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Inhibition of Urease Enzyme Production and some Other Virulence Factors Expression in *Proteus mirabilis* by *N*-Acetyl Cysteine and Dipropyl Disulphide

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Abstract

Proteus mirabilis is one of the important pathogens that colonize the urinary tract and catheters resulting in various complications, such as blockage of the catheters and the formation of infective stones. **Purpose:** In this study we evaluated the effect of *N*-acetyl cysteine (NAC) and dipropyl disulphide on some virulence factors expressed by a *Proteus mirabilis* strain isolated from a catheterized patient. **Methods:** Antibacterial activity of both compounds was determined by broth microdilution method. Their effect on different types of motility was determined by LB medium with variable agar content and sub-MIC of each drug. Their effect on adherence and mature biofilms was tested by tissue culture plate assay. Inhibitory effect on urease production was determined and supported by molecular docking studies. **Results:** The minimum inhibitory concentration (MIC) of NAC and dipropyl disulphide was 25 mM and 100 mM, respectively. Both compounds decreased the swarming ability and biofilm formation of the tested isolate in a dose-dependent manner. NAC had higher urease inhibitory activity (IC50 249 $\pm$0.05 mM) than that shown by dipropyl disulphide (IC_{50} 10$\pm$0.2 mM). Results were supported by molecular docking studies which showed that NAC and dipropyl disulphide interacted with urease enzyme with binding free energy of -4.8 and -8.528 kcal/mol,

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respectively. Docking studies showed that both compounds interacted with Ni ion and several amino acids (His-138, Gly-279, Cysteine-321, Met-366 and His-322) which are essential for the enzyme activity. **Conclusion:** NAC and dipropyl disulphide could be used in the control of *P. mirabilis* urinary tract infections.

Keywords
P. mirabilis • Urease • Biofilm • NAC • Dipropyl Disulphide • Molecular Docking • Motility

1 Introduction

Infective stones are responsible for approximately 15% of urinary stone diseases. The main cause for the formation of infective stones is the presence of urease positive organisms associated with the urinary tract infection mainly from *Proteus* species. *P. mirabilis* is second to *E. coli* as a common cause of urinary tract infections, especially in the elderly catheterized population (Peerbooms et al. 1985). UTIs associated with *P. mirabilis* usually lead to urinary tract obstruction, stone formation, blockage of urinary catheters, and bacteriuria. This may be due to the production of several virulence factors including urease, hemolysin, surface adherence and biofilm formation (Rózalski et al. 1997; Coker et al. 2000).

Urease enzyme is one of the most important virulence factors identified until today (Rózalski et al. 1997). Urease is a nickel containing enzyme, it generates ammonia from urea increasing the alkalinity of urine which favors formation of struvite and carbonate apatite crystals. The aggregation of these crystals is considered one of the primary causes of urinary stone formation. Once a stone begins to form, bacteria stick to the stone and live within its layers protecting them from antibiotics. In addition, urease is involved in the chemotactic reactions of the bacterium and reduces opsonization by human complement (Nakamura et al. 1998; Rokita et al. 1998; Hu and Mobley 1990). *Proteus mirabilis* urease enzyme differs from other bacterial ureases in which it is not only found in the cytoplasm of the cell but also on the bacterial surface (Mobley et al. 1995; Hawtin et al. 1990).

Swarming ability of *P. mirabilis* is another factor that can play a role in pathogenesis. Swarmer cells have been shown to move rapidly over catheter surfaces (Stickler and Hughes 1999). Therefore initiation of infection could possibly be mediated by the migration of the organism. *P. mirabilis* showed three types of movement: swimming, swarming and twitching. *Proteus* bacteria use swimming motility (SM) in liquid media. Swarming motility (SW) is used for movement on solid surfaces and twitching motility (TM), to twitch between two solid surfaces (Rózalski et al. 1997; Rashid and Kornberg 2000).

Biofilm formation facilitates survival and adaptation to the unfavorable conditions of the external environment and protects organisms from the host immune system and antimicrobials (Costerton 1999; Jacobsen and Shirtliff 2011). Biofilms, in particular crystalline biofilms, lead to encrustation and blockage of indwelling catheters in patients with UTIs (Mobley and Warren 1987).

Furthermore, the development of multiple drug resistant strains made treating *P. mirabilis* infections more difficult (Rózalski et al. 1997; Mokracka et al. 2012). So, finding antibiotics with new modes of action is urgently needed. Also, Neutralizing or suppressing the expression of the pathogen's virulence factors is needed to attenuate the pathogenicity of the bacterium and make it easier for the host innate immune system to clear the infection (Fernebro 2011).

Enzyme inhibitors, such as urease enzyme inhibitors, have attracted great attention of biomedical scientists the last couple of decades. In addition, inhibition of other virulence factors such as adherence, biofilm production and swarming ability of *P. mirabilis* will facilitate the organism's treatment and decrease its virulence.

Based on the previous data and the known urease inhibition properties of thiol and disulphide compounds (Mobley et al. 1995; Todd and Hausinger 1989), our study was done using NAC and dipropyl disulphide due to the presence of thiol group in NAC and disulphide moiety in dipropyl disulphide which may affect the urease activity. Also, NAC plays an important role in destroying biofilm due to its mucolytic properties(Millea 2009; Olofsson et al. 2003) by cleaving disulfide bonds which crosslink glycoproteins (Dodd et al. 2008). We aimed to test the urease inhibitory activity of *N*-acetyl cysteine (NAC) and dipropyl disulphide (a major component of onion oil) against *Proteus mirabilis* urease enzyme. Their effect on *P. mirabilis* adherence, biofilm formation and ability to inhibit different types of movements was also investigated. Finally, molecular docking and simulation studies were carried out to improve the reliability, accuracy of biological tests, and show possible interactions between both compounds and urease enzyme.

2 Materials and Methods

2.1 Bacterial Strain, Chemicals and Media

Two *Proteus mirabilis* isolates were collected from a urine sample and the stent of a catheterized patient undergoing a ureteral stent removal. Isolation and identification were performed according to standard procedures (Benson 2002). Dienes typing for the discrimination between strains of *Proteus mirabilis* was performed as previously described (Sabbuba et al. 2003). Isolates of *P. mirabilis* were plated for single colonies on CLED agar and incubated overnight at 37 °C. Single colonies from both isolates were then inoculated as macro-colonies onto tryptone soy agar plates. After overnight incubation at 37 °C, no zone of inhibition (dienes demarcation line) developed between the last waves of swarming of both isolates. Thus both isolates were regarded as the same strain. Biofilm formation assay was conducted by tissue culture plate method (TCP) as described by Christensen et al., stationary (18-h) trypticase soy broth(TSB) cultures of *P. mirabilis* were diluted 1:100 with fresh TSB .Individual wells of sterile, polystyrene, 96-well, flat-bottomed tissue culture plates were filled with 0.2 ml aliquots of the diluted culture. The tissue culture plates were incubated for 18 h at 37 °C. The contents of each well were gently aspirated by tipping the plate and placing the aspirator tip in the lowest corner of the well. With an automatic hand pipette, the wells were washed four times with 0.2 ml of phosphate-buffered saline (pH 7.2). Then 25 µl of 1% solution of crystal violet was added to each well (this dye stains the cells but not the polystyrene) plates. The plates were incubated at room temperature for 15 min, rinsed thoroughly and repeatedly with water. After drying, the ODs of stained adherent bacterial films were read with a micro ELISA auto reader (model 680, Bio rad) wavelength of 570 nm, and the wavelength of values was considered as an index of bacteria adhering to surface and forming biofilms. Biofilm production is considered high (OD $>$ 0.240), moderate (OD = 0.120–0.240), or weak (OD $<$ 0.120) (Christensen et al. 1985).The isolate was cultured on trypticase soy agar (TSA, Difco) slants for daily use and stored in a trypticase soy broth medium (TSB, Difco) along with 15% glycerol, at −80 °C for subsequent uses. *N*-acetylcysteine was obtained from Sedico, Egypt. Dipropyl disulphide $\geq$ 97% was purchased from Sigma-Aldarich. About 163.19 mg of NAC was dissolved in 10 ml distilled water to prepare a final concentration

of 100 mM stock solution while 300.638 mg of dipropyl disulphide was dissolved in 10 ml of dimethyl sulphoxide (DMSO) to prepare a stock solution of 200 mM concentration.

2.2 Scanning Electron Microscopy (SEM)

The removed ureteral stent was examined by scanning electron microscope (SEM) for crystalline biofilm. The stent was fixed in 2.5% (vol/vol) glutaraldehyde in Dulbecco phosphate buffered saline PBS (pH 7.2) for 1.5 h, rinsed with PBS, and then dehydrated through an ethanol series. Samples were dried using critical-point drying method (Bray 2000) and gold-palladium coated. SEM examinations were made on a JSM-840 SEM (JEOL Ltd., Tokyo, Japan) (Soboh et al. 1995).

2.3 Evaluation of the Antibacterial Effect of NAC and Dipropyl Disulphide and Determination of their MIC Using Broth Microdilution Method

Briefly, twofold serial dilutions of both agents were prepared in sterile Mueller Hinton Broth (MHB, Oxoid) for a testing concentration range of 1.5–200 mM. Then 100 µl from each dilution was transferred into the well of a microtiter plate and inoculated with 5 µl of standardized cell suspension (1.5×10^7CFU/ml). Plates were incubated at 37 °C overnight, and the lowest concentration of NAC and dipropyl disulphide that prevented visible growth was recorded as the MIC (CLSI 2011). Minimum bactericidal concentration (MBC) was determined by sub-culturing of 10 µl of broth from wells with no visible growth on TSA plates. The lowest concentration of the tested agents that killed 99.9% of the original inoculum was considered as MBC.

2.4 Effect of NAC and Dipropyl Disulphide on Different Virulence Factors of the Tested Strain

(a) *Effect on swarming (SW), Swimming (SM) and Twitching (TM) motility of tested isolate:* Luria-bertani (LB) medium was used with agar content appropriate for the type of motility being tested. Swarming medium contained 0.7% agar and the tested isolate was cultured by picking on the agar surface. Swimming medium contained 0.3% agar and the isolate was applied by puncture into the medium. Twitching medium contained 1.0% agar and the isolate was inoculated with a micropipette under the agar layer (10 µL of bacterial suspension). Sub-MIC concentrations of the tested agents were added to LB media to test their ability to inhibit different forms of movement. After cultivation for 24 h at 37 °C, the diameters of the formed zones were measured (SM and SW) (Jones et al. 2005). Also swarming of *P.mirabilis* over the tested catheter was performed using LB swarming medium. A strip of the medium was aseptically cut away from the agar plate. The prepared gap was bridged with a piece of the tested catheter. The tested strain was applied on the surface using a pick on one half of the agar. After 24 h cultivation at 37 °C, the ability to swarm over the tested catheter was determined (Hola et al. 2012).

(b) *Effect on adherence of the tested isolate:* 100 µl of the suspension (OD600) was inoculated into individual wells of polystyrene 96-well plates (flat bottom; Nunc). TSB broth was used as a negative control. The plates were incubated at 37 °C for 90 min (adhesion period). Supernatants including planktonic cells were discarded and wells were gently washed with phosphate buffer saline (PBS) twice to remove any non-adherent cells. 100 µl of fresh TSB

broth containing one of the following: 1/4MIC, 1/2MIC, MIC, 2MIC and 4MIC concentrations of NAC and dipropyl disulphide (each alone) were added to each well. The plates were covered to prevent evaporation and incubated at 37 °C for 24 h. Liquid media containing the non-adherent cells were discarded and plates washed twice with PBS buffer. Adherent cells to the plastic surfaces were quantified using crystal violet assay (Wei et al. 2006). Experiment was performed in triplicate.

(c) *Effect on mature biofilms:* 100 µl of the suspension (OD600) was inoculated into individual wells of polystyrene 96-well plates (flat bottom; Nunc). The plates were incubated at 37 °C for 48 h. After the incubation period, the supernatants from each well were aspirated and the wells washed twice with PBS without disturbing the biofilms at the bottom of the wells, then 100 µl of 1/4MIC, 1/2MIC, MIC, 2MIC and 4MIC concentrations of NAC and dipropyl disulphide (each alone) were added to the wells. Normal saline without any agents was added to the control wells. The plates were incubated at 37 °C for 24 h. Supernatants were discarded and plates washed twice with PBS buffer. Cells adherent to the plastic surfaces were quantified using crystal violet assay (Wei et al. 2006). Experiment was performed in triplicate.

(d) *Effect on urease production:* We determined the change of pH value of TSB containing 500 mM of urea (pH adjusted to 7.3) in presence of different concentrations (1/32, 1/16, 1/8, 1/4, 1/2 X MIC) of NAC and dipropyl disulphide (Bibby and Hukins 1992). Media containing urea were inoculated with standardized cell suspension (5×10^5 CFU/ml). *Proteus mirabilis* was considered as positive control while uninoculated media was used as negative control. The pH values of TSB containing urea (with the tested agents and control) were screened at 2 h interval for 18 h along the experiment using digital pH-meter (Elmetron CP-215). The increase in pH indicates the activity of urease enzyme.

Urease Activity Assay A total of 50 µl of overnight culture of the isolated *P. mirabilis* strain in MHB were transferred into 10 ml sterile MHB and additionally incubated 18 h at 37 °C with constant shaking. The cells were pelleted by centrifugation at 1258 *g* for 15 min (4 °C). The pellet was washed three times with 10 mM K_2HPO_4 solution and resuspended in 2 ml of the same solution. Thereafter, to release the urease, bacteria were sonicated for 90 s with 0.5 cycles at 100% amplitude using an ultrasonicator (UP200H, Hielscher Ultrasonics, Teltow, Germany) in an ice container. The resulting bacterial lysate was used for determination of urease activity in a microtiter plate using the phenol red colorimetric method. The assay mixture contained 10 mM K_2HPO_4 solution (pH 6.2), 0.002% phenol red and 500 mM urea (assay reagent). The increase in absorbance at 570 nm was recorded using a microplate reader (BioTek, USA) (Goldie et al. 1989; Tanaka et al. 2003). To determine the concentration of urease enzyme in the lysate, the lysate was centrifuged for 3 min. Then the absorbance (A) of the upper solution was determined in $\lambda = 278$ nm. By using the following eq. $A = \lambda bc$, where c is the concentration of solution (mol/L), b the length of the UV cell (Golbabaei et al. 2013; Akhtar et al. 2014). *P. mirabilis* ATCC 12453 reference strain served as a control during the procedure.

Determination of IC_{50} of the Tested Compounds In vitro inhibitory studies on urease were determined using indophenols method, which measures the liberation of ammonia from the reaction (Weatherburn 1967). The urease enzyme was dissolved in 20 mM sodium phosphate buffer and the pH was adjusted to 7 using 1 N NaOH. The assay mixture contained 50 µl

(2 mg/ml) of enzyme and 100 µl of different concentrations of the tested agents(NAC 0.39–50 mM and dipropyl disulphide 1.56–100 mM).The mixture was then added to 850 µl of 25 mM urea and pre-incubated for 0.5 h in water bath at 37 °C. The urease reaction was stopped after 30 min incubation by procedure. Urease activity was determined by measuring ammonia production using the indophenol method as described by Weatherburn 1967. After pre-incubation, 500 µl of phenol reagent (1% w/v phenol and 0.005% w/v sodium nitroprusside) and 500 µl of alkali reagent (1% w/v NaOH and 0.075% active chloride NaOCl) were added to 100 µl of incubation mixture and kept at 37 °C for 30 min. The absorbance was measured at 625 nm. All experiments were performed in triplicate in a final volume of 1 ml, and acetohydroxamic acid was used as a standard urease inhibitor.

Percentage inhibition was calculated using the formula (100 – (OD sample/OD control) × 100). The concentration that provokes an inhibition halfway between the minimum and maximum response of each compound (relative IC_{50}) was determined by monitoring the inhibition effect of various concentrations of compounds in the assay.

2.5 Protocol of the Docking Study

The automated docking simulation study is performed using Molecular Operating Environment (MOE®) version.09, 2014, at Assuit University Faculty of Pharmacy, Chemical Computing Group Inc., Montreal, Canada. The X-ray crystallographic structure of the target urease (1E9Y) was obtained from Protein Data Bank (PDB). The target compounds were constructed into a 3D model using the builder interface of the MOE program. After checking their structures and the formal charges on atoms by 2D depiction, the following steps were carried out: the target compounds were subjected to a conformational search, all conformers were subjected to energy minimization, all the minimizations were performed with MOE until a RMSD gradient of 0.01 Kcal/mole and RMS distance of 0.1 Å with MMFF 94 X force-field and the partial charges were automatically calculated. The enzyme was prepared for docking studies as follows: hydrogen atoms were added to the system with their standard geometry, the atoms connection and type were checked for any errors with automatic correction and selection of the receptor and its atoms potential were fixed. MOE alpha site finder was used for the active site search in the enzyme structure using all default items. Dummy atoms were created from the obtained alpha spheres.

2.6 Statistical Analysis

One-Way ANOVA was employed to evaluate any significant difference between the values of optical densities (OD) obtained from biofilm formed by control (untreated) and the values obtained in the presence of different concentrations of NAC and dipropyl disulphide. Differences were done using SPSS, 17 statistical software (SPSS Inc., Chicago, IL).

3 Results & Discussion

E. coli, *Enterococcus faecalis*, *P. mirabilis* are more commonly associated with urinary tract infections (UTIs) in those individuals with structural or functional abnormalities, especially ascending infections in patients undergoing urinary catheterization (O'May et al. 2008; Garsin and Willems 2010; Goller and Seed 2010). *P. mirabilis* was found to have the greatest ability out of all gram-negative organisms to attach to many catheter materials (Hawthorn and Reid 1990; Roberts et al. 1990). Its high pathogenicity is due to the expression of many virulence factors. So, it is necessary to understand these virulence factors and their role in infection in order to find agents that can interact with these factors to prevent the establishment of infection and facilitate treatment.

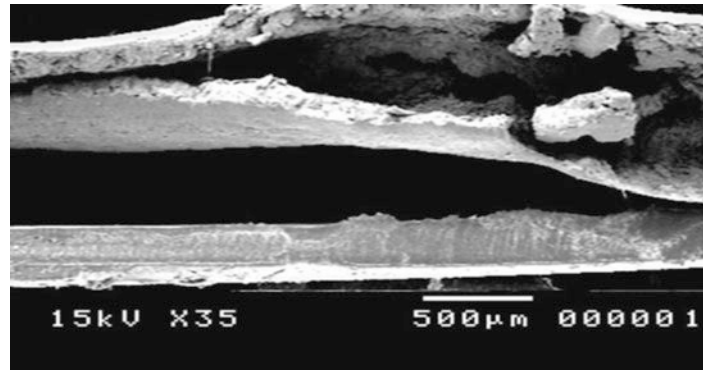

Fig. 1 Scanning electron micrograph showed the lumen of a ureteral stent obtained from a catheterized patient (×35). It showed a dense mass of biofilm and a high level of encrustation

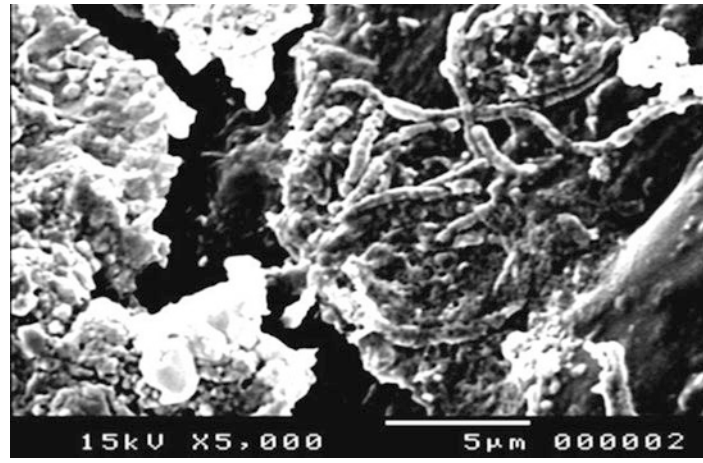

Fig. 2 Scanning electron micrograph showed the lumen of the ureteral stent covered with a dense mass of biofilm containing bacteria (*P. mirabilis*) and crystalline patches (white patches) (×5000)

In this work, the same *P. mirabilis* strain was isolated from two clinical samples taken from the same patient, which was proven by negative dienes reaction between both isolates. The *P. mirabilis* strain was negative for hemolysis, urease positive and strong biofilm producer according to tissue culture plate method (OD = 0.6). Stent removed from the patient was examined by scanning electron microscopy. SEM micrograph showed a dense mass of biofilm blocking the lumen of the stent (Fig. 1). Also, a high level of encrustation (crystalline patches) and a dense mass of biofilm containing *P. mirabilis* rods were observed using 2 different magnification powers (Figs. 2 and 3). Many studies reported the ability of *P. mirabilis* to encrust and block the lumen of catheters (Jones et al. 2005; Morris et al. 1997; Stickler and Sabbuba 2007; Vlamakis 2011).

The antibacterial activity, MIC and MBC of the tested agents were evaluated. The MIC of NAC was 25 mM while MBC was 50 mM. For dipropyl disulphide, MIC was 100 mM while MBC was > 200 mM. The antimicrobial and the anti-biofilm activity were previously reported by many researchers (Marchese et al. 2003; El-Feky et al. 2009; Abd El-Baky et al. 2014; Mohsen et al. 2015). They all showed that NAC has powerful effect on the disruption of mature biofilms formed by several types of microorganisms and can inhibit adherence. Also, it can increase the therapeutic activity of several antimicrobials helping in the inhibition and eradication of biofilms. Dipropyl disulphide

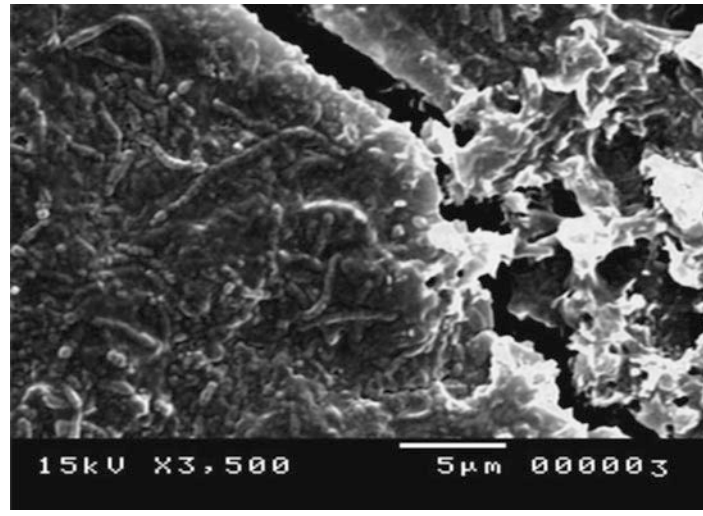

Fig. 3 Scanning electron micrograph showed the surface of the ureteral stent (×3500). It showed a dense mass of biofilm containing microorganisms and a high level of encrustation

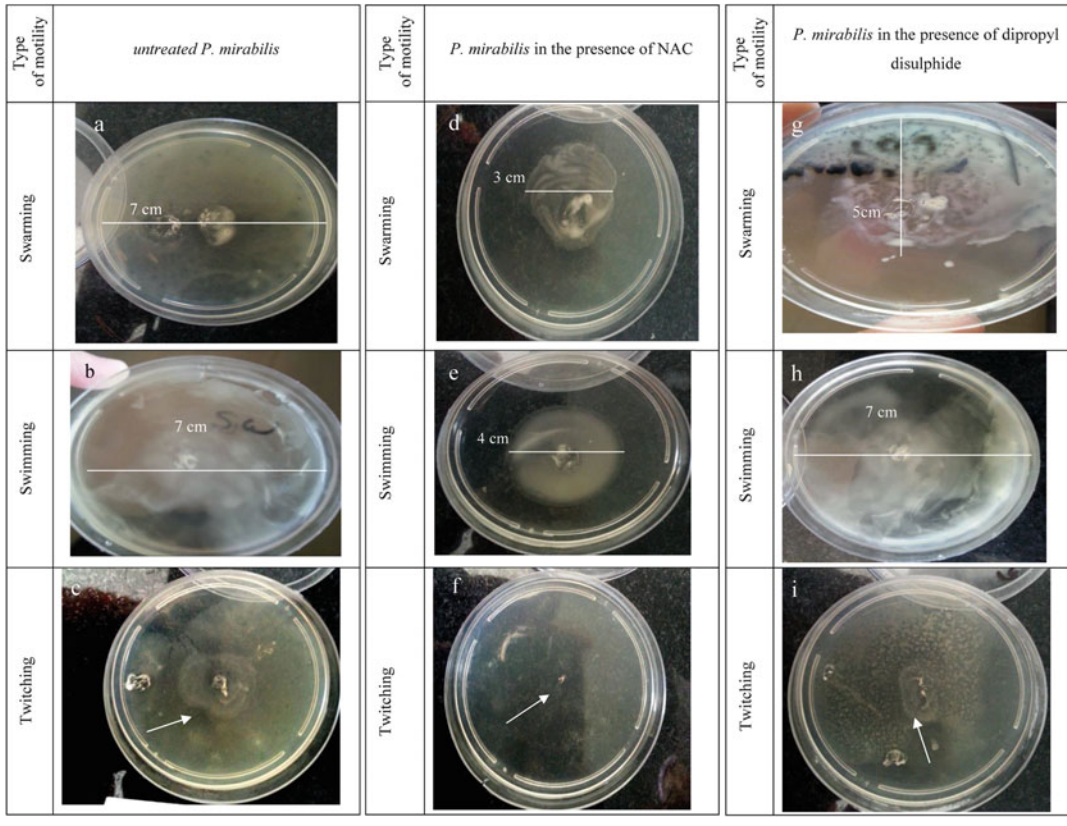

Fig. 4 (**a**–**c**) The ability of the tested strain to show different types of motility (swarming (*SW*), Swimming (*SM*) and Twitching (*TW*). (**d**–**f**) The effect of NAC on the ability of *P. mirabilis* to show the different types of motility. (**g**–**i**) The effect of dipropyl disulphide on the ability of *P. mirabilis* to show the different types of motility

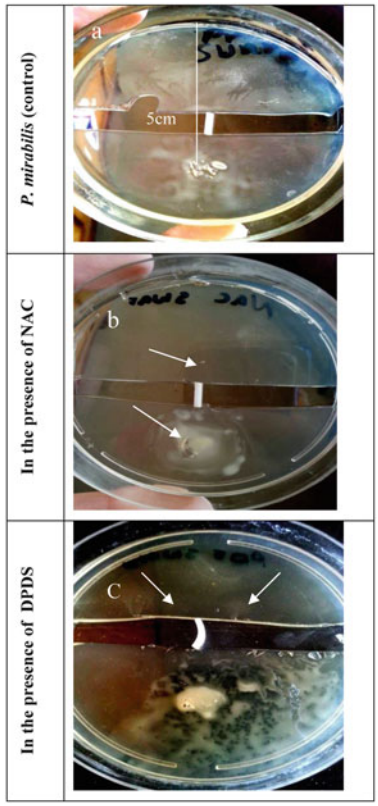

Fig. 5 (**a**) Swarming of the tested strain over the stent segment , (**b**) NAC inhibited the swarming motility of *P. mirabilis* , (**c**) dipropyl disulphide did not inhibit the swarming over the stent segment

is one of the main components of onion. Several studies showed that its antibacterial activity was scarce which agrees with our results (Kim et al. 2004; Mnayer et al. 2014).

The tested strain showed the ability to swarm over the whole plate. In addition, it was positive for swimming on 0.3% agar plate and twitching motility using 1% agar plates (Fig. 4a–c). Its ability to swarm over the stent was shown in Fig. 5a which indicates the ability of *P. mirabilis* to switch from swimmer cells in liquid media (urine) to swarmer cells on solid surfaces which helps in the dissemination of infection to other locations in the urinary tract. Naturally, the mucous layer covering the epithelial surfaces is highly viscous; it can trap many motile bacteria and prevent them from ascending the tract. But in case of *P. mirabilis*, entrapment of the organism in the mucus layer inhibits the flagellar rotation of trapped swimmer cells which induces swarmer cells' differentiation. Swarming motility may then facilitate the migration of this organism through the mucous layer and expand the population to new locations (Williams and Schwarzhoff 1978; Mobley and Belas 1995; Rather 2005).

By testing the changes in the ability of the organism to move in the presence of NAC and dipropyl disulphide, results revealed that NAC decreased the swarming ability of the tested isolate. As *P. mirabilis* showed a swarming zone of 3 cm while dipropyl disulphide decreased swarming zone to 5 cm in comparison to the control (untreated) strain that produced swarming zone of 7 cm (Fig. 4a, d, g). For swimming motility, the untreated strain showed swimming motility over the whole plate (Fig. 4b). Dipropyl disulphide didn't affect the swimming motility (Fig. 4h) while NAC decreased its ability to swim producing swimming zone of 4 cm (Fig. 4e). Figure 5f showed that twitching motility was blocked by NAC but no effect was observed by dipropyl disulphide (Fig. 4i). In the presence of NAC, *P. mirabilis* lost its ability to swarm over the stent surface while dipropyl disulphide didn't show any effect on its swarming activity (Fig. 5). Surface-associated swarming motility is implicated in enhanced bacterial spreading and virulence. Many studies tested different compounds that can block the swarming ability of motile microorganisms. As O'May et al. 2012 reported that tannic acid (TA) and epigallocathecin gallate (EGCG) and undefined cranberry powder (CP) can block swarming motility of *Pseudomonas aeruginosa*. Dusane et al. 2014 reported that Piperin and reserpine alkaloids decreased the swarming and the swimming ability of *E. coli*. Also, Ulrey et al. 2014 found that Cranberry proanthocyanidins reduced swarming and can disrupt biofilm of *P. aeruginosa*.

Stones are characteristic features of the biofilms developed during *P. mirabilis*-associated UTIs (Jones et al. 1990). The majority of patients with recurrent *P. mirabilis* catheter encrustation (62%) developed bladder stones, which led to the colonization of replacement

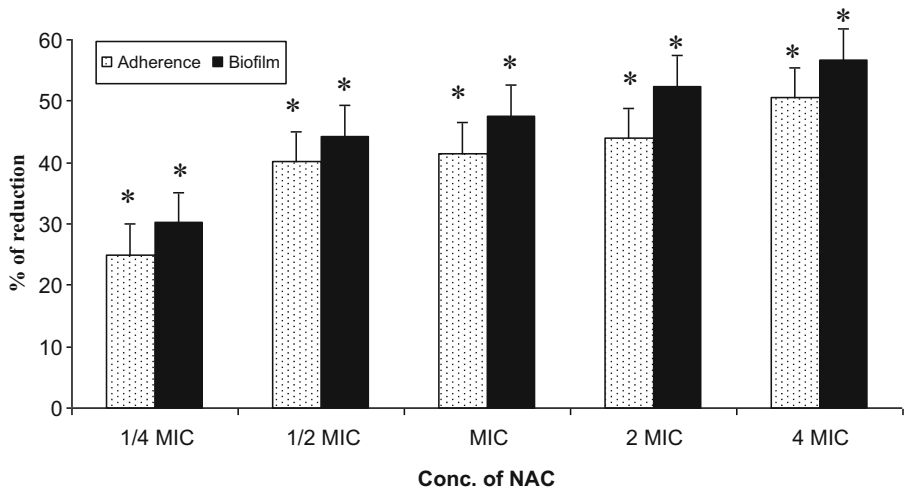

Fig. 6 Effect of different conc. of NAC on the adherence and mature biofilms of *P. mirabilis*. Results were obtained from three independent replicates. % of reduction was calculated in comparison to control and expressed as mean ± SD
*statistically significant ($P < 0.05$) compared with control

catheters with these organisms (Sabbuba et al. 2004). Both physical and chemical factors play a role in the initiation and development of the crystalline biofilms observed during *P. mirabilis* colonization. The pH of urine can be essential for bacterial attachment to polymer surfaces as macroscopic aggregates of cells and for crystals of calcium and magnesium phosphate to settle on the polymer surface and initiate crystalline biofilm development that blocks the flow of urine through the catheter (Jones et al. 1990). Therefore, the control of urine pH and subsequent crystallization could be critical in preventing biofilm formation on indwelling devices inserted in the urinary tracts of patients infected with *P. mirabilis*. Figures 6 and 7 showed the effect of *N*-acetyl cysteine and dipropyl disulphide on the adherence and mature biofilms of *P. mirabilis* isolate using tissue culture plate (TCP) assay. It was found that the inhibitory effect of both NAC and dipropyl disulphide was concentration dependent. The disruptive effect of NAC against mature biofilms (% of reduction ranged from 30.2 to 56.8 in comparison to control) was more than its ability to decrease the adherence of the tested isolate (% of reduction ranged from 25 to 50.5 in comparison to control). Dipropyl disulphide showed weak inhibitory effect on both the adherence and the mature biofilm of the tested isolate (% of reduction ranged from 12.2 to 28.5 and 11.3 to 26.1, respectively in comparison to control). All values were statistically significant ($P < 0.05$) compared with the control.

Urease inhibition by potent and specific compounds could provide an invaluable addition for the treatment of infections caused by urease-producing bacteria. Although several potent inhibitors of this enzyme have been reported, more effective, safe and potent inhibitors are considered necessary for the control of urease-related infections (Amtul et al. 2002, 2004). It was found that NAC and dipropyl disulphide had the ability to inhibit urease production at sub–MIC concentrations of the tested compounds. Change in pH value due to the production of ammonia from urea was determined at 2 h interval for 18 h. *P. mirabilis* incubated with urea showed an increase in the pH value ranged from 7.3 to 9. In the presence of increasing concentration of NAC (1/32 MIC-1/2 MIC), a decrease in the pH value was observed (7.1–6.2). Dipropyl disulphide caused a slight increase in the pH value from 7 (at 1/32 MIC) to 8 (at 1/2 MIC).

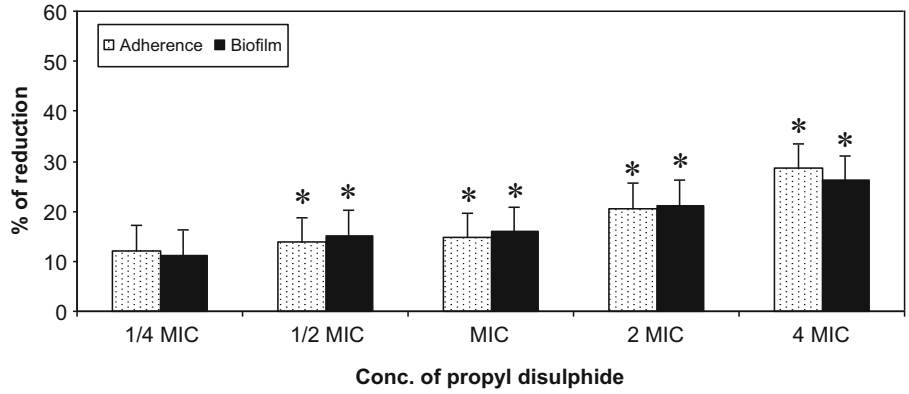

Fig. 7 Effect of different conc. of dipropyl disulphide on the adherence and mature biofilms of *P. mirabilis*. Results were obtained from three independent replicates. % of reduction was calculated in comparison to control and expressed as mean ± SD
*statistically significant ($P < 0.05$) compared with control

The urease inhibitory activity and IC_{50} of the tested compounds at different concentrations were evaluated. The results obtained were compared to that obtained by acetohydroxamic acid (standard urease inhibitor). It was found that the inhibitory activity ranged from 61.4 to 96.9% for NAC, 26 to 72.4% for dipropyl disulphide while acetohydroxamic acid (AHA) showed inhibitory activity ranging from 90 to 98% in comparison to control (without inhibitor). It was found that NAC had higher inhibitory activity (IC_{50} 249 ± 0.05 mM) than that shown by dipropyl disulphide (IC_{50} 10 ± 0.2 mM) when it is compared to the standard inhibitor acetohydroxamic acid (IC_{50} 120 ± 0.06 μM).

The role of urease enzyme in the degree of virulence was reported by many studies. These findings are further supported by the fact that treating the animals with acetohydroxamic acid, a potent urease inhibitor, reduces the severity of *P. mirabilis* infection (Musher et al. 1975). Despite the difference in the number of subunits forming urease enzyme among Jack bean (one), helicobacter (three) and all other bacterial species (two), the amino acid sequences are well conserved. The active site of the enzyme is found in the UreB subunit and comprises amino acid residues which are His-136, His-138, Lys-219, His-248, His-274, and Asp-362. These amino acids come in direct contact with the two nickel ions, urea, or a water molecule within the active site of the urease enzyme of *H. pylori* (Labigne et al. 1991). In addition, His-322 is near the active site and acts as a general base in the catalysis (Dixon et al. 1980). Urea binds in *O*-coordination to one nickel ion aided by His-221. His-322 (as an active base) activates a water molecule bound to the other nickel ion. An attack by the metal-coordinated hydroxide on the substrate carbon atom results in a tetrahedral intermediate that bridges the two nickel sites, a proton transfer to the intermediate with accompanying ammonia release, and a displacement to the carbamate by water to complete the cycle (Mobley 2001).

The tested compounds were studied by docking them into the crystal structure of *Helicobacter pylori* urease enzyme (1E9Y) obtained from Protein Data Bank (PDB) to observe the common behavior of interaction of these compounds with the enzyme. In order to validate the docking reliability, the ligand acetohydroxamic acid was removed from the active site, and the Grid Box was set large enough to do blind docking. Figure 8a showed the docking studies of acetohydroxamic acid (standard urease inhibitor) with the active site. NAC interacts with urease with binding free energy of −4.8 kcal/mol. Docking studies of *N*-acetyl cysteine with the active site of the enzyme

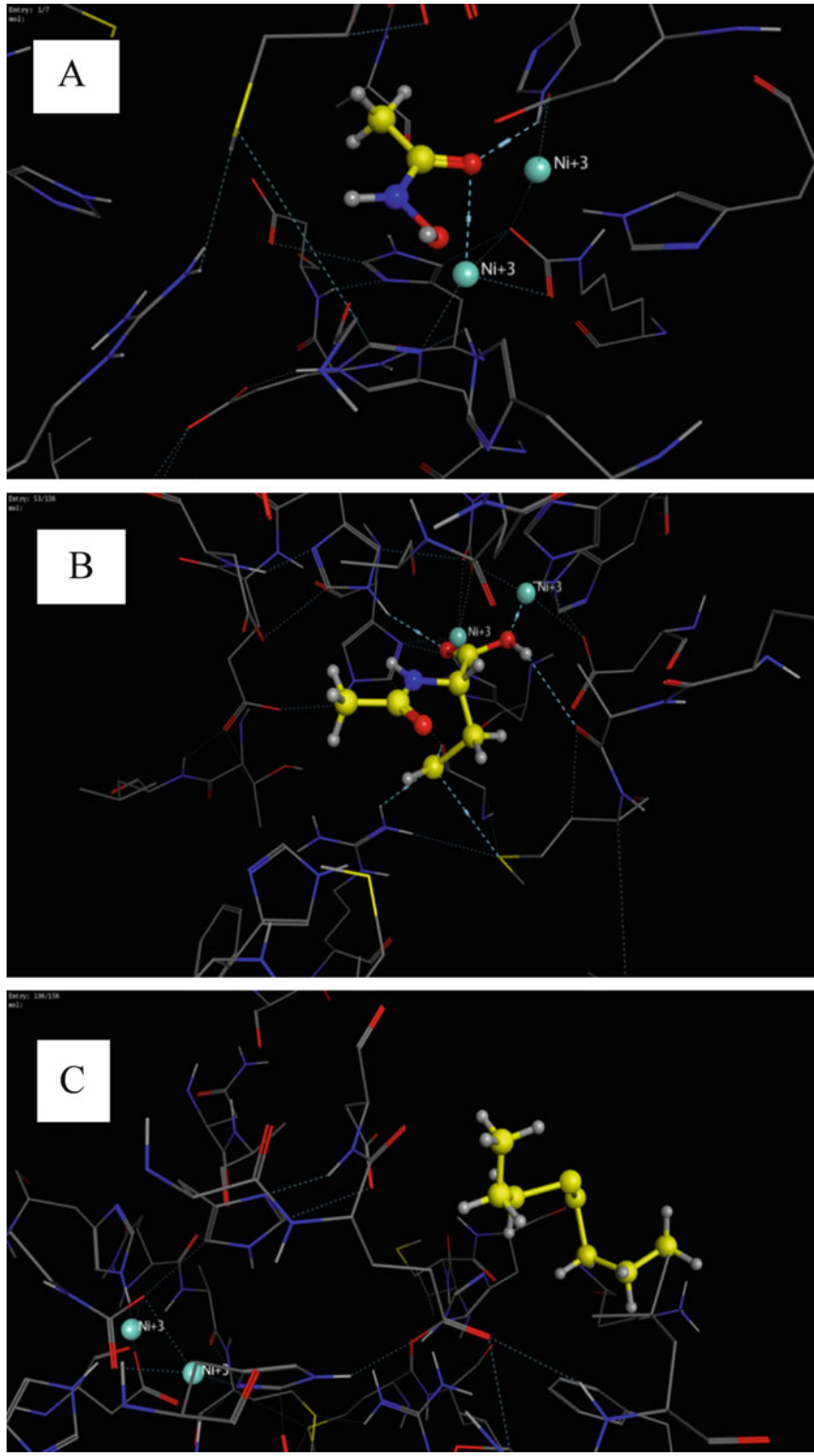

Fig. 8 Molecular docking of Acetohydroxamic acid AHA (**A**) NAC (**B**) and dipropyl disulphide (**C**) and their interactions with different residues of urease enzyme active site involved in interaction

(Fig. 8b) showed an *O*-coordinated bond with nickel 3002 and a hydrogen bond with His-138 which is in direct contact with Ni ion. The oxygen atom in the OH group makes a hydrogen bond with Gly 279. The thiol group forms two disulfide bonds with SH group of Cysteine 321 (Cys 321) and S-CH3 of methionine (Met366). So, these compounds lock the enzyme into a single conformation by covalently modifying the Cys residue in the flap. Kühler et al. reported that covalent modification of Cys321 will lead to an inactive enzyme, which indicated that Cys321 may be one of the key residues for the catalytic activity of urease (Kühler et al. 1995). Xiao et al. showed that quercetin inhibits urease enzyme due to the formation of hydrogen bonding with Cys 321, Met366 and Gly367 (Xiao et al. 2012). On the other hand, dipropyl disulphide interacts with urease with binding free energy of −8.528 kcal/mol. Diporpyl disulfide forms a hydrogen bond with His-322 located near to the active site (Fig. 8c); it plays an important role in the catalysis process and the release of ammonia. Many studies use several natural and chemical compounds to inhibit urease enzyme. Ranjbar-Omid et al. found that allicin extracted from garlic was able to inhibit urease, hemolysin and biofilm formation of *P. mirabilis* (Ranjbar-Omid et al. 2015). Prywer and Torzewska reported that curcumin may inhibit the activity of urease, not influencing the bacterium's vitality (Prywer and Torzewska 2012). Also, they noticed that in the presence of curcumin, pH of the urine increases much more slowly compared with the absence of curcumin which is similar to results obtained by dipropyl disulphide in our study.

4 Conclusion

NAC and dipropyl disulphide exhibited antimicrobial properties and can be used for the inhibition of crystalline biofilm formation by *P. mirabilis*. Both compounds decreased the swarming ability of the tested isolate in comparison to the untreated strain. NAC and dipropyl disulphide showed variable inhibitory activity against urease enzyme produced which was also supported by docking studies. Using NAC with antibiotic therapy may have an additive therapeutic effect in eradication of *P. mirabilis* and the protection of patients suffering from urinary tract infections or catheterized patients from developing infective stones.

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