

Involvement of oxidative stress in bactericidal activity of 2-(2-nitrovinyl) furan against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*

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ARTICLE INFO

Article history:

Received 20 September 2015

Received in revised form

11 November 2015

Accepted 19 November 2015

Available online 2 December 2015

Keywords:

2-(2-nitrovinyl)furan

Oxidative stress

Escherichia coli

Pseudomonas aeruginosa

Staphylococcus aureus

Reactive oxygen species

ABSTRACT

The involvement of reactive oxygen species and oxidative stress in 2-(2-nitrovinyl) furan mediated bacterial cell death was investigated in *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Time kill assay resulted in significant decrease in the optical density and colony-forming unit (CFU) of *E. coli*, *P. aeruginosa* and *S. aureus*. The level of superoxide anion radical and nitric oxide increased significantly in concentration dependent when compared with dimethyl sulfoxide (DMSO) treated bacteria. Similar concentration dependent increase in the activity of superoxide dismutase and catalase were recorded. The non-enzymatic antioxidant glutathione decreased significantly with a concomitant increase in glutathione disulfide. The level of malondialdehyde and fragmented DNA increased significantly in the bacterial cells treated with 2-(2-nitrovinyl) furan when compared with DMSO treated cells. The CFU of *E. coli*, *P. aeruginosa* and *S. aureus* following exposure to 2-(2-nitrovinyl) furan increased significantly ($p < 0.05$) in the presence of 2,2' bipyridyl, an Fe chelator, significantly when compared with only 2-(2-nitrovinyl) furan suggesting the involvement of hydroxyl radical in the cell death. The available data from this study showed that 2-(2-nitrovinyl) furan induced oxidative stress in *E. coli*, *P. aeruginosa* and *S. aureus* as evident from elevated levels of superoxide anion radical nitric oxides and antioxidant enzymes.

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1. Introduction

Increasing death cases arising from bacterial infection calls for development of new, effective and safe antimicrobial drugs. While the development of effective antibiotics cannot be overemphasized owing to increasing resistance to the arrays of available antibiotics [1]. Pharmaceutical companies are not encouraging development of new antibiotics [2]. Antibiotics are grouped as bactericidal or bacteriostatic depending on interaction with cellular targets. Bacteriostatic antibiotics inhibit ribosome function by targeting both the 30S and 50S ribosome subunits [3–5], while bactericidal antibiotics interfere with protein translation [6,7], cell-wall

synthesis, DNA replication and repair [8,9]. In addition, there are accumulating evidences suggesting the involvement of oxidative stress in bactericidal activities of all classes of bactericidal antibiotics [9–11]. Also, some antibacterial natural products have been reported to affect the activities of antioxidant enzymes, an evidence of redox imbalance mediated antibacterial activities [12,13]. Compounds such as 2-(2-nitrovinyl) furan having β -nitrostyrene moiety have attracted interest owing to ready synthesis and significant antibacterial/antifungal activity [14].

2-(2-nitrovinyl) furan (Fig. 1) is a lipid soluble derivative of nitrovinyl furan. The antimicrobial activities of this group have been documented against gram-positive and gram-negative bacteria, with gram-positive bacteria highly susceptible to this compound [14–16]. β -nitrostyrene derivatives inhibit bioenergetics processes and modify thiol groups of enzymes [17]. Furthermore, vinyl furans inhibit glyceraldehyde-3-phosphate dehydrogenase, glucose-6-phosphate dehydrogenase (Glc 6-PD),

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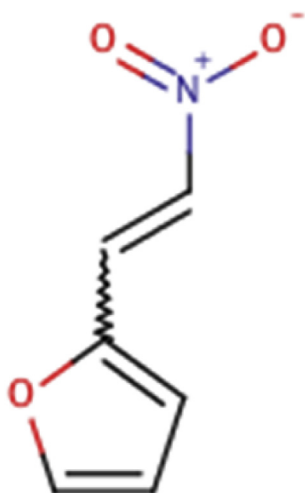


Figure 1. Structure of 2-(2-nitrovinyl) furan.

malate dehydrogenase, and glutathione reductase (GSH-red) [17]. Recently, 2-(2-nitrovinyl) furan was reported to mediate its toxicity in rats by inducing oxidative stress [18]. Despite the established antibacterial activities and inhibitory effect of vinylfuran group on bioenergetics process and redox enzymes, there is no report linking this to its bactericidal activity. Arising from these facts, this study evaluated possible involvement of oxidative stress in the antibacterial activities of 2-(2-nitrovinyl) furan against *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

2. Materials and methods

2.1. Bacterial strains

E. coli (ATCC 25922), *P. aeruginosa* (ATCC 2783) and *S. aureus* (ATCC 29213) were procured from American Type Culture Collection and propagated on a lysogeny broth (LB) at 37 °C.

2.2. Materials

Diphenylamine, 5,5'-dithiobis(2-nitrobenzoic acid), guanidine hydrochloride, and N-ethyl-maleimide were procured from Research Organics (Cleveland, OH). 2-(2-nitrovinyl) furan, epinephrine, trichloroacetic acid, Hydrogen peroxide (H₂O₂), nitroblue tetrazolium, 2,2'-bipyridyl are product of Sigma–Aldrich, St. Louis, MO. All other reagents are of analytical grades.

2.3. Methods

2.3.1. Time kill bacterial susceptibility study

Susceptibility of *E. coli*, *P. aeruginosa* and *S. aureus* against 2-(2-nitrovinyl) furan was investigated using the procedure described by Samoilova et al. [12] with some modifications. Briefly, organisms were grown overnight in a liquid medium, lysogeny broth (LB), harvested by centrifugation and resuspended in 50 mL fresh medium (LB) to OD₆₀₀ = 0.1 and grown aerobically at 37 °C in 250 mL flask. At mid-log phase (OD₆₀₀ = 0.5), 2-(2-nitrovinyl) furan (prepared in dimethyl sulfoxide) was added to obtain concentrations (0.02, 0.04, 0.06, 0.08 and 0.10 mg/mL) or dimethyl sulfoxide (DMSO) and incubated at 37 °C for 3 h. Absorbance of the incubation medium was read at 600 nm for every 20 min interval of 3 h incubation time. For colony formation, samples of control culture (DMSO treated culture) and cultures treated with 2-(2-nitrovinyl)

furan were removed at intervals (0, 20, 40, 60, 80, 100, 120, 140, 160 and 180 min) and centrifuged to collect the cells as pellet. The cells were washed and diluted with 0.9% NaCl, mixed with molten soft LB-agar (0.8%) at 42 °C and poured onto agar plates containing solid LB-agar (1.5%). Colonies were counted after 24 h at 37 °C.

2.3.2. Preparation of cell free extract

Cell free extract was prepared from the samples obtained after 3 h incubation of organisms with 2-(2-nitrovinyl) furan at the varying concentrations (0.02–0.10 mg/mL). Cells were harvested by centrifugation, washed twice and suspended in sucrose-Tris buffer (25 mmol/L sucrose solution, 10 mmol/L Tris–HCl, pH 7.4). Glass beads (2 g) was added to the bacterial suspension, homogenized and centrifuged at 3000 g for 10 min at 4 °C to obtain the cell free extract as the supernatant.

2.3.3. Oxidative stress assay

2.3.3.1. Superoxide radical anion and nitric oxide assay. Superoxide anion content of the cell free extract was determined using the procedure described by Becerra and Albasa [10] with little modifications. Briefly, cell free extract (0.5 mL) was incubated with 2.5 mL nitroblue tetrazolium (1 mg/mL) for 30 min at 37 °C. Then 0.5 mL of 0.1 M HCl was added. The blank was constituted same way except that the cell free extract was replaced with 0.25 M sucrose solution. The blue colour was read at 575 nm. The amount of superoxide radical anion generated was calculated using the molar extinction coefficient of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide formazan (17,000 M⁻¹ cm⁻¹ at pH 7.4–8.0).

The nitric oxide (NO) content of the cell free extract was determined using the colorimetric method based on the Griess reaction [19].

2.3.3.2. Superoxide dismutase. The activity of superoxide dismutase (SOD) was determined according to Misra and Fridovich [20]. Briefly, 0.2 mL of cell free extract was added to 2.5 mL of 0.05 mol/L carbonate buffer (pH 10.2) to equilibrate and the reaction was started by addition of 0.3 mL of freshly prepared 0.3 mmol/L epinephrine. The increase in absorbance at 480 nm was recorded every 30 s for 150 s. One unit of enzyme activity was defined as 50% inhibition of the rate of autooxidation of pyrogallol as determined by change in absorbance min⁻¹ at 480 nm.

2.3.3.3. Catalase. Catalase (CAT) activity was determined using the method described by Aebi [21]. Briefly, 50 µL of homogenate was added to a cuvette containing 2 mL of phosphate buffer (pH 7.0) and 1 mL of 30 mmol/L H₂O₂. The absorbance at 240 nm was measured for 1 min using a spectrophotometer. A molar extinction coefficient of H₂O₂ of 43.6 L mol⁻¹ cm⁻¹ was used to determine the CAT activity.

2.3.3.4. Glutathione reduced (GSH) and glutathione disulfide (GSSG). The level of GSH in the cell free extract was determined using the procedure described by Ellman [22]. Briefly, 1.0 mL of cell free extract was added to 0.1 mL of 25% trichloroacetic acid (TCA) and precipitate was removed by centrifuge at 5000 g for 10 min. Supernatant (0.1 mL) was added to 2 mL of 0.6 mM DTNB prepared in 0.2 M sodium phosphate buffer (pH 8.0). The absorbance was read at 412 nm.

GSSG level was determined using the procedure described by Hissin and Hilf [23]. Cell free extract (50 µL) was mixed with 20 µL of 0.04 M N-ethylmaleimide (NEM) to prevent oxidation of GSH to GSSG. It was incubated at room temperature for 30 min and 1.68 mL of 0.3 M Na₂HPO₄ solution was added to it followed by 250 µL of DTNB reagent. The absorbance of the sample was measured at 412 nm.

2.3.3.5. Malondialdehyde. The level of malondialdehyde was determined according to Reilly and Aust [24].

2.3.3.6. Fragmented DNA. The percentage fragmented DNA in the cell free extract was determined using the procedure described by Burton [25]. Briefly, cell free extract was centrifuged at $15,000 \times g$, for 15 min at 4 °C. The supernatant was separated from the pellet and treated with trichloroacetic acid (1.50 mL, 10%). The pellet was also treated with trichloroacetic acid (0.65 mL, 5%). The reaction mixtures were allowed to precipitate overnight (≥ 4 h) in a refrigerator (4 °C), centrifuged at 2500 g for 10 min. The reaction mixtures were boiled at 100 °C for 15 min, cooled to room temperature and centrifuged at 2500 g for 5 min. The supernatants (0.5 mL) were treated with diphenylamine reagent (1 mL) and incubated at 37 °C for 4 h. Absorbance was read at 600 nm using spectrophotometer. The fragmented DNA was calculated using the following expression:

$$\text{Fragmented DNA(\%)} = \frac{\text{Absorbance of the supernatant}}{\text{Absorbance of the supernatant} + \text{Absorbance of pellet}}$$

2.3.4. Hydroxyl radical mediated bactericidal activity study

E. coli, *P. aeruginosa* and *S. aureus* were grown overnight in a liquid medium (LB), harvested by centrifugation and resuspended in 50 mL fresh medium (LB) to $OD_{600} = 0.1$ and grown aerobically at 37 °C in 250 mL flask. At mid-log phase ($OD_{600} = 0.5$), 2,2'-bipyridyl (Fe chelator) and 2-(2-nitrovinyl) furan (0.1 mg/mL) were added and incubated at 37 °C for 3 h. Absorbance of the incubation medium was read at 600 nm for every 20 min interval of 3 h incubation time. In addition, samples of control culture and treated culture were removed at intervals of (0, 20, 40, 60, 80, 100, 120, 140, 160 and 180 min) and centrifuged to collect the cells as pellet. The cells were washed and diluted with 0.9% NaCl, mixed with molten soft LB-agar (0.8%) at 42 °C and poured onto agar plates containing solid LB-agar (1.5%). Colonies were counted after 24 h at 37 °C.

2.4. Statistical analysis

Results were expressed as the mean of three independent experiments $\pm$ standard deviation. One-way analysis of variance (ANOVA) followed by Student's *t*-test was used to detect any significant difference ($p < 0.05$) between the treatments using Statplus, 2011 (AnalystSoft Inc., Alexandria, VA, USA).

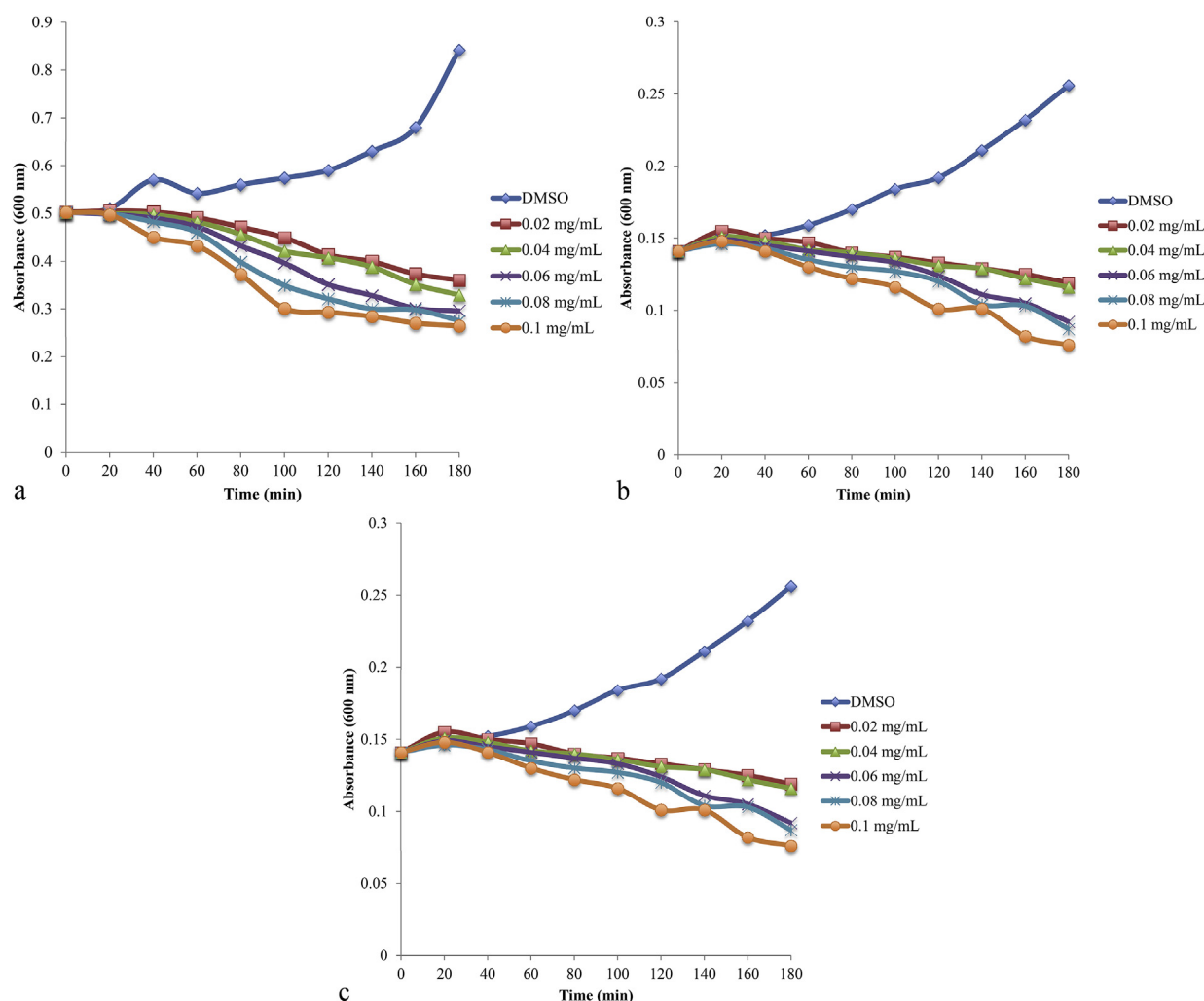


Figure 2. Optical densities of (a) *Escherichia coli* (ATCC 25922), (b) *Pseudomonas aeruginosa* (ATCC 27853) and (c) *Staphylococcus aureus* (ATCC 29213) treated with 2-(2-nitrovinyl) furan. Values are mean $\pm$ SEM of three determinations and are statistically significant at $p < 0.05$.

3. Results

3.1. Time kill assay

The absorbance of *E. coli*, *P. aeruginosa* and *S. aureus* following exposure to various concentrations of 2-(2-nitrovinyl) furan decreased steadily when compared to control that received no treatment except the vehicle (DMSO) (Fig. 2a–c). These decrease were very profound at the first 60 min and becomes steady at 140–180 min. Similar pattern of decrease in CFU/mL of *E. coli*, *P. aeruginosa* and *S. aureus* was recorded (Fig. 3a–c). The inhibition of bacteria growth was more profound at 0.1 mg/mL, with no growth being recorded for *E. coli* (Fig. 3a–c).

3.2. Oxidative stress biomarkers

3.2.1. Superoxide anion radical and nitric oxide

2-(2-nitrovinyl) furan significantly increased ($p < 0.05$) the level of superoxide anion radical in the cell free extract of bacterial. The highest concentration (0.1 mg/mL) produced 82.32, 78.11 and 78.03% increase in superoxide anion radical in *E. coli*, *P. aeruginosa*

and *S. aureus* when compared to the DMSO treated group (Fig. 4a). In a similar vein, the level of nitric oxide significantly ($p < 0.05$) increased in a concentration dependent manner when compared to the control, with the highest concentration producing 78.60, 84.52 and 77.64% increase in *E. coli*, *P. aeruginosa* and *S. aureus* respectively (Fig. 4b).

3.2.2. Superoxide dismutase and catalase

The activities of superoxide dismutase and catalase in the cell free extract of the bacterial cells treated with 2-(2-nitrovinyl) furan (0.02–0.1 mg/mL) increased significantly ($p < 0.05$) (Fig. 5a and b). 2-(2-nitrovinyl) furan (0.1 mg/mL) produced 85.18, 83.11 and 82.88% increase in the activity of superoxide dismutase in *E. coli*, *P. aeruginosa* and *S. aureus* respectively. Catalase activity in cell free extract of *E. coli*, *aeruginosa* and *S. aureus* increased significantly following treatment with 2-(2-nitrovinyl) furan in a concentration dependent manner (Fig. 5b).

3.2.3. Glutathione reduced and glutathione disulfide

GSH level in cell free extract of *E. coli*, *P. aeruginosa* and *S. aureus* decrease significantly ($p < 0.05$) following treatment with 2-(2-nitrovinyl) furan in concentration dependent manner (Fig. 6a).

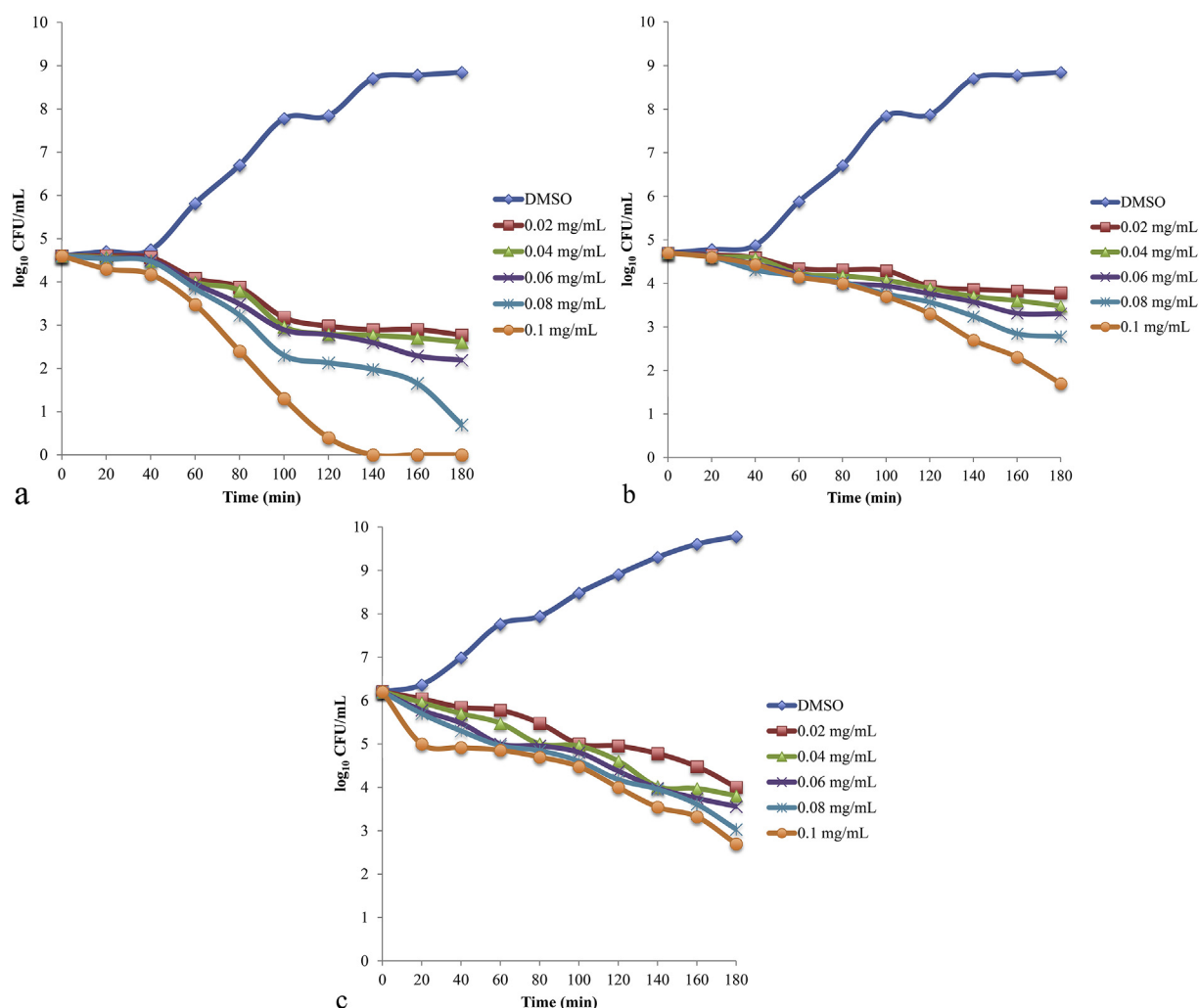


Figure 3. CFU/mL of (a) *Escherichia coli* (ATCC 25922), (b) *Pseudomonas aeruginosa* (ATCC 27853) and (c) *Staphylococcus aureus* (ATCC 29213) treated with 2-(2-nitrovinyl) furan. Values are mean $\pm$ SEM of three determinations and are statistically significant at $p < 0.05$.

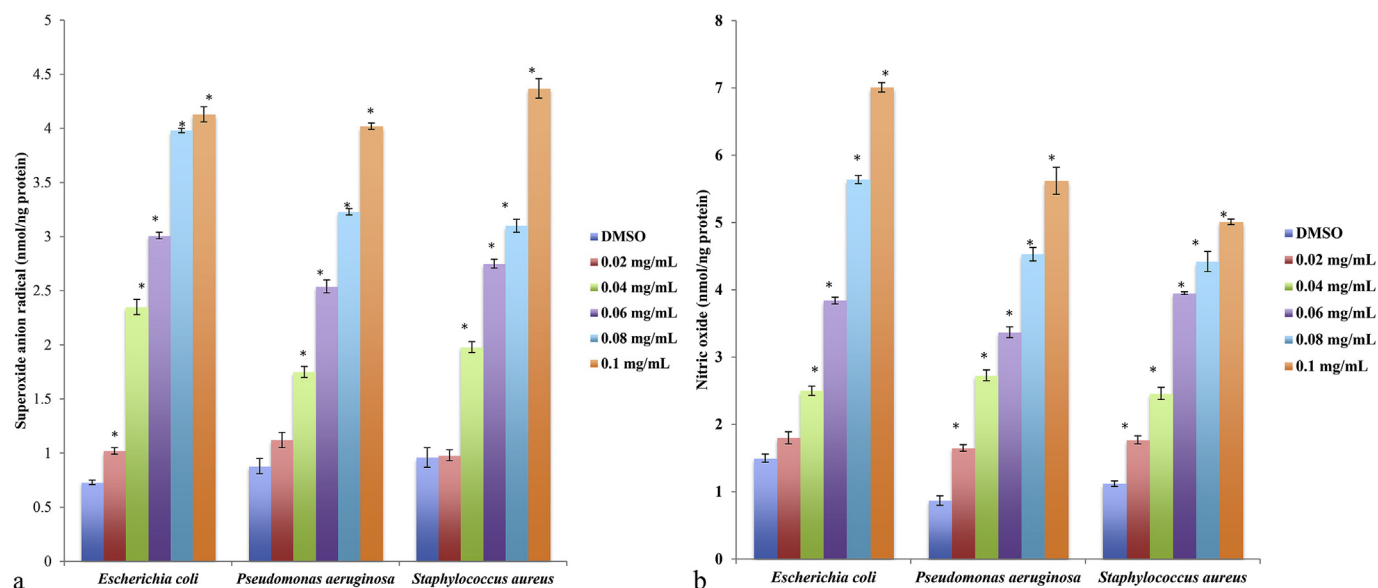


Figure 4. (a) superoxide anion radical and (b) nitric oxide content of 2-(2-nitrovinyl) furan treated *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 29213). Values are mean $\pm$ SEM of three determinations and are statistically significant at $p < 0.05$. * $p < 0.05$ vs DMSO. DMSO: Dimethyl sulfoxide. Superoxide anion radical and nitric oxide are expressed in nmol/mg protein.

Conversely, 2-(2-nitrovinyl) furan significantly ($p < 0.05$) increased the level of GSSG in *E. coli*, *P. aeruginosa* and *S. aureus*. This increase was about 2, 3.5 and 5 folds in *E. coli*, *P. aeruginosa* and *S. aureus* respectively at the highest concentration (0.1 mg/mL) investigated in this study (Fig. 6b).

3.2.4. Malondialdehyde and fragmented DNA

Malondialdehyde, a biomarker of lipid peroxidation, increased significantly in the cell free extract of *E. coli*, *aeruginosa* and *S. aureus* treated with 2-(2-nitrovinyl) furan (Fig. 7a). 2-(2-nitrovinyl) furan at 0.1 mg/mL produced about 5 fold increase in malondialdehyde after 3 h exposure. Similarly, there was

concentration dependent increase in fragmented DNA (%) following treatment of *E. coli*, *P. aeruginosa* and *S. aureus* with 2-(2-nitrovinyl) furan (Fig. 7b).

3.3. Hydroxyl radical involvement in bactericidal

There was significant increase in the absorbance and CFU/mL of *E. coli*, *P. aeruginosa* and *S. aureus* incubated with 2,2'-bipyridyl and 2-(2-nitrovinyl) furan (0.1 mg/mL) when compared with bacterial cells treated with only 2-(2-nitrovinyl) furan (Fig. 8a–c). This increase compared significantly with bacterial cells treated with only DMSO (Fig. 8a–c).

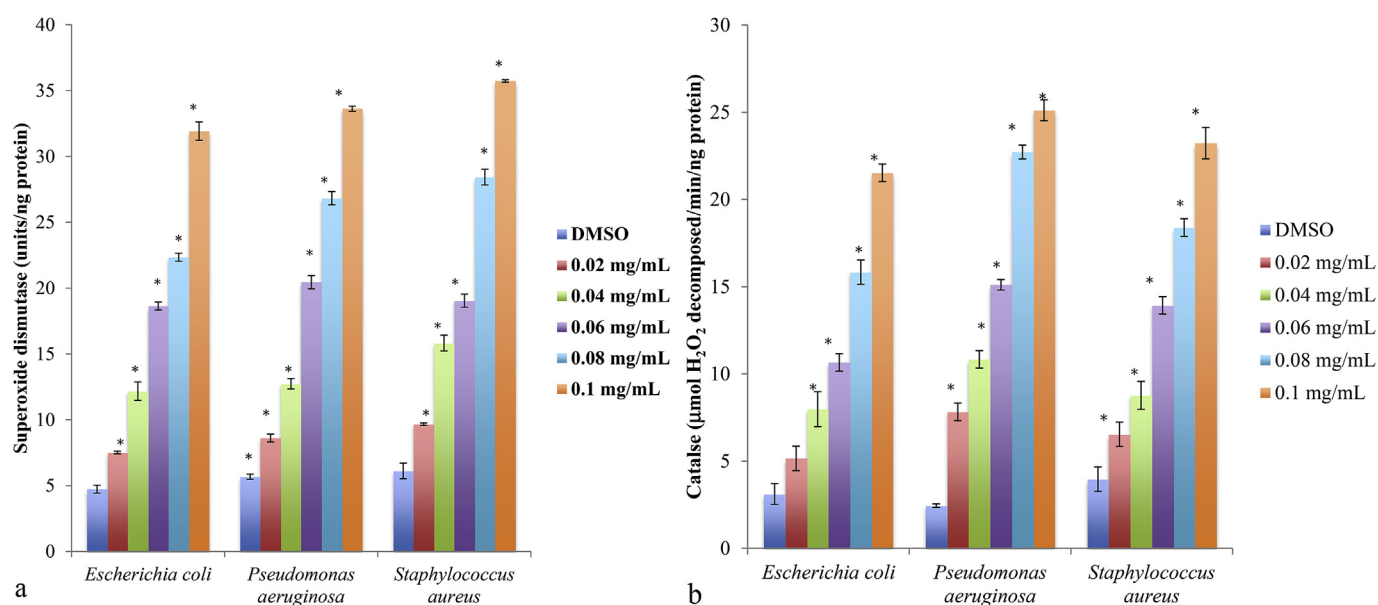


Figure 5. Activity of (a) catalase and (b) superoxide dismutase in 2-(2-nitrovinyl) furan treated *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 29213). Values are mean $\pm$ SEM of three determinations and are statistically significant at $p < 0.05$. * $p < 0.05$ vs DMSO. DMSO: Dimethyl sulfoxide. Catalase and superoxide dismutase are expressed in nmol/min/mg protein.

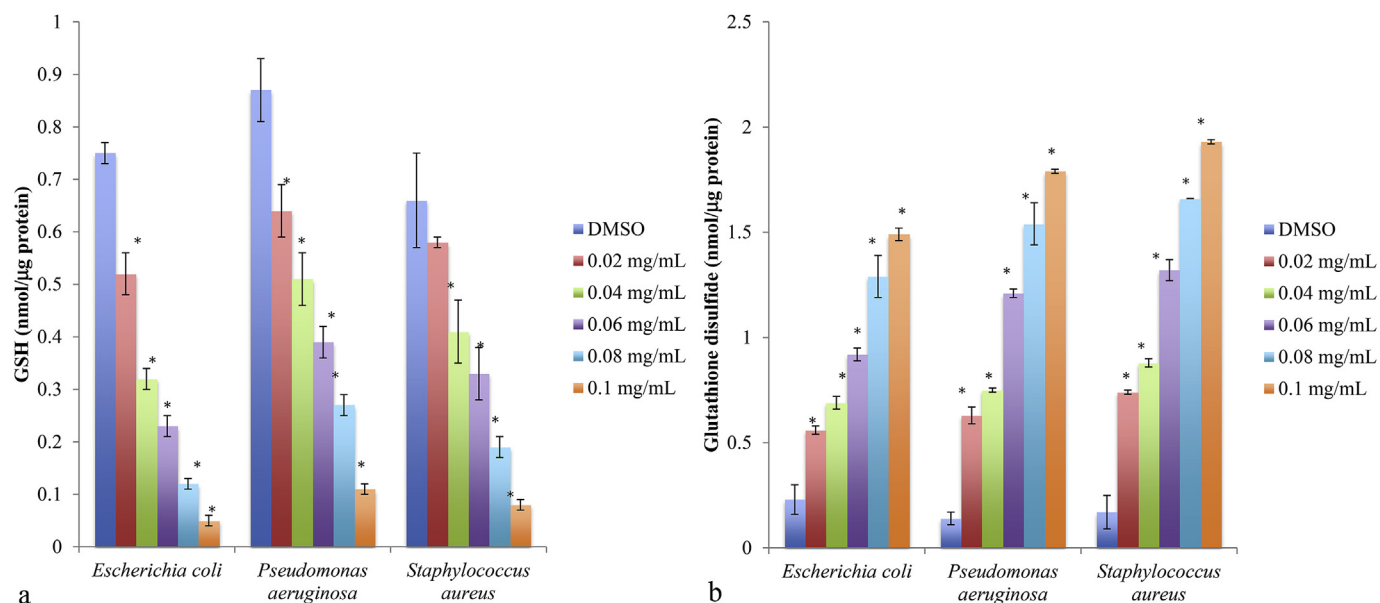


Figure 6. Concentrations of (a) glutathione reduced and (b) glutathione disulfide in 2-(2-nitrovinyl) furan treated *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 29213). Values are mean $\pm$ SEM of three determinations and are statistically significant at $p < 0.05$. * $p < 0.05$ vs DMSO. DMSO: Dimethyl sulfoxide.

4. Discussion

Oxidative stress mediated bactericidal activity of antimicrobial agents is becoming appreciated and documented as a mechanism of antimicrobial agents. This occurs via the inhibition of cellular enzymes involved in redox cycling leading to generation of superoxide anion radical and hydrogen peroxide, which is later, converted to hydroxyl radical. This study presents the possible involvement of reactive oxygen species (superoxide anion radical and nitric oxide) and oxidative stress in 2-(2-nitrovinyl) furan mediated bactericidal activity against *E. coli*, *P. aeruginosa* and *S. aureus*.

In the time kill assay, absorbance and CFU/mL of bacterial cells following exposure to 2-(2-nitrovinyl) furan significantly decrease in concentration and time dependent manner indicating the anti-bacterial activity against *E. coli*, *P. aeruginosa* and *S. aureus*. Similar bactericidal activity of β -nitrostyrene, nitrovinylfuran and 2-(2-nitrovinyl) furan have been previously reported [14–16]. Thus, the decrease in CFU/mL of *E. coli*, *aeruginosa* and *Staphylococcus aureus* reported in this study indicates antibacterial property.

In order to understand the involvement of reactive oxygen species in 2-(2-nitrovinyl) furan-mediated bactericidal activity, the effect of this compound on superoxide anion radical and nitric oxide were investigated. Studies have reported superoxide anion

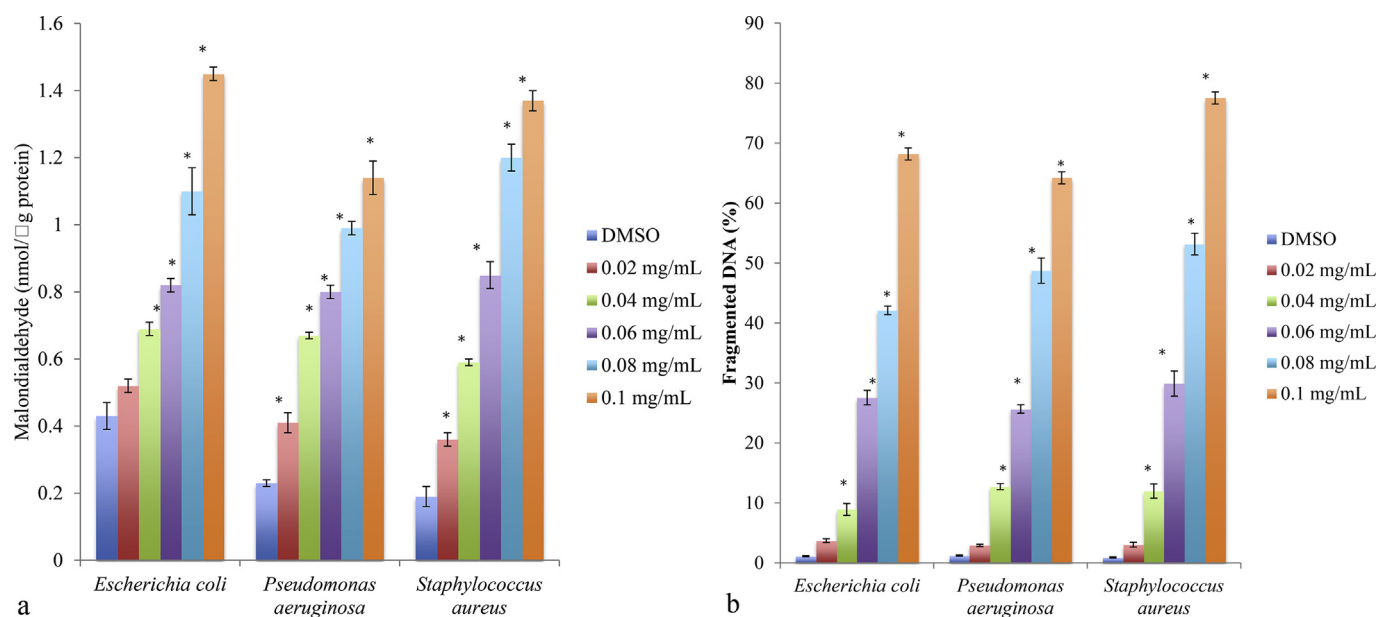


Figure 7. Concentration of (a) malondialdehyde and (b) Fragmented DNA in 2-(2-nitrovinyl) furan treated *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 29213). Values are mean $\pm$ SEM of three determinations and are statistically significant at $p < 0.05$. * $p < 0.05$ vs DMSO. DMSO: Dimethyl sulfoxide.

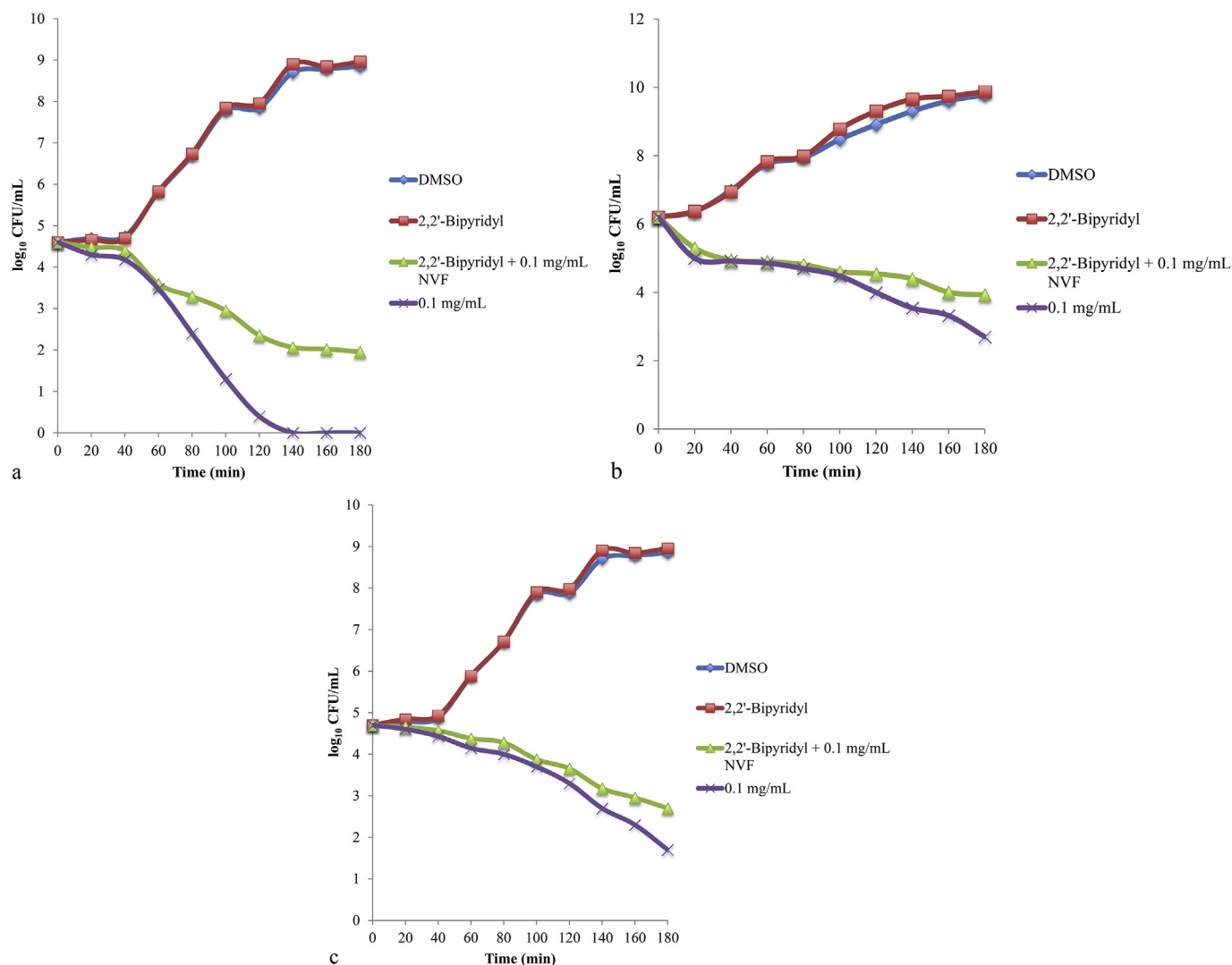


Figure 8. CFU/mL of (a) *Escherichia coli* (ATCC 25922), (b) *Pseudomonas aeruginosa* (ATCC 27853) and (c) *Staphylococcus aureus* (ATCC 29213) treated with 2-(2-nitrovinyl) furan in the presence of 2,2'-bipyridyl. Values are mean $\pm$ SEM of three determinations and are statistically significant at $p < 0.05$.

radical production as one of the mechanism by which ciprofloxacin, a quinolone, mediate antibacterial action [10]. The enhanced level of superoxide anion radical and nitric oxide in cell free extracts of *E. coli*, *P. aeruginosa* and *S. aureus* exposed to 2-(2-nitrovinyl) furan support report of Ajiboye et al. [18], which reported increased superoxide anion radical and nitric oxide in rats.

Superoxide dismutase and catalase are antioxidant enzymes responsible for the detoxification of superoxide anion radical and hydrogen peroxide, which can be converted to a more potent antimicrobial agent, hydroxyl radical, in the presence of iron through Fenton reaction. Increased activities of superoxide dismutase and catalase in *E. coli*, *P. aeruginosa* and *S. aureus* following exposure to 2-(2-nitrovinyl) furan indicates enhanced superoxide and hydrogen peroxide production in bacterial cells. This could have occurred in an event to counteract/detoxified superoxide anion radical and hydrogen peroxide.

The redox state of cells are often assessed using glutathione system, such that a decrease in GSH, with a concomitant increased in GSSG indicates redox imbalance tending to oxidized state [26–28]. This imbalance leads to oxidative stress and damage, arising from attack on cellular macromolecules by reactive radicals [29–32]. Recent study revealed decreased GSH level with

concomitant increased GSSG in 2-(2-nitrovinyl) furan treated rats [18]. Similar alterations in the level of GSH and GSSG were observed in this study, indicating redox imbalance likened to oxidative stress.

Cellular biomolecules such as proteins, lipids and DNA are primary targets of reactive oxygen species (superoxide anion radical, hydroxyl radical, hydrogen peroxide and nitric oxide) [33,34]. The elevated level of malondialdehyde in the cell free extracts of 2-(2-nitrovinyl) furan treated *E. coli*, *P. aeruginosa* and *S. aureus* indicate peroxidation of lipid layer of cell membrane. This could results to distorted membrane organization and induces functional loss and modification of proteins and DNA bases [35]. Similar increase in the level of fragmented DNA in the cell free extracts of 2-(2-nitrovinyl) furan treated *E. coli*, *P. aeruginosa* and *S. aureus* is an indication of oxidative stress, which supports the previous report that 2-(2-nitrovinyl) furan could induced DNA fragmentation [18].

Involvement of hydroxyl radical generation possibly via Fenton reaction has been reported as a unified mechanism for bactericidal antibiotics [9]. While Hassett and Imlay [36] reported that the mechanism of bactericidal antibiotics is far more complicated than the radical approach, it is still one of the widely accepted mechanisms of bactericidal antibiotics mediated cell death [37–39]. The increase in the CFU/mL of *E. coli*, *P. aeruginosa* and *S. aureus*

following incubation with 2-(2-nitrovinyl) furan in the presence of 2,2'-bipyridyl when compared to 2-(2-nitrovinyl) furan treated bacterial indicates that hydroxyl radical is involved in the bactericidal activity, supporting the report of Kohanski et al. [9].

5. Conclusion

Based on the findings from this study, it can be concluded that 2-(2-nitrovinyl) furan induced oxidative stress in *E. coli*, *P. aeruginosa* and *S. aureus* as evident from elevated levels of superoxide anion radical nitric oxides and antioxidant enzymes.

Conflict of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors received no financial support for the research, authorship, and/or publication of this article.

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