Germany #AHO1232), AMPK (Invitrogen, Carlsbad, Germany #AHO1332), AKT (Invitrogen, Carlsbad, Germany #MA5-14898), Beta-actin

(Proteintech, China #66009-1-Ig) primary antibodies were examined. Following TBS-T washing, the membranes were treated with either an anti-mouse secondary antibody (Bio-Rad, Hercules, CA, USA, #170–6515) or horseradish peroxidase-conjugated anti-rabbit antibody (Bio-Rad, Hercules, CA, USA). In order to regulate loading, β-actin was employed. TBS-T with 5% dry milk was used to dilute all of the antibodies. Chemiluminescence detection was carried out with the Clarity Western ECL Substrate (Bio-Rad, Hercules, CA, USA). and the ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA) was used to view the blots. The ChemiDoc MP Imager application programme (Bio-Rad, Hercules, CA, USA) was used to quantify the blots using a densitometer (7-9).

GC

c-MYC is an important transcription factor that regulates expression of gene involved in processes such as cell proliferation, differentiation, and apoptosis (10), and it enhances the progression of gastric tumor (11,12). TRAIL was inversely linked to the risk of GC (13, 14). mTOR is the downstream target of the PI3K/AKT and AMPK pathways, which are potential therapeutic targets for cancer. mTOR is activated by AKT and PI3K/AKT/mTOR signalling pathway increases cell growth and survival through regulating cell growth, proliferation and apoptosis cancer cells (15, 16). Whereas the major negative regulator of mTOR is AMPK which is tumor suppressor protein in cancer cells (17, 18). The number of studies indicating

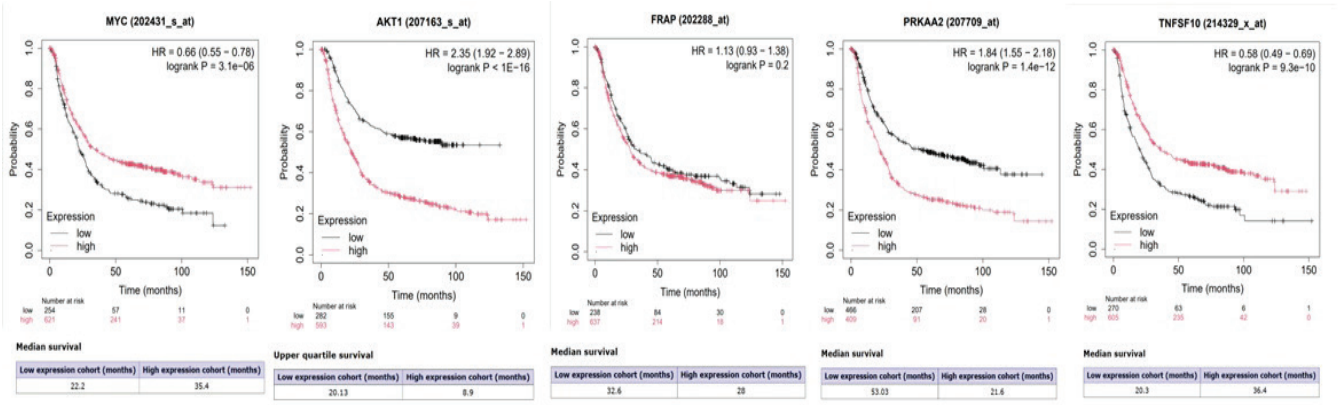

Figure 1: The Kaplan-Meier survival curves of c-MYC, mTOR (FRAP), AMPK (PRKAA2), AKT, and TRAIL (TNFSF10)

2.2. Statistical analysis

The IBM SPSS Statistics version 24.0 (IBM Corp., Armonk, NY) and GraphPad Prism version 5.01 (GraphPad Software, Boston, MA) was used for calculations and graphs. The results were summarized as means with standard deviations. Also, NCI-The Cancer Genome Atlas (TCGA) analysis was performed. The Kaplan-Meier method was used for the log-rank test in order to estimate survival curves and examine the survival distribution. The student t-test was used to establish statistical significance. Statistical significance was defined as p values below 0.05.

3. Results

3.1. c-MYC, mTOR, and AKT are overexpressed, while AMPK and TRAIL are down expressed in

that the AMPK-mTOR signaling pathway plays a significant role in the metabolism and tumorigenesis of GC is increasing day by day (19-24). To determine the clinical importance of c-MYC, mTOR (FRAP), AMPK (PRKAA2), AKT, and TRAIL (TNFSF10), Kaplan-Meier survival curves (www.KMplotter.com) were used to evaluate the prognosis of patients with GC in the NCI-The Cancer Genome Atlas (TCGA) GC database (Fig. 1). Among the patients with GC, the overall survival rate was significantly higher than those with high TRAIL (TNFSF10) gene expression but much worse for those with high mTOR and AKT gene expressions. On the other hand, patients with a high c-MYC gene had a better overall survival rate, and patients with a low AMPK (PRKAA2) level had a significantly greater survival rate.

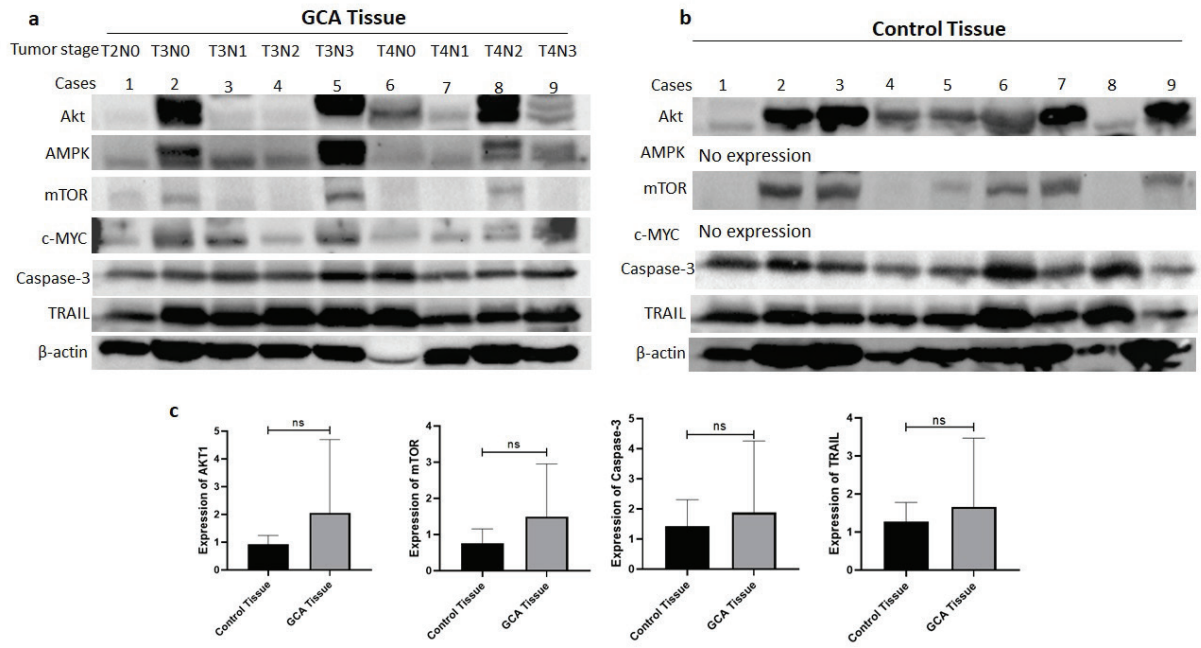

Figure 2: The western blot results of tumoral and non-tumoral samples. The results of gastric tumors (a). The results of non-tumoral samples (b). Comparison of the groups (c)

3.2. c-MYC, mTOR and AKT AMPK and TRAIL protein expression levels in tumor and non-tumorous tissue samples

The expression levels of c-MYC, mTOR, AKT, AMPK, and TRAIL proteins were analyzed in tumors and non-tumorous tissue samples of GC patients. There was no difference between the expressions of mTOR, AKT, TRAIL, and total caspase-3 proteins. However, c-MYC and AMPK proteins have not been expressed in non- tumorous tissue samples (Figure 2a-c). In contrast to Kaplan-Meier survival curves, the western blot results of this study suggest that c-MYC expression in patient tumor tissue may contribute to tumor initiation and progression by participating in processes such as proliferation and differentiation of gastric cells.

4. Discussion

The findings of the present study provide significant insights into the molecular mechanisms underlying GC, focusing on c-MYC, mTOR, AKT, AMPK, and TRAIL expressions. Compared with non-tumorous tissues, Western blot analysis showed upregulation of c-MYC, mTOR and AKT in gastric cancer tissues, although not statistically significant. In contrast, AMPK showed decreased expression, highlighting the disturbed balance of GC between oncogenic and tumor suppressor pathways.

The upregulation of c-MYC in tumor tissues is consistent with its established role as an oncogenic driver. Specifically, the increased expression of c-MYC correlates with AKT and mTOR activation, supporting the hypothesis that c-MYC promotes tumorigenesis through AKT/mTOR pathway activation. The findings of this study are parallel to the finding of the previous studies demonstrating that c-MYC overexpression triggers gastric adenoma development via AKT/mTOR signaling (25).

AMPK, a key metabolic regulator, exhibited reduced expression in tumor tissues. This is significant as AMPK acts as a tumor suppressor by inhibiting the mTOR pathway and reducing c-MYC expression (26,27). The observed downregulation of AMPK in GC tissues likely contributes to the activation of mTOR and the subsequent promotion of tumorigenesis. Furthermore, Kaplan-Meier survival analysis revealed that patients with high PRKAA2 (AMPK) expression had better overall survival, underscoring the prognostic significance of AMPK in GC.

The results of the present study suggest that interactions between TRAIL and the AMPK/mTOR pathway may influence the TRAIL sensitivity in GC cells. Similar observations have been reported by

who found that modulation of the AMPK pathway could enhance TRAIL-mediated apoptosis in cancer cells (28) TRAIL which is a pro-apoptotic ligand, showed variable expression across the tumor and non-tumor tissues. Although its apoptotic potential is well-documented, the findings of the present study suggest that TRAIL's efficacy may be influenced by the status of the AMPK/mTOR pathway. Kaplan-Meier analysis further highlighted that high TNFSF10 (TRAIL) expression is related with improved survival, indicating its potential as a therapeutic target in GC.

Interestingly, while AKT and mTOR upregulation was evident in tumor tissues, Kaplan-Meier survival analysis showed that high AKT expression is associated with poorer survival outcomes. This highlights the dual role of these pathways in promoting both tumor growth and resistance to therapy.

Overall, our findings underscore the intricate interplay between c-MYC, mTOR, AKT, AMPK, and TRAIL in GC pathogenesis. Together with decreased AMPK expression, the increase of c-MYC, mTOR, and AKT indicates that focusing on these pathways may have therapeutic advantages. For example, techniques to suppress mTOR signaling or activate AMPK may improve GC therapy results and increase TRAIL sensitivity. Despite these discoveries, the present study has limitations, including limited sample size and no functional tests to confirm causation. Future research should look at combinatory therapy approaches that target these pathways and confirm these findings in bigger cohorts.

The deregulation of important molecular pathways in GC is highlighted by this study, which also suggests c-MYC, mTOR, AKT, AMPK, and TRAIL as possible biomarkers and treatment targets. A detailed comprehension of their interactions may shed light to more individualized and successful therapy strategies in GC.

Conflicts of interest: The authors have declared no conflicts of interest.

Ethics approval: This study was approved by the ethics committee of the Istanbul University, Faculty of Medicine, Istanbul,Türkiye (issue no: 1471) and conducted in accordance with the principles of the Declaration of Helsinki.

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Original Article

Alterations in Redox System Parameters in Obsessive-Compulsive Disorder

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Abstract

Introduction: Obsessive-compulsive disorder (OCD) is a chronic neuropsychiatric condition characterized by intrusive obsessions and repetitive compulsions, significantly impairing daily functioning and the quality of life. Increasing evidence suggests that oxidative stress may play an important role in the pathophysiology of OCD, but the biochemical mechanisms underlying this relationship require further elaboration. This study aims to investigate the oxidant-antioxidant status in OCD patients compared to the healthy controls.

Methods: A total of 40 OCD patients diagnosed according to the Yale-Brown Obsessive-Compulsive Scale (Y-BOCS) and 40 age- and sex-matched healthy controls were included in the study. Blood samples were collected from all the participants and a spectrophotometer was used to measure levels of lipid peroxidation (LPO), superoxide dismutase (SOD) activity, and glutathione S-transferase (GST).

Results: The results of our study showed that LPO levels increased ($p \leq 0.0001$) while SOD and GST activities decreased ($p \leq 0.0001$) in OCD patients when compared to the healthy controls. These findings suggest that oxidative stress imbalance may contribute to the neurobiological mechanisms of OCD, supporting the hypothesis that increased oxidative damage and impaired antioxidant defense systems are involved in the disorder's pathophysiology of OCD.

Conclusions:. This study contributes to the growing literature on oxidative stress in psychiatric disorders and highlights the importance of investigating the biochemical pathways that may underline OCD pathogenesis. Understanding these biochemical alterations could provide valuable insights into the molecular basis of OCD and may led to the new approaches for potential therapeutic targets.

Keywords: Obsessive-compulsive disorder, oxidative stress, antioxidant enzymes, lipid peroxidation

1. Introduction

Obsessive-compulsive disorder (OCD) is a chronic and debilitating mental health condition characterized by the presence of obsessions and/or compulsions, as defined by the American Psychiatric Association in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-V) (1). Obsessions are recurrent, persistent, and intrusive thoughts, urges, or images that cause considerable anxiety or distress. Compulsions, on the other hand, are repetitive behaviors or mental actions performed in response to an obsession or according to strict rules, aimed at reducing distress or preventing a feared event or situation (2). Common obsession types include contamination fears, harm-related thoughts, and symmetry or ordering concerns, while compulsions often involve excessive cleaning, checking, or counting behaviors (3). The prevalence of OCD is estimated to be approximately 1-3% globally, with significant variations across populations, and it frequently leads to substantial impairments in social, occupational, and overall quality of the life (3, 4).

The etiology of OCD is multifactorial, involving a complex interaction of environmental, familial, and genetic-epigenetic factors (5). Environmental triggers such as infections, trauma, or stress have been associated with the onset or exacerbation of symptoms in predisposed individuals. Familial studies indicate a higher prevalence of OCD among first-degree relatives of affected individuals, suggesting a strong hereditary component (6). Furthermore, genetic and epigenetic mechanisms, including variations in genes related to serotonergic, dopaminergic, and glutamatergic systems, have been implicated in the pathophysiology of the disorder (7). Emerging evidence highlights the role of oxidative stress and neuroinflammation in neuropsychiatric disorders. These findings provide new pathways for understanding the biological basis of OCD (8, 9).

On the otherhand, oxidative stress arises from an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense systems (10). This imbalance can lead to lipid peroxidation, protein oxidation, and DNA damage, which have been linked to neuropsychiatric disorders, including OCD (11). Malondialdehyde (MDA), a marker of lipid peroxidation, serves as an indicator of oxidative stress levels. Enzymatic antioxidants, such as glutathione S-transferase (GST), and superoxide dismutase (SOD), play pivotal roles in mitigating oxidative damage by neutralizing ROS (12). Dysregulation of these systems has been reported in various psychiatric conditions and it is hypothesized to contribute to the pathophysiology of OCD through mechanisms such as neuronal damage, synaptic dysfunction, and altered neurotransmitter metabolism (9).

The investigation of oxidant-antioxidant enzyme systems and lipid peroxidation levels in OCD patients is crucial for elucidating the potential role of oxidative stress in the OCD. By comparing these parameters between OCD patients and healthy controls, the present study aims to provide insights into the biochemical alterations associated with OCD. Understanding these mechanisms may not only enhance our knowledge of the etiology of the disease, but may also pave the way for the development of novel therapeutic strategies targeting oxidative stress pathways. This study may contribute to the literature investigating the relationship between oxidative stress and psychiatric disorders and may offer potential biomarkers for monitoring the diagnosis and treatment of OCD in the future.

2. Methods

2.1. Selection of study groups

The patient group consisted of 40 OCD patients which admitted to Istanbul University-Cerrahpasa, Cerrahpasa Faculty of Medicine, Department of Psychiatry, Istanbul, Türkiye, and diagnosed with OCD according to DSM-V diagnostic criteria (1). The ethnic identity of the OCD patients included in the study was Turkish. OCD patients with comorbid psychiatric disorders were not included in the study. The patient group consisted of 22 female and 18 male volunteers, and the mean age was 30.32 ± 10.38 years. The severity of the disease was determined by the Yale-Brown Obsessive

Compulsive Scale (Y-BOCS) administered to OCD patients (3). The control group was selected among healthy individuals who applied to Istanbul University-Cerrahpasa ,Cerrahpasa Faculty of Medicine Hospital for regular health screening. The control group consisted of 40 healthy Turkish volunteers among of whom 25 were females and 15 males, with a mean age of 32.00 ± 10.92 years. Volunteers with a previous diagnosis of psychiatric disorder were excluded from the control group. The study groups consisted of volunteers aged 18-55 years. The procedures to be performed on the blood samples were explained to each individual in the patient and control groups and informed consent forms were obtained to voluntarily agree to participate in this study.

2.1.1. Yale-Brown obsessive compulsive scale (Y-BOCS)

Y-BOCS is a 10-question scale designed to measure the severity and type of symptoms in people with OCD. This scale was validated by Goodman et al. who reported that the Y-BOCS was significantly correlated with OCD. According to the result of this scale, the patient has a disease severity score between 0–40. Total scores can be split five categories, based on the severity of the symptoms. A score of less than 7 subclinical, 8–15 mild, 16–23 moderate, 24–31 severe, and 32–40 extreme OCD severity (3).

2.1.2. Sociodemographic questionnaire

A sociodemographic questionnaire was applied to the study groups, questions such as age, ethnicity, age of onset of OCD, medication use and family history were asked.

2.2. Biochemical analysis

Venous blood samples were collected in EDTA tubes from each volunteer in the study groups. The blood in the anticoagulated tubes was centrifuged at 2500 rpm, 4 °C for 20 minutes. The plasma remaining at the top of the tube at the end of centrifugation was collected in 1.5 ml Eppendorf tubes. It was stored at -80 °C until biochemical methods were performed.

2.2.1. Assay of the Total Protein Content

The plasma protein levels were measured in order to evaluate the biochemical analyses per mg of protein by Lowry method (13).

2.2.2. Determination of LPO

LPO measurement was made based on the absorbance obtained by reading the color change resulting from the reaction of Malondialdehyde (MDA), the product of this reaction, with thiobarbituric acid at 532 nm spectrophotometrically (14).

2.2.3. Measurement of SOD and GST Enzyme Activities

SOD activity was measured depending on the increase in the photooxidation rate of riboflavin-sensitized o-dianisidine. Under the influence of fluorescence light, the superoxide radical formed by riboflavin was converted to H_2O_2 by the catalysis of SOD in the environment. The formed H_2O_2 reacts with o-dianisidine to form a colored product, and the absorbance was measured at 460 nm spectrophotometrically depending on this color (15).

Glutathione *S*-transferase (GST) activity was evaluated by measuring the absorbance of the product formed by the conjugation of Glutathione (GSH) with 1-chloro-2,4-dinitro-benzene at 349 nm spectrophotometrically (16).

2.3. Statistical Analysis

Data was presented as mean ± standard deviation. The differences between the parameters investigated were tested by using unpaired samples Student t-test. Differences with p-values of 0.05 or less were considered significant. Statistical analysis was performed with Graph-Pad version 10.

3. Results

LPO was evaluated by measuring malondialdehyde (MDA) levels in plasma. The result of LPO comparison between the groups was-given in Fig 1. MDA levels were significantly higher in the OCD group when compared with the control group ($p \leq 0.0001$).

The results of SOD activity were given in Fig 2. SOD activity decreased significantly in the OCD group when compared to the control group (p≤0.0001).

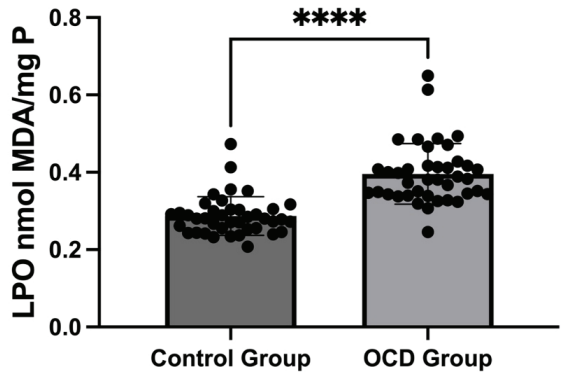

Figure 1. Comparison of lipid peroxidation (LPO) in terms of malondialdehyde (MDA) levels in plasma samples. n=40; ****p≤0.0001 significantly different.

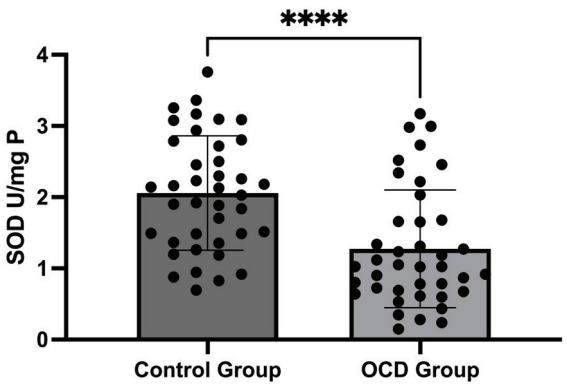

Figure 2. Comparison of superoxide dismutase activities in plasma samples. n=40; ****p≤0.0001 significantly different.

A comparison of GST activity between the two groups was in Fig 3. Consistent with increased LPO and decreased SOD activities, decreased GST activity was found in the OCD group when compared with the control group (p≤0.0001).

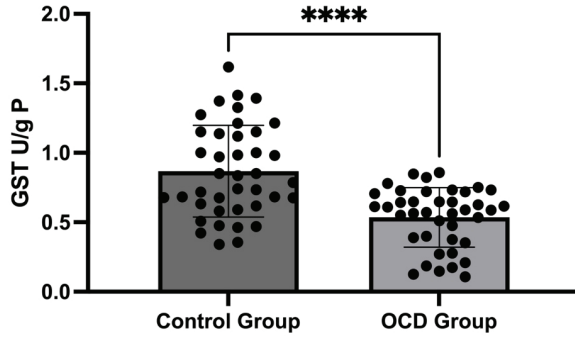

Figure 3. Comparison of glutathione S-transferase activities in plasma samples. n=40; ****p≤0.0001 significantly different.

4. Discussion

The results of the present study showed that the oxidant-antioxidant status in OCD patients was disrupted. In the literature, conflicting results have been reported regarding MDA levels in studies on OCD, and these studies also have various limitations (17). Jiménez-Fernández et al. and Liu et al. showed that MDA levels were higher in groups with depression when compared to the healthy controls (18, 19). Other studies have also reported that MDA levels were higher in groups with psychological disorders when compared to the healthy controls (20, 21). However, unlike these studies, Ranjekar et al. showed that MDA levels in the serum of schizophrenia patients did not reveal any difference when compared to the healthy controls (22). Furthermore, Talarowska et al. also stated that plasma MDA levels of depressive disorders was not statistically significant from the healthy controls (23). In the present study, it was found that LPO was increased through MDA levels in the plasma of OCD patients. In conclusion, it was found that LPO increased in the plasma of OCD patients when compared to the healthy control groups.

As it is known, ATP production by electron transport chain in mitochondria or respiratory burst of macromolecules in macrophages and neutrophils causes oxidative stress. Overcoming antioxidant capacity by ROS causes damage to macromolecules, neurotransmitters, and DNA. Furtherly, neurotransmitters such as dopamine, serotonin, gamma-aminobutyric acid (GABA), and glutamate have a regulatory role in OCD (21). A change in the synthesis or mechanism of these neurotransmitters could increase oxidative stress in the central nervous system (24). Brambilla et al. reported that in their study that MDA levels reduce serotonin levels (25). These results may allow the establishment of a relationship between MDA and OCD by associating MDA levels with neurotransmitters that are important in OCD.

The production of ROS can physiologically cause neurophysiological responses by causing neurotransmitter disruption and increased blood–brain barrier permeability. This results in neuroinflammation and cell death (26, 27). OCD is commonly seen in individuals with mitochondrial

dysfunction due to genetic disorders, and it has been reported that oxidative stress is high in these patients. In addition, increased ROS may cause peroxidation in membrane lipids and disrupt membrane integrity.

Under normal conditions, there is a balance between the oxidant-antioxidant system. In order to ensure this balance, various antioxidant enzymes such as SOD and CAT are present in the organism. With the increase in the oxidant species, the defense system in the organism is triggered and antioxidant enzymes are activated. Özdemir et al. repoted that SOD activity increased in OCD patients compared to controls (17). It is thought that there is an increase in the levels of oxidative species in OCD, and the antioxidant system is triggered in response to this situation and there may be an increase in the activities of antioxidant enzymes such as SOD. In the present study, the decrease in SOD activity in the plasma of OCD patients compared to the healthy control group may be an indication that the defense mechanism in the body is active but insufficient when compared to ROS.

Also it is kown that, GST is an important enzyme that catalyzes the conjugation of reduced GSH to xenobiotic substrates for detoxification purposes (28, 29). The activation of the antioxidant system in response to increased oxidative stress may also cause changes in GST activity. Quadros et al. revealed increased GST activity in anxiety-like behaviors induced zebrafish brains compared to the control group (30). In contrast to this study, the current study found that GST activity was decreased in OCD groups compared to the control group. As far as we know, GST activity has not been measured in OCD patients before. Therefore, the decrease in the GST activity measured in the plasma of OCD patients in our study may be important data for future studies.

5. Conclusion

In our study, redox system parameters were evaluated by measuring MDA levels, GST, and SOD activities in the plasma of OCD patients. As a result of our study, it could be stated that the oxidant-antioxidant balance in the plasma of OCD patients is disrupted. In future studies, the pathways of various neurotransmitters (serotonin, glutamate, GABA and dopamine), which are thought to have an important role in OCD, can be investigated and the relationships between these neurotransmitters and oxidant-antioxidant balance can be evaluated.

Conflicts of interest: The authors declare no conflicts of interest related to this work

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Ethics approval: This study was approved by the ethics committee of the Cerrahpaşa Faculty of Medicine, Istanbul, Türkiye (issue no: E-83045809-604.01.02-70153) and conducted in accordance with the principles of the Declaration of Helsinki.

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