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In Vitro Anti-bacterial Effect of Ox-bile Against Some Important Gram-positive and Gram-negative Bacteria

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Abstract

Background: Ox-bile has been recommended as a natural remedy with several therapeutic potentials in traditional Persian medicine. It has had efficacy against inflammation and infection according to traditional medicine. Evidence revealed that bile disrupts bacterial cell membrane and degrades DNA structure, so it has anti-bacterial effects. However, there is no evidence of any approved medication composed of ox-bile in Iran.
Objectives: The aim of this study was to evaluate the in vitro anti-bacterial effects of ox-bile.
Methods: Ox-bile was obtained under aseptic conditions and sterilized with a 0.22 μm syringe filter, then examined for their sterility status through culture on different media. Following incubation under aerobic cultures for 48 hours and the anaerobic cultures for one week. Two different kinds of antimicrobial susceptibility tests, including well-diffusion method and serial dilution test were employed to characterize the inhibitory effect of ox-bile extraction on *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Propionibacterium acnes*.
Results: Based on our study, no anti-bacterial effect of ox-bile was observed against selected gram-positive and gram-negative bacteria.
Conclusions: No in-vitro evidence of inhibitory effect was observed against studied gram-positive and gram-negative bacteria. Though further evaluation of the anti-bacterial effects of different preparations of ox-bile seems is still required.

Keywords: Anti-bacterial Effect, Gram-positive Bacteria, Gram-negative Bacteria, Ox-bile

1. Background

Bile is the exocrine secretion of the liver, which is a product of cholesterol metabolism (1-3). It is stored in the gallbladder and enters into the duodenum via the common bile duct (1, 2). Its major components include various amino acids such as glycine and taurine, as well as bile acids, cholesterol, phospholipids and bile pigments, biliverdin, and bilirubin (4, 5). Bile contains proteins, especially mucin glycoproteins, and a wide range of mineral salts ions, and anti-oxidants (4, 6). According to this structure, bile has a wide range of functions throughout the body (7).

Evidence suggest that anti-bacterial effects could be a potential effect of ox-bile (2, 5). In this regard, some studies have mentioned the bactericidal role of bile salts (8, 9), and its bacteriostatic function has been explained (10, 11). Bile salts provoke intestinal cell proliferation and prevent apoptosis, decrease mucosal damage, and improve survival after intestinal injury (1, 12). Bile acid may protect the intestine from injury or infection and help with compensation (12). It inhibits bacterial overgrowth and endotoxin absorption too (13). Concomitantly, bile has anti-inflammatory, immunomodulatory, and anti-oxidant effects. Indeed, it offers protection against oxidative stress and detoxifies a wide range of free radicals to control bacterial infections (14-16). The balance of bile acids and microbiota is also important for human health since dysbiosis and secondary bile acid deficiency can cause intestinal inflammation to progress (17).

Zootherapy is an important alternative therapy in

some countries (18). This is because three million years ago animal bile's was used in traditional Chinese medicine for the treatment of different infections and inflammations such as ocular infections and skin diseases, suppurative otitis media, sinusitis, rhinitis, tonsillitis, gingivitis, laryngitis as well as pharyngitis, bronchitis, pneumonia, cystitis, and hepatitis (19, 20).

In previous studies of traditional Persian medicine, several therapeutic indications have been mentioned for bile. For example, a study reported topical application of bile for the treatment of ear infections, purulent ulcer, and smallpox (21). In addition, some studies demonstrated that gram-positive and gram-negative bacteria have different rates of resistance or sensitivity to bile, gram-positive bacteria seem to be more sensitive to bile (2). To the best of our knowledge, although numerous uses of animal bile, especially ox-bile (bile of caw) were reported, there is no evidence of approved ox-bile related drugs in Iran.

2. Objectives

The aim of this study was to evaluate the anti-bacterial effects of ox-bile on some clinically important gram-positive and gram-negative bacteria.

3. Methods

3.1. Ox-bile Preparation

The bovine gallbladder was obtained from Marvdasht slaughterhouse (Marvdasht, Iran). Immediately after slaughtering and evisceration of the male cattle (1.5 - 2 years old), under aseptic conditions, their gallbladder was removed from the liver junction, the bile duct was ligated, and they were transported to the laboratory in plastic bags under cold conditions. In the laboratory, the surface of the gallbladders was sterilized with a hot spatula, their contents were sucked with a 50 mL syringe, collected in a sterile beaker, and then sterilized with a 0.22 μm syringe filter.

3.2. Sterility Status

Ox-bile samples were then examined for their sterility status through culture on different media, including EMB agar (MERCK, Germany), Blood agar (MERCK, Germany), Thioglycolate broth (MERCK, Germany), and TSB broth (MERCK, Germany) following incubation under both aerobic and anaerobic conditions. The aerobic cultures were checked for 48 hours and the anaerobic cultures for one week.

3.3. Minimum Inhibitory Concentration

The well diffusion and micro-broth dilution tests were performed to determine the minimum inhibitory concentration (MIC) of ox-bile against gram-positive and gram-negative bacteria, including *Pseudomonas aeruginosa* (PAO1), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis*, and *Propionibacterium acnes* (ATCC 6919).

Through the well diffusion method, bacterial suspension of each species (equal to 0.5 McFarland turbidity) was cultured on distinct Muller Hinton agar plate wells were made, four different concentrations of ox-bile (3, 1/5, 0/75, 0/375%) according to two folds serial dilution with primary concentration of 6% bile were prepared and 50 μL of each prepared concentration of ox-bile was inoculated in the wells. In each plate, one well was dedicated to penicillin as a positive control. This test was done twice for each examined bacterium.

For the MIC test, the procedure was done based on CLSI guidelines (22). This test was also done twice for each bacterium

Finally, the plates were incubated in the proper atmosphere and temperature given the bacterium they contained. *P. aeruginosa*, *E. coli*, *S. aureus*, and *S. epidermidis* were incubated aerobically, but *P. acnes* was incubated anaerobically.

3.4. Ethical Statement

The study was approved by the Research Ethics Committee of School of Medicine- Shiraz University of Medical Sciences (IR.SUMS.MED.REC.1400.117).

4. Results

After 24 - 48 hours of incubation, plates of well diffusion assay were examined for inhibition zone, no inhibition zone was detected in any of the plates. Microplates were examined for the well with no growth, but no inhibitory effects were detected for any concentrations of ox-bile against the examined bacteria.

5. Discussion

The results of our study revealed that ox-bile did not show any anti-bacterial effect against evaluated gram-positive and gram-negative bacteria, including *P. aeruginosa*, *E. coli*, *S. aureus*, *S. epidermidis*, and *P. acnes* in the in-vitro setting.

Not concurring with our results, previous studies have mentioned anti-bacterial effects of bile and several mechanisms, especially damaging the bacterial cell membranes,

DNA and RNA via oxidative stress mechanisms, resulting in alteration of protein expression and cell wall disruption (23, 24). Also, it has been reported that it affects the lipid bilayer structure of bacterial membranes due to hydrophobicity and the amphipathic nature of the membrane (25, 26). Further, there are reports that indicate the detergent role of bile acids and potential antimicrobial activity (27, 28).

Some evidences showed that ox-bile has an antibacterial effect on gram-positive and gram-negative bacteria as Kandell and Bernstein reported that bile salts could induce damage to the DNA of *E. coli* (9, 29). Yan and Zou mentioned that bile powder has a significant inhibitory effect on *S. aureus* and *E. coli* (30). In another study, the zone of inhibition against *S. aureus* was 28 mm at 100 mg/mL and for *E. coli* it was 29 mm (31). Prieto et al. reported that the MICs of ox-bile for *Salmonella typhimurium* and *Salmonella typhi* were 18% and 12%, respectively (32). Amine et al. explored the mechanism of action of ox-bile on *Salmonella typhimurium* at the molecular level. They revealed that alteration of seq A and its protein changed the phospholipids and fatty acids and enhanced bacterial sensitivity to bile acid (25). In another study, both *E. coli* and *Salmonella enterica* were sensitive to bile due to the alteration of cellular functions (32, 33).

On the other hand, some investigations reported different information and mentioned that *E. coli* is considered to be bile-resistant or *Listeria monocytogenes* strains are inherently bile-resistant (2, 34). These differences in bile tolerance can be related to the concentration of bile, exposure to various pH and temperatures, or the type and structure of bile (35, 36). Other factors include alteration of the cell membrane structure and composition, especially different lipopolysaccharides of the outer membrane, as well as changes in membrane electric charge and hydrophobicity (6). Capacity of cell wall to maintain intracellular homeostasis and altered activity of critical enzymes (2, 37), differences in gene transcription and protein expression are important too (9, 38).

There were some limitations in our study. First, we selected some positive and negative micro-organisms to test their sensitivity against ox-bile in this study. Therefore, it is not possible to generalize these results to all gram positive and negative micro-organisms. In this regard, evaluation of the anti-bacterial efficacy of ox-bile against other micro-organism and fungal agents are necessary. Next, it is possible that the animal race is a factor that can affect the results, so we recommend that this factor will be considered in further studies. Our suggestion for other researchers is to work on different organisms of specific diseases and to evaluate its efficacy on bacteria apart from the bacterial strains examined in this study or detecting other mechanisms for ox-bile such as anti-fungal effects. Another suggestion is to combine ox-bile with herbal medication for evaluating the other possible effects of ox-bile such as enhancing the penetration of drugs to the bacterial structures or their synergistic anti-bacterial effects.

5.1. Conclusions

According to the results of this study, the use of ox-bile to inhibit the growth or kill of the evaluated organisms is not recommended, and the evaluation of the antibacterial utility of ox-bile warrants further research.

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Footnotes

Authors' Contribution: S. S. conceived and designed the evaluation and drafted the manuscript. A. M. J participated in designing the evaluation, performed parts of the statistical analysis and helped to draft the manuscript. Z. S. re-evaluated the clinical data, revised the manuscript and performed the statistical analysis and revised the manuscript. M. H., N. H. S. collected the clinical data, interpreted them and revised the manuscript. M. M. re-analyzed the clinical and statistical data and revised the manuscript. All authors read and approved the final manuscript.

Conflict of Interests: There is no conflicts of interest.

Ethical Approval: The study was approved by the Research Ethics Committee of School of Medicine- Shiraz University of Medical Sciences IR.SUMS.MED.REC.1400.117.

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References

1. Alzawqari M, Moghaddam HN, Kermanshahi H, Raji AR. The effect of desiccated ox bile supplementation on performance, fat digestibility, gut morphology and blood chemistry of broiler chickens fed tallow diets. *J Appl Anim Res*. 2011;**39**(2):169–74. https://doi.org/10.1080/09712119.2011.580999.
2. Begley M, Gahan CG, Hill C. The interaction between bacteria and bile. *FEMS Microbiol Rev*. 2005;**29**(4):625–51. [PubMed ID: 16102595]. https://doi.org/10.1016/j.femsre.2004.09.003.
3. Calamita G, Delporte C. Aquaporins in glandular secretion. *Adv Exp Med Biol*. 2023;**1398**:225–49. [PubMed ID: 36717498]. https://doi.org/10.1007/978-981-19-7415-1_16.

4. Singh N, Bhattacharyya D. Identification of the anti-oxidant components in a two-step solvent extract of bovine bile lipid: Application of reverse phase HPLC, mass spectrometry and fluorimetric assays. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2016;**1019**:83–94. [PubMed ID: 26639449]. https://doi.org/10.1016/j.jchromb.2015.11.020.
5. Faiz M. Chemical composition. *J Wildl Ecolo*. 2022;**6**(4):172–7.
6. Urdaneta V, Casadesus J. Interactions between bacteria and bile salts in the gastrointestinal and hepatobiliary tracts. *Front Med (Lausanne)*. 2017;**4**:163. [PubMed ID: 29043249]. [PubMed Central ID: PMC5632352]. https://doi.org/10.3389/fmed.2017.00163.
7. Chiang JY. Bile acids: Regulation of synthesis. *J Lipid Res*. 2009;**50**(10):1955–66. [PubMed ID: 19346330]. [PubMed Central ID: PMC2739756]. https://doi.org/10.1194/jlr.R900010-JLR200.
8. Dubuisson JF, Vianney A, Hugouvieux-Cotte-Pattat N, Lazzaroni JC. Tol-Pal proteins are critical cell envelope components of Erwinia chrysanthemi affecting cell morphology and virulence. *Microbiol (Reading)*. 2005;**151**(Pt 10):3337–47. [PubMed ID: 16207916]. https://doi.org/10.1099/mic.0.28237-0.
9. Merritt ME, Donaldson JR. Effect of bile salts on the DNA and membrane integrity of enteric bacteria. *J Med Microbiol*. 2009;**58**(Pt 12):1533–41. [PubMed ID: 19762477]. https://doi.org/10.1099/jmm.0.014092-0.
10. Lorenzo-Zuniga V, Bartoli R, Planas R, Hofmann AF, Vinado B, Hagey LR, et al. Oral bile acids reduce bacterial overgrowth, bacterial translocation, and endotoxemia in cirrhotic rats. *Hepatology*. 2003;**37**(3):551–7. [PubMed ID: 12601352]. https://doi.org/10.1053/jhep.2003.50116.
11. Kim H, Rwubuzizi R, Fugaban JII, Holzapfel WH, Todorov SD. Beneficial properties and evaluation of survival in model systems of LAB isolated from oral cavity. *Acta microbiol bulg*. 2023;**39**(1):36–50.
12. Perrone EE, Chen C, Longshore SW, Okezie O, Warner BW, Sun CC, et al. Dietary bile acid supplementation improves intestinal integrity and survival in a murine model. *J Pediatr Surg*. 2010;**45**(6):1256–65. [PubMed ID: 20620329]. [PubMed Central ID: PMC2904360]. https://doi.org/10.1016/j.jpedsurg.2010.02.094.
13. Sheen-Chen SM, Chen HS, Ho HT, Chen WJ, Sheen CC, Eng HL. Effect of bile acid replacement on endotoxin-induced tumor necrosis factor-alpha production in obstructive jaundice. *World J Surg*. 2002;**26**(4):448–50. [PubMed ID: 11910478]. https://doi.org/10.1007/s00268-001-0247-5.
14. Stocker R. Antioxidant activities of bile pigments. *Antioxid redox signal*. 2004;**6**(5):841–9. [PubMed ID: 15345144]. https://doi.org/10.1089/ars.2004.6.841.
15. Vitek L, Ostrow JD. Bilirubin chemistry and metabolism; harmful and protective aspects. *Curr Pharm Des*. 2009;**15**(25):2869–83. [PubMed ID: 19754364]. https://doi.org/10.2174/138161209789058237.
16. Adam AH, Verdegem M, Soliman AA, Zaki M, Khalil RH, Nour AM, et al. Effect of dietary bile acids: Growth performance, immune response, genes expression of fatty acid metabolism, intestinal, and liver morphology of striped catfish (Pangasianodon hypophthalmus). *Aquaculture Reports*. 2023;**29**. https://doi.org/10.1016/j.aqrep.2023.101510.
17. Sinha SR, Haileselassie Y, Nguyen LP, Tropini C, Wang M, Becker LS, et al. Dysbiosis-induced secondary bile acid deficiency promotes intestinal inflammation. *Cell Host Microbe*. 2020;**27**(4):659–670 e5. [PubMed ID: 32101703]. [PubMed Central ID: PMC8172352]. https://doi.org/10.1016/j.chom.2020.01.021.
18. Alves RR, Rosa IL. Why study the use of animal products in traditional medicines? *J Ethnobiol Ethnomed*. 2005;**1**:5. [PubMed ID: 16270931]. [PubMed Central ID: PMC1277085]. https://doi.org/10.1186/1746-4269-1-5.
19. Feng Y, Siu K, Wang N, Ng KM, Tsao SW, Nagamatsu T, et al. Bear bile: Dilemma of traditional medicinal use and animal protection. *J Ethnobiol Ethnomed*. 2009;**5**:2. [PubMed ID: 19138420]. [PubMed Central ID: PMC2630947]. https://doi.org/10.1186/1746-4269-5-2.
20. Romano N, Fischer H, Rubio-Benito MM, Overtuf K, Sinha AK, Kumar V. Different dietary combinations of high/low starch and fat with or without bile acid supplementation on growth, liver histopathology, gene expression and fatty acid composition of largemouth bass, Micropterus salmoides. *Comp Biochem Physiol A Mol Integr Physiol*. 2022;**266**:111157. [PubMed ID: 35093523]. https://doi.org/10.1016/j.cbpa.2022.111157.
21. Exir-e-Azam CH. Tehran, Iran: Tehran University of Medical Sciences. *Ins Islamic Complemen Med*. 2008.
22. Wayne PA. *Clinical and Laboratory Standards Institute (CLSI)*. 28th ed. CLSI document M100-S20; 2018.
23. Kristoffersen SM, Ravnum S, Tourasse NJ, Okstad OA, Kolsto AB, Davies W. Low concentrations of bile salts induce stress responses and reduce motility in Bacillus cereus ATCC 14579 [corrected]. *J Bacteriol*. 2007;**189**(14):5302–13. [PubMed ID: 17496091]. [PubMed Central ID: PMC1951874]. https://doi.org/10.1128/JB.00239-07.
24. Ruiz L, Coute Y, Sanchez B, de Los Reyes-Gavilan CG, Sanchez JC, Margolles A. The cell-envelope proteome of Bifidobacterium longum in an in vitro bile environment. *Microbiol (Reading)*. 2009;**155**(Pt 3):957–67. [PubMed ID: 19246766]. https://doi.org/10.1099/mic.0.024273-0.
25. Amine A, Mouadh M, Sahbani Saloua K, El Alya M, Ahmed L. Effects of ox bile extract on the phospholipids and fatty acids membrane composition of Salmonella enterica serovar typhimurium seqA mutant strain. *Current Chemical Biology*. 2011;**5**(3):189–96.
26. De Boever P, Wouters R, Verschaeve L, Berckmans P, Schoeters G, Verstraete W. Protective effect of the bile salt hydrolase-active Lactobacillus reuteri against bile salt cytotoxicity. *Appl Microbiol Biotechnol*. 2000;**53**(6):709–14. [PubMed ID: 10919331]. https://doi.org/10.1007/s002530000330.
27. Herold BC, Kirkpatrick R, Marcellino D, Travelstead A, Pilipenko V, Krasa H, et al. Bile salts: Natural detergents for the prevention of sexually transmitted diseases. *Antimicrob Agents Chemother*. 1999;**43**(4):745–51. [PubMed ID: 10103175]. [PubMed Central ID: PMC89201]. https://doi.org/10.1128/AAC.43.4.745.
28. Wang DQ, Carey MC. Therapeutic uses of animal biles in traditional chinese medicine: An ethnopharmacological, biophysical chemical and medicinal review. *World J Gastroenterol*. 2014;**20**(29):9952–75. [PubMed ID: 25110425]. [PubMed Central ID: PMC4123376]. https://doi.org/10.3748/wjg.v20.i29.9952.
29. Kandell RL, Bernstein C. Bile salt/acid induction of DNA damage in bacterial and mammalian cells: Implications for colon cancer. *Nutr Cancer*. 1991;**16**(3-4):227–38. [PubMed ID: 1775385]. https://doi.org/10.1080/01635589109514161.
30. Yan HY, Zou CC. Determination of total cholic acid in bear bile powder, porcine gall powder, ox bile powder and chicken bile powder and study on the bacteriostatic action of these bile powder. *Chin J Hospital Pharma*. 2012;**32**(3):175–9.
31. Habila JD, Achika JI, Habila AJ, Abubakar M. *Bile chemical substance from northern nigerian red goat inhibit microbes*. Fuw Trends Sci Technol J; 2016. Available from: http://www.ftstjournal.com/uploads/docs/52%20Article%2053.pdf.
32. Prieto AI, Ramos-Morales F, Casadesus J. Bile-induced DNA damage in Salmonella enterica. *Genetics*. 2004;**168**(4):1787–94. [PubMed ID: 15611156]. [PubMed Central ID: PMC1448704]. https://doi.org/10.1534/genetics.104.031062.
33. Ramos-Morales F, Prieto AI, Beuzon CR, Holden DW, Casadesus J. Role for Salmonella enterica enterobacterial common antigen in bile resistance and virulence. *J Bacteriol*. 2003;**185**(17):5328–32. [PubMed ID: 12923112]. [PubMed Central ID: PMC181002]. https://doi.org/10.1128/JB.185.17.5328-5332.2003.
34. Olier M, Rousseaux S, Piveteau P, Lemaitre JP, Rousset A, Guzzo J. Screening of glutamate decarboxylase activity and bile salt resistance of human asymptomatic carriage, clinical, food, and environmental isolates of Listeria monocytogenes. *Int J Food Microbiol*. 2004;**93**(1):87–99. [PubMed ID: 15135585]. https://doi.org/10.1016/j.ijfoodmicro.2003.10.010.
35. Kurdi P, Kawanishi K, Mizutani K, Yokota A. Mechanism of growth inhibition by free bile acids in lactobacilli and bifidobacteria. *J Bacte-*

riol. 2006;**188**(5):1979–86. [PubMed ID: 16484210]. [PubMed Central ID: PMC1426545]. https://doi.org/10.1128/JB.188.5.1979-1986.2006.

36. Ahmadi A, Khezri A, Norstebo H, Ahmad R. A culture-, amplification-independent, and rapid method for identification of pathogens and antibiotic resistance profile in bovine mastitis milk. *Front Microbiol*. 2022;**13**:1104701. [PubMed ID: 36687564]. [PubMed Central ID: PMC9852903]. https://doi.org/10.3389/fmicb.2022.1104701.
37. Piddock LJ. Multidrug-resistance efflux pumps-not just for resistance. *Nat Rev Microbiol*. 2006;**4**(8):629–36. [PubMed ID: 16845433]. https://doi.org/10.1038/nrmicro1464.
38. Horackova S, Vesela K, Klojdova I, Bercikova M, Plockova M. Bile salt hydrolase activity, growth characteristics and surface properties in Lactobacillus acidophilus. *Eur Food Res Technol*. 2020;**246**:1627–36.