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Graphene oxide, GO, as immunostimulator in controlling Motile Aeromonad Septicemia (MAS) due to *Aeromonas hydrophila* in red hybrid tilapia, *Oreochromis* sp. farming

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Abstract

In the present study, the potential of graphene oxide, GO, was evaluated as immunostimulator in controlling Motile Aeromonad Septicemia (MAS)due to Aeromonas hydrophila infected in Red Hybrid Tilapia. MAS due to A. hydrophila was recognized as main constraint in red hybrid tilapia farming and may lead to mass mortality of the infected fish either marketable size or fish fry. Many of commercial antibiotics were found no longer effective to control this bacterial disease. The fish farmers were left with no option and have to continue use the antibiotics in order to overcome this disease. Therefore, this study was conducted to investigate the potential of GO to control this bacterial disease problem. In the present study, minimum inhibitory concentration (MIC) of GO extract against A. hydrophila was determined via two fold microbroth dilution method. The effectiveness of GO as an immunostimulator agent was evaluated. The experimental fish were fed with medicated feed at three different concentrations (25 mg kg^{-1}; GO-25, 50 $mgkg^{-1}$; GO-50 and100 $mgkg^{-1}$of fish; GO-100) of GO for one week before they were intraperitoneally exposed to A. hydrophila. Enzyme linked immunosorbent assay (ELISA) was carried out to determine the value of antibody response to A. hydrophila in fish from group of fish that received medicated fish and the percentage of total cumulative mortality of the experimental fish were observed at the end of the experiment. The results of the present study showed the value of antibody response to A. hydrophila in fish from group of fish which received medicated feed (GO-25, 0.138 ± 0.012 OD; GO-50, 0.112 ± 0.013 OD; GO-100, 0.141 ± 0.12 OD) were found significantly higher (P< 0.05) compared to fish did not received medicated fish (0.00 OD). Whereas, percentage cumulative mortality of fish from all groups of fish received medicated feed (GO-25, 22.0 ± 1.1 %; GO-50, 23.2 ± 1.8 %; GO-100, 21.7 ± 1.3 %) were found significantly lower (P< 0.05) compared to group of fish did not received medicated feed (70.2 ± 3.2 %). The findings of the present study indicated the huge potential of GO to replace commercial antibiotic as immunostimulator agent for aquaculture uses.

Keywords: *Aeromonashydrophila*, motile aeromonad septicemia, immunostimulator, red hybrid tilapia, graphene oxide.

Introduction

Motile Aeromonad Septicemia (MAS) due to *Aeromonas hydrophila* was recognized as one of the major bacterial diseases that responsible to disease outbreak in many commercial fish farm and caused mass mortality of farmed fish. Hence, MAS posed a significant economic loss in aquaculture industry. In some cases, the virulent of the disease can devastated whole fish farm and killed all of the farmed fish and led to fish farmer suffered bankruptcy. This bacterial was found frequently attacked freshwater ornamental fish in aquarium shop[1]and reported responsible to the mass mortality of mantis shrimp, *Squila* sp.[2]. Based on the literature survey, *A. hydrophila* was successfully isolated from Malaysian Giant Prawn, *Macrobrachium rosenbergii*[3], African catfish, *Clarias gariepinus*[4], American bullfrog, *Rana catesbeina*[5], Asian seabass, *Lates calcarifer*[6], golden pompano, *Trachinotus blochii*[7], Silver catfish, *Pangasius sutchi* and red hybrid tilapia, *Oreochromis* sp.[8]. Red hybrid tilapia is one of commercial freshwater fish that can be found and farmed in world wide. However, this fish species was also reported susceptible to this disease and mass mortality of red hybrid tilapia was found due to the infection of the bacterium. The worst case scenario of the disease outbreak due to *A. hydrophila* was devastated whole red hybrid tilapia farm.

At present, antibiotic is the best solution to overcome MAS infection in red hybrid tilapia. Unfortunately, the misuse and overuse of antibiotic led to the occurrence of antibiotic resistance case among pathogenic bacteria including *A. hydrophila* which responsible to MAS infection in red hybrid tilapia. Subsequently, fish farmers have no option and continue apply commercial antibiotic in managing fish health. Hence, it gave an alarm to the fish farmer to find alternative antimicrobial

agent in controlling fish disease at aquaculture sites.One of the suggested alternative antimicrobial agent is graphene oxide (GO) where it was documented well the huge potential in inhibiting the growth of a various bacteria derived from aquaculture sites[9]. Therefore, in the study was conducted to reveal the potential ofGO extract as an alternative antimicrobial agent for aquaculture uses.

Materials and Methods

Bacterial isolate: *Aeromonashydrophila* isolated from diseased red hybrid tilapia, *Oreochromis* sp., at commercial farms in Kelantan, Malaysia was used in the experiment. The bacterial isolate was cultured using brain heart infusion broth (Oxoid, England) for 24 h at room temperature. The bacterial pellet was harvested by centrifugation at 13,500 rpm for 10 min. The harvested bacterial pellet was washed twice using physiological saline and the concentration of the bacterial isolate was adjusted to 10^9 colony forming unit (cfu) mL^{-1} by using ELISA plate reader (Biorad, USA) and using forchallenged by intaperitoneal (i.p) injection of 100 µL of each inoculum, at a dose causing 50 % mortality (LD_{50})[9].

Graphene oxide (GO) preparation: Sulfuric acid(H_2SO_4) (320 mL):phosphoric acid (H_3PO_4) (80 mL), graphite flakes and $KMnO_4$ (18g) were mixed using a magnetic stirrer in order to allow the process of graphite oxidation. The process was continued for 3 daysto complete the graphite oxidation activity. Once the mixture changed color to dark brown from dark purplish green, oxidation process was stopped by adding H_2O_2 solution. The color of the mixture will change to bright yellow whereshowing a high graphite oxidation level. The graphite oxide (GO) formed was then washed three times with 1 M of HCl aqueous solution and followed with deionized water until a pH of 4-5 is achieved. The sample was subjected to centrifugation at 10,000 g and freeze dried. GO was keep in freezer – 20^{o}C for further uses[9].

Determination of Minimum Inhibitory Concentration (MIC) values: Minimum inhibitory concentration (MIC) values were determined using two fold micro broth dilution method in 96-wells microliter plate format. Bacterial suspensions were inoculated into wells in the presence of graphene oxide with concentration start from 0.244 to 500 mg/L and positive control, kanamycin[10]. The growth of bacteria was checked after 24 h (s) incubation. MIC value is determined as the lowest concentration of antimicrobial agent inhibits the visible growth of the inoculated bacteria[11].

Medicated feed: The fish pellets (Cargills, Malaysia) were purchased commercially before they were mixed with crude extract of graphene oxide. Medicated feed was consisted of three different concentrations (25 mg kg^{-1}; GO-25, 50 mg kg^{-1}; GO-50 and 100 mgkg^{-1} of fish; GO-100) of graphene oxide. The extract was coated with fish pellet at a desired concentration and oven dried at 30^{0}C for 24 h. The prepared fish pellet was then kept at -20^{o}C for further use.

Efficacy of medicated feed experiment: The efficacy if medicated feed experiment was conducted as described by Lee et al. (2014)[12]. Antimicrobial agent efficacy test was carried out to determine the effectiveness of GO in preventing and controlling MAS in red hybrid tilapia due to *A. hydrophila*. A total of 15 groups of fish, where each group contain 10 fish were maintained in 20 L aquaria. Six groups of fish were used as control which each three groups served as negative and positive control. 9 groups of fish were used as treatment for three different concentration of GO (25 mg kg^{-1}; GO-25, 50 mg kg^{-1}; GO-50 and 100 mg kg^{-1} of fish; GO-100) which each treatment contained a triplicate. The experimental fish were given medicated fish pellet at 2 % body weight of fish per day for one week before the fish were exposed to *A. hydrophila* by intraperitoneal injection. The mortality of the infected fish was observed and recorded for four weeks. Simultaneously, the medicated and unmediated fish pellet was continuously given to the fish for four weeks. Fish from each treatment was randomly sampled for enzyme linked immunosorbent assay (ELISA) for every week.

Indirect enzyme linked immunosorbent assay (ELISA): ELISA was carried out as described by Shelby et al.[13] and Lee et al.[12] with few modification. Briefly, fish were bled from the caudal vein and the blood was collected into micro centrifuged tube. The blood was then allowed to clot for 1 h at 25^{0}C. The fish serum was harvested through centrifuged at 300 *g* and stored at – 80^{0}C for further use. The motile aeromonad septicemia antigen was prepared by diluted whole cell of *A. hydrophila* with carbonate buffer to 500 µg mL^{-1}. A hundred µL of motile aeromonad septicemia antigen was added into each well of microtitre plate for 1 h at 25^{0}C. The wells were then blocked with 3 % bovine serum albumin (Sigma, USA) for 1 h at 25 ^{o}C. After the incubation period, the wells were washing 5 times with PBS plus teewn-20 (PBS-T). A hundred µL of a serum sample (1 µL of serum diluted in 999 µL of PBS-T) was added to three replicate wells of plate followed by 30 min incubation at 25^{0}C. The wells were then washed 3 times with PBS-T. After washing, a hundred µL of goat anti-tilapia immunoglobulin serum (diluted 1 : 5000 in PBS-T) was added into the wells followed by 30 min incubation at 25^{0}C. After 3 times washing again with PBS-T, a hundred µL of rabbit anti-goat peroxidase conjugate (diluted 1: 5000 PBS-T) was added into the wells. Finally, the wells were washed again with PBS-T followed by a hundred µL of *o*-phenylenediamine in urea-peroxide buffer was added to each well. The elisa reaction was stopped at 15 min by adding 50 µL of 3 M H_2SO_4. The optical densities (O.D) of the reactions were read with microplate reader (Bio Rad, USA) at 490 nm. Negative controls consisted with wells coated with antigen and no sample serum, and wells with no antigen and a serum sample. The control reactions gave an OD of 0.04 or less.

Statistical analysis: Statistical differences between mortality

and ELISA values were analyzed with one-way analysis of variance using Tukey post hoc multiple comparison tests at 5% of significant level.

Results and Discussion

In the present study, GO was found can inhibit the growth of *A. hydrophila* with the MIC value was 31.5 mg/L. The value of antibody response to *A. hydrophila* in fish from group of fish which received medicated feed (GO-25, 0.138 ± 0.012 OD; GO-50, 0.112 ± 0.013 OD; GO-100, 0.141 ± 0.12 OD) were found significantly higher ($P< 0.05$) compared to fish did not received medicated fish (0.00 OD). Whereas, percentage cumulative mortality of fish from all groups of fish received medicated feed (GO-25, 22.0 ± 1.1 %; GO-50, 23.2 ± 1.8 %; GO-100, 21.7 ± 1.3 %) were found significantly lower ($P< 0.05$) compared to group of fish did not received medicated feed (70.2 ± 3.2 %).

Based on the literature survey, antimicrobial property of GO was widely studied and well documented. For instance, Chen et al.[14] reported that GO can inhibit the growth plant pathogen consists of fungi and bacteria by causing cell lysis to the pathogen. Antimicrobial activity of GO was also revealed by Liu et al.[15] where GO was found performed inhibitory activity against *E. coli*. The study was successfully showed that GO was the best antimicrobial agent among graphite, graphene oxide and GO towards inhibiting the growth of *E. coli*. Satish et al.[16] reported that GO was able to inhibit the growth of *Klebsiella* sp. and *Staphylococcus* sp. Recently, GO was found to be able to inhibit various pathogenic bacteria isolated from aquaculture sites including *A. hydrophila*[12]. To our knowledge, there is no study was conducted to revealed the potential of GO as immune stimulator in controlling fish disease. Hence, the present study was the first attempt to document the promising outcomes where GO was found can increase red hybrid tilapia immune and prevent from MAS infection. Therefore, we may suggest to fish farmer to apply GO as antimicrobial agent and immune stimulator to combat MAS infection in tilapia farming.

Although the present study revealed the huge potential of GO as antimicrobial agent where other than antibiotic compound or substance that can prevent from fish disease infection, however, many studies were reported antimicrobial activity of plant which also can be used against pathogenic bacteria from aquaculture sites. For example, *Allium sativum* extract[17], *Cymbopogon nardus* essential oil[18], *Ficus deltoidea* leaf extract[11], *Andrographis paniculata* leaf extract[19], *Michelia chempaca* seed and flower extracts[20], *Colocasia esculenta* extract[21], *Sauropus androgynous* stem extract[10], *Syzygium aromaticum* flower bud (Clove)[22], *Citrus microcarpa*[23]; *Murdannia bracteata* leaf extract[24], *Phyllanthus urinaria* Linn. Leaf[25] and many more. However, the mentioned studies were only focus on the *in vitro* antimicrobial activity of the plants. On the other hand, in the present study highlighted the mechanism and mode of action of the GO in stimulating the immune system of the fish against infection of MAS due to *A. hydrophila*.

Conclusion

Based on the finding of the present study showed the huge potential ofGO as immunostimulator in controlling Motile Septicemia Motile (MAS) due to *A. hydrophila* infected in red hybrid tilapia, *Oreochromis* sp. Further study should be carried out to expand the potential of GO in other fish species before the present finding can come to a commercial sense.

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References

1. Najiah M., Lee S.W., Faizah S. and Wendy W. (2008). Surveillance of bacteria species in diseased freshwater ornamental fish from aquarium shop. *J. World App. Sci*., 3 (6), 903-905.

2. Najiah M. and Lee S.W. (2008). Outbreak Vibriosis of Mantis Shrimp (*Squilla* sp.). *World J. Agric. Sci.*, 4 (2), 137-139.

3. Lee S.W., Najiah M., Wendy W., Zarol A. and Nadirah M. (2009). Multiple antibiotic resistance and heavy metal resistance profile of bacteria isolated from giant freshwater prawn (*Macrobrachiumrosenbergii*) hatchery. *Agric. Sci. China* 8(6), 740-745. doi:10.1016/S1671-2927(08)60273-4

4. Najiah M., Lee S.W., Wendy W. and Nadirah M. (2009). Bacterial diseases outbreak of African catfish (*Clariasgariepinus*) from Manir River, Terengganu, Malaysia. *J. Life Sci*., 3 (5): 10 – 13.

5. Lee S.W., Najiah M., Wendy W., Nadirah M. and Faizah S.H. (2009). Occurrence of heavy metals and antibiotic resistance in bacteria from organs of American bullfrog (*Ranacatesbeiana*) cultured in Malaysia. *J. Ven. Anim. Tox. Incl. Trop. Dis.,* 15 (2), 353-358. dx.doi.org/10.1590/S1678-91992009000200016

6. Lee S.W., Najiah M. and Wendy W. (2010). Bacterial flora from a healthy freshwater Asian sea bass (*Latescalcarifer*) fingerling hatchery with emphasis on their antimicrobial and heavy metal resistance pattern. *Vet. Arhiv.,* 80(3), 411-420.

7. Lee S.W., Najiah M. and Wendy W. (2010). Bacteria associated with golden pompano (*Trachinotusblochii*) broodstock from commercial hatchery in Malaysia with emphasis on their antibiotic and heavy metal resistances. *Front. Agric. China.,* 4(2), 332-336. Doi10.1007/s11703-010-0106-y

8. Lee S.W., Najiah M., Wendy W. and Nadirah M. (2010). Antibiogram and heavy metal resistance of pathogenic bacteria isolated from moribund cage cultured silver catfish (*Pangasiussutchi*) and red hybrid tilapia (*Tilapia* sp.). *Front. Agric. China.,* 4(1), 116-120. Doi10.1007/s11703-009-0085-z

9. Lee S.W., Mustakim M.T, An'amt M.N., Wendy W. and Huang N.M. (2015). The potential of graphene oxide as antimicrobial agent against pathogenic bacteria isolated from aquaculture sites. *Ann. Res. Rev. Biol.*, 6 (4), 264

10. Lee S.W., Wendy W., Julius Y.F.S. and Desy F.S. (2011). Characterization of antimicrobial, antioxidant, anticancer property and chemical composition of *Sauropusandrogynus* stem extract. *Acta Med. Lit.,* 18 (1), 12-16.

11. Lee S.W., Wendy W., Julius Y.F.S. and Desy F.S. (2011). Characterization of antioxidant, antimicrobial, anticancer property and chemical composition of *Ficusdeltoidea* Jack. leaf extract. *J. Biol. Active Prod. Nat.* 1 (1), 1-6.Doi 10.1080/22311866.2011.10719067

12. Lee S.W., Wendy W. and Zulhisyam A.K. (2014). *Citrus microcarpa* extract as immunostimulator in controlling Edwardsiellosis in African catfish culture. *J. Trop. Res. Sus. Sci*., 2(2): 68-71.

13. Shelby R.A., Klesius P.H., Shoemaker C.A. and Evans J.J. (2002). Passive immunization of tilapia, *Oreochromisniloticus* (L.), with anti-*Streptococcus iniae* whole sera. *J. Fish. Dis.,* 25, 1-6. DOI: 10.1046/j.1365-2761.2002.00327.x

14. Chen J., Peng H., Wang X., Shao F., Yuan Z. and Han H. (2014). Graphene oxide exhibits broad-spectrum antimicrobial activity against bacterial phytopathogens and fungal conina by intertwining and membrane perturbation. *Nanoscale*., 6(3), 1879-1889. doi: 10.1039/c3nr04941h.

15. Liu S., Zeng T.H., Mario H., Ehdi B., Wei J., Jiang R., Kong J. and Chen Y. (2011). Antibacterial activity of Graphite, Graphite Oxide, Graphene Oxide, and Reduced Graphene Oxide: Membrane and Oxidative Stress. *ACS Nano.,* 5(9), 6971-6980. doi:10.1021/nn202451x

16. Satish B., Venkateswara R.K., Shilpa C.C.H. and Tejaswi T. (2013). Synthesis and characterization of grapheme oxide and its antimicrobial activity against *Klebsiella* and *Staphylococcus*. *Int. J. Adv. Biotech. Res.*, 4(1), 142-146.

17. Lee S.W. and Najiah M. (2008). Inhibition of *Edwardsiellatarda* and other fish pathogens by *Allium sativum* L. (Alliaceae) extract. *American-Eurasian J. Agric. Environ. Sci*., 3 (5), 692-696.

18. Lee S.W. and Wendy W. (2013). Chemical composition and antimicrobial activity of *Cymbopogonnardus* citronella essential oil against systemic bacteria of aquatic animals. *Iranian J Microbiol.,* 5 (2), 147-152.

19. Lee S.W., Wendy W., Julius Y.F.S. and Desy F.S. (2011). Characterization of antimicrobial, antioxidant, anticancer properties and chemical composition of Malaysian *Andrographispaniculata* leaf extract. *Pharmacologyonline* 2, 996-1002.

20. Lee S.W., Wendy W., Julius Y.F.S. and Desy F.S. (2011). Characterization of Antimicrobial, Antioxidant, Anticancer property and Chemical Composition of *Micheliachampaca* Seed and Flower Extracts. *S.J. Pharm Sci*., 4(1), 19-24.

21. Lee S.W., Najiah M. and Wendy W. (2010). *In vitro* antimicrobial activities of *Colocasiaesculenta* extract against *Vibrio* spp. *Agricultura* 7(1), 5-7.

22. Lee S.W., Najiah M., Wendy W. and Nadirah M. (2009). Chemical composite on and antimicrobial activity of the essential oil of *Syzygiumaromaticum* flower bud (Clove) against fish systemic bacteria isolated from aquaculture sites. *Front. Agric. China*., 3 (3), 332-336. Doi 10.1007/s11703-009-0052-8

23. Lee S.W. and Najiah M. (2009). Antimicrobial property of 2-hydroxypropane-1,2,3-tricarboxylic acid isolated from *Citrus microcarpa*extract. *Agric. Sci. China*, 8 (6), 740-745. doi:10.1016/S1671-2927(08) 60291-6.

24. Lee S.W., Wendy W., Julius Y.F.S., Desy F.S. and Ahmad A.I. (2010). Characterization of antioxidant, antimicrobial, anticancer property and chemical composition of *Murdanniabracteata* leaf extract. *Pharmacologyonline*, 3, 930-936.

25. Lee S.W., Wendy W., Julius Y.F.S. and Desy F.S. (2012). Characterization of Anticancer, Antimicrobial, Antioxidant property and chemical composition of *Phyllanthusurinaria* Linn. leaf extract. *Pharmacology Online,* 49 (10), 670-674