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Contribution of reactive oxygen species to (+)-catechin-mediated bacterial lethality

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ABSTRACT

The contribution of reactive oxygen species to (+)-catechin-mediated bacterial lethality was investigated. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC) of (+)-catechin against *E. coli*, *P. aeruginosa* and *S. aureus* were investigated using 96-well microtitre plate. MIC and MBC of (+)-catechin against *E. coli*, *P. aeruginosa* and *S. aureus* are 600 and 700; 600 and 800; 600 and 800 µg/mL respectively. The optical densities and colony forming units of (+)-catechin-treated bacteria decreased. (+)-Catechin (4× MIC) significantly increased the superoxide anion content of *E. coli*, *P. aeruginosa* and *S. aureus* compared to DMSO. Superoxide dismutase and catalase in (+)-catechin treated *E. coli*, *P. aeruginosa* and *S. aureus* increased significantly. Conversely, level of reduced glutathione in (+)-catechin-treated *E. coli*, *P. aeruginosa* and *S. aureus* decreased significantly while glutathione disulfide increased significantly. Furthermore, malondialdehyde and fragmented DNA increased significantly following exposure to (+)-catechin. From the above findings, (+)-catechin enhanced the generation of reactive oxygen species (superoxide anion radical and hydroxyl radical) in *E. coli*, *P. aeruginosa* and *S. aureus*, possibly by autoxidation, Fenton chemistry and inhibiting electron transport chain resulting into lipid peroxidation and DNA fragmentation and consequentially bacterial cell death.

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1. Introduction

Reactive oxygen species (ROS) including superoxide anion radical, hydrogen peroxide, peroxylnitrite and hydroxyl radical have longed been reported to possess antimicrobial activity [1,2]. Microorganisms are exposed to ROS via endogenous and exogenous means. The endogenous source of ROS in bacteria is largely the mitochondria electron transport chain, where leakage of electron leads to the production of superoxide anion radical [3]. Environmental toxicants contribute largely to the exogenous sources of ROS. The primary targets of ROS are cellular macromolecules including protein, lipids and DNA. More importantly, ROS kill bacteria through DNA damaging effect of hydroxyl radical, produced through the catalytic action of ferrous ion on hydrogen peroxide

(Fenton chemistry) [4]. Recent experimental evidences have proposed ROS hypothesis in the killing of bacterial [5–7]. This hypothesis further provides link between bacterial tricarboxylic acid cycle, NADH depletion, destabilization of iron-sulfur cluster and formation of hydroxyl radical (via Fenton reaction) in both gram-negative and gram positive bacteria [8–10].

(+)-Catechin (Fig. 1) is a flavanol that is found in many plants [11–13]. It is non-toxic, cheaper and natural compound with broad-spectrum antibacterial activity against Gram-positive and Gram-negative bacteria such as *Staphylococcus aureus* and *Escherichia coli* [13]. The catechol group in ring B and resorcinol group in ring A as well as hydroxyl group at position 3 in ring C are the pharmacophore of this flavanol [14]. Pharmacological activities including antioxidant, antibacterial, antitumor etc have been reported for (+)-catechin. Although, the antibacterial activity of (+)-catechin is unrelated to antioxidant activity [15] hydrogen peroxide generation and interaction with modeled membrane are responsible for its antibacterial action [16,17]. The antibacterial action was enhanced by heating and addition of CuSO₄ [18]. In this study, we present the

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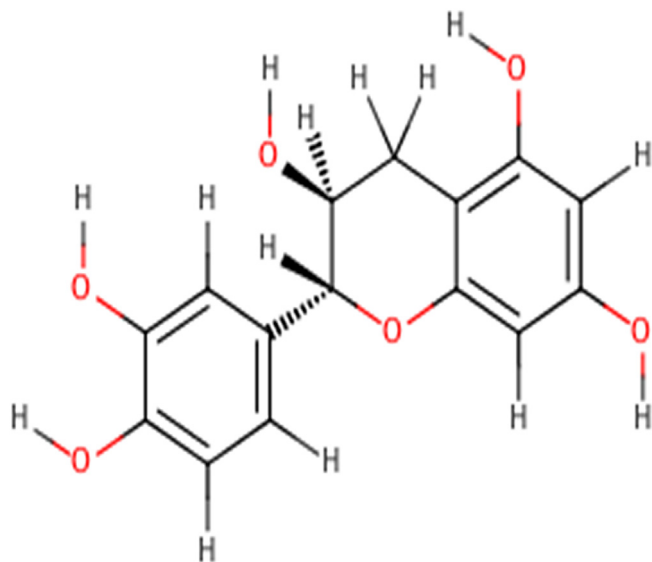


Fig. 1. (+)-Catechin.

contribution of ROS in (+)-catechin-mediated bacterial lethality.

2. Materials and methods

2.1. Bacteria strains

Escherichia coli (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 29213) were procured from American Type Culture Collection and propagated on a Luria-Bertani (LB) at 37 °C.

2.2. Chemicals

Dimethyl sulfoxide, diphenylamine, 5,5'-dithiobis(2-nitrobenzoic acid), epinephrine, guanidine hydrochloride, hydrogen peroxide, *N*-ethyl-maleimide, sodium chloride, thio-barbituric acid were procured from Sigma-Aldrich (St. Louis, MO). (+)-Catechin, nitroblue tetrazolium, 2,2'-dipyridyl and L-glutathione (reduced) are products of Santa Cruz Biotechnology. All other reagents are products of Sigma-Aldrich (St. Louis, MO).

2.3. Minimum inhibitory concentration and minimum bactericidal concentration

Minimum inhibitory concentration (MIC) of (+)-catechin was determined using 96-well micro titre plate as described by Balouiri et al. [19]. Inocula (10^4 CFU/mL) were mixed with (+)-catechin in 96-well microtitre plate to give concentrations ($1-10^9$ ng/mL). The culture medium containing 0.04% DMSO in sterile distilled water served as control. Plates were incubated for 24 h at 37 °C. MIC was expressed as the lowest concentration which inhibited growth, judged by lack of turbidity in the well. The turbidity in each well was then determined using Versamax microplate reader (Molecular Devices, California, USA). The experiment was performed in triplicate and repeated three times along with a reference antibiotic, ciprofloxacin, whose concentration ranged from 0.001 to 0.099 mg/mL [20]. The content of wells lacking turbidity were aspirated into eppendorf tubes, and centrifuged to collect the cells. The cells were washed to aseptically remove (+)-catechin, 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. The concentration that completely inhibited colony formation after the removal of (+)-catechin was considered as MBC.

2.4. Time-kill bacterial susceptibility assay

Susceptibility of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* to (+)-catechin was investigated using the procedure described by Ajiboye et al. [6]. Briefly, organisms were grown overnight in Luria-Bertani (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 to $OD_{600} = 0.2$. (+)-Catechin was added to the culture to obtain concentration ($4\times$ MIC) or dimethyl sulfoxide (DMSO) and incubated at 37 °C for 3 h. Absorbance of the incubation medium was read at 600 nm for every 30 min interval of 3 h incubation time. For colony formation, samples of control culture (DMSO treated culture) and cultures treated with (+)-catechin ($4\times$ MIC) were removed at intervals (0, 30, 60, 90, 120, 150 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.

2.5. Preparation of cell free extract

Cell free extract was prepared from the samples obtained after 3 h incubation of organisms with (+)-catechin ($4\times$ MIC). 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.

2.6. Oxidative stress biomarkers

2.6.1. Superoxide anion radical assay

Superoxide anion radical in the cell free extract was determined as described by Ajiboye et al. [6]. Briefly, cell free extract (20 μ L) was incubated with 100 μ L nitroblue tetrazolium (1 mg/mL) for 30 min at 37 °C. Then 20 μ L of 0.1 M HCl was added. The blank was constituted same way except that the cell free extract was replaced with sucrose-Tris buffer. The blue colour was read at 575 nm using Versamax microplate reader (Molecular Devices, California, USA). 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 ($17,000\text{ M}^{-1}\text{ cm}^{-1}$ at pH 7.4–8.0).

2.6.2. Superoxide dismutase

The activity of superoxide dismutase (SOD) was determined according to Misra and Fridovich [21]. Briefly, 10 μ L of cell free extract was added to 125 μ L of 0.05 mol/L carbonate buffer (pH 10.2) to equilibrate and the reaction was started by adding 15 μ L of freshly prepared 0.3 mmol/L epinephrine. The increase in absorbance at 480 nm was recorded every 30 s for 150 s using Versamax microplate reader (Molecular Devices, California, USA). One unit of enzyme activity was defined as 50% inhibition of the rate of autoxidation of epinephrine as determined by change in absorbance/min at 480 nm.

2.6.3. Catalase

Catalase activity in the cell free extract was evaluated using the procedure described by Chen et al. [22]. Briefly, cell free extract (10 μ L) was mixed thoroughly with cold 6 mM H_2O_2 (100 μ L). The

reaction was stop by mixing with 3 M H₂SO₄ (20 µL) followed by 0.01 KMnO₄. Reaction mixture was vortexed and absorbance read at 480 nm within 30–60 s using Versamax microplate reader (Molecular Devices, California, USA).

2.6.4. Reduced glutathione and glutathione disulfide

The level of GSH in the cell free extract was determined using the procedure described by Ellman [23]. Cell free extract (20 µL) was mixed with 170 µL of 0.1 M potassium phosphate buffer (pH 7.4). The reaction was started by adding 10 mM DTNB (10 µL) and incubated for 30 min at room temperature. Absorbance of the reaction mixtures was read at 412 nm using Versamax microplate reader (Molecular Devices, California, USA).

GSSG level was determined using the procedure described by Hissin and Hilf [24]. 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.

2.6.5. Malondialdehyde

Malondialdehyde content of cell free extract was determined as described by Reilly and Aust [25]. Briefly, cell free extract was mixed with TBA/TCA/HCl (15%, 0.37%, 0.2 N) at a reagent/sample ratio of 2:1 (v/v), placed in a boiling water bath for 15 min, cooled to room temperature and centrifuged at 1000 g for 10 min at room temperature. The absorbance of the solution was read at 535 nm against the blank (containing all reagents except cell free extract) using Versamax microplate reader (Molecular Devices, California, USA). MDA content was determined using the extinction coefficient of 1.56×10^6 .

2.6.6. Fragmented DNA

The percentage fragmented DNA in the cell free extract was determined using the procedure described by Burton [26]. Briefly, cell free extract was centrifuged at 15,000×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 (50 µL) were treated with diphenylamine reagent (100 µL) and incubated at 37 °C for 4 h. Absorbance was read at 600 nm using Versamax microplate reader (Molecular Devices). The fragmented DNA was calculated using the following expression:

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

2.7. Involvement of superoxide anion radical and hydroxyl radical in bacterial lethality

Escherichia coli, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were grown overnight in 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'-dipyridyl (500 µM), thiourea (150 mM) and or (+)-catechin

(4× MIC) was 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 using Versamax microplate reader (Molecular Devices). 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 using digital colony counter after 24 h at 37 °C.

2.8. Molecular docking analysis

Molecular docking analysis was done to determine the possible binding mode of (+)-catechin to NADH: Quinone oxidoreductase, succinate dehydrogenase, topoisomerase II (DNA gyrase), penicillin binding protein and DNA-directed RNA polymerase as potential inhibitor using CLC Drug Discovery Workbench 3 Software (Qiagen, Waltham MA, USA). The crystal structures of NADH: quinone oxidoreductase (Protein Data Bank (PDB 1ozk), succinate-quinone oxidoreductase (PDB 1nek), DNA gyrase (PDB 4kcl), penicillin binding protein (PDB 4bjp) and DNA-directed RNA polymerase (PDB 3n4m) were downloaded from RCSB Protein Data Bank, imported to CLC Drug Discovery Workbench 3 and the binding site was set up. (+)-Catechin drawn with Marvin sketch (ChemAxon, Budapest, Hungary) were optimized, docked and evaluated for possible interactions at the binding pocket of these proteins.

2.9. Statistical analysis

Results were expressed as mean of three independent experiments ± 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).

3. Results

3.1. Minimum inhibitory concentration and minimum bactericidal concentration

The MIC and MBC of (+)-catechin against *E. coli*, *P. aeruginosa* and *S. aureus* are presented in Table 1.

3.2. Time-kill bacterial susceptibility

The optical densities of *E. coli*, *P. aeruginosa* and *S. aureus* treated with (+)-catechin (4× MIC) decreased significantly when

compared with those treated with only DMSO (Fig. 2a). While this decrease was time dependent, it was more pronounced at 180 min. Ciprofloxacin, a reference antibiotic produced similar alteration in the absorbance of these bacterial cells (Fig. 2a–c). Furthermore, the decreased change in absorbance was supported by the reduction in CFU/mL of the organisms following incubation with the (+)-catechin (Fig. 3a–c).

Table 1

Minimum inhibitory concentration and minimum bactericidal concentration of (+)-catechin ($\mu\text{g/mL}$).

	MIC	MBC
<i>Escherichia coli</i> ATCC 25922	600	700
<i>Pseudomonas aeruginosa</i> ATCC 27853	600	800
<i>Staphylococcus aureus</i> ATCC 29213	600	800

3.3. Oxidative stress biomarkers

(+)-Catechin significantly ($p < 0.05$) increased superoxide anion radical contents of *E. coli*, *P. aeruginosa* and *S. aureus* when compared to the DMSO treated cells (Fig. 4a). (+)-Catechin produced 15.72-, 12.31 and 42.4-fold increase in superoxide contents of *E. coli*, *P. aeruginosa* and *S. aureus* respectively. Although ciprofloxacin produced similar increase in superoxide anion radical, it

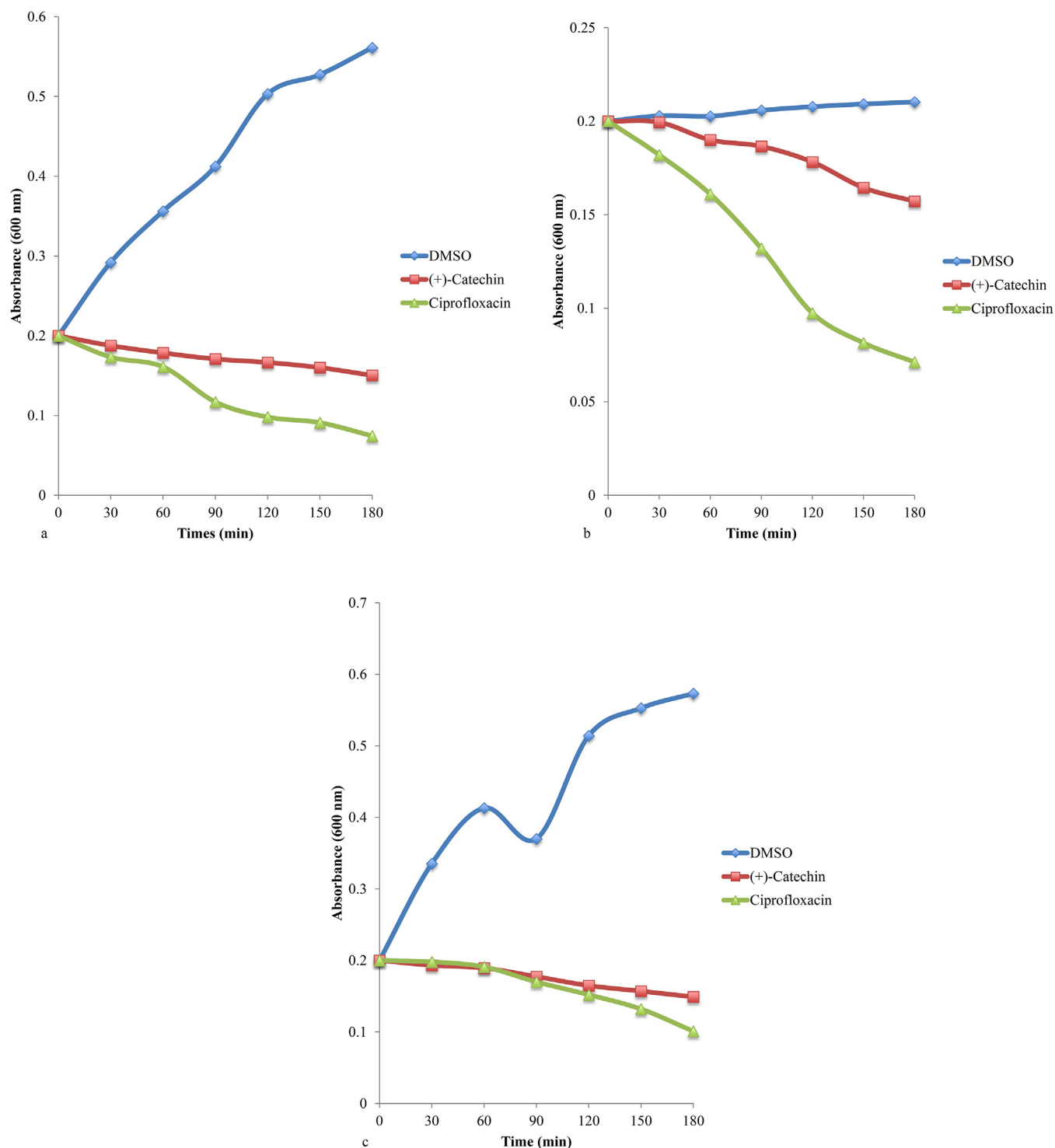


Fig. 2. Optical densities of (a) *Escherichia coli* (ATCC 25922), (b) *Pseudomonas aeruginosa* (ATCC 27853) and (c) *Staphylococcus aureus* (ATCC 29213) exposed to (+)-catechin ($4 \times \text{MIC}$). Values are mean $\pm$ SEM of three determinations and are statistically significant at $p < 0.05$.

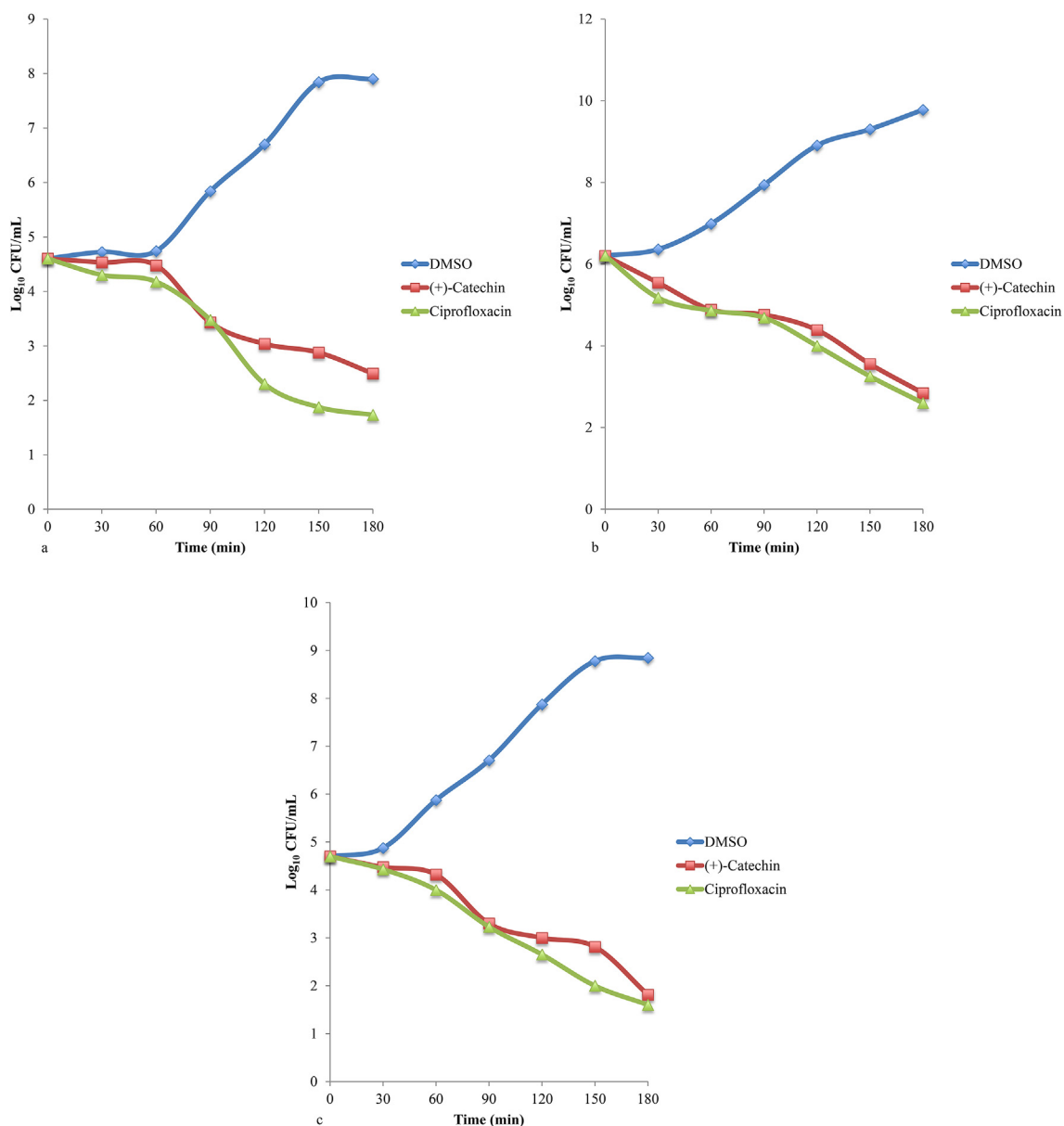


Fig. 3. Viabilities (CFU/mL) of (a) *Escherichia coli* (ATCC 25922), (b) *Pseudomonas aeruginosa* (ATCC 27853) and (c) *Staphylococcus aureus* (ATCC 29213) exposed to (+)-catechin (4× MIC). Values are mean ± SEM of three determinations and are statistically significant at $p < 0.05$.

did not compare with (+)-catechin.

Similar increase ($p < 0.05$) in the activities of superoxide dismutase and catalase in response to elevated superoxide anion radical was observed in the cell free extracts of (+)-catechin (4× MIC) treated-*E. coli*, *P. aeruginosa* and *S. aureus* when compared to DMSO treated organisms (Fig. 4b–c).

The level of reduced glutathione in *E. coli*, *P. aeruginosa* and *S. aureus* exposed to (+)-catechin (4× MIC) decreased significantly ($p < 0.05$) by 70.42, 69.04 and 47.62% respectively (Fig. 5a). Conversely, glutathione disulphide increased significantly in the cell free extracts of (+)-catechin treated organisms. This increase compared significantly with ciprofloxacin treated organisms (Fig. 5b).

Malondialdehyde, a product of lipid peroxidation, increased significantly ($p < 0.05$) by 20.68-, 7.15- and 17.67-fold respectively in the cell free extracts of *E. coli*, *P. aeruginosa* and *S. aureus* treated with (+)-catechin (4× MIC; Fig. 6a). There was similar increase in the fragmented DNA of (+)-catechin (4× MIC) treated organisms

(Fig. 6b).

3.4. Involvement of hydroxyl radical

There was significant increase in CFU/mL of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* treated with only 2,2'-bipyridyl (500 μM) and thiourea (150 mM) when compared with (+)-catechin treated organisms (Fig. 7a–c). Although, (+)-catechin in combination with 2,2'-bipyridyl or thiourea decreased the number of viable cells, it did not compare with organisms treated with only (+)-catechin (Fig. 7a–c).

3.5. Molecular docking

(+)-Catechin bound NADH: ubiquinone oxidoreductase (PDB: 1ozk) by hydrogen bonding amino acid residues, Ser 123, Ser 125 and Lys 134, in the binding pocket (Fig. 8a–b). There was no hydrogen bond interaction between (+)-catechin and cofactors,

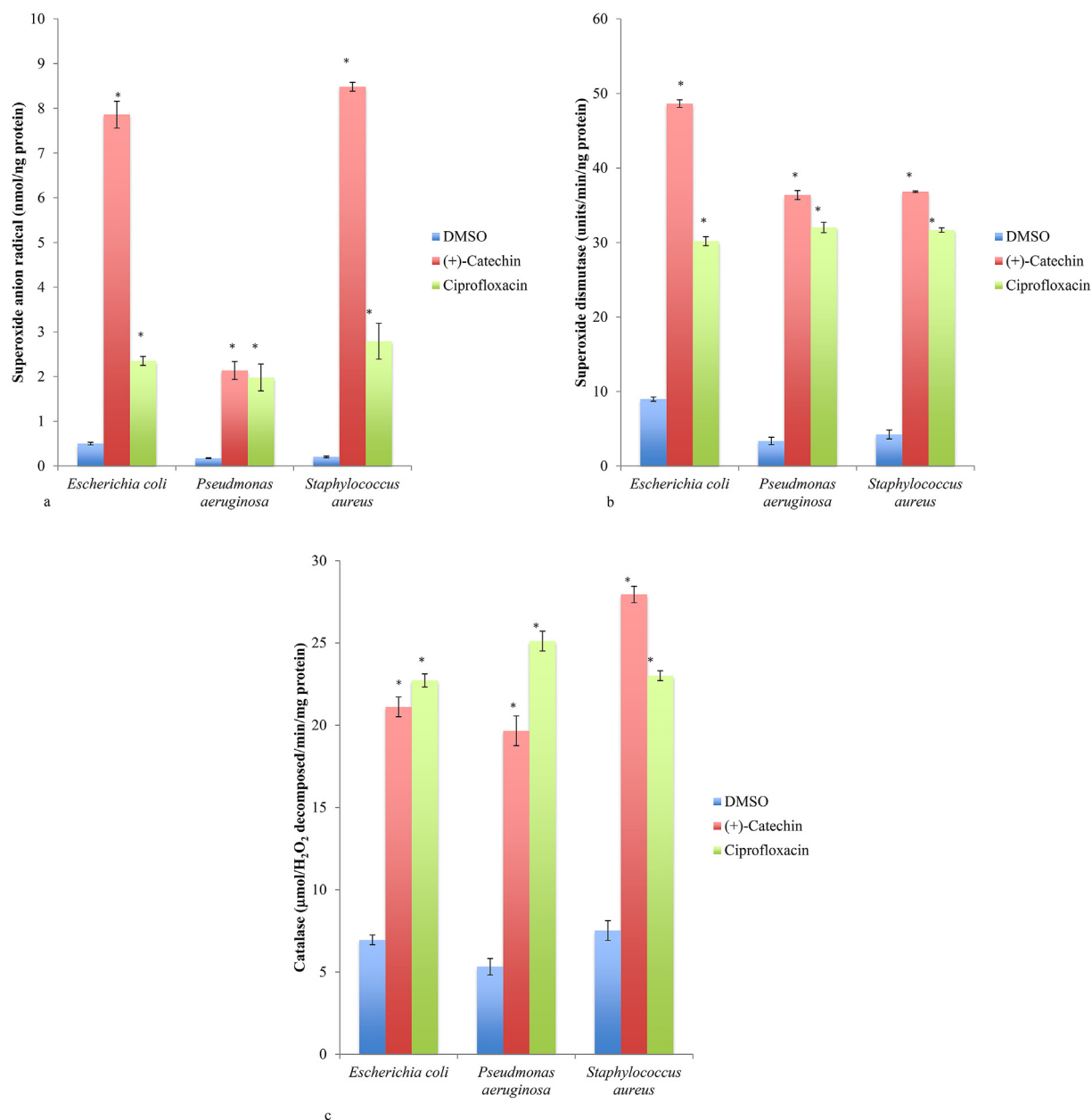


Fig. 4. (a) Level of superoxide anion radical of (b) activity of catalase and (c) activity of superoxide dismutase in *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 29213) exposed to (+)-catechin (4× MIC). Values are mean $\pm$ SEM of three determinations and are statistically significant at $p < 0.05$. * $p < 0.05$ vs DMSO. DMSO: Dimethyl sulfoxide.

FAD and NAD. (+)-Catechin share common interaction with Lys 134 as amytal and rotenone, known inhibitor of NADH: ubiquinone oxidoreductase (Fig. 8c–f). Furthermore, (+)-Catechin hydrogen bond amino acid residues, Val 47, Ala 49, Thr 213, Asn 214 and Leu 252 (Fig. 9a–b) in the binding pocket of succinate:quinone oxidoreductase. There was no interaction with cofactor, FAD.

In the binding pocket of DNA gyrase, (+)-catechin interacted with Ala 33, Asp 82, Ala 84, Thr 88 and Arg 91 amino acid residues via hydrogen bond (Fig. 10a–b). (+)-Catechin shares similar interaction with amino acid residues, Ala 33, as nalidixic acid (Fig. 10c–d). Amino acid residue, Tyr 507, is involved in hydrogen bonding (+)-catechin in the binding pocket of penicillin binding protein (Fig. 11a–b). This amino acid residue, Tyr 507, is also hydrogen bound by carbapenem (Fig. 11c–d). Hydrogen bond with amino acid residue Thr 127 in the binding pocket of DNA-directed

RNA polymerase is the point of similar interaction shared by (+)-catechin and rifampin (known inhibitor of DNA-directed RNA polymerase; Fig. 12a–d).

4. Discussion

Redox perturbations leading to oxidative stress and cell death have been proposed as possible mechanism of bactericidal agents. In this study, we present the contribution of reactive oxygen species generation to (+)-catechin-mediated bacterial lethality.

4.1. Bacterial susceptibility

The antibacterial activity of (+)-catechin have been reported in literature and the MIC and MBC values, as obtained in this study, are

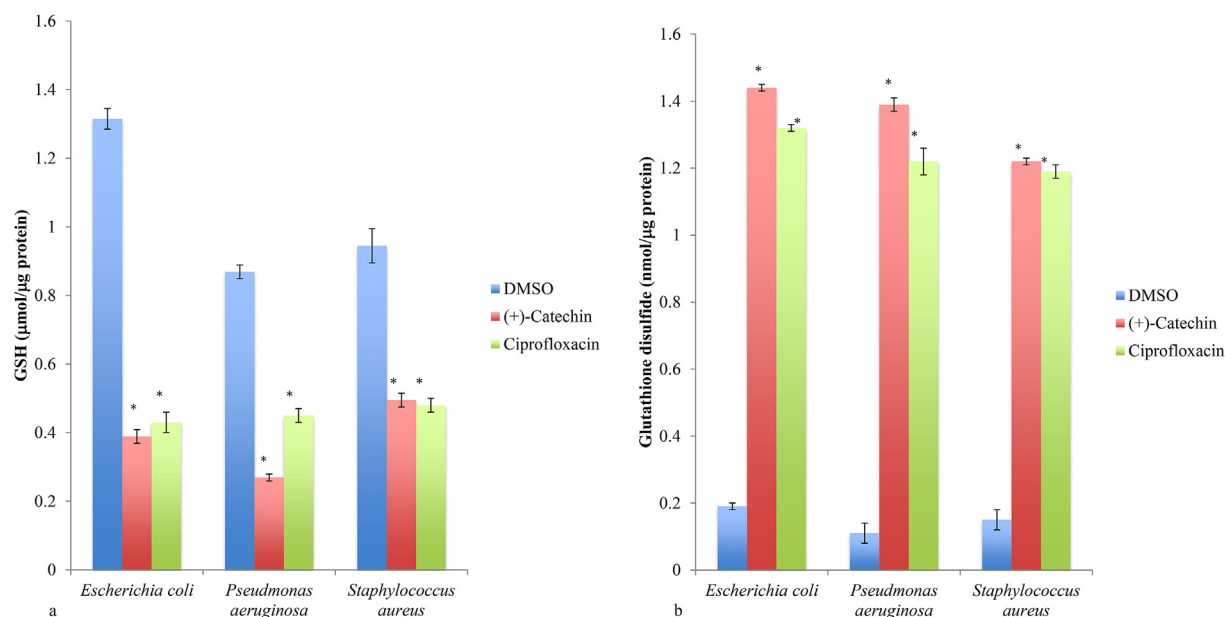


Fig. 5. Concentrations of (a) reduced glutathione and (b) glutathione disulfide in *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 29213) exposed to (+)-catechin (4× MIC). Values are mean ± SEM of three determinations and are statistically significant at $p < 0.05$. * $p < 0.05$ vs DMSO. DMSO: Dimethyl sulfoxide.

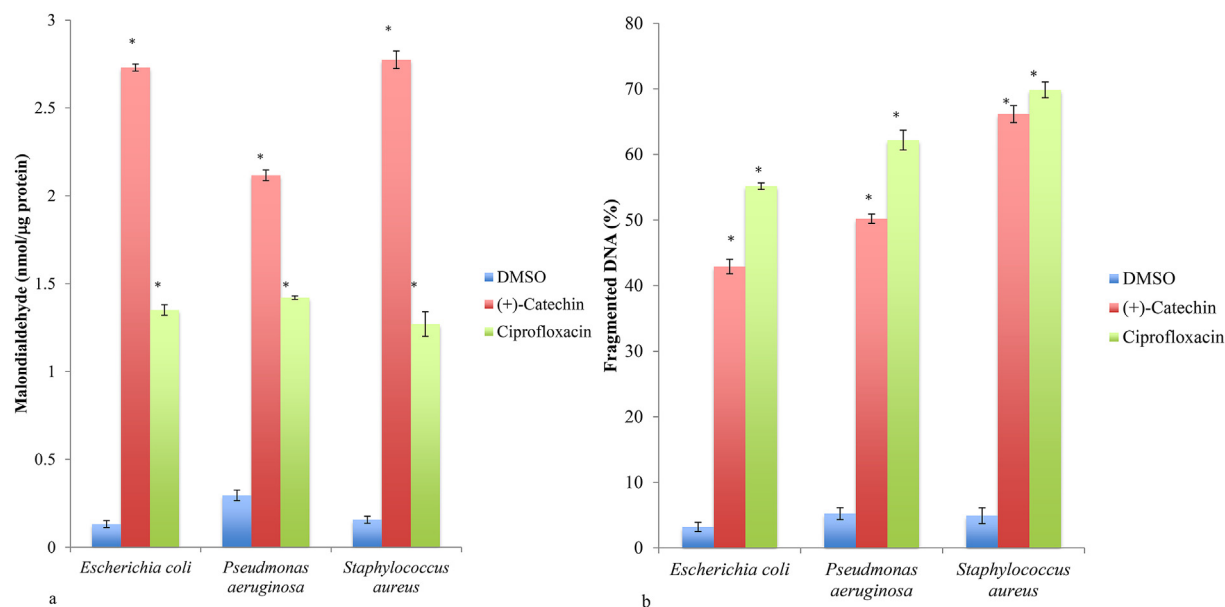


Fig. 6. (a) Malondialdehyde and (b) fragmented DNA in (+)-catechin-treated *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC 29213). Values are mean ± SEM of three determinations and are statistically significant at $p < 0.05$. * $p < 0.05$ vs DMSO. DMSO: Dimethyl sulfoxide.

inline with previous studies [16,27]. These values are indicative of its antibacterial activity and are supported by decrease in absorbance and CFU/mL for 3 h incubation. Similar antibacterial activity of this compound was reported with the pure form and derivatives [16,28].

4.2. Oxidative stress

Bacteria are equipped with antioxidant machineries to cope with ubiquitous ROS generation in cells growing in aerobic environments. This is further supported by recent reports on the involvement of ROS and oxidative stress in the bactericidal activity

of antibiotics, natural and synthetic compounds [6–8,29–33]. Studies have reported the capability of catechins including (+)-catechin to autoxidized [14] and enhance ROS (H_2O_2 and $\cdot O_2^-$) generation [16,34,35]. Increased level of $\cdot O_2^-$ as obtained in this study indicates enhanced ROS generation possibly from autoxidation of (+)-catechin or electron leakage in electron transport chain. The increased activities of SOD and CAT are possible bacterial response to detoxify elevated ROS (H_2O_2 and $\cdot O_2^-$) levels.

Bacterial GSH complements cellular antioxidants by detoxifying H_2O_2 [36]. The depleted level of GSH could have resulted from conjugation with semiquinone [37], product of (+) catechin oxidation. This depletion could alter redox status and result in cell

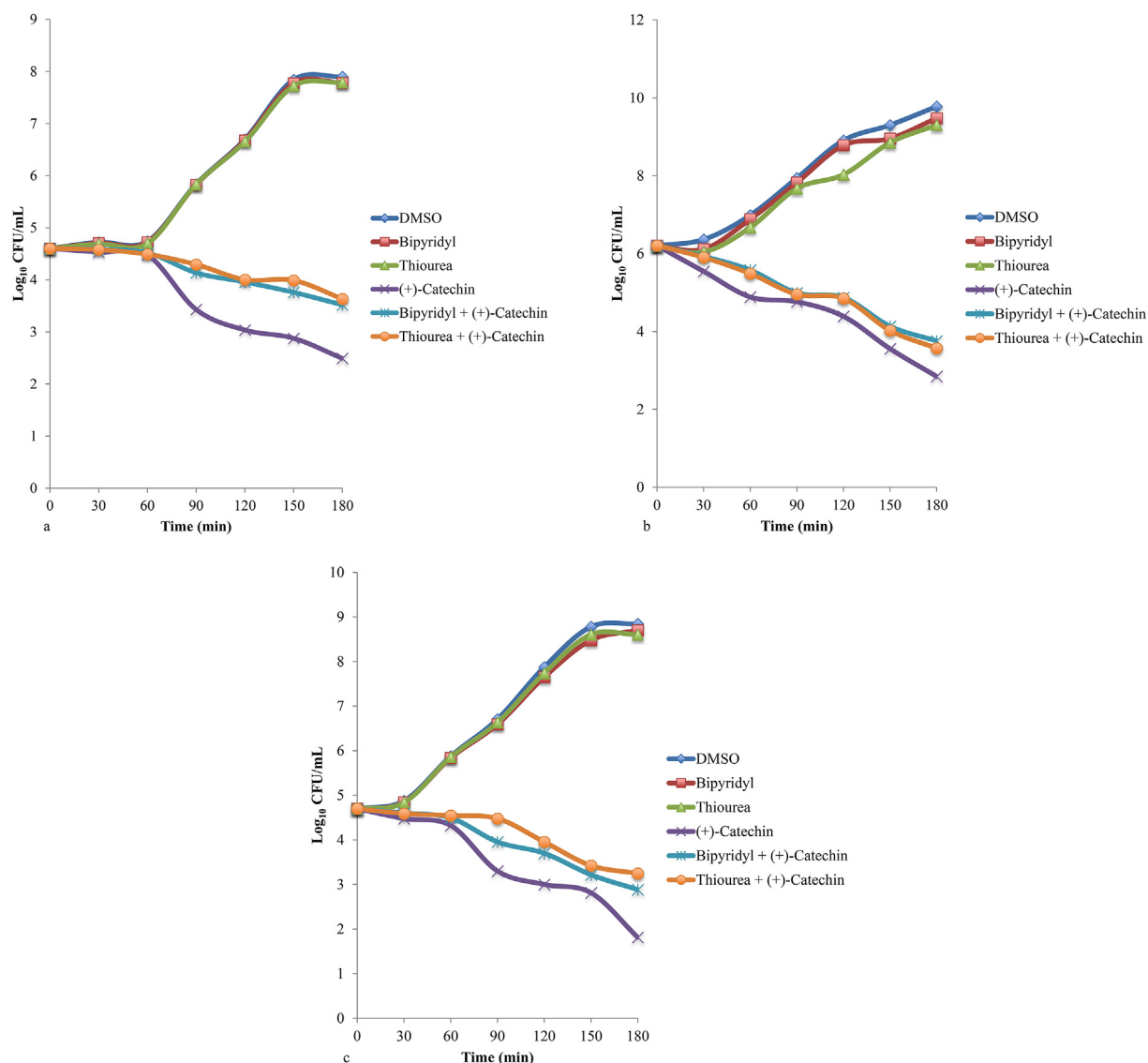


Fig. 7. Viability (CFU/mL) of (a) *Escherichia coli* (ATCC 25922), (b) *Pseudomonas aeruginosa* (ATCC 27853) and (c) *Staphylococcus aureus* (ATCC 29213) treated with (+)-catechin (4× MIC) in the presence of 2,2'-bipyridyl (500 μM) and thiourea (150 mM). Values are mean ± SEM of three determinations and are statistically significant at $p < 0.05$.

death. The elevated level of GSSG could have resulted from the reaction of thiol radical of GSH (product of semiquinone reaction with GSH) and with GSH [37]. Reduction of disulfide radical formed by molecular oxygen also contributes to superoxide anion radical generation [37], leading to redox imbalance.

Deleterious effects of ROS on cellular macromolecules, DNA and lipids, results to DNA fragmentation and lipid peroxidation [38,39]. Products of these processes, malondialdehyde and fragmented DNA, are useful indicator of oxidative status in bacteria. The elevated level of MDA and fragmented DNA in (+)-catechin treated *E. coli*, *P. aeruginosa* and *S. aureus* suggest oxidative onslaught of cellular macromolecules, possibly resulting from enhanced ROS generation.

4.3. Involvement of hydroxyl radical

Evidences have reported the involvement of hydroxyl radical in antimicrobials and antibiotics-mediated bacterial lethality [5,6,40]. We investigated the contribution of hydroxyl radical in (+)-catechin mediated bacterial lethality by treating *E. coli*, *P. aeruginosa*

and *S. aureus* with (+)-catechin and or 2,2' dipyridyl (Fe chelator) and thiourea (hydroxyl radical) scavenger. Increased CFU/mL of *E. coli*, *P. aeruginosa* and *S. aureus* following incubation with (+)-catechin in the presence of 2,2' dipyridyl or thiourea suggest the involvement of hydroxyl radical in bacterial lethality. This support previous studies that have established the involvement of hydroxyl radical as mechanism of antibacterial agents [5,6].

4.4. Molecular docking

The primary sites of ROS, in particular $\cdot\text{O}_2^-$ and H_2O_2 , generation in the mitochondria is the complexes I and III. To know if respiratory chain complexes I–III contributes to (+)-catechin-mediated ROS generation, we performed *in silico* analysis. Moini et al. [41] reported no inhibitory effect of (+)-catechin on electron complexes. However, *in silico* study shows that (+)-catechin interacted with Lys 134 residue via hydrogen bonding as rotenone and amyltal, known inhibitors of complex I. Other hydrogen bond interactions in the binding pocket of NADH:quinone oxidoreductase are with Ser 123 and Ser 125. The non-existence of interactions

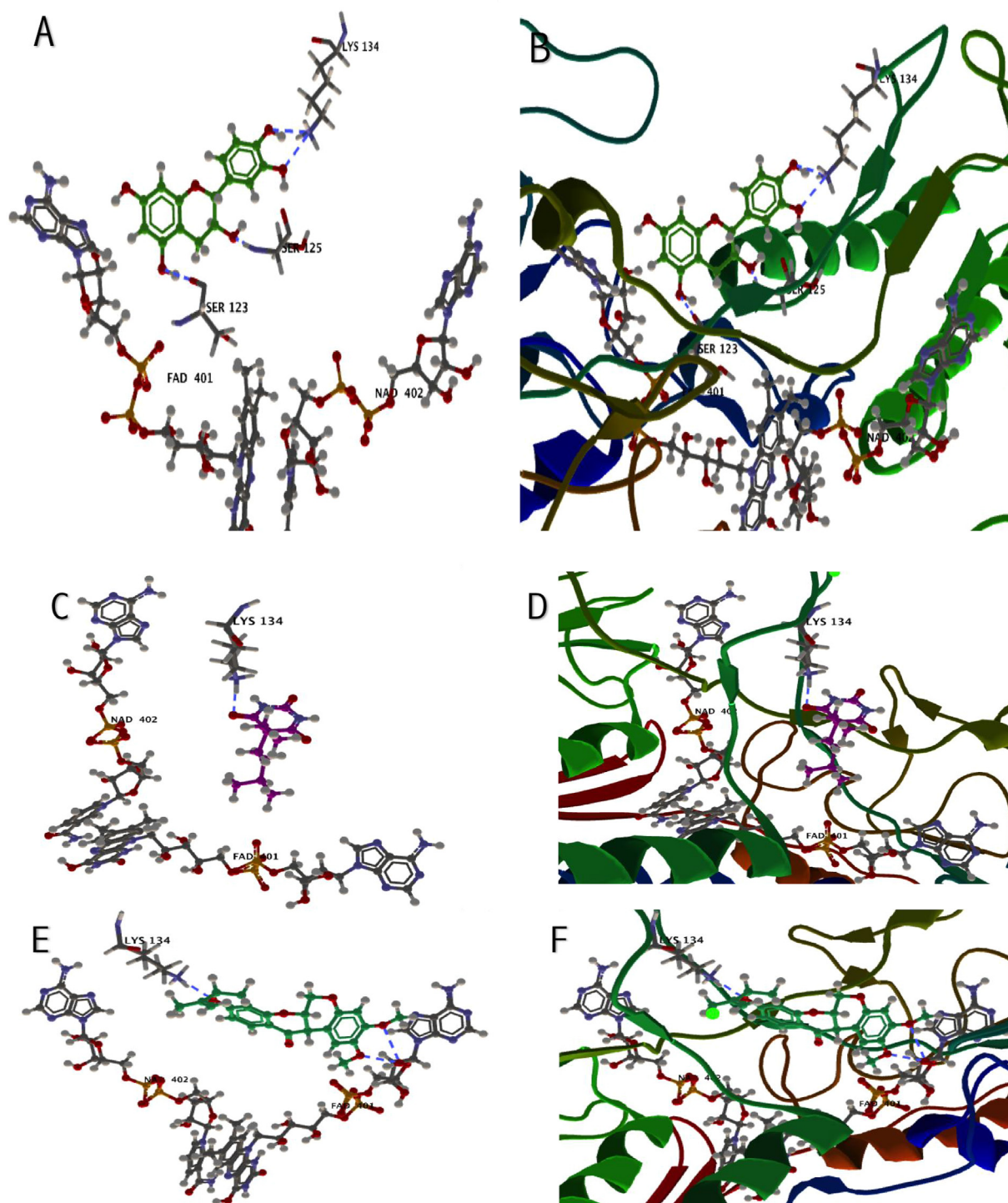


Fig. 8. Molecular docking analysis showing (A) interaction of (+)-catechin with amino acid residues; (B) crystal structure representation of (+)-catechin interaction with amino acid residues; (C) interaction of amtyl with amino acid residues; (D) crystal structure representation of amtyl interaction with amino acid residues; (E) interaction of rotenone with amino acid residues; (F) crystal structure representation of rotenone with amino acid residues in the binding pocket of NADH:quinone oxidoreductase (PDB:1ozk).

with amino acid residues involve in substrate binding (His A242, His A354, Arg A399, Gly A51 and Gly A402), proton shuttle (Arg A286), substrate polarization (Arg A286 and Arg 399) and transition state stabilization (Thr A254 and GluA255) indicates that complex II is not involved in (+)-catechin-mediated bacterial death. It is also evident that (+)-catechin does not alter the substrate binding, substrate polarization, proton shuttle and transition state stabilization of succinate quinone oxidoreductase activity.

DNA gyrase (topoisomerase II) is the primary target of quinolone-based antibiotics. It (quinolone-based antibiotics) traps DNA gyrase during DNA cleavage stage, preventing strand rejoining and inducing bacterial death [42]. The similar interaction (hydrogen bonding with Ala 33) shared by (+)-catechin and nalidixic acid is an indication that (+)-catechin could produce a sort of interaction like nalidixic acid. This could prevent strand rejoining and kill bacteria in a similar way as nalidixic acid [42].

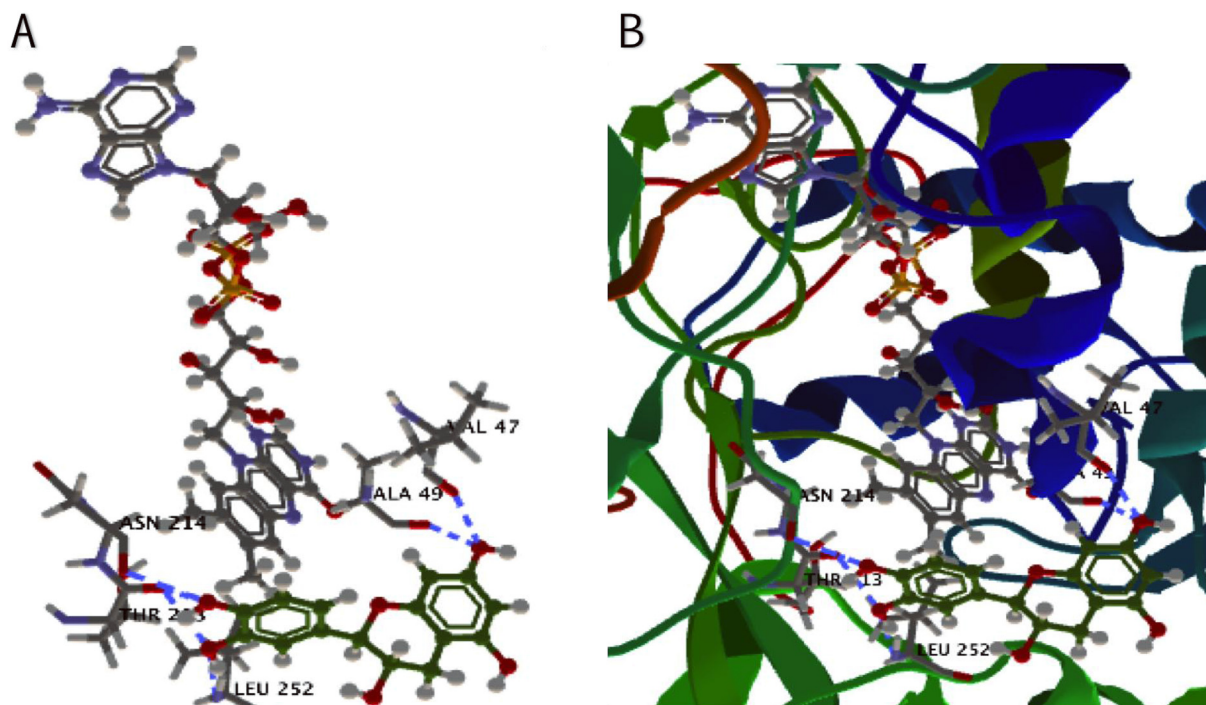


Fig. 9. Molecular docking analysis showing (A) interaction of (+)-catechin with amino acid residues; (B) crystal structure representation of (+)-catechin interaction with amino acid residues in the binding pocket of succinate:quinone oxidoreductase (PDB ID:1nek).

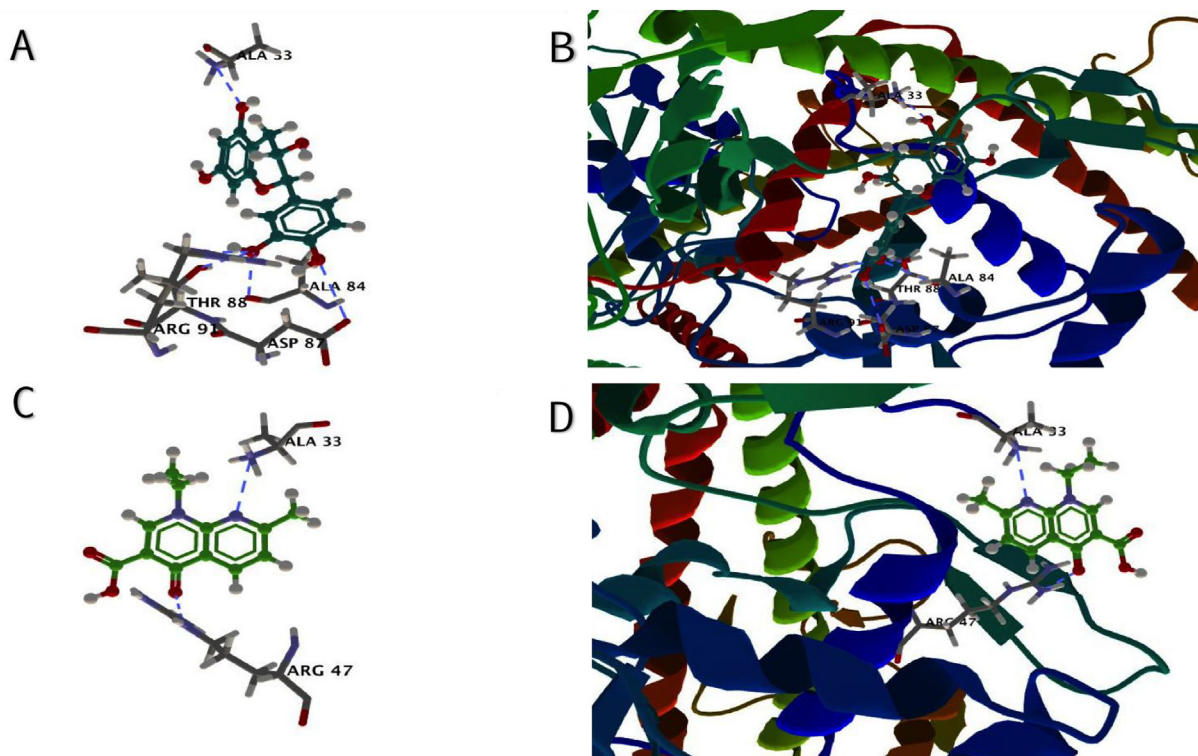


Fig. 10. Molecular docking analysis showing (A) interaction of (+)-catechin with amino acid residues; (B) crystal structure representation of (+)-catechin interaction with amino acid residues; (C) interaction of nalidixic acid with amino acid residues (D) crystal structure representation of nalidixic acid interaction with amino acid residues in the binding pocket of DNA gyrase (PDB ID:4kcl).

Ampicillins, cephalosporins and penicillins inhibit bacterial cell wall synthesis, cell division and enhanced ROS formation leading to cell death [42]. The amino acid residue, Tyr 507, involved in

hydrogen bonding (+)-catechin is same as carbapenem. *In silico* prediction shows that (+)-catechin could mediate bacterial lethality through the inhibition of cell wall synthesis. Epicatechin

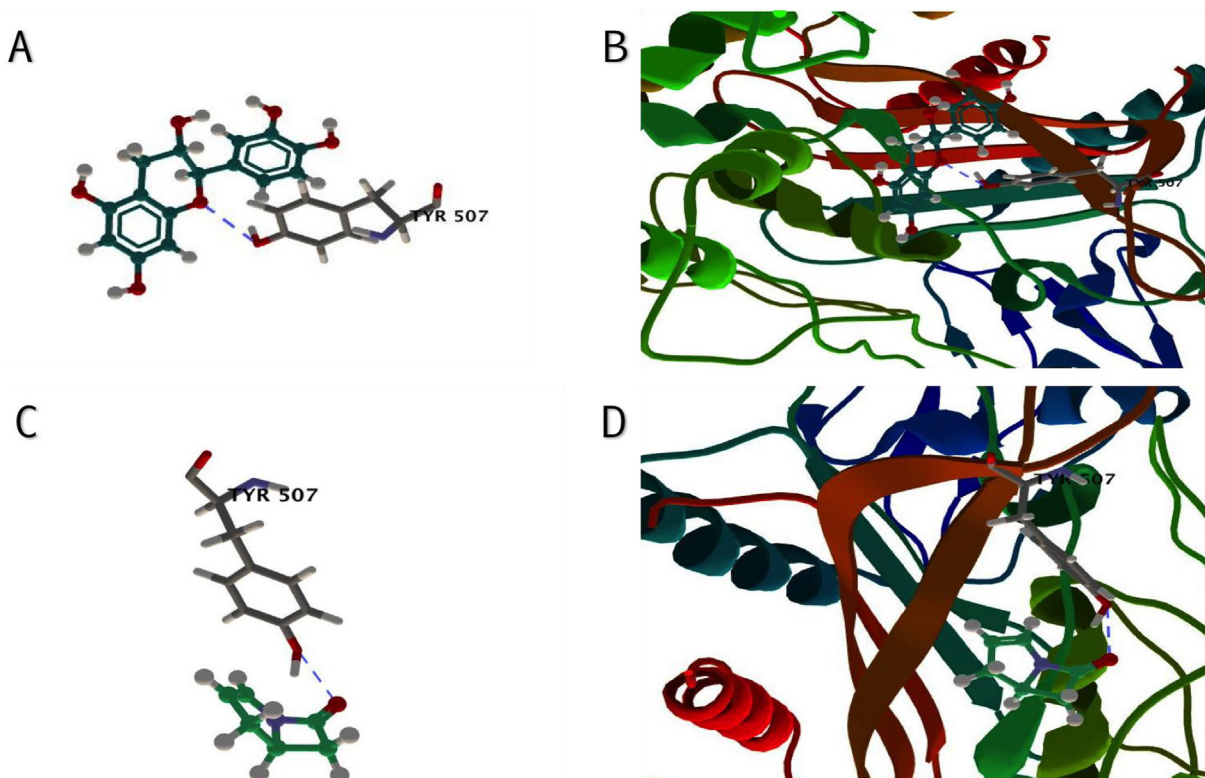


Fig. 11. Molecular docking analysis showing (A) interaction of (+)-catechin with amino acid residues; (B) crystal structure representation of (+)-catechin interaction with amino acid residues; (C) interaction of carbapenem with amino acid residues; (D) crystal structure representation of carbapenem interaction with amino acid residue in the binding pocket of penicillin binding protein (PDB ID:4bjp).

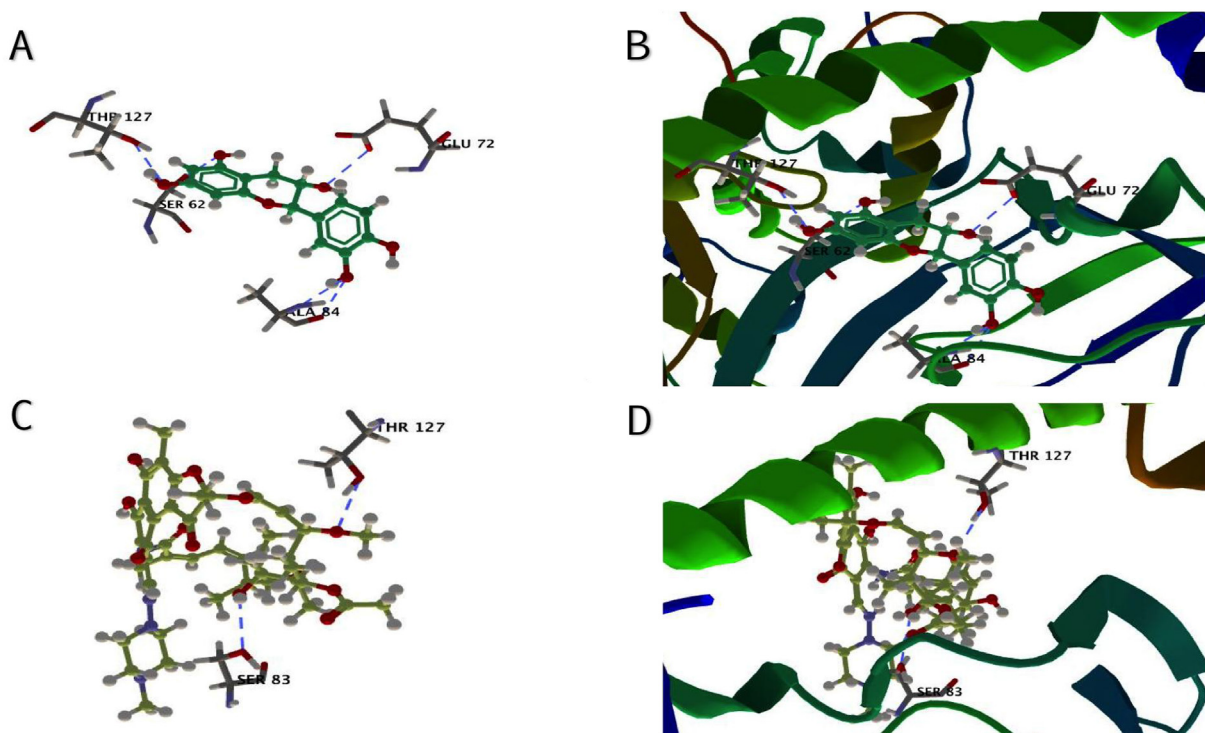


Fig. 12. Molecular docking analysis showing (A) interaction of (+)-catechin with amino acid residues; (B) crystal structure representation of (+)-catechin interaction with amino acid residues; (C) interaction of rifampin with amino acid residues and (D) crystal structure representation of rifampin interaction with amino acid residues in the binding pocket of DNA-directed RNA polymerase (PDB ID:3n4m).

gallate insertion into the cytoplasmic membrane of methicillin-resistant *Staphylococcus aureus* have been reported to disrupt penicillin binding protein (PBP) 2a-mediated beta-lactam resistance [43]. Inhibition of RNA synthesis is noted to induce bacterial cell death by inhibiting nucleic acid metabolism [42]. The capability of (+)-catechin to hydrogen bound Thr 127 in the binding pocket of DNA-directed RNA polymerase indicates that it could halt RNA synthesis in a similar way as rifampin (a known inhibitor of RNA synthesis).

5. Conclusion

It is evident from the data generated from this study that oxidation of (+)-catechin resulted in redox imbalance. This is evident from increased superoxide anion radical generation, enhanced antioxidants enzymes, depleted reduced glutathione, increased malondialdehyde and decreased respiratory chain activity.

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