PLAI OIL EXTRACTION BY ENZYME-ASSISTED METHOD AND INCORPORATION INTO ELECTROSPUN NANOFIBERS

By

Miss Tuddao Chuchote

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree
Master of Pharmacy Program in Pharmaceutical Technology
Graduate School, Silpakorn University
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การสกัดน้ำมันไพลโดยใช้เอนไซม์ช่วยและการบรรจุลงในเส้นใยนาโนซึ่งเตรียมโดยวิธีอิเล็กโตรสปิน

โดย

นางสาวทัดดาว ชูโชติ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต

สาขาวิชาเทคโนโลยีเภสัชกรรม

บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร

ปีการศึกษา 2554

The Graduate School, Silpakorn University has approved and accredited the Thesis title of "Plai Oil Extraction by Enzyme-Assisted Method and Incorporation into Electrospun Nanofibers" submitted by Miss Tuddao Chuchote as a partial fulfillment of the requirements for the degree of Master of Pharmacy in PHARMACEUTICAL TECHNOLOGY.

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51353202 : MAJOR : PHARMACEUTICAL TECHNOLOGY
KEY WORDS : PLAI / TERPINEN-4-OL / ENZYME-ASSISTED / EXTRACTION / ELECTROSPUN NANOFIBERS

TUDDAO CHUCHOTE : PLAI OIL EXTRACTION BY ENZYME-ASSISTED METHOD AND INCORPORATION INTO ELECTROSPUN NANOFIBERS. THESIS ADVISORS : ASSOC. PROF. PRANEET OPANASOPIT, Ph.D., AND ASSOC. PROF. THEERASAK ROJANARATA, Ph.D. 87 pp.

The objectives of this study were to investigate the use of cell wall degrading enzymes for the enhancement of the extraction efficiency of oil from rhizomes of Plai (*Zingiber cassumunar* Roxb.) and to prepare the Plai oil-loaded electrospun nanofibers for topical application. The conventional extraction methods i.e. soxhlet extraction, water distillation, solvent extraction combined with ultrasonication were performed and the extraction yields were compared based on the content of pharmacological active compound terpinen-4-ol in the extract. The result revealed that the yields from these methods were not statistically different. However, in the aspects of the ease and time consumed, hexane extraction combined with ultrasonication was superior and was thus chosen for the subsequent studies. To further improve the extraction, commercial enzymes capable of digesting plant cell wall components i.e. cellulase, hemicellulase and pectinase, as single enzyme and in combination, were used for the treatment of plant materials prior to the selected conventional extraction. Factors including the type and concentration of enzymes as well as the pH, temperature and time of enzyme treatment were investigated for the influence on the extraction yield. It was found that the optimum pH, temperature and time of enzyme pretreatment were pH 5.5 and incubation in an orbital shaker at 40 ± 0.5°C, 100 rpm for 24 h. Among the use of single enzyme at the weight ratio between the enzyme: plant sample of 3:100, cellulase gave the highest extraction yield of terpinen-4-ol followed by pectinase and hemicellulase. The increase of cellulase ratio to 6:100 and 9:100 slightly lowered the yield. However, at all weight ratios tested, cellulase treatment gave higher extraction yield than the conventional extraction without enzymatic treatment. In the case of combined enzymes, cellulase/pectinase showed the highest extraction efficiency yield followed by cellulase/hemicellulase and hemicellulase/pectinase, respectively. This finding demonstrated that the enzymatic treatment promoted the hydrolysis of the cell walls, facilitated the release of oil from the plant sample and finally improve the extraction yield. Plai oil-loaded electrospun nanofibers of PVP/HPβCD were successfully prepared using electrospinning technique. Plai oil was loaded in a different amount of 10%, 20% and 30% to polymer. The morphology, diameter and structure of the electrospun nanofibers were investigated. SEM images showed that nanofiber were observed. The average diameter of fibers was 225-486 nm, and decreased with the increasing of Plai oil content. The release study showed the initial fast release in the first few hours, followed by the slow release rate over the experimental time of 24 h about 67-79%. The release rate ranged was in the order of 10 % > 20% > 30% Plai oil-loaded electrospun fibers. 10 % Plai oil-loaded electrospun fibers provided the highest release and also showed the highest physical stability over 9 months. At high concentration of initial Plai oil (20% and 30%), the instability of morphology was observed. Hence, this 10 % Plai oil-loaded electrospun nanofibers have a potential for the use as an alternative topical application.

Program of Pharmaceutical Technology Graduate School, Silpakorn University
Student's signature ..... Academic Year 2011
Thesis Advisors' signature 1. ..... 2. .....

51353202 : สาขาวิชาเทคโนโลยีเภสัชกรรม
คำสำคัญ : น้ำมันไพล / เทอพิเนน-4-ออล / เอนไซม์ช่วย / การสกัด / อิเล็กโตรสปิน

ทัดดาว ชูโชติ : การสกัดน้ำมันไพลโดยใช้เอนไซม์ช่วยและการบรรจุลงในเส้นใยนาโนซึ่งเตรียมโดยวิธีอิเล็กโตรสปิน. อาจารย์ที่ปรึกษาวิทยานิพนธ์ : ภญ.รศ.ดร.ปราณีต โอปณะโสภิต และ ภก.รศ.ดร.ธีรศักดิ์ โรจนราธา. 87 หน้า.

วัตถุประสงค์ของงานวิจัยนี้คือเพื่อศึกษาความเป็นไปได้ในการนำเอนไซม์ซึ่งสามารถย่อยผนังเซลล์ของพืชมาช่วยเพิ่มประสิทธิภาพการสกัดน้ำมันจากเหง้าไพล (*Zingiber cassumunar* Roxb.) และเพื่อเตรียมเส้นใยระดับนาโนเมตรที่บรรจุน้ำมันไพลโดยกระบวนการอิเล็กโตรสปิน วิธีการสกัดแบบดั้งเดิม ทำโดยวิธี การสกัดแบบต่อเนื่องด้วยเครื่องซอกท์เลต การกลั่นด้วยน้ำ และการสกัดด้วยตัวทำละลายร่วมกับอัลตร้าโซนิค แล้วเปรียบเทียบปริมาณสารเทอร์พิเนน-4-ออล ซึ่งมีฤทธิ์ทางเภสัชวิทยาในน้ำมันที่สกัดได้ ผลการศึกษาพบว่าปริมาณสารที่สกัดได้ในแต่ละวิธีไม่แตกต่างกันอย่างมีนัยสำคัญ อย่างไรก็ตามในแง่ของความง่ายและเวลาที่ใช้ในการสกัด การสกัดด้วยตัวทำละลายเฮกเซนร่วมกับอัลตร้าโซนิคมีความเหมาะสม ดังนั้นจึงเลือกใช้วิธีนี้ในการศึกษาต่อไป เพื่อเพิ่มประสิทธิภาพการสกัดเอนไซม์ซึ่งสามารถย่อยส่วนประกอบของผนังเซลล์ของพืช เช่น เซลลูเลส เฮมิเซลลูเลสและเพคติเนส โดยใช้ชนิดเดียวและใช้เอนไซม์มากกว่าหนึ่งชนิดร่วมกันใช้ในการย่อยพืชก่อนสกัดด้วยวิธีการแบบดั้งเดิมต่อเพื่อศึกษาผลของปัจจัยต่าง ๆ ได้แก่ ชนิดและความเข้มข้นของเอนไซม์ พีเอช อุณหภูมิและระยะเวลาของการบ่ม พบว่าสภาวะที่เหมาะสมคือพีเอช 5.5 บ่มในตู้เขย่าที่ควบคุมอุณหภูมิที่ 40±0.5 องศาเซลเซียส ความเร็ว 100 รอบต่อนาที นาน 24 ชั่วโมง การใช้เอนไซม์ชนิดเดียวที่อัตราส่วนน้ำหนักเซลลูเลสต่อตัวอย่างพืช 3:100 พบว่าเซลลูเลสมีประสิทธิภาพในการสกัดสารเทอร์พิเนน-4-ออลสูงที่สุดตามด้วยเพคติเนสและเฮมิเซลลูเลส การเพิ่มอัตราส่วนน้ำหนักของเซลลูเลสต่อตัวอย่างพืชเป็น 6:100 และ 9:100 ทำให้ปริมาณสารสกัดที่ได้ลดลงเล็กน้อย อย่างไรก็ตามพบว่าในทุกอัตราส่วนให้ปริมาณสารสกัดสูงกว่าการสกัดที่ไม่ใช้เอนไซม์ช่วย ในกรณีที่ใช้เอนไซม์มากกว่าหนึ่งชนิดร่วมกัน เซลลูเลส/เพคติเนสมีประสิทธิภาพในการสกัดสารเทอร์พิเนน-4-ออลสูงที่สุดตามด้วยเซลลูเลส/เฮมิเซลลูเลสและเฮมิเซลลูเลส/เพคติเนสตามลำดับ ผลที่ได้แสดงให้เห็นว่าเอนไซม์ช่วยส่งเสริมการไฮโดรไลซิสของผนังเซลล์ทำให้ได้สารสกัดออกมาเพิ่มขึ้น นอกจากนี้ยังได้เตรียมแผ่นเส้นใยนาโนจากพีวีพี/ไฮดรอกซีโพรพิลไซโคเด็กตรินที่บรรจุน้ำมันไพลโดยวิธีอิเล็กโตรสปินได้สำเร็จ โดยบรรจุน้ำมันไพลปริมาณต่างๆ คือ 10%, 20% และ 30% โดยน้ำหนักต่อพอลิเมอร์ และทำการศึกษาสัณฐานภายนอก ขนาดเส้นผ่านศูนย์ของเส้นใย ภาพถ่ายจากกล้องจุลทัศน์อิเล็กตรอนแบบส่องกราดแสดงให้เห็นว่าเส้นใยที่ได้มีขนาดเส้นผ่านศูนย์กลางระดับนาโนเมตร โดยขนาดเส้นผ่านศูนย์กลางเฉลี่ย 225-486 นาโนเมตร ซึ่งลดลงเมื่อเพิ่มปริมาณของน้ำมันไพล จากการศึกษาการปลดปล่อยน้ำมันไพลพบว่ามีอัตราการปลดปล่อยเร็วในช่วงต้นชั่วโมงแรกของการศึกษาและตามด้วยอัตรการปลดปล่อยที่ช้า ที่ 24 ชั่วโมงการปลดปล่อยน้ำมันไพลประมาณ 67-79% โดยเรียงลำดับดังนี้ เส้นใยนาโนที่บรรจุน้ำมันไพล 10% >20% > 30% เส้นใยบรรจุน้ำมันไพล 10% ให้การปลดปล่อยที่สูงสุด อีกทั้งในเวลาที่ศึกษา 9 เดือนมีความคงตัวทางกายภาพมากที่สุด ในขณะที่เส้นใยบรรจุน้ำมันไพล 20% และ 30% พบความไม่คงตัวทางกายภาพ ดังนั้นแผ่นเส้นใยบรรจุน้ำมันไพล 10% เตรียมด้วยวิธีอิเล็กโตรสปินอาจเป็นทางเลือกหนึ่งที่น่าสนใจในการใช้นำส่งยาทางผิวหนัง

สาขาวิชาเทคโนโลยีเภสัชกรรม บัณฑิตวิทยาลัย มหาวิทยาลัยศิลปากร

ลายมือชื่อนักศึกษา ..... ปีการศึกษา 2554

ลายมือชื่ออาจารย์ที่ปรึกษาวิทยานิพนธ์ 1. ..... 2. .....

ACKNOWLEDGEMENTS

I would like to express my deep sincere appreciation to every people who contributed in diverse ways to the completion of this research. First of all, I would like to express my deepest gratitude to my thesis advisor, Assoc. Prof. Dr. Praneet Opanasopit for her supervision, valuable guidance, support, kindness and encouragement throughout the course of my study. She provided me good opportunities and support in various ways. My sincere gratitude also goes to my thesis co-adviser, Assoc. Prof. Dr. Theerasak Rojanarata for his helpful support, invaluable suggestion, and kindness given to me during my study. I am very much indebted to both of them.

Furthermore, I express my sincere gratitude to Assoc. Prof. Dr. Tanasait Ngawhirunpat and Assist. Prof. Dr. Warisada Sila-on for their kindness and valuable guidance in my research work.

I would like to manifest the gratefulness to every teachers and staffs in Faculty of Pharmacy, Silpakorn University for the knowledge and generous support. I also would like to pass my heartfelt thanks to all my friends and members of the Pharmaceutical Development of Green Innovation Group (PDGIG) for their scientific discussion and friendship. I am very appreciated with you all. Our friendship will certainly last long in my heart.

I would like to thank Mr. Natthan Charernsriwilaiwat and Miss Areerut Sripattanaporn for their assistant in electrospinning study. I also would like to thank Miss Sureewan Duangjit and Dr.Nanthida Wonglertnirant for their help and support.

I would like to thank Bangkok Lab and Cosmetic Co., Ltd. for providing and supporting time for my graduate study and my colleagues for their support and encouragement.

Finally, I wish to give my special thanks to my beloved family for their all along support, love, caring, blessing, understanding and encouragement. Finally, an apology is offered to those whom I cannot mention personally one by one here.

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Ø	fiber diameter
%RH	percent relative humidity
%w/v	percent weight by volume
%wt	percent weight
°C	degree Celsius
Δ*H*	enthalpy
μg	microgram(s)
μL	microliter(s)
μm	micrometer(s)
AFM	atomic force microscopy
ANOVA	analysis of variance
CDs	cyclodextrins
cm	centimeter(s)
cm^{-1}	wavenumbers
compound D	(*E*)-4-(3,4-dimethoxyphenyl)but-3-en-l-ol
Cont.	continue
COX-2	cyclooxygenase
cPs	centipoise(s)
D-acetate	(*E*)-4-(3,4-dimethoxyphenyl)but-3-en-yl acetate
DMPBD	(*E*)-1-(3,4-dimethoxyphenyl)butadiene
DMPBDMS	(*E*)-3-(3,4-dimethoxyphenyl)-4[(*E*)-3,4-dimethoxystyryl]cyclohex-1-ene
DSC	differential scanning calorimeter
e.g.	exemplī grātiā (Latin); for example
et al.	and others
etc.	et cetera (Latin); and other things/ and so forth
EtOH	ethyl alcohol
FE-SEM	field emission scanning electron microscope

LIST OF ABBREVIATIONS

FTIR	fourier transform infrared spectroscopy
G	(needle) gauge
GC	gas chromatography
GC-MS	gas chromatography-mass spectoscopy
g	gram(s)
h	hour(s)
HPβCD	2-hydroxypropyl-β-cyclodextrin
IC_{50}	Inhibition concentration at 50%
i.d.	inner diameter
i.e.	id est (Latin); that is
J	joule(s)
kV	kilovolt(s)
L	liter(s)
LOX	lipoxygenase
m	meter(s)
mg	milligram(s)
min	minute(s)
mJ	millijoule(s)
mL	milliliter(s)
mm	millimeter(s)
mM	millimolar(s)
mS	millisiemens
M.W.	molecular weight
n.d.	not defined
nm	nanometer(s)
n.pag.	no page
PVP	Polyvinylpyrrolidone
pH	potentia hydrogenii (Latin); power of hydrogen
R^2	coefficient of determination

LIST OF ABBREVIATIONS

rpm	revolutions per minute
S.D.	standard deviation
SEM	scanning electron microscope
SFE	supercritical fluid extraction
t	time
T	temperature
TEM	transmission electron microscopy
THF	tetrahydrofuran
v/v	volume by volume
XRD	X-ray diffraction

CHAPTER 1
INTRODUCTION

1. Statement and significance of the research problem

Medicinal plants are natural resources which are important in both economic and health care system in developing countries including Thailand. Nowadays the domestic and global popularity of natural product trends to increase, leading to an urgent demand for herbal product development and production. To achieve this, not only the clinical research and scientific evidences to support the activities, but also the efficient isolation method and novel and effective dosage forms should be investigated. In many Asian countries, Plai (*Zingiber Cassumunar* Roxb.) is widely used in folklore remedies as a single plant or as a component of herbal recipes for the treatment of muscle pain and sprain (Wiroon Laupattarakasem et al., 1993: 159–164), gastrointestinal distress (Supaporn Pitiporn, 2000: 56–57) and smooth muscle relaxation (Vanlapa Anantasan, 1982: 1–27). It has been reported later that active constituents in the rhizome extract has biological activities for the treatment of inflammation, sprain, muscular pain, wounds and asthma, and as a mosquito repellent, a carminative, a mild laxative and an anti-dysentery agent (Pithayanukul, P., Tubprasert, J. and Wuthi-Udomlert, M., 2007: 164–169). As a result, several products from Plai have been formulated and commercially available. To obtain the essential oil from this plant, several extraction methods have been reported and employed, mostly involving the use of organic solvents and heat. As a consequence, it generates plentiful waste in the liquid and vapours emerging to environment or needs further treatment. Steam distillation was the most useful method but requires a large amount of sample and long extraction time and may give rise to the degradation of unsaturated compounds, the loss of some volatile compounds and hydrolysis effect (Thanaporn Poonsukchareon et al, 2004: 1–105). As a large amount of sample required and long extraction time, reflex to high price of

Plai oil in the market (8–10 L of Plai oil obtained by 1 ton of fresh rhizome of Plai) owing to the limitation of use and product development of Plai oil.

Over the last few decades, a novel alternative method namely enzymatic extraction or enzyme-assisted extraction which has been proposed. It involves the breakdown of plant cell wall components such as cellulose, hemicelluloses and pectin by the use of cell wall degrading enzymes into small pieces and facilitation of the release of active constituents when conventional extraction methods are subsequently performed. By enhancing the extraction efficiency, it helps eliminate the organic solvent consumption and lowers the investment costs, energy requirements and adverse consequences on health and the environment (Rosenthal, Pyle and Niranjan, 1996: 402–403). A recently studied gave an interesting account of enzymatic pre-treatment applied to different plants, fruits and oilseeds such as grape seed, soy bean, citrus peels and palm kernel on the enhancement of extraction yield compared to those conventional extraction methods (Domínguez, Núñez and Lema, 1995: 223–225; Gaur et al., 2007: 696–699; Li, Smith and Hassian, 2006: 189–196, Passos et al., 2009: 48–53; Tano-derbrah and Ohta, 1996: 173–179).

The production of polymer filaments using electrostatic force was known for more than a 100 years. This technique became well known since 1934 when Formhals patented “method and apparatus for spinning” (Formhals, 1934: n. pag.). The work of Taylor (1969: n. pag.) on electrically driven jets has laid the groundwork for electrospinning, the hemi-spherical surface of the fluid at the tip of the capillary tube elongates to form a conical shape known as the Taylor cone (Ming Huang et al., 2003: 2223–2224). The popularity of the electrospinning process can be realized by the fact that over 200 universities and research institutes worldwide are studying various aspects of the electrospinning process and the fiber it produces and also the number of patents for applications based on electrospinning has grown in recent years (Bhardwaj and Kundu, 2010: 325–327). Electrospinning, a spinning technique, is a unique approach using electrostatic forces to produce fine fibers from polymer solutions or melts and the fiber thus produced have a thinner diameter and larger surface area than those obtained from conventional spinning processes. The electrospinning process offers a simplified technique and for fiber formation, low investment costs of spinning apparatus. The non-wovens nanofibrous mats produced

by this technique are mimics extracellular matrix components much closely as compared to the conventional techniques. The sub-micron range spun fibers produced by this process, offer various advantages like high surface area to volume ratio, tunable porosity and the ability to manipulate nanofibers composition in order to get desired properties and function. In the past years, electrospinning method has gained widespread interest as a potential polymer processing technique for application in tissue engineering scaffold, wound dressing, drug delivery carrier, biosensor/chemosensor, protective cloth, material reinforcement, air filtration, and electronic and semi-conductive materials, etc. (Sill and Recum, 2008: 1989–2006; Chronakis, 2005: 283; Huanga, 2003: 2223–2253).

From the aforementioned advantages of enzyme-assisted extraction and fiber fabrication via electrospinning method, their application to improve the extraction process of Plai oil and formulate it as a novel dosage form is of interest. However, there are no reports has been found in the literatures on the use of both techniques for Plai oil. The objectives of this study were therefore to enhance the Plai oil extraction yield by using enzyme-assisted method and to incorporate it into electrospun nanofibers for topical application.

2. Objectives of this research

2.1 To investigate the use of plant cell wall degrading enzymes for the enhancement of Plai oil extraction efficiency

2.2 To investigate the influence of type of enzymes and extraction procedures on the extraction yield

2.3 To prepare the Plai oil-loaded electrospun nanofibers and investigate their physicochemical properties intended for topical application

3. The research hypothesis

3.1 The enzyme-assisted method increases the efficiency of the extraction of Plai oil.

3.2 Factors including the type of enzyme as well as extraction method influence on the extraction yield.

3.3 Plai oil can be incorporated into electropun nanofibers for topical application.

CHAPTER 2
LITERATURE REVIEWS

1. Plai (*Zingiber cassumunar* Roxb.) and its biological activities

Plai (*Zingiber cassumunar* Roxb.) a medicinal plant widely cultivated in Thailand and tropical Asia. In Thailand, its vernacular names called in the northern part are Plai, Puu Loi, Puu Loie, whilst in the central part is Waan Fai. In many Asian countries, Plai is wildly use in folklore remedies as a single plant or as a component of herbal recipes for a treatment of condition such as treatment of muscle pain and sprain (Wiroon Laupattarakasem et al., 1993: 159–164), gastrointestinal distress (Supaporn Pitiporn, 2000: 56–57) and smooth muscle relaxation (Vanlapa Anantasan, 1982: 1–27). Ancient people used its rhizome as medicine the directly rubbed into the muscle pain area. It has been reported later that active constituents in the rhizome extraction has biological activities for the treatment of conditions such as inflammation, sprain, muscular pain, wounds and asthma, and as a mosquito repellent, a carminative, a mild laxative ad an anti-dysentery agent (Pithayanukul, P. et al., 2007: 164–169). Especially potent anti-inflammatory activity has proven to be extremely useful for human health and has thus been developed into products for various medical uses (Pithayanukul, P et al., 2007: 164–169).

1.1 Botanical data of Plai

Plant name: Plai

Family name: *Zingiberaceae*

Botanical name: *Zingiber cassumunar* Roxb.

Part use: Rhizome, Leave, Flower

Description of the plant: The plant is perennial herb, consisting of underground rhizome (Figure 1) which is bright yellow inside, and leafy stem rising

up to 80–150 cm. Leaves are simple; oblong-lanceolate (Figure 2); having apex acute; base narrowing and clasping the stem by their long shealth; membranaceous texture. Inflorescence is on a separate shoot without normal leaves (Figure 3); consisting of spike cylindric rising up from the rhizome, 20–30 cm long; flower white, each subtended by reddish green bracts, tightly closed when young; calyx-tube cylindric, shortly 3-lobed; colla-tube cylindric, segment whitish, lip yellowish white with a deeply bifid midlobe; fertile stamen 1; overy 3-loculed, many ovules, style filiform, stigma subglobose. Fruit is a globose capsule, 1–1.5 cm in diameter.

Figure 1 Underground rhizome which is bright yellow inside of Plai.

Source: **Plai, more than herb. (2011).** Accessed March 14, 2012. Available from http://eureka.bangkokbiznews.com/detail/379776

Figure 2 Leafy stem of Plai.

Source: Taweesak Suntornthanasart. (2009). **Plai, Thai herbal for muscular pain.** Accessed March 14, 2012. Available from http://blog.eduzones.com/futurecareer/30074

Figure 3 Inflorescence with white flower of Plai.

Source: **Plai, more than herb.** (2008). Accessed March 14, 2012. Available from http://www.eldercarethailand.com/content/view/519/54/

1.2 Chemical constituents of Plai

In general essential oil extraction from Plai can be classified into three groups: terpene, oxygenated derivatives and benzene derivatives. Most compounds extracted from Plai are monoterpene presented as acyclic, monocyclic or bicyclic. Terpene exhibits the least volatility among all constituents such as α-pinene, β-pinen, sabinene, α-terpinene, and γ-terpinene. Oxygenated derivatives are the second group carrying the least one oxygen atom, giving characteristic odor of essential oil. A remarkable oxygenated compound containing in the rhizome is terpinene-4-ol. The last constituent is derivatives of benzene which has utilized as flavor and fragrance (Table 1). Most medicinal compounds are also classified into this group, e.g. (*E*)-1-(3,4-dimethoxyphenyl)butadiene (or DMPBD) widely accepted as a well-known active compound. It has been reported that the anti-inflammatory active constituents in the rhizome extract DMPBD is one of the most effective compounds that has 11 times more potent than diclofenac in anti-inflamation and it inhibits cyclooxygenase-2 (COX-2) and lipoxygenase (LOX) (Rattima Jeenapongsa et al., 2003: 143–148). Other compounds such as (*E*)-4-(3,4-dimethoxyphenyl)but-3-en-l-ol (Compound D), (*E*)-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl] cyclohexene-1-ene, (*E*)-3-(4-hydroxy-3-methoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl] cyclohexene-1-ene (Han et al., 2005: 1446–1468), terpinene-4-ol, curcumin, cassumunarin A, B, and C also have anti-inflammatory activity (Masuda, Jitoe and Mabry, 1995: 1053–1057; Thanaporn Poonsukchareon, 2004: 1–27).

1.3 Anti-inflammatory activities of Plai

Among active constituents from the rhizome extraction, many constituents seemed to possess a potent anti-inflammatory activity. Pongprayoon et al. (1997: 319-322) report that the essential oil of the rhizome of *Zingiber cassumunar* was found to exhibit a topical anti-inflammatory effect, when tested using the model of carrageenan-induced hind paw edema in rats (ID50 = 22 mg oil/paw) (Table 2). Individual assessment of topical anti-inflammatory activity of the five major components of the oil demonstrated that DMPBD, terpinen-4-ol, and α-terpinene

significantly inhibited edema formation, whereas sabinene and γ-terpinene were inactive up to 6 mg/paw (Pongprayoon et al., 1997: 319–321).

Table 1 The chemical constituents of Plai (*Zingiber cassumunar* Roxb.).

Compound name	References
α-pinene, β-pinene, sabinene, mycene, α-terpinene, Limonene, γ-terpinene, ρ-cymene, terpinolene, Terpinene-4-ol, α-thujene, ocimene, β-phellandrene, *Z*)-sabinen hydrate, (2-carene, (*E*)-piperitol, borneol, Bornyl acetate, Germacrene D, γ-selinene, α-selinene, β-bisabolene, α-bergamotene, β-sesquiphellandrene, Methyl eugenol, δ-cadinene and juniper camphor	Jantan, et al., 2003: 392-397
Zerumbone	Kishore and Dwivedi, 1992: 155-159
Curcumin, Cassumunin A, B, C, Cassumunarin A, B, C	Masuda, T. and A. Jitoe., 1995: 459-461 Nagano et al., 1997: 363-370
8-(3,4-dimethoxyphenyl)-2 methoxynaphtho-1,4-quinone, 8-(2,4,5-dimethoxyphenyl)-2 methoxynaphtho-1,4-quinone, 4-(2,4,5-trimethoxyphenyl)-but-1,3-diene	Farnsworth and Bunyapraphatsara, 1992: 35-46
(*E*)-1-(3,4-dimethoxyphenyl)butadiene(DMPBD), (*E*)-4-(3,4-dimethoxyphenyl)but-3-en-l-ol(Compound D), (*E*)-4-(3,4-dimethoxyphenyl)but-3-en-yl acetate (D-acetate), (*E*)-4-(3,4-dimethoxyphenyl)but-3-en-l-*o*-β-D-glucopyranoside, (*E*)-3-(3,4-dimethoxyphenyl)but-l-ene	Jeenapongsa et al., 2003: 143-148
(*E*)-3-(3,4-dimethoxyphenyl)-4[(*E*)-3,4-dimethoxystyryl]cyclohex-1-ene (DMPDMS), (*E*)-3-(4-hydroxy-3-methylphenyl)-4-[(*E*)-3,4-dimethoxystyryl]cyclohex-1-ene, (*Z*)-3-(3,4-dimethoxyphenyl)-4[(*E*)-3,4-dimethoxystyryl]cyclohex-1-ene, (*Z*)-3-(3,4-dimethoxyphenyl)-4[(*E*)-2,4,5-dimethoxystyryl]cyclohex-1-ene, (*Z*)-3-(2,4,5-dimethoxyphenyl)-4[(*E*)-2,4,5-trimethoxystyryl]cyclohex-1-ene	Han et al., 2005

Table 2 Anti-inflammatory activity of extracts and compounds of Plai.

Extract/Compounds	IC_{50}(µg/mL)
Compound D	43.9
D-acetate	21.7
DMPBD	10.7
DMPDMS	15.1
Volatile oil	21.5

Source: Kaewchoothong, A., Tewtrakul, S. and Panichayupakaranant, P. (2009) "HPLC Quantitative determination of anti-inflammatory active compounds in Zingiber cassumuna extracts." **The 8th Joint Seminar NRCT – JSPS.** 231-232, Bangkok, Thailand, February 3-4, 2009.

1.4 Extraction of active constituents from Plai

Over the past decade, studies about the extraction of active constituents from the rhizome of Plai have attracted special attention. Conventional extraction methods of active constituents from medicinal plant involve liquid-liquid extraction, solid-solid extraction, Soxhlet extraction, solvent sonication and steam distillation. The first four methods utilize high organic solvent consumption, as a consequence, it generates plentiful waste emerging to environment or needs a further treatment. Steam distillation requires a large amount of sample and long extraction time. Furthermore, it may give rise to degradation of unsaturated compounds, the loss of some volatile compounds and hydrolysis effect. To the last few decades, a novel alternative method named supercritical fluid extraction (SFE) was employed, since the method allowed the use of highly volatile solvent, such as carbon dioxide, pentane, and etc., to replace other less favorable conventional organic solvent, leaving virtually no waste for further treatment. One drawback for SFE occurred from the properties of the extractant that is non-polar. SFE then is primarily useful technique for mostly non-polar, but less advantageous for the polar compounds. Therefore,

several disadvantages of the conventional methods, including SFE, brought about a finding in new solvent for extraction process.

2. Enzyme-assisted extraction of bioactive from plants

Over the last few decades, a novel alternative method named enzymatic extraction or enzyme-assisted extraction which is expected to break cell walls of plants such as cellulose, hemicelluloses and pectin into small pieces and ready to facilitate the flow of active constituent yield prior the extraction by conventional extraction method. The use of enzymes as a pre-treatment in extraction process appears potentially attractive compared to the conventional method since it offers many advantages. For instance, it eliminates solvent consumption with reportedly may also lower the investment costs and energy requirements (A. Rosenthal, D.L. Pyle and K.Niranjan, 1996: 402–403). A recently studied gave an interesting account of enzymatic pre-treatment applied to different plants, fruits and oilseeds such as grape seed, soy bean, citrus peels and palm kernel on the enhancement of extraction yield compared to those conventional extraction methods (Gaur, R. et al., 2007: 696–699; Domínguez, Núñez and Lema, 1995: 223–225; Li, Smith, Hassian, 2006: 189–196; Passos et al., 2009: 48–53; Tano-derbrah and Ohta, 1996: 173–179).

2.1 General composition of cell wall

The cell wall of most of the vegetable matrixes is composed of three layers differing in polysaccharide composition; the primary cell wall, the middle lamella and the secondary cell wall (Figure 4).

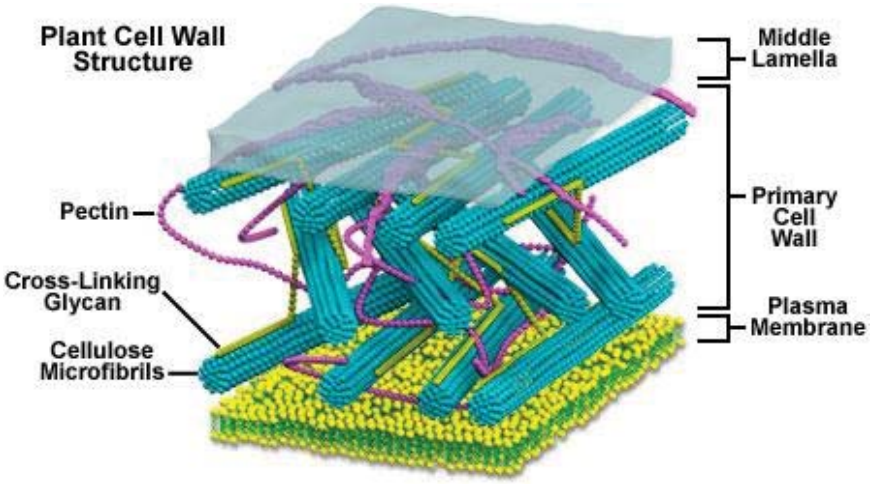

Figure 4 Plant cell wall structure.

Source: **Plant cell wall.** (2005). Accessed March 16, 2012. Available from http://micro.magnet.fsu.edu/cells/plants/cellwall.html

The primary cell wall, generally a thin, flexible and extensible layer formed while the cell is growing. The main chemical components of the primary plant cell wall include: (1) cellulose, a polysaccharide composed of 1,4-linked β-D-glucose residues (in the form of organized microfibrils; see Figure 5), a complex carbohydrate made up of several thousand glucose molecules linked end to end; (2) pectins, a family of complex polysaccharides that all contain 1,4-linked α-D-galacturonic acid. To date three classes of pectic polysaccharides have been characterized: homogalacturonans, rhamnogalacturonans, and substituted galacturonans; (3) hemicelluloses, branched polysaccharides that are structurally homologous to cellulose because they have a backbone composed of 1,4-linked β-D-hexosyl residues. The predominant hemicellulose in many primary walls is xyloglucan. Organized into a network with the cellulose microfibrils, the cross-linking glycans increase the tensile strength of the cellulose, whereas the coextensive network of pectins provides the cell wall with the ability to resist compression. In addition to these networks, a small amount of protein can be found in all plant primary cell walls. Some of this protein is thought to increase mechanical strength and part of it consists of enzymes, which initiate reactions that form, remodel, or

breakdown the structural networks of the wall. Such changes in the cell wall directed by enzymes are particularly important for fruit to ripen and leaves to fall in autumn.

The middle lamella is a layer rich in pectins. This outermost layer forms the interface between adjacent plant cells and glues them together (see Figure 4). The middle lamella is shared by neighboring cells and cements them firmly together. Positioned in such a manner, cells are able to communicate with one another and share their contents through special conduits.

The secondary cell wall (Figure 5) a thick layer formed inside the primary cell wall after the cell is fully grown. It is not found in all cell types. Some cells, such as the conducting cells in xylem, possess a secondary wall containing lignin, which strengthens and waterproofs the wall. The secondary plant cell wall, which is often deposited inside the primary cell wall as a cell matures, sometimes has a composition nearly identical to that of the earlier-developed wall. More commonly, however, additional substances, especially lignin, are found in the secondary wall. Lignin is the general name for a group of polymers of aromatic alcohols that are hard and impart considerable strength to the structure of the secondary wall. Lignin is what provides the favorable characteristics of wood to the fiber cells of woody tissues and is also common in the secondary walls of xylem vessels, which are central in providing structural support to plants. Lignin also makes plant cell walls less vulnerable to attack by fungi or bacteria, as do cutin, suberin, and other waxy materials that are sometimes found in plant cell walls.

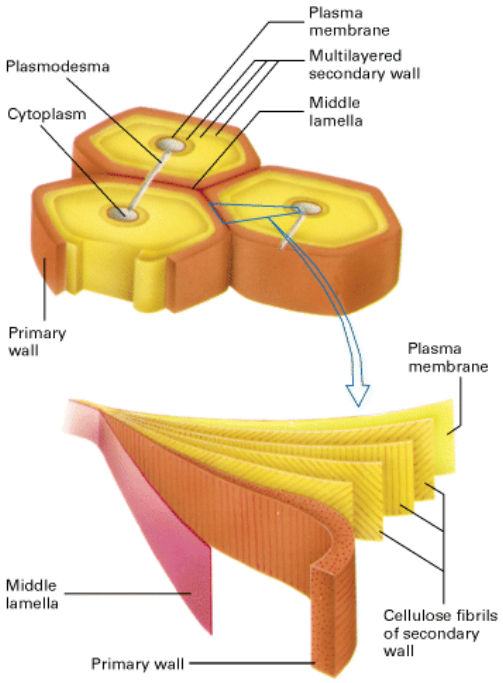

Figure 5 Secondary cell wall structure.

Source: Shakib. (2012). **General biology in context.** Accessed March 16, 2012. Available from http://shakib-biology.blogfa.com/8708.aspx

2.2 Cell wall degrading enzymes

The application of enzymes for complete extraction of bioactive from plants is an attractive proposition since the compositions of plant cell wall were studied. Cellulolytic, pectinolytic and other enzymes able to catalyst the hydrolysis of bonds in plant cell polysaccharides resulting in the decomposition of the structure of polysaccharides in the different layers of the cell wall structure.

Many researchers reported significant differences in the amount of total bioactive when cell wall degrading enzymes were used as pretreatment prior the conventional extraction. Table 3 shows the list of bioactive compounds of industrial importance obtained by enzyme-assisted extraction from plants (Puri, Sharma and Barrow, 2012: 37–44).

To date, cellulase, hemicellulase and pectinase have a wide range of potential applications in enzyme-assisted extraction of bioactive from plants. The last two decades, the use of cellulase, hemicellulase and pectinase has increased. These

enzymes account for approximately 20% of the world enzyme market (Bhat, M.K., 2000: 344-383).

2.2.1 Cellulase

Cellulase enzymes are produced primarily by fungi, bacteria, and protozoans. Cellulases are a mixture of slightly different enzymes that include endo-1,4-β-glucanase enzymes, which attack internal bonds, exo-1,4-β-glucanase enzymes, which cleave two to four units from the ends of cellulose strands and cellobiase, which cleaves the disaccharide cellobiose into two glucose moieties. Cellulase enzyme catalyzes the hydrolysis of cellulose into glucose (Figure 6 and 7).

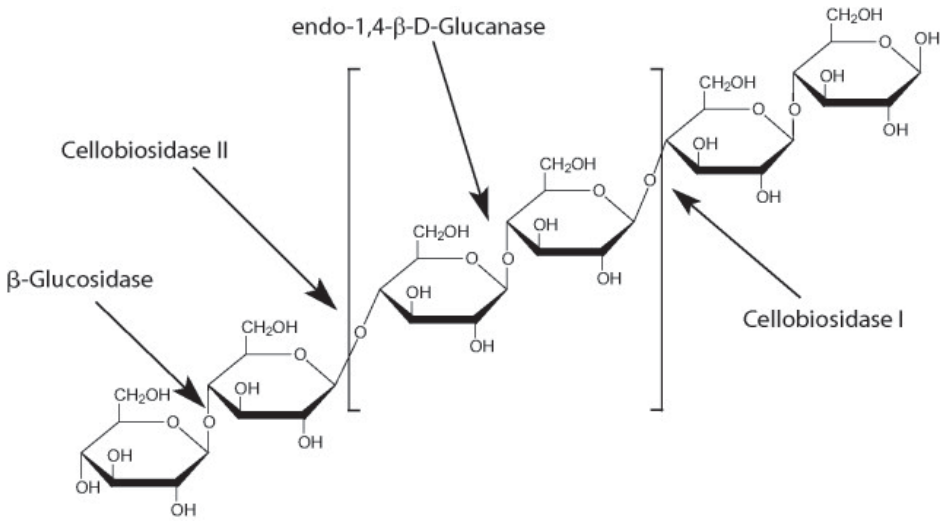

Figure 6 Structure of cellulose and its digestion to glucose.

Source: **Cellulase and related enzymatic activities.** (2000). Accessed March 20, 2012. Available from http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/analytical-enzymes/enzymes-for-aer.html

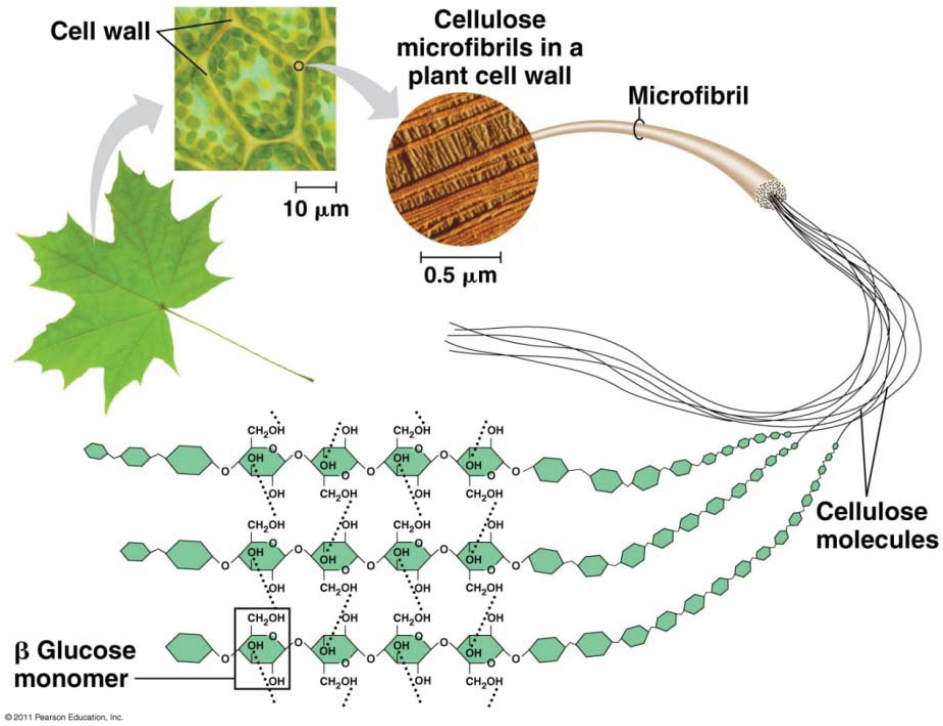

Figure 7 Hydrolysis of cellulose microfibrils of cell wall.

Source: **Biomolecule.** (2009). Accessed March 20, 2012. Available from http://www.thaigoodview.com/node/21065?page=0%2C4

Table 3 List of bioactive compounds of industrial importance obtained by enzyme-assisted extraction from plants.

Product type	Product	Source	Enzyme used	Maximum yield (%)
Oil and carotenoids	Oil	Grape seed	Cellulase, protease, xylase and pectinase	17.5
	Carotenoids	Marigold flower	Viscozyme, Pectinex, neutrase, corolase and HT-proteolytic	97
	Volatile oil	Mandarin peel	Xylan-degrading enzyme	15
	Carotene	Carrot pomace	Pectinex Ultra SP-L	0.0064
	Lycopene	Tomato	Pancreatin	2.5 fold
		Tomato	Cellulase and pectinase	206
	Capsaicin	Chilli	Cellulase, hemicellulase and pectinase	n.d.[a]
	Colourant	Pitaya	Pectinolytic, hemicellulolytic and cellulolytic enzymes	83.5
	Anthocyanin	Grape skin	Pectinex BE3-L	n.d.[a]
Glycosides	Sugar	Grape fruit peel waste	Cellulase and pectinase	0.6377
	Oligosaccharide	Rice bran	Cellulase	39.9
	Inulin	Jerusalem artichoke	Inulinase	n.d.[a]
	Strach	Cassava	Pectinase enzyme	45.6

Product type	Product	Source	Enzyme used	Maximum yield (%)
Glycosides (Cont.)	Pectin	Pumpkin	Xylase, cellulase, β-glucosidase, endopoly-galacturonase and pectinesterase	14.0
Others	Vanillin	Vanilla green pods	β-glucosidase and pectinase	14-21
	Flavonoid (naringin)	Kinnow peel	Recombinant rhamnosidase	n.d.[a]
	Phenolics	Citrus peel	Celluzyme MX	65.5
	Proteins	Lentils and white beans	Glucoamylase	50.3
	Polyphenols	Grape pomace	Pectinolytic	98.1
	Catechins	Tea beverage	Pepsin	80
	Lignans	Flax	Cellulase and glycosidase	40.75 mg/g
	Soluble fibre	Carrot pomace	Cellulase-rich crude preparation	77.3

[a]Abbreviation: n.d., not defined.

Source: Puri, Munish, Deepika Sharma and Colin J. Barrow. (2012). "Enzyme-assisted extraction of bioactives from plants." **Trends Biotechnol** 30, 1: 37-44.

2.2.2 Hemicellulase

Hemicellulase is a group of enzymes as a mixture of glycolytic enzymes usually containing xylanase (Figure 8), mananase and other activities that degrade the plant cell wall hemicellulose. Hemicellulase enzymes are produced primarily by fungi.

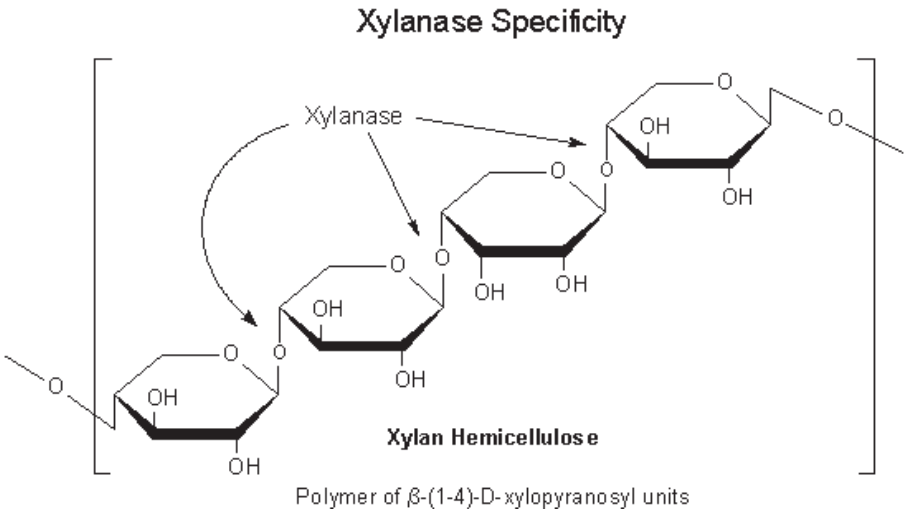

Figure 8 Structure of xylan hemicellulose and its digestion.

Source: **Hemicellulose and xylane.** (2000). Accessed March 20, 2012. Available from http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/learning-center/carbohydrate-analysis/carbohydrate-analysis-ii.html

2.2.3 Pectinase

Pectinase enzymes are produced primarily by fungi, bacteria, and protozoans. Pectinase catalyzes the random hydrolysis of 1,4-α-D-galactosiduronic linkages in pectin and other galacturonans (Figure 9).

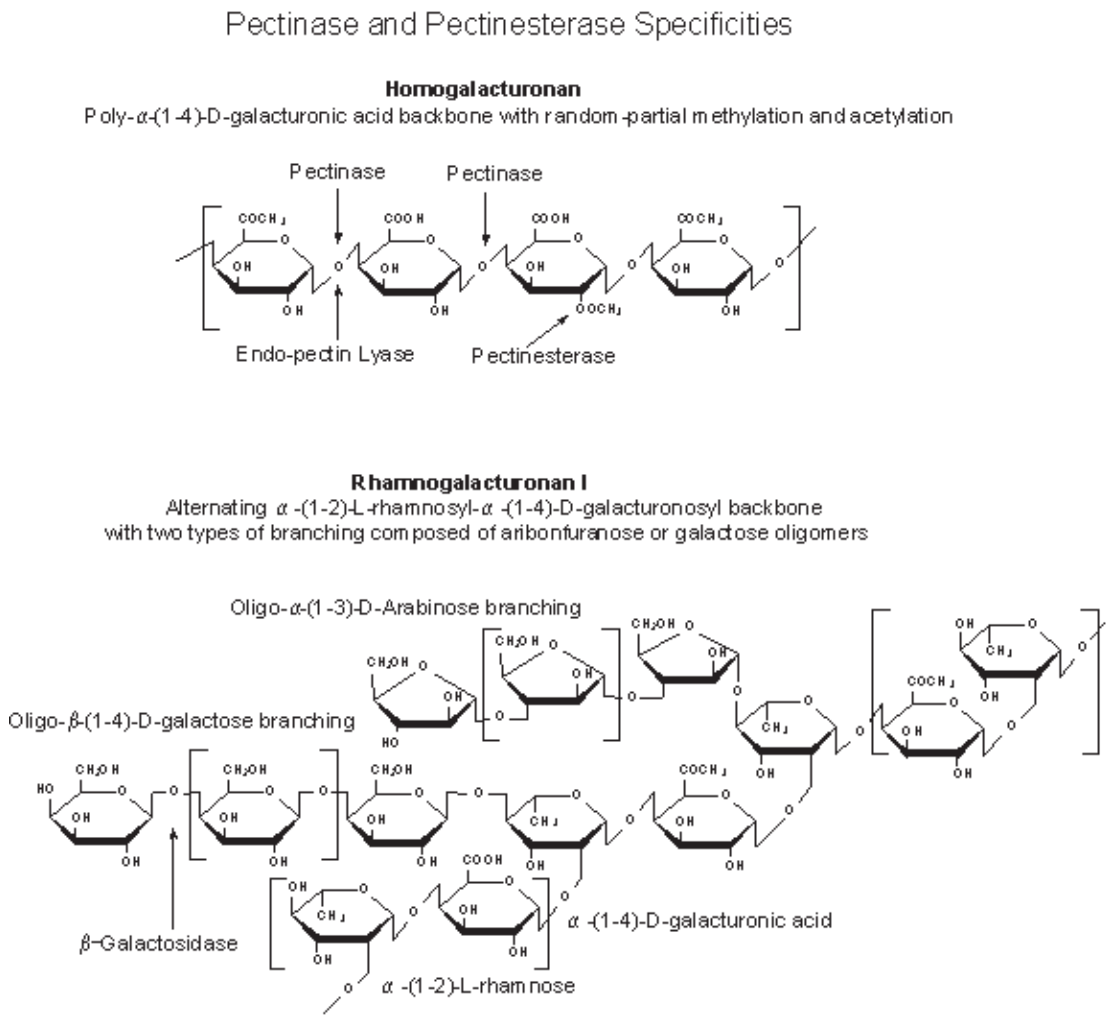

Figure 9 Structure of Pectinase and its digestion.

Source: **Pectinase and pectin.** (2000). Accessed March 20, 2012. Available from http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/learning-center/carbohydrate-analysis/carbohydrate-analysis-iii.html

2.3 Factor effecting the efficiency of the enzyme-assisted extraction of bioactive from plants

There are several factors conditioning directly the effect of the enzymes on the degradation of the cell walls and release of the bioactive compounds can be modified by manipulation and finding the optimal conditions. The time-temperature regime of the enzymatic treatment is possibly one of the most important; to perform

the enzymatic treatment at optimum conditions of temperature and also pH will result in the maximal activity of enzyme. Different enzyme treatments with mixed enzyme preparations have also been show to affect differently the efficiency of the extraction bioactive from plants. The type of extraction solvent employed as well as the enzyme/plant ratio is one of the most studied variables (Pinelo et al., 2008: 432-440).

Passos et al. (2009: 48-53) measured the effect of reaction time, temperature, pH, particle size and enzyme concentration on the enzyme activities. The following set of parameters was optimized to enhanced the yield of grapes seed oil as: time = 24 h, pH 4, temperature 30-40ºC, particle diameter 1.0-1.4 mm and cocktail combination of: cellulase = 29, protease = 1191, xylanase = 21 and pectinase = 569 unit/g of seed sample. The extraction yield was 13.7% which represents an increment 106% over non-treated sample. For 120 h the yield achieved was 17.5%, and increment reach 163% indicated that a prolonged enzymatic treatment may certainly be used to enhance oil extraction (Passos et al., 2009: 48-53).

Waliszewski et al. (2007: 1267-1273) reported the effect of pH and temperature of enzymatic pretreatment condition of vanillin beans extraction by three cellulolytic enzymes: Crystalzyme PML-MX, Zymafilt L-300 and Novozym 342. They found that the optimum condition for cellulose rich food products hydrolysis were pH 3.5 and T = 50ºC for Crystalzyme PML-MX, pH 5 and T = 50ºC for Novozym 342 and pH 6.5 and T = 50ºC for Zymafilt. The result exhibited the influent of pH and temperature on the extraction yield. Furthermore, the used of enzyme combination (Viscozyme L and Celluclast) can enhanced the extraction yield of vanillin extraction (Waliszewski et al., 2007: 1267-1273).

3. Application of electrospun fiber in drug delivery system

The production of polymer filaments using electrostatic force was known for more than a 100 years. This technique became well known since 1934 when Formhals patented "method and apparatus for spinning" (Formhals, 1939: n. pag.). The work of Taylor (1969: n. pag.) on electrically driven jets has laid the groundwork for electrospinning, the hemi-spherical surface of the fluid at the tip of the capillary tube elongates to form a conical shape known as the Taylor cone (Ming Huang et al.,

2003: 2223–2224). The popularity of the electrospinning process can be realized by the fact that over 200 universities and research institutes worldwide are studying various aspects of the electrospinning process and the fiber it produces and also the number of patents for applications based on electrospinning has grown in recent years (Bhardwaj and Kundu, 2010: 325–327). Electrospinning, a spinning technique, is a unique approach using electrostatic forces to produce fine fibers from polymer solutions or melts and the fiber thus produced have a thinner diameter and larger surface area than those obtained from conventional spinning processes. The electrospinning process offers a simplified technique and for fiber formation, low investment costs of spinning apparatus. The non-wovens nanofibrous mats produced by this technique are mimics extracellular matrix components much closely as compared to the conventional techniques. The sub-micron range spun fibers produced by this process, offer various advantages like high surface area to volume ratio, tunable porosity and the ability to manipulate nanofibers composition in order to get desired properties and function. In the past years, electrospinning method has gained widespread interest as a potential polymer processing technique for application in tissue engineering scaffold, wound dressing, drug delivery carrier, biosensor/chemosensor, protective cloth, material reinforcement, air filtration, and electronic and semi-conductive materials, etc. (Sill and Recum 2008:1989-2006; Chronakis 2005: 283; Huanga 2003: 2223–2253).

3.1 Electrospinning process

A typical electrospinning set up (Figure 10) consists of a capillary through which the liquid to be electrospun is forced, a high voltage source with positive or negative polarity, which injects charge into the liquid; and a grounded collector. A syringe pump, gravitational forces, or pressurized gas are typically used to force the liquid through a small-diameter capillary forming a pendent drop at the trip. An electrode from high voltage source is then immersed in the liquid or can be directly attached to the capillary if a metal needle is used. The voltage source is then turned on and charge is injected into the polymer solution. Increasing the electric field strength causes the repulsive interactions between like charges in the liquid and the

attractive forces between the oppositely charged liquid and collector to begin to exert tensile forced on the liquid, elongating the pendant drop at the trip of the capillary. As the electric field strength is increased further a point will be reached at which the electrostatic forces balance out the surface tension of the liquid leading to the development of the Taylor cone. If the applied voltage is increased beyond this point a fiber jet will be ejected from the apex of cone and be accelerated toward the grounded collector.

While the fiber jet is accelerated through the atmosphere toward the collector it undergoes a chaotic bending instability thereby increasing the transit time and the path length to the collector and aiding in the fiber thinning and solvent evaporation process. The solid polymer fibers are then deposited onto a grounded collector (Sill et al., 2008: 1989-2006).

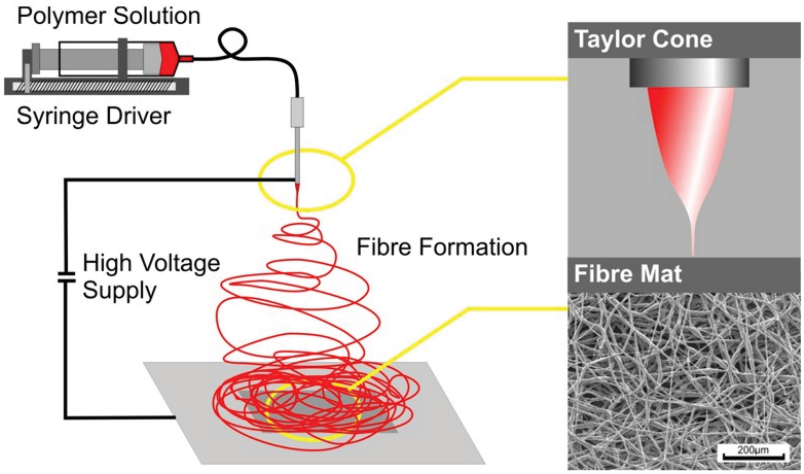

Figure 10 Electrospinning set up.

Source: Stevens, Shaffer and George. (2006). **Nanofibrous material for tissue engineering: Investigating the cellular response to multiwalled carbon nanotubes.** Accessed March 19, 2012. Available from http://www.centropede.com/UKSB2006/ePoster/background.html

3.2 Effect of various parameters on electrospinning

In order to carry out electrospining, polymer must first be in a liquid form, the properties of polymer solution as well as the process parameter play a significant role in electrospinning process and the resultant fiber morphology. Many researchers studied the effect of those parameters in fiber morphology (Table 4). While a number of general relationships between processing parameters and fiber morphology can be draw, it is important to realize that the exact relationship will differ for each polymer/solvent system. Depending on a number of solution parameters very different results can be obtained using the same polymer and electrospinning set up. Thus, it is difficult to give quantitative relationships that can be applied across a broad range of polymer/solvent system. This being said, there are general trends which are useful when determining the optimum conditions for certain system.

Table 4 Effect of electrospinning parameters on fiber morphology.

Parameter	Effect on fiber morphology
Applied voltage ↑	Fiber diameter ↓ initially, then ↑ (not monotonic)
Flow rate ↑	Fiber diameter ↑ (beaded morphologies occur if the flow rate is too high)
Distance between capillary and collector ↑	Fiber diameter ↓ (beaded morphologies occur if the distance between the capillary and collector is too short)
Polymer concentration (viscosity) ↑	Fiber diameter ↑ (with optimum range)
Solution conductivity ↑	Fiber diameter ↓ (broad diameter distribution)
Solvent volatility ↑	Fiber exhibit microtexture (pores on their surfaces, which increase surface area)

Source: Sill, Travis J. and Horst A. von Recum. (2008). "Electrospinning: Applications in drug delivery and tissue engineering." **Biomaterials** 29, 13: 1989-2006.

3.3 Polymer used in electrospinning

There are a wide range of polymers that used in electrospinning and able to form fine nanofibers within the submicron range and used for varied applications. Various synthetic polymers, natural polymers and a blend of both including protein, nucleic acids and polysaccharides have been used in the process. More than 200 polymers have been electrospun successfully, over the years (Bhardwaj and Kundu, 2010: 325-347). Various polymers used in electrospinning with their characterization method and applications have been listed in Table 5.

Table 5 Polymers used in electrospinning process.

No.	Polymer	Solvent	Conc.	Applications
1	Polyurethanes	Diethyl formamide	10% wt.	Protective clothing
2.	Polycarboate	Dichloromethane	10% wt.	Sensor, filter
3.	Polyvinyl alcohol	Distilled water	8-16% wt.	Protective clothing
4.	Polylactic acid	Dichloromethane	14% wt.	Drug delivery
5.	Collagen-Polyethylene oxide	Hydrochloric acid	1-2% wt.	Wound healing, tissue engineering
6.	Silk-like polymer with fibronectin functionality	Formic acid	0.8-16.2% wt.	Implantable device
7.	Polystyrene	Tetrahydrofuran	15% wt.	Catalyst, filter
8.	Cellulose acetate	Acetone	12.5-20% wt.	Membrane
9.	Polyvinylpyrrolidone	Tetrahydrofuran	20% wt.	Antimicrobial agent
10.	Polycaporactone	Toluene:Methanol (1:1)		Scaffold for tissue engineering
11.	Polyethylene glycol	Chloroform	0.5-30% wt.	Biomedical

Source: Zheng-Ming Huang. et al. (2003). "A review on polymer nanofibers by electrospinning and their applications in nanocomposites." **Composites Science and Technology** 63, 15: 2223-2253.

3.3.1 Polyvinylpyrrolidone

Polyvinylpyrrolidone (PVP) (Figure 11) also commonly called Polyvidone or Povidone, is a water-soluble polymer made from the monomer *N*-vinylpyrrolidone. PVP is commonly used various pharmaceutical applications. When dry it is a light flaky powder, which readily absorbs up to 40% of its weight in atmospheric water. In solution, it has excellent wetting properties and readily forms films. This makes it good as a coating or an additive to coatings. PVP is a branched polymer, meaning its structure is more complicated than linear polymer, though it too lies in a two-dimensional plane. In the recent year, PVP have been successfully electrospinning. Various concentrations and type of solvent were studied. Jie Bai (2008: 251-254) have been successfully prepared PVP/β-CD composite nanofibers by using EtOH/water (6/4) as solvent. And PVP also successfully used to stabilize silver chloride nanoparticles in electrospinning nanofibers (Jie Bai 2008: 4520-4523).

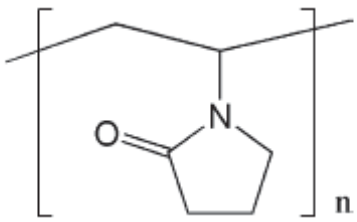

Figure 11 Chemical structure of PVP.

Source: Jie Bai et al. (2008). "Synthesis of poly(N-vinylpyrrolidone)/β-cyclodextrin composite nanofibers using electrospinning techniques." **Journal of Materials Processing Technology** 208: 251-254.

3.4 HPβCD used in electrospinning process

Cyclodextrins (CDs) are non-toxic cyclic oligosaccharides constituted by six (α-cyclodextrin), seven (β-cyclodextrin) and eight (γ-cyclodextrin) glycopyranose unit linked by α-(1,4) bonds. Cyclodextrin structure provides a hydrophilic outer surface and hydrophobic interior hallow. The inner part of cyclodextrin molecules is made apolar by glycosidic oxygens and methane protons, while external surface is polar by presence of secondary and primary hydroxyls at the edge of the ring (Figure 12). 2-hydroxypropyl-β-cyclodextrin (HPβCD) is a hydroxyalkyl derivative, prepared by reactive β-CD with propylene oxide in alkaline aqueous solutions. HPβCD can increase the aqueous solubility compared with α-, β- and γ-CD due to hydrogen bonds was replaced by hydroxyl group. As a result, CDs are able to form inclusion complexes with large number of hydrophobic compounds and these complexes have been successfully used to improve chemical stability, solubility and bioavailability of many compound (Huahua Wu et al., 2010: 613-617, Szjtli, J., 1998: 1743-1753).

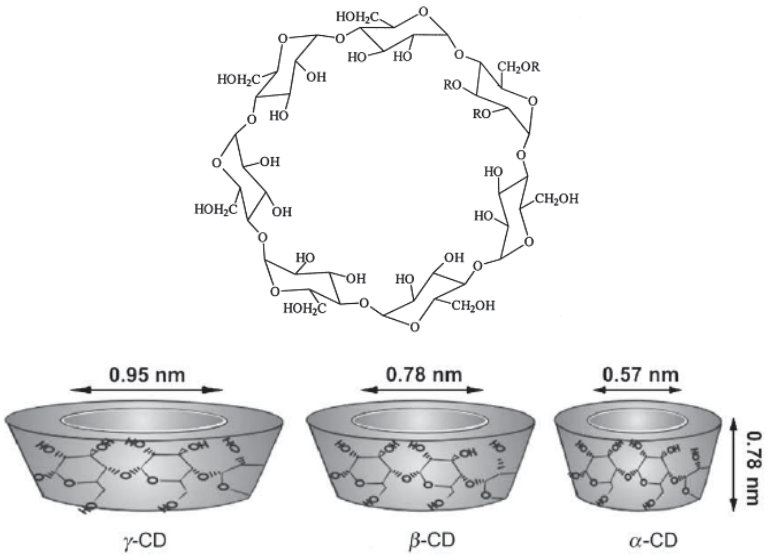

Figure 12 Chemical structure of HPβCD and approximate dimension of α-, β-, and γ-CD.

Source: Jie Bai et al. (2008). "Synthesis of poly(N-vinylpyrrolidone)/β-cyclodextrin composite nanofibers using electrospinning techniques." **Journal of Materials Processing Technology** 208: 251-254.

3.5 Characterization of electrospun nanofibers.

In order to empirically understand electrospinning process, assessment of the entire process from polymer selection to mechanical testing needs to be carried out accurately. Generally in electrospinning, the basic properties of nanofibers are (1) morphology, (2) molecular structure and (3) chemical properties.

3.5.1 Morphology

The morphology of electrospun nanofibers can be characterized by scanning electron microscope (SEM), field emission SEM (FE-SEM), transmission electron microscope (TEM) and atomic force microscopy (AFM) (Bhardwaj and Kundu, 2010: 325-347; Bai, J., 2008: 251–254). Geometric properties of nanofibers included fiber diameter, diameter distribution, fiber orientation and fiber morphology.

3.5.2 Molecular structure

Amongst conventional research works, a few papers discussed the crystalline structure and organic group detection of electrospun nanofibers from the viewpoint of size effect, processing parameters and solution properties. When polymer shrink from solution to electrospun nanofibers, a question arises on how the crystalline structure is created in nanofibers and how different are they in comparison with the crystalline structure of bulk polymers. Another concern is how processing parameters influence the crystalline structure of the nanofibers. The crystalline structure of electrospun nanofiber has been investigated by many researchers using X-ray Diffraction (XRD), Differential Scanning Calorimeter (DSC) and Transmission Electron Microscope (TEM) (Uyar, T. et al., 2009: 475–480).

3.5.3 Mechanical properties

Electrospun polymer nanofibers have several possible applications such as filtration, tissue scaffolds, protective clothing, gas sensors etc. In order to meet the long-life durability in those applications, mechanical properties of nanofibrous membrane as well as a single nanofibers have to be discussed (Ramakrishna et al., 2005: 234-246). Mechanical characterization is achieved by applying tensile test loads. A variety of approaches has been applied towards mechanical characterization of nanofibers and nanowires by employing

nanoidentation, bending tests, resonance frequency measurements and microscale tension tests. A commercial nano tensile testing system (Nano Bionix System, MTS, TN, USA) is being used to conduct the tensile test for the evaluation of mechanical properties of single ultrafine polymeric fibers of polycaprolactone (PCL) (Bhardwaj and Kundu, 2010: 325-347).

CHAPTER 3
MATERIALS AND METHODS

1. Materials

- 2-hydroxypropyl-β-cyclodextrin (HPβCD, Fluka, Sigma-Aldrich Chemie, Germany)
- Cellulase, 1.4 unit/mg solid (Sigma-Aldrich Chemie, Germany)
- Citric acid (Carlo Erba Reagenti, Italy)
- Ethyl alcohol (EtOH; Scharlau Chemie S.A., Spain)
- Hemicellulase, 1.5 unit/mg solid (Sigma-Aldrich Chemie, Germany)
- Hexane (Labscan Ltd., Ireland)
- Pectinase, 1.32 unit/mg solid (Fluka BioChemica, Switzerland)
- Plai oil (Thai China flavours & Fragances industry Co., Ltd., Thailand)
- Polyvinylpyrrolidone (PVP), M.W. ~1,300,000 by LS, Aldrich chemistry, Sigma-Aldrich, Spain)
- Sodium hydroxide (P. C. Drug center Co., Ltd., Thailand)
- Standard terpinen-4-ol (Fluka analytical, Sigma-Aldrich, Spain)

2. Equipments

- Analytical balance (Satorius CP224S; Scientific promotion Co., Ltd., Germany)
- Centrifuge (Microfuge® 16 centifuge, Beckman coulter, Inc., Germany)
- Conductivity meter (ECTestr 11^+ conductivity meter, Eutech instrument, Malaysia)
- Climate chamber (Binder KBF720, Binder Inc., USA)
- Differential Scanning Colorimeters (Pyris diamond DSC, Perkin Elmer Inc., USA)
- Distillation apparatus

- Fourier Transform Infrared Spectroscopy (FTIR, Shimadzu IR solution 1.30,
  Shimadzu Corporation, Japan)
- Gas chromatography (GC-MS model 6890N-5973, Agilent Technologies Inc., USA)
- Gas chromatography column: HP-5 capillary column 30 m x 0.32 mm x 0.25 μm (5% phenyl-methylpolysiloxane) (Agilent Technologies Inc., USA)
- High voltage supply (Gamma high voltage, Ormond Beach, Finland)
- Magnetic stirrer and magnetic bar (Hotplate stirrer, Diahan Latech Co., Ltd., Korea)
- Measuring pipette (5 and 10 mL)
- Micropipette 20–100 μL, 100–1000 μL, 1–5 mL (Biohit®; Gibthai Co., Ltd.)
- Micropipette tip
- Orbital shaker (model WY-200, Bio-active Co., Ltd., Spain)
- pH meter (Sartorius Professional meter PP-15, Germany)
- Rotary evaporator (Buchi rotavapor R-205, Buchi Laboratory Equipment, USA)
- Scanning Electron Microscope (SEM, JEOL, JSM-6400, Japan)
- Separating funnel
- Shaker (Intelli-Mixer RM-2L, ELMI Ltd., Latvia)
- Sonication bath (Transsonic cleaner T890/H, Elma GmbH & Co KG, Germany)
- Soxhlet extraction apparatus
- Speed control motor (Oriental motor Co., Ltd., Japan)
- Vacuum pump
- Viscometer (Brookfield DV-III programmable viscometer, Brookfield Engineering Lab, USA)
- Vortex mixer (VX100, Labnet International Inc., USA)

3. Methods

3.1 Sample preparation

Fresh rhizomes of Plai were purchased from the local market in Kampangsan district, Nakhon Pathom province. They were washed with water to remove soil and other contaminants, then chopped into small pieces and mixed to homogeneity and stored in a refrigerator.

3.2 Extraction method

3.2.1 Conventional method

3.2.1.1 Soxhlet extraction

Soxhlet extraction was performed in a simple Soxhlet apparatus using 30 g of chopped rhizome as starting sample. The organic solvent used was 400 mL of hexaxe. After heating in hexane at the boiling point of 69°C for 3 h, the extractant was collected and further evaporated to remove the solvent. Then, 2 mL of EtOH was added to dissolve the residue.

3.2.1.2 Water distillation

Water distillation was performed on a simple distillation laboratory apparatus. Ten grams of sample was added into 100 mL of round bottom flask filled with 40 mL water. After the water was boiled, the stream passed through a condenser and condensed into the receiving flask. The volume of collected distillate was 40 mL and it was further extracted individually with 4 mL of hexane for 3 times. The organic layer was collected and adjusted to volume of 25 mL with hexane.

3.2.1.3 Solvent extraction in combined with ultrasonication

Ten grams of sample was weighed into a glass bottle and extracted with 20 mL of hexane (or EtOH). The glass bottle was placed in a sonicating bath at 25°C for 30 min. Then the extract was separated by vacuum filtration through 0.45 µm membrane filter. Finally, the volume of organic layer was adjusted to 25 mL with hexane (or EtOH) and filtered through 0.10 µm membrane filter prior to analysis of terpinen-4-ol with GC.

3.2.2 Enzyme-assisted extraction method

3.2.2.1 The optimization of pH, temperature and time of enzyme treatment

In this study, to access the effect of commercial plant cell wall degrading enzymes (Table 6) on the enhancement of Plai oil extraction, experiments were carried out using the same extraction conditions prior the extraction by the hexane ultrasonication method. The enzyme-assisted extraction conditions were as following; extraction time 24 h, extraction temperature 40±0.5ºC, speed of rotation 100 rpm and control pH at 5.5.

Table 6 Commercial enzymes used in the experiment.

Name	Source	Specified activity (units/mg)	Optimum pH	Optimum temperature	Company
Cellulase	*Aspergillus niger*	1.40	5–6	37–40	Sigma-Aldrich Chemie
Hemicellulase	*Aspergillus niger*	1.50	4–6	30 to 75	Sigma-Aldrich Chemie
Pectinase	*Aspergillus niger*	1.32	3.5–6	40–55	Fluka BioChemica

3.2.2.2 Enzyme concentration

Ten grams of sample was weighed into glass bottle then cellulase enzyme solutions prepared in 95.65 mM citrate buffer pH 5.5 was added in the weight ratio of enzyme to sample of 3:100, 6:100 and 9:100, respectively. The sample was incubated in an orbital shaker at 40±0.5°C, 100 rpm for 24 h as described in 3.2.2.1 and the reaction was stopped by removing enzyme solutions from the sample by vacuum filtration through 0.45 μm membrane filtered. The filtrate were

kept as fraction 1 and then the digested sample was further extracted with 20 mL EtOH for 20 min and separated by vacuum filtration through 0.45 μm membrane filtered and kept as fraction 2. Finally, the sample was extracted with hexane in combined with ultrasonication as the method described in 3.2.1.3. The final part was kept as fraction 3. The quantity of terpinen-4-ol was analyzed by gas chromatography (GC). The schematic of extraction was shown in figure 13.

3.2.2.3 Types of enzyme

Ten grams of sample were weighed into glass bottle then enzyme solution was added as a single enzyme, double enzyme and triple enzyme mixtures at the enzyme:sample weight ratio of 3:100, 3:3:100 and 3:3:3:100, respectively. The formula of each treatment was shown in table 7. Then the sample was incubated and extracted as described in 3.2.2.1.

Table 7 Formulation of the enzyme mixture.

Formula	**Weight ratio of enzyme to 100 parts of sample**		
	Cellulase	Hemicellulase	Pectinase
T1	3	–	–
T2	6	–	–
T3	9	–	–
T4	–	3	–
T5	–	–	3
T6	3	3	–
T7	3	–	3
T8	–	3	3
T9	3	3	3

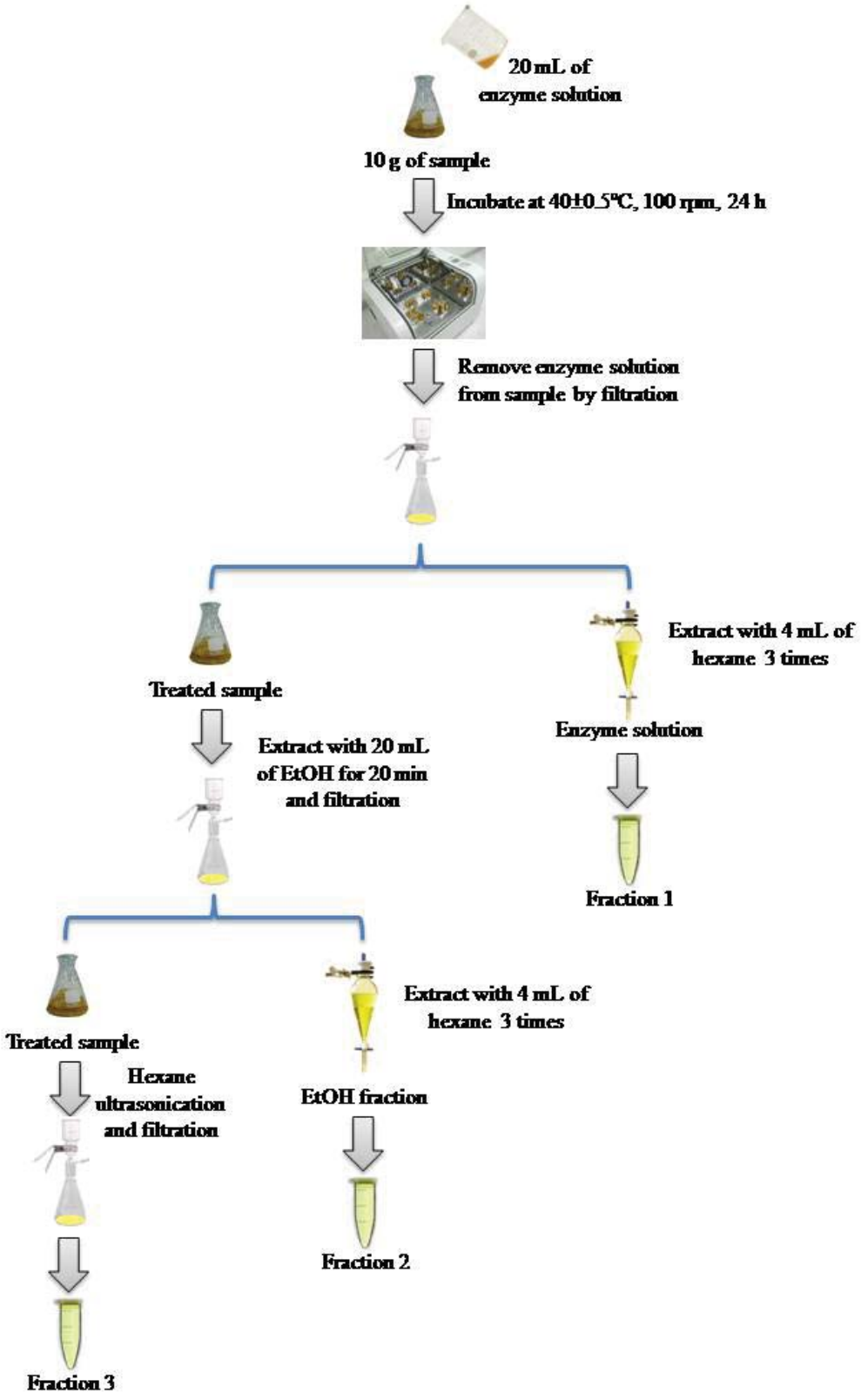

Figure 13 Schematic drawing of the enzyme-assisted extraction method.

3.3 Preparation of Plai oil loaded electrospun nanofibers

3.3.1 PVP electrospun nanofibers

1.6 g of PVP (as a fixed concentration of 8% w/v) was added in 20 mL of 70:30 (v/v) EtOH:H_2O solution and stirred for 24 h at room temperature. Then Plai oil (0%, 5%, 10% and 20% by weight of Plai oil to polymer) was added and stirred by magnetic stirrer for 12 h until homogenous solution was obtained. Then electrospun fibers were prepared by the following procedures. Firstly, the PVP solution was filled in a 5-mL glass syringe with a blunt-ended aluminium needle (Number 20G) as a nozzle. The distance from a tip to aluminium collector was 15 cm. The feeding rate of the solution was kept constant at 0.3 mL h^{-1} by means of a Kd Scientific syringe pump. The sample solution was electrospun at a positive voltage of 15 kV from high voltage supply (Gamma high voltage, Ormond Beach, FL) onto aluminium foil on a drum which acted as a ground electrode. All of electrospinning procedures were carried out at 25°C. The schematic drawing of the electrospinning apparatus is illustrated in Figure 14.

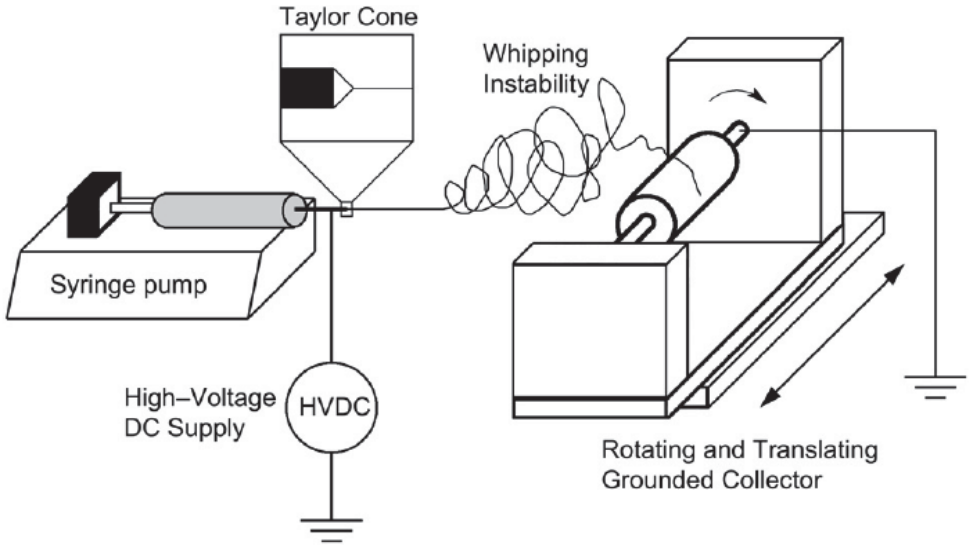

Figure 14 Schematic drawing of the electrospinning apparatus.

Source: Sill, Travis J. and Horst A. von Recum. (2008). "Electrospinning: Applications in drug delivery and tissue engineering." **Biomaterials** 29, 13: 1989-2006.

3.3.2 PVP/HPβCD electrospun nanofibers

3.3.2.1 Equilibrium time of HPβCD

27.6 mg of HPβCD was dissolved in 20 mL of 70:30 (v/v) EtOH:H_2O solution and stirred for 30 min to obtained 1 mM of HPβCD. Then excess amount of Plai oil was added into solution and stirred by magnetic stirrer. The experiment was carried out at 25ºC. At time interval of 0, 12, 24, 36, 48 and 72 h, 0.5 mL of solution were withdrawn and determined the quantity of terpinen-4-ol.

3.3.2.2 The loading of plai oil in HPβCD

According to Plai oil consists of various constituents, ratio of of HPβCD:Plai oil was applied from the continuous variation Job's method (Job, 1928: n.pag.). 10, 20 and 30 mM of HPβCD was prepared in 3 mL of 70:30 (v/v) EtOH:H_2O solution and stirred for 30 min. Then excess amount of Plai oil (0.05 g) was added into solution and stirred for 24 h at room temperature. Then, 0.5 mL of solution were withdrawn and determined the quantity of terpinen-4-ol.

3.3.2.3 HPβCD functionalized PVP nanofibers

10, 30, 50, 70, 90 and 110 mM of HPβCD was prepared in 20 mL of 70:30 (v/v) EtOH:H_2O solution and stirred for 30 min. Then 1.6 g of PVP (as a fixed concentration of 8% w/v) was added into solution and stirred for 24 h at room temperature. Then PVP/HPβCD electrospun fibers were prepared by the procedures described in 3.3.1.

3.3.2.4 PVP/HPβCD electrospun nanofibers

70 mM of HPβCD was prepared in 20 mL of 70:30 (v/v) EtOH:H_2O solution and stirred for 30 min. Then Plai oil (0%, 10%, 20% and 30% by weight of Plai oil to polymer) was added into solution and mixed for 12 h until homogenous solution was obtained. 1.6 g of PVP (as a fixed concentration of 8% w/v) was added into solution and stirred for 24 h at room temperature. Before electrospinning, viscosity and conductivity of all solutions were measured by using a Brookfield DV-III programmable viscometer and a ECtestr11+ conductivity, at 25°C,

respectively. Then PVP/HPβCD electrospun fibers were prepared by the procedures described in 3.3.1.

3.4 Characterization of Plai oil loaded electrospun nanofibers

3.4.1 Morphology and diameters (SEM)

The morphological appearance and size of the electrospun fibers were determined by using scanning electron microscope (SEM). Electrospun fibers were cutting and then coating with a thin layer of gold using a JEOL JFC-1100E sputtering device. Diameter and thickness of fibers were directly measured from selected SEM images using a jMicroVision software.

3.4.2 Chemical characterization (FTIR)

The FTIR spectra of all samples were obtained by a Shimadzu IR solution 1-30 FTIR spectrophotometer (Shimadzu Corporation, Japan). The spectral values of the samples were obtained by scanning from 4000 to 750 cm^{-1} at a resolution of 4 cm^{-1}. FTIR spectral parameters of the samples were obtained using a IR Solution 1.3 (Shimadzu Corporation, Japan).

3.4.3 Thermal analysis (DSC)

The thermal behavior of electrospun fibers was evaluated by DSC under an atmosphere of nitrogen. Approximately 10-20 mg of samples were hermetically sealed into an aluminium pan. The samples were heated over the temperature range of 50-250 $^{\circ}C$ at a heating rate 10 $^{\circ}C\ min^{-1}$.

3.5 Entrapment efficiency

Electrospun fibers were approximately weighed (0.1 g) into glass bottle. 0.5 mL of EtOH was added and mixed by using shaker (Intelli mixer Rm-22, Rose Scientific Ltd.) for 24 h at 25°C. After that sample was taken into microcentrifuge tube and extracted with 1 mL hexane by vortex mixing for 5 min and then centrifuged at 120,000xg for 30 min. The upper layer sample was used for quantitative analysis by GC using terpinen-4-ol as a marker. The percentage entrapment efficiency and loading capacity are calculated by using equation (1) and (2), respectively.

$$\% \text{ Entrapment efficiency} = (P_t / L_t) \text{ X } 100 \quad (1)$$

where P_t is the amount of Plai oil embedded in nanofibers, and L_t is the theoretical amount of Plai oil (Obtained from feeding condition) incorporated into nanofibers.

$$\text{Loading capacity} = P_t \text{ (mg)} / M_t \text{ (g)} \quad (2)$$

where P_t is the amount of Plai oil embedded in nanofibers, and M_t is the total amount of nanofibers.

3.6 *In vitro* release

In vitro release studies of Plai oil loaded electrospun fibers were performed by weighing the fibers (0.1 g) on the pettri dish and put into a climate chamber (Binder KBF720, Binder Inc) to control temperature and humidity at 32°C and 75%RH, respectively. To determine the released amount of Plai oil, after a given time (0, 1, 2, 4 and 24 h), the fiber sample was taken and analyzed the quantity of remained Plai oil by gas chromatography (GC).

3.7 Stability study

Plai oil loaded electrospun fibers were kept at in room temperature for certain period of time. After 3, 6 and 9 months storage, electrospun fibers were characterized by SEM in order to determine the morphology and diameter.

3.8 Analysis of terpinen-4-ol

Gas chromatography (GC) with flame ionization detector was used for quantitative analysis. The separation was performed on a 30 m x 0.32 mm i.d. x 0.25 µm film thickness fused silica capillary HP-5 column (Agilent Technologies Inc.). One microliter of each extract was injected into the column. The oven temperature was programmed at 55°C for 2 min and then heated up at 4°C/min to 150°C followed by 10°C/min to 200°C for 17.47 min. A completion time of one chromatogram was 32.83 minute per injection. The temperature of injector and detector was kept

constant at 250°C, respectively. Helium gas was used as a carrier gas with a flow rate of 1.4 mL/min. Identification and quantitative analysis of extraction was carried out by comparison with library spectra (Wiley7n) and standard curve of terpinen-4-ol (Figure 15).

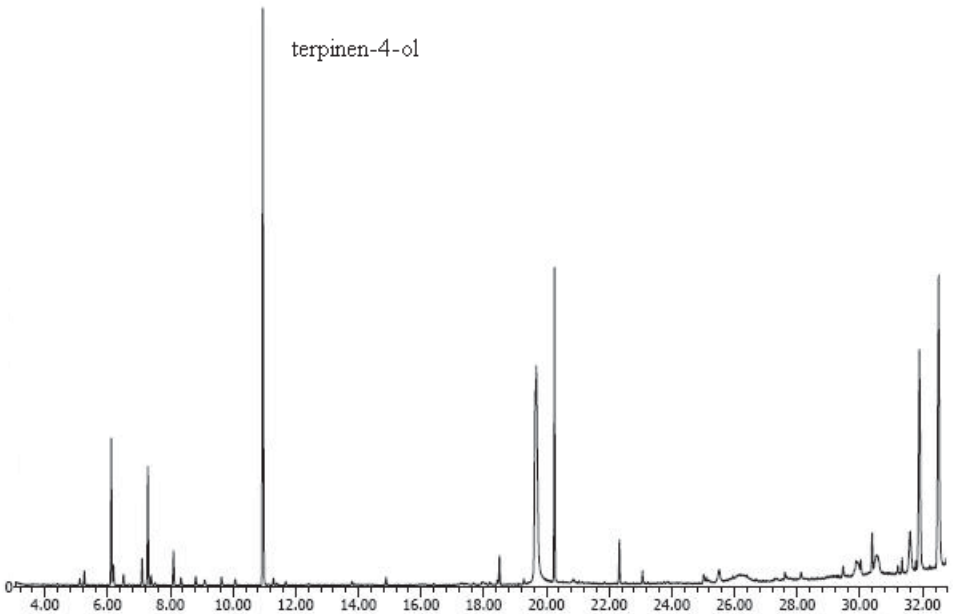

Figure 15 Chromatogram of active constituents from Plai oil.

3.8 Statistical analysis

All experimental measurements were performed in triplicate. Result values were expressed as mean value ± standard deviation (S.D.). Statistical significance of differences were examined using one-way analysis of variance (ANOVA) followed by LSD post hoc test. The significance level was set at $p < 0.05$.

CHAPTER 4
RESULTS AND DISCUSSION

1. Optimization of extraction method

The influences of various parameters on the extraction yield of terpinen-4-ol from the rhizomes of Plai were studied. These parameters included the different extraction methods, enzyme pretreatment, and the concentration and type of enzymes added. The chopped rhizome was prepared as previously described and used in all experiments.

The typical conventional extraction methods i.e. soxhlet extraction, water distillation and solvent extraction combined with ultrasonication were compared interms of the potential extraction of terpinen-4-ol from the rhizomes of Plai. As shown in Table 8 and Figure 16, the extraction yield of terpinen-4-ol obtained from the different extraction methods was in the increasing order of hexane ultrasonication (0.96 ± 0.12 μg/g), Soxhlet extraction (0.81 ± 0.55 μg/g), alcohol ultrasonication (0.72 ± 0.01 μg/g) and water distillation (0.70 ± 0.14 μg/g). However, no significant difference on the terpinen-4-ol extraction yield were observed. From the results, the hexane ultrasonication method showed the greatest extraction yield of terpinen-4-ol due to its lowest polarity (2.0) compared to those of EtOH (24) and water (80). Thus, this method was selected for the further study of enzyme pretreatment method.

Table 8 Extraction yield of terpinen-4-ol from conventional extraction methods.

Extraction method	Terpinen-4-ol (μg/g)
Soxhlet extraction	0.81 ± 0.55
Water distillation	0.70 ± 0.14
Hexane ultrasonication	0.96 ± 0.12
Alcohol ultrasonication	0.72 ± 0.01

Mean $\pm$ S.D. from 3 measurements.

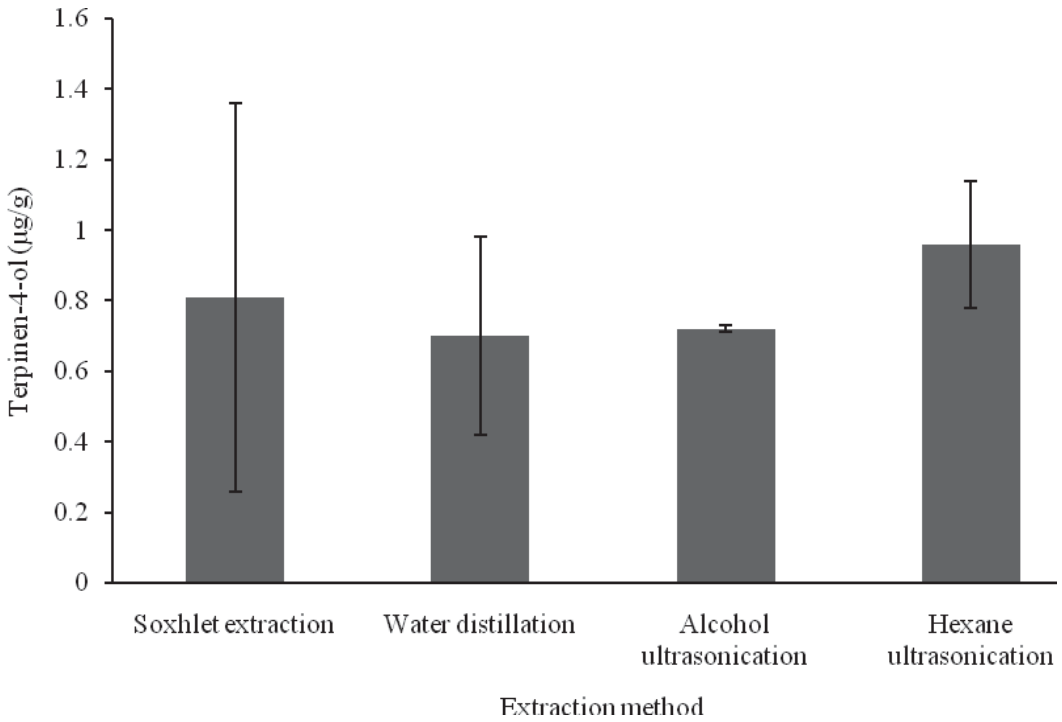

Figure 16 Effect of extraction method on the extraction yield of terpinen-4-ol. Average values are shown with standard deviation bars based on the triplicate measurements.

2. Enzyme-assisted extraction method

2.1 The optimization pH, temperature and time of enzyme pretreatment

In this study, to access the effect of commercial plant cell wall degrading enzymes (Table 6) on the enhancement of Plai oil extraction, experiments were carried out using the same extraction conditions prior the extraction by the hexane ultrasonication method. The enzyme-assisted extraction conditions were as following; extraction time 24 h, extraction temperature 40 $\pm$ 0.5ºC, speed of rotation 100 rpm and control pH 5.5 which is the optimum condition for all three commercial enzymes. Table 9 shows the extraction yield of terpinen-4-ol from the hexane ultrasonication method alone and following a cellulase pretreatment. It was observed that the amount of terpinen-4-ol extracted with cellulase pretreatment was 59.4%

(1.59 times) greater than that with the hexane ultrasonication alone. Cellulase is said to has a activity in degrading cell wall polysaccharides (Puri et al., 2012: 37–44). Therefore, the treatment with cellulase would lead to loss of integrity and disintegration of the cell wall, thus, improving the solvent access to terpinen-4-ol in the rhizomes of Plai. In other studies, the enzymes pretreatment of plant materials prior to conventional extraction methods were also shown an increase the extraction yields of oil from soya beans (Domínguez, Núñez, and Lema, 1995: 223–231), phenolic contents from citrus peels (Li, Smith, and Hossain, 2006: 189–196), vanillin from vanilla beans (Waliszewski, Ovando, and Pardio, 2007: 1267–1273), and garlic volatile oil from garlic (Sowbhagya et al., 2009: 1234–1238). These results suggest the enzyme-assisted extraction method as a promising tool to improve the extraction efficiency.

Table 9 Extraction yield of terpinen-4-ol from cellulase pretreatment and hexane ultrasonication.

Treatment	Weight ratio of cellulase:sample	Terpinen-4-ol (μg/g)
Hexane ultrasonication	–	0.96 $\pm$ 0.12
Cellulase	3:100	1.53 $\pm$ 0.26

Mean $\pm$ S.D. from 3 measurements.

2.2 Enzyme concentration

From the previous section, cellulase gave the superior result to those obtained using other enzymes acting separately as well as their combinations. Therefore, cellulase was chosen for the following study of their optimal extraction concentration. Three levels of cellulase concentration were examined in this research as shown in Table 10. Other extraction conditions i.e. pH, temperature, incubation time, and agitation rate were constant fixed to be constant for all cellulase concentrations. The results of the effects of varied concentrations of cellulase on the terpinen-4-ol extraction yield are shown in Figure 16. It was observed that the use of cellulase at all concentrations resulted in an increase of terpinen-4-ol content. A significant difference was observed with the use of cellulase at the weight ratio of

enzyme:sample of 3:100 and 6:100 compared to the control ($p < 0.05$). However, the statistical analysis of the extraction yields showed no significant differences among the three enzyme concentrations. The highest extraction yield with cellulase was at the weight ratio of cellulase:sample of 3:100, with a terpinen-4-ol yield of 1.53 $\pm$ 0.26 µg/g. This extraction yield was 1.6 times greater than the terpinen-4-ol yield from the control experiment. Further increase in terpinen-4-ol yields were not observed with the higher cellulase levels. Similar behavior was also observed for the extraction of stevioside from *Stevia rebaudiana* leaves (Puri et al., 2012: 37–44). These results may be attributed to the fact that cellulolytic enzymes tend to be inhibited by soluble products of cellulolysis. When the cells are disrupted, the materials released from the cells, such as sugars, are known to form a complex with enzymes, thus resulting in product inhibition (Puri et al., 2012: 37–44). These results revealed that the optimal concentration of cellulase used in the extraction system of terpinen-4-ol from the rhizomes of Plai was the weight ratio of cellulase:sample at 3:100.

Table 10 Extraction yield of terpinen-4-ol from different concentrations of cellulase pretreatment extraction.

Treatment	Weight ratio of cellulase:sample	Terpinen-4-ol (µg/g)
Control	–	0.96 $\pm$ 0.12
T1	3:100	1.53 $\pm$ 0.26*
T2	6:100	1.25 $\pm$ 0.26*
T3	9:100	1.13 $\pm$ 0.25

Mean $\pm$ S.D. from 3 measurements.

* $p < 0.05$ compared with control.

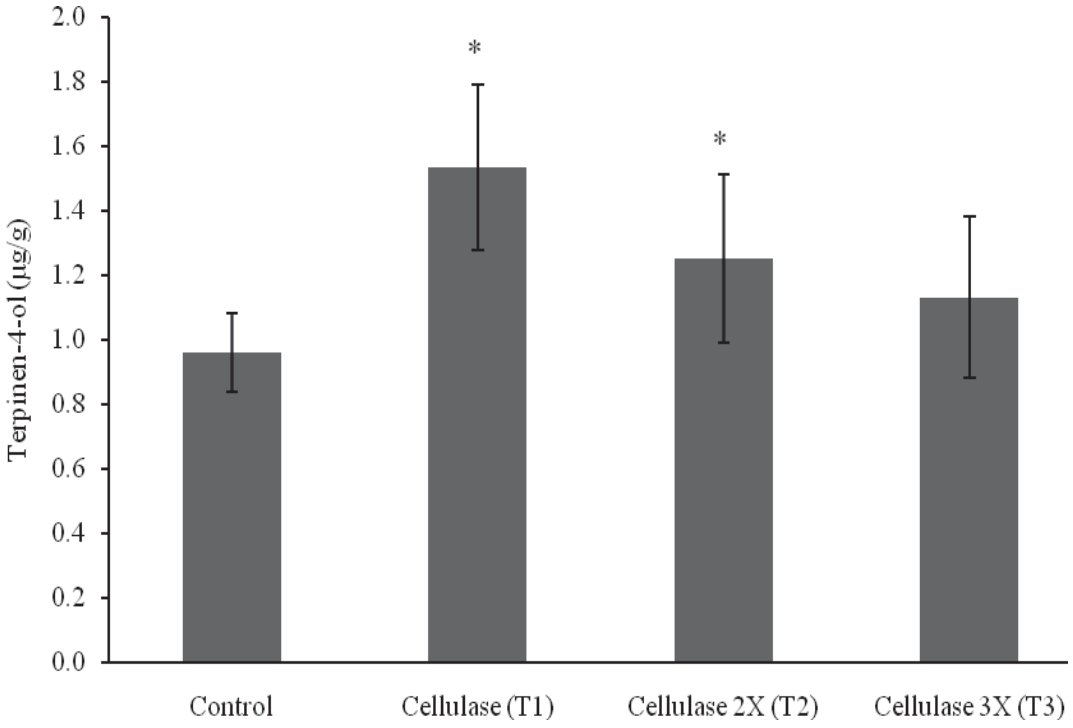

Figure 17 Effect of cellulase concentration on the extraction yield of terpinen-4-ol. Average values are shown with standard deviation bars based on the triplicate measurements.

2.3 Type of enzymes

Different enzymes may have different capabilities in extracting the bioactive compounds from plant materials because of their characteristic nature, (Rosenthal, Pyle, and Niranjan, 1996: 402–420; Sowbhagya et al., 2009: 1234–1238; Zúñiga et al., 2003: 51–57). In this work, three enzymes i.e. cellulase, hemicellulase, and pectinase were examined for their capability in extracting terpinen-4-ol from the rhizomes of Plai. The enzymes were used either as an individual or as a combination. Other extraction conditions remained the same for all three enzymes (as previously described in Section 3.2.2 of Chapter 3). The weight ratio of enzyme:sample used and the obtained extraction yield were expressed in Table 11. The hexane ultrasonication method was served as a control. From Figure 18, it was clearly shown that all the tested enzymes enhanced the extraction yield of terpinen-4-ol by 9.4% to 59.4% (1.09 to 1.59 times), comparing with the hexane ultrasonication method

without enzyme pretreatment (control group). A significant increase in terpinen-4-ol extractability was observed with the use of cellulase (T1), hemicellulase (T4), pectinase (T5), and a mixture of cellulase and pectinase (T7) in the comparison with the control ($p < 0.05$). Cellulase (T1) showed the greatest effect on the extraction yield while the mixture of hemicellulase and pectinase (T8) exhibited the least effect. These results implied that enzymes such as cellulases, hemicellulases, and pectinases can be used to improve the yield of terpinen-4-ol extraction from the rhizomes of Plai by promoting the hydrolysis of cell walls and facilitating the bioactive release from the plant material.

Table 11 Extraction yield of terpinen-4-ol from different enzymes.

Treatment	Weight ratio of enzyme:sample			Terpinen-4-ol (μg/g)
	Cellulase	Hemicellulase	Pectinase	
Control	–	–	–	0.96 ± 0.12
T1	3:100	–	–	1.53 ± 0.26*,**
T4	–	3:100	–	1.21 ± 0.18*
T5	–	–	3:100	1.22 ± 0.20*
T6	3:100	3:100	–	1.12 ± 0.17
T7	3:100	–	3:100	1.34 ± 0.27*,**
T8	–	3:100	3:100	1.05 ± 0.12
T9	3:100	3:100	3:100	1.16 ± 0.16

Mean ± S.D. from 3 measurements.

* $p < 0.05$ compared with control.

** $p < 0.05$ compared among the enzyme pretreatment groups.

A significant enhancement in the extraction yield of terpinen-4-ol among the enzyme pretreatment groups was observed with the treatment of cellulase (T1) and cellulase/pectinase combination (T7) ($p < 0.05$). Since there are several parameters conditioning directly the effect of the enzymes on the degradation of cell walls, and the release of targeted compounds can be modified by manipulation and finding the optimal conditions. Thus, these results may be explained by the extraction conditions

used in this study that might be the most suitable for the cellulase activity. Moreover, the differences in the targeted plant composition also determine, the choice of the enzymes to be used for each plant material.

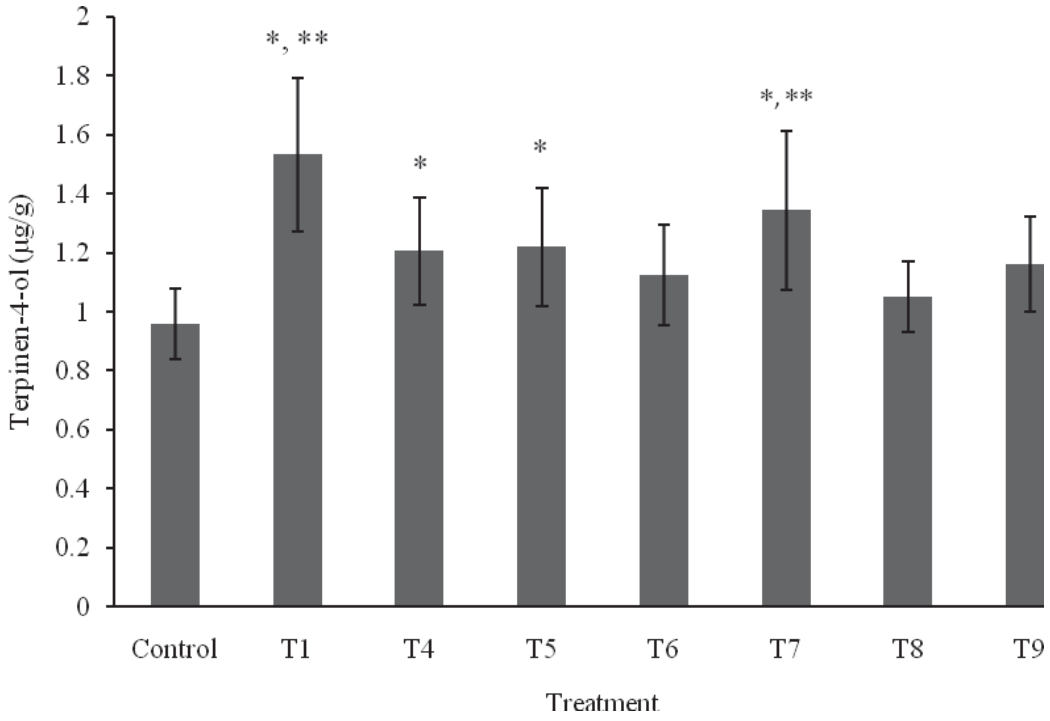

Figure 18 Effect of different enzymes on the extraction yield of terpinen-4-ol. Average values are shown with standard deviation bars based on 3 replicate measurements. The data were compared using one-way ANOVA followed by LSD post hoc test and differences were considered significant at $p < 0.05$. Symbols: *, comparing with the control; ** comparing among the enzyme pretreatment groups.

3. Preparation of Plai oil loaded electrospun nanofibers

3.1 PVP electrospun nanofibers

During the electrospinning process, the electrifield jet (solution of PVP and Plai oil) under a high electrostatic field was continuously stretched and splitted into multiple branches due to the complicated action such as bending instability and

high frequency of whipping. With the solvent evaporation, the PVP were solidified and became filaments. The sample solutions were successfully electrospun at the set condition. A white sheet of non-woven fibers were observed by visual inspection. However the morphology of the PVP nanofibers trend to have instability. Figure 19 shows the SEM images of 0%, 5%, 10% and 20% of Plai oil in PVP nanofibers. It was observed that pure PVP electrospun fibers fuse fused together under the room temperature.

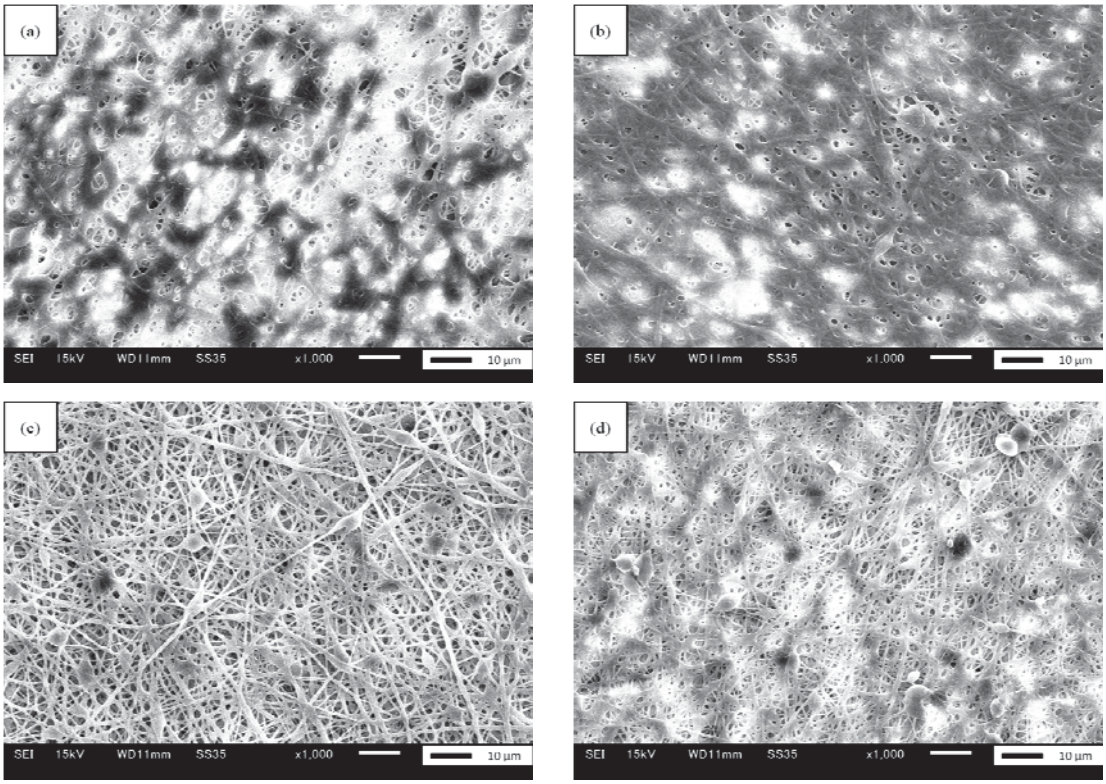

Figure 19 SEM photographs (1,000x) of PVP electrospun fibers with (a) 0%, (b) 5%, (c) 10%, and (d) 20% Plai oil loaded.

3.2 PVP/HPβCD electrospun nanofibers

3.2.1 Equilibrium time of HPβCD

The excess amount of Plai oil was added into solution of 1 mM of HPβCD. At the interval (0, 12, 24, 36, 48 and 72 h) 0.5 mL of solution were withdraw and determine the quantity of terpinen-4-ol. After 24 h, the quantity of

dissolved terpinen-4-ol reached the plateau and slightly increased at 48 and 72 h (0.41 and 0.46 μg/g, respectively), however no significant difference of dissolved terpinen-4-ol was observed. The complex of Plai oil within the cyclodextrin is a dynamic equilibrium reached for 24 h, as shown in Figure 20.

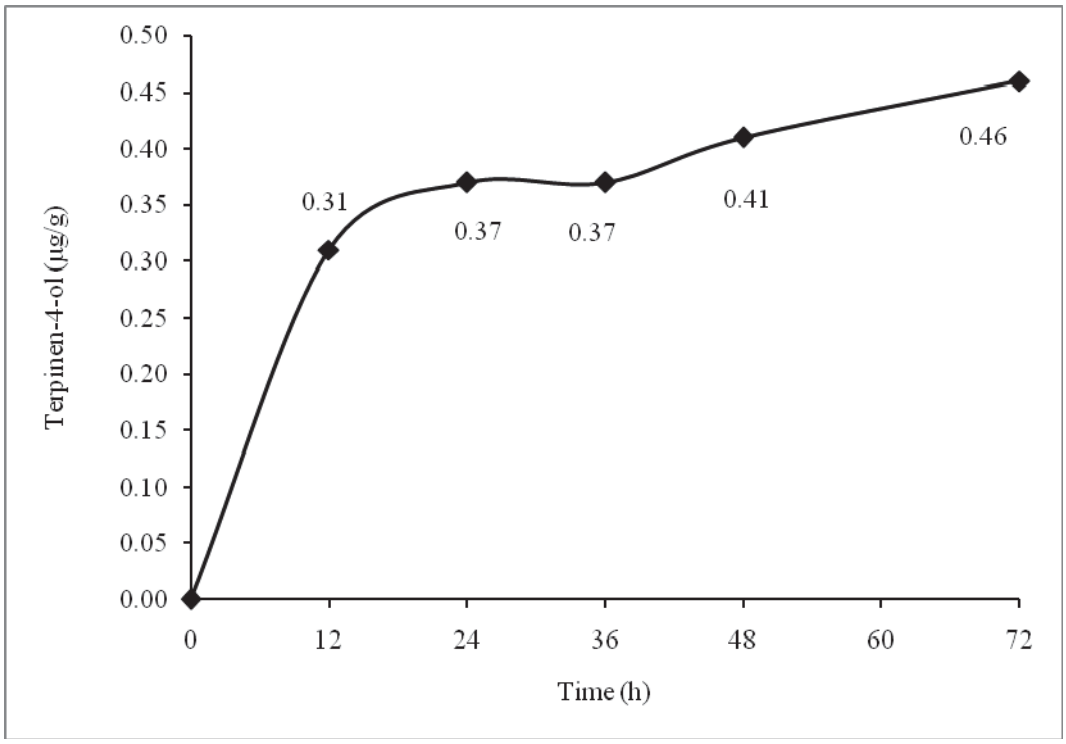

Figure 20 Quantitative amount of terpinen-4-ol in 1 mM HPβCD solution at 0, 12, 24, 36, 48, and 72 h.

3.2.2 The loading of plai oil in HPβCD

The loading of plai oil in HPβCD was determined by adding an excess amount of Plai oil (0.05 g) into 0, 10, 20 and 30 mM HPβCD solution. After stirring for 24 h, solutions were taken and determined the quantity of terpinen-4-ol. The maximum concentration of terpinen-4-ol was observed at 10 mM HPβCD (Figure 21).

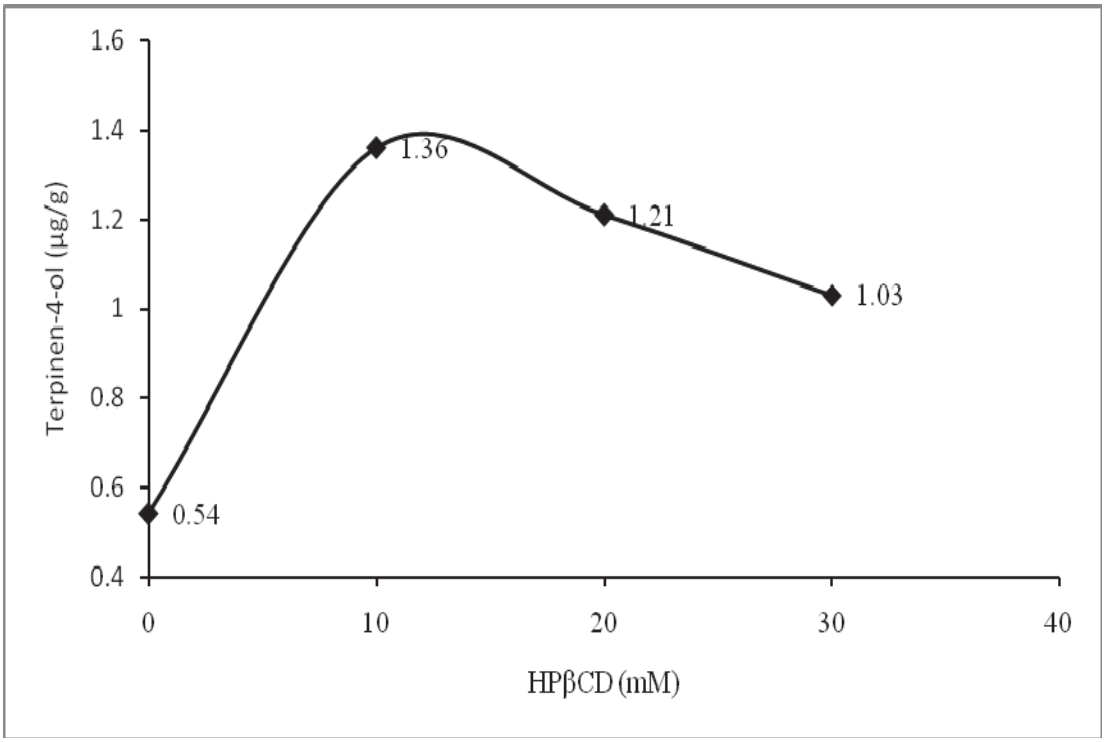

Figure 21 Quantitative amount of terpinen-4-ol in 0, 10, 20, and 30 mM HPβCD solution after stirring for 24 h.

3.2.3 HPβCD functionalized PVP nanofiber

Difference concentrations of HPβCD were used to develop stable and uniform electrospun PVP/HPβCD nanofibers. 10, 30, 50, 70, 90 and 110 mM of HPβCD were dissolved in the PVP solution and electrospinning process was performed at the same condition. By visual inspection, a white sheet of non-woven fibers were observed. Figure 22 shows SEM images of PVP/HPβCD electrospun fibers. HPβCD at 10 mM (Figure 22a) showed the same result as previous observed in PVP electrospun fibers. The solution was unable to form the stable fibers for 30 and 110 mM HPβCD (Figure 22b and f). The beads formation was observed. This might be resulted from the high viscosity of this solution. Meanwhile 50, 70 and 90 mM were successfully electropun at the set condition. Figure 22c, d and e, 50, 70 and 90 mM HPβC showed the desirable morphology with the uniform strings ~~and few beads were observed~~. 70 mM HPβCD gave the uniform strings with the smaller range fibers diameter, thus 70 mM HPβCD were selected for the further study.

It was found that before addition of HPβC, the PVP electrospun nanofibers were instable and melted after keeping for 1 week. Therefore HPβC was incorporated into polymer nanofibers. Nowadays, the preparation of composite nanofibers has become a hotspot study in many fields. Some functional materials were incorporated into polymer nanofibers, and the result composites had many excellent chemical and physical properties (Bai et al., 2008: 251-254). The HPβC is considered as one of the supermolecules pioneers which has been widely applied in electrospinning process (Bai et al., 2008: 205-208, Bai et al., 2008: 251-254, Uyar et al., 2009: 475-480). Figure 22c, d and e, 50, 70 and 90 mM HPβC showed desired morphology; the uniform strings and few beads were observed. And 70 mM HPβC gave the uniform strings with smaller range fibers diameter, thus 70 mM HPβC were used in further study.

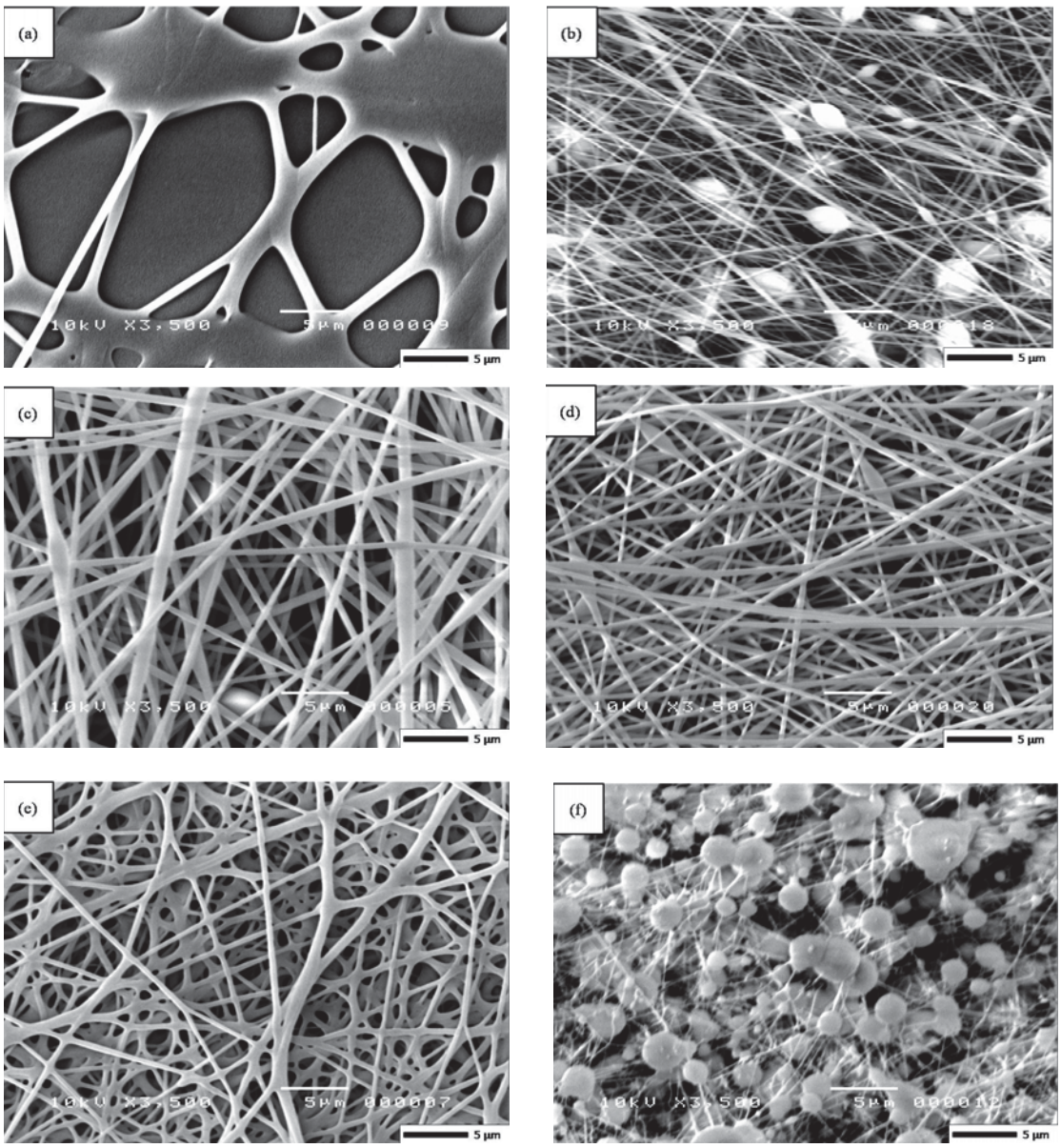

Figure 22 SEM images (3,500 x) of PVP electrospun fibers with (a) 10 mM, (b) 30 mM, (c) 50 mM, (d) 70 mM, (e) 90 mM, and (f) 110 mM of HPβCD.

3.2.4 PVP/HPβCD eletrospun nanofibers

For prepared PVP/HPβCD eletrospun nanofibers, 70 mM HPβCD with Plai oil (0%, 10%, 20% and 30% to polymer) was added into 8 % PVP solution. Since there has been reported that both of the viscosity and the conductivity of the electrospun solution influenced the morphology of the electrospun nanofibers and electrospinability. Therefore, prior to electrospinning process, the polymer solutions

were measured for their electrical conductivity and viscosity. The results were summarized in Table 12. The presence of Plai oil in the polymer solution decreased the viscosity of the PVP solutions, in which the higher the level of Plai oil concentration incorporated the lower the viscosity of the resulting solutions. The viscosity of the solutions affected the morphology and diameter of the electrospun fibers. The viscosity of the solution decreased, the formation of beads in fibers and the diameter of the nanofiber mats decreased (Charernsriwilaiwat et al., 2010: 675–680). The conductivities of the PVP solutions containing Plai oil slightly decreased when the Plai oil content was increased from 10 to 30 %wt of PVP. Therefore, the decrease in the diameter of the nanofibers was mainly due to the viscosity of the electrospun solution. When the viscosity of a solution increased, the diameter of the nanofibers decreased. The characteristics of electrospun fibers were shown in Table 13. Electrospinning process was performed at a fixed electrical field of 15 kV, distance from the tip to collector of 15 cm, and a constant feeding rate at 0.3 mLh^{-1}.

Table 12 The electrospun solutions and their characteristics.

Electrospun solution	PVP (%w/v)	HPβCD (mM)	Plai oil loaded (%wt of PVP)	Viscosity (cPs)	Conductivity (mS/cm)
0% Plai oil	8	70	–	254.27 $\pm$ 0.46	6.30 $\pm$ 0.10
10% Plai oil	8	70	10	254.53 $\pm$ 2.68	6.07 $\pm$ 0.25
20% Plai oil	8	70	20	234.38 $\pm$ 1.66	5.97 $\pm$ 0.15
30% Plai oil	8	70	30	239.71 $\pm$ 8.29	5.37 $\pm$ 0.06

Mean $\pm$ S.D. from 3 measurements.

4. Characterization of Plai oil loaded electrospun nanofibers

4.1 Morphology and diameter (SEM)

The morphology and diameter of electrospinning fibers were investigated by SEM. Table 13 and Figure 23 show the characteristics and the SEM images of neat and Plai oil-loaded electrospun fibers. It was found that the neat PVP/HPβCD fibers were straight and uniform with a few beads formation. The average diameter

was about 0.561 ± 0.094 μm, while those of the Plai oil-loaded electrospun fibers were ranged between 0.225 and 0.486 μm (Table 13). The presence of Plai oil caused a decrease in the average fiber diameter. SEM images clearly show that the incorporation of Plai oil affected the morphology and diameter of PVP/HPβCD electrospun fibers (Figure 23). The increasing the Plai oil concentration, the more beads formation were found. This could be attributed to the reduction of shear viscosity of spinning solutions when the Plai oil was added. In many previously studies, there have been shown that within an optimal range of the polymer concentrations the fiber diameter increases with increasing the polymer concentration (as the shear viscosity increases). Quan et al. (2011: 304–309) found that the increasing the concentration of the PVP spinning solutions beyond 5%, the diameter of the fibers increased due to the high viscosity of spinning solutions. Their result was agreed with that of the Megelski and his group study (Megelski et al., 2002: 8456–8466). They found that the increasing the concentration of polystyrene in tetrahydrofuran (THF) the fiber diameter increased and the distribution of pore sizes became narrower. Deitzel et al. (2001: 261–272) reported the relation between the polyethylene oxide (PEO) fiber diameter and the PEO concentration by power law relationship.

Table 13 The characterization of electrospun fibers.

Sample	Fibers characteristic		
	Ø (μm)	Thickness (mm)	Morphology
0% Plai oil	0.561 ± 0.094	0.38 ± 0.03	Fibers
10% Plai oil	0.486 ± 0.076	0.44 ± 0.04	Fibers
20% Plai oil	0.225 ± 0.016	0.54 ± 0.06	Fibers
30% Plai oil	0.258 ± 0.013	0.63 ± 0.05	Fibers

Mean ± S.D. from 3 measurements.

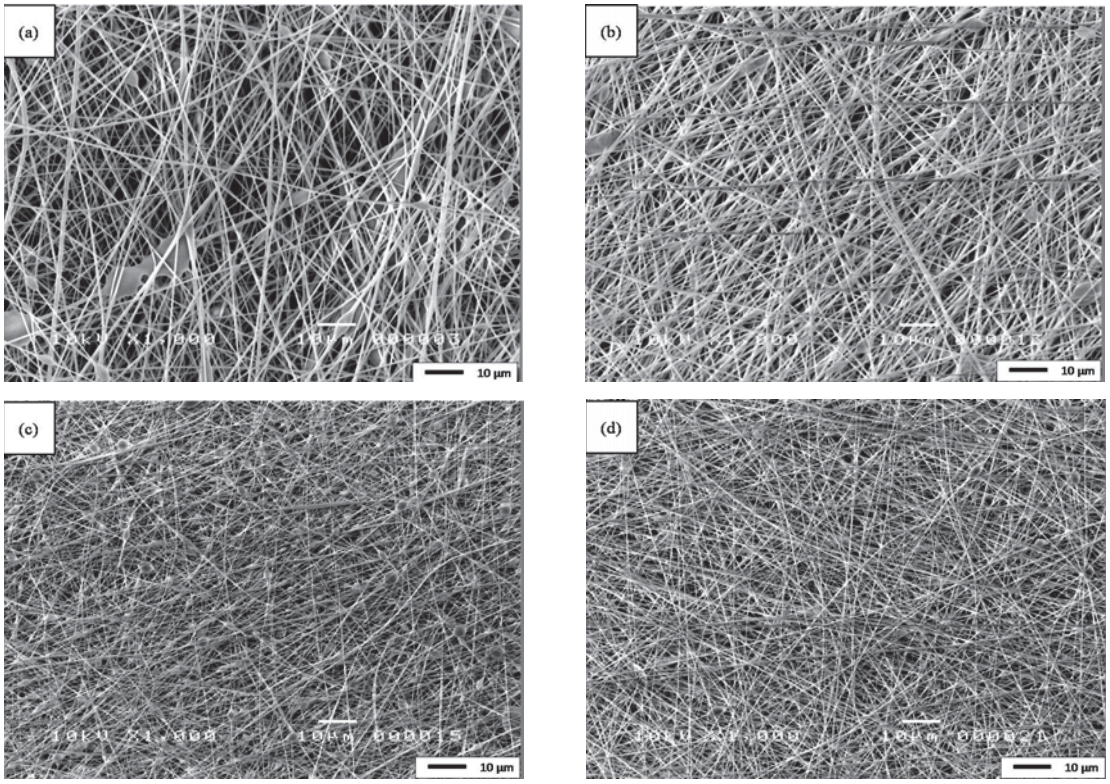

Figure 23 SEM images (1,000x) of PVP/HPβCD electrospun fibers with (a) 0%, (b) 10%, (c) 20%, and (d) 30% Plai oil to polymer

4.2 Chemical characterizations (FTIR)

To confirm the presence of Plai oil in electrospun fibers, the infrared (FTIR) spectra of wavenumber from 4000 to 750 cm^{-1} of Plai oil, PVP, HPβCD, physical mixture of PVP-HPβCD, and Plai oil-loaded electrospun fibers (0%, 10%, 20%, and 30%) were investigated. Figure 24 shows the FTIR spectra of active constituents (terpinen-4-ol and Plai oil). The spectrum of Plai oil showed the peaks whose wave numbers were in the region at 3074, 2956, 2933, 2870, 2635, 1653, 1265, and 1028 cm^{-1}, while spectrum of terpinen-4-ol showed the peaks whose wave numbers were in the region at 3442, 3010, 2960, 2921, 2887, 2854 and 2837 cm^{-1}.

Figure 25 shows the FTIR spectra of PVP, HPβCD, and their physical mixture. The characteristic absorption peak of PVP appeared at 1666.50 cm^{-1} corresponding to the carbonyl on the pyrrolyl ring stretching vibration. In the spectrum of HPβCD, it exhibited a broad peak of O−H stretching in the range of 3000−3500 cm^{-1} and 1363.67 (δ(C−H)), 1026.13 (δ(O−C−H), δ(C−C−H), and

δ(C−C−O) and 948.98 cm^{-1}, corresponding to the skeleton vibration involving α−1,4 linkage. The physical mixture of PVP and HPβCD showed a broad peak of O−H stretching in the range of 3000−3500 cm^{-1} indicating the presence of HPβCD in the mixture, while the peak at 1666 cm^{-1} of carbonyl group of PVP showed only a weak intensity.

When Plai oil was incorporated in electrospun fibers (Figure 26), the FTIR spectra exhibited a broad peak of O−H stretching in the range of 3000−3500 cm^{-1} and also the peak of carbonyl group at 1666 cm^{-1}, corresponding to the PVP/HPβCD fibers. The peaks at a fingerprint region of Plai oil around 2800−2900 cm^{-1} shifted and their intensities were also changed when Plai oil was loaded into the fibers. These may indicate that the presence of Plai oil in the inclusion complex of PVP/HPβCD electrospun fibers.

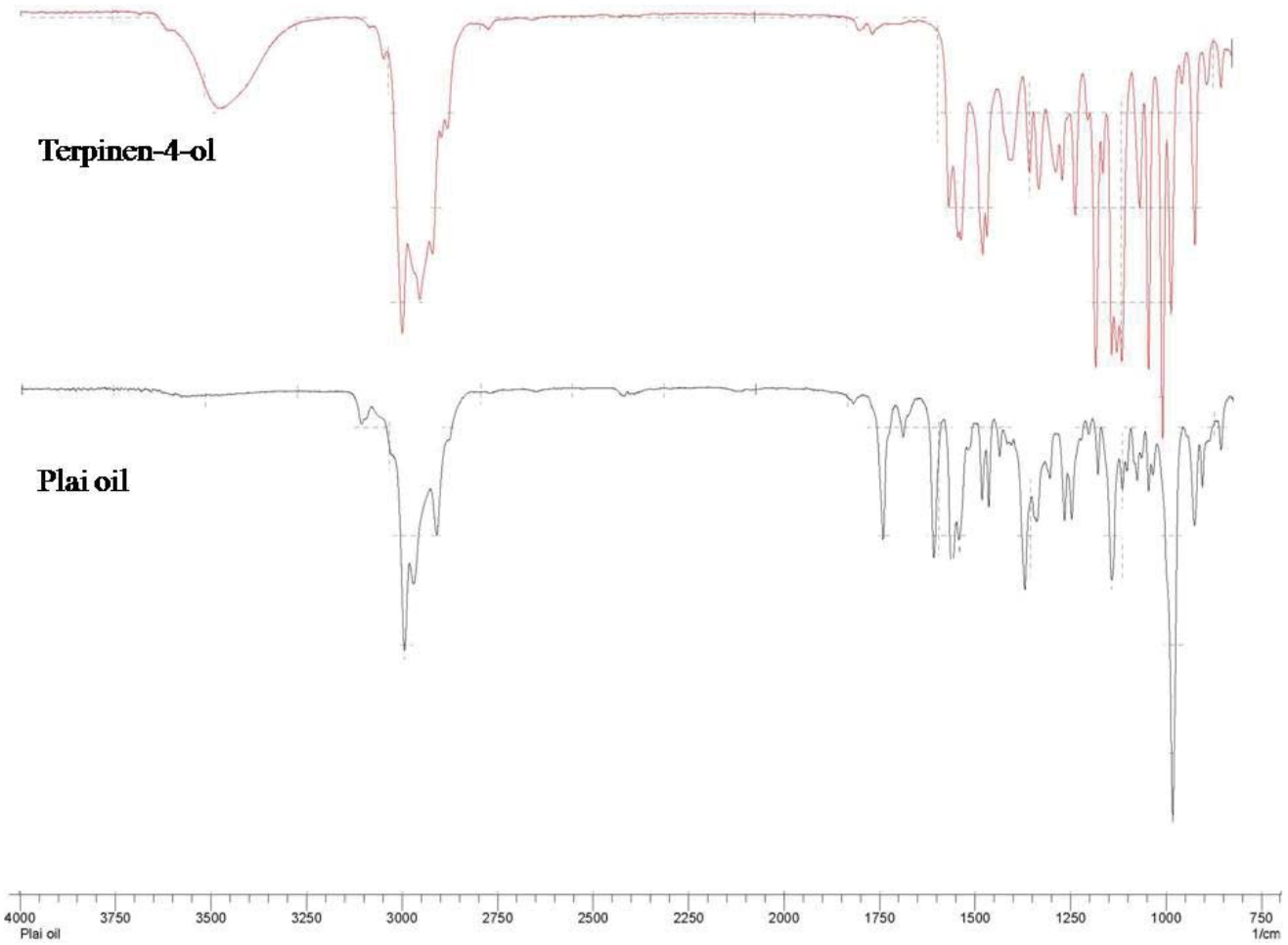

Figure 24 FTIR spectra of active constituents (terpinen-4-ol and Plai oil).

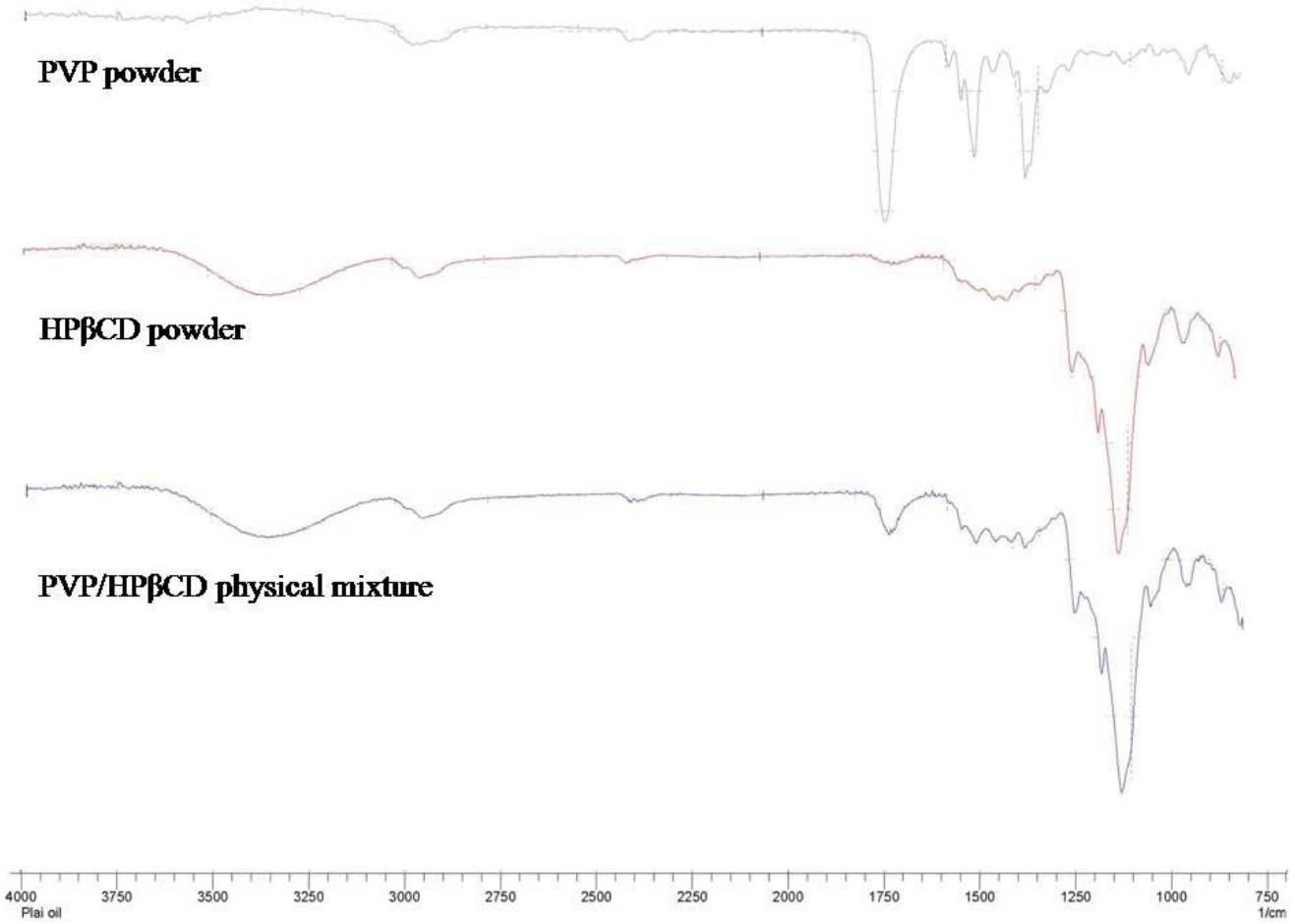

Figure 25 FTIR spectra of polymer base (PVP, HPβCD, and HPβCD-PVP physical mixture).

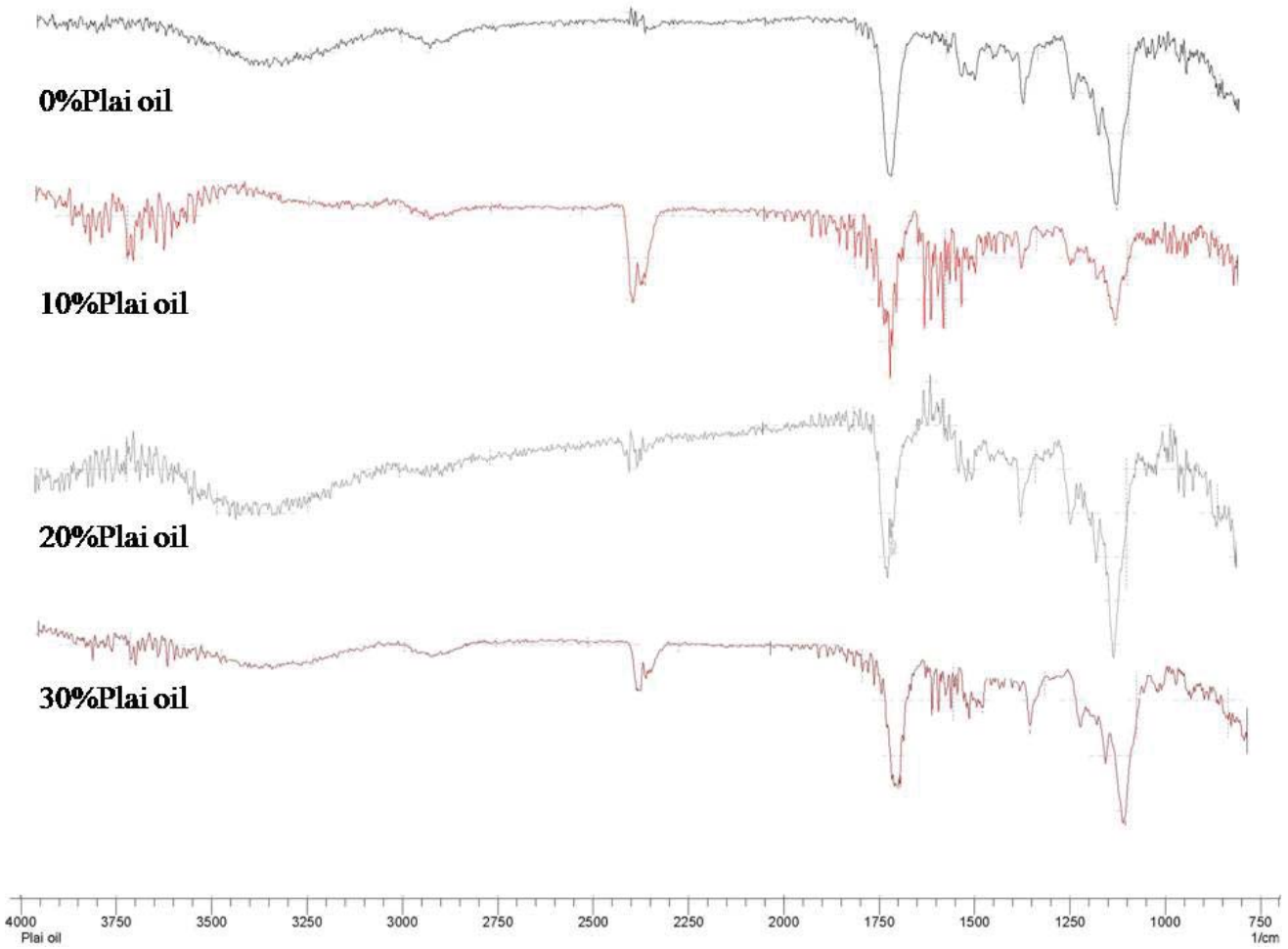

Figure 26 FTIR spectra of Plai oil and electrospun fibers incorporated Plai oil (0%, 10%, 20%, and 30%).

4.3 Thermal analysis (DSC)

The DSC thermograms of pure components, physical mixture, and electrospinning fibers are shown in Figure 27 and 28. During scanning of PVP, a broad endothermic transition over the temperature range of 60–140ºC was observed (Figure 25), indicating the loss of water due to the extremely hygroscopic nature of PVP polymer whereas HPβCD exhibited a sharp endothermic peak at 164.47 ºC corresponding to the transconformation of the molecule (Kohata, Jyodoi, and Ohyoshi, 1993: 187–198). The physical mixture of PVP and HPβCD also showed the similar thermogram characteristic between PVP and HPβCD (Figure 27). This result indicated that the physical mixture of PVP and HPβCD showed the partial interaction between these two polymers, thus, the thermogram revealed the combination of thermodynamic properties between each pure polymer.

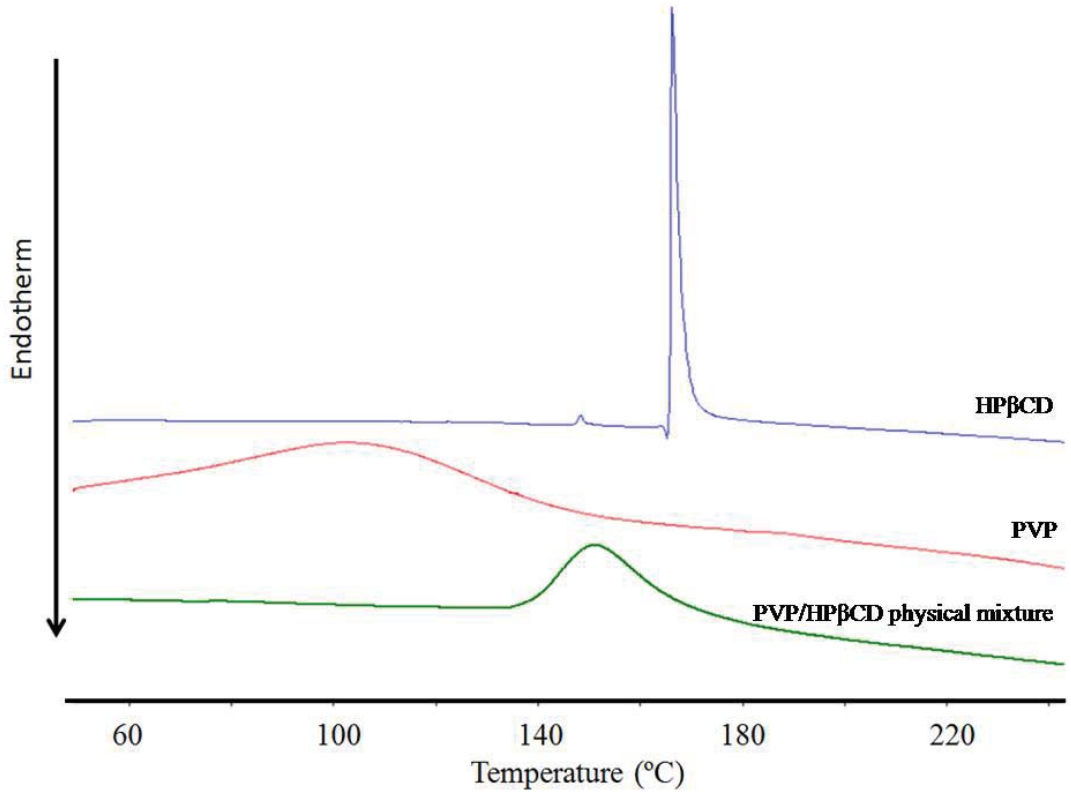

Figure 27 DSC thermograms of polymer base (PVP, HPβCD, and HPβCD-PVP physical mixture).

The transition temperature endotherms of Plai oil and of Plai oil-loaded electrospun fibers at different concentrations are shown in Figure 28. A sharp endothermic peak of Plai oil was detected at 206.82ºC. When Plai oil was loaded into nanofibers, their thermal properties were shifted. The thermograms of all Plai oil-loaded fibers exhibited the sharp endothermic peaks at 127.88ºC, 170.73ºC, 155.57ºC, and 166.12ºC according to 0%, 10, 20%, and 30% Plai oil loaded, respectively. The thermodynamic parameters of Plai oil, polymers and their physical mixture, and electrospun fibers are summarized in Table 14. The thermograms of Plai oil-loaded electrospun fibers exhibited the sharp endothermic peaks similar to that of Plai oil and of HPβCD. These results indicated that Plai oil was incorporated in electrospun fibers.

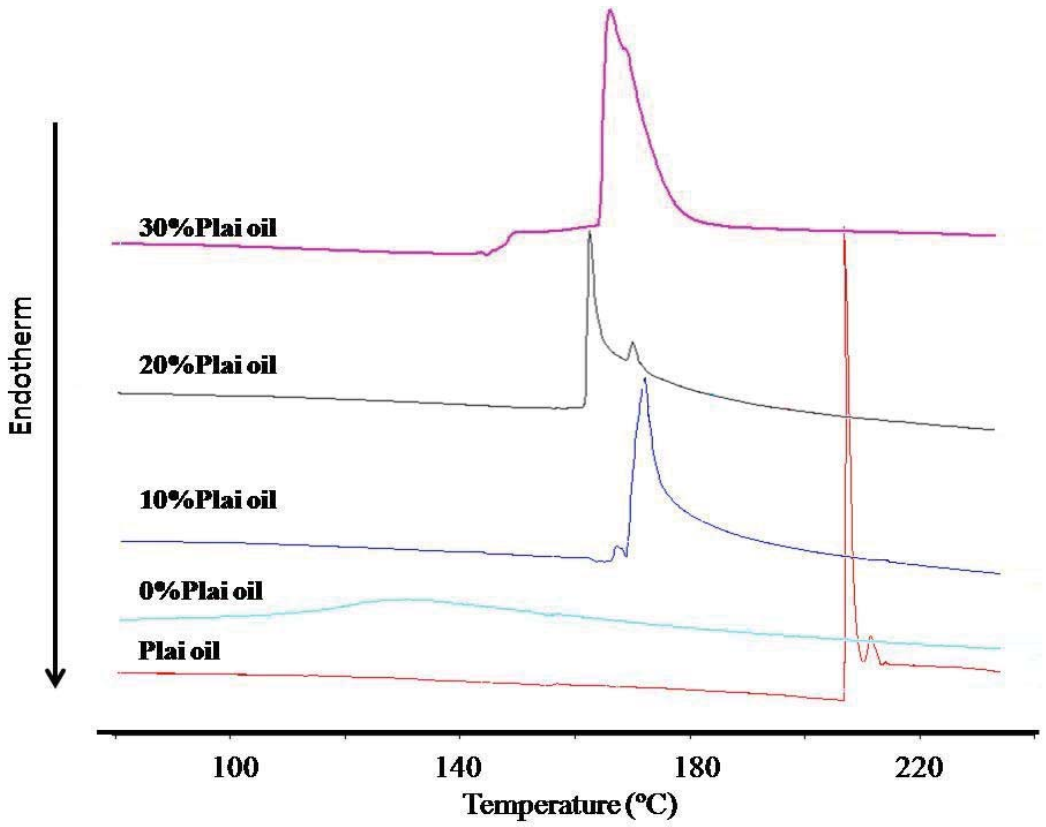

Figure 28 DSC thermograms of electrospun fibers incorporated Plai oil (0%, 10%, 20%, and 30%).

Table 14 Thermodynamic parameters of each composition in electrospun fibers.

Sample	T (ºC)	Δ*H* (J/g)	Area (mJ)	Weight (mg)
Plai oil	206.82	137.2784	1400.240	10.200
PVP powder	103.66	372.9891	7124.093	19.100
HPβCD powder	164.47	207.1181	2899.653	15.6
Physical mixture of HPβCD-PVP	151.63	180.0530	2808.826	14.0
0% Plai oil	127.88	160.1195	2129.589	13.3
10% Plai oil	170.73	284.7389	3758.553	13.2
20% Plai oil	155.57	197.0684	2384.527	12.1
30% Plai oil	166.12	265.0390	3684.034	13.9

5. Entrapment efficiency (The percentage entrapment efficiency and loading capacity)

The total amount of Plai oil loaded in the electrospun fibrous mats was quantified by a gas chromatography. Table 15 showed the Plai oil content in the nanofibers with the different Plai oil loadings. 10% and 20% Plai oil-loaded electrospun fibers showed the high drug entrapment efficiency values (between 90.84% and 91.20%). The initial Plai oil concentration increased up to 30 %wt of polymer, the final amount of Plai oil incorporated into the electrospun fibers decreased. This might be due to the capacity of nanofibers was maximum at 20% of Plai oil. The high variation observed in the % loading might be explained by the result of solvent volatility during the electrospinning process. As its volatile property, Plai oil may evaporate during the electrospinning process.

Table 15 Percentage of entrapment efficiency and loading capacity of Plai oil loaded in electrospun fibers.

Electrospun fibers	% Entrapment efficiency	Loading capacity (mg/g)
10% Plai oil	90.84 ± 19.34	0.10 ± 0.02
20% Plai oil	91.20 ± 11.84	0.20 ± 0.02
30% Plai oil	55.53 ± 5.70	0.18 ± 0.02

Mean ± S.D. from 3 measurements.

6. *In vitro* release

In vitro release studies of Plai oil-loaded electrospun fibers were performed and the amount of Plai oil released was determined at the predetermined time of 0, 1, 2, 4, and 24 h. The release behavior of Plai oil from electrospun fibers showed the initial fast release in the first few hours followed by slower release rates as shown in Figure 29. More than 46%, 67%, and 60% of Plai oil was released from 10%, 20%, and 30% Plai oil-loaded electrospun fibers, respectively, within the first 4 h at 32ºC (Table 16). A total release of Plai oil over the experimental time of 24 h was observed at 78.8%, 70.0%, and 66.9% of its loading content from 10%, 20%, and 30% Plai oil-loaded electrospun fibers, respectively (Table 16).

Table 16 Percentage of Plai oil release from Plai oil-loaded electrospun fibers.

Sample	% Plai oil released			
	1 h	2 h	4 h	24 h
10% Plai oil	46.47 ± 0.00	46.50 ± 0.00	46.89 ± 0.00	78.84 ± 0.00
20% Plai oil	50.80 ± 0.10	69.44 ± 0.00	67.61 ± 0.00	70.00 ± 0.00
30% Plai oil	0.00 ± 0.03	12.33 ± 0.09	60.66 ± 0.00	66.94 ± 0.00

Mean ± S.D. from 3 measurements.

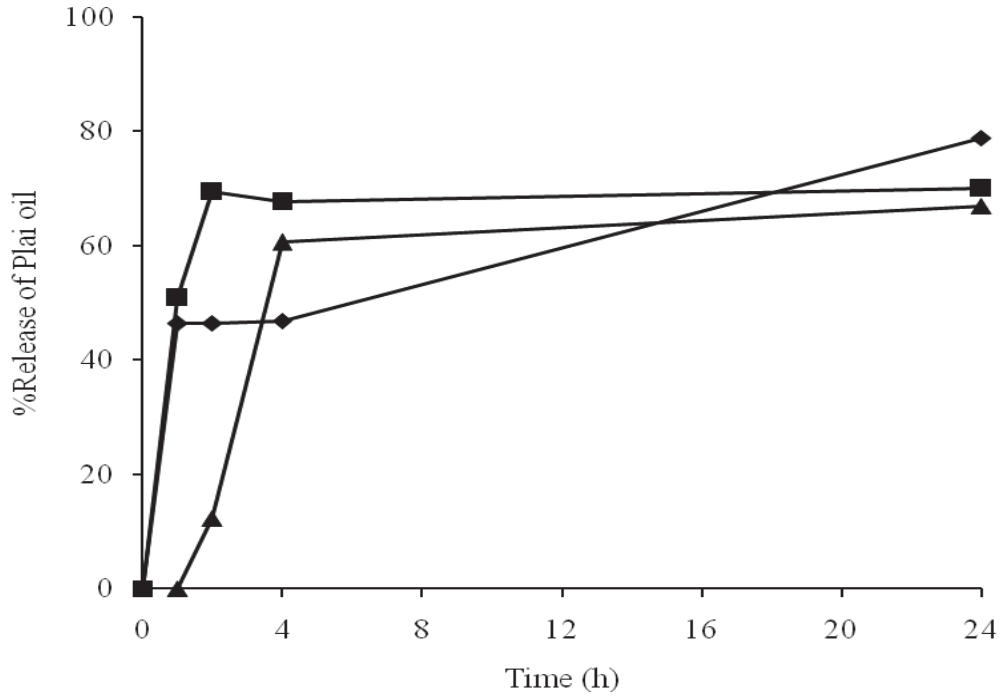

Figure 29 Release profiles of Plai oil from Plai oil-loaded electrospun fibers. Symbols: ◆, 10%; ■, 20%; ▲, 30%. Each point represents the mean ± S.D. of 3 experiments.

7. Stability study

In this study, the Plai oil loaded electrospun fibers were kept at the room temperature for 9 months. Figure 30 shows the SEM images of the 0% Plai oil-loaded electrospun fibers at the initial, 3, 6, and 9 months. The results were found that the neat PVP/HPβCD fibers slightly changed in the diameter and morphology, in which the diameter size increased to 1.149 ± 0.070, 1.080 ± 0.047, and 0.856 ± 0.166 μm for 3, 6, and 9 months, respectively (Table 17). The storage time up to 9 months did not affect the morphology of the neat polymer fibers since the fibers formation was also straight and uniform as previously described (Figure 30). The results of the neat PVP/HPβCD fibers were correspondent with the 10% Plai oil-loaded nanofibers as shown in Table 17 and Figure 31. The fibers diameter was increased in the range of 0.551–1.104 μm (Table 17) but no significant changes in the fibers morphology were observed (Figure 31). Figure 32 and 33 show the SEM images of 20% and 30% Plai

oil-loaded electrospun fibers. Unlike 0% and 10% Plai oil-loaded nanofibers, it was observed that when the time passed by the diameter of fibers showed the significant increase at 9 months storage from 0.225 $\pm$ 0.016 to 4.199 $\pm$ 0.409 µm and 0.258 $\pm$ 0.013 to 3.920 $\pm$ 0.446 µm for 20% and 30% Plai oil loaded, respectively (Table 17). SEM images clearly indicated that at 9 months storage the 20% and 30% Plai oil-loaded nanofibers showed the larger diameter sizes than the initial states and the fibers became flat formation (Figure 32 (d) and 33 (d)). These results implied that the concentration of Plai oil incorporated influenced on the stability of nanofibers.

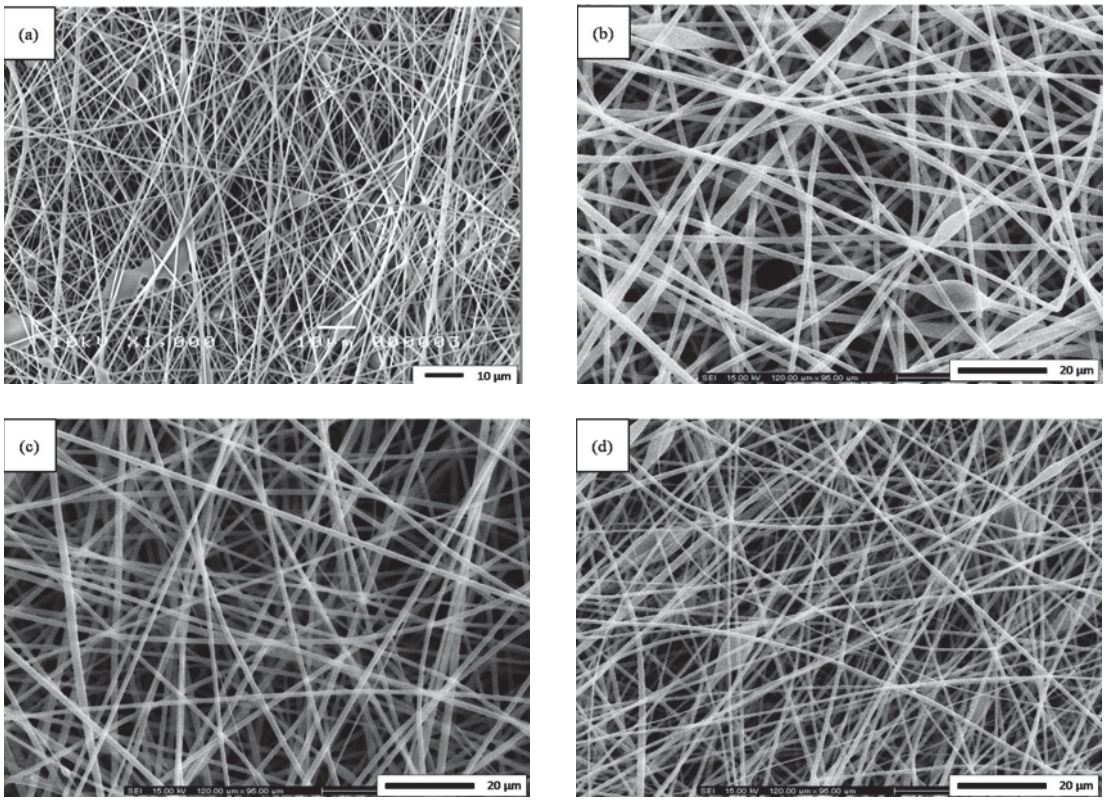

Figure 30 SEM images (1,000x) of PVP/HPβCD electrospun fibers at (a) initial, (b) 3 months, (c) 6 months, and (d) 9 months.

Table 17 Stability data of Plai oil loaded electropsun nanofibers.

Sample	Fiber characteristic					
	3 months		6 months		9 months	
	Ø (μm)	Morphology	Ø (μm)	Morphology	Ø (μm)	Morphology
0%Plai oil	1.149 ± 0.070	Fibers	1.080 ± 0.047	Fibers	0.856 ± 0.166	Fibers
10%Plai oil	0.551 ± 0.117	Fibers	1.104 ± 0.172	Fibers	0.839 ± 0.214	Fibers
20%Plai oil	0.600 ± 0.024	Fibers	0.637 ± 0.075	Fibers	4.199 ± 0.409	Flat fibers
30%Plai oil	0.646 ± 0.158	Fibers	1.025 ± 0.061	Fibers	3.920 ± 0.446	Flat fibers

Mean ± S.D. with 3 measurements.

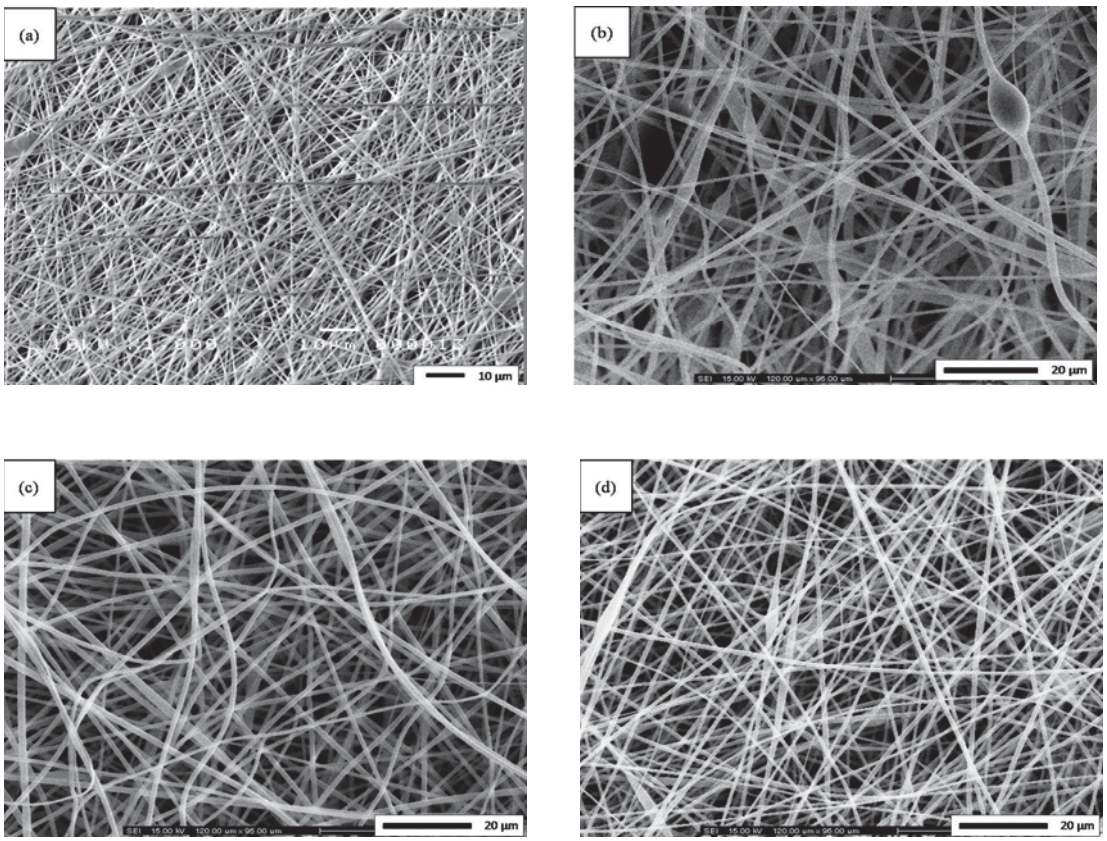

Figure 31 SEM images (1,000x) of PVP/HPβCD electrospun fibers with 10% Plai oil loaded at (a) initial, (b) 3 months, (c) 6 months, and (d) 9 months.

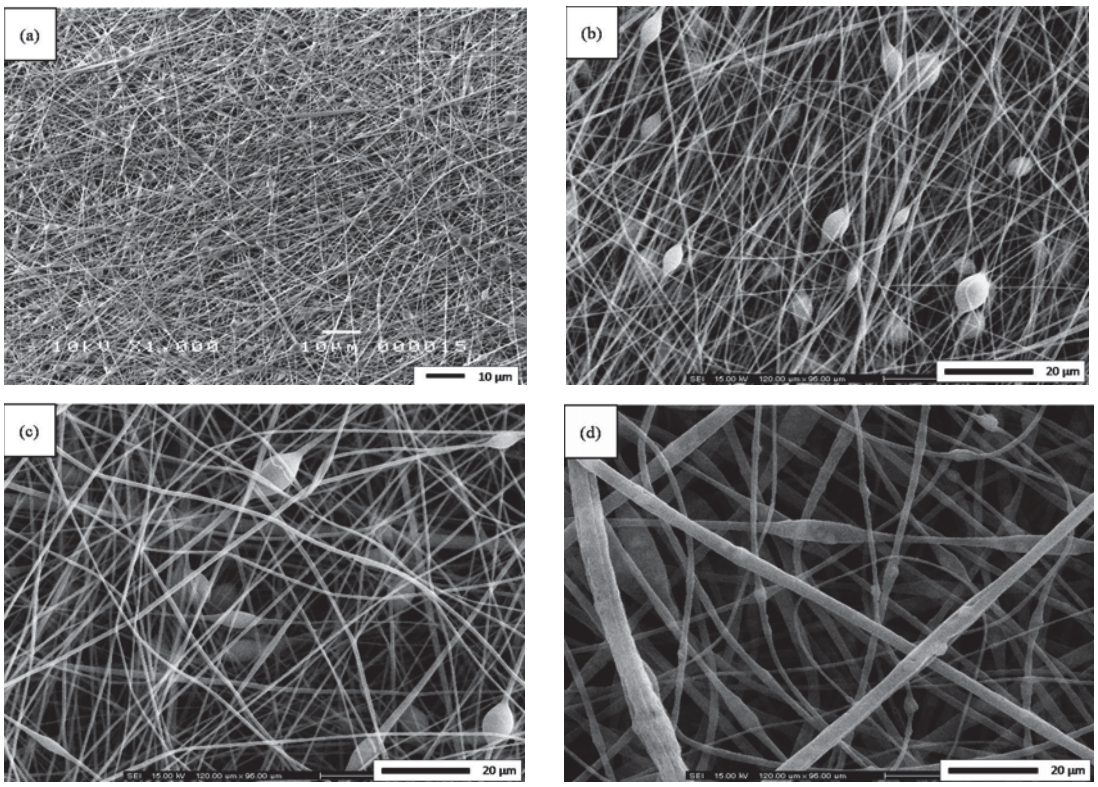

Figure 32 SEM images (1,000x) of PVP/HPβCD electrospun fibers with 20% Plai oil loaded at (a) initial, (b) 3 months, (c) 6 months, and (d) 9 months.

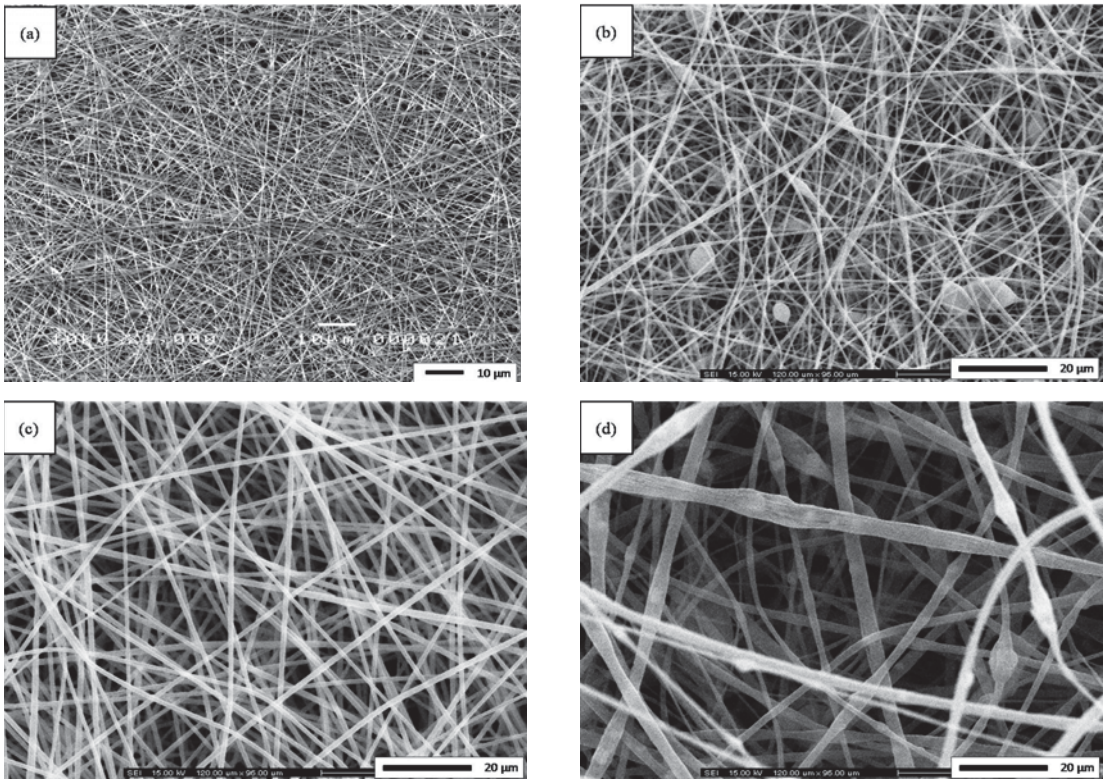

Figure 33 SEM images (1,000x) of PVP/HPβCD electrospun fibers with 30% Plai oil loaded at (a) initial, (b) 3 months, (c) 6 months, and (d) 9 months.

CHAPTER 5
CONCLUSIONS

1. The optimum extraction method

In this study, the typical conventional extraction methods i.e. soxhlet extraction, water distillation, solvent extraction combined with ultrasonication were selected and compared for the extraction of potential terpinen-4-ol from the rhizomes of Plai (*Zingiber cassumunar* Roxb.). Hexane extraction in combination with ultrasonication gave the highest extraction yield among those conventional extraction method (Soxhlet extraction, water distillation and alcohol extraction in combined with ultrasonication).

2. Enzyme-assisted method

Factors including the type and concentration of enzymes as well as the pH, temperature and time of enzyme treatment were investigated. The optimum pH, temperature and time of enzyme pretreatment was pH 5.5 and incubated in an orbital shaker at 40 ± 0.5°C, 100 rpm for 24 h.

The effect of amount of cellulase on the extraction yields were performed at the weight ratio of cellulase: sample; 3:100, 6:100 and 9:100. The result revealed that all the weight ratios gave the higher extraction yield than that without the enzyme pretreatment. A significant difference was observed with the use of cellulase at the weight ratio of 3:100 (1.53 ±0.26 μg/g) and 6:100 (1.25 ± 0.26 μg/g) compared to the control ($p < 0.05$). However, the statistical analysis of the extraction yields showed no significant differences among the three weight ratios. Therefore, the optimal weight ratio used in the extraction system of terpinen-4-ol from the rhizomes of Plai was 3:100.

The different type of enzymes used (cellulase, hemicellulase and pectinase) had an influence on extraction yield. At the weight ratio of enzyme: sample

3:100, cellulase showed the highest extraction efficiency yield of terpinen-4-ol (1.53 ± 0.26 μg/g) followed by pectinase (1.22 ± 0.20 μg/g) and hemicellulase (1.21 ± 0.18 μg/g), respectively. In the case of mixed enzymes, cellulase/pectinase showed the highest extraction efficiency yield of Terpinen-4-ol (1.34 ± 0.27μg/g) followed by cellulase/hemicellulase (1.12 ± 0.17μg/g) and hemicellulase/pectinase (1.05 ± 0.12μg/g), respectively. It could be concluded that the use of cellulase as a single enzyme and combination with pectinase exhibited significant higher extraction yield compare with to the other enzyme treatment.

3. Plai oil loaded electrospun nanofibers

Plai oil (8.89, 17.79 and 26.69% terpinen-4-ol) was successfully loaded into PVP/HPβCD nanofibers using electrospinning method. 10%, 20% and 30% Plai oil loaded electrospun nanofibers were in the nanometer size range. The presence of Plai oil in the polymer solution decreased the viscosity of the resulting PVP/HPβCD solutions, resulting in decrease of the diameter. The neat PVP/HPβCD fibers were straight and uniform with a few beads formation. The average diameter was about 561 ± 94 nm, while those of the 10-30 % Plai oil-loaded electrospun fibers were between 225 and 486 nm.

The initial Plai oil content affected the loading capacity of Plai oil in fiber. As the initial Plai oil concentration from 10, 20 and 30 % to polymer was performed, the incorporation efficiency of Plai oil loaded of the formula was 90.84±19.34%, 91.20±11.84% and 55.53±5.70% with 10, 20 and 30 % of the initial Plai oil, respectively.

The DSC thermogram and FTIR spectrum of the fibers confirm that the Plai oil can be loaded in the electrospun nanofibers. However, the incorporation method of Plai oil in the formulation must be concerned because of its volatility property.

In vitro release behavior of Plai oil from electrospun nanofibers showed the initial fast release in the first few hours followed by slower release rates over the experimental time of 24 h. The release of Plai oil from loaded- electrospun fibers were about 67-79%. The release was in the order of 10 % > 20% > 30% Plai oil-

loaded electrospun fibers. The highest release was observed in 10 % Plai oil-loaded electrospun fibers and also showed the highest physical stability over 9 month. At high concentration of initial Plai oil (20% and 30%), the instability of morphology was observed. This study indicated that 10 % Plai oil loaded electropun nanofibers have a potential for use as topical application.

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APPENDIX

APPENDIX A

Standard curve for analysis of terpinen-4-ol

Standard	:	terpinen-4-ol
Method	:	Gas chromatography
Detector	:	Flame Ionization Detector
Concentration (µg/mL)	:	0.25, 0.50, 1.50, 2.50 and 3.50

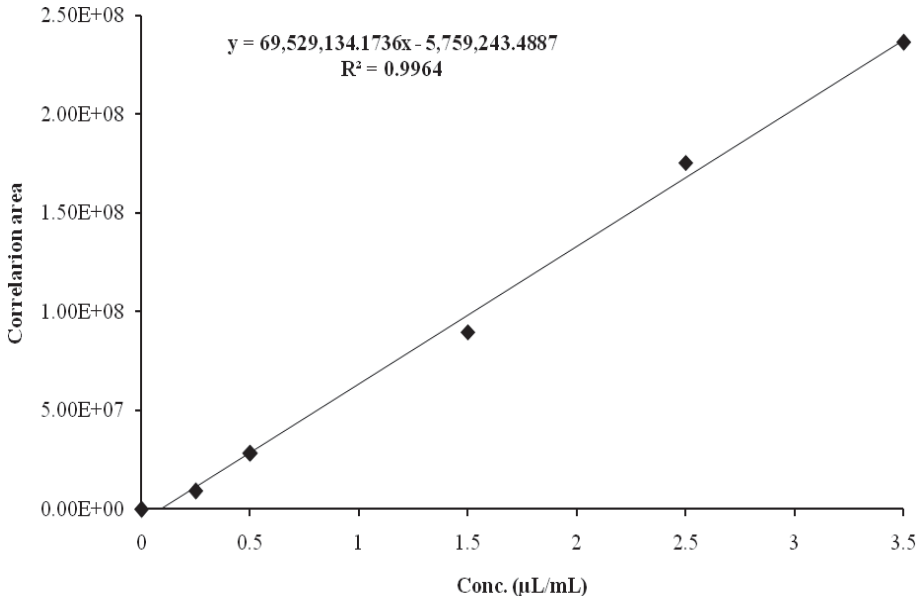

Figure 34 Standard curve for analysis of terpinen-4-ol.

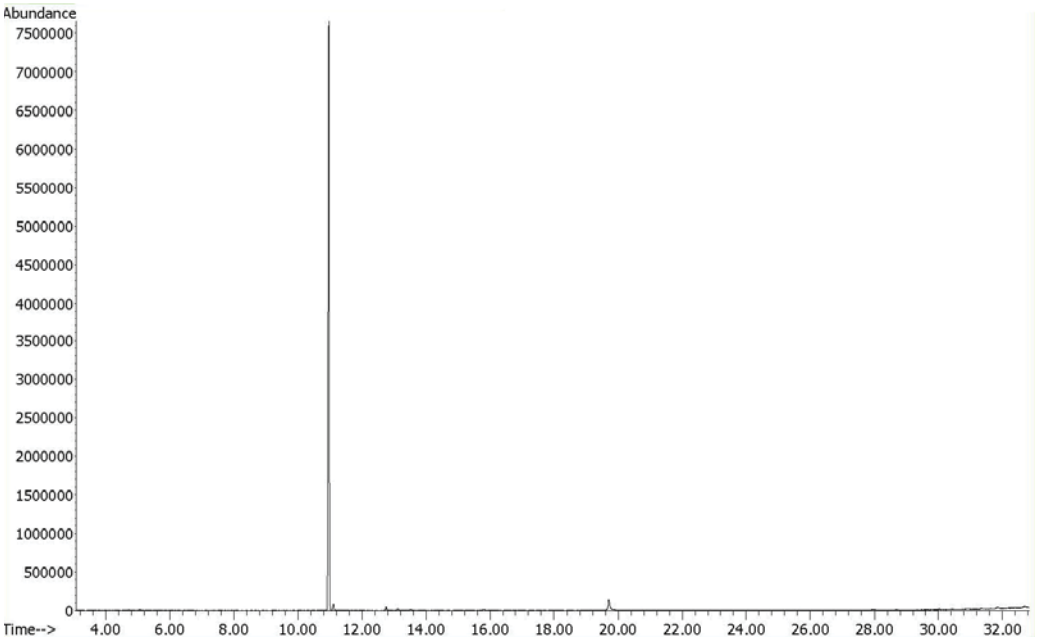

Figure 35 Chromatogram of standard terpinen-4-ol.

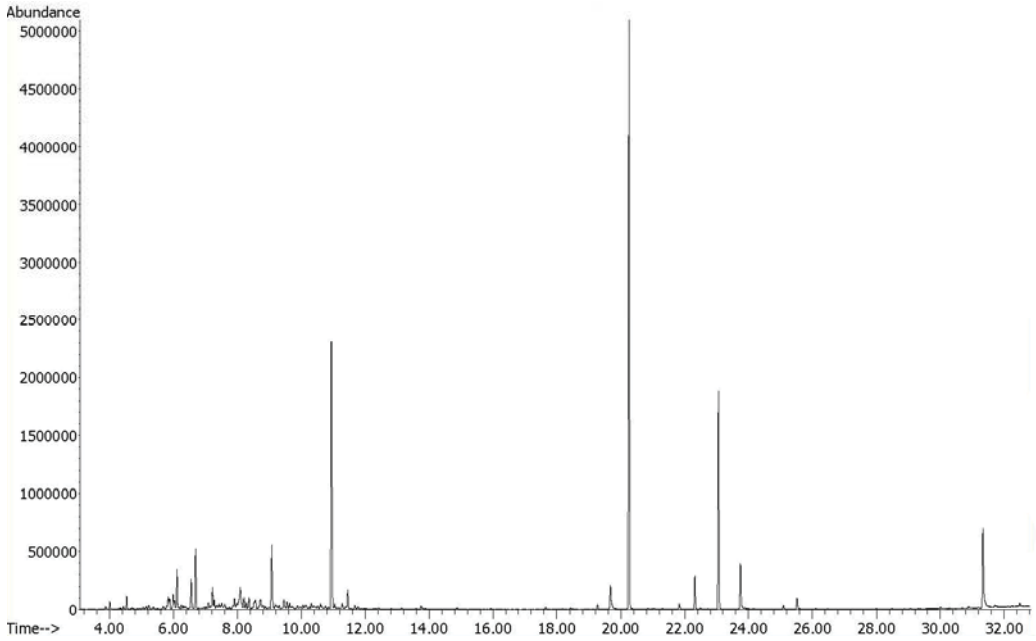

Figure 36 Chromatogram of Plai oil extract.

APPENDIX B

PVP/HPβCD electospun nanofibers preparation

Table 18 Quantitative amount of terpinen-4-ol in 1mM HPβCD solution at 12, 24, 36, 48, and 72 h.

Time	12 h	24 h	36 h	48 h	72 h
Terpinen-4-ol (μg/g)	0.31±0.08	0.37±0.01	0.37±0.05	0.41±0.01	0.41±0.01

Mean ± S.D. from 3 measurements.

Table 19 Quantitative amount of terpinen-4-ol in 0, 10, 20 and 30 mM HPβCD solution after stirring for 24 h.

HPβCD (mM)	10 mM	20 mM	30 mM
Terpinen-4-ol (μg/g)	1.36±0.03	1.21±0.04	1.0.3±0.10

Mean ± S.D. from 3 measurements.

APPENDIX C

***In vitro* release**

Table 20 Quantitative amount of remained Plai oil in electrospun nanofibers at initial, 1, 2, 4, and 24 h.

Sample	Remained Plai oil (g)				
	initial	1 h	2 h	4 h	24 h
10% Plai oil	0.130±0.054	0.070±0.000	0.070±0.003	0.070±0.002	0.038±0.004
20% Plai oil	0.294±0.038	0.145±0.099	0.090±0.003	0.095±0.002	0.088±0.001
30% Plai oil	0.267±0.027	0.027±0.049	0.234±0.086	0.105±0.084	0.088±0.002

Mean ± S.D. from 3 measurements.

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Presentation

Poster

1. Tud-dao Chuchote, Praneet Opanasopit and Theerasak Rojanarata. (2009). Enzyme-assisted extraction of Plai (*Zingiber cassumunar* Roxb.). The 35th congress on Science and Technology of Thailand (STT35) at The Tide beach resort, Chonburi, Thailand. (October 15-17, 2009)

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