CHEMICAL CONSTITUENTS, ANTIOXIDANT AND CYTOTOXICITY PROPERTIES OF *RHODOMYRTUS TOMENTOSA* ROOT EXTRACTS

ROZIASYAHIRA BINTI MUTAZAH

Master of Science

UNIVERSITI MALAYSIA PAHANG

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_____

(Supervisor's Signature)

Full Name : Dr. Hazrulrizawati Bt Abd Hamid

Position : Lecturer of Faculty of Industrial sciences and Technology, UMP

Date :

_____

(Co-supervisor's Signature)

Full Name : Assoc. Prof. Dr Ahmad Faizal Abdull Razis

Position : Lecturer of Faculty of Food Science & Technology, UPM

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ROZIASYAHIRA BINTI MUTAZAH

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ABSTRAK

Satu kajian telah dijalankan terhadap juzuk kimia, antioksidan dan sitotoksik aktiviti ekstrak akar *Rhodomyrtus tomentosa*. Pengekstrakan, pemeringkatan dan pemisahan campuran menggunakan teknik kromatografi yang berbeza (kromatografi kolum dan kromatografi lapisan nipis) telah digunakan dalam proses pengasingan sebatian tulen. Juzuk fitokimia daripada akar *R. tomentosa* tertakluk kepada prestasi cecair ultra tinggi kromatografi-quadrupole masa penerbangan / spektrometri jisim (UPLC-QToF / MS) bagi menentukan komposisi aktif di dalam tumbuhan tersebut. Potensi sitotoksik ekstrak *R. tomentosa* dari pelarut extrak yang berbeza telah dinilai dalam bahagian sel HepG2, MCF-7 dan HT 29 dan aktiviti antioksidan dipantau oleh perangkap radikal bebas (DPPH), pengurangkan kapasiti antioksidan melalui tembaga (CUPRAC) dan pelunturan β- karotena. Setiap ekstrak dan pecahan campuran dari *R. tomentosa* menghalang pertumbuhan sel kanser dan bergantung kepada kepekatan extrak dan pecahan campuran. Menurut IC_{50} yang diperolehi, keputusan memaparkan kegiatan yang penting terhadap titisan sel kanser dalam kadar masa selama 72 jam pengeraman dengan nilai $IC_{50} < 30$ μg / mL untuk etil asetat pada HepG2, methanol dan etil asetat pada MCF-7 dan HT 29. Ekstrak methanol menunjukkan aktiviti antioksidan yang signifikan pada DPPH, CUPRAC dan β-karotena kerana kehadiran jumlah kandungan fenolik dan flavonoid yang tinggi di dalam *R. tomentosa*. Pemisahan campuran daripada ekstrak etil asetat yang paling aktif dalam sitotoksik membawa kepada pengasingan sebatian lupeol dan pemisahan ekstrak kloroform menghasilkan β-sitosterol. Struktur sebatian tulen tersebut dikenalpasti melalui kaedah spektroskopi seperti spekroskopi ultra lembayung, spektroskopi inframerah, spekroskopi 1D RMN, spekroskopi korelasi RMN, spektroskopi jisim serta perbandingan data dengan tinjauan kajian. Hasil kajian sitotoksik dan antioksidan menunjukkan bahawa *R. tomentosa* sebagai sumber potensi sebagai antioksidan dan antikanser dan lupeol dikenal pasti sebagai sebatian semula jadi dengan sitotoksik yang tinggi.

ABSTRACT

A study was carried out on the chemical constituents, antioxidant and cytotoxicity properties of *Rhodomyrtus tomentosa* root extracts. Solvent–solvent extraction, fractionation and separation using different chromatographic techniques (column chromatography and thin layer chromatography) were used for isolation of pure compounds. The chemical constituents of the roots of *R. tomentosa* were subjected to ultra-high performance liquid chromatography-quadrupole time-of-flight/mass spectrometry (UPLC-QToF/MS) for the determination of compound composition present in the plant. The potential cytotoxicity of *R. tomentosa* extracts from different solvents were evaluated in vitro on HepG2, MCF-7 and HT 29 cell lines and antioxidant activity was monitored by radical scavenging assay (DPPH), copper reducing antioxidant capacity (CUPRAC) and β-carotene bleaching assay. Each extract from *R. tomentosa* inhibited proliferation on all cancer cell lines in a concentration-dependant manner. According to the IC_{50} obtained, the results exhibited significant activity against cancer cell line under 72h incubation with IC_{50} value < 30 µg/mL for ethyl acetate on HepG2, methanol and ethyl acetate on both MCF-7 and HT 29. Methanol extracts show significant antioxidant activities in DPPH, CUPRAC and β-carotene due to the presence of high total flavonoid and total phenolic content of *R. tomentosa*. Bioassay guided fractionation of ethyl acetate extract led to the isolation of lupeol and fractionation of chloroform extract led to the isolation of β-sitosterol. The structure of the isolated compounds were elucidated by spectroscopic methods such as 1D (^{1}H, ^{13}C, DEPTQ), 2D (COSY, HMQC, HMBC) NMR, MS, UV, FTIR and also by comparison with the literature. Collectively, the results show that *R. tomentosa* is a potential source of antioxidant and cytotoxicity and identified lupeol being a possible promising chemical lead for further development as anticancer agent.

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LIST OF SYMBOLS

β	beta
δ	chemical shift
°C	degree Celsius
g	gram
Hz	hertz
λ	wavelength
µ	micro
µL	microliter
%	percentage
mL	mililiter
ppm	part per million

LIST OF ABBREVIATION

^{13}C NMR	carbon nuclear magnetic resonance
BHT	butylated hydroxytoluene
CC	column chromatography
$CDCl_3$	deuterated chloroform
$CHCl_3$	chloroform
COSY	correlation spectroscopy
CUPRAC	cupric reducing antioxidant capacity
d	doublet
DEPTQ	distortionless enhancement by polarization transfer with retention of quaternaries
DPPH	2,2,-diphenyl-1-picrylhydrazyl
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
EIMS	electron impact mass spectrometry
EA	ethyl acetate
EC_{50}	concentration required to obtain a 50 % antioxidant effect
EtOAc	ethyl acetate
GAE	gallic acid equivalents
GCMS	Gas Chromatography-Mass Spectroscopy
HepG2	human liver cancer cells
^{1}H NMR	proton nuclear magnetic resonance
HMBC	heteronuclear multiple bond coherence
HSQC	heteronuclear single quantum coherence
HT 29	human colorectal adenocarcinoma cell
Hx	hexane
IC_{50}	concentration of drug required to inhibit cell growth by 50 %
m	multiplet
M^+	molecular ion
MCF-7	breast cancer cell
MeOH	methanolic
MS	mass spectrometry
MTT	3-(4,5-Dimethylthiaozol-2-yl)-2,5-diphenyltetrazolium bromide

m/z	mass to charge ratio
NMR	nuclear magnetic resonance
RE	Rutin equivalents
s	singlet
t	triplet
TFC	total flavonoid content
TLC	thin layer chromatography
TPC	total phenolic content

CHAPTER 1

INTRODUCTION

1.1 Research Background

In this era, humans around the world have extensive concerns with regards to their health. Air pollutions, water pollutions, and unhealthy food consumption are the causes of many diseases including cancer, diabetes, hypertension, cataract, immune system decline, and Alzheimer. Due to this, new drug discovery and the development of supplements has a very high potential and demand for consumers around the world. Throughout the history of civilization, humans survived and relied on the healing process from natural products. There was a great deal of folkloric knowledge about general medicinal properties of plants. Natural products have served human beings as a primary source of medicine in many cultures. An estimation of around 80 - 85% of global population rely on traditional medicines for their primary health care needs. One of the major approaches in developing new drugs from natural products is to examine the uses claimed for a traditional preparation (Farnsworth, N. R., 1988; Farnsworth, Norman R., Akerele, Bingel, Soejarto, & Guo, 1985).

Medicinal plants are known to be in use throughout the history of civilisation. Due to an advent of synthetic drugs and antibiotics, usage of medicinal plants has seen a decline and is considered as an alternative medicine only. However, the toxicity level and harmful effects associated with antibiotics and synthetic drugs have brought the medicinal plants species again to the forefront of healthcare system. Plants provide an extensive reservoir of natural products and not only that, it demonstrates important structural diversity and also offers a wide variety of novel and exciting chemical entities in modern medicine. Plants remain as an important source of new drugs and new chemical entities. The plants usually are composed of versatile matrix compounds with different functional groups and polarity. Thus, a lot of

techniques are needed to extract, purify and even isolate the plant compound from the diverse groups.

Nowadays, medicinal plants play important roles and are widely used, for the preparation of various products either as an alternative medicine or as the main source in pharmaceutical industry. Due to inadequate and inaccessible health facilities, majority of the people living in sub-Saharan Africa are heavily depended on traditional medicine in treating malaria (Mukungu, Abuga, Okalebo, Ingwela, & Mwangi, 2016). Asia is one of the largest continent that has medicinal plants in abundance. In Thailand, lots of patients with cancer are still using traditional medicine from plants as their alternative medicine (Kummalue, Suntiparpluacha, & Jiratchariyakul, 2012). Today, people are starting to realize and have gained knowledge on the potential of medicinal natural plants on their health care. Malaysia is in the tropical country with an abundance of sources on medicinal plants which has a promise to reduce and cure diseases.

Research of drug discovery from plants involves a multifaceted approach combining botanical, phytochemical, biological, and molecular techniques (Balunas & Kinghorn, 2005). From identifying plant of interest, preparation of extracts, biological screening of extract, isolation process till the characterization of active compounds is required in an attempt to discover drugs from plants. Medicinal plant drug discovery continues to provide new and important leads against various pharmacological targets including cancer. The investigation of bioactive compounds from natural products are mainly the study of bioactive constituents existed in natural products either from plants or living organisms. The stage of investigating bioactive compound starts from the detection of plants, the isolation of compounds, and the structural elucidation of natural products. The extraction, purification, analysis and quantification of bioactive properties have stimulated on the development of research on natural plants (Dai & Mumper, 2010).

Cancer is a multi-lateral disease incorporating chemical, physical, genetic, metabolic, and environmental factors. All of these factors play a role in the induction and deterioration of cancers. Breast and colon cancers are the leading causes of death from cancer worldwide. Interest in cancer research, especially chemotherapy, intensified with the increase in cancer deaths. Due to the need of anticancer agents, many researchers are intensively investigating

the chemistry and biology of anticancer agents. A wide array of researches focusing on natural plants as potential targets towards anticancer drug development has arisen (Aboul-Enein, El-Ela, Shalaby, & El-Shemy, 2014; Ahmed Hassan, Khadeer Ahamed, Abdul Majid, Iqbal, Al Suede et al., 2014; Banno, Akihisa, Tokuda, Yasukawa, Higashihara et al., 2004; Casagrande, Macorini, Antunes, Santos, Campos et al., 2014). Major advances have been made in understanding the nature and vulnerability of cancerous cells, resulting in development of novel screens and approaches.

Several plant-derived compounds are currently successfully employed in cancer treatment due to its novel anticancer properties (Shah, Shah, Acharya, & Acharya, 2013). Vinblastin and Vincristine of the rosy periwinkle *Catharanthus roseus* are potent anticancer agents and are the first agents that have been developed for clinical use for cancer treatment (Cragg & Newman, 2005; Ganie, Upadhyay, Das, & Prasad Sharma, 2015). The discovery of paclitaxel derived from extracts of Pacific yew, *Taxus brevifolia* after being tested in the National Cancer Institute's (NCI) screening program yielded anticancer compounds showed another evidence of the success in natural product drug discovery (Pezzuto, 1997; Shoeb, 2006; Wani, Taylor, & et al., 1971). Camptothecin, an alkaloid exhibiting potent antileukemic and antitumor activities in animal has been isolated from the tree *Camptptheca acuminata* (Wall, Wani, Cook, Palmer, McPhail et al., 1966). Promising new anticancer agents are in clinical development based on selective activity against cancer-related molecular targets, including flavopiridol and combretastin A4 phosphate (Cragg & Newman, 2005).

Brewer (2011) believed that beneficial effect of plants is mainly due to its number of phenolic compounds and their ability to promote antioxidant effect. Halliwell and Gutteridge (2007) stated that oxidative stress is involved in the development and secondary pathology of various human diseases. Progression of cancer cells development is characterised by redox imbalance with a shift towards oxidative conditions. Antioxidant properties from plants can help in scavenging reactive oxygen species. Studies have shown that not only phenolic but flavonoids may also exert anticancer effects as well (Anter, Romero-Jiménez, Fernández-Bedmar, Villatoro-Pulido, Analla et al., 2011; Ferry, Smith, Malkhandi, Fyfe, Anderson et al., 1996). Hence, the validation of antioxidant effect of medicinal plants nowadays routinely

supplemented with analysis of anticancer activity against various types of cancer cell lines (Malta, Tessaro, Eberlin, Pastore, & Liu, 2013). Bhuvaneswari and Nagini (2005) provided evidences that by improving the antioxidant capacity, it effectively lowers the risk of reactive oxygen species (ROS)-mediated diseases such as cardiovascular disease and cancer. An understanding of antioxidants in different cancer subtypes provides an opportunity for further research on the targeted anticancer therapy.

Rhodomyrtus tomentosa also known as Kemunting in Malaysia is an ornamental, evergreen shrub that can grow up to four meters tall. This plant is native to southern and south eastern Asia. Ethnomedicinal studies on *R. tomentosa* have been documented by several researchers. All part of this plant (leaves, roots, buds, and fruits) has been used traditionally in Vietnamese, Chinese and Malaysian medicine. It has been reported that *R. tomentosa* is one of anti-tubercular plants used to treat tuberculosis (Arya, 2011). *R. tomentosa* has been employed in traditional medicine in treating colic diarrhea (Ong & Nordiana, 1999), abscesses, haemorrhage, and gynaecopathy (Wei, 2006a). It also has been used in traditional medicine as an antipyretic, antidiarrheal, and antidysentery (Chuakul, 2005). *R. tomentosa* has been manufacture in traditional Chinese medicine in treating urinary tract infection (Wei, 2006b).

1.2 Problem Statement

Globally, there has not been any research conducted with regards to the anticancer activity of *R. tomentosa*. Recently, *R. tomentosa* was identified as one of the 240 ‘‘Neglected and Underutilised Crop Species’’ of Vietnam, China, Thailand and Cambodia by the scientific project ‘‘Agrofolio’’. Literature and art work indicates that *R. tomentosa* played an important holistic role in the daily lives of some ancient cultures, providing medicinal benefits. *R. tomentosa* with a wide spectrum of pharmacological effects has been used to treat colic diarrhoea, wound treatments, pain killer, heart burn, abscesses and gynaecopathy. Almost 42 compounds isolated from this plant and identified comprises of phloroglucinol, flavonoid, terpenoid, antracene glycoside, tannin and other compounds. A few studies on biological activities have been documented including antibacterial, antifungal, antimalarial, osteogenic, antioxidant and anti-inflammatory properties. Cancer remains as one of the major health threats to Malaysian people with yearly mortality rate of cancer patients that has

consistently increased throughout the years. Consequently there is an important need for new natural anticancer compounds in chemotherapeutics. *R. tomentosa* is widely used in traditional medicine, however only few studies have provided the evidence on the relationship between its chemical compounds and medicinal properties of the plants. Furthermore, the toxicity of extracts to validate pharmacological activities is needed. Moreover, the potentiality of anticancer activity from *R. tomentosa* is not well understood and known yet. There are many more bioactive compounds waiting to be isolated from *R. tomentosa*.

1.3 Objectives of research

The objectives of this research are:

1. To extract and identify the chemical constituents of the root extracts of *R. tomentosa*.
2. To evaluate the antioxidant and cytotoxicity properties of the root extracts of *R. tomentosa*.
3. To isolate and characterize the bioactive compounds from *R. tomentosa*.

1.4 Scope of Study

There are five main approaches in this study which are the extraction of *R. tomentosa* root extracts via solvent-solvent extraction method, identification of chemical constituents of *R. tomentosa* root extracts via UPLC-QToF/MS, the evaluation of biological activities of *R. tomentosa* root extracts, the isolation of compounds, and elucidation of the isolated compounds. Evaluation of the biologically active extracts was done to screen for cytotoxic and antioxidant activities. The elucidation of isolated compounds was carried out by spectroscopic technique such as NMR (^{1}H, ^{13}C, DEPTQ, COSY, HSQC, HMBC), UV-Vis, FTIR and GC-MS. The isolated compound lupeol was then chosen to be screen for cytotoxic and antioxidant activities.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The purpose of this review is not only to provide a review on previous research efforts related to current research but also to link between the chemical compositions present in the plant with its biological activities. As the current research is exploring on the root of *R. tomentosa*, this review studies are organized to offer insight on previous research efforts toward both chemical composition and bioactivities of the plant.

2.2 Botanical Overview

Rhodomyrtus tomentosa is classified under family Myrtaceous of the order Myrtales (Table 2.1).

Table 2.1 Scientific classification of species *Rhodomyrtus tomentosa*

Scientific Classifications	
Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Myrtales
Family	Myrtaceae
Genus	*Rhodomyrtus*

2.2.1 Myrtaceae

The Myrtaceae family (Myrtle or guava family), is the eighth largest flowering plant family. Myrtaceae, which consists of evergreen trees or shrubs, have a wide distribution in tropical and warm-temperate regions such as Asia, Australia, Madagascar, Pacific Islands

and South America (Jie & Craven, 2007). The Myrtaceae can be distinguished from other families by its persistent leaves with entire margin, whitish flowers with inferior ovaries and abundant stamens, and unlignified portions dotted with glands containing viscous aromatic substances. The family Myrtaceae have traditionally been divided into two subfamilies based on their fruit structure, Myrtoideae with fleshy berry fruits and Leptospermoideae with dry capsular fruits (Wilson, O' Brien, Gadek, & Quinn, 2001). Fleshy-fruited Myrtoideae genera have their greatest concentrations in Australia and in Neotropics. Capsular-fruited Leptospermoideae genera such as *Eucalyptus*, *Leptospermum* and *Metrosideros* are absent in America (Lucas, Belsham, NicLughadha, Orlovich, Sakuragui et al., 2005).

Many Myrtaceous plant are cultivated as an ornamental plant, plantation trees for subtropical and tropical areas, and for its abundant flowers and sweet edible fruits (Jie & Craven, 2006, 2007). Economically, Myrtaceae is a very important family; some species are cultivated for its important in the spices, fruits, honeys, timbers, pharmacology industries and its potential due to the presence of vitamin-rich soft fruits and its bioactive compounds. *Eucalyptus* is the most widely planted genera and a large genus of aromatic trees comprise of more than 900 species (Tian, Zhang, Wang, Lai, & Yang, 2009). *Eucalyptus* is widely cultivated, from which the wood is used widely in pulp industries, producing paper, lamppost, timber and charcoal (Grattapaglia, Vaillancourt, Shepherd, Thumma, Foley et al., 2012; Okun, Kenya, Oballa, Odee, & Muluvi, 2008; Tian et al., 2009).

Pimenta dioica (allspice) and *Syzygium aromatica* (clove) are important in the spice industry (Cortés-Rojas, de Souza, & Oliveira, 2014; Dharmadasa, Abeysinghe, Dissanayake, & Fernando, 2015; Panawala, Abeysinghe, & Dharmadasa, 2016; Rao, Navinchandra, & Jayaveera, 2012; Soysa, Abeysinghe, & Dharmadasa, 2016). *Eucalyptus*, *Syzygium aromatic*, *Pimenta*, and *Leptospermum* provide essential oils for flavour and fragrance industries (Aurore, Abaul, Bourgeois, & Luc, 1998; Brophy, Goldsack, Bean, Forster, & Lepschi, 1999; Chaieb, Hajlaoui, Zmantar, Kahla-Nakbi, Rouabhia et al., 2007; Chalchat, Kundakovic, & Gomnovic, 2001; Chaverri & Cicció, 2015; Pragadheesh, Yadav, Singh, Gupta, & Chanotiya, 2012). Most of the fleshy-fruited Myrtaceae are edible either raw or for juice, sweets, and jams, such as *Psidium guajava*, *Syzygium jambos*, *Feijoa sellowiana*, *Myrciaria cauliflora*, *Eugenia uniflora*, and *Campomanesia lineatifolia*.

Myrtaceous plant has also been used in traditional medicine. The leaves and stems from Brazilian species of *Campomanesia xanthocarpa* are employed in a remedy for dysentery, stomachache, and as an anti-inflammatory agent. In Brazil, the skin from the stem of *Myrciaria cauliflora* has been employed traditionally in treating asthma and diarrhea. The leaves from *Melaleuca leucadendron* are used as pain relieving agents in Taiwan. In New Zealand, the Maori tribe uses *Leptospermum scoparium* to treat fever and pain. *Rhodomyrtus tomentosa* has been used as treatment for wound and diarrhea (Tung, Ding, Choi, Kiem, Minh et al., 2009).

2.2.2 Genus *Rhodomyrtus*

Rhodomyrtus is a group of trees and shrubs in the family of Myrtaceae described as a genus since 1841. Genus *Rhodomyrtus* comprises of around 21 species which are widely distributed around tropical Asia, Australia and Pacific Island (Jie & Craven, 2007).Previous chemical studies on *Rhodomyrtus* genus is concentrated on essential oils, with less work done on non-volatiles from *R. macrocapa* and *R. tomentosa*. *R. canescens*, *R. effussa, R. macrocarpa, R. pervagata, R. psidioides, R. sericea,* and *R. trineura* from Australian spesies of *Rhodomyrtus* has produced various of essential oils (Brophy, Goldsack, & Forster, 1997). Isomers of the natural dibenzofuran derivative, rhodomyrtoxin, from fruits of *R. macrocarpa* have been isolated by Igboechi, Parfitt, and Rowan (1984). Previous studies on *Rhodomyrtus* genus have investigated and resulted in the isolation of several compounds including acylphloroglucinols (Dachriyanus, S., Sargent, Skelton, Soediro, Sutisna et al., 2002; Hiranrat, Asadhawut & Mahabusarakam, 2008; Liu, H.-X., Tan, & Qiu, 2016), anthocyanins (Cui, Zhang, You, Ren, Luo et al., 2013; Lowry, 1976), dibenzofurans (Anderson, Ollis, Underwood, & Scrowston, 1969; Carvalho & Sargent, 1984; Igboechi et al., 1984), flavonoids (Dachriyanus, Fahmi, Sargent, Skelton, & White, 2004; Hou, Wu, & Liu, 1999), naphthalenoids (Tung et al., 2009), stilbenoids (Shiratake, Nakahara, Iwahashi, Onodera, & Mizushina, 2015) , tannins (Liu, H.-X., Zhang, Xu, Chen, Tan et al., 2016), and terpenoids (Brophy et al., 1997; Hui & Li, 1976; Hui, Li, & Luk, 1975).

Active compound of *Rhodomyrtus* genus can only be investigated and identified on *R. tomentosa*. Hmoteh, Syed Musthafa, Pomwised, and Voravuthikunchai (2016) evaluated the potential of ethanolic leaf extract of *R. tomentosa* in enhancing the activity of human

neutrophils against E. coli. Jeenkeawpieam, Phongpaichit, Rukachaisirikul, and Sakayaroj (2012) studied 213 culturable endophytic fungi isolated mostly from leaves and branches of Thailand species of *R. tomentosa,* exhibited inhibitory activity against human pathogenic fungi. Methanol extract from *R. tomentosa* showed significant *in vitro* and *in vivo* anti-inflammatory properties by suppressing Syk/Src/NF-kB and IRAK1/IRAK4/AP-1 pathways (Jeong, Yang, Yang, Nam, Kim et al., 2013). Lavanya, Voravuthikunchai, and Towatana (2012) indicated that acetone extract from *R. tomentosa* showed potent natural antioxidant properties assessed by lipid peroxidation inhibition capacity, ferric reducing antioxidant power, and metal chelating activity.

2.2.3 *Rhodomyrtus tomentosa*

R. tomentosa (Figure 2.1) or Kemunting is a wild shrub that fruits throughout the year. It is a plant native to Southern and South Eastern Asia, India, China, Taiwan, Philippines, Malaysia, Thailand, and Sulawesi. Due to this, it has many common names such as rose myrtle (English-Florida), hill gooseberry (English), feijoa (French), Isenberg bush (English-Hawaii). In Thailand, the plant is mostly known as Toh, Pruad, and Pruad Yai (Smitinand, 2001).

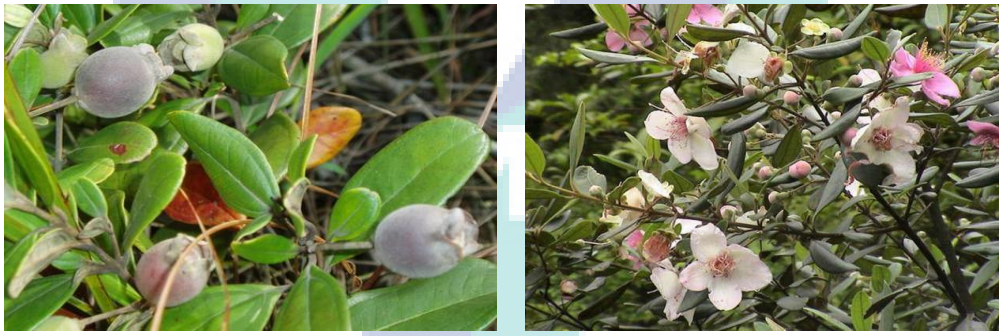


Figure 2.1 Flowers and fruits of *R. tomentosa*

Source: Baharudin, Abdullah, Shariff, Kamal, and Abu Bakar (2014)

As a tropical plant, it can resist heat and high humidity temperature of tropical climate and rough soil especially in salty and acidic soil. The flowers of *R. tomentosa* are pinkish white and sometimes in red colour. The berries of *R. tomentosa* are roundish, purplish black when ripe, sweet and edible. Fresh berries are delicious eaten fresh with sweet and tad tangy.

Due to its sweet characteristic, *R. tomentosa* fruits are a favourite for birds and wild small animals. They are similar in taste to blueberries and raspberries. The berries can be made into pastries like pies and jams. In Malaysia, *R. tomentosa* plants are widely found in east coast of Malaysia especially in Terengganu and Johor where the soil is fertile for this kind of plant. The abundance of this kind of plant especially in remote areas is highly appreciated by the native people or the Orang Asli as they use this plant as daily consumption intake. The botanical features of *R. tomentosa* is depicted in Figure 2.2.

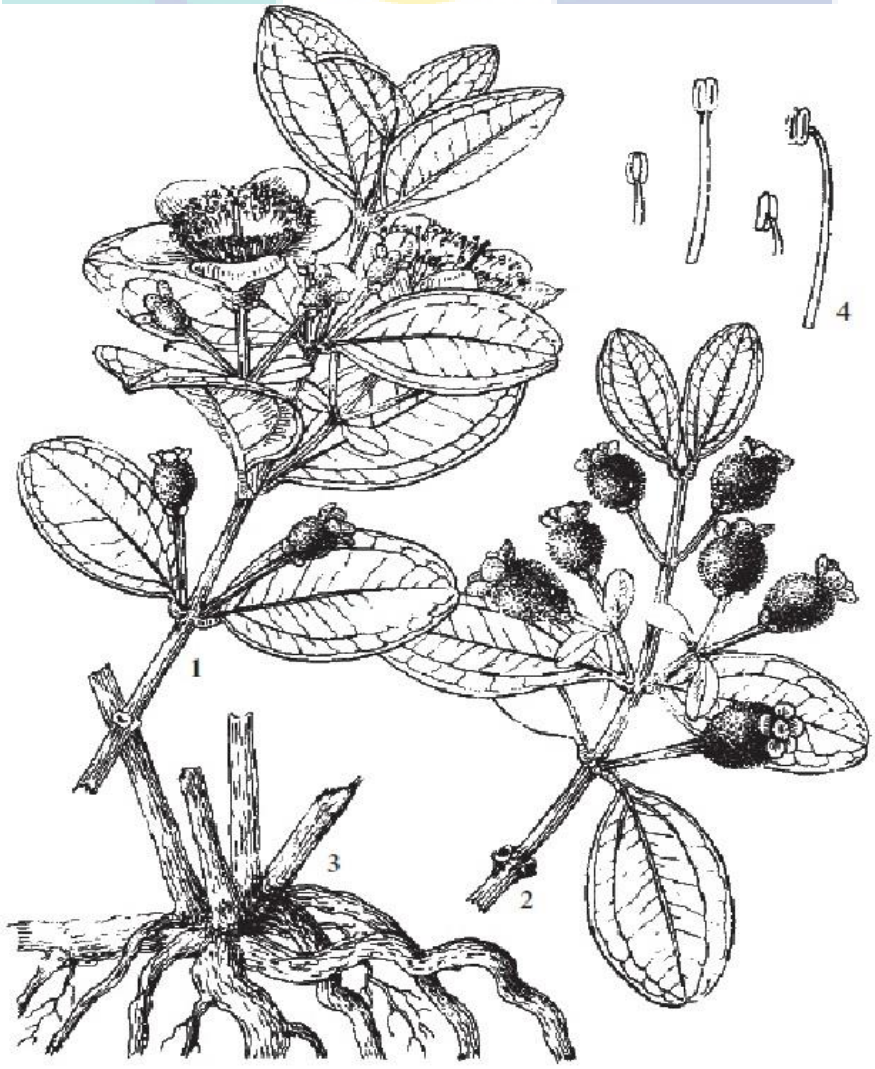


Figure 2.2 *Rhodomyrtus tomentosa*, 1. Flowering branch; 2. Fruiting branch; 3. Root; 4. Stament

Source: Hu and Wu (2008)

2.3 Medicinal Uses

All parts of this plant (leaves, roots, buds, and fruits) have been used traditionally in Vietnamese, Chinese, Thais, and Malaysian medicine. In this medicinal plant, all parts of *R. tomentosa* are used to treat various digestive problems and diseases. The fruits of *R. tomentosa* have been used in a Vietnamese traditional medicine in treating diarrhoea and wounds (Ho, 1999). The Chinese have used the leaves as a pain killer, root to treat heart burn and seeds as a tonic for digestion and to treat snake bites since centuries ago (Gayathri & Kiruba, 2014). *R. tomentosa* has also been manufactured in traditional Chinese medicine in treating urinary tract infections, abscesses, haemorrhage, and gynaecopathy. In Thailand, *R. tomentosa* is often used as traditional medicine by folk people (Gayathri & Kiruba, 2014; Ruqiang Sr & Yonglu Sr, 2006). Native people in Thailand have used *R. tomentosa* to treat oral, gastrointestinal, urinary tract infections, and used it as an antiseptic wash for wounds (Limsuwan, Hesseling-Meinders, Voravuthikunchai, van Dijl, & Kayser, 2011). In southern Thailand, the plant has been used in traditional medicine as antipyretic, antidiarrheal, and antidysentery agent (Chuakul, 2005). *R. tomentosa* has been employed in Malaysian traditional medicine in treating colic diarrhoea (Ong & Nordiana, 1999). Based on *R. tomentosa* ethnomedicinal uses, it has been sold as herbal supplementary product in America (Jeong et al., 2013).

2.4 Phytochemical Constituents of *R. tomentosa*

Several studies on the constituents of *R. tomentosa* have been conducted and has led to isolation of varies compounds from the plant. The chemical study on *R. tomentosa* was concentrated on essential oils, with little work having been published on non-volatiles from this species. Table 2.2 demonstrates a literature survey in the isolation of several representative components on *R .tomentosa* including acylphloroglucinols (rhodomyrtone), flavanoids (myricitrin, quercertin, combretol), naphthalenoids, stilbenoids, and triterpenes.

Table 2.2 Phytochemical constituents of *R. tomentosa*

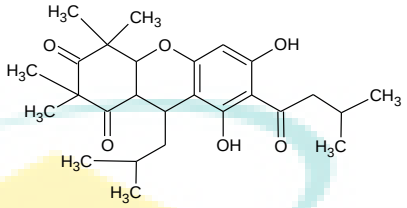
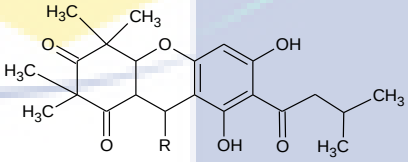
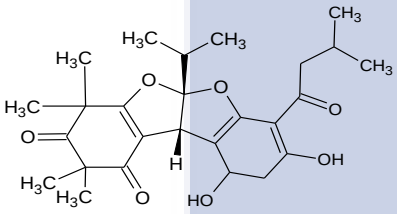
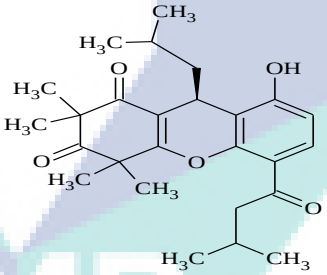
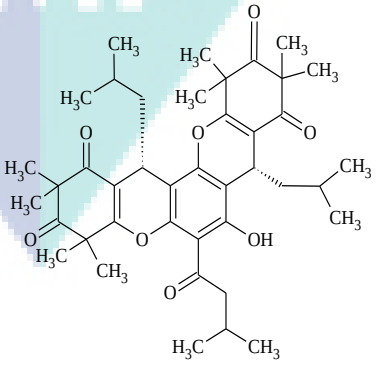
Compound Class	Compound	Structure	References
Phloroglucinol	rhodomyrtone (1)		Dachriyanus, S. et al. (2002); Limsuwan, Trip, Kouwen, Piersma, Hiranrat et al. (2009)
	rhodomyrtosone I (2)	 R = isobutyl	Hiranrat, A, Chitbankluoi, Mahabusarakam, Limsuwan, and Voravuthikunchai (2012)
	rhodomyrtosones A (3)		Hiranrat, Asadhawut and Mahabusarakam (2008)
	rhodomyrtosones B (4)		Hiranrat, Asadhawut and Mahabusarakam (2008)
	rhodomyrtosones C (5)		Hiranrat, Asadhawut and Mahabusarakam (2008)

Table 2.2 Continued

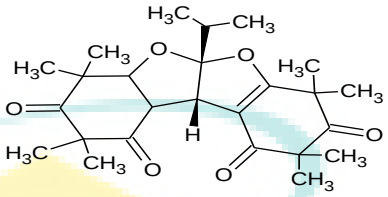
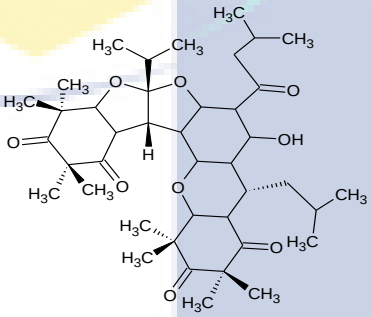
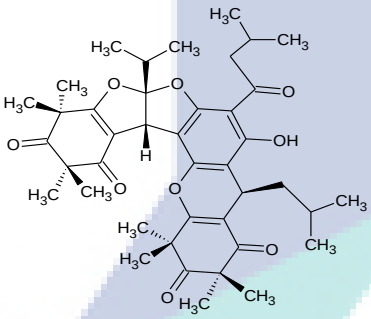
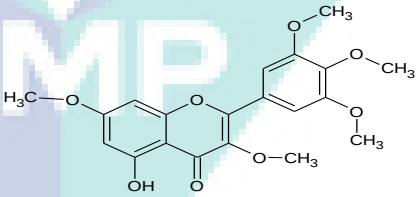
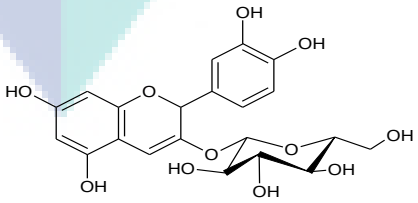
Compound Class	Compound	Structure	References
Phloroglucinol	rhodomyrtosone D (6)		Dachriyanus et al. (2004)
	tomentosone A (7)		Dachriyanus et al. (2004)
	tomentosone B (8)		Hiranrat, A et al. (2012)
Flavonoid	combretol (9)		Dachriyanus et al. (2004)
	cyanidin-3-galactoside (10)		Hou et al. (1999)

Table 2.2 Continued

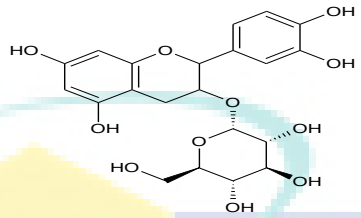
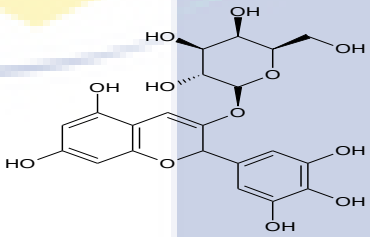
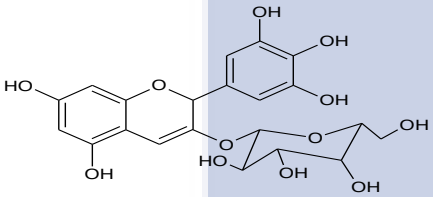
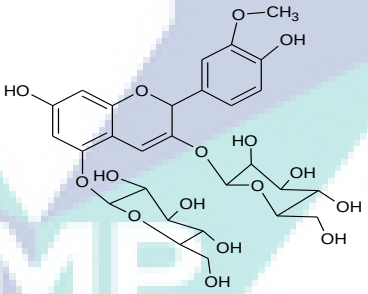
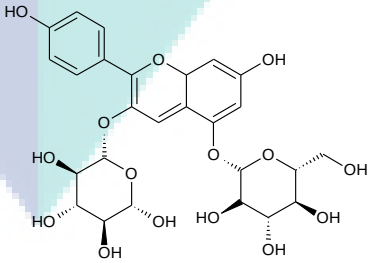
Compound Class	Compound	Structure	References
Flavonoid	cyanidin-3-O-glucoside (11)		Cui et al. (2013)
	delphinidin-3-galactoside (12)		Cui et al. (2013)
	delphinidin-3-O-glucoside (13)		Cui et al. (2013)
	peonidin-3-O-glucoside (14)		Cui et al. (2013)
	pelargonidin-3,5-biglucoside (15)		Cui et al. (2013)

Table 2.2 Continued

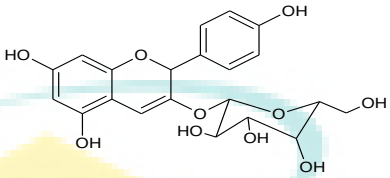
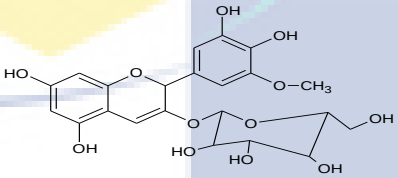
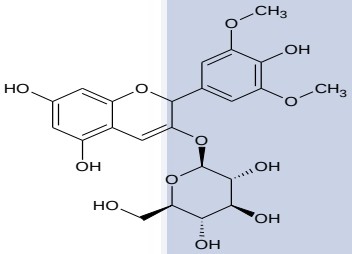
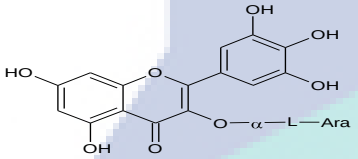
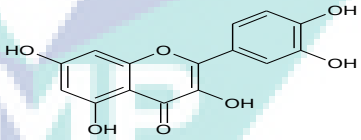
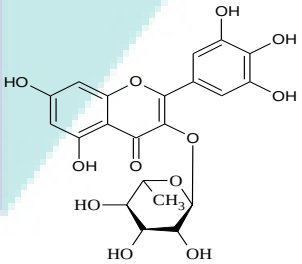
Compound Class	Compound	Structure	References
Flavonoid	pelargonidin-3-glucoside (16)		Cui et al. (2013)
	petunidin-3-O-glucoside (17)		Cui et al. (2013)
	malvidin-3-O-glucoside (18)		Cui et al. (2013)
	myricetin 3-o-α-L-furanoarabinoside (19)		Hou et al. (1999)
	quercertin (20)		Tung et al. (2009)
	myricitrin (21)		Tung et al. (2009)

Table 2.2 Continued

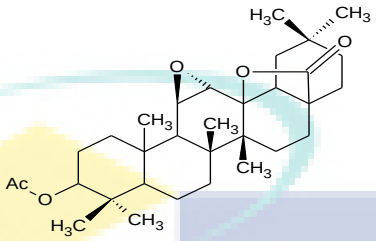
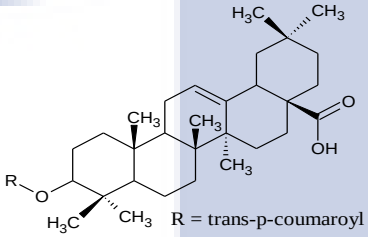
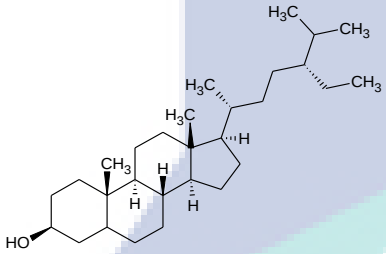
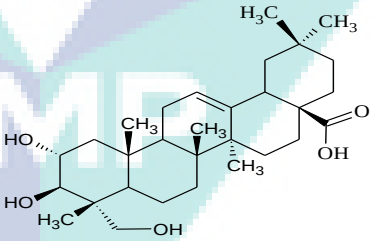
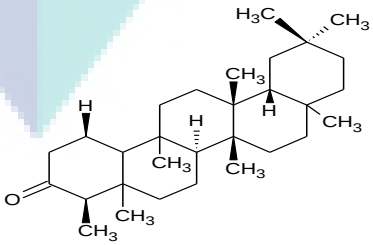
Compound Class	Compound	Structure	References
Terpenoid	3β-Acetoxy-11α,12α-epoxyoleanan-28, 13β-olide (22)		Hui and Li (1976)
	3-O-(E)-coumaroyloleanolic acid (23)		Hiranrat, A et al. (2012)
	β-sitosterol (24)		Hui et al. (1975)
	arjunolic acid (25)		Hiranrat, A et al. (2012)
	friedelin (26)		Hui et al. (1975)

Table 2.2 Continued

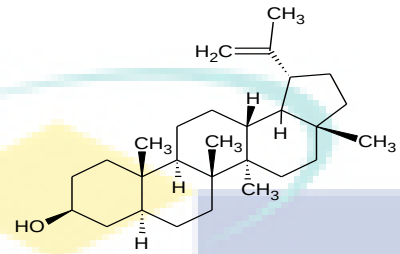
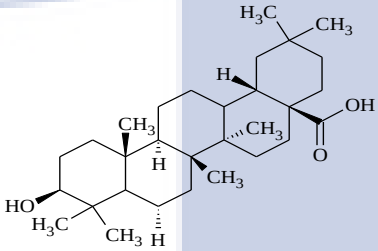
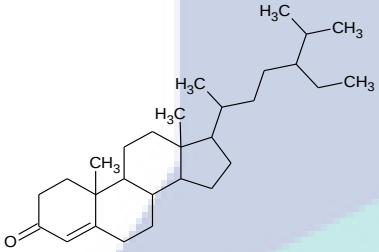
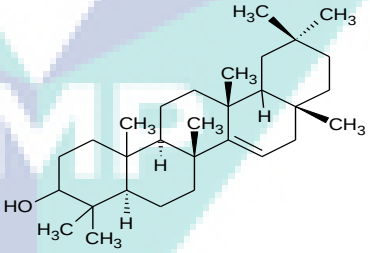
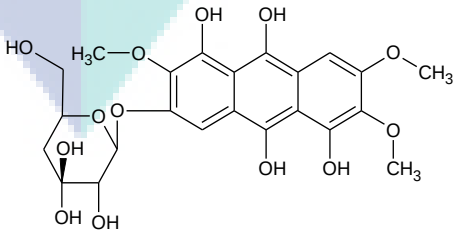
Compound Class	Compound	Structure	References
Terpenoid	lupeol (27)		Hui et al. (1975)
	oleanolic acid (28)		Hiranrat, A et al. (2012)
	stigmast-4-en-3-one (29)		Hiranrat, A et al. (2012)
	taraxerol (30)		Hui et al. (1975)
Anthracene glycoside	4,8,9,10-Tetrahydroxy-2,3,7-trimethoxy-anthracene-6-o-β-D-glucopyranoside (31)		Tung et al. (2009)

Table 2.2 Continued

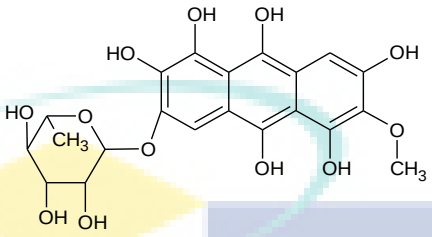
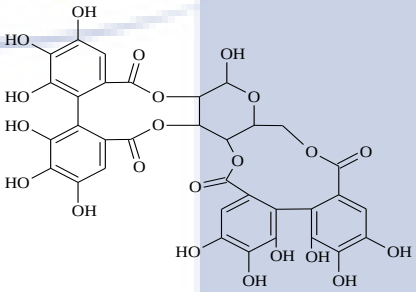
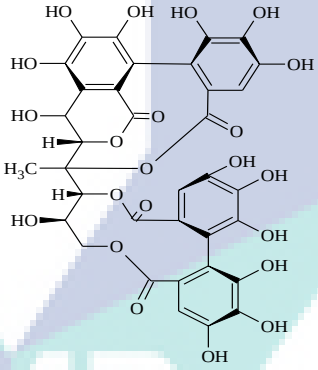
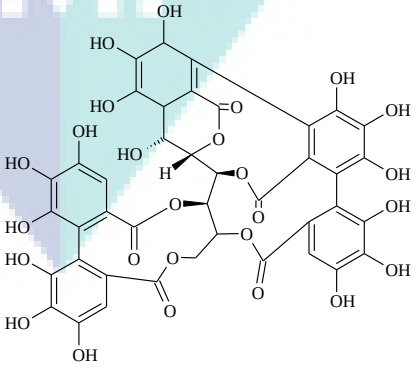
Compound Class	Compound	Structure	References
Anthracene glycoside	2,4,7,8,9,10-hexahydroxy-3-methoxyanthracene-6-O-α-L-rhamnopyranoside (32)		Tung et al. (2009)
Tannin	pedunculagin (33)		Liu, Y., Hou, Ji, and Wu (1998)
	casuarin (34)		Liu, Y. et al. (1998)
	castalagin (35)		Liu, Y. et al. (1998)

Table 2.2 Continued

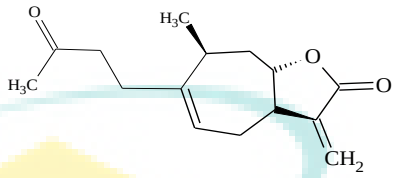
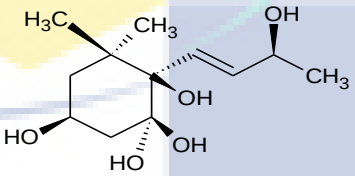
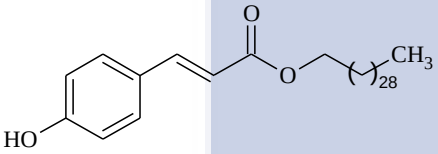
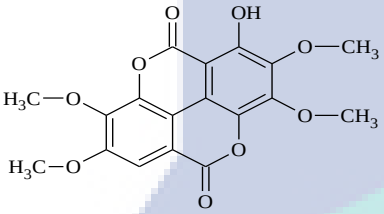
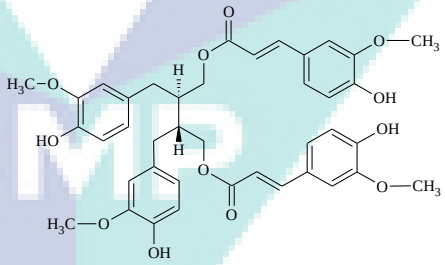
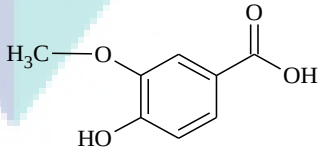
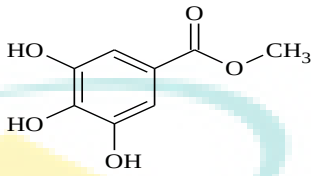
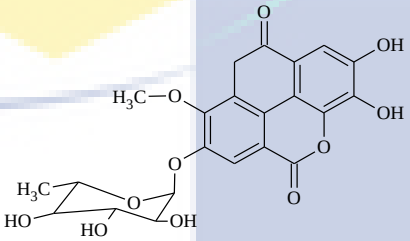
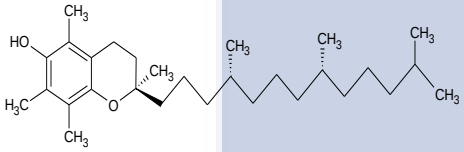
Compound Class	Compound	Structure	References
Other compounds	tomentosin (36)		Liu, Y. et al. (1998)
	(3S,5R,6R,7E,9S)-megastiman-7-ene-3,5,6,9-tetrol (37)		Tung et al. (2009)
	trans-triacontyl-4-hydroxycinnamate (38)		Hiranrat, A et al. (2012)
	3,3',4,4'-tetra-O-methylflavellagic acid (39)		Hiranrat, A et al. (2012)
	(-)-(2R,3R)-1,4-O-diferuloylsecoisolariciresinol (40)		Hiranrat, A et al. (2012)
	4-hydroxy-3-methoxybenzoic acid (41)		Hiranrat, A et al. (2012)

Table 2.2 Continued

Compound Class	Compound	Structure	References
Other compounds	methyl gallate (42)		Hiranrat, A et al. (2012)
	3-O-methylellagic acid 4-O-rhamnopyranoside (43)		Hiranrat, A et al. (2012)
	α-tocopherol (44)		Hiranrat, Asadhawut and Mahabusarakam (2008)

2.5 Biological Activities of *R. tomentosa*

The methanolic extract from aerial part of *R. tomentosa* revealed the isolation of two new anthracene glycosides named 4,8,9,10-Tetrahydroxy-2,3,7-trimethoxy-anthracene-6-o-β-D-glucopyranoside and 2,4,7,8,9,10-hexahydroxy-3-methoxanthracene-6-o-α-L-rhamno-pyranoside) that significantly increases the alkaline phosphate activity, collagen synthesis, and mineralization of the nodules of MC3T3-E1 osteoblastic cells (Tung et al., 2009). The crude ethanolic extract from *R. tomentosa* showed good antibacterial activities against Gram-positive bacteria. Isolation of ethanolic extract using antibacterial bioguided fractionation revealed the active compound, rhodomyrtone that has powerful *in vitro* activity against broad range of Gram-positive bacteria with antibiotic resistant strains (Limsuwan et al., 2009). Rhodomyrtone showed potential antibiotic and anti-infective effects on bacterial pathogen Streptococcus pygenes (Limsuwan et al., 2011). Methanolic extract from the leaves of *R. tomentosa* showed significant *in vitro* and *in vivo* anti-inflammatory effect which inhibits the production of inflammatory mediators (nitric oxide and prostaglandin E_2) (Jeong et al., 2013).

2.5.1 Anticancer Property

Cancer is one of the major causes of death across the world. Cancer can begin from one part of the body and then metastasize if not treated. Cancer can occur when the genes in a cell become abnormal and the cells started to divide and grow uncontrollably. A mass of tissue or tumor forms when process of cell division becomes uncontrollable. Phenolic compounds constitute one of the most numerous groups in plant metabolites. They can be categorized as simple phenolic, phenolic acids, hydroxycinnamic acid derivatives and flavonoids. Phenolic compounds play a vital role as antibacterial, anticarcinogenic, anti-inflammatory, anti-viral, anti-allergic, estrogenic, and immune-stimulating agents. In addition to phenolic compound to their primary antioxidant activity, it also displays variety of biological functions related to carcinogenesis modulation (Dai & Mumper, 2010) and as chemopreventive agents (Fresco, Borges, Diniz, & Marques, 2006).

Phenolic extract from natural plants have been studied in a number of cancer cell lines with different stages of cancer. Among them, flavonoid extract from *Pandanus amaryllifolius Roxb.* possess anticancer activity against breast cancer cells, MCF-7 (Ghasemzadeh & Jaafar, 2013). Phenolic compounds of *Momordica charantia* extract has shown a remarkable anticancer effect especially in leukemia (Kumar, Sharathnath, Yogeswaran, Harani, Sudhakar et al., 2010; Semiz & Sen, 2007). An antiproliferative activity of the ethanol extract between 10 edible berries on HL-60, human leukemia cancer cells and HCT-116 cells showed that bilberry extract was the most effective anticancer agent (Katsube, Iwashita, Tsushida, Yamaki, & Kobori, 2003). Piceantannol, a stilbene has been shown to have potent biological activities including anticancer activities (Kita, Mitsuoka, Kaneko, Nakata, Yamanaka et al., 2012). Myricitrin is another phenolic compound that have been studied due to its highly potent health promoting properties including antioxidant, anticancer, antidiabetic, and anti-ageing activities. Quercetin also shows chemopreventive and anticancer effect (Ren, Boulikas, Lundstrom, Soling, Warnke et al., 2003). It states that quercetin can induce apoptosis in a caspase-3-dependent pathway (Niu, Yin, Xie, Li, Nie et al., 2011). Piceantannol, myricitrin, and quercetin are some of the phenolic compounds that can be found in *R. tomentosa* (Lai, Andre, Chirinos, Nguyen, Larondelle et al., 2014; Tung et al., 2009).

A systematic search regarding anticancer activity in natural plants has already begun in 21th century by using the application of biological screening assays. Anticancer activities are investigated via screening of plants extracts against human cancer cell lines (Fadeyi, Fadeyi, Adejumo, Okoro, & Myles, 2013; Ghasemzadeh & Jaafar, 2013; Tiwary, Bihani, Kumar, Chakraborty, & Ghosh, 2015; Yoo, Park, & Kwon, 2007). Cell viability assay or cytotoxicity assay is used in detecting the viability of the cells. There have been many established methods for the purpose of detecting the viability of cells such as Colony Formation method, Crystal Violet method, Tritium-Labeled Thymidine Uptake method, MTT, and WST methods. All of these methods are used for counting the number of live cells. Screening of methanol extract of 11 Cameroonian medicinal plants for their antiproliferative activity against four cell lines were done by using SBR assay (Tagne, Telefo, Nyemb, Yemele, Njina et al., 2014). Calcein-AM release assay and Hoechst 33342 was used in assessing cytotoxicity of ethanolic extract from *Zingiber Officinale* (Plengsuriyakarn, Viyanant, Eursitthichai, Tesana, Chaijaroenkul et al., 2012).

Among the enzyme-based assays, the MTT assay is the best known method for determining mitochondrial dehydrogenase activities in living cells due to its easy-to-use, safe, has a high reproducibility, and is widely used in both cell viability and cytotoxicity tests. In the method, MTT is reduced to a purple formazan by NADH. However, MTT formazan is insoluble in water, and it forms purple needle shaped crystals in the cells. Therefore prior to measuring the absorbance, an organic solvent is required to solubilize the crystals. Additionally, the cytotoxicity of MTT formazan makes it difficult to remove cell culture media from the plate wells due to floating cells with MTT formazan needles, giving significant well-to-well error. The cytotoxic effects of Thai herbal remedy and anticancer drugs on cell growth were assessed by MTT assay in triplicate manner (Kummalue et al., 2012). Cytotoxicity studies of endophytic fungus of Trichothecium sp. was performed by MTT assay (Taware, Abnave, Patil, Rajamohananan, Raja et al., 2014). Cell proliferation of *Lantana camara* for the studies on induction apoptosis by Bcl-2 family and its caspases activation was evaluated by performing the MTT assay (Han, Chang, Jung, & Kim, 2015). The effect of piceantannol on numbers of viable cells was determined via an MTT assay for the studies of piceantannol and its effect on prostate cancer cells (Kwon, Jung, Song, Woo, Jun et al., 2012).

2.5.2 Antioxidant Activities

Assessments and studies of antioxidant properties of natural compounds from plants is crucial in the application of medicines, foods, and cosmetics. Antioxidants are defined as a compound that can not only delay or inhibit, but also prevent the oxidation of oxidisable materials by scavenging the free radicals and diminishing oxidative stress. Oxidative stress is important especially for the development of chronic degenerative diseases (Ames, Shigenaga, & Hagen, 1993; Dai & Mumper, 2010).

A variety of antioxidant assays have been used in an attempt to screen the potentiality of its antioxidant activities such as oxygen radical absorbance capacity (ORAC), total radical-trapping antioxidant parameter (TRAP), Trolox equivalent antioxidant capacity (TEAC), cupric ion reducing antioxidant capacity (CUPRAC) and ferric ion reducing antioxidant power (FRAP) assays. ORAC and TRAP assays measure the capacity of an antioxidant to quench free radicals by hydrogen atom donation. TEAC, CUPRAC, and FRAP assays measure the capacity of an antioxidant in reduction of an oxidant probe. The change of colour in these assays is proportional to the concentration of antioxidant (Dai & Mumper, 2010; Huang, D., Ou, & Prior, 2005).

The CUPRAC assay uses the copper (II)-neocuproine reagent as the chromogenic oxidant. The method is based on reduction of Cu (II) to Cu (I) by reductants or antioxidants present in a plant sample. It involves mixing the antioxidant solution with CUPRAC reagent consisting of 10 mM Copper (II) chloride, 7.5 mM neucoproine and ammonium acetate buffer (1% (v/v) acetic acid, 0.1 M ammonium acetate at pH 7 and measuring the absorbance at 450 nm after 30 min (Apak, Guclu, Birsen, Ozyurek, Celik et al., 2007; Karadag, Ozcelik, & Saner, 2009).

A method based on the reduction of 2,2-diphenyl-1-picrylhydrazyl (DPPH) is a colorimetric method that can also be carried out in order to detect antioxidant activity of compounds. The reducing ability of antioxidants towards DPPH can be evaluated by electron spin resonance or by monitoring the absorbance decrease at 515 – 528 nm until the absorbance remains stable in organic media, its free radical property is lost due to chain

breakage and its colour changes from purple to light yellow (Badarinath, Mallikarjuna RAo, Sudhana hetty, Ramkanth, Rajan et al., 2010; Karadag et al., 2009).

β-carotene bleaching assay is routinely practised for assessment of lipid peroxidation activity of extracts. The β-carotene bleaching assay measures the decrease in the rate of β-carotene or crocin decay provided by antioxidants. An addition of an antioxidant or plant extracts causes the inhibition of β-carotene bleaching. Linoleic acid reacts with ROS and O_2 to form an unstable peroxy radical. β-carotene acts as an antioxidant will react with the unstable peroxy radical to form a stable epoxide causing the bleaching of yellow solution. A competition will occur when plant sample is present resulting in a slower bleaching solution reacting with peroxy radical detected at 470 nm (Barreira, Ferreira, Oliveira, & Pereira, 2008; Karadag et al., 2009; Mogana, Teng-Jin, & Wiart, 2013).

A systematic research on antioxidant activity in natural plants has already been widely done by using the application of biological screening assays. The antioxidant activities of chloroform extract of *Cymbopogon citratus* was screened to determine its free radical scavenging activities. Three different methods were used to test the antioxidant activity of the extract, including FRAP assay, DPPH radical scavenging assay, and *β*-carotene bleaching assay (Lu, Khoo, & Wiart, 2014). Foon, Ai, Kuppusamy, Yusoff, and Govindan (2013) determined the antioxidant activity of *Eucheuma cottonii* and *Padina* sp. by DPPH, FRAP, and *β*-carotene bleaching assays. All of the antioxidant assays done by Foon et al. (2013) showed positive correlation with antioxidant activities. Mogana et al. (2013) investigated the bioassay guided fractionation of an ethanol extract of leaves of *Canarium patentinervium* and its antioxidant activities by ABTS, DPPH, FRAP, and *β*-carotene bleaching assay with EC_{50} values equal to 5.62 ± 0.03 µM, 0.19 ± 0.01 mM, 0.25 ± 0.03 mM and 0.65 ± 0.07 mM, respectively. Orhan, Yilmaz, Altun, Saltan, and Sener (2011) assess the antioxidant activity of the extracts of the ethyl acetate, methanol, and water extracts from the bulbs of Turkish *Sternbergia Waldst*. & Kit. (Amaryllidaceae) species; *S. candida*, *S. clusiana*, *S. fisheriana*, *S. lutea* sub sp. *lutea*, and *S. lutea* sub sp. *Sicula* evaluated by DPPH radical scavenging activity, ferrous ion-chelating capacity, ferric-reducing antioxidant power, and beta-carotene bleaching assays at 500, 1000, and 2000 µg mL^{-1}.

Extraction from sim fruits of *R. tomentosa* presented a total phenolic level (49.21 ± 0.35 mg GAE/g DW) (Table 2.3) (Lai, Andre, Rogez, Mignolet, Nguyen et al., 2015). This result if compared with other fruits, *R. tomentosa* had a similar total phenolic content as berries that are good sources of phenolic compounds (Wu, X., Beecher, Holden, Haytowitz, Gebhardt et al., 2004). Phenolic compounds bioactivity is mostly linked and studied for its antioxidant capacity. The oxygen radical absorbance capacity (ORAC) value of *R. tomentosa* fruits (431.17 ± 14.56 μmol TE/g DW) (Table 2.3) is higher than commonly consumed fruit (Lai et al., 2015).

Table 2.3 Total phenolic content and ORAC value of *R. tomentosa*

	Per 100 g DW	Per serving	RDI	% CS
Total phenolic (mg GAE)	4.92 ± 0.04	1.81	-	-
ORAC value (μmol TE)	43117 ± 1456	15858.43	3000-5000	>100

GAE: Gallic acid equivalent

TE: Trolox equivalent

Wu, P., Ma, Li, Deng, Yin et al. (2015) demostrated the *in vivo* and *in vitro* antioxidant activity of the flavonoids rich extract of *R. tomentosa* berries. The study revealed that the flavonoid-rich extract from *R. tomentosa* berries has potent antioxidant activities in reducing power, superoxide radical, hydroxyl radical, inhibition of peroxidation, and DPPH radical scavenging activities. Maskam, Mohamad, Abdulla, Afzan, and Wasiman (2014) investigated methanol and water extracts of *R. tomentosa* fruits possesses higher scavenging activities than the chloroform and petroleum ether extracts of the fruits. The EC_{50} values of the methanol and water extract were found to be 107 μg/mL and 154 μg/mL, respectively. FRAP assay from the study by Maskam et al. (2014) also proved that *R. tomentosa* leaves extract have strong antioxidant activity, and all extracts from *R. tomentosa* were shown to have significantly weaker chelating ability than the control ethylenediaminetetraacetic acid (EDTA) in a metal chelating ability assay.

CHAPTER 3

METHODOLOGY

3.1 Introduction

Overall methodology of this research started with the roots of *R. tomentosa* were extracted through solvent-solvent extraction method. Extracts of *R. tomentosa* root were then proceed toward UPLC-QToF/MS for the identification of chemical constituents. *R. tomentosa* root extracts war further screening for its antioxidant and cytotoxicity properties. The biologically active extracts were promoted to be chromatographed, fractionated and isolated. The isolated compounds were characterized by spectroscopy techniques with comparison with previous reported spectral data. Isolated compound lupeol was further supported with bioassay activities.

3.2 Materials

3.2.1 Plant Material

The roots of *R. tomentosa* (5.0 kg) were collected in two stages in September 2014 and March 2015 from Bodywell My Marketing (JR0042311-M), Johor, Malaysia and identified by Dr. Shamsul Kamis, Botanist at Institute of Bioscience, University Putra Malaysia. A voucher specimen was deposited at the Herbarium Unit of University Putra Malaysia, Malaysia.

3.2.2 Extraction and Isolation Materials

Industrial grade solvents hexane (Hx), chloroform ($CHCl_3$), ethyl acetate (EtOAc), and methanol (MeOH) were used for extraction and isolation purposes. Analytical grade

solvents were used in purification and chromatography. All solvent were purchased from BT Science SDN BHD, Selangor, Malaysia. The spraying reagent used for TLC was dilute sulphuric acid reagent. Silica gel 60 (0.063 - 0.020 mm) (70 - 230 mesh ASTM) was used in column chromatography. Aluminium sheets of silica gel 60 F254 was used for TLC. Both silica gel and TLC aluminium sheets were purchased from Merck Millipore, Malaysia.

3.2.3 Cell Culture Materials

Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Aldrich) was used for media with supplement of 10% foetal calf serum (Sigma-Aldrich). Penicillin-streptomycin (Sigma) and fungizone (Sigma) is used for cells maintenance purpose. Dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiaozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent were both purchased from Impian Z Enterprise, Selangor, Malaysia. The cells used in screening extracts and isolated compounds are HepG2, human liver cancer cells, MCF-7, breast cancer cells, and HT 29 human colorectal adenocarcinoma cell lines. All cell lines were a courtesy of MAKNA Lab, Universiti Putra Malaysia.

3.3 *Rhodomyrtus tomentosa* Extraction

The roots of *R. tomentosa* were dried in an oven at 60 °C for 48 hours and grounded by grinder. The dried ground roots of *R. tomentosa* (968.87 g) were percolated at room temperature for a day with hexane and methanol consecutively. Both extracted plant sample were sonicated for 30 minutes at below 60 °C (thrice). Hexane and methanol extracts were filtered and each filtrate was evaporated to dryness using vacuum rotary evaporator at 60°C. The concentrated methanol extract was suspended with water and then fractionated successfully with chloroform and ethyl acetate, consecutively following evaporating in vacuo to give chloroform and ethyl acetate extract. Figure 3.1 shows the procedure for extraction and fractionation of *R. tomentosa* roots.

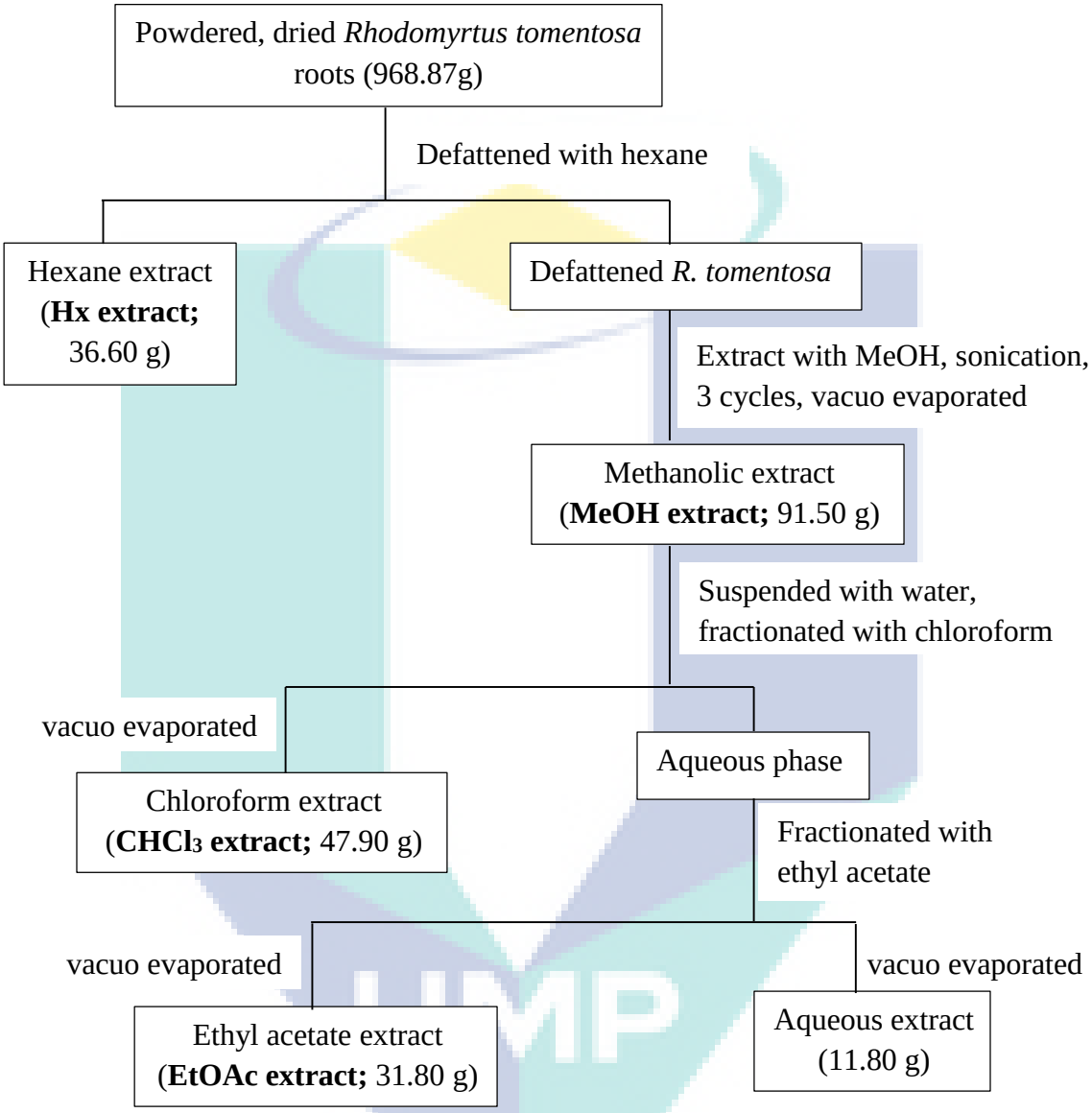


Figure 3.1 Extraction and fractionation of *R. tomentosa* roots

3.4 Phytochemical Analysis

1 g of each extracts were dissolved in 1 mL methanol and subjected to ultra-high performance liquid chromatography-quadrupole time-of-flight/mass spectrometry (UPLC-QToF/MS) for the determination of active composition present in roots of *R. tomentosa*. HSS BEH C18 column (2.1 x 100 mm, 1.8 µm) was used. The HPLC data was obtained on Waters Xevo G2-S QToF. Water with 0.1 % formic acid (A) and a mixture of methanol with 0.1 % formic acid (B) was used as a mobile phase. Injection volume was 2 µL, while the flow rate was set at 0.4 mL/min. The QToF MS was operated in the range of 10 - 1500 Da, in negative and positive modes.

3.5 Total Flavonoid Contents

The total flavonoid contents were determined by aluminium calorimetric method. A stock solution of plant extracts was prepared in different aliquots (31.25, 62.5, 125, 250, 500, and 1000 µg/mL). 150 µL of plant extracts and 15 µL of sodium nitrate solution were transferred in 96-well plates. Then, 15 µL of 10 % aluminium chloride solution was added; after 5 min followed by 75 µL of 4 % sodium hydroxide (NaOH) solution. The absorbance of the mixture was immediately recorded at 518 nm. To minimize standard error, the reaction was performed in triplicate and the results were expressed in milligrams of rutin equivalents per gram of sample (mg RE/ g sample).

3.6 Total Phenolic Contents

Total phenolic contents were determined by using Folin-Ciocalteu method. The solution was diluted to 25 mL with distilled water. Concisely, 2 mL of each plant extracts were mixed with Folin-Ciocalteu reagent (10-fold diluted with distilled water, 20 mL). After a 5 min interval, 8 mL of 7.5 % (w/v) sodium carbonate (Na_2CO_3) solution was added. After 2 hours, the absorbance was measured at 765 nm against a blank prepared as described above with distilled water (100 µL), Folin-Ciocalteu reagent, and sodium carbonate solution. The content of total phenolics was expressed as mg of gallic acid equivalents per g of sample (mg GAE/ g sample).

3.7 Bioactivity Assay

3.7.1 DPPH Radical Scavenging Assay

Free radical scavenging ability was determined by using the stable radical DPPH method. Plant extracts sample were prepared at 1 mg/mL and 100 µL was added to 200 µL of 2 mg/mL methanolic solution of DPPH, mixed well and allowed to incubate at 30 °C for 15 min. The absorbance was measured at 517 nm. The percentage of DPPH radical scavenging activity was theoretically calculated by the following formula:

$$\%\ DPPH\ radical\ scavenging\ activity = \frac{(A_{control} - A_{sample})}{A_{control}}\ x\ 100 \qquad 3.1$$

3.7.2 Cupric Reducing Antioxidant Capacity (CUPRAC) Assay

CUPRAC assay was performed to measure the scavenging activity of *R. tomentosa* extracts. To each well, in a 96-well plate, 150 µL CUPRAC reagent consisting of 10 mM Copper(II)chloride, 7.5 mM neucoproine and ammonium acetate buffer (1 % (v/v) acetic acid, 0.1 M ammonium acetate, pH7) solution were added. 50 µL of extract at different concentrations (31.25, 62.5, 125, 250, 500, and 1000 µg/mL) was mixed to the initial mixture and incubated for 30 minutes at room temperature before absorbance readings were obtained at 450 nm. Results were given as absorbance and compared with ascorbic acid used as antioxidant standard.

3.7.3 β-Carotene Bleaching Assay

To assess lipid peroxidation activity of extracts, β-carotene bleaching assay was carried out. 1 mL of β-carotene solution (2 mg/mL in chloroform) was transferred into round bottom flask (50 mL) containing 20 µL of linoleic acid and 200 µL of Tween 80 solution. After evaporating to dryness, 50 mL of distilled water was added to the residue to form emulsion after vigorous agitation. The emulsion (150 µL) was mixed with 50 µL of different concentration of extracts and BHT standard (31.25, 62.5, 125, 250, 500, and 1000 µg/mL) in each well of the 96-well plate, where methanol was used as control. The absorbance was immediately measured at 470 nm using Tecan Infinite M200 PRO microplate reader. The

reaction mixture was incubated at 50 °C for 120 min before the absorbance was measured again. The assay was calculated by using the following equation:

$$\beta - carotene\ bleaching\ assay = 1 - \frac{(A_o - A_t)}{(A_{o'} - A_{t'})} x\ 100 \qquad 3.2$$

Where:

A_o *and* A_t = The absorbance values measured at initial time of the incubation for samples and control, respectively.

$A_{0'}$ *and* A_t' = The absorbance values measured in the samples and control at t = 120 min.

3.7.4 Cell Culture for Cytotoxicity

A cryovial consisting of cancer cells were thawed in constant temperature water bath at 37 °C with a light swirling. The cells were then were transferred into 15 mL ampoule centrifuge at 3000 rpm for 5 minutes for the purpose of removing cryopreservative, dimethylsulfoxide (DMSO) by centrifuging. The cells were introduced into falcon flask T-25 and were cultivated in DMEM medium (Sigma-Aldrich) supplemented with 10 % (v/v) foetal serum (Sigma-Aldrich), penicillin-streptomycin (Sigma), and fungizone (Sigma) to increase and stimulate cells survival and its proliferation. Penicilin-streptomycin was added to preferentially kill the bacteria (*Streptococcus lactic and Bacillus cereus*) that might hinder the cells while fungizone hinder the growth of fungal (Mycoplasma). The growth medium was complimented with the presence of pH indicator, phenol red. The maintenance of pH is important and requisite for the sustenance of exponentially growth of cells. Cells were incubated at 37 °C, 5 % CO_2. The medium was replaced on every alternate day until the cells were confluent and ready to be sub-cultivated.

The media in the flask was discarded when the cells reached its confluency. The cells were then washed several times with isotonic solution (PBS). The solution allows the removal of any residue of culture media and dead cells. Cells were detached by adding 0.025 - 0.040 mL/cm^2 of 0.25 % (v/v) trypsin and then incubated for 5 - 10 minutes. The process of trypsinization was enhanced by gently tapping tapping the flask a few times. Addition of 1 - 2 mL of 10 % FCS-DMEM was added to the cells as soon as the cells appear to be rounded

and solitary under the microscope, to inactivate the trypsin. The cells suspension was transferred to 15 mL falcon tube and then centrifuged at 1800 rpm for 5 minutes. The cells were then resuspended in culture media.

3.7.5 Cell Viability Assay

The effect of *R. tomentosa* extracts on the viability of various cancer cell lines was determined by an MTT-based assay. Briefly, exponential-phase cells were collected and transferred to a microtitre 96 plate ($1 \times 10^4 - 10^5$ cells per well). The cells were then incubated for three days in the presence of various concentrations of the extracts. Tamoxifen was used as positive control and untreated media was the negative control. After incubation, 50 µL of MTT (2 mg/mL MTT in plain culture medium) was added to each well and the cells were incubated at 37 °C for 4 h. The plates were then centrifuged at 1,500 rpm for 20 min and the medium was carefully removed. DMSO (200 µL) was then added to each well to dissolve the formazan crystals. The plates were read immediately at 540 nm on a microplate reader. The concentration of *R. tomentosa* extracts at which growth was inhibited by 50 % (the IC_{50} value) was determined in triplicates for each well.

In preparing all *R. tomentosa* extracts and tamoxifen (positive control), 10 mg of each sample were dissolved in 1 mL distilled water as a stock solution of 10 mg/mL. The testing saluting were made by 2x serial dilution for 10 points ranged from 0.2 µg/mL to 100 µg/mL (0.1 mg/mL, 0.05 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 6.25×10^{-3} mg/mL, 3.125×10^{-3} mg/mL, 1.563×10^{-3} mg/mL, 7.81×10^{-4} mg/mL, 3.91×10^{-4} mg/mL, 1.95×10^{-4} mg/mL). Cell concentration was determined by trypsin blue before plating. 80 - 90 % confluent culture were harvested with 0.05 % (v/v) trypsin 0.02 % (v/v) EDTA and plated onto 96 well plates at initial density of approximately $1 \times 10^4 - 10^5$ cells per well and were left to be attach, overnight. After overnight incubation, the medium was removed and replaced with fresh media (198 µL/well) with 2 µL of each concentrations of *R. tomentosa* fractions prepared. Tamoxifen was used as a control drug and was dissolved in DMSO. The control culture cells received the same treatment concentration of solvent. Final concentration of DMSO was 1 % (v/v) or less. Each concentration of the extracts was assayed in triplicates. The cells were incubated at 37 °C, 5 % CO_2 in the incubator. The assay was terminated at 72 hours. The number of surviving cells were determined by MTT staining assay. After incubation, 50 µL

of MTT solution (2 mg/mL MTT in plain culture medium) was added to each well. The plate was then incubated for 4 hours. MTT assay was then removed. Purple formation crystal forming at the bottom of the wells was dissolved with 200 µL DMSO before being placed on the shaking microplate for 20 minutes. The proportion of surviving cells was calculated as:

$$\frac{(OD\ of\ drug\ treated\ sample - OD\ of\ blank)}{(OD\ of\ sample - OD\ of\ blank)}\ x\ 100 \qquad 3.3$$

The dose-response curves were constructed to obtain the IC_{50} values.

3.8 Isolation of Compounds

3.8.1 Compound (27)

R. tomentosa ethyl acetate extract showing notable antiproliferative activity was chromatographed on silica gel column with gradient of hexane-ethyl acetate and chloroform-methanol. Fractions showing similar TLC profile were pooled to give 8 fractions (A1 - A8) as shown in Table 3.1. Repeated silica gel column chromatography of fraction A3 (223.89 mg) with gradient hexane-chloroform yielded 10 fractions (A3.1 - A3.10). A3.8 (46.56 mg) was chromatographed with hexane-ethyl acetate yielded compound **(27)** (10.45 mg).

Table 3.1 Fractions from column chromatography of *R. tomentosa* ethyl acetate extract

Eluent	Ratio (%)	Fraction	Weight (mg)
Hx : EtOAc	80 : 20	A1	174.56
Hx : EtOAc	60 : 40	A2	140.23
Hx : EtOAc	40 : 60	A3	223.89
Hx : EtOAc	10 : 90	A4	352.21
$CHCl_3$	100	A5	431.25
$CHCl_3$: MeOH	90 : 10	A6	211.56
$CHCl_3$: MeOH	70 : 30	A7	378.20
$CHCl_3$: MeOH	50 : 50	A8	425.76

3.8.2 Compound (24)

Chloroform extract from *R. tomentosa* showing notable anticancer and antioxidant activities was chromatographed on silica gel column with gradient of hexane-ethyl acetate and chloroform-methanol to give 12 fractions (B1 - B12) as shown in Table 3.2. Repeated

silica gel column chromatography of fraction B2 (253.91 mg) with gradient hexane-chloroform yielded 10 fractions (B2.1 - B2.10). B2.6 (66.57 mg) was chromatographed with hexane-ethyl acetate yielded compound **(24)** (8.04 mg).

Table 3.2 Fractions from column chromatography of *R. tomentosa* chloroform extract

Eluent	Ratio (%)	Fraction	Weight (mg)
Hx : EtOAc	90 : 10	B1	196.73
Hx : EtOAc	80 : 20	B2	253.91
Hx : EtOAc	60 : 40	B3	325.19
Hx : EtOAc	30 : 70	B4	472.56
$CHCl_3$	100	B5	376.87
$CHCl_3$: MeOH	90 : 10	B6	523.74
$CHCl_3$: MeOH	80 : 20	B7	432.17
$CHCl_3$: MeOH	70 : 30	B8	357.73
$CHCl_3$: MeOH	60 : 40	B9	421.97
$CHCl_3$: MeOH	50 : 50	B10	503.79
$CHCl_3$: MeOH	20 : 80	B11	491.26
MeOH	100	B12	573.41

3.9 Statistical Analyses

All analytical values shown represent the means of triplicates. The data are expressed as mean ± standard deviation. Statistical significant difference was determined using One-way ANOVA and differences were considered to be significant if $p < 0.05$.

CHAPTER 4

RESULT AND DISCUSSIONS

4.1 Introduction

The roots of *R. tomentosa* were extracted to give four extracts. *R. tomentosa* root extracts were investigated for chemical composition via UPLC-QToF/MS. The preliminary screening of bioactivity assay found that *R. tomentosa* root extracts exhibited strong antioxidant and cytotoxicity properties. Fractionation and chromatography of EtOAc and $CHCl_3$ extracts from roots of *Rhodomyrtus tomentosa* resulted in the isolation of lupeol **(27)** and β-sitosterol **(24)**. Structure elucidation of compounds were determine by analyses of spectral data in association comparison with literature. Isolated compound lupeol exhibit significant antioxidant and cytotoxicity properties.

4.2 Extraction of *R. tomentosa*

The roots of *R. tomentosa* was productively extracted via solvent-solvent extraction method with hexane, EtOAc, $CHCl_3$, and MeOH to give bright yellowish gums of Hx extract (36. 60 g), dark yellow gums of EtOAc extract (31.80 g), dark orange gums of CHCl3 extract (47.90 g), and dark brownish gums of MeOH extract (91.50 g).

4.3 Profiling of *R. tomentosa* Compounds through UPLC-QToF/MS

The combination between mass spectrometry and chromatography is considered to be an important progress on analytical technology. Mass spectrometry commonly used due to its reproducibility and its ability in analysing complex biological samples. Liquid Chromatography-Mass Spectrometry (LC-MS) analysis is used to interpret non-volatile

compounds whereas gas chromatography-mass spectrometry (GC-MS) is generally for analysing volatile compounds (Halket, Waterman, Przyborowska, Patel, Fraser et al., 2005; Lee, Jung, Shin, Kim, Moon et al., 2014).

In this study, LC-MS based profiling was used to analyse compounds of *R. tomentosa*. In an effort to investigate the relationship between the extracts composition and its activities, ultrahigh-performance liquid chromatography–quadrupole time-of-flight mass spectrometry (UPLC-QToF/MS) was used to determine content of the active components in samples from *R. tomentosa*. UPLC-QToF/MS in plants analysis is relatively new and increasingly used technique in the field due to its effectiveness in detecting chemical compounds with high sensitivity (Farag, Gad, Heiss, & Wessjohann, 2014; Grata, Boccard, Guillarme, Glauser, Carrupt et al., 2008). The compounds in MeOH extract, $CHCl_3$ extract EtOAc extract were identified by their characteristic mass fragments.

The peak identification was performed by comparison of the retention time (RT), mass error, ion response, and fragmentation pattern for each compound. Since all fragment ions have been automatically elucidated by Mass Fragment, the verification process for the components become easier. Based on the data processing in UNIFI software, the components are classified as a good match with ± 5 mDa error and poor match with ± 10 mDa error. In order to verify if match is reasonable, we looked at the adduct ions as well as the fragmentation ions. The element 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 5 mDa and high ion response. Then by checking on MS/MS fragmentation ions and relevant reference literatures, their chemical structures were further verified.

Figure 4.1 (negative) and Figure 4.2 (positive) show the UPLC-QToF/MS base peak ion (BPI) chromatogram of *R. tomentosa* MeOH extract. Based on the confirmed component plotted, major compounds that can be found in MeOH extract (Figure 4.3) was kaempferol 3-*O*-β-D-glucuronopyranoside. The compound detected in MeOH extract *R. tomentosa* at higher concentrations included catechin 7-*O*-β-D-glucopyranoside, methylellagic acid and khellol-β-D-glucoside. Despite the relatively low sensitivity of (+)ESI compared to (-)ESI, positive mode (+)ESI-MS was effective in terms of identifying methylellagic acid and

quercetin-3-*O*-(6''-*O*-acetyl)-β-D-glucopyranoside which are most abundant in MeOH extract of *R. tomentosa* (Figure 4.4).

Appendix A1 and Appendix A2 shows the data of confirmed compounds on (-)ESI and (+)ESI of MeOH extract from *R. tomentosa*. The precursor ions of *R. tomentosa* in MeOH extract were as $[M - H]^-$ at *m*/*z* 461.0728 for kaempferol 3-*O*-β-D-glucuronopyranoside, 343.0462 for methylellagic acid, $[M - HCOO]^-$ at *m*/*z* 497.1306 for catechin 7-*O*-β-D-glucopyranoside, 453.1036 for kallol-β-D-glucoside and $[M - H]^+$ at *m*/*z* 345.0604 for methylellagic acid.

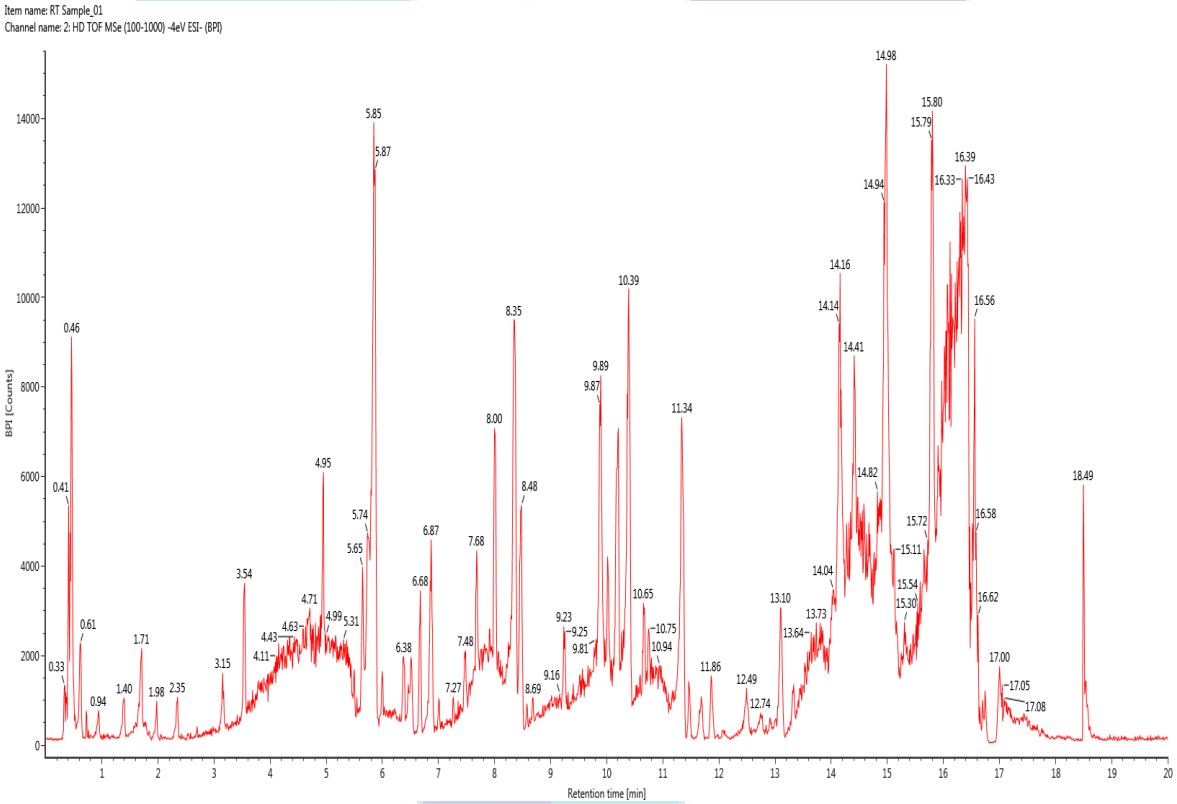


Figure 4.1 UPLC-QToF/MS negative BPI chromatogram of *R. tomentosa* methanolic extract

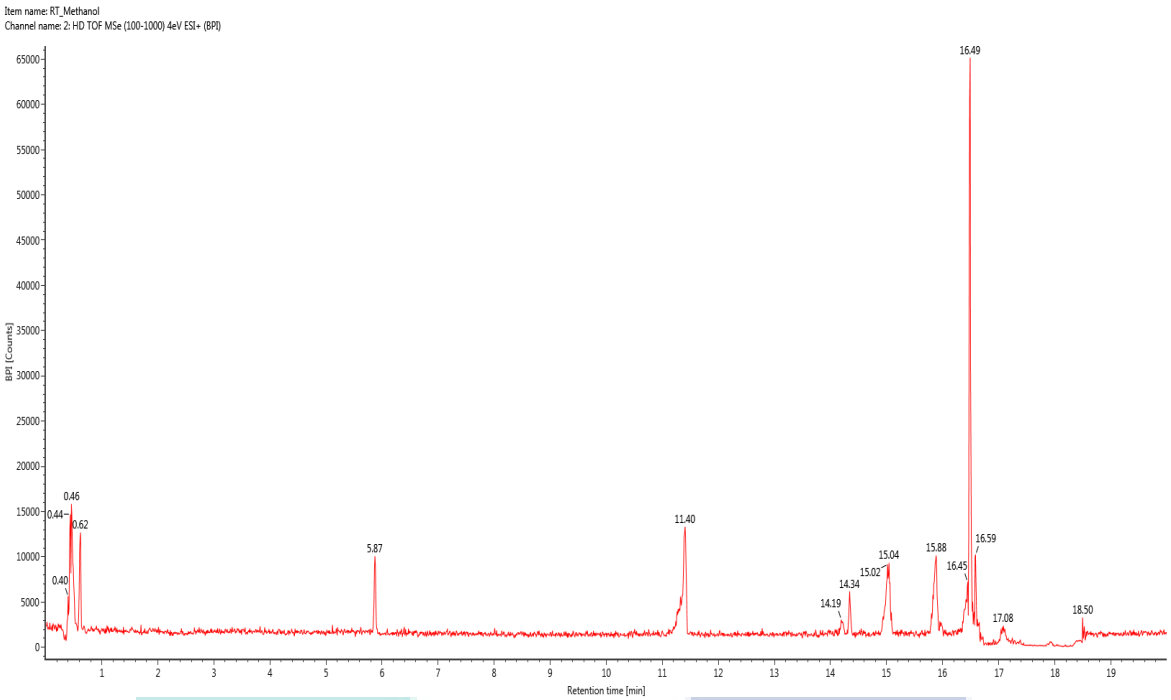


Figure 4.2 UPLC-QToF/MS positive BPI chromatogram of *R. tomentosa* methanolic extract

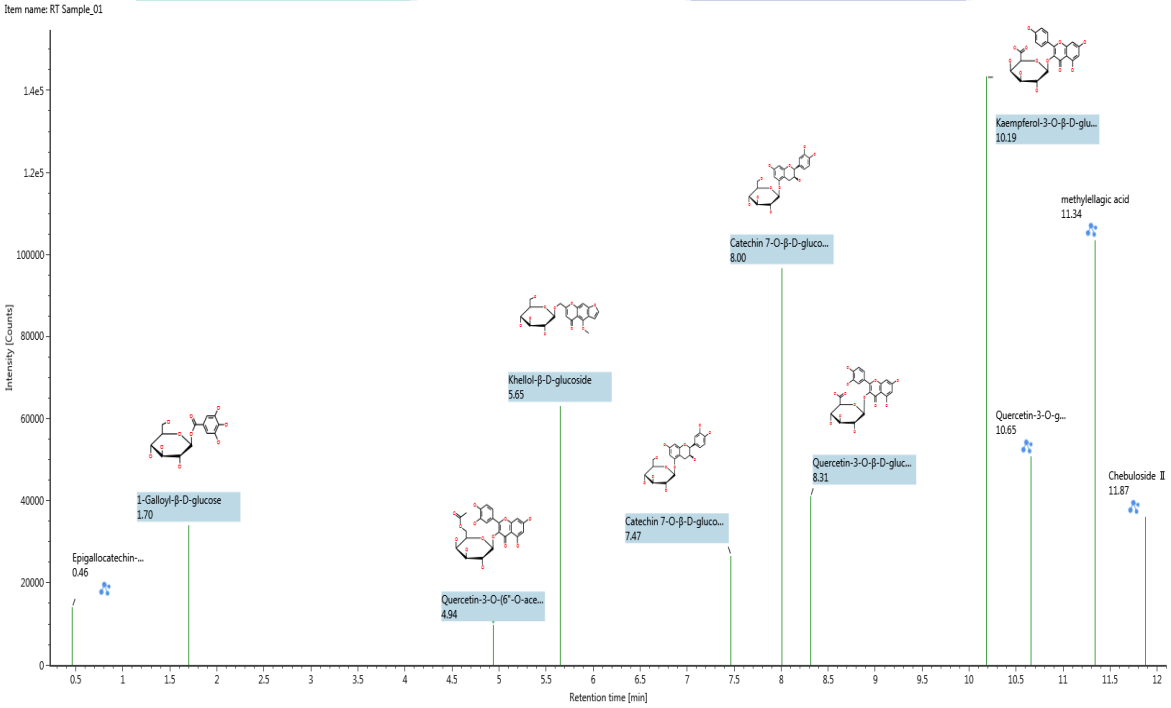


Figure 4.3 Confirmed major compounds plotted on (-)ESI-MS of methanolic extract

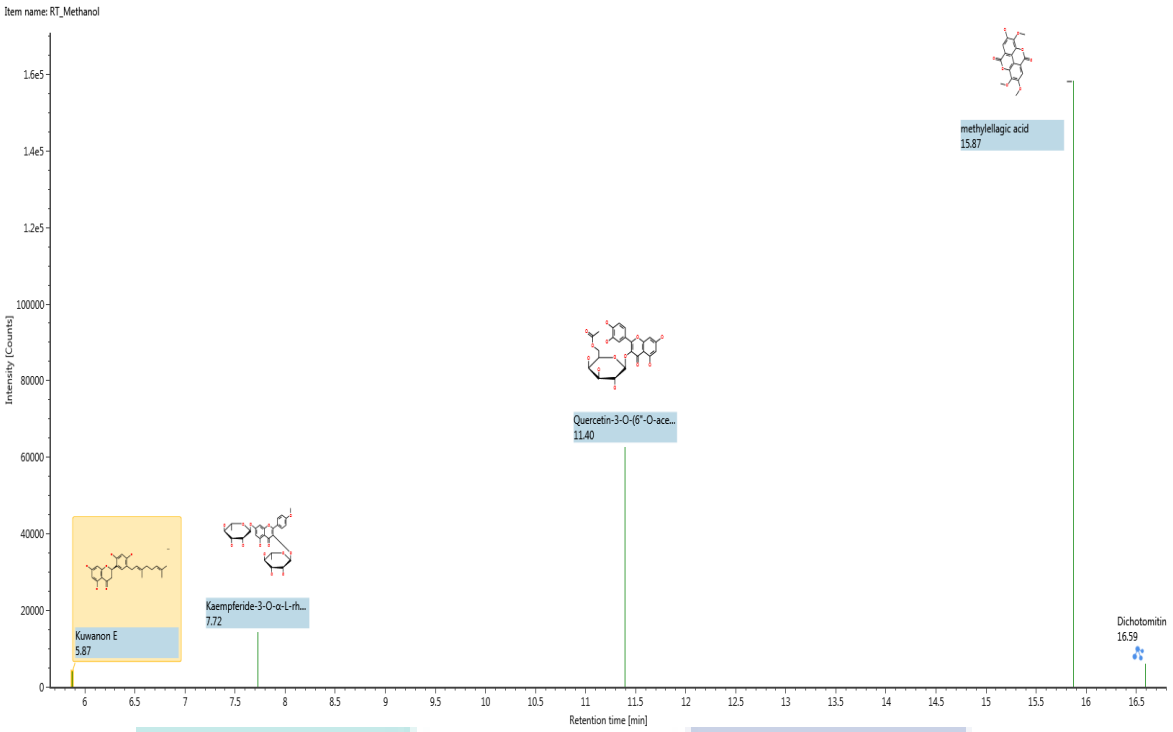


Figure 4.4 Confirmed major compounds plotted on (+)ESI-MS of methanolic extract

Appendix B1 shows the UPLC-QToF/MS base peak ion (BPI) chromatogram of $CHCl_3$ extract from *R. tomentosa*. Compound dichotomitin and methylellagic acid are the most abundant in $CHCl_3$ extract (Appendix B2).Compounds detected in $CHCl_3$ extract of *R. tomentosa* also included rhodomyrtosone C, (2S)-3',4'-methylenedioxy-5,7-dimethoxyflavane, and 4′,7-dimethyltectori-genin. Appendix B3 shows data of confirmed compounds on (+)ESI-MS of *R. tomentosa* $CHCl_3$ extract. The abundant ions of *R. tomentosa* in $CHCl_3$ extract were detected as $[M - H]^+$ at *m/z* 345.0608 for methylellagic acid and $[M - H]^-$ at *m/z* 359.076 for dichotomitin. Other ions that were also detected from $CHCl_3$ extract as $[M - H]^+$ *m/z* 329.1017 for 4′,7-dimethyltectori-genin, 675.3893 for rhodomyrtosone C, and $[M - Na]^+$ at *m/z* 337.1046 for (2S)-3',4'-methylenedioxy-5,7-dimethoxyflavane.

Appendix C1 shows the UPLC-QToF/MS base peak ion (BPI) chromatogram of EtOAc extract from *R. tomentosa*. Based on the confirmed component plotted, major compound from EtOAc extract (Appendix C2) was rhodomyrtosone D. 3-hydroxynaringenin, quercetin 3-*O*-α-L-arabinoside, dihydrokaempferol-5-*O*-β-D-glucopyranoside and isoxanthohumol are compounds that are also be detected at higher

concentrations in EtOAc extract of *R. tomentosa*. Appendix C3 shows data of confirmed compounds on (+)ESI-MS of *R. tomentosa* EtOAc extract. A major compound found in EtOAc extract was annotated as rhodomyrtosone D, $[M - Na]^+$ at *m*/*z* 471.2202. Other compounds were also detected as $[M - H]^+$ at *m*/*z* 289.0707 for 3-hydroxynaringenin, 355.1488 for isoxanthohumol, $[M - Na]^+$ at *m*/*z* 459.0925 for quercetin 3-*O*-a-L-arabinoside, and 473.1154 for dihydrokaempferol-5-*O*-β-D-glucopyranoside.

The classification of major compounds established on ESI-MS of *R. tomentosa* root extracts (EtOAc, $CHCl_3$ and MeOH) between either flavonoid or phenolic compound were exhibited in Table 4.1.

Table 4.1 Classification of major compounds on ESI-MS of *R. tomentosa* root extracts

Compound Class	Compound
Flavonoid	kaempferol 3-*O*-β-D-glucuronopyranoside
	catechin 7-*O*-β-D-glucopyranoside
	(2S)-3',4'-methylenedioxy-5,7-dimethoxyflavane
	4′,7-dimethyltectori-genin
	quercetin 3-*O*-α-L-arabinoside
	dihydrokaempferol-5-*O*-β-D-glucopyranoside
	3-hydroxynaringenin
	isoxanthohumol
Phenolic	methylellagic acid
	khellol-β-D-glucoside
	dichotomitin
	rhodomyrtosone C
	rhodomyrtosone D

4.4 Total Flavonoid and Phenolic Contents

The results obtained from preliminary analysis of flavonoid and phenolic compound is shown in Table 4.2. The preliminary analysis showed that methanolic extract has highest value of both TFC and TPC (110.822 ± 0.017 mg RE/g; 190.467 ± 0.009 mg GAE/g) when compared with other samples. Hexane extract showed lowest values for both TFC and TPC (65.422 ± 0.004 mg RE/g; 23.483 ± 0.008 mg GAE/g). Chloroform extract (78.678 ± 0.005 mg RE/g; 65.789 ± 0.004 mg GAE/g) and ethyl acetate extract (65.856 ± 0.002 mg RE/g; 29.317 ± 0.008 mg GAE/g) had moderate flavonoid and phenolic contents.

Table 4.2 Quantitative analysis of total flavonoid and phenolic contents of *R. tomentosa*

Sample	Total flavonoid content, (mg RE/g)	Total phenolic content, (mg GAE/g)
Hx extract	65.422 ± 0.004	23.483 ± 0.008
MeOH extract	110.822 ± 0.017	190.467 ± 0.009
$CHCl_3$ extract	78.678 ± 0.005	65.789 ± 0.004
EtOAc extract	65.856 ± 0.002	29.317 ± 0.008
Standard	256.011 ± 0.001*	265.894 ±0.001[#]

* α-Rutin

[#] Gallic acid

Results were mean ± SD (n=3)

The solubility of phenolics and flavonoids varies with the degree of conjugation of the aromatic rings, glycosidic form and side chain such as hydroxyl, methoxy or methyl. Molecules with a high number of hydroxyl group tends to be relatively polar and dissolve in alcohols or aqueous. In principle, polar molecules are soluble in polar solvents and vice versa. Polar solvent has a property of dipole-dipole interaction forces, particularly hydrogen-bond formation to which solvating molecules become soluble (Visht & Chaturvedi, 2012). The results indicate that phenolics and flavonoids from *R. tomentosa* were polar compounds due to its highest TPC and TFC values in methanol extract. The results from the study were similar to previous studies (Addai, Abdullah, & Mutalib, 2013; Ghasemzadeh, Jaafar, & Rahmat, 2011) where methanol was most effective in extracting phenolic and flavonoid compounds from plants.

Phenolic compounds play a vital role as an antioxidant due to their redox properties that acts as hydrogen donor, singlet oxygen quenchers, reducing agent and free radical scavenging properties (Chang, Wu, Wang, Kang, Yang et al., 2001; Khan, Rahman, Sardar, Arman, Islam et al., 2016). They can be categorized as simple phenolic, phenolic acids, hydroxycinnamic acid derivatives and flavonoids. The phenolic contents of methanolic and chloroform in this study is higher than the value reported from previous work of Lai et al. (2015) for sim fruits of *R. tomentosa* (49.21 ± 0.35 mg GAE/g) and Huang, W.-Y., Cai, Corke, and Sun (2010) (24.00 ± 0.04 mg GAE/g). The difference could be due to part of the plant sample used, genetic variations, environmental conditions, or extraction methods.

Flavonoids, which are believed to be another determinant in possessing antioxidant property (Harborne & Williams, 2000), are also known to possess anticancer activities due to its ability in scavenging free radical (Bennett, Rojas, & Seefeldt, 2012; Jomova & Valko, 2011; Prasad, Phromnoi, Yadav, Chaturvedi, & Aggarwal, 2010; Rossi, Bosetti, Negri, Lagiou, & La Vecchia, 2010; Yao, Xu, Shi, & Zhang, 2011). High intakes of flavonols and flavanones consumption may be associated with lower risk in ovarian cancer (Cassidy, Huang, Rice, Rimm, & Tworoger, 2014). A study by Wu, P. et al. (2015) stated that extracts from *R. tomentosa* berries are rich in flavonoids which possess 20 times more TFC than those in cranberry and even higher amounts than those in other berries and vegetables. Previous studies by Shen, Jin, Xiao, Lu, and Bao (2009) correlated the relation between TPC and TFC with antioxidant activity. To the best of our knowledge, this is the first study of phenolic and flavonoid on the roots of *R. tomentosa.*

4.5 Bioactivities

4.5.1 Antioxidant Activities

The antioxidant properties of the extracts were obtained through different mechanisms of action such as radical scavenging effects (DPPH), reducing power (CUPRAC) and β-carotene bleaching assays.

Radical scavenging of DPPH measures the decrease in DPPH after exposure to radical scavenger. The changes in colour occurred in the presence of proton donating substances from the formation of diamagnetic molecules by receiving hydrogen radical (Baratzadeh, Asoodeh, & Chamani, 2013; Cheng, Wang, & Xu, 2006). Reducing power measures the reduction of Cu^{2+} to Cu^{+} by reductant, and higher absorbance indicates a higher cupric ions (Cu^{2+}) reducing power. In the β-carotene bleaching assay, linoleic acid reacts with reactive oxygen species (ROS) and O_2 to form an unstable peroxy radical (Mogana et al., 2013). β-carotene bleaching can be limited by the presence of an antioxidant that can help to overcome linoleic acid free radicals and other radicals formed. Lower absorbance of β-carotene indicated higher lipid peroxidation inhibition (Memarpoor-Yazdi, Asoodeh, & Chamani, 2012).

Figure 4.5 depicts the ability of different *R. tomentosa* extracts to scavenge DPPH radicals compared with the ability of ascorbic at different concentrations ranging from 31.25 to 1000 µg/mL. Increasing the concentrations of *R. tomentosa* extracts resulted in the increase of the radical scavenging activities. The order of the scavenging activity for the different extracts is methanolic > chloroform > ethyl acetate > hexane. These can be seen from decreasing slopes between chloroform extract, ethyl acetate extract, and hexane extract respectively. The various slopes of different extracts are due to capability of solvents to extract different types of compounds. Different concentration of compounds present in the extracts might affect the ability of donating hydrogen atom and reaction with free radicals. In general, the higher polyphenol yield corresponds to a higher antioxidant activity and the presence of glycoside in the flavonoids may decrease the antioxidant activity (Siddhuraju & Becker, 2003). The presence of polyphenol compounds in *R. tomentosa* contribute to high antioxidant activity. In a study done by Liu, Y. et al. (1998), extract from *R. tomentosa* yield polyphenol compounds such as pedunculagin **(33)**, casuarin **(34)**, and castalagin **(35)**.

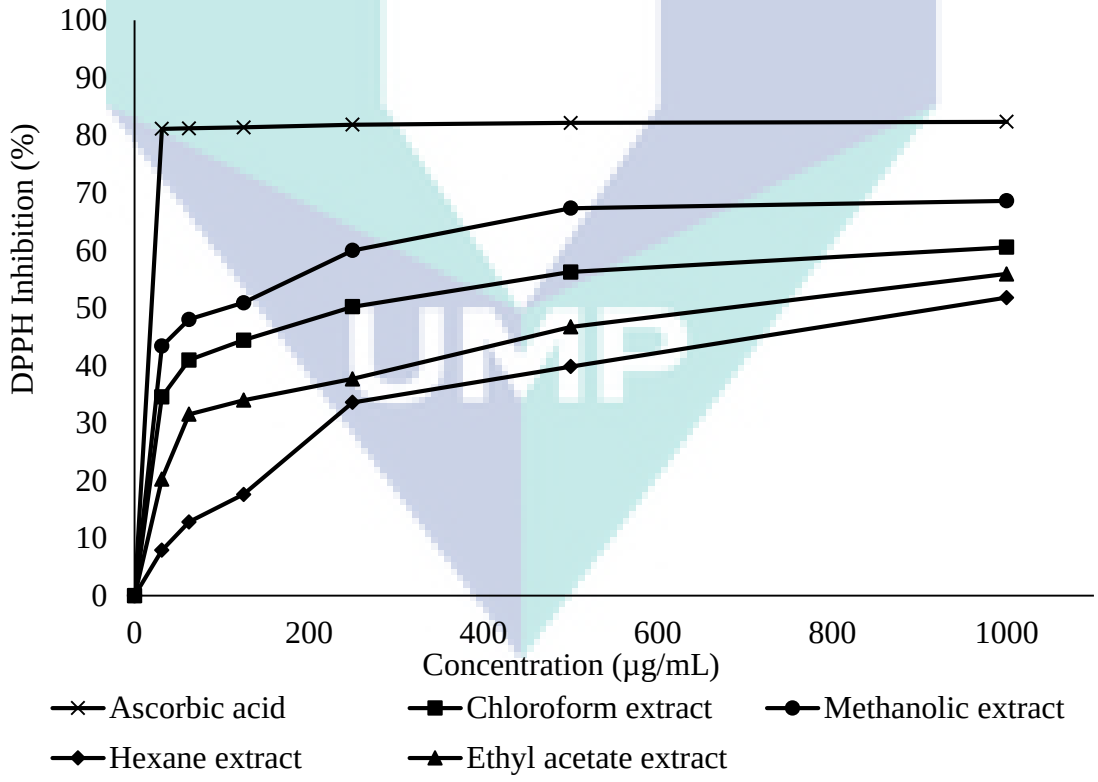


Figure 4.5 The DPPH free radical scavenging activity of *R. tomentosa*

The results are in tandem with previous studies which indicate high antioxidant activity in DPPH assay from methanol extract (Maskam et al., 2014). The features of both phenolic and flavonoid compounds are correlated and responsible for antioxidant activities. The capacity of phenolics to scavenge free radicals may be due to their possession of many phenolic hydroxyl groups. Phenols play an important role in antioxidant activity, because they transfer hydrogen to radicals and produce phenoxide radical, which stabilizes products. Hence, in this study, the higher activity of antioxidant from methanolic extract (110 ± 0.005 µg/mL) shows the relationship between TPC and TFC, which is 190.467 mg GAE/g and 110.822 mg RE/g respectively. Bazzaz, Khayat, Emami, Asili, Sahebkar et al. (2011) indicated that antioxidant capacity is based on composition of phenolic contents in the extracts.

The reduction capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The reducing power increases with concentration, and the absorbance values obtained for methanolic and chloroform extracts were above 1.5 nm (Figure 4.6). Ethyl acetate and hexane extracts show a lower absorbance value at concentration of 1000 µg/mL with 0.577 and 0.473 respectively. From the above findings, the results correlate with the amount of phenolic constituents, which are present in abundance in methanol extracts. The profiling result from UPLC-QToF/MS also support presence of phenolic compound in methanolic extract. The phenolic compound acts as a reductant by providing a hydrogen atom, thus breaking the free radical chain. The results are similar with previous studies that show the existence of natural antioxidants in plants that are mainly from phenolic compounds (Chanwitheesuk, Teerawutgulrag, & Rakariyatham, 2005; Nijveldt, van Nood, van Hoorn, Boelens, van Norren et al., 2001; Parr & Bolwell, 2000; Sghaier, Skandrani, Khochtali, Bhouri, Ghedira et al., 2012). In addition, the antioxidant activity of phenolic compounds is not only due to their redox properties, which allows them to act as reductant, but also act as hydrogen donors and singlet oxygen quenchers.

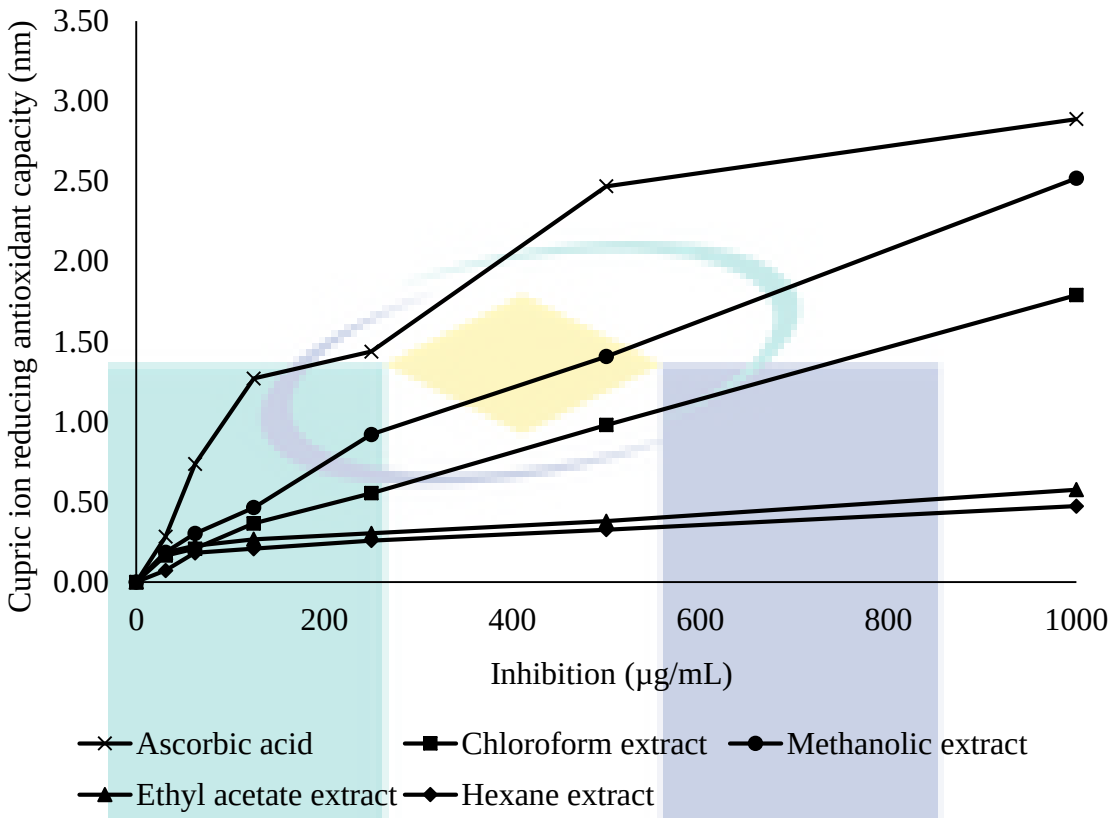


Figure 4.6 Cupric ion reducing antioxidant capacity of *R. tomentosa*

Figure 4.7 shows the antioxidant activity of the extracts, compared with BHT, using β-carotene. Methanolic extract showed the highest inhibition value at concentration of 1000 µg/mL with 85.903 % followed by chloroform extract, 77.837 %, ethyl acetate extract, 60.527 % and hexane extract, 48.757 % respectively. Bleaching inhibition increased as the concentration was high, and the value were all above 50 % except for hexane extract. An antioxidative components in the *R. tomentosa* extracts prevent the bleaching of β-carotene by neutralizing the linoleate-free radical and other free radicals formed in the system. The profiling of phenolic and flavonoid compound achieved through UPLC-QToF/MS highly influenced the inhibition values of bleaching assay.

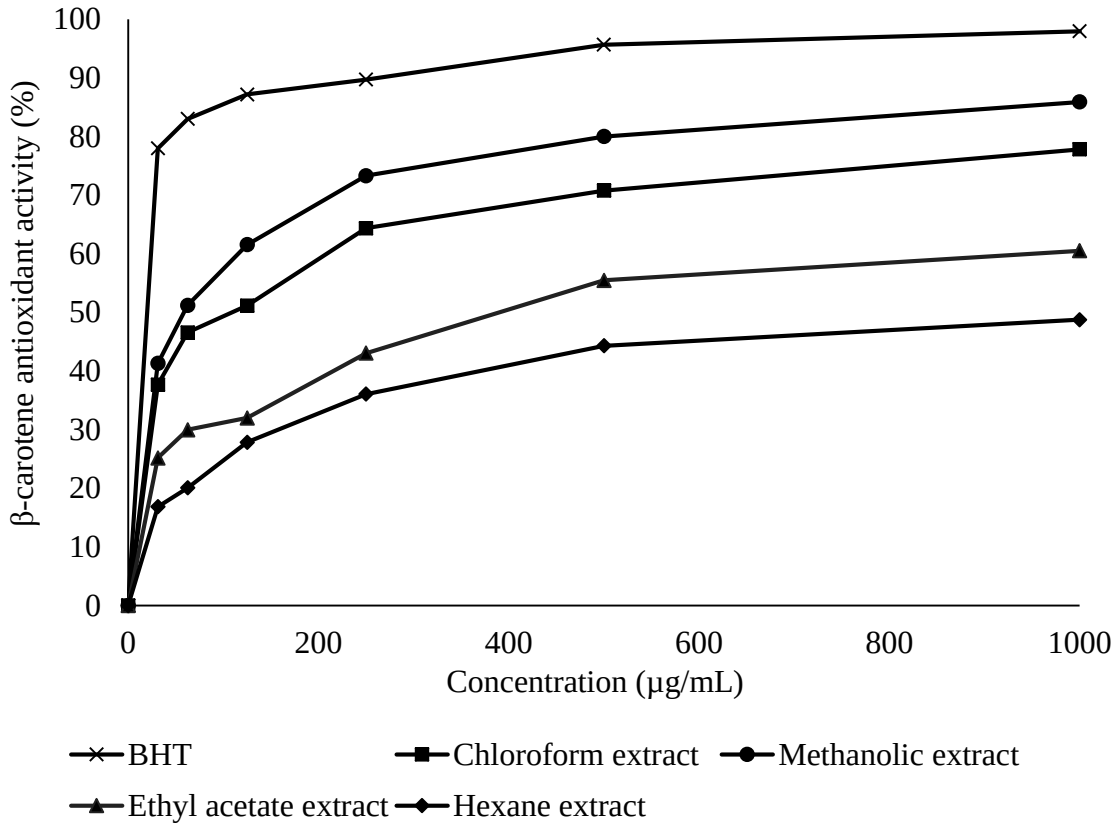


Figure 4.7 β-carotene bleaching inhibition of *R. tomentosa*

Table 4.3 shows antioxidant activity with EC_{50} value of different extracts measured by different antioxidant assays. The lower the EC_{50}, the better the antioxidant activity. Overall, methanolic extract demonstrated the strongest antioxidant properties for all the tested methods. The EC_{50} values obtained for methanolic extract were excellent, particularly for reducing power and β-carotene bleaching assays which were less than 60 µg/mL. The hexane extract revealed very poor antioxidant properties with EC_{50} more than 200 µg/mL for DPPH assay and reducing power assay. The EC_{50} values obtained for chloroform and ethyl acetate extracts were revealed to possess moderate antioxidant properties for all antioxidant assays. The obtained results are similar to the phenol and flavonoid contents determined for each sample shown in Table 4.2.

Table 4.3 Antioxidant activities (DPPH, CUPRAC and β-carotene) of *R. tomentosa*

Sample	IC_{50} µg/mL		
	DPPH assay	**CUPRAC assay**	**β-carotene assay**
MeOH extract	110 ± 0.005	53.84 ± 0.004	58.62 ± 0.001
$CHCl_3$ extract	240 ± 0.001	90.32 ± 0.001	108.93 ± 0.001
EtOAc extract	650 ± 0.005	112.24 ± 0.005	389.49 ± 0.001
Hx extract	920 ± 0.005	247.99 ± 0.001	-
*Ascorbic acid	81.20 ± 0.001	26.25 ± 0.004	-
*BHT	-	-	20.04 ± 0.001

*Standard control

'-' : Not detected

Results are means ± standard deviation of duplicate analysis of three replications

The antioxidant activities achieved can also be correlated with profiling result of *R. tomentosa* through UPLC-QToF/MS. The phenolic and flavonoid compounds present from the methanol extract of *R. tomentosa* were proved to be responsible for its bioactivities. This was proven on the high antioxidant activity is correlated with its high TPC and TFC. Based on the confirmed component plotted by UPLC-QToF/MS, compounds that can be found in MeOH extract (Appendix C) were kaempferol 3-*O*-β-D-glucuronopyranoside, quercetin-3-*O*-(6"-*O*-acetyl)-β-D-glucopyranoside and catechin 7-*O*-β-D-glucopyranoside. The presence of all three phenolic compounds influenced the TPC and TFC values hence strongly influencing the antioxidant activities. Cathechin has also been reported to possess antioxidant activity (Said, Abuotabl, Raoof, Huefner, & Nada, 2015; Sang, Tian, Wang, Stark, Rosen et al., 2003; Sano, Yoshida, Degawa, Miyase, & Yoshino, 2003; Santos-Buelga & Scalbert, 2000).

4.5.2 Cytotoxic activity

The cytotoxicity effect of *R. tomentosa* on cancer cell lines were evaluated by MTT assay. Treatment of cell lines with *R. tomentosa* extracts at various concentrations inhibited cell viability in a dose dependent manner. The extracts concentration (1.5 – 100 µg/mL) was based on suggestions by Houghton, Fang, Techatanawat, Steventon, Hylands et al. (2007) and previous cytotoxicity studies of medicinal plants on MTT assay by Charoensin (2014); Dahham, Al-Rawi, Ibrahim, Abdul Majid, and Abdul Majid (2016) and Wang, Liu, Zou, Lu, Chen et al. (2011) with some minor modifications.

The preliminary process of screening showed that *R. tomentosa* extracts possessed anticancer promoting activity against HepG2, MCF-7 and HT 29 cancer cell lines. Maximum HepG2 cell line inhibition was observed in *R. tomentosa* ethyl acetate extract with values of 81.43 % (Appendix D1). HepG2 cell line treated with tamoxifen (positive control) at the same concentration showed 75.28 % inhibition. In terms of cytotoxicity of MCF-7 cell line, *R. tomentosa* methanolic extract showed maximum inhibition of 82.36 % with tamoxifen at 90.86 % inhibition (Appendix D2). HT 29 cell line maximum inhibition was observed in *R. tomentosa* methanolic extract with values of 83.64 %. HT 29 cell line treated with tamoxifen showed maximum inhibition at 90.05 % (Appendix D3). Meanwhile, with the increase of extracts concentration, cell viability of cell lines decreased in all extracts. *R. tomentosa* ethyl acetate extract is the most active in inhibiting HepG2, MCF 7 and HT 29 cell lines with 54.46 %, 68.58 % and 39.39 % inhibition rate (Appendix D1-3), respectively at a concentration of 12.5 µg/ mL. This suggests that high anticancer activity in *R. tomentosa* ethyl acetate extract may be attributed to its high concentration of anticancer component such as quercetin, kaempferol and lupeol as stated on profiling result through UPLC-QToF/MS (Appendix C3). Methanolic extract from *R. tomentosa* is the next in line after ethyl acetate extract to be active in inhibiting HepG2 (14.85 %), MCF-7 (52.50 %) and HT 29 (37.30 %) cell lines at the same concentration of 12.5 µg/ mL. The anticancer effect of methanolic extract can also be correlated with the profiling of *R. tomentosa* compounds, possessing catechin, quercetin and kaempferol the profiling results of *R. tomentosa* methanolic extract (Appendix A5 - 6).

The results of the effect of *R. tomentosa* extracts on HepG2, MCF-7 and HT 29 cell lines at different concentrations show significant ($p < 0.05$) decrease in the IC_{50} values in a concentration-dependent manner after a 24, 48 and 72 h incubation period (Table 4.4). According to the results of IC_{50} in Table 4.4, ethyl acetate extract is the most active in inhibiting HepG2, MCF-7and HT 29 cell lines. In a study done by Dachriyanus, S. et al. (2002), the ethyl acetate extract of the leaves of *R. tomentosa* yield a compound called rhodomyrtone. This compound was found to have significant antibacterial activity, especially against *Escherichia coli* and *Staphylococcus aureus*. The presence of rhodomyrtone in ethyl acetate extract may contribute to a synergetic anticancer activity. Furthermore, strong cytotoxic effect obtained from methanol extract against MCF-7 and HT 29 cells may be attributed to high abundance of phenolics and flavonoids compounds. Chloroform extract

exhibited moderate anticancer activities (IC_{50} > 50µg/mL) while hexane extract exhibited weak anticancer activities. This could be due to the nature of the extracted bioactive compounds. Generally, non-polar crude extracts may contain carbohydrates and lipids that dilutes the concentration of phenolics and other bioactive compounds. Although non-polar compounds contain lipophilicity properties, the anticancer activities are weak because of other factors, such as reduction potential and structural characteristics, length of the alkyl chain and degree of hydroxyl group (Fiuza, Gomes, Teixeira, Girão da Cruz, Cordeiro et al., 2004).

Table 4.4 IC_{50} µg/mL values after 24, 48 and 72 hours of incubation for extracts of *R. tomentosa* against HepG2, MCF-7 and HT 29 cancer cell lines

Extract	**HepG2 (IC_{50} µg/mL)**			**MCF-7 (IC_{50} µg/mL)**			**HT 29 (IC_{50} µg/mL)**		
	24	48	72	24	48	72	24	48	72
MeOH extract	10.38 ± 0.005	39.27 ± 0.015	33.30 ± 0.020	25.95 ± 0.179	23.07 ± 0.206	10.48 ± 0.230	54.27 ± 0.825	31.52 ± 0.611	18.61 ± 0.743
$CHCl_3$ extract	-	-	99.98 ± 0.015	29.08 ± 0.650	46.05 ± 0.635	33.63 ± 0.340	71.80 ± 0.599	50.14 ± 0.864	35.54 ± 0.573
EtOAc extract	4.84 ± 0.010	16.01 ± 0.029	11.47 ± 0.280	57.84 ± 0.040	41.25 ± 0.021	2.68 ± 0.529	49.15 ± 0.885	27.41 ± 0.800	16.18 ± 0.538
Hx extract	8.55 ± 0.026	41.26 ± 0.012	-	45.35 ± 0.031	88.58 ± 0.667	-	-	95.23 ± 0.112	73.18 ± 0.089
Tamoxifen (positive control)	2.97 ± 0.02	4.58 ± 0.01	3.55 ± 0.03	42.16 ± 0.02	17.86 ± 0.01	10.67 ± 0.02	10.16 ± 0.02	7.89 ± 0.02	6.02 ± 0.01

'-': Not detected

Results are means ± standard deviation of duplicate analysis of three replications

The criterion of cytotoxicity for the crude extract, established by U.S National Cancer Institute, is IC_{50} < 30 µg/mL in the preliminary screening assay Roslen, Alewi, Ahamada, and Rasad (2014) while Houghton et al. (2007) suggested IC_{50} < 40 µg/mL as a suitable concentration for anticancer effect of either extract or compound. The comparisons of IC_{50} values after incubation at 72 h is summarized in Figure 4.8. Tamoxifen was used as positive control which was able to inhibit cancer cell proliferation. It had an IC_{50} of 3.55 ± 0.03, 10.67 ± 0.02 and 6.02 ± 0.01 µg/mL at 72 h for HepG2, MCF-7 and HT 29, respectively. The results exhibit significant activity against cell lines under 72 h incubation with IC_{50} value < 30 µg/mL are ethyl acetate extract and methanolic extract on both MCF-7 (2.68 ± 0.529 µg/mL, 10.48 ± 0.230 µg/mL) and HT 29 (16.18 ± 0.538 µg/mL, 18.61 ± 0.743 µg/mL). Ethyl acetate extract is the only extract that shows significant anticancer activity against HepG2 cell line with IC_{50} 11.47 ± 0.280 µg/mL.

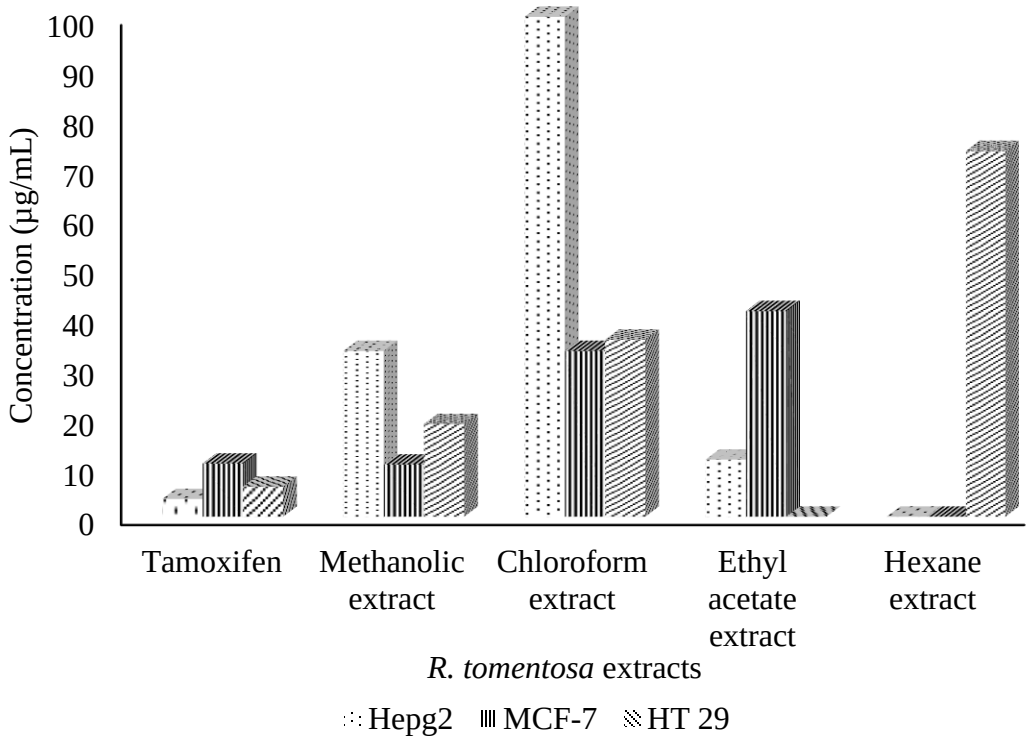


Figure 4.8 IC_{50} (µg/mL) values of *R. tomentosa* extracts on cancer cell lines under 72 hours incubation as determined by the MTT assay

The anticancer activity of the compounds is dependent on their structure which is related to the balance between its lipophilicity and hydrophilicity solubility (Moosavi-Movahedi, Hakimelahi, Chamani, Khodarahmi, Hassanzadeh et al., 2003). Several studies on different potential antitumor agents have shown that a maximum cytotoxic activity is often achieved for intermediate lipophilicity and water solubility values (Fiuza et al., 2004). The synergistic effect of ethyl acetate extract and methanolic extract can be correlated to lipophilicity of compound and their ability to penetrate the cell membrane.

The anticancer effect can also be correlated with the profiling result of *R. tomentosa* compounds through UPLC-QToF/MS. Both methanolic extract and ethyl acetate extract possess quercetin in their profiling result. Dajas (2012) concluded that pharmacological activities of quercetin that modulate antioxidation/oxidation/kinase-signaling pathways ultimately promoting cell survival or death, exhibit potential as therapeutic modalities in neuropathology and in cancer. The phloroglucinol compound present from ethyl acetate extract of *R. tomentosa* might be responsible for its high anticancer activity. Naturally occurring phloroglucinol compound have shown anticancer and antitumor activities (Ghisalberti, 1996; Nagpal, Shah, Arora N, Shri, & Arya, 2010; Sidana, Rohilla, Roy, Barrow, Foley et al., 2010; Singh & Etoh, 1997; Soliman, Fathy, Salama, Al-Abd, Saber et al., 2014). Kim, Uddin, Hyun, Kim, Suh et al. (2015) implicated phloroglucinol compound as a good candidate in targeting breast cancer stem-like cells and to prevent the disease relapse.

Previous studies done by Chakravarti, Maurya, Siddiqui, Bid, Rajendran et al. (2012) on ethanolic extract of *Wrightia tomentosa* suggested that the plant has significant anticancer signs against breast cancer cells due to induction of apoptosis pathway with IC_{50} of 50 µg/mL (MCF-7) and 30 µg/mL (MDA-MB-231). Crude extracts of *Petunia punctata*, *Alternanthera sessilis*, and *Amoora chittagonga* show cytotoxicity in three cancer cell lines (Panc-1, Mia-Paca2 and Capan-1) with IC_{50} values ranging between 20.3 - 31.4 µg/mL, 13.08 - 34.9 µg/mL, and 42.8 - 49.8 µg/mL, respectively (George, Bhalerao, Lidstone, Ahmad, Abbasi et al., 2010). The difference between the study conducted by Chakravarti et al. (2012) on *Wrightia tomentosa* and recent research indicates that *R. tomentosa* has low cytotoxicity values. The

present study also contributes to the evidence that the roots extracts of *R. tomentosa* does indeed possess anticancer importance.

Plant-derived extracts containing antioxidant principle shows cytotoxicity toward tumor cells (Gupta, Mazumder, Kumar, Sivakumar, & Vamsi, 2004). Cells are equipped with enzymatic and non-enzymatic antioxidant mechanisms play an important role in the elimination of free radicals. In the defense against oxidative stress, the antioxidant enzyme system of cells plays an important role. The primary antioxidative enzyme, superoxide dismutase (SOD) and reduced glutathione (GSH), a powerful antioxidant, are among the most important antioxidative system parameters of the organism.

4.6 Compound Isolation

Fractionation and chromatography of ethyl acetate and chloroform extracts from *R. tomentosa* resulted in the isolation of lupeol and β-sitosterol. The isolated compounds were characterized by spectroscopy techniques.

4.6.1 Lupeol (27)

Compound **(27)** (10.45 mg) was obtained as white needles from the purification of ethyl acetate extract of *R. tomentosa*. λmax in $CHCl_3$: 230 nm (Appendix E1) suggesting the presence of a highly conjugated aromatic system. IR absorption bands (Appendix E2) appeared at 3226 cm^{-1} (OH), 2927 cm^{-1} (sp^3) and 1078 cm^{-1} (C-O).

The ^1H NMR spectrum (Appendix E3) displayed the presence of seven tertiary methyl protons at δ 0.76, 0.79, 0.83, 0.94, 0.97, 1.03, and 1.66 integrated to three protons each. A pair of singlet at δ 4.69 and 4.57 (1H each) was indicatives of olefinic protons at C-29. This indicated that compound **(27)** belong to lupine class of triterpenoids. The H-3 proton showed a doublet of doublet at δ 3.20 due to the presence of oxymethine proton at C-3. A sextet at δ 2.37 was assigned to 19β-H as characteristic of lupeol. The structural assignment of compound **(27)** was further substantiated by the ^{13}C NMR (Appendix E4) spectrum which revealed the presence of thirty carbons. This included seven methyls, eleven methylenes, six quaternary carbons and six methine carbons. Among these, exomethylene group appeared at δ 109.35 (C-29) and 151.03 (C-20) were assigned with the aid of DEPTQ experiment

(Appendix E5). The signal at δ 79.03 was due to the hydromethine proton attached to C-3. The complete structural assignment of ^{1}H, ^{13}C, DEPTQ, COSY (Appendix E6), HSQC (Appendix E7), and HMBC (Appendix E8) NMR is summarized in Table 4.5. This data was further supported by mass spectrum (Appendix E9), showing a molecular ion peak at *m/z* 426 corresponding to the molecular formula $C_{30}H_{50}O$. Based on spectral data and in comparison of its physical and spectral data reported (Jain & Bari, 2010; Sánchez-Burgos, Ramírez-Mares, Gallegos-Infante, González-Laredo, Moreno-Jiménez et al., 2015), led to propose the structure of compound **(27)** as lupeol (Figure 4.9).

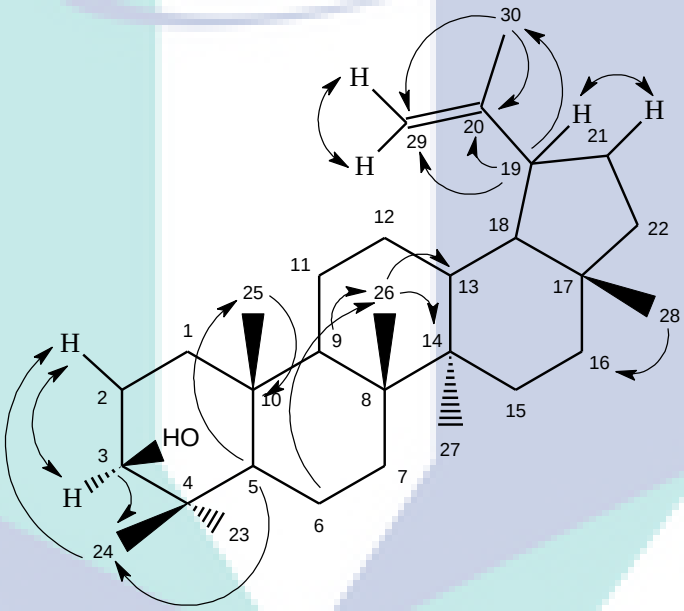


Figure 4.9 Lupeol **(27)**

Table 4.5 ^{1}H (500 MHz, $CDCl_3$), DEPTQ, COSY, HMQC and HMBC of Lupeol **(27)**

Carbon number	Lupeol isolated compound				Literature	
	δ_C (ppm)	δ_H (ppm) Int. Mult. J	COSY	HMBC	δ_C (ppm) (1a)	δ_H (ppm) (1b)
1	38.70				38.76	
2	29.75	1.58 (2H, m)	H3		27.42	
3	79.03	3.2 (1H, t, 7.9)	H2		78.98	3.21 (1H, dd, 4.7, 11.0)
4	38.87				38.84	
5	55.27				55.26	
6	18.33	1.39 (1H, m)		C26	18.30	1.39 (1H, m)
7	34.31				34.25	
8	40.84				40.80	
9	50.42	1.25 (1H, overlapped)			50.40	1.28 (1H, s)
10	37.19				37.14	
11	20.93				20.90	
12	25.12				25.10	
13	38.03				38.02	
14	42.81				42.81	
15	27.43				27.39	
16	35.60				35.56	
17	43.03				42.98	
18	48.30				48.27	
19	48.01	2.37 (1H, m)	H21	C20, C29, C30	47.96	2.37 (1H, m)
20	151.03				150.96	
21	29.87	1.92 (1H, m)	H19	C30	29.82	1.91 (1H, m)
22	40.01				39.98	
23	28.02	0.97 (3H,s)			28.08	0.98 (3H, s)
24	15.41	0.76 (3H, s)		C2, C3, C5	15.36	
25	16.12	0.83 (3H, s)		C5, C10	16.11	0.84 (3H, s)
26	15.93	1.03 (3H, s)		C9, C13, C14	15.95	
27	14.58	0.94 (3H, s)			14.53	0.97 (3H, s)
28	18.01	0.79 (3H, s)		C16	17.99	0.79 (3H, s)
29	109.35	4.69 (1H, s) 4.57 (1H,s)	H29	C19, C30 C19, C30	109.32	4.71 (1H, s)
30	19.31	1.68 (3H, s)		C19, C20, C29	19.28	1.69 (3H, s)

1a: Sánchez-Burgos et al. (2015)

1b: Jain and Bari (2010)

4.6.2 β-Sitosterol (24)

Compound **(24)** (8.04 mg) was yielded from the purification of chloroform extract as white crystalline needles. λmax in $CHCl_3$: 230 nm (Appendix F1) suggesting the presence of a highly conjugated aromatic system. IR absorption bands (Appendix F2) appeared at 3420 cm^{-1} (OH), 2966 cm^{-1} (sp^3) and 1052 cm^{-1} (C-O).

The ^1H NMR spectrum (Appendix F3) displayed singlet at δ 0.68 (H-21), 1.01 (H-26) and 1.25 (H-18, H-19) attributed to four methyl groups. The structural assignment of compound **(24)** was further substantiated by the ^{13}C NMR spectrum (Appendix F4) which revealed the presence of twenty-nine carbons. This included six methyl, eleven methylene and three quaternary carbons with a hydroxyl group. The carbons of alkenes conjugated at δ 140.80 ppm (C-5) and 121.73 ppm (C-6). The complete structural assignment of ^1H, ^{13}C and DEPTQ (Appendix F5) NMR is summarized in Table 4.6. This data was further supported by mass spectrum (Appendix F6), showing a molecular ion peak at *m/z* 414 corresponding to the molecular formula $C_{29}H_{50}O$. Based on spectral received and previous reported (Patra, Jha, Murthy, & Sharone, 2010), led to proposing of structural compound **(24)** as β-sitosterol (Figure 4.10).

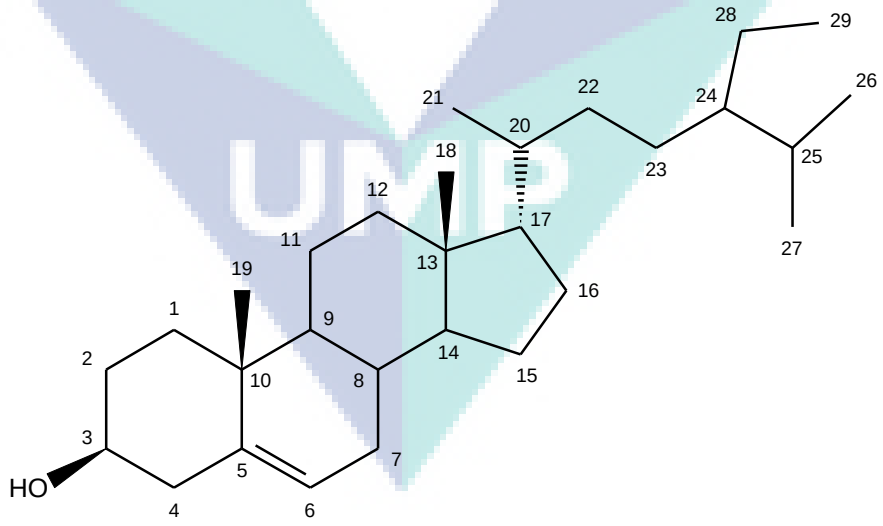


Figure 4.10 β-sitosterol **(24)**

Table 4.6 ^{1}H (500 MHz, $CDCl_3$), and DEPTQ of β-sitosterol **(24)**

Carbon number	β-sitosterol isolated compound		Literature (2a)	
	δ_C (ppm)	δ_H (ppm) Int. Mult. J	δ_C (ppm)	δ_H (ppm)
1	37.26		37.28	
2	31.69		31.69	
3	71.79	3.53 (1H, m)	71.82	3.52 (1H, m)
4	42.33	2.27 (2H, m)	42.33	2.28 (2H, m)
5	140.80		140.70	
6	121.73	5.35 (1H, m)	121.72	5.36 (1H, m)
7	29.74		31.69	
8	31.93		31.93	
9	50.12		50.17	
10	36.53		36.52	
11	21.12		21.10	
12	39.80		39.80	
13	42.31		42.33	
14	56.76		56.79	
15	24.32		24.37	
16	28.27		28.25	
17	56.12		56.09	
18	11.88	1.25 (6H, s)	11.86	0.68 (3H, s)
19	19.42	1.25 (6H, s)	19.40	1.02 (3H, s)
20	36.17		36.52	
21	18.80	0.68 (3H, d, 7 Hz)	18.79	0.94 (3H, d, 7 Hz)
22	33.97		33.98	
23	26.07		26.14	
24	45.83		45.88	
25	29.16		28.91	
26	19.84	1.01 (3H, d, 7 Hz)	19.80	0.83 (3H, d, 7 Hz)
27	19.05	0.81 (3H, d, 7 Hz)	18.79	0.85 (3H, d, 7 Hz)
28	23.09		23.10	
29	12.60	0.83 (3H, t, 7 Hz)	11.99	0.88 (3H, t, 7Hz)

2a: Patra et al. (2010)

4.7 Bioactivities of Isolated Compound Lupeol

Two types of bioactivity studies which are antioxidant activities and cytotoxic activity has been carried out on isolated compound from *R. tomentosa*. The antioxidant properties of isolated compound were obtained through radical scavenging effects (DPPH), reducing power (CUPRAC) and β-carotene bleaching assays. The cytotoxicity activity of isolated compound on three cancer cell lines (HepG2, MCF-7 and HT 29) were evaluated via MTT assay. The bioactivities assay of isolated compound were only focusing on lupeol **(27)**. This is due to isolated compound lupeol from *R. tomentosa* root extract has never been tested with antioxidant and cytotoxicity assays, to the best of our knowledge. Isolated compound lupeol can be detected by UPLC-QToF/MS with low intensity in $CHCl_3$ (Appendix B3) and EtOAc (Appendix C3) extracts. β-sitosterol was omitted in this bioactivities study due to an absent present of β-sitosterol detected via UPLC-QToF/MS. This occurrence may be due to β-sitosterol having lower intensity compared with other isolated compound, lupeol.

4.7.1 Antioxidant Activities

The antioxidant property of the lupeol as an isolated compound was obtained through three antioxidant assays including radical scavenging effect (DPPH), reducing power (CUPRAC) and β-Carotene bleaching assays. Table 4.7 shows percentage of antioxidant activities of radical scavenging effect (DPPH), reducing power (CUPRAC) and β-Carotene bleaching assays. Radical scavenging activities of lupeol increased with increasing concentration. It depicts the ability of lupeol scavenge DPPH radicals compared with the ability of ascorbic at different concentrations ranging from 31.25 to 1000 µg/mL. Different concentration of lupeol present affect the ability of donating hydrogen atom and reaction with free radicals. The reducing power increases with concentration, and the absorbance values obtained for lupeol increased. The antioxidant activity of lupeol might be due to its redox properties, which allows them to act as reductant, hydrogen donors and singlet oxygen quenchers. Bleaching inhibition of lupeol increased as the concentration was increased, compared with BHT, using the β-carotene. This phenomenon is probably due antioxidative component of lupeol by preventing the bleaching of β-carotene by neutralizing the linoleate-free radical and other free radicals formed in the system. The results (Table 4.7) exhibit

significant antioxidant activity of lupeol for all three assays. Hence, this indicates that lupeol possess high antioxidant property

Table 4.8 shows antioxidant activity with EC_{50} value of lupeol purified from *R. tomentosa* measured by different antioxidant assays. The lower the EC_{50}, the better the antioxidant activity. Overall, lupeol demonstrated to possess antioxidant properties for all the tested methods. The EC_{50} values obtained from lupeol were excellent for all three antioxidant assays which were less than standard controls of antioxidant activities. Previous study by Li, Ruan, Huang, Chen, and Chen (2012) validate the IC_{50} value of DPPH for lupeol from *Leucaena leucocephala* was 102.3 ± 4.1 μg/mL. Previous study indicated that oral administration of lupeol can change the tissue redox system scavenging the free radicals and by improving the antioxidant status of the rat liver (Sunitha, Nagaraj, & Varalakshmi, 2001).

Table 4.7 Percentage of radical scavenging effect (DPPH), reducing power (CUPRAC) and β-Carotene bleaching assays

Concentration (µg/mL)	**Radical scavenging activity (%)**		**Reducing antioxidant capacity (%)**		**β-carotene bleaching inhibition (%)**	
	Ascorbic acid	**Lupeol**	**Ascorbic acid**	**Lupeol**	**BHT**	**Lupeol**
31.25	80.15 ±.0.004	83.57 ± 0.001	54.47 ± 0.008	57.02 ± 0.001	77.99 ± 0.001	78.59 ± 0.001
62.50	80.99 ± 0.002	82.54 ± 0.001	82.50 ± 0.002	84.59 ± 0.002	83.02 ± 0.002	82.27 ± 0.002
125.00	81.40 ± 0.001	82.35 ± 0.001	89.86 ± 0.001	90.64 ± 0.008	87.19 ± 0.001	86.44 ± 0.001
250.00	81.75 ± 0.001	82.18± 0.001	90.96 ± 0.002	91.55 ± 0.002	89.73 ± 0.001	91.02 ± 0.001
500.00	82.14 ± 0.001	81.57 ± 0.001	94.86 ± 0.006	95.06 ± 0.006	95.67 ± 0.002	96.81 ± 0.002
1000.00	82.16 ± 0.001	81.19 ± 0.001	95.54 ± 0.004	95.69 ± 0.004	97.96 ± 0.002	98.69 ± 0.002

Results were expressed as mean ± SD

Table 4.8 Antioxidant activities value, EC_{50} (DPPH, CUPRAC and β-carotene) of lupeol from *R. tomentosa*

Sample	**EC_{50} µg/mL**		
	DPPH assay	**CUPRAC assay**	**β-carotene assay**
Lupeol	76.05 ± 0.001	27.40 ± 0.002	19.88 ± 0.001
*Ascorbic acid	76.78 ± 0.002	28.68 ± 0.004	-
*BHT	-	-	20.04 ± 0.001

*Standard control

ND: Not detected

Results are means ± standard deviation of duplicate analysis of three replications

4.7.2 Anticancer Activities

An interesting observation on the potential cytotoxicity effect on ethyl acetate extract from *R. tomentosa* on all cell lines were observed in this research. The compounds responsible for the properties observed in the ethyl acetate extract from *R. tomentosa* was fractionated and isolated resulted in isolated compound, lupeol **(27)**.

The cytotoxicity of isolated compound, lupeol from *R. tomentosa* was evaluated by MTT assay on three cancer cell lines: HepG2, MCF-7 and HT 29. A decrease in the cell count was observed with increase in lupeol concentration (Appendix G1 - 3). Anticancer activities of lupeol from *R. tomentosa* illustrated an inhibition effect on the cell proliferation in a dose dependent manner. These results were compared with tamoxifen treated and untreated cells. As shown in Appendix G1 - 3, proliferation of lupeol treated HepG2, MCF-7 and HT 29 cells was significantly suppressed compared to untreated cells. Maximum HepG2, MCF-7 and HT 29 cell line inhibition was observed in lupeol with values of 71.38 %, 75.28 % and 81.92 %, respectively. Maximum HepG2, MCF-7 and HT 29 cell line inhibition observed in tamoxifen (positive control) was 75.28 %, 90.86 % and 90.05 %, respectively. The cell viability at concentration of 12.5 µg/ mL of lupeol was measured in HepG2, MCF-7 and HT 29 cells to be 51.79 %, 39.39 % and 52.13 %, respectively.

According to the results of IC_{50} in Table 4.9, lupeol showed dose-dependent inhibition of cell proliferation in cancer cell lines HepG2, MCF-7 and HT 29 after 24, 48 and 72 hours of incubation. The highest toxicity (IC_{50}) was observed for both HepG2 (24h: 4.17 ± 0.02 µg/mL, 48h: 7.29 ± 0.01 µg/mL, 72h: 6.85 ± 0.09 µg/mL) and HT 29 (24h: 8.5 ± 0.18 µg/mL, 48h: 6.21 ± 0.19 µg/mL, 72h: 5.95 ± 0.92 µg/mL) cell lines indicating the synergistic behaviour of compound lupeol in inhibition of cell proliferation. Tamoxifen was used as positive control which was able to inhibit cancer cell proliferation. The toxicity of tamoxifen (IC_{50}) observed for HepG2 (24h: 2.97 ± 0.02 µg/mL, 48h: 4.58 ± 0.01 µg/mL, 72h: 3.55 ± 0.03 µg/mL), MCF-7 (24h: 42.16 ± 0.02µg/mL, 48h: 17.86 ± 0.01µg/mL, 72h: 10.67 ± 0.02 µg/mL) and HT 29 (24h: 10.16 ± 0.02µg/mL, 48h: 7.89 ± 0.02µg/mL, 72h: 6.02 ± 0.01 µg/mL).

Suitable concentration for anticancer effect for compounds suggested by Houghton et al. (2007) is 40 µg/ml. Hence, inhibition of MCF-7 (24h: 54.03 ± 0.05 µg/mL, 48h: 28.21 ± 0.41 µg/mL, 72h: 24.71 ± 0.09 µg/mL) by lupeol can also be accepted due to its inhibition for 48 and 72 hours of MCF-7 is below than 40 µg/mL.

Table 4.9 IC_{50} µg/mL values after 24, 48 and 72 hours of incubation of lupeol from *R. tomentosa* against HepG2, MCF-7 and HT 29 cancer cell lines

Sample	**HepG2 (IC_{50} µg/mL)**			**MCF-7 (IC_{50} µg/mL)**			**HT 29 (IC_{50} µg/mL)**		
	24	48	72	24	48	72	24	48	72
Lupeol	4.17 ± 0.02	7.29 ± 0.01	6.85 ± 0.09	54.03 ± 0.05	28.21 ± 0.41	24.71 ± 0.09	8.5 ± 0.18	6.21 ± 0.19	5.95 ± 0.92
Tamoxifen	2.97 ± 0.02	4.58 ± 0.01	3.55 ± 0.03	42.16 ± 0.02	17.86 ± 0.01	10.67 ± 0.02	10.16 ± 0.02	7.89 ± 0.02	6.02 ± 0.01

Results are means ± standard deviation of duplicate analysis of three replications

Lupeol is a compound that has an ability in exerting anticancer and antitumor activity by cell cycle regulation and inducing apoptosis (Chaudhary, Chandrashekar, Pai, Setty, Devkar et al., 2015). Previous studies regarding the relationship between lupeol and anticancer has been reported in a dose and time-dependent manner (Lambertini, Piva, Khan, Lampronti, Bianchi et al., 2004; Nigam, Prasad, & Shukla, 2007; Prasad, Nigam, Kalra, & Shukla, 2008; Saleem, 2009). Studies done by Pitchai, Roy, and Ignatius (2014) on lupeol isolated from *Elephantopus scaber* displayed anticancer activity against MCF-7 cancer cell line. Prakash, Schilling, Miller, Andriantsiferana, Rasamison et al. (2003) investigated that lupeol isolated from *Vepris p unctate* screening exhibit IC_{50} 26.4 µg/mL on human ovarian cancer cell line A2780.

The comparisons of lupeol against three cell lines of the IC_{50} values after incubation at 72 h is summarized in Figure 4.11 with tamoxifen act as positive control. The results exhibit significant activity of lupeol against all three cancer cell lines under 72 h incubation with IC_{50} value < 30 µg/mL on all cancer cell lines HepG2 (6.85 ± 0.09 µg/mL), MCF-7 (24.71 ± 0.09 µg/mL) and HT 29 (5.95 ± 0.92 µg/mL). From the results, lupeol isolated from *R. tomentosa* shows significant anticancer activity against all three cancer cell lines.

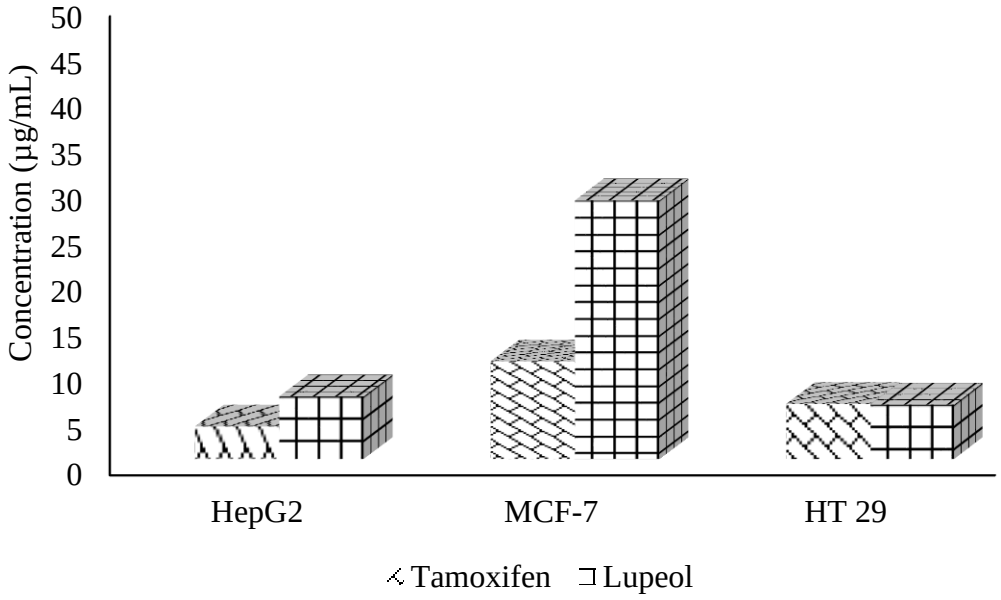


Figure 4.11 IC_{50} (mg/mL) values of lupeol from *R. tomentosa* on cancer cell lines under 72 hours incubation as determined by the MTT assay

Lupeol **(27)** is classified under pentacyclic triterpenes group and is commonly found in fruits, vegetables and other parts of several medicinal plants. Lupeol can function by modulating processes associated with xenobiotic biotransformation, with the protection of cellular elements from oxidative damage, or with the promotion of a more differentiated phenotype in target cells (Chaturvedi, Bhui, & Shukla, 2008). Furthermore, lupeol has been shown to stimulate apoptosis by intervening in several signalling pathways or signalling cascades (Chaturvedi et al., 2008). Lupeol's ability as an anticancer was also attributed to its ability to inhibit topoisomerase II (topo II), an essential enzyme in eukaryotic cells. Lupeol is also able to inhibit lyase activity of DNA polymerase β. Both of these ability by lupeol is expected to sensitize cancer cells to DNA-damaging agent hence potentiating their cytotoxicity, thus can be regarded as a promising target for the development of novel chemotherapeutic agent for the prevention and/or treatment of cancer (Chaturvedula, Schilling, Miller, Andriantsiferana, Rasamison et al., 2004; Moriarity, Huang, Yancey, Zhang, Setzer et al., 1998; Sobol, Prasad, Evenski, Baker, Yang et al., 2000).

CHAPTER 5

CONCLUSIONS

5.1 Conclusion

The relationship between the extracts composition and its biological activities was determined by UPLC-QToF/MS. Major compounds classified as flavonoid that can be found existed in *R. tomentosa* were kaempferol 3-*O*-β-D-glucuronopyranoside, catechin 7-*O*-β-D-glucopyranoside, (2S)-3',4'-methylenedioxy-5,7-dimethoxyflavane, 4′,7-dimethyltectorigenin, quercetin 3-*O*-α-L-arabinoside, dihydrokaempferol-5-*O*-β-D-glucopyranoside, 3-hydroxynaringenin, and isoxanthohumol. Methylellagic acid, khellol-β-D-glucoside, dichotomitin, rhodomyrtosone C, and rhodomyrtosone D were some of major compounds detected in *R. tomentosa* root extracts. In medicinal plant, using UPLC-QToF/MS is relatively new and is increasingly being used in the field due to its effectiveness in detecting chemical compounds with high sensitivity. The present investigation on *R. tomentosa* also resulted in isolation of lupeol and β-sitosterol. Both lupeol and β-sitosterol belong to the class of terpenoids. From UPLC-QToF/MS result, isolated compound lupeol (27) (10.45 mg) was presence in $CHCl_3$ and EtOAc extracts while β-sitosterol (24) (8.04 mg) was not detected due to its lower intensity than lupeol.

Two types of bioactivity studies which are antoxidant activities and cytotoxic activity has been carried out on *R. tomentosa* extracts and isolated compound lupeol. The antioxidant properties of the extracts and isolated compound lupeol were obtained through different mechanisms of action of radical scavenging effects (DPPH), reducing power (CUPRAC) and β-carotene bleaching assays. All of the extracts from *R. tomentosa* showed antioxidant activity whether highest, moderately or weak. The EC_{50} values obtained from lupeol were

excellent for all three antioxidant assays which were less than standard controls of antioxidant activities. The cytotoxicity activity of *R. tomentosa* on cancer cell lines were evaluated by MTT assay. Treatment of cells with extracts from *R. tomentosa* and lupeol compound at various concentrations inhibited cell viability in dose dependent manner. All of the extracts from *R. tomentosa* shows a significant anticancer activity. From present investigation, ethyl acetate extract from *R. tomentosa* is the most active in inhibiting HepG2, MCF-7and HT 29 cells. The IC_{50} values of isolated compound lupeol shows significant cytotoxicity activity against all three cancer cell lines.

5.2 Recommendation

Plants are an important source for the development of new chemotherapeutic and antioxidant agents. This study indicates both antioxidant and cytotoxicity properties of the traditional plant *R. tomentosa* are important. The anticancer effect of *R. tomentosa* on HepG2, MCF-7 and HT 29 cells suggest that the presence of lupeol and antioxidant potential may be due to the high contents of phenolic and flavonoid compounds in *R. tomentosa* root extracts. The present results help in providing a base for further investigation of *R. tomentosa* and potential identification of novel bioactive compounds with therapeutic, anticancer properties. Current studies of *R. tomentosa* mostly focus on the leaves and fruits and their rich bioactive secondary metabolites, but these studies are still unclear and insufficient. Pharmacological studies using different chemical constituents from the roots, and other parts of *R. tomentosa* is required. It is also necessary to combine studies of the biological activity with research on clinical applications that explore the material basis of their efficacy. Present investigation on *R. tomentosa* provided a new foundation for further research on its mechanism of action and the development of better understanding of *R. tomentosa*. A systematic phytochemical investigation of *R. tomentosa* and its pharmacological properties, to illustrate its ethnomedicinal use, and support further healthcare product development will undoubtedly be the focus of further research.

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APPENDIX A1

DATA OF CONFIRMED COMPOUNDS ON (-)ESI-MS OF R. TOMENTOSA METHANOLIC EXTRACT

No.	Component Name	Neutral mass (Da)	Observed neutral mass (Da)	Observed m/z	Mass error (mDa)	Mass error (ppm)	Obs. RT (min)	Obs. drift (ms)	Obs. CCS (Å²)	Response	Adducts
1	Epigallocatechin-3-O-gallate	458.0849	458.086	457.079	1.2	2.6	0.46	7.34	198.05	13983	$[M - H]^-$
2	1-Galloyl-β-D-glucose	332.0744	332.074	331.067	0.1	0.2	1.7	6.13	171.15	34109	$[M - H]^-$
3	Quercetin-3-O-(6-O-acetyl)β-D-glucopyranoside	506.1060	506.103	505.096	-2.8	-5.6	4.94	7.87	210.32	9772	$[M - H]^-$
4	Khellol-β-D-glucoside	408.1057	408.105	453.104	-0.3	-0.6	5.65	7.15	193.5	63152	$[M + HCOO]^+$
5	Catechin 7-O-β-D-glucopyranoside	452.1319	452.132	511.146	0.2	0.4	7.47	7.73	207.07	26653	$[M + CH_3COO]^+$
6	Catechin 7-O-β-D-glucopyranoside	452.1319	452.132	497.131	0.6	1.1	8	7.93	211.87	96733	$[M + HCOO]^+$
7	Quercetin-3-O-α-D-glucuronide	478.0747	478.075	477.068	0.6	1.2	8.31	7.51	201.9	41133	$[M - H]^-$
8	Kaempferol 3-O-β-D-glucuronopyranoside	462.0798	462.080	461.073	0.2	0.5	10.19	7.36	198.55	143291	$[M - H]^-$
9	Quercetin-3-O-glucuronide 6″-methylester	492.0904	492.091	491.084	0.7	1.3	10.65	7.75	207.64	50814	$[M - H]^-$
10	methylellagic acid	344.0532	344.054	343.046	0.2	0.7	11.34	6.08	169.95	103486	$[M - H]^-$

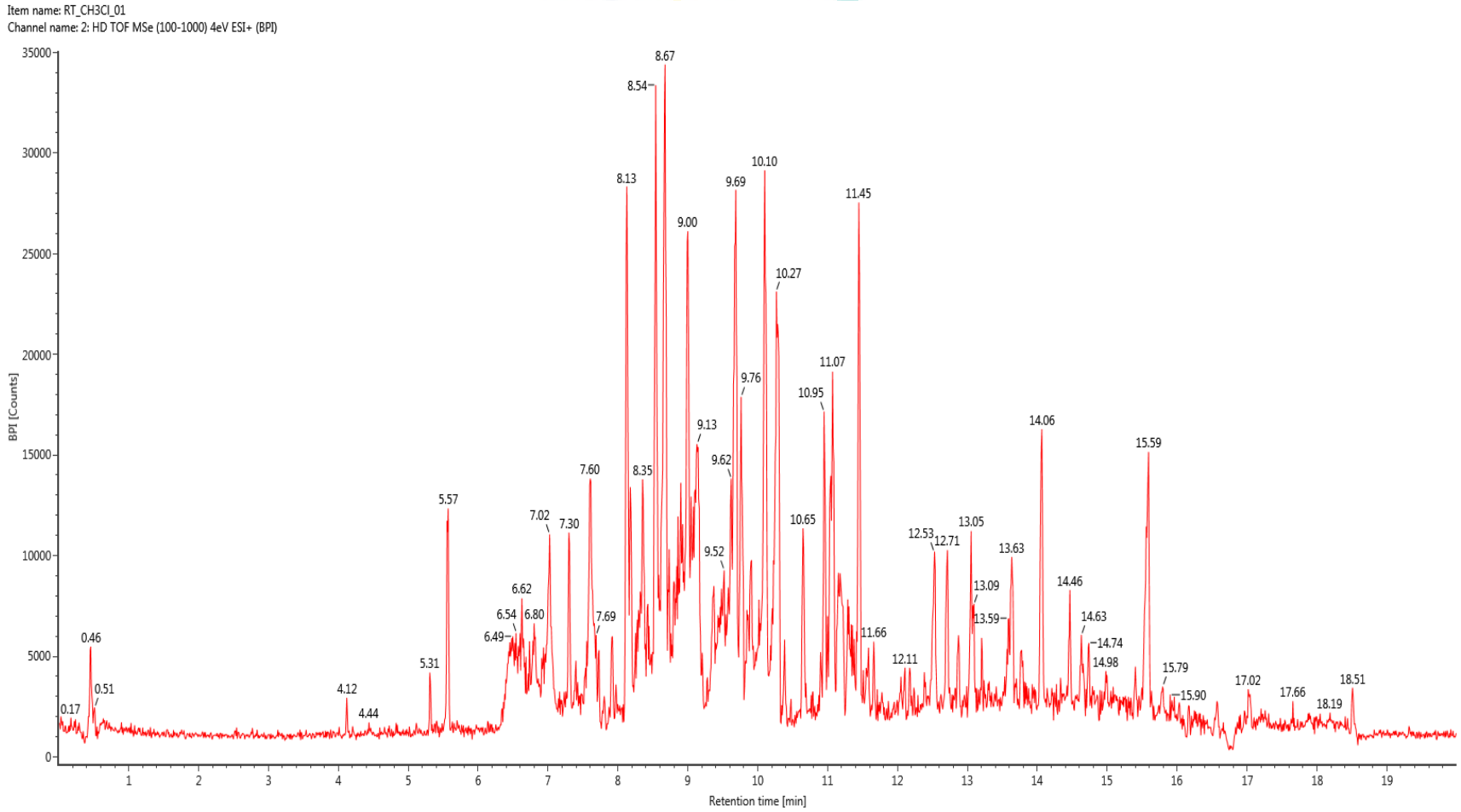
APPENDIX A2

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

No.	Component Name	Neutral mass (Da)	Observed neutral mass (Da)	Observed m/z	Mass error (mDa)	Mass error (ppm)	Obs. RT (min)	Obs. drift (ms)	Obs. CCS (Å²)	Response	Adducts
1	Kuwanon E	424.18859	424.1925	425.1998	4	9.3	5.87	7.3	199	3967	$[M + H]^+$
2	Kaempferide-3-O-α-L-rhamnosyl-7-O-α-L-rhamnoside	592.17921	592.1769	593.1842	-2.3	-3.9	7.72	8.31	222.94	14329	$[M + H]^+$
3	Quercetin-3-O-(6''-O-acetyl)-β-D-glucopyranoside	506.10604	506.1058	529.095	-0.3	-0.5	11.4	8.14	219.01	62748	$[M + Na]^+$
4	Methylellagic acid	344.05322	344.0531	345.0604	-0.1	-0.2	15.87	5.95	167.84	158281	$[M + H]^+$
5	Dichotomitin	358.06887	358.0693	359.0766	0.5	1.3	16.59	6.15	172.32	5947	$[M + H]^+$

APPENDIX B1

UPLC-QToF/MS POSITIVE BPI CHROMATOGRAM OF *R. TOMENTOSA* CHLOROFORM EXTRACT



APPENDIX B2

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

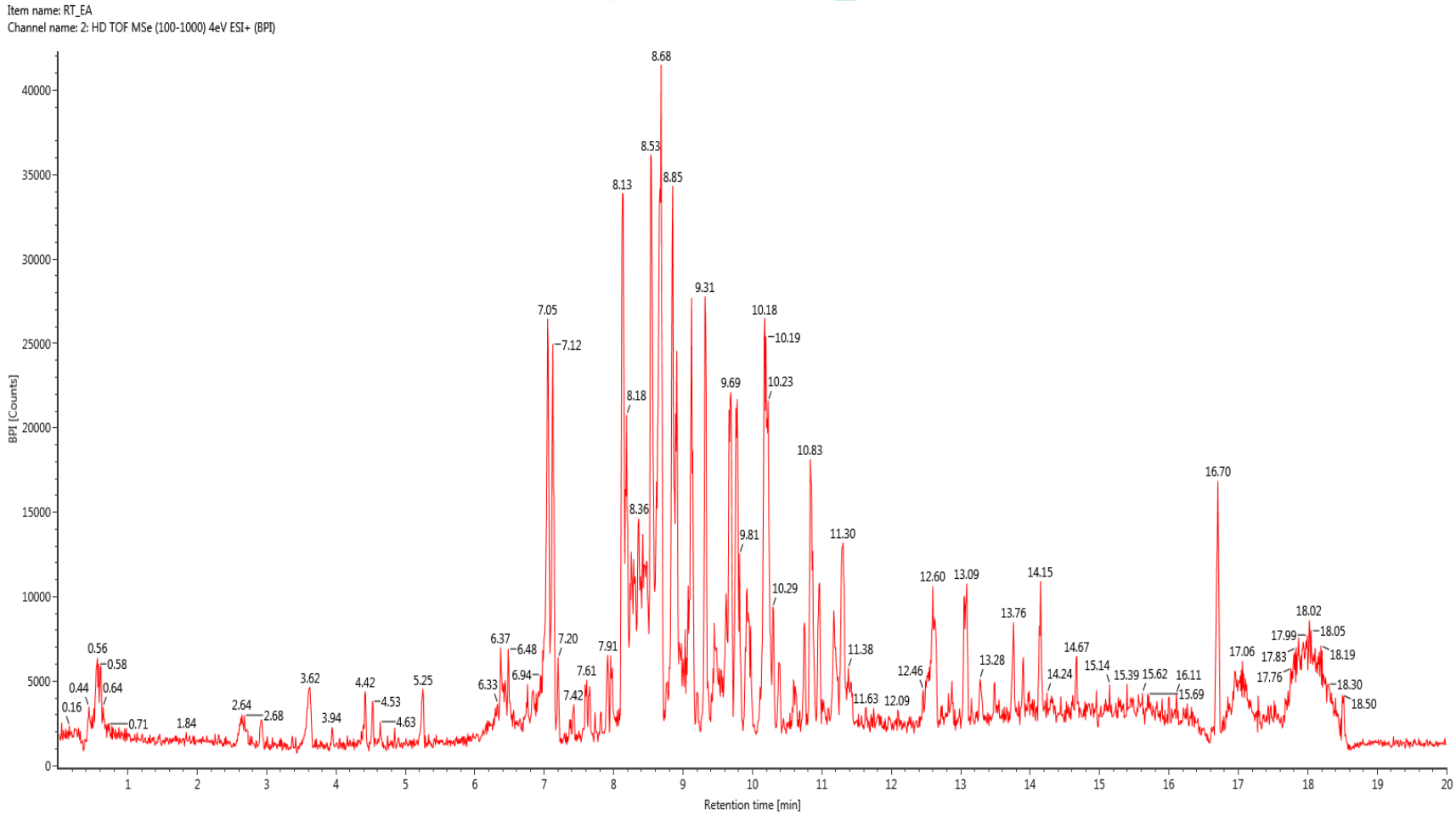
APPENDIX B3

DATA OF CONFIRMED COMPOUNDS ON (+)ESI-MS OF *R. TOMENTOSA* CHLOROFORM EXTRACT

No.	Component Name	Neutral mass (Da)	Observed neutral mass (Da)	Observed m/z	Mass error (mDa)	Mass error (ppm)	Obs. RT (min)	Obs. drift (ms)	Obs. CCS (Å²)	Response	Adducts
1	Quercetin-3-O-(6''-O-acetyl)-β-D-glucopyranoside	506.10604	506.1035	507.1108	-2.5	-5	0.56	7.61	205.75	2718	$[M + H]^+$
2	Methylellagic acid	344.05322	344.0535	345.0608	0.3	0.8	5.56	5.94	167.67	100632	$[M + H]^+$
3	Dichotomitin	358.06887	358.0688	359.076	-0.1	-0.3	8.89	6.18	172.88	105825	$[M + H]^+$
4	Rhodomyrtosone A	444.2148	444.2246	445.2319	9.8	22.1	9.52	7.56	205.11	6476	$[M + H]^+$
5	(2S)-3',4'-Methylenedioxy-5,7-dimethoxyflavane	314.11542	314.1154	337.1046	0	-0.1	9.6	6.25	175.03	5837	$[M + Na]^+$
6	Rhodomyrtosone C	674.38187	674.3821	675.3893	0.2	0.3	9.96	10.37	278.08	2440	$[M + H]^+$
7	Tomentosone b	688.36113	688.3637	689.371	2.6	3.8	10.05	9.29	248.36	3035	$[M + H]^+$
8	Ledebouriellol	374.13655	374.1359	397.1251	-0.7	-1.7	10.25	6.66	183.75	2330	$[M + Na]^+$
9	4′,7-Dimethyltectorigenin	328.09469	328.0944	329.1017	-0.3	-0.8	11.05	6.03	169.9	2956	$[M + H]^+$
10	Lupeol	398.35487	398.361	421.3502	6.1	14.5	13.65	7.88	213.63	3289	$[M + Na]^+$
11	Rhodomyrtosone D	432.25119	432.2494	433.2566	-1.8	-4.2	14.2	7.68	208.44	4930	$[M + H]^+$

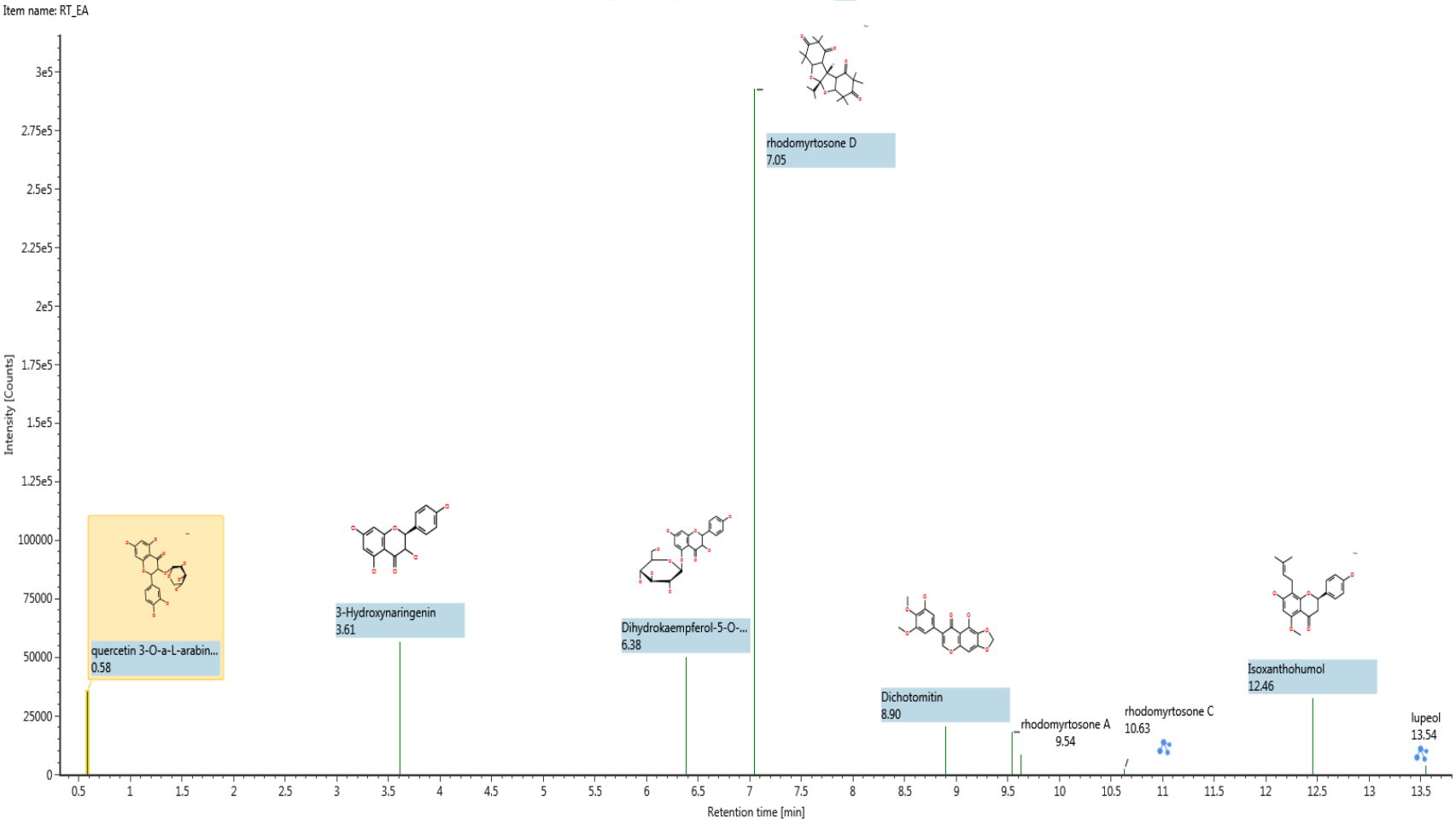
APPENDIX C1

UPLC-QToF/MS POSITIVE BPI CHROMATOGRAM OF *R. TOMENTOSA* ETHYL ACETATE EXTRACT



APPENDIX C2

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



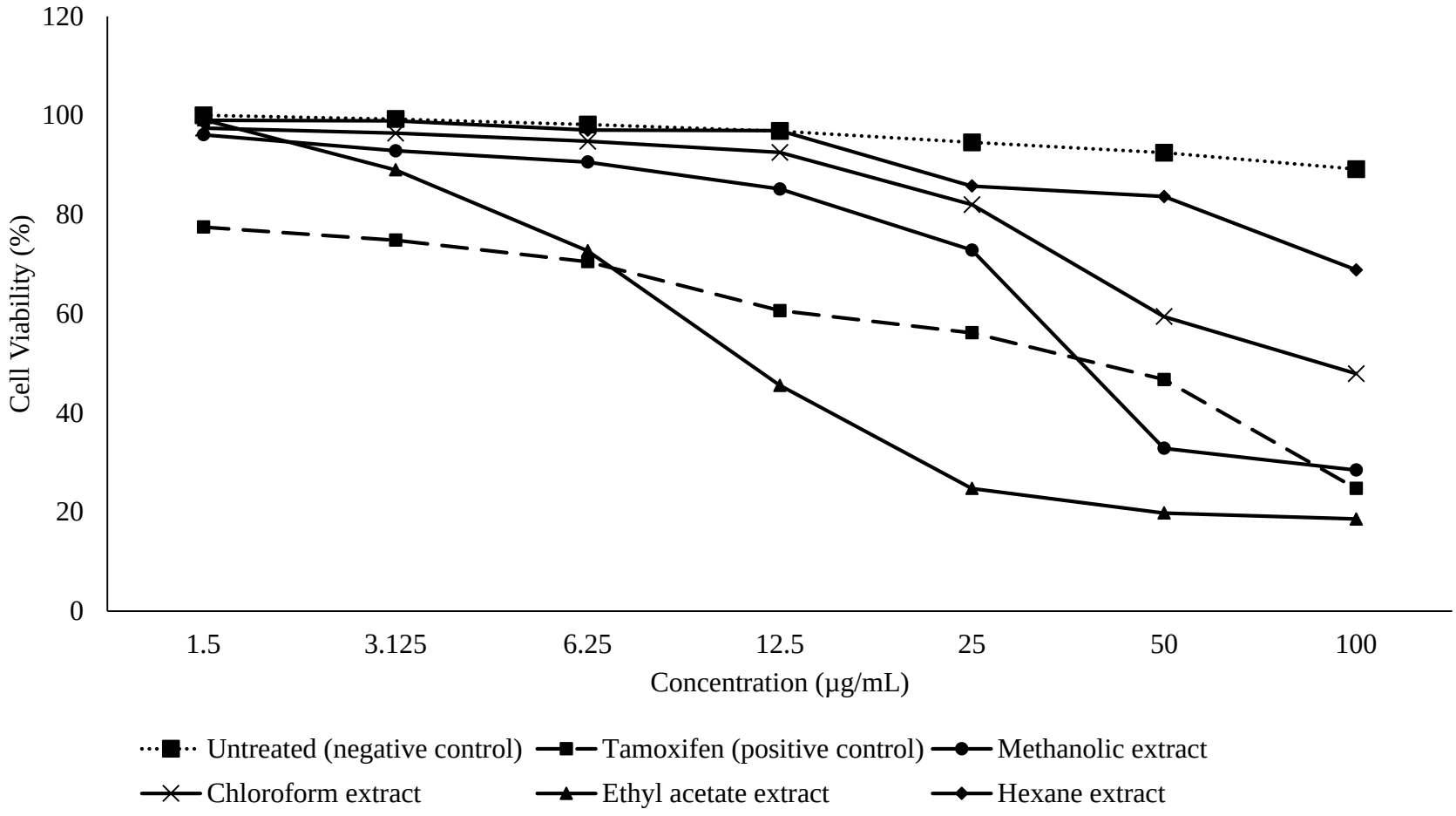
APPENDIX C3

DATA OF CONFIRMED COMPOUNDS ON (+)ESI-MS OF *R. TOMENTOSA* ETHYL ACETATE EXTRACT

No.	Component Name	Neutral mass (Da)	Observed neutral mass (Da)	Observed m/z	Mass error (mDa)	Mass error (ppm)	Obs. RT (min)	Obs. drift (ms)	Obs. CCS (Å²)	Response	Adducts
1	Quercetin 3-O-a-L-arabinoside	436.10056	436.1032	459.0925	2.7	5.8	0.58	7.46	202.61	35428	$[M + Na]^+$
2	3-Hydroxynaringenin	288.06339	288.0635	289.0707	0.1	0.2	3.61	5.61	161.13	89716	$[M + H]^+$
3	Dihydrokaempferol-5-O-β-D-glucopyranoside	450.11621	450.1261	473.1154	9.9	21	6.38	6	167.55	50062	$[M + Na]^+$
4	Rhodomyrtosone D	432.25119	432.2571	471.2202	5.9	12.4	7.05	7.32	198.86	292458	$[M + K]^+$
5	Dichotomitin	358.06887	358.0689	359.0762	0	0.1	8.9	6.19	173.18	20255	$[M + H]^+$
6	Rhodomyrtosone A	444.2148	444.2054	467.1946	-9.4	-20.2	9.54	7.56	204.91	18033	$[M + Na]^+$
7	6-Methoxy-2-[2-(4'-methoxyphenyl) ethyl] chromone	326.15181	326.152	327.1593	0.2	0.6	9.63	8.75	238.1	8358	$[M + H]^+$
8	Rhodomyrtosone C	674.38187	674.387	697.3763	5.2	7.4	10.63	9.11	243.36	2165	$[M + Na]^+$
9	Isoxanthohumol	354.14672	354.1415	355.1488	-5.2	-14.7	12.46	6.77	186.96	32253	$[M + H]^+$
10	Lupeol	398.35487	398.363	421.3522	8.1	19.3	13.54	7.76	210.6	3691	$[M + Na]^+$

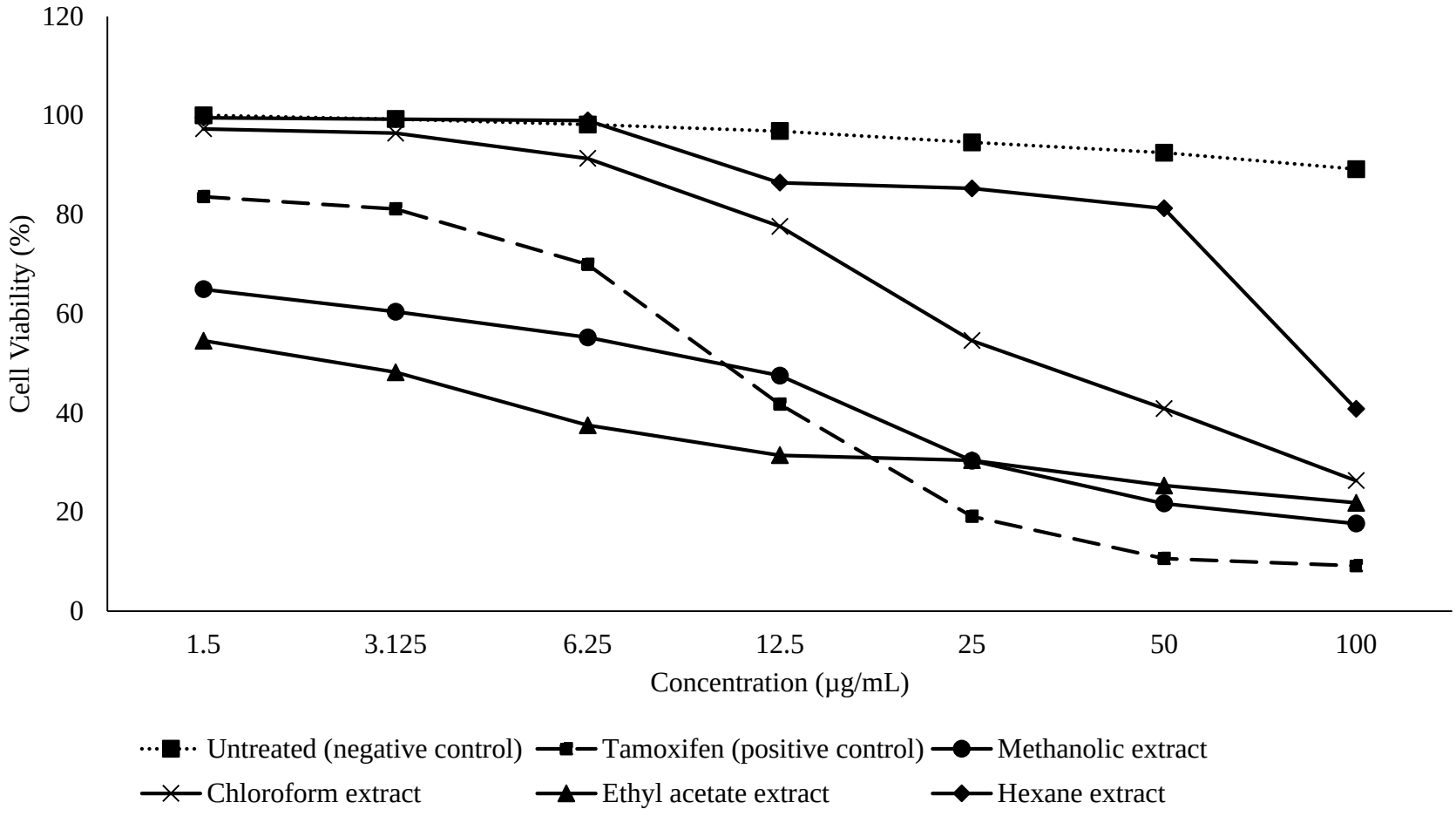
APPENDIX D1

DOSE-DEPENDENT ANTICANCER OF *R. TOMENTOSA* EXTRACTS ON HepG2 CELL LINE



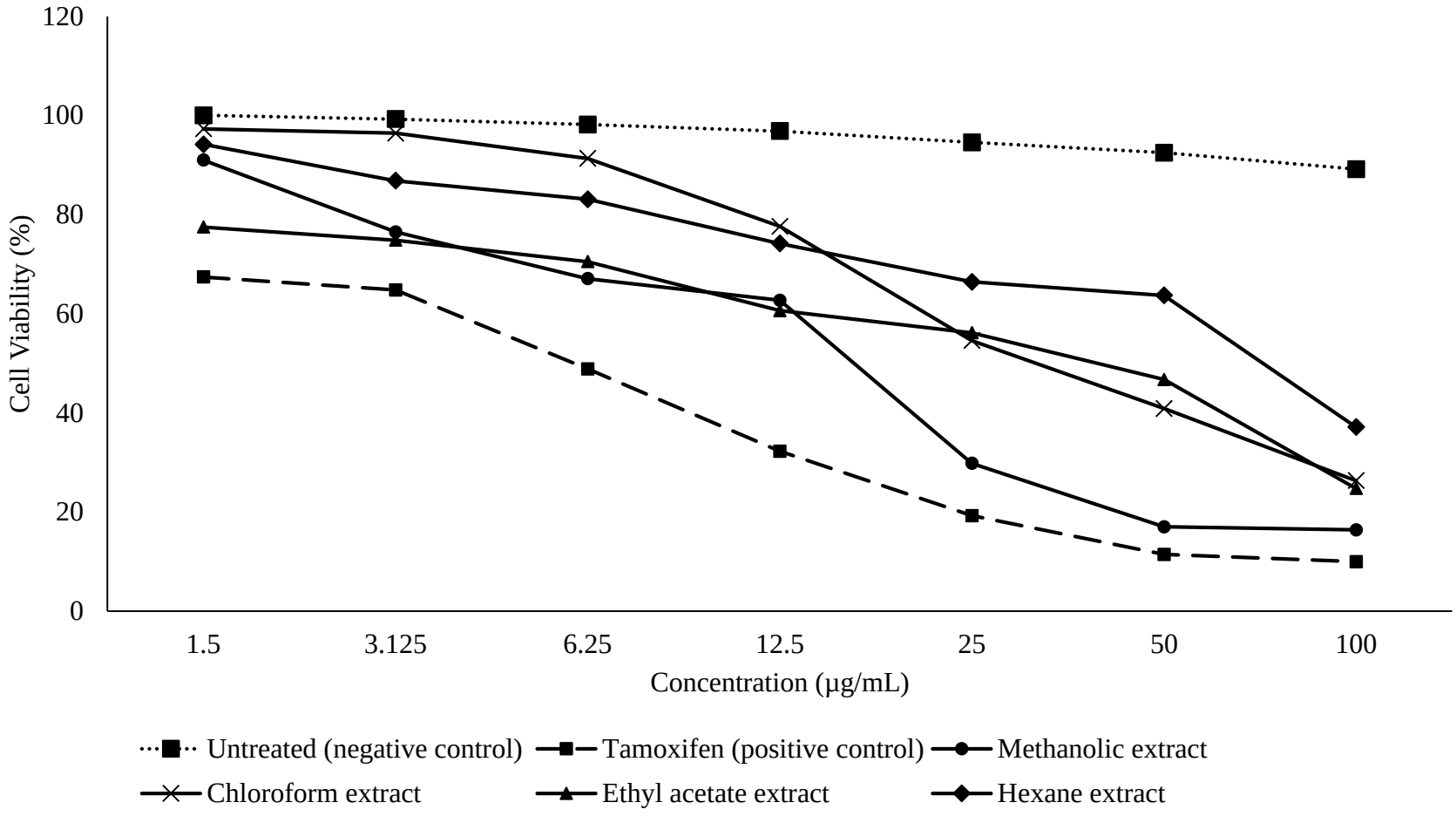
APPENDIX D2

DOSE-DEPENDENT ANTICANCER OF *R. TOMENTOSA* EXTRACTS ON MCF-7 CELL LINE



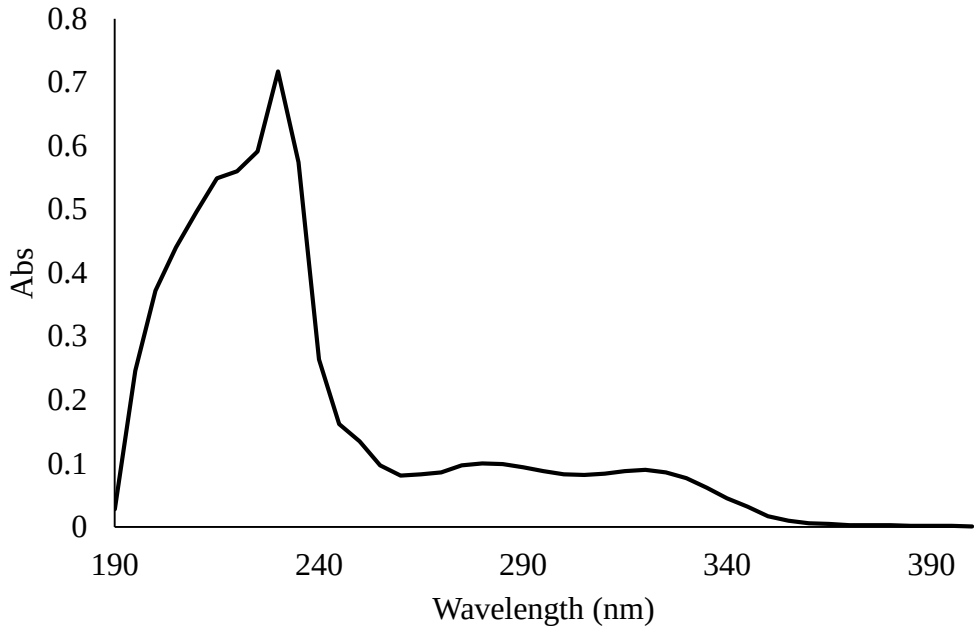
APPENDIX D3

DOSE-DEPENDENT ANTICANCER OF *R. TOMENTOSA* EXTRACTS ON HT 29 CELL LINE



APPENDIX E1

UV SPECTRA OF LUPEOL (27) FROM *R. TOMENTOSA*

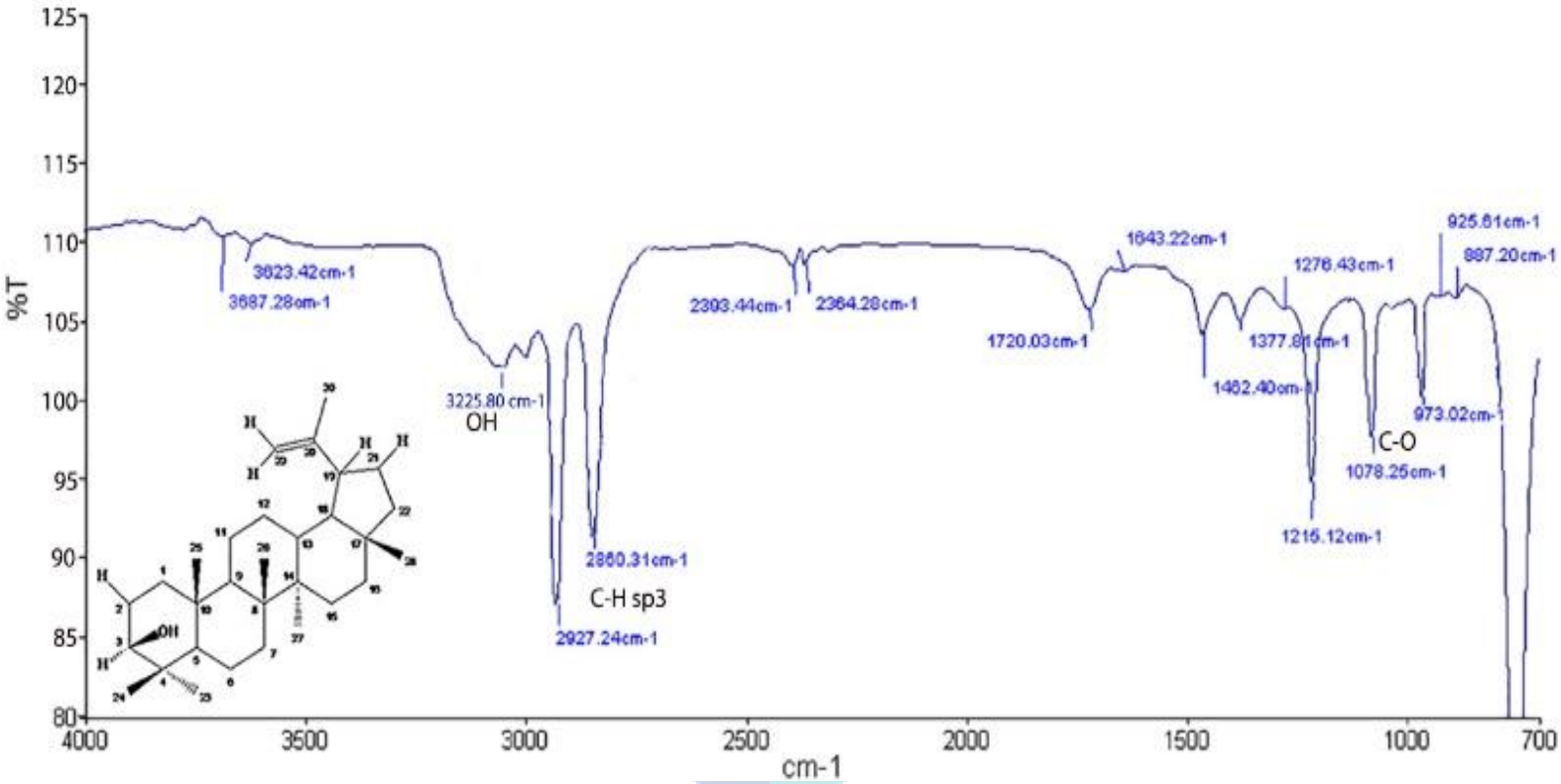


Compound **(27)** ($CHCl_3$)

No	Wavelength (nm)	Abs.
1.	215	0.549
2.	230	0.717

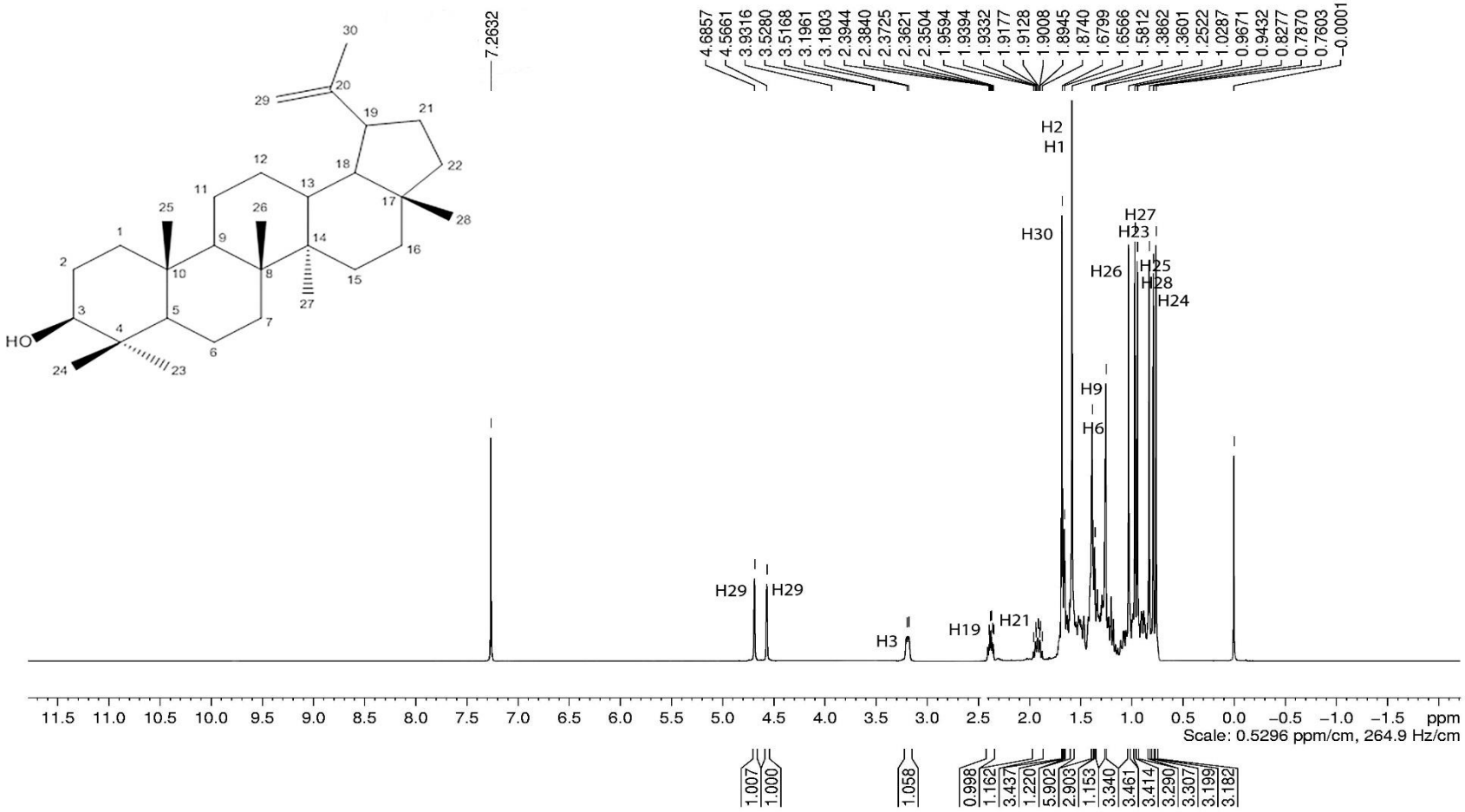
APPENDIX E2

IR SPECTRUM OF LUPEOL (27) FROM *R. TOMENTOSA*



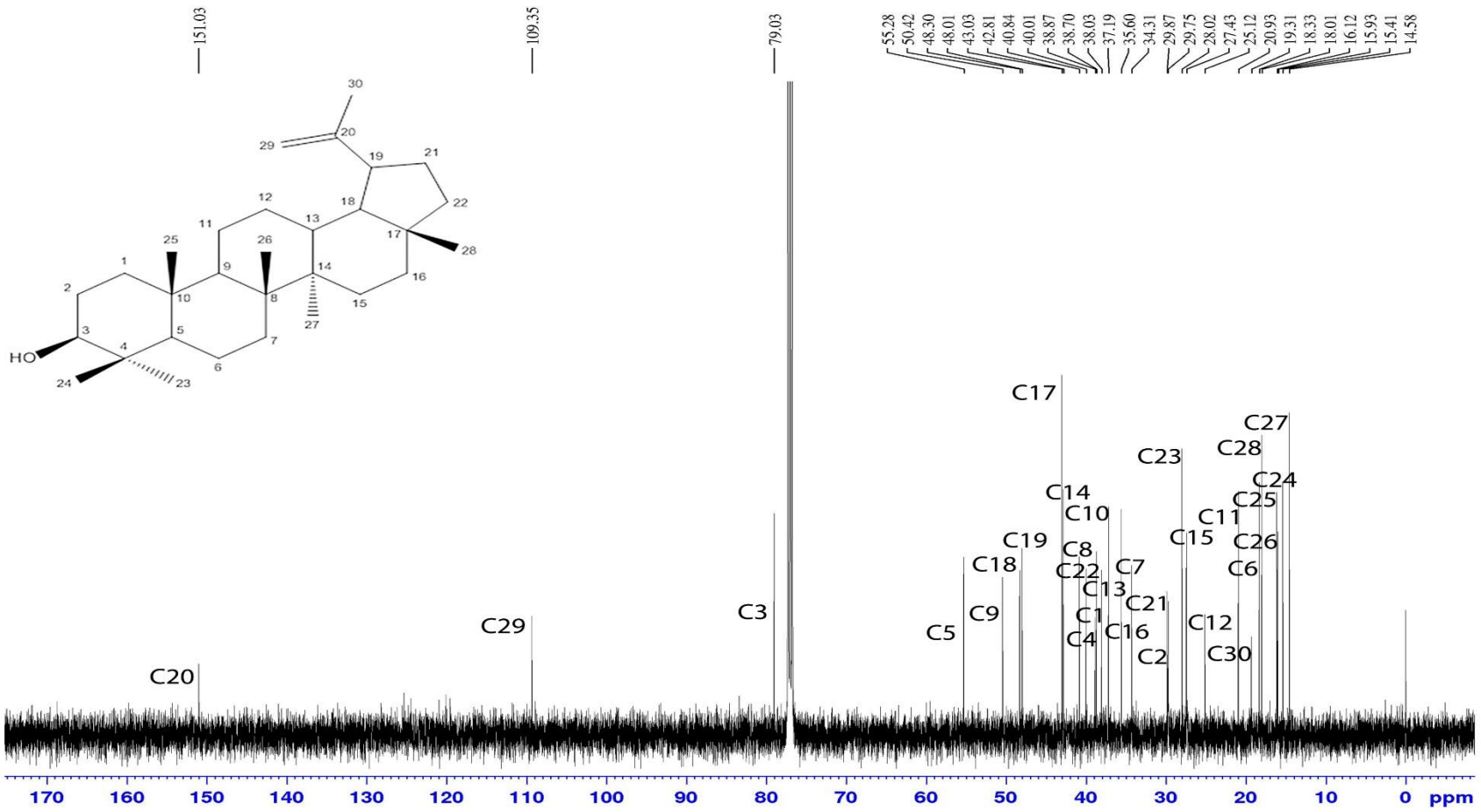
APPENDIX E3

^{1}H NMR SPECTRUM OF LUPEOL (27) FROM *R. TOMENTOSA*



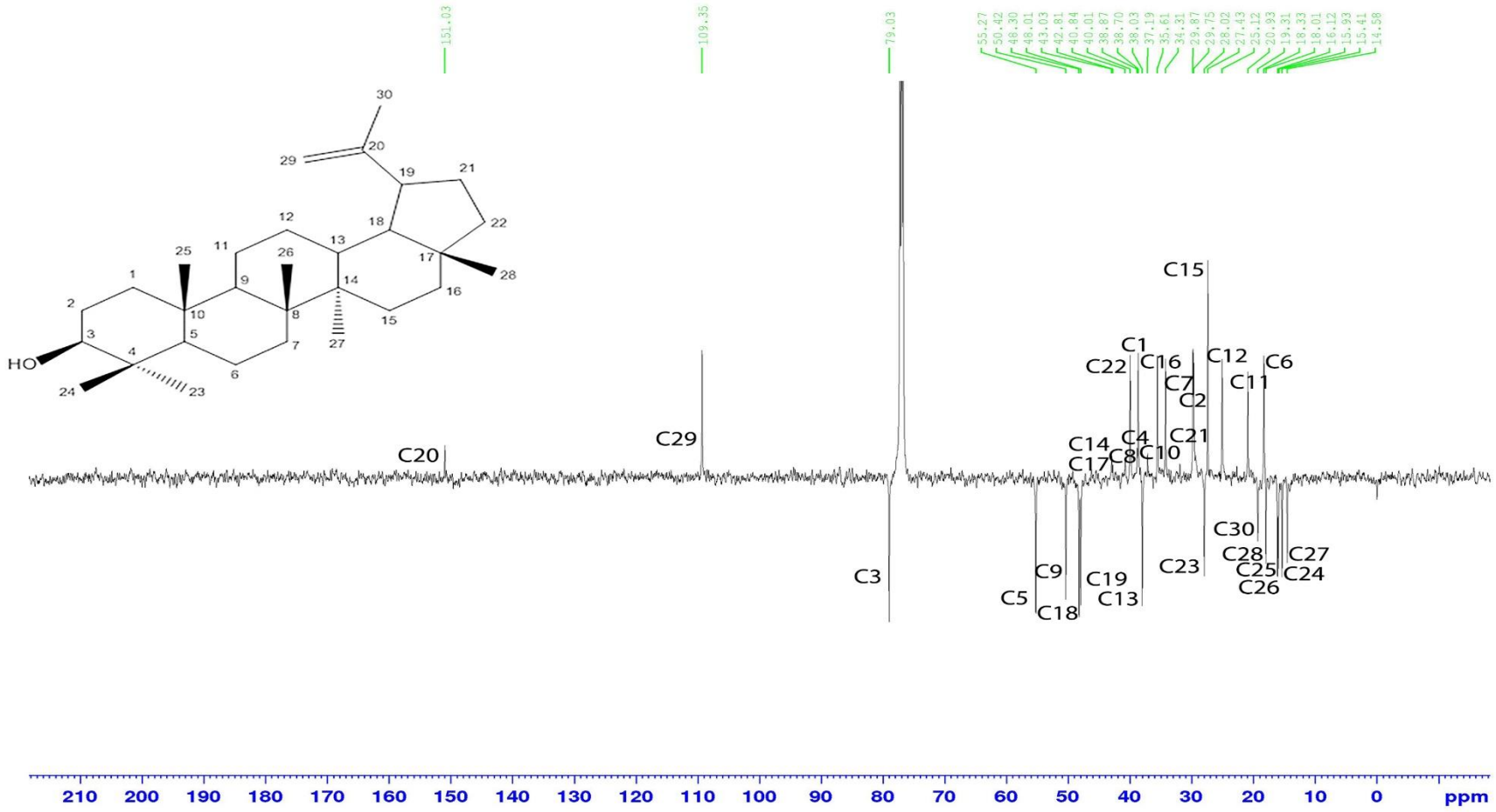
APPENDIX E4

^{13}C NMR SPECTRUM OF LUPEOL (27) FROM *R. TOMENTOSA*



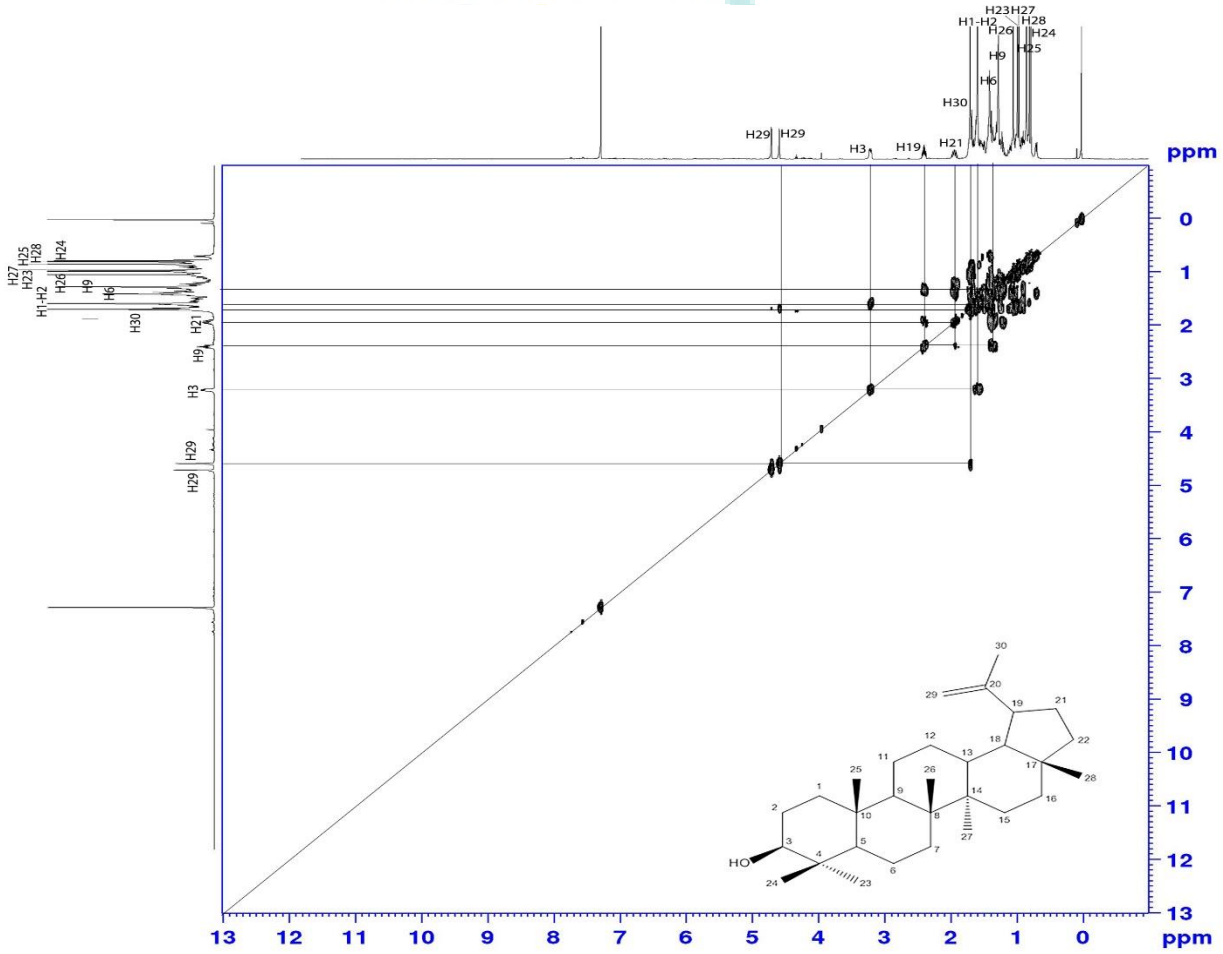
APPENDIX E5

DEPTQ SPECTRUM OF LUPEOL (27) FROM *R. TOMENTOSA*



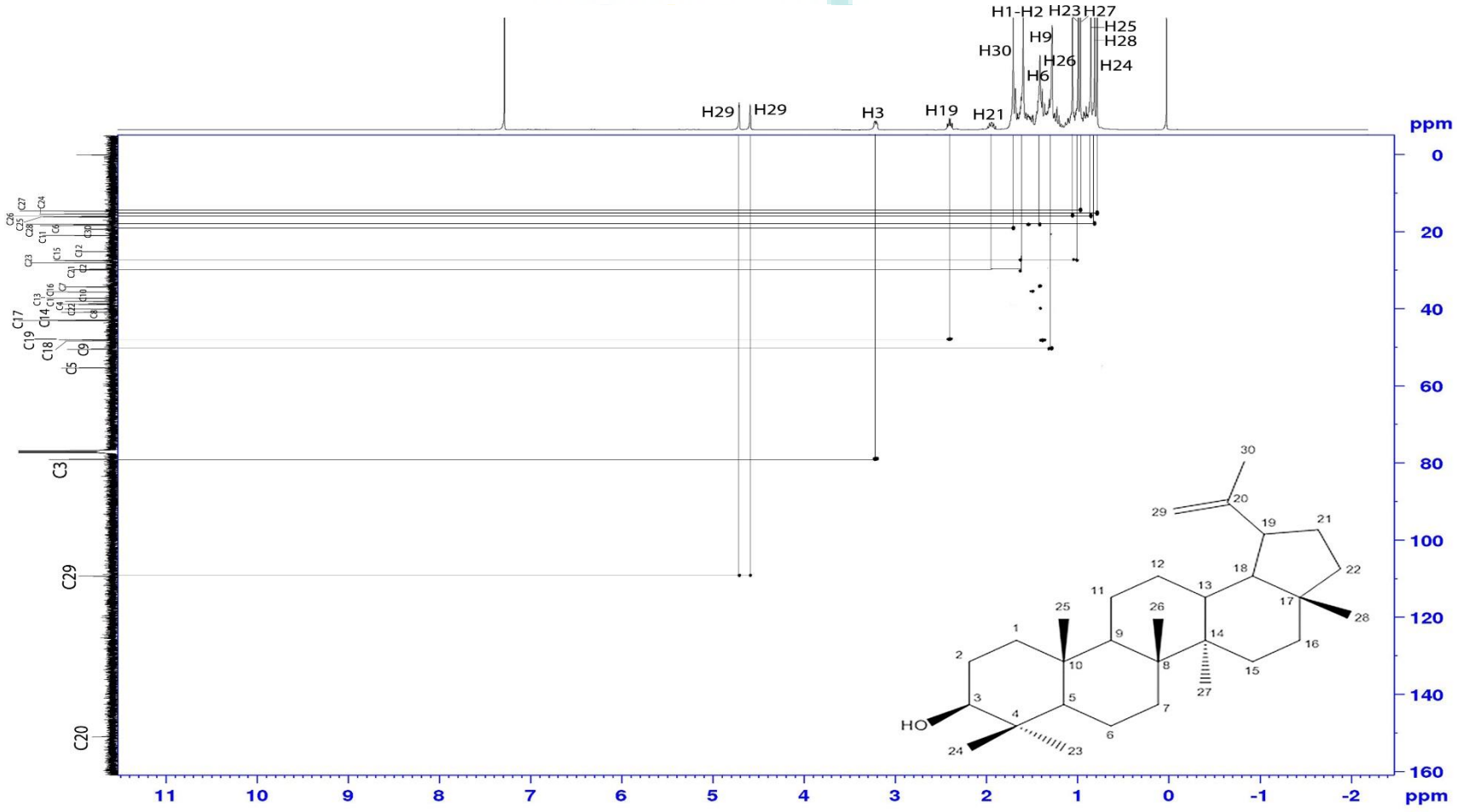
APPENDIX E6

COSY NMR SPECTRUM OF LUPEOL (27) FROM *R. TOMENTOSA*



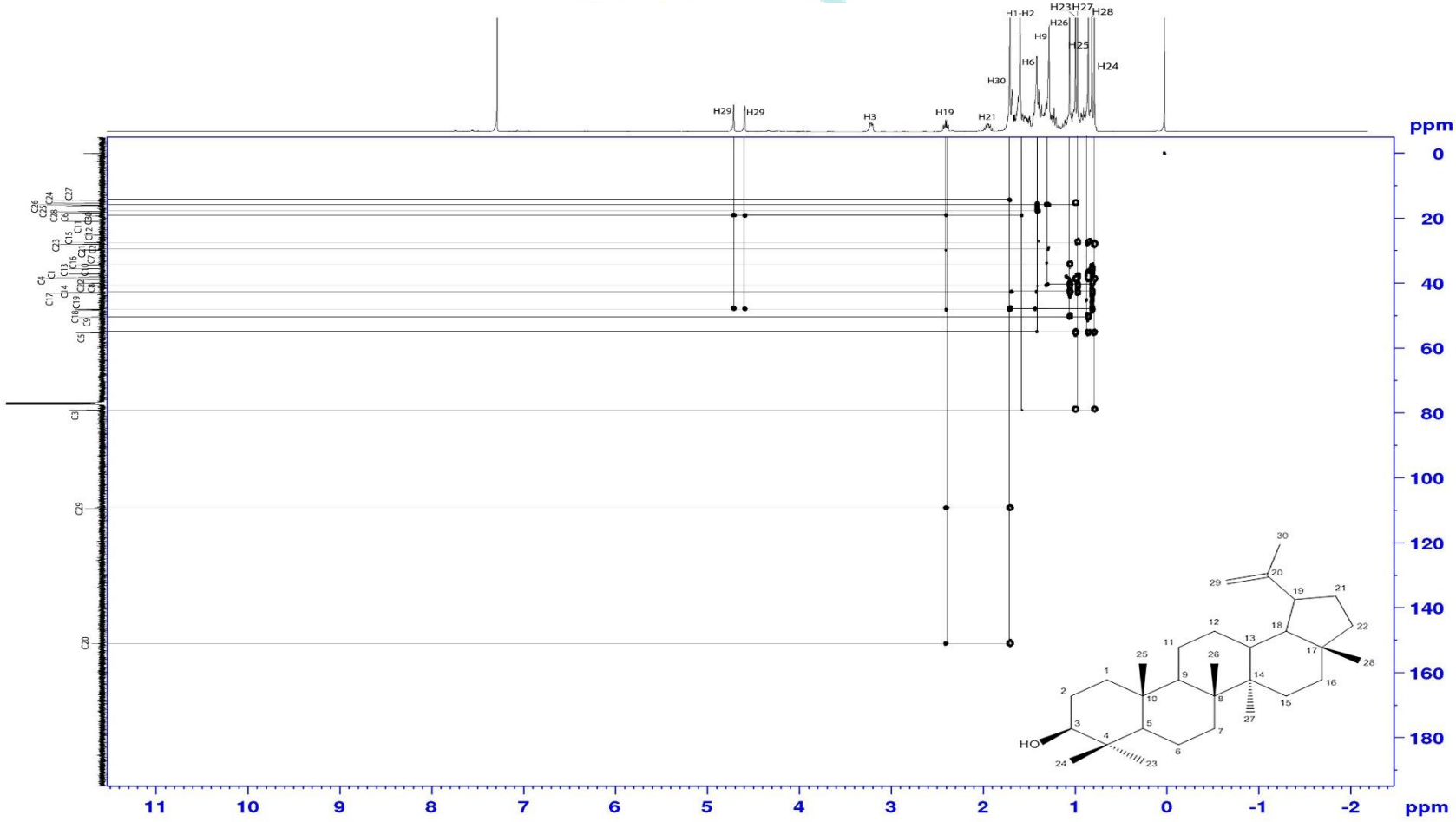
APPENDIX E7

HSQC NMR SPECTRUM OF LUPEOL (27) FROM *R. TOMENTOSA*



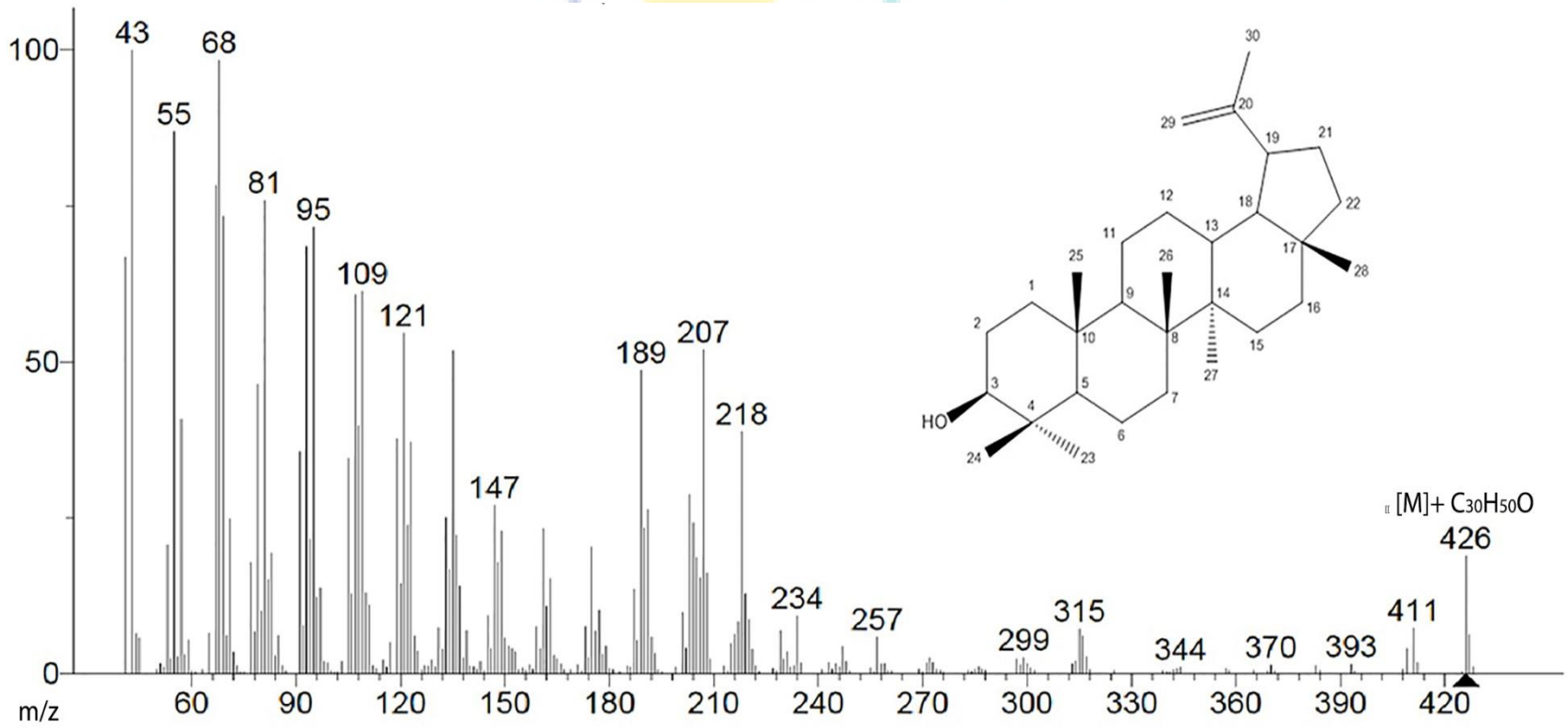
APPENDIX E8

HMBC NMR SPECTRUM OF LUPEOL (27) FROM *R. TOMENTOSA*



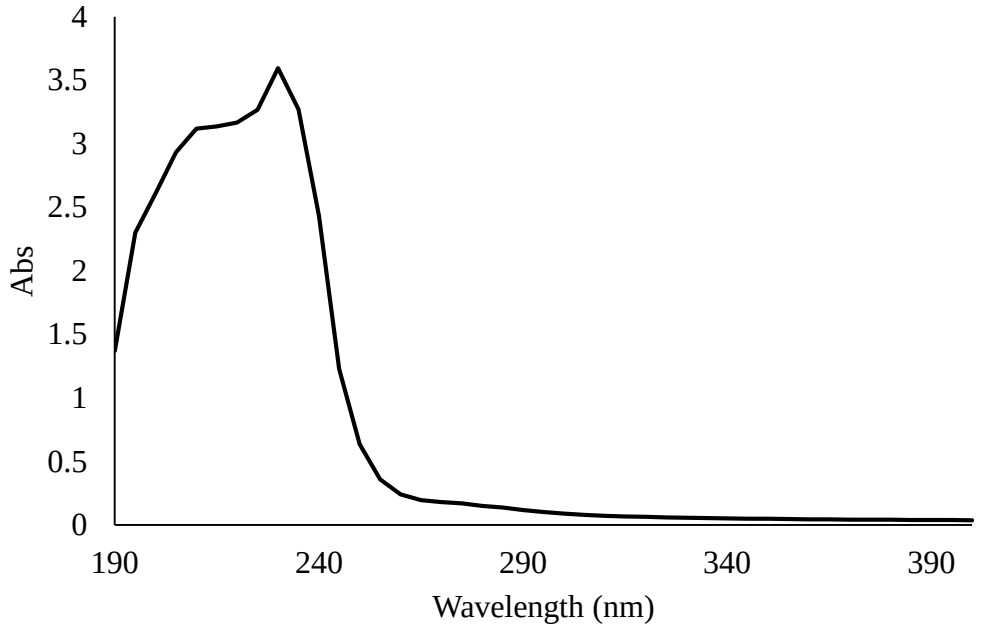
APPENDIX E9

EIMS SPECTRUM OF LUPEOL (27) FROM *R. TOMENTOSA*



APPENDIX F1

UV SPECTRA OF B-SITOSTEROL (24) FROM *R. TOMENTOSA*

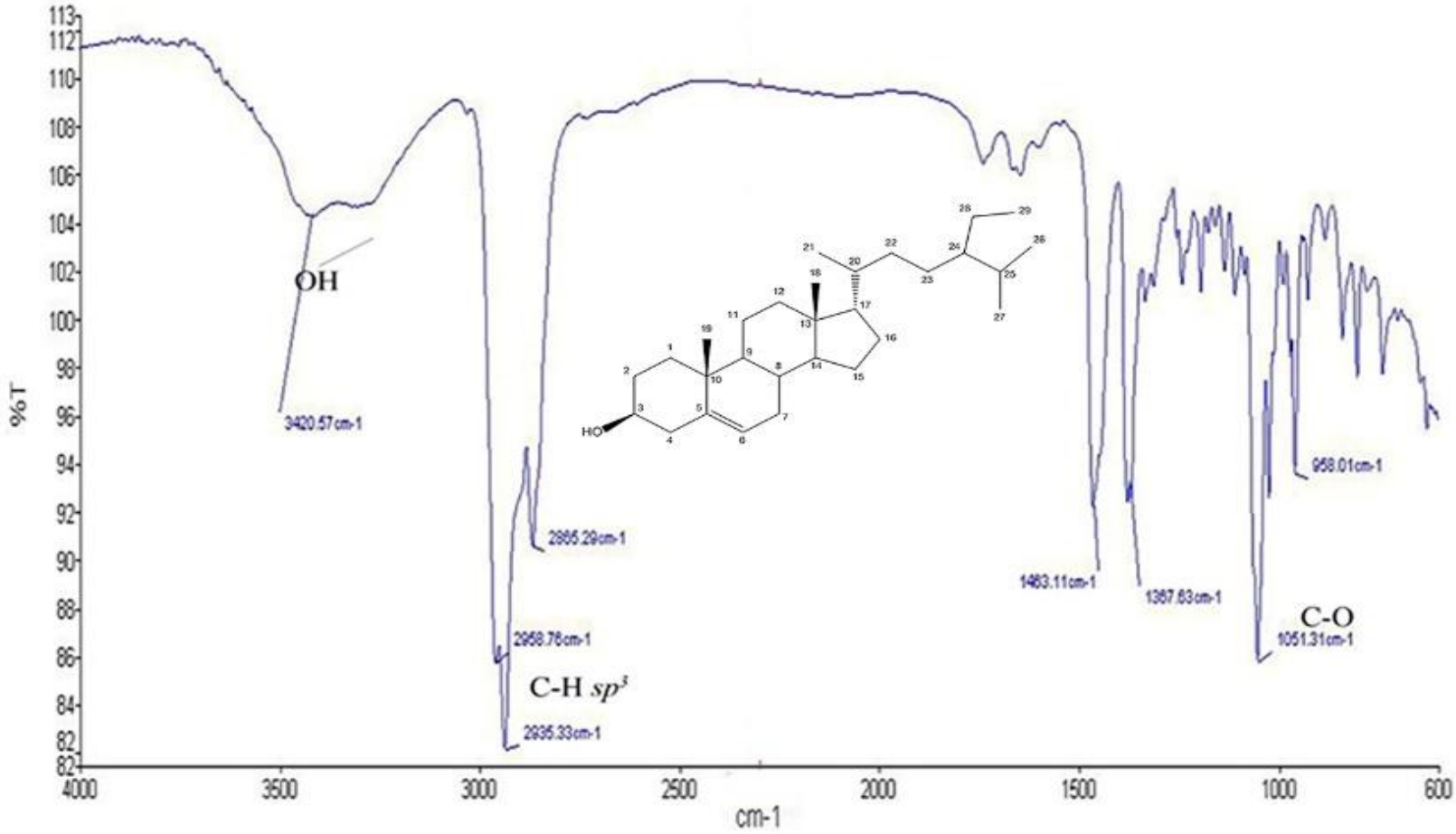


Compound **(24)** ($CHCl_3$)

No	Wavelength (nm)	Abs.
1.	210	3.118
2.	230	3.596

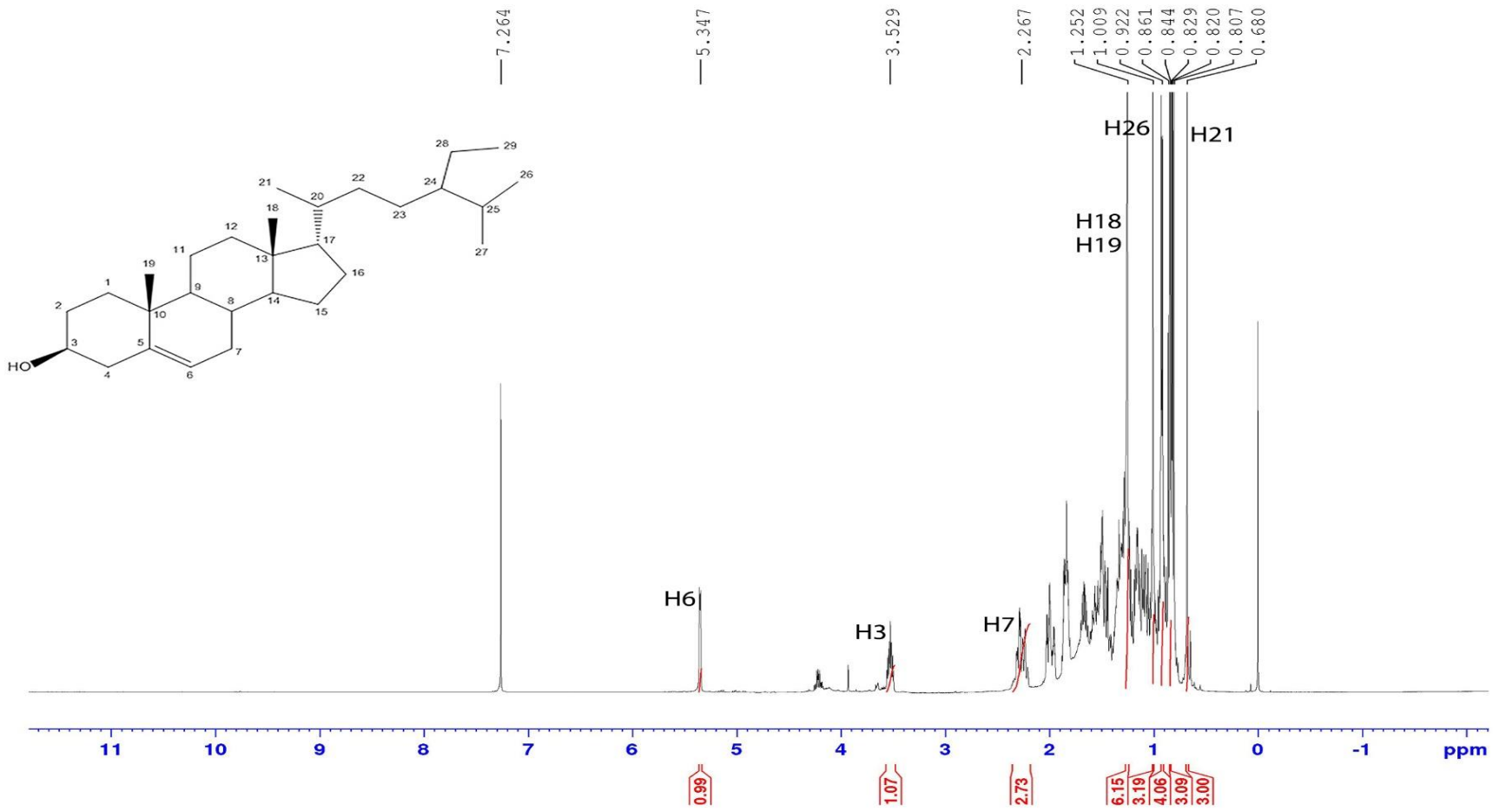
APPENDIX F2

IR SPECTRUM OF β-SITOSTEROL (24) FROM *R. TOMENTOSA*



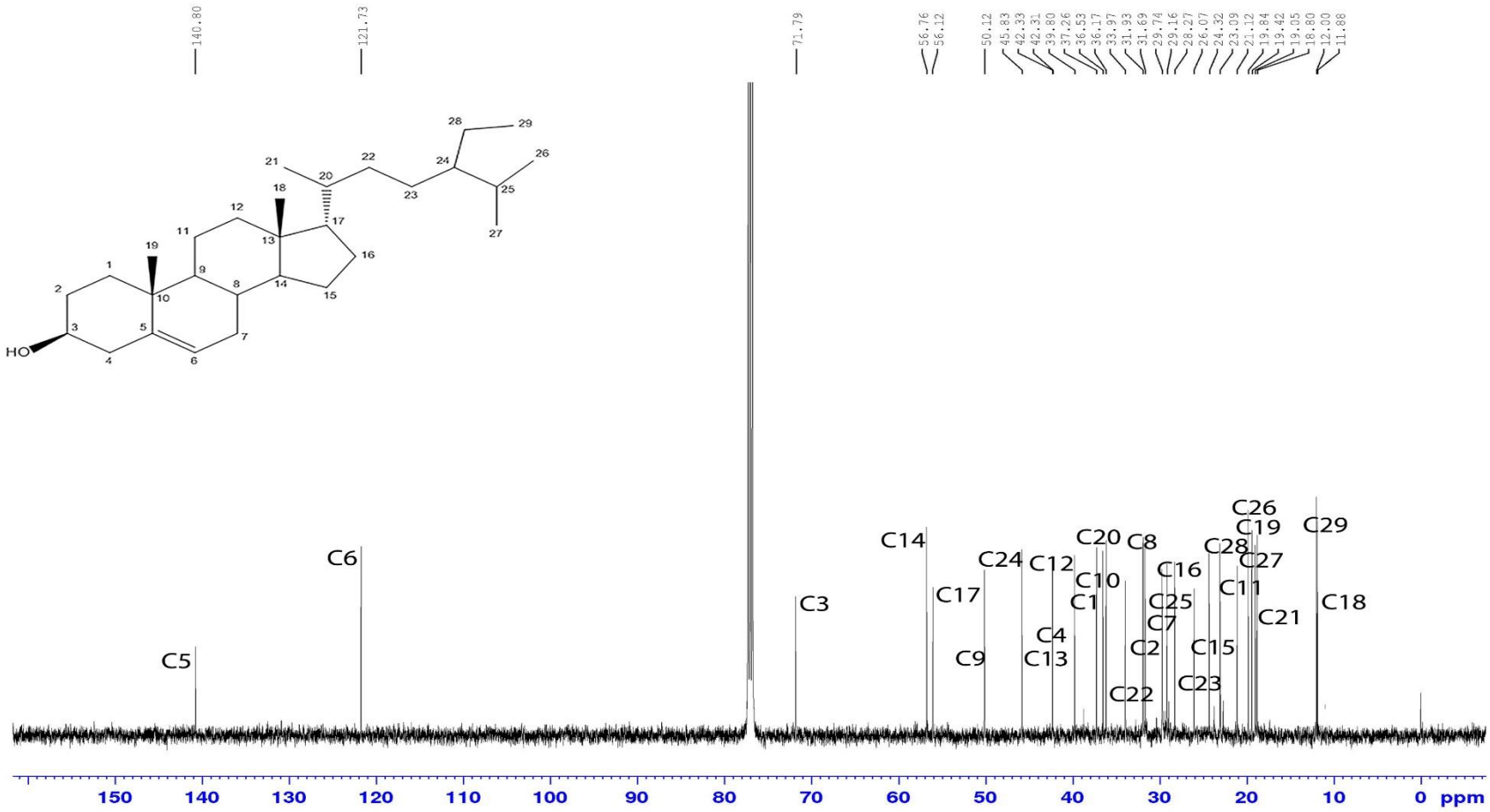
APPENDIX F3

^{1}H NMR SPECTRUM OF β-SITOSTEROL (24) FROM *R. TOMENTOSA*



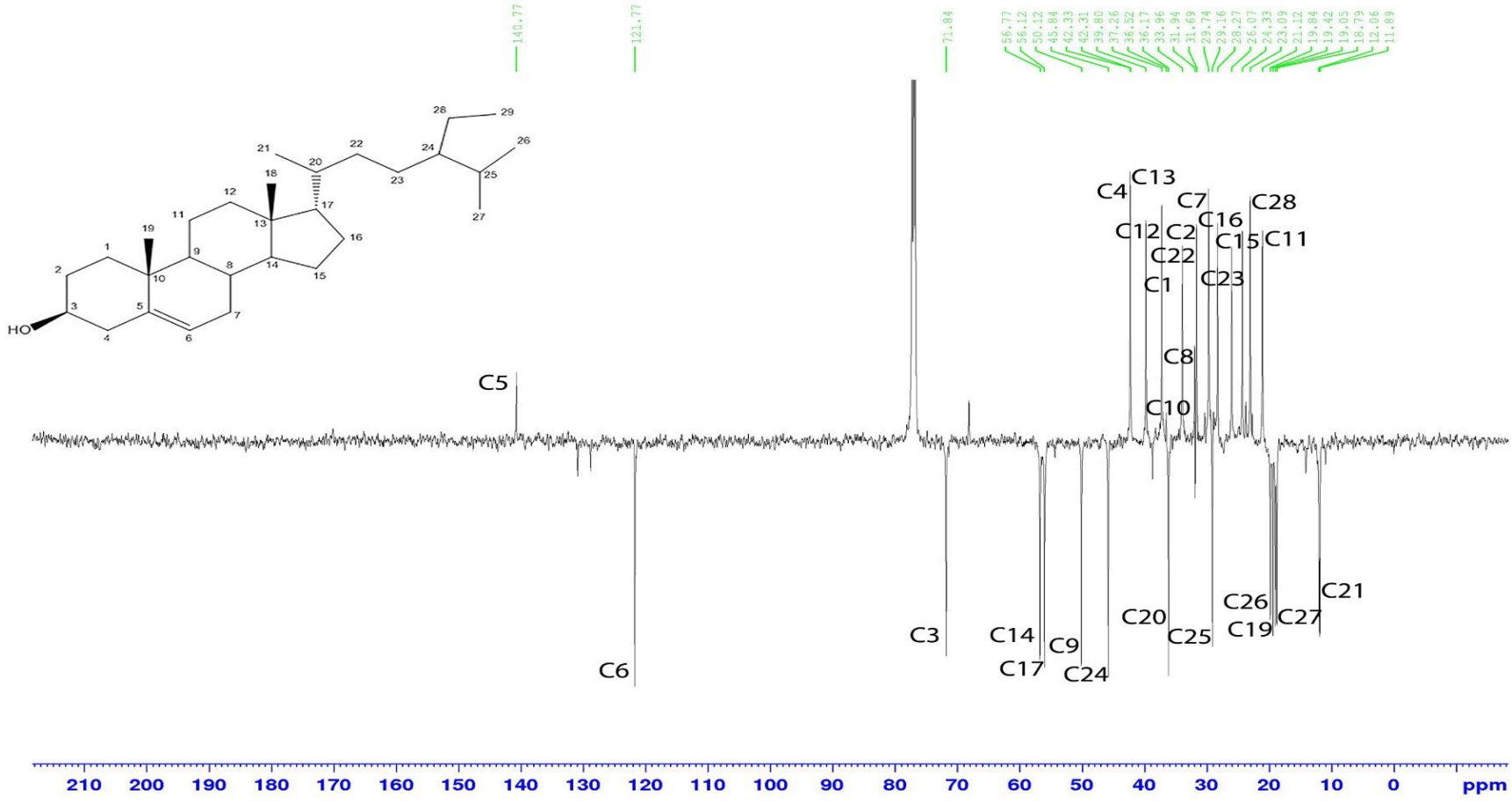
APPENDIX F4

^{13}C NMR SPECTRUM OF β-SITOSTEROL (24) FROM *R. TOMENTOSA*



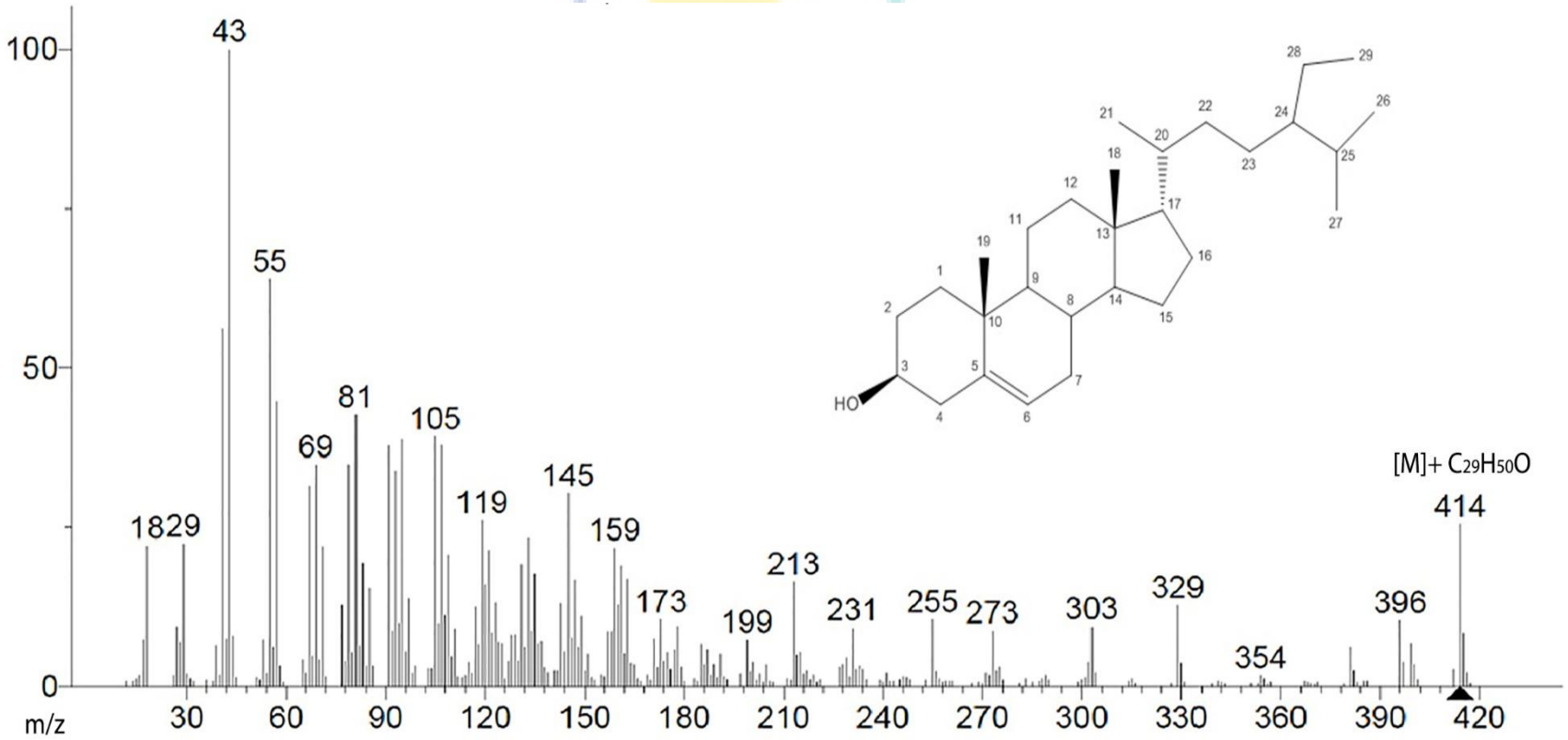
APPENDIX F5

DEPTQ SPECTRUM OF β-SITOSTEROL (24) FROM *R. TOMENTOSA*



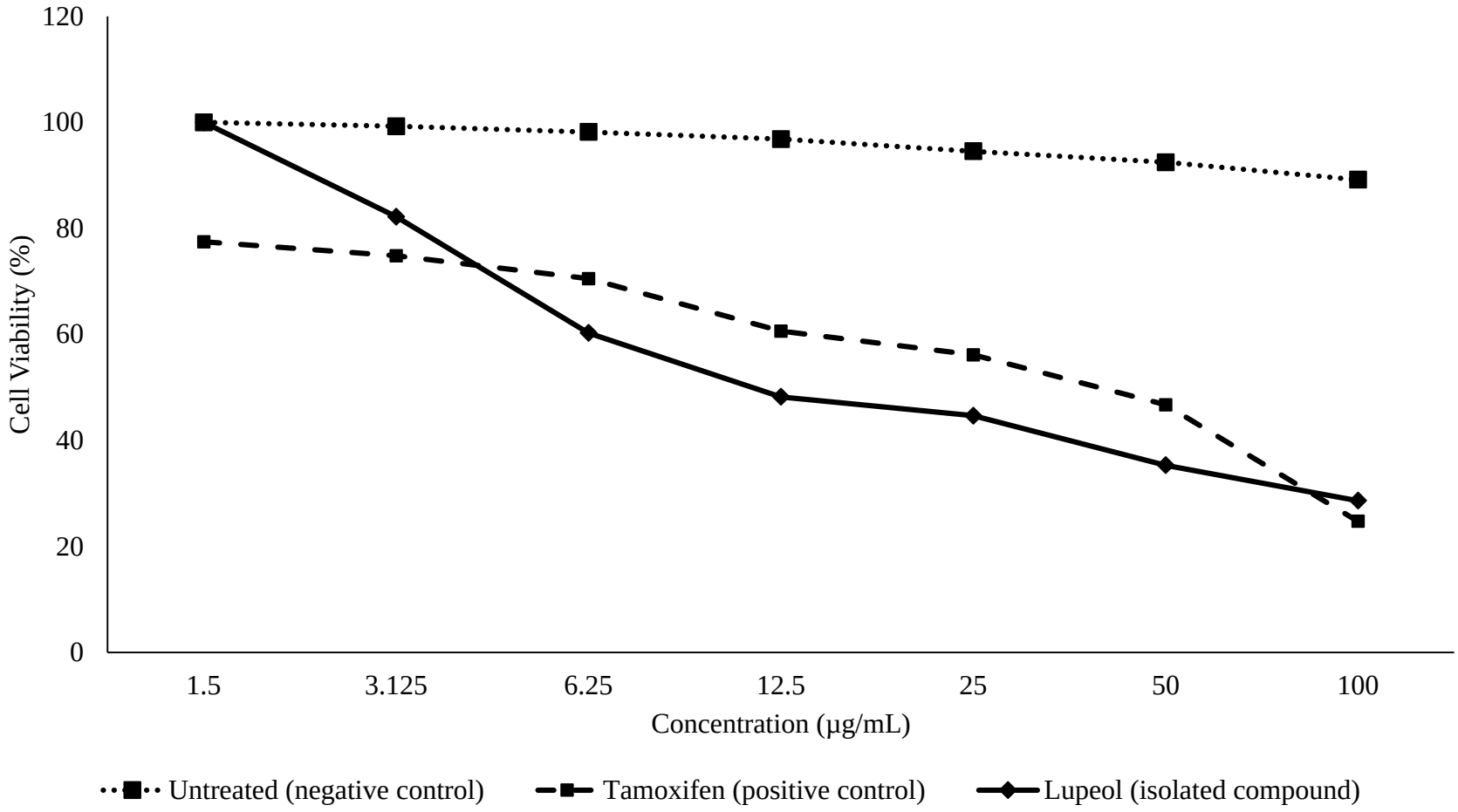
APPENDIX F6

EIMS SPECTRUM OF β-SITOSTEROL (24) FROM *R. TOMENTOSA*



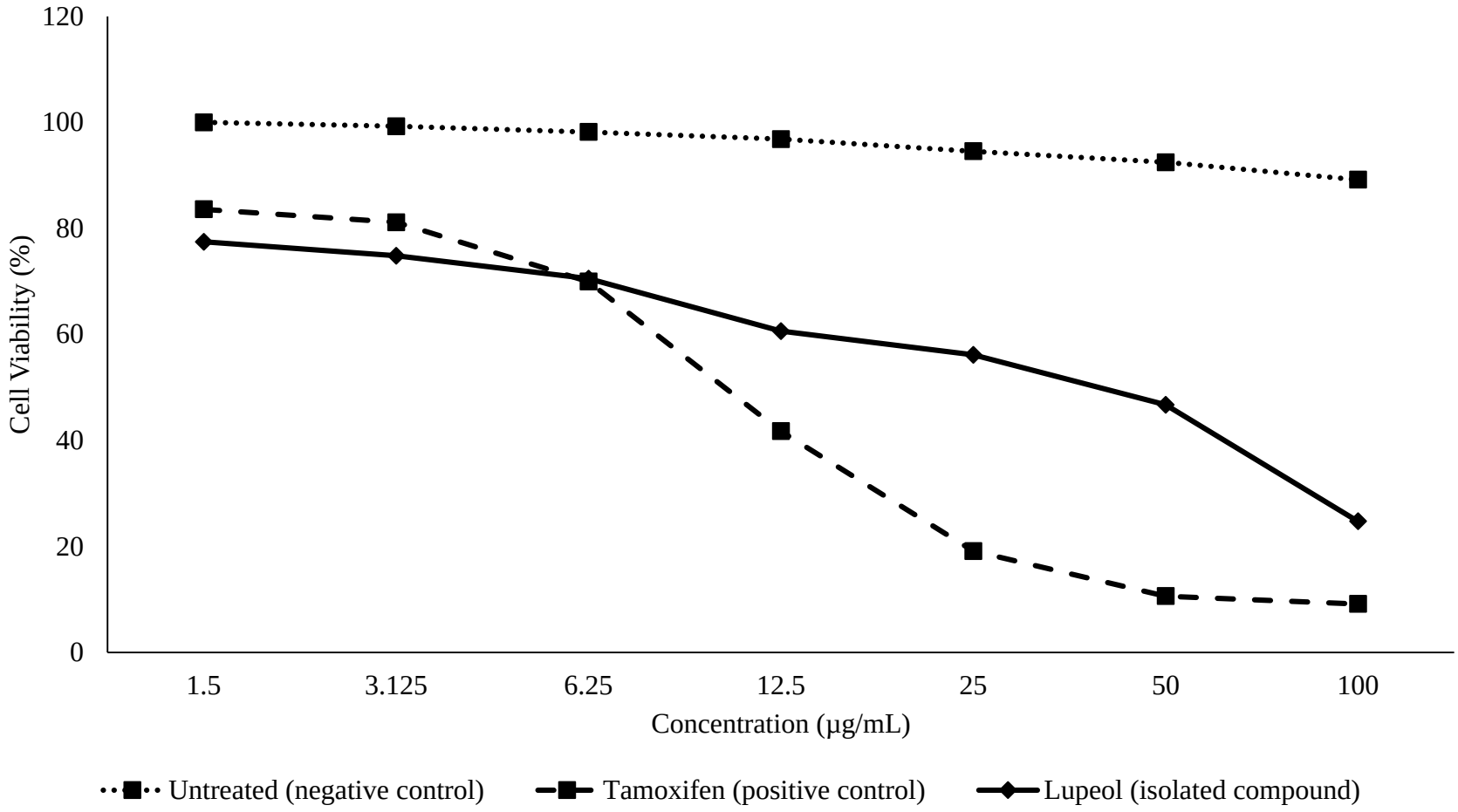
APPENDIX G1

DOSE-DEPENDENT ANTICANCER OF LUPEOL ON HepG2 CELL LINE



APPENDIX G2

DOSE-DEPENDENT ANTICANCER OF LUPEOL ON MCF-7 CELL LINE



APPENDIX G3

DOSE-DEPENDENT ANTICANCER OF LUPEOL ON HT 29 CELL LINE