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Intrinsic insights to antimicrobial effects of Nitrofurantoin to multi drug resistant *Salmonella enterica* serovar Typhimurium ms202

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ABSTRACT

Emerging multidrug resistant (MDR) serovar of *Salmonella* has raised the concern of their impactful effect on pathogenic infection and mortality in human lead by the enteric diseases. In order to combat the battle against these MDR *Salmonella* pathogen, new drug molecules need to be evaluated for their potent antibacterial application. This study evaluates the mechanistic antimicrobial effect of nitrofurantoin against a MDR strain of *Salmonella* named *S. enterica* Typhimurium ms202. The antimicrobial effect of nitrofurantoin was studied through experimental and computational approach using standard microbiological and molecular techniques like growth curve analysis, live-dead analysis, oxidative stress evaluation using high throughput techniques like flow cytometry and fluorescent microscopy. The result showed a potent dose dependent antibacterial effect of nitrofurantoin against *S. enterica* Typhimurium ms202 with a MIC value of 64 μg/ml. Moreover, the mechanistic excavation of the phenomenon described the mechanism as an effect of molecular interaction of nitrofurantoin molecule with membrane receptor proteins OmpC of *S. enterica* Typhimurium ms202 leading to internalization of the nitrofurantoin heading towards the occurrence of cellular physiological disturbances through oxidative stress impeded by nitrofurantoin-Sod1 C protein interaction. The results indicated towards a synergistic effect of membrane damage, oxidative stress and genotoxicity for the antibacterial effect of nitrofurantoin against *S. enterica* Typhimurium ms202. The study described the potent dose-dependent application of nitrofurantoin molecule against MDR strains of *Salmonella* and guided towards their use in further discovered MDR strains.

1. Introduction

Food-borne infection has been one of the major global threat to human health [1]. Many enteric and non-enteric microbes have been discovered to be responsible for this type of infection [2]. Among plethora of microbes, *Salmonella* has got its name as a cause of global food-borne infections threat. It is a gram negative entero-pathogenic bacteria leading to one of the pathogenic diseases named as Salmonellosis [3]. Many species of *Salmonella* have been recognized by microbiologist; majority of which lead to typhoidal fever in human [4]. However, the non-typhoidal *Salmonella* (NTS) cause acute/chronic gastroenteritis. Recent reports have suggested about ~ 93.8 million infection cases and ~ 155,000 deaths annually due to effect of non-typhoidal *Salmonella* [5]. The NTS infections symptomize as the occurrence of diarrhea and non-specific febrile illness [6].

The cause of major food-borne diseases has been found to be the variable serovar of *Salmonella* enterica [7]. The serovar of this subspecies embrace more than 2600 serovar which are identified by their unique somatic (O) and flagella (H) antigen [8]. Among different countries, India has reported ~ 53 serovar till now, and the counting is

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still progressing with the time. Classified among different types of serovar, *Salmonella* enterica Enteritis and *Salmonella* enterica Typhimurium (S. Typhimurium) have been known to the lead pathogens responsible for gastroenteritis in humans [9]. These pathogenic serovar have also been found to be associated with chicken, cattle, and many other hosts relating it with zoonotic pathogens [10]. Remarkably, the pathogenicity of S. Typhimurium has been noted with unknown information about the mechanism of pathogenicity because of large portion of their genome related with different physiological functions [8].

With an enhancement of knowledge about increasing number of S. Typhimurium serovar, microbiologist and clinicians have been regularly in quest of different drugs for their prognosis and treatment [11–14]. Many types of antibiotics and drugs have been regularly prescribed to the patients suffering from the gastroenteritis caused by S. Typhimurium [15]. However, with increase in consumption of different antibiotics with the time, the knowledge about new multidrug resistant (MDR) serovar of S. Typhimurium have also been increased. Recently, one of our reports have detailed about one of such MDR serovar of S. Typhimurium called as *Salmonella* enterica subsp. enterica serovar Typhimurium ms202 (termed as *S. enterica* Typhimurium ms202) which was isolated from feces of a patient admitted to a hospital in Odisha, India and complaining about gastroenteritis [9]. The phenomenon of MDR in serovar of *Salmonella* has been reasoned to the horizontal gene transfer according to the antibiotics used [16].

The choice antibiotics for the bactericidal activity against *Salmonella* strains are based on the mechanism of the antibiotics action [17]. Some of the renowned antibiotics that have been known to use against the *Salmonella* are ampicillin, streptomycin, sulfonamides, tetracycline, and chloramphenicol [18]. However, MDR version of the *Salmonella* against these antibiotics has been largely discovered known as ASSuT and ACSSuT [16]. With references to the records till now, there is high demand of evaluation of new drug as an antibiotic against *Salmonella* MDR strains along with their detailed study of mechanism.

In references to the quest of new compounds and mechanism of their action, the objective of this study was to identify the potential of Nitrofurantoin against *S. enterica* Typhimurium ms202. Nitrofurans are a group of synthetic antimicrobials that are effective against both Gram-negative and Gram-positive parasites and bacteria [19]. These compounds have been extensively utilized in human and veterinary medicine, as well as in animal growth promoters for human consumption. Nitrofurantoin has been recognized as one of the important drug now a day to be used in diseases related with urinary tract infection[20]. Nitrofurantoin is a bactericidal antibiotic with a which can target a broad spectrum of microbial infection [20]. Nitrofurantoin's ability to act at many sites is one of its strongest points [21]. This includes the non-specific action on bacterial ribosomal proteins, which inhibits bacterial enzymes involved in carbohydrate synthesis as well as, to a greater extent, DNA, RNA, and total protein synthesis [22]. Bacterial dehydrogenases convert nitrofurantoin into extremely reactive electrophilic intermediates, which leads to bacterial inhibition [22]. Since nitrofurantoin does not dramatically alter gut flora and accumulates in the lower urinary tract while retaining a low serum concentration, it is favorable antibiotic in many cases. With a plasma half-life of 0.5–1 h, nitrofurantoin is metabolized in renal tissue and promptly eliminated in the urine through both glomerular filtration and tubular secretion [22]. The synthetic antibacterial called nitrofurantoin is made from the chemical compound furan with a nitro group added and hydantoin as a side chain [21].

Nitrofurantoin have been reported to have antimicrobial effects against Salmonella [23]. Owing to the information from literature, it can be hypothesized nitrofurantoin can be an effective antimicrobial gent against this MDR strain *S. enterica* Typhimurium ms202. Moreover, the mechanistic action of the nitrofurantoin needs to be understand to explore its possibility for other MDR strains related to the Salmonella family. Hence, this study was intended to explore the antimicrobial efficacy of Nitrofurantoin against the *S. enterica* Typhimurium ms202 and evaluate the mechanistic details of the action of the compound. A detail study of the antimicrobial action was done using experimental microbial assay like grown curve analysis, Dead/live analysis, oxidative stress analysis and genomic break analysis using high end techniques like flow cytometer and fluorescent microscopy. Moreover, computational approach was taken to understand the molecular interaction of the drug with the membrane proteins and oxidative stress proteins for mechanistic action of the drug. The study provided details for the use of nitrofurantoin as a potent drug against the *S. enterica* Typhimurium ms202 serovar.

2. Materials and methods

2.1. Bacterial strains, growth conditions and antibiotic

The bacterial strain *S. enterica* Typhimurium ms202 was obtained through the isolation from fecal sample of a patient admitted in Odisha hospital, India. The detailed isolation process and the functional and genomic charactersation was performed according to the previous study done and reported by our group [9]. The isolated strain was cultured in M9 minimal media (20% 5X M9 salts) (G013, HiMedia, India). The overnight culture inoculum was then sub-cultured in minimal media and incubated at 35 ± 2^0 C and 150 rpm. The optical density was measured by UV–Vis spectrophotometer (cary 80, Agilent, USA) at 600 nm. Nitrofurantoin was purchased form Merck India. *S. enterica* Typhimurium SB300 was used as reference strain was procured from.

2.2. Antimicrobial assays

2.2.1. Determination of minimum inhibitory (MIC)

The MICs of nitrofurantoin against the S. enterica Typhimurium ms202 were evaluated by macrobroth dilution technique in 96 well plate. One ml of the stock solution (5 mg/ml) of nitrofurantoin was serially diluted in test tubes to obtain concentrations of 1024, 512, 256, 128, 64, 32, 8, 4, 2, and 1 μg/ml. 50 μL of bacterium with an OD of 0.5 was added to each of the solution. One row with only nutrient broth was inoculated with bacterial isolate to serve as control. The plate was incubated aerobically at 35 ± 2^0 C for 24 h, after which the tubes were examined for microbial growth by observing for turbidity. The least concentration of the solution at which inhibited growth was observed read as MIC after 24 h' incubation.

2.2.2. Time kill assay

Time kill analysis was done using a modified procedure described by Appiah et al [24]. The bacterial strain *S. enterica* Typhimurium ms202 and *S. enterica* Typhimurium SB300 (reference strain) were sub-cultured and diluted to 0.5 McFarland standard. With reference to the concentration obtained from the MIC analysis, 10, 25, 50, 100, 250 and 500 μg/ml of nitrofurantoin was added into sterile broth in test tubes, with bacterium of an inoculum size of 1.0×10^6 cfu/ml and the test tubes incubated for 35 ± 2 °C. Followed by incubation, Aliquots (1.0 ml) of the medium were taken at time intervals of 0, 2, 4, 6, 8, and 10 for bacteria was inoculated into nutrient agar in sterile Petri dishes. The Petri dishes with settled agar containing inoculum were further incubated at 37 °C for 24 h. A control test was done for the organisms only. The colony-forming unit (cfu) was calculated with the procedure performed in triplicate. A graph of log CFU/ml was plotted against time. The data were statistically analysed using one-way ANOVA followed by Dunnett's post hoc test from Graph Pad Prism Version 9 for windows (Graph Pad Software Inc., San Diego, CA, USA) [24].

2.2.3. Growth curve analysis

The growth analysis was done using optical density measurement [25]. The growth curve of *S. enterica* Typhimurium ms202 was determined in presence of nitrofurantoin to evaluate their quantitative antibacterial activity. The overnight culture of *S. enterica* Typhimurium

ms202 was sub-cultured for 4 h at 35 ± 2^0 C in a shaker incubator. Followed by incubation, the culture was suspended with different concentration (10, 25, 50, 100, 250 and 500 μg/ml) of nitrofurantoin in 500 μL volume of phosphate buffer saline (PBS). The set of culture was then incubated at 35 ± 2^0 C in an orbital shaker incubator (New Brunswick Scientific, USA) at 160 rpm to determine the antibacterial effect of nitrofurantoin its minimum inhibitory concentration at both temperatures. Culture without nitrofurantoin were taken as control. The optical density (OD) was measured at 600 nm in an interval of 1 h till 8 h. The measurements were taken in triplicates and the statistical analysis and graph was prepared using GraphPad prism 9.

2.2.4. Live-dead analysis using fluorescent microscopy

Bacterial sample was prepared by diluting overnight culture of *S. enterica* Typhimurium ms202 to approximately 1 × 10^{-4} to 10^{-5} CFU/ml. After obtaining the desire concentration the bacterial sample was centrifuged and suspended in PBS buffer. Prepared bacterial sample was incubated for 8 h with nitrofurantoin at different concentration. After incubation the bacterial sample was centrifuged, and the pallet was washed with PBS buffer. The washing step was repeated twice and then the final pellet was resuspended in PBS buffer followed by staining the sample with Syto 9 and PI dye and incubated further for 20 min. The glass slide for microscopic observation was prepared by making a smear of sample on the slide with help of a pipette. With the use of an EVOS fluorescence microscope from AMG, (Mill Creek, Washington), the images were observed and captured in fluorescent filters with red, green and blue channel.

2.2.5. Dead-live analysis using Flow cytometry

Dead-live analysis was done by two-color fluorescence assay in flow cytometry [26]. Bacterial sample was prepared by diluting overnight culture of *S. enterica* Typhimurium ms202 to approximately 1 × 10^{-4} to 10^{-5} CFU/ml. After incubation with nitrofurantoin at different concentration and 37 ^{o}C temperature, cells were stained by stain using Syto 9 dye and Propidium iodide (PI). The fluorescence of single cell suspension was then detected in flow cytometer by Attune (Life technologies). Compensation was done accordingly to compensate two dyes detection. Syto 9 fluorescence emission was collected in the BL1channel using the 530/30 bandpass filter, and propidium iodide (PI) fluorescence emission was collected in the BL3 channel using the 640 LP filter. The data was analyzed using FCSExpress7.

2.2.6. Bacterial morphology analysis by SEM

The untreated and treated nitrofurantoin bacterial strain was imaged using SEM to analyses the morphology of bacteria after treatment [26]. For imaging of bacteria treated with nitrofurantoin. The experimental set up was similar to growth kinetics analysis experiment as described above. After incubation, the cells were washed three times with Phosphate buffer solution and were fixed with 4% paraformaldehyde (PFA) for 25 min. Followed by fixation, dehydration of cells were done with increasing concentration of alcohol (10%, 30%, 50%, 70%, 90%, 100%). Soon after completion of dehydration cells were layered on silicon substrate, air dried and were analyzed for imaging in SEM (Carl Ziess, EVOS 18). Images were processed for their proper contrast and resolution in Image J imaging software.

2.2.7. Oxidative stress analysis

The mechanistic antibacterial activity of the nitrofurantoin was evaluated by understanding the oxidative stress in *S. enterica* Typhimurium ms202 induced on treatment with nitrofurantoin [27]. The oxidative stress was checked by measuring the reactive oxygen species (ROS) indicated by the green fluorescence of DCFDA stain using flow cytometry. The bacterial stain was treated with different concentration of nitrofurantoin (10, 25,50,100, 200 μg/ml). Followed by incubation at 37 ^{o}C, the untreated and treated *S. enterica* Typhimurium ms202 were stained with 2 μg/ml of DCFDA suspended in PBS. The stained samples were incubated in dark for 20 min and washed thrice after incubation to extrapolate the extra stain. The fluorescent intensity of bacterial cells was measured in BL1 filter of flow cytometer (Attune acoustic flow cytometer, Thermofisher Scientific, USA). The debris cells were gated out and 10,000 cells were counted in the flow-cytometer. The data were analyzed using FacsXpress 7 (Denovo, USA).

2.2.8. Genotoxicity analysis

The genotoxic effect of nitrofurantoin was analyzed by measuring the cell death induced by DNA damage indicated by the green fluorescence of Acridine orange stain using flow cytometry [28]. The bacterial stain was treated with different concentration of nitrofurantoin (10, 25, 50,100, 200 μg/ml). Followed by incubation at 37 ^{o}C, the untreated and treated *S. enterica* Typhimurium ms202 were stained with 2 μg/ml of Acridine Orange suspended in PBS. The stained samples were incubated in dark for 20 min and washed thrice after incubation to extrapolate the extra stain. The fluorescent intensity of bacterial cells was measured in BL2 filter of flow cytometer (Attune acoustic flow cytometer, Thermofisher Scientific, USA). The debris cells were gated out and 10,000 cells were counted in the flow-cytometer. The data were analyzed using FacsXpress 7 (Denovo, USA).

2.3. In silico molecular docking analysis

In order to determine the preferred binding positions that confer the minimum binding energy (generally in negative energies) on the docking complex, molecular docking studies are used to analyze the interactions between chemical compounds and proteins; molecular docking analysis was carried out using Autodock 4.2 and Autodock Vina v. 1.2.2 using Nitrofurantoin drug as ligand with sodc1 and OmpC receptor proteins. As the whole genome annotations of a multi-drug resistant *Salmonella enterica subsp. enterica serovar* Typhimurium ms202 has been submitted to the Comprehensive Genome Analysis service and is not yet available on databases, sequences of the most similar *Salmonella enterica subsp. enterica serovar* was used to perform the docking and analyze the molecular interactions of both the ligand and receptors. During the genome analysis and virulence gene expression profiling, the similarity index was highest with *S. enterica sub sp enterica* strain LT2 [9]. So, for the docking purpose, we have used the OmpC and sod1c protein of *S. enterica sub sp enterica* strain LT2 for the close proximity analysis. Due to the unavailability of 3-dimensional structures of the OmpC receptor proteins from *S. enterica sub sp enterica* strain LT2, we have obtained the modeled structures from the AlphaFold database (UniProt ID: P0A263); 3-dimensional structure for sodc1 was retrieved from Protein Data Bank (PDB-ID-6D52). The structure of nitrofurantoin was obtained from the PubChem database and its geometry was optimized using Gaussian 03 program. The receptor proteins were subjected to energy minimization and prepared using the Chimera and AutoDock tools program. The parameters for nitrofurantoin have been set for Autodock 4.2 while the Grid dimensions were set to 82x62x62 for OmpC and 72x48x118 for sodc1 with a spacing of 1 Å. The exhaustiveness was set to 8 for both docking parameters. The post-docking analysis was performed using Autodock 4.2 analysis tools using conformations and clustering and visualized using ChimeraX. For nitrofurantoin, the ligands were docked against the receptor proteins using Vina 1.2.2, and the interactions were visualized using Discovery Studio Visualizer.

2.4. Statistical analysis

All statistical analyses were done using GraphPad Prism and assessed by ANOVA. The data were expressed as mean ± SEM, and $p < 0.05$ or below was considered for significant difference.

3. Results and discussion

3.1. Antimicrobial effect of nitrofurantoin with S. enterica Typhimurium ms202

The antibacterial effect of Nitrofurantoin against the MDR strain *S. enterica* Typhimurium ms202 was evaluated by analyzing the effect of nitrofurantoin on growth of *S. enterica* Typhimurium ms202 through different slandered microbiological assays. As shown in Fig. 1A, MIC analysis showed an inhibition of growth of the bacteria at a range of 32–64 μg/ml of nitrofurantoin (Table 1). The time analysis showed a decrease in CFU of the bacteria with increase in concentration specifically after 50 μg/ml, the growth was further found to be decreasing with increase in concentration to 500 μg/ml (Fig. 1B). Similar declination in growth was observed in time kill analysis doen with the reference wild type strain of *S. enterica* Typhimurium SB300 (Fig. 1C). Interestingly, the inhibition in growth was observed from the 6th hour of incubation. As shown in Fig. 1D, the growth curve of the bacteria was found to be decreased with increase in concentration treatment of the nitrofurantoin. At the very low concentration of 10 μg/ml, the growth curve was found to be proceeded in an equivalence of the untreated control. The result can be reasoned to the minimal effect of nitrofurantoin which was unable to inhibit the division of the bacterial strain at any phase [29]. With increase in concentration form 10 μg/ml to 500 μg/ml, a consistent steep in the growth was observed which indicated towards the bacteriostatic and bactericidal effect of nitrofurantoin. The data can be reasoned to the bacteriostatic effect of nitrofurantoin leading to the death of the bacteria and inhibiting them for any further growth. Similar effects have been reported for the action of Nitrofurantoin against other bacterial strains like *E. coli* and *S. typhi* [29,30].

3.2. Cellular effect of Nitrofurantoin to S. enterica Typhimurium ms202

To excavate the cellular effect and mechanism of action of nitrofurantoin against *S. enterica* Typhimurium ms202, a quantitative and qualitative dead-live analysis was performed using the syto9 and Propidium dye. Syto 9 is meant to exhibit green fluorescence on binding with DNA of the bacteria while PI is meant to fluoresce red [31]. However, due to higher molecular size, it was estimated that the exhibition of PI fluorescence will occur only in case of bacterial membrane damage because of their penetration through the membrane after damage done by the nitrofurantoin treatment. As shown in Fig. 2, the bacterial strain treated with lower concentration of nitrofurantoin (10, 25 μg/ml) showed a higher green fluorescence compared to the higher doses. Interestingly, at the treatment concentration of 50 μg/ml and 100 μg/ml, a yellow fluorescence was found to be prominent which can be reasoned to the combined fluorescence intensity of Syto 9 and PI occurring due to their binding after the membrane damage done by the higher doses nitrofurantoin. Consequently, at higher dose (200 μg/ml), red fluorescence was found to be prominent indicating towards the greater membrane damage of the bacterial strain treated with nitrofurantoin. The data indicated to the membrane damage effect of nitrofurantoin leading to the internalization of nitrofurantoin inside the bacterial cell for further physiological consequences. The qualitative microscopy analysis was supported by a flow cytometry quantitative analysis. As shown in Fig. 3, the fluorescence intensity of PI indicating the membrane damage was found to be increasing from 0.05% in control bacteria to 0.20%, 0.26%, 2.61%, 9.65%, 60.80% in bacterial strain treated with 10, 20, 50, 100 and 200 μg/ml of nitrofurantoin. The data confirmed the results of fluorescence microscopy and affirmed the membrane damage effect in *S. enterica* Typhimurium ms202 possessed

Table 1
MIC of nitrofurantoin with different strains of Salmonella,.

Antimicrobials	MIC (μg/ml)	
	S. Typhimurium ms202	S. Typhimurium SB300
Nitrofurantoin	**64 μg/ml**	**32 μg/ml**

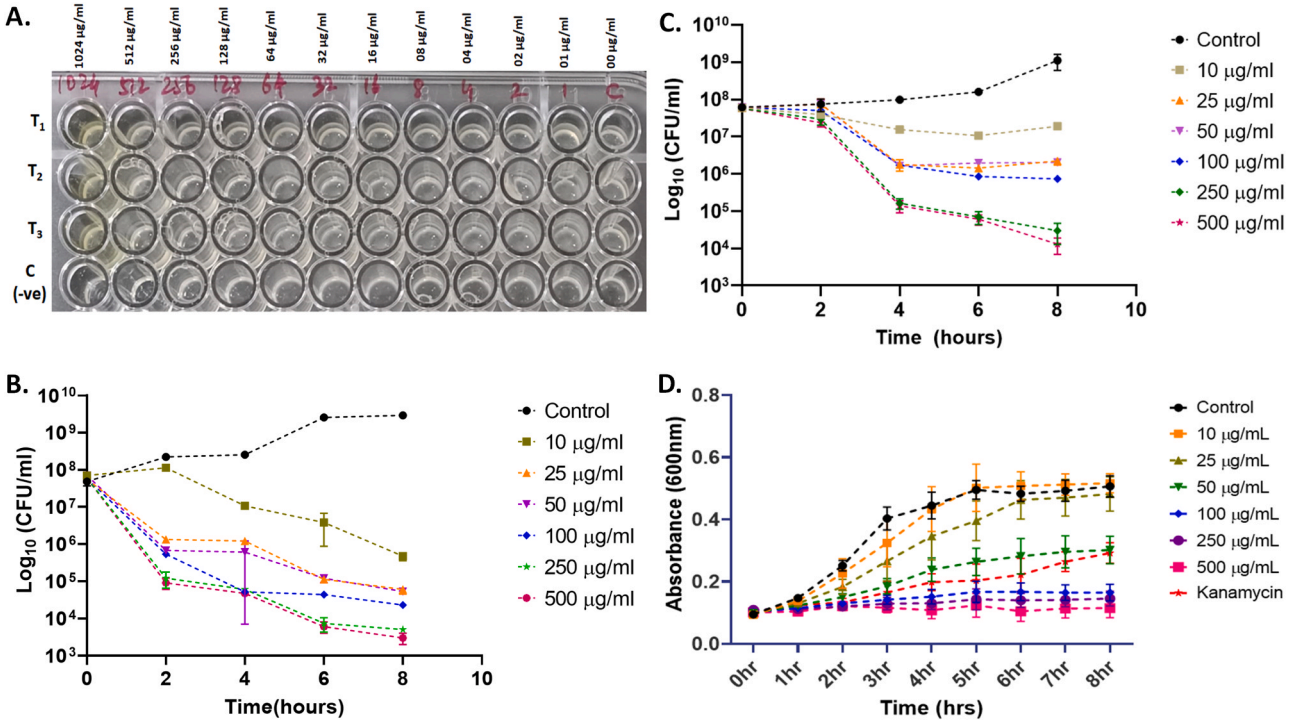

Fig. 1. : Antibacterial effect of nitrofurantoin with *S. enterica* Typhimurium ms202; (A) MIC analysis through 96 well plate broth dilution method. (B) CFU analysis of *S. enterica* Typhimurium ms202 in presence of different concentration of nitrofurantoin determined by Tome kill assay (C) CFU analysis of *S. enterica* Typhimurium SB300 in presence of different concentration of nitrofurantoin determined by Tome kill assay (D) Growth curve of *S. enterica* Typhimurium ms202 in presence of different concentration of nitrofurantoin.

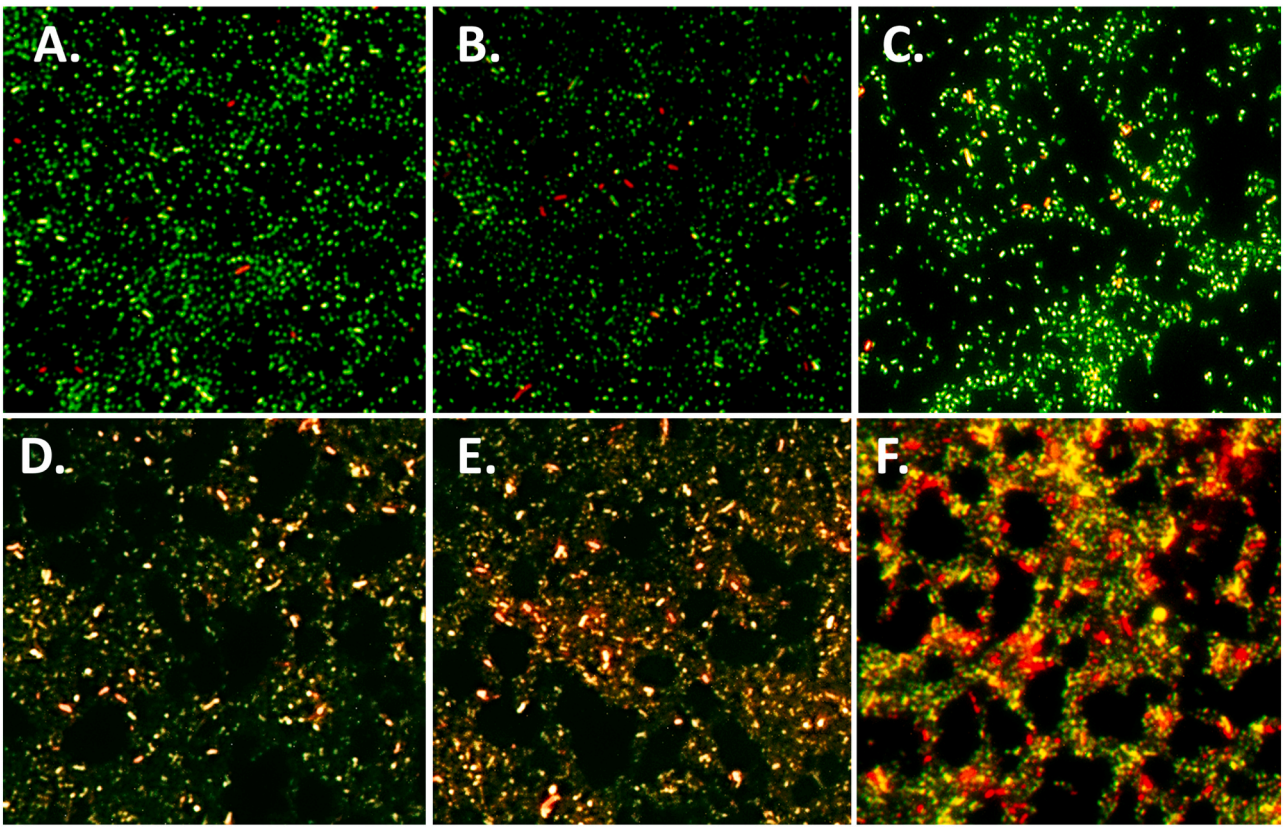

Fig. 2. : Microscopic Live-dead analysis of *S. enterica* Typhimurium ms202 exposed to different concentration of nitrofurantoin; (A) control (B) 10 μg/ml (C) 20 μg/ml (D) 50 μg/ml (E) 100 μg/ml (F) 200 μg/ml. The bacterial strain was stained with Syto9 and counterstained by Propidium Iodide (PI).

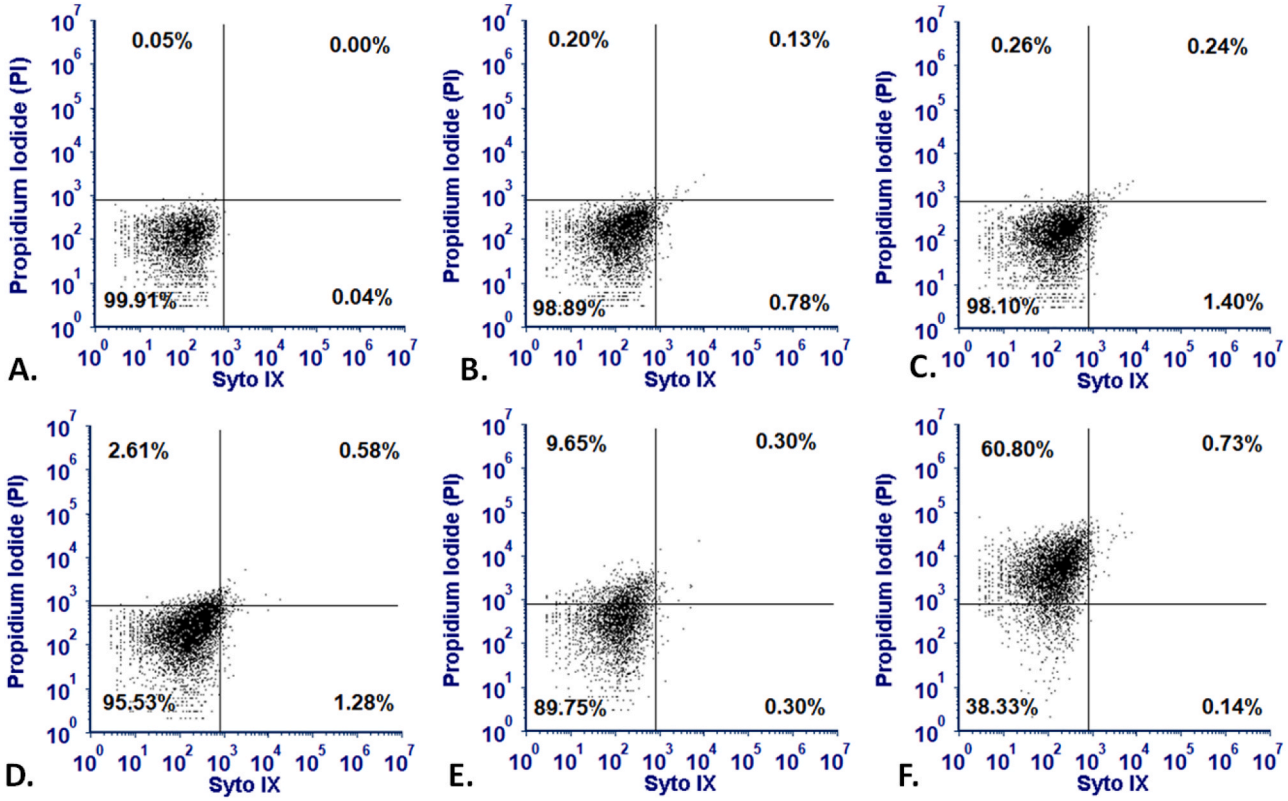

Fig. 3. : Flow cytometry analysis of Live-dead of *S. enterica* Typhimurium ms202 exposed to different concentration of nitrofurantoin; (A) control (B) 10 μg/ml (C) 20 μg/ml (D) 50 μg/ml (E) 100 μg/ml (F) 200 μg/ml. The bacterial strain was stained with Syto9 and counterstained by Propidium Iodide (PI).

by nitrofurantoin treatment.

The visual affirmation of the membrane damage was further done by electron microscopy of the bacterial strain treated with nitrofurantoin. As shown in Fig. 4, compared to the integral structure of control bacterial cell (Fig. 4A), the membrane of the nitrofurantoin treated bacterial cells were seen damaged and shrink. The effect was prominent and higher in case of bacterial cells treated with higher doses of nitrofurantoin. The data was in correlation with the results obtained by flow cytometry and previous literature reports of other bacterial strains like *E. coli* treated with nitrofurantoin [30].

The live-dead analysis affirmed the induction of membrane damage by nitrofurantoin hypothesizing their internalization inside the bacterial cells leading to influential cellular and molecular changes in the bacterial cells treated with nitrofurantoin. It has been reported that the nitrofurantoin induces oxidative stress changes in the bacterial strains like *E. coli* upon their exposure [32,33]. Hence, it was hypothesized that the internalized nitrofurantoin would have been inducing major molecular changes through the induction of oxidative stress [34]. The hypothesis was crisscrossed by flow cytometry analysis of DCFDA dye exhibiting green fluorescence in bacterial cells treated with nitrofurantoin. As shown in Fig. 5A, the green fluorescence intensity of DCFDA was found to be increased in bacterial cells treated with nitrofurantoin with increase in exposure concentration of nitrofurantoin to the *S. enterica* Typhimurium ms202 cells. The result confirmed the induction of oxidative stress in the nitrofurantoin treated bacterial cells indicating to the lethal effect like cell death. The induction of oxidative stress has been reported to be a major key cellular and molecular phenomenon for the induction of cell death in different bacterial cells [25]. Further, it was hypothesized that the induced oxidative stress and the membrane damage leads to the genotoxic effect in nitrofurantoin treated bacterial cells. The genotoxicity was further evaluated in nitrofurantoin treated bacterial cells by analyzing the acridine orange fluorescence intensity through flow cytometry [28]. As shown in Fig. 5B, the acridine orange green fluorescence intensity was found to be enhanced with increase in exposure concentration of the nitrofurantoin confirming the dose dependent genotoxic effect of nitrofurantoin to *S. enterica* Typhimurium ms202 cells. Previous reports have suggested similar results in other bacterial strains treated with nitrofurantoin [32].

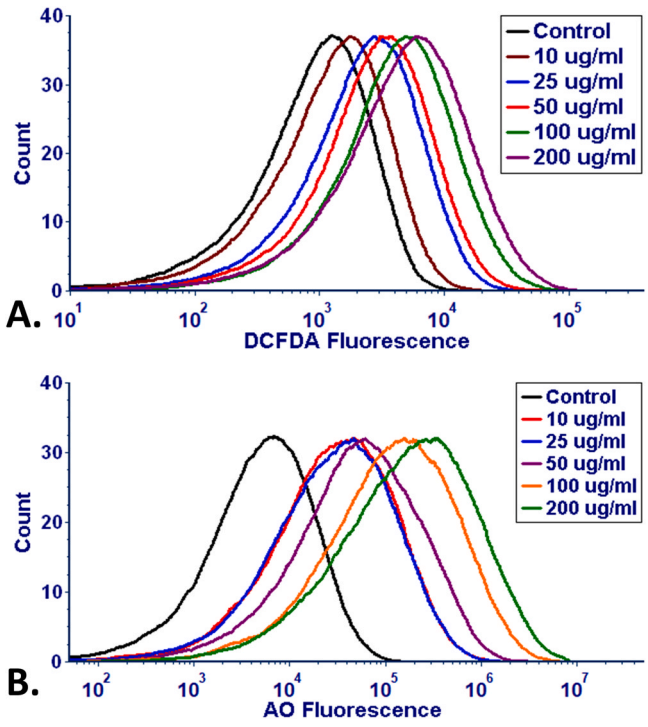

Fig. 5. : Cellular and molecular changes in *S. enterica* Typhimurium ms202 exposed to different concentration of nitrofurantoin; (A) Induced oxidative stress, the cells were stained with DCFDA (B) Induced Genotoxicity; the cells were stained with Acridine orange.

3.3. *In silico analysis*

The experimental analysis of antimicrobial effect of nitrofurantoin indicated a membrane damage impact of nitrofurantoin to *S. enterica* Typhimurium ms202 cells along with induction of oxidative stress and genotoxicity. However, the molecular mechanism remains unclear about the initial interaction of nitrofurantoin with bacterial membrane

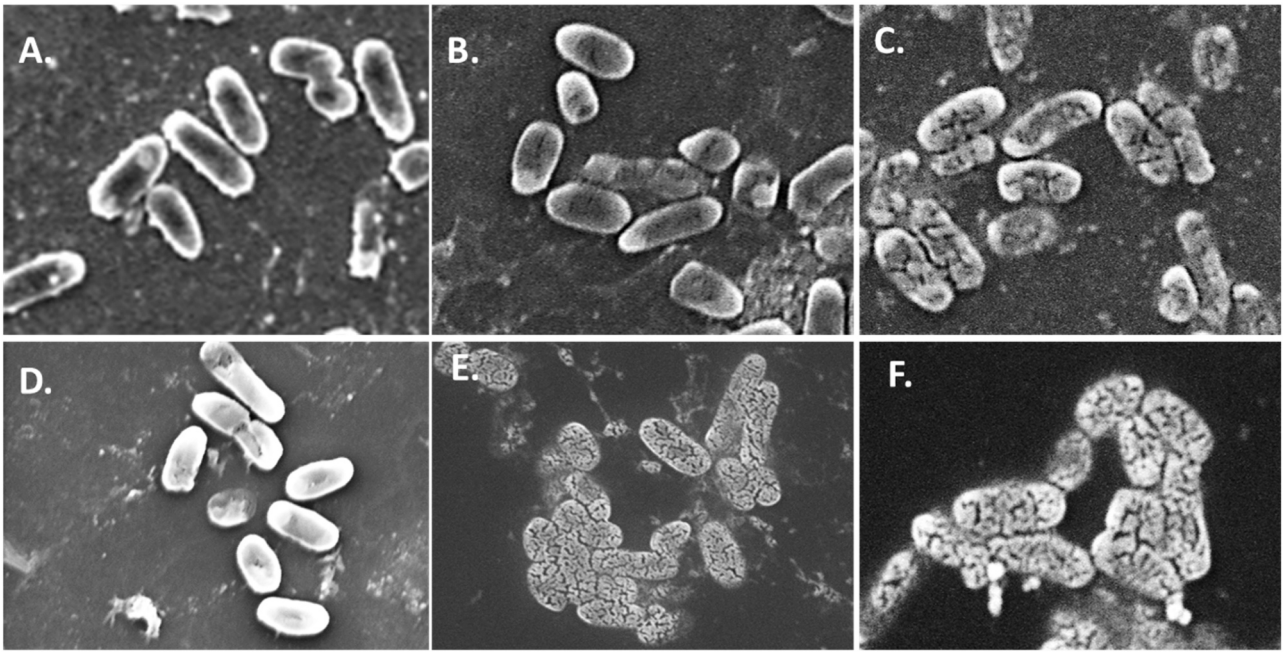

Fig. 4. : SEM images of *S. enterica* Typhimurium ms202 exposed to different concentration of nitrofurantoin; (A) control (B) 10 μg/ml (C) 20 μg/ml (D) 50 μg/ml (E) 100 μg/ml (F) 200 μg/ml.

leading to their internalization. Moreover, it was argued that the induction of oxidative stress was resulted due to molecular interaction of nitrofurantoin with oxidative stress regulating proteins in bacterial cells like Sod1c [25,34]. Hence, to understand the molecular mechanism, an *in silico* molecular docking analysis was done. OmpC receptor proteins has been annotated to be one of the important membrane receptor protein in S. Typhimurium cells [35]. OmpC was chosen to understand the interaction of nitrofurantoin with membrane proteins through molecular docking approach. As shown in Fig. 6, nitrofurantoin was found to be interacted with amino acids like serine, arginine, threonine, alanine and lysis via strong hydrogen bond. Moreover, interestingly it was observed that the oxygen atom of the nitrofurantoin was playing a major role in the interaction with the proteins amino acids. The analysis indicated towards an influential structural changes in the membrane protein of bacterial cells due to interaction with nitrofurantoin and reasoned towards the fact of their internalization inside the bacterial cells for disturbing the cellular physiological phenomenon. Further, it was argued that the internalized nitrofurantoin molecule interacts with oxidative stress protein like Sod1c to bring oxidative stress changes at molecular level [25]. As shown in Fig. 7, the computational analysis showed a hydrogen bond interaction of nitrofurantoin with threonine, asparagine and glycine through oxygen and nitrogen atom of the nitrofurantoin. The result indicated to a firm influential structural and functional changes in the Sod1c protein leading to oxidative stress. The interaction pathway as predicted from the string analysis showed a predictive role of many proteins like SodA, SodB, katG in the channel of induction of oxidative stress phenomenon (Fig. 8).

3.4. Mechanism

The experimental and computational analysis indicated towards a predictive mechanism of the antibacterial activity of the nitrofurantoin against *S. enterica* Typhimurium ms202. With reference to previous literature and the obtained results the mechanism can be elucidated as: the exposure of nitrofurantoin to the bacterial cells leads to their accumulation and internalization at the surface of the bacteria. The accumulated nitrofurantoin molecule interacts with the bacterial membrane receptor proteins like OmpC through different amino acid residues. The interaction leads to destabilization in the structure of the receptor molecules due to an imbalance in energy level of the amino residues molecules. The structure destabilization and modification further leads to membrane damage and the membrane leakage resulting in the internalization of the nitrofurantoin molecule inside the bacterial cells. The internalized nitrofurantoin molecule then interacts with metabolic proteins like oxidative stress proteins Sod 1c and others to induce abnormal reactive oxygen species (ROS) production. The abnormal rise in ROS in the cell also affect the functionality of other organelles. A synergistic effect of membrane damage, DNA damage and the oxidative stress further leads to the bacterial cell death accomplishing the antibacterial effect of nitrofurantoin.

The study showed a firm antibacterial effect of nitrofurantoin against *S. enterica* Typhimurium ms202 and indicated towards a dose dependent potent application. Moreover, the information obtained through the mechanistic analysis described a potent pathway for the excavation and evaluation of other drug and antibiotics against the MDR strains of *S. enterica* Typhimurium serovar.

4. Conclusion

In brief, the present study described and evaluated the mechanistic antibacterial effect of nitrofurantoin against *S. enterica* Typhimurium ms202. The MIC analysis and Time kill assays determined the growth inhibitory effect of nitrofurantoin at a concentration range of 32–64 μg/ml. The growth curve analysis confirmed a dose dependent effect of nitrofurantoin for its antimicrobial effect. The cellular and molecular analysis with experimental and computational approach described a dose-dependent bacteriostatic and bactericidal action of nitrofurantoin with a speculative mechanistic action through membrane damage and oxidative stress. It was caricatured as the interaction of the molecule with receptor protein leads to their internalization and disturbance of cellular physiology through oxidative stress and membrane leakage leading to bacterial cells death. The data suggested nitrofurantoin as a potent molecule for the treatment of modalities associated with pathogenesis of *S. enterica* Typhimurium ms202.

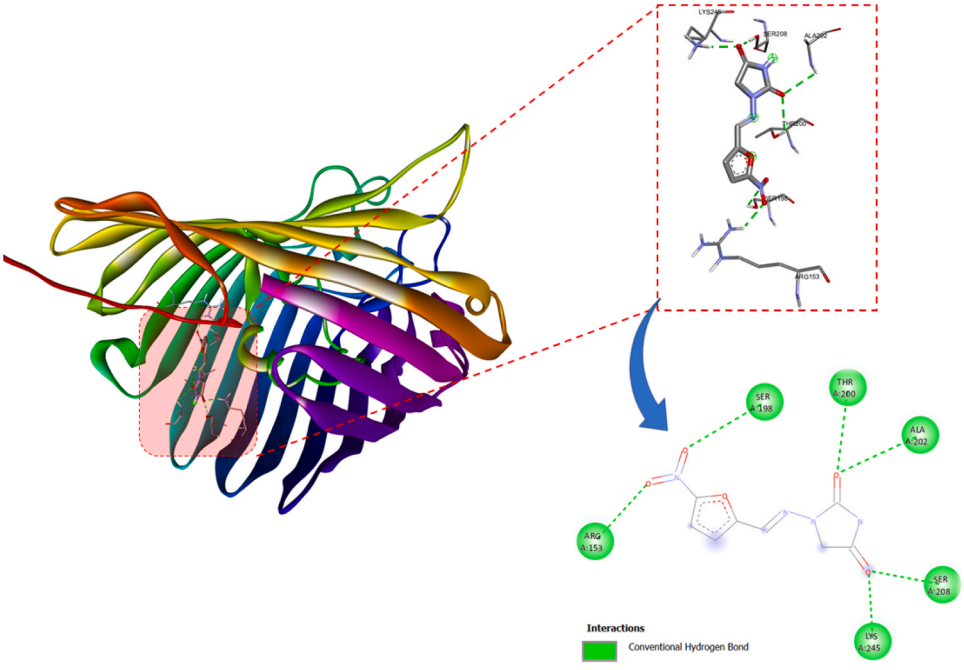

Fig. 6. : Molecular docking analysis of interaction of nitrofurantoin with OmpC membrane receptor protein of S. Typhimurium. The image shows molecular interaction and bonding of nitrofurantoin with amino acids of protein.

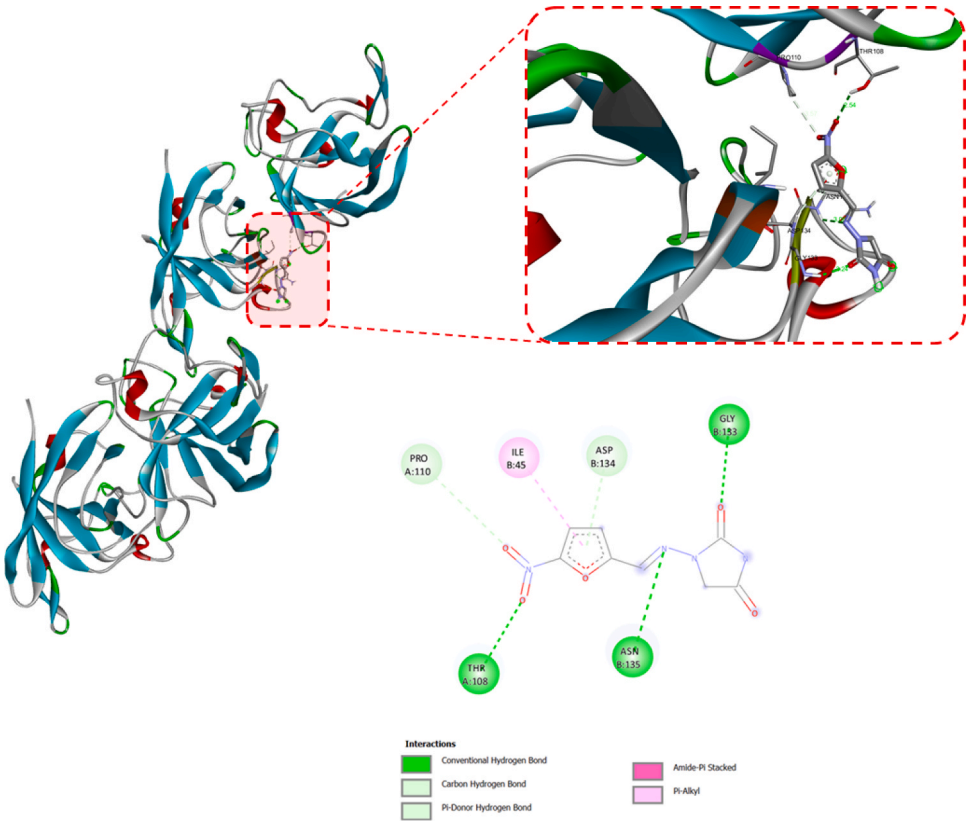

Fig. 7. : Molecular docking analysis of interaction of nitrofurantoin with Sod1C protein of S. Typhimurium. The image shows molecular interaction and bonding of nitrofurantoin with amino acids of protein.

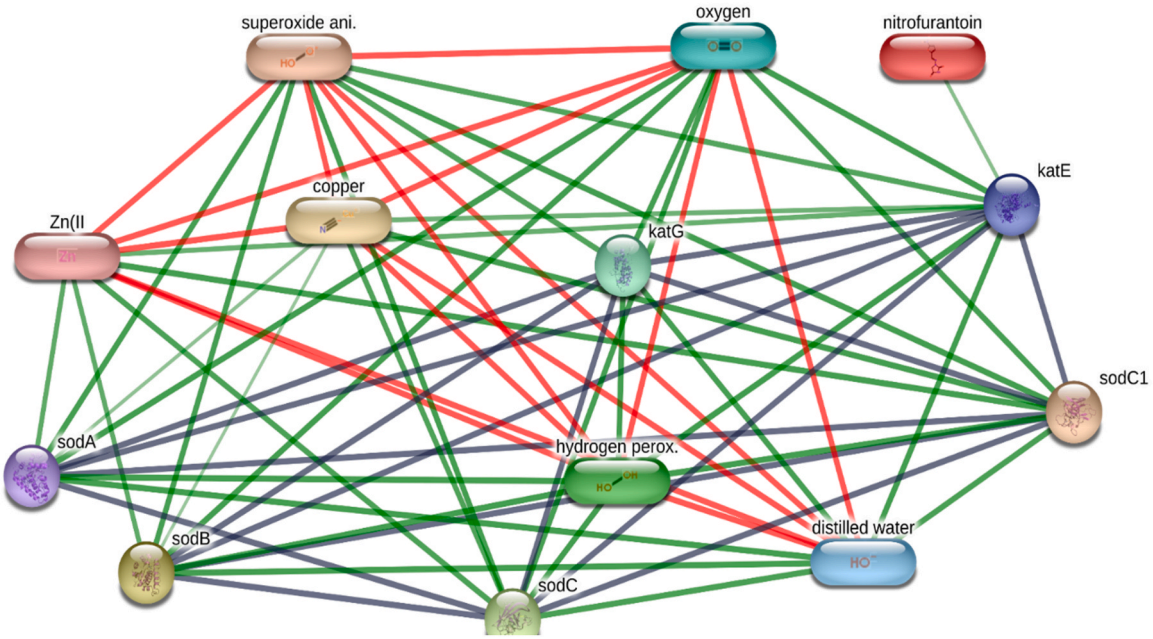

Fig. 8. : String analysis showing channel of interactive proteins in interactive pathway of nitrofurantoin with S. Typhimurium proteins.

CRediT authorship contribution statement

Nirmal Kumar Mohakud: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing. **Rakesh Kumar Panda:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing. **Dibyangshee Singh:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing. **Saumya Darshana Patra:** Investigation, Methodology, Formal analysis, Writing – review & editing. **Faizan Zarreen Simnani:** Investigation,

Methodology, Formal analysis, Writing – review & editing. **Adrija Sinha:** Methodology, Formal analysis, Software, Investigation, Validation, Writing – review & editing. **Aditya Nandi:** Methodology, Investigation, Formal analysis, Validation, Writing – review & editing. **Ealisha Jha:** Methodology, Investigation, Validation, Writing – review & editing. **Sarita Singh:** Methodology, Investigation, Validation, Writing – review & editing. **Nagendra Kumar Kaushik**: Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Pritam Kumar Panda:** Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Deobrat Singh:** Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Suresh K. Verma:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition**. Mrutyunjay Suar:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – review & editing, Supervision, Funding acquisition**.**

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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