ANTIMICROBIAL ACTIVITIES OF THE CRUDE EXTRACTS AND ELECTROSPUN NANOFIBERS CONTAINING *RHODOMYRTUS TOMENTOSA* (KEMUNTING) ROOTS

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Thesis submitted in fulfillment of the requirements
for the award of the degree of
Master of Science

Faculty of Industrial Sciences & Technology
UNIVERSITI MALAYSIA PAHANG

MAY 2018

ACKNOWLEDGEMENTS

I would like to praise God for the opportunities granted and blessings bestowed upon me in achieving my goals. First and foremost, my appreciation goes to my supervisor, the pillar and backbone of my research work, Dr. Hazrulrizawati Abd Hamid, Head, department of industrial chemistry. Her guidance, support, patience, friendship and credible motivation instilled huge confidence to continue my search from the stage of identifying problem to the accomplishment of goals. I would like to thank you once again for helping me to understand and appreciate research as an enjoyable duty for which scientists are called. I also thank my co supervisor, Dr. Farah Hanani Zulkifli, whose valuable input was most appreciated.

I would like to thank Faculty of Industrial Sciences & Technology, Universiti Malaysia Pahang, for offering me all the facilities that I have used to complete the research. I wish to thank all staff and fellow students of the Faculty specially to Mr. Muhamad Husaini Sulaiman, person in-charge of the microbiology lab, for his unconditional support offered for the project.

My parents deserve special mention for their devoted support and prayers. I would like to express my heartfelt thanks and great debts of gratitude to my father in law, mother in law, brothers in law, brothers, sister and friends for being supportive and caring. My gratitude and respect to my husband, Dr. Ftwi Yohannes Hagos whose dedication, love and persistent confidence in me, has taken the load off my shoulder. He helped me in different ways that I shared my joys & sorrows with him. My special thanks to my two beautiful souls, Eyoas Ftwi and Elelta Ftwi for their huge support and tolerance in allowing me to proceed my duty till the day of my accomplishment.

My appreciation goes to everyone who contributed in the successful completion of this project from every possible aspect.

ABSTRAK

Tumbuhan perubatan tradisional boleh digunakan untuk merawat penyakit kronik dan berjangkit. Akibatnya, amalan menggabungkan ekstrak tumbuhan perubatan ke dalam sebatian polimer untuk menghasilkan bioaktif electrospun nanofibers menjadi semakin meningkat. Dalam kajian ini akar Rhodomyrtus tomentosa telah diekstrak dengan menggunakan heksana, kloroform, etil asetat dan metanol. Analisis fitokimia awal telah dilakukan dengan menggunakan kaedah ultra-high-performance liquid chromatography-quadrupole time-of-flight/mass spectrometry (UPLC QToF/MS) dan aktiviti antimikrob telah dinilai terhadap mikroorganisma patogen manusia biasa, dua strain bakteria gram negative (Escherichia coli, Pseudomonas aeruginosa), dua strain bakteria gram positif (Bacillus subtilis, Enterococcus faecalis) dan satu strain kulat (Aspergillus niger) dengan menggunakan kaedah penyebaran cakera dan zon perencatan ditentukan. Ekstrak yang paling aktif, ethyl acetate extract (EAE), telah digabungkan dengan sebatian 10% Polyvinyl alcohol (PVA) (W/V) pada kepekatan yang berlainan (0.25, 0.5, 1.5 dan 2.5% W/V) dan nanofiber ethyl acetate extract of Rhodomyrtus tomentosa roots incorporated in 10% PVA (RTE/PVA) telah direka dengan menggunakan proses elektrospinning. Aktiviti antimikrobial elektrospun nanofibers telah di nilai menggunakan prosedur yang sama diikuti dengan analisis untuk semua ekstrak. Berdasarkan analisis UPLC QToF / MS, ekstrak akar *Rhodomyrtus tomentosa* telah disahkan mengandungi flavonoid, tanin, xanthenes, phenol, glikosida, feromon, kromon, triterpenes dan pyranes. Ekstrak etil asetat telah didapati mempunyai aktiviti antibakteria yang baik dengan zon perencatan antara 9.33 ± 0.21 mm hingga 13.67 ± 0.32 mm. Ujian aktiviti antimikrob ekstrak etil acetae yang dimuatkan PVA nanofiber telah diperiksa dan menunjukkan aktiviti antibakteria yang baik. Kesemua empat pelarut yang diekstrak dari tumbuhan dan ekstrak yang dimuatkan oleh serat nano PVA telah menunjukkan tidak mempunyai sebarang aktiviti antikulat. *Rhodomyrtone*, yang dikenali sebagai antibiotik semulajadi, dipercayai telah menyumbang kepada aktiviti antibiotik terhadap strain bakteria di atas. Morfologi yang dimuatkan ekstrak nanofibers telah dicirikan menggunakanspektrum FESEM, FTIR dan XRD di mana hasilnya menunjukkan kesemua nisbah berat ekstrak yang dimuatkan dengan sebatian PVA dan 10% sebatian PVA telah menghasilkan nanofiber dengan garis pusat dalam lingkungan 76.3 nm dan 388.6 nm masing-masing dan ekstrak itu bergabung dengan baik bersama gentian.

ABSTRACT

Traditional medicinal plants can be used to treat chronic and infectious diseases. As a result, the practice of incorporating medicinal plant extracts into polymeric solutions to produce bioactive electrospun nanofibers is rising progressively. In this study Rhodomyrtus tomentosa roots were extracted with hexane, chloroform, ethyl acetate and methanol. Preliminary phytochemical analysis was done by using ultra-high-performance liquid chromatography-quadrupole time-of-flight/mass spectrometry (UPLC QToF/MS) method and antimicrobial activity was evaluated against common human pathogenic microorganisms, two Gram-negative bacterial strains (Escherichia coli, Pseudomonas aeruginosa), two Gram-positive bacterial strains (Bacillus subtilis, Enterococcus faecalis.) and one fungal strain (Aspergillus niger) by using disk diffusion method and zone of inhibition was determined. The extract with better activity, ethyl acetate extract (EAE), was incorporated with 10% Polyvinyl alcohol (PVA) (W/V) solution at different concentrations (0.25, 0.5, 1.5 and 2.5% W/V) and Ethyl acetate extract of Rhodomyrtus tomentosa roots incorporated in 10% PVA (RTE/PVA) nanofiber were fabricated using electrospinning process. Morphology of the extract loaded nanofibers were characterized using FESEM, FTIR and XRD spectrums. Antimicrobial activity of the electrospun nanofibers was evaluated using same procedures followed during the analysis of all extracts. Based on the UPLC QToF/MS analysis, it is confirmed that the root extract of *Rhodomyrtus tomentosa* contains flavonoids, tannins, xanthenes, phenols, glycosides, pheromones, chromones, triterpenes and pyranes. Ethyl acetate extract has been found to have good antibacterial activity with zone of inhibition ranging from 9.33 ± 0.21 mm to 13.67 ± 0.32 mm. Antimicrobial activity assay of ethyl acetate extract loaded PVA nanofiber was examined and showed good antibacterial activity. All the four solvent extracts of the plant material and the extract loaded PVA nano-fiber have shown not to have any antifungal activity. Rhodomyrtone, known as a natural antibiotic, must contribute to the antibiotic activity against the above bacterial strains. Characterization of nanofibers indicates all weight ratios of the extract loaded PVA slution and the 10% PVA solution has made a nanofiber with a diameter in a range of 76.3 nm and 388.6 nm, respectively and the extracts were well incorporated with in the fibers.

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α	Alpha radiation
Å	Ångström
d	Spacings of the atomic planes
θ	Diffracted beam angle
λ	Incident X – rays

LIST OF ABBREVIATIONS

CC	Column Chromatography
CCS	Collision cross- section
CE	Chloroform extract of Root of *R. tomentosa*
C/H	Carbon/Hydrogen
$CHCl_3$	Chloroform
Da	Unified atomic mass unit, Dalton
DNA	Deoxyribonucleic acid
EAE	Ethyl acetate extract of Root of *R. tomentosa*
ECM	Extra Cellular Matrix
EDX	Energy dispersive X-ray
EFSA	European Food Safety Authority
ESI	Electrospray Ionisation
EtOAc	Ethyl acetate
FDA	US Food and Drug Administration
FESEM	Field emission scanning electron microscopy
FTIR	Fourier-transform infrared spectroscopy
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Gas Chromatography
HE	Hexane extract of Root of *R. tomentosa*
HMBC	Hetero Nuclear Multiple - Correlation
HMQC	Hetero Nuclear Multiple – Quantum Correlation
HPLC	High Performance Liquid Chromatography
HREIMS	High-Resolution Electron Ionization Mass Spectrometry
IR	Infrared
kV	kilo Volt
LC	Liquid Chromatography
MBC	Minimum Bactericidal Concentration
mDa	Milli Dalton
ME	Methanol extract of Root of *R. tomentosa*
MeOH	Methanol
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-Resistant *S. aureus*
MS	Mass Spectrometry
m/z	Mass-to-Charge ratio
NC	Negative Control
NMR	Nuclear Magnetic Resonance
PC	Positive Control
PCEC	Poly(ε-caprolactone) -oly(ethylene glycol)-poly(ε-caprolactone)
PCL	Polycaprolactone
PEO	Polyethylene oxide
PGA	Poly(DL-lactide-co-glycolic acid)
PLGA	Polyglycolic acid
Ppm	Parts per million

PVA	Polyvinyl alcohol
PVP	Polyvinylpyrrolidone
QTOF	Quadrupole time-of-flight
RNA	Ribonucleic acid
RT	Retention time
RTE	Ethyl acetate extract of *Rhodomyrtus tomentosa* roots
RTE/PVA	Ethyl acetate extract of *Rhodomyrtus tomentosa* roots incorporated in 10% PVA solution
SE	Secondary Electron
SFP	Silk Fibroin Protein
TLC	Thin Layer Chromatography
TOF	Time Of Flight
XRD	X-ray Diffraction
UMP	Universiti Malaysia Pahang
UPLC	Ultra-high-Performance Liquid Chromatography
UV	Ultra-Violate
2D	2 Dimensional

CHAPTER 1

INTRODUCTION

1.1 Background

Exploring the nature especially plants in search of medications has brought the use of substantial number of medicinal plants with curative properties for treatment of different diseases. In recent years, nearly 80% of the world's population relies on traditional medicines for primary health care while most of it involves the utilization of plant extracts Tadesse *et al.* (2012). As a result of the unmatched availability of chemical diversity, standardized plant extracts or the pure compounds isolated from the extracts are offering broad opportunity of findings Cos *et al.* (2006). Bioactive compounds which play a vital role in treating illnesses in plants are described as the secondary plant metabolites eliciting pharmacological or toxicological effects in man and animals Bernhoft (2010). An extensive variety of substances are found from different traditional medicinal plants which have an ability to treat chronic and infectious diseases Sasidharan *et al.* (2011). Plants may provide alternate drugs which can replace or substitute currently available antibiotics to defeat resistant microbial pathogens Limsuwan *et al.* (2011). In addition, as the unwanted effects of modern synthetic drugs become common, a huge interest is observed towards medicinal studies of natural products.

Rhodomyrtus tomentosa (*R.tomentosa*) is found abundantly in South East Asia. The roots, leaves and fruits of the plant have been utilized in folk medicine. In Peninsular Malaysia, the fruits, roots and leaves have been used to treat dysentery, diarrhoea, stomach aches and as a tonic after childbirth. In addition, an infusion of the roots was recommended for spouting into the eyes to treat scars on the cornea. In Singapore, the Chinese have used the seeds as a tonic for digestion, and to treat snake bites whereas

Indonesians use the leaves to treat wounds Lim (2012). The extract from aerial parts of *R. tomentosa* are known to have antimicrobial activity against several species of gram-positive bacteria including *Streptococcus pneumoniae* and *Bacillus cereus* Limsuwan *et al.* (2009; Saising *et al.* (2011). In addition, ethanolic extract of leaf and endophytic fungi isolates of *R. tomentosa* have shown antifungal activity against plant and human pathogenic fungi strains respectively Jeenkeawpieam *et al.* (2012; Plodpai *et al.* (2013). The above information clarified that *R. tomentosa* would be a promising agent for preventing infections and bring effective wound healing.

The field of nanotechnology is a rapidly emerging cross-discipline program that utilizes assorted synthetic and naturally occurring materials in nanoscale dimensions Sridhar *et al.* (2015). The term "nanotechnology" refers to the "application of scientific knowledge to manipulate and control matter at the nanoscale in order to make use of size and structure dependent properties and phenomena, as distinct from those associated with individual atoms, molecules or bulk materials" ISO (2015).

The outstanding properties such as large surface area to volume ratio, flexibility in surface functionalities, and superior mechanical performance make the polymer nanofibers to be optimal candidates for many important applications Z.-M. Huang *et al.* (2003) such as carriers for cell/tissue regeneration, scaffolds in wound dressing, and drug delivery systems Sridhar *et al.* (2015). Studies revealed that, the European Food Safety Authority (EFSA) Nano Inventory indicated 276 nanomaterials are available on the market and the number increases to 633 if applications at the research and development stage are considered Belluco *et al.* (2016).

The emergence of antibiotic-resistant bacteria and advancement of tissue engineering led to introduction of novel approaches of using natural product based scaffolds that act as drug carrier and can mimic extracellular matrix to overcome the wound healing drawbacks Venugopal *et al.* (2014; Wang & Vermerris (2016). Scaffolds are highly porous solids that are used to synthesize tissue or organ *in vitro* or *in vivo.* One of the techniques that had attracted a great attention in production of scaffolds is electrospinning, a technique that can produce large amounts of nanofibers from polymeric solutions with diverse molecules encapsulated within Heunis & Dicks (2010). The topology of electrospun products can mimic the Extra Cellular Matrix (ECM) and

enhance cell migration and proliferation Ngiam *et al.* (2009). As a result, electrospun fibrous materials are found to be suitable for biomedical applications as in wound dressings, drug delivery materials and tissue engineering scaffold Hsu *et al.* (2010). This study will reveal the antimicrobial activity and phytochemicals content of *R. tomentosa* root extracts. The extract with better activity against the test microbes will be incorporated with Polyvinyl alcohol (PVA) for fabrication of nanofibers by electrospinning process. PVA, a biocompatible synthetic polymer, is known for its solubility in water, nontoxicity, biodegradability and its enhanced fibre forming ability Charernsriwilaiwat (2012). Therefore, it is well matched to be a guest polymer in blending with *R. tomentosa* extract.

To the best of our knowledge, studies on biological activities of root extract of *R. tomentosa* and application in nanotechnology are none; thus, the results from its research will have significant contribution to the scientific knowledge.

1.2 Problem statement

Recently, microbials are experiencing resistance against different anti-microbial drugs, thus great emphasis should be given to a search for new antimicrobial compounds from natural products. In Malaysia, a wide range of medicinal plants is available which, are used in folk medicine. Further investigation on searching of bioactive compounds from medicinal plants may contribute to a finding of potential antimicrobial agents. Most of the literature available are on the study of phytochemical investigation of the aerial parts of *R. tomentosa* and its antibacterial and antifungal activities but none was reported from the root part of *R. tomentosa*. Incorporation of bioactive crude extracts with polymeric nanofibers can contribute to the benefits gained from the advances of modern medicine without serious complications or increasing fatality rates. Thus, application of antibacterial scaffolds in topical treatment for wound healing can overcome drawbacks of current treatment of skin infections such as overuse of antibiotics that leads to development of antibiotic resistance due to inadequate penetration for deep skin infections and biofilm formation associated with the generation of secondary resistance that makes the treatment difficult to penetrate into the biofilm to completely eliminate the organisms Qureshi *et al.* (2015). Besides, most topical skin infection treatments must be applied twice daily. In which, bandages must be changed daily if incorporated in

wound dressings. This phenomenon may expose the wound to further infection Heunis & Dicks (2010).

1.3 Objective of the study

The aim of this study is:

1. To extract and analyse the phytochemical constituents of *R. tomentosa* roots
2. To develop and characterise *R. tomentosa* extract loaded PVA nano-fibers
3. To evaluate antimicrobial activity of *R. tomentosa* root extracts and RTE/PVA nanofibers

1.4 Scope of the study

The root of *R. tomentosa* was extracted using solvent-solvent extraction method with solvents of different polarity such as hexane, ethyl acetate, chloroform and methanol. Phytochemical analysis of different *R. tomentosa* extracts was performed by ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC/QTOF MS).

Preliminary antimicrobial assay was done using disc diffusion method against two Gram negative bacterial strains (*Escherichia coli*, *Pseudomonas aeruginosa*), two Gram positive bacterial strains (*Bacillus subtilis*, *Enterococcus faecalis*) and one human pathogenic fungal strain, *Aspergillus niger*. The most active antimicrobial extract had been chosen to be incorporated with PVA solution for further fabrication of nanofiber by using a manual electrospinning method. Characterization of the nanofiber was undergone using Field Emission Scanning Electron Microscopy (FESEM), Fourier-transform Infrared Spectroscopy (FTIR) and X-ray diffraction (XRD) techniques. The antimicrobial analysis of PVA/RTE nanofibers is conducted using disc diffusion methods against the same four bacterial strains and one fungal strain used for the preliminary assay.

CHAPTER 2

LITERATURE REVIEW

2.1 *Rhodomyrtus tomentosa*

R. tomentosa berries became a popular regional fruit as it is known for its excellent nutritional properties and delicious flavour Zhao *et al.* (2017). As well, it is one of the traditional medicinal plants which is highly used as folk medicine in the southeastern part of Asia such as Sabah, Indonesia and Brunei Ahmad & Holdsworth (1995).

R. tomentosa or locally known as Kemunting in Bahasa Melayu is a large evergreen shrub native originated from South East Asia Verheij & Coronel (1992). The small evergreen shrub 1–2 m high is found in the family *Myrtacea* and is native to tropical and subtropical Asia – Southern Asia and Southeast Asia, from India, East to Southern China, Taiwan and the Philippines, and South to Malaysia and Sulawesi Lim (2012).

In Malaysia *R. tomentosa* plants are widely found in the east coastal areas of the country especially in Terengganu and Johor where the fertile nature of the soil is favourable for the growth of this type of plants Maskam (2011).

The word Rhodomyrtus is derived from Greek word, Rhodon for red and myrtose for myrtle. In different areas *R. tomentosa* is also known as Australia Murta, Ceylon Hill Cherry, Ceylon Hill Gooseberry, Downy Myrtle, Downy Rose Myrtle, Fluffy Blueberry, Hill Guava, Isenberg Bush, Rhodomyrtus, Rose Myrtle and Tomentose Rose Myrtle Lim (2012).

Kemunting usually grows up to a length of 12 feet with three veined from the base. It has 2-3 inches long piercing leaves. The 10-15 mm long blueberry-like fruits are edible and are well known for its sugar, vitamin, and mineral contents. Each *R. tomentosa* flower has five petals in clusters of two or three, 2.5-3 cm diameter which are tinged white outside with purplish-pink or all pink Maskam (2011). Table 2.1 shows the scientific classification of *R. tomentosa* and parts of the plant that are presented in Figures 2.1, 2.2 and 2.3.

Table 2.1 Scientific classification of *R. tomentosa (Aiton) Hassk.* Rose myrtle

Rank	**Scientific name and common name**
Kingdom	*Plantae* – Plants
Subkingdom	*Tracheobionta* – Vascular plants
Superdivision	*Spermatophyta* – Seed plants
Division	*Magnoliophyta* – Flowering plants
Class	*Magnoliopsida* – Dicotyledons
Subclass	*Rosidae*
Order	*Myrtales*
Family	*Myrtaceae* – Myrtle family
Genus	*Rhodomyrtus (DC.) Rchb.* – rhodomyrtus
Species	*Rhodomyrtus tomentosa (Aiton) Hassk.* – rose myrtle

Source: serrvice (N.d.)

Figure 2.1 Flowers, buds and leaves of *R. tomentosa*

Source: Starr & Starr (2012)

Figure 2.2 Dried roots of *R. tomentosa*

Source: InnerPath (2017)

Figure 2.3 *Rhodomyrtus tomentosa,* 1. Flowering branch; 2. Fruiting branch; 3. Root; 4. Stamen

Source: Mutazah (2017)

2.2 Phytochemical constituents of *Rhodomyrtus Tomentosa*

Several studies have been reported findings of bioactive compounds from plant materials. In the past decade, researchers have made different investigations on the chemical components and biological activities of extract of *R. tomentosa.* The most commonly investigated parts of the plant are its leaf, stem and the fruit. The foregoing paragraphs are details of the bioactive compounds of the plant.

Hiranrat and co-authors have further investigated its chemical components when a negative result on the preliminary test for antibacterial activity of stem extract of *R. tomentosa* was found. Along with other six known compounds, they have isolated a new flavellagic acid derivative (1) from CH_2Cl_2 and Me_2CO extract of the stem and one new phloroglucinol (rhodomyrtosone I) (2) as indicated in Table 2.2. Other seven compounds are also isolated from the MeOH extract of the dried fruits of the plant A Hiranrat *et al.*

(2012). Based on the Ultraviolate/Infrared (UV/IR) spectrum and carbon/hydrogen (C/H) Nuclear Magnetic Resonance (NMR) data, the new flavellagic acid derivative and rhodomyrtosone I, are assigned as 3,3',4,4'-tetra-O-methylflavellagic acid and 6,8-dihydroxy-2,2,4,4-tetramethyl-7-(2-methylbutanoyl)-9-phenyl-4,9-dihydro-1H-xanthene-1,3(2H)-dione, respectively. All the compounds isolated in this study are shown in Table 2.3.

Table 2.2 Two new compounds isolated by Hiranrat.

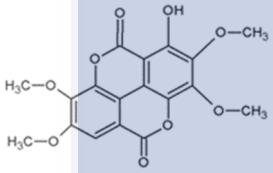
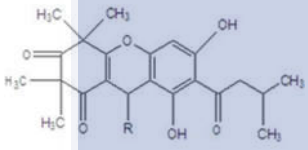
Chemical classifications	Compound	Chemical structure	Source
Pyran	3,3',4,4'-tetra-O-methylflavellagic acid	 (1)	A Hiranrat *et al.* (2012)
Phloroglucinol	rhodomyrtosone I	 R = isobutyl (2)	

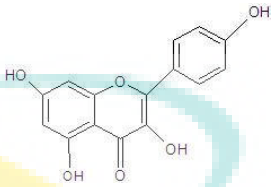
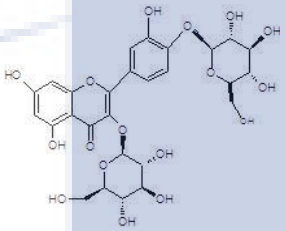
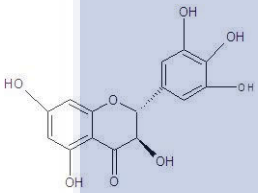
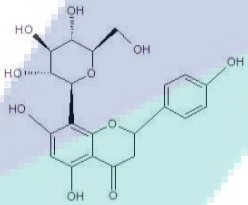
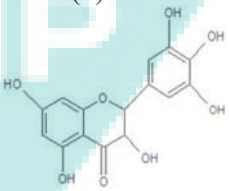
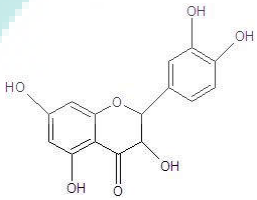
Table 2.3 Compounds isolated from the stem and fruit extract of *R. tomentosa*

Crude Extracts	CH_2Cl_2 and Me_2CO extract of the stem	MeOH extract of the dried fruits
Identified Compounds	trans-triacontyl-4-hydroxycinnamate	stigmast-4-en-3-one
	3,3',4,4'-tetra-O-methylflavellagic acid	rhodomyrtone
	3-O-(E)-coumaroyloleanolic acid	rhodomyrtosone I
	(-)-(2R,3R)-1,4-O-diferuloylsecoisolariciresinol	rhodomyrtosone D
	arjunolic acid	oleanolic acid
	4-hydroxy-3-methoxybenzoic acid	methyl gallate
	gallic acid	3-O-methylellagic acid 4-O--rhamnopyranoside

Source: A Hiranrat *et al.* (2012)

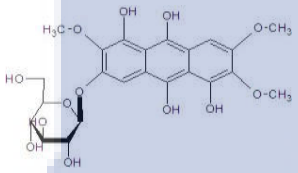
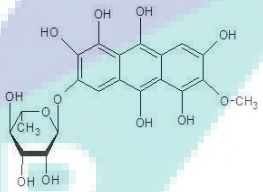
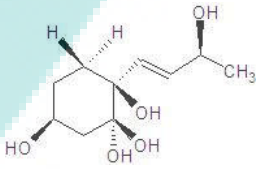
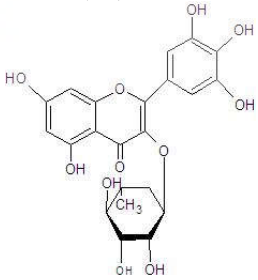
According to Wu et al. (2015), the dried berry is extracted with 95% ethanol-petroleum ether and is further purified with 40% ethanol and it is revealed that the extract from *R. tomentosa* berries is rich in flavonoids by possessing more than 20 times of total flavonoids content (62.09 rutin equivalent) than those in cranberry P. Wu *et al.* (2015). Using the ultra-performance liquid chromatography (UPLC)-time of flight (TOF)- Mass Spectrometry (MS) techniques, the same study has determined the composition of six flavonoids (Table 2.4) from the extract as, Kaempferol (3), Quercetin-7,4'-diglucoside (4), Dihydromyricetin (5), Vitexin (6), Myricetin (7), Quercetin (8) with molecular formula as ($C_{15}H_{10}O_6$), ($C_{27}H_{30}O_{17}$), ($C_{15}H_{12}O_8$), ($C_{21}H_{20}O_{10}$), ($C_{15}H_{10}O_8$), ($C_{15}H_{10}O_8$), respectively.

Table 2.4 Flavonoids identified from *R. tomentosa* extract

Chemical classifications	Compound	Chemical structure	Source
Flavonoids	kaempferol	 (3)	P. Wu *et al.* (2015)
	quercetin-7,4'-diglucoside	 (4)	
	dihydromyricetin	 (5)	
	vitexin	 (6)	
	myricetin	 (7)	
	quercetin	 (8)	

Tung *et al*. (2009) also reported the identification of Quercetin from MeOH extract of the aerial part of the plant is also reported in another study. The ^{1}H and ^{13}C NMR, Hetero Nuclear Multiple - Correlation (HMBC), Hetero Nuclear Multiple - Quantum Correlation (HMQC) and Gas Chromatography (GC) spectrum analysis of the study have revealed that another two new anthracene glycosides are isolated from the crude extract as 4,8,9,10-tetrahydroxy-2,3,7-trimethoxyanthracene-6-O-β-D-glucopyranoside (9) and 2,4,7,8,9,10-hexahydroxy-3-methoxyanthracene-6-O-α-L-rhamnopyranoside (10) Tung *et al.* (2009) as shown in Table 2.5. In addition to the above compounds, two additional compounds (*3S, 5R, 6R, 7E, 9S*)-megastigman-7-ene-3,5,6,9-tetrol) (11) and myricitrin (12) are also isolated in the study.

Table 2.5 New anthracene glycoside and other compounds identified from *R. tomentosa*

Chemical classifications	**Compound**	**Chemical structure**	**Source**
Anthracene glycoside	4,8,9,10-tetrahydroxy-2,3,7-trimethoxyanthracene-6-O-β-D-glucopyranoside	 (9)	Tung *et al.* (2009)
	2,4,7,8,9,10-hexahydroxy-3-methoxyanthracene-6-O-α-L-rhamnopyranoside	 (10)	
Monoterpenes and sesquiterpenes	(*3S, 5R, 6R, 7E, 9S*)-megastigman-7-ene-3,5,6,9-tetrol	 (11)	
Flavonoid	myricitrin	 (12)	

Phytochemical investigation on the leaf extract of *R. tomentosa* has led to an isolation of some phloroglucinols. The CH_2Cl_2, Me_2CO and MeOH extract of the leaves have resulted on isolation of four new acylphloroglucinols known as rhodomyrtosones (A-D) (13-16). Six other known compounds are also identified as rhodomyrtone, combretol, 3,3',4-tri-O-methylellagic acid (17), endoperoxide G3 (18), (6R,7E,9R)-9-hydroxy-4,7-megastigmadien-3-one (19) and α-tocopherol (20) (Table 2.6). The data obtained from the High-Resolution Electron Ionization Mass Spectrometry (HREIMS) spectrum has shown that rhodomyrtosone B is a structural isomer of rhodomyrtone. The b-triketone moiety, which has given a unique structure to these compounds, is rare but commonly found in the *Myrtaceae* family. Out of these compounds, rhodomyrtosone A is reported to be the first example with a novel bisfuran fused-ring skeleton and rhodomyrtosone D as a lepstospermone derivative Asadhawut Hiranrat & Mahabusarakam (2008). Rhodomyrtone (6,8-dihydroxy-2,2,4,4-tetramethyl-7-(3-methyl-1-oxobutyl)-9-(2-methylpropyl)-4,9-dihydro-1*H* –xanthen-1,3(2*H*)-di-one) (21) is isolated and identified for the first time from the ethyl acetate extract of leave of *R. tomentosa* Salni *et al.* (2002). Similarly, combretol (22) is isolated and characterized from leaves of the plant Fahmi *et al.* (2004). Table 2.7 shows summary of these constituents.

Further investigation by Hiranrat *et al*. (2008) has reported the isolation of two phloroglucinols named as tomentoson A (23) and tomentosone B (24) from the CH_2CL_2 extract of the leaves. Each of these phloroglucinols is known to contain six continuous novel hexacyclic rings that deduce the structure to be new to science. Elucidation of their structure from the two dimensional (2D) NMR spectroscopic data and further correlations made in the study suggested that, tomentosone B is a diastereomer of tomentosone A Asadhawut Hiranrat *et al.* (2011).

Table 2.6 Newly identified Acylphloroglucinols and other constituents of *R. tomentosa*

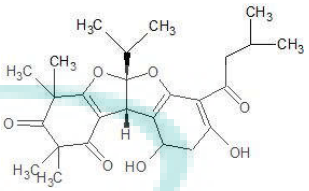
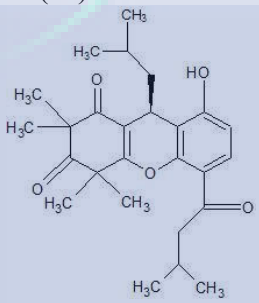
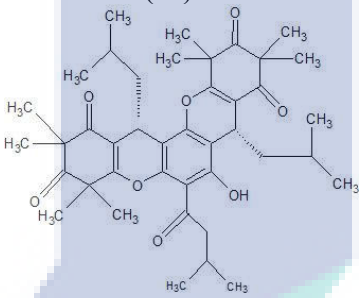
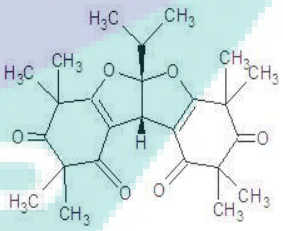
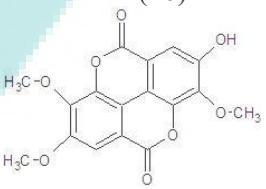
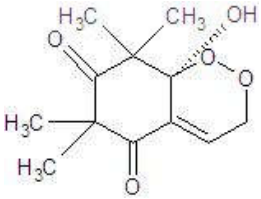
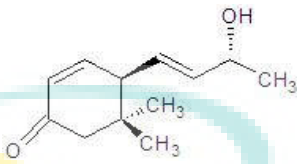
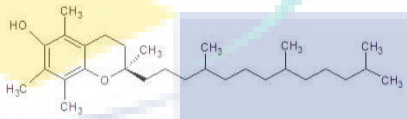
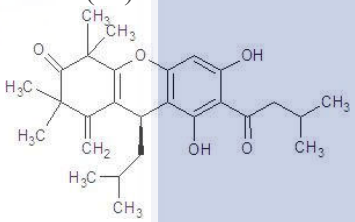
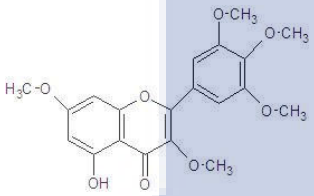
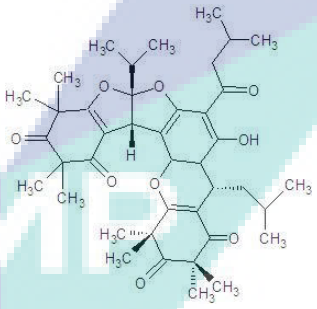
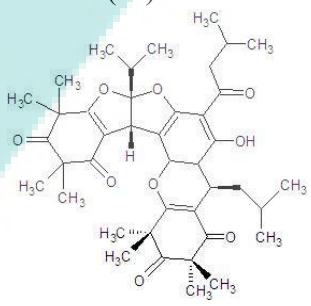
Chemical classifications	Compound	Chemical structure	Source
Acylphloroglucinol	rhodomyrtosone A	 (13)	Asadhawut Hiranrat & Mahabusarakam (2008).
	rhodomyrtosone B	 (14)	
	rhodomyrtosone C	 (15)	
	rhodomyrtosone D	 (16)	
Tannin	3,3',4-tri-O-methylellagic acid	 (17)	
Hormone	endoperoxide G3	 (18)	

Table 2.7 Rhodomyrtone, and other constituents of *R. tomentosa*

Chemical classifications	Compound	Chemical structure	Source
Monoterpenes and sesquiterpene	9-hydroxy-4,7-megastigmadien-3-one	 (19)	Asadhawut Hiranrat & Mahabusarakam (2008).
Pyran	α-tocopherol	 (20)	
Xanthene	rhodomyrtone	 (21)	Salni *et al.* (2002).
Flavonoid	combretol	 (22)	Fahmi *et al.* (2004)
Phloroglucinol	tomentoson A	 (23)	Asadhawut Hiranrat *et al.* (2011)
Phloroglucinol	tomentoson B	 (24)	Asadhawut Hiranrat *et al.* (2011)

As a major phenolic component, a stilbene component known as Piceatannol (3,5,3',4'-tetrahydroxy-trans-stilbene) (25) was identified from the $(CH_3)_2CO$: H_2O: $C_2H_4O_2$ (50:49:1 V/V/V) extract of a freeze dried fruit of the plant Lai *et al.* (2013). However, other phenolic compounds are also identified with lesser concentration than that of piceatannol. The phenolic components reported by Lai et al. (2013) are shown in Table 2.8.

Table 2.8 Phenolic compounds identified from the sim fruit of *R. tomentosa*

Di-HHDP-galloyl-glucose	**astringin**	**myricetin-pentoside**	**gallic acid**	**delphinidin-3-glucoside**
HHDP-galloyl-glucose	piceatannol	miricitrin		cyanidin-3-0-glucoside
HHDP-digalloyl-glucose	piceatannol-galloyl-hexoside	rhamnetin-deoxyhexoside		[elargonidin-3-hexoside
Furosin	resveratrol			petunidin-3-glucoside
HHDP-trigalloyl-glucose				malvidin-3-glucoside
				peonidin-3-glucoside

Source: Lai *et al.* (2013)

In a similar study, the extraction of the dried fruits with CF_3CO_2H: MeOH (1:99) and with further purification by High Performance Liquid Chromatography (HPLC) and column chromatography (CC) has yielded to an identification of six major anthocyanins as cyanidin-3-O-glucoside (26), peonidin-3-O-glucoside (27), malvidin-3-O-glucoside(28), petunidin-3-O-glucoside (29), delphinidin-3-O-glucoside (30) and pelargonidin-3-glucoside (31) Cui *et al.* (2013). The study confirmed that pelargonidin-3-glucoside was identified in addition to the five anthocyanins identified previously by Liu *et al.* (2012) whereby the five anthocyanins are identified for the first time from the plant *R. tomentosa* G. L. Liu *et al.* (2012). Cyanidin-3-O-glucoside is known to be found in higher concentration than the rest of the anthocyanins Cui *et al.* (2013; Lai *et al.* (2013; G. L. Liu *et al.* (2012).

Table 2.9 Phenolic constituents of *R. tomentosa*

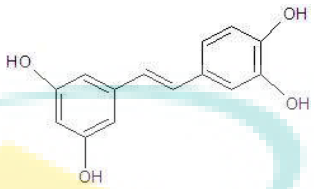
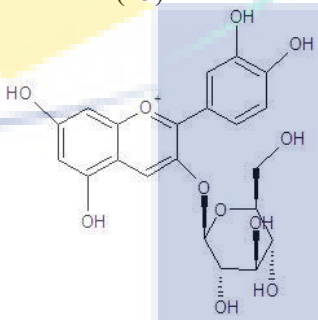
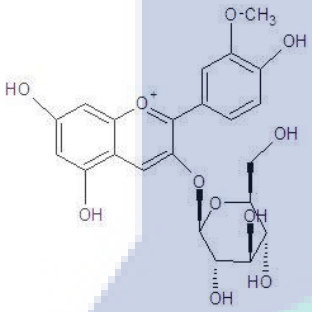
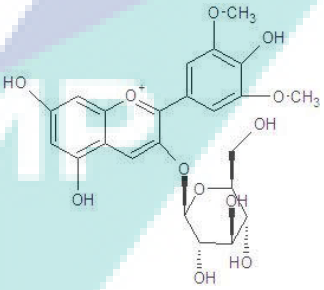
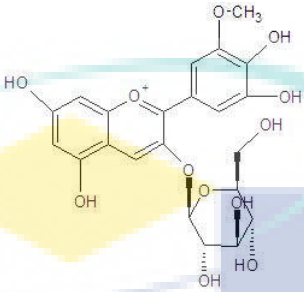
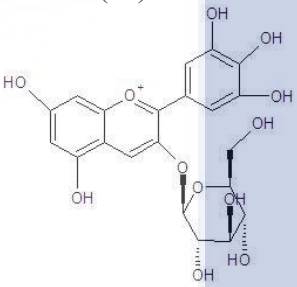
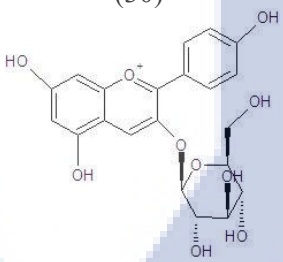
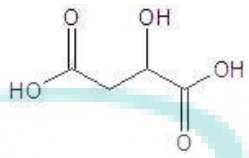
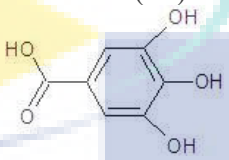
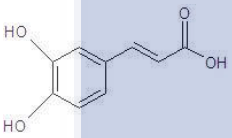
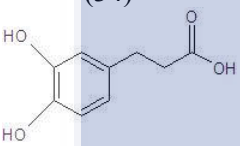
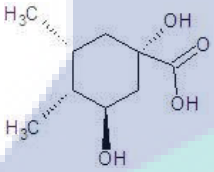
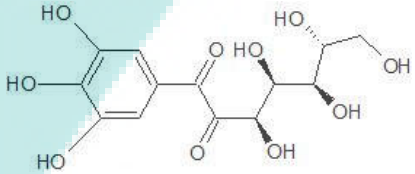
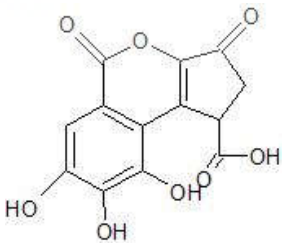
Chemical classifications	Compound	Chemical structure	Source
Stilbene	piceatannol	 (25)	Lai *et al.* (2013).
Anthocyanins	cyanidin-3-O-glucoside	 (26)	Cui *et al.* (2013)
	peonidin-3-O-glucoside	 (27)	
	malvidin-3-O-glucoside	 (28)	

Table 2.9 Continued

Chemical classifications	Compound	Chemical structure	Source
Anthocyanins	petunidin-3-O-glucoside	 (29)	Cui *et al.* (2013).
	delphinidin-3-O-glucoside	 (30)	
	pelargonidin-3-O-glucoside	 (31)	

The crude extracts from four solvents (water, methanol, chloroform, petroleum ether) were further incorporated to Thin Layer Chromatography (TLC)/HPLC/GCMS techniques for separation of bioactive compounds. In all the solvent extracts, alkaloids, phenols and terpenoids were identified. Compounds identified from the water extract are malic acid (32), gallic acid (33), caffiec acid (34), dihydrocafeic acid (35), quinic acid (36), octadecenoic acid (37), galloyl glucose (38) and brevifolin carboxylic acid (39). It was reported that the total phenolic content is much higher in the water extract than in the other extracts. The HPLC analysis also shows the presence of quercetin, tannic and gallic acids Maskam (2011).

Table 2.10 Carboxylic acid constituents of *R. tomentosa*

Chemical classifications	Compound	Chemical structure	Source
Carboxylic acid	malic acid	 (32)	Maskam (2011)
	gallic acid	 (33)	
	caffiec acid	 (34)	
	dihydrocafeic acid	 (35)	
	quinic acid	 (36)	
	octadecenoic acid	 (37)	
Tannin	galloyl glucose	 (38)	
Carboxylic acid	brevifolin carboxylic acid	 (39)	

Forty years ago, Hui *et al* (1975) examined the plant *R. tomentosa* for its triterpenoids and steroids. The study has reported an isolation of 18 different known triterpenoids and 3 steroids Hui *et al.* (1975). After one-year, further investigation on the same plant has revealed isolation of two new triterpenoids besides to the other known ones as 3*β*-hydroxy-2l*α*-*H*-hop-22(29)-en-30-al and 3*β*-acetoxy-12-oxo-oleanan-28,13*β*-olide (40, 41) from the leaves and stem part of *R. tomentosa,* respectively. Extraction was made with petrol and subsequently with ethanol and diethyl ether. Compounds are presented in Table 2.12 *Wai-Haan & Man-Moon (1976).* The known compounds isolated from the ethanolic extract of stem and leaves reported in the study are listed in Table 2.11.

Table 2.11 Chemical compounds isolated by Hui from the extracts of leaves and stem of *R. tomentosa*

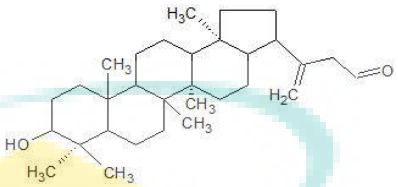
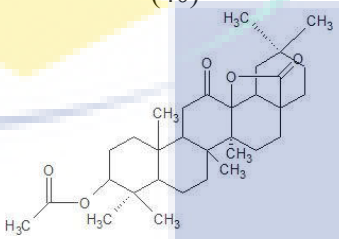
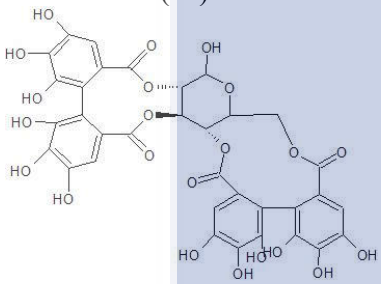
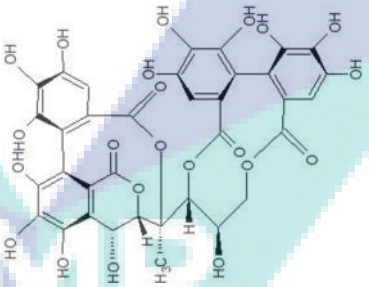
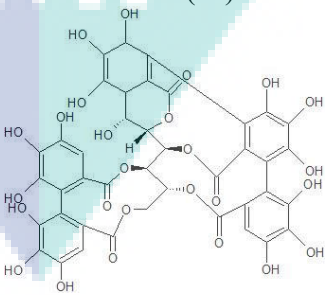
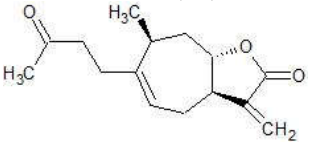
Plant parts	**Leaves**	**Stem**
Identified Chemical Compounds	betulinic acid ursolic acid aliphitolic acids	betulonic acid betulinic acid oleanolic acid

Source: Hui *et al.* (1975)

Another study on the nutritional composition of the acetone: water: acetic acid (50:49:1 v/v/v) extract of the sim fruit has revealed for its low proteins and lipids content. An essential poly unsaturated fatty acid and linoleic acid are found in relatively higher amount than the other fatty acids whereas the content of vitamins and phenolic compounds are reported to have the highest value. Important elements including manganese, copper, chromium , and iron are also found in high content when compared to that of other fruits Lai *et al.* (2015).

Four hydrolysable tannins are isolated from leaves and root extract of *R. tomentosa* for the first time by Y. Liu *et al.* (1997). Out of the four tannins, three are known to be C-glycosidic hydrolysable tannins. The spectroscopic analysis has revealed that the compounds are identified as pedunculagin (42), casuariin (43), castalagin (44), and new C-glycosidichydrolysable tannin, named tomentosin (45).

Table 2.12 Tannin and triterpenoid constituents of *R. tomentosa*

Chemical classifications	Compound	Chemical structure	Source
Triterpenoid	3β-hydroxy-2lα-*H*-hop-22(29)-en-30-al	 (40)	Wai-Haan & Man-Moon (1976)
	3β-acetoxy-12-oxo-oleanan-28,13β-olide	 (41)	
Tannins	pedunculagin	 (42)	Y. Liu *et al.* (1997)
	casuariin	 (43)	
	castalagin	 (44)	
	tomentosin	 (45)	

The phytochemical constituents isolated so far from different parts of the plant *R. tomentosa* are summerised in Table 2.13.

Table 2.13 Summery of isolated phytochemicals from different parts of *R. tomentosa*

Plant part extracts	**Isolated phytochemicals**	**source**
Fruits	stigmast-4-en-3-one, rhodomyrtone, rhodomyrtosone D	A Hiranrat *et al.* (2012)
	oleanolic acid, methyl gallate, 3-O-methylellagic acid 4-O--rhamnopyranoside	
Leaves	rhodomyrtosones (A-D)	Asadhawut Hiranrat & Mahabusarakam (2008)
	tomentoson A	
	tomentoson B	
	rhodomyrtone	Salni *et al.* (2002)
	combretol	Fahmi *et al.* (2004)
	3β-hydroxy-2lα-H-hop-22(29)-en-30-al and 3β-acetoxy-12-oxo-oleanan-28,13β-olide	Wai-Haan & Man-Moon (1976)
	pedunculagin, casuariin, castalagin, and tomentosin	Y. Z. Liu *et al.* (1997)
Stems	3,3',4,4'-tetra-O-methylflavellagic acid, rhodomyrtosone I	A Hiranrat *et al.* (2012)
Aerial part	4,8,9,10-tetrahydroxy-2,3,7-trimethoxyanthracene-6-O-β-D-glucopyranoside	Tung *et al.* (2009)
	2,4,7,8,9,10-hexahydroxy-3-methoxyanthracene-6-O-α-L-rhamnopyranoside	
	(3S, 5R, 6R, 7E, 9S)- megastigman-7-ene-3,5,6,9-tetrol,	

2.3 Anti-infective activities of *Rhodomyrtus tomentosa*

2.3.1 Antibacterial activity

Limited numbers of publications have investigated the antibacterial activity of different extracts and isolated pure compounds of *R. tomentosa*. The rhodomyrtone, an isolated compound from ethyl acetate extract of the leaves of *R. tomentosa*, is shown to have significant antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* Salni *et al.* (2002). This compound is found to be the most effective antibiotic compound against different bacterial strains Leejae, Hasap*, et al.* (2013; Limsuwan *et al.* (2011; Limsuwan *et al.* (2009; Saising *et al.* (2008). Leejae *et al*. (2013) incubated *Staphylococcus aureus* with rhodomyrtone at 0.5× Minimum Inhibitory Concentration (MIC) (0.25 µg ml^{-1}) to assess susceptibility of rhodomyrtone-treated cells compared with untreated cells at different concentrations of oxidants (H_2O_2) *in vitro*, and the result has shown that the untreated normal *S. aureus* cells survived much better than these cells which are treated with rhodomyrtone as a result of reduced pigmentation of the cells after incubation with rhodomyrtone Leejae, Hasap*, et al.* (2013). It is also indicated that main target of rhodomyrtone is not on the bacterial cell membrane and cell lysis as there is no significant effect of the compound observed on either of them

The crude ethanolic extract and its pure compound, rohodomyrtone, is shown to have substantial antibacterial activity against gram positive bacteria, *Bacillus cereus, Bacillus subtilis, Enterococcus faecalis, Staphylococcus aureus,* methicillin-resistant *S. aureus* (MRSA), *Staphylococcus epidermidis, Streptococcus gordonii, Streptococcus mutans, Streptococcus pneumoniae, Streptococcus pyogenes, and Streptococcus salivarius* Leejae, Taylor*, et al.* (2013; Limsuwan *et al.* (2009). The study has further approved that rhodomyrtone has stronger antibacterial activity than the reference antibiotic vancomycin, in which rhodomyrtone has shown 2–3 times lower MIC values and 160–320 times lower Minimum Bactericidal Concentration (MBC) values than vancomycin. As rhodomyrtone has shown no bacteriolytic activity on the pathogenes, further study is conducted to prove that the antibacterial effect of rhodomyrtone is due to inhibition of *S. pyogenes* toxin by altering the metabolic pathway of the bacterium Limsuwan *et al.* (2011). Similarly, the study has presented the good anti-infective activity of rhodomyrtone as it inhibite the expression of the *S. pyogenes* fibronectin-binding

protein, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that leads to inability of *S. pyogenes* to adhere to mammalian cells and mucosal surfaces.

On the other hand, another study has reported that rhodomyrtone is very effective against *Staphylococcus aureus* with MIC value at 0.5 µg/ml which is close to that of vancomycin. However the study challenges the use of rhodomyrtone as an alternative in treating staphylococcal cutaneous infections Saising *et al.* (2008). Same studies have assessed the antibacterial activity of the crude ethanolic extract of *R. tomentosa* on staphylococci isolated from acne lesions and it has shown antibacterial activity against all isolated coagulase-positive and coagulase-negative staphylococci. Based on their previous study, the same authors have conducted further investigation on antibacterial activity of the ethanolic extract of the plant and rhodomyrtone against propionibacterium acnes. The result has revealed that both have exhibited good antibacterial activity. As both the extract and rhodomyrtone have also shown very low toxicity on skin cells, the researchers proposed them as potential candidate for acne therapeutic agents Saising & Voravuthikunchai (2012).

The ethanolic crude extract of leaves of *R. tomentosa* is tested for its antibacterial activity against sixty-five samples of *Bacillus cereus* previously isolated from foods. The crude extract has demonstrated large inhibition zones (10.00 to 18.00 mm) against all tested isolates. The study has found that the extract could affect both cells and endospores Voravuthikunchai *et al.* (2010). The study has suggested for the use of the extract as food preservative or food additive agent for controlling both the growth of the pathogen and reducing its incidence and it would make no compromise on the safety of food. Similar study is assessed for the effect of the crude ethanol extract of *R. tomentosa* leaf and its isolate rhodomyrtone on forty-seven clinical isolates of *Streptococcus pyogenes* isolated from tonsillitis /pharyngitis patients. The crude extract and its isolated compounds, rhodomyrtone, have shown significant antibacterial activity against forty seven and fourteen clinical isolates Limsuwan *et al.* (2012). The study has further reported that the extract has produced no effect on bacterial cell lysis at all concentrations.

R. tomentosa plant extract has showed significant inhibithion of biofilm formation in both *Streptococcus pyogenes* and *Staphylococcus aureus* isolates Limsuwan & Voravuthikunchai (2008; Saising *et al.* (2011). The former study confirmed that ethanol

extract is able to reduce biofilm formation more readily than rhodomyrtone and vancomycin while the later one indicated the *R. tomentosa* extract has also strong quorum-sensing inhibition effect. A compound identified as tomentosenol A from the leaves of *R. tomentosa* exhibited potent antibacterial activity with a MIC value of 4.74 mM against *S. aureus*, comparable to the positive control substance vancomycin (MIC ¼ 1.23 mM) H.-X. Liu *et al.* (2016).

2.3.2 Antimalarial activity

In general, there is limited study of *R. tomentosa* plant extract and their isolates for the antimalarial activity as compared to the studies on antibacterial activities. The two phloroglucinols named tomentosones A and B, are assessed for their activity against growth of chloroquine-sensitive and chloroquine -resistant strains of the malaria parasite, plasmodium falciparum. While tomentosone A inhibited the growth of both the strains, insignificant inhibitory activity is observed on tomentosone B Asadhawut Hiranrat *et al.* (2011).

2.3.3 Fungicidal activity against animal and plant pathogenic fungi strains

At a concentration of 200 µg/ml, ethanolic extract of leaf of *R. tomentosa* has exhibited antifungal activity against three pathogenic fungi (*B. Setariae, C. Oryzae, R. oryzae-sativa*) of rice plant with mycelial inhibition of above 50% Plodpai *et al.* (2013).

About 617 extracts of 213 fungal isolates from *R. tomentosa* are tested for antifungal activity against five strains of human pathogenic fungi (Candida albicans, flucytosine-sensitive Cryptococcus neoformans, flucytosine- resistant Cryptococcus neoformans, Penicillium marneffei and Microsporum gypseum). Approximately 83.1% of the total isolates has shown antifungal activity against at least for one test fungus Jeenkeawpieam *et al.* (2012).

The highest inhibitory activity of the crude extract from endophytic fungi of the plant against *Penicillium marneffei* is reported as the first active endophytic fungi. As a result, the researchers have suggested that *R. tomentosa has* high level of endophytic fungi producing sufficient antifungal activity.

2.4 Nanofibrous biomaterials

2.4.1 Biomaterials

Biomaterials are natural or synthetic materials that can incorporate a living structure or biomedical device, which performs, supplements, or substitutes a natural process. The American National Institute of Health Consensus Development Conference defined a biomaterial as ‘‘any substance or combination of substances (other than drugs) synthetic or natural in origin, which can be used for any period of time, which augments or replaces partially or totally any tissue, organ or function of the body, in order to maintain or improve the quality of life of the individual”Kar (2016). Application of biomaterials dates back into ancient civilizations. Artificial eyes, ears, teeth, and noses were found on Egyptian mummies Sanchez *et al.* (2005) and was first reported at around 2000 BC where Egyptians applied elephants tusks, Walrus teeth and linen in bone replacement, missing teeth and wound closure respectively Williams & Cunningham (1979). Thus far, a wide range of materials surrounding the classical materials such as metals, ceramics, composite and polymers have been investigated as biomaterials Patel & Gohil (2012). An ideal biomaterial should be biocompatible, biomimetic, non-toxic, and non-immunogenic Mantovani *et al.* (2013).

Fabrication and design of biomaterial scaffolds are an important subjects of tissue engineering and regenerative medicine research Dhandayuthapani *et al.* (2011). Scaffolds are defined as three-dimension porous solid biomaterials designed to perform some functions as promoting cell-biomaterial interactions, cell adhesion, permit sufficient transport of gases, nutrients, and regulatory factors to allow cell survival, proliferation, and differentiation Langer & Tirrell (2004), to facilitate the delivery of non-cellular biomaterials, like drugs and other bioactive compounds into the body Edgar *et al.* (2016). An ideal scaffold should meet the physiological demands of native extracellular matrix to promote excellent host-cell mediated healing Sell *et al.* (2007) and primarily scaffolds should be biocompatible whereby neither the biomaterials nor its degradation should incite any inflammation or toxicity *in vivo* Chen *et al.* (2002). In general, the structural and behavioural characteristics of scaffolds as physical, mechanical, chemical and biological are critical factors to ensure normal host cell activities and performance Karp *et al.* (2003).

2.4.2 Electrospinning

Nanotechnology is the rapid evolving science of manufacturing and utilizing extremely small particles, as small as single atoms and molecules. Electrospinning is a simple, cost effective and suitable method for the fabrication of uniform and very long fibers ranging from 10 μm down to 5 nm. Sayed & Asran (2011). As a result, electrostatic spinning (electrospinning) has gained much attention among many different processing techniques. The process was patented by Antonin Formhals in 1934 for the first time Formhals (1934) followed by another patent for the fundamental idea of the term "electrostatic spinning or electrospinning" in 1902 by J.F Cooley and W.J. Morton Cooley (1902; Morton (1902).

Electrospinning is carried out by applying a high voltage, in the range of 5–60 kV Zhang *et al.* (2017), to a capillary filled with polymer fluid, which is ejected out toward a collector. The liquid jet undergoes a whipping process and expansion occurs in a region where the repulsive force from the electric charges carried by the jet becomes larger than its cohesive force. Splaying and solvent evaporation, together with a large elongation of fibers (because of the acceleration of the polymer jet by the electric force) are responsible for formation of the nanometer-sized polymeric fibers Hohman *et al.* (2001). Size and the morphology of the electrospun fibres can be controlled by acting on the material viscosity and conductivity, and on the operational parameters (voltage, needle-collector distance, environmental humidity, and temperature). To date, electrospinning of synthetic and natural proteins, composites, and nanocomposite materials has been demonstrated Zhang *et al.* (2017).

Due to the extremely high surface area to volume ratio characteristics of electrospun nanofibers, they have attracted great attention and become the nanomaterials of choice in applications such as sensors, energy, biomedicine, and filtration Liang *et al.* (2007; J. Wu *et al.* (2013). Research towards the development fibres encapsulating plant extracts by using electrospinning has been increased specially in the last 10 years Sridhar *et al.* (2015). Some studies of electrospun nanofibers regarding the application of nanocomposites Shao *et al.* (2003), drug delivery Zeng *et al.* (2005) , wound healing Khil *et al.* (2003) and vascular grafts Buttafoco *et al.* (2005) were recorded.

The apparatus used for electrospinning must contain a high voltage power supply, a syringe, as polymer reservoir, with needle spinneret and a conducting flat plate or rotating drum acting as a ground collector (Figure 2.4). The polymer solution or melt is held by its surface tension in the form of a droplet at the needle tip (spinneret). After an electric potential is applied between the needle of syringe and collector, charge is induced on the fluid surface and the suspended droplet of the polymer solution at the needle tip that is deformed into a conical shape (Taylor cone). When the intensity of the electrical field surpasses a critical value, the electrostatic forces will overcome the surface tension of the polymer solution. As a result, a fine charged jet is ejected from the tip of the cone. The solvent will evaporate, and the jet solidifies as nanofibers deposited on the collector plate.

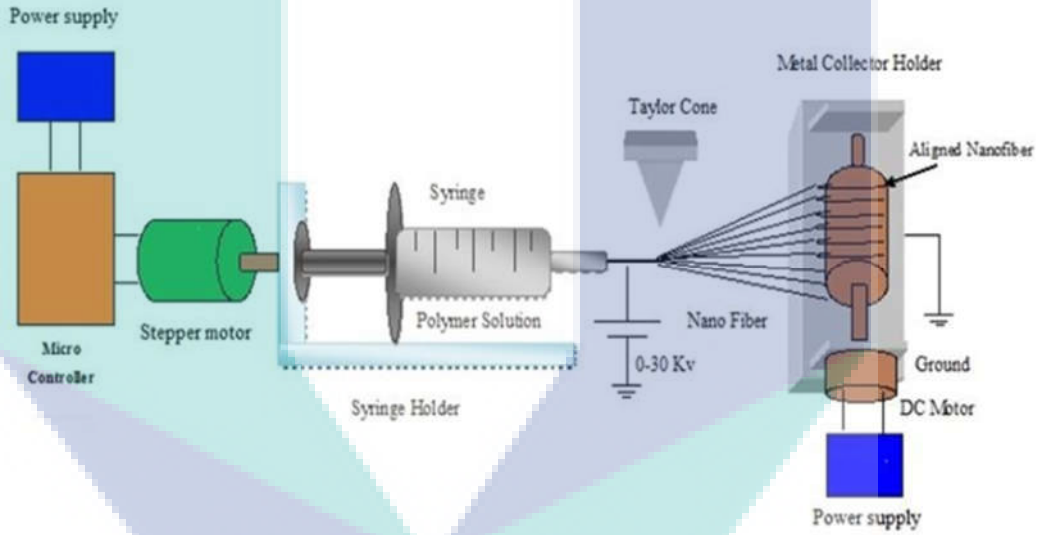


Figure 2.4 Schematic diagram of electrospinning nanofiber generator

Source: Thangavel *et al.* (2015).

2.4.3 Poly (vinyl) Alcohol (PVA)

PVA is a water-soluble 1,3-diglycol polymer with a chemical formula of $[CH_2CH(OH)]_n$, the chemical structure is showed in Figure 2.5. PVA, as nontoxic substance (included in the US Food and Drug Administration (FDA) inactive database) Rowe *et al.* (2012), is known for its desirable behaviours such as good film forming, high hydrophilicity, biocompatibility Gimenez *et al.* (1996; R. Huang & Rhim (1993). In addition, it has good chemical resistance and physical properties that makes it easily

processable to have a broad industrial uses Krumova *et al.* (2000) after it had put into commercial use in Germany in the 1920s Wong & Parasrampuria (1996) for the first time . The properties led PVA to be studied intensively in different areas of research Shao *et al.* (2003). As advancements in medical and pharmaceutical research surpasses, PVA is now being used in transdermal patches Aina *et al.* (2014) in the field of nanotechnology where PVA is incorporated with other natural products to produce electrospun nanofiber mat. Electrospinning of PVA usually performed from aqueous solutions leads to generation of homogenous nanofiber webs Supaphol & Chuangchote (2008). A study recommend that chemical crosslinking of PVA nanofibers can provide a higher stabilization of the fiber mats towards aqueous surroundings Voigt *et al.* (2007).

Furthermore, some studies have revealed that PVA was an effective emulsifying agent in the preparation of polylactide and Poly(DL-lactide-co-glycolic acid) (PLGA) polymeric nano/micro-particles/scaffolds formulations for the treatment of age-related diseases of the heart, muscle and bones Ionescu *et al.* (2010; Lee *et al.* (1999; Puppi *et al.* (2010; Sahoo *et al.* (2002; Westedt *et al.* (2007). Fine polymeric nanofibers were achieved in the size range of 100-500 nm for pure PVA fiber and 100-700 nm for PVA/silica in an indigenously designed electrospinning setup that intended to ease rapid harvesting of nanofibers Sasipriya *et al.* (2013).

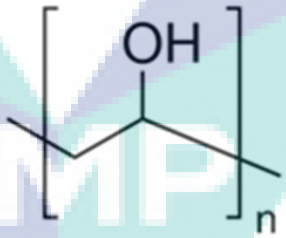


Figure 2.5 Chemical structure of PVA polymer

2.5 Medical applications of biomaterials

2.5.1 Biomaterials in wound care

Skin is the principle exterior defence that protects the inner body systems from microorganism's attack, contamination, infection, and the effect of external environment. Skin is composed of three multi-histological layers, the epidermis, the

dermis, and the hypodermis or subcutaneous layer. The epidermis upper layers or the stratum corneum layers builds barrier to percutaneous penetration of any external invasion. Dermis layer exists next to epidermis layer and consist of connective tissues matrix which provide the elasticity and deformation resistance for skin. In addition, the dermis layer contains blood vessels which give the layers with nutrients and oxygen. The hypodermis layer, subcutaneous fat tissue followed the epidermis and dermis layers, providing thermal isolation and mechanical protection to the body Potts (1997).

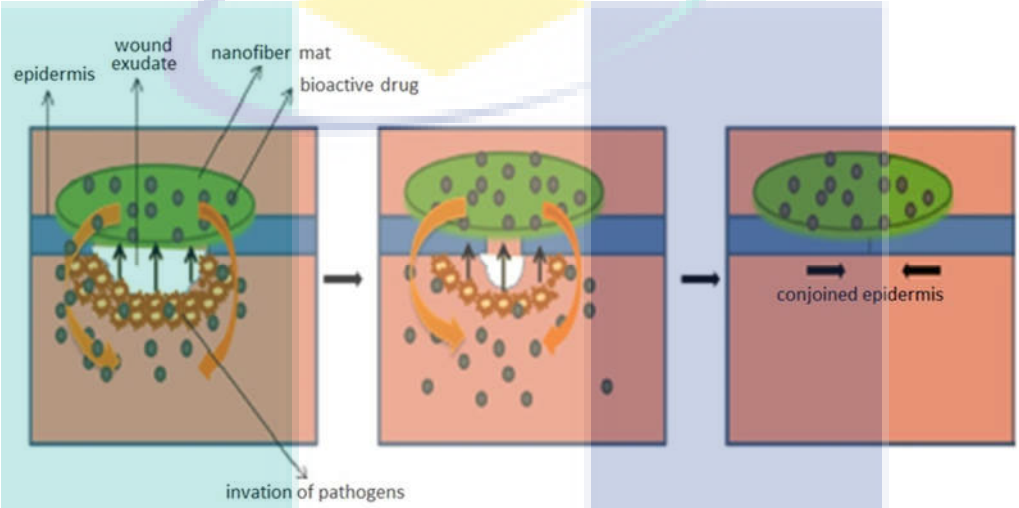


Figure 2.6 Mode of action of plant extract loaded polymeric mats

Source: Qureshi *et al.* (2015)

Defect or break in the skin is described as a wound, which can be formed due to physicochemical or thermal damage. Some chronic wounds need a long healing time including months and leaves serious scars. Reasons which delay the healing of these wounds may include diabetes, wound strong dryness and infections Boateng *et al.* (2008). The healing of wound passes during four stages, homeostasis, inflammation, granulation tissue formation and remodelling. These healing phases can be affected by some individual factors as nutrition, patient age, diseases, size, depth and cause of wound Babu (2000). The ability of the skin to regenerate itself following minor epidermal injury is notable. However, in such cases as severe injury, bacterial infection and loss of epidermis and dermis that result in full-thickness skin wounds, the damaged skin may not initiate the healing process leading to life threatening complications. The World Health Organization stated that, more than 30,000 deaths per year occur due to scalds and burns

forms Kamoun *et al.* (2015). Figure 2.6 shows plant extract loaded polymeric films in wound healing process.

Table 2.14 Summary of miscellaneous herbal medicines in wound care

Herbal medicine	Properties
Aspilia africana	Haemostatic properties on wounds, inhibits the growth of microbial organisms, accelerates wound healing, treatment of rheumatic pain, bee and scorpion stings, remove corneal opacities and foreign bodies from the eyes
Bridelia ferruginea, Parkia biglobosa Jacq	Increased the proliferation of dermal fibroblast
Elaeis guineensis leaf extract	Improved tissue regeneration
Cedrus libani, Abies cilicica subsp. Cilicica	Improved wound healing and anti-inflammatory properties
Hippophae rhamniodes L	Improved wound healing
Melaleuca alternifolia	Anti-microbial, anti-septic, anti-inflammatory, anti-fungal and anti-viral properties

Source: Dorai (2012).

Wounds dressings, which are loaded with antibiotic drugs, may speed up the wound healing but are suspicious to generate resistance of bacteria. Furthermore, they result in toxicity and de-pigmentation to the wound area Qureshi *et al.* (2015). As a result, demand for herbal drugs is increasing in developed as well as developing countries because they are safer and well tolerated as compared to those allopathic drugs Dorai (2012). In the search for new therapeutic options in wound dressing, plants and their metabolites are a great source of novel bio-molecules. Table 2.14 shows the summary of the use of miscellaneous herbal medicines in wound care.

2.5.2 Electrospun Nanofibers Containing Natural Products

Nanofibers can be fabricated from a wide variety of natural as well as synthetic polymers by electrospinning as the process is flexible in nature. Chitosan and cellulose are among the famous natural polymers which are suitable to produce electrospun nanofibers Wang & Vermerris (2016). PVA, Polyethylene oxide (PEO), Polyglycolic acid (PGA) and Polycaprolactone (PCL) are also some of the synthetic polymers which are reported to electrospun successfully L. Manea *et al.* (2016). As the most important field, biomedical applications of polymeric nanofibers obtained through electrospinning process are numerous L. R. Manea *et al.* (2015; Mitchell (2015). Progresses have been made during the recent years in this field that synthetic polymers could be blended with natural polymers to produce biocompatible fibers for medical applications. For instance, nanofibers of collagen type I blended with PCL were used to grow human dermal fibroblast cells since it has improved cell development, proliferation and migration inside human dermatic fibroblasts matrix Lazarescu *et al.* (2009)

Natural products, specially crude plant extracts such as, Baicalein green tea Sadri *et al.* (2015), *Garcinia mangostana* Suwantong *et al.* (2014), *aloe vera* Agnes Mary & Giri Dev (2015; Fu *et al.* (2014), *Grewia mollis* Al-Youssef *et al.* (2013), *chamomile* Motealleh *et al.* (2014), grape seed Lin *et al.* (2016), *Indigofera aspalathoides, Azadirachta indica, Memecylon edule and Myristica andamanica* Jin *et al.* (2013) have been successfully encapsulated into electrospun fibres.

Gelatin nanofibres were loaded with *Centella asiatica*, a medicinal herb from Asia that is composed of triterpenoids (Asiatic acid, madecassic acid and asiaticoside), was reported to have wound healing activity Yao *et al.* (2017). Another herbal extract, Baicalein, Chinese herb with antibacterial, anti-inflammatory, antioxidant properties and wound healing property, was incorporated into Silk Fibroin Protein (SFP) and Polyvinylpyrrolidone (PVP) fibres and the mats were evaluated for its antibacterial activity against *S. aureu*. The result exhibited a reduction of bacteria viability in the range of 88%–99% Chan *et al.* (2017). Crude bark extracts of *Tecomella undulata* incorporated PCL and PVP embedding nanofibers was proposed by Suganya et al, 2011 for the treatment of skin infections. The potent antibacterial activity of the bark extracts released from the electrospun mats was showed to have good antibacterial activity against *P.*

aeruginosa, S. aureus, and E. coli, attaining an inhibition zones of 30, 24, and 28 mm in diameter, respectively Suganya *et al.* (2011). Similarly, a Curcumin-loaded poly(ε-caprolactone)-poly(ethylene glycol)-poly(ε-caprolactone) (PCEC) scaffolds were fabricated to repair wounded dermal defect in rats and the covered PCEC/Curcumin membrane fell off naturally because of the formation of a scab and a slightly smaller defect that can be seen after the membrane was covered with PCEC/curcumin for 21 days Fu *et al.* (2014).

Nanofibers, especially made of biocompatible or biodegradable materials, show immense potential in biomedical and healthcare sector due to their unique combination of properties and innate functionalities. One of the first studies to report the incorporation of an antibiotic in nanofibers through electrospinning for the production of antibacterial nanofibers, was made by Kenawy et al. though they had not examined the antibacterial activity of the resulting nanofiber Kenawy *et al.* (2002). Many research showed that biocompatible nanofibers were used to construct post-burn wound protective dressing. Recent results obtained with nanofibers was derived from bio-based polymers (chitosan, cellulose, antimicrobial proteins) and from synthetic nanofibers containing antimicrobial compounds of biological origin, have been shown to be effective against microbes, and are being utilized to minimize bacterial adherence to surfaces Wang & Vermerris (2016).

Nanofibers were applied in cases as construction of fluorescent probes for bio-diagnostics, constructing post-burn wound protective dressing, successfully tested on mouse skin in prevention of finger liaisons as well as to facilitate maintenance of skin implants Ciesielski *et al.* (). producing biodegradable, biocompatible and non-toxic functional nanofibers using organic natural products are seen as a novel candidate and a shining star for many medical applications and tissue engineering Balta (2010).

The use of natural antimicrobial agents is rapidly growing as it provides antimicrobial surfaces that tend to be non-toxic and environmentally benign. These natural antimicrobial compounds are mostly extracted from plants Wang & Vermerris (2016). A nanofiber composite of PVA containing *Juniperus chinensis* extract has good efficiency for antibacterial activity against both the Gram-positive and Gram-negative bacteria, even in lower amount of the extract Kim *et al.* (2016). Yao and co-workers have produced gelatine nanofiber loaded with *Centella asiatica* extract that has wound healing

activity. They have assessed *Centella asiatica* extract previously and was found effective against Staphylococcus aureus, Escherichia coli, and Pseudomonas aeruginosa Yao *et al.* (2017). Furthermore, *in vitro* antibacterial tests showed the efficacy of green tea extract loaded chitosan and polyethylene oxide electrospun fibers against *E. coli* and *S. aureus* with inhibition zone on agar plates of 4 and 6 mm in diameter, respectively Sadri *et al.* (2015)

CHAPTER 3

MATERIALS AND METHODS

3.1 Introduction

The overall methodology of this research emphasis on the extraction of *R. tomentosa* roots by using different polarity solvents and identification of their phytochemicals. Antimicrobial activity of all crude extracts was determined. The extract with better activity was incorporated with PVA solution and the *R. tomentosa* loaded PVA electrospun nanofiber was further tested on antimicrobial activity. Physicochemical Characterization of the fabricated nanofibers was done using FESEM, FTIR spectroscopy and XRD techniques. Details of the general methodology is presented in Figure 3.1.

3.2 Collection and preparation of plant material

The roots of *R. tomentosa* were collected from Bodywell My Marketing (JR0042311-M), Johor, Malaysia. And identified by Dr. Shamsul Kamis, Botanist at Institute of Bioscience, Universiti Putra Malaysia. A voucher specimen was deposited at the Herbarium Unit of Universiti Putra Malaysia, Malaysia. The dried roots of *R. tomentosa,* which were previously grounded into small pieces from the source, was stored for further study.

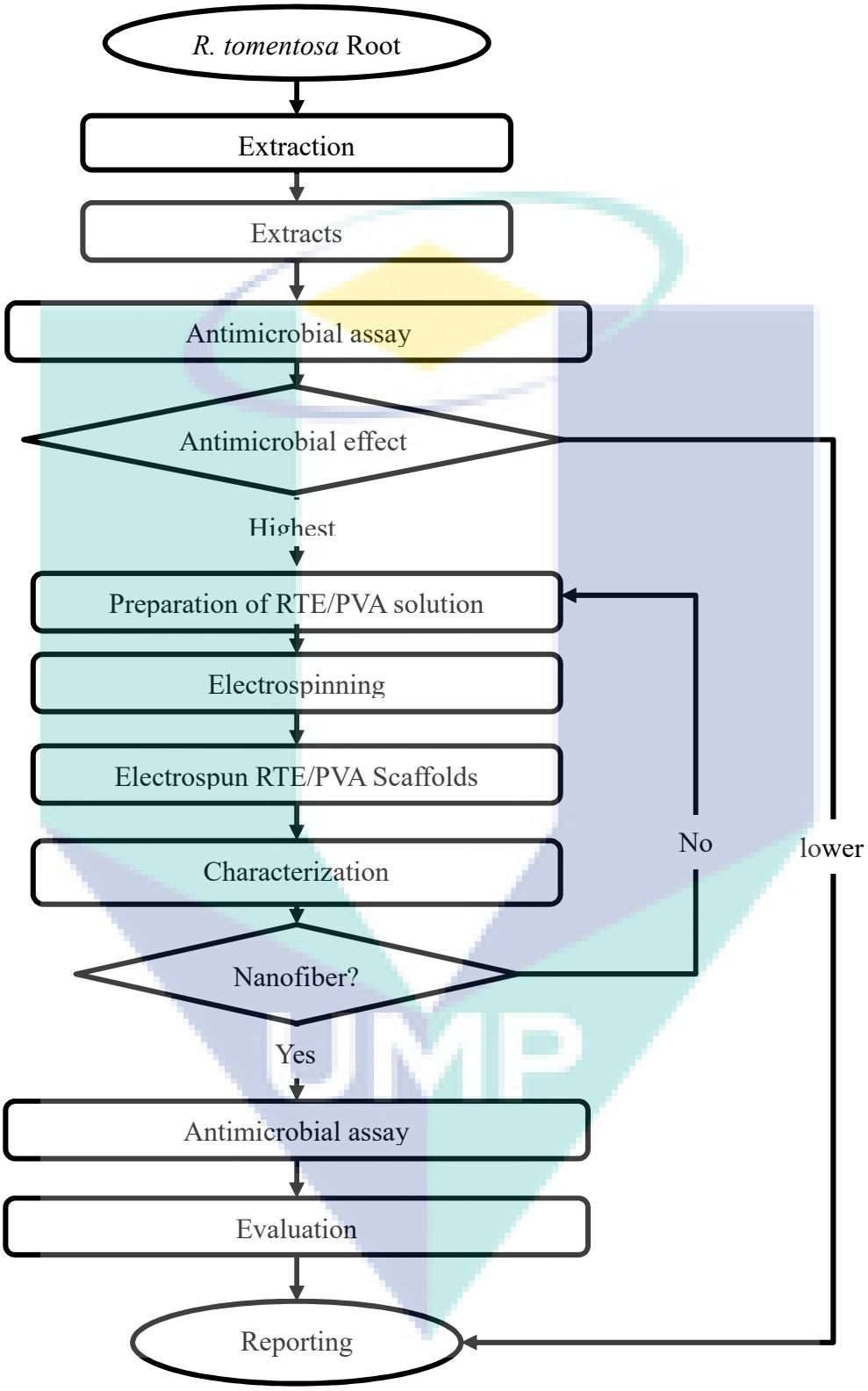


Figure 3.1 Methodology flowchart

3.3 Extraction

The dried and crushed roots of *R. tomentosa* (2.9 kg) were soaked overnight in hexane and methanol subsequently. The soaked plant material was then sonicated at 60ºC for 30 minutes for three cycles in a Branson (8510-DTH) sonicator Azrulrizawati (2013).

The sonicated solution was filtered with Whatman Number 1 filter paper to insure the solution from any plant remain. Both the filtrates were concentrated by rotary evaporator, Buchi Rotavapour R-3, under reduced pressure and at a temperature of 45ºC to prevent bioactive compounds from being destroyed Maskam (2011).

Concentrated methanol extract in water (3:1) was suspended in a separatory funnel and fractionated with chloroform and ethyl acetate accordingly. After 24 hours, the clearly separated layers were placed in different containers. Dried methanol, chloroform and ethyl acetate extracts were obtained by totally evaporating the solvents under appropriate conditions. Maceration and liquid-liquid solvent extraction methods were followed during the overall extraction process. The dried extracts were kept for further analysis. Figure 3.2 shows the methodology flowchart of the crude extraction.

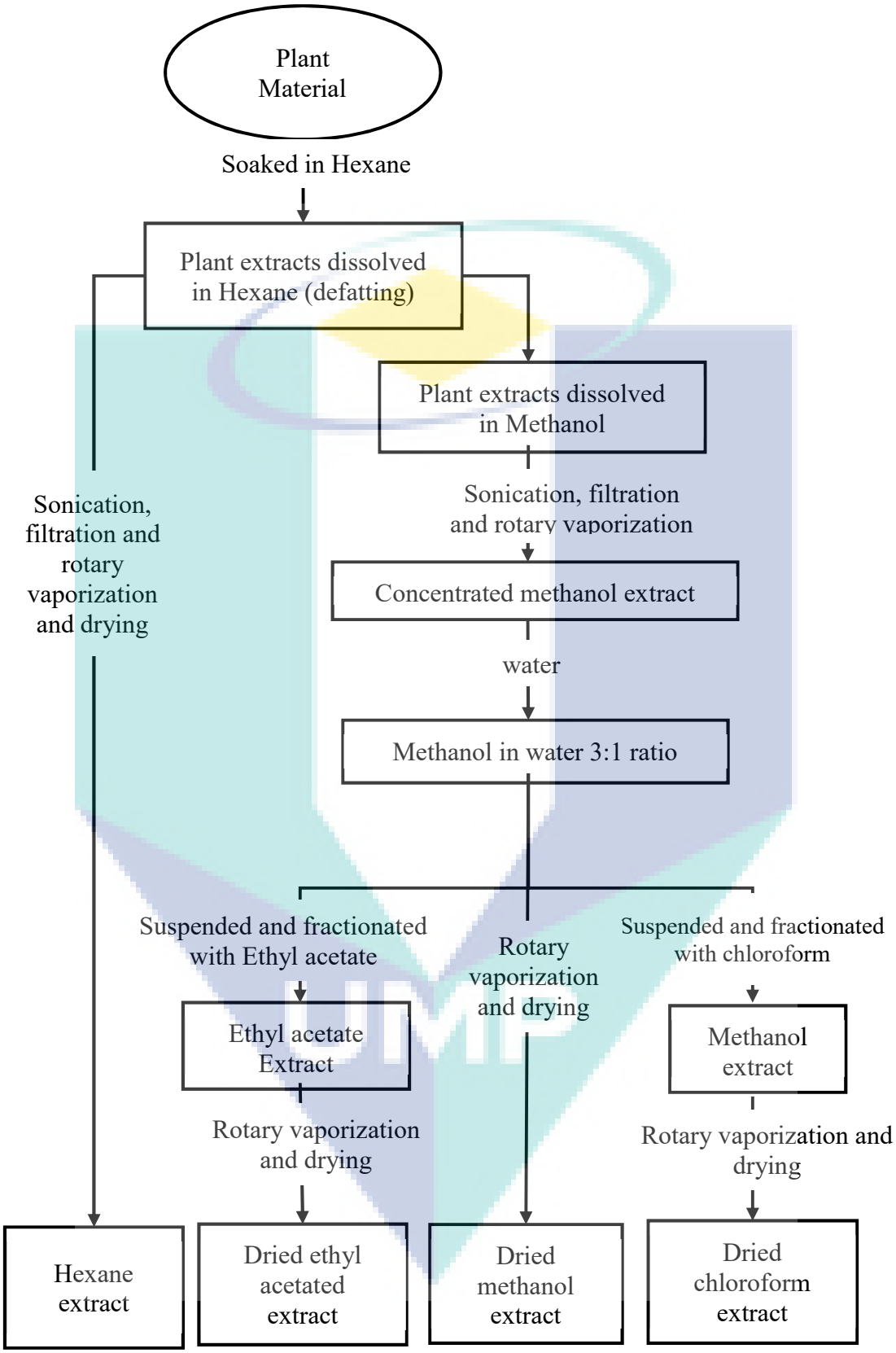


Figure 3.2 Extraction steps of *R. tomentosa* flowchart

3.4 Ultra-high-performance liquid chromatography-quadrupole time-of-flight/mass spectrometry (UPLC QToF/MS)

A high-performance liquid chromatography method is applied for the simultaneous qualitative determination of multiple components Song *et al.* (2010).

The dried extracts of root of *R. tomentosa* (1gm each) were dissolved in 1 ml of respective solvents just before subjected to ultra-high-performance liquid chromatography-quadrupole time-of-flight/mass spectrometry (UPLC QToF/MS) for the determination of active components present in the roots of *R. tomentosa*. Analysis was performed using HSS BEH C18 column (2.1 x 100 mm, 1.8 µm) at column temperature of 40ºC. Mobile phases were performed by the dual pumping system, Mobile A (Water + 0.1%Formic Acid) and mobile phase B (acetonitrile) with flow rate at 0.6 ml/min and injection volume was 2 µL. The UPLC analysis data was obtained on G2-S QToF while The QToF MS was operated in the range of 10-1500Da, in negative and positive modes. Detections of compounds were done by comparing retention times of the peaks detected with those in the library of Waters Traditional Medicine, UMP Centeral Laboratory database, and *R. tomentosa* samples. UNIFI software was used to process all data where the components are classified as a good match with ± 5 mDa error and poor match with ± 10 mDa error.

3.5 Antimicrobial activity of crude extracts

3.5.1 Antibacterial test

The disk diffusion method was used to determine the antibacterial activity of the crude extracts. The assay was conducted against human pathogens, *Escherichia coli* (ATCC 10536), *Pseudomonas aeruginosa* (ATCC 1542), *Bacillus subtilis* (ATCC 11774) and *Enterococcus faecalis*. Inoculum was prepared in a nutrient broth and was incubated for 24 hours at 37ºC. The bacterial suspension was adjusted to the 0.5 McFarland turbidity standards Doughari *et al.* (2007).

The inoculum was spread to the entire surface of the nutrient agar plates. Gentamicine was used as positive control (PC) and solvent was used as negative control (NC). Solution of the dried extracts with their respective solvents was prepared at

concentration of 0.2gm/ml. Each 50µl of all different solvent extracts, positive control and negative control were applied on the 6mm filter paper discs and the agar plates were incubated for 24 hours at 37ºC. The clear zones were considered as positive antibacterial activity and the areola diameter was measured to analyse the antibacterial potential of each extract. The antibacterial assay for each extract was repeated for three times.

3.5.2 Antifungal test

Potato dextrose agar-based disk diffusion method was used to determine the activity of crude extracts against fungal strain, *Aspergillus niger*. Nystatin was used as a positive control and solvents were used as a negative control. The crude extracts and solvents were applied on filter paper discs (6mm). Agar plates were incubated for seven days at 30º C. The diameter of the clear zone around the reservoir was measured at the end of the incubation period Scorzoni *et al.* (2007).

3.6 Preparation of *R. tomentosa* incorporated polymer solution

3.6.1 PVA Solution preparation

50 gm of PVA, (= 95,000 $gm \cdot mol^{-1}$) was added to 450 ml of de-ionized water pre-heated to 40°C, under continuous stirring. The resulting solution was continuously stirred and heat up to 65°C without any interruption until the PVA is completely dissolved Aina *et al.* (2014). The 10% PVA (W/V) solution is kept at room temperature for further procedures.

3.6.2 RTE/PVA Solution preparation

RTE stock solution was prepared by dissolving the dried crude extract in the solvent, ethyl acetate, which was later used in the preparation of RTE/PVA solution for electrospinning. Aqueous solutions of *R. tomentosa* extracts in PVA was prepared at four different concentrations (0.25, 0.5,1.5 and 2.5% (W/V)) of the extract with respect to the previously prepared 10% PVA solution. All the solutions were stirred vigorously for 24 hours to fully dissolve the samples at room temperature Kim *et al.* (2016).

3.7 Electrospinning

High voltage supplied manual electrospinning apparatus (**kd*S***cientific®), as displayed in Figure 3.3, was installed to which a positive and negative electrode were attached to the ejection syringe needle and rotating collector respectively. The variable electro-static high voltage of (0–30 kV), applied across the syringe needle and Grounded collector system which consists of a rotatable flat metallic plate covered with aluminium sheet, was used to extract high surface tension across needle tip and the collector Thangavel *et al.* (2017). Nanofiber was made because of the forward movement of needle plunger and the applied high voltage. The process parameters of indigenous electrospinning set up was optimized using 8.5 cm of Tip-to-collector distance, Voltage of 26 kV, and 0.09 ml/hr of feed rate for both the pure PVA and RTE/PVA solutions fiber production. The electrospun nanofibers were collected on rotating collector rolled with aluminium foil.

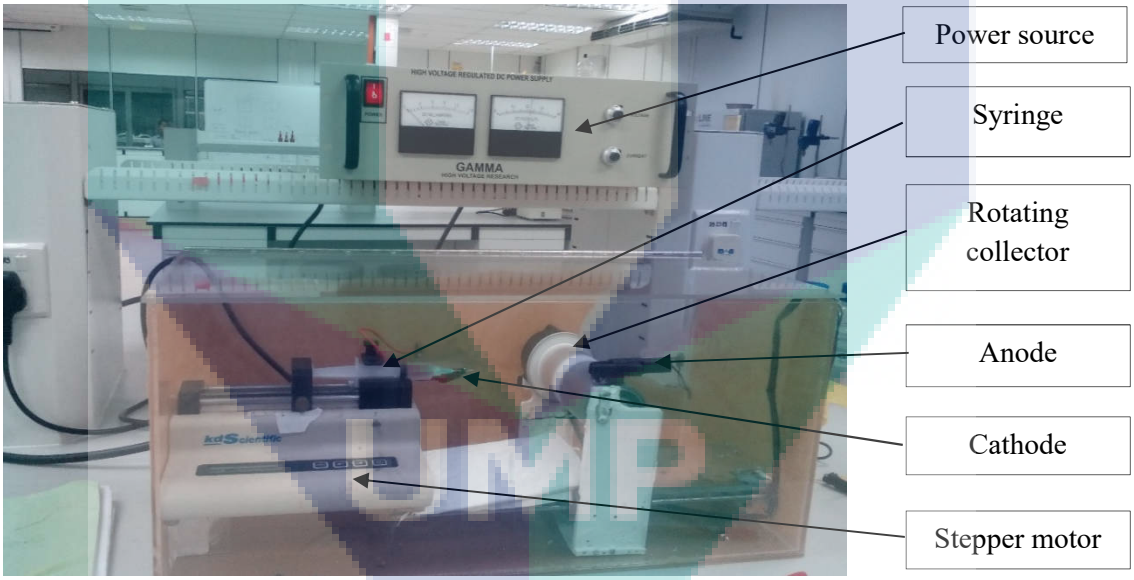


Figure 3.3 Manual electrospinning apparatus

3.8 Characterization of Electrospun Nanofiber

The scaffolds of pure PVA and RTE/PVA nanofibers were kept in a desiccator to avoid exposure to humidity and were characterized by standard analytical tools: XRD analysis, FESEM, and FTIR absorption spectroscopy methods. The operation parameters for the respective instruments are described as follows:

3.8.1 FESEM

The 10% PVA solution and (0.25, 0.5,1.5 and 2.5%) RTE/PVA nanofiber samples were subjected to morphological characterization using FESEM (Hitachi S-7400, Hitachi, Japan), at an accelerating voltage up to 30 kV, a magnification of 40kX and at resolution of 1.3 nm. Fiber diameters were measured with the aid of image software (ImageJ, National Institutes of Health, USA). For each sample, average fiber diameter and distribution were determined using micrographs from about 100 random measurements taken as representative of fibre morphology.

3.8.2 FTIR

In the present study, FTIR spectroscopy was carried out to analyse any structural changes that might have occurred due to incorporation of RTE to PVA nanofibers and to elucidate the interaction (hydrogen bonding) in the blend nanofibers which consequently can affect the compatibility of RTE/PVA nanofibers Asran *et al.* (2010). The spectra of PVA and RTE/PVA nanofiber scaffolds were obtained using an FTIR spectroscopic analysis done on Spectrum 100, (Perkin–Elmer, USA) spectrometer, over a range of 700 – 4000 cm^{-1} at a resolution of 2 cm^{-1}. Furthermore, this characterization was important to ensure the functional group available in the samples and the effect of incorporating *R. tomentosa* extract on intensities and peaks of the FTIR spectrum of PVA nanofibers.

3.8.3 XRD

In the present study, the structural characterization of PVA nanofiber in contrast with RTE/PVA nanofiber scaffolds was done by X-ray spectroscopy (XRD) technique using a Rigaku Miniflex II, diffractometer with CuKα radiation at (λ = 1.54Å) to generate diffraction patterns from the nanofiber scaffolds at ambient temperature over the 2θ range of an angle of 2° to 80°and at a scan rate of 2°/min using Ni-filtered.

3.9 Antimicrobial activity of RTE/PVA nanofibers

The same antibacterial/antifungal test methods and procedures on same microorganisms were done as that of the procedures followed for antimicrobial test of the crude extracts. The zone of inhibition was measured for qualitative determination of the antimicrobial activity of the extract incorporated nanofibers.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Extraction yield of *R. tomentosa* roots

Extraction yields were obtained from each solvent. A reddish-brown gummy crystal with relatively higher yield was obtained from extraction by methanol (21.4 gm) and solvent fractionation with chloroform and ethyl acetate yielded (9.8gm) dark and (4.7 gm) light brownish shining crystals respectively. The higher yield upon extraction with methanol may indicate the availability of highly polar constituents in the root extract of *R. tomentosa* as polarity of methanol (polarity index = 5.1, Burdick & Laboratories (1984)) ranks the highest in the group. Defatting of the plant material with hexane yielded yellowish green extract with gummy nature. The defatting process was intended for effectiveness of the extraction of the bioactive constituents by the higher polar solvents as most of the bioactive components contained in a plant matrix are medium-sized molecules that have aromatic delocalized μ-electrons. As a result, the molecules are highly polarizable, and their high polarizability makes the molecules to be highly reactive to specific interactions with polar solvents Anokwuru *et al.* (2012).

4.2 Phytochemical analysis by UPLC-QToF/MS on *R. tomentosa* extracts

In this study, UPLC-QToF/MS was used to identify the active components in samples from *R. tomentosa* with intention to relate the extracts composition and bioactivities. The compounds in MeOH, $CHCl_3$ and EtOAc extracts of root of *R. tomentosa* were identified by their characteristic mass fragments and are shown to contain constituents under chemical classification of flavonoids, tannins, xanthenes, phenols, glycosides, pheromones, chromones, triterpenes and pyranes.

The retention time(RT), mass error, ion response and fragmentation pattern were compared to perform the peak identification of each compound. The verification process for the components became easier, as Mass Fragment has automatically elucidated fragmentations. The adductions and the fragmentation ions were considered to verify if match is reasonable of elemental compositions were inferred in the light of high accuracy molecular ions such as $[M + H]^+$, $[M + Na]^+$ and $[M + K]^+$ within the mass accuracy of 5mDa and high ion response.

As it can be seen from the chromatograms in Appendixes A2, A3 and B3, methylellagic acid was detected in the first 12 minutes in all (-) ESI methanol, (+) ESI methanol extract. The response in the data of confirmed compounds (Appendix A1) signified that due to the relative high sensitivity of (-) ESI compared to (+) ESI, negative mode (-) ESI-MS was effective in terms of identifying methylellagic acid which is found to be most abundant in MeOH extract of *R. tomentosa*. Similarly, previous studies revealed that hydrolysable tannins were identified as one of the major components found in the extracts of *R. tomentosa* Y. Z. Liu *et al.* (1997). The triterpenoid compound 3B-Acetoxy-11a,12a-epoxyoleanan-28,13B-olide is the major compound found in chloroform extract (Appendixes D2, D3). More flavonoid constituents than other class of chemicals are found in the plant material that includes myricetin, naringenin, kushinol, kaempferol and leucodelphinidin. That could be the reason that the *R. tomentosa* is known for good antioxidant property.

Rhodomyrtone, which is known as a natural antibiotic Limsuwan *et al.* (2009) is found abundantly in the ethyl acetate extract of *R. tomentosa* at retention time of 11.8 minutes (Appendixes C2, C3). This might contribute to higher antibacterial activity that the extract attained in this study. Figure 4.1 shows the chromatogram of rhodomyrtone on ethyl acetate extract of *R. tomentosa*. Acylphloroglucinols, rhodomyrtosone A (Appendixes C1, C2) rhodomyrtosone B (Appendixes B1, B2), were found in the ethyl acetate and methanol extracts respectively while tomentosone A and tomentosone B are found abundantly in the chloroform and methanol extracts respectively. Table 4.1 shows the phytochemical analysis and chemical classification of major components of *R. tomentosa* root extract.

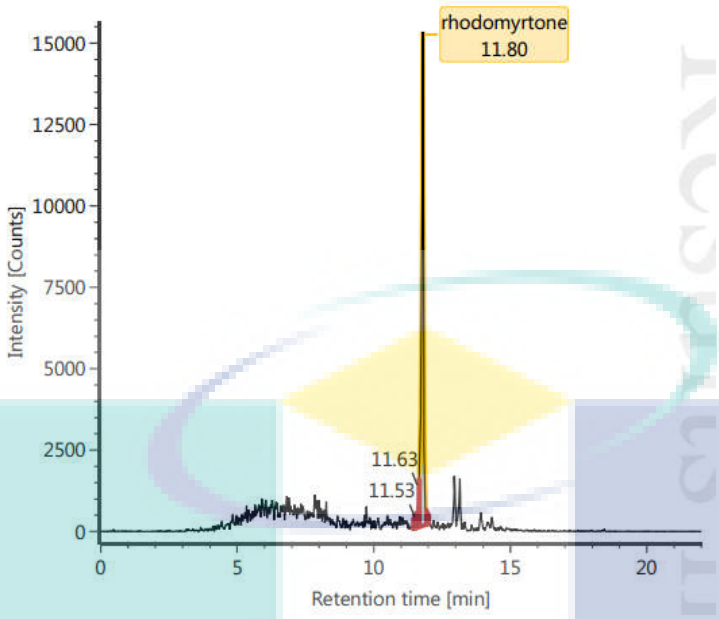


Figure 4.1 UPLC chromatogram of rhodomyrtone in ethyl acetate extract of *R. tomentosa*

Table 4.1 Qualitative phytochemical analysis and chemical classification of Major components of *R. tomentosa* root extract

		***R. tomentosa* root extract constituents**
Chemical classifications	Flavonoids	myrcetine, naringenin, kushinol, glabrol, kaempferol, leucodelphinidine.
	Tannins	methylellagic acid, 1-galloyl-β-D-glucose
	Xanthenes	rhdomyrtone,
	Phenols	tomentosone A, tomentosone B,
	Chromones	aloeresin G
	Triterpenes	3B-Acetoxy-11a,12a-epoxyoleanan-28,13B-olide
	Acylphloroglucinols	rhodomyrtosone A, rhodomyrtosone B,

The precursor ions of *R. tomentosa* in (-) ESI and (+) ESI methanol, ethyl acetate and chloroform extract for rhodomyrtone were as $[M + CH_3COO]^+$ at m/z 503.2622, $[M + H]^+$ at m/z 445.2583, $[M + H]^-$ at m/z 445.2568 and $[M + H]^+$ at m/z 445.2549. The summary of chemical constituents identified in each extract is listed in Table 4.2.

Table 4.2 chemical costituents identified from root extract of *R. tomentosa* in respective solvents.

***R.tomentosa* root extract**	**Constituents**
Methanol (+ve)	methylellagic acid, leucodelphinidine, aleoresin G, (3S,5R,6R,7E,9S)-megastiman-7-ene-3,5,6,9-tetrol, rhodomyrtosone B, 7-hydroxy-1-methoxy-2-methoxyxanthone
Methanol (-ve)	tomentosone B, methylellagic acid, 1-galloyl-β-D-glucose
Ethyl acetate	myrcetine, naringenin, kushinol B, rhodomyrtone, 3B-Acetoxy-11a,12a-epoxyoleanan-28,13B-olide
Chloroform	kaempferol-3-O-α-L-arabinoside, kushinol B, tomentosone A, 3B-Acetoxy-11a,12a-epoxyoleanan-28,13B-olide

4.3 Characterization of RTE/PVA nanofibers

4.3.1 FESEM characterization

Surface morphology was studied by a field emission scanning electron microscope (FESEM, Hitachi S-7400, Hitachi, Japan) equipped with energy dispersive X-ray (EDX). The fundamental principle in characterization capabilities of EDX is due in majority that an element has a unique atomic structure that allows a unique set of peaks on its electromagnetic emission spectrum Goldstein (2003). In the study of nanoparticles, FESEM gives information about distribution and size of the nanoparticles within the continuous matrix, and the possible orientation of nanoparticles Choo *et al.* (2016). Whereas, in regard to nanofibers, FESEM result determines the morphological structure and fiber diameter of electro spun nanofiber Abdullah *et al.* (2014). In this study, the FESEM result indicates all weight ratios of the extract loaded PVA solution and the 10% PVA solution has made a nanofiber with a diameter in between 76.3 nm and 388.6 nm respectively. That has assured that the fiber patches loaded with *R. tomentosa* extracts were in the nanoscale range. As shown in Figure 4.3, the RTE/PVA solution has formed a nanofiber that did not interfere with formation of beads. Besides, no crystals of *R. tomentosa* extract was observed on the structures of the nanofibers. This result indicated that the *R. tomentosa* extract was well incorporated in the fibers. Figure 4.2 shows the FESEM pictures of 10% PVA, 0.25% RTE/PVA, 0. 5% RTE/PVA, 1.5% RTE/PVA, 2.5% RTE/PVA, 0.25% RTE/PVA nanofibers at 40 K magnification. The picture of nanofiber maths of 10% PVA solution and that of 2.5% RTE/PVA solution are shown in appendix F1.

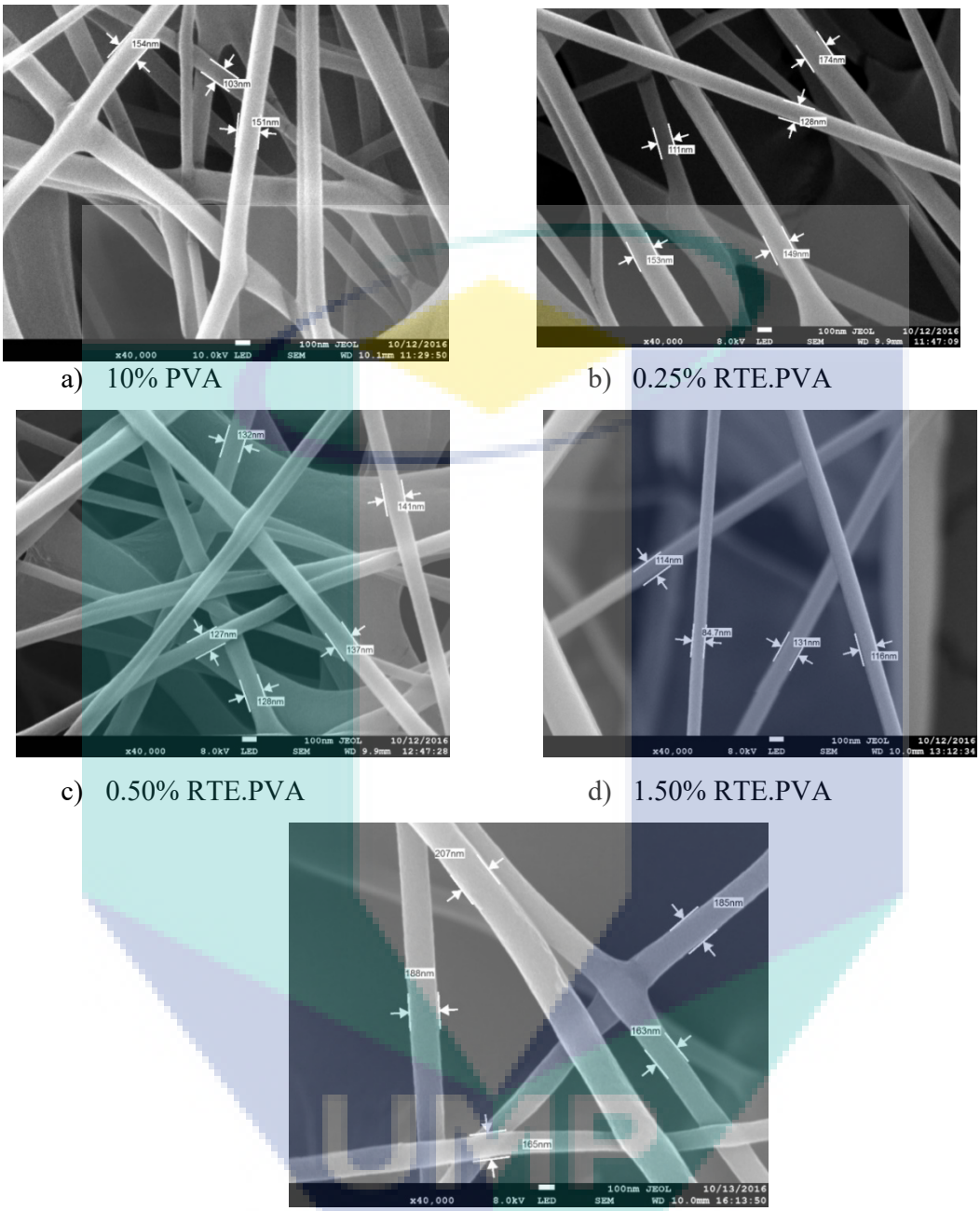


Figure 4.2 FESEM image of RTE/PVA nanofibers at magnification 40k X

Table 4.3 Average diameter of different 10%PVA, 0.25%RTE/PVA, 0.50% RTE/PVA, 1.50% RTE/PVA, 2.50% RTE/PVA solution nanofibers. Data are expressed as mean + SD (n = 100)

Nanofibers	**Average diameter (nm)**
10% PVA	119 ± 22.63
0.25% RTE/PVA	120 ± 30.47
0.50% RTE/PVA	153 ± 42.20
1.50% RTE/PVA	168 ± 45.21
2.50% RTE/PVA	214 ± 60.13

Since nanoscale fibers are defined as fibers with diameter range of 50 nm to 500 nm Zhou (2006), the diameter of all weight ratios of RTE/PVA fibers in this study were in the range of nanometer scale. The average diameter of all ratio of RTE/PVA extract and that of 10% PVA solution is shown in Table 4.3 and Figure 4.4 shows the diameter distribution of these fibers. The average diameter of the fibers increases as the concentration of *R. tomentosa* increases. The difference in diameter became higher as the amount of the extract in the 10 % PVA solution increases. The reason may be same as it was reported by some studies that a nanofiber diameter increases with increase in concentration and viscosity of a solution Deitzel *et al.* (2001; Yang *et al.* (2004; Zhou (2006).

The increase in concentration of RTE in the 10% PVA solution not only increased the average diameter (from 120 nm (Fig. 4.3 (a)) to 214 nm (Fig. 4.3 (d)) but also it broadens the fiber size distribution. This can be clearly observed that the percentage frequency of fibers in the range of 200-250 nm increases as the concentration of RTE/PVA increases. Two factors which account for this effect are the concentration of RTE in PVA solution, which leads to an increased diameter and viscosity that leads to broader distribution of the nanofibers' diameter due to nonuniform ejection of the jet Imran *et al.* (2013; H. Wu & Pan (2006).

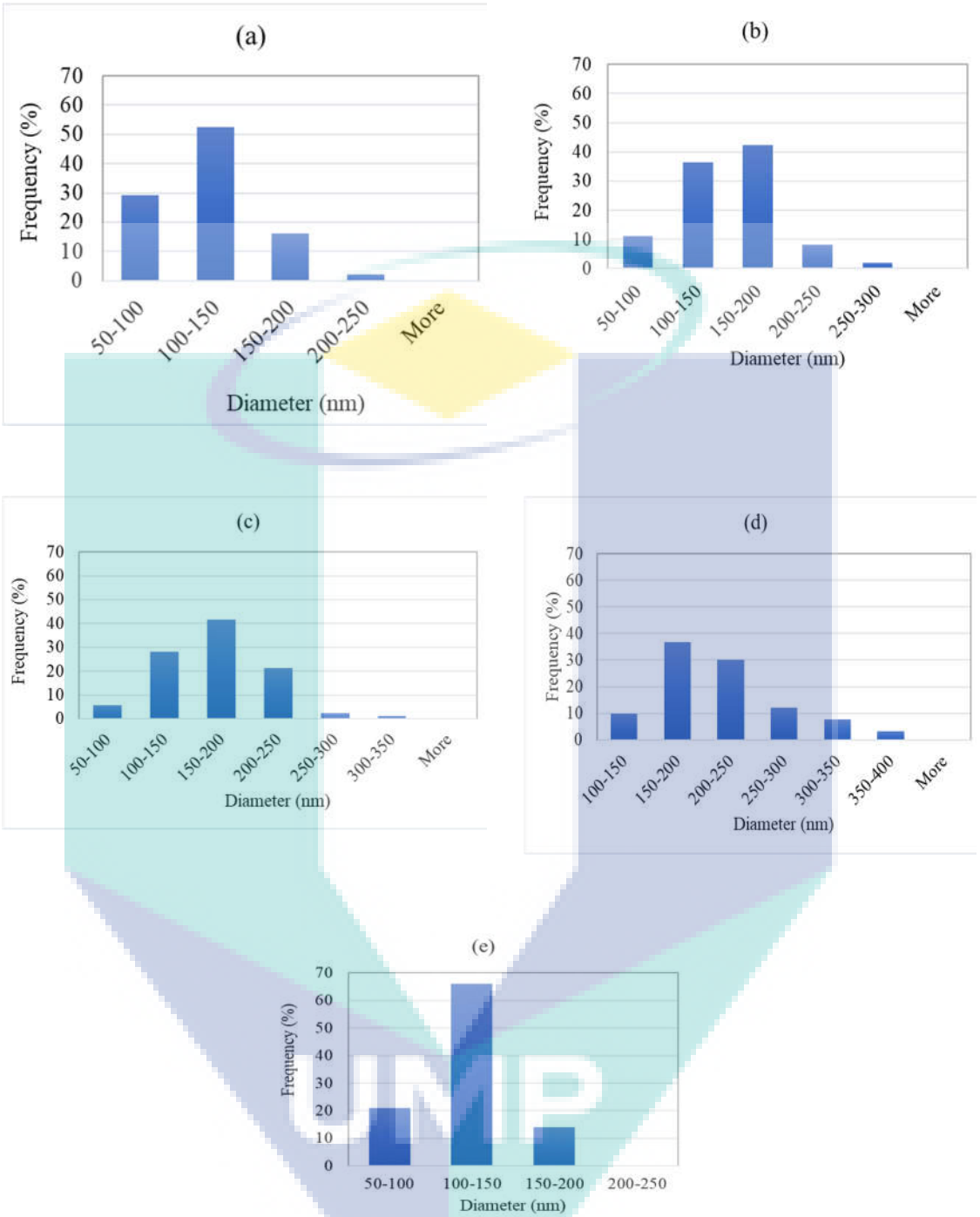


Figure 4.3 Histogram of diameter distribution of PVA and Extract loaded PVA nanofiber (a) 0.25% RTE/PVA, (b) 0.5% RTE/PVA, (c) 1.5% RTE/PVA, (d) 2.5% RTE/PVA and (e) 10% PVA.

4.3.2 XRD characterization

The XRD pattern of the RTE/PVA nanofibers were analysed using a Rigaku Miniflex II, X-ray diffraction (XRD) using copper Kα radiation (wavelength, λ = 0.154 nm) and at an angle 2θ from 2–80° to confirm the loading of *R. tomentosa* extract on the PVA nanofibers.

Though the crystalline structure and chain orientation in polymer nanofibers electrospun is dependent on process variables as molecular weight, polymer-solvent interactions, and process timescale Oliveira *et al.* (2013), The sharp peaks obtained in XRD pattern confirms the formation of highly good crystallinity materials Jesudoss *et al.* (2017). As the interpretation of XRD is based on positions and intensities of diffraction peaks which are unique to a given chemical compound Nobarzad *et al.* (2014), The orange line in Figure 4.4 diffractogram corresponds to pure PVA fiber which has strong intensive peak at an angle 2θ, 20° Nakane *et al.* (1999).

All the RTE/PVA nanofibers possess a pick that lies at 20° to imply that the *R. tomentosa* extract was successfully loaded in PVA nanofiber. A few peaks for RTE/PVA nanofibers at an angle of around 5°, 23° and 40° were also observed that might be due to crystallization of phytochemicals present in *R. tomentosa* extracts. The change in intensities may imply it is obvious that the *R. tomentosa* extract affect the physical structure of PVA nanofiber Shankar *et al.* (2014). The peaks showed considerable broadening is due to the Nano phase formation with less internal stress Thangavel *et al.* (2015).

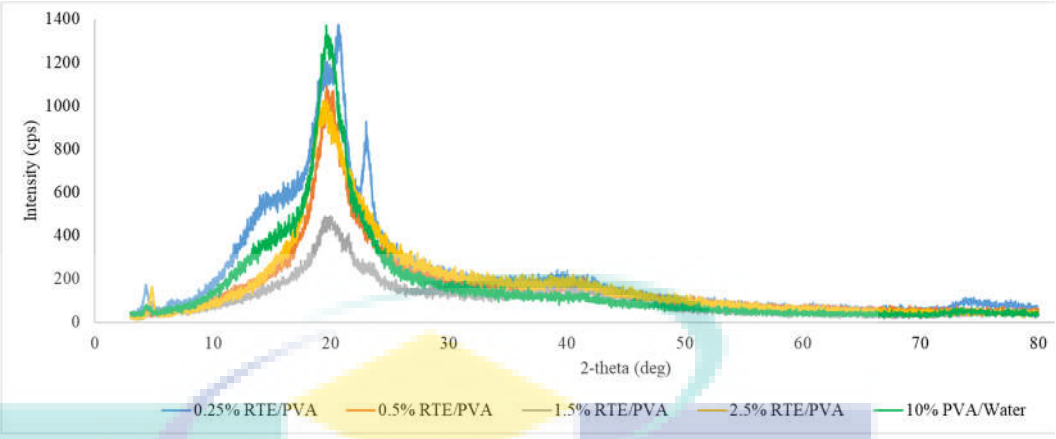


Figure 4.4 XRD patterns of nanofibers 10%PVA solution and RTE/PVA concentrations at 0.25%, 0.5%,1.5% and 2.5%.

4.3.3 Fourier transform infrared (FTIR) characterization

Spectroscopic characterization has been investigated by a Fourier transform infrared (FT-IR), the spectra were recorded as KBr pellets using Varian FTS 1000 FT-IR, Mid-IR spectral range.

FTIR spectra of electrospun fibers of PVA and different concentration of RTE/PVA solutions were recorded in the 700–4,000 cm^{-1} region. Figure 4.5 shows an overlap FTIR spectrum of PVA and RTE/PVA solutions of different concentration.

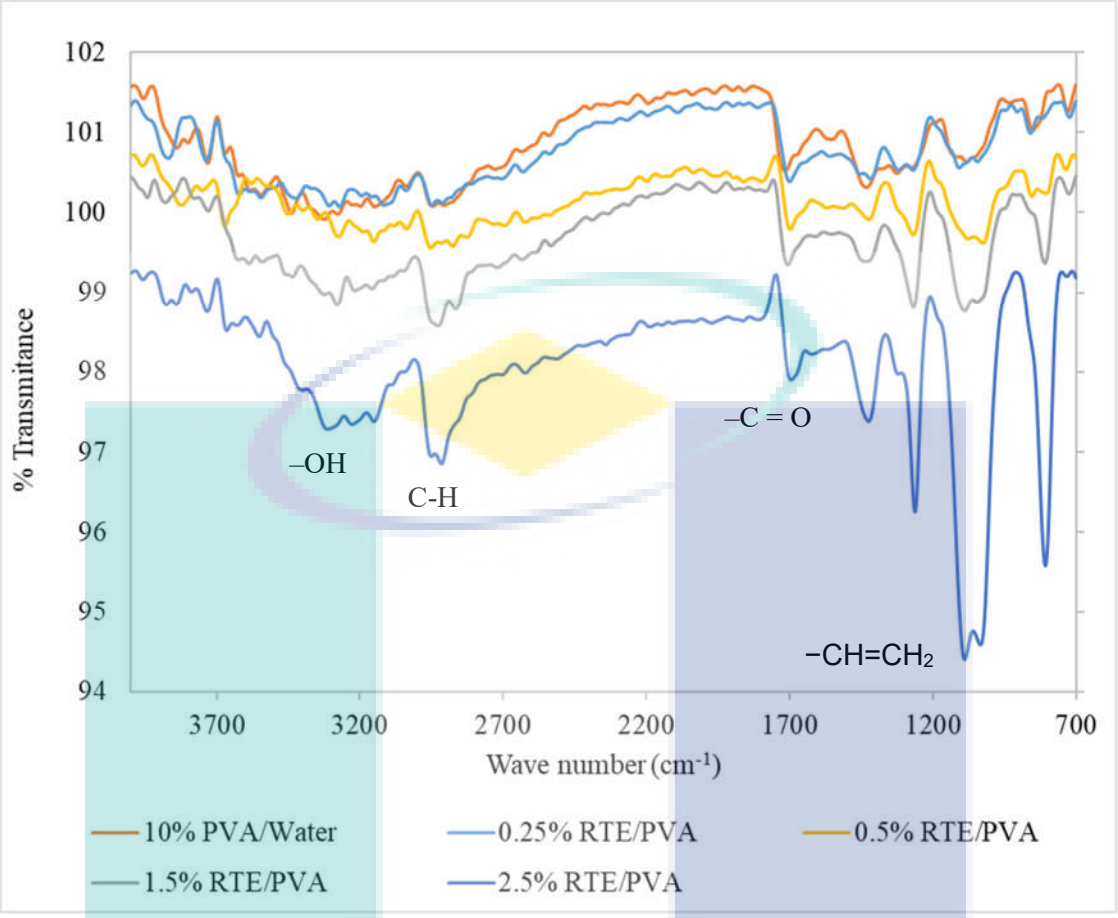


Figure 4.5 Fourier Transform Infrared Spectroscopy (FTIR) Spectra of PVA and RTE/PVA nanofibers in different concentrations of weight ratio.

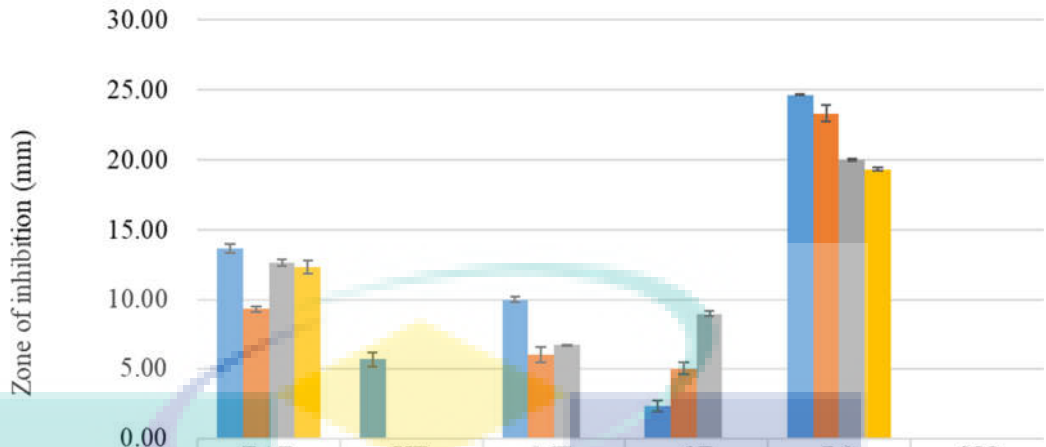
The basic composition of PVA is – $(CH_2–CHOH)_n$ and the monomer structure is (CH_2=CHOH). Intra and intermolecular hydrogen bindings are expected to occur among PVA chains due to high hydrophilic forces Reis *et al.* (2006). The peak observed at approximately 1081 cm^{-1}, where the area is considered to be a fingerprint for most chemical compounds Silverstein *et al.* (2014), is attributed to the presence of terminal poly vinyl groups. The pick at around 1715 cm^{-1} indicates the –C = O carbonyl stretching bond. It is also observed that the band obtained at 2900-3000cm^{-1} indicates C-H stretching bond and the peak between 3325 and 3840 $cm^{-1}$is hydrogen bonded –OH group. In addition, peak intensities at around 2947, 1431, 1081 cm^{-1} are assigned to the aliphatic CH group vibration of different modes in OH, CH_2 and CH, respectively Alarifi *et al.* (2015).

Overlapped FTIR spectra of PVA and RTE/PVA solution nanofibers are recorded as well in the spectral range of 700-4000 cm^{-1} as shown in Figure 4.5. Significant changes observed between the vibrational frequencies and intensity of nanofibers formation. Obviously with the increase of concentration of RTE in RTE/PVA nanofiber, the relative intensity of peak at around 1700 cm-1 which belongs to carbonyl group (–C = O) in PVA was increased as the correlation between absorbance and concentration is clearly stated in the beer-lambert law Swinehart (1962). This might be due to the carbonyl groups in phytochemicals like acylphloroglucinols, chromones, triterpenes and xanthenes. The broad bands noticed at 2916 -3732 cm^{-1} is due to stretching vibrations involving the hydroxyl groups of PVA and phytochemical constituents of *R. tomentosa* as flavonoids and phenols. From the FTIR result, the pick that was observed for PVA nanofibers were also observed in spectra of the different concentration of RTE/PVA nanofibers. The result indicates that the *R. tomentosa* extracts were well incorporated in to the PVA nanofiber patches.

4.4 Antimicrobial analysis

4.4.1 Antimicrobial test of *R. tomentosa* extracts

All the crude extracts were evaluated for their antimicrobial activity using the paper disc diffusion method. The inhibition zones for both the crude extracts, positive and negative controls were measured to compare the antibacterial effect of each of the extracts. Figure 4.6 shows antibacterial activity of root extracts of *Rhodomyrtus tomentosa* on *Bacillus subtilis* (Gram +ve), *Escherichia coli* (Gram -ve), *Pseudomonas aeruginosa* (Gram -ve) *and Enterococcus faecalis* (Gram +ve). The ethyl acetate extract (EAE) has recorded relatively higher (14mm) antibacterial activity than the other extracts. Besides, EAE was the only extract that had an activity against the gram-positive bacteria, *Enterococcus faecalis.* Hexane extract (HE) shows no activity against three bacterial strains, *E. coli, P. aeruginosa* and *E. faecalis*. Methanol extract (ME) has relatively good antibacterial activity next to the EAE whereas, all the four extracts showed zero activity against the antifungal test conducted on the fungal strain *Aspergillus niger.* Appendix E1-E4 shows the inhibition zone of the extracts within the reservoir in the Petri dishes.



	EAE	HE	ME	CE	PC	NC
B. subtilis	13.67	5.67	10.00	2.33	24.67	0.00
E. coli	9.33	0.00	6.00	5.00	23.33	0.00
P. aeurogenosa	12.67	0.00	6.67	9.00	20.00	0.00
E. faecalis	12.33	0.00	0.00	0.00	19.33	0.00

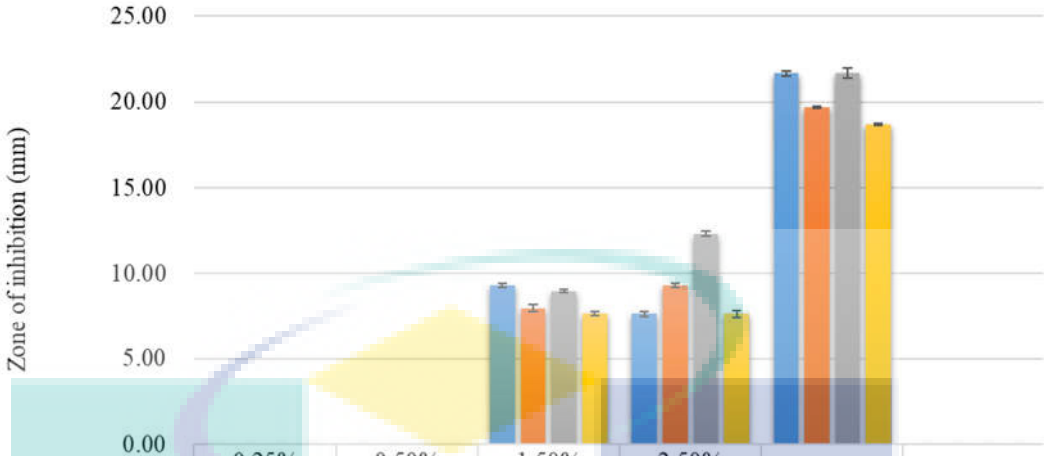
Figure 4.6 Antibacterial activity of root extracts of *R. tomentosa* on the test organisms

Crude extracts and their isolates of aerial parts of *R. tomentosa* were reported to show good antibacterial activity against different gram positive and gram negative bacterial strains Salni *et al.* (2002; Voravuthikunchai *et al.* (2010). Similarly, this study showed the root extract of the plant as well, was sensitive against the four bacterial strains evaluated. Flavonoids as kaempferol, myricetin, quercetin could contribute to the antibacterial activity of *R. tomentosa* as they have been reported to possess antimicrobial activity Cushnie & Lamb (2005) against human pathogenic microorganisms with some mechanisms of action such as inhibition of nucleic acid synthesis, cytoplasmic membrane function and energy metabolisms Hendra *et al.* (2011). The inhibitory action of quercetin and myricetin on DNA and RNA synthesis could be due to hydrogen bonding of B ring of the flavonoids with nucleic acid bases of the bacterial strains Cushnie & Lamb (2005). In addition, rhodomyrtone, which is found in all the methanol, ethyl acetate and chloroform extracts, was reported to have a strong antibacterial activity against *Streptococcus pneumoniae* Mitsuwan *et al.* (2017). Rhodomyrtone was reported to be non-harmful to human erythrocytes which is a highly desirable characteristic of an antibacterial agent Leejae, Taylor, *et al.* (2013). From the result in section 4.2, it was discussed that, rhodomyrtone was more abundant in the ethyl acetate extract than in methanol and chloroform extracts. Which could be the factor for its higher antibacterial

activity than the other extracts. As a result, ethyl acetate extract was selected to be incorporated to PVA nanofiber.

4.4.2 Antimicrobial test of ethyl acetate extract of *R. tomentosa* roots loaded PVA nanofiber

The antibacterial activity test result of RTE/PVA nanofibers with the relatively higher concentration of the extract (1.5 and 2.5%) showed good antibacterial activity against all test organisms where a clear zone of inhibition of, 7–12 mm, was observed indicating the effectiveness Gao *et al.* (2014) of the RTE/PVA nanofiber leached into the agar during the incubation period. The antibacterial effect could be associated with the constituents described in the above section mainly of rhodomyrtone. This result indicates that the RTE retained its antibacterial activity after the process of electrospinning. The RTE/PVA nanofibers with lower concentration (0.25 and 0.5%) of the extract showed no activity at all in all the test organisms (Appendixes E5-E8). This showed that the antibacterial activity of the elctrospun nanofibers is dependent on the concentration of the *R. tomentosa* extract loaded to PVA solution. Previous studies revealed that PVA nanofiber didn't have any antibacterial activity, instead it incubated some bacterial strains Kim *et al.* (2016). Based on that, PVA nanofiber was taken as a negative control in this study. The nanofibers show no activity as that of the pure extracts against the fungal strain, *Aspergillus niger*. Figure 4.7 shows antibacterial activity of root extracts of *R. tomentosa* loaded PVA nanofiber on the test organisms.



	0.25% W/V RTE/PVA	0.50% W/V RTE/PVA	1.50% W/V RTE/PVA	2.50% W/V RTE/PVA	PC	NC
B. subtilis	0.00	0.00	9.33	7.67	21.67	0.00
E. coli	0.00	0.00	8.00	9.33	19.67	0.00
P. aeurogenosa	0.00	0.00	9.00	12.33	21.67	0.00
E. faecalis	0.00	0.00	7.67	7.67	18.67	0.00

Figure 4.7 Antibacterial activity of root extracts of *R. tomentosa* loaded PVA nanofiber on the test organisms

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

The aerial part of *Rhodomyrtus tomentosa* is known for its good antibacterial activity. Based on this, the root of *R. tomentosa* was extracted with hexane, chloroform, ethyle acetate and methanol for phytochemical analysis and antimicrobial assay. The phytochemical analysis was done using the UPLC-QToF/MS technique which is known for its accuracy and efficiency in identification of constituents in a sample. Methanol, ethyl acetate and chloroform extract were analysed in intention to relate the available active constituents with the antimicrobial effect of the extracts. The result showed that majority of the constituents belongs to the group of chemicals compounds, flavonoids. These include, myricetin, eupatelitine, naringenin, rhamnetin, kushinol, koparine, glabrol, flavonochromon, kaempferol, dichotomitine, quercetine, bavachinin, leucodelphinidin. The hydrolysibile tannin, Methylelllagic acid is found to be most abundant compound in all the extracts except in chloroform extract. Rhodomyrtone, which is known to have a good antibacterial activity, is also identified from this root extract of *Rhodomyrtus tomentosa*. In addition to the flavonoids myricetin and quercetine, rhodomyrtone might be the reason behind the antibacterial activity of root of *R. tomentosa*. The class acylphloroglucinols, rhodomyrtosone A, rhodomyrtosone B and rhodomyrtosone C, were also identified from the root extract of *R. tomentosa*.

In recent decades, studies assessing the utilization of electrospun nanofibers containing natural products for the application in different fields are increasing. The electrospun RTE/PVA nanofibers were characterized by using XRD analysis, FESEM,

and FT-IR absorption spectroscopy methods. From the fiber morphology test by FESEM, it is found that the 10% PVA and (0.25, 0.5, 1.5 and 2.5%W/V) RTE/PVA electrospun fibers were successfully fabricated using electrospinning process to yield fibers with in a nanoscale range (76.3 - 388.6nm) and with average diameter size ranging from 119 – 215 nm. As the sharp peak at an angle of 20^0 in XRD pattern indicates that of PVA, the sharp peaks of RTE/PVA nanofibers at an angle of 2θ, 23^o and 40^o shows there could be crystallization of some phytochemicals in the extracts. While the peaks in the FTIR spectra indicate that the *R. tomentosa* extract is well merged in the PVA nanofiber.

Based on the disc diffusion assay result, it is assured that the root extract of *R. tomentosa* have good antibacterial activity against two gram positive (*Bacillus subtilis, Enterococcus faecalis*) and two gram negative (*Escherichia coli*, *Pseudomonas aeruginosa*) bacterial strains. However, the extract was very inactive against the fungal test organism, *Aspergillus niger.* This is the first work that assessed the root of *R.tomentosa* for its antimicrobial activity. In addition, the extract with better activity, ethyl acetate extract, was successfully loaded to 10% PVA solution at different concentrations (0.25, 0.5, 1.5 and 2.5%). The electrospun *R. tomentosa* loaded PVA nanofiber mats were also found to be active against the four bacterial strains with an inhibition zone ranging 7-12 mm. The production of *R. tomentosa* root extract loaded PVA nanofiber, which have good antibacterial activity, is primary to the area.

As a concluding remark, the root extract of *Rhodomyrtus tomentosa* is shown to have a good antibacterial activity against the bacterial strains: *P. aeruginosa, S. aureus, E. coli* and *B. subtilis.* Ethyle acetate extract showed a relatively higher antibacterial activity While all extracts showed no activity against the fungal strain *A. niger.* and a known antibacterial active constituent, rhodomyrtone, was detected from the root extract of *R. tomentosa. R. tomentosa* root extract loaded PVA nanofibers were fabricated successfully by electrospinning method and the nanofiber patches were assessed for the antimicrobial activity. The electrospun nanofiber with relatively higher concentration (2.5% W/V) of the extract show good antibacterial activity against all the test microorganisms. The characterization of the electrospun mats showed that the fibers have nanoscale diameter and ethyl acetat extract was incorporated well in the PVA nanofibers.

5.2 Recommendation

Studies investigated the crude extract of *R.tomentosa* for its biological activities and promising results have been achieved. However, emphasis should be given to further studies on separation and isolation of the pure compounds, which can be responsible for pharmaceutical/biological activities. This will give huge support in development of new alternative drugs. On the other hand, the existing studies made on *R. tomentosa* are limited to its aerial part. There are very limited studies on the root part of the plant. Therefore, the root extracts and their secondary metabolites could be investigated as incorporate in nanofibers for further biological activities. Cross-linking of the RTE/PVA nanofiber mats should be considered for further study so that stabilized antibacterial fibres against aqueous environments could be achieved. These active RTE/PVA nanofibers should be designed for further study in medical applications such as wound dressing, from the fact that electrospun scaffolds possess several advantages over traditional dressings for the treatment of chronic and acute wounds Hanna & Giacopelli (1997; Zahedi *et al.* (2010), high absorption of exudates from the wound site, efficient exchange of gases and nutrients to promote cells proliferation, physical protection of the injured tissue and the possibility to release functional molecules Zhang *et al.* (2017).

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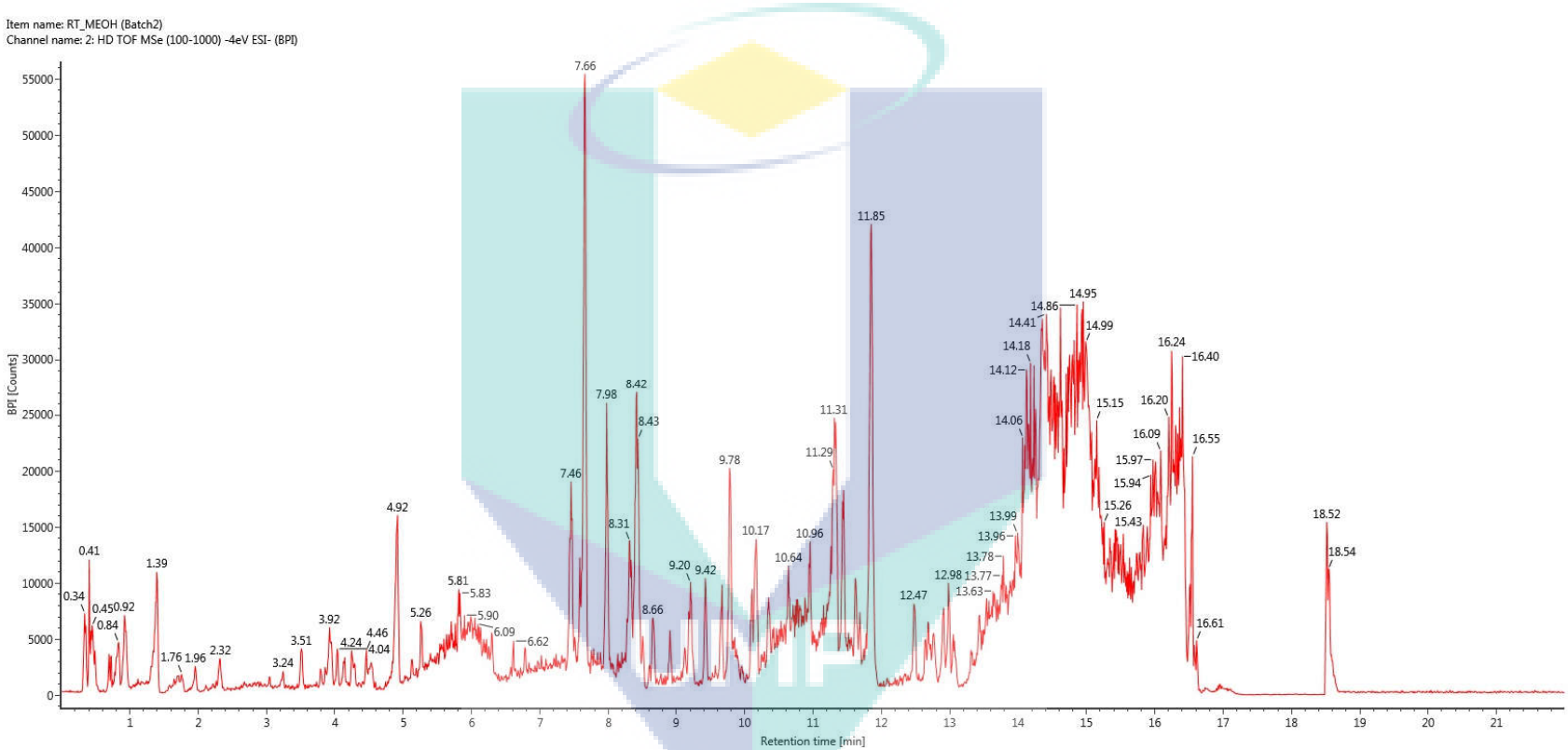
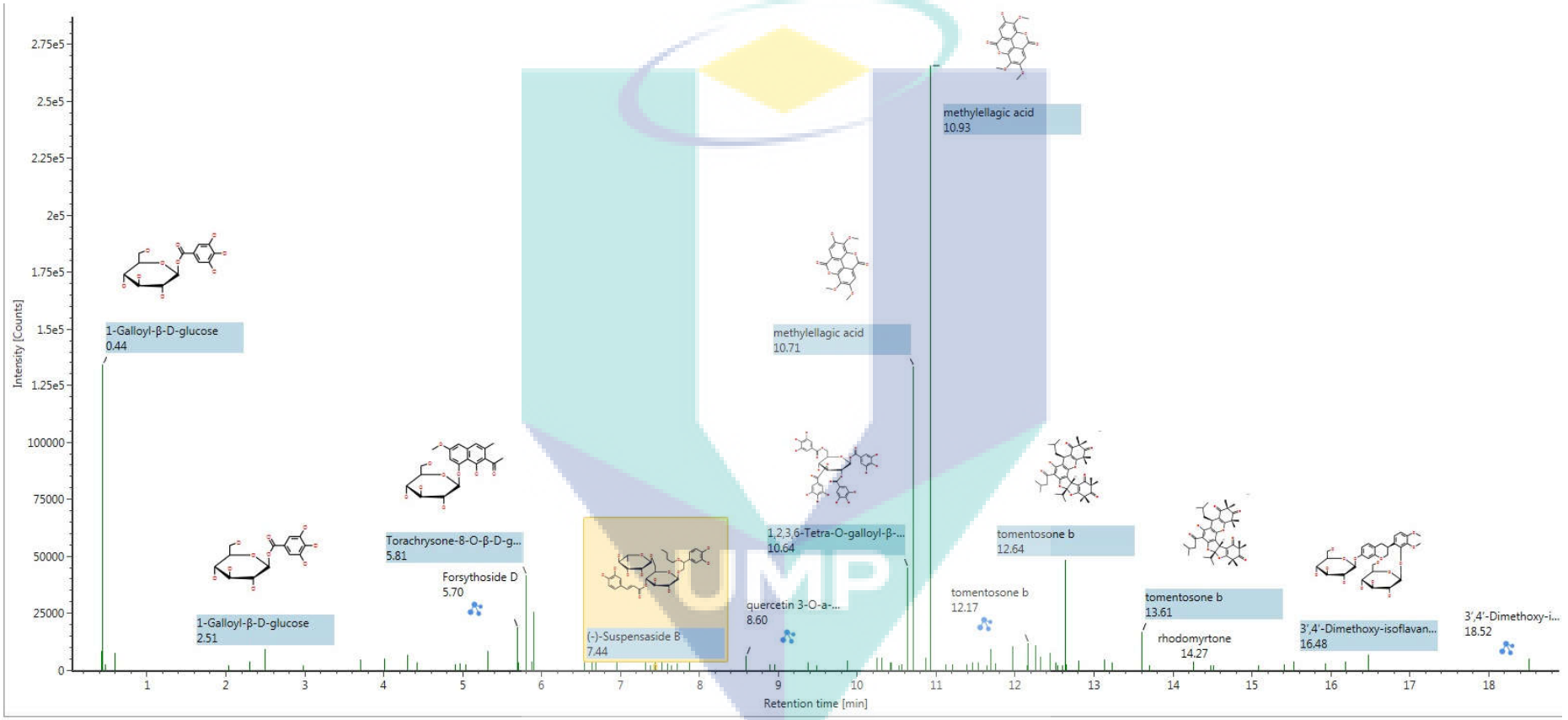
APPENDIX A1
UPLC-QTOF/MS NEGATIVE BPI CHROMATOGRAM OF *R. TOMENTOSA* METHANOLIC EXTRACT



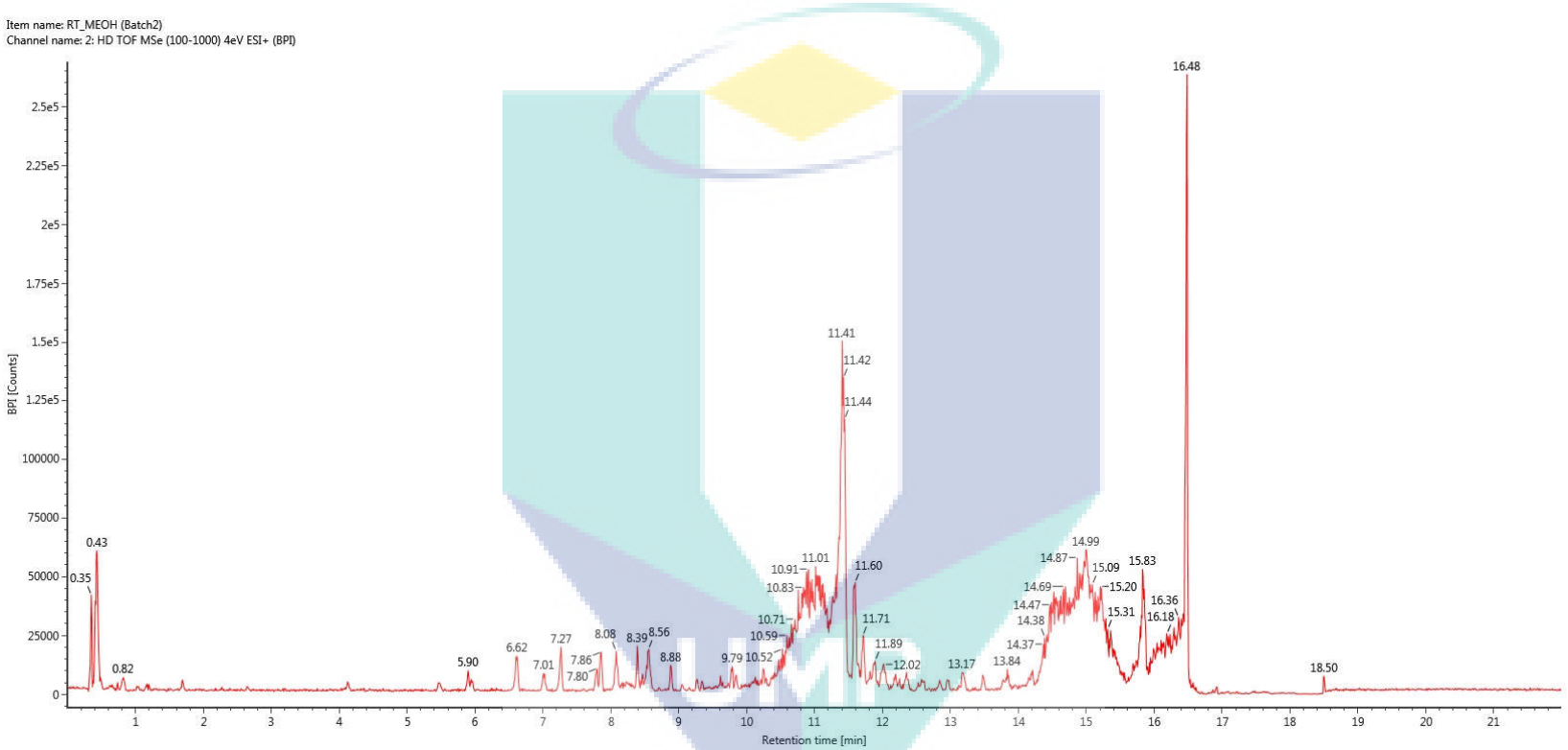
APPENDIX A2
DATA OF CONFIRMED COMPOUNDS ON (-) ESO-MS OF *R. TOMENTOSA* METHANOLIC EXTRACTS

Component name	Identification status	Neutral mass (Da)	Observed neutral mass (Da)	Observed m/z	Mass error (mDa)	Mass error (ppm)	Observed RT (min)	Observed drift (ms)	Observed CCS (Å²)	Response	Adducts
rhodomyrtosone C	Identified	674.38187	674.3728	673.3656	-9	-13.4	16.49	9.34	256.59	2718	-H
rhodomyrtone	Identified	444.25119	444.2484	503.2622	-2.8	-5.6	14.27	7.99	222.8	3917	+CH3COO
Forsythoside E	Identified	462.17373	462.1697	507.1679	-4	-8	4.93	8.28	230.35	10013	+HCOO
1-Galloyl-β-D-glucose	Identified	332.07435	332.0706	331.0633	-3.7	-11.3	1.4	5.97	174.67	16593	-H
3,7-Dihydroxy-2,4-dimethoxyphenanthrene-3-O-glucoside	Identified	432.14203	432.1381	477.1363	-4	-8.3	8.66	8.39	233.95	24531	+HCOO
tomentosone b	Identified	688.36113	688.3556	687.3483	-5.5	-8	12.64	9.58	263.09	48212	-H
methylellagic acid	Identified	344.05322	344.0512	343.0439	-2	-5.8	11.32	8.27	234.59	513301	-H

APPENDIX A3
CONFIRMED MAJOR COMPOUNDS PLOTTED ON (-) ESI-MS OF METHANOL EXTRACT



APPENDIX B1
UPLC-QTOF/MS POSITIVE BPI CHROMATOGRAM OF *R. TOMENTOSA* METHANOLIC EXTRACT

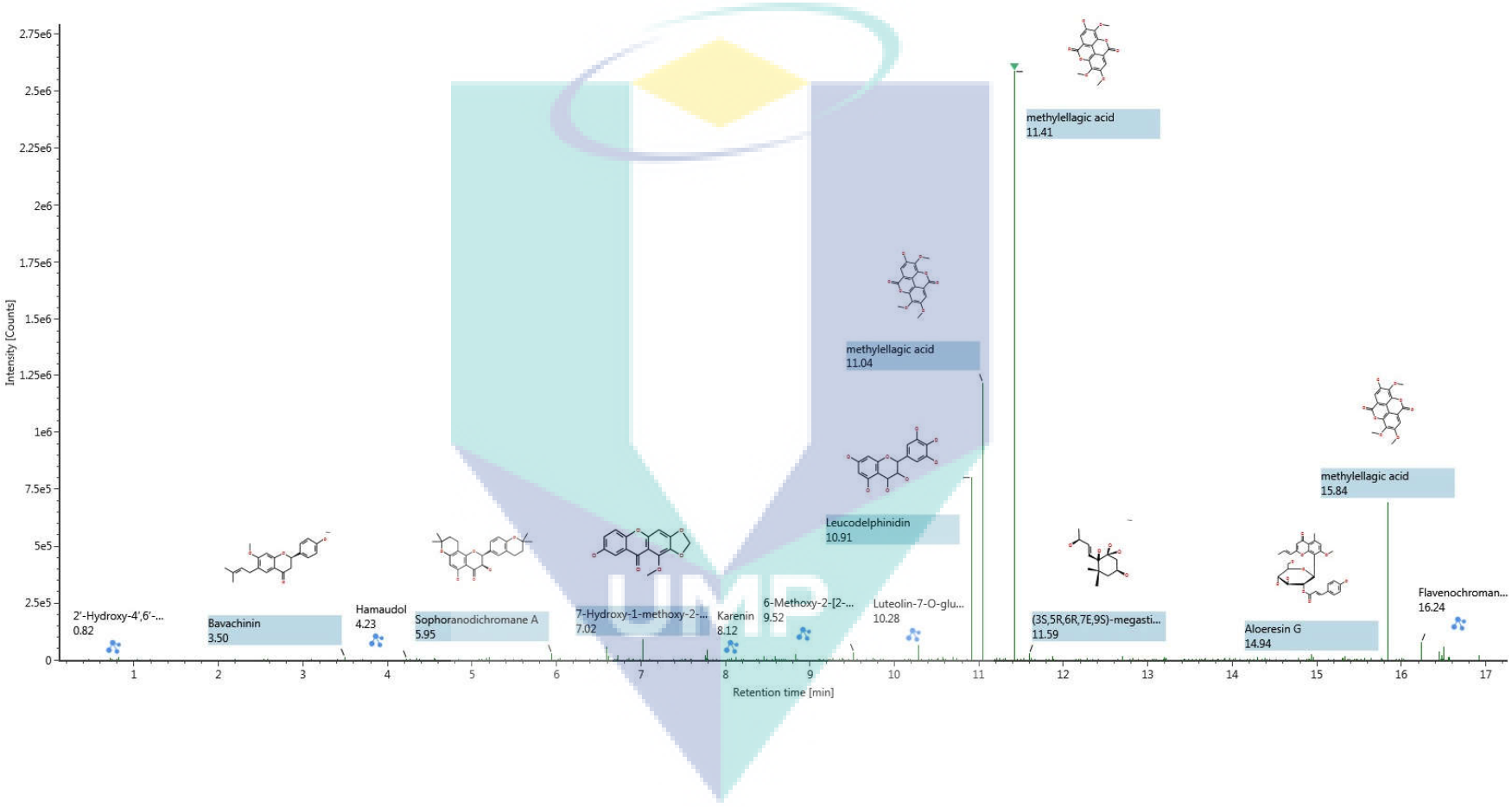


APPENDIX B2
DATA OF CONFIRMED COMPOUNDS ON (+) ESI-MS OF *R. TOMENTOSA* METHANOLIC EXTRACTS

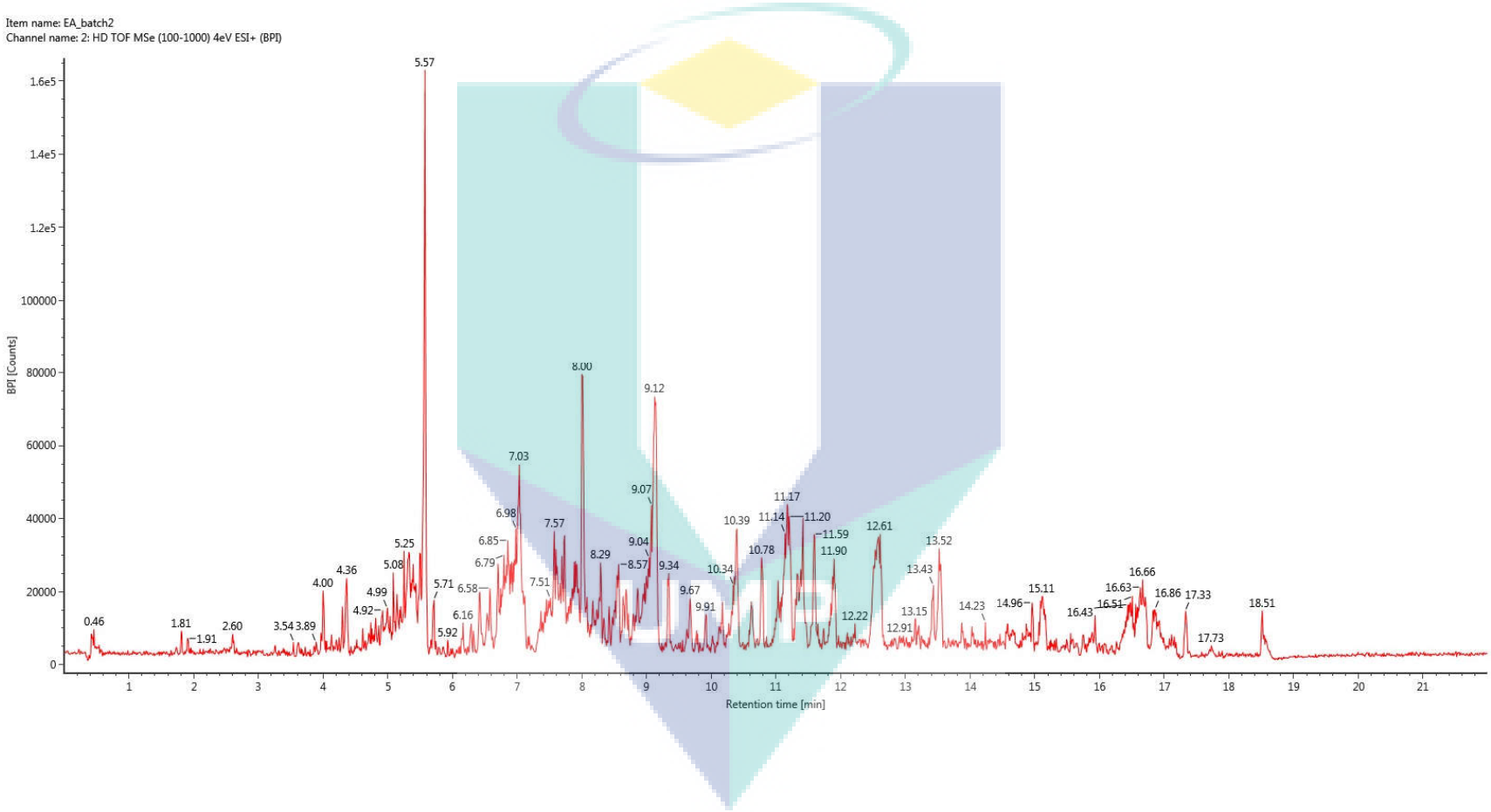
Component name	Identification status	Neutral mass (Da)	Observed neutral mass (Da)	Observed m/z	Mass error (mDa)	Mass error (ppm)	Observed RT (min)	Observed drift (ms)	Observed CCS (Å²)	Response	Adducts
7-Hydroxy-1-methoxy-2-methoxyxanthone	Identified	286.04774	286.0478	287.0551	0.1	0.3	7.02	5.37	163.82	90244	+H
Leucodelphinidin	Identified	322.06887	322.0714	345.0606	2.5	7.2	10.91	5.78	172.73	802163	+Na
methylellagic acid	Identified	344.05322	344.0537	345.061	0.5	1.4	11.41	10.11	291.89	186888	+H
(3S,5R,6R,7E,9S)-megastiman-7-ene-3,5,6,9-tetrol	Identified	246.14672	246.147	269.1363	0.3	1.1	11.59	5.2	160.21	30714	+Na
rhodomyrtosone B	Identified	450.29814	450.3004	473.2897	2.3	4.8	14.39	7.53	215.51	8680	+Na
rhodomyrtone	Identified	444.25119	444.251	445.2583	-0.2	-0.5	14.88	7.17	206.39	2546	+H
Aloeresin G	Identified	538.1839	538.1838	539.1911	0	-0.1	14.94	8.43	239.43	23433	+H

APPENDIX B3

CONFIRMED MAJOR COMPOUNDS PLOTTED ON (+) ESI-MS OF METHANOL EXTRACT



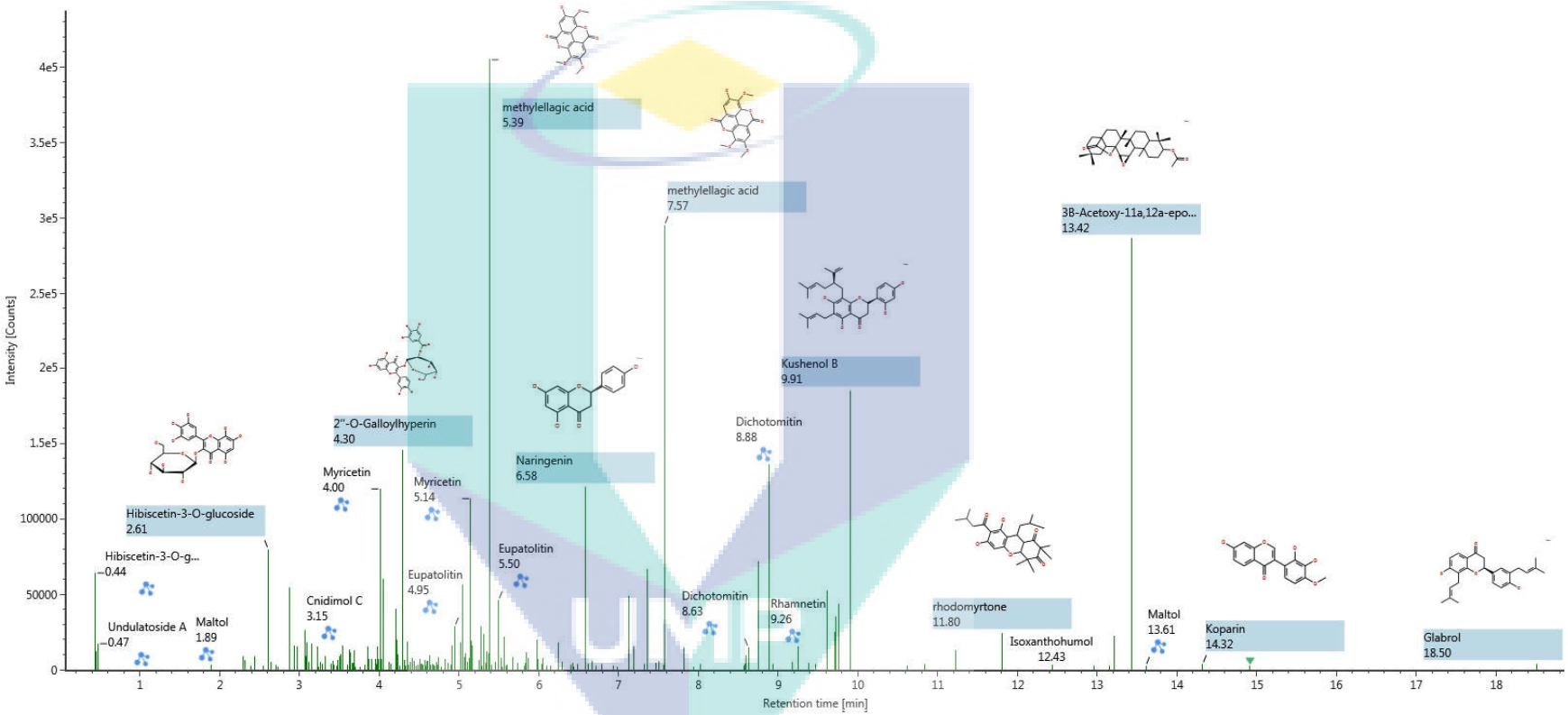
APPENDIX C1
UPLC-QTOF/MS POSITIVE BPI CHROMATOGRAM OF *R. TOMENTOSA* ETHYL ACETATE EXTRACT



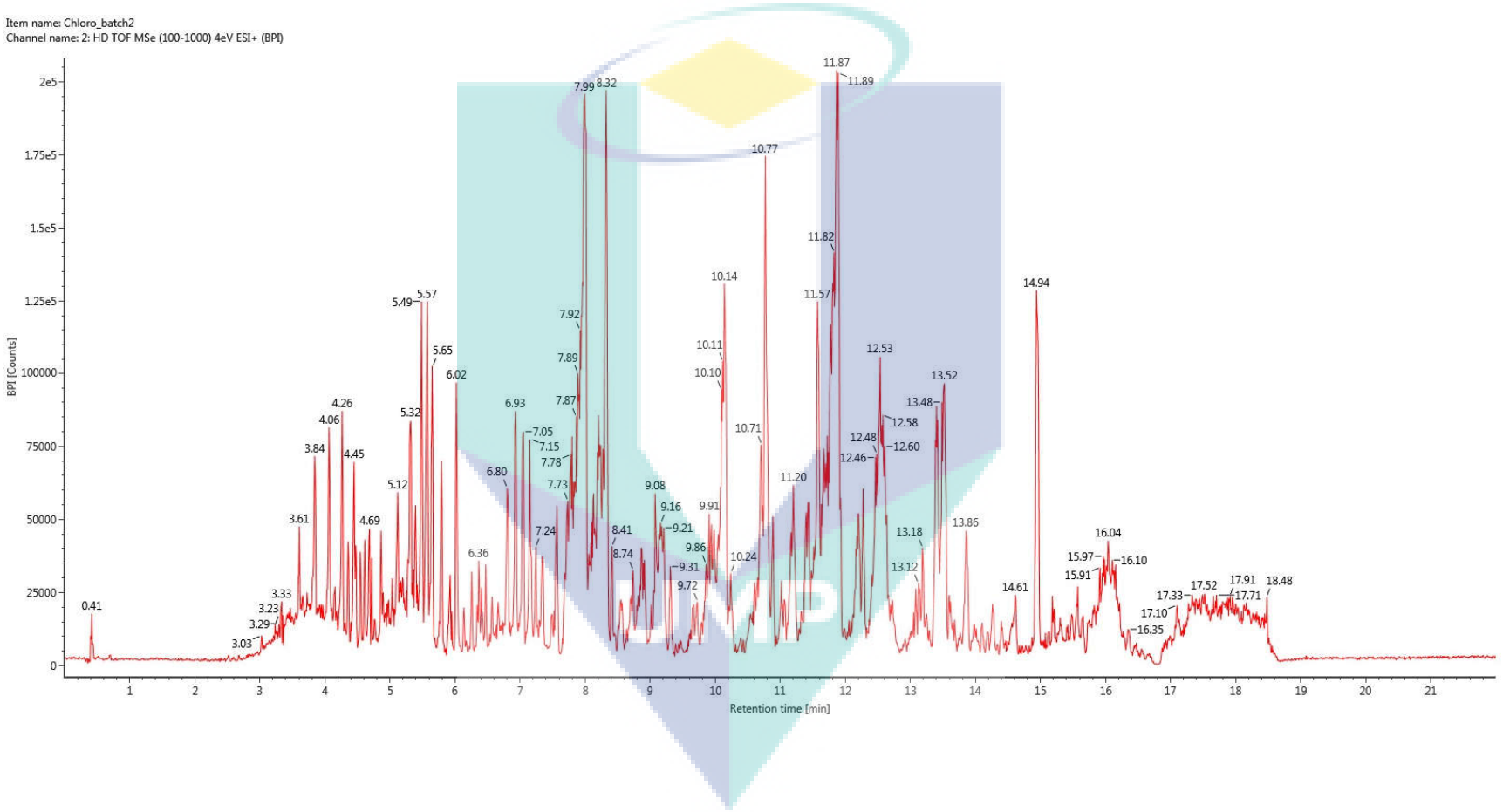
APPENDIX C2
DATA OF CONFIRMED COMPOUNDS ON *R. TOMENTOSA* ETHYL ACETATE EXTRACTS

Component name	Identification status	Neutral mass (Da)	Observed neutral mass (Da)	Observed m/z	Mass error (mDa)	Mass error (ppm)	Observed RT (min)	Observed drift (ms)	Observed CCS (Å²)	Response	Adducts
Myricetin	Identified	318.03757	318.0377	319.045	0.1	0.5	5.14	5.62	169.24	113670	+H
methylellagic acid	Identified	344.05322	344.0534	345.0607	0.2	0.7	5.57	5.75	171.93	1029098	+H
Naringenin	Identified	272.06847	272.0688	273.0761	0.3	1.1	6.58	5.3	162.62	120977	+H
rhodomyrtosone A	Identified	444.2148	444.2138	445.2211	-1	-2.3	7.82	7.34	210.75	11219	+H
Kushenol B	Identified	492.25119	492.2509	493.2582	-0.3	-0.6	9.91	7.76	221.49	185039	+H
rhodomyrtone	Identified	444.25119	444.2495	445.2568	-1.7	-3.7	11.8	7.46	214.08	24463	+H
3B-Acetoxy-11a,12a-epoxyoleanan-28,13B-olide	Identified	512.35017	512.3507	513.3579	0.5	1	13.42	8.01	228.18	286453	+H

APPENDIX C3
CONFIRMED MAJOR COMPOUNDS PLOTTED ON ESI-MS OF ETHYL ACETATE EXTRACT



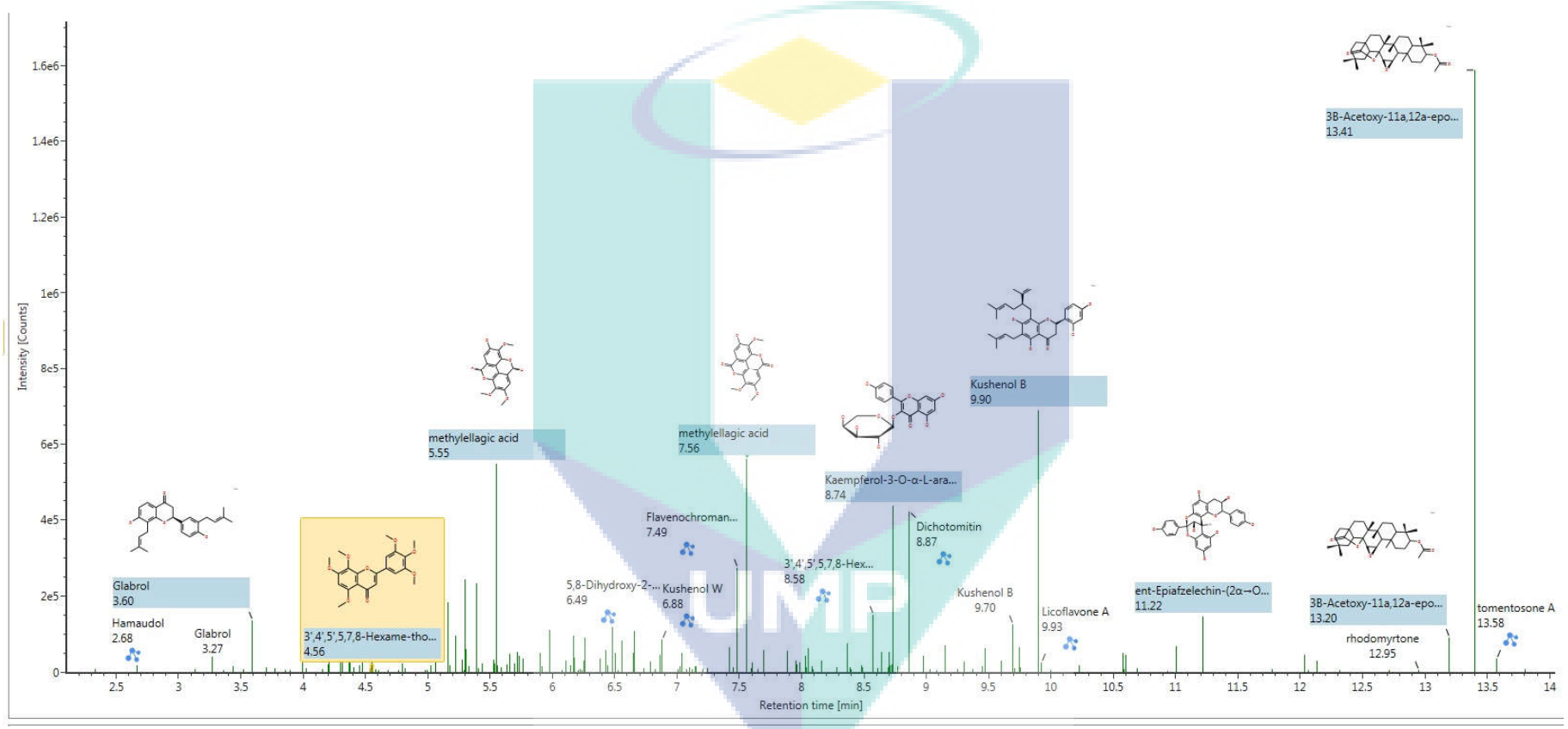
APPENDIX D1
UPLC-QTOF/MS POSITIVE BPI CHROMATOGRAM OF *R. TOMENTOSA* CHLOROFORM EXTRACT



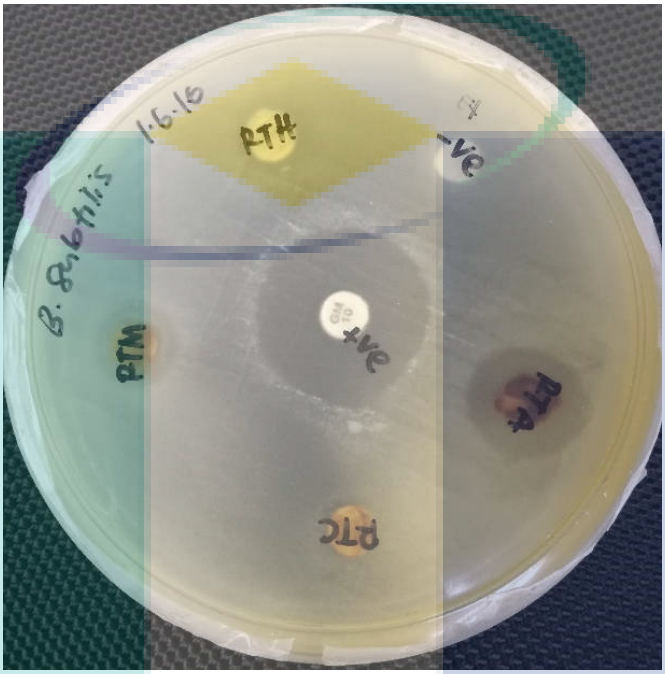
APPENDIX D2
DATA OF CONFIRMED COMPOUNDS ON *R. TOMENTOSA* CHLOROFORM EXTRACTS

Component name	Identification status	Neutral mass (Da)	Observed neutral mass (Da)	Observed m/z	Mass error (mDa)	Mass error (ppm)	Observed RT (min)	Observed drift (ms)	Observed CCS (Å²)	Response	Adducts
Glabrol	Identified	392.19876	392.2029	393.2101	4.1	10.4	3.6	6.05	178.64	134906	+H
Leucodelphinidin	Identified	322.06887	322.0715	345.0608	2.7	7.8	5.39	5.73	171.44	232561	+Na
methylellagic acid	Identified	344.05322	344.0535	345.0608	0.3	0.8	5.55	5.73	171.52	548637	+H
Kaempferol-3-O-α-L-arabinoside	Identified	418.09	418.09	419.0972	0	-0.1	8.74	6.39	186.76	437323	+H
Kushenol B	Identified	492.25119	492.2512	493.2585	0	0	9.9	7.74	221.05	687777	+H
rhodomyrtone	Identified	444.25119	444.2476	445.2549	-3.6	-8.1	12.95	7.5	215.05	5041	+H
3B-Acetoxy-11a,12a-epoxyoleanan-28,13B-olide	Identified	512.35017	512.3509	513.3582	0.7	1.4	13.41	7.99	227.64	1588913	+H
tomentosone A	Identified	698.43938	698.4412	737.4044	1.9	2.5	13.58	9.5	268.69	35546	+K

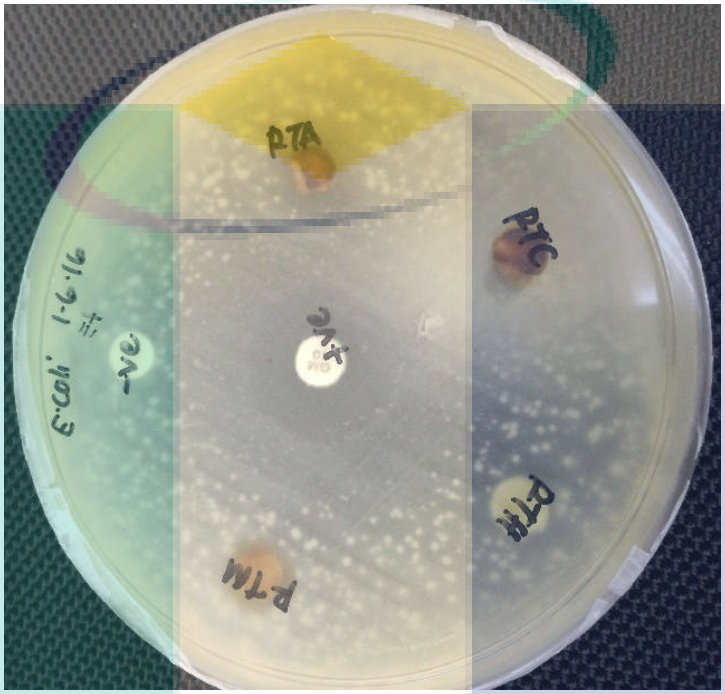
APPENDIX D3
CONFIRMED MAJOR COMPOUNDS PLOTTED ON ESI-MS OF CHLOROFORM EXTRACT



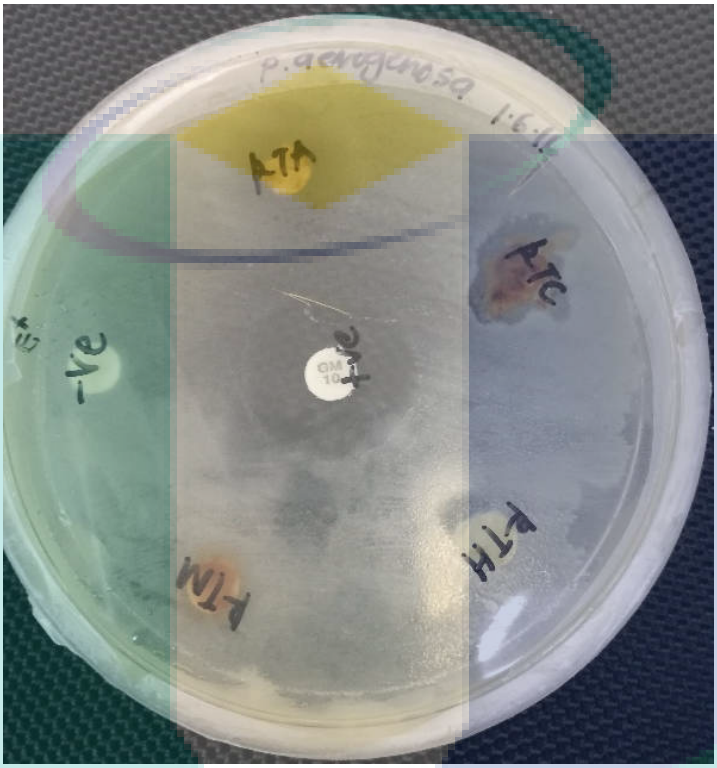
APPENDIX E1
SAMPLE PETRI DISHES OF EXTRACTS WITH INHIBITION ZONE AGAINST B. *SUBTILIS*



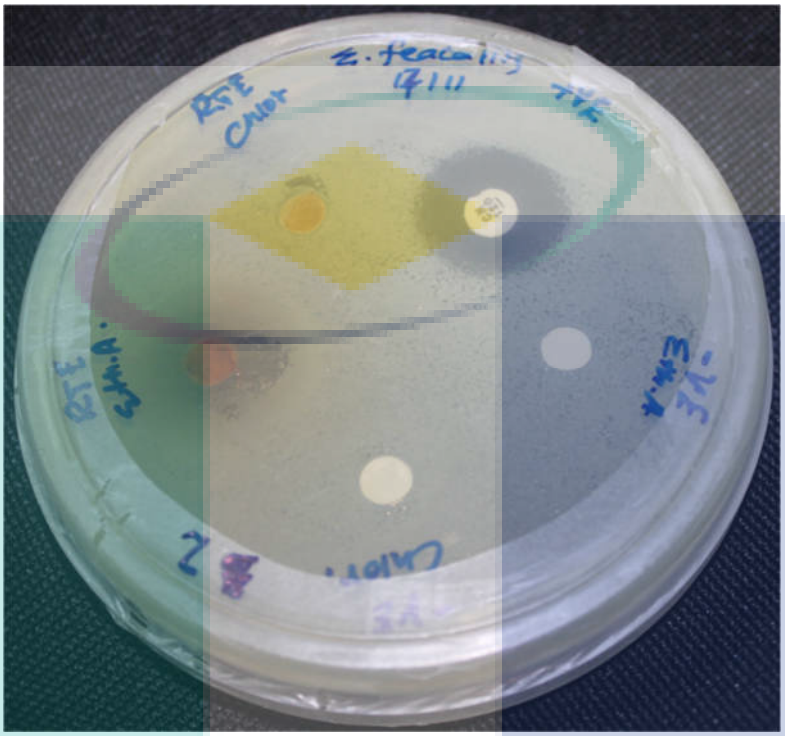
APPENDIX E2
SAMPLE PETRI DISHES OF EXTRACTS WITH INHIBITION ZONE AGAINST *E. COLI*



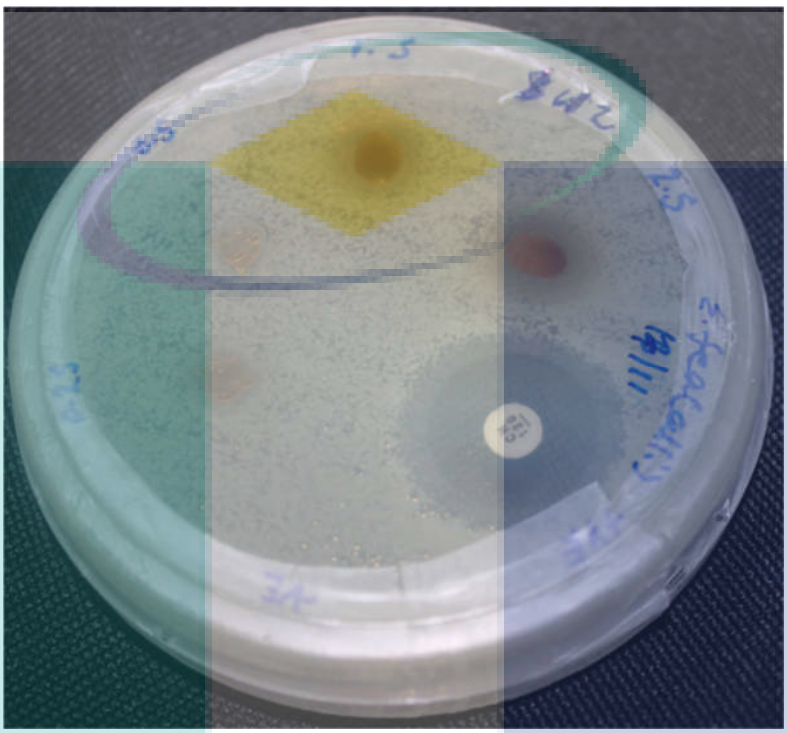
APPENDIX E3
SAMPLE PETRI DISHES OF EXTRACTS WITH INHIBITION ZONE AGAINST *P. AERUGINOSA*



APPENDIX E4
SAMPLE PETRI DISHES OF EXTRACTS WITH INHIBITION ZONE AGAINST *E. FAECALIS*

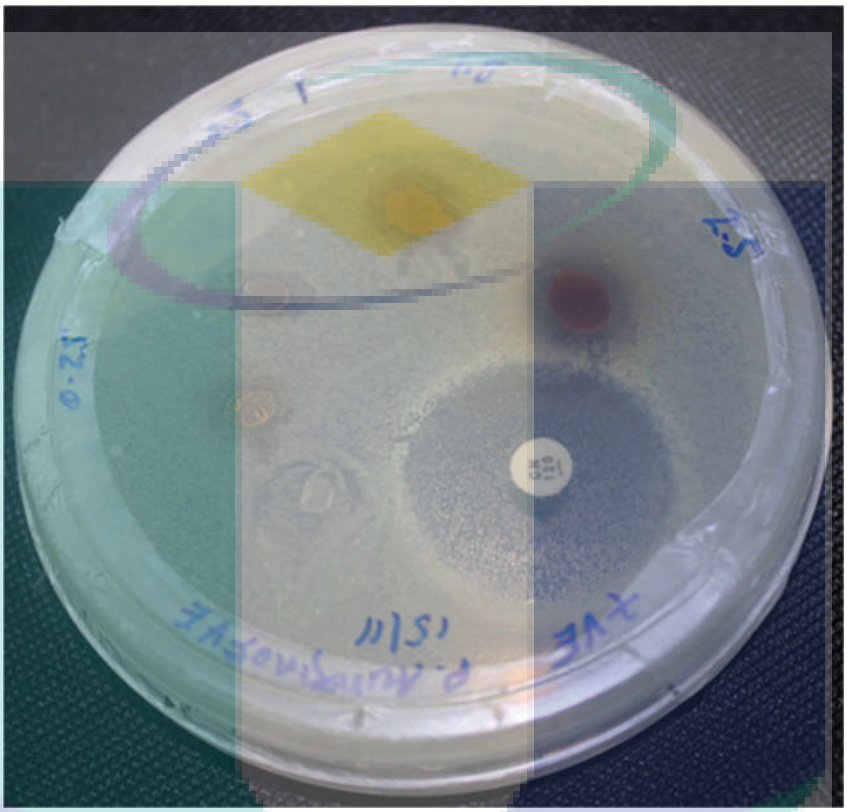


APPENDIX E5
PETRI DISH OF RTE/PVA NANOFIBER (0.25%, 0.5%, 1.5%, 2.5%) WITH INHIBITION ZONE AGAINST E. *FEACALIS*

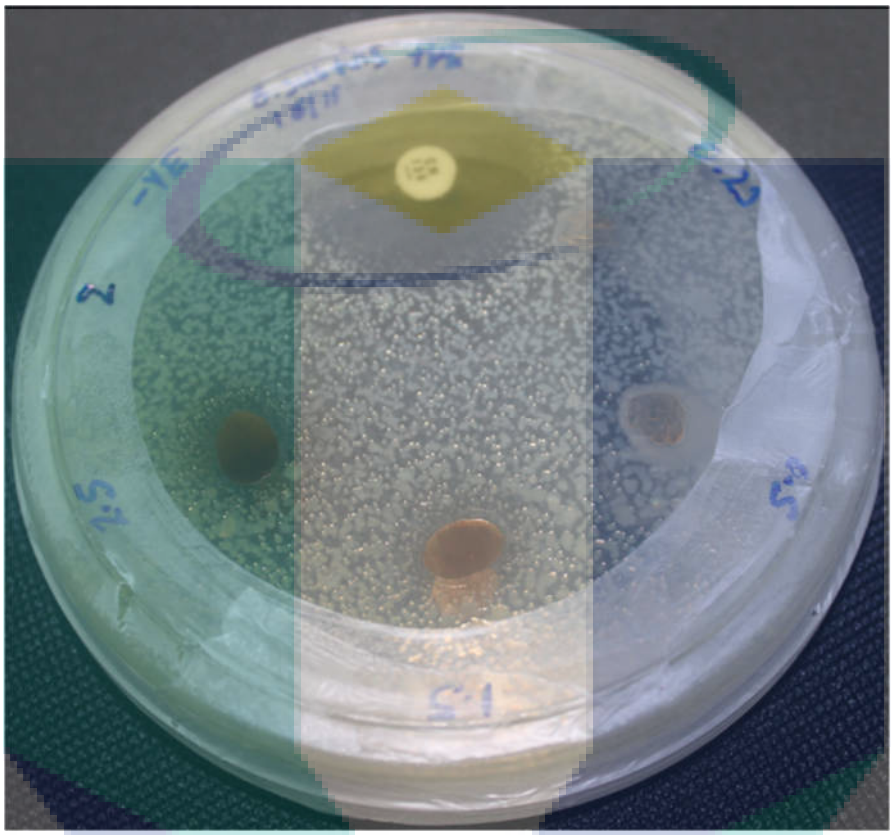


APPENDIX E6

PETRI DISH OF RTE/PVA NANOFIBER (0.25%, 0.5%, 1.5%, 2.5%) WITH INHIBITION ZONE AGAINST *P. AERUGINOSA*

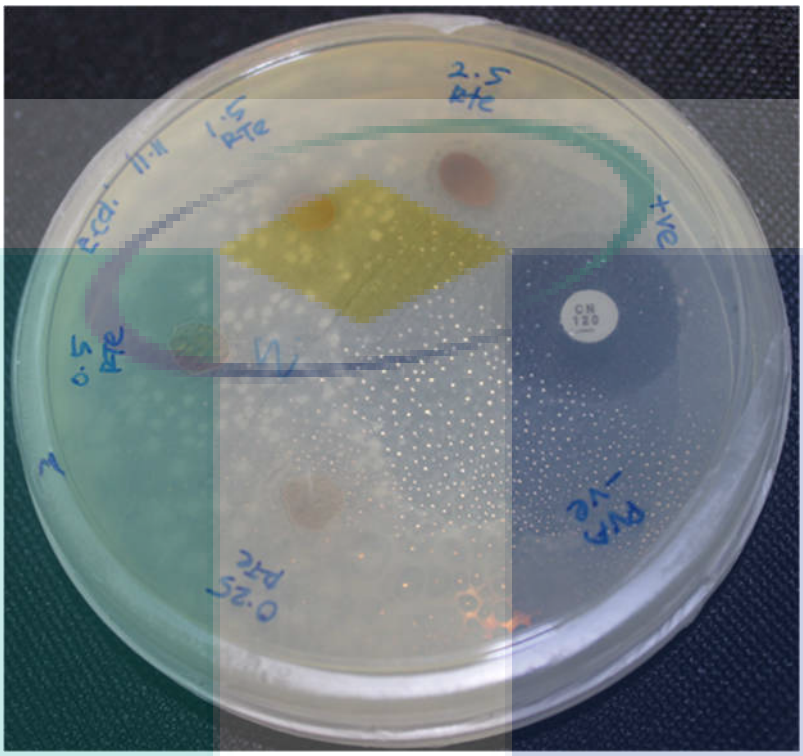


APPENDIX E7
PETRI DISH OF RTE/PVA NANOFIBER (0.25%, 0.5%, 1.5%, 2.5%) WITH INHIBITION ZONE AGAINST *B. SUBTILIS*

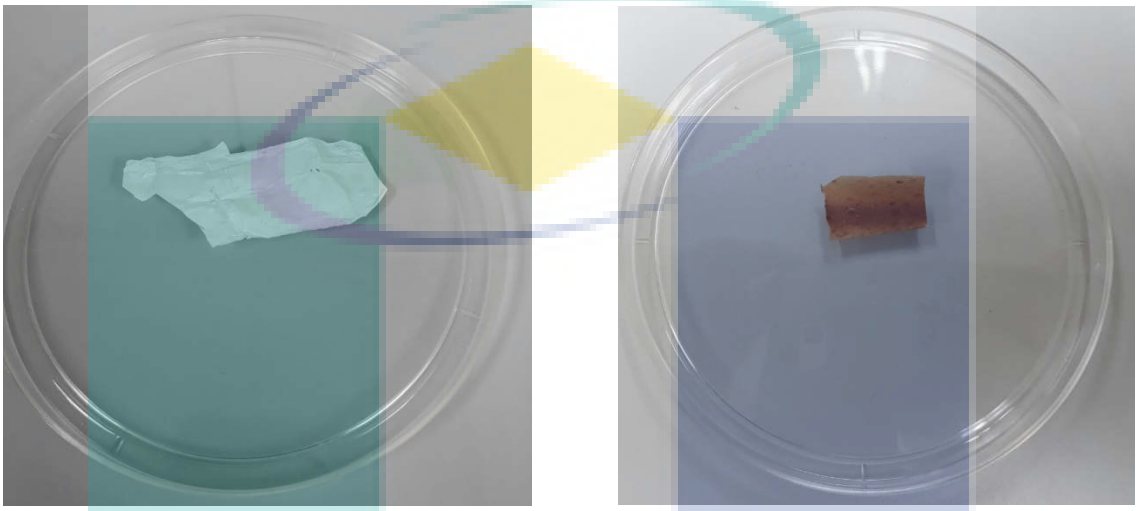


APPENDIX E8

PETRI DISH OF RTE/PVA NANOFIBER (0.25%, 0.5%, 1.5%, 2.5%) WITH INHIBITION ZONE AGAINST *E. COLI*



APPENDIX F1
SAMPLE PICTURE OF FIBER MATS OF 10% PVA (A) AND 2.5% RTE/PVA NANOFIBER (B)



(A) (B)