UNIVERSITI PUTRA MALAYSIA

GASTROPROTECTIVE AND ANTI-INFLAMMATORY ACTIVITIES OF A MIXTURE OF Melastoma malabathricum L. AND Muntingia calabura L. LEAVES

SITI ZAWANAH BINTI HALIM

FPSK(M) 2017 66

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By

SITI ZAWANAH BINTI HALIM

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

June 2017

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Master of Science

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June 2017

Chairman : Associate Professor Zainul Amiruddin Zakaria, PhD
Faculty : Medicine and Health Sciences

Gastric ulcer is a part of the peptic ulcer disease which well acknowledged as one of major human illness. Basically, the gastric ulcer is occurred due to the imbalance between the protective factors and aggressive factors in the stomach. Recently, there were a few of medicines being used to treat the gastric ulcer. However, those medicines had caused certain adverse effects. At the same time, medicinal plants have proved to give positive results as the treatment for many diseases including gastric ulcer. *Melastoma malabathricum* (MM) and *Muntingia calabura* (MC) were two plants which have been studied for their pharmacological activities. In addition, the previous study had also reported the markedly gastroprotective effect of both those plants leaves individually via *in vitro* and *in vivo* experiments. Thus, in attempt to develop a pharmaceutical product with antiulcer potential, it is necessary to make sure it is effective at lower dose range, so the present study was aimed to identify the gastroprotective activity of methanol extract of a mixture of *Melastoma malabathricum* and *Muntingia calabura* (MMMC) at various ratio using rats models and further investigate whether combination of those plant at various ratio will exert synergistic effect by enhancing or reducing the observed antiulcer activity. The small particle of MM and MC grinded leaves were mixed together based on three different ratios (1:1, 1:3 and 3:1; w/w) and soaked in methanol in ratio 1:20 for 72 hours, filtered and evaporated using the rotatory evaporator to get the concentrated crude extract. By using the crude extract obtained from the evaporation process, the doses (15, 150 or 300 mg/kg) were prepared. Ranitidine (100 mg/kg) was used as a reference drug while 2% Tween 80 was used as a negative control. The Sprague-Dawley rats were given the test solutions orally for seven consecutive days and were subjected to the ethanol-induced gastric ulcer. The rats were euthanized; macroscopic and histological observations of the gaster were done. The ulcer area (UA) was determined and the percentage of the inhibition exhibited by the MMMC was calculated. The gaster was subjected to antioxidant studies including the superoxide dismutase (SOD), catalase

(CAT), glutathione (GSH), prostaglandin E_2 (PGE_2) and malondialdehyde (MDA) assay. The MMMC was tested for antioxidant study using the total phenolic content (TPC), oxygen radical absorbance capacity assay (ORAC) and 2,2- diphenyl-1-picrylhydrazyl radical scavenging assay (DPPH). The antisecretory potential of MMMC was investigated using the pyloric ligation model while the role of nitric oxide and endogenous sulfhydryl group were measured by testing the extract against L-NG-Nitroarginine Methyl Ester ($_L$-NAME) and N-Ethylmaleimide (NEM) using the ethanol-induced model. Ratio 1:1 of MMMC showed the most effective gastroprotective activity in which it significantly ($P<0.05$) reduced the gastric lesion when assessed using the ethanol-induced gastric ulcer models. The macroscopic findings were validated by the microscopic observations. Furthermore, except for the pH and total acidity, the extract also significantly ($P<0.05$) reduced the volume of gastric content whereas the mucus content was significantly ($P<0.05$) increased in MMMC-treated rats in the pyloric-ligation test, where by ratio 1:1 constantly exhibited the best result among the three ratios. Besides that, the gastroprotection effect of MMMC significantly ($P<0.05$) involved the participation of nitric oxide and sulfhydryl compounds. Ratio 1:1 of MMMC showed the highest value of TPC and ORAC activity compared to the other ratios, meanwhile ratio 3:1of MMMC exhibited the strongest IC_{50} of DPPH scavenging activity. The *in-vitro* anti-inflammatory effect of MMMC evaluation showed moderate NO inhibitory activity at IC_{50} of ratio 1:1 and 1:3 of MMMC, compared to ratio 3:1 which showed weak NO inhibitory activity. While the antioxidants studies of the stomach revealed the improvement of CAT, SOD, GSH, PGE_2 and MDA activities by MMMC with ratio 1:1 showed the best result among the three ratios. Besides that, the result obtained from the GC-MS analysis showed several peaks detected at the different real time suggested that MMMC contained several compounds that have been proven by other studies to show antioxidant, anti-inflammatory and gastroprotective effect. The UHPLC analysis suggested that the presence of quercetin and gallic acid in the most effective ratio of MMMC, which is 1:1 was confirmed in MMMC. In conclusion, MMMC at the ratio of 1:1, exerted the best gastroprotective activity against the ethanol-induced gastric ulcer assay partly by activating its antisecretory and antioxidant activities, together with modulation of the gastric tissue endogenous antioxidant system.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

AKTIVITI PERLINDUNGAN GASTRO DAN ANTI-INFLAMASI OLEH CAMPURAN DAUN-DAUN *Melastoma malabathricum* L.DAN *Muntingia calabura* L

Oleh

SITI ZAWANAH BINTI HALIM

Jun 2017

Pengerusi : Profesor Madya Zainul Amiruddin Zakaria, PhD
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Ulser gastrik adalah sebahagian daripada penyakit ulser peptik yang telah sedia maklum merupakan salah satu penyakit manusia yang major. Secara asasnya, ulser gastrik berlaku disebabkan oleh ketidakimbangan di antara faktor-faktor perlindung dan faktor-faktor agresif di dalam perut. Baru-baru ini, terdapat beberapa ubat-ubatan yang digunakan untuk merawat ulser gastrik. Namun, ubat-ubatan tersebut telah menyebabkan kesan-kesan buruk yang tertentu. Pada masa yang sama, tumbuh-tumbuhan perubatan telah terbukti memberi keputusan-keputusan positif sebagai rawatan untuk pelbagai penyakit termasuk ulser gastrik. *Melastoma malabathricum* (MM) dan *Muntingia calabura* (MC) merupakan dua tumbuh-tumbuhan yang telah dikaji mengenai aktiviti-aktiviti farmakologi mereka. Tambahan pula, kajian sebelum ini telah melaporkan kesan perlindungan gastro yang signifikan oleh daun daripada kedua-dua tumbuhan tersebut secara berasingan melalui eksperimen *in vitro* dan *in vivo*. Oleh itu, dalam percubaan mengembangkan sesuatu produk farmasiutikal, ianya menjadi keperluan untuk memastikan produk tersebut berkesan pada kadar dos yang lebih rendah, maka kajian ini dijalankan bertujuan untuk mengenalpasti aktivit sinergi perlindungan gastro oleh ekstrak metanol dari campuran *Melastoma malabathricum* dan *Muntingia calabura* (MMMC) pada nisbah yang berbeza menggunakan model tikus dan seterusnya dikaji samada gabungan kedua-dua tumbuhan tersebut pada nisbah yang berbeza mampu menghasilkan kesan sinergi melalui peningkatan atau penurunan aktivit anti ulser yang diperhatikan. Zarah-zarah kecil daripada daun-daun MM dan MC yang telah dikisar, dicampur bersama-sama berdasarkan tiga nisbah yang berbeza (1:1, 1:3 dan 3:1; w/w) dan direndam di dalam metanol dalam nisbah 1:20 selama 72 jam, ditapis dan disejat menggunakan pemutar penyejat untuk mendapatkan mentah ekstrak yang pekat. Dengan menggunakan mentah ekstrak yang diperoleh daripada proses penyejatan, dos-dos (15, 150 atau 300 mg/kg) disediakan. Ranitidine (100 mg/kg) telah digunakan sebagai dadah rujukan sementara 2% Tween 80 telah digunakan sebagai kawalan negatif. Tikus-tikus jenis *Sprague-Dawley* tersebut telah

diberikan solusi –solusi yang diuji melalui oral untuk tujuh hari berturut-turut dan telah di arah kepada ulser gastrik rangsangan etanol. Tikus-tikus tersebut telah dieutanasia; pemerhatian makroskopik dan histologi ke atas gaster telah dijalankan. Kawasan ulser (UA) telah dikenalpasti dan peratusan perencatan yang telah ditunjukkan oleh MMMC turut dikira. Gaster telah di arah kepada kajian-kajian antioksidan termasuk eksperimen superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), prostaglandin E_2 (PGE_2) dan malondialdehyde (MDA). MMMC telah diuji untuk kajian antioksidan menggunakan jumlah kandungan fenolik (TPC), eksperimen kapasiti penyerapan oksigen radikal (ORAC) dan eksperimen memerangkap radikal 2,2- diphenyl-1-picrylhydrazyl (DPPH). Potensi anti-rembesan oleh MMMC telah dikaji menggunakan model ikatan pilorik sementara peranan nitrik oksida dan kumpulan sulfhydryl dalaman disukat dengan menguji ekstrak terhadap L-NG-Nitroarginine Methyl Ester ($_L$-NAME) dan N-Ethylmaleimide (NEM) menggunakan model rangsangan etanol. Nisbah 1:1 MMMC menunjukkan aktiviti perlindungan gastro yang paling efektif di mana ia mengurangkan luka gastrik secara signifikan ($P<0.05$) apabila diuji menggunakan model gastrik ulser rangsangan etanol. Penemuan makroskopik telah disahkan menggunakan pemerhatian mikroskopik. Tambahan pula, kecuali pH dan jumlah keasidan, ekstrak tersebut turut menggurangkan isipadu kandugan gastrik secara signifikan ($P<0.05$) sedangkan kandungan mukus telah meningkat secara signifikan ($P<0.05$) dalam kumpulan tikus yang dirawat oleh MMMC dalam kajian ikatan pilorik, di mana nisbah 1:1 secara terus mempamerkan keputusan terbaik di antara ketiga-tiga nisbah. Selain itu, kesan perlindungan gastro oleh MMMC telah melibatkan penyertaan nitrik oksida dan sebatian sulfhydryl secara signifikan ($P<0.05$). Nisbah 1:1 MMMC telah menunjukkan nilai TPC dan aktiviti ORAC yang paling tinggi berbanding nisbah yang lain, sementara nisbah 3:1 MMMC mempamerkan IC_{50} aktiviti memerangkap DPPH yang paling kuat. Penilaian kesan anti-keradangan secara *in-vitro* oleh MMMC menunjukkan bahawa aktiviti penyekatan NO pada IC_{50} adalah sederhana bagi nisbah 1:1 dan 1:3 MMMC, berbanding nisbah 3:1 yang menunjukkan aktiviti penyekatan NO yang lemah. Sementara kajian-kajian antioksidan terhadap perut mendedahkan penambahbaikan aktiviti-aktiviti CAT, SOD, GSH, PGE_2 dan MDA oleh MMMC dengan nisbah 1:1 telah menunjukkan keputusan paling baik di antara ketiga-tiga nisbah. Selain itu, keputusan yang diperoleh daripada analisa GC-MS menunjukkan beberapa puncak-puncak telah dikesan pada masa sebenar yang berbeza yang mencadangkan bahawa MMMC mengandungi beberapa sebatian yang telah terbukti oleh kajian-kajian lain menujukkan kesan antioksidan, anti-keradangan dan perlindungan gastro. Analisa UHPLC mencadangkan bahawa kehadiran quercetin dan gallic acid di dalam nisbah MMMC yang paling efektif iaitu 1:1 adalah pasti di dalam MMMC. Kesimpulannya, MMMC pada nisbah 1:1, telah menunjukkan aktiviti perlindungan gastro yang terbaik terhadap eksperimen ulser gastrik yang dirangsang oleh etanol, sebahagiannya melalui pengaktifan aktiviti-aktiviti anti-rembesan dan antioksidanya, bersama-sama pemodulasian sistem antioksida di dalam tisu gastrik.

ACKNOWLEDGEMENTS

Alhamdulillah, praise to Allah S. W. T for His blessing finally I can complete all my labwork and this precious thesis. Foremost, I would like to express my sincere gratitude to my supervisor, Associate Professor Dr. Zainul Amiruddin Zakaria for the continuous support of my Master study and research, for his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my Master study.

Besides my supervisor, I would like to thank the rest of my thesis committee: Dr. Norhafizah Mohtaruddin and Prof. Muhammad Nazrul Hakim Abdullah for their encouragement, insightful comments and precious advice. I also want to thank my beloved lecturer from Department of Science Biomedical: Dr Siti Farah Md Tohid, Dr Fezah, Dr Hasiah, Dr Abdah Akim, Dr Latifah Saiful Yazan, Dr Suhaili, Dr Manraj, Prof Daud Asraf Ali, Dr Tham, Dr Khairi and Dr Roslida for their supports and advises. I also would like to acknowledge Dr. Ikarastika Rahayu (UMK), Dr. Maizatul Hasyima (IMR) and Prof Rahim (Faculty of Veterinary, UPM) for their support and guidance.

My countless thank to my beloved fellow seniors in Pharmacology Laboratory: Khusairi, Wahida Ismail, Tavamani Balan, Liana Nasir, Diyana Mahmood, Farah Hidayah, Syariah Mamat, Erin, Farhana Yahya, Hafiz Abd. Rahim, Sallahuddin, Hijjaz and Fauzi Fahmi for always been right there, helping out wherever and whenever needed for these past few years.

To my beloved and supportive labmates: Baizura Borhan, Rushduddin Al Jufri, Aifaa Akmal, Qais Jarrar, Muhammad Luqman Nordin, Said, Najwa Najihah and Syaz, thank you for helping me in my labwork, for the stimulating discussions, guidances and precious advises. All of you are valued members of our team, and I truly appreciate your kindness. Besides that, so many thanks to all my beloved friends who always being with me through thick and thin of my life: Aminah Suhaila, Anis Syafiqah, Arina Ahmad Jelani, Fatin Najwa, Fatin Hannani, Afifah Jalil, Amirah Salwani, Farizatul Akmawati, Ana Masara, Amielia, Sarah Sapuan, Nazmi Firdaus, Aida, Hui Min, Elson, Hana, Arina Soberi, Maira Hafitz, Lyana Bukhari, Asyikin, Zaidi, Najwa Raz, Shakimah, Erma Suriyana, Mahirah and Syazrain.

My appreciation also goes to all undergraduate students, suppliers and staffs who helped me a lot throughout my research. They are the final year student: Faezah Zainol, Hanini, Nadzirah, Izyan, Faizah Talib, Cha Qairunnicaa, Tiq, Syu, Yusof; suppliers: Faizi and Saleh (Alchemy Supplies), Puan Hayati, En. Zain, En. Kufli, En. Mat Noor, En. Firdaus, En Abul and Puan Huda (IBS).

Special thanks to my beloved parents, Maliah Omar and Halim Bakar, my family members, Zahirah, Sumaiyah, ‘Izzah, ‘Iffah, Sa’idah, Husna, ‘Umair, Khairul Fazli, Hakim Zakaria, Auni, Ulya, Iris, Iman and Alief for all their unstoppable encouragement, endless love and prayer.

Last but not least, I would like to thank scholarship Jabatan Perkhidmatan Awam (JPA) and Postgraduate Student Grant under Faculty of Medicine and Health Sciences, UPM.

I certify that a Thesis Examination Committee has met on 5 June 2017 to conduct the final examination of Siti Zawanah binti Halim on her thesis entitled "Gastroprotective and Anti-Inflammatory Activities of a Mixture of *Melastoma malabathricum* L. and *Muntingia calabura* L. Leaves" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

AAPH	2,2'-Azobis 92-methylpropionamidine dihydrochloride
AchE	Acetylcholinesterase
ANOVA	Analysis of variance
ASA	Acetylsalicylic acid
cAMP	Cyclic adenosine monophosphate
CAT	Catalase
$CuSO_4$	Copper Sulphate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
GAE	Gallic acid equivalent
GC-MS	Gas Chromatography – Mass Spectral
GSH	Glutathione
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High-performance liquid chromatography
H_2O_2	Hydrogen peroxide
IACUC	Institutional Animal Care and Use Committee
IFN-γ	*Interferon gamma*
IHME	Institute for Health Metrics and Evaluation
LC-MS	Liquid chromatography- mass spectrometry

$_{L}$-NAME	N^{G}-ω-nitro-L-arginine methyl ester
LPS	*Lipopolysaccharide*
MDA	Malondialdehyde
MgCl	Magnesium Chloride
MMMC	*Melastoma malabathricum Muntingia calabura*
NaCl	Sodium Chloride
NaOH	Natrium Hydroxide
Na_2CO_3	Sodium carbonate
NEM	N-ethylmaleimide
nm	Nanometer
NO	Nitric oxide
NOS	*Nitric oxide* synthase
NO_2^-	Nitrite ions
NSAIDs	Non Steroidal anti-inflammatory drug
OD	Optical Density
ORAC	Oxygen radical absorbance capacity
PAF	Platelet-activating factor
PGE_2	Prostaglandins
PGs	Prostaglandins
PUD	Peptic ulcer disease
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpm	Revolutions per minute
SEM	Standard Error of Mean
SH	Sulfhydryl

SOD	Superoxide Dismutase
TBA	Thiobarbituric acid
TE	Trolox Equivalent
TPC	Total phenolic content
UA	Ulcer area
UHPLC-ESI	Ultra High Performance Liquid Chromatography-Electrospray Ionizaton
UV	Ultraviolet

CHAPTER 1

INTRODUCTION

Peptic ulcers have become one of the major human illness affecting nearly 8-10% of the global population (Calam and Baron, 2001), and of these number, 5% suffer from gastric ulcers (Bandyopadhyay *et al*., 2001). Diverse factors such as alcohol consumption, a stressful lifestyle, use of steroidal and non-steroidal anti-inflammatory drugs (NSAIDs) and drugs which stimulate gastric acid and pepsin secretion, *Helicobacter pylori* infections and smoking contribute to the pathogenesis of gastric ulcers (Rang *et al*., 2014). An imbalance between the aggressive factors such as acid and pepsin secretion, *Helicobacter pylori*, refluxed bile, release of leukotrienes and reactive oxygen species (ROS) and mucosal defensive factors that include bicarbonate secretion, mucus-bicarbonate barrier, surface active phospholipids, prostaglandins (PGs), mucosal blood flow, cell renewal and migration, non-enzymatic and enzymatic antioxidants and some growth factors (De Lira Mota *et al*., 2009) leads to gastric damages.

The prevention or cure of peptic ulcers has become an important challenge in the current medicine world. Although gastric ulcer is linked to many causative factors, secretion of gastric acid is still believed to remain as the central component of this disease (De Lira Mota *et al*., 2009). Thus, inhibition of gastric acid secretion is the key therapeutic target for ulcer diseases (Jain *et al*., 2007). Therefore, current medicinal treatment of gastric ulcers available is generally based on the inhibition of gastric acid secretion by histamine H_2-antagonists, proton pump inhibitors, and antimuscarinics, as well as on acid-independent therapy provided by sucralfate and bismuth cholinergic (Bighetti *et al*., 2005). However, gastric ulcer therapy faces a major drawback nowadays as most of the drugs available in the market are often associated with adverse effects such as bradycardia and pancreatitis (Bandyopadhyay *et al*., 2002; Rang *et al*., 2014; Herrmann *et al*., 1990; Johnson and Miller, 1988).

In this context, the use of medicinal plants has gained the interest of many researchers. The natural product is in continuous expansion all over the world and became the most attractive source of new drug for the treatment and prevention of many diseases. A diverse range of bioactive molecules isolated from plant natural product has been shown to produce promising results for the treatment of gastric ulcer (Borrelli and Izzo, 2000).

Two of the plants that are currently under investigation for their potential pharmacological activities in the laboratory are *Melastoma malabathricum* L. (family Melastomataceae) and *Muntingia calabura* L. (family Muntingiaceae). *Melastoma malabathricum* also was known as *senduduk ungu* in Malaysia. It is one of the most common herbs or small shrubs found throughout the tropic in the moist land mostly from India, Thailand and Malaysia. *Melastoma malabathricum*'s leaves, shoots, barks, seeds and roots have been used as a folk remedy to treat diarrhea, dysentery, hemorrhoids, cuts and wounds, toothache, and stomachache (Joffry *et al*., 2011). Scientific evaluations on

Melastoma malabathricum have revealed several pharmacological activities possessed by the plant. *Melastoma malabathricum* leaves have been reported to exhibit significant antinociceptive, anti-inflammatory, wound healing, cytotoxic, antidiarrheal and antioxidant activities (Joffry *et al*., 2011). Flavonoids, tannins, saponins, triterpenes and steroids have been detected in the leaves of *Melastoma malabathricum* (Mamat *et al*., 2013).

While *Muntingia calabura*, commonly known as Jamaican cherry or *kerukup siam* in Malaysia, is widely cultivated in warm areas of the Asian region, including Malaysia (Chin, 1989). In Asia and tropical America, various parts of this tree have been documented for several medicinal uses. The leaves, flowers, barks and roots of *Muntingia calabura* have been used as a folk remedy to treat headaches, fever and incipient cold. Besides, they are also employed as antiseptic, antispasmodic, and antidyspeptic agent (Kaneda *et al*., 1991; Nshimo *et al*., 1993). It also has been scientifically validated to possess several pharmacological activities. Significant antinociception (Zakaria *et al*., 2006b, 2007a, 2007b), antitumor (Kaneda *et al*., 1991; Su *et al*., 2003), anti-inflammatory, antipyretic (Zakaria *et al*., 2007b), antibacterial (Sulaiman *et al*., 2006), antiproliferative and antioxidant (Zakaria *et al*., 2011b) activities have been exhibited by the leaves of *Muntingia calabura.* Several types of flavonoids have been isolated and identified from the leaves, roots and stem barks of *Muntingia calabura* (Kaneda *et al*., 1991; Nshimo *et al*., 1993; Su *et al*., 2003; Chen *et al*., 2005; Sufian *et al*., 2013).

The previous study has reported the significant antiulcer activity of methanol extract of *Melastoma malabathricum* and *Muntingia calabura* leaves individually using several animal models as well as *in vitro* antioxidant and anti-inflammatory activities (Balan *et al*., 2014; Zakaria *et al*., 2015). Therefore, in an attempt to develop a pharmaceutical product with gastroprotective potential, it is necessary to make sure it is effective at lower dose range. Since no study has been done to determine whether there is gastroprotective action between combination ratio of these two plants, which might demonstrate gastroprotective activity at lower dose range, the present study was aimed to investigate whether the combination of these plants at various ratio will exert a gastroprotective effect by enhancing or reducing the observed gastroprotective activity.

Justification for studying the gastroprotective and anti-inflammatory activity of methanol extract of *Melastoma malabathricum* and *Muntingia calabura* leaves

To date, there are many approaches (Konturek *et al*., 2011) and advanced development of modern medicine. However, these scenario comes together with several obstacles, such as the presence of drugs adverse effect that might stop the patients with a certain health condition to consume the drugs, unaffordable and descend of drug availability. Because of that, there is calling for a research that can produce a product which is safe, effective in a low dose of consumption and affordable. The methanol extract of *Melastoma malabathricum* and *Muntingia calabura* leaves individually have been reported to show significant gastroprotective activity using several animal models as well as *in vitro* experiment of antioxidant and anti-inflammatory activities (Balan *et al*., 2015; Mamat *et al*., 2013; Mazura *et al*., 2007; Zakaria *et al*., 2011a; Zakaria *et*

al., 2006a). However, in an attempt develop a pharmaceutical product with gastroprotective potential, it is necessary to make sure it is effective at lower dose range since there were many commercial drugs reported to cause an adverse effect when consumed at a certain dose which is considering high. In this study, the combination of those plant at the various ratio with gastroprotective activity will be investigated.

Hypothesis

There are gastroprotective and anti-inflammatory activities of methanol extract of a mixture *Melastoma malabathricum* and *Muntingia calabura* leaves through *in vivo* and *in vitro* experiments.

General objectives

- To identify the gastroprotective and anti-inflammatory activities of methanol extract of a mixture *Melastoma malabathricum* and *Muntingia calabura* leaves through *in vivo* and *in vitro* experiments.

Specific objectives

- To determine the best ratio and lowest dose among the three combination ratios of mixture of *Melastoma malabathricum* and *Muntingia calabura* that gives the most effective gastroprotective and anti-inflammatory activities.
- To elucidate the physical changes through pyloric ligation model, L-NAME and NEM pre-treated ethanol-induced in rats.
- To determine the level of antioxidant activities of the extract as a part of the gastroprotective pathway.
- To screen for the compounds present in MMMC using Gas Chromatography-Mass Spectrometry (GC-MS) and Ultra High Performance Liquid Chromatography-Electrospray Ionization (UHPLC-ESI).

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