AN INEXPENSIVE & SAFE METHOD FOR PREPARATION OF CARYOPHYLLENE OXIDE,

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The thesis submitted in fulfillment of the
requirement of the award of the degree of
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I hereby declare that I have checked this thesis and in my opinion this thesis is adequate in terms of scope and quality for the award of the degree of master of Science in Industrial Chemistry

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Dedicated to my parents, and all my family

ACKNOWLEDGEMENT

Interpendency makes human superior than other living beings. From cradle to funeral we depend upon family members and friends in all aspects of life. This thesis would never have happened without the constant support of my family and friends.

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ABSTRACT

Caryophyllene oxide which exists as white crystalline solids with melting points of 62 °C, is widely used as an important material in perfumery industry and recently had been patented as antitumor agent. This 99% pure oxide compound is very much more expensive than the original caryophyllene due to the difficulty in production. Experiments were carried out to produce caryophyllene oxide by using inexpensive and safe method. In this study, four objectives were studied; extraction, separation, purification of caryophyllene from clove buds which were later used in this synthesis of caryophyllene oxide in this study. In extraction, four methods were used, cleaner- ultrasonic, hydrodistillation, steam distillation and microwave to obtain clove oil and found that, hydro distillation method was found to give the highest yield of clove oil. For separation process the cayophyllene and the other non-polar molecules in clove oil were separated from eugenol, the highest yield for both clove bud and clove leaf were achieved at a ratio of clove oil: sodium hydroxide of 1: 2. Purification of caryophyllene by using vacuum distillation consist 94% component of a mixture of caryophyllene, and 5.8% alpha-cubebene by using GC/MS analyzer at 136 °C. Caryophyllene oxide was then synthesized by using four different acids, formic acid, acetic anhydride, 3-chloroperbenzoic acid, and acetic acid. The highest percentage of caryophyllene oxide formed in solution analysed by GC/MS was by formic acid (86.47%), followed by 3-chloroperbenzoic acid (81.47%), acetic anhydride (77.04%), and acetic acid (75.33%). Caryophyllene oxide was then crystallized at low temperature until subsequent analysis showed that it is 99% pure.

ABSTRAK

Kariofillin Oksida (C.O.) hadir sebagai pepejal kristal berwarna putih dengan takat lebur pada suhu 62 °C, digunakan secara meluas sebagai bahan penting dalam industri pewangi dan kebelakangan ini, C.O. dipatenkan sebagai agen antitumor. Oksida ini yang berkepekatan 99% ketulenannya adalah lebih mahal daripada kariofillin asalnya disebabkan kerumitan dalam proses pengeluaran. Beberapa eksperimen dijalankan untuk menghasilkan C.O. dengan menggunakan kaedah murah serta selamat dikendalikan. Projek penyelidikan ini merangkumi empat objektif pengajian; proses pengekstrakan, proses pengasingan, proses penulenan kariofilin daripada putik cengkih yang kemudiannya digunakan dalam sintesis kariofilin oksida dalam pengajian ini. Bagi proses pengekstrakan, empat kaedah telah digunakan iaitu ultrasonic, penyulingan dengan air, penyulingan dengan stim dan gelombang mikro untuk mendapatkan minyak cengkih, kaedah penyulingan dengan air didapati mempunyai nilai hasil minyak cengkih yang tertinggi. Dalam proses pengasingan, kariofilin dan molekul bukan polar yang lain dalam minyak cengkih telah di asingkan daripada eugenol, yield tertinggi diperolehi daripada kedua-dua putik cengkih dan daun cengkih yang dicapai oleh nisbah minyak cengkih: natrium hidroksida iaitu 1: 2. Penulenan kariofillin menggunakan penyulingan vakum mengandungi 94% komponen campuran kariofillin, dan 8% alfa-cubebene dengan menggunakan GC/MS pada 136 °C. kariofillin oksida kemudiannya disintesiskan dengan menggunakan empat jenis asid berlainan, asid formic, asetik anhidrat, asid 3-kloroperbenzoik dan asid asetik. Peratusan tertinggi kariofillin oksida yang terbentuk dalam larutan yang dianalisa oleh GC/MS adalah menggunakan asid formic (86.47%); diikuti dengan menggunakan asid 3-kloroperbenzoik (81.47%), asetik anhidrida (77.04%), dan asid asetik (75.33%). Kariofillin oksida seterusnya dikristalkan pada suhu yang rendah dan menunjukkan 99 % tulen.

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LIST OF SYMBOLS

%	Percent
º C	Degrees Celsius
g	Gram
kg	Kilogram
h	Hour
MHz	Mega hertz
KHz	Kilo hertz
W	Watt
mL	Mililitre
L	Litre
min	Minute
mL/g	Mililitre per gram
β	Beta
mg	Miligram
H_2O	Water
µm	Micrometer
±	Plus minus
R	Factor (crystallography)
a	Crystallography axis
b	Crystallography axis
c	Crystallography axis

z	Crystallography axis
º	Degree
w/v	Weight per volume
mm	Milimeter
m	Meter
º C/min	Degree celcius per min
ml/min	Mililitre per minute
eV	Electron volt
s	Second
g/mol	Gram per mol
C.O	Caryophyllene oxide
V/V	Volume per volume
:	Ratio
cm^{-1}	Per centimeter
ppm	Part per million

LIST OF ABBREVIATIONS

GC/MS	Gas Chromatography Mass Spectrometer
RM	Ringgit Malaysia
et al.	And others
SFE	Supercritical Fluid Extraction
GC	Gas Chromatography
SFME	Supercritical Fluid Microvawe Extractor
MAHD	Microvawe assist Hydrodistillation
HSCCC	High speed counter current chromatography
ELSD	Evaporative light scatter detector
HeLa	Human cervical adenocarcinoma cells
HepG2	Human leukemia cancer cells
AGS	Human lung cancer cells
SNU-1	Human gastric cancer cell
SNU-16	Human stomach cancer
TLC	Thin layer chromatography
NEOS	Network-Enabled Optimization System
USA	United States of America
S80H	Elma model (plug-in mains supply)
He	Helium gas
N2	Nitrogen gas
H2	Hydrogen gas

DB-5	Colum phase composition
NMR	Nuclear magnetic resonance
^{1}H	Proton 1
^{13}C	Carbon 13
AM 400	Atomic mass 400
$CDCl_3$	Dichloromethane
AMU	Atomic mass unit
FTIR	Fourier transform infrared
IR	Infrared
pKa	Primary knock-on atom
C=C	Carbon double bonded to carbon
=CH	Double bonded to 1 carbon, 1 hydrogen
=CH2	Double bonded to 1 carbon, 2 hydrogen
C-H	Carbon bonded to hydrogen
C-O	Carbon bonded to oxygen

CHAPTER 1

INTRODUCTION

1.1 Background

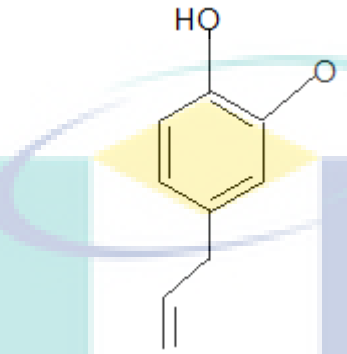
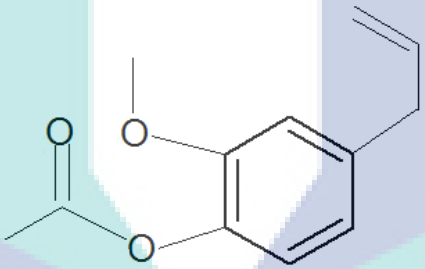
Clove yields three types of crude essential oil which can be extracted from the leaves, the stems and the buds (Alma et al., 2007) namely clove leaf, clove stem and clove bud oils. The clove oil that is produced from these raw materials differs considerably in yield and quality. The yield and compositions of the oil obtained are influenced by its origin, season, variety and quality of raw material, maturity at harvest, pre- and post-distillation treatments and finally the method of distillation. Clove oil contains eugenol (Myint et al., 1996), caryophyllene as the major compositions and other minor compounds such as eugenol acetate (Huston and Li, 1991).

The best quality essential oil from the clove oil contains (80- 90%) eugenol, (15%) eugenol acetate and (5-12%) β-caryophyllene (Alma et al., 2007). Alma et al. (2007) identified 18 chemical compositions of the essential oil from Turkish Clove Buds which is produced or extracted by steam distillation method.

The analysis of clove bud oil extracted with liquid and supercritical carbon dioxide showed significant qualitative and quantitative compositional differences compared to oil obtained by the conventional hydrodistillation process. Wengqiang et al. (2007) reported clove bud oil obtained by supercritical fluid extraction (SFE)

and hydrodistillation contained (53.8-55.9%) and (48.82%) percentage of eugenol respectively. The extraction of the bud flavor from the spice indicated different result by the parameters of pressure, temperature, contact time (Gopalakrishnan et al., 1990). The essential oils can be extracted by three methods hydrodistillation, microwave and ultrasonification. GC-MS analysis of the clove oils obtained by different methods showed that the composition of the clove oil was almost similar, but the relative concentration of the identified compounds was apparently different. The oil yield was influenced largely by particle size while the caryophyllene content by temperature (Wengqiang et al., 2007). Table 1.1 contains molecule structures of eugenol, eugenol acetate, caryophyllene and caryophyllene oxide and description of its properties respectively.

Table 1.1: Molecule structures and properties of eugenol, caryophyllene and its derivatives.

compounds	Molecule structure	Properties
Eugenol		Molecular formula = $C_{10}H_{12}O_2$ Molar mass = 164.20 g/mol Physical state = clear to pale yellow oily liquid Boiling point = 254 °C Soucre: Chemicalland, 2011.
Eugenol acetate		Molecular formula = $C_{12}H_{14}O_3$ Molar mass = 206.24 g/mol Physical state = clear to pale yellow oily liquid Boiling point = 281-286 °C Source: Chemicalland, 2011.

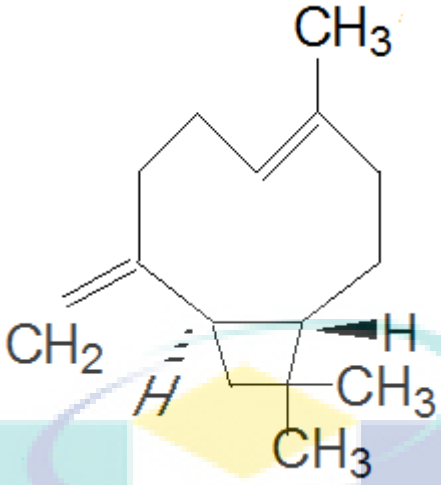
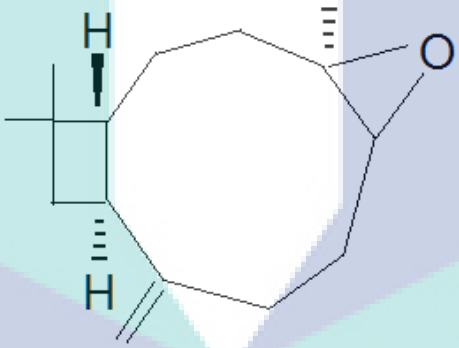
Caryophyllene		Molecular formula = $C_{15}H_{24}$ Molar mass = 204.36 g/mol Boiling point = 254 °C Category = fragrance and flavor agent Appearance = colorless clear oily liquid Odor type = spicy Source: The Good Scents Company,2012.

Table 1.1: Continued.

Caryophyllene oxide		Molecular formula = $C_{15}H_{24}O$ Molar mass = 220.36 g/mol Category = fragrance and flavor agent Appearance = white crystalline solid Odor type = woody Melting point = 60- 63 °C Source: The Good Scents Company, 2012.

Caryophyllene and their derivatives such as caryophyllene oxide, caryophyllene alcohol (Bhatia et al., 2008 and Mussinan et al., 1980) and caryophyllene acetate are widely used in flavour and fragrance compositions (Kaiser et al., 1976). In industries all caryophyllene derivatives are produced synthetically using caryophyllene as the raw material and therefore they are more expensive than caryophyllene. Some are very much more expensive because of the cost of the other materials used as well as other reasons (Kaiser et al., 1976).

Caryophyllene oxide is very much more expensive than caryophyllene itself or any other eugenol derivatives because of the difficulty in producing it. Just for comparison the price of 1 gram caryophyllene oxide of 99 % purity is RM 279.50 compared to the price of caryophyllene which is RM 10 per kilogram at the time of this work. The price of the most expensive eugenol derivative, dihydroeugenol synthesized using eugenol extracted from clove oil is only over RM 800 per kilogram. Figure 1.1 shows the molecule structure of caryophyllene and caryophyllene oxide. Table 1.2 shows the list of price of clove oil, caryophyllene and its derivatives.

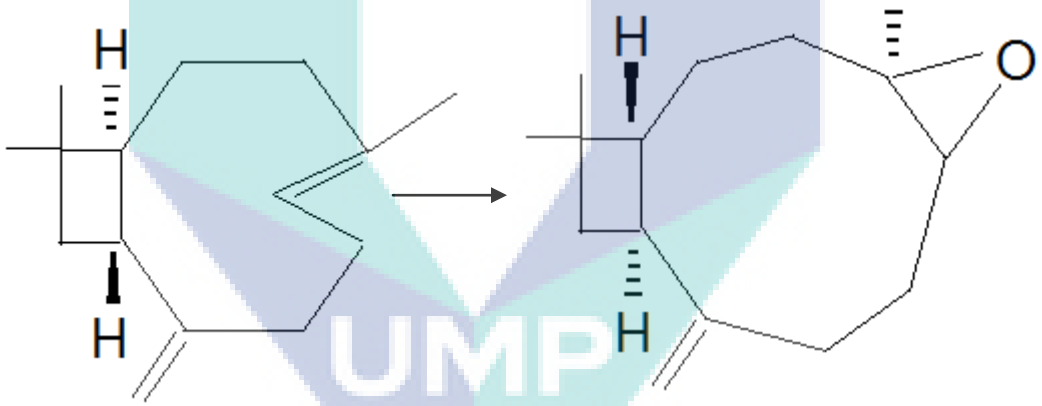


Figure 1.1: Conversion from caryophyllene to caryophyllene oxide.

Table 1.2: Prices of clove oil, caryophyllene and its derivatives.

No	Compound	Price
1	Clove oil	RM 10 / kg
2	Crude caryophyllene	RM 10 / kg
3	Caryophyllene oxide 99% pure	RM 279.50 / gram

Source: SIGMA ALDRICH MALAYSIA June 2009.

Caryophyllene oxide besides commercially applied in perfume industry (Sapra et al., 2010) and as synthetic flavoring substances (Kaiser et. al, 1976 and Yang et. al, 1999) this oxygenated terpenoid has recently been patented as antitumor agent (Choudary et. al, 2006 and Pichette et al., 2002). In previous studies, caryophyllene oxide was used as antifungal against dermatophytes (Yang et al., 1999). Other derivatives such as α-humulene and isocaryophyllene (γ-caryophyllene) are also known to have antitumor properties as shown in figure 1.2.

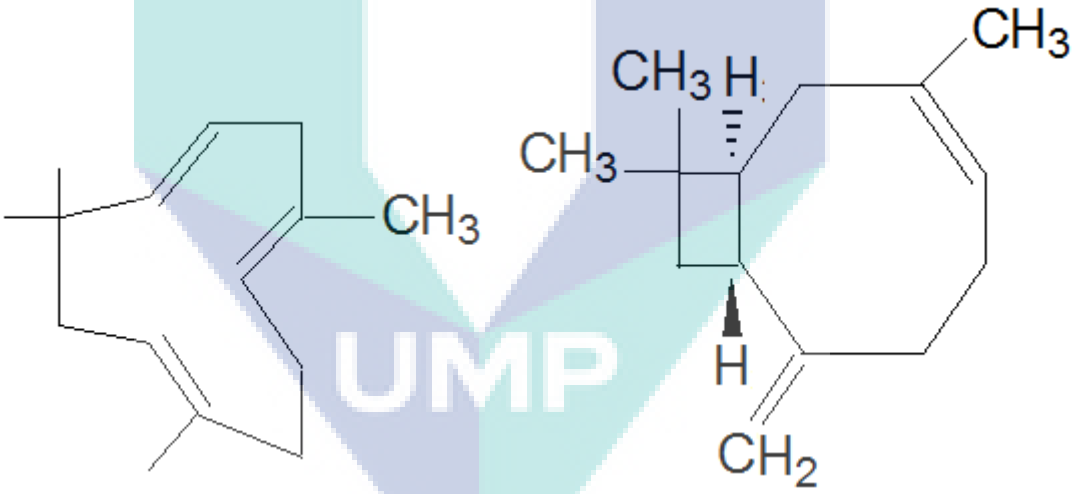


Figure 1.2: α-humulene and isocaryophyllene molecule structure.

Moreover, gluthatione-S-transferase enzyme and anticarcinogenic agent of β-caryophyllene, β-caryophyllene oxide and α-humulene have been shown to increase the activity of the detoxification which could prevent the formation of cancers (Pichette et al., 2002). Other study by Kubo et al. (1996) decribed β-caryophyllene and β-caryophyllene oxide being isolated from *Asteraceae* exhibits an antitumoral activity against solid tumor cell lines.

1.1.1 Caryophyllene oxide special properties

Plants which belonging to the *Psidium* genus possess caryophyllene oxide have been shown to exhibit several therapeutic properties, including antibacterial, hypoglycemic, anti-inflammatory, analgesic, antipyretic, spasmolytic, and central nervous system-depressant activities, and are used as a popular medicine (Begum et al., 2002).

Lapcik et al. (2005) and Pino et al. (2001) found the isoflavonoids and volatile compounds were detected from the leaf and fruit oil respectively, of *P. cattleianum*. GC/MS analysis showed the oil was composed at different percentages of the following primary components caryophyllene-pinene, myrcene-thujene, 1, 8-cineole, epi-muurolol, cadinol, epi-cadinol and caryophyllene oxide, at different percentages (Pino et al., 2001). The leaf extract have antimicrobial specific properties (de Souza et. al, 2004 and Brighenti et al., 2008) and anti-caries effects in rats (de Menezes et al., 2010). Joseph and Priya (2010) and Arima and Danno (2002) reported there was antimicrobial activity in the essential oil *Psidium guajava L*. leaf.

1.2 Problem Statement

Research had explored about the caryophyllene oxide which can be isolated from the selected plants. Hubert and Wiemer (1984) claimed the epoxide of caryophyllene had been detected in the essential oil of sage, *Salvia sclarea.* However he did not isolate this compound.

The first study by Jun explained the caryophyllene oxide was isolated from the leaves of the Jeju guava (Jun et al., 2011) and this useful sesquiterpene existed about 5.1% in oil of guava leaves (Dweck, 2001). Isolation of caryophyllene oxide from essential oil of *L. citriodora* contains 6.60% caryophyllene oxide (Meshkatalsadat et al., 2010). Judzentiene et al. (2010) study on chemical

composition of essential oil of Artemisia found that the content of caryophyllene oxide in the oil ranged from 8.5% - 38.8%. In addition, GC-MS analysis of bioactive components found the composition of 19.25% caryophyllene oxide from the ethanolic leaf extract of *Canthium dicoccum* (Rajeswari et al., 2011). The highest composition of caryophyllene oxide (24.14%) was found in the essential oil from inflorescences of *Spilanthes calva* (Begum et al., 2008). The Afsharypuor and Azarbayejany (2006) investigation of chemical constituents in *Lavandula officinalis* flowers resulted a very low caryophyllene oxide percentage (0.5%).

In commercial the methods of preparation for all of these derivatives are well kept secret. It is only known that the raw material used for production of caryophyllene oxide in industry is caryophyllene derived from the clove oil which is cheap, widely abundant essential oil and can be commonly obtained by steam distillation of clove leaves or clove buds (Kaiser et al., 1976).

Only certain multinational manufacturers such as Firmenich, International Flavor and Fragrance (IFF), Rhone Poulenck and Givaudan are known to produce all these derivatives.

1.3 Research Aim

In this study caryophyllene oxide will be prepared from caryophyllene using an inexpensive safe method with peracid prepared in situ at a various carboxylic acid reagents with various parameters. The parameter includes pH, reaction time, reagent concentration, reaction temperature and also solvent temperature reaction which will be fully discussed in chapter 4. The cost of these reagents used in this method is less than RM 2 for 1 kg of caryophyllene oxide produced at the time of this work.

1.4 Research Objectives

The objectives of the research project are:

-To extract crude clove bud oils by using different methods of extraction.

-To separate and purify of caryophyllene from crude clove oil.

-To synthesise caryophyllene oxide using caryophyllene and other inexpensive reagents.

It is known that propylene oxide which is produced by Petronas in Kerteh Terengganu using the method in which propylene is epoxidized using hydrogen peroxide and acetic acid under certain conditions. It is interesting to know if similar method can be applied to this caryophyllene an cyclic alkene of natural product organic chemical.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This chapter indicates the methods relevant to the present studies. It consist of several eugenol extraction methods, separation and purification of caryophyllene from the plants, isolation of caryophyllene oxide and synthesis of caryophyllene oxide. The chateristics of caryophyllene oxide also briefly discussed included caryopyllene oxide as antitumor, fragrance, molecule structure and also crystallization studies.

2.2 Extraction of clove oil

2.2.1 Clove

Clove or scientifically named *Syzygium aromaticum* (Huston and Li, 1991) is native to Indonesia and cultivated commercially in many countries such as Madagascar, Zanzibar, Sri Lanka and South of China (Baseri et al., 2008). These aromatic herb flower buds can grow to a height 10-20 m of range and the buds are harvested as they reach 1.5-2 cm in length. It is commercially used as a flavoring agent, and in cigarette industry. Clove oil and eugenol can act as antifungal and is beneficial to the digestion system (Woldeamanuel, 2011). This essential oil was traditionally used as a flavoring agent and also as an anti microbial in food due to the presence of high percentage of eugenol in the clove oil. Currently, clove oil, shown in figure 2.1 is in great demand in Korea as it is used as medicine in the pharmaceutical industry (Baseri et al., 2008).

There are several extraction methods for clove oil established by previous works. These are hydrodistillation, steam distillation, microwave and Supercritical fluid extractions (SFE).

Figure 2.1: Clove bud oil.

2.2.2 Supercritical fluid extraction (SFE)

This method is a beneficial sample preparation technique because its extraction system provides minimal solvent disposal and it has a short extraction time (Huston and Li, 1991).

Wright et al. (1987) reported that the coupling of SFE to high resolution capillary gas chromatography (GC) acts as a superior analyzer in sample preparation and analysis system. The clove is extracted by using either column interface, as shown in figure 2.2 or solvent recovery. In order to obtain a high efficiency result, the column interface was better than solvent recovery method because of having high component selectivity in potential resolution for many complex natural products analysis.

Both steam distillation method and SFE have significant effects in extraction time. The maximum yield obtained by SFE method was 24% followed by 19% of Gopalakrishnan et al. (1990) work. However, there is no research which has found a distinct difference between steam distillation and SFE method.

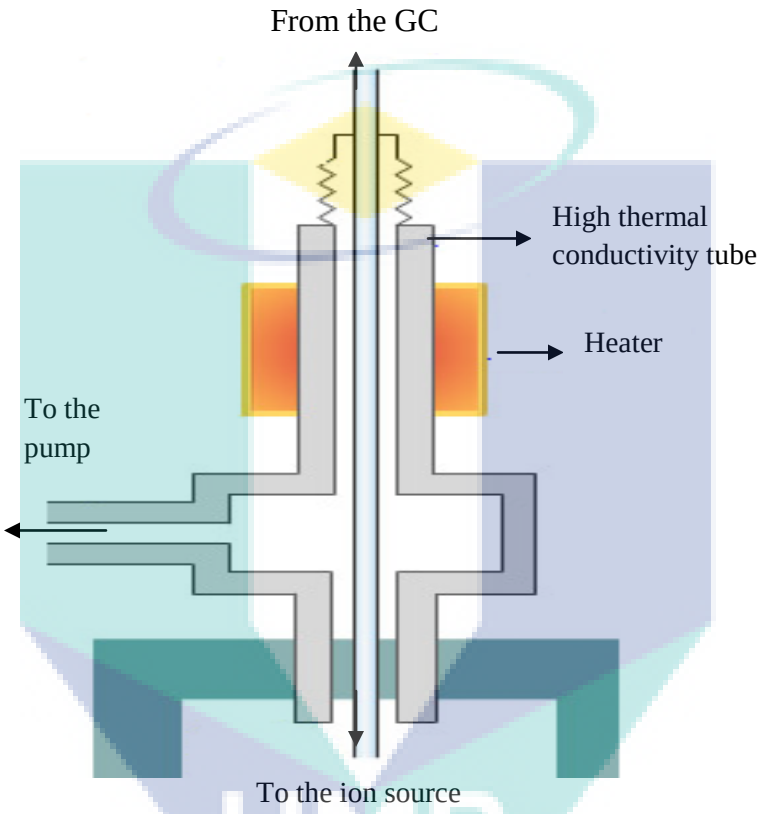


Figure 2.2: Direct column interface.

Source: Huston and Li (1991).

2.2.3 Hydrodistillation and Steam Distillation Extraction

The traditional and simplest methods for extraction of clove oil are hydrodistillation, as shown in figure 2.3 and steam distillation methods, as shown in figure 2.4. For hydrodistillation, the plant material was fully dipped in the water which was then boiled. The oil obtained by this extraction can be easily isolated.

Both hydrodistillation and steam distillation methods could use either dried clove buds or clove leaves to produce clove oil. The plant for hydrodistillation was submitted for 4–6 h and steam distillation for 8–10 h. The oil was easily isolated using this simple technique. Clove oil obtained by steam distillation had the highest percentage of eugenol (58.2%), compared to SFE (53.8–55.9%). Clove oil obtained by hydrodistillation had the lowest percentage of eugenol (48.82%). However, clove oil obtained by SFE contained highest percentage of eugenol acetate (20.32–21.75%) (Wengqiang et al. 2007).

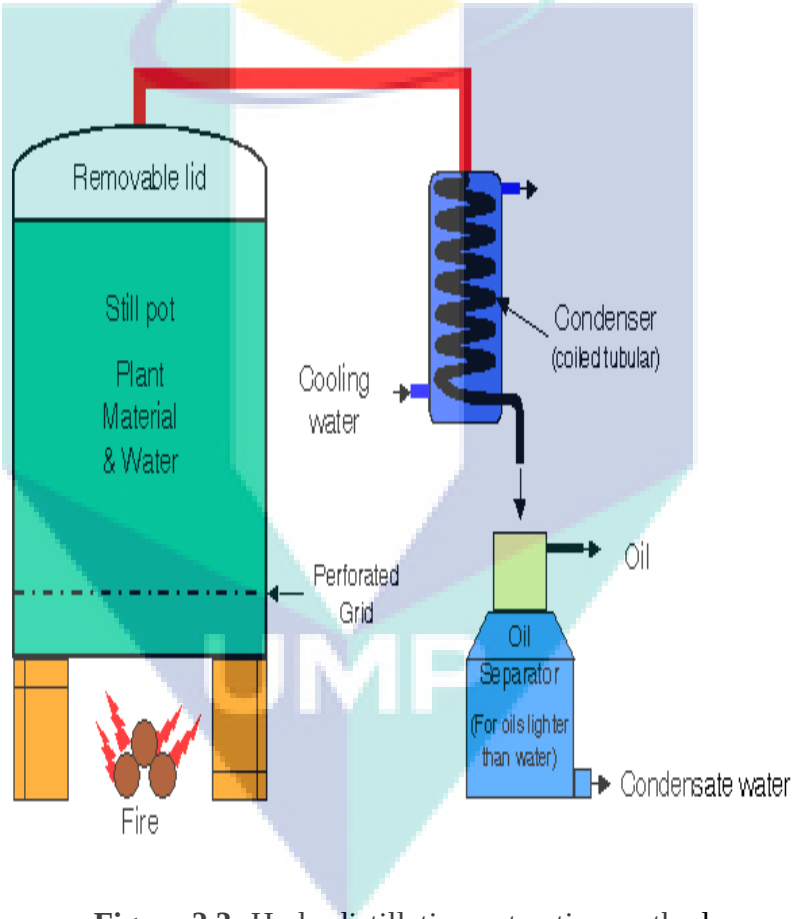


Figure 2.3: Hydrodistillation extraction method.

Source:

http://www.google.com.my/search?num=10&hl=en&site=imghp&tbm=isch&source=hp&biw=1137&bih=548&q=Ultrasonic+Extractor+equipment.

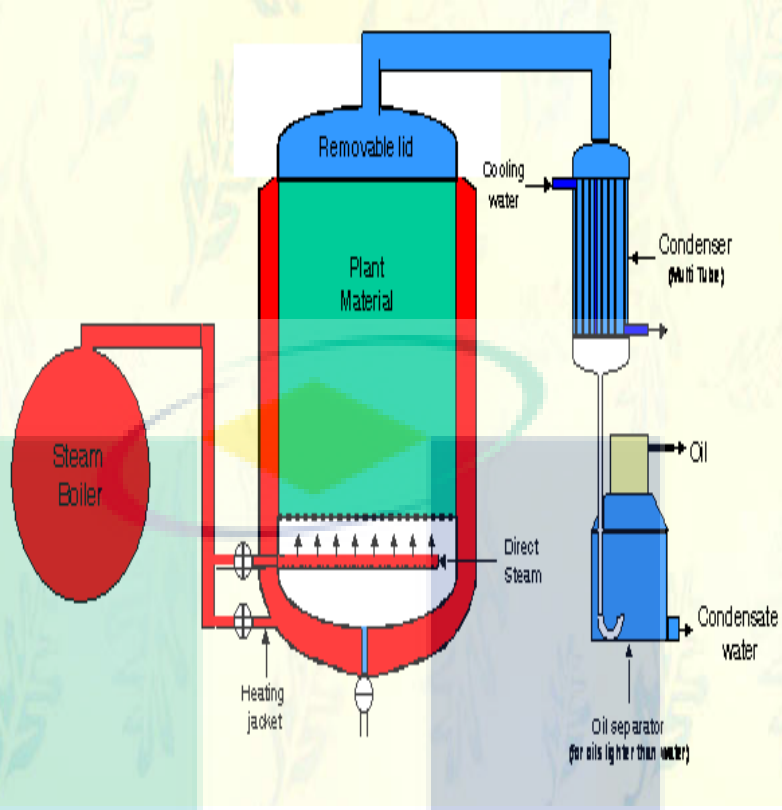


Figure 2.4: Steam distillation method.

Source:

http://www.google.com.my/search?num=10&hl=en&site=imghp&tbm=isch&source=hp&biw=1137&bih=548&q=Ultrasonic+Extractor+equipment.

2.2.4 Microwave Extraction

In the literature, two types of microwave extractors were reported. First, solvent Free Microwave Extractor (SFME), as shown in figure 2.5 which was experimentally used to extract the oregano. Oregano (*origanum vulgare*) is a shrubby herb cultivated in Mediterranean and in some regions in Siberia (Sahin et al., 2004). Another type of microwave extractor is Microvawe Assist Hydrodistillation (MAHD) which had been used for extracting rosemary oils (Fadel et al., 2011). Both had the same experimental set up to study the effect of microwave power and

microwave level. SFME was suspended with 2450 MHz frequency with a maximum power of 622W. The microwave oven was connected to Clavenger apparatus and the essential oil was collected and kept at 4 °C until it was analyzed. For SFME, a dried plant was soaked in 700ml distilled water for 1 h at room temperature being before extracted.

For MAHD, dried leaves were hydrodistilled in 500ml water at 460W energy of 2450 MHz at atmospheric pressure for 80 min. The system was equipped by Clevenger apparatus and the produced essential oil was stored in the dark at 5°C until analysis. The result shows SFME gave essential oil yield (0.054 ml/ g oregano) (Bayramoglu et al., 2008) while MAHD produced 0.015 ml/ g rosemary (Fadel et al., 2011). These results cannot be compared as they used different plants.

Figure 2.5: Solvent Free Microwave Extractor (SFME).

Source:

http://www.google.com.my/search?num=10&hl=en&site=imghp&tbm=isch&source=hp&biw=1137&bih=548&q=Solvent+Free+Microwave+Extractor.

2.2.5 Ultrasonic Extraction

Ivana et al. (2006) reported the Ultrasonic Extraction of tobacco (*Nicotiana tabacum*) oil was investigated. The tobacco seeds, belongs to the Solanaceae family (Zhao et al., 2007) and as a byproduct of tobacco leaves (Giannelos et al., 2002) were ground and performed in 2.5-60 min using Ultrasonic Cleaner at 40 KHz. The equipment of ultrasonic extractor was shown in figure 2.6.

The sonication was carried out at 25, 40 °C and at a boiling point temperature. At the end of extraction, the liquid extracted was separated from the solid residue by vacuum filtration. The seed cake was twice washed with solvent and evaporated in rotary vacuum evaporator. The best condition was obtained at 25 °C in 20 min using ratio of solid to solvent of 1:3 with the yield of 1.5g oil/ 100g tobacco (Ivana et al., 2006).

Figure 2.6: Ultrasonic Extractor equipment.

Source:

http://www.google.com.my/search?num=10&hl=en&site=imghp&tbm=isch&source=hp&biw=1137&bih=548&q=Ultrasonic+Extractor+equipment.

2.3 Separation and purification of caryophyllene

2.3.1 Caryophyllene

Caryophyllene, a common sesquiterpene widely distributed in plants (Matasyoh et al., 2007) is naturally found in essential oil, primarily in clove oil (*Eugenia caryophyllata*) (Alma et al., 2007 andMd. Nazrul et al., 2010) and essential oil of many liverworts (Fricke et al., 2005). Caryophyllene can also be found in

Artemisia annua plant with sweet warmwood odor (Cai et al., 2002) which has anti-inflammory effects (Fernandes et al., 2007) and cytotoxic activity (Kubo et al., 1996).

This unsaturated alipathic hydrocarbon was traditionally used in cosmetic fields and fragrance industry (Reinsvold et al., 2010 and Skold et al., 2006). Caryophyllene also has special behavior properties as it can attract two types of herbivore enemies, entomopathogenic nematodes and parasitic wasps (Crocoll 2010).

The chemistry of caryophyllene is a rich source of interesting reactions which is not yet intensively explored (Warnhoff, 1964). Kaiser et al. (1976) claimed that 10% humulene in the crude extract of caryophyllene can be isolated to produce a pure caryophyllene. In commercial caryophyllene production, column chromatography on Kieselgel was used. The process of column chromatography was illustrated in figure 2.7. Other study reported that caryophyllene extract obtained from clove oil consist some α-humulene as well as β-elemene (Cheng et al., 2007) which can be separated in order to obtain pure caryophyllene.

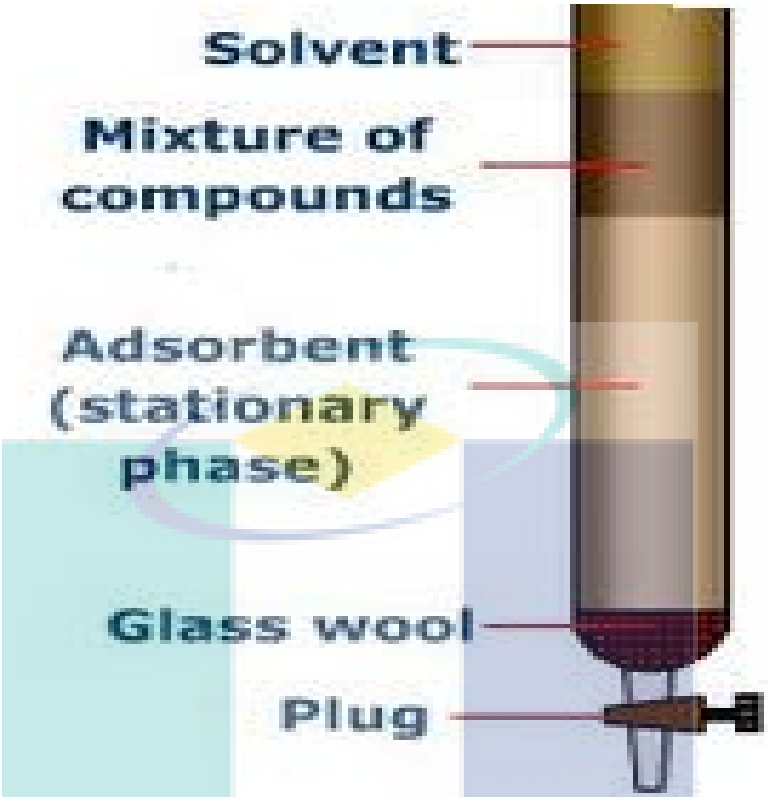


Figure 2.7: Column chromatography process.

Source:

http://www.google.com.my/search?num=10&hl=en&site=imghp&tbm=isch&source=hp&biw=1137&bih=548&q=Column+chromatography+process.

2.3.2 Caryophyllene separation

To isolate and purify caryophyllene, high speed countercurrent chromatography (HSCCC) coupled with an evaporative light scatter detector (ELSD) was successfully applied as shown in figure 2.8. Two ratio of n-hexane-chloroform-acetonitrile (6:2:5) or *n*-hexane-dichloromethane-acetonitrile (10:3:7) was used and the result shows application of *n*-hexane-dichloromethane-acetonitrile (10:3:7) gives a higher purity of 97.3% by GC. 85mg of pure caryophyllene obtained yielded from

600 mg crude essential oil when eluted with the lower phase at a flow rate of 1.5 ml/ min (Xie et al., 2008).

Figure 2.8: High speed countercurrent chromatography (HSCCC) equipment.

Source:

http://www.google.com.my/search?num=10&hl=en&site=imghp&tbm=isch&source=hp&biw=1137&bih=548&q=High+speed+countercurrent+chromatography.

Fractional distillation work also was famously applied in caryophyllene purification. Starting from the crude caryophyllene, this mixture was fractionally distilled through a vacuum jacketed spinning band column. Then the distillate was analysed by TLC which showed that it contained isocaryophyllene, caryophyllene and humulene. Next, these three components was re-distillated and the TLC result showed only caryophyllene and humulene produced. The caryophyllene and humulene then underwent treatment with petroleum and silver nitrate to remove

humulene together with some caryophyllene. The petroleum ether residual was washed and then percolated first through silica gel column and finally through a neutral alumina column. Distillation gave a 97% pure of caryophyllene with the remaining five other components impurities (Warnhoff and Srinivasan, 1972).

2.4 Caryophyllene oxide

2.4.1 Caryophyllene oxide background

Caryophyllene represents a natural medium-ring olefeins containing a nine-membered ring. Caryophyllene can undergo typical reaction to produce caryophyllene derivatives such as caryophyllene alcohol, caryophyllene acetate. The first caryophyllene derivative found in nature was caryophyllene oxide, an important sesquiterpenoids which is widespread in many plants in small amounts (Yang and Deinzer 1994). Caryophyllene oxide appears as white amorphous powder and shows analgesic and anti-inflammatory activity (Chavan et al., 2010).

2.4.2 Caryophyllene oxide sources

(i) Synthesis

Tkachev (1987) reported that the caryophyllene oxide can be produced by oxidation of caryophyllene by acid-catalyzed cyclization (Collado et al., 1997) although no detail of method preparation was presented. Caryophyllene oxide was produced by pervanadic acid-catalyzed oxidation method, but no detail experiment as reported (Treibs, 1947). A direct x-ray structural study of caryophyllene oxide was reported by Gatilov et al. (1983) which confirmed the structure of this epoxide. In University of Western Ontario Research, some experiments on the trans-cyclononene caryophyllene were carried out. One of them was epoxidation of caryophyllene. Caryophyllene in chilled ether was reacted with perphthalic acid in ice cold water bath. The reaction was kept at 5 °C for 9 hours when no peracid remained. Then the decanted ethereal solution was treated

and the solvent was removed. The product was then analysed by using TLC and gave 94% of monoepoxide and 3% of bis-epoxide (Warnhoff and Srinivasan, 1972).

(ii) Isolation

A small amount of caryophyllene oxide had been isolated from certain essential oils such as verbena oil by distillation and column chromatography. However as mentioned earlier this essential oil is very expensive and is very difficult to obtain especially for commercial production due to climatic conditions during the plants cultivation (Kaiser et al., 1976).

Jun et al. (2011) reported that caryophyllene oxide was successfully isolated from the leaf of Jeju guava (strawberry guava). The Jeju guava (*P. cattleianum*) leaf samples were air-dried, chopped, and extracted with methanol. The 80% methanol solvent was evaporated to dryness with a rotary evaporator to obtain the crude methanol extract. A portion of the crude methanol extract was then suspended in H_2O and fractionated with the organic solvents hexane. The hexane fraction was then subjected to column chromatography over silica gel to obtain pure caryophyllene oxide.

2.4.3 Caryophyllene oxide as Cytotoxic and antitumor agent

The main components of Guava leaf, caryophyllene oxide is reported to be cytotoxic. Scientist found that this oxide performed cytotoxic activity (Sibanda et al., 2004) but was inactive against tumor cell lines (Pichette, 2002).

Cytotoxic activities against several human cancer cell lines were evaluated to determine whether or not caryophyllene oxide is a cytotoxic compound and compared with quercetin, a well known antioxidant of natural product (Kanokmedhakul and Lekphrom, 2007). Cancer cell lines, including HeLa (human cervical adenocarcinoma cells), HepG2 (human leukemia cancer cells), AGS (human lung cancer cells), SNU-1 (human gastric cancer cell) and SNU-16 (human stomach cancer) were incubated in the presence of various concentrations of caryophyllene

oxide. The effect of 50 µM concentration of caryophyllene oxide on the growth of HeLa, HepG-2, AGS, SNU-1 and SNU-16 as showed in table 2.1 below.

Table 2.1: Caryophyllene oxide and quercetin effect on the cell lines growth.

cell lines	caryophyllene oxide	quercetin
HepG-2	3.95 ± 0.23	97.3 ± 2.17
HeLa	13.55 ± 0.45	166.3 ± 2.23
AGS	12.6 ± 0.86	126.03 ± 1.94
SNU-1	16.79 ± 1.2	19.64 ± 1.3
SNU-16	27.39 ± 1.4	62.53 ± 1.8

Source: Jun et al., (2011)

The results proved caryophyllene oxide excellent performed excellently as a cytotoxic activity compared to natural standard quercetin. The findings demonstrated the caryophyllene oxide showed the highest cytotoxic activity against HepG-2 cell cancer rather than previous studies (Jun et al., 2011).

2.4.4 Caryophyllene oxide as a fragrance

In the field of odorant manufacturing, the caryophyllene oxide was widely used as an important component such as in eau de cologne. A woody and balsamic odour characteristic of caryophyllene oxide compound was added to many odorant compositions resulting in a strong modifying action. The combination of individual odorant like citral odorant generally used by manufacturers, in perfuming technical and cosmetic products such as detergents, aerosol, lotion cream and many others. The ideal composition of caryophyllene oxide in perfume ranges from 2 to 10 weight

% and in the finished products presence between 0.01 and 0.5 weight % (Kaiser et al., 1976).

2.4.5 Caryophyllene oxide structure

The structure of caryophyllene oxide isolated from guava species (Jun et al 2011) in figure 2.9 was confirmed after comparison of its NMR and Mass. 1H-NMR and 13C-NMR at 400 and 100 MHz, respectively, were conducted on a Bruker AM 400 spectrometer in CDCl3.

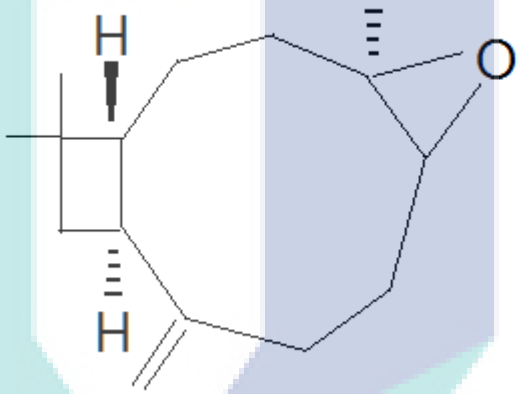


Figure 2.9: Structure of caryophyllene oxide isolated from Jeju guava leaf

Source: Jun et al., (2011)

2.4.6 Caryophyllene oxide crystal study

Gatilov et al. (1983) reported in his x-ray study on caryophyllene oxide. The temperature of 98-100 °C used resulted (1182 reflections with R = 0.051). The caryophyllene oxide crystals of the orthorhombic system gave the value of a = 8.975, b = 10.160, c = 14.882 A, z = 4 with the 62-63 °C of melting point.

Figure 2.10 shows the structure of the caryophyllene oxide molecule, the bond lengths, and some torsion angles. The values of the valence angles are given in table 2.2.

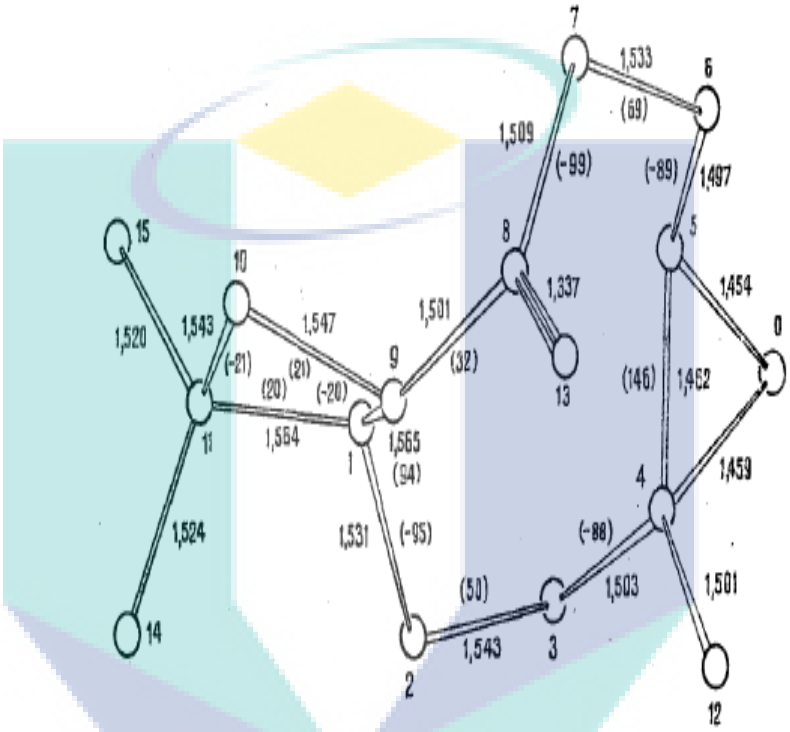


Figure 2.10: Structure of the caryophyllene oxide molecule in the crystal.

Source: Gatilov et al. (1983)

Table 2.2: The values of valence angle of caryophyllene oxide crystal.

Angle	Value (°)	Angle	Value (°)
$C_2C_1C_9$	120.6	$C_6C_7C_8$	113.2
$C_3C_1C_{11}$	119.0	$C_7C_8C_9$	121.7
$C_9C_1C_{11}$	88.3	$C_8C_9C_{13}$	119.2
$C_1C_2C_3$	114.8	$C_9C_8C_{13}$	119.0
$C_2C_3C_4$	112.2	$C_1C_9C_8$	125.6
$C_3C_4C_5$	115. 4	$C_1C_9C_{10}$	86.7
$C_3C_4C_{12}$	116.4	$C_8C_9C_{10}$	117.3
C_3C_4O	115.0	$C_9C_{10}C_{11}$	89.8
$C_5C_4C_{12}$	123. 7	$C_1C_{11}C_{10}$	87.2
C_5C_4 O	59.7	$C_1C_{11}C_{14}$	113.9
$C_{12}C_4$ O	113.3	$C_1C_{11}C_{15}$	115.4
$C_4C_5C_6$	125.3	$C_{10}C_{11}C_{14}$	111.9
C_4C_5 O	60. 0	$C_{10}C_{11}C_{15}$	116.2
C_6C_5 O	120.6	$C_{14}C_{11}C_{15}$	110.5
$C_5C_6C_7$	108.9	C_4OC_5	60.3

Source: Gatilov et al. (1983)

CHAPTER 3

MATERIALS AND METHODS

3.1 GENERAL

3.1.1 Reagents

The reagents used in this study are formic acid 98% (w/v), dichloromethane, acetic acid 99.8% (w/v), acetic anhydride 98.5% (w/v), 3-chloroperbenzoic acid 77% (w/v), sodium formate, hydrogen peroxide 30% (w/v); which were purchased from Sigma Aldrich. Crude caryophyllene from PT Djasula Wangi Jakarta, clove buds were purchased locally from a spice shop (Kuantan, April 2010).

3.1.2 Apparatus and instruments

The apparatus used were microwave extractor (NEOS Milestone Incorporation), laboratory blender (Waring USA), Cleaner Ultrasonic (Elmasonic S80H model), thermometer, steam distillation glass apparatus unit, pH meter, universal bottle, membrane filter, beaker, micro pipette, 3 neck round bottom flasks, magnetic stirrer, hot plate, vacuum pump, Buchner flask, Buchner funnel, and volumetric flask.

(i) Gas Chromatography Mass Spectrophotometer (GC/MS)

GC/MS is a method to identify different components within a sample. This detector is composed of two ideal combination systems which is gas chromatogram and the mass spectrometer (Harvey, 2005). The gas chromatography provides a capillary column to separate the constituents as the sample travels the length of the column. The separation of molecules refers to the different chemical properties of different molecules in the mixture.

As the gaseous sample exits the column and enters the Mass Spectrometer, the gaseous are bombarded with electrons and the molecules become unstable and break down into charged fragments. The positive ions are collected and separated at different retention time the on the basis of their mass charge ratio (Hawavitharana, 2008). Both these compartments are used together to obtain higher efficiency in the identification of molecule composition rather than being used separately. Figure 3.1 shows the GC/MS schematic diagram with the column and detector.

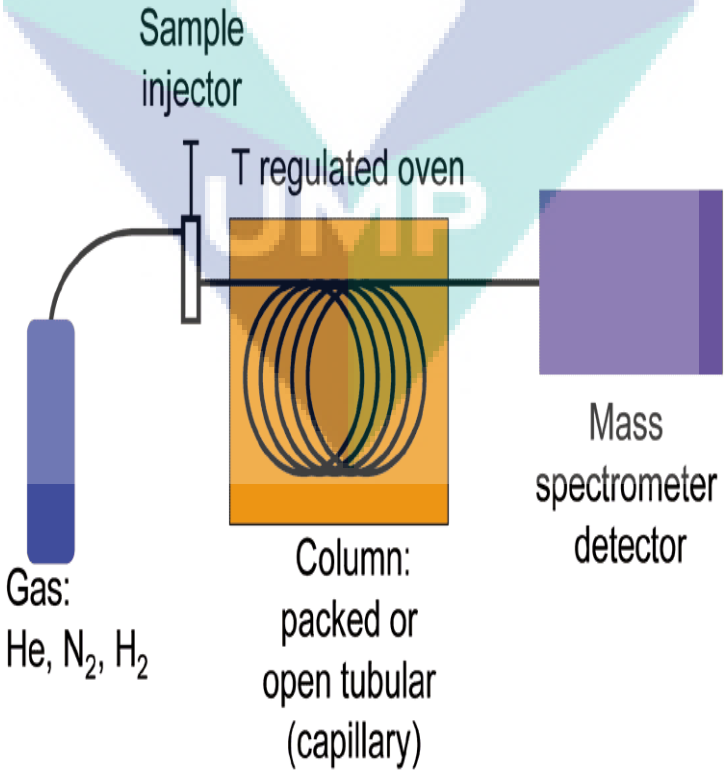


Figure 3.1: GC-MS Schematic Diagram

Instruments which were used are Gas Chromatography Mass Spectrophotometer Agilent Technologies having a DB-5 fused-silica column (30 m · 0.25 mm i.d., film thickness 0.25 µm, Agilent). The GC/MS was set up with having oven temperature conditions were 40 °C for 1 min, with programmed heating from 40 °C to 200 °C at a rate of 6 °C/min and from 200 °C to 280 °C at a rate of 30 °C/min. Injector temperature was 250 °C. The carrier gas, helium, was adjusted to a linear velocity of 1 ml/min. Ion source temperature was 250 °C. The ionization energy was 70 eV with a scan time of 1 s and mass range of 20–500 AMU (Wenqiang et al., 2007).

3.1.3 Characterization

Fourier Transform Infrared Spectroscopy (FTIR) and Nuclear magnetic resonance spectroscopy (NMR) were also used as research equipments in this study to characterize the sample.

(i) Fourier Transform Infrared Spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) is an analytical technique used to identify mainly organic materials. FTIR analysis results in absorption of spectras which provides information about the chemical bonds and molecular structure of a material. FTIR method is not suitable for obtaining the precise and quantitative analysis (Swann and Patwardhan, 2011).

The FTIR spectrum is equivalent to the "fingerprint" (Duygu et al., 2009) of the material absorption, the peaks represent the frequencies vibrations between the bonds of the atom. The frequency of the peaks of every sample can be compared with cataloged FTIR spectra to identify the material. The size of the frequency peaks indicated the amount of material present. Interestingly, there are no exactly same

peaks frequency trend due to its unique combination of atoms in all the different materials.

The instrumental process starts with the sources of infrared energy passing through an aperture. Then the beam enters the interferometer where the spectral encoding takes place. The resulting interferogram signal then exits the interferometer. The beam enters the sample compartment where it is transmitted through or reflected off of the surface of the sample, depending on the type of analysis being accomplished. This is where specific frequencies of energy, which are uniquely characteristic of the sample, are absorbed. The beam finally passes to the detector for final measurement. The detectors used are specially designed to measure the special interferogram signal. The measured signal is digitized and sent to the computer where the Fourier transformation takes place. The final infrared spectrum is then presented to the user for interpretation and any further manipulation as shown in figure 3.2. (Thermo Nicolet Corporation, 2001).

In this work, Fourier Transformation Infrared (FTIR) Perkin Elmer Spectrum Express Version 1.03.02 was used to identify functional groups in sodium formate, caryophyllene and caryophyllene oxide

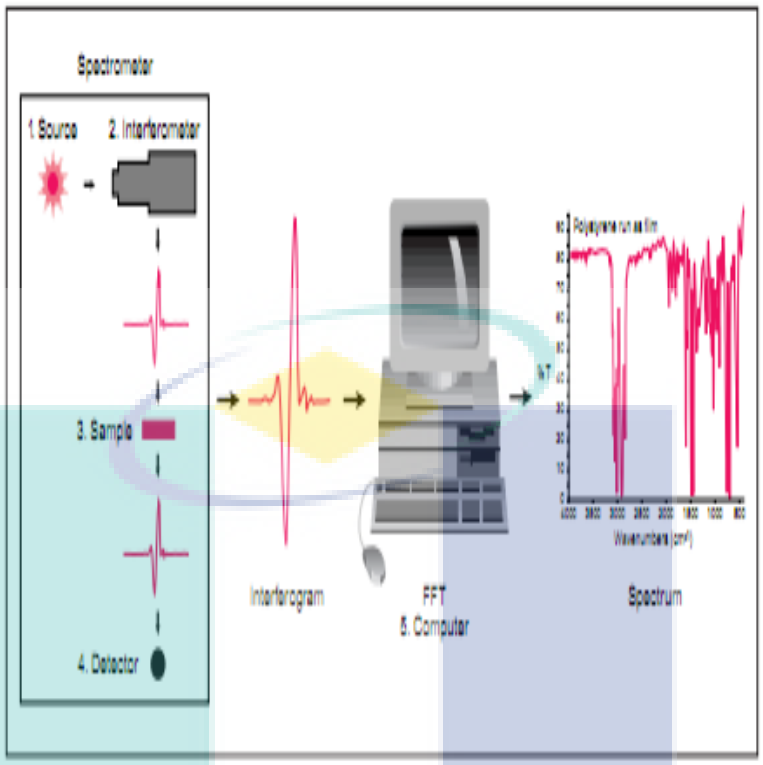


Figure 3.2: FTIR Process Analysis Diagram.

Source: Thermo Nicolet Corporation, 2001.

(ii) Nuclear magnetic resonance spectroscopy (NMR)

Nuclear magnetic resonance spectroscopy (NMR) in figure 3.3, is a research technique to determine physical and chemical properties of atoms or the molecule resin which they contain. The phenomenon of nuclear magnetic resonance and can provide detailed information about the structure, dynamics, reaction state, and chemical environment of molecules. This equipment is a non destructive technique that enables analysis of organic and some inorganic compounds (Central Lab University Malaysia Pahang, 2011). NMR is inherently a quantitative and non-selective equipment, thus more chemical information can be analyzed from single NMR spectrum (Chang et al., 2007).

Nuclear magnetic resonance spectra were recorded on (NMR) BRUKER Ultrashield 500 Plus using TMS as internal standard. Chemical shifts are reported in ppm and coupling constants are reported in Hz in this research.

Figure 3.3: Nuclear magnetic resonance spectroscopy (NMR) BRUKER Ultrashield 500 Plus.

3.2 METHODS

3.2.1 Extraction of clove oil

The first objective for this research is to extract clove buds, to obtain clove oil by using the following extraction methods, Cleaner Ultrasonic Extraction, Steam Distillation, Hydrodistillation and Microvawe Extraction.

(i) Cleaner Ultrasonic Extraction

2.5 g of clove bud powder were ground, sieved through Vibratory Siever Shaker (45µm) and poured into a 100 mL volumetric flask. Distilled water was added up the mark level. The sample was placed in the cleaner ultrasonic (Elmasonic S80H model) and the reaction started as shown in figure 3.4. This equipment was degased for 10 min to remove the balance of gas inside the equipment. Then the reaction was initiated by turning this equipment to sweep mode. Two variables were used for this extraction, the temperature and the time of the extraction. After the extraction, the mixture was cooled to room temperature. The sample was filtered by refrigerated centrifugal for 10 min with 8000 rpm. The supernatant was then removed.

The mixture was poured into separating funnel and dichloromethane was then added to separate water from the organic layer. The vacuum distillation was set up with the temperature of round bottom flask was kept in the range of 100- 200 °C. The dichloromethane from the solution was removed by vacuum distillation. The drops of oil were collected and kept in the centrifuge tubes and stored in refrigerator for analysis.

Figure 3.4: Cleaner Ultrasonic (Elmasonic S80H model).

(ii) Steam distillation

The second method for this extraction is the steam distillation method as showed in figure 3.5. 50 g ground clove which had been sieved through sieve shaker was placed in the 2.5L round bottom flask of the steam distillation unit. The small hole at the bottom of flask was covered by the gauze material to prevent powder from falling down into lower flask. The lower flask was filled with 2 L of water. The water was heated by the heating mantle and when the water boiled, the time was recorded as the starting point. Although the temperature was maintained at a maximum, it was controlled based on the temperature system to avoid the samples from being burnt and the oil being damaged. The steam was allowed to pass through the powder and volatile components from the clove were condensed in the condenser system.

After the extraction was completed, the clove oil was collected at the bottom of burette and kept in a universal bottle as shown in figure 3.5 below. Before storing in refrigerator for the analysis, the water impurities were removed by adding some anhydrous sodium sulphate powder which will coagulate water at the bottom of the flask. After sodium sulphate was filtered and the oil was then refrigerated prior to analysis.

Figure 3.5: Steam distillation method.

(iii) Dean-Stark Hydrodistillation

The Dean-Stark Distillation or Hydrodistillation process as showed in figure 3.6 was run for 6 and 8 h. In order to complete this extraction, the apparatus was set up as shown in figure 3.6 below with the powder samples fully submerged in the boiled water.

The water level was ensured at the half of flask and some pieces of glass were inserted to make sure that the water does not splash out during heating. The oil produced appeared at the upper layer in the burette as shown in figure 3.6 and collected several times. The oil was then poured into the universal bottle and some anhydrous sodium sulphate powder was added before analysis to remove any water present.

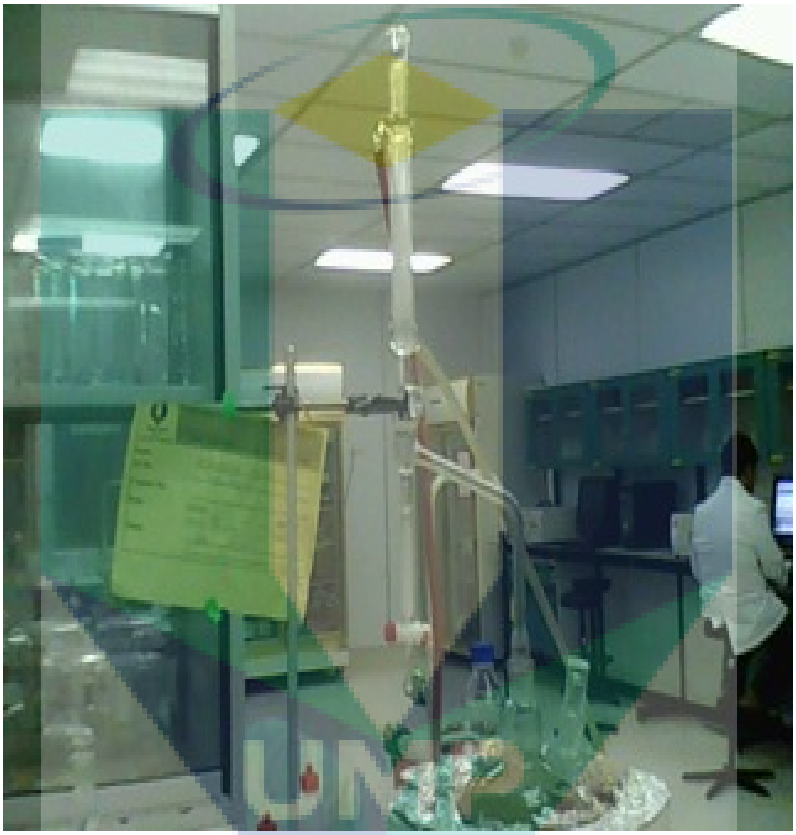


Figure 3.6: Dean-Stark Distillation.

(iv) Microwave Distillation

The start up procedure of microwave extractor was as follows. A 40 g of cloves bud powder was poured into extractor flask followed by 500 mL of distilled water. The microwave was set under these operating conditions, temperature of 110 ˚C, time of 30 min and power of 450 watt. The cloves oil produced was collected

from the oil collector from the distillation unit connected with the microwave extractor (figure 3.7). The extraction was repeated with different extraction times.

Figure 3.7: Microwave Extractor.

3.2.2 Separation of caryophyllene from clove oil

The clove oil obtained by all the extraction methods used in this study contains eugenol and caryophyllene as two major components, as well as other minor components. The eugenol was separated chemically from clove oil by using aqueous solution of sodium hydroxide and dichloromethane leaving caryophyllene and other non-polar organic compounds.

Into a solution which contained 5 g of sodium hydroxide in 30 mL distilled water in a 100 mL volumetric flask, 10.3 g of clove oil was slowly poured and

stirred for 45 min. The mixture was then filtered and placed in the separating funnel. Then 5 mL (10% w/v) of sodium hydroxide solution was added followed by 20 mL of dichloromethane. The mixture was shaken for 100 s to get two layer phases as shown in figure 3.8. The separation occurred here where the caryophyllene dissolved in dichloromethane separated at the bottom layer, and the eugenol in sodium hydroxide solution as the top layer. The crude caryophyllene was then easily separated from eugenol. This step was repeated by adding 10 mL dichloromethane to make sure all of caryophyllene was separated from eugenol. 5 mL of 25% (w/v) NaOH solution was then added to the solution of caryophyllene in dichloromethane and stirred for 15 min.

Figure 3.8: Eugenol and caryophyllene phases.

After that, the solution was transferred into separatory funnel to separate the bottom layer which contained caryophyllene and other non-polar molecules in

dichloromethane. 5mL of 25% (w/v) NaOH solution was added into this caryophyllene solution. The mixture was shaken before leaving it aside for 24 h. The bottom layer was then collected to be distilled to remove dichloromethane. The caryophyllene obtained was then analyzed using GCMS. The experiment was repeated for the series of sodium hydroxide ratio for both clove bud and clove leaf oils.

3.2.2 Purification of caryophyllene

The crude caryophyllene that was produced from the separation process undergoes purification process by using vacuum distillation unit as showed in figure 3.9. First, the crude caryophyllene was poured into 50 mL round bottom flask equipped with vacuum distillation unit. During distillation, when each fraction of distillate is collected, the heating and vacuum were stopped before the next fraction was collected in order to avoid all the liquid in the round bottom flask coming out to the receiver. Better result should be obtained by using fractional distillation.

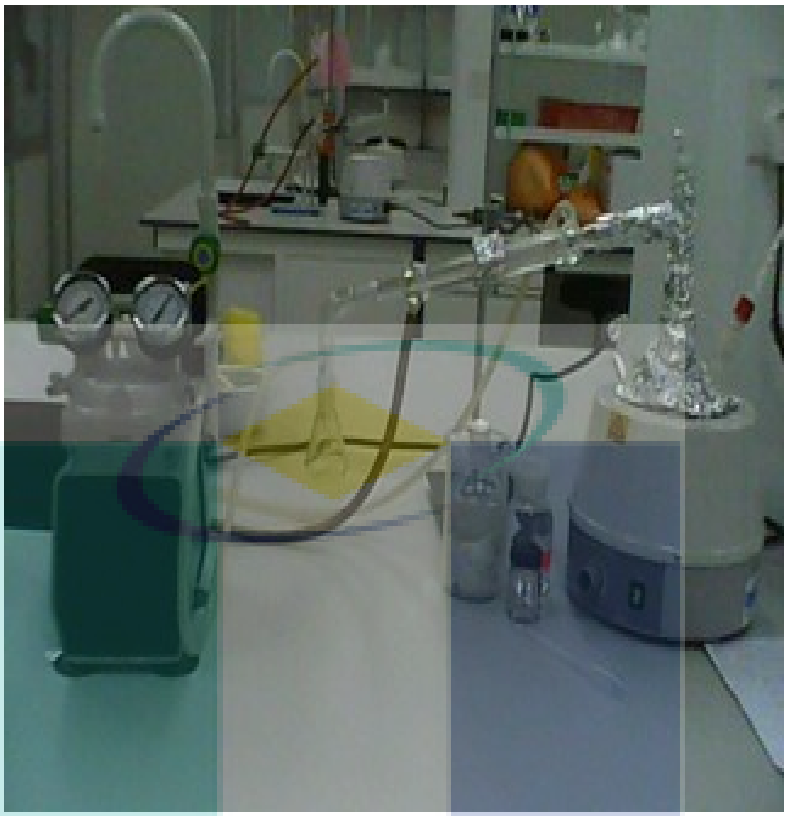


Figure 3.9: Vacuum distillation unit

3.3 CARYOPHYLLENE OXIDE SYNTHESIS

Firstly, highly reactive peracid was formed in situ through the reaction between carboxylic acid and hydrogen peroxide. Percarboxylic acid is very unstable and has to be made in situ. It is expected that, peracid reacts with alkene to form an epoxide by transferring one of its oxygen atoms.

3.3.1 Formic acid

(i) pH determination of reagent

Formic acid which is carboxylic acid was used in this synthesis as shown in figure 3.10. Formic acid aqueous solutions with variable pHs were made by

adding 25% (w/v) of sodium formate solution to 98% (w/v) of formic acid solution until the pH required (pH 0.2- 4.2) was obtained.

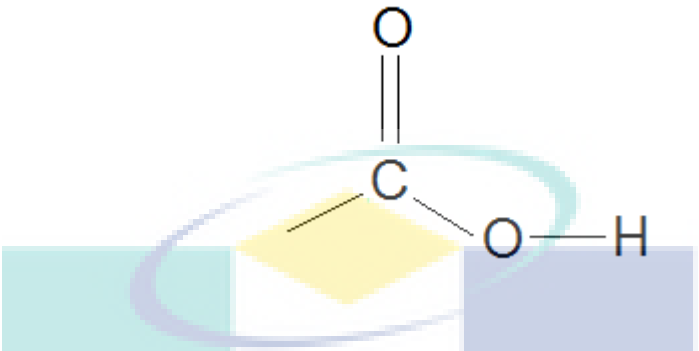


Figure 3.10: Formic Acid.

(iii) Performic Acid Reaction

30% (w/v) hydrogen peroxide solution was added into a well stirred mixture consisting of solution of formic acid of certain pH and crude caryophyllene in 100 mL of 3 neck round bottom flask. Reaction was carried out with several variables. The mixture was allowed to stir continuously. Next, 10 mL dichloromethane were added into the solution and the mixture was gently stirred in order to terminate the reaction. Then, the solution was poured into 100 ml separatory funnel and left to settling down. Finally, two separate layers of solution, organic and water were observed as shown in figure 3.11. The organic solution was then collected, and heated at varied temperatures in order to study the heat stability of caryophyllene oxide in solution.

Figure 3.11: Layers of organic and water.

(iii) Variables

The experiments were carried using 5 different variables namely pH, reaction time, ratio of reagents, reaction temperature, and caryophyllene oxide solution temperature. For the pH, the other variables such as time, reaction temperature, ratios and caryophyllene oxide solution temperature were kept constant as shown in table 3.1. For the time variable, pH, reaction temperature, ratio and caryophyllene oxide solution temperature were kept constant as shown in table 3.2. For the reaction temperature variable, time, pH, ratio and caryophyllene oxide solution temperature were kept constant as shown in table 3.3. For the ratio variable, time, reaction temperature, pH and caryophyllene oxide solution temperature were kept constant as shown in table 3.4. The ratio of reagents included formic acid, caryophyllene and hydrogen peroxide. For the caryophyllene

oxide solution temperature variable, time, reaction temperature, ratio and pH were kept constant as shown in table 3.5. The experiment also were carried out to study variables of reaction temperature at 8 h, 16 h and 20 h as shown in table 3.6, 3.7 and 3.8. For reaction temperature variable at 16 h and 20 h, the temperature of 15 ºC were not used due to ineffective result. All the experiment was duplicated for each variable.

Table 3.1: pH variable.

pH	time(h)	reaction temperature(°C)	ratio(mol)			C.O. solution temperature(°C)
			formic acid	caryophyllene	hydrogen peroxide	
0.2	40	24	0.8	1	1.2	35
1.2	40	24	0.8	1	1.2	35
2.2	40	24	0.8	1	1.2	35
3.2	40	24	0.8	1	1.2	35
4.2	40	24	0.8	1	1.2	35

Table 3.2: Time variable.

pH	time(h)	reaction temperature(°C)	ratio(mol)			C.O. solution temperature(°C)
			formic acid	caryophyllene	hydrogen peroxide	
2.2	30	24	0.8	1	1.2	35
2.2	35	24	0.8	1	1.2	35
2.2	40	24	0.8	1	1.2	35
2.2	45	24	0.8	1	1.2	35
2.2	50	24	0.8	1	1.2	35

Table 3.3: Ratio variable.

pH	time(h)	reaction temperature(°C)	ratio(mol)			C.O. solution temperature(°C)
			formic acid	caryophyllene	hydrogen peroxide	
2.2	40	24	0.8	1	1.2	35
2.2	40	24	1.0	1	1.4	35
2.2	40	24	0.8	1	1.3	35
2.2	40	24	0.8	1	1.4	35
2.2	40	24	0.6	1	1.4	35

Table 3.4: Reaction temperature variable.

pH	time(h)	reaction temperature(°C)	ratio(mol)			C.O. solution temperature(°C)
			formic acid	caryophyllene	hydrogen peroxide	
2.2	40	30	0.8	1	1.3	35
2.2	40	35	0.8	1	1.3	35
2.2	40	40	0.8	1	1.3	35
2.2	40	45	0.8	1	1.3	35
2.2	40	50	0.8	1	1.3	35

Table 3.5: C.O solution temperature variable.

pH	time(h)	reaction temperature(°C)	ratio(mol)			C.O. solution temperature(°C)
			formic acid	caryophyllene	hydrogen peroxide	
2.2	40	30	0.8	1	1.3	35
2.2	40	30	0.8	1	1.3	55

Table 3.5: Continued.

2.2	40	30	0.8	1	1.3	65
2.2	40	30	0.8	1	1.3	75
2.2	40	30	0.8	1	1.3	95

Table 3.6: Reaction temperature variable at 8 hours.

pH	time(h)	reaction temperature(°C)	ratio(mol)			C.O. solution temperature(°C)
			formic acid	caryophyllene	hydrogen peroxide	
2.2	8	15	0.8	1	1.3	55
2.2	8	24	0.8	1	1.3	55
2.2	8	35	0.8	1	1.3	55
2.2	8	45	0.8	1	1.3	55
2.2	8	55	0.8	1	1.3	55

Table 3.7: Reaction temperature variable at 16 hours.

pH	time(h)	reaction temperature(°C)	ratio(mol)			C.O. solution temperature(°C)
			formic acid	caryophyllene	hydrogen peroxide	
2.2	16	24	0.8	1	1.3	55
2.2	16	35	0.8	1	1.3	55
2.2	16	45	0.8	1	1.3	55
2.2	16	55	0.8	1	1.3	55

Table 3.8: Reaction temperature variable at 20 hours.

pH	time(h)	reaction temperature(°C)	ratio(mol)			C.O. solution temperature(°C)
			formic acid	caryophyllene	hydrogen peroxide	
2.2	20	24	0.8	1	1.3	55
2.2	20	35	0.8	1	1.3	55
2.2	20	45	0.8	1	1.3	55
2.2	20	55	0.8	1	1.3	55

3.2.3 Types of acid used: acetic acid, acetic anhydride, 3-chloroperbenzoic acid.

For these three types of acids (figure 3.12- 3.14), no pH determination of reagent was needed. First crude caryophyllene was poured into the acid; next 30 % (w/v) hydrogen peroxide was added into a well stirred mixture of acid and crude caryophyllene in a 100 mL 3 neck round bottom flask. Special caution for 3-chloroperbenzoic acid, the caryophyllene must be added drop by drop due to its rapid increase of temperature which may cause explosion if not controlled, as shown in the table 3.9 consisting the characteristic of these three acids.

Reaction was carried under different temperatures. The mixture was stirred continuously till desired time period. Next, 10 mL dichloromethane were added into the solution with continuous stirring. Then, the solution was poured into 100 mL separatory funnel. Finally, two separate layers of solution, organic and water were observed. The organic solution was then collected, and heated at varied temperatures in order to study the heat stability of caryophyllene oxide in solution.

Table 3.9: Characteristic of Acids.

Types of acid	Acetic Acid	Acetic Anhydride	3-chloroperbenzoic acid
	acetic acid is highly dangerous to skin although it is classified as a weak acid.	simpler acid anhydride, spontaneously decomposes, especially once removed from solution.	strong oxidizing agent that may cause fire upon contact with flammable material.
Preparation	produced by methanol carbonylation. In this process, methanol ancarbon monoxide react to produce acetic acid.	produced by carbonylation of methyl acetate. Carbonylation of the methyl iodide in turn affords acetyl iodide, which reacts with acetate salts or acetic acid to give the product.	prepared by reacting *m*-chlorobenzoyl chloride with hydrogen peroxide in the presence of magnesium sulfate, aqueous sodium hydroxide, and dioxane, followed by acidification.
	colourless liquid	clear liquid	white powder
Melting point	16-17 °C	−73.1 °C	92 - 94 °C
Molecular formula	$C_2H_4O_2$	$C_4H_6O_3$	$C_7H_5ClO_3$
Molar mass	60.05 g mol^{-1}	102.09 g mol^{-1}	172.57 g/mol
Solubility in water	miscible	2.6 g/100 mL	immiscible

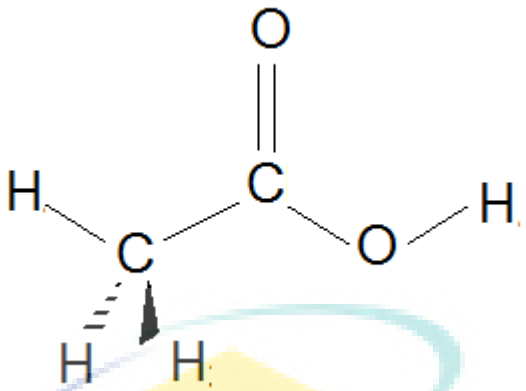


Figure 3.12: Acetic Acid.

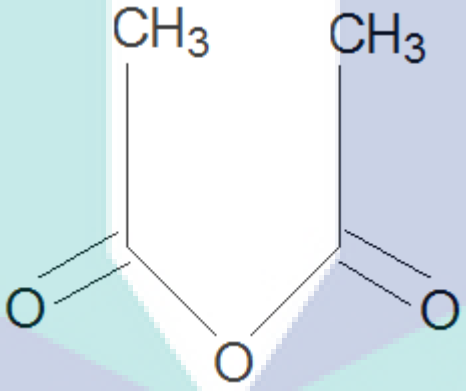


Figure 3.13: Acetic Anhydride.

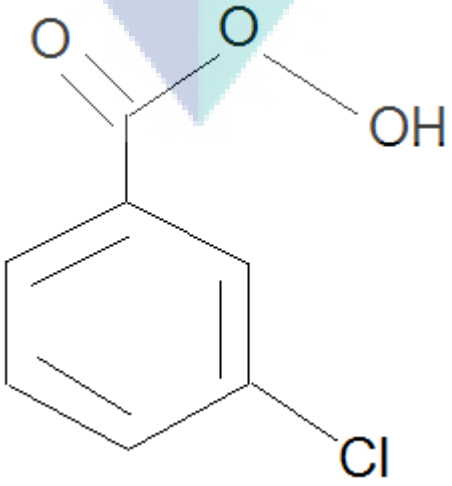


Figure 3.14: 3-chloroperbenzoic acid

The experiment was carried out using 3 different variables. For the pH variable and C.O solution temperature variable were not related for these acids. For the reaction temperature variable, time, and ratio were kept constant as shown in table 3.10. For the time variable, reaction temperature and ratio were kept constant as shown in table 3.11and 3.12. For the ratio variable, time, and reaction temperature were kept constant as shown in table 3.13. The ratio of reagents included acid, caryophyllene and hydrogen peroxide. The experiment was duplicated for each variable.

Table 3.10: Temperature variable.

Types of acid	reaction temperature(°C)	time (h)	ratio(mol)		
			acid	caryophyllene	hydrogen peroxide
Acetic acid	24	16	0.8	1	1.3
	45	16	0.8	1	1.3
	50	16	0.8	1	1.3
	55	16	0.8	1	1.3
Acetic anhydride	24	16	0.8	1	1.3
	45	16	0.8	1	1.3
	50	16	0.8	1	1.3
	55	16	0.8	1	1.3
3-chloro perbenzoic acid	24	16	0.8	1	1.3
	45	16	0.8	1	1.3
	50	16	0.8	1	1.3

Table 3.10: Continued

	55	16	0.8	1	1.3

Table 3.11: Time variable at best temperature.

Types of acid	reaction temperature(°C)	time (h)	ratio(g)		
			acid	caryophyllene	hydrogen peroxide
Acetic acid	55	16	0.8	1	1.3
	55	20	0.8	1	1.3
	55	24	0.8	1	1.3
Acetic anhydrate	45	16	0.8	1	1.3
	45	20	0.8	1	1.3
	45	24	0.8	1	1.3
3-chloro perbenzoic acid	50	16	0.8	1	1.3
	50	20	0.8	1	1.3
	50	24	0.8	1	1.3

Table 3.12: Hydrogen peroxide ratio variable.

Types of acid	reaction temperature(°C)	time (h)	ratio(mol)		
			acid	caryophyllene	hydrogen peroxide
Acetic acid	55	16	0.8	1	1.2
	55	16	0.8	1	1.3
	55	16	0.8	1	1.4
Acetic anhydrate	45	16	0.8	1	1.2

Table 3.12: Continued

	45	16	0.8	1	1.3
	45	16	0.8	1	1.4
3-chloroperbenzoic acid	50	16	0.8	1	1.1
	50	16	0.8	1	1.2
	50	16	0.8	1	1.3
	50	16	0.8	1	1.4

Table 3.13: Time variable at room temperature.

Types of acid	reaction temperature(°C)	time (h)		ratio(g)	
			acid	Caryophyllene	hydrogen peroxide
Acetic acid	24	35	0.8	1	1.3
	24	40	0.8	1	1.3
	24	45	0.8	1	1.3
Acetic anhydride	24	35	0.8	1	1.3
	24	40	0.8	1	1.3
	24	45	0.8	1	1.3
3-chloroperbenzoic acid	24	35	0.8	1	1.3
	24	40	0.8	1	1.3
	24	45	0.8	1	1.3

3.2.4 GC/MS Analysis

The caryophyllene oxide solution obtained by synthesis process was cooled to room temperature before being injected into 1.5 mL vial using a hexane as a solvent with a dilution of 0.01% (V/V). Samples were analysed by using Gas Chromatography Mass spectrophotometer (GCMS) to check the yield and the compositions of the caryophyllene oxide solution.

3.2.5 Crystallization

The solution of caryophyllene oxide was slowly cooled to get crystals formed. Then the solution was taken out and filtered using vacuum filter as shown in figure 3.15 to give white crystalline solid as the product. The crystals were analyzed using GCMS to check the purity of caryophyllene oxide.

Figure 3.15: Vacuum Filtration of Crystal.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 INTRODUCTION

This chapter presents the experimental data and analysis based on data analysis. It consists of the various processes: an extraction, separation, purification, synthesis and characterization.

4.2 SAMPLE COLLECTION AND DATA ANALYSIS

In this study, samples were collected to meet the four objectives of research, that is to extract clove oil from the clove buds, to separate the caryophyllene from the eugenol, to purifiy the caryophyllene. Learning about extraction, separation and also purification gives an idea on how to pursue other projects for natural product organic chemical research. The last objective is on the synthesis of caryophyllene oxide. Characterization of pure caryophyllene oxide by using NMR and FTIR also were carried out. FTIR characterized the functional group of caryophyllene, sodium formate and caryophyllene oxide.

All samples were analyzed by using Gas Chromatography Mass Spectrophotometer (GCMS) to check the percentage of composition of eugenol, caryophyllene and also caryophyllene oxide.

4.3 Extraction

The four types extraction methods of clove bud powder, which are Cleaner Ultrasonic, Steam Distillation, Hydrodistillation and also Microwave Extraction produce different yields and compositions of the clove oil.

(i) Cleaner-Ultrasonic

Table 4.1, 4.2, and 4.3 show the yield and the eugenol content of clove oil produced by cleaner- ultrasonic method.

Table 4.1: Yield and the eugenol content of oil at different times.

Time (min)	temperature (°C)	yield (mL)	% eugenol by GC/MS
20	80	0.1	70.53
40	80	0.1	84.01
60	80	0.2	74.47
80	80	0.2	85.00
120	80	0.1	24.91
240	80	0.1	27.62
360	80	0.1	14.28

Table 4.2: Yield and the eugenol content of oil at different temperature.

Time (hour)	temperature (°C)	yield (mL)	% eugenol by GC/MS
6	40	0.1	22.12
6	60	0.2	54.35

Table 4.2: Continued.

6	80	0.2	19.31

(ii) Steam distillation

Table 4.3 shows the yield and the eugenol content of clove oil produced by steam distillation method.

Table 4.3: Yield and the eugenol content of oil at different times and constant temperature.

Time (h)	temperature (ºC)	yield (mL)	eugenol (%)
6	80	2.1	65.8
8	80	2.5	26.41
10	80	3.5	29.20
12	80	5	69.75

(iii) Hydrodistillation

Table 4.4 shows the yield and the eugenol content of clove oil produced by hydrodistillation method.

Table 4.4: Yield and the eugenol content of oil at different times and constant temperature.

Time (h)	temperature (ºC)	yield (mL)	eugenol (%)
6	80	4	62.98

Table 4.4: Continued.

8	80	8.3	62.42
10	80	8.4	57.55
12	80	9.2	29.80

(iv) Microwave Extraction

Table 4.5 shows the yield and the eugenol content of clove oil produced by Microwave Extraction method.

Table 4.5: Yield and the eugenol content of oil at different times.

Time (min)	yield (mL)	eugenol (%)
30	2.5	54.51
45	3.6	60.18
60	6	64.82
90	6.5	61.77
120	6.6	29.14

The above four types of extraction method have been studied. Maximum yield of clove oil was obtained by hydrodistillation method with a yield of 9.2 mL at 12 h process, while cleaner- ultrasonic method showed a minimum yield with a few drops of clove oil amongs 6 h of extraction. Result obtained by GC/MS showed that maximum eugenol composition was 85 % at 80 min extraction by cleaner-ultrasonic. The GC/MS chromatogram of eugenol composition can be found in appendix A.

4.4 Separation of Caryophyllene from Eugenol

Caryophyllene was successfully separated chemically. Table 4.6 shows the caryophyllene and eugenol contents of standard of crude clove leaf oil and clove bud oil.

Table 4.6: Caryophyllene and eugenol composition of clove oils.

Types of clove oil	caryophyllene (%)	eugenol (%)
leaf	18.54	79.41
bud	10.34	86.45

The percentage of caryophyllene in the standard clove leaf oil was higher than caryophyllene in the standard clove bud oil. The GC/MS chromatogram of composition crude clove leaf oil and clove bud oil located in appendix B1 and B2. Table 4.7 and 4.8 showed the yield and the composition of caryophyllene with different ratio of sodium hydroxide for bud and leaf.

Table 4.7: Yield of caryophyllene with different ratio of sodium hydroxide for bud.

Ratio oil : sodium hydroxide	oil (g) : sodium hydroxide (g)	caryophyllene and others (ml)	caryophyllene composition (%)
1 : 2	10.3 : 5	0.70	74.06
1 : 1.7	10.02 : 4.06	0.50	61.88
1 : 1.5	10.2 : 3.74	0.42	60.43
1 : 1.2	9.97 : 2.92	0.40	44.90

Table 4.8: Yield of caryophyllene with different ratio of sodium hydroxide for leaf.

Ratio oil : sodium hydroxide	oil (g) : sodium hydroxide (g)	caryophyllene and others (ml)	caryophyllene composition (%)
1 : 2	10.3 : 5	0.9	80.38
1 : 1.7	10.02 : 4.06	0.7	75.11
1 : 1.5	10.2 : 3.74	0.6	70.08
1 : 1.2	9.97 : 2.92	0.5	60.38

The maximum yield of caryophyllene and others are at the ratio of 1: 2 with 0.9 ml from 10 ml of clove leaf oil. It can be concluded from the results obtained, the yield of caryophyllene successfully separated from the eugenol is favoured by the amount of sodium hydroxide that was consumed during its contact with eugenol. GC/MS result shows that the there is higher percentage of caryophyllene also by clove leaf at the ratio of 1: 2. These results showed that the percentage of caryophyllene in the clove leaf oil is higher than in clove bud oil. The GC/MS chromatogram of caryophyllene separation at the ratio of 1: 2 for both clove leaf and clove bud located in appendix C1 and C2.

4.5 Purification of Caryophyllene

Table 4.9 and table 4.10 show the commercial caryophyllene composition and caryophyllene at different of temperature obtained by vacuum fractional distillation. The GC/MS chromatogram of crude caryophyllene situated in appendix D.

Table 4.9: Composition of standard caryophyllene (Sigma Aldrich) analyzed by GC/MS

caryophyllene (%)	alpha-caryophyllene (%)	other components (%)
86.86	13.14	0

Table 4.10: Composition of caryophyllene at different temperature distillate analyzed by GCMS.

Temperature distillate (°C)	caryophyllene (%)	alpha-caryophyllene (%)	other components (%)
136	75.14	19.07	5.80
137	74.07	18.79	7.14
138	70.14	11.18	18.68
139	67.04	11.78	21.18
140	59.89	12.93	27.18
142	59.10	11.64	29.26
144	48.88	11.97	39.15
145	30.16	8.51	61.33

Among the several distillate components obtained, the highest caryophyllene percentage 75% by GC/MS analysis was obtained at 136 °C. By comparing to the standard caryophyllene, distillates of caryophyllene still contain the alpha-cubebene and some minor mixtures. This phenomenon result factored by the method used was categorized as a laboratory scale.

By using the laboratory scale method, the compositions of the caryophyllene, the vapors of distilling compounds and the distillate change during the distillation was determined. The percentage of caryophyllene content of various distillate of caryophyllene obtained in these experiments still cannot match the percentage of caryophyllene standard.

4. 6 Synthesis of caryophyllene oxide

The caryophyllene was successfully converted into caryophyllene oxide by epoxidation reaction by using either formic acid or acetic acid or acetic anhydride or 3-chloroperbenzoic acid as a peracid. The conversion percentage of caryophyllene to caryophyllene oxide is based on the equation below.

$$\% \text{ conversion} = \frac{\% \text{ caryophyllene oxide formed}}{\% \text{ of crude caryophyllene}} \times 100$$

4.6.1 Performic Acid

Figure 4.1 to 4.7 illustrated the graph of percentage of caryophyllene oxide against the variables. Tables in Appendix E1 provided the experiment conditions at different variables, percentage of caryophyllene oxide in solution, caryophyllene oxide crystal yield and also conversion of caryophyllene oxide value. The percentage of caryophyllene oxide produced was based on the GC/MS result analysis. The GC/MS chromatogram of maximum caryophyllene oxide formed for each variables can be found in appendix E2 to E7.

An experiment was conducted to analyse the composition and yield of caryophyllene oxide (CO). Samples were analyzed using GC/MS and the result showed that yield gradually increases from pH 0.2 - 2.2 and then decreases as shown in figure 4.1. The maximum yield of caryophyllene oxide was pH 2.2 (84.54%). The pH effect depended on the acidity or the alkalinity of the medium (Gangwal et al., 2005). This phenomenon may be due to the strong absorption of oxygen atom by the alkenes in acidic medium especially around pH 2.2 during epoxidation process.

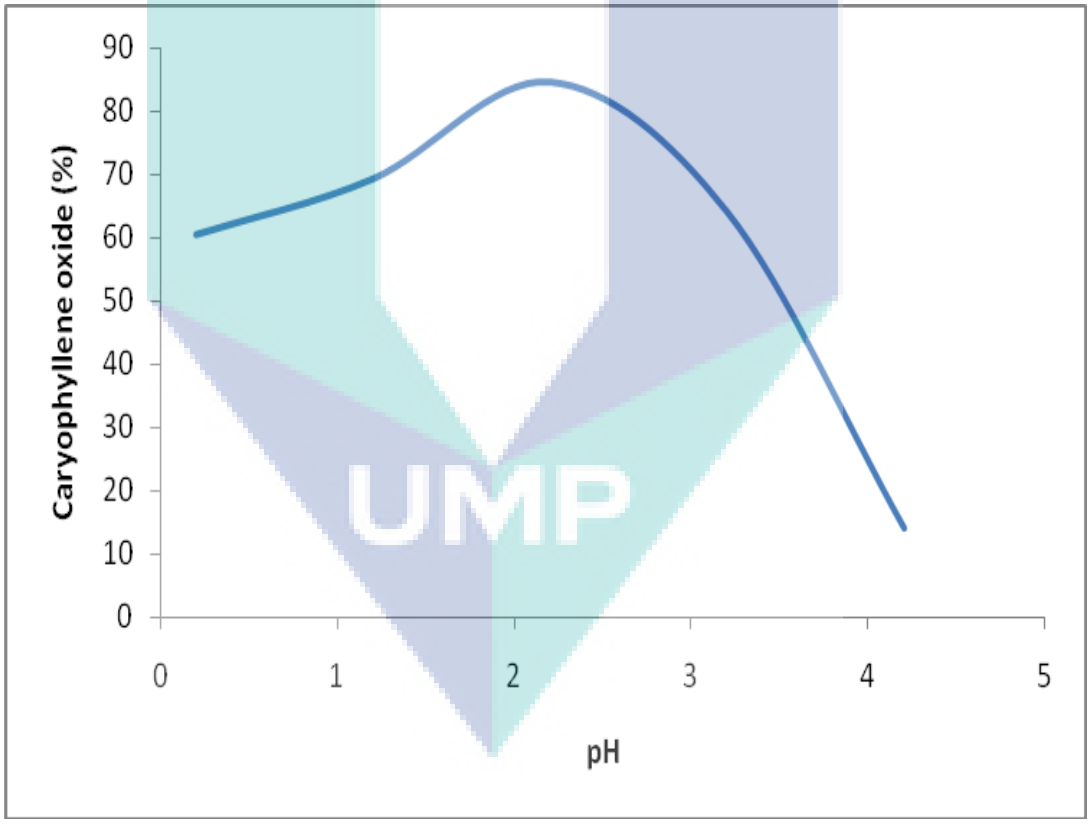


Figure 4.1: Percentage of caryophyllene oxide formed at different pH, same ratio (0.8, 1, 1.2 mol), time (40h), reaction temperature (24 °C), and C.O. solution temperature (35 ºC).

For the variable of time, the CO yield increases as reaction time increases from 30 to 40 hours as shown in figure 4.2. As the oxygen atom's migration rate from peracid molecule to alkene increases with time, the yield increases too. Electron donating substituents also enhances the oxidation rate through nucleophilic attack of hydrogen peroxide onto the alkene double bond (Corma et al., 2004). However as time inecreases from 40 to 50 hours, the yield suprisingly decreases. This may be due to overoxidation and other by products hence were produced. The maximum yield of caryophyllene oxide was at 40 h reaction time (84.07%).

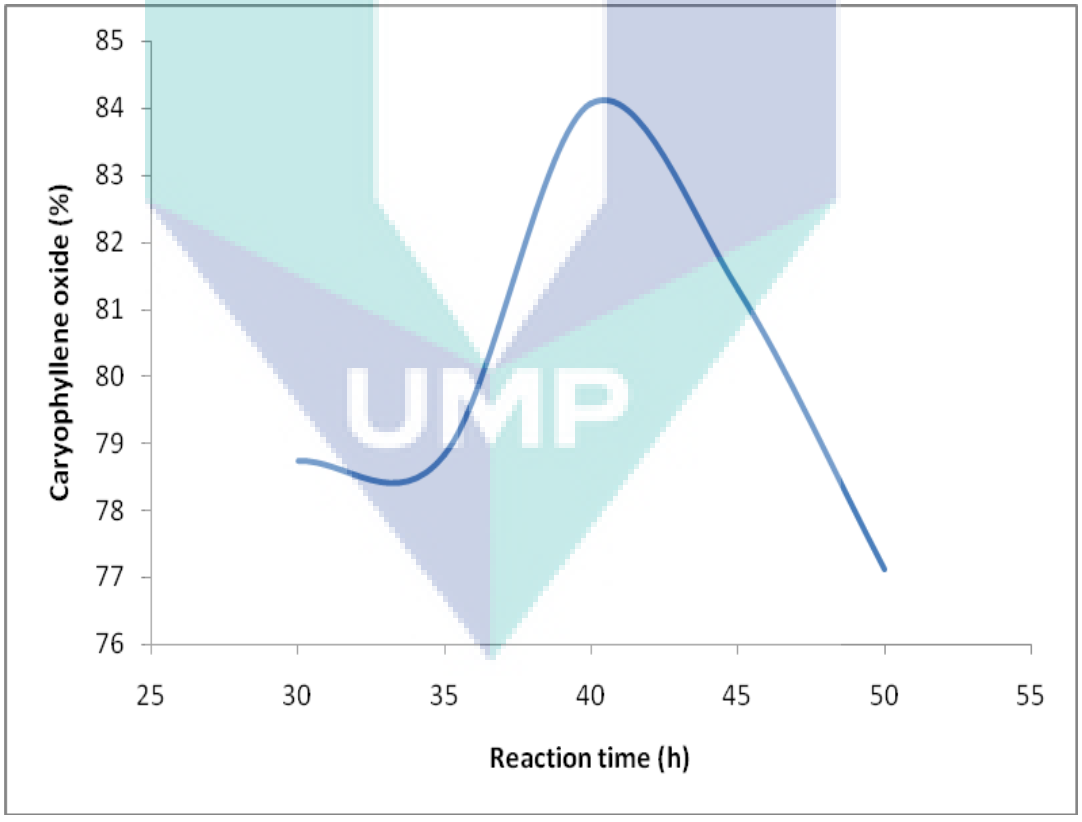


Figure 4.2: Percentage of caryophyllene oxide formed at different reaction time same ratio (0.8, 1, 1.2 mol), pH 2.2, reaction temperature (24 °C), and C.O. solution temperature (35 °C).

Figure 4.3 shows the maximum yield of caryophyllene oxide (80.01%) was obtained using 0.80 mol of formic acid. The increasing of caryophyllene oxide formation may be caused by the increase of performic acid used. In the reaction, formic acid acts as a catalyst for oxygen atom which attacks the double bond at caryophyllene molecule structure. Surprisingly, for mol ratio of 1.0: 1: 1.4, the percentage of caryophyllene oxide obtained decreases.

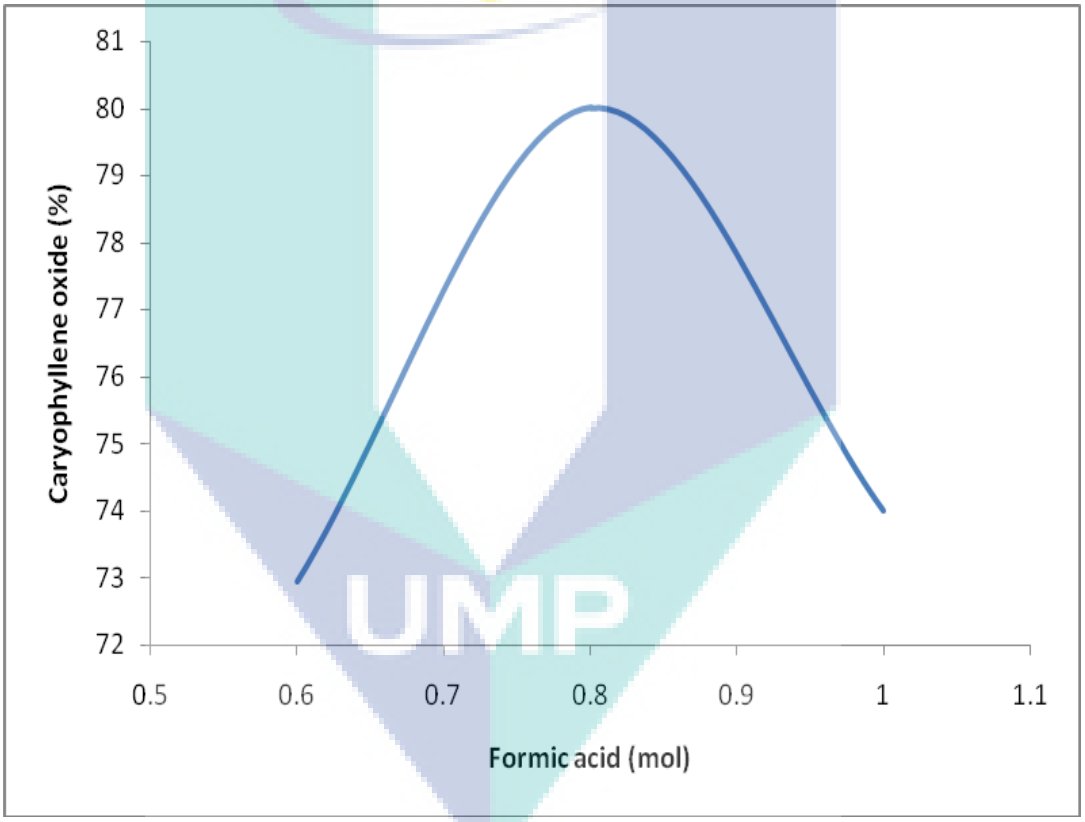


Figure 4.3: Percentage of caryophyllene oxide formed at different formic acid mol used same time (40h), pH 2.2, reaction temperature (24 °C), and C.O. solution temperature (35°C).

Percentage of caryophyllene oxide formed also increased as the concentration of hydrogen peroxide increased as shown in figure 4.4. Hydrogen peroxide as an oxygen carrier provides oxygen atom to the peracid during the reaction, as more hydrogen peroxide was used, more oxirane was produced. The result showed mol ratio of 0.8: 1: 1.3 gave the 85.11 % a maximum yield of caryophyllene oxide. The caryophyllene oxide formation observed for mol ratio of 0.8: 1: 1.4 had decreased. The stability of the oxirane ring at this moment condition was poor (Derawi and Salimon, 2010) as well as some impurities formed from over-oxidation of the caryophyllene.

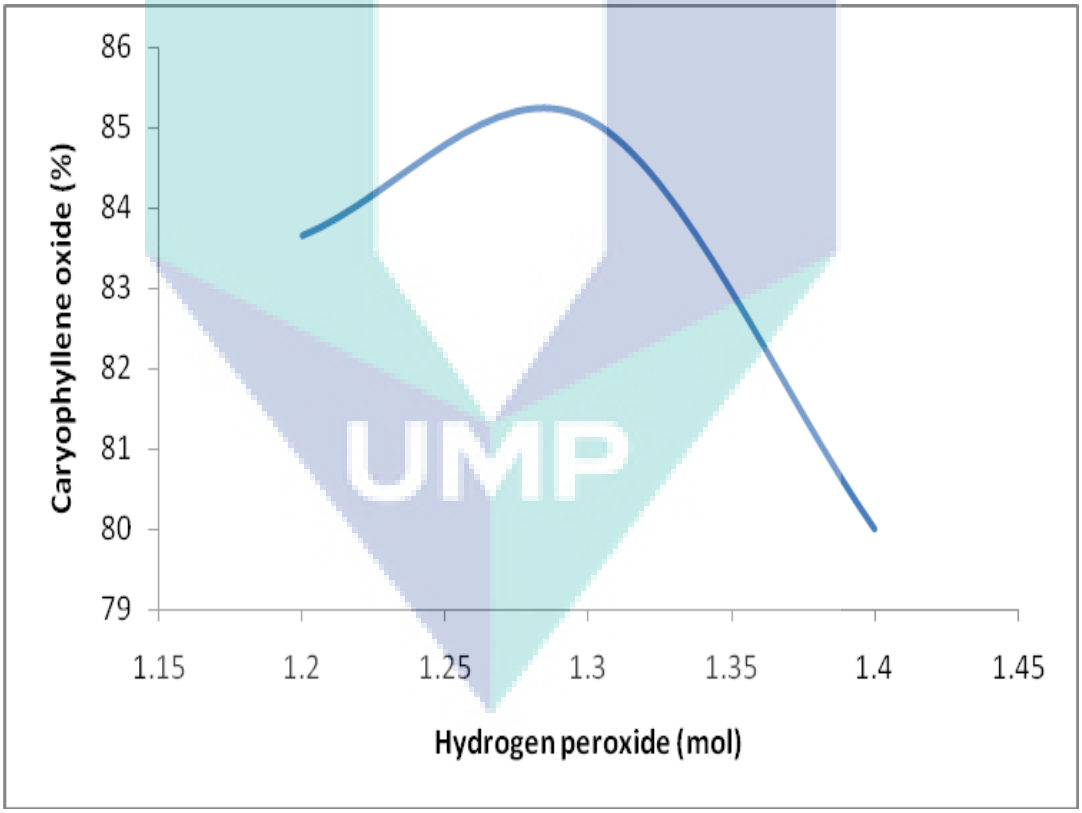


Figure 4.4: Percentage of caryophyllene oxide formed at different hydrogen peroxide mol same time (40h), pH 2.2, reaction temperature (24 °C), and C.O. solution temperature (35 °C).

Experiments were conducted to analyse the composition and yield of caryophyllene oxide (CO). Samples were analyzed using GC/MS and the results in figure 4.5 showed epoxide yield was gradually increasing from room temperature to 30 °C and then started to decrease at higher temperature. This phenomenon showed a favourable effect on the performic acid formation. Increasing the temperature provides more rapid epoxidation and also higher rate of hydrolysis (Derawi and Salimon, 2010). Furthermore, hydrogen peroxide decomposes at high reaction temperature (Li et al., 2008).

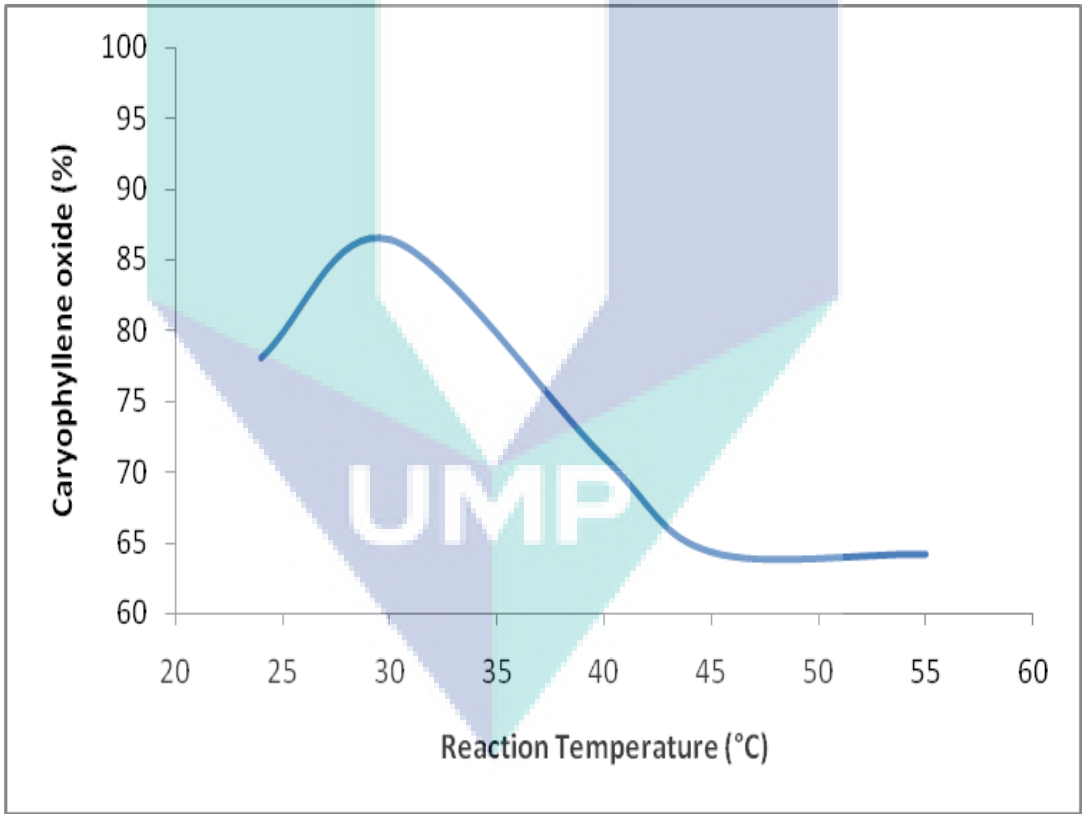


Figure 4.5: Percentage of caryophyllene oxide formed at different reaction temperature same ratio (0.8, 1, 1.3 mol), pH 2.2, time (40h), and C.O. solution temperature (35 °C).

From the figure 4.6, results show that caryophyllene oxide in solution is stable at temperature less than 75 °C. The yields for 35 to 75 °C are almost the same in this range of temperature.

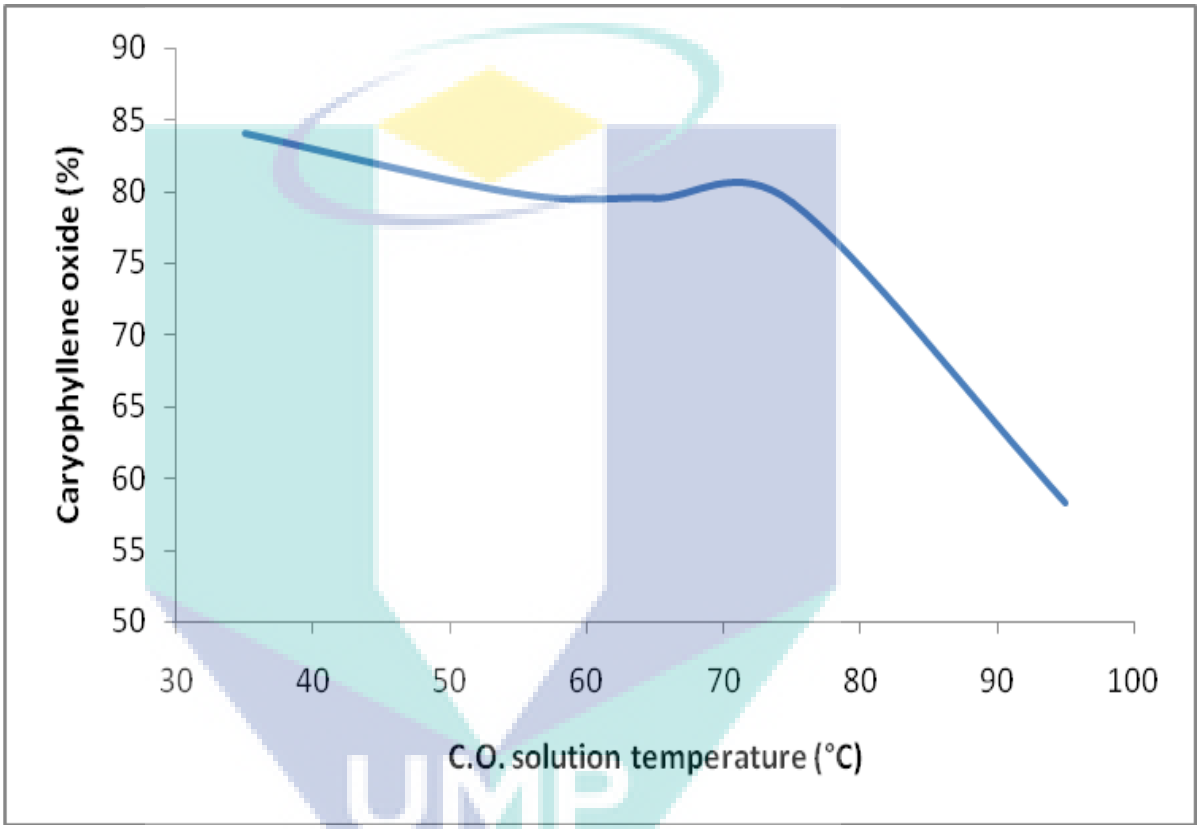


Figure 4.6: Percentage of caryophyllene oxide formed at different caryophyllene oxide solution temperature same ratio (0.8, 1, 1.2 mol), pH 2.2, time (40h), and reaction temperature (24 °C).

The reaction temperature had a significant impact on epoxidation of caryophyllene. To investigate the effect of reaction temperature, reactions were carried out under three different reaction times which are 8 h, 16 h and 20 h respectively as shown in figure 4.7. The yield of caryophyllene oxide formed for 8 h reaction at 15 °C was very low (14.62 %). The caryophyllene oxide formation was hindered at lower temperature. For the reaction times of 8 h, 16 h and 20 h, the amount of caryophyllene oxide formed increases with the increasing of reaction temperature with its maximum yield of 80.84 %, 81.13 % and 85.23 % respectively. Epoxidation of caryopyhllene will become faster due to acceleration in collision speed of reagent molecules at high reaction temperature. Once it reached over 45 °C of reaction temperature, the percentage of caryophyllene oxide formed dropped may be due to a by-product formation.

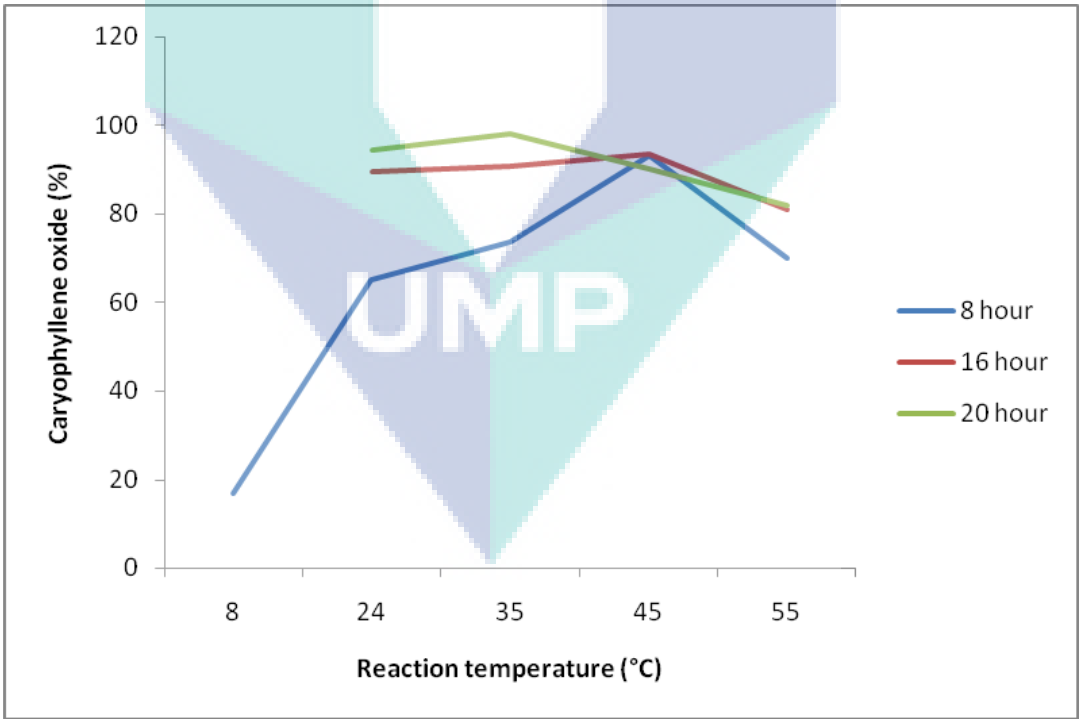


Figure 4.7: Percentage of caryophyllene oxide formed at 8, 16 and 20h at different reaction temperature same ratio (0.8, 1, 1.3 mol), pH 2.2, and C.O. solution temperature (55 °C).

4.6.2 Acetic acid, Acetic Anhydride, 3-chloroperbenzoic Acid

The influence of the acetic acid, acetic anhydride, and 3-chloroperbenzoic acid on the caryophyllene oxide formation was investigated. There were three variables or parameters that were successfully examined, namely reaction temperature, reaction time and hydrogen peroxide molar ratio. Figure 4.8 to 4.17 illustrated the graph of percentage of caryophyllene oxide against the variables. Table in Appendix F1 describes the experiments condition, the amount caryophyllene oxide produced and also the caryophyllene oxide percentage of conversion based on the GC/MS analysis. The GC/MS chromatogram of maximum caryophyllene oxide formed for each acids can be found in appendix F2 to F4.

Acetic acid:

The percentage of epoxidation of caryophyllene by peracetic acid at various temperatures, caryophyllene oxide production increases as reaction temperature increases. In figure 4.8 at 55 °C (74.56%), more epoxide was formed. This may be because acetic acid is weaker acid than formic acid so that to make it more reactive it would need higher temperature compared with formic acid. Higher temperature was not studied as it is not applicable for industry, even at this 55 ° C there is a danger of explosion that may occur.

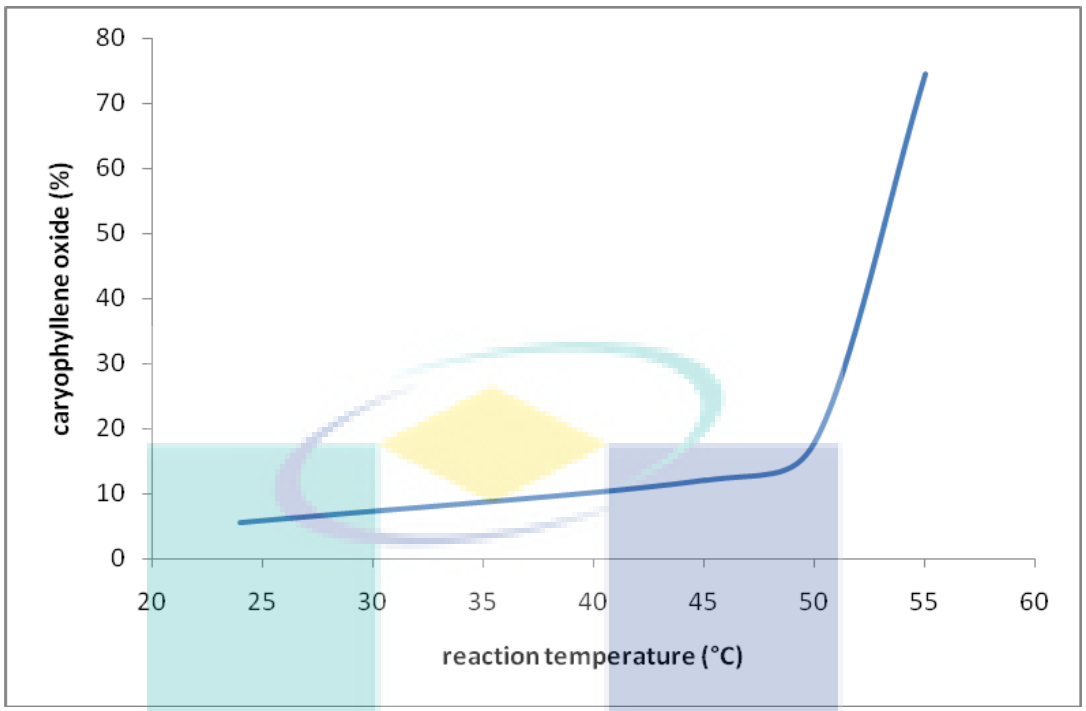


Figure 4.8: Percentage of caryophyllene oxide formed at different reaction temperature by using acetic acid, same ratio (0.8, 1, 1.3 mol), time (16h), and C. O solution temperature (55°).

Based on the previous experiment by using acetic acid, the temperature at 55°C was selected as a best condition for this experiment. However, at 55 °C of reaction temperature, the caryophyllene oxide produced decreased as reaction time increased showed in figure 4.9. The optimum time reaction was 16 h with 74.33% caryophyllene oxide formed. Higher temperature provides a good electrophile of acetic acid, thus increases the acidity of the system.

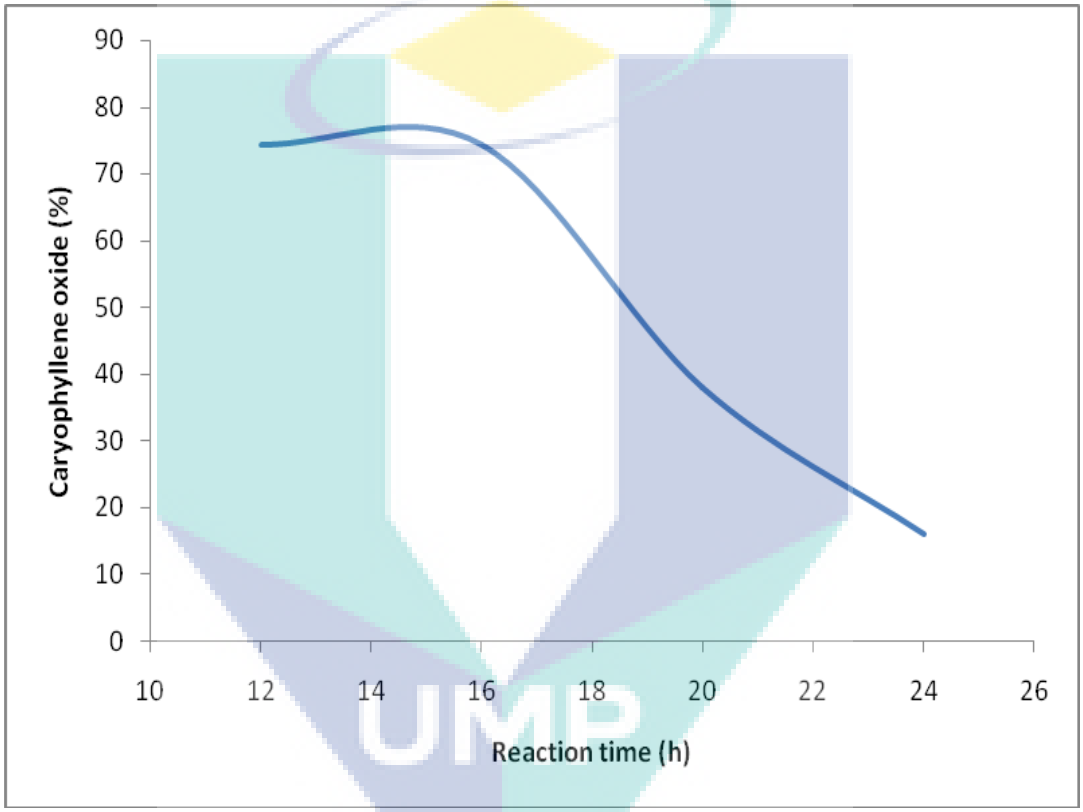


Figure 4.9: Percentage of caryophyllene oxide formed at 55°C at different reaction time by using acetic acid, same ratio (0.8, 1, 1.3 mol), reaction temperature (55° C), and C.O solution temperature (55° C).

In figure 4.10, as the amount hydrogen peroxide used increased, there was a progressive increase of formation of caryophyllene epoxide. This may be attributed to the higher hydrogen peroxide concentration which produced higher amount of peracid being formed leading to an accelerated rate of oxirane ring decomposition (Goud et al., 2007). The maximum relative conversion to oxirane was attained with 1.3 molar ratio (86.50%). However, the epoxide formation decreased at 1.4 molar ratio which may be due to instability of the oxirane ring at this ratio.

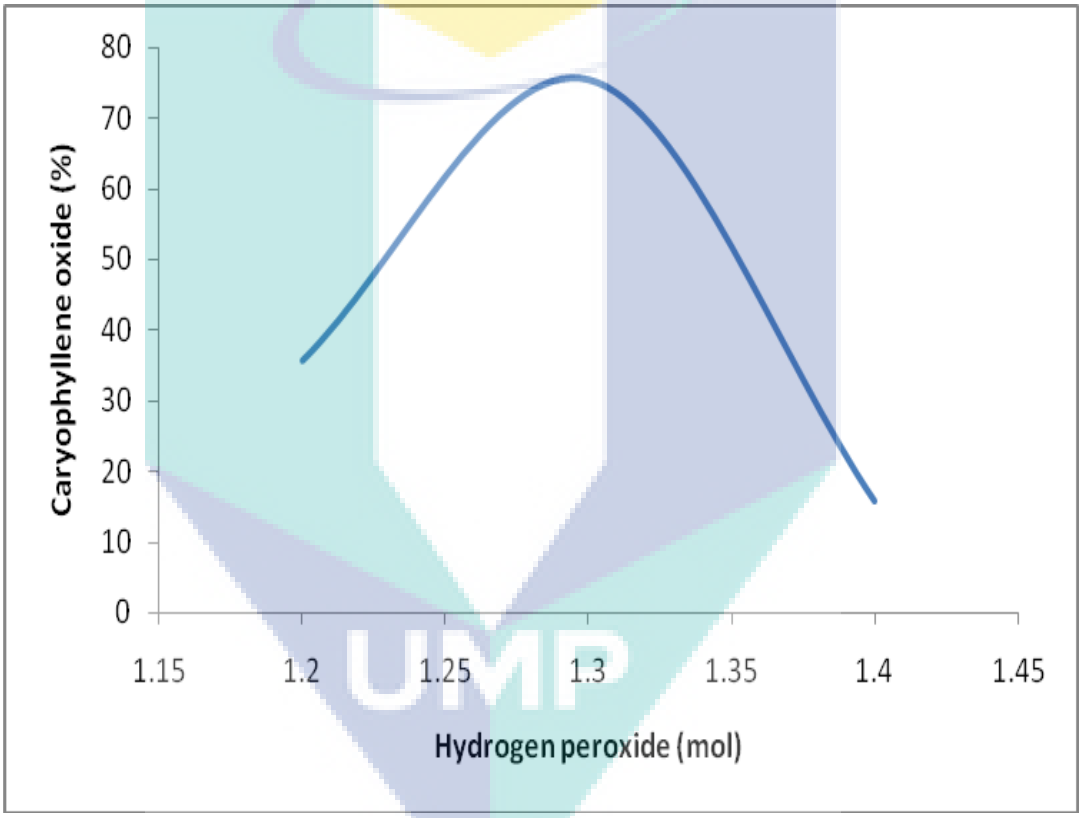


Figure 4.10: Percentage of caryophyllene oxide formed at 55 °C at different hydrogen peroxide molar ratio by using acetic acid, time (16h), reaction temperature (55° C), and C.O solution temperature(55° C).

Acetic anhydride:

Figure 4.11 shows the graph of caryophyllene oxide composition versus reaction temperature of epoxidation of caryophyllene by peracetic generated in situ at various temperatures. The epoxide formed was shown to increase with temperature of 24 °C until it achieved the optimum value at 45 °C (77.02%). Beyond 45 °C of reaction temperature, the percentage of caryophyllene oxide composition started to decrease. The increase in temperature reaction has caused the instability in electron donation process.

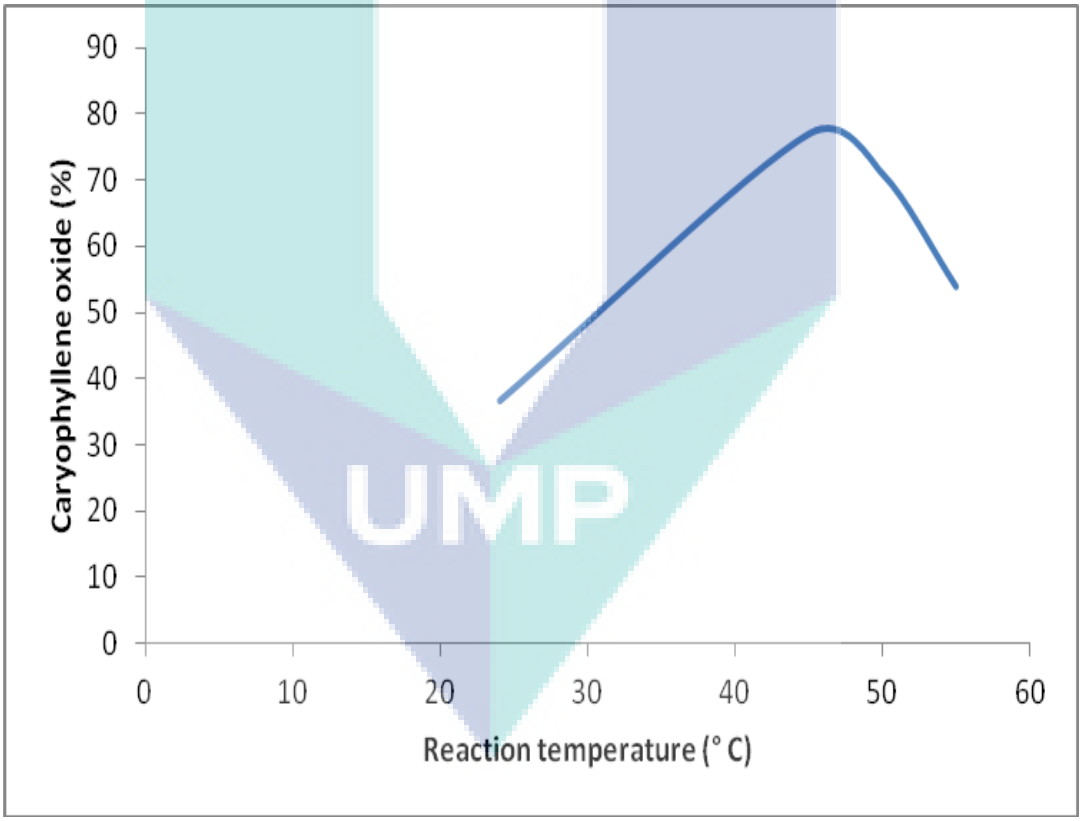


Figure 4.11: Percentage of caryophyllene oxide formed at different reaction temperature by using acetic anhydride, same ratio (0.8, 1, 1.3 mol), time (16h), and C.O solution temperature (55° C).

Based on the temperature parameter by using acetic anhydride experiment, the temperature at 45 °C was selected as the best condition for this experiment. Based on figure 4.12, the caryophyllene oxide produced decreased as reaction time increased. At 45° C of reaction temperature, for 16 h of reaction time the amount of caryophyllene oxide produced was the highest at 75.77 %. At higher reaction time, the caryophyllene oxide produced was lower as this may be due to decomposition of caryophyllene oxide. GC/MS shows that there are higher number of other chemicals present at 24 h compared to at 16 h reaction time.

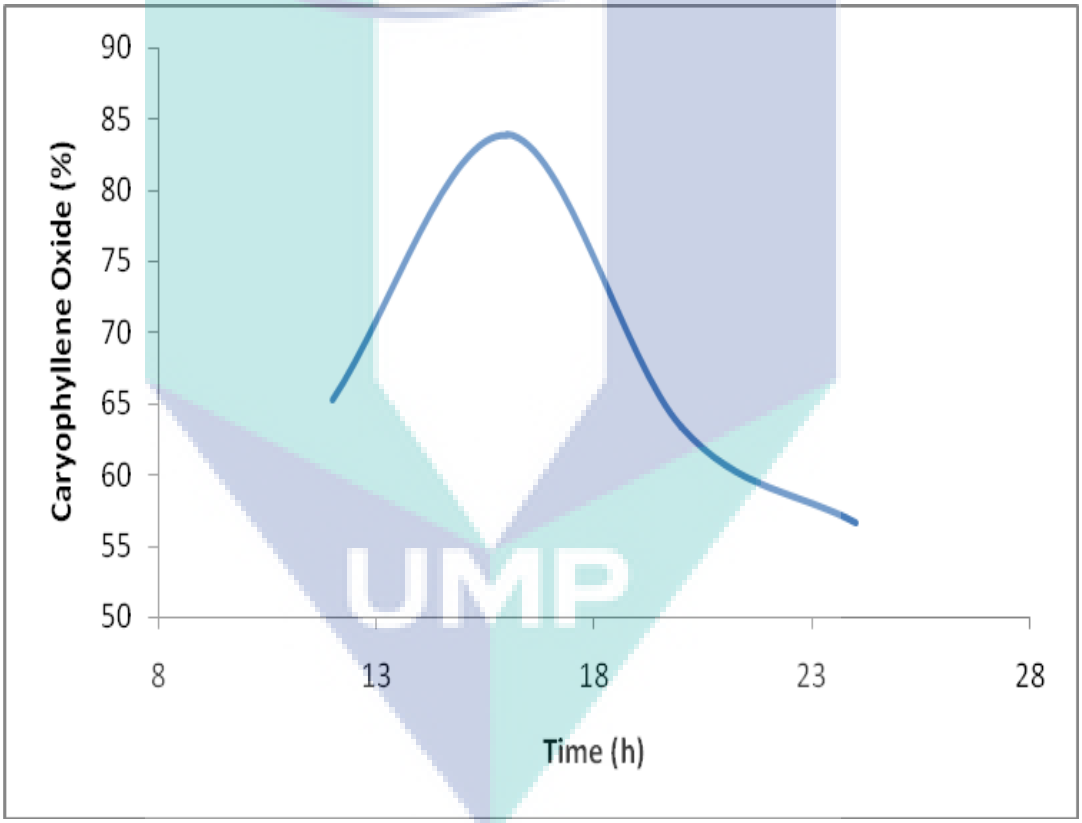


Figure 4.12: Percentage of caryophyllene oxide formed at 45°C at different reaction time by using acetic anhydride , same ratio (0.8, 1, 1.3 mol), reaction temperature (45 ºC), and C.O solution temperature (55° C)**.**

Figure 4.13 shows as hydrogen peroxide molar ratio increased, caryophyllene oxide composition also increased, but the value of caryophyllene oxide dropped to 44.19% at 1.4 molar ratios. The maximum value was 73.62% obtained at 1.3 molar ratio of hydrogen peroxide. This 1.3 molar ratio was the best ratio to make the one side of carbonyl carbon very reactive to donate its electrons.

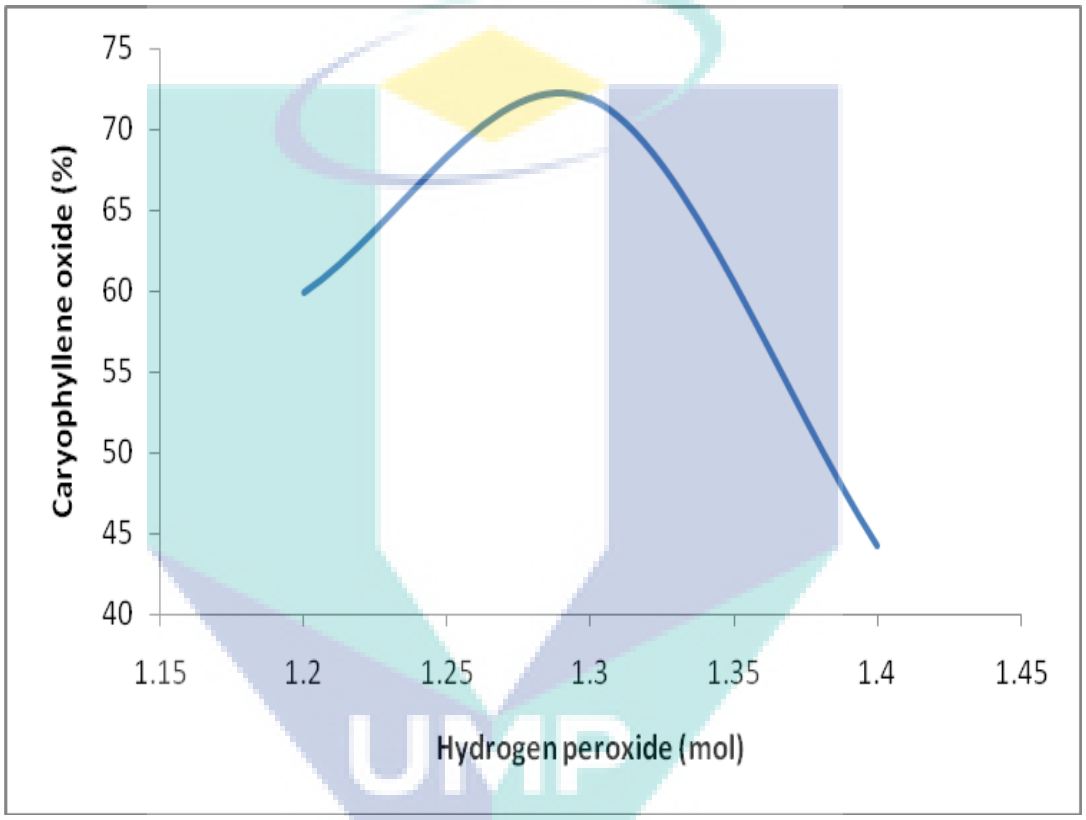


Figure 4.13: Percentage of caryophyllene oxide formed at 45 °C at different hydrogen peroxide molar ratio, time (16h), reaction temperature (55° C), and C.O solution temperature (55° C).

3-Chloroperbenzoic acid:

Figure 4.14 shows the percentage of caryophyllene oxide formed at different reaction temperature. The caryophyllene oxide produced decreased after it reached 50 °C at which the percentage of caryophyllene oxide produced was 79.18%. Flora (2011) showed in their paper the same phenomenon which shows that the reactant did not processed sufficiently fast.

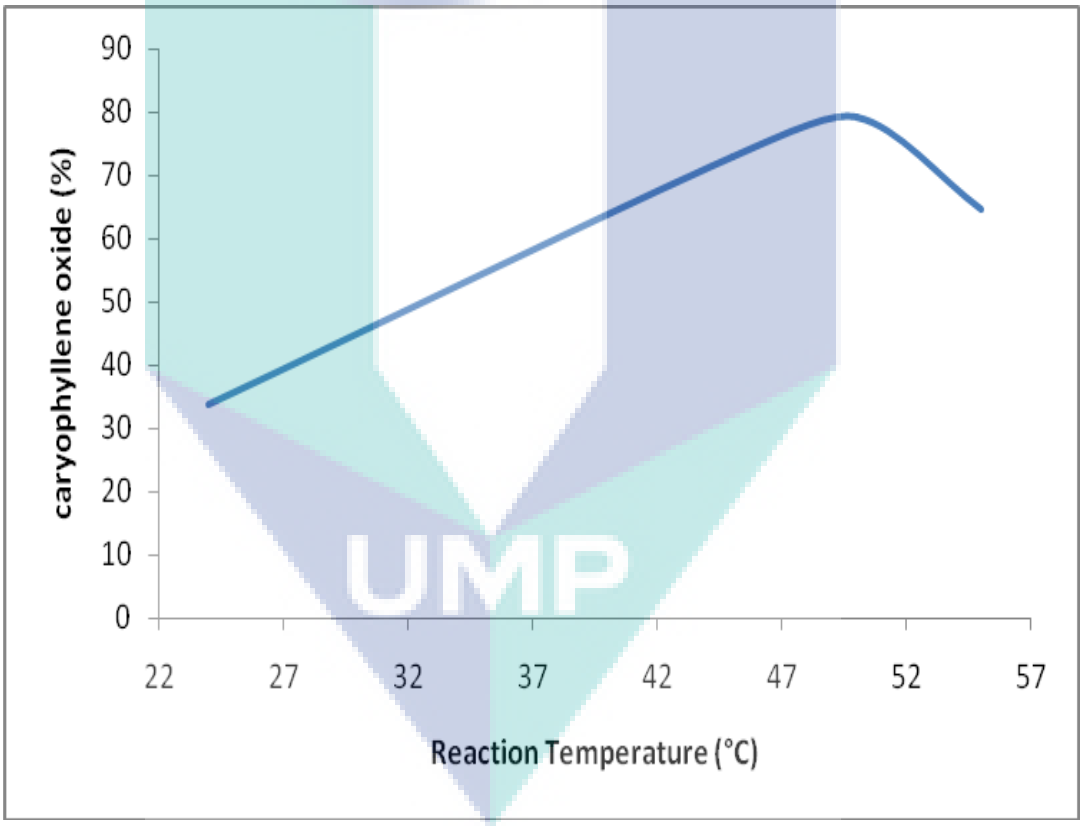


Figure 4.14: Percentage of caryophyllene oxide formed at different reaction temperature by 3-chloroperbenzoic acid, same ratio (0.8, 1, 1.3 mol), time (16h), and C.O solution temperature (55° C).

On these experiments with varied temperatures, the temperature at 50 °C was selected as a best condition for this experiment. Figure 4.15 shows the caryophyllene oxide produced decreased as reaction time was increased. The optimum condition was 16 h with 79.22% caryophyllene oxide value. As increasing time at 50 °C of reaction temperature, this complex structure of 3-chloperbenzoic acid may produce side products that contribute to less production of epoxide.

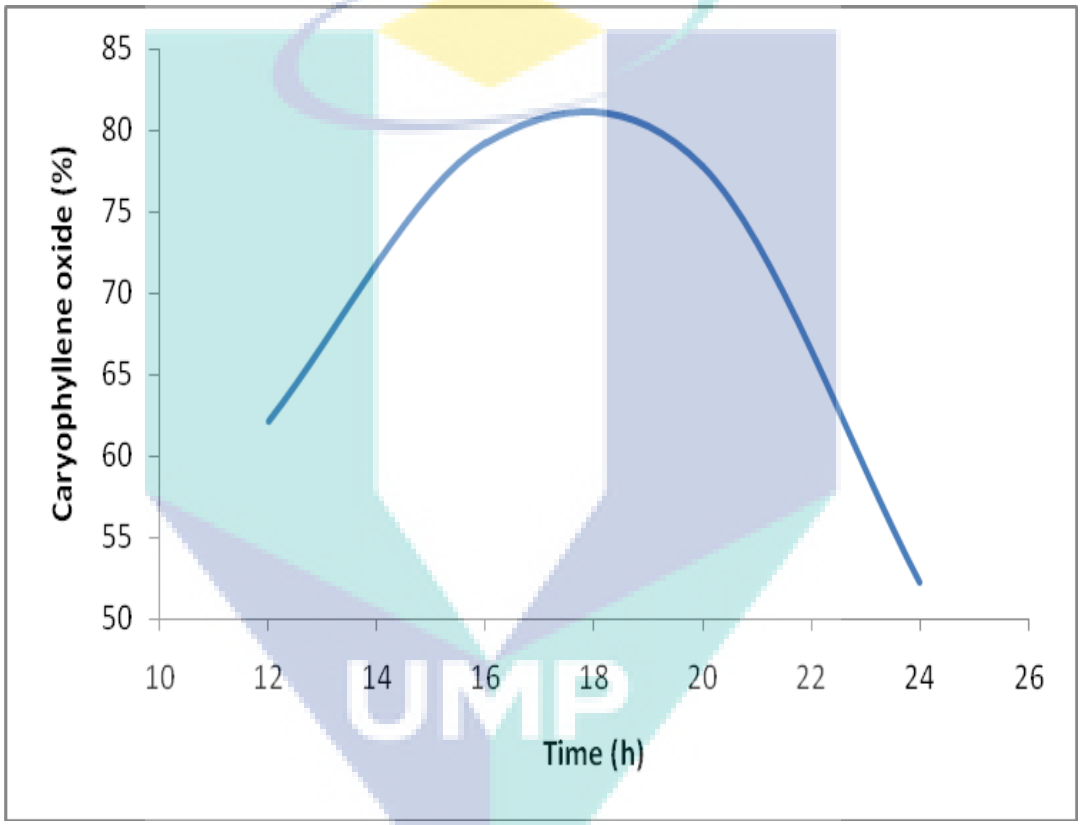


Figure 4.15: Percentage of caryophyllene oxide formed formed at 50°C at different reaction time by using 3-chloroperbenzoic acid , same ratio (0.8, 1, 1.3 mol), reaction temperature (50° C), and C.O solution temperature (55° C).

From the figure 4.16, it can be concluded that as hydrogen peroxide molar ratio was greater than 1.2, the caryophyllene oxide formation decreased. The maximum yield obtained was 81.47% at 1.2 molar ratio of hydrogen peroxide. As concentration of hydrogen peroxide in the system increased, the alkalinity of the system also increased therefore reduced the epoxidation reaction.

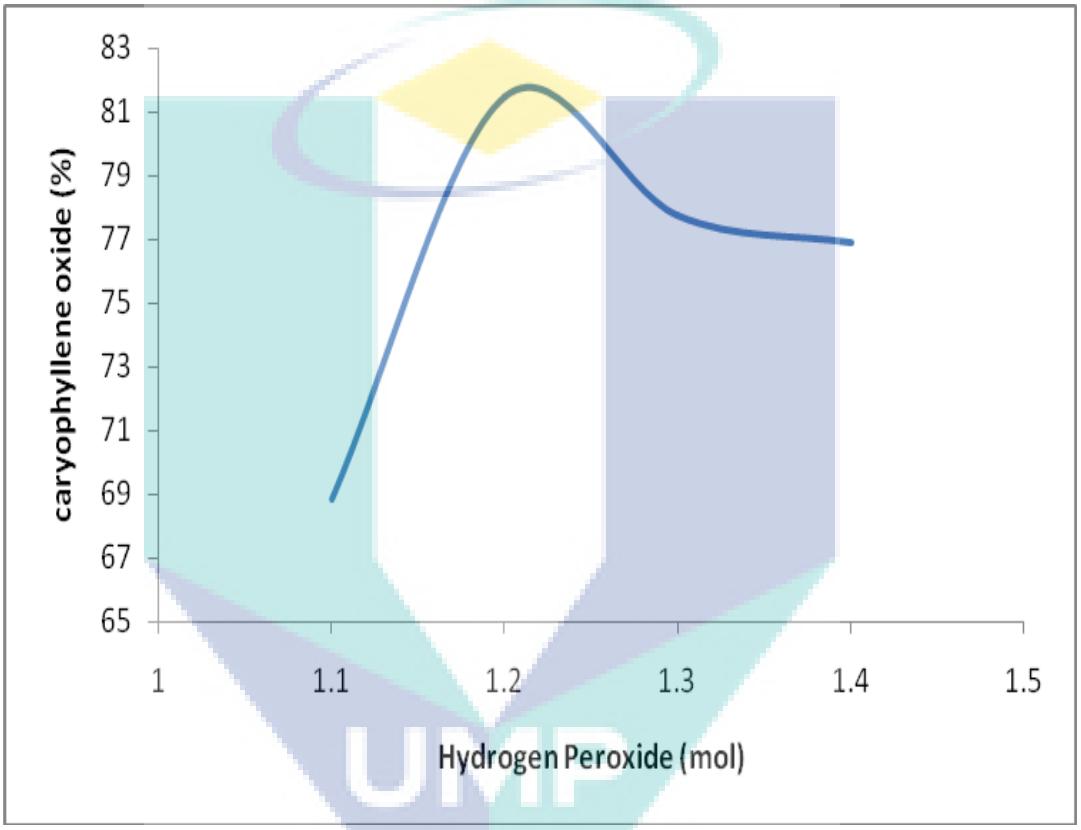


Figure 4.16: Caryophyllene oxide formed at 50 °C at different hydrogen peroxide molar ratio, time (16h), reaction temperature (55° C), and C.O. solution temperature (55° C).

Room temperature reaction:

Figure 4.17 shows the trend of epoxidation of caryophyllene using acetic acid, acetic anhydride and 3-chloroperbenoic acid. The reaction time for these three different acids were chosen to be 35, 40 and 45 hours. Based on previous experiments, higher reaction time were not carried out. The maximum amount of caryophyllene oxide formed were 37.10%, 40.95% and 60.49% respectively. Caryophyllene oxide formation increased as increasing the reaction time from 35 h to 45 h. These acids promoted donation electron to the alkene during epoxidation process to form epoxide. Increasing the reaction time means more electron are donated to the caryophyllene structure. The orbital energies of peroxo bond of protonated peracids are lower than those of neutral peracids, which greatly increase the epoxidation reactivity of peracids (Shi et al., 2005).

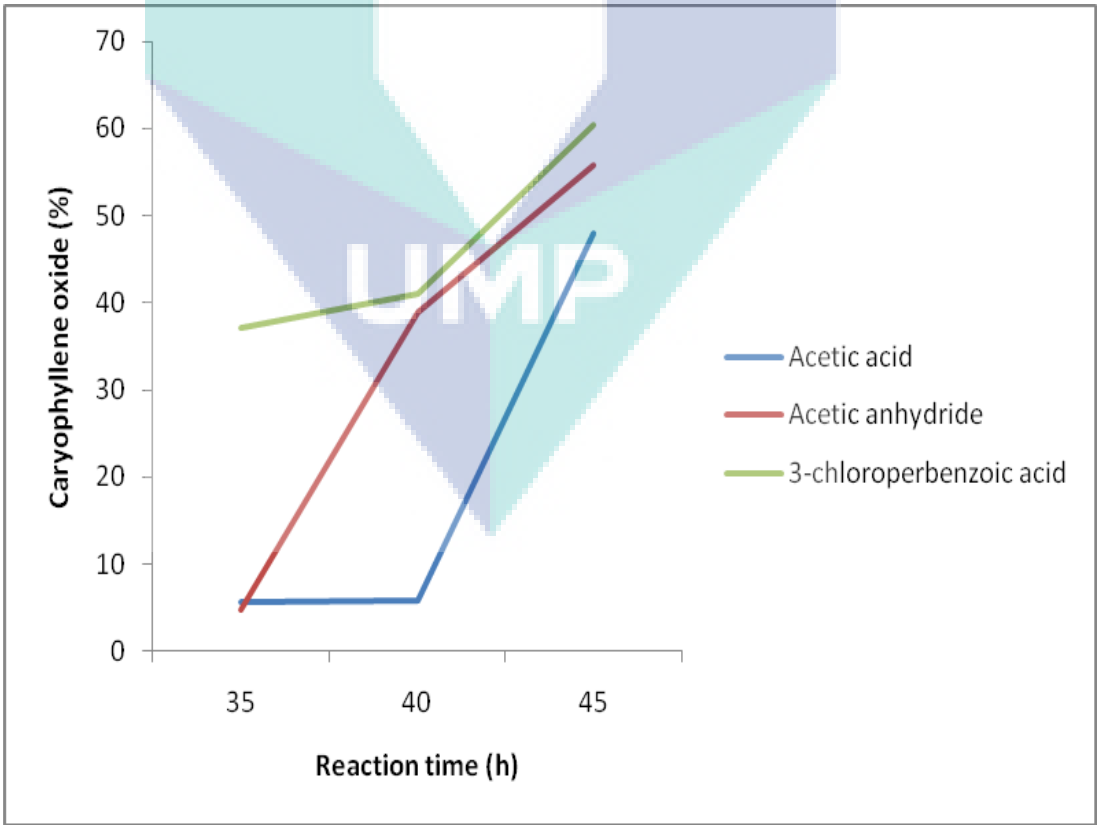


Figure 4.17: Percentage of caryophyllene oxide formed at room temperature at different time reaction, same ratio (0.8, 1, 1.3 mol), and C.O solution temperature (55° C).

The experiments among the formic acid, acetic acid, acetic anhydride and 3-chloroperbenzoic acid showed that the pKa value play a role determining the best lowest temperature to produce the maximum yield of caryophyllene oxide. This can be summarized at table 4.33 below.

Table 4.11: pKa values and best temperature production.

Acids	pKa value	best temperature production (° C)
Formic acid	3.77	30
Acetic acid	4.76	55
Acetic anhydride	Not mentioned but assumed closed to acetic acid	45
3-chloroperbenzoic acid	3.82	50

4.7 Pure Caryophyllene Oxide

The pure caryophyllene oxide was obtained by crystallization of the caryophyllene oxide solution using simple known method. The crystals formed were filtered using vacuum filter as shown in figure 4.18. This product appears as white crystalline solid with a sweet odor. The caryophyllene oxide crystal was then analyzed to compare with the standard caryophyllene oxide of 99% purity from Sigma & Aldrich. Physical properties of standard and produced sample were listed in table 4.34.

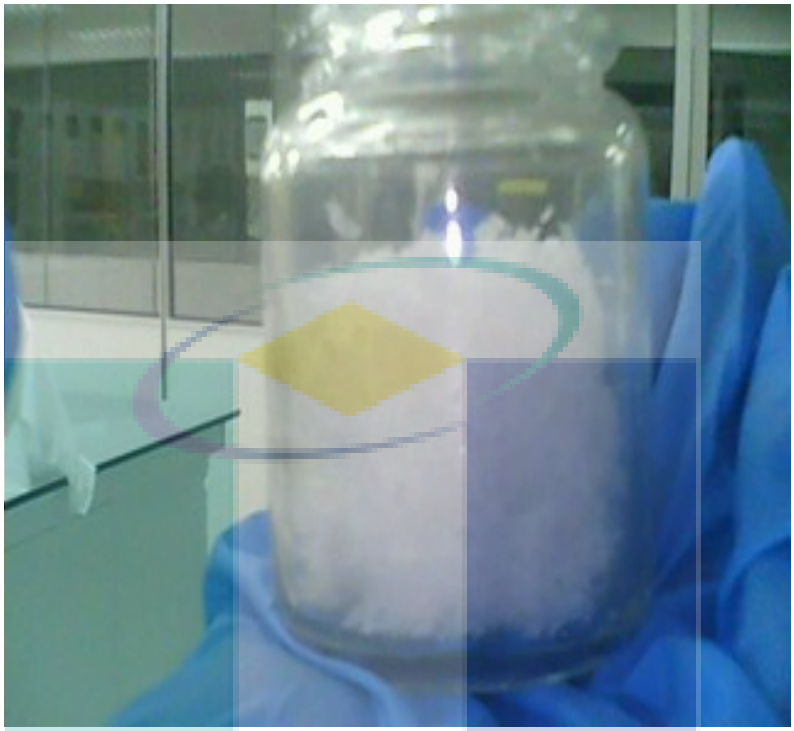


Figure 4.18: Pure caryophyllene oxide.

Table 4.12: Physical properties of standard and produced sample.

Types of sample	Odor	Appearance
Standard caryophyllene oxide	woody	short needle
Synthesized caryophyllene oxide	sweety	long needle

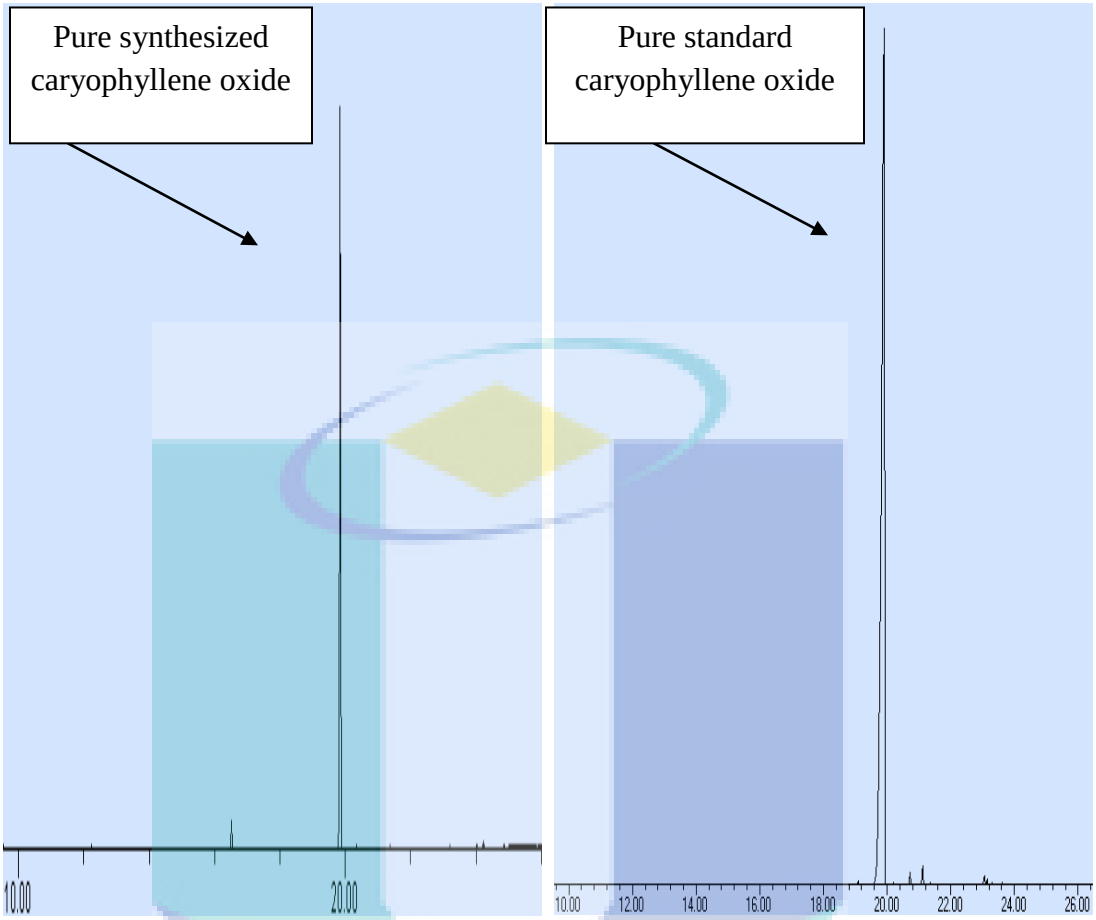


Figure 4.19: GC/MS Comparison between pure caryophyllene oxide sample and standard.

GC/MS analysis in figure 4.19 shows that the pure caryophyllene oxide crystal obtained in the reaction is similar with the standard that was purchased from Sigma Aldrich. Both appear at retention time range of 19-20 min. Raja Rajeswari et al., (2011) reported that the retention time obtained for the caryophyllene oxide extracted from the ethanolic leaves appeared at 29.19 min. The difference here may be due to diffrent columns used in GC/MS analysis.

4.8 Characterization

In order to prove the purity of caryophyllene oxide in the sample product, FTIR and NMR were used to analyse the caryophyllene oxide crystals produced.

(i) Fourier Transform Infrared Spectroscopy (FTIR)

Infrared radiation is absorbed by organic molecules and converted into energy of molecular vibration, either stretching or bending. Different types of bonds and functional groups absorb infrared radiation of different wavelengths. IR spectrum is a plot of wavenumber (X-axis) versus percent transmittance (Y-axis). Caryophyllene, sodium formate and caryophyllene oxide were analyzed and should give information about its compound's structure.

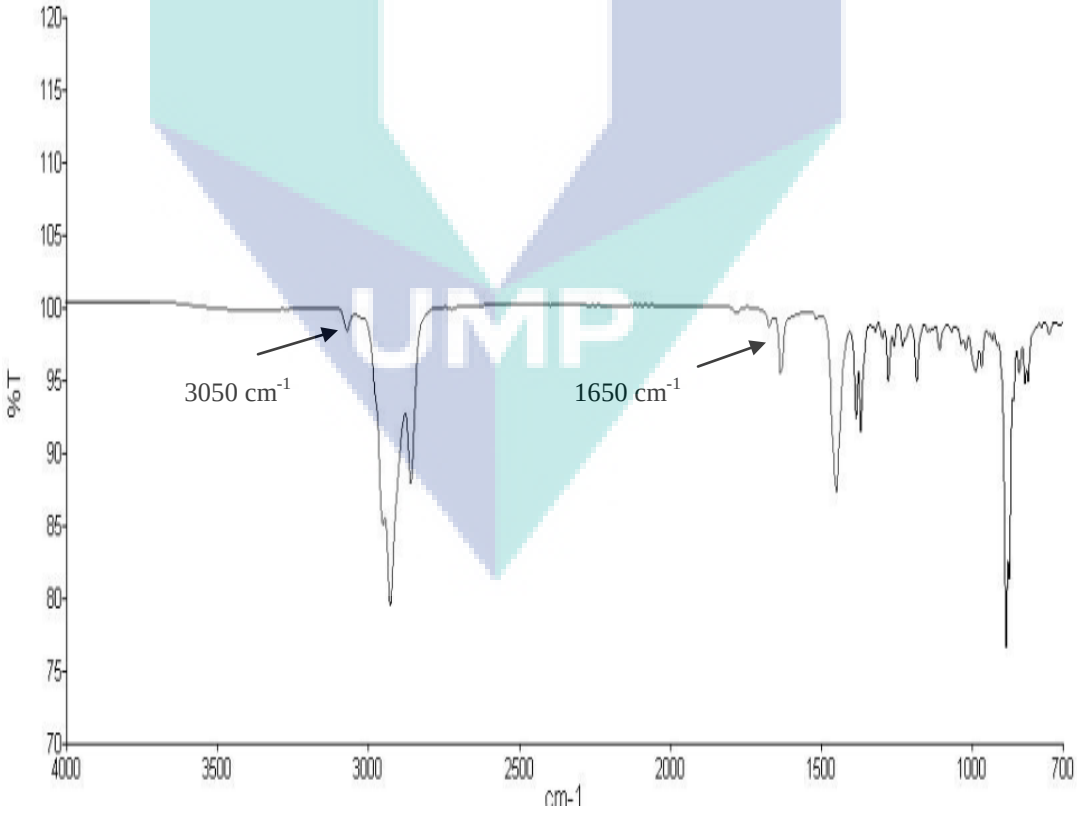


Figure 4.20: FTIR of caryophyllene spectrum.

Figure 4.20 illustrated the spectrum of crude caryophyllene (Sigma Aldrich). The caryophyllene structure has C=C and =CH bond belongs to an alkene group which is allocated at 1650 $cm^{-1}$and 3050 cm^{-1} respectively.

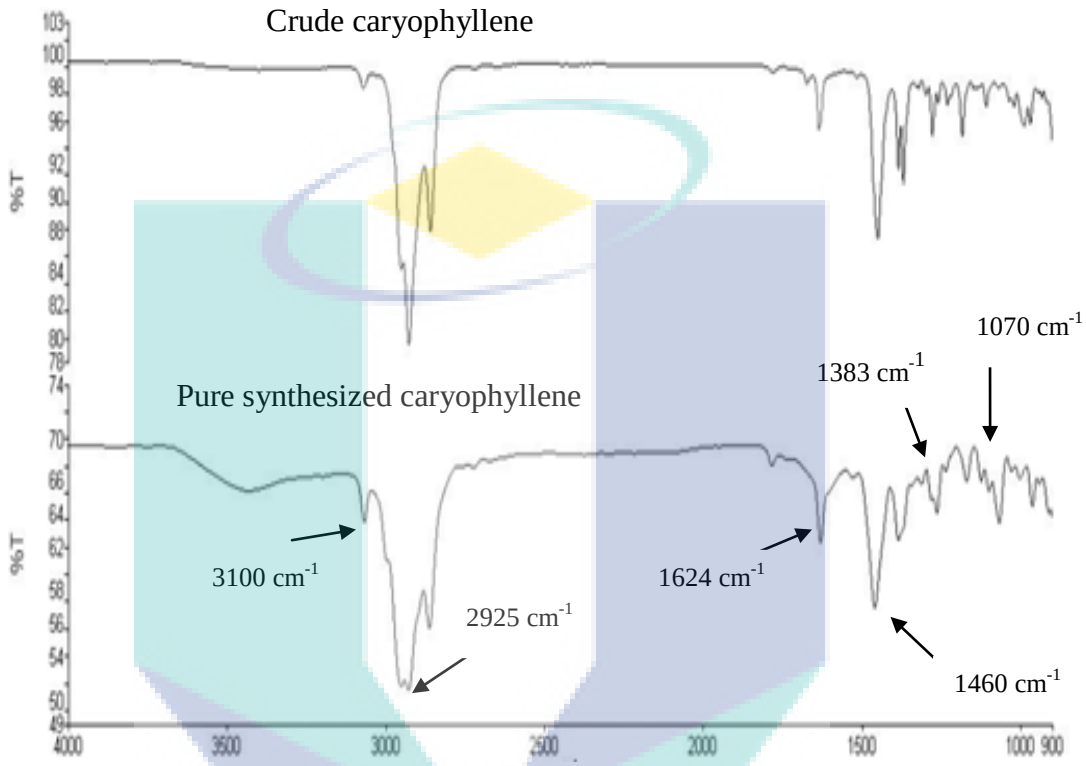


Figure 4.21: IR overlaid spectra of crude caryophyllene and caryophyllene oxide.

The broadband at 3100 cm^{-1} correspond to $=CH_2$ stretching. The peak at 2925 cm^{-1} belong to C-H stretch. A sharp peak at 1624 cm^{-1} was caused by C=C stretching. The peaks observed at 1460 cm^{-1} and 1383 cm^{-1} were the CH_2 and CH_3 bend.The caryophyllene oxide displays a peak characteristic of C-O stretching vibration at 1070 cm^{-1}.

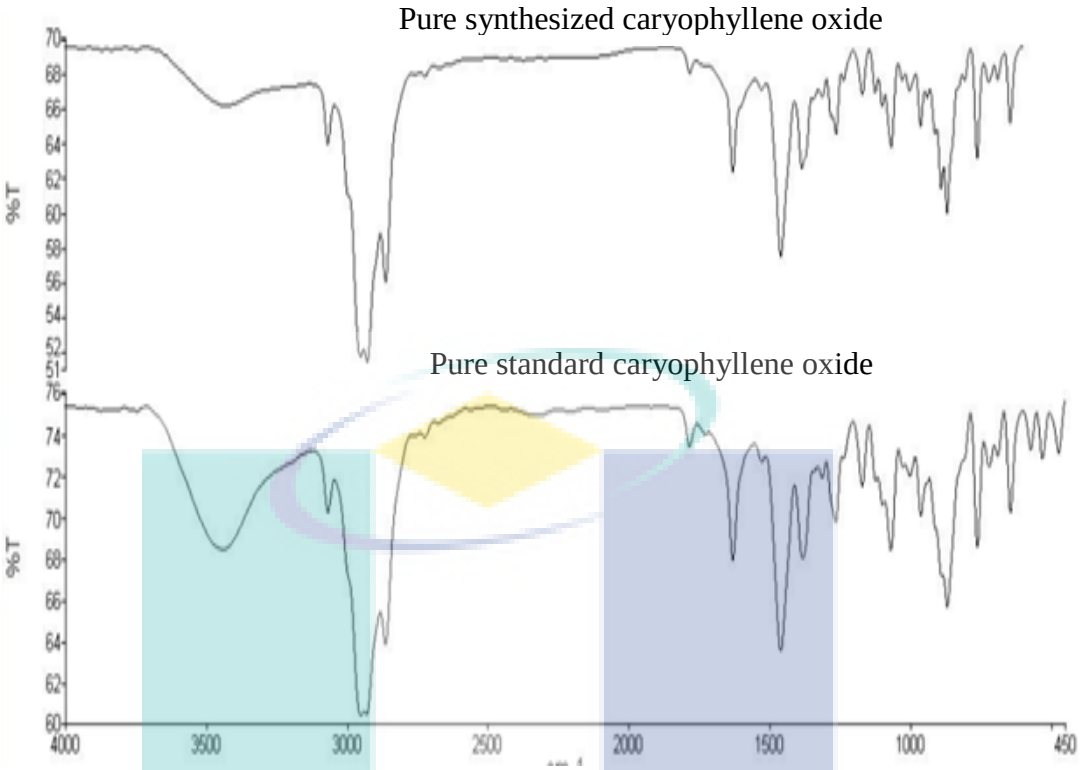


Figure 4.22: Overlaid spectra of CO sample and CO standard.

Overlaid FTIR spectra of caryophyllene oxide sample and caryophyllene oxide standard was shown in figure 4.22. The result showed both spectra appear as a same trend, where C-O bond of this oxide existed in the region of 1300-1080 cm^{-1}.

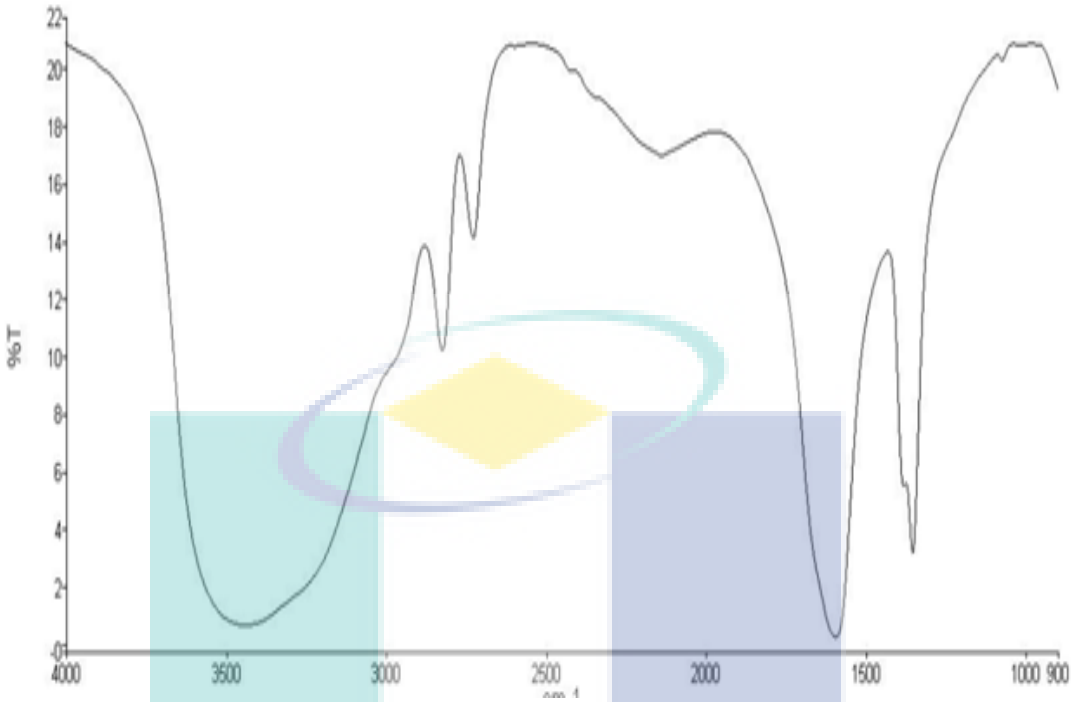


Figure 4.23: FTIR sodium formate spectrum.

Sodium formate spectrum also was obtained to confirm the region's band of this carbonyl was in the range of 1200 - 1700 cm^{-1}. From the spectroscopy spectrum illustrated in figure 4.23 it can be seen that the product CO was free from impurities of sodium formate.

(ii) Nuclear Magnetic Resonance (NMR)

In this study, NMR analysis was carried out to confirm the existence of C-O bond at the carbon 4 and 5 in the caryophyllene oxide molecule structure as shown in figure 4.24.

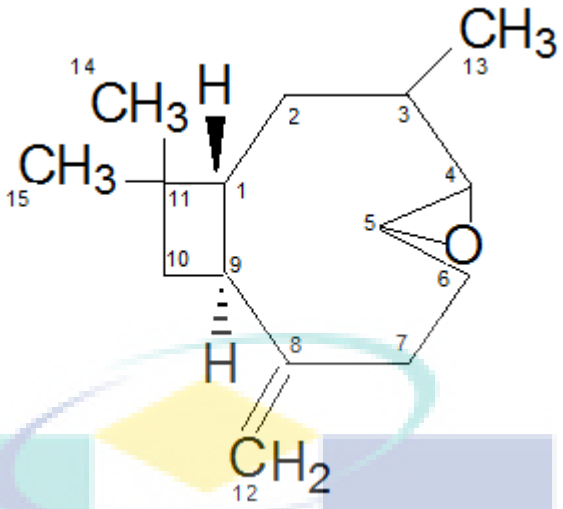


Figure 4.24: Carbon position of caryophyllene oxide.

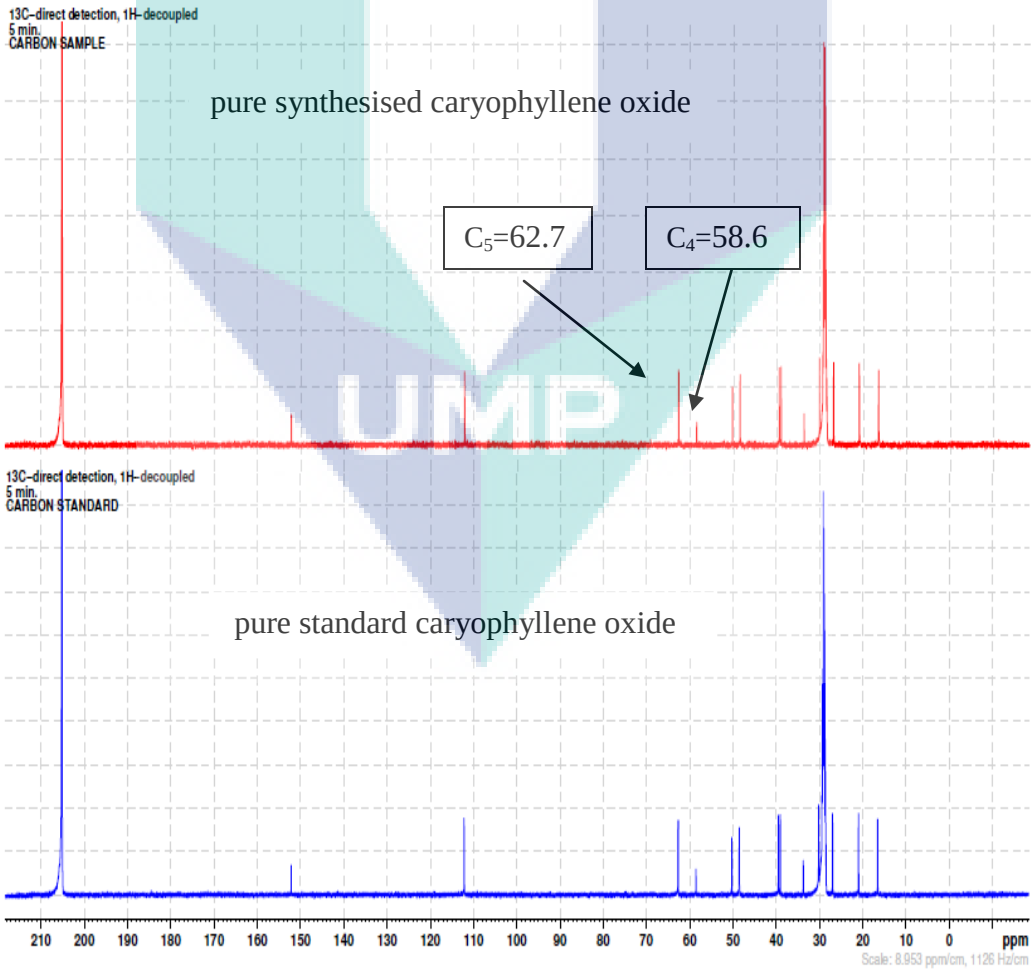


Figure 4.25: IR overlaid spectra of crude caryophyllene and caryophyllene oxide.

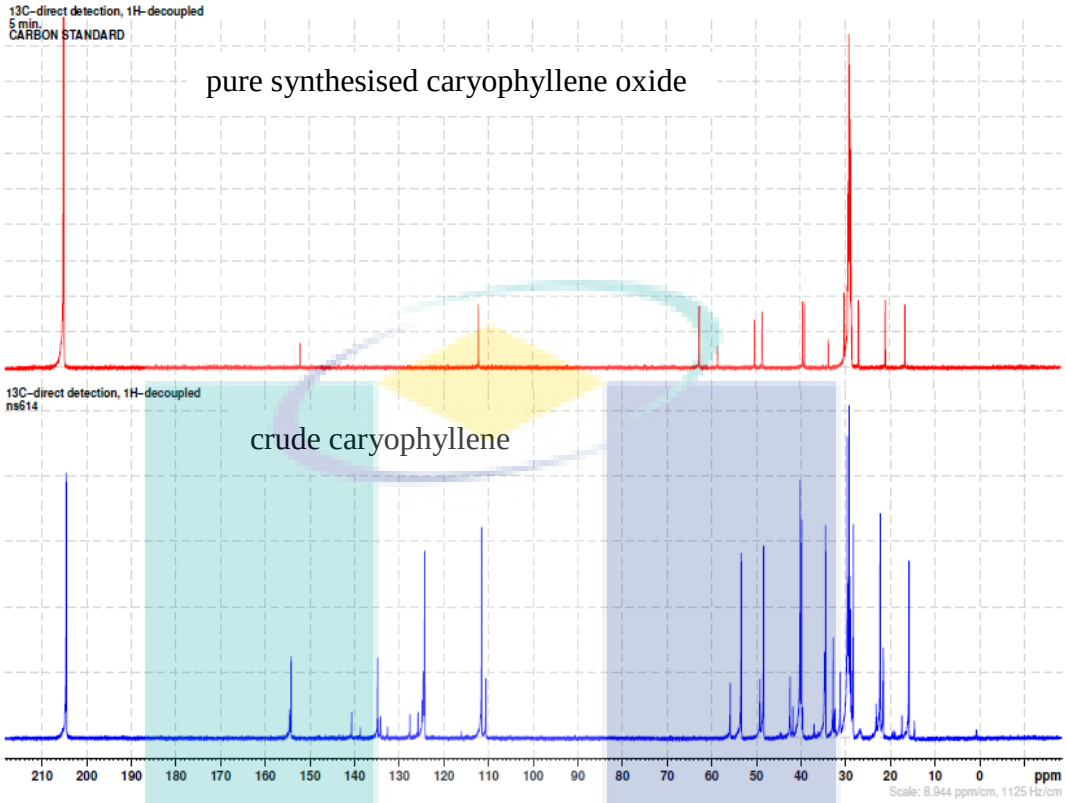


Figure 4.26: ^{13}C NMR overlaid spectra of crude caryophyllene and pure synthesised caryophyllene oxide.

Figure 4.25 shows the spectra of overlaid ^{13}carbon standard and the product that was successfully overlapped. From the overlaid carbon spectrum obtained, the C-O bond was found at 58.6 and 62.71 ppm at the position of carbon 4 and 5 respectively as shown in figure 4.25. Choudary et al., (2006) reported in data compound of caryophyllene oxide that C-O bond at position 4 and 5 was found at 59.8 and 63.7 ppm respectively as shown in figure 4.24.

Figure 4.26 illustrated the spectra of overlaid ^{13}carbon of caryophyllene, starting material to produce caryophyllene oxide and the product. Both spectra of carbon 4 and 5 which belongs to C-O bond does not appear in caryophyllene spectrum. This proves the caryophyllene has been converted to caryophyllene oxide.

^{1}H spectra were indicated the spectra of overlaid ^{1}H standard and the product that was successfully overlapped**.** From the overlaid proton spectrum obtained, the position of proton 1 to 15 were summarized in the table 4.25. Table 4.25 indicated that the proton at the carbon 5 was present at 2.83 ppm. Choudary et al. (2006) was reported the proton at carbon 5 of caryophyllene oxide structure was present at chemical shift of 2.85 ppm. Chemical shift is signal frequency of the nucleus that is detected by NMR

However, the ^{1}H NMR spectrum of pure standard caryophyllene oxide and pure synthesized caryophyllene oxide can be found in appendix G1 and G2.

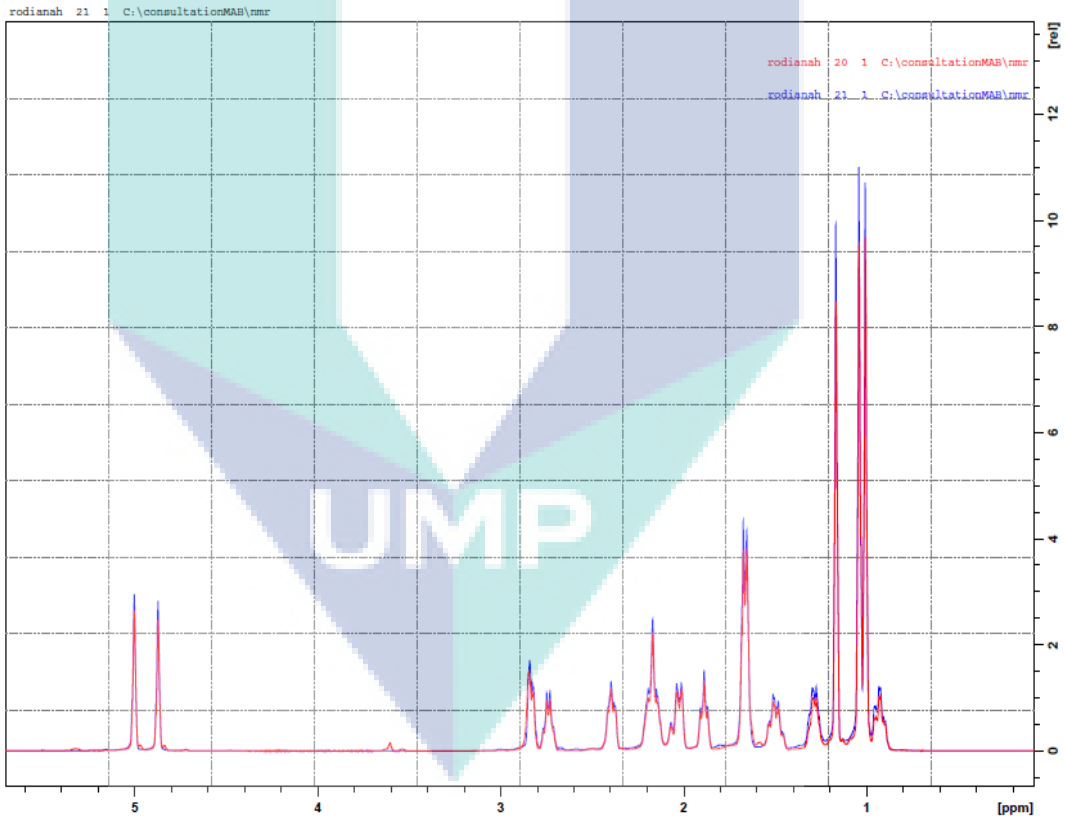


Figure 4.27: ^{1}H NMR overlaid spectra of crude caryophyllene and pure synthesized caryophyllene.

Table 4.13: ^{1}H NMR Data for caryophyllene oxide compound

Position	Chemical shift
1	1.87
2	1.45, 1.65
3	2.14, 0.93
5	2.83
6	2.37, 2.19
7	2.40, 2.15
9	2.41
10	1.65, 1.67
12	4.87, 5.00
13	1.17
14	0.95
15	0.96

CHAPTER 5

CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH

5.1 Introduction

This chapter discusses the findings of extraction of eugenol, separation and purification of caryophyllene and synthesis of caryophyllene oxide.

5.2 Conclusion

Thus from this study it can be concluded that in the extraction of clove bud oil, hydro distillation method was found to produce the highest yield followed by microwave extraction, steam distillation and the lowest value in yield was the cleaner- ultrasonic extraction method. However, the method that produced clove oil which has the highest percentage of eugenol was from ultrasonic method (85%), the steam distillation (70%), microwave extraction (65%), and hydro distillation (63%). By comparing these four extraction method result, ultrasonic method is better extraction method to produce high yield of caryophyllene oxide.

The separation of caryophyllene from eugenol was successfully obtained from clove bud and clove leaf oils. The highest yield was achieved at a ratio of clove oil: sodium hydroxide of 1: 2 and the lowest yield was at 1: 1.2 for both clove bud and clove leaf oils. Similar to the yield obtained, both sources gave the highest

purity at ratio clove oil: sodium hydroxide of 1: 2 followed by 1: 1.7, 1: 1.5, and 1: 1.2.

Purification of caryophyllene from the other minor components gave the highest purity of caryophyllene at 136 °C. This fraction distillate consists of 75 % component caryophyllene, 19.07 % alpha-caryophyllene and 5.8 % alpha-cubebene by using GC/MS analyzer. Standard caryophyllene showed 86.9 % composition of caryophyllene and 13.14 % of alpha caryophyllene.

The experiments of caryophyllene oxide synthesis using some carboxylic acids such as, formic acid, acetic acid, acetic anhydride, and 3-chloroperbenzoic acid were conducted within the variables of pH, reaction time, ratio of reagents, reaction temperature and caryophyllene oxide solution temperature. The GC/MS analysis showed that the highest percentage of caryophyllene oxide in solution, 86.47% equals to 99.55% conversion of caryophyllene to caryophyllene oxide, was obtained at pH 2.2, 40 hours of reaction time, 0.8 mol formic acid, 1.3 mol hydrogen peroxide, 30 °C reaction temperature, and 35 °C caryophyllene oxide solution temperature. For all the experiments were carried out, formic acid is the best carboxylic acid for this method as it works at room temperature and therefore less possibility of explosion to occur and also it is the cheapest among the rest and it gave the highest yield of caryophyllene oxide.

This result will be useful for the industry because this method did not consume large amount of energy and high maintenance cost in producing this valuable product.

5.3 Recommendation for future research

It is shown that caryophyllene oxide crystals produced by this inexpensive and safe process is quite pure. Commercial pure caryophyllene oxide (99%) is very expensive compared to the starting materials. It would be interesting to know if this method of preparation for the lab scale can be used for the pilot scale for industry.

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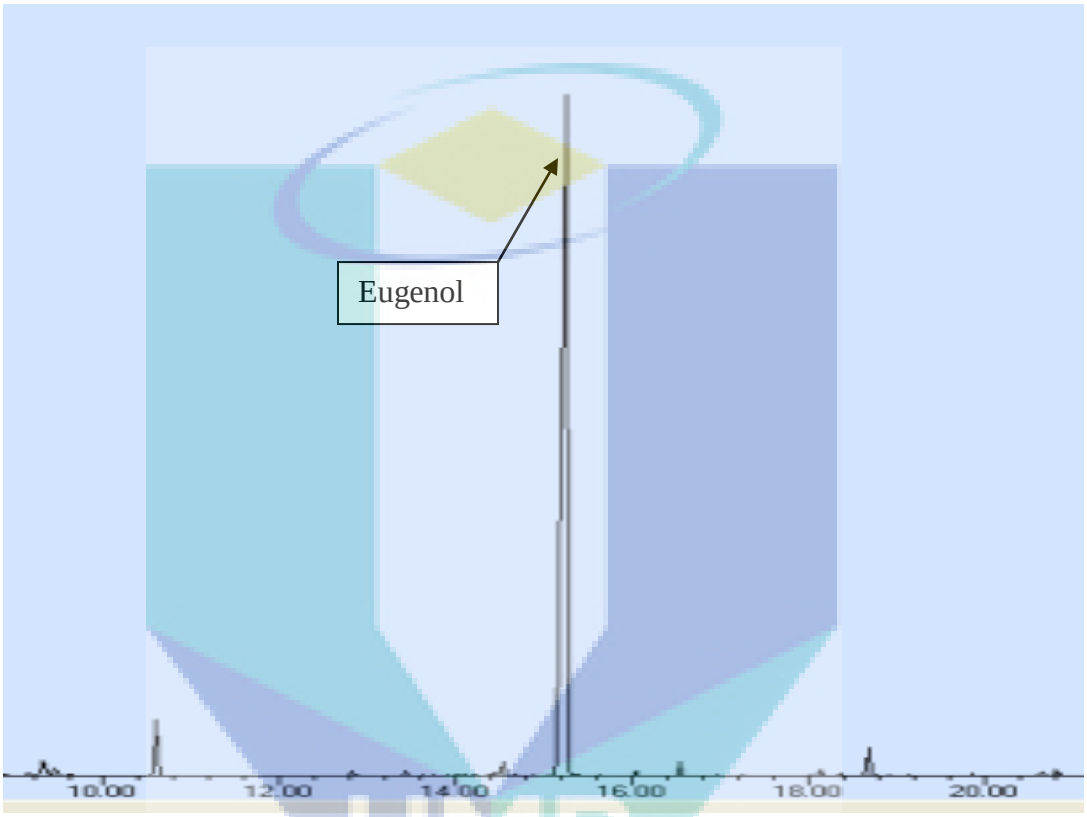
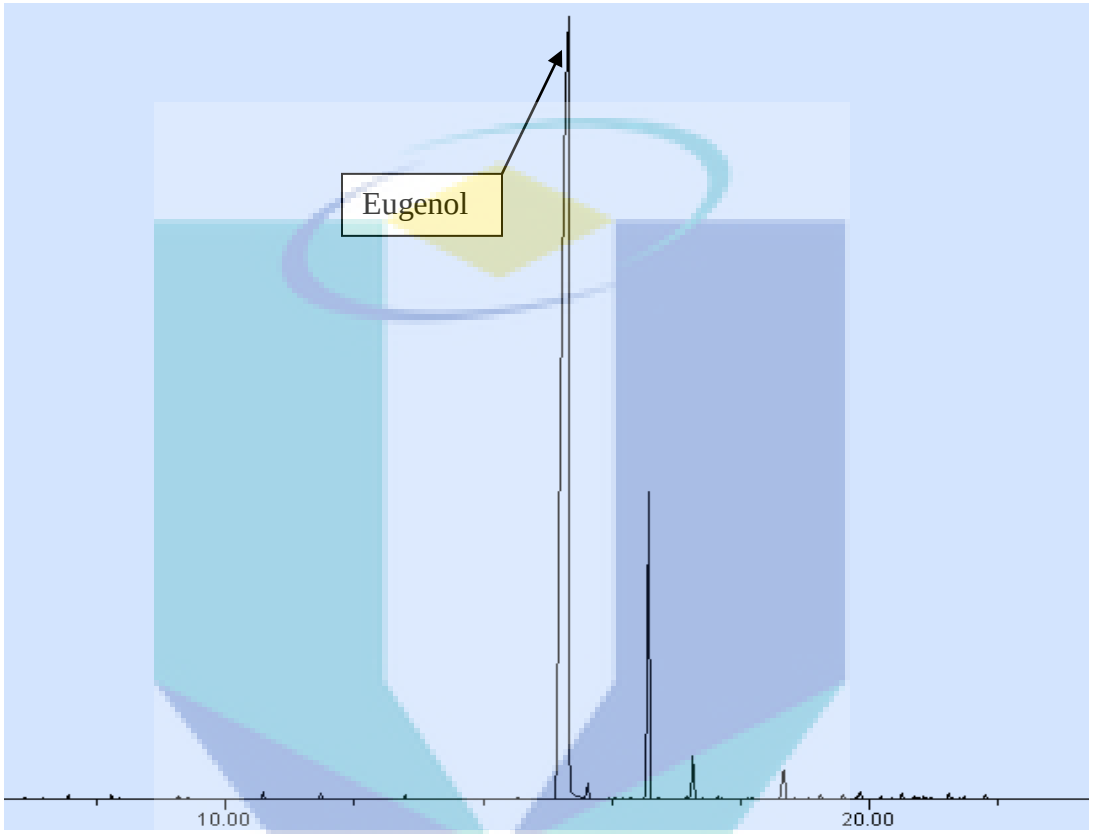
APPENDIXES A

Chromatogram of extraction by Ultrasonic Extractor at reaction temperature of 80 °C



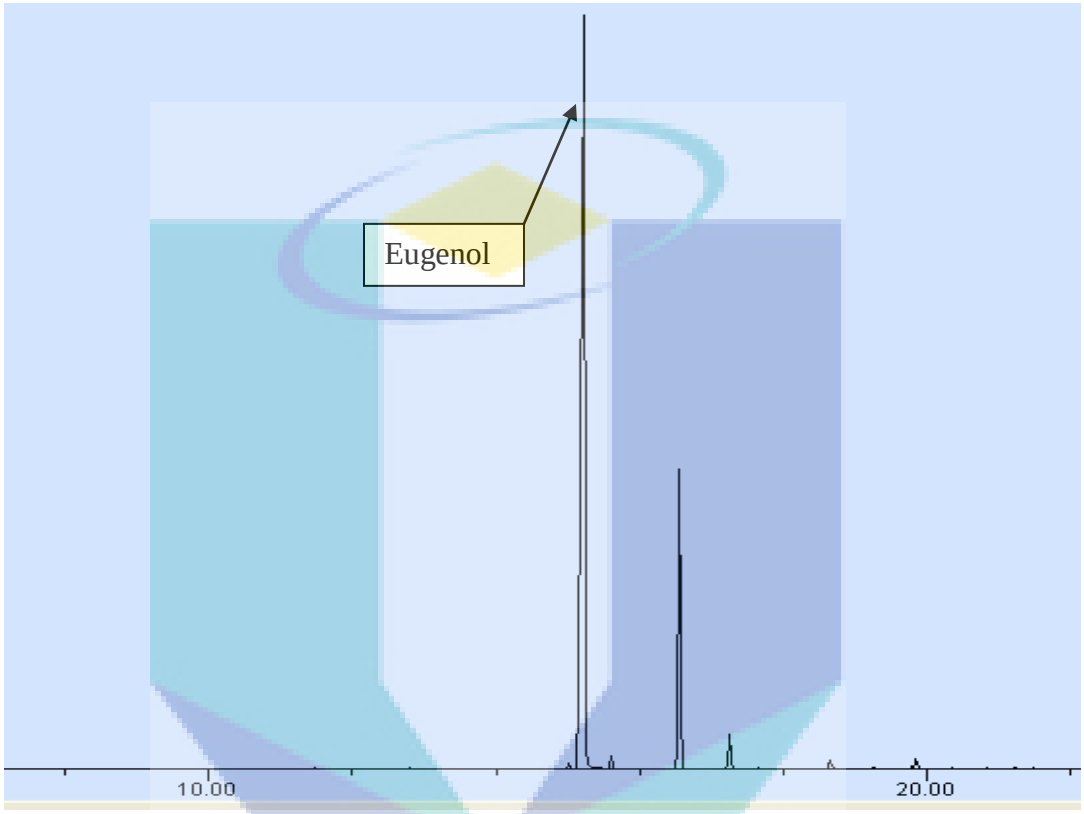
APPENDIXES B1

Chromatogram of clove bud oil standard



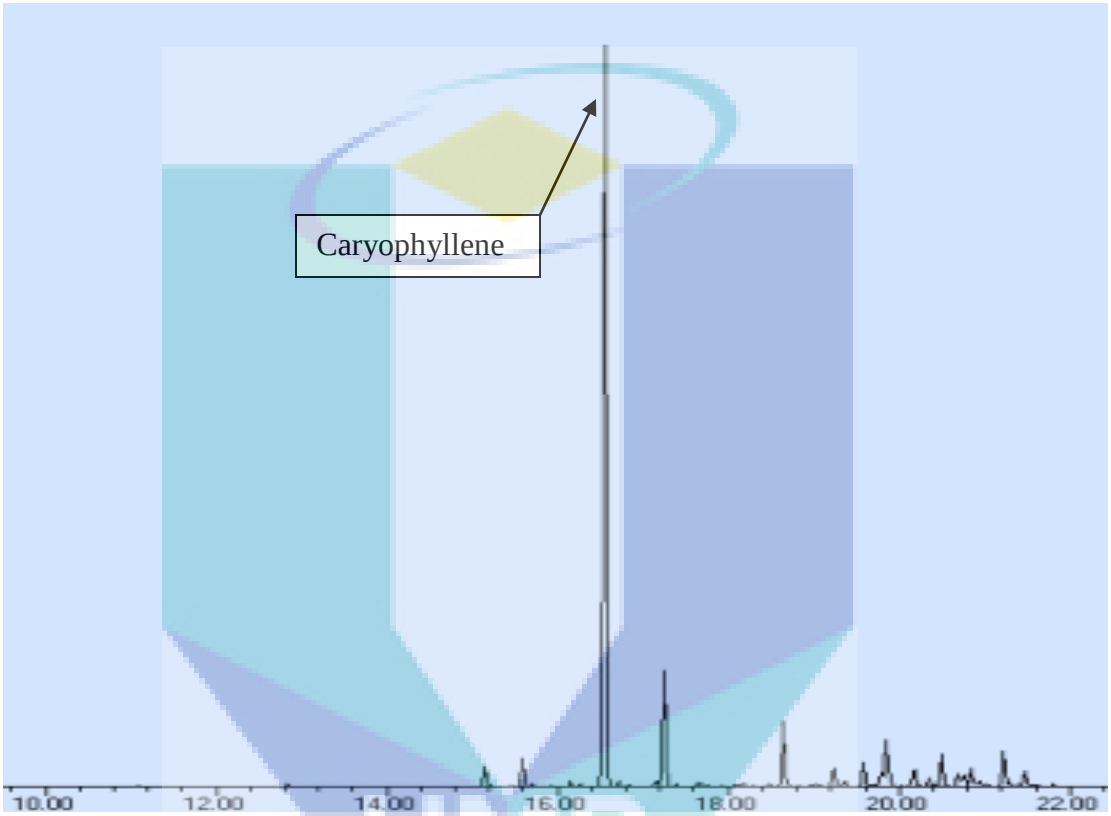
APPENDIXES B2

Chromatogram of clove leaf oil standard



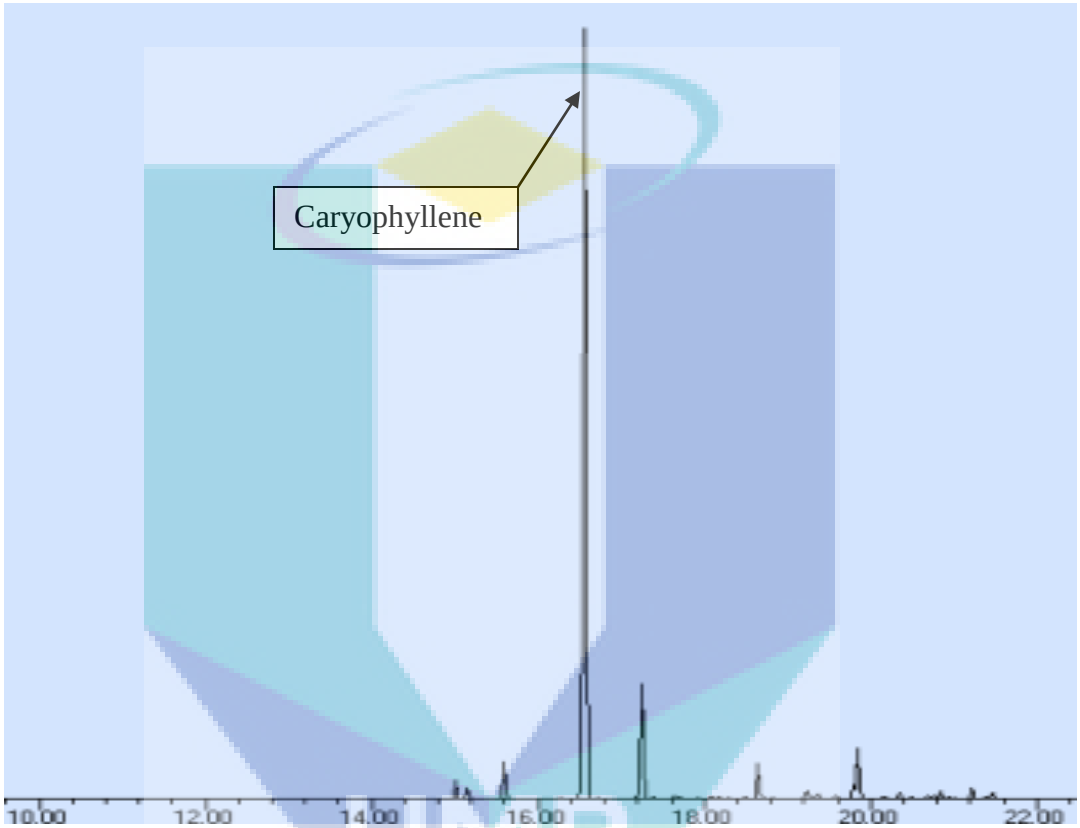
APPENDIXES C1

Chromatogram of caryophyllene obtained by separation of clove bud oil at ratio of 1:2



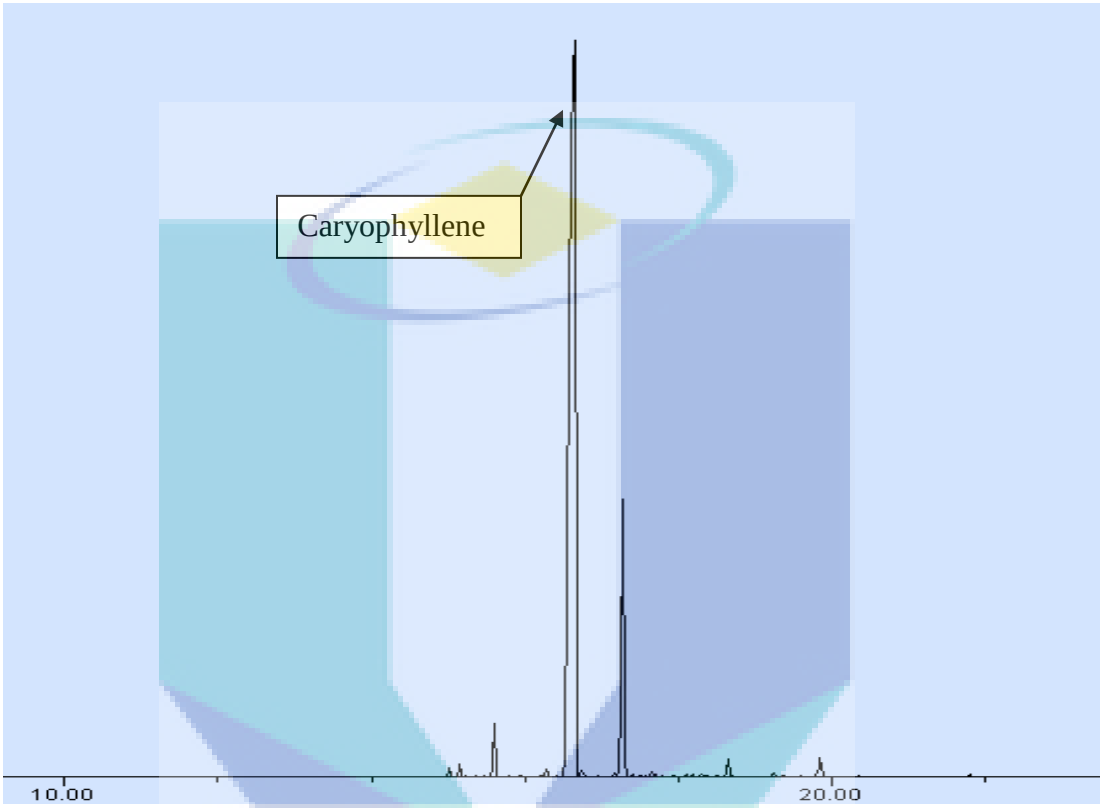
APPENDIXES C2

Chromatogram of caryophyllene obtained by separation of clove leaf oil at ratio of 1:2



APPENDIXES D

Chromatogram of standard crude caryophyllene



APPENDIXES E1

Experiment conditions at different variables, percentage in solution, crystal yield and conversion of caryophyllene oxide by using formic acid.

Ratio (mol)			pH	yield (g)	% of caryo phyllene oxide in solution by GCMS	% conversion
formic acid	caryo phyllene	hydrogen peroxide				
0.8	1	1.2	0.2	5.95	60.45	69.59
0.8	1	1.2	1.2	6.85	69.27	79.75
0.8	1	1.2	2.2	8.31	84.54	97.33
0.8	1	1.2	3.2	6.37	64.04	73.73
0.8	1	1.2	4.2	1.41	14.07	16.20

Ratio (mol)			time (h)	yield (g)	% of caryo phyllene oxide in solution by GC/MS	% conversion
formic acid	caryo phyllene	hydrogen peroxide				
0.8	1	1.2	30	7.71	78.73	90.64
0.8	1	1.2	35	7.74	78.82	90.74
0.8	1	1.2	40	8.40	84.07	96.79
0.8	1	1.2	45	35	8.11	81.30
0.8	1	1.2	50	35	7.65	77.11

Ratio (mol)			reaction temperature (°C)	yield (g)	% of caryo phyllene oxide in solution by GC/MS	% conversion
formic acid	caryo phyllene	hydrogen peroxide				
0.6	1	1.4	24	7.11	72.94	82.89
0.8	1	1.4	24	7.96	80.01	92.11
1.0	1	1.4	24	7.22	73.99	85.18

Ratio (mol)			reaction temperature (°C)	yield (g)	% of caryo phyllene oxide in solution by GC/MS	% conversion
formic acid	caryo phyllene	hydrogen peroxide				
0.8	1	1.2	24	8.28	83.66	96.32
0.8	1	1.3	24	8.43	85.11	97.98
0.8	1	1.4	24	7.96	80.01	92.11

Ratio (mol)			reaction temperature (°C)	yield (g)	% of caryo phyllene oxide in solution by GC/MS	% conversion
formic acid	caryo phyllene	hydrogen peroxide				
0.8	1	1.3	24	7.77	78.06	89.87
0.8	1	1.3	30	8.56	86.47	99.55
0.8	1	1.3	40	7.01	71.08	81.83
0.8	1	1.3	45	6.41	64.32	74.05
0.8	1	1.3	55	6.33	64.16	73.87

Ratio (mol)			reaction temperature (°C)	yield (g)	% of caryo phyllene oxide in solution by GC/MS	% conversion
formic acid	caryo phyllene	hydrogen peroxide				
0.8	1	1.3	24	7.77	78.06	89.87
0.8	1	1.3	30	8.56	86.47	99.55
0.8	1	1.3	40	7.01	71.08	81.83
0.8	1	1.3	45	6.41	64.32	74.05

0.8	1	1.3	55	6.33	64.16	73.87

Ratio (mol)			C.O. solution temperature (°C)	yield (g)	% of caryo phyllene oxide in solution by GC/MS	% conversion
formic acid	caryo phyllene	hydrogen peroxide				
0.8	1	1.3	35	8.31	84.87	97.71
0.8	1	1.3	55	7.86	79.85	91.93
0.8	1	1.3	65	7.91	79.54	91.57
0.8	1	1.3	75	7.90	79.24	91.23
0.8	1	1.3	95	5.72	58.26	67.07

Ratio (mol)			reaction temperature (°C)	yield (g)	% of caryo phyllene oxide in solution by GC/MS	% conversion
formic acid	caryo phyllene	hydrogen peroxide				
0.8	1	1.3	15	1.44	14.62	16.83
0.8	1	1.3	24	5.56	56.49	65.04
0.8	1	1.3	35	6.34	63.81	73.46
0.8	1	1.3	45	8.00	80.84	93.07

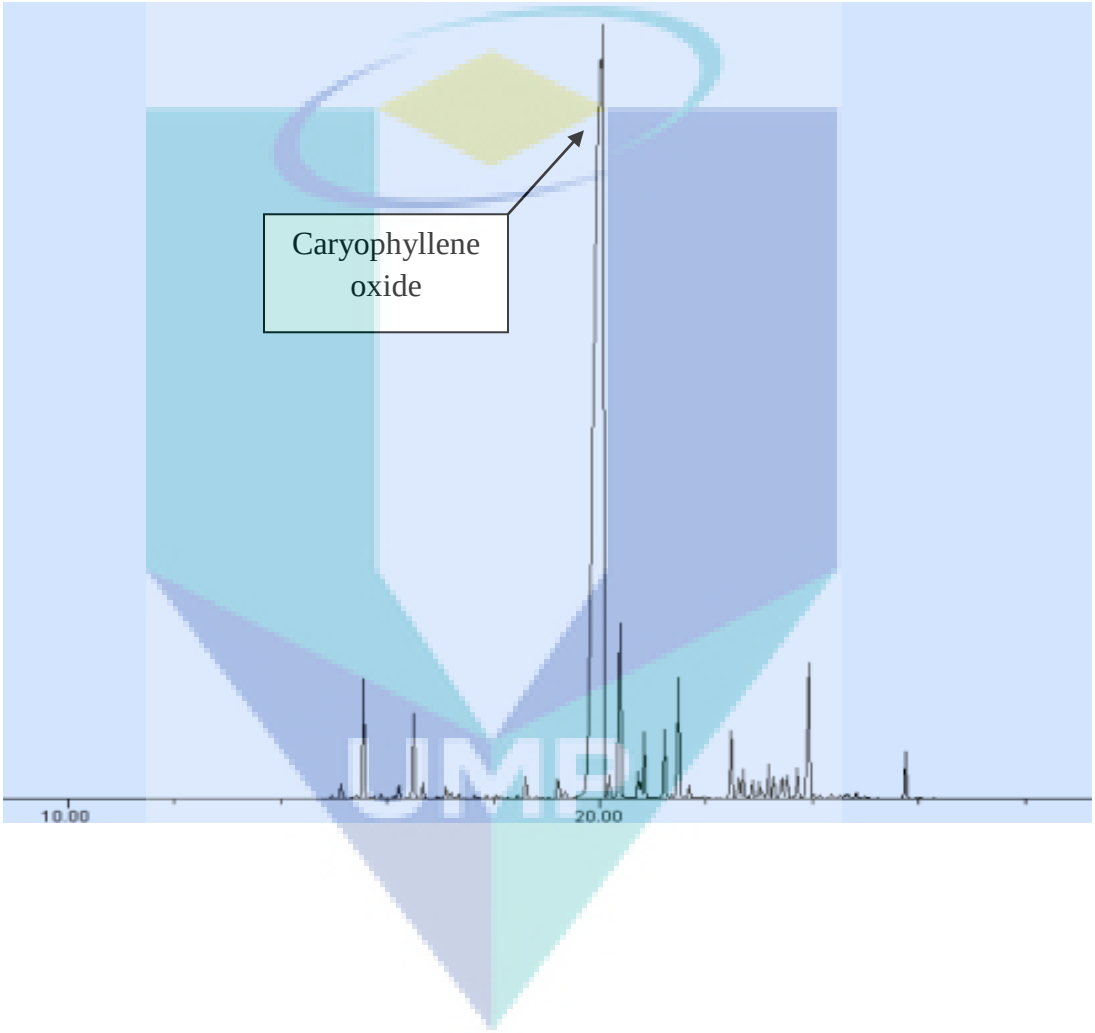
0.8	1	1.3	55	5.98	60.74	69.93

Ratio (mol)			reaction temperature (°C)	yield (g)	% of caryo phyllene oxide in solution by GC/MS	% conversion
formic acid	caryo phyllene	hydrogen peroxide				
0.8	1	1.3	24	7.65	77.74	89.50
0.8	1	1.3	35	7.79	78.73	90.64
0.8	1	1.3	45	8.09	81.13	93.40
0.8	1	1.3	55	6.97	70.25	80.88

Ratio (mol)			reaction temperature (°C)	yield (g)	% of caryo phyllene oxide in solution by GC/MS	% conversion
formic acid	caryo phyllene	hydrogen peroxide				
0.8	1	1.3	24	8.13	82.01	94.42
0.8	1	1.3	35	8.42	85.23	98.12
0.8	1	1.3	45	7.76	78.36	90.21
0.8	1	1.3	55	7.04	71.12	81.88

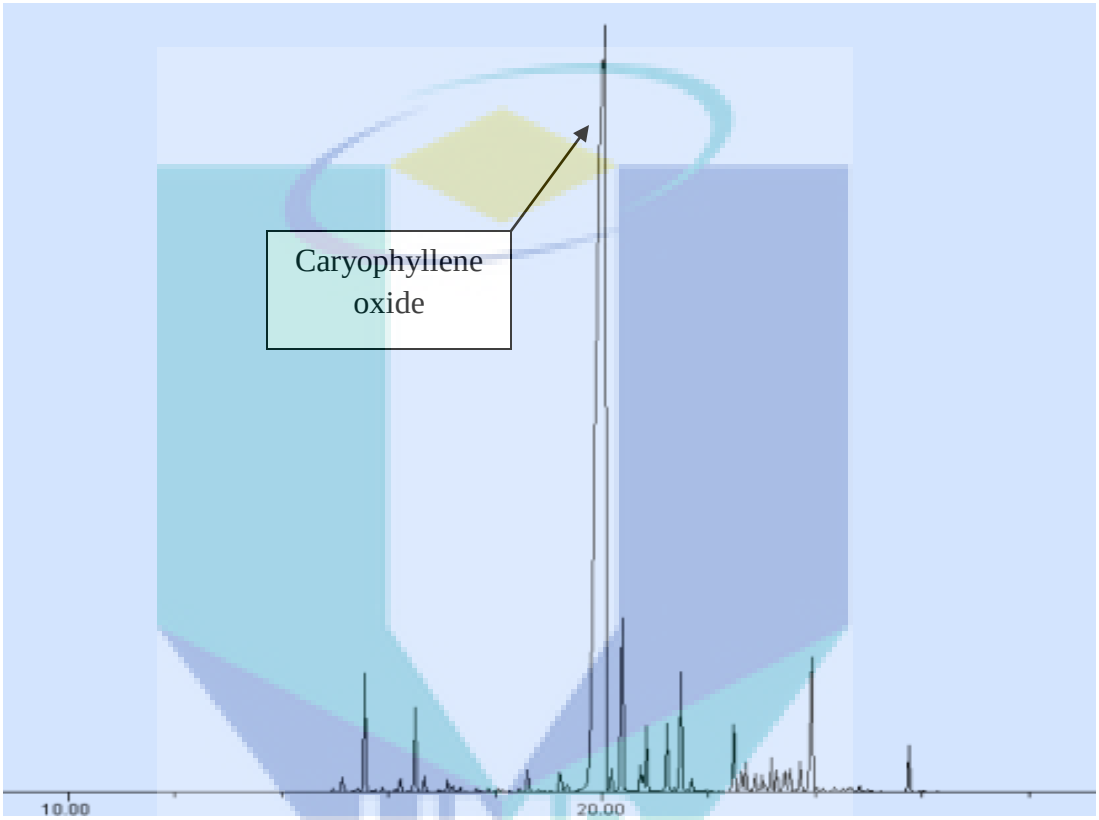
APPENDIXES E2

Chromatogram of caryophyllene oxide solution formed at pH 2.2, ratio (0.8, 1, 1.2 mol), time (40 h), reaction temperature (24 °C), and C.O solution temperature (35 ºC).



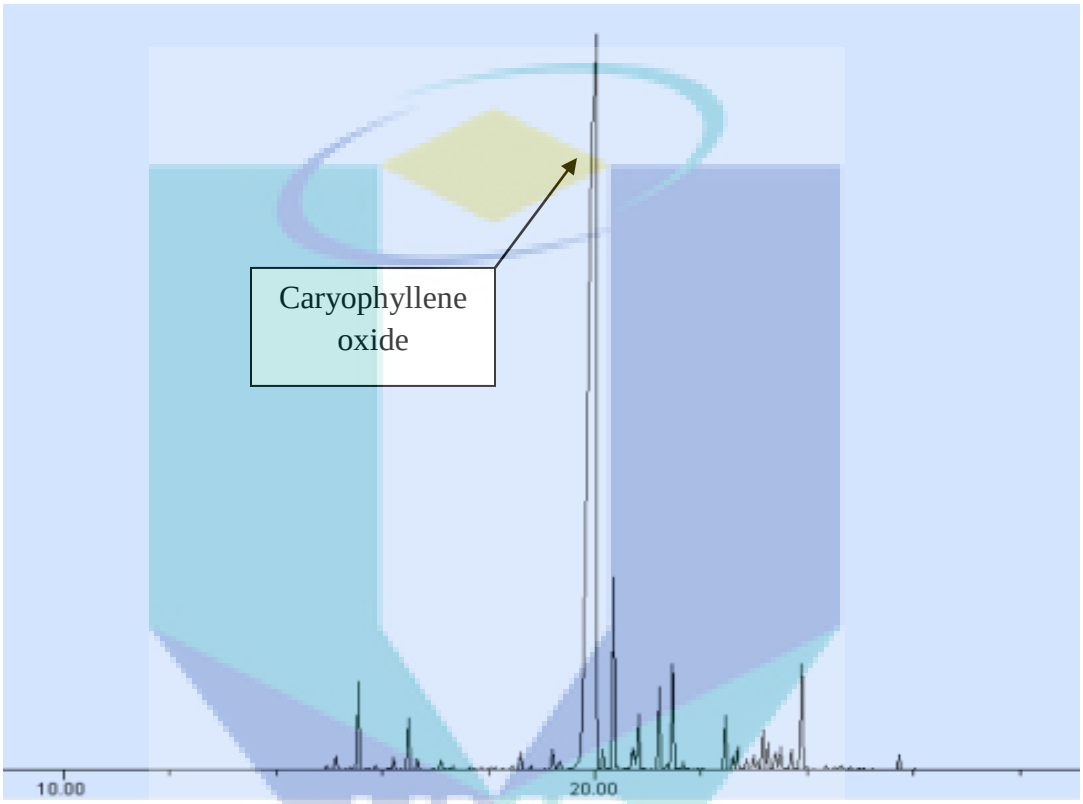
APPENDIXES E3

Chromatogram of caryophyllene oxide formed at 40 h reaction time, ratio (0.8, 1, 1.2 mol), pH 2.2, reaction temperature (24 °C), and C.O. solution temperature (35 °C).



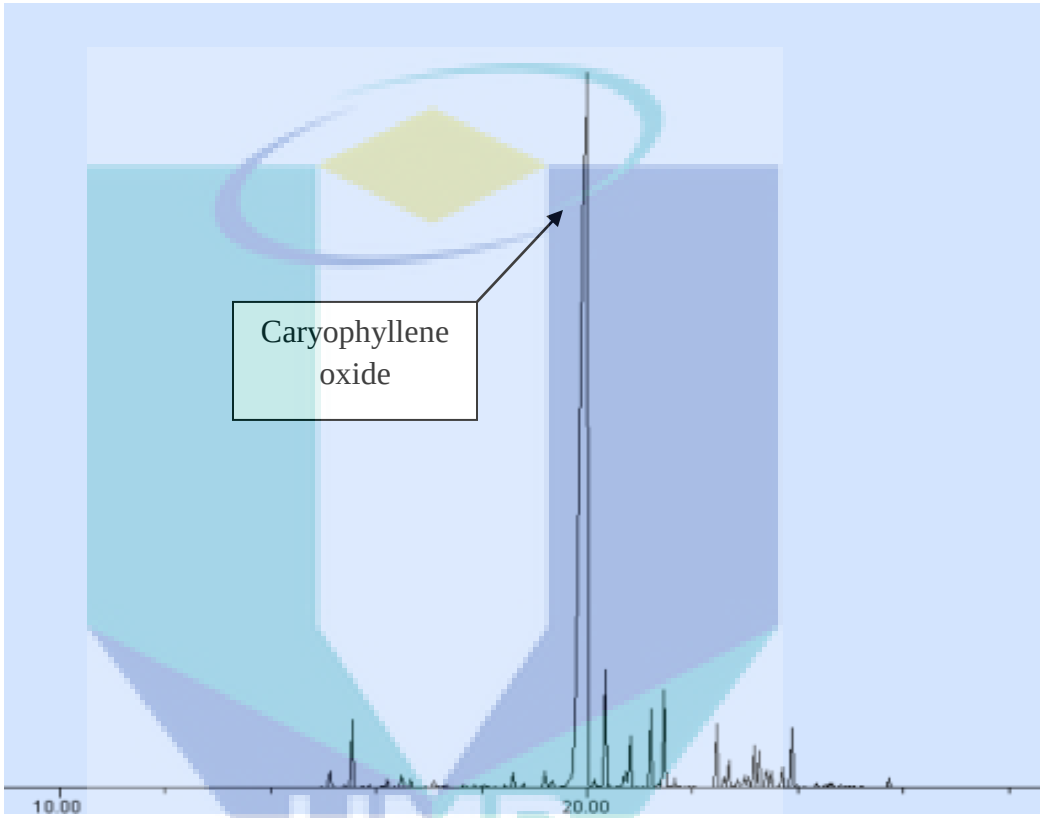
APPENDIXES E4

Chromatogram of caryophyllene oxide formed at 0.8 mol formic acid used, time (40h), pH 2.2, reaction temperature (24 °C), and C.O. solution temperature (35°C).



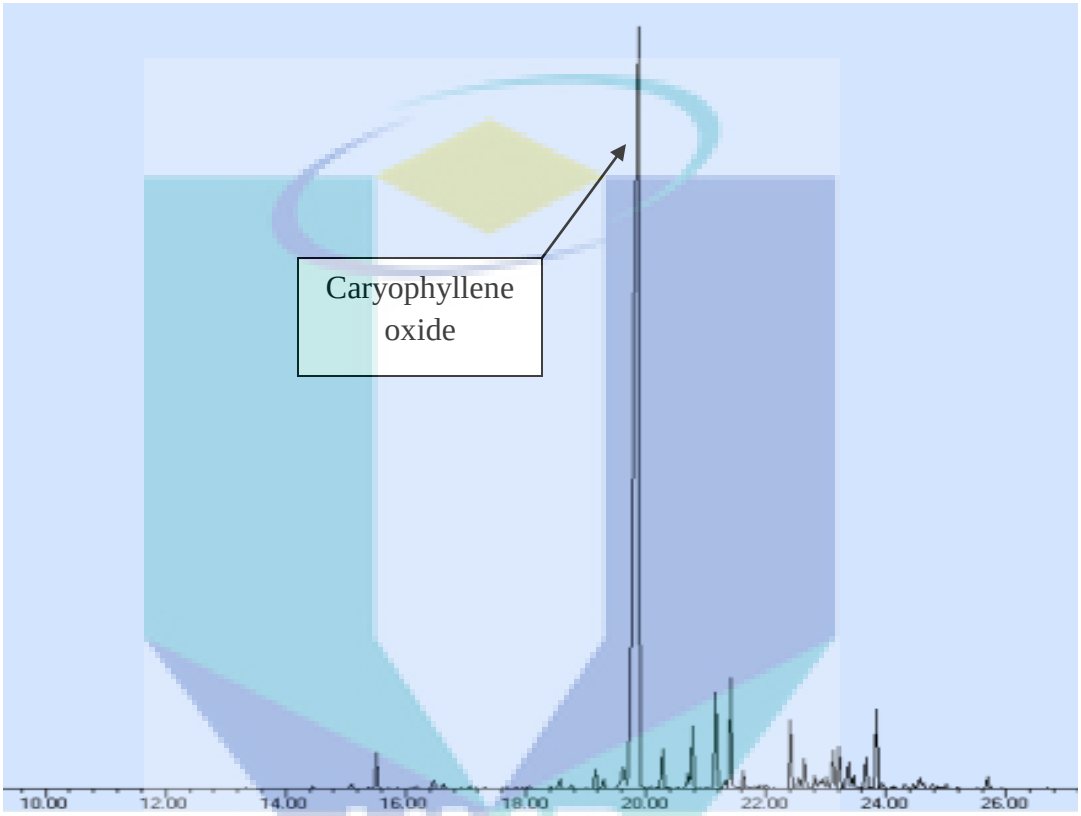
APPENDIXES E5

Chromatogram of caryophyllene oxide formed at 1.3 mol hydrogen peroxide, time (40h), pH 2.2, reaction temperature (24 °C), and C.O. solution temperature (35 °C).



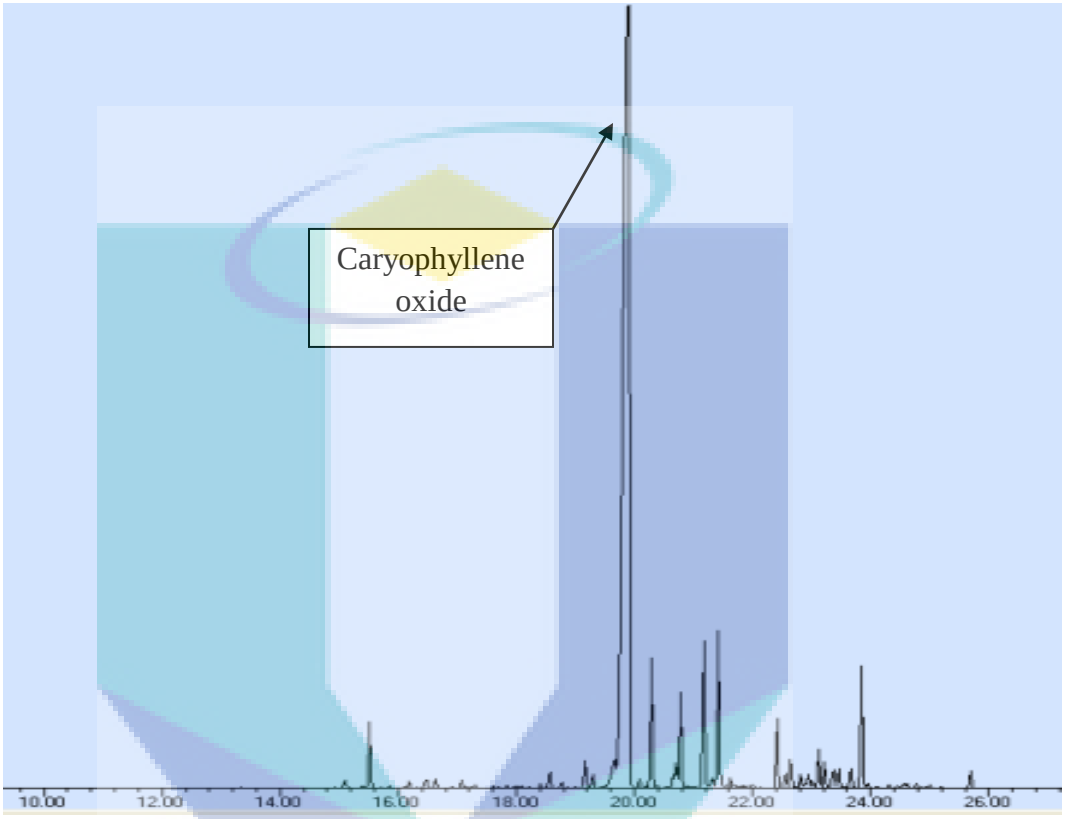
APPENDIXES E6

Chromatogram of caryophyllene oxide formed at 30 °C reaction temperature, ratio (0.8, 1, 1.3 mol), pH 2.2, time (40h), and C.O. solution temperature (35 °C).



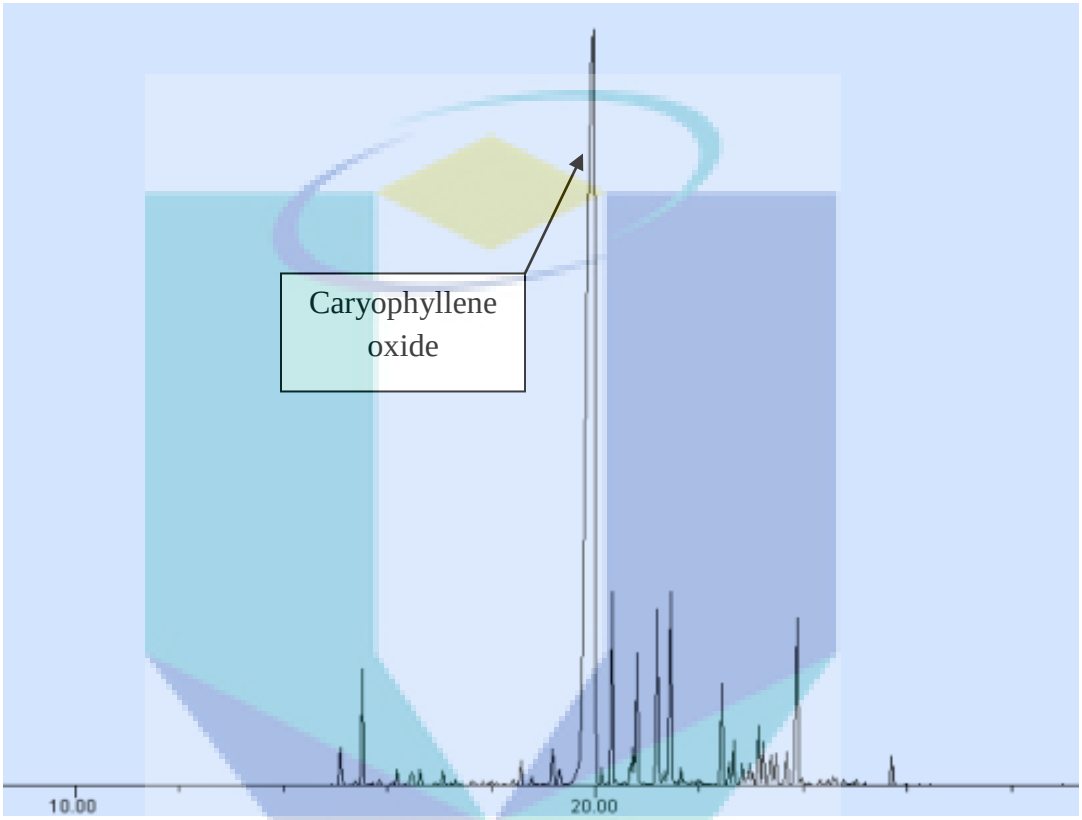
APPENDIXES E7

Chromatogram of caryophyllene oxide formed at different caryophyllene oxide solution temperature same ratio (0.8, 1, 1.2 mol), pH 2.2, time (40 h), and reaction temperature (24 °C).



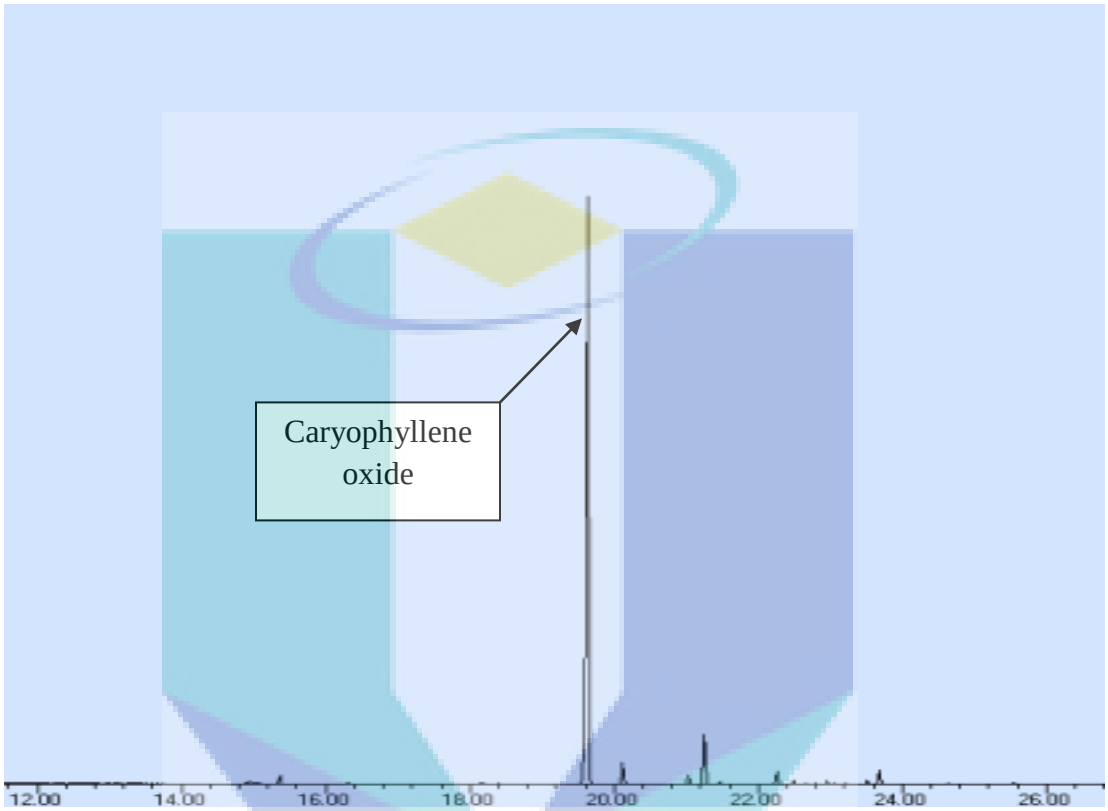
APPENDIXES E8

Chromatogram of caryophyllene oxide formed at 8 h at 45 °C reaction temperature same ratio (0.8, 1, 1.3 mol), pH 2.2, and C.O. solution temperature (55 °C).



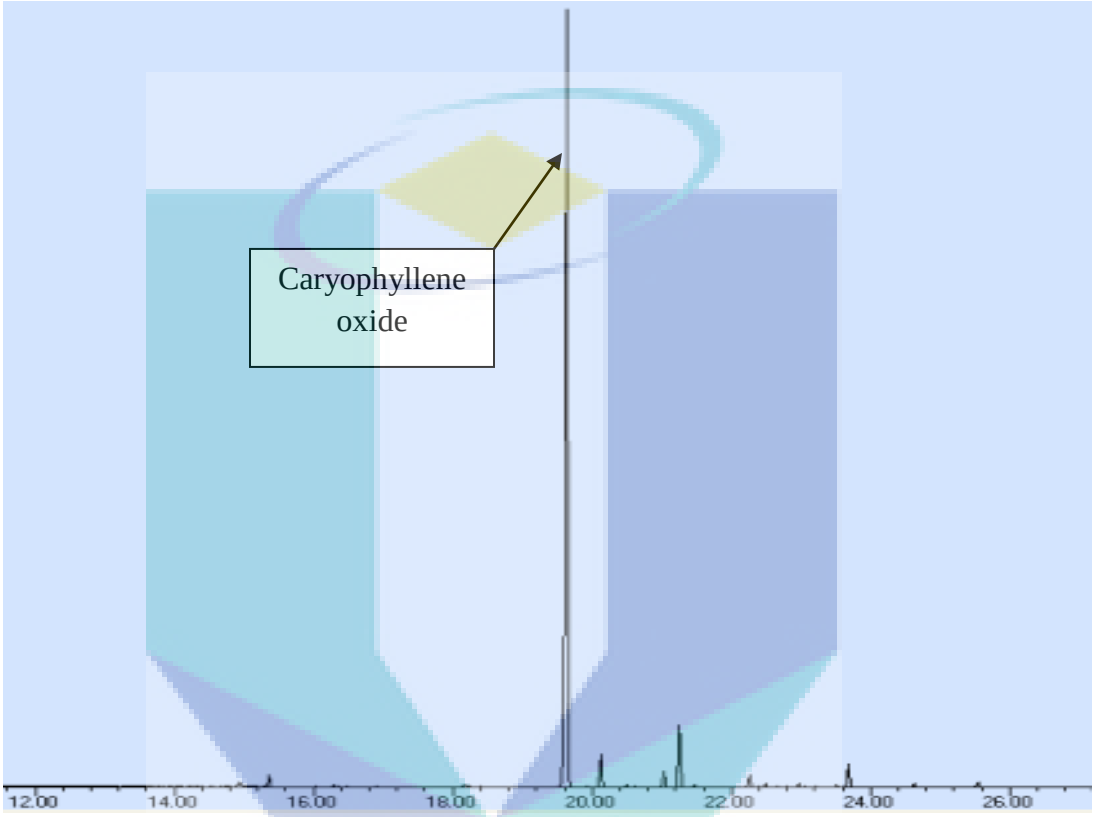
APPENDIXES E9

Chromatogram of caryophyllene oxide formed at 16 h at 45 °C reaction temperature same ratio (0.8, 1, 1.3 mol), pH 2.2, and C.O. solution temperature (55 °C).



APPENDIXES E10

Chromatogram of caryophyllene oxide formed at 20 h at 35 °C reaction temperature same ratio (0.8, 1, 1.3 mol), pH 2.2, and C.O. solution temperature (55 °C).



APPENDIXES F1

Experiment conditions at different variables, percentage in solution, crystal yield and conversion of caryophyllene oxide by using acetic acid, acetic anhydride, and 3-chloroperbenzoic acid.

ratio(mol)			reaction temperature (°C)	yield (g)	% of caryo phyllene oxide in solution by GCMS	% conversion
acid	caryo phyllene	hydrogen peroxide				
0.8	1	1.3	24	0.46	5.53	6.37
0.8	1	1.3	45	1.08	11.97	13.78
0.8	1	1.3	50	1.71	17.94	20.65
0.8	1	1.3	55	7.32	74.56	85.84

ratio(mol)			time (h)	yield (g)	% of caryo phyllene oxide in solution by GC/MS	% conversion
acid	caryo phyllene	hydrogen peroxide				
0.8	1	1.3	12	7.31	74.30	85.54
0.8	1	1.3	16	7.32	74.33	85.57
0.8	1	1.3	20	3.77	38.08	43.84
0.8	1	1.3	24	1.59	16.11	18.55

ratio(mol)			C.O. solution temperature (°C)	yield (g)	% of caryo phyllene oxide in solution by GC/MS	% conversion
acid	caryo phyllene	hydrogen peroxide				
0.8	1	1.2	55	3.44	35.69	41.09
0.8	1	1.3	55	7.49	75.13	86.50
0.8	1	1.4	55	1.49	15.85	18.25

ratio(mol)			reaction temperature (°C)	yield (g)	% of caryo phyllene oxide in solution by GC/MS	% conversion
acid	caryo phyllene	hydrogen peroxide				
0.8	1	1.3	24	3.51	36.73	42.29
0.8	1	1.3	45	7.71	77.02	88.67
0.8	1	1.3	50	6.76	71.01	81.75
0.8	1	1.3	55	5.28	53.96	62.12

ratio(mol)			time (h)	yield (g)	% of caryo phyllene oxide in solution by GC/MS	% conversion
acid	caryo phyllene	hydrogen peroxide				
0.8	1	1.3	12	6.43	65.26	75.13
0.8	1	1.3	16	7.53	75.77	87.23
0.8	1	1.3	20	6.21	63.35	72.93
0.8	1	1.3	24	5.52	56.64	65.21

Table 4.26: Percentage of caryophyllene oxide formed at 45 °C at different hydrogen peroxide molar ratio using acetic acid, time (16 h).

ratio(mol)			C.O solution temperature (°C)	yield (g)	% of caryo phyllene oxide in solution by GC/MS	% conversion
acid	caryo phyllene	hydrogen peroxide				
0.8	1	1.2	55	5.89	59.90	68.96
0.8	1	1.3	55	7.22	73.62	84.76
0.8	1	1.4	55	4.39	44.19	50.87

ratio(mol)			reaction temperature (°C)	yield (g)	% of caryo phyllene oxide in solution by GC/MS	% conversion
acid	caryo phyllene	hydrogen peroxide				
0.8	1	1.3	24	3.22	33.84	38.96
0.8	1	1.3	45	7.14	72.91	83.94
0.8	1	1.3	50	7.82	79.18	91.15
0.8	1	1.3	55	6.44	64.53	74.29

ratio(mol)			time (h)	yield (g)	% of caryo phyllene oxide in solution by GC/MS	% conversion
acid	caryo phyllene	hydrogen peroxide				
0.8	1	1.3	12	6.11	62.18	71.59
0.8	1	1.3	16	7.84	79.22	91.20
0.8	1	1.3	20	7.64	77.79	89.55
0.8	1	1.3	24	5.10	52.26	60.17

ratio(mol)			C.O. solution temperature (°C)	yield (g)	% of caryo phyllene oxide in solution by GC/MS	% conversion
acid	caryo phyllene	hydrogen peroxide				
0.8	1	1.1	55	6.73	68.83	79.24
0.8	1	1.2	55	7.99	81.47	93.79
0.8	1	1.3	55	7.61	77.73	89.49
0.8	1	1.4	55	7.60	76.89	88.52

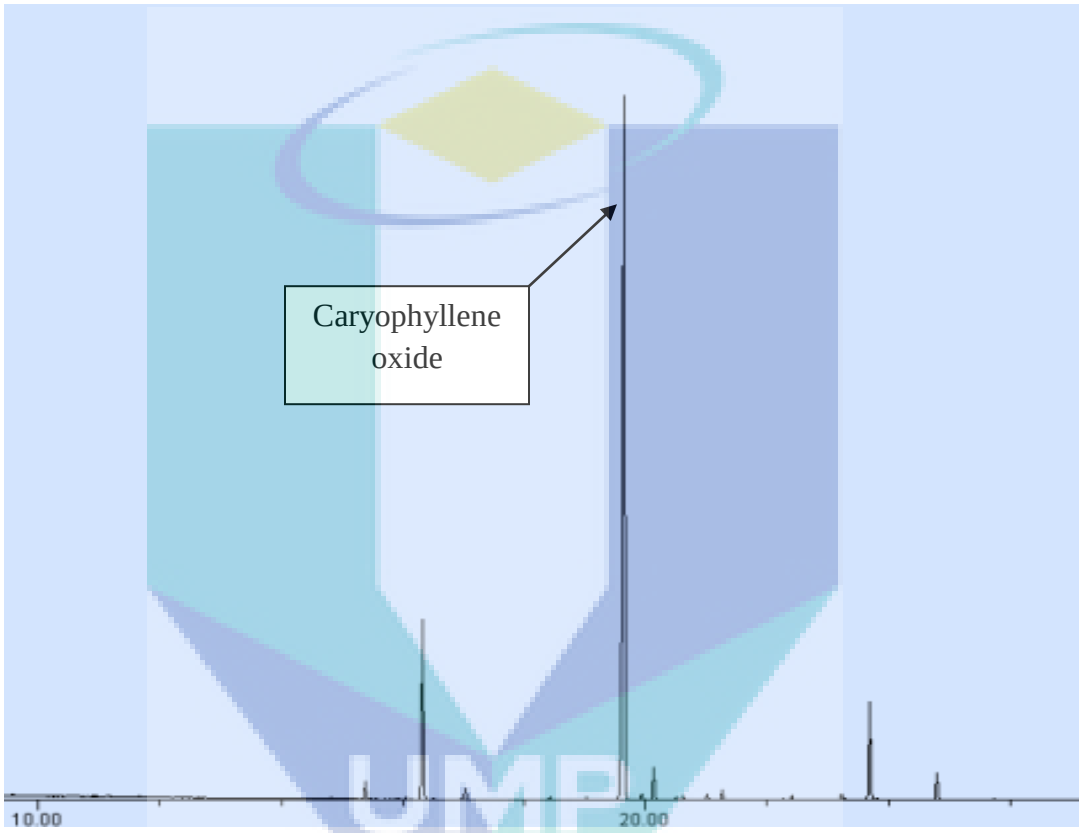
ratio(mol)			time (h)	yield (g)	% of caryo phyllene oxide in solution by GC/MS	% conversion
acid	caryo phyllene	hydrogen peroxide				
0.8	1	1.3	35	0.43	5.61	6.46
0.8	1	1.3	40	0.32	4.82	5.55
0.8	1	1.3	45	3.67	37.10	42.71

ratio(mol)			time (h)	yield (g)	% of caryo phyllene oxide in solution by GC/MS	% conversion
acid	caryo phyllene	hydrogen peroxide				
0.8	1	1.3	35	0.56	5.72	6.58
0.8	1	1.3	40	3.74	38.95	44.84
0.8	1	1.3	45	0.45	40.95	47.14

ratio(mol)			time (h)	Yield (g)	% of caryo phyllene oxide in solution by GC/MS	% conversion
acid	caryo phyllene	hydrogen peroxide				
0.8	1	1.3	35	4.63	47.91	55.16
0.8	1	1.3	40	5.56	55.83	64.28
0.8	1	1.3	45	5.97	60.49	69.64

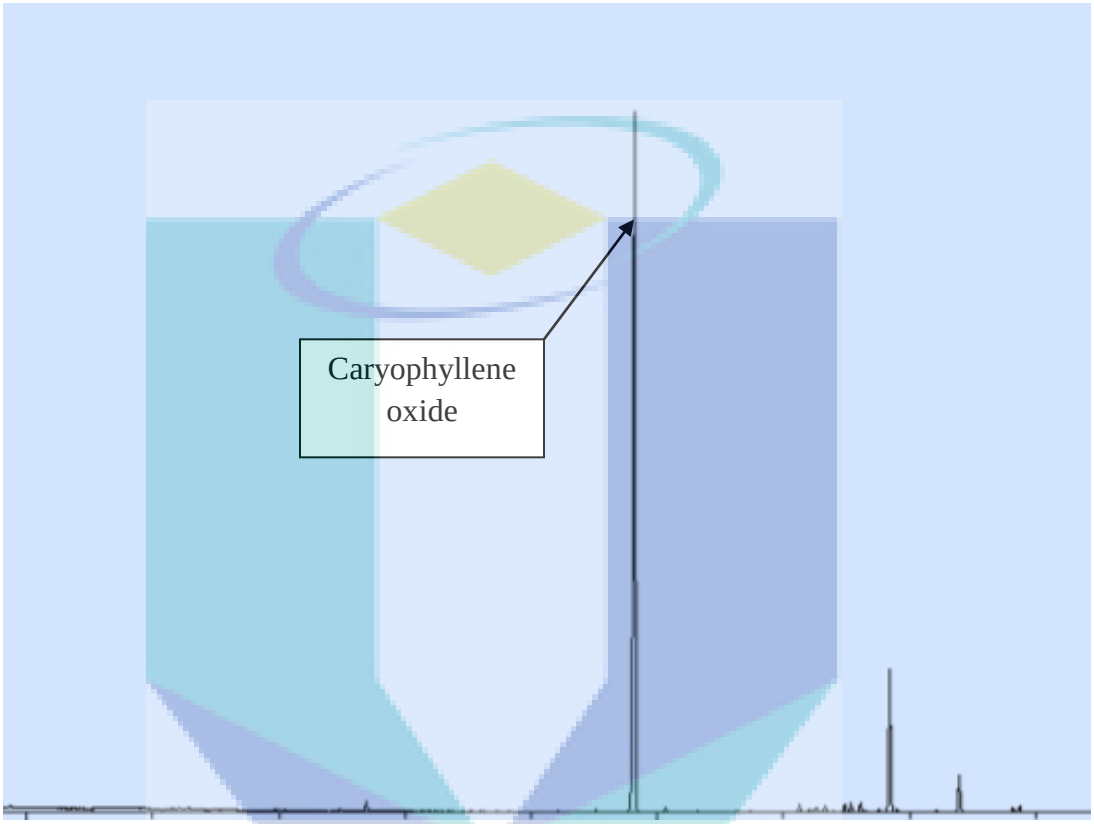
APPENDIXES F2

Chromatogram of caryophyllene oxide formed at 55 °C reaction temperature by using acetic acid, same ratio (0.8, 1, 1.3 mol), time (16h), and C. O solution temperature (55°C).



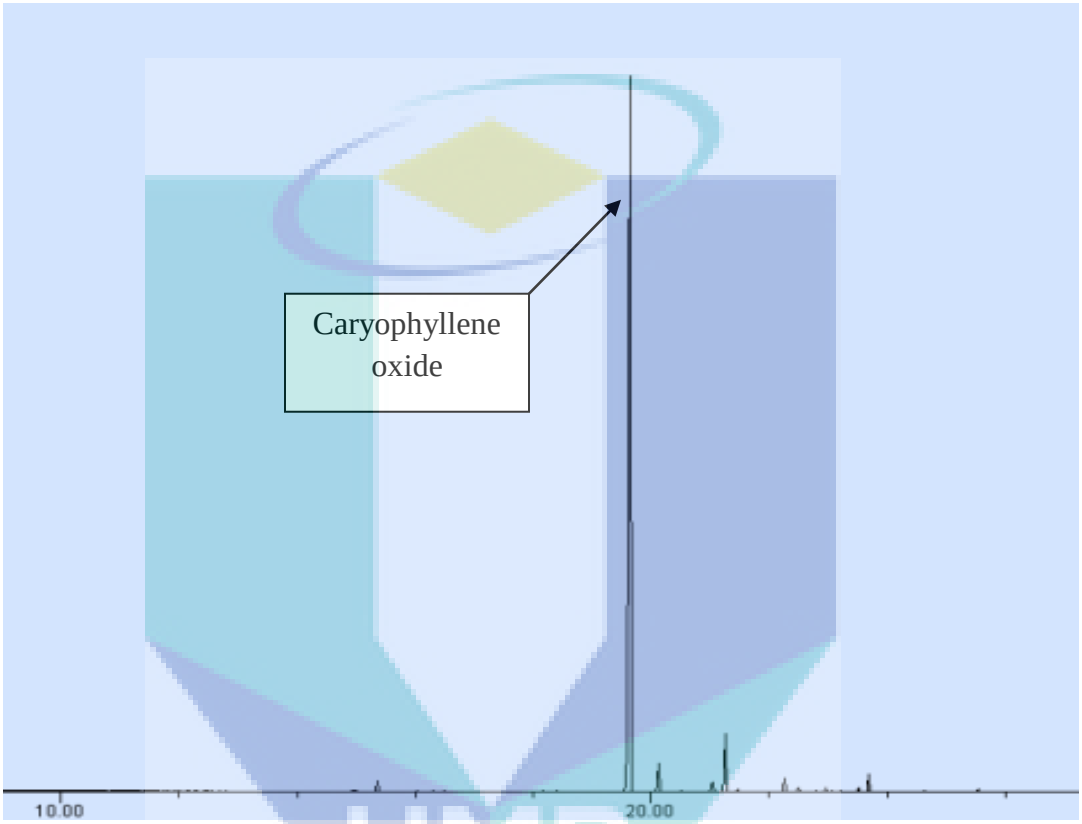
APPENDIXES F3

Chromatogram of caryophyllene oxide formed at 45° reaction temperature by using acetic anhydride, same ratio (0.8, 1, 1.3 mol), time (16h), and C.O solution temperature (55° C).



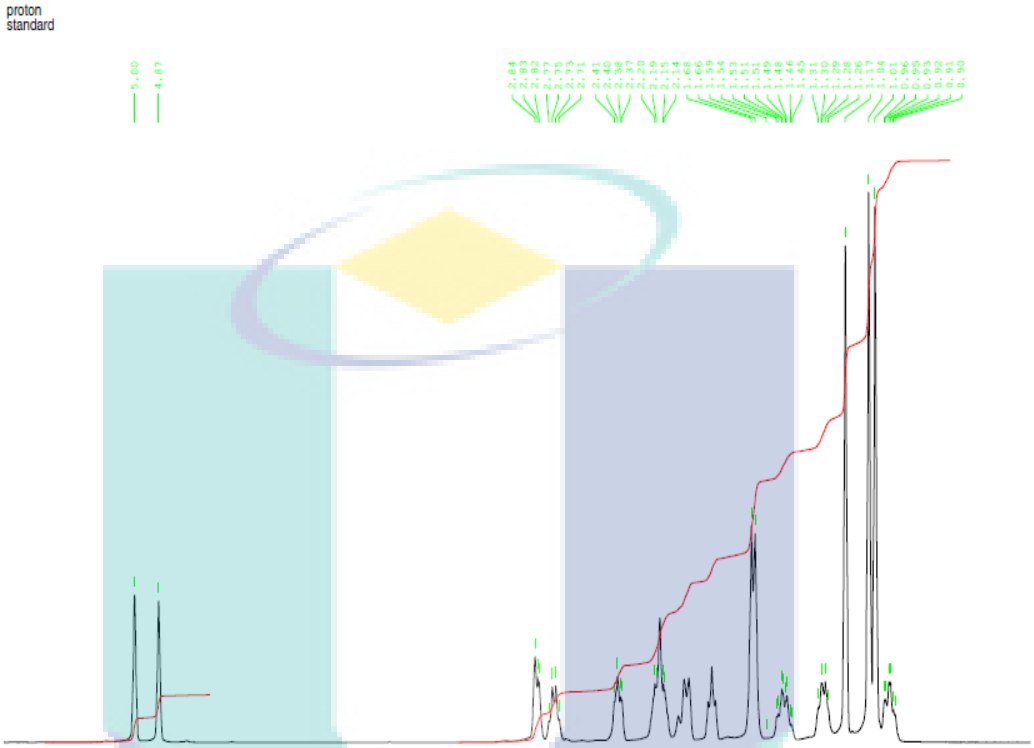
APPENDIXES F4

Chromatogram of caryophyllene oxide formed at 50 °C at 1.2 hydrogen peroxide molar ratio by using 3-chloroperbenzoic acid, time (16h), reaction temperature (55° C), and C.O. solution temperature (55° C).



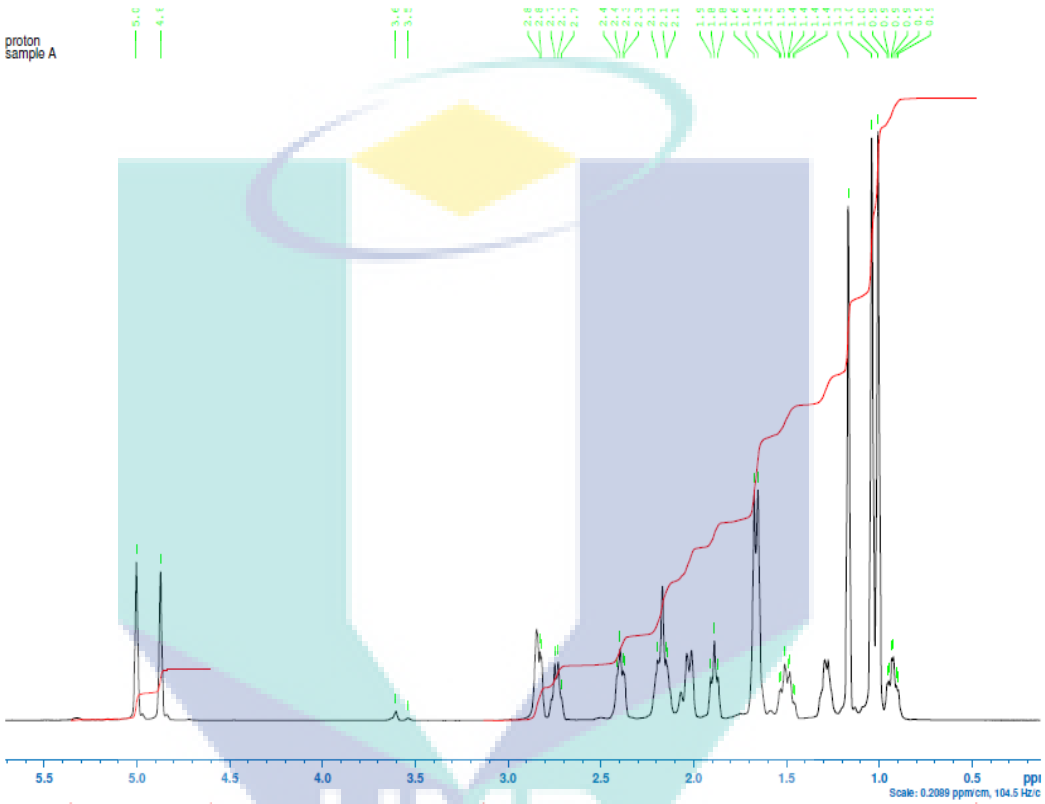
APPENDIXES G1

^{1}H NMR spectrum of pure standard caryophyllene oxide



APPENDIXES G2

^{1}HNMR proton spectrum of pure synthesised caryophyllene oxide



APPENDIXES H

Patent files

TRADEMARK2U
INTELLECTUAL PROPERTY

Ref : 2011/PT/TMI/PTA3.91/APP/0482/HNM
Date : 9th JUNE 2011

By Email, Fax: 09-549 2424
(Without enclosure) & post

UNIVERSITI MALAYSIA PAHANG
JABATAN PENYELIDIKAN DAN INOVASI,
UNIVERSITI MALAYSIA PAHANG,
LEBUHRAYA TUN RAZAK,
26300 KUANTAN, PAHANG DARUL MAKMUR,
MALAYSIA.

[Attn: Ms. Nor Ilma Mustafa Kamal]

Dear Madam/Sir,

Title	**: A METHOD FOR PRODUCING PURE CARYOPHYLLENE OXIDE**
Application Number	**: PI 2011002210**
Filing Date	**: 18 MAY 2011**
Country	**: MALAYSIA**
Applicant	**: UNIVERSITY MALAYSIA PAHANG**
Inventor	**: (1) ASSOC. PROF. BENJAMIN LUKAS**
	(2) RODIANAH BINTI ALIAS

We refer to the above matter.

Kindly find enclosed herewith copies of:

1. Certificate of Filing
2. Preliminary Examination- Clear Formalities Report
3. Patent Form 5 - Request for Substantive Examination

We shall attend to the necessary and keep you duly informed. Should you require further information, please do not hesitate to contact us.

Thank You.

Yours sincerely,

TRADEMARK2U SDN BHD

ROHANIM IBRAHIM
[PATENT DEPARTMENT]

I/We hereby acknowledge receipt of the above stated document(s)

.....
(Signature & Company stamp)

Name :
NRIC No :
Date :

TRADEMARK2U SDN BHD (670910-M)
Registered Trademark, Industrial Design, Patent Agent/Consultant
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Perbadanan Harta Intelek Malaysia
Intellectual Property Corporation of Malaysia
(Diperbadankan)
Unit 1-7 & Mezzanine, Unit 12, 12A, 13, 15, 16, 17, 18 dan 19
Tower B, Menara UOA Bangsar, No.5, Jalan Bangsar Utama 1,
59000 Kuala Lumpur
Tel : 603-2299 8400 Fax : 603-2299 8989 Website : www.myipo.gov.my

CERTIFICATE OF FILING

APPLICANT	:	UNIVERSITI MALAYSIA PAHANG
APPLICATION NO	:	PI 2011002210
REQUEST RECEIVED ON	:	18 MAY 2011
FILING DATE	:	18 MAY 2011
AGENT'S/APPLICANT'S FILE REF.	:	PTA3.91

Please find attached, a copy of the Request Form relating to the above application, with the filing date and application number marked thereon in accordance with Regulation 25(1).

Date : 01 JUNE 2011

(ABDUL RAHMAN RAMLI)
For Registrar of Patents
rahman@myipo.gov.my
03 – 22998814

To : YIP JIUN HANN
TRADEMARK2U SDN BHD.
WISMA MANJALARA, SUITE 2-8, 8TH FLOOR,
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52200 KUALA LUMPUR
MALAYSIA

Perbadanan Harta Intelek Malaysia
Intellectual Property Corporation of Malaysia
(Diperbadankan)
Unit 1-7 & Mezzanine, Unit 12, 12A, 13, 15, 16, 17, 18 dan 19
Tower B, Menara UOA Bangsar, No.5, Jalan Bangsar Utama 1,
59000 Kuala Lumpur
Tel : 603-2299 8400 Fax : 603-2299 8989 Website : www.myipo.gov.my

APPLICATION NO.	:	PI 2011002210
APPLICANT	:	UNIVERSITI MALAYSIA PAHANG
FILING DATE	:	18 MAY 2011
APPLICANT'S OR AGENT'S REF.	:	PTA3.91

PRELIMINARY EXAMINATION - CLEAR FORMALITIES REPORT

Please find attached a copy of the Examiner's clear report under Section 29 of the Patents Act.

A request for Substantive Examination should be made on Form 5 or a request for Modified Substantive Examination should be made on Form 5A, together with the appropriate prescribed fee, within 2 years from the filing date of the application, otherwise the application may be treated as withdrawn.

Date : 01 JUNE 2011

(ABDUL RAHMAN RAMLI)
For Registrar of Patents
✉rahman@myipo.gov.my
☎03 – 22998814

To : The Registrar of Patents

APPLICATION NO. : PI 2011002210

PRELIMINARY EXAMINATION - CLEAR FORMALITIES REPORT

I have examined the above application in accordance with Section 29(1) of the Patents Act and report that the application complies with the formal requirement of the Act.

Date : 01 JUNE 2011

(ABDUL RAHMAN RAMLI)
Formalities Examiner
rahman@myipo.gov.my
03 – 22998814

Patents Form No.1 PATENTS ACT 1983 REQUEST FOR GRANT OF PATENT [Regulation 7 (1)] To : The Registrar of Patents Patent Registration Office Kuala Lumpur Malaysia	**For Official Use** APPLICATION NO : Filing Date : Request received on: * Fee received on: Amount: * Cheque/Postal Order/Money Order/Draft/Cash No:
Please submit this Form in duplicate together with the prescribed fee.	Applicant's or Agent's file reference PTA3.91

THE APPLICANT (S) REQUEST (S) THE GRANT OF A PATENT IN RESPECT OF THE FOLLOWING PARTICULARS.

I. TITLE OF INVENTION : **A METHOD FOR PRODUCING PURE CARYOPHYLLENE OXIDE**

II. APPLICANT(S) (the data concerning each applicant must appear in this box or, if the space is insufficient in the space below):

Name : **UNIVERSITI MALAYSIA PAHANG**

I.C. / Passport No: -

Address : **JABATAN PENYELIDIKAN DAN INOVASI, UNIVERSITI MALAYSIA PAHANG, LEBUHRAYA TUN RAZAK, 26300 KUANTAN, PAHANG DARUL MAKMUR, MALAYSIA.**

Address for service in Malaysia : **WISMA MENJALARA BUSINESS AVENUE, SUITE 2-8, 8TH FLOOR, JALAN 7A/62A, BANDAR MENJALARA, 52200 KUALA LUMPUR, MALAYSIA.**

Nationality: **MALAYSIAN**

* Permanent residence or principal place of business:

JABATAN PENYELIDIKAN DAN INOVASI, UNIVERSITI MALAYSIA PAHANG, LEBUHRAYA TUN RAZAK, 26300 KUANTAN, PAHANG DARUL MAKMUR, MALAYSIA.

Telephone Number (if any)	Fax Number (if any)
03 – 6274 5352	03 – 6274 4795

Additional Information (if any):

Diterima 18 MAY 2011

2011002210

III. INVENTOR

Applicant is the inventor: YES [ ] NO [X]

If the applicant is not the inventor:

Name of inventor: PLEASE REFER TO ATTACHMENT 1

Address of inventor: PLEASE REFER TO ATTACHMENT 1

A statement justifying the applicant's right to the patent accompanies this Form:

YES [X] NO [ ]

Additional Information (if any):

IV. AGENT OR REPRESENTATIVE

Applicant has appointed a patent agent in accompanying Form No. 17: YES [X]

NO [ ]

Agent's Registration No: PA/2004/0128

Applicants have appointed YIP JIUN HANN (TRADEMARK2U SDN BHD) to be their common representative.

V. DIVISIONAL APPLICATION [ ]

This application is a divisional application

The benefit of the filing date [ ] priority date [ ]

of the initial application is claimed in as much as the subject-matter of the present application is contained in the initial application identified below :

Initial Application No :

Date of filing of initial application:

Diterima
18 MAY 2018

VI DISCLOSURES TO BE DISREGARDED FOR PRIOR ART PURPOSES

Additional Information is contained in supplemental box:

(a) Disclosure was due to acts of applicant or his predecessor in title ☐

Date of disclosure :

(b) Disclosure was due to abuse of rights of applicant or his predecessor in title ☐

Date of disclosure:

A statement specifying in more detail the facts concerning the disclosure accompanies this Form: YES ☐ NO ☐

Additional Information (if any):

VII PRIORITY CLAIM (If any)

The priority of an earlier application is claimed as follows:

Country (if the earlier application is a regional or international application, indicate the office with which it is filed):

.....

Filing Date: .....

Application No: .....

Symbol of the International Patent Classification:

If not yet allocated, please tick ☐

The priority of more than one earlier application is claimed:

YES ☐ NO ☐

The certified copy of the earlier application (s) accompanies this Form:

YES ☐ NO ☐

If No, it will be furnished by ..... (date)

Additional Information (if any)

3

Diterima 18 MAY 2011

VIII. CHECK LIST

A. This application contains the following:

1. request		
2. description	7	sheets
3. claim	2	sheets
4. abstract	1	sheets
5. drawings	2	sheets
TOTAL	12	sheets

B. This Form, as filed, is accompanied by the items checked below (please tick where appropriate):

(a) signed Form No. 17	X
(b) declaration that inventor does not wish to be named in the patent	
(c) statement justifying applicant's right to the patent	X
(d) statement that certain disclosures be disregarded	
(e) priority document (certified copy of earlier application)	
(f) cash, cheque, money order, banker's draft or postal order for the payment of application fee	X
(g) other documents (specify)	

IX. SIGNATURE

.....

** (Applicant / Agent)

18 MAY 2011

(Date)

If Agent, indicate Agent's Registration No : PA/2004/0128

For Official Use

1. Date application received: .....
2. Date of receipt of correction, later filed papers or drawings completing the application: .....

* Delete where inapplicable.
** Type name under signature and delete where inapplicable

Dilerima 18 MAY 2011

ATTACHMENT 1

The following are the list of inventors of the invention:-

1. Name : Assoc. Prof. Benjamin Lukas

   Address : Universiti Malaysia Pahang,
   Jabatan Penyelidikan dan Inovasi,
   Universiti Malaysia Pahang,
   Lebuhraya Tun Razak,
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   Jabatan Penyelidikan dan Inovasi,
   Universiti Malaysia Pahang,
   Lebuhraya Tun Razak,
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