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Antibacterial Activity of Bee Venom Against Multidrug Resistance *Staphylococcus Aureus* From Milk of Cow and Buffaloes

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ABSTRACT

Bacteriological study includes bacteria isolated from Buffaloes milk, and Cow at Wassit Province was done in Technical institute. 120 milk samples were collected randomly from different places in Wassit Province included 60 samples from Buffaloes milk and 60 samples from Cow milk during the period October to December 2018. A bacteriological study was conducted for isolation and identification *S. aureus* by morphological and biochemical tests. The results showed that (20%) of Buffaloes milk and (13.33%) in Cow's milk (13.33 %) of Cow milk contaminated with *S. aureus*. Anti-biogram pattern of *S. aureus* was carried out by using a diffusion method from an antibiotic saturated disk, Results showed more effect in *S. aureus* isolated from Buffaloes milk was COT in efficiency ratio (78.33 %), CLR in efficiency ratio (61 %), SPX in efficiency ratio (51.66 %), and L in efficiency ratio (15 %). Also the results appeared more effective in *S. aureus* isolated from Cow milk was COT in efficiency ratio (56.66 %), CLR in efficiency ratio (35 %), SPX in efficiency ratio (30 %), and L in efficiency ratio (8.33 %), while *S. aureus* appeared resistant in all milk samples from Buffaloes and Cow for antibiotic OX (100%), also MIC to staphylococcal (0.70 - 3.10 μg/ml) While MBC concentration was (0.12-0.101 μg/ml).

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INTRODUCTION

Milk is a good medium for growth of various microorganisms that are responsible for changing taste and smell as well as milk composition. The main cause of milk contamination with bacterial pathogens is the treatment method, the workers hygene and the unhealthy environment. The presence of bacterial pathogens in milk can cause significant health risks to the people because the milk contains high nutritional value and complex chemical composition (Cappuccino and Sherman, 2005; Al-Safar *et al.*, 2018).

When the milk is exposed to different levels of the temperatures, or stored at temperatures ranging from 37C° - 42 C ° noted the presence of the toxins of *S.aureus* in it (Altekruse *et al.*, 1994).

Warming the milk to the normal temperature of the conventional cooking may kill *S.aureus*, but their toxins remain effective, because toxins are more resistant to high heat in food (Balaban and Rasooly, 2000; Bergdoll, 1983). The symptoms of food poisoning in the *S.aureus* include sudden symptoms in digestive system. In addition, the toxins produced by *Staphylococcus* bacteria play vital role in damaged cells of host (Bhatia and Zahoor, 2007; Bonfoh

Table 1: Results of Biochemical tests for the diagnosis *S.aureus* isolates from milk of cow and buffalo

Biochemical Test	Result
Gram stain	+
Growth on Mannitol Salt Agar	+
Catalase test	+
Oxidase test	-
Indole test	-
Methyl Red test	+
Vocus Proscauer test	+
Coagulase test	+
Hemolysis on Blood Agar	Complete hemolysis

Table 2: Types and concentration of antibiotics that usedin antibiotic susceptibility test against *S. aureus* isolates

Antibiotic	Abbreviate Antibiotic and its concentration (micro-gram)
Oxacilin	Ox (5 μgm.)
Lincomycin	L (2 μgm.)
Sparfloxacin	SPX (5 μgm.)
Clarithromycin	CLR (15 μgm.)
Co-Trimoxazol	COT (25 μgm)

Table 3: Antibiotic sensitivity test of *S. aureus* isolate in Buffalo´s Milk and Cow's milk

Number of Samples		Antibiotic type	The Percentage %	
Buffalo´s Milk	Cow's milk		Buffalo´s Milk	Cow's milk
47	34	COT (25)	78.33	56.66
37	21	CLR (15)	61	35
31	18	SPX (5)	51.66	30
9	5	L (2)	15	8.33

et al., 2003).

Many studies have indicated a significant similarity among isolated of *S.aureus* from bovine spongiform encephalitis with species isolated from food poisoning (Choi *et al.*, 2015).

The rate of infection is still in high level, particularly in India, due to increase temperature of climate and humidity (Dingwell *et al.*, 2003).

Infection of the cattle with mastitis led to significant economic losses and also led to a decrease in milk production as well as treatment cost. Where economic losses in England 187 million Euros annually, and in America, 180 million dollars annually (Ekici *et al.*, 2004) Bee Venom is a natural complex material consisting of two major compound are peptides and proteins with active biochemical substance such as melittin, histamine, and dopamine (Han *et al.*, 2013). Phospholipase A and Hyaluronidase in venom play important role in analyze Hyaluronic acid to simple unite, so use the bee venom against gram positive *S.aureus* mention by (Han *et al.*, 2016).

MATERIALS AND METHODS

Samples

120 Milk samples from cow and buffalo at variety place in Wasit province included (60 milk buffalo with 60 milk samples from cow) during period October to December 2018 by using sterile collection containers.

Isolation and identification of *S. aureus*

Isolation of *S.aureus* was adopted (Hegazi *et al.*, 2014). Pepton water medium was used in which 10 ml of the homogeneity milk samples were taken with 90 ml of sterile Pepton water overnight at temperature of laboratory. Then samples inocu-

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lated at the selective medium MSA agar (Himedia /India) at 35C° for 18 hrs. Isolates of *S.aureus* determined based on morphological specifications and biochemical tests. Bacterial smears were carried out of the isolates on clean, free grease glass slides, and then stained by Gram stain, the isolates were Gram positive and arranged in irregular clusters similar to the grape cluster. Biochemical tests were performed based on (Min *et al.*, 2013) as shown after being compared with turbidity of McFarland Table 2.

Antibiotics susceptibility test

Antibiotics susceptibility test of the *S.aureus* was performed in a diffusion method from the antibiotic-saturated disc in the Muller-Hinton Agar (Himedia /India) Lawn method by using five antibiotics discs as shown in (Table 3) were then fixed and incubated at 37 C° for 18-24 hrs (Moroni *et al.*, 2005).

Collect and Purification solution of Bee Venom

Bee venom was collected from Iraqi bees in farm that is located in Wasit by utilising electro-stimulate apparatus inside the hive, that generate pulses in voltage at maximum 27V at 1-2 seconds, all venom produced by bee accumulate on Flat glass then dry by exposure to air for 5-10 min with sharp scalpel then collected by sterile tube and stored at a refrigerator until usage. Prepared solution of Bee Venom (BV) use for the detect activity of bee venom against *S.aureus* dissolved 250 mg from bee venom in 1ml of distilled water (Nelson and Stephen, 2003) then added solution of BV in centrifuge apparatus approximately 10-12min. at 14,000 x g then worked serial dilution for next test according to (Normanno *et al.*, 2007).

Bactericidal assay

Bacteria are collected at absorption A600=0.5 and added to a buffer phosphate solution (PBS) pH = 7.2 at (10cfu/ml) followed by incubation of different serial dilution of bee venom solution with bacterial samples at 25C° for 1800 sec. The subsequence of dilution is then taken and grown on the blood agar (Himedia /India) plates for 18 hrs. At 37C° to calculate the numbers of remaining bacteria according to (Novoslavskij *et al.*, 2018).

Minimum Inhibitory Concentrations (MIC) assay

To determine MIC for BV towards bacteria, method of micro-dilution broth used the concentration of cells adjust to (1x10cell/ml) in phosphate buffer solution at pH = 7.0 for bee venom dissolved in phosphate buffer solution at pH = 6.0 before dilution, then (10 μl) solution of BV is added to (190 μl) of diluted bacteria, and incubate for 18-20 hrs. At 35-37 C° MIC read as the less BV concentration inhibits growth of bacteria, and determines a clear optical by ELISA reader (Human reader HS /Germany) depending on (Payne and Wood, 1974).

Minimum Bactericidal Concentrations (MBC) assay

To determine MBC for BV Versus antibiotic resistant bacteria, depend on following method was adopted by adding 0.1 ml of the minimum inhibitory concentration mixture, which showed no bacterial growth, inoculated in liquid medium brain heart infusion broth (Himedia/India) and incubated for 2 days at 37 C° Thus, the value of the MBC is less concentration of bee venom that requires reduction of 99.9% from the accumulation of live bacteria according to (Presscott *et al.*, 2002).

RESULTS AND DISCUSSION

The results showed that the percentage of positive isolates of the *S.aureus* under study of buffalo milk is (20%), and cow's milk is (13.33%) (Figure 1). Table 1 explains the consequences of biological tests for the diagnosis *S.aureus* isolates from milk of cow and buffalo. Table 2 depicts categories and concentration of antibiotics that were adopted for antibiotic susceptibility test against *S. aureus* isolates.

The isolates appeared at different levels of antibiotics resistance. The highest sensitivity of the antibiotic (COT) was 78.33%, CLR (61%), SPX (51.66%) and (L) 15 %.

All isolates exhibited resistance of OX according to CLSI (Presscott *et al.*, 2002), (Table 3), and (Figures 2 and 3).

The milk is sterile in buffalo and cow that does not suffer from mastitis, but if it is infected with mastitis, it will result in the presence of large numbers of Gram positive bacteria, such as *S.aureus* in milk when it is out of the udder (Singh and Prakash, 2008). Milk is not only a food source, but suitable media to appear staphylococcal bacteria that can cause milk damage, leading to foodborne diseases and the spread of gastrointestinal diseases in hot places (Soomro *et al.*, 2003).

The lack of hygiene of milk collection containers, cleanliness of workers to collect milk, and atypical storage of milk increase the presence of Gram-positive bacteria and Gram-negative bacteria in milk containers (Tambekar and Bhutda, 2006). The percentage of *S.aureus* isolates in buffalo milk was 20% slightly higher than conducted by (Tambekar and Bhutda, 2006; Wikler, 2006) which was 18.18 % and 17.39 % respectively, while the percentage of *S.aureus* isolates in cow's milk was 13.33%, which is almost consistent with (Wayne, 2012) which was 12.8%.

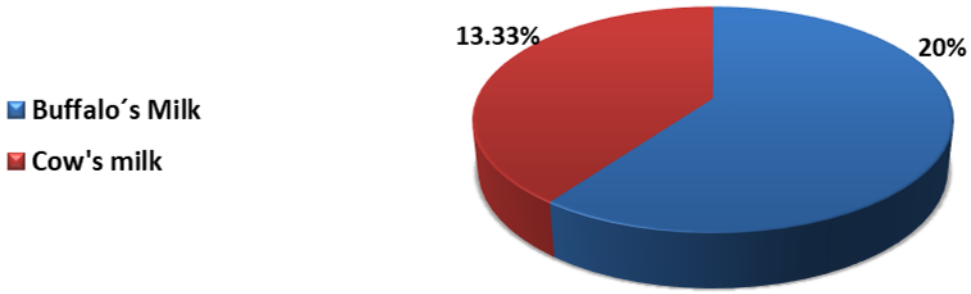

Figure 1: Percentage isolates from cow and buffalo milk's

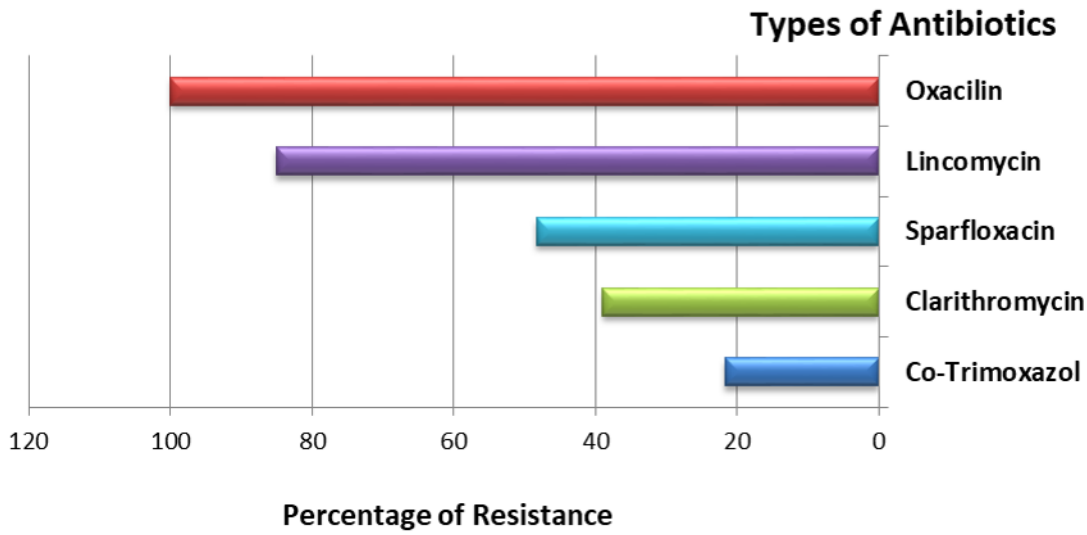

Figure 2: Percentage resistant of *S. aureus* isolated from Buffalo´s Milk and Cow's milk against antibiotics

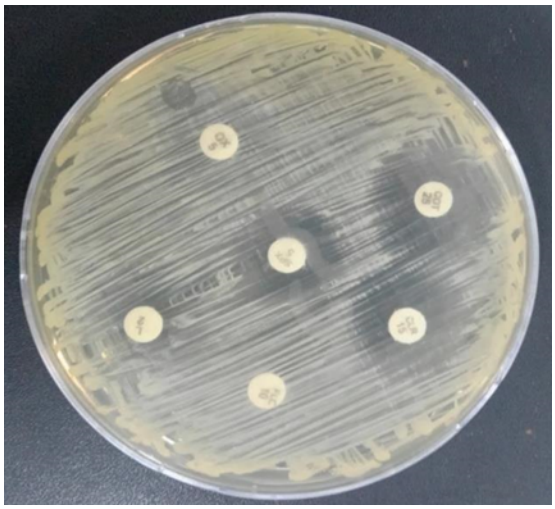

Figure 3: Resistance of *S. aureus* isolate In the three left disc on Muller-Hinton agar

The results of Antibiogram showed sensitivity of the *S.aureus*; it isolates against antibiotics in different levels which was (78.33%) of Co-Trimoxazol, 61% of Clarithromycin, (51.66%) of Sparfloxacin, and 15% of Lincomycin. While all *S.aureus* isolates showed resistance to the antibiotic Oxacilin (100%). The ability of *S.aureus* isolates to resist antibiotics due to the possession of a number of resistance mechanisms, like production of large-spectrum Beta-lactamase enzymes that may occur because of genetic mutations in the sequences genes that are encoded of this protein Beta-lactamase enzymes analyze the Beta-lactam antibiotics and transformed into ineffective compounds in addition to efflux pump mechanism, change metabolic pathways and change in the target location is recognized by the antibiotic also randomly using of antibiotics or the use of insufficient concentrations to kill bacteria, which increase ability of bacteria to resist antibiotics.

The results of bactericidal effect of (BV) concentration treatment for 30 min against MRSA in our study showed bacterial viability decrease in > 90% at BV concentration among (1.25-12.25μg/ml) and no bacteria survived incubation with more than (12.5 μg/ml) concentration of BV that results sufficient agreement with the study of (Jenkins *et al.*, 2011), while disagree with another study (Seo *et al.*, 2012) that was BV reduced bacteria viability at concentration (0.85), also minimum inhibitory concentration to *staphylococcal* bacteria approximately among (0.70 - 3.10 μg/ml). This shows that bee venom contains anti-bacterial molecules targeted MRSA isolates agree with study achieved (Jenkins *et al.*, 2011), and not agree with study (Seo *et al.*, 2012) because concentration that inhibits growth were ranged between (0.17-0.85 μg/ml). BV a natural peptides substances and most important component that plays an active role in its effectiveness is phospholipase and melittin, where the two components work together to kill a wide range of bacteria such as the strains of MRSA (Jenkins *et al.*, 2011), while results showed MBC concentration against MRSA strain were rang between (0.12-0.101 μg/ml) As expected higher (BV) MIC concentration as compared to the MBC concentration were observed in our study that indicates the higher concentration of bee venom kill the strain of MRSA that results opposite to result from the study (Seo *et al.*, 2012).

CONCLUSIONS

S.aureus was a contaminated sample of milk collected from infected animals with mastitis. Percentage of its presence in buffalo milk reached 20% and in the milk of cows was 13.33%. The most effective antibiotic against the bacteria was Co-Trimoxazol in 78.33% of buffalo isolates and 56.66% of bovine isolates. All bacterial isolates were resistant to Oxacilin 100%. All those lead to making the milk that distributed in the market carrier for the disease and, Bee venom can inhibit the growth and survival of MRSA, and thus can be used as a supplement alternative agent at limited concentrations.

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