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# Fluoride triggers oxidative stress and cell damage in antibiotic-resistant *Klebsiella pneumoniae*

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## ABSTRACT

**Purpose:** Due to the critical threat of antibiotic-resistant *K. pneumoniae*, there is an urgent need for novel therapeutic strategies. This study aimed to evaluate the antibacterial efficacy of sodium fluoride (NaF) against *K. pneumoniae* by investigating changes in both morphology and biochemical profiles.

**Methods**: The study investigated the antibacterial and antibiofilm effects of different concentrations of NaF against the *K. pneumoniae* strain. Additionally, it examined alterations in morphology using scanning electron microscopy (SEM), metabolic changes through Fourier Transform Infrared (FTIR) Spectroscopy, biochemical responses, and antioxidant defense mechanisms by assessing stress markers in NaF-exposed *K. pneumoniae* cells.

**Results:** *K.pneumoniae* strains exhibited sensitivity, with inhibition zones of 13±1.0 mm, 20±1.5 mm, and 25±1.0 mm for NaF concentrations of 10%, 20%, and 30%, respectively. Remarkable morphological and biochemical alterations were noted in NaF-treated cells in a dose-dependent manner. NaF-exposed *K. pneumoniae* cells demonstrated elevated levels of oxidative markers such as lipid peroxidation and ascorbic acid, and a decrease in both enzymatic and non-enzymatic antioxidants, particularly reduced glutathione (GSH) and glutathione-S-transferase (GST)

**Conclusions:** NaF induces oxidative stress and causes morphological changes in antibiotic-resistant *K. pneumoniae* strains.

***Keywords:*** *Antibacterial; K. pneumoniae; Morphology; NaF stress; Oxidative stress.*

## INTRODUCTION

The mouth and nasal passages in humans are frequently inhabited by pathogenic microorganisms capable of causing acute respiratory infections [1]. Some pathogens colonize asymptomatically to facilitate their spread within human populations, particularly those carrying genes for antibiotic resistance, which present substantial clinical difficulties [2]. The *Klebsiella* genus, in particular, is frequently associated with hospital-acquired infections and exhibits resistance to multiple medications [3,4]. Klebsiella bacteria are mainly found in the human digestive system and are spread by contact [5]. Enterotoxins are produced by certain strains, which makes therapy more difficult [6,7]. *Klebsiella pneumoniae* typically colonizes asymptomatically in human oral, skin, respiratory, and gastrointestinal tracts. However, it can also act as an opportunistic pathogen, causing a range of infections like pneumonia, meningitis, liver

abscesses, urinary tract infections, and sepsis [8.] With the global increase in antibiotic resistance, *K. pneumoniae* strains have become particularly concerning [9.] The World Health Organization (WHO) has identified *K. pneumoniae* as a critical priority pathogen requiring new therapeutic approaches [10]. *K. pneumoniae* strains in oral and nasal cavities are resistant to multiple drugs and persist longer than other strains [11]. Pathogenic strains of *K. pneumoniae* have been identified in dental caries and saliva of patients with oral infections [12, 13]

Fluoride acts as a potent antibacterial agent by disrupting bacterial metabolism [14]. It inhibits bacterial cell growth by directly interfering with glycolysis and energy metabolism in vitro [15]. Additionally, fluoride induces oxidative stress and disrupts glutathione metabolism[16]. It is reported that water fluoridation has contributed to a decline in dental cavities [17]. However, accidental ingestion of fluoride, particularly during early childhood has also been linked to dental fluorosis. Fluoride not only aids in preventing dental cavities but is also used therapeutically to deactivate early-stage carious lesions. Its effectiveness is maximized when applied directly to the teeth, especially when combined with proper dental hygiene practices. Using topical fluoride varnish, typically formulated with 5% sodium fluoride, every six months is recommended to prevent tooth decay, reducing its occurrence by one-third [17-19]. This treatment significantly decreases the presence of harmful bacteria in dental plaque. Medicated mouthwashes leverage antimicrobial properties as anti-plaque agents without inducing bacterial resistance in the oral cavity [20]. Fluoridated mouthwashes containing 0.1 to 0.2 % sodium fluoride are designed for weekly use, whereas those with 0.02% sodium fluoride are suggested for daily rinsing to prevent dental caries [21, 22]. However, information about the effect of fluoride-containing oral products on *K. pneumoniae* is limited. The response of bacteria to NaF can be characterized by metabolic changes influenced by various internal and external factors, which may lead to the generation of oxidative stress. This study is the first to describe the defense mechanisms employed by a clinically isolated strain of *K. pneumoniae* against NaF. Our investigation focuses on understanding the mechanisms involved in K. *pneumoniae* to tolerate NaF, studying changes in cell morphology, and compositional and structural changes under varying NaF concentrations. Additionally, we examine the biochemical responses of *K. pneumoniae* cells to NaF, specifically assessing oxidative damage markers and the production of antioxidant metabolites and enzymes.

## MATERIAL AND METHODS

### *Sodium Fluoride (NaF) solution*

Aqueous solutions of NaF were prepared by using Anhydrous NaF powder with different concentrations and used directly

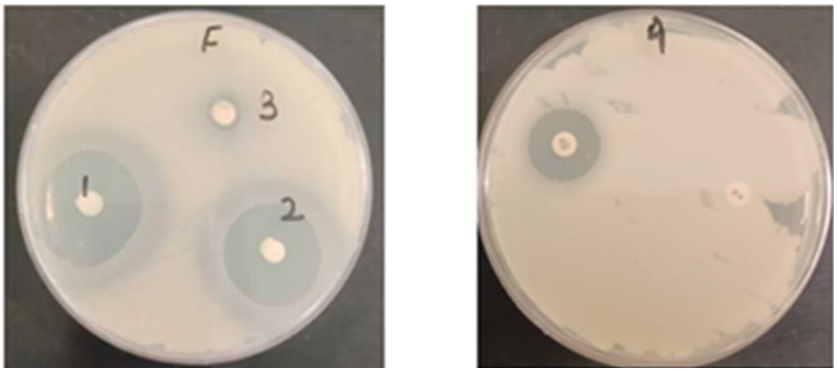
### *Antimicrobial and anti-biofilm activity*

#### *Bacterial culture*

King Khalid Hospital, Riyadh Saudi Arabia provided gram-negative strains of *Klebsiella pneumoniae.* The strain was reactivated on Mueller-Hinton Agar (MHA) plates, before use.

#### *Antibacterial activity*

The disk diffusion method was used to measure the antibacterial activity of three different concentrations of NaF by using 6mm paper discs. Antibacterial activity was assessed by measuring the zone of inhibition in millimeters (mm) at the bottom of test agar plates incubated at 37 °C for 24 hours.cm (Figure 1).




1=30% NaF; 2=20% NaF; 3=10% NaF. Gentamicin/Penicillin

**Figure 1.** Disk diffusion assay demonstrating the antibacterial activity of different percent solutions of sodium fluoride against *K. pneumoniae*

### *Minimum inhibitory (MIC) and Minimum Bactericidal Concentrations (MBCs)*

Broth microdilution method was used to determine MIC and MBC using 96-well microliter plates through serially diluted NaF solutions with Mueller Hinton broth (MHB) to achieve final concentrations ranging from 0.156 % to 20% for NaF *K. pneumoniae* culture adjusted to approximately 106 CFU/mL. The turbidity of bacterial growth was read at 600nm after 24 hours of incubation at 37°C. The lowest concentration of NaF required to prevent K. *pneumoniae* growth was noted as MIC and to kill 99.9% of *K. pneumoniaee* showing no growth was reported as MBC.

### *Biofilm formation*

The microtiter plate method was used to determine the effect of NaF on the biofilm formation of *K. pneumonia* grown in MHA incubated at 37 °C for 48 hours. 0.1% solution of crystal violet was used for staining biofilms and read at 570 nm to quantify biofilm formation.

## *K. pneumoniae growth curve, microscopic and metabolic changes under NaF stress.*

### *Growth curve*

*K. pneumoniae* cultures were grown under different concentrations of NaF (0.2%; 1% &2% of NaF using sterilized Mueller-Hinton (MH) broth and incubated overnight at 37°C in a shaker incubator (shaken at 180 rpm) for 48 hours. *K. pneumoniae* cultures without NaF serve as a control to monitor normal microbial growth. Optical density at 600 nm was taken hourly using a spectrophotometer (Thermo Scientific, Waltham, MA, USA) to monitor *K. pneumoniae* growth. The growth rate of *K. pneumoniae* cells in the presence of NaF was determined by plotting the optical density against time.

### *Scanning electronic microscope (SEM) and Fourier Transform Infrared (FTIR) Spectroscopic Analyses*

*K. pneumoniae* exposed to three different concentrations of NaF (0.2%; 1% &2% of NaF) for 24 hours were harvested and examined under SEM for morphology changes. High-resolution three-dimensional images were generated by scanning *K. pneumonia* cells with an electron beam using the JSM-7610F Field Emission Scanning Electron Microscope.

FTIR was used to examine functional groups of various metabolites in *K. pneumoniae* cells. Control and NaF-exposed *K. pneumoniae* cells were encapsulated with potassium bromide for FTIR analysis using a Perkin Elmer FTIR Spectrometer. The FTIR spectra were in a range from 4000 to 400 cm−1.

## *Antioxidant defense system in K. pneumoniae under NaF-induced stress*

The *K. pneumoniae* cells grown with different concentrations of NaF (0.2%; 1% &2% of NaF) in MHB were harvested after 24 hours. The cells were

centrifuged at 12,000 g for 10 min and washed with ice-cold Phosphate Buffer Saline (PBS) buffer. Due to insufficient cell count following treatment with 1% and 2% NaF, the experiment investigating stress markers proceeded using only a 0.2% NaF concentration treatment. Harvested cells from the 0.2% NaF treatment batch were sonicated in ice-cold PBS and centrifuged at 15,000 x g for 10 min to pellet debris. The supernatant was collected in a sterile tube to measure the stress markers.

***Markers of oxidative stress were determined as follows:***

Lipid oxidation was assessed using the thiobarbituric acid reactive substances (TBARS) assay, which measures malondialdehyde and other products of lipid peroxidation.

Total protein was estimated via the Bradford method, employing bovine serum albumin as a standard.

***Antioxidant metabolites were evaluated as follows:***

Reduced Glutathione levels were determined using 5, 5'-dithiobis(2-nitrobenzoic acid) (DTNB), which forms a yellow-colored product upon reacting with sulfhydryl groups.

Vitamin C levels were determined using the Folin-Ciocalteu reagent, generating a blue-colored complex.

***Enzymatic antioxidant activity was assessed through:***

Glutathione S-Transferase (GST) activity, evaluated by its catalytic reaction involving glutathione (GSH), a GST substrate, and CDNB (1-chloro-2,4-dinitrobenzene)

A UV–visible spectrophotometer (Thermo Scientific, Waltham, MA, USA) was employed to measure the absorbance for all the assays used to measure oxidative stress markers

***Statistical Analysis***

The results were presented as the mean ± standard deviation (SD) with each test performed in triplicate.

## RESULTS

### *Antimicrobial and anti-biofilm activity*

Diffusion tests were conducted to assess the primary antibacterial efficacy of three concentrations of NaF against *K. pneumoniae*, as depicted in Figure 1. The antibacterial activities were quantified by measuring inhibition zone diameters (IZ), minimum inhibitory concentrations (MIC), and minimum bactericidal concentrations (MBC), detailed in Tables 1 and 2. Gentamicin and Penicillin served as standard antibiotics. *K. pneumoniae* strains exhibited sensitivity to NaF, with IZs of 13±1.0 mm, 20±1.5 mm, and 25±1.0 mm for NaF concentrations of 10%, 20%, and 30%, respectively (Table 1). Furthermore, NaF demonstrated a bacteriostatic effect against *K. pneumoniae* with MIC values of 2.5% and MBC values of 5.0% (Table 2). Figure 2 illustrates the biofilm formation by *selected K. pneumoniae* strains in the presence of varying NaF concentrations, showing a dose-dependent effect with complete biofilm eradication observed at 20% and 10% NaF concentrations

**Table 1.** Zone of Inhibition (mm) of different percent sodium fluoride solutions by disk diffusion method.

| Strain | Zone of Inhibition (mm) | | | | |
|---|---|---|---|---|---|
| | 10% NaF | 20% NaF | 30% NaF | Std1 | Std 2 |
| *K. pneumonia* | 13±1.0 | 20±1.5 | 25±1.0 | 18 | Resistant |

Standard antibiotic (Std) 1- Gentamycin; 2- Pencillin

**Table 2.** The MIC and MBC values of sodium fluoride solution against K. pneumoniae

| Strain | MIC (% solution ) | MBC (% solution) |
|---|---|---|
| *K . pneumonia* | 2.5 | 5 |

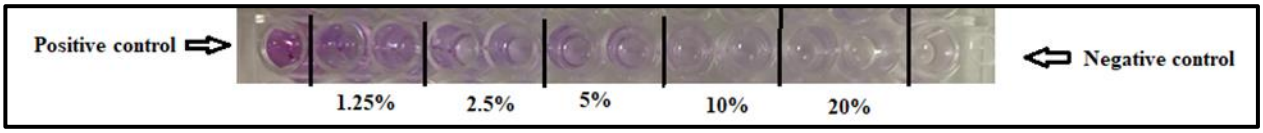



**Figure 2**- Microliter plates demonstrating the anti-biofilm activity of sodium fluoride solution against K. pneumoniae

## *K. pneumoniae growth curve, microscopic and metabolic changes under NaF stress.*

*K. pneumoniae* strains were evaluated for their susceptibility to NaF at concentrations (0.2%, 1%, and 2% NaF) below the MIC values. Figure 3 illustrates the results of bacterial growth rates in the presence and absence of NaF. In growth curve experiments, the inhibition of bacterial growth was observed to be concentration-dependent, with remarkable suppression observed at 1% and 2% concentrations. Even at 0.2% NaF, early suppression of growth compared to the control was observed.

SEM analyses examined the concentration-dependent morphological changes induced by NaF in *K. pneumoniae* (Figure 4*). K. pneumoniae* cells grown in normal media served as controls for comparison. Cells treated with different NaF concentrations displayed characteristic wrinkled cell surfaces in a dose-dependent manner. Specifically, Figure 4a shows regular smooth rod-shaped *K. pneumoniae* cells; Figure 4b depicts slight irregularities and wrinkling of cell structures with 0.2% NaF treatment; Figure 4c shows irregular shapes and cell aggregation with 1% NaF treatment; and Figure 4d illustrates cell rupture, aggregation, and destruction with 2% NaF treatment.

Exposure to NaF induced specific FTIR spectroscopic changes in *K. pneumoniae* cells, as illustrated in Figure 5. FTIR spectra typically exhibit four distinct regions for most bacteria: Region I (3000-2800 $cm^{-1}$) represents cell membrane fatty acids, Region II (1700-1500 $cm^{-1}$) displays the amide I (1650 $cm^{-1}$) and amide II (1550 $cm^{-1}$) bands of proteins and peptides, Region III (1500-1200 $cm^{-1}$) corresponds to fatty acids, proteins, and phosphate-carrying molecules, and Region IV (1200-900 $cm^{-1}$) shows absorption bands typical of polysaccharides or carbohydrates in microbial cell walls. Our findings indicated shifts in peak positions across nearly all these regions (Figure 5), suggesting alterations in the biochemical composition of cells following NaF exposure. Remarkable shifts were particularly noted in Regions I and IV, indicating cell membrane fluidity and phospholipids changes.

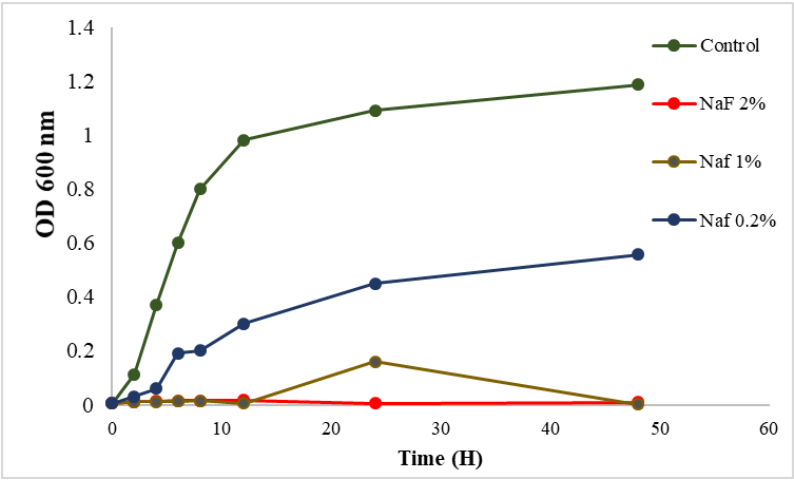



**Figure 3**. Growth curves of *K. pneumoniae* incubated with three different concentrations of NaF for 48 hours

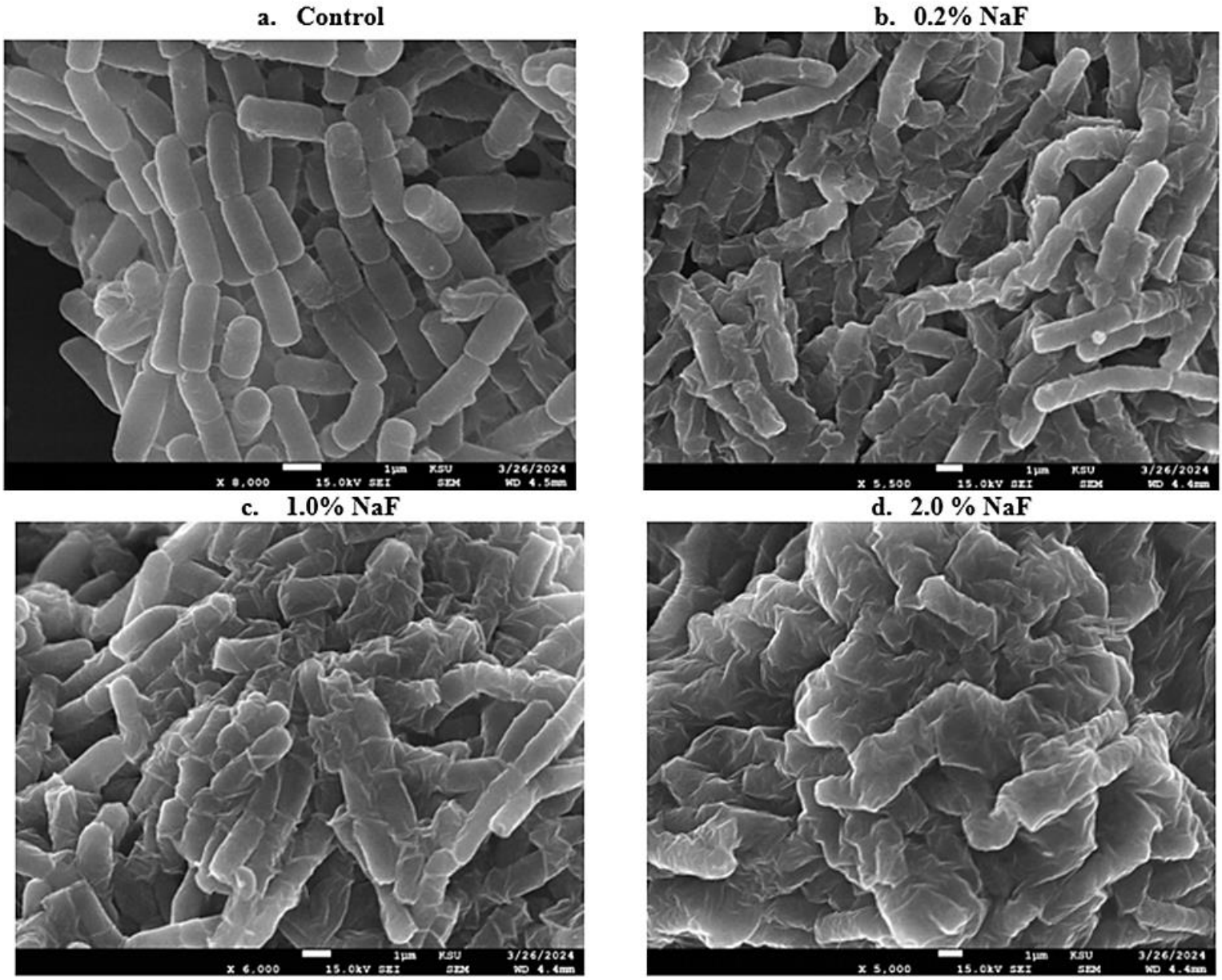



**Figure 4.** SEM micrograph showing the *K. pneumoniae* cells with various levels of degeneration when treated with different concentrations of NaF. **a** –shows regular smooth rods; **b** –shows irregular shape; **c**- shows irregular shape and aggregation; **d**- shows rupture, aggregation and destruction of cells

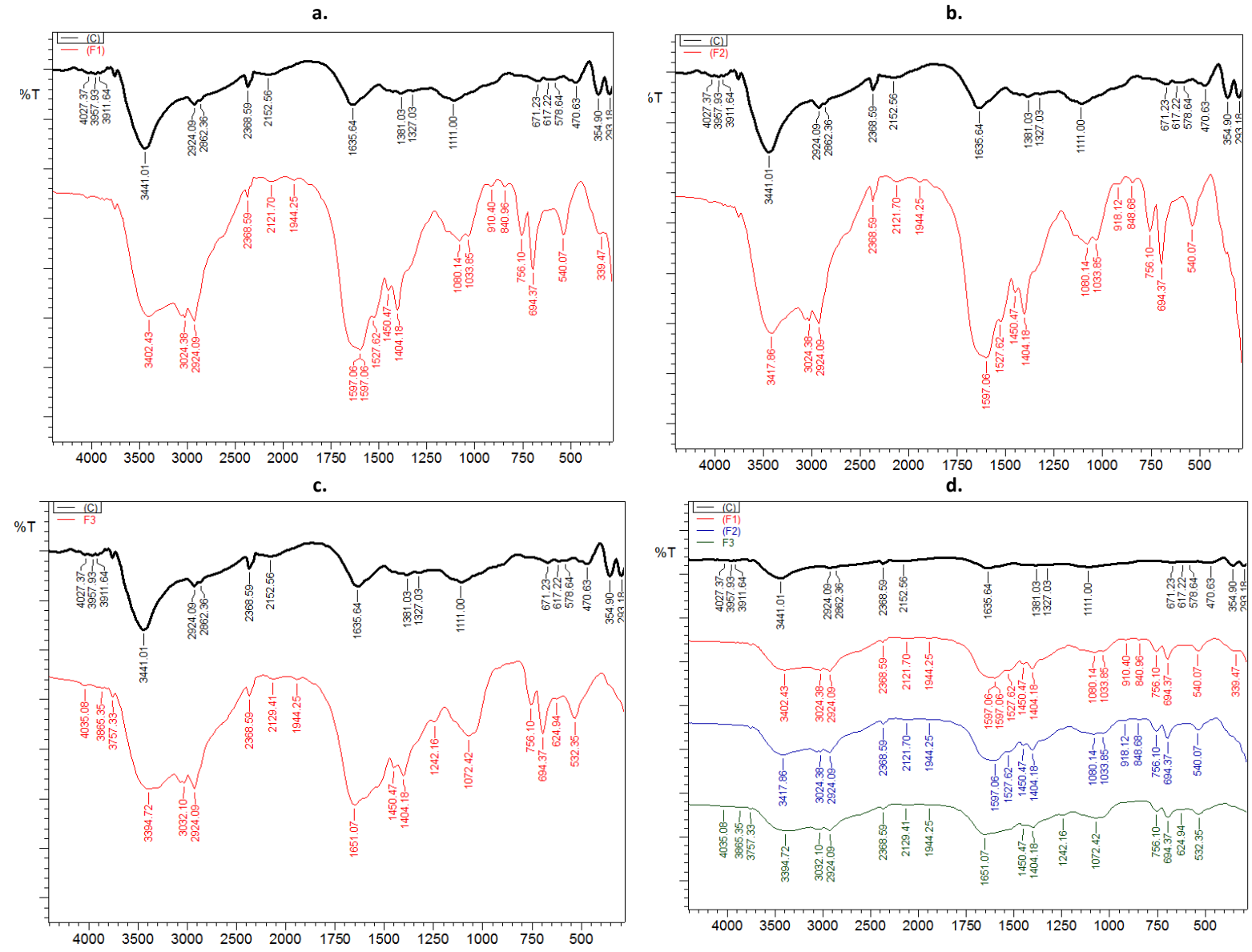



**Figure 5**. IF-IR Spectra Analysis of *K pneumoniae* Exposed to Various Sodium Fluoride Concentrations. a. 0.2% NaF; b. 1.0% NaF; c. 2.0% NaF; d. Comparison of 0.2% NaF, 1.0% NaF, and 2.0% NaF with the control group

## *Antioxidant defense system in K. pneumoniae under NaF-induced stress*

*K. pneumoniae* cells treated with 0.2% NaF were assessed for stress markers, as depicted in Figures 6 and 7. In Figure 5, it was observed that NaF stress for 24 hours led to a reduction in glutathione (GSH) levels and an increase in ascorbic acid levels in *K. pneumoniae* cells. Exposure to NaF also resulted in elevated lipid peroxidation values, while glutathione S-transferase (GST) activity and total protein levels decreased upon exposure.

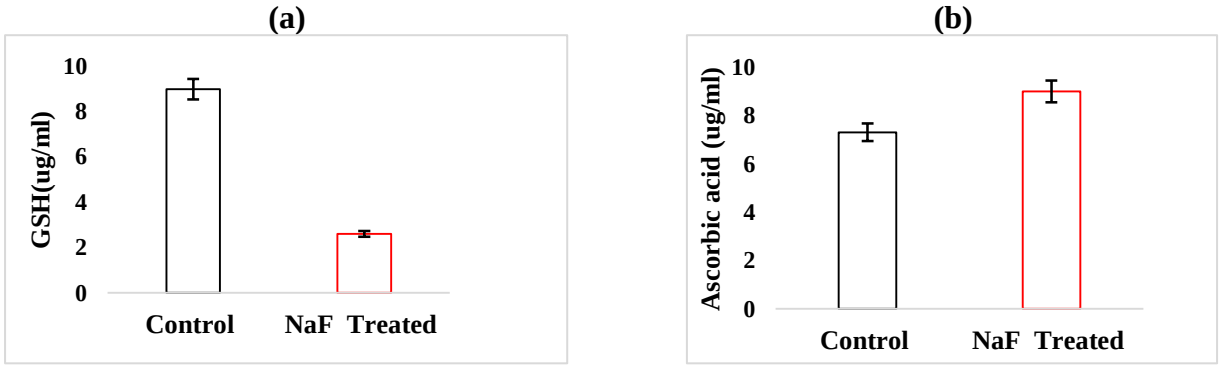



**Figure 6.** The antioxidant metabolites of the K. pneumoniae strain include (a) Reduced GSH, (b) Ascorbic acid under control, and NaF treatment. Data are represented by the mean of at least 3 replicates ± standard error.

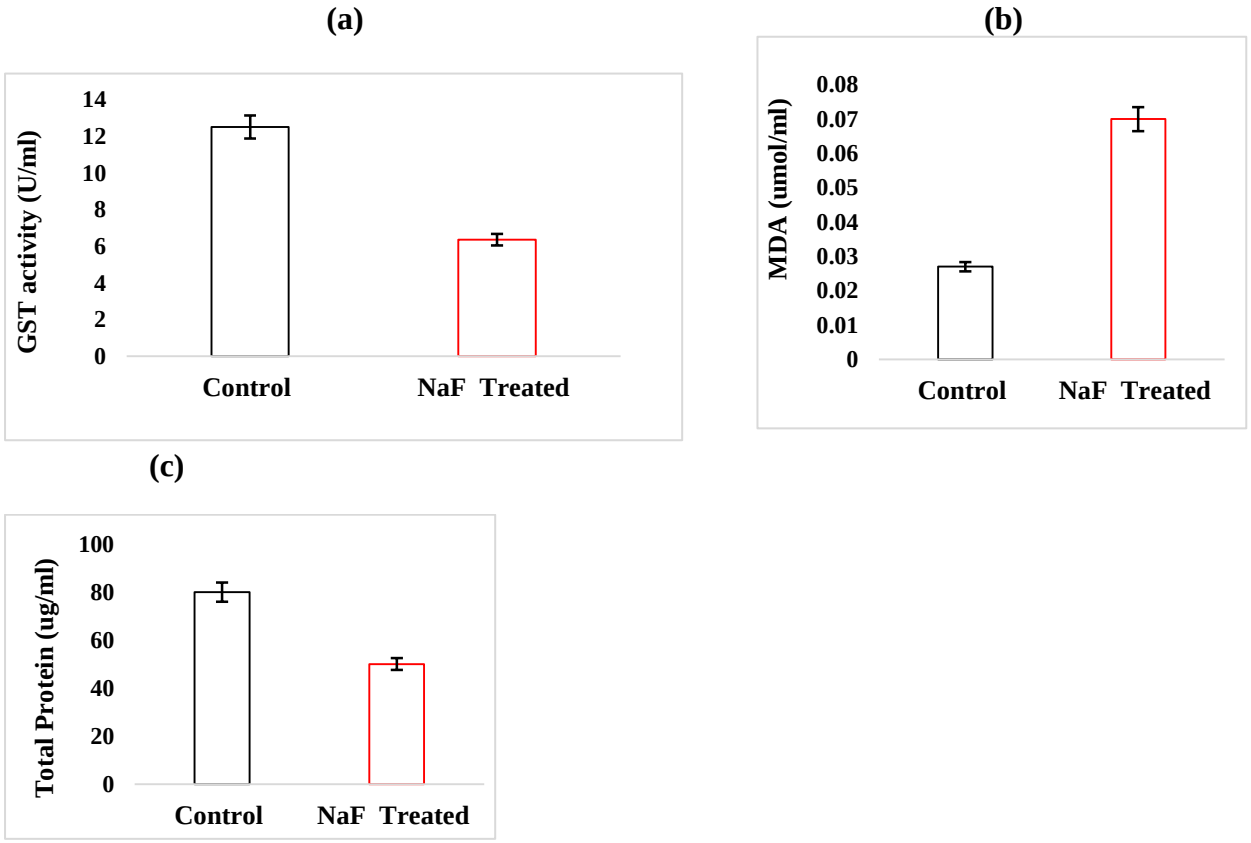



**Figure 7.** The antioxidant enzyme activities include (a) Glutathione S-transferase activity and the oxidative damage markers include (b) Lipid peroxidation (MDA), (c) Total crude protein of the K. pneumoniae strain under control, and NaF treatment. Data are represented by the mean of at least 3 replicates ± standard error.

## DISCUSSION

Many studies have reported the antibacterial activity of fluoride. [14-16] However the complex mechanism behind this is still not very clear. In the present study, *K. pneumoniae* was used to explore the effects of different concentrations of NaF fluoride on growth, biofilm formation, and biochemical and morphological changes related to oxidative stress.

*K. pneumoniae* is a prevalent antibiotic-resistant bacterium presenting a significant global public health challenge [29.] Infections can spread through respiratory routes, environmental contamination, or via contaminated medical devices [30]. The discovery of new therapeutic agents is crucial for effectively addressing *K. pneumoniae* infections [31.] Particularly concerning is the resistance of *K. pneumoniae* strains associated with aspiration pneumonia and healthcare-associated pneumonia to Chlorhexidine, a commonly used hospital

disinfectant [32]. Researchers are actively investigating oral antiseptics suitable for use as mouthwashes that can effectively eradicate such strains when used for gargling [33, 34].

The growth of bacterial cells treated with NaF was inhibited as the concentration increased, showing minimal growth at 2% NaF treatment. Bacterial genomes possess entire regulons dedicated to managing stress [35]. Stress markers are tightly regulated to ensure bacterial survival. Although bacteria can respond to stress, their ability to manage it has limits [36]. Stress exceeding the bacterial response capacity can lead to growth suppression as clearly shown in Figure 3.

While previous studies have focused on the antibacterial properties of NaF, our research primarily investigated the biochemical and morphological alterations induced in *K. pneumoniae* cells upon treatment. SEM was employed to examine the surface morphology of *K. pneumoniae* as it is the most widely used technique to visualize bacterial morphology [37]. Untreated cells displayed a clear cell wall structure without noticeable changes, whereas cells treated with increasing concentrations of NaF exhibited a distinct pattern of alterations (Figure 4). Specifically, K. pneumoniae cells treated with 2% NaF showed irregular shapes and remarkable cell lysis compared to those treated with a 0.2% solution. These observations suggest that NaF induces dose-dependent morphological changes by damaging bacterial membranes and causing leakage of cytoplasmic material. Our SEM findings were further corroborated by FT-IR spectroscopic analysis of treated cells, which also demonstrated notable shifts in Regions I and IV, confirming the morphological changes observed in SEM. FT-IR spectroscopy can be used to analyze the complete molecular composition of microbial cells. It can describe all cellular components, including the cell wall and membrane (comprising phospholipid bilayers, peptidoglycan, and lipopolysaccharides), as well as the cytoplasm (containing fatty acids, water, nucleic acids, proteins, and polysaccharides) [38, 39]. This technique provides a rapid and non-invasive approach to study changes or damage occurring in biological samples and requires minimal sample preparation [40, 41].

Exposure of *K. pneumoniae* cells to NaF resulted in oxidative damage, evidenced by increased levels of oxidative markers such as Lipid peroxidation and ascorbic acid, along with depletion of both enzymatic and non-enzymatic antioxidants, notably GSH and GST. GSH is primarily consumed through GST-catalyzed reactions, representing a significant detoxification mechanism in bacterial cells [16, 42]. These adaptations highlight the defense mechanisms employed by *K. pneumoniae* to counteract oxidative stress induced by NaF exposure [43,44]. Lipid peroxidation indicates intracellular damage that affects the cell membrane structure in bacteria [45]. This process involves the removal of electrons from membrane lipids, triggering a cascade that affects the bacterial membrane and leads to morphological changes [46, 47]. The increased lipid peroxidation observed in our results (Figure 7) is linked to the wrinkled structures seen in SEM images (Figure 4) and the band shifts in Region I of FT-IR analysis (Figure 5) in bacterial cells treated with NaF.

## CONCLUSIONS

In the present study, NaF demonstrated antibacterial and anti-biofilm activity against *K. pneumoniae*, a prominent antibiotic-resistant bacterium NaF demonstrated a dose-dependent effect, achieving complete biofilm eradication at a 20% NaF solution. It also caused significant morphological changes, resulting in wrinkled cell surfaces in a dose-dependent manner. Biochemical alterations were observed in NaF-exposed cells, including decreases in GSH, GST, and total protein levels, alongside increases in lipid peroxidation and ascorbic acid levels. Fluoride-containing mouthwashes can effectively eradicate *K. pneumoniae* when used for gargling. However, accidental fluoride ingestion, in young children, can be harmful.

Due to insufficient cell count following treatment with 1% and 2% NaF concentrations, the study was limited to only a 0.2% NaF concentration for investigating stress markers. This limit the ability to assess the dose-response relationship of NaF on stress markers.

Future research exploring various metabolites and stress markers, particularly in bacteria subjected to high concentrations of NaF, could provide deeper insights into how fluoride impacts these markers.

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## CONFLICT OF INTERESTS

"None"

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