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Synthesis and Antibacterial Activity Studies of Monosubstituted Metal Cholates Against *P. Aeruginosa* and *S. Aureus* Isolated from Clinical Samples

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Abstract

Clinical specimens from burns, wounds and UTI`s, were collected from four hospitals in Baghdad area, and local isolates of *P. aeruginosa* and *S. aureus* were obtained. Five monosubstituted metal cholates were prepared by reacting monoequivalents of sodium cholate with metal salts in two different solvent, viz. 95% ethanol or water. Ten monosubstituted metal cholates were obtained, and characterized by their physical and spectrophotometric properties. It is the first time to report systematic preparation, characterization and study of such monosubstituted metal cholate. The growth curves for *P. aeruginosa* and *S. aureus* in the presence of metal cholates, revealed that the time for logarithmic phase were reduced from 25-30 to 5-25 minutes for both bacteria, while the stationary phase from 90-100 to 40-60 minutes. The antibacterial activities of the prepared metal cholates against *P. aeruginosa* and *S. aureus* were determined by broth dilution method in the presence of metal cholates. It was found that in the presence of the metal cholates, the MBC values were found to be within the range 8.0×10^{-5} - 8.35×10^{-5} M.

Keywords: Burns, wounds, UTI`s, *P. aeruginosa*, *S. aureus*, monosubstituted metal cholates, MBC values.

Introduction

Cholic acid, (IUPAC as 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) is a primary bile acid is a white crystalline substance. Cholic acid, along with chenodeoxycholic acid, is one of the two major bile acids produced by the liver, where it is synthesized from cholesterol. These two major bile acids are roughly equal in concentration in humans [1]. The bactericidal action was shown on the examples of cholic acids derivatives to a broad spectrum of gram-negative and gram-positive organisms. Other cholic acid derivatives were weakly active against gram-negative organisms, but effectively permeabi-lized the outer membranes and sensitized the bacteria for hydro-phobic antibiotics such as erythromycin and rifampicin [2]. Cholic acid, a natural bio detergent has been reported to exhibited an anti-bacterial effect [3]. Since cholic acid is a suitable building block for new molecules or in other words, it is a leading substance for the development of various compounds. Cholic acid and their complexes were found to have more active antibacterial activity because of synergistic effect of cholic acid as well as metal ions [4].

The antimicrobial activity of several metal cholates under the codes viz. BSN I, CN II, ZA III, MCL IV, NA V, MA VI, CA were studied by well diffusion method and considered as a new compounds with distinguished antibacterial effects. MIC is usually derived by means of tests in solid media, whereas both MIC and MLC can be determined in broth dilution assays [5]. The only published article deals with the preparation and study of

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antimicrobial activity of metal cholates is that of [6], and no published study has been reported anywhere about their biological effects. As matter of fact, no preparation procedure and no chemical structures were given to the products mentioned in this article. The aim of the present study is to establish procedures to prepare several monosubstituted transition metal cholates and to investigate their antibacterial effects against *S. aureus* and *P. aeruginosa*.

MAREIALS AND METHODS

a) Materials and instruments:

Cholic acid, sodium hydroxide (97%), ethyl acetate and anhydrous ether, dimethyl formamide, dimethyl sulphoxide were obtained from BDH, England. Copper (II) chloride, CuCl2, chromium sulfate, CrSO45H2O, nickel (II) chloride, NiCl2, cobalt (II) chloride, CoCl2, Zinc (II) chloride, ZnCl2 were from Sigma-Aldrich, Germany. Ethanol 99%, ethanol 95% and ammonia solution, acetic acid, and hydrochloric acid were from GCC, UK.

Culture media used throughout this study are Blood agar base, Mannitol salt agar, Mueller Hinton (MH) agar and broth, Nutrient agar from Hi media, India, Brain heart infusion (BHI) agar from Oxoid, England, and Cetrimide agar from Hardy Diagnostics, USA.

Among the instrument used are HPLC, Fourier Transform Infra-red spectrophotometer, Atomic absorption spectrophotometer, UV-visible spectrophotometer, model 1800 from Shimadzu, Japan, and VITEK 2 compact system from biomeruex, France.

b) Reagents and solutions:

The following reagents and solutions used throughout this work were prepared according to literature: Normal saline [7], Catalase tests [8], Oxidase test [9].

c) Identification specimens of P. aerugenosa and S. aureus collection:

Seventy five clinical specimens from different sources, including burn, wound, UTI, and skin infections were collected from four hospitals in Baghdad area. Baghdad Teaching Hospital, Burn Hospital and Teaching Laboratories in Medical City, and Protect Children Hospital during the period September 2015 to November 2015. These samples were examined for the presence of *S. aureus* and P. aerugenosa from their culture characteristic, microscopy, biochemical test, and finally confirmed by VITEK 2 compact system. The bacterial isolates were preserved according to the procedure recommended by literature [10].

d) Antibiotics susceptibility test:

The antibiotics susptibility test was performed according to Kirby-Bauer method were used, and the results of the experiment presented in Table 1[11].

e) Metal cholate synthesis:

Metal cholates were prepared by following two different procedures by using different solvents, 95 % ethanol and only water. Metal cholates of copper, chromium, cobalt, nickel, and zinc were prepared as the following procedures.

1. Metal cholates from 95% ethanol: cholic acid was dissolved in 95% ethanol containing one equivalent of sodium hydroxide then, treated with one equivalent of metal salt solution in same solvent. The ethanol will help to remove water by isotropic effect.
2. Metal cholates preparation in aqueous solvent: cholic acid was dissolved in aqueous sodium hydroxide solution, then treated with one equivalent of aqueous metal salt solution. It was washed with ethanol to remove absorbed water, and ethanol will help to remove water by isotropic effect.

f) Antibacterial activity of metal cholates:

1. Preparation of bacterial inoculums: the accurate measurement of antibacterial activities of a serial dilution of metal cholates, it is

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necessary to use defined bacterial inoculate recommended by Swenson [12]. The bacteria, *P. aeruginosa* or *S. aureus*, were cultured on brain heart infusion agar for 24 hours at 37ºC. The entire bacterial colonies were harvested with help of sterilized metal loop, and transferred to plain tube containing 10 mL of normal saline. This solution is considered as the stock bacterial solution. Stock bacterial suspension (1.0 mL) was added to a plain tube containing normal saline (9.0 mL), and the solution was homogenized on vortex for 2 minute. The resulted solution is considered as 10^{-1} dilution. Bacterial suspension of the above 10^{-1} dilution (1.0 mL) was added to another plain tube containing normal saline (9.0 mL), homogenized physically as the above solution. The resulted solution is considered as 10^{-2} dilution. Bacterial suspension of the above 10^{-2} dilution (1.0 mL) was added to another plain tube containing normal saline (9.0 mL), homogenized physically as the above solution. The resulted solution is considered as 10^{-3} dilution. The procedure is repeated until five serial dilutions of 1, 10^{-1}, 10^{-2}, and 10^{-3}, 10^{-4}, and 10^{-5}. A volume of 1 mL of the bacterial serial dilutions were spreaded over the surface of brain heart infusion agar and incubated at 37ºC for 24 hour. The colony count should be between 30-300 colonies to carry on the bacterial activity measurements. The calculation of the colony forming unite, CFU according to the following equation:

CFU = (# of colonies × dilution factor)/volume of the media

Where the number of colonies were practically measured with help of colony counter. It was found that solution with dilution 10^{-5} is convenient for the use in this experiment because it contain the required CFU.

2. Serial Dilutions of cholate salts: to determine the MIC & MBC by spectrophotometric method a serial dilutions of five metal cholates. Serial dilutions were prepared by suspending 25.0 mg of each salt in 100 mL deionized water, followed by warming to 70ºC, then replacing the mixture on vortex for 5 minute. Turbidity measurements were performed at wave length 696.0 nm, by using double beam spectrophotometer against the broth as blank (5.0 mL diluted with 10.0 mL of distilled water) [13].

Results and Discussion

Seventy five clinical specimens from different sources, including burn, wound and UTI, were collected from four hospitals in Baghdad area. Baghdad Teaching Hospital, Burn Hospital and Teaching Laboratories in Medical City, and Protect Children Hospital during the period September 2015 to November 2015. Of the total 75 samples obtained from 28 burns and 22 wounds patients, 25 UTI patients, as shown in Table 2. The confirmatory diagnosis of bacteria by VITEK 2 Compact System considered a decisive confirmation for *S. aureus* isolates by using Gram-positive card.

Twenty nine isolates of *P. aeruginosa* from burn, wound, and UTI patients were tested for antibiotic sensitivity by Disc Diffusion Method (Kirby Bauer Test) [14]. Among *P. aeruginosa* specimens, it was found that 29 of them were 100% resistant to carbenicillin and doxycycline. High resistance was found for amoxicillin/ clavulanic acid, 82.7%, cefipime, 79.3%, netilmicin, 72.4% and azetreonam, 55.1%. Moderate resistance were obtained for gentamycin, tobramycin and ciprofloxacin of 24.1, 20.6, and 17.2%, respectively, while imipenem was found to be the least resistant antibiotic, as shown in Figure 1. Forty six isolates of *S. aureus* were tested for antibiotic sensitivity by Disc Diffusion Method. The results showed high resistance towards azithromycin, vancomycin, cefoxitin, clathromycin and chloramphenicol with frequencies of 89.1, 85.6, 84.7, 76.0 and 76.0% respectively, while moderate resistance towards ciprofloxacin and doxycycline with frequencies of 73.9, and 71.7%, respectively. *S. aureus* showed low resistance to clindamycin, tobramycin and gentamycin with frequencies of 58.6, 60.8, 63.1 and 19.0%, respectively.

Cholic acid (IUPAC name 3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) is a bile acid [15], is a white crystalline water insoluble substance (soluble in alcohol and acetic and acid), and its salts called cholates. Cholic acid, along with chenodeoxycholic acid, is one of the two major bile acids are roughly equal in

concentration in humans [16]. Following the two previously mentioned procedures will end with ten metal cholates having similar chemical structure and different physical properties, as shown in Figure 1.

There is an apparent difference in their colors and melting points, when they are prepared in different solvents. Copper cholate gives light blue crystals when precipitated from ethanol, while it gives orange color when precipitated from water. The physical properties, which include melting points, colors, UV-visible and FT-IR absorption spectrophotometry, their purity by HPLC, atomic absorption spectrometry for the presence of the metals, as presented in Table 3. They are colored compounds, and their colors is dependent on the attached metal atom, and not the cholate moiety. Cholates prepared in ethanol were analyzed by HPLC and found to have good purity (> 90%). In general, the melting points of the cholates prepared in 95% ethanol are higher than that prepared in water, except for copper cholate. The UV-visible absorption spectrum of cholic acid showed absorption band with maxima at 239 nm, while all metal cholates gave single band with maxima at 259.2 nm. All the bands of metal cholate stand for shifted absorption of C=O bond of the carboxylate group, to higher wave length. Only copper cholate gave a value of 264 nm when prepared in ethanol, while at water 215.5 nm from water. It was difficult to spot the forbidden d-d electronic transitions of the central transition metal atoms, due to the low percentage of the metal in compound, as well as their low solubility. The FT-IR spectra of all the metal cholates were recorded for the range 500-4000 cm-1, and all the spectra were coincide with the formation of the sought compounds. The FT-IR absorption bands for the carboxylic group C=O, C-O, and the paraffinic C-C bonds were obtained as similar fingerprint. The presence of the metal atom with 1:1 proportion was indicated from atomic absorption spectrometry measurements. Generally, the obtained ratio was almost 1:1 for all the prepared metal cholates. This is the first time to report the preparation of monosubstituted metal cholates. It worth mentioning that Chakrabarty et al. report the preparation of disubstituted metal cholate hydrogel without any proper identification of products [17].

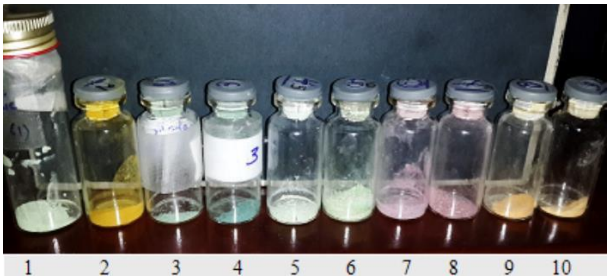

Figure 1: Monosubstituted metal cholates obtained from ethanol and H2O solvents; 1 & 2. Copper, 3 & 4. Chromium, 5 & 6. Nickel, 7 & 8 Cobalt, and 9 & 10. Zinc.

Chemical reaction of these products with four reagents viz. hot 1;1 HCl, hot 0.5 N NaOH, 3% $AgNO_3$, and (35% NH_4OH, concent-rated ammonia solution, and the visual observation were presented in Table 7. All metal cholates reacted with silver nitrate reagent, with the formation of white precipitates of silver chloride (or silver sulfate for chromium sulfate), indicating the presence of the chloride ions ions. In case of chromium a white precipitate of silver sulfate was formed, indication of the presence of ionic sulfate. All metal cholate dissolved in concentrated ammonia, except chromium and nickel cholate, which are prepared in ethanol. When copper, cobalt, and zinc cholate reacted with hot 1:1 HCl, the metal-cholic acid bond break ending with cholic acid and metal chloride mixture, which appeared as a faint precipitates, but chromium and nickel cholate did not respond to this reagent. Hot 5N NaOH solution destroy the salt with the formation of sodium cholate and the corresponding metal hydroxides with their known colors.

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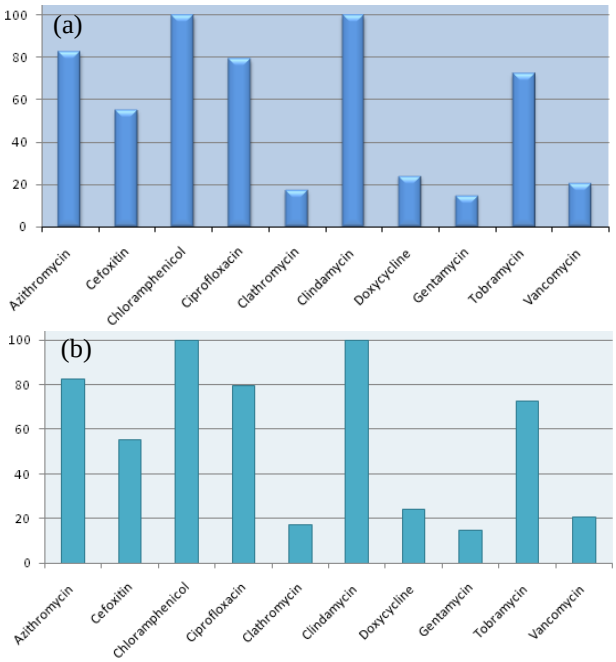

Figure 2: The percentage of the resistance of (a) *P. aeruginosa* and (b) *S. aureus* against ten antibiotic were used in this study

The antibacterial activity of the prepared metal cholate were determined by following the method of serial dilution of metal cholates mentioned in the experimental part [13]. Turbidity measurements were performed at wave length 696.0 nm after 24 hour, by using double beam spectrophotometer against the broth as blank (5.0 mL diluted with 11.0 mL of distilled water), as shown in Table 5 and the results were presented in Figures 2-6. Positive control solutions for *S. aureus* and for *P. aeruginosa* were prepared by mixing 1.0 mL of bacterial inoculums, 5.0 mL broth, and 10.0 distilled water. The optical density of these solutions were measured after 24.0 hours, against blank which contain the broth solution only. The optical density recorded for *S. aureus* and for *P. aeruginosa* were 2.5 and 2.22, respectively. The data obtained for the serial dilutions of ten metal cholates prepared in ethanol and water solvent, recorded for both bacteria, and compared with that of the positive control. The antibacterial effect of serial dilution of each salt (prepared in EtOH and H_2O) were plotted as the change in the optical density against the change in the molar concentration, as shown in Figures 3-7.

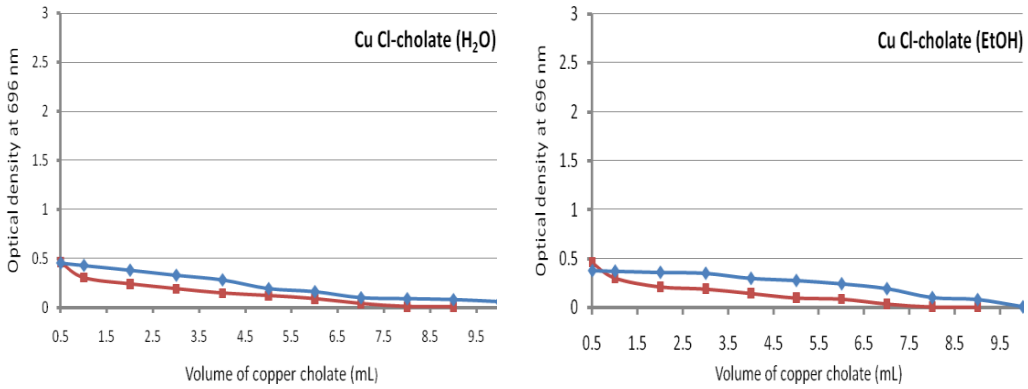

Figure 3: The antibacterial effect of serial dilution of copper chloride cholate (prepared in EtOH and H_2O) on *P. aeruginosa* (♦) and *S. aureus* (■) at Brain Heart broth.

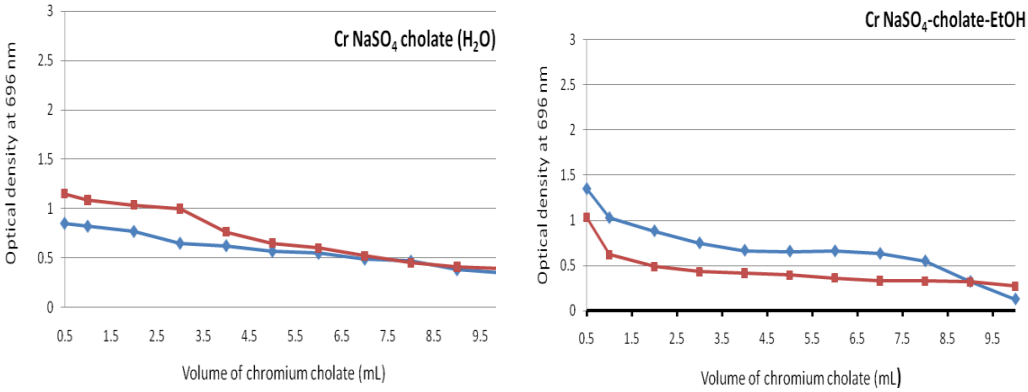

Figure 4: The antibacterial effect of serial dilution of sodium chromium sulfate cholate (prepared in EtOH and H_2O) on *P. aeruginosa* (♦) and *S. aureus* (■) at Brain Heart broth.

The value of the optical density at the lowest concentration as well as that for two of the highest dilutions, were presented in Table 5. The highest optical density recorded for the serial of dilutions is that of the positive control solution free from any metal cholates, with a value of 2.2 for *P. aeruginosa*, and 2.5 for *S. aureus*. The MBC and MIC values were considered for both bacteria, when the optical density reach a value of 0.006 and less. All the ranges of concentration showed significant value of inhibition for *P. aeruginosa* with maximum value of optical density for chromium sodium sulfate cholate at a molarity of 1.43×10^{-5} with optical density of 1.35. The molarity of serial dilution solution were presented in Table 6. The minimum value of optical density belong to copper cholate at a molarity of 1.63×10^{-5} with optical density of 0.376.

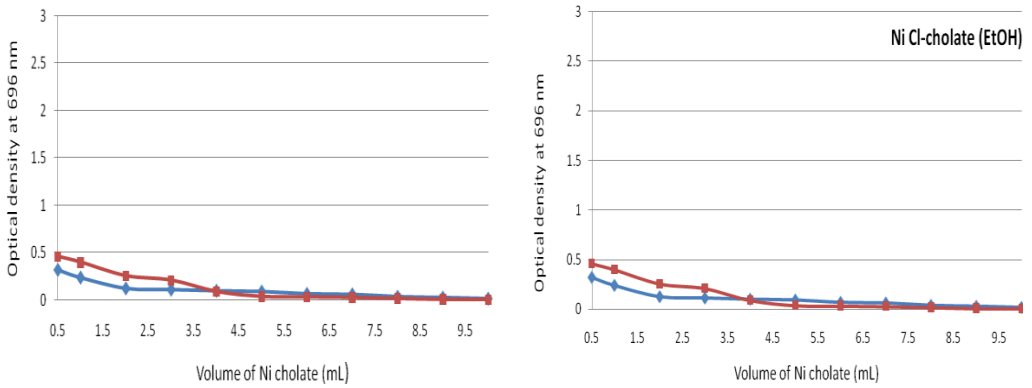

Figure 5: The antibacterial effect of serial dilution of Nickel chloride cholate (prepared in EtOH and H_2O) on *P. aeruginosa* (♦) and *S. aureus* (■) at Brain Heart broth.

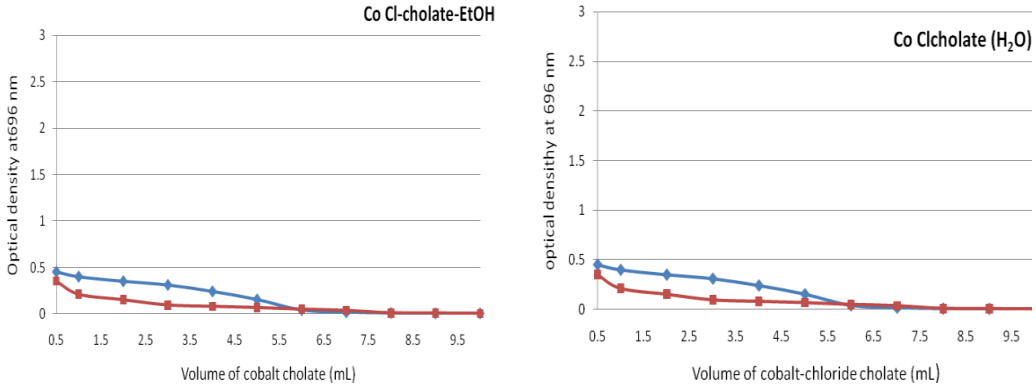

Figure 6: The antibacterial effect of serial dilution of cobalt chloride cholate (prepared in EtOH and H_2O) on *P. aeruginosa* (♦) and *S. aureus* (■) at Brain Heart broth

All the ranges of concentration showed significant value of inhibition for *S. aureus* with maximum value of optical density for chromium at a molarity of 1.43×10^{-5} with optical density of 1.122. The minimum value of optical density belong to cobalt cholate at a molarity of 1.66×10^{-5} with optical density of 0.35. Four values were recorded for the MBC`s of metal cholates against *P. aerugenosa* were found in the cases of copper, cobalt, and zinc prepared in ethanol. Only nickel cholate prepared in water have MBC value. Chromium cholate did show an MBC within this range of concentration for both solvent and for both bacteria. Seven values were recorded for the MBC`s of metal cholates against *S. aureus* were found in the cases of copper, cobalt, and

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nickel prepared in both solvents, as well as zinc cholate prepared in water.

It worth mentioning that the only similar work found in literature is that of Kishu [6], who prepared a set of what they call organometallic complexes of cholic acid. Organometallic compounds should contain carbon-metal bond (C-M bond), metal cholates did not contain such bonds. No procedures were presented in their work for how they prepare their products, nor they mentioned the chemical structures of their fifteen products. They concluded that their products, with metals ions have larger antibacterial activity with synergistic effect from cholic acid and the metal ions. They use the agar disc diffusion assay [14], and gave values for Zone of inhibition (mm ± S.E) in the range from 7.83 (± 0.44) to 19.00 (± 0.57) mm for five bacteria viz. *Micrococcus luteus, Bacillus subtilis, Streptococcus pneumonia, Klebsiela pneumonia,* and *P. aeruginosa.*

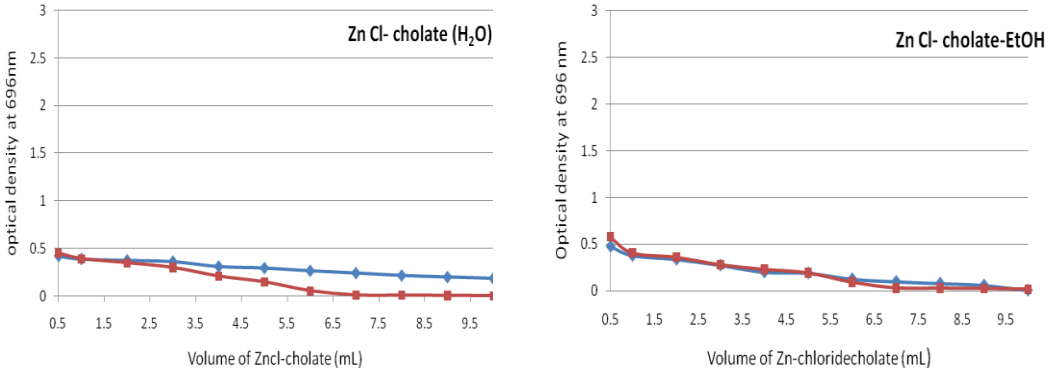

Figure 7: The antibacterial effect of serial dilution of zinc chloride cholate (prepared in EtOH and H_2O) on *P. aerugenosa* (♦), and *S. aureus* (■) at Brain Heart broth.

The growth curves of *P. aeruginosa* and *S. aureus* were studied according to experimental part, and the results were presented in Figures 7-12. The data of the logarithmic and stationary phases were presented in Tables 7-8 for *P. aeruginosa* and *S. aureus,* respectively. The growth curves for both bacteria were recorded in absence of metal cholates, and it's possible to note that the time for the logarithmic phase were 25 and 30 minute for *P. aeruginosa* and *S. aureus,* respectively. The recorded time for the stationary phase in the absence of metal cholates were 100 and 90 minute for *P. aeruginosa* and *S. aureus,* respectively. The recorded time for Lag phase in the absence of metal cholates were 10 and 70 minute for *P. aeruginosa* and *S. aureus,* respectively. There is much longer time needed for later to start growing. The addition of metal cholates were added before the start of the logarithmic phase, and the time count start since. This action is necessary to overcome the development of bacterial resistance (Belly *et al.*, 1982) [18].

The recorded time for logarithmic phase for *P. aeruginosa* in the presence of the five metal cholates were in the range 5-25 minutes. It is shorter than the recorded time in their absence, while the recorded optical density in the presence of metal cholates is ten times lower than that for the free bacteria. However, chromium sodium sulfate cholate (prepared in EtOH and H_2O), possess the least optical density, and hence the least antibacterial effect, as well as Nickel chloride cholate (prepared in EtOH), zinc chloride cholate (prepared in H_2O). Accordingly, the highest antibacterial effect belong to cobalt chloride cholate and copper chloride cholate (both prepared in EtOH and H2O).

The recorded time for the stationary phase in the presence of metal cholates were within the range 20-55 minute, a value less than half that in their absence, while the observed optical density in presence of metal cholates is ten times lower than that for the free bacteria. Consequently, the antibacterial effect is apparent in the presence of metal cholates. The recorded time for logarithmic phase for *S. aureus* in the presence of metal cholates were in the range 5-25 minutes. It is shorter than that in their absence, while the observed optical density in presence of metal cholates is ten

times lower than that for the free bacteria. However, chromium sodium sulfate cholate and Nickel chloride cholate (prepared in EtOH and H_2O), as well as cobalt chloride cholate (prepared in H_2O), having the least antibacterial effect. The highest antibacterial effect belong to cobalt chloride cholate (prepared in EtOH) and copper chloride cholate (prepared in H_2O). In similar way, the recorded time for the stationary phase for *S. aureus* in the presence of metal cholates while the observed optical density in presence of metal cholates is ten times lower than that for the free bacteria. The highest antibacterial effect is related to decrease in optical density.

There are work on the action of certain chemicals effect on bacterial enzymes by inhibiting the growth when deposited in vacuole and cell wall as granules [19]. Silver ion was reported to inhibit Silver ion was reported to inhibit cell division and damaged the cell envelope and contents of bacteria. Bacterial cells increased in size, and the cytoplasmic membrane, cytoplasmic contents, and outer cell layers all exhibited structural abnormalities. Accordingly, metal cholate are likely to behave in similar way. Possibly the remarkable change in the logarithmic phase and stationary phase of the bacterial growth, as shown in Figures 7-12, were proceeded in similar mechanism. Further work is needed to investigate the action of metal cholates on growth curves of *P. aeruginosa* and *S. aureus*.

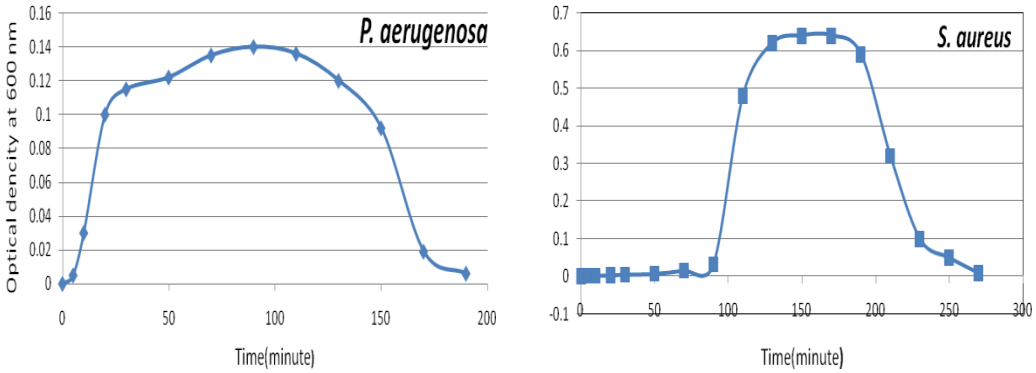

Figure 7: The growth curve of *P. aeruginosa* (Top, ♦) and *S. aureus* (Bottom, ■) in Brain Heart broth in absence of metal cholate.

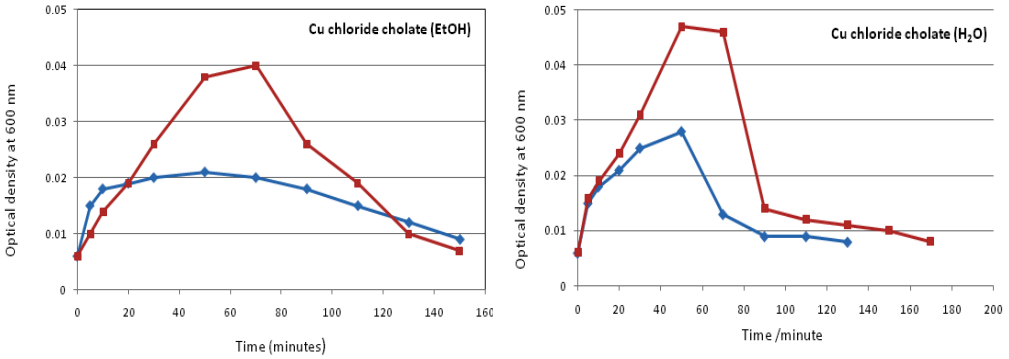

Figure 8: The growth curve of *P. aeruginosa* (♦) and *S. aureus* (■) in Brain Heart broth and 8.15×10^{-5} M copper chloride cholate (prepared in EtOH and H_2O).

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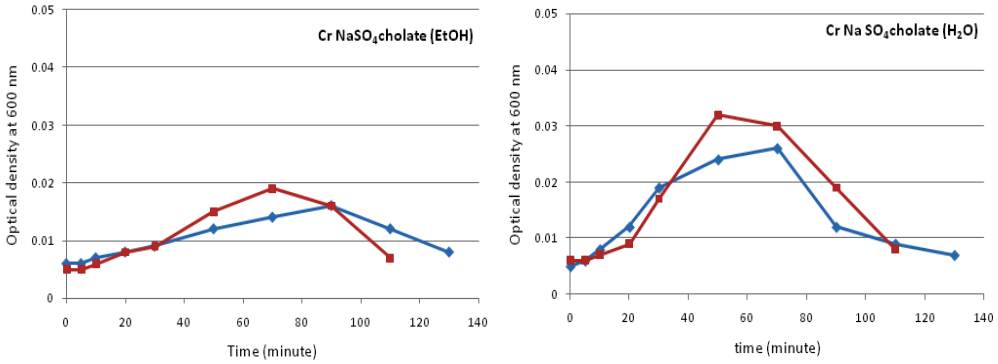

Figure 9: The growth curve of *P. aeruginosa* (♦) and *S. aureus* (■) in Brain Heart infusion broth with 7.15×10^{-5} M sodium chromium sulphate cholate (prepared in EtOH and H_2O).

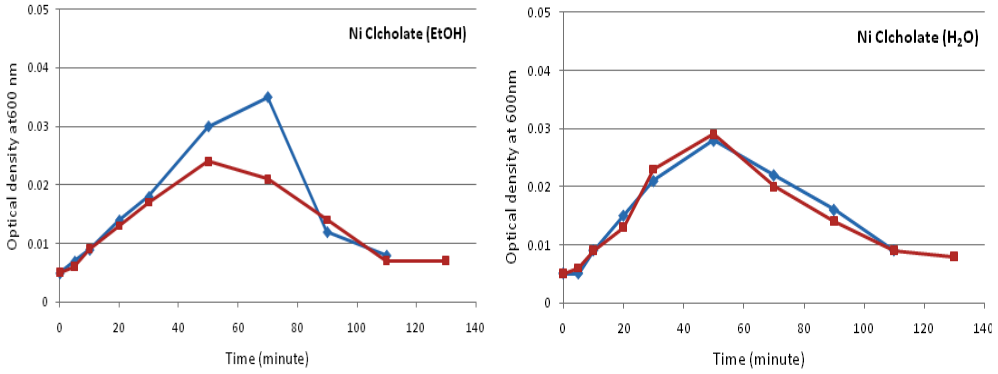

Figure 10: The growth curve of bacteria *P. aeruginosa* (♦) and *S. aureus* (■) at Brain Heart broth in 8.35×10^{-5} M nickel chloride cholate (prepared in EtOH and H_2O).

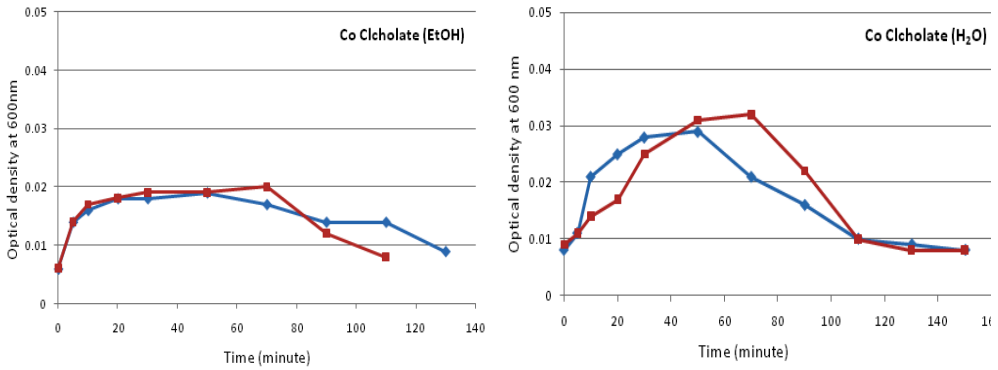

Figure 11: The growth curve of bacteria *P. aeruginosa* (♦) and *S. aureus* (■) at Brain Heart broth in 8.0×10^{-5} M cobalt chloride cholate (prepared in EtOH and H_2O).

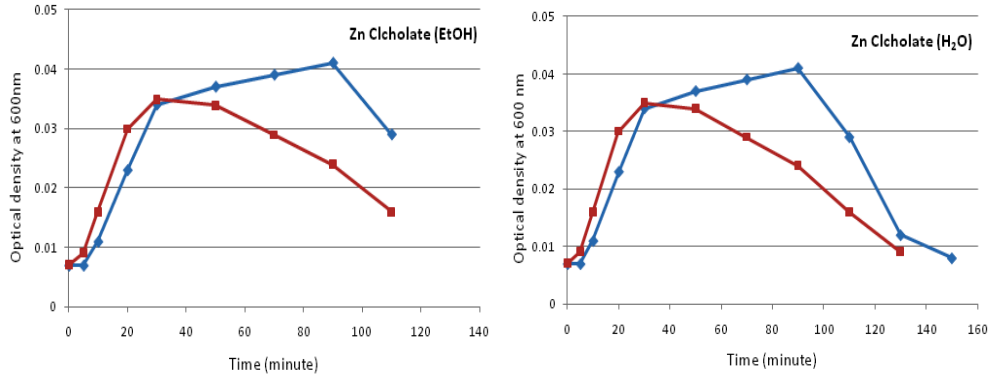

Figure 12: The growth curve of bacteria *P. aeroginosa* (♦) and *S. aureus* (■) at Brain Heart broth in 8.2×10^{-5} M zinc chloride cholate (prepared in EtOH and H_2O).

Conclusions

It was found the most common pathogen isolated from burn and wound infections is *P. aeruginosa*, 38.6%, while *S. aureus* represent 61.4%, and all isolates of *P. aeruginosa* and *S. aureus* are multidrug Resistance to more than one class of antibiotics. This is the first time to report the preparation of monosubstituted metal cholate, and there is no previous work on their antibacterial activity against *P. aeruginosa* and *S. aureus*. The metal cholates have the ability to inhibit growth of these bacteria, and in certain concentrations, the antibacterial activity of these metal cholates increases directly proportional with increase in their concentrations.

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