ATATÜRK ÜNİVERSİTESİ YAYINLARI
ATATURK UNIVERSITY PUBLICATIONS

Research Article *Araştırma Makalesi* DOI: 10.5152/AUAF.2023.23074

# Optimization of Real-Time Polymerase Chain Reaction Conditions of Some Superoxide Dismutase Genes for Analysis in Wheat

## Buğdayda Gerçek Zamanlı Polimeraz Zincir Reaksiyonu Analizi için Bazı Süperoksit Dismutaz Genlerinin Optimizasyonu

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This study was presented as an oral presentation at the 5. International Conference on Life and Engineering Science (19–22 May–2022, Alanya, Türkiye) and its summary was published in the abstract booklet.

Received/Geliş Tarihi: 08.02.2023
Accepted/Kabul Tarihi: 22.08.2023
Publication Date/Yayın Tarihi: 23.09.2023

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Cite this article as: Bildirici, A.E., Tatar, M., Ölmez, F., Mustafa, Z., & Hajyzadeh, M. (2023). Optimization of real-time PCR conditions of some superoxide dismutase (SOD) genes for analysis in wheat. *Research in Agricultural Sciences*, 54(3), 153-158.



**ABSTRACT**

Environmental stress causes a significant decrease in crop quality and losses of productivity every year. One of the important mechanisms by which plants are damaged in adverse environmental conditions is the overproduction of reactive oxygen species such as superoxide, hydrogen peroxide, and hydroxyl radicals. In addition, such oxidative stresses have been shown to occur in plants exposed to high and low temperatures, particularly to high light intensities, drought, the presence of air pollutants such as ozone or sulfur dioxide, ultraviolet light, and herbicides. Hydroxyl radicals instantly react with proteins, lipids, and deoxyribonucleic acid, causing rapid cell damage. Therefore, plants have developed enzymatic and nonenzymatic mechanisms that efficiently scavenge oxygen radicals. However, hydroxyl radicals are too reactive to be eliminated enzymatically, so the formation of radicals is limited by the release of $O_2$ and $H_2O_2$. Superoxide dismutases, which are also the subject of this study, are key enzymes that scavenges superoxide radicals ($2O_2 + 2H^+ \rightarrow H_2O_2 + O_2$), contain metals, and act as the first line of antioxidant defense. In this study, eight superoxide dismutase genes were chosen for real-time polymerase chain reaction optimization. Specific primers were designed, and annealing temperature optimization was performed for real-time polymerase chain reaction analysis using deoxyribonucleic acid from wheat sample. In addition, annealing temperature optimization for β-actin specific primers were performed using the same deoxyribonucleic acid sample. β-actin is a housekeeping gene with a constant expression profile that is commonly used as a normalizing factor in expression profile studies. Evaluation of the relative and absolute values of superoxide dismutase gene expressions and the changes of superoxide dismutase gene expressions over time and under different conditions can be easily studied using the established real-time polymerase chain reaction protocols. These studies can provide important information on wheat coping mechanisms under different stress conditions.

**Keywords:** β-Actin, reactive oxygen radicals, *Triticum aestivum*

**ÖZ**

Çevresel stress her yıl mahsul kalitesinde önemli düşüşlere ve verimlilik kayıplarına neden olmaktadır. Olumsuz çevre koşullarında bitkilerin zarar görmesine neden olan mekanizmalardan biri de süperoksit, hidrojen peroksit ve hidroksil radikalleri gibi reaktif oksijen türlerinin aşırı üretimidir. Ayrıca, bu tür oksidatif streslerin, yüksek veya düşük sıcaklıklara, özellikle yüksek ışık yoğunluklarına, kuraklığa, ultraviyole ışığa, herbisitlere ve ozon veya kükürt dioksit gibi hava kirleticilerin varlığına maruz kalan bitkilerde meydana geldiği gösterilmiştir. Hidroksil radikalleri, proteinler, lipitler ve DNA ile anında reaksiyona girerek hızla hücre hasarına neden olur. Bu nedenle bitkiler, oksijen radikallerini verimli bir şekilde temizleyen enzimatik ve enzimatik olmayan mekanizmalar geliştirmiştir. Bununla birlikte, hidroksil radikalleri enzimatik olarak elimine edilemeyecek kadar reaktiftir, bu nedenle radikallerin oluşumu $O_2$ ve $H_2O_2$ salınımı ile sınırlandırılır. Bu çalışmanın da konusu olan süperoksit dismutazlar (SOD), $O_2$ radikallerini ortadan kaldıran ($2O_2 + 2H^+ \rightarrow H_2O_2 + O_2$), metal içeren ve antioksidan savunmanın ilk hattı olarak görev yapan anahtar enzimlerdir. Bu çalışmada, real-time PCR optimizasyonu için sekiz SOD geni seçilmiştir. Spesifik

primerler dizayn edilmiş ve buğday örneğinden elde edilen DNA kullanılarak, real-time PCR analizi için annealing (bağlanma) sıcaklığı optimizasyonu yapılmıştır. Ayrıca, aynı DNA örnekleri kullanılarak β-aktin spesifik primerleri için de bağlanma sıcaklığı optimizasyonu yapılmıştır. β-aktin, ekspresyon profili çalışmalarında normalizasyon faktörü olarak yaygın bir şekilde kullanılan, sabit ekspresyon profiline sahip bir housekeeping gendir. Mevcut real-time PCR protokolleri kullanılarak; SOD gen ekspresyonunun bağıl ve mutlak değerlerinin değerlendirilmesi ve SOD gen ekspresyonlarının zaman içinde ve farklı koşullar altında değişimlerinin incelenmesi kolayca gerçekleştirilebilir. Bu tür çalışmalar, buğdayın farklı stres koşulları ile başa çıkma mekanizmaları hakkında önemli bilgiler sağlayabilir.

**Anahtar kelimeler:** β-aktin, reaktif oksijen radikalleri, *Triticum aestivum*

## Introduction

Plants are constantly exposed to biotic and abiotic stresses that negatively affect their growth and yield, and this causes great losses in agriculture around the world. Among stressors are pathogenic infections, drought, salinity, and temperature extremes. These factors lead to the accumulation of excessive reactive oxygen species (ROS) such as superoxide anion ($O_2^-$), hydroxyl radical (OH), hydrogen peroxide ($H_2O_2$), singlet oxygen ($^1O_2$), ozone ($O_3$), and nitric oxide (NO) (Bowler et al., 1992).

In order to cope with ROS toxicity, plants have developed effective enzymatic [superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), glutathione peroxidase (GPX), and ascorbate peroxidase (APX)] and nonenzymatic (nonenzymatic scavengers and ascorbic acid, glutathione [GSH], vitamins E and C, β-carotenoids, and low-molecular-weight compounds such as phenols) antioxidative mechanisms that adapt to different types of stress imposed by adverse environments (Novo & Parola, 2008).

Superoxide dismutase (EC1.15.1.1), a family of antioxidant enzymes, is an important defense system against oxidative stress in plants and is ubiquitous in every cell of all plant species. As the first line of defense against oxidative damage, SOD catalyzes the conversion or dismutation of toxic superoxide radicals to $H_2O_2$ and molecular oxygen ($O_2$). Then, $H_2O_2$ is detoxified into the water by CAT and GPX (McCord & Fridovich, 1968, 1969).

Detailed expression analyses in *Nicotiana plumbaginifolia*, tomato, and maize showed that SOD genes are differentially regulated throughout development and in response to various stress conditions (Perl-Treves & Galun, 1991; Scandalios, 1993; Tsang et al., 1991). A study of wheat SOD genes revealed that the family contains 26 members and that wheat SOD genes also regulated stresses due to NaCl, mannitol, and polyethylene glycol (Jiang et al., 2019). From this point of view, it is possible to say that changes in SOD expression have an important place in the response of plants to physical stresses.

Plants respond to environmental stress with physiological, morphological, and metabolic changes in all their organs (Dudley & Shani, 2003). Stress tolerance involves multiple mechanisms for regulating gene expression at the genetic level (Knight & Knight, 2001). Plant engineering approaches to abiotic stress tolerance often take advantage of regulatory genes that control biochemical and physiological responses. Stress-resistant genotypes may be valuable in evaluating resistance genes in functional studies to understand the molecular basis of adaptation to abiotic stresses (Zhang et al., 2016). Gene expression research methods such as microarray and quantitative reverse transcription polymerase chain reaction (qRT-PCR) play a critical role in biology research (Kavousi et al., 2009). qRT-PCR is a technique used to quantitatively analyze gene expression, and target gene expression levels which must be normalized by internal reference genes to obtain definitive results (Bustin et al., 2009; Gachon et al., 2004). Housekeeping genes are often used as reference genes because of their stable expression (Vandesompele et al., 2002). These genes are often involved in vital processes of cell metabolism and architecture such as cytoskeleton formation, protein folding, and ribosomal subunit synthesis (Huggett et al., 2005; Gutierrez et al., 2008).

In the last decade, housekeeping genes such as actin (Act), glyceraldehyde-3 phosphate dehydrogenase (GAPDH), 18S rRNA, β-tubulin 2 (β-TUB2), and eukaryotic elongation factor 1-α (eEF-1α) have been evaluated as suitable reference genes for qRT-PCR (Mittler, 2006). Actins, which have three major isoforms called α, β, and γ, are the most abundant proteins in eukaryotic cells. These proteins play a key role in cell motility and cytoskeleton (Hunter & Garrels, 1977). Stable reference genes have been validated for some economically important cereal crops, including bread wheat (Jain et al., 2006; Manoli et al., 2012; Paolacci et al., 2009).

Based on the knowledge that SOD genes expressions change depending on various stress conditions, expression studies with these genes can explain how plants respond to stress conditions. The first step of all these studies is the synthesis of appropriate primers, and in this study, it was aimed to obtain preliminary data for further studies by optimizing the specific primers synthesized.

## Methods

### Subject

In this study, a bread wheat deoxyribonucleic acid (DNA) was used for the annealing temperature optimization of the selected primers. Deoxyribonucleic acid was obtained from the DNA collection in Sivas University of Science and Technology, Agricultural Sciences Laboratory. The wheat DNA used was isolated according to the DArTSeq protocol. Accordingly, after the samples were homogenized with liquid nitrogen, 1 mL of extraction buffer was added, and the tubes were inverted gently and vortexed for 10 seconds. The tubes were incubated at 65°C for 1 hour by inverting every 10 minutes. Afterward, the tubes were centrifuged at 13.500 *g* for 15 minutes at room temperature and the supernatant was transferred to 2 mL Eppendorfs and 1 mL of chloroform:isoamyl alcohol (24:1) was added. Then, the tubes were gently inverted at room temperature for 30 minutes and then centrifuged at 13.500 *g* for 15 minutes at room temperature. The supernatant was transferred into new 1.5 mL Eppendorf tubes and 800 µL cold isopropanol was added. Repeatedly the tubes were inverted very slowly and incubated for 10 minutes at room temperature. The tubes were centrifuged at 13.500 *g* for 30 minutes at room temperature and the supernatant was carefully transferred to

a new tube and 1 mL of cold 70% ethanol was added. Similarly, these tubes were centrifuged at 13.500 *g* for 10 minutes at room temperature and the supernatant was carefully discarded. Pellets were dried overnight at room temperature with the lid open and then dissolved in 100 µL double-distilled $H_2O$.

Preparation of fresh buffer (30 mL): Add 0.15 g sodium bisulfite and 0.6 g polyvinylpyrrolidone 40 to 12.5 mL extraction buffer and dissolve; add 12.5 mL lysis buffer stock and 5 mL sarcosyl stock.

Preparation of extraction buffer: 0.35 M sorbitol, 0.1 Tris–HCl (pH 8.0), and 5 mM EDTA (pH 8.0).

Preparation of lysis buffer: 0.2 M Tris–HCl (pH 8), 0.05 M EDTA (pH 8.0), 2 M NaCl, and 2% CTAB.

Samples isolated in this way can be stored for extended periods at –20°C or –80°C.

**Primer Selection and Design**

Within the scope of the study, 27 SOD genes belonging to bread wheat were examined and primers for eight SOD genes (SOD-1 [7AL_E14A72218.2], SOD-6 [2AS_529825553.1], SOD-10 [2BL_7BF340BAE.1], SOD-11 [7BL_A42D6C984], SOD-13 [3B_2650F3AB3.2], SOD-18 [2DL_F48387F4E.1], SOD-20 [2BL_1724AE8A9.2], and SOD-21 [7DS_1CE2F67B7.1]) were designed for real-time PCR using NCBI primary BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and Benchling (https://benchling.com) programs. Housekeeping gene β-actin primers were obtained from previously published studies (Wei et al., 2015). Designed forward and reverse primer sequences and amplicon sizes are given in Table 1.

**Polymerase Chain Reaction**

Specific primers synthesized for SOD genes and β-actin as a reference gene were used in the reactions. Each reaction tube was loaded with 7.55 µL of PCR master mix (ThermoScientific, Massachusetts, USA), 1 µL each of forward and reverse primers and 0.5 µL 100 ng DNA sample. PCR reaction was performed in three amplification steps consisting of 35 cycles following a 3-minute denaturation step at 95°C: (i) denaturation: 15 seconds at 95°C, (ii) annealing: 15 seconds at corresponding annealing temperature, and (iii) elongation: 10 seconds at 72°C.

Polymerase chain reaction was run a gradient and annealing temperatures for each primer were by evaluating annealing temperature stepwise up to ±5°C from the estimated annealing temperature. Polymerase chain reaction results were evaluated in 1% agarose gel electrophoresis. To determine the specific annealing temperatures for the designed primers, 0.5 g of agarose was weighed and placed in 50 mL TBE (Tris–boric acid–ethyl enediaminetetraacetic acid [EDTA]) buffer, and then dissolved by heating for about 3 minutes (until bubbles form) in the microwave oven. The gel solutions were then poured onto plates with 10 wells placed and left at room temperature for 20 minutes for polymerization. Deoxyribonucleic acid ladder 100 bp (Vivantis, Selangor, Malaysia) and then PCR products according to the increasing temperature values were loaded into the wells of the gels. Samples run for 90 minutes at 120 V were visualized under ultraviolet light. For imaging, GelDoc Go Imaging System (Bio-Rad, Dubai, United Arab Emirates) brand gel imaging technology was used and gels were photographed.

## Results

Gradient PCR was applied to determine the optimum annealing temperature for the designed primers and results were visualized on 1% agarose gel. Annealing temperatures were determined between 60 and 68°C. Since the members of the SOD gene family have high sequence homology, attention was paid to finding the most specific annealing temperatures of the primer designed for each gene. Therefore, even 0.1°C sensitivities have been considered for the determination of temperatures.

The annealing temperature of SOD-21 was found to be the lowest (60°C). The annealing temperature of SOD-20 was found to be 60.7°C with a difference of 0.7°C. Optimum annealing temperatures for SOD-6 and SOD-10, and SOD-1 and SOD-18 were 64.5°C and 66.4°C, respectively. The annealing temperature for SOD-11

**Table 1.**
*Eight SOD Genes and Housekeeping β-Actin*

| Gene | Sekans | Amplicon size (bp) |
|---|---|---|
| SOD-1 (7AL_E14A72218.2) | F: 5′-TCAGAGCCTCCTCTTTGCCG-3′<br>R: 5′-CGCCTTCTGGGGTGCAGACAAT-3′ | 98 |
| SOD-6 (2AS_529825553.1) | F: 5′-GGATGGTGTTGCTAGCATCAATA-3′<br>R: 5′-CAGCACGGCCAACAATTGAATTT-3′ | 77 |
| SOD-10 (2BL_7BF340BAE.1) | F: 5′-AGGTGCAGAAAGTACTGGCAA-3′<br>R: 5′-CCTGAAAACTGGGCTTCTCTTT-3′ | 111 |
| SOD-11 (7BL_A42D6C984.1) | F: 5′-CCGAATTGTCTCCACTCCAGG-3′<br>R: 5′-TCTTCCTGGGTGAGCGTGA-3′ | 135 |
| SOD-13 (3B_2650F3AB3.2) | F: 5′-TGGGCGTTCGGCATCTACTTCTGC-3′<br>R: 5′-TGGCACAAAAGGAACGGGGTCG-3′ | 121 |
| SOD-18 (2DL_F48387F4E.1) | F: 5′-GGAAGAACCTCAAGCCCATC-3′<br>R: 5′-TCCTTGTAAAGCAGCACCCT-3′ | 137 |
| SOD-20 (2BL_1724AE8A9.2) | F: 5′-CCACCTACGTCGCCAACTA-3′<br>R: 5′-GGAGGCATATCCAGAGGAGAG-3′ | 148 |
| SOD-21 (7DS_1CE2F67B7.1) | F: 5′-AGACCAAGCTGCCGATGATA-3′<br>R: 5′-TCCACCGTCTCCTTGCTTAT-3′ | 144 |
| β-Actin | F: 5′-GGAGAAGCTCGCTTACGTG-3′<br>R: 5′-GGGCACCTGAACCTTTCTGA-3′ | 135 |

Note: F, forward primer; R, reverse primer; SOD, superoxide dismutase.

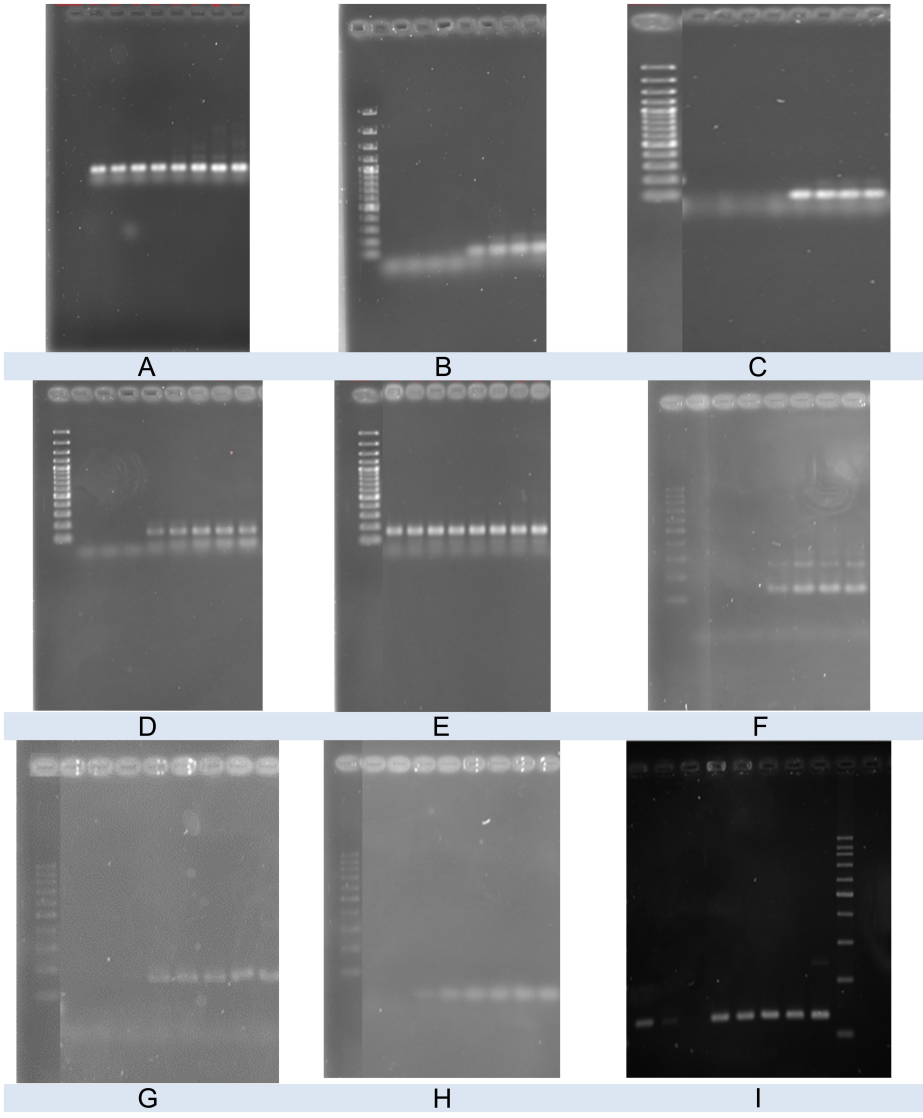


**Figure 1.**
*Gradient PCR Results. (A) SOD-1 gene, 68–60°C; (B) SOD-6 gene, 72–60°C; (C) SOD-10 gene, 72–60°C; (D) SOD-11 gene, 70–60°C; (E) SOD-13 gene, 70–60°C; (F) SOD-18 gene, 70–60°C; (G) SOD-20 gene, 70–60°C; (H) SOD-20 gene, 70–60°C; and (I) β-actin gene, 67–65°C.*

was found to be 63.8°C. The annealing temperature of SOD-13 was found to be the highest (68°C). However, the annealing temperature was optimized considering the laboratory and device changes. Accordingly, β-actin annealing temperature was found to be 65.8°C. Gel images showing optimum temperature for each studied gene primer are given in Figure 1 and Table 2.

It seems that the determined annealing temperatures can be used without requiring an extra optimization step in studies carried out using PCR and real-time PCR methods.

## Discussion

Plants have developed appropriate defensive response systems to prevent or minimize stress-induced damage. In the long term, we need to understand how plants sense oxidative stress in their various compartments and how this stress signal is transmitted to turn on appropriate defense systems.

**Table 2.**
*SOD Genes and β-actin Gene Optimum Annealing Temperatures*

| Gene | Optimum Annealing Temperature |
|---|---|
| SOD-1 (7AL_E14A72218.2) | 66.4 |
| SOD-6 (2AS_529825553.1) | 64.5 |
| SOD-10 (2BL_7BF340BAE.1) | 64.5 |
| SOD-11 (7BL_A42D6C984.1) | 63.8 |
| SOD-13 (3B_2650F3AB3.2) | 68 |
| SOD-18 (2DL_F48387F4E.1) | 66.4 |
| SOD-20 (2BL_1724AE8A9.2) | 60.7 |
| SOD-21 (7DS_1CE2F67B7.1) | 60 |
| β-actin | 65.8 |

Note: SOD = superoxide dismutase.

Superoxide dismutase genes play a central role in protecting plants against the toxic effects of reactive oxygen species produced during normal cellular metabolic activity or because of various environmental stresses (Wang et al., 2016). Superoxide dismutase overexpression in some transgenic plants has been reported to increase tolerance to stress (Tseng et al., 2007). Similarly, it has been reported that some exogenous substances such as β-estradiol and spermidine can induce SOD expression and modify SOD activity, thus improving the stress tolerance of plants (Wang et al., 2016). Jiang and colleagues identified 26 SOD genes (TaSOD) in wheat and found that the propensity to change SOD gene expression in wheat roots and leaves following drought exposure was different (Jiang et al., 2019). These and similar results may have implications for agricultural and industrial production and are clearly findings worthy of further investigation.

Polymerase chain reaction as an in vitro technique in which a specific DNA segment stretching between two known parts of a DNA chain is enzymatically amplified forms the basis of many molecular methods used today. Regardless of the purpose of the reaction, it is necessary to optimize the reagents and PCR parameters to be used for each gene region (Kahya et al., 2013). In this sense, optimization of primers, which are one of the basic components of PCR, is an extremely important step. Fine-tuning the binding temperature is very important in order to reproduce the correct DNA fragment, especially when working with gene families with high sequence homology. Therefore, it is possible to say that optimization of the annealing temperature is crucial to obtain correct PCR products.

## Conclusion and Recommendations

Global warming and changes in climate cause significant decreases in crop quality and productivity every year as plants undergo higher environmental stress conditions. Finding the difference in gene expression levels due to these factors are important in the field of breeding. In the breeding process, it is important to establish standards to optimize them for use in molecular studies. Since oxidative stress occurs in all aerobic organisms, it can be expected that the basic defense systems would be preserved throughout the evolutionary process, and based on this, it can be said that SOD genes have an important place in engineering applications used to obtain more stress-resistant crops. This study is a preliminary data for future research.

**Peer-review:** Externally peer-reviewed.

**Author Contributions:** Concept – F.Ö., M.H.; Design – Z.M.; Analysis and Interpretation – A.E.B., M.T.; Writing Manuscript – A.E.B.; Critical Review – Z.M., M.T.

**Declaration of Interests:** The authors declare that they have no competing interest.

**Funding:** The authors declare that this study has received no financial support.

**Hakem Değerlendirmesi:** Dış bağımsız.

**Yazar Katkıları:** Fikir – F.Ö., M.H.; Tasarım – Z.M.; Analiz ve/veya Yorum – A.E.B., M.T.; Yazıyı Yazan – A.E.B.; Eleştirel İnceleme –Z.M., M.T.

**Çıkar Çatışması:** Yazarlar çıkar çatışması bildirmemişlerdir.

**Finansal Destek:** Yazarlar bu çalışma için finansal destek almadıklarını beyan etmişlerdir.

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