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Antimicrobial effect of 2-phenylethynyl-butyltellurium in *Escherichia coli* and its association with oxidative stress

short title: Antimicrobial effect of PEBT and its association with oxidative stress

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ABSTRACT

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 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 disk diffusion test, with 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-hour exposure. PEBT at 3.84, 1.92, and 0.96 mg/ml increased extracellular ROS production, decreased intracellular NPSH level, and reduced SOD and CAT activities. GSH and AA 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 (AR) 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. AR 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$_2$O$_2$). 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 (SOD) (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 researches [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 glutathione 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 glutathione 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.

### 2. MATERIALS AND METHODS

#### 2.1 Chemicals

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

#### 2.2 Bacterium and growth conditions
*Escherichia coli* (CCBHI 7961) was obtained from Oswaldo Cruz Foundation–FIOCRUZ. 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.

2.3 Antimicrobial activity

### 2.3.1 Disk diffusion method

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 saline solution 0.85% 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) were soaked with 10 µl PEBT at concentrations 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. Halo was measured in centimeters from one extremity to the other. Paper disks soaked with DMSO (10 µl) was used as a negative control. Each experiment was performed in triplicate.

### 2.3.2 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 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 solutions 10 mM of GSH or AA solution was poured into sterilized petri dishes, where the inoculum were subsequently spread on. The 6-mm paper disks were soaked with 10 µl PEBT at concentrations 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.

### 2.3.3 Broth macrodilution assay for minimum inhibitory concentration
The MIC of PEBT was determined by the broth macrodilution method according to the [27]. Seventeen micrograms standard bacterial inoculum of $5 \times 10^5$ 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. MIC was defined as the lowest concentration of compound that completely inhibited visible growth. The experiment was performed in triplicate.

### 2.3.4 Kill-time curve assay

The kill-time curve assay method [30] was used to investigate the bactericidal effects of PEBT against E. coli that 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 Muller-Hinton Agar plates for colony count determination. Data from triplicate runs was averaged and plotted as log CFU/ml versus time (h) for each time point.

### 2.4 Extracellular reactive oxygen species (ROS) 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 OD600. Subsequently, 67 μl E. coli was incubated with 10 μl 2′,7′-dichlorofluorescein diacetate (DCHF-DA; 10 nM) and PEBT at of 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. ROS level was expressed as unit of fluorescence. The method described above was performed according to a previous study [31] with modifications.

### 2.5 Incubation and preparation of lysates

Bacterial cells (50 ml) harvested from nutrient broth were centrifuged at 2822.4 $\times g$ for 10 min. The supernatant was removed, whereas the pellet was weighed, transferred to microtubes, and resuspended in 1 ml 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 0.5MIC, MIC, and 2MIC, respectively), or DMSO (control group). Afterwards, 0.9 g glass beads were added to the microtubes, which were then mixed by a vortexer 6 times for 5 min and incubated for 2 min in an ice bath to complete a 30-minute
cycle [32]. Debris was removed from the suspension by centrifugation at 2822.4 × g for 10 min, after which the supernatant was collected for biochemical assays.

2.6 Non-protein thiols levels (NPSH)

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

2.7 Superoxide dismutase (SOD) activity

The activity of superoxide dismutase 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 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].

2.8 Catalase (CAT) activity

CAT activity was measured in accordance with a published method [35]. A solution was prepared using 0.25 M KPi buffer, 2.5 mM EDTA pH 7.0, 30 % H₂O₂, and X-100 water triton. For the test, 2 ml of the prepared solution was added to 60 μl supernatant. The absorbance was monitored at 240 nm for 1 min.

2.9 Protein concentration

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

2.10 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 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.
3. RESULTS

3.1 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 to 0.128 mg/disk in a dose-dependent 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 than that of without AA. The antioxidant GSH also decreased the antimicrobial effect of 1.28 and 0.128 mg/disk PEBT.

3.2 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 PEBT concentration in the subsequent examination of antioxidant enzymes activity, non-protein thiols levels, and reactive species generation.

3.3 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.

3.4 PEBT exposure increases oxygen reactive species (ROS) production

ROS level was used to monitor formation of extracellular ROS in *E. coli* treated with various concentrations of PEBT (0.96, 1.92, and 3.68 mg/mL). PEBT at all tested concentration 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.

3.5 PEBT exposure decreased non-protein thiols (NPSH) 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.

### 3.6 PEBT exposure decreased superoxide dismutase (SOD) and catalase (CAT) activities

To determine the effect of PEBT on the RS-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 SOD and CAT activities compared with control, as shown in Figure 5A and 5B, respectively.

## 4. 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 ER, 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 bacteria species have been increasingly in 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 a bactericidal action against *E. coli*, whereas PEBT at 0.96 mg/ml, 1.92 mg/ml, and 3.84 mg/ml exerted bactericidal activity, as indicated by the significant reduction in viable cell count after 9 h of exposure. We proposed that both bactericidal and bacteriostatic actions of PEBT were dependent on its concentration and time of exposure.
The present study also reported an increase in the production of ROS in \textit{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 level of ROS leads to damage to cellular components, including DNA as well as membrane lipids and proteins. In addition, we observed a decrease in NPSH level in \textit{E. coli} exposed to PEBT. Taken together, these two results clearly showed the pro-oxidant effect of. 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 to the antimicrobial activity of PEBT observed in this study. In this context, other studies showed that \textit{E. coli} exposed to tellurium compounds exhibited a decrease in ATP level, increase in ROS generation, carbonylation of proteins, and decrease in cellular reduced-thiol content [38,39,17,14,15]. The oxidation of thiols is associated with cell toxicity and death [23, 24, 17].

Previous studies on have shown that \textit{E. coli} can function efficiently in the presence of substances that alter O$_2$ levels due to the excellent activity of SOD [6, 12, 10]. In fact, \textit{E. coli} has three different O$_2$-metabolizing SOD enzymes encoded by \textit{sodA}, \textit{sodB}, and \textit{sodC} genes, as well as two catalases, hydroperoxidase I and II (encoded by \textit{katG} and \textit{katE}, respectively), which are involved in the detoxification of intracellular H$_2$O$_2$ [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, SOD and CAT activities were decreased in presence of PEBT at various concentrations. In fact, the activity of these enzymes was associated to the elevated level of RS. Previous studies have shown that SOD and CAT are key enzymes in the defense against oxidative stress [42, 43, 20]. 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 relation 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 \textit{E. coli}. The presence of GSH and 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 \textit{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 [47, 6, 44], norfloxacin, ampicillin, kanamycin A [7], and 2,2′-dithiienyl 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 reactive species 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.

**Conflict of Interest**

The authors declare that they have no competing interests.

**Acknowledgments**

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**References**


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

<table>
<thead>
<tr>
<th>PEBT (mg/disk)</th>
<th>Diameter of zone inhibition zone (nm)</th>
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<tr>
<td></td>
<td>Medium</td>
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<td>0</td>
<td></td>
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<td>0.00128</td>
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<td>0.0128</td>
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<td>0.128</td>
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<td>1.28</td>
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Data are reported as mean ± SD and was analyzed using one-way ANOVA, followed by Newman–Keuls test. *Numerical values are significantly different from the values of the corresponding control (p < 0.05).
<table>
<thead>
<tr>
<th></th>
<th>0.5 MIC (mg/mL)</th>
<th>MIC (mg/mL)</th>
<th>2MIC (mg/mL)</th>
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<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>0.96</td>
<td>1.92</td>
<td>3.68</td>
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Table 2. Minimum inhibitory concentration (MIC) of 2-phenylethynyl-butyltellurium (PEBT) in *Escherichia coli*. 
Fig. 1. Figure 1: Chemical structure of 2-phenylethynyl-butyltellurium (PEBT).
Fig. 2. Figure 2: The time course of various concentrations of 2-phenylethynyl-butyltellurium (PEBT) at 0.5 minimum inhibitory concentration (MIC), MIC, and 2MIC. Data are presented as mean.
Fig. 3. Figure 3: Total extracellular reactive oxygen species (ROS) in Escherichia coli exposed to 2-phenylethynyl-butyltellurium (PEBT). Data are presented as mean.
Fig. 4. Figure 4: Levels of non-protein thiols (NPSH) in Escherichia coli exposed to 2-phenylethynyl-butyltellurium (PEBT). Data are reported as mean.
Fig. 5A. Figure 5: Activity of superoxide dismutase (A) in Escherichia coli exposed to 2-phenylethynyl-butyltellurium (PEBT). Data are reported as mean.
Fig. 5B. Figure 5: Activity of catalase (B) in Escherichia coli exposed to 2-phenylethynyl-butyltellurium (PEBT). Data are reported as mean.