UNIVERSITI PUTRA MALAYSIA

ANTINOCICEPTIVE ACTIVITY OF THE ESSENTIAL OILS OF ZINGIBER OFFICINALE ON FORMALIN INDUCED PAW LICKING IN MICE

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A PROJECT PAPER SUBMITTED AS PARTIAL REQUIREMENT FOR THE DEGREE OF BACHELOR SCIENCES (BIOMEDICAL SCIENCE)

DEPARTMENT OF BIOMEDICAL SCIENCES
FACULTY OF MEDICINE AND HEALTH SCIENCES
UNIVERSITI PUTRA MALAYSIA
SERDANG, SELANGOR

2020

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ABSTRACT

Antinociceptive Activity of the Essential Oils of *Zingiber officinale* on Formalin Induced Paw Licking in Mice

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Introduction: *Zingiber officinale* or locally known as ginger belongs to Zingiber genus as well as the family of *Zingiberaceae.* The origin of this plant is uncertain since it does not grow in the wild forest. It has been commonly used as a spice and herbal medicine for hundreds of years. It is also claimed to be useful in traditional medicinal practices in order to treat colds, headaches, nausea and emesis. Previously, studies of this plant reported that it has various medicinal properties including anti-inflammatory, antimicrobial, anticancer and antioxidant. **Objective:** This study generally aims to investigate the analgesic effects of essential oil of *Zingiber officinale* on formalin induced paw licking in mice. **Hypothesis:** It is hypothesized that formalin induced paw licking in mice can be inhibited by oral administration of essential oil of *Zingiber officinale* for nociception. **Methodology:** The rodent mice were pre-treated with different concentrations of essential oil of ginger (GEO) at 10, 30 and 100 mg/kg by oral administration for one hour. The mice were injected with 2.5% formalin subcutaneously into the plantar surface of one of hind paws after one hour. The observation of the paw licking of mice were comprised with two phases which phase 1 (early phase) at 0-5 minutes and phase 2 (late phase) at 15-30 minutes. **Results:** The three different concentrations at 10, 30 and 100 mg/kg of GEO significantly reduced the time spent of each mouse on licking and biting of the injected hind paw during the early/neurogenic phase by 27.82%, 29.75% and 35.21% as well as late/inflammatory phase by 37.92%, 43.42% and 60.91%. **Conclusion:** Based on the findings, the antinociceptive activity that produces by this plant is possibly by the constituents of numerous bioactive compounds of ginger in exhibiting various medicinal properties to treat a wide variety of ailments.

Keywords: Zingiber officinale, nociception, analgesic, pain

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ABSTRAK

Aktiviti Antinosiseptif Minyak Pati daripada *Zingiber officinale* Terhadap Formalin yang Menyebabkan pada Penjilatan Tapak Kaki Mencit

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Pengenalan: *Zingiber officinale* atau lebih dikenali sebagai halia berasal daripada genus Zingiber dan keluarga *Zingiberaceae.* Asal usul tumbuhan ini tidak boleh dikenal pasti kerana ia bukan berasal dari hutan liar. Ia selalu digunakan sebagai rempah dan ubatan herba selama ratusan tahun lampau. Ia juga berguna untuk amalan perubatan tradisional untuk merawat selsema, sakit kepala, loya dan muntah. Kajian sebelum ini telah melaporkan tumbuhan ini mengandungi ciri-ciri perubatan seperti anti-keradangan, antibakteria, antikanser dan antioksidan. **Objektif:** Kajian ini bertujuan untuk mengkaji kesan analgesik minyak pati halia terhadap formalin yang menyebabkan pada penjilatan tapak kaki mencit. **Hipotesis:** Hipotesis kajian menunjukkan penyuntikan formalin untuk penjilatan tapak kaki mencit boleh disekat apabila dirawat dengan oral minyak pati halia untuk nosisepsi. **Kaedah:** Mencit telah dipra-rawat dengan kepekatan minyak pati halia yang berbeza iaitu pada 10, 30 dan 100 mg/kg melalui kaedah oral selama sejam. Mencit telah disuntik dengan 2.5% formalin secara subkulitan pada salah satu telapak kakinya selepas sejam. Pemerhatian terhadap penjilatan tapak kaki oleh mencit mengandungi dua fasa yang berbeza iaitu fasa pertama (fasa awal) pada 0-5 minit dan fasa kedua (fasa lewat) pada 15-30 minit. **Keputusan:** Tiga kepekatan minyak pati halia yang berbeza iaitu pada 10, 30 dan 100 mg/kg telah menunjukkan masa mencit menjilat atau menggigit tapak kaki menurun dengan bererti semasa fasa pertama/neurogenik sebanyak 27.82%, 29.75% dan 35.21% juga pada fasa kedua/radang sebanyak 37.92%, 43.42% dan 60.91%. **Konklusi:** Keputusan kajian ini menunjukkan bahawa aktiviti antinosiseptif yang dihasilkan oleh tumbuhan ini adalah berkemungkinan kerana halia yang mempunyai pelbagai komponen bahan bioaktif sehingga boleh menghasilkan ciri-ciri perubatan untuk merawat pelbagai jenis penyakit.

Kata kunci: *Zingiber officinale, nosisepsi, analgesik, sakit*

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ACKNOWLEDGEMENT

In the name of Allah, the Most Gracious and the Most Merciful

First and foremost, I would like to express my deepest gratitude to my supervisors, Dr. Ahmad Akira Omar Farouk and Prof. Dr. Mohd Roslan Sulaiman for their guidance, advice and patience throughout this project. Without their guidance and assistance, this research would have never been accomplished.

Besides, I would like to thank my project mates, Yap Zhiying, Nazatul Sahira and Siti Norizzaty for their help and support throughout this journey. Not to forget, my sincere gratitude goes to postgraduate student, Ong Hui Ming for her great help, support and guidance in completing this project. My appreciation also goes to all the laboratory staffs for their kind help and allow me to access all the facilities provided in the laboratory.

Last but not least, I would like to thank both of my parents (Salleh bin Ahmad and Rohana binti Mohddin) for their endless support and encouragement for me throughout this journey. Finally, I also would like to thank to everyone who were directly or indirectly contributing to the completion of this study.

MIMI ATHIRAH BINTI SALLEH

Universiti Putra Malaysia

August 2020

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ABBREVIATIONS

Aδ fiber	A delta fiber
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
ASA	Acetylsalicylic acid
CGRP	Calcitonin gene-related peptide
CNS	Central nervous system
COX	Cyclooxygenase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EOs	Essential oil
EC_{50}	The half maximal effective concentration
GEO	Ginger essential oil
H_2O_2	Hydrogen peroxide
HeLa	Henrietta lacks
HepG2	Human liver cancer cell line
HL-60	Human promyelocytic leukaemia cell line
IC_{50}	The half maximal inhibitory concentration
i.p.	Intraperitoneal
kg	Kilogram
MCF-7	Michigan cancer foundation-7
min	Minutes (s)
mg	Milligram
ml	Millilitre
MTT	Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NGF	Nerve growth factor
NR	Neutral red
NSAIDs	Nonsteroidal anti-inflammatory drug
p	p-value
p.o.	orally
PBS	Phosphate-buffered saline
PMA	Progressive muscular atrophy
PNS	Peripheral nervous system
s	Seconds (s)
SiHa	Studies on a new human cell line
SEM	Standard error of mean
TRPA1	Transient receptor potential ankyrin 1
TRPV1	Transient receptor potential vanilloid 1

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CHAPTER 1

INTRODUCTION

1.1 Background

Plants that are derived from natural products are known as great important sources of food and medicines (Che & Zhang, 2019). Natural products components have been discovered as a source of healing agents and structural diversity for many years (Koparde et al., 2019). The consumption of this natural product, especially natural plants is not only for human's nutritional support yet also for their health benefits because majority of people in this world still depend on herbal remedies to make their own traditional medicines or as complementary and alternative medicines (Che & Zhang et al., 2019). According to Ekor (2014), the usage of herbal remedies and supplement products by the majority of the world population has been rising extremely for more than thirty years because these products have been used as treatment for different health problems in various national health management programmes.

Traditional medicines are very important for modern drug discovery because they consist with several phytochemical constituents like flavonoids, alkaloids, terpenoids etc. In general, these components can be worked together to exhibit any pharmacological effect that people desire (Parasuraman, Thing & Dhanaraj, 2014).

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Besides, Koparde et al. (2019) demonstrated that these natural plants are therapeutic because of the existence of various bioactive properties. The ability of these traditional medicines in combating several diseases make it functional to use as a natural drug because of its high effectiveness and less toxic elements thus, making these biological and chemical components into potent drugs by several processes (Koparde et al., 2019).

Antinociceptive properties from natural sources could be a potential medicine in the therapy for pain and healing processes. Thus, there are various medicinal plants that have been well documented in relieving pain as well as to improve healing. An investigation of medicinal plants that comprises with phytochemical diversity which can portray the properties of analgesic or antinociceptive effects is indeed necessary and beneficial. In fact, the phytoconstituents of the natural plants with antinociceptive activity are worth to be studied because the current treatment for pain in terms of analgesic drugs can exhibit potential side effects. Therefore, the discovery of plant-based medicinal products that constitute with high therapeutic effects especially for pain management that could have no or low potential side effects could become beneficial as substitutes for conventional analgesics like opiates and NSAIDs (Sen et al., 2010).

Ginger *(Zingiber officinale)* which belongs to the family of Zingiberaceae known as a famous herb in the world that is most used in Asian countries (Gao & Zhang, 2010). It is one of the beneficial plants that have been used as herbs in

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traditional medicine for more than hundred years worldwide (Ajayi, 2013). The consumption of rhizome parts has been proved to relieve the general health issues such as nausea, pain and vomiting (Li et al., 2019). Hence, the benefits of ginger that used in folk medicine showed that it comprises a lot of medicinal properties like antimicrobial (Kumar et la., 2014), anti-inflammatory (Zhang et al., 2016), anticancer (Citronberg et al., 2013) as well as antioxidants (Nile & Park, 2015).

Therefore, further study on this plant should be conducted in order to investigate the chemical composition that contributes to the antinociceptive activity of ginger. Thus, this research has been carried out to determine the antinociceptive activity of the essential oil extracted from *Zingiber officinale* in mice model of nociception.

1.2 Objectives

1.2.1 General Objective:

To investigate the analgesic effects of the essential oils of ginger (GEO) of *Zingiber officinale* on chemically induced mice of nociception.

1.2.2 Specific Objective:

To identify the effects of GEO by measuring the time spent of each mouse on licking and biting on the injected hind paw of mice in both phase I and phase II of formalin test.

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1.3 Hypothesis

The formalin induced paw licking in mice can be inhibited by oral administration of the essential oils of *Zingiber officinale* for nociception.

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CHAPTER 2
LITERATURE REVIEW

2.1 Medicinal Plants

Natural plants have been always playing a huge role in traditional medicines and practices as it has been a source for the treatment of numerous human diseases for centuries (Saranraj et al., 2014). This is because Li et al. (2019) reported that most medicinal plants have been used as long-term clinical practice worldwide. There are various benefits of medicinal plants that have been proved to cure various illnesses due to certain conditions like mild side effects, less depletion of active compounds as well as highly curative effects. A huge number of studies reported that most plants consisted with numerous biologically active phytochemical constituents which some of the active compounds contribute to the remedy of the health problem by its traditional claim. One of these useful plants is called *Zingiber officinale.*

2.1.1 *Zingiber officinale*

Ginger or scientifically known as Zingiber officinale belongs to the family of Zingiberaceae as well as Zingiber genus. It is usually consumed as a spice and herbs for medicinal practices since the ancient times (Han et al., 2013). It is also known as a rhizomatous perennial of herbaceous plants which is largely grown in warm climate

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regions in certain parts of the world like Taiwan, Jamaica, Bangladesh, India, Nigeria as well as the United States of America (Ajayi 2013). The main portion that is consumed by people is the rhizome part from the horizontal stem (Benzie & Wachtel-Galor, 2011). It consists with many divided swells which are horizontally grown, and the colour of the outer membrane is yellow or brown while the internal part is yellowish brown. It consists with several vessels cells and elements that comprise oleoresin (Arablou et al., 2014). Besides, ginger is added as an indispensable curry powder or sauce to a wide selection of food. Because of its aroma and flavour, it is often used to flavor bread, tea, carbonated beverages, biscuits, pickles and other confectionaries (Ajayi, 2013).

According to Benzie & Wachtel-Galor (2011), ginger is used in a wide range of forms such as dried, fresh, preserved, candied, crystallized, pickled, powdered or ground. The flavor comprises slightly peppery and sweet and the aroma is somewhat strong and spicy. The essential oil that is extracted from ginger's rhizome (GEO) has a pale yellow to light-amber colour that consists with sweet-smelling and strong compound. It can be extracted in the range of 1.5-3.0% from the plant but depends on its crop's quality (Bellik, 2014). The concentration of essential oils rises as ginger ages thus, the usage of rhizome is determined by the time when it is harvested. If people intended to use the rhizome as its for oil extraction, the ginger shall be harvested for 9 months or longer. The harvesting of ginger about 8-9 months or more has a resilient skin that have to get rid before eating. The part of the root has a strong smell and often used dried or crushed it into ground ginger. The production of ginger into this form has been used in the cakes, cookies as well as curry dishes. In the production of sugar

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syrup, the candied or crystallized ginger is cooked and shielded with granulated sugar. The 5 months old of harvesting ginger is still immature and has very slim skin. However, the rhizomes are tender and have a mild flavour and are best applied in fresh or preserved form.

Figure 2.1: *Zingiber officinale* (Ryan & Morrow, 2010).

2.1.2 Bioactive Compounds of Ginger

As stated by Prasad & Tyagi (2015), there are numerous active compounds in ginger such as phenolic and terpene elements. The main phenolic constituents that can be identified are shogaols, gingerols and paradols. Gingerols are known as the major polyphenols in fresh ginger including 6-gingerol, 8-gingerol, and 10-gingerol. Besides, gingerols can modify into corresponding shogaols if kept and treated with a heat in a longer time. Then, shogaols can also change into paradols after the hydrogenation process (Stoner, 2013). The other phenolic compounds that can be discovered are

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zingerone, gingerenone-A, and 6-dehydrogingerdione (Ji et al., 2017). Besides, the terpene compounds such as α-curcumene, β-bisabolene, α-farnesene, zingiberene and β-sesquiphellandrene are known as main components in ginger essential oils. The other components that are also present in ginger include raw fibers, lipids, polysaccharides and organic acids (Prasad & Tyagi, 2015).

As mentioned by Srinivasan (2017), ginger's characteristic flavour is caused by zingerone, shogaols, gingerols and volatile (essential) oils, which comprise up to 3 percent of fresh weight ginger. The main constituents in the volatile fragrant essential oil of ginger is sesquiterpenoids that consists of α-zingiberene (30–70%) as the main element (Srinivasan, 2017). The strong smell like the pungent of the fresh ginger rhizome is because of the presence of 6-gingerol (1-[4′-hydroxy-3′-methoxyphenyl]-5-hydroxy-3-decanone) which is the main components for the pungent characteristics. It is an oily liquid that exhibits most in the gingerols. However, the presence of shogaols which is nonvolatile phenylpropanoid-derived compounds from gingerols contributes to the production of the pungency of dried or cooked ginger. The less pungency of ginger is derived from zingerone which is also exhibited from gingerols. This component is produced from the drying process that exhibits a smell like spicy and sweet. The other component that is present in the ginger is known as acrid resinous substances (5–8%).

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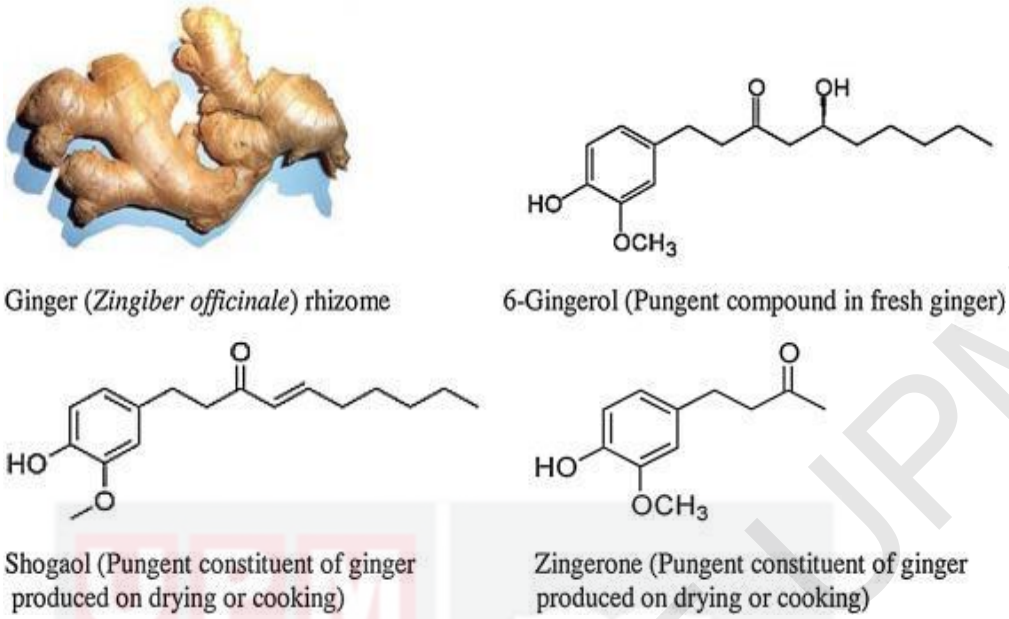

Figure 2.2: The bioactive components of ginger rhizome (Srinivasan, 2017).

2.1.3 Chemical Components of Ginger Essential Oils

As mentioned by Messomo et al. (2013), the Zingiber genus is commonly used worldwide in a wide range of form as it consists of various medicinal and biological elements. Zingiber officinale is the most popular genus that people studied as it can possess the health benefit effects (An et al., 2016). Previously, studies of this ginger essential oils reported that it can exhibit a lot of pharmacological properties including antioxidant, antimicrobial, insecticidal and cytotoxic (Barbarinde et al., 2016) as well as anti-inflammatory activities (De Melo et al., 2011). Besides, it has an element of food preservatives as well (Azizi et al., 2016).

The chemical constituents of ginger essential oils mainly consist of monoterpenes and sesquiterpenes hydrocarbons. Alpha-zingiberene is the richest

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compound in this essential oil which is responsible for its aroma and flavor. Besides, other compounds that can be identified are β-bisabolene, neral, geranial, β-sesquiphellandrene and ar-curcumene (Sharifi-Rad et al, 2017). Gingerol and shogaol are other pungent constituents found in lesser amounts. However, the compositions and quantities of bioactive compounds in ginger essential oils may be different according to several factors like the geographical location, extraction methods as well as the freshness and dryness of rhizomes (Mahboubi, 2019).

According to Mahboubi (2019), the chemical composition of Nigerian fresh ginger oil (1.02% w/v) consist of numerous bioactive compounds like 1,8-cineole, limonene, β-phellandrene (10.5%) β-zingiberene (12.2%), geraniol (15%), neral (8.9), β-bisabolene (5.6%) and β-sesquiphellandrene (6.5%). However, the main component that can be found in the extraction of ginger from dry rhizomes (1.84% w/v) is β-sesquiphellandrene (10.6%). The other active components are known as 1,8-cineole, limonene, β-phellandrene (4.5%), geraniol (9.0%), neral (5.3%), β-bisabolene (8.4%) and β-zingiberene (28.1%). Furthermore, the extraction methods used in order to obtain the other ginger essential oil which derived from Nigeria was a hydro-distillation process (2.4% w/w). As a result, the bioactive compounds that can be identified are zingiberene (29.5%), sesquiphellandrene (18.4%), farnesene (6.46%), germacrene D (3.6%), neral (2.5%), geranial (3.56%), neryl acetate (1.2%), and α-farnesene (1.9%). Thus, based on comparison between those fresh and dried rhizomes of ginger above, the dried one contained higher essential oil as well as β-zingiberene content than the fresh ginger rhizomes (Mahboubi, 2019).

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In Bangalore market, the main chemical compositions that can be found in ginger EOs from fresh rhizomes was zingiberene + zingiberol (38.9%) which contain the highest amount in that essential oil. The other compounds were ar-curcumene (17.7%), β-sesquiphellandrene+β-bisabolene (11%), β-phellandrene (4.9%) which were extracted from a simultaneous distillation method. Besides, the ginger extraction from dried rhizome by hydro-distillation method (1.2% w/w) in Iran market consists of Zingiberene (32%), β-sesquiphellandrene (15.6%), β-bisabolene (9.3%), and ar-curcumene (15.9%) as the main components. Meanwhile, the essential oil from fresh ginger rhizomes extracted by hydro-ditillation process had α-zingiberene (23.9%) and citral (21.7%) (Mahboubi, 2019).

2.1.4 Pharmacological Effects of Ginger Essential Oils

As stated by Mahboubi (2019), even though the chemical constituents of ginger essential oils are caused by several factors however, different literatures reported for different pharmacological and biological activities of ginger essential oils. Previous study reported that this essential oil consists of various pharmacological effects including antimicrobial, anticancer, anti-inflammatory and antioxidant.

2.1.4.1 Antimicrobial Properties

The essential oil of ginger from Brazil which consist of α-zingiberene, β-sesquiphellandrene, α-farnesene, ar-curcumene, β-bisabolene and gerania as the major components had the higher inhibition zone diameters for certain bacterias like *Staphylococcus aureus*, *Listeria monocytogenes* and *Pseudomonas aeruginosa.*

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However, there were several bacterias that had resistance to ginger essential oils such as *Escherichia coli, Shigella flexneri* and *Salmonella typhimurium* (Mesomo et al., 2013). Besides, Vietnamese ginger essential oil reported to have antifungal properties against *Aspergillus niger, Botrytis cinerea, Penicillium sp*. following *Candida albicans, Saccharomyces cerevisiae* and *Rhizopus nigricans* which consisted of β-sesquiphellandrene, ar-cucumene, α-zingiberene and β-bisabolene. However, there are some bacterias that had less sensitivity towards this essential oil such as *Bacillus pumilus, Staphylococcus epidermidis, Bacillus subtilis, Escherichia coli* as well as *Salmonella abony*. Other bacteria that had resistance towards the essential oil was known as *Pseudomonas aeruginosa* (Stoyanova et al., 2006).

Furthermore, the essential oil's ginger that contained citral (30.8%) and zingiberene (17.07 %), β-bisabolene, geranyl acetate (6.7%), β-sesquiphellandrene (5.9%), 1,8-cineol (6.1%) and geraniol (6.1%) also had antimicrobial properties towards *Streptococcus pyogenes, Bacillus subtilis, Staphylococcus aureus, Salmonella typhi, Aspergillus niger* and *Pseudomonas aeruginosa*. *Proteus mirabilis, Escherichia coli* and *Citrobacter koseri* had less activity to the essential oil from ginger (Meliani, Nair & Bensoltane, 2014). Based on the literature data above, the ginger essential oil exhibited antimicrobial effects better on gram positive bacteria compared to gram negative bacteria. The existence of antimicrobial properties in ginger essential oil could be due to the bioactive components that presence in the essential oil itself (Mahboubi, 2019).

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2.1.4.2 Anticancer Properties

Anticancer effects is one of the pharmacological effects that can be found in ginger essential oil. It can be proved by the presence of the IC_{50} values for 46.2-172 μg/ml which consisted α-zingiberene as the major constituents in the essential oil of ginger that had 60.6, 46.2, 172 and 80.3 μg/ml for HeLa, SiHa, MCF-7 including HL-60 cell lines. Besides, α-zingiberene capable of causing apoptosis, nucleosomal deoxyribonucleic acid (DNA) fragmentation, elevated the percentage of sub-diploid cells and triggered the caspases in SiHa cells as well. In fact, the IC_{50} values of the essential oil of ginger were in the ranges of 38.6-82 μg/ml towards the cell lines which contained bioactive components like α-zingiberene by 35.0%, ar-curcumene by 15.3% and β-sesquiphellandrene (12.3%) (Lee, 2016).

The cytotoxic effects of ginger essential oils had been proved from the result of MTT assays of neutral red (NR) and tetrazolium which consisted of neral, geranial, β-phellandrene, 1,8-cineole and camphene compounds against HepG2 and HeLa cells. For these cells, the MTT-IC_{50} (μl / ml) values for ginger essential oil were 635.1 and 141.4 respectively. The anti-proliferative activities of ginger essential oil were shown towards the HeLa cervical cancer cells by having condensation of chromatin, blebbing and protrusions of cell membrane. The ginger essential oil can have a similar function as camptothecin if it increased the concentration up to 1928 μl/ml thus, leading to amorphous cells, blebbing and condensation of chromatin which eventually resulted in apoptosis (Santos et al., 2016).

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2.1.4.3 Anti-Inflammatory Properties

Inflammation has an important role in the body. The ginger essential oil exhibited the anti-inflammatory activities in female Lewis arthritis by assessing the streptococcal cell wall-induced rheumatoid arthritis. The animal models had been injected intraperitoneally daily with 28 mg/kg ginger essential oil. As a result, the granuloma formation in the streptococcal cell wall was inhibited after being treated with this ginger essential oil. It produced no effects in the initial acute phase of chronic joint inflammation as well in the animal models. The essential oil of ginger acts as a phytoestrogen in a way that produces no effects on the oestrogen target organ (Funk et al., 2016).

Besides, other studies reported that the ginger essential oil (100, 500 and 1000 mg/kg) can produce anti-edema effects in carrageenan induced paw edema of mice in a dose dependent manner. The results were shown as 27.8, 44.4 and 61.1% vs. 55.6% for 10 mg/kg diclofenac. Furthermore, the ginger essential oil of 100, 500 and 1000 mg/kg can exhibit the inhibitory activities on formalin induced inflammation as well. It reduced the inflammation by 54.17, 62.5 and 70.8%, respectively vs. 54.8% for 10 mg/kg diclofenac. During this phase, the anti-inflammatory mediators were released like histamine, bradykinins and prostaglandins. The anti-inflammatory effects of ginger essential oil resulted in the inhibition of prostaglandin released in order to suppress the inflammation in paw edema of mice (Jina, Liju & Kuttan, 2013).

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2.1.4.4 Antioxidant Properties

Scientists nowadays developed interest to investigate any natural plants that can exhibit the antioxidant activities as production of free radicals in the body which have a relation to various human diseases. Thus, the potential of antioxidant activities in ginger essential oil was evaluated in the previous study. The evaluation of antioxidant effects on Chinese ginger essential oil exhibited EC_{50} (mg/ml) of 63.23, 11.68 and 0.118 in reducing power, DPPH scavenging as well as H_2O_2 scavenging assays. Meanwhile, the EC_{50} for ascorbic acid showed as 0.025, 0.005, 0.478, respectively and for quercetin were 0.017, 0.002, 0.078 (Bellik et al., 2013). Besides, the ginger essential oil produced antioxidant activities through intraperitoneal injection by scavenging the hydroxide radicals, superoxide as well as suppressing tissue lipid peroxidation. The ginger essential oil (250mg/kg) also inhibited (18.25%) phorbol 12-myristate 13-acetate (PMA) evoked superoxide radicals in macrophages.

The ginger essential oil that had been administered orally (100 or 200 mg/kg) in mice for 30 days also elevated the enzymes of antioxidant like superoxide dismutase, catalase, glutathione, glutathione reductase in blood when compared to paraffin oil group. In liver, the essential oil of ginger also produced antioxidant effects by increasing the amount of superoxide dismutase, glutathione peroxidase and glutathione-s-transferase enzymes (Jina, Liju & Kuttan, 2013). Based on the findings from the previous study above, the ginger essential oil reported to exhibit a role in protecting the cells from any free radicals that can cause damage to healthy cells by increasing the antioxidant enzymes as well as the serum level (Mahboubi, 2019).

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2.2 Pain and Nociception

According to Cascella et al. (2016), the capability of an individual to feel pain is a vital component in the capacity of the body to heal. Thus, pain is the way the body tells us there is an injury, and we have to do something about it to make sure the process of healing can occur. As mentioned by Yam et al. (2018), pain is recognized to be a human primate instinct and can be characterized as a distressing sensation and an emotional experience which is linked to real or possible tissue damage. It is also capable of giving impact to an individual by reducing the life's quality (Orr, Shank & Black, 2017). The motive of the pain is to notify the defence mechanism in the body as to respond to a stimulus in order to prevent further harm to the affected tissues (Yam et al., 2018).

Pain can be classified into acute and chronic types (Dydyk & grande, 2020). As studied by Armstrong & Herr (2019), the pain sensation can be classified into 4 large types which are acute pain, nociceptive pain, chronic pain as well as neuropathic pain. However, this studied grouping the acute and nociceptive pain together. Nociceptive pain originates from the destruction of physical tissue by physical or chemical elements by the activation of nociceptive nerves (Thai & Fainsinger (2011). Acute noxious stimuli are responsible to signal nociceptors like cold, heat, chemical stimulation as well as mechanical force. Acute pain capable of developing into inflammatory pain if the noxious stimulus experienced longer time in order to let on the nociceptive neurons to exhibit pro-inflammatory markers to trigger the responsive cells (Woller et al., 2017).

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Hence, nociception is recognized as the process that involved the central nervous system (CNS) as well as peripheral nervous system (PNS) that triggered the nociceptors and their pathway that derived from the noxious stimuli like tissue injury and intense heat (Kendroud et al., 2020). Both CNS and PNS are important in carrying out their role for pain perception. PNS that located outside of the spinal cord and brain consisted with nerves and ganglia. It functions to connect the CNS to the organ and limbs in the body which CNS comprises with spinal cord and brain. The function of CNS is to integrate and interpret the information that derived from PNS thus, coordinate all the activities in the body before transmitting signals to the effector organs (Yam et al., 2018).

2.2.1 Nociceptors of Pain

As mentioned by Pereira & Goudet (2018), the receptors in charge of sending the nociceptive signals are called nociceptors and they can be found on skin, joint, muscles as well as viscera. These receptors are activated by a various range of chemical components, including globulin and protein kinases, histamine, arachidonic acid, substance P, potassium, calcitonin gene-related peptide (CGRP), acetylcholine, nerve growth factor, serotonin, acetylcholine, lactic acid, low-pH solutions and adenosine triphosphate (ATP). Besides, the other factors that can also activate the receptors including extreme pressures and temperature as well as tissue injury that can lead to inflammation.

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Nociceptors can be subdivided into a few types according to the information that they received. For example, there are several types of skin nociceptors like thermal receptors (thermal and mechanical stimulation), high threshold mechanoreceptors (intense mechanical stimulation), polymodal receptors (high-intensity mechanical, thermal, and chemical stimulation) and chemical receptors. Joint nociceptors are categorized as high threshold mechanoreceptors, polymodal and silent receptors. Silent receptors are not responsive to initial heat or pressure stimuli but only react after tissue damage and causes inflammatory molecules to be released. Moreover, visceral receptors are categorized into mechanoreceptors, chemical, thermal as well as silent receptors (Kendroud et al., 2020).

Zieglgänsberger (2019) analysed that the perception of pain starts with free nerve endings derived from the primary neuron branches that are surrounded by Schwann cells in which the nerve tips are unsheathed. The different classification of receptors transmit signals that connect with neuronal cell bodies that originate from dorsal root ganglion (stimulus from the body) as well as trigeminal ganglia (stimulus derived from head and face). Furthermore, A delta and C fibers are two major types of nociceptive nerve fibers. A delta fibers have small receptive fields and mildly myelinated that allow them to be alerted when the body experiences pain. Those fibers are responsible for the initial perception of pain due to the higher degree of myelination compared to C fibers. In fact, C fibers are unmyelinated which allow them to transfer the intensity of pain because it has a large receptive field.

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Yam et al. (2018) demonstrated that pain sensation is associated with receptor activation in the primary afferent fibers, which includes the unmyelinated C-fibers and myelinated Aσ-fibers. During the absence of pain, both these nociceptors remain silent in homeostasis and only activate when there is a potential of noxious stimulus. In order for the brain to sense the pain and to exhibit a response to the harmful stimulus as well, the series of events on the sensory perception is required. Generally, there are three main phases in the pain perception. The first phase involves the sensitivity of pain whereas the second phase involves the transmission of signals from the periphery to the dorsal horn. This process takes place in the spinal cord through the peripheral nervous system (PNS). The third phase involves when the signals transferred to the brain through the central nervous system. There are two pathways in order to transmit the signals which are the ascending and descending pathway. The ascending pathway is defined when the sensory information from the body goes upward to the brain via the spinal cord whereas the descending pathway is when the signal goes downward from the brain to the reflex organs through the spinal cord as well (Yam et al., 2018).

2.2.2 Basic Mechanism of Pain

Generally, there are three types of events in the basic pain mechanism including transduction, transmission and modulation when the noxious stimuli is present. For example, transduction process begin in the three following order which firstly, the stimulus events are converted to chemical tissue events. Secondly, chemical tissue and synaptic cleft events are then, converted into electrical events in the neurons whereas thirdly, at the synapses, the electrical events are translated into chemical

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events. The transmission process started after the completion of the previous transduction process. This process involves the transmission of electrical events along the neuronal pathways including the involvement of the transmission of neurotransmitters that are located in the synaptic cleft from a post-synaptic terminal of one cell to pre-synaptic terminal of another cell. The modulation process occurs through the primary afferent neuron, dorsal horn and higher brain centre via up-or down-regulation at all levels of nociceptive pathways (Yam et al., 2018).

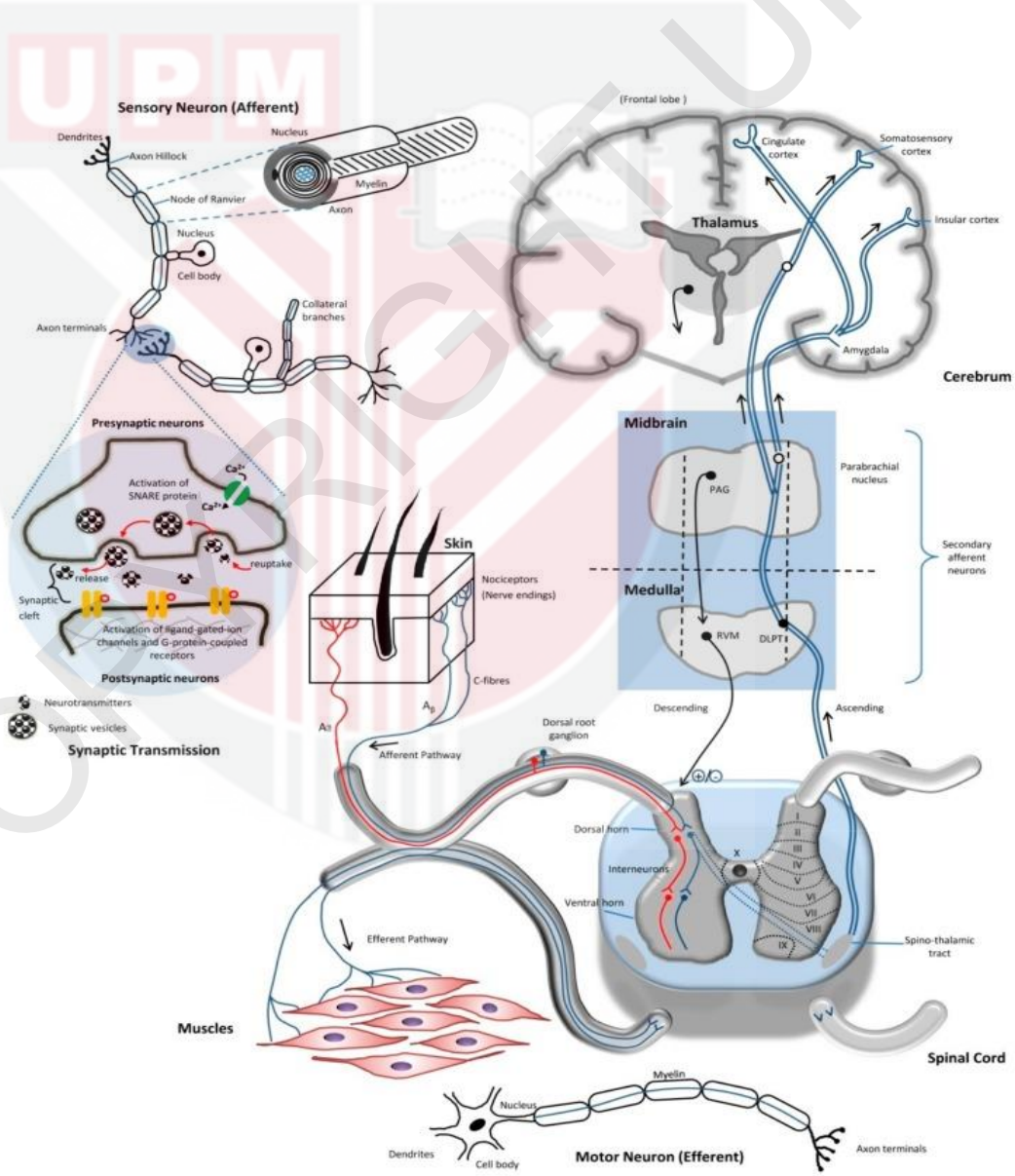

Figure 2.3: The basic route of transmission of pain (Yam et al., 2018).

2.2.2.1 The Ascending System of Pain

According to Garland (2012), generally, as the body receives information that is derived from the noxious stimuli regarding the tissue injury, the stimuli are transduced through neural pathways as well as transmitted through the peripheral nervous system then to the central and autonomic nervous system. This process is recognized as nociception. Nociception is the process by which the body sends signals about the actual tissue damage to the brain. When an intense mechanical stimulation such as cutting, stretching as well as pinching or exposed to any noxious chemical, these events activate the nociceptors. The nociceptors like thin myelinated Aδ and unmyelinated C fibers are located at the end of the dorsal horn in the spinal cord.

These primary afferent nociceptors are responsible to transmit the noxious stimuli to the neurons in the dorsal horn of the spinal cord within the ascending pathway. Thus, these neurons transfer these sensory information up to the thalamus through the spinothalamic tract in order to reach the somatosensory cortex. This process leads to the informing about the intensity and location of the noxious stimuli. The spinothalamic tracts consist of two different parts which each part carries a different function. There are known as lateral and anterior spinothalamic tracts which are located in the white matter of the spinal cord. The lateral spinothalamic tract is responsible for pain and temperature sensation while the anterior spinothalamic tract focuses on the crude touch and firm pressure sensation. These signals then transmit to the thalamus in the brain (Yam et al., 2018).

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In thalamus, the sensory nerves transmit to the cerebral cortex. The nociceptive pathway ends in discrete subdivisions of thalamic nuclei which are the ventral posterolateral nucleus and the ventromedial nucleus. Nociceptive information is relayed from these nuclei to multiple cortical and subcortical regions, including the amygdala, hypothalamus, periaqueductal grey, basal ganglia and cerebral cortex area. When the nociceptors are stimulated by the noxious stimuli, the insula and anterior cingulate cortex are activated thus, resulting in the pain experience. These integrated thalamocortical and corticolimbic structures, collectively called the "neuromatrix" of pain, access the somatosensory input and output neural impulses that influence the nociception and perception of pain (Garland, 2012).

2.2.2.2 The Descending System of Pain

The pain information from the body is not directly transmitted to the brain instead the sensory transmission is regulated by stimulating the spinal dorsal horn through the descending pathway from the medulla. In Gate Control theory of pain, the substantia gelatinosa of the dorsal horn controls the perception of noxious stimuli by integrating the upward afferent sensory neurons that derived from peripheral nervous system with downward modulation from the brain. Interneurons in the dorsal horn act to inhibit and enhance the pain signals to the higher brain centers which lead to the central nervous system where it controls the transmission of impulse into consciousness (Garland, 2012).

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The modulation of pain system influenced the nociceptive information from the spinal cord. There are various brain structures that involve with this descending pain modulation including prefrontal cortex, anterior cingulate cortex, insula, amygdala, hypothalamus, periaqueductal grey, rostral ventromedial medulla, and dorsolateral pons/tegmentum. The nociceptive signals are regulated by the activity of these brain structures through the descending projections to the dorsal horn of the spinal cord thus, the central nervous system can selectively control the transmission of signals from particular parts of the body (Garland, 2012).

2.2.2.3 Neurochemistry of Pain

As mentioned by Garland (2012), there are numerous molecular messengers that are involved in signal transduction via peripheral and central nervous system during nociception. The nociceptors which are activated by the mechanical, thermal, or chemical stimulus directly transmit the input through the excitatory neurotransmitter glutamate. Besides, the inflammatory mediators are also released at the site of the tissue injury in order to activate the nociceptors. This inflammatory mediator or also known as inflammatory soup consists of bradykinin, serotonin, prostaglandin as well as NGF. The existence of these molecules initiated the neurogenic inflammation by exciting the nociceptors or decreased the activation threshold thus, leading to the transmission of afferent nerves to the spinal dorsal horn.

Garland (2012) demonstrated that neurogenic inflammation occurs when the nociceptors activate, thus, releasing the neurotransmitter like substance P that derived

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from the peripheral terminal into the extracellular space of the terminal end of the nociceptor. The secreting of substance P induces vasodilation, leak fluids as well as proteins and stimulate the immune cells by releasing the inflammatory soup that is caused by the tissue injury. Therefore, these events lead to the increased activation of Aδ and C fibers as well as the sensitization of the peripheral nerve endings.

The pain signals from the spinothalamic tract results in an increased secretion of norepinephrine from the locus coeruleus neurons towards the thalamus which transmit the nociceptive input to the area of somatosensory cortex, hypothalamus, and hippocampus. This norepinephrine then, relayed the informative signals in the cortical and subcortical brain areas for processing. In addition, the pain can inhibit when opioid receptors in the peripheral and central nervous systems are stimulated by opiates or endogenous opioids such as endorphin, enkephalin, or dynorphin. The release of endogenous opioids majorly occurred in the descending pain modulatory pathway. A host of other neurochemicals also participated in perception of pain such as the neurochemistry of nociception however, the central and peripheral modulation of pain is highly complex (Garland, 2012).

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CHAPTER 3

MATERIALS AND METHODS

3.1 Plant collection and preservation

Ginger rhizomes were the plant materials used in this experiment. They were obtained from the same environmental and growth conditions in order to avoid any differences between samples thus, we bought from the local producers in Bentong, Pahang (GPS coordinates: 3.8126° N, 103.3256° E) in February 2019. Then, the ginger rhizomes were immediately transported back to the lab by packing them into ice to prepare for the extraction process of oil essentials.

3.2 Preparation and extraction of essential oils

The rhizomes were performed for the extraction of essential oils. Firstly, in order to remove the dirt of the plant, they were washed under the running tap of water as well as with distilled water. They were cut into small pieces, then grounded by using a blender.

The apparatus used for the extraction was known as Clevenger-type apparatus which was a hydro-distillation process. It consisted with an electric boiler, condenser as well as decanter in order to split the oil from the condensation of water. The plants

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were pre-treated with distilled water in the ratio of 3:5. This process took almost 5 hours. Then, the process of performing essential oils (EOs) began by boiling the ground ginger rhizomes that were placed in the 10 litres flask. It allowed the aromatic compound that originated from EOs to be free which was from the hot steam. The boiling process took about 20 minutes for 100°C however, it was reduced to 45°C for 5hours. The EOs from the hot steam were condensed through a cooling system which the liquid presented that comprises of EOs and water that were collected and later to be separated. The EOs stored at -20°C after the extraction process as previously described (Fitriady et al., 2017).

3.3 Animals care and handling

The type of mice that were used in this study were male ICR mice (20-30g). All mice were kept at the animal house facility, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia with a 12 h light/12 h dark cycle as well as provided with standard pellets and water. The experimental protocols were approved by Animal Care Unit Committee, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. Prior to experiment, the mice were divided into groups of 10 mice each and fasted for 12 hours with free access to water. In the experiment each animal was used only once.

3.4 Preparation of drug and mode of administration

The following chemicals and reagents that were used are essential oils, 1% DMSO, 2.5% formalin, distilled water and saline. The preparation of 1% DMSO was by dissolving in saline while 2.5% formalin in distilled water. There were three

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different concentrations of essential oils (10mg/kg, 30mg/kg and 100mg/kg) that were dissolved in 1% DMSO. The mice were pre-treated for 1 hour with essential oils by oral administration prior to nociceptive testing while formalin was injected subcutaneously into the plantar surface of one hind paw of the mice after treatment with essential oils.

3.5 Formalin-induced spontaneous nociceptive behaviour

The animals were divided into 6 groups with each group consisting of 6 mice. The experiment was conducted for months with 6 mice per group for a day (30 animals total). All mice were gently restrained in order to inject the 2.5% formalin subcutaneously into the plantar surface of one of hind paw by using a 30-gauge needle together with a micro syringe. Prior to treatment, one mouse was used as a control that only received formalin injection. The mice were pre-treated orally with GEO (10, 30 and 100 m/kg) 1 hour before the formalin injection. Then, the paw of the mice was injected with a solution of formalin and immediately placed in the transparent observation chamber that consisted with mirror at 45◦ angle in order to have a clear observation of the pain reaction for 30 minutes. The spontaneous nociceptive behaviour of mice was indicated as licking and lifting of the injected paw in the first 5 minutes then, for 15 to 30 minutes. The first 5 min was defined as early phase while 15-30 min as late phase or neurogenic phase. The pain responses were measured for both phases (Ong et al., 2011).

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CHAPTER 4

RESULTS

4.1. Formalin-induced Paw Licking Test

The three different concentrations of GEO at doses of 10, 30 and 100 mg/kg were significantly decreased the time spent of each mouse on licking and biting the injected of right hind paw in both neurogenic (early, 0-5 mins) as well as inflammatory phases (late, 15-30 mins). Based on the figure 4.1, the late or inflammatory phase produced better inhibition than the early or neurogenic phase. In neurogenic phase, three concentrations of GEO were identified to have the inhibition of 27.82%, 29.75% and 35.21% whereas in inflammatory phase were 37.92%, 43.42% and 60.91%. However, acetylsalicylic acid (ASA) only showed better reduction of licking time during the inflammatory phase by 62.47% compared to the neurogenic phase, 0.81%. Meanwhile, morphine significantly suppressed the formalin-evoked nociceptive behaviour of mice in both phases by 76.20% and 99.99%

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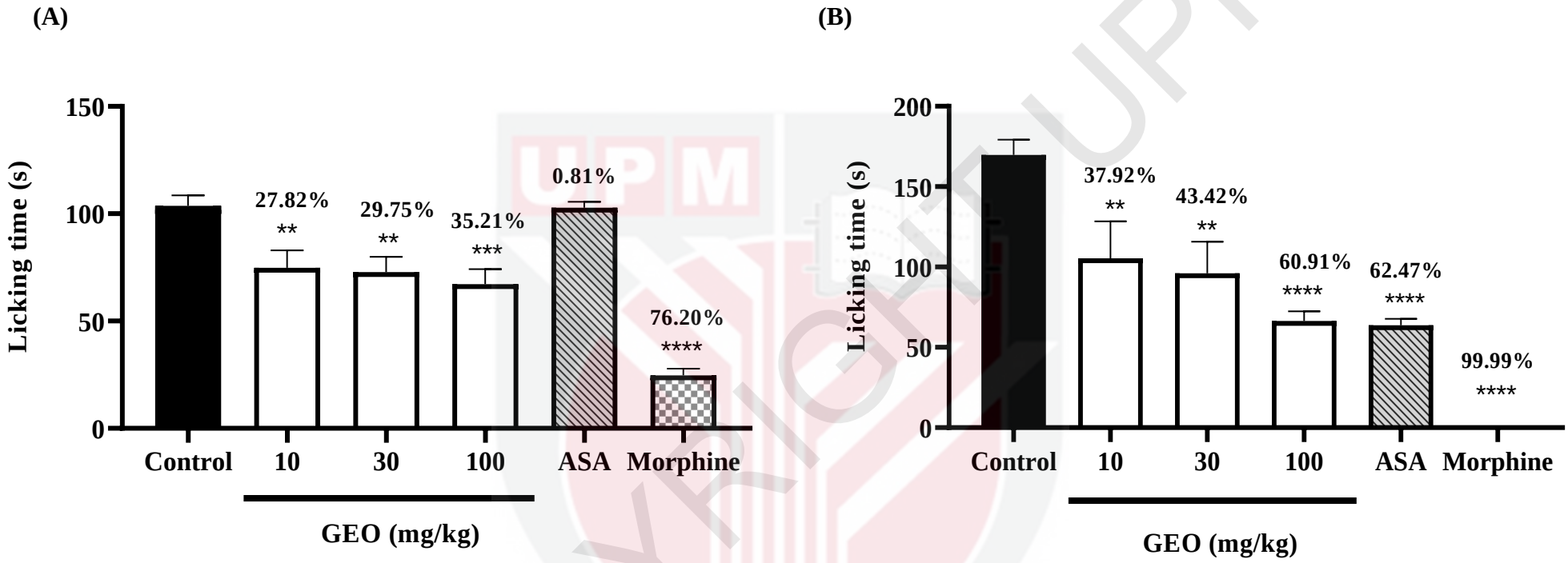

Figure 4.1: Effects of GEO against 2.5% formalin-induced paw licking behaviour in mice.

(A) Early/neurogenic phase (B) Late/inflammatory phase. The mice were pre-treated with groups of control, acetylsalicylic acid (ASA, 100mg/kg, i.p.), morphine (5mg/kg, i.p.) and GEO (10, 30, 100mg/kg, p.o.). Data were expressed as mean ± SEM (n=6). Results were analysed using one-way ANOVA and Dunnett's post hoc test. The asterisks (*) means significance level as compared to the vehicle group, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

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CHAPTER 5

DISCUSSION

The purpose of this study is to investigate the antinociceptive properties of *Zingiber officinale* by using animal models induced nociception. In this present study, the animal models chemically induced nociception were used to determine the potential antinociceptive effects of essential oil of rhizome's *Zingiber Officinale* (GEO). Based on the findings, the oral administration of GEO can produce the antinociceptive effects by inhibiting the mechanism of pain that mediated both centrally and peripherally. In fact, the orally GEO proved to exhibit an effective response because it was well absorbed through the gastrointestinal tract. This is because drugs that have an excellent absorption properties are one of the best selection criteria for new drug discovery.

In this present study, the administration of formalin into the hind paw was used to evoke nociceptive behaviour in mice. It exhibited biphasic behaviours of licking or biting the injected hind paw of mice with phase I (early/neurogenic phase) and phase II (late/inflammatory phase). According to Ismail et al. (2016), the first phase reflects the centrally mediated pain that involves direct stimulation of the primary afferent fibres via C-fibers. As a result, the chemicals such as substance P, serotonin, kinins and calcitonin gene-related peptide (CGRP) are released. The activation of transient

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receptor potential vanilloid 1 (TRPV1) is caused by pain in injected hind paw during 0-5 min (neurogenic phase) (Wani et al., 2012). Transient receptor potential (TRP) channel family is known as the biggest group of nociceptive ion channels including TRPV1 and transient receptor potential ankyrin 1 (TRPA1) members (Jardin et al., 2017). Kamarudin et al. (2018) demonstrated that the nonsteroidal anti-inflammatory drugs (NSAIDs) which specifically blocked the action of cyclooxygenase (COX) during this phase was ineffective in reducing the pain behaviour in mice compared to other drug such as morphine which is known as centrally acting analgesic drug. Based on the results above, ASA which is one of the NSAIDs did not suppress the nociceptive behaviour by having the highest licking or biting time during the early phase. In fact, three different doses of GEO exhibited better reduction of licking or biting time than ASA. However, the effectiveness of morphine in blocking the nociceptive behaviour was incomparable during this phase.

As mentioned by Ismail et al. (2016), the second phase (late phase) reflects peripherally mediated pain which is due to the inflammation response in peripheral tissue. There are several inflammatory mediators involved during this phase such as serotonin, histamine, prostaglandin, bradykinin as well as excitatory amino acids which are released when the tissue is damaged by formalin. Based on the findings above, three doses of GEO (10, 30 and 100mg/kg) had significantly decreased the licking or biting time which was similar with positive control (ASA). The reduction of licking time indicated that the release of inflammatory mediators during this phase may decrease or suppress as it involves in modulating the transduction pathways for

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pain control at peripheral level. Therefore, this action decreases the sensation of pain in mice (Kamarudin et al., 2018).

In addition, all doses of GEO showed a significant reduction of licking time in mice for both phases of the formalin test. Similar cases with another centrally acting drug like opiates which can suppress the nociceptive behaviour in both phases while for peripherally acting drugs it can only function in late phase (Kamarudin et al., 2018). For example, in this study, morphine is known as a centrally acting drug as it can reduce the nociceptive behaviour in both phases while ASA which is known as peripherally acting drug as it can only have a reduction of licking time during the late phase. It means the oral administration of GEO is effective in decreasing the pain sensation not only at peripheral level but central level as well. As GEO is effective in both neurogenic and inflammatory phases in this study, it indicated that the essential oil of *Zingiber officinale* consists not only of antinociceptive but also anti-inflammatory properties.

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CHAPTER 6

CONCLUSION AND FUTURE RECOMMENDATION

6.1 Conclusion

The present study demonstrated that the antinociceptive activity of the essential oils of ginger *(Zingiber officinale)* did produce a significant reduction in nociceptive behaviour of mice. It can be proved by the oral administration of three doses of GEO decreased the time spent of mice on licking or biting the injected hind paw with formalin in both early (neurogenic phase) and late phases (inflammatory phase). This is because ginger has numerous bioactive compounds like shogaols, gingerols and paradols that may contribute to the exhibition of excellent antinociceptive properties in nociception. Besides, GEO did participate in reducing the pain at both central and peripheral level. Therefore, the essential oils of ginger *(Zingiber officinale)* did exhibit the central and peripheral antinociceptive activity of mice induced nociception.

6.2 Future Recommendation

This present study showed that GEO can exhibit the antinociceptive activities in both central and peripheral pathways. Thus, the other specific tests are recommended to validate the previous results of formalin test by conducting the

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abdominal writhing and hot plate test in order to confirm the presence of peripheral and central antinociceptive effects.

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