IN VITRO AND *IN VIVO* STUDIES ON THE POTENTIAL EFFECTS OF *MELALEUCA CAJUPUTI* AND *ARDISIA ELLIPTICA* ON LYMPHATIC FILARIASIS: A STRATEGY TARGETING THE *WOLBACHIA*

NAZEH MOHAMMED AL-ABD ALI

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ABSTRACT

Lymphatic filariasis (LF) is caused by parasitic filarial nematodes. Currently available therapeutics are largely effective against the microfilaria stage, and they have a limited influence on the adult worms that are actually causing the disease. The present study aims to investigate the knowledge, attitudes, and practice with regards to LF among the population of an endemic area in Terengganu, Malaysia, and to explore the antifilarial activity of local medicinal plant extracts. *In vitro* antioxidant, antibacterial, and antifilarial activity tests were performed to assess the potential activities of these extracts. In order to develop and study the infection *in vitro*, Mongolian gerbils were infected intraperitoneally with *B. pahangi* larvae (L3) and they were used to host adult worms. The potential anti-filarial activities of the plant extracts of *Melaleuca cajuputi* and *Ardisia elliptica* were evaluated *in vitro* using methyl-thiazolyl-tetrazolium (MTT) assays, electron microscopy, and polymerase chain reaction (PCR). The extracts were fractionated using high-performance liquid chromatography (HPLC). The effect of each fraction was tested against *Wolbachia in vitro* using *Aedes albopictus* cells line (Aa23) cells. The active fractions were further subjected to liquid chromatography-mass spectrometry (LC-MS) to identify the active ingredients. The toxicities of the active extracts and isolated compounds were investigated. The identified compounds were then tested *in vitro* and *in vivo*, against adult worms and *Wolbachia*. RT-PCR was used to measure the expression levels of the *Wolbachia* surface protein (*wsp*) and Filamenting temperature-sensitive mutant Z (*Fstz*) and the *B. pahangi* genes. Baseline results have demonstrated that more than 80% of the respondents have become cognizant of LF, and additionally 70% of these individuals have grasped the fact that the LF is not devoid of problems. Estimatedly, 77% indicated that filariasis was transmitted by mosquitoes. Only 35% of the participants in this research were well aware of the local Mass Drug

Administration (MDA) program held in the area. All plant extracts showed good antioxidant activity, free radicals scavenging and antibacterial activity. A methanolic extract of *M. cajuputi* flowers significantly reduced adult worms' viability and release of microfilariae. Caffeic acid phenyl ether (CAPE) was identified as the active constituent in *M. cajuputi*. It showed significant micro and macrofilaricidal activities *in vitro* and *in vivo*. In summary, it has been discovered in this study that most respondents did acknowledge LF, in spite of the fact that their knowledge of MDA was rather limited. There is a need to enhance the delivery of health education and information programs, as well as mass mobilization campaigns, in order to improve understanding of the LF in the affected population for the control program to be successful. In the present study, the methanol extract of *M. cajuputi* flowers, and CAPE possesed anti-filaricidal activites. The mechanism of action of the CAPE is suggested to be due to its anti-*Wolbachia* activity; this compound has been shown to significantly downregulated the expression of the *wsp*, *fstz*, and *Shp-1* genes. Therefore, CAPE could be a promising candidate drug for the treatment of LF.

ABSTRAK

Filariasis Limfatik (FL) berpunca daripada nematod filarial parasit yang berbentuk seakan bebenang dan merupakan salah satu penyakit bawaan nyamuk yang paling diabaikan. Terapeutik yang ada pada masa kini sebahagian besarnya berkesan pada peringkat mikrofilaria, dan ia mempunyai pengaruh yang terhad pada cacing dewasa yang menyebabkan penyakit tersebut. Kajian ini bertujuan untuk menyiasat pengetahuan, sikap dan amalan berhubung dengan FL di kalangan penduduk kawasan endemik di Terengganu, Malaysia, serta meneroka aktiviti antifilaria bagi ekstrak tumbuhan herba tempatan. Ujikaji-ujikaji secara *in-vitro* bagi antioksidan, anti-bakteria, dan aktiviti antifilaria telah dijalankan untuk menilai keupayaan aktiviti ekstrak-ekstrak ini. Dalam usaha untuk membangunkan dan mengkaji jangkitan secara *in vitro*, gerbil Mongolia telah dijangkitkan secara intraperitoneum dengan larva *B. pahangi* (L3) dan telah digunakan untuk menempatkan cacing dewasa. Keupayaan aktiviti anti-filarial ekstrak tumbuhan *Melaleuca cajuputi* dan *Ardisia elliptica* telah dikaji secara *in vitro* menggunakan cerakin methyl-thiazolyl-tetrazolium (MTT), mikroskopi elektron, dan teknik-teknik PCR. Ekstrak-ekstrak telah dibahagikan kepada lima bahagian menggunakan kromatografi cecair berprestasi tinggi (HPLC). Kesan bagi setiap bahagian telah diuji terhadap *in vitro Wolbachia* menggunakan sel-sel Aa23. Kromatografi cecair dilakukan ke atas bahagian aktif dan spektrometer jisim untuk mengenal pasti bahan-bahan aktif. Tahap ketoksikan ekstrak-ekstrak aktif dan sebatian terpencil telah disiasat mengikut pendedahan tunggal (akut) dan dos harian berulang-ulang (sub-akut). Sebatian yang telah dikenal pasti kemudiannya diuji secara *in vitro* dan *in vivo*, terhadap cacing dewasa dan *Wolbachia*. Reaksi rantai polimerase transkripsi berbalik (RT-PCR) telah digunakan untuk mengukur kesan potensi rawatan ke atas *Wolbachia* dan ke atas peringkat ekspresi gen-gen *Wolbachia wsp* dan *fstz* serta gen-gen *B. pahangi*. Keputusan

garis pangkal menunjukkan bahawa lebih daripada 80% responden menyedari akan FL, dan 70% daripada individu tersebut memahami bahawa FL adalah bermasalah. Kira-kira 77% menyatakan bahawa filariasis disebarkan oleh nyamuk. Hanya 35% daripada peserta dalam kajian ini menyedari program MDA telah dilaksanakan kawasan tersebut. Semua ekstrak-ekstrak tumbuhan menunjukkan antioksidan dan anti-bakteria aktiviti. Satu ekstrak bermetanol bagi bunga *M. cajuputi* telah mengurangkan kebolehhidupan cacing dewasa dan pembebasan mikrofilaria dengan ketara. Fenil eter asid kafeik (CAPE) telah dikenal pasti sebagai bahan aktif dalam ekstrak ini. Kompaun ini menunjukkan aktiviti mikrofilarsidal dan makrofilarisidal *in vitro* dan *in vivo* yang berkesan/efektif. Mekanisme tindakan CAPE adalah disebabkan oleh aktivitinya yang bersifat anti-*Wolbachia*; sebatian ini telah diperhatikan dapat menurunkan ekspresi gen-gen *wsp*, *fstz*, dan *Shp-1* dengan ketara. Kesimpulannya, kajian ini telah menunjukkan bahawa majoriti responden menyedari akan FL, termasuk mod penghantaran dan gejalanya; namun mereka mempunyai pengetahuan yang kurang tentang MDA, yang berlaku di kawasan kajian. Terdapat keperluan untuk meningkatkan cara penyampaian pendidikan kesihatan dan program penerangan, serta kempen mobilisasi secara besar-besaran, dalam usaha untuk meningkatkan pemahaman FL dalam populasi yang terkesan supaya program kawalan berjaya. Hasil kajian tentang ekstrak tumbuhan menunjukkan bahawa ekstrak bermetanol bunga *M. cajuputi*, dan juzuk aktifnya (CAPE), tidak menyebabkan sebarang tanda keracunan dan menunjukkan kesan filarisidal yang berkesan. Kesan ini mungkin disebabkan oleh aktiviti anti-*Wolbachia* yang terdapat pada sebatian ini. Oleh itu, CAPE boleh menjadi ubat yang berpotensi bagi rawatan FL dan layak dipertimbangkan untuk menjalani pembangunan kajian praklinikal dan farmakologikal lanjutan.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
/	Divided by
^{0}C	Degree Celsius
±	Plus minus
<	Less than
µ	Micro
Aa23	*Aedes albopictus* cells line
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
ATCC	American type culture collection
Bp	*Brugia pahangi*
bp	Base pair
cDNA	Complementary desoxyribonucleic acid
CAPE	Caffeic acid phenethyl ester
DEC	Diethylcarbamazine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Desoxy nucleotide triphosphate
DPPH	2,2-diphenyl-1-picrylhydrazyl
FAD	Favin adenin dinucleotide
FBCS	Fetal bovine serum
Fstz	Filamenting temperature-sensitive mutant Z

FRAP	Ferric reducing antioxidant power
GCMS	Gas Chromatography Mass Spectrometry
GPELF	Global Programme to Eliminate Lymphatic Filariasis
H&E	Haematoxylin-eosin stain
HPLC	High performance liquid chromatography
HSP	Heat shock protein
i.p.	Intraperitoneally
IVM	Ivermectin
LCMS	Liquid chromatography Mass Spectrometry
LF	lymphatic filariasis
M	Molar
Mb	Mega base
MDA	Mass drug administration
Mf	Microfilariae (the first stage larvae L1)
Mg	Milligram
min	Minutes
ml	Millilitre
mRNA	Messenger ribonucleic acid
NADH	Nicotinamide Adenine Dinucleotide dehydrogenase
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
p.i.	Post infection
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
OD	Optical density
VEGF	Vascular endothelial growth factor

WHO	World Health Organization
WSP	*Wolbachia* surface protein
wBp	*Wolbachia* of *Brugia pahangi*
w/v	Weight per volume

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Research background

Lymphatic filariasis (LF) is a disease caused by filarial parasites, namely *Wuchereria bancrofti, Brugia malayi*, and *B. timori* of the *Onchocercidae* family. It is transmitted to humans by mosquito vectors. Several mosquitoes, such as *Anopheles, Culex, Mansonia* and *Aedes,* have specifically been reported to be responsible for the transmission of the disease (Ottesen *et al.*, 1997). LF is prevalent in tropical and subtropical regions of Africa, Asia and the Americas. In rural Africa, the *Anopheles* mosquito is said to be the primary carrier of LF, while in South East Asian countries and India, the *Culex* species is the major vector of *W. bancrofti* and *B. malayi*. In the Pacific Islands, *Aedes aegypti* and *Mansonia* were shown to be the main vectors for *B. malayi*. However, the efficiency of parasite transmission to humans differs based on the species of mosquito. For instance, *Anopheles* mosquitoes are reported to be generally less efficient vectors of *W. bancrofti* than *Culex* mosquitoes (USAID, 2009).

Commonly known as elephantiasis, LF is a painful and profoundly disfiguring disease. It is usually acquired in childhood, but the majorly deforming visible manifestations of the disease occur later in life. This situation usually results in a crippling condition of permanent disability whereby the limbs or other parts of the body are grotesquely swollen or enlarged and results in lymphedema, a swelling of the lymph nodes based on fluid build-up in the lymphatic system, and elephantiasis, a syndrome from the long-term obstruction of the lymphatic vessels that leads to engorgement and thickened skin resulting in disfigurement, quite often of the leg (Burnand & McGuiness, 2000).

According to World Health Organization (WHO) fact sheets, more than 1.3 billion people in 72 countries worldwide are threatened by LF, with over 120 million people currently infected and roughly 40 million disfigured and incapacitated by the disease (WHO., 2013b). In these endemic regions, the public health and economic burden caused by the disease is significant to the extent that it adversely affect productivity and quality of life (Krishna Kumari *et al.*, 2005). LF is said to have been a disease of antiquity, with reported documentation dating back to 2000 BC in ancient Egyptian hieroglyphics and in the Nok civilization of West Africa as early as 500 BC (Cashin-Garbutt, 2013). Although the disease has been around for ages, valid scientific documentation of its symptoms only appeared in the 16th century in areas like Goa, Asia and Africa (Cashin-Garbutt, 2013). However, a true understanding of the disease did not even begin to develop until centuries later.

As far as Malaysia is concerned, LF is still a very real public health burden, especially in the states of Pahang, Sarawak, Johor, Terengganu, Kedah, Perak, Kelantan, Sabah and Selangor (MOH, 2012). In 2011 about of 387 filariasis cases were reported (52 % among local and 48 % among immigrant) showing an increased number of cases compared to the previous year (156 cases). In 2011, the incidence rate of filariasis was 1.36 per 100, 000 population (Figure 1.1). The microfilaria rate for the last 5 years ranges from 2.14 - 1.41 per 1000 people. In 2011, *W. bancrofti* are the predominant parasite species which contributes to 45.5 % followed by *B. malayi* (periodic) making up to 36.2% and *B. malayi* (subperiodic) 18.3 %. Aslo, in 2011, it was reported that about 652,000 people required preventive therapy against LF in those areas (WHO, 2013b). Unfortunately, there is no recent data of LF clinical manifestations evaluated in sentinel sites.

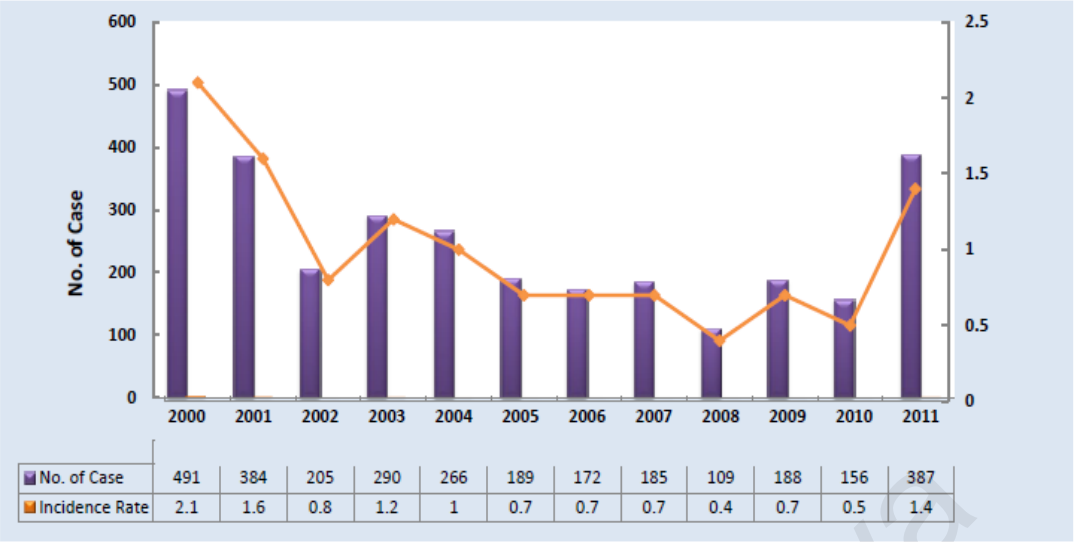

Figure 1.1: Filariasis cases and incidence rate trends in Malaysia, 2000-2011 (Source: vector born disease section, Disease control Division, MOH)

The mosquito species, *Mansonia* (*M. uniformis, M. bonnea* and *M. dives*), account for a large proportion of transmissions of filariasis in Malaysia (MOH, 2012). However, Vythilingam *et al.*, (1996) reported observing infective larvae of *B. malayi* in *Anopheles donaldi*. Presently, Malaysia participates in the Global Programme to Eliminate Lymphatic Filariasis (GPELF) and has already completed the fifth cycle of the programme. Malaysia hopes to eliminate filariasis by the year 2020 (Noordin, 2007).

Despite the numerous efforts and interventions undertaken to eradicate LF in Malaysia, there is still great concern over the existence of the disease as a public health problem in rural areas of the country. These encompass a population of 1,169,610 people and comprise 139 *mukims* or implementation units (IU) (Noordin, 2007; Tan *et al.*, 2011). The annual incidence rate of chronic elephantiasis is about 5 to 10 cases, with 2.9 million people susceptible to the disease (Noordin, 2007).

All across the world, several initiatives that serve as form of intervention in the transmission and which seek to eradicate LF infection from the public health domain have been spear-headed. Mass therapy campaigns and other public health strategies, including

vector (mosquito) control, were some of the reported methods that have been adopted and actively executed at both national and international stages. Mass therapy campaigns involve several drugs as the first line drugs of choice, including (1) diethylcarbamazine (DEC), a piperazine derivative, (2) ivermectin, said to be a broad spectrum antiparasitic agent and (3) albendazole, a benzimidazole derivative. These therapies, however, were found to be mostly effective against the microfilarial (Mf) stage and not particularly effective on filarial adult worms. In addition, their administration was illustrated to have heavy systemic and inflammatory reaction side effects (Saini et al., 2012).

Another strategy employed in LF treatment is to target *Wolbachia*, an intracellular bacterial symbiont. The endosymbiont persists throughout the worm life cycle, although its abundance varies between life cycle stages, and among individual worms (Hoerauf *et al.*, 1999; Hoerauf *et al.*, 2000; Ghedin *et al.*, 2009). Filariasis control by targeting *Wolbachia* has been successfully demonstrated in humans using the antibiotics doxycycline and rifampicin, which inhibit embryogenesis and induces long-term sterility in the adult worm (Bandi *et al.*, 1999; Taylor *et al.*, 2012). The antibiotics also inhibit the molting process of both third (L_3) and fourth (L_4) larvae (Hoerauf *et al.*, 2000). Unfortunately, antibiotic regimens are a long process, and are contra-indicated for children not more than eight years old and for pregnant or breast-feeding women. Therefore, alternative drugs active against *Wolbachia* and without these contraindications will leave a remarkable impact.

In accordance with WHO goals of the global programme to eliminate LF by 2020, WHO has realized that it is pertinent to study and understand the knowledge, attitudes and practice of those people living in endemic areas. It is anticipated that findings from such inquiry would help improve the efficiency of epidemiologic strategy design to successfully suppress and eradicate LF (Ottesen, 2000).

Obviously, the knowledge, attitudes and practices (KAP) concerning LF differs from one region to another as it is heavily influenced by specific socio-cultural settings. In fact, there was little information documented formally about how communities incorporate LF, dwell into its origins and impact, into local knowledge systems (Wynd *et al.*, 2007).The role played by mosquitoes in transmitting the disease is also not really known by many endemic communities, and thus we should expect to find that there is little awareness in these areas in terms of the importance of minimizing mosquito contact for the prevention of infection. For example, a KAP study in Malaysia revealed that the majority of the respondents were aware of filariasis; however, with regards to filarial transmission, 14.8% of infected people and 20.4% of uninfected people involved in the study thought that filarial worms entered the human body through the consumption of unhygenically prepared foods and drinks (Riji, 1986). Regardless of the fact that these groups were aware of the presence of mosquitoes in their village, only a very small number (n= 9) out of 108 respondents associated filariasis with mosquitoes (Riji, 1986). The vast majority did not associate this factor with the host's susceptibility to filarial infections. Rather, they were of the opinion that personal hygiene, such as walking barefoot on the dirty ground or consuming contaminated food and drink, was commonly implicated as the source of infection (Riji, 1986).

Based on the associated debilitating side effects and other medical complications of the synthesized therapeutic agents mentioned earlier, many have opted to search for alternative therapies through innovative and extensive phyto-medicinal research. The antioxidant capacity of certain phyto-chemical extracts inhibiting or suppressing the initiation or propagation of oxidizing radical species have made them attractive in complementary medicine (Velioglu *et al.*, 1998). With this, myriads of phyto-chemical extracts have been evaluated. Many literature has described *Melaleuca cajuputi*, or tea tree, that belongs to the Myrtaceae family as plant found to exhibit anti-inflammatory

(Liu, 1995), antimicrobial (Doran *et al.*, 1999), anticancer (Wolter *et al.*, 2002), and hepatoprotective (Saravanan & Pugalendi, 2006) activities. *Ardisia elliptica* (marlberry) belongs to the plant family of Primulaceae, widely distributed throughout Asia (Kobayashi & de Mejía, 2005) was described in several instances within the literature, as to have utero-contracting (Siti-Azima *et al.*, 2013), antitumor (Newell *et al.*, 2010), anti-inflammatory & antihyperalgesic (Ching *et al.*, 2010), antipyretic (Moongkarndi *et al.*, 2004), and anti-plasmodial (Noor Rain *et al.*, 2007) activities.

The present study aimed to address the issue of filarial nematode in two stages. First, a study on the KAP of LF among people living in the filarial endemic areas in order to provide a set of platform as a guide for the stakeholders in the fight against LF was undertaken. Secondly, there was an evaluation of the preliminary tests on selected Malaysian flora for potential anti-filarial activity directly or indirectly in both *in vitro* and *in vivo* experimental models.

It is hoped that the work presented herein will be of significance in the exploring of a new anti-filarial therapy based on Malaysian traditional medicinal plants.

1.2 Objectives

The following are the objectives of this study:

1.2.1 General objectives

1. To evaluate the Knowledge, Attitude and Practices (KAP) with regards to LF transmission, treatment, control and mass drug administration among residents of an endemic area.

2. To collect and state/document the ethno-botanical information about Malaysian medicinal plants that have potential anti-filarial activity.

3. To assess the anti-filarial and anti-*Wolbachial* activities of selected Malaysian medicinal plants, essential for further evaluation of their efficacy as anti-filarial remedies.

1.2.2 Specific objectives

1. To study preventive measures against LF used by the interviewees.
2. To investigate the management of LF with traditional plant remedies, including their use, preparation and administration by interviewees.
3. To investigate the acute toxicity, antibacterial, antioxidant and phytochemical characterization of plant extracts.
4. To evaluate the *in vitro* anti-filarial activity of different concentrations of plant extracts *in vitro* and *in vivo* against filarial parasites (ex. *Brugia pahangi).*
5. To evaluate the bioactivities of phytochemical extracts on filarial worms, and *Wolbachia*.

1.3 Problem statement

LF is one of the most neglected mosquito-borne diseases and is caused by parasitic thread-like filarial nematodes, such as *Wuchereria bancrofti*, *Brugia malayi* and *B. timori*. The disease is mostly distributed within the tropical and subtropical regions of the world, having a global prevalence of more than 120 million people (Michael *et al.*, 1996; Michael & Bundy, 1997).

LF has been identified as a major health problem since 1907 (Cashin-Garbutt, 2013). Based on WHO estimates, 1.2 billion people in about 73 countries are at risk, while 750

million people are exposed every year (WHO, 2012).Globally, LF is the second leading cause of permanent and long-term disability (Taylor *et al.*, 2010).

In 2004, the WHO held an inspiration session that addressed the issue of combating the disease. It concluded that among endemic communities, there appeared to be lack of knowledge with regards to this issue. It is essential for people to be well informed that although the disease is severe, controlling it is definitely possible. As information about the knowledge, attitude, and practice with regards to LF is scarce in Malaysia, assessment of these matters is crucial in order to plan a successful implementation of the elimination programme. It is in view of these motivations that this investigation is required to measure the level of KAP toward the disease.

In line with the implementation of Malaysia's filarial elimination programme by 2018, the study here was conducted to evaluate the community's awareness with regards to filarial transmission, treatment and vector control in rural communities within the district of Kemaman in Terengganu, which still has one of the highest proportions of filaria cases in Peninsular Malaysia.

The current study was also carried out to explore the possibility of antifilarial activity in traditional plants. The therapy used against LF in the current WHO Mass Drug Administration programme involving diethylcarbamazine (DEC), ivermectin and albendazole are known to be predominantly active against Mf, with DEC showing only partial activity against adult worms (Ottesen, *et al.*, 1997; Ndjonka *et al.*, 2013). Although there has been a significant reduction in infection prevalence, these drugs do not fully eradicate the adult worms. As well, the treatment needs to be repeated annually or biannually for many years to have a desired reduction of Mf levels in the endemic population so that transmission through the vector gets impaired. Furthermore, threat of development of resistance to mainstay drugs, especially ivermectin and albendazole, as

evidence in veterinary settings has advocated, means that sincere efforts are needed to develop new macrofilaricidal (adulticidal) agents for long-term reduction of filarial pathology.

There have been several attempts to use alternative drugs against filariasis. The most successful involve the novel use of antibiotics that target *Wolbachia*, a mutualistic bacterial endosymbiont of filariae that is essential for worm development, fertility and survival, and is a component of inflammatory disease pathogenesis (Taylor *et al.*, 2010).

Hoerauf *et al.,* (1999) observed that tetracycline treatment managed to eliminate *Wolbachia* and lead to filarial growth retardation and infertility in a *Litomosoides sigmodontis* filarial model. A year after, Hoerauf *et al.,* (2000) saw that administration of doxycycline at 200 mg for 6 weeks resulted in depletion of *Wolbachia,* long-term amicrofilaremia and 80% of worm nests from scrotal areas of infected men examined by ultrasonography. Doxycycline has been demonstrated to have both microcidal and macrocidal activity with exhibited efficacy in both *W. bancrofti* and *B. malayi* species (Hoerauf *et al.,* 2000; Taylor, 2000). Regardless of being rather effective against adult and Mf of filarial parasites, doxycycline has influences on developing cartilage and bone and is therefore not advisable for use in children less than eight years of age or pregnant women (Taylor *et al.*, 2010).

1.4 Expected outcomes

The expected outcomes of this study are:

1. Provide information on KAP of resident population in study area, to be used as a basic data for control programme.
2. Providing scientific information on antimicrobial, and antioxidant activities of selected plants.

3. Providing new source of antifilarial agent that could be used as an alternative therapeutic.

CHAPTER 2: LITERATURE REVIEW

2.1 Lymphatic Filariasis

Lymphatic filariasis (LF) is a disease caused by the parasitic thread-like filarial nematodes of the family *Onchocercidae*, which is comprised of *Wuchereria bancrofti*, *Brugia malayi* or *Brugia timori*. The disease is prevalent in tropical and subtropical regions of the world and is transmitted to humans *via* mosquito bites during blood meals (Brady, 2014). Several species of mosquitoes are known to transmit the disease as mentioned earlier.

As the effect of LF is determined by the intensity and the duration of the infection, and it has been reported that the greatest impact of LF is on older age groups (USAID, 2009). In general, about 90% of LF infection worldwide is caused by *W. bancrofti*, while most of the remaining infections are caused by *B. malayi* and *B. timori* (WHO., 2012).

In 1866, Timothy Lewis, who developed the work of Jean-Nicolas Demarquay and Otto Henry Wucherer, made a sound linkage between Mf and elephantiasis, simultaneously establishing the course of research that would finally justify the disease. In 1876, Joseph Bancroft discovered the adult form of the worm. In 1877, the first report of *W. bancrofti* was published in the Lancet by Cobbold naming the worm as '*Filaria bancrofti'* in honour of parasitologist, Joseph Bancroft. Later on, in the same year, the life cycle which involved an arthropod vector was mentioned by Patrick Manson who later demonstrated the presence of the worms in mosquitoes. Manson had made an inaccurate hypothesis that the disease was transmitted via skin contact with water in which the mosquitoes had laid eggs. In 1900, George Carmichael Low determined the actual transmission method by discovering the presence of the worm in the proboscis of

the mosquito vector (Cox, 2002). The scientific name, *W. bancrofti*, was crafted after physician Otto Wucherer and Joseph Bancroft, both of whom extensively studied filarial infections.

The first identification of *B. malayi* was in 1927 by Lichentenstein and Brugia (Cashin-Garbutt, 2013). They reported the occurrence of a species of human filariae in North Sumatra that was both physiologically and morphologically distinct from the *W. bancrofti* Mf common place in Jakarta and thus, it was named pathogen *Filaria malayi* (Sutaone, 2013). However, despite epidemiological studies identifying *F. malayi* in India, Sri Lanka, China, North Vietnam, and Malaysia in the 1930s, Lichentenstein and Brug's hypothesis was dismissed until the 1940s, when Rao and Mapelstone made an identification of two adult worms in India. The outstanding similarity of those newly found worms with *W. bancrofti* had led Rao and Mapelstone to name the parasite *Wuchereria malayi* (Sutaone, 2013). The new name, *B. malayi*, came into existence in 1960 when Buckley proposed to divide the old genus *Wuchereria* into two genera, *Wuchereria* and *Brugia*, and renamed *F. malayi* as *B. malayi* (Edeson & Wilson, 1964). Although Timor filariasis was described in 1965 (David & Edeson, 1965), the identity of the causative parasite as *B. timori* was only established in 1977 (Atmosoedjono *et al.*, 1977). In that same year, *Anopheles barbirostris* was shown to be its primary vector (Atmosoedjono *et al.*, 1977).

The *W. bancrofti* adult worm is noted as long, slender and smooth with rounded ends, with a short cephalic region and dispersed nuclei throughout its body cavity, with no nuclei at the tail tip. The male worm is 40 mm long and 100 μm wide, and it also has a curved tail. Quite differently, the female is 60 mm to 100 mm long and 300 μm wide, nearly three times larger than the male, diameter-wise. Females are ovoviviparous and they can produce thousands of juveniles known as Mf which retain the egg membrane as a sheath and are often accounted for as advanced embryos (Dissanaike & Mak, 1980).

The adult worms of *B. malayi* are typically smaller than adult *W. bancrofti*. Adult females of *B. malayi* are twice (50 mm) larger than male worms (25mm) (Muller, 2002). The Mf is 200-275µm in length and has a round anterior end along with a pointed posterior end (Muller, 2002). The Mf is sheathed, which stains heavily with Giemsa and is also retained until it is digested in the mosquito midgut (John *et al.*, 2006). *B. malayi* Mf has a resemblance with *W. bancrofti* Mf, with little differences that can facilitate laboratory diagnosis. *B. malayi* Mf can be distinguished by the presence of two terminal nuclei distinctly separated from the other nuclei in the tail, whereas the tail of *W. bancrofti* has no nuclei (John *et al.*, 2006). The main differentiating feature of *B. timori* Mf from the other two parasites is that they are longer and morphologically distinct from those of *B. malayi* and *W. bancrofti* with a cephalic space length-to-width ratio of about 3:1 (Purnomo, 1977)

2.2 Epidemiology, Pathology, and clinical manifestations of lymphatic filariasis

As mentioned before, filariasis is endemic to tropical and sub-tropical regions of Asia, Africa, Central and South America and Pacific Island nations (Figure 2.1). Among the infected, almost 25 million men have genital disease (most commonly hydrocele) and nearly 15 million, mostly women, have lymphoedema or elephantiasis of the leg (WHO, 2012). Approximately 66% of those at risk of infection live in the South East Asia Region and 33% in the African Region (WHO, 2012). In communities where LF is endemic, as many as 10 percent of women can be afflicted with swollen limbs, and 50 percent of men can suffer from mutilated genital symptoms.

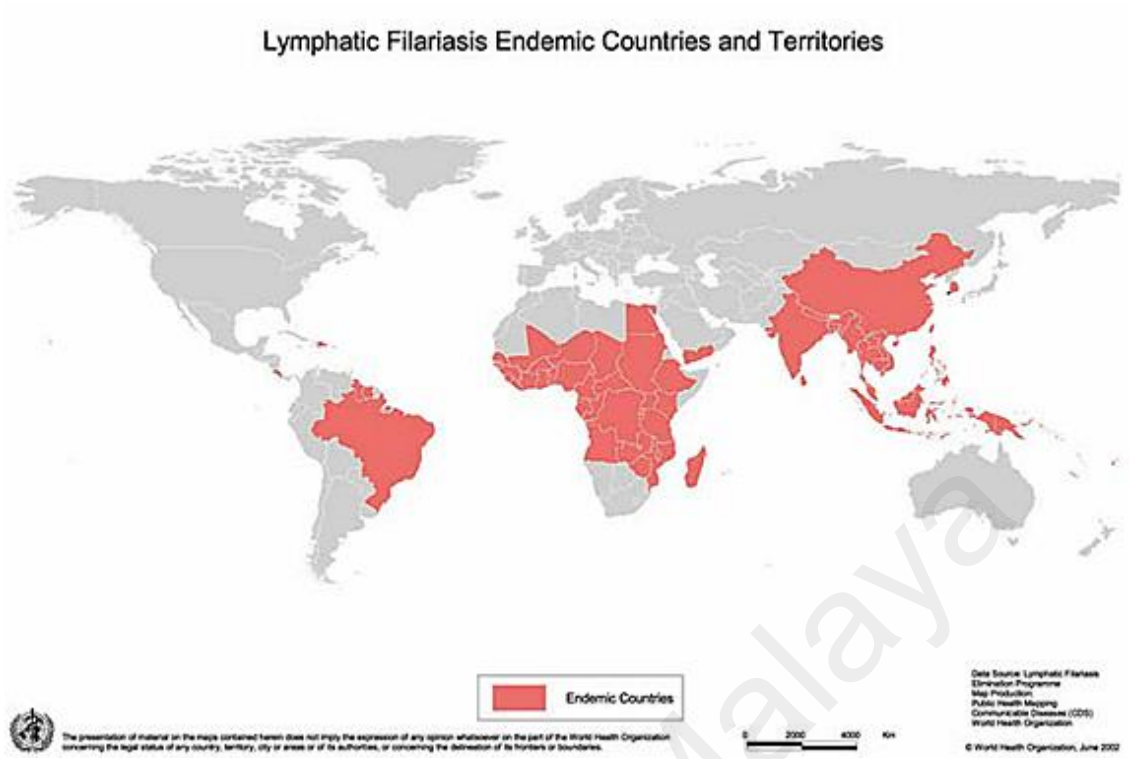

Figure 2.1: Lymphatic filariasis (LF) endemic countries (adopted from WHO, 2014).

As mentioned earlier, LF is caused by nematodes of different taxonomic genera (Figure 2.2) that inhabit the lymphatic vessels and lymph nodes

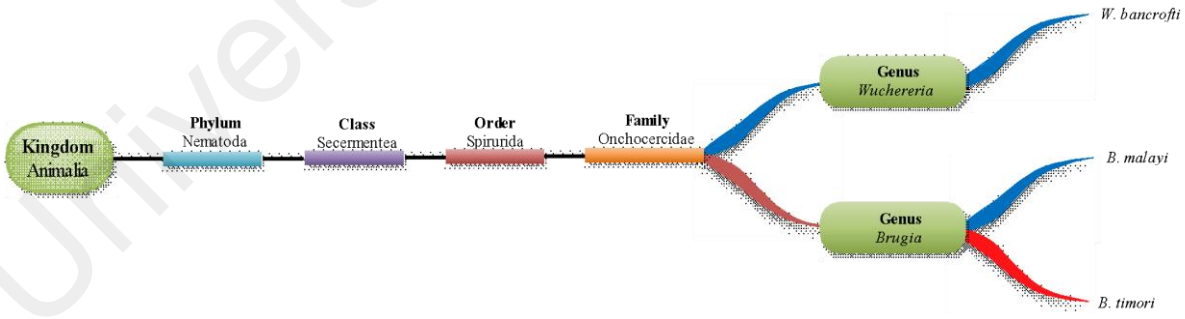

Figure 2.2: Taxonomy of filarial parasites

As covered earlier, the pathogenesis of the disease starts with infective larvae, L_3, that are transmitted by infected mosquitoes during a blood meal (Figure 2.3 and Figure 2.4). The larvae migrate to lymphatic vessels and lymph nodes where they develop into Mf - producing adults (Rajamanickam & Babu, 2013). In these areas, the adults dwell and can

live for several years, with the female worms producing Mf that circulates throughout the blood (Feasey *et al.*, 2010).

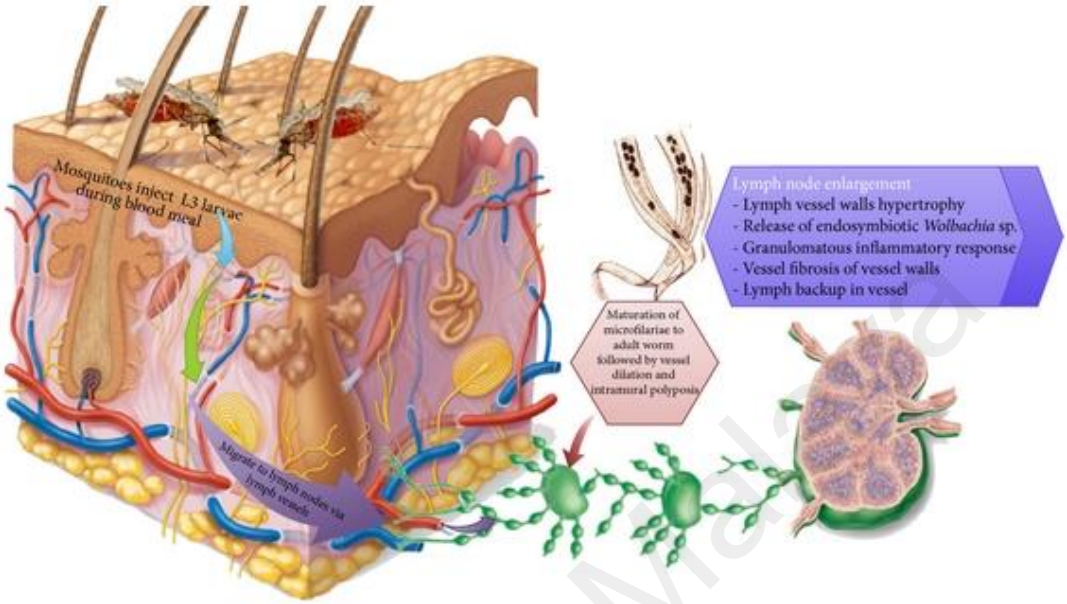

Figure 2.3: Inflammatory responses in lymphatic filariasis (LF)

The life cycle (Figure 2.4), starts when the infected mosquito deposits larvae into the skin during the biting, and L3 enter onto the bite wound❶. Larvae migrate to lymphatic system and there they develop into adult worms❷. Adult worms, damage the lymphatic system, causing infections that result in blockages, swelling, and fevers, and produce microfilariae which migrate into lymph and blood channels moving actively through lymph and blood❸. A mosquito, feeding on the blood of an infected person, ingests the microfilariae during a blood meal and becoming infected❹. After ingestion, the microfilariae lose their sheaths and some of them work their way through the wall of the proventriculus and cardiac portion of the mosquito's midgut and reach the thoracic muscles muscles❺. There the microfilariae develop into first-stage larvae (L1) ❻ and subsequently into infectious larvae (L3)❼. The L3 infective larvae migrate to the mosquito's head and prosbocis ❽ and can infect another human when the mosquito takes

a blood meal and continues the cycle❶. Mosquitoes become infected when they take up Mf during the blood meal. Inside the mosquito, the Mf develops in 1 to 2 weeks into infective filariform third-stage larvae, L3. During a subsequent blood meal, L3 infects the vertebrate host for continuation of the life cycle (Ramaiah *et al*., 2000).

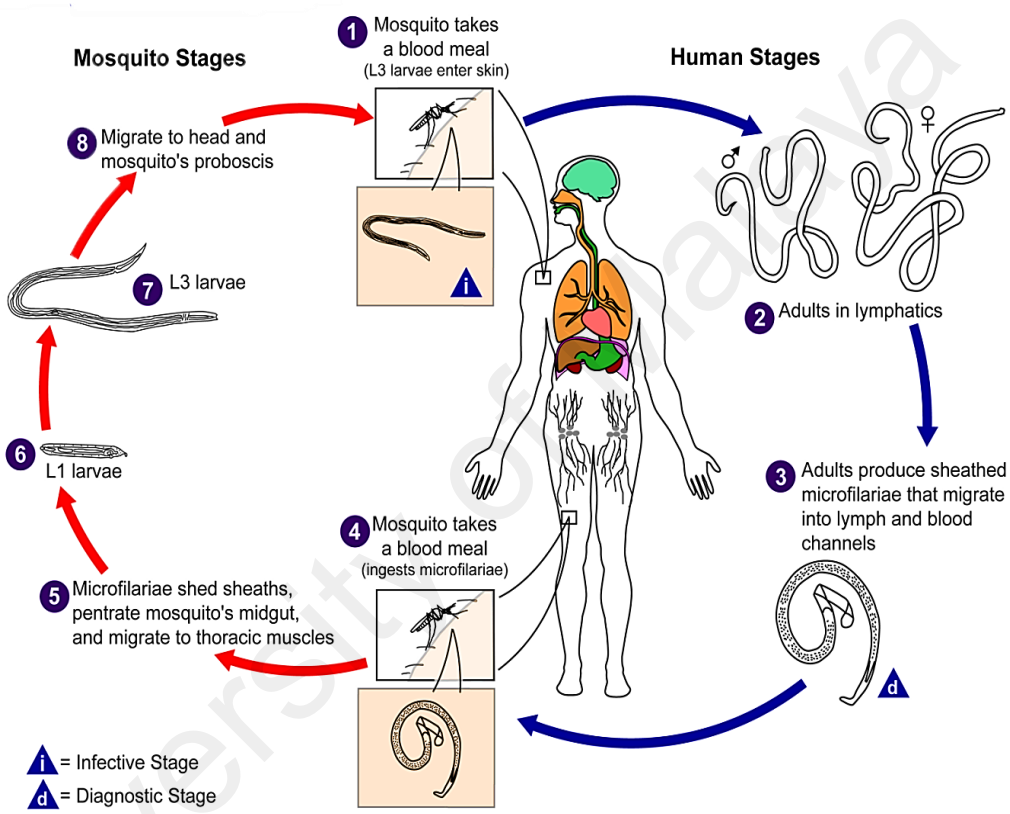

Figure 2.4: Life-cycle of *W. bancrofti* (CDC, 2010)

Filarial infection results in a wide spectrum of clinical findings. In general, however, LF has both an acute and chronic phase. Acute manifestations are often characterized by episodic attacks of fever, malaise, inflammatory nodules in breast, scrotum or subcutaneous tissue (Partono, 1987). Chronic manifestations include hydrocele being the most common chronic manifestation in bancroftian filariasis, followed by lymphodaema of the extremities and genitals in females (vulva, clitoris), scrotal skin in males and

chyluria (the presence of chyle in the urine) following the rupture of dilated lymphatic vessels into the urinary excretory system (Ottesen, 1984). Tropical pulmonary eosinophilia (TPE), otherwise referred to as "occult filariasis", is the least common clinical manifestation of filariasis with low frequency in most endemic areas containing LF (Partono, 1987). This manifestation occurs because of immunological hyper responsiveness to Mf in the lungs and is characterized primarily by coughing and asthmatic breathing.

Lymphatic filarial infection usually results in crippling condition of permanent disability in which limbs or other parts of the body are grotesquely swollen or enlarged resulting in lymphedema, a swelling of lymph nodes from fluid build-up in the lymphatic system and elephantiasis, a syndrome based on long-term obstruction of lymphatic vessels that leads to engorgement and thickened skin resulting in disfigurement, often of the leg (Chandrasena *et al.*, 2007). Approximately 65% of those infected live in the South East Asia Region, 30% in the African Region, and the remainder in other tropical areas (WHO, 2013b). In these endemic regions, the psychological, economic and social impact associated with the disease is significant to the extent that it adversely affects productivity and quality of life.

The many species of filarial worms tend to leave an impact on the various parts of the body, whereby for instance *W. bancrofti* can affect the legs, arms, vulva, and breasts, while *Brugian* filariasis rarely affects the genitals.Therefore, the main difference in the clinical manifestations between *Bancroftian* and *Brugian* filariasis is the rarity of hydroceles and other genital lesions in areas endemic for *B. malayi*.

Chyluria is another sign there is no association with *B. malayi*. Elephantiasis of the legs in *B. malayi* infections is often confined to below the knee, whereas in infections with *W. bancrofti* are present in the lower legs as well as the thigh (Babu & Nutman,

2012). *B. timori* infections produce similar clinical manifestations to those seen in infections from *B. malayi*, i.e. scrotal lesions, are almost absent while elephantiasis tends to be below the knee.

Disease pathogenesis can be associated with the host inflammation invoked by the death of the parasite that causes hydrocoele, lymphoedema and elephantiasis. Vascular endothelial growth factor (VEGF)-A is suggested to be the marker of filarial hydrocoele pathogenesis and VEGF-C as a marker of lymphedema. VEGF-A concentrations have been found to have increased in all infected patients and also in patients suffering from hydrocoele disease (Taylor *et al*., 2010). Most filarial species that infect people co-exist in a mutualistic symbiosis with *Wolbachia* bacteria, where they are necessary for growth, development, and survival of their nematode hosts, endosymbiotically becoming a contributor to inflammatory disease pathogenesis. *Wolbachia* lipoproteins have been identified as inflammatory ligands that bind toll-like receptor (TLR) 2 and TLR-6 to activate innate and adaptive inflammatory responses further leading to the classical activation of macrophages and boost the activation of neutrophils. Exposure to *Wolbachia* lipoproteins can lead to the innate and adaptive immune-mediated expression of VEGFs, simultaneously promoting lymphangiogenesis, lymphatic endothelial proliferation and dilation of lymphatic vessels to expedite the development of chronic disease (Taylor *et al.*, 2010). Anuradha *et al.*, (2012) wrote a report on the use of circulating microbial products and acute phase proteins as pathogenesis markers in LF disease.

2.3 Diagnosis of LF

The identification of Mf in a blood smear by microscopic examination has been the standard method in diagnosing LF. Based on the periodicity phenomenon of the parasite, blood collection should be performed at a time coinciding with the appearance of Mf in circulation (Rajan, 2008). The periodicity of both *W. bancrofti* and *B. malayi* in most areas is nocturnal, with the highest concentration at the midnight, and few or none during midday, and thus, the parasites are transmitted by night-biting mosquitoes in these areas (Edeson & Wilson, 1964). Mf has been found mainly in the peripheral blood and can be established at peak amounts from 8 p.m. to 2 a.m. Yet, the reason behind this periodicity fails to be detected, although some attributed physiological changes such as lowered body temperature, oxygen tension and adrenal activity are linked with sleeping, and an increased carbon dioxide tension to facilitate the rhythm (McNulty *et al.*, 2013b). The presence of Mf in the peripheral blood during these hours increases the possibility of the vector transmitting the Mf elsewhere. However, the South Pacific *W. bancrofti* shows diurnal periodicity (Mandal, 2015). Clearly, the mechanism of periodicity is very important in the disease diagnosis and transmission.

A thick smear of collected blood should be made and stained with Giemsa or hematoxylin and eosin (H & E) (Fritsche & Selvarangan, 2011). The stains permit morphological identification of the filarial species; however, this method has the drawbacks of loss of Mf through the staining procedure. In order to avoid this disadvantage, the collection of blood in heparinised capillary tubes is more sensitive than thick blood smearing (Partono & Idris, 1977). With this method, the blood sample is transferred to 1 ml of 3% acetic acid and then examined microscopically in the laboratory by using a counting chamber technique (McMahon *et al.*, 1979). Both methods are inappropriate for the patient and scientist because of the time needed for blood collection, it is labour intensive and time-consuming and there is great difficulty in distinguishing

one filarial species from another (Simonsen *et al.*, 2008). Moreover, the sensitivity of the method is overall poor as they fail to recognize individuals with very low Mf levels.

Serological techniques such as the immune chromatographic test (ICT) (for *W. bancrofti*) and PanLF *Rapid* and *Brugia Rapid* tests provide an alternative method for the diagnosis of LF regardless of periodicity (Chanteau *et al.*, 1991). Patients with active filarial infections typically have elevated levels of antifilarial IgG4 in the blood and these can be detected using routine assays. In line with this, the use of antigen-antibody based assays have been established in the literature (Lammie *et al.*, 2004). Omar *et al.* (2001) has described the use of a dipstick colloidal dye immunoassay (DIA) in LF diagnosis. The authors found the method to be robust and rapid as it can be read in approximately 2 hours. The use of PCR-ELISA in filarial infection diagnosis has also been evaluated (Wijegunawardana *et al.*, 2013). Previously, a study on the comparison of conventional versus real-time PCR methods in diagnosing LF using dried blood smear samples was also reported (Rahmah *et al.*, 2010).

2.4 Therapeutic management of lymphatic filariasis

2.4.1 Chemotherapeutic intervention

The various efforts to interrupt transmission and do away with LF as a public health issue are certainly depend on effective mass therapy campaigns and other public health strategies, including vector control where it is deemed suitable. Among the more popular chemotherapies, including albendazole, ivermectin or diethylcarbamazine (Figure 2.5 (a-c)), the drug of choice is diethylcarbamazine (DEC), which can eliminate Mf from the blood with a dosage of 6 mg/kg semi-annually or annually (Eberhard *et al.*, 1997). A polytherapy/combination treatment that includes ivermectin with DEC or albendazole has been shown to be more effective than individual drug regimens (De Kraker *et al.*, 2006). One other strategy is to employ an antibiotic, doxycycline (Figure 2.5 (d)), which targets

the *Wolbachia* bacterial endosymbionts, resulting in long-term sterility and macrofilaricidal activity (Taylor *et al*., 2010). A 6-week course of doxycycline 100 mg per day can also work well in mitigating *Wolbachia* and Mf in Brugian filariasis. A year after this treatment, microfilarial prevalence was able to be reduced by 77% in patients given doxycycline monotherapy and 88% in those given doxycycline with diethylcarbamazine-albendazole, as opposed to 27% in those given placebo and diethylcarbamazine-albendazole (Supali *et al*., 2008).

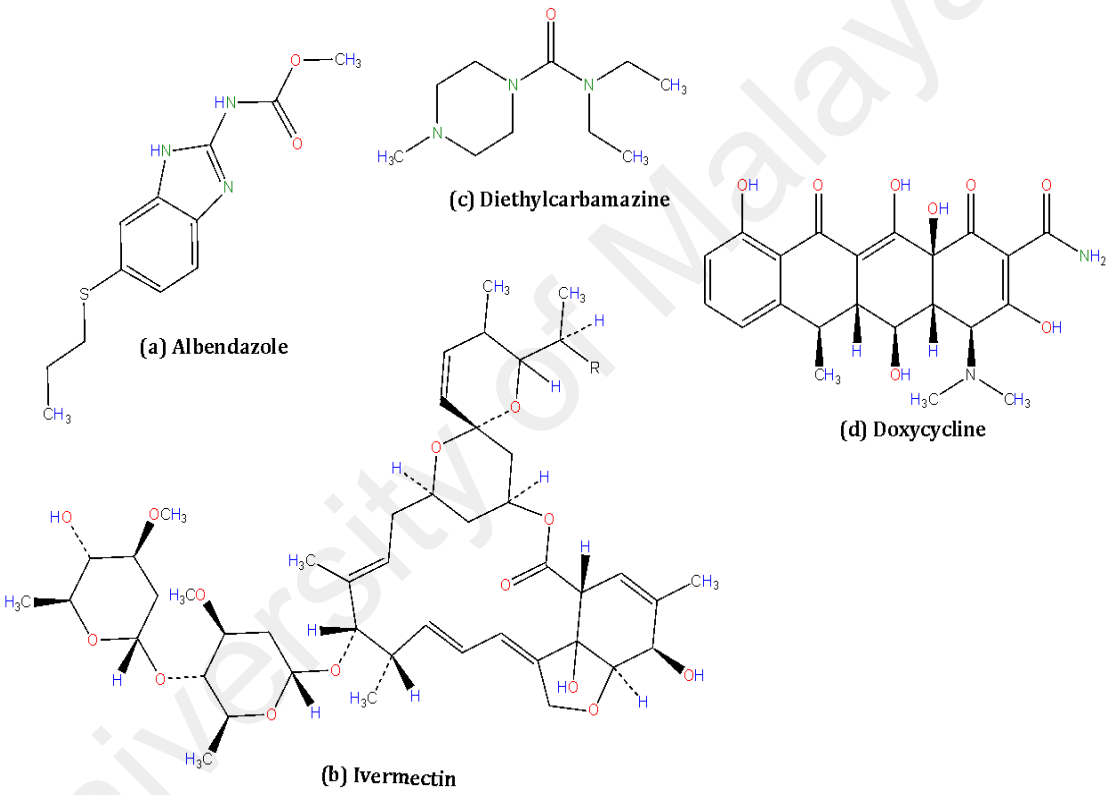

Figure 2.5: Chemical structures of LF drugs

Albendazole is a broad-spectrum anthelmintic consumed orally; it is effective against nematodes, cestodes and flatworms (Horton, 2002). It is shown to inhibit the polymerisation of β-tubulin and microtubule formation (Hoerauf *et al.*, 2011). 400 mg given to treat most intestinal helminth infections decreases *W. bancrofti* Mf progressively for half a year to a year (Gyapong *et al.*, 2005). There are claims that ivermectin is a highly effective, well-tolerated drug at doses of 100–200 μg/kg for Mf reduction in LF,

but it also leads to the hyperpolarisation of glutamate-sensitive channels and immobilisation of Mf (Wolstenholme & Rogers, 2005). At an early stage, infection can be treated with drugs such as diethylcarbamazine citrate (DEC), albendazole or a combination of albendazole and ivermectin (Pani *et al.*, 2002). However, chronic conditions may not be curable with the use of anti-filarial drugs and they require other measures such as exercising to increase the lymphatic drainage in lymphedema (WHO, 2013a).

While DEC is considered the first choice in LF therapy, various shortcomings have been noted in individuals with infection, particularly those who are parasitaemic leaning on adverse reactions post-DEC. These reactions have to do with the fast annihilation of adult worms, which, for example, can lead to scrotal pain in men, dizziness, nausea, fever, headache and pain in muscles or joints. Rapid killing of Mf starts the systemic inflammation due to *Wolbachia*'s release.

The low rate of severe adverse reactions originating from slow parasite elimination, high degree of antifilarial effectiveness and improvement of urogenital pathological outcomes and lymphoedema in LF makes doxycycline an option that is naturally solid therapeutically. Despite this, its use in community-based control is compromised by the logistics of the treatment duration and contraindications in both children and pregnant women (Taylor *et al*., 2010). In an attempt to evade these undesirable side effects, research on exploration and development of alternative therapeutic drugs are increasingly gaining momentum (Gupta *et al*., 2012).

2.4.2 Phyto-therapeutic intervention

The application of phyto-chemicals to treat diseases are among the oldest forms of healthcare known to mankind. Ancient Chinese characters and Egyptian papyrus hieroglyphs have documented the use of herbal medicine dating back as early as 3000 B.C (Chevallier & Chevallier, 1996). In fact, herbal remedies had been in use by all cultures throughout human history. Plant extracts such as those from *Azadirachta indica* (Mishra *et al.*, 2005), *Polyalthia suaveolens* (Zaridah *et al.*, 2001), *Andrographis paniculata* (Sheeja *et al.*, 2012), *Bauhinia racemosa* (Sashidhara *et al.*, 2012) and *Haliclona oculata* (Gupta *et al.*, 2012) were reported to have a bioactivity on either the filarial parasites or their vectors.

Anti-filarial activity of the alcoholic extract of *A. paniculata* was reported by Kumarappan *et al.* in 2009. Another study reported that aqueous extract of leaves showed microfilaricidal activity on *Dipetalonema reconditum* within 40 min, both *in vitro* and *in vivo* (Dutta & Sukul, 1982). Administration of the extract (0.06 ml/Kg body weight) reduced the number of Mf in infected dogs by more than 85% (Dutta & Sukul, 1982). Earlier, Zaridah *et al.* (2001), documented the filaricidal activity of *A. paniculata* aqueous leaf extract against *B. malayi*. The authors analyzed the anti-filarial activity of the extract using relative movability (RM) values of the adult worm over a period of 24 hr. Specifically, they utilised 5 or 10 mg/ml of the extract resulting in a 0% RM value, signifying total death of the parasite. Lowering the concentration of the extract to 1 mg/ml, however, failed to produce a similar effect (mean RM value was 35%).

The filaricidal activity of the Mermaid's glove sponge (*Haliclona oculata*) has also been detailed in the literature. Gupta *et al.*, (2012) reported the anti-filarial activity of *H. oculata* against experimental lymphatic filariidae, *B. malayi*. Employing methanolic extract, chloroform fractions of the methanolic extract and a fraction from the

chromatographic eluent, at 100 mg/kg for five consecutive days by subcutaneous route, demonstrated significant macrofilaricidal efficacy of 51.3%, 64% and 70.7%, respectively. In all samples, about 45–50% macrofilaricidal activity with moderate embryostatic effect was observed. Further analysis of the chromatographic fraction revealed that it contained a mixture of four alkaloids, namely mimosamycin, xestospongin-C, xestospongin-D and araguspongin-C together with few minor compounds (Gupta *et al.*, 2012). Work done by Lakshmi *et al*., (2009) on anti-filarial activity of another species, *H. exigua,* against lymphatic *B. malayi in vitro* and *in vivo* showed that 31.25 µg/ml concentrations of the crude methanolic extract and butanol soluble fraction were able to rid the samples of the adult worm. With this, the chloroform extract was found to be effective at lower concentrations (15.6µg/ml). According to the authors, such a finding could be attributed to the single bioactive molecule, 'araguspongin-C' (Lakshmi *et al.*, 2009).

Mathew *et al.,* (2008) studied the effect of the methanolic extract of *Trachyspermum ammi* fruits against adult bovine filarial *Setaria digitata* worms at a concentration of 0.01-0.5 mg/ml for a period of 24-48 hr and found that both the crude extract and the active fraction showed significant activity against the adult *S. digitata* through both worm motility and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assays. They also isolated a compound and classified it as phenolic monoterpene, described as 2-isopropyl-5-methyl phenol, and tested it *in vivo* for anti-filarial activity against the human filarial worm, *B. malayi*. In *Mastomys coucha,* it showed macrofilaricidal activity where female worm sterility was detected.

Shanmugapriya & Ramanathan (2012), noticed that the filaricidal effect of the organic solvent extract of *Ricinus communis* (castor oil) seed against filarial parasite *B. malayi* using various dosages (10, 50 and 100 µg/ml of extract) for the period of 24 hours. Their findings suggested dose-dependent filaricidal activity (40-90%). Previously, Satapathy

(2007) showed the filaricidal efficacy of noni (*Morinda citrifolia*) against *W. bancrofti* in an *in vitro* study and ultimately demonstrated that adding noni fruit extract to cultured media containing *W. bancrofti* Mf at a concentration of 2000 Mf/ml killed the parasite within 20 hours as compared to the control group without the extract that survived for up to 60 hours. A similar study by Misra, *et al.,* (2011) showed an *in vivo* filaricidal activity of *Xylocarpus granatum* extract against *B. malay*i (both adult worm and Mf). When testing the crude aqueous ethanolic extract *in vitro*, they noted an IC_{50} of 15.46 and 13.17 ug/ml in both adult worms and Mf, respectively. On the other hand, investigation of the ethyl acetate-soluble fraction revealed anti-filarial activity to be moderate (IC_{50} of 8.5 and 6.9 μg/ml) in adult worms and Mf, respectively. They discovered further that this extract showed adulticidal (52.8%) and embryostatic (62.7%) effects against *B. malayi* upon exposure it to *Mastomys* orally at 50 mg/Kg. It was thought before that this species had filaricidal activity against *B. pahangi (*Wan Omar *et al.,* 1997*)*. Filaricidal activity of *X. granatum* leaves aqueous extract against *B. malayi* was also reported by Zaridah *et al.*, (2001) where administration of 10 mg/ml resulted in total elimination of the parasite after 24 hrs exposure.

Kushwaha *et al.*, (2011), reported for the first time filaricidal activity of an ethanolic extract of *Hibiscus sabdariffa* leaves against *B. malayi*. The efficacy of filaricidal activity was assessed using both the *in vivo* and *in vitro* motility and MTT reduction assays on Mf and adult worms. The authors found that administering the leaf extract at 500 mg/ml for 5 days incurred about 30% macrofilaricidal efficacy and 42% sterilization of female worms in *Meriones unguiculatus*. Conversely, feeding *M. coucha* with the extract (1g/kg for 5 days) exerted a 57.0% macrofilaricidal influence with a 64% sterilizing effect on female worms.

Cardiospermum halicacabum ("love in a puff") is a climbing plant widely distributed in tropical and subtropical regions of Africa and Asia. Khunkitti *et al.* (2000) previously

reported the *in vitro* filaricidal activity of ethanolic and aqueous extracts of this plant against *B. pahangi*. They saw that the activity on adult worms and the amount of Mf released by female worms was concentration and time dependent. For example, using 500µg/ml, the authors observed the aqueous extract to significantly reduce motility of adult females after 24 h exposure, the release of Mf from female worms on day 2 and the motility of the adult males after 3 days. The *in vitro* and *in vivo* filaricidal activities of four bioactive compounds isolated from *Alnus nepalensis* leaves against human lymphatic filariasis (*B. malayi*) were investigated for the first time (Yadav *et al.*, 2013). The researchers isolated the diarylheptanoids phyto-chemical compounds as shown in Figure 2.6 (a-d).

Shanmugapriya & Ramanathan (2012) has demonstrated filaricidal activity of galactolipid compounds isolated from the ethanolic extract of *Bauhinia racemose* leaves. The n-butanol fraction of the extract revealed a promising adulticidal (IC_{50} 5.46 mg/mL) and microfilaricidal (IC_{50} 4.89 mg/mL) activity with a minimum inhibitory concentration (MIC) of 15.6 mg/mL. Among the identified isolated galactolipids was (2S)-1,2-di-O-linolenoyl-3-O-a-galactopyranosyl-(1/6)-O-b-galactopyranosyl glycerol (Figure 2.7), which they found in particular to have a filaricidal efficacy that rivalled the standard treatment drug, ivermectin (IC_{50} 1.61 mg/ml; MIC 7.8 mg/ml in adult and IC_{50} 3.62 mg/ml; MIC 125 mg/ml in Mf), in terms of dose and efficacy.

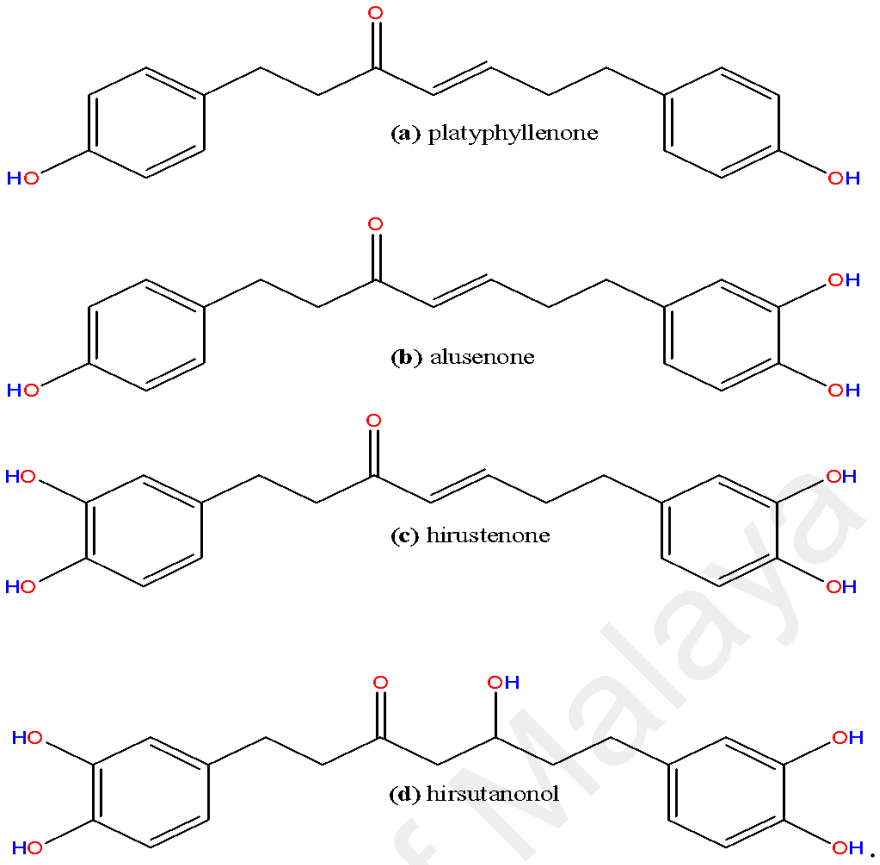

Figure 2.6: Chemical structure of bioactive phytochemicals extracted from *A. nepalensis*

In traditional Malay medicine, *Ardisia elliptica* (Myrsinaceae) is employed to treat various ailments associated with inflammation, chest pains, fever, diarrhoea, liver poisoning and parturition complications. The plant's hexane fraction is known to inhibit the progression of several diseases associated with inflammation (Ching *et al.*, 2010). Similarly, a triterpenoid extracted from *Melaleuca cajuputi* (Myrtaceae) identified as ursolic acid was shown to possess anti-inflammatory properties by inhibiting histamine release from mast cells (Liu, 1995). Based on their anti-inflammatory efficacy, both *A. elliptica* and *M. cajupati* could provide an alternative phyto-therapeutic agent against filarial induced inflammation.

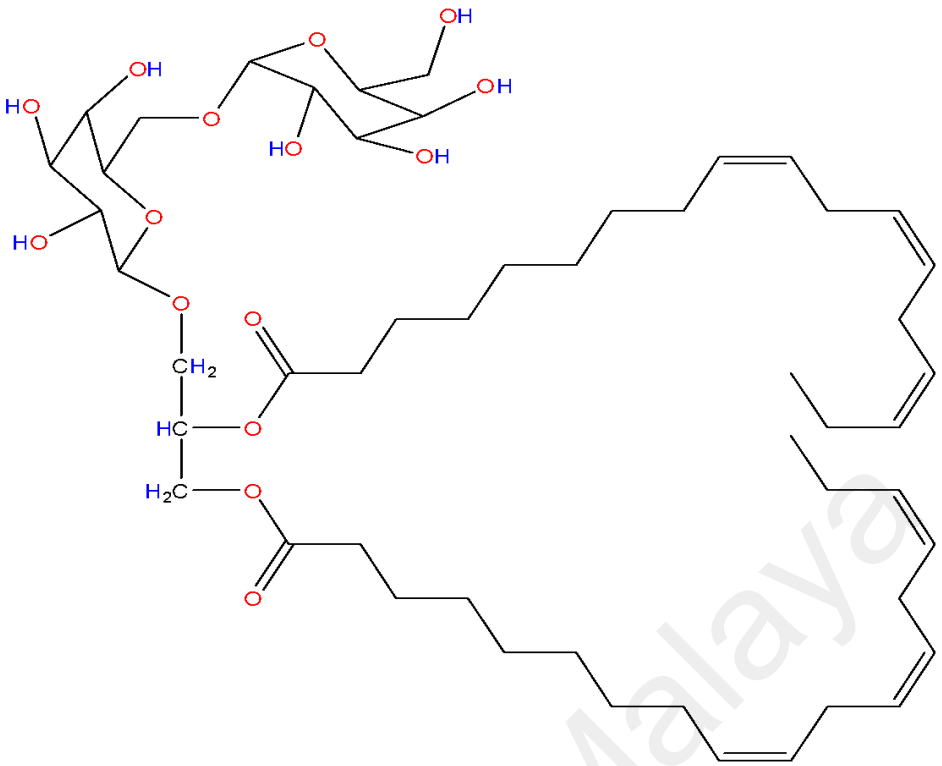

Figure 2.7: Chemical structure of (2S)-1,2-di-O-linolenoyl-3-O-a-galactopyranosyl-(1/6)-O-b-galactopyranosyl glycerol, the bioactive phytochemical extracted from *B. racemose*

2.5 Control of mosquito vector

One of the most common filarial preventive methods is mosquito vector control. As filariasis is transmitted by mosquito vectors, controlling these insects could lead to reduce incidence of the disease. Research on the insecticidal and larvicidal efficacy of phyto-chemical extracts in regulating these filarial vectors has been described in the literature. For example, the essential oil extracted from *M. cajuputi* exhibited 12.5% mortality at 50ppm and 27% mortality at 125ppm concentrations when tested against mosquito *A. albopictus* first stage larvae (Ul Hassan *et al.*, 2003). The oil of *M. cajuputi* was also found to be successful in providing repellency against *A. aegyti, A. stsphensi* and *C. qumquefasciatus* where it provided a protection time of 8 h at the maximum and a 100% repellency against all three species (Amer & Mehlhorn, 2006).

Ul Hassan *et al.* (2003) employed the use of novel phytosynthesis to diminish silver nitrate to biogenic silver nanoparticles in the presence *Cocos nucifera* extracts against *A. stephensi* and *C. quinquefasciatus*. Approximately 100% and 92% 4th instar larval motility inhibition at 4 mg/ml dosage strength in both *A. stephensi* and *C. quinquefasciatus* after 72 hours, respectively, was seen. In addition, Subarani *et al.*, (2012) observed the larvicidal activity of *Vinca rosea* aqueous leaf extract biosynthesized-silver nanoparticles against *A. stephensi Liston* and *C. quinquefasciatus*. On exposure for 72 hours, larvicidal activity reached maximum efficacy in synthesized silver nanoparticles against the fourth instar larvae of *A. stephensi* (LC_{50} = 16.84 mg/ml) and against *C. quinquefasciatus* (LC_{50} = 43.80 mg/ml). Hadis *et al.,* (2003) further elaborated on the repellent activity of essential oils of lemon eucalyptus (*Eucalyptus maculatacitrodion*), rue (*Rutachalepensis*), oleoresin of pyrethrum (*Chrysanthemum cinerariaefolium*) and neem (*Azadiractaindica*) solutions in coconut oil against mosquitoes consisting mainly of *Mansonia* in Gambella, in the western part of Ethiopia. 50% concentrations had the highest repellency recorded with the protection being 91.6%, 87.0% and 96.0% for rue, neem and pyrethrum, respectively. *Lemon eucalyptus* was found to show significantly better performance than neem and pyrethrum at 75%.

In 2012, Kovendan *et al.,* (2012) uncovered the larvicidal effect of noni (*M. citrifolia*) leaf extract against three medically important mosquito vectors, specifically of the *Anopheles, Aedes* and *Culex* genera. After 24 hours of exposure, larvicidal activity appeared to be concentration and extraction-solvent dependent. Govindarajan *et al.*, (2008) demonstrated the larvicidal and ovicidal activity of *Cassia fistula* Linn methanolic leaf extract against *C. quinquefasciatus* and *A. stephensi*. The activity of the extract (10-50 mg/l) was concentration dependent, and at 40 mg/l, the percent mortality was 89.33 and 100 in both *Culex* and *Anopheles*, respectively, with LC_{50} values recorded at 17.97 and 20.57 mg/l, respectively.

In contrast to this, (Rajkumar & Jebanesan, 2009) observed a LC_{50} value of 52.2 mg/l in *A. staphensi* using ethanolic leaf extract of *Cassia obtusifolia*. Moreover, the insecticidal efficacy of *Carica papaya* leaf extracts against *A. aegypti* larvicidial and pupicidal properties was also reported (Kovendan *et al.*, 2012). In particular, the leaf extract showed both a larvicidal and pupicidal efficacy after 24 h of exposure. In all extracts tested, the methanolic extract had the highest larval and pupal mortality against the larvae and pupae with values of LC_{50} at 51.76, 61.87, 74.07, 82.18 and 440.65 ppm for 1^{st}, 2^{nd}, 3^{rd} and 4^{th} instar larvae and pupae, respectively. Larvicidal activity of *Acalypha alinifolia* leaf extract against *A. stephensi*, *A. aegypti* and *C. quinquefasciatus* was also evaluated (Kovendan *et al.*, 2012). Exposing the larvae to extracts of different solvents for 24 hours resulted in methanol extract being the most bioactive (98.4% larval mortality) while hexane extract was found to be the least. Ngente *et al.*, (2012) have been described on larvicidal, adulticidal and repellent activity of *Hiptage benghalensis* acetone root bark extracts against mosquitoes of the *Anopheles*, *Culex* and *Aedes* genera. Overall, the extract was effectively larvicidal with a low LC_{50} (11.15–16.78 ppm) and lethal time LT_{50} (1.25–4.84 h at 200 and 400 ppm). Further, this lethal time was found to be concentration dependent.

Indigofera suffruticosa Mill (*Fabaceae*) is a plant found in tropical and subtropical areas and is well adapted to growth in semiarid regions with soil of low fertility. It has been known for its medicinal efficacy against bacterial and fungal infections (Vieira *et al.*, 2011). Vieira *et al.*, (2011) have demonstrated the oviposition and embryotoxicity of *I. suffruticosa* leaves extract against *Aedes aegypti*. Repellent activity was uncovered to be concentration dependent. Exposing the larvae to a concentration of 250 µg/ml for 72 hours showed a 93.3% growth inhibition in L_2 instar.

2.6 Knowledge, attitudes and practices (KAP) on lymphatic filariasis

It is important to realise that the knowledge gap regarding the disease and general attitudes and perceptions towards the eradication programme was the basis of the major causes of lower compliance (Rath *et al.*, 2006) and very likely resulted in continued endemicity of this disease in the areas concerned. This is a proven phenomenon in Kenya (Njomo *et al.*, 2012) and in India (Mukhopadhyay *et al.*, 2008; Ghosh *et al.*, 2013), where low compliance during MDA programme existed because of poor knowledge in the target population on LF.

Little formal documentation was true, in light of about how communities integrate LF, its origins and impact into the local knowledge systems (Wynd *et al*., 2007). The role of mosquitoes in the disease transmission is also less appreciated in many endemic communities, and thus there is little awareness on the significance of minimizing mosquito contact for avoiding infection. In 1997, the WHO organized the Global Programme aiming at discarding LF as a public health issue by the year 2020, mainly via the administration of annual mass drug administration (MDA) programs for the people residing in endemic areas.

In Malaysia, Lymphatic filariasis elimination programme (LFEP) had started in 2001 targeting at achieving filariasis elimination by 2013. Nevertheless, after evaluation and revision of the WHO strategy in 2011, the target elimination was looked into and revised to 2018. Five round of Mass drug administration program were completed in all endemic areas in the period of 2004 and 2008, using more than 80% coverage of Diethylcarbamazine and albendazolewith. The MDA program in Malaysia was conducted with strict adherence to WHO guidelines. The success of a MDA programme is reliant on people's knowledge- those responsible for the acceptance or rejection of such a strategy and who are dependent on the programme delivery system. Knowledge also plays an

essential role in prevention of the disease and awareness is the most suitable way to avoid the disease and remain healthy as illness misunderstanding and health-seeking behaviour may improve or interfere with the effectiveness of control measures (Vanamail & Gunasekaran, 2011).

Incorporation of KAP surveys has been recommended by the WHO as a cornerstone for health promotion campaigns, where they help programmes to adjust health education message to improve public knowledge and attitudes (Mathieu *et al.*, 2004).

2.7 Endosymbiotic relationship between *Wolbachia* and filarial parasite

Technological advancements in electron microscopy in the 1970s led to the first report of the presence of *Wolbachial* endosymbionts in filarial nematodes by Mclaren *et al.*, (1975). Microscopic analyses of the filarial tissue section have revealed the bacteria to normally be contained in the host vacuole (Taylor *et al.*, 1999) and are found individually or in clusters of different sizes that could almost entirely fill their cellular environment (Taylor & Hoerauf, 1999).

The association and dependency of filarial nematodes on the endosymbiont bacteria make them unique among other nematode infections (Taylor *et al.*, 2005). In fact, molecular techniques based on 16S rDNA or ftsZ sequences and phylogenetic analysis have revealed all filarial *Wolbachia* are closely related species, but an entirely different group from the *Wolbachia* of arthropods (Taylor *et al.*, 1999). Based on ftsZ gene analysis, it was illustrated that filarial *Wolbachia* could be placed into two clusters (C and D) which diverge from the A and B clusters recognized for arthropod *Wolbachia* (Werren, 1997).

The genomic sequencing and annotation of the metabolic pathways of *Wolbachia* from *B. malayi* have revealed the bacteria to be dependently symbiotic in the filarial host

(Foster *et al.*, 2005). This being said, the mechanism of the symbiotic relationship remains elusive, and it has been suggested that the bacteria provide riboflavin, flavin adenine dinucleotide (FAD), heme and nucleotides to the host (Taylor & Hoerauf, 1999). On the other hand, the host nematode provides amino acids required for bacterial growth with the exception of meso-diaminopimelate, the only amino acid synthesized by the bacteria.

In keeping with what has been mentioned, the bacteria have been reported to be found in all developmental stages of the nematode, especially in adult worms, where it is found abundantly (Taylor *et al.*, 2005). In adult female filarial worms, the organisms are normally found in the hypodermis and reproductive tissues (McNulty *et al.*, 2013a). Thus, this suggests the organisms are probably transmitted vertically via the egg cytoplasm in a comparable manner to *Wolbachia* of athropods (Kozek, 1977). Since their discovery, it has been suggested that these endosymbionts might contribute to the pathogenesis of filarial infection, and thus could be targeted via an alternative chemotherapeutic approach (Taylor & Hoerauf, 1999). Extensive series of human trials have demonstrated success in managing the filarial infection by chemotherapy targeting *Wolbachia* that in turn cause the worm's loss of fertility and viability (Slatko *et al.*, 2010). Previously, a lipoprotein inhibitor (globomycin) was used as anti-*Wolbachia* agent in managing a *B. malayi* filarial infection by interfering with the signal peptidase II (LspA) pathway (Johnston *et al.*, 2010). Others have targeted the bacteria by inhibiting the *de novo* heme biosynthesis and therefore the parasite will lack the heme based on its absence in the genome sequence of the host nematode, thus losing fertility (Slatko *et al.*, 2010). Similarly, whole mount confocal microscopy was used by Landmann *et al.*, (2011) to demonstrate that killing *Wolbachia* resulted in extensive apoptosis in the adult worm and in the somatic cells of the embryos, and Mf.

Molecular techniques were used to explore the mutualistic relationship between *Wolbachia* and the filarial parasite. Foster *et al.*, (2005) have indicated that *Wolbachia*

provide riboflavin, flavin adenine dinucleotide, heme and nucleotides to the worm, and in return is provided with amino acids required for its growth. Hence, eliminating the *Wolbachial* endosymbiont is considered a promising alternative for controlling filarial infection (Townson *et al.*, 2000).

The molecular DNA analysis of *Wolbachia*-specific surface protein (*wsp*) gene revealed it to be a gene of about 590 bp (Gunawardena *et al.*, 2005; Zhang *et al.*, 2010). Others have shown that filarial infection induces the body to produce antibodies against *wsp* (Bazzocchi *et al.*, 2000), and thus, the presence of the *wsp* gene appears tantamount to filarial infection.

Previous studies have illustrated a similar reduced intensity of the *wsp* gene amplicon upon treatment with tetracycline (Gunawardena *et al.*, 2005). This observation is further accorded with reports that exposing *Brugia* spp. to tetracycline treatment for a period of less than two weeks is not sufficient to completely clear out the *wsp* DNA amplicon (Rao & Weil, 2002). The *Wolbachia* FtsZ gene was said to be a single copy gene, and so amplification is proportional to the quantification of the bacteria (Simoncini *et al.*, 2001). Normally, Quantitative PCR (qPCR) is applied to the amplification of the FtsZ gene. In this kind of analysis, it is expected that the gene concentration should be dependent on the *Wolbachia* present (Simoncini *et al.*, 2001). A similar reduction in *Wolbachia* population based on real-time PCR analysis upon treatment with tetracycline is seen in the literature (Casiraghi *et al.*, 2002). Additionally, Bazzocchi *et al.*, (2008) reported a significant decrease in *Wolbachia* upon treatment with ivermectin and deoxycycline.

CHAPTER 3: METHODOLOGY

3.1 STRATEGY APPROACH OF THE STUDY

A systematic approach was used in this study in order to identify and select the plants used to treat filaria in endemic communities, such as in the Kemaman district of Terengganu state, Malaysia. The study began with investigation of people's knowledge, attitude and practices (KAP) regarding filarial infection. However, no ethnobotanical information on plants that are locally employed to treat filarial in these communities was available. As a result of the absence of this information in the study area, the selected plants utilised in this study were based on literature reports. Two plants (*Melaleuca cajuputi* and *Ardisisa elliptica*) were selected and tested for their antioxidant, antibacterial and antifilarial activities against *Brugia pahangi*. The present study was conducted according to the flow chart shown in Figure 3.1.

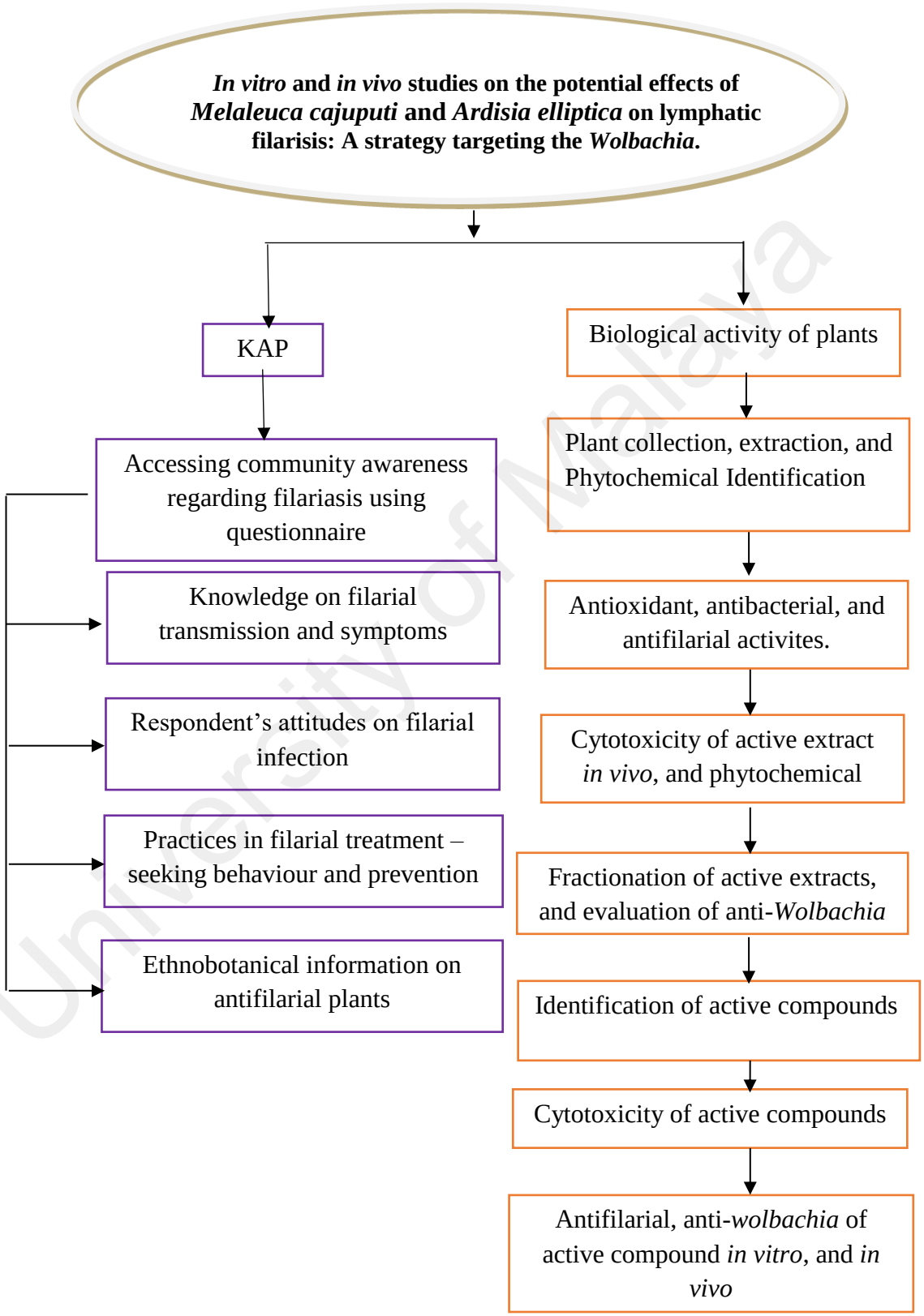

Figure 3.1: Flow chart of this study

3.2 Community awareness regarding the filarial and ethnobotanical study

3.2.1 Study area

The Kemaman district is located in a coastal area of Terengganu, which is one of the states in Malaysia (Figure 3.2). The area was selected based on the established occurrence of LF within the region. The Ministry of Health (MOH) stated that the Mf rate in the endemic areas of the country ranged from 1.41 to 2.14 per 1000 people, with 387 cases reported in 2011(MOH, 2012). The capital of the district is Chukai town, located on the coast at latitude $4^0$14'N and longitude $103^0$25'E. The district was inhabited by 167,824 people based on the 2010 population census of Malaysia, with Chukai town accommodating 82,425 people. There are also other settlements in the district namely Hulu Chukai, Kijal, Seri Bandi, and Ibok. The residents embark into fishing, subsistence farming, transportation, industrial labor, and public service.

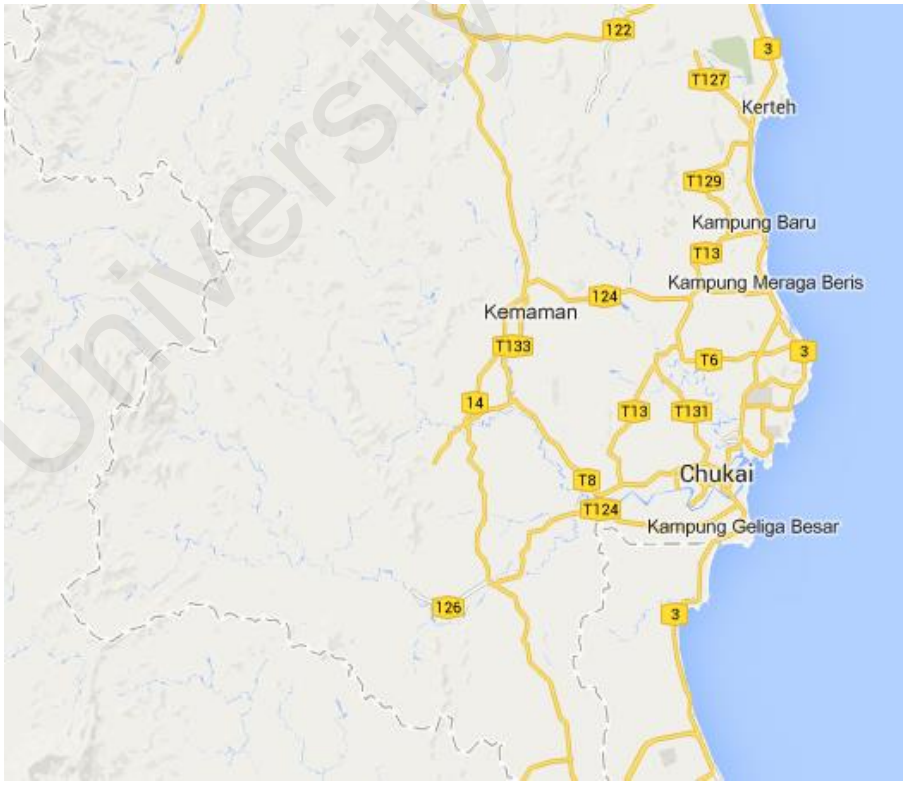

Figure 3.2: Map of the study area

3.2.2 Study population

The period of the survey was between August and October 2012. The study population consisted of all adolescents and adults who were also the attendants of the participating health clinics within the study area during the survey period and at the same time, those who agreed to participate in the survey. The clinics were: 1) the Sri Bandi health clinic; 2) the Ibok health clinic; and 3) the Kijal health clinic. These clinics are the main public health facilities in the area of study. In order to participate in the study, respondents had to have stayed in the area for at least 5 years and they also had to be 15 years or older. Additionally, pregnant women were dismissed from the study to avoid partial or non-compliance. 230 voluntary respondents aged ≥ 15years had fully participated in the survey. The survey was conducted with the support and cooperation obtained from local community leaders and medical personnel in the area.

3.3 Study Instruments

3.3.1 Questionnaire survey

The questionnaire comprised of four sections. Questions in these sections were created to find out sufficient amount of information about the participant's knowledge on filariasis (covering aspects like the prevention, treatment, symptoms & transmission). In addition, there were questions with the aim to ascertain the respondents' attitude towards the disease. Moreover, another section of the questionnaire presented a set of questions which had gauged the knowledge of the participant with regard to the practices, such as participation in MDAs, or the source of information about the MDA.

The questionnaire used in this survey was written in Bahasa Malaysia, or the first language (Appendix A) for the local population to better understand the questions, allowing ample information to be extracted from the answers during the interview. Some

of the questions were open ended, giving the respondents the opportunity to provide greater details while others are more restricted, specifically with 'yes' or 'no'.

3.3.2 Structured questionnaire

3.3.2.1 Study questionnaire

A semi-structured questionnaire was developed with the help from researchers in medical anthropology. During fieldwork, questionnaires were administered with the help of a medical doctor and a nurse who were indigenous to the area. The study participants were interviewed, using the local language, to determine the extent of each participant's knowledge of LF, including prevention, treatment, symptoms and transmission, as well as the attitude of the participants towards the disease. Additional questions, such as those about the MDA programme and participation in it, were also added. A number of questions were open-ended and allowed the respondents the chance to give greater details while others were restricted to a 'yes' or 'no' answer.

3.3.2.2 Validation of the questionnaire

The questionnaire was validated and pre-tested with 20 individuals to ensure reliability and validity before embarking on the actual field work. The validation of questionnaires was based on the following criteria:

i. Attempting to cover all possible information about KAP and MDA toward LF, the questionnaire was written in English by the principal investigator and translated to Bahasa Malaysia.

ii. To confirm the authenticity of the translation, the translated copy was given to a scientist doctor and was asked to translate it to English. The later was compared with the former for accuracy.

iii. Retesting of the questionnaire was also conducted among the same people after three weeks of the pre-testing. It was noticed that most of respondents were able to give the same or a very close answer to each of the pre-test questions.

iv. Statistical analysis was carried out using SPSS version 13 to test consistency (Cronbach's alpha) and validity was tested by a face validity test. However, the results of statistical tests for the consistency of the questionnaire is Cronbach's Alpha = 0.828. Overall, these showed that the questionnaire had consistency and high retesting reliability.

3.3.3 Distribution of questionnaire

During the field work, the questionnaires were administered to the respondents with the help of a medical doctor and a nurse who were indigenous to the area. Based on time constraints, house-to-house distribution of the questionnaires could not be completed. However, they were distributed in public health centres and clinics. The study was conducted within three months from August to October 2012. The semi-structured questionnaire approach used here was according to standard qualitative communication research methods (Lindlof & Taylor, 2010).

3.3.4 Data management and statistical analysis

3.3.4.1 Data Processing

Data was entred by double-entry by two different researchers into Microsoft Office Excel version 2009. Next, a third researcher cross-checked the two data sets for accuracy and created a single data set.

3.3.4.2 Statistical analysis

The computed data in an Excel file was imported into SPSS software version 13. The data was cleaned and checked thoroughly by another person to ensure correctness of the entries before embarking upon analysis. The demographic and socio-economic characteristics of the respondents were presented in percentages and frequencies.

3.4 Ethical Consideration

During the survey, the objectives of the research were discussed with the health personnel, the community leaders, as well as each respondent. All respondents were fully notified that participation was voluntary and that it was possible to withdraw from the research without notice. Those who wished to participate were required to sign a consent form (Appendix B) prepared in accordance with the guidelines of the Malaysian Department of Health and Human Services prior to the administration of the questionnaires. Moreover, permission for this research was granted by the Medical Ethics Committee of the University of Malaya Medical Centre, Malaysia. Ethics No. PAR/21/11/2011/ZMN (R).

3.5 Plants selections and extraction

3.5.1 Plants selection criteria

Malaysia is rich in its traditional knowledge in using of medicinal plants for combating diseases. Several plant extracts or plant-derived molecules in the past have been shown to battle several diseases. However, there are few studies on Malaysian plant that have been conducted to evaluate their antifilarial activity, so many still remain unexploited. Likewise, *Melaleuca cajuputi* and *Ardisia elliptica* are widely consumed by human population in Malaysia, however, reports of their anti-filarial properties are either unavailable or scanty. In the present work, the plants were selected based on their ethno-medicinal use. The study aimed at exploration of anti-filarial activities of these plants and detailing their activities against the lymphatic filarial parasite *Brugia pahangi* in a rodent model.

The criteria used for selecting plants for anti-filarial investigation were based on traditional medicinal information and scientific literature on the selected plants.

3.5.1.1 *Melaleuca cajuputi*

M.cajuputi, the Powell or Gelam tree, locally known in Malaysia as Kayu Putih (It has also been referred to as *M. leucadendron*), belongs to the family of Myrtaceae. In Malaysia, local people living around *M. cajuputi* forests use these plants to relieve a myriad of diseases. It is used as an ointment to relieve pain, rheumatism, stiff joints and even as mosquito repellent (Susanto *et al.*, 2003). In addition, *M. cajuputi* is used as an antiseptic to heal fresh wound, a disinfectant drug, hypoglycaemic agent and to treat a number of ailments, including intestinal parasites, asthma, viruses, and to treat several inflammatory conditions (Liu, 1995; Ul Hassan *et al.*, 2003), and other health problems such as dandruff. Different parts of the plant tend to have various uses in the food

industry, such as for flavouring meat and sauces. Several studies have been conducted to investigate their activities and the extracts that possess antiseptic, analgesic, vermifuge (Hussein *et al*., 2007), anti-inflammatory (Liu, 1995), anticancer (Wolter *et al.,* 2002), and hepatoprotective acitivities (Saravanan & Pugalendi, 2006). There have been several articles on the use of *M. cajuputi* extract to control the filarial vector of *Anopheles*, *Aedes* and *Culex* genera (Elango *et al.*, 2009; Elango *et al.*, 2010; Warikoo *et al.*, 2011).

3.5.1.2 *Ardisia elliptica*

A. elliptica Thunberg is an evergreen tree, locally known in Malaysia as the Mata pelandok (or Mata ayam) is from the family of Myrsinaceae and traditionally it was a medicinal plant used for alleviating chest pains, treatment of fever, diarrhoea, liver poisoning and parturition complications, as well as treating herpes, measles, scabies and intestinal worms. In Thai traditional medicine, the fruits are used to cure diarrhoea and fever (Koh, 2009). In Southeast Asia, the fruits are used to treat intestinal worms and the leaves to treat scabies (Giesen *et al.*, 2007).

A.elliptica has been studied scientifically for different biological activities, including antiplatelet aggregation (Ching *et al.,* 2010; Jalil *et al.,* 2004), antiviral (Kobayashi & de Mejia, 2005), antiproliferative (Moongkarndi *et al.,* 2004), antibacterial (Alias *et al*., 2014), antifungal (Kobayashi & de Mejía, 2005), anti-inflammatory (Gupta *et al*., 2011), anti-plasmodial (Noor Rain *et al.* (2007), anti-hyperalgesic and anti-pyretic agents (Ching *et al.*, 2010; Raga *et al.*, 2013).

As is best known presently, no research is available on anti-filarial activity of either on the filarial parasite or the endosymbiont bacterium, *Wolbachia*. Therefore, it is pertinent on the part of this work to fill in this gap of the knowledge.

3.5.2 Plants collection

M. cajuputi and *A. elliptica* were collected in September 2013 from Kedah state, Malaysia, and identified at the Faculty of Science, University Malaya. Voucher specimens were deposited at the Herbarium of Rimba Ilmu, Institute of Biological Sciences, University of Malaya, Kuala Lumpur with KLU 048231 and KLU048232 voucher number respectively (Appendix C).

3.5.3 Preparation of methanolic extract

The plant parts used were leaves and flowers of *M. cajuputi* (Gelam), fruits and leaves of *A. elliptica* as samples. Each collected sample was washed independently with distilled water, then ground to pieces and dried in the shade for seven days. The dried pieces were then grounded into a fine powder. For the methanolic extracts, weighted powder (100 g) of either the Gelam leaves (G.L), Gelam flower (G.F) powder, *A. elliptica* leaves (A.L) or *A. elliptica* fruit (A.F) was added to 1 L of absolute methanol in a conical flask and left to stand for 72 hours at room temperature. The extracts were filtered out of the mixture using Whatman filter paper (No.1). This procedure was repeated three times, then the mixture concentrated *in vacuo* at 40ºC using a rotary evaporator and finally dried under high vacuum pressure for 3 hours to remove the last traces of the solvent. Consequently, G.F, G.L, A.L, and A.F extracts were obtained, respectively. The freeze dried extracts were kept at 4ºC until use.

3.6 Phytochemical screening

In this study, methanolic extracts were subjected to preliminary screening of phytochemical constituents. The screenings were carried out following series of chemical qualitative analyses as described earlier (Trease, 1989; Sofowora, 1996). This qualitative screening of plants is required in order to predict the chemicals that may have caused

effective suppression against filarial activity. The chemicals most known for this are alkaloids, flavonoids, and terpenoids, possessing robust activity against a filarial parasite (Misra *et al.*, 2007; Lakshmi *et al.*, 2010) Anthraquinones and their chemical constituents have recently shown significant anti-filarial effects, and these include saponin, tannin, glycosides and steroids (Chatterjee *et al.*, 1992; Rajan *et al.*, 1994; Mengome *et al.*, 2010; Khanna *et al.*, 2011). The results that were obtained from the phytochemical screening tests painted a clear picture for the subsequent isolation and characterization of active constituents. All the extracts were evaluated for phytochemical constituents based on the following:

i. Test for alkaloids

A sample of plant extract (0.5) g was dissolved in 10 mL acid alcohol and then boiled for 5 minutes and filtered. After that, 2 mL of diluted ammonia followed by 5 mL of chloroform were added to the filtrate and mixed gently. Following this, 10 ml of acetic acid was added to the chloroform layer. Finally, Draggendorff's reagent (potassium iodide-bismuth nitrate) was added to the mixture. The presence of alkaloids was observed by the appearance of a reddish-brown precipitate.

ii. Test for terpenoids (Salkowski test)

Two mL of chloroform was added to 0.5 g of each plant extract. Then, 3 mL of concentrated sulfuric acid was carefully added to the mixture. The presence of terpenoids was indicated by the presence of a reddish-brown layer.

iii. Test for flavonoids

Plant extract (0.5 g) was boiled for three minutes in 10 mL of ethylacetate and filtered. Aliquots (1 mL) of 1 % ammonia solution was added to 4 mL of the filtrate. The mixture was allowed to stand for some time in order for phase separation to take place. Presence of flavonoids in the sample was confirmed by the appearance of a yellow coloration in the ammonia phase.

iv. Test for saponins (Froth test)

5 mL of distilled water was added to 0.5 g of each plant and boiled for five minutes. The hot mixture was filtered and then 4 mL of distilled water was added to 1 mL of the filtrate. After that, the solution was mixed for 30 min. The existence of saponins was identified by the persistence of a sable for more than 30 minutes.

v. Test for steroid

The plant extract (0.5 g) was mixed with 2 ml of chloroform and concentrated sulfuric acid was added carefully to the mixture. The presence of steroid was revealed by the formation of a reddish-brown layer at the interface.

vi. Test for glycosides

5 mL of dilute sulfuric acid was added to 0.5 g of each plant extract and boiled for 15 min. The mixture was neutralized with 20 % potassium hydroxide. An aliquot (10 mL) of the mixture was mixed with an identical quantity of Fehling's solution A and Fehling's solution B (Sigma-Aldrich, St Louis, MO, USA). The mixture was then heated for 5 minutes. The appearance of a red dense precipitate confirmed the presence of glycosides.

vii. Test for phenols

2 ml of each extract was added into water and warmed to 45-50 ^{0}C. Then, 2 ml of 3% $FeCl_3$ was added. Formation of a blue or green colour indicated the presence of phenols.

viii. Total flavonoid assay

Total flavonoid content of a methanolic extract was determined photometrically by the aluminium chloride assay (Ghasemzadeh *et al.,* 2011). Briefly, an aliquot (1 mL) of extract (1 mg/ml) or standard solution of quercetin (31.5, 62.5, 125, 250, 500 and 1 mg/L) was added to a volumetric flask (10 ml). The sample was then diluted with 4 mL of double distilled water at zero time, followed by the addition of 0.3 mL of 5% (W/v) $NaNO_2$ to the flask and after 5 min, 0.6 mL $AlCl_3$ (10%) was added. At the 6th min, 2 mL of NaOH (1 M) was added to the mixture. Finally, the total volume was made to 10 mL with double distilled water. The solution was mixed completely and the absorbance was measured against a prepared reagent blank in triplicate at 430 nm. Total flavonoid content was expressed as quercetin equivalents in mg/100 g of dry weight.

ix. Total phenol content

Total phenolic content of the methanolic extract was conducted based on the methods of the Folin-Ciocalteu reagents (Merck, Darmstadt, Germany) as described by Kim *et al.*, (2003) with slight modifications. Briefly, 100 µL of extract or standard solutions of gallic acid (16-1000 µg/mL in 80% methanol) was mixed with (200 µl) of Folin-Ciocalteu reagent (Sigma-Aldrich, St Louis, MO, USA), followed by adding 2 ml of deionized water and 1 ml of 15 % Na_2Co_3. Thereafter, the mixture was incubated for 120 min at room temperature; the absorbance level was measured at 765 nm in triplicate using a UV-Visible spectrophotometer (GBC, Cintra 40). Total phenolics were quantified by a calibration curve obtained from measuring the standard gallic acid concentrations. The

total phenolic content was expressed as mg gallic acid equivalent (GAE) per mg of extract weight.

3.7 Antioxidant activity

The methanol extracts of all the samples were evaluated for their antioxidant potential activities using a di(phenyl)-(2,4,6-trinitrophenyl) **(**DPPH) Radical Scavenging Assay, Ferric Reducing Power Assay (FRAP), and Metal Chelating Assay.

3.7.1 Determination of antioxidant activity with the di(phenyl)-(2,4,6-trinitrophenyl)iminoazanium (DPPH) radical scavenging method

The antioxidant activity of the extracted compounds was measured in terms of hydrogen donating or radical scavenging ability using the radical DPPH as reported previously (Mensor *et al.*, 2001). An aliquot sample (120 µl) of 0.25 mM DPPH solution in methanol and 30 µl of extract at different concentrations (31.3, 62.5, 125, 250, 500 and 1000 µg/ml) were mixed vigorously together, and the mixture was allowed to stay at room temperature in darkness. The absorbance was measured at 518 nm after 30 min against different concentration of extracts in methanol as blank or DPPH in methanol without extract as a control. The standard synthetic antioxidant, butylhydroxytoluene, was used as a positive control. The percentage of antiradical activity (AA %) of the extract was calculated according to the following formula reported by (Ghasemzadeh *et al.*, 2011).

$$AA\% = \left(100 - \left(\frac{Abs_{sample} - Abs_{blank}}{Abs_{control}}\right) \times 100\right) \quad \text{(Eq. 3.1)}$$

Where Abs is the Absorbance

3.7.2 Ferric-reducing antioxidant power (FRAP) Assay

This assay was employed to evaluate the ability of test extracts to reduce Fe^{3+} to Fe^{2+}.The reducing power of the compound was assessed based on the developed blue coloration from the formation ferrous ion from the reduction of ferric as described previously (Loizzo *et al.*, 2012). Extract solution was prepared by dissolving 0.1 µg/ml extract in ethanol. Aliquot samples (0.2 ml) of the extract solution were added to test tubes containing 1.8 ml of freshly prepared FRAP reagent that contained: 2.5 ml of 10 mM tripyridyl-s-triazine (TPTZ) solution in 40 mM HCl and 2.5 ml of 20 mM $FeCl_3.6H_2O$ in 25 ml of 0.3 M acetate buffer (pH 3.6). The mixture was incubated at 37°C for 5 min. Spectrophotometric absorbance was recorded at 593 nm. The reducing power was ascertained by comparing the spectrophotometric absorbance of each sample against a standard curve obtained from iron (II) sulphate (Fe_2SO_4).

3.7.3 Fe^{2+}-chelating activity assay

The chelating activity of extracts for ferrous ions (Fe^{2+}) was measured based on the method previously described by (Dinis *et al.*, 1994). 0.05 mL of $FeCl_2$ (2 mM), 1.6 mL of deionized water were added to different concentrations of extracts (31.3, 62.5, 125, 250, 300 and 500 µg/mL). After 30 s, 0.20 mL of ferrozine (5 mM) was added and the mixture was shaken vigorously for 15 s. Ferrozine reacted with divalent iron to form a stable magenta complex species that was very soluble in water. After 10 min at room temperature, the absorbance of the Fe^{2+}-Ferrozine complex was measured at 562 nm. The chelating activity of the extract for Fe^{2+} was calculated using the following equation:

$$\%\text{Chelating rate} = \frac{A0-A1}{A0} \times 100 \qquad \text{(Eq. 3.2)}$$

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

3.7.4 Statistical analysis

Data were expressed as mean ± SD of triplicate determinations. The half maximal inhibitory concentration (IC_{50}) values were estimated from the AA% versus concentration plot using a non-linear regression algorithm.

3.8 Antibacterial activity

3.8.1 Test organisms

The *in vitro* antibacterial activities of the extracts were evaluated against eight strains of bacteria that were provided by Department of Medical Microbiology, University of Malaya comprising four Gram positive bacteria: (*Staphylococcus epidermidis* (MTCC 3615), *Staphylococcus aureus* (RF 122), *Bacillus cereus* (ATCC11778), *Streptococcus pneumonia* (*ATCC10015),* and four Gram negative *Escherichia coli* (UT181), *Salmonella typhimurium* (*ATCC14028), Klebsiella pneumonia* (ATCC13883), *and Pasteurella multocida* (a clinically isolated strain). All strains were stored in the appropriate medium before use.

3.8.2 Inoculum preparation by direct colony suspension method

The colony suspension method was used to prepare the inoculum of the test organisms. The bacterial strains were grown on nutrient agar (NA) at 37°C for 18 h and adjusted to a turbidity of 0.5 relative to the Mac Farland standard (10^6 CFU/ml) based on optical density (O.D.) measurement at 620 nm. After 24 h of NA culture, colonies were picked and cultured in a nutrient broth (NB) medium for 24 hour at 37ºC. Susceptibility tests were performed by the nutrient agar-well diffusion method.

3.8.3 Screening for antibacterial activity

3.8.3.1 Disc Diffusion Assay

The disc diffusion method is a widely acclaimed method of screening for a crude extract's antibacterial activities, and in this study, antibacterial activity was measured based on a previously described modified method (Carbonnelle *et al.*, 1987). Briefly, crude extract at a concentration of 0.1 g/mL was dissolved in 100 % dimethyl sulphoxide (DMSO, Merck, Darmstadt, Germany) and sterilized by filtration with a 0.20 mm millipore disposable filter (Minisart, Sartorius Biotech, GmbH, Germany). Autoclaved sterilized (121ºC for 20 min) Mueller Hinton agar (MHA) medium (Merck, Darmstadt, Germany) was used for the disc diffusion assay. 50 µl of the filter sterilized plant extract was loaded onto a sterile paper disc (6 mm in diameter). The disc was placed onto the surface of the agar plate (nutrient agar) previously inoculated with bacteria. A disc prepared in the same condition with only 50 µl of DMSO was used as a negative control. Meanwhile, another similar disc but loaded with the reference antibiotic (streptomycin) as positive control at a concentration of 20 mg of drug per disc was prepared and used as described above. Both samples were allowed to diffuse for 1 h into the agar plates, where they were then inverted and incubated at 37^0C for 18 h. Antibacterial activity was determined by measuring the diameter of the growth inhibition zones (millimetres) surrounding each disc. Each assay was performed in triplicate with two repetitions, and the results were expressed as average values.

3.8.3.2 Determination of the minimal inhibitory concentration (MIC)

MIC values, which represent the lowest plant extract concentrations that completely inhibit the growth of microorganisms, were determined by a micro-well dilution method as described previously (Wiegand *et al.*, 2008). Briefly, extracts were dissolved at 100 mg/ml in 100% DMSO and then two field serial dilutions were

prepared in a 96-well dilution microplate. The antibiotic, streptomycin, was included as a reference for each assay. Extract-free solution was used as a blank control. Each well of the microplates included 40 µl of the growth medium, 10 µl of the inoculum (10^6 cfu/ml) and 50 µl of the diluted sample extracts. Then, the microplates were incubated overnight at 37°C. Afterwards, they were examined for bacterial growth by observing turbidity. The first tube showing no growth (lowest concentration that inhibited growth) was the MIC (Kariba & Houghton, 2001). The determination of MIC values was done in triplicate.

3.8.3.3 Minimum bactericidal concentration (MBC)

MBC values refer to the lowest concentration of an antibacterial agent that is required to prevent growth of a particular bacteria after subculture onto antibiotic free media MBC of the active extracts was determined by sub-culturing 0.1 ml (100µl) of all the tubes from the MIC assay that showed no growth onto nutrient agar. After 24 hr incubation at 37°C, the first plate showing no growth was the MBC (Ramage *et al.*, 2003).

3.9 Antifilarial activity study

3.9.1 Preperation of parasite

Brugia pahangi, a sub-periodic strain of filarial parasite, was used in the present study to evaluate the antifilarial activities of the extracts or compounds demonstrating promising antifilarial activity.

3.9.1.1 Host parasites system

The study was conducted in two areas, Bukit kasing and Pulau cary, to identify the hosts infected with the filarial parasite. Trapping of cats was performed from January to May 2013. In this study, cat was used as the definitive host of parasite and was maintained in Animal Room of the Department of Parasitology, Faculty of Medicine, University of Malaya, Kuala Lumpur. Briefly, a cat was approached slowly and grabbed by its neck. Once caught, the cat was held for a few minutes to calm it down.

Blood samples were collected from each cat after it was calm and comfortable, and 60 µl of blood was collected from each cat via its ear. All blood samples were collected using a graduated capillary pipette fitted with a simplified non-breakable Sinton pipette. This procedure was conducted according to the approved guidelines of the Ethics Committee of the Faculty of Medicine at University Malaya, Kuala Lumpur, Malaysia.

3.9.1.2 Staining of the blood films

Giemsa stain according to the Innenkorper technique was employed based on the literature (Sivanandam & Fredericks, 1966). Thick blood films were prepared on glass slides and allowed to dry at room temperature for 24-48 hours. The blood films were lysed in tap water for 1 min and air dried. The slides were then fixed with absolute methanol for about 30 sec to min. The dried blood films were then stained with 2% Giemsa (Merck, Darmstadt, Germany) in a phosphate buffer of pH 7.2 for 35 min and then rinsed with tap water.

3.9.1.3 Microscopic examination of blood films

Each blood film was examined under a light microscope by two individuals. The target Mf for this part of study was *B. pahangi*. Stained blood films were examined for mf using an Olympus CX40 microscope (Olympus, Japan). Mf were observed at 100x and 400x magnification. The pictures of Mf detected were captured using an onboard Olympus DP12 digital microscope camera (Olympus, Japan).

3.10 Experimental maintenance of *B. pahangi*

3.10.1 Rearing and breeding of mosquito vector (*Aedes togoi*)

The filarial parasite model used in this study was *B. pahangi*, and laboratory bred *Aedes togoi* mosquitoes were used as the vector. Eggs of *A. togoi* were maintained in the insectarium of the Department of Parasitology, Faculty of Medicine, University Malaya, Kuala Lumpur. The growth conditions were first optimized to produce healthy mosquitoes for the high yield of infective larvae. Briefly, filter paper with eggs of *A. togoi* was placed in a bowl containing 0.85 % NaCl solution and were incubated at 37ºC and humidity (75±5%) in the regulated insectarium in order to speed up the hatching process. The eggs hatched into first stage larvae (L_1) within 24-72 h. Yeast extract tablets and dog biscuits were mixed in 3:1 ratio and used as a feed for growing mosquito larvae. After 5-6 days, the hatched larvae entered into the pupae phase and transferred using a broad-tip pipette to small crystallizing glass dishes containing water to be kept inside the nylon netted mosquito cages. Then, after 2-3 days the pupae emerged into adult mosquitoes. Small Petri dishes containing cotton soaked in 3% glucose solution covered with a moist filter paper disc of the size of Petri dish were kept inside each mosquito cage for feeding and a small beaker containing water was kept for females to lay eggs. 7 to 10 day old mosquitoes were collected and were fed on *B. pahangi* infected cat (Mf density: 500-700 Mf /10 µl of tail blood) between 10.00 and 11.45 h, the time when the maximum number

of Mf appear in peripheral blood circulation. Mf were observed to develop into infective stage larvae (L_3) within 10–14 days post-feeding. The larvae were recovered using Baermann's technique by gently crushing the mosquitoes to isolate the L_3 larvae (Bandi *et al.*, 1999).

3.10.2 Experimental transmission of infection to jirds

The host animal model used here was the Mongolian jird/gerbil (*Meriones unguiculatus,* family - Gerbillinae), and was proven to be a superb choice for a permissive rodent model for the study of LF using *B. pahangi (*Appendix H). Eight week old male jirds (*Meriones unguiculatus*) weighing between 30 and 35 g were intra-peritoneally inoculated with 150 infective larvae L_3 of *B. pahangi* that were recovered from the *A. togoi* mosquitoes. All the animals were housed under standard conditions of temperature (23 ± 1 °C), relative humidity (55 ± 10 %) and 12/12 h light/dark cycles and fed with standard rodent chow pellets and water.

3.10.3 Parasite Recovery

For *in vitro* studies, the parasite (*B. pahangi* adult worms) were obtained at necropsy by washing the peritoneal cavity of infected jirds between 4 to 6 months of post infective period. These worms were free of any contaminants by washing 3-4 times in RPMI 1640 medium containing 20 mM HEPES, 100 µg/ml pencillin and 100 unit/ml streptomycin.

3.11 Filaricidal evaluation of the plants extracts

3.11.1 Sample preparation

For the stock suspension of the plant samples, the standard drug ivermectin (10 mg/ml) was prepared in DMSO (25 and 400 µg/ml concentrations) and used as a standard filarcidal for the *in vitro* screen. The *in vitro* activity of the crude extract was assessed by

serial two-fold dilutions of the stock starting from 1 mg/ml to the lowest concentration at 65. 25 µg/ml.

3.11.2 Assessment of cytotoxicity of the active test samples

The *in vitro* cytotoxicity assay was carried out using the fluorescent dye, Alamar blue (Sigma-Aldrich, St Louis, MO, USA) for testing the plant extracts for cytotoxicity following the methods that have been previously described by Misra *et al.*, 2011. Briefly, Vero cells (green monkey kidney cell line) obtained from the American Type Culture Collectionin (ATCC), were grown in tissue culture flasks, once cells had reached confluency were rinsed with 10% PBS 3 times, and then added to 3 ml of 0.1% trypsin EDTA and counted using a Neubauer Chamber. For a single 96-well plate, cells were plated onto each well except the third, sixth, ninth and twelfth columns (which had cell free medium to serve as negative control) and incubated at 37°C in a CO_2 incubator to allow cell adherence. Firstly, the medium was removed from all the wells and substituted by 100 µl of fresh medium. 150 µl of media with the test samples (2000 µg/ ml) were added to row H in triplicate i.e. sample 1 from columns 1 to 3, sample 2 from columns 4 to 6, sample 3 from columns 7 to 9 and sample 4 from columns 10 to 12. Serial dilutions (2:1) were prepared, with a multi-channel pipette by transferring 100 µl from row H to row G, mixing it and re-transferring 100 µl in the same way to each consecutive row up to row B. Row A was kept sample free as it functioned as a positive control and the plate was incubated for 48 h at 37°C in 5% CO_2 in air. After 72 h of the exposure of the sample, 10 µl/well of cell viability marker dye, Alamar Blue (stock solution 12.5 mg/100 ml PBS), was added to each well and the plate was re-incubated for 2-4 hours more The non-fluorescent dye, Resazurin, as it was exposed to living cells, gets reduced into a highly fluorescent product called resorufin (De Fries & Mitsuhashi, 1995) that is measured at an excitation wavelength of 536 nm and an emission of 588 nm in a fluorometric plate reader. Data was transferred to Excel and plotted according to the template using the

fluorescent signal against corresponding sample concentration. IC_{50} values were determined in a direct way.

3.11.3 Assessment of antifilarial activity of the extract

The *in vitro* evaluation of the antifilarial activity of GF, GL , AF, and AL extracts on *B. pahangi* adult worms was assessed by introducing 4 adults worms (2 female, 2 male worms) in triplicate of *B. pahangi* that were recovered from the peritoneal cavity into each well of a 6-well microtitre plate (TPP Techno Plastic Products, Switzerland), containing different concentrations (62.5-1000 µg/ml) of the extracts in completed media (CM) (comprised of 4 ml of RPMI medium supplemented with antibiotics, HEPES and 10 % heat inactivated FBS) and incubated at 37ºC, 5 % CO_2 and humidity over 90 % for 7 days. These experiments were conducted in triplicate.

The extract's lethal concentration assessment was evaluated based on the inhibition of the parasite's motility on a daily basis as follows: each worm was transferred to a new well containing the culture medium that is antibiotic-free. An hour after the transfer, the worms were microscopically observed for motility and death score. The scoring was recorded in comparison to the activity and motility of the control group, i.e. worms cultured in CM without added antibiotics or extracts. Immobility or death of the parasites was scored as 0, slightly active worms are allocated 1, moderately active and motile were recorded as 2, and those scored 3 were highly active and motile. The total number of Mf released into the culture medium by adult females on days 2, 4 and 7 were calculated. Results were expressed as percentages of production of Mf release relative to number of Mf released by control worms cultured without test drugs. Each experiment was carried out in triplicate.

3.11.4 Post-treatment analyses

3.11.4.1 MTT viability assay

The viability of the worms after 7 days exposure in the extracts or antibiotics media was assessed using the 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT; (Sigma-Aldrich, St Louis, MO, USA) reduction assay as described by Saini *et al.*, (2012) with slight alterations. In brief, adult worms were introduced into the well and incubated for 30 min in 0.5 ml of PBS (pH7.4) containing 0.5 mg/ml of MTT and the plates were then incubated in the dark for 2 h at 37°C. The solid formation of dark-blue formazan crystal was solubilized upon addition of 100-200 µl of DMSO into the well and allowed to stand for 1 h at room temperature with occasional gentle shaking to dissolve the developed colour. The absorbance of the resulting formazan solution was then determined to be 492 nm in a microplate spectrophotometer using DMSO as blank. Positive controls were set up with adult females not treated with extract but exposed to DMSO. Adult worms that had previously been heat-killed (heating at 56°C for 30 min) were separately incubated as negative controls for background absorbance. All experiments were executed in triplicate and the total loss of motility and percent inhibition in MTT reduction in treated parasites compared to untreated controls was estimated with the following equation:

$$\% \text{ inhibition (parameter)} = \frac{100 - (T - H)}{(C - H)} \times 100 \qquad \text{(Eq. 3.3)}$$

where *T*, *C* and *H* are absorbance values obtained for formazan produced in treated, control and heat killed samples, respectively.

3.11.5 Assessment of activity of extracts on filarial *Wolbachia*

3.11.5.1 *Wolbachia* DNA extraction and polymerase chain reaction (PCR)

12 adult worms in triplicate incubated for 7 days in different concentrations of extracts were recovered and the DNA extracted using the DNeasy Blood & Tissue kit (catalog number: 69504, Qiagen, GmbH, Hilden, Germany) DNA extraction was performed following manufacturer's guideline. Polymerase chain reaction (PCR) was conducted to amplify the extracted DNA according to the protocols described by Gunawardena *et al.,* (2005). A set of primers (forward 5'-3';GCAGCGGGTGAGTAATGTATA and reverse 5'-3'; CCACTGGTG-TTCCTCCTAATA) were used. The reaction conditions were operated in 40 cycles of 95°C for 30 s, then 50°C for 30 s and 72°C for 1 min. 6 fresh females without incubation were also tested as a positive control. The negative control was with water for DNA. Amplification products from 6 worms were combined according to the treatment regimen, and were electrophoresed on 1.5% agarose gel and stained with SYBR Safe DNA gel stain (Invitrogen Corp., Carlsbad, CA, USA). The experiments were conducted in triplicate.

3.11.6 Transmission electron microscopy

The structural morphology characteristics of both treated and non-treated *B. pahangi* female worms (after 7 days of culture) were observed by modifying the reported protocols (Ghedin *et al.,* 2009). Briefly, the female worms were fixed in 3% glutaraldehyde with 0.1 M cacodylate buffer (pH 7.4) for 2 hrs at 25±1°C and post-fixed in 1 % osmium tetroxide in cacodylate buffer. Then, dehydration via increasing concentrations of ethanol (30-95%) and embedding in low-viscosity embedding resin (Eponate 812, Ted Pella, Inc., Redding, CA) took place with ultra-thin sections (65-70 nm) stained with uranyl acetate and Reynold's lead citrate and viewed on a JEOL 1200EX transmission electron microscope (JEOL USA, Peabody, MA).

3.11.7 Statistical analysis

Data were expressed as mean ± SD of triplicate determinations. One way analysis-of-variance (ANOVA) was used to identify differences among treatment groups compared with controls. *P* values < 0.05 were considered to be significant. The Prism software programme (GraphPad Software, San Diego, CA) was used to analyze results.

3.12 Assessment of cytotoxicity *in vivo* of the active test samples

The plant extracts which exhibited antifilarial activity *in vitro* against *B. pahangi* were tested *in vivo* for their cytotoxicity profile.

3.12.1 Animal maintenance

The study was conducted in the Parasitology Department, University Malaya, Malaysia. The use of the laboratory animals and the procedures in this work was approved by Ethics Committee for Animal Experimentation, Faculty of Medicine, University of Malaya, Malaysia Ethics No. PAR/21/11/2011/ZMN (R). Adult jirds weighing 20-30 g were housed in large spacious polypropylene cages (4 gerbils per cage), maintained in a controlled environment (12 h dark/light cycle, 25 ± 2°C temperature with 50-70% humidity). The jirds were made familiar to the laboratory conditions for 7 days before the experiments were carried out. All animals were given commercial food pellets and water *ad libitum*. The jirds had been made to fast for approximately 12 hours before dosed, but allowed to freely drink at any time. All animals received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences (Barthold *et al.*, 2011).

3.12.2 Acute toxicity

Acute toxicity was evaluated by giving a single dose of extract for 14 days following the Organization for Economic and Cooperation Development (OECD) guidelines for Testing of Chemicals 420 (OCED, 2002). Healthy adult jirds were equally divided into 3 groups of 8 jirds each (4 male and 4 female), each labelled as vehicle (0.01 % DMSO), 2 g/kg and 4 g/kg of plant extract preparation, respectively. The animals were observed continuously for first 2 h for any gross change in behaviour or any other symptoms of toxicity and mortality, if any, and the next 24 h for any lethality or death.

3.12.3 Subacute toxicity

The subacute toxicity study was carried out by providing daily repeat doses following the OECD guidelines (OCED, 2002). The jirds were divided into 4 groups of 8 gerbils each (4 male and 4 female). Group I was given 5 ml/kg vehicle (5% DMSO) and served as negative control. Groups II, III and IV were the test groups and received various concentrations of active extract at 100, 500 and 1000 mg/kg of body weight. Each dosage was administrated daily for 28 days and at each instance, food was withdrawn 3 to 4 hours after dosing. The animals were observed for 30 min 2, 4, 8, 24 and 48 h after the administration of extracts for the onset of clinical or toxicological symptoms. Toxic manifestations and mortality, if any, was observed daily for 4 weeks and body weight changes were recorded every week. Mortality, body weight, as well as observations for signs of general toxicity in the animals were evaluated daily for 28 days.

3.12.4 Blood biomarker assay

On the 15^{th} and 29^{th} days for the acute and subacute, respectively, after overnight fasting, the jirds were anesthetized with ketamine/xylazine 0.55 ml/100 g body weight (0.5 ml of 100 mg/ml ketamine combine with 0.05 ml of 20 mg/ml xylazine) and blood

samples were collected from each jird by heart puncher for further haematological and biochemical evaluation. Haemoglobin, red blood cell count, white blood cell count and platelet count were determined using an automatic analyser Cell-Dyne 4000 cell counter (Abbott Laboratories). The serum biochemical analysis was used to evaluate the liver function test by observing the activities of liver enzymes, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), serum levels of total bilirubin (TBIL) and total protein. Renal function tests were also evaluated by measuring the level of creatinine and urea. Also, histopathological evaluation was conducted on both the liver and kidney tissues obtained from the samples.

3.12.5 Histology assay (preparation of tissues and slides procedures)

(i) Fixation

The anesthitised jirds were dissected to remove its kidney and liver. The kidney and the liver were isolated from jirds and was cut into small pieces, estimated at 2 to 3 mm thickness and rinsed with normal saline. The tissues were then fixed individually into 10% formaldelhyde (formalin) for 24 hours. A solution of formaldelhyde (formalin) was used to react with proteins and other organic molecules to stabilize cell structures. This solution was buffered and osmotically balanced to minimize shrinkage or swelling. All the fixation procedures were done quickly to minimize post-mortem changes in cell structure. After 24 hours of fixation, the tissues were submerged into 70 % alcohol.

(ii) Dehydration and clearing

Based on a large fraction of the tissue being composed of water, a graded series of alcohol baths, beginning with alcohol 70% and progressing in graduated steps to 95% alcohol, was used to remove the water. The tissues were then immersed in 70% alcohol for 30 minutes. Thereafter, the tissues were transferred into 85% alcohol for another 30

minutes. Next, they were submerged in alcohol 95% for another 30 minutes, and this step was repeated twice. The tissues were then treated with terpineol for 30 minutes, this step also being repeated twice. After 30 minutes, the tissues were transferred into a mixture of terpineol: paraffin (1:1) for 30 minutes at 60ºC.

(iii) Infiltration and embedding

After 30 minutes in terpineol: paraffin (1:1), tissues were immersed in paraffin for 60 minutes. This step was repeated for another 60 minutes until the tissues were completely infiltrated. Once the tissues were impregnated with paraffin, they were placed into a small receptacle, covered with melted paraffin and allowed to harden, forming a paraffin block containing the tissue.

(iv) Sectioning and mounting

After blocks of tissue were trimmed of excess embedding material, they were mounted for sectioning. The task was performed by a microtome (Leica, Germany) and the thickness of each section was 8 μm. The slides were labelled and a drop of Mayers' albumin was placed on the centre of the slide and spread as thin as possible. A drop of distilled water was also placed on the centre of the slide. A layer of sectioned tissue was placed on the slide and the slide was heated on a hot plate (40 – 45 ºC) to ensure the tissue was fixed to the slide for as long as it took for the distilled water to be dried up.

(v) Staining (hematoxylin & eosin method)

Staining for light microscopy was performed mostly with water-soluble stains. Therefore, the slides were stained following scheme of the flow chart (Appendix D). After staining, the section was labelled and permanently affixed with a coverslip by a mounting medium known as Canada Balsam.

The slides were next analysed under a light microscope and the magnified images of the tissue structures were captured for histopathological evaluation.

3.12.6 Statistical analysis

Statistical evaluation of the acquired Data was performed using Statistical Package for Social Sciences (SPSS) version 13. Data are given as the mean ± SEM; the statistical significance of differences between groups were performed with one-way Analysis-of-Variance (ANOVA) followed by Tukey's post-hoc analysis. *P* values less than 0.05 ($p < 0.05$) were considered to be statistically significant between the groups.

3.13 High-performance liquid chromatography (HPLC) profile of *M. cajupati* flowers and collection of fractions

In bioassay-guided isolation, the HPLC profiling was initially carried out on the *M. cajupati* flower methanolic extract determined to be active. An analytical scale was setup on the high-performance liquid chromatograph (Agilent 1100 Series (Agilent, Waldbronn, Germany) HPLC system) and then the extract was further fractionated by an optimized method followed by screening for bioactive fractions. The process of fractionation was repeated several times using the optimized method until sufficient amounts of fractions were obtained. The fractions were collected using a fraction collector, pooled and screened for anti-*Wolbachia* activity.

3.13.1 Optimization of HPLC method for chemical profiling

The HPLC profiling of *M. cajupati* flower crude extract was carried out using an Agilent 1100 Series (Agilent, Waldbronn, Germany) HPLC system. 15 µl of *M. cajupati* flower crude extract (10 mg/ml) was injected into the HPLC machine. The compounds were detected using a DAD detector and monitored at 214 nm. The column utilised was a reversed-phase C_{18} column, Zorbax ODS C_{18} (4.6 X 250 mm, 5.0 µm, and 70A). The

mobile phases were acetonitrile, HPLC grade (solvent A) and deionized water (solvent B) at constant solvent flow rate of 1 mL/min. The following gradient was used: 100% water (B) was flowed through the column isocratically with 0% solvent (A) for 15 min which was then increased to 48% water (B) for 20 min, to 0% water (B) for 20 min and then followed by isocratic elution with 100% methanol (A) for 8 min.

3.13.2 Fractionation of *M. cajupati* flower crude extract

M. cajupati flower crude extract was resolved by maximum peaks using methanol: water as mobile phase at 254 nm. Therefore, this mobile phase was selected for the fractionation of *M. cajupati* flower crude extract. Crude extract was eluted by using acetonitrile: water as the mobile phase. The gradient used for HPLC fractionation was slightly modified from the optimized method in Section 3.13.1 to reduce the elution retention time. The following gradient used was modified: 80% water (B) was flowed through the column isocratically with 0% solvent (A) for 15 min, which was then increased to 48% water (B) for 20 min, to 0% water (B) for 20 min and then followed by isocratic elution with 100% acetonitrile (A) for 8 min.

3.14 *In vitro* Anti-*Wolbachia* activity

3.14.1 Cell culture conditions

Aedes albopictus (Aa23) (Appendix H), cell lines were derived from a culture which was a generous gift by Prof. O'Neill and adapted and subsequently maintained according to cultivation methods previously described by O'Neill *et al.* (1997). Briefly, the Aa23 cell line was grown in 25cm^2 cell culture flasks at 26°C and originally maintained in 5 ml of growth medium which contained equal volumes of Mitsuhashi-Maramorosh (Appendix E) and Schneider's insect media (Invitrogen Corp., Carlsbad, CA, USA) and supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen Corp., Carlsbad, CA, USA). The cell line was passed every 3-4 days in the prevention of overgrowth and

at the time of passage, cells were detached by shaking the flasks gently and 0.5 or 1.5ml of the resulting cell suspension was added to fresh medium for a total volume of 5 ml and transferred to a new flask.

A stock solution of Tetracycline (1 mg/ml) was prepared in water, filter-sterilized, and diluted into the culture medium to a final concentration of 10 μg/ml and used as a control drug to eliminate the *Wolbachia* infection in Aa23.

3.14.2 Cytotoxicity of fractions in cell line and MTT assays

MTT ([3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide was utilised for the measurement of cell survival/proliferation. In this experiment, the cytotoxicity of the fractions and compounds was evaluated against the Aa23 cell line using the MTT reduction assay as previously described (Ferrari *et al.*, 1990). In brief, Aa23 cells were cultured as mentioned above. Cultures were incubated at 26°C in a humidified atmosphere. The cells were harvested upon reaching confluence. The harvested cells were then fruited into 24-well culture plates containing 2 ml culture medium (with 10% fetal bovine serum) per well, and the cells were allowed to adhere overnight. Next, the fractions were dissolved in DMSO with a final concentration of DMSO less than 0.01 % and diluted to different concentrations ranging from 3-100 μg/ml. Afterwards, the fractions were added to the cells and incubated for 48 hr (3 wells on a plate for each concentration). Blank 5% DMSO was used as a control. Thereafter, 100 μl of MTT (5 mg/ml) was added to each well and the plates were incubated at 26°C for 4 h. Following that, the cells were directly resuspended in the culture medium using a Pasteur pipette and transferred to a 2 ml microcentrifuge tube. A purple pellet was collected by centrifugation at 10,000 rpm for 4 min. The supernatant was aspirated and discarded; pellets were mixed with 1 ml of acid isopropanol (0.04 N HCl in isopropanol), and the MTT formazan reaction product was leached from the pellet by incubation at 50°C for 15 to 30 min, with occasional

vortexing. When the pellet lost colour, tubes were centrifuged at 10,000 rpm for 4 min to obtain the coloured supernatant that was read in a spectrophotometer at 570 nm using a GF-M3000 microplate reader against a blank of acid isopropanol. This experiment was performed in triplicate. The percentage of cell viability was calculated according to the equation:

$$\text{Cell viability \%} = \frac{Abs\ 570\ treated}{Abs570\ untreated} \times 100 \qquad \text{(Eq. 3.4)}$$

3.14.3 Fraction susceptibility of *Wolbachia in vitro*

3.14.3.1 Preparation of test samples

Stock solutions of fractions were dissolved in DMSO and diluted with culture media to give the required final concentration 5 ml/kg vehicle (5% DMSO) and filtered before each use. The samples were prepared fresh each day prior to administration.

3.14.3.2 Assessment the effect of fractions on *Wolbachia*

The susceptibility of *Wolbachia* toward the fractions were investigated as previously described (Brennan *et al*., 2008). Briefly, the cells were plated at 1×10^5 cells/well of Aa23 cells and were cultured in 25 cm^2 plastic tissue culture flasks containing 5 ml of equal volumes of Mitsuhashi-Maramorosh (Appendix E) and Schneider's insect media (Invitrogen Corp., Carlsbad, CA, USA), supplemented with 20% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen Corp., Carlsbad, CA, USA).The drugs were administrated based on 10 µg/mL of tetracycline and 50 µg/mL of fractions and vehicle (DMSO) controls in duplicate for 7 days. Duplicate cultures were maintained for each concentration of extracts. Culture without treatment was used as a *Wolbachia* positive control. The medium was replaced every third day. Cells undergoing treatment were harvested by removing the cells to a 15 ml conical tube and centrifuged at 14,000 rpm for 10 minutes. The supernatant was removed by aspiration and the pellet was resuspended

in 1 ml of fresh medium to use for extraction of DNA. Depletion of *Wolbachia* was monitored by PCR.

3.14.3.3 DNA Extraction

The extraction of genomic DNA was performed using QIAamp kit (Qiagen, GmbH, Hilden, Germany, category number: 69504) according to the manufacturer's instructions. DNA concentration and quality were determined using the Nanodrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). Nucleic acid purity is determined by the ratio of absorbance at 260 nm compared to 280 nm (~ 1.8) and 260nm compared to 230 nm (1.8-2.2). Samples with ratios significantly outside of these ranges (± 0.4) were not used for analysis.

3.14.3.4 PCR diagnosis for *Wolbachia* detection

In order to ascertain the presence of *Wolbachia* in Aa23, the extracted DNA was amplified via PCR using *Wolbachia wsp* gene-specific primers of 590 bp (O'Neill *et al.*, 1992; Zhou *et al.*, 1998) to confirm the presence of *Wolbachia*. The primer's forward sequence was 5'-TGGTCCAATAAGTGATGAAGAAAC-3' and the reverse sequence was 5'-AAAAATTAAACGCTACTCCA-3'. Universal Primers for 28S nuclear ribosomal DNA (28sF3633/28sR4076) (Baldo *et al*., 2006) produced a band at 400 bp and was used as a control for template quality (i.e. to confirm that high quality DNA was present in samples).

Each reaction was performed in a total volume of 20 µl that contained 200 µM dNTPs (PE Applied Biosystems, Norwalk, CT, USA), 300 nM *wsp* primers, 0.5 unit AmpliTaq DNA polymerase (PE Applied Biosystems, Norwalk, CT, USA), 2 µl PCR buffer (PE Applied Biosystems, Norwalk, CT, USA), 1.5 mM $MgCl_2$ and 1 µl of DNA template. The thermal cycling profile used in the GeneAmp PCR system 9700 (PE Applied

Biosystems, Norwalk, CT, USA) worked as follows: 94ºC for 10 min, followed by 35 cycles of 94º C for 1 min, 55º C for 1 min and 72º C for 1 min with a final extension at 72º C for 7 min. 10 uL of amplified product was electrophoresed through 2 % agarose gel and stained with SYBR Safe DNA gel stain to visualize the amplified DNA fragments under a gel documentation system. Negative controls containing DNA-free water and positive controls containing untreated Aa23 were included with each set to scrutinize PCR conditions and contamination.

3.15 Identification of the compounds in the active fraction

The active methanolic fraction was further subjected to purification by chromatography to obtain the major pure molecules which were characterized by spectral analysis using liquid chromatography-mass spectrometry (LCMS), and Nuclear magnetic resonance (NMR). In brief, the active fraction (80.0 g) was subjected to repeated column chromatography over silica gel (60-120 mesh) and eluted with gradient solvents using the method described earlier (Yadav *et al.*, 2013). The F2 fraction yielded one known pure compound identified by comparing their spectroscopic data with that previously reported in the literature.

3.15.1 LCMS

An Agilent 1290 infinity UHPLC coupled with an Agilent 6410 Triple Quad LCMS was used to separate compounds from the extract. The mass detector was equipped with an electrospray ionization (ESI) interface and controlled by Mass Hunter software. 2 µl of all samples prepared were loaded on a 2.1 mm (*i.d*) Narrow-BoreSB- C_{18} (length150 mm) analytical column (particle size 3.5 mM) with a flow rate of 0.5 mL/min in solution A (0.1% formic acid in water) and solution B (100% acetonitrile in water with 0.1% formic acid). The gradient was run as follows: 3% B for 0.5 min, 3–100% B for 5.5 min, and 100% for 4 minutes. The total gradient time for the LCMS run is 11 min. The

ionization conditions were adjusted at 350°C and 4000 V for capillary temperature and voltage, respectively. The nebulizer pressure was 45 psi and the nitrogen flow rate was 11 L/min.

3.15.2 Nuclear magnetic resonance (NMR)

Nuclear magnetic resonance (NMR) spectra were recorded for ^{1}H NMR at 400 MHz on a JEOL LA 400 MHz spectrometer. For ^{1}H NMR, 8 mg of sample was dissolved in $CDCl_3$ and was sonicated at 60°C to fully dissolve. Tetra-methylsilane (TMS) served as an internal standard (δ=0) and data were reported as follows: chemical shift, integration, multiplicity (s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet) and coupling constant in Hz.

3.15.3 HPLC for comparison between identified compound with authentic standard

The compound present in GF2 was identified by comparing the chromatographic retention time of the GF2 fraction solution with authentic standard caffeic acid phenethyl ester (CAPE) under identical analytical conditions used in the HPLC method in Section 3.13.1. The column utilised was a reversed-phase C18 column, Zorbax ODS C-18 (4.6 X 250 mm, 5.0 µm,70 A). The mobile phases were methanol, acetonitrile HPLC grade (solvent A) and deionized water (solvent B) at a constant solvent flow rate of 1 mL/min. The following gradient was used: 100% water (B) was flowed through the column isocratically with 0% solvent (A) for 15 min which was then increased to 48% water (B) for 20 min, to 0% deionized water (B) for 20 min and then followed by isocratic elution with 100% acetonitrile (A) for 8 min. The retention time and response of the peak in the chromatogram of the standard CAPE solution with chromatogram of GF2 was then compared.

Then, *M. cajupati* flower crude extract was fractionated using the same method describe in Section 3.13.2 to produce more CAPE from fraction 2 (GF2). Higher volumes

(100 ml) and higher concentration of crude (100 mg/ ml) were injected and the fractionation was repeated 100 times. The following gradient used was modified: 80% water (B) was flowed through the column isocratically with 0% solvent (A) for 15 min which was then increased to 48% water (B) for 20 min, to 0% water (B) for 20 min and then followed by isocratic elution with 100% acetonitrile (A) for 8 min.

3.16 Assessment of the effect of caffeic acid phenyl ester (CAPE) on *Wolbachia*

3.16.1 Cytotoxicity of caffeic acid phenyl ester (CAPE) *in vitro*

Different concentration of CAPE (5-100 µg/ml) were administrated into the Aa23 cell line to assess the cytotoxicity of compounds as previously described in Section 3.14.2.

After evaluating the cytotoxicity of CAPE, the compounds were tested on the cells to evaluate their effects on *Wolbachia*. Different concentrations (2-30 µg/ml) were administrated to the cells for 9 days, as previously mentioned in Section 3.14.3.2 Depletion of *Wolbachia* was monitored by PCR, live-dead cells and the FISH technique.

3.16.2 DNA Extraction and PCR analyses

As described in Sections 3.14.3.3 and 3.14.3.4.

3.16.3 *Wolbachia* purification

Aa23 cells were recovered from the culture flask by making vigorous shakes of the dislodge cells. The recovered cells were then pelleted by centrifugation at 2,500 x *g* and 4°C for 10 min and the supernatant was later removed. Afterwards, the cells were resuspended in 10 ml media in a 50 ml conical tube. Cells were vortexed for 5 min with approximately 100 sterile 3 mm borosilicate glass beads to lyse cells. The lysate was centrifuged at 2,500 x *g* at 4°C for 10 min to pellet large cellular debris. The supernatant was decanted and then it was passed through a 5µm Millex syringe filter (Millipore,

Billerica, MA, USA) and centrifuged at 18,400 x *g* at 4°C for 5 min on a 250 mM sucrose cushion to pellet *Wolbachia*. The *Wolbachia* pellet was resuspended in 1 ml Schneider's media with 10% FBS and passed through a 2.7µm syringe filter (Whatman, Florham Park, NJ, USA) to eliminate residual cellular debris.

3.16.4 *Wolbachia* viability assay

The viability of purified *Wolbachia* suspended in Schneider's media with 10% FBS was assessed at the end of treatment by BacLight live-dead (Invitrogen, Molecular Probes, Carlsbad, CA, USA) staining and FISH and DAPI stain. All assays were performed in triplicate.

3.16.4.1 BacLight live-dead assay

BacLight live-dead staining first came into use to examine the survival of purified *Wolbachia* (Rasgon *et al.*, 2006). The BacLight assay serves as a differential stain that uses the cell membrane integrity to dwell into bacterial viability in which the SYTO 9 (green) stain penetrates all cells while the propidium iodide (red) stain only penetrates cells with destroyed cell membranes. Consequently, live cells (intact) appear green while dead cells (non-intact) look red.

Fifty microliters of purified *Wolbachia* suspension was stained following the manufacturer's suggested protocol and viewed on an Olympus BX-41 compound microscope fitted with epifluorescent optics. The test was done, with adherence with the manufacturer's instructions. In brief, equal volumes of Component A and Component B of BacLight live-dead staining were brought together in a microfuge tube and mixed thoroughly. Next, 3 µL of the dye mixture for each mL of the bacterial suspension was added. The mixture was mixed in an extensive manner and incubated at room temperature in the dark for 15 minutes. 5 µL of the stained bacterial suspension was trapped between

a slide and an 18 mm square coverslip. *Wolbachia* viability was estimated by dividing the number of green *Wolbachia* bacteria which could be seen in the field by the total number visible (green plus red). As control, the assay was repeated using *Wolbachia* bacteria that had been killed by performing the heating at 95°C for 10 min. As confirmation that what was observed by BacLight was really *Wolbachia*, purified bacteria were placed on an 8-well chamber slide (Nalge Nunc International, Rochester, NY, USA), where they were allowed to air dry, fixed for 15 min with 4% formalin and viewed using *Wolbachia*-specific fluorescence *in situ* hybridization (FISH) as shall be described next.

3.16.4.2 FISH and DAPI staining

Cell monolayers were grown on sterile cover slips (Fisherfinest™ Premium Cover Glass, Fisher Scientific, Pittsburgh, PA, USA) and they were coated with concanavalin A (Vector Laboratories, Burlingame, CA, USA). Cells (0.5ml) were fruited onto the coated cover slips in sterile 6-well plates (Greiner Bio-One, Monroe, NC, USA) and fresh medium (1.5ml) was added into the wells. Cells were incubated at 28°C for 18h without having to change the medium.

FISH staining is similar to that mentioned in the literature (Xi *et al.*, 2005). Briefly, the medium was taken out and cells on cover slips were washed with PBS and fixed with cold 4%formaldehyde in PBS at room temperature (RT) for 15 min. The cover slips were then rinsed with PBS plus 0.1% Tween 20 and pre-hybridized for 1h at RT with a buffer composed of: 50% deionized formamide, 20% 20×SSC (3 M sodium chloride, 0.3M sodium citrate, pH7.0), 1% 50×Denhardts (Invitrogen, Carlsbad, CA, USA), 10% 1M dithiothreitol (DTT; BioChemika, Buchs, Switzerland), 0.25 mg/ml t-RNA (Sigma-Aldrich, St Louis, MO, USA), and 0.25 mg/ml poly A (Sigma-Aldrich, St Louis, MO, USA). Cells were hybridized in a moist chamber made up of an air tight plastic container with wet paper towels for 18h at 37°C with the pre-hybridization buffer plus 0.25 mg/ml

ssDNA (Sigma-Aldrich, St Louis, MO, USA), 0.2 g/ml dextran sulfate (Invitrogen, Carlsbad, CA, USA) and 10 ng/ml of fluorescent-labeled oligonucleotide *Wolbachia* 16S rDNA probes, 5′-FACC AGA TAG ACG CCT TCG CC-3′ (Xi *et al.*, 2005) and 5′-FCTT CTG TGA GTA CCG TCATTA-3 (Heddi *et al.*, 1999). Post-hybridization, cells were washed twice with 1× SSCD (SSC augmented with 10mM DTT) at 42°C, twice with 0.5×SSCD at 42°C, once with 0.5×SSCD at 25°C and again with deionized water at 25°C. Following FISH staining, cells were stained with 0.03% DAPI (Invitrogen, Carlsbad, CA, USA)) for 5 min at RT, and then they were rinsed with water and air dried. The cover slips were mounted onto glass slides with VECTASHIELD® Mounting Medium (Vector Laboratories, Inc., Burlingame, CA).

3.17 *In vitro* screening of the caffeic acid phenethyl ester (CAPE) for antifilarial activity

Adult worms of *B. pahangi* (2 male and 2 female) (with three replicates simultaneously) were incubated in 5 mL of complete media , CM (RPMI-1640 supplemented with 25 mM HEPES buffer, 2 mM glutamine, 100 U/mL streptomycin, 100 µg/mL penicillin, 0.25 µg/mL of amphotericin B and 10% fetal bovine serum), alone and in combination with CAPE at 2, 5, 10, 15 and 20 µg/mL in a 24-well flat-bottomed culture plate (Greiner Bio-One,GmbH,Germany). Cultures were maintained for 7 days at 37°C in a humidified atmosphere of 5% CO2 (Rao & Weil, 2002). The cultures were carried out in triplicate and repeated 3 times. Ivermectin (25 µg/mL) was used as a standard filaricide for *in vitro* screening purposes. The activity of CAPE against *B. pahangi* were assessed by the motility assay and MTT reduction assay, as mentioned earlier.

3.18 Caffeic acid phenethyl ester (CAPE) influence on *B. pahangi* and *Wolbachia in vivo*

3.18.1 Assessment of cytotoxicity of CAPE *in vivo*

Acute and subacute cytotoxicity study of administration CAPE *in vivo* was evaluated as mentioned in Section 3.12 with a low dose concentration group (0.5 g/kg), and for acute toxicity (1 g/kg) while the subacute toxicity groups were given 50, 100 and 200 mg/kg.

3.18.2 Treatment schedules

Analysis was done by daily intraperitoneal injections of 0.01% DMSO (vehicle control), 50 mg/kg/day of doxycycline (Merck, Darmstadt, Germany) and 50 and 20 mg/kg/day of CAPE for 14 days. The injection was given to 120 day old post-infection *B. pahangi* infected male jirds that infected with introduce 150 L3 subcutaneously. The efficacy of the drug on the filarial parasite was evaluated according to Misra *et al.* (2011). In brief, a total of 10 µl of blood was taken from the tail vein of each animal (treated and control animals), and spread as thick smears which then was air dried. The smear were dehaemoglobinized with water and later stained with Geimsa stain. Blood was collected from each animal prior to the starting of treatment at day 0 and on days 8, 15 and 30 as well as regular intervals of 15 days until day 90 post-treatment. Microfilaricidal efficacy in an individual animal at each interval was expressed as the percent change in the density of Mf compared to the pre-treatment level. On day 90 after the start of treatment, worms were recovered from the pleural cavity by PBS lavage. Treated and untreated animals were killed and the heart, lungs and testes were taken out and squeezed carefully to recover the adult parasites of both sexes. Macrofilaricidal/adulticidal efficacy of the extract was carried out by evaluating the percent reduction in adult worm recovery in the

treated group compared to untreated animals as described previously (Mathew *et al.*, 2008).

3.18.3 Influence of the treatment on *Wolbachia* by evaluation of *wsp* gene copy Number

3.18.3.1 DNA extraction

DNA from *B.pahangi* worms was extracted by using Qiagen extraction kit following the manufacturer's instructions.

3.18.3.2 Cloning of DNA and synthesis of plasmid

A primer pair was used to amplify a short (226 bp) fragment of the *wsp* gene of *B.pahangi Wolbachia*: *Bpwsp-forward 5'* TTGGTCTTGGTGTAGCATATA 3', *and Bpwsp-reverse 5'*ACTTTTGTTTCTTTATCCTCA 3'. To verify the presence of a single amplification product, the primers were tested using conventional PCR reactions performed on DNA extracted from *B. pahangi.* Reaction mixtures for this PCR were carried out as follows: 3 mins at 98^{o}C, 10 sec at 98^{o}C, 15 sec at 57^{o}C and 30 sec at 72^{o}C for 30 cycles. The amplification products were analysed by 1.7 % agarose gel electrophoresis.

The PCR amplified 226 bp *wsp* gene specific sequence was ligated into the pJET 1.2 vector (Thermo Scientific) following the manufacturer's instruction to generate standard plasmids to be used in Q-PCR. The cloned amplicon-sequence was verified by automatic DNA sequencing and followed by a BLAST2 analysis (National Centre for Biotechnology Information [NCBI (www.ncbi.nlm. nih.gov), Bethesda, MD, USA] with their corresponding sequences in GenBank. The concentrations of the purified plasmids that spanned the target regions for the forward and reverse primers were measured by

using NanoDrop™ 1000 Spectrophotometer. These measured plasmids were converted to copy numbers/µL according to the formula (Eq. (3.5)):

$$\text{DNA copy number} = \frac{[6.02 \times 10e23\ (\text{copy/mol}) \times \text{DNA amount (g)}]}{[\text{DNA length (dp)} \times 660\ (\text{g/mol/dp})]} \qquad \text{(Eq. 3.5)}$$

3.18.3.3 Colony PCR

Colony PCR was performed to identify clones with the expected size of insert cloned into the pJET 1.2 vector (Thermo Scientific) (Figure 3.3). Reactions were performed in 20 µl and consisted of 1x PCR buffer, 1x YellowSub, 1.5 mM MgCl2, 0.2 mM dNTPs, 0.1 mM pJET 1.2 forward primer, 0.1 mM pJET 1.2 reverse primer and 0.25 U HotStart Taq Polymerase. A single colony was selected using a toothpick and dissolved into the PCR reaction. 10 white colonies were chosen for analysis. Cycling conditions were as described in Section 3.14.3.4. As pJET 1.2 primer bound to the vector, 120 bp of the vector sequence was amplified and had to be added to the insert length to identify the expected PCR product length on the agarose gel.

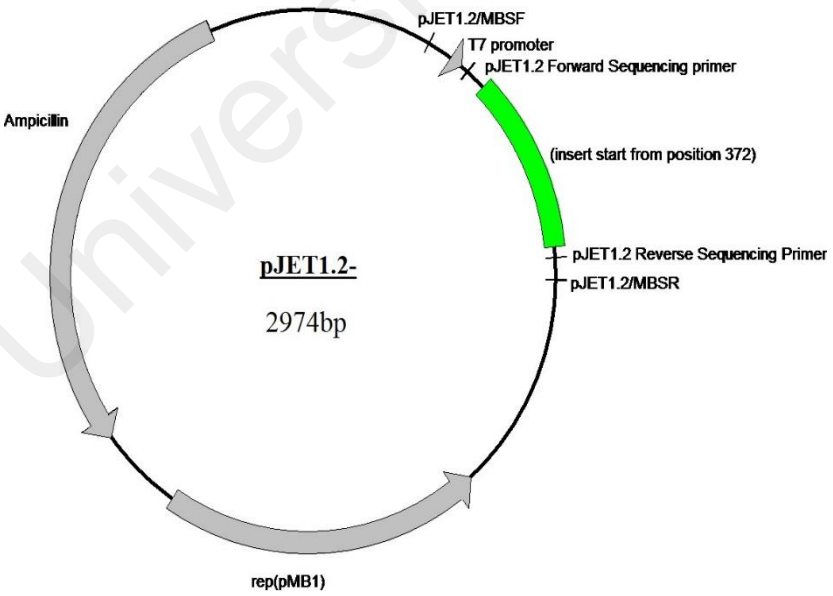

Figure 3.3: Pjet11.2 cloning vector used in this study (http://www.bioinfo.pte.hu/f2/pict_f2/pJETmap.pdf)

3.18.3.4 Plasmid purification

For purification of plasmids, *E. coli* were grown in 5 ml LB medium with 50 µg/ml ampicillin overnight in a shaker at 37^0 C and 250 rpm. Bacteria were centrifuged at 6000 rpm for 10 min and the plasmids were purified from the pellet using the DNA MiniPrep kit (Qiagen, GmbH, Hilden, Germany) following the manufacturer's protocol. The plasmid purification kit was either applied by hand or by the Automated Plasmid Purification System (Qiagen, GmbH, Hilden, Germany). The concentration and purity of the plasmids was assessed spectrophotometrically with a Nanodrop spectrophotometer. Part of the plasmid solution was used for sequence confirmation and the remaining stored at -20°C until further use as a plasmid standard in Q-PCR.

3.18.3.5 Calculation of plasmid copy numbers

The copy numbers/µl of the gene specific plasmids used for generating standard curves can be estimated as shown in the following example:

Length of plasmid: 2974 bp (pJET 1.2 vector) + 226 bp (insert) = 3200 bp molecular mass × 660 Da/bp = 2.1×10^6 g/mol

DNA concentration: 300 ng/µl

Copy numbers can now be calculated with:

Copy number $= (3.0 \times 10^{-7}$ g/µl $\times 6 \times 10^{23}$ copies/mol) / $(2.1 \times 10^6$ g/mol) = 8.6×10^{10} copies/ µl

3.18.3.6 Quantitative PCR (Q-PCR)

The Q-PCR reaction mixtures comprised of 12.5 µl SYBR green solution (2x SYBR Green qPCR Master Mix, Universal), 8.5 µl of nuclease-free water, and 1 µl of each primer (sense and anti-sense at 7.5 µM concentrations) and 2 µl of DNA template in a final volume of 25 µl. Assays were carried out on Bio-Rad iCycler apparatus (Bio-Rad Laboratories, Hercules, Calif., USA) under the following conditions for all the samples: UDG pre-treatment for 2 min at 50 ^{o}C; denaturation cycle of 10 min at 95 ^{o}C; 40 cycles of PCR (95 ^{o}C for 15 sec; 58 ^{o}C for 30 , and 72 ^{o}C foe 30 sec); a melting curve analysis, which was started from 95 °C to 60 °C with a 0.2°C/sec transition rate; and a final holding temperature of 10°C. The testing of each sample was in triplicate.

All samples, including the non-template negative control, were run in triplicate and compared to a standard curve developed by a 20-fold serial dilution of the synthesized plasmid- this was equivalent to 300,000,000 to 1,875 copies. The 20-fold serial dilution ran with each Q-PCR session and produced an equation for a linear regression line that served to determine the gene copy number. The mean threshold cycle (CT) value of the triplicate produced by each sample was entered into the equation for the linear regression line and solved for Y, and this gave the copy number for the sample.

3.18.3.7 Sample quantification

10 ng of each tested sample was subjected to real-time qPCR and the thermal cycling and reaction conditions used were same as the standard curve construction qPCR in Section 3.18.3.6. The quantification of *wsp* gene copies was calculated by reference to the constructed standard curve and its equation, “y = -0.32x + 13.01”. y referred to log DNA copies of *wsp* gene and x referred to the CT value obtained based on the average of the 3 replicates.

3.18.3.8 Evaluation of efficiency

A standard curve was constructed for *wsp* gene copy number analysis to test the efficiency of the qPCR reactions and it was used as a reference for *wsp* gene copy number analysis. A series of 20-fold dilutions starting from 300,000,000 to 1,875 copies (corresponding to 2.1e-10 g/ul - 1.31e-15 g/ul of the initial B. *pahangi* preparation) were prepared from lysates of *B. pahangi*. Thermal cycling and reaction conditions were as just described. Each point of dilution was tested with 3 replicates.

3.19 Gene expression analysis by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

After the experiment was completed, recovery adult worms were immediately snap frozen for later use. 16 adult males and females from each animal group were pooled prior to RNA extraction. Samples were crushed in liquid nitrogen using a mortar and pestle. Then, total RNA was extracted from samples by using the RNeasy plus Mini Kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's instructions. The integrity and concentration of RNA was determined with using a RNA LabChip Agilent Bioanalyzer RNA 6000 Nano assay. Prior to proceeding with cDNA synthesis, RNA samples were treated with DNase I to remove any genomic DNA contamination. Next, complementary cDNA was synthesized from 500 ng of the same purified RNA samples according to ImProm-II™ Reverse Transcription System (Promega Corporation, Fitchburg, WI, USA). Relative quantification of *Wolbachia* gene expression was analysed using Quantitect™ SYBR Green (Qiagen, GmbH, Hilden, Germany).The reaction mixtures were prepared with a mixed 12.5 µl 2X SYBR Green PCR master with 1 µl each of 7.5 µM gene specific sense and anti-sense primers (Table 3.1) and 2 µl the cDNA (50 ng/µl) template added last for a total of a 25 µl reaction. The PCR conditions were: initial activation step was for 10 min at 95^0C followed by 50 cycles of denaturation (10 s at 95^0C), annealing (10 s at 56^0C) and extension (10 s at 72^0C) on the StepOnePlus Real-

time PCR System (Applied Biosystems, Foster City, CA, USA). Melt curve was run after the end of the PCR cycles; temperature was ramped from 60-95^{0}C at a rate 0.2^{0}C/s to characterize PCR amplification specificity.

These experiments were performed in triplicate (from 3 separate RNA pools with 2 batches of worms) and each sample was replicated 3 times within each experiment on a 96-well optical plate. Reactions containing template generated without the reverse transcriptase enzyme were included as controls to ensure that no genomic DNA contamination in cDNA and no primer dimer artifacts was present. As well, water was also tested in each reaction as a "no template" control (NTC). The same amount of total RNA was used for reverse transcription of control and treated RNAs, and the same amount of cDNA was used for each real-time reverse transcription PCR (RT-PCR) reaction. Primer pairs were *Wolbachia fstz* and *wsp* as described (Fenn & Blaxter, 2004), while primers for *B. pahangi* were designed from sequences obtained from GenBank with Primer Express 3.0 software (Applied Biosystems, Foster City, CA, USA). Sequence specific primer pairs were purchased from (iNtRON Biotechnology, Seoul, Korea). Specific primers were used to amplify the selected genes shown in Table 3.1.

Table 3.1: Sequences and product sizes of primers

Primer name	Sequence	Product size (bp)	Accession Number
FtsZ	Fstz-F 5'-GCAAAAGCAACAAGAGAGGCAAG-3' Fstz-R 5'-CCAAGTTCCGCAATACGCATAC-3'	135	-
WSP	Wsp-F 5'-ATCAGCAACCCTGCAAAGGCAC -3' Wsp-R 5' CGCCATAAGAACCAAAGTAGCGAG -3'	145	-
NADH	NADH-F 5'- GGGTGGCACTCAGTGTCGTA-3' NADH-R 5'-ACAACGCCTGAAAAATACCAGAGTA-3'	58	-
HSP-90	HSP-90-F 5' TCTCGAAATCAACCCTGACC-3' HSP-90-R 5' AACCAGAAGAAAGCAAGGCA-3'	128	AJ005784.1
SHP	SHP-F 5' GCAGTGCATTTGGGACATTA- 3' SHP-R 5' CATAATCTGCGGTCCCATTC-3'	111	X82314.1
COX-1	COX-1-F 5' GAATCCGTTGCTGTATCAACAT-3' COX-1-R 5' TTTGACCAAATAGGCGATCC-3'	134	EF406112.1

3.19.1 Comparative C_T method

Differences in the relative expression between treatments groups and controls were calculated using the ΔΔCt method (Ghedin *et al.*, 2009), where the difference of a given target gene in treated groups versus the control is computed. The Normfinder and genNorm algorithm (MultiD Analyses AB, Göteborg, Sweden) were used to evaluate and calculate the expression stability of three endogenous references genes - NADH, beta actin and histone 3. The expression of the target genes were normalized to more stable endogenous reference genes. The calculation was based on the difference between Ct values of the samples of interest (test group) with a control (non- treated sample) and

where ΔΔCt = ΔCt (treated) - ΔCt (control). ΔCt is the Ct value for any sample, treated or untreated, normalized to the endogenous housekeeping gene. Then, ΔΔCt values were calculated by subtracting the ΔCt value of the control group from that of treated samples. Finally, the fold expression of a given target gene in treated samples over the control worms was calculated using the formula ($2^{-\Delta\Delta Ct}$).

3.19.2 Statistical analysis

Statistical analyses took place with GraphPad Prism 4.0 (GraphPad Software, San Diego, USA). Data sets were tested for data normality using Kolmogorov–Smirnov's one sample test. qRT- PCR results for individual genes were analysed by one-way Analysis-of-Variance (ANOVA) to examine the significance of differences in gene expression between treated and control groups. The criterion for significance in all statistical tests was $P < 0.05$.

CHAPTER 4: RESULTS

4.1 Knowledge, attitude, and practice (KAP) towards LF

4.1.1 General characteristics of subjects

A total of 230 people participated voluntarily in this survey and were interviewed face-to-face in order to fill in a questionnaire about their KAP towards LF. The general socio-demographic characteristics of the respondents are presented in Table 4.1.

Out of the 230 respondents, 68.7% were females and 31.3% were males. Age distribution of the respondents showed that the most represented age group was 30-39 years old, while the least was ≥ 60 years age group. Of the respondents, 87.8 % had received education (37.4 % at the primary level, 39.1% at the secondary level, and 11.3% at the tertiary level). Almost half of the respondents (47%) were employed, with 73.5 % of the employed respondents earning more than RM500 (approximately US$165) per month. Of those not employed, the majority were housewives. There was an approximately equal split in the percentage of respondents who owned wood/bamboo-based houses and brick-based houses (Table 4.1).

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Table 4.1: General socio-demographic characteristics of the study population (N = 230)

Variable	Frequency	%
Gender		
Male	72	31.3
Female	158	68.7
Age		
15-29 years	67	29.1
30-39 years	90	39.1
40-49 years	58	25.2
50-59 years	10	4.3
≥60 years	5	2.2
Educational level		
No formal education	28	12.2
Primary	86	37.4
Secondary	90	39.1
Tertiary	26	11.3
Occupation		
Employed	108	47.0
Unemployed/Housewives	122	53.0
Types of houses		
Bamboo/wood	131	57.0
Bricks/rock	99	43.0
Income Level		
≤RM500.00	61	26.5
>RM500.00	169	73.5

4.1.2 Knowledge of LF and LF transmission

Table 4.2 presents the results of the knowledge survey on LF and its transmission.As observed clearly, most respondents (83.9%) had heard about LF and they reported that the source of LF information was either school (30.1%), mass media (21.8%) or both (8.8%). Others had heard about LF from various places- the health centers (12.9%), mass media and health centers (11.4%) or from other people (15.0%). The majority (77.2%) of respondents were aware that LF is transmitted by mosquitoes. Approximately 20.2 % did not know how the transmission of LF takes place and 1 respondent mentioned bacteria as the transmission agent. Slightly more than half (59.6%) reported that the main symptom of LF was evident in swollen legs while 10.9 % admitted that they did not know any symptoms of LF.

Table 4.2: Perceived knowledge of the respondents about LF and its transmission (N=230)

Variable	Frequency	%
Knowledge about filariasis		
Yes	193	83.9
No	37	16.1
Source of information		
Mass Media	42	21.8
School	58	30.1
Health centre	25	12.9
Mass Media & School	17	8.8
Mass Media & Health Centre	22	11.4
Other people	29	15.0
Transmission		
Bacteria	1	0.5
Mosquito	149	77.2
Worms	4	2.1
Don't know	39	20.2
Symptoms		
Fever	8	4.1
Swollen legs	115	59.6
Body Pain	1	0.5
Gland Enlargement	3	1.6
Fever & Swelling	29	15.0
Body weakness	11	5.7
Headache	5	2.6
Don't know	21	10.9

4.1.3 Attitudes and practices towards LF, its vector and control measures

Respondents' attitude towards several aspects in regard of the LF, its vector and control measures are presented in Table 4.3. 67.4 % of those respondents were unanimous to say that LF did cause a problem. 41.5% of the respondents regarded LF to be a medical problem which brought about symptoms that include pain, fever, itching and the failure to walk. Meanwhile, 40% of respondents opined that the LF is an economic problem that occurs following the inability to work, not employed and expenditures incurred for certain purposes such as medication and/or transport to a health facility. 11.5% of respondents highlighted that LF is a social problem as those infected often shy away and opt to not interact with the community. The remaining 7 % of survey respondents did not make specific the kind of problems they perceived LF to be in association with.

In terms of the treatment of illness, more than half of respondents (60.9%) had stated their preference of hospital treatment. About 2% had faith in a traditional healer (*bomoh/dukun*) when it comes to treating the illness. The rest of the respondents (37%) had decided to combine both the forms of treatments. Almost all respondents lacked the knowledge of the drug used in LF treatment, as 96.1% indicated that they had no knowledge of the drug used for LF treatment and 3.9 % of respondents mentioned paracetamol as the potential drug. Moreover, when the respondents posed questions about the participation in an MDA programme, only 11.7 % admitted that they had taken part previously or had family member(s) undergoing treatment for LF through an MDA programme.

To prevent the transmission of LF, more than 39.1 % of respondents confirmed that they did use some protective clothes and had slept under bed nets to protect themselves from mosquito bites. However, 16.5 % failed to indicate any specific protection against mosquitoes. Most respondents (76.5%) were aware and did take some preventive

measures- cleaning their water containers, making sure that they have good drainage, using chemical sprays or a combination of these activities.

Table 4.3: Attitude of the respondents towards mosquitoes and treatment-seeking behavior (N=230)

Variable	Number	Percent (%)
Do you consider LF to be a problematic disease? (N = 193)		
Yes	130	67.4
No	5	2.6
Don't know	58	30.0
Type of problem (N = 130)		
Medical	54	41.5
Economical	52	40
Social	15	11.5
Unspecified	9	7
Preferred treatment method (N = 230)		
Hospital	140	60.9
Bomoh/dukan/traditional healers	5	2.1
Presumed drug in the treatment of LF		
Paracetamol	9	3.9
Don't know	221	96.1
Participation of respondent or family member(s) in mass drug administration programme (MDA)		
Yes	27	11.7
No	203	88.3
Protection from mosquito bites		
Wear clothes	25	10.9
Use of bed nets	77	33.5
Wear clothes and use of bed nets	90	39.1
No response	38	16.5
Control of mosquitoes		
Cleaning of water containers	30	13.0
Good water drainage	12	5.2
Use of chemical spray	15	6.5
Cleaning and drainage	42	18.3
Cleaning, drainage, and chemical spray	77	33.5
No response	54	23.5

4.1.4 Perception of respondents towards MDA programme

The results of the respondent's knowledge regarding the MDA programme is presented on Table 4.4. It was found that the majority of the respondents (64.8%) were not aware of the existence of the MDA programme. The remaining 35% of the respondents indicated that they had in fact heard about it. For those who indicated that they heard of the MDA programme, 39.5 % stated that they had learnt about it from schools, 32.1 % from mass media, and 22.2 % from a health centre.

Table 4.4: Knowledge of the respondents about MDA programme (N=230)

Variable	Frequency	%
Have you heard about MDA programme?		
Yes	81	35.2
No	149	64.8
Source of information		
Mass Media	26	32.1
School	32	39.5
Health Centre	18	22.2
Mass Media & School	1	1.2
Mass Media & Health Centre	4	5.0

4.1.5 The association of demographic factors of respondents and knowledge of MDA and LF

The association between demographic factors and knowledge about LF and the MDA programme among the study population was determined using Chi-square tests (Table 4.5). The results showed that although females had a higher knowledge of LF than male

respondents, the difference was not statistically significant (χ^2 =1.749; P > 0.05). Moreover, the respondents aged >40 years had a greater knowledge of LF compared to those aged ≤40years, and again, the difference was not statistically significant (χ^2 = 2.083; P > 0.05). Similarly, educated respondents were more knowledgeable about LF compared to the non-educated. However, the difference was also not statistically significant (χ^2 = 1.876; P > 0.05). Further, employed subjects had more knowledge of LF than their unemployed counterparts, though the difference was not statistically significant (χ^2 = 1.918; P > 0.05). Assessment of monthly income level of the respondents with knowledge of LF interestingly showed that those with income level ≤RM500.00/month had higher knowledge of the disease than those with monthly income >RM500.00. However, the difference was not statistically significant (χ^2 = 0.095; P > 0.05). Note: RM 4.3 = US$1.00.

Table 4.5: Association of knowledge of LF with some demographic factors among the respondents

Variables	Knowledge of LF		
	Prevalence (%)	**OR (95% confidence interval)**	***P***
Gender			
Female	86.1	1.19 (0.895, 1.570)	0.130
Male	79.2	1	
Age Group			
≥ 40 years	89.0	1.56 (0.818, 2.968)	0.104
< 40 years	81.5	1	
Education Level			
≥ 6 years formal education	85.1	1.10 (0.934, 1.294)	0.138
No formal education	75.0	1	
Occupation			
Employed	87.4	1.33 (0.859, 2,055)	0.114
Unemployed/housewives	80.7	1	
Monthly income			
≤ RM500.00	90.9	1.36 (0.188, 9,828)	0.611
> RM500.00	87.7	1	

4.2 Phytochemical identification

The weight yields of extracts from *M. cajuputi* leaves and flowers obtained after extraction were 11g and 9.5 g, respectively, while that of *A. elliptica* leaves and fruit were 17 g and 15.8 g, respectively.

The results of the phytochemical screening of the plant extracts are presented in Table 4.6. Flavonoids and alkaloids were found in all samples. On the other hand, saponins were observed in all samples except *A. elliptica* fruit. However, glycosides was only observed to be present in the samples of *M. cajuputi* extracts, whereas presence of terpeniods occured only in *A. elliptica* leaf extracts, and *M. cajuputi* flower extracts, however steroids was presence only in *A. elliptica* fruit extracts .

Table 4.6: Phytochemical constituents present in the extracts

Extracts	*A. elliptica*	*A. elliptica*	*M. cajuputi*	*M. cajuputi*
Part used	leaf	fruit	leaf	flower
flavonoids	+	+	+	+
alkaloids	+	+	+	+
phenolic	+	+	+	+
saponins	+	-	+	+
terpenoids	+	-	-	+
glycosides	-	-	+	+
steroid	-	+	-	-

4.3 Antioxidant activity of *M. cajuputi* extract

4.3.1 Total phenolic and flavonoid content

The results obtained from total phenolic content (TPC) analysis of the *M. cajuputi* leaves and flower extracts are presented in Table 4.7. Comparing the TPC contents of the extracts, floral extract was found to show a higher TPC value (155 ±0.05 GAE/mg extract

dry weight) than the leaves extract (137±0.05 GAE/mg extract dry weight). A similar trend was also observed in comparing the total flavonoid content between the extracts (Table 4.7), i.e. *M. cajuputi* flowers > *M. cajuputi* leaves extracts. There were no significant differences between either extract in terms of total phenolic and flavonoid content.

Table 4.7: Crude extract activity of the investigated plants

Plant extract and standard	DPPH IC_{50} (µg/ml)	FRAP (µmol Fe ((II)/g)	Iron chelating IC_{50} (µg/ml)	TPC value (mg Gallic acid/g of extracts)	Flavonoid (mg Quercetin/ g of extract)
***M. cajuputi* flower**	40.0 ±1.4[a]	0.24 ± 0.9[b]	130.5 ± 1.3[c]	155 ± 0.03[d]	106 ± 0.4[e]
***M. cajuputi* leaves**	85.0±1.2[a]	0.12 ±0.4[b]	55.6 ± 0.8 [e]	137 ± 1.8[d]	91.9 ± 6.2[e]
***A. elliptica* fruit**	45.0±2.3[a]	0.12±0.3[b]	90.0 ± 1.1[c]	71 ± 1.3[g]	5.6 ± 0.4[f]
***A. elliptica* leaves**	95.0±6. 1[a]	0.16 ±0.1[b]	90.3± 0.05[c]	37 ± 2.2[h]	3.4 ± 0.2[f]
BHT	65.0±0.031[b]	188.8±24.8[c]	-	-	-
EDTA	-	-	100.0± 0.53[c]	-	-

GAE= Gallic acid equivalent

QE= Quercetin equivalent

Data are represented in mean ± SEM of triplicates; means followed by the same letters are not statistically significant.

4.3.2 DPPH free radical scavenging assay

Antioxidant activity is the measure of the capacity of the tested extracts to reduce the generation of free radicals. The scavenging capability of the *M. cajuputi* leaves extract and *M. Cajuputi* flower extract increase with the rise in the concentration of the extract as shown in Figure 4.1. The results were found to be in accordance with the total phenolic content data - DPPH radical scavenging capacity revealed the flower extract of *M. cajuputi* (G.F) exhibited the highest inhibition activity (80.16±0.4%) at a concentration of 500 µg/ml. In contrast, leaves extract (G.L) showed 65.77±0.0% at a concentration of 500 µg/ml. The percentage of radical inhibition of *M. cajuputi* flowers extract seemed to be higher (80.16±0.4%), than that of *M. cajuputi* leaves extract (65.77±0.0%). This demonstrates that both *M. cajuputi* flowers and *M. cajuputi* leaves extract exhibit DPPH radical scavenging activity in a concentration-dependent manner. In addition, a significant difference ($P < 0.05$) was observed between the IC_{50} of the flower and leaves extract with 40.0 ±1. 4 and 85.0±1.2, respectively, in comparison to the IC_{50} of the synthetic antioxidant standard BHT at 65.0 ± 0.031 (Table 4.7). For both samples, however, increasing the concentration beyond 250 µg/ml resulted in negligible increases in radical scavenging activity (Figure 4.1).

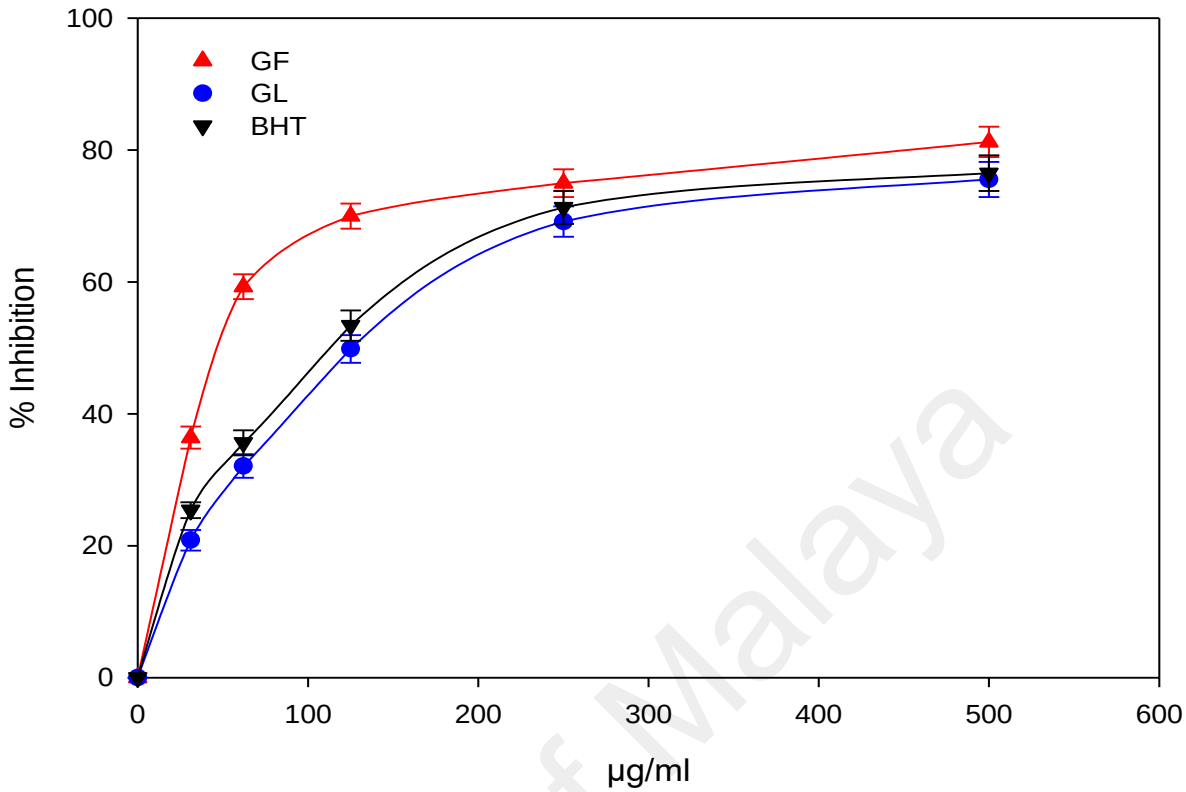

Figure 4.1: DPPH radical scavenging activity of the *M. cajuputi* extract

4.3.3 Metal chelating power

Figure 4.2 shows the chelating effect of the *M. cajuputi* extracts. An activity of about 50% was observed when extract concentrations were around 0.4 mg/ml. The chelating activity increased with elevated sample concentration. As expected, the activity of the flower extract was observed to be higher than that displayed by the leaves extract with maximum chelating activity found to be at 1 mg/ml with 75.94±0.2% and leaves extract was 59.85±0.11% (Figure 4.2). In all samples tested, there was an observed logarithmic increase in chelating power with increasing concentration to 0.25 mg/ml, and thereafter, the percentage of chelating activity seemed to rise gradually. However, in comparison with the IC_{50} of the positive EDTA control (100.0 ±0.53), there were no significant differences between it with the IC_{50} of flowers at 130.8 ± 1.3. Conversely, the IC_{50} of the methanol extract of leaves (55.6 ± 0.8) showed a significant difference compared to

EDTA (Table 4.7). That suggested *M. cajuputi* flower methanolic extract possesses strong chelating activity.

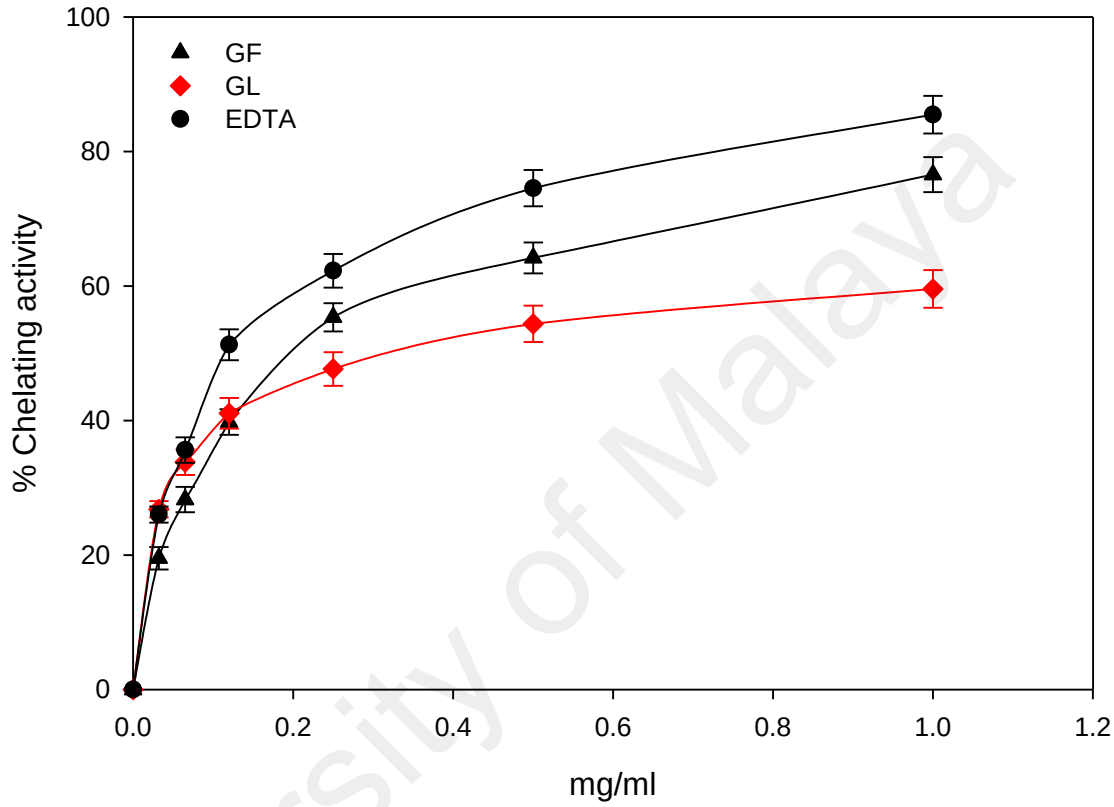

Figure 4.2: Metal chelating activity of *M. cajuputi* extract

4.3.4 Ferric reducing antioxidant power (FRAP) assay

The FRAP antioxidant ability of *M. cajuputi* extracts to reduce the Fe^{3+}–TPTZ reagent was evaluated. In the present study, both extracts of *M. cajuputi* flowers and *M. cajuputi* leaves demonstrated some level of reducing power, achieving FRAP values of 0.24 and 0.12 µM Fe(II)/g , respectively.

4.4 Antioxidant activity of *A. elliptica* extract

4.4.1 Total phenolic content

The results obtained from total phenolic content (TPC) analysis of the *A. elliptica* leaves and fruit extracts are presented in Table 4.7. Comparing the TPC contents of the extracts, fruit extract was found to possess a higher TPC value (71 ±1. 3 GAE/mg extract dry weight) than the leaves extract (37± 2. 2 GAE/mg extract dry weight). A similar trend was also observed when comparing the flavonoid content between the extracts (Table 4.7), i.e. *A. elliptica* fruit > *A. elliptica* leaves extracts.

4.4.2 DPPH radical scavenging activity

The free radical scavenging activities of both extracts from *A. elliptica* (fruit and leaves) is shown in Figure 4.3. Generally, *A. elliptica* fruit extract exhibited DPPH percentage inhibition activity with (63.16±0.4%) at a concentration of 500 µg/ml with IC_{50} = 45.0 µg/ml, followed by leaves extract with 58.0±0.1% at a concentration of 500 µg/ml and an IC_{50} of 95.0 µg/ml. However, there were significant differences in IC_{50} of both extracts in comparison to standard BHT (Table 4.7).

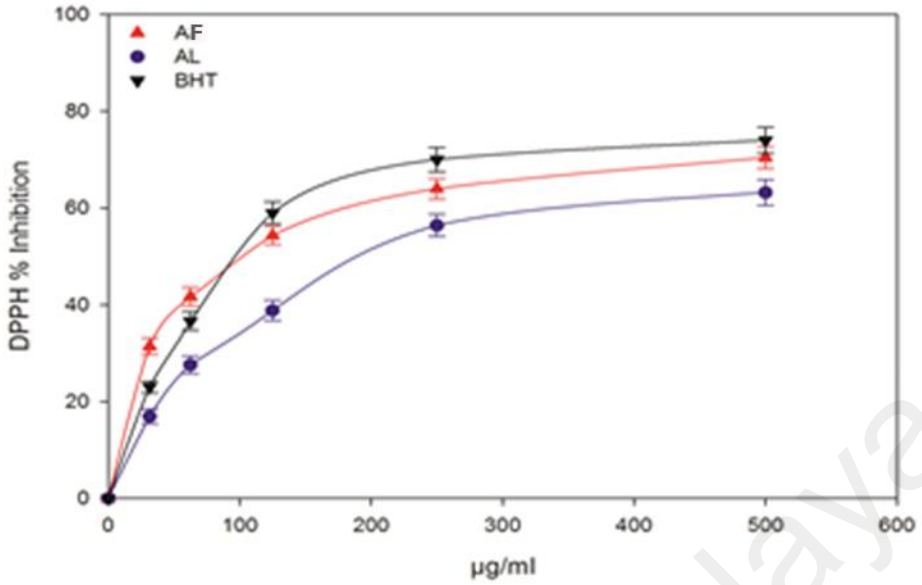

Figure 4.3: DPPH radical scavenging activity of *A. elliptica* extract

4.4.3 Metal chelating power

The metal chelating activities of *A. elliptica* extracts were also evaluated (Figure 4.4), and as expected, the fruit extract was found to show higher chelating power (43.6±0.13%) with an IC_{50} of 90. 0 ± 1.1, versus the leaves extract that exhibited lower chelating activity (27.4±0.13%) with an IC_{50} of 90.3± 0.05, and both extracts showed significant differences in comparison with EDTA (Table 4.7). Moreover, the chelating activity was observed to be concentration dependent.

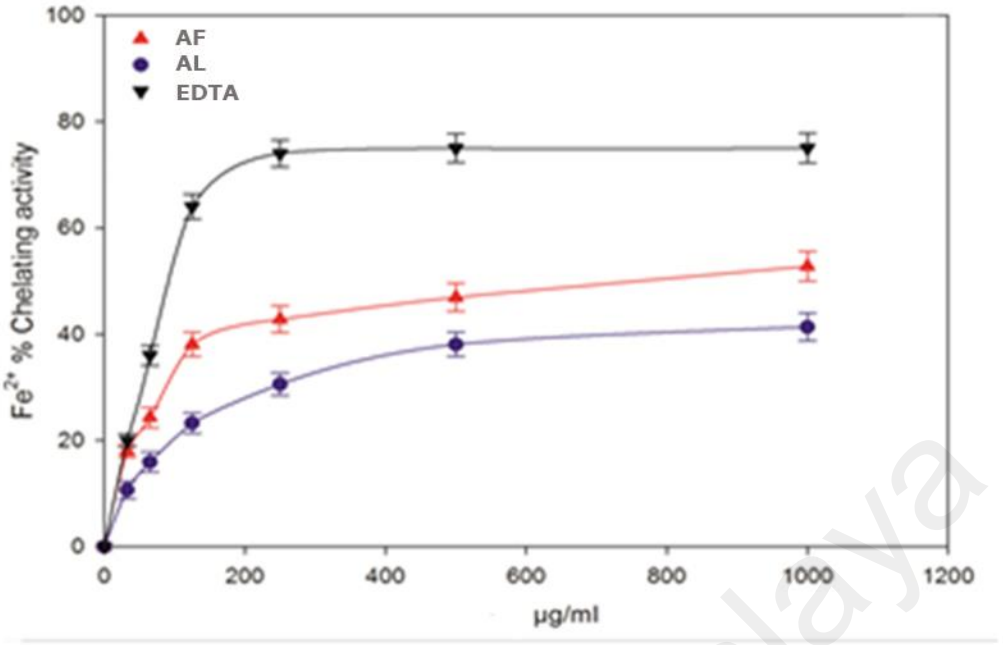

Figure 4.4: Metal chelating activities of *A. elliptica* fruits and leaves extracts

4.4.4 Ferric reducing antioxidant power (FRAP)

The FRAP antioxidant ability of *A. elliptica* extracts to reduce Fe^{3+}–TPTZ reagent was evaluated. Both extracts (i.e. *A. elliptica* leaves and *A. elliptica* fruit) demonstrated some level of reducing power achieving FRAP values of 0.12 and 0.16 µM Fe (II)/g, respectively.

4.5 Antibacterial activity of the extracts

The antibacterial activity of the extracts was studied and the result was presented in Table 4.8. As can be seen with the extracts from *M. cajuputi* leaves and flowers, both were found to be potent against *S. epidermidis, S. aureus* and *B. cereus*. The overall mean inhibition zone of *M. cajuputi* flower methanol extract against *S. epidermidis* and *B.cereus* is significantly different ($P < 0.05$) versus the other tested extracts materials. In comparison to *M. cajuputi,* leaves extract from *A. elliptica* was effective against *K. pneumonia* only. While the fruit extract from *A. elliptica* showed a broader activity

spectrum against *P. multocida*, *K. pneumonia* and *S. pneumonia*, none of the extracts were found to be effective against *E. coli* or *S. typhi*. Contrarily, relative to the overall mean inhibition zone of the standard drug against all the tested organisms, there were no extracts that showed the same activity as the standard drug ($P < 0.05$).

Table 4.8: Antibacterial activity of crude extracts

	Inhibition diameter (mm ± SD)							
Sample	***S.epidermidis*** **(Gram +ve)**	***S.aureus*** **(Gram +ve)**	***B.cereus*** **(Gram +ve)**	***P.multocida*** **(Gram +ve)**	***K.pneumonia*** **(Gram+ve)**	***S. pneumonia*** **(Gram +ve)**	***E. coli*** **(Gram - ve)**	***S. typhi*** **(Gram -ve)**
M.cajuputi leaves	13.66±0.43	12.33±0.57	6.33±0.33	-	-	-	-	-
M.cajuputi flowers	17.33±0.36	12.33±0.31	12.33±0.48	-	-	-	-	-
A. elliptica leaves	-	-	-	-	9.66 ± 0.31	-	-	-
A. elliptica fruit	-	-	-	9.66 ± 0.35	12.66±0.26	7.66 ± 0.45	-	-
Streptomycin sulfate [a]	20.33 ± 0.38	18.0 ± 0.2	21.0 ± 0.25	21.0 ± 0.05	20.0 ± 0.1	22.0 ± 0.08	15.0 ± 0.1	10.0 ± 0.2

- No inhibition was observed. Doses of the samples were 1 mg/mL per disc, streptomycin sulfate 10 µg per disc. [a] Positive control

Similarly, the minimum inhibitory concentration (MIC) and minimum bacteriocidal concentration (MBC) of both extracts were tested (Table 4.9). As shown, there were no observed values for MIC and MBC in *B. cereus* when exposed to *M. cajuputi* leaves extract. Likewise, no MIC or MBC was recorded when *P. multocida* was exposed to *A. elliptica* leaves extract (Table 4.10). The MBC for the GF extract against *S. epidermidis* was 25 mg/mL, which was more effective than the GL extract that was at 50 mg/ml. However, exposing *S. aureus* to both extracts elicited similar MIC and MBC values (12.5 mg/ml, 25 mg/ml, respectively). Nonetheless, *K. pneumonie* was found to be more susceptible to *A. elliptica* extract in terms of both MIC and MBC analysis.

Table 4.9: MIC and MBC of *M. cajuputi* extracts

SAMPLE	MIC (mg/mL)			MBC (mg/mL)		
	S. aureus	***S.epidermidis***	***B.cereus***	***S. aureus***	***S. epidermidis***	***B.cereus***
G.L.	12.5	12.5	NA	25	50	NA**
G.F.	12.5	25	12.5	25	25	50
Streptomycin*	1.95	1.95	> 1.0	1.95	1.95	> 1.0

MIC, Minimum inhibitory concentrations; MBC, Minimum bactericidal concentrations
* Doses of Streptomycin were 1mg/ml.
** No Activity observed.

Table 4.10: MIC and MBC of *Ardisiac elliptica*

SAMPLE	MIC (mg/mL)		MBC (mg/mL)	
	Pasteurella multocida	***Klebsiella pneumoniae***	***Pasteurella multocida***	***Klebsiella pneumoniae***
A.L.	NA**	12.5	NA**	50
A.F.	25	12.5	50	25
*Streptomycin	1.95	1.95	1.95	1.95

* Doses of Streptomycin was 1mg/ml.
** No Activity observed.

4.6 Scouting infective host

Brugia-infected zoonotic hosts (cats) were obtained from the two study areas in Selangor state via field trapping.

4.6.1 Identification of parasite species detected in blood films

The morphology of Mf detected in the study is portrayed in Figure 4.5. Identification is based on the size and proportions of the innenkorper (inner body) to the whole length of the Mf. As can be seen in Figure 4.5, the innenkorper length relative to the overall length of the Mf in *B. pahangi* was about 21% (44-63µm).

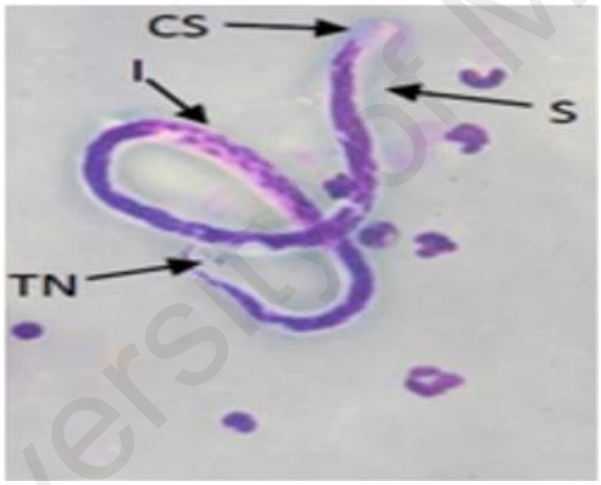

Figure 4.5: Micrograph of microfilariae (Mf): *B. pahangi* showing the presence of marked innenkorper (I), sheath (S), cephalic space (CS) and 2 two terminal nuclei (TN)

4.7 Assessment of *in vitro* cytotoxicity and antifilarial activity of test samples against *B. pahangi*

4.7.1 *In vitro* cytotoxicity of test samples

To further ensure that the activity of the extracts (if any) were not because of their cytotoxic effects, the cytotoxicity of all tested samples were evaluated using the fluorescent dye, resazurin, to determine the inhibitory concentration at which 50% of the cells under evaluation became dead (IC_{50}) as described earlier in Chapter 3 (Materials and Methods). All test samples indicated that extracts tested did not cause any death of cells at the tested concentrations *in vitro*. The standard antifilarial drug, ivermectin, showed IC_{50} values of 65.0 µg/ml. Tetracycline showed IC_{50} values of 100.0 µg/ml.

4.7.2 Effect of extracts on microfilariae (Mf) release, motility and viability of the filarial parasite

A stable release of Mf was observed in control female worms throughout the 7 day period (Figure 4.6 (a-d)). Compared with the control group, the extract treated groups of A.L (Figure 4.6 a), A.F (Figure 4.6 b), G.L (Figure 4.6 c) and ivermectin revealed a non-significant ($P > 0.05$) decrease in Mf release within the period of study (7 days). A progressive decrease in Mf release pattern was observed upon G.F treatment (Figure 4.6 d). This reduction in Mf release with increasing extract concentration was found to be related with the observed decrease in Mf release when increasing the concentration of the reference drug (tetracycline). With the present analysis, treatment with G.F extracts was found to cause complete cessation of Mf release (1000 µg/ml) at day 6.

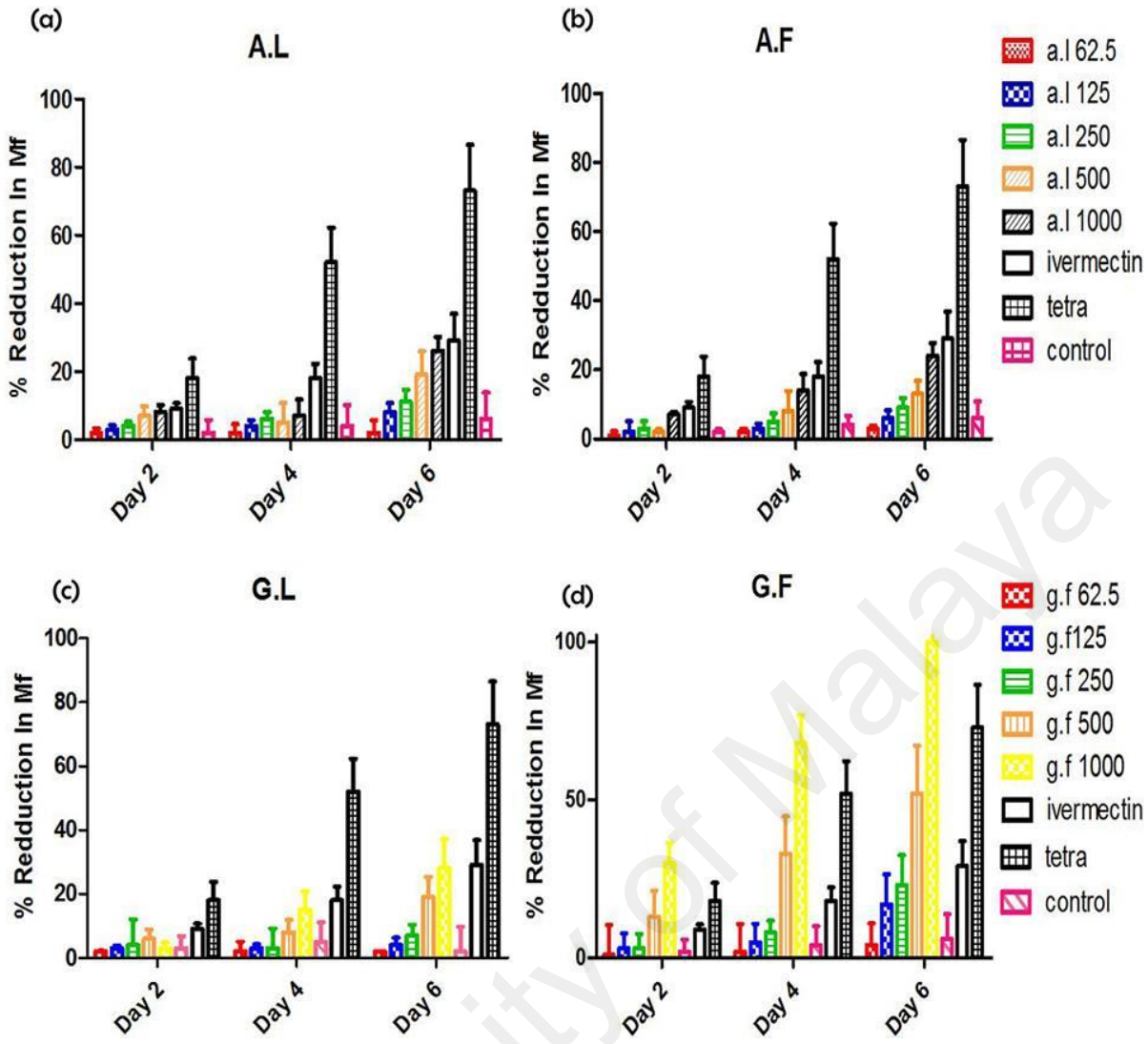

Figure 4.6: Effects of *M. cajuputi* and *A. elliptica* extracts *in vitro* on the percentage of microfilariae (Mf) released by *B. pahangi* female worms at days 2, 4 and 6. (a) *A. elliptica* leaves extract and (b) *A. elliptica* fruit extract (c) *M. cajuputi* leaves extract (d) *M. cajuputi* flower extract. All data points were plotted as S.E.M.±5%

As expected there was no observable mortality in the control group. Thus, this revealed a stable mean motility score during the 7 day period with a motility score of 3 (Figures 4.7 a-d) in comparison to control worm, the treatment with extract of A.L (Figure 4.7 a), A.F (Figure 4.7 b), G.L (Figure 4.7 c) or the reference drug (Figure 4.7 a-d) did not exhibit significant reduction (P >0.05) in the mean motility score. While in treatment with the extract of G.F (Figure 4.7 d), the reduction in filarial motility was observed to be

concentration dependent. Generally, 1000 µg/ml was found to cause complete termination of worm motility at day 6.

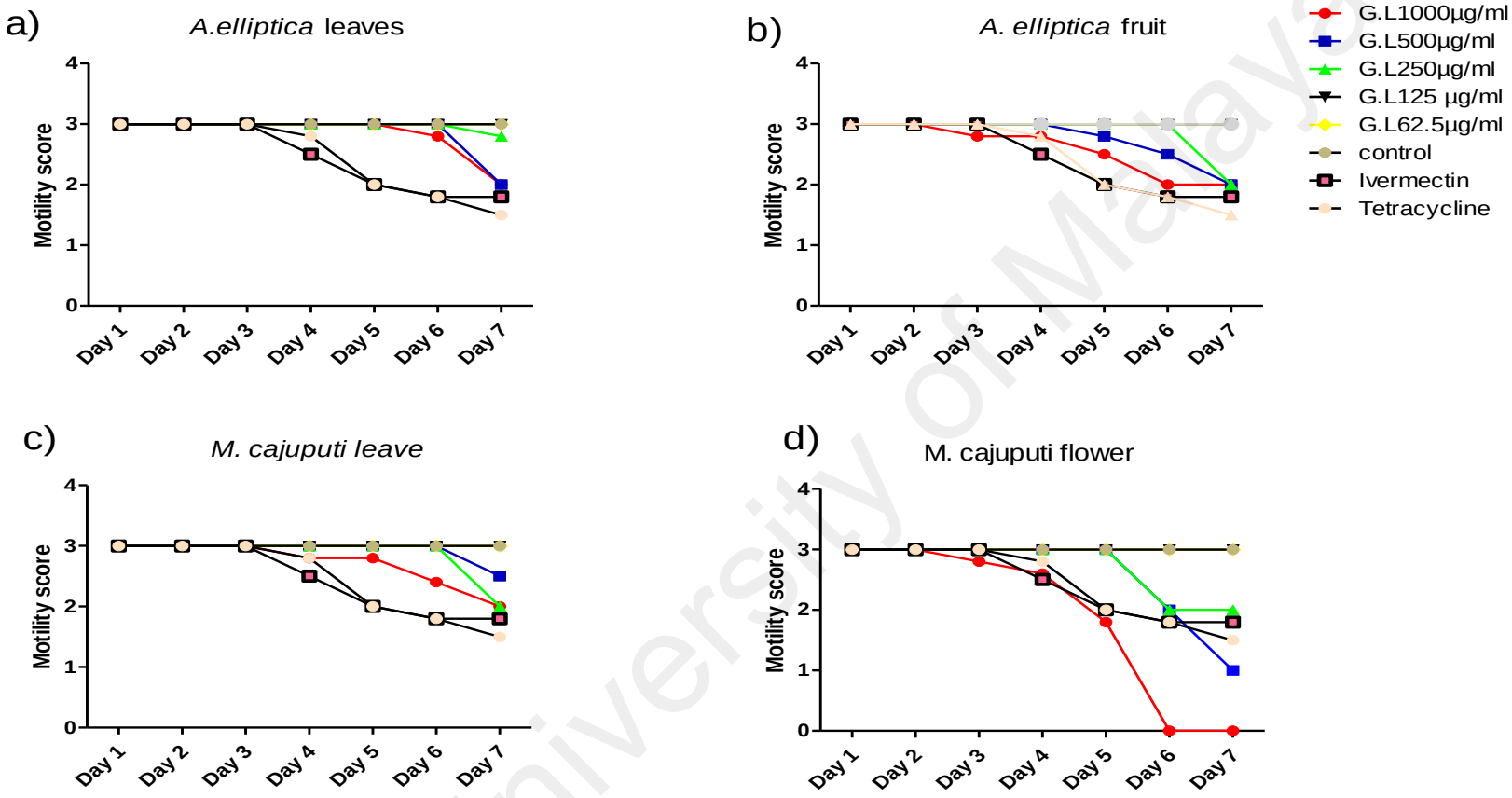

Figure 4.7: Effects of *M. cajuputi* and *A. elliptica* extracts *in vitro* on the motility of adult *B. pahangi*. (a) *A. elliptica* leaves extract (b) *A. elliptica* fruit extract (c) *M. cajuputi* leaves extract (d) *M. cajuputi* flower extract. All data points were plotted as S.E.M.±5%

The effect of the crude extract on the viability of adult worm (Figure 4.8) was evaluated using the MTT reduction assay. After incubating the adult worm for 7 days with 62.5 -1000µg/ml of the extracts, a significant (P <0.05) and dose dependent decrease in the adult worm at higher extract concentrations (≥500 µg/ml) of G.F extract was seen. On the other hand, A.F, A.L and G.L were found not to have any significant reduction in filarial viability upon exposure. In fact, treatment with G.F extract at 1000 µg/ml was found to incur a reduction in the Mf release and adult worm viability by 100%, an inhibition that was found to be almost greater than that displayed by the standard reference drug, ivermectin (25 µg/ml), along with tetracycline at 40 µg/ml.

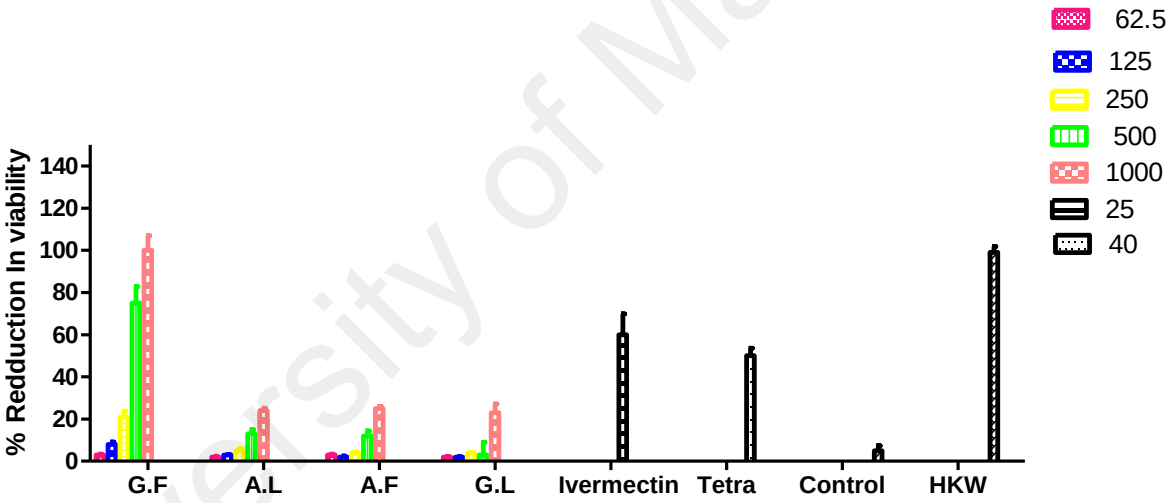

Figure 4.8 : Effect of crude extract of *M. cajuputi* (G.L, G.F) and *A. elliptica* (A.F, A.L) on the viability of adult worms of *B. pahangi*. The parasite viability was assayed using a MTT reduction assay. Results were expressed as percent reduction viability (reduction of formazan production by treated parasites) and observed values were contrasted with untreated parasites, positive controls, standard ivermectin and tetracycline groups. All experimental values were expressed as ±S.E.M. (P<0.05). HKW= heat-killed worm

4.7.3 Molecular analysis

The activity of the extracts on *Wolbachia* endosymbionts was further evaluated using molecular DNA analysis of *Wolbachia*-specific surface protein (*wsp*) gene on a 1.5% agarose gel electrophoresis (Figure 4.9 A and B). The *Wolbachia wsp* gene was reported to be a gene of about 590 bp (Gunawardena *et al.*, 2005; Zhang *et al.*, 2010). In both figures, extracts-treated groups displayed the effect of treatment on the PCR quantity product 590 bp. Upon treatment with A.F, there does not appear to be a reduction in *Wolbachia*-specific products (Figure 4.9 A), and the same can be said for G.L and A.L (Figure 4.9 C, and D). Yet, treatment with G.F showed a typical dose-dependent disappearance of the *wsp* gene amplicon. Interestingly, when compared to the positive control sample, the intensity of the *wsp* gene amplicon of G.F treated samples at concentrations ≥ 500µg/ml was observed to be greatly diminished with increasing concentrations of the treatment (Figure 4.9 B). This clearly makes evident the antimicrobial effect of the extract on the *Wolbachia* population in the filarial parasite.

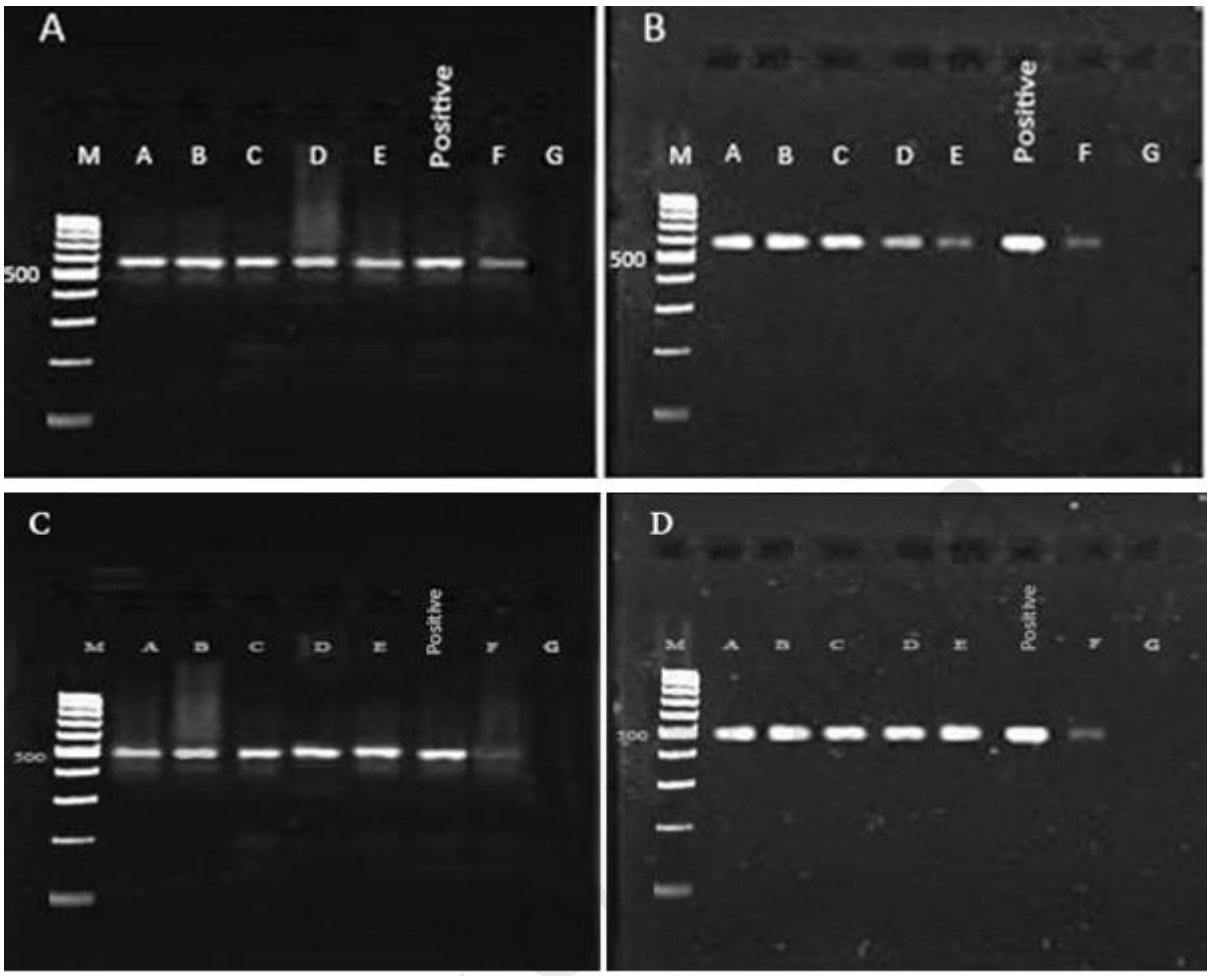

Figure 4.9: Agarose gel electrophoresis of PCR products of *Wolbachia wsp* gene obtained from *B. pahangi* showing the effects of (A) *A. elliptica* fruit extracts , *M. cajuputi* flower extracts (B), *A. elliptica* leave extracts (C) and *M. cajuputi* leave extracts (D) treatment on the quantity of *Wolbachia wsp* PCR products (590 bp) using *Wolbachia*-specific primers. *B. pahangi* adult females (n=6) were exposed to varied extract concentrations (µg/ml): A=62.5, B=125, C=250, D=500 and E=1000, Positive = Fresh females without incubation were used as positive control, F = Tetracycline treatment, G = negative control with water only**)**

4.7.4 Post-treatment TEM evaluation

The effect of G.F extracts on a filarial parasite was further evaluated based on the ultrastructural analysis of intracellular *Wolbachia* in *B. pahangi* (Figure 4.10). After treatment for 7 days, *B. pahangi* worms were collected and prepared for structural analysis using transmission electron microscopy (TEM). It may be observed when reviewing Figure 4.10 A that there were many intracellular *Wolbachia* cells (WBp) in the hypodermal cord of the worm's section obtained from the positive control groups. On the

other hand, a degenerated *Wolbachia* (dWBp) was observed in the sample's hypodermal sections with visible phagocytized bacterial remnants in the cellular vacuoles (black arrow) in the group treated with the G.F extract (Figure 4.10 B) and tetracycline (Figure 4.10 C). The total number of *Wolbachia* was observed to generally decrease in the G.F extract-treated samples compared to the control sample, signifying the anti-*Wolbachial* activity of the extracts, in turn enhancing antifilarial efficacy.

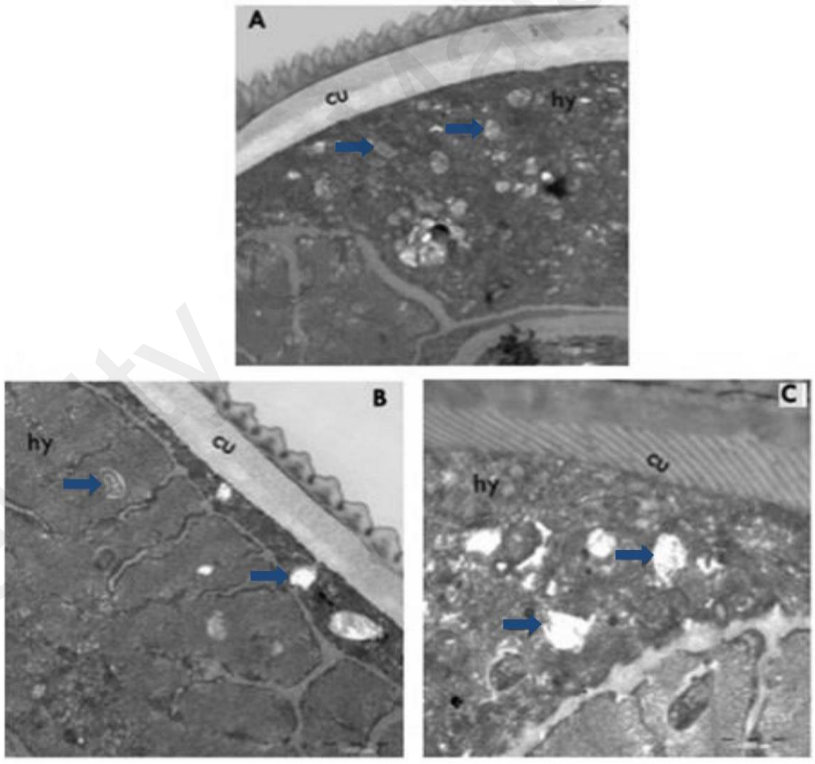

Figure 4.10: Ultra-structural observation of *Wolbachia* of *B. pahangi* within the hypodermis of normal and treated worms. In comparison to the normal worm (A), in G.F-treated worms, the number of *Wolbachia* appeared to be reduced and the endobacteria seemed to be degenerated (see arrow in B). The same can be said for tetracycline (40 µg/ml) treatment. (C) The endobacteria look to be dead and the vacuoles contain only remnants of bacteria or membrane whorls (see arrow in C). The bar is 1000 nm cu = cuticle; hy = hypodermis

Based on these results, further work was carried out on the methanol extracts of the *M. cajuputi* flower.

4.8 Assessment of cytotoxicity of *M. cajuputi* flowers *in vivo* (acute and subacute toxicity study)

4.8.1. General Signs and Behavioural Analysis

The oral toxicity of methanolic extracts was investigated here in this study to determine the safety parameters of the extracts with respect to the general behavioural pattern of jirds. There were no behavioural changes and all animals were alive up to 14 days after oral administration of single dose (2 and 4 g/kg body weight). In addition, the animals were alive up to 28 days after oral administration of the daily dose (100, 500 and 1000 mg/kg body weight). The animals in both the vehicle and the extracts-treated groups were normal and did not exhibit any significant changes in behavior, breathing, skin effects, food or water intake or hair loss. No deaths were observed for any dose during the 14 days and 28 days of plant extract treatment.

4.8.2 Haematology, biochemistry and histology parameters

Data obtained from observing the haematology parameters, serum biochemistry, histology of the liver and kidneys and body weights were presented in Tables 4.11 to 4.16. In general, the toxicological studies revealed no significant differences between groups compared to the normal control group. Therefore, the tested extracts seemed rather safe even at high doses and displayed no toxicity on the animal model used.

Table 4.11: Hematology parameters of treated animal groups with *M. cajuputi* flower extracts (acute toxicity study)

parameters	Control 0.01 DMSO	Group 1 2 g/kg	Group 2 4 g/kg
WBC ($\times 10^3/\mu l$)	7.55±0.25	7.4±0.9	6.8±0.2
RBC ($\times 10^6/\mu l$)	7.69±0.27	7.66±0.79	7.66±0.13
Haemoglobin (g/dl)	12.8±0.98	12.6±3.4	12.6±0.33
Platelets ($\times 10^3/\mu l$)	681±7.45	698±14.8	660±11.5

Values expressed as mean ± S.E.M. There were no statistically significant differences between the measurements in different groups. The significance value was set as $P < 0.05$.

Table 4.12: Biochemistry parameters of treated animal groups with *M.cajuputi* flower extracts (acute toxicity study)

Parameters	**Flower**		
Groups	**Control**	**Group 1**	**Group 2**
	0.01 DMSO	**2g/kg**	**4g/kg**
ALP (U/L)	118.8±16.9	109.0±4.5	116.7±9.6
ALT(U/L)	118.67±14.1	96.6±2.5	97.7±7.8
AST (U/L)	98.8±2.5	86±5.6	87.7±3.6
T.Billirubin(umol/L)	3.3±0.87	3.6±0.67	3.7±0.33
T. protein (g/L)	53±5.0	51±3.8	54±4.7
Urea (mmol/L)	7. 93±2.3	8.1±0.32	8.0±0.4
Creatinine (umol/L)	18.8±2.5	17.3±1.6	17.7±1.4

Values expressed as mean ± S.E.M. There were no statistically significant differences between the measurements in different groups. The significance value was set as $P < 0.05$.

Table 4.13: Hematology parameters of treated animal groups with *M. cajuputi* flower extracts (subacute toxicity study)

Parameters	***M. cajuputi* Flower**			
	Control	**Group II**	**Group III**	**Group IV**
Groups	**5% DMSO**	**100 mg/kg**	**500 mg/kg**	**1000 mg/kg**
WBC ($\times 10^3/\mu l$)	7.55±0.25	6.13±1.10	6.03±0.3	6.3±1.1
RBC ($\times 10^6/\mu l$)	7.69±0.27	7.62±0.44	7.66±0.13	6.63±0.79
Haemoglobin (g/dl)	12.8±0.98	12.5±3.9	12.6±0.33	12.1±2.2
Platelets ($\times 10^3/\mu l$)	681±7.45	693±18.8	660±11.5	694±1.8

Values expressed as mean ± S.E.M. There were no statistically significant differences between the measurements in different groups. The significance value was set as $P < 0.05$.

Table 4.14: Biochemistry parameters of treated animal groups with *M. cajuputi* flower extracts (subacute toxicity study)

Parameters	***M. cajuputi* Flower**			
	Control	**Group II**	**Group III**	**Group IV**
Groups	**5% DMSO**	**100 mg/kg**	**500mg/kg**	**1000 mg/kg**
ALP (U/L)	118.1±16.9	109.0±4.5	116.3±9.6	107.6±7.8
ALT(U/L)	118.67±14.1	96.6±2.5	97.7±7.8	97.3±1.1
AST (U/L)	98.8±2.5	86.5±5.6	87.7±3.6	92.0±6.5
T. billirubin(umol/L)	3.3±0.87	3.6±0.67	3.7±0.33	3.8±0.4
T. protein (g/L)	53±5.0	51±3.8	54±4.7	49±2.8
Urea (mmol/L)	7. 93±2.3	8.1±0.32	8.0±0.4	8.0±0.5
Creatinine (umol/L)	18.8±2.5	17.3±1.6	17.7±1.4	18.0±0.6

Values expressed as mean ± S.E.M. There were no statistically significant differences between the measurements in different groups. The significance value was set as $P < 0.05$.

Table 4.15: Body weight of animal groups treated with *M. cajuputi* flowers extracts (acute toxicity)

Week of treatment (n=8)	**Control** 5% DMSO	***M. cajuputi* flower** **2g/kg**	**4g/kg**
W0	25.7± 1.5	25.6±1.8	24.3±1.3
W1	26.6±2.0	25.2±2.3	26.5±1.8
W2	27.7±2.6	26.4±1.6	27.8±2.5

Values expressed as mean ± S.E.M. There were no statistically significant differences between the measurements in different groups. The significance value was set as $P < 0.05$.

Table 4.16: Body weight of animal groups treated with *M. cajuputi* flower extracts (subacute toxicity)

Week of treatment (n=8)	Control 5% DMSO	G.flower 100mg/kg	G.flower 500mg/kg	G.flower 1000mg/kg
W0	23.8±1.2	25.3±1.1	25.8±1.0	25.9±1.5
W1	24.7±0.9	26.2±2.3	26.9±1.8	27.0±1.3
W2	27.3±1.7	26.4±1.6	27.7±2.5	27.8±1.0
W3	29.03±2.4	27.3±1.0	28.2±1.4	29.1±0.9
W4	31.5±2.3	30.7±0.9	31.1±1.1	32.0±1.1

Value expressed as mean ± S.E.M. there were no statistically significant differences between the measurements in different groups. The significance value was set at $P < 0.05$.

4.8.3 Histology of the kidney and liver

The histopathological study of the kidney and liver sections of treated groups are presented in Figures 4.11 to 4.14. The results are in agreement with the observed normal values of biochemical enzymes,haematological parameters, and body weights. From the H & E micrographs, it can be seen that in comparison to the normal vehicle control group, there is no observable toxicological implication as a result of the extract treatments even at higher dosages ranging all the way to 4000 mg/kg.

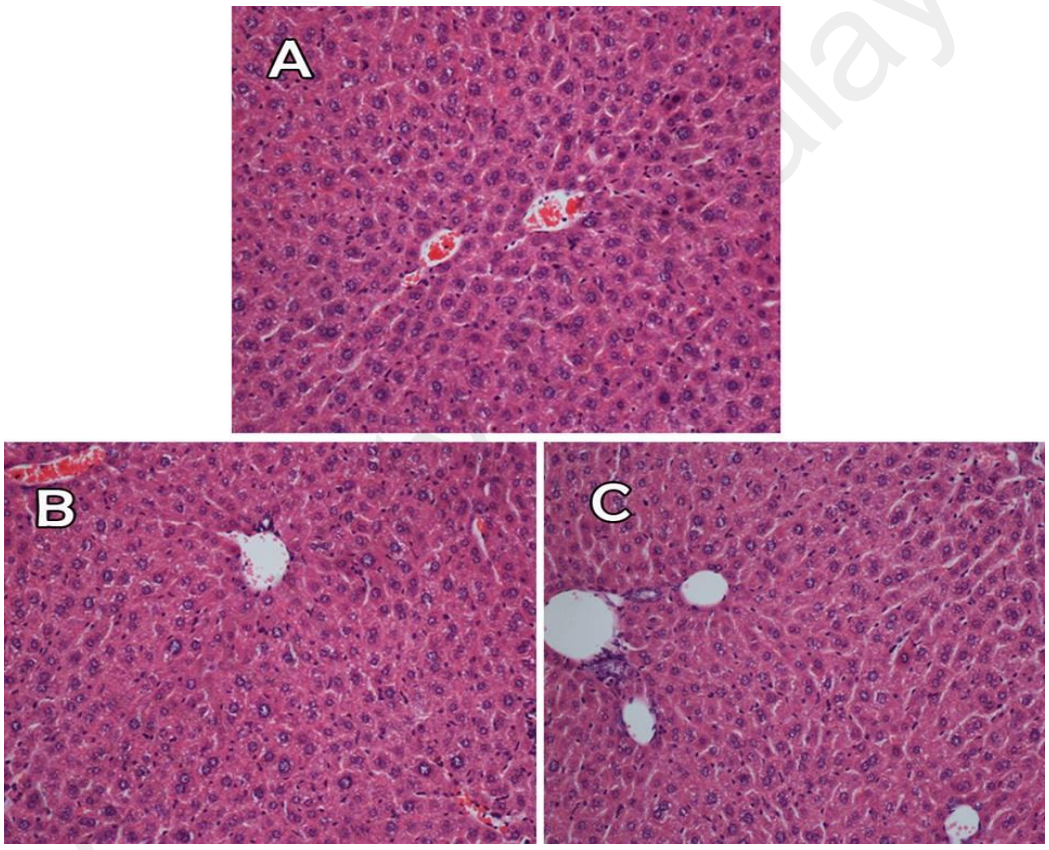

Figure 4.11: Histological micrographs section from Jird's liver treated with (A) control treated with 5 ml/kg (5% DMSO) (vehicle control), (B) methanolic extracts of *M. cajuputi* flowers (**2** g/kg) and (C) methanolic extract of *M. cajuputi* flowers **4**g/kg. No structural differences were seen between the plants-treated groups and the control group (H & E stain 10x)

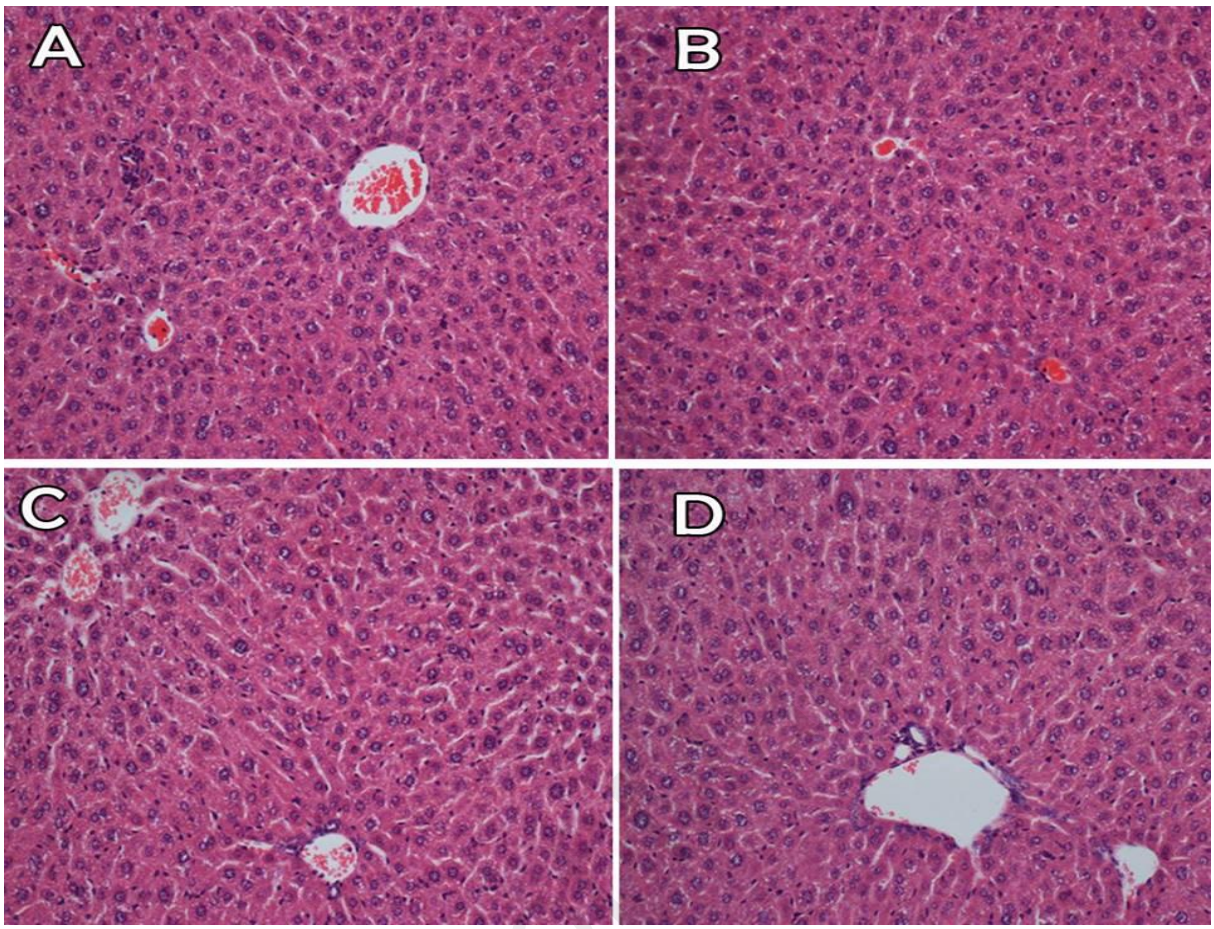

Figure 4.12: Histological micrographs of Jird's liver section treated with (A) 5 ml/kg (5% DMSO) (vehicle control), (B) methanolic extracts of *M. cajuputi* flowers (100 mg/kg) (C) methanolic extract of *M. cajuputi* flowers (500 mg/kg), and (D) methanolic extracts of *M. cajuputi* flowers (1000mg/kg) (H & E stain 10x)

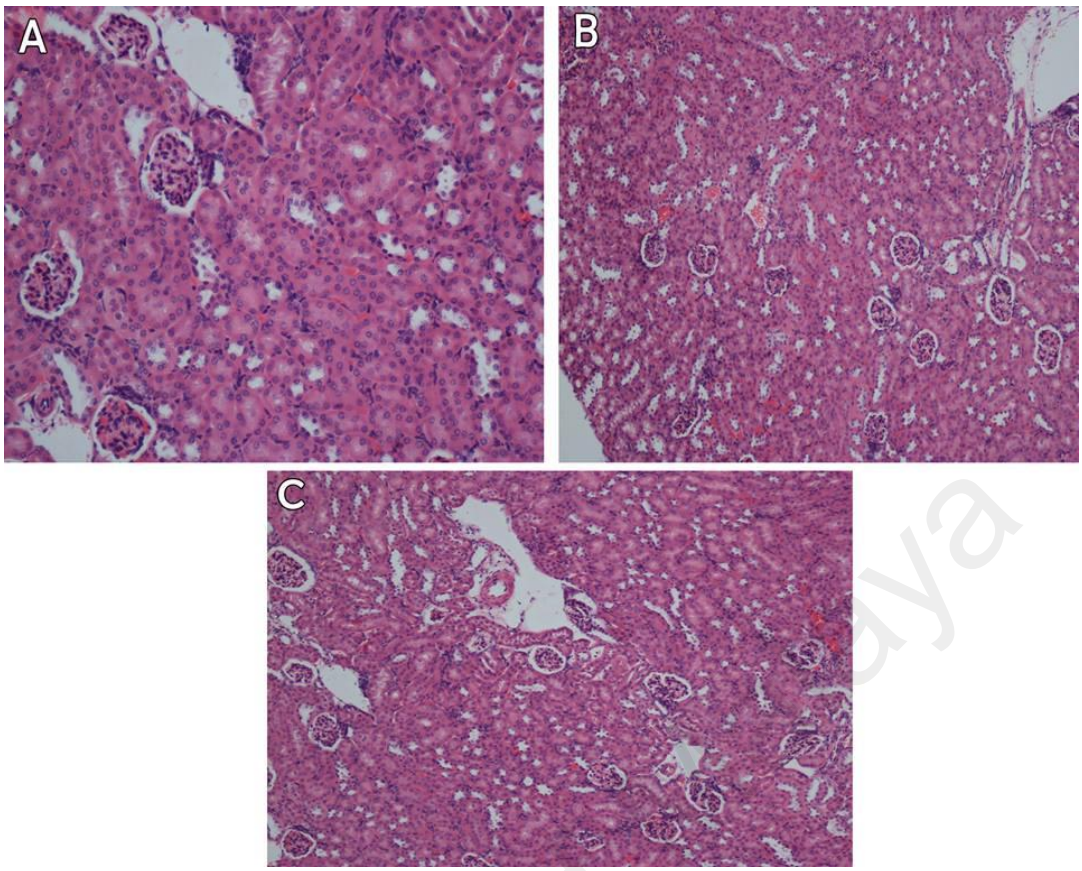

Figure 4.13: Histological micrographs of Jird's kidney section treated with (A) 5 ml/kg (5% DMSO) (vehicle control), (B) methanolic extracts of *M. cajuputi* flowers (2 g/kg), (C) methanolic extract of *M. cajuputi* flowers (4g/kg) (H & E stain 10x)

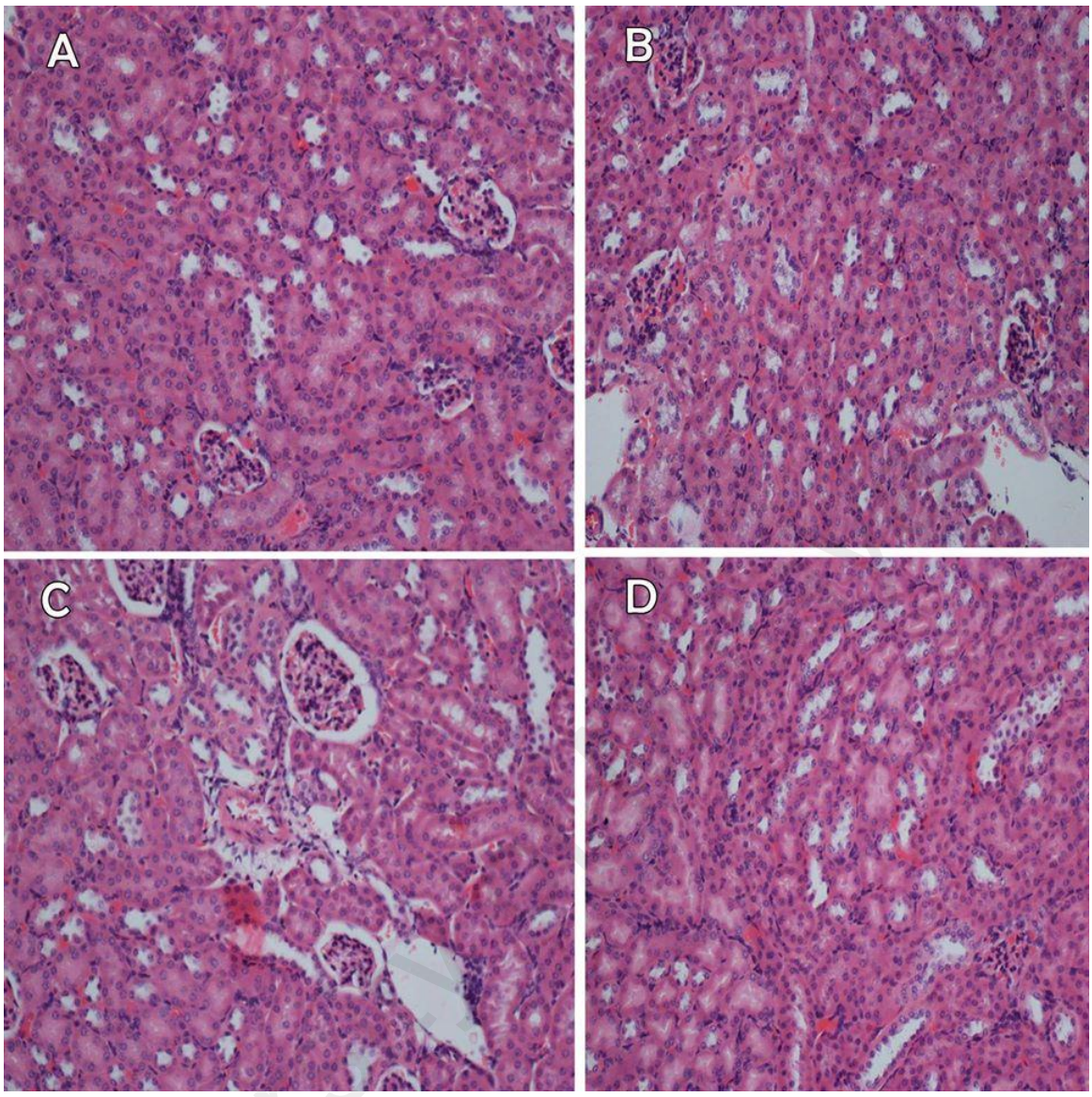

Figure 4.14: Histological micrographs of jird's kidney section treated with (a) 5 ml/kg (5% DMSO) (vehicle control), (b) methanolic extracts of *M. cajuputi* flowers (100 mg/kg), (c) methanolic extract of *M. cajuputi* flowers (500mg/kg) and (d) methanolic extract of *M. cajuputi* flowers (1000mg/kg) (H & E stain 10x)

4.9 Phytochemical identification and characterisation of *M. cajuputi* flowers

The phytochemical identification and characterisation of the flowers of *M. cajuputi* was performed using GCMS and LCMS analysis. Subsequent fraction identification was observed using LCMS. In either of the analyses, mass fragmentation data obtained were parsed via the National Institute of Standard and Testing (NIST, USA) database for

individual compound identification. The results obtained are presented in Table 4.17 and 4.18.

4.9.1 GCMS analysis of the methanol extracts from the flower of *M. cajuputi*

The phytochemical identification and characterisation of the crude extracts was performed using GCMS analysis. The relative retention times (Rt) and mass spectra of the extract components were compared with mass spectra from a data library. As shown in Table 4.17, GCMS analysis of the *M. cajuputi* flower extract revealed the major compounds with more than 90% similarity with the standard mass spectra in the library, representing 72.9 % of the relative area of the methanolic extract. This extract is characterized by the presence of fatty acids, including octadecanoic acid (0.68%), hexadecanoic acid (0.60%) and 9,12-octadecadienoic acid (Z,Z) (1.90%) as well as phenolic compounds, such as alpha tocopherol (vit E) (0.47%), ethanone (4.34%) and 1,4-naphthalenedione (1.07%), terpenoid compounds like urs-12-en-28-al (6.40%), and aromatic compounds like naphthalene (7.92%) and alpha-tetralone (6.24%).

4.9.2 LCMS analysis of the methanol extracts from the flower of *M. cajuputi*

The methanol extracts of the flowers of *M. cajuputi* were further analysed by LCMS. The major compounds of the main peaks are presented in Table 4.18 and characterized on the basis of fragmentation mass charge. Most of them belonged to typical hydroxy cinnamic acid and phenolic acid derivatives. Specifically, LCMS analysis indicated the presence of CAPE, gingerol, aspidin, methylorsellinic acid, ethyl ester, trans-2,3,4-trimethoxycinnamate and metyrosine.

Table 4.17: Phytochemical compounds identified based on GCMS analysis

ID	Compound Name	Class of compound	Mol. formula	Mol mass	Rt time (min)	%
1	3-Cyclohexen-1-ol	Terpenoid	C18H18O	154	6.151	1.07
2	Cyclohexane, 1-ethenyl-1-methyl-2-(1methylethenyl)-4-(1-methylethylidene)-	Terpenoid	C15H24	204	7.482	0.68
3	Copaene	Terpenoid	C15H24	204	7.997	1.66
4	Cyclohexane, 1-ethenyl-1-methyl-2,4bis(1-methylethenyl)	Terpenoid	C15H24	204	8.107	2.60
5	Caryophyllene Bicyclo[7.2.0]undec-4ene	Terpenoid	C15H24	204	8.588	6.14
6	1,6-Cyclodecadiene	Terpenoid	C15H24	204	8.690	1.49
7	Caryophyllene *	Terpenoid	C15H24	204	9.059	3.16
8	Naphthalene	Aromatics	C15H24	204	9.536	3.26
9	Naphthalene*	Aromatics	C15H24	204	9.616	2.66
10	Naphthalene *	Aromatics	C15H24	204	9.851	1.97
11	1H-Cycloprop[e]azulen-7-ol	Terpenoid	C15H24O	220	10.906	1.29
12	Caryophyllene oxide	Terpenoid	C15H24O	220	11.176	1.73
13	Alpha.-Tetralone	Aromatics	C12H13FO3	224	11.279	6.24
14	2-Naphthalenemethanol	Aromatics	C15H26O	222	12.318	2.84
15	2-Naphthalenemethanol*	Aromatics	C15H26O	222	15.545	0.98
16	Hexadecanoic acid	Fatty acid	C17H34O2	270	17.648	0.60
17	1,4-Naphthalenedione	Phenolic	C11H8O5	220	18.672	1.07
18	4H-1-Benzopyran-4-one	Flavonoids	C16H20O4	276	19.168	2.12
19	Ethanone	Phenolic	C16H14O4	234	19.563	4.34
20	9,12-Octadecadienoic acid (Z,Z)-,	Fatty acids	C19H34O2	294	21.327	1.90
21	Octadecanoic acid	Fatty acid	C19H38O2	298	22.779	0.68
22	1-Heptacosanol	Straight chain	C27H56O	396	40.363	0.85
23	Alpha Tocopherol (vit E)	Phenolic	C29H50O2	430	40.600	0.47
24	Sitosterol, Stigmast-5-en-3-ol	Terpenoids	C29H50O	414	42.438	0.57
25	Sitosterol, Stigmast-5-en-3-ol*	Terpenoids	C29H50O	414	43.399	3.88
26	Urs-12-en-28-al	Terpenoids	C30H48O	424	44.480	1.47
27	Urs-12-en-28-al*	Terpenoids	C30H48O	424	45.816	4.93
28	Urs-12-en-28-al, 3-(acetyloxy)-,	Terpenoids	C32H50O3	482	47.628	1.49
29	Betulin	Terpenoids	C30H50O2	442	47.949	3.68
30	Urs-12-en-28-al *	Terpenoids	C30H48O	424	48.690	3.00
31	3.beta.-Myristoylolean-12-en-28-ol	Terpenoids	C44H76O3	652	50.431	1.67
32	Urs-12-en-28-al*	Terpenoids	C30H48O	424	50.867	2.41

***** Unknown compound isomers

Table 4.18: Phytochemical compounds identified based on LCMS analysis

Compound Name	GF extract		RT	m/z
	Presence	% Abundance		
Metyrosine	+	0.6	13.072	194.08203
Methylorsellinic Acid, Ethyl Ester	+	0.74	11.18	209.0822
Hydroxyibuprofen	+	1.2	10.759	221.1184
Trans-2, 3, 4-Trimethoxycinnamate	+	0.7	10.386	237.07724
Gingerol	+	0.45	11.768	293.17629
Catharanthine	+	2.1	20.202	371.15374
calicoferol D	+	1.1	21.489	409.31181
Caffeic acid Phenethyl ester (CAPE)	+	18.69	18.451	283.30206
Aspidin	+	0.65	12.819	459.20282
Cucurbitacin F	+	1.2	20.886	517.31874
Kurilensoside G	+	2.2	20.89	633.33896
1α,22,25-trihydroxy-26,27-dimethyl-23,23,24,24-tetrahyro-24a, 24b, 24c-trihomovitami	+	9.68	20.869	497.36525

4.9.3 Collection of fractions by HPLC

The methanol extracts of the flowers of *M. cajuputi* were further subjected to HPLC to collect different fractions for *in vitro* testing. The fractions were collected per minute using a fraction collector. A total of 42 fractions were collected in 42 minutes HPLC runtime and later combined based on their retention times outlined in Table 4.19 and Figure 4.15. A total of five fractions were pooled together from different retention times shown in Table 4.19. These fractions were subjected to bioactivity evaluation (anti-

Wolbachia activity *in vitro*). The most superior anti-*Wolbachia* activity, in fraction No. 2, was collected at 16-18 min.

Table 4.19: Five fractions obtained at different retention times

Fractions	Retention times
GF1	2-16 min
GF2	16-18 min
GF3	18-20 min
GF4	20-40 min
GF5	40-42 min

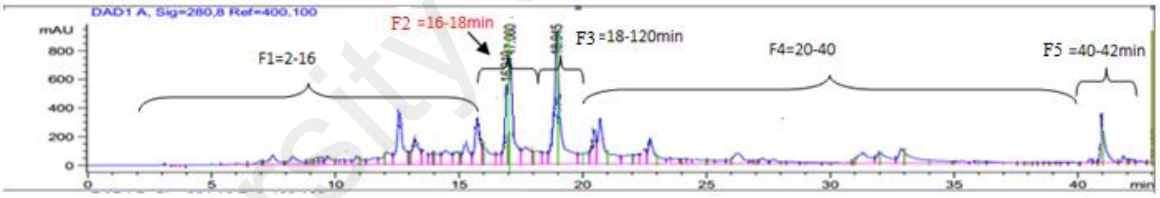

Figure 4.15: HPLC profile of fractionation of *M. cajuputi* flowers

4.10 Evaluation of the *in vitro* activity of the extract fractions obtained from *M. cajuputi* flower

Wolbachia-infected (Aa23) cell line was exposed to extract's fractions (5 fractions) over a period of 7 days. DNA from the aforementioned cell line was extracted and amplified using *wsp*-specific primers and 28S arthropod primers to validate DNA template quality. The results obtained from the PCR analysis can be viewed in Figure 4.16. In the PCR electrophoretic macrographs, it was noticed that all DNA extracts

produced a band at approximately 590 bp, attributable to a *Wolbachia wsp* gene signal. However, this band was observed to be less pronounced in *M. cajuputi* fraction No. 2 (Figure 4.16 A). Thus, this signified the potency of these fractions on *Wolbachia* upon *in vitro* exposure.

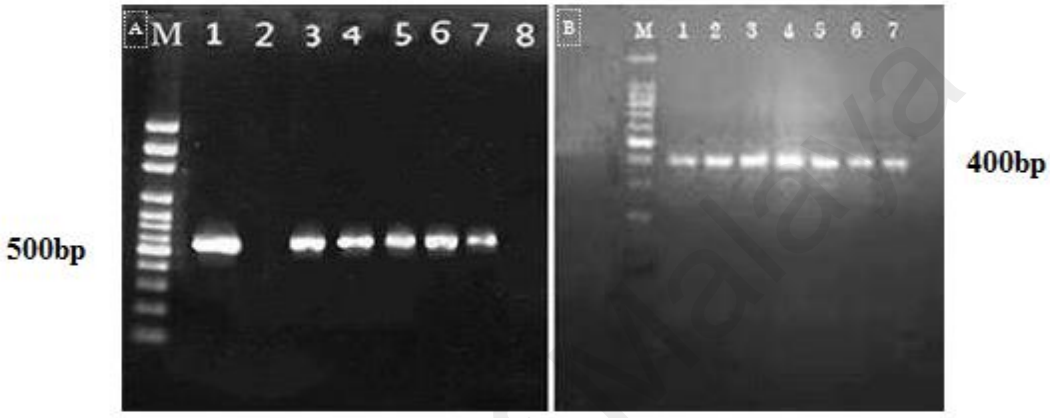

Figure 4.16: PCR analysis of *wsp* DNA obtained from Aa23 cells exposed to different fractions of *M. cajuputi* flower extracts for 7 days. Primers used were: *Wolbachia wsp* primers (A) and arthropod 28S primers (B). Lane M: molecular ladder 100 bp, lane 1 through 5: cells treated with Fraction 1, Fraction 2, Fraction 3, Fraction 4 and Fraction 5, respectively. Lane 6: non-treated cell lines (positive control), lane 7: cells treated with tetracycline, lane 8: negative control (only water - no cell line)

The best anti-*Wolbachia* activity was again in Fraction No. 2. Therefore, this fraction underwent LCMS which showed that it contained CAPE, and so it was also characterized by spectral analysis through NMR (Appendix F).

4.10.1 LCMS of Fraction No. 2

The MS parameters were optimized and 10 mg/ml of *M. cajuputi* flowers Fraction No. 2 was injected and scanned in negative mode. Fraction No. 2 was eluted as a single compound after 5.5 minutes (Figure 4.17 a) and showed that ESI negative ion provided a reliable signal where the unprotonated molecular ion $[M\text{-}H]^-$ peak was observed at m/z

283.3000 (Figure 4.17 b). The compound identity was predicted by the mass library software and the compound was matched with CAPE.

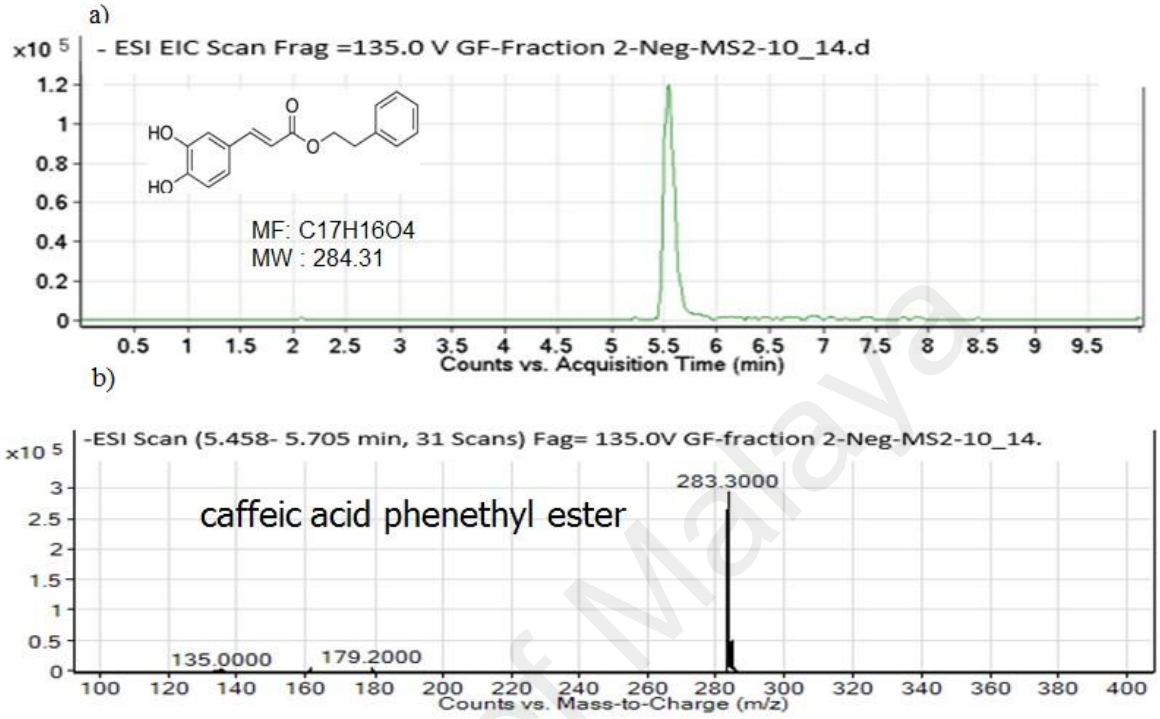

Figure 4.17: Chromatogram of *M. cajuputi* flowers Fraction No. 2

4.10.2 NMR

The NMR that is used is a JEOL 400 MHz. CAPE (8 mg) - 1H NMR ($CDCl_3$): 2.95 (t, 2H,CH_3), 4.40 (t, 2H, CH_2), 6,22 (d, 1H, CH), 6.65 (t, 1H, aromatic H), 7.20 (m, 2H, aromatic H), 7.30 (m, 5H, aromatic H), 7.54 (d, 1H, aromatic H). The structure was determined by comparison with the published data for naturally occurring CAPE (Jung *et al.*, 2008).

4.10.3 HPLC to collect more CAPE

A total of 8 mg of CAPE was isolated from 1 g of crude extract. The compound isolated was confirmed by HPLC running with a CAPE standard.

4.14.3.1 HPLC identification of CAPE vs *M. cajuputi* flowers Fraction No. 2

The retention times of both *M. cajuputi* flowers Fraction No. 2 and the CAPE standard are identical at 22.5 min (Figure 4.18).

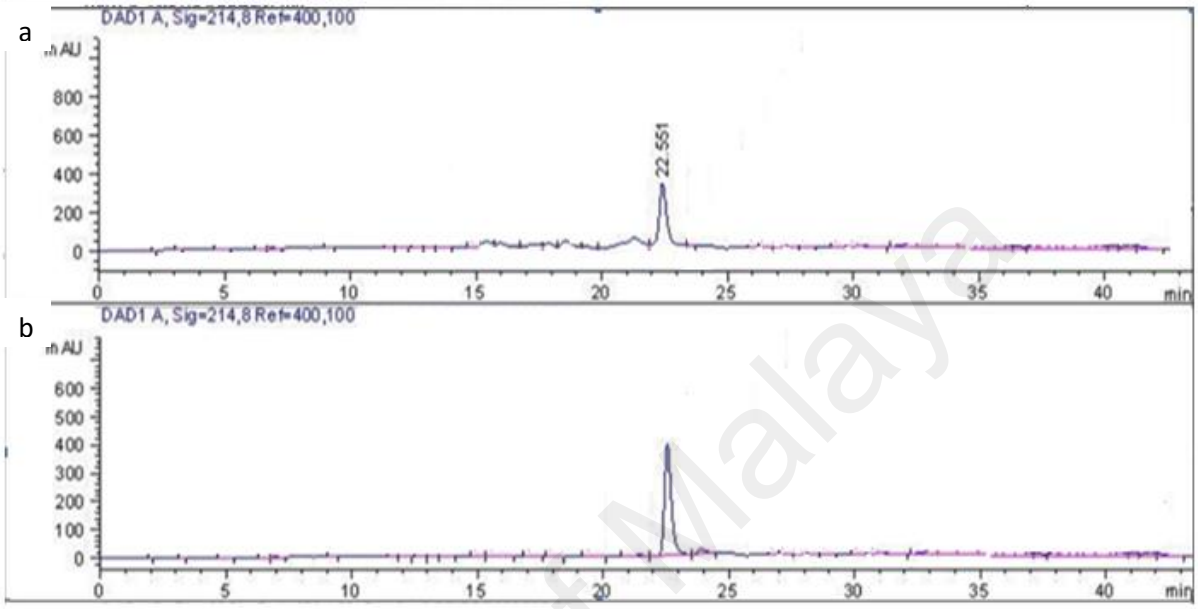

Figure 4.18: Chromatogram of a) M. cajuputi flowers Fraction No. 2 and b) standard compound CAPE under same analytical conditions.

4.10.4 Cytotoxicity of CAPE in Aa23 cell line

The *in vitro* cytotoxicity of CAPE against the Aa23 cell line was evaluated using the MTT reduction assay. The result obtained can be seen in Figure 4.19. With increasing the CAPE concentration from 0 to 100 (μg/ml), beyond 40 μg/ml resulted in a decreased cell line proliferation.

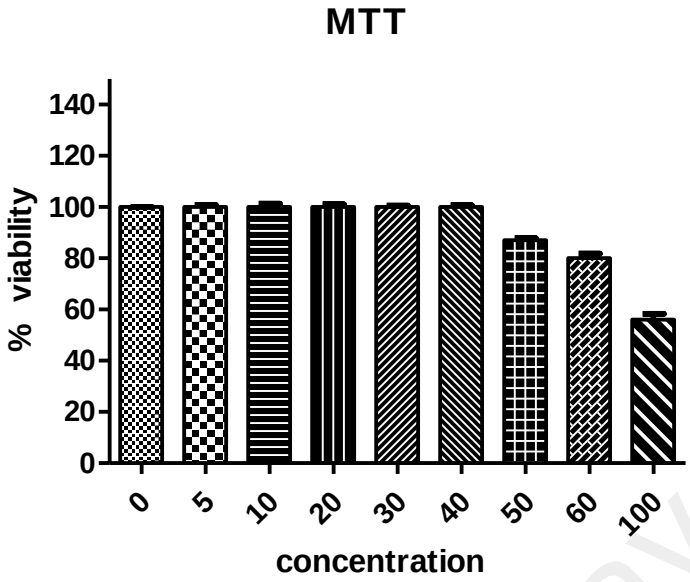

Figure 4.19: The *in vitro* cytotoxic effect of caffeic acid phenethyl ester on Aa23 cell line using MTT reduction assay

4.11 Molecular analysis of *in vitro* caffeic acid phenethyl ester (CAPE) activity against *Wolbachia*

The PCR results obtained from *Wolbachia*-infected (Aa23) cell lines that were exposed to different concentrations of CAPE (2-20 µg/ml) for a period of 7 days was presented in Figure 4.20. As expected, from the PCR electrophoretic macrographs, the *Wolbachial* DNA band signal at 590 bp was observed to strongly fade out with increasing CAPE concentration up to 20 µg/ml (Figure 4.20, top) denoting its *Wolbachial* potency at higher concentrations. DNA extracts produced a band at approximately 400 bp using 28S arthropod primers, confirming DNA template quality (Figure 4.20, bottom).

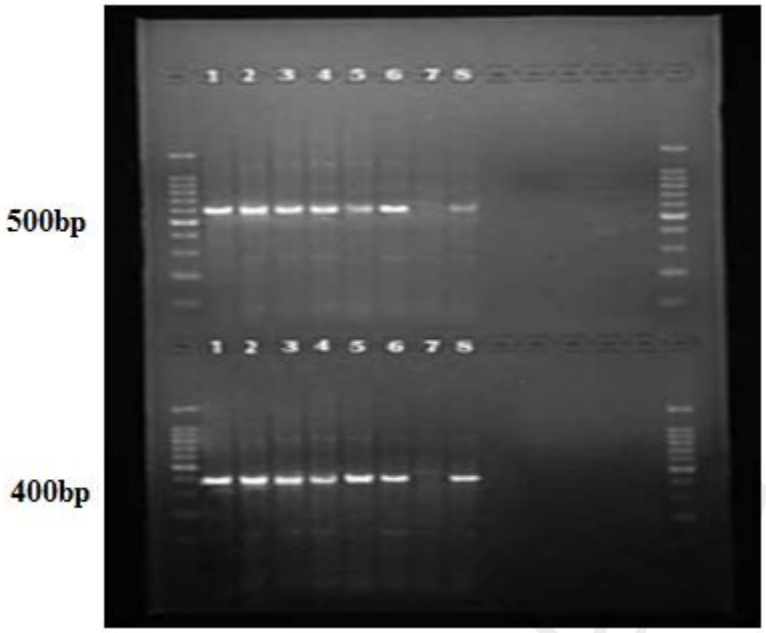

Figure 4.20: PCR analysis of *wsp* DNA obtained from Aa23cells exposed to different concentrations of caffeic acid phenethyl ester (CAPE) for 7 days. Primers used are: *Wolbachia wsp* primers (top) and arthropod 28S primers (bottom). M=leader 100 bp, in rows 1-5, CAPE concentrations are 2, 5, 10, 15 and 20 µg/ml, respectively. Lane 6: non-treated cell line (positive control); Lane 7: no cell line (negative control); Lane 8: tetracycline-treated cell line

4.12 *In vitro* caffeic acid phenethyl ester (CAPE) activity against *Wolbachia* using BacLight™ Live/Dead and FISH assays

Further validation of the effect of CAPE (20 µg/ml) on the viability of *Wolbachia* was observed using Baclight™ Bacterial Viability assay as shown in Figure 4.21. In the negative control micrographs, the cells were observed to be alive by their being stained green (Figure 4.21 a). Contrarily, the CAPE-treated sample (Figure 4.21 b) was a display of a number of dead cells (stained red).

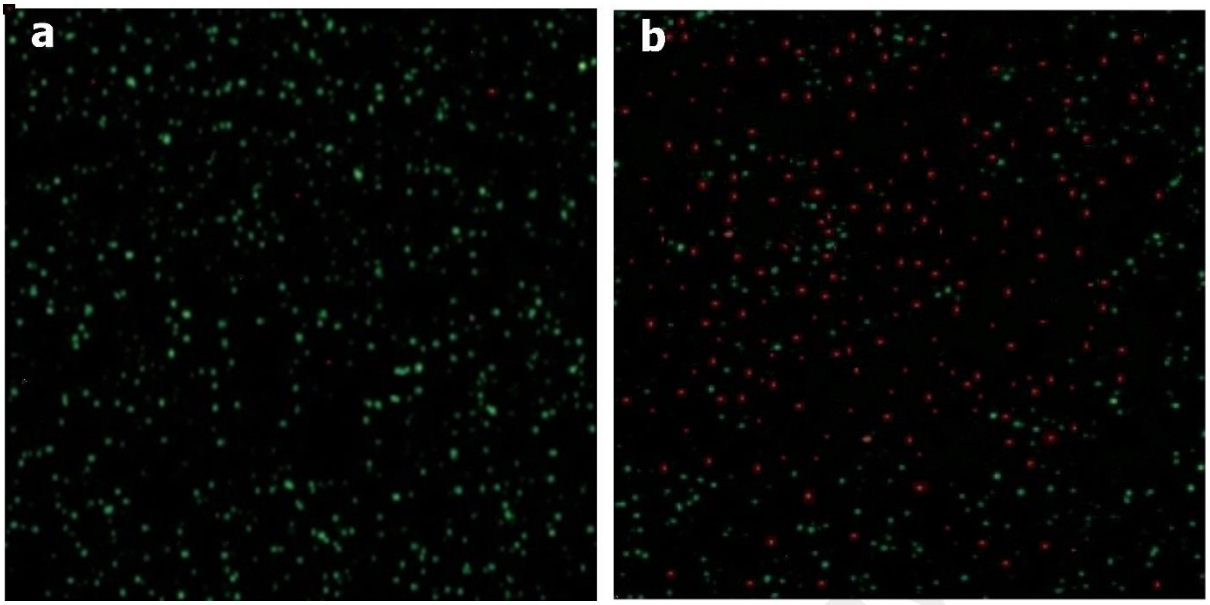

Figure 4.21: Purified *Wolbachia* organisms visualized by the BacLight live-dead assay. (a) control sample; live *Wolbachia* organisms are stained green; (b) caffeic acid phenethyl ester (CAPE) (20 μg/ml) sample; dead *Wolbachia* organisms are stained red. (Magnification x 1,000)

Further analysis using FISH ascertained the observed Baclight dead cells are really *Wolbachia*. The results obtained from this analysis (Figure 4.22) reiterated the *in vitro* anti-*Wolbachial* activity of CAPE at 20 μg/ml.

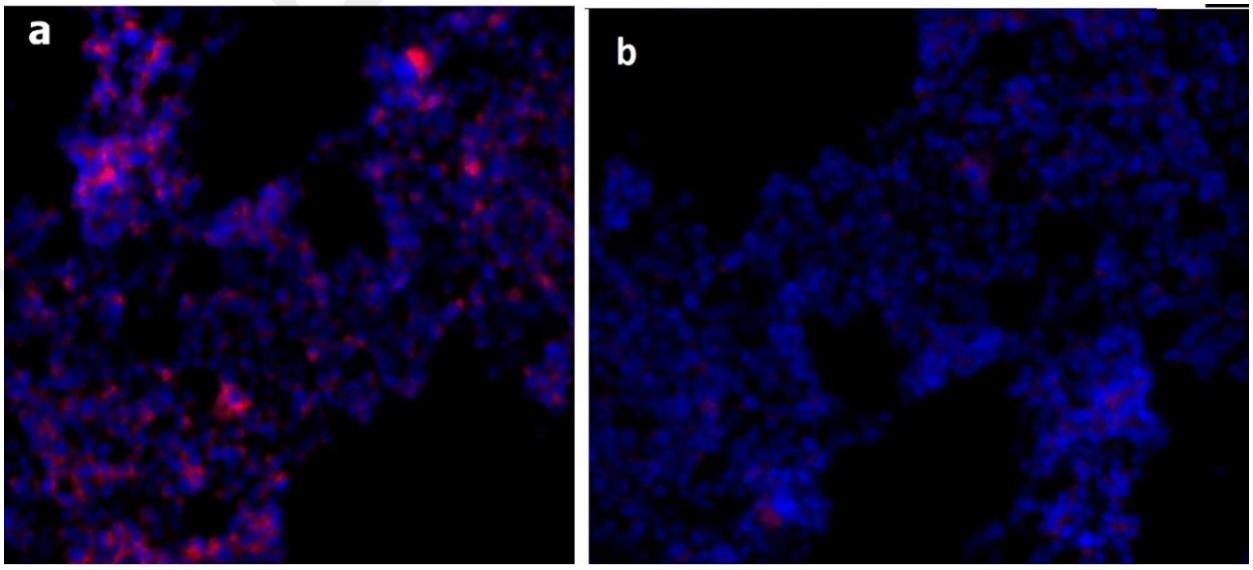

Figure 4.22: FISH (a) Aa23 negative control, (b) caffeic acid phenethyl ester (CAPE) (20 μg/ml)-treated group. Red stain - live *Wolbachia*; blue stain - cell nuclei stained with 4', 6' diamidino-2-phenylindole (DAPI)

4.13 Cytotoxicity of CAPE *in vivo* (acute and subacute toxicity)

There were no clinical signs of toxicity observed, and no death was recorded. All animals were alive up to 14 days after a single dose administration of CAPE (0.5 and 1 g/kg body weight), and up to 28 days after oral administration of the daily dose of CAPE (50, 100 and 200 mg/kg body weight). Oral administration of CAPE did not produce any noticeable toxic effects on the behavior, breathing, skin, food intake or water intake or hair of the animals. The hematologic parameters (Table 4.20) of the treated groups did not differ from those of the control group. A biochemical evaluation confirmed these results; there were no significant differences in liver function, kidney function, and body weight between the control group and treated animals (Table 4.21 - 4.25). Additionally, no changes were observed in the histopathology between the control group and the treated animals (Figure 4.23 - 4.26). These results demonstrated that the tested doses in acute and subacute study did not cause any signs of toxicity in the treated animals.

Table 4.20: Hematology parameters of treated animal groups with caffeic acid phenethyl ester (CAPE) (acute toxicity)

Parameter	Control	Caffeic acid phenethyl ester 500mg/kg	Caffeic acid phenethyl ester 1000mg/kg
WBC($\times10^3$/µl)	8.5±1.3	87.8±2.0	9.2±0.98
RBC ($\times10^6$/µl)	7.2±0.23	7.0±0.34	6.8±0.1
Haemoglobin (g/dl)	11.6±0.2	11.3±0.87	10.8±0.8
WBC($\times10^3$/µl)	8.5±1.3	87.8±2.0	9.2±0.98
RBC ($\times10^6$/µl)	7.2±0.23	7.0±0.34	6.8±0.1

Values expressed as mean±S.E.M. There were no statistically significant differences between the measurements in different groups. The significance value was set at $P < 0.05$.

Table 4.21: Biochemistry parameters of treated animal groups with caffeic acid phenethyl ester (CAPE) (acute toxicity)

Parameter	Control 0.01 DMSO	Caffeic acid phenethyl ester 500 mg/kg	Caffeic acid phenethyl ester 1000mg/kg
ALP (U/L)	107.0±1.6	103.1±2.1	106.3±0.9
ALT(U/L)	112.4±3.1	103.3±1.4	104.0±1.6
AST (U/L)	93.4±1.9	91.3±1.4	88.7±0.8
T.Billirubin (umol/L)	3.3±0.25	3.2±0.75	3.2±1.2
T.protein (g/L)	55.6±1.2	53.4±1.3	53.5±2.0
Urea (mmol/L)	8.3±2.1	8.1±0.9	8.1±0.87
Creatinine (umol/L)	18.0±1.3	17.8±1.6	17.6±1.6

Values expressed as mean ±S.E.M. There were no statistically significant differences between the measurements in different groups. The significant value was set at $P < 0.05$.

Table 4.22: Haematology parameters of treated animal groups by caffeic acid phenethyl ester (CAPE) (subacute toxicity)

Parameters	Control 0.01 DMSO	Group II 50 mg/kg	Group III 100mg/kg	Group IV 200mg/kg
WBC (×103/µl)	7.55±0.25	7.4±2.2	6.9±0.9	6.1±2.3
RBC (×106/µl)	7.69±0.27	7.69±0.23	7.0±0.29	7.6±0.12
Haemoglobin (g/dl)	12.8±0.98	12.9±3.5	11.7±1.3	12.8±2.5
Platelets (×103/µl)	681±7.45	599±21	620±12.0	613±13.4

Values expressed as mean ±S.E.M. There were no statistically significant differences between the measurements in different groups. The significance value was set at $P < 0.05$.

Table 4.23: Biochemistry parameters of treated animal groups by caffeic acid phenethyl ester (CAPE) (subacute toxicity)

Parameters	**Control 0.01 DMSO**	**Group I 50 mg/kg**	**Group II 100 mg/kg**	**Group II 200 mg/kg**
ALP (U/L)	110±4	106±11	103±12	120±15.8
ALT(U/L)	100±13.33	109±0.9	95±0.2	104± 6.9
AST (U/L)	90.6±2.46	77.6±4.79	74.8±0.13	80±13.2
T.Billirubin(umol/L)	3.33±0.87	3.6±0.67	3.67±0.33	3.6±1.1
T.protein (g/L)	54±3.2	51±2.0	54±3.8	52±2.9
Urea (mmol/L)	9.95±1.15	8.5±0.98	8.3±1.7	9.2±3.4
Creatinine (umol/L)	18.83±2.55	18.8±2.5	18.67±2.33	18.9±2.9

Values expressed as mean ±S.E.M. There were no statistically significant differences between the measurements in different groups. The significance value was set at $P < 0.05$.

Table 4.24: Body weight of animal groups treated by caffeic acid phenethyl ester (CAPE) (acute toxicity)

Week of treatment	Control	500 mg/kg	1000 mg/kg
W0	25.0± 1.5	25.9±1.8	26.4±1.3
W1	26.0±2.0	26.7±2.3	27.3±1.8
W2	27.0±2.6	27.5±1.6	27.9±2.5

Values expressed as mean ±S.E.M. There were no statistically significant differences between the measurements in different groups. The significance value was set at $P < 0.05$.

Table 4.25: Body weight of animal groups treated by caffeic acid phenethyl ester (CAPE) (subacute toxicity)

Week of treatmente (n=6)	Control	Caffeic acid phenethyl ester 50mg/kg	Caffeic acid phenethyl ester 100mg/kg	Caffeic acid phenethyl ester 200mg/kg
W0	26.3± 1.0	27.2±1.8	26.8±2.3	27.7±4.6
W1	27.2±1.1	27.9±2.3	27.0±1.8	28.0±3.2
W2	28.3±0.9	28.8±1.6	28.9±0.9	29.6±3.4
W3	29.0± 1.3	29.9±1.6	29.6±0.4	30.3±1.4
W4	30.1±1.6	30.8±1.9	30.9±1.0	31.09±3.6

Values expressed as mean ±S.E.M. There were no statistically significant differences between the measurements in different groups. The significance value was set at $P < 0.05$.

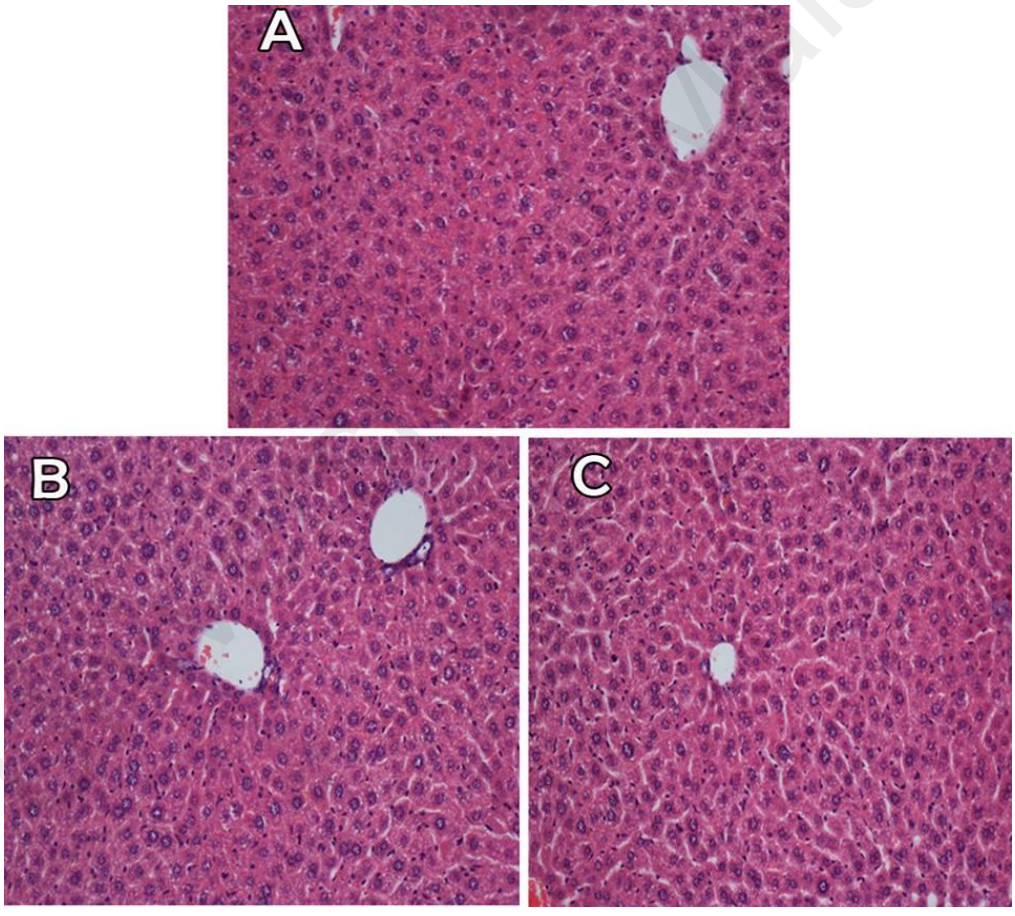

Figure 4.23: Histological micrographs of Jird's liver section treated with (A) 5 ml/kg (5% DMSO) (vehicle control), (B) CAPE (500 mg/kg) and (C) CAPE (1000 mg/kg) (H & E stain 10x)

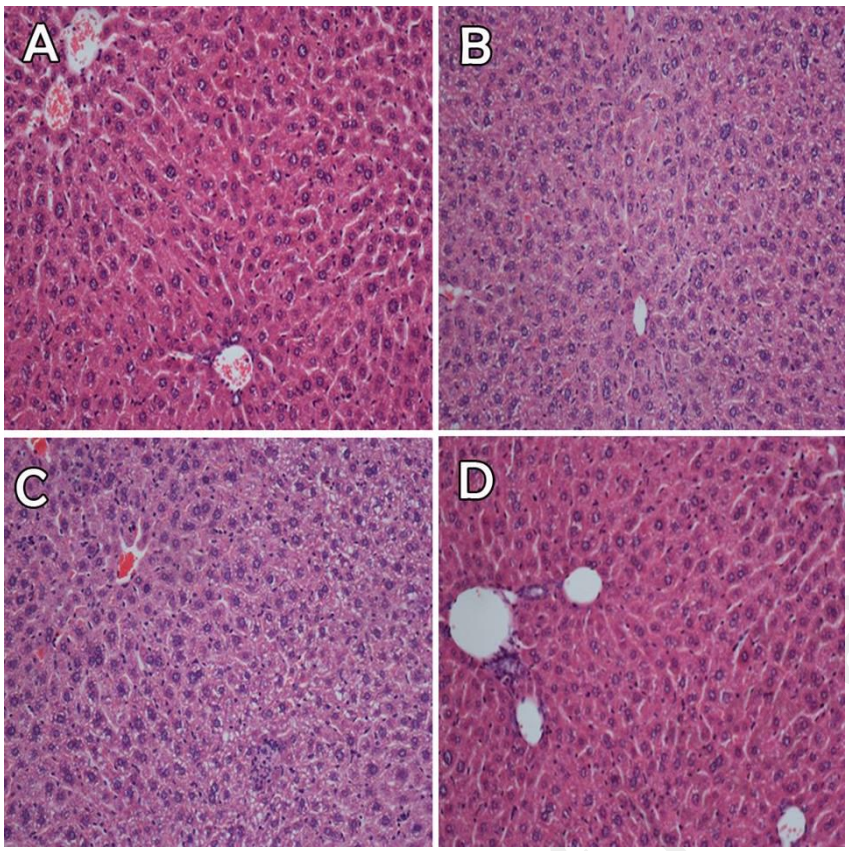

Figure 4.24: Histological micrographs of Jird's liver section treated with (A) 5 ml/kg (5% DMSO) (vehicle control), (B) CAPE (50 mg/kg), (C) CAPE (100 mg/kg) and (D) CAPE (200 mg/kg) (H & E stain 10x)

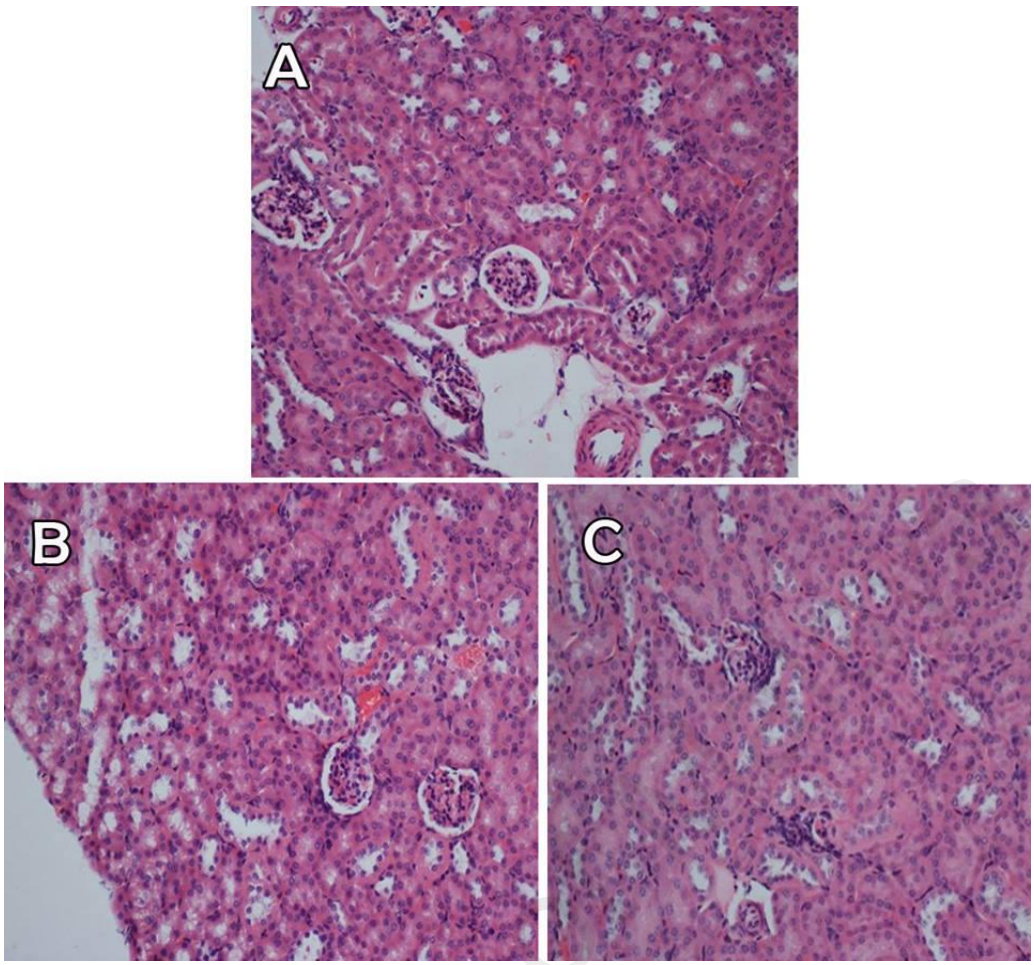

Figure 4.25: Histological micrographs of Jird's kidney section treated with (A) 5 ml/kg (5% DMSO) (vehicle control), (B) CAPE (500 mg/kg) and (C) CAPE (1000 mg/kg) (H & E stain 10x)

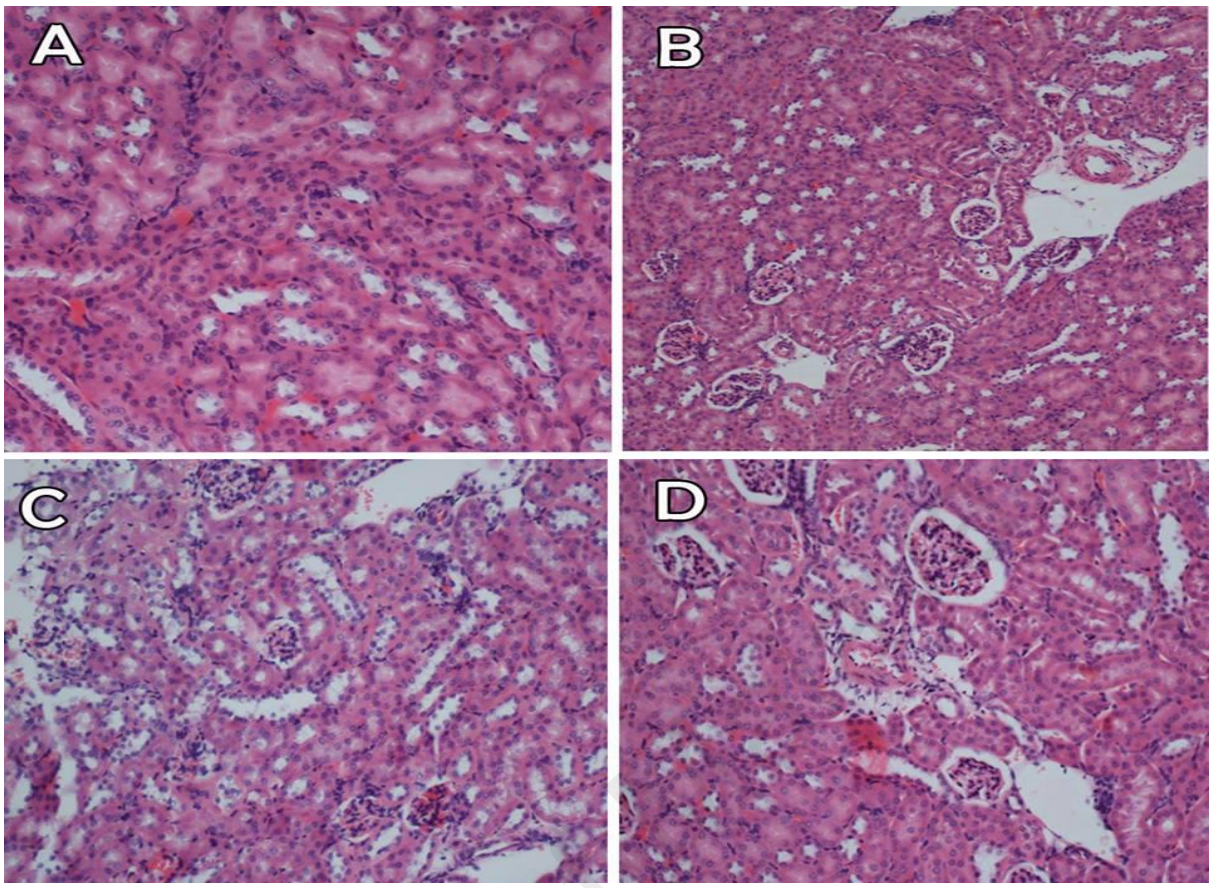

Figure 4.26: Histological micrographs of Jird's kidney section treated with (A) 5 ml/kg (5% DMSO) (vehicle control), (B) CAPE (50 mg/kg), (C) CAPE (100mg/kg) and (D) CAPE (200mg/kg) (H & E stain 10x)

4.14 Antifilarial activity of CAPE *in vitro*

The *in vitro* anti-filarial activity of CAPE was also evaluated on *B. pahangi*. The results are displayed in Figures 4.27 to 4.29. As is shown, the mean motility score (Figure 4.27) was found to be concentration dependent. This agrees with the analysis of worm viability that was tested within a 7 day period (Figure 4.28). As expected, the reduction in worm viability was observed to increase with increasing concentration. Similarly, Mf release was also found to be reduced in a concentration-dependent fashion (Figure 4.29). Furthermore, the reduction in Mf release was observed to be risen with prolonged incubation time from day 2 to day 6. The motility of CAPE (20µg/ml)-treated worms differed significantly ($P < 0.05$) from that of the positive control (ivermectin) at the 5^{th},

6th and 7th days of the experiment. The motility between other concentrations and ivermectin did not differ significantly after exposure to either treatment.

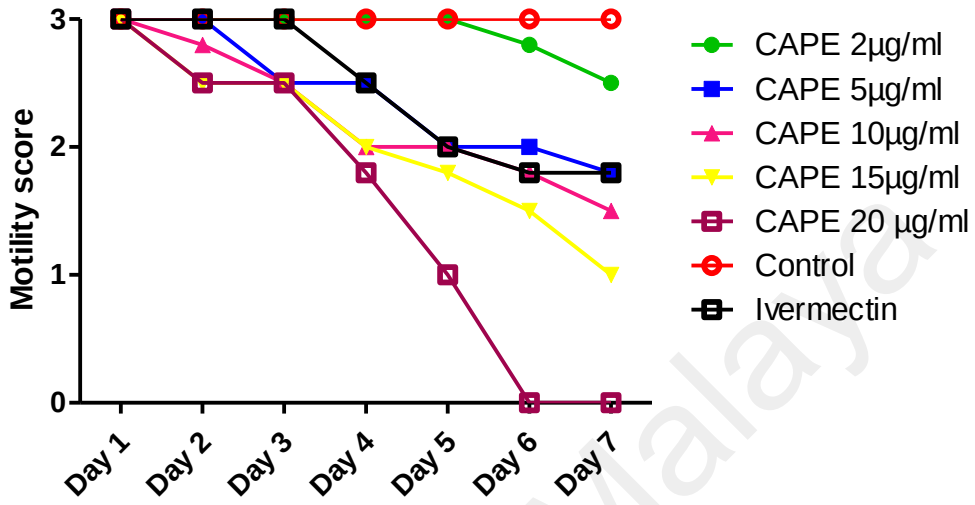

Figure 4.27: Motility score of adult worms of *Brugia pahangi* exposed to CAPE (2-20µl/ml) and control worms

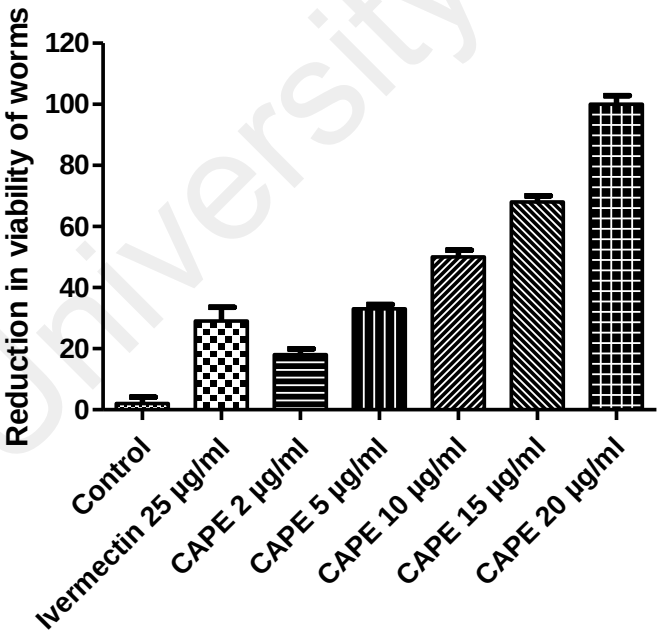

Figure 4.28: Effects of CAPE on *B. pahangi* adult worm viability. Worms were cultured with CAPE (2–20 µg/ml) for 7 days. Viability was measured by the MTT reduction assay. Results shown are as percent reduction in formazan production relative to control cultured worms

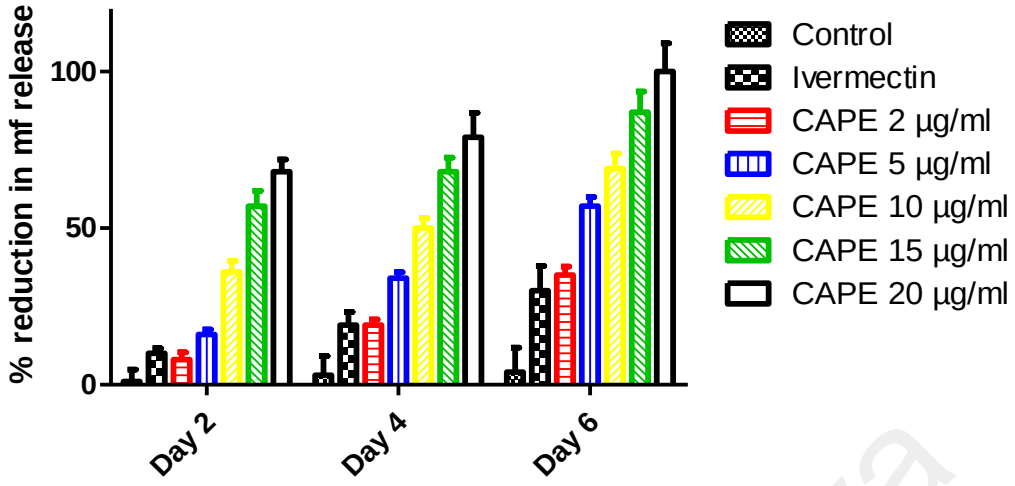

Figure 4.29: Effects of CAPE on the number of Mf released by *B. pahangi* female worms *in vitro*. Results shown are percent reduction in Mf release in treated worms relative to untreated control worms

4.15 *In vivo* study

The *in vivo* effects of CAPE in comparison to DEC and doxycycline on Mf release in *B. pahangi* within a period of 90 days was observed and the results are depicted in Table 4.26. Regarding CAPE treatment, the effect was also found to be concentration dependent.

The CAPE treatment regimen (50 mg/kg/day) for 14 days was capable of highly reducing the number of Mf that started gradually decreasing from day 45, where a 28.9% reduction at day 60 compared to pretreatment levels was observed and that subsequently decreased further by day 90, where Mf levels were reduced by 63.9 %. Conversely, 20 mg/kg CAPE treatment did not show a noticeable reduction in Mf density versus untreated infected controls. Similarly, the regimen of doxycycline using the same route and dose had no significant effect on circulating Mf in infected gebrils. DEC treatment on day 15 also exerted a significant reduction in Mf counts, with about a 34.7% drop in Mf levels remaining below pre-treatment levels temporarily with significant differences

up to day 45. Similarly, the number of worms recovered was also found to be reduced with the increase in CAPE concentration (Figure 4.30). In fact, treatment with CAPE at 50 mg/kg resulted in a significant decrease in the number of recovered worms ($P < 0.05$) in comparison to other treated groups.

Table 4.26: *In vivo* effects of CAPE in comparison to DEC and doxycycline on *Brugia pahangi* microfilaria (Mf)

	Day 8	Day 15	Day 30	Day 45	Day 60	Day 75	Day 90
Doxy 50mg/kg	+73.6±8.5	+96.8±11.3	+93.5±11.4	+64.8±13.6	+122.9±10.1	+113±20.9	+81±23.8
DEC 50mg/kg	+70.6±11.8	-34.7±9.4**	-39.8±3.5**	-27.8±6.9**	+9.0±3.9	+23.8±9.8	+108.9±15.7
CAPE 20mg/kg	+75.9±7.8	+88.8±20.5	+107.0±3.9	+149.8±8.9	+204.8±18.7	+328.8±42.9	+612.9±38.9
CAPE 50mg/kg	+68.9±26.7	+34.8±30.4	+16.9±30.9	-17.9±30.0**	-28.9±20.8**	-39.6±11.9**	-63.9±13.2**
Control	+98.8±14.9	+154.9±21.9	+228±28.6	+323.8±17.3	+393±23.6	+487±13.5	+889.9±78.8

Gerbils (n = 5) were treated with CAPE (20 mg/kg/bw), CAPE (50 mg/kg/bw), DEC (50mg/kg/bw) and Doxy (doxycycline) (50 mg/kg/bw). Controls were untreated. During two weeks of treatment, Mf density was examined on days 8, 15, 30, 45, 60, 75 and 90. ** $P < 0.01$.

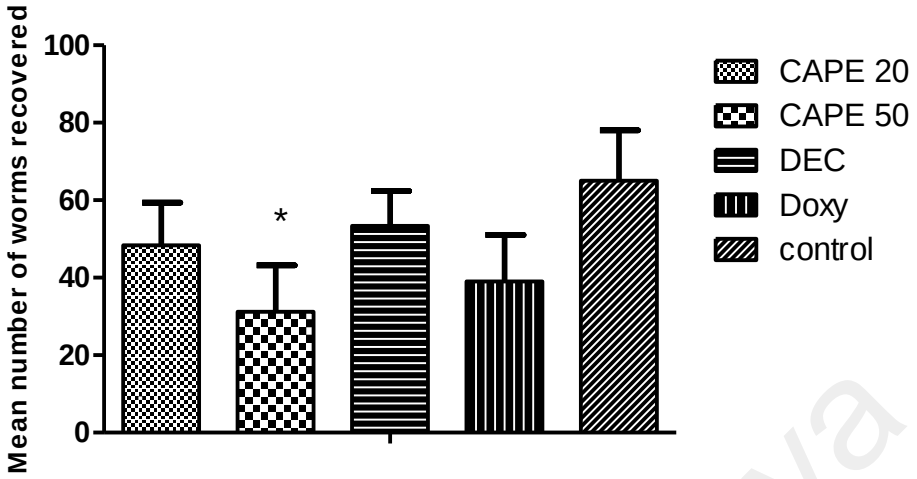

Figure 4.30: Mean number of worms recovered from *B. pahangi*-infected gerbils at necropsy. Data are presented as mean numbers of worms ±S.E.M. * Differences at CAPE 50 were significant at ($P < 0.05$) in comparison to the untreated control

4.15.1 Copy number of *Wolbachia*

The determination of the quantification of *Wolbachia* in *B. pahangi* based on a single copy *wsp* gene of these bacteria is thus assumed to correspond to the quantification of *Wolbachia*.

The results for *wsp* gene copy number analysis are presented in Figures 4.31 and 4.32, respectively. Amplification curves of *wsp* A 5-point 20x dilution series of plasmid DNA standards can be seen in Figure 4.31 A. They show a steady decrease in the threshold cycle (CT) with increasing sample concentration. In order to verify the linearity of the PCR reaction and to determine the efficiency of the system, a standard curve from serial dilutions of crude *B. pahangi* preparation was constructed (Figure 4.31 B). Amplification, data acquisition and analysis was carried out using a LightCycler instrument. The y-axis units are arbitrary fluorescence units. The mean threshold cycle (CT) value of the triplicate produced by each sample is entered into the equation for the line and solved for y, giving the copy number for the sample. The efficiencies during the exponential phase were 100% for the gene.

In Reference to the negative sample with no template (Figure 4.31 D), the melting curve analysis exhibited that there was only one single peak for the amplified products and no specific amplification product or primer dimers (Figure 4.31 C).

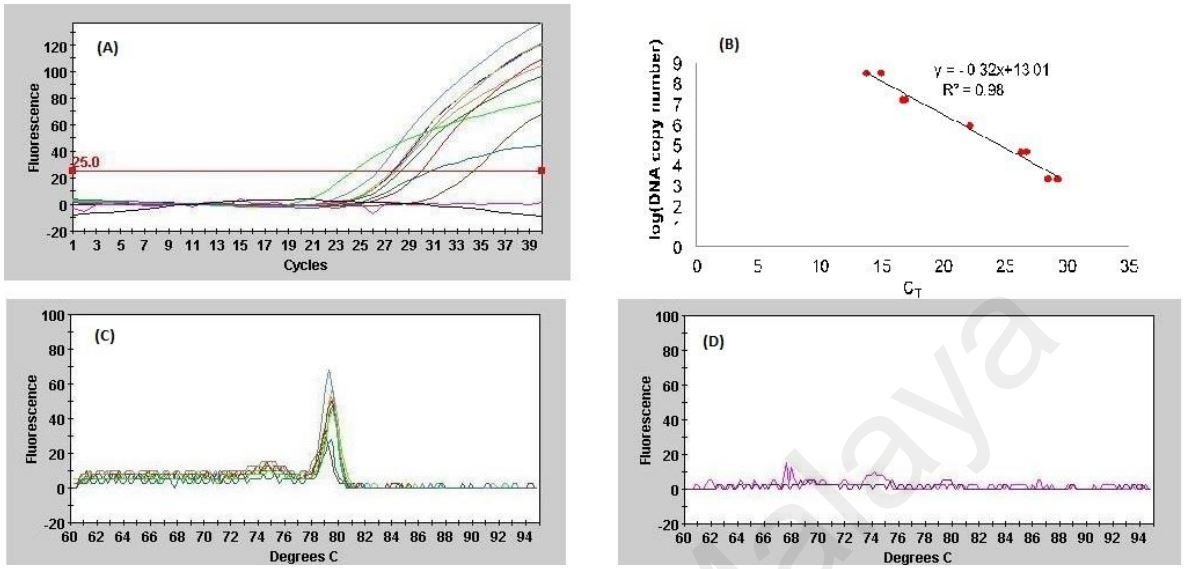

Figure 4.31: *wsp* plasmid DNA standard curve. (A) Amplification curves of *wsp* A 5-point 20x dilution series of plasmid DNA standards over 5 orders (300,000,000 – 1,875 copies). The resulting amplification curves showed a steady decrease in the threshold cycle (CT) with increasing sample concentration. (B) *wsp* plasmid DNA standard curve plotting the log copies/µL (y) of *wsp* plasmid DNA against C_T (x). The equation was calculated by linear regression of the amplification curve data derived from the dilution series generating a standard curve. qPCR efficiency was calculated using -1 + 10 (-slope) (r^2=0.98 and 109% of PCR efficiency). Assay specificity was showed in (C) the melting curve of the PCR products showing a single peak for the amplified products. (D) Negative sample (no template control) did not show any product or primer dimers

The copy number of the *wsp* gene of *Wolbachia* in all groups are shown in (Figure 4.32). Interestingly, the highest dose of CAPE was related to the highest significant reduction of *wsp* among all groups. In worms exposed to CAPE 50 and doxycycline, *wsp* expression was clearly shown to exhibit a potent reduction of *wsp* copy number with significant decrease ($P < 0.01$) compared to the untreated group and the standard DEC drug group. However, in the CAPE 20 treated group, *wsp* expression was significantly diminished at ($P < 0.05$) versus the control group, though there were no significant differences in the constitutive expression of *wsp* in the DEC treated group with respect to the control group ($P > 0.05$).

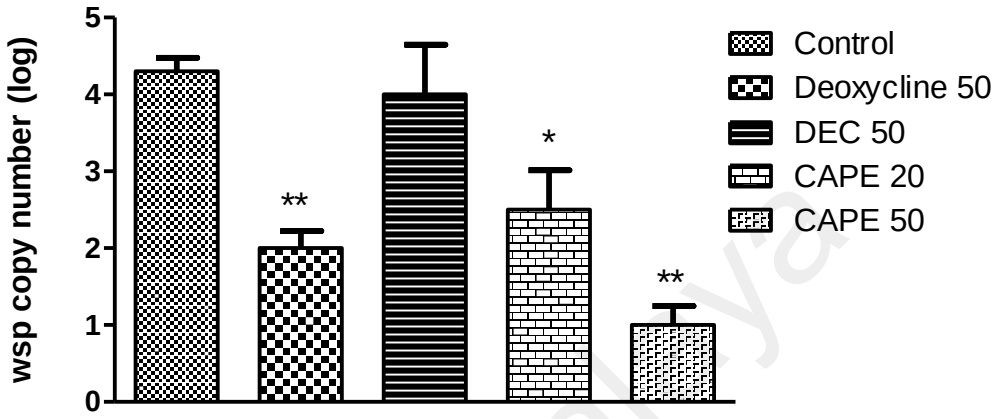

Figure 4.32: Copy number comparisons between treated and control (untreated) groups. *Wolbachia* are depleted from *B. pahangi* after CAPE treatment. The depletion of *Wolbachia* from CAPE treatment based on *wsp* copies were measured by qPCR of gDNA extracted from treated and control worms. PCR reactions were performed in triplicate. All results are expressed in mean ±S.D. * $P < 0.05$, ** $P < 0.01$

4.15.2 Gene expression analysis

The integrity and concentration of RNA was also determined. The ratio of the absorbance readings at 260 nm and 280 nm were used to evaluate the quality of RNA. In this study, the 260/280 ratio for the RNA preparation spanned the range of 1.8 – 2.1, thus indicating appropriate quality RNA (Appendix G).

According to the Normfinder and genNorm algorithms, the endogenous reference genes that show the lowest variability and the most consistent RNA expression levels prepared from worms was NADH in comparison to beta actin and histone 3, in line with the previous findings (Ghedin *et al.*, 2009). However, b-actin and histone 3 showed differential expression, and therefore the transcriptional levels of the target genes were normalized using

NADH that was consistently expressed in worm tissue and in all experiments in the study (Appendix G). The gene expression levels of the target genes in worm tissue samples were normalized using *NADH* as a reference gene. The expression levels of *wsp*, *fstz*, *Shp-1*, cytochrome c oxidase, *Hsp-90* and the reference gene *NADH* were subsequently validated by RT-PCR. According to the standard curve (Appendix G), all genes demonstrated that the efficiency lies between 90-110% and the slope between (-3.1) and (-3.5) are within the reference criteria to run the quantitative RT-PCR (Table 4.27).

Table 4.27: Measured efficiency slope and R^2 of target and endogenous reference genes using the Ct method with 5 concentrations points

Genes	*NADH*	*wsp*	*fstz*	*Hsp-90*	*Shp-1*	Cytochrome c oxidase	Reference criteria
Effeiciency (%)	100.7%	103	103.1	97.8	101	99.8	90-110
Slope	-3.3	-3.24	-3.25	-3.17	-3.2	-3.16	(-3.1) –(-3.5)
R^2	0.953	0.984	0.963	0.99	0.99	0.998	$\geq$ 0.98

All measured mRNAs showed a significantly different expression in comparison to the control group. Figure 4.33 represents the result for real-time PCR analysis, and as can be seen, there is a clear increase in the level of expression of cytochrome c oxidase in doxycycline at $P < 0.01$ (8.56± 1.20-fold increase), CAPE 25 at $P < 0.05$ (6.26 ± 0.60-fold increase), and CAPE 50 at $P < 0.01$ (9.38±0.4-fold increase) groups versus than the DEC group which did not show any significant difference (5.01 ±0.7) in comparison to the normal control group.

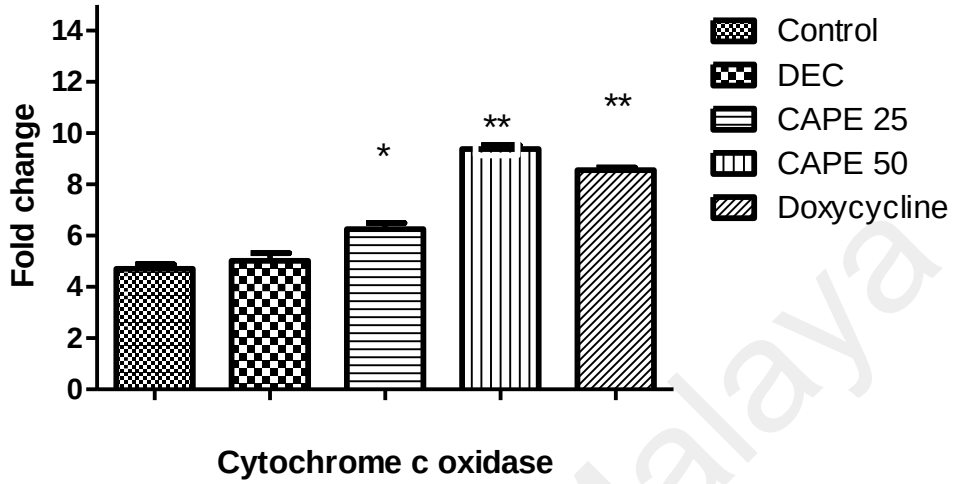

Figure 4.33: Real-time PCR showing relative-fold changes in cytochrome c oxidase in treated groups in comparison to control groups. Values represent mean ±S.D. * indicates significance versus control group P <0.05. ** indicates significance versus control group at $P < 0.01$

The analysis for the relative-fold changes of *Hsp*-90 in the treated group is seen in Figure 4.34. Relative to the control group, there is a significant increase in the difference in mRNA expression ($P < 0.01$) with a 9.948± 2.021-fold increase in the CAPE 50 group compared to the other treated groups that showed no significant difference.

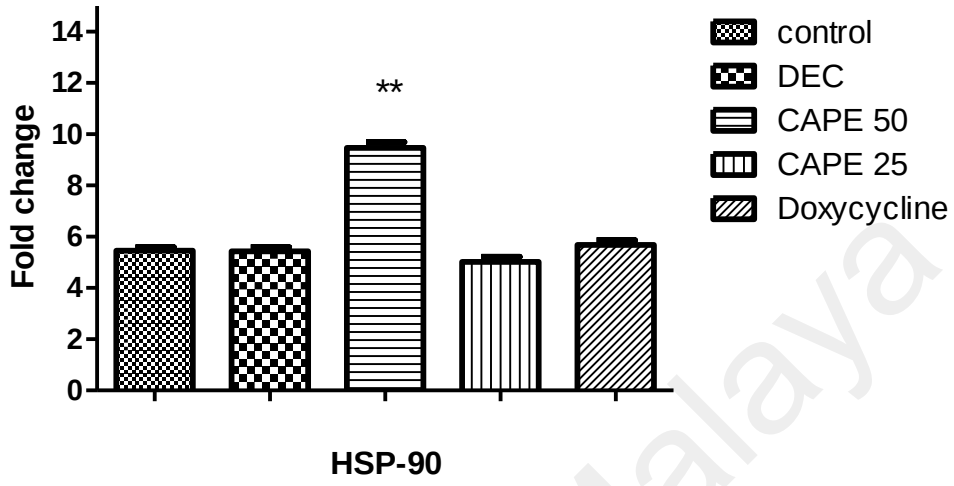

Figure 4.34: Real-time PCR showing relative-fold changes in *Hsp*-90 treated groups in comparison to the control group. Values represent mean ±S.D. * indicates significance versus control group P <0.05. ** indicates significance versus control group at $P < 0.01$

In contrast to heat shock protein analysis, RT-PCR result relative to the *wsp* gene (Figure 4.35) suggest a significant at ($P < 0.01$) decrease in *wsp* in CAPE 50 (2.38±0.8-fold change), CAPE 25 (4.3 ± 1.0-fold change) and doxycycline (3.5 ± 0.6-fold change). However, the decrease was observed to be non-significant in DEC (10.9± 0.9)-treated groups. A like trend of decrease ($P < 0.01$) in fold changes of *B. pahangi* sheath protein was observed (Figure 4.36) and *fstz* analysis (Figure 4.37). When viewing both figures, there was no significant decrease in the fold changes of either Bp-*shp* 1 (7.43 ± 0.7) or *fstz* (15.4 ± 0.9) in the DEC-treated group compared to the control group. Meanwhile, the mRNA expression of the Bp-*shp* 1 gene in the CAPE 25-, CAPE 50- and doxycycline-treated groups showed a significant reduction at $P < 0.01$ (9.47±0.54-, 5.8±1.74-, and 7.52± 0.7-fold changes, respectively) as shown in Figure 4.36.

For the *fstz* gene; CAPE 50, CAPE 25 and doxycycline showed significant decrease in the mRNA expression at $P < 0.01$ with fold declines of 5.01 ± 0.9, 5.5 ± 1.0, and 3.8 ± 0.8, respectively.

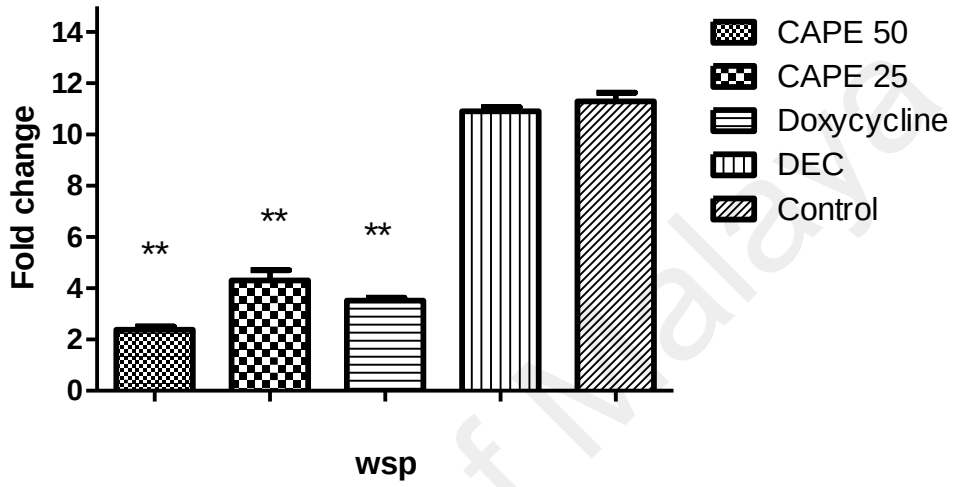

Figure 4.35: Real-time PCR showing relative fold changes in *Wolbachia* surface proteins in treated groups in comparison to the control group. Values represent mean ±S.D. ** indicate significance versus control group at $P < 0.01$

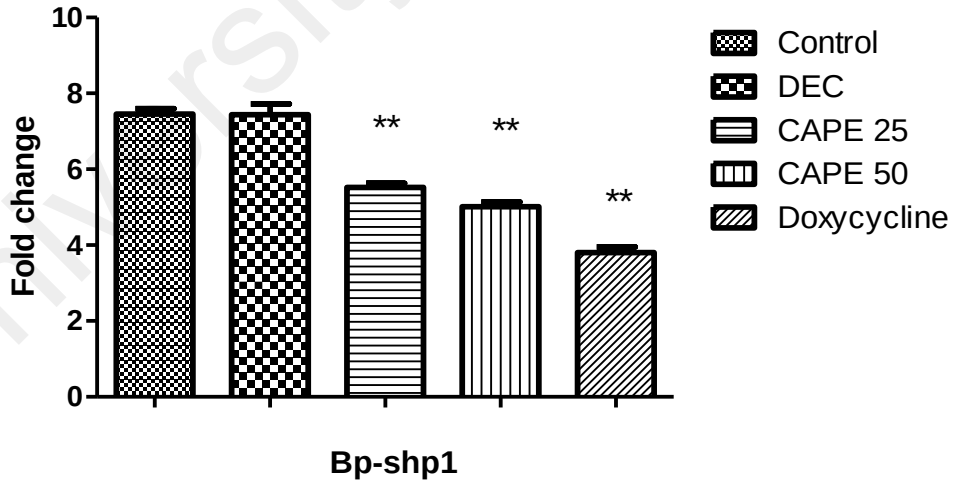

Figure 4.36: Real-time PCR showing relative fold changes in *B. pahangi* sheath protein in treated groups compared to the control group. Values represented in mean ±S.D. ** indicate significance versus control group at $P < 0.01$

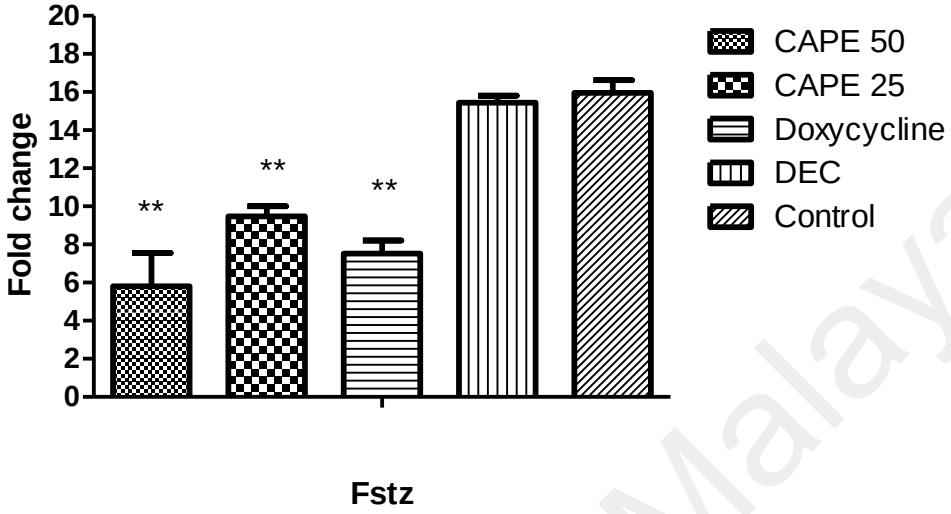

Figure 4.37: Real-time PCR showing relative fold changes in *fstz* in treated groups compared to control group. Values represent mean ±S.D. ** indicates significance versus control group at $P < 0.01$

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CHAPTER 5: DISCUSSION

5.1 Knowledge, Attitude, and Practice on LF

Incorporation of KAP surveys was recommended by the WHO as a cornerstone for health promotion campaigns as they consider it important in helping programmes to adjust health education messages to improve public knowledge and attitudes (Mathieu *et al*., 2004). The KAP concerning LF infection differs from one region to another and is heavily influenced by socio-cultural settings. Little information has been formally documented about how communities incorporate LF, its origins and impact into local knowledge systems (Wynd *et al.*, 2007). As is best presently understood, this is the first study on KAP in relation to LF among people living in LF endemic areas in peninsular Malaysia.

The survey was conducted among residents of Kemaman district in the state of Terengganu in Malaysia. The respondents included 158 (68.7%) females and 72 males (31.3%).

Overall, the results had demonstrated that most respondents were women, and they did pose some degree of potential bias. However, this is not intentional, and we did not expect that the findings were to be affected as both women and men have equal chances of getting the infection. Moreover, some medical personnel with ample experience were used to help collect the data, especially in light of the administration of the questionnaires. This was the effect of these personnel being mostly indigenous to the area - the language they speak is the same with that of the respondents and they also perform direct interactions with the target population, so involving them would facilitate compliance and cooperation and subsequently,

the most honest information will be able to be gathered. Furthermore, all aspects of the survey were conducted with close scrutiny of the researchers**.**

This survey done on indigenous adults who have lived in the area for at least 5 years was the pioneering work in an LF endemic area of Malaysia. The limitation of the survey rests on only those who attended the clinics on various reasons; all pregnant women however, were not included in the study. Most of the survey respondents were female, as it has been found that women make more hospital visits or they are naturally more cooperative and willing to become volunteers for surveys. After receiving training from the researchers, medical doctors and nurses had distributed the questionnaires to ensure that there would be no unbiased reporting and responses from the subjects.

The findings here showed that although there was an MDA programme among the endemic communities in the area some years back, about 16% of the respondents still had no prior knowledge of LF. As a result, the people had poor knowledge of the disease. This is in agreement with previous findings from endemic communities in Thailand (Rauyajin *et al.*, 1995), Ghana (Ahorlu *et al.*, 1999) and India (Babu & Kar, 2004). Prior knowledge regarding any disease is paramount to its successful control. The survey of this work indicates that the major source of information in regards to knowledge about LF were schools, health centers and the mass media. This is in line with previous findings (Aswathy *et al.*, 2009). Moreover, in a survey of KAP towards LF in the Plateau state of Nigeria, Azzuwut *et al.*, (2013) found that majority of the respondents obtain their knowledge of LF through health centres and mass media (Azzuwut *et al.*, 2013).

In our survey, the majority of the respondents knew that mosquitoes are responsible for transmitting the disease. This coincides with the findings of (Mukhopadhyay *et al.*, 2008;

Azzuwut *et al.*, 2013). However, in similar research performed in India (Rath *et al.*, 2006; Karmakar *et al.*, 2011), the majority of respondents did not know that mosquitoes transmit LF. The implication of this poor knowledge is that families may not take appropriate measures to protect their loved ones, which could serve as an impediment towards efforts to control the disease.

In the present work, the majority of the respondents recognized the common symptoms of LF, such as swelling of the legs and fever. This is consistent with the findings from (Hopkins *et al.*, 2002; Mathieu *et al.*, 2004; Mukhopadhyay *et al.*, 2008; Azzuwut *et al.*, 2013). On the contrary, Karmakar *et al.* (2011) reported in his study in endemic areas in India that the majority of respondents did not know the symptoms of LF.

The findings from this survey revealed that the majority of respondents preferred hospital treatment whenever they are sick. This alludes to a high degree of awareness of the usefulness of hospitals. However, about 40% of respondents still consider traditional methods in the treatment of their illnesses. This correlates to the reports in Nigeria (Azzuwut *et al.*, 2013), where the majority of survey participants were reported to prefer hospital treatment, while others still cling to traditional methods. Although most of the respondents preferred hospital treatment, their knowledge of the drug used for the treatment of filariasis was poor, and largely indicated that they did not know the drug used. This finding is in accordance with previous literature from India (Azzuwut *et al.*, 2013). Ultimately, this attitude reflects poor awareness regarding the disease among the respondents in this endemic area. Further, despite the fact that the research area has been tagged as the most endemic for LF in Malaysia, the current work's survey regarding treatment-seeking behavior found that only about 12% of participants, including their families, had ever received treatment for LF. This therefore suggests that people are still ignorant about the disease and its treatment.

The findings that concern with the attitudes of the respondents towards LF showed that most had viewed LF to be a problematic disease. Furthermore, the respondents had raised different views with regard to the significance of LF as a problem, where they viewed LF as a medical, social or an economic problem. This is in line with one of the latest Indonesian surveys (Krentel *et al.*, 2006). With only two-thirds of the survey participants stating that they viewed LF to be a problem, this suggests that there is poor level of awareness and knowledge of the disease in general among the residents of this endemic community. That said, there is a need to increase the various efforts to improve education to ensure that LF can be controlled effectively.

Prevention from mosquito bites is an important step to take in order to ensure eradication of LF. Most respondents also indicated using protective clothes or bed nets. However, approximately one-sixth of the respondents did not provide any indication that they had used any form of protection. Some respondents did not mention any protective measures and this only indicates that the respondents did not value the need to get protection or that they were at a discomfort in taking preventive measures. Some other barriers, such as the cost, availability or ease of use of the materials can also come into the picture. Either way, the lack of knowledge on the transmission of LF is quite evident.

Interestingly, this survey revealed that most of the respondents were aware of several ways used to control mosquito breeding, demonstrating some understanding of vector control strategies, although this knowledge was not necessarily translated to be part of an effective eradication programme, similar to the findings of (Azzuwut *et al.*, 2013). However, our findings contradict the reports by (Eberhard *et al.*, 1996; Krentel *et al.*, 2006; Wynd *et al.*, 2007), where the majority of subjects do not know the importance of minimizing mosquito contact in preventing infection.

Of the work presented herein, the results showed that about 64.8% of respondents were not even aware of the programme that took place in the area and roughly 16. 1 % had not heard of LF, meaning that large proportions of people that took drugs during the MDA campaign consumed it without proper knowledge of the reason it was administered.

Thus, the knowledge gap regarding the disease and general attitudes and perceptions towards the programme may be the basis of the major causes for lower compliance (Rath *et al.*, 2006). This probably accounted for the continued endemicity of this disease in the area because the poor knowledge of the MDA programme resulted in low compliance rates. Poor knowledge is the precursor to poor participation, and poor participation leads to low coverage and persistence in the disease transmission. Moreover, it was observed that the MDA programme in Malaysia had primarily focused on distributing the drugs to people, with little emphasis on ensuring that they actually took the drugs or that they are educated on preventive measures, such as knowing how to use the bed nets. Hence, suspected patients kept on making visits to the hospitals with various complaints.

However, in this survey, no admitted cases of LF in any clinic were encountered. In previous studies in Kenya (Njomo *et al.*, 2012) and in India (Mukhopadhyay *et al.*, 2008; Ghosh *et al.*, 2013), there was low compliance to MDA as a result of the poor knowledge in the target population of LF. Moreover, in this survey, of those that indicated having knowledge of an MDA programme, the majority heard of it via schools, mass media and health centres. This is consistent with a report from a previous similar survey (Azzuwut *et al.*, 2013). In contrast with Ghosh *et al* (2013), who reported that the major forms of information were leaflets and posters, Chattopadhyay *et al*. (2012) noted that personal communication was a critical source. The use of appropriate means of communication based on prevailing conditions is always imperative to ensure that messages get to the target

audience. The success of the MDA programme to treat LF is dependent on the knowledge of the target population. It cannot be assumed that the distribution of information in schools, health centers and by the mass media is sufficient to convey information successfully. Recent studies have shown that the distribution of information leaflets and posters are effective (Ghosh *et al.*, 2013).

As evidenced by the findings of a previous survey, the knowledge gap regarding LF, as well as the general attitudes towards and perceptions of the eradication programme, was the basis of the major causes of lower compliance (Rath et al., 2006), and this could have likely resulted in the continued endemicity of LF in specific areas of Malaysia. This has already been shown to be the case in Kenya (Njomo et al., 2012), Papua New Guinea (Graves et al., 2013), and in India (Mukhopadhyay et al., 2008; Ghosh et al., 2013), where low compliance existed for an MDA programme based on the poor knowledge of LF in the target population. With this, there is also sometimes the problem of poor drug delivery. This was observed to be among the hindrances encountered in Malaysia after the completion of TAS−1 when MDA was continued (Graves et al., 2013).

Furthermore, as documented by others, no single formula can ensure the success of MDA in all settings as compliance may be negatively affected by other factors, including the community's perceptions of the potential benefits of participation, the possible risk of adverse events as well as their fear of the unknown (MOH, 2012).

In this survey, assessment of the respondents' knowledge of LF with some demographic factors among the respondents revealed no significant association between knowledge with gender, occupation, age or educational status. Although the results showed that the females had a greater knowledge of LF, the elderly also had a higher knowledge than the young, those

who acquired formal education had higher knowledge of LF than the illiterate and the employed had more knowledge versus the unemployed. The findings here agree with that seen in Nigeria (Azzuwut *et al.*, 2013). However, these findings are contrary to the reports from the Philippines (Wynd *et al.*, 2007), where a significant association was in place between gender, age and educational status of people regarding their knowledge of filariasis.

The present study showed that the elderly people had a higher awareness of the disease, and this is probably because they grew up before the major step was taken by the country towards enhanced socioeconomic and infrastructural development. Most of them had lived with the victims for quite a long while, and some had suffered from the disease themselves. Moreover, the more literate respondents are likely to read about the disease in books or have heard about it during their school days. Similarly, the working/employed respondents are likely to acquire the knowledge of the disease in their places of work or during encounters with working colleagues during gatherings.

The results from these findings also exhibited that although the less privileged respondents had more knowledge of the disease compared to those with higher income, the difference was not statistically significant. This is in contrast to the report by Babu & Kar (2004), who noted that among urban people in Orissa, India, those from lower socioeconomic groups had significantly lower knowledge of the disease. Most of the neglected diseases are associated with poverty; hence, poverty eradication is a positive step towards their elimination.

5.2 Phytochemical extracts and antioxidant activity

Cells of living organisms generate free radicals as a result of pathophysiological and biochemical processes that occur as a result of environmental pollutants, radiation, chemicals, toxins etc. (Dröge, 2002). This causes an imbalance between formation and neutralization of prooxidants that seek stability through electron pairing with biological macromolecules, like proteins, lipids and DNA incurring oxidative stress in the physiological system (Nagmoti *et al.*, 2012). Consequently, this leads to protein and/or DNA damage, in addition to lipid peroxidation in healthy human cells. Eventually these changes lead to many chronic diseases such as cancer, diabetes, aging, atherosclerosis, cardiovascular diseases, inflammatory diseases and other degenerative diseases in human (Nagmoti *et al.*, 2012).

The ability of certain phytochemical extracts to inhibit or delay the oxidation of other molecules by suppressing the initiation or propagation of oxidizing chain reactions have made them an attractive alternative in complementary medicine. Such kinds of naturally occurring antioxidant chemicals have been reported to composed of phenolic compounds (such as flavonoids, phenolic acids and tocopherols), nitrogen compounds (alkaloids, chlorophyll derivatives, amines and amino acids), carotenoids as well as ascorbic acid (Velioglu *et al.*, 1998). In fact, phytochemical extracts such as plant-derived vitamins, flavonoids, alkaloids carotenoids, terpenoids, polyphenols and phenolic compounds such as caffeic, vanillin, ferulic, and ellagic acids have been reported to exhibit antioxidant and anticancer activities (Madhuri & Pandey, 2009).

In phytochemical analyses, the plant's extracts are normally characterised using diverse quantitative and qualitative analytical techniques spanning from chromatography to spectroscopy (Nicoletti *et al.*, 1984). In this preliminary screening, flavonoids and alkaloids

were commonly present in all samples, while the occurrence of terpenoids, saponins, glycosides and steroids depends on the type of plant extract.

A surfeit of phenolic metabolites containing aromatic arene (phenyl) ring with one or more acidic hydroxyl residues attached to it are known to be produced in plants (Ainsworth & Gillespie, 2007). Studies have shown that flavonoids, tannins, etc. were among the major phenolic contents present in plant extracts. Phenolic radicals are less reactive and possess lower electron reduction potential than oxygen radicals (Ainsworth, & Gillespie, 2007; Gillespie *et al.*, 2007). Owing to these properties, phenolic compounds are considered excellent radical scavengers. Consequently, phenolic compounds have the prospect of scavenging reactive oxygen intermediates without invoking further oxidative reactions. Therefore, it has been among the norms of phytochemical research to evaluate the total phenolic content (TPC) as a measure to ascertain the antioxidant activity of extracts. The current study evaluated the TPC of extracts and found that in *M. cajuputi*, the floral extract showed the highest TPC, while in *A. elliptica*, it was fruit extract that revealed the highest TPC. Similarly, the documented direct and rapid reaction between DPPH radicals and antioxidants has been utilized as a measure for antioxidant analysis (Milardović *et al.*, 2006). The higher the percentage DPPH radical scavenging of a compound, the better. In the current work, DPPH was also found to be associated with TPC observation, and thus the higher the TPC value, the higher the percentage of DPPH radical scavenging activity. Additionally, metal chelating analyses were found to coincide with the increase in TPC of the extracts. It is, however, not surprising that the higher TPC values confer higher antioxidant ability. This is because the substitution of 5,7,3′,4′-hydroxy flavonoids is said to possess very efficient radical scavenging power (Tsimogiannis & Oreopoulou, 2006). So, the more the content, the

better the antioxidant. Similarly, higher antioxidant activity was reported in *Ardisia* (Siti-Azima et al., 2013) and *Melaleuca* (Batubara et al., 2009).

5.3 Antibacterial activity

Much inquiry has gone into understanding the antimicrobial activities of both *Ardisia* and *Melaleuca* extracts (Hammer *et al.*, 1999; Carson *et al.*, 2006; Ramírez-Mares *et al.*, 2010; Alias *et al*., 2014). Here, *Melaleuca* flower extract was found to be active against several species of *Staphylococci* and *Bacillus cereus*, while the leaves extracts were found to be inactive against the *Bacillus cereus*. Hammer *et al.* (1999) reported a similar observation of the antimicrobial activity of *M. cajuputi* extract against *Staphylococcus aureus*. On the other hand, *Ardisia* fruit proved to be potent against both *Pasteurella* and *Klebsiella*, while the leaves extract was found to only be active against *Klebsiella* not *Pasteurella*. This observation is akin to the demonstrated antimicrobial activity of *Ardisia* extract on *Klebsiella* (Ramírez-Mares *et al.*, 2010).

Overall, plants are rich in a wide variety of phytochemical compounds, including flavonoids, alkaloids carotenoids, terpenoids, polyphenols and phenolic compounds such as caffaiec, vanillin, ferulic, and ellagic acids, and all have been reported to engage in antibacterial activities (Madhuri & Pandey, 2009)

Phytochemicals extracts have been used in disease management and therapeutics for centuries (Vadivel, 2011). Despite their promising medicinal efficacy, quite a few of these phytochemical compounds are known to incur a certain level of toxicity in living things depending on their chemical structure and type (Lagarto Parra *et al.*, 2001). Among them are those that exert their toxicity once taken, while others can only be toxic when taken at a particular concentration.

It is known that inflammatory chemical mediators released by the cells during infection are the reason behind host oxidative stress (Mogensen, 2009). In fact, previous literature has documented a marked increase in the concentration of free radical species during several pathological states, including LF (Pal *et al.*, 2006) and bacterial infections (Koedel & Pfister, 1999; Pinto *et al.*, 2012). A similar reason was given by El Abed *et al.* (2014) when correlating the capacity of antioxidant and antimicrobial activity in *Thymus capitata* essential oil. As such, and as expected in our extracts, those with higher antioxidant capacity had better antimicrobial and parasitical activity against *Wolbachia* and filarial infection. Besides that, it is important for the extract used as remedies to treat disease to possess broad spectrum activity, and moreover, it has been shown that patients with LF are predisposed to secondary bacterial infections and triggering inflammatory reactions in the skin. As a consequence, antioxidant and bacteria screening of the extracts were carried out in this study.

5.4 Infected-host trapping and parasite identification

The microscopic examination of Mf from prepared stained blood samples is said to be among the most widely used method for the diagnosis of filarial infections (Nuchprayoon *et al.*, 2005). As expected, here, the microscopic examination of Giemsa stained blood-smear slides demonstrated the presence of *B. pahangi*. In Figure 4.5, the Giemsa stained micrograph showed the presence of a clearly sheathed microfilaria (S) with an observed cephalic space (CS), innenkorper (I) and 2 terminal nuclei (TN), indicating the to be *B. pahangi*. The observed morphological characteristics of microfilarial identification in Giemsa stained slides were comparable to that described on *B. pahangi* by (Sivanandam & Fredericks, 1966).

5.5 Effect of extracts exposure on the filarial parasite

Exposing adult filarial parasites to certain external conditions resulted in low viability and decrease in Mf release. Exposing *B. malayi* to antibiotics was reported to inhibit growth and caused a significant reduction in worm viability (Rao *et al.*, 2002). In another study, the authors found that exposing the worms to gamma radiation not only reduced viability, but also Mf release (Rao *et al.*, 2005). In this study, as expected stable release of Mf and mean motility score were observed in control female worms throughout the 7 day period. However, compared to the control group, the methanol extract of *M. cajuputi* flower-treated groups uncovered a significant (P <0.05) and dose-dependent decrease in Mf release and mean motility of the worms within the period of the study (7 days). The illustrated decrease in Mf release with increasing extract concentrations was found to be related to the observed decrease in Mf release with increasing concentrations of the reference drug (tetracycline). These observations are in suitable agreement with Gunawardena *et al.* (2005), who detailed a decrease in Mf release upon treatment with tetracycline in *B. pahangi*.

The reduction in the filarial motility was further observed to be concentration and extract-type dependent. A similar trend of significant decreases in the worm's motility was observed when *Seteria cervi* was treated with *Azadrachta indica* (Mishra *et al.*, 2005), the same also observed when ethyl acetate extract from *Vitex negundo* leaves were used on *S. cervi* (Sahare & Singh, 2013).

The MTT reduction assay is a standard method used for evaluating cellular proliferation and viability (Roehm *et al.*, 1991; Vistica *et al.*, 1991; Fotakis & Timbrell, 2006). Here, the effect of the crude extract on the viability of adult worm was evaluated using it. After incubating the adult worm for 7 days with the extracts, a significant (P <0.05) and dose-dependent

decrease in both Mf release and adult worm viability, especially at higher extract's concentrations (≥ 500 µg/ml) of *M. cajuputi* flower, was observed. Furthermore, the filarial viability inhibition was observed to highly be determined by the type of extract. For example, *M. cajuputi* flower extract significantly (P <0.05) exhibited higher percentage inhibition of filarial viability versus other extracts. With this, *A. elliptica* fruit and leaves and *M. cajuputi* leaves extracts did not show a marked reduction in filarial viability. In fact, with *M. cajuputi* flower extract, exposure with extracts at 1000 µg/ml was found to incur a reduction in Mf release and adult worm viability by 100%. Similar drops in Mf and filarial adult worm viability had been seen in the literature in *B. malayi* to extract from *Bauhinia racemosa* (Sashidhara *et al.*, 2012), and in *Setaria cervi* when treated with extracts from *Hibiscus mutabilis* (Saini *et al.*, 2012).

5.5.1 Ultra-structural analysis

The filarial nematode endosymbiont bacteria, *Wolbachia,* has been described as essential in filarial larval development and fertility of the adult worm (Langworthy *et al.*, 2000; Stolk *et al.*, 2005). The effect of the extract in comparison to both negative and positive control (tetracycline) exposed with *in vitro* treatment was evaluated based on the ultrastructural analysis of intracellular *Wolbachia* in *B. pahangi* through TEM. From the TEM micrographs, the intracellular *Wolbachial* seem degenerated, signifying the anti-*Wolbachial* activity of the GF extract, which in turn enhances antifilarial efficacy. This observation corroborates similar degeneration of *Wolbachia* in *B. malayi* (Ghedin *et al.*, 2009) and *Onchocerca ochengi* (Langworthy *et al.*, 2000) upon treatment with tetracycline. In fact, *Wolbachia* are said to be major stimulus for innate inflammatory reactions, and reactivity of antibody to *Wolbachia* surface proteins are a vital factor in the pathology of lymphoedema and hydrocele in patients

with bancroftian filariasis (Langworthy *et al.*, 2000). Indeed, removal of *Wolbachia* was shown to be associated with the worm mortality and fecundity (Chirgwin *et al.*, 2003a).

5.5.2 Molecular analysis

The filaricidal activity of the extracts on *Wolbachia* endosymbionts was further evaluated using molecular DNA analysis of *Wolbachia*-specific surface protein (wsp) gene, reported to be a gene of about 590 bp (Gunawardena *et al.*, 2005; Zhang *et al.*, 2010). In both analyses, flower extracts-treated groups displayed a typical dose-dependent disappearance of the *wsp* gene amplicon. Interestingly, when the positive control lane was compared with that of the treated groups, the intensity of the *wsp* gene amplicon was observed to become greatly reduced with increasing concentrations of the treatment. This noticeably demonstrated the antimicrobial effect of the extract on the *Wolbachia* population in the filarial parasite.

Molecular techniques were used to explore the mutualistic relationship between *Wolbachia* and the filarial parasite. Foster *et al.* (2005) have indicated that *Wolbachia* provide riboflavin, flavin adenine dinucleotide, heme and nucleotides to the worm, which in return, is provided with amino acids required for its growth. In consequence, eliminating the *Wolbachial* endosymbiont was proposed as an alternative for killing the filarial parasite (Townson *et al.*, 2000).

Previous works have illustrated a similarly reduced intensity of the *wsp* gene amplicon upon treatment with tetracycline (Gunawardena *et al.*, 2005). This observation was also found to be in accordance with a report that exposing *Brugia spp*. to tetracycline treatment for a period of less than two weeks is not sufficient to completely clear the *wsp* DNA amplicon (Rao & Weil, 2002). Additionally, Bazzocchi *et al.* (2008) reported a significantly diminished *Wolbachia* population upon treatment with ivermectin and deoxycycline. In

general, the results presented in this study confirm previous reports of worm death following the chemotherapeutic elimination of *Wolbachia* endosymbionts that are said to be essential in the worm's reproduction process (Rao & Weil, 2002).

5.6 Chemical constituents of *M. cajuputi* flower

As mentioned before, in phytochemical analyses, the plant's extracts are normally identified using diverse quantitative and qualitative analytical techniques spanning from chromatography to spectroscopy (Nicoletti *et al.*, 1984). Previously, Li and Sheu (1995) employed the use of a micellar electrokinetic capillary chromatographic (MEKC) method to assess the phytochemical contents of a scute-coptis herb couple. Near infrared spectroscopy was also used to screen the content of *M. cajuputi* foliar oil (Schimleck *et al.*, 2003). In another study, GCMS was used to characterize the chemical content of *Melaleuca* essential oils (Sakasegawa *et al.*, 2003; Silva *et al.*, 2007). Silica gel chromatography has been employed to isolate a new chromonone from *M. cajuputi* leaves extracts (Rattanaburi *et al.*, 2013). Using a combination of spectroscopic techniques, the study characterized the newly isolated chromonone as melachromone (Rattanaburi *et al.*, 2013). A combined technique of HPLC, MS and NMR analyses were used to distinguish phytochemical compounds in plant extracts (de Mejía *et al.*, 2006).

Likewise, in this study, preliminary compound identification and quantification was performed using GCMS, and LCMS. The occurrence of the identified compounds were also reported in *Melaleuca* extracts (Ireland *et al.*, 2002; Padalia *et al.*, 2015). Previous studies have demonstrated that a number of the identified compounds in *M. cajuputi* flower extracts, such as alpha tocopherol (Chapple *et al.*, 2013) and hexadecanoic acid (Soumya *et al.*, 2014) possess antioxidant activities. Similarly, myriads of previous studies have reported the

antioxidant activities of some of the other compounds (Kraujalis & Venskutonis, 2013; Singh, 2013).

5.7 Acute and subacute toxicity of *M. cajuputi* flower

Owing to these reasons, it is of primary importance to evaluate the safety of the intended plant's extracts either *in vitro* or *in vivo* before being tested for the proposed medicinal application. Therefore, in this research, the acute and subacute toxicity of the GF extracts were also evaluated in experimental animal models for 14 and 28 days, respectively. And based on the conducted study, no visible signs of toxicity were observed even at the highest tested concentration. Additionally, data obtained from haematological, biochemical and histopathological analyses revealed an absence of toxicity upon oral ingestion of the extracts as well as the active compound CAPE. In both extracts and CAPE treatment, no observable significant difference between the control group and the treated groups in terms of WBC, RBC, haemoglobin and platelets levels, were present. Additionally, the levels of liver and kidney function tests, in the treated groups were found to be similar to control groups. Furthermore, no observable loss of weight during the treatment as the body weight in all treatments were found to be normal and comparable to the control group.

5.7.1 Histomorphometric study of kidney and liver

Hematoxylin and Eosin (H&E) staining is one of the principal staining techniques that is widely popular in clinical histology. This staining techniques have been in use for almost a century and continued to be an essential technique for recognizing various tissue types and the morphologic changes that form the basis of pathological diagnosis (Fischer *et al.*, 2008). The technique has been widely explored in evaluating the nature of phytochemical cytotoxicity (Mohd Zohdi *et al.*, 2011; Aziz *et al.*, 2014; Bai *et al.*, 2014; Hamzah *et al.*,

2014). The use of H&E was employed by Hamzah *et al.* (2014) to analyse the effect of *M. cajuputi* of alveolar bone loss in experimental periodontitis. A similar technique was used by Aziz *et al.* (2014) while observing the effect of *M. cajuputi* on inflammatory mediators in periodontitis. Therefore, in this study, H & E was also used to evaluate the cytotoxic effects of both the extracts and CAPE using tissue samples obtained from the kidney and liver of the treated jirds. In both samples, the histological micrographs revealed normal topographical structure with perfect cellular orientation in both liver and kidney samples. Thus, this confirmed the safety of the extract and CAPE at those tested concentrations.

5.8 *In vitro* activity of the *M. cajuputi* flower extract fraction and the phytochemical constituent

In this study, *Aedes albopictus* (Aa23) cell line was used to eavalute the potentinal activities of *M. cajuputi* on *Wolbachia in vitro*. DNA obtained from the infected cell line was amplified and the analysis revealed that the *wsp* gene was totally lost in cells treated with 50 µg/mL flower extract for 7 days, this indicates further anti-*Wolbachia* activity of this extract. The anti-*Wolbachial* activity of the extract has to be the result of the presence of a certain phytochemical compound in the extract. Thus, crude extract phytochemical charaterisation using GCMS and LCMS were carried out. In particular, the active component of the fraction in *M. cajuputi* flower extract was found to be CAPE. Using MTT reduction assay *in vitro* to assess the cytotoxicity of CAPE on Aa23 cell lines showed that at concentrations of $\geq$ 50 µg/ml, there was a significantly ($P < 0.05$) reduced cell line proliferation. Thus, for studying its effect on *Wolbachia* a concentrations of 0 to 20 µg/ml was used. Based on molecular analysis, a similar trend of fading of the *Wolbachia wsp* gene signal (590 bp) was observed. A similar situation is seen in the *Wolbachia wsp* gene in the Aa23 cell line upon treatment with rifampicin was reported (Brennan *et al.*, 2008).

Baclight live/dead cell testing as well as FISH assays were also performed to confirm the results that were observed by PCR. In comparison to the uniform presence of the live cells in the control sample, Baclight analysis uncovered contrasting data concerning the CAPE treatment (20 μg/ml), exhibiting the presence of dead cells. This was clearly displayed, as well, with FISH analysis, whereby the CAPE treated group had most of the *Wolbachia* removed versus the negative control group that showed many *Wolbachia* cells.

5.9 *In vitro* and *in vivo* antifilarial activity of CAPE

Exposing adult filarial parasites to CAPE resulted in low viability and a decrease in Mf release. *B. pahangi* treatment with CAPE was observed to cause a decrease in mean motality score in a concentration dependent fashion. As expected, worm viability was also found to be reduced considerably with increasing dosages of CAPE. Similarly, reduction in percentage Mf release was also observed to increase with increasing concentration, indicating that the compound has a mode of action by arresting of the worm viability *in vitro* that lead to cease the production of Mf.

The *in vivo* effect of CAPE was also evaluated. A comparison study between the effect of CAPE and DEC in reference to the control group on *B. pahangi* with *in vivo* treatment demonstrated a significant ($P < 0.01$) reduction of *B. pahangi* after 90 days of the exposure to CAPE. Additionally, the reduction was also found to be concentration dependent. Notably, the number of worms recovered was also found to be reduced with the increase in CAPE concentration (Figure 4.30). However, *B. pahangi* treatment with CAPE at 50 mg/kg (CAPE 50) harboured a significant decrease ($P < 0.05$) in the number of recovered worms compared to other groups.

It was also observed that there was a decrease in the mean number of worms recovered from gerbils treated with CAPE 50, suggesting that *B. pahangi* may survive for only a short time following the eradication of *Wolbachia*. This is steadily in agreement with (Chirgwin *et al.*, 2003b), who found that worm death followed a decrease in the number of *Wolbachia* in worms. Further, these results are in line with other instances in the literature (Bazzocchi *et al.*, 2008; Dangi *et al.*, 2010), where the conclusions that the treatment of *D. immitis* and *B. malayi* worms with doxycycline and DEC elicited no significant detrimental influence on the recovered numbers of adult worms at the post-treatment timepoint.

An effective depletion of *Wolbachia* in the present study was further evaluated by qPCR that evaluated absolute *wsp* copy numbers. A steady decrease in *Wolbachia wsp* copies with an increase in treatment dosages was observed. In fact, a significant (P <0.01) decrease in gene copy number was observed upon treatment with doxycycline and CAPE 50, and also CAPE at 20 mg/kg (CAPE 20), where a significant reduction in copy number of *Wolbachia* at (P < 0.05) compared with control groups took place. Treatment with DEC did not show a significant difference (P > 0.05) relative to the control groups. This finding is in accord with previous reports of an observed reduction in *Wolbachia* copy number in *Onchocerciasis* upon treatment with deoxycycline (Hoerauf *et al.*, 2008). A similar drop in *Wolbachia* copy number was also reported in *B. malayi* (Supali *et al.*, 2008).

In the current study, a real-time PCR assay was used to evaluate the presence of *wsp* as an indicator of the presence of *Wolbachia* by determining the number of *wsp* transcripts present. In employing this technique, it was not possible to determine whether if CAPE has a direct effect on *Wolbachia*. Besides, the death of *B. pahangi* is not known to be the result of the loss of *Wolbachia* or an alternative explanation, like the direct effect of CAPE on worms as is shown *in vitro*. Also, the products that release *Wolbachia* bacteria that known to be toxic

to the nematode could be the cause. It has been reported that the filarial nematode and *Wolbachia* are in coexistence with an obligatory mutualistic relationship, meaning each organism requires the other to survive (Taylor *et al.*, 2001). Yet another explanation for parasite death following *Wolbachia* elimination could be that products emitted by *Wolbachia* upon its death may be toxic to the nematode (Chirgwin *et al.*, 2003a). Therefore, to rule out the mechanism of action of CAPE, further study is necessary on *Wolbachia*-free filarial parasite, such as *Acanthocheilonema viteae.*

5.10 Gene expression profile

Assessing the integrity of RNA is said to be a critical step in obtaining meaningful gene expression data (Fleige & Pfaffl, 2006). Experimental work with low-quality RNA may compromise the results, especially in downstream applications which are often labour-intensive, time-consuming and highly expensive. In this study, the observed results of the RNA concentration indicated that the RNA to be of a robust quality.

Wolbachia are said to be major stimulus for innate inflammatory reactions and reactivity of antibodies to *Wolbachia* surface proteins has been reported to be a vital factor in the pathology of lymphoedema and hydrocele in patients with bancroftian filariasis (Langworthy *et al.*, 2000). As a matter of fact, removal of *Wolbachia* was shown to be associated with the worm's mortality and fecundity (Chirgwin *et al.*, 2003a).

The filaricidal activity of CAPE on *Wolbachia* endosymbionts was further measured using RT-qPCR to assess the level of expression of the *Wolbachia*-specific surface protein (*wsp*) gene and cell cycle (ftsz). The results here suggest that CAPE effects on the level of expression of both of *Wolbachia* genes in relation to the controls indicates that CAPE treatment reduces *Wolbachia* bacteria in female worms upon treatment *in vivo*. In both

analyses, a significant (P <0.05) decrease in the *wsp* gene was observed upon treatment. Similar reports of gene expression decrements in *wsp* gene were reported earlier (Rao *et al.*, 2012). Similarly, a significant reduction in the relative fold change of bacterial cell division protein (*fstz*) was observed in treatment groups versus the control or DEC groups. This particular protein is said to play a prominent role in cytokinesis (de Boer *et al.*, 1992). Interestingly, FtsZ is a GTPase, thereby making the protein an attractive *Wolbachia* drug target.

This study found a striking increase after treatment in expression of the cytochrome c-oxidase gene, reported to be associated with oxidative phosphorylation in energy metabolism and electron transfer, observed to increase in relative fold upon treatment (Rao *et al.*, 2012). A similar increase in relative fold of cytochrome c-oxidase has been described (Ghedin *et al.*, 2009). Upregulation of cytochrome c-oxidase gene after treatment suggests that CAPE treatment may partially protect filarial worms from the loss of *Wolbachia* so that they can adapt responses to stress (Ghedin *et al.*, 2009). As a point of interest, CAPE has been shown to affect host mitochondrial metabolism and reduce cytochrome c oxidase in adult worms. This may reflect some sort of direct effect of CAPE on host metabolism rather than an indirect effect because of bacteria death (Ghedin *et al.*, 2009).

Heat shock proteins (*HSPs*) are among the most important cytoprotection factors (Ahmed *et al.*, 2012). *HSPs* are a group of genetically conserved proteins that serve as molecular chaperons, functioning to ensure accurate and functional folding or refolding of bioactive proteins. They were first documented by (Ritossa, 1962), who observed them in *Drosophila melanogaster* chromosomes after heat treatment of flies. This group of proteins are said to be expressed in stress-related conditions to protect cells (Targosz *et al.*, 2012) and are located in almost every cellular compartment.

HSPs are also important in the maintenance of cell integrity during normal cellular growth as well as during pathophysiological conditions (Otaka *et al.*, 2009). Over expression of *HSPs* may protect multiple cellular compartments and prevent protein damage from oxidative stress. In particular, the cytosolic 90 kDa molecular chaperon (*HSP*-90) is said to be expressed in response to environmental changes, such as heat, oxidants and various viral and bacterial infections (Muralidharan & Mandrekar, 2013).

HSP-90 plays a role in preventing damage in the cellular repair process after injury (Otaka *et al.*, 2007). In this study, PCR analyses revealed a relative fold change in the *HSP*-90 gene upon treatment with CAPE 50 compared to the other treated groups. Our results in accordance with Ghedin *et al.* (2009), they reported that the upregulation of this gene upon treatment of *B. malayi* with tetracycline. Upregulation of this gene was attributed to the disruption of homeostasis of the endosymbionts and the attempt of the worm to adapt with this situation via a general stress response (Ghedin *et al.*, 2009).

The third gene selected of *B. pahangi* was *SHP*-1, an essential polypeptide constituent of the sheath that codes for the microfilarial sheath-protein-1 (Bp-*shp*-1). Downregulation of this gene was predicted to affect the structure of the Mf sheath and cause complete inhibition in Mf released by adult female parasites (Aboobaker & Blaxter, 2003).

In the current work, it was discovered that downregulation of development-associated transcripts, like *shp-1*, were also significantly (P <0.05) downregulated in treated groups compared to controls. Similar downregulation in sheath protein has already been observed (Ghedin *et al.*, 2009; Rao *et al.*, 2012).

CHAPTER 6: CONCLUSION

The objectives of this research have been succeefully achieved.

6.1 The main finding of this study

1. The research on lymphatic filariasis is still scanty versus several other relatively neglected tropical diseases, despite the ever-increasing burden in terms of public health and economics. Poor of knowledge in the community of LF and MDA programmes, as indicated by the current study, is still a major issue of concern. Hence, adequate training and motivation for people in endemic areas would be rather valuable.
2. *M. cajuputi* and *A. elliptica* have a significant amount of phenolic and flavonoid contents and exhibited potent antioxidant activity *in vitro* by virtue of DPPH, FRAP, and iron chelating activity.
3. *M. cajuputi* and *A. elliptica* possess significant *in vitro* antibacterial activity.
4. *M. cajuputi* methanol flower extract possess significant filaricidal activity *in vitro,* molecular evidence gathered demonstrated that macrofilaricidal activity of *M. cajuputi* flower extracts might be effective because of their anti-*Wolbachia* effects
5. *M. cajuputi* flower extracts were toxicological safe *in vivo* by oral administration.
6. Caffeic acid phenyl ester (CAPE) was the active principle component responsible for this filaricidal activity in flower of *M. cajuputi,* also found that CAPE possess macro and micro filaricidal activity, and anti-*Wolbachia* activity *in vitro,* and *in vivo*. From analyses using the Aa23 cell line and the *B. pahangi* parasite model, it may be concluded that CAPE acts through targeting *Wolbachia*. The specific mechanism of action has not yet been clarified.

6.2 LIMITATIONS

In this present study, several limitations were encountered throughout the duration of the experiments. The ethnobotanical survey took longer than expected, and no plant was identified during this study used by the participant as remedies against filarial disease. Researcher faced a variety of difficulties such as finding the infective host for filarial, as well with the breeding of mosquitoes and transinfection to animals.

As a consequence of financial support, time constrain those limitation did not allow the study to go further to study the immune response, and antioxidant activity of the plants utilised *in vivo*. As well, microarray or proteomic techniques were not carry out to further elaborate the mechanism of action of CAPE.

6.3 RECOMMENDATINOS

LF is a serious risk, despite this fact there seems to be a little research on it in comparison to other tropical diseases. It is hope that the findings of this study will assist policy makers of health authorities to improve and design more appropriate and successful control measures to reduce the prevalence of LF infections, as well as to improve MDA delivery.

The following are the main recommendation of this study:

Public health authorities need to provide proper health education to those living in endemic areas of LF on the dangers posed by the disease in terms of loss of The disability-adjusted life year (DALYs), causing permanent incapacitation to patients and huge economic loss from treatment, all leading to generally low productivity.

The effect of LF is also very serious on governments, as considerable funds are needed for both MDA administration and case management in endemic areas. There an urgent need for

continued research on methods of eliminating LF infection among endemic and vulnerable communities. This could be achieved by an effective education programme that focuses on LF transmission and prevention via public media awareness or by strategic advocacy of vector control.

The provision of appropriately innovative methods of educating residents of endemic and vulnerable communities, include incorporating public health professionals, audio-visual campaigns, and the running of workshops and seminars, is essential. In addition, participation in activities or exhibits that promote the adoption of policies regarding prevention and control of the disease will increase public awareness. Nevertheless, it is important that the information is presented in a concise, informative and easy to understand fashion.

It is recommended that an awareness campaign regarding the importance of MDA be stressed in all endemic areas of Malaysia before embarking on subsequent MDA rounds for successful implementation and control. Further ethnobotanical surveys in the Malaysian states endemic to LF to enhance the documentation of plants that are used against filarial disease and to give priority to them to be investigated for their activities against the LF is also required. Further evaluation of CAPE on *Wolbachia*-free filarial worm such as *Acanthocheilonema viteae*, in order to exclude a direct effect of CAPE on the worms, is of value. Additional evaluation of the effects of CAPE on *B. malayi* through the recommendation to use more advanced techniques, such as microarray, flow cytometry, proteomics, to ascertain the changes in gene expression to supply more molecular information about the mechanism of action of CAPE on both worms and their intracellular bacteria *Wolbachia*, is also necessary. Finally, it would be valuable to perform more extensive studies on potential synergistic interactions of CAPE with other filarial chemotherapeutic agents.

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APPENDIX

Appendix A: Questionnaire

BORANG SOAL SELIDIK

A. BIODATA:

Nama: ..... **Umur:** .....

Jantina: ..... **Suku kaum:** .....

Agama: ..... **Pendidikan:** .....

Pekerjaan: ..... **Pendapatan bulanan:** .....

Daerah/Kampong: ..... **Jenis rumah:** .....

Sanitasi.....

B. PENGETAHUAN MENGENAI FILARIASIS:

1. Anda tahu filariasis/penyakit untut/kaki gajah**?**

YA ☐ **TIDAK** ☐

(Jika jawapan adalah "TIDAK", tunjukkan gambar pesakit dan tanya soalan semula)

2. Apakah tanda dan gejala penyakit ini?

…….

…….

…….

3. Apakah penyebab penyakit ini?

…….

…….

4. Apakah yang menyebarkan penyakit ini?

…….

C. SIKAP DALAM MENDAPATKAN RAWATAN:

1. Anda mendapatkan rawatan apabila sakit terhadap Filariasis?

YA ☐ **TIDAK** ☐

2. Di mana anda mendapatkan rawatan? Where you go for treatment?

Hospital/klinik ☐ **ilmu sihir** ☐

Traditional healer ☐ **Sendiri** ☐

3. Apakah ubat yang diberi? (sekiranya ubat berupa tumbuhan, teruskan dengan soalan-soalan selanjutnya)

……

3.1 Nama tumbuhan:

……

3.2 Bahagian dari tumbuhan yang digunakan sebagai ubat:

……

3.3 Bentuk yang digunakan:

……

3.4 Kaedah aplikasi:

……

3.5 Jumlah yang diambil:

……

3.6 Tanda-tanda kesan sampingan (jika ada):

……

……

D. SIKAP TERHADAP PENULARAN PENYAKIT:

1. Bagaimana anda menghalang dari digigit nyamuk?

Memakai pakaian ☐ Menggunakan kelambu semasa tidur ☐

Lain-lain ☐ Nyatakan: …….

2. Di mana nyamuk membiak?

……

3. Apakah langkah yang anda lakukan untuk menghalang nyamuk dari membiak?

Membersih kawasan ☐ Menghapus kawasan berair ☐

Menggunakan Abate ☐

Lain-lain ☐ Nyatakan: ……

E. PENGETAHUAN MENGENAI PROGRAM ELIMINASI FILARIASIS:

1. **Anda pernah dengar mengenai** "*mass drug administration"* **(MDA)?**

YA ☐ TIDAK ☐

(Jika jawapan adalah "YA", pergi ke soalan seterusnya)

2. Dari mana anda mendapatkan informasi mengenainya?

Surat khabar ☐ Guru di sekolah ☐

Pusat Kesihatan ☐ Radio/TV ☐

Lain-lain ☐ Nyatakan: ……

3. Apa yang anda faham tentang MDA?

……..

……

……

APPENDIX B: Consent form

Course name: PhD programme, Department of Parasitology, Faculty of Medicine, University of Malaya.

Project title: _____

I, _____ (participant's name), understand that I am being asked to participate in this survey activity that forms part of PhD programme required in the University of Malaya. It is my understanding that this survey has been designed to gather information about the above project. I have been given information about this project and I understand that my participation in this project is completely voluntary and that I am free to decline to participate, without consequence, at any time prior to or at any point during the activity. I understand that the results of this survey will be used by University of Malaya PhD programme and will be published in journals and conference proceedings. I also understand that there are no risks involved in participating in this activity, beyond those risks experienced in everyday life.

I have read the information above. By signing below and returning this form, I am consenting to participate in this survey project as designed by the below named University Malaya student.

Participant name: _____

Signature: _____

Adress: _____

Date: _____

Please keep a copy of this consent form for your records. If you have other questions concerning your participation in this project, please contact me at:

Student name: _____

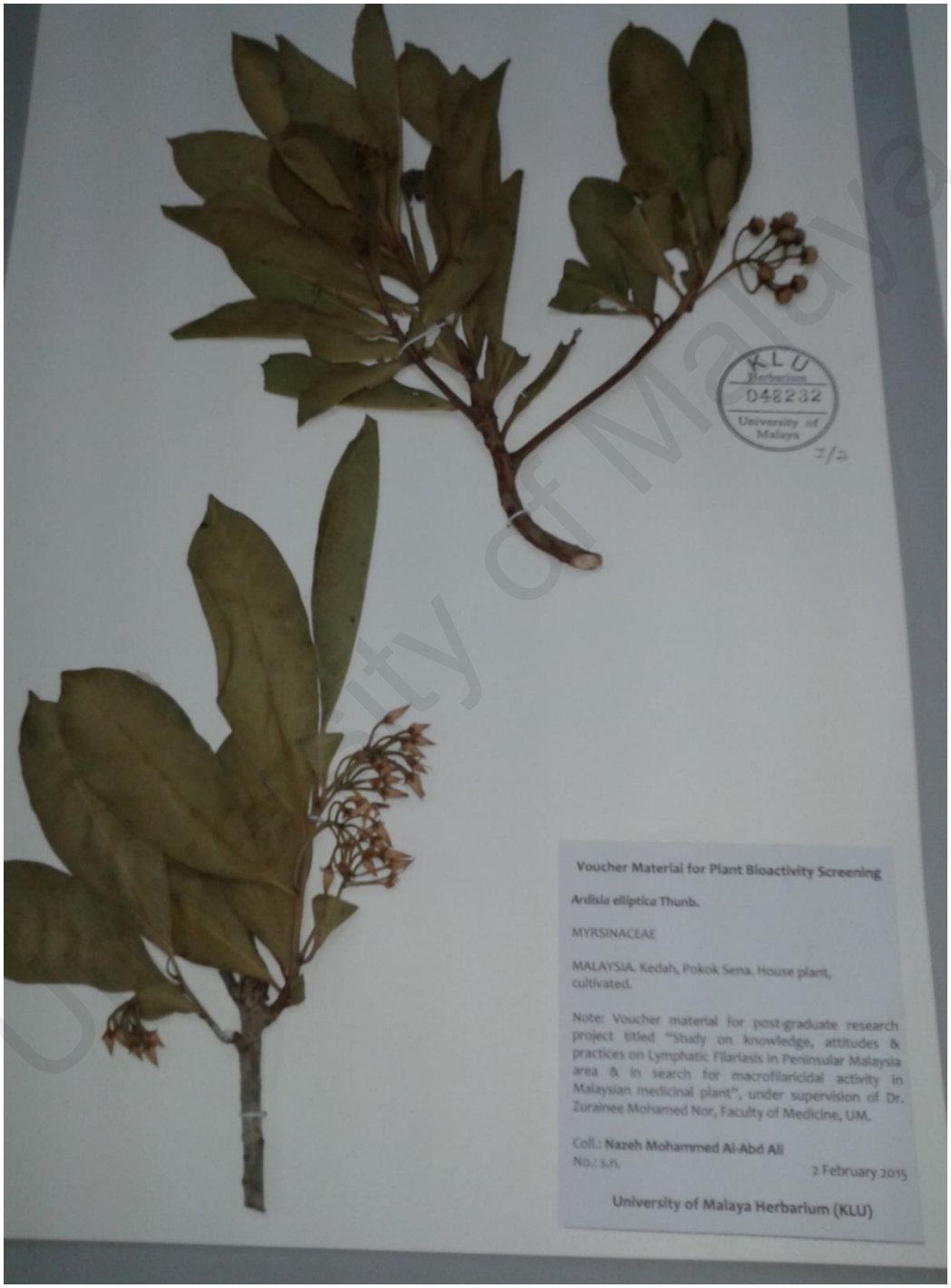
Telephone number: email address:

Principal Investigator name: _____

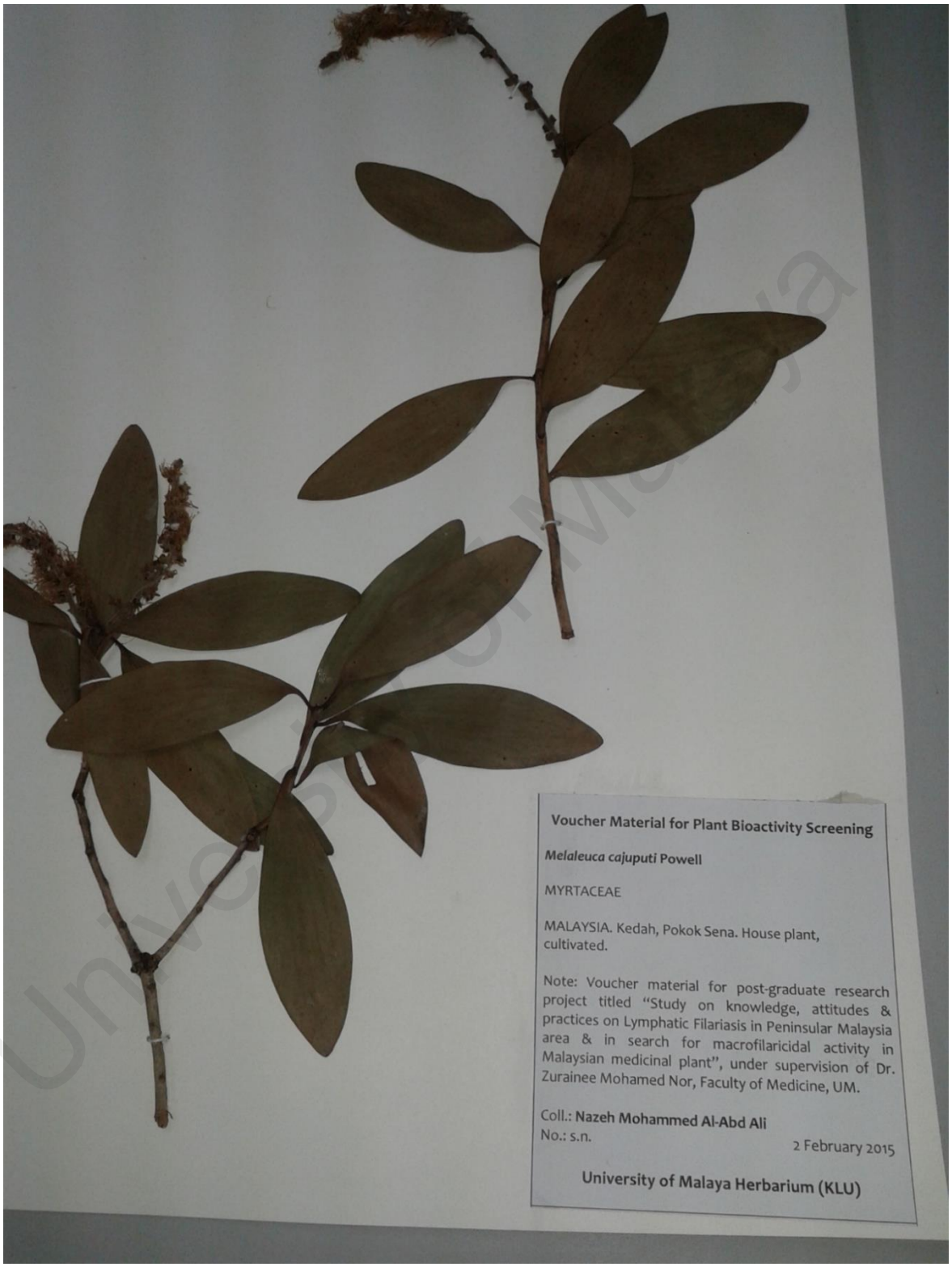
Telephone number: email address:

Thank you for agreeing to participate in my project.

APPENDIX C: Voucher speciements

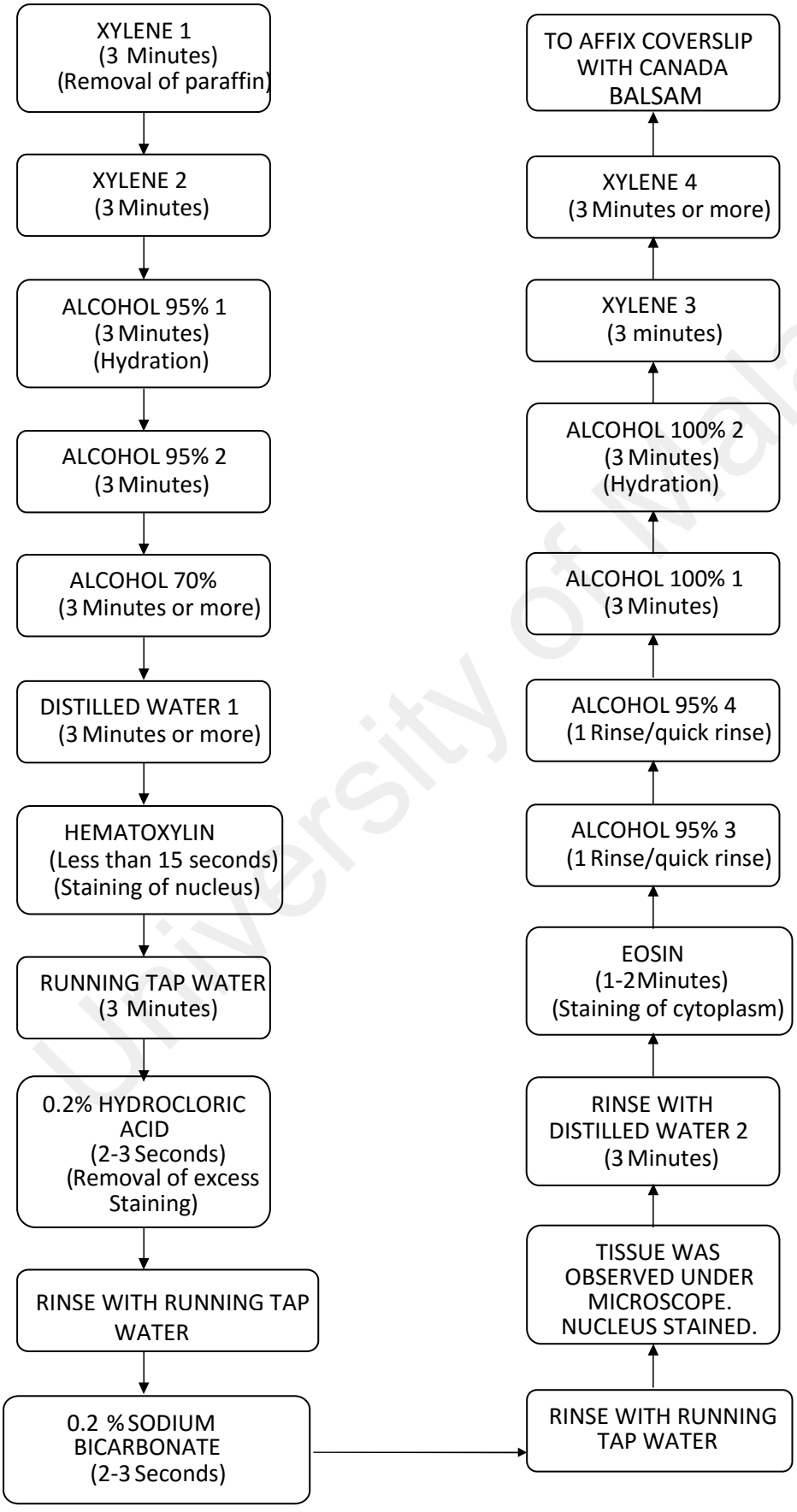


Voucher specimens of *Ardisia elliptica*



Voucher specimens of *Melaleuca cajuputi*

APPENDIX D: Time of staining



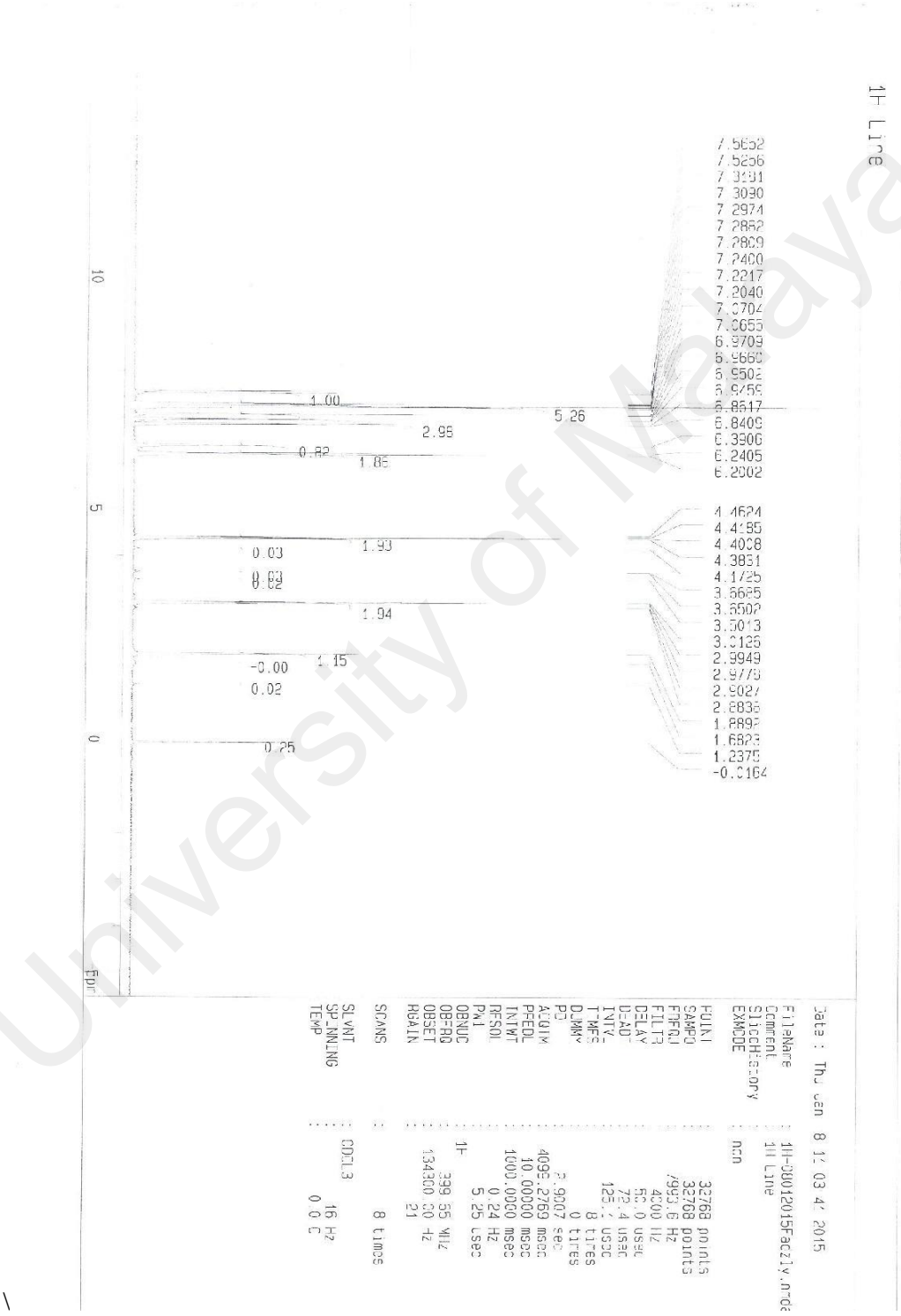
APPENDIX E: Culture medium recipe

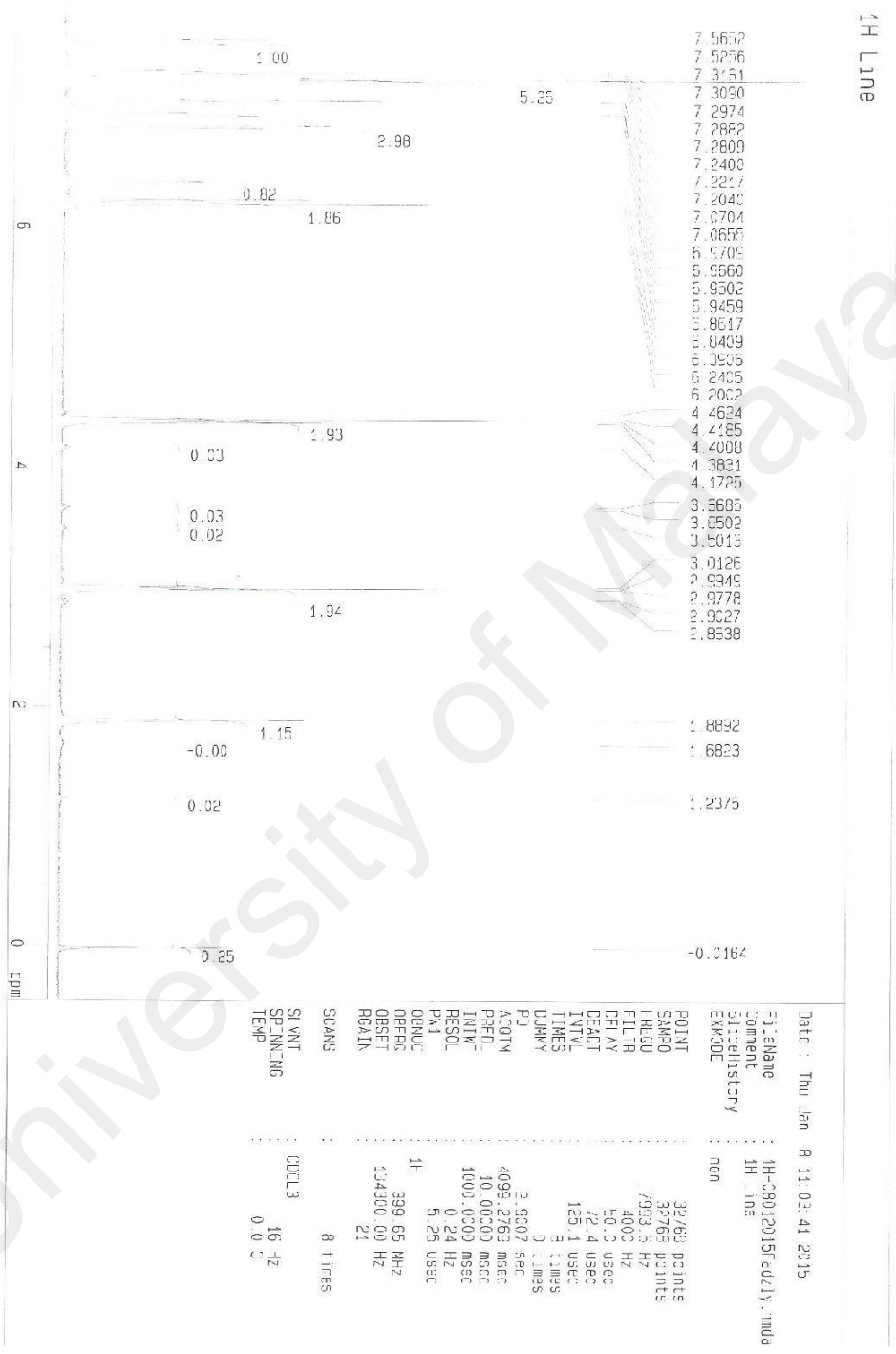
Mitsuhashi-Maramorosch Insect Medium (Handmade)

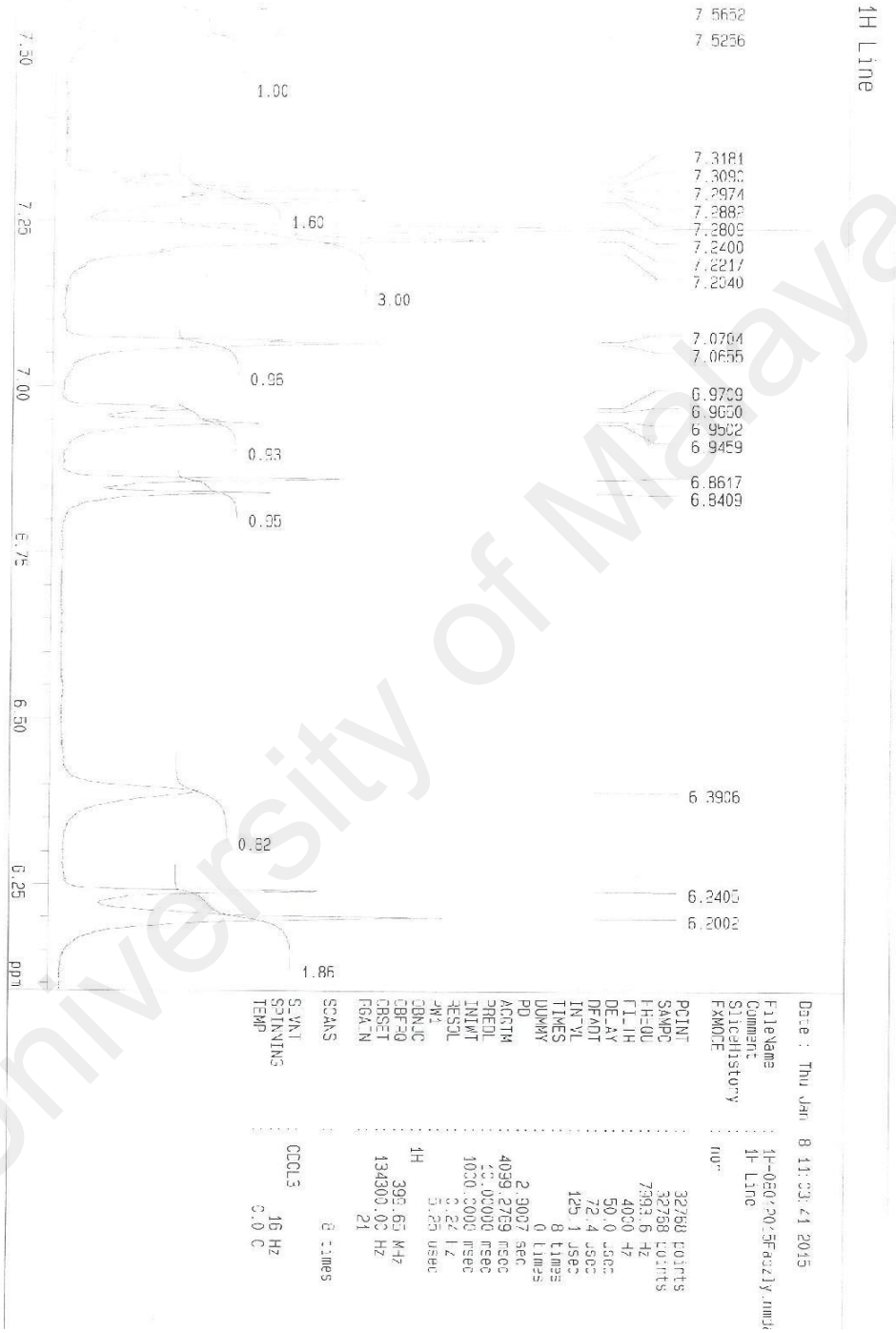
Recipe according to (Mitsuhashi and Maramorosch, 1964)

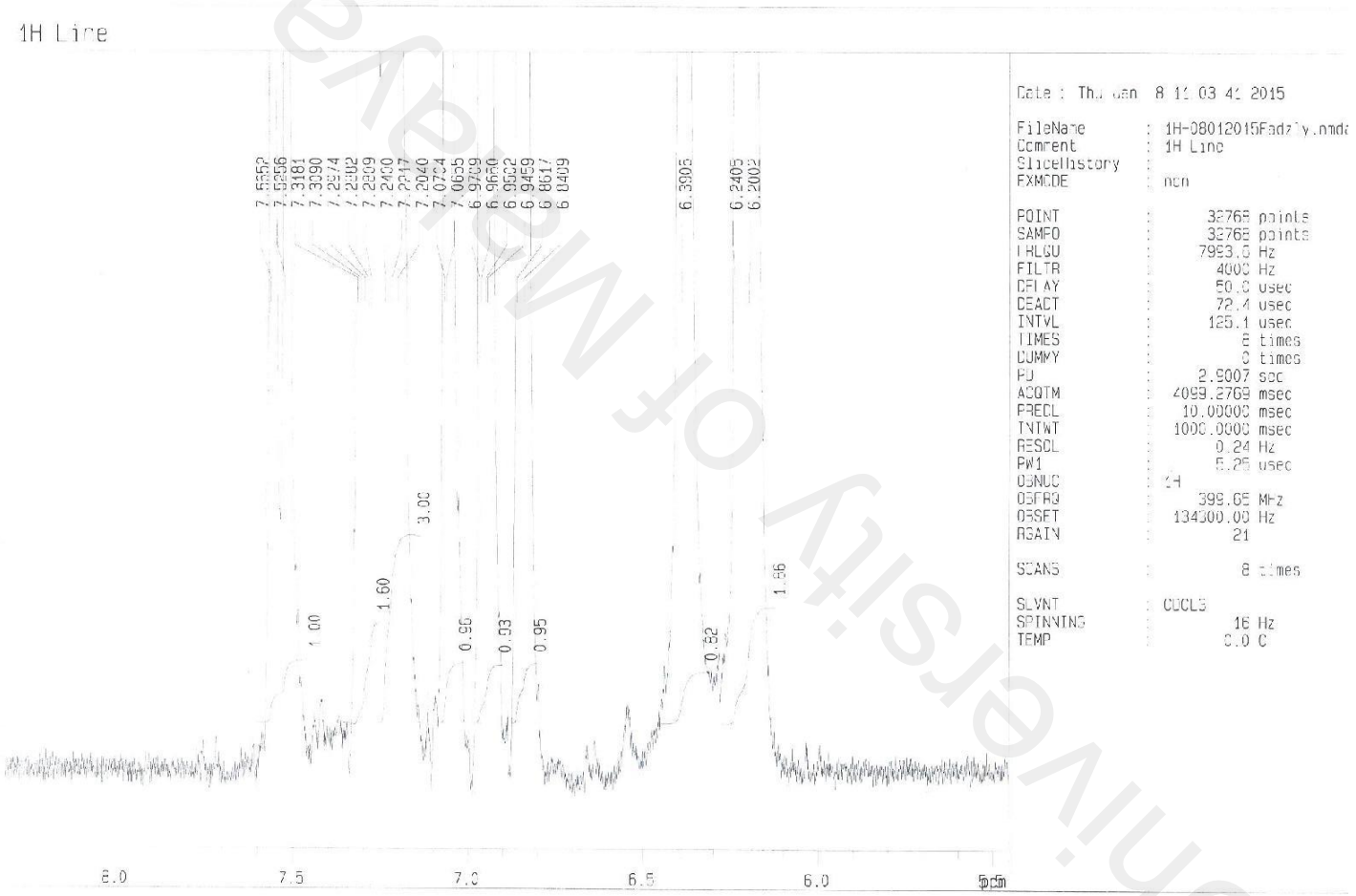
COMPONENT	g/L
INORGANIC SALTS	
Calcium Chloride (anhydrous)	0.151
Magnesium Chloride•6H2O	0.1
Potassium Chloride	0.2
Sodium Chloride	7.0
Sodium Phosphate Monobasic	0.174
Sodium bicarbonate	0.12
OTHER	
D(+)-Glucose	4.0
Lactalbumin Hydrolysate	6.5
Yeast Extract	5.0
pH at RT	6.5 ± 0.3

APPENDIX F: NMR spectrum







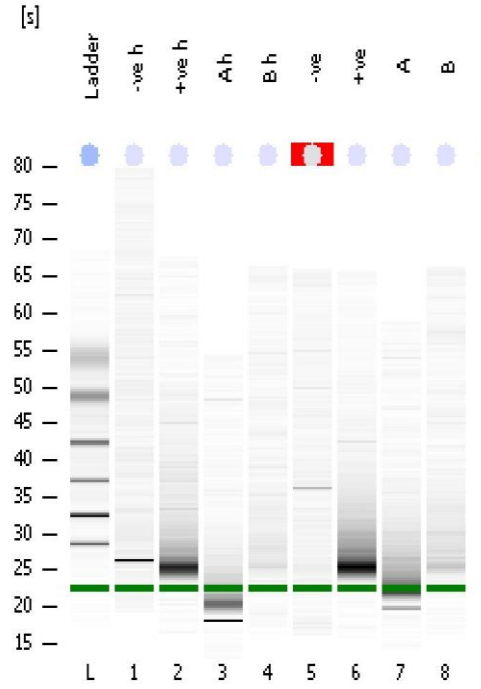


APPENDIX G: Supplementary Data

G1: RNA concentration and integrity of some samples

Sample Name	Concentration ng/µl	260/280	260/230	230	260	280	320
N1	603	1.993	1.81	1.28	1.95	1.20	0.45
N2	625	2.08	1.818	0.668	1.371	0.56	0.191
N3	496	2.091	2.191	0.372	1.046	0.339	0.194
N4	600	2.059	2.062	0.529	1.301	0.53	0.198
N5	764	1.833	1.99	1.335	2	1.417	0.375
T1	145	2.017	1.276	0.8	0.95	0.825	0.584
T2	567	2.052	2.026	0.7	0.414	0.691	0.004
T3	272	2.07	1.616	0.443	0.717	0.346	0
T4	416	1.964	1.512	1.093	1.445	0.935	0.406
T5	690	1.977	2.029	1.077	1.952	1.116	0.227
T2	243	2.082	1.906	0.32	0.609	0.293	0.001
T2	224	2.102	0.809	0.63	0.562	0.269	0.003
T3	105	1.13	0.25	1.13	0.251	0.114	0.001
T4	494	2.057	1.836	1.726	1.288	0.654	0.054
T5	308	2.059	2.059	0.38	0.776	0.38	0.006
K1	130	2	1.5	0.23	0.33	0.168	0.001
K2	102	2.1	1.81	0.2	0.35	0.21	0.098
K3	417	2	2	0.5	1.05	0.5	0.01
K4	262	2	2	1.01	1.35	1.018	0.69
K5	385	2	1.9	0.5	0.96	0.47	0.007

G2: Normalization



Instrument Information:

Instrument Name: DE54700222

Serial#: DE54700222

Assay Information:

Assay Origin Path:

Assay Class:

Version: 2.6

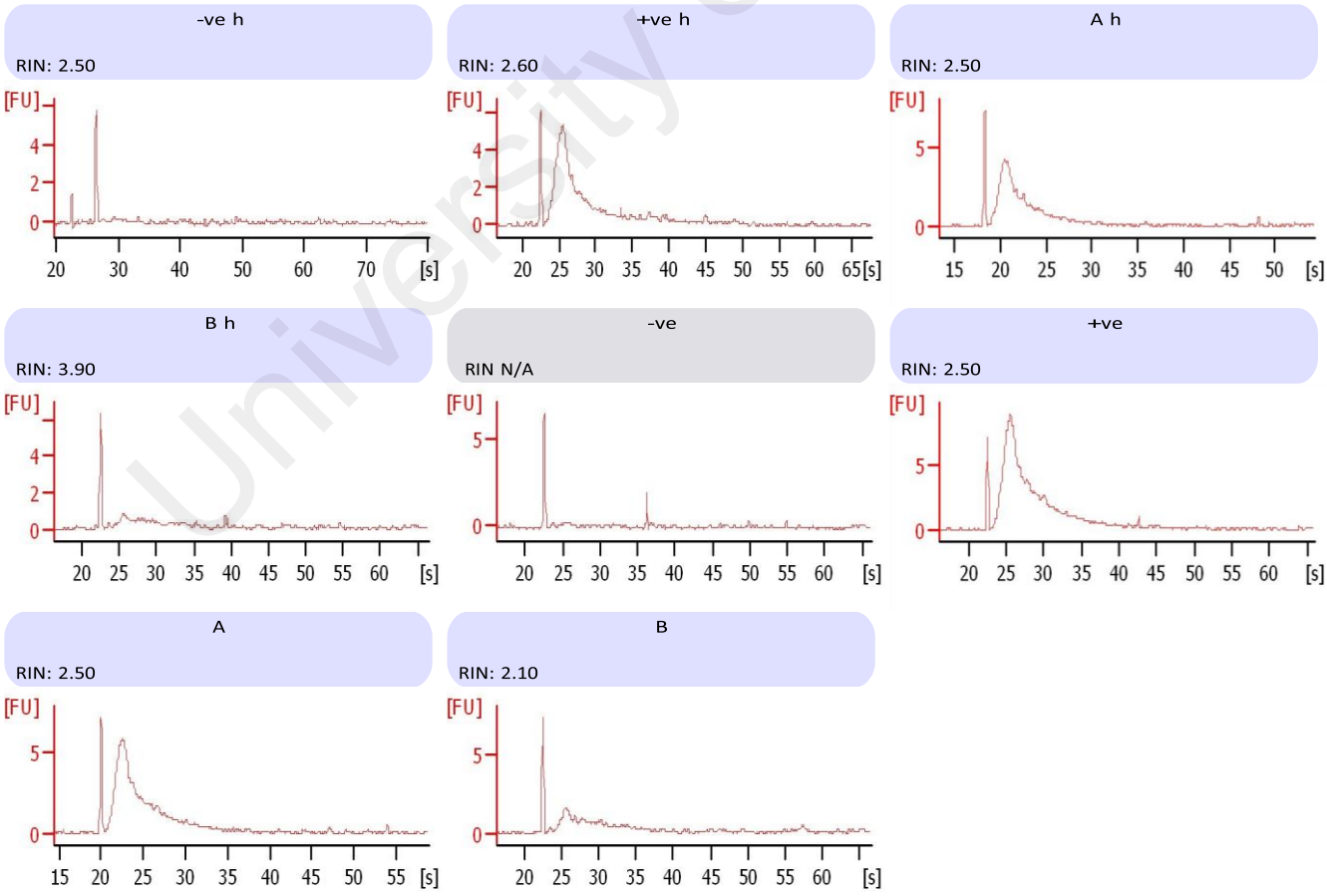
Assay Comments:

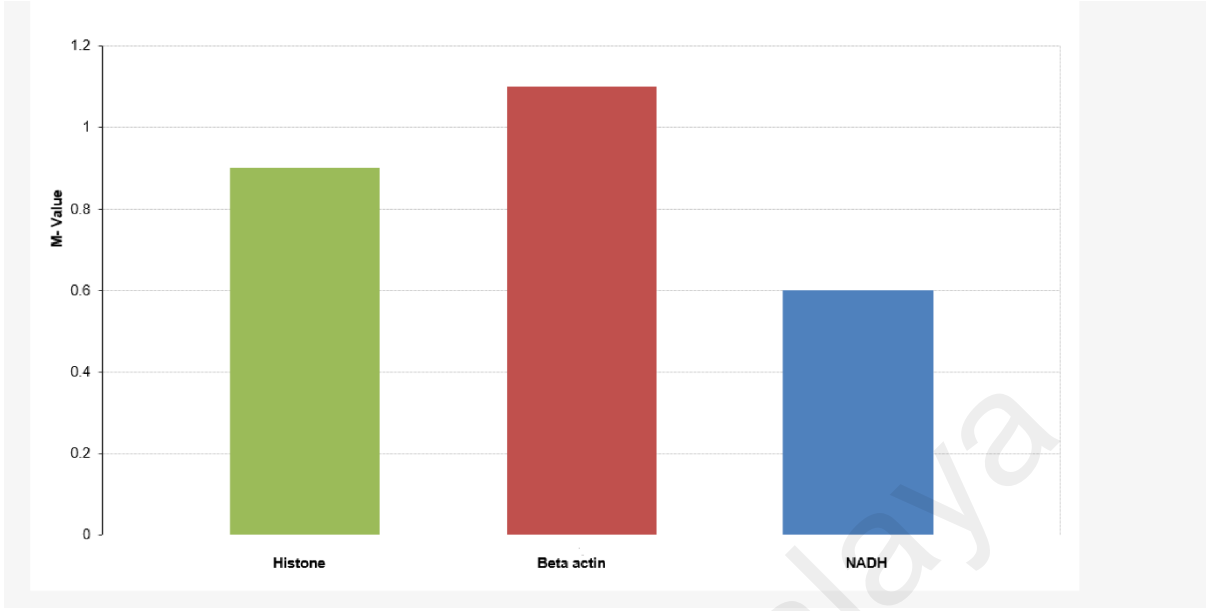
Chip Information:

Chip Lot #:

Reagent Kit Lot #: 1327

Chip Comments:





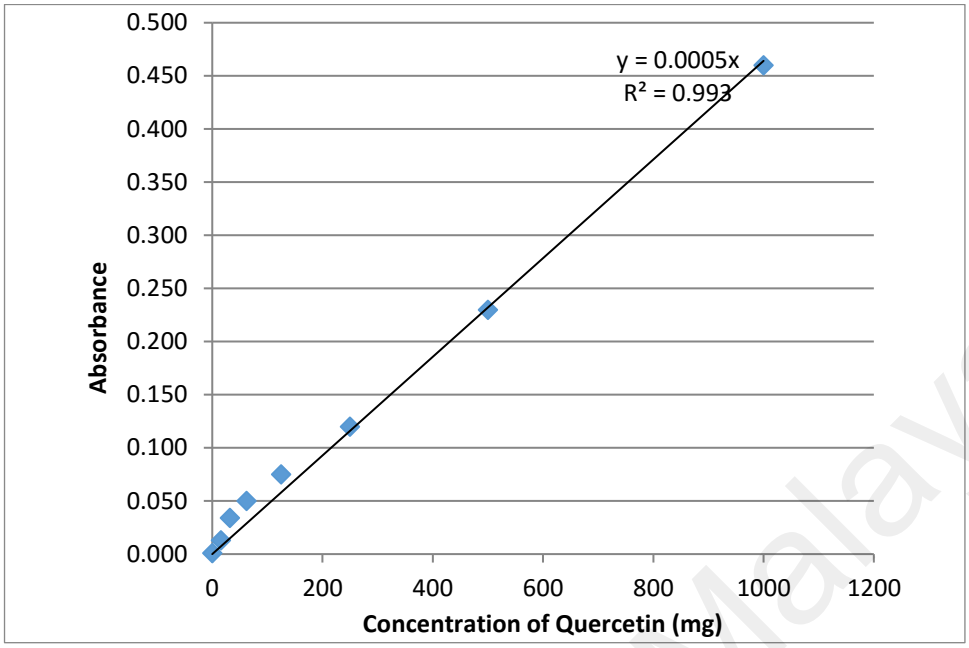
Gene Name	M-value
Histone	0.9
Beta actin	1.1
NADH	0.619

Output summary

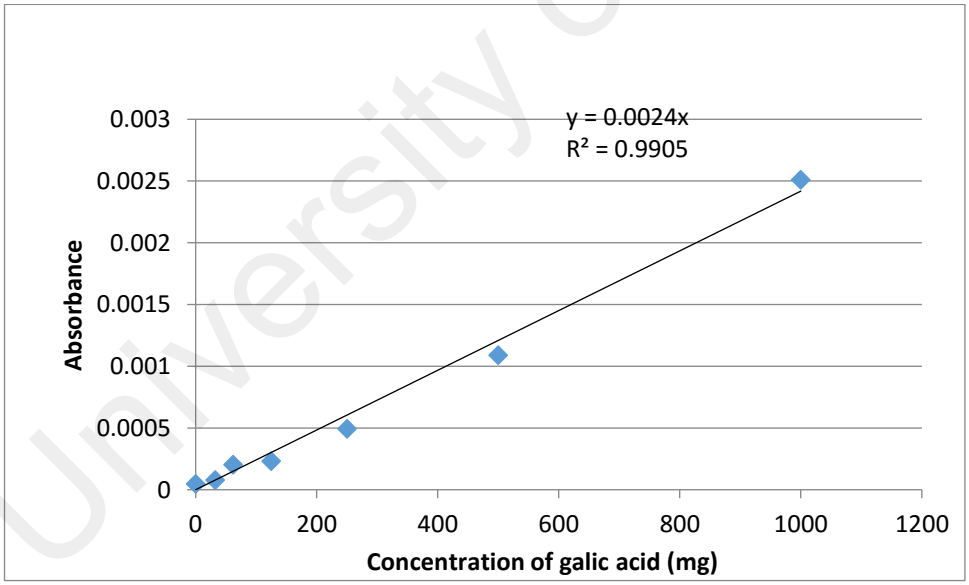
Best gene NADH

M value: 0.619 (stable expression)

G3: Standard curve of antioxidant



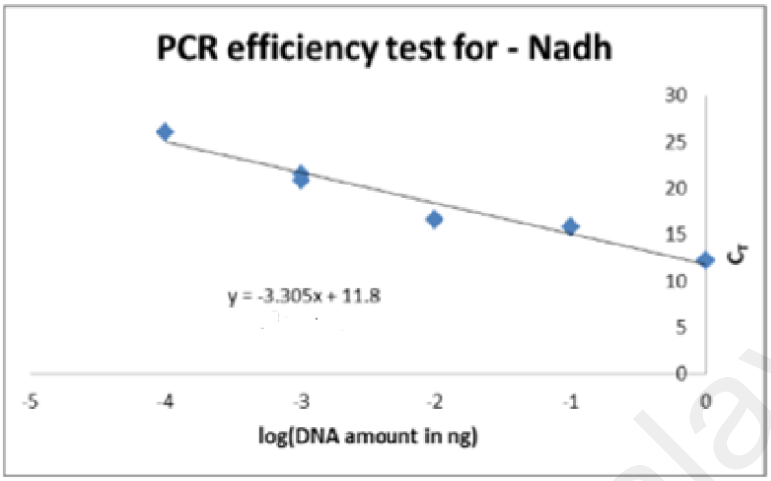
Total flavonoid content (TFC): Quercetin calibration plot



Total phenolic content: Gallic acid calibration plot

G4: Gene's efficient and standard curves:

Standard Curve for Nadh gene



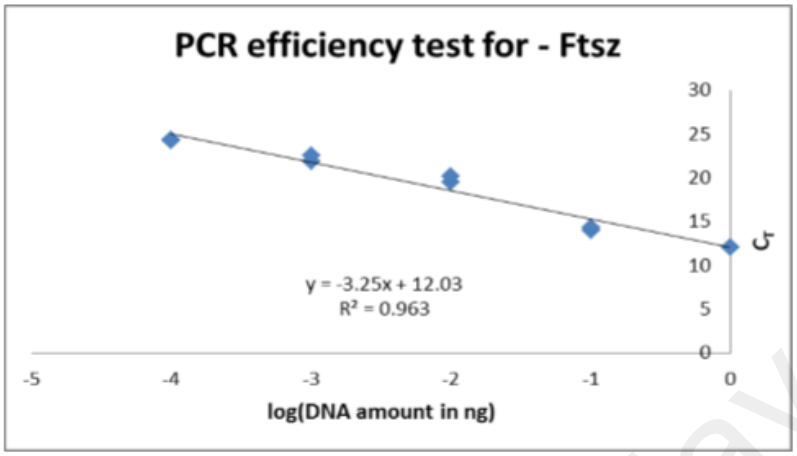
Slope: -3.305

R^2: 0.99

qPCR efficiency: 100.7%

NADH standard curve.

Standard Curve for Ftsz gene



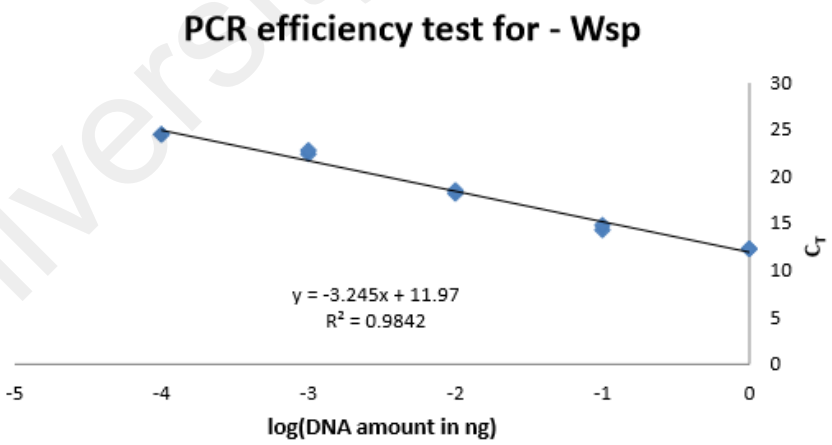
Slope: -3.25

R^2: 0.963

qPCR efficiency: 103.1%

Ftsz standard curve.

Standard Curve for Wsp gene

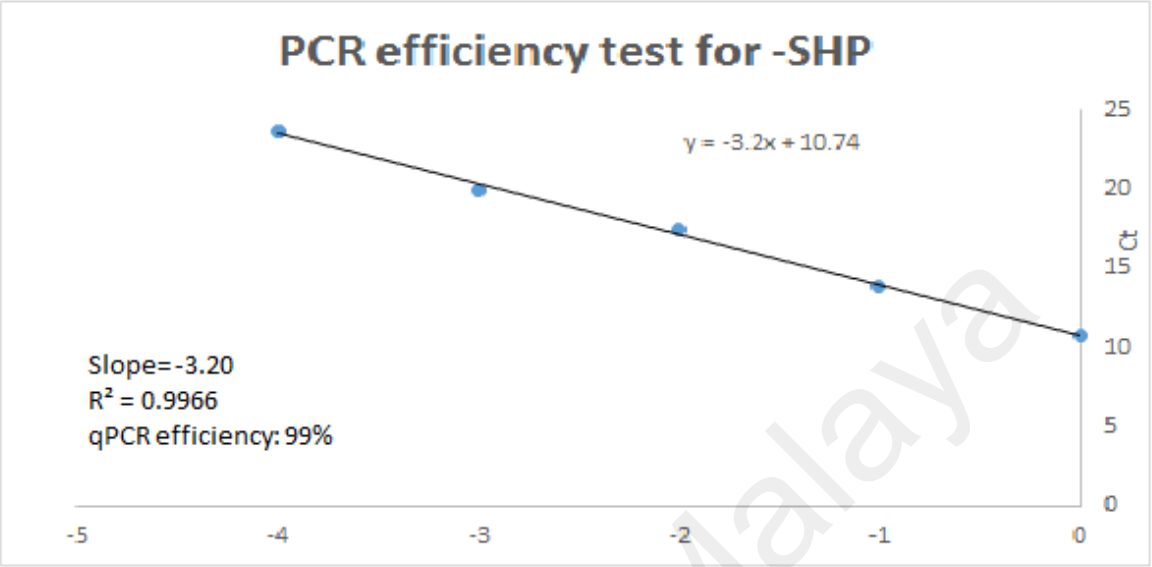


Slope: -3.245

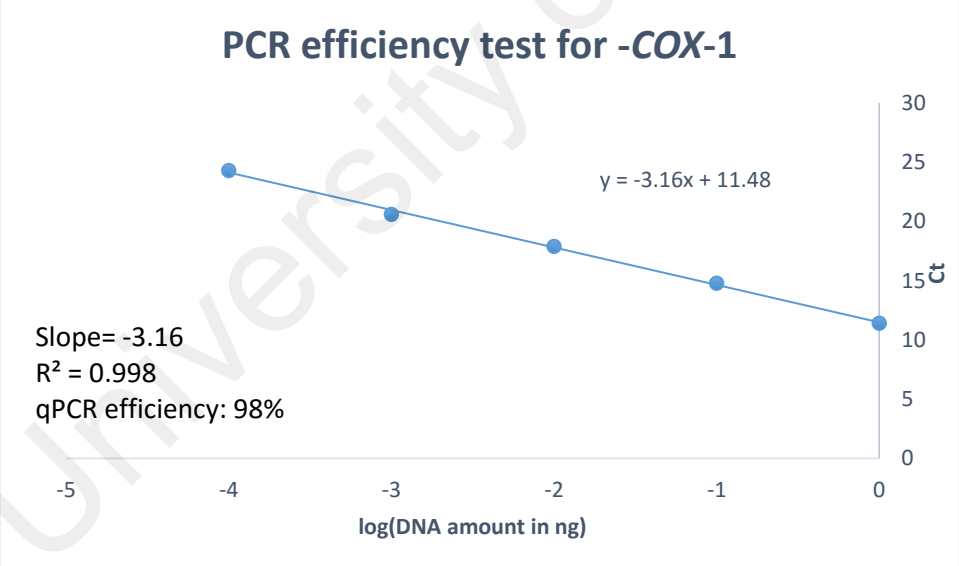
R^2: 0.9842

qPCR efficiency: 103%

wsp standard curve.

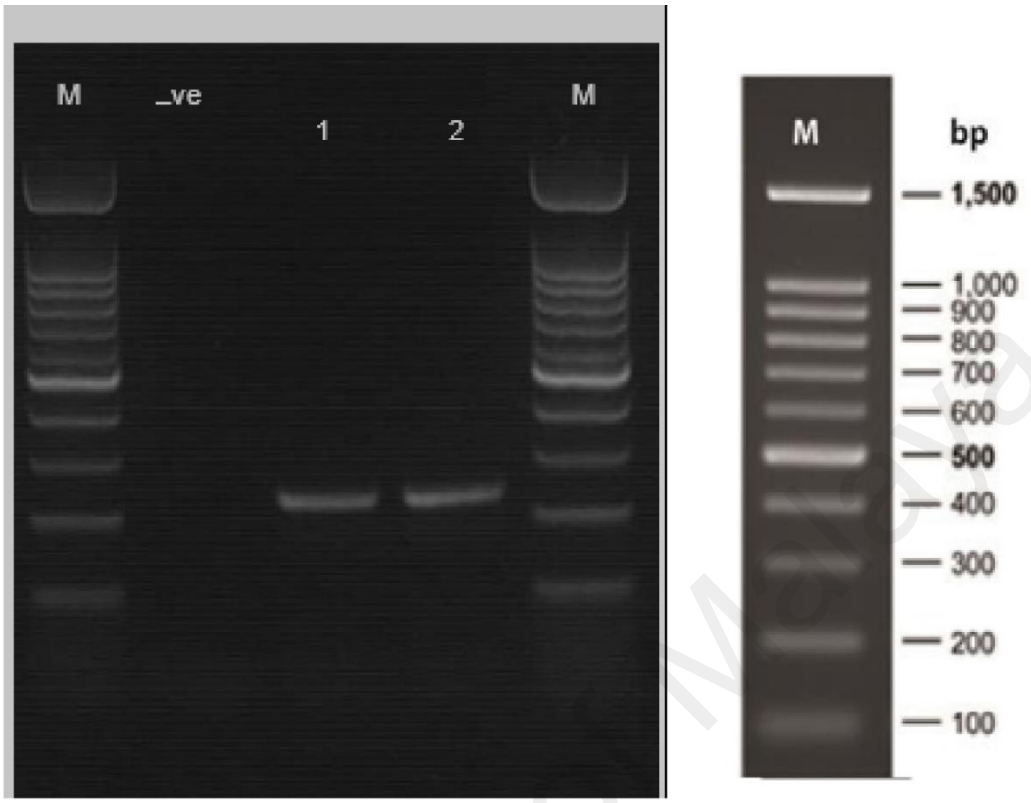


SHP-1 standard curve.

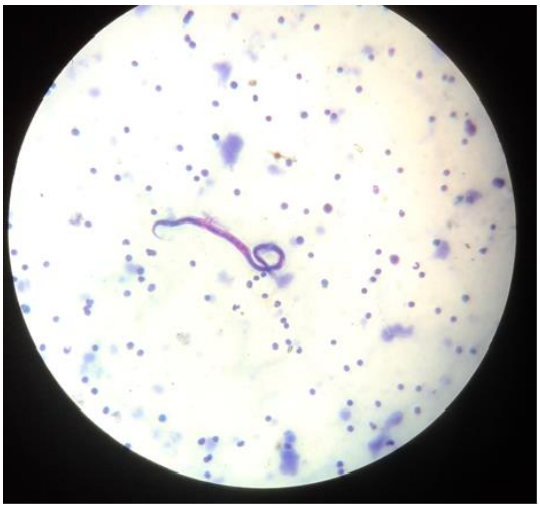


Cox-1 standard curve.

G5: Gel electrophoresis of copy number confirmation of insertion of *wsp* gene



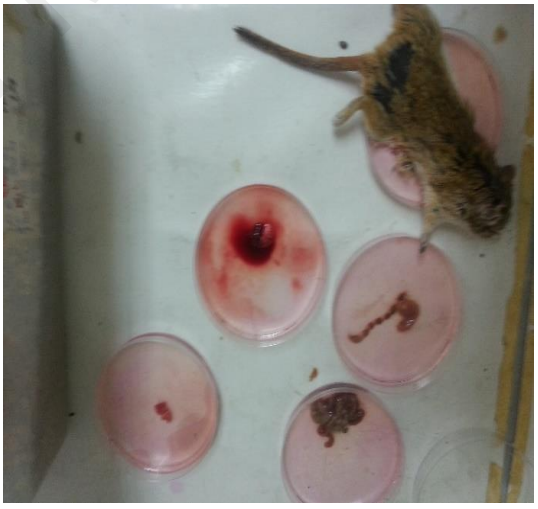
APPENDIX H: Some photographs of the study



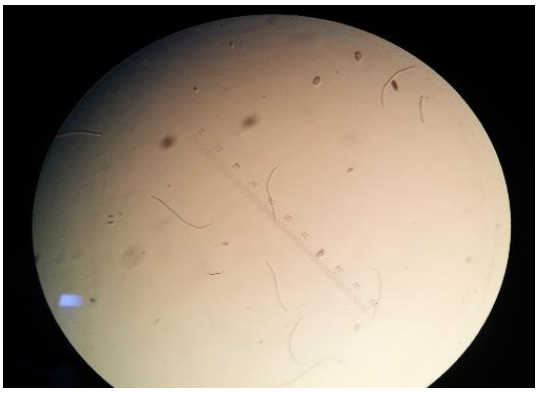
B. pahangi Mf stained with Giemsa stain (innerkupper techniques).

Dose preparation for killing of the jirds.

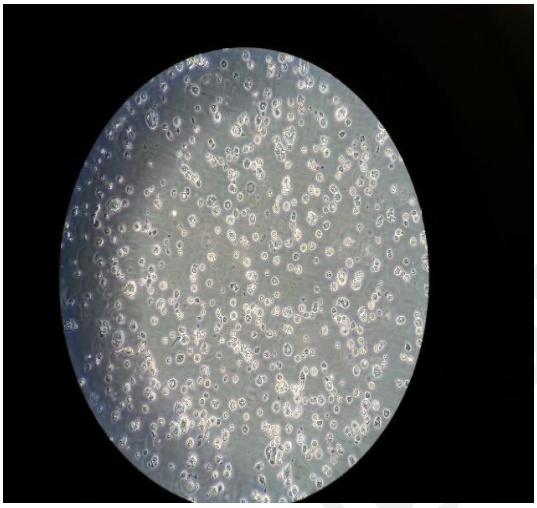
Experimental male jird, *Meriones unquiculatus*, used in this study.



Dissection of jirds, and recovery of adult worms from various organs.



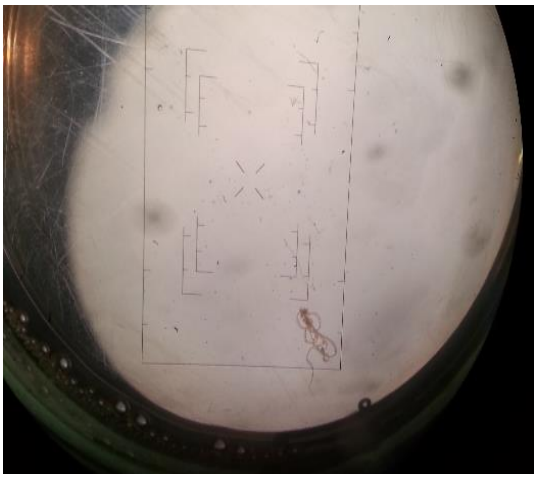
Mf count under microscope



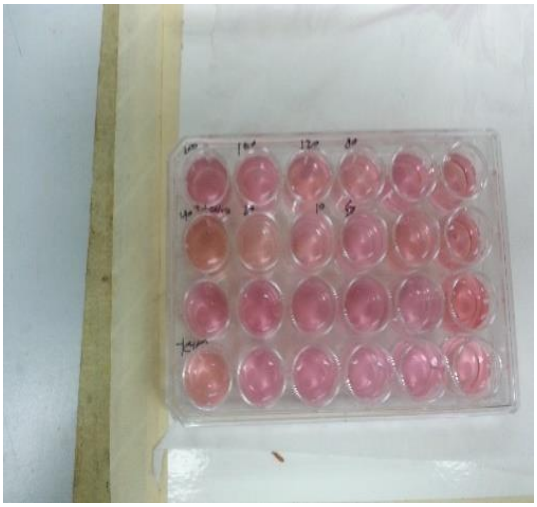
Aa23 cell line.

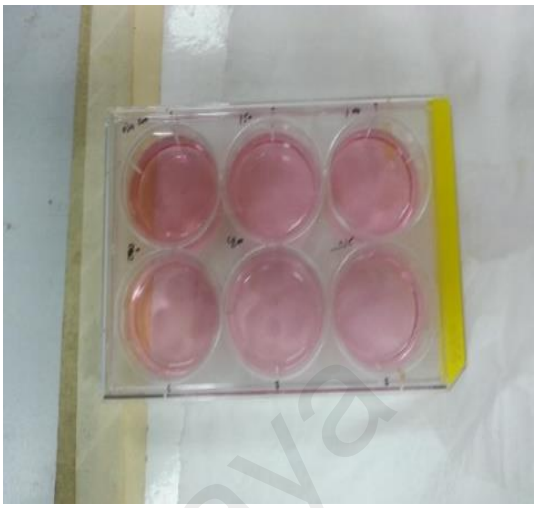
Reading of slides by Electron microscope

Cassettes embedded in fresh paraffin wax



Scoring of adult worm motility





Treatment of adult worms *in vitro*

LIST OF PUBLICATIONS AND PAPERS PRESENTED

A. Publication directly arising from this thesis

1. Nazeh M Al-Abd, Zurainee Mohamed Nor, Abdulelah H Al-Adhroey, Anwar Suhaimi, S Sivanandam (2013). Recent Advances on the Use of Biochemical Extracts as Filaricidal Agents. *Evidence-Based Complementary and Alternative Medicine. 5;2013*

2. Nazeh M Al-Abd, Zurainee Mohamed Nor, Abdulhamid Ahmed, Abdulelah H Al-Adhroey, Marzida Mansor, Mustafa Kassim (2014). Lymphatic filariasis in Peninsular Malaysia: a cross-sectional survey of the knowledge, attitudes, and practices of residents.*Parasites & Vectors*, *7*(1), 1-9.

3. Nazeh M Al-Abd, Zurainee Mohamed Nor, Marzida Mansor, Fadzly Azhar, MS Hasan, Mustafa Kassim (2015). Antioxidant, antibacterial activity, and phytochemical characterization of *Melaleuca cajuputi* extract. *BMC Complementary and Alternative Medicine*,*15* (1), 385

4. Nazeh M Al-Abd, Zurainee Mohamed Nor, Marzida Mansor, MS Hasan, Mustafa Kassim (2016). Antifilarial and antibiotic activity of methanolic extract of *Melaleuca cajuputi* flowers. *The Korean Journal of Parasitology*, *54(3), 273-280.*

B. Publication related, but not directly arising from this thesis

1. Nazeh M. Al-Abd , Zurainee Mohamed Nor, Mustafa Kassim, Marzida Mansor, Abdulelah H. Al-Adhroey, Romano Ngui, Sinnadurai Sivanandam (2015). Prevalence of filarial parasites in domestic and stray cats in Selangor State, Malaysia. *Asian Pacific Journal of Tropical Medicine*, *8*(9), 705-709.

C. Publication ready for submission

1. Nazeh M. Al-Abd, Zurainee Mohamed Nor, Mustafa Kassim, Marzida Mansor. Antifilarial activity of Caffeic acid phenyl ester.

2. Nazeh M. Al-Abd, Zurainee Mohamed Nor, Mustafa Kassim, Fadzly Azhar, MS Hasan, Marzida Mansor. Phytochemical constituents, antioxidant and antibacterial activities of methanolic extract of *Ardisia elliptica*.

D. Conference presentation made during the candidature period

1. Nazeh M Al-Abd, Zurainee Mohamed Nor, Mustafa Kassim. Knowledge, Attitudes and Practices (KAP) On Lymphatic Filariasis Among Population Of Terengganu State, Malaysia. 6th ASEAN Congress of Tropical Medicine and Parasitology (2014). Kuala Lumpur , Malaysia.

2. Nazeh M Al-Abd, Zurainee Mohamed Nor, Mustafa Kassim. Antioxidant activity, free-radical scavenging capacity and phyto-chemical characterization of *Melaleuca cajuputi* extract. International Conference on Natural Products 2014 (ICNP 2014), Putrajaya, Malaysia.

Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2013, Article ID 986573, 13 pages
http://dx.doi.org/10.1155/2013/986573

Review Article

Recent Advances on the Use of Biochemical Extracts as Filaricidal Agents

Nazeh M. Al-Abd,[1] Zurainee Mohamed Nor,[1] Abdulelah H. Al-Adhroey,[1] Anwar Suhaimi,[2] and S. Sivanandam[1]

[1] *Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia*
[2] *Department of Rehabilitation Medicine, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia*

Correspondence should be addressed to Nazeh M. Al-Abd; nazehali78@yahoo.com

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Academic Editor: Yew-Min Tzeng

Lymphatic filariasis is a parasitic infection that causes a devastating public health and socioeconomic burden with an estimated infection of over 120 million individuals worldwide. The infection is caused by three closely related nematode parasites, namely, *Wuchereria bancrofti*, *Brugia malayi,* and *B. timori*, which are transmitted to human through mosquitoes of *Anopheles, Culex,* and *Aedes* genera. The species have many ecological variants and are diversified in terms of their genetic fingerprint. The rapid spread of the disease and the genetic diversification cause the lymphatic filarial parasites to respond differently to diagnostic and therapeutic interventions. This in turn prompts the current challenge encountered in its management. Furthermore, most of the chemical medications used are characterized by adverse side effects. These complications urgently warrant intense prospecting on bio-chemicals that have potent efficacy against either the filarial worms or thier vector. In lieu of this, we presented a review on recent literature that reported the efficacy of filaricidal biochemicals and those employed as vector control agents. In addition, methods used for biochemical extraction, screening procedures, and structure of the bioactive compounds were also presented.

1. Introduction

Lymphatic filariasis is a disease that is caused by parasitic helminthes, namely, *Wuchereria bancrofti*, *Brugia malayi*, and *B. timori*. The parasites are transmitted by several mosquito species [1, 2], and the disease is reported to constitute serious public health and socioeconomic issues. In fact, it is said to be a major cause of acute and chronic morbidity in humans within tropical and subtropical areas of Africa, Asia, the Western Pacific, and some parts of the Americas [3]. It has been characterized with long-term infection through suppression of host immunity [1]. The pathogenesis of lymphatic filariasis is linked to host inflammation invoked by the death of the parasite (Figure 1), resulting in an altered lymphatic system and the abnormal enlargement of body for example, hydrocoele, lymphedema, and elephantiasis, causing pain and severe disability. The filarial species that infect people are known to coexist in a mutualistic endo-symbiotic relationship with *Wolbachia* bacteria, which are reported to be essential for the growth, development, and survival of the nematode hosts [1]. These endosymbionts are said to be among the factors that contribute to the inflammatory effect of this disease [1]. According to World Health Organization (WHO) fact sheets, more than 1.3 billion individuals in 72 countries worldwide are threatened by lymphatic filariasis, with over 120 million individuals being currently infected, and about 40 million being disfigured and incapacitated by the disease [2].

Currently, the chemotherapeutic drugs used to treat filariasis include doxycycline therapy, which targets the endosymbionts, delivers macrofilaricidal activity and improves pathological outcomes. Interestingly, the drug is said to be effective, even when used as monotherapy [1]. Combined therapeutic dosage of diethylcarbamazine (DEC), ivermectin, and

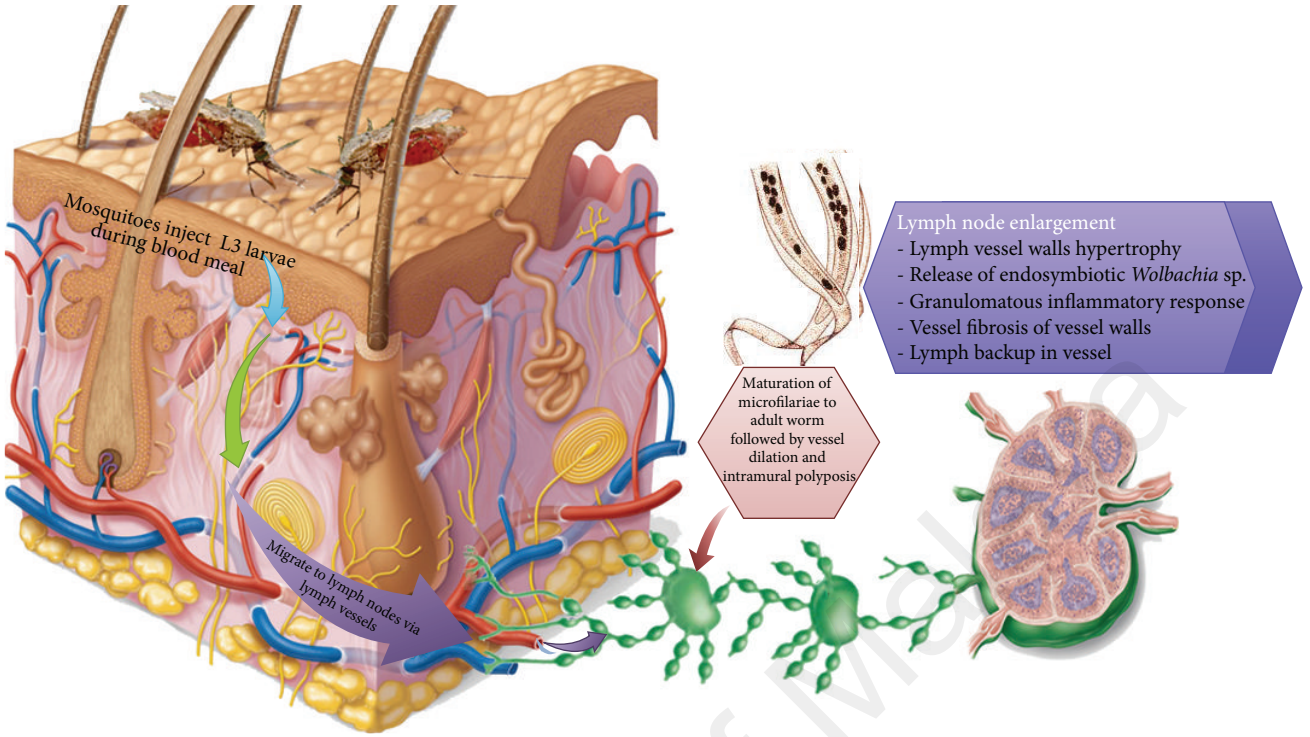

Figure 1: Inflammatory response in lymphatic filariasis.

albendazole effectively reduces microfilariae in blood [1, 4]. Unfortunately, most of these chemical medications are characterized by adverse side effects. For example, DEC has been around and in use since 1947 and is considered a good medication against microfilarial infection. Regrettably, this drug is reported to have a detrimental side effect [4]. Ivermectin, though reported to significantly lower the concentration of the microfilaria in the blood, was found to be associated with recurrence of microfilaraemia after treatment [4]. Hence, considering the public health and socioeconomic burden due to this disease, research on exploration and development of alternative therapeutic drugs, especially placid and/or less hazardous drugs of natural "organic" origin, is highly recommended.

Among the complementary alternative therapies that have been investigated is the exploration of biomedicine (botanical medicine) for possible filaricidal activities. The application of biochemicals to treat disease is among the oldest forms of healthcare known to humankind. Ancient Chinese characters and Egyptian papyrus hieroglyphs have documented the use of herbal medicine dating back to as early as 3000 B.C. [5]. In fact, herbal remedies had been in use by all cultures throughout the human history.

In recent years, it is not uncommon to find literature reported on the use of biochemical extracts against lymphatic filariasis [6–11]. Biological extracts such as those from *Azadirachta indica* [12], *Polyalthia suaveolens* [13], *Andrographis paniculata* [14], *Bauhinia racemosa* [15], and *Haliclona oculata* [16] were reported to have a bioactivity on either the filarial parasites or thier vectors. These extracts were believed to exert their bioefficacy through immunomodulatory elicitation of Th1/Th2 response, either by single (Th1, Th2) or mixed adjuvant activity. This paper presents a detailed review on the recent literature that reported the efficacy of direct filaricidal biomedicine or those employed as vector control agents. In addition, methods used for biochemical extraction, screening procedures, biochemical structure of the bioactive compounds and attempts made by researchers to evaluate the efficacy of the bio-chemical extract were also discussed.

2. Biomedicinal Agents Used against Filarial Parasites

2.1. Azadirachta indica (Family: Meliaceae). Popularly called "neem tree," the plant is a large evergreen tree with its height reaching up to 50 ft. [17]. It is widely distributed within the hot tropical regions especially in India and West Africa [18]. The leaves and bark of this tree have a bitter taste of triterpenoid bio-chemical component described as azadirachtin (Figure 2(a)) [19]. Almost every part of this tree is reported to be used in complementary medicine for the cure of different ailments such as antimicrobial [20], anti-inflammatory [21], anticancer [22], antimalarial [23], antiulcerogenic [24], and antifilarial [17] activities. Al-Rofaai et al. [12] reported the effect of *A. indica* leaf extract against the helminth *Teladorsagia circumcincta* (Table 1). Employing organic solvents extraction and aqueous fractionation methods, they found that the first stage larvae (L_1) were shown to be more sensitive having the lowest LC_{50} at 7.15 mg/mL of the extract as compared to 24.91 mg/mL on infective stage

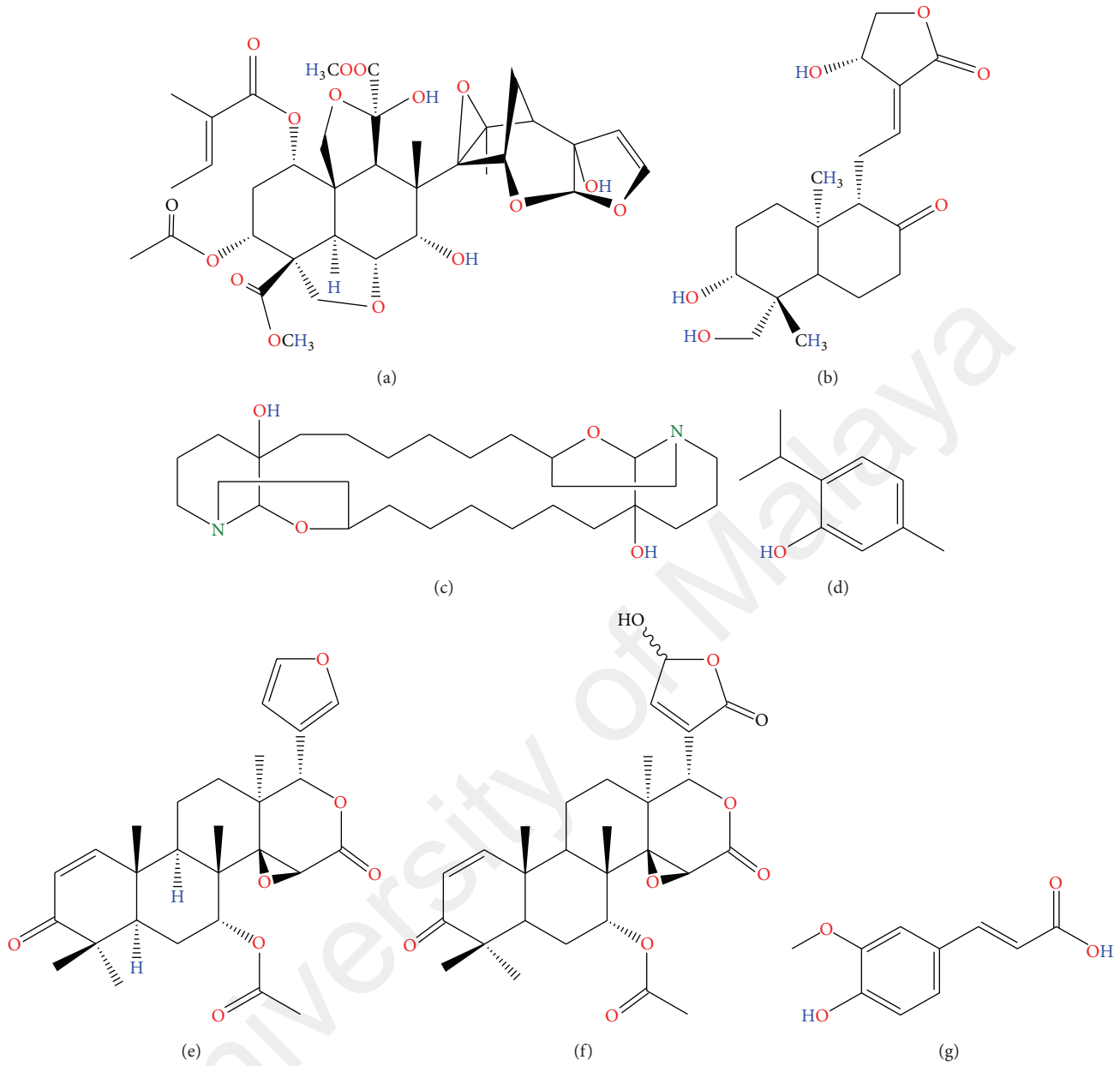

FIGURE 2: Chemical structure of (a) azadirachtin [19], (b) andrographolide [25], (c) araguspongin C [26], (d) 2-isopropyl-5-methyl phenol [27], (e) gedunin [28], (f) photogedunin [28], and (g) ferulic acid [29].

larvae (L_3). Other workers employing distilled alcoholic and aqueous extracts of *A. indica* flowers showed that they have potential antifilarial activity against microfilariae of *Setaria cervi* [17]. The study also showed that the inhibition was concentration dependent, and both extracts were found to have almost similar lethal effect on the microfilariae of *S. cervi*, with LC_{50} being 15 and 18 ng/mL, respectively [17].

2.2. Andrographis paniculata Linn. (Family: Acanthaceae). *Andrographis paniculata* (Kalmegh) is an annual herbaceous plant belonging to the family Acanthaceae, native to Southeast Asia especially China, India, and Sri Lanka. It has been traditionally used for centuries in Ayurvedic medicine. The herb has been revered for treating infectious diseases and highly regarded as having a preventative effect from many diseases, due to its powerful immune strengthening benefits [35]. Extensive research literature has revealed that *A. paniculata* has a broad range of pharmacological activities in different ailments such as being antianalgesic [36], antioxidant [37], antibiofilm [38], gastroprotective [39], wound healer [40], hepatoprotective [41], antifilarial [14], antimicrobial [42, 43], anticancer agent [44], antimalarial [45], and antitermitic [46]. It has been reported that the prophylactic effect of *Andrographis* was its ability to stop the catastrophic effect of the deadly flu virus of 1919 global epidemic from reaching India [47]. In fact, its bioactive diterpenoid andrographolide

Table 1: Bioproduct with reported filaricidal activity.

Name	Family	Part used	Extraction method	Filarial pathogen	Dosage	Reference
Azadirachta indica	Meliaceae	Leaf	Organic solvent and water fractionation	*Teladorsagia circumcincta*	3.1–50 mg/mL	[12]
		Flower	Distilled ethanol Distilled water	*Setaria cervi*	5–25 ng/mL	[17]
Andrographis paniculata	Acanthaceae	Leaf	Water decoction	*Dipetalonema reconditum*	0.06 mL/Kg	[30]
		Leaf	Aqueous extract	*B. malayi*	0.5–10 mg/mL	[31]
Heliclona oculata	Chalinidae	Sponges	Methanol extract solvents fractionation	*B. malayi*	100 mg/Kg	[16]
Haliclona exigua	Chalinidae	Sponges	methanolic extract and butanol fraction	*B. malayi*	15.6–31.2 μm/mL	[26]
Trachyspermum ammi L.	Apiaceae	Fruits	Methanolic extract	*Setaria digitata* *B. malayi*	0.01–0.5 mg/mL	[27]
Ricinus communis	Euphorbiaceae	Seed	Methanolic extract ethanol fractionation	*B. malayi* *S. digitata*	10–100 μg/mL 1 mg/mL	[32] [33]
Morinda citrifolia	Rubiaceae	Fruits	Aqueous extract	*W. bancrofti*	0.02–0.04 noni : media	[34]
Xylocarpus granatum	Meliaceae	Leaf	Ethanolic extract Aqueous extract	*B. malayi* *B. pahangi*	100 mg/kg 0.5–10 mg/mL	[28] [31]
Hibiscus sabdariffa	*Malvaceae*	Leaf	Ethanolic extract	*B. malayi*	500 mg–1 g/mL	[8]

(Figure 2(b)) and its analogs were reported to block the MCF-7 breast cancer cells cycle at the G0-G1 phase [25].

Kumarappan et al. [48] studied the antifilarial activity of alcoholic extract of *A. paniculata*. Another study reported that aqueous extract of the leaves showed microfilaricidal activity on *Dipetalonema reconditum* within 40 min (Table 1), both *in vitro* and *in vivo* [30]. Administration of the extract (0.06 mL/Kg body weight) reduced the number of the microfilariae in infected dogs by more than 85% [30]. Earlier, Zaridah et al. [31] reported the filaricidal activity of *A. paniculata* aqueous leaf extract against *B. malayi*. The authors analyzed the antifilarial activity of the extract using relative movability (RM) value of the adult worm over a period of 24 hr. The use of 5 or 10 mg/mL of the extract resulted in 0% of RM value signifying total death of the parasite. Lowering the concentration of extract to 1 mg/mL, however, failed to produce similar effect (mean RM value was 35%).

2.3. Haliclona sp. (Family: Chalinidae). *Haliclona oculata* does not belong to plant kingdom; it is a marine demospone of family Chalinidae in the animal kingdom. It is known to possess a variety of bioactivities against many diseases such as cancer [49], neurodegeneration [50], type 2 diabetes [51], and fungal and microbial infections [52, 53]. The biological activity of these sponges is said to be due to the presence of novel sterols, metabolites including steroids, terpenoids, alkaloids, cyclic peptides, and unsaturated fatty acids [16]. Gupta et al. [16] reported the antifilarial activity of *H. oculata* against experimental lymphatic filaria *B. malayi*. Employing methanolic extract, chloroform fraction of the methanolic extract, and a fraction from the chromatographic eluent, at 100 mg/kg for five consecutive days by subcutaneous route, demonstrated significant macrofilaricidal efficacy of 51.3%, 64%, and 70.7%, respectively. In all the samples, about 45–50% macrofilaricidal activity with moderate embryostatic effect was observed. Further analyses on the chromatographic fraction revealed that it contained a mixture of four alkaloids, namely, mimosamycin, xestospongin C, xestospongin D, and araguspongin C together with few minor compounds [16]. Work done by Lakshmi et al. [26] on antifilarial activity of another species, *H. exigua* against lymphatic *B. malayi in vitro* and *in vivo* study, showed that 31.25 μg/mL concentrations of the crude methanolic extract and butanol soluble fraction were able to kill the adult worm, whereas the chloroform extract was found to be effective at lower concentration (15.6 μg/mL). According to the authors, such finding could be attributed to the single bioactive molecule "araguspongin C" (Figure 2(c)).

2.4. Trachyspermum ammi (Family: Apiaceae). *Trachyspermum ammi* (Ajwain) is a native of Egypt and is cultivated in Iraq, Iran, Afghanistan, Pakistan, and India [54]. It has been reported to possess various pharmacological activities like being anti-fungal, antioxidant, antimicrobial, antinociceptive, cytotoxic, hypolipidemic, anti-hypertensive, antispasmodic, bronchodilating, anti-lithiasis agent, diuretic, abortifacient, antitussive, nematicidal, anti-helmintic, and antifilarial [54]. Studies on its bio-chemical composition and characterization revealed the presence of various bio-chemical constituents, mainly carbohydrates, glycosides,

saponins, phenolic compounds, volatile oil (thymol, γ-terpinene, para-cymene, and α- and β-pinene), protein, fat, fiber, and mineral matter containing calcium, phosphorous, iron, and nicotinic acid [54]. Mathew et al. [27] studied the effect of methanolic extract of *T. ammi* fruits against adult bovine filarial *Setaria digitata* worms at a concentration of 0.01–0.5 mg/mL for a period of 24–48 hr (Table 1) and found that both the crude extract and the active fraction showed significant activity against the adult *S. digitata* by both worm motility and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assays. They also isolated a compound and characterized it as phenolic monoterpene described as 2-isopropyl-5-methyl phenol (Figure 2(d)). When tested *in vivo* for antifilarial activity against the human filarial worm *B. malayi* in *Mastomys coucha*, it presented a macrofilaricidal activity where female worm sterility was detected.

2.5. Ricinus communis (Family: Euphorbiaceae). *R. communis* (castor oil plant) is commonly found in both the tropical and temperate climates of the world. The seed extract was reported to have efficacy in the treatment of warts, cold tumours, indurations of the mammary glands, corns, and moles, as well as being widely used as a human laxative-cathartic agent [32]. Ramanathan and Shanmugapriya [32] observed the filaricidal effect of organic solvent extract of *R. communis* seed against filarial parasite *B. malayi* using different dosages (10, 50, and 100 μg/mL of the extract) for the period of 24 hours. Their findings indicated dose-dependent filaricidal activity (40–90%). Nisha et al. [33] reported that treatment with ethanol fraction (1 mg/mL) of *R. communis* seed extract caused a complete suppression of *S. digitata* microfilarial growth within 1 hr and 40 min (Table 1). Based on worm motility and MTT reduction assay, the authors found the seed extract to cause about 72.39% growth inhibition within 4 hr of exposure [33].

2.6. Morinda citrifolia L. (Family: Rubiaceae). *M. citrifolia* (noni) is plant that could be found in a wide variety of habitats including volcanic terrains, lava-strewn coasts, and clearings or limestone outcrops. It can grow up to 9 m (30 ft) tall due to its tolerance to adverse agricultural conditions such as saline soils, drought conditions, and secondary soils. It has large, simple, dark green, shiny, and deeply veined leaves. The medicinal applications of this plant span over all kinds of ailments involving both modern and complementary medicines. Satapathy [34] reported the filaricidal activity of noni against *W. bancrofti* in an *in vitro* study. The study showed that adding noni fruit extract to culture media containing *W. bancrofti* microfilariae at a concentration of 2000 microfilaria per mL (Table 1) killed the parasite within 20 hours as compared to the control group without adding the extract, which survived for up to 60 hours [34].

2.7. Xylocarpus granatum (Family: Meliaceae). *Xylocarpus granatum* (nyireh bunga) is a mangrove swamp species with reported medicinal importance [31]. Misra et al. [28] found an *in vivo* filaricidal activity of *X. granatum* extract against *B. malay*i (both adult worm and microfilaria). On testing the crude aqueous ethanolic extract *in vitro*, they observed IC_{50} of 15.46 and 13.17 μg/mL in both adult worm and microfilariae, respectively. On the other hand, study on the ethyl acetate-soluble fraction revealed the antifilarial activity to be moderate (IC_{50} of 8.5 and 6.9 μg/mL) in both adult and microfilariae, respectively. They further found that on testing the extract's efficacy *in vivo* by administering it to Mastomys orally at 50 mg/Kg, it showed adulticidal (52.8%) and embryostatic (62.7%) effects against *B. malayi*. Isolation of the bioactive biochemical components of this plant revealed eight pure molecules with two of these compounds, namely, gedunin (Figure 2(e)) and photogedunin (Figure 2(f)), at 100 mg/kg by subcutaneous route revealing excellent adulticidal efficacy resulting in the death of about 80% *B. malayi* [28]. Previously, this species has been reported to have filaricidal activity against *B. pahangi* [55]. Filaricidal activity of *X. granatum* was further evaluated somewhere else.

2.8. Hibiscus sp. (Family: Malvaceae). *H. sabdariffa* (roselle) is a native of tropics, used for the production of fiber and infusions that are normally used as beverages. It is a woody shrub of annual to perennial seasoning. It is reported to have variant medicinal efficacy, especially on hypertensive patients [56]. The plant is said to be rich in anthocyanins, as well as dihydroxybenzoic acid. Daphniphylline forms the major pigment, while the dried calyces contain the flavonoids gossypetin, hibiscetine, and sabdaretine. In addition, small amounts of delphinidin-3-monoglucoside, cyanidin-3-monoglucoside, and delphinidin were also present [56]. Moreover, the seeds were reported to be a good source of lipid-soluble antioxidants, particularly gamma-tocopherol [57]. Recently, Saxena et al. [8] reported for the first time the filaricidal activity of ethanolic extract of *H. sabdariffa* leafs against *B. malayi* (Table 1). The efficacy of the plant extract filaricidal activity was assessed using both the *in vivo* and *in vitro* motility and MTT reduction assays on the microfilariae (mf) and adult worms. The authors found that administering the leaf extract at 500 mg/mL for 5 days incurred about 30% macrofilaricidal efficacy and 42% sterilization of female worms in *Meriones unguiculatus*. On the other hand, feeding *M. coucha* with the extract (1 g/kg for 5 days) exerted 57.0% macrofilaricidal activity with 64% sterilizing effect on female worms [8]. In similar studies, the crude methanolic extract of *H. mutabilis* (confederate rose) and the isolated bioactive molecule "ferulic acid" were tested against bovine *S. cervi* [29]. The authors reported that both the extract and the bioactive molecule "ferulic acid" (Figure 2(g)) showed significant microfilaricidal as well as macrofilaricidal activities against *S. cervi*. Using 500 μg/mL of the aqueous fraction of ethyl acetate extract, the authors reported about 50% microfilarial motility inhibition within 24 hours and more than 80% within 48 hours, while adult worms motility inhibition was observed to be about 40% and 70% within 24 and 48 hours, respectively. Test on the bioactive compound ferulic acid was shown to be more effective. At a concentration of 400 μg/mL, it caused 100%

and 90% motility inhibition in both micro- and macrofilariae after 48 hours, respectively [29].

2.9. Cardiospermum halicacabum (Family: Sapindaceae). *Cardiospermum halicacabum* (love in a puff) is a climbing plant widely distributed in tropical and subtropical regions of Africa and Asia [58]. This plant has been reported to have bioactivity, such as being homoeopathic [58], having anti-diarrheal efficacy [59], and being antimicrobial [60, 61]. Khunkitti et al. [62] previously reported the *in vitro* filaricidal activity of ethanolic and aqueous extracts of this plant against *B. pahangi*. The researchers found activity on the adult worms and the amount of microfilariae released by female worms was concentration and time dependent. For example, using 500 μg/mL (Table 1), the authors observed that the aqueous extract significantly reduces motility of adult females after 24 h of exposure, the release of microfilariae from female worms on day 2, and the motility of the adult males after 3 days. However, the aqueous extract at this concentration (500 μg/mL) did not affect the motility of microfilariae, with the exception of those in higher concentration extracts. In contrast, 500 μg/mL of the ethanol extract was found to rapidly reduce the motility of microfilariae on day 2. Furthermore, higher concentrations of ethanol extracts (2 mg/mL) inhibit both the motility of adult worms and the release of microfilariae from females [62].

2.10. Excoecaria agallocha L. (Family: Euphorbiaceae). *Excoecaria agallocha* is a small tree species that inhabits the mangrove swamps of Southeast Asia and that can grow up to 15 m height. The tree has a well-developed chemical defense mechanism composing of diterpenoids, triterpenoids, and flavonoids [63]. The extract of this tree is reported to possess depressant action on the central nervous system [64], antimicrobial efficacy [65], and anti-viral and anti-carcinogenic activities [64]. Patra et al. [66] reported the antifilarial activities of methanolic and aqueous extracts of *E. agallocha* leaf against *S. digitata* were dose dependent at concentrations of 10, 50, and 100 μg/mL for 24 hrs at 37°C in 5% CO_2 incubation. The study showed reduction in percentage of motility by about 20, 60, and 83%, respectively. Testing the radical scavenging activity of the extract, the authors found the aqueous extract to be effective in 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, reducing power, and hydrogen peroxide scavenging activity, which increased with the increase in concentration of the extract. Based on their observations, the authors concluded that *Excoecaria agallocha* can be a potential source of bioactive chemicals that can be used not only for meeting the oxidative stress generated during chronic manifestation of lymphatic filariasis in human beings but also for blocking embryogenesis in filarial parasites, which in turn can potentially affect their transmission and survival in host communities [66].

2.11. Alnus nepalensis (Family: Betulaceae). *Alnus nepalensis* (Nepalese Alder) is widely found in the subtropical highland of Himalaya. It is a fast growing deciduous tree that reaches up to 30 m in height, and it can grow on different kinds of soils especially in wet areas. The leaves are naturally shallow with dimension of 7–16 cm long and 5–10 cm broadness. Occasionally, the tree is used for making boxes, in light construction, and as firewood by the local people. Furthermore, it is sometimes planted as erosion control on hillsides and for land recovery in shifting cultivation. Recently, the *in vitro* and *in vivo* filaricidal activities of four bioactive compounds isolated from *A. nepalensis* leaves against human lymphatic filariasis (*B. malayi*) have been investigated for the first time [11]. The researchers designated the isolated diarylheptanoid biochemicals as compounds (a–d), that is, (a) platyphyllenone, (b) alusenone, (c) hirustenone, and (d) hirsutanonol, as shown in Figure 3. The authors reported that compounds (a) and (c) showed better efficacy as indicated by about 60% mean inhibition in motility of adult worms (Figure 4). Comparing the mean percentage MTT inhibition with the control synthetic drug diethylcarbamazine (DEC) in Figure 4, the authors concluded that *A. nepalensis* extract especially compound (c) showed a promising antifilarial activity against both the macro- and microfilarial worms of *B. malayi*.

2.12. Bauhinia racemosa Lam. (Family: Fabaceae). *Bauhinia racemosa* (mountain ebony) is a small deciduous tree native to the tropics especially Southeast Asia. Almost each and every part of this tree has some medicinal values [67]. The stem bark of the tree has analgesic activity [68], antidiabetic activity [69], and anti-helmintic activity [70] and is used in the treatment of headache, fever, skin diseases, blood diseases, dysentery, and diarrhea [67]. An extract of the leaves has been reported to show anti-histaminic effect [71], antimicrobial efficacy [72], and hypolipidemic activity [73], while a decoction of the dried fruits has antiulcer activity [74]. The tree is demonstrated to have antioxidant and hepatoprotective effects [75]. Sashidhara et al. [15] recently reported the filaricidal activity of the galactolipids compounds isolated from ethanolic extract of *B. racemosa* leaves. Results were based on the fractions tested (Figure 5); the n-butanol fraction of the extract revealed promising adulticidal (IC_{50} 5.46 mg/mL) and microfilaricidal (IC_{50} 4.89 mg/mL) activity, with minimum inhibitory concentration (MIC) of 15.6 mg/mL [15]. Among the characterized isolated galactolipid is *(2S)*-1,2-di-*O*-linolenoyl-*3*-*O*-a-galactopyranosyl-*(1/6)*-*O*-b-galactopyranosyl glycerol (Figure 6), which they found to have filaricidal efficacy that rivals the standard drug ivermectin (IC_{50} 1.61 mg/mL; MIC 7.8 mg/mL in adult and IC_{50} 3.62 mg/mL; MIC 125 mg/mL in microfilariae) in terms of dose and efficacy [15].

2.13. Cocos nucifera (Arecaceae). The coconut palm is widely distributed within the tropical and subtropical regions, growing up to 30 meters tall, with pinnate leaves 4–6 meters long. The tree has versatile importance spanning from domestic, commercial to medicinal applications. The biochemical analysis of the endosperm revealed the presence of terpenoids, alkaloids, resins, glycosides, steroids, and flavonoids [76, 77]. Medically, coconut is reported to have efficacy against prostatic hyperplasia [78], anti-helmintic activity

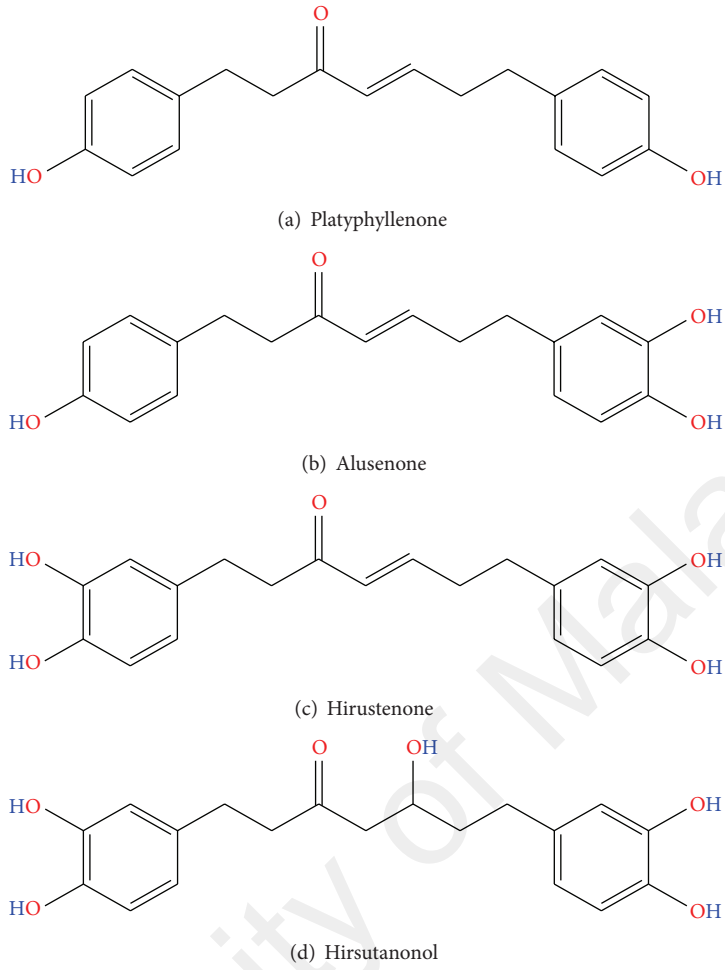

Figure 3: Chemical structure of bioactive biochemicals extracted from *A. nepalensis* [11].

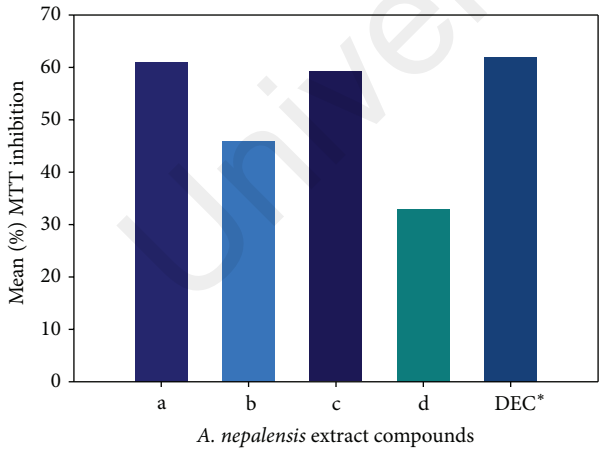

Figure 4: Effect of *A. nepalensis* extract on adult female worm motility inhibition, DEC* (diethylcarbamazine used as control).

[79], and antimicrobial and antiviral activities [80], vasorelaxant, antimalarial [81], and anti-hypertensive; activities, and inhibitory effect on oral microflora [82]. Furthermore, its bioactive compounds were found to have extended efficacy on agropest control [83]. Al-Adhroey et al. [81] studied the antimalarial efficacy of *C. nucifera* endocarp methanolic extract against *Plasmodium berghei* (NK65) infections in mice. The antimalarial activity was evaluated using different doses (50, 100, 200, and 400 mg/kg) of the extract in reference to chloroquine (20 mg/kg) and pyrimethamine (1.2 mg/kg) drugs. Although, at 200 and 400 mg/kg doses, the extract revealed significant reduction of parasitaemia, it failed to increase the survival time of the infected mice.

3. Medicinal Plants Used as Agents for Vector Control

Among the methods used to manage filariasis is control its vectors, since filariasis is transmitted by mosquito vectors of the genera *Aedes*, *Anopheles*, *Culex*, and *Mansonia*. In this section, a review on the recent use of bio-chemical extracts in the control of these filarial vectors is presented.

Cocos nucifera (Arecaceae). Recently, Roopan et al. [84] employed the use of novel biosynthesis that reduced silver nitrate to biogenic silver nanoparticles in the presence

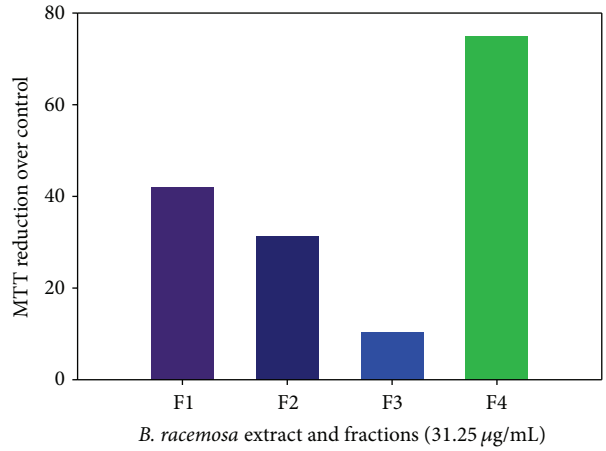

Figure 5: *In vitro* motility inhibition by MTT reduction assay as a function of *B. racemosa* extract and fractions exposure (F1: crude ethanolic extract; F2: n-hexane fraction; F3: chloroform fraction; F4: n-butanol fraction).

C. nucifera extracts against *A. stephensi* and *C. quinquefasciatus*. The researchers reported about 100% and 92% 4th instar larval motility inhibition at 4 mg/mL dosage in both *A stephensi* and *C. quinquefasciatus* after 72 hours, respectively.

Subarani et al. [85] observed the larvicidal activity of *Vinca rosea* (Apocynaceae) aqueous leaf extract biosynthesized silver nanoparticles also against *A. stephensi* Liston and *C. quinquefasciatus*. On exposure for 72 hours, the researchers found that the larvicidal activity showed maximum efficacy in synthesized silver nanoparticles against the fourth instar larvae of *A. stephensi* (LC_{50} = 16.84 mg/mL) and against *C. quinquefasciatus* (LC_{50} = 43.80 mg/mL).

Kovendan et al. [86] recently reported the larvicidal effect of *M. citrifolia* leaf extract against three medically important mosquito vectors of *Anopheles*, *Aedes*, and *Culex* genera. After 24 hours of exposure, the authors found that the larvicidal activity was concentration and extraction-solvent dependent (Figure 7).

Govindarajan et al. [87] reported the larvicidal and ovicidal activity of *Cassia fistula* Linn. (Fabaceae) methanolic leaf extract against *C. quinquefasciatus* and *A. stephensi*. The researchers found the activity of the extract (10–50 mg/L) was concentration dependent. At 40 mg/L the percent mortality was 89.33 and 100 in both *Culex* and *Anopheles*, respectively, with LC_{50} values recorded as 17.97 and 20.57 mg/L, respectively, signifying that the extract is more potent to *Anopheles* than to the *Culex* larvae. Their investigation on the ovicidal activity of the extract, based on percentage of hatchability, showed that it was inversely proportional to the concentration (25–200 mg/L) used. The authors also investigated the bioefficacy of *C. fistula* leaf extract on *A. aegypti*. They reported that the activity was concentration and extraction-solvent dependent. In methanol extract, exposure to 25 mg/L for 24 hours resulted in 98% larval mortality. The percentage of mortality was reduced up to about 31% when the concentration was brought down to 5 mg/L. Exposure to 20 mg/L of methanol extract for 24 hours resulted in about 85% larval mortality. By using the same concentration, only 55% and 41% larval mortality were observed when employing benzene and acetone extracts, respectively.

In contrast to the earlier study, Rajkumar and Jebanesan [88] reported LC_{50} value of 52.2 mg/L in *A. stephensi* using ethanolic leaf extract of Chinese Senna (*Cassia obtusifolia* Linn.; Fabaceae). As for ovicidal activity, the researchers used an extract concentration of 100–400 mg/L, and they found the oviposition deterrent activity of the leaf extract to be concentration dependent. At extract concentration of 400 mg/L, oviposition effective repellency of 92.5% was indicated while at 300, 200, and 100 mg/L the effective repellency of 87.2%, 83.0%, and 75.5% was observed, respectively.

Recently, the bioefficacy of *Carica papaya* (Caricaceae) leaf extracts against *A. aegypti* larvicidal and pupicidal properties was reported [89]. The authors found that the leaf extract showed both larvicidal and pupicidal types of efficacy after 24 h of exposure. In all the extracts tested, the methanolic extract has the highest larval and pupal mortality against the larvae and pupae with values of LC_{50} of 51.76, 61.87, 74.07, 82.18, and 440.65 ppm for 1st, 2nd, 3rd, and 4th instar larvae and pupae, respectively.

The author also evaluated the larvicidal activity of *Acalypha alnifolia* Klein ex Willd. (Euphorbiaceae) leaf extract against *A. stephensi*, *A. aegypti,* and *C. quinquefasciatus* [90]. Exposing the larvae to extract of different solvents for 24 hours, methanol extract was shown to be the most bioactive (98.4% larval mortality) while hexane extract was found to be the least bioactive as shown in Figure 8.

A combined bioefficacy of the fruit extracts of *Solanum xanthocarpum* and copepods of *Mesocyclops thermocyclopoides* was assessed for the control of *A. aegypti*, respectively [91]. The *S. xanthocarpum* fruit extract revealed significant larval mortality to *A. aegypti* 1st–4th instar larvae exposed to dosage of 100–300 ppm (the highest LC_{50} = 253.18 ppm). The authors reported an increase in the percentage of copepod predatory efficiency in the extract treated sample (8.7%) as compared to 6.5 % in samples without the extract. This increase in predation efficiency was opined by the authors to possibly be due to the detrimental effects of the extract active principle compound (solanocarpine and solanocarpidine) on the mosquito larvae [91].

Lalrotluanga et al. [92] recently reported the larvicidal, adulticidal, and repellent activity of *Hiptage benghalensis* L. Kruz (Malpighiaceae) acetone root bark extracts against mosquitos of *Anopheles*, *Culex,* and *Aedes* genera. The extract was found to be effective as larvicide with low LC_{50} (11.15–16.78 ppm) and lethal time LT_{50} (1.25–4.84 h at 200 and 400 ppm). The lethal time was found to be concentration dependent.

Kovendan et al. [93] reported their work on the larvicidal efficacy of *Sphaeranthus indicus*, *Cleistanthus collinus,* and *Murraya koenigii* organic-solvent leaf extracts against the third instar larvae of *C. quinquefasciatus*. Using a dosage of 750 ppm, significant mortality of larvae was observed. *S. indicus* extracts showed mortality of 78.62% in chloroform, 100% in ethyl acetate, and 60.16% in hexane extracts within 72 hours. *C. collinus* extract, on the other hand, showed 100% mortality in chloroform and 78.09% in hexane. Exposure to

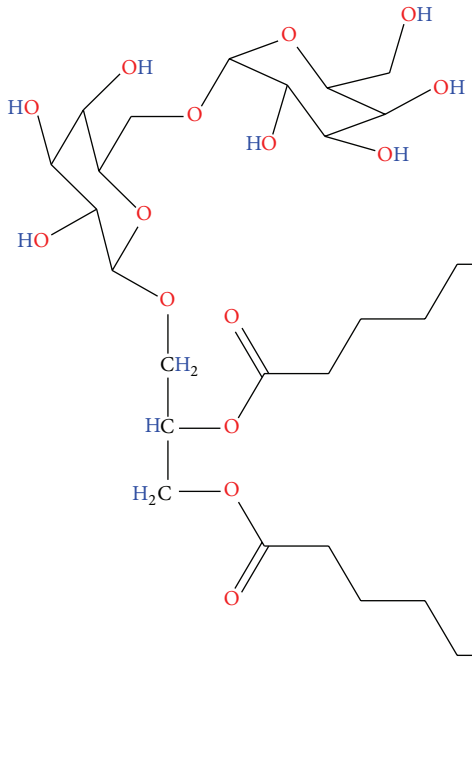

Figure 6: Chemical structure of the bioactive biochemical extracted from *B. racemosa* [15].

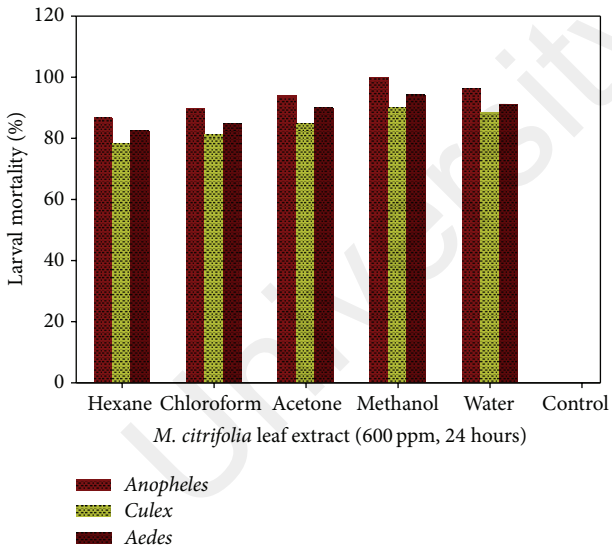

Figure 7: *M. citrifolia* solvent extract as a function of percent larval mortality.

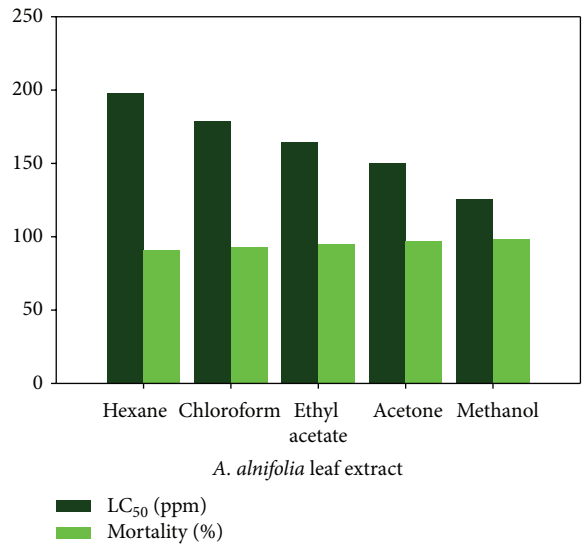

Figure 8: Influence of extraction solvent on larval percent mortality and LC_{50} during exposure to *A. alnifolia* extract.

M. koenigii extract for 72 hours showed mortality of 91.24% (ethyl acetate), 89.03% (chloroform), and 86.35% (hexane), respectively [93].

Indigofera suffruticosa Mill. (Fabaceae) is a plant found in tropical and subtropical areas and well adapted to growth in semiarid regions and low fertile soil. The plant has been known for its medicinal efficacy against bacterial and fungal infections [94]. Vieira et al. [94] reported their study on the oviposition and embryotoxicity of *I. suffruticosa* leaves extract against *Aedes aegypti*. The authors found the repellent activity to be concentration dependent. Exposing the larvae to concentration of 250 μg/mL for 72 hours showed 93.3% growth inhibition in L2 instar [94].

4. Conclusion

Ancient biomedicine described the use of plants in traditional system of medicine for the treatment of several human ailments, including filarial infections. This kind of complementary medicine provides an avenue for therapeutic treatment in a more benign approach, with plant materials that are mostly available and easily assessable. The present report is a survey of literature indicating the screenings of crude plant extracts, essential oils, and isolated active principles for *in vitro* and *in vivo* filaricidal activities to substantiate those folklore claims. It is worth mentioning that despite the fact that infection with *W. bancrofti* accounts for major incidence cases (91%) of total lymphatic filariasis infections while *B. malayi* and *B. timori* are responsible for only 9% in South and Southeast Asia, literature on biomedicinal efficacy against bancroftian filariasis is highly scarce; specifically the research on that area seems to be neglected. Hence, we opined that, in future studies, more research on filaricidal biochemicals with efficacy on *W. bancrofti* is needed.

Furthermore, proper control of filarial vector can be achieved via careful design of extraction and administration processes such as use of efficient bio-chemical solvent extraction, preferably hydrophilic solvent, and logically controlled dosage.

Conflict of Interests

The authors have declared that no conflict of interests exists either financial or otherwise from any company or other entities.

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Parasites & Vectors

RESEARCH Open Access

Lymphatic filariasis in Peninsular Malaysia: a cross-sectional survey of the knowledge, attitudes, and practices of residents

Nazeh M Al-Abd[1*], Zurainee Mohamed Nor[1], Abdulhamid Ahmed[2], Abdulelah H Al-Adhroey[1], Marzida Mansor[3] and Mustafa Kassim[3]

Abstract

Background: Lymphatic filariasis (LF) is a major cause of permanent disability in many tropical and sub-tropical countries of the world. Malaysia is one of the countries in which LF is an endemic disease. Five rounds of the mass drug administration (MDA) program have been conducted in Malaysia as part of the Global Program to Eliminate Lymphatic Filariasis (GPELF) by year 2020. This study investigated the level of awareness of LF and the MDA program in a population living in an endemic area of the country.

Methods: A descriptive cross-sectional survey that involved 230 respondents (≥15 years old) living in the LF endemic communities of Terengganu state in Peninsular Malaysia was performed. Demographic, socioeconomic, and knowledge, attitudes and practices (KAP) data of the respondents were obtained using pre-tested questionnaires and were analyzed using SPSS software version 13.0.

Results: More than 80% of the respondents were aware of LF and the common symptoms of the disease. Moreover, about 70% of the respondents that were aware of LF indicated that it is a problematic disease. Approximately 77% of the respondents indicated that filariasis is transmitted by mosquitoes. Two-thirds of respondents preferred hospital treatment for illness; however, only 12% had participated and/or received treatment for LF during an MDA program. Only 35% of the respondents that participated in this research were aware of the MDA program that had taken place in the area. None of the respondents had knowledge of the drug used in the treatment of LF. The findings from this research indicated that there was no significant association between LF awareness and with gender, age group, educational status, occupation, or socio-economic status of the respondents ($P > 0.05$).

Conclusion: A good proportion of the respondents are aware of LF, its mode of transmission and symptoms, however they demonstrated a poor knowledge of MDA which took place in the study area. For greater understanding of LF in the Malaysian population, there is a need for an enhancement in the delivery of health education and information programs and mass mobilization campaigns in endemic communities.

Keywords: Lymphatic filariasis, Mass drug administration, Control program, Mosquito

Background

Lymphatic filariasis (LF), often called elephantiasis, is considered by the WHO as the second most common debilitating mosquito-transmitted disease caused by filarial parasites [1]. It is classified as one of the neglected tropical diseases (NTDs) by the World Health Organization (WHO) and is the second leading cause of permanent long-term disability in the world [1,2]. The prevalence of LF continues to increase, and LF is a major public health concern that is associated with significant socio-economic obstacles [3]. Recent estimates suggest that approximately 1.4 billion people living in 73 tropical and sub-tropical countries are at risk of infection [4]. It has been estimated that approximately 120 million people have been infected globally by the disease and that approximately 40 million have become incapacitated due to

* Correspondence: Nazehali78@yahoo.com
[1]Department of Parasitology, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603, Malaysia
Full list of author information is available at the end of the article

the disease [4,5]. Approximately 65% of those infected live in South-East Asia, 30% in Africa, and the remainder in other tropical areas [6]. Approximately 90% of LF infections are caused by *Wuchereria bancrofti* and the rest are caused by *Brugia malayi* and *B. timori* [4]. In these endemic regions, the psychological, economic, and social impact associated with the disease is significant, adversely affecting productivity and quality of life. The most common manifestations of LF are hydrocele, lymphedema, and elephantiasis. In 1997, the WHO organized the Global Programme with the aim of eliminating LF as a public health crisis by the year 2020, mainly through the institution of annual mass drug administration (MDA) programs for those people living in endemic areas [7,8].

In Malaysia, LF is caused by *W. bancrofti* and *B. malay*i and is transmitted by mosquitoes of the genus *Anopheles* and *Mansonia* [9]. It occurs only in very small pockets in Malaysia: Sabah, Sarawak, and several states of the Peninsular Malaysia including Terengganu, Kelantan, Pahang, Selangor, and Johor [9]. In Malaysia, two phases of transmission-assessment survey (TAS) were performed during 2010–2011, with the goal of eliminating LF by 2015 [10]. According to the Ministry of Health Malaysia, five rounds of MDA program have been completed in all endemic areas between 2004 and 2008, with >80% coverage, using diethylcarbamazine (DEC) and albendazole [10]. According to Dr. Rose Faiza Hanim, the manager of the LF control program in Malaysia, the MDA program was strictly conducted according to WHO guidelines. TAS survey in Malaysia was conducted mainly in the Sabah state. After TAS-1, it was observed that the number of positive cases still exceed the critical cut-off value. Hence, MDA was continued before re-testing in TAS-2, but only one round of the drug administration was conducted due to DEC supply problems. TAS-2 was thus conducted after administering the Brugia rapid test (BmR1) and the result still showed values higher than the critical cut-off values and therefore it was recommended that MDA should continue in Malaysia (8). Despite these efforts, reports indicate increasing incidence of the disease. Thus, knowledge, attitudes, and practices (KAP) studied and additional TAS and MDA programs are required in the study area and other LF endemic areas of the country.

The success of the MDA program is dependent on the knowledge of the intended recipients of the program and is dependent on the program delivery system. Knowledge plays an important role in the prevention of LF. Awareness of LF is a suitable method to avoid the disease and remain healthy, as it is known that misunderstanding of illness and health-seeking behavior may improve or interfere with the effectiveness of control measures [11]. Therefore, we conducted a study of the population living in an LF endemic area in Terengganu state, Malaysia. The aim of the study was to assess the knowledge, attitudes, and practices of the study population with respect to LF, as well as knowledge of the MDA program. The results of this survey will aid in the design and implementation of educational strategies, as well as in the development of disease control and interventional methodologies that require active community participation.

Methods

Description of the study area

This cross-sectional study was conducted in Kemaman district, which is located in a coastal area of Terengganu state in Malaysia (Figure 1). The area was selected on the basis of an established occurrence of LF within the region. According to the Ministry of Health [10], the microfilariae (mf) rate in the endemic areas of the country ranges from 1.41 to 2.14 to per 1000 people, with 387 cases reported in 2011. The capital of the district is Cukai town, which is a coastal town located at latitude 4°14′N and longitude 103°25′E and is at an elevation of 42 feet above sea level. According to the 2006 population census of Malaysia, the Kemaman district had a population of 174,876 people, with Cukai town having a population of 82,425 people. Other settlements in the district include Hulu Cukai, Kijal, Seri Bandi, and Ibok. The main occupations of the residents include fishing, subsistence farming, transportation, industrial labor, and public service.

Study population

The study was conducted between August and October 2012. The study population consisted of all adolescents and adults who attended the participating health clinics within the study area during the survey period and agreed to participate in the survey. The clinics were the Sri Bandi health clinic, the Ibok health clinic, and the Kijal health clinic, which are the main public health facilities in the study area. In order to participate in the study, respondents had to have lived in the area for at least 5 years and had to be 15 years or older. In addition, pregnant women were excluded from the study to avoid partial or non-compliance. A total of 230 volunteered respondents aged ≥15 years participated fully in the survey. The survey was conducted with the support and cooperation from the local community leaders and medical personnel in the area.

Study questionnaire

A semi-structured questionnaire was developed by the researchers with input from a medical anthropologist. The questionnaire was validated and pre-tested with 20 individuals to ensure reliability and validity prior to initiating the fieldwork. During the fieldwork, questionnaires were administered with the help of a medical doctor and a nurse who were indigenous to the research area. The

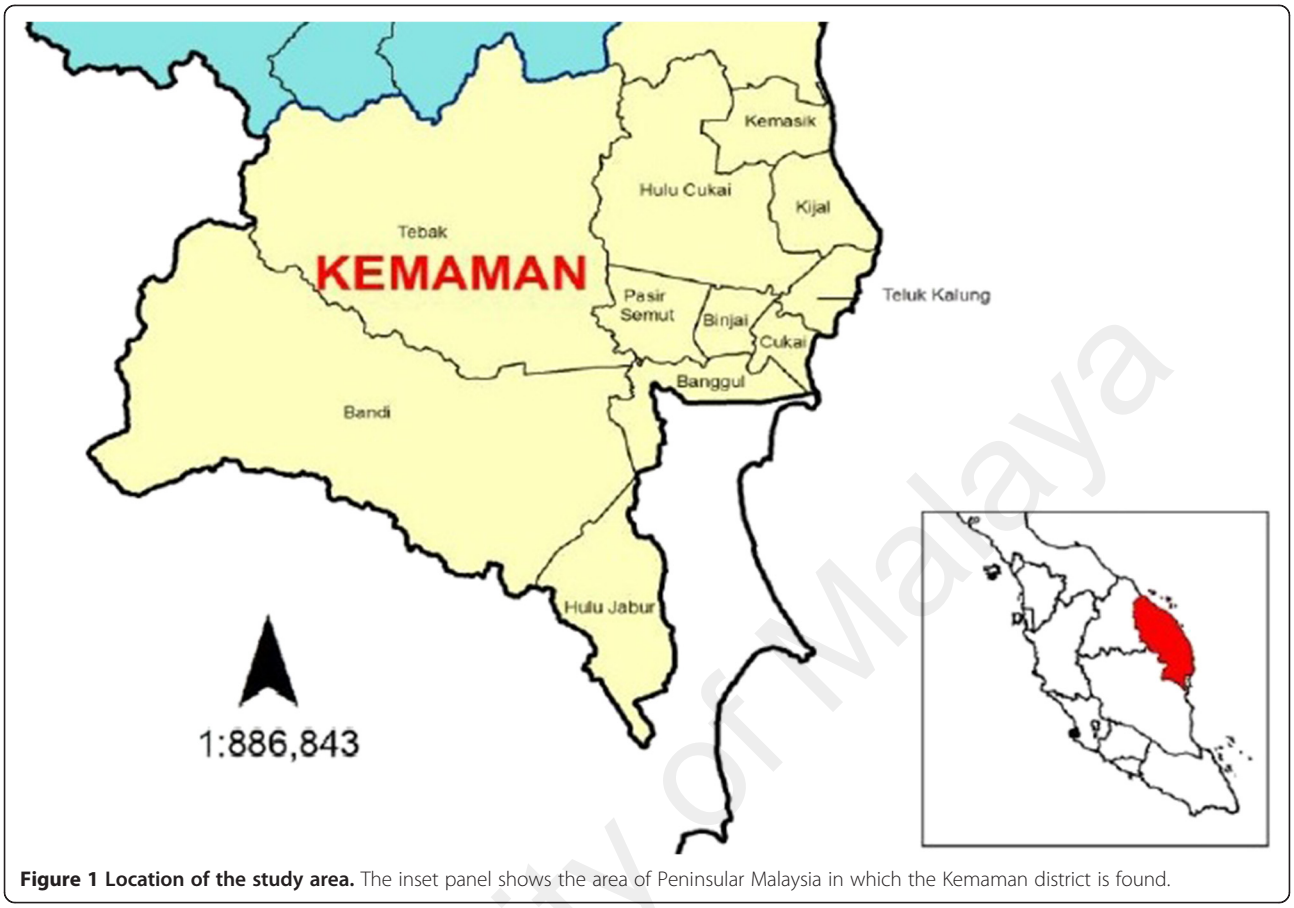

Figure 1 Location of the study area. The inset panel shows the area of Peninsular Malaysia in which the Kemaman district is found.

study participants were interviewed, using the local language, to determine the extent of each participant's knowledge of LF, including prevention, treatment, symptoms, and transmission, as well as the attitude of the participants towards the disease. Additional questions included those about the MDA program, such as participation in the MDA program and the source of information about the MDA program. Some questions were open ended and allowed the respondents the chance to give greater details while others were restricted to a yes or no answer. The questionnaire was not distributed house-to-house. The questionnaire used in the survey was written in Bahasa Melayu, the national language of Malaysia.

Ethical consideration

All respondents were fully notified that participation was voluntary and that it was possible to withdraw from the research without notice. Those who wished to participate were required to sign a consent form prepared in accordance with the guidelines of the Malaysian Department of Health and Human Services prior to the administration of the questionnaires.

Statistical analysis

The data obtained for this research was analyzed using SPSS (Statistical Package for Social Sciences, Version 13.0; SPSS, Chicago, IL, USA; 2004). The data was cleaned and checked thoroughly to ensure correctness of entries before the initiation of analysis. The demographic and socio-economic characteristics of the respondents were presented in percentages and frequencies. Association of the knowledge of filariasis with demographic factors of the respondents was assessed using the chi-square test. A *P*-value of less than 0.05 was considered to be significant in the determination of association between the variables.

Results

Study population characteristics

A total of 230 people voluntarily participated in the survey; 68.7% of respondents were female and 31.3% of respondents were male. The general socio-demographic characteristics of the respondents were presented in Table 1. Most patients were aged 30–39 years, while the age group ≥60 years had the least number of patients. Of the respondents, 90% had received education: 37% at the primary level, 39.1% at the secondary level, and 11.3% at the

Table 1 Socio-demographic characteristics of the study population

Variable	Number (N = 230)	Percent (%)
Sex		
Male	72	31.3
Female	158	68.7
Age		
15–29 years	67	29.1
30–39 years	90	39.1
40–49 years	58	25.2
50–59 years	10	4.3
≥60 years	5	2.2
Educational level		
No formal education	28	12.2
Primary	86	37.4
Secondary	90	39.1
Tertiary	26	11.3
Occupation		
Employed	108	47.0
Unemployed or housewife	122	53.0
Type of house		
Bamboo/wood	131	57.0
Bricks/rock	99	43.0
Income level		
≤ RM 500.00 ($160.00)	61	26.5
> RM 500.00 ($160.00)	169	73.5

tertiary level. Approximately 12% of the respondents had not received any formal education. Almost half of the respondents (47%) were employed, with 70% of the employed respondents earning more than RM500 (about US$165) per month. Of those not employed, the majority were housewives. There was an equal split in the percentage of respondents who owned wood/bamboo-based and the percentage of respondents who owned brick-based houses.

Knowledge of lymphatic filariasis

Almost all of the respondents (83.9%) had heard about LF and reported that the source of LF information was school (30.1%), mass media (21.8%), or both (8.8%). Others had heard about LF from health centers (12.9%), mass media and health centers (11.4%), or from other people (15.0%). The majority (77.2%) of respondents knew that LF is transmitted by mosquitoes. Approximately 20% did not know how LF is transmitted and 1 respondent mentioned bacteria as the agent of transmission. Slightly more than half (59.6%) reported that the main symptom of LF was swollen legs, while 11% admitted not knowing any symptoms. The knowledge of the respondents regarding LF and its transmission is shown in Table 2.

Attitudes and practices towards LF and its treatment

Approximately 68% of the respondents that exhibited LF knowledge in the survey perceived LF to be a problem. Furthermore, approximately 41.5% of the respondents perceived LF to be a medical problem that results in symptoms including pain, fever, itching, and the inability to walk. In contrast, approximately 40% of respondents viewed LF to be an economic problem that occurs as a result of inability to work, loss of employment, and expenditures for medication and/or transport to health facility. Approximately 11.5% of respondents viewed LF to be a social problem, as those infected are unable to interact with the community. The remaining 8% of survey respondents did not specify the kind of problem they perceived LF to be.

When asked about treatment of illness, the majority of respondents (60.9%) preferred hospital treatment.

Table 2 Respondents' knowledge of lymphatic filariasis, its transmission and MDA

Variable	Number (N = 230)	Percent (%)
Knowledge about lymphatic filariasis		
Yes	193	83.9
No	37	16.1
Source of knowledge		
Mass media	42	21.8
School	58	30.1
Health center	25	12.9
Mass media and school	17	8.8
Mass media and health center	22	11.4
Other people	29	15.0
Assumed method of transmission		
Bacteria	1	0.5
Mosquito	149	77.2
Worms	4	2.1
Don't know	39	20.2
Knowledge of mass drug administration		
Yes	81	35.2
No	149	64.8
Source of knowledge		
Mass media	26	32.1
School	32	39.5
Health centre	18	22.2
Mass media and school	1	1.2
Mass media and health centre	4	5.0

Approximately 2% preferred to use "bomoh/dukun" (traditional healer) to treat illness. The remaining respondents (37%) preferred to combine the 2 forms of treatments. Almost all respondents had poor knowledge of the drug used in the treatment of LF, as 96.1% of the respondents indicated having no knowledge of the drug used for treatment of LF and 4% of respondents mentioned paracetamol as the possible drug used for LF treatment. Moreover, when the respondents were asked about participation in an MDA program, only 12% admitted having participated in an MDA program previously or had family member(s) treated for LF through an MDA program.

To prevent transmission of LF, more than 40% of respondents reported using protective clothes and sleeping under bed nets to protect themselves from mosquito bites. However, 15% did not indicate specific protection against mosquitoes. Most respondents were aware that cleaning of water containers, provision of good drainage, the use of chemical sprays, or a combination of these activities were the correct methods to control the mosquito population. The attitudes of the respondents towards mosquitoes are presented in Table 3.

Table 3 Respondents' understanding of the symptoms of LF, treatment seeking behavior and attitudes towards mosquitoes

Variable	Number	Percent (%)
Known symptoms of LF (N = 193)		
Fever	8	4.1
Swollen legs	115	59.6
Body pain	1	0.5
Gland enlargement	3	1.6
Fever and swelling	29	15.0
Do you consider LF to be a problematic disease? (N = 193)		
Yes	130	67.4
No	5	2.6
Don't know	58	30.0
Type of problem (N = 130)		
Medical	54	41.5
Economical	52	40
Social	15	11.5
Unspecified	9	7
Preferred treatment method (N = 230)		
Hospital	140	60.9
Bomoh/dukan/traditional healers	5	2.1
Hospital and traditional healer	85	37.0
Presumed drug in the treatment of lymphatic filariasis		
Paracetamol	9	3.9
Don't know	221	96.1
Participation of respondent or family member(s) in mass drug administration program		
Yes	27	11.7
No	203	88.3
Protection from mosquito bites		
Wear clothes	25	10.9
Use of bed nets	77	33.5
Wear clothes and use of bed nets	90	39.1
No response	38	16.5
Control of mosquitoes		
Cleaning of water containers	30	13.0
Good water drainage	12	5.2
Use of chemical spray	15	6.5
Cleaning and drainage	42	18.3
Cleaning, drainage, and chemical spray	77	33.5
No response	54	23.5

Knowledge of MDA program among the respondents

The majority of the respondents (65%) were not aware of the existence of the MDA program (Table 2). The remaining 35% of respondents, who had previous knowledge of the MDA program, stated that they had learnt about it from schools (40%), mass media (32%), and health centers (22%).

Association between some demographic factors of respondents and knowledge of MDA and LF

The association between demographic factors and knowledge about LF and the MDA program among the study population was determined using chi-square tests (Table 4). No significant association was detected between the demographic factors examined and knowledge of LF or the MDA program.

Discussion

This survey was conducted in the state of Terengganu which is known to be endemic for LF. The information gathered for the purpose of this survey was obtained from visitors to the clinics in the survey area. The results showed that the majority of the respondents were women which may pose potential bias. However this is not the plan of the researchers, and is not expected to affect the findings since both the women and men have equal chances of getting infected. Moreover, experienced medical personnel were used to assist in data collection especially the administration of the questionnaires. This is done because these personnel were mostly indigenes of the area, they speak the local language of the respondents and we feel that because they interact directly with the target population, involving them will facilitate compliance and cooperation of the respondents to give honest

Table 4 Association between lymphatic filariasis knowledge and respondents' demographic factors

Variables	Knowledge of lymphatic filariasis		
	Prevalence (%)	Odds ratio (95% confidence interval)	*P*-value
Sex			
Female	86.1	1.19 (0.895, 1.570)	0.130
Male	79.2	1	
Age group			
≥40 years	89.0	1.56 (0.818, 2.968)	0.104
<40 years	81.5	1	
Education level			
≥6 years formal education	85.1	1.10 (0.934, 1.294)	0.138
No formal education	75.0	1	
Occupation			
Employed	87.4	1.33 (0.859, 2,055)	0.114
Unemployed or housewife	80.7	1	
Monthly income			
≤ RM 500.00 ($160.00)	90.9	1.36 (0.188, 9.828)	0.611
> RM 500.00 ($160.00)	87.7	1	

information required [10]. Furthermore, all aspects of the survey were conducted in close supervision by the researchers.

The WHO has recommended the implementation of knowledge, attitudes, and practices (KAP) surveys as a cornerstone for health promotion campaigns, as the surveys help programs adjust health education messages to increase public knowledge and awareness [12]. The KAP related to LF infection differs between regions and is heavily influenced by socio-cultural settings. Little is known about how individual communities incorporate knowledge of the origins and impacts of LF into local knowledge systems [13]. To the best of our knowledge, this is the first KAP study of LF in residents of LF endemic areas of Peninsular Malaysia.

This survey of indigenous adults who have lived in the area for at least 5 years was the first to be performed in this LF endemic area of Malaysia. The survey was limited to only those who attended the clinics for any reason; however, all pregnant women were excluded from the study. The majority of the survey respondents were female respondents, likely due to the fact that women in the area make more hospital visits or that women in the area are more cooperative and willing to volunteer for surveys. After receiving training from the researchers, medical doctors and nurses administered the questionnaires to ensure unbiased reporting and responses from the subjects.

Our study revealed that although the study area is categorized as an LF endemic area, the majority of the respondents were not aware of that status, revealing that information about the disease was not effectively conveyed to the general public. Thus, there were people who had poor or no knowledge of LF. This finding is in agreement with several previous studies performed on the population of endemic areas in Thailand [14], Ghana [15], Tanzania [16], and India [17,18].

In the control or elimination of a disease, the population involved must have prior knowledge of the disease for the control measure to be successfully implemented. Our survey, as well as others [19,20], indicated that the major sources of information were schools, health centers, and the mass media. In order to achieve greater awareness in the community, additional informational campaigns should be considered, including house-to-house visits.

In our survey, the majority of the respondents indicated knowing that LF is transmitted by mosquitoes. This is in agreement with the findings of previous studies [20,21], however, several other studies [18,22,23] have reported that the majority of respondents did not know that mosquitoes are the vectors that transmit LF. The implication of this deficit of knowledge is that families may not take appropriate measures to protect their family members, which could counteract efforts to control the disease.

In our study, the majority of the respondents recognized that the common symptoms of LF include swelling of the legs, as well as other symptoms including fever. This is consistent with previous studies [12,20,21,24,25]. In contrast, it has also been, reported that the majority of respondents in 1 study did not know the symptoms of LF [17].

Our findings with respect to the attitudes of the respondents towards LF showed that the majority of respondents view LF to be a problematic disease. Furthermore, the

respondents had differing views in terms of the significance of LF as a problem, with respondents viewing LF as a medical, a social, or an economic problem. This finding is in agreement with the findings from a recent Indonesian survey [26]. The fact that only two-thirds of the respondents indicated that they view LF to be a problem shows that awareness and knowledge of the disease in general is lacking among the residents of this endemic community. Thus, there is a need to increase efforts to improve education of the residents to ensure effective control of LF.

Our survey revealed that the majority of respondents preferred hospital treatment during illness, indicating that there is awareness of the usefulness of hospitals. However, approximately 40% of the respondents still consider traditional methods when treating illnesses. Similarly, in Nigeria, the majority of respondents were reported to prefer hospital treatment, while a small portion preferred traditional treatment methods [20]. Although, most of the respondents preferred hospital treatment, their knowledge of the drug used in the treatment of LF was poor, similar to what has been observed previously in India [18].

Despite the fact that the study area is known to be endemic for LF and an MDA program was previously conducted in the area, our survey showed that only a small proportion of respondents had obtained treatment for LF. This result could suggest that the respondents are either ignorant of or are taking for granted the treatment of LF and the MDA program. It could also be possible that the drug deliverers do not strictly observe the people taking their drugs directly. As approximately two-thirds of the respondents were not aware of the MDA program that took place in the area and approximately one-fifth had not heard of LF before the survey, it is likely that a large proportion of people did not participate in the MDA program. Similarly, the poor awareness of the people regarding the MDA program results in poor participation. Thus, the success of an MDA program depends upon the target population's knowledge of the benefits. Knowledge is therefore a vital component in the success or failure of any MDA program [11]. Poor knowledge leads to poor participation, and poor participation leads to low coverage and persistence in transmission of the disease. Moreover, we observed that the MDA program in Malaysia concentrated mainly on distributing the drugs to people, with less emphasis on ensuring that they actually swallowed the drugs or that they are educated on preventive measures such as the use of bed nets and care of enlarged limbs. Hence, suspected patients kept on going to the hospitals with one complain or the other. However, in this survey we did not encounter any admitted case of LF in any clinic.

One of the most important preventative measures in the eradication of mosquito-borne diseases, such as filariasis, is the prevention of mosquito bites. Our study indicated that the majority of respondents use protective clothes or sleep under bed nets to protect against mosquito bites; however, approximately one-sixth of the respondents did not mention using any form of protection. The fact that a proportion of respondents did not mention any protective measures probably indicates that the respondents did not see the need for protection or that they are not comfortable taking preventative measures. There could be some other barriers too such as cost, availability or ease of use of the materials. Either way, the lack of knowledge with respect to the transmission of LF is apparent. Interestingly, our survey revealed that most of the respondents were aware of several ways used to control mosquito breeding, demonstrating some understanding of vector control strategies, although this knowledge was not necessarily translated to be part of an effective eradication program. While our results are in agreement with a previous study [20,27], a number of previous studies demonstrated that the majority of their subjects did not know the importance of minimizing mosquito contact in preventing infection [13,16,23,26].

As evidenced from the findings of a previous survey, the knowledge gap regarding LF, as well as general attitudes towards and perceptions of the eradication program, was the basis of the major causes of lower compliance [28], this could have likely resulted in the continued endemicity of LF in the endemic areas of Malaysia. This has been shown to occur in Kenya [29,30], Papua New Guinea [31], and in India [25,32], where it was reported that there was low compliance for an MDA program due to poor knowledge of LF by the target population. On one hand there is sometimes the problem of poor drug delivery. This was observed to be among the hindrance encountered in Malaysia after the completion of TAS −1 when MDA was continued [8].

Furthermore, as observed by some other researchers, no single formula can ensure success of MDA in all settings as compliance may be negatively affected by other factors such as the perceptions of the potential benefits of participation, the possible risk of adverse events as well as the fear of the unknown by the target population [10].

The success of the MDA program to treat LF is dependent on the knowledge of the target population. It cannot be assumed that the distribution of information from schools, health centers, and mass media is sufficient at conveying the information effectively. Recent studies have shown that the distribution of information leaflets and posters [25] are effective. The use of appropriate means of communication based on prevailing conditions is important in ensuring that messages reach the target audience.

There was no significant association found between LF knowledge and gender, occupation, age, educational status, or income of the respondents; however, our results

did indicate that females, older respondents, employed respondents, and respondents with higher income had greater knowledge of LF. This finding is in contrast to the results of a study performed in the Philippines [13] that found significant associations between gender, age, and educational status of respondents with LF knowledge.

Despite the fact that LF is an increasing burden from the perspective of both public health and economics, there seems to be little research on LF in comparison to other neglected tropical diseases. Public health authorities therefore have a great role to play in educating the people living in endemic areas for LF on the dangers posed by the disease in terms of loss of DALYs, causing permanent incapacitation to patients and huge economic loss on treatment etc., which leads to a general low productivity. The effect of LF is also very serious on the part of the governments, as considerable funds are needed for both MDA administration and case management in endemic areas. There is thus, an urgent need for continued research on methods of elimination of LF infection among endemic and vulnerable communities. This could be achieved by an effective education program that focuses on LF transmission and prevention, via public media awareness, or by strategic advocacy on vector control. Other innovative methods of educating residents of endemic and vulnerable communities include incorporating public health professionals, audio-visual campaigns, and the running of workshops and seminars. In addition, participation in activities or exhibits that promote the adoption of policies regarding prevention and control of the disease would increase public awareness. Nevertheless, it is important that the information is presented in a concise, informative, and easy to understand form. Finally, it is recommended that an awareness campaign regarding the importance of MDA be stressed in all endemic areas of Malaysia before embarking on subsequent MDA rounds for successful implementation and control.

Conclusion

The findings from this survey showed that there was some awareness regarding LF among people in Kemaman district of Malaysia, although knowledge of the MDA program was poor. Pre-MDA campaigns would help in improving residents' knowledge of LF and of the purpose of MDA programs and would increase the likelihood of participation in the MDA program, thereby improving the general wellbeing of the people in the area.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NMA was involved in all phases of the study, including data collection, data analysis, interpretation, and write-up of the manuscript; ZMN designed, supervised the study, and revised the analysis and manuscript. AH were involved in data collection; AA, MM and MK were involved in the analysis and revised the manuscript. All authors read and approved the final version of the manuscript.

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Author details

[1]Department of Parasitology, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603, Malaysia. [2]Department of Biology, Faculty of Natural and Applied Sciences, Umaru Musa Yar'adua University, Katsina, Katsina State, Nigeria. [3]Department of Anesthesiology, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603, Malaysia.

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Antioxidant, antibacterial activity, and phytochemical characterization of *Melaleuca cajuputi* extract

Nazeh M. Al-Abd[1*], Zurainee Mohamed Nor[1], Marzida Mansor[2], Fadzly Azhar[3], M. S. Hasan[2] and Mustafa Kassim[2]

Abstract

Background: The threat posed by drug-resistant pathogens has resulted in the increasing momentum in research and development for effective alternative medications. The antioxidant and antibacterial properties of phytochemical extracts makes them attractive alternative complementary medicines. Therefore, this study evaluated the phytochemical constituents of *Melaleuca cajuputi* flower and leaf (GF and GL, respectively) extracts and their antioxidant and antibacterial activities.

Methods: Radical scavenging capacity of the extracts was estimated using 2,2-diphenyl-2-picrylhydrazyl and Fe^{2+}-chelating activity. Total antioxidant activity was determined using ferric reducing antioxidant power assay. Well diffusion, minimum inhibitory concentration, and minimum bactericidal concentration assays were used to determine antibacterial activity against eight pathogens, namely *Staphylococcus aureus, Escherichia coli, Bacillus cereus, Staphylococcus epidermidis, Salmonella typhimurium, Klebsiella pneumonia, Streptococcus pneumoniae, and Pasteurella multocida*. We identified and quantified the phytochemical constituents in methanol extracts using liquid chromatography/mass spectrometry (LC/MS) and gas chromatography (GC)/MS.

Results: This study reports the antioxidant and radical scavenging activity of *M. cajuputi* methanolic extracts. The GF extract showed better efficacy than that of the GL extract. The total phenolic contents were higher in the flower extract than they were in the leaf extract (0.55 ± 0.05 and 0.37 ± 0.05 gallic acid equivalent per mg extract dry weight, respectively). As expected, the percentage radical inhibition by GF was higher than that by the GL extract (81 and 75 %, respectively). A similar trend was observed in Fe^{2+}-chelating activity and β-carotene bleaching tests. The antibacterial assay of the extracts revealed no inhibition zones with the Gram-negative bacteria tested. However, the extracts demonstrated activity against *B. cereus, S. aureus*, and *S. epidermidis*.

Conclusions: In this study, we found that *M. cajuputi* extracts possess antioxidant and antibacterial activities. The results revealed that both extracts had significant antioxidant and free radical-scavenging activity. Both extracts had antibacterial activity against *S. aureus*, *S. epidermidis*, and *B. cereus*. The antioxidant and antimicrobial activities could be attributed to high flavonoid and phenolic contents identified using GC/MS and LC/MS. Therefore, *M. cajuputi* could be an excellent source for natural antioxidant and antibacterial agents for medical and nutraceutical applications.

Keywords: *Melaleuca cajuputi*, Antioxidant capacity, Total phenol content, Gas chromatography/mass spectrometry (GC/MS), Liquid chromatography/mass spectrometry (LC/MS)

* Correspondence: Nazehali78@yahoo.com
[1]Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia
Full list of author information is available at the end of the article

Background

The cells of living organisms generate free-radicals as a result of pathophysiological and biochemical processes in response to factors such as environmental pollutants, radiation, chemicals, and toxins. This creates an imbalance in the formation and neutralization of prooxidants that subsequently seek stability through electron pairing with biological macromolecules such as proteins, lipids, and DNA, leading to oxidative stress in the physiological system [1]. Furthermore, these effects lead to lipid peroxidation as well as protein or DNA damage or both in human cells. Moreover, the cellular damage consequently lead to aging and several chronic diseases such as cancer, diabetes, and atherosclerosis as well as cardiovascular, inflammatory, and other degenerative diseases in humans [1]. The ability of certain phytochemical extracts to inhibit or delay the oxidation of other molecules by suppressing the initiation or propagation of oxidizing chain reactions have made them active alternatives in complementary medicine. These naturally occurring antioxidant chemicals have been reported to be composed of phenolic (such as flavonoids, phenolic acids, and tocopherols) and nitrogen compounds (alkaloids, chlorophyll derivatives, amines, and amino acids) as well as carotenoids and ascorbic acid [2]. In fact, phytochemical extracts containing constituents such as plant-derived vitamins, flavonoids, alkaloids carotenoids, terpenoids, polyphenols, and phenolic compounds such as caffeic, vanillic, ferulic, and ellagic acids have been reported to exhibit antioxidant and anticancer activities [3].

Although chemically synthesized antioxidant compounds such as butylated hydroxytoluene and hydroxyanisole have been used for several decades, the safety of their continued use is currently being questioned due to reports of their carcinogenicity [4]. Therefore, alternative effective antioxidants that have benign or minimal side effects are highly needed.

Infectious diseases caused by microorganism are a major cause of mortality and morbidity in humans. Although several antibiotics have been developed to manage these diseases with optimum efficacy, their mismanagement and maladministration, as well as microbial mutation have led to the emergence of drug-resistant strains. As a result, over the past decades, antibiotics that are known to cure specific diseases have lost their effectiveness. Therefore, the search for new antimicrobial drugs from natural sources is warranted.

Traditional medicine practices in ancient human civilizations worldwide have demonstrated that plants are one of the most promising sources of effective medicinal agents. Therefore, scientific studies have been carried out on the antimicrobial activities of plant extracts against different types of microorganisms, which have resulted in the development of alternative plant-based antimicrobial drugs.

Numerous phytochemical extracts have been evaluated in the process of searching for plant-based antimicrobial agents and some recently reported studies include those on *Syzygium gratum, Justicia gangetica*, and *Limnocharis flava* [5], *Buglossoides purpurocaerulea* [6], Nymphaea nouchali [7], and Polygonum hydropiper [8]. *Melaleuca cajuputi* is commonly known as the Gelam tree and is used to cure cholera as well as muscle and joint pain in folk medicine. It is a member of the Myrtaceae family with reported anti-inflammatory [9], anticancer [10], hepatoprotective [11], and anthelmintic activities [12]. Studies have revealed the antibacterial activity of essential oils of *M. caguputi* against Gram-positive and Gram-negative bacterial strains in the disc diffusion and minimum inhibitory concentration (MIC) assays. The extracted oil inhibited the growth of *Enterococcus faecalis, Escherichia coli, Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella enterica*, *Staphylococcus aureus,* and *Streptococcus pyogenes* [13, 14].

In this study, we evaluated the *in vitro* antioxidant and antibacterial activities of methanolic extracts of the leaves and flowers of *M. cajuputi*. In addition, we analyzed the phytochemical constituents of the extracts using liquid chromatography (LC)/mass spectrometry (MS) and gas chromatography (GC)/MS.

Methods

Chemical and reagents

Methanol, sodium hydroxide (NaOH), iron (II) sulfate ($FeSO_4$), iron (II) chloride ($FeCl_2$), sodium nitrite, iron (II) chloride ($FeCl_3$), chloroform, hydrochloric acid (HCL), β-carotene, quercetin, chloragenic acid, tripyridyl-s-triazine (TPTZ), butylated hydroxytoluene (BHT), propyl gallate, 2,2-diphenyl −1- picrylhydrazyl (DPPH), linoleic acid, Tween 20, Folin-ciocalteu reagent, acetate buffer, ferrozine, ethylenediaminetetraacetic acid (EDTA), ascorbic acid, and all other reagents and solvents used in this study were of analytical grade purchased from Merck (Merck, Darmstadt, Germany).

Plant materials

The plant material used in this study was collected from Kedah State, Malaysia, in September 2013, and Identity was confirmed at the Herbarium of Rimba Ilmu, Institute of Biological Sciences, University of Malaya, Kuala Lumpur with voucher number KLU048231.

Preparation of methanolic extracts

The leaves and flowers of the Gelam tree (*M. cajuputi*) were washed separately with distilled water, ground to a powder, and then dried in the shade for seven days. The methanolic extracts were prepared by adding 100 g of either the Gelam leaves (GL) or Gelam flower (GF) powder to 1 L of absolute methanol in a conical flask and leaving it

for 72 h at 25 °C. The mixtures were then filtered using Whatman filter paper (No: 1) to obtain the extract and this procedure was repeated thrice, followed by *in vacuo* concentration at 40 °C using a rotary evaporator to obtain the GF and GL extracts.

GC/MS analysis

The GC/MS analysis of the methanol extract was carried out by sonicating a 10-mg sample for 15 min in 2.5 mL of dichloromethane at 40 °C in a sealed vial. Then, 1 mL of the treated extract sample was filtered through a 0.20-μm nylon filter into a standard GC 2-mL vial for analysis. The GC/MS analyses were performed at an ionization energy of 70 eV while separation of the hydrocarbons and other volatile compounds were determined using a GCMS-QP2010 series GC system (Shimadzu, Japan) equipped with a DB-5MS Agilent nonpolar column (30 mm × 0.25 mm, 0.25 mm) (Agilent Technologies Inc., Tokyo, Japan). The oven was initially programmed to run at a temperature of 60 °C for 2 min, followed by an increase of 7 °C/min to 150 °C, with a final hold at 310 °C for 15 min. The injector and detector temperatures were kept at 300 (split) and 310 °C, respectively. The analysis was performed with He as the carrier gas at a linear flow rate of 40 cm/s, and the MS detector was operated at 200 °C while the scan range was from 50–1000 m/z at a rate of 0.50 scan/s. To check the purity of each GC peak, the MS was recorded at various parts of each peak. All compounds were putatively identified using a mass spectral database search (National Institute of Standards and Technology/ Environmental Protection Agency/National Institutes of Health, NIST/ EPA/NIH) followed by a comparison with the acquired MS data to determine the degree of matching. The compounds that showed mass spectra with match factors ≥90 % were included on the "positive list" of tentatively identified metabolites.

LC/MS analysis

The LC/MS experiments were conducted to chemically profile the methanolic extracts. The system used to analyze the samples was comprised of an Agilent 1290 Infinity LC system coupled to an Agilent 6520 Accurate-Mass quadrupole time-of-flight mass spectrometer with dual electrospray ionization (ESI) source. The LC separations were performed using a 2.1 mm (i.d) Narrow-BoreSB-C18 (length 150 mm, particle size 3.5 mM) analytical column. The LC parameters used were: autosampler temperature, 25 °C; injection volume, 0.5 μL; column temperature, 25 °C; and flow rate, 0.4 mL/min. A gradient system consisting of solvents A (0.1 % formic acid in water) and B (0.1 % formic acid in acetonitrile) was employed. The mass spectra data were acquired using an ESI capillary voltage of (+) 4000 V and (−) 4000 V in the positive and negative ion modes, respectively with the fragmentor at 125 V. For the other conditions, the liquid nebulizer was set to 45 psi, the nitrogen drying gas was set at a flow rate of 10 L/ min with the drying gas and vaporizer temperatures maintained at 300 °C, and the ionization interface was operated in both positive and negative modes. The data were acquired at a rate of 1.03 spectra/s with a stored mass range of 100–3200 and 115–3200 m/z for the positive and negative modes, respectively. The data were collected using the Agilent Mass Hunter Workstation Data acquisition software. LC/MS data files were processed using the Agilent Mass Hunter Qualitative Analysis B.05.00 software. Feature finding was achieved by using the molecular feature extraction and correlation algorithms, which located the groups of covariant ions in each chromatogram. In the positive-ion mode, this included adducts H^+, Na^+, K^+, and NH_4^+ and in the negative-ion mode, adducts H^- and Cl^-.

Total flavonoid assay

The total flavonoid content of the methanolic extracts was determined photometrically using the aluminum chloride ($AlCl_3$) assay [15]. Briefly, a 1-mL aliquot of each extract (1 mg/mL) or standard solution of quercetin (31.5, 62.5, 125, 250, 500, and 1 mg/L) was added to a volumetric flask (10-mL) and diluted with 4 mL double distilled water at time 0. Then, 0.3 mL of 5 % (w/v) sodium nitrite ($NaNO_2$) was added and after 5 min, 0.6 mL $AlCl_3$ (10 %) was added. At 6 min, 2 mL of sodium hydroxide (NaOH, 1 M) was added to the mixture, and the final total volume was made up to 10 mL with double-distilled water. The solution was mixed completely, and the absorbance was measured against a prepared reagent blank in triplicate at 430 nm. The total flavonoid content was expressed as quercetin equivalents in mg/100 g of dry extract weight.

Total phenolic content (TPC)

The TPC of the methanolic extracts was determined using the Folin-Ciocalteu reagent (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) as previously described by Kim et al. [16], with slight modifications. Briefly, 100 μL of each extract or standard solution of gallic acid (16–1000 μg/mL in 80 % methanol) was mixed with 200 μL of Folin-Ciocalteu reagent, followed by 2 mL of deionized water and 1 mL of 15 % sodium bicarbonate (Na_2CO_3). Then, the mixture was incubated for 120 min at room temperature, and the absorbance was measured at 765 nm in triplicate using an ultraviolet (UV)-Visible (Vis) spectrophotometer (GBC, Cintra 40). The total phenolics were quantified using a calibration curve constructed from measurements of the standard gallic acid concentrations and expressed as mg gallic acid equivalent (GAE) per mg of extract weight.

Determination of antioxidant activity using DPPH radical scavenging

The antioxidant activity of the extracts was measured by determining the hydrogen donating or radical scavenging ability, using the stable radical, DPPH as reported previously [17]. An aliquot (120 μL) of 0.25 mM DPPH solution in methanol and 30 μL of each extract at increasing concentrations (31.3, 62.5, 125, 250, 500, and 1000 μg/mL) were mixed vigorously together and left at room temperature in the dark. The absorbance was measured at 518 nm after 30 min against different concentrations of the extracts in methanol as blanks and DPPH in methanol without extract as the control. The standard synthetic antioxidant, butylhydroxytoluene was used as the positive control. The percentage antiradical activity (AA%) of the extracts was calculated using the following formula [15],

$$AA\% = \left(100 - \left(\frac{Abs_{sample} - Abs_{blank}}{Abs_{control}}\right) \times 100\right)$$

Where, Abs_{sample}, Abs_{blank}, and $Abs_{control}$ are the absorbance values of the extract, blank, and control samples, respectively.

β-Carotene bleaching test

The β-carotene bleaching test was used to evaluate the antioxidant activities based on the β-carotene lenolate system model [18], with slight modifications. Briefly, 1 mL of β-carotene solution (0.2 mg/mL in chloroform) was added to 0.02 mL of linoleic acid and 0.2 mL of 100 % Tween 20. Then, 5 mL samples of this emulsion were transferred into test tubes containing 0.2 mL of test samples in 80 % methanol at increasing concentrations (62.5, 125, 250, 500, and 1000 μg/mL). These mixtures were then incubated in a water bath at 40 °C for 120 min. All determinations were performed in duplicate and the mean values calculated. The absorbance was measured at 470 nm using a Perkin–Elmer Lambda 40 UV/Vis spectrophotometer against a blank consisting of the emulsion without β-carotene. The measurements were carried out at initial and final times (t = 0 and 120 min, respectively) with propyl gallate as the positive control. The AA% was measured and expressed as the percentage of inhibition of β-carotene oxidation using the following equation:

$$AA\% = \left[\left(\frac{AS_0 - AS_{120}}{AC_0 - AC_{120}}\right)\right] \times 100$$

Where AS_0 and AS_{120} are absorbance values of the samples and AC_0, and AC_{120} are the controls at 0 and 120 min, respectively.

Fe^{2+}-chelating activity assay

The Fe^{2+}-chelating activity of GL and GF extracts was measured as follows: The extract was treated with ferrozine (5 mM), which reacted with the divalent iron to form a stable and highly water-soluble magenta complex species. After 10 min at room temperature, the absorbance of the Fe^{2+}–ferrozine complex was measured at 562 nm. The Fe^{2+}-chelating activity of the extract was calculated using the following equation:

$$\%\text{Chelating rate} = \frac{A0 - A1}{A0} \times 100$$

Where, A_0 and A_1 are the absorbance values of the control (blank without extract) and in the presence of the extract, respectively.

Ferric reducing antioxidant power (FRAP) assay

The ferric reducing power of the extracts was assayed based on the blue coloration that developed due to the reduction of ferric iron to the ferrous form as described previously [18]. Extract solutions were prepared by dissolving about 0.1 μg/mL of extracts in ethanol. An aliquot (0.2 mL) of each extract solution was added to a test tube containing 1.8 mL of freshly prepared FRAP reagent that consisted of 2.5 mL of 10 mM TPTZ solution in 40 mM of HCl and 2.5 mL of 20 mM $FeCl_3.6H_2O$ in 25 mL of 0.3 M acetate buffer (pH 3.6). The mixture was incubated at 37 °C for 5 min. The spectrometric absorbance was recorded at 593 nm. The reducing power was ascertained by comparing the spectrophotometric absorbance of each sample against a standard curve obtained from Fe_2SO_4.

Antimicrobial activity

Test organisms

The *in vitro* antibacterial activities of the *M. cajuputi* extracts were evaluated against eight strains that were supplied by the Microbiology Laboratory of the University of Malaya Medical Centre. They comprised of four Gram-positive (*Staphylococcus epidermidis*, MTCC 3615; *Staphylococcus aureus,* RF 122; *Bacillus cereus*, ATCC 11778*;* and *Streptococcus pneumoniae*, ATCC 10015) and four Gram-negative (*Escherichia coli*, UT181; *Salmonella typhimurium*, ATCC 14028; *Klebsiella pneumonia*, ATCC13883; and *Pasteurella multocida*, a clinically isolated strain) bacterial strains. All the strains were stored in the appropriate medium before use.

Inocula preparation

The colony suspension method was used to prepare the inocula of the test organisms. The bacterial strains were grown on nutrient agar (NA) at 37 °C for 18 h, and then adjusted to a turbidity of 0.5 McFarland standards (10^6

colony forming units, CFU/mL) based on the optical density (OD) measurement at 620 nm. After being cultured for 24 h on NA, the colonies were collected, and cultured in nutrient broth medium for 24 h at 37 °C. The susceptibility tests were subsequently performed using the NA-well diffusion method.

Bacterial cultures and disc diffusion assay

The disc diffusion method is a widely acclaimed method used in screening crude extracts for antibacterial activities. In this study, the antibacterial activity was determined based on the method previously described [19] with modifications. Briefly, the crude extract at a concentration of 0.1 g/mL was dissolved in 100 % dimethyl sulfoxide (DMSO, Merck, Germany) and sterilized by filtration using a 0.20-mm Millipore disposable filter (Minisart, Sartorius Biotech, Germany). Autoclave-sterilized (121 °C for 20 min) Mueller Hinton Agar (MHA) medium (BioLab) was used in the disc diffusion assay. A 50-μL sample of the filtration-sterilized plant extract was loaded onto a sterile paper disc (6 mm in diameter), which was then placed on the surface of the agar plate (NA) previously inoculated with the bacteria. A disc prepared under the same conditions with only 50 μL of DMSO was used as a negative control. In addition, a similar disc was loaded with the reference antibiotic (streptomycin) at a concentration of 20 mg of drug per disc and used as described above. Both samples were allowed to diffuse into the agar plates for 1 h and were then inverted and incubated at 37 °C for 18 h. Antibacterial activity was determined by measuring the diameter of the growth inhibition zones (IZs, mm) surrounding each disc. Each assay was performed in triplicate with two repetitions and the results were expressed as average values.

MIC and minimal bactericidal concentration (MBC) assays

The MIC values, which represent the lowest plant extract concentration that completely inhibits the growth of microorganisms, were determined using a micro-well dilution method as described previously [20]. In addition, the MBC values refer to the lowest concentration of an antibacterial agent required to prevent the growth of a particular bacterium after subculture in an antibiotic-free medium. Briefly, the extracts were dissolved in DMSO at 100 mg/mL, and then twofold serial dilutions were prepared in a 96-well dilution microplate. The antibiotic streptomycin was included as reference agent in each assay while the extract-free solution was used as a blank control. Each well of the microplates contained 40 μL of growth medium, 10 μL of inoculum (10^6 CFU/ml), and 50 μL of diluted sample extracts. Then, the microplates were incubated overnight at 37 °C. As an indicator of microorganism growth, 40 μL of p-iodo nitro tetrazolium violet (INT) dissolved in water was added to the wells, and the plates were incubated at 37 °C for 30 min. The colorless tetrazolium salt acts as an electron acceptor and is reduced to a red-colored formazan product by biologically active organisms [20]. A Tecan microplate reader (Infinite M200PRO) was used to quantify the OD of the reactants in each well. Where microbial growth was inhibited, the solution in the well remained clear after incubation with INT. The determination of MIC values was performed in triplicate. The MBC of the extracts was determined by subculturing samples from the MIC assay tubes onto NA plates from wells that showed growth inhibition, and then subsequently determining the dilution at which growth was arrested, which was considered the MBC.

Statistical analysis

The data were expressed as mean ± standard deviation (SD) of triplicate determinations. The half-maximal inhibitory concentrations (IC_{50}) values were estimated from the AA% versus concentration plots using a non-linear regression algorithm.

Results and discussion

In this study, we evaluated the chemical constituents of GL and GF using preliminary GC/MS and LC/MS analysis. Furthermore, we determined their antioxidant and antibacterial activities using various *in vitro* methods.

GC/MS and LC/MS analysis of GF and GL from *M. cajuputi*

The phytochemical analyses of plant extracts are normally performed using diverse quantitative and qualitative analytical techniques spanning from chromatography to spectroscopy [21]. Previously, K-L Li and S-J Sheu [22] used a micellar electrokinetic capillary chromatographic method to analyze the phytochemical constituents of scute-coptis, a dual herbal combination. Using this method, the researchers identified six scute flavonoids namely baicalin, wogonin 7-*O*-glucuronide, oroxylin A 7-*O*-glucuronide, baicalein, wogonin, and oroxylin A as well as four coptis alkaloids comprising of berberine, palmatine, coptisine, and epiberberine.

In another study, GC/MS was used to characterize the chemical content of *Melaleuca* essential oils [23–25]. Similarly, silica gel chromatography was used to isolate a new chromone from *M. cajuputi* leaf extracts [26]. Using a combination of spectroscopic techniques, the study characterized the newly isolated chromonone as melachromone [26].

Similarly, in this study, preliminary compound identification and quantitation was performed using GC/MS and LC/MS. As expected, flavonoids and alkaloids were consistently present in all samples. Furthermore, the occurrence of terpenoids, saponins, glycosides, and steroids depended on the type of plant part extract analyzed. The occurrence of the identified compounds mentioned in Tables 1, 2 and 3

Table 1 List of major compounds identified from *M. cajuputi* Laef extract

ID	Posibble Compound Name	Class of compound	Mol. formula	Mol mass	Rt time (min)	%
1	3-Cyclohexen-1-ol	Terpenoids	C18H18O	154	6.152	1.08
2	Cyclohexane, 1-ethenyl-1-methyl-2,4bis(1-methylethenyl)	Terpenoids	C15H24O4	204	8.109	1.83
3	Caryophyllene Bicyclo[7.2.0]undec-4ene	Terpenoids	C15H24	204	8.589	3.64
4	Caryophyllene 1,4,8-Cycloundecatriene	Terpenoids	C15H24	204	9.061	2.32
5	Naphthalene	Terpenoids	C15H24	204	9.537	2.13
6	Naphthalene	Terpenoids	C15H24	204	9.619	1.66
7	1H-Cycloprop[e]azulen-7-ol	Terpenoids	C15H24O	220	10.914	2.27
8	Caryophyllene oxide 5-Oxatricyclo[8.2.0.0(4,6)-]dodecane	Terpenoids	C15H24O	220	11.046	2.0
9	Alpha.-Tetralone	Aromatic	C12H13FO3	224	11.308	7.0
10	2-Naphthalenemethano	Sesquiterpene	C15H24O	222	12.325	2.38
11	Spathulenol 1H-Cycloprop[e]azulen-7ol	Sesquiterpene	C15H24O	222	13.636	1.31
12	Ethanone	Phenolic	C10H10O5	210	15.181	2.83
13	3,7,11,15-Tetramethyl-2-hexadecen-1ol $$ (2E)-3	Fatty acid	C20H40O	296	15.708	2.50
14	3-Eicosyne 3-Icosyne	Straight chain	C20H38	278	16.634	0.87
15	4H-1-Benzopyran-4-one	Flavone	C11H10O4	206	16.791	1.38
16	1,4-Naphthalenedione	Aromatic	C11H8O5	220	18.750	4.53
17	4H-1-Benzopyran-4-one	Flavonoids	C11H8O5	220	19.298	6.09
18	Ethanone	phenolic	C16H14O4	234	19.713	8.81
19	Methyl lathodoratin	Flavonoids	C12H12O4	220	20.921	0.57
20	Phytol 2-Hexadecen-1-	Fatty acids	C20H40O	296	21.666	0.57
21	Octadecanoic acid	Fatty acid	C22H44O2	340	27.517	1.31
22	1-Heptacosanol	Straight chain	C27H56O	396	33.437	0.27
23	Squalene	Straight chain alkene	C30H50	410	35.458	2.05
24	1-Heptacosanol	Straight chain	C27H56O	396	33.437	0.27
25	2H,6H-Pyrano[3,2-b]xanthen-6-one	Flavonoids	C18H14O6	326	39.607	0.41
26	Alpha Tocopherol (vit E)	Phenolic	C29H50O2	430	43.406	2.37
27	Sitosterol, Stigmast-5-en-3-ol	Terpenoids	C29H50O	414	43.406	2.37
28	Urs-12-en-28-al	Terpenoids	C30H48O	424	44.485	0.95
29	Dammarane-3,12,25-triol	Terpenoids		562	47.086	0.55
30	Betulin Lup-20(29)-ene-3,28-diol	Terpenoids	C30H50O2	482	47.947	1.15
31	Urs-12-en-28-al	Terpenoids	C30H48O	424	50.881	0.96

in this study has been previously reported in *Melaleuca* extracts [27, 28].

As it can be seen in Table 1, the GC/MS analysis of the GF revealed that the major compounds are essential oils, characterized by the presence of fatty acids including octadecanoic (0.68 %), hexadecanoic (0.60 %), and 9,12-octadecadienoic acids (1.90 %). Additionally, there were phenolic compounds such as alpha-tocopherol (vitamin E, 0.47 %), ethanone (4.34 %), 1,4-naphthalenedione (1.07 %), and terpenoid compounds such as Urs-12-en-28-al (6.40 %). Furthermore, aromatic compounds such as naphthalene (7.92 %) and alpha-tetralone (6.24 %) were detected. Previous studies have demonstrated that some of the identified compounds in the GF extract such as alpha-tocopherol [29] and hexadecanoic acid [30] possess antioxidant activities.

GC/MS analysis of the GL extract revealed the presence of 31 phytochemical compounds. As shown in Table 2, the methanol extract predominantly contained aromatic compounds such as alpha-tetralone (7 %) and 1,4-naphthalenedione (4.53 %); phenolic compounds such as ethanone (11.6 %); terpenoids such as caryophyllene bicyclo [7.2.0] undec-4ene (3.64 %), naphthalene (3.79 %), and sitosterol (2.37 %); and flavonoids such as 4H-1-benzopyran-4-one (6.09 %). In addition, squalene (2.05 %) and octadecanoic acid (1.31 %) were also present. Furthermore, numerous

Table 2 List of major compounds identified from Gelam flower extract

ID	Posibble Compound Name	Class of compound	Mol. formula	Mol mass	Rt time (min)	%
1	3-Cyclohexen-1-ol	Terpenoid	C18H18O	154	6.151	1.07
2	Cyclohexane, 1-ethenyl-1-methyl-2-(1methylethenyl)-4-(1-methylethylidene)-	Terpenoid	C15H24	204	7.482	0.68
3	Copaene	Terpenoid	C15H24	204	7.997	1.66
4	Cyclohexane, 1-ethenyl-1-methyl-2,4bis(1-methylethenyl)	Terpenoid	C15H24O4	204	8.107	2.60
5	Caryophyllene Bicyclo[7.2.0]undec-4ene	Terpenoid	C15H24	204	8.588	6.14
6	1,6-Cyclodecadiene, 1-methyl-5methylene-8-(1-methylethyl)-,	Terpenoid	C15H24	204	8.690	1.49
7	Caryophyllene 1,4,8-Cycloundecatriene	Terpenoid	C15H24	204	9.059	3.16
8	Naphthalene	Aromatics	C15H24	204	9.536	3.26
9	Naphthalene	Aromatics	C15H24	204	9.616	2.66
10	Naphthalene	Aromatics	C15H24	204	9.851	1.97
11	1H-Cycloprop[e]azulen-7-ol	Terpenoid	C15H24O	220	10.906	1.29
12	Caryophyllene oxide 5-Oxatricyclo[8.2.0.0(4,6)-]dodecane	Terpenoid	C15H24O	220	11.176	1.73
13	Alpha.-Tetralone	Aromatics	C12H13FO3	224	11.279	6.24
14	2-Naphthalenemethanol	Aromatics	C15H26O	222	12.318	2.84
15	2-Naphthalenemethanol	Aromatics	C15H26O	222	15.545	0.98
16	Hexadecanoic acid	Fatty acid	C17H34O2	270	17.648	0.60
17	1,4-Naphthalenedione	Phenolic	C11H8O5	220	18.672	1.07
18	4H-1-Benzopyran-4-one	Flavonoids	C16H20O4	276	19.168	2.12
19	Ethanone	Phenolic	C16H14O4	234	19.563	4.34
20	9,12-Octadecadienoic acid (Z,Z)-,	Fatty acids	C19H34O2	294	21.327	1.90
21	Octadecanoic acid	Fatty acid	C19H38O2	298	22.779	0.68
22	1-Heptacosanol	Straight chain	C27H56O	396	40.363	0.85
23	Alpha Tocopherol (vit E)	Phenolic	C29H50O2	430	40.600	0.47
24	Sitosterol, Stigmast-5-en-3-ol	Terpenoids	C29H50O	414	42.438	0.57
25	Sitosterol, Stigmast-5-en-3-ol	Terpenoids	C29H50O	414	43.399	3.88
26	Urs-12-en-28-al	Terpenoids	C30H48O	424	44.480	1.47
27	Urs-12-en-28-al	Terpenoids	C30H48O	424	45.816	4.93
28	Urs-12-en-28-al, 3-(acetyloxy)-,	Terpenoids	C32H50O3	482	47.628	1.49
29	Betulin $$ Lup-20(29)-ene-3,28-diol,	Terpenoids	C30H50O2	442	47.949	3.68
30	Urs-12-en-28-al	Terpenoids	C30H48O	424	48.690	3.00
31	3.beta.-Myristoylolean-12-en-28-ol	Terpenoids	C44H76O3	652	50.431	1.67
32	Urs-12-en-28-al	Terpenoids	C30H48O	424	50.867	2.41

previous studies have reported the antioxidant activities of some of these compounds [31, 32].

The results of the LC/MS analyses are presented in Table 3. Most of the observed compounds were typical hydroxycinnamic acid and phenolic acid derivatives (Fig. 1). The LC/MS analysis of the GF extract indicated the presence of caffeic acid phenyl ester, gingerol, aspidin, methyl orsellinic acid ester, ethyl ester, trans-2,3, 4-trimethoxycinnamate, and metyrosine. In addition, the GL extract contained epigallocatechin 3-O-(4-hydroxybenzoate), 5,6,3'-trimethoxyflavone, metyrosine, gingerol, polygonolide, and trans-2, 3, 4-trimethoxycinnamate.

Total phenolic, total flavonoids contents, and antioxidant activity

Numerous phenolic metabolites containing an aromatic arene (phenyl) ring with one or more acidic hydroxyl residues attached to it are known to be produced by plants. A previous study showed that compounds such as flavonoid and tannins were among the major phenolic constituents present in plant extracts [33]. The phenolic radicals were reported to be less reactive and with a lower electron reducing potential than the oxygen radicals had [33, 34]. Because of these properties, phenolic compounds are considered excellent radical scavengers.

Table 3 Chemical composition comparison of methanol extract from flower, and leaves extract of *M. cajuputi* based on LCMS

Phytochemical extract	GF extract		GL extract		RT	m/z
	presence	% Abundance	presence	% Abundance		
Metyrosine	+	0.6	+	0.39	13.072	194.08203
Methylorsellinic Acid, Ethyl Ester	+	0.74	+	8.02	11.18	209.0822
Hydroxyibuprofen	+	1.2	+	0.74	10.759	221.1184
Trans-2, 3, 4-Trimethoxycinnamate	+	0.7	+	11.61	10.386	237.07724
Gingerol	+	0.45	+	0.28	11.768	293.17629
Catharanthine	+	2.1	+	1.0	20.202	371.15374
calicoferol D	+	1.1	+	0.65	21.489	409.31181
Caffeic acid Phenethyl ester (CAPE)	+	18.69	-	-	18.451	283.30206
Aspidin	+	0.65	+	0.59	12.819	459.20282
Cucurbitacin F	+	1.2	+	1.2	20.886	517.31874
Kurilensoside G	+	2.2	+	3.1	20.89	633.33896
1α,22,25-trihydroxy-26,27-dimethyl-23,23,24,24-tetradehydro-24a,24b, 24c-trihomovitami	+	9.68	+	9.48	20.869	497.36525

Rt Retention time (as min), m/z mass

Furthermore, phenolic compounds are able to scavenge reactive oxygen intermediates without invoking further oxidative reactions. Therefore, one of the current standards of phytochemical research is the evaluation of the TPC as a measure of determining the antioxidant activity of extracts. Therefore, the current study evaluated the TPC of the extracts and found that the GF extract showed a higher value than the GL extract did (55 ± 0.05 and 37 ± 0.05 GAE/mg extract dry weight, respectively). A similar trend was also observed with the flavonoids content of both extracts (Table 4, GF > GL extracts). However, it is not surprising that the higher TPC value of the GF confers a stronger antioxidant ability than that of the GL. Therefore, the higher the TPC content of an extract, the higher its antioxidant activity will be. This is because the substituted 5,7,3′,4′-hydroxy flavonoids are believed to possess a very efficient radical scavenging power [35]. This observation was in agreement with the report of a direct relationship between the flavonoid and phenolic contents and the biological activities of plant extracts [36–38].

DPPH radical scavenging activity

The direct and rapid reaction between DPPH radicals and antioxidants has been utilized as a measure of antioxidant activity [39] and a high percentage DPPH radical scavenging of a compound indicates excellent activity. In this present study, the free-radical scavenging activity of the GL and GF methanolic extracts, which was evaluated using DPPH, was found to agree with TPC observation. Therefore, the extract with the higher TPC value also showed a higher percentage DPPH radical scavenging activity (Fig. 2). Furthermore, in agreement with the TPC and flavonoid observations, the percentage radical inhibition by GF extract was also higher than that of the GL extract was (81 and 75 %, respectively). This observation confirmed that both the GF and GL extracts exhibited DPPH radical-scavenging activity concentration-dependently, although only the GL extract showed a scavenging power that was greater than the values obtained with the BHT positive control. For both samples, however, increasing the concentration beyond 250 μg/mL resulted in a negligible increase in the radical scavenging activity (Fig. 2). Based on the calculated IC_{50} values, only the GL extract revealed a higher scavenging effect than the GF extract by demonstrating a lower IC_{50} value (10 μg/mL) than the positive control BHT (13 μg/mL); the GF extract showed an IC_{50} value of 25 μg/mL. Several reports have indicated that free radical scavenging activity is greatly influenced by the phenolic contents of the sample, flavonoid, and the presence of hydroxycinnamic acids such as caffeic acid phenyl ester [40–42]. Similarly, high antioxidant activity was previously reported in *Melaleuca* [43].

Fe^{2+}-chelating activity

In cellular lipid peroxidation determination, the Fenton reaction is used in metal chelating activity assays to reduce the concentration of the catalyzing transition metal. This kind of chelating reaction is considered significant in reducing the oxidative stress generated by reactive oxygen species. The chelating effect of the methanolic extracts was around 50 % at an extract concentration of around 0.4 mg/mL, and the chelating activity was concentration-dependent (Fig. 3). As expected, the activity of the GF extract was higher than that of the GL extract was, with maximum chelating activities of ~75 and 59 %, respectively (Fig. 3). The presence of a significant amount of caffeic acid phenyl ester in the GF may have contributed to this activity

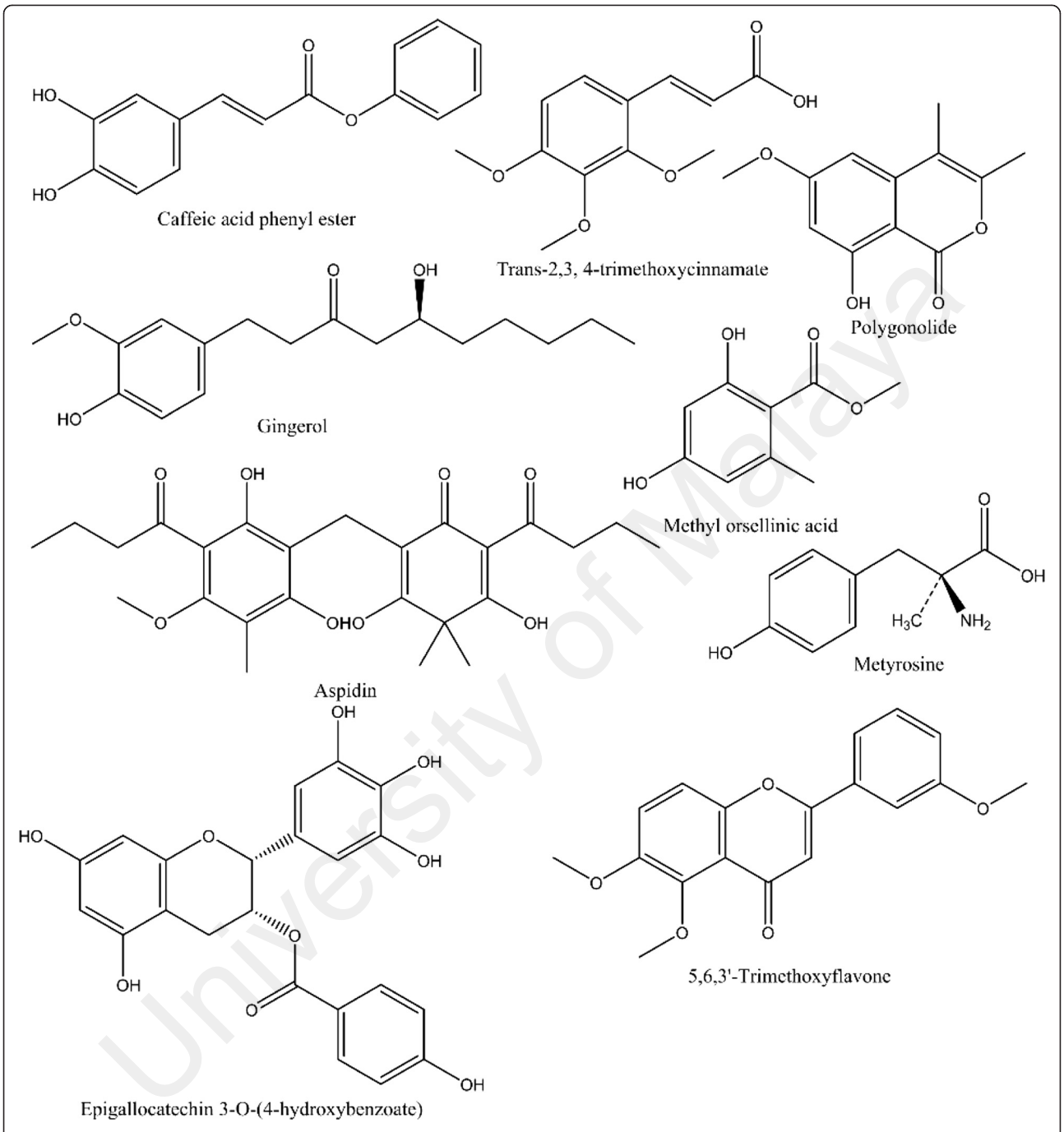

Fig. 1 Chemical structure of the observed phytochemical compounds in *M. cajuputi* flower and leaves extracts

Table 4 A comparison of total phenolic and flavonoids contents

Methanolic Extract	Total phenolic content (GAE/mg dw)	Total flavonoid content (QE/mg dw)
M. cajuputi flower extract	55 ± 0.03	19.6 ± 0.4
M. cajuputi leaves extract	37 ± 0.02	10.2 ± 0.2

GAE Garlic Acid Equivalent
QE Qurcetin Equivalent

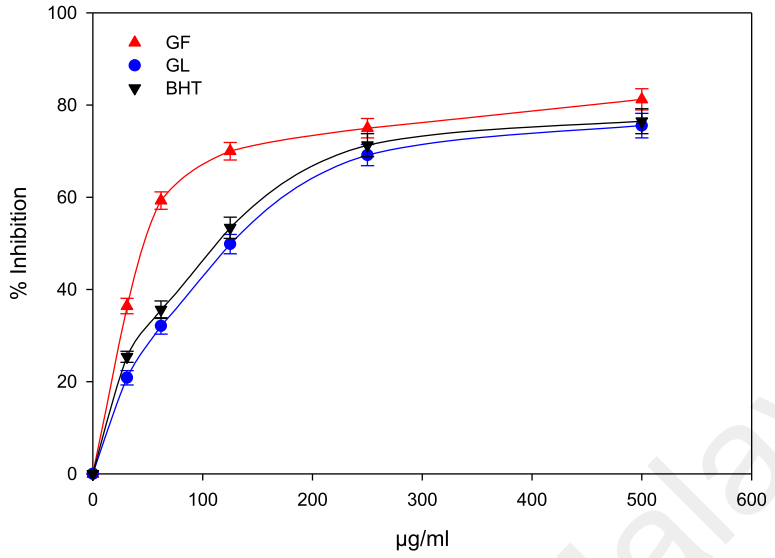

Fig. 2 Antioxidant activity of *M. cajuputi* flower and leaves extracts following DPPH radical scavenging assay

as these substances have been reported to exhibit strong chelating activity [44].

All the samples tested exhibited a logarithmic increase in chelating power with increasing concentrations up to 0.25 mg/mL and, thereafter, the percentage chelating activity appeared to increase gradually.

β-Carotene bleaching test

Carotenoids are among the most common natural pigments and are responsible for most of the red, orange, and yellow coloration of plant leaves, fruits, and flowers [45]. Currently, more than 600 different carotenoid compounds were reported to have been characterized [45]. In animals, carotenoids act as antioxidants. Furthermore, carotenoids have attracted much attention because numerous studies have revealed that their consumption is correlated with a diminished risk for several degenerative disorders including various types of cancer and cardiovascular or ophthalmological diseases [45]. This effect is attributed to their antioxidant activity, which protects cells and tissues from oxidative damage [45]. In this study, all the analyzed samples inhibited the discoloration of β-carotene in a concentration-dependent manner (Fig. 4). Generally, an increase in the percentage inhibition was observed with

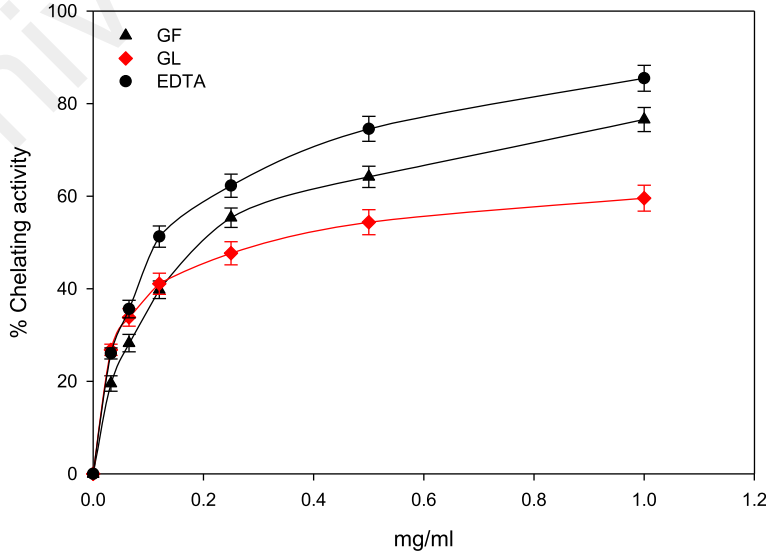

Fig. 3 Metal chelating activity of *M. cajuputi* flower and leaf extracts

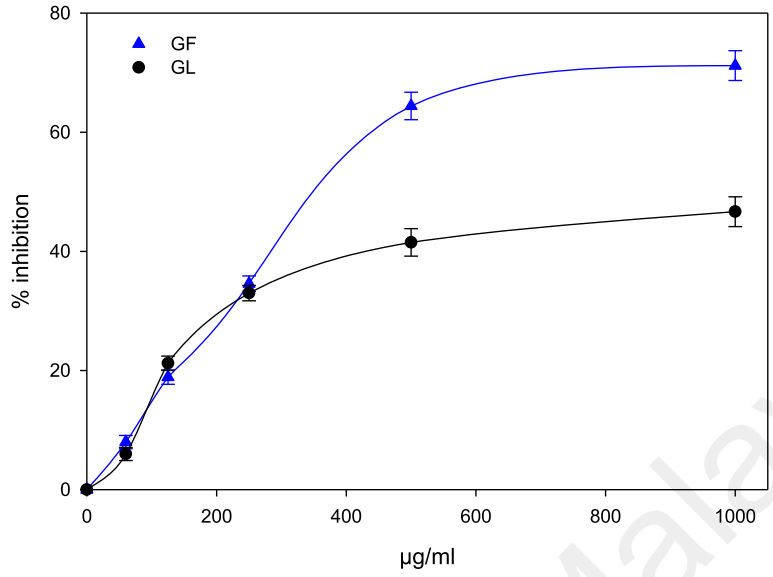

Fig. 4 Antioxidant activity of *M. cajuput* flower and leaf extracts determined by β-carotene bleaching test

all the samples tested. As expected, the GF extract was more effective than the GL extract was with inhibition rates of 71 and 47 %, respectively.

FRAP assay

The FRAP assay is primarily based on the principle of reduction of ferric ions to their ferrous form at a lower pH, which results in the formation of a chromatic ferrous-tripyridyltriazine complex [46]. This assay is considered to be an accurate method for testing the antioxidant power of therapeutic compounds [47]. In this research study, the FRAP antioxidant ability of the *M. cajuputi* extracts in reducing the Fe^{3+}–TPTZ reagent was evaluated and both extracts (GL and GF) demonstrated some reducing power with FRAP values of 0.12 and 0.14 μM Fe(II)/g, respectively. The presence of a significant amount of caffeic acid phenyl ester in the GF extract likely contributed to its higher FRAP value than that of GL extract since this compound is known to possess strong FRAP antioxidant activity [44]. Zunjar and colleagues [36] reported that the reducing capacity of a compound may serve as a remarkable indicator of its antioxidant activity and ability to ameliorate oxidative stress by reacting with certain precursors.

Antibacterial activity

The antibacterial activity of the *M. cajuputi* extracts was evaluated against four Gram-positive (*B. cereus*, *S. epidermidis*, *S. aureus*, and *S. pneumoniae*) and four Gram-negative (*E. coli*, *P. multocida*, *K. pneumoniae,* and *S. typhimurium*) bacteria. The antibacterial activity of the extracts was assessed by determining their IZ, MIC, and MBC values (Tables 5 and 6).

The results revealed that both the GL and GF extracts potently inhibited *S. epidermidis*, *S. aureus*, and *B. cereus* (Table 5). The GF extract was more effective than GL against *S. epidermidis* and *B. cereus,* while both extracts showed comparable activity against *S. aureus*. However, both extracts had no effect against the tested Gram-negative organisms and *P. multocida.* The most susceptible

Table 5 Antibacterial activity of crude extracts

Inhibition diameter (mm ± SD)								
Sample	*Staphylococcus epidermidis* (Gram + ve)	*Staphylococcus aureus* (Gram + ve)	*Bacillus cereus* (Gram + ve)	*Pasteurllamultocida* (Gram + ve)	*Klebsiella pneumonia* (Gram + ve)	*Streptotococcus pneumonia* (Gram + ve)	*Esherichia coli* (Gram -ve)	*Salmonella typhimurium* (Gram -ve)
M.cajuputi Leaves	13.66 ± 0.43	12.33 ± 0.57	6.33 ± 0.33	-	-	-	-	-
M.cajuputi flower	17.33 ± 0.36	12.33 ± 0.31	12.33 ± 0.48	-	-	-	-	-
Streptomycin sulfate[a]	20.33 ± 0.38	18.0 ± 0.2	21.0 ± 0.25	21.0 ± 0.05	20.0 ± 0.1	22.0 ± 0.08	15.0 ± 0.1	10.0 ± 0.2

[a]Doses of Streptomycin was 1 mg/ml
-No Activity observed

Table 6 MIC and MBC of *M. cajuputi* extracts

	MIC (mg/mL)			MBC (mg/mL)		
SAMPLE	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Bacillus cereus*	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Bacillus cereus*
G.L.	12.5	12.5	NA	25	25	NA**
G.F.	12.5	25	12.5	50	25	50
Streptomycin*	1.95	1.95	>1.0	1.95	1.95	>1.0

*Doses of Streptomycin was 1 mg/ml
**NA No Activity observed

bacteria to the GF extract were *S. aureus*, *S. epidermidis*, and *B. cereus* with MIC values of 12.5, 12.5, and 25 g/mL, respectively. Furthermore, the results showed there were no observed MIC and MBC values against *B. cereus* exposed to the GL extract (Table 6). However, the exposure of *S. aureus* to both extracts resulted in identical MIC, and MBC values 12.5, and 25 mg/mL, respectively.

The MBC for the GF extract against S. epidermidis was 25 mg/mL, which was more effective than the GL extract was at 50 mg/ml. These findings are of great significance, especially in the case of *S. aureus* and *B. cereus* that are well-known for being resistant to numerous antibiotics. In addition, these organisms are capable of producing several types of enterotoxins that can cause septicaemia and several forms of enteritis. In general, the GF extract was found to be active against some species of *Staphylococci* and *Bacilli* while the GL extract was inactive against the tested *Bacilli*. Therefore, the antibacterial activity of the extracts could be correlated with their phenolic and flavonoids contents. KA Hammer, C Carson and T Riley [13] reported a similar observation for the antimicrobial activity of *M. cajuputi* extract against *S. aureus*. In contrast, it was reported that the hexane, dichloromethane, and acetone extracts of *M. cajuputi* leaves showed no activity against *S. aureus*, methicillin-resistant *S. aureus*, *E. coli*, and *P. aeruginosa* [26].

Conclusions

We attempted to explore the diverse phytochemical efficacy of *M. cajuputi* against several diseases and oxidative stress, by evaluating the antioxidant and antibacterial activities of its GF and GL methanolic extracts. Furthermore, to the best of our knowledge, this is the first comprehensive study of the antioxidant and antibacterial potential of the GF and GL extracts. Generally, the GF extract showed a higher efficacy than the GL extract did. In addition, the TPCs were higher in the GF extract than they were in the GL extract, and these results were in agreement with the percentage radical inhibition results, which were higher for the GF extract than they were for the GL extract. The same trend was also observed in the Fe^{2+}-chelating activity, flavonoid contents, and β-carotene bleaching test. Both extracts showed promising evidence of antibacterial activity against *S. aureus*, *S. epidermidis*, and *B. cereus*. Finally, the observed antioxidant and antibacterial activities of these extracts could be attributed to the high content of phenolics and flavonoids identified using LC/MS and GC/MS.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NMA participated in all face of the study, collection plant, conceived of the study, design and wrote the manuscript, ZMN design the study and rewrote the final one. MM, MSH supervised part of the study and reviewed the manuscript. MK participated in the design and coordination. FA participated in the chemistry part. All authors read and approved the final manuscript.

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Author details

[1]Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia. [2]Department of Anesthesiology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia. [3]Department of Chemistry, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia.

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ORIGINAL ARTICLE

Antifilarial and Antibiotic Activities of Methanolic Extracts of *Melaleuca cajuputi* Flowers

Nazeh M. Al-Abd[1,*], Zurainee Mohamed Nor[1], Marzida Mansor[2], MS Hasan[2], Mustafa Kassim[2]

[1]Department of Parasitology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia; [2]Department of Anesthesiology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia

Abstract: We evaluated the activity of methanolic extracts of *Melaleuca cajuputi* flowers against the filarial worm *Brugia pahangi* and its bacterial endosymbiont *Wolbachia*. Anti-*Wolbachia* activity was measured in worms and in *Aedes albopictus* Aa23 cells by PCR, electron microscopy, and other biological assays. In particular, microfilarial release, worm motility, and viability were determined. *M. cajuputi* flower extracts were found to significantly reduce *Wolbachia* endosymbionts in Aa23 cells, *Wolbachia* surface protein, and microfilarial release, as well as the viability and motility of adult worms. Anti-*Wolbachia* activity was further confirmed by observation of degraded and phagocytized *Wolbachia* in worms treated with the flower extracts. The data provided in vitro and in vivo evidence that *M. cajuputi* flower extracts inhibit *Wolbachia*, an activity that may be exploited as an alternative strategy to treat human lymphatic filariasis.

Key words: *Brugia pahangi*, *Wolbachia*, lymphatic filariasis, *Melaleuca cajuputi*, natural product

INTRODUCTION

Lymphatic filariasis is one of the most neglected mosquito-borne diseases. It affects more than 150 million people in tropical and subtropical regions, and is caused by parasitic filarial nematodes [1-4]. It has high morbidity and heavy socio-economic burdens [5,6]. The drugs frequently used to manage lymphatic filariasis include albendazole, a benzimidazole derivative; diethylcarbamazine, a derivative of piperazine; and ivermectin, which is believed to be a broad-spectrum anti-parasitic agent [7]. However, these drugs are effective mostly against microfilariae, but have limited activity against adult worms, which cause the disease [8]. In addition, the drugs elicit systemic and inflammatory side effects [5] and other medical complications [7]. Hence, alternative therapies have been sought through innovative and extensive phytomedicinal research that relies on traditional medicine [3].

Melaleuca cajuputi (Gelam tree), is used as a traditional medicine to treat cholera, muscular pain, diarrhea, scabies, and intestinal worms [9]. It has anti-inflammatory [10], anti-microbial [11], anti-cancer [12], and hepatoprotective [13] activities. A more recently adopted strategy to treat filarial infection is to target *Wolbachia*, an intracellular bacterial symbiont, the numbers of which increase dramatically (up to 600-fold) and peak shortly after infection of a mammalian host [14]. Filariasis control by targeting *Wolbachia* has been successfully demonstrated in humans using the antibiotics doxycycline and rifampicin, which inhibit embryogenesis and induce long-term sterility in the adult worm [7,15]. Antibiotics also inhibit the molting process of both third (L_3) and fourth (L_4)-stage larvae [14,16]. However, antibiotic regimens are lengthy and are contraindicated for children under 8 years old and for pregnant or breast-feeding women. Therefore, alternative drugs active against *Wolbachia* and without these contraindications will have significant impact. In this study, we present in vitro and in vivo evidence that methanolic extracts of *M. cajuputi* flowers inhibit *Wolbachia* and is active against *Brugia pahangi* worms.

MATERIALS AND METHODS

Chemicals

Roswell Park Memorial Institute medium (RPMI 1640 with L-glutamine), PBS (pH 7.2), fetal bovine serum (FBS), HEPES, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), tetracycline, and ivermectin were purchased from Sigma (St. Louis, Missouri, USA).

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* Corresponding author (Nazehali78@yahoo.com)

Plant materials

Flowers of *Melaleuca cajuputi* were collected in September 2013 from Kedah State, in the northwestern part of the Malaysian peninsula. Identity was confirmed at the Herbarium of Rimba Ilmu, Institute of Biological Sciences, University of Malaya, Kuala Lumpur with the voucher no. KLU048231.

Flower extracts

M. cajuputi flowers were washed with distilled water, ground to powder, and dried in the dark for 7 days. Several independent extracts were obtained by macerating 100 g dried powder for 72 hr at room temperature in 1 L absolute methanol. Extracts were filtered into a Schott bottle through Whatman (No. 1) filter paper. The residue was macerated and filtered in the same manner another 2 times. Extracts were then pooled, concentrated in vacuo at 40˚C using a rotary evaporator, and finally dried for 3 hr under high vacuum to remove residual solvent.

Sample preparation

M. cajuputi flower extracts, ivermectin, and tetracycline were dissolved in pre-filtered DMSO to final concentrations of 10 mg/ml, 1 mg/ml, and 1 mg/ml, respectively. The antibiotics were then diluted in PBS (pH 7.2) to obtain 25 µg/ml ivermectin and 40 µg/ml tetracycline. Flower extracts were serially diluted 2-fold to prepare working concentrations between 1,000 µg/ml and 62.5 µg/ml for use against worms, and 10-50 µg/ml for use in Aa23 cells.

Anti-*Wolbachia* activity in Aa23 cells

Cell culture: *Aedes albopictus* Aa23 cells were derived from a culture generously provided by Prof. Scott O'Neill at Monash University, Clayton, Australia. Cells were cultured according to published methods [17]. Briefly, cells were grown at 26˚C in 25 cm^2 flasks containing 5 ml medium consisting of equal parts of Mitsuhashi-Maramorosch and Schneider's insect medium (Invitrogen, Carlsbad, California, USA) and supplemented with 20% heat-inactivated fetal bovine serum (Invitrogen). Cells were passaged every 3-4 days to prevent overgrowth. Triplicate cultures were used for each treatment.

Treatment with flower extracts: *M. cajuputi* flower extracts were dissolved at 1 mg/ml in DMSO, filter-sterilized, and stored in aliquots at -20˚C. Cells were treated in triplicate with 10-50 µg/ml extracts, 40 µg/ml tetracycline, or DMSO. Untreated cultures were used as the positive control. Media were replaced every 3 days with fresh media supplemented with fresh treatment. Cells were harvested after 7 days by centrifugation at 14,000 rpm for 10 min in 15 ml conical tubes, and resuspended in 1 ml fresh medium.

DNA Extraction

Total DNA was extracted from the cells using QIAamp Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. DNA concentration and quality were determined by measuring absorbance on a Nanodrop ND-1000 spectrophotometer. Nucleic acid purity was determined by the ratio of absorbance at 260 nm to 280 nm (~1.8), and to 230 nm (1.8-2.2). Samples with ratios significantly different from these values (±0.4) were discarded.

Detection of *Wolbachia* by PCR

The presence of *Wolbachia* in Aa23 cells was verified by PCR using primers specific for the 590 fragment of *Wolbachia* surface protein [18,19]. The forward and reverse primers had sequence 5′-TGGTCCAATAAGTGATGAAGAAAC-3′ and 5′-AAAA ATTAAACGCTACTCCA-3′, respectively. Primers for 28S nuclear ribosomal DNA (28sF3633/28sR4076) [20] were used as the control for template quality.

Each reaction was 20 µl in total volume, and contained 200 µM dNTPs, 300 nM primers, 0.5 U AmpliTaq DNA polymerase, 1×PCR buffer, 1.5 mM $MgCl_2$, and 1 µl template. Reactions were denatured at 94˚C for 10 min, and then amplified over 35 cycles at 94˚C for 1 min, 55˚C for 1 min, and 72˚C for 1 min with final extension at 72˚C for 7 min. Amplification products (10 µl) were electrophoresed on 2% agarose, and stained with SYBR. Negative controls containing DNA-free water as template were used to test potential contamination in reaction components, while DNA from untreated Aa23 cells was used as the positive control.

Ethics statement

Experimental animal models were used according to guidelines approved by the Ethics Committee for Animal Experimentation, Faculty of Medicine, University of Malaya, Malaysia, Ethics no. PAR/21/11/2011/ZMN (R).

Isolation of infective larvae

Conditions were first optimized to obtain high yields of infective *B. pahangi* larvae from healthy, laboratory-bred *Aedes togoi* mosquitoes, eggs of which are maintained in the insectari-

um of the Department of Parasitology, Faculty of Medicine, University of Malaya. Thus, a filter paper with *A. togoi* eggs was immersed in a bowl containing 0.85% NaCl, and incubated at 37˚C to accelerate hatching. Hatched larvae were allowed to develop into pupae within 10 days, which were then transferred into an enclosed plastic jar containing clean water, and allowed to grow into adult mosquitoes within 2-3 days. Adult mosquitoes were then collected between 7-10 days and blood-fed on a naturally parasitized cat found in Pulau Carey, Selangor, Malaysia. *B. pahangi* infective stage larvae (L_3) were collected from mosquitoes 10-14 days post-feeding using the Baermann's technique [17].

Experimental infection and isolation of adult parasites from animal hosts

Male gerbils (*Meriones unguiculatus*) were intraperitoneally inoculated with 150 infective L3 larvae isolated from *A. togoi* mosquitoes. Infected animals were fed rodent chow and water. Adult worms for in vitro studies were obtained from the peritoneal cavity by necropsy 4-6 months after infection. Contaminants were removed from worms by washing 3-4 times in RPMI 1640 medium containing 20 mM HEPES, 100 µg/ml penicillin and 100 U/ml streptomycin. On the other hand, microfilariae were obtained by passing the peritoneal suspension through a 5 µm filter membrane (Millipore), and resuspending in RPMI 1640 medium.

Effect of flower extracts on microfilarial release, worm motility, and viability

The antifilarial activity of *M. cajuputi* flower extracts was evaluated in vitro by incubating adult worms for 7 days at 37˚C, 5% CO_2, and 90% humidity in 4 ml RPMI medium supplemented with antibiotics, HEPES, 10% heat-inactivated FBS, and 6.25-1,000 µg/ml flower extracts. Antiparasitic activity was measured based on microfilarial release, worm motility, and viability.

The number of microfilariae released was determined in 1 ml medium on days 2, 4, and 6 post-treatment. Antifilarial activity is expressed as percent reduction in microfilariae released relative to the number released by untreated worms. Motility was measured daily by moving worms to fresh medium without antibiotics or flower extracts and observing under an inverted microscope. Mobility or death was scored according to a published scale [20], in which worms immobile for 10 sec were considered dead and scored 0, while slightly, moderately, and highly active worms were scored 1, 2, and 3, respectively. Treated worms were scored in comparison to the activity and motility of untreated worms. Results were reported as the mean motility score over 7 days. Data were collected in triplicate.

Worm viability was evaluated in duplicate after 7 days in culture using MTT (Sigma), as described by Saini and coworkers [5], with slight modification. After viability was assessed by microscopic examinations, worms were then transferred to a 24-well flat-bottomed plate, and incubated for 30 min in 0.5 ml PBS (pH 7.4) containing 0.5 mg/ml MTT. Subsequently, plates were incubated in the dark for 2 hr at 37˚C [17]. Formazan crystals formed were solubilized with occasional gentle shaking for 1 hr at room temperature in 100-200 µl DMSO. The absorbance at 492 nm of the resulting formazan solution was measured in a microplate spectrophotometer using DMSO as blank. Adult females treated with DMSO only were used as the positive control, while worms killed by heating at 56˚C for 30 min were used as the negative control. Percent inhibition of formazan production was estimated according to the following equation:

$$\%\ inhibition = \frac{100-(T-H)}{(C-H)} \times 100,$$

where T, C, and H are absorbance values obtained from treated, control, and heat-treated worms, respectively.

Transmission electron microscopy

Treated and untreated *B. pahangi* female worms were examined by transmission electron microscopy after 7 days in culture, following published protocols [16], with some modification. Briefly, worms were fixed for 2 hr at 25±1˚C with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). Samples were then treated with 1% osmium tetroxide in cacodylate buffer, dehydrated in increasing concentrations of ethanol (30-95%), and embedded in Eponate 812 low-viscosity resin (Ted Pella, Redding, California, USA). Ultrathin sections of 65-70 nm were obtained, stained with uranyl acetate and Reynold's lead citrate, and examined on a Zeiss 912 Omega transmission electron microscope (Oberkochen, Germany).

Molecular analysis

DNA was extracted from worms using DNeasy Blood and Tissue Kit (Catalog no. 69504, Qiagen) following the manufacturer's protocol. *Wolbachia* surface protein was amplified by PCR as described previously [21], with modifications. The for-

ward and reverse primers had sequence 5′-GCAGACGGGTGA GTAATGTATA-3′ and 5′-GCCACTGGTGTTCCTCCTAATA-3′. The reaction consisted of 40 cycles at 95℃ for 30 sec, 50℃ for 30 sec, and 72℃ for 60 sec. Newly developed worms were used as the positive control, and water was used as the negative PCR control. Amplification products from 6 worms in the same treatment regimen were pooled, separated on 1.5% agarose, and stained with SYBR Safe.

Statistical analysis

Data are reported as mean±SD of triplicate experiments and were analyzed in Prism (GraphPad Software, San Diego, California, USA). One-way ANOVA was used to test differences among treatment groups, and *P*-values <0.05 were considered significant.

RESULTS

In vitro effects of flower extracts against *Wolbachia* sp.

PCR amplification of *Wolbachia* surface protein generated a 590-bp band from *Wolbachia*-colonized Aa23 cells (Fig. 1A). The gene was totally lost in cells treated with 50 μg/ml flower extracts for 7 days, denoting anti-bacterial activity. Presumably, this effect was not due to general cytotoxicity, as MTT assays indicated that *M. cajuputi* flower extracts were not cytotoxic in Aa23 cells up to 200 μg/ml (data not shown). In addition, all DNA preparations produced a band at approximately 400 bp when amplified with primers for 28S ribosomal DNA, verifying template quality (Fig. 1B). Thus, the effects of flower extracts were investigated in vivo.

Effects of flower extracts against the filarial worm *B. pahangi*

Untreated female worms released microfilariae at comparable rates throughout 7 days in culture (Fig. 2). However, a progressive decrease in microfilariae was observed in worms treated with *M. cajuputi* flower extracts (Fig. 2). The decrease was

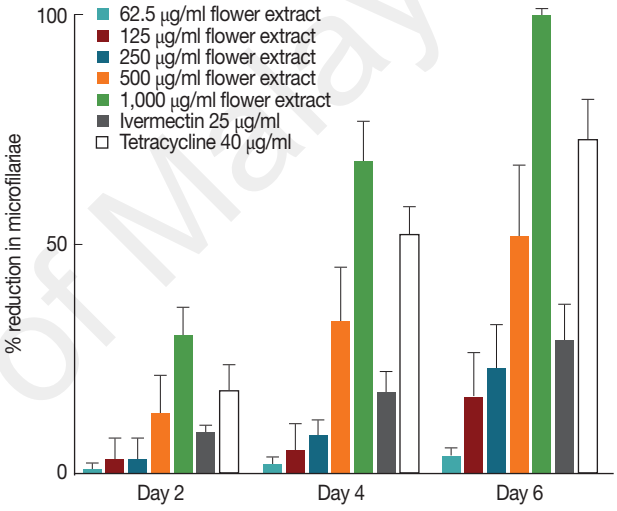

Fig. 2. Percent reduction at days 2, 4, and 6 in microfilariae released by *Brugia pahangi* female worms treated with *Melaleuca cajuputi* flower extracts. Data are mean±SE.

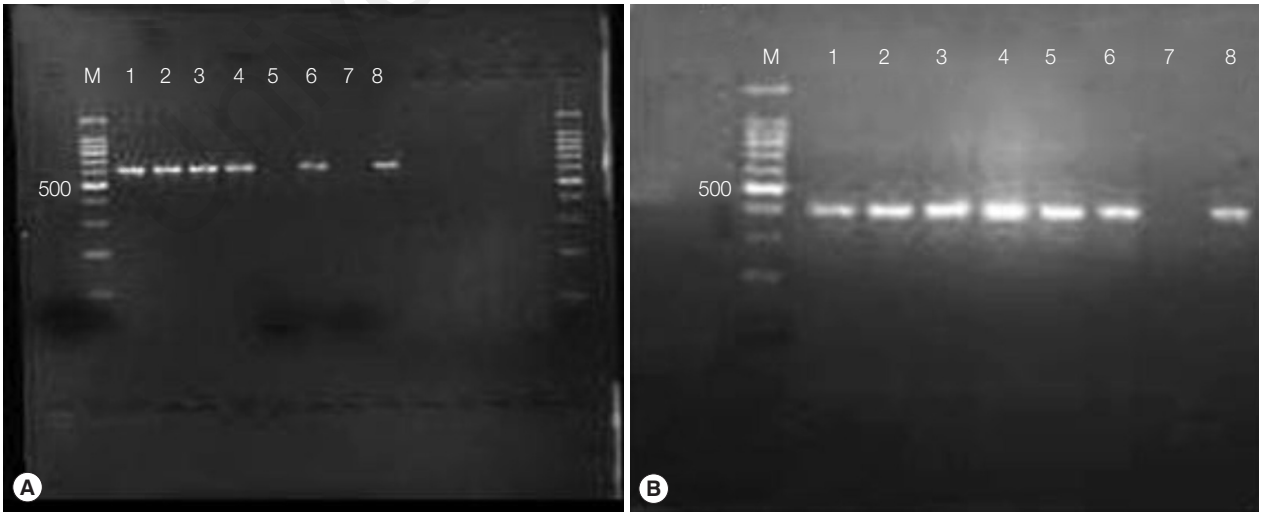

Fig. 1. PCR amplification of *Wolbachia* surface protein (A) and 28S ribosomal DNA (B) in Aa23 cells treated with *Melaleuca cajuputi* flower extracts for 7 days. Lane M, 100 bp molecular ladder; lanes 1-5, cells treated with 10, 20, 30, 40, and 50 μg/ml flower extracts, respectively; lane 6, cells treated with tetracycline; lane 7, PCR using water as template; lane 8, untreated cells.

dose-dependent, such that the release completely ceased at day 6 in worms treated with 1,000 μg/ml flower extract. These observations were in line with the effects of the reference drug tetracycline (Fig. 2).

As expected, there was no observable change in the motility of untreated worms, which had a constant motility score of 3 over 7 days. Notably, 62.5-500 μg/ml *M. cajuputi* flower extracts, tetracycline, and the reference drug ivermectin did not significantly reduce ($P>0.05$) the mean motility score. However, 1,000 μg/ml flower extract was found to cause complete immobility at days 6 and 7.

The effect of *M. cajuputi* flower extracts on worm viability was also evaluated using MTT (Fig. 3). In worms treated with 62.5-1,000 μg/ml flower extracts for 7 days, viability decreased significantly ($P<0.05$) in a dose-dependent manner, especially at concentrations above 500 μg/ml. In fact, 1,000 μg/ml *M. cajuputi* flower extracts reduced microfilarial release and worm viability more drastically than the standard reference drugs

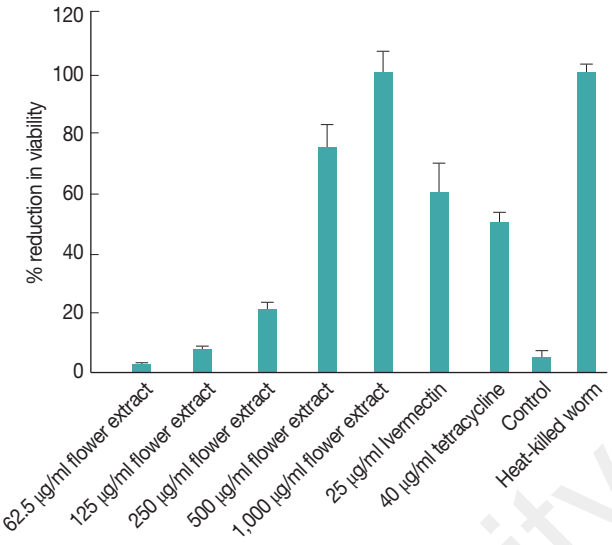

Fig. 3. Percent reduction in the viability of adult *Brugia pahangi* worms treated with *Melaleuca cajuputi* flower extracts, as measured by MTT reduction assay. Untreated parasites were used as negative control, while the reference drugs ivermectin and tetracycline were used for comparison. Data are mean ± SE. There is statistically significant difference among treatments ($P<0.05$).

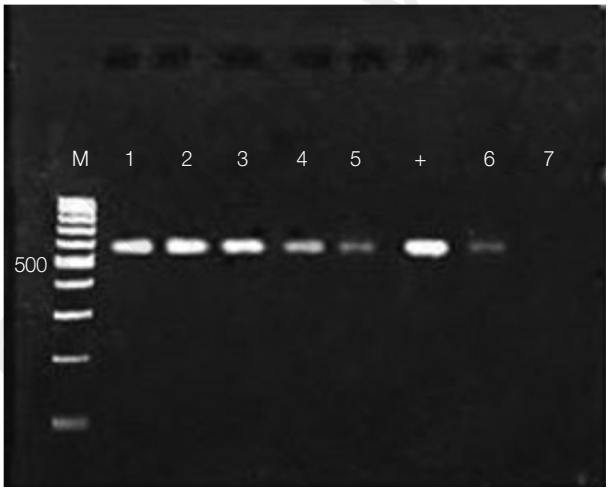

Fig. 4. Agarose gel electrophoresis of *Wolbachia* surface protein (590 bp) amplified from female *Brugia pahangi* worms (n=6) treated with 62.5, 125, 250, 500, and 1,000 μg/ml *Melaleuca cajuputi* flower extracts (lanes 1-5) and tetracycline (lane 6). Newly developed adult worms (+) were used as the positive control, while water was used as the negative PCR control (lane 7).

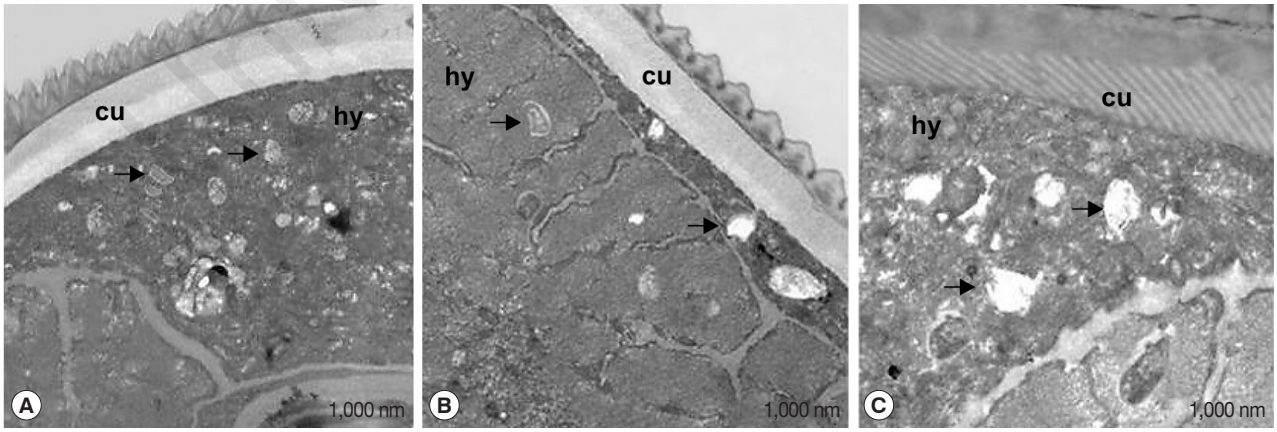

Fig. 5. Transmission electron microscopy of *Wolbachia* in the hypodermis of untreated *B. pahangi* worms (A), or of worms treated with *Melaleuca cajuputi* flower extracts (B), and 40 μg/ml tetracycline (C). In (B), *Wolbachia* was reduced in abundance and appeared degenerated (arrow). In (C), the endosymbiont appeared dead, and vacuoles contained only remnants or membrane whorls (arrow). Scale bar = 1,000 nm. cu, cuticle; hy, hypodermis.

ivermectin and tetracycline at 25 μg/ml and 40 μg/ml, respectively (Fig. 3).

Effects of flower extracts against *Wolbachia* endosymbionts in filaria adults

The activity of flower extracts against *Wolbachia* endosymbionts in adult worms was evaluated by amplifying *Wolbachia* surface protein, which has been reported to be about 590 bp [29,30]. A fragment of comparable size was amplified using DNA preparations from adult worms (Fig. 4). As in Aa23 cells, the amplicon diminished in dose-dependent fashion with exposure to *M. cajuputi* flower extracts, indicating antibacterial activity in vivo, especially at concentrations beyond 500 μg/ml.

Ultrastructural effects of flower extracts against *Wolbachia* endosymbionts

The effect of flower extracts on filarial parasites was further evaluated by transmission electron microscopy (TEM) (Fig. 5). Numerous intracellular *Wolbachia* cells were detected in the hypodermal cord of untreated worms (Fig. 5A). On the other hand, degraded *Wolbachia* cells were observed in worms treated with flower extracts (Fig. 5B) and tetracycline (Fig. 5C), along with phagocytized bacterial remnants in cellular vacuoles (black arrows).

DISCUSSION

Antibiotics against *Wolbachia* have been shown in extensive human trials to also induce loss of filarial worm fertility and viability. In line with these observations, our results in vitro and in vivo also indicated that *M. cajuputi* flower extracts reduced microfilarial release, worm motility, and viability by potently inhibiting *Wolbachia*, as indicated by the reduction in *Wolbachia* abundance and the total loss of the gene encoding *Wolbachia* surface protein. Anti-*Wolbachia* activity was further confirmed by the observation of degraded and phagocytized *Wolbachia* in worms treated with flower extracts. In turn, this antibacterial activity enhances antifilarial activity, as evidenced by the significant reduction in worm viability after 7 days of treatment. However, short-term treatment with flower extracts did not reduce *Wolbachia* to the same extent as tetracycline. Nevertheless, flower extracts and tetracycline at the highest doses reduced microfilariae release and worm motility more drastically than standard antiparasitic drugs.

Whereas untreated female worms released microfilariae throughout 7 days in culture, a progressive decrease in microfilariae was observed in worms treated with *M. cajuputi* flower extracts. The release completely ceased at day 6 in worms treated with 1,000 μg/ml flower extracts. These results were similar to the effects of the reference drug tetracycline, which was demonstrated by Gunawardena et al. [21]. It was of note that 1,000 μg/ml flower extracts were found to cause complete immobility of worms at days 6 and 7, which was consistent with microfilariae release and with earlier studies [22-27]. Our results were similar to published data describing the antifilarial activity of extracts from *Bauhinia racemosa* [28] and *Hibiscus mutabilis* [5] against *B. malayi* and *Setaria cervi*, respectively.

The activity of flower extracts against *Wolbachia* endosymbionts in adult worms was evaluated by amplifying *Wolbachia* surface protein of about 590 bp [29,30]. Our results indicated antibacterial activity in vivo, especially at concentrations beyond 500 μg/ml. Previous studies have demonstrated similar effects with tetracycline [29]. It is of note, however, that flower extracts did not reduce *Wolbachia* abundance to the same extent as tetracycline, presumably because the treatment regimen was too short. Indeed, tetracycline treatment for less than 2 weeks is insufficient to clear *Wolbachia* from *Brugia* worms [31].

When the effects were evaluated by transmission electron microscopy, numerous intracellular *Wolbachia* cells were detected in the hypodermal cord of untreated worms, whereas degraded *Wolbachia* cells were observed in worms treated with flower extracts and tetracycline, along with phagocytized bacterial remnants. The data are in line with reports of *Wolbachia* degeneration in *B. malayi* [14] and *Onchocerca ochengi* [31] treated with tetracycline. The total number of endosymbionts was generally lower in worms treated with flower extracts than in untreated worms, indicating anti *Wolbachia* activity. In turn, this antibacterial activity should enhance antifilarial activity, because the endosymbiont has been reported to be essential for development and fertility [32,33]. Notably, *Wolbachia* is known to elicit innate inflammatory reactions in the human host, and antibodies against its surface proteins are a key to the development of lymphedema and hydrocele in patients with bancroftian filariasis [32].

In conclusion, the data provide evidence that *M. cajuputi* flower extracts are active against both microfilariae and adult worms. The extracts significantly reduce microfilarial release and viability of adult worms by targeting endosymbiotic *Wolbachia*, as indicated by DNA and TEM analysis. However, mechanistic details remain unclear, and will be investigated in future studies.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interests exists, financial or otherwise, from any company or other entities.

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Prevalence of filarial parasites in domestic and stray cats in Selangor State, Malaysia

Nazeh M. Al-Abd[1*], Zurainee Mohamed Nor[1], Mustafa Kassim[2], Marzida Mansor[2], Abdulelah H. Al-Adhroey[1], Romano Ngui[1], Sinnadurai Sivanandam[1]

[1]*Department of Parasitology, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603, Malaysia*

[2]*Department of Anesthesiology, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603, Malaysia*

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ABSTRACT

Objective: To determine the prevalence of the filarial parasites,*ie.*,*Brugia malayi, Brugia, Brugia pahangi*(*B. pahangi*), *Dirofilaria immitis*and*Dirofilaria repens* (*D. repens*) in domestic and stray cats.

Methods: A total of 170 blood sample were collected from domestic and stray cats and examined for filarial worm parasites in two localities, Pulau Carey and Bukit Gasing, Selangor State, Malaysia.

Results: The overall prevalence of infection was 23.5% (40/170; 95% *CI* = 17.4–30.6). Of this, 35% (14/40; 95% *CI* = 22.1–50.5) and 50% (20/40; 95% *CI* = 35.2–64.8) were positive for single *B. pahangi* nd *D. repens*, respectively. The remaining of 15% (6/40; 95% *CI* = 7.1–29.1) were positive for mixed *B. pahangi* and *D. repens*. In addition, 75% of the infected cats were domestic, and 25% were strays. No *Brugia malayi* and *Dirofilaria immitis* was detected. Eighty-four cats were captured at Pulau Carey, of which 35.7% (30/84) were infected. Among the cats determined to be infected, 93% (28/30; 95% *CI* = 78.7–98.2) were domestic, and only 6.7% (2/30; 95% *CI* = 19.0–21.3) were strays. Conversely, the number of infected cats was three times lower in Bukit Gasing than in Pulau Carey, and most of the cats were stray.

Conclusions: *B. pahangi* and *D. repens* could be the major parasites underlying filariasis in the study area. Adequate prophylactic plans should be administrated in the cat population in study area.

1. Introduction

Cats, dogs, and leaf monkeys are among the known animal hosts that serve as reservoirs for *Brugian* filarial parasites [1]. Numerous published reports on zoonotic filariae involving cats have originated from several countries including Thailand [2], Indonesia [3], the Philippines [4], and other Southeast Asian countries [5,6]. In the endemic regions, both domestic and stray cats have been reported to be infected with several filarial parasites, such as *Brugia malayi*(*B. malayi*), *Brugia pahangi*(*B. pahangi*),*Dirofilaria immitis*(*D. immitis*) and*Dirofilaria repens*(*D. repens*) [2,7,8].

In Malaysia, domestic cats and leaf monkeys have been established as the primary reservoir hosts for these parasites [2]. The current investigation was prompted by the close association and proximity of cats and humans in Malaysia, as well as the evidence of possible natural infections of *B. pahangi* in man [3,9,10] and the possibility that *B. pahangi* infection in humans may be underestimated.

In humans, infection with *B. malayi* causes lymphatic filariasis [11]. Reports of experimental transmission of *B. pahangi* in humans have indicated that volunteers inoculated with *B. pahangi* not only developed microfilaria but also suffered from episodes of lymphangitis, lymphadenitis, and edema in the inoculated limb, each of which began approximately 1 month after the inoculation [12]. Human infection with *D. immitis*, on the other hand, is very rare, but when infection occurs, it is usually associated with pulmonary lesions or radiological coin lesions in the lung [13,14]. Moreover, humans may become infected with *D. repens* and present with subcutaneous nodules, pruriginous urticarioid patches, eosinophilia, photophobia, conjunctival irritation, and nodules or cysts in the eye or in the periocular tissues. Infection with

*Corresponding author: Nazeh M. Al-Abd, Department of Parasitology, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603, Malaysia.
E-mail: Nazehali78@yahoo.com
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D. repens is always amicrofilaremic; thus, it was assumed that larvae introduced into humans died and did not reach maturity [15].

There are limited data available on the prevalence of the filarial parasite in cats in Malaysia, but we believe that a study of the prevalence of these filarial parasites, particularly *B. malayi, B. pahangi,* and *D. immitis,* in both domestic and stray cats could have an impact on public health, as such information may help veterinarians to assess the risk of these parasites and establish a control program for zoonotic infections, thereby eliminating infection in cats and reducing the exposure to humans. In addition, estimation of the regional prevalence of filarial infection could increase awareness of this serious problem.

2. Materials and methods

2.1. Study area

Two geographical sites in Selangor state, Malaysia, were included in the current study. Bukit Gasing (3°6′0″ N, 101°41′1″ E) is a residential area located in Kuala Lumpur and is a hilly and forested area with individual bungalow houses. Pulau Carey (2°8′53″N, 101°39′4″E), in contrast, is an Orang Asli (Aborigines) village situated east of Kuala Lumpur (Figure 1) that is also forested, and most of the houses are constructed of wood. Both sites included on the study have a tropical climate with high humidity and frequent rainfall throughout the year. The temperature for the sites ranges between 30 °C and 36 °C.

2.2. Collection of domestic and stray cats

In this study, domestic cat refers to a cat living in close proximity to a human household, from which the animal obtains all of its basic requirements for survival (*eg*., food, water, andshelter). In contrast, stray cat refers to cat living separately from a household, and depending only in part on humans for provision of shelter and sustenance. Trapping of the cats was performed from January to May 2013. Briefly, each cat was approached slowly and restrained by scruffing the loose skin on the back of its neck. Once caught, the cat was allowed a brief period of acclimation and was allowed to regain composure while under humane restraint. The Ethics Committee of the Faculty of Medicine at University Malaya, Kuala Lumpur, Malaysia, approved all procedures involving the cats performed on the current study [Ethical number: PAR/21/11/2011/ZMN (R)].

2.3. Collection of blood

When each cat was calm and comfortable, blood collection was performed by a well-trained veterinarian. With the animal restrained humanely, blood was collected from the ear vein of each cat. All blood samples were collected in EDTA tubes by using a graduated capillary pipette fitted with a simplified non-breakable Sinton pipette. Samples were stored at 4 °C until direct transport within 1 d to the laboratory for analysis.

2.3.1. Staining of blood films

Staining of all blood films was performed using Giemsa stain (Innenkorper technique) as previously described [16]. Briefly, thick blood films were prepared on glass slides and allowed to dry at room temperature for 24–48 h. Before staining, the slides were dehemoglobinized in water for 1 min and air-dried. The slides were then fixed with absolute methanol for approximately 30–60 s. The dried blood films were then stained with 2% Giemsa (Merck, NJ) in pH 7.2 phosphate buffer for 35 min, and then rinsed with tap water.

2.3.2. Microscopic examination of blood films

Two independent individuals, using an Olympus CX40 light microscope (Olympus, Japan), examined the stained blood films for microfilaria. The microfilariae were observed at 100× and 400× magnification, and images of the microfilaria detected were captured using an on-board Olympus DP12 digital microscope camera (Olympus, Japan). Data entry and analysis were performed using Microsoft Excel 2010.

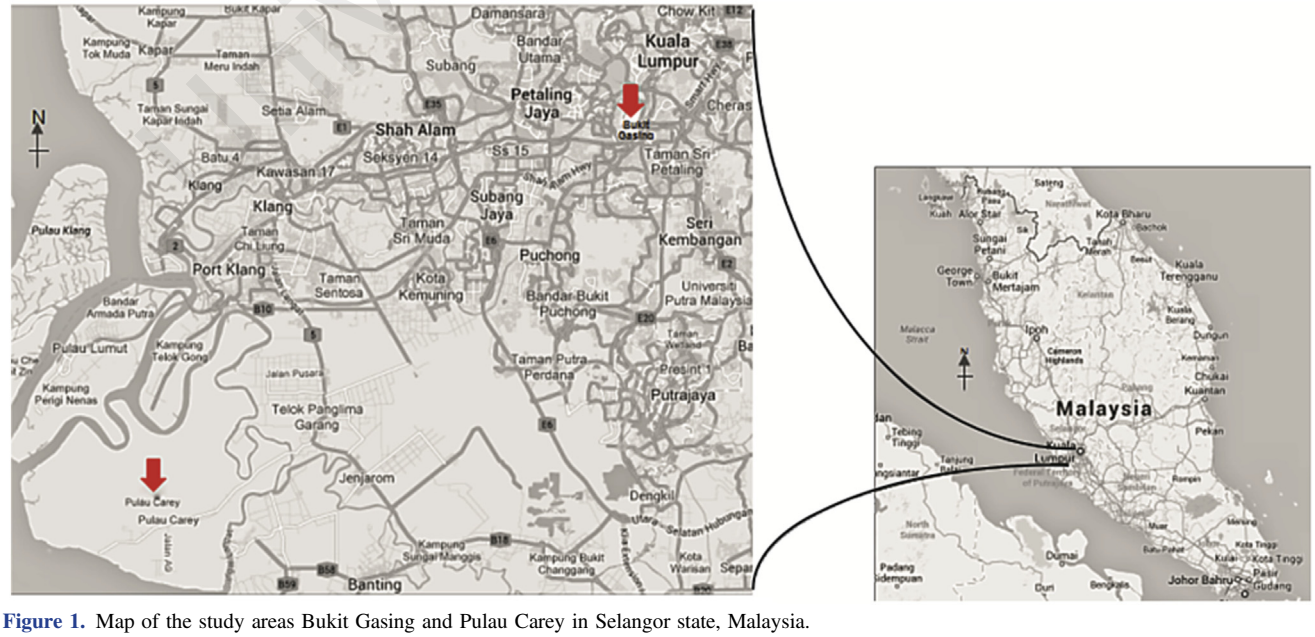

Figure 1. Map of the study areas Bukit Gasing and Pulau Carey in Selangor state, Malaysia.

3. Results

3.1. Cat population and infection status

The sites selected for this study are detailed in the map in Figure 1. In total, 170 domestic and stray cats (average age, 2 years) were collected. Eighty-four cats were caught from Pulau Carey, including 54 domestic cats and 30 stray cats, whereas 86 cats, including 52 domestic cats and 34 stray cats, were caught from Bukit Gasing. Of the 170 cats included on the study, 49.4% were male and 50.6% were female. Eighty-four cats were captured at Pulau Carey, of which 35.7% (30/84) were infected. Among the cats determined to be infected, 93% (95% *CI* = 78.7–98.2) were domestic, and only 6.7% (95% *CI* = 19.0–21.3) were stray. Conversely, the number of cats infected from Bukit Gasing was three times lower than the number of infected cats from Pulau Carey, and most of the cats were stray (Table 1).

3.2. Identification of parasite species detected in blood films

The morphology of the *B. pahangi* and *D. repens* microfilaria detected in the study is shown in Figure 2. Identification of *B. pahangi* microfilaria (Figure 2a) was based primarily on the size of the innenkorper relative to the whole length of the microfilaria, which was approximately 21% (44–63 μm). Further, the proportional size of the *B. pahangi* innenkorper was slightly larger than that of *B. malayi* (subperiodic form), which was approximately 13.7%. Other characteristics of this microfilaria, which were similar to those of other *Brugia* species, included the presence of two terminal nuclei at the posterior end and a 2:1 cephalic space ratio. Identification of *D. repens* microfilaria (Figure 2b) was mainly based on the presence of two distinct nuclei in the cephalic space region and the observation that the microfilariae of this parasite lacked a sheath.

Table 1
Number and the type of cats found infected in the study.

Study site	Number of cats captured	Domestic cats infected			Stray cats infected		
		n	%	95% *CI*	*n*	%	95% *CI*
Pulau Carey	84	28	33.3	24.4–44.0	2	2.4	0.6–8.3
Bukit Gasing	86	2	2.3	0.6–8.1	8	9.3	4.8–17.3

n: number of infected cats; %: percentage of infected cats; 95% *CI*: 95% confidence interval.

3.3. Distribution of the filarial parasites within the cat population

In total, 35% (14/40; 95% *CI* = 22.1–50.5) of the cats determined to have a filarial parasite infection were infected with *B. pahangi*. Furthermore, all of the cats infected with *B. pahangi* were from Pulau Carey, and all were domestic. Approximately 50% (20/40; 95% *CI* = 35.2–64.8) of the cats tested positive for *D. repens,* and an equal number of cats was infected by this parasite at each of the study sites. In Pulau Carey, infection with *D. repens* was detected in eight domestic and two stray cats, whereas in Bukit Gasing, the numbers of infected cats were reversed (two domestic and eight stray cats). Mixed infection with both *B. pahangi* and *D. repens* was observed in 15% (6/40; 95% *CI* = 7.1–29.1) of the cats, all of which were from Pulau Carey and included domestic cats only. Similarly, infection occurred in both sexes, with 40% of the infected cats being male (16/40; 95% *CI* = 26.4–55.4) and 60% female (24/40; 95% *CI* = 44.6–73.7). None of the cats in this study were infected with *B. malayi* or *D. immitis* (Table 2). Infection rates were higher in the domestic cat population (75%) than in the stray cat population (25%).

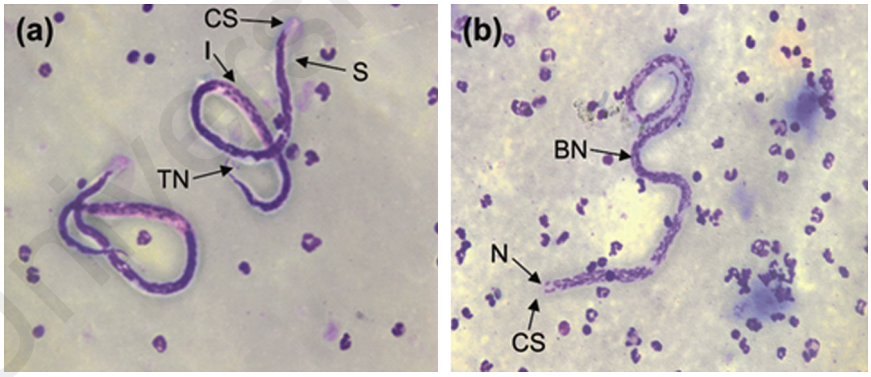

Figure 2. Micrograph of microfilariae.
(a) Microfilaria of *B. pahangi,* showing the presence of marked innenkorper (I), sheath (S), cephalic space (CS) and two terminal nuclei (TN). (b) Microfilaria of *D. repens* lacked a sheath and had two nuclei (N) present in the cephalic space (CS).

Table 2
Species of parasites detected in the infected cats.

Study site	Number of cats infected	Number of domestic cats infected					Number of stray cats infected				
		B. malayi	*B. pahangi*	*D. immitis*	*D. repens*	Mix	*B. malayi*	*B. pahangi*	*D. immitis*	*D. repens*	Mix
Pulau Carey	30	0	14 (4 M, 10 F)	0	8 (4 M, 4 F)	6 (2 M, 4F)	0	0	0	2 (1 M, 1 F)	0
Bukit Gasing	10	0	0	0	2 (1 M, 1 F)	0	0	0	0	8 (4 M, 4 F)	0
Total	40	0	14 (4 M, 10 F)	0	10 (5 M, 5 F)	6 (2 M, 4F)	0	0	0	10 (5 M, 5 F)	0

M = male; F = female.

4. Discussion

Cats are known to serve as the host for zoonotic filarial infections, including *B. pahangi* and *D. repens* [7,8,10]. However, it is a common practice among humans to keep a cat as a domestic household pet. Because many stray cats roam areas near human populations in search of food, the possibility exists that these cats could serve as hosts and contribute to zoonotic infections [17]. In the current study, 170 cats were captured and evaluated for the presence of microfilaremia. The blood film analyses indicated that 40 of the cats were infected with filarial worms, of which 30 were domestic cats and 10 were strays. The predominant animal filarial parasite species identified included *B. pahangi* and *D. repens*. Both *B. pahangi* and *D. repens* were detected in domestic cats, but only *D. repens* was detected in the stray cats. Furthermore, some domestic cats were infected simultaneously with both parasite species, which was not apparent in the stray cat population. Additionally, infection with other filarial parasites, including *B. malayi* and *D. immitis,* was not observed in the cat population included in the current study. These results were not unexpected, considering that *D. immitis* is a parasite found primarily in canine species, whereas *B. malayi* is a human filarial parasite. Nonetheless, both parasites have been reported to infect cats.

The overall infection rate was determined to be 23.5% for the total cat population examined, where 35% of the affected animals were infected with *B. pahangi,* 50% were infected with *D. repens,* and 15% were infected with both *B. pahangi* and *D. repens*. Additionally, the results of the current study were similar to those of a previously published report [2]. A prior study that employed polymerase chain reaction–restriction fragment length polymorphism of internal transcribed spacer regions, ITS1, to determine the filarial distribution in 52 domestic cats of south Thailand demonstrated the existence of parasites in 9.5% of the cats, of which 7.6% were infected with *B. pahangi*. The study also determined that 1.9% of the cats were infected with *D. immitis*.

A study conducted in Petaling Jaya, another district in Selangor state, reported the occurrence of *B. pahangi* microfilaria in blood samples from 5 out of 12 domestic cats [10]. In that study, absence of *B. malayi* in the blood samples of domestic cats was also reported. Based on this report and our findings, we surmised that cats are not the main host for *B. malayi* infection and that these cats were not from areas endemic for human filariasis. However, our findings contradicted an observation of the occurrence of *B. malayi* in a cat obtained from an area with endemic lymphatic filariasis in southern Thailand [8].

Reports of several cases of natural human infection with *B. pahangi* in a suburb of Kuala Lumpur, Malaysia [10], clearly indicated the presence of zoonotic infections, which led the authors to conclude that domestic cats may be the source of *B. pahangi*. The results of our study support this conclusion, based on the detection of *B. pahangi* in 35% of the infected cats and the finding that 15% of the cats had mixed infections (*B. pahangi* and *D. repens*). In addition, all of the cats infected with *B. pahangi* were domestic cats.

All of the cats infected with *B. Pahangi* (singly, or mixed with *D. repens*) were caught at Pulau Carey. None of the cats caught at Bukit Gasing were infected with *B. pahangi*. The life cycle of *B. pahangi* involves an intermediate mosquito vector and a primary mammalian host. The *Mansonia annulata* and *Mansonia dives* mosquito species are known to be natural vectors of *B. pahangi* [18], and to thrive in a forest environment similar to that of Pulau Carey, but not in a suburban area such as Bukit Gasing [9].

A previous study revealed the occurrence of *D. repens* microfilaria in cats and reported that the infected cats exhibited lesional pruritus, concurrent hemobartonellosis, and cutaneous lesions [19]. In our study, *D. repens* was detected in cats from both areas and affected both domestic and stray cats. Furthermore, although 50% of the affected cats were infected with *D. repens* and an additional 15% had mixed infections (*B. pahangi* and *D. repens*), none of the cats presented conditions such as those described in the previous study [19]. Human infection by this parasite is of great concern because it has become increasingly recognized worldwide as an inadvertent human pathogen [20,21].

In the current study, although the number of cats infected with *B. pahangi* was lower than the number of cats infected with *D. repens,* several cats infected with *B. pahangi* presented a higher number of circulating microfilaria, which increased the likelihood for human infection to occur in the presence of a suitable vector. One of the limitations of the current study was the use of microscopy as the method of diagnosis, as microscopy has low sensitivity compared to other techniques such as the polymerase chain reaction [2,22]. Therefore, it is strongly recommended to use polymerase chain reaction in a future study to accurately determine the true prevalence of filariasis in the area.

The type of filarial parasites reported in the current study, and the associated microfilaria count of each species detected in both domestic and stray cats, could provide insight on the status of zoonotic transmission of parasites of concern in Malaysia. Although *B. malayi* is the most prominent parasite among all of the filarial parasites in peninsular Malaysia, *B. pahangi* and *D. repens* were found to be the major parasites of involved in filariasis in the included study areas. Hence, some attention should be given to *B. pahangi* because this parasite could potentially pose a threat to the human population.

In conclusion, the data from this study suggest that *B. pahangi* and *D. repens* could be the major parasites underlying filariasis in the study area. The identification of these parasites in cats could provide an insight into public health, particularly with regard to the extent of the danger of zoonotic infection in the research, as previously reported for several cases of natural human infection with *B. pahangi* in a suburb of Kuala Lumpur, the capital city of Malaysia. Further studies that will elucidate the role of cats in the zoonotic transmission of microfilarial parasites in the research area are important and are expected to suggest that appropriate prophylaxis should be administered to cats throughout the study area.

Conflict of interest statement

We declare that we have no competing interests.

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