

## Biochemical Antioxidant Effects of Ellagic Acid Against Di-(n)-butyl Phthalate–Induced Oxidative Stress in Rat Brain Tissue

### Sıçan Beyin Dokusunda Di-(n)-bütil Ftalat Kaynaklı Oksidatif Strese Karşı Elajik Asidin Biyokimyasal Antioksidan Etkileri

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#### Abstract

Di-n-butyl phthalate (DBP), a widely used plasticizer, induces oxidative stress and neurotoxicity through the generation of reactive oxygen species. Ellagic acid (EA), a natural polyphenolic antioxidant, may counteract these effects. This study aimed to evaluate the protective role of EA against DBP-induced oxidative brain injury. Thirty-two male rats were randomly divided into four groups: Control, DBP, EA, and DBP+EA. DBP was administered orally at a dose of 500 mg/kg/day, while EA was given by gavage at 2 mg/kg/day for four weeks. Oxidative stress parameters, including thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) activities were analyzed in brain tissue. DBP exposure significantly increased TBARS levels while decreasing GSH and SOD activities, indicating lipid peroxidation and antioxidant depletion. In contrast, CAT activity was elevated, suggesting a compensatory enzymatic response to excess hydrogen peroxide. EA treatment markedly attenuated oxidative damage by restoring GSH and SOD levels and normalizing CAT activity. These findings indicate that DBP disrupts redox homeostasis by inducing lipid peroxidation, while EA mitigates this effect through restoration of antioxidant defenses.

**Keywords:** Antioxidant Enzymes, Di-n-butyl Phthalate, Ellagic Acid, Oxidative Stress, Rat Brain.

#### Özet

Di-n-butil ftalat (DBP), yaygın olarak kullanılan bir plastikleştirici olup, reaktif oksijen türlerinin (ROS) oluşumu yoluyla oksidatif stres ve nörotoksositeye neden olur. Doğal bir polifenolik antioksidan olan elajik asit (EA), bu etkileri dengeleyebilir. Bu çalışmada, EA'nın DBP kaynaklı oksidatif beyin hasarına karşı koruyucu rolü araştırılmıştır. Otuz iki erkek sıçan rastgele dört gruba ayrılmıştır: Kontrol, DBP, EA ve DBP+EA. DBP, oral yolla 500 mg/kg/gün dozunda, EA ise dört hafta boyunca gavaj yoluyla 2 mg/kg/gün dozunda uygulanmıştır. Beyin dokusunda tiyobarbitürik asit reaktif maddeleri (TBARS), indirgenmiş glutatyon (GSH), süperoksit dismutaz (SOD) ve katalaz (CAT) aktivitelerini kapsayan oksidatif stres parametreleri analiz edilmiştir. DBP uygulaması, lipid peroksidasyonunu ve antioksidan tükenmesini yansıtarak TBARS düzeylerini anlamlı şekilde artırmış, GSH ve SOD aktivitelerini azaltmıştır. Buna karşın, CAT aktivitesi artmış olup aşırı hidrojen peroksitine karşı gelişen kompanse edici bir enzimatik yanıtı düşündürmektedir. EA tedavisi, GSH ve SOD düzeylerini geri kazandırarak ve CAT aktivitesini normale döndürerek oksidatif hasarı belirgin şekilde hafifletmiştir. Bu bulgular, DBP'nin lipid peroksidasyonu yoluyla redoks homeostazını bozduğunu, EA'nın ise antioksidan savunmayı yeniden düzenleyerek oksidatif nörotoksositeye karşı koruma sağladığını göstermektedir.

**Anahtar Kelimeler:** Antioksidan Enzimler, Di-n-butil Ftalat, Elajik Asit, Oksidatif Stres, Sıçan Beyni.

## Introduction

Phthalates, esters of phthalic acid (1,2-benzenedicarboxylic acid), are extensively used as plasticizers in industrial and consumer products, including building materials, toys, paints, pharmaceutical packaging, personal care items, and food containers (1, 2). Di-(n)-butyl phthalate (DBP) is among the most common, present in vinyl flooring, paints, plastic bags, gloves, shoes, and a variety of food packaging materials (3, 4). As it is not covalently bound to plastics, DBP can easily leach into the environment, leading to human exposure via ingestion, inhalation, or dermal absorption (3). DBP has been detected in multiple tissues and can cross the blood-brain barrier (5, 6). Its exposure has been linked to reproductive toxicity, developmental defects, respiratory disorders, and more recently, neurotoxicity (7-10).

Although research is still limited, accumulating evidence indicates that DBP can also exert neurotoxic effects (10). For instance, DBP has been shown to impair neural progenitor cell proliferation, disrupt hippocampal neurogenesis, and impair spatial learning and memory in animal models (11). In zebrafish embryos, DBP alters the expression of neurotoxicity biomarkers (12). In *Caenorhabditis elegans*, DBP exposure increases reactive oxygen species (ROS) production, implicating oxidative stress as a possible mechanism (13). Similarly, DBP induces neuronal cell death through aryl hydrocarbon receptor-mediated ROS generation (14) and reduces cell viability in mouse embryonic stem cells via intracellular ROS accumulation (15). Collectively, these findings suggest that oxidative stress is a major contributor to DBP-induced neurotoxicity.

Oxidative stress reflects an imbalance between the generation of reactive oxygen species (ROS) and the capacity of endogenous antioxidant defenses to neutralize them (16). Lipid peroxidation, a hallmark of ROS-mediated cellular damage, results in the formation of malondialdehyde (MDA), which is commonly quantified as thiobarbituric acid reactive substances (TBARS) to assess oxidative injury in tissues (17). Thus, an increase in TBARS levels indicates enhanced lipid peroxidation and oxidative damage to cellular membranes. Reduced glutathione (GSH) is a major non-enzymatic antioxidant that directly scavenges free radicals and serves as a substrate for glutathione-dependent enzymes, so depletion of GSH reflects impaired cellular antioxidant capacity (18). Superoxide

dismutase (SOD) and catalase (CAT) are key enzymatic antioxidants that work cooperatively to detoxify ROS. SOD catalyzes the dismutation of superoxide radicals into hydrogen peroxide, which is subsequently decomposed into water and oxygen by CAT (19, 20). Alterations in the activities of SOD and CAT thus provide insight into the enzymatic antioxidant response to oxidative insults. Together, these biochemical parameters are widely accepted markers for evaluating oxidative stress and antioxidant defense mechanisms in toxicological studies. Strengthening the antioxidative defense system is a key strategy for reducing oxidative stress, leading to increased interest in natural compounds, especially polyphenols, for their potential protective effects (21).

Natural polyphenols have attracted attention for their antioxidative and neuroprotective potential. Ellagic acid (EA), a naturally occurring polyphenol abundant in fruits such as pomegranates, blueberries, raspberries, black mulberries, blackberries, strawberries, and in nuts such as walnuts, exhibits strong antioxidant potential (22). EA possesses a wide range of pharmacological activities, including anti-inflammatory, anticancer, and neuroprotective effects, largely attributed to its ability to neutralize ROS (23, 24). Although EA has been identified as a promising neuroprotective agent (25), studies on its neuroprotective role remain limited. Previous studies report that EA alleviates oxidative brain injury in models of Parkinson's disease and toxin-induced neurotoxicity (26, 27). Given the widespread presence of DBP in the environment and the potent antioxidant properties of EA, this study aimed to investigate the protective effects of EA against DBP-induced oxidative stress in rat brain tissue.

## Material and Method

### Chemicals

DBP (CAS: 84-74-2) was obtained from ABCR GmbH, and EA (CAS: 476-66-4) from Alfa Aesar. All other reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and were of analytical grade or the highest available purity.

### Animals

All experimental protocols were approved by the Animal Research Ethics Committee of Burdur Mehmet Akif Ersoy University (protocol no. 2024/1286) and conducted in accordance with the

National Institutes of Health Guide for the Care and Use of Laboratory Animals. Thirty-two healthy adult male Wistar albino rats (3–4 months old, 280–320 g) were housed in sterilized polypropylene cages under a 12-h light/dark cycle at ~23°C, ~55% humidity, with free access to standard pellet diet and water ad libitum.

The rats were randomly assigned to four groups (n = 8 per group)

- Group 1 (Control): Received the vehicle (carboxymethyl cellulose, CMC) both intraperitoneally (i.p.) and by oral gavage.
- Group 2 (DBP): Received DBP i.p. at 500 mg/kg/day and vehicle of EA by oral gavage.
- Group 3 (EA): Received EA 2 mg/kg/day by oral gavage and vehicle of DBP by i.p.
- Group 4 (DBP + EA): Received DBP i.p. at 500 mg/kg/day and EA 2 mg/kg/day by oral gavage.

At the end of the 4-week treatment, rats were anesthetized, and brain tissues were rapidly removed and dissected over ice. Samples were stored at –86 °C until analysis.

#### *Biochemical Assays*

Brain tissues were homogenized in a Teflon–glass homogenizer with 150 mM potassium chloride (pH 7.4) to obtain a 1:10 (w/v) dilution. The homogenates were centrifuged at 18,000 × g for 30 min at 4 °C to determine TBARS, GSH levels, and CAT activity, and at 25,000 × g for 50 min to assess copper/zinc-SOD activity. Lipid peroxidation was evaluated by measuring TBARS levels using the thiobarbituric acid (TBA) method described by Yagi, with absorbance read at 532 nm and results expressed as nmol/mg tissue protein (17). GSH content was determined according to the method of Sedlak and Lindsay at 412 nm and expressed as nmol/mg tissue protein (18). SOD activity was measured by the inhibition of

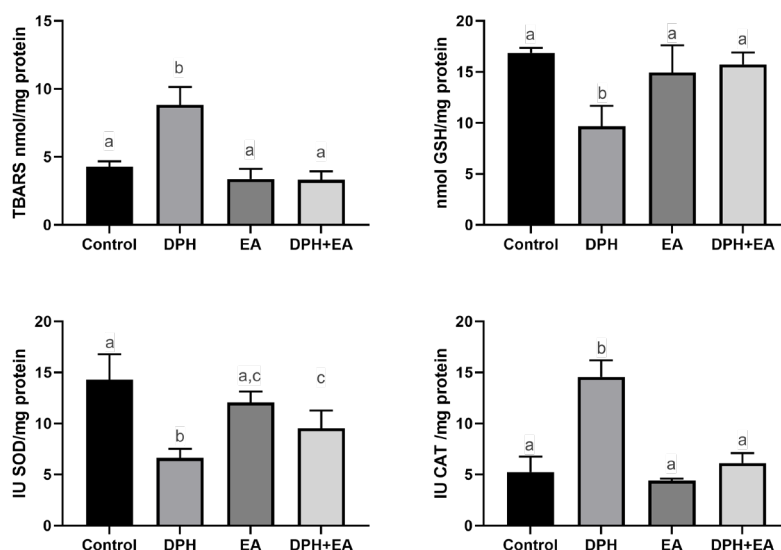
nitroblue tetrazolium (NBT) reduction via the xanthine/xanthine oxidase system, as described by Sun et al., with one unit defined as the amount of protein causing 50% inhibition of NBT reduction; results were expressed as IU/mg protein at 560 nm (19). CAT activity was determined according to Aebi by monitoring the decomposition of hydrogen peroxide at 240 nm, expressed as IU/mg protein (20). Total protein concentration in tissue homogenates was determined using the Lowry method with bovine serum albumin as the standard (28).

#### *Statistical Analysis*

Data are expressed as mean ± standard deviation (SD), and differences were considered statistically significant at  $P < 0.05$ . The normality of data distribution was assessed using the Shapiro–Wilk test, which confirmed that all variables exhibited a normal distribution. Intergroup comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Statistical analyses were conducted using GraphPad Prism, version 9 (GraphPad Software Inc., San Diego, CA, USA).

#### **Results**

Analysis of biochemical parameters revealed statistically significant variations between the experimental groups (Table 1 and Figure 1). The level of TBARS, a widely used marker of lipid peroxidation, was significantly elevated in the brain tissue of rats exposed to DBP compared to the control group. This increase indicates enhanced lipid peroxidation following DBP administration. In the group receiving EA alongside DBP, TBARS levels were significantly reduced relative to the DBP-only group, approaching values observed in the control animals.



Levels of thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) in rat brain tissue across the Control, DBP, EA, and DBP+EA groups. DBP: di-n-butyl phthalate, EA: ellagic acid. Data are presented as mean  $\pm$  SD. Statistical analyses were performed using one-way ANOVA followed by Tukey's post-hoc test. Different letters (a, b, c) indicate statistically significant differences between groups for each biochemical parameter ( $p < 0.05$ ).

**Figure 1.** Effects of DBP and ellagic acid on biochemical parameters in rat brain tissue

**Table 1.** Comparison of oxidative stress and antioxidant parameters among experimental groups.

| Groups (n:8)            | TBARS<br>(nmol/mg protein)   | Reduced GSH<br>(nmol/mg protein) | SOD<br>(U/mg protein)           | CAT<br>(U/mg protein)         |
|-------------------------|------------------------------|----------------------------------|---------------------------------|-------------------------------|
| Control                 | 4.27 $\pm$ 0.41 <sup>a</sup> | 16.86 $\pm$ 0.49 <sup>a</sup>    | 14.30 $\pm$ 2.49 <sup>a</sup>   | 5.25 $\pm$ 1.51 <sup>a</sup>  |
| DBP                     | 8.83 $\pm$ 1.32 <sup>b</sup> | 9.67 $\pm$ 2.01 <sup>b</sup>     | 6.62 $\pm$ 0.90 <sup>b</sup>    | 14.57 $\pm$ 1.62 <sup>b</sup> |
| EA                      | 3.39 $\pm$ 0.74 <sup>a</sup> | 14.96 $\pm$ 2.65 <sup>a</sup>    | 12.06 $\pm$ 1.09 <sup>a,c</sup> | 4.43 $\pm$ 0.19 <sup>a</sup>  |
| DBP+EA                  | 3.32 $\pm$ 0.63 <sup>a</sup> | 15.74 $\pm$ 0.16 <sup>a</sup>    | 9.52 $\pm$ 1.76 <sup>c</sup>    | 6.13 $\pm$ 0.98 <sup>a</sup>  |
| P values of comparisons |                              |                                  |                                 |                               |
| Control vs. DPH         | <b>0.001</b>                 | <b>0.001</b>                     | <b>0.001</b>                    | <b>0.001</b>                  |
| Control vs. EA          | 0.299                        | 0.282                            | 0.129                           | 0.714                         |
| Control vs. DPH+EA      | 0.238                        | 0.700                            | <b>0.001</b>                    | 0.670                         |
| DPH vs. EA              | <b>0.001</b>                 | <b>0.001</b>                     | <b>0.001</b>                    | <b>0.001</b>                  |
| DPH vs. DPH+EA          | <b>0.001</b>                 | <b>0.001</b>                     | <b>0.034</b>                    | <b>0.001</b>                  |
| EA vs. DPH+EA           | 0.999                        | 0.872                            | 0.071                           | 0.163                         |

Levels of thiobarbituric acid reactive substances (TBARS), reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) levels in rat brain tissue from the Control, DBP, EA, and DBP+EA groups. DBP: di-n-butyl phthalate; EA: ellagic acid. Values are expressed as mean  $\pm$  SD. Different letters (a, b, c) indicate statistically significant differences between groups for each biochemical parameter, as determined by one-way ANOVA followed by Tukey's post-hoc test ( $p < 0.05$ ).

Analysis of GSH content revealed a significant decrease in the DBP-treated group compared to controls. The group co-treated with EA and DBP exhibited significantly higher GSH levels than the DBP-only group, although GSH concentrations did not fully reach control levels.

SOD activity, a key enzymatic antioxidant defense against superoxide radicals, was significantly reduced in the brains of DBP-exposed rats compared to controls. In the EA-only group, SOD activity did not differ significantly

from the control group. Co-administration of EA with DBP increased SOD activity relative to the DBP group; however, the levels did not fully reach those of the control group and remained lower.

CAT activity, responsible for hydrogen peroxide decomposition, was significantly increased in the DBP group, whereas EA administration restored catalase levels in the DBP+EA group to values comparable to the control group, with no statistically significant difference observed between them.

## Discussion

This study investigated the effects of DBP exposure on oxidative stress parameters in rat brain tissue and evaluated the potential protective role of EA. Our biochemical findings demonstrated that DBP induces oxidative damage, as evidenced by increased lipid peroxidation and decreased antioxidant enzyme activities, while EA co-administration mitigates these effects by enhancing antioxidant defenses.

The observed elevation of TBARS levels in DBP-exposed rats aligns with previous studies reporting increased lipid peroxidation as a key feature of phthalate-induced neurotoxicity. A significant increase in malondialdehyde levels in brain regions following DBP exposure has been reported, indicating enhanced lipid peroxidation as measured by the TBARS assay (29). Similarly, an increase in ROS generation has been observed in neuronal cells exposed to DBP in a concentration and time-dependent manner, corroborating the role of oxidative stress in DBP-induced neurotoxicity (14).

GSH depletion observed following DBP exposure is consistent with studies highlighting compromised intracellular antioxidant capacity resulting from phthalate toxicity. In isolated human lymphocytes, DBP-induced oxidative stress has been reported to cause marked GSH depletion, accompanied by mitochondrial and lysosomal dysfunction (30). Similarly, a study utilizing mouse brain tissue reported that DBP exposure led to significant GSH depletion, supporting the involvement of impaired antioxidant defense mechanisms in DBP-mediated neurotoxicity (31). Importantly, EA has been shown to counteract such oxidative damage by restoring intracellular redox balance and enhancing GSH-dependent antioxidant defenses. EA has been reported to significantly mitigate arsenic-induced neuronal ferroptosis and cognitive impairment by activating the Nrf2/GPX4 signaling pathway, thereby preventing GSH depletion and suppressing lipid peroxidation in brain tissue (32). Moreover, EA has been shown to restore GSH levels via modulation of the Nrf2 pathway, which may contribute to the reversal of oxidative damage induced by toxic insults (33). These findings support the notion that EA's multi-targeted antioxidant and anti-inflammatory actions could effectively attenuate DBP-induced neurotoxic effects by preserving GSH levels and maintaining redox homeostasis.

The suppression of the key antioxidant enzyme SOD by DBP observed in this study is consistent with previous reports, indicating a general impairment of enzymatic antioxidant defense systems under phthalate-induced oxidative stress. Wójtowicz et al. demonstrated that DBP exposure leads to increased ROS levels via aryl hydrocarbon receptor-mediated oxidative stress pathways, suggesting a mechanistic link between phthalate toxicity and redox imbalance at the cellular level (14). Similarly, in a 12-week study using rat models, DBP exposure significantly decreased both SOD and CAT activities in the brain, reflecting a pronounced oxidative burden and weakened antioxidant defense (5). Comparable reductions in SOD and CAT activities were also reported in *Cyprinus carpio* following DBP exposure, along with increased lipid peroxidation and reduced brain cholinesterase activity (34). Although these studies support the oxidative and neurotoxic effects of DBP, our results diverge in terms of CAT response, while SOD activity decreased, CAT activity was elevated in our 4-week exposure model. On the other hand, our results are consistent with studies reporting increased CAT activity in response to agents that promote ROS generation (35, 36). This finding aligns with literature indicating that under mild to moderate oxidative stimuli, CAT expression or activity can be induced as part of an adaptive defense response, whereas prolonged or excessive ROS exposure may ultimately suppress or inhibit the enzyme (37, 38). Therefore, the CAT activity observed in our study likely represents the early, compensatory phase of a biphasic response, an initial increase during short- to mid-term oxidative exposure, potentially followed by downregulation under prolonged stress. If the exposure were extended, CAT activity would likely decline, consistent with the patterns reported in the aforementioned DBP studies.

In the present study, the restoration of antioxidant enzyme activities following EA administration aligns with the neuroprotective properties previously attributed to this compound in experimental models of neurotoxicity. Ríos et al. (2018) emphasized EA's dual mode of antioxidant action, involving both the upregulation of endogenous defense enzymes through activation of the Nrf2/ARE signaling pathway and direct scavenging of ROS, thereby alleviating oxidative burden (23). This bidirectional mechanism may account for the reversal of DBP-induced enzymatic suppression observed in our study, suggesting that EA not only replenishes

cellular antioxidant capacity but also provides sustained protection against ongoing oxidative insults in neural tissues. Similarly, a recent study in an Alzheimer's disease mouse model reported that EA alleviated oxidative stress in brain tissue by enhancing SOD activity (39). Consistent with our findings, EA reversed DBP-induced reductions in SOD activity, further supporting its role in reinforcing enzymatic antioxidant defenses within neural tissue.

One limitation of the present study is the absence of histopathological and behavioral assessments that could complement the biochemical data with structural and functional evidence. However, the study provides biologically meaningful findings by demonstrating clear alterations in well-established oxidative stress markers associated with neurotoxicity and antioxidant defense. These results not only strengthen the evidence for DBP-induced oxidative brain injury and the neuroprotective potential of ellagic acid but also offer a solid scientific basis for future investigations. Accordingly, our findings have the potential to stimulate further studies incorporating histopathological and behavioral evaluations to achieve a more comprehensive understanding of the underlying mechanisms.

Overall, our findings contribute to the growing body of evidence implicating oxidative stress as a key mechanism underlying DBP-induced neurotoxicity. Moreover, they highlight the therapeutic potential of natural polyphenols such as EA as promising neuroprotective agents. Future research exploring molecular signaling pathways including mitochondrial redox regulation, post-translational modifications of antioxidant enzymes, and the time-course kinetics of CAT expression and inactivation could provide deeper insights into the precise mechanisms underlying EA-mediated protection against phthalate neurotoxicity.

## Conclusion

In conclusion, the present study demonstrates that DBP induces marked oxidative stress in rat brain tissue, as reflected by increased lipid peroxidation and altered antioxidant enzyme activities. These findings strengthen the evidence linking DBP exposure to neurotoxic outcomes mediated through excessive ROS generation. Notably, co-administration of EA effectively mitigated DBP-induced oxidative injury by restoring redox balance, normalizing glutathione

levels, and modulating the activities of key antioxidant enzymes such as SOD and CAT. These findings suggest that EA may exert protective effects through free radical scavenging activity and modulation of antioxidant defense mechanisms. Further studies, particularly long-term and mechanistic investigations, are warranted to elucidate the molecular pathways underlying EA's protective effects and to explore its translational potential in preventing environmental pollutant-related neurological disorders.

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None

## Conflict of interest statement

The authors do not declare any conflicts of interest/competing interests.

## Ethics Committee Approval

Experimental protocols were approved by the Animal Research Ethics Committee of Burdur Mehmet Akif Ersoy University on 25.04.2024 with protocol no. 2024/1286.

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