Antibiofilm and Antibacterial Activity of Sodium Deoxycholate on *P. aeruginosa* and *Staph. aureus*

Suzan Saadi Hussain
College of science, Al-Mustansiriya University
m12_moon@yahoo.com

Abstract

Background: Burn and wound infections are one of the most important problem in Iraq because my country's vulnerability to terrorism, and the repeated explosions in most areas of it, most causing of these infections were fungi, viruses and both Gram negative bacteria and positive bacteria as *Pseudomonas aeruginosa* and *Staphylococcus aureus* which form biofilm that causing multiple resistance drug for this bacteria.

Methods: Forty clinical samples (24 wounds and 16 burns) were collected as wound and burn swabs from patients suffering from different types of wounds and burns patients who attending Medical City at a period between (November 2015 to January 2016).

Culturing all swabs on MacConkey agar and Blood agar also used differential selective media as (Pseudomonas agar for *Pseudomonas aeruginosa* and Mannitol salt agar for *Staphylococcus aureus*). As well as used Vitek system for diagnosis and identificated bacteria.

Results : This is the first study on sodium deoxycholate as antibiofilm and antibacterial activity against the bacteria (*P. aeruginosa* and *S. aureus*) , where the results of the study showed sodium deoxycholate inhibits *P. aeruginosa* in both concentration 10^{-2} and 0.5×10^{-3} M. ,whilst its inhibit growth of *S. aureus* at a concentration higher than 1.0×10^{-4} M.

Conclude: *Staph. aureus* is more sensitive to sodium deoxycholate than *P. aeruginosa*

Keywords: Antibacterial activity, sodium deoxycholate ,*P. aerugenosa*, *S. aureus*

Introduction

Burns can be defined as the damage of the skin by the variety of the non-mechanical sources as heat ; chemicals ; electricity and nuclear radiation or sunlight , as well as the burn severity are determined according of degree of the tissue damage also the size of area affected (Church *etal*., 2006) ,bacteria and fungi are common pathogens of burns and wound infections which form biofilms on them within 48-72 hours of injury, biofilm is microbial city that attached to biotic and a biotic surface which covering by exopolysaccharide matrix (Toole *etal.*,2000).

Persistent infection and resistant these bacteria to antibiotics correlated with biofilm form (Simon and Robertson ,2008), as form by *P. aerugenosa* and *S. aureus* which constituted most common bacterial infected of burn and wounds (Saaiq, *et al.*, 2015), so *P. aeruginosa* is important bacterium that responsible for the most severe nosocomial infection ; life-threatening infections in the immune compromised patients as well as the chronic infection in the cystic fibrosis patients , that has many important virulence, which are depending on many numbers of the cell-associated and extracellular factors , these virulence factors are playing important pathological role in colonization ; survival of the bacteria as well as invasion tissues (Williams *et al*., 2000) , also *P. aeruginosa* within biofilm shows more resistant to the host immune system clearance and stiff environmental factors (Quinm, 2003).

Staphylococci.aureus carries a riche of pathogenic determinants that promote colonization tissue ; damage in tissue and the distant diseases , so these bacteria have ability surviving inside host cells and invade in vitro a variety of the nonprofessional phagocytes such as fibroblasts ; osteoblasts ; endothelial and epithelial cells (Jevon *etal.*, 1999) , also *S. aureus* may escaping or persist defenses of the host and antibacterial agents (Yan *et al.*, 2013).

Deoxycholic acid (DCA or ATX-101), is one of secondary of bile acids, that are metabolic by the product of the intestinal bacteria , these two primary bile acids are secreting by the liver.

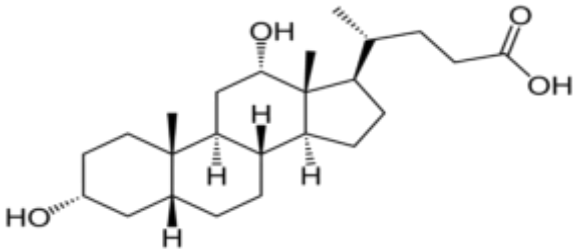


Figure (1): Deoxycholic acid

Metabolized of chenosodium deoxycholate by bacteria to the secondary bile acid lithocholic acid also metabolize of cholic acid to the deoxycholic acid, as well as secondary bile acids , as ursosodium deoxycholate .sodium deoxycholate is soluble in alcohol and acetic acid , also when pure, it comes in a white to off-white crystalline powder form, Sodium deoxycholate has been used since its discovery in various fields of human medicine, so in human body sodium deoxycholate is used in emulsification of fats for the absorption in intestine(Streuli, *et al.*, 1992), whilst outside body can be used in the basis of experimental of the cholagogues , also to prevent and dissolve gallstones , so it can be used it as the mild detergent to isolation the membrane associated proteins(Neugebauer, 1990).

Deoxycholic acid is endogenous secondary bile acid which help to emulsify and solubilize fat, as well as its breakdown and absorption within the gut(Rotunda , 2004) ,so the sodium salt of the deoxycholic acid, is usually used as the biological detergent for lysing cells and solubilise the cellular and components of the membrane (Duncan and Rotunda, 2011), as well as the sodium deoxycholate can be mixed with the phosphatidylcholine, is used in mesotherapy injection for producing lipolysis, and it used as alternative for surgical excision in the treatment of the lipomas (Jin-Baek Kim *etal.*, 2000) , so deoxycholates and bile acid derivatives in general are actively being studied as structures for incorporation in nanotechnology (Tamminen and Kolehmainen, 2001).

As well as they may be found application in the microlithography as the components of photoresistant , In the United States the deoxycholic acid is named under the trade name : Kybella®, also its approved by drug Administration to reduced severe to moderate fat below the chin and used in food (Xin *etal.*, 2002), also if injected it in the submental fat, the sodium deoxycholate helping to destroy the fat cell , but no study about sodium deoxycholate as Antibacterial activity and antibiofilm against pathogenic bacteria.

Sodium Deoxycholate is soluble in water , ionic detergent that used for cell lysis , also used in supplement culture media and most effective reagent for removing LPS from immobilized poly mixin B, remove endotoxin , prevent nonspecific binding in affinity chromatography, primary of bile acids are chenodeoxycholic acid and cholic acid that synthesized from the cholesterol in liver (Okoli *et al.*, 2007) , in liver of metabolism, in formation of the conjugated bile salts via attachment of the glycine or taurine to side chain of various of bile acid , also concentrated of bile salts and stored in the gall bladder until the activated of entero-hepatic circulation (Ridlon *etal.*, 2006) , Bile acids are involving in the regulation of glucose ; lipid also energy metabolism, so known to mediate drug metabolism and detoxification, indicating a central role for bile acids in maintaining gut health(Li *etal.*,2014) .

Materials and methods

Solutions:

(a) Preparation of stock of 0.4 M sodium hydxide:

(b) Sodium hydroxide (0.4 g, 10 mmole) was dissolved in 100 mL distilled water.

(c) Preparation of stock 10^{-2} M sodium deoxycholate:
Sodium deoxycholate (0.392 g, 10 mmole) was dissolved in 20 mL from 0.40 M of NaOH solution, warmed solution to compete dissolution, filtered and completed in volumetric flask to 25.0 mL.

(d) Preparation of working sodium deoxycholate solutions:
Prepare of following sodium deoxycholate (SD) working solutions in 25.0 mL volumetric flask:

Resulting Working SD solutions	**Stock 10^{-2} M SD (mL)**	**Volume of Working 10^{-3} M SD**	**Distilled water (mL)**
Stock 10^{-2} M	2.50	-	22.50
0.5×10^{-3} M	-	12.5 mL	12.50
1.0×10^{-4} M	-	2.5 mL	22.50
0.5×10^{-4} M	-	0.5 Ml	24.50
0.1×10^{-4} M	-	100 µL	24.90
1.0×10^{-5} M	-	10 µL	24.99

Clinical Samples Collections:

Forty samples were collected from burn and wound patients who attending Medical City at a period between (November 2015 to January 2016) , total 24 samples obtained from burns and 16 sample from wound patients.

Sample culturing

All specimens were cultured on MacConkey agar, blood agar, as well as selective media (For *P. aeruginosa* used the pseudomonas agar and Mannitol salt agar for *S. aureus).* and incubated aerobically for 24 hour at 37°C, then Identification of *P. aeruginosa* by Using API 20E System and VITEK system.

Method

Determined the Antimicrobial Activity of sodium deoxycholate using Wells Diffusion methods.

Antimicrobial activities of sodium deoxycholate determined against *P. aeruginosa* and *S. aureus* by modifying Kirby Bauer disc Diffusion Method.

In brief, prepared lawn of bacterial culture by spreading bacterial suspension (100 µL), each test organism having (10^6 CFU/mL) on the nutrient agar and allowed absorption by stand for 10-15 minute , then by used head of sterile micropipette tips, punched 8 mm size wells into the agar , and loading all the wells with 100 µL of different concentrations (200, 400, 800, 1600 and 3200) µg/mL of sodium deoxycholate suspension , then incubated for 24 hour at 37°C and measured the size of inhibition zone (I.Z) (Azam*et al*., 2012).

Quantitative biofilm by tissue culture plate assay (TCP)

By spectrophotometric method determine the adhesion and form biofilm (Stepanovic *et al*., 2003) as the following:

1. prepared the working cultures by inoculation this bacterial isolate on the columbia agar, which supplemented with 5% blood after then incubated aerobically for 24 hr. at 35°C.
2. Preparation the standardized bacterial suspension (0.5 McFarland turbid standard reaching 10^8 CFU/ml), then inoculated these suspensions into the Brain Heart Infusion broth (with and without) sodium deoxycholate .
4. Added 200 µL of these cultures (with and without sodium deoxycholate) to all wells of polystyrene Microtiter plate, then incubated for 18 hr. at 37°C
5. after then aspirated the content of each well and washed with distilled water for 3-4 times.
6. Added 200 µL of methanol per each well to fixed the remaining attached bacteria for 15 minutes.
7. After then empted each well of the plate and left to air dry.
8. By 160 µL of crystal violet (0.25 %) staining each well for 5 min, then by tap water excess staining and dried the plate.
9. Resolubilized by 160 µL (33 %) of glacial acetic acid per each well.
10. Finally, measured optical density (OD) for each well by Elisa microplate reader Reader (at 570 nm).

The biofilm degree was calculated as follows:

Biofilm degree = Mean OD570 of testing bacteria- Mean OD570 of control.

And classified the isolates according to biofilm production by Christensen *et al*., (1985) as the following: non-producer less than 0.120 ; moderate producer between 0.125-0.250 whilst strong producer more than 0.240.

Data Analysis

To comparison between samples, data was analysed by one-way analysis of the variance (ANOVA). In all cases statistically significant considered p values < 0.05.

Results and Discussion

Table (1): Number and percentage of *Staphylococcus aureus and P. aeruginosa* from burn and wound infections.

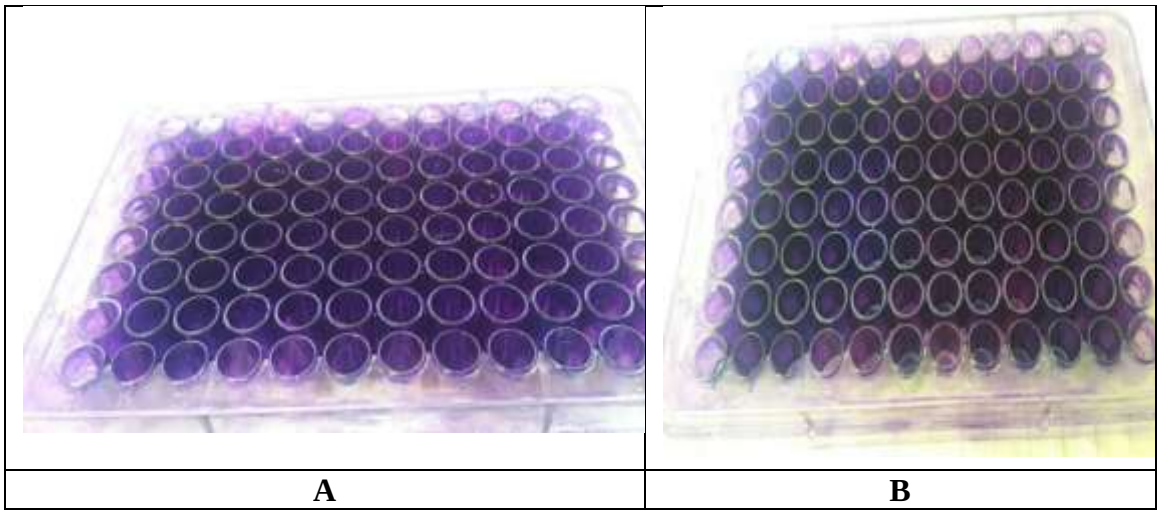
Type of infections	No. of samples (positive growth)	No. of *S.aureus* (%)	No. of *P.aeruginosa* (%)	No.of others(%)
Burn infections	24(60)	8(20)	12(30)	4(10)
Wound infections	16(40)	7(17.5)	8(20)	1(2.5)
Total	40(100)	15(37.5)	20(50)	5(12.5)

from wound and burn infections , isolated 40 bacterial isolates , *P.aeruginosa* were most commonly found in both burn and wound infections (30 , 20) % respectively ,whilst *S.aureus* (20 , 17.5) % respectively , in the same time *P.aeruginosa* were isolated from Burn infections (30%) more them from wound infections (20%), also from Burn infections isolate *S.aureus* are more frequent(20%)than isolate from wound infections (17.5%) (Table 1).

Table (2) :Antimicrobial activities of the sodium deoxycholate on *P. aeruginosa* and *Staph. aureus.*

Concentration of SD solutions (M)	Mean diameter of Inhibition zone (mm)	
	P. aeruginosa	***S. aureus***
10^{-2}	6.0	10.0
0.5×10^{-3}	4.0	8.0
1.0×10^{-4}	2.0	6.0
0.5×10^{-4}	0.0	4.0
0.1×10^{-4}	0.0	2.0
1.0×10^{-5}	0.0	0.0

Results in table -2 showed that antibacterial activity of sodium deoxycholate against *P. aeruginosa* is limited only in concentration of 10^{-2}, 0.5×10^{-3} M. That's mean this bacteria can resist the antibacterial effect of sodium deoxycholate up to a concentration of 0.5×10^{-3} M. ,also deoxycholate acids showed has ability inhibiting growth of *Staph. aureus* at concentration higher than 1.0×10^{-4} M. as well as *S. aureus* is more sensitive to sodium deoxycholate than *P. aeruginosa*, No research and previous studies about sodium deoxycholate as antibacterial because this study is the first study in this area.



A **B**

Figure.1: Ability of bacteria (*P. aeruginosa* and *Staph. aureus*) to form biofilm on microtiterplate wells (A: microtiter plate (M.t.p) treated with Sodium deoxycholate , B: microtiter plate (M.t.p) without treated Deoxycholat acid.

Biofilm formation assays were performed using (0.5×10^{-3} M) concentrations of sodium deoxycholate (Figure 1).Also these results showed out of 20 ,15 isolates to *P. aeruginosa* and *Staph. aureus* 18,13 isolate (52, 37.5)% respectively were biofilm producers. as 18,10 isolate (52,28.5) % strong produced biofilm in both bacteria ,also 1, 3 isolate (3,8)% as moderate biofilm , whilst 1,2 isolate (3 ,6)% as non produced biofilm in both *P. aeruginosa* and *Staph. aureus* isolate, but in untreated with Sodium deoxycholate, the produced biofilm was decreased as 16, 9 isolate (46 , 26)% strong produced biofilm in both bacteria ,also 2,4 isolate (6,11.5)% as moderate biofilm , whilst 2 isolate (6)% as non produced biofilm in both *P. aeruginosa* and *Staph. aureus* isolate.

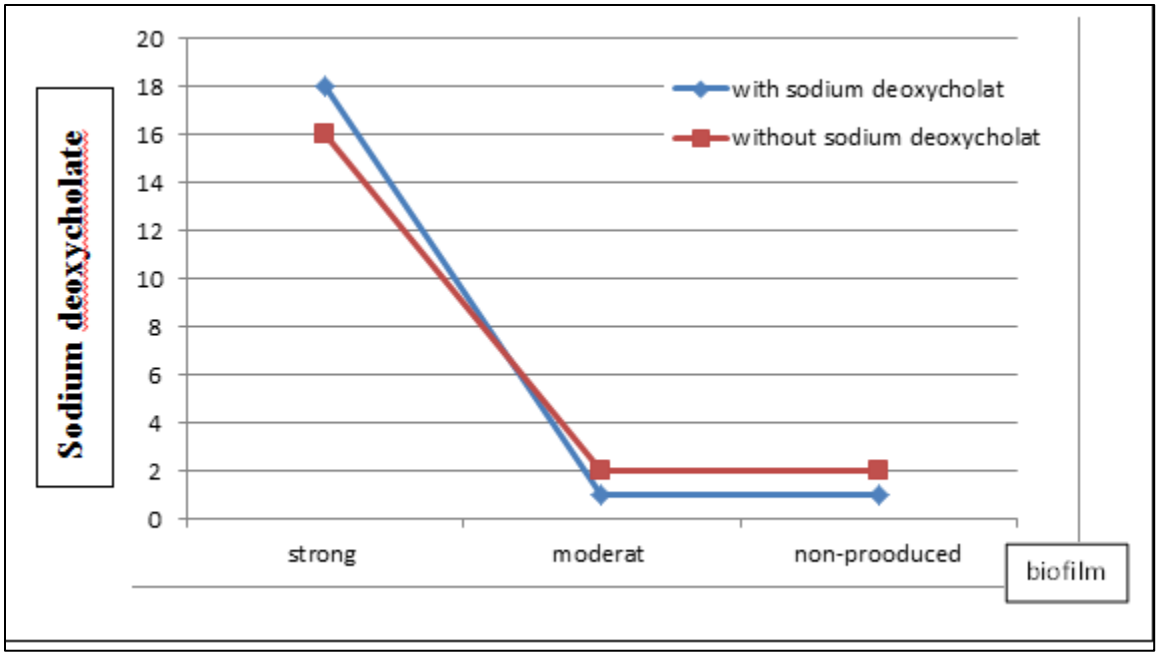


Figure (2): Effect of Sodium deoxycholate on biofilm production by microtiterplate assay (M.t.p) among strong and moderate and non -biofilm produced all isolates of the *Pseudomonas aeruginosa*. (SP:-Strong biofilm producer, M:- Moderate producer, NP:- Non produce biofilm).

High differences observed in the readings of optical density at 570 nm with treatment and non- treatment of Sodium deoxycholate among isolates of *P. aeruginosa* and *Staph. aureus* (Fig. 2).

Our results appeared with treat sodium deoxycholate 18 isolates of *Pseudomonas aeruginosa* (52 %) were produced biofilm. Of these, 16 (46%) isolates were produced biofilm strongly while 2 (6%) isolates as both moderate and non -produced biofilm, On the other hand, without treated Sodium deoxycholate, 18 (51.5 %) were strong biofilm producers ,whilst only 1 (3 %) were both moderate and non- produced biofilm, also there is statistically significant differences in the reading of optical density at 570 nm with the presence and absence of Sodium deoxycholate among isolates of *Pseudomonas aeruginosa* (Fig. 2).

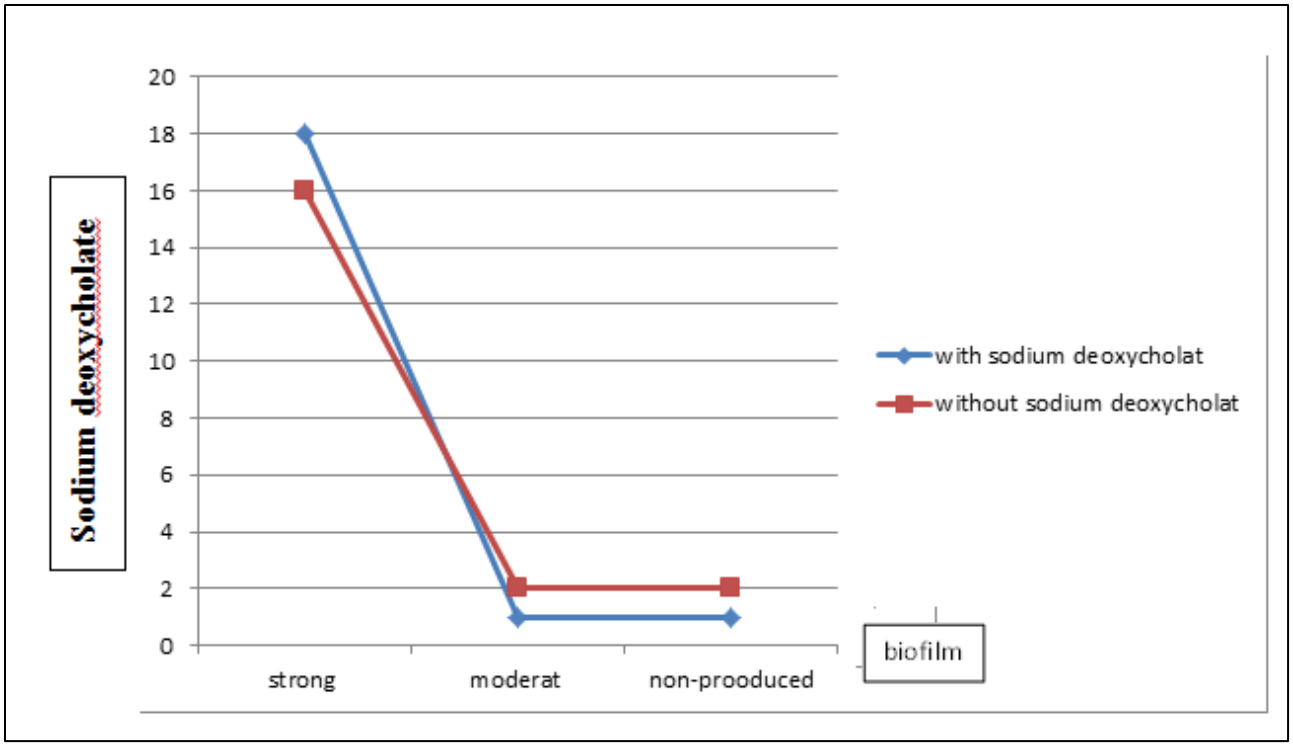


Figure (3): Effect of Sodium deoxycholate on biofilm production by microtiterplate assay (M.t.p) among strong and moderate and non -biofilm produced of *staph.aureus* (SP:-Strong biofilm producer, M:- Moderate producer, NP:- Non produce biofilm).

Whilst the results appeared without treated Sodium deoxycholate, 13 isolates of *staph.aureus*, (37.5%) were produced biofilm. Of these, 10 (28.5 %) isolates were produced biofilm strongly whilst 3,2 (8,6)% respectively moderate and non-produced biofilm .

On the other hand, in treated by Sodium deoxycholate, 13 (37.5 %) out of 15 isolates were produced biofilm of them 9 (26 %) as strong biofilm produced and 4(11.5 %) as moderate biofilm produced , but 2(6%)non produced biofilm .Also, there is statistically significant differences in the reading of optical density at 570 nm with the presence and absence of Sodium deoxycholate among isolates of *staph.aureus* .

Discussion

Results of this study showed that *S. aureus* and *P. aeruginosa* isolates which isolated from infected burn and wound patients were produce biofilm at strong and moderate degrees this may be due the fact that debridement and irrigation of an infected burn and wound removes most of the bacteria (any remaining bacteria may replicate and produce a new biofilm)as well as some antibiotics are effective in preventing biofilm development but not in disrupting an already established one(Hammond *etal.*, 2011).

Pseudomonas aeruginosa and *Staph. aureus* that produced strong biofilm (84%) and highly resistant to many antibiotics(Rewatkar,2013).

This result is close to a study by Moteeb,(2008) who was indicated that *P. aeruginosa* isolates (87.5%) had ability to form biofilm whilst this rate decreased to(66%) and (68.7%) in a study by Salah (2012) and Mahmmod (2013), these differences due to the fact that the bacterial isolates differ in their ability to develop biofilm.

Effect of sodium deoxycholate as inhibitor biofilm (antibiofilm)that produced by *P. aeruginosa* and *staph.aureus* isolates , that's mean the biofilm form by these bacteria were decreasing when treated with sodium deoxycholate **,** as well as Sodium deoxycholate may benefit in healthcare and hospitals facilities as biofilm control agents for preventing contamination in the medical devices, as well as in the 1970s and 1980s , the clinical studies conducted confirm the expectation, that sodium deoxycholate was involved in natural healing processes of the local *inflammations*(Vlček ,1972 and Chyle , 1988).

Bile and bile acid constituents have been associated with a variety of physiological effects on gut/ microbe interactions, including the ability to modulate gut bacteria implicated in irritable bowel syndrome associated with high fat diets(Devkota *etal.,* 2012), so D'mello and Yotis appearance in his study only 0.1% of deoxycholate, may be inhibition form flagellum and also responsible for decreased spreading of these bacteria (D'mello and Yotis 1987).

Conclusion

The results showed that for burn and wound infections that antibacterial activity of the obtained sodium deoxycholat to *P. aeruginosa* and *Staphylococcus aureus* estimated by using Well Diffusion method , so *Staphylococcus aureus* can resist the antibacterial effect of sodium deoxycholate up to a concentration of 0.5×10^{-3} M, while *P. aeruginosa* is limited only to the concentration of 10^{-2} and 0.5×10^{-3} M , as well as in this study conclude sodium deoxycholate can be used for the treatment of ailments caused by infectious agents and as antibacterial and antibiofilm against both *P. aeruginosa* and *Staphylococcus aureus* .

Recommendations

1. Preparation of topical ointments and lotions containing sodium deoxycholate to treat wound and burn infections especially those caused by *Staphylococcus aureus* and *P. aeruginosa*
2. Further works on the biological effect of sodium deoxycholate to other microorganisms such as another pathogenic bacteria, viruses and fungi.
3. Study the effect of Sodium deoxycholate as antibacterial and antibiofilm against other pathogenic bacteria .
4. The *in vivo* and *in vitro* biological effect of sodium deoxycholate on human tissue need further investigations.

Refernces

Al-Habib, H.M.; Al-Gerir, A. Z. and hamdoon , A. M. (2011). Profile of *pseudomonas aeruginosa* In burn infection and their antibiogram Study. *Ann. Coll. Med. Mosul*, 37(182).

Alharbi, **S. A. and Zayed, M. E**. (2014).Antibacterial susceptibility of bacteria isolated from burns and wounds of cancer patients. *Journal of Saudi Chemical Society*, 18: 3-11.

Anderson and Cindy (2013). Great Adventures in the Microbiology laboratory Pearson. pp:175-176.

Anderson, G.G.; and O'Toole G.A.(2008). Innate and induced resistance mechanisms of bacterial biofilm. *Curr. Top. Microbiol. Immunol.,* 322:85–105.

Atlas, R.M.; Brown,A.E. and Parks, L.C . (1995) . Laboratory Manual of Experimtal Microbiology.1st ed.Mosby, St.Louis, U.S.A.

Azam, A.; Ahmed, A.; Oves, M.; Khan, M. and Memic, A. (2012). Size- dependent antimicrobial propertiesofCuO nanoparticles against Gram-positiveand-negative bacterial strains.*International journal of nanomedicine,* 7:3527-3535.

Branski ,L., K. ; Al-Mousawi , A. ; Rivero, H. ; Jeschke,M.G. ;Arthur, P. S.and David, N. H. (2009). Emerging Infections in Burns. Surg infect (larchmt) .(5): 389–397.

Brooks, F.G.;Carroll, K.C.;Butel, S.J. and Morse, A.S.(2007). Medical Microbiology, Editors, Jawetz, E., Melnic, J. L. and Adelberg, E. A. 24th ed. McGraw-Hill. New York.

Church, D.; Elsayed, S.; Reid, O.; Winston, B. and Lindsay, R.(2006). Burn wound Infections. *Clin. Microbiol. Rev.,* 19(2):403-434.

Duncan D., Rotunda A. M (2011). "Injectable therapies for localized fat loss: state of the art". Clin. Plast. Surg., 38 (3): 489–501.

D'mello ,A. and yotis ,W. .(1987).AnthonyThe Action of Sodium Deoxycholate on Escherichia coli. American Society for Microbiology, 53.(8):1944- 1946

Gallagher JJ. Williams-Bouyer N. Villarreal C.(2007). Treatment of infection in burns. In: Herndon DN, editor. Total Burn Care. 3rd. Philadelphia: WB Saunders; pp. 136–176.

Harris,L.G.;Foster,S.J.andRichards,R.G.(2002). An introduction to *Staphylococcus aureus* and techniques for identifying and quantifying *Staphylococcus aureus*adhesins in relation to adhesion to biomaterials: *Review. European Cells and Materials*., 4:39-60.

Holt, J. G.; Krieg, N. R.; Sneath, P. H.; Staley, J. T. and Williams, S. T. (1994). Bergey's manual of determinative bacteriology. 9th ed. Williams, and Wilkins, USA.

Hospenthal, D.R. (2014). Burn wound infections. emedicinehttp.emedicine. Medscape com ,article, 213595, overview.

Japoni, A., Farshad, S. and Alborzi, A. (2009.) *Pseudomonasaeruginosa*: Burn Infection,Treatment and Antibacterial Resistance.*Iranian Red. Crescent Med. J*. 11: 244-253.

Jevon, M.C. and Guo, B. (1999).Mechanisms of Internalization of *Staphylococcusaureus* by Cultured Human Osteoblasts.*Infection and Immunity.*, 67(5): 2677-2681.

Neugebauer, J. M.Detergents.(1990).An Overview, in: M.P. Deutscher, Guide to Protein Purification (Methods in Enzymology Vol. 182), Academic Press, San Diego .

Quinm, J.P.(2003). *Pseudomonasaeruginosa* infection in intensive care unit. Seminar in respiratory and critical care medicine. 24(1): 61-68.

Rotunda. (2004). Further in vitro and in vivo studies subsequently confirmed that, when injected**.**

Rewatkar,A. R and B. J. (2013). Staphylococcus aureus and Pseudomonas aeruginosa-Biofilm formation Methods .IOSR Journal of Pharmacy and Biological Sciences (IOSR-JPBS) 8,(5): 36-40.

Saaiq,M. ; Ahmad, S, and Zaib,M.S.(2015).Burn Wound Infections and Antibiotic Susceptibility Patterns at Pakistan Institute of Medical Sciences, Islamabad, Pakistan.World J Plast Surg.; 4(1): 9–15

Simon A.L., Robertson GT.(2008).Bacterial and fungal biofilm infections. Annual review of Medicine. 59:415-428.

Stepanovic S, Vulcovic D, Hola V, Bonaventura GD, Djukic S, Cirkovic I, Ruzicka F.(2007). Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by *staphylococci*. Acta Path Micro Im A. , 115: 891-899. 10.

Streuli, H. (1992). SLMB -SchweizerLebensmittelbuch, chapter 58, 4/3.

Todar, K. (2008). *Pseudomonasaeruginosa*. Todar's Online. Textbook of Bacteriology. University of WisconsinMadison-Department of Bacteriology.

Toole, G.O. ; Kaplan, H.B. and Kolter, R.(2000). Biofilm formation as microbial development. Annual review of Microbiology; 54:49-79.

Williams, P.; Camara, M.; Hardman, A.; Swift, S.; Milton, D.; Hope, V. J.; Winzer, K.; Middleton, B.; Pritchard, D. I. and Bycroft, B. W. (2000). Quorum sensing and the population dependent control of virulence. *Philos. Trans. R. Soc. London. B. Biol. Sci*., 355: 667- 680.

Yan, M. ;Pamp, S.J.; Fukuyma, P.H. (2013). Nasal micro environment and inter specific interactions influence nasal microbiota complexity and *S.aureus* carriage. *Cell Host microbe*, 14(6):631-640.

Yilmaz, M.; Sunar, H. and Merta, A. (2013). Community acquired left-Sided *Pseudomonasaeruginosa*endocarditis in apatient with intravenous drug use. *Springer Link*, 41(1):243-245.

Devkota, S.; Wang, Y.W.;Musch,M.W ;Leone, V.; Fehlner-Peach, H. and Nadimpalli,A.(2012). Dietary-fat-induced taurocholic acid promotes pathobiont expansion and colitis in Il10(-/-) mice. Nature.;487(7405):104