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Antimicrobial Effect of 2-Phenylethynyl-Butyltellurium in *Escherichia coli* and Its Association with Oxidative Stress

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This study aimed to evaluate the antimicrobial activity of 2-phenylethynyl-butyltellurium (PEBT) in *Escherichia coli* and the relation to its pro-oxidant effect. For this, we carried out the disk diffusion test, minimum inhibitory concentration (MIC) assay, and survival curve analysis. We also measured the level of extracellular reactive oxygen species (ROS), activity of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT), and level of non-protein thiols (NPSH). PEBT at 1.28 and 0.128 mg/disk exhibited antimicrobial capability in the disk diffusion test, with an MIC value of 1.92 mg/ml, whereas PEBT at 0.96, 1.92, and 3.84 mg/ml inhibited bacterial growth after a 9-h exposure. PEBT at 3.84, 1.92, and 0.96 mg/ml increased extracellular ROS production, decreased the intracellular NPSH level, and reduced the SOD and CAT activities. Glutathione or ascorbic acid in the medium protected the bacterial cells from the antimicrobial effect of PEBT. In conclusion, PEBT exhibited antimicrobial activity against *E. coli*, involving the generation of ROS, oxidation of NPSH, and reduction of the antioxidant defenses in the bacterial cells.

Keywords: Antibacterial, tellurium, oxidative stress, pro-oxidant activity, thiol groups

Introduction

The emergence, propagation, accumulation, and maintenance of antimicrobial-resistant pathogenic bacteria have become a health concern in human and veterinary medicine worldwide [1]. Antibiotic resistance is one of the main causes of the difficulty in curing infectious diseases [2]. The abusive and indiscriminate use of antimicrobial drugs over the years is the main factor responsible for the appearance of antibacterial resistance [3]. This imposes severe limitations on therapeutic options, implying a threat to public health [4].

Pathogenic strains of *Escherichia coli*, a gram-negative rod-shaped bacterium found as a normal flora in the gastrointestinal tract of animals and humans, have emerged by the acquisition of virulence factors through transposons, plasmids, bacteriophage, and/or pathogenicity islands [5]. The level of antimicrobial resistance in *E. coli* is a useful indicator of the level of resistance expected in pathogenic bacteria. Antimicrobial-resistant bacteria and antimicrobial resistance genes can be exchanged between the animal reservoir and the human reserve, as a consequence of direct contact with animals or their environment or indirect contact through the food chain [1]. Studies have shown the effect of oxidative stress on the antimicrobial activity of drugs, such as fluoroquinolones and ciprofloxacin, whose antimicrobial activities are affected by the production of reactive oxygen species (ROS) [6]. Additionally, norfloxacin, ampicillin, and kanamycin showed an ability to induce oxidative stress and cell death in *E. coli* [7]. We have previously described ciprofloxacin as one of several antibiotics that induce oxidative stress in bacteria [8].

Substances that affect the generation of ROS in bacterial cells have the capability to undergo redox cycling, resulting

in the generation of superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). Moreover, the production of ROS in *E. coli*, including superoxide anion, hydrogen peroxide, and hydroxyl radicals, are mainly related to the activity of the respiratory chain [9], and the increase in ROS level causes oxidative stress and thiol oxidation. Thiol oxidation has multiple damaging effects on cellular macromolecules. Some of these thiols form a part of cellular proteins, such as the *OxyR* transcriptional regulator, which is transitorily activated by the formation of disulfide linkage under oxidative stress [10, 11].

To cope with oxidative stress, microorganisms use various defense mechanisms involving complementary action in different pathways. These include the evolution of specific enzymes targeted against a particular adverse substance [12]. For example, three superoxide dismutases (SODs) (encoded by *sodA*, *sodB*, and *sodC* genes) and two catalases, hydroperoxidase I and II (encoded by *katG* and *katE*, respectively), have been described in *E. coli* [13].

Several studies showed that compounds derived from tellurium exhibit toxic effects against microorganisms. For example, oxyanion tellurite (TeO_3^{2-}) is extremely toxic to most microorganisms, particularly gram-negative bacteria [14, 15]. It has been suggested that tellurite toxicity is due to its strong oxidizing ability, which might interfere with many cellular enzymatic processes [16]. Bacteria turn black upon exposure to tellurite because of the deposition of elemental tellurium (Te) within the cell [17]. Studies on the biological effects of inorganic and organic tellurium compounds have led to various interesting and promising applications [18].

The pharmacological properties of organotellurium compounds have been the subject of many research studies [19–22]. A telluroacetylene compound, 2-phenylethynyl-butyltellurium (PEBT), at a low concentration has shown pharmacological effects in animal models of neurotoxicity and memory [20–22]. In contrast, several studies have established the toxicity of organotellurium compounds, including PEBT, which is associated with the oxidation of thiol groups in bioactive molecules, inhibiting sulfhydryl enzymes (δ-aminolevulinate dehydratase and Na^+ K^+ ATPase), or even decreasing the glutathione (GSH) concentration [20, 21, 23–25].

Several studies have also shown the involvement of oxidative stress in the antimicrobial activity of drugs. Antibiotics, such as fluoroquinolones and ciprofloxacin, exert antibacterial activity by inducing the generation of ROS [6]. In addition, it has been reported that the non-enzymatic antioxidant GSH exhibits a protective effect against ciprofloxacin-induced ROS production in microorganisms [4]. Based on the above considerations, the present study aimed to verify the antimicrobial activity of PEBT in *E. coli* and examine the relation between its antimicrobial action and its pro-oxidant effect.

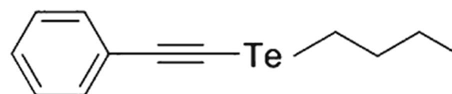

Fig. 1. Chemical structure of 2-phenylethynyl butyltellurium.

Materials and Methods

Chemicals

The compound PEBT (Fig. 1) was prepared according to a previous method [26]. Analysis of the ^1H NMR and ^{13}C NMR spectra showed that the synthesized PEBT exhibited analytical and spectroscopic data consistent with its designed structure. All other chemicals were of analytical grade and were purchased from standard commercial suppliers.

Bacterium and Growth Conditions

Escherichia coli (CCBH 17961) was obtained from the Oswaldo Cruz Foundation–FIOCRUZ (Brazil). The colonies were kept frozen in 10% glycerol until use. For each experiment, the colonies were transferred to nutrient broth and incubated for 24 h at 37°C.

Antimicrobial Activity

Disk diffusion method. The disk diffusion method of antimicrobial resistance assay was carried out according to a previous method [27] with modifications. Isolates were cultivated in nutrient broth at 36°C and adjusted in a series of 0.85% saline solution at 0.5 McFarland scale. The culture was subsequently spread on a plate containing Mueller-Hinton Agar (MHA). Paper disks of ±6 mm in diameter (Laborclin, Ltda, Brazil) were soaked with 10 µl of PEBT at concentrations of 1.28, 0.128, 0.0128, and 0.00128 mg/disk diluted in dimethyl sulfoxide (DMSO) and were placed on the seeded plates. The plates were incubated at 37°C for 24 h. The halo formed was measured in centimeters from one extremity to the other. Paper disks soaked with DMSO (10 µl) were used as a negative control. Each experiment was performed in triplicate.

Effects of antioxidants in the disk diffusion method. To examine the relation between the pro-oxidant and antimicrobial activities of PEBT, we performed the agar disk diffusion method using the antioxidants GSH and ascorbic acid (AA). *E. coli* was subcultured in nutrient agar and incubated at 37°C for 24 h to prepare the inoculums. The inoculum was dissolved in a series of sterile saline solution to a final concentration of approximately 1.5×10^8 CFU/ml adjusted at 0.5 McFarland scale. MHA with or without 10 mM solutions of GSH or AA was poured into sterilized

petri dishes, where the inoculum was subsequently spread on. The 6-mm paper disks were soaked with 10 μl of PEBT at concentrations of 1.28, 0.128, 0.0128, and 0.00128 mg dissolved in DMSO and were placed on the seeded plates. The plates were incubated at 37°C for 24 h. The inhibition of the bacterial growth was determined by measuring the inhibition zone around the disks by a digital caliper. DMSO was used as a negative control. The method above was performed according to previous studies [28, 29] with modifications. Each experiment was performed in triplicate.

Broth macrodilution assay for minimum inhibitory concentration (MIC). The MIC of PEBT was determined by the broth macrodilution method according to the CLSI [27]. Seventeen micrograms of a standard bacterial inoculum of 5×10^5 colony forming units (CFU)/ml was diluted serially in various concentrations of PEBT (3.84, 1.92, 0.96, 0.48, 0.24, 0.12, 0.06, and 0.030 mg/ml) dissolved in DMSO, and the inoculum was incubated for 24 h at 36°C. The MIC was defined as the lowest concentration of compound that completely inhibited visible growth. The experiment was performed in triplicate.

Kill-time curve assay. The kill-time curve assay method [30] was used to investigate the bactericidal effects of PEBT against *E. coli* at 0.5 MIC, MIC, and 2 MIC. Tests were performed in triplicate at 37°C. At the predetermined time points (0, 3, 6, 9, 12, and 24 h), 15 μl of sample was removed from each test suspension, diluted in sterile saline 0.9%, and plated on MHA plates for colony count determination. Data from triplicate runs were averaged and plotted as log CFU/ml *versus* time (h) for each time point.

Extracellular Reactive Oxygen Species Assay

E. coli was cultured in nutrient agar for 24 h at 37°C and suspended in sterile 0.9% saline, pH 7.4. Its absorbance was adjusted to 0.8 at OD_{600}. Subsequently, 67 μl of *E. coli* was incubated with 10 μl of 2′,7′-dichlorofluorescein diacetate (DCHF-DA; 10 nM) and PEBT at 0.96, 1.92, and 3.84 mg/ml (corresponding to 0.5 MIC, MIC, and 2 MIC, respectively). DCF fluorescence intensity emission was recorded at 520 nm excitation (with 488 nm emission) for 30 min for the detection of ROS in *E. coli*. The ROS level was expressed as units of fluorescence. The method described above was performed according to a previous study [31] with modifications.

Incubation and Preparation of Lysates

Bacterial cells (50 ml) harvested from nutrient broth were centrifuged at 2,822.4 ×*g* for 10 min. The supernatant was removed, whereas the pellet was weighed, transferred to microtubes, and resuspended in 1 ml of 0.1 mM sodium phosphate buffer. The suspension was incubated for 30 min at 37°C in the presence of PEBT at 0.96, 1.92, or 3.84 mg/ml (corresponding to 0.5 MIC, MIC, and 2 MIC, respectively), or DMSO (control group). Afterwards, 0.9 g of glass beads was added to the microtubes, which were then mixed 6 times by a vortexer for 5 min each time and incubated for 2 min in an ice bath to complete a 30-min cycle [32]. Debris was removed from the suspension by centrifugation at 2,822.4 ×*g* for 10 min, after which the supernatant was collected for biochemical assays.

Non-Protein Thiol Levels

The levels of intracellular non-protein thiols (NPSHs) in *E. coli* were estimated using spectrophotometry according to a previous method [33] with adaptations. To prepare the sample, 200 μl of supernatant was added to 200 μl of 10% TCA. The mixture was vortexed for 1 min and centrifuged at 705.6 ×*g* for 10 min. To 50 μl of supernatant, 750 μl of 1 M potassium phosphate buffer (TFK) and 50 μl of 10 mM 5,5′-dithiobis(2-nitrobenzoic acid) were added. The absorbance was measured at 412 nm immediately after the addition of the reagents.

Superoxide Dismutase Activity

The activity of SOD was determined at 406 nm as the inhibition of quercetin oxidation in a reaction medium containing 30 mM Tris-HCl buffer (pH 9.0), 0.3 mM EDTA, 0.8 mM TEMED, 14 μM quercetin, and 30 μl of supernatant in a final volume of 2 ml. One unit of SOD activity was defined as the amount of supernatant protein that inhibited the maximum rate of quercetin oxidation by 50% [34].

Catalase (CAT) Activity

CAT activity was measured in accordance with a published method [35]. A solution was prepared using 0.25 M potassium phosphate buffer, 2.5 mM EDTA (pH 7.0), 30% H_2O_2, and aqueous Triton X-100. For the test, 2 ml of the prepared solution was added to 60 μl of supernatant. The absorbance was monitored at 240 nm for 1 min.

Protein Concentration

The protein concentration was determined by the Coomassie brilliant blue G-250 dye-binding method using bovine serum albumin as a standard [36].

Statistical Analysis

The data were analyzed using Prism 5 (GraphPad) software. Comparisons between the experimental and control groups were performed by one-way analysis of variance (ANOVA), followed by the Newman–Keuls post-hoc test. Data are expressed as the mean ± SD. Probability values less than 0.05 ($p < 0.05$) were considered statistically significant.

Results

PEBT Caused Inhibition of *E. coli* Growth and the Presence of GSH and AA Blocked the Antimicrobial Effect of PEBT

In the present study, *E. coli* was sensitive to PEBT at the concentrations of 1.28−0.128 mg/disk in a dose-dependent

Table 1. Effect of glutathione (GSH) or ascorbic acid (AA) on 2-phenylethynyl-butyltellurium (PEBT) inhibition of *Escherichia coli* growth.

PEBT (mg/disk)	Diameter of the inhibition zone (nm)		
	Medium	Medium + GSH	Medium + AA
0			
0.00128			
0.0128			
0.128	1.067 ± 0.3055*		
1.28	1.267 ± 0.3055*		

Data are reported as the mean ± SD and were analyzed using one-way ANOVA, followed by the Newman–Keuls test. *Numerical values are significantly different from the values of the corresponding control ($p < 0.05$).

manner. In contrast, it showed no sensitivity to PEBT at 0.0128 and 0.00128 mg/disk, as shown in Table 1.

The presence of 10 mM GSH or AA in the growth medium lowered the susceptibility of *E. coli* to PEBT ($p < 0.05$), as shown in Table 1. The presence of AA in the culture medium blocked the antimicrobial effect of 1.28 and 0.128 mg/disk PEBT, indicated by the significantly reduced inhibition zone compared with that without AA. The antioxidant GSH also decreased the antimicrobial effect of 1.28 and 0.128 mg/disk PEBT.

Minimum Inhibitory Concentrations

The MIC of PEBT as an antimicrobial agent was 1.92 mg/ml, as shown in Table 2. This MIC value was used as the PEBT concentration in the subsequent examination of antioxidant enzyme activity, NPSH levels, and ROS generation.

Kill-Time Curve

E. coli was incubated for 24 h in the presence of PEBT at concentrations relative to its MIC, namely 0.5 MIC, MIC, and 2MIC, which corresponded to 0.96, 1.92, and 3.68 mg/ml, respectively, in order to determine the time-response effect of the compound. Following incubation, no viable cells of *E. coli* were observed (Fig. 2) in the PEBT-treated cultures.

PEBT Exposure Increases Reactive Oxygen Species Production

The ROS level was used to monitor the formation of extracellular ROS in *E. coli* treated with various concentrations

Table 2. Minimum inhibitory concentration (MIC) of 2-phenylethynyl-butyltellurium in *Escherichia coli*.

	0.5 MIC (mg/ml)	MIC (mg/ml)	2 MIC (mg/ml)
Escherichia coli	0.96	1.92	3.68

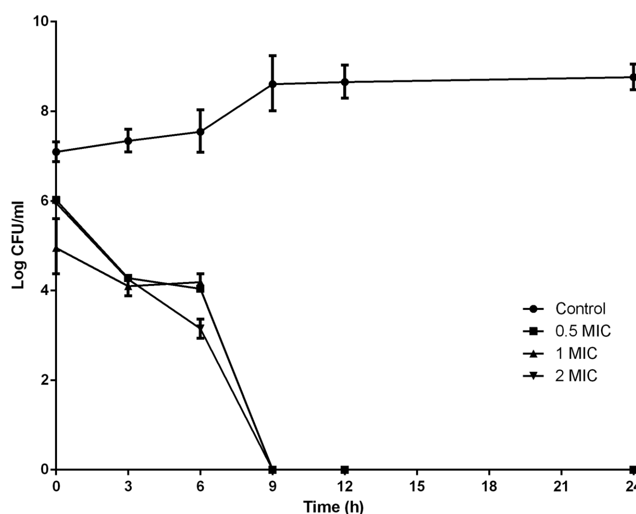

Fig. 2. Time-course of different concentrations of PEBT after exposure to *E. coli* 0.5 MIC (0.5-fold minimum inhibitory concentration), MIC (1-fold minimum inhibitory concentration), and 2 MIC (2-fold minimum inhibitory concentration).
Data are reported as the mean ± SD and were analyzed by one-way ANOVA, followed by the Newman–Keuls test when appropriate.

of PEBT (0.96, 1.92, and 3.68 mg/ml). PEBT at all tested concentrations had pro-oxidant activity in *E. coli*, as indicated by the increase in ROS level compared with the control group (Fig. 3). This result suggested that the pro-oxidative effects of PEBT are mediated, at least in part, by an increase in extracellular ROS concentration.

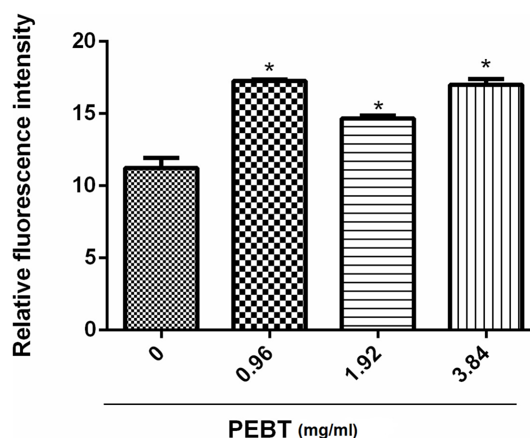

Fig. 3. Total extracellular reactive oxygen species in *E. coli* exposed to PEBT.
Data are reported as the mean ± SD and were analyzed by one-way ANOVA, followed by the Newman–Keuls test when appropriate. *Numerical values are significantly different from the values of the corresponding control ($p < 0.05$).

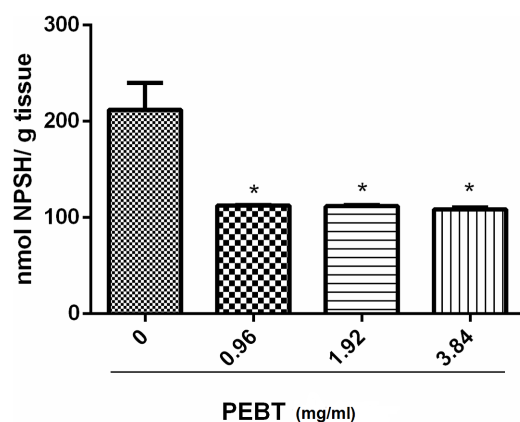

Fig. 4. Levels of non-protein thiols (NPSHs) in *E. coli* exposed to PEBT.

Data are reported as the mean ± SD and were analyzed by one-way ANOVA, followed by the Newman–Keuls test when appropriate. *Numerical values are significantly different from the values of the corresponding control ($p < 0.05$).

PEBT Exposure Decreased Non-Protein Thiol Levels

NPSH levels were measured in *E. coli* exposed to PEBT at 0.96, 1.92, and 3.68 mg/ml. Significant reduction in the NPSH levels was observed in the cultures exposed to PEBT at all concentrations (Fig. 4) compared with the control.

PEBT Exposure Decreased Superoxide Dismutase and Catalase Activities

To determine the effect of PEBT on the ROS-responsive enzymatic behavior of *E. coli*, the activities of CAT and SOD were examined in *E. coli* cultures treated with PEBT at 0.96, 1.92, and 3.68 mg/ml. PEBT treatment significantly decreased the SOD and CAT activities compared with control, as shown in Figs. 5A and 5B, respectively.

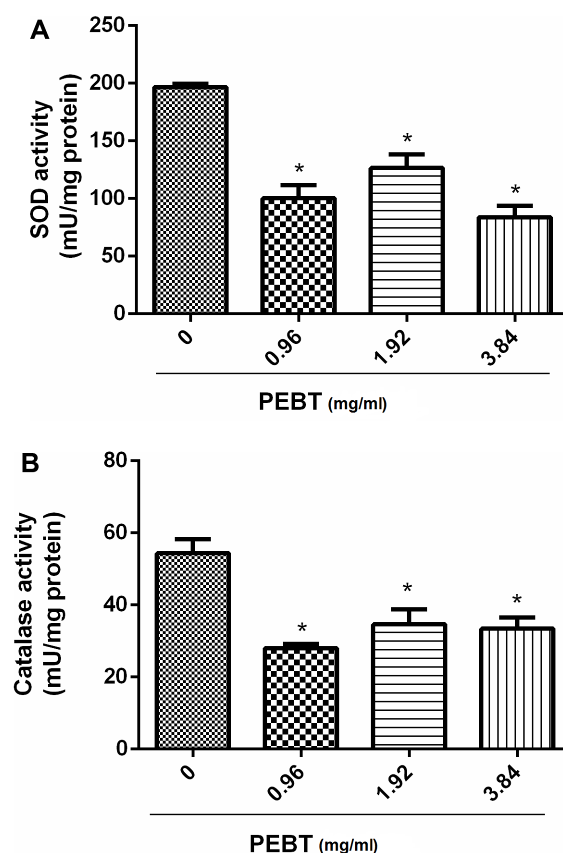

Fig. 5. Activity of superoxide dismutase (**A**) and catalase (**B**) in *E. coli* exposed to PEBT.

Data are reported as the mean ± SD and were analyzed by one-way ANOVA, followed by the Newman–Keuls test when appropriate. *Numerical values are significantly different from the values of the corresponding control ($p < 0.05$).

Discussion

Tellurium compounds have been proven to exert toxic effects in most microorganisms, especially in gram-negative bacteria [14, 15]. In the present study, we verified the antimicrobial activity of the telluroacetylene compound PEBT against *E. coli*. Our results indicated that the pro-oxidant effects of PEBT were a possible mechanism behind its antimicrobial activity. It was suggested that PEBT acted by inducing the generation of ROS, oxidation of thiol groups, and reduction of SOD and CAT activities, which led to cellular damage and cell death.

PEBT was positive for antimicrobial activity, as indicated by its consistent inhibitory effect against the growth of *E. coli* in the disk diffusion, MIC, and survival curve tests. In the disk diffusion test, PEBT at 0.128 and 1.28 mg/disk successfully produced zones of inhibition, which showed the susceptibility of *E. coli* to this compound. This result was of high importance since *E. coli* and other bacterial species have become increasingly resistant to antibiotics, which are associated with 95% of the cases of infection in healthcare professionals. Studies have shown that other tellurium compounds exhibit antibacterial activity against gram-positive (*Bacillus subtilis, Staphylococcus aureus*) [37] and gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa*, and *Salmonella* sp.) [14]. In the present study, PEBT at 1.92 mg/ml exerted bactericidal action against *E. coli*, whereas PEBT at 0.96, 1.92, and 3.84 mg/ml exerted bactericidal activity, as indicated by the significant reduction in viable cell count after 9 h of exposure. We

propose that both the bactericidal and bacteriostatic actions of PEBT were dependent on its concentration and time of exposure.

The present study also observed an increase in the production of ROS in *E. coli* treated with PEBT at 0.96, 1.92, and 3.84 mg/ml, which represented 0.5 MIC, MIC, and 2 MIC, respectively. It is well known that increased levels of ROS lead to damage to cellular components, including DNA as well as membrane lipids and proteins. In addition, we observed a decrease in NPSH level in *E. coli* exposed to PEBT. Taken together, these two results clearly showed the pro-oxidant effect of PEBT. Previous studies showed that PEBT catalytically oxidizes glutathione sulfhydryl groups of various proteins and enzymes [20−22]. This effect, which can lead to cellular toxicity, is potentially associated with the antimicrobial activity of PEBT observed in this study. In this context, other studies showed that *E. coli* exposed to tellurium compounds exhibited a decrease in ATP levels, an increase in ROS generation, carbonylation of proteins, and a decrease in cellular reduced-thiol content [14, 15, 17, 38, 39]. The oxidation of thiols is associated with cell toxicity and death [17, 23, 24].

Previous studies have shown that *E. coli* can function efficiently in the presence of substances that alter O_2 levels owing to the excellent activity of SOD [6, 12, 10]. In fact, *E. coli* has three different O_2-metabolizing SOD enzymes encoded by *sodA*, *sodB*, and *sodC* genes, as well as two catalases, hydroperoxidase I and II (encoded by *katG* and *katE*, respectively), which are involved in the detoxification of intracellular H_2O_2 [40, 41]. The various responses of SOD and CAT to oxidative stress suggest that oxidative stress is one of the most important aspects of chemical stress [42]. In the present study, the SOD and CAT activities were decreased in the presence of PEBT at various concentrations. In fact, the activity of these enzymes was associated with the elevated level of ROS. Previous studies have shown that SOD and CAT are key enzymes in the defense against oxidative stress [20, 42, 43]. In this contest, the decrease in antioxidant activity of SOD and CAT observed in the present study can lead to insufficient enzymatic responses, resulting in poor defense against oxidative stress and cell death.

To determine the relationship between the pro-oxidant activities and the antimicrobial effect of PEBT, we added GSH and AA, two well-known antioxidants, in the culture medium of *E. coli*. The presence of GSH or AA in the medium was effective in decreasing the diameter of PEBT-induced inhibition zone. It is well known that GSH removes oxygen radicals [44] and some studies showed that antioxidants such as GSH and AA protect mutants of *E. coli* against pro-oxidant compounds [4]. The results in the present study were consistent with other studies that reported the association between oxidative stress and the antimicrobial effect of ciprofloxacin [6, 44, 47], norfloxacin, ampicillin, kanamycin A [7], and 2,2′-dithienyl diselenide [4].

In conclusion, the present study revealed that PEBT exhibited bactericidal and bacteriostatic actions against *E. coli*. Additionally, we confirmed that the pro-oxidant activity of PEBT is involved in the mechanism of its antimicrobial effect. PEBT oxidized the thiol groups of biomolecules, which consequently raised the levels of ROS and lowered the activities of SOD and CAT. These alterations led to a decrease in the decomposition of free radicals in *E. coli*, causing cellular damage and eventual cell death.

Acknowledgments

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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