ISOLATION AND CHARACTERISATION OF CHEMICAL CONSTITUENTS WITH ANTI-METHICILIN RESISTANT STAPHYLOCOCCUS AUREUS ACTIVITY FROM MESUA FERREA LEAF EXTRACT

SANGEETA VANI ALAGASAMY

UNIVERSITI SAINS MALAYSIA

2018

ISOLATION AND CHARACTERISATION OF CHEMICAL CONSTITUENTS WITH ANTI-METHICILIN RESISTANT STAPHYLOCOCCUS AUREUS ACTIVITY FROM MESUA FERREA LEAF EXTRACT

by

SANGEETA VANI ALAGASAMY

Thesis submitted in fulfilment of the requirements

for the degree of

Master of Science

May 2018

ACKNOWLEDGEMENT

First and foremost, I would like to humbly offer this dissertation as an offering of love to the lotus feet of Their Lordships Sri Sri Radha Krishna Kanhaiya, Sri Sri Nitai Gauranga Rai and Srila Prabhupada. Without Their causeless mercy, I would not have able to finish this project. As Lord Krishna has stated in Chapter 9 text 27 of the holy Bhagavad Gita:

yat karosi yad asnasi yaj juhosi dadasi yat

yat tapasyasi kaunteya tat kurusva mad-arpanam

"O son of Kunti, all that you do, all that you eat, all that you offer and give away, as well as all austerities that you may perform, should be done as an offering unto Me." My utmost gratitude goes to my spiritual master (diksa guru), HH Jayapataka Swami Guru Maharaj for always showing me sheer determination and love in serving Srila Prabhupada and his mission. By following his great example, I can only humbly pray to his lotus feet to make me a tool in the service of Srila Prabhupada's movement in the future. As the famous poet Narottama das Thakur says, "The lotus feet of the spiritual master are the abode of pure devotional service. I bow down to those lotus feet with great care and attention."

I would like to convey my deepest gratitude to my supervisor and siksa guru, Prof. Dr. Surash Ramanathan @ HG Surabala das for allowing me a place to learn and grow under him. Without his patience in paving the way for me, I would not have completed this thesis. He has provided professional and spiritual guidance for me throughout these years and for that I can't thank him enough. I would like to thank my co-supervisors Dr. Lai Choon Sheen and Assoc. Prof. Dr. Vickneswaran

Murugaiyah for guiding and teaching me throughout this project. I am truly indebted to them for always lending me a helping hand during hard times.

A special thanks to my godsisters Dr. Yasodha Ponnusamy @ HG Yasodha Yasomathi dd and Dr. Suhanya Partasarathy @ HG Sulochana Radhika dd for their encouragement and invaluable advises. I would like to thank Center for Drug Research for giving me the opportunity to pursue my graduate studies and all the supporting staff and colleagues who have created a cheerful and happy environment for me. Special thanks to Mr.Asokan, Mr.Arunacalam, and Mr.Hilman for assisting me to operate the analytical instruments.

Last but not least, I would like to take this opportunity to thank my one and only family for all the sacrifices that they have made for me. My father HG Adharma Satru das, my mother HG Rupavathi Sundari dd and my siblings, HG Dr.Simharaj Narahari das, HG Srivasudha Subasini dd and HG Vrajasri Radha dd who have been very supportive towards my mental, emotional and spiritual being all these years. Without their prayers and moral support, I would not have able to finish this project.

Finally, one has to understand that the ultimate knowledge is to attain the Supreme Personality of Godhead as Lord Krishna says to Arjuna in the Bhagavad Gita, Chapter 10 Text 32:

sarganam adir antas ca madhyam caivaham arjuna

adhyatma-vidya vidyanam vadah pravadatam aham

"Of all creations I am the beginning and the end and also the middle, O Arjuna. Of all sciences I am the spiritual science of the Self, and among logicians I am the conclusive truth!" Hare Krishna!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
°C	Degree Celcius
A	Alpha
B	Beta
µ	Micro
µg/mL	Microgram per milliliter
µL	Microliter
ALT	Alanine aminotransferase
AMX	Amoxicillin
APCI	Atmospheric-pressure chemical ionization
AR	Analytical grade
AST	Aspartate aminotransferase
ATCC	American type culture collection
B. subtilis	*Bacilllus subtilis*
CAT	Catalase
CFU	Colony forming unit

$CHCl_3$	Chloroform
CI	Chemical ionization
cm	Centimeter
CO_2	Carbon dioxide
COSY	Homonuclear correlation spectroscopy
CPK	Creatinine phosphorkinase
DMSO	Dimethyl sulfoxide
dd	Doublet of doublet
DEPT	Distortionless enhancement by population transfer
E. coli	*Escherichia coli*
EI	Electron impact ionization
ESI	Electrospray ionization
EtOAc	Ethyl Acetate
F3	Fraction F3
FAB	Fast atom bombardment
FDA	Food and drug administration, United states
FICI	Fractional inhibitory concentration indices
FT	Fourier Transform

g	Gram
GC-MS	Gas chromatography-mass spectrometry
GPX	Glutathione peroxidase
GR	Glutathione reductase
H_2O	Water
H_2O_2	Hydrogen peroxide
HMBC	Heteronuclear Multiple Bond Coherence
hr	Hour
HSQC	Heteronuclear Single Bond Coherence
HTS	High throughput screening
Hz	Hertz
IC_{50}	Concentration of a test substance required for 50 % inhibition *in vitro*
INT	*para* iodonitrotetrazolium
IR	Infrared Radiation
J	Coupling constant
KBr	Potassium Bromide
khz	Kilohertz
L	Liter

LC-MS	Liquid chromatography-mass spectrometry
m	Meter
m	Multiplet
MALDI	Matrix-assisted laser desorption/ionization
MBC	Minimum bactericidal concentration
M. ferrea	*Mesua ferrea*
MeOH	Methanol
mg/kg	Milligram per kilogram
mg/mL	Milligrams per milliliter
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
MIC	Minimum inhibitory concentration
min	Minute
mL	Milliliter
mL/min	Milliliter per minute
mm	Millimeter
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectrometry

MSSA	Methicillin-sensitive *Staphylococcus aureus*
n-BuOH	n-Buthanol
NMR	Nuclear magnetic resonance
P. aeruginosa	*Pseudomonas aeruginosa*
ppm	Parts per million
prep-TLC	Preparative thin layer chromatography
R_f	Retention factor
Rpm	Revolution per minute
s	Singlet
S4	Sub-fraction S4
S. aureus	*Staphylococcus aureus*
SCCmec	Staphylococcal cassette chromosome, mec
SFD	Staphylococcal foodborne diseases
SOD	Super oxide dismutase
t	Triplet
T8	Sub-fraction T8
TLC	Thin layer chromatography
TMS	Tetramethylsilane

UV	Ultraviolet
v/v	Volume over volume
WHO	World Health Organization
w/v	Weight over volume

PEMENCILAN DAN PENCIRIAN SEBATIAN DENGAN AKTIVITI ANTI-METHICILIN TAHAN STAPHYLOCOCCUS AUREUS DARI EKSTRAK DAUN MESUA FERREA

ABSTRAK

Tujuan kajian ini adalah untuk mengasingkan kompaun yang mempunyai aktiviti antimikrobial dari daun *Mesua ferrea* (*M. ferrea*) dengan menggunakan pendekatan berpandukan bioesei. Memandangkan ini, satu prosedur pengekstrakan dan pemencilan sebatian berasaskan bioesei antimikrobial telah dijalankan ke atas ekstrak metanolik daun *M. ferrea* dengan bakteria Gram positif dan negatif. Bagi permulaan, teknik pengekstrakan daun *M. ferrea* dioptimumkan dengan kaedah berpandukan esei antimikrobial. Serbuk beku kering daun *M. ferrea* (saiz partikel 0.5 mm) dalam methanol (1:15 w/v) dengan bantuan ekstraksi ultrasonik dapat menghasilkan ekstrak mentah dengan aktiviti antibakteria yang baik terhadap *S. aureus*, MRSA, *P. aeruginosa*, *B. subtilis* dan *E. coli*. Ekstrak mentah metanol, seterusnya dipartisikan dengan pelbagai pelarut untuk mendapatkan pecahan aktif daun *M. ferrea*. Fraksi (T8) menunjukkan aktiviti antibakteria yang paling kuat terhadap *S. aureus* dan MRSA dan terus tertakluk kepada pemencilan berpandu esei antibakteria. Empat sebatian telah diasingkan di mana stigmasterol dan caryophyllene oksida menunjukkan aktiviti antibakteria yang baik terhadap *S. aureus* dan MRSA dengan nilai MIC 31.25, 62.5 µg/mL dan 15.625, 31.25 µg/mL masing-masing. Nilai MBC > 500 µg/mL mencadangkan bahawa kedua-dua stigmasterol dan caryophyllene oksida adalah sebatian bakteriostatik. Baki dua kompaun mempuyai nilai MIC melebihi 500 µg/mL. Kedua-dua sebatian ini tidak dipertimbangkan untuk

penentuan struktur. Aktiviti antibakteria stigmasterol dan caryophellene oksida adalah jauh lebih baik apabila diberikan dalam kombinasi dengan antibiotik daripada digunakan sebagai sebatian tunggal. Kedua-dua sebatian ini didapati menunjukkan kesan sinergi dengan antibiotik konvensional, penisilin G, ampicillin dan chloramphenicol terhadap *S. aureus* dan MRSA. Interaksi sinergistik menunjukkan bahawa aktiviti antibakteria antibiotik telah dipertingkatkan dan gabungan produk semulajadi dengan agen antibakteria ini boleh digunakan sebagai ubat terhadap bakteria yang rintangan terhadap pelbagai ubat. Sebagai kesimpulan, kajian ini pertama kali melaporkan kaedah pengekstrakan dan pemencilan sebatian dari ekstrak metanolik daun *M. ferrea* berpandukan esei antibakteria. Sinergi antara bioaktiviti daun *M. ferrea* (stigmasterol dan caryophyllene oksida) dan antibiotik konvensional terhadap *S. aureus* dan MRSA juga pertama kali ditunjukkan. Fakta yang menarik ialah, stigmasterol diasingkan daripada daun *M. ferrea* buat pertama kali. Kajian ini juga mengesahkan kehadiran caryophyllene oksida dalam daun *M. ferrea* melalui kajian NMR.

ISOLATION AND CHARACTERISATION OF CHEMICAL CONSTITUENTS WITH ANTI-METHICILIN RESISTANT STAPHYLOCOCCUS AUREUS ACTIVITY FROM MESUA FERREA LEAF EXTRACT

ABSTRACT

The present study aims to isolate compounds with antimicrobial activity from *Mesua ferrea* leaf employing bioassay guided approach. An antibacterial assay guided extraction and compound isolation procedure was carried out on *Mesua ferrea* methanolic leaf extract with a number of Gram positives and negatives bacteria. Firstly, *M. ferrea* leaf extraction technique was optimized using antimicrobial assay guided method. Powdered freeze dried leaves of *M. ferrea* (particle size 0.5mm) (1:15w/v) using ultrasonic-assisted technique yielded methanol crude extract with a good antibacterial activity against *Staphylococcus aureus*, MRSA, *Pseudomonas aeruginosa, Bacillus subtilis* and *Escherichia coli*. The methanolic crude extract was further partitioned with various solvents to obtain active fractions of *M. ferrea* leaf. Sub-fraction (T8) demonstrated the strongest antibacterial activity against *S. aureus* and MRSA and was further subjected to antibacterial assay guided isolation. Four compounds were isolated of which stigmasterol and caryophyllene oxide demonstrated good antibacterial activity against *S. aureus* and MRSA with MIC values of 31.25, 62.5 µg/mL and 15.625, 31.25 µg/mL respectively. The MBC values of > 500 µg/mL suggested that both stigmasterol and caryophyllene oxide are bacteriostatic compounds. As for the remaining two compounds the MIC values were above 500 µg/mL. These

compounds were not considered for structural elucidation. The antibacterial activity of stigmasterol and caryophyllene oxides was far better when given in combination with antibiotics than used as a single compound. Both compounds were found to show synergism with conventional antibiotics, ampicillin, chloramphenicol, and penicillin G against *S. aurues* and MRSA. The synergistic interactions indicated that the antibacterial activities of antibiotics were improved and combining natural products with these antibacterial agents could be useful against infectious multi-drug resistant bacteria. In conclusion, this study first reported the bioassay guided extraction and isolation of bioactives from *M. ferrea* methanolic leaf extract. The synergism between the *M. ferrea* leaf bioactives (stigmasterol and caryophyllene oxide) and the conventional antibiotics against *S. aureus* and MRSA were also first demonstrated. Interestingly, stigmasterol was first isolated from the leaves of *Mesua ferrea*. This study also confirmed the presence of caryophyllene oxide in the leaves of *M. ferrea* by NMR assignment.

CHAPTER 1

BACKGROUND

Infectious diseases have become the leading source of death world-wide. The emergence of new multidrug-resistant pathogens is threatening the many existing antibiotics clinical effectiveness (Bandow *et al.*, 2003). Scientific articles describing the prevalence of resistance of *S. aureus* against the conventional antibiotics, reported approximately 90–95% of the isolated *S. aureus* was penicillin resistant while 70–80% of the same microorganism was methicillin resistant (Casal *et al*., 2005; Chambers, 2001).Hence, antibiotic resistance has become an interest globally (Wesch *et al.*, 2004). In addition, the increasing failure of chemotherapeutics and the toxicity of the allopathy drugs had prompted researchers to look for an alternative therapy. (Iwu *et al.*, 1999).

Natural product research appear to be a promising option for exploring new compounds with antimicrobial activity. These new compounds with varied chemical structures and unique action mechanisms provides a bright platform to fight the current and recurring infectious diseases (Rojas *et al.*, 2003). Natural products can cater boundless opportunities either as standardized herbal extracts or as pure isolated compounds, for new drug establishment because of the immeasurable availability of chemical diversity. As a result of this, folk medicine has been increasingly examined, in pursuit for new drug leads to develop a better treatment against microbial diseases (Tambekar *et al*., 2010; Benkeblia, 2004).

Natural product research does not only emphasis on finding new leads, but also focus on the effort to revamp regular antibiotics with better efficiency. Several

researches have disclosed unique findings such as the synergistic effects of standardized plant extracts when used together with regular antibiotics. For example pomegranate extract, pyridine and myricetin that were isolated from *Jatropha elliptica*, displayed compelling synergistic effect when employed together with regular antibiotics such as gentamicin, oxacillin, tetracycline, chloramphenicol, and ampicillin (Braga *et al*., 2005; Lin *et al*., 2005). Synergy studies have been testified to be a good alternative strategy to development of new antibacterial agent as the latter has been proven to be expensive and time consuming. Development of resistance towards regular antibiotics are somewhat easy being that these antibiotics have been largely of microbial roots and therefore susceptible to random mutation. Unlike to these regular antibiotics, natural products have a more diverse and novel structures that are not of typical to microbes (Cowan, 1999). Therefore, there has been an upsurge of recent studies that focuses on the synergistic effect of plant extracts or compounds with regular antibiotics in order to lessen the future prospects of developing antibiotic-resistant bacterial strains. Besides perpetuating the potent lifespan of an antibiotic, this can also scale down the side effects that are caused by these antibiotics.

In Malaysia, the traditional practitioners use medicinal plants and plant juices to treat illness and this practice is still in use (Samuel *et al*., 2010). Since there is a high demand on herbal drugs and traditional plants that have antibacterial activity, study and research are enormously taking place. One of those local medicinal plants of interest is *Mesua ferrea* Linn. Past studies have shown *M. ferrea* Linn bark, seed and flowers extracts to have antibacterial properties against a number of Gram positives and negatives bacteria (Ali *et al.*, 2004 and Prashanth *et al*., 2006).

Aruldass *et al.* (2013) reported antibacterial activity of *M. ferrea* methanolic leaf extract against *S. aureus*. A similar study was carried out by Adewale *et al.* (2012) against a spectrum of bacteria using *M. ferrea* leaf methanolic and ethanolic extracts and found antibacterial activities. Ali *et al.* (2004) tested the chloroform, petroleum and ethanolic extracts of *M. ferrea* leaf against various bacteria and reported promising antimicrobial activities.

However most of the previous studies reported on *M. ferrea* were conducted on the crude extracts instead of its active fraction or compounds. Studies to further isolate the bioactives responsible for the antibacterial properties are lacking. Hence, in recognition of its antibacterial properties, the current study was undertaken to examine the antibacterial activity of *M. ferrea* leaf extracts, fractions and sub fractions. After then, a systemic bioassay guided isolation of compounds from *M. ferrea* leaf extract was carried out. Finally, the synergism between the conventional antibiotics and the isolated bioactives of *M. ferrea* leaf extract were determined.

With this in view and present work focussed on the following objectives :

1. To optimize the extraction method for *M. ferrea* leaf by bioassay guided method with antibacterial activity.
2. To isolate, characterize and elucidate the structure of the bioactives or compounds that are responsible for the antibacterial activities found in the active fractions of *M. ferrea* leaf.
3. To evaluate the antibacterial activity and any synergistic effect of the isolated compounds with regular antibiotics.

CHAPTER 2

LITERATURE REVIEW

2.1 *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive, round-shaped bacterium relatively 1 μm in diameter. Being widely known as "golden staph" due to its formation of golden colonies, the cells are often found on skins, and noses. Cell division usually takes place in more than one plane, *S. aureus* commonly appears as a grape-like clusters. (Crossley & Archer, 1997). Approximately 20–30% of the general population has been estimated to be carriers of *S. aureus* (Heyman, 2004). Colonies of *S. aureus* on sheep blood agar plates, often cause β-hemolysis (Ryan & Ray, 2004). In order to protect the pathogen, the presence of careotenoids has been disclosed to be a virulence factor against oxidants produced by the immune system and its presence is also the cause for the golden pigmentation of *S. aureus* colonies (Liu *et al.*, 2005). Staphyloccoci are capable to yield lactic acid by fermentation, besides generating energy by aerobic respiration.

Staphylococcus sp. is catalase-positive as they are able to produce the enzyme catalase, a trait discerning them from *Streptococcus* sp. They are also oxidase-negative and requirement of complex nutrients, e.g., vitamins B and many amino acids, is much needed for growth. *S. aureus* has a very high tolerance for sodium chloride, as it is able to tolerate concentration up to 1.7 molar. Besides this, another prominent trait of this genus is the peptidoglycan structure of the cell wall which contains multiple glycine residues in the crossbridge. This trait is that which causes susceptibility to lysostaphin (Crossley & Archer, 1997). *S. aureus* produces coagulase that enables the conversion of fibrinogen into fibrin. This conversion is

done when coagulase interacts with prothrombin in the blood resulting in plasma to coagulate. Most of the members of the genus are generally entitled as coagulase-negative staphylococci, so blood coagulation is therefore a trait used to differentiate *S. aureus* from the others. (Ryan & Ray, 2004). Due to being a common ethological agent of human ailments and the ability to exhibit resistance to an increasing amount of healing agents, *S. aureus* is therefore one of the most extensively investigated bacterial strains.

2.1.1 *S. aureus* infections

S. aureus is a commensal bacteria and a pathogen. The mucosal surfaces and skin are the main sites of colonization. Approximately 20–30% of humans are tenacious carriers of *S. aureus*, and 30% are intermittent carriers. The stubborn carriers are always colonized by this bacteria strain while the intermittent ones are colonized transiently (Wertheim *et al*., 2005). When host defence is compromised, colonization provides a source of the pathogen from which bacteria are brought in and thus significantly increasing the possibility of infections (Kluytmans *et al*., 1997). *S. aureus* infected patients are normally affected by the same commensal strain that they carry (Williams *et al*., 1959).

Diekema *et al.* (2001) reported that *S. aureus* is an important source for hospital- and community-acquired infections that may develop severe problem. Nosocomial *S. aureus* infections can badly influence the skin, bloodstream, lower respiratory tracts and soft tissues. Besides causing serious deep rooted infections like osteomyelitis and endocarditis, it can also be the reason for ventilator-assisted pneumonia and central venous catheter-associated bacteremia (Schito, 2006). Not only that, *S. aureus* is also usually responsible for diseases that are toxin-mediated,

like staphylococcal foodborne diseases (SFD), scalded skin syndrome and toxic shock syndrome. Due to their constant catheter insertions and injections and their compromised immune system, patients in the hospitals are normally the ones that are notably prone to *S. aureus* infections (Lindsay & Holden, 2004). During a two- year period of survey, the SENTRY Surveillance Program that investigated worldwide *S. aureus* infections reported that this pathogen is the leading cause for skin or soft tissues, lower respiratory tract and bloodstream infections in all the regions that were studied (Diekema *et al*., 2001). Besides its capability of creating life-endangering infections, this human pathogen has also shown an exceptional potential to develop an antimicrobial resistance.

2.1.2 Prevalence of *S. aureus* resistance

The European Centre for Disease Prevention and Control carried out a research in 2009 and reported that in Europe Member States hospitals, approximately 380,000 healthcare associated infections annually are caused by bacteria that are resistant to antibiotics. Chambers and colleagues (2001) study showed that in most Asian countries, relatively 70-95% of *S. aureus* strains were reported to be methicillin and penicillin-resistant. Apart from that, 75% of morbidity post-injuries is also reported to be related to infections (Church *et al*., 2006), that is commonly caused by *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus* (MRSA) (Vindenes & Bjerknes, 1995). Saba *et al.* (2017) study showed that the isolates of *S. aureus* and MRSA had high rates of resistance to the antibiotics used in Ghana.

2.2 Methicillin resistant *Staphylococcus aureus* (MRSA)

After introducing β-lactamase-insensitive Penicillin into medical settings, MRSA strains were first notably spotted in hospitals, where they remain to be a severe risk in health care. This is caused by their capability to amass multidrug resistance determinants. Besides MRSA, Methicillin-sensitive *S. aureus* (MSSA) is also capable of causing outbreaks of diseases in a hospital (Kurlenda *et al*., 2009), but it is the infections that are caused by MRSA which are spread throughout the hospitals with ease. Therefore, without proper establishment of surveillance program with controlled method and techniques, a high risk of an epidemic in such hospitals is inevitable (Kurlenda *et al*., 2007). MRSA at molecular level is characterized by the existence of staphylococcal cassette chromosome, mec (SCCmec), a large mobile genetic element. It bears the mecA gene which codes PBP2a, a penicillin binding protein alternate that has a low attachment affinity to all β-lactams (Ito *et al.*, 1999). Globally, MRSA has become a crucial cause of nosocomial infections and is currently the most commonly known antibiotic-resistant pathogen in the hospitals of United States (Monaco *et al*., 2016).

2.2.1 Strategies to Fight MRSA

There are a couple of healing strategies introduced in which some are still under progress, to fight MRSA such as therapeutic vaccines, antibiotic-free treatments, antibiotic-based treatments, alternative treatment, and immunotherapy. (McKenna, 2012: Zhao *et al.*, 2015 and Bal *et al.*, 2005). Among these treatments, antibiotics usage has been proven to be effective and most important historically. Unfortunately in recent decades, an alarming trend has been observed in which there

is an upsurge of antibiotic resistance but a decline in antibiotic study and development (Boucher *et al.*, 2009 and Spellberg *et al.*, 2004).

Spellberg *et al*. (2004) reported that since 1968 only two novel classes of systemic antibiotics, linezolid (2000) and daptomycin (2003) were developed due to the failure of garnering interest for the development of antibiotics. Some of the reasons contributing to this scenario are lack of commercial interests, short-term usage and low profit motivations (Arias, 2009). Besides that, the level of difficulty for conducting clinical trials against these drug-resistant strains is high and therefore diminishes the hope of many researchers for final approval (Boucher *et al.*, 2009). However, an emerging trend is noticed when the efforts of the U.S. FDA (Food and Drug Administration) to restart the antibiotic development (Shlaes *et al.*, 2013) has quicken the drug approvals and documented development of antibiotics.

In the effort to look for an effective treatment for MRSA, some of the existing established drug had been extensively researched. Simvastatin that used predominantly for cardiovascular disease has been anticipated to exhibit antimicrobial activity against skin bacterial disease. It is found to exhibit wide range of antibacterial activity including a few MRSA stains, anti-staphylococcal biofilm potential, anti-inflammatory and wound healing activities (Thangamani *et al.*, 2015). In addition, simvastatin was also found to have synergistic effect with antimicrobials. Apart from simvastatin, tamoxifen, is also broadly used for the treatment of breast cancer. Tamoxifen is a selective estrogen receptor modulator that has been relocated to boost MRSA clearance by enhancing the neutrophil bactericidal capacity (Thangamani *et al.*, 2015).

2.2.2 Ethnomedical Remedies against MRSA

Antibiotics are progressively being defeated in the battle against microbes globally. A widespread adversity can be observed in this ever evolving world. This dire need compel people to reconsider the "old-fashioned" but still in practise, ethnomedicine, which originates from rational, constructive, empirical and "almost forgotten" knowledge about natural products. The resources are mainly based on herbs in which many active compounds have been isolated. Nevertheless, many remedies and treatments are yet to be verified, and therefore more proofs are needed to substantiate their usage in modern treatments. Unfortunately, there is a passive transition of ethnomedicine to evidence-based medicine due to the non-fitting capability of natural product-centered paradigms in the ethnomedical research scope. The complexity form of the active compounds in these ethnomedicinal materials due to pleiotropic effects produces a wide but unclear spectrum of their ethnomedical indications. In addition to this, the prescribed recipes may have different formulas to enhance their healing effectiveness known as Bianzheng lunzhi in Chinese folk medicine, which directly means "pattern differentiation and treatment determination" (Temrangsee *et al.*, 2011). The general populace in the developing world, continue to use healing agents that are naturally derived in context to historical context and theory of ethnomedicine procedures.

Ethnomedical drugs may fight against drug-resistant strains through their anti-biofilm activities. This is because, formation of biofilms confers antibiotic resistance to bacteria. For instance, one medicinal plant that has displayed inhibition towards MRSA is the plant *Duabanga grandiflora.* This plant is capable to inhibit the formation of biofilm in MRSA by reducing the cell surface attachment and the

attenuation of the level of PBP2a (Santiago *et al.*, 2015). Encoded by mec A, PBP2a encourages the emergence of MRSA, due to it being a protein that presents β-lactam antibiotic resistance to *S. aureus* (Kreiswirth *et al.*, 1993). In addition to that, naturally derived substances may encompass some inhibitors of multidrug efflux pumps (Bharate *et al.*, 2015), which in turn are treated to detoxify antibiotics by multi- and pan-resistant *S. aureus* (Costa *et al*., 2013). Besides that, naturally derived substances are also used to reverse the effect of microbial antibiotic resistance and by doing so helps to limit the over usage of antibiotics. Nevertheless, whether these naturally derived materials have a low resistance capability by themselves remains vague.

2.3 *Mesua ferrea* Linn.

2.3.1 Botanic Description

Mesua ferrea (Figure 2.1) is an average-sized tree with a height up to 36 m and a trunk that is 95 cm in diameter. The trunk is also usually cylindrical to poorly-shaped. The bark surface is unwrinkled to sometimes scaly. It is has a brown colour with a bright orange layer below. The shiny leaves on the other hand are simple and opposite, normally elliptical and glabrous with plenty of secondary veins, curving parallel of the border. The flowers are axillary, solitary and bisexual. It has 4 sepals that are decussate, persistent and suborbicular. It has an up to 9-flowered open panicle and pedicel that has small paired bracts. Besides that, the flower is either pink or white with 4 petals, as shown in Figure 2.2. It has plenty of stamens, either free or united but only at the base. The fruit is normally globose and appears like a capsule, beaked, commonly seen as thin and woody, usually cleaving with 2(-4) valves before falling, that normally exudes resinous droplets. One fruit may have one

to four seeds (Dassanayake, 1980). The generic name for the plant is given after J. Mesue (777-857) and the specific descriptive name is originated from Latin which also means ‘belonging to iron’, notable to its famous hard, durable timber. It is generally known in Malaysia as Penaga Lilin.

Figure 2.1: *M. ferrea* tree in Universiti Sains Malaysia.

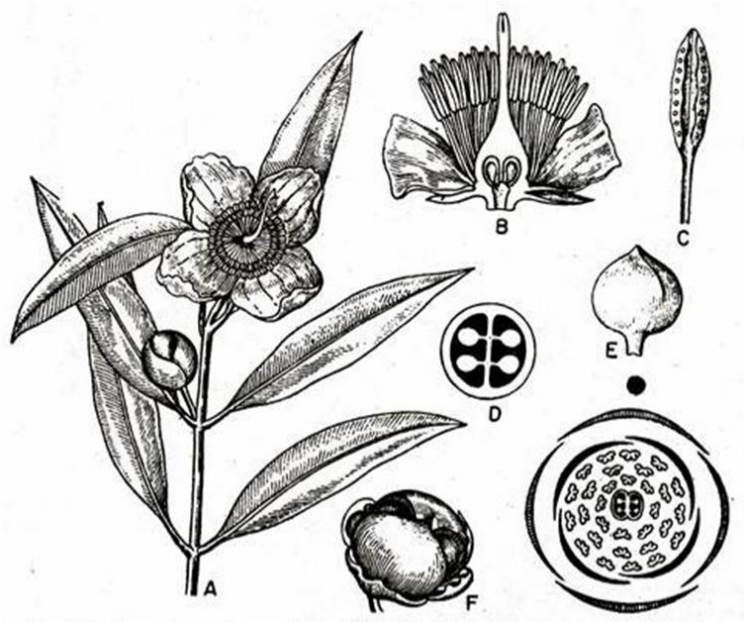


Figure 2.2: *Mesua ferrea* Linn. – A. Small branch with a flower (x1); B. L.s through a flower showing floral parts (x2); C. Part of a filament with anther; D. T.s through ovary; E. A small fruit; F. Bursting fruit (x1/2)
(Source:http://www.biologydiscussion.com/angiosperm/dicotyledonae/classification-of-parietales-11-families-dicotyledonae/42464)

2.3.2 Biology and Ecology

M. ferrea blossoms during the dry season and flushes of new leaves that are usually formed right after the flowering at the start of the rainy season. The flowers of *M. ferrea* blooms for one day, where it opens at 3 or 4 a.m. and closes around sunset. *M. ferrea* is usually associated with dipterocarps in Borneo. In mountainous evergreen forest, it commonly observed as an understorey tree whereas in the lowland forest, it is usually seen as a canopy component. *M. ferrea* needs a rather rich, well-drained soil.

2.3.3 Distribution

The tree is commonly found at a height up to 1500 m elevation in tropical evergreen forests throughout Southeast Asia (Dassanayake, 1980). It is largely distributed in countries such as Myanmar, Malaysia, Thailand, India, New Guinea and Sri Lanka (Kritikar and Basu, 1981). In the tropical India, the distribution of this plant is mainly observed in the montane range of Andaman, Assam, Eastern Himalaya and East Bengal (Anonymous, 2004) where it is also locally known with a different name. *M. ferrea* is called in English as Cobra's saffron, in Hindi as Nagakeshara, in Tamil as Nagachampakam, in Assam as Nageshwar and in Kannada as Nagasampige.

2.3.4 Uses

M. ferrea is acknowledged for improving human thermal relief by its shade production and radiation modification. The seed oil has also been proven to be a

good alternative for petroleum gasoline, where a distillation of the fraction between 200 and 300°C can produce fuel for diesel engines (Konwer *et al*., 1984; Kallappa *et al*., 2003). Besides that, the seed oil can also produce polymers that are used to prepare resins (Dutta *et al*., 2005; Mahapatra *et al*., 2004; Das *et al.,* 2010).

Konwarh *et al*. (2010) has reported that the aqueous leaf extract was used in the preparation of silver Nano particles whereas Sahni (1998) investigated that the seeds are brunt like candles, the stamens and flowers are normally used to stuff pillows for the bridles bed, while the wood is commonly used for the heads of golf clubs.

2.3.5 Ethno pharmacological relevance of *Mesua ferrea* Linn

Numerous parts of the *M. ferrea* plant have tremendous values in the Indian folk medicine for the remedy of a variety of ailments. Parts of the trees such as the flowers and leaves are used to cure scorpion strings and snake bites. Besides being used as astringents, the barks are also commonly used as a sudorific when in combination with ginger. The flowers as well are used as astringents besides being stomachic and expectorant, whereas the unripe fruits are sudofiric. The seed oil is externally used to cure cutaneous affections while the flower buds are commonly used in dysentery (Satyavati *et al*., 1987). Apart from this, several varying aerial parts of *M. ferrea* are traditionally used in the folk medicines to prepare unguents and cosmetics. The kernels of the seeds are pounded and externally applied to poultice cuts and all designs of skin inflammations.

Rai *et al*. (2000) observed that the plant is capable to treat septic and inflammation conditions whereas Parukutty *et al*. (1984) reports that the tribal of Assam uses this plant widely for its medicinal properties such as purgative,

antiseptic, worm controller, blood purifier and tonic properties. Apart from that, *M. ferrea* is also used to cure cold, asthma and fever in Thai folk medicine. (Foundation of Resuscitate and Encourage Thai Traditional Medicine, 2005). The ashes of *M. ferrea* leaves are used to treat sore eyes. (Kumar *et al*., 2006). *M. ferrea* is also an important component in many formulations of Ayurvedic practice that are used to treat various ailments (Roshy *et al*., 2010) such as in various "churnas" (Sharangadhara, 2000), mahakaleshwara rasa (Das *et al*., 2001) and dasamoolarishta (Nishteshwar *et al*., 2008). Not only that, there is also another formulation of Ayurvedic practice that incorporates *M. ferrea* where the recipe displayed haemostatic and astringent characteristics and therefore is notably helpful in the bleeding of uterus (Joy *et al*., 1992). In Unani folk medicine, it is a component of a wide amount of formulations such as, "Halwa-i-supari pack" a general tonic, "Hab Pachaluna", an appetiser and "Jawarish Shehryaran" a stomach and liver herbal medicine (Joy *et al*., 1992).

2.3.6 Pharmacological Activities

2.3.6 (a) Disinfection studies

M. ferrea seed kernel oil was investigated by Adewale *et al.* (2011) to have an impressive disinfection capability and the studies of disinfection kinetics pointed out that the seed kernel oil fits the first-order model with a k value of -0.040.

2.3.6 (b) Antioxidant and hepatoprotective activity

The flowers of *M. ferrea* were dried and the methanolic extract of the flowers (100 and 200 mg/kg) was investigated in female Wistar rats for hepatoprotective and in vivo antioxidant activity. The study showed a notable increase in liver for super

oxide dismutase (SOD) and aspartate aminotransferase (AST) in treated groups. Apart from this, there was a sharp decline in catalase (CAT), glutathione peroxidise (GPX), glutathione reductase (GR) and alanine aminotransferase (ALT) activity and there was no substantial difference that was noticed in Creatinine and Creatinine phosphorkinase (CPK) activity (Garg *et al.*, 2009).

Makchuchit *et al.* (2010) investigated the ethanolic extract of *M. ferrea* flowers and observed an activity of strong inhibition (96.03%) against nitric oxide (NO) assay at 100 μg/ml whereas Yadav (2010) reported that the ethanol-water (1:1) leaf extract of *M. ferrea* exhibited strong inhibition on peroxidation of lipid. The natural food supplement Maharishi AK-4 (Cullen *et al.,* 1997) and Brahma rasayana (Ramnath *et al.,* 2009) (refer to Appendix Table 7.1) which is formulated in Ayurveda consisting *M. ferrea* as a potent ingredient have been recorded to display notable antioxidant activity in isolated rat and cold stressed chicken heart respectively.

2.3.6 (c) Analgesic activity

Hassan *et al.* (2006) studied the analgesic activity of the *M. ferrea* leaves extracts from methanol, ethyl acetate and n-Hexane (125 and 250 mg/kg). Each extract displayed a potent analgesic activity in acetic acid induced writhing response in mouse. The declination in writhing feedback for for higher dosage of above extracts was 17.06, 19.63, and 42.21%, respectively whereas the lower dosage was 10.21, 16.33 and 36.08%, respectively.

2.3.6 (d) Antispasmodic activity

The antispasmodic activity of *M. ferrea* seed oil petroleum extract was assessed in vitro by Prasad *et al*. (1999) on isolated rat ileum. Kymograph was used to evaluate the contraction of rat ileum. Carbachol and Acetycholine caused a contraction of 3.20 and 2.61 cm, respectively. A reduction of 55% was observed for the response of acetylcholine in the presence of atropine.

2.3.6 (e) Anti-venom activity

The aqueous extract of this plant's leaves was investigated for its anti-venom activity against the viability of fibroblast cells. After treating the cells with 0.706 mg/ml *M. ferrea* extracts that was pre-incubated with *Heterometrus laoticus* scorpion venom, the study showed that *M. ferrea* leaf's aqueous extracts was efficient in protecting the fibroblast cells against the scorpion venom induced lysis. (Uawonggul *et al*., 2006).

2.3.6 (f) Anti-ulcer activity

Using pyloric ligation procedure in albino rats, the antiulcer activity for the xanthones from *M. ferrea* were evaluated. The animals that were pre-treated with xanthones displayed only dispersed sites of hyperemia and sporadic haemorrhagic spots while the control animals observed sustained extensive ulceration, perforation and haemorrhage. A distinctive lower value (3.50 ± 0.27) of ulcer scoring for the gum acacia treated rats was noticed comparative to that of standards. (Gopalakrishnan *et al*., 1980).

2.3.6 (g) Anti-microbial activity

Mazumder *et al*. (2004) studied the methanolic extract of *M. ferrea* flowers in vivo and in vitro experiments. A declination of mice mortality was observed when

the methanolic extract of protected mice was stimulated with *S. typhimurium* ATCC 6539.2 in the in vivo experiment. Apart from this, an obvious depletion of the viable bacteria of blood, spleen and liver was noticeable in the extract-treated mice. In the in-vitro experiment, the methanolic extract was also capable to inhibit at 50 μg/mL of concentration, all the approved strains of *Pseudomonas* spp., *Salmonella* spp., *Bacilllus* spp., *Lactobacillus arabinosus Proteus mirabilis, Sarcina lutea, Streptococcus pneumonia* and the 30 strains of *Staphylococcus aureus* in the in vitro experiment.

Complete inhibition at 500 and 1000 μg/ml were shown against all tested bacteria by dichloromethane and methanol (1:1 v/v) extracts of *M. ferrea* flowers. The study was carried out by using the agar dilution-streak method against 14 bacterial strains (Prashanth *et al.*, 2006).

Ali *et al.* (2004) reported that the *M. ferrea* leaves extracts of light petroleum ether, chloroform and ethanol were found to have mediocore activity against many of the tested bacteriums. The chloroform extract of *M. ferrea* stem bark was investigated and it showed significant strong activity against Gram-negative *E. coli* (19 mm) and Gram-positive *S. aureus* (16 mm) using disk diffusion method. All these *M. ferrea* extracts did not show much antifungal activity against the tested fungal strains.

In another report by Parekh *et al.* (2007) where the alcoholic and aqueous extracts of *M. ferrea* seeds were evaluated for antibacterial activity using the agar disc diffusion and agar well diffusion techniques, the ethanolic or methanolic extracts had a better antibacterial activity compared to aqueous extracts. From this study, it is also observed that the extracts had the strongest antibacterial activity against *K. pneumoniae* (20 mm) and *P. mirabilis* (23 mm).

The flowers of *M. ferrea* also showed interesting activity against Gram positive Staphylococcus, Enterococcus, Gram negative bacteria, and a strain of *S. durans*, and fungi *P. falciparum*. 4-Alkyl and 4-phenyl coumarins that were isolated from this part of the plant displayed a high minimum inhibitory concentration (MIC) of 2 to 4 μg/ml against all strains except for *P. falciparum* where a weak response was noticed (Verotta *et al*., 2004).

Methanolic and ethanolic extracts of *M. ferrea* leaves were investigated by Adewale *et al*. (2012) for their antibacterial activity by evaluating the minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) of these extracts. Both methods showed an interesting result for these extracts where the MIC range and MBC value for the Gram-negative bacteria was reported to be 2.5 to 0.625 mg/ml and 5 mg/ml, respectively whereas for the Gram-positive bacteria the result obtained showed that the MIC range was 1.3 to 0.313 mg/ml while the MBC value was 2.5 mg/ml.

2.3.7 Phyto-constituents

Interestingly, *M. ferrea* has been the only species from the genus *Mesua* to be widely investigated chemically (Rao *et al*., 1981) and there are many reports on the phytochemical research on this plant which has resulted on varying classes of secondary metabolites isolated from *M. ferrea*. These secondary metabolites comprising of xanthones, triterpenoids and phenylcoumarins (Raju *et al*., 1976). From the seed oil of *M. ferrea*, 4-Phenylcoumarins such as mesuol **(1)**, mesuagin **(2)**, mammeisin **(3)**, mammeigin **(4)** and mesuone were isolated. Apart from this, an oil called Nahor was also separated from the kernel seeds. (Joy *et al.*, 1998).

Compounds such as 4-alkylcoumarins ferruols A and B, mesuaxanthones A **(5)** and B **(6)** and 1, 7-dihydroxyxanthone were isolated out of the bark of the *M. ferrea* trunk whereas β-sitosterol, biflavonoids- mesuaferrones A **(7)** and B **(8)**, α and β-amyrin, mesuaferrol, and mesuanic acid were isolated from the stamens of the plant which is responsible for yielding the drug Nagakeshara according to Ayurvedic texts. (Handa *et al*., 1992).Apart from this, other compounds that were isolated include euxanthone and leuco anthocyanidin (Sharma *et al*., 2002).

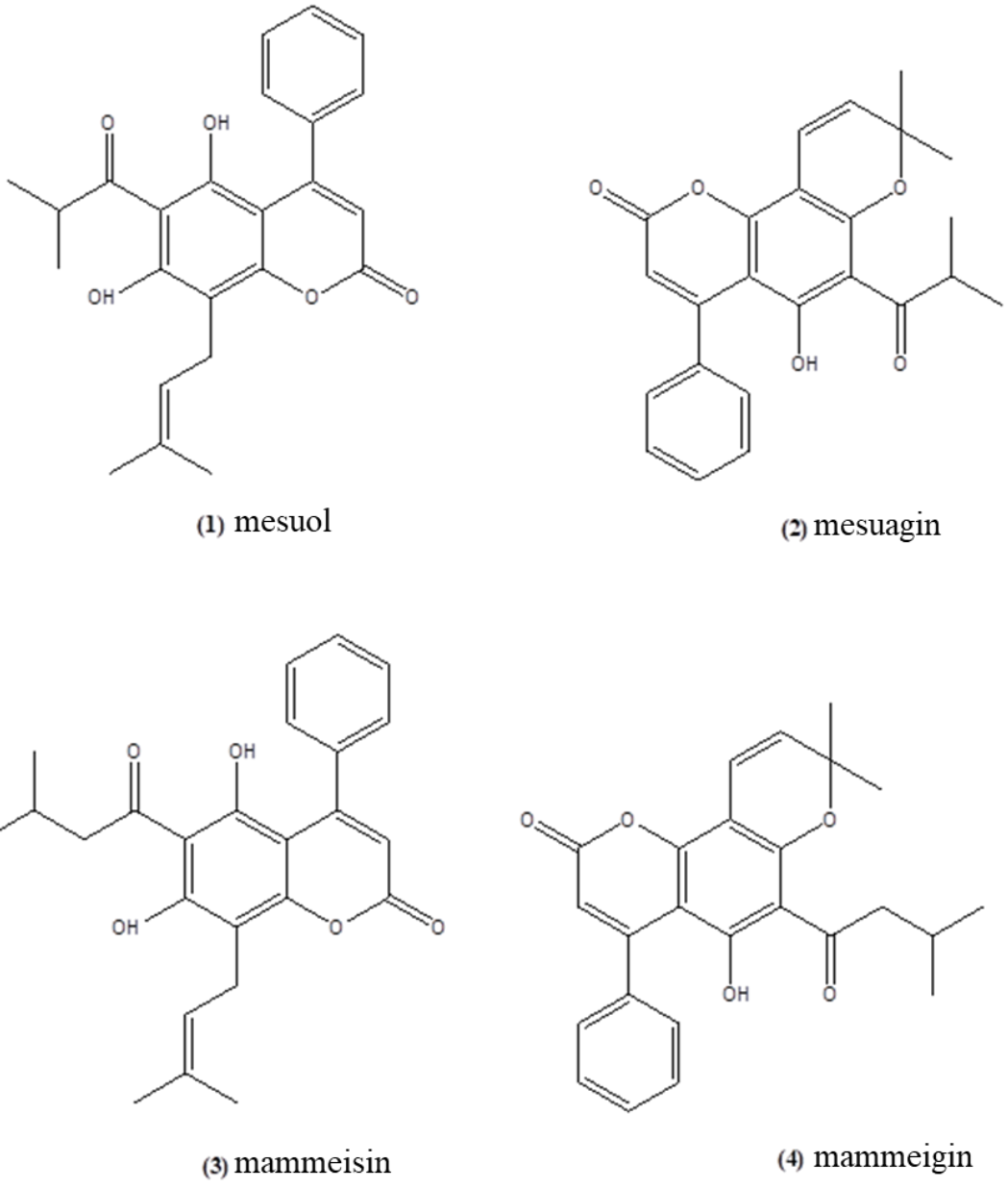
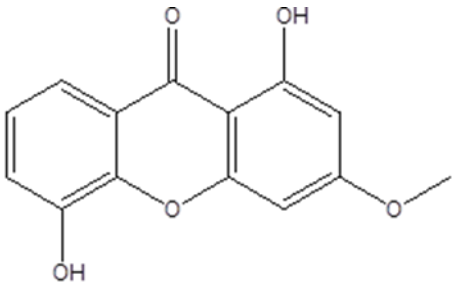
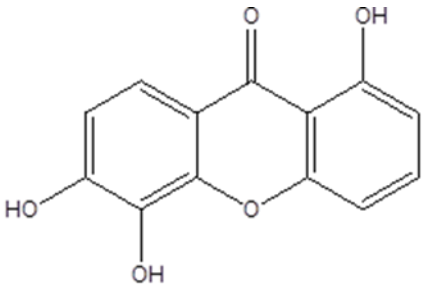


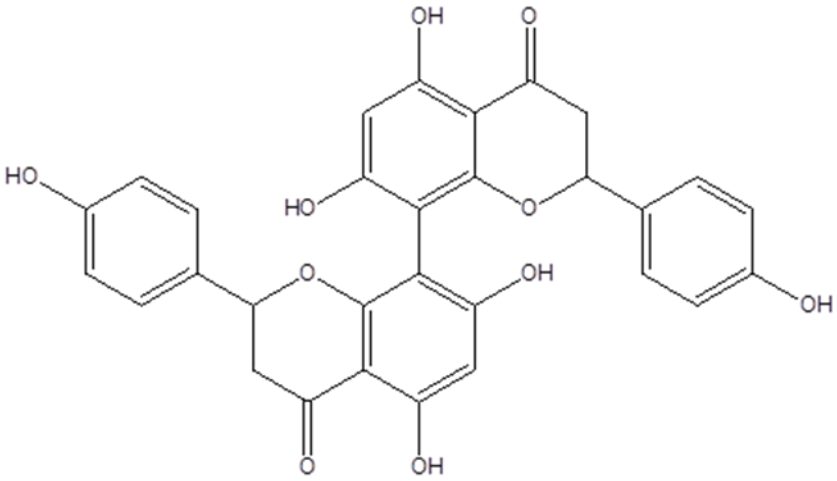
Figure 2.3: Chemical structures of compounds isolated from *M. ferrea.*



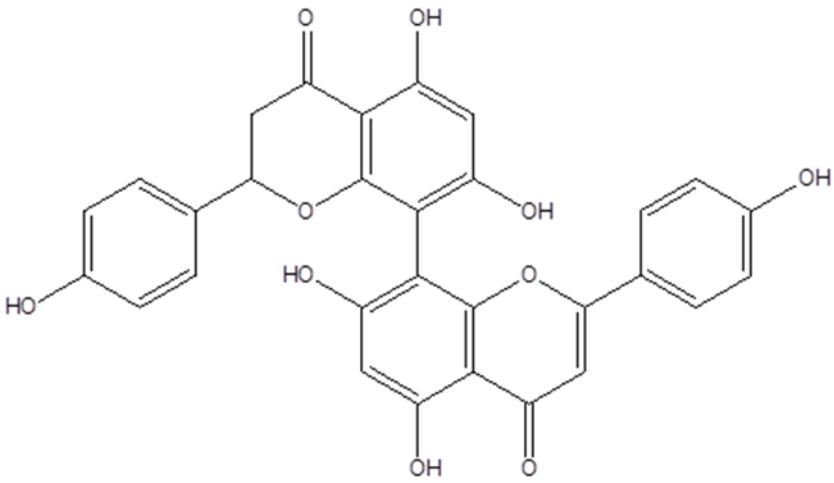
(5) mesuaxanthones A



(6) mesuaxanthones B



(7) mesuaferrones A



(8) mesuaferrones B

2.4 Herbal mixtures versus isolated compounds

For centuries products that are naturally derived from plants have played a dominant role in the field of medicine. The general populace has been dependant on herbal and folk medicines to cure diseases. However, in 1897 the discovery of aspirin, a drug synthetically obtained from salicylic acid, has changed the medical trend to mono-drug treatment. This approach of employing a singular synthetic drug or naturally derived single compound, to cure ailments shifted the paradigm in drug discovery (Williamson, 2001). This mono-drug therapeutic approach was further strengthened by the creation of high throughput screening (HTS) and structure activity-guided organic synthesis. Some of the many benefits of utilizing this approach is that the study on pharmacodynamics and pharmacokinetics properties, its side effects and mechanism of actions are made easier to comprehend. Some of the various early examples of these mono-drugs such as pilocarpine, penicillin, morphine and quinine were isolated mainly from natural products. For instance, morphine was derived from opium that was isolated from the poppy plant (*Papaver somniferum*) (Katiyar *et al*., 2012; Harvey, 2008).

On the contrary, herbal preparations contains a mixture of different compounds. The mixture of ingredients for these preparations is either found in a single plant or a number of different plants. In most of the folk medicines practised, herbal treatment has become a daily basis of curing diseases. Comparative to mono-drug treatment, herbal preparations are better because the multicomponent preparation is able to increase the medicinal properties of the treatment, such as increasing the bioavailability of the pharmacological activities of each component and reducing the toxicity of the preparations in whole (Schmidt *et al.,* 2008). According to Cravotto *et al.* (2010) the effect of combining a few compounds

together or the potency of one whole plant itself has high healing activity. In most cases, the therapeutic effect is reduced with repeated fractionation and isolation as loss of key components that is active against other pharmacological activity (Raskin & Ripoll, 2004). This “synergism” between different components in a preparation is the main cause for the boost in the healing potential of herbal mixtures. With a sharp increase of pathogenic resistance and the variety of response towards a single treatment has made the large populace to revert back to herbal concoctions. It is also noteworthy to mention that many researchers are also looking back to this “synergistic” approach to enhance the pharmacological activity of a treatment. The usage of different isolated compounds or drugs in a combination to target different ailments in a whole are in fact the apparent benefit of herbal medicines.

2.5 Separation techniques

According to the chemical nature of the active compounds, there are several techniques to separate out active compounds from the plant extract. The technique of having the mobile phase and the stationary phase selectively collecting the compounds is the primary method used to isolate and separate the compounds.

Since many decades ago, thin layer chromatography (TLC) has become the most commonly used separation technique as it is famed for its quick separation techniques and cost effectivity. TLC is a crucial step to determine the perfect combinations of solvents that is needed to separate out the compounds from the extract or fraction for further isolation and purification. The stationary phase (adsorbents) for TLC such as silica or reverse phase silica is coated on a glass or aluminum plates. The original size that these TLC plates are commonly distributed are of 20 cm × 20 cm size but these plates are normally cut into smaller sizes to fit

the choice of users. In order to prepare for the mobile phase for the TLC technique, two or more solvents with different polarity are mixed to give a variety of eluting strength to different mobile phases. A spot of extract is usually developed at the origin limit of a TLC plate and then placed in a glass jar that has an atmosphere which is saturated with the eluting solvent. As different compounds have different polarity, they are attracted towards the different polarity of the eluting solvents. Separation of the extract on TLC happens along with the movement of the eluting solvent upwards that is caused by the capillary action. As a result, at the end of the elution of the mobile phase, a well separated image of the extract is observed on the TLC either with naked eye or by viewing the plate under ultraviolet (UV) illumination. Further chemical nature of the compounds can be attained by spraying the TLC plate with appropriate reagents (Gilbert & Martin, 1998).

After many trial and errors, once the right separation has been obtained on a TLC plate, the same mobile phase is prepared for Preparative thin layer chromatography (Prep-TLC). Prep-TLC is a technique that is used at a larger scale but normally this technique is only preferred as the final step of isolation or purification. Like TLC, the separation on Prep-TLC is identified by UV rays or reaction with a specific reagent. Once the bands of different compounds are confirmed, the adsorbent of the specific bands are scraped out and later desorbed by a solvent that is able to dissolve the compound needed.

Apart from this, another separation technique that has been proven to benefit the research in natural product vastly is the column chromatography. Column chromatography can be divided into two types, flash column and open column. Flash column functions by using external air pressure to elute compounds whereas open column performs by engaging gravitational force for elution of compounds.

Recently, flash column chromatography has been preferred to open column because of its capability to elute compounds in a shorter span of period. Not only that, the efficiency of flash column to give a better separation has made it replace the cost effective open column. Selection of certain parameters in column chromatography is important in order to isolate out compounds from an extract or fraction. Options such as diameter and length of the column, the size of sample load and the composition of mobile phase is crucial and is usually determined by analyzing the complex nature of the sample and its interaction with stationary phase (Bohen *et al.*, 1973).

Another alternative to open and flash chromatography is Dry Column Vacuum chromatography (DCVC). DCVC functions with lesser amount of silica and solvent compared to the other columns using techniques of gradient fractionation where after each step of solvent elution, the sorbent bed is dried out using a vacuum pump before the next solvent system is added. It is common to use this technique in separating crude mixtures that are complex into simpler fractions (Pedersen & Rosenbohm, 2001).

Besides these chromatographic methods, other chromatographic techniques such as supercritical fluid chromatography, counter current chromatography and high performance liquid chromatography (HPLC) is also being commonly used to separate mixtures these days. Recently, these techniques have become very popular among researchers as they provide automated control over the separation of compounds besides being specific to its separation (Coskun *et al.*, 2016).