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BY

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Anti-Inflammatory Effect of *Alternanthera sessilis* Red (ASR) Aqueous Extract on RAW 264.7 Cells

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ABSTRACT

Introduction: Inflammation is a normal and protective physiological response towards foreign body such as injury, infection, infiltration and irritation. Macrophages play an important role in the initiation, maintenance, and resolution of inflammation. Its main functions including antigen presentation, phagocytosis and immunomodulation through production of cytokines and growth factors, thus able to inhibit pathogen replication by releasing a variety of effector molecules, including nitric oxide (NO). However, high level of NO production may induce toxic reactions against other tissue of the host during inflammation particularly in cancer. Thus, NO become a potential molecular therapeutic target for anti-inflammatory therapy. *Alternanthera sessilis* red (ASR), a cultivar of *A. sessilis* green has red instead of green aerial parts proved to exhibits anti-inflammatory properties in protein denaturation in vitro. However, the anti-inflammatory mechanism still remains largely unclear. **Objective:** The aim of this study is to determine the anti-inflammatory effect of ASR aqueous extract on lipopolysaccharides (LPS)-induced RAW 264.7 cells. **Methodology:** Non-toxic concentration of the ASR was determined by MTT assay on RAW 264.7 cells. In addition, the morphology changes of the cells were observed. Meanwhile, anti-inflammatory effect was determined by measuring NO production from RAW 264.7 cells induced by LPS using Griess reagent. Data was analysed by using one-way analysis of variance (ANOVA) followed by a post hoc Dunnet test. **Results and Discussion:** MTT assay revealed that only 1000 µg/mLsignificantly ($p = 0.018$) decreased cell viability. The cells also exhibited no significant changes in its morphology when compared to untreated cells. In NO assay, LPS significantly increased NO production by 3.8-fold compared to untreated group. However, all concentration of ASR significantly ($P < 0.001$) inhibited NO production induced by LPS. The highest percent of inhibition is exhibited by 500 µg/mL concentration with 71.3%. **Conclusion:** ASR aqueous extract is found to have no cytotoxic effects and possesses anti-inflammatory effect on RAW 264.7 cells.

Keywords: Inflammation, Alternanthera sessilis red, nitric oxide, macrophage, lipopolysaccharide

Kesan Anti-Radang Ekstrak Akueus *Alternanthera sessilis* Red (ASR) Terhadap Sel RAW 264.7

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ABSTRAK

Pengenalan: Keradangan ialah suatu proses yang normal dan berfungsi sebagai respons fisiologikal pertahanan terhadap benda asing seperti kecederaan, jangkitan, infiltrasi dan iritasi. Makrofag memainkan peranan yang penting dalam pemulaan, penyelenggaraan dan kesudahan proses keradangan. Antara fungsi utamanya adalah termasuk penyajian antigen, fagositosis dan modulasi imunisasi melalu penghasilan sitokin dan faktor tumbuh, lalu boleh menghentikan pembiakan patogen dengan melepaskan pelbagai jenis molekul efektor termasuklah nitrik oksida (NO). Walau bagaimanapun, tahap penghasilan NO yang tinggi boleh menyebabkan kesan toksik terhadap tisu badan yang lain ketika keradangan terutamanya ketika kanser. Justeru, NO menjadi suatu sasaran terapi molekular yang mempunyai potensi untuk terapi anti-radang.. *Alternanthera sessilis* red (ASR), kultivar untuk *A. sessilis* green mempunyai bahagian aerial berwarna merah terbukti mempamer sifat anti-radang dalam denaturasi protein secara in vitro. Walau bagaimanapun, mekanisme anti-radang ini masih lagi tidak jelas. **Objektif:** Tujuan eksperimen ini adalah untuk menentukan kesan anti-radang ekstrak akueus ASR terhadap sel RAW 264.7 yang dirangsang lipopolisakarida (LPS). **Metodologi:** Konsentrasi tidak toksik bagi ASR ditentukan menggunakan asai MTT terhadap sel RAW 264.7. Tambahan lagi, perubahan morfologi sel juga sel juga dilihat. Manakala, kesan anti-radang pula ditentukan dengan mengukur penghasilan NO oleh sel RAW 264.7 yang dirangsang oleh lipopolisakarida menggunakan reagen Griess. Data pula telah dianalisa menggunakan analisis varians (ANOVA) diikuti dengan ujian post hoc Dunnett. **Keputusan dan Perbincangan:** Asai MTT menunjukkan bahawa cuma konsentrasi 1000 µg/mL mengurangkan daya hidup sel dengan ketara ($p = 0.018$). Sel – sel juga menunjukkan tiada perbezaan yang ketara berbanding dengan sel yang tidak dirawat. Dalam asai NO, LPS telah meningkatkan penghasilan NO dengan ketara sebab 3.8 kali ganda berbanding kumpulan terkawal. Walau bagaimanapun, semua konsentrasi berjaya merencat penghasilan NO yang dirangsang LPS dengan ketara ($p < 0.001$). Peratus perencatan yang tertinggi ditunjukkan oleh konsentrasi 500 µg/mL dengan 71.3%. **Kesimpulan:** Ekstrak akueus ASR didapati tidak mempunyai kesan kesitotoksikan dan mempunyai kesan anti-radang terhadap sel RAW 264.7.

Kata kunci: Keradangan, Alternanthera sessilis red, nitrik oksida, makrofag, lipopolisakarida

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ASG	Alternanthera sessilis green
ASR	Alternanthera sessilis red
COX-2	Cyclooxygenase-2
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
eNOS/NOS3	Endothelial nitric oxide synthase
FBS	Foetal bovine serum
GC-MS	Gas chromatography-mass spectrometry
IL-1β	Interleukin-1β
IL-6	Interleukin-6
iNOS/NOS2	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
MTT	3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl-tetrazolium bromide
NF- κB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
nNOS/NOS1	Neuronal nitric oxide synthase
NO	Nitric oxide
NO_2^-	Nitrite
NO_3^-	Nitrate
NOS	Nitric oxide synthase
NSAID	Non-steroidal anti-inflammatory drug
PAMP	Pathogen-associated molecular pattern

PBS	Phosphate buffer saline
PGE2	Prostaglandin
PRR	Pattern-recognition receptor
SEM	Standard error of mean
TFC	Total flavonoid content
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-alpha
TPC	Total phenolic content

CHAPTER 1

INTRODUCTION

1.1 Background

Inflammation is a biological defensive mechanism of the immune system that reacts towards a variety of stimuli such as pathogens, injury and chemical irritations (Chen et al., 2018) . It protects our system and organs by removing the stimuli and start the healing process, minimizing further unwanted effects at the site of inflammation (Ferrero-Miliani et al., 2007). Inflammation can be differentiated into two categories; acute and chronic inflammation. Acute inflammation is the initial rapid response by the immune system to the stimuli, efficiently resolving the issue. However, this can then lead to chronic inflammation, if prolonged. The consequences of chronic inflammation are that it is involved pathologically in the development of many chronic diseases such as cardiovascular diseases, autoimmune diseases and cancers (Furman et al., 2019).

During an inflammation, there are various of pro-inflammatory mediators released, for example, interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-α (TNF- α), inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 (Walsh et al., 2005). These mediators are upregulated during the process of inflammation. This is due to stimuli for instance, lipopolysaccharide (LPS) activated inflammatory cells such as macrophages and release these inflammatory cytokines and enzymes due to signalling to facilitate the inflammation process (Fujiwara &

Kobayashi, 2005). Cytokines like iNOS and COX-2 are also in charge of for producing other inflammatory mediators like nitric oxide (NO) and prostaglandin (PGE2). Increase in these mediators can help in positive feedback manner where for more inflammatory cells are recruited and activated, accumulating more mediators. However, prolonged inflammation can cause damage to the site of inflammation and the local tissue area, making it detrimental to the system. Overproduction of NO specifically has been associated with inflammation tumorigenesis and various other chronic diseases (Mann et al., 2005). Thus, controlling the upregulation of these inflammatory mediators, especially NO is the important target in controlling inflammations.

However, many anti-inflammatory agents available in the markets nowadays such as NSAIDs have increased risk of gastrointestinal such as bleeding (Dhikav et al., 2003). Hence, there is a need in the market for an anti-inflammatory option with less side effects and more compatible with patients with different backgrounds. *Alternanthera sessilis* red, a cultivar of *Alternanthera sessilis* has been showing multiple usage in ethnobotanical purposes to promote health and boost the immune system (Siew et al., 2014). Besides, it also has been proved to contain anti-diabetic (Tan & Kim, 2013) and anti-oxidant properties (Khan et al., 2016). Its green coloured cultivar also has been determined to contain anti-inflammatory properties (Muniandy et al., 2018). Therefore, in this study, *Alternanthera sessilis* red will be tested for its anti-inflammatory properties by determining its inhibition of nitric oxide production on LPS-stimulated RAW 264.7 murine macrophages.

1.2 Objectives

1.2.1 General objective

To evaluate anti-inflammatory effect of *Alternanthera sessilis* red (ASR) aqueous extract on RAW 264.7 cells.

1.2.2 Specific objectives

1) To determine the cytotoxicity level of different concentration of ASR aqueous extract on RAW 264.7 macrophages.
2) To observe the morphological changes in RAW 264.7 cells induced by ASR aqueous extract.
3) To investigate effect of ASR aqueous extract in nitric oxide production in RAW 264.7 cells induced by LPS.

1.3 Hypothesis

ASR aqueous extract significantly reduce nitric oxide in RAW 264.7 cells induced by LPS.

CHAPTER 2

LITERATURE REVIEW

2.1 Inflammation

The term inflammation is derived from Latin word “inflammare” which means to burn. In general, inflammation is an immune mechanism in order to cope with the endogenous and exogenous stimuli. As it is considered as biological process, inflammation can be triggered by various factors such as infections, toxins, pathogens and damaged cells. It is considered as a complex protective reaction in healing mechanism. As inflammation react to the harmful stimuli, it will act to remove the injurious stimuli and trigger the healing process as part of the defence mechanism. As it considered as defence system, inflammation can be divided into two types; acute and chronic inflammation (Altameemi & Mohammed, 2019).

2.1.1 Acute inflammation

Acute inflammation is defined as an immediate response to the injurious agents and has short duration, lasting for few hours. It is known to cause the immediate change to the vascular and causes the influx of inflammatory cells mainly neutrophils. Acute inflammatory response causes the widespread effect of the inflammatory mediators for healing mechanism (Pahwa et al, 2020).

Several signs of acute inflammation include redness (rubor), heat (calor), swelling (tumor), pain (dolor) and loss of function. These signs are known as cardinal sign of inflammation and each of the signs explain the mechanism behind the pathophysiology of acute inflammation. In instance, redness occurred at the site of inflammation is due to the dilation of blood vessel within the damaged cells and tissue while heat is resulted from the hyperaemia, the increase of blood flow to the inflamed region. In addition, swelling occurred due to the accumulation of the fluid in the extravascular space in which caused by the vascular hyperpermeability. As inflammation caused the stretch and destruction of the tissue, one will feel pain as the result of the inflammation process and lastly, as the inflamed area is already inhibited by pain, it will cause the immobilization of the inflamed tissue and lead to the loss of function (Altameemi & Mohammed, 2019).

2.1.2 Chronic inflammation

Chronic inflammation is due to the infiltration of various inflammatory cells such as macrophages, lymphocytes and fibroblast. It is a long-term inflammation and usually last for several months and years due to the excessive inflammation and uncontrol healing mechanism. Chronic inflammation response will cause severe implications such as persistent inflammation, fibroblast proliferation and scar formation. Chronic inflammation can be due to the several causes such as persistent infections, prolonged exposure to the non-degradable toxic substances, progression from acute inflammation and autoimmune diseases.

In acute inflammation, the cardinal signs are considered as important signs and symptoms for acute inflammatory response but in chronic inflammation, it can lead to the systemic effects of inflammation such as fever, weight loss, leukopenia, leucocytosis and autonomic response such as increase of blood flow and decrease in sweating (Tseung, 2005). Chronic inflammation can be divided into two major classifications; non-specific chronic inflammation and specific inflammation in which each classification is based on the histologic features (Pahwa et al, 2020).

2.2 Mechanisms of inflammation

The mechanism of inflammation can be a bit complicated as it is considered as an immune response and body defence. The course of the inflammation can be influenced by the immune condition and other genetic factors. In short, the mechanism of inflammation can be classified into two main types; acute and chronic inflammation.

2.2.1 Mechanism of acute inflammation

The pathophysiology of acute inflammation can be divided into early and late vascular cellular response. In early vascular acute inflammatory response, inflammation caused vasodilation to the arterioles and venule (Altameemi & Mohammed, 2019). This resulted into the increase of blood flow which explain the redness on the inflamed part. After an episode of increase blood flow, the blood become more slower due to the increase of the vascular permeability and lead into stasis. Once the stasis happens, the dilated blood vessels are packed with red blood cells and cause the exudation and swelling (Altameemi & Mohammed, 2019)..

Next, in the cellular response, there are several stages involve in the inflammatory response which consist of migration of leukocytes, transmigration of leukocytes, chemotaxis and phagocytosis as illustrated in Figure 2.2.1. As vascular permeability increase, the inflammatory cells mainly neutrophils tend to accumulate at the endothelial lining. In migration of leukocytes, neutrophils roll slowly along the endothelium and lined up. This process is known as pavementing. The binding between the leukocytes and endothelium is due to the interaction between the adhesion molecules such as selectin and integrins (Sahlmann & Ströbel, 2016).

The leukocytes will eventually escape from the venules and capillaries in which it will extend the pseudopodia through the vascular wall. This whole process is called transmigration of leukocytes. As in, chemotaxis will take place and lead the leukocytes into the site of inflammation within the tissue due to the chemical gradients. The receptors on the leukocyte's membrane will react to the chemo-attractant and trigger the movement of leukocytes towards the stimulus (Sahlmann & Ströbel, 2016).

In order to remove the injurious stimuli, the leukocytes will undergo phagocytosis. It is a process that engulf the invading materials such as damaged cells, pathogens and tissue debris. Phagocytosis involves in three steps which include recognition and attachment, engulfment and killing or degradation (Fujiwara & Kobayashi, 2005). The whole inflammation process is mediated by the chemical mediators. These chemical mediators can be derived from plasma and cells as it enhances the binding between the receptors and target. The important inflammatory

mediators are classic mediators such as histamine and prostaglandins while oxidant agents include superoxide derivatives and nitric oxide.

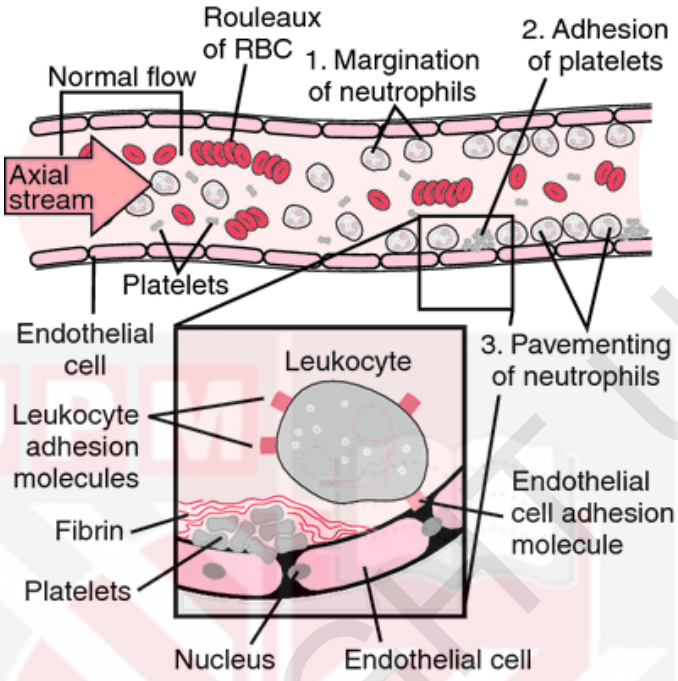

Figure 2.2.1: Cellular changes in inflammation, (Erler, 2000)

2.2.2 Mechanism of chronic inflammation

The mechanism of the chronic inflammation is much simpler compared to the acute inflammation. The main parameter for chronic inflammation is the presence of granuloma. There are two types of granulomas which are foreign body granuloma and immune granuloma and both involved in the pathogenesis of chronic inflammatory response. In foreign body granuloma, these granulomas have the presence of foreign body reaction (FBR). FBR consists of various materials such macrophages, fibroblast and it is considered large to be phagocytes by a single phagocytic cell (Anderson et al., 2008). In immune granuloma, the immune cells mainly antigen presenting cells such as macrophages are able to distinguish the foreign materials but unable to eliminate them. These foreign materials include bacteria, fungi, keratin and suture fragments (Pahwa et al, 2020).

2.3 LPS and inflammation

Liposaccharide (LPS) is a crucial component of outer membrane of gram-negative bacteria. Various study showed that LPS can be recognised by the inflammatory cells mainly by the monocytes and macrophages as foreign materials and trigger inflammatory response (Guha & Mackman, 2001) (Fujiwara & Kobayashi, 2005) (Shalova et al., 2017). LPS can triggers the activation of interleukin 8 (IL-8) and TNF-α in order to destroy the pathogens. LPS induce inflammatory response by activating the toll-like receptor 4 (TLR4) (Shalova et al., 2017). Subsequently, the binding between the TLR4 with the LPS cause the activation of various cytokines such as NF-κB. NF-κB is known for its crucial role in the inflammatory response and study

revealed that NF-κB is important in regulating the transcription gene related to the innate immunity and inflammation as it is a central for the pro-inflammatory mediator (Liu et al., 2017).

2.4 Nitric oxide as major inflammatory mediator

Nitric oxide (NO) is one of the important inflammatory mediators in oxidative agent class (Sharma et al., 2007). It is a free radical, a member of group known as reactive oxygen species. It is very reactive with oxygen and it is readily reduced to more stable nitrate form. This reactivity causes NO to have short lifetime, and can only act as paracrine mediators. It is synthesized from L-arginine by enzymes called nitric oxide synthases (NOSs). Its small size allows it to penetrate and diffuse into cell membranes of more than several microns.

There is three type of NOS; neuronal NOS (nNOS/NOS1), inducible NOS (iNOS/NOS2), and endothelial NOS (eNOS/NOS3) (Tripathi et al., 2007). All NOS require calmodulin binding for its activity, although only iNOS activity is independent of level of calcium, unlike other NOS. This will then allow iNOS to produce more NO than other isoforms of NOS and the NO production also will last much longer.

In inflammation, pro-inflammatory cytokines or LPS induction can lead to the expression of iNOS by several different inflammatory cells such as macrophages and neutrophils. This induction then leads to an increase in NO production, exceeding the normal level up until 1000-fold (Cook & Cattell, 1996). Its function may include killing of infectious agents, killing tumor cells, and regulating expression of other inflammatory cytokines (Tripathi et al., 2007). Upregulation of other pro-inflammatory cytokines by NO will create a positive feedback where it will keep encouraging the both recruitment of inflammatory cells and release of cytokines.

However, overproduction of NO can lead to tissue destruction such as in neurological diseases and other inflammatory disorders. Overproduction of NO in chronic inflammation involving activated glial cells has been said to be involved with neurodegeneration and neuroprotection (Moncada & Higgs, 2006).

NO help in host defences by inhibiting metabolic enzymes and destroying DNA inside invading organisms. However, overproduction of NO can lead to septic shock, damaging the host tissues instead (Sharma et al., 2007). Enhanced NO production has also been observed in serum and synovial fluid of patients with rheumatoid arthritis (Nagy et al., 2010). These act as evidences that overproduction of NO does bring detrimental effects to the host and lead to many chronic diseases.

2.5 *Alternanthera sessilis* Red (ASR)

ASR is an edible medicinal plant that belongs to the family Amaranthaceae. It is one of the two cultivars of *Alternanthera sessilis,* with red aerial parts instead of green, explaining its name. The locals know this cultivar by the name of "bayam keremak merah" in Malay, and "hong tian wu" in Chinese language (Tan & Kim, 2013). Some also may know them as "pudoh rumput aoh" or "serapat", depending on state and dialect (Khan et al., 2018). Meanwhile, it is called "red sessile joyweed" in English. This particular cultivar is more popular in Southeast Asia, specifically Malaysia and Singapore (Tan & Kim, 2013).

2.5.1 Description

ASR or red-carpet weed is a perennial prostrate herb that can grow up to 0.5m in height. Its stem has a smooth surface with reddish-purple colour with rooting at the nodes. The leaves have a narrow and elongated shape about 6 cm long with the entire leaf is coloured similar to its stem. Regarding the flowers, it is small in size, pinkish white in colour and have inflorescence bracts as can be seen in Figure 2.5.1 (Quattrocchi, 2012) (Mohd Hazli et al., 2019). It can grow in various soil and climate conditions although it will grow best on a wet soil condition. However, it is found more frequently in marshy areas and wetlands (Vennila and Nivetha, 2015). It is considered weed and pest to paddy, sugarcane and bananas in tropical areas (Das, 2014).

Figure 2.5.1: ***Alternanthera sessilis*** **Red (ASR)** (Singapore National Parks Board, 2020)

2.5.2 Phytoconstituent

ASR does have a potential of containing phytochemicals such as polyphenols and betalain due to its green counterpart ability to have analgesic properties (Hossain et al., 2014). This is proved by a study where ethanol extract of ASR has shown higher amount of phenolic content such as ferulic acid, rutin, quercetin and apigenin when processed through high performance liquid chromatography (Mohd Hazli et al., 2019).. Meanwhile, in the same study, ethyl acetate extract of ASR has a high amount of flavonoid, betalain, and carotenoid but lower phenolic content. This trend is also supported by a different study, but with varying result in total content detected due to different parts of plant tested (Yap et al., 2019).

Furthermore, ASR has been found to contain sesquiterpenes, ketones, diterpenes, fatty acid and esters based on gas chromatography-mass spectropmetry (GC-MS) analysis of essential oils extracted from ASR (Khan et al., 2016). Another study using GC-MS has also confirmed that ASR has high total phenolic content (TPC) and total flavonoid content (TFC) (Khan et al., 2018). When comparing between ASR and *A. sessilis* green (ASG), ASR is found to have the higher phenolic compound and antioxidant capacity (Azizah et al., 2015). ASR also have high content of ferulic acid and vanillic acid while ASG has high vanillic acid and catechin (Othman et al., 2016).

2.5.3 Ethnomedical uses

There is a record of this particular plant is consumed as “ulam” in raw or boiled form (Bachok et al., 2014). According to this study, “ulam” is a functional food with known significant medicinal properties and rich in macro and micronutrients. This study also listed *A. sessilis* as 32 most popular “ulam” vegetables in Malaysia alongside “pegaga” (*Hydrocotyle asiatica*) and “paku (pucuk)” (*Athyrium esculentum*). In addition, a study on ethnobotanical uses of different plants in Singapore has mentioned several usages of ASR whole plants and aerial parts being used in decoction and tea by 14 records (Siew et al., 2014). These records explained the application of these plants includes boosting immunity, promoting blood circulation, promoting health and detoxifying body system in general (Siew et al., 2014).

In Bangladesh, there is a study on usage of ethnomedicinal plants being used as food during famine. In this study, ASR is found out to possess multiple ethnomedicinal properties such as anti-inflammatory, hematinic, wound healing and anti-diabetic (Azam et al., 2014). Other than that, ASR is also believed to be able to reduce risks of cardiovascular diseases (Tan & Kim, 2013). In China, ASR is being used as herbal vegetable that is a part of Chinese food therapy called “Yao Shan”. The leaves of ASR have also been used to treat eye and skin diseases with an ability to heal wounds (Konda et al., 2013).

2.5.4 Pharmacological uses

A. sessilis in general has been investigated in past studies to contain nootropic activity, cytotoxic effects on pancreatic cancer cell line and free radical-scavenging ability (Tan & Kim, 2013). There are few different phytochemicals that are responsible for anti-diabetic activity for *Alternanthera sessilis* such as triterpenoids, phytosterols and glycosides. Moreover, ASR ethyl acetate fraction has been demonstrated to possess anti-hyperglycaemic, anti-triglyceridemic and pancreatic protective effects on obese type 2 diabetic rats (Tan & Kim, 2013).

In addition, sesquiterpenes contained inside ASR also suggests that the plants have anti-inflammatory properties (Khan et al., 2016) (da Silveira e Sá et al., 2015). Quercetin contained in ASR ethanol extract is suggested show anti-inflammation and immune-enhancement *in vitro* and *in vivo* (Li et al., 2016). Last but not least, there are multiple studies supporting ASR extract does have significant anti-oxidant activity *in vitro* and biochemicam tests (Khan et al., 2016) (Othman et al., 2016) (Khan et al., 2018) (Mohd Hazli et al., 2019) (Yap et al., 2019).

CHAPTER 3

MATERIALS AND METHOD

3.1 Chemicals and reagents

All reagents and solvents used in this experiment are bought from common supplier available and listed in Table 3.1.

Table 3.1: List of chemicals and reagents used.

Chemicals & reagents	Manufacturer
Dulbecco's Modified Eagle Medium (DMEM)	Invitrogen Co., (California, USA)
Foetal bovine serum (FBS)	Invitrogen Co., (California, USA)
Phosphate buffer saline (PBS)	Sigma-Aldrich Co. (Missouri, USA)
3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl-tetrazolium bromide (MTT) reagent	Fisher Scientific Co. (England, UK)
Trypan Blue dye	Sigma-Aldrich Co. (Missouri, USA)
Griess reagent	Sigma-Aldrich Co. (Missouri, USA)
Lipopolysaccharide (LPS)	Invitrogen Co., (California, USA)

3.2 Sample water extraction

The whole plants of ASR used are collected and obtained at Ipoh, Perak, Malaysia. The plants then authenticated by a Chinese medicine practitioner also a senior lecturer from Universiti Tunku Abdul Rahman, Dr. Teh Lai Kuan. The fresh plants were found in good condition and washed thoroughly in running water. Then, the plants were dried and grinded into powder form for extraction process for testing purposes using water as solvent. For water extraction, the powdered sample was extracted using an incubator shaker with 40 ml water as solvent at 50 °C for 24 hours. It is then transferred to an orbital shaker for another 2 hours at 200 rpm and 50 °C. The ratio of sample and extraction media used was 1:10 (w/v). Next, the product was immediately kept in -80 °C freezer and lyophilized in a freeze dryer to remove the water. After lyophilization, the remaining extract was kept at -20 °C until further usage for experiments.

3.3 Cell culture

3.3.1 Cell thawing

RAW 264.7 murine macrophages cell line obtained from American Type Culture Collection (ATCC) were cryopreserved inside a vial containing DMEM media with 10% of dimethyl sulfoxide (DMSO) and stored in -80 °C freezer. For thawing, the vial of cells was thawed using the water bath at 37 °C. Then, the content of the vial is transferred into a 15 mL centrifuge tube and 4 mL of enriched DMEM (DMEM with 10% FBS) was added slowly to avoid osmotic shock. The centrifuge tube was then centrifuged at 1200 RPM for 10 minutes. The supernatant resulted from the centrifuge is discarded and the pallet was resuspended inside 1 mL of enriched DMEM. Finally, the cell suspension was transferred into T25 culture flask with 4 mL of enriched DMEM ready for incubation at 37 °C.

3.3.2 Cell maintenance

RAW 264.7 was maintained using humidified incubator with 5% CO2 at 37 °C. The cells were cultured using sterile cell culture flasks until reaching confluency (80-100%). The media was changed accordingly every few days to provide enough nutrients and suitable environment for the cells. Mechanical detachment using scrapper is the method of cell detachment used in removing the confluent cells. The scrapped cells were transferred into a 15 ml centrifuge tube and centrifuged at 1200 RPM for 10 minutes. For subculture, pellet of cells from the centrifugation was suspended in 1 mL of enriched media and transferred equally into 5 new T25 flasks with 5 ml of enriched DMEM with FBS. The ratio of 1:5 is due to the cells ability to

proliferate quickly. Then, the flasks were observed under inverted microscope for distribution and morphology.

3.3.3 Cell counting

To prepare the cells for experiment use, the scrapped cells have to be quantified for equal distribution in seeding. This can be done by counting the total concentration of cells after centrifugation of removed cells mentioned in the previous procedure. First, the cell pellet was suspended in 1 mL of enriched DMEM. Next, 10 µL of the suspension was mixed with 10 µl of Trypan Blue dye, resulting in dilution factor of 2. Then, 10 µL was pipetted and loaded into a haemocytometer, a counting chamber device used for cell counting. Haemocytometer has 4 different sets of 16 squares that was covered evenly with the cell suspension loaded. Total number of cells observed inside all these squares will be used inside a formula to calculate the true number of cells inside the 1 mL of media.

$$Total\ number\ of\ cells\ inside\ 1\ mL\ of\ media = \frac{Set\ A + Set\ B + Set\ C + Set\ D}{4} \times 10^4 \times 2\ (Dilution\ factor)$$

3.4 Treatment preparation

As the ASR extract is available in stock form with very high concentration, it needs to be diluted in order to be used during experiments. The target concentration of extracts for MTT assay are as follows; 1000 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL and 62.5 µg/mL. The concentrations can be prepared using 2-fold serial dilution, so the stock need to be diluted to 1000 µg/mL to be diluted to other concentrations. The stock amount that needs to be used can be calculated using:

$$M1 \times V1 = M2 \times V2$$

For Griess assay, the concentrations used are similar except without the concentration of 1000 µg/mL but with addition of LPS. The desired concentration of LPS is 10 µg/mL, so the same formula can be used to calculate stock volume needed to be diluted.

3.5 MTT assay and morphological observation

MTT assay is employed in this experiment to determine the non-toxic concentration of ASR aqueous extract on RAW 264.7 cells by calculating the percentage of cell viability after treatment. This is done by measuring the absorbance of dissolved purple formazan crystals, reduced from tetrazole from cells.

RAW 264.7 cells were seeded in 96-well plate with concentration of 1 X 10^4 in 100 µL of enriched DMEM in each well. After a 24-hour incubation inside humidified CO2 incubator, the cells were treated with five different concentrations of ASR aqueous extract; 62.5 µg/mL, 125 µg/mL, 250 µg/mL, 500 µg/mL and 1000 µg/mL. The cells were then incubated for another 24 hours. Next, the cells were observed under an inverted microscope to determine if there are any changes to the cells induced by the treatment. The experimental design for this assay is shown in Table 3.4.1.

Then, 10 µL of 5 mg/mL MTT reagent is added into the wells and the cells were incubated for 4 hours. Last, the absorbance of the plate was measured using a microplate reader at 570 nm. The absorbance recorded was calculated to determine the cell viability presented in percentage of control using this formula:

$$Cell\ viabilty = \frac{OD\ treatment}{Average\ OD\ control} \times 100$$

Table 3.5: Experimental design of MTT assay of ASR aqueous extract on RAW 264.7 cells.

GROUP	CELLS (RAW 264.7)	ASR EXTRACT *(*62.5, 125, 250, 500 and 1000 µg/mL*)*	MTT REAGENT
NORMAL	+	-	+
TREATMENT	+	+	+

3.6 Griess assay

Griess assay is used in this experiment to determine if there is any inhibition effect on NO production by measuring nitrite concentration, an end product of metabolism of NO. With this, we can indirectly measure NO produced by the macrophages cell.

First, RAW 264.7 cells were seeded in a 96-well plate with 100 µL of enriched DMEM inside each well. After the cells reaches confluence, the cells were treated with different concentrations of ASR (62.5 µg/mL, 125 µg/mL, 250 µg/mL and 500 µg/mL) except for two wells for control. After two hours, all wells were added with LPS (10 µg/mL) to induce inflammation. One well remains untreated without LPS is used for control group. After a 24-hour incubation, the culture media was collected and transferred for assay with Griess reagent (1% sulfanilic acid, 0.1% N-1-naphthyl-ethylenediamine dihydrochloride, and 5% phosphoric acid). Then, the mixture was

stored in the dark for 10 minutes and the absorbance is measured at microplate reader at 540 nm. Table 3.5.1 shows the experiment design for Griess assay used in this experiment.

The absorbance measured was then used to calculate the percentage of inhibition of NO production in percentage of control.

$$Percentage\ of\ NO\ production\ (NOP)(\%) = \frac{OD\ treatment}{Average\ OD\ control} \times 100$$

$$Percentage\ of\ inhibition\ of\ NO\ production\ (\%)$$

$$= \left[1 - \frac{\left(Average\ NOP^{LPS} - Average\ NOP^{Control}\right) - \left(Average\ NOP^{LPS} - NOP^{Tx}\right)}{\left(Average\ NOP^{LPS} - Average\ NOP^{Control}\right)}\right]$$

$$\times 100$$

Table 3.6: Experimental design of Griess assay of ASR aqueous extract on LPS-induced RAW 264.7 cells.

GROUP	RAW 264.7 CELLS	GRIESS REAGENT	LPS (10 µg/mL)	ASR EXTRACT (62.5, 125, 250 and 500 µg/mL)
NORMAL	+	+	-	-
TREATMENT	+	+	+	+
NEGATIVE CONTROL	+	+	+	-

3.7 Statistical Analysis

Results obtained from the experiments were analysed using software IBM SPSS Statistics Client version 26.0.0.1 and graphs were generated using GraphPad Prism version 8.4.3. The data were analysed using one-way analysis of variance (ANOVA) followed by post hoc Dunnett test. The experiments held were done in triplicate in three independent experiments and the data were presented in mean ± SEM with $p < 0.05$ as significant level.

CHAPTER 4

RESULTS

4.1 Cytotoxic effect of ASR aqueous extract on RAW 264.7 cells

It is very important to determine the suitable range of non-cytotoxic concentration of ASR aqueous extract to the cells in order to study its anti-inflammatory properties. In order to do that, a simple assay such as MTT assay can be used to evaluate the cell viability of RAW 264.7 macrophages when treated with ASR aqueous extract. MTT reduction assay is a colorimetric assay that is able to measure the cell proliferation and metabolism by reducing purple formazan into coloured formazan product that is measurable using spectrophotometer.

As shown in Figure 4.1., there is variability in the cell viability with decreasing trend as the concentration increases. The acceptable threshold for cell viability of RAW 264.7 macrophages is 80% (Muniandy et al., 2018). The highest concentration, 1000 µg/mL has significantly decreased cell viability with 82.5% ($p<0.05$). Therefore, there is possibility or risk that 1000 µg/mL may be cytotoxic to the macrophages. Meanwhile, concentration of 62.5, 125, 250 and 500 µg/mL only showed minor changes to the cell viability, and not significant different compared to control, thus, considered as non-cytotoxic to the macrophages. Thus, these four concentrations were chosen as concentrations to be used for anti-inflammatory analysis.

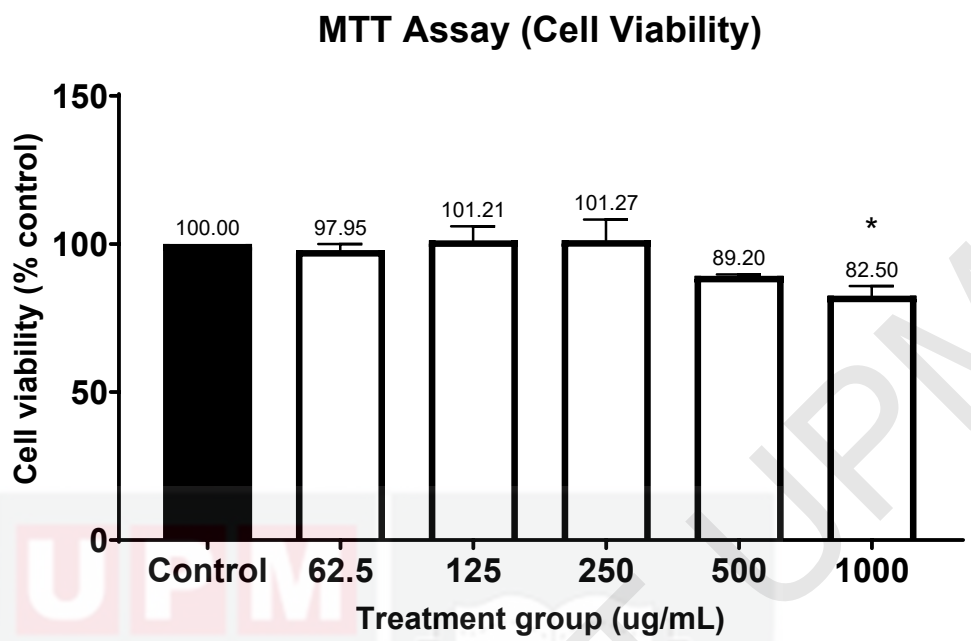

Figure 4.1: MTT reduction assay of ASR aqueous extract on RAW 264.7 macrophages cells. RAW 264.7 macrophages were treated with different concentrations concurrently for 24 hours. Cell viability is presented as percentage of compared to the control group. *$p < 0.05$ when 1000 µg/mL group is compared to control group. All values as expressed as mean ± SEM from three independent experiments.

4.2 Cell morphology

RAW 264.7 macrophages morphology are also observed at the same time as MTT reduction assay is done. This is done to observe if there are any changes to the macrophages due to ASR extract that may lower or inhibit the cell proliferation. Figure 4.2 shows the morphology of RAW 264.7 macrophages after 24 hours of incubation with ASR aqueous extract.

Even in different concentrations, RAW 264.7 cells displayed similar characteristics branching and projections, similar when compared to control group. The macrophages also showed polygonal or non-circular shape, similar to control.

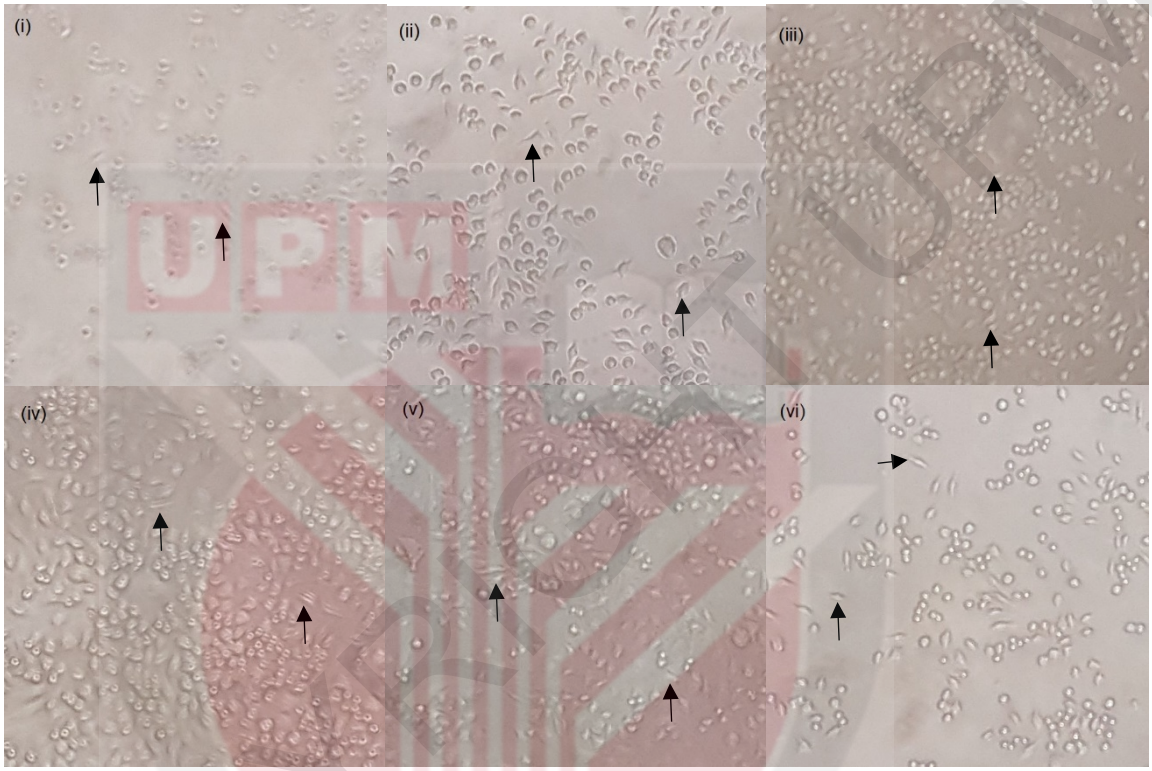

Figure 4.2: Morphological observation of RAW 264.7 macrophages after 24-hour treatment with ASR aqueous extract. The morphology is visualized under inverted microscope with 20X magnification. (i) 62.5 µg/mL, (ii) 125 µg/mL, (iii) 250 µg/mL, (iv) 500 µg/mL, (v) 1000 µg/mL, and (vi) control.

4.3 Inhibition of NO production of LPS-induced RAW 264.7 macrophages by ASR aqueous extract

Nitric oxide (NO) is an inflammatory mediator released from macrophages that plays an important role in the development both acute and chronic inflammation. Therefore, controlling its production is one of the first target in an anti-inflammatory study. The purpose of Griess assay is to determine the nitric oxide level indirectly by measuring nitrite concentration, produced by RAW 264.7 macrophages.

Figure 4.3.1 has shown that LPS-induced macrophages has produced NO by 3.8-fold compared to normal macrophages. Also, all treatment groups have shown decreased NO production significantly ($p < 0.001$) when compared to LPS only group..The lowest NO production by the treatment group is by concentration of 500 µg/mL with a mean of 180.49%, almost doubling the unstimulated macrophages NO production.

Meanwhile, in Figure 4.3.2, the percentage of inhibition of NO production for each group when compared to LPS only group is shown. Every treatment group has able to inhibit the NO production by LPS-stimulated macrophages significantly ($p < 0.001$). Moreover, it is clear that the inhibition percentage increased in dose-dependent manner except for 250 µg/mL at 26.09%. Of equal importance, 500 µg/mL group has recorded the highest inhibition percentage by 71.3%.

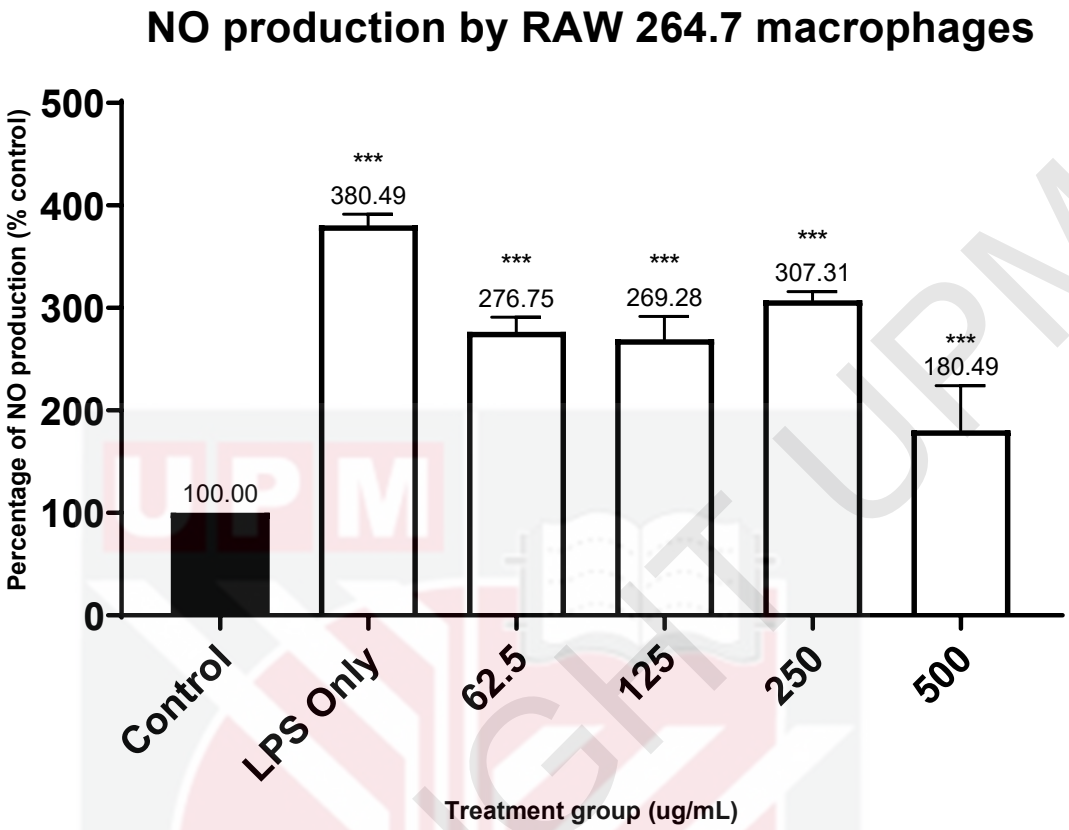

Figure 4.3.1: Effect of ASR aqueous extract on the percentage of NO production by LPL-stimulated RAW 264.7 macrophages. RAW 264.7 cells are treated with 4 different concentrations and stimulated with LPS to induce inflammation. The percentage NO production is expressed in percentage of control and calculated based on reaction with Griess reagent and comparison with control group. Statistical significance: ***$p < 0.001$, every treatment group vs control and LPS only (negative control) vs control. The values are presented in mean ± SEM.

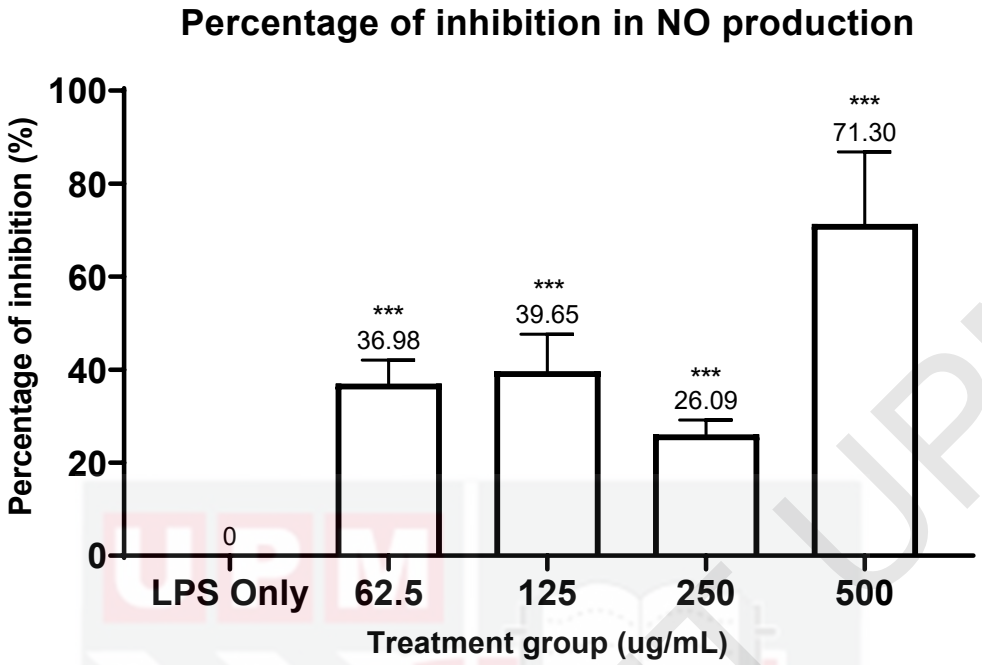

Figure 4.3.2: Percentage of inhibition in NO production by ASR aqueous extract on LPS-stimulated RAW 264.7 macrophages. The percentage of inhibition is calculated based on comparing NO production level between interest group and LPS only group. The values are presented in percentage and mean value ± SEM. ***p < 0.001, the treated group is significantly different than LPS only group.

CHAPTER 5

DISCUSSION

Inflammation is the general defensive response of our body towards any foreign substances yet it is responsible for development of multiple chronic diseases such as autoimmune diseases, cardiovascular diseases, and cancer (Libby, 2007). Many researchers have done countless of studies on different herbal medicine in vitro and in vivo to find new anti-inflammatory agents, with minimal side effects. In this study, to determine ASR extract effects on inflammation, a model of inflammation using RAW 264.7 macrophages cells stimulated by LPS has been used to mimic inflammation process.

LPS is an endotoxin, a component of gram-negative outer membrane. It is used in this study to stimulate inflammation, where LPS can initiate inflammation due to its release from bacteria lysis caused by immune system. Substances like LPS is known as pathogen-associated molecular pattern (PAMP), which can trigger inflammation by activating pattern-recognition receptors (PRR) expressed on the cell membrane (Chen et al., 2018). Signalling through these PRRs such as Toll-like receptors (TLRs) will activate intracellular signalling cascade leading to translocation of transcription factor into nucleus, such as nuclear factor kappa-light-chain-enhancer of enhanced B cells (NF-κB).

These transcription factors are important in activating several primary inflammatory mediators and enzymes that includes interleukin-1β, interleukin-6, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (Chen et al., 2018). iNOS is the enzyme responsible for generation of NO from L-arginine, free radicals that have deleterious effects and present on various inflammatory diseases and also every step of inflammation development. Thus, NO as a major inflammatory marker has been the target in studying anti-inflammation properties.

However, before measuring anti-inflammatory properties of ASR extract, its cytotoxicity must be determined first to ensure it is safe to RAW 264.7 cells. 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide or MTT assay is a colorimetric assay used to measure metabolic activity of the cells (Riss et al., 2004). As only living cells can possess metabolic activity, it can be used to measure the cell viability. MTT reduction assay works based on the ability of the cell to reduce yellow tetrazolium into insoluble purple formazan product. This reduction is due to the activity of NAD(P)H-dependent mitochondrial dehydrogenase enzymes. Thus, instead of only used for cell viability and cytotoxicity, MTT reduction assay can also be used to measure mitochondrial function of the cells. However, the crystal particles formed during the reduction may cause additional background interference, reducing its accuracy (Aslantürk, 2018).

MTT assay results have demonstrated that only concentration of 1000 µg/mL has shown cytotoxic effects to the macrophages based on acceptable threshold of 80%

cell viability. Hence, it is deemed unsafe and not considered to be used in anti-inflammatory analysis.

Since overproduction of NO is the results of chronic inflammation, inhibition to the production would be a promising strategy to tackle this problem. For this purpose, ASR extract is evaluated for its effects on NO production on LPS-stimulated RAW 264.7 cells. However, NO is known for its short half-life, making it difficult to quantify. One of the easiest methods to measure NO production is by measuring its end product, nitrite (NO_2^-) and nitrate (NO_3^-). This can be done by employing Griess assay, a common and validated method to measure nitrite ion concentration inside a target solution (Sun et al., 2003).

Griess reagent usually consists of 1% sulfanilic acid, 0.1% N-1-naphthyl-ethylenediamine dihydrochloride, and 5% phosphoric acid although different kit may differ in concentration. In Griess reaction, nitrite ions converted into diazonium salt when reacted with sulfanilic acid. Addition of N-1-naphthyl-ethylenediamine dihydrochloride will cause azo coupling reaction, forming red azo dye that can be measured using spectrophotometer.

From here on, based on Griess assay results, all concentration of ASR extracts has significantly inhibited NO production by LPS-stimulated RAW 264.7 cells. NO production induced by LPS increased significantly by almost 4-fold when compared to non-stimulated group. This result is expected as LPS is known to be strong inducer

for RAW 264.7 cells to produce higher level of NO due to stronger induction for inflammation.

The inhibition increases in dose-dependent manner except for concentration 250 µg/mL. This abnormal reading might have been cause due to abnormal amount of cell inside that concentration group. Higher amount of cell will lead to higher NO production, thus leading to lower amount of inhibition in 250 µg/mL group. To remedy this problem, operator may require better skills for to conduct the experiments.

However, inhibition of NO production can be caused by several reasons. For example, NO production can be inhibited due to suppression on the expression or activity of iNOS. It also can happen due to the suppression on the expression of NF-κB protein itself. Therefore, further testing is needed to understand the true target and mechanisms of this inhibition to properly utilize ASR as potential anti-inflammatory agent. As per this experiment, it is confirmed that ASR extract are able to inhibit NO production by LPS-stimulated RAW 264.7 cells, although there is no confirmation on the mechanism of the inhibition.

CHAPTER 6

CONCLUSION

Different concentrations of ASR aqueous extract have been tested for anti-inflammatory properties on LPS-stimulated RAW 264.7 cells. LPS is useful in inducing macrophages to release inflammatory mediators to be used in model in testing inflammations. From preliminary testing, ASR extract is considered non-cytotoxic to RAW 264.7 macrophages until concentrations of 500 µg/mL with above 80% cell viability. Anti-inflammatory testing using nitrite assay has shown significant inhibition on NO production of RAW 264.7 macrophages when stimulated with LPS showing huge potential in a new anti-inflammatory therapeutical option.

6.1 Future recommendations

As per this experiment, a study to understand the mechanism and target of inhibition of ASR extract in vitro such as using protein expression is a great start to further support this research. Evaluation of this extract in form of *in vivo* is also necessary to observe if there is any anti-inflammatory effect on more complex real-life model such as carrageenan-induced paw oedema model.

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APPENDICES

APPENDIX 1: Absorbance reading of MTT assay of ASR extract on RAW 264.7 cells.

SET 1

	CONTROL	62.5 µg/mL	125 µg/mL	250 µg/mL	500 µg/mL	1000 µg/mL
	0.679	0.661	0.72	0.696	0.599	0.554
	0.66	0.65	0.763	0.699	0.561	0.538
	0.631	0.696	0.742	0.699	0.577	0.502
AVERAGE	0.656	0.669	0.741	0.698	0.579	0.531
PERCENTAGE OF VIABILITY (%)	100	101.878	112.944	106.294	88.172	80.913

SET 2

	CONTROL	62.5 µg/mL	125 µg/mL	250 µg/mL	500 µg/mL	1000 µg/mL
	0.691	0.652	0.578	0.76	0.601	0.443
	0.634	0.619	0.61	0.812	0.557	0.522
	0.606	0.638	0.573	0.719	0.59	0.462
AVERAGE	0.643	0.636	0.587	0.763	0.582	0.475
PERCENTAGE OF VIABILITY (%)	100	98.860	91.196	118.643	90.523	73.899

SET 3

	CONTROL	62.5 µg/mL	125 µg/mL	250 µg/mL	500 µg/mL	1000 µg/mL
	1.055	1.161	1.129	0.955	1.077	1.021
	1.162	1.167	1.233	0.915	0.935	1.03
	1.184	1.03	1.183	1.105	1.03	0.967
AVERAGE	1.133667	1.119	1.181	0.991	1.014	1.006
PERCENTAGE OF VIABILITY (%)	100	98.735	104.234	87.474	89.444	88.738

SET 4

	CONTROL	62.5 µg/mL	125 µg/mL	250 µg/mL	500 µg/mL	1000 µg/mL
	0.919	1.007	1.037	1.002	0.957	0.938
	1.165	1.029	1.026	1.138	1.086	0.907
	1.156	0.955	1.063	0.862	0.829	0.956
AVERAGE	1.08	0.997	1.042	1.000	0.957	0.933
PERCENTAGE OF VIABILITY (%)	100	92.314	96.481	92.654	88.641	86.450

TOTAL AVERAGE PERCENTAGE VIABILITY (%)

MTT TEST	PERCENTAGE OF VIABILITY (%)					
	CONTROL	62.5 µg/mL	125 µg/mL	250 µg/mL	500 µg/mL	1000 µg/mL
SET 1	100	101.878	112.944	106.294	88.172	80.913
SET 2	100	98.860	91.196	118.643	90.523	73.899
SET 3	100	98.735	104.234	87.474	89.444	88.738
SET 4	100	92.314	96.481	92.654	88.641	86.450
AVERAGE PERCENTAGE OF VIABILITY (%)	100	98.80	100.4	99.47	89.04	83.68

APPENDIX 2: Univariate analysis of MTT assay of ASR extract on RAW 264.7 cells.

ANOVA

Absorbance

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1202.982	5	240.596	4.149	.011
Within Groups	1043.916	18	57.995		
Total	2246.899	23			

Multiple Comparisons

Dependent Variable: Absorbance

Dunnett t (2-sided)[a]

		Mean Difference (I-J)			95% Confidence Interval	
(I) Treatment_Group	(J) Treatment_Group		Std. Error	Sig.	Lower Bound	Upper Bound
62.5	Control	-2.05266750	5.38494874	.994	-16.9227242	12.8173892
125	Control	1.21401250	5.38494874	1.000	-13.6560442	16.0840692
250	Control	1.26655000	5.38494874	.999	-13.6035067	16.1366067
500	Control	-10.80452500	5.38494874	.205	-25.6745817	4.0655317
1000	Control	-17.49938500*	5.38494874	.018	-32.3694417	-2.6293283

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

APPENDIX 3: Absorbance reading on Griess assay of ASR extract on LPS-stimulated RAW 264.7 cells.

	CONTROL	LPS ONLY	62.5 µg/mL	125 µg/mL	250 µg/mL	500 µg/mL
	0.145	0.531	0.557	0.398	0.389	0.424
	0.139	0.525	0.514	0.371	0.345	0.441
	0.134	0.518	0.488	0.392	0.381	0.434
	0.141	0.511	0.513	0.412	0.401	0.405
	0.129	0.508	0.547	0.373	0.37	0.43
	0.144	0.518	0.523	0.371	0.407	0.414
	0.134	0.51	0.513	0.368	0.326	0.452
	0.131	0.553	0.488	0.351	0.335	0.464
AVERAGE	0.137	0.521	0.517	0.379	0.369	0.433
PERCENTAGE OF INHIBITION (%)			36.984	39.649	26.090	71.303

APPENDIX 4: Univariate analysis of variance of Griess assay of ASR extract on LPS-stimulated RAW 264.7 cells.

ANOVA

Inhibition_Percentage

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	20935.000	4	5233.750	71.113	.000
Within Groups	2355.114	32	73.597		
Total	23290.114	36			

Multiple Comparisons

Dependent Variable: Inhibition_Percentage

Dunnett t (2-sided)[a]

(I) Treatment_Group	(J) Treatment_Group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
62.5	LPS	36.98407625*	4.28944350	.000	25.9304284	48.0377241
125	LPS	39.64901000*	4.28944350	.000	28.5953621	50.7026579
250	LPS	26.09034600*	4.89071807	.000	13.4872483	38.6934437
500	LPS	71.30321750*	4.28944350	.000	60.2495696	82.3568654

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.