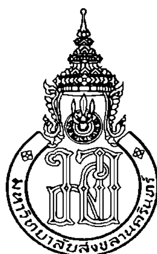

Preparation and quality control of *Zingiber cassumunar* extract with high-yielded anti-inflammatory active compounds

Arpaporn Kaewchoothong

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Herb Sciences (International Program)

Prince of Songkla University

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ชื่อวิทยานิพนธ์	การเตรียมและการควบคุมคุณภาพสารสกัดไพลที่มีสารออกฤทธิ์ต้านการอักเสบปริมาณสูง
ผู้เขียน	นางสาว อาพาภรณ์ แก้วชูทอง
สาขาวิชา	วิทยาศาสตร์สมุนไพร (นานาชาติ)
ปีการศึกษา	2552

บทคัดย่อ

สาร phenylbutanoids สี่ชนิดได้แก่ (*E*)-4-(3,4-dimethoxyphenyl)but-3-en-l-ol (compound D), (*E*)-4-(3,4-dimethoxyphenyl)but-3-en-l-yl acetate (D-acetate), (*E*)-1-(3,4-dimethoxyphenyl)butadiene (DMPBD) และ (*E*)-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl] cyclohex-1-ene (DMPDMS) ซึ่งแยกได้จากสารสกัดไพล เมื่อนำมาทดสอบฤทธิ์ต้านการอักเสบโดยการยับยั้งการหลั่ง Nitric oxide (NO) จากเซลล์ macrophage พบว่าสาร phenylbutanoids ทั้งสี่ชนิดออกฤทธิ์ยับยั้งการหลั่ง NO ด้วยค่า IC_{50} เท่ากับ 211.1, 86.8, 56.3 และ 39.7 µM ตามลำดับ โดย DMPDMS เป็นสารที่ออกฤทธิ์ต้านการอักเสบได้ดีที่สุด โดยมีค่า IC_{50} เท่ากับ 39.7 µM ดังนั้นจึงใช้สาร phenylbutanoids ทั้งสี่ชนิดนี้เป็น indicative markers ในการควบคุมคุณภาพสารสกัดไพล การวิเคราะห์ปริมาณ phenylbutanoids โดยใช้เทคนิค reversed-phase HPLC ที่ได้ถูกสร้างขึ้นเพื่อใช้ในการวิเคราะห์ปริมาณสาร phenylbutanoids ในสารสกัดไพล วิธีวิเคราะห์นี้ใช้คอลัมน์ชนิด TSK-gel ODS-80Tm และใช้ตัวทำละลาย 2% acetic acid กับ methanol ชะด้วยระบบ gradient ดังนี้ ที่ 0-20 นาที อัตราส่วนระหว่าง 2% acetic acid กับ methanol เท่ากับ 52:48, ที่ 22-38 นาที ชะด้วยอัตราส่วน 50:50 และที่ 40-60 นาที ชะด้วยอัตราส่วน 52:48 และตรวจวัดที่ความยาวคลื่น 254 nm และทำการตรวจสอบความถูกต้องของวิธีการวิเคราะห์นี้ โดยการประเมิน linearity, accuracy, intraday- and interday-precision, specificity และ sensitivity พบว่าวิธีการวิเคราะห์นี้มี accuracy ดี โดยให้ % recovery ในช่วง 95-105% และมี linearity ที่ดี ($r^2 \geq 0.9996$) มีความเที่ยงตรง (R.S.D. < 5%) และความจำเพาะสูง และปริมาณสารต่ำสุดที่สามารถตรวจวัดและวิเคราะห์ปริมาณได้มีค่าเท่ากับ 0.13, 0.18, 0.18, 0.72 และ 0.18, 0.60, 0.90, 1.45 µg/ml ตามลำดับ การศึกษาตัวทำละลายที่เหมาะสมในการสกัดสาร phenylbutanoid จากไพลพบว่า hexane เป็นตัวทำละลายที่สามารถสกัดสาร phenylbutanoid ได้อย่างจำเพาะเจาะจงสูงสุด จึงใช้ hexane เป็นตัวทำละลายในการสกัดสาร phenylbutanoid จากไพล เมื่อนำสารสกัดไพลที่ได้มาผ่านกระบวนแยกสารด้วย silica gel vacuum column chromatography โดยชะด้วยตัวทำละลายผสมระหว่าง hexane และ ethyl acetate (8:2) จะ

ได้สารสกัดที่มีปริมาณสาร phenylbutanoids สูง จากการวิเคราะห์ด้วย HPLC พบว่าปริมาณ phenylbutanoids ในสารสกัดที่เตรียมได้มีค่าสูงกว่า ปริมาณ phenylbutanoids ในสารสกัดหยาบด้วย hexane ประมาณ 8% และเมื่อทดสอบฤทธิ์ต้านการอักเสบพบว่าสารสกัดที่มีปริมาณสาร phenylbutanoids ในปริมาณสูงมีฤทธิ์ต้านการอักเสบสูงกว่าสารสกัดหยาบด้วย hexane, น้ำมันหอมระเหย และสาร phenylbutanoids บริสุทธิ์ที่แยกได้จากไพล การศึกษาค่าการละลายของสกัดที่มีปริมาณสาร phenylbutanoids ในปริมาณสูง พบว่าสารสกัดละลายได้ดีใน methanol, ethyl acetate, chloroform และ hexane แต่ไม่ละลายในน้ำ

Thesis Title	Preparation and quality control of *Zingiber cassumunar* extract with high-yielded anti-inflammatory active compounds
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ABSTRACT

Four phenylbutanoids including (*E*)-4-(3,4-dimethoxyphenyl)but-3-en-l-ol (compound D), (*E*)-4-(3,4-dimethoxyphenyl)but-3-en-l-yl acetate (D-acetate), (*E*)-1-(3,4-dimethoxyphenyl)butadiene (DMPBD) and (*E*)-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl] cyclohex-1-ene (DMPDMS) that isolated from *Z. cassumunar* extract were evaluated for their anti-inflammatory activity *via* inhibitory effect against nitricoxide (NO) production by macrophage. The results showed that all four compounds possessed inhibition effect on NO production with IC_{50} values of 211.1, 86.8, 56.3 and 39.7 µM, respectively. Among these compounds, DMPDMS exhibited the highest inhibitory activity with IC_{50} value of 39.7 µM. These phenylbutanoids were therefore used as the indicative markers for standardization of *Z. cassumunar* extracts. A reversed-phase HPLC method was established for the simultaneous quantitative determination of the phenylbutanoids in *Z. cassumunar* extract. The method involved the use of a TSK gel ODS 80 Tm with the mixture of 2% aqueous acetic acid-methanol (gradient elution as follow 0-20 min; 52:48, 22-38 min; 50:50, 40-60 min; 52-48) as the mobile phase and detection at 254 nm. Defining the linearity, accuracy, intraday- and interday-precision, specificity and limits of detection and quantitation validated the HPLC method. The recovery of the method was 95-105% and linearity ($r^2 \geq 0.9996$) was obtained for all phenylbutanoids. A high degree of precision (R.S.D. values less than 5%) as well as specificity were achieved. The lower limits of detection and quantification were 0.13, 0.18, 0.18, 0.72 and 0.18, 0.60, 0.90, 1.45 µg/ml, respectively. The solvents for extraction were examined in order to maximize the phenylbutanoid content in *Z. cassumunar* extract. The results indicated that hexane was the most specific for phenylbutanoid extraction. Hexane was therefore used as the solvent for extraction of phenylbutanoids from *Z. cassumunar*. The hexane extract was further fractionation using silica

gel vacuum chromatography eluted with a mixture of hexane and ethyl acetate (8:2) to produce a phenylbutanoid rich extract. On the basis of HPLC, the phenylbutanoid content of the obtained extract was about 8% higher than that of the crude hexane extract. On the basis of inhibition of NO production assay, the phenylbutanoid rich *Z. cassumunar* extract exhibited anti-inflammatory activity stronger than those of the crude hexane extract, volatile oil and the pure phenylbutanoids from *Z. cassumunar*. Solubility determination of the phenylbutanoid rich extract indicated that the extract is freely soluble in methanol, ethyl acetate, chloroform and hexane. It is practically insoluble in water.

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LIST OF ABBREVIATIONS AND SYMBOLS

α-	alpha-
β-	beta-
p-	para-
δ-	delta-
ºC	degree Celcius
cm	centimeter
d	doublet (for NMR signals)
g	gram
kg	kilogram
μM	micromolar
μmol	micromole
μg	microgram
μl	microliter
μm	micrometer
δ	chemical shift in ppm
ν	wave number
IC_{50}	inhibitory concentration at 50% of tested subject
IR	infrared
J	coupling constant
ml	milliliter
M	Molar
mmol	millimole
m	meter
nm	nanometer
m	multiplet (for NMR signals)
MS	mass spectroscopy
m/z	mass-over-charge ratio
nM	nanomolar

LIST OF ABBREVIATIONS AND SYMBOLS

NMR	nuclear magnetic resonance
ppm	part per million
q	quartet (for NMR signals)
s	singlet (for NMR signals)
SD	standard deviation
t	triplet (for NMR signals)
w	weight

CHAPTER 1

INTRODUCTION

1.1 General introduction

Zingiber cassumunar Roxb. (Zingiberaceae family) is a medicinal plant which commonly known in Thai as "Plai". It distributes mainly in India, Indochina and tropical Southeast Asia (Chirangini and Sharma, 2005). The plant has been used in folk medicine for the treatment of various disorders such as inflammation, sprains, rheumatism, muscular pain, wounds and asthma. It has also been used as a mosquito repellant, a carminative, a mild laxative and an antidysenteric agent as well as a cleansing solution for skin diseases (Bhuiyan, *et al*., 2008).

To develop the high quality of *Z. cassumunar* extract as a material for medicinal preparation, the high content of the active compounds and the consistency of the extracts between batch to batch are required. In this study, the anti-inflammatory activity of *Z. cassumunar* extract is focused. In Thai Herbal Pharmacopoeia, the volatile oil content is used for quality control of *Z. cassumunar* rhizome (not less than 2% v/w) (Subcommittee on the establishment of the Thai Herbal Pharmacopoeia, 1998). In addition, *Z. cassumunar* cream that used for treatment of joint pain and relieve inflammation, should has the volatile oil content not less than 14% v/w (National Drug Committee, 2006). If the volatile oil content in *Z. cassumunar* cream is higher than 14% v/w, it may cause skin irritation, whereas if the volatile oil content is lower than 14% v/w, it may be not effective. Recently, it has been reported that the anti-inflammatory active constituents in the rhizome extract are (*E*)-4-(3,4-dimethoxyphenyl) but-3-en-1-ol, (*E*)-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl] cyclohexene-1-ene, (*E*)-3-(4-hydroxy-3-methoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl] cyclohex-1-ene, (*E*)-1-(3,4-dimethoxyphenyl) butadiene (Han *et al*., 2005), curcumin and cassumunarins A, B and C (Masuda *et al*., 1995). The quantitative analysis method for standardization of the active compounds in *Z. cassumunar* extract is necessary for the development of the anti-inflammatory medicine. There was a report on the use of HPLC to determine (*E*)-1-(3,4-dimethoxyphenyl) butadiene and (*E*)-4-(3,4-dimethoxyphenyl) but-3-en-l-yl acetate. The method involved the use of a Zorbax Extend-C18 column (150 mm × 4.6 mm I.D., 5 μm, Agilent) and gradient elution with a mixture of methanol and water, increasing methanol

from 50% to 90% in 40 minutes. The flow rate was 1 ml/min and the quantification wavelength was set at 254 nm (Lu *et al*., 2005). In addition, determination of major components, including (*E*)-4-(3,4-dimethoxyphenyl)-3-butene-1,2-diol, (*E*)-4-(3,4-dimethoxy-phenyl)-but-3-en-1-ol, (*E*)-4-(3,4-dimethoxyphenyl)-but-3-en-1-yl-acetate, (*E*)-3-(3,4-di-methoxyphenyl) propenal, (*E*)-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl]cyclohex-1-ene and (*Z*)-3-(3,4-dimethoxy-phenyl)-4-[(*E*)-3,4-dimethoxystyryl] cyclohex-1-ene) in the ethanol extract of *Z. cassumunar* using HPLC has also been reported. The HPLC condition was performed with Zorbax XDB-C18 column (150 mm × 4.6 mm I.D., 5 µm, Agilent) and gradient elution with a mixture of methanol and water, increasing methanol from 40% up to 90% in 40 minutes. The flow rate is 0.8 ml/min and the quantification wavelength was set at 254 nm (Lu *et al*., 2008). However, the reported HPLC methods had several defect such as time consuming (total run time of 40 minutes), two cyclohexene derivatives had poor resolution and validation of the HPLC methods was not yet established. Therefore, there is a need to develop and validate a method for simultaneous quantification of the active compounds against inflammation in order to be used as a valuable informative tool for the quality control. In addition, determination of the optimal extraction solvent and fractionation method for preparation of *Z. cassumunar* extract containing high active constituent concentration as well as solubility of the extract are also necessary for the development of herbal medicines.

1.2 **Objectives**

1.2.1. To isolate compounds, which exhibit anti-inflammatory activity from *Zingiber cassumunar* extract and use them as the markers for quality control

1.2.2 To establish an HPLC quantitative analysis method for standardization of *Zingiber cassumunar* extract with high-yielded anti-inflammatory active compounds

1.2.3 To determine optimal solvent and method for preparation of *Zingiber cassumunar* extract with high-yielded anti-inflammatory active compounds and compare with the volatile oil isolated from *Zingiber cassumunar* rhizomes

1.2.4 To determine solubility of *Zingiber cassumunar* extract with high-yielded anti-inflammatory active compounds

CHAPTER 2

REVIEW OF LITERATURES

2.1 Botanical description of *Zingiber cassumunar* Roxb.

Figure 2-1 *Zingiber cassumunar* Roxb.

Scientific name: *Zingiber cassumunar* Roxb.

Family name: Zingiberaceae

Synonym: *Z. purpurium* Roscoe, *Z. montanum* (Koen.) Theilade

Common name: ไพล, ปูลอย, ปูเลย, มิ้นสะล่าง, ว่านไฟ และ ginger cassumunar

This plant is a perennial herb, consisting of underground rhizome, which is bright yellow inside, and leafy stem rising up to 80-150 cm. Leaves are simple; oblong-lanceolate; having apex acute; base narrowing and clasping the stem by their long sheath; membranaceous texture. Inflorescence is on a separate shoot without normal leaves; 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-loped; corolla-tube cylindric, segment whitish, lip yellowish white with a deeply bifid midlops; fertile stamen 1; ovary 3-loculed, many ovules, style filiform, stigma subglobose capsule, 1-1.5 cm in diameter (Figure 2-1) (Farnsworth and Bunyapraphatsara, 1992).

2.2 Medicinal properties of *Z. cassumunar* Roxb.

The rhizome has been traditionally used as antidote to snakebite, carminative, stimulant, and also used for treatment of diarrhea and colic (Chirangini and Sharma, 2005). In addition, it has been topically used as muscle relaxant and treatment of joint pain in Thai traditional massage. Moreover, it is well known that the essential oil has been shown to cure acne, bruises, burnt skin, skin inflammation, muscle pain, insect bite, and asthmatic symptoms. Various lotions and decoctions made from *Z. cassumunar* rhizome have been used by applying to swelling, rheumatism, bruise, numb feet and other painful parts. Thus, it has been proven to be extremely useful for human health (Rukachaisirikul *et al*., 1983).

2.3 Chemical constituents of *Z. cassumunar*

The chemical constituents of *Z. cassumunar* such as monoterpenoids, sesquiterpenoids, curcuminoids, cyclohexene derivatives, napthoquinone derivatives and phenylbutenoid derivatives have been reported as shown in Table 2-1 (Subcommittee on the Establishment of the Thai Herbal Pharmacopoeia, 1998).

Table 2-1 The chemical constituents of *Z. cassumunar* Roxb.

Chemical class	Compound name	References
Monoterpenoids	α-pinene, β-pinene, sabinene, myrcene, α-terpinene, limonene, γ-terpinene, p-cymene, terpinolene, terpinene-4-ol, α-thujene, ocimene, β-phellandrene , (*Z*) sabinene 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; Bhuiyan, *et al*., 2008
Sesquiterpenoids	zerumbone	Kishore and Dwivedi, 1992

Chemical class	Compound name	Reference
Curcuminoid	Curcumin, Cassumunin A, B, C Cassumunarin A, B, C	Masuda *et al*., 1995; Nagano *et al*., 1997
Napthoquinone derivatives	8-(3,4-dimethoxyphenyl)-2 methoxynaphtho-1,4-quinone 8-(2,4,5- dimethoxyphenyl)-2 methoxynaphtho-1,4-quinone	Farnsworth and Bunyapraphatsara, 1992
Phenylbutenoid derivatives	4-(2,4,5-trimethoxyphenyl)-but-1,3-diene (*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-l-yl acetate (*E*)-4-(3,4-dimethoxyphenyl)but-3-en-l-*O*-β-D-glucopyranoside (*E*)-1-(3,4-dimethoxyphenyl)but-1-ene	Jeenapongsa *et al*., 2003; Han *et al*., 2005
Cyclohexene derivatives	(*E*)-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4 dimethoxystyryl]cyclohex-1-ene (*E*)-3-(4-hydroxy-3-methoxyphenyl)-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-trimethoxystyryl] cyclohex-1-ene (*Z*)-3-(2,4,5-trimethoxyphenyl)-4-[(*E*)-2,4,5-trimethoxystyryl] cyclohex-1-ene	Han *et al*., 2005

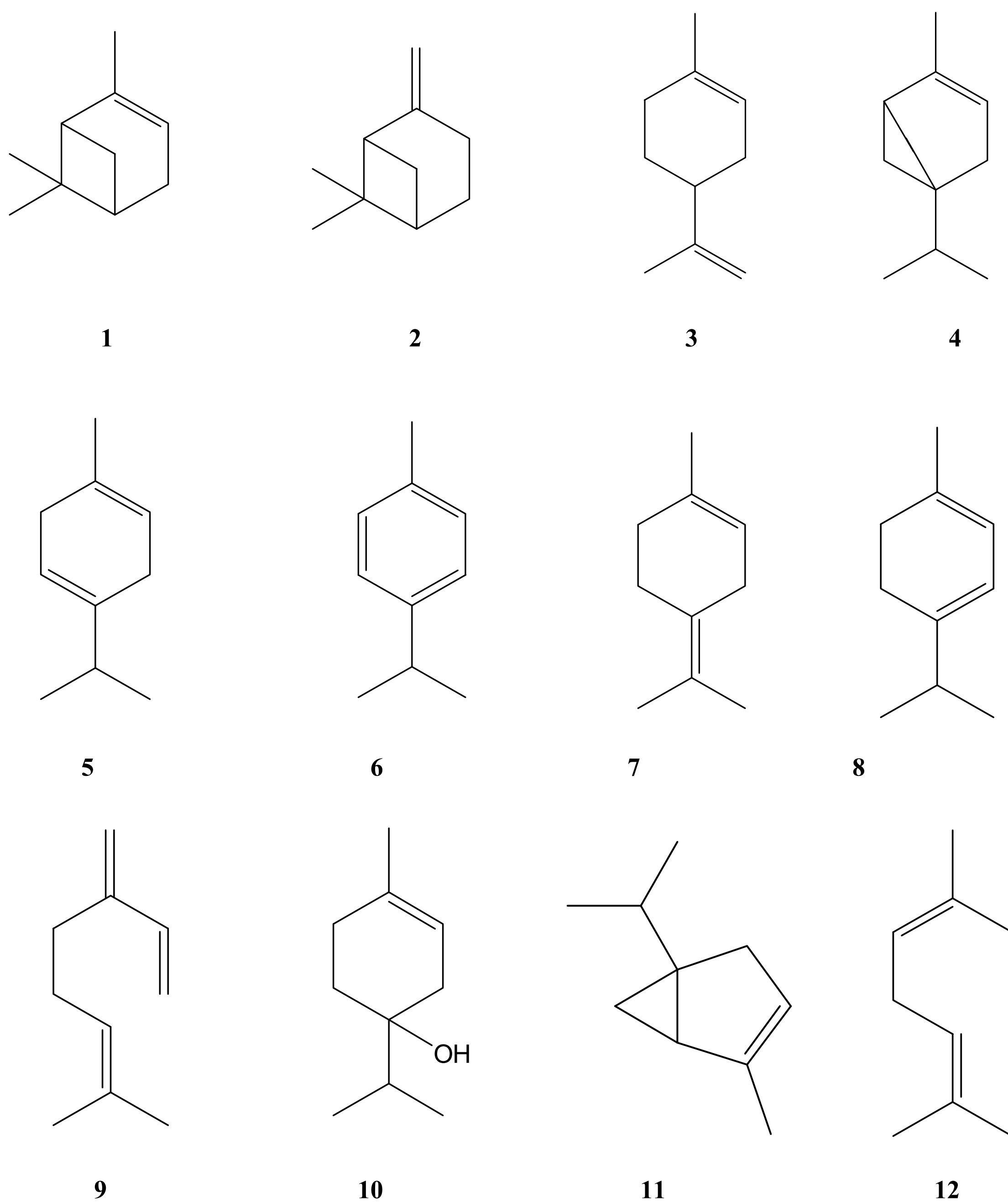
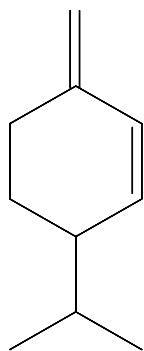
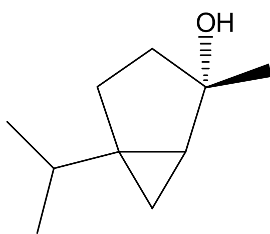
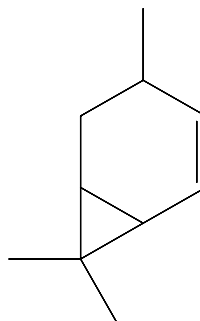
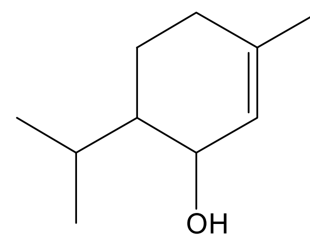


Figure 2-2 Chemical structure of monoterpenoids: [α-pinene (**1**), β-pinene (**2**), limonene (**3**), sabinene (**4**), γ-terpinene (**5**), p-cymene (**6**), terpinolene (**7**), α-terpinene (**8**), myrcene (**9**) terpinene-4-ol (**10**), α-thujene (**11**), ocimene (**12**)]

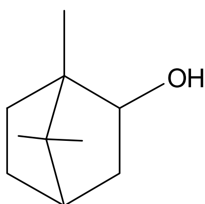


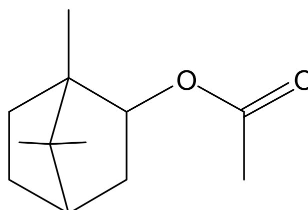


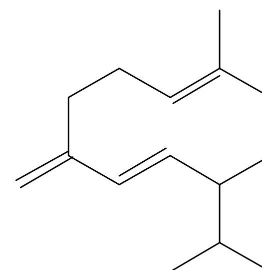




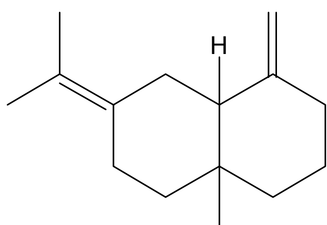
13 **14** **15** **16**

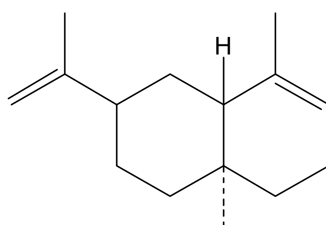


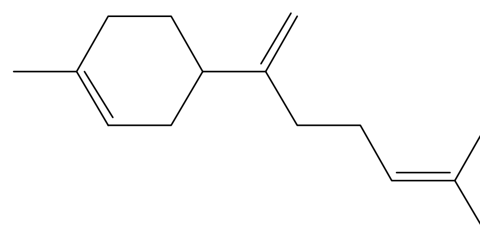




17 **18** **19**

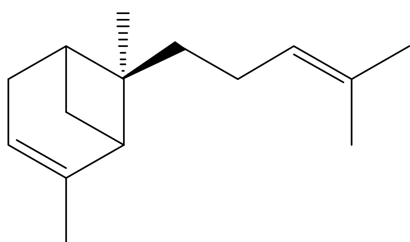


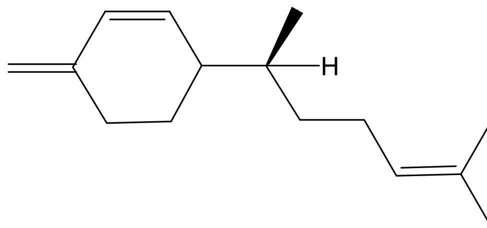




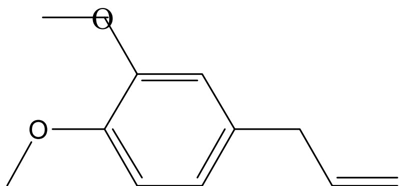
20 **21** **22**

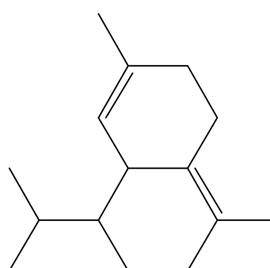
Figure 2-3 (continued) Chemical structure of monoterpenoids**:** [*β*-phellandrene (**13**), (*Z*) sabinene hydrate (**14**), 2-carene (**15**), (*E*)-piperitol (**16**), borneol (**17**), bornyl acetate (**18**), germacrene D (**19**), *γ*-selinene (**20**), *α*-selinene (**21**) and *β*-bisabolene (**22**)]

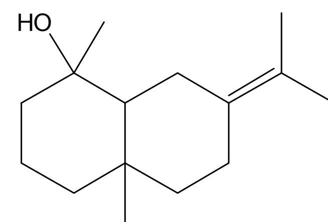




23 **24**







25 **26** **27**

Figure 2-4 (continued) Chemical structure of monoterpenoids: [*α*-bergamotene (**23**), *β*-sesquiphellandrene (**24**), methyl eugenol (**25**), *d*-cadinene (**26**) and juniper camphor (**27**)]

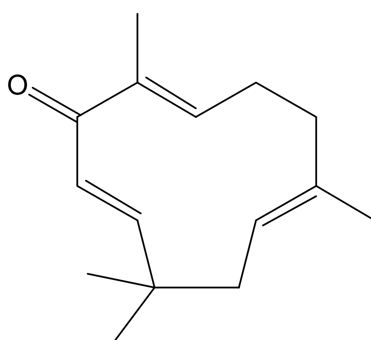


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Figure 2-5 Chemical structure of sesquiterpenoid: [zerumbone (**1**)]

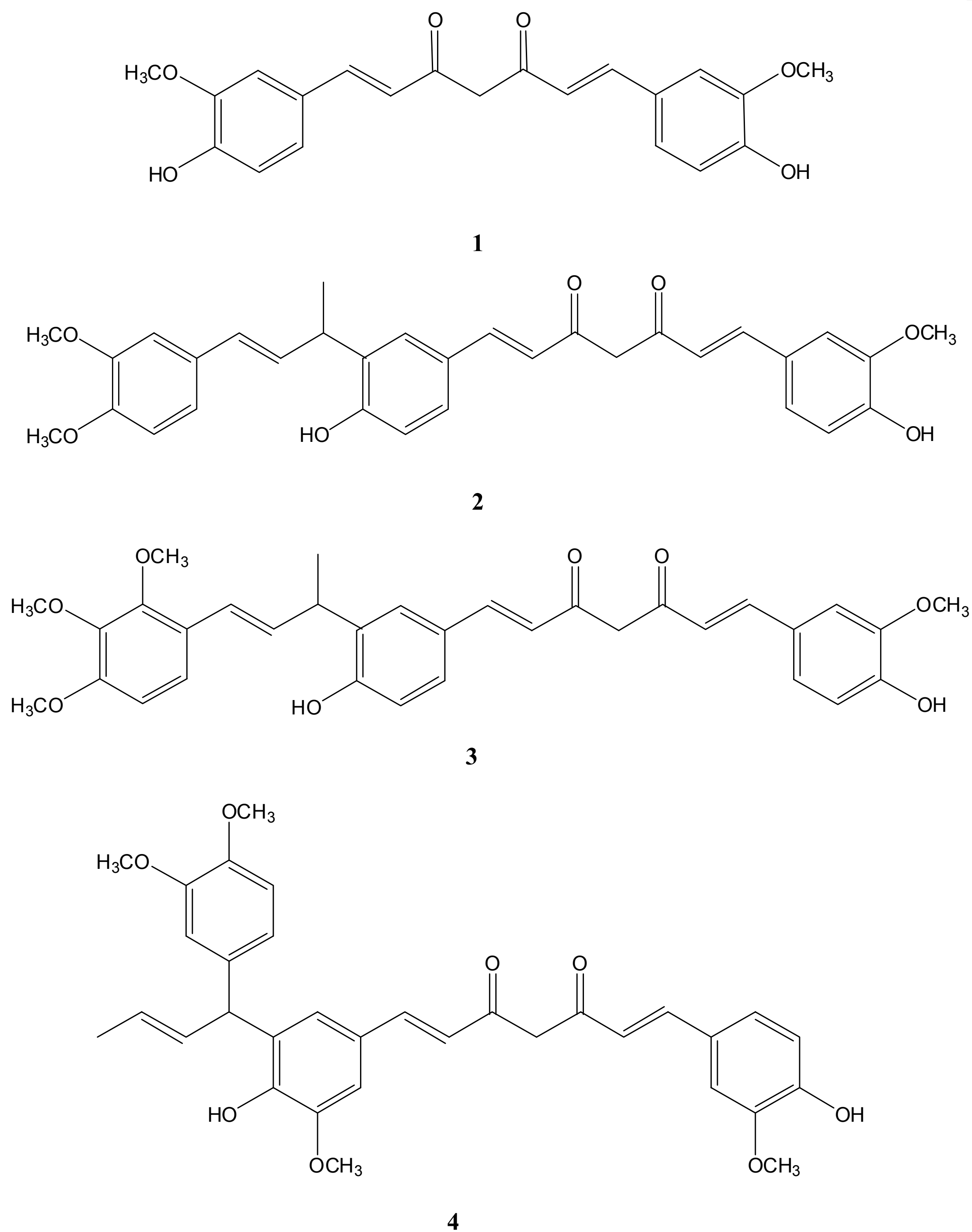
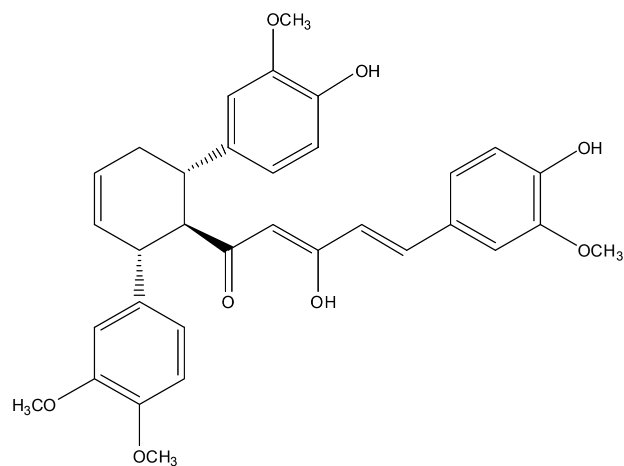


Figure 2-6 Chemical structure of curcuminoids: [curcumin (**1**), cassumunin A (**2**), cassumunin B (**3**), cassumunin C (**4**)]



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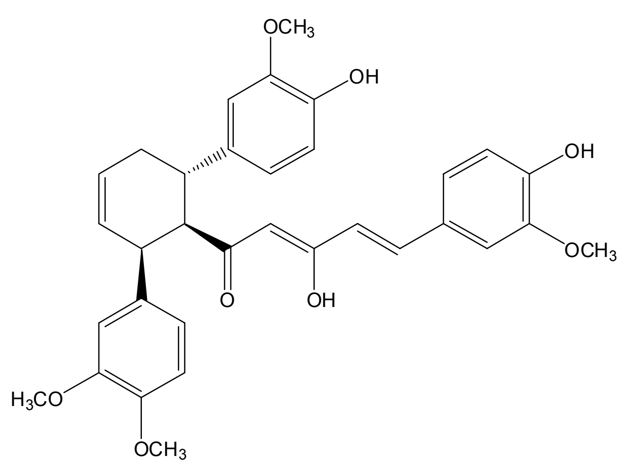

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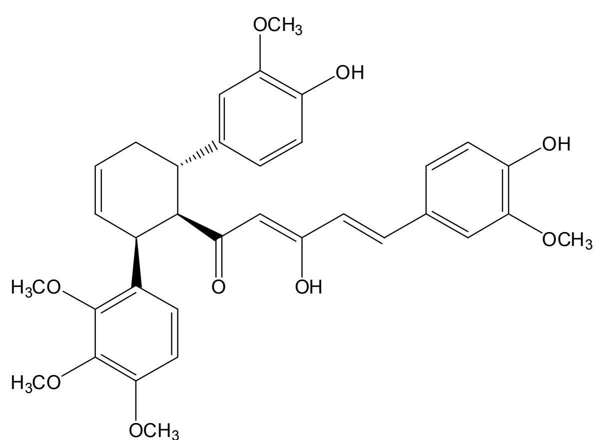

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Figure 2-7 (continued) Chemical structure of curcuminoids: [cassumunarin A (**5**), cassumunarin B (**6**) and cassumunarin C (**7**)]

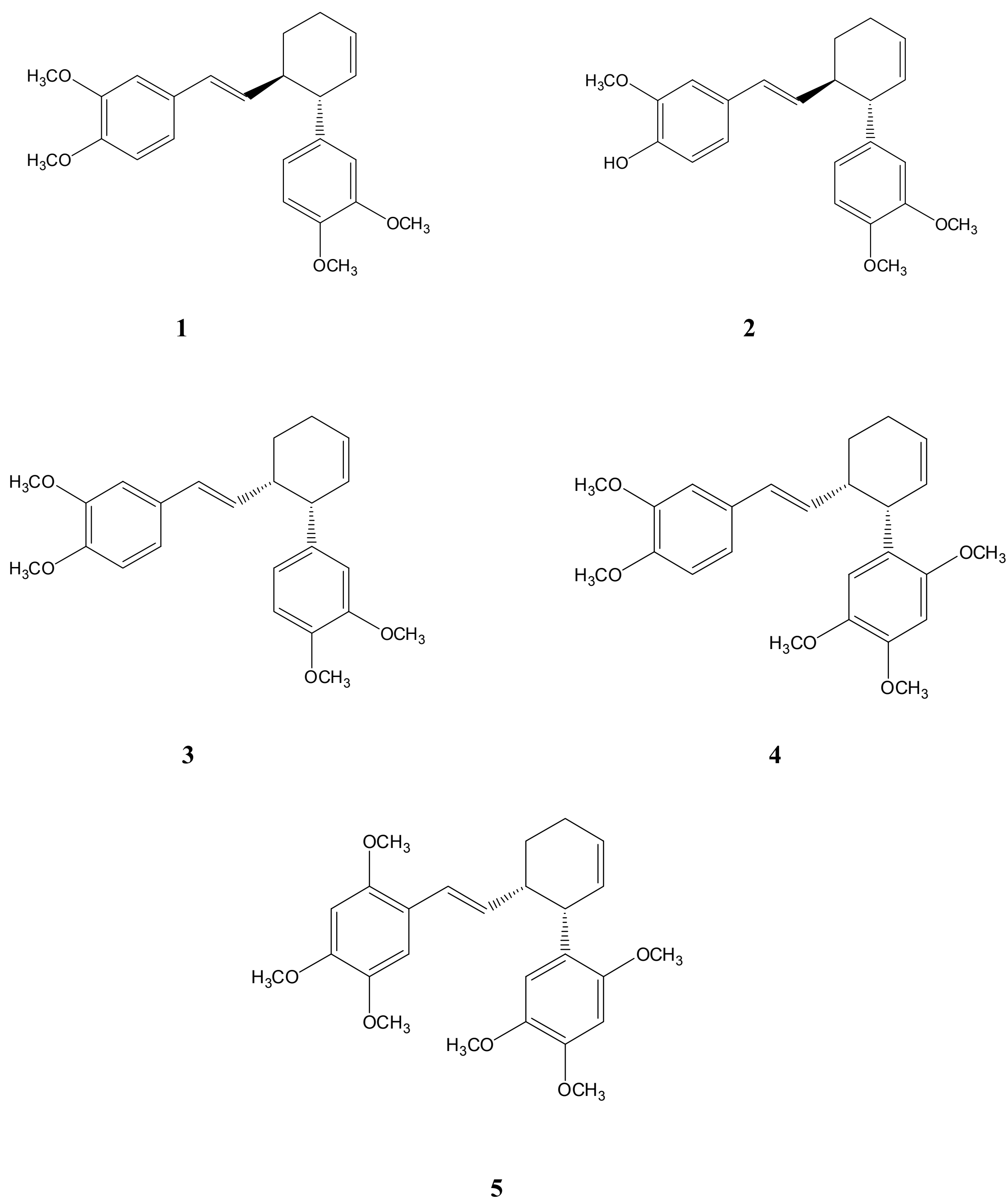


Figure 2-8 Chemical structure of cyclohexene derivatives: [(*E*)-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl]cyclohex-1-ene (**1**), (*E*)-3-(4-hydroxy-3-methoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl]cyclohex-1-ene (**2**), ((*Z*)-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxy-styryl]cyclohex-1-ene (**3**), (*Z*)-3-(3,4-dimethoxyphenyl)-4-[(*E*)-2,4,5-trimethoxystyryl] cyclohex-1-ene (**4**) and (*Z*)-3-(2,4,5-trimethoxyphenyl)-4-[(*E*)-2,4,5-trimethoxystyryl] cyclohex-1-ene (**5**)]

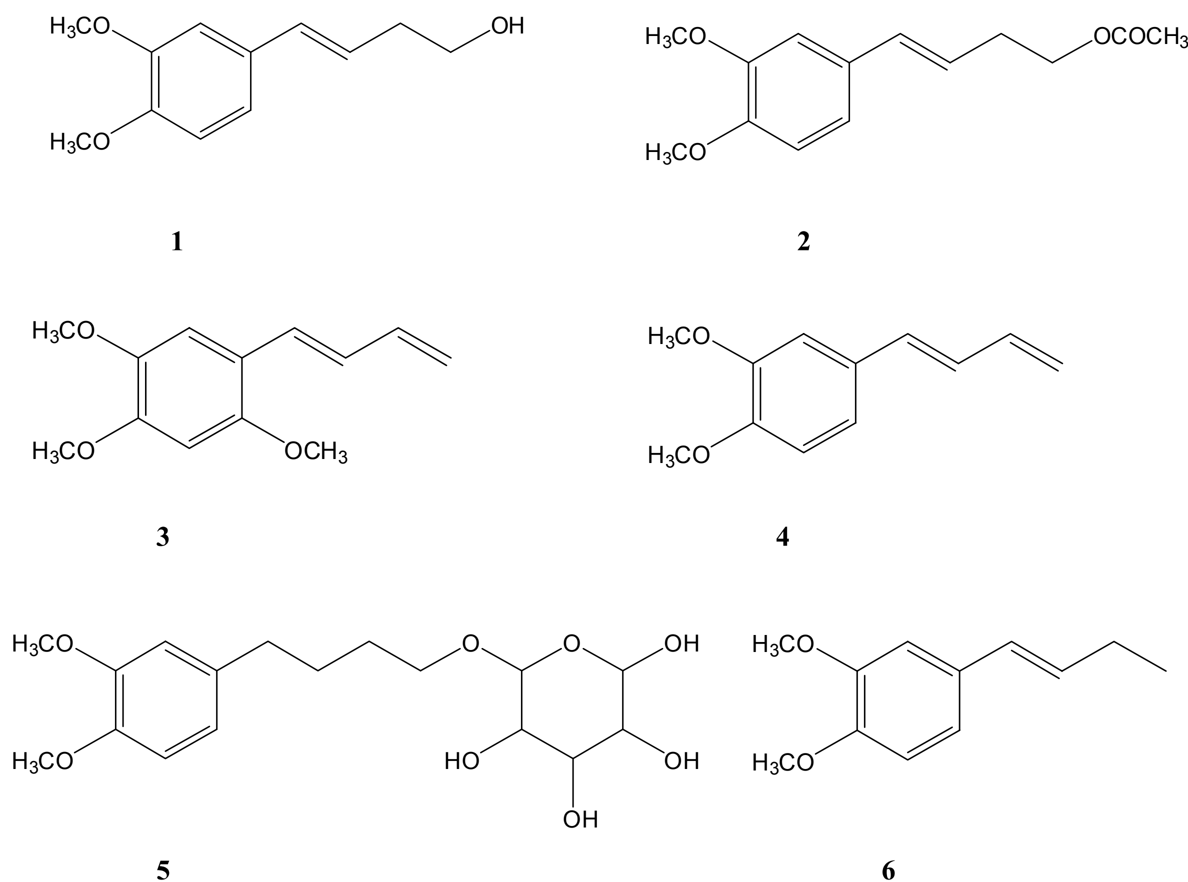


Figure 2-9 Chemical structure of phenylbutenoid derivatives: [(*E*)-4-(3,4-dimethoxyphenyl) but-3-en-l-ol (compound D) (**1**), (*E*)-4-(3,4-dimethoxyphenyl) but-3-en-l-yl acetate (**2**), (4-(2,4,5-trimethoxyphenyl)-but-1,3-diene (**3**), (*E*)-1-(3,4-dimethoxyphenyl) butadiene (DMPBD) (**4**), (*E*)-4-(3,4-dimethoxyphenyl) but-3-en-l-*O*-*β*-D-glucopyranoside (**5**) and (*E*)-1-(3,4-dimethoxy-phenyl) but-1-ene (**6**)]

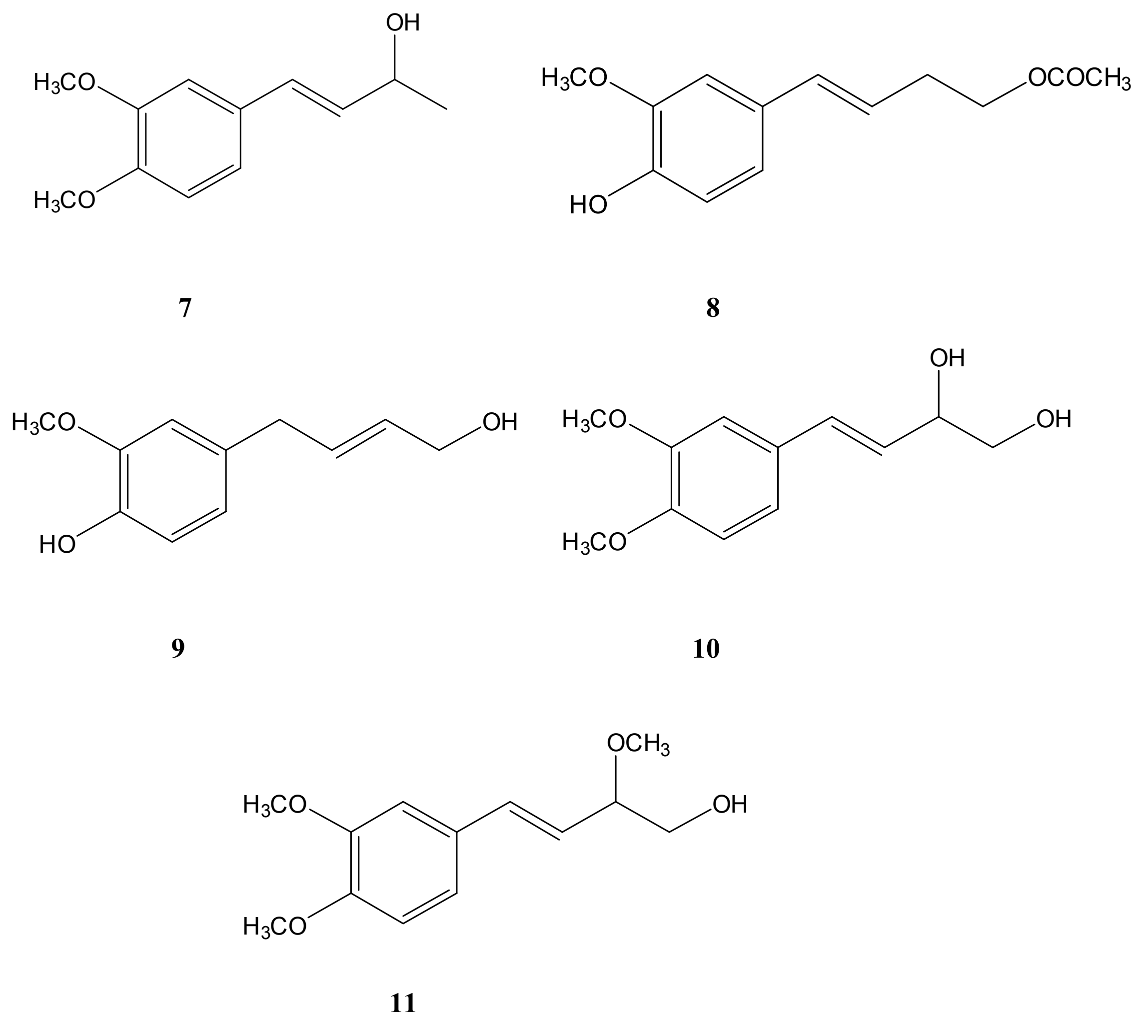


Figure 2-10 (continued) Chemical structure of phenylbutenoid derivatives**:** (*E*)-3-hydroxy-l-(3,4-dimethoxyphenyl)but-l-ene (**7**), (*E*)-4-(4-hydroxy-3-methoxyphenyl) but-3-en-l-yl acetate (**8**), (*E*)-4-(4-hydr oxy-3-methoxyphenyl) but- 2-en- 1-ol (**9**), (*E*)-2-hydroxy-4-(3,4-dimethoxy-phenyl)but-3-en- 1-ol (**10**) and (*E*)-2-methoxy-4-(3,4-dimethoxyphenyl)but-3-en-1-ol (**11**)]

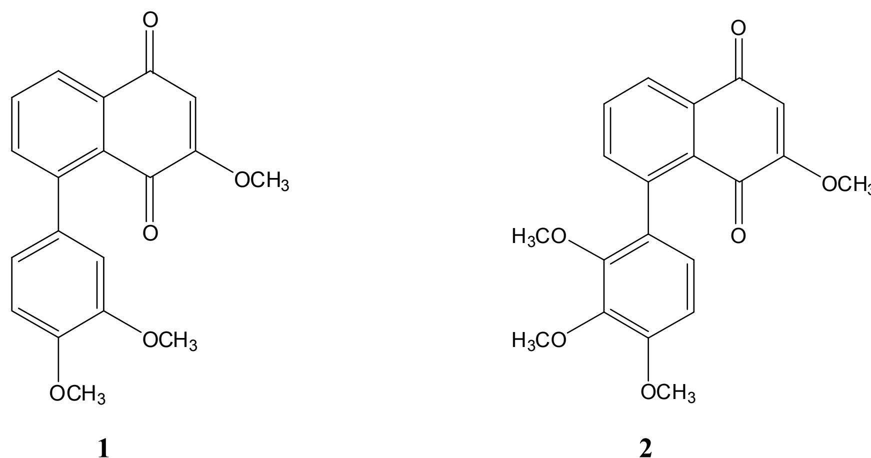


Figure 2-11 Chemical structure of napthoquinone derivatives**:** [(8-(3,4-dimethoxyphenyl)-2 methoxynaphtho-1,4-quinone (**1**) and 8-(2,4,5- dimethoxyphenyl)-2 methoxynaphtho-1,4-quinone (**2**)]

Several biological activities of *Z. cassumunar* rhizome extracts have been reported, such as anti-inflammatory, analgesic, antipyretic, smooth muscle relaxation, hypotensive, smooth muscle stimulation, anti-arrhythmic, insecticidal, local anesthetic, antibacterial, spermicidal, antifungal and anti-allergic activities.

2.4 Biological activities of *Z. cassumunar* Roxb.

Anti-inflammatory activity

It has been reported that some phenylbutenoid derivatives have anti-inflammatory activities and can be used as insecticidal constituents (Nugroho *et al*., 1996). The hexane extract seemed to possess a potent anti-inflammatory activity. Compound D, [(*E*)-4-(3,4-dimethoxy phenyl)but-3-en-1-ol] has been isolated from the hexane extract and exhibited a strong inhibitory activity on the edema formation in carageenan-induced rat paw edema (Jeenapongsa *et al*., 2003). In addition, DMPBD [(*E*)-1-(3,4-dimethoxyphenyl)butadiene] has also been isolated from hexane extract and exhibited markedly inhibitory activity on EPP-induced ear edema. This compound also exhibited moderate COX-2 inhibitory activity with the IC_{50} value of 20.68 μM (Han *et al*., 2005).

Two phenylbutenoid dimers (cyclohexene derivatives), (*E*)-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl]cyclohexene-1-ene and (*E*)-3-(4-hydroxy-3-methoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl]cyclohex-1-ene have been isolated from the chloroform extract of *Z. cassumunar* rhizomes. These two compounds exhibited the most potent COX-2 inhibitory activity with the IC_{50} values of 2.71 and 3.64 μM, respectively (Han *et al*., 2005).

The effects of methanol extract of *Z. cassumunar* rhizomes on ethanol, 0.6 M HCl, and indomethacin induced gastric mucosal lesion in rats were examined. It was found that the extract markedly inhibited the ethanol induced gastric mucosal lesions (ED_{50} = 17.8 mg/kg). In addition, this extract also significantly inhibited the lesions induced by 0.6 M HCl and indomethacin with ED_{50} values of 31.3 and 29.5 mg/kg, respectively. Similarly, a reference drug omeprazole, significantly inhibited the gastric mucosal damage produced by ethanol, 0.6 M HCl, and indomethacin with ED_{50} values of 10.9, 39.5 and 3.5 mg/kg, respectively. These results indicated that the extract of *Z. cassumunar* played a role of action similar to omeprazole in the experimentally model and probable for further developing a new anti-gastric ulcer (Pongpiriyadacha *et al*., 2003).

Cassumunarins A, B, and C were isolated from the rhizomes of *Z. cassumunar*, and the structures were determined by spectroscopic methods to be complex curcuminoids. The anti-inflammatory effect was measured by inhibition of edema formation on mouse ear induced by 12-*O*-tetradecanoylphorbol-I 3-acetate. The anti-inflammatory activity of cassumunarins was stronger than that of the curcumin (Masuda *et al*., 1995).

Analgesic and antipyretic activities

Compound D exhibited an antipyretic effect in yeast-induced hyperthermia rats and possessed comparable inhibitory activity as acetylsalicylic acid on acetic acid-induced writhing response in mice, but elicited only a weak effect on the tail flick test (Panthong *et al.,* 1990).

Smooth muscle relaxation activity

There were reports on the smooth muscle relaxant action of isolated rat smooth muscle. The doses, which completely relaxed the uterine, intestinal and fundus strips were 8, 16 and 64 mg/ml, respectively. However, the effects of water extracts on rhythm, tension and human umbilical cord were not prominent. The uterine and intestinal smooth muscle relaxing effect of the water extract could be antagonized by acetylcholine, calcium chloride and Syntocinon®. However, it could not be prevented and antagonized by either α- or β-adrenergic blocking agents (Regitine® and Inderal®) or histamine. The report suggested that a water extract did not exert its relaxation effect through α-or β-adrenergic or histaminic receptors (Anantasan, 1982).

Compound D exhibited a dose-related relaxant effect on rat and rabbit uterus with the ED_{50} values of 95 and 117 mg/kg, respectively. The activity was less than that of isoproterenol and papaverine but higher than aminophylline. The effects of compound D were not blocked by propanolol. It was suggested that compound D does not act via β-adrenergic receptors but probably by mechanisms similar to that of papaverine (Kanjanapothi *et al*., 1987).

Antiasthmatic activity

A solution of compound D in 10% alcohol also antagonized the action of histamine, acetylcholine, nicotine and serotonin. Compound D (0.067 mg/ml) antagonized the guinea pig bronchospasm induced by histamine at concentration of 7×10^{-6} g/ml. Relaxation of smooth muscle (100%) could be affected by compound D at a concentration of 0.133 mg/ml. Compound D also had a dose-dependent relaxation effect on diaphragm contraction induced by electric stimulation of phrenic nerve. At a concentration of 1.23 mg/ml, the stimulated muscle contraction was abolished (Kiatyingungsulee *et al*., 1979).

There were reports on a clinical study of *Z. cassumunar* rhizomes for antiasthmatic activity in children. Eight patients, 5 males and 3 females at ages of 9-13 years, had asthmatic attacks and never received medicine before. The studies were carried out by oral administration of powdered rhizome at a dose of 260 mg. The physical condition, heart rate, breathing ability and lung function were examined before and after receiving the drug at 30 minutes interval for 2 hours. The result showed that after receiving the drug, the patient physical condition and lung function were improved while the heart rate and blood pressure were

unaffected. In severe cases, the patients were not improved. In long-term treatment, 12 patients (boys 8, girls 4) at the age of 9-14 years, received 130 mg of powdered rhizome 2 times a day after meals for 3 months. The results showed that all patients were improved with no complication or toxicity being observed (Tuchinda *et al*., 1985).

An open study was carried out on 22 patients with moderate degree of asthma to assess the prophylactic efficacy and tolerance of the rhizomes. The results showed that the rhizomes provided a significant protection against asthmatic attacks. Statistically significant reduction in asthmatic symptoms as well as a reduction in the use of bronchodilators and steroids was noted between 1-3 and 2-4 months after treatment, respectively. Statistically significant improvement in peak expiratory flow rate and forced expiratory volume in one second were also noted in the second month of treatment. Slightly increased frequency in bowel movement was noted in 5 patients and one patient complained of mild headache (Yongchaiyud *et al*., 1985).

Pharmacokinetic studies of a preparation prepared from an ethanolic extract of *Z. cassumunar* rhizome were performed in rats and monkeys. The apparent first order transfer rate constant, using *in situ* intestinal loop technique, was found to be 0.0731 min^{-1}. *In vivo* study by oral administration of drug to rats and determination of the concentration of bronchodilation of this plant in plasma also showed rapid absorption with a rate constant of 0.0385 min^{-1}. Maximum plasma level of 0.75 µg/ml was observed at 1.04 hours after drug administration. Elimination half-life in monkeys (2.31 hours) was found to be not statistically different from rat (2.00 hours). Prolong half-life after multiple dosing was observed in monkeys. An accumulation ratio of 3.13 was found after long term drug administration. The pharmacokinetic parameters obtained from this study provide valuable information for future use in clinical evaluation of *Z. cassumunar* for the treatment of asthmatic patients (Koysooko *et al*., 1988).

Hypotensive activity

The sodium salt of compound D at a dose of 100 mg/kg and 1 g/kg could diminish the activity of nicotine in dogs by 46.7 and 66% respectively. The water extract of rhizomes had a hypotensive activity when applied to anesthetized mice with pentobarbital sodium. The activity was dose-dependent. An alcoholic extract of *Z. cassumunar* rhizomes

administered intravenously to dogs at various doses, was reported to be equivocal (Mokkhasmit *et al*., 1971).

Anti-arrhythmic activity

A water extract of *Z. cassumunar* rhizomes showed antiarrythmic activity in normal mice when intravenously injected at dose of 1.33 g/kg. There were effects on the EKG by reducing the height of T-wave, S-T segment but increasing the P-R interval QRS complex, followed by ventricular tachycardia, ventricular fibrillation and finally, cardiac arrest (Anantasan and Kruanak 1976).

The water extract of *Z. cassumunar* rhizomes at concentration of 14% (w/v) exhibited a depression of the contractility and rhythmicity of isolated atrium strips. This activity could be antagonized by calcium chloride, adrenaline and atropine. Other than the above activities, the water extract depressed excitability and contractility of isolated right ventricular strips of mice, but increased the conduction time and effective refractory period, similar to the actions of quinidine. Tests on cardiac arrhythmia-induced mice showed that the water extract could not depress ventricular fibrillation, while quinidine did (Anantasan, 1977).

The effects of the rhizome extract on atrial activity was investigated and reported that the extract caused negative inotropic effects on both rhythmicity and contractility of turtle myocardial. The effects could not be blocked by atropine, but were blocked by calcium (Veerasarn *et al*., 1971).

Compound D (0.04 mg/ml) exhibited positive inotropic and chronotropic effects on guinea pigs. There was an increase in contraction and rate of 100 - 200% and 20%, respectively. When the concentration was increased to 0.64 mg/ml, cardiac arrest was observed (Kiatyingungsulee *et al*., 1979).

Insecticidal activity

A methanol extract of *Z. cassumunar* was found to be considerably lethal to *Spodoptera litura*. The active principles were a mixture of terpenes containing β-pinene and sabinene as the major components (Rukachachaisirikul *et al*., 1983).

Local anesthetic activity

A water extract of *Z. cassumunar* exhibited local anesthetic action on the sciatic nerve of toads. The activity was similar to that of lidocaine (Anantasan and Asayakun, 1975).

Antimicrobial activity

The essential oil of *Zingiber cassumunar* (Plai oil) exhibits antimicrobial activity against a wide range of *Staphylococcus aureus* ATCC 29737, *S. pyrogenes*, *S epidermidis* ATCC 12228, *Propionibacterium acnes*, *Escherichia coli* ATCC 10536, *Salmonella typhi*, *Pseudomonas aeruginosa* ATCC 25619, *Klebsiella pneumoniae* ATCC 10031, *Proteus vulgaris*, *Epidermophyton occosum*, *Microsporum gypseum*, *Trichophyton mentagrophytes*, *Trichophyton rubrum*, *Candida albicans* and *Cryptococcus neoformans*. Dermatophytes were found to be the most susceptible microorganisms followed by yeasts, whereas bacteria were the least susceptible. The mean diameter of the inhibition zone determined by the disc diffusion screening method increased with increasing Plai oil concentration between 6.25 and 50 vol %. The minimum bactericidal concentration (MBC) determined by the broth macrodilution method ranged from 0.62 to 2.5 vol % for Plai oil and from 52 to 79 mg/ml for the 5 wt % Plai oil gel, whereas the minimum fungicidal concentration (MFC) ranged from 0.31 to 1.25 vol % for Plai oil and from 13.8 to 39.5 mg/ml for the 5% Plai oil gel (Pithayanukul *et al*., 2007).

The essential oil of *Z. cassumunar* rhizome was rich in terpinen-4-ol (37.7%), β-pinene (20.8%), (*E*)-1(3,4-dimethoxyphenyl)but-1-ene (13.3%), γ-terpinene (5.1%) and (*E*)-1-(3,4-dimethoxyphenyl)butadiene (4.7%). The presence of a high concentration of terpinen-4-ol in this oil could explain its moderate activity against the yeasts. A previous study on the antifungal assay of standard sample of terpinen-4-ol showed that the compound exhibited moderate to strong activities against *Saccharomyces cerevisiae* (ATCC 9783), *Cryptococcus neoformans*, *Candida albicans*, *C. tropicalis*, *Torulopsis glabrata*, *Trichophyton mentagrophytes, T. rubrum, Microsporum canis, M. nanum, Epidermophyton floccosum, Aspergillus niger, A. fumigatus* and *Mucor* sp. (Jantan, *et al*., 2003).

The rhizomes of *Z. cassumunar* exhibited strong fungitoxic action against *Rhizoctonia solani*, the damping-off pathogen. On chemical and spectral investigations, the antifungal compound was found to be zerumbone as a sesquiterpene. Its minimum effective dose

against *R. solani* was 1000 ppm, much lower than some commercial fungicides. Zerumbone had fungistatic activity and was not phytotoxic. Moreover, when used as a seed treatment, zerumbone could control damping-off disease of *Phaseolus aureus* caused by *Rhizoctonia solani* by 85.7% (Kishore and Dwivedi, 1992).

Spermicidal activity

The screening of contraceptive medicinal plants indicated that *Z. cassumunar* extract possessed spermicidal effect *in vitro* (Wasuwat *et al*., 1982). Terpinen-4-ol, an active ingredient extracted from essential oil of *Z. cassumunar* showed 100% spermicidal activity at concentration 0.016% (Wasuwat *et al*., 1982).

The study on the spermicidal effect, *in vitro*, on ox sperm, of the fraction and pure compound isolated from *Z. cassumunar*, revealed that fraction no. 3 and 4 and terpinen-4-ol isolated from fraction no. 4, possessed ED_{100} at the concentrations of 0.05, 0.025 and 0.015%, respectively, equivalent to that of nonylphenoxy polyethoxy ethanol (Wasuwat *et al*., 1986).

Anti-allergic activity

The anti-allergic effect of *Z. cassumunar* ethanol extract (IC_{50} = 12.9 µg/ml) was two-fold higher than that of ketotifen fumarate (IC_{50} = 20.2 µg/ml), the positive control. It is suggested that the plant extract may contain some certain compounds that have IC_{50} values considerably lower than that of ketotifen fumarate. The active compound of *Z. cassumunar* is likely to be useful as lead compounds for the development of a novel class of anti-allergic agents (Tewtrakul and Subhadhirasakul, 2007).

A study on the antihistamine effect by skin testing of the rhizomes was carried out on 24 asthmatic children who were not treated with medicine for 24 hours. There were 13 males and 11 females, age ranged between 8-16 years. Twelve subjects were tested for effect of “Plai” by using intracutaneous test with 0.02 ml of 1 : 100 histamine solution before and 1 hours after taking 500 mg of “Plai” orally. The results revealed that 500 mg of rhizomes had less antihistaminic effect than 0.1 mg/kg chlorpheniramine when orally administered (Piromrat *et al*., 1986).

Gelatinase activity

The extract of *Z. cassumunar* inhibited the gelatinase activity from stimulated oral epithelial cells at the mRNA level. The activity of Matrixmetalloproteinase-9 (MMP-9) was increased in oral epithelial cells stimulated with 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), interleukin-1β (IL-1β), and retinoic acid (RA) (21577.00±11.88 mm, 19922.67±5.62 mm, and 19100.67±17.76 mm, respectively), when compared with untreated control cells (14105.33±5.81 mm). Consistently, induction of MMP-9 mRNA by TPA, IL-1β, and RA was inhibited by the extract in a dose-dependent fashion. Similar to MMP-9, induction of MMP-2 mRNA was inhibited by the extract. However, no inhibitory effect on the MMP-2 and MMP-9 activity by the extract was found in oral fibroblasts. There was no significant increase in cytotoxicity in treated oral fibroblasts and epithelial cells as compared with untreated cells ($P > 0.05$). The findings from this *in vitro* study will be essential for future development of a new drug in the management of inflammatory oral diseases (Chotjumlong *et al*., 2005).

Antioxidant activity

New antioxidants, cassumunarins A, B, and C, were isolated from the rhizomes of *Z. cassumunar*, and the structures were determined by spectroscopic methods to be complex curcuminoids. The antioxidant efficiency of cassumunarins was determined by inhibitory activity of autooxidation of linoleic acid in a buffer-ethanol system. The antioxidant activity of cassumunarins (ED_{50} = 2.7 µmol) was stronger than curcumin (Masuda *et al*., 1995).

Effect of new complex curcuminoids (cassumunin A and cassumunin B) isolated from *Z. cassumunar*, were examined in dissociated rat thymocytes suffering from oxidative stress induced by 3 nM hydrogen peroxide by using a flow cytometer and ethidium bromide. The effects were compared with those of curcumin, a natural antioxidant, whose chemical structure is included in those of cassumunins A and B. Pretreatment of rat thymocytes with the respective cassumunins at concentrations ranging from 100 nM to 3 µM dose-dependently prevented the hydrogen peroxide (H_2O_2)-induced decrease in cell viability. The potencies of cassumunins A (ED_{50} = 1 µM) and B (ED_{50} = 3 µM) in protecting the cells suffering from H_2O_2-induced oxidative stress were greater than that of curcumin (ED_{50} = 300 nM - 3 µM). It is suggested that

cassumunins A and B may possess a potent protective activity on living cells suffering from oxidative stress (Nagano *et al*., 1997).

Anti-tumor activity

The chloroform extract of *Z. cassumunar* showed strong inhibitory activity against Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) in Raji cells. At all concentrations tested (20-640 µg/ml), the chloroform extract of *Z. cassumunar* demonstrated complete inhibitory activity (IE = 100%), the petroleum ether extract showed complete inhibitory activity (IE = 100%) at concentration 20-80 µg/ml and the ethanol extract demonstrated 70-100% IE at concentrations 40-640 µg/ml. All these extract concentrations significantly inhibited EBV activation (Vimala *et al*., 1999).

Anticancer activity

(*E*)-3-(3,4-dimethoxyphenyl)-4-[(E)-3,4 dimethoxystyryl]cyclohex-1-ene (PSC) isolated from *Z. cassumunar*, inhibited proliferation of various human cancer cells with the IC_{50} values ranging from 10 to 30 µM. Prompted by these anti-proliferative effects, it was performed using A549 human lung cancer cells which exhibited the most potent inhibitory activity with IC_{50} of 15.6 mM at 72h. PSC arrested cell cycle progression at the G0/G1 phase in a concentration- and time-dependent manner. PSC dose-dependently induced cyclin-dependent kinase (CDK) inhibitor p21 expression, whereas the expression of cyclin D1, cyclin A, CDK4, CDK2, and proliferating cell nuclear antigen (PCNA) were decreased by treatment with PSC. These results suggest that one of the anti-proliferative mechanisms of PSC is to suppress cell cycle progression by increasing p21 expression and down-regulating cyclins and CDKs (Lee *et al*., 2007).

Toxicity assessment

Acute toxicity was not observed using both 50% alcohol extract (either subcutaneously or orally administered to mice at a dose of 10 g/kg) and compound D suspended in 2% tween 80 [either intraperitoneally or orally administered to mice at a dose (calculated as rhizome) of 30 g/kg)]. Toxicity was observed when the sodium salt of compound D was administered at a dose of 450 mg/kg by the intraperitoneal route. The mice had quick and deep

breathing, slow movement and fatigue of the legs but all of the mice survived through the experiment. By gross and histopathological examinations, there were no chronic toxicities (Kiatyingungsulee *et al*., 1979).

LD_{50} of a water extract of *Z. cassumunar* in mice was 4.00 mg/kg. The observed toxicities were lowering of body temperature, convulsion, difficult breathing and finally death. The administration of quinidine at a dose of 5.6 mg/kg with propanolol at a dose of 2 mg/kg decreased the mortality rate and the LD_{50} was increased to 6.00 mg/kg (Kiatyingungsulee *et al*., 1979). Another report showed that a 50% alcohol extract of *Z. cassumunar*, either orally or subcutaneously administered to mice at a dose of 10 g/kg, showed no toxicity (Mokkhasmit *et al*., 1971).

The toxicity of the powdered rhizomes was also investigated. The LD_{50} in mice was more than 10 g/kg and a hepatotoxic effect was observed after administration for a period of 1 year. Administration of up to 50 times the therapeutic dose to monkeys for 6 months showed no change in internal organ morphology (Koysooko *et al*., 1987).

2.5 Biological significance of nitric oxide

Nitric oxide (NO, formula N=O) is a simple, inorganic, gaseous free radical whose predominant functions are that of a messenger and effector molecules. In mammals, NO is synthesized by a family of enzymes referred as the nitric oxide synthases (NOS). NO is physiologically significant for its role in regulating vascular tone and in signaling neurotransmission Moncada *et al*., 1991). Nitric oxide is also an important component of the antineoplastic and antimicrobial armament of macrophages (Coleman *et al*., 2001). This highly labile and noxious gas is produced in large and sustained quantities by macrophages following exposure to a variety of immunologic and inflammatory mediators. The high-output production of nitric oxide is dependent on induction and expression of the inducible nitric oxide synthase (iNOS) expressed in macrophages (Fang *et al*., 1997). Endothelial cells and neurons also express unique forms of nitric oxide synthase as eNOS and nNOS, respectively. However, the expressions of both enzymes are constitutive and nitric oxide is produced at lower steady state levels than iNOS (Hevel *et al*., 1991). It is this low level of production that is biological significance, while overproduction may lead to circulatory shock, chronic inflammation and carcinogenesis (Hidaka

et al., 1997). NO and its functions have been shown to be more and more complex in physiological and pathological processes, for example, NO can regulate vascular tone, smooth muscle cell relaxation, neurotransmission, neuromodulation, apoptosis (Kim *et al*., 2004) and modulate mitochondrial energy generation (Moncada and Erusalimsky, 2002). NO also has been implicated in different mechanisms of diseases such as atherosclerosis (Barton and Haudenschild, 2001), asthma (Fischer *et al*., 2002), neurologic disorders and septic shock (Thiemermann *et al*., 1997). The isoforms of nitric oxide synthase and their major physiological functions and implications in various diseases are summarized in Figures 2-12 and 2-13.

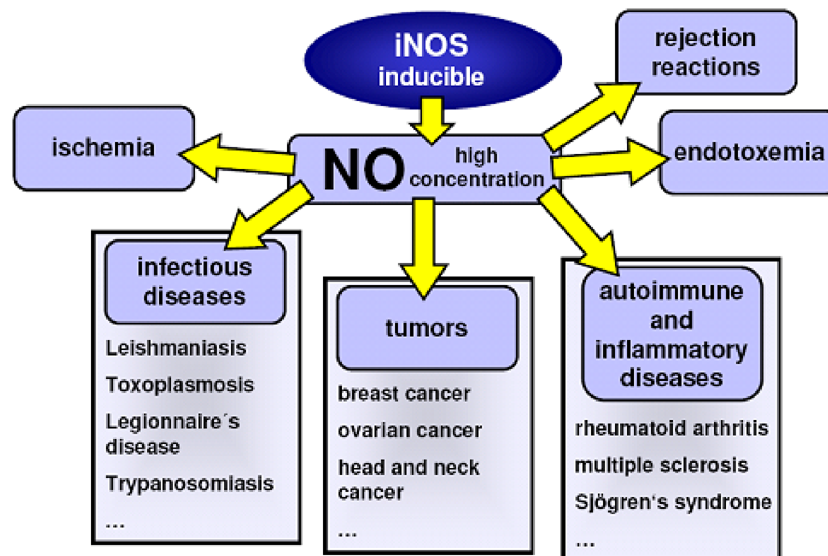


Figure 2-12 The isoforms of nitric oxide synthases. Two cNOS enzymes (eNOS, nNOS) are contrasted by a third, inducible NOS (iNOS) (Hemmrich *et al*., 2003).

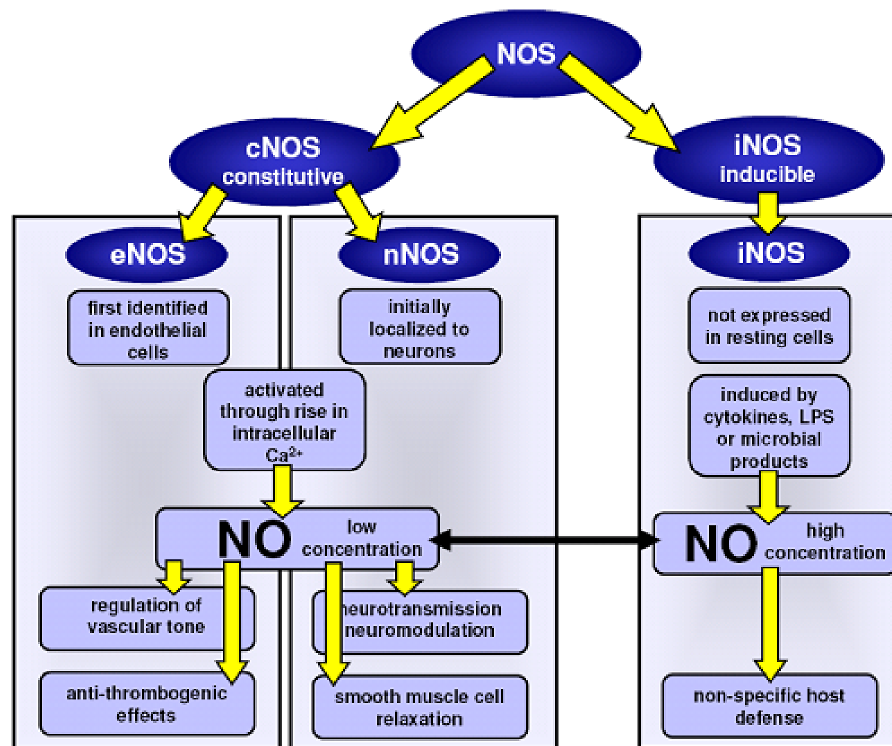


Figure 2-13 Implication of iNOS-derived NO in various human diseases. NO demonstrates its key roles in the human defense against adverse factors from the environment. Furthermore, many chronic inflammatory diseases are associated with sustained iNOS expression (Hemmrich *et al.*, 2003).

Nitric oxide (NO) is one of the inflammatory mediators causing inflammation in many organs. This inorganic free radical has been implicated in physiologic and pathologic processes, such as vasodilation, non-specific host defense and acute or chronic inflammation. NO is produced by the oxidation of L-arginine by NO synthase (NOS). In the family of NOS, inducible NOS (iNOS) is involved in pathological aspects, and can be expressed in response to pro-inflammatory agents such as tumor necrosis factor-α (TNF-α), interleukin 1-β (IL-1β) and lipopolysaccahride (LPS) in various cell types including macrophages. NO acts as a host defense by damaging pathogenic DNA and as a regulatory molecule with homeostatic activities. However, excessive production of this free radical is pathogenic to the host tissue itself, since NO can bind with other superoxide radicals and acts as a reactive radical which directly damages the function of normal cell (Tewtrakul and Itharat, 2007).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant material

The rhizomes of *Z. cassumunar* were collected from Songkhla province in Thailand in October 2007. The voucher specimen (specimen No. SKP 206 26 03 01) was deposited at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. The rhizomes were washed and dried at 50 °C in hot air oven for 24 hours and then ground into powder.

3.1.2 Chemicals and reagents

3.1.2.1 Extraction and purification

- Methanol, commercial grade (Lab scan Asia, Thailand)
- Ethyl acetate, commercial grade (Lab scan Asia, Thailand)
- Chloroform, commercial grade (Lab scan Asia, Thailand)
- Hexane, commercial grade (Lab scan Asia, Thailand)
- Silica gel 60 (SiO_2 60, 230-400 mesh) (Merck, Germany)
- TLC plate F_{60} 254 (Merck, Germany)
- Anisaldehyde-Sulfuric Acid TS (consisted of anisaldehyde 0.5 ml, gracial acetic acid 10 ml, methanol 85 ml and sulfuric acid 5 ml)

All solvents were redistilled before use.

3.1.2.2 HPLC analysis

- HPLC column TSK gel ODS 80Tm (Biosciences, Japan)
- Distilled water (Faculty of Pharmaceutical Sciences, Thailand)
- Methanol, HPLC grade (Lab scan Asia, Thailand)
- Acetic acid, glacial AR grade (Lab scan Asia, Thailand)

3.1.2.3 Anti-inflammatory activity assay

- Lipopolysaccharide (LPS, from *Escherichia coli*) (Gibco, USA)
- RPMI 1640 medium (Gibco, USA)
- 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazodium bromide (MTT) (Gibco, USA)
- L-nitroarginine (L-NA) (Gibco, USA)
- Caffeic acid phenethylester (CAPE) (Sigma, USA)
- Phosphate-buffered saline (PBS) (Sigma, USA)
- Foetal calf serum (FCS) (Gibco, USA)
- Trypan blue (Gibco, USA)
- Trypsin-EDTA (Gibco, USA)
- Dimethyl sulfoxide (DMSO) (Lab scan Asia, Thailand)
- Isopropanol (Lab scan Asia, Thailand)

3.1.3 Instruments

The equipments used in this study were listed in Table 3-1.

Table 3-1 General information of equipments

Instrument	Model	Company
IR spectrophotometer	JASCO IR-810	Japan Spectroscopic, Japan
Hot air over	DIN 12880-KI	Memmert, Germany
HPLC	SN 4000	Fortune Scientific, Thailand
Rotary evaporator	N-N Series	EYELA, Japan
NMR spectrometer	UNITY INOVA	Varian, USA
Laminar air flow	Faster Ultrasafe 48	FASTER, Italy
CO_2 incubator	Shel LAB	GIBTHAI, Thailand

3.2 Methods

3.2.1 Preparation of *Z. cassumunar* extract

The dried powder of *Z. cassumunar* rhizomes (0.5 kg) (Figure 3-1) was refluxed with hexane (2 L x 3) for 1 hour. The pooled extract was then evaporated to dryness *in vaccuo*. The yield of the hexane extract was recorded.

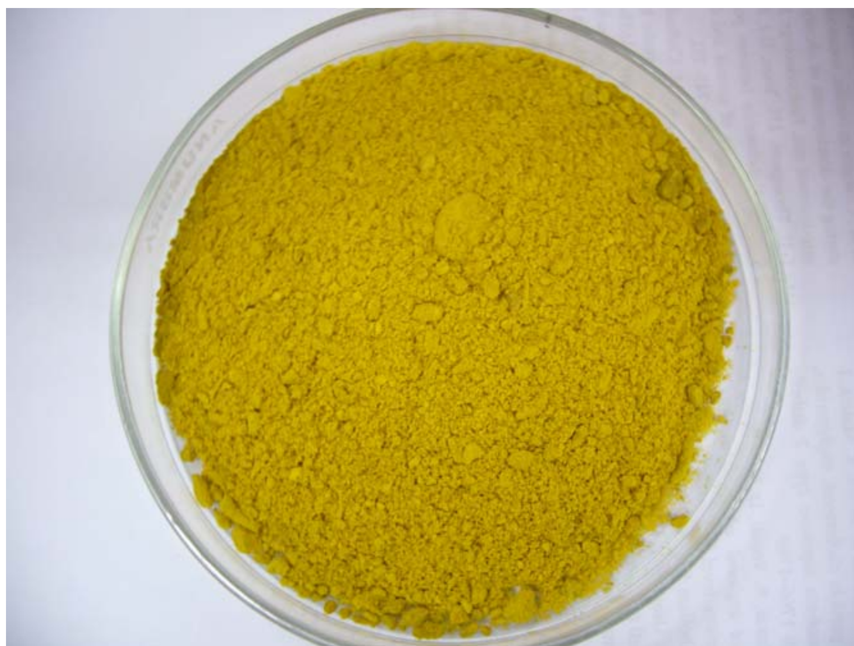


Figure 3-1 Dried powder of *Z. cassumunar* rhizomes

3.2.2 Preparation of essential oil from *Z. cassumunar* rhizomes

The essential oil of *Z. cassumunar* rhizomes was prepared, using water distillation method (modified from Subcommittee on the Establishment of the Thai Herbal Pharmacopoeia, 1998). The fresh rhizomes were cut to small pieces (500 g) and placed in the round-bottomed flask of suitable apparatus. A sufficient was added and heated between 130°C and 150°C to boiling. Continue boiling for 5 hours and allowed it to stand for some time, open the stopper of the apparatus and draw off the water until obtain the volatile oil.

3.2.3 Isolation of the active compounds from *Z. cassumunar* extract

The hexane extract of *Z. cassumunar* was subjected to silica gel vacuum chromatography. A sintered glass column (13 cm in diameter) was packed with silica gel approximately 6 cm high. The hexane extract (25 g), which pre-adsorbed on silica gel, was loaded as a thin layer on the surface of column. The column was eluted with hexane and mixtures

of hexane and chloroform (9:1, 8:2 and 7:3) (500 ml x 2) with the aid of a vacuum pump. The pooled fractions of *Z. cassumunar* extract were then dried *in vaccuo* (figure 3-2).

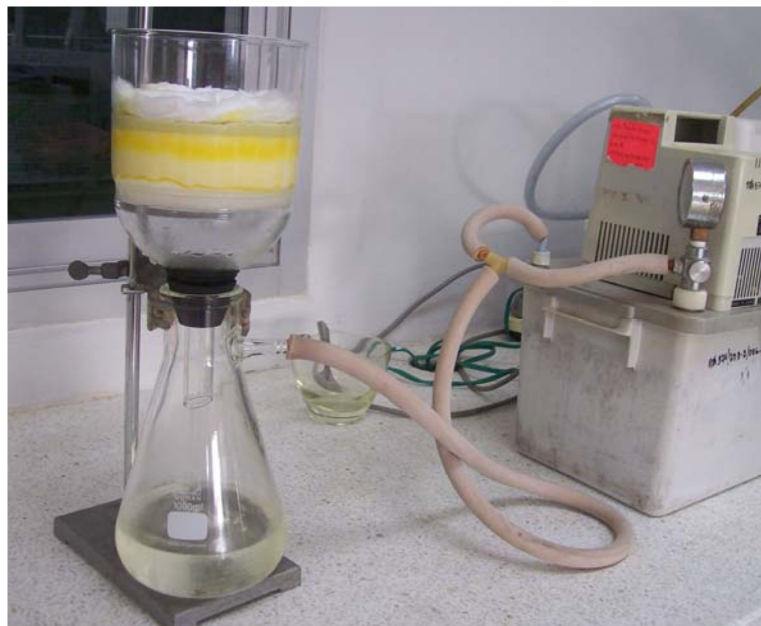


Figure 3-2 Silica gel vacuum chromatography

The pooled hexane fractions were further isolated by silica gel column chromatography eluted with a mixture of hexane and ethyl acetate (95:5). The fractions of each 30 ml were collected. The pooled fractions 1-7 were rechromatographed with the same chromatographic conditions. A yellowish oily liquid (P4) was obtained from the pooled fractions 4-11 (Figure 3-3). The pooled fractions 6-10 (3 g) were isolated by silica gel column chromatography eluted with a mixture of hexane and ethyl acetate (7:3). The fractions of each 30 ml were also collected. The pooled fractions1-6 and 7-11 were rechromatographed with the same chromatographic conditions. The yellowish oily liquids (P1 and P2) were obtained from the pooled fractions 1-3 and 1-5, respectively (Figure 3-3).

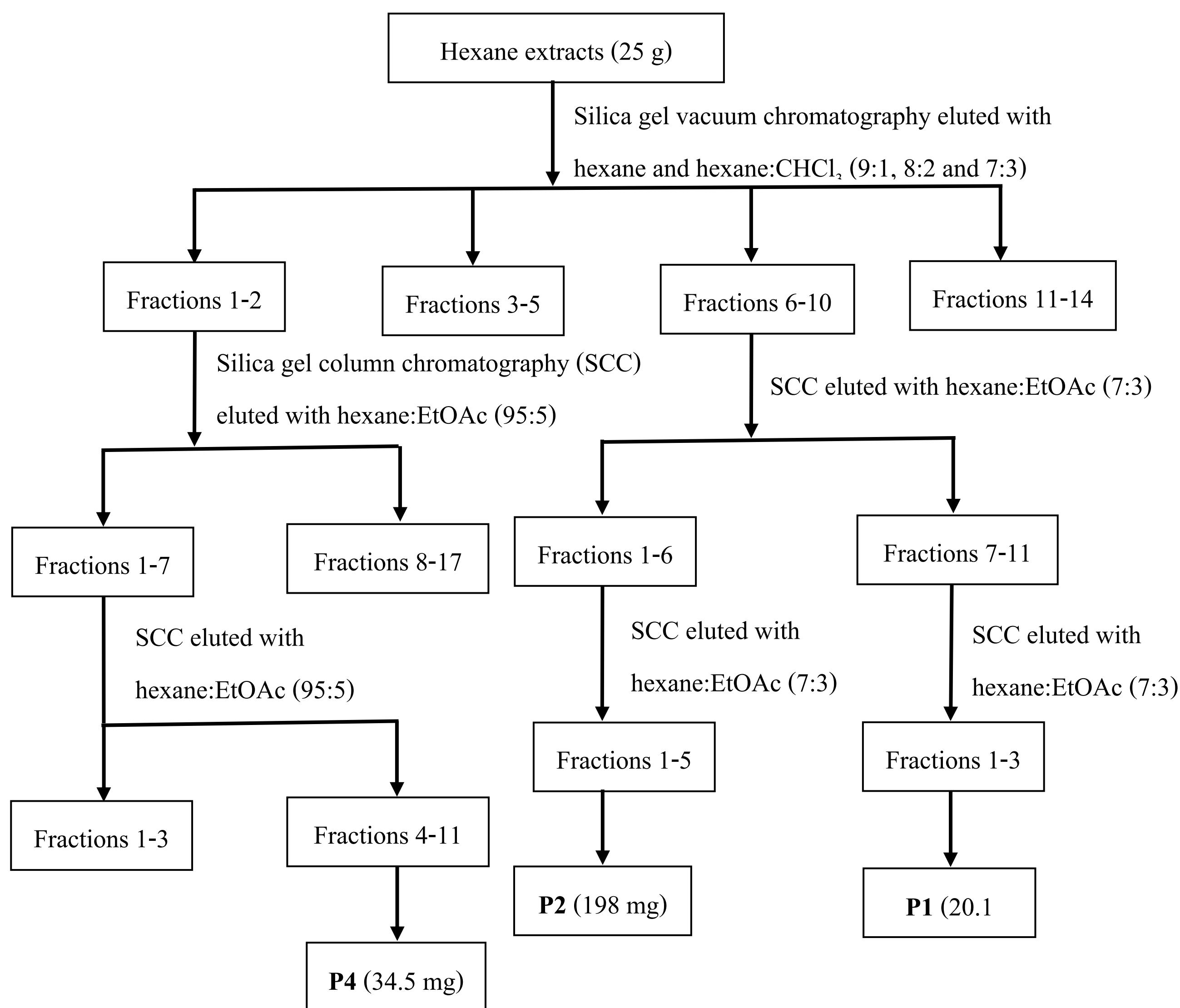


Figure 3-3 Purification of P1, P2 and P4 from *Z. cassumuna* extract

The essential oil of *Z. cassumunar* rhizome (1 g) was isolated by silica gel column chromatography eluted with a mixture of hexane and ethyl acetate (95:5). The fractions of each 30 ml were collected. The pooled fractions 13-24 were rechromatographed with a mixture of hexane and ethyl acetate (96:4). The fractions of each 30 ml were also collected. A yellowish oily liquid (P3) was obtained from the pooled fractions 6-11 (Figure 3-4).

P1, P2, P3 and P4 were determined by Thin-layer chromatography with a mixture of hexane and ethyl acetate (7:3) as the mobile phase, allowing the solvent front to ascend 15 cm above the line of application. After removed the plate from the chromatographic chamber, allowed it to dry in air and detected under ultraviolet light (254 nm), marking the

quenching spots and then sprayed the plate with anisaldehyde-sulfuric acid TS and heat at 110°C for 10-15 minutes. The spots of different color were observed (Subcommittee on the Establishment of the Thai Herbal Pharmacopoeia, 1998).

The structures of P1, P2, P3 and P4 were determined by NMR, MS and IR. The pure compounds were then determined for evaluation of anti-inflammatory activity.

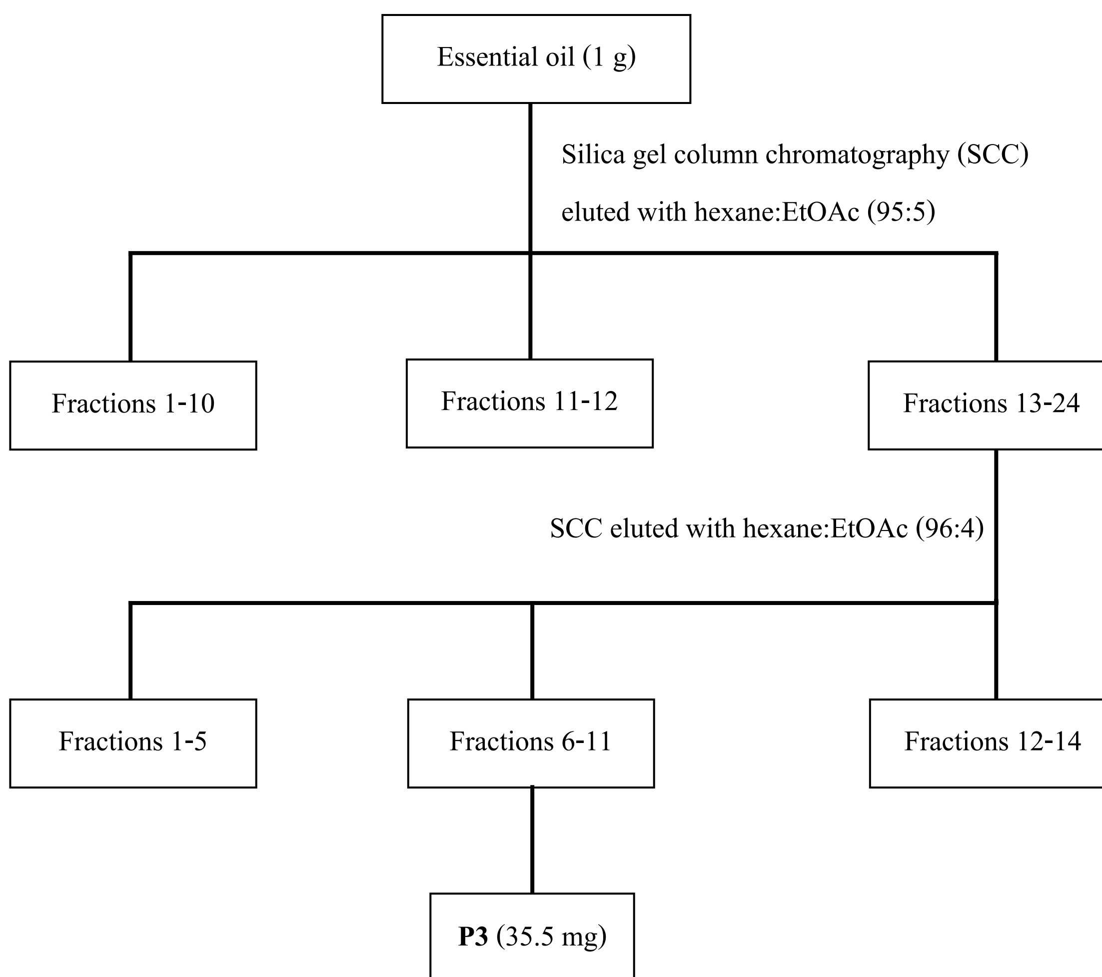


Figure 3-4 Purification of P3 from the essential oil of *Z. cassumunar* rhizome

3.2.4 Evaluation of anti-inflammatory activity

Inhibitory effect on NO production by murine macrophage like RAW 264.7 cells was evaluated using a modified method from that previously reported (Tewtrakul and Itharat,

2007). Briefly, the RAW 264.7 cells (purchased from Cell Lines Service, Heidelberg, Germany) were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 0.1% sodium bicarbonate, 2 mM glutamine, penicillin G (100 U/ml), streptomycin (100 μg/ml) and 10% fetal cafe serum. The cells were harvested with trypsin-EDTA and diluted to a suspension in fresh medium. The cells were seeded in 96-well plates with $1x10^5$ cells/well and allowed to adhere for 1 h at 37 °C in a humidified atmosphere containing 5% CO_2. After that the medium was replaced with fresh medium containing 100 μg/ml of lipopolysaccharide together with test samples at various concentrations and then incubated for 48 h. NO production was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent.

Cytotoxicity was determined using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) colorimetric method (Dong *et al*., 2001). Briefly, after 48 h incubation with test samples, MTT solution (10 μl, 5 mg/ml in phosphate buffered saline) was added to the wells. After 4 h incubation, the medium was removed, and isopropanol containing 0.04 M HCl was then added to dissolve the formazan production in the cells. The absorbance of formazan solution was measured with a microplate reader at 570 nm. The test compounds were considered to be cytotoxic when the absorbance at 570 nm of the sample-treated group was less than 80% of that in the control (vehicle-treated) group. L-nitroarginine and caffeic acid phenethylester were used as positive controls. The stock solution of each test sample was dissolved in DMSO, and the solution was added to the RPMI medium (final DMSO is 1%). Inhibition (%) was calculated using the following equation and IC_{50} values were calculated from the graph plotted of % inhibition against standard compound concentrations.

$$\text{Inhibition (\%)} = [A - B/A - C] \times 100$$

A - *C*: NO_2^- concentration (μM) [*A*: LPS (+), sample (-); *B*: LPS (+), sample (+); *C*: LPS (-), sample (-)].

For statistical analysis, the values were expressed as mean ± S.E.M of four determinations. The statistical significance was calculated by one-way analysis of variance (ANOVA), followed by Dunnett's test ($P < 0.05$).

3.2.5 HPLC method

3.2.5.1 Standard solution

Separate stock solution of the reference standards, (*E*)-4-(3,4-dimethoxyphenyl)but-3-en-l-ol (compound D), (*E*)-4-(3,4-dimethoxyphenyl)but-3-en-l-yl acetate (D-acetate), (*E*)-1-(3,4-dimethoxyphenyl)butadiene (DMPBD) and (*E*)-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl]cyclohex-1-ene (DMPDMS) were made in methanol. A working solution of the combined standards was subsequently prepared in methanol and diluted to provide a series of the standards solution of 12.5, 6.25, 3.125, 1.56 and 0.78 µg/ml. The calibration curves were constructed for each of the target analyzes.

3.2.5.2 Sample preparation

Z. cassumunar dried powder (100 mg) was extracted with hexane (30 ml) under reflux conditions for an hour. The extract was then filtered and the solvent was evaporated to dryness *in vacuo*. The sample was reconstituted and adjusted to 10 ml with methanol. The samples were filtered through 0.45 µm membrane filter and analyzed immediately after extraction in order to avoid possible chemical degradation. The experiments were in triplicate.

3.2.5.3 Apparatus and chromatographic conditions

HPLC analysis was carried out using Thermo Spectranet SN 4000. Data analysis was performed using Spectranet software ChromQuest. Separation was achieved at 25ºC on a 150 mm × 4.6 mm i.d. TSK-gel ODS-80Tm column. The mobile phase consisted of methanol and 2% acetic acid, gradient mode (0-20 min, 52% v/v methanol, 22-38 min, 50% v/v methanol and 40-60 min, 52% v/v methanol) with a flow rate of 1 ml/min. The injection volume was 20 µl. The quantitation wavelength was set at 254 nm.

3.2.5.4 Validation of analytical method (Association of Official Analytical Chemists, 2002)

Linearity

Calibration curves were constructed on three consecutive days by analysis of a mixture containing each of reference standard compounds at five concentrations (0.78, 1.56, 3.125, 6.25 and 12.5 µg/ml) using the HPLC conditions as described in section 3.2.4.3 and plotting peak area against the concentration of each reference standard. The linearity of the detector response for the standards was determined by means of linear regression.

Accuracy

Mixture of compound D, Compound D-acetate, DMPBD and DMPDMS were prepared at 3 different concentrations (12.5, 6.25 and 3.125 µg/ml) and mixed with sample portions at a ratio of 1:1 (v/v). The three injections for each concentration were done per day (3 injections x 3 concentrations) and percentage recoveries of each active compound were then calculated.

Precision

The chromatographic system was tested by performing intra- and inter-day multiple injections of sample solution. Six injections were performed each day for 3 consecutive days. The precision of the extraction procedure was validated by repeating the extraction procedure on the same sample of *Z. cassumunar*. An aliquot of each extract was then injected and quantified. This parameter was evaluated by repeating the extraction in triplicate on 3 different days with freshly prepared mobile phase and samples.

Specificity

Peak identification was carried out using the standards and diode-array detector. The UV spectra were taken at various points of the peaks to check peak homogeneity.

Limit of detection (LOD) and Limit of quantification (LOQ)

LOD and LOQ for the analytical method were studied. The dilutions of sample were made with methanol and were then analyzed with HPLC method. LOD and LOQ were obtained as the ratio of signal to noise equal to 3 and 10, respectively.

3.2.6 Determination of solvent for extraction

To optimize the solvent for extraction, *Z. cassumunar* rhizome powder (100 mg) was separately extracted with hexane, chloroform, ethyl acetate and methanol (30 ml) under reflux conditions for an hour (3 times). The extract was then filtered and the solvent was evaporated to dryness *in vacuo*. The sample was adjusted to 10 ml with methanol and subjected to HPLC analysis. The experiments were performed in triplicate.

3.2.7 Preparation of *Z. cassumunar* extract with high-yielded anti-inflammatory active compounds

After the solvent for extraction of *Z. cassumunar* rhizomes was optimized, the hexane extract was further fractionation using silica gel vacuum chromatography to produce a high-yielded anti-inflammatory active compound extract of *Z. cassumunar*. The appropiate method is described as followed. Dried powder rhizomes of *Z. cassumunar* (250 g) were refluxed with hexane (3 times x 3 L) for 1 hour then the combined extract was evaporated to dryness *in vaccuo*. The resulting residue was weighed. The hexane extract (15 g) was further fractionated using silica gel vacuum chromatography with a mixture of hexane and ethyl acetate (8:2, 500 ml). The combined extracts were evaporated to dryness *in vaccuo* to produce a phenylbutanoid rich extract.

3.2.8 HPLC analysis of *Z. cassumunar* extracts and volatile oil

The hexane extracts and volatile oil of *Z. cassumunar* were accurately weighed about 5 mg and dissolved in 10 ml methanol. The solutions were filtered through 0.45 µm membrane filter and subjected to the HPLC analysis.

3.2.9 Determination of solubility (British Pharmacopoeia Commission, 2001)

Zingiber cassumunar extract with high-yielded anti-inflammatory active compounds was accurately weighed to 10 mg and placed in a vessel of at least 100 ml capacity. The vessel was placed in a constant temperature device, maintained at a temperature of 25 ± 0.2 ºC. Various solvents (water, methanol, ethyl acetate, chloroform and hexane) were examined by adding of the solvents by increments of 10 µl, shaking frequently and vigorously for 10 minutes. The volume of solvent was recorded when a clear solution was obtained. If the solution was become cloudy or non-dissolve, the sample was continuously added until 10 ml. After addition of 10 ml of solvents, the sample or parts of it remained non-dissolve; the experiment had to be repeated in a 100 ml volumetric flask. At lower solubility, the time required to dissolve a substance can be considerably longer, at least 24 hours should be allowed.

Descriptive term of solubility and approximate volume of solvents required to completely dissolve a solute (in milliliters per gram of solute) were drawn as follow.

Table 3-2 Solubility criteria of the extract in various solvents

Solubility term	Volume of solvent required to dissolve 1 g of solute (ml)
Very soluble	less than 1
Freely soluble	from 1 to 10
Soluble	From 10 to 30
Sparingly soluble	from 30 to 100
Slightly soluble	from 100 to 1000
Very slightly soluble	from 1000 to 10,000
Practically insoluble	more than 10,000

The term partly soluble is used to describe a mixture of which only some of the components dissolve.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Isolation of active compounds in *Z. cassumunar* extract

Z. cassumunar rhizomes were collected from Songkhla province in Thailand in October 2007, the dried powder of *Z. cassumunar* rhizome were refluxed with hexane to yield hexane extract 25 g (5%). The hexane extract was fractionated by silica gel vacuum chromatography and silica gel column chromatography to produce three yellowish oily liquids, P1 (20.1 mg), P2 (198 mg) and P4 (34.5 mg). P3 (35.5 mg) was isolated from the *Z. cassumunar* essential oil by silica gel chromatography, and also obtained as a yellowish oily liquid. P1, P2, P3 and P4 were determined by thin-layer chromatography with a mixture of hexane and ethyl acetate (7:3) to give hR_f values of 0.13, 0.52, 0.68 and 0.75, respectively. P1, P2, P3 and P4 were sprayed with anisaldehyde-sulfuric acid TS which gave red, dark pink, dark-violet and violet, respectively. The hR_f values and the color of P1 and P2 were corresponded to (*E*)-4-(3,4-dimethoxyphenyl)but-3-ene-1-ol (compound D) and (*E*)-4-(3,4-dimethoxyphenyl)but-3-en-l-yl acetate when compared with Thin-layer chromatogram as described by Subcommittee on the Establishment of the Thai Herbal Pharmacopoeia, 1998. These pure compounds were subjected to determination of their chemical structures by IR, NMR and MS.

4.1.1 Structure identification

4.1.1.1 Identification of P1

The IR spectrum of P1 (Figure 4-1) exhibited the absorption spectrum at ν 3400, 1600, 1580, 1515, 1260, 1240 cm^{-1}. The absorptions at ν 3400 cm^{-1} is consistent with the presence of the alcohol functionality.

The ^{1}H NMR spectrum of P1 (Table 4-1, Figure 4-2) exhibited a phenyl group bearing two methylene protons [δ 2.45 (2H, q), 3.75 (2H, t)], two methoxyl groups on aromatic ring [δ 3.86 (3H, s), 3.88 (3H, s)], two olefinic proton [δ 6.06 (1H, dt), 6.41 (1H, d)] and three aromatic protons [δ 6.81 (1H, d), 6.89 (1H, dd), 6.92 (1H, d)]. The ^{13}C NMR spectrum of P1 (Table 4-1, figure 4-3) revealed 12 carbons, two methoxy, three quarternary, five methines and two methylenes. P1 was identified as (*E*)-4-(3, 4-dimethoxyphenyl) but-3-en-l-ol (compound D) (Figure 4-4) when compared with the spectral data as described by Lu *et al*., (2008).

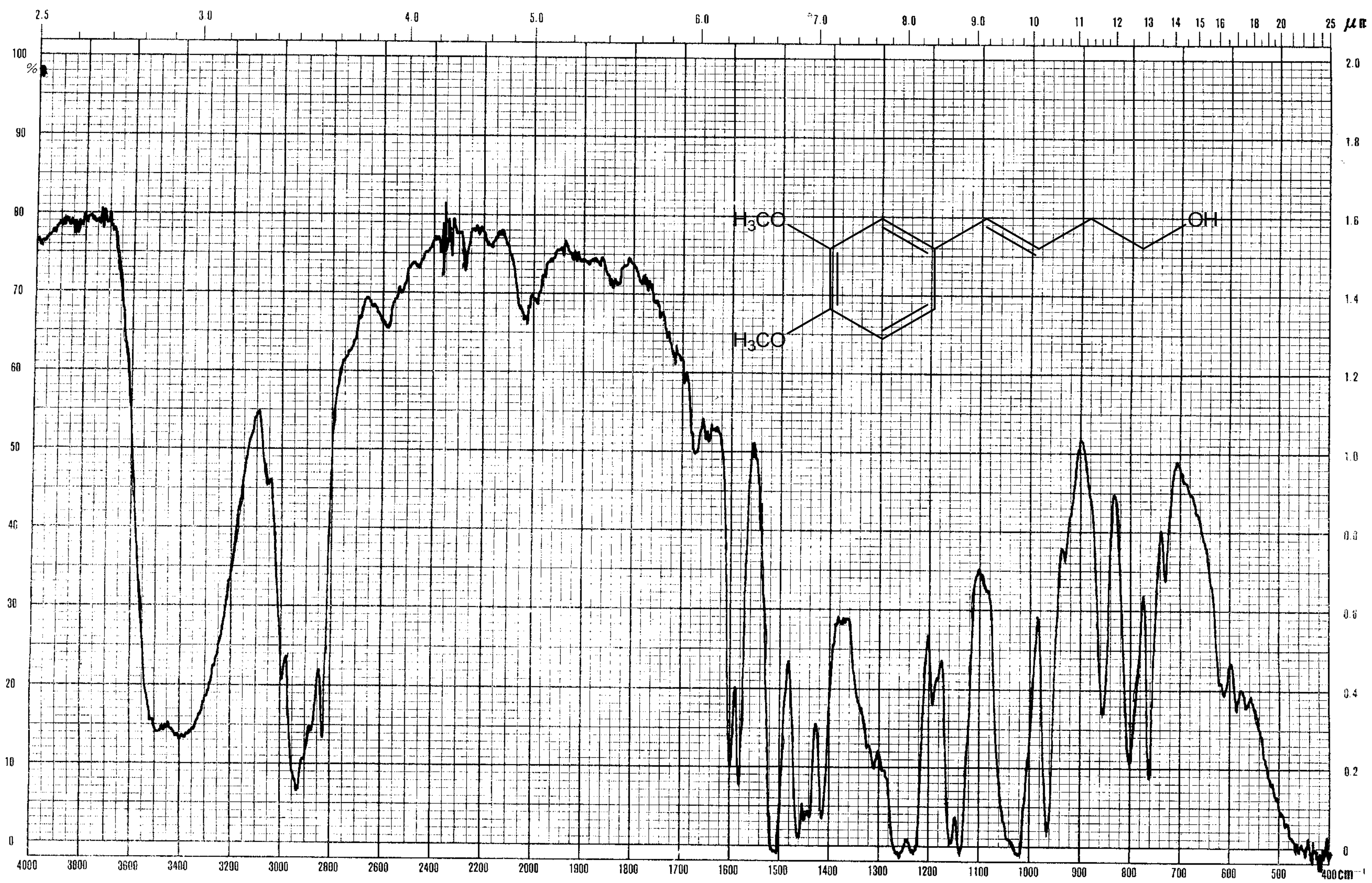


Figure 4-1 IR spectrum of P1 (neat)

Table 4-1 ^{1}H NMR ($CDCl_3$; 500 MHz) and ^{13}C NMR ($CDCl_3$; 125 MHz) spectral data of P1

Positions	**P1**		**Compound D** (Lu *et al*., 2008)	
	^{1}H (mult.; J in Hz)	**^{13}C**	**^{1}H (mult.; J in Hz)**	**^{13}C**
1	-	132.4 (C)	-	131.4 (C)
2	6.92 (d; 2.2)	108.4 (CH)	6.89 (d; 1.5)	111.0 (CH)
3	-	148.5 (C)	-	148.0 (C)
4	-	148.9 (C)	-	148.6 (C)
5	6.81 (d; 8.2)	111.0 (CH)	6.84 (d; 8.5)	112.2 (CH)
6	6.89 (dd; 8.2, 2.0)	119.0 (CH)	6.87 (dd; 8.5, 1.5)	121.4 (CH)
7	6.41 (d; 15.5)	130.3 (CH)	6.51 (d; 15.5)	130.2 (CH)
8	6.06 (dt; 15.8, 7.3)	124.3 (CH)	5.61 (dt; 15.5, 6.5)	127.1(CH)
9	2.45 (q; 6.2)	36.3 (CH_2)	2.63 (q; 6.5)	32.1 (CH_2)
10	3.75 (t; 6.2)	62.1 (CH_2)	3.75 (t; 6.5)	62.6 (CH_2)
11	3.86 (s)	55.8 (OCH_3)	3.87 (s)	55.9 (OCH_3)
12	3.88 (s)	55.9 (OCH_3)	3.88 (s)	56.0 (OCH_3)

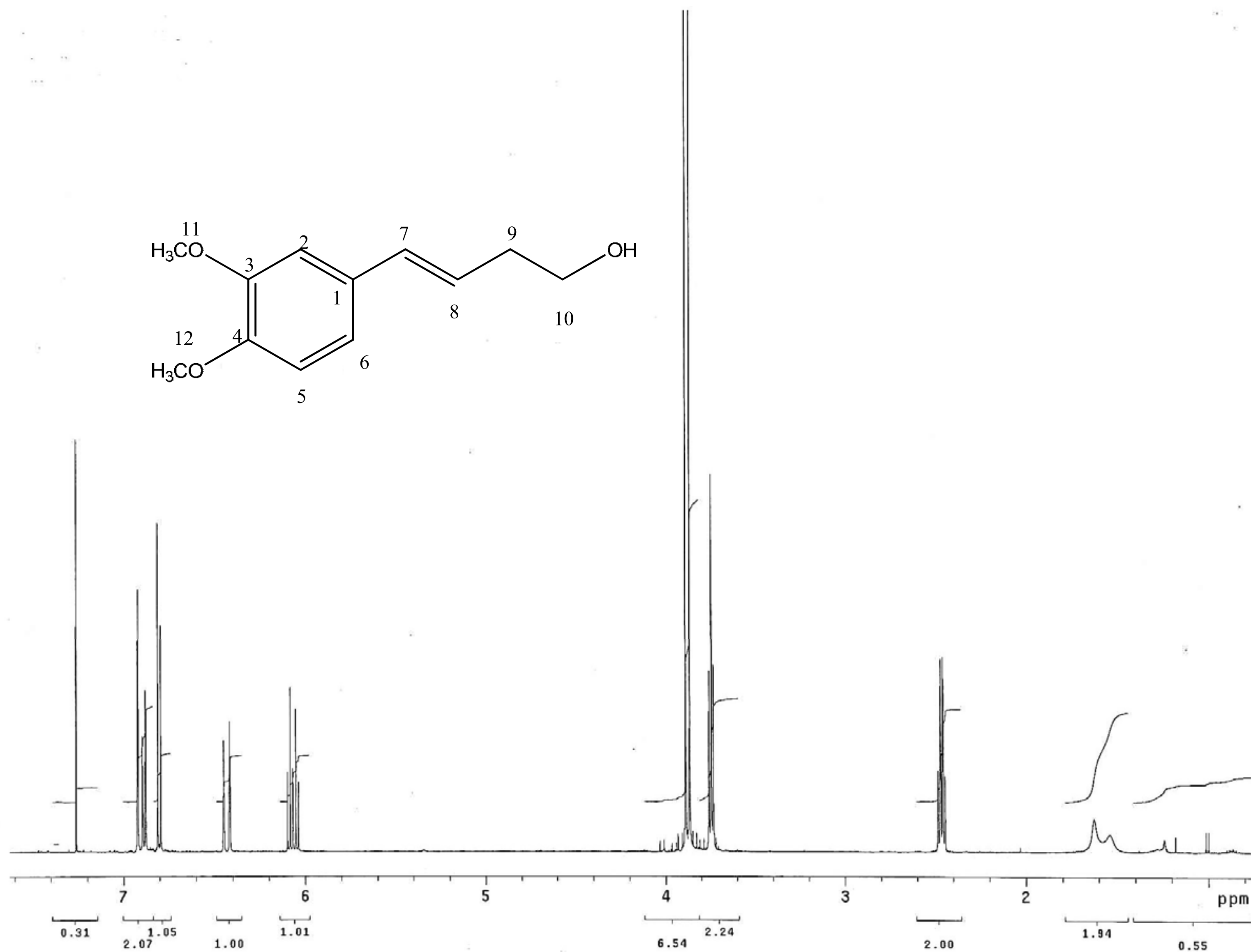


Figure 4-2 ^{1}H NMR spectrum of P1 ($CDCl_3$; 500 MHz)

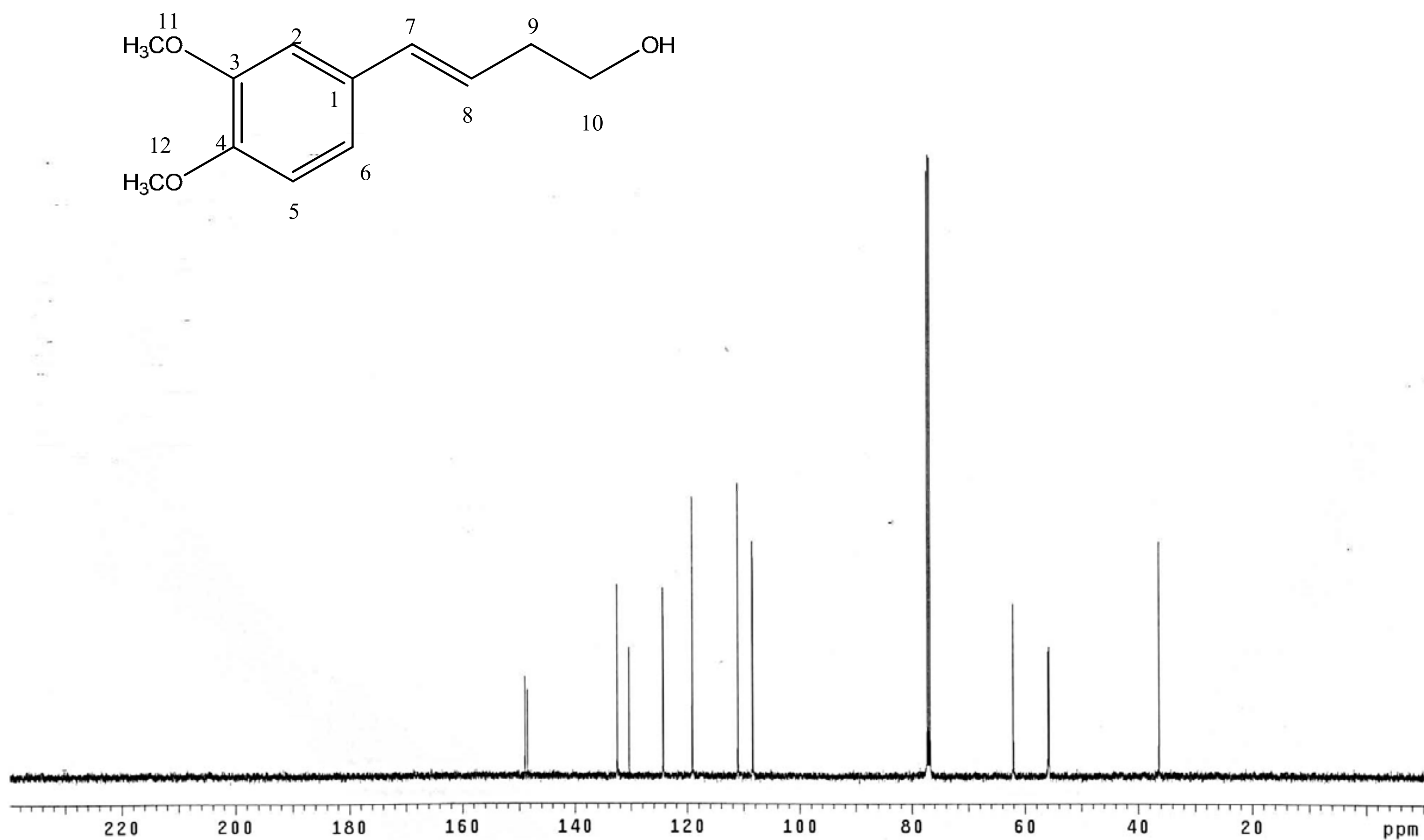


Figure 4-3 ^{13}C NMR spectrum of P1 ($CDCl_3$; 125 MHz)

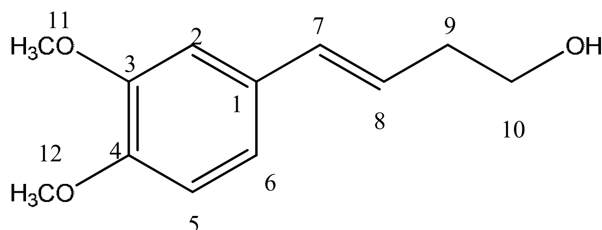


Figure 4-4 Chemical structure of (*E*)-4-(3,4-dimethoxyphenyl)but-3-ene-1-ol (compound D)

4.1.1.2 Identification of P2

The IR spectrum of P2 (Figure 4-5) exhibited the absorption spectrum at ν 1720, 1600, 1580, 1520, 1380, 1245 cm^{-1}. The absorptions at ν 1720 cm^{-1} is consistent with the presence of the ester carbonyl functionality.

The ^{1}H NMR spectrum of P2 (Table 4-2, Figure 4-6) exhibited a phenyl group bearing one methyl proton [δ 2.04 (3H, s)], two methylene proton [δ 2.5 (2H, q), 4.16 (2H, t)], two methoxyl groups on aromatic ring [δ 3.86 (3H, s), 3.88 (3H, s)], two olefinic protons [δ 6.01 (1H, dt), 6.40 (1H, d)] and three aromatic protons [δ 6.83-6.88 (3H, m)]. The ^{13}C NMR spectrum of P2 (Table 4-2, figure 4-7) revealed 14 carbons, two methoxy, three quarternary, five methines, two methylenes, one methyl and one acetate group. P2 was identified as (*E*)-4-(3,4-dimethoxyphenyl) but-3-en-l-yl acetate (D-acetate) (Figure 4-8) when compared with the spectral data as described by Lu *et al*., (2008).

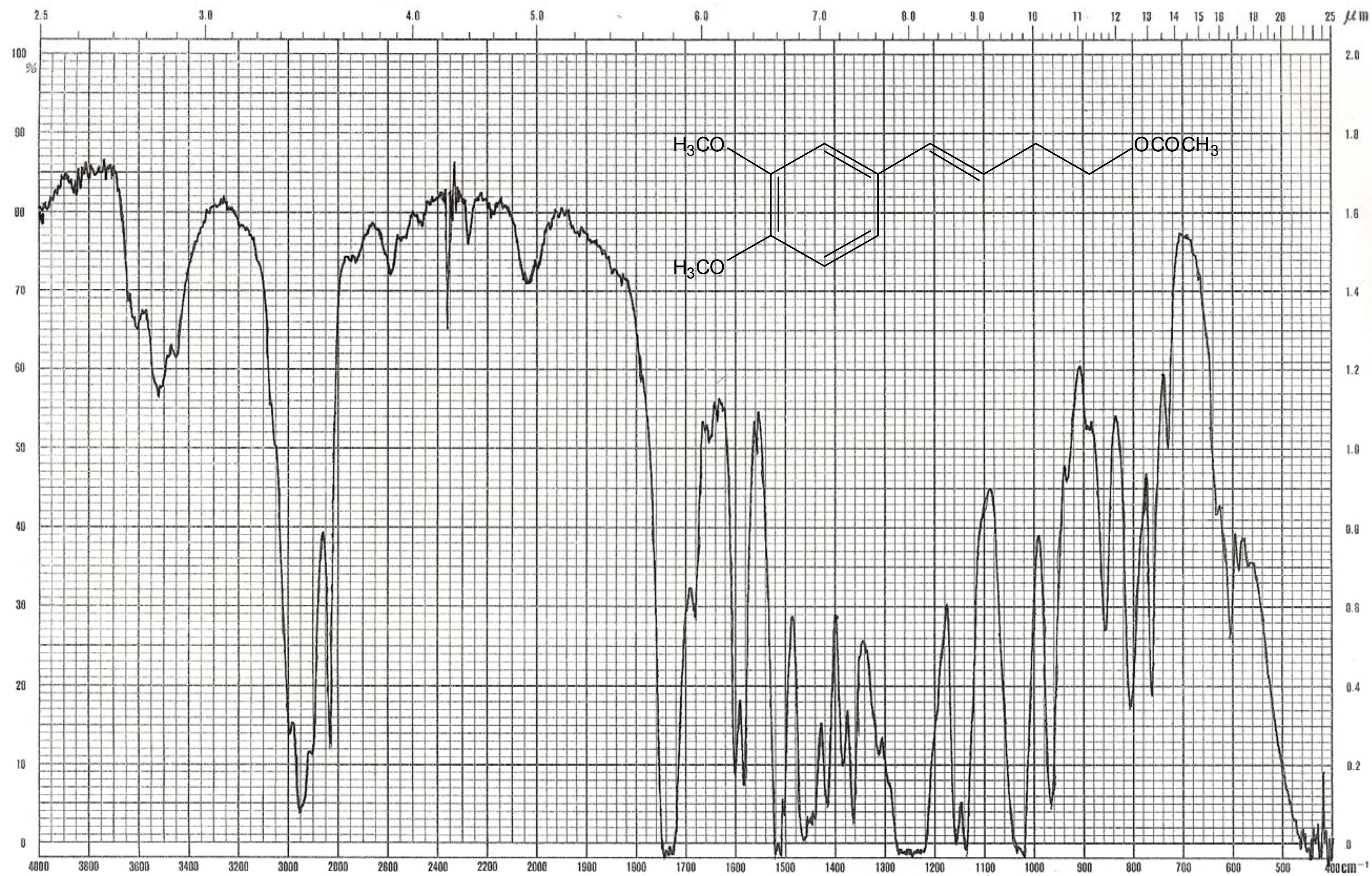


Figure 4-5 IR spectrum of P2 (neat)

Table 4-2 ^{1}H NMR ($CDCl_3$; 500 MHz) and ^{13}C NMR ($CDCl_3$; 125 MHz) spectral data of P2

Positions	**P2**		**D-acetate** (Lu *et al*., 2008)	
	^{1}H (mult.; *J* in Hz)	**^{13}C**	**^{1}H (mult.; *J* in Hz)**	**^{13}C**
1	-	132.0 (C)	-	132.2 (C)
2	6.83-6.88 (m)	108.5 (CH)	6.88-6.90 (m)	108.7 (CH)
3	-	148.5 (C)	-	148.7 (C)
4	-	149.0 (C)	-	149.1 (C)
5	6.78 (d; 8.2)	111.1 (CH)	6.81 (d; 8.0)	111.2 (CH)
6	6.83-6.88 (m)	119.0 (CH)	6.88-6.90 (m)	119.2 (CH)
7	6.40 (d; 16)	130.3 (CH)	6.41 (d; 15.5)	130.5 (CH)
8	6.01 (dt; 14.6, 7.3)	123.5 (CH)	6.03 (dt; 15.5, 6.5)	123.7 (CH)
9	2.50 (q; 6.9)	32.3 (CH_2)	2.53 (q; 6.5)	32.4 (CH_2)
10	4.16 (t; 6.9)	63.8 (CH_2)	4.18 (t; 6.5)	64.0 (CH_2)
11	-	171.1 (-C=O)	-	171.4 (-C=O)
12	2.04 (s)	21.0 (CH_3)	2.06 (s)	21.1 (CH_3)
13	3.86 (s)	55.8 (OCH_3)	3.88 (s)	55.9 (OCH_3)
14	3.88 (s)	55.9 (OCH_3)	3.9 (s)	56.0 (OCH_3)

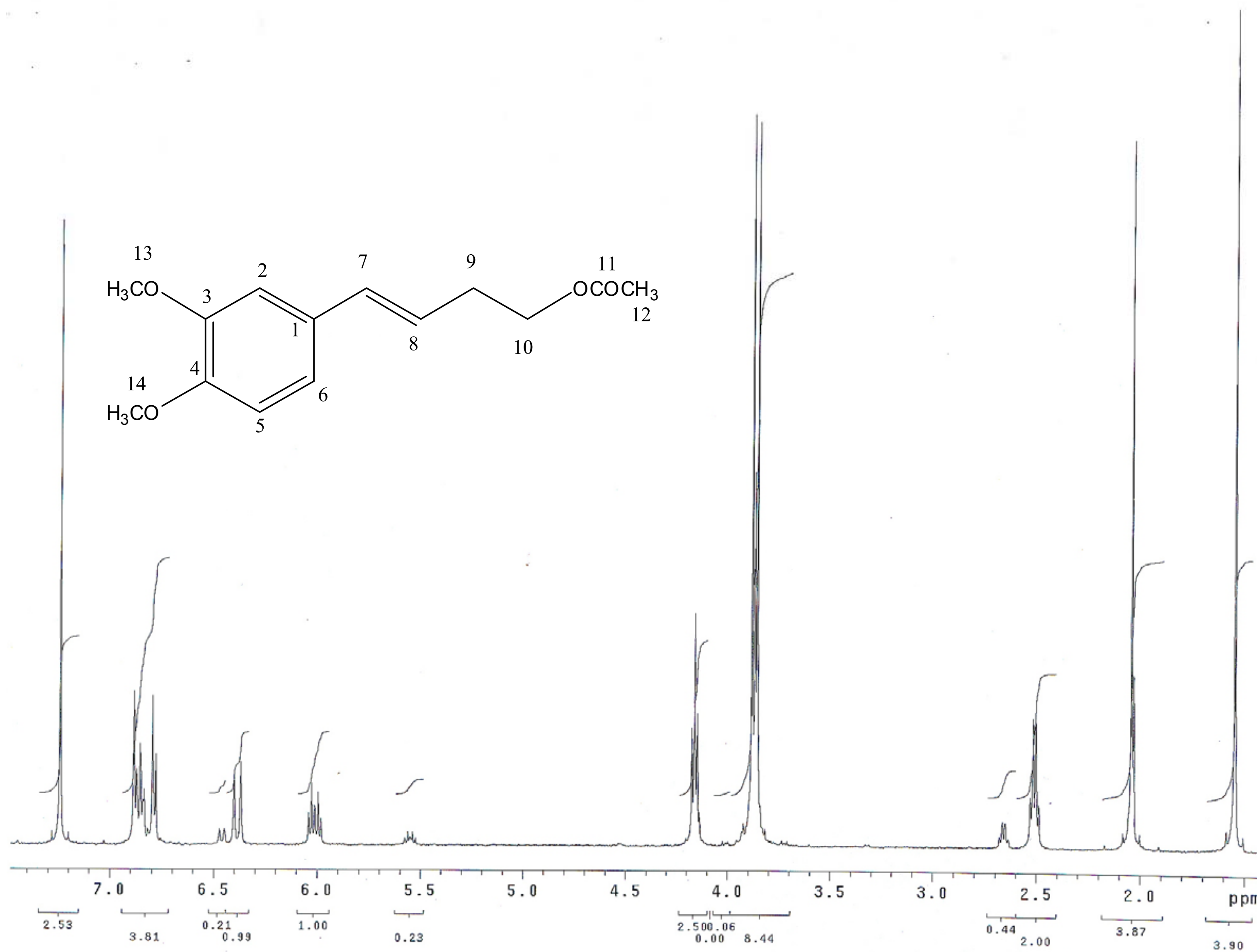


Figure 4-6 ^{1}H NMR spectrum of P2 ($CDCl_3$; 500 MHz)

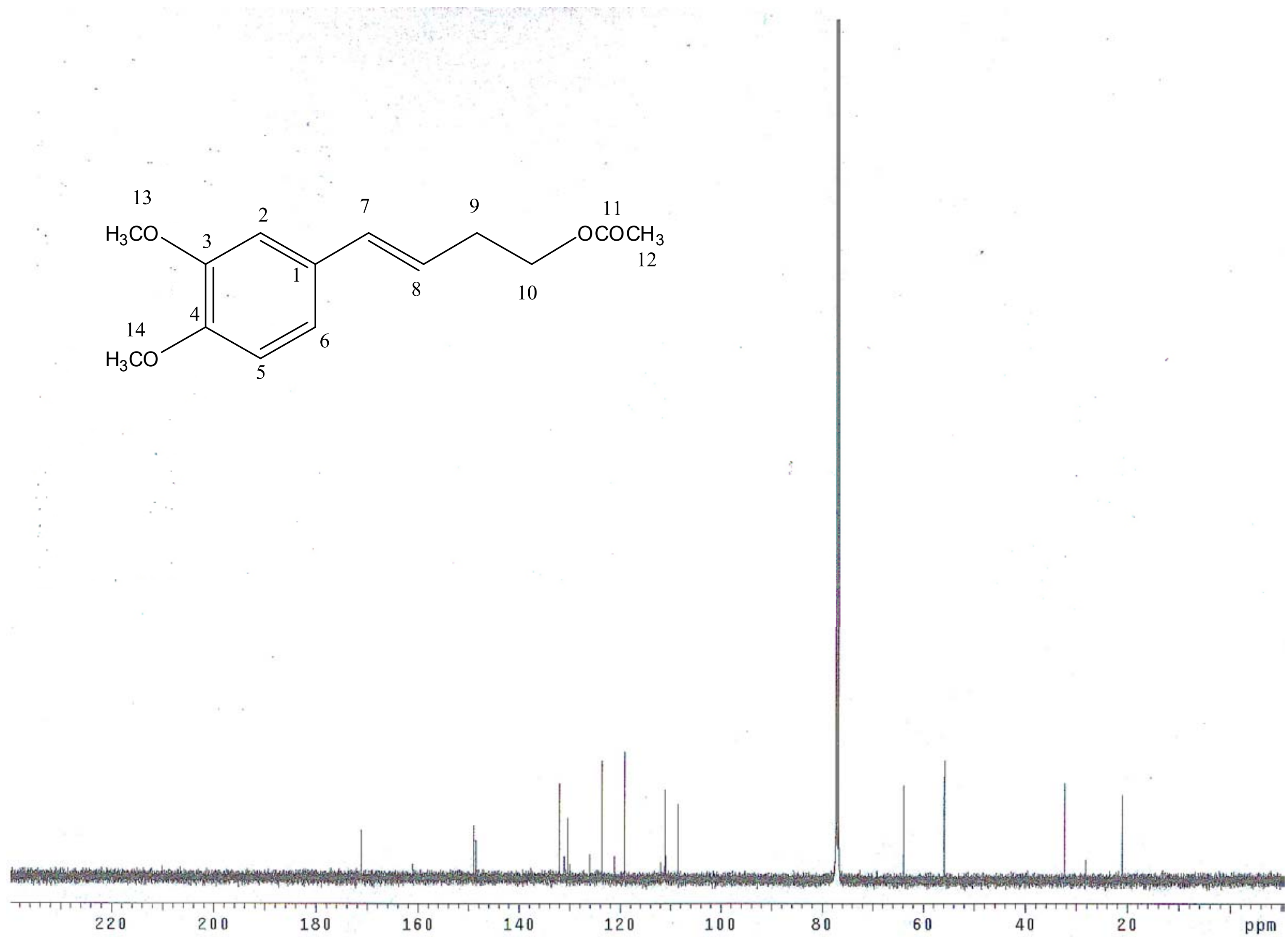


Figure 4-7 ^{13}C NMR spectrum of P2 ($CDCl_3$; 125 MHz)

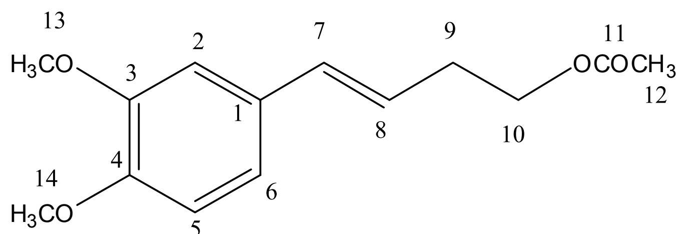


Figure 4-8 Chemical structure of (*E*)-4-(3,4-dimethoxyphenyl)but-3-en-l-yl acetate (D-acetate)

4.1.1.2 Identification of P3

P3 has a molecular formula of $C_{12}H_{14}O_2$ as established by mean of the EI mass spectrum, which shows a molecular ion peak (M^+) at *m/z* 190. The fragment ion at m/z 175 is due to a facile loss of the methyl group, while the fragment ion at *m/z* 159 is due to a loss of the methoxy group ($[M^+ - OCH_3^\bullet]$). The fragment ion at *m/z* 128 is due to a loss of dimethoxy group ($[M^+ - 2OCH_3^\bullet]$). The fragment ion at *m/z* 91 is $C_7H_7^\bullet$ (Figure 4-9).

The ^{1}H NMR spectrum of P3 (Table 4-3, Figure 4-10) exhibited a phenyl group bearing one methylene proton [δ 5.11 (1H, d)], two methoxyl groups on aromatic ring [δ 3.86 (3H, s), 3.89 (3H, s)], three olefinic protons [δ 5.29 (1H, d), 6.45 (1H, dd), 6.80 (1H, d)] and three aromatic protons [δ 6.48 (1H, dd), 6.91 (1H, d), 6.94 (1H, m)]. The ^{13}C NMR spectrum of DMPBD (Table 4-3, Figure 4-11) revealed 12 carbons, two methoxy, three quarternary, six methines, and one methylene. The spectral data of this compound was identified as (*E*)-1-(3,4-dimethoxyphenyl) butadiene (DMPBD) (Figure 4-12) when compared with the spectral data as described by Kuroganagi *et al.*, (1980).

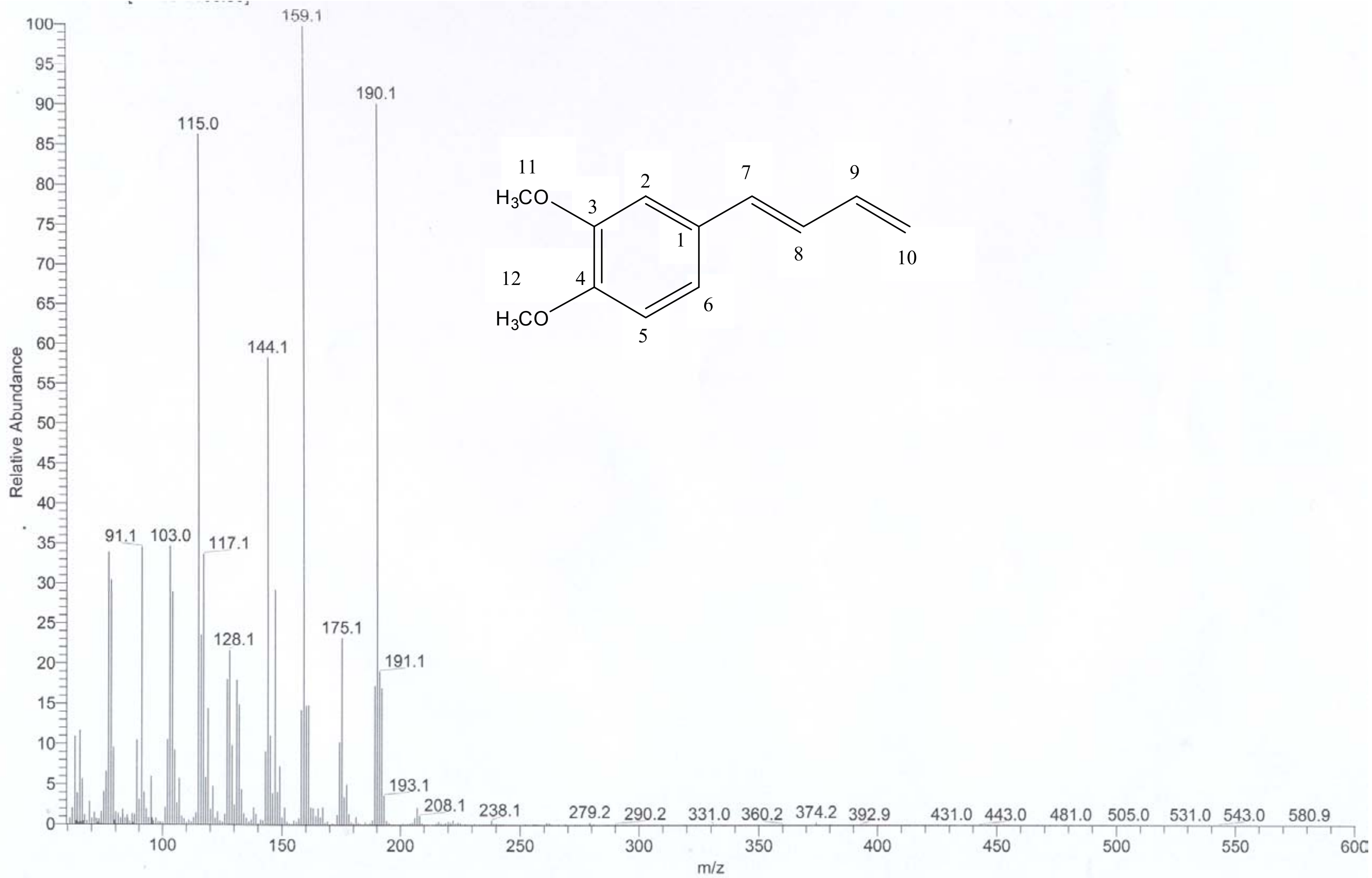


Figure 4-9 EI Mass spectrum of P3

Table 4-3 ^{1}H NMR ($CDCl_3$; 500 MHz) and ^{13}C NMR ($CDCl_3$; 125 MHz) spectral data of P3

Positions	**P3**		**DMPBD** (Kuroganagi *et al*., 1980)	
	^{1}H (mult.; *J* in Hz)	**^{13}C**	**^{1}H (mult.; *J* in Hz)**	**^{13}C**
1	-	127.8 (C)	-	
2	6.94 (d; 2.0)	108.6 (CH)	6.70-6.93 (m)	No report
3	-	148.9 (C)	-	
4	-	149.0 (C)	-	
5	6.80 (d; 8.0)	111.1 (CH)	6.70-6.93 (m)	
6	6.91 (dd; 8.3, 2.0)	119.8 (CH)	6.70-6.93 (m)	
7	6.65 (dd; 10.7, 4.6)	137.2 (CH)	6.00-6.22 (m)	
8	6.48 (dd; 10.0, 6.6)	130.2 (CH)	6.00-6.22 (m)	
9	6.43 (dt; 10.0, 6.6)	132.6 (CH)	6.00-6.22 (m)	
10a	5.29 (dd; 15.4, 1.7)	116.7 (CH_2)	4.95-5.39 (m)	
10b	5.11 (dd; 10.0, 0.7)	-	4.95-5.39 (m)	
11	3.86 (s)	55.8 (OCH_3)	3.81 (s)	
12	3.89 (s)	55.9 (OCH_3)	3.83 (s)	

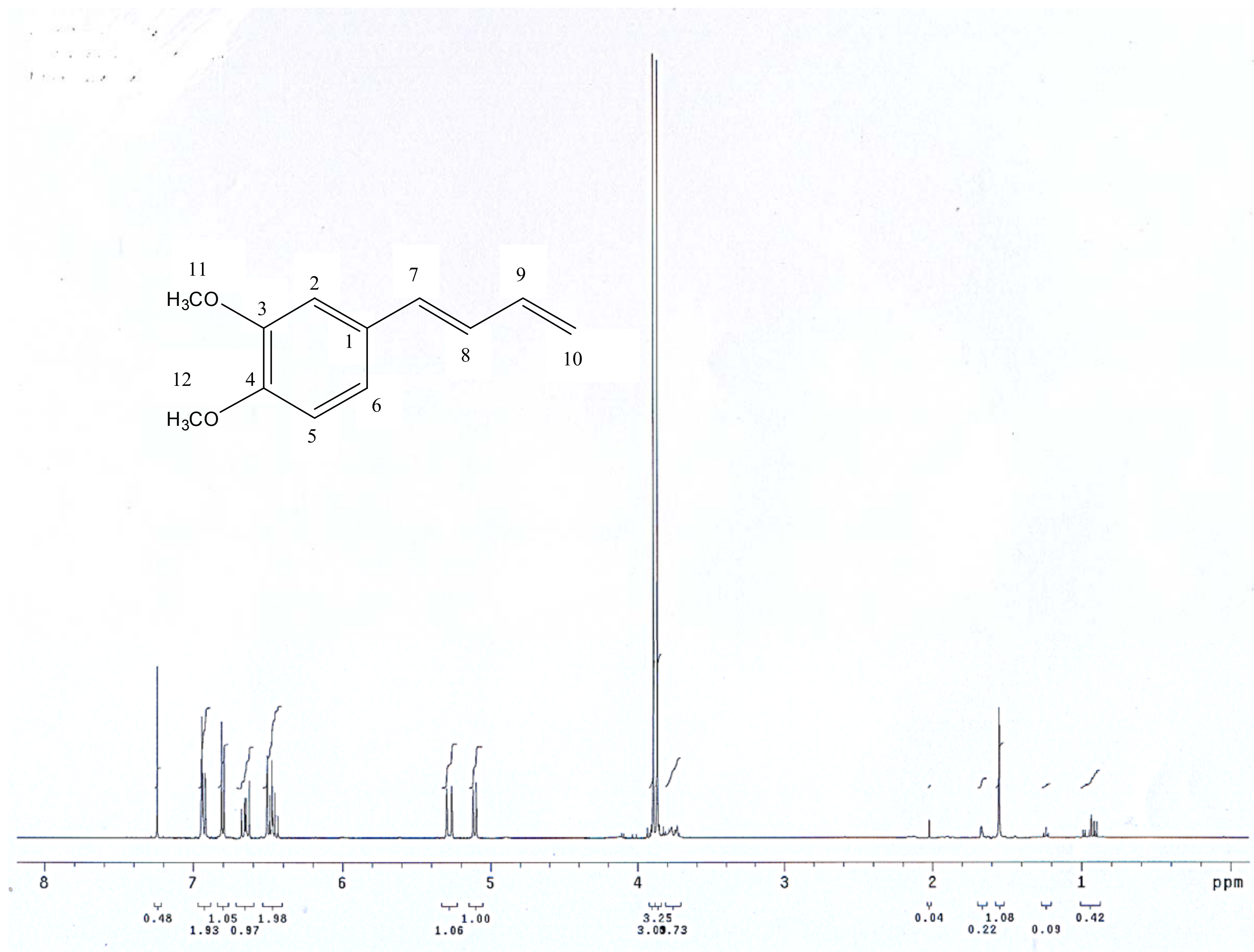


Figure 4-10 ^{1}H NMR spectrum of P3 ($CDCl_3$; 500 MHz)

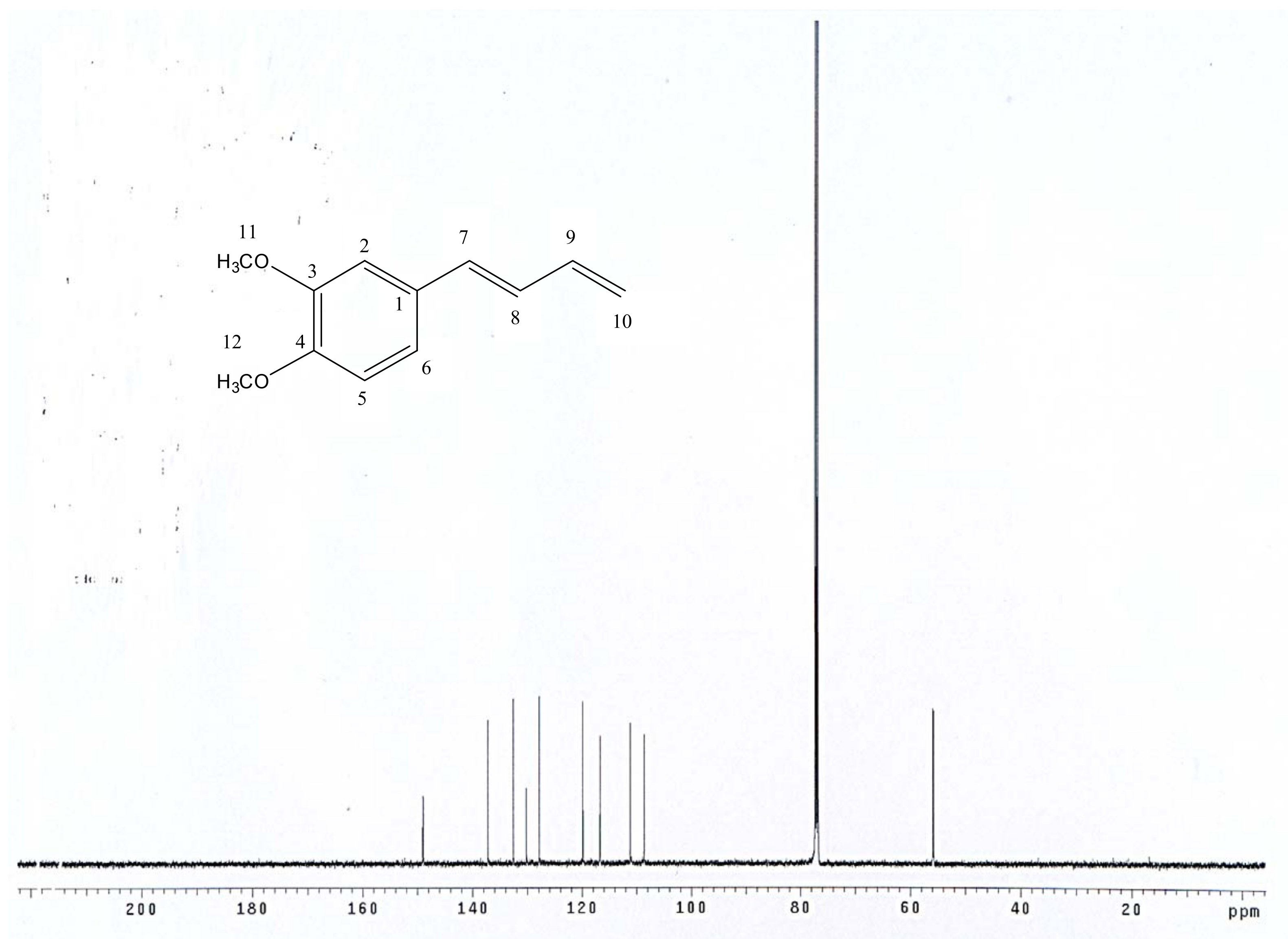


Figure 4-11 ^{13}C NMR spectrum of P3 ($CDCl_3$; 125 MHz)

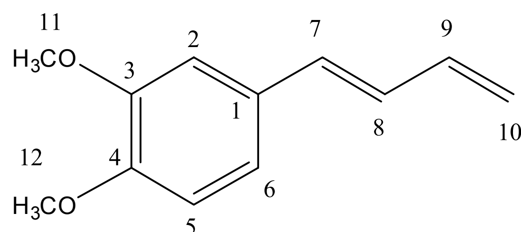


Figure 4-12 Chemical structure of (*E*)-1-(3,4-dimethoxyphenyl)butadiene (DMPBD)

4.1.1.2 Identification of P4

The IR spectrum of P4 (Figure 4-13) exhibited the absorption spectrum at ν 1600, 1580, 1520, 1265, 1235, 1140 cm^{-1}. Aromatic hydrocarbons showed absorptions in the regions ν 1580-1600 cm^{-1} due to carbon-carbon stretching vibrations in the aromatic ring. Bands in the region ν 1000-1250 cm^{-1} were due to C–H in-plane bending. The pattern of overtone bands in the region ν 1665-2000 cm^{-1} was reflected the substitution pattern on the ring.

The ^{1}H NMR spectrum of DMPDMS (Table 4-4, Figure 4-14) exhibited diphenyl group bearing two methylene proton [δ 1.95 (1H, m), 2.17 (1H, m), 2.21 (2H, m), 2.22 (1H, m)], four methoxyl groups on aromatic ring [δ 3.87 (3H, s), 3.87 (3H, s), 3.88 (3H, s), 3.88 (3H, s)], four olefinic protons [δ 5.53 (1H, m), 6.11 (2H, dt), 6.29 (1H, d)] and six aromatic protons [δ 6.70 (1H, d), 6.79 (1H, d), 6.84 (2H, dd), 6.89 (2H, d)]. The ^{13}C NMR spectrum of DMPDMS (Table 4-4, Figure 4-15) revealed 24 carbons, four methoxy, six quarternary, twelve methines, and two methylenes. The spectral data of this compound was identified as (*E*)-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl]cyclohex-1-ene (DMPDMS) (Figure 4-16) when compared with the spectral data as described by Lu *et al*., (2008).

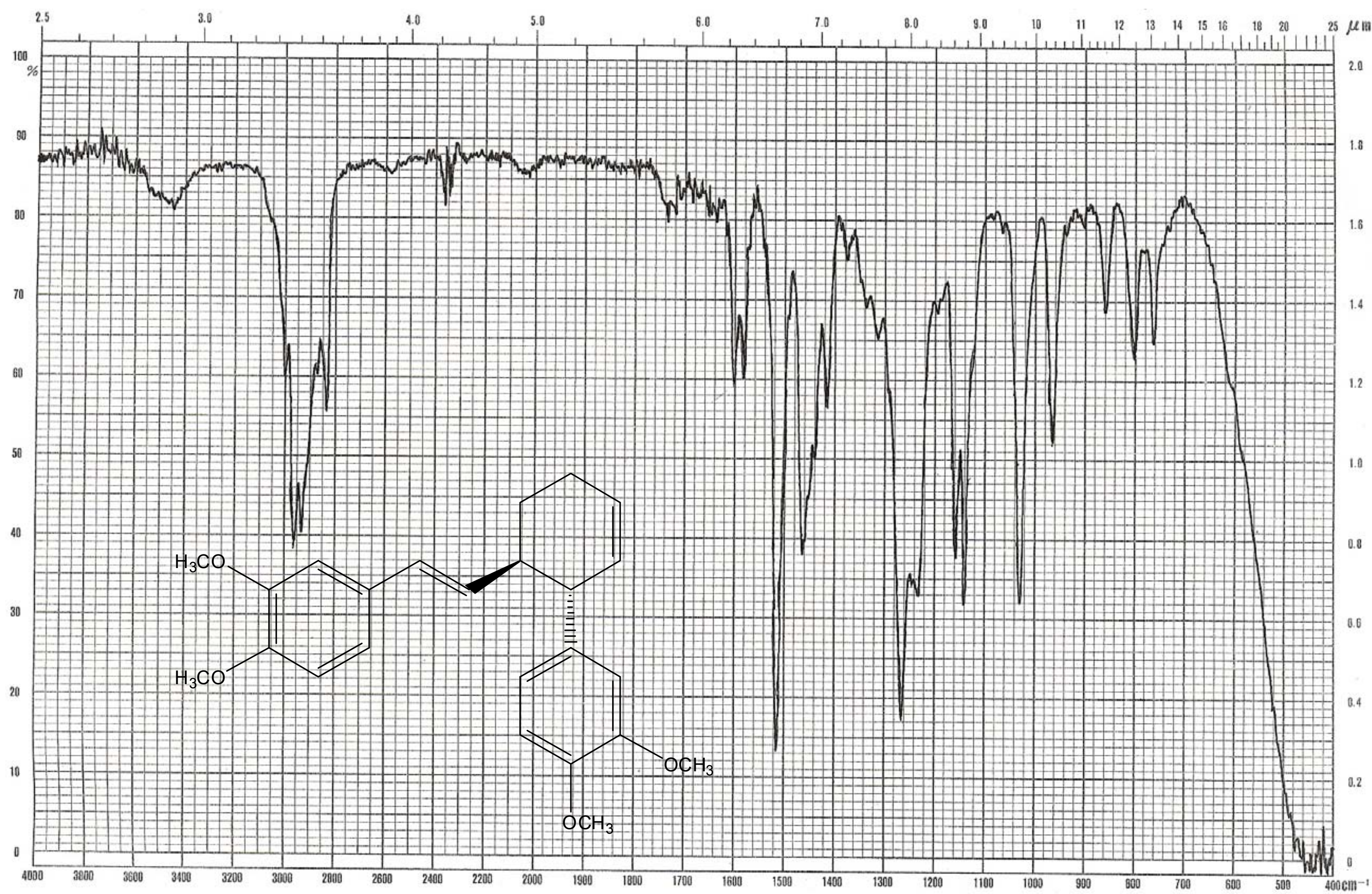


Figure 4-13 IR spectrum of P4 (neat)

Table 4-4 ^{1}H NMR ($CDCl_3$; 500 MHz) and ^{13}C NMR ($CDCl_3$; 125 MHz) spectral data of P4

Positions	**P4**		**DMPDMS** (Lu *et al*., 2008)	
	^{1}H (mult.; J in Hz)	**^{13}C**	**^{1}H (mult.; J in Hz)**	**^{13}C**
1	-	128.4 (CH)	-	129.0 (CH)
2	6.89 (d; 2.0)	108.5 (CH)	6.62 – 6.73 (m)	108.8 (CH)
3	-	149.0 (C)	-	149.1 (C)
4	-	148.2 (C)	-	148.7 (C)
5	6.70, 6.79 (d; 8.3)	111.2 (CH)	6.62 – 6.73 (m)	111.0 (CH)
6	6.84 (dd; 8.3, 2.0)	111.8 (CH)	6.62 – 6.73 (m)	111.8 (CH)
7	6.29 (d; 15.9)	120.2 (CH)	6.01 (d; 15.9)	120.6 (CH)
8	6.11 (dt; 15.9, 7.4)	130.8 (CH)	5.93 (dd; 15.9, 7.3)	130.4 (CH)
9	2.22 (m)	36.8 (CH)	2.27 (m)	45.6 (CH)
10	1.95, 2.17 (m)	30.8 (CH_2)	1.59, 1.84 (m)	28.0 (CH_2)
11	2.21 (m)	26.0 (CH_2)	2.13 (m)	24.7 (CH_2)
12	5.53 (m)	126.2 (CH)	5.6 (dd; 10.0, 2.1)	127.7 (CH)
13		118.8 (CH)	5.81 (dt; 10.0, 2.3)	118.9 (CH)
14	3.23 (d; 6.3)	38.6 (CH)	3.10 (m)	48.2 (CH)
15	-	131.1 (C)	-	132.3 (C)
16	6.84 (dd; 8.3, 2.0)	118.4 (CH)	6.62 – 6.73 (m)	118.9 (CH)
17	6.79 (d; 8.3)	111.2 (CH)	6.62 – 6.73 (m)	111.3 (CH)
18	-	133.7 (C)	-	147.5 (C)
19	-	133.9 (C)	-	148.4 (C)
20	6.89 (d; 2.0)	130.3 (CH)	6.62 – 6.73 (m)	130.4 (CH)
21	3.87 (s)	55.8 (OCH_3)	3.74 (s)	55.9 (OCH_3)
22	3.87 (s)	55.8 (OCH_3)	3.76 (s)	56.0 (OCH_3)
23	3.88 (s)	55.9 (OCH_3)	3.77 (s)	56.1 (OCH_3)
24	3.88 (s)	55.9 (OCH_3)	3.79 (s)	56.1 (OCH_3)

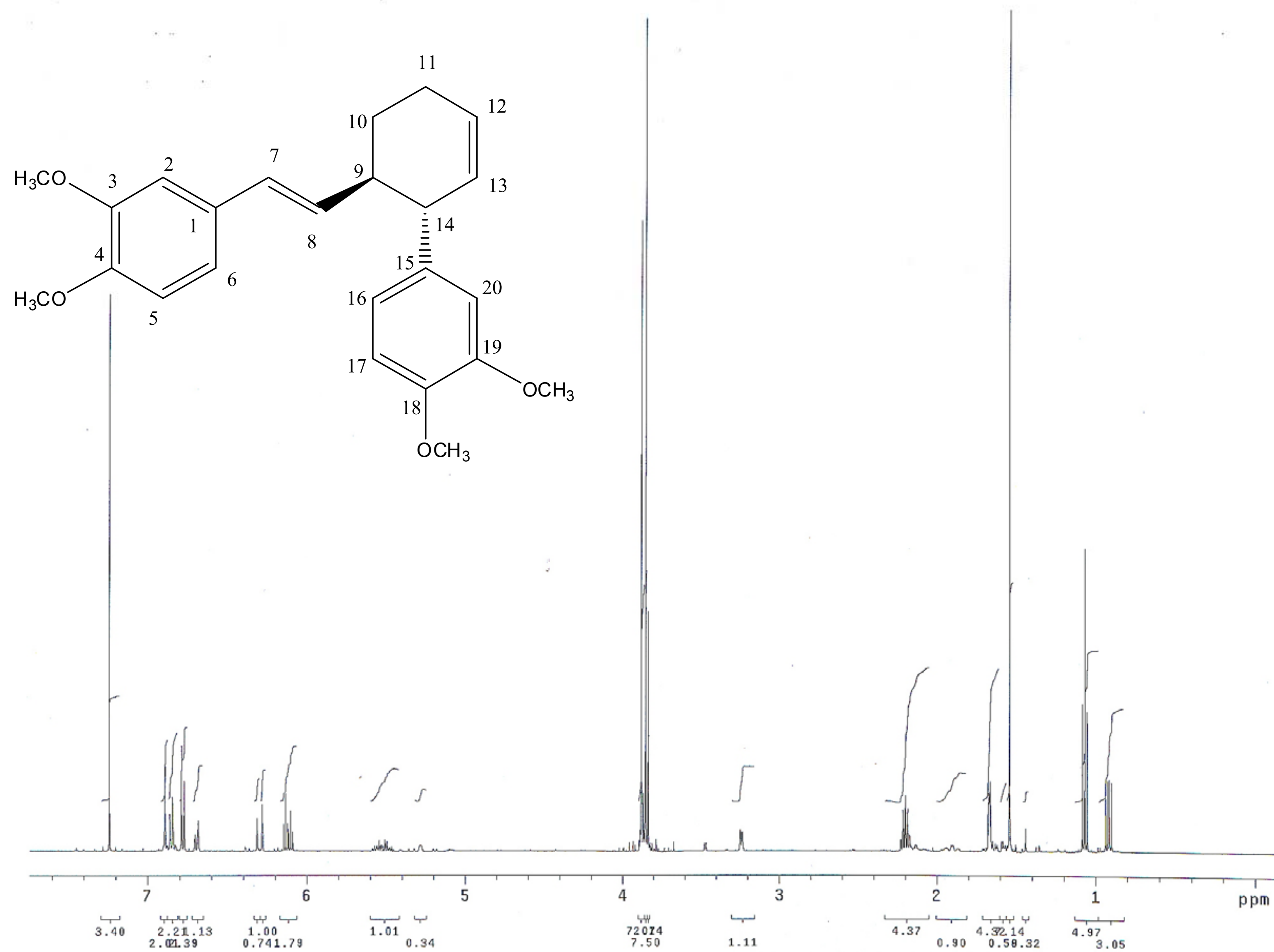


Figure 4-14 ^{1}H NMR spectrum of P4 ($CDCl_3$; 500 MHz)

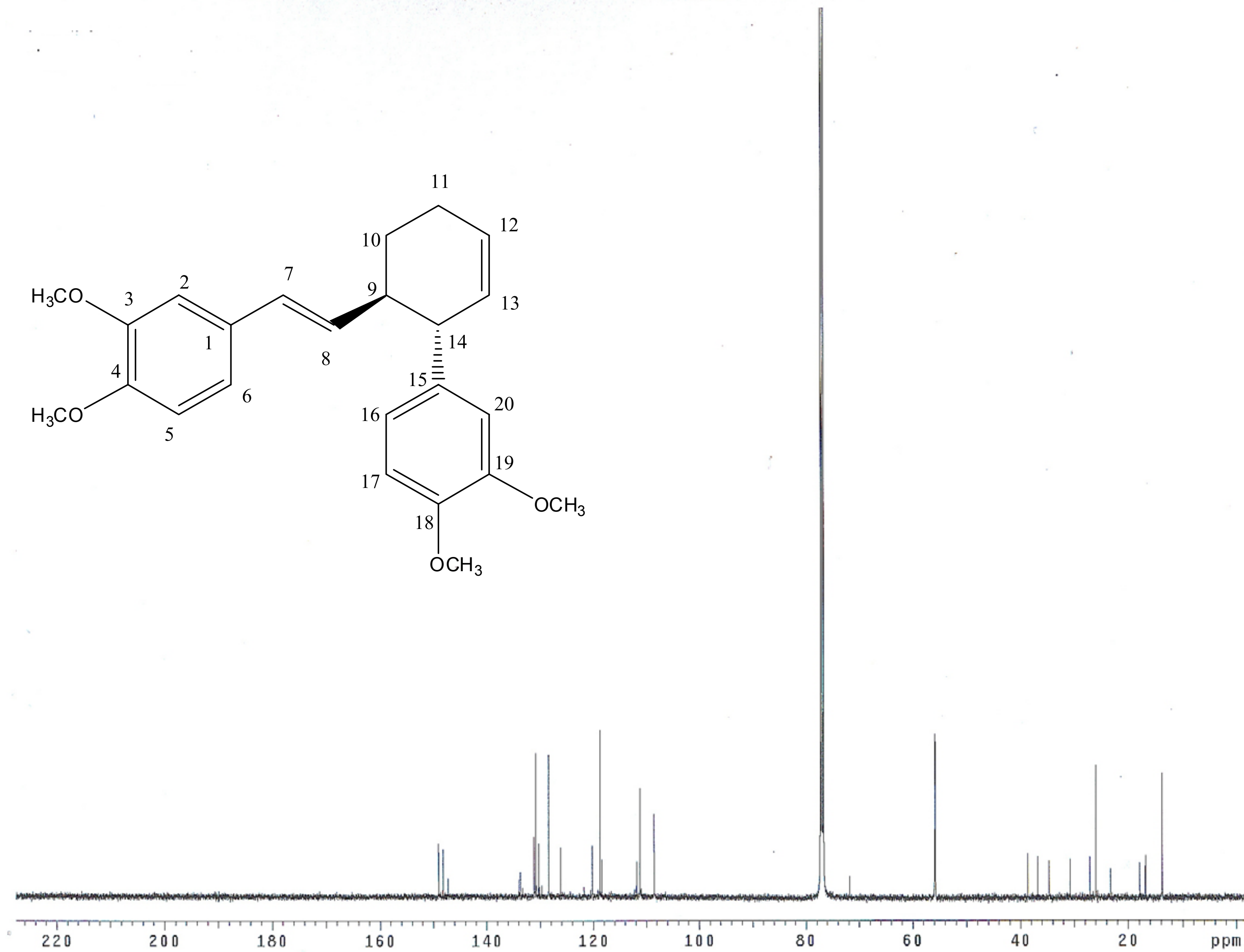


Figure 4-15 ^{13}C NMR spectrum of P4 ($CDCl_3$; 125 MHz)

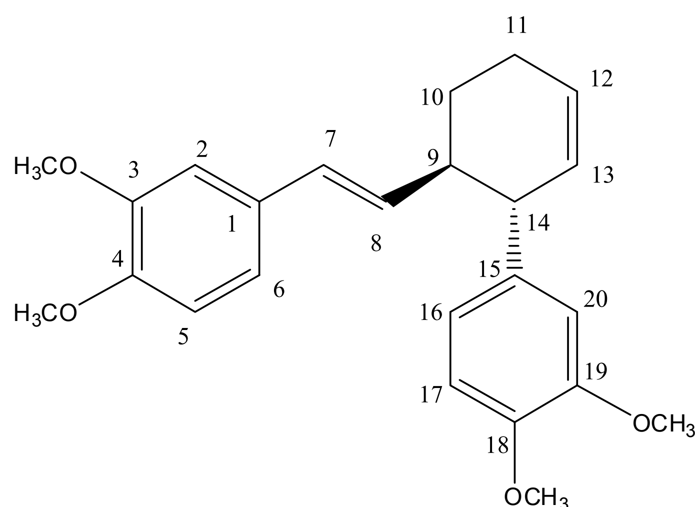


Figure 4-16 Chemical structure of (*E*)-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4dimethoxystyryl] cyclohex-1-ene (DMPDMS)

Compound D, D-acetate, DMPBD and DMPDMS were isolated and used them as indicative marker for quality control of *Z. cassumunar* extract. Four compounds were then subjected to evaluate of anti-inflammatory activity.

4.2 Evaluation of anti-inflammatory activity

Four pure compounds (compound D, D-acetate, DMPBD and DMPDMS) that isolated from *Z. cassumunar* extract were examined for their inhibitory activities against nitricoxide (NO) production. The results showed that all four compounds possessed anti-inflammatory activity via inhibition of NO production with IC_{50} values of 211.1, 86.8, 56.3 and 39.7 µM, respectively (Table4-5). Among these compounds, DMPDMS exhibited the highest inhibitory activity with IC_{50} value of 39.7 µM. The structure-activity relationship of these phenylbutanoids indicated that substitution on the position -1 of the butenyl group with more polar moiety could decrease in NO production inhibitory activity which could be observed from IC_{50} of compound D and D acetate and DMPBD (IC_{50} = 211.1, 86.8, 56.3 µM, respectively). However, the activity was increased when the two phenylbutanoids were formed as a dimeric compound at position -1 of the butenyl group (DMPBD) which could be observed from IC_{50} of

DMPDMS (IC_{50} = 39.7 μM). These finding suggest that these compounds possess anti-inflammatory activity via inhibition of NO production. In addition, it has been reported that compound D and D-acetate possessed the inhibitory activity on edema formation, when was evaluated using the carageenin-induced rat paw edema model at the same dose as acetylsalicylic acid (300, 150-600 mg/kg). The result showed that compound D exhibited the strongest inhibitory activity on edema formation (Panthong *et al.*, 1990). DMPBD and DMPDMS exhibited the COX-2 inhibitory activity with the IC_{50} values of 20.68 and 2.71 μM, respectively (Han *et al.*, 2005).

Table 4-5 Inhibitory effect against LPS-induced NO production of compound D, D-acetate, DMPBD and DMPDMS

Compounds	IC_{50} (μg/ml)	IC_{50} (μM)
Compound D	43.9	211.1
D-acetate	21.7	86.8
DMPBD	10.7	56.3
DMPDMS	15.1	39.7
Caffeic acid phenethyl ester	1.6	5.6

4.3 HPLC quantitative determination of *Z. cassumunar* extract

The optimal conditions for quantitative determination of *Z. cassumunar* extract were performed using gradient reverse phase HPLC system. The four phenylbutanoid compounds were used as the indicative markers for quantitative analysis of *Z. cassumunar* extract as these compounds have maximum absorption at 254 nm, this wavelength was then used for quantification. Mixtures of methanol and 2% aqueous acetic acid were examined as the mobile phase, and the ratios as well as gradient elution system were optimized. The amount of methanol in 2% aqueous acetic acid was maintained at 52% v/v for 20 minutes and reduced from 50 to 52% v/v for 16 minutes and increased to 52% at 38 minutes until total run at 60 minutes (Figure 4-17). Phenylbutanoids were eluted with the retention time of 50 minutes (Figure 4-18) with satisfactory

resolution. The previously reported HPLC method analyzed (*E*)-4-(3,4-dimethoxyphenyl)-3-butene-1,2-diol, (*E*)-4-(3,4-dimethoxy-phenyl)-but-3-en-1-ol, (*E*)-4-(3,4-dimethoxyphenyl)-but-3-en-1-yl-acetate, (*E*)-3-(3,4-di-methoxyphenyl) propenal, (*E*)-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl]cyclohex-1-ene and (*Z*)-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl] cyclohex-1-ene) in the *Z. cassumunar* extract with a poor resolution of DMPDMS. In addition, this method can not determine DMPBD content in the extract and validation of the analytical procedure is not yet established (Lu *et al*., 2008).

On the basis of HPLC analysis, all four phenylbutanoids were found in *Z. cassumunar* extract. Compound D, D-acetate, DMPBD and DMPDMS were detected as the major active compounds.

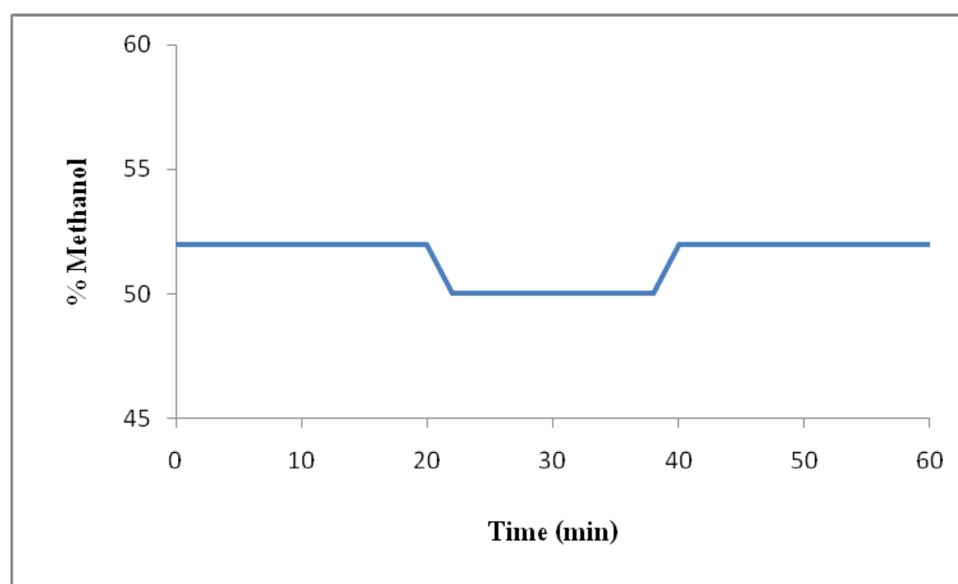


Figure 4-17 Gradient profile of the mobile phase (2% aqueous acetic acid: methanol)

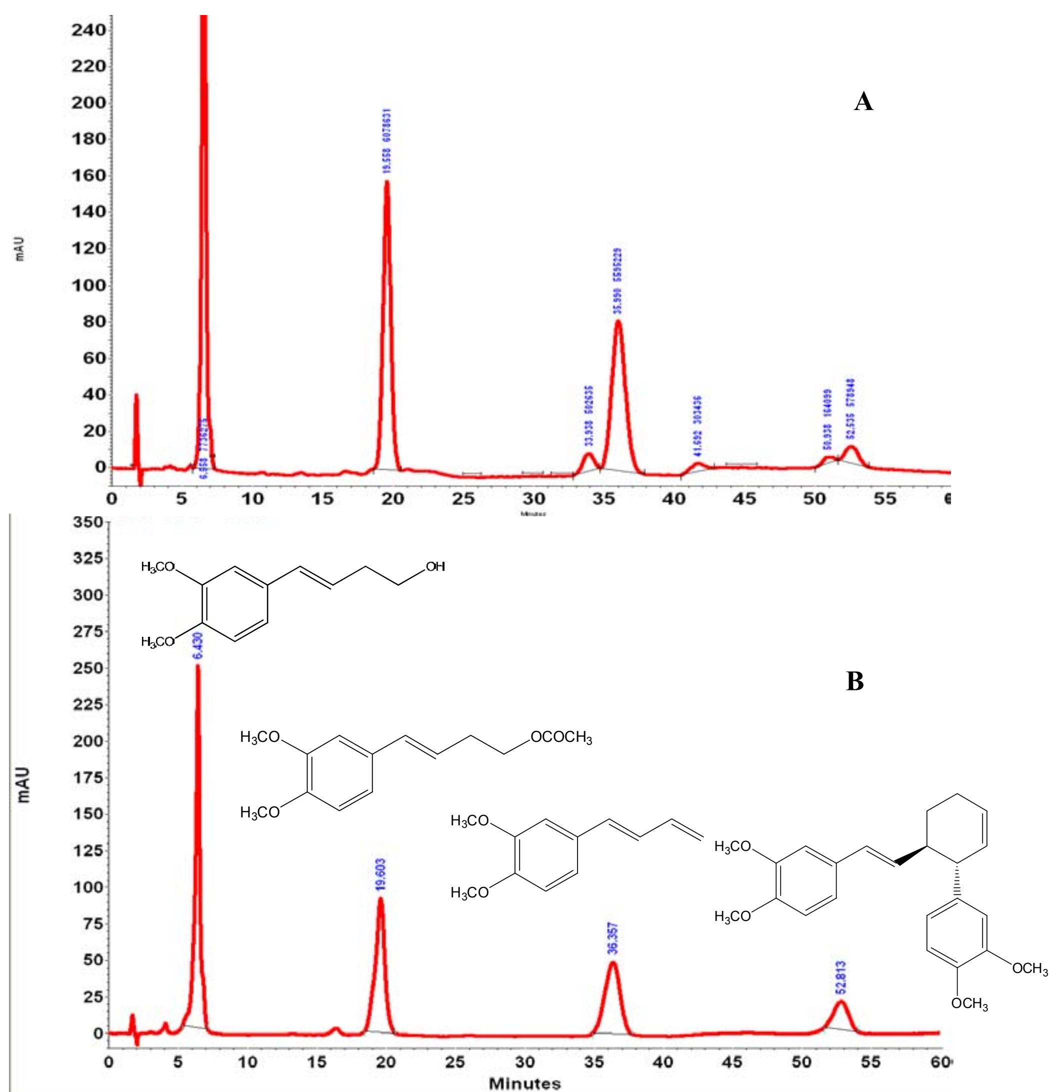


Figure 4-18 HPLC-chromatograms of (A) *Z. cassumunar* extract and (B) standard compounds

4.3.1 Validation of analytical method

Validation of HPLC analytical method was examined for linearity, precision, accuracy, specificity, LOD and LOQ.

4.3.1.1 Linearity

Linearity was evaluated using the standard solutions over five calibration points (from 0.78-12.5 µg/ml) with three measurements for each calibration points. Excellent linearity was observed for all standard compounds over the evaluated ranges with the correlation coefficients of 0.9999, 0.9996, 0.9998 and 0.9999 for compound D, D-acetate, DMPBD and DMPDMS, respectively (Table 4-6).

Table 4-6 Calibration curves of phenylbutanoid compounds from *Z. cassumunar*

Compounds	Linear range (µg/ml)	t_R (min)	Equation[a]	Linearity (R^2)
Compound D	0.79-12.5	6.3	y = 418.0300x + 54.0078	0.9999
D-acetate	0.79-12.5	18.7	y = 321.1270x + 45.9839	0.9998
DMPBD	0.79-12.5	34.0	y = 355.8780x + 42.3301	0.9996
DMPDMS	0.79-12.5	50.0	y = 213.0669x + 8.4696	0.9999

[a] Y= AX + B, where Y is peak area, X is the concentration of the analyzed material.

4.3.1.2 Accuracy

The accuracy of the analytical method was studied by spiking technique. Compound D, D-acetate, DMPBD and DMPDMS solution at concentrations of 12.5, 6.25 and 3.125 µg/ml were spiked into *Z. cassumunar* sample solutions to evaluate recoveries of the standard compounds. The recoveries closed to 100% (Table 4-7) indicate a good accuracy of this method.

Table 4-7 Recovery data of *Z. cassumunar*

Compounds	**Spiked level (µg/ml)**	**Recovery (%)[a] (n=3) (mean ± SD)**
	3.125	101.47 ± 0.10
Compound D	6.25	102.14 ± 0.09
	12.5	98.63 ± 0.12
	3.125	104.90 ± 0.05
Compound D-acetate	6.25	105.85 ± 0.07
	12.5	97.61 ± 0.14
	3.125	101.40 ± 0.15
DMPBD	6.25	105.35 ± 0.23
	12.5	96.06 ± 0.03
	3.125	99.93 ± 0.05
DMPDMS	6.25	101.09 ± 0.09
	12.5	96.05 ± 0.10

[a] All values were mean ± S.D. obtained by triplicate analyses.

4.3.1.3 Precision

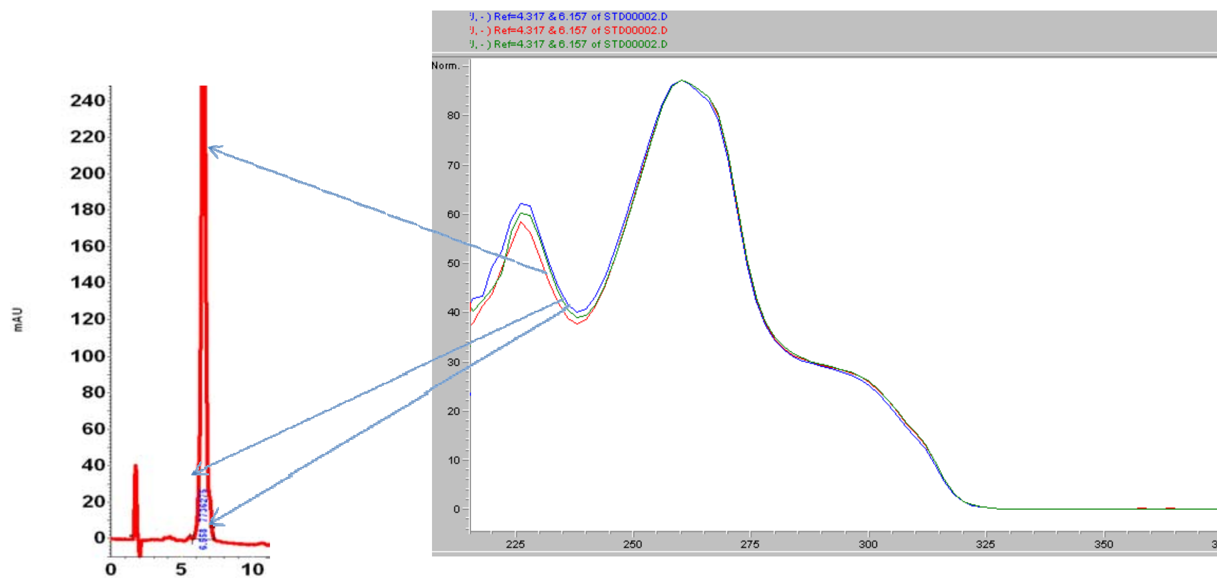
The precision of the chromatographic system was tested by performing intra- and inter-day multiple injections of sample solutions. The percentage relative standard deviation (%R.S.D.) was determined. The %R.S.D. values of intra- and inter-day analysis of all compounds were less than 5% (Table 4-8) indicated the high precision of HPLC method.

Table 4-8 Intra-day and inter-day presision data of *Z. cassumunar* extract

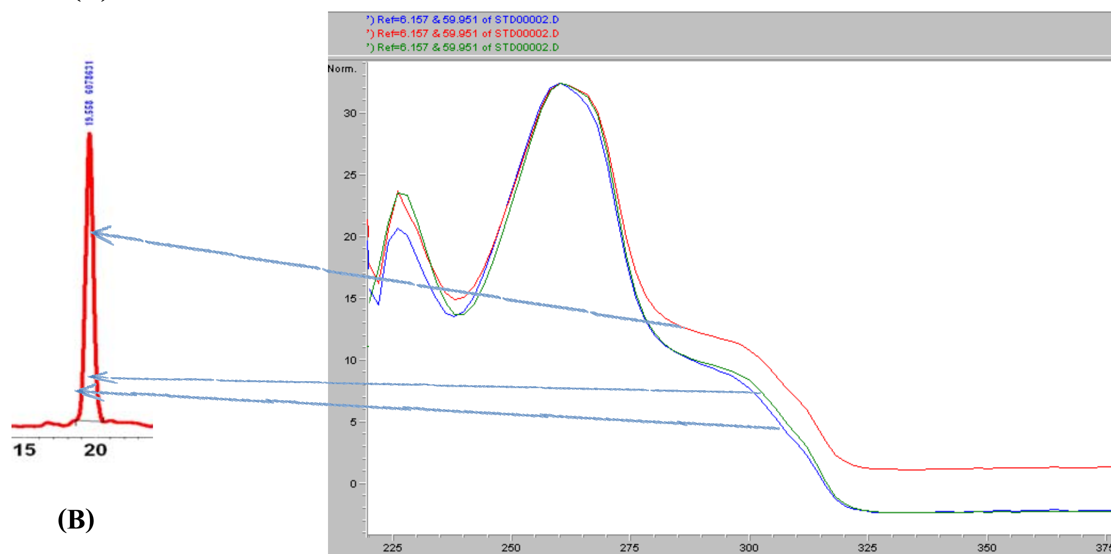
Compounds	Inter-day (n=3)						Intra-day (n=6)	
	Day 1		Day 2		Day 3			
	Content (%w/w)	R.S.D. (%)	Content (%w/w)	R.S.D. (%)	Content (%w/w)	R.S.D. (%)	Content (%w/w)	R.S.D. (%)
Compound D	8.22	0.19	8.18	0.06	7.82	0.75	8.07	0.33
D-acetate	4.07	0.96	5.04	0.24	4.58	0.35	4.56	0.52
DMPBD	10.34	0.96	10.40	0.10	7.74	1.45	9.49	0.84
DMPDMS	0.75	1.74	0.76	1.61	0.68	1.26	0.73	1.54

4.3.1.4 Specificity

Utilizing the PDA makes it possible to obtain the UV spectra of the analyzed compounds. Specificity of the method was evaluated using UV-absorption that were taken at three points of the peaks and compared to those of the standard compounds. The results indicated that the UV absorbtion spectra of the samples were similar to their authentic compounds and the homogenity of the three spectra that taken at three different points, of the peaks was observed (figure 4-19, 4-20). These results suggested that the HPLC method possessed good specificity.

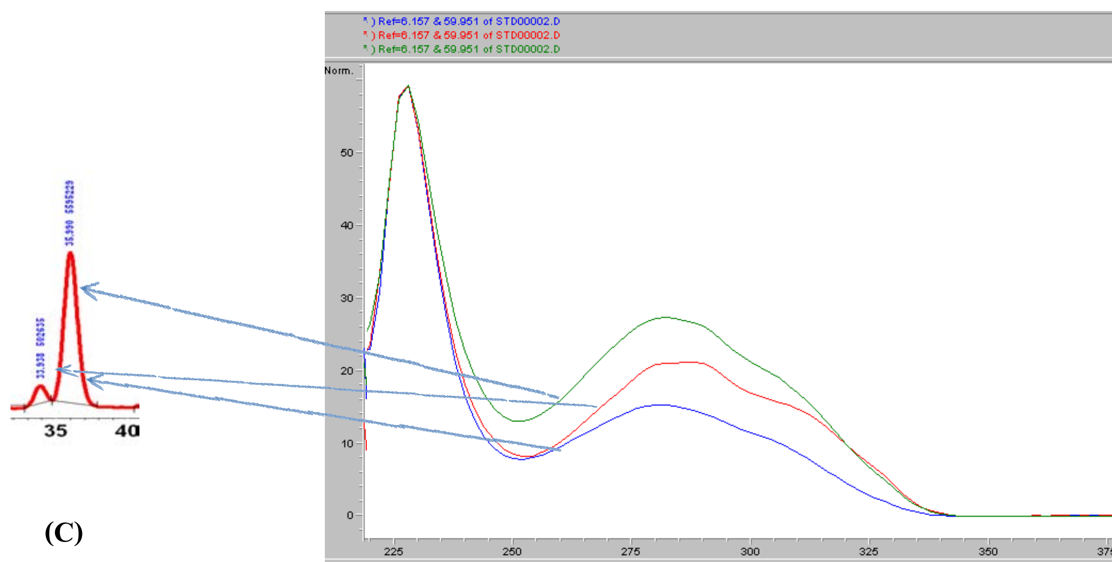


(A)

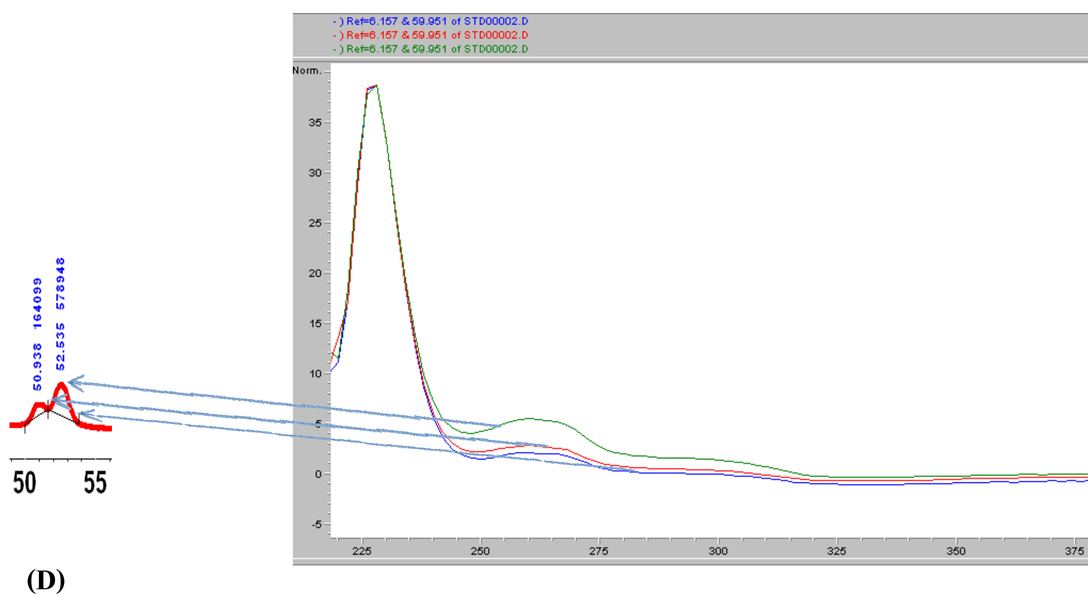


(B)

Figure 4-19 Specificity of compound D (A) and D-acetate (B)



(C)



(D)

Figure 4-20 (continued) Specificity of DMPBD (C) and DMPDMS (D)

4.3.1.5 LOD & LOQ

The limit of detection represents the lowest concentration of compound D, compound D-acetate, DMPBD and DMPDMS that can be detected by the analytical method, whereas the limit of quantitation represents the lowest concentration of the active compounds that can be determined with acceptable precision and accuracy by the instrument and method. The results of LOD and LOQ analysis for compound D, compound D-acetate, DMPBD and DMPDMS (Table 4-9) indicate that the established HPLC method is sufficiently sensitive for determination of these four active compounds in *Z. cassumunar* rhizome extract.

Table 4-9 LOD and LOQ of *Z. cassumunar* extract

Compounds	LOD[a] (μg/ml)	LOQ[b] (μg/ml)
Compound D	0.13	0.18
D-acetate	0.18	0.60
DMPBD	0.18	0.90
DMPDMS	0.72	1.45

[a] Limit of detection (LOD): signal to noise ratio = 3

[b] Limit of quantification (LOQ): signal to noise ratio = 10

4.4 Determination of solvent for extraction

A few different extraction solvents were examined to produce the highest content of the phenylbutanoid compounds in *Z. cassumunar* extract. Four major active compound found in *Z. cassumunar* rhizome possess quite different polarity. Thus, hexane, chloroform, ethyl acetate and methanol were examined as the extraction solvents. The result showed that although methanol produced highest yield of the extract. Hexane produced the extract with the highest content of all active compounds (Table 4-10). In contrast, hexane was appropriately used for the extraction of all four active compounds. In addition, determination of the active compounds in the volatile oil isolated from *Z. cassumunar* rhizome found that the volatile oil contained high content of DMPBD. However, the content of DMPBD is lower than in the hexane extract.

Compound D, D-acetate and DMPDMS were not found in the volatile oil. This finding suggests that the hexane extract is more suitable for use as anti-inflammatory agent than volatile oil.

Table 4-10 The content of active compounds in *Z. cassumunar* extracts extracted under reflux conditions with various solvents and compare with the volatile oil

Extracts	Yield (% w/w)	Content (% w/w; Mean ± S.D.)			
		Compound D	D-acetate	DMPBD	DMPDMS
Hexane ext.	6.4	8.74 ± 1.047	4.29 ± 0.759	14.58 ± 1.192	2.49 ± 0.358
$CHCl_3$ ext.	8.7	6.20 ± 0.919*	4.04 ± 0.796	9.41 ± 1.785*	1.79 ± 0.261*
EtOAc ext.	18.9	3.72 ± 0.421*	2.05 ± 0.372*	5.30 ± 0.545*	0.84 ± 0.072*
MeOH ext.	41.7	2.39 ± 0.885*	1.21 ± 0.513*	3.80 ± 1.046*	0.67 ± 0.203*
Volatile oil	0.6	0.00*	0.00*	12.23 ± 0.488	0.00*

* Significant difference (*P<0.05*) when compare with the hexane extract within the same column

4.5 Preparation of high-yield anti-inflammatory active compounds extract

After the solvent for extraction of *Z. cassumunar* rhizomes was optimized, the hexane extract was further fractionation using silica gel vacuum chromatography to produce a phenylbutanoid rich extract of *Z. cassumunar*. The appropiate method is described as followed. Dried powder rhizomes of *Z. cassumunar* (250 g) were refluxed with hexane (3 times x 3 L) for 1 hour then the combined extract was evaporated to dryness *in vaccuo*. Yellowish oily liquid was obtained after evaporated to dryness (Figure 4-8) and the yield of the extract was 5.9 ± 0.253 % w/w when compared to the weight of the dried powder (Table 4-11). The hexane extract (15 g) was further fractionated using silica gel vacuum chromatography with a mixture of hexane and ethyl acetate (8:2). The combined extracts were evaporated to dryness *in vaccuo* to produce a phenylbutanoid rich extract. Phenylbutanoids were found as the major compound in the extract with the total phenylbutanoid content of 39.63 ± 0.301 % w/w (Table 4-11). The phenylbutanoid content was 48.29 ± 0.379 % w/w, which the phenylbutanoid content was increased to 8.66

%w/w (Table 4-12). The phenylbutanoid rich *Z. cassumunar* extract were then subjected to evaluation of anti-inflammatory activity compared with the hexane extract, volatile oil and the pure active compounds. The result showed that anti-inflammatory of phenylbutanoid rich *Z. cassumunar* extract was higher than those of the hexane extract, volatile oil and the pure phenylbutanoid compounds. In addition, its activity was closed to those of a positive control and indomethacin (IC_{50} values of 1.6 and 8.9 µg/ml) (Table 4-13). This may be due to a synergistic effect of the phenylbutanoid compounds on anti-inflammatory activity. Thus, the phenylbutanoid rich *Z. cassumunar* extract is more suitable for use as anti-inflammatory agent.

Table 4-11 Yield and phenylbutanoid content of the hexane extract of *Z. cassumunar*

Lot No.	**Extraction yield (%w/w)**	**Content (% w/w; Mean ± S.D.)**				**Total active compounds content (%w/w)**
		Compound D	**D-acetate**	**DMPBD**	**DMPDMS**	
1	6.02	13.56 ± 0.116	12.90 ± 0.073	11.19 ± 0.040	1.85 ± 0.056	39.50 ± 0.285
2	6.09	12.34 ± 0.023	9.88 ± 0.032	10.65 ± 0.145	1.72 ± 0.040	34.58 ± 0.241
3	5.62	9.21 ± 0.080	11.86 ± 0.093	22.54 ± 0.159	1.21 ± 0.044	44.82 ± 0.376
Mean ± SD	5.91± 0.253	11.70 ± 0.073	11.55 ± 0.066	14.79 ± 0.115	1.59 ± 0.047	39.63 ± 0.301

Table 4-12 Phenylbutanoid content in the phenylbutanoid rich *Z. cassumunar* extract

Lot No.	**High-yielded active compounds extract (%w/w)**	**Content (% w/w; Mean ± S.D.)**				**Total active compounds content (%w/w)**
		Compound D	**D-acetate**	**DMPBD**	**DMPDMS**	
1	4.46	15.54 ± 0.195	15.78 ± 0.146	13.20 ± 0.174	2.21 ± 0.050	46.74 ± 0.565
2	4.43	16.17 ± 0.034	14.34 ± 0.036	14.89 ± 0.187	2.55 ± 0.099	47.95 ± 0.357
3	3.66	10.27 ± 0.041	13.05 ± 0.093	25.45 ± 0.069	1.40 ± 0.012	50.18 ± 0.215
Mean ± SD	4.18 ± 0.455	13.99 ± 0.090	14.39 ± 0.092	17.85 ± 0.143	2.05 ± 0.054	48.29 ± 0.379

Table 4-13 Anti-inflammatory activity of the extracts and compounds of *Z. cassumunar*

Extract/Compounds	IC_{50} (µg/ml)
Compound D	43.9
D-acetate	21.7
DMPBD	10.7
DMPDMS	15.1
Hexane ext. Lot 1	10.9
Hexane ext. Lot 2	9.5
Hexane ext. Lot 3	11.9
Volatile oil	21.5
High-yield active compounds extract Lot 1	4.9
High-yield active compounds extract Lot 2	4.8
High-yield active compounds extract Lot 3	4.6
Caffeic acid phenethyl ester	1.6 (5.6 µM)
Indomethacin	8.9 (25 µM)

4.6 Determination of solubility

Solubility is commonly expressed as a maximum equilibrium amount of a solute that can normally dissolve per amount of solvent or a maximum concentration of a saturated solution. These maximum concentrations are often expressed as grams of solute per 100 ml of solvent. The solubility test of the phenylbutanoid rich extract is used to estimate the dissolution of the extract in various solvents. The result showed that the phenylbutanoid rich extract is freely soluble in methanol, ethyl acetate, chloroform and hexane. It is practically soluble in water (Table 4-14). The phenylbutanoid rich extract contains most likely high, moderate and non-polar compounds therefore the suitable solvents for the phenylbutanoid rich extract should be a moderate and non- polar solvents.

Table 4-14 Solubility property of the phenylbutanoid rich extract

Solvent	Volume of solvent in ml/g of solute	Level of solubility
Methanol	1	Freely soluble
Ethyl acetate	1	Freely soluble
Chloroform	1	Freely soluble
Hexane	4	Freely soluble
Water	>10,000	Practically insoluble

CHAPTER 5

CONCLUSION

1. Compound D, D-acetate, DMPBD and DMPDMS were isolated from *Z. cassumunar* extract and used as indicative markers for quality control of *Z. cassumunar* extracts.

2. HPLC quantitative analysis method for *Z. cassumunar* extract were achieved by using the mobile phase consisted of methanol and 2% acetic acid, gradient mode (0-20 min, 52% v/v methanol, 22-38 min, 50% v/v methanol and 40-60 min, 52% v/v methanol) with a flow rate of 1 ml/min. The injection volume was 20 µl. The quantitation wavelength was set at 254 nm. This method simple, specific, precise, accurate and reproducible HPLC method has been developed to quantify the phenylbutanoid compounds in *Z. cassumunar*. The simultaneous quantitative determination for compound D, D-acetate, DMPBD and DMPDMS provides useful marker information for the quality control of *Z. cassumunar* extract.

3. Hexane is a suitable solvent for the phenylbutanoids from *Z. cassumunar* rhizome.

4. Fractionation of the hexane extract of *Z. cassumunar* was capable of producing the phenylbutanoid rich *Z. cassumunar* extract to 48.29 ± 0.38 % w/w. This method was also capable of producing the strong anti-inflamatory activity with IC_{50} value of 4.7 µg/ml compare to the positive control, indomethacin, and active compounds (IC_{50} = 1.6, 8.9, 43.9, 21.7, 10.7 and 15.1 µg/ml, respectively).

5. The phenylbutanoid rich *Z. cassumunar* extract contains most likely moderate and non polar compounds. Therefore, the suitable solvents to dissolve the extract should be moderate polar solvent such as methanol, ethyl acetate, chloroform and hexane.

6. The standard specification of the phenylbutanoid rich extract of *Z. cassumunar* was established that the phenylbutanoid rich extract should contain the total phenylbutanoid content not less than 45% w/w.

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