UNIVERSITI PUTRA MALAYSIA

ANTI-INFLAMMATORY ACTIVITY OF PARKIA SPECIOSA IN RATS

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FPV 2020 9

ANTI-INFLAMMATORY ACTIVITY OF *PARKIA SPECIOSA* IN RATS

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A project paper submitted to the

Faculty of Veterinary Medicine, Universiti Putra Malaysia

In partial fulfillment for the requirement for

THE DEGREE OF DOCTOR OF VETERINARY MEDICINE

Universiti Putra Malaysia

Serdang, Selangor

2020/2021

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It is hereby certified that we have read this project paper entitled "Anti-inflammatory Activity of *Parkia Speciosa* in Rats" by Norazlin binti Yasin and in our opinion it is satisfactory in term of score, quality and presentation as partial fulfillment for the course VPD 4999- Project.

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ACKNOWLEDGEMENTS

I wish to express my sincere and deepest appreciation to my supervisor, Professor Madya Dr. Mohd Hezmee B. Mohd Noor and my co-supervisor, Professor Madya Dr. Hazilawati binti Hj Hamzah and Dr Mazlina binti Mazlan for their guidance, valuable advice, support, motivation and constructive criticisms in the course of preparing this project.

Thanks to Prof. Rasedee Abdullah for allowing me to use Haematology & Clinical Pathology Laboratory. My appreciation also goes to Puan Darulmuqaamah, Encik Johari and Puan Jamilah for assisting me in the Pharmacology Laboratory, Haematology & Clinical Pathology Laboratory and Histopathology laboratoty and also for their technical supports.

Special thanks to En. Aiman, En. Qayyum, Cik Atira, En. Usamah, Cik Aliaa, En, Thaqif and my coursemates, who helped me in many ways to accomplish this study, for their valuable comments and suggestions.

To my family, I would like to dedicate my heartiest gratitude for their support and encouragement throughout my academic life. Last but not least, thanks to all that helped me directly or indirectly throughout the project.

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ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek

AKTIVITI ANTI-KERADANGAN *PARKIA SPECIOSA* DALAM TIKUS

Oleh

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2020

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Kata kunci : *Parkia speciosa, anti-keradangan, edema kaki*

Perkia speciosa (P. speciosa) atau lebih dikenali sebagai *petai papan* oleh orang tempatan ialah salah satu tumbuhan perubatan tradisional yang dijumpai di Asia Tenggara dan mengandungi 'polyphenol'. Tumbuhan yang mempunyai

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kandungan 'polyphenol' yang tinggi dilaporkan mempunyai kesan anti-keradangan. Kajian secara *vivo* untuk menilai potensi anti-keradangan tumbuhan ini sangat kurang dijalankan. Oleh itu, kajian ini bertujuan untuk menunjukkan anti-inflamsi *P. speciosa* pada tiga dos yang berbeza terhadap tikus-tikus yang telah diberikan lipopolisakarida (LPS) pada kaki yang menyebabkan pembentukan edema, untuk melihat perbezaan anti-keradangan antara rawatan ekstrak *P. speciosa* dan flunixine meglumine, jumlah sel darah putih, pembezaan kiraan sel darah putih, parameter darah merah, biokimia plasma dan penilaian histologi pada kaki edema. Sejumlah 36 ekor tikus dibahagikan secara rawak dan sama rata kepada enam kumpulan. Kaki kanan tikus diberikan LPS (100 µg/100 µL) secara akut setelah diberikan ubatan daripada ekstrak petai pada tiga kepekatan (100 mg/kg, 200mg/kg, 400mg/kg) bagi kumpulan satu sehingga kumpulan tiga dan untuk kumpulan empat, flunixine meglumine (2.5 mg/kg) diberikan yang berfungsi sebagai kawalan positif, manakala bagi kumpulan lima, tiada rawatan akan diberikan dan berfungsi sebagai kawalan negatif. Kumpulan enam berfungsi sebagai kawalan dimana tiada LPS diberikan pada kaki mahupun rawatan. Ketebalan kaki edema, jumlah dan pembezaan kiraan sel darah putih, parameter darah merah, biokimia plasma dan penilaian histologi pada kaki edema telah dinilai. Hasil kajian menunjukkan penurunan ketara ($p < 0.05$) dalam pembentukan edema 5 jam selepas induksi pada semua dos *P. speciosa* (100, 200 dan 400 mg/kg). Penurunan ketara dapat dilihat pada ratio neutrophil dan limfosit (N:L) selepas kajian berbanding sebelum bagi kumpulan rawatan 200 mg/kg *P. speciosa*. Walau bagaimanapun, tiada kesan ketara dilihat pada jumlah dan pembezaan sel darah putih, parameter darah merah dan biokimia plasma. Penilaian histologi kaki edema menunjukkan perbezaan ketara ($p < 0.05$) pada kumpulan tikus

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yang dirawat dengan semua dos *P. speciosa* berbanding kumpulan kawalan negatif. Sebagai kesimpulan, *P. speciosa* dapat menghasilkan kesan anti-keradangan.

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ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfillment of the VPD 4999 – Project.

ANTI-INFLAMMATORY ACTIVITY OF *PARKIA SPECIOSA* IN RATS

by

Norazlin binti Yasin

2020

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Co-supervisor I : Associate Professor Dr. Hazilawati binti Hj Hamzah

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Parkia speciosa (P. speciosa) or stink beans and locally known as “petai papan” is one of the traditional medicinal plants found in Southeast Asia that contains polyphenol. It is reported that the plant contains high concentration of polyphenol compound and possess anti-inflammatory properties. Currently, there is lack of in vivo study has been conducted to evaluate its potential as an anti-inflammatory substance. Hence, the objectives of this study are to determine the anti-inflammatory activity of *P. speciosa* extract using three different dosages on

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lipopolysaccharide (LPS)-induced paw oedema in rats, and to compare the inflammatory activity between the extract and flunixine meglumine in those rats, by evaluating the numbers of total white blood cell (TWBC) and differential WBC, erythron parameters, plasma biochemistry values and also histological scoring of the oedematous paw. Thirty-six rats were divided randomly and equally into six groups. The rat hind paw was injected with LPS (100 µg/100 µL) 30 minutes after the rats were orally administered with the extract at three different concentrations (100 mg/kg, 200mg/kg, 400mg/kg). The groups administered with the extract were named as groups I to III, group IV was flunixine meglumine (2.5 mg/kg) group which served as the positive control, group V acted as the negative control and group VI as control. Paw oedema thickness, total WBC number and its differential, erythron parameters, plasma biochemistry and histology of the paw were evaluated. Results showed significant reduction ($p < 0.05$) in the paw oedema thickness at all concentrations of *P. speciosa* extract (100, 200 and 400 mg/kg) starting at 5 hours after LPS was infected, and the results were comparable with flunixine meglumine. Significant reduction in the ratio of neutrophils to lymphocytes was observed in rats given 200 mg/kg P. speciosa extract at the end of the experiment compared to the other groups. Histological scoring of the inflammatory reaction and oedematous lesion in the paws showed significant reduction ($p < 0.05$) in rats treated with three concentrations of *P. speciosa* extract compared to negative control group. The findings conclude *P. speciosa* extract possesses anti-stress and anti-inflammatory activities in rats.

Keywords : *Parkia speciosa, anti-inflammatory, paw oedema*

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1.0 INTRODUCTION

Stink bean, *Parkia speciosa* Hassk, is a plant that belongs to the pea or bean family Fabaceae and placed in Leguminosae and Mimosaceae (Lim, 2016). It is a classic Malaysian favourite and is commonly grown and cultivated in Southeast Asian countries e.g. Indonesia, Malaysia, and some parts of northeastern India (Al Batran et al., 2013). The seeds can be eaten raw as "ulam" (a Malay word for uncooked) or cooked and have a peculiar smell. Half-riped seeds are also usually pickled in brine. The seeds of this plant have been used by the locals to treat illnesses such as diabetes, kidney disorders and headache (Samuel et al. 2010; Milow et al 2011; Azliza et al. 2012).

P. speciosa contain several phytochemical compounds such as polyphenols and flavonoids (Ghasemzadeh et al., 2018; Sonia et al., 2018), alkaloids (Ghasemzadeh et al., 2018; Hasim et al., 2018; Sihombing et al. 2015), terpenoids (Ghasemzadeh et al., 2018; Hasim et al., 2018; Musa et al. 2012), saponins (Hasim et al., 2018; Sihombing et al. 2015), steroids (Hasim et al., 2018; Musa et al, 2012; Sihombing et al. 2015)¬, tannins (Hasim et al., 2018; Balaji et al. 2015) and phytosterol (Ko et al., 2003). It also contains three natural sugars, which are sucrose, fructose and glucose together with fiber. The combination of these substances can provide instant energy boost. *P. speciosa* has been reported to exhibit many pharmacological effects include antioxidant and antimicrobial activities (Sonia et al., 2018), anti-tumor effect (Aisha et al., 2012) and as an anti-stress effect (Shuib & Hassan et al., 2017). According to

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previous studies (Diaz-Rivas et al., 2015; Tuzcu et al., 2017), plants with polyphenol compounds possess anti-inflammatory properties. Thus it is possible that *P. speciosa* treatment will also possess anti-inflammatory effect. To demonstrate this, lipopolysaccharide (LPS)-induced paw oedema in rats (Bargavi et al., 2004) can be used as a model to evaluate its anti-inflammatory effect. Currently, there is an absence of study in-vivo in evaluating the medicinal effects of *P. speciosa* (Azizul et al., 2019). The present study was therefore undertaken to investigate further the anti-inflammatory effect of *P. speciosa* at different dosages on LPS-induced paw oedema on the total white blood cell count (WBC), differential count of WBC, erythron parameter, plasma biochemistry and histological evaluation of the paw of the treated rats.

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2.0 LITERATURE REVIEW

2.1 Nutritional values of *Perkia speciosa*

The *P. speciosa* seeds are rich in carbohydrates (68.3-68.7%), proteins (6–27.5%), fats (1.6–13.3%), fibers (1.7–2.0%) and minerals (0.5–0.8%) content and it also contains abundant amount of minerals such as calcium (108–265.1 mg), magnesium (29 mg), potassium (341 mg), phosphorous (115 mg), iron (2.2–2.7 mg) per 100 g of the edible portion of the plant (Kamisah et al., 2013).

The pods contains 84.24 mg GAE/g of phenolic compound while the seeds contains 51.9 mg GAE/g of phenolic compound. The seeds contain 20.3 mg retinol equivalent (RE)/g of flavonoid content (Zaini et. al., 2017) and also 19.3 mg vitamin C and 4.15 mg α-tocopherol (vitamin E) per 100 g (Kamisah et al., 2013). The seed coats and pods being being detected to contain high concentration of tannin compared to other fruit vegetables (Tunsaringkarn, 2012).

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2.2 Phytochemical compound of *Perkia speciosa*

Several phytochemical compound can be found in *P. speciosa* (stinky beans) such as phenolics, flavonoids, alkaloids and terpenoids in all parts of the plants that account for its diverse health benefits (Chhikara, 2018). In fresh *P. speciosa* seeds seventy-seven chemicals were also identified and the major constituents are ethanol, H2S (Hydrogen Sulphide) and C2H4S3 (1,2,4-trithiolane), CH3CH2COOH (propanoic acid) and C30H58O4S (3,3-thiobis-didodecyl ester) (Salman et al. 2006). The pods that contain polysaccharide from 17–18% yield is found to contain 97–99 mg/g uronic content and high antioxidant property (48–50% DPPH scavenging activity) while the pods also found to have high phenolic and flavonoid contents accompanied by high antioxidant properties (Gan et al, 2010).

Phenolic compound of *P. speciosa* was being concluded to have antioxidant substances by the action of oxidation termination by scavenging free radicals to form stabilized radicals (Rice-Evans et al, 1997). Flavonoid is also being reported to have antioxidant activity by inhibiting xanthine oxidase and having superoxide scavenging activities (Cos et al., 2000) while it also shows to have antibacterial, antifungal, hepatoprotective, anti-inflammatory, anti-diabetic effects, etc. (Tapas et al., 2008).

Another compound is terpenoids which include β-sitosterol, stigmasterol, lupeol, campesterol and squalene. Synergistic action of β-sitosterol and stigmasterol was shown to have hypoglycaemic effect (Fathaiya et al., 1994).

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The current analysis of alkaloids in Perkia speciosa being reported was only at screening stage (Ahmad et al, 2019). Alkaloids can be classified into different types inclusive indole, tropane, piperidine, purine, imidazole, pyrrolizidine, pyrrolidine, quinolizidine and isoquinoline alkaloids (Kaur et al., 2015; Matsuura et al., 2015; Roy, 2017) and different alkaloids have their own specific properties. Utilization of pure extracted plant alkaloids and their synthetic derivatives is commonly use as common medicinal ingredients for their analgesic, antispasmodic, bactericidal effects and some alkaloids are used as an antiseptic due to its antibiotic activity (Roy, 2017).

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2.3 Anti-inflammatory activity of *Perkia speciosa* Hassk

Phenolics consist of the aromatic ring (C6) bonded directly to at least one (phenol) or more (polyphenol) hydroxyl group (-OH) and other substituents such as methoxyl (CH3O−) or carboxyl (COOH) groups which these substituents either give the hydrophilic or hydrophobic character to the compounds (Kulbat, 2016). These compounds occurred in conjugated forms with one or more sugar residues that occurred in these compounds linked to hydroxyl group inclusive direct linkages of sugar to an aromatic carbon and there is also association forms with other compounds as well, such as carboxylic and organic acids, amines, lipids and other phenol (Pandey et al., 2009). Such structural diversity resulted of these compounds often referred to as polyphenols.

Polyphenols is one of the phytochemical compunds that can be found in *P. Speciosa* (Ghasemzadeh et al., 2018; Sonia et al., 2018). Polyphenols contain at least one aromatic ring and one or more hydroxyl groups, in addition to other components of them. The main groups of polyphenols are; flavanoids, lignans, stilbenes and phenolic acids. (Ahmad et al., 2019). According to the previous studies (Diaz-Rivas et al., 2015; Tuzcu et al., 2017), plants with polyphenol compounds possess anti-inflammatory properties.

Mustafa et al., 2018 reported that empty pod extract of *P. speciosa* possess anti-inflammatory activity in human umbilical vein endothelial cells (HUVECs) after exposing the cells to TNF- α. *P. speciosa* empty pod extract attenuate TNF- α -

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induced inflammatory responses by blocking the activation of NFκB p65 and thus reduces the iNOS, COX-2 and VCAM-1, as determined with Western blot analysis expressions as well as decreasing reactive oxygen species (ROS) and nitric oxide (NO) level which were comparable with quercetin which act as positive control thus this shows that *P. speciosa* possess anti-inflammatory activity.

A relatively similar study design was performed by Gui et al., (2019) found that NFκB p65 and p38 MAPK expression were reduced as well as iNOS, COX-2 and VCAM-1 expression and also NO and ROS levels were also reduced in cardiomyocytes pre-treated with *P. speciosa* extract or quercetin. This effect confirmed the postulation and could be attributable to the polyphenol content of *P. speciosa*, specifically quercetin.

Other study reported by Sonia et al. (2018) found out that *P. speciosa* empty pod extract produce anti-inflammatory activity through the action of inhibition of lipoxygenase activity, proteinase inhibitory activity and RBC membrane stabilization activity suggested that this action is due to presence of high levels of phenolics and flavonoids.

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2.4 Inflammation

Inflammation is categorized into acute and chronic stage. Inflammation is also classified into peracute, subacute and granulomatous inflammation (Damjanov, 2009; Ward, 2010). The Roman medical writer Aulus Cornelius Celsus in A.D 30 described the four clinical signs of inflammation which include color (heat), dolor (pain), tumor (swelling) and rubor (redness) (Serhan et al., 2011). In 19th century, Rudolf Virchow added the fifth signs of inflammation which is *function laesa* (loss of function) (Liu et al. 2017).

Acute inflammatory response is triggered when there is direct injury in the tissues caused by chemical, physical or biological agents manifested by a series of vascular events such as vasodilation, vascular leakage, oedema and leukocyte emigration (Medzhitov, 2008). Contraction and retraction of endothelial cells, and alteration in transcytosis are early phase of acute inflammation that initiate vascular events. In order to increase blood flow to the site of injury, blood vessel will dilate, thus causing the affected area to be redden or warmer. Then, blood vessels become permeable and allow blood plasma to diffuse into the extravascular tissue. As result, oedema will be formed due to the accumulation of plasma components and fluid into the affected tissue. In addition, several chemotactic factors such as chemokines and C5a resulted in concurrent emigration of neutrophil and leukocytes to the site of injury (Ward, 2010).

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When tissues are damaged or stimulated, arachidonic acid will be released by the action of phospholipase that act on the phospholipids in cell walls (Figure 1). After that, arachidonic acid is further metabolized through two pathways either cylooxygenation, with subsequent production of prostaglandins and thromboxanes by the action of cyclooxygenase enzyme; or through lipoxygenation, to form leukotrienes and lipoxins by the action of lipoxygenase enzyme. Collectively, prostaglandins, thromboxanes, leukotrienes and lipoxins are eicosanoids that contribute to inflammatory process and regulate a diverse set of homeostatic (Fung, 2001; Buczynski, 2009). Besides, other compounds such as histamine, kinins, substance P and superoxide radicals also contribute to the inflammation (Bill, 1997).

Corticosteroids are widely used to suppress tissue destruction associated with many inflammatory diseases by inducing synthesis of an inhibitor of PLA2 and block release of arachidonic acid in inflammatory cells, however prolonged administration can have significant harmful effects, including increased risk of infection, damage to connective tissue, and adrenal gland atrophy. Nonsteroidal antiinflammatory drugs (NSAIDs) inhibit cyclooxygenase, and to varying degrees, lipoxygenase thus this action will further reduce production of eicosanoids and eventually reduce inflammation, pain, and hyperthermia, however NSAIDs treatment in long term can damage the stomach mucosa and kidneys, possibly, because of a decreased cytoprotection by prostaglandin E2 (Hanna and Hafez, 2018).

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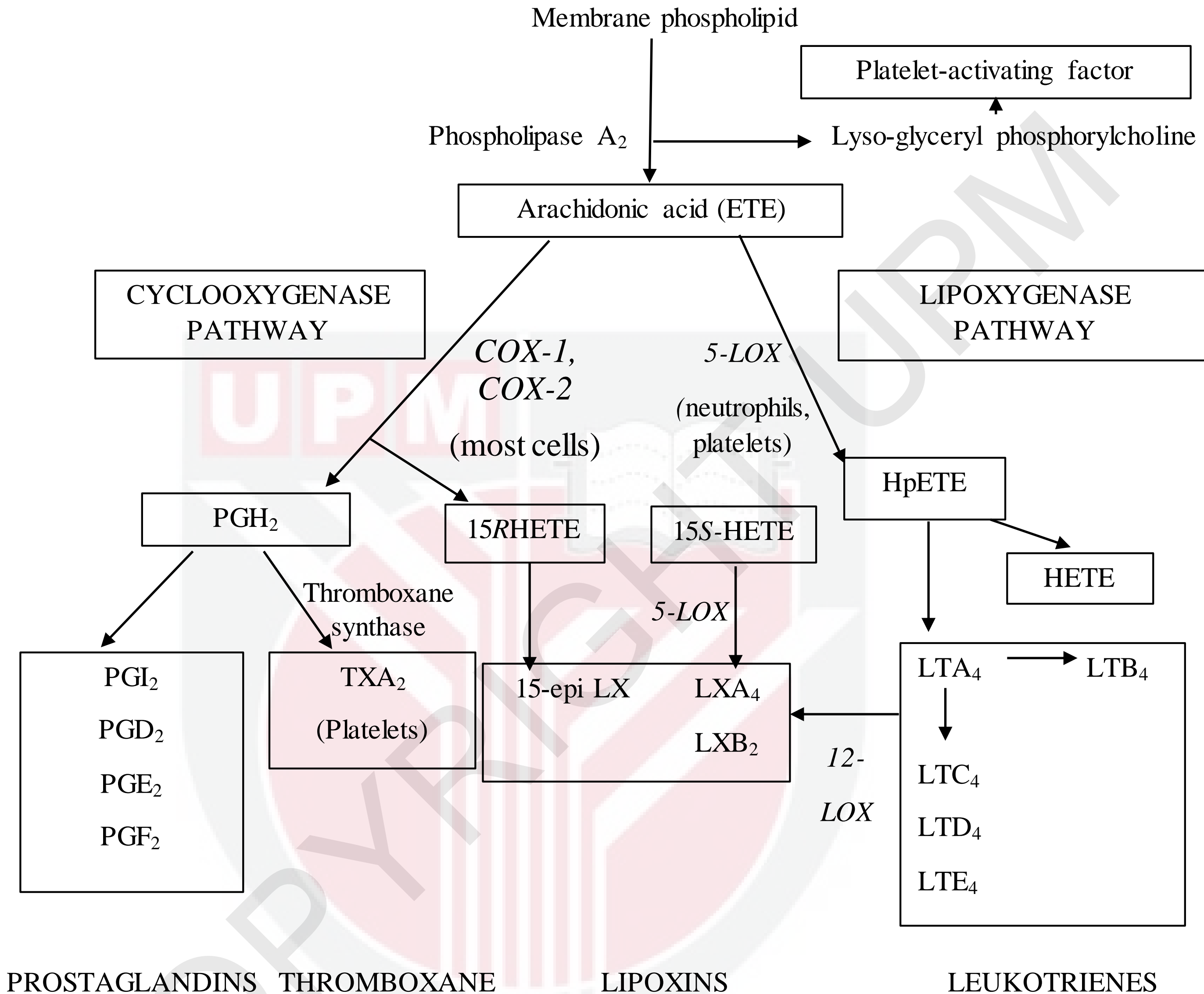

Figure 1 : The arachidonic acid cascade for production inflammatory mediators (Hanna and Hafez, 2018). Biologically active arachidonic acid metabolites. The cyclo-oxygenase pathway of arachidonic acid metabolism generates prostaglandins (PG) and thromboxane (TXA_2). The lipoxygenase pathway forms lipoxins (LX) and leukotrienes (LT); COX, cyclooxygenase; HETE, hydroxyeicosatetraenoic acid; HpETE, 5-hydroperoxyeicosatetraenoic acid; LOX, Lipoxygenase

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2.5 Lipopolysaccharide (LPS)

Potential anti-inflammatory agents in the pharmaceutical industry can be evaluated by using various method either in vivo or in vitro. Agents such as carrageenan, histamine, bradykinin, dextran and lipopolysaccharide have been used to induce inflammation in animal models and mechanisms for screening of anti-inflammatory. Acute localized inflammatory reaction and swelling of injected paw can be induced by subplantar injection of LPS into the rat hind paws (Calil et al., 2014; Vajja et al., 2004).

LPS is a molecule derived from the outer cell membrane of gram-negative bacteria. LPS preparations have been used in research for the elucidation of LPS structure, metabolism, immunology, physiology, toxicity and biosynthesis. They have also been used to induce synthesis and secretion of growth promoting factors such as interleukins. Because of its connection to septicemia, lipopolysaccharide has been studied to identify possible targets for antibodies and inhibitors to LPS biosynthesis (Merck, 2020).

LPS is a potent activators of macrophages, as well as of endothelial cells, smooth muscle cells and leukocytes. LPS stimulates host cells to produce and release endogenous mediators that include bioactive lipids such as platelet-activating factor and thromboxane A_2, reduced oxygen species, NO and also LPS activates cells via specific receptors, either directly or after binding a serum LPS-binding protein (LBP). It is a potent stimulus for production of TNF-a and interleukins (IL-1, IL-6,

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IL-8, IL-12, and others). Macrophage-derived cytokines modulate endothelial cell leukocyte adhesion (TNF-a), leukocyte recruitment (IL-8), the acute phase response (IL-6, IL-1), and immune functions (IL-1, IL-6, IL-12) (Schletter, 1995).

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2.6 Flunixine meglumine

Flunixin meglumine is a nonsteroidal anti-inflammatory drug (NSAID) and cyclooxygenase inhibitor. It is a potent analgesic, antipyretic, and anti-inflammatory. NSAIDs work by inhibiting the body's production of prostaglandins and other chemicals that stimulate the body's inflammatory response. Flunixin meglumine is a non-selective inhibitor of enzymes cyclooxygenases (COX1/ COX-2), which control the production of different prostanoids (prostaglandins and thromboxanes) from arachidonic acid being released from cell membrane phospholipids (Ciofalo et al., 1977; Beretta et al., 2005).

Several standard animal models of inflammation to establish the anti-inflammatorry effects of flunixine meglumine have been used and reported. Flunixine meglumine being reported to depress increases of TNFa, IL-1b and IL-10 concentrations in lipopolysaccharide-induced endotoxic mice (Yazar et al., 2007) while it also reduced the levels of thromboxane B2 and 6-keto-prostaglandin F1 in equine with endotoxaemia (Jackman et al., 1994; Baskett et al., 1997). Other than that, flunixin meglumine also reported to show anti-inflammatory activity by reducing in edema in laboratory animals four hours after treatment (Merck Animal Health, n.d).

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3.0 MATERIALS AND METHODS

3.1 Animals

Thirty-six Sprague Dawley male rats with an average weight of 150g to 200g, 6 week old, were used in this study. The animals were maintained on standard pellet diet with water ad libitum and kept in animal house. All experiments and care involving laboratory animal utilization were performed in accordance to the Universiti Putra Malaysia (UPM) Institutional Animal Care and Use Committee (IACUC) guidelines with the approval certificate number UPM/IACUC/AUP-U025/2020).

3.2 Herbs, Lipopolysaccharide and Drug

P. speciosa extract was extracted using an ethanol extraction method according to the procedure described by Fithri et al. (2019). The extract was diluted at three different concentrations (100mg/kg, 200mg/kg and 400mg/kg) based on the procedure described by Santha-Maria et al. (2015). Flunixine meglumine (Norbrook Laboratories) (50 mg/mL) was used as a positive control drug and lipopolysaccharide (LPS) from Escherichia coli O55:B5 (Sigma-Aldrich) was used to induce acute inflammation of rat hind paw.

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3.3 Experiment protocol

The rats were randomly allocated into six groups according to their body weight with each group consist of six animals. The groups were named as in table below:

Group Number	Treatment
I	100 mg/kg of *P. speciosa* extract
II	200 mg/kg of *P. speciosa* extract
III	400 mg/kg of *P. speciosa* extract
IV	2.5 mg/kg of Flunixine meglumine (positive control)
V	No treatment (negative control)
VI	No induction of paw oedema and no treatment (control)

Rats of the above-mentioned groups were fasted for 18 hours prior to isoflurane anaesthesia (1-3%) and commencement of the experiment. A volume of 100µl/100µg of LPS was injected into the subplantar of the right hind paw of the rats, 30 minutes after the P. speciosa extracts and flunixine meglumine treatment. *The P. speciosa* extracts was given orally through stomach gavage while the flunixine meglumine was administered subcutaneously using a 26-G needle. The rats only being treated once. The subplantar region of the right hind paw of the rats was measured before LPS injection, using a digital vernier caliper then measuredly again immediately after the injection followed by every hour for 6 hours and at 12th hour according to the method described by Kamaruddin et al. (2018). Increase in the paw

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thickness was taken as an indicator of inflammation. The rats were kept under a light anaesthesia during the assessment of the paw.

Blood was collected from tail vein by using 26-G needle and 1 mL syringe before the experiment was started and 12 hours after LPS injection. The blood was collected into EDTA tubes and was stored at 4°C until further analysis. The blood samples were analyzed within 24 hours after collection. The rats were euthanized at the end of the experiment using overdose carbon dioxide (CO2). Right paws were collected for further histology evaluation.

3.4 Analytical procedure

LPS-induced oedema was measured which paw oedema thickness is the different of paw thickness before treatment with LPS and after treatment with LPS at injection time, followed by every hour until six hours then at 12 hours. Percentage of inhibition was calculated by using the following formula described by Kamaruddin, N. et al (2018) :

Equation to calculate the inhibition of inflammation:

$$\text{Percentage reduction of edema} = \frac{(Ct - C0)\text{ in control mice} - (Ct - C0)\text{ in treated mice}}{(Ct - C0)\text{ in control mice}} \times 100$$

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where Ct = average paw thickness after LPS treatment at time t and C0 = average initial (basal) paw thickness for each group.

The complete blood count (CBC) was analysed using ABC Vet haematology analyzer (Scilvet, Germany) for the total white blood cell (WBC), erythron parameters that include evaluation red blood cells (RBC) and haemoglobin (Hb). The packed cell volume (PCV) was 32 L/L, and mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were calculated which were 61.2 fl and 353 g/L in average. The differential WBC was determined by counting five different types of leukocytes on a Wright-stained blood smear. Plasma obtained from the EDTA-blood was used to analyze plasma biochemistry test to evaluate liver and kidney parameters by using an automated biochemistry analyzer (Raj Biosis Pvt. Ltd., Rajasthan, India).

For histological examination, paws were taken 12 h after edema was induced by lipopolysaccaride (LPS). Tissue slices were fixed in 10% neutral-buffered formaldehyde, embedded in paraffin, and sectioned. The sections were stained with hematoxylin and eosin (H & E) (Cantley et. al., 2011). For the specimens, histological analysis was reported as a 0–3 score grade based on the edema and inflammatory cell infiltrate observed in epithelial and connective tissues. The degree of inflammation was evaluated by two different pathologists who were blinded to the treatments. Inflammation scoring (score 0–3) was performed on the muscle within the paws, based on the number of inflammatory cells (lymphocytes, plasma cells, neutrophils or macrophages), as follows: normal tissue (<5% inflammatory cells)

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was scored 0, mild inflammation (5–20% inflammatory cells) was scores as 1, moderate inflammation (21–50% inflammatory cells) as 2 and severe inflammation with a massive immune cell infiltration (>50% of cells) as 3. Discrepancies in scoring were resolved by discussion, with a third examiner being consulted when consensus could not be reached.

3.5 Statistical analysis

All data were analyzed using GraphPad Prism 8 software and presented as mean and standard error mean (S.E.M). $p < 0.05$ means the data is statistically significant. One-way ANOVA test followed by Tukey's multiple comparison test were used to determine significant differences between different time for paw oedema for each group while one-way ANOVA test followed by Dunnett's multiple comparison test being used to determine the significant different between different treatment groups. Significant differences between before and after treatment for white blood cell, differential count of white blood cells, erythron parameters, kidney and liver parameter from plasma biochemistry were tested by paired t-test. For histological evaluation, non-parametric Kruskal-Wallis H-Test followed by Dunn's multiple comparison tests were used.

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4.0 Results

4.1 Paw oedema

Results in Table 1 revealed treatment that being given once to the rats by oral administration) of *P. speciosa* at three different doses (100, 200 and 400 mg/kg) showed significant decrease ($p < 0.05$) in paw oedema formation. At all doses, significant decrease ($p < 0.05$) in the paw oedema can be seen starting at 5 hours with inhibition levels of 60.18%, 39.53% and 25.24%, respectively while at dose of 100 mg/kg significant decrease of paw oedema can be seen starting at 2 hours with inhibition level of 32.92% and for 400 mg/kg significant reduction can be seen started at 4 hours. For flunixine meglumine, significant ($p < 0.05$) decreased of paw edema starting at 2 hours can be seen after lipopolysaccharide (LPS) injection with inhibition level of 47.75%, 37.71%, 68.28%, 70.98%, 83.97% and 85.17% respectively.

Group treated with 100, 400 mg/kg *P. speciosa* and 2.5-mg/kg flunixine meglumine significantly different ($p < 0.05$) from negative control group started at 2 hours after injection (Table 2). Groups in all treatment groups (100, 200, 400 mg/kg *P. speciosa*, 2.5 mg/kg flunixine meglumine) was significantly different ($p < 0.05$) from negative control group at 12 hours after injection, however there is no different between group treated with dose of 400 mg/kg *P. speciosa* and positive control group, 2.5 mg/kg flunixine meglumine. This suggests that *P. speciosa* at high dose

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could have comparable effect with flunixine meglumine at the suggested dose used in this study.

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Table 1 : Changes in the paw thickness (mm^2) of rats injected with 100µl/100µg of LPS from at injection time then every hour until 6 hours and at 12 hrs following oral administration of various doses of *P. speciosa* extract (100-400 mg/kg) or intramuscular injection of 2.5 mg/kg flunixine meglumine (n = 6).

Treatment	Dose (mg/kg)	Paw thickness ± S.E.M (mm^2)/ inhibition (%)							
		At injection	1 h	2 h	3 h	4 h	5 h	6 h	12 h
P. speciosa	100	31.645 ± 1.787	57.257 ± 1.184	50.390 ± 1.573* (32.92%)	59.858 ± 4.862 (-18.13%)	44.99 ± 2.588* (36.79)	37.652 ± 2.850*** (60.18%)	33.642 ± 2.757**** (72.58%)	32.282 ± 1.887**** (77.37%)
	200	34.36 ± 1.744	52.097 ± 2.857	55.955 ± 4.576 (23.59%)	61.848 ± 3.765 (-15.78%)	58.332 ± 4.167 (-5.90%)	44.697 ± 2.756* (39.53%)	37.878 ± 1.053* (62.74%)	33.4283 ± 1.462** (67.69%)
	400	25.055 ± 1.176	51.218 ± 4.041	47.847 ± 3.382 (22.34%)	46.968 ± 4.458 (8.70%)	37.618 ± 0.472* (42.63%)	39.100 ± 4.179* (25.24%)	36.767 ± 2.276* (18.23%)	26.86 ± 0.6383**** (89.97%)
Flunixine meglumine	2.5	26.02 ± 2.545	51.453 ± 3.857	39.935 ± 4.152* (47.75%)	39.402 ± 4.696* (37.71%)	31.372 ± 4.857*** (68.28%)	24.823 ± 2.738*** (70.98%)	25.905 ± 1.830**** (83.97%)	26.637 ± 1.421**** (85.17%)
Negative control (no treatment)	-	29.982 ± 2.511	57.257 ± 1.184	64.733 ± 4.202	56.932 ± 3.528	56.042 ± 4.658	52.393 ± 6.575	47.493 ± 4.712*	45.127 ± 2.246*

*p <0.05, **p <0.01, ***p <0.001, ****p <0.0001 significantly different compared to 1 hour after injection (One way – ANOVA followed by Tukey's post hoc test)

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Table 2 : Comparison between administration of various doses of *P. speciosa* extract (100-400 mg/kg) or intramuscular injection of 2.5 mg/kg flunixine meglumine in changes in the paw thickness (mm^2) of rats injected with 100µl/100µg of LPS from at injection time then every hour until 6 hours and at 12 hrs (n=6).

Treatment	Dose (mg/kg)	Paw thickness ± S.E.M (mm^2)							
		At injection	1 h	2 h	3 h	4 h	5 h	6 h	12 h
P. speciosa	100	31.645 [a] ± 1.787	57.257 [a] ± 1.184	50.390 [b] ± 1.573	59.858 [a] ± 4.862	44.99 [a] ± 2.588	37.652 [b] ± 2.850	33.642 [b] ± 2.757	32.282 [b] ± 1.887
	200	34.36 [a] ± 1.744	52.097 [a] ± 2.857	55.955 [a] ± 4.576	61.848 [a] ± 3.765	58.332 [a] ± 4.167	44.697 [a] ± 2.756	37.878 [a] ± 1.053	33.4283 [b] ± 1.462
	400	25.06 [a] ± 1.176	51.218 [a] ± 4.041	47.847 [b] ± 3.382	46.968 [b] ± 4.458	37.618 [b] ± 0.472	39.100 [b] ± 4.179	36.767 [b] ± 2.276	26.86 [c] ± 0.6383
Flunixine meglumine	2.5	26.02 [a] ± 2.545	51.453 [a] ± 3.857	39.935 [b] ± 4.15	39.402 [b] ± 4.696	31.372 [b] ± 4.857	24.823 [b] ± 2.738	25.905 [b] ± 1.830	26.637 [c] ± 1.421
Negative control (no treatment)	-	29.982 [a] ± 2.511	57.257 [a] ± 1.184	64.733 [a] ± 4.202	56.932 [a] ± 3.528	56.042 [a] ± 4.658	52.393 [a] ± 6.575	47.493 [a] ± 4.712	45.127 [a] ± 2.246

(One way – ANOVA followed by Dunnett's post hoc test). [a,b,c]Means within column with different superscripts differed significantly at $p < 0.05$.

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4.2. White blood cells (WBC)

There were no significant differences in the TWBC and differential WBC counts of animals treated with *P. speciosa* at all doses and in group treated with flunixine meglumine, before and after the experiment (Table 3). Meanwhile, the neutrophil to lymphocyte ratio (N:L) (Figure 2) in negative control group was significantly increased ($P < 0.05$) after LPS injection, and reduction in the ratio were observed in groups treated with *P. speciosa* at doses of 200 and 400 mg/kg, however the result was only significant in group administered with the extract at 200 mg/kg.

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Table 3 : Total white blood cell (WBC) and WBC differential count through the blood smear (mean ± SEM) of Sprague Dawley (SD) rat before and at the end of experimental period after the paw being injected with 100µl/100µg of LPS following administration of different doses of *P. speciosa* (100, 200 and 400 mg/kg) and flunixine meglumine (2.5 mg/kg) before and at the end of experimental period.

Parameter /Group	**100 mg/kg *P. speciosa***		**200 mg/kg *P. speciosa***		**400 mg/kg *P. speciosa***		**2.5 mg/kg Flunixine meglumine**		**Negative control (no treatment)**		**Control**	
	B	A	B	A	B	A	B	A	B	A	B	A
WBC (x 10^9/L)	11.2 ± 0.313	9.70 ± 2.533	10.60 ± 0.700	10.13 ± 4.533	11.70 ± 1.200	10.80 ± 1.200	11.33 ± 0.233	9.03 ± 0.233	9.60 ± 1.200	8.30 ± 1.200	11.03 ± 0.233	11.10 ± 0.400
Band Neutrophils (x 10^9/L)	0.00 ± 0.000	0.00 ± 0.000	0.00 ± 0.000	0.00 ± 0.000	0.00 ± 0.000	0.00 ± 0.000	0.00 ± 0.000	0.00 ± 0.000	0.00 ± 0.000	0.00 ± 0.000	0.00 ± 0.000	0.00 ± 0.000
Segmented Neutrophils (x 10^9/L)	3.92 ± 0.109	3.58 ± 0.297	3.85 ± 0.199	3.13 ± 0.125	4.45 ± 0.244	3.55 ± 0.236	4.125 ± 0.107	3.98 ± 0.040	3.65 ± 0.244	3.13 ± 0.056	4.01 ± 0.00	4.10 ± 0.100
Lymphocytes(x 10^9/L)	5.50 ± 0.224	5.75 ± 0.067	7.00 ± 6.65	6.65 ± 0.291	7.13 ± 0.192	7.00 ± 0.134	7.20 ± 0.313	7.05 ± 0.335	7.80 ± 0.089	8.10 ± 0.045	6.20 ± 0.134	6.30 ± 0.134
Monocytes (x 10^9/L)	0.22 ± 0.048	0.29 ± 0.060	0.27 ± 0.360	0.36 ± 0.004	0.22 ± 0.004	0.22 ± 0.009	0.215 ± 0.007	0.27 ± 0.004	0.255 ± 0.020	0.39 ± 0.007	0.26 ± 0.011	0.32 ± 0.001
Eosinophils (x 10^9/L)	1.00 ± 0.447	0.00 ± 0.000	1.50 ± 0.224	1.00 ±0.447	0.00 ± 0.000	0.00 ± 0.000	0.00 ± 0.000	0.00 ± 0.000	0.00 ± 0.000	1.00 ±0.000	0.00 ± 0.000	0.00 ± 0.000
Basophils (x 10^9/L)	0.00 ± 0.000	0.00 ± 0.000	0.50 ± 0.224	0.50 ± 0.224	0.00 ± 0.000	0.00 ± 0.000	0.00 ± 0.000	0.00 ± 0.000	0.00 ± 0.000	0.00 ± 0.000	0.00 ± 0.000	0.00 ± 0.000
N : L ratio	0.63 ± 0.011	0.63 ± 0.018	0.67 ± 0.002	0.60 ± 0.029*	0.62 ± 0.001	0.58 ± 0.013	0.58 ± 0.006	0.60 ± 0.017	0.63 ± 0.004	0.69 ± 0.006*	0.63 ± 0.006	0.62 ± 0.002

*p < 0.05 compared with before treatment, (B : Before; A : After)

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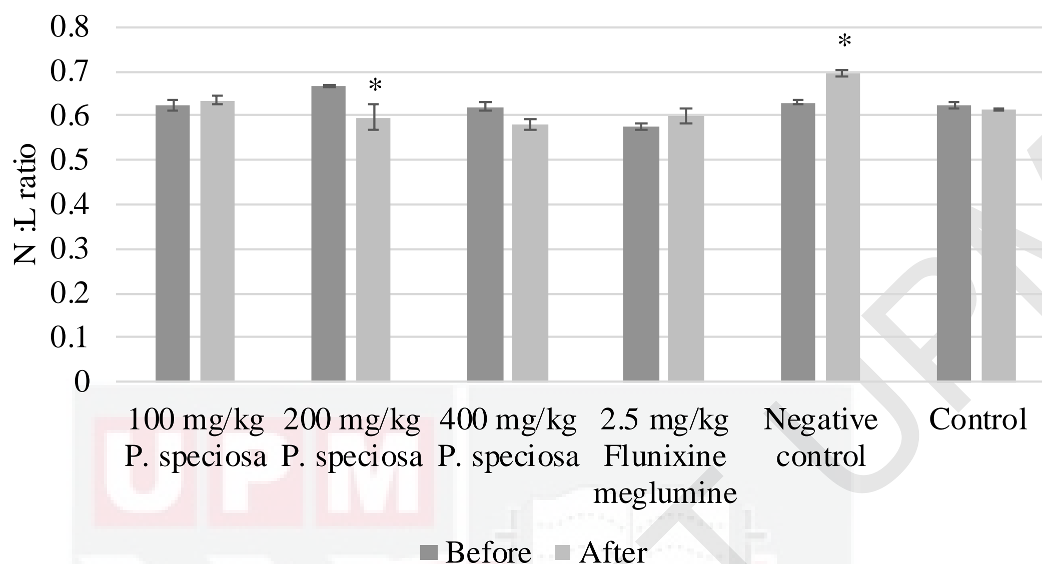

Figure 2 : Effect on Neutrophil : Lymphocyte (N : L ratio) in rats before and after the experiment after the paw being injected with 100µl/100µg of LPS following treatment of *P. speciosa* at different doses and flunixine meglumine. Values are expressed as mean ± S.E.M (n = 6). *p < 0.05 compared with before treatment.

4.3. Erythron parameters

Results (Table 4) of RBC, PCV, Hb, MCV and MCHC did not show any significant differences (p > 0.05) in all groups before and after experiment.

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Table 4 : The erythron parameters (mean ± SEM) of Sprague Dawley (SD) rat before and after the experiment after the paw being injected with 100µl/100µg of LPS following administration of *P. speciosa* at different doses and flunixine meglumine.

Group /parameter	RBC (10^{12}/L)		Hb (g/L)		PCV (L/L)		MCV (Fl)		MCHC (g/L)	
	B	A	B	A	B	A	B	A	B	A
100 mg/kg *P. speciosa*	6.25 ± 0.309	5.96 ± 0.112	133.50 ± 7.379	124.50 ± 2.012	31.50 ± 1.118	28.50 ± 0.671	60.55 ± 0.291	59.70 ± 0.179	352.00 ± 0.447	350.50 ± 0.224
200 mg/kg *P. speciosa*	6.63 ± 0.047	5.57 ± 0.134	133.50 ± 0.224	123.00 ± 1.789	25.50 ± 1.565	29.00 ± 0.447	58.50 ± 0.089	57.95 ± 0.157	345.00 ± 3.578	350.50 ± 2.012
400 mg/kg *P. speciosa*	7.33 ± 0.045	6.56 ± 0.045	148.00 ± 1.789	123.00 ± 1.789	33.00 ± 0.000	32.00 ± 0.000	58.00 ± 0.134	58.00 ± 0.134	348.00 ± 1.342	350.00 ± 1.342
2.5 mg/kg Flunixine meglumine	6.19 ± 0.121	6.26 ± 0.121	128.00 ± 1.789	124.00 ± 1.789	27.00 ± 0.894	30.00 ± 1.342	57.25 ± 0.246	57.40 ± 0.224	346.00 ± 1.789	350.00 ± 0.447
Control (no treatment)	5.815 ± 0.127	6.41 ± 0.114	141.00 ± 1.789	120.00 ± 1.789	35.50 ± 0.224	33.50 ± 0.224	58.70 ± 0.402	58.80 ± 0.402	356.00 ± 0.447	353.00 ± 0.447
Negative control	7.00 ± 0.382	6.28 ± 0.101	144.00 ± 7.603	128.00 ± 0.894	32.00 ± 3.578	32.50 ± 0.224	57.95 ± 0.157	58.70 ± 0.224	355.50 ± 1.565	348.00 ± 1.789

*significantly different, $p < 0.05$ compared with before treatment

(B : Before, A : After)

4.4. Plasma biochemistry

Liver and kidney parameters showed no significant differences in all groups (Table 5) before and after experiment.

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Table 5 : Plasma biochemistry parameter (mean ± SEM) of Sprague Dawley (SD) rat before and after the experiment after the paw being injected with 100µl/100µg of LPS following administration of *P. speciosa* at different doses and flunixine meglumine.

Parameter /Group	**100 mg/kg *P. speciosa***		**200 mg/kg *P. speciosa***		**400 mg/kg *P. speciosa***		**2.5 mg/kg Flunixine meglumine**		**Control (no treatment)**		**Negative control**	
	B	A	B	A	B	A	B	A	B	A	B	A
Urea (mmol/L)	8.12 ± 0.80	9.93 ± 0.49	7.56 ± 0.22	8.42 ± 0.51	7.59 ± 0.15	9.33 ± 0.18	7.95 ± 0.12	9.30 ± 0.26	7.09 ± 0.10	8.07 ± 0.08	7.78 ± 0.27	8.18 ± 0.28
Creatinine (µmol/L)	57.02 ± 2.79	56.13 ± 1.95	52.95 ± 1.44	55.38 ± 1.76	49.57 ± 2.22	58.15 ± 1.77	52.36 ± 1.07	59.11 ± 1.07	57.07 ± 1.39	59.20 ± 0.87	50.35 ± 3.32	48.43 ± 4.48
ALT (U/L)	27.78 ± 3.01	31.82 ± 1.661	23.76 ± 1.48	24.78 ± 1.49	20.74 ± 1.13	22.63 ± 0.96	24.38 ± 0.80	25.14 ± 0.84	25.58 ±1.89	26.21 ± 1.69	29.76 ± 2.58	26.53 ± 2.13
GGT (U/L)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ±0.00	0.00 ± 0.00	0.00 ±0.00
AST (U/L)	153.31 ± 4.94	167.90 ± 0.88	158.82 ± 9.05	158.82 ± 2.09	171.37 ± 2.39	160.91 ± 4.28	151.71 ± 1.93	166.88 ± 3.66	152.89 ± 7.77	158.81 ± 4.30	153.31 ± 4.94	155.95 ± 4.04
CK (U/L)	482.19 ± 47.13	550.9 ± 83.29	586.58 ± 19.08	498.36 ± 62.62	575.11 ± 62.76	629.23 ± 50.69	549.63 ± 34.81	535.59 ± 89.86	465.45 ± 62.61	653.16 ± 72.00	649.66 ± 114.54	535.58 ± 89.86

*significantly different, $p < 0.05$ compared with before treatment, (B : before; A : after)

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4.5. Histological findings

Results showed (Figure 3 II) rats treated with 100, 200, 400-mg/kg *P. speciosa* and 2.5-mg/kg flunixine meglumine had significant lower scoring ($P<0.05$) for inflammation and oedema ($p < 0.05$) compared to negative control group. There were no differences between 200 and 400 mg/kg *P. speciosa* with 2.5-mg/kg flunixine meglumine suggesting a similar anti-inflammatory effects between the extract and the drug.

The severity of oedema was significantly different ($P<0.05$) all treated groups and negative control group. Negative control group (Figure 3: I (negative control)) showed severe oedema while experimental groups show mild to moderate degree of oedema (Figure 3: I (100, 200, 400 mg/kg *P. speciosa*; 2.5 mg/kg flunixine meglumine).

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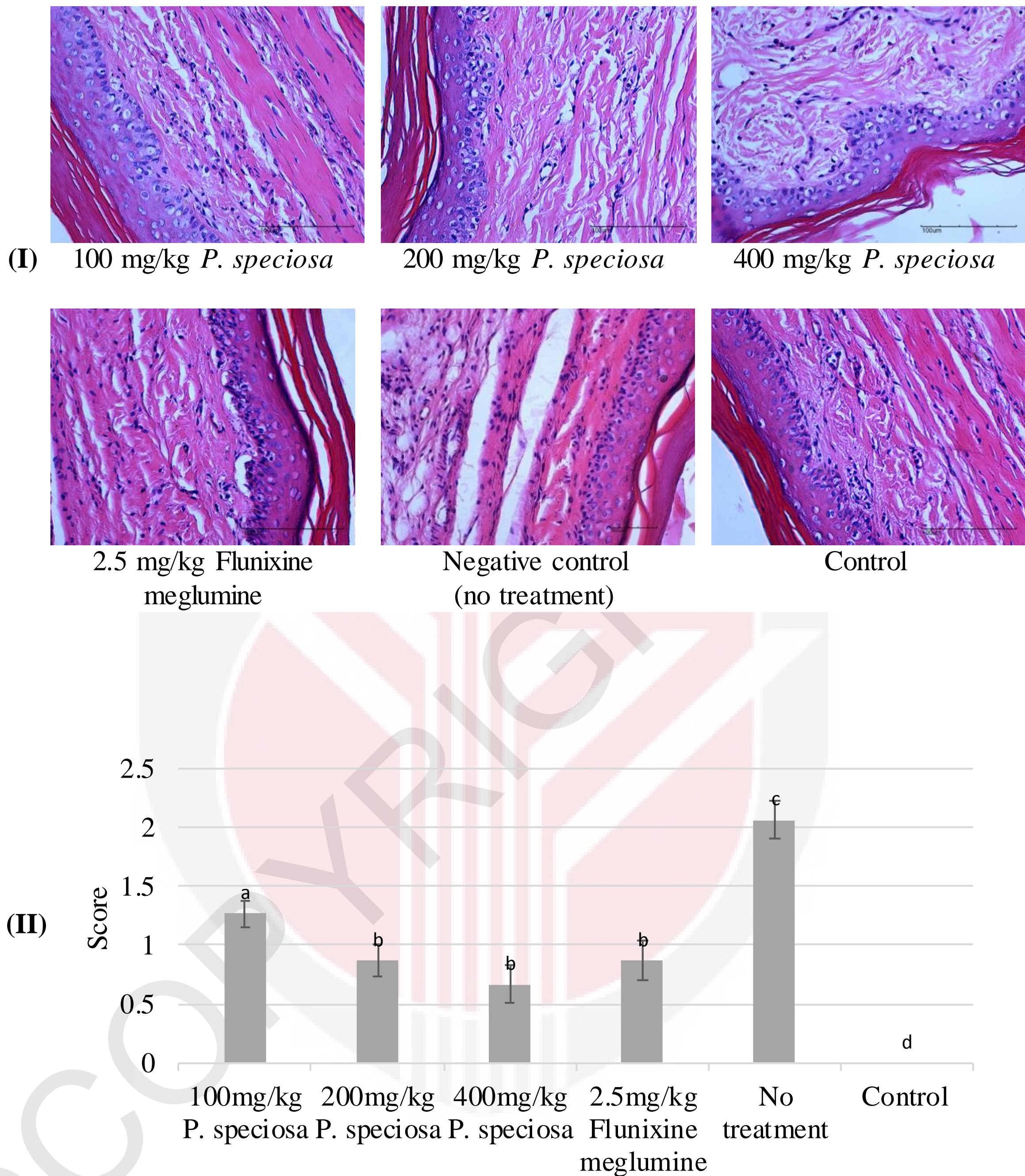

Figure 3 : Histological evaluation of anti-inflammatory effects of *P. speciosa* at different concentration and flunixine meglumine in paw tissue after being injected with 100µl/100µg of LPS. **(I)** Each photo is representative of six specimens for each group. HE stain (40 x magnification). **(II)** Score of inflammation in rat paw tissues by HE staining on a scale from 0 to 3. Data are presented as the mean ± SEM. [a,b,c,d]Means within group with different superscripts differed significantly at $p < 0.05$

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5.0 Discussion

After tissue is damage, acute inflammation can develop in less than an hour. Inflammation occurs as a defensive response of an organism against invasion by the foreign bodies like bacteria, parasites, and viruses. Acute inflammatory response is manifested as redness, heat, swelling, pain, and the loss of function whereas increased vascular permeability, accelerated blood flow, and nerve fiber sensitization are associated with swelling, redness, and pain respectively (Calixto, 2003). In this study, paw oedema thickness being used to evaluate one of the cardinal signs, which is swelling to evaluate the effect of *P. speciosa* treatment at different doses.

Fluid accumulation is the causes of oedema formation that happened during acute tissue inflammation. Increased vascular permeability mediated by vasoactive molecules released by damaged tissues such as histamine, serotonin, prostaglandins and nitric oxide causes fluid to enter the tissue (Rubin & Reisner, 2009).

The result of this study revealed that all *P. speciosa* extract at different doses were able to reduce the oedema formation induced by LPS as compared at injection time. Oral administration of *P. speciosa* extract at all doses (100, 200 and 400 mg/kg) showed reduction of oedema started at 4 hours after injection and caused significant decrease ($p < 0.05$) in paw oedema formation at 5, 6 and 12 hours after LPS administration. However there was no significant difference between *P. speciosa* at dose of 400-mg/kg and flunixine meglumine at 12 hours after the experiment suggesting a similar anti-inflammatory effect of the extract with the drug

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at high dose. This anti-inflammatory effect could be due to the presence of phenolic compounds which include phenolic acid and flavonoid that being reported to have anti-inflammatory property (Ahmad et al., 2019) and hence reducing the fluid accumulation that causes paw oedema after LPS injection. The percentages of inhibition produced by *P. speciosa* at doses of 100, 200 and 400 mg/kg at 12 hours were 77.37%, 67.69% and 89.97%, respectively whereas flunixine meglumine (2.5 mg/kg) was 85.17%. This shows *P. speciosa* at highest dose inhibit oedema more than flunixine meglumine at 12 hours, however this could be due to inhibition of flunixine meglumine on oedema that started to be significant starting at 4 hours of injection. Flunixine meglumine have been reported to have a potent anti-inflammatory property (Barbara, 2017).

According to Bargavi et. al (2004), intraplantar injection of 100 µg lipopolysaccharide (LPS) induced a time dependent swelling at the injected paw similar to carrageenan. Subplantar injection of LPS into the rat paw causes an acute localized inflammatory reaction and swelling of injected paw (Calil et. al, 2004; Vajja et. al, 2004). It involves time-dependent rise in the TNF-α, IL-1β expression and myeloperoxidase activity in the mouse paw where the swelling is peak at 2 hr after injection and reduced at consequence hours (Calil et. al, 2004). LPS-induced paw edema assist in the identification of drugs that are effective against TNF-α mediated inflammation. According to Yazar et. al (2007) flunixine meglumine inhibited increases of cytokine levels (including TNF-α, ILN-1β) that being induced by LPS. The significant reduction of oedema in *P. speciosa* treatment after being injected with LPS suggested the involvement of inhibition of cytokine levels. Apart

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from that, Mustafa et al., 2018 reported that empty pod extract of *P. speciosa* possess anti inflammatory activity in human umbilical vein endothelial cells (HUVECs) after exposing the cells to TNF-α.

Treatment with LPS to induce paw oedema can induce stress to the rats. Neutrophils to lymphocytes (N : L) ratio from standard blood smears made before and after a stressful event, is positively related to the magnitude of the stressor and to the circulating glucocorticoids (Davis et al., 2008). From this study, results showed reduction of N : L ratio in group treated with *P. speciosa* at doses of 200 and 400 mg/kg, however the results were only significantat dose of 200 mg/kg. In addition, Abdullah et al. (2011) reported that from a survey undertaken among people suffering from stress, lots of them felt much better after eating *P. speciosa*. This might happens because of *P. speciosa* contain tryptophan, a type of protein which can be converts into serotonin that will make people relax, improve mood and generally make people happier. Other than that, herbs that contain flavonoid, oligosaccharide, polysaccharide, alkaloid, organic acid being reported to have antistress effect (Velraj et al., 2009). Thus, this reflects that *P. speciosa* has potential to reduce stress that is causing by inflammation.

Decrease in hemoglobin (Hb) levels within 2-3 days being reported to be seen after an inflammatory response to an infection that is caused by decrease in erythrocyte survival (Leb et al., 1987). In this study, the normal values of haematology parameters indicate extract of *P. speciosa* at doses of 100, 200 and 400 mg/kg did not induce anaemia. This may because the acute inflammation that only

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happened within 12 hours. Besides, there is no study related to the effect of *P. speciosa* extract on erythron parameter being conducted.

Treatment with different doses of extract may cause side effect to the animal. Plasma biochemistry shows no significant findings in changes of liver and kidney parameters. Kamisah et al. (2013) reported there is lacking of toxicity study on *P. speciosa* extract. A cytotoxicity study by Aisha et al. (2012) of the plant using HUVEC shows the methanolic extract of the fresh pods (100 μg/mL) did not show any significant cytotoxic effect on the cell lines. A recent study by Puspitahati (2019) shows high doses of ethanolic extract of *P. speciosa* starting at doses 600 mg/kg started to show toxicity effect where it can be seen in the increase in SGOT levels also SGPT levels. However, the full thesis is not published yet.

From this study, LPS causes infiltration of inflammatory cells and causes formation of oedema. Histopathological findings by Wu et al. (2017) showed LPS causes infiltration of inflammatory cells into lung tissue of rats. Histological findings in this study shows statistically significant different between 200, 400 mg/kg P. speciosa, 2.5 mg/kg flunixine meglumine in comparison with negative control group. However, there is no significant different between treatment with *P. speciosa* and flunixine meglumine suggesting the extract have similar effect with the drug.

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6.0 Conclusion

These results support the potential anti-inflammatory activity of *P. speciosa* extract. *P. speciosa* extract exerts its pharmacological activity on acute inflammation induced by 100µl/µg of LPS in rat paw by causing significant reduction of paw oedema thickness at all doses (100, 200 and 400 mg/kg) started at 5 hours of experiment, which comparable with flunixine meglumine.

Significant decrease of neutrophils to lymphocytes (N : L) after the experiment in comparison with before experiment at dose of 200 mg/kg *P. speciosa*, indicate that *P. speciosa* have potential anti-stress activity that being induced by inflammation. However, *P. speciosa* extract did not cause any reduction in total white blood cell (WBC). *P. speciosa* also did not produce effect on the erythron parameters. There were no significant changes in plasma biochemistry for liver and kidney that reflect *P. speciosa* for entire doses used in this experiment did not produce any side effect.

Histological findings also support the anti-inflammatory activity of *P. speciosa* where significant different can be seen in rats that being treated with 100, 200, 400 mg/kg *P. speciosa* compared to the negative control group.

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7.0 Recommendation

The results need to be verified on other experimental models in order to be aunthetic. Histological evaluation of liver and kidney can be done in order to verify the plasma biochemistry results. Phytochemical investigation is needed in order to isolate the active fraction of *P. speciosa* Hassk extract and eventually the pure compound that can exert anti-inflammatory action. More studies need to be conducted to investigate the anti-inflammatory activity of the plant on specific inflammatory diseases in animals and human.

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APPENDICES

Appendix 1 :

Table 6 : The degree of inflammation from histology of paw oedema on a scale from 0 to 3. Values are expressed ± standard error of the mean (SEM). $p < 0.05$

Treatment groups	Mean ± S.E.M
100 mg/kg *P. speciosa*	1.267 [a] ± 0.118
200 mg/kg *P. speciosa*	0.867 [b] ± 0.133
400 mg/kg *P. speciosa*	0.667 [b] ± 0.159
2.5 mg/kg Flunixine meglumine	0.867 [b] ± 0.165
Negative control (no treatment)	2.067 [a] ± 0.153
Control	0.000 [c] ± 0.000

Inflammation scoring : normal tissue (<5% inflammatory cells) was scored 0, mild inflammation (5–20% inflammatory cells) = 1, moderate inflammation (21–50% inflammatory cells) = 2 and severe inflammation with a massive immune cell infiltration (>50% of cells) = 3 (Cantley et al, 2011).

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Appendix 2 :

White Blood Cells (WBC) Differential Counts

Materials :

Glass slide

Wright stain

Phosphate buffer (pH 6.5)

1. **Blood smear preparation**
    a. A small drop of blood is placed on the midline at the end of a glass slide.
    b. Second slide (narrower than smear slide, to avoid spreading the cells over the edge) is placed on the smear slide in front of the blood spot in a way that it forms a 30-45°angle on the side of the blood drop.
    c. Second slide is pulled back into the blood drop, so that the blood spreads along its edge, and then pushed in other direction to the end of the smear slide. The last push being done with rapid single movement and minimal downward pressure.
    d. The smear approximately 2/3 of the length of the slide which is the ideal length.
    e. Let the blood smear dry.

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2. Wright stain staining procedure

a. Flood the blood smear with Wright stain for 3 minutes.

b. Add phosphate buffer and let stand for 8 minutes.

c. Rinse stained smear with water until the edges show faintly pinkish-red.

d. Let the stained blood smear dry.

e. View and count under 10x magnification (segmented neutrophil, band neutrophil, monocyte, lymphocyte, eosinophil and basophil)

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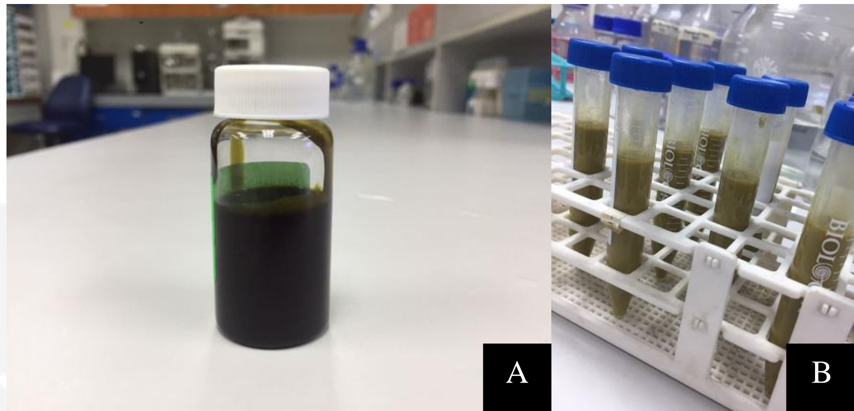
Appendix 3:

Plate 1 : *P. speciosa* extract (ethanol extraction)



a. *P. speciosa* extract collected after undergoing rotary evaporator

b. *P. speciosa* extract 10% concentration

Plate 2 : Lipopolysaccharide (LPS), 1mg/ml (Escherichia coli O55:B5, by Sigma-Aldrich)

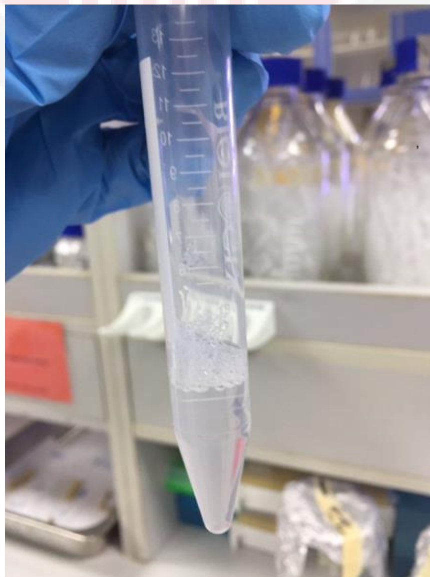


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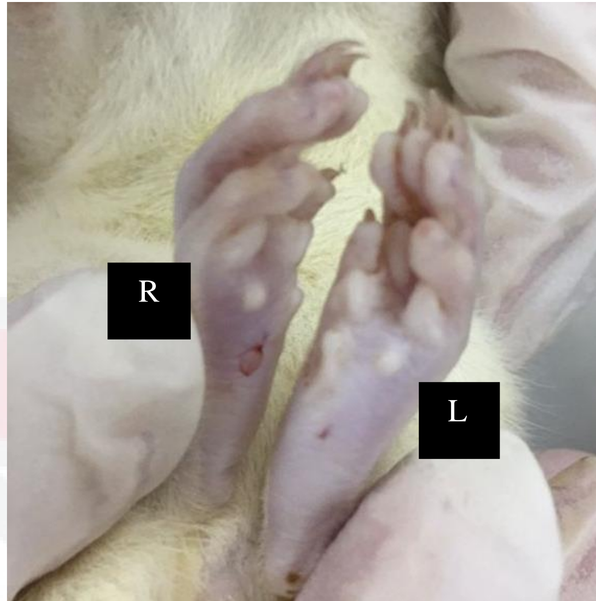
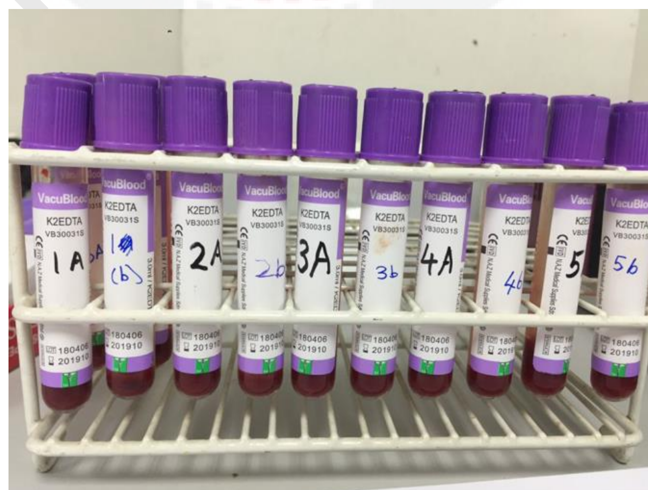
Plate 3 : Paw oedema at 1 hr after lipopolysaccharide injection



R. Right paw injected with 0.1ml lipopolysaccharide

L. Left paw injected with 0.1ml normal saline

Plate 4 : Blood sample in EDTA tubes for white blood cell count (WBC), differential WBC count, erythron parameters and plasma biochemistry.



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