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Antimicrobial activity of copper sulphate and zinc sulphate on major mastitis causing bacteria in cattle

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Abstract
A study was undertaken on bovine mastitis in Ganderbal district of Kashmir valley so as to identify major mastitis causing pathogens and evaluate the antimicrobial efficacy of copper sulphate and zinc sulphate solutions *in vitro* on pure cultures of major mastitis causing pathogens. The major mastitis causing bacteria isolated from clinical cases were *Staphylococcus* (46.4%), *Streptococcus* (18.4%) *and E.coli* (14.4%). *In-vitro* antimicrobial property of copper sulphate and zinc sulphate was carried out using aqueous solutions of different concentrations of copper sulphate and zinc sulphate (2.5%, 5%, 7.5% and 10%). The antimicrobial property of copper sulphate and zinc sulphate was established and increase in zones of inhibition against each organism were found with increasing concentrations of these compounds. The zones of inhibition were compared with the standard antibiotic enrofloxacin. It is concluded that the use of these solutions as teat dips could prove beneficial in terms of efficacy and economy in comparison to conventional teat dips in prophylaxis of bovine mastitis.

Keywords: Bovine mastitis, copper sulphate, zinc sulphate, major mastitis causing pathogens

Introduction
Mastitis is an infectious disease characterized by parenchymal inflammation of the mammary gland with a range of physical and chemical changes in the milk along with pathological alterations in the glandular tissue (Constable *et al*., 2017) [4]. Different microbes are responsible for such insult, but usually bacteria invade the udder, multiply and produce toxins which are harmful to the mammary gland (Sharma *et al*., 2006) [6]. Mastitis is a primary problem in dairy industry and is one of the most prevalent, challenging diseases of dairy animals, causing heavy economic losses in terms of quality and quantity of milk (Zadoks and Fitzpatrick, 2009) [7]. Approximately 2 billion dollars in USA are lost yearly in the dairy sector due to mastitis and 526 million dollars in India, subclinical mastitis being the primary cause for approximately 70% of such losses (Varshney and Naresh, 2004). Mastitis is a complex disease and the pathogenesis is highly complex because of various microbial pathogens, stress, management and environmental hygiene.

The antimicrobial activities of copper have been studied and application of it in the prevention of bovine mastitis is a novel area of research. The minimum inhibitory concentration of copper (MIC-Cu) as low as 250 ppm has been reported to inhibit the majority of mastitis causing pathogens (Reyes-Jara *et al.*, 2016) [9]. Studies have revealed that copper is able to eliminate variety of bacteria (i.e., *S. aureus, Enterobacter aerogenes*, MRSA, *Pseudomonas aeruginosa* and *E. coli* O157:H7). Antibacterial effect of copper has already been proved for *E. coli* and *S. aureus*, two of the main bacterial species involved in mastitis (Noyce *et al*., 2006, Santo *et al*., 2011) [10, 11]. The antibacterial property of copper is due to the ability of damaging the microbial DNA, altering bacterial protein synthesis and membrane integrity (Warnes *et al*., 2010, Grass *et al*., 2011, Chaturvedi and Henderson, 2014) [13, 12, 3]. Copper may be an attractive alternative to apply as a teat dip to control bovine mastitis in milk farms (Reyes-jara *et al.*, 2016) [9].

Zinc is one of the elements used for keratin production that lines the interior of the teat canal and operates as a plug to entrap bacteria and prevents their entry into the udder (Spain *et al*., 2005) [14]. The antimicrobial effect of zinc has been attributed to reactive oxygen species (like OH, H_2O_2 and O^{2-}) released on the surface which causes bacterial killing. Antimicrobial activity against *E. coli* is reported which is attributed to prolonging of lag phase of growth cycle and generation time of microorganisms (Atmaca *et al.*, 1998) [1]. Zinc acts by production of reactive oxygen species (ROS) because of the semi-conductive properties, the destabilization of microbial membranes upon direct contact of zinc particles to the cell walls and the intrinsic antimicrobial properties of Zn^{2+} ions released by zinc salts in aqueous medium (Pasquet *et al.*, 2014) [156].

Materials and Methods

The antimicrobial activity of Copper Sulphate and Zinc Sulphate was evaluated on pure cultures of *Staphylococcus, Streptococcus and E. coli* by agar well diffusion method. Freshly prepared sterilized Muller Hinton Agar petriplates were seeded with pure cultures of bacteria (*Staphyllococcus, Streptococcus and E. coli*). Ten plates were used for each organism and four wells in each plate were cut in Muller Hinton agar with base of microtip (1 ml). Each well was 5 mm in diameter and the cut out of the agar was removed using a sterile needle. 100 µl of different concentrations of aqueous solutions of Copper Sulphate and Zinc Sulphate (2.5%, 5%, 7.5% and 10%) were poured in each well of single agar plates. Enrofloxacin standard discs were used as a control to ensure the agar medium was able to support the growth of microorganism beyond the zone of inhibition. The Enrofloxacin standard disc was placed and pressed gently onto the same inoculated agar plate by using a sterile forcep. The plates were then incubated at 37 °C for 24 hours. The antibacterial activity was assayed by using measuring scale to read out the diameter of the inhibition zone formed around the wells (NCCLS, 1993).

Statistical Analysis

The data were analyzed using students t-test. All values were expressed as mean ± SE.

Results and Discussion

Study was ascertained by measuring the diameter of the zone of inhibition on the inoculum agar plate. The results of the study showed that all the concentrations of Copper sulphate and zinc sulphate (2.5%, 5%, 7.5% and 10%) had antibacterial activity against *Staphylococcus, Streptococcus* and *E. coli* with dose dependent increase in zone of inhibition with maximum zone at 10% and minimum at 2.5% on agar plates. The average zone of inhibition in 10 wells for each organism with different concentrations of copper sulphate and zinc sulphate against *Staphylococcus, Streptococcus* and *E. coli* is given in table 1, 2 and 3 respectively and represented in Plate 1 and 2. The results indicate that there is statistically significant difference in zones of inhibition at different concentrations of copper sulphate and zinc sulphate proving that copper sulphate and zinc sulphate have an antibacterial activity against *Staphylococcus, Streptococcus* and *E. coli*. *Staphylococcus, Streptococcus* and *E. coli* showed some variability in terms of zones of inhibition in response to different concentrations of copper sulphate. This variability can be due to inoculum size, which also affects the inactivation time of the microorganism (Aspridou and Koutsoumanis, 2015) [2]. Studies have reported that copper surfaces can eliminate bacteria (*S. aureus, Enterobacter aerogenes*, MRSA, *Pseudomonas aeruginosa* and *E. coli* O157:H7) usually causing nosocomial infections (Faundez *et al.*, 2004, Wilks *et al.*, 2005) [5, 16]. Lately, the efficacy of copper has been tested in other microorganisms such as viruses, fungi, and other bacterial pathogens (Noyce *et al.*, 2006, Grass *et al.*, 2011) [10, 12]. The antibacterial activity of zinc sulphate is attributed to prolonging of lag phase of growth cycle and generation time of microorganisms (Atmaca *et al.*, 1998) [1]. In a study conducted by (Sodeberg *et al.*, 1990) [17], gram positive bacteria were most susceptible to zinc ion compared to gram negative which were not inhibited even at the highest concentration (1024 µl/ml). Our results are in agreement with the findings of (Surjawidjaja *et al.*, 2004) [18], who observed inhibitory effect of $ZnSO_4$ against enteric bacteria.

Table 1: Zone of inhibition (mm) shown by Copper Sulphate and Zinc Sulphate against *Staphylococcus* (Mean±SE)

% solution	Copper Sulphate	Zinc Sulphate
2,5	20 ± 1.140^{aB}	10 ± 0.548^{bB}
7.5	22 ± 0.707^{aBC}	12 ± 0.548^{bC}
10	24 ± 0.707^{aC}	13 ± 0.316^{bC}
Standard Antibiotic (Enrofloxacin)	20 mm	

Values with different superscript differ significantly ($P < 0.05$); capital alphabets represent column-wise and small alphabets represent row-wise.

Table 2: Zone of inhibition (mm) shown by Copper Sulphate and Zinc Sulphate against *Streptococcus* (Mean±SE)

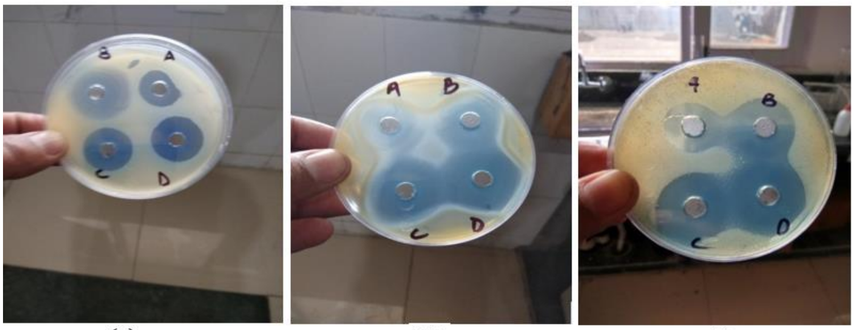
% solution	Copper Sulphate	Zinc Sulphate
2.5	14 ± 0.707^{aA}	10 ± 0.837^{bA}
5	19 ± 0.547^{aB}	12 ± 0.447^{bB}
7.5	22 ± 0.316^{aC}	13 ± 0.447^{bBC}
10	25 ± 0.548^{aD}	14 ± 0.707^{bC}
Standard antibiotic (Enrofloxacin)	24 mm	

Values with different superscript differ significantly ($P < 0.05$); capital alphabets represent column-wise and small alphabets represent row-wise

Table 3: Zone of inhibition shown by Copper Sulphate and Zinc Sulphate against *E.coli* (Mean±SE)

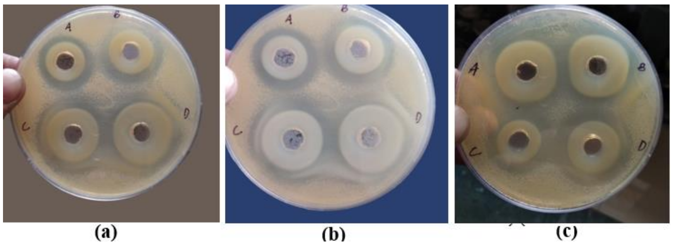
% solution	Copper Sulphate	Zinc Sulphate
2.5	14 ± 0.316^{aA}	10 ± 0.548^{bA}
5	20 ± 0.548^{aB}	12 ± 0.316^{bB}
7.5	22 ± 0.447^{aC}	13 ± 0.447^{bBC}
10	24 ± 0.316^{aD}	14 ± 0.548^{bC}
Standard antibiotic (Enrofloxacin)	23 mm	

Values with different superscript differ significantly ($P < 0.05$); capital alphabets represent column-wise and small alphabets represent row-wise



(a) (b) (c)

Plate 1: Zones of inhibition produced by (A) 2.5% Copper Sulphate (B) 5% Copper Sulphate (C) 7.5% Copper Sulphate (D) 10% Copper Sulphate against (a) *Staphylococcus* (b) *Streptococcus* and (c) *E.coli*



(a) (b) (c)

Plate 2: Zones of inhibition produced by (A) 2.5% Zinc Sulphate (B) 5% Zinc Sulphate (C) 7.5% Zinc Sulphate (D) 10% Zinc Sulphate against (a) *Staphylococcus* (b) *Streptococcus* and (c) *E.coli*

Conclusions

This study advocates the use of copper sulphate and zinc sulphate solutions as an alternative to disinfectants and envisages use of these solutions as teat dips for prevention of bovine mastitis which is a major disease of economic importance affecting the dairy sector.

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