PATHOLOGICAL CHANGES IN THE ORGANS OF RATS EXPERIMENTALLY INFECTED WITH MANNHEIMIOSIS AND TREATED WITH SEA CUCUMBER (*STICHOPUS HORRENS*) EXTRACT

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CERTIFICATION

This is to certify that we have read this research paper entitled **'Pathological Changes in the Organs of Rats Experimentally Infected with Mannheimiosis and Treated with Sea Cucumber (*Stichopus Horrens*) Extract'** by Shafiqah Syahirah Binti Hanafi, and in our opinion, it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement for the course DVT 5436 – Research Project.

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This thesis is dedicated to:

My Mother (Che Samsiah Binti Mohamad)

My Father (Hanafi Bin Yasim)

My beloved family

Dr. Mohd Farhan Hanif Bin Reduan

Prof. Dr. Jasni Bin Sabri

Dr. Fathin Faahimaah

Friends

Classmates of 2017/2022

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ABSTRACT

An abstract of the research paper presented to the Faculty of Veterinary Medicine, University Malaysia Kelantan, in partial requirement on the course DVT 5436- Research Project.

Mannhemiosis caused by *Mannheimia hemolytica* is a respiratory disease that is highly infectious, often lethal and causes a high economic impact on the large animal sector. This is an experimental study to study the pathological changes in the organs of the mannheimiosis rat model treated with sea cucumber extract, *Stichopus horrens*. Group 1 was a positive control group and was inoculated with bacteria but did not receive any treatment. Group 2 was inoculated with bacteria and received sea cucumber extract treatment seven days before inoculation and 14 days post-inoculation All rats were euthanized on day 15 of post-inoculation and post mortem was done. Lung and liver organ samples were observed for gross lesions and submitted for histopathology evaluation. Based on gross lesion and histopathological findings, the severity of lesions of group 2 rats were significantly ($p<0.04$) decrease than group 1. In conclusion, sea cucumber extract, *Stichopus horrens* reduced the severity of organ lesions in mannheimiosis rat model and had potential as a therapeutic and prophylactic agent against mannheimiosis.

Keywords: Mannheimiosis, Sea cucumber extract (*Stichopus horrens*), *lung*, *liver*, *histopathology*

ABSTRAK

Abstrak kertas penyelidikan yang dibentangkan kepada Fakulti Perubatan Veterinar, Universiti Malaysia Kelantan, sebagai keperluan sebahagian daripada kursus DVT 5436- Projek Penyelidikan.

Mannhemiosis yang disebabkan oleh *Mannheimia hemolytica* adalah penyakit pernafasan yang sangat berjangkit, selalunya membawa maut dan menyebabkan kesan ekonomi yang tinggi kepada sektor haiwan yang besar. Ini adalah kajian eksperimen untuk mengkaji perubahan patologi dalam organ model tikus mannheimiosis yang dirawat dengan ekstrak gamat, *Stichopus horrens*. Lapan ekor tikus telah digunakan dalam kajian ini dan dibahagikan kepada dua kumpulan yang terdiri daripada 4 ekor tikus dalam setiap kumpulan. Kumpulan 1 adalah kumpulan kawalan negatif dan telah disuntik dengan bakteria tetapi tidak menerima sebarang rawatan. Kumpulan 2 telah disuntik dengan bakteria dan menerima rawatan ekstrak gamat tujuh hari sebelum inokulasi dan 14 hari selepas inokulasi. Tikus telah diperhatikan untuk perubahan dalam tanda klinikal sebelum dan selepas selepas inokulasi. Semua tikus telah dieuthanized pada hari ke-15 selepas inokulasi dan bedah siasat dilakukan. Sampel organ paru-paru dan hati diperhatikan untuk lesi kasar dan diserahkan untuk penilaian histopatologi. Terdapat kehadiran penyatuan dalam semua paru-paru tetapi berbeza dalam lobus yang terlibat dan keterukan. Sementara itu, hepatomegali dan kesesakan hati diperhatikan pada semua tikus untuk hati. Berdasarkan penemuan histopatologi, terdapat penurunan ketara ($p<0.05$) dalam keterukan lesi untuk pendarahan, edema dan trombus paru-paru pada tikus kumpulan 2 berbanding tikus kumpulan 1. Di samping itu, terdapat penurunan ketara ($p<0.05$) dalam keterukan lesi untuk trombus, pendarahan dan nekrosis hati dalam kumpulan 2 tikus berbanding tikus kumpulan 1. Kesimpulannya, ekstrak gamat, *Stichopus horrens* mengurangkan keterukan lesi organ dalam model tikus mannheimiosis dan berpotensi sebagai agen terapeutik dan profilaksis terhadap mannheimiosis.

Kata kunci: Mannheimiosis, ekstrak gamat (*Stichopus horrens*), paru-paru, hati, histopatologi

1.0 INTRODUCTION

Mannhemiosis is one of the respiratory tract infections caused by *Mannheimia hemolytica* that commonly occurs in various farm and domestic animals (Mohamed & Abdelsalam, 2008). The disease is highly infectious, often lethal, and has a significant economic impact on the livestock sector in its typical clinical state (Mohamed & Abdelsalam, 2008). One of the common commensal organisms of the nasopharynx and tonsils of healthy sheep and goats is *Mannheimia haemolytica* (Abdullah & Chung, 2014). They are non-motile gram-negative rod bacteria that cause cranioventral bronchopneumonia affecting ruminants of all ages (Cynthia and Scott, 2012).

Mannheimiosis is a disease that requires several underlying stress conditions as it does not emerge on its own and can lead to secondary problems (Laishevtsev, 2020). In 1998, Brogden et al. indicated if there is breakage in the barrier integrity, Mannheimia haemolytica can become pathogenic and migrate from its commensal status in the nasopharynx, allowing it to colonise and grow throughout the upper respiratory system and affect lung parenchyma. Poor nutrition, climatic stress, poor husbandry, transportation stress, sudden environmental changes, bacterial infection, and viral infection are the risk factors that depress the immune system, causing pneumonic mannheimiosis in sheep and goats to develop (Leite-Browning, 2007).

Pasteurellosis was once a catch-all word for various illnesses in domestic animals caused by Gram-negative non-motile facultative anaerobic rods or coccobacilli previously, but with more advances in molecular biology, *Pasteurella hemolytica* biotype A was assigned to a new genus and renamed *Mannheimia* (Mohamed & Abdelsalam, 2008). The most common clinical signs in mannheimiosis are fever, dyspnea, mucopurulent oculonasal discharge, anorexia, and depression. This disease can be diagnosed based on the history, clinical signs, bacteria culture, polymerase chain reaction, and immunohistochemical technique (Leite-Browning, 2007).

According to the data by the European Committee on Antimicrobial Susceptibility Testing, the most effective antibiotics used to treat mannheimiosis are amoxiclav, danofloxacin, marbofloxacin, lincomycin, oxytetracycline, spectinomycin, tilmicosin, tylosin, cefazolin, and cephalexin (Laishevtsev, 2020).

Sea cucumbers are blind cylindrical marine invertebrates that live in ocean intertidal beds. They come in various morphologies and colours and are found worldwide (Masre, 2018). Sea cucumbers belong to the Holothuroidea family of echinoderms and Holothuroidea animals have soft body tissue that is bilaterally symmetrical which lies on one side with an elongated body axis between the mouth and the anus. They also have a coelom and three tissue layers (Moore, 2006). The most popular and well-studied gamat species is *Stichopus horrens*, which has been economically exploited for its lipid extracts (minyak gamat), bodily fluid extracts (air gamat) as well as gamat-based nutritional supplements and health foods products made with modern technology (Kamarudin *et al*., 2015).

Pathogenic bacterial infections face a significant problem due to pathogens' multi-resistant nature to antibiotics. As a result, finding new antimicrobial compounds from multiple natural sources has become a critical and essential requirement (Hassanshahian *et al*., 2020). On the other hand, there is a constant need to extract metabolites from sea cucumbers with a range of chemical structures that may have antibacterial properties (Hamayeli *et al*., 2019). Sea cucumbers are a vast genus of aquatic organisms that can be found all over the world's oceans (Barnes, 1987).

According to studies on the biological properties of marine invertebrates, sea cucumber possesses the majority of chemical compounds with biological properties (Farjami *et al*., 2013). Several unique biological and pharmacological activities, including anticancer, anticoagulant, antimicrobial, antioxidant, antihyperlipidemic, antihyperglycemic, anti-inflammatory antihypertension and radioprotective, have been ascribed to various compounds isolated from sea cucumbers (Shi *et al.*, 2015).

In addition, a variety of bioactive compounds, such as essential fatty acids, glycosaminoglycans (GAGs), lectins, phenolics, sulphated polysaccharides, chondroitin sulphates, glycoproteins, terpenoids, glycosphingolipids, triterpene glycosides (saponins), and sterols (glycosides and sulphates) can be effective on pharmacological and therapeutical properties of sea cucumbers (Datta *et al*., 2015). Sea cucumber sulfated fucans are part of a group of well-studied sulfated polysaccharides that are derived from non-mammals but have biological action in mammalian systems.

FuCS and sulfated fucans from the sea cucumbers belong to a group of sulfated polysaccharides exerting biological activity in mammalian systems that possess heparin-like anticoagulants and antithrombotic activities (Khotimchenko, 2018). Phenol compounds were the most abundant in *S. horrens* extracts and it is found that brown phenolic compounds such as eckol, dieckol, and phloroglucinol contributed to antibacterial activity (Suleria *et al.* 2015). In 2021, Movahed *et al.* also mentioned that their study revealed the ethyl acetate extracts of *S. horrens* displayed the best spectrum of bactericidal effects. Based on Moelyono *et al*. 2018, in their research, they concluded that ethanolic extract of *Stichopus horrens* has the best antiinflammatory activity. Therefore, this study aims to investigate the potential of the sea cucumber (*Stichopus horrens*) extract as a treatment for Mannheimiosis.

2.0 PROBLEM STATEMENT

Mannheimiosis is endemic in Malaysia, causing economic losses to goat and sheep industry through high morbidity and mortality. Frequent usage of antimicrobial has led to antimicrobial resistance. Plant and animal-derived natural medicines can be the alternative way to encounter the issue and Sea cucumber is one of these natural products that has been demonstrated to have many pharmacological actions that can help treat and lower the severity of various conditions. There is currently no evidence on the prophylactic and therapeutic effects of sea cucumber (*Stichopus horrens*) extract on pneumonic pasteurellosis in a rat model, to our knowledge.

3.0 RESEARCH QUESTION

Is the severity of gross and histpathology of organ lesions less in mannheimiosis rats treated with sea cucumber (*Stichopus horrens*) extract compared to untreated mannheimisosis rats?

4.0 RESEARCH HYPOTHESIS

The gross and histopathology lesion of the organs in mannheimiosis rats treated with sea cucumber (*Stichopus horrens*) extracts is less severe compared to untreated mannheimiosis rats.

5.0 RESEARCH OBJECTIVES

To differentiate the gross and histopathology lesion between mannhemiosis rat treated with Sea cucumber extract (*Stichopus horrens*) and untreated mannheimiosis rat.

6.0 LITERATURE REVIEW

6.1 Pneumonic Pasteurellosis

Pneumonic Pasteurellosis is the most common type of infection involving respiratory system in small ruminants, including goats, and is caused by *Pasteurella multocida* and *Mannhemia hemolytica*. The interaction between host immunity, bacteria, and environmental stress will causes infection (Mohamed & Abdelsalam, 2008). Due to expensive treatment costs, decreased daily weight growth, carcass condemnation, and decreased meat and milk output, the disease can result in significant economic losses. Diagnosis is conducted based on the history, clinical signs, bacteria identification and isolation, molecular techniques such as polymerase chain reaction, post mortem examination and histopathological findings (Mohamed & Abdelsalam, 2008). A few antimicrobial treatments are suggested to be effective for pneumonic pasteurellosis, such as ampicillin, penicillin, ceftiofur, ceftriaxone, and enrofloxacin (Rahal *et al.*, 2014). Preventive methods include improvement of housing conditions, sanitary and hygiene management, and biosecurity practices are vital to prevent the disease. The farmer must follow the right transporting system of the animal to avoid unnecessary stress, which can lead to immune suppression.

6.2 Different Phases of Disease

Based to Rhadistits *et al.*, 2000, there are three phases of disease occurrence for mannheimiosis. The first stage entails the onset of acute fever with a 1-2^{o}C deviation from the physiological norm in body temperature. A manifestation of respiratory failure related to fibrinous bronchopneumonia, fibrinous pleurisy, occurs in the second phase. Clinical signs of lung insufficiency in adult animal start to show within 10-14 days following pathogen invasion, however, in some condition, the time it takes for clinical signs to appear is decreased to a few days. The animal dies in the third phase as a result of septic processes. The clinical course of the disease in adult animals lasts for 2-3 days, followed by death or a chronic form.

6.3 Virulence factor involved in Manheimiosis infection

A method of experimentation Yates (1982) reviewed many findings of field workers and researchers who indicated that, in the absence of a well-defined predisposing factor, *P. haemolytica* could not act as the disease's causal pathogen. It also proves that the pathogenicity of the bacteria is influenced by endogenous factors which allow rapid invasion and destruction of the vulnerable host's target tissues (Mohamed & Abdelsalam, 2008). These factors are known as virulence factors and are found on the surface of bacterial cells and their products (Mohamed & Abdelsalam, 2008). Capsular structure, fimbriae, endotoxin, and leukotoxin are virulence factors that promote the organism's adhesion, ccolonization and proliferation, all of which are important in the pathogenesis of mannheimiosis (Marru *et al.,* 2013). In 2008, Mohamed and Abdelsalam mentioned that the *Mannheimia haemolytica* cell wall has a lipopolysaccharide (LPS) endotoxin, the same as all other Gram-negative bacteria. This endotoxin is one of the essential virulence factors in pneumonic pasteurellosis pathogenesis.

According to experimental findings, the endotoxin is directly toxic to endothelial cells, capable of changing leukocyte activities and triggering blood platelet lysis (Breider *et al*., 1990). Endotoxins produced in diseased lung lobules cause intravascular thrombosis which is supported by a strong inflammatory response dominated by fibrinous exudate (Per *et al.*, 2010). Microbial activity can be inhibited by the airway's serous and mucinous fluids. Lysozyme, lactoferrin, phospholipase A2, surfactant proteins, peroxidases, secretory leukoprotease inhibitor, serprocidins, lysozyme, bactericidal permeability-inducing factor, cathelicidins, , defensins, and anionic peptides are some of the factors involved. Gram-negative bacteria products, including *Mannheimia haemolytica* mammalian bronchial and bronchiolar epithelial cells, are the first line of defence (Shanthalingam *et al.*, 2014).

The mucociliary apparatus lowers microbial attachment and multiplication within the respiratory tract's airways. (Zamri *et al.*, 2006). Fimbriae are bacterial cell wall surface features that allow or enhance adherence to and ccolonizationof the susceptible animal's target epithelium and *Mannheimia haemolytica* produces two forms of fimbriae, both of which are capable of enhancing the organism's mucosal adhesion and ccolonizationof the lower respiratory tract epithelium of cattle and sheep (Mohamed & Abdelsalam, 2008). As a result of successful ccolonization the number of bacteria seeded in lung tissue will be significantly increased beyond the level of normal lung capacity that can efficiently resolve (Gonzalez & Maheswaran, 1993). The cell capsule's pathogenicity is primarily related to its capacity to protect the invading organism from the host's cellular and humoral defensive mechanisms (Mohamed & Abdelsalam, 2008). To evade phagocytosis by macrophages and polymorphonuclear leukocytes and shield the organism from the complement-mediated breakdown of the outer membrane in serum, each serotype of *M. haemolytica* generates a distinct polysaccharide capsule (Brogden *et al*, 1998).

The leukotoxin is a 102 kDa protein secreted by *M. haemolytica* during its logarithmic phase of growth. It is also regarded as the bacteria's principal weapon or virulence factor (Zecchinon *et al*, 2005). For its great specificity to ruminant leukocytes, *M. haemolytica* has also been demonstrated to create a soluble heat-labile exotoxin known as leukotoxin (Shewn & Wilkie, 1983). Leukotoxin inhibits phagocytosis and lymphocyte proliferation at low concentrations, but it has a cytotoxic impact at higher concentrations, resulting in cell death secondary to lysis and the lysis of the cell leads to the transmission pore formation in the target cell, allowing the movement of the calcium, sodium and potassium through transmembrane gradients (Clinkenbeard & Upton, 1991).

6.4 Pathological changes related to *Mannheimia haemolytica* infection

Mannheimiosis is a disease that affects animals that have a compromised pulmonary defence system. A severe respiratory illness with fibrinopurulent bronchopneumonia and pleurisy is commonly recognized. (Alemneh *et al.,* 2016). The most visible lesions in infected animals are generally confined to one area of the lung in postmortem examinations. There is 'marbling' of the lung on the surface, with lobules ranging from normal to grey to red, where the entire lobule undergoes coagulation necrosis or bleeding. Fibrin-rich edema fluid and interlobular lymphatics inflated the interlobular septa, which may include fibrin thrombi. There was also an excess of straw-coloured exudate in the thorax, slightly inflated intralobular septa, a red area of consolidation, pleural adhesion, and encapsulated foci containing whitish material known as sequestrate (Griffin *et al.,* 2010). Similar observations were seen in an adult goat with pasteurellosis, where the left and right craniolateral lung lobes were congested with foamy exudates in the trachea, bronchi, and cut surface of the lungs (Abdullah *et al.*, 2014).

In the lungs of sick goats, histopathological abnormalities such as bleeding, edoema, necrosis, and white blood cell infiltration were found (Boukahil *et al.,* 2016). Acute cranioventral fibrinous to fibrinopurulent pleuropneumonia is a symptom of *Mannheimia haemolytica*-induced pneumonia. Fibrin, edema, red blood cells, and dense clusters of primary macrophages and neutrophils were seen in damaged sections of the lung, bronchioles, and alveolar spaces, according to histological examinations (Odugbo *et al.,* 2004). The alveoli were filled with fibrin, macrophage, neutrophil, typical 'oat cells,' compacted and streaming necrotic macrophages inside the damaged alveoli. Lobules with appear as infarcts with thick neutrophilic infiltrate surrounding the margin. Vasculitis with fibrin thrombi is common, and fibrin-rich exudates are frequently discharged from the bronchioles, which can spread from the alveoli (Hussain *et al.,* 2017).

6.5 Diagnosis for mannheimiosis

The diagnosis for mannheimiosis includes clinical observation, microbial isolation and identification, complete blood count, serum biochemistry, gross and histopathological changes, and Polymerase chain reaction. Upon clinical examination, the animal will show clinical signs of pyrexia, anorexia, dyspnea, open mouth breathing, nasal discharge and frothy salivation (Hussain *et al*., 2017) . The gross findings for animal infected with *Mannheimia hemolytica* are fibrinous pleuropneumonia, pulmonary edema, tracheitis, fibrinous pericarditis, multifocal whitish necrohepatitis and severe pneumonic lesion in cranioventral portion (Hussain *et al.*, 2017). Petechial haemorrhages of subcutaneous connective tissue, pericarditis and hemorrhagic injury to the heart ventricles are all symptoms of mannheimiosis (Laishevtsev, 2020).

Based on Laishevtsev 2020, diffuse capillary congestion, interstitial or alveolar edoema, and vascular thrombosis of capillaries, small blood vessels, and pulmonary lymph vessels are all found upon histological investigation. Alveolar necrosis is ccharacterizedby a significant amount of fibrinous exudate and inflammatory cells within the alveoli, which can be observed throughout the affected area. For the bacterial isolation, *Mannheimia hemolytica* could be isolated from nasal swabs and lung tissue which grow as grey colonies on blood agar with a musty odour and appear as gram-negative coccobacillus on microscopic examination (Hussain *et al*., 2017). DNA sequence that was done following the PCR procedure reveals the sequence related to *Mannheimia hemolytica*.

6.6 Treatment of mannhemiosis

The most common treatment for mannheimiosis was by admistration of antibiotics such as penicillin, ampicillin, tetracycline, oxytetracycline, tylosin, florfenicol and ceftiofur which reported to be most effective (Abdullah & Chung, 2014). Other than that, vaccination is also part of the prevention of mannheimiosis, such as the recombinant DNA vaccines that give significant protection and antibody response (Sabri *et al*., 2013). The other medication given aside from antibiotics is non-steroidal antiinflammatory drugs such as Flunixine Meglumine and fluid therapy as supportive treatment in mannheimiosis animals such as Flunixine Meglumine fluid therapy as supportive treatment in mannheimiosis animals (Venkatasivakumar *et al*., 2016). In one study, the affected goat was given Anistamin as an antihistamine at the dose of 2 ml through I.M, twice a day for two days and a calcium supplement at a dose of 1 ml per animal, three times a week for two weeks to treat the clinical manifestations (Venkatasivakumar *et al*., 2016).

6.7 Sea cucumber industry in Malaysia

Sea cucumbers are commonly uutilizedin traditional medicine in Malaysia and other regions of Asia due to their health advantages and functional features. Sea cucumbers belong to the Holothurioidea and Stichopodidae families of marine animals. They are echinoderms with leathery skin and a long, flexible, gelatinous body. Sea cucumbers are nutrient-dense, with plenty of vitamins, minerals, fatty acids, bioactive peptides, and other useful components. These advantages could be attributed to numerous functional chemicals in Sea Cucumbers. (Bordbar *et al.,* 2011). Sea cucumbers belonging to the Stichopus genus are frequently processed into medicinal and health goods such as oil, lotion, cream, pills, and soap (Kamarudin *et al.,* 2009). Sea cucumbers or Gamat-based products are utilized for Malaysia's wound healing and overall wellness. Mothers used gamat products after childbirth for their therapeutic effects. Gamat-based products are common in Malaysian traditional medicine, and they can even be combined with other herbs to make cough medicine or medicinal oil.

Skulphated Glycosaminoglycans (GAGs) are responsible for the sea cucumber's therapeutic properties. Mucopolysaccharides, or glycosaminoglycans, are large complex carbohydrate molecules that interact with various proteins involved in physiological and pathological processes (Masre *et al.,* 2012). The ossicles of sea cucumbers belonging to the Stichopodidae family are fashioned like C-, S. Sea cucumber has branching rods and has a square or trapezoidal cross-section. The primary approach for species identification is morphological characteristics of the sea cucumber, such as body shape, body colour, and the presence and shape of papillae on both the dorsal and ventral sections of the sea cucumber. There are four species of sea cucumber in Malaysia that are commonly called 'gamat' *Stichopus chloronatus, Stichopus horrens (gamat emas), Stichopus ocellatus,* and *Stichopus vastus* (Kamarudin *et al.*, 2009). The bottom of the Stichopus genus is somewhat flattened and covered with podia or tube feet. Tubercules and papillae also cover the body. (Moriarty, 1982).

6.8 Pharmacologic properties of *Stichopus horrens*

From Malaysia to the Society Islands, around French Polynesia, and from southern Japan and Hawaii to New Caledonia, *Stichopus horrens* can be found in the Pacific Ocean. *S. horrens* has a grey-brown body with irregular grey-white markings. Sea cucumbers have been studied for their pharmacological effects, and it has been discovered that they have antibacterial, aantiinflammatory anti-fungal, anti-cancer, and wound healing properties. In a study, Nurjehan Mohamed Ibrahim *et al* (2018) discovered that the sea cucumber *S. horrens* possesses antibacterial properties against *Streptococcus mutans*. *S. horrens* contains promising amounts of antioxidant and cytotoxic natural compounds that could be employed for cancer prevention and treatment, according to findings from Althunibat *et al.*, 2013. Moelyono *et al*. (2018)

reported that an ethanolic extract of *S. horrens* possesses aantiinflammatoryproperties. Compared to the standard (Povidone iodine ointment) and the control, *Stichopus horrens* has wound healing activity, as it showed a higher percentage of wound contraction (Subramaniam *et al.*, 2013). MTS assay results did not show the potential effect on inhibiting the proliferation of cancer cells, MCF7 and MDA MB 231 cells, which might give antiapoptotic activity in a study conducted by Adila (2014) on the effect of sea cucumber, *S. horrens*, in its ability to induce cell death (apoptosis) in cancer cell lines.

Various chemicals derived from sea cucumbers have been attributed to a range of distinct biological and pharmacological activities, including anticancer, anticoagulant, antibacterial, antioxidant, antihyperlipidemic, antihyperglycemic, aantiinflammatory antihypertension, and radioprotective (Shi *et al.*, 2016). The presence of a wide range of bioactive, including triterpene glycosides, acid mucopolysaccharide, sphingoid bases, glycolipids, fucosylated chondroitin sulphate, polysaccharides, phospholipids, cerebrosides, phosphatidylcholines, and other extracts and hydrolysates, can be linked to the therapeutic properties and medicinal benefits of sea cucumbers (Shi *et al.*, 2016).

Moreover, sea cucumbers have been recorded to display curative effects on many liver and kidney diseases (Dakrory *et al.*, 2015). In 2021, based on Movahed *et al.*, the antimicrobial potential of sea cucumber extract can be attributed to the presence of antimicrobial agents or accumulation of several bioactive compounds such as steroidal saponins, polyunsaturated fatty acids (PUFAs) , glycolipids , polyamines , carotenoids, and sterols which act as the bioactive compounds. A recent study found that the best spectrum of bactericidal effect was found in the ethyl acetate extracts of *S. horrens*, with a ratio of MBC/MIC4 obtained from four bacteria strains evaluated by both disk-diffusion and well-diffusion methods. Thus, ethyl acetate extracts of *S. horrens* at a 12 mg/g can be used as bioactive chemicals to impede bacteria growth and eventually kill them (Movahed *et al*., 2021). Based on the study conducted by Moelyono *et al*., 2018, they were 20 rats divided into 4 groups treated with diclofenac sodium and ethanol extract of *Schitopus horrens* to check for the aantiinflammatoryproperties and they concluded that ethanolic extract of *Stichopus horrens* has best aantiinflammatoryactivity.

6.9 Effect of sea cucumber on various animals

The *Stichopus horrens* contains spingoid bases that will cause the reduction of cell viability, induction of apoptosis and increasing caspase-3 activity, which helps in the treatment of tumors (Khotimchenko, 2018). In 2018, Khotimchenko mentioned that after receiving the saponin fraction from the sea cucumber, rats with streptozotocin-induced hyperglycemia and insulin insufficiency showed a significant drop in blood glucose levels and a rise in blood insulin. In addition to lower blood glucose levels, Saponin administration resulted in lower levels of adiponectin, pro-inflammatory cytokines interleukin-6 and tumour necrosis factor-alpha in serum, and L-malondialdehyde in the liver. It was linked to a higher amount of glycogen stored in the liver.

Sea cucumber protein, specially produced from the body wall, is rich in glycine, glutamic acid and arginine. Glycine can stimulate IL-2 and B cell antibody production and release, thus enhancing phagocytosis. Glycine and glutamic acid are essential components for cells to synthesize glutathione which can stimulate the activation and proliferation of NK cells. Arginine can enhance cell immunity by promoting the activation and proliferation of T-cells. Due to these amino acid components, sea cucumbers have remarkable functions in immune regulation (Bordbar *et al*., 2011). Amino acid's pharmacological effects as an eicosanoids precursor and a substantial component of cell membrane phospholipids are well recognized. It is known to play a function in the growth and clotting of blood that helps in wound healing (Bordbar *et al*., 2011).

7.0 MATERIALS AND METHODS

7.1 Isolation and Identification of field isolates

Field and local isolates of *Mannheimia haemolytica* was obtained from a clinical case of Pneumonic Pasteurellosis. The bacteria were cultured in blood and MacConkey's agar plates and incubated at 37°C for 48 hours. Biochemical tests (catalase, nitrate reduction, urease, triple sugar iron), ornithine decarboxylase and O-nitrophenyl B, D-galactosidase (ONPG) disc test as well culture in purple agar were conducted for further confirmation of the bacterium.

7.2 Sea cucumber extract collection and antimicrobial evaluation

Sea cucumber extract was purchased from a local supplier. The extract was tested for its sterility by inoculating 1 ml of the extract in 1 ml of sterile nutrient broth. Incubation was done at 37 °C for 24 hours. The absence of turbidity was observed after the incubation.

7.3 Inoculum Preparation and Colony Forming Unit Counts

Few colonies from the blood agar plate were inoculated into a brain-heart infusion broth (BHI) and were incubated in an incubator shaker set at 37°C, 150 rpm for 48 hours. The BHI broth was serially diluted (10-fold). One millilitre of 10^7 dilutions was used as inoculum and simultaneously plated onto a blood agar plate for colony-forming unit (CFU) count.

7.4 Animal management & experimental design

The experimental procedure was conducted under the approval of the Animal Care and Use Ethics Committee (ACUC), Faculty of Veterinary Medicine, Universiti Malaysia Kelantan. Eight rats were used in this study. The rats were kept in separate cages and fed with commercial rat pellets. Drinking water was provided *ad libitum*. The rats were acclimatized for one weeks before the experimental study. The rats were divided into 2 groups (Groups 1 and 2). All rats were inoculated with 10^7 cfu/1mL of *Mannheimia haemolytica* intranasally. Group 1 was kept in negative control and did not receive any treatment. Rats in group 2 received sea cucumber extract during acclimatization and post inoculated orally every day at a dose of 2ml/day for 14 days of post-inoculation.

7.5 Daily clinical observation and post mortem evaluation

All rats were observed daily for any clinical signs such as mucopurulent discharge, signs of lethargy, and inappetence. The respiratory effort and behaviour were also observed. All rats were sacrificed on day 14 post-infection. Photographs of all organs were taken for further evaluation. All organ samples from rats were collected for gross and histopathological examinations.

7.6 Histopathological evaluation of organs

For the histopathological evaluation, all of the following organs, including the heart, lung, liver, kidney, and spleen, were collected and fixed in 10% formalin for 48 hours. After fixation, the samples were sliced to 0.5 cm thickness and placed in plastic cassettes for dehydration by using an automated processor (Leica ASP300, Germany). The tissue samples were later embedded with paraffin. The tissue blocks were trimmed and sectioned at 4 μm thickness by using a rotary microtome (Leica RM 2155, Germany). The tissue sections were mounted on glass slides using a hot plate (Leica H11220, Germany) at 42°C and allowed to dry overnight. Subsequently, the tissue sections were deparaffinizedby xylene for 5 minutes and rehydrated by two changes of different ethanol dilutions (100% and 80%) for 5 minutes each, respectively. The tissue sections were then rinsed in tap water and stained with Haemotoxylin and Eosin (H&E) staining. The histopathological scoring was performed on the organ section of all rats. About ten microscopic areas for each slide will be observed at different magnifications (200x and 400x). The lesions were scored on a scale of 0–3.

7.7 Data analysis

Data were statistically analysed by using Statistical Package for Social Science (SPSS) software version 23. The values were expressed as mean ± standard deviation (SD) for different parameters. Analysis of variance (ANOVA) tests was done to compare data differences between and within groups. Post hoc analysis using the Duncan test was used to determine the level of statistical significance which was set at $p<0.05$. The histopathology results was expressed as mean ± SD and analysed using Kruskal-Wallis test for global comparison of groups of all parameters. Non-parametric Mann-Whitney-U test was used for comparison between the two groups.

8.0 RESULT

8.1 General Observation

After sea cucumber extract administration, all rats in group 2 were observed for any clinical signs of adverse effects for the first 30 minutes, followed by 6 hours intermittently for one day. After that, daily observation was made before the bacteria inoculation for one week during the acclimatization period. There was no adverse effect observed. After one week of acclimatization, all rats were inoculated with *Mannheimia hemolytica* at 10^7 CFU and were observed daily for the clinical signs. The results revealed that all rats in both groups were dull but still responsive. However, those rats from the treated group show improvement and are more active than the non-treated group. There were no adverse clinical signs such as oculonasal discharge or difficulty in breathing.

8.2 Gross lesions and histopathology evaluation of lung and liver in rats

<u>8.2.1 Gross lesion of the lung</u>

The images of gross lesions of the lung and liver were taken for evaluation between the treated and non-treated groups. There was a presence of consolidation in the lung regions and the distribution of the lesions differed depending on the lung lobes involved. For the group of rats that received the extract; there was consolidation at the caudal part of the left lobe and all of the right lobes (G2 A1), consolidation at all lobes (G2 A2) and consolidated area interspersed with normal lung appearance (G2 A3 and G2 A4).

For the group of rats that did not receive any treatment, there was a consolidated area at all lung lobes (G1 B1), consolidation lesion at the lobes but still intermixing with normal lung appearance (G1 B2 and G1 B3), consolidation area on the left side and right caudal lobes (G1

B4). In general, lung lesions were observed in both groups and the difference in the severity of organ lesions between groups was not differentiated.

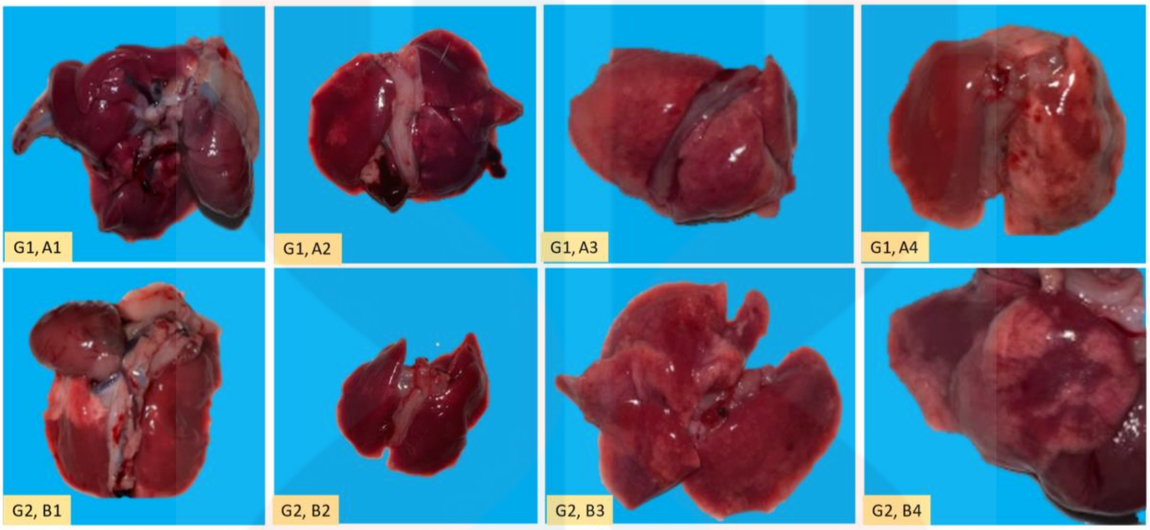

Figure 1: G1 A1 to G1 A4 shows the gross lesion of the lung in the non-treated group rat, while G2 B1 to G2 B4 shows the gross lesion of the lung in the treated group.

8.2.2 Histopathological evaluation of lung

Based on Table 1 and Figure 2, the haemorrhage, edema and thrombus lesions in the lung showed a significant ($p<0.05$) decrease in the lesions scoring of group 2 compared to group 1. Histopathologically, the haemorrhage lesion is more severe in group 1compared to group 2 as many regions show extravasation of red blood cells. For the edema lesion, group 1 shows more severe fluid extravasation than group 2. Then, many of the blood vessels in group 1 revealed the presence of homogenous pinkish material (thrombus) compared to group 2.

Table 1: Lung lesion scoring

Parameters	Group 1	Group 2	Asymptotic significant (p<0.05) Kruskal-Wallis test	Asymptotic significant (p<0.05) Mann-Whitney test
Inflammation	1.50 ± 1.00	0.50 ± 0.58	0.13	1.00
Edema	2.25 ± 0.5	0.75 ± 0.50	0.04	0.01
Hemorrhage	2.50 ± 0.58	1.5 ± 0.82	0.02	0.03
Thrombus	2.50 ± 0.58	2.50 + 0.00	0.01	0.04

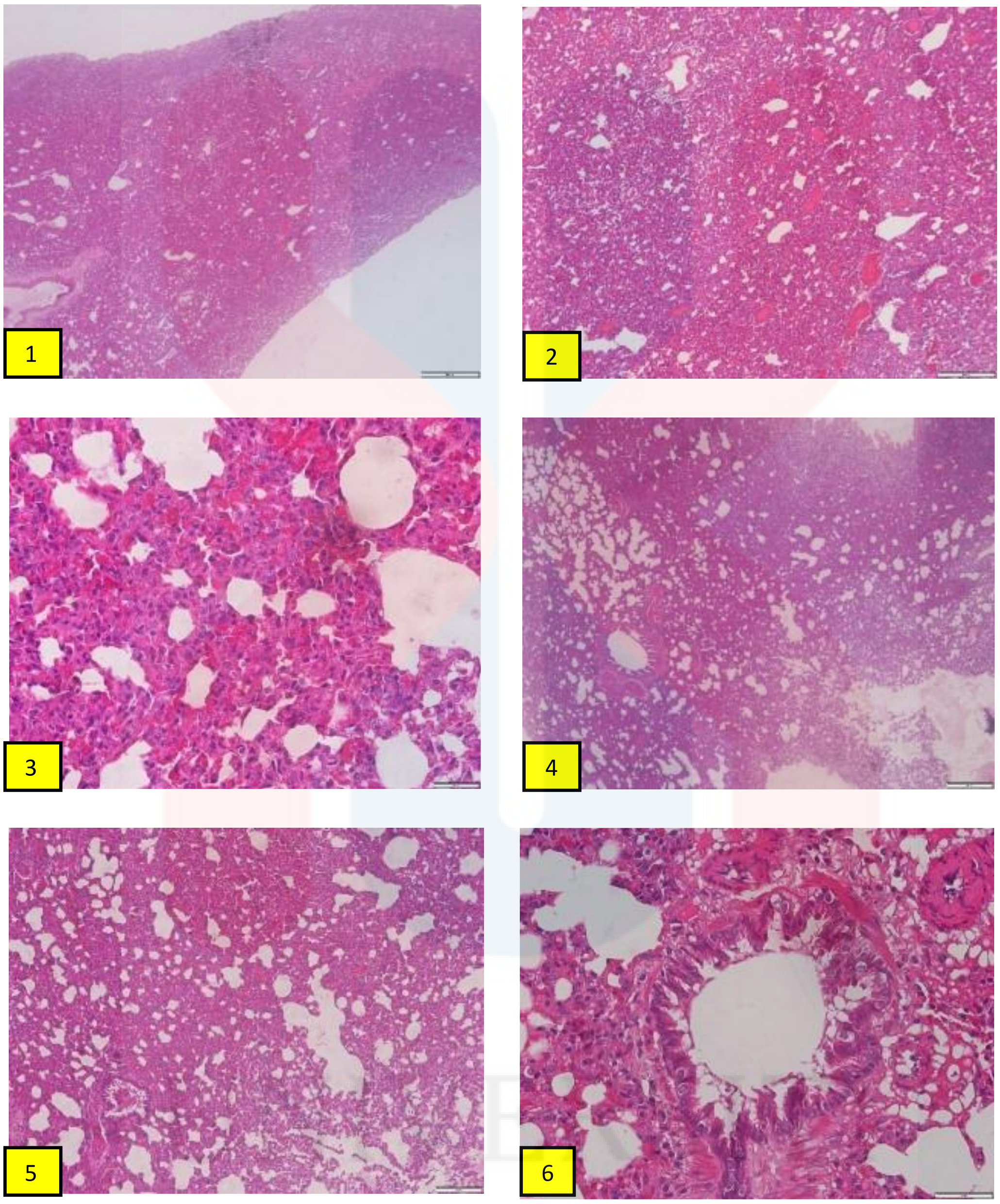

Figure 2 (1): Cross sections of lungs of the treated group rat at 4X magnification, Figure 2 (2): Lung section of treated group rat at 10X magnification, Figure 2 (3): Lung section of treated group rat at 40X magnification, Figure 2 (4): Lung section of treated group rat at 10X magnification, Figure 2 (5): Lung section of treated group rat at 10X magnification, Figure 2 (6): Lung section of treated group rat at 40X magnification (H&E staining).

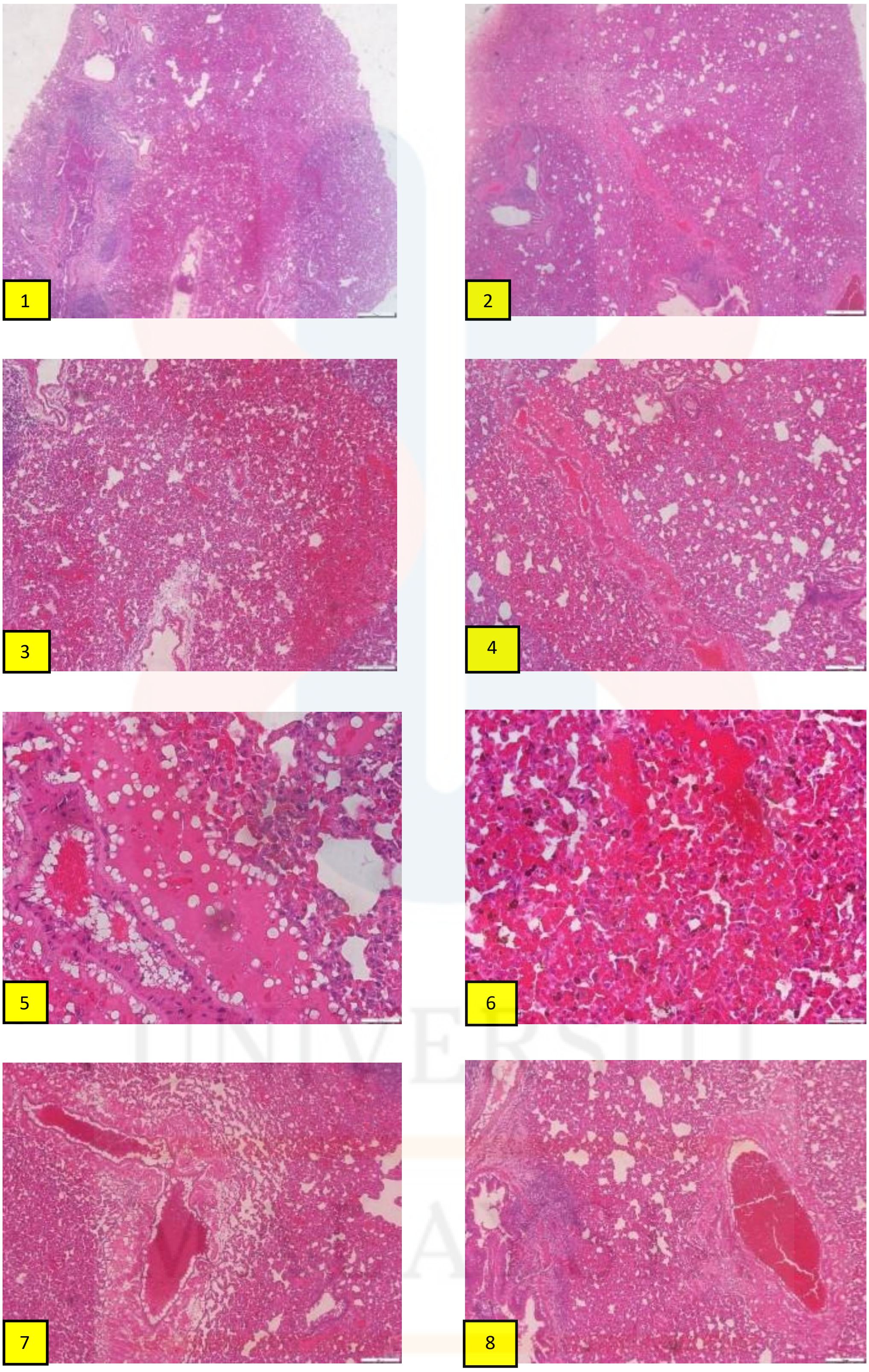

Figure 3 (1): Lung section of non-treated group rat at 4X magnification, Figure 3 (2): Lung section of non-treated group rat at 10X magnification, Figure 3 (3): Lung section of non-treated group rat at 40X magnification, Figure 3 (4): Lung section of non-treated group rat at 10X magnification, Figure 3 (5): Lung section of non-treated group rat at 40X magnification, Figure 3 (6): Lung section of non-treated group rat at 40X magnification (H&E staining).

8.2.3 Gross lesion of the liver

All liver shows the presence of hepatomegaly and hepatic congestion. The lesion of hepatic congestion was more evident in the non-treated group 1 than in treated group 2. All liver lobes were involved, but the distribution of the lesions was more severe in group 1.

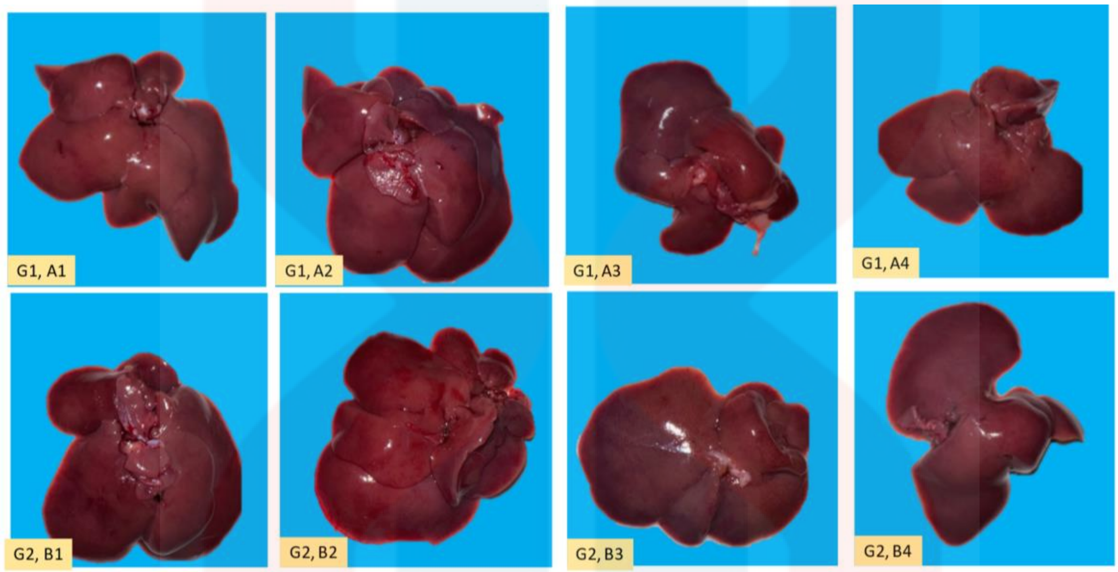

Figure 4: G1 A1 to G1 A4 shows the gross lesion of the lung in the non-treated group rat, while G2 B1 to G2 B4 shows the gross lesion of the lung in the treated group.

8.2.4 Histopathological evaluation of liver

Histopathology evaluation of the liver in treated group 2 shows less severity of thrombosis and hemorrhagic lesions than in group 1. The thrombotic, edematous and hemorrhagic lesions in the lung show a significant decrease in the lesion scoring ($p<0.05$) of group 2 compared to group 1 (Table 2).

Table 2: Liver lesion scoring

Parameters	Group 1	Group 2	Asymptotic significant (p<0.05) Kruskal-wallis	Asymptotic significant (p<0.05) Mann-whitney
Inflammation	0.00 ± 0.00	0.00 ± 0.00	1.000	1.00
Edema	3.00 ± 0.00	1.25 ± 0.50	0.01	**0.03**
Hemorrhage	2.00 ± 0.82	0.50 ± 0.58	0.04	**0.05**
Thrombus	1.75 ± 0.50	0.75 ± 0.50	0.04	**0.05**

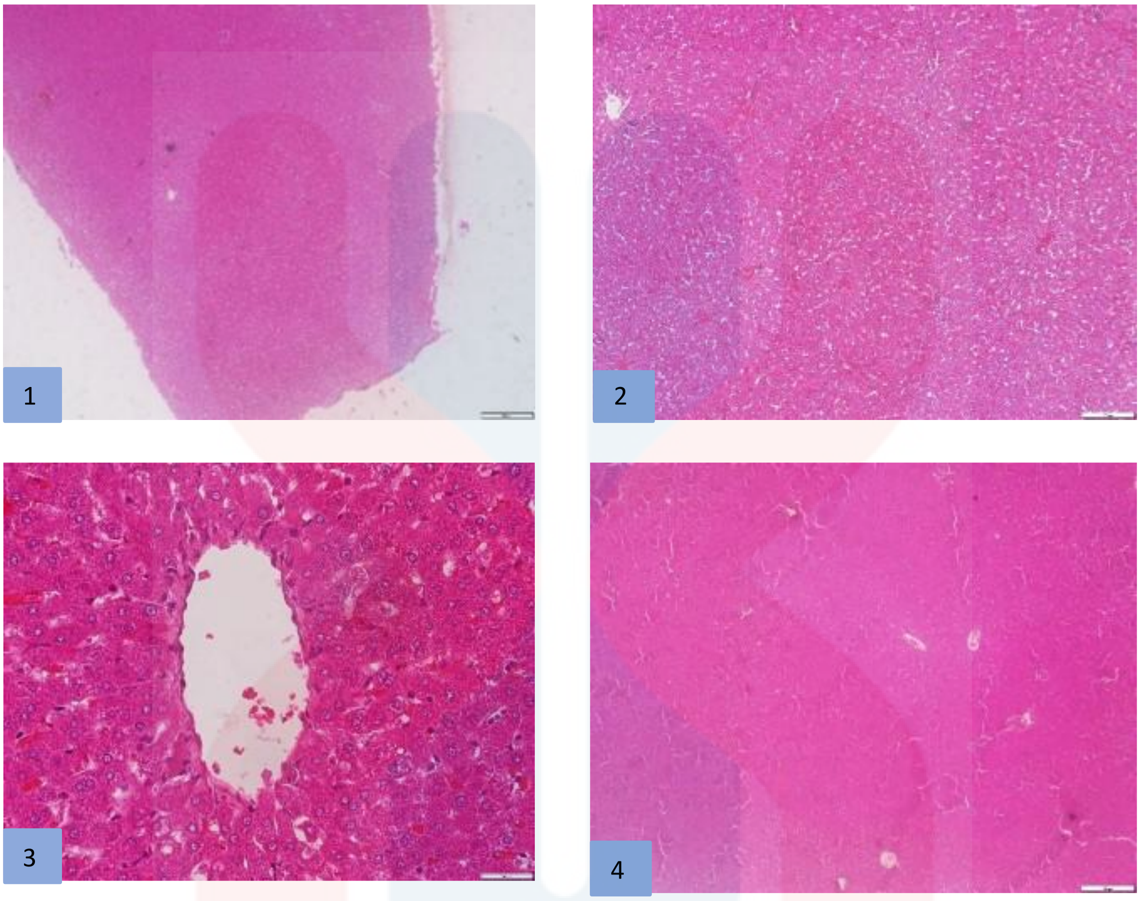

Figure 5 (1): Cross section of liver from treated group rat at 4X magnification, Figure 5 (2): Liver section of treated group rat at 10X magnification, Figure 5 (3): Liver section of treated group rat at 40X magnification, Figure 5 (4): Liver section of non-treated group rat at 4X magnification (H&E staining).

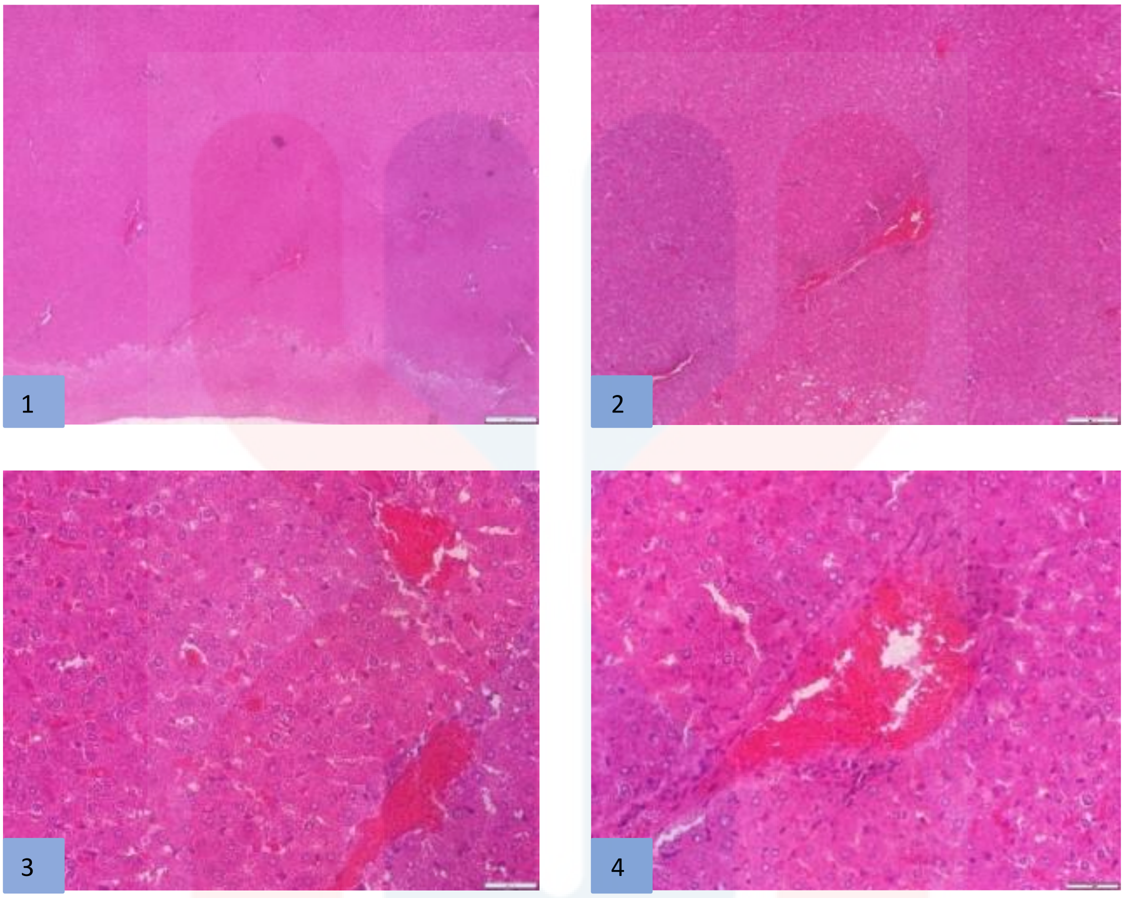

Figure 6 (1): Liver section of non-treated group rat at 10X magnification, Figure 6(2): Liver section of non-treated group rat at 10X magnification, Figure 6 (3): Liver section of non-treated group rat at 40X magnification, Figure 6 (4): Liver section of non-treated group rat at 40X magnification (H&E staining).

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9.0 DISCUSSION

Mannheimiosis caused by *Mannheimia hemolytica* is a highly infectious respiratory disease that has a significant economic impact on the livestock industry (Mohamed & Abdelsalam, 2008). The overuse of antibiotics in controlling this disease leads to antimicrobial resistance, where most treatments are not successful. Alternatively, natural sources containing antimicrobial properties can be used to treat a bacterial infection, and it has become an essential requirement (Hassanshahian *et al*., 2020).

From the results of this study, the lung and liver in group 2, where the rats were treated with sea cucumber extract, showed less severe pathological lesions than in group 1 (negative control). Grossly, the most prominent lesions shown in the lungs were consolidation and appeared dark red and firm upon palpation. The findings were similarly consistent with another study of Mannheimosis, where the affected lung region showed hepatization with crusty consistency (Dutta *et al.*, 2020*)*. Meanwhile, some areas of the lung fields appeared normal in the treated group (pinkish colour).

Mannheimia hemolytica, in the present study, was inoculated orally using oral gavage; hence the distribution of the lesions was different compared to the animal infected through the nasal route. If the portal of entry is through nasal route, the lesion in the lung would be in cranioventral distribution affecting the cardiac and apical lobes and if the portal of entry was through oral route, the distribution of the lesion in the lung would be bilaterally.

For the animal being infected through the oral route, as in this study, the lesions appeared to be more generalized and diffused because the bacteria travel into the gastrointestinal system, affecting the mesenteric lymph node and travel in the blood circulation towards the heart and lung. The spreading of the bacteria was hematogenous, which is septicemia. Spreading of the bacteria causes injury to the blood vessel leading to the formation thrombus that further obstructs the blood vessel and leads to organ congestion. Hence, the liver becomes reddening in colour, indicating hepatic congestion.

The bacteria would then spread to the heart and the lung. Once the bacteria reach the lung, it triggers the inflammation to occur. There would be an increase in the permeability of the blood vessel and extravasation of fluid and red blood cells into the interstitial and alveolar spaces. This condition leads to pulmonary consolidation, inflammation, edematous and hemorrhagic, where the alveolar spaces are filled with inflammatory cells, proteinaceous fluid and red blood cells. The lung and liver gross lesions between groups 1 and 2 were difficult to distinguish, except through histopathological evaluation.

As for the histopathology lesions, there are significant differences between lung lesions in group 2 of rats treated with sea cucumber extract compared to group 1 rats (negative control). Group 2 rats show a significant ($p < 0.5$) decrease in lung lesion scoring; hemorrhage, edema, and thrombus. While for the liver, group 2 also demonstrated a significant ($p < 0.5$) reduction in thrombotic, edematous and hemorrhagic lesions l compared to the group 1. The virulence factor for *Mannheimia hemolytica* are capsule, fimbriae, adhesin, leukotoxin, proteinase, neuraminidase, lipopolysaccharides and outer membrane protein which contribute to the severity of lesions observed in this study (Laishevtsev, 2020).

From the experimental study that we have conducted and based on the data analysis of histopathological scoring, the use of sea cucumber extract *Stichopus horrens* is quite effective in treating Mannheimiosis as there is a significant difference between the lesion severity in group 2 and group 1. From a previous study, they found out that *Stichopus horrens* has broad-spectrum antimicrobial activities which contain antimicrobial agents or accumulation of several bioactive compounds such as steroidal saponins, polyunsaturated fatty acids (PUFAs) , glycolipids, polyamines , carotenoids, and sterols which act as bioactive compound (Mohaved *et al*,.2021). These findings supported the fact that *Stichopus horrens* inhibit the growth of *Mannheimia hemolytica* in treated group rats, making them less severe lesions than the non-treated group.

10.0 CONCLUSION

In conclusion, sea cucumber (*Stichopus horrens)* extract was demonstrated to decrease the severity of lung and liver lesion of rats inoculated with *Mannheimia hemolytica*. From the experimental study that we have conducted and based on the data analysis of histopathological scoring, the use of sea cucumber extract *Stichopus horrens* is quite effective in treating Mannheimiosis as there is a significant difference between the lesion severity in group 2 and group 1

11.0 RECOMMENDATION

There are a few recommendations to improve the study in the future. First of all, a negative control group which is not inoculated and treated needs to be established. Second, it is recommended to expose the rat with *Mannheimia hemolytica* through the nasal route as the lesion distributions at the lung and other organs would be different. more studies need to be done with respect to the dose of the extract. The duration of sea cucumber administration can be extended to investigate the distribution and organ lesion changes further.

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