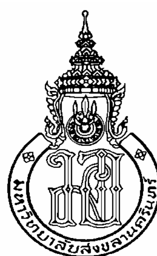

Chemical Determinations, Antimicrobial and Antioxidant Activities of Thai Wood Vinegars

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Pharmacy in Pharmaceutical Sciences

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ชื่อวิทยานิพนธ์	การศึกษาคุณสมบัติทางเคมี ฤทธิ์ต้านจุลินทรีย์ และฤทธิ์ต้านอนุมูลอิสระของน้ำส้มควันไม้ไทย
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บทคัดย่อ

น้ำส้มควันไม้เป็นของเหลวสีน้ำตาล มีกลิ่นควันไฟและมีคุณสมบัติเป็นกรด ซึ่งเป็นผลพลอยได้จากกระบวนการผลิตถ่าน น้ำส้มควันไม้เป็นที่รู้จักและถูกนำมาใช้ประโยชน์อย่างกว้างขวางในด้านการเกษตร ใช้เป็นส่วนผสมในผลิตภัณฑ์สุขภาพ และใช้ในการรักษาเนื้อไม้ ในประเทศไทยนอกจากการนำน้ำส้มควันไม้มาใช้ในด้านการเกษตรยังได้มีการนำไปใช้ในการรักษาโรคผิวหนังที่มีการติดเชื้อและรักษารังแค แต่ยังไม่มีงานวิจัยรับรองประสิทธิภาพดังกล่าว ดังนั้นงานวิจัยนี้จึงมีวัตถุประสงค์ที่จะศึกษาองค์ประกอบทางเคมีและฤทธิ์ทางชีวภาพของน้ำส้มควันไม้ที่มีการผลิตในประเทศไทย

ในการศึกษานี้ใช้ตัวอย่างน้ำส้มควันไม้ที่ผลิตจาก ไม้ไผ่ ไม้กระถิน ไม้ยูคาลิปตัส และไม้ยางพารามาใช้ในการทดสอบ การศึกษาองค์ประกอบทางเคมีของน้ำส้มควันไม้โดยเทคนิค คาร์ลฟิชเชอร์ ไตเตรชั่น พบว่าตัวอย่างน้ำส้มควันไม้มีปริมาณน้ำอยู่ในช่วง 78-88% v/v และเมื่อนำตัวอย่างไปศึกษาด้วยเทคนิค แก๊สโครมาโทกราฟี – แมสสเปกโตรเมททรี พบว่าองค์ประกอบหลักของน้ำส้มควันไม้เป็นกรดอินทรีย์ เช่น กรดอะซิติก กรดโพรพาโนอิก และกรดบิวทาโนอิก เป็นต้น และสารประกอบฟีนอล เช่น ฟีนอล และครีซอล เป็นต้น ซึ่งชนิดและปริมาณจะแตกต่างกันไปตามชนิดของไม้ และเมื่อวิเคราะห์หาปริมาณของสารประกอบฟีนอลโดยเทคนิคฟอลลิน – ซีโอคาลทู พบว่าตัวอย่างที่นำมาศึกษามีปริมาณฟีนอลอยู่ในช่วง 0.9-9.15 ปริมาณสมมูลย์กับกรดแกลลิค mg/g WV ตัวอย่างที่ผ่านกระบวนการไลโอฟิลไลซ์ พบว่า องค์ประกอบทางเคมีของน้ำส้มควันไม้เข้มข้นขึ้นแต่กรดอินทรีย์ส่วนใหญ่ก็จะถูกกำจัดออกไปด้วย ในการสกัดน้ำส้มควันไม้โดยใช้ตัวทำละลายอินทรีย์ ได้แก่ ไดคลอโรมีเทน ไดเอทิลอีเทอร์ และไอโซบิวทานอล พบว่าน้ำส้มควันไม้ที่สกัดด้วยไดคลอโรมีเทนและไดเอทิลอีเทอร์ สารสกัดจะมีปริมาณของกรดอินทรีย์และสารประกอบฟีนอลที่สูงกว่าการไลโอฟิลไลซ์ ในขณะที่ไอโซบิวทานอล สกัดได้ทั้งกรดอินทรีย์และฟีนอลน้อยกว่า ในการทดสอบฤทธิ์ต้านจุลินทรีย์โดยวิธีการซึมผ่านดิสต์ และการหาความเข้มข้นต่ำสุดที่ใช้ยับยั้งการเจริญของจุลินทรีย์ โดยเชื้อแบคทีเรียที่ใช้ทดสอบได้แก่ *Staphylococcus aureus, Staphylococcus*

epidermidis และ *Propionibacterium acne*s ส่วนเชื้อราที่ใช้ทดสอบได้แก่ *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Microsporum gypseum* และ *Candida albicans* ซึ่งเป็นเชื้อแบคทีเรียและเชื้อราที่เป็นสาเหตุของโรคผิวหนัง นอกจากนี้ก็ทำการทดสอบฤทธิ์ต้านเชื้อแบคทีเรียก่อโรคในระบบทางเดินอาหารด้วย ได้แก่ *Streptococcus faecalis* และ *Escherichia coli* ซึ่งพบว่าตัวอย่างน้ำส้มควันไม้ทุกตัวอย่างมีฤทธิ์ในการต้านเชื้อแบคทีเรียและเชื้อราทุกชนิดที่นำมาทดสอบ หลังจากการไลโอฟิลไลซ์พบว่า น้ำส้มควันไม้มีฤทธิ์เพิ่มขึ้น โดยพบว่าฤทธิ์การต้านจุลินทรีย์จะเพิ่มมากขึ้นในตัวอย่างน้ำส้มควันไม้ที่สกัดด้วยไดคลอโรมีเทนและไดเอทิลอีเทอร์ น้ำส้มควันไม้ไผ่ที่สกัดด้วยไดเอทิลอีเทอร์มีฤทธิ์ในการต้านเชื้อแบคทีเรียดีที่สุดโดยมีค่า MIC ต่ำสุดที่ 31.3 μg/ml ต่อเชื้อ *S. epidermidis* และน้ำส้มควันไม้ไผ่ที่สกัดด้วยไดคลอโรมีเทนมีฤทธิ์ต้านเชื้อราดีที่สุดโดยมีค่า MIC ต่ำสุด 62.5 μg/ml ต่อเชื้อ *T. rubrum* ส่วนฤทธิ์ต้านออกซิเดชันของน้ำส้มควันไม้ที่ทำการทดสอบโดยใช้ 3 วิธีคือ 1, 1-diphenyl-2-picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Power (FRAP) และ Trolox Equivalent Antioxidant Capacity (TEAC) โดยมี Butylated hydroxytoluene (BHT) เป็นสารต้านออกซิเดชันมาตรฐานเปรียบเทียบ ซึ่งการทดสอบทั้ง 3 วิธีให้ผลการทดสอบไปในแนวทางเดียวกัน พบว่าน้ำส้มควันไม้ทุกตัวอย่างมีฤทธิ์ต้านออกซิเดชัน โดยน้ำส้มควันไม้ที่สกัดด้วยไดคลอโรมีเทนมีฤทธิ์ต้านออกซิเดชันสูงที่สุดเมื่อเทียบกับน้ำส้มควันไม้อื่นๆ ไม่ว่าจะทดสอบด้วยวิธีใดๆ

จากผลการทดลองแสดงให้เห็นว่าน้ำส้มควันไม้ไทยทั้งหมดที่นำมาทดสอบมีฤทธิ์ต้านเชื้อแบคทีเรียและเชื้อราที่ก่อโรคผิวหนัง รวมทั้งมีฤทธิ์ต้านเชื้อแบคทีเรียที่ก่อโรคระบบทางเดินอาหารด้วย นอกจากนี้ยังมีฤทธิ์ต้านออกซิเดชัน ซึ่งฤทธิ์ต้านออกซิเดชันมีบทบาทในการต้านการอักเสบจากบาดแผลหรือร่องรอยจากการติดเชื้อ ดังนั้นน้ำส้มควันไม้ที่นำมาทดสอบจึงมีศักยภาพที่จะนำไปใช้เป็นสารต้านเชื้อแบคทีเรียและเชื้อราได้ และสามารถนำไปพัฒนาเพื่อใช้ในการรักษาโรคผิวหนังจากการติดเชื้อได้ ซึ่งการสกัดด้วยไดคลอโรมีเทนเป็นวิธีที่สามารถเพิ่มประสิทธิภาพของน้ำส้มควันไม้ได้ดีที่สุด ในการที่จะนำไปพัฒนาต่อเพื่อใช้เป็นผลิตภัณฑ์

Thesis Title	Chemical Determinations, Antimicrobial and Antioxidant Activities of Thai Wood Vinegars
Author	Miss Jaruporn Rakmai
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ABTRACT

Wood vinegar is a brown condensed acidic liquid with smoky odor which is the by-product in the process of producing wood charcoal. It has been widely used in agriculture, in health promotion products and as wood preservative. In Thailand, wood vinegar was not only utilized in agriculture, it was also used to treat skin infection and dandruff, however, none of any scientific report published to support this application. Thus, the aims of this research were to evaluate the chemical components and bio-efficacies of wood vinegars produced in Thailand.

In this research four types of wood vinegars produced from bamboo, white popinac, eucalyptus and rubber wood were utilized. Karl Fischer titration was used to determine water content in wood vinegar samples. The results showed that wood vinegar samples contained water in the range of 78-88% v/v. Gas chromatography-mass spectrometry results demonstrated that the main chemical components were organic acids such as acetic acid, propanoic acid and butanoic acid, etc. and phenolic components such as phenol and cresol, etc., which types and amount were varied depending on wood species. Folin–Ciocalteu method was used to determine total phenolic content and found that wood vinegar samples contained phenolic content in the range of 0.9-9.15 mg of gallic acid equivalents/g WV. Total amounts of chemical compositions in lyophilized wood vinegars were concentrated, however, the main organic acids lost during the process. Wood vinegars were then extracted by organic solvents, dichloromethane, diethyl ether or isobutanol. Dichloromethane and diethyl ether extracts could preserve the main chemical components better than lyophilized products. Moreover, isobutanol was not a good extraction solvent since less phenolic and organic acids were not well dissolved in this solvent. The antimicrobial activity was

determined by the disc diffusion and the minimal inhibitory concentration methods. The tested dermatitis bacterial species were Staphylococcus aureus, Staphylococcus epidermidis, and *Propionibacterium acne*s; and dermatitis fungal species were *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Microsporum gypseum* and *Candida albicans*. *Streptococcus faecalis* and *Escherichia coli*, the gastrointestinal tract disorder bacterial species, were also teseted. All types of wood vinegar gave antibacterial and antifungal effects. Lyophilized wood vinegars showed higher antibacterial and antifungal activities. Strong antimicrobial activities presented in dichloromethane and diethyl ether wood vinegar extracts. The strongest antibacterial activity could be observed in bamboo wood vinegar extracted by diethyl ether with the lowest MIC value of 31.3 μg/ml against *S. epidermidis*. The highest antifungal property could be found in bamboo wood vinegar extracted by dichloromethane with the lowest MIC value of 62.5 μg/ml against *T. rubrum*. Antioxidant activities were tested by three different methods; 1, 1-diphenyl-2-picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Power (FRAP) and Trolox Equivalent Antioxidant Capacity (TEAC). Butylated hydroxytoluene (BHT) was used as the positive standard for comparison. These three methods were found to give similar antioxidant activity values. All wood vinegar samples gave antioxidant activity. Wood vinegar extracted by dichloromethane presented strong antioxidant activity compared to the other wood vinegars.

The results from this study indicated that all tested Thai wood vinegar samples showed antibacterial and antifungal activities against dermatitis bacteria and fungi and bacteria that cause gastrointestinal tract disorder. In addition, they presented antioxidant activity. Since, antioxidant agent may play an important role in anti-inflammatory caused by wounds or skin diseases. Therefore, wood vinegars could be applied as antibacterial and antifungal agents and presented the potential for using as skin diseases treatment caused by dermatitis bacteria and fungi. The results suggested that dichloromethane was a suitable solvent for wood vinegar extraction which could increase efficiency of the original wood vinegars. The extract will be potentially developed in product formulation in the future.

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LIST OF ABBREVIATIONS AND SYMBOLS

A	=	Absorbance
ABTS	=	2, 2’-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)
Amu	=	Atomic mass unit
BHI	=	Brain Heart Infusion
BHT	=	Butylated Hydroxyl Toluene
CFU	=	Colony Forming Unit
cm	=	Centimeter
°C	=	Degree Celsius
DPPH	=	1, 1-Diphenyl-2-picrylhydrazyl
EI	=	Electron Ionization
EQ	=	Equivalent
FRAP	=	Ferric Reducing Antioxidant Power
g	=	Gram
GC-MS	=	Gas Chromatography coupled Mass Spectrometry
HP	=	Hewlett Packard
hr	=	Hour
I	=	Inhibition
IC_{50}	=	Half Maximal Inhibitory Concentration
KFT	=	Karl Fischer titration
LL	=	Lyophilized
LL-WV	=	Lyophilized wood vineagar
µg	=	Microgram
µg/disc	=	Microgram per disc
µg/ml	=	Microgram per milliliter
µl	=	Microliter
µm	=	Micrometer
m-	=	Meta
M	=	Molarity

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

MBC	=	Minimal Bactericidal Concentration
MFC	=	Minimal Fungicidal Concentration
mg	=	Milligram
mg/ml	=	Milligram per milliliter
MHA	=	Müller-Hinton Aar
MIC	=	Minimal Inhibitory Concentration
min	=	Minute
ml	=	Milliliter
ml/min	=	Milliliter per minute
mm	=	Millimeter
mM	=	Millimolar
N	=	Normality
NIST	=	National Institute of Standards and Technology
nm	=	Nanometer
o-	=	Ortho
OH	=	Hydroxyl
p-	=	Para
PA	=	Pascal
%	=	Percent
% v/v	=	Percent volume by volume
% w/w	=	Percent weight by weight
R^2	=	Coefficient of Determination
RT	=	Room temperature
S.D.	=	Standard deviation
SDA	=	Sabouraud Dextrose Agar
SDB	=	Sabouraud Dextrose Broth
TE	=	Trolox Equivalent
TEAC	=	Trolox Equivalent Antioxidant Capacity

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

TISTR	=	Thailand Institute of Scientific and Technological Research
TPTZ	=	2, 4, 6-Tris(2-pyridyl)-1, 3, 5-Triazine
TSA	=	Tryptic Soy Agar
TSB	=	Tryptic Soy Broth
V	=	Volt
WV	=	Wood Vinegar

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Wood vinegar is a condensed acidic liquid obtained in the process of producing wood charcoal. It has a special smoky odor and its color is light-yellow to brown. There are over 200 kinds of chemical components involved and these components mainly include acetic acid, formaldehyde, methanol, acetone and tar compounds (Mu et al., 2003; Kadota and Niimi, 2004).

They have been used in the traditional remedy of the Japanese over 400 years. It has very complex and numerous chemical constituents. Depending on the process conditions and type of the starting wood material, wood vinegars may contain fermentable sugars, furan compounds, various phenolic compounds, organic acids, etc. Phenolic compounds are generally formed by degradation of lignin and wood sugars while acetic acids as organic acid are believed to originate from acetyl groups in the hemicelluloses. Apart from wood vinegar, tar and oil, gases, and wood alcohols are typical products of wood carbonization (Fengel and Wegener, 1984).

Wood vinegar is a good source for organic production in agriculture (Mu et al., 2003). It has been widely used in agriculture and daily life in Japan. There are about 4×10^7 L produced every year and over half of it is being used in agriculture (Higashino et al., 2005).

In agriculture, wood vinegar can be used as plant growth acceleration agent. It was found to have an effect on improving soil quality against plant diseases and insect pests, promoting plant growth and decreasing the necessity for fertilizer utilization as well as improving the quality of agriculture plants without any toxicity to people and animals (Ikeshima, 1999). Beside, wood vinegar can retard the growth of phytopathogenic fungi, such as *Fusarium*, *Pythium* and *Rhizoctonia* (Yagi and Tsukamoto, 1991), and promote the growth of plant roots (Tsuzuki et al., 1989; Kadota et al., 2002).

The mixture of charcoal and wood vinegar was used in plant cultivation to improve growth and yield (Katoda et al., 2004). It was used to improve quantity and quality of some agricultural products such as rice (*Oriza sativa*) (Tsuzuki et al., 1989), sweet potato (*Ipomoea*

batatas) (Du et al., 1998), sugar cane (*Saccharum officinarum*) (Uddin et al., 1995[a,b]) and melon (*Cucumis melo*) (Du et al., 1997). Moreover, wood vinegar was found to promote the sweet taste of some fruits (จิรพงษ์ คูหากาญจน์, 2548).

Wood vinegar can be used as smell and bugs controlling agent in animal farm (http://www.ata.or.th), deodorant (Kasai, 2001), prebiotics (Watarai et al., 2008; Tana et al., 2003), detoxificant (http://www.kenrico.com/sapsheet.html), smoke flavoring agent in food processing (Miyakawa et al., 2003), and promote coagulating agent for natural rubber sheet production (ยอดธง ใบมาก, 2549; Ferreira et al., 2005). It was also found to have termidicidal activity (Yatagai et al., 2002), antifungal property against fungi that cause wood decay such as *Trametes versicolor* and *Tyromyces palustris* (Nakai et al., 2005) and anti plant pathogenic microorganisms for example *Ralstonia solanacearum*, *Phytophthora capsici*, *Fusarium oxysporum*, and *Pythium splendens* (Hwang et al., 2005).

Wood vinegar can be useful for soil improvement, vermin extermination, and deodorant (Kartal et al., 2004). The complex chemical compositions of wood vinegar from wood carbonization process might be expected to protect the wood from fungal and termite attack. Previous studies by Sameshima and co-workers (2002) and Yatagai's team (2002) showed the correlated results on termiticidal activity of wood vinegars and concluded that the termiticidal property of wood vinegars may cause by phenolic and acetic acid content from charcoal production.

Wood vinegar has long traditionally been used as insect repellant, deodorizer, antibacterial agent, sterilizer, alkali bath, and food additive. It was approved by US FDA to use as smoke flavoring agent in food production and cosmetic additives. It has been used to promote digestion of animals when mixed in animal food. Ikegami and co-workers (1998) found that Japanese commercially available wood vinegar under trade name " Mokusaku-eki" obtained from *Quercus* spp. have anti-dermatophyte bacteria that cause eczema.

There are a number of wood vinegars available in Thailand. Most of those wood vinegars were obtained from wood grown in Thailand. They were usually utilized in agricultural process, by diluting 1 to 100 parts of water to treat skin infection and dandruff. However, none of any

scientific report was published to support this application. Thus, the aim of this research was to evaluate the chemical components and bio-efficacies of wood vinegars produced in Thailand.

In this study four types of wood vinegars produced in Thailand were utilized. Wood vinegars obtained from bamboo (*Bambusa* spp.), white popinac (*Leucaena leucocephala*), eucalyptus (*Eucalyptus* spp.) and rubber (*Hevea brasiliensis*) were investigated for their chemical components and pharmacological properties.

Gas chromatography-Mass spectroscopy (GC-MS), Karl Fisher Titration and Folin–Ciocalteu method were used for determine chemical components of wood vinegars. Their pharmacological properties were determined for antibacterial, anti-fungal and antioxidant activities.

Wood vinegar samples were concentrated by lyophilization, and extracted by organic solvents and further used for chemical composite and bio-efficacy studies.

1.2 Objectives

The objectives of this study are:

1. To determine chemical composition of Thai wood vinegars
2. To investigate antibacterial and antifungal activity of Thai wood vinegars
3. To investigate antioxidant activity of Thai wood vinegars
4. To evaluate bio-efficacies of Thai wood vinegars for use as antibacterial and antifungal agents as well as study potential utilization for skin diseases treatment caused by dermatitis bacteria and fungi.

CHAPTER 2

REVIEW OF LITERATURES

2.1 Definition of wood vinegars

Wood vinegar is a condensed acidic liquid obtained in the process of producing wood charcoal. It has a special smoky odor and its color is light-yellow to brown. There are over 200 kinds of chemical components involved and these components mainly include acetic acid, formaldehyde, methanol, acetone and tar compounds (Mu et al., 2003; Kadota and Niimi, 2004).

2.2 Production of wood vinegars

Wood vinegar is produced during carbonization process of wood charcoal using the instrument as displayed in diagram shown in Figure 2-1. At the beginning of the process, dry woods were placed in the furnace, closed and heated to 300 – 600 °C. During burning process, the smoke from the burning wood flows through a condenser, condenses to liquid and drops into a container. The liquid obtained at the temperature less than 300 °C was discarded. Once the temperature rises to 300-600°C, distillate was collected.

To obtain wood vinegar, the distillate was stored in a container around 90 days. The distillate will separate into three layers, which are light oil on the top, wood vinegar in the middle and tar at the bottom. Then the middle layer was collected and filtered through a filter cloth or filter paper to remove unwanted materials, resulting in raw wood vinegar. Wood vinegar should be clear, light yellow to brown liquid. Purified wood vinegars could be obtained by distillation to separate required compounds especially for pharmaceutical industry. However, distillation needs to be performed after sedimentation. The overall carbonization process gave charcoal in 20-32% and collected distillate in 30% compare to the weight of the fresh wood. This process needs 1400 kilocalories per kilogram of fresh wood.

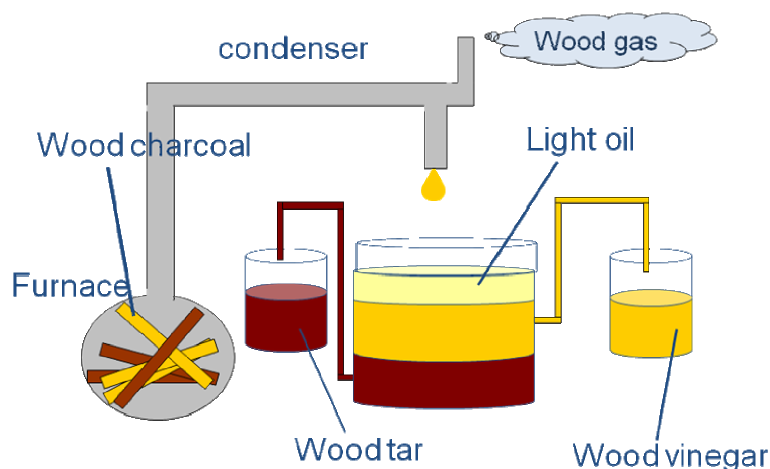


Figure 2-1 Instrument of producing wood vinegars (นิคม แหลมศักดิ์, 2549)

2.3 Components and specifications of wood vinegars

Raw wood vinegar composed of more than 200 chemicals derived from cellulose, hemi-cellulose and lignin carbonization. The main components of wood vinegar are water (85%), organic acids (3%) and organic substances (12%) such as alcohols, neutral substances, phenols and basic substances. The quality of wood vinegar was specified by several criterias such as pH should be about 3.0, specific gravity should be in a range of 1.005-1.050, color should be pale yellow, bright brown or reddish brown, odor should be smoky odor, dissolved tar content should be less than 3%, loss on drying residue should be less than 0.2% by weight and it should be transparency (นิคม แหลมศักดิ์, 2549).

Nakai and co-workers (2005) characterized the chemical components in the wood vinegars obtained by carbonization using solid wood or wood-based composites such as particleboard, plywood and medium density fiberboard (MDF) with phenol or urea type adhesive as the wood materials. The plywood was manufactured by using red meranti (*Shorea* sp.). Particleboards made from mixed species of hardwood and softwood, and MDF comprised of mixed tropical hardwood as raw material. Solid woods were used as reference. They found that the chemical compositions of the obtained wood vinegars were greatly different depend on the type of wood materials. Type of adhesive also contributed to the differences in chemical compositions. When plywood or particleboard bonded with urea-type adhesive was pyrolyzed, the characteristic components such as acetamide or pyrrole were identified in the liquid, and these were assumed to be derived from the glue adhesive. A large number of these nitrogenous compounds possibly due to the thermal

degradation of the adhesive were detected in wood vinegar liquid at the relatively low heating temperature zone of RT – 300 °C. When phenol-type adhesive-bonded plywood and particleboard were pyrolyzed, the yield of aromatic compounds including various types of phenol compounds increased along with the rise in heating temperature, and the maximum yield was obtained at the range of 400 – 500 °C.

The determination of water content of the pyrolysis liquids could be performed by Karl Fischer titration method. It has been found that the water content of the liquids obtained from plywood, particleboard and MDF with urea type adhesive composites slightly increased as the temperature rose. However, the water content of the pyrolysis liquids obtained from plywood and particleboard with phenol type adhesive composites as well as meranti solid wood did not depend on the temperature in carbonization process. Water content of the distillate collected at RT – 300 °C was around 73 – 80 %. When the temperature rose to 300 – 400 °C, water content of the collected distillate was decrease to 56-57 %. At the higher temperature (400 – 500 °C), water content of pyrolysis liquids obtained from solid wood and particle board raised to 80 % and 86 % respectively. While, water content of pyrolysis liquid from plywood with phenol type adhesive was only 65 %, greatly decreased from distillated collected at RT-300 about 15 %.

Physical and chemical properties of the wood vinegar were also reported by Kartal and co - workers (2004). Contents of wood vinegars showed variability depending on wood species and process conditions. In this study, sugi and acacia woods were utilized. In general, when temperature during carbonization process increased, concentrations of phenolic compounds in the wood vinegar increased. The result demonstrated that acacia wood gave higher concentration of phenolic compounds in the wood vinegar compared to those obtained from sugi wood. Therefore, the differences in the concentration of phenolic compounds were due to both wood species and process temperatures. In contrast to phenolic compounds, concentration of organic acid in the wood vinegar from sugi wood decreased with increased in carbonization temperature. Lignocellulosic materials like wood are mainly composed of cellulose, hemicellulose, and lignin. Lignin composed of phenylpropane units (*p*-Coumaryl alcohol, Coniferyl alcohol, and Sinapyl alcohol) linked into a three dimensional structure through a variety of different chemical bonds such as ring-side chain, ring-ring and side chain-side chain (Figure 2-2).

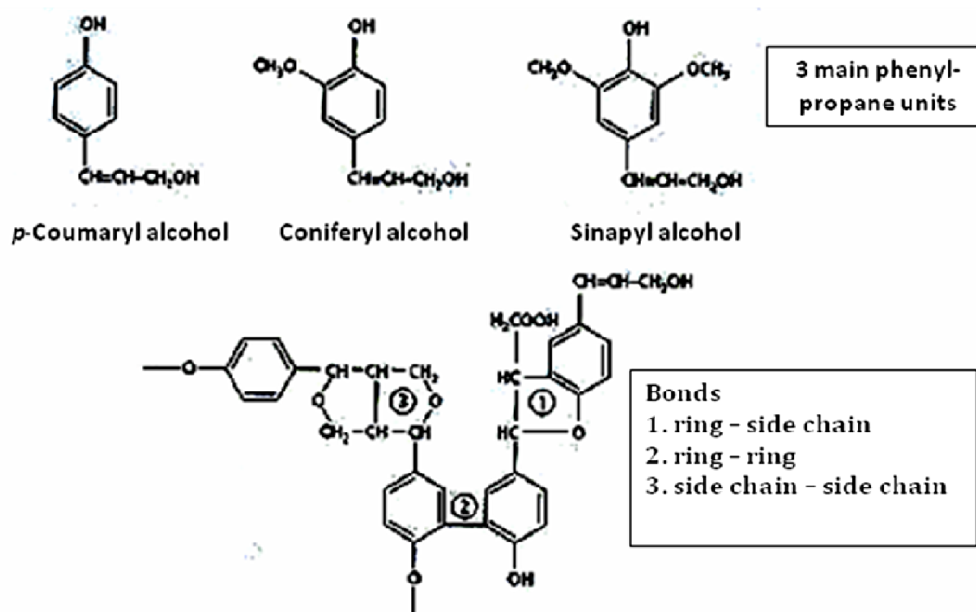
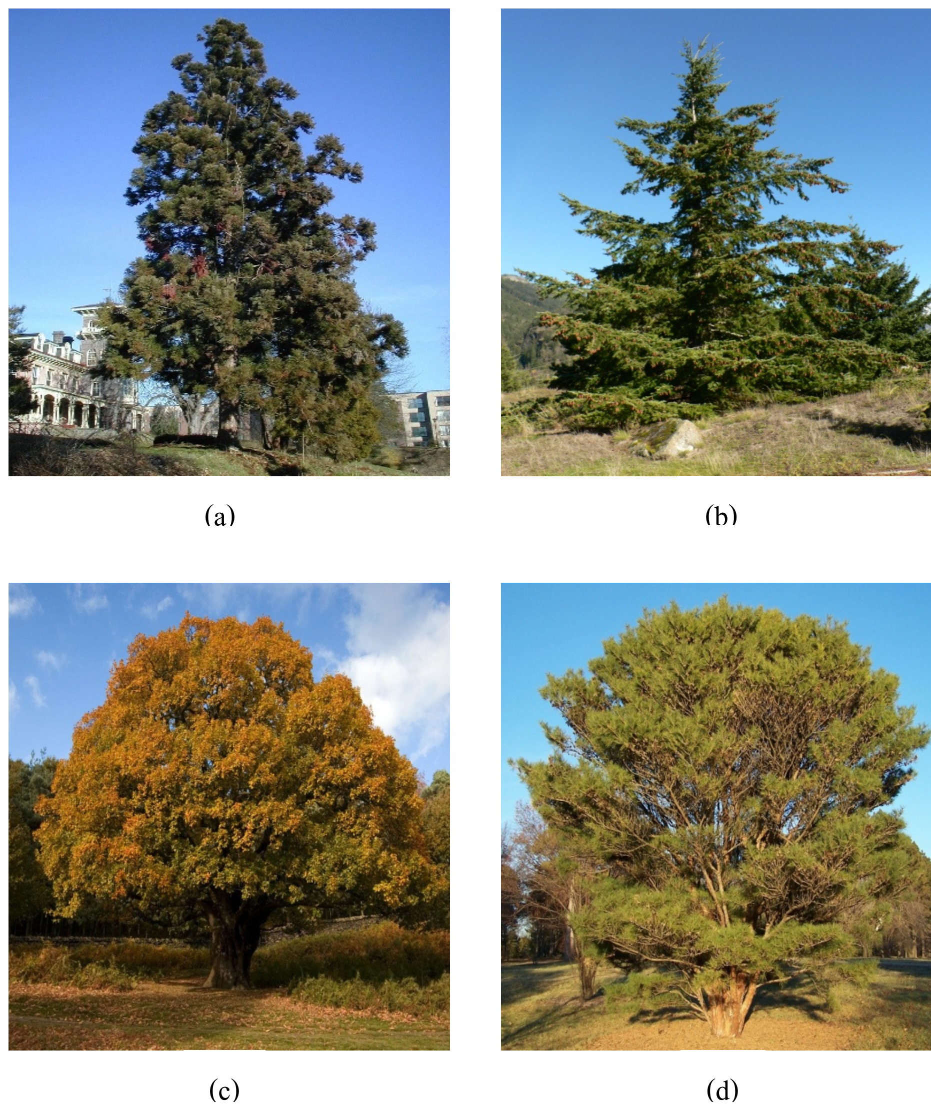


Figure 2-2 Simplified structure of lignin showing the three main phenyl-propane units and 3 of the many types of bond (From: Deacon, 1997).

At the temperature above 200 °C, lignin degrades into gas, liquid tar, and solid char. The liquid tar fraction generally contains water, alcohols, acetic acid and other organic acids, ketones, aldehydes, and phenolic compounds (Fengel and Wegener, 1984; Grandmaison and Kaliaguine, 1991; Chen et al., 2001). Chen et al. (2001) also stated that the main chemical reaction in the transformation of biomass to phenols starts where the dehydration of OH^- groups in the alkyl chain of phenylpropane basic unit of lignin followed by the cleavage of interaromatic bonds. Fengel and Wegener (1984) proposed that thermal degradation products of hemicelluloses were acetic acid, methanol, furfurol, aldehydes and ketones. In addition, O-acetyl groups had an important influence on the thermal stability of the hemicelluloses.

The increasing in acidity of the wood vinegar was assumed to be caused by the removal and leaching of organic acids such as acetic and lactic acid from wood. Acetic acid was derived from acetyl groups in the hemicelluloses (Kubinsky and Ifju, 1972; Fengel and Wegener, 1984; Yilgor et al., 2001). Besides phenolic compounds from lignin, acetic acid formation in wood vinegars from charcoal production with several wood species was also shown by Yatagai et al. (2002). Yatagai and co-workers studied the relationship between chemical components of wood vinegars such as acids and phenols, with their termidicidal activity. Three types of wood vinegar

were studied, wood vinegar A obtained from mixed chips of *Cryptomeria japonica* (Figure 2-3; a) and *Pseudotsuga menziesii* (Figure 2-3; b), wood vinegar B from *Quercus serrata* (Figure 2-3; c), and wood vinegar C from *Pinus densiflora* (Figure 2-3; d).



(a) (b)

(c) (d)

Figure 2-3 Four types of wood species which were used to produce wood vinegars, *Cryptomeria japonica* (a), *Pseudotsuga menziesii* (b), *Quercus serrata* (c) and *Pinus densiflora* (d) (From: http://www.pabigtrees.com; http://commons.wikimedia.org and http://georgiachronicles.wordpress.com)

Wood vinegars A and B contained about 84% of water content and about 16% of organic fraction, whereas wood vinegar C contained about 88% of water and about 12% of organic fraction. Furthermore, acetic acid, the highest contents in wood vinegars, seems to be the reason

for the termidicidal activity of wood vinegars A and B which were higher than that of wood vinegar C.

Table 2-1 Contents of main acids and phenols of wood vinegars (Yatagai et al., 2002)

Components	Wood vinegar A	Wood vinegar B	Wood vinegar C
Water	84.22	84.68	88.13
Acetic acid	45.9	48.9	27.64
n-Propionic	4.65	2.75	1.85
Butanoic	3.38	1.85	1.83
n-Valeric	0.25	0.15	0.27
Total acidic	53.47	53.65	31.89
Phenol	3.52	1.82	1.66
2-methylphenol	-	-	0.92
2-ethylphenol	0.12	-	0.86
2-methoxyphenol	7.32	2.12	4.73
2-methoxy-4-methylphenol	3.60	1.20	3.49
4-methylphenol	0.92	0.48	1.44
2-methoxy-4-ethylphenol	0.71	0.57	1.66
2,6-dimethylphenol	0.05	6.58	8.87
Total phenolic	16.24	12.77	23.63

2.4 Applications of wood vinegars

Due to wood vinegars composed of more than 200 chemical substances, the main components were organic acids and phenols, therefore, wood vinegar could be applied in several fields such as plant growth acceleration, weed control, bugs and parasites control, deoderization, detoxification, prebiotics, termidicidal and antifungal agents.

2.4.1. Plant growth acceleration agent. Wood vinegars have been found to enhance the growth of many kinds of plants.

Since the components of wood vinegar were naturally occurring organic compounds, therefore, it was highly suitable for use in organic and hydrophonic farming, as well as for

conventional farming. The improvement of activities of bacteria and small living creatures in soil was observed when apply wood vinegar to the soil.

For using wood vinegar as plant growth accelerator, it should be diluted 1 part with water 500-1000 parts by volume before spraying on the soil. For young plants a solution of wood vinegar 1 part with 1000 parts of water was applied by spraying onto the leaves. For mature plants, wood vinegar solution 1 part: 1000 parts of water was applied by spraying onto the leaves and sprinkled onto the soil once or twice a month. When plants become weak or their health begin to fail, 1 part of wood vinegar should be diluted with 300 parts of water and sprinkle onto the plants. Repeat this procedure once or twice every two weeks (http://www.the-organic-gardener.com/organic-weed-control.html).

Utilizing chemical fertilizers was not only imposing heavy loads and pollution on the environment but also threaten our health. Long-term application of chemical fertilizers exposed the following problems: exhaustion of soil organics, lower conservation of water and nutrition, deterioration of the soil structure and heavy losses of water and soil. Excessive chemical fertilization not only polluted the soil, water and air but also kept most residues in vegetables, which decreases the quality and security of our food supply. Therefore, it was very important to find and develop natural materials for vegetable production. Wood vinegar was one such good source, given the principle of organic production in agriculture. Mu and co-workers (2003) studied the effects of moso bamboo (*Phyllostachys pubescense*) vinegar with different diluents on the growth of lettuce, cole and cucumber based on field tests. The results showed that moso bamboo vinegar with 500–800 times dilution gave good effect on the growth of tested vegetables. The mass production of vegetables increased 18.8 %–20.2 % compared with a control. The height and the weight of vegetables tested also increased.

Wood vinegar has been widely used in agriculture and daily life in Japan. There were about 4×10^{7} L produced every year and over half of it was used in agriculture (Higashino et al., 2005).

The mixture of charcoal with wood vinegar (4:1) have been used for rice (*Oriza sativa*) (Tsuzuki et al., 1989), sweet potato (*Ipomoea batatas*) (Du et al., 1998), sugar cane (*Saccharum officinarum*) (Uddin et al., 1995[a,b]) and melon (*Cucumis melo*) (Du et al., 1997) production. This mixture was found to promote growth and yield for those field cultivation crops (Katoda et al., 2004).

Wood vinegar could also induce the metabolism of some plants resulted in increasing of sugar level and the sweet taste of fruits (จิรพงษ์ คูหากาญจน์, 2549).

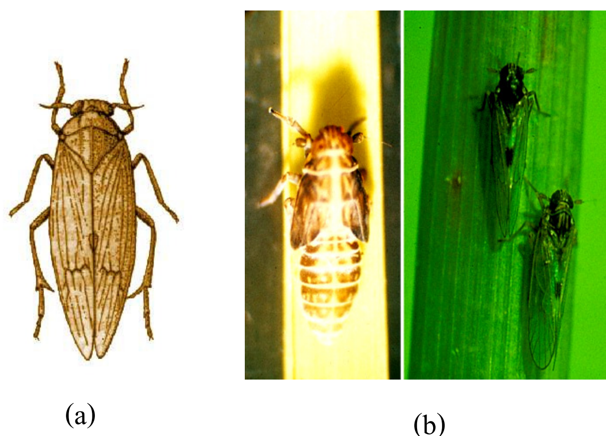
2.4.2 Weed controlling agent. Since, wood vinegar contained mainly acetic acid (70% of all chemical components), therefore, to use wood vinegar as weed controlling agent, it should be diluted to contain about 5-20% of acetic acid to give the best effect. Using higher concentration (more than 20%) gave better affect but mainly destroys surface growth; therefore, it should be used as a diluted solution and respraying when needed. Wood vinegars have been found to be an effective non-selective contact herbicide (weed killer). Some commercial herbicide products available in the market contained wood vinegar as an active ingredient as ready-to-use formulations. The concentration of acetic acid in these products was found to be about the same amount as in regular household vinegar. However, to use wood vinegar as herbidicidal agent might have some disadvantages such as, it is less effective than the commercial products because the commercial products contain a surfactant that allowed the products to wet the leaves better. Another disadvantage of utilizing wood vinegar was that is does not have an EPA-approved (EPA = environmental protection agency) label with directions and safety information. Wood vinegar might also kill desirable plants such as grass or decorated plants. It also only affected some parts of plants which direct contact with it, therefore, spraying it on leaves or stems would not affect the roots (http://www.the-organic-gardener.com/organic-weed-control.html).

Wood vinegar was found to promote the weed control activity of two types of sulfonylurea-based herbicides (Rico et al., 2007). A mixture of equal amount of wood vinegar and herbicide (contains bensulfuron-methyl, butachlor or imazosulfuron ethyl, thiobencarb) could increase efficacy of herbicides and improve mass production of rice (*Oryza sativa* L.).

2.4.3 Bugs and parasites controlling agents. Wood vinegar has been found to have an effect against plant diseases cause by insect pests, improving the quality of agriculture without any toxicity to people and animals (Ikeshima, 1999). Wood vinegar could reduce the growth of phytopathogenic fungi such as *Fusarium*, *Pythium* and *Rhizoctonia* (Yagi and Tsukamoto, 1991), and promote growth of plant roots (Tsuzuki et al., 1989; Kadota et al., 2002). Wood vinegar also enhanced the activity of insecticides. Kim et al. (2008) studied the effect of the mixture of insecticides, such as 2-*sec*-butylphenyl *N*-methylcarbamate (BPMC), dinotefuran, imidacloprid and carbosulfan, and wood vinegar against two species of rice planthoppers, *Nilaparvata lugens*

(Figure 2-4; a) and *Laodelphax striatellus* (Figure 2-4; b). The mixture of wood vinegar and carbosulfan gave the activity greatly higher than the other treatments.

In order to use wood vinegar to control bugs and parasites, it (1 part) should be diluted with 1000 parts of water and sprinkled onto the leaves and soil once or twice a month (http://www.the-organic-gardener.com/organic-weed-control.html).



(a) (b)

Figure 2-4 Rice planthoppers, *Nilaparvata lugens* (a) and *Laodelphax striatellus* (b)
(From: http://www.ento.csiro.au/aicn/name_s/b_2799.htm
http://hampyeong.jares.go.kr/vvkham/sub/sub0302020201.htm?book=3&part=2&chapter=2)

To control bugs in animal farm, wood vinegar should be diluted with water 100-200 parts and sprayed around animals living area.

2.4.4 Deodorant: Wood vinegars demonstrated deodorization property. Strong smell of compounds such as ammonia and triethylamine were reduced after treatment with wood vinegar from Hiba wood (*Thujopsis dolabrata*). It displayed better activity than the oil from the same wood but showed similar activity to that of 10% acetic acid solution (Kasai, 2001). Wood vinegar was applied as an ingredient in shower cream and body lotion which were the products of Kiengmool co., Ltd., Thailand (Figure 2-5).

2.4.5 Detoxificating agent: For detoxification, wood vinegar was believed to promote equilibrium and greater healing in the body. Since, toxin could be accumulated in the body from a number of sources such as chemical pesticides and fertilizers from food, the polluted air and as a by-product from our metabolism, etc.

Figure 2-5 Wood vinegar shower cream and body lotion products of Kiengmool co., Ltd.
(From: http://www.kiengmool.com)

The result of continued accumulation of toxins was poor health as manifested by weakness, pains and aches, disease and sickness. Common examples of illnesses caused by bodily toxins were gout, arthritis, rheumatism, and back pains. Therefore, regular removal of toxins from our bodies may resulting in good health.

Detoxification pad is the products of sap sheet containing wood vinegar available in Japan, Korea, America and China (Figure 2-6).

Figure 2-6 Commercial detoxi-pads (From: http://www.fifija.com/detoxipad/tour.htm)

Wood vinegars were used in preparation of detoxification pad. The direction of using detoxification pad is by placing at the bottom of both feet before go to bed. The detoxification pad will directly attach to the reflex points on the feet (Figure 2-7). It was believed to promote equilibrium and greater healing in the body.

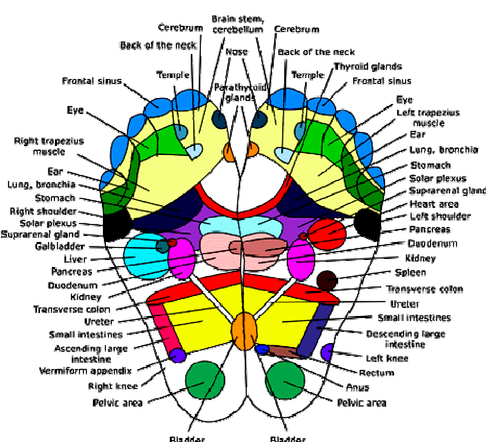


Figure 2-7 Reflex points at the bottom of the feet

(From: http://www.kenrico.com/sapsheet.html)

The sap sheet was believed to help by cleaning out waste and toxic materials that were excreted in the form of the sweat under the feet (Figure 2-8). A clinical study of the effectiveness of using sap sheet or detox pad are under investigation.

Figure 2-8 The sap sheet before and after use

(From: http://www.kenrico.com/sapsheet.html)

2.4.6 Wood vinegars were utilized as prebiotics. Prebiotics are defined as non digestible food ingredients that beneficially affect the host by selectively stimulating the growth and activity of one or a limited number of bacteria in the colon, and thus improve host health. Consumption of food containing less fiber, more meat and carbohydrate, and toxin may reduce good bacteria in large intestine. Wood vinegar was a source of acids help to promote acidity in large intestine,

resulting in inhibition the growth of bad bacteria, reducing absorption of alkaline carcinogen, enhancing calcium and magnesium absorption and increasing blood circulation.

Wood vinegar was not only consumed as prebiotics for human but it also used in animal. It could promote digestion system, increase nutrient adsorption, and reduce diarrhea. Wood vinegar liquid could reduce the number of *Cryptosporidium parvum oocyst* (Watarai et al., 2008).

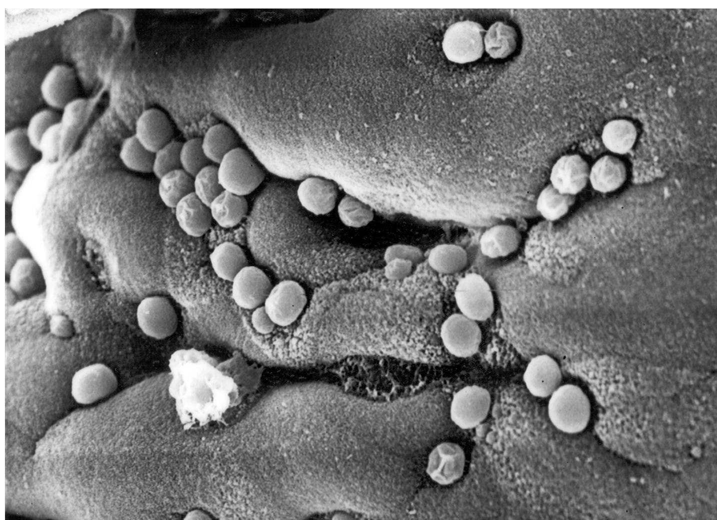


Figure 2-9 Electron Microscopy Images of Human gut infected with *Cryptosporidium parvum* (From: http://www.lshtm.ac.uk/immu/wcbf/Images/Images.html)

Cryptosporidium parvum (Figure 2-9) is an intestinal protozoa parasite that causes diarrhea in both humans and domestic animals (Casemore et al., 1997; Anderson, 1998; Xiao et al., 2004). Because the feces of calves were a recognized source of *Cryptosporidium,* fecal contamination in drinking water was a potential means of transmission of this protozoa (Smith, 1998; Fayer et al., 2000). Others have reported that organic acids could inhibit the growth of enteropathogenic bacteria (Anderson, 1992; Hsiao and Siebert, 1999; Nakai and Siebert, 2003) as well as the viability of *C. parvum* (Kniel et al., 2003). Nekka-Rich (Miyazaki- Midori Pharmaceuticals Inc., Miyazaki, Japan) was a product made by mixing activated charcoal and wood vinegar liquid that contains organic acids. The activated charcoal and wood vinegar liquid of Nekka-Rich were both obtained from the bark of evergreen oak (*Castanopsis cuspidate* and *Quercus acuta*) by carbonization. Beside, Nekka-Rich had a stimulatory effect on the growth of

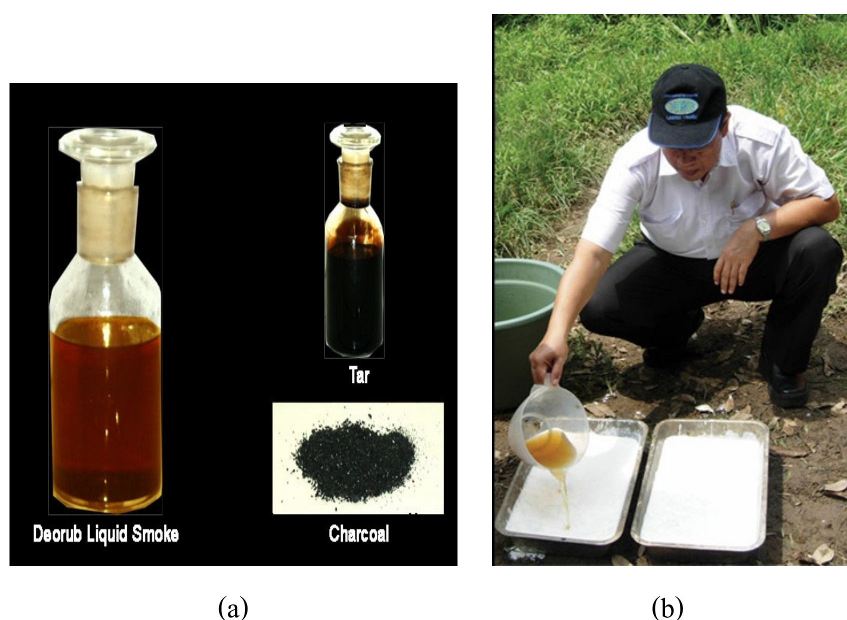
Enterococcus faecium and *Bifidobacterium thermophilum*, both of which acted as probiotics (Tana et al., 2003).

It was suggested that wood vinegar liquid which contains organic acids could inhibit the growth of enteropathogenic microbes and reduce the viability of *C. parvum* in dose dependent manner. Wood vinegar could also be mixed with charcoal and added in animal food to use as animal prebiotics. It has been found to promotes digestion, inhibit gas and metal adsorption in animal stomach, resulting in increasing meat quality. When adding this to chicken food, eggs contain higher vitamin and lower cholesterol were obtained. When adding this to cow food, quantity of milk was increased. Beside, this mixture could decrease ammonia and sulfur dioxide, products from animal metabolism which cause bad smell (http://www.ata.or.th).

2.4.7 Smoke flavoring agent in food processing. An application of wood vinegar as a food additive was studied by Miyakawa and co-workers (2003). Wood vinegars were extracted by organic solvents to obtain antioxidative smoke flavors. Wood vinegar from drift wood were extracted by benzene and fractionated to acidic, phenolic and neutral fractions using 5% $NaHCO_3$ and 5% NaOH. The extracted wood vinegars were analyzed using GC-MS to identify the chemical components; twenty-three main smoke flavors were identified. Antioxidant of extracted wood vinegar was tested by DPPH assay. Phenolic fraction showed most strong antioxidant activity. This study showed that wood vinegar using drift wood as raw material could be manufactured without consuming forest resources compared with conventional wood vinegar. In addition, the smoke flavors extracted from wood vinegar had possibility for application to food as a safer product than conventional wood vinegar.

2.4.8 Anti-allergy composition: Imamura and Watanabe (2007) found that wood vinegar from moso bamboo (*Phyllostachys heterocycla*) which carbonized at 350 to 450 ° C and distilled at a low temperature of 50 to 60 ° C under reduced pressure could be applied as an effective ingredient of the anti-allergy composition. The anti-allergy composition contained a distilled solution of wood vinegar as an effective ingredient, which could inhibit allergic reaction, in particular, Type I allergic reaction by oral administration. This composition was indicated for preventing allergic rhinitis, hay fever, allergic conjunctivitis, atopic dermatitis, allergic asthma, urticaria and food allergy. In addition, the composition could be also applied on skin. Moreover,

since the distilled solution of a wood vinegar contains large amount of polyphenols, an effect in a liver disease or an adult disease such as arteriosclerosis and diabetes were expected. In order to use safely by oral administration, the authors suggested that all carcinogenic substances such as benzopyrene, di-benz-anthracene and methylcholanthrene and cresol should be previously removed. Additionally, various components of organic acid presented in this wood vinegar were useful in a living body.



(a) (b)

Figure 2-10 "Deorub® Liquid Smoke", wood vinegar product of Badja Baru Co., Ltd and Balai Penlitian Sembawa Co., Ltd (a) used as coagulating agent in natural rubber sheet production (b) (Kamis, 2009).

2.4.9 Coagulating agent in rubber production. Wood vinegar could be applied instead of formic acid to promote coagulation in natural rubber sheet production (Figure 2-10; b). The obtained rubber sheets have special smoky odor and their color is yellow-brown. Recently, the Indonesian company, Badja Baru Co., Ltd and Balai Penlitian Sembawa Co., Ltd., are the co-ordinate owner of the research patent to produce and sell coagulating agent wood vinegar obtained from rubber, coconut peel and palm seed in the commercial name of "Deorub® Liquid Smoke" (Figure 2-10; a). They found that wood vinegar could also inhibit the growth of bacteria that cause of bad smell and fungi growth on surface of rubber sheet and can be used as antioxidative agent to

increase the flexion of rubber (ยอดธง ใบมาก, 2549; Ferreira et al., 2005). The load and extension properties of natural rubber sheet were increased when using a mixture of wood vinegar with formic acid. Moreover, using wood vinegar diluted with formic acid could increase tensile strength and stain of natural rubber sheet.

2.5 Bio-efficacies of wood vinegars

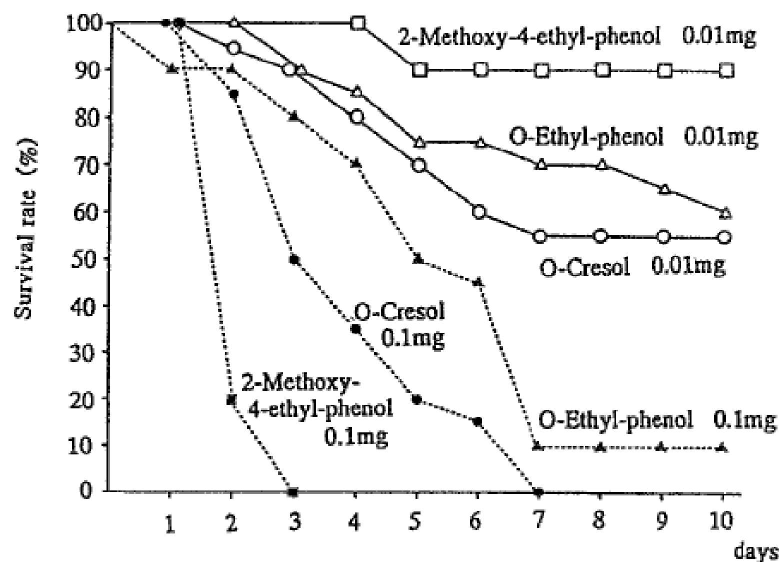
Nakai and co-workers (2005) studied the effectiveness of the wood vinegars to control fungal growth in vitro with consideration of the bioactive components included in the wood vinegars. *Trametes versicolor* and *Tyromyces palustris* were tested. Fungicidal tests showed a significant difference in the effectiveness of controlling fungi between wood vinegars obtained from solid wood and the wood composites. The liquids from the wood composites revealed higher effectiveness against the fungi tested. It was assumed that the chemical components derived from the adhesive in the wood composites contributed to the increase in controlling the growth of the microorganisms. Type of adhesives also played an important role in differentiation of the components in the liquids. Carbonization temperature was also an important factor in characterization of the components. A large number of nitrogenous compounds from thermal degradation of urea type adhesive-bonded plywood and particle board were detected at low heating temperature zone of room temperature – 300 °C. When phenol-type adhesive-bonded plywood and particleboard were carbonized, the yield of aromatic compounds including various types of phenol compounds increased along with the rise of heating temperature, 400 - 500 °C. Kartal et al. (2004) showed that higher concentrations of wood vinegars were critical for inhibition of fungal growth. Wood vinegars which contain components such as phenolic from lignin degradation gave antifungal activity against brown rot fungi. The liquids derived from phenol-bonded particleboard and plywood at the range of 400 - 500 °C which had high phenolic content may have contributed in increasing inhibition of fungal growth.

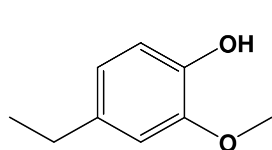
The termiticidal activity of wood vinegars, their components, and their homologues have been studied (Yatagai et al., 2002). Three kinds of wood vinegar made from the mixed chips of *Cryptomeria japonica* and *Pseudotsga menziesii* (wood vinegar A), *Quercus serrata* (wood vinegar B), and *Pinus densiflora* (wood vinegar C) exhibited high termiticidal activities against *Reticulitermes speratus* at a dosage of 0.1 ml. The activity of wood vingar C was slightly weaker

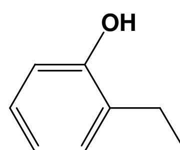
than A and B. The difference in the activities among these three wood vinegar samples appeared at the diluted dosage (at 0.01 ml). Wood vinegar A gave a survival rate of 0% at 3 days after the test was started, whereas wood vinegar B and C gave survival rates of 65% and 67%, respectively, after 7 days. Acetic acid, which was the largest content of wood vinegar played important role in termiticidal activity. The difference in contents of organic fraction of wood vinegars and acetic acid were responsible for the differences in termiticidal activities among wood vinegars. The chemical structure and termiticidal activity relationship of phenols were studied. Phenols containing substituents on aromatic ring revealed higher termiticidal activity than non-substituted derivatives with dose dependent. It has been found that high termiticidal activity was not due to a phenolic hydroxyl group alone but also the aromatic ring substituents, especially an ortho position to a phenolic hydroxyl group. The bulkiness of the substituent at the ortho position participated in termiticidal activity such as activity decreased as the size of an ortho substituent increased. Therefore the interaction at the receptor site of termites might be affected by the increased size of the ortho substituent. Figure 2-11 shows the time course of the termiticidal activity of phenols. The activities of o-ethyl phenol and o-cresol at a dose of 0.01mg were mild, and the differences in the activities between 0.10 and 0.01 mg were relatively small, whereas the activity of 2-methoxy-4-ethyl phenol at a dose of 0.10 mg was extremely high but extremely low at 0.01 mg. The activities have been found to be varied over the wide range between 0.10 and 0.01 mg.

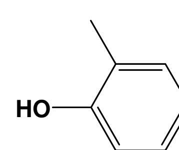
Fungicidal and termiticidal properties of wood vinegars from Biomass Slurry Fuel (BSF) produced from sugi (*Cryptomeria japonica*) and acacia (*Acacia mangium*) woods were evaluated for wood decay and termite resistance tests (Kartal et al., 2004). Wood blocks treated with wood vinegars showed increased in resistance against brown-rot fungus, *Fomitopsis palustris*. However, only wood vinegar derived at 270 ºC from sugi wood increased the resistant against white-rot fungus.

The result indicated that phenolic compounds of wood vinegars seemed to play a role in increased decay resistance against brown-rot fungus, while wood vinegars did not increase the durability of the wood blocks against subterranean termites, *Coptotermes formosanus*. Despite high acetic and lactic acid content of wood vinegars, it was found that vanillin content of wood vinegars may promote termite attack.









2-methoxy-4-ethyl-phenol o-ethyl-phenol o-cresol

Figure 2-11 Time course of termiticidal activities of phenols (From: Yatagai et al., 2002)

The antimicrobial effect of the wood vinegar of *C. japonica* sapwood and its constituents was evaluated against *Ralstonia solanacearum*, *Phytophthora capsici*, *Fusarium oxysporum*, and *Pythium splendens*. Phenols and guaiacols had a strong antimicrobial effect against tested microorganisms, but methanol and acetic acid exhibited little or no antimicrobial activity (Hwang et al., 2005).

Ikegami and co-worker (1998) studied anti-dermatophyte activity of wood vinegar under trade name 'Mokusaku-eki' that obtained from *Quercus* spp. This wood vinegar was used as a folk medicine to treat water eczema. It contained several phenolic compounds such as 4-ethyl-2-methoxyphenol and 2, 6-dimethoxyphenol which could not be found in the fresh woods. Among these compounds, 4-ethyl-2-methoxyphenol showed the highest anti-dermatophyte activity against *Trichophyton mentagrophytes* at a minimum inhibitory concentration (MIC) 150 μg/mL.

(1) (2)

Figure 2-12 Wood vinegar products in Japan, 'Chikusaku-eki'(1) and 'Mokusaku-eki'(2)

(From: http://www.e-kanekoya.com/s_zakka_ni-mokusaku.htm)

Kimura and co-workers (2002) also found that wood vinegar products under trade name 'Chikusaku-eki', produced from bamboo species, such as *Phyllostachys pubescens* or *Phyllostachys bambusoides*, that widely used in Japan as mokusaku-eki (Figure 2-12). Chikusaku-eki and mokusaku-eki were frequently diluted with water and mixed into the bath as folk remedy for scabies, eczema, atopic dermatitis, and other skin diseases. These were gaining widespread popularity in Japan.

2.6 Skin infection

Skin infection is a skin disease caused by infection of pathogen. Skin infections can be divided into 3 groups (Figure 2-13), (1) the most common superficial infection that effect only on the outer layers of the skin, (2) cutaneous infection that extends to the dermis of skin, and (3) an invasive infection that extends in adjacent tissues and may spread to other organs (Karen et al., 2005).

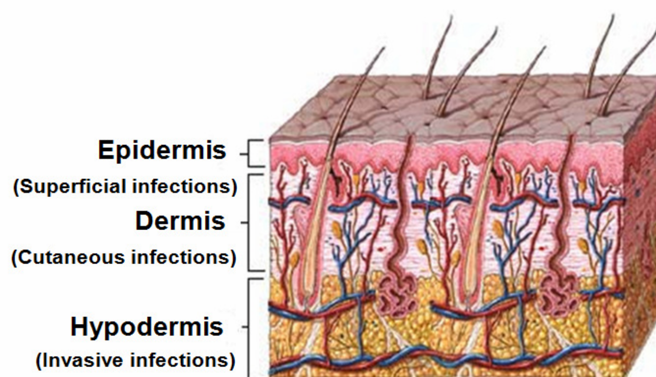


Figure 2-13 Infection of skin layer (ADAM, Inc. 2001)

Microorganisms which are the causes of skin infection are bacteria, fungi, virus and protozoa. In this review, skin infections caused by bacteria and fungi were focused.

2.6.1 Bacterial Skin Infections: Bacteria are commonly found on the human skin. Infections may result from skin injury, insect bites, but often arise spontaneously by bacterial invasion of hair follicles and skin glands (Karen et al., 2005). Bacterial skin infections are very common, and they can range from merely annoying to deadly. Most bacterial infections of the skin are caused by *Staphylococcus aureus* and a form of *Streptococcus* (Brannon, 2009).

1) Streptococci skin infections: Skin infection can range from folliculitis (infection of the hair follicles), cellulitis (a deep infection of the skin cells, producing red, swollen skin which is hot to the touch), and impetigo (a vesicular, blistered, eruption, most common in children, that becomes crusty and flaky and is frequently found around the mouth). Major skin infections are almost exclusively caused by *Steptococcus pyogenes* (Group A beta hemolytic).

2) Staphylococci skin infection: Staphylococci are commonly found under foot, nasopharynx and skin. About 50% of people have this type of bacteria. Three major pathogenic species are *Staphylococcus aureus*, *Stapphylococcus epidermidis*, and *Staphylococcus saprophyticus*. *Staphylococcus epidermidis*, a normal bacterial flora, is widely found on the body. This bacteria normally lives peacefully on our skin without causing disease. However, patients in hospital with Foley urine catheters or intravenous lines can be infected when this bacteria migrates from the skin along the tubing.

Skin infections caused by staphylococci or streptococci usually follow a major or minor break in the skin along with scratching resulting in spreading of the infection. Four types of symptom may be the result of bacterial infections, a) Impetigo: This contagious infection usually occurs on the face, especially around the mouth. Small vesicles lead to pustules, which crust over to become honey-colored, wet, and flaky, b) Cellulitis: This is a deeper infection of the cells. The tissue becomes hot, red, shiny and swollen, c) Local abscesses, Furuncles, and Carbuncles: An abscess is a collection of pus. Infection of a hair follicle produces a single pus-filled crater with a red rim. This infection can penetrate deep into the subcutaneous tissue and become a furuncle. These may lead to produce multiple contiguous, painful lesions under the skin called "carbuncles". Significant abscesses must be surgically drained, d) Wound infections: Any skin wound can be infected with *Staphylococcus aureus*, resulting in an abscess, cellulitis, or both. When a sutured post-surgical wound becomes infected, it must be reopened and often left open to prevent secondary infection (Gladwin and Trattler, 1997).

3) Anaerobic bacteria skin infection

3.1) ***Propionibacterium acnes*****:** *Propionibacterium acnes* is an anaerobic, Gram-positive bacillus that produces propionic acid as a metabolic byproduct. This bacterium resides in the sebaceous glands, derives energy from the fatty acids of sebum, and is susceptible to ultraviolet radiation due to the presence of endogenous porphyrins. The most well-known ailment associated with *P. acnes* is the skin condition known as acne vulgaris. In the sebaceous gland, *P. acnes* produces free fatty acids as a result of triglyceride metabolism. These byproducts can irritate the follicular wall and induce inflammation through neutrophil chemotaxis to the site of residence. Inflammation due to host tissue damage or production of immunogenic factors by *P. acnes* subsequently leads to cutaneous infections. *P. acnes* may cause postoperative infections similar to *S. epidermidis*. Prosthetic joints, catheters and heart valves may transport the cutaneous microflora into the body resulting in sepsis and endocarditis. Another common port of entry for *P. acnes* is through eye injuries or operations. *P. acnes* can cause endophthalmitis (inflammation of the interior of the eye causing blindness) weeks or months after trauma or eye surgery (Cogen et al., 2008).

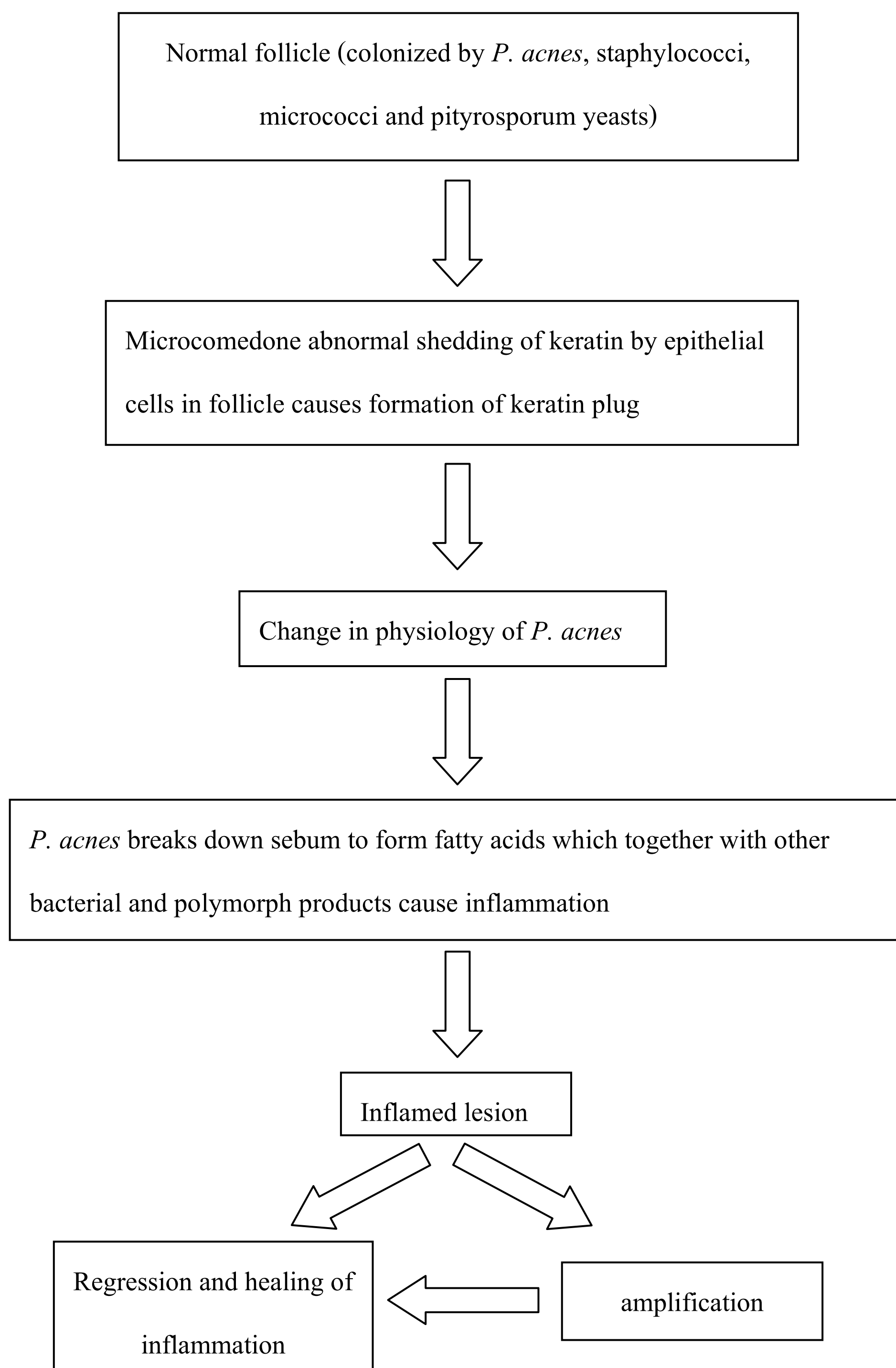


Figure 2-14 The mechanism of the pathogenesis of acne (From: Mims et al., 1998)

An increased responsiveness to androgenic hormones leads to increased sebum production plus increased keratinization and desquamation in pilosebaceous ducts. Blockage of ducts turns them into sacs in which *P. acnes* and other member of the normal flora (e.g. micrococci, yeasts, staphylococci) multiply. *P. acnes* acts on sebum to form fatty acids and peptides, which together

with enzymes and other substances released from bacteria and polymorphs, cause the inflammation. Comedones are greasy plugs composed of a mixture of keratin, sebum and bacteria and capped by a layer of melanin (Figure 2-14).

Hormonal changes in the host initiate the formation of comedones from normal follicles and thereby change the environment of *Propionibacterium acnes* and its physiologic properties (Mims et al., 1998).

3.2) Clostridium species: Gas gangrene or clostridial myonecrosis can be caused by several species of clostridium, *Clostridium perfringens* is the most common. This organism and its spores can be found in the soil and feces (in human and animal). It access to traumatized tissues by contamination from these sources. The organisms multiply in the subcutaneous tissues producing gas and an anaerobic cellulitis. A characteristic feature of clostridial infection is that the organisms invade deeper into the muscle, cause necrosis and produce bubbles of gas. The infection proceeds very rapidly and causes acute pain. Much of the damage is due to the production of a lecithinase (also known as alpha toxin) by *Cl. perfringens*, which hydrolyzes the lipids in cell membranes resulting in cell lyses and death.

4) Mycobacterial skin infection: There are several types of mycobacteria causing skin diseases such as *Mycobacterium leprae*, *Mycobacterium marinum, Mycobacterium ulcerans and Mycobacterium tuberculosis*.

M. marinum and M. ulcerans are slow-growing mycrobacterial species that prefer cool temperatures and cause skin lesions. *M. marinum* was normally found in water and marine organisms. Human infections follow trauma, often minor such as a graze acquired while climbing out of a swimming pool or while cleaning out an aquarium, which becomes contaminated with mycobacteria from the wet environment. After an incubation period of 2-8 weeks, initial lesions appear as small papules, which enlarge and suppurate and may ulcerate. The lesions are granulomas therefore the name 'swimming pool granulomas' or fish-tank granulomas'. Sometimes the nodules follow the course of the draining lymphatic and produce an appearance that may be mistaken for sporotrichosis. *M. ulcerans* causes chronic, relatively painless cutaneous ulcers known as 'Buruli ulcers'. This disease is prevalent in Africa and Australia, but is rarely found elsewhere. Finally, tuberculosis of the skin is exceedingly uncommon. Infection can occur

by direct implantation of *M. tuberculosis* during trauma to the skin (lupus vulgaris) or may extend to the skin from an infected lymph node (scrofuloderma) (Mims et al., 1998).

2.6.2 Fungal Skin Infections: Fungi could be found everywhere and yeasts are one the skin normal flora. Fungal infections of the skin, hair and nails are common skin diseases. Fungi can infect the skin of people of all ages. Moreover, increased incidence occurs in patients who have AIDS or are being treated with chemotherapeutic agents and therapy directed at reducing inflammation. Diabetic patients and elderly people usually have more chance to skin infections (Karen et al., 2005).

2.6.2.1 Superficial fungal infections

Pityriasis versicolor and tinea nigra are superficial fungus infections, whose primary manifestation is pigment change of the skin. Pityriasis versicolor (also called tinea versicolor) is a chronic superficial fungal infection which leads to hypopigmented or hyperpigmented patches on the skin. When skin expose to the sunlight, skin around the patched will tan, but the patches will remain white. This infection is caused by *Malassezia furfur*. Tinea nigra is a superficial fungal infection that causes dark brown to black painless patches on the soles of the hands and feet. This infection is caused by *Exophiala werneckii*.

Diagnosis of both infections is based on microscopic examination of skin scrapings, mixed on a slide with potassium hydroxide (KOH). This will reveal hyphae and spherical yeast, as the KOH digests nonfungal debris. Malassezia look like spaghetti (hyphae) with meatballs (spherical yeast). Treatment of both fungal infection consists of using anti-dandruff shampoo containing selenium sulfide or apply topical imidazoles antifungal agents.

2.6.2.2 Cutaneous fungal infections of the skin, hair, and nails

There are many types of fungi cause cutaneous fungal infections. Dermatophytoses and *Candida albicans* are the main causes of cutaneous fungal infections

1) Dermatophytoses are a category of cutaneous fungal infections caused by more than 30 species of fungi. The dermatophytic fungi live in the dead, horny layer of the skin, hair, and nails. These fungi secrete an enzyme called keratinase which digests keratin. Since keratin is the primary structural protein of skin, nails and hair, the digestion of keratin manifests as scaling of the

skin, loss of hair, and crumbling of the nails. The common dermatophytes include *Microsporum*, *Trichophyton*, and *Epidermophyton*.

1.1) Tinea corporis (body): This type of infection caused by infected fungi invade the horny layer of the skin, followed by the fungi spread, forming a ring shape with a red, raised border. This expanding raised red border represents areas of active inflammation with a healing center. This type of infection called “ringworm”.

1.2) Tinea cruris (jock itch): Patients develop itchy red patches on the groin and scrotum.

1.3) Tinea pedis (athlete’s foot): This infection commonly found between the toes, and causes cracking and peeling of the skin. This type of infection requires warmth and moisture, therefore, most commonly found in those wearing close shoes.

1.4) Tinea capitis (scalp): This type primarily occurs in children. The infecting organisms grow in the hair and scalp, resulting in scaly red lesions with loss of hair. The infection appears as an expanding ring.

1.5) Tinea unguium (onychomycosis) (nails): The nails are thickened, discolored, and brittle after infection.

2) *Candida albicans* can infect all areas of the skin as well as the mucous membranes but it prefers warm moist places. Infections by *C. albicans*, especially the variants that are found in the mucous membranes or the genitals, are contagious. They can be spread from person to person by direct contact, by sexual contact and indirectly by damp towels or flannels (Rutherford, 2009). *Candida* can infect the mouth (oral thrush), groin (diaper rash), and the vagina (Candida vaginitis). It can also cause opportunistic systemic infections (Gladwin and Trattler, 1997).

2.6.3 Antibacterial antibiotics

A variety of bacterial pathogens and types of skin infections necessitate multiple therapeutic options. Topical antibacterial agents are extremely important and highly versatile in antimicrobial therapy. They are used to treat multiple skin infections, traumatic and surgical wounds, and are also used as prophylaxis to prevent infection. The following is an overview of topical antibacterial agents for uncomplicated skin infections (Bajaj and Gupta, 1986; Gallenkemper et al., 1998; Hirschmann, 1998; Kaye, 1995 and Thestrup-Pedersen, 1998).

(a)

(b
)

(c
)

(d)

(e)

Figure 2-15 Topical antibacterial drugs (From: http://www.wikipedia.org)

1. Bacitracin (Figure 2-15; a): Bacitracin, the polypeptide drug, blocks bacterial cell wall formation by complexes with C55-prenol pyrophosphate which is the constituent of bacterial cell wall. Bacitracin has effective against gram-positive bacteria (streptococci, staphylococci, clostridia and corynebacteria) and some type of *Neisseria*.

2. Mupirocin (Figure 2-15; b); Mupirocin can inhibit bacterial isoleucyl tRNA synthetase which involved in protein synthesis important for protein and cell wall synthesis. Mupirocin is active against gram positive bacteria (except enterococci) but inactive against many organisms of normal flora. The side effects of this drug appears in less than 1% of patients, such as burning, stinging, pain, swelling and nausea.

3. Neomycin (Figure 2-15; c); Neomycin is a bactericidal antibiotic which binds 30S subunit of bacterial ribosome (involved in protein synthesis), interferes with bacterial DNA polymerase. This drug is effective against most gram-negative bacilli (except *Pseudomonas aeruginosa), staphylococci.* The resistance of neomycin has been reported in staphylococci, *E. coli*, *Klebsiella* and *Proteus*.

4. Polymyxin; Polymyxin is a cationic decapeptide acts as surfactant to disrupt cell membrane. This drug is effective against *P. aeruginosa, E. coli, Enterobacter* spp, *Klebsiella* spp. but ineffective against *Proteus, Serratia* and gram-positive bacteria. It is very infrequently cause allergic contact dermatitis (possible cross-reactivity with bacitracin), and frequently used with bacitracin, zinc and neomycin in a petroleum base. There are two types of polymyxin, polymyxin B (Figure 2-15; d) and polymycin E or Colistin (Figure 2-15; e).

2.6.4 Antifungal antibiotics

In this review, only the topical antifungal drugs were focus. Topical antifungal drugs are common in treatment of skin infections that are caused by fungi. Topical antifungal drugs not only relieve the symptoms of fungal infection, such as itching, burning, and cracked skin, but they also eliminate the fungus.

Commonly used topical antifungal drugs include nystatin, ciclopirox, haloprogin, naftifine, tolnaftate and azole family.

<u>Nystatin</u> (Figure 2-16; a): Nystatin binds to ergosterol, causes increasing the permeability of the cell membrane and resulting in cell lysis. It is only used topically on the skin and mucous

membranes. Since it is not absorbed from the gastrointestinal tract, oral nystatin can be used to treat oral and esophageal infections by yeast or fungi. It is also given topically for vaginal candidiasis as vaginal suppository.

The azole family: The azole family may be classified into 2 groups of drugs, the imidazoles and the triazoles.

1. Imidazoles: There are 3 types of imidazoles, ketoconazole, miconazole and clotrimazole.

Ketoconazole (Figure 2-16; b) is the drug of choice for chronic mucocutaneous candidiasis, but not used for systemic candidiasis. Ketoconazole is currently used for the treatment of fungal infections as topical and oral applications.

Miconazole (Figure 2-16; c) and Clotrimazole (Figure 2-16; d) are too toxic for systemic use, therefore, they are available only in topical preparation and used for topical fungal infections, including pityriasis versicolor, cutaneous candidiasis, and the dermatophytosis (tinea pedis, corporis, etc.).

2. Triazoles: There are 2 types of triazoles, fluconazole and itraconazole.

Fluconazole (Figure 2-16; e) is less toxic and has broader antifungal activity than ketoconazole. It is used for cutaneous Candida infections but it is a second-line drug behind amphotericin B for systemic candidiasis and cryptococcal meningitis. In AIDS patients who have had cryptococcal meningitis, maintenance with fluconazole will prevent relapses.

Itraconazole (Figure 2-16; f) is now used as the first-line treatment for chromoblastomycosis, histoplasmosis, coccidioidomycosis, blastomycosis, and possibly for invasive aspergillosis (Gladwin and Trattler, 1997).

Other topical antifungal agents: A variety of other agents such as Whitfield's ointment (a mixture of benzoic and salicylic acids), tolnaftate, ciclopirox, haloprogin and naftifine, are available as creams for topical treatment of superficial mycoses (Mims et al., 1998).

1. Tolnaftate (Figure 2-17; a); Tonaftate is a synthetic over-the-counter anti-fungal agent. It may present as a cream, powder, spray, or liquid aerosol, and is used to treat jock itch, athlete's foot and ringworm. However it has been found to be generally slightly less effective than azoles when used to treat tinea pedis (Crawford et al., 2001).

Figure 2-16 Topical antifungal drugs

(From: http://www.wikipedia.org; http://www.dic.academic.ru)

2. Ciclopirox (Figure 2-17; b); Ciclopirox is a broad-spectrum antifungal agent with activity against a broad spectrum of dermatophytes, yeasts, actinomycetes, molds, other fungi, and a variety of Gram-positive and Gram-negative bacteria (Jue et al., 1985). It also exhibits anti-inflammatory and antibacterial activity (Gupta and Skinner, 2003).

3. Haloprogin (Figure 2-17; c); Haloprogin is a halogenated phenolic ether administered topically for dermotaphytic infections. It is used as a topical ointment or cream in the treatment of tinea infections. (http://www.drugbank.ca/cgi-bin/getCard.cgi?CARD=APRD01011.txt)

4. Naftifine (Figure 2-17; d); Naftifine is a topical fungicidal drug against broad spectrum of dermatophyte fungi and provides good activity against Candida and Aspergillus species. It is also effective against gram-negative and gram-positive bacteria (Gupta et al., 2008)

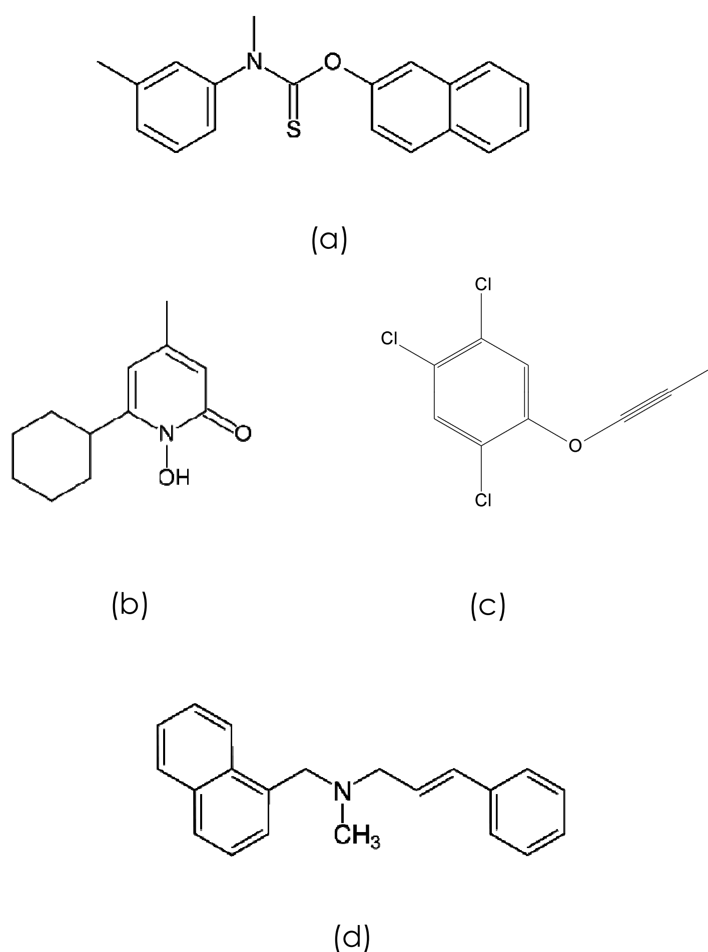


Figure 2-17 Other topical antifungal drugs (From: http://www.wikipedia.org)

2.7 Inflammation

Inflammation is a protective response to infection by the immune system that requires communication between different classes of immune cells to coordinate their actions. Acute inflammation is an important part of the immune response, but chronic inappropriate inflammation can lead to destruction of tissues in autoimmune disorders and perhaps neurodegenerative or cardiovascular disease. Secreted cytokine proteins provide signals between immune cells to coordinate the inflammatory response. Some cytokines such as interleukin-1 (IL- 1) and interleukin-6 (IL-6) and tumor necrosis factor (TNF) act to broadly provoke the inflammatory response; while, others act on specific types of immune cells. Macrophages and other phagocytotic cells provide a front-line defense against bacterial infection. Macrophages stimulate the inflammatory responses of neutrophils, fibroblasts, and endothelial cells in response infection by secreting IL-1 and TNF. IL-1 and TNF cause fever through alteration of the body temperature set-point in the hypothalamus. Fibroblasts and endothelial cells respond to IL-1 and TNF by recruiting more immune cells to the site of inflammation. Secreted interleukin- 8 (IL-8) is a chemokine that attracts neutrophils to sites of infection. Macrophages also present antigen to T helper cells that play a central role in coordinating immune responses. T helper cells induce clonal expansion of T cells that respond to antigen, with interleukin- 2 (IL-2) as a key mediator of T cell proliferation and activation. Transforming growth factor (TGF)-beta is a negative regulator of proliferation in many cells, have anti-inflammatory actions in some settings. The cytotoxic activity of Natural Killer cells (NK cells) and lymphokine activated killer cells (LAK cells) toward viral infected or tumor cells is stimulated by IL-2 and other cytokines. T helpers secrete interleukin- 3 (IL-3) and interleukin- 5 (IL-5) to stimulate eosinophil proliferation and activation. Eosinophils are involved in the immune response to parasitic infection. T helper cells are required to stimulate B cell responses as well, with the cytokines interleukin- 10 (IL-10), interleukin- 4 (IL-4) and other cytokines regulating the clonal selection and differentiation of antigen-specific B cells to form antibody-secreting plasma B cells and memory cells. In addition to inducing activation and proliferation of specific differentiated immune cells, cytokines act on hematopoeitic stem cells, causing their proliferation and differentiation into the full range of immune cells. (http://www.biocarta.com/pathfiles/h_inflamPathway.asp)

Inflammation is the complex biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants (Ferrero-Miliani et al., 2007). It is the body's first defense against infection, which is biological process that turns the tissue around a splinter pain, edema, erythema and hyperthermia (Gorman and Park, 2004). Most of the time, inflammation is a lifesaver that enables our bodies to fend off various disease-causing bacteria, viruses and parasites (Gorman and Park, 2004).

There are two basic types of inflammation:

1. Acute inflammation is a short duration type, which could be from a few minutes to a few days. Such inflammation is caused by foreign substances entering the body, or by physical damage. A viral infection may also precipitate in acute inflammation. The process of acute inflammation is initiated by cells which already present in all tissues, mainly resident macrophages, dendritic cells, histiocytes, Kuppfer cells and mastocytes. Once activated by infection, burn, or other injuries, the cells undergo activation and release inflammatory mediators responsible for the signs of inflammation.

There are 3 steps of acute inflammation

1) Vasodilatation: Vasodilatation is resulting in increased blood flow causes the redness (*rubor*) and increased heat (*calor*).

2) Increased vascular permeability: Increased permeability of the blood vessels results in an exudation (leakage) of plasma proteins and fluid into the tissue (oedema), manifesting as swelling (*tumor*). Some of the released mediators such as bradykinin increase the sensitivity to pain (hyperalgesia, *dolor*).

3) Extravasation: The mediator molecules also alter the blood vessels to permit the migration of leukocytes, mainly neutrophils, outside of the blood vessels (extravasation) into the tissue. The neutrophils migrate along a chemotactic gradient created by the local cells to reach the site of injury. The loss of function (*functio laesa*) is probably the result of a neurological reflex in response to pain.

In addition to cell-derived mediators, several acellular biochemical cascade systems consisting of preformed plasma proteins act in parallel to initiate and propagate the inflammatory response. These include the complement system activated by bacteria, and the coagulation and fibrinolysis systems activated by necrosis, e.g. a burn or a trauma (Cotran et al., 1998).

The acute inflammatory response requires constant stimulation to be sustained. Inflammatory mediators have short half lives and are quickly degraded in the tissue. Hence, inflammation ceases once the stimulus has been removed (Cotran et al., 1998).

2. Chronic inflammation, on the other hand, is a long lasting process. It may persist for weeks, months or even years. Chronic inflammation may be brought on by acute inflammation or it may be the result of an auto - immune disease.

Morphologic Features:

1) Infiltration with mononuclear cells (macrophages, lymphocytes and plasma cells) indicates persistent reaction to injury.

2) Tissue destruction

3) Repairing which involves in angiogenesis and fibrosis attempt to replace lost tissue

Mechanisms of macrophage accumulation during chronic inflammation:

1) Continued recruitment of monocytes from the circulation which are the most important source for macrophages

2) Local prolifetration of macrophages from the blood stream

3) Immobilization of macrophages within the site of inflammation. Cytokines and oxidized lipids can cause immobilization.

2.7.1 Inflammation caused by skin infections and antioxidant activity

Wound and skin infections represent the invasion of tissues by one or more species of microorganisms. This infection induces the immune system of the body causes inflammation and tissue damage, and slows the healing process. Many infections could effect in the small area, such as an infected scratch or hair follicle. Others may persist and untreated causing increase in severity and spread further and deeper into the body. Some infections spread to other organs or cause septicemia.

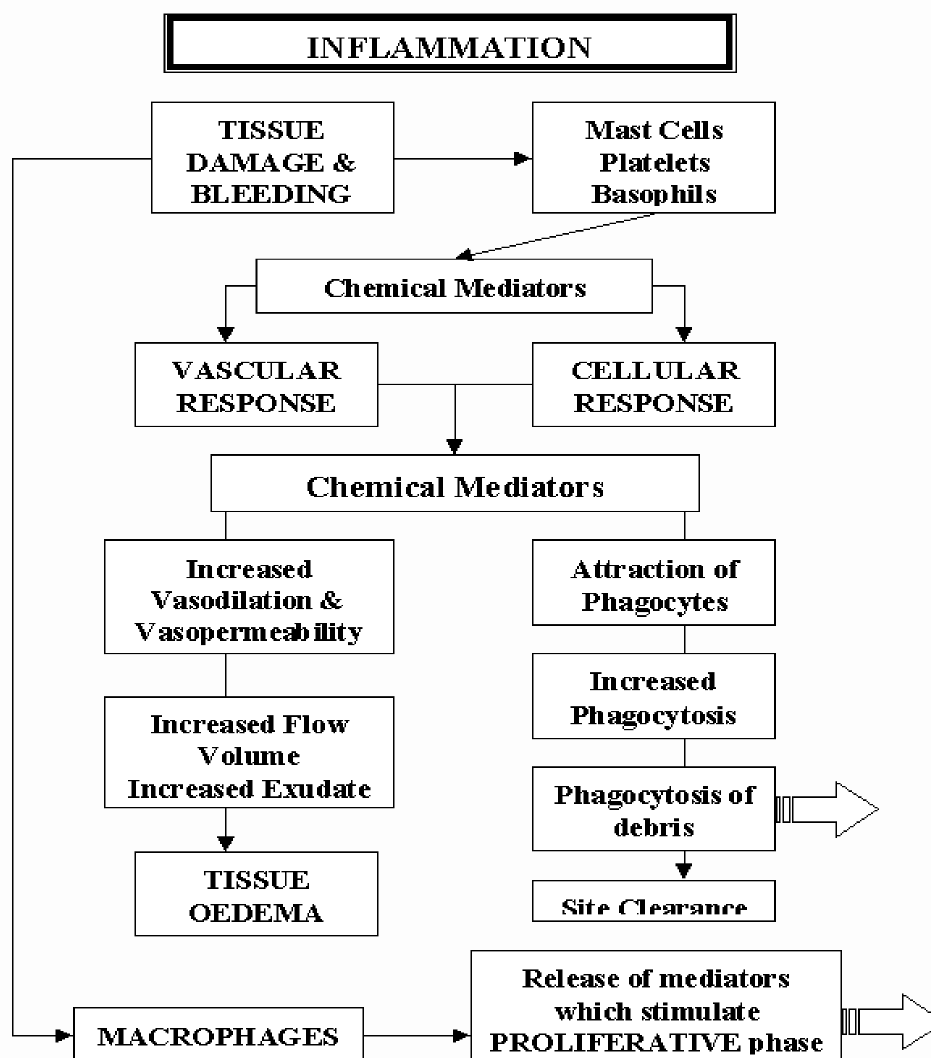


Figure 2-18 Mechanism of inflammation (Wagner, et al. 2003)

Skin is the largest organ of the body and its first line of defense. It is populated with a mixture of microorganisms called normal flora. This normal flora forms a dynamic barrier that helps to keep other more harmful microorganisms (pathogens). At any one time, a certain percentage of the general population will be carriers of a pathogen that displaces some of their normal flora and "colonizes" locations like the mucous membranes of the nose. Most of the time normal flora and colonizing pathogens do not cause illness and do not stimulate the immune system. If there is a break in the skin or if the immune system becomes compromised, then any of the microorganisms present can cause a wound or skin infection.

Wounds of the skin and tissues may be superficial cuts, scrapes or scratches but also include punctures, burns or may be the result of surgical or dental procedures. The microorganisms likely to infect them depend on the wound's extent and depth, the environment in which the wound occurs, and the microorganisms present on the skin. The skin has three layers: the outer epidermis, the dermis – where many hair follicles and sweat glands are located, and the fatty subcutaneous layer. Below these layers are membranes that protect connective tissues, muscle, and bone. Wounds can penetrate to any of these layers and skin. Infections can spread into them. Wound healing is a complex process that involves many related systems, chemicals, and cells are working together to clean the wound, seal its edges, and to produce new tissues and blood vessels.

The inflammatory response is the second line of the body defense against invasion by phathogens which are 4 steps (Figure 2-19).

1. Damaged tissue release histamines, increasing blood flow to the area.
2. Histamines cause capillaries to leak, releasing phagocytes and clotting factors into the wound.
3. Phagocytes engulf bacteria, dead cells and cellular debris.
4. Platelets move out of the capillary to seal the wounded area.

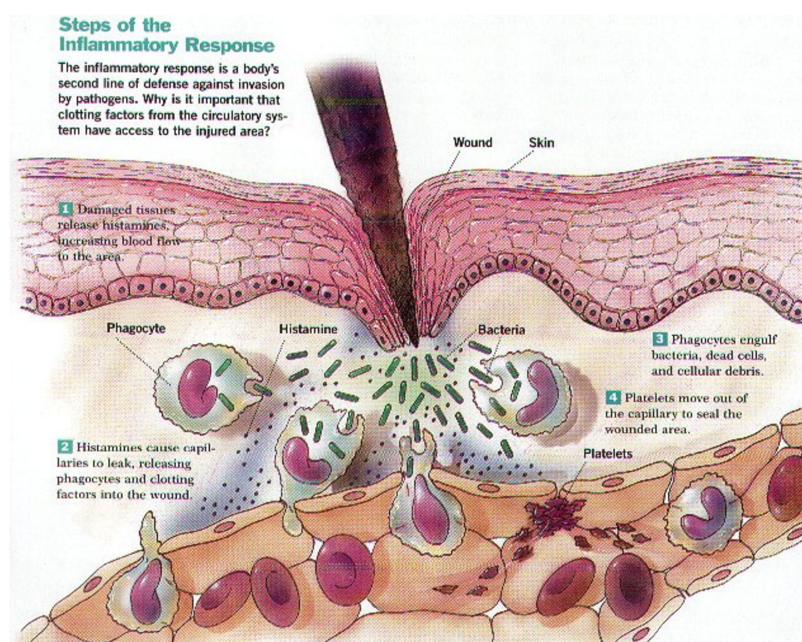


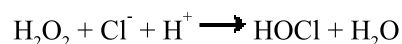
Figure 2-19 Step of the inflammatory response

(From: http://www.healthyfutures4all.com/images/img_inflammation.jpg)

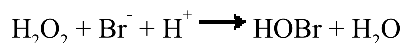
2.7.2 Oxidation reaction during inflammation

Reactive oxygen species have been linked to such a variety of diseases because of their potential for causing wide-ranging tissue damage. Reactive oxygen species can damage DNA and membranes and the oxidation products can induce protein damage, apoptosis, and the release of pro-inflammatory cytokines, leading to serious tissue damage if antioxidant capacity is insufficient (Briganti and Picardo, 2003). The modulatory effects of antioxidant on inflammation are high affinity for divalent ions of heavy metals which catalyze process involved in free radical generation (Younes and Siegers, 1981) and inhibit the production of reactive oxygen species by activated neutrophils by inhibiting either the release of the enzyme myeloperoxidase or its activity (Hart et al., 1990).

Inflammatory cells, particularly neutrophils, are an abundant source of highly reactive oxidants that are able to react with many biological targets. On stimulation, neutrophils generate a variety of oxidants through the action of the haem enzyme myeloperoxidase that can oxidise either chlorine anion or bromine anion to generate hypohalous acids as display in the scheme below.



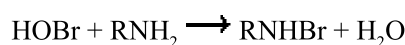
$$H_2O_2 + Cl^- + H^+ \longrightarrow HOCl + H_2O$$



$$H_2O_2 + Br^- + H^+ \longrightarrow HOBr + H_2O$$

HOCl is the reactive component of household bleach, and is famous for its anti-microbial activity. It is, however, indiscriminate in its action, can react with most cells and tissues, and is thought to be responsible for many deleterious effects of chronic and acute inflammation. Both HOCl and HOBr react readily with compounds containing amino group to generate chloramines or bromamines, which are potent oxidants.

$$HOCl + RNH_2 \longrightarrow RNHCl + H_2O$$



$$HOBr + RNH_2 \longrightarrow RNHBr + H_2O$$

Together with hypothiocyanous acid (HOSCN), these oxidants form can intiate a diverse range of effects on cells and tissues surrounding the stimulated neutrophil (Figure 2-20).

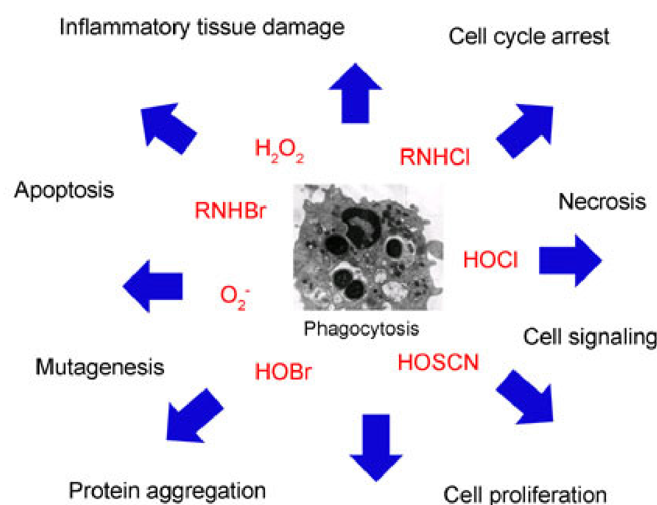


Figure 2-20 Reactive oxygen species of inflammation process

(From: http://www.chmeds.ac.nz/research/freerad/margreet.htm)

The participation of superoxide in the inflammatory response has been inferred from the anti-inflammatory effect of parenterally administered superoxide dismutase (superoxide: superoxide oxidoreductase) (Menander-Huber, 1977; McCord and Wong, 1979) upon activation by complement, aggregated immunoglobulin (Goldstein et al., 1975), endotoxin, lymphokines (Mccord and Salin, 1975), or phagocytosis of opsonized particles (Babior et al., 1973), neutrophils produce and release superoxide. This free radical or the oxidative species derived from it are toxic to cells and tissues (Babior et al., 1975).

During inflammation, neutrophils produce and release superoxide, a property presumably acquired as an antimicrobial mechanism. The superoxide produced by stimulated neutrophils reacts with the plasma precursor. Superoxide is unreactive toward purified polyunsaturated fatty acids. However, it can reduce hydroperoxides, presumably to alkoxy radicals (RO-) which may abstract hydrogen to become hydroxy fatty acids (Thomas et al., 1978). Neutrophils may have been drawn to the site initially by complement or bacterial or other chemotactic factors. The superoxide-dependent activity perpetuates and amplifies the arrival of inflammatory cells by continuously providing a neutrophil chemo-attractant for the duration of superoxide production. Upon elimination of the inflammatory stimulus, superoxide production would subside and thus preclude further generation of chemotactic activity, allowing the situation to resolve and return to normalcy.

Antioxidants are molecules that can neutralize free radicals by accepting or donating an electron to eliminate the unpaired condition. Typically this means that the antioxidant molecule becomes a free radical in the process of neutralizing a free radical molecule to a non-free-radical molecule. But the antioxidant molecule will usually be a much less reactive free radical than the free radical neutralized. The antioxidant molecule may be very large, it may be readily neutralized by another antioxidant and it may have another mechanism for terminating its free radical condition. Molecules with loosely-held hydrogen atoms can use those hydrogen atoms like electrons to neutralize free radicals. The hydrogen atoms are called reducing equivalents, and the molecules having such hydrogen atoms are said to be in a reduced state.

An antioxidant can neutralize a free radical by donating one of its electrons without jeopardizing its own chemical stability. In the human skin antioxidants are normally present in concentrations to deal with physiologically produced oxidative stresses. Notably these antioxidants decrease in concentration with age. The reason for antioxidants in the skin is that reactive oxygen species (ROS) can damage important skin components such as lipids and collagen. UV light also causes the formation of ROS. Examples of free radicals are the superoxide anion (O^{2-}) hydroxyl (OH^{-}) and nitric oxide (NO). The reactive oxygen species (ROS) indicates not only free radicals based on oxygen, but also some non-radical by-products of oxygen, like hydrogen peroxide (H_2O_2) and singlet oxygen (O_2) (http://www.alltracel.org/cc-antioxidation.html).

Phenolic compounds have been known to possess a capacity to scavenge free radicals. They are commonly found in both edible and nonedible plants, and have multiple biological effects, including antioxidant activity (Kähkönen et al., 1999; Valenzuela et al., 2003). The antioxidant activity of phenolic compounds is principally due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers. In addition, they have a metal chelation potential (Rice-Evans et al., 1996). Phenolic compounds, such as flavonoids, phenolic acids, stilbenes, lignans, lignin and tannins, are especially common in leaves, flowering tissues, and woody parts such as stems and barks (Larson, 1998). Larson has proposed that they played an important preventive role in the development of cancer, heart disease and ageing-related diseases.

Phenol antioxidants are known to interrupt the chain of free-radical oxidation through formation of phenoxyl radical. The main way of irreversible death of the radicals is the reaction of disproportionation. However, phenoxyl radicals are also able to form unstable ketodimers, yielding phenoxyl radical upon decomposition (Polyanskii and Muranov, 1994).

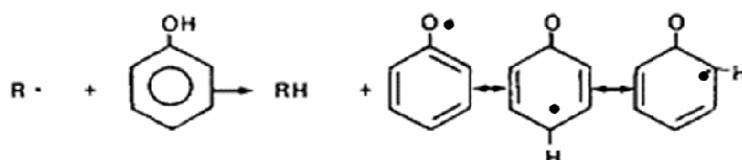
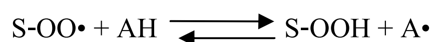


Figure 2-21 Antioxidant mechanism of phenolic antioxidants (From: Buck, 1981)

A primary mechanism for a phenolic antioxidant includes the trapping and stabilizing of species generated from the radical chain oxidation. The chain-breaking antioxidation process could be divided into two stages as follows:

(1) Radical trapping stage



(2) Radical termination stage

(Where S is oxidation substrate; AH is antioxidant; A• is antioxidant radical; A-A is dimer of A; SOOA is substrate antioxidant peroxide; Aox is oxidized A)

Although the first stage is a reversible process, the second stage is irreversible and must produce stable radical termination compounds. The structure of the termination compound would afford important information to reveal the antioxidant mechanism of the phenolic antioxidant (Masuda et al., 2001).

2.8 Bio-efficacies of chemical components of wood vinegar

2.8.1 Phenolic compounds

Phenolic compounds such as phenol, guaiacol and cresols were found to give antimicrobial, antioxidant and smoke flavor properties.

Phenol

Phenol which was obtained from coal tar has been widely used as a disinfectant for industrial and medical applications. It has antiseptic properties, and was used by Sir Joseph Lister (1827–1912) in his pioneer technique of antiseptic surgery, though the skin irritation caused by continue exposure to phenol eventually led to the substitution of aseptic techniques in surgery (Lister, 1867).

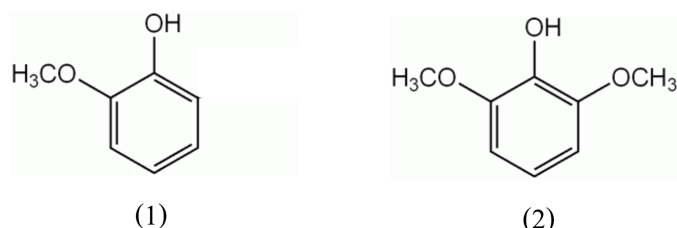
Phenol was also used as active ingredient in some oral analgesics such as Chloraseptic® spray. Phenol was also the main ingredient of the Carbolic Smoke Ball, a device marketed in London in the 19th century as protectant against influenza and other ailments. It was also used in the production of drugs (starting material in the industrial production of aspirin), herbicides, and synthetic resins. It was the desired chemical to use for embalming bodies for anatomical study because of its ability to preserve tissues for extended periods of time.

Phenol was also used in the preparation of cosmetics including sunscreens (Svobodova' et al., 2003), hair dyes, and skin lightening preparations (DeSelms, 2008). Compounds containing phenol moieties can be used to prevent ultraviolet light-induced damage to hair and skin due to the UV-absorbing properties of the aromatic ring of the phenol. It was also used in cosmetic surgery as an exfoliant, to remove layers of dead skin. It was also used in phenolization, a surgical procedure to treat an ingrown nail, in which it was applied to the nail bed to prevent regrowth of nails.

Guaiacol

Guaiacol (2-methoxyphenol) has a smokey flavor, found in roasted coffee, whisky, and smoke (http://en.wikipedia.org/wiki/Guaiacol). It is a naturally occurring organic compound with the formula $C_6H_4(OH)(OCH_3)$ (Figure 2-22). It is colourless aromatic oil which is derived from guaiacum or wood creosote. Guaiacol was commonly found in wood smoke which resulting from the pyrolysis of lignin. Roasted coffee also contains the compound, which contributes to the flavor

of the coffee (Dorfner et al., 2003). In preparation of smoked food, guaiacol was the main chemical responsible for the smoky taste, whereas syringol is responsible for the smoky aroma. Guaiacol has been used medicinally as an expectorant, antiseptic, and local anesthetic (http://en.wikipedia.org/wiki/Guaiacol).



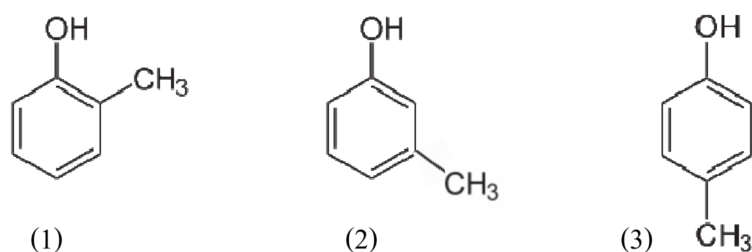
(1) (2)

Figure 2-22 Chemical structure of guaiacol (1) and syringol (2)

Cresols

Cresols are organic compounds which have a methyl group substituted onto the benzene ring of a phenol molecule. There are three forms of cresols that are slightly different in their chemical structure (Figure 2-23):

1. *ortho*-cresol (2-methylphenol)
2. *meta*-cresol (3-methylphenol)
3. *para*-cresol (4-methylphenol)



(1) (2) (3)

Figure 2-23 Chemical structures of cresols (1) *o*-cresol (2) *m*-cresol and (3) *p*-cresol

Cresols were used to dissolve other chemicals, as disinfectants and deodorizers, and to make specific chemicals that kill insect pests. Cresol solutions have been used as household cleaners and disinfectants, under the trade name Lysol®. In the past, cresol solutions have been used as antiseptics in surgery, but they have been largely displaced this role by less toxic

compounds. Lysol was also advertised as a disinfecting vaginal douche in mid-twentieth century in America. Besides, cresols are found in many grilled foods, wood and tobacco smoke, crude oil, coal tar, and in brown mixtures such as creosote, cresolene and cresylic acids, which are wood preservatives (Williams and Whitten, 1983).

Phenolics are membrane active antibacterial agents on the biochemical activities of bacterial cells. Effects of phenols on bacterial respiration have been reviewed by Hugo (1967). In general it was considered that, phenolic compounds at bactericidal concentrations inhibit the activity of respiratory enzymes, which led to the leakage of cytoplasmic constituents from the cell. At bacteriostatic concentrations of phenolic compounds, it must affect either oxidative or substrate level phosphorylation, or inhibit utilization of energy in metabolic reactions.

Many membrane- active antibacterial agents were known to cause uncoupling of oxidative phosphorylation in mitochondria1 preparations of animal cells including gramicidin (Lardy and Elvejham, 1945). 2, 4-Dinitrophenol (Loomis & Lipman, 1948), 3, 3', 4', 5-tetrachlorosalicylanilide (TCS) and other salicylanilides (Hamilton, 1968; Williamson and Metcalfe, 1967) which may suggest that oxidative uncoupling is responsible for inhibitory effects in bacterial cells.

The antifungal ability of phenolic compounds were depended on the fungal enzyme inhibition which contains thiol (SH) groups in their active sites. The antifungal activity of phenolic compounds might be influenced by the water soluble properties of phenolic compounds (Cowan, 1999). Since cell membrane acts as selective barrier for the passage of solution between cytoplasm and cell surface area, phenols can easily interact with cell membrane and damage it. This causes the cell burst and release of cell constituents from cytoplasm which leads the cell death (Park et al., 2001). The hydroxyl group of phenolic compounds can easily react with enzymes and form hydrogen bonds. These bonding formations can extent the inhibition of phenolic compounds (Farag et al., 1989). Ikeda found some correlation between the reduction potential and the fungicidal activity of 1,4-naphthoquinone derivatives and assumed that the binding ability of fungicides with SH group in the body of fungi may be the main factor determining the fungicidal activity (Ikeda, 1955).

2.8.2 Organic acids

Acetic acid

Acetic acid has been commonly used as medicine for more than 6000 years for the disinfection of wounds and, especially, as an antiseptic agent in the treatment and prophylaxis of the plague.

The use of acetic acid in the treatment of wound infection was found in 1916, when Taylor found that application of a 1% solution to war wounds led to the elimination of *Bacillus pyocyaneus* after 2 weeks treatment (Taylor, 1916).

In 1968, Phillips reported about the use of a 5% solution of acetic acid in superficial wounds could inhibit the growth of *P. aeruginosa*, however, the side effects, itching and pain, was reported (Phillips et al., 1968). Sloss et al. (1993) fould that acetic acid concentration between 1% and 5% showed effectiveness and clinical feasibility in would healing but itching and pain side effect still remain. Most effective was achieved by soaking the wounds twice daily for 15 min.

Other organic acids

Propionic acid and propionates (0.32%) have long been used as antifungal agent in bread, cake and Swiss cheeses. Benzoic and benzoates (0.1%) have been used as antifungal agent in margarine, cider, relishes and soft drinks. Sorbic acid and sorbates (0.2%) have been used as antifungal agent in cheeses, jellies, syrups and cakes. In addition wood smoke which contains organic acids could prevent microbial spoilage of meats, fish, etc (Todar, 2000).

2.8.3 Other organic components

Butyrolactone, corylone and malton, organic compounds found in wood vinegar, were tested for antimicrobial and smoke flavor properties.

Butyrolactone

Three synthetic butyrolactone derivatives which were metabolites from fungus *Aspergillus terreus* Thorn var. *terreus* were assessed for antimicrobial activity against Gram-positive and Gram-negative bacteria and fungi as well as for seed germination and seedling growth (Cazar et al., 2005). γ-Butyrolactone was a very common structural feature of a number of organic

compounds and was presented in about 10% of all natural products (Hoffmann and Rabe, 1985; Devon and Scott, 1975). These compounds displayed a broad range of biological ability including antibiotic, cytotoxic and antimicrobial activities (Yang et al., 1993; Davidson and Ireland, 1990).

Corylone and malton

Corylone (2-hydroxy-3-methyl-2-cyclopenten-1-one), malton (3-hydroxy-2-methyl-pyran-4-one) were the active ingredients which were used to produce the tobacco-like flavor (Anderson and Calder, 1975).

O OH O OH O O

(1) (2) (3)

Figure 2-24 Chemical structure of γ-Butyrolactone (1), corylone (2) and malton (3)

These organic acids and phenolic compounds gave an important role in bio-efficacies of wood vinegars were evaluated in previous studies. Yatagai and co-workers (2002) found that wood vinegar obtained from *Cryptomeria japonica* and *Pseudotsuga menziesii, Quercus serrata,* and *Pinus densiflora* contained acetic acid as the main organic acids and phenolic compounds which were phenol, 2-methoxyphenol (guaiacol), 2-methoxy-4-methyl phenol, 4-methylphenol (*p*-cresol), 2-methoxy-4-ethylphenol, 2,6-dimethylphenol (syringol), gave termiticidal activity. Phenolic components of wood vinegar obtained from sugi (*Cryptomeria japonica*) and acacia (*Acacia mangium*) which were 2-methoxyphenol, 2-methoxy-4-methylphenol, 4-ethyl-2-methoxyphenol, 2,6-dimethoxyphenol, 2,6-dimethoxy-4-methylphenol, *p*-cresol, *m*-cresol, *o*-cresol and phenol, play roles in antifungal activity against the brown-rot fungus, *Fomitopsis palustris* and the white-rot fungus, *Trametes versicolor* (Kartal et al., 2004).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

Bamboo (*Bambusa* spp.) wood vinegar, white popinac (*Leucaena leucocephala*) wood vinegar and eucalyptus (*Eucalyptus* spp.) wood vinegar were obtained from Pasak co. Ltd., Chon Buri, Thailand. Rubber (*Hevea brasiliensis*) wood vinegar was a gift from Assoc. Prof. Dr. Somchai Choochom, Faculty of Engineering, Prince of Songkla University. Karl Fischer reagent was obtained from Sigma, Steinheim, Germany. Analytical grade Folin reagent, analytical grade gallic acid, Na_2CO_3, 1, 1-diphenyl-2-picrylhydrazyl (DPPH), sodium acetate trihydrate ($C_2H_3NaO_2 \cdot 3H_2O$), 2, 4, 6-Tris(2-pyridyl)-1, 3, 5-Triazine (TPTZ), 6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox), 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)(ABTS), Potassium persulfate and Butylated hydroxytoluene (BHT) were obtained from Fluka, Buchs, Switzerland. $FeCl_3$ anhydrous, Tryptic soy agar (TSA), Tryptic soy broth (TSB), Müller-Hinton agar (MHA), Sabouraud dextrose agar (SDA), Sabouraud dextrose broth (SDB) and dried methanol SeccoSolv® (used for Karl Fischer titration) were obtained from Merck, Darmstadt, Germany. $MgSO_4$ anhydrous was obtained from *Panreac* (*Barcelona*, Spain). Brain Heart Infusion (BHI) *was obtained from Becton*- Dickinson, Meylan, *France*. Filter-paper discs (6.0 mm), 30 µg/disc tetracycline disc and 10 µg/disc norfloxacin disc were obtained from Oxoid, Hampshire, England. Isobutanol was obtained from *Carlo Erba*, *Milan*, Italy. Methanol was obtained from Labscan Ltd., Bangkok, Thailand. Ketoconazole (raw material), dichloromethane and diethyl ether were commercial grade and purchased from High Science Ltd. Songkhla, Thailand.

3.1.2 Microorganisms

The tested microorganisms used for biological evaluation consisted of bacteria and fungi. Four Gram-positive bacteria: *Staphylococcus aureus* (ATCC 29213)*, Staphylococcus epidermidis* (ATCC 1228), *Streptococcus faecalis* (ATCC 19212) and *Propionibacterium acne*s (DMST 14916) and a Gram-negative bacteria: *Escherichia coli* (ATCC 25922) were provided by department of Pathology, Faculty of Medicine, Prince of Songkla University. Fungi including one yeast: *Candida albicans* (TISTR 5799) was purchased from the Thailand Institute of Scientific and Technological Research (TISTR), *Trichophyton rubrum* (SH-MU-2), *Trichophyton mentagrophytes* (SH-MU-3) and *Microsporum gypseum* (SH-MU-4) were obtained from Songklanagarind *Hospital*.

3.2 Methods

3.2.1 Lyophilization of wood vinegar

Lyophilized wood vinegars were obtained by using freeze-drying method (Eyela FD-1, Tokyo, Japan) (Figure 3-1). They were obtained as dark brown viscous liquid with smoky odor. The products were kept in opacity container, protect from light until needed.

Figure 3-1 Freeze-dryer Eyela FD-1 (Rikakikai, Tokyo)

3.2.2 Extraction of wood vinegar

Extracted wood vinegars were obtained by extraction procedure using three different types of organic solvents, dichloromethane, diethyl ether and isobutanol. Aliquot of 300 ml of each wood vinegar were added to 500 ml separatory funnel. Organic solvent (50 ml) was then added to the funnel. The mixture was shaken and left standing until two phases separation was achieved (Figure 3-2). Each layer was down off separately. The aqueous phase was extracted again for another two times with the same organic solvent (50 ml each). The organic layer was dried over magnesium sulfate anhydrous. The organic solvent was then removed using rotary evaporator (Eyela, Japan) under reduced pressure to yield the extracts. The extracts were kept in opacity container, protect from light until needed.

Figure 3-2 Two phases (organic and aqueous phase) separation after extraction using organic solvents

3.2.3 Chemical analysis of wood vinegars

3.2.3.1. Karl Fischer titration (KFT) for determination of water contents in wood vinegars

A Karl Fischer Titrator (Mettler DL18, Figure 3-3) from Mettler Instruments, Greifensee, Switzerland) was used to determine amounts of water in the wood vinegar samples. The titrator was calibrated with dried methanol. Then 200 µL of original wood vinegar were drop in a container and titrated with the Karl Fisher reagent until end point. Results are the mean water contents ± S.D. (% mg of water/ml sample) of 3 different measurements of each sample.

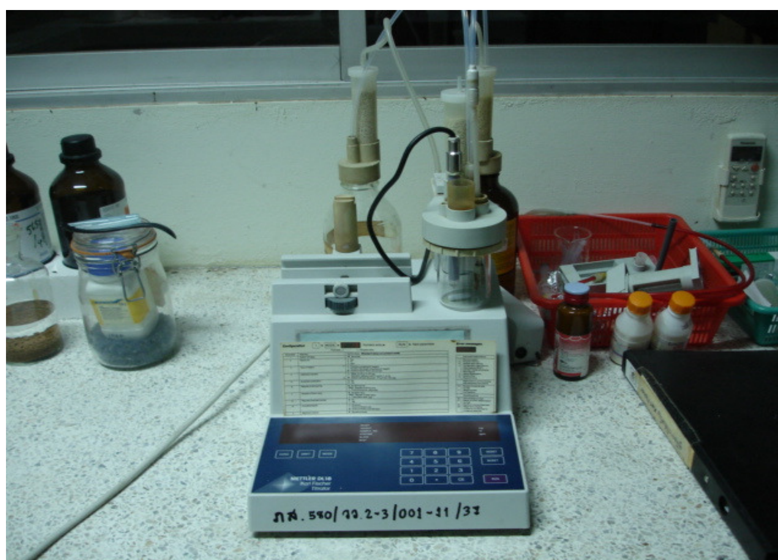


Figure 3-3 A Karl Fischer Titrator (Mettler DL18)

3.2.3.2 Determination of the chemicals composition of wood vinegars

The chemical compositions of wood vinegar samples were performed by gas chromatography-mass spectroscopy (GC-MS) (HP) (HP 5890-GC/5972 Mass Selective Detector) from Palo Alto, California, USA. The GC column was a 30 m × 0.25 mm capillary coated with cross linked polyethyleneglycol (film thickness 0.25 µm) (Stabilwax) from Saunderton, Buckinghamshire, England. The GC was programmed as follow: started at 70 °C, held 3 min, then increased to 240 °C at 6°C/min and held for 3 min. A splitless injector of 1 µL sample was used. The carrier gas was helium (flow rate 1.0 mL/min). MS was EI mode with acquisition scan mode

at 25-500 amu. Peak identify was confirmed by comparisons with standards in National Institute of Standards and Technology (NIST) library data base.

3.2.3.3 Phenol determination

Total phenolics content was determined by the Folin–Ciocalteu method (McDonald et al., 2001) using gallic acid as a standard comparison. The 0.2 mL of sample (original wood vinegars, 1 mg/mL of lyophillized wood vinegars or extract solution in methanol) and 1 mL of 0.25 N Folin reagent were combined in a 10 mL tube and mixed well using a vortex. The mixture was allowed to react for 3 min then 0.8 mL of 1 N Na_2CO_3 solution was added and mixed. An aliquot (150 µL) of each mixed was transfered with micropipette into each well of micro 96 well plate (COSTAR) and incubated at room temperature in the dark for 20 min. Absorbance was then measured at 765 nm using a spectrophotometer (PowerWaveX, Biotek, Maryland, USA) (Figure 3-4) and the results were expressed in gallic acid equivalents (GE; mg/mL of original wood vinegars, or mg/g mass of lyophilized wood vinegars or extracted wood vinegars).

Figure 3-4 A microplate reader pectrophotometer (PowerWaveX, Biotek, Maryland, USA)

The standard curve of gallic acid was prepared using an equal volume of methanol and water as the solvent. The gallic acid stock solution was prepared by accurately weigh about 25 mg

of gallic acid and dissolve in 50% aqueous methanol 25 ml to give 1 mg/mL solution. The gallic acid stock solution was further diluted in 50% methanol to obtain several concentrations (0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/mL). The phenol content of gallic acid solutions (0.2 mL) were tested as wood vinegar samples. Absorbance was then measured at 765 nm using a spectrophotometer (PowerWaveX, Biotek, Maryland, USA). The absorbance was plotted against concentrations (µg/mL) as a standard curve.

Additional dilution of sample (original, lyophilized and extracted wood vinegars) was carried out if the absorbance of tested samples was over the linear range of the standard curve.

3.2.4 Evaluation of bio-efficiencies of wood vinegars

3.2.4.1 Assessment of the inhibition of bacterial growth (Lorain, 1996)

Preparation of culture media

Tryptic soy agar (TSA) solutions were prepared by dissolve 40 g TSA with 1000 mL water and boiled. Melted media (10 mL) was filled in each tube (25 mL) and sterilized with autoclave (HV 110, Hirayama, Japan) (Figure 3-5) at 121 ºC, 15 PA for 15 min. Brain heart infusion media (BHI) were prepared by dissolve 37 g BHI and 1.5 % agar powder with 1000 mL of water and boiled. Melted media (25 mL) was filled in each tube (50 mL) and sterilized with autoclave at the same condition with TSA. Then the melted media were poured in sterile petridish (90 mm × 1.5 mm). Müller-Hinton agar (MHA) were prepared by dissolve 34 g MHA with 1000 mL water and boiled. Melted media (25 mL) was filled in each tube (50 mL) and sterilized with autoclave at the same condition with TSA and BHI. Then the melted media were poured in sterile petridish (90 mm ×1.5 mm).

Tested microorganisms

S. aureus, *S. epidermidis*, *S. faecalis* and *E. coli* cultures were incubated aerobically on tryptic soy agar (TSA) at 35 ºC for 24 hours. *P. acnes* was incubated anaerobically on brain heart infusion (BHI) media at 35 ºC for 3 days. These microorganisms were used for the preparation of inocula.

Antibacterial activity screening

Disc diffusion method was used to test the susceptibility for antibacterial activity. A colony of tested bacteria was taken to suspend in 0.85% normal saline solution. The cell suspension was diluted with 0.85% normal saline solution to achieve 0.5 McFarland standard suspensions (10^6 CFU/mL).

A sterile cotton swab was dipped in the inoculums and excess was removed by rotating the swab several times against inside wall of the tube above the fluid level. The surface of MHA and BHI plates were inoculated by streaking the swab over the surface. Streaking was repeated three times and each time the plate was rotated 60º to ensure an even distribution of inoculate.

Figure 3-5 An autoclave (HV 110, Hirayama, Japan)

Sterile paper discs (diameter 6 mm) were impregnate with 20 µL of original wood vinegar or 20 µl of reconstituted lyophilized wood vinegar (concentration 100 mg/mL in methanol) or 20 µl of reconstituted extracted wood vinegar (concentration 100 mg/mL in methanol) and were applied and placed on the surface of bacterium inoculated MHA or BHI. Control disc were prepared similarly using 20 µL of methanol or sterile water as negative control. Standard

antimicrobial drugs composed of tetracycline (30 µg/disc) for Gram-positive bacteria, norfloxacin (10 µg/disc) for Gram-negative bacteria. Each sample was tested in triplicate. Tested agar plates were incubated aerobically at 35 °C for 24 hours and anaerobically for *P. acne*s at 35 °C for 3 days. Inhibition zone were recorded as the diameter of growth-free zone, included the diameter of the disc, in cm at the end of the incubation period.

Determination of minimal inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC) value was determined using micro agar dilution assay. This experiment was modified from the method of Lorian (1996). A sequential two-fold dilution method was used in MIC test. Equal volume of samples (original wood vinegar, 16 mg/mL of lyophilized wood vinegar in methanol and 16 mg/mL of extracted wood vinegar in methanol) and broth culture media (TSB for aerobic bacteria and BHI for *P. acne*s) were mixed to obtain the first stock solution. The first stock solutions were further diluted with broth culture media sequentially in two-fold dilution manner to give the final tested solutions (1:1, 1:2, 1:4, 1:8, 1:16 ml wood vinegar: mL TSB of original wood vinegar; 16,000, 8,000, 4,000, 2,000, 1,000, 500, 250, 125, 62.5, 31.3, 15.6, 7.8 µg/mL of lyophilized wood vinegar and 16,000, 8,000, 4,000, 2,000, 1,000, 500, 250, 125, 62.5, 31.3, 15.6, 7.8 µg/mL of extracted wood vinegar). Tetracycline hydrochloride, a positive control was used as a reference standard. The tetracycline hydrochloride was dissolved and diluted in sterile water to have a concentration of 5,000 µg/mL and then filtered through 0.45 µm sterile filter paper (stock solution). The stock solution of tetracycline hydrochloride was diluted with TSB to give the concentration of 1000 µg/mL. Equal volume of 1000 µg/mL tetracycline solution and broth culture media (TSB and BHI) was mixed to obtain the first stock solution. The first stock solutions were further diluted with broth culture media sequentially in two-fold dilution manner to give the final tested solutions (500, 250, 125, 62.5, 31.3, 15.6, 7.8, 3.9, 1.95, 0.97, 0.48, 0.24, 0.12, 0.06 µg/mL). All solutions (150 µL) were transferred into each well of micro 96-well plate. The inoculums were prepared and adjusted as noted previously to contain approximately 1.0×10^6 CFU/mL, by adjusting the turbidity of saline culture to match the 0.5 McFarland standard. The inoculum (3 µL) was added to each well which was previously filled with broth culture media containing tested samples and tetracycline standard. The cultures were then incubated at 35 °C for 24 hours for *S. aureus*, *S. epidermidis*, *S. faecalis* and *E. coli* under aerobic condition, and at 35 °C for 3 days for *P. acnes* under anaerobic condition.

The experiment was performed in triplicate. A number of wells were reserved in each plate for sterile control (no inoculums added), inoculums viability control (no sample or tetracycline added) and the solvent control. MIC was defined as the lowest concentration of sample at which no visible growth was observed after incubation.

The minimal bactericidal concentration (MBC)

The MBC test known as the minimal lethal concentration (MLC), is the most common estimation of bactericidal activity and is defined as the lowest concentration of antimicrobial agent needed to kill 99.9% of the initial inoculums after incubation (Lorain, 1996).

In this experiment, the MBC was defined as the lowest concentration at which no turbidicity was visible after 24 hours of aerobic incubation at 35 ºC for *S. aureus*, *S. epidermidis*, *S. faecalis* and *E. coli* and after 3 days of anaerobic incubation at 35 ºC for *P. acnes*. The MBC was determined by swabbing of broth from each clear well onto MHA for *S. aureus*, *S. epidermidis*, *S. faecalis* and *E. coli or* BHI for *P. acnes*. The MBC was designated as the lowest concentration of bacterial growth after incubation at noted above. MBC of standard antibiotics, norfloxacin and tetracycline were performed similarly.

3.2.4.2 Assessment of the inhibition of the fungi-dermatophytes growth (Lorain, 1996)

Preparation of culture media

Sabouraud dextrose agar (SDA) solutions were prepared by dissolve 65 g SDA with 1000 mL water and boiled. Melted media (10 mL) were filled in each tube (25 mL) and sterilized with autoclave (HV 110, Hirayama, Japan) at 121 ºC, 15 PA for 15 min.

Tested fungi-dermatophytes

T. rubrum, *T. mentagrophytes* and *M. gypseum* cultures were grown aerobically on Sabouraud dextrose agar (SDA) at room temperature (27 °C + 2 °C) for 10 days and *C. albicans* culture were grown aerobically on SDA medium at 35 °C for 24 hours. These fungi were used for the preparation of inocula.

Antifungal susceptibility test

The disc diffusion method (Lorain, 1996) was used to screen for antifungal activity of wood vinegar samples.

The tested fungi were taken to suspend in sterile 0.85% normal saline solution. The cell suspension was further diluted to achieve 0.5 McFarland standard suspensions (10^6 CFU/mL).

One milliliter of standard suspensions (0.5 McFarland) of log phase growth cells of each fungus (*T. rubrum*, *T. mentagrophytes* and *M. gypseum*) was mixed with 25 mL melted (45 °C) SDA in 50 ml test tubes. The mixed medium was poured into Petri dishes (90 mm×15 mm) and left in a clean conditioning room (27 °C + 2 °C) for cooling and setting. Sterile filter-paper discs (diameter 6 mm) containing 20 µL of original WV or 0.2 mg/disc of lyophilized wood vinegar and extracted wood vinegar were then placed on the medium. The plates were incubated aerobically in the air-conditioned room for 3 days at room temperature (27 °C ± 2 °C). Any zone of inhibition occurring around the disc was then measured and compared with 25 µg/disc ketoconazole as the positive control.

For *C. albicans*, a sterile cotton swabs was submerged into the suspension and spread them onto the surface of SDA agar plates to form a lawn. Sterile filter-paper discs containing 20 µL of original WV or 2 mg/disc of lyophillized wood vinegar and extracted wood vinegar were then placed on the dry lawn and the plates were incubated aerobically at 35 °C for 24 hours. The film of *C. albicans* inoculated onto the plate produce just confluent growth after incubation. Any zone of inhibition occurring around the disc was then measured and compared with positive control (25 µg/disc ketoconazole). The assessments were performed in triplicate for each sample.

Determination of anti-dermatophytes minimal inhibitory concentrations (MIC)

The minimal inhibitory concentration (MIC) value was determined using micro agar dilution assay. This experiment was modified from the method of Lorian (1996). A sequential two-fold dilution method was used in MIC test.

Stock solution of wood vinegar samples was prepared by mixing an equal volume of original wood vinegar, 2000 µg/mL of lyophilized wood vinegar solution in methanol or extracted wood vinegar in methanol, with broth culture media (SDB) to obtain 1:1 (v/v) of original wood vinegar solution, 1000 µg/mL of lyophilized wood vinegar solution in SDB and 1000 µg/mL of extracted wood vinegars in SDB.

The stock solutions in SDB were further diluted with broth culture media (SDB) to give the test samples of original wood vinegar (1:1, 1:2, 1:4, 1:8 and 1:16 v:v), lyophilized wood vinegars (16,000, 8,000, 4,000, 2,000, 1,000, 500, 250, 125, 62.5, 31.3, 15.6 and 7.8 µg/mL) and

extracted wood vinegar (16,000, 8,000, 4,000, 2,000, 1,000, 500, 250, 125, 62.5, 31.3, 15.6 and 7.8 µg/mL).

Ketoconazole was used as positive control. It was prepared by accurately weigh about 10 mg and dissolved in 2 N HCl to have a concentration of 1 mg/mL as the stock solution. The two-fold serial dilution was utilized to prepare ketoconazole test solutions by first mixing an equal volume of ketoconazole stock solution with broth culture media (SDB) to give a solution having concentration of 500 µg/mL in SDB. The latter was further diluted with SDB to give a series of ketoconazole solution in SDB having concentrations of 500, 250, 125, 62.5, 31.3, 15.6, 7.8, 3.9, 1.95, 0.97, 0.48, 0.24, 0.12 and 0.06 µg/mL.

Minimal inhibitory concentration (MIC) evaluation was performed by using 3µl of fungal suspension (0.5 McFarland standard) aerobically incubated with 150 µL of test samples or positive control in each well of micro 96 well plate at room temperature for 7 days for *T. rubrum*, *T. mentagrophytes* and *M. gypseum* and 35 °C for 24 hours for *C. albicans*. The lowest concentration of each sample solution that inhibits fungal growth was used to determine the MIC.

The minimal fungicidal concentration (MFC)

The MFC test known as the minimal lethal concentration (MLC), is the most common estimation of fungicidal activity and is defined as the lowest concentration of antimicrobial agent needed to kill 99.9% of the initial inoculums after incubation (Lorain, 1996).

In this experiment, the MFC was defined as the lowest concentration at which no turbidicity was visible after 7 days of aerobic incubation at room temperature for *T. rubrum*, *T. mentagrophytes* and *M. gypseum* and after 24 hours at 35 ºC for *C. albicans*. The MFC was determined by swabbing of broth from each clear well onto SDA. The MFC was designated as the lowest concentration of fungal growth after incubation at noted above. MFC of standard antibiotics, ketoconazole was performed similary.

3.2.4.3 Antioxidant activity determinations

1) DPPH (1, 1-diphenyl-2-picrylhydrazyl) assay

The DPPH assay was carried out according to the method of Brand-Williams et al. (1995) with some modifications. The stock solution of DPPH (1, 1-diphenyl-2-picrylhydrazyl) was

prepared by dissolving 24 mg of DPPH in 100 mL methanol and stored at -20 °C until required. The working solution (240 µg/mL) was obtained by mixing 10 mL stock solution with 45 mL methanol to obtain an absorbance of 1.1 $\pm$ 0.02 units at 515 nm using a spectrophotometer. Wood vinegar samples (concentration range of 50-2000 µg/mL) were prepared using methanol as a solvent. Original wood vinegar was diluted in methanol to obtain concentration of 100, 500, 1000, 1500 and 2000 µg/mL. Lyophilized wood vinegar and extracted wood vinegar was dissolved in methanol to obtain the stock solution of sample (1000 µg/mL). Sample stock solutions were further diluted with methanol to obtain several concentrations; 100, 200, 300, 400 and 500 µg/mL for lyophilized wood vinegar; 200, 400, 600, 800 and 1000 µg/mL for alkalinized lyophilized wood vinegar; 50, 100, 200, 300 and 400 µg/mL for wood vinegar extracted by dichloromethane; 50, 100, 200, 300 and 400 µg/ml for wood vinegar extracted by diethyl ether; 200, 400, 600, 800 and 1000 µg/mL for wood vinegar extracted by isobutanol.

Butylated hydroxytoluene (BHT) was used as a positive standard. The stock solution of standard BHT was prepared by dissolved 50 mg of BHT in 50 mL of methanol to obtain 1 mg/mL of stock solution. The stock solution was further diluted with methanol to obtain several concentrations of BHT standard solutions (1, 5, 10, 50, 100, 150 µg/mL).

Aliquots (150 µL) of these solutions (wood vinegar sample and BHT standard) were allowed to react with 2.85 mL of the DPPH working solution for 24 h in the dark. The absorbance was then measured at 515 nm. Sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph plotted between % inhibitions against sample concentrations. The percentage of inhibition was calculated according to the following equation:

$$I\,\% = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

Where I = inhibition (%), $A_{control}$ = absorbance of DPPH solution, A_{sample} = absorbance of DPPH react with sample. Tests were carried out in triplicate for each sample.

2) Ferric Reducing Antioxidant Power (FRAP) assay

The Ferric Reducing Antioxidant Power (FRAP) assay was performed according to Benzie and Strain (1999) with some modifications. The stock reagent contained 300 mM acetate buffer pH 3.6 (3.1 g sodium acetate trihydrate ($C_2H_3NaO_2{\cdot}3H_2O$) and 16 mL glacial acetic acid

($C_2H_4O_2$), 10 mM TPTZ (2, 4, 6-Tris(2-pyridyl)-1, 3, 5-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The fresh working reagent (FRAP solution) was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3 \cdot 6H_2O$ solution and then warmed at 37°C before using.

Trolox standard curve was used as a reference. It was prepared by using 62.6 mg Trolox dissolved in 25 mL of 95% ethanol to obtain 0.01 M of the first stock solution. Ten mL of the first stock solution was dissolved in 100 mL of distilled water to obtain 1×10^{-3} M of the second stock solution. The second stock solution (0.25, 0.5, 1.0, 2.0, 4.0 and 5.0 mL) was transferred to each 10 mL volumetric flask and 10 mL of water was added to each flask to obtain the final concentrations of 0.025, 0.05, 0.1, 0.2, 0.4 and 0.5 mM respectively. Trolox solutions (150 µL) were allowed to reacted with 2.85 mL of the FRAP solution at 37 ºC for 30 min in the dark. Readings of the colored product [ferrous tripyridyltriazine complex] were then taken at 593 nm. The absorbance of product was plot against concentration (mM) to obtain the linear curve of a reference standard trolox solution.

One mg/mL of original wood vinegar (150 µL), 1 mg/mL of lyophilized wood vinegar solution in methanol (150 µL) or 1 mg/mL of extracted wood vinegar in methanol (150 µL) were allowed to react with 2.85 mL of the FRAP solution at 37ºC for 30 min in the dark and the absorbance was measure at 593 nm as standard trolox. Results are compared and expressed in Trolox equivalents (TE mg/g sample). Additional dilution was needed if the FRAP value measured was over the linear range of the standard curve of water soluble vitamin E (Trolox).

3) Trolox Equivalent Antioxidant Capacity (TEAC) assay

For Trolox Equivalent Antioxidant Capacity (TEAC) assay, the determination followed the method of Arnao et al. (2001) with some modifications. The stock solutions included 7.4 mM 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)(ABTS) solution and 2.6 mM potassium persulfate solution. The working reagent (ABTS working solution) was then prepared by mixing the two stock solutions in equal volumes and allowing them to react for 12 hours at room temperature (27$\pm$2 °C) in the dark. The ABTS test solution was then prepared by mixing 1 mL ABTS working solution with 60 mL methanol to obtain an absorbance of 1.1$\pm$ 0.02 units at 734 nm using the spectrophotometer. Fresh ABTS test solution was prepared for each assay. One

mg/mL of original wood vinegar (150 µL), 1 mg/mL of lyophilized wood vinegar solution in methanol (150µL) or 1 mg/ml of extracted wood vinegar in methanol (150µL) were allowed to react with 2.85 mL of the ABTS test solution for 2 hours in a dark condition. Then the absorbance was taken at 734 nm using the spectrophotometer. Results are expressed in Trolox equivalents (TE mg/g sample). Additional dilution was needed if the ABTS value measured was over the linear range of the standard curve of water soluble vitamin E (Trolox).

3.3 Statistical analysis

Statistical data analyses were performed using SPSS 11.5 software package. All experiments were performed in triplicate. The mean and standard deviation of at least three experiments were determined. A significant difference was considered at the level of $p < 0.05$ using Scheffe's multiple comparison. Correlations among data obtained were calculated using regression analysis. The results were analyzed by Pearson's correlation with a significant difference was considered at the level of $p < 0.01$.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Components of wood vinegars

4.1.1 Water content of wood vinegars

The result from Karl Fischer titration revealed that high amounts of water were found in all types of tested wood vinegar samples. Little difference was observed in the water content of the white popinac (87.9%) and eucalyptus wood vinegars (87.0%), followd by bamboo wood vinegar (82.2%). Rubber wood vinegar contained the lowest amount of water which only 78.3% (Table 4-1).

Table 4-1 Water contents of wood vinegars tested by Karl Fisher Titration

Wood vinegar	Water content (%) (Mean ± SD), n=3
Bamboo	82.2 ± 1.3
White popinac	87.9 ± 0.4
Eucalyptus	87.0 ± 0.4
Rubber	78.3 ± 0.5

Qualities of wood vinegar have been continually researched and developed for many years in Japan and China. The international qualifications of wood vinegar should be therefore based upon Japanese and Chinese standard which stated that wood vinegar should contain 80-90% of water (http://bantanthai.tarad.com). Water content in bamboo, white popinac and eucalyptus wood vinegars were in the acceptance criteria of the international standard qualification. However amount of water in rubber wood vinegar (78.3%) wass slightly lower than the standard. That may be due to the instrument used for preparation rubber wood vinegar was different from those prepared by Pasak co. Ltd.

4.1.2 GC-MS analysis of wood vinegars

4.1.2.1 GC-MS analysis of original wood vinegars

According to the Karl Fischer titration result (section 4.1.1), about 78-88% was water. So only about 12-22% were organic compounds. The chemical compositions of organic content were analyzed by gas chromatography with mass spectroscopy detector and the results are summarized in Table 4-2.

Table 4-2 Chemical components of original wood vinegars determined by GC-MS

Chemical components of original wood vinegars	**Relative quantity (% w/w)**			
	bamboo	**White popinac**	**eucalyptus**	**rubber**
Acetic acid	77.3	74.7	77.6	65.1
propanoic acid	2.0	2.1	2.2	-
Butanoic acid		0.9	0.9	-
Formic acid	-	-	-	1.8
Benzoic acid	-	-	0.3	0.1
Total organic acid content	79.3	77.7	81.0	67.0
phenol	2.0	3.5	1.9	14.1
2,6-dimethoxyphenol	1.3	1.4	3.2	-
4-methylphenol	0.3	0.3	-	-
2-methoxyphenol	1.3	1.3	0.6	1.5
3,4-dimethoxyphenol	5.9	-	-	1.2
Total phenolic content	10.8	6.5	5.7	16.8
1,2-cyclopentanedione	1.0	1.1	-	-
butyrolactone	2.1	2.1	2.0	-
2-hydroxy-3-methylcyclopent-2-en-1-one (Corylone)	-	-	-	0.8
furanmethanol	-	5.7	-	-
1,4:3,6-dianhydro-α-d-glucopyranose	-	-	0.3	-
1,4-banzenediol	-	-	-	0.2
Unidentified compounds	6.8	6.9	11.0	15.2
Total	100	100	100	100

Two main groups of compounds were found in all type of wood vinegars, organic acid (67.0-79.3%), and phenolic compounds (5.7-16.8%). Other compounds could be detected in small amount such as ketones and alcohols. It is however about 6.8-15.2% were compounds which could not be identified since they could not match well with the library data.

The GC-MS results revealed that the contents of the wood vinegar showed variability depending on original wood species (Table 4-2). Data in table 2 refers to peak areas for each component obtained from GC-MS chromatograms, divided by total area, times 100 to give a relative quantity in unit of % weight by weight.

From GC-MS analysis of bamboo WV, about 93% of the observed peaks were able to be identified. Among the identifiable compounds, about 79.3% were organic acid components. Acetic acid was found to be the major compound, while propanoic acid was a minor component. Phenolic compounds of about 10.8% were found in bamboo wood vinegar with 3,4-dimethoxyphenol being the major component. 1,2-Cyclopentanedione and butyrolactone were found in 1.0 and 2.1% w/w, respectively.

White popinac wood vinegar samples contained 77.7% organic acid components and 6.5% w/w phenolic compounds. Acetic acid was the main acidic components; propanoic acid was the minor component, with only small amount of butanoic acid. Phenol was the main component (3.5% w/w) of phenolic compounds found in white popinac wood vinegar. Other phenolic compound such as 2,6-dimethoxyphenol, 2-methoxyphenol and 4-methylphenol were found in 1.4, 1.3 and 0.3% w/w respectively. 1,2-Cyclopentanedione and butyrolactone were found similarly in this type of wood vinegar as in bamboo wood vinegar. Furanmethanol was found only in white popinac wood vinegar in 5.7% w/w. Other compounds which were not been identified was about 6.9%.

GC-MS analysis of eucalyptus wood vinegar resulted in peaks that were almost all identified (93.1%). Eucalyptus wood vinegar contained the highest amount of organic acid compound compared to other wood vinegar, acetic acid again as the main component (77.6%). In contrast, eucalyptus wood vinegar contained the lowest amout of phenolic content (5.7% w/w) with 2,6-dimethoxyphenols as a major constituent (3.2%). Phenol and 2-methoxy phenol were found in 1.9 and 0.6% w/w, respectively. 4-Methylphenol and 1,2-cyclopentanedione which were found in bamboo and white popinac wood vinegars were not detected in this type of wood vinegar. Butyrolactone was found in 2.0 % w/w. Moreover, 1,4:3,6-dianhydro-α-d-glucopyranose which

was not found in other wood vinegars was detected only in this type of wood vinegar in 0.3% w/w. About 11.0% w/w of compounds found in this type of wood vinegar were not be identified, and other neutral substances presented only in trace amounts. Finally, 84.8 % of the observed peaks of rubber wood vinegar were identified, with 67% were acidic components. Acetic acid was the major acidic component (65.1%) with 0.1% benzoic acid. Formic acid which not was presented in other wood vinegars was found only in trace amounts in rubber wood vinegar in 1.8%. The highest amount of phenolic contents was detected in rubber wood vinegar in 16.8%. Phenol was the major components (14.1%). Other phenols such as 2-methoxyphenol and 3,4-dimethoxyphenol were found in 1.5 and 1.2% w/w respectively. Ketones and other neutral substances were found only 1%.

The results indicated that chemical components of all four wood vinegars showed difference in the concentrations and types of components, which may be due to the source of wood used in production. Since lignocelluloses, the main unit components of wood materials are mainly composed of cellulose, hemicelluloses, and lignin (Nakai et al., 2005) which were degraded during carbonization process. The components in the wood vinegars were assumed to consist of products from carbonization of lignin which one of the main unit components of lignocelluloses composed of phenylpropane units and it degrades into gas, liquid and solid charcoal above the temperatures of 200 ºC. The liquid fraction generally contains water, alcohols, organic acids, ketones, aldehydes and phenolic compounds (Fengel and Wegener, 1984; Grandmaison and Kaliaguine, 1991; Chen et al., 2001). Therefore chemical components in liquid fraction of wood vinegar derived from wood carbonization were depended on unit components of wood materials which varied along type of wood species.

4.1.2.2 GC-MS analysis of lyophilized wood vinegars

The chemical compositions of the lyophilized wood vinegars were found greatly different from the original WVs (Table 4-3). Acidic compounds, the main components of original wood vinegars, were lost or reduced in all lyophilized wood vinegar samples during the lyophillization process, since alkyl carboxylic acids have low boiling point (acetic acid; 118.1 ºC, propanoic acid; 141 ºC, butanoic acid; 163.5 ºC, formic acid; 101 ºC and benzoic acid; 249 ºC) (Budavari et al., 2001).

Table 4-3 Chemical components of lyophilized wood vinegars determined by GC-MS

Chemical components of lyophilized wood vinegars	Relative quantity (% w/w)			
	bamboo	White popinac	eucalyptus	rubber
Acetic acid	-	9.2	41.5	3.5
Benzoic acid	-	3.5	1.7	1.2
Total organic acid contents	0	12.7	43.2	4.7
Phenol	6.7	5.4	4.1	36.6
2,6-Dimethoxyphenol	11.3	3.8	14.0	-
2-Methylphenol	-	-	-	2.4
2-Methoxyphenol	-	-	-	3.3
Total phenolic contents	18.0	9.2	18.1	42.3
2-Hydroxy-3-methylcyclopent-2-en-1-one (corylone)	-	-	-	3.0
Furanone	-	4.3	-	-
Butyrolactone	-	-	-	2.7
Pyridinone	-	-	-	1.2
Tetrahydrofurfuryl alcohol	-	36.5	25.2	-
Benzaldehyde	-	-	-	0.7
Unidentified compounds	82	37.3	13.5	45.4
Total	100	100	100	100

All acidic components were not detected in lyophilized bamboo wood vinegar. However, the bioactive residues responsible for their bio-efficacies were believed to be the phenolic components and these still remained in all the lyophilized wood vinegar samples. Phenol and 2,6-dimethoxyphenol was now the main compound in all lyophilized wood vinegar samples. 2-Methylphenol and 2-methoxyphenol were found only in rubber wood vinegar. Rubber wood vinegar presented the highest amount of total phenolic contents about 42%. Furanone, the ketonic compound was found only in white popinac WV. Corylone, butyrolactone and pyridinone were again found only in rubber wood vinegar. Neutral compound, tetrahydrofurfuryl alcohol was found in white popinac and eucalyptus wood vinegar, and benzaldehyde was found only in rubber wood vinegar.

4.1.2.3 GC-MS analysis of alkalinized lyophilized wood vinegars

Due to the loss of some chemical components, especially acidic fraction, wood vinegars were then alkalinized priror to lyophilization. That was expected to protect the acidic components during freeze-dry process. After lyophilization, the resulting residues were adjusted pH back to acid before testing. The result showed that alkyl carboxylic acid could be preserved, but most of other components were lost by this process. Therefore, alkalinized lyophilization could preserve volatile acidic components during lyophilize process. However, the other compositions, such as phenolics, ketonic, basidic and neutral components could not be recovered (Table 4-4).

Table 4-4 Chemical components of alkalinized lyophilized wood vinegars determined by GC-MS

Chemical components of alkalinized lyophilized wood vinegars	Relative quantity (% w/w)			
	bamboo	White popinac	eucalyptus	rubber
Acetic acid	57.7	76.5	76.1	72.0
Propanoic acid	4.7	-	3.6	-
Total organic acid content	62.4	76.5	79.7	72.0
Phenol	6.5	-	-	28.0
4-Methylphenol	4.9	-	-	-
Total phenolic content	11.4	0	0	28
Butyrolactone	1.0	-	-	-
Unidentified compounds	25.2	23.5	20.3	0
Total	100	100	100	100

The products from alkalinized lyophilized wood vinegars contained organic acids as the major content. Acetic acid was the major compound in all types of wood vinegars. Product from white popinac wood vinegar contained the highest amount of 76.5% w/w followed by product from eucalyptus wood vinegar (76.1%), rubber wood vinegar (72.0%) and bamboo wood vinegar (57.7%). Propanoic acid was detected in products from bamboo wood vinegar (4.7%) and eucalyptus wood vinegar (3.6%). Phenolic compounds were now detected only in products from bamboo wood vinegar (6.5% phenol and 4.9% methylphenol) and rubber wood vinegar (28%

phenol). Butyrolactone was detected (1.0%) only in product from bamboo wood vinegar. Products from bamboo, white popinac and eucalyptus wood vinegar contained 25.2%, 23.5% and 20.3% of unidentified compounds, respectively. Whereas, all peak of product from rubber wood vinegar could be able to identify.

4.1.2.4 GC-MS analysis of wood vinegars extracted by organic solvents

Another method which was used to concentrate the chemical compounds from wood vinegar samples was solvent extraction. In this study, three type of organic solvents used were dichloromethane, diethyl ether and isobutanol.

The GC-MS results revealed that the contents of the extracted wood vinegar showed variability depending on extraction solvent and types of original wood vinegar (Table 4-5, 4-6 and 4-7). Data in table 4-5, 4-6 and 4-7 refers to peak areas for each component obtained from GC-MS chromatograms, divided by total area, times 100 to give % relative quantity. Table 4-5 shows chemical components of wood vinegars extracted by dichloromethane. From GC-MS chromatogram, 65.2% of the observed peaks were identifiable. Among these about 20.3% were organic acids, acetic acid (14%) being the major acidic component, followed by propanoic acid (4.4%) and butanoic acid (1.9%) as a minor components. Total organic acid component decreased from 79.3 to 20.3% which may be due to the low solubility properties of these compounds in dichloromethane and the lost during solvent removing process. Phenolic compounds account for about 30.7% with phenol (10.9%) being the major component.

Other phenolic compounds found in dichloromethane extracted wood vinegars were 2,6-dimethoxyphenol (7.9%), o-methoxyphenol (6.2%), *p*-cresol (2.4%), m-cresol (1.0%) and *p*-ethylphenol (2.3%). Phenol, 2,6-dimethoxyphenol and 2-methoxyphenol which were previously detected in original bamboo wood vinegar were increased about 4-5 times. *p*-Cresol, m-cresol and *p*-ethylphenol which were not detected in original bamboo wood vinegar, were detected in dichloromethane extracted bamboo wood vinegar. Total phenolic compounds in dichloromethane extracted bamboo wood vinegar increased from 10.8% to 30.7% which may be because of the better solubility of these compounds in dichloromethane and the removing water from organic parts resulted in high organic components concentrations in the extract. 1,2-Cyclopentanedione which was found in original bamboo wood vinegar was not remained in dichloromethane extracted wood vinegar. Butyrolactone was found about 3.5 times higher than in original bamboo wood

vinegar. Ethanone, corylone, pyridine were now detected in dichloromethane extracted wood vinegar.

Table 4-5 Chemical components of wood vinegars extracted by dichloromethane analyzed by GC-MS

Components of wood vinegar extracted with dichloromethane	**Relative quantity (% w/w)**			
	bamboo	**White popinac**	**eucalyptus**	**rubber**
Acetic acid	14.0	18.3	12.2	26.5
Propanoic acid	4.4	4.9	2.9	3.2
Butanoic acid	1.9	3.1	2.1	1.1
Benzoic acid	-	0.1	1.3	1.5
Total organic acid content	20.3	26.4	18.5	32.3
Phenol	10.9	5.9	4.8	37.2
2,6-Dimethoxyphenol	7.9	7.2	-	5.0
3,4-Dimethoxyphenol	-	-	22.8	-
2-Methoxyphenol (*o*-methoxyphenol)	6.2	8.2	-	-
4-Methoxyphenol (*p*-methoxyphenol)	-	-	3.6	-
4-Methylphenol (*p*-cresol)	2.4	1.9	1.5	1.3
3-Methylphenol (*m*-cresol)	1.0	1.7	-	-
2-Methylphenol (*o*-cresol)	-	-	2.3	-
2-Methoxy-4-methylphenol	-	0.5	-	-
4-Ethylphenol	2.3	-	-	-
Total phenolic content	30.7	25.4	35.0	43.5
Ethanone	2.0	-	-	-
2-Hydroxy-3-methylcyclopent-2-en-1-one (corylone)	4.6	-	-	-
Butyrolactone	7.3	-	-	-
Pyridine	0.3	2.0	1.3	-
Furfural	-	-	-	3.6
Moltal	-	-	5.2	-
Unidentified compounds	34.8	46.2	40.0	20.6
Total	100	100	100	100

In dichloromethane extracted white popinac wood vinegar, GC-MS analysis resulted in 53.8% of all were identified. Organic acids and phenolic compound being the major components. Acetic acid was the major component for acidic fraction and 2,6-dimethoxy phenol was the major component for phenolic fraction. Propanoic and butanoic acids were still remined in white popinac dichloromethane extracted wood vinegar in about 2-3 times higher than in original white popinac wood vinegar. Benzoic acid which was not detected in original white popinac wood vinegar was now found in 0.1%. Similary to the bamboo wood vinegar, organic acid fractions reduced about 3 times compared to that found in original white popinac wood vinegar. Total phenolic content in white popinac dichloromethane extracted wood vinegar was found in about 4 times higher than in original white popinac wood vinegar. In this sample, 2-methoxyphenol (8.2%) was found the highest content, followed by 2,6-dimethoxyphenol (7.2%), phenol (5.9%), *p*-cresol (1.9%), m-cresol (1.7%) and p-creosol (0.5%), respectively. *p*-Creosol was found only in this sample. Pyridine was found the highest compared to other types of wood vinegar which was 2%. In dichloromethane extracted eucalyptus wood vinegar, 60% of the observed peaks were identifiable. Phenolic compounds (total of 35.0%) being the major components followed by organic acids (18.5%). 3,4-Dimethoxyphenol (22.8%) was the highest content of phenolic components. Phenol, 4-methoxyphenol, *o*-cresol and *p*-cresol were found in eucalyptus dichloromethane extracted wood vinegar in 4.8, 3.6, 2.3 and 1.5% w/w, respectively. *o*-Cresol was detected only in this extract. Acid components account for about 18.5%, having acetic acid (12.2%) as the main component, followed by propanoic acid (2.9%), butanoic acid (2.1%) and benzoic acid (1.3%). Pyridine, basic substance, was found in 1.3%. Moltal, a neutral substance was found only in this extract in 5.2%. Dichloromethane extracted rubber wood vinegar had 79% of the observed peaks were identified. Phenolic compounds (43.5%) again as the major component with phenol (37.2%) as the main phenolic substances followed by 2,6-dimethoxyphenol (5.0%) and *p*-cresol (1.3%). Acidic fraction (32.3%) was the minor component; acetic acid (26.5%) was the major organic acid component. Propanoic acid, butanoic acid and benzoic acid were found in 3.2, 1.1 and 1.5% w/w respectively. Furfural, a neutral substance, was found only in rubber wood vinegar in 3.6% w/w. Pyridine was not detected in dichloromethane extracted rubber wood vinegar.

The chemical compositions of wood vinegar extracted by diethyl ether are summarized in Table 4-6. The results were in contrast to the wood vinegar extracted by dichloromethane, acidic

fractions were the main components for all types of wood vinegar. From GC-MS analysis found that 67.9% of the observed peaks of bamboo wood vinegar were identifiable compounds. Among those about 46.2% were organic acid components with acetic acid being the major component followed by propanoic (7.4%) and butanoic (2.4%) acids.

Table 4-6 Chemical components of wood vinegars extracted by diethyl ether analyzed by GC-MS

Components of wood vinegar extracted with diethyl ether	**Relative quantity (% w/w)**			
	bamboo	**White popinac**	**eucalyptus**	**rubber**
Acetic acid	36.4	55.8	52.1	37.1
Propanoic acid	7.4	5.8	3.6	2.8
Butanoic acid	2.4	-	2.8	-
Benzoic acid	-	1.9	-	-
Total organic acid content	46.2	63.5	58.5	39.9
phenol	-	5.7	1.8	35.2
2,6-dimethoxyphenol	-	4.6	13.8	4.3
2-methoxyphenol (*o*-methoxyphenol)	6.7	-	2.6	4.8
4-methylphenol (*p*-cresol)	2.4	1.5	0.6	1.1
3-methylphenol (*m*-cresol)	-	-	-	0.6
2-Methoxy-4-methylphenol (*p*-creosol)	-	-	2.0	-
4-ethylphenol (*p*-ethylphenol)	3.5	-	-	-
4-ethyl-2-methoxyphenol	1.3	-	-	-
Total phenolic content	13.9	11.8	20.8	46.0
corylone	3.7	-	-	-
butyrolactone	2.0	2.2	-	-
1,2-butanone	-	-	1.6	-
benzene	2.1	-	-	-
furfural	-	-	-	2.0
Unidentified compound	32.1	22.5	19.1	12.1
Total	100	100	100	100

In this sample, phenolic compounds accounted for only 13.9% with 2-methoxyphenol as the major phenolic component (6.7%). The others phenolic compounds were *p*-ethylphenol (3.5%), *p*-cresol (2.4%), and 4-ethyl-2-methoxyphenol (1.3%). Corylone and benzene were detected only in this sample in 3.7% and 2.1%, respectively. Butyrolactone was found in 2.0%. White popinac wood vinegar contained the highest amount of organic acid in 63.5%. Acetic acid was the major organic acid components (55.8%). Diethyl ether extracted white popinac wood vinegar was found to have the lowest amount of phenolic content (11.8%) compared to others samples. Phenol (5.7%) was found the most followed by 2,6-dimethoxyphenol (4.6%) and *p*-cresol (1.5%). Butyrolactone was detected in 2.2%. Eucalyptus wood vinegar extract contained organic acids as the main components (58.5%). Acetic acid was the major acidic fraction (52.1%). Propanoic and butanoic acids were found in 3.6 and 2.8% respectively. Phenolic compounds was detected in 20.8% with 2,6-dimethoxy phenol was the major phenolic content (13.8%). Phenol was detected in the highest amount compared to other samples (35.2%). Other detected phenolic compound were 2,6-dimethoxyphenol (4.3%), *o*-methoxyphenol (4.8%), *p*-cresol (1.1%) and *m*-cresol (0.6%). Of the identifiable peaks about 39.9% was acidic fraction, acetic acid again as the major acid component. All of wood vinegar samples extracted by diethyl ether, neutral components were found in trace amounts. Corylone and benzene were found only in bamboo wood vinegar. Butyrolactone was found in bamboo and white popinac wood vinegar, and 1,2-butanone was found only in eucalyptus wood vinegar.

Table 4-7 shows the chemical compositions of wood vinegars extracted by isobutanol. GC-MS analysis of bamboo wood vinegar revealed that 42.3% of the observed peaks were identifiable. Among these about 15.6% were organic acids, acetic acid (14.2%) being the main organic acid components and benzoic acid (1.4%) was a minor component. Phenolic compounds account for about 13.1% with 2,6-dimethoxyphenol (11.4%) being the major phenolic component and phenol (1.7%) as a minor component. Other substances, about 10.8% were pyridine (2.8%), heptene (4.1%) and pyrazine (6.7%). GC-MS analysis of white popinac wood vinegar revealed that only 19.9% of all peaks were identifiable. Acetic acid was only organic acid found in this sample (8.6%). 2,6-Dimethoxyphenol, a phenolic compound was detected in 8.4%. 2-Propanol, was detected in 2.9%.

Table 4-7 Chemical components of wood vinegars extracted by isobutanol analyzed by GC-MS

Components of wood vinegar extracted with isobutanol	**Relative quantity (% w/w)**			
	bamboo	White popinac	eucalyptus	rubber
Acetic acid	14.2	8.6	1.1	-
Benzoic acid	1.4	-	-	-
Total organic acid	15.6	8.6	1.1	0
phenol	1.7	-	-	-
2,6-dimethoxyphenol	11.4	8.4	-	8.5
2-methoxyphenol	-	-	3.7	-
Total phenolic compounds	13.1	8.4	3.7	8.5
ethanone	-	-	-	3.4
pyridine	2.8	-	-	
2-propanol	-	2.9	-	-
butene	-	-	1.1	-
Oxirane	-	-	-	19.0
1,2-diethyldiborane	-	-	-	40.2
Benzene	-	-	8.6	3.7
benzaldehyde	-	-	-	0.9
heptene	4.1	-	-	-
pyrazine	6.7	-	-	-
Unidentified compound	57.7	80.1	85.5	24.3
Total	100	100	100	100

In isobutanol extracted eucalyptus wood vinegar, 14.5% of the observed peaks were identifiable. Acetic acid (1.1%) was found as an organic acid component. 2-Methoxyphenol (3.7%) could be detected as a phenolic compound. Benzene and butane were detected in 9.7% and 1.1%, respectively. None of organic acid was detected in rubber wood vinegar. 2,6-Dimethoxy phenol, a phenolic compound was found in 8.5%. Other substances, accounted for about 63.8% of the identified compounds, which were ethanone (3.4%), oxirane (19.0%), 1,2-diethyldiborane (40.2%), benzene (3.7%) and benzaldehyde (0.9%).

According to the GC-MS analysis, chemical compositions of wood vinegar varied among types of wood species and concentrate treatments. Among treatments, lyophilized wood vinegars showed the lost of many important components, such as organic acids and phenolics compounds, especially organic acids were almost lost on lyophilize process. While wood vinegar extracted by organic solvents presented higher amounts of important chemical components. Among organic solvents extracted wood vinegars, wood vinegar extracted by dichloromethane contained the highest amount of phenolics (average about 32%). That might be due to dichloromethane was not only has high efficiency in extracting polar compounds such as phenol, but also had high volatility (Guille'n and Manzanos, 1996; Yrieix et al., 1996). Wood vinegar extracted by diethyl ether presented higher amounts of acids components while most of chemical components were lost on extraction by isobutanol.

4.1.3 Phenolic components determined by the Folin-Ciocalteu method

The concentration of phenolic compounds was calculated using a calibration curve of gallic acid (concentration range of 0.02 - 0.4 µg/ml) as a polyphenol reference (n = 3) (Figure 4-1). The results were expressed in gallic acid equivalents (GE; mg/g mass of wood vinegars). These values were obtained from the absorbance of wood vinegar sample reacted with Folin-Ciocalteu reagent.

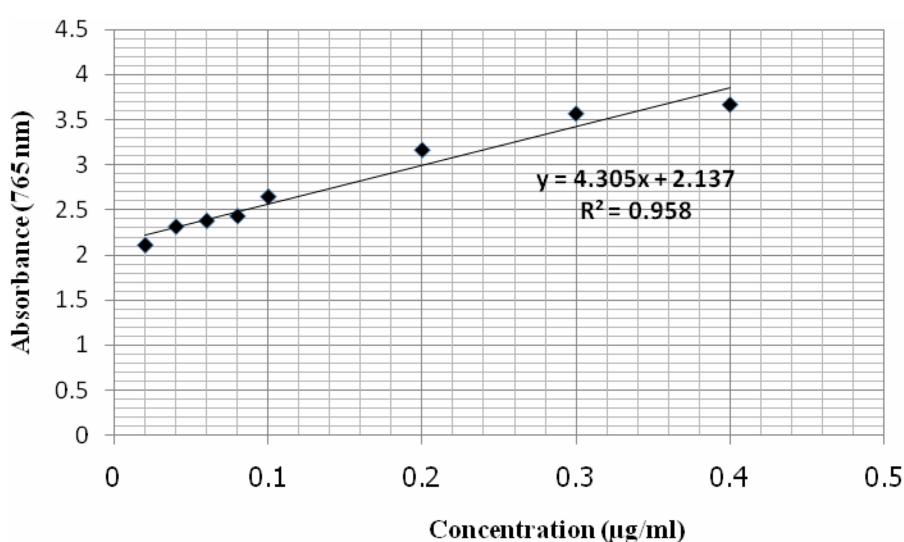


Figure 4-1 Standard curve of gallic acid determined by Folin-Ciocalteu method

Total phenolic contents of wood vinegars are shown in Figure 4-2. All of original wood vinegars contained phenolic contents less than 10 mg GE/g WV which were 7.5, 0.9, 2.7 and 9.2 mg GE/g WV in bamboo, white popinac, eucalyptus and rubber wood vinegars, respectively. After wood vinegars were subjected to lyophilization, the total amounts of phenolic compounds were greatly higher about 5 – 20 times than the original wood vinegars which were 42.9, 20.2, 44.7 and 83.9 mg GE/g WV in bamboo, white popinac, eucalyptus and rubber wood vinegars, respectively. If lyophilize process removed almost water content which was existed around 78 - 88% in wood vinegar samples; consequently, chemical components including phenolic contents should increase compared with original wood vinegar in 5-8 times. Conversely phenolic contents determined by Folin-Ciocalteu method demonstrated 6-20 times phenolic contents increased from original wood vinegars. Eventhough, some compounds were lost during lyophilize process, according to GC-MS analysis results, a number of unidentified compounds in GC-MS analysis could be the interfering substances and reacted with Folin reagent in Folin-Ciocalteu method. Wood vinegar treated by alkalinized lyophilization gave phenolic contents only in 0.5-3 times higher than original wood vinegars which were 11.7, 5.7, 7.9 and 17.6 GE mg/g WV; in bamboo, white popinac, eucalyptus and rubber wood vinegar, respectively. Beside phenolic contents of white popinac and eucalyptus wood vinegar found by Folin-Ciocalteu, could not be detected by GC-MS. It might be due to a number of interfering substances such as particularly sugars, aromatic amines, sulfur dioxide, ascorbic acid, etc. could react with Folin reagent (Phipps et. al., 2007). Otherwise, unidentified compounds of GC-MS analysis might be some of phenolic components.

Higher amounts of total phenolic contents could be found in wood vinegars extracted by organic solvents. Wood vinegar extracted by dichloromethane contained significantly high amounts of total phenolic contents which were 70.5, 55.6, 68.4 and 87.6 mg GE/g WV in bamboo, white popinac, eucalyptus and rubber wood vinegar, respectively. Wood vinegar extracted by diethyl ether also presented high total phenolic contents. The highest total phenolic contents contained in rubber wood vinegar with 90.7 mg GE/g WV, while, others showed lower total phenolic contents compared with wood vinegar extracted by dichloromethane with 37, 30 and 45 mg GE/g WV in bamboo, white popinac and eucalyptus wood vinegar, respectively. Among treatments, wood vinegar extracted by dichloromethane contained the highest amount of phenolics

(average around 70.5 mg GAE/g of WV) and wood vinegar extracted by diethyl ether contained total phenolic contents in average about 50 GAE/g of WV.

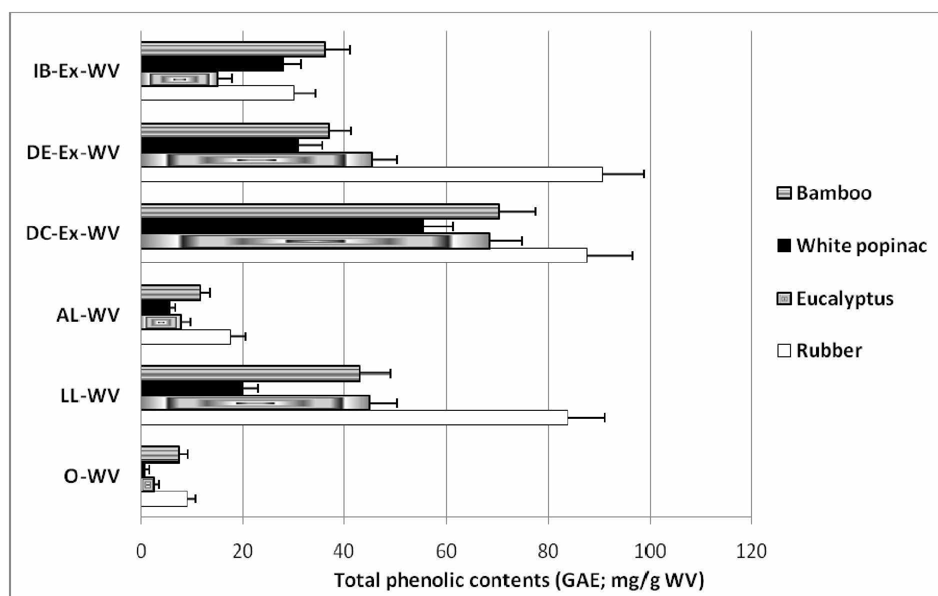


Figure 4-2 Total phenolic contents of wood vinegars (GAE; mg/gWV) (Mean $\pm$ S.D., n=3)

Original wood vinegar (O-WV); Lyophilized wood vinegar (LL-WV); Alkalinized lyophilized wood vinegar (AL-WV); Wood vinegar extracted by dichloromethane (DC-Ex-WV); Wood vinegar extracted by diethyl ether (DE-Ex-WV); Wood vinegar extracted by isobutanol (IB-Ex-WV)

Lower total phenolic content was found in wood vinegar extracted by isobutanol which ranged from 15-36 mg GAE/g of WV and in average of 27 mg GAE/g of WV. Therefore, concentrated treatments and types of wood vinegar had influences in amount of total phenolic compounds (Table 4-8 and 4-9). Table 4-8 shows four groups of variation for the observed mean of phenolic contents among concentrate treatments of Scheffe's multiple comparison with the mean difference reaching the most significant level at 95% ($p < 0.05$). However, there was no difference between total phenolic contents of original wood vinegar and alkalinized lyophilized wood vinegar, also between lyophilized wood vinegar and wood vinegar extracted by diethyl ether.

Table 4-8 Homogeneous subsets of total phenolic contents among concentrate treatments of all wood vinegar types

Wood vinegar samples	Total phenolic contents (mg GAE/gWV)*			
	1[a]	2[a]	3[a]	4[a]
Original WV	5.0625[b]			
Alkalinized lyophilized WV	10.7167[b]			
Isobutanol extracted WV		27.4333		
Lyophilized WV			47.8750[b]	
Diethyl ether extracted WV			51.0000[b]	
Dichloromethane extracted WV				70.5583

* Means for groups in homogeneous subsets (n = 12)

[a] Significant differences ($p < 0.05$) among treatments

[b] No significant differences ($p > 0.05$) among treatments

Table 4-9 Homogeneous subsets of total phenolic contents among wood vinegar types of all concentrate treatments

Wood vinegar samples	Total phenolic contents (mg GAE/gWV)*		
	1[a]	2[a]	3[a]
White popinac WV	23.5333		
Eucalyptus WV		30.7222[b]	
Bamboo WV		34.3389[b]	
Rubber WV			53.1694

* Means for groups in homogeneous subsets (n = 18)

[a] Significant differences ($p < 0.05$) among treatments

[b] No significant differences ($p > 0.05$) among treatments

In all types of wood vinegars, wood vinegar extracted by dichloromethane (subset 4) presented the highest estimated marginal means of total phenolic contents (around 70.6 mg GAE /g

WV). Followed by wood vinegar extracted by diethyl ether (around 51.0 mg GAE /g WV) and lyophilized wood vinegar (around 47.9 mg GAE /g WV) (subset 3), wood vinegar extracted by isobutanol (around 27.4 mg GAE /g WV) (subset 2), as well as alkalinized lyophilized wood vinegar (around 10.7 mg GAE /g WV) and original wood vinegar (around 5.1 mg GAE /g WV) (subset 1).

Type of wood vinegar was also an important factor following concentrate treatment playing roles in total phenolic contents. Table 4-9 shows four groups of variation for the observed mean of phenolic contents among wood vinegar types of Scheffe's multiple comparison with the mean difference reaching the most significant level at 95% ($p < 0.05$). In all concentrate treatments of wood vinegars, rubber wood vinegar (subset 4) displayed the highest estimated marginal means of total phenolic contents (around 53.2 mg GAE /g WV). Followed by similarity of bamboo wood vinegar (around 34.3 mg GAE /g WV) and eucalyptus wood vinegar (around 30.7 mg GAE /g WV) in subset 2, and the lowest total phenolic contents presented in original wood vinegar (around 23.5 mg GAE /g WV) (subset 1).

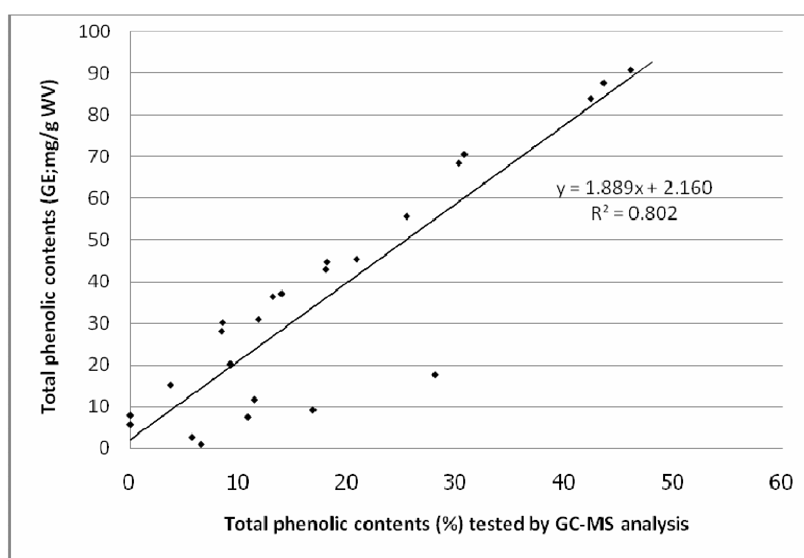


Figure 4-3 Correlation between total phenolic contents detected by GC-MS analysis and Folin-Ciocalteu method

The correlation between total phenolic contents detected by GC-MS analysis, and total phenolic contents tested by Folin-Ciocalteu method were displayed in Figure 4-3. It can be inferred from Figure 4-3 that positive correlation existed between total phenolic contents observed

using GC-MS and Folin-Ciocalteu method ($R^2 = 0.082$), with the correlation coefficient reaching the most significant level ($p < 0.01$), indicating that the total phenolic contents detected by GC-MS analysis was related to the total phenolic contents detected by Folin-Ciocalteu method.

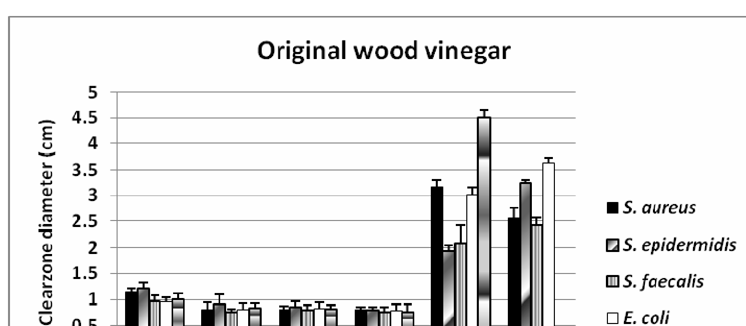
4.2 Inhibition of bacterial growth

The antibacterial activity of wood vinegars was determined on MHA and BHI agar plates for screening inhibitory activity and evaluates the inhibition value in TSA and BHI broth solution. Growth of *S. aureus*, *S. epidermidis, S. faecalis, E. coli* and *P. acne*s film was inhibited by all wood vinegars types. Clear zones could be observed around 6 mm paper discs containing 20 µl of all the kinds of wood vinegars. However, water and methanol showed no activity against all tested bacteria. Antibacterial activities of wood vinegars were similar among wood species with inhibition zone ranged from 0.75-1.2 cm (Figure 4-4; a) and MIC values ranging of 1:4 – 1:8 ml wood vinegar: ml TSB. Bamboo wood vinegar gave higher clear zone size (0.95-1.2 cm) against all five bacterial species compared to the other three wood vinegars which afforded similar results, correlated with MIC value. The lowest MIC value presented in bamboo wood vinegar against all tested bacteria (1:8 ml wood vinegar: ml TSB) while MIC value of the other three wood vinegars was 1:4 ml wood vinegar: ml TSB. The biggest clear zone size was 1.2 cm, presented in bamboo wood vinegar against *S. epidermidis*. However, the antibacterial activity of wood vinegars was lower than standard antibiotics (tetracycline and norfloxacin) about 50-80%. Inhibition zone of tetracycline and norfloxacin was in the range of 1.9-4.5 cm. It is worth to note that all types of original wood vinegars displayed antibacterial activity against all types of tested bacteria; however, tetracycline and norfloxacin presented different activities with different types of bacteria. The standard antibiotics clearly show selectivity, with tetracycline gave the highest activity against *P. acne*s with clear zone size was 4.5 cm and MIC was 0.24 µg/ml as well as the lowest activity against *S. epidermidis* with clear zone size was 1.9 cm and MIC was 7.8 µg/ml. Similarly norfloxacin gave the highest activity against *E. coli* with clear zone size was 3.6 cm and the lowest activity against *S. faecalis* with clear zone size was 2.4 cm. While there appeared to be little selectivity of the four wood vinegars for five different tested organisms. Since wood vinegars composed of 78-88% of water, biological activities much less than the standard antibiotics,

therefore, putative active principles in the bacterial testing protocol, wood vinegars were concentrated by removing water by lyophilize process.

Better antibacterial activity was observed in lyophilized wood vinegars. The 2 mg/disc of lyophilized wood vinegar gave bigger size of inhibition zones with were ranged from 0.8-1.8 cm (Figure 4-4; b) and with MIC values ranging of 125-1000 μg/ml. However, the activity profiles changed significantly after lyophilizing. Although almost of water was removed during lyophilize process, GC-MS analysis reported that organic acids were also lost, and phenolic compounds presented in the main chemical components. In original wood vinegars, the content of acetic acid was particular high but their antimicrobial effect was extremely weak. Ketone and the other organic acids had a weak antimicrobial effect. Matsuda and co-workers (1994) reported that organic acids such as acetic and propionic acids showed weak activities of growth inhibition against yeasts and bacteria (MIC 10.0-50.0 mg/ml). Phenolic compounds such as phenol and cresols have been well known as an antimicrobial agent (Nishimura, 1987[a,b]).

Phenolic compounds might considerably contribute to the antimicrobial activities of the wood vinegars, because the total content of the phenolic compounds was presented as the main chemical components. Total phenolic contents of bamboo, white popinac, eucalyptus and rubber lyophilized wood vinegars presented in 18.0, 9.2, 18.1 and 42.3% w/w, respectively; which already reported in GC-MS result. Rubber wood vinegar showed much more phenolic compounds and gave higher clear zone size compared to others against *S. aureus*, *S. faecalis, E. coli* and *P. acnes* which clear zone size was 1.5, 1.25, 1.3 and 1.4 cm, respectively and MIC value was 250 μg/ml against all four bacteria. And much more active against *S. epidermidis* with gave the biggest clear zone size was 1.8 cm and MIC value was 125 μg/ml. Clearly the change in ratios of various chemical constituents that occur during the lyophilize process affected on the activity profiles of the lyophilized wood vinegar. Because of around 80-90% of water presented in the original wood vinegar, after lyophilization antibacterial activity should increased 9-10 times from original wood vinegars, while; clear zone size of lyophilized wood vinegars increased less than 2 times from original wood vinegars. It might be due to the loss of some chemical components, especially, acids and some of phenolic components on lyophilize process.



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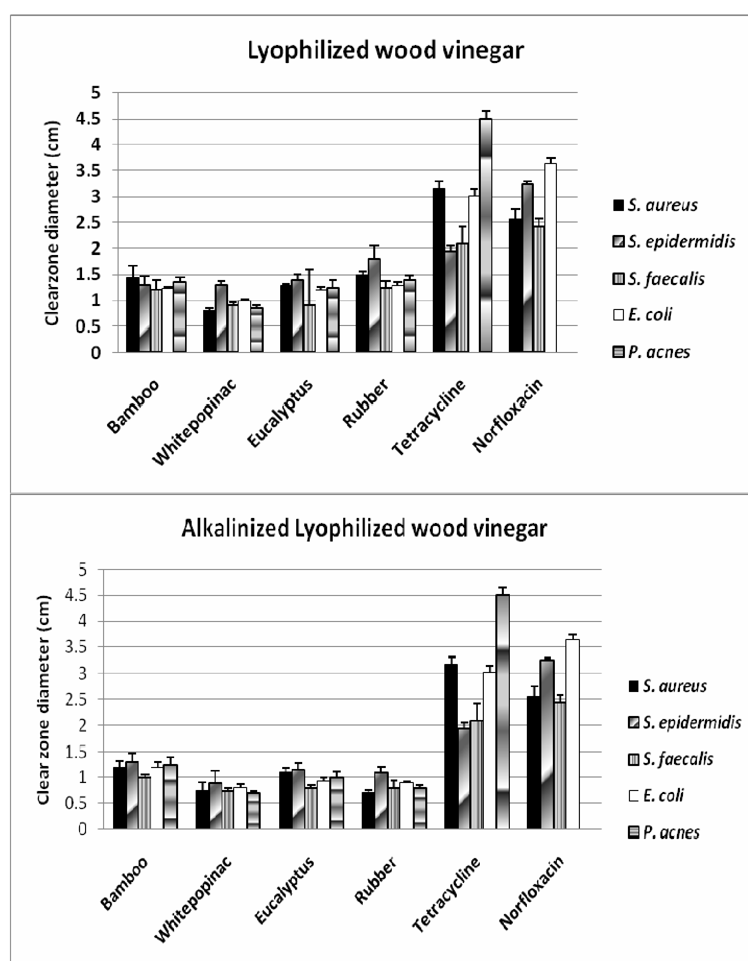


Fig 4-4 Antibacterial activity of original WVs (a), LL-WVs (b) and alkalinized LL-WVs (c) tested by disc diffusion assay (20 µl/disc Original WV; 2 mg/disc LL-WV and alkalinized LL-WV; 30 µg/disc Tetracycline; 10 µg/disc Norfloxacin

Table 4-10 Minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of original wood vinegars against tested bacteria

Bacteria / WV	Bamboo		White popinac		Eucalyptus		Rubber		Tetracycline	
	MIC (ml : ml)	MBC (ml : ml)	MIC (ml : ml)	MBC (ml : ml)	MIC (ml : ml)	MBC (ml : ml)	MIC (ml : ml)	MBC (ml : ml)	MIC (ml : ml)	MBC (ml : ml)
S. aureus	1:8	-	1:4	-	1:4	-	1:4	-	0.48	31.3
S. epidermidis	1:8	-	1:4	-	1:4	-	1:4	-	7.8	31.3
S. faecalis	1:8	-	1:4	-	1:4	-	1:4	-	7.8	62.5
E. coli	1:8	-	1:4	-	1:4	-	1:4	-	0.48	250
P. acnes	1.8	-	1:4	-	1:4	-	1:4	-	0.24	15.6

Table 4-11 Minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of lyophilized wood vinegars against tested bacteria

Bacteria / WV	Bamboo		White popinac		Eucalyptus		Rubber		Tetracycline	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
S. aureus	250	500	1000	2000	250	500	250	1000	0.48	31.3
S. epidermidis	500	500	250	2000	250	500	125	500	7.8	31.3
S. faecalis	500	1000	1000	2000	1000	2000	250	500	7.8	62.5
E. coli	500	2000	1000	4000	500	1000	250	1000	0.48	250
P. acnes	250	500	1000	2000	500	1000	250	500	0.24	15.6

Table 4-12 Minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of alkalinized lyophilized wood vinegars against tested bacteria

Bacteria / WV	Bamboo		White popinac		Eucalyptus		Rubber		Tetracycline	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
S. aureus	500	1000	2000	4000	500	1000	2000	4000	0.48	31.3
S. epidermidis	500	1000	1000	2000	500	500	500	1000	7.8	31.3
S. faecalis	1000	2000	2000	4000	2000	4000	2000	4000	7.8	62.5
E. coli	500	2000	2000	8000	1000	4000	1000	4000	0.48	250
P. acnes	500	1000	2000	4000	1000	2000	1000	2000	0.24	15.6

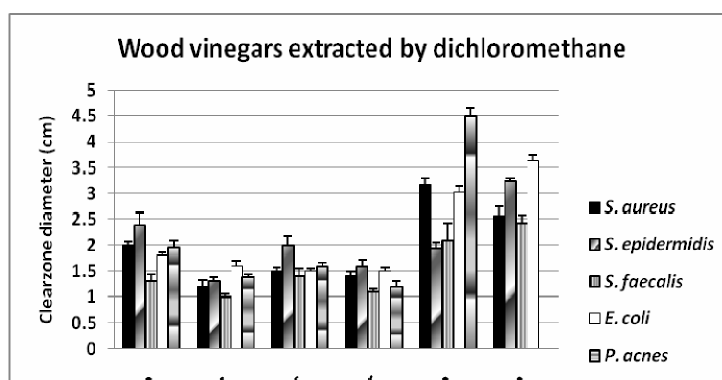
Consequently, wood vinegar should be preserved the chemical components during removing water in lyophilize process. We expected that wood vinegar might be alkalized with a strong basic compound, such as NaOH before lyophilizing. The basic substances would form

complexes with acids in wood vinegar. We expected that the salt complexes could be fixed and preserved during the lyophilize process.

After lyophilize process, the alkalinized lyophilized wood vinegars produced clear zone size ranged from 0.7-1.3 cm (Figure 4-4; c) and MIC value ranging of 500-2000 µg/ml (Table 4-12). Bamboo wood vinegar gave bigger clear zone size than the others against all tested bacteria, which ranged from 1.0-1.3 cm and MIC value ranging of 500-1000 µg/ml. The largest clear zone was 1.3 cm against *S. epidermidis*. The antibacterial activity was just slightly better than original wood vinegars, and exhibited weaker than lyophilized wood vinegars. In this process, most of organic acids could be preserved by the alkalinized lyophilize method; however, phenolic compounds and other substances were lost on this process. Although organic acids in alkalinized lyophilized wood vinegars presented much more than organic acids in wood vinegars from lyophilize method before, they gave lower potential in antibacterial property. Due to organic acids such as acetic and propionic acid were exhibited weak antibacterial activity (Matsuda, 1994). Phenolic compounds contributed the antibacterial activity. Phenolic compounds such as phenol and cresols have been well known as an antimicrobial agent (Nishimura, 1987[a,b]).

Since the chemical compositions of all wood vinegars were mainly phenolic compounds and organic acids (Fengel and Wegener, 1984), wood vinegars were then concentrated by using solvent extraction procedure. The solvents used in this study were dichloromethane, diethyl ether and isobutanol. The extracted residues were then utilized for antibacterial activity and the results were summarized in Figure 4-5.

After extraction of wood vinegars by organic solvents, antibacterial activities were higher than lyophilized wood vinegars. The results revealed that dichloromethane and diethyl ether extracts of wood vinegar exhibited strong antibacterial activity against all five tested bacterial species. The inhibition zones were in the range of 1.0-2.4 cm with MIC values were 31.3-1000 µg/ml of dichloromethane extracted wood vinegars, and 1.5-2.8 cm of inhibition zone with MIC values ranging of 31.3-125 µg/ml of diethyl ether extracted of wood vinegars.



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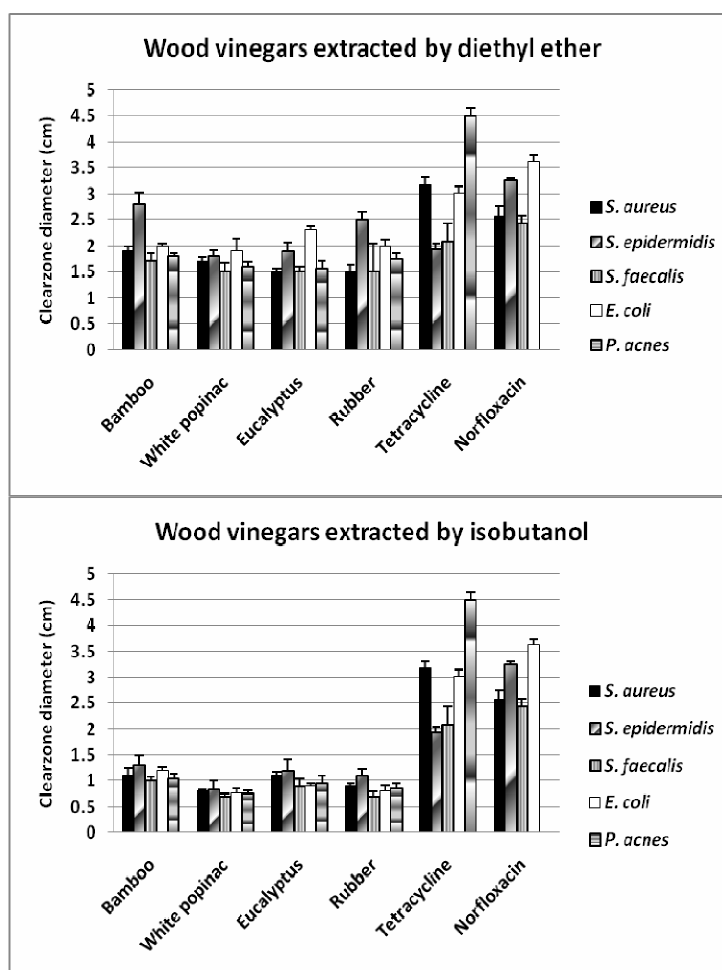


Fig 4-5 Antibacterial activity of wood vinegars extracted by organic solvents, dichloromethane (a), diethyl ether (b) and isobutanol (c) tested by disc diffusion assay (2 mg/disc WV; 30 µg/disc Tetracycline; 10 µg/disc Norfloxacin)

Table 4-13 Minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of wood vinegars extracted by dichloromethane against tested bacteria

Bacteria / WV	Bamboo		White popinac		Eucalyptus		Rubber		Tetracycline	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
S. aureus	62.5	125	500	1000	125	250	250	500	0.48	31.3
S. epidermidis	31.3	62.5	250	500	62.5	62.5	125	250	7.8	31.3
S. faecalis	125	250	1000	1000	125	250	500	1000	7.8	62.5
E. coli	62.5	250	125	500	125	500	125	500	0.48	250
P. acnes	62.5	125	125	500	125	250	500	1000	0.24	15.6

Table 4-14 Minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of wood vinegars extracted by diethyl ether against tested bacteria

Bacteria / WV	Bamboo		White popinac		Eucalyptus		Rubber		Tetracycline	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
S. aureus	62.5	125	125	250	125	250	125	250	0.48	31.3
S. epidermidis	31.3	62.5	62.5	125	62.5	62.5	31.3	125	7.8	31.3
S. faecalis	62.5	125	250	500	125	250	125	250	7.8	62.5
E. coli	62.5	125	62.5	250	31.3	125	62.5	250	0.48	250
P. acnes	62.5	125	125	250	125	250	125	250	0.24	15.6

Table 4-15 Minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of wood vinegars extracted by isobutanol against tested bacteria

Bacteria / WV	Bamboo		White popinac		Eucalyptus		Rubber		Tetracycline	
	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)
S. aureus	500	1000	1000	2000	500	1000	1000	2000	0.48	31.3
S. epidermidis	250	500	1000	2000	500	500	500	2000	7.8	31.3
S. faecalis	1000	2000	2000	4000	1000	2000	2000	4000	7.8	62.5
E. coli	500	2000	1000	4000	1000	2000	1000	4000	0.48	250
P. acnes	500	1000	1000	2000	1000	2000	1000	2000	0.24	15.6

Diethyl ether extracted bamboo wood vinegars exhibited stronger antibacterial activity against *S. aureus, S. epidermidis*, *S. faecalis* and *P. acnes* than other wood vinegars with clear zone sizes were 1.9, 2.8, 1.7 and 1.8 cm and MIC values were 62.5, 31.3, 62.5 and 62.5 µg/ml,

respectively. The inhibition zones were in the range of 1.0-2.4 cm with MIC values were 31.3-1000 μg/ml of dichloromethane extracted wood vinegars, and 1.5-2.8 cm of inhibition zone with MIC values ranging of 31.3-125 μg/ml of diethyl ether extracted of wood vinegars. Diethyl ether extracted bamboo wood vinegars exhibited stronger antibacterial activity against *S. aureus, S. epidermidis*, *S. faecalis* and *P. acnes* than other wood vinegars with clear zone sizes were 1.9, 2.8, 1.7 and 1.8 cm and MIC values were 62.5, 31.3, 62.5 and 62.5 μg/ml, respectively. The largest clear zone size (2.8 cm) and the lowest MIC value (31.3 μg/ml) could be observed in antibacterial activity of bamboo wood vinegar against *S. epidermidis*. While eucalyptus wood vinegar exhibited stronger antibacterial activity than others against *E. coli* with clear zone size of 2.3 cm and MIC value was 31.3 μg/ml. Although phenolic components play a role in antibacterial activity more than organic acids, total phenolic contents of wood vinegar extracted by diethyl ether (23% w/w) was less than of wood vinegar extracted by dichloromethane (32% w/w), however, antibacterial activity of wood vinegar extracted by diethyl ether were higher than wood vinegar extracted by dichloromethane. It might be due to the different in types of phenolic compounds may specific to different kinds of bacteria. In diethyl ether extracted wood vinegars, bamboo and rubber wood vinegars exhibited strong antibacterial activity against *S. epidermidis* and *P. acnes*. 2-Methoxyphenol was observed as a major phenolic compound in bamboo wood vinegar and presented as a minor phenolic component in rubber wood vinegar. Therefore 2-methoxyphenol might be contributed to inhibit *S. epidermidis* and *P. acnes*. Eucalyptus wood vinegar contained 2,6-dimethoxyphenol as a main phenolic component, and exhibited strong antibacterial activity against *E. coli*. Therefore 2,6-dimethoxyphenol might be contributed to inhibit the growth of *E. coli*. Although a large number of phenol presented in both rubber wood vinegars extracted by dichloromethane (37.2% w/w) and by diethyl ether (35.2% w/w), antibacterial activity of rubber wood vinegar was weaker than bamboo wood vinegar. It could be assumed that the original phenol with a hydroxyl group alone exhibited lower antibacterial activity than phenols with substituent, such as methyl and methoxy groups. Beside, phenols that have a substituent at the ortho position showed better antibacterial activity than phenols that have a substituent at the other positions. In addition, phenols with methoxy groups substituent might be contribute to inhibit the tested bacteria higher than phenol with methyl groups. Wood vinegars extracted by isobutanol showed much lower antibacterial activities against all tested bacteria than wood vinegar extracted by dichloromethane; wood vinegars extracted by diethyl ether and lyophilized wood vinegars with

clear zones in the range of 0.7-1.3 cm and MIC values in the range of 250-2000 µg/ml. It might be due to the chemical components which have antibacterial properties such as organic acids and phenolic components which were not dissolved well in isobutanol.

Consequently, variation of antibacterial activities was depending on types of wood vinegars and concentrate treatments. Table 4-16 shows five groups of variation for the observed mean of clear zone sizes among concentrate treatments of Scheffe's multiple comparison with the mean difference reaching the most significant level at 95% ($p < 0.05$). However, there was no difference between clear zone sizes of wood vinegars extracted by isobutanol and alkalinized lyophilized wood vinegars. In all types of wood vinegars, wood vinegar extracted by diethyl ether (subset 5) presented the highest estimated marginal means of inhibition zone size (around 1.8 cm). Followed by wood vinegars extracted by dichloromethane (around 1.5 cm) (subset 4), lyophilized wood vinegars (around 1.2 cm) (subset 3), alkalinized lyophilized wood vinegars and wood vinegars extracted by isobutanol (around 0.96 and 0.95 cm, respectively) (subset 2), and the smallest clear zone sizes were observed in original wood vinegars (around 0.87 cm) (subset 1). Types of wood vinegar also gave a variable in antibacterial activities (Table 4-17). Three groups of variation for the mean of clear zone sizes among types of wood vinegar of Scheffe's multiple comparison with the mean difference reaching the most significant level at 95% ($p < 0.05$) could be observed. Bamboo wood vinegar displayed the biggest clear zone sizes (around 1.4 cm) (subset 3). Followed by eucalyptus and rubber wood vinegars (around 1.23 and 1.20 cm, respectively) (subset 2), and white popinac wood vinegar gave the smallest clear zone sizes (around 1.06) (subset 1).

The variation of antibacterial activities were depended on concentrate treatments and types of wood vinegar. Figure 4-6 shows the estimate marginal means of the inhibition zones of all wood vinegars against all tested bacteria. This variable plot indicated that bamboo wood vinegar gave the highest potency to use as antibacterial agent and extraction by diethyl ether could increase antibacterial efficiency better than other treatment. Extraction by dichloromethane could also increase high efficacy but slightly lower than extraction by diethyl ether. While, lyophilization, alkalinized lyophilization and extraction by isobutanol were scarcely increased antibacterial activity.

Table 4-16 Homogeneous subsets of inhibition zone size among concentrate treatments of wood vinegars

Wood vinegar samples	Clear zone size (cm)*				
	1[a]	2[a]	3[a]	4[a]	5[a]
Original WV	0.8677				
Isobutanol extracted WV		0.9457[b]			
Alkalinized lyophilized WV		0.9588[b]			
Lyophillyzed WV			1.2380		
Dichloromethane extracted WV				1.5383	
Diethyl ether extracted WV					1.8355

* Means for groups in homogeneous subsets (n = 60)

[a] Significant differences ($p < 0.05$) among treatments

[b] No significant differences ($p > 0.05$) among treatments

Table 4-17 Homogeneous subsets of inhibition zone size among types of wood vinegars

Wood vinegar samples	Clear zone size (cm)*		
	1[a]	2[a]	3[a]
White popinac WV	1.0568		
Rubber WV		1.1970[b]	
Eucalyptus WV		1.2323[b]	
Bamboo WV			1.4366

* Means for groups in homogeneous subsets (n = 90)

[a] Significant differences ($p < 0.05$) among treatments

[b] No significant differences ($p > 0.05$) among treatments

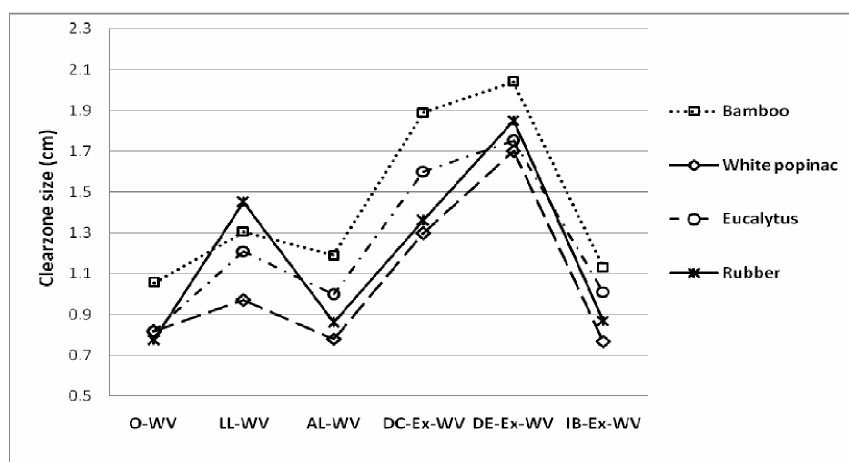


Figure 4-6 Estimate marginal means of the inhibition zone of all wood vinegars against all tested bacteria; Original wood vinegar (O-WV); Lyophilized wood vinegar (LL-WV); Alkalinized lyophilized wood vinegar (AL-WV); Wood vinegar extracted by dichloromethane (DC-Ex-WV); Wood vinegar extracted by diethyl ether (DE-Ex-WV); Wood vinegar extracted by isobutanol (IB-Ex-WV)

4.3 Inhibition of fungal growth

The inhibitory activities of all wood vinegars against three different species of dermatophyte, *T. rubrum*, *T. mentagrophytes* and *M. gypseum* and yeast, *C. albicans*, were determined on SDA agar plate for screening inhibitory activity and evaluate the inhibition value in SDB broth solution. Growth of *T. rubrum*, *T. mentagrophytes*, *M. gypseum* and *C. albicans* was inhibited by all types of wood vinegar. Clear zones could be observed around 6 mm paper discs containing 20 µl of all kinds of wood vinegars, while water and methanol showed no activity against the tested fungal. Antifungal activities of wood vinegars were similar among wood species with inhibition zone ranged from 0.7-0.95 cm (Figure 4-7; a) and MIC values ranging of 1:4 – 1:16 ml wood vinegar: ml SDB. However, bamboo wood vinegar gave slightly higher clear zone sizes (0.85-0.95 cm) against all tested fungal species compared to the other three wood vinegars which afforded similar results, correlated with MIC value, the lowest MIC value presented in bamboo wood vinegar against all tested dermatophyte (1:16 ml wood vinegar: ml SDB) and *C. albicans* (1:8 ml wood vinegar: ml SDB). However, the antibacterial activity of wood vinegars was lower than standard antibiotics about 60-80% with inhibition zones of ketoconazole were in the range of 2.3-4.5 cm.

(a)

(b)

(c)

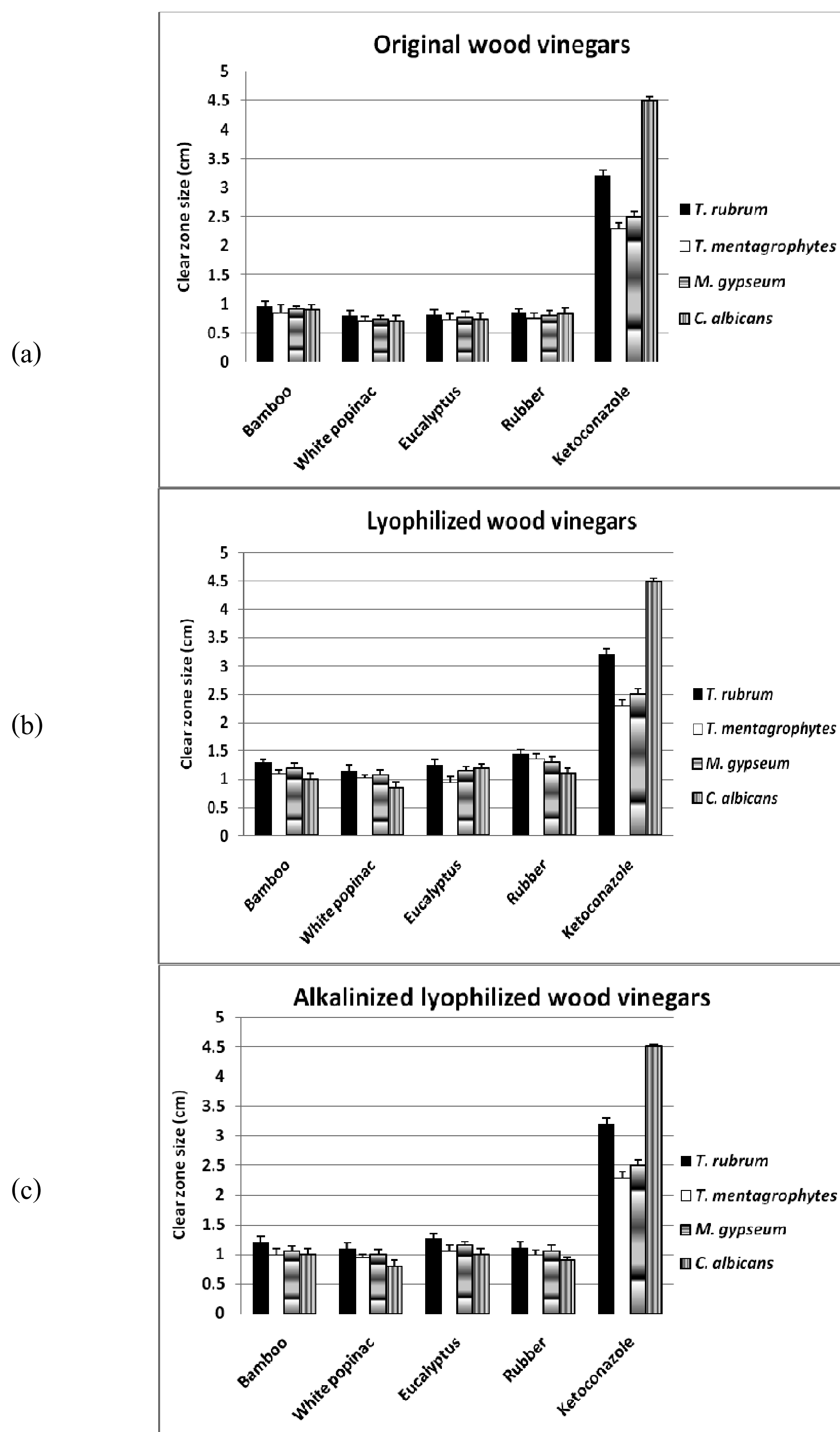


Figure 4-7 Antifungal activities of original WV (a), LL-WV (b) and alkalinized LL-WV (c) tested by disc diffusion assay (20 µl/disc original WV; 2 mg/disc LL-WVs and alkalinized LL-WV; 25 µg/disc Ketoconazole)

Table 4-18 Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of original wood vinegars against tested fungi

	Bamboo		White popinac		Eucalyptus		Rubber		Ketoconazole	
	MIC (ml : ml)	MFC (ml : ml)	MIC (ml : ml)	MFC (ml : ml)	MIC (ml : ml)	MFC (ml : ml)	MIC (ml : ml)	MFC (ml : ml)	MIC (ml : ml)	MFC (ml : ml)
T. rubrum	1:16	ND	1:16	ND	1:16	ND	1:16	ND	0.97	7.8
T. mentagrophytes	1:16	ND	1:8	ND	1:8	ND	1:8	ND	7.8	31.3
M. gypseum	1:16	ND	1:8	ND	1:8	ND	1:8	ND	7.8	15.6
C. albicans	1:8	ND	1:4	ND	1:4	ND	1:4	ND	0.24	3.9

Table 4-19 Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of lyophilized wood vinegars against tested fungi

Fungi / WV	Bamboo		White popinac		Eucalyptus		Rubber		Ketoconazole	
	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)
T. rubrum	250	500	500	1000	500	1000	250	250	0.97	7.8
T. mentagrophytes	500	1000	500	2000	500	1000	250	1000	7.8	31.3
M. gypseum	500	1000	500	2000	500	1000	250	500	7.8	15.6
C. albicans	500	1000	1000	2000	500	1000	500	1000	0.24	3.9

Table 4-20 Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of alkalinized lyophilized wood vinegars against tested fungi

Fungi / WV	Bamboo		White popinac		Eucalyptus		Rubber		Ketoconazole	
	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)
T. rubrum	500	1000	500	1000	500	1000	500	2000	0.97	7.8
T. mentagrophytes	500	2000	1000	4000	500	1000	500	1000	7.8	31.3
M. gypseum	500	1000	500	1000	500	1000	500	1000	7.8	15.6
C. albicans	500	1000	1000	4000	500	2000	500	2000	0.24	3.9

The standard antibiotics, clearly showed selectivity, ketoconazole gave the highest activity against *C. albicans* with clear zone size of 4.5 cm and MIC of 0.24 µg/ml while the lowest activity

against *T. mentagrophytes* with clear zone size of 2.3 cm and MIC of 7.8 µg/ml. There appear to be little selectivity of the wood vinegars for four different tested fungal. Since biological activities of original wood vinegars were much less than the standard antibiotics about 60-80%, therefore, lyophilized wood vinegars were then used for antifungal activity determination.

Disc containing 2 mg of lyophilized wood vinegar gave bigger sizes of clear zone, in the range of 0.85-1.45 centimeters with MIC values ranging of 250-1000 µg/ml against all tested fungal. Rubber wood vinegar exhibited the strongest antifungal activity against *T. rubrum*, *T. mentagrophytes* and *M. gypseum* with clear zone sizes of 1.45, 1.35 and 1.3 cm respectively, and eucalyptus wood vinegar exhibited the strongest antifungal activity against *C. albicans* with clear zone size of 1.2 cm.

In spite of lyophilize process removed almost water content, clear zone sizes were increased from original wood vinegars less than 50%. Alkalinized lyophilize wood vinegars gave clear zone sizes in the range of 0.8-1.27 cm with MIC values ranging from 500-1000 µg/ml. That showed antifungal activity less than lyophilized wood vinegar and just slightly higher antifungal property than original wood vinegar. Therefore organic solvent extracted wood vinegars were utilized for antifungal activity determinations.

After extraction of wood vinegars by organic solvents, antifungal activities were better than lyophilized wood vinegars. The results revealed that dichloromethane and diethyl ether extracted wood vinegars exhibited strong antifungal activities against all four tested fungal species. The inhibition zone were in the range of 1.1-1.9 cm with MIC values ranging from 62.5-500 µg/ml of dichloromethane extracted wood vinegars, and 1.0-1.63 cm with MIC values ranging of 125-500 µg/ml of diethyl ether extracted wood vinegars. Wood vinegars extracted by dichloromethane presented higher antifungal property than other wood vinegars with bamboo wood vinegar exhibited stronger antifungal activity against *T. rubrum, T. mentagrophytes* and *C. albicans* than other dichloromethane extracted wood vinegars with clear zone size of 1.9, 1.5 and 1.7 cm and MIC values were 62.5, 125 and 125 µg/ml; respectively. The largest clear zone size (1.9 cm) and the lowest MIC value (62.5 µg/ml) could be observed in antifungal activity of bamboo wood vinegar against *T. rubrum*. Eucalyptus wood vinegar exhibited stronger antifungal activity against *M. gypseum* with clear zone size was 1.75 cm and MIC value was 125 µg/ml. According to GC-MS analysis, phenolic components presented as the main chemical composites of wood vinegars extracted by dichloromethane, therefore, phenolic contents might contribute to antifungal property.

As the recent study, antifungal activities of plant phenols were studied by Pereira and co-workers (2007).

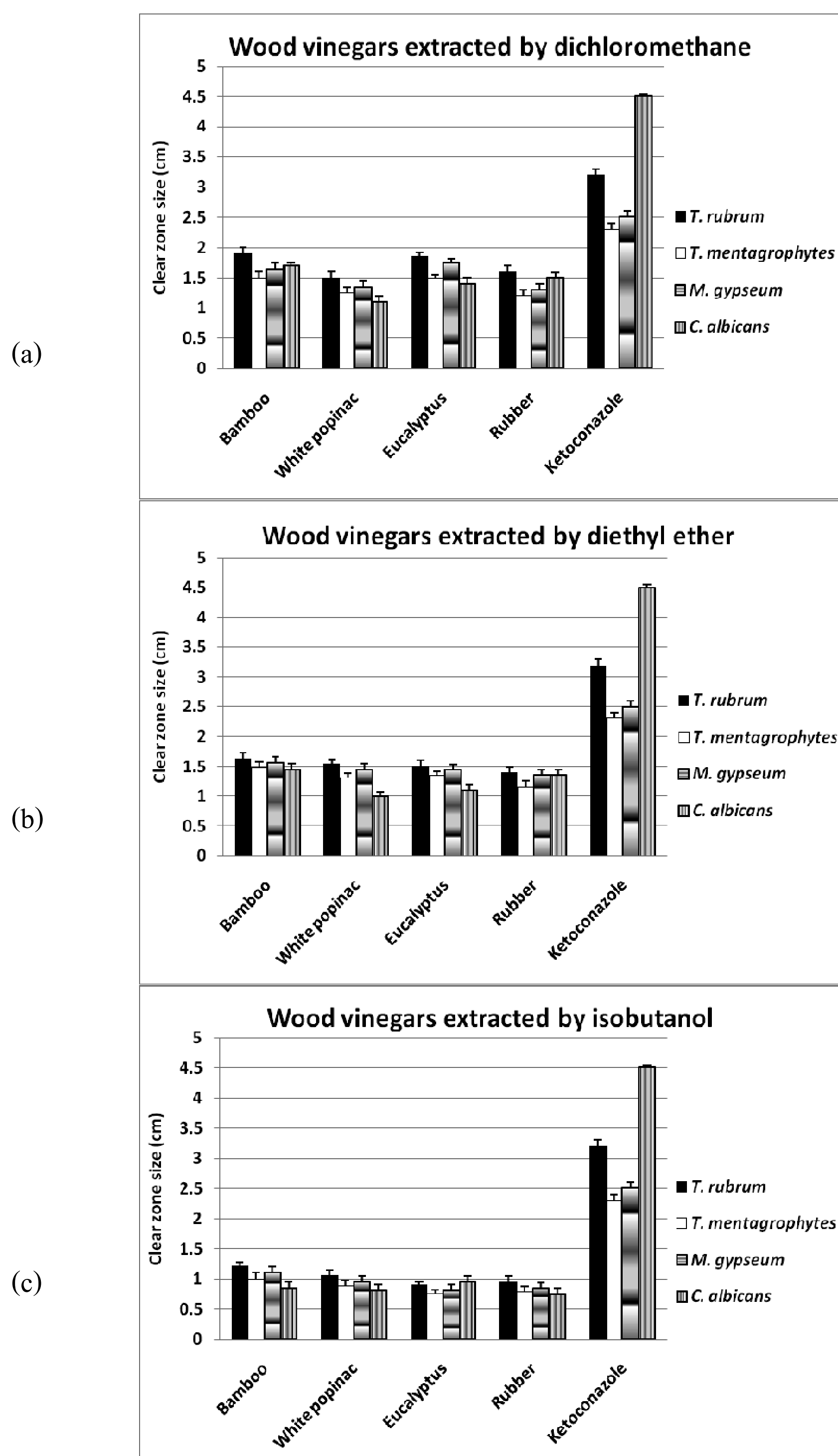


Figure 4-8 Antifungal activities of WV extracted by organic solvents, dichloromethane (a), diethyl ether (b) and isobutanol (c) tested by disc diffusion assay (2 mg/disc WVs; 25 µg/disc Ketoconazole)

Table 4-21 Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of wood vinegars extracted by dichloromethane against tested fungi

Fungi / WV	Bamboo		White popinac		Eucalyptus		Rubber		Ketoconazole	
	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)
T. rubrum	62.5	125	125	500	125	125	125	500	0.97	7.8
T. mentagrophytes	125	250	250	500	125	250	500	1000	7.8	31.3
M. gypseum	125	500	250	500	125	250	250	1000	7.8	15.6
C. albicans	125	250	500	1000	250	500	125	500	0.24	3.9

Table 4-22 Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of wood vinegars extracted by diethyl ether against tested fungi

Fungi / WV	Bamboo		White popinac		Eucalyptus		Rubber		Ketoconazole	
	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)
T. rubrum	125	250	125	250	125	250	250	500	0.97	7.8
T. mentagrophytes	250	250	250	500	250	500	500	1000	7.8	31.3
M. gypseum	125	500	250	1000	250	500	250	2000	7.8	15.6
C. albicans	250	250	500	1000	500	1000	250	500	0.24	3.9

Table 4-23 Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of wood vinegars extracted by isobutanol against tested fungi

Fungi / WV	Bamboo		White popinac		Eucalyptus		Rubber		Ketoconazole	
	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)	MIC (µg/ml)	MFC (µg/ml)
T. rubrum	500	1000	500	1000	500	2000	500	2000	0.97	7.8
T. mentagrophytes	500	1000	500	2000	1000	2000	500	2000	7.8	31.3
M. gypseum	500	1000	500	1000	500	2000	500	2000	7.8	15.6
C. albicans	500	2000	1000	2000	500	2000	1000	2000	0.24	3.9

Thus, variation of antifungal activity was depending on types of wood vinegars and concentrate treatments. Table 4-24 shows six groups of variation for the observed mean of clear zone sizes among concentrate treatments of Scheffe's multiple comparison with the mean difference reaching the most significant level at 95% ($p < 0.05$). In all types of wood vinegars, wood vinegars extracted by dichloromethane (subset 6) presented the highest estimated marginal means of inhibition zone sizes (around 1.52 cm). Followed by wood vinegar extracted by diethyl ether (around 1.38 cm) (subset 5), lyophilized wood vinegars (around 1.17 cm) (subset 4), alkalinized lyophilized wood vinegars (around 1.04 cm) (subset 3), wood vinegars extracted by isobutanol (around 0.91 cm) (subset 2) and original wood vinegars gave the smallest clear zone size (around 0.80 cm) (subset 1). Types of wood vinegar also gave a variable in antifungal activity (Table 4-25). Two groups of variation for the mean of clear zone sizes among types of wood vinegar could be observed. Bamboo wood vinegars displayed the biggest clear zone sizes (around 1.22 cm) (subset 2). There was no difference among eucalyptus, white popinac and rubber wood vinegars, with the mean difference reaching the most significant level at 95% ($p < 0.05$) of Scheffe's multiple comparisons (around 1.1 cm).

Table 4-24 Homogeneous subsets of inhibition zone size among concentrate treatments of wood vinegars

Wood vinegar samples	Clear zone size (cm)*					
	1[a]	2[a]	[a]	4[a]	5[a]	6[a]
Original WV	0.7952					
Isobutanol extracted WV		0.9075				
Alkalinized lyophilized WV			1.0435			
Lyophilized WV				1.1669		
Diethyl ether extracted WV					1.3846	
Dichloromethane extracted WV						1.5160

* Means for groups in homogeneous subsets (n = 48)

[a] Significant differences ($p < 0.05$) among treatments

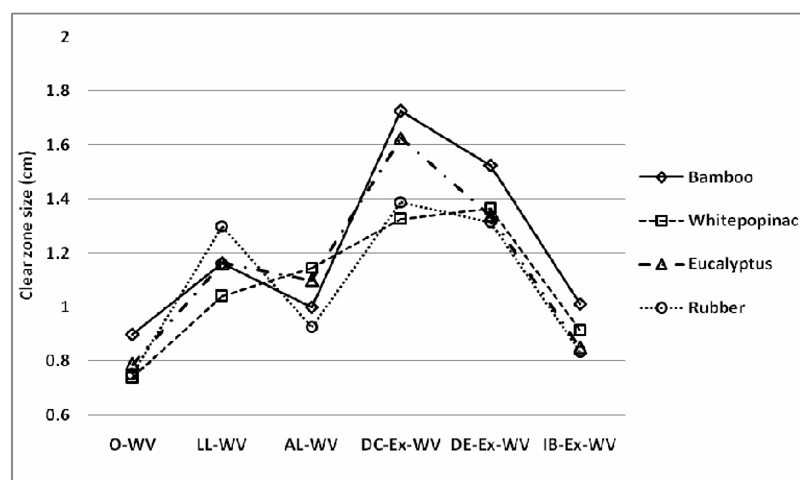


Figure 4-9 Estimate marginal means of the inhibition zones of all wood vinegars against all tested fungi; Original wood vinegar (O-WV); Lyophilized wood vinegar (LL-WV); Alkalinized lyophilized wood vinegar (AL-WV); Wood vinegar extracted by dichloromethane (DC-Ex-WV); Wood vinegar extracted by diethyl ether (DE-Ex-WV); Wood vinegar extracted by isobutanol (IB-Ex-WV)

Table 4-25 Homogeneous subsets of inhibition zone size among types of wood vinegars

Wood vinegar samples	Clear zone size (cm)	
	1[a]	2[a]
Rubber WV	1.0857[b]	
White popinac WV	1.0876[b]	
Eucalyptus WV	1.1443[b]	
Bamboo WV		1.2249

* Means for groups in homogeneous subsets (n = 72)

[a] Significant differences ($p < 0.05$) among treatments

[b] No significant differences ($p > 0.05$) among treatments

The variation of antifungal activities were depended on concentrate treatments and types of wood vinegar. Figure 4-9 shows the estimate marginal means of the inhibition zones of all

wood vinegars against all tested fungal. The variable plotted indicated that bamboo wood vinegar gave the highest potency to use as antifungal agent and extraction by dichloromethane could increase antifungal efficiency higher than other treatments. Extraction by diethyl ether gave antifungal efficacy slightly lower than extraction by dichloromethane; followed by lyophilization, alkalinized lyophilization, extraction by isobutanol and none treatment.

The antifungal abilities of phenolic compounds were depended on the fungal enzyme inhibition which contains SH groups in their active sites and the antifungal activity of phenolic compounds might be influenced by the water soluble properties of phenolic compounds (Cowan, 1999). Since cell membrane acts as selective barrier for the passage of solution between cytoplasm and cell surface area, phenols can easily interact with cell membrane and damage it. This causes the cell burst and release of cell constituents from cytoplasm which leads the cell death (Park et al., 2001). The hydroxyl group of phenolic compounds can easily react with enzymes and form hydrogen bonds. These bonding formations can extent the inhibition of phenolic compounds (Farag et al., 1989).

These antibacterial and antifungal properties determination of all wood vinegars indicated that wood vinegar have a potential to be used for treatment of wound infection and skin diseases caused by bacteria and fungi. However wood vinegars should be concentrated for higher efficiency. Although original wood vinegars used in this study contained high amount of organic acids, especially acetic acid which their antimicrobial effect was extremely weak. After liophillization, high amounts of acids were lost on this process, however; antibacterial and antifungal activities were still higher than that original. Other components such as phenolic compounds, ketonic compounds and neutral compounds might be key compounds in bio-efficacies. Phenolic compounds such as phenol and cresols have been well known to have antimicrobial property and the relationship between the structures and antimicrobial activities of substituted phenols were already reported (Nishimura et al., 1987[a,b]). This study showed that phenolic would be the components contribute to the antibacterial and antifungal activities.

4.4 Antioxidant activities

Antioxidant activities of wood vinegars were evaluated by DPPH redical scavenging activity, ferric reducing antioxidant power (FRAP) and Trolox Equivalent Antioxidant Capacity (TEAC).

4.4.1 Antioxidant activity tested by DPPH assay

The DPPH method has been one of the oldest and most frequently used methods (Brand-Williams, 1995) which rapid and simple to measure antioxidant capacity of sample involves the use of the free radical, 1, 1-diphenyl-2-picrylhydrazyl (DPPH). It has been widely used to test the ability of compounds to act as free radical scavenger or hydrogen donors, and to evaluate antioxidant activity. It is based on the ability of antioxidant to give hydrogen radical to synthetic long-lived nitrogen radical compounds (Stratil et al., 2006) according to the following reaction:

The reduction capacity of DPPH radicals was determined by the decrease in its absorbance at 515 nm (Brand-Williams et al., 1995). A blue-violet color changes gradually to green and yellow (absorption maximum at 405 nm), and the decrease in absorbance at 515 nm during the reaction in neutral medium (Roginsky, 2005).

The H-donor potential was expressed in IC_{50} value, amount of antioxidant needed for decrease of DPPH• concentration by 50%. All types of wood vinegars showed a concentration-dependent antiradical activity by reducing the stable radical DPPH to a yellowish colored diphenlypicrylhydrazine derivative.

All of original wood vinegars showed free radical scavenging activity; however antioxidant activity was greatly lower than standard BHT with greatly high IC_{50} in the range of 412-510 µg/ml. Lyophilized wood vinegars presented higher antioxidant activity with lower IC_{50} in the range of 125-223 µg/ml. Rubber wood vinegar gave the highest antioxidant with the lowest IC_{50} (125.8 µg/ml), followed by bamboo wood vinegar (182 µg/ml) and afforded similar results in white popinac and eucalyptus wood vinegars (218 and 223 µg/ml respectively). However their

antioxidant activities were lower about 20-50 % than standard BHT. Much lower free radical scavenging activity of alkalinized lyophilized wood vinegars with IC_{50} in the range of 239-455 µg/ml. Therefore wood vinegars extracted by organic solvents were then examined. Wood vinegar extracted by dichloromethane showed high potential in antioxidant activity with IC_{50} values ranged from 73-158 µg/ml. Bamboo and eucalyptus wood vinegars showed stronger free radical scavenging activities compared with standard BHT with IC_{50} of 73 and 96 µg/ml, respectively. Wood vinegars extracted by diethyl ether gave IC_{50} values ranged from 120-156 µg/ml. While, wood vinegars extracted by isobutanol gave greatly lower antioxidant activities than wood vinegars extracted by the previous two solvents with IC_{50} ranged from 335-528 µg/ml (Figure 4-10).

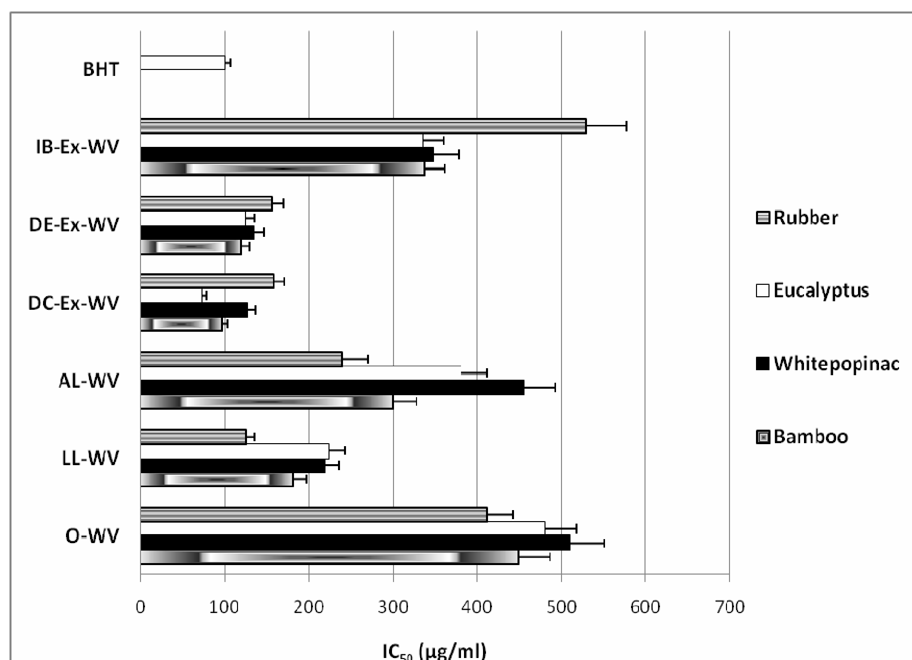


Figure 4-10 Antioxidant activities of wood vinegars tested by DPPH assay; Original wood vinegars (O-WV); Lyophilized wood vinegars (LL-WV); Alkalinized lyophilized wood vinegars (AL-WV); Wood vinegars extracted by dichloromethane (DC-Ex-WV); Wood vinegars extracted by diethyl ether (DE-Ex-WV); Wood vinegars extracted by isobutanol (IB-Ex-WV)

4.4.2 Antioxidant activity tested by FRAP assay

The FRAP method is based on the ability of antioxidant to reduce (electron transfer) Fe^{3+} to Fe^{2+} ion in the presence of 2, 4, 6-Tris(2-pyridyl)-1, 3, 5-Triazine (TPTZ) forming an intense blue Fe^{2+}- TPTZ complex with an absorption maximum at 593 nm which herein, a ferric salt, $Fe(III)(TPTZ)_2Cl_3$, is used as an oxidant (Stratil et al., 2006). This assay takes advantage of electron-transfer reaction (Benzie and Strain, 1999), the reaction detects species with redox potentials < 0.7 V [the redox potential of $Fe(II)(TPTZ)_2$], therefore FRAP is a reasonable screening method for the ability to maintain redox status in cells or tissues. This test measures the reducing potential of an antioxidant reacting with a ferric 2,4,6-tripyridyl-*S*-triazine [Fe(II)-TPTZ] complex by a reductant at low pH, was adopted. This complex has an intense blue color that can be monitored at 593 nm. The reduction capacity of the compound may serve as a significant indicator of its potential antioxidant activity (Meir et al., 1995). Results were expressed in trolox equivalent (mg Trolox/g WV) (Figure 4-11).

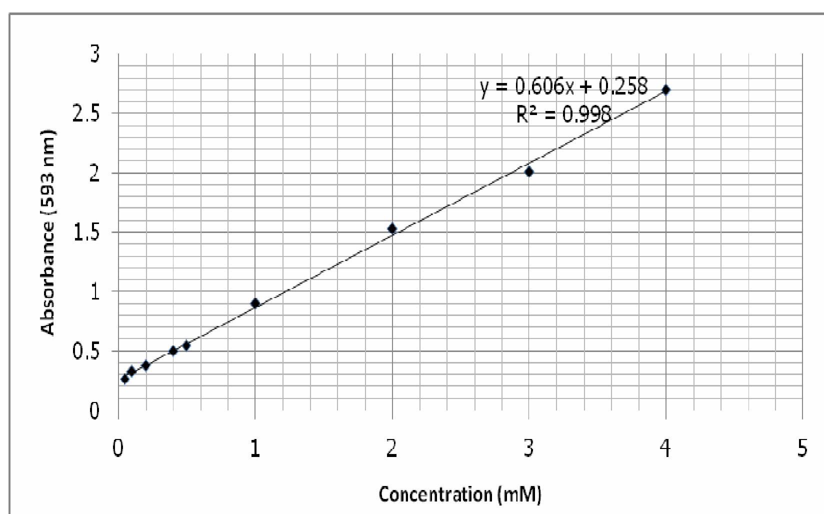


Figure 4-11 Calibration curve of standard Trolox solutions from FRAP assay

Original wood vinegars gave greatly low reduction capacity (33-106 mg TE/g WV) compare to BHT (control) with a reduction capacity was 670 mg TE/g WV. After lyophilization, higher reducing efficiency presented with ranged from 521-650 mg TE/g WV. While, alkalinized lyophilized wood vinegars presented lower antioxidant activity with ranged from 77-350 mg TE/g WV. Organic solvents extracted wood vinegars were then determined again. As the DPPH assay,

the reduction capacity was higher. Wood vinegars extracted by dichloromethane showed strong reducing power which were 605-745 mg TE/g WV. The strongest reducing power presented in eucalyptus wood vinegars with 745 mg trolox/g WV, followed by the reduction capacity of wood vinegars extracted by diethyl ether which ranged from 580-658 mg TE/g WV, and wood vinegars extracted by isobutanol with the reducing power were 60-83 mg TE/g WV (Figure 4-12).

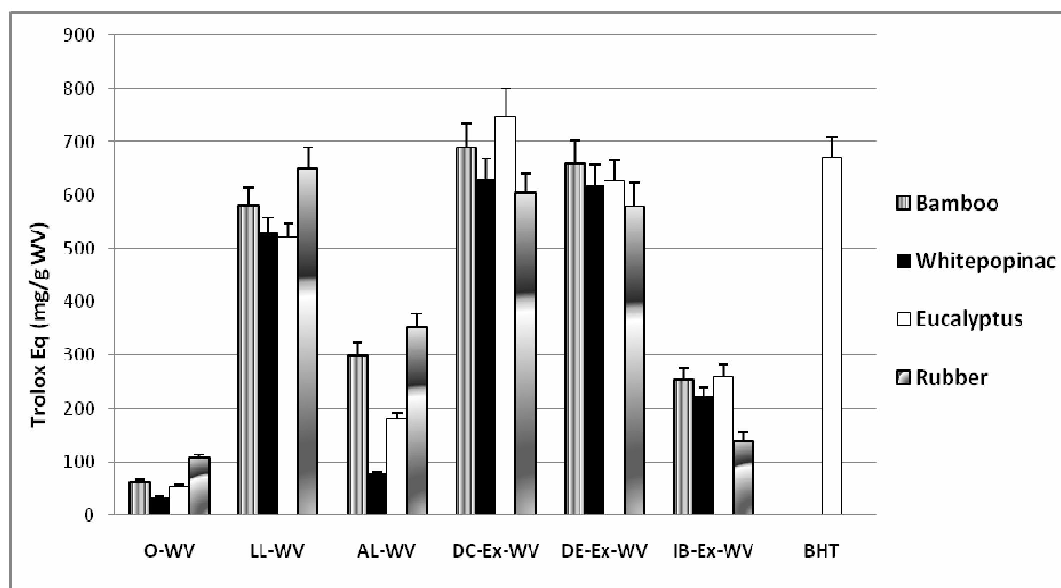
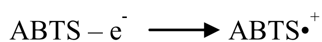


Figure 4-12 Antioxidant of wood vinegars tested by FRAP assay; Original wood vinegars (O-WV); Lyophilized wood vinegars (LL-WV); Alkalinized lyophilized wood vinegars (AL-WV); Wood vinegars extracted by dichloromethane (DC-Ex-WV); Wood vinegars extracted by diethyl ether (DE-Ex-WV); Wood vinegars extracted by isobutanol (IB-Ex-WV)

4.4.3 Antioxidant activity tested by TEAC assay

TEAC assay has been used in many research laboratories for studying antioxidant capacity (Prior et al., 2005). It is based on a neutralization of radical cation formed, ferrylmyoglobin radical (from reaction of metmyoglobin with H_2O_2), by a single-electron oxidation of a synthetic ABTS chromophore [2,2'-azinobis(3-ethylbenzothiazoline-6)-sulfonic acid] to a strongly absorbing $ABTS•^+$ radical at 700-750 nm (Van den Berg et al., 1999) according to the reaction:



ABTS$^{•+}$ radical reacts rapidly with antioxidants to form colorless ABTS, typically within 30 min. It can be used over a wide pH range and can be applied to study effects of pH on antioxidant mechanisms. ABTS$^{•+}$ is soluble in both aqueous and organic solvents and is not affected by ionic strength, so it can be used in multiple media to determine both hydrophilic and lipophilic antioxidant capacities of extracts and body fluids (Phipps et al., 2007).

ABTS$^{•+}$ is intensively colored, and antioxidant capacity is measured as the ability of the test species to decrease the color by reacting directly with the ABTS$^{•+}$ radical. The results were expressed in trolox equivalent (mg Trolox/g WV) (Figure 4-13).

Antioxidant activity presented in all tested wood vinegars samples (Fig 4-13), with ABTS radical cation scavenging capacities ranged from 6-270.4 mg trolox/g WV. Original wood vinegars presented greatly weak radical scavenger (6-61mg trolox/g WV) compare to standard BHT control 250 mg trolox/g WV. Lyophilized wood vinegars showed higher antioxidant activity than original wood vinegars with radical scavenger ranged from 159-215 mg trolox/g WV. Rubber wood vinegar gave the highest antioxidant activity among lyophilized wood vinegars with 215 mg trolox/g WV. Alkalinized lyophilized wood vinegars showed lower antioxidant activity which in the range of 77-350 mg TE/g WV.

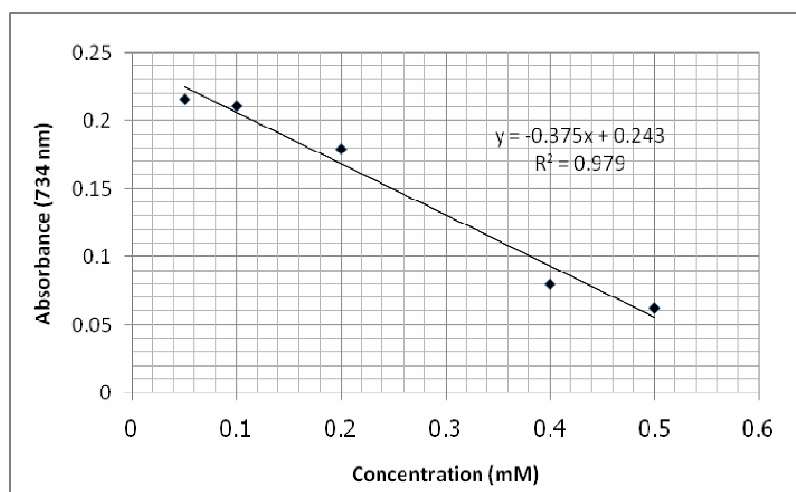


Figure 4-13 Calibration curve of standard Trolox solutions from TEAC assay

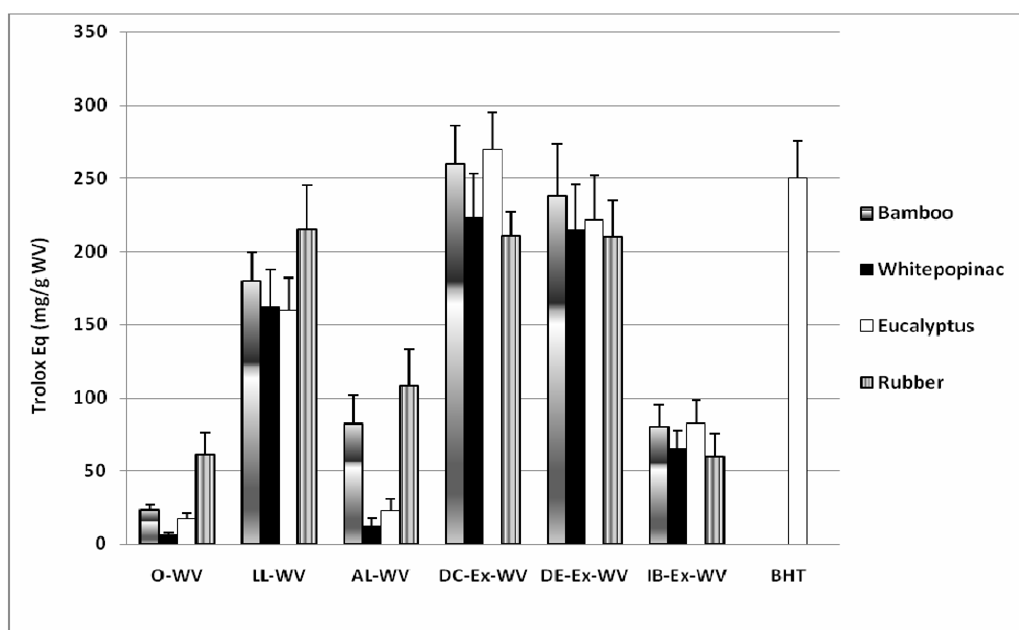


Figure 4-14 Antioxidant activity of wood vinegars tested by TEAC assay; Original wood vinegars (O-WV); Lyophilized wood vinegars (LL-WV); Alkalinized lyophilized wood vinegars (AL-WV); Wood vinegars extracted by dichloromethane (DC-Ex-WV); Wood vinegars extracted by diethyl ether (DE-Ex-WV); Wood vinegars extracted by isobutanol (IB-Ex-WV)

Better radical cation scavenging capacities presented in wood vinegars extracted by organic solvents. Wood vinegars extracted by dichloromethane showed strong ABTS radical cation scavenging capacities with ranged from 210-270 mg trolox/g WV. Bamboo and eucalyptus wood vinegars gave antioxidant activities higher than standard BHT which was 260 and 270 mg trolox/g WV, respectively. Wood vinegars extracted by diethyl ether presented ABTS radical cation scavenging capacities in the range of 209-238 mg trolox/g WV, while wood vinegars extracted by isobutanol showed very low radical scavenger (60-83 mg trolox/g WV) (Figure 4-14).

The results indicated that wood vinegars extracted by dichloromethane gave the highest antioxidant activity among the other wood vinegars; especially, eucalyptus and bamboo wood vinegars extracted by dichloromethane presented antioxidant activity higher than BHT control. Followed by, wood vinegars extracted by diethyl ether, lyophilized wood vinegars, wood vinegars extracted by isobutanol, alkalinized lyophilized wood vinegars and original wood vinegars, respectively. Wood vinegars extracted by dichloromethane showed the highest efficiency to obtain

the important chemical components that play a role to antioxidant. Therefore solvent extraction should bemethod of choice used for isolation the antioxidant. Both extraction yield and activity of extracts were strongly dependent on the type of solvent, since the different in antioxidant potential of compounds may have different polarity (Julkunen-Tiito, 1985; Marinova and Yanishlicha, 1996). Dichloromethane was not only have high efficiency in extracting polar compounds, but also present high volatility (Guille'n and Manzanos, 1996; Yrieix et al., 1996). Similar to the previous work which has been done and evidently shown that the dichloromethane extract of the wood vinegars of *Rhizophora apiculata* was a rich source of antioxidants. It has been found to contain high total phenolic contents which gave superior free radical scavenging activity and ferric reducing power (Loo et al., 2007).

Table 4-26 Homogeneous subsets of antioxidant activity among concentrate treatments of wood vinegars

Wood vinegar samples	Mean of antioxidant activity (TE; mg/g and IC_{50}; μg/ml) *			
	1[a]	2[a]	3[a]	4[a]
Original WV	184.6167			
Alkalinized lyophilized WV		209.3333[b]		
Isobutanol extracted WV		226.1111[b]		
Lyophilized WV			312.6111[b]	
Diethyl ether extracted WV			325.3889[b]	325.3889
Dichloromethane extracted WV				340.9722

* Means for groups in homogeneous subsets (n = 36)

[a] Significant differences ($p < 0.05$) among treatments

[b] No significant differences ($p > 0.05$) among treatments

Table 4-26 shows four groups of variation for the observed mean of antioxidant activity among tested samples of Scheffe's multiple comparison with the mean difference reaching the

most significant level ($p < 0.05$). However, there was no difference between antioxidant activities of alkalinized lyophilized wood vinegars and isobutanol extracted wood vinegars (subset 2); lyophilized wood vinegars and diethyl ether extracted wood vinegar (subset 3); dichloromethane extracted wood vinegars and diethyl ether extracted wood vinegars (subset 4). Wood vinegars extracted by dichloromethane presented the highest estimated marginal means of antioxidant values (around 341) (subset 4). Followed by wood vinegars extracted by diethyl ether and lyophilized wood vinegars (around 325.4 and 312.6, respectively) (subset 3); wood vinegars extracted by isobutanol and alkalinized lyophilized wood vinegars (around 226.1 and 209.3, respectively) (subset 2); and original wood vinegars (around 184.6) (subset 1).

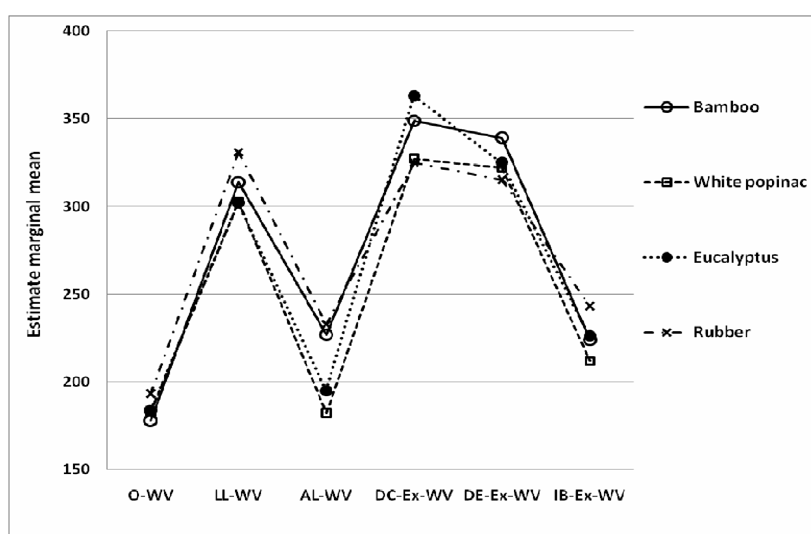


Figure 4-15 Estimate marginal mean of antioxidant values tested by all three assays; Original wood vinegar (O-WV); Lyophilized wood vinegars (LL-WV), Alkalinized lyophilized wood vinegars (AL-WV); Wood vinegars extracted by dichloromethane (DC-Ex-WV); Wood vinegars extracted by diethyl ether (DE-Ex-WV); Wood vinegars extracted by isobutanol (IB-Ex-WV)

Figure 4-15 indicated that extraction by dichloromethane or diethyl ether or treated by lyophillization gave high antioxidant potencies (over 300); while, extraction by isobutanol or alkalinized lyophillization gave lower chemical components that play an important role in antioxidant activity. Antioxidant activities were not so different among types of wood vinegars (Table 4-27).

Table 4-27 Homogeneous subsets of antioxidant activity among types of wood vinegars

Wood vinegar samples	Mean of antioxidant activity (TE; mg/g and IC_{50}; μg/ml) *	
	1[a]	2[a]
White popinac WV	254.7241[b]	
Eucalyptus WV	265.8889[b]	265.8889[b]
Bamboo WV		272.0741[b]
Rubber WV		273.3352[b]

* Means for groups in homogeneous subsets (n = 54)

[a] Significant differences ($p < 0.05$) among treatments

[b] No significant differences ($p > 0.05$) among treatments

The correlation between antioxidant activities tested by FRAP and DPPH assay; FRAP and TEAC; and DPPH and TEAC are displayed in Figures 4-16, 4-17 and 4-18, respectively. Closely correlation existed of antioxidant determination between and FRAP and DPPH (R^2= 0.957), FRAP and TEAC (R^2= 0.970.) and TEAC and DPPH assay (R^2= 0.930), respectively; with the correlation coefficient reaching the most significant level ($p < 0.01$), indicating that antioxidant determination the methods used in this study were related among tested methodology.

Recent studies have shown that polyphenols contribute significantly to the total antioxidant activity of many fruits and vegetables (Luo et al., 2002). The correlation between phenols and antioxidant shows in Figures 4-19, 4-20 and 4-21.

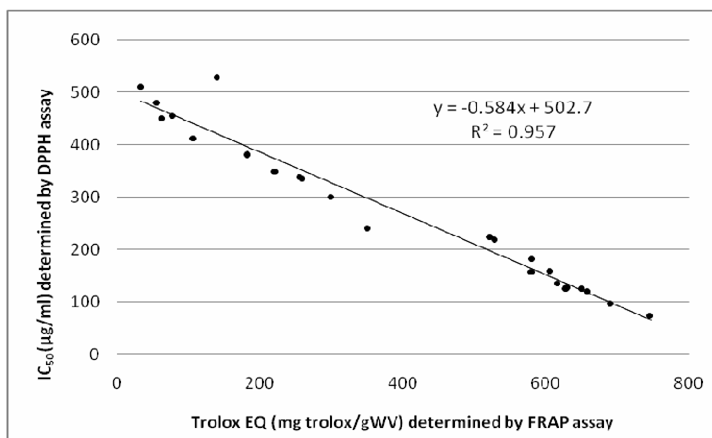


Figure 4-16 The correlation between antioxidant activities tested by FRAP and DPPH assay

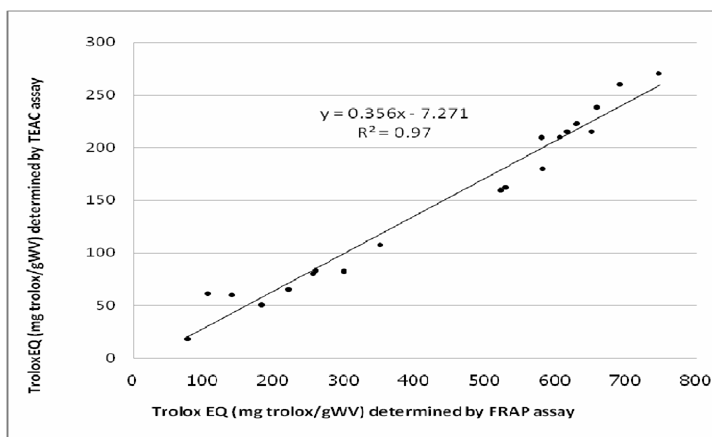


Figure 4-17 The correlation between antioxidant activities tested by FRAP and TEAC assay

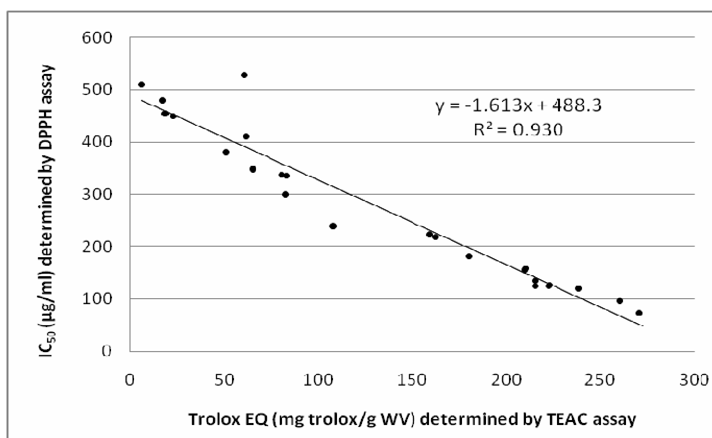


Figure 4-18 The correlation between antioxidant activities tested by TEAC and DPPH assay

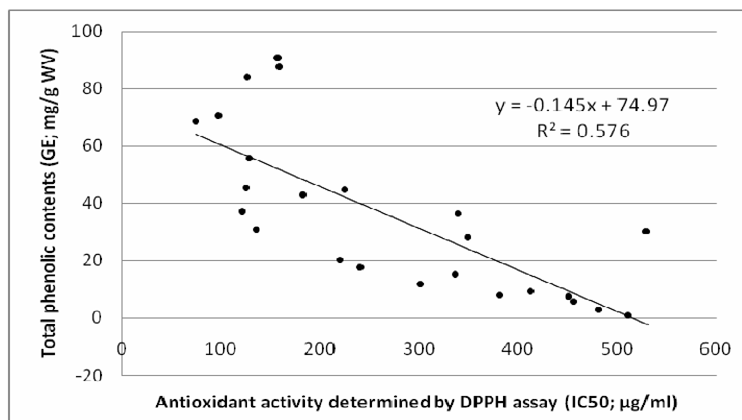


Figure 4-19 The correlation between total phenolic contents and antioxidant activity tested by DPPH assay

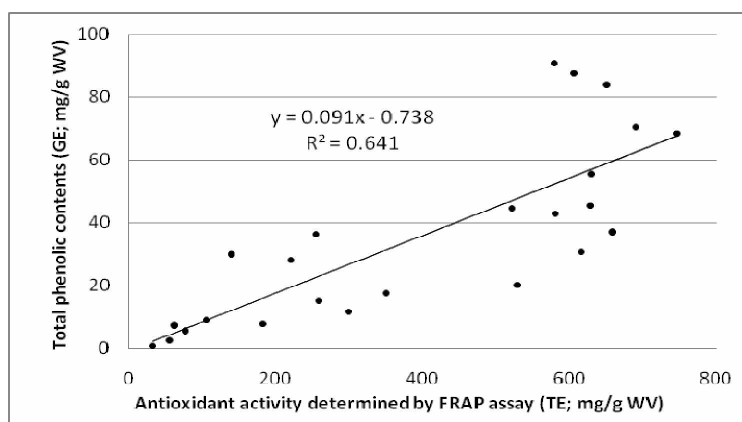


Figure 4-20 The correlation between total phenolic contents and antioxidant activity tested by FRAP assay

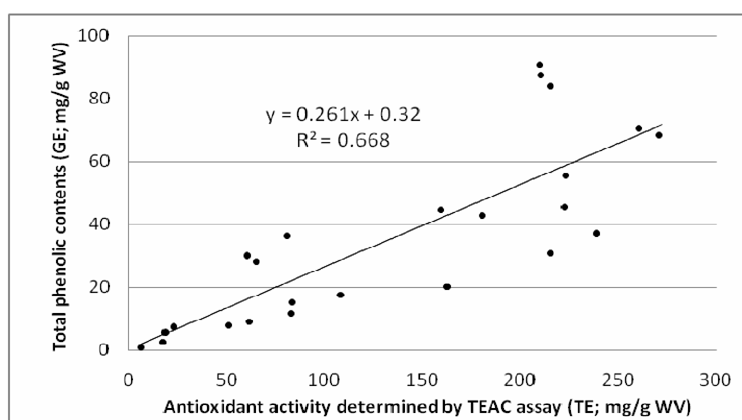


Figure 4-21 The correlation between total phenolic contents and antioxidant activity tested by TEAC assay

The results from Figures 4-19, 4-20 and 4-21 demonstrated that total phenolic contents correlated with antioxidant activity tested by DPPH, FRAP and TEAC assay with R^2 was 0.576, 0.641 and 0.668, respectively, with the correlation coefficient reaching the most significant level ($p < 0.01$), indicating that antioxidant determinations in this study were related among tested methodology.

It has been well known that the antioxidant properties of the polyphenols are correlated with the delocalization on the aromatic ring of the phenoxyl unpaired electron, which stabilizes the free radical (Stevanato et al., 2004).

CHAPTER 5

CONCLUSIONS

Four types of wood vinegar samples (Bamboo, White popinac, Eucalyptus and Rubber wood vinegars) were utilized in this study. They contained around 78-88 % of water. The main chemical compositions were organic acids and phenolic components. The chemical components of wood vinegars determined by GC-MS were found to be varied depending on wood species that were used in wood vinegar production. Since tested wood vinegars contained high amounts of water, they were then concentrated and tested for their bio-efficacies. Lyophilization and organic solvent extraction methods were selected to concentrate wood vinegar samples. After lyophilizing, total amounts of chemical compositions were concentrated and type of some chemical components found to be changed from original wood vinegars, however, almost organic acids were less detectable. Organic solvents extraction was considered using dichloromethane, diethyl ether and isobutanol as organic solvents. The main chemical components such as organic acids and phenolic compounds in dichloromethane and diethyl ether extracts were higher than lyophilized wood vinegars.

The bio-efficacy studies demonstrated that all types of wood vinegar gave antibacterial and antifungal activities. Lyophilized wood vinegars showed higher antibacterial and antifungal than original wood vinegars with the inhibition zones were in the range of 0.8-1.8 cm and 0.85-1.45 cm and the minimal inhibitory concentrations (MIC) were in the range of 125-1000 µg/ml and 250 µg/ml against tested bacteria and fungi, respectively. Stronger antimicrobial activities presented in organic solvent extracted wood vinegars. Dichloromethane and diethyl ether extracted wood vinegars exhibited strong antibacterial and antifungal activities against all tested bacteria and fungi. The strongest antibacterial activity could be observed in bamboo wood vinegar extracted by diethyl ether which gave the largest clear zone size (2.8 cm) and the lowest MIC value (31.3 µg/ml) against *S. epidermidis*. The highest antifungal property could be found in bamboo wood vinegar extracted by dichloromethane with the largest clear zone size (1.9 cm) and the lowest MIC value (62.5 µg/ml) against *T. rubrum*. Antioxidant activities were tested by three different methods; 1, 1-diphenyl-2-picrylhydrazyl (DPPH) assay, Ferric Reducing Antioxidant

Power (FRAP) assay and Trolox Equivalent Antioxidant Capacity (TEAC) assay. The results indicated that wood vinegar extracted by dichloromethane presented strong radical scavenging capacities tested by all three methods, compared with Butylated hydroxytoluene (BHT) positive control. Both bamboo and eucalyptus wood vinegars showed stronger antioxidant capacities than standard BHT. Wood vinegar extracted by diethyl ether showed slightly weaker antioxidant activity than wood vinegar extracted by dichloromethane, followed by lyophilized wood vinegars. However wood vinegars extracted by isobutanol, alkalinized lyophilized wood vinegars and original wood vinegars showed weak antioxidant activity. Therefore, dichloromethane extract wood vinegars could be an interesting material for further study on the development to the products used as antimicrobial agents and may be used in treatment of skin diseases caused by dermatitis microorganisms.

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APPENDIX

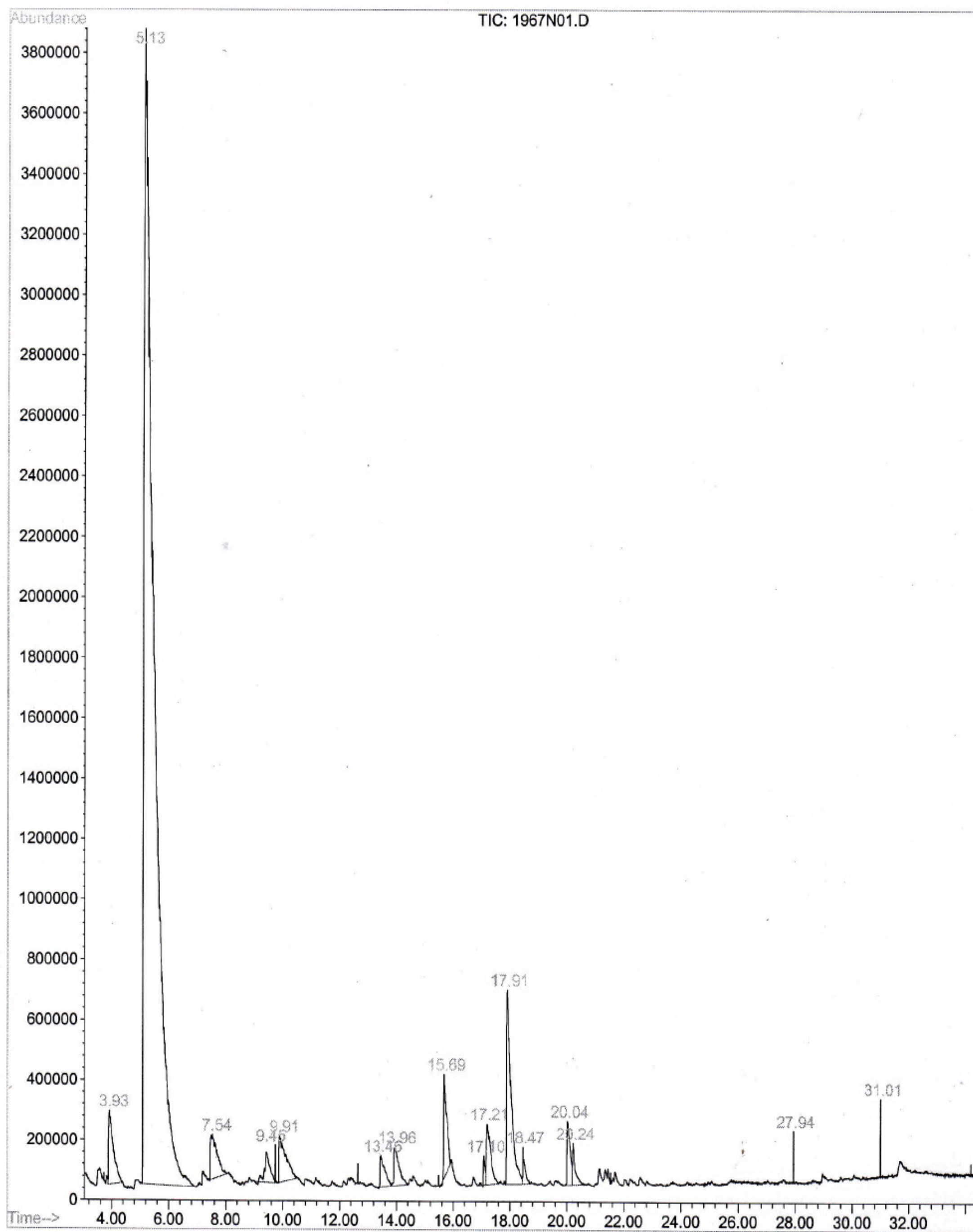

Figure A-1 GC-MS chromatogram of bamboo original wood vinegar

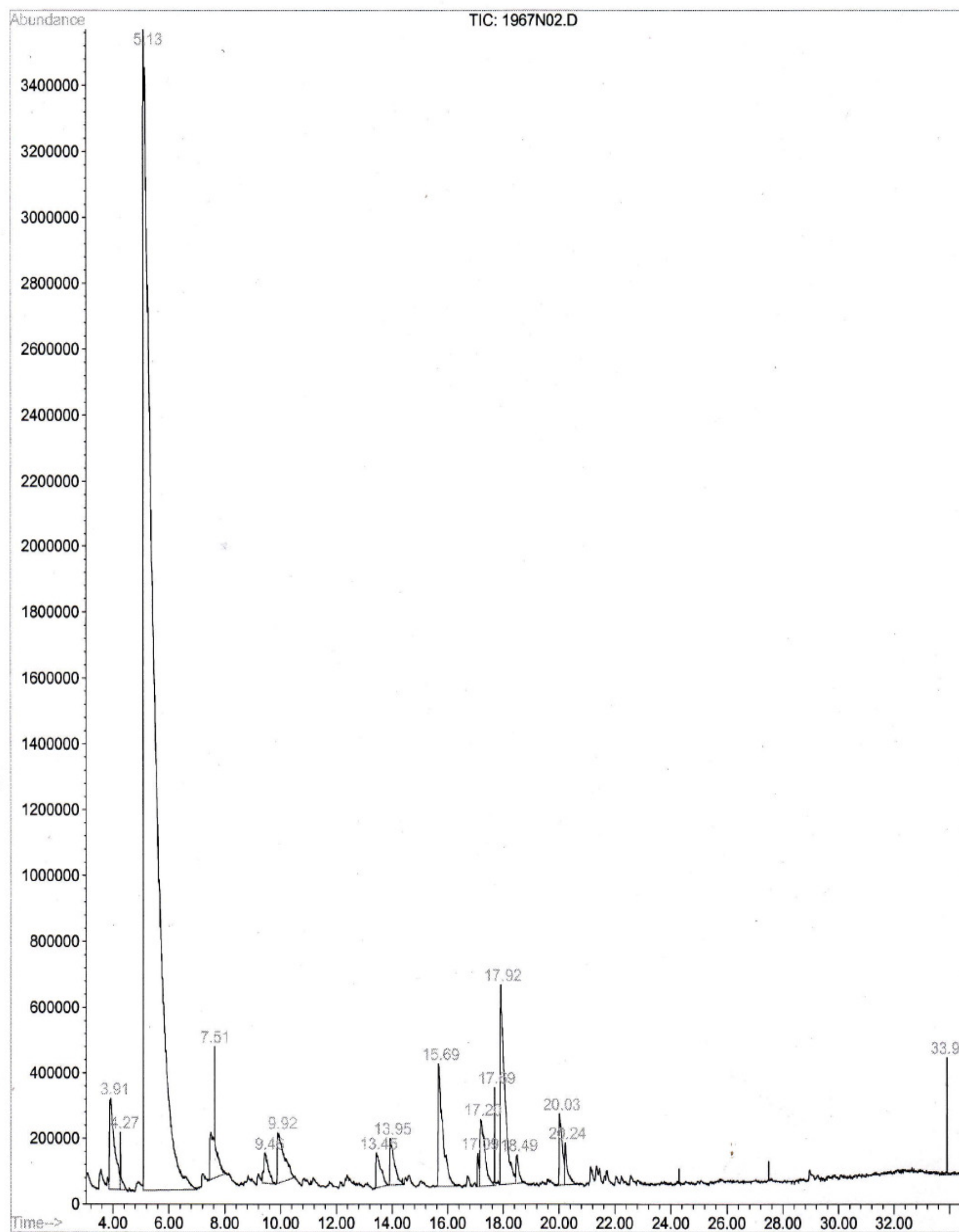

Figure A-2 GC-MS chromatogram of white popinac original wood vinegar

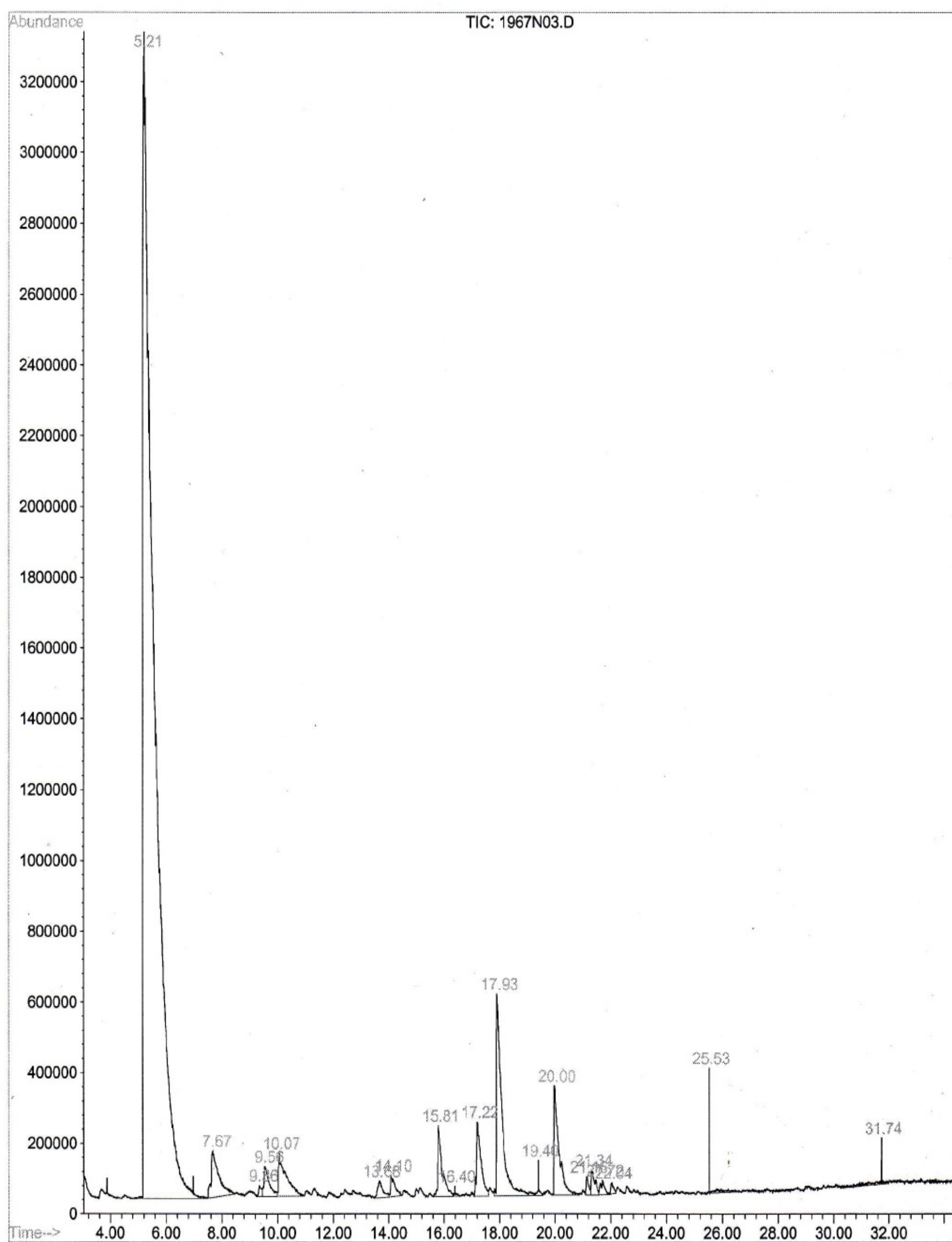

Figure A-3 GC-MS chromatogram of eucalyptus original wood vinegar

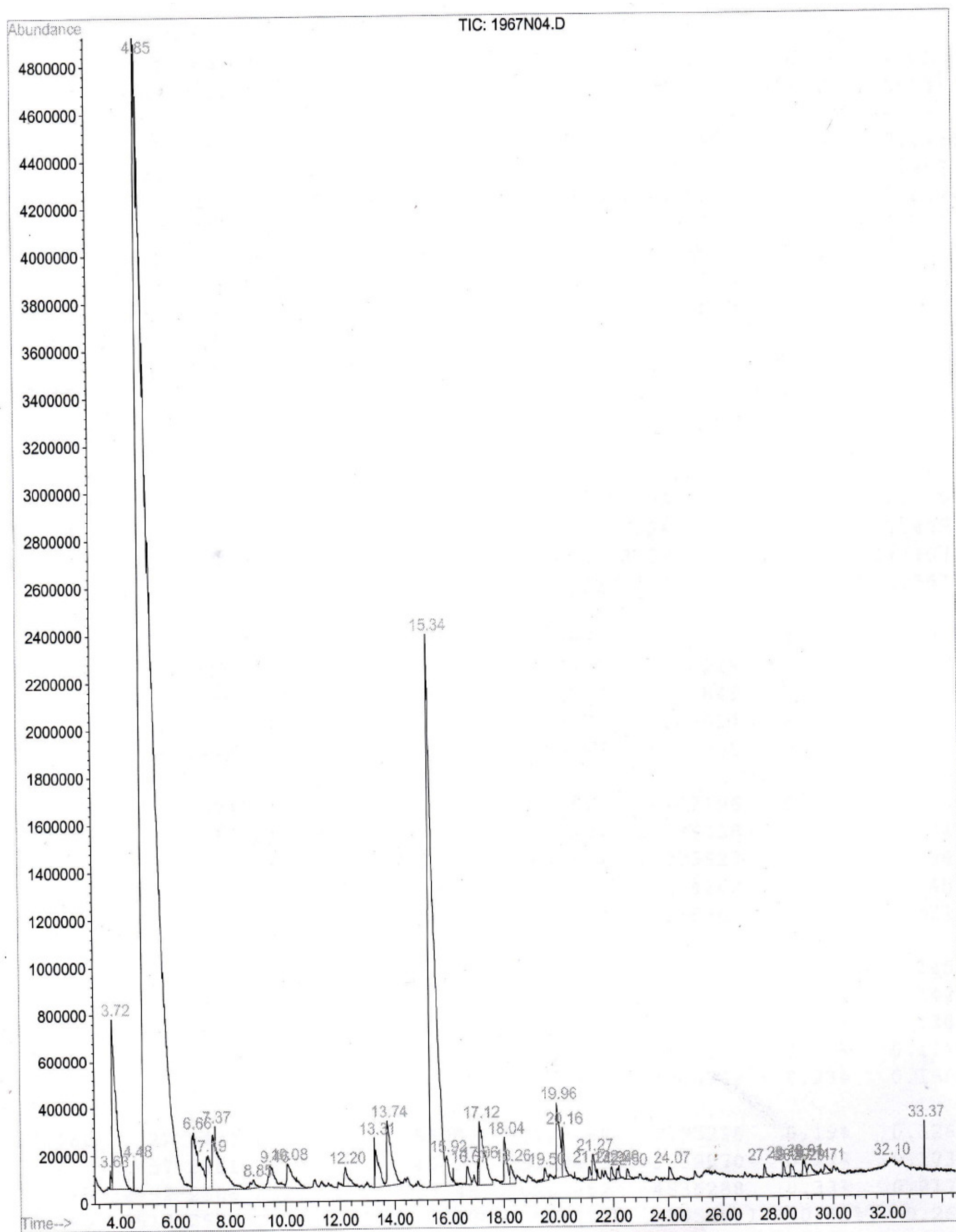

Figure A-4 GC-MS chromatogram of rubber original wood vinegar

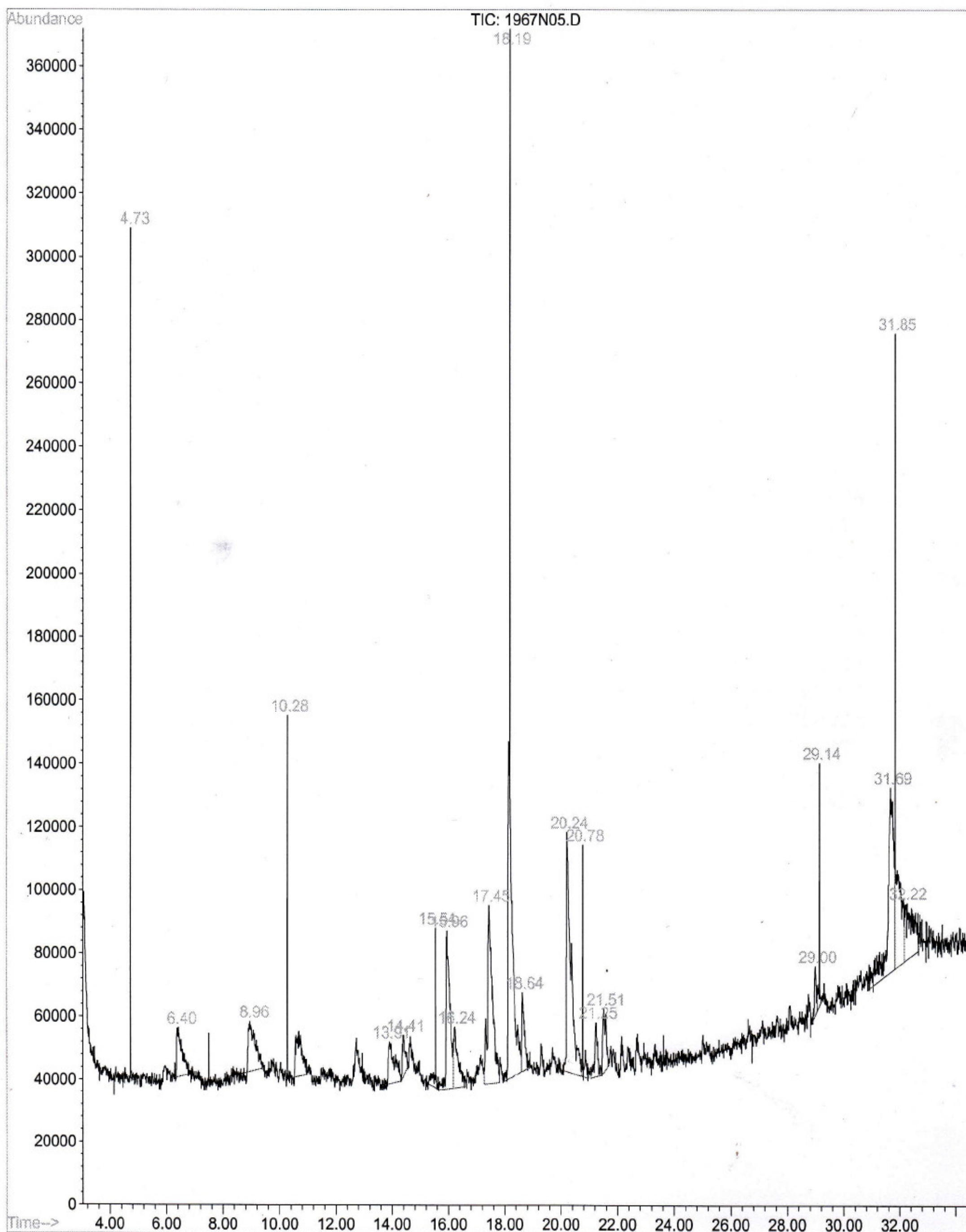

Figure A-5 GC-MS chromatogram of bamboo lyophilized wood vinegar

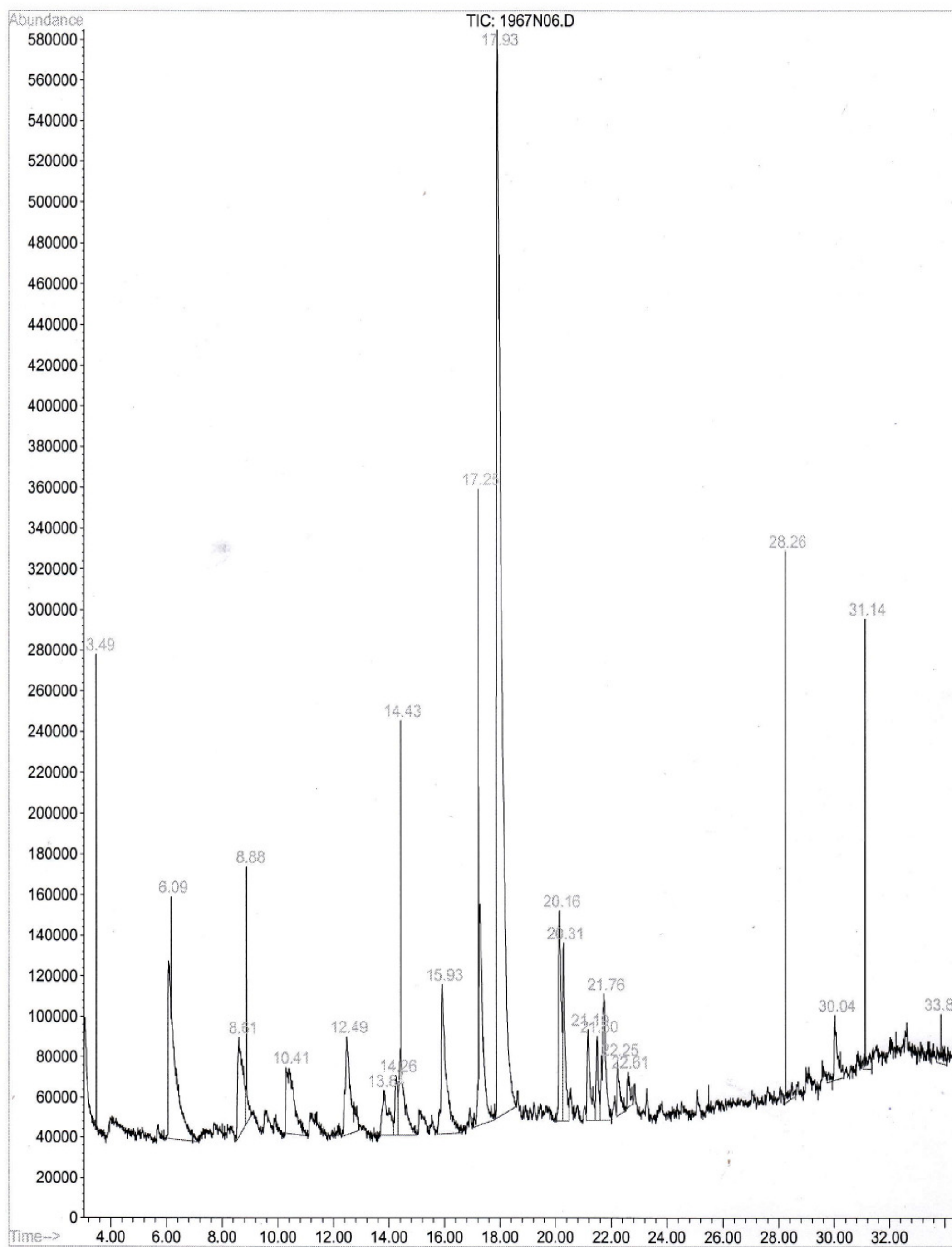

Figure A-6 GC-MS chromatogram of white popinac lyophilized wood vinegar

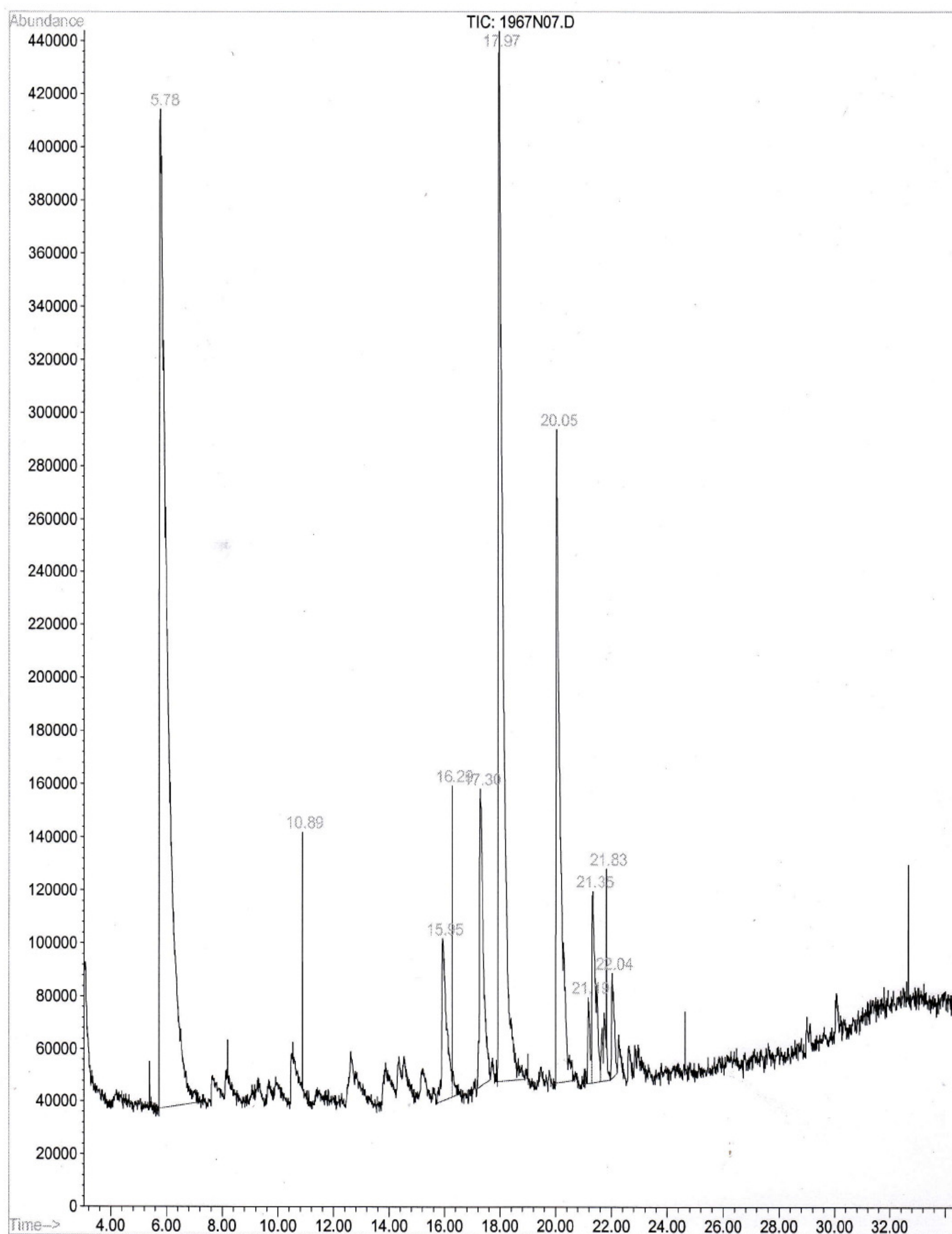

Figure A-7 GC-MS chromatogram of eucalyptus lyophilized wood vinegar

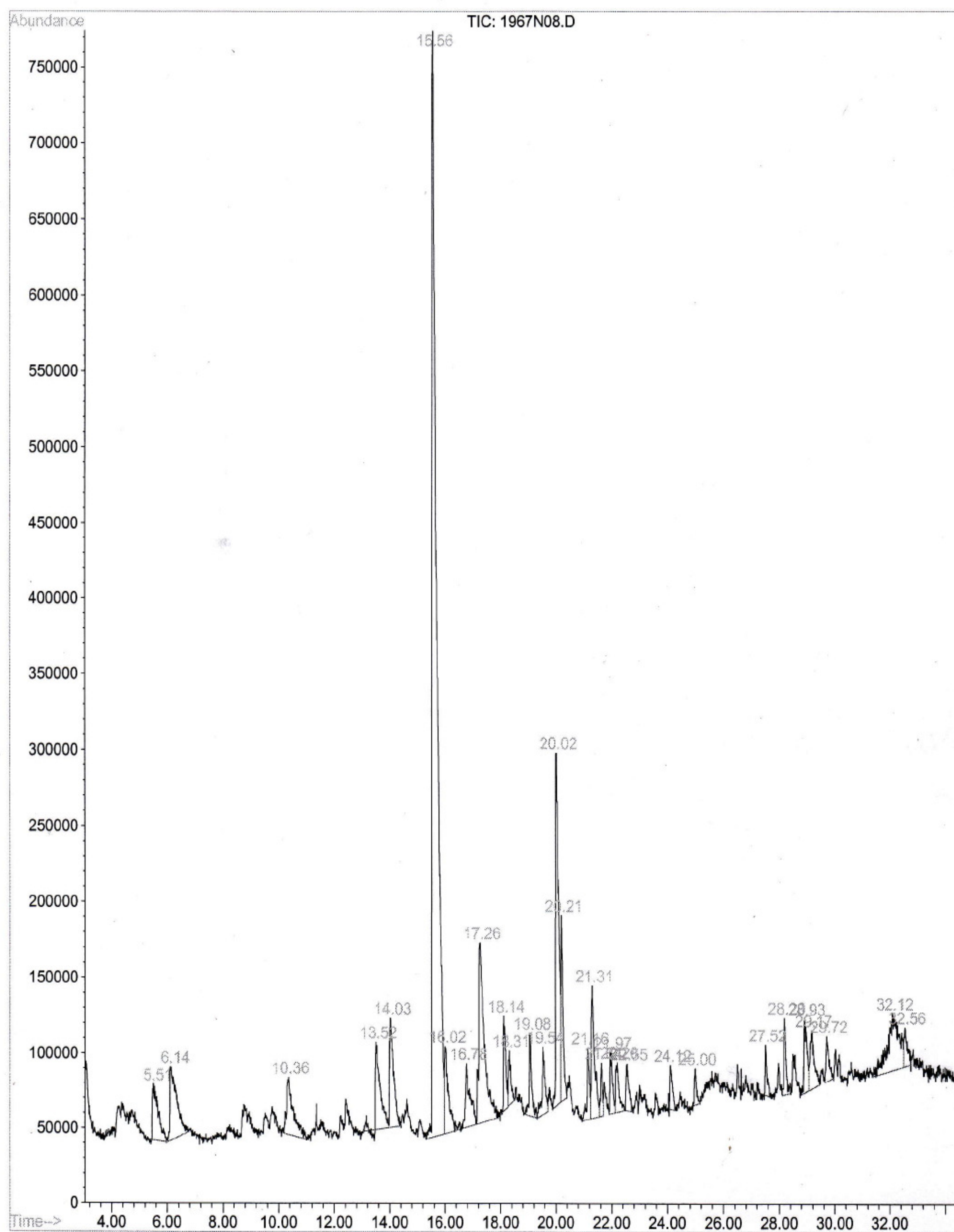

Figure A-8 GC-MS chromatogram of rubber lyophilized wood vinegar

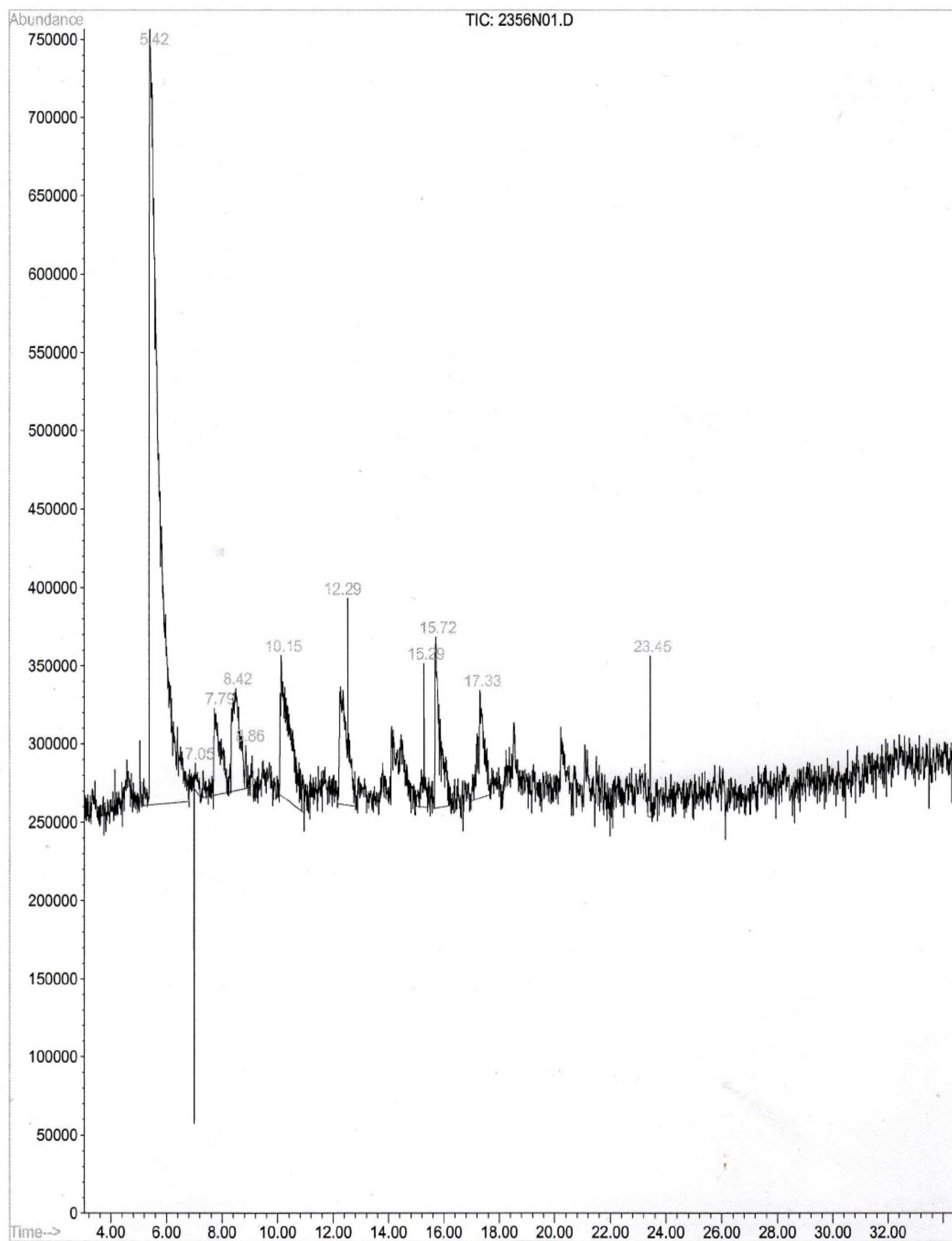

Figure A-9 GC-MS chromatogram of bamboo alkalinized lyophilized wood vinegar

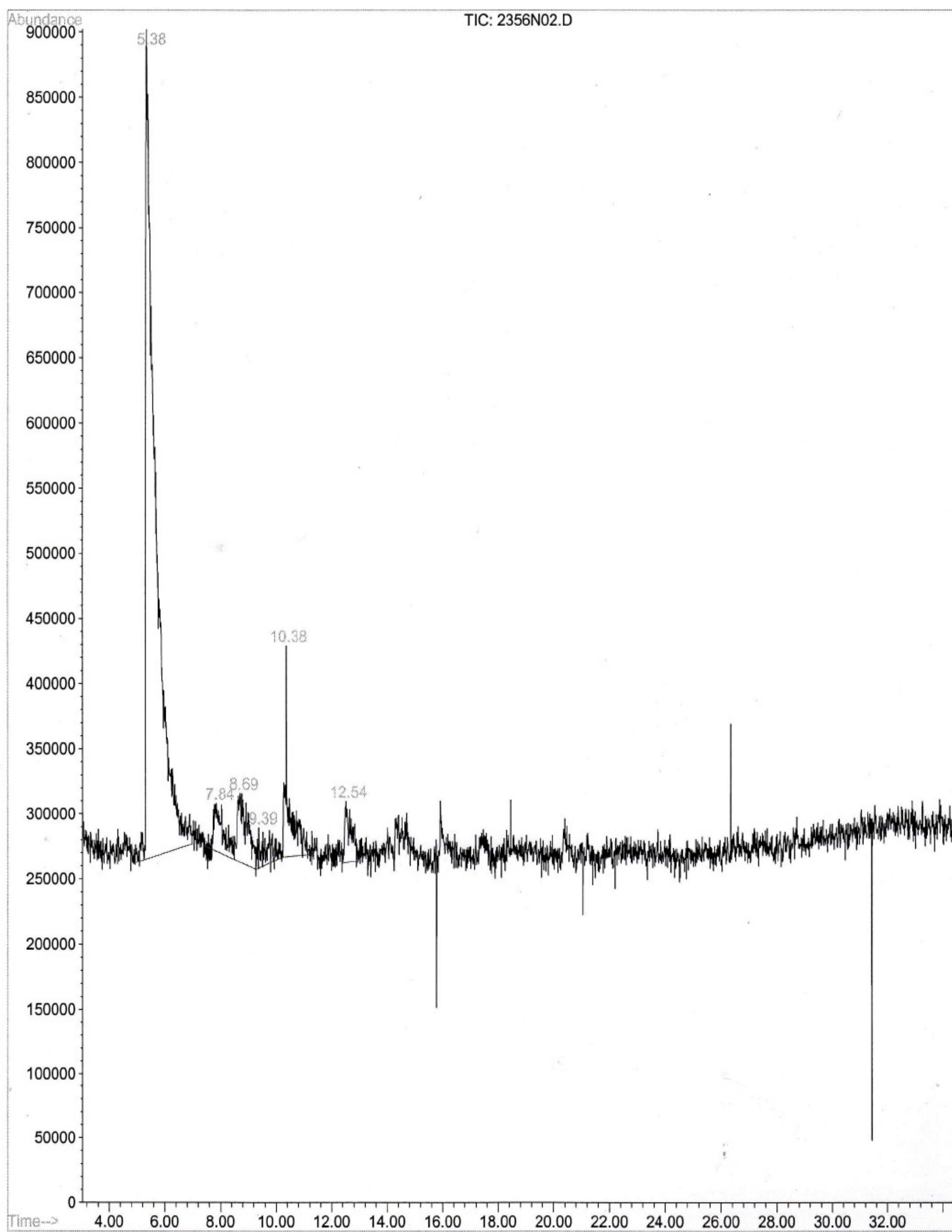

Figure A-10 GC-MS chromatogram of white popinac alkalinized lyophilized wood vinegar

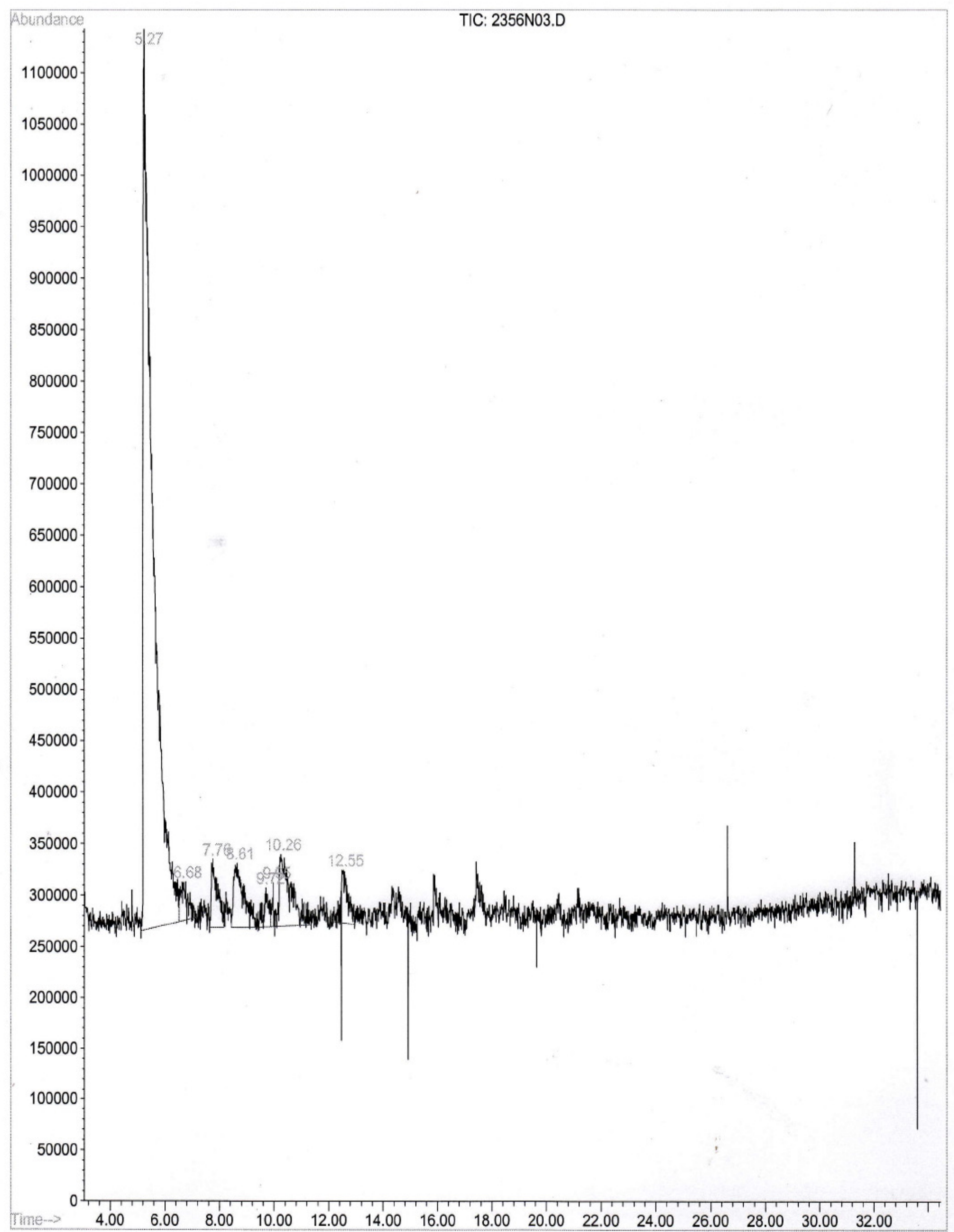

Figure A-11 GC-MS chromatogram of eucalyptus alkalinized lyophilized wood vinegar

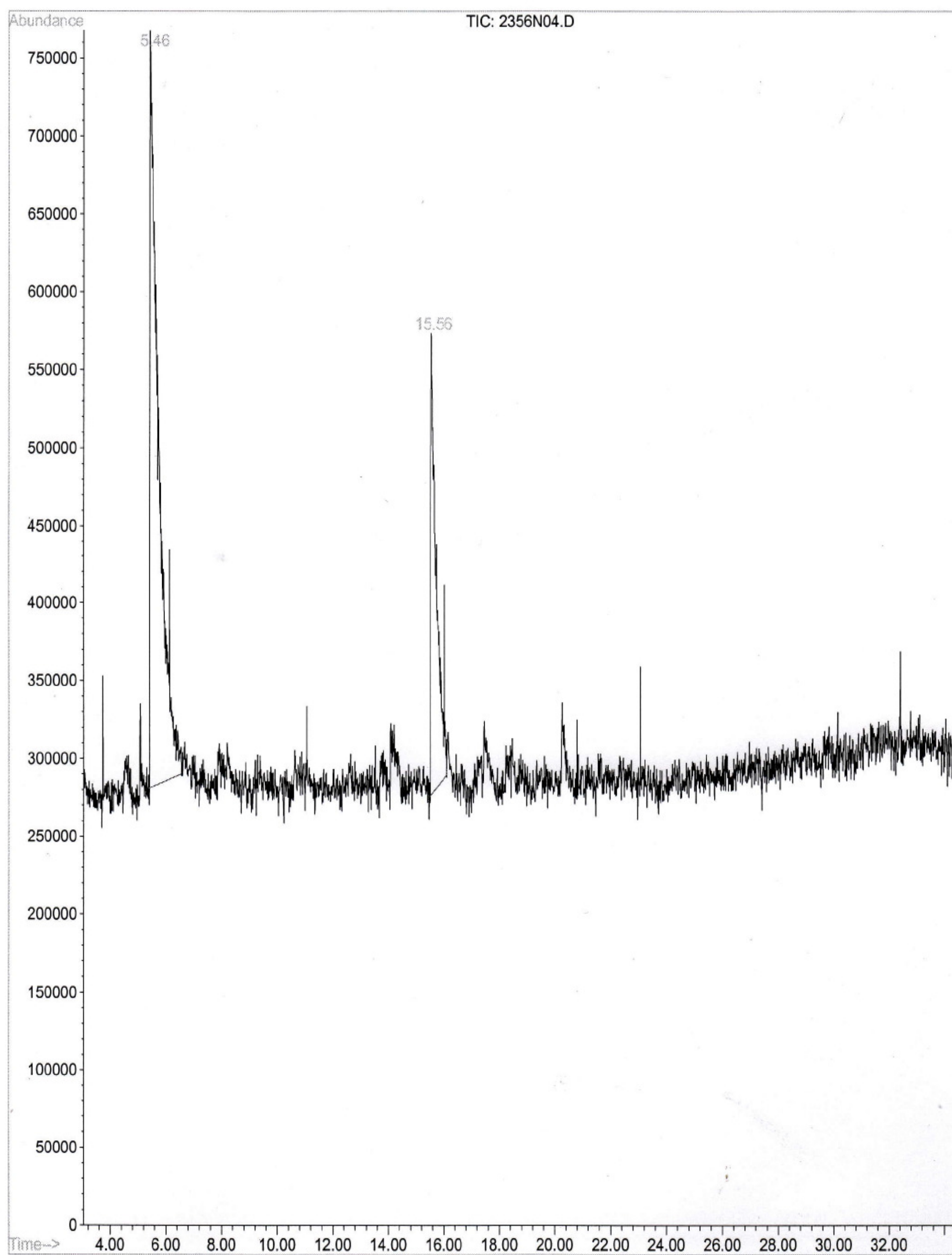

Figure A-12 GC-MS chromatogram of rubber alkalinized lyophilized wood vinegar

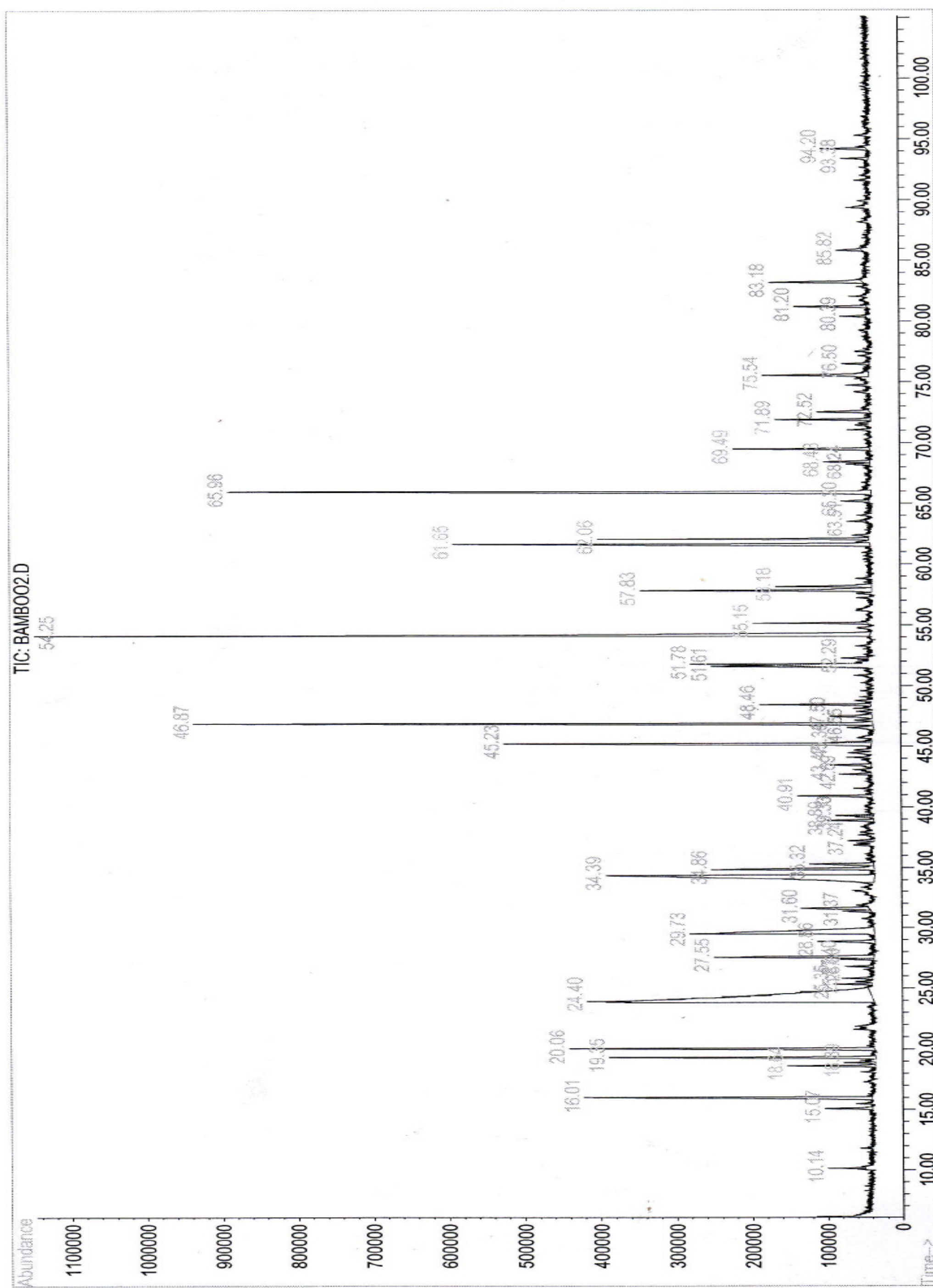

Figure A-13 GC-MS chromatogram of bamboo wood vinegar extracted by dichloromethane

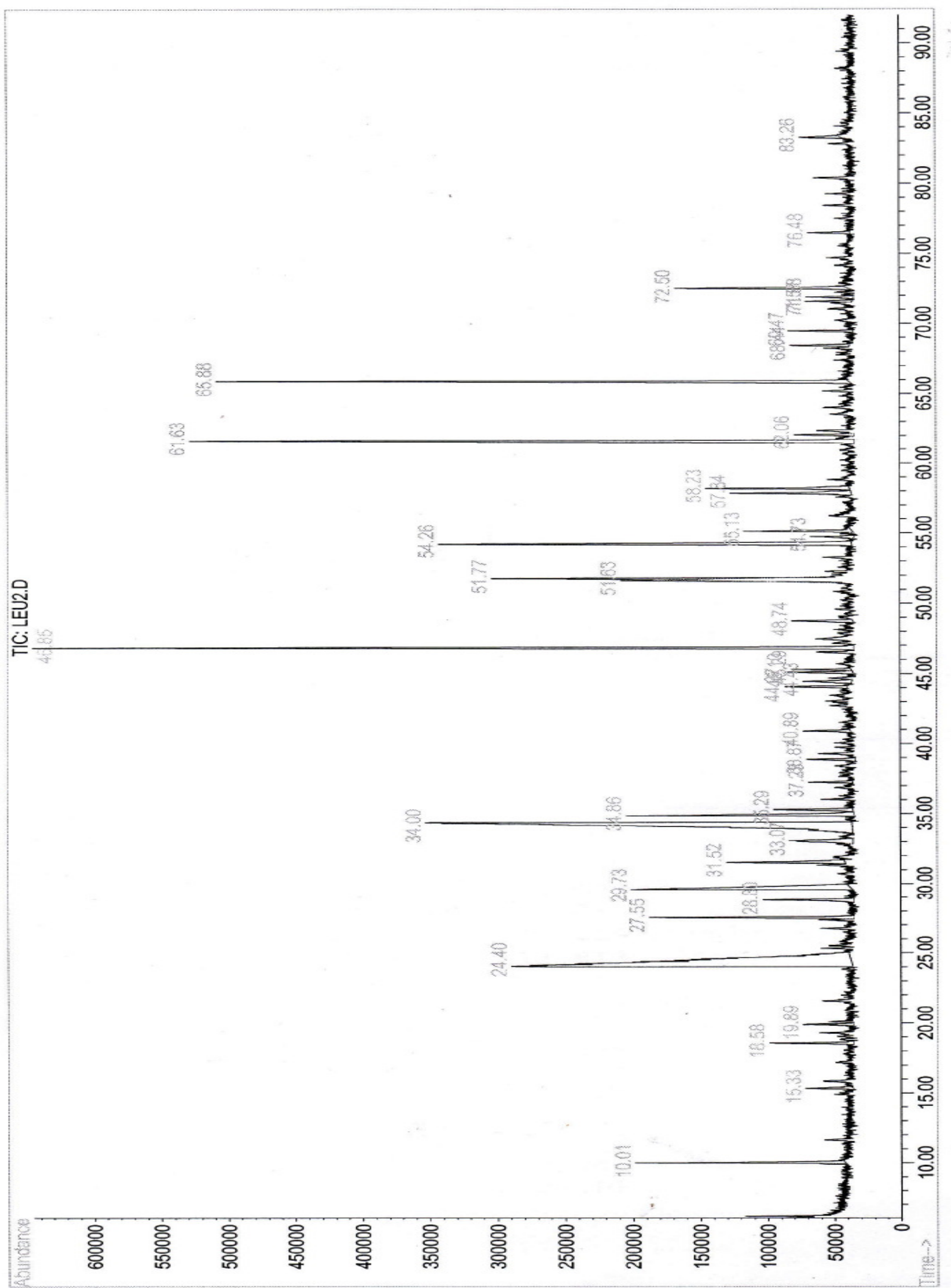

Figure A-14 GC-MS chromatogram of white popinac wood vinegar extracted by dichloromethane

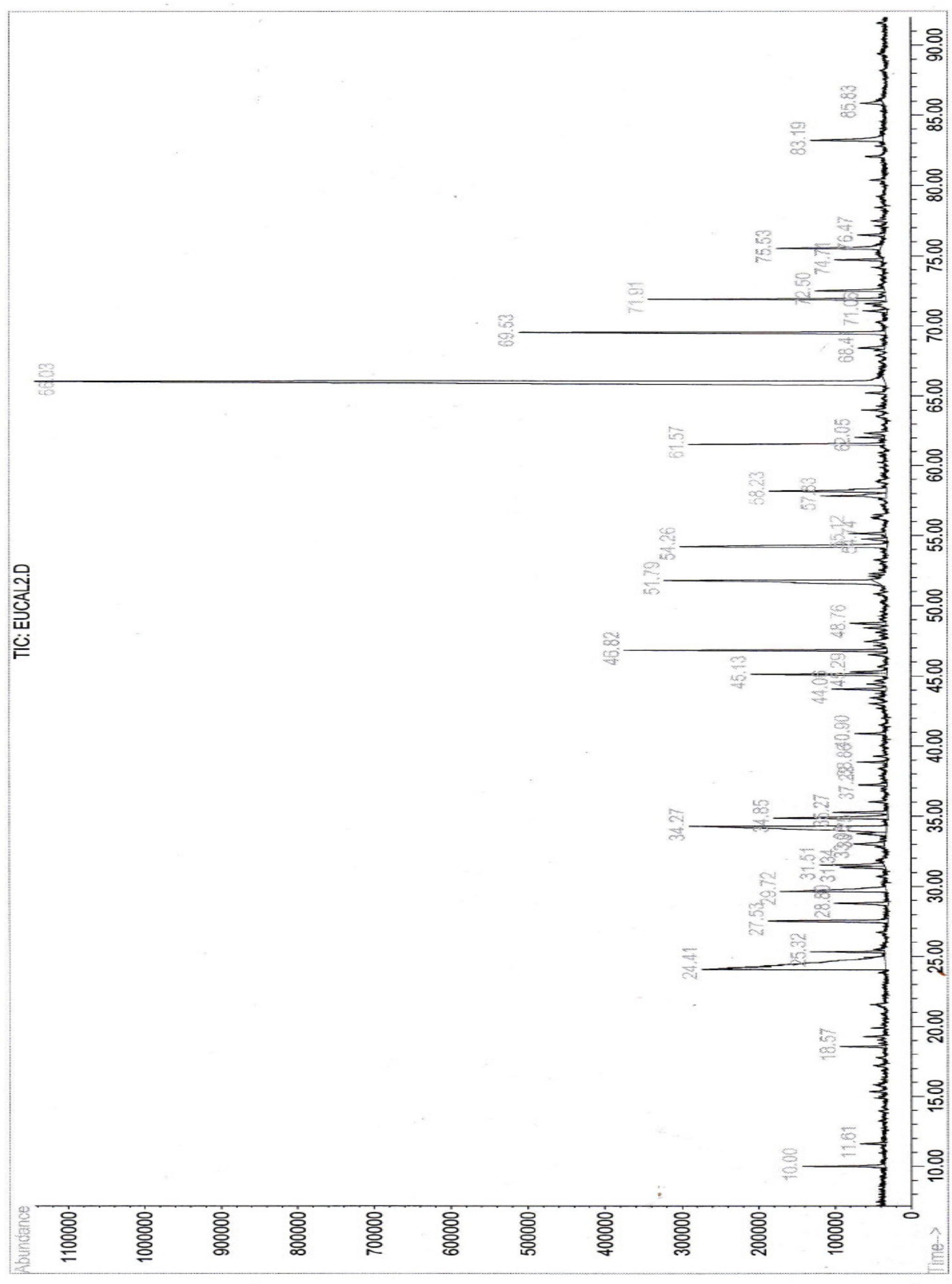

Figure A-15 GC-MS chromatogram of eucalyptus wood vinegar extracted by dichloromethane

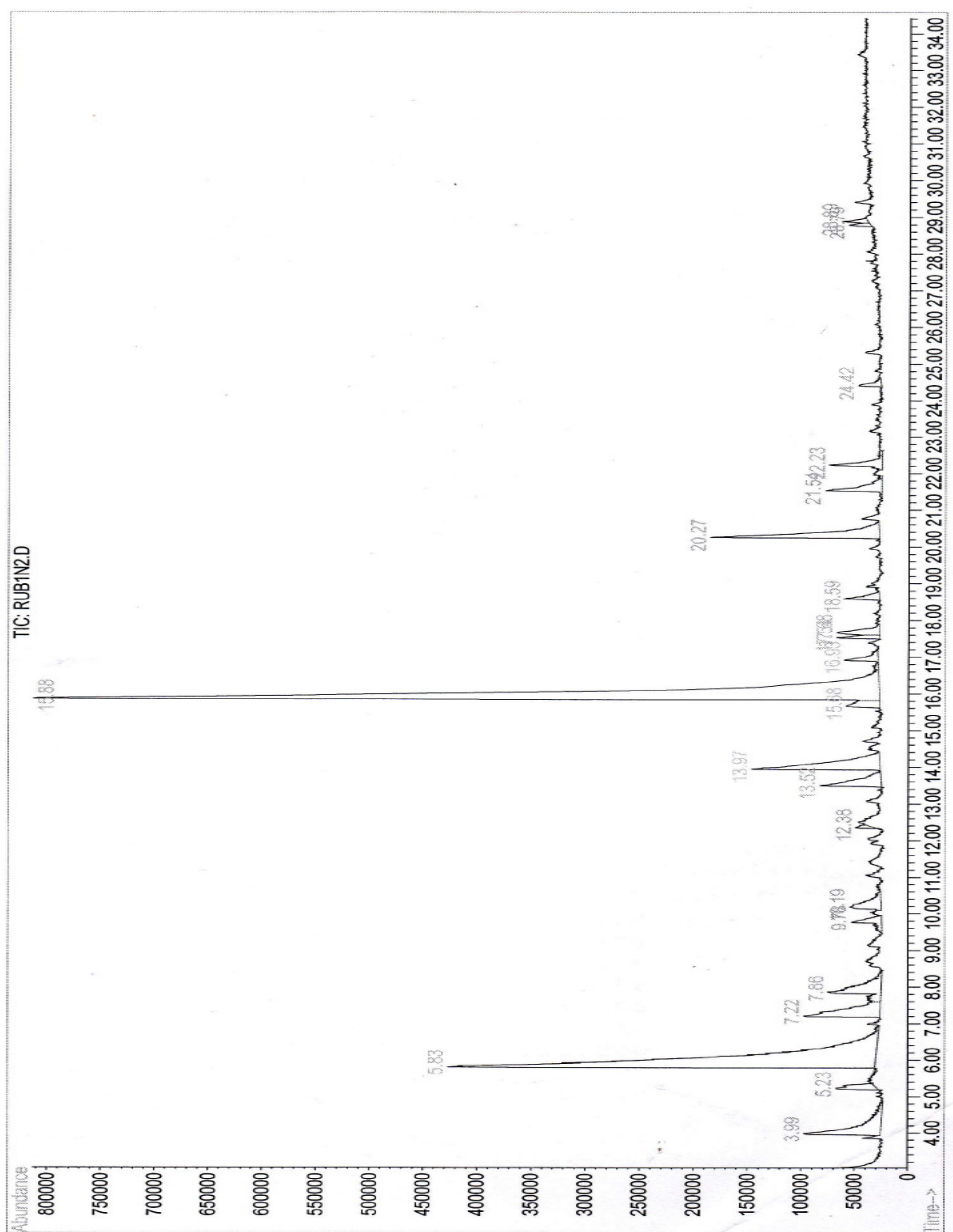

Figure A-16 GC-MS chromatogram of rubber wood vinegar extracted by dichloromethane

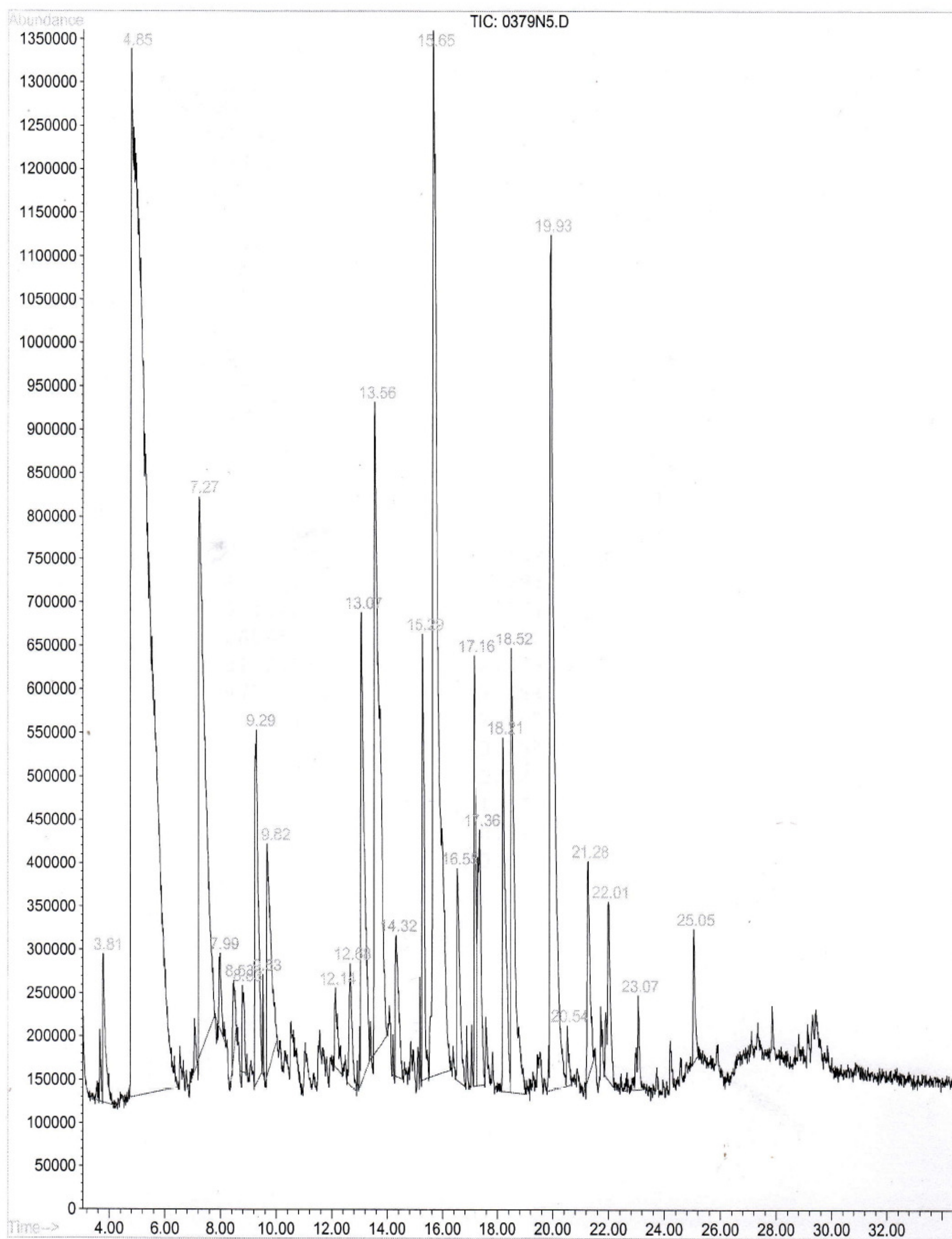

Figure A-17 GC-MS chromatogram of bamboo wood vinegar extracted by diethyl ether

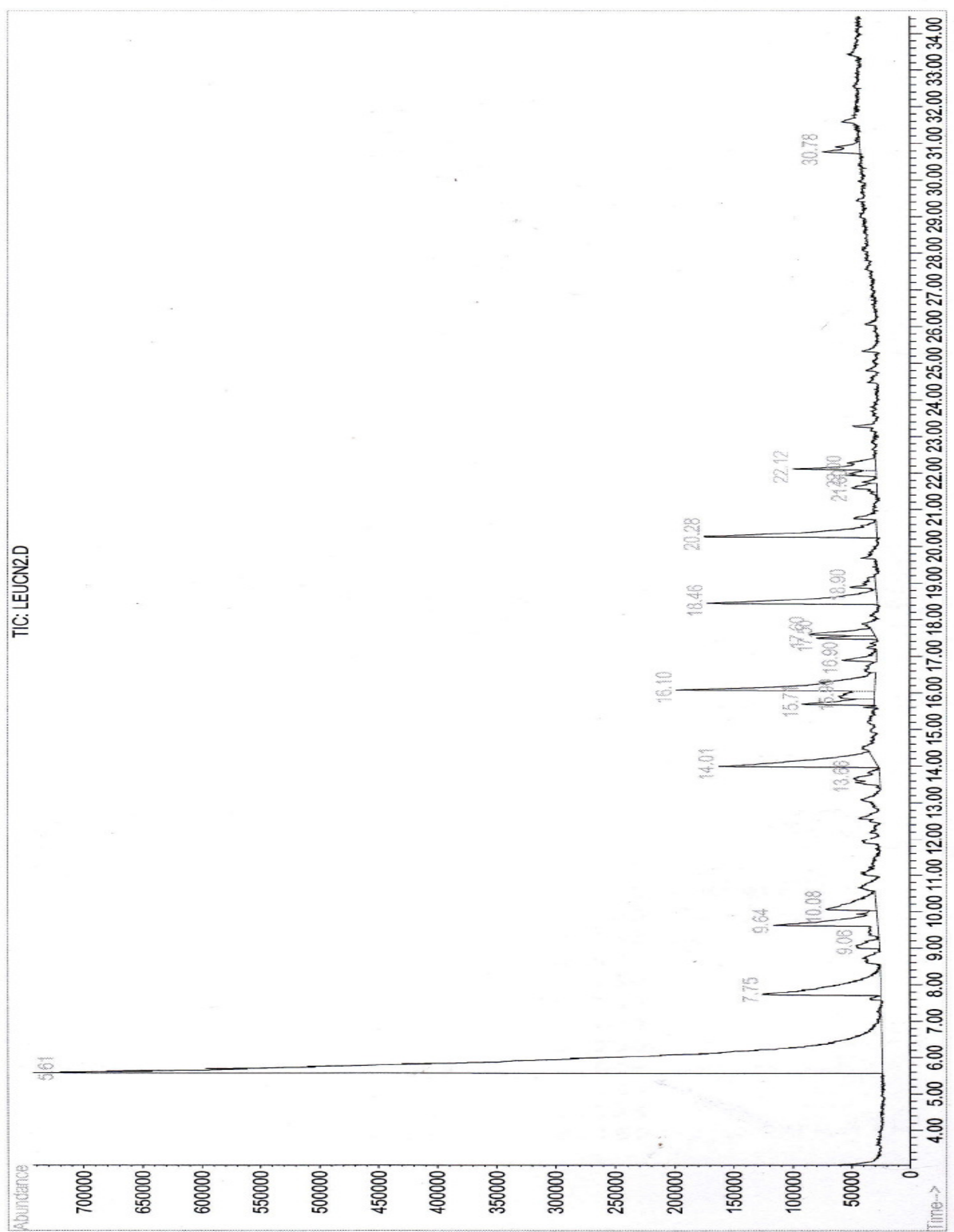

Figure A-18 GC-MS chromatogram of white popinac wood vinegar extracted by diethyl ether

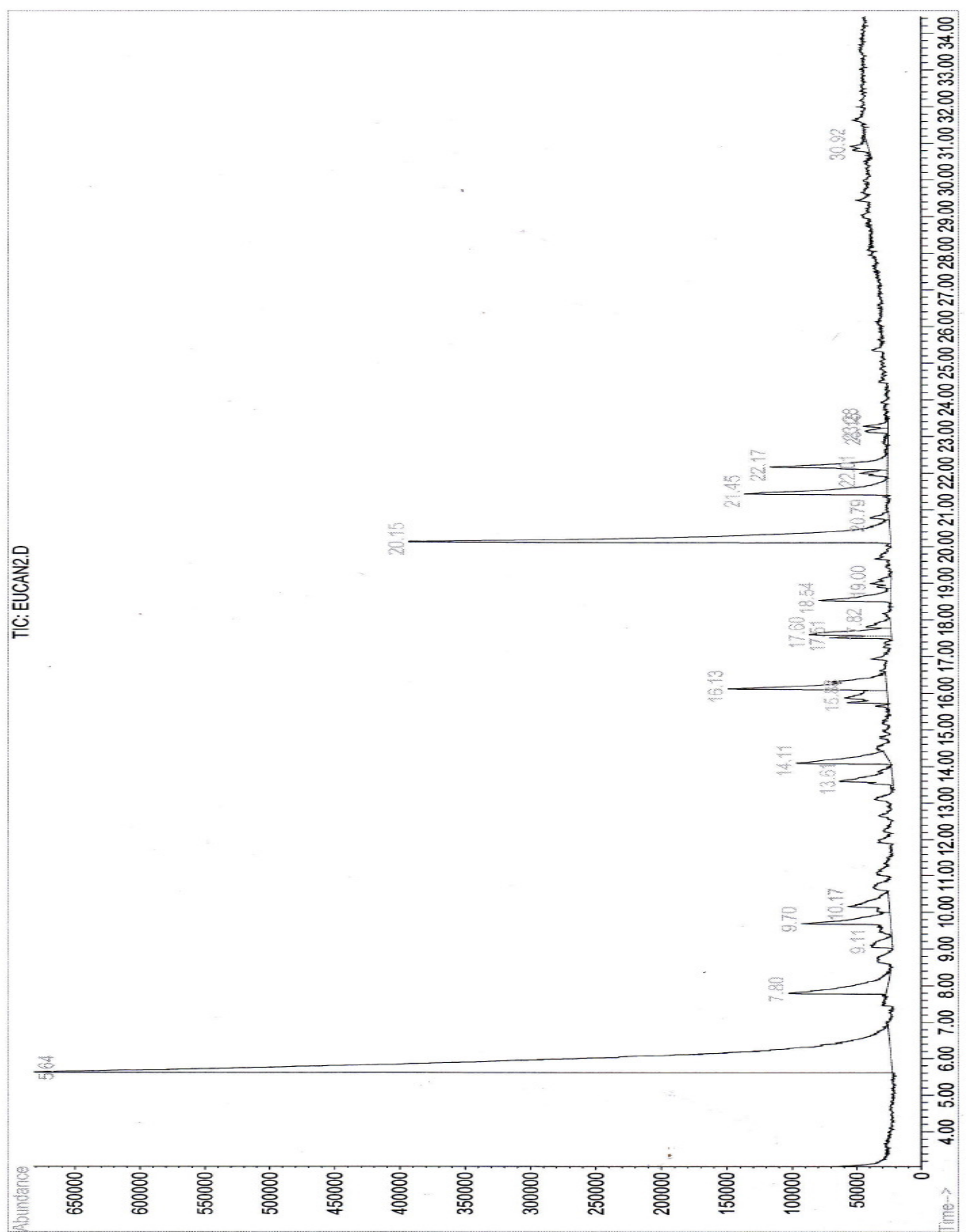

Figure A-19 GC-MS chromatogram of eucalyptus wood vinegar extracted by diethyl ether

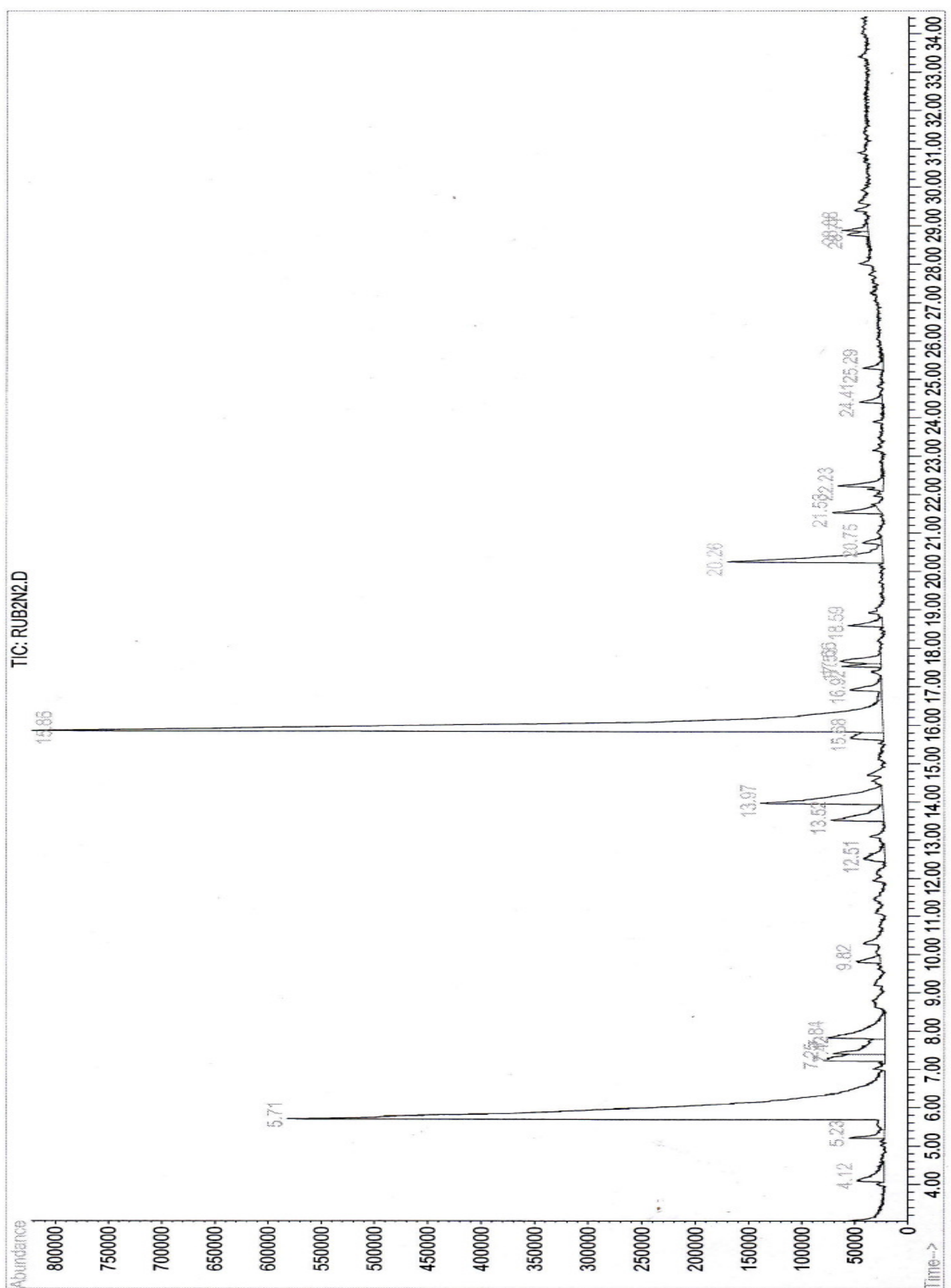

Figure A-20 GC-MS chromatogram of rubber wood vinegar extracted by diethyl ether

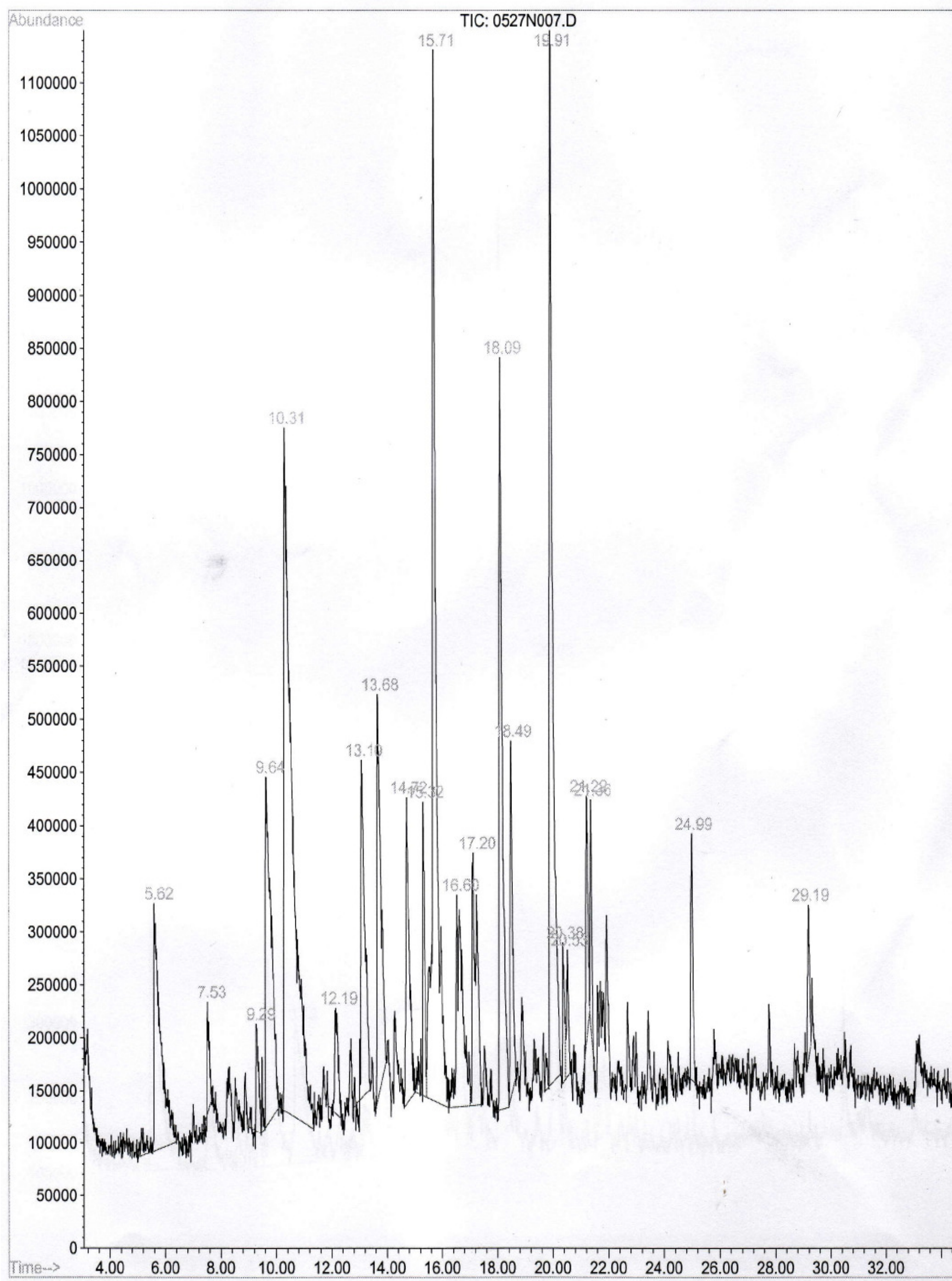

Figure A-21 GC-MS chromatogram of bamboo wood vinegar extracted by isobutanol

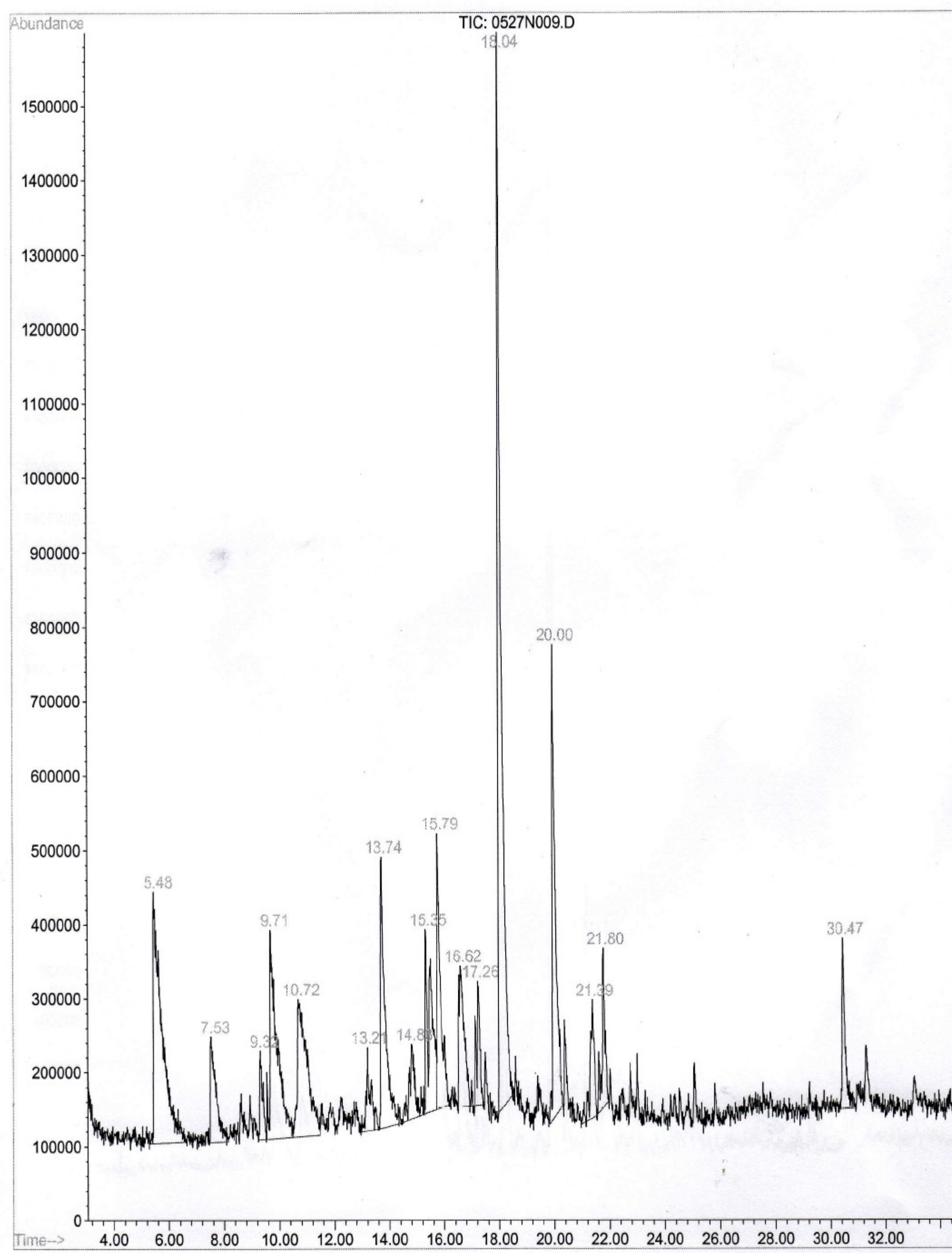

Figure A-22 GC-MS chromatogram of white popinac wood vinegar extracted by isobutanol

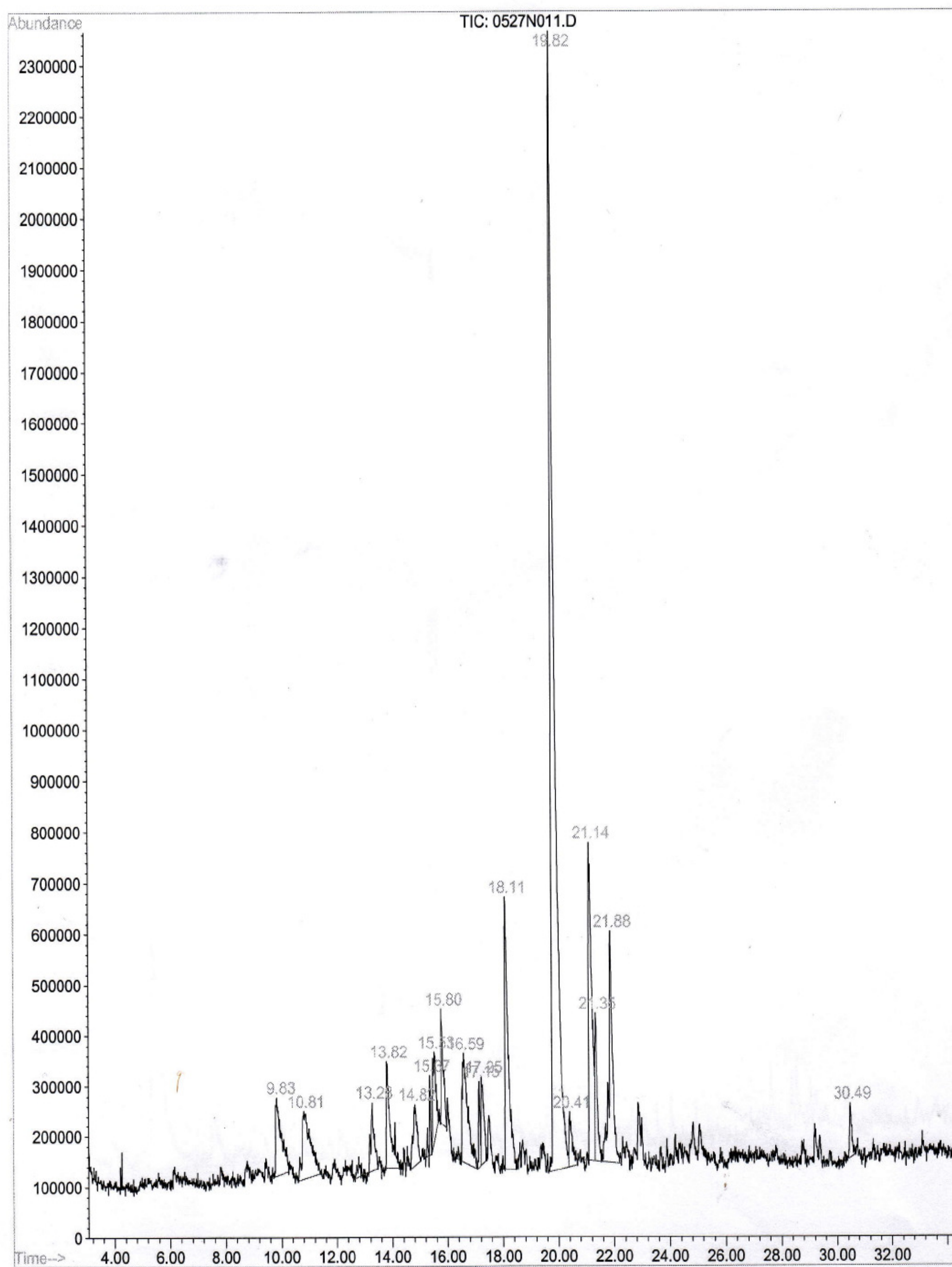

Figure A-23 GC-MS chromatogram of eucalyptus wood vinegar extracted by isobutanol

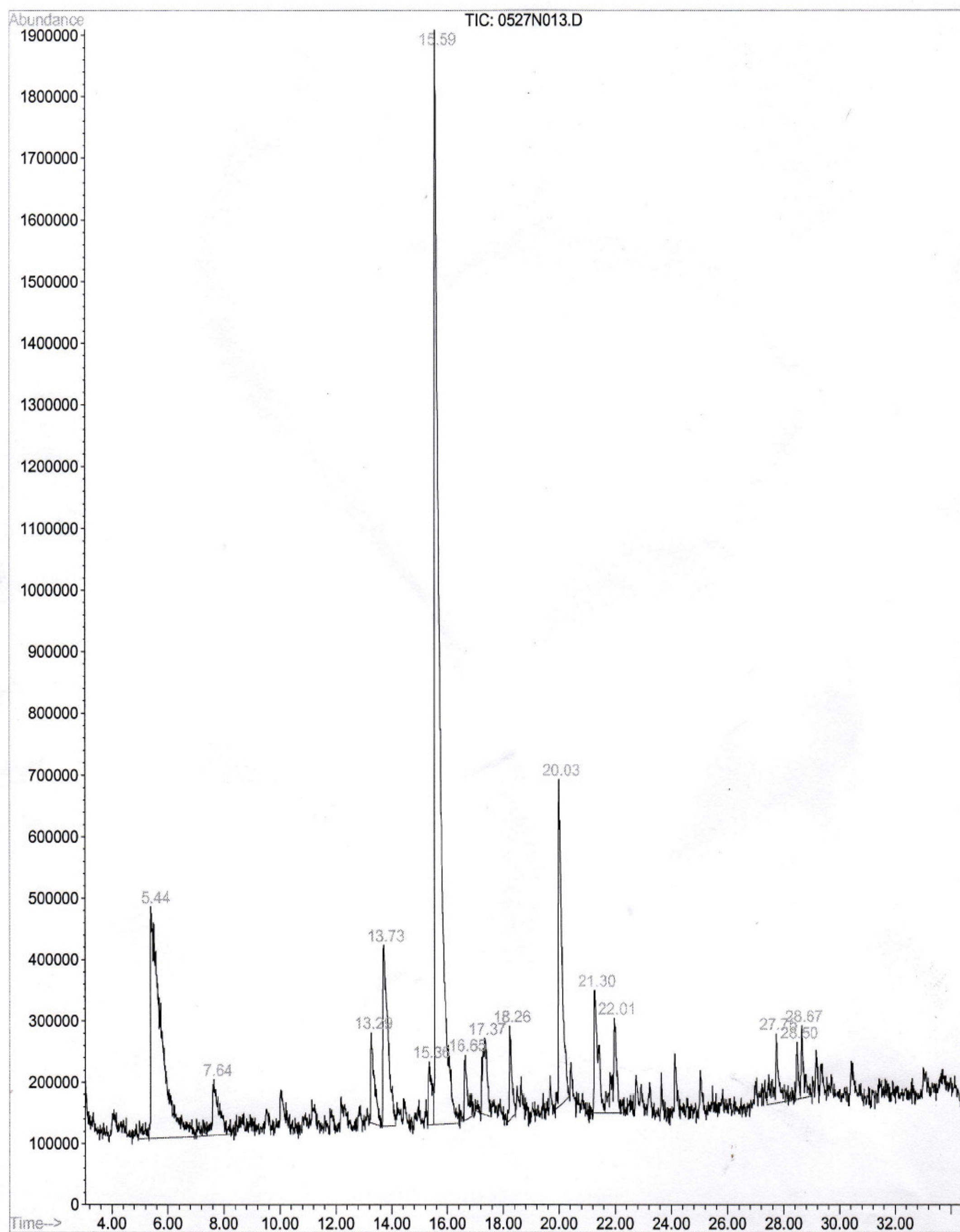

Figure A-24 GC-MS chromatogram of rubber wood vinegar extracted by isobutanol

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List of Publication and Proceedings

Rakmai, J., Ovatlarnporn, C., Kaewnopparat, S. and Amnauykit C. 2008. Chemical and biological properties determinations of wood vinegars and potential utilization studies for skin diseases treatment. *Proceeding of the 9th National Grad Research Conference*, 14-15 March, 2008 at Burapha University, Chonburi, Thailand (oral presentation)

Rakmai, J., Ovatlarnporn, C. and Kaewnopparat, S. 2009. Antibacterial and antifungal properties of wood vinegars and their potential use in the treatment of skin diseases. *Proceeding of the 3rd TRF-Master Research Conference*, 1-3 April, 2009 at Jomtien Palm Beach Resort, Pattaya Beach, Chonburi, Thailand (oral presentation)

Rakmai, J., Ovatlarnporn, C. and Kaewnopparat, S. 2009. Antibacterial properties against dermatitis bacteria of wood vinegars. *Proceeding of the 35th Congress on Science and Technology of Thailand (STT35)*, 15-17 October, 2009 at the Tide Resort, Bangsaen Beach, Chonburi, Thailand (oral presentation)