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ASSESSMENT OF THE ANTIMICROBIAL AND CYTOTOXIC POTENTIAL OF ALUMINUM SULPHATE (ALUM) AGAINST SOME FISH PATHOGENS

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ABSTRACT

Aluminum sulphate (alum) is one of the most widely used natural chemical compounds in aquaculture, with variety of bioactivities. This study aimed to assess the antibacterial effect of alum against four microorganisms that cause frequent outbreaks in aquaculture (*Pseudomonas fluorescens*, *Streptococcus agalactiae*, *Aeromonas hydrophilia* and *Photobacterium damsel*), as well as investigating the cytotoxic activity using a brine shrimp lethality assay. The antibacterial activity of alum was evaluated by agar well diffusion assay. Results revealed that alum possess a strong antibacterial activity against the four tested bacterial strains with a minimum bactericidal concentration of 7.8 mg/ml. Brine shrimp lethality assay was evaluated against alum concentration range of 31.25, 15.62, 7.81, 3.9, 1.95 mg/ml. The number of brine shrimp that survived after 24 h was counted, and the mortality percentage was calculated. Alum showed a strong cytotoxic impact, killing all larvae after 30 min of exposure to all concentrations, even 1.95 mg/ml, which is below the MBC value. In conclusion, alum is a potent antibacterial agent with substantial cytotoxic activity. Further detailed studies are required to determine its toxicity to various fish species and whether it is suitable to use as an in-water treatment agent.

KEYWORDS: Alum, Antibacterial activity, Cytotoxicity, Fish.

INTRODUCTION

Aquaculture drugs and chemicals play a critical role in fish treatment and reduction of fish mortalities and are important for increasing fish growth rate, immunity and reproduction **(Gesamp, 1997)**. Drugs and chemicals are frequently used in aquaculture to control parasitic infestations like EDTA for vibriosis and copper sulphate for filamentous bacterial disease **(Roy et al. 2021)**. Intensification in aquaculture has led to increased incidence of several bacterial diseases that resulted in use of a wide range of aqua–medicines, drugs, and chemicals to control the production loss (**Burridge et al. 2010).** Out of 364 aqua-drugs and chemicals used, the majority (31%) are drugs and chemicals used as feed supplements and growth promoters, followed by probiotics (24%), water quality improvement products (18%), antiseptics and sanitizers (13%), anti-parasitic medications (10%), and antibiotics (4%) (**Mishra et al., 2017)**. Farmers use these chemicals and drugs randomly without knowing their necessity, effective dose, side effects and its proper method of application (**Roy et al., 2021)**. Serious concern has been raised by different international organizations over misuse or abuse of these chemicals, often leading to development of antimicrobial resistance (AMR) leading to public health hazard **(FAO, 2005)**.

Most bacterial infections of fish are caused by Gram negative organisms as *Aeromonas*, *Citrobacter*, *Edwardsiella, Pseudomonas, and Vibrio* (**Palmeiro and Roberts, 2009).** *A. hydrophila* and other motile *Aeromonads* are among the most common bacteria in a variety of aquatic environments worldwide and are frequently associated with severe disease among cultured and feral fishes and crustaceans **(Martin and Joseph, 2005).** *Pseudomonas* species are also involved as common disease-causing genera in crustaceans, finfish, and bivalve molluscs **(Alexopoulos et al., 2011)**. *Ph. damselae* ssp. *piscicida* infection is now recognized as one of the most threatening bacterial diseases in mariculture worldwide due to its wide host range, massive mortality, ubiquitous geographical distribution, widespread antibiotic resistance and lack of efficient vaccines **(Barnes et al. 2005)**. Depending on the environmental factors, streptococcosis can cause high mortality, sometimes more than 50%, over a period of 3 to 7 days **(Raissy et al., 2012)**.

Potassium aluminium sulphate (PAS), with the formula KAl (SO4)2.12H2O, often known as potash alum (PA), alum, or tawas, is an effective, commercially available and cost-effective inorganic chemical with various activities. It is a great agent for the biotransformation of a variety of organic and inorganic chemicals as well as for medical, cosmetic, pharmacological, pharmaceutical, and food systems. Moreover, both Gram-positive and Gram-negative bacteria, as well as yeasts and moulds are very susceptible to the antibacterial effects of alum. This action depends on the dosage and the incubation period and is greatly enhanced when used in combination with plant extracts, inorganic compounds, or antibiotics **(Amadi, 2020)**. Potassium alum is reported to be one of the most traditional chemicals of natural source used in aquaculture. **(Attarnejad, 2007)**, and was used to get rid of the growth of nuisance algae in United States lakes and there have been no reported incidents of fish kills or other evidence of aquatic toxicity **(Barry, 2004)**.

Therefore, this study aimed to determine the antimicrobial effect of alum against *Pseudomonas fluorescens*, *Streptococcus agalactiae*, *Aeromonas hydrophilia* and *Photobacterium damsel* as well as investigating the cytotoxic activity using brine shrimp lethality assay.

MATERIAL AND METHODS

Bacterial strains

Aeromonas hydrophila (ATCC 13037), *Pseudomonas fluorescens* (ATCC 13525) and *Streptococcus agalactiae* (ATCC 13813) were purchased from Faculty of Agriculture, Ain Shams University, Egypt. *Photobacterium damselae* was kindly provided by Microbiology Lab., Marine Environment Division, National Institute of Oceanography and Fisheries, Egypt. These strains were previously isolated and identified from fishes **(Abu-Elala et al., 2015)**.

Preparation of the bacterial inoculum

Bacterial strains were adjusted at a concentration of 10^8 cells/ml using 0.5 MacFarland standard **(Andrews, 2001).** Cell turbidity was adjusted from 0.08 to 0.13 optical densities at wavelength 625 nm **(Wiegand et al., 2008).** The bacterial suspension was prepared from bacterial cells cultured overnight on a non selective solid or liquid medium**.** The inoculum was added to the liquid media or placed on solid media within 30 min of preparation so that cell density (CFU/ml) is maintained **(Andrews, 2001; Kowalska-Krochmal and Dudek-Wicher, 2021)**.

Aluminum sulphate (Alum)

Aluminum sulphate (Al_2 $(SO4)_3$.16H2O) was purchased from Elnasr Pharmaceutical Chemicals Company, Egypt. Alum stock solution was prepared by dissolving alum in distilled water at a concentration of 500 mg/ml, then sterilized by filtration with 0.2 µm filter. Fifty µl of alum stock solution was added to the wells.

Brine shrimp larvae

Brine shrimp larvae (20-24 h old) were obtained from Ghalioun Project-National Company for Fishery and Aquaculture, Egypt.

Assessment of the antimicrobial activity of alum

The antimicrobial activity was determined using agar well diffusion method according to **Kowalska-Krochmal and Dudek-Wicher, (2021)** with some modifications. Mueller Hinton agar (Oxoid, UK) was melted and cooled to 45ºC. A standardized inoculum of each bacterial strain at a concentration of 1×10^8 CFU/ml, 0.5 McFarland was added aseptically to the cooled media. The medium was carefully poured into agar plates and allowed to solidify. After solidifying, circular 8 mm wells were punched and bottoms were sealed with soft agar media. Alum stock solution (500 mg/ml, 50 µl / well) was loaded in the wells. Sterile phosphate buffer saline was used as a control negative. The plates were incubated at 37°C for 48 h. Alum antibacterial potency was assessed by the presence or absence of inhibition zone surrounding the wells. The assay was performed in triplicate.

Minimum Bactericidal Concentration of alum (MBC)

The quantitative antibacterial activity of alum was evaluated according to **Bonev et al, (2008).** Alum stock solution was two-fold serially diluted in distilled water up to 10 concentrations (250, 125, 62.5, 31.25, 15.625, 7.81, 3.90, 1.95, 0.97 and 0.48 mg/ml). Fifty µl of the different concentrations were added to its representative well in the previously inoculated agar of the four tested bacterial strains. After incubation at 37°C for 24 h, plates were observed for inhibition. The lowest concentration showing an inhibition zone after 48 h was recorded as the minimum bactericidal concentration of the alum.

Determination of cytotoxic activity of alum (Brine shrimp lethality bio-assay)

Cytotoxic effect of alum was evaluated using brine shrimp lethality bioassay, a preliminary toxicity-screening test according to **Sarah et al., (2017)**. Alum (500 mg/ml) was serially diluted in filtered seawater, and several concentrations were tested (31.25, 15.62, 7.81, 3.9, 1.95 mg/ml). Ten nauplii freshly hatched (20-24 h old), were exposed to the different concentrations of alum in a glass container using a Pasteur pipette. For each concentration, three replications were used. Ten larvae exposed to natural filtered seawater were used as a control negative. The average number of nauplii survivors and the percentage of death was calculated within 24 h. Larvae were considered dead when they stop to move completely.

RESULTS

Qualitative screening of antibacterial activity of alum

Preliminary detection of the antibacterial effect of alum at concentration of 500 mg/ml against *Pseudomonas fluorescens, Streptococcus agalactiae, Aeromonas hydrophilia* and *Photobacterium damsel* revealed effective bactericidal effect against the four tested strains at 500 mg/ml (Fig. 1).

Figure 1: Qualitative antibacterial activity of Alum against *Streptococcus agalactiae* as an example.

Quantitative antibacterial activity of alum (Minimum Bactericidal Concentration (MBC))

The lowest concentration of alum showed a peripheral inhibition zone after 48 h against *Pseudomonas fluorescens, Streptococcus agalactiae, Aeromonas hydrophilia* and *Photobacterium damsel* was recorded as the minimum bactericidal concentration of alum, which was 7.81 mg/ml of alum against the four tested strains (Fig. 2).

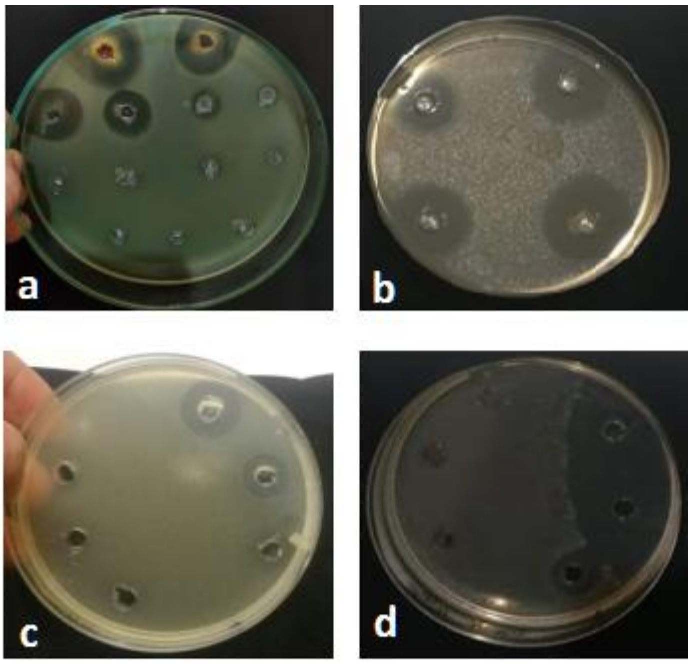

Figure 2: Minimum Bactericidal Concentration of Alum against (a) *Pseudomonas fluorescence*, (b) *Streptococcus agalactiae*, (c) *Aeromonas hydrophilia* and (d) *Photobacterium damselae*.

Alum cytotoxic activity (Brine shrimp lethality bioassay)

Alum exhibited a significant cytotoxic effect where 100% mortality was observed in brine shrimp within 30 min at all alum concentrations (31.25, 15.62, 7.81, 3.9, 1.95 mg/ml). However, all the naupli larvae in the control group survived up to 48 h of observation (Table 1).

Table (1): Biological screening of cytotoxicity of alum using the brine shrimp bioassay.

Conc. of alum (mg/ml)	31.25	15.62	7.81	3.90	1.95	Control
Total nauplii number	30	30	30	30	30	30
Number of survivors	0	0	0	0	0	30
Time elapsed to death (min)	30	30	30	30	30	48*
Mortality (%)	100	100	100	100	100	0

*48 hours

DISCUSSION

Recently, numerous studies confirmed various bioactivities of natural products. the findings here revealed that alum is a strong bactericidal water-soluble salt against the four examined bacterial fish pathogens (*P. fluorescens, S. agalactiae, A. hydrophila and Ph. damselae*) with a minimum bactericidal concentration of 7.8 mg/mL. In this regard, **Dutta et al., (1996), Ahmed (2011)**, and **Bnyan, (2014)** showed the broad-spectrum antibacterial activity of potassium salt of alum against several types of Gram-negative and Gram-positive bacteria (with more efficiency against Gram-negative bacteria). **Amadi and Ngerebara, (2017)** also reported the potential ability of potassium salt of aluminum sulphate to combat spoilage of raw and processed oysters and it was considered as a broad-spectrum antibacterial with a high level of sensitivity on *P. aeruginosa* and *Vibrio* species. Although the mechanism of bactericidal effects of alum is not exactly well-known, **Khan et al., (1984), Osuala et al., (2009) and Ali (2018)** reported that the acidification is the main mechanism of alum activity to damage bacterial cell walls. In this concern, **Miller et al., (1984)** suggested that sulphuric acid is produced when alum reacts with water, which could change the pH value and affect the optimum conditions needed for the physiological functions in fish body. Moreover, alum minimum bactericidal concentration possessed a high toxicity hazard where it kills artemia salina larva within just 30 min of exposure. The alum salts toxicity was previously reported by **Martyn et al., (1989)** and **Najm et al., (1998)**. **Mallinckreod, (2009)** documented that ingestion of 30 grams of potassium salts of alum could kill an adult human where, high level of potassium alum solution could cause distraction of gum tissue, kidney damage and high mortality rate due to intestinal bleeding. On the other hand, aluminum sulfate is an EPA-approved pesticide **(Fifra, 1996)**. Indeed, a value of 0.2 mg/L alum is safely used in drinking water treatment **(Dutta et al., 1996)**. With regard to the safety concerns, **Oneda et al. (1994)** reported that a concentration above 3 % of potassium salt of alum is considered as non-cytotoxic and antitumorigenic for both animals and humans. Furthermore, because aluminum is the major component in alum, it may be associated with aquatic toxicity (**Mortula, 2009)**. However, alum could be considered as a harmless material with low toxicity in laboratory animals because **the** body does not absorb aluminum **(Ilham et al., 2014).** Moreover, aluminum concentrations determined to be toxic to algae were far below those reported to cause lethal toxicity to fish and ceriodaphnids (**Boyd, 1979; McCauley et al., 1986** and **George et al., 1995)**, they also added that the aluminum toxicity is dependent on the water chemistry. The potential risk of aquatic toxicity should be negligible if alum treated water entering the tested lake has a pH > 6.0 (**Pilgrim** and **Brezonik, 2005)**. The sensitivity to alum toxicity may differ between different types of live organisms as well as the condition of water chemistry. The MBC reported in this study may exceed its lethal dose fifty (LD50) in fish and other different organisms. Hence, further studies are necessary to examine alum LD50 therapeutic and toxic effect for different types of aqua organisms.

CONCLUSIONS

Alum is a non-cost traditionally used material that is already used in various aquaculture requirements. The results revealed that alum possess a strong antibacterial activity against four of the most outbreak causing bacteria in aquaculture (*Pseudomonas fluorescens, Streptococcus agalactiae, Aeromonas hydrophilia and Photobacterium damsel*). Moreover, alum had a potential toxicity hazard. Therefore, further detailed studies on its toxicity, long-term effects, mode of treatment and withdrawal time against different types of aquaculture organisms are required.

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