CHAPTER 3

RESULTS

3.1) SCREENING OF TOTAL PHENOLIC CONTENT, ANTIOXIDANT, AND ANTIMICROBIAL ACTIVITIES FROM INDIGENOUS PLANTS

3.1.1) Total phenolic content

The determination of total phenolic content was carried out by Folin-Ciocalteu's method, referring to the calibration curve using tannic acid solution as a standard solution (Figure 3.1). The content of total phenolics in five tested plants ranged from 57.66 to 1924.42 mg/100 g dried weight (Table 3.1). The highest amount was observed in the plant *C. mimosoides*, followed by *P. oderatum*, *G. inodorum*, *C. grandis*, and *P. anisum*, respectively.

3.1.2) Antioxidant activity

In this study, the antioxidant activity of the methanolic extracts of five plant species was determined by *β*-carotene bleaching method. The results are also shown in Table 3.1. It was found that the methanolic extracts of all plants possessed antioxidant activity. Stronger activity is indicated by a higher antioxidant index. The antioxidant index of the tested plants varied between 2.87-14.79. The methanolic extract of *G. inodorum* exhibited the highest level of the activity using the highest index of 14.97, followed by *C. mimosoides*, *P. anisum*, *P. oderatum*, and *C. grandis*, respectively.

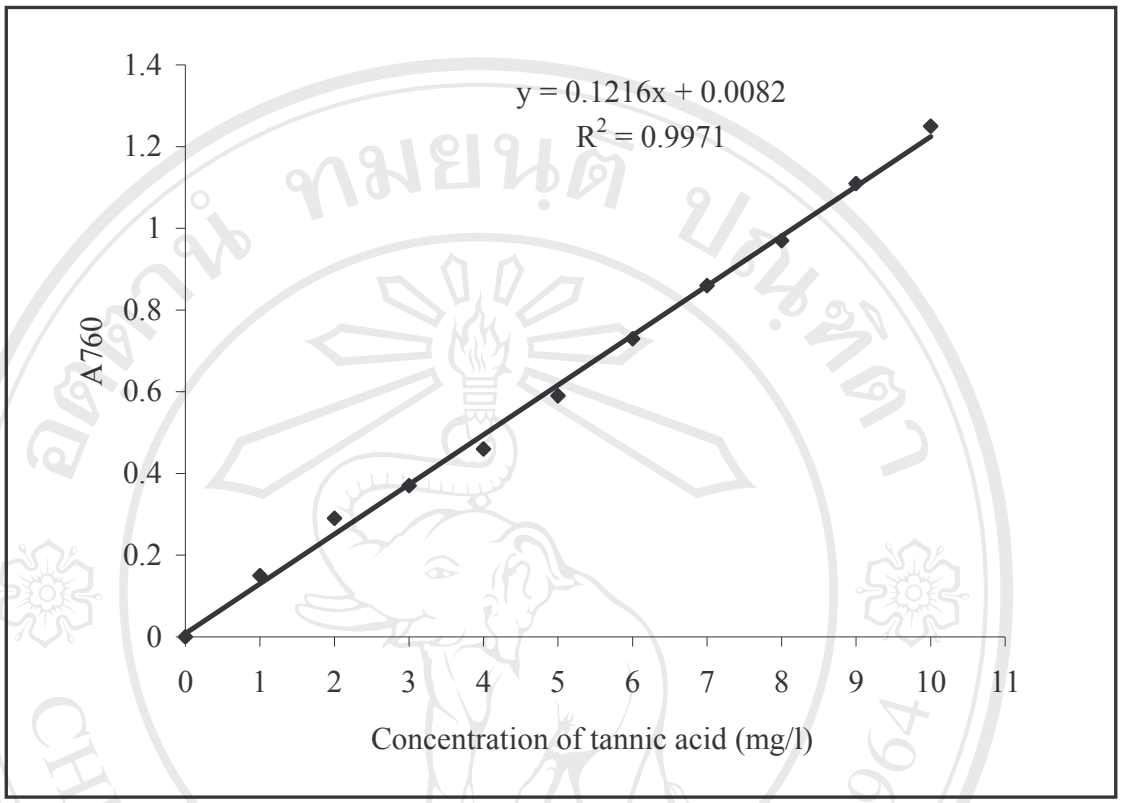


Figure 3.1 Calibration curve of tannic acid solution

Table 3.1 Total phenolic content and antioxidant index of five indigenous plants

Plant	Total phenolic content (mg%)	Antioxidant index
Caesalpinia mimosoides	1924.42	5.43
Coccinia grandis	74.67	2.87
Gymnema inodorum	188.30	14.79
Pimpinella anisum	57.66	4.47
Polygonum oderatum	329.44	3.80

3.1.3) Antimicrobial activity

In order to screen the antimicrobial activity of the test plants, they were extracted with several solvents including acetone, ethanol, chloroform, and water. The percentage yields of the crude extracts are shown in Table 3.2. The antimicrobial activities of various crude extracts of the plants were investigated in term of inhibition zones against some pathogenic bacteria and fungi. The results are summarized in Table 3.3-3.7 for the plant *C. mimosoides*, *C. grandis*, *G. inodorum*, *P. anisum*, and *P. oderatum*, respectively.

As shown in Table 3.3, the aqueous extract of *C. mimosoides* exhibited a potent activity against all of the test bacteria and dermatophytic fungi. The ethanolic extract also showed a high level of the activity against the same microorganisms except *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. The chloroform extract exhibited lower activity against some species (*Vibrio cholerae*, *Staphylococcus aureus*, and *S. epidermidis*), whereas, the acetone extract demonstrated a moderate activity against *V. cholerae*, Gram positive bacteria, and dermatophytic fungi.

The plants *C. grandis* and *G. inodorum* exhibited a low level of activity. Antibacterial activity of *C. grandis* was found in the aqueous extract against *K. pneumoniae*, and the chloroform extract against *S. epidermidis* (Table 3.4), whereas the activity of *G. inodorum* was observed in the aqueous extract against *V. cholerae* only (Table 3.5). Moreover, the antifungal activity of both plant species was not found in this study.

The potent antimicrobial activity was also observed in the extracts of *P. anisum* (Table 3.6); although its aqueous extract exhibited the activity against *V. cholerae* only. The ethanolic extract of this plant demonstrated the higher activity

against all the test microbial strains except *P. aeruginosa* and *Trichophyton rubrum*. The chloroform and the acetone extracts also showed the activity against some species.

The antimicrobial activity of *P. oderatum* was found in all extracts except the aqueous extract (Table 3.7). The activity of the ethanolic extract was higher than other extracts.

Table 3.2 Percentage yield of various crude extracts of the tested plants

Plant	%Yield			
	AcE	AqE	CE	EE
Caesalpinia mimosoides	6.56	9.60	5.96	7.00
Coccinia grandis	8.16	9.60	4.96	2.30
Gymnema inodorum	5.90	24.40	3.90	4.00
Pimpinella anisum	7.00	7.80	4.00	3.00
Polygonum oderatum	7.48	9.40	2.08	4.20

AcE: acetone extract; AqE: aqueous extract; CE: chloroform extract; EE: ethanolic extract

Table 3.3 Inhibition zones of the extracts of *Caesalpinia mimosoides* against some pathogenic microorganisms

Microorganism	Inhibition zone (mm)			
	AcE	AqE	CE	EE
Gram negative bacteria				
Escherichia coli	-	12.67	-	18.67
Klebsiella pneumoniae	-	8.33	-	-
Pseudomonas aeruginosa	-	14.33	-	-
Salmonella typhi	-	25.33	-	18.67
Vibrio cholerae	11	29.67	8.33	20
Gram positive bacteria				
Enterococcus faecalis	10	7.33	-	12.67
Staphylococcus aureus	14	39	11.33	14.33
Staphylococcus epidermidis	12.33	35	8.33	15
Yeast				
Candida albicans	-	-	-	-
Filamentous fungi				
Aspergillus sp.	-	-	-	-
Fusarium sp.	-	-	-	-
*Microsporum gypseum**	15	42.33	-	24.33
Penicillium sp.	-	-	-	-
*Trichophyton rubrum**	11.67	40.67	-	13.33

AcE: acetone extract; AqE: aqueous extract; CE: chloroform extract; EE: ethanolic extract; *: dermatophytic fungi; and "-": no inhibition zone

Table 3.4 Inhibition zones of the extracts of *Coccinia grandis* against some pathogenic microorganisms

Microorganism	Inhibition zone (mm)			
	AcE	AqE	CE	EE
Gram negative bacteria				
Escherichia coli	-	-	-	-
Klebsiella pneumoniae	-	9.33	-	-
Pseudomonas aeruginosa	-	-	-	-
Salmonella typhi	-	-	-	-
Vibrio cholerae	-	-	-	-
Gram positive bacteria				
Enterococcus faecalis	-	-	-	-
Staphylococcus aureus	-	-	-	-
Staphylococcus epidermidis	-	-	8.33	-
Yeast				
Candida albicans	-	-	-	-
Filamentous fungi				
Aspergillus sp.	-	-	-	-
Fusarium sp.	-	-	-	-
*Microsporum gypseum**	-	-	-	-
Penicillium sp.	-	-	-	-
*Trichophyton rubrum**	-	-	-	-

AcE: acetone extract; AqE: aqueous extract; CE: chloroform extract; EE: ethanolic extract; *: dermatophytic fungi; and "-": no inhibition zone

Table 3.5 Inhibition zones of the extracts of *Gymnema inodorum* against some pathogenic microorganisms

Microorganism	Inhibition zone (mm)			
	AcE	AqE	CE	EE
Gram negative bacteria				
Escherichia coli	-	-	-	-
Klebsiella pneumoniae	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-
Salmonella typhi	-	-	-	-
Vibrio cholerae	-	7.67	-	-
Gram positive bacteria				
Enterococcus faecalis	-	-	-	-
Staphylococcus aureus	-	-	-	-
Staphylococcus epidermidis	-	-	-	-
Yeast				
Candida albicans	-	-	-	-
Filamentous fungi				
Aspergillus sp.	-	-	-	-
Fusarium sp.	-	-	-	-
*Microsporum gypseum**	-	-	-	-
Penicillium sp.	-	-	-	-
*Trichophyton rubrum**	-	-	-	-

AcE: acetone extract; AqE: aqueous extract; CE: chloroform extract; EE: ethanolic extract; *: dermatophytic fungi; and "-": no inhibition zone

Table 3.6 Inhibition zones of the extracts of *Pimpinella anisum* against some pathogenic microorganisms

Microorganism	Inhibition zone (mm)			
	AcE	AqE	CE	EE
Gram negative bacteria				
Escherichia coli	7.33	-	-	19.67
Klebsiella pneumoniae	-	-	-	10
Pseudomonas aeruginosa	-	-	-	-
Salmonella typhi	7	-	7.33	16.67
Vibrio cholerae	11.33	15.33	12.67	25.67
Gram positive bacteria				
Enterococcus faecalis	-	-	-	11.67
Staphylococcus aureus	9.33	-	14	23.33
Staphylococcus epidermidis	8.33	-	13.33	13.67
Yeast				
Candida albicans	-	-	-	13.67
Filamentous fungi				
Aspergillus sp.	-	-	-	8.33
Fusarium sp.	-	-	-	14
*Microsporum gypseum**	-	-	-	40.33
Penicillium sp.	-	-	-	7.67
*Trichophyton rubrum**	-	-	9.33	-

AcE: acetone extract; AqE: aqueous extract; CE: chloroform extract; EE: ethanolic extract; *: dermatophytic fungi; and "-": no inhibition zone

Table 3.7 Inhibition zones of the extracts of *Polygonum oderatum* against some pathogenic microorganisms

Microorganism	Inhibition zone (mm)			
	AcE	AqE	CE	EE
Gram negative bacteria				
Escherichia coli	-	-	-	7.17
Klebsiella pneumoniae	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-
Salmonella typhi	-	-	-	7
Vibrio cholerae	7.67	-	8	10.33
Gram positive bacteria				
Enterococcus faecalis	-	-	-	-
Staphylococcus aureus	9.33	-	8.67	8.33
Staphylococcus epidermidis	8	-	8.33	8.33
Yeast				
Candida albicans	7.33	-	-	7.67
Filamentous fungi				
Aspergillus sp.	-	-	-	7.67
Fusarium sp.	-	-	-	-
*Microsporum gypseum**	10.67	-	-	10.67
Penicillium sp.	-	-	-	-
*Trichophyton rubrum**	-	-	-	12.33

AcE: acetone extract; AqE: aqueous extract; CE: chloroform extract; EE: ethanolic extract; *: dermatophytic fungi; and "-": no inhibition zone

3.2) SEPARATION AND PARTIAL PURIFICATION OF ANTIMICROBIAL COMPOUNDS FROM *Caesalpinia mimosoides* AND *Pimpinella anisum*

Based on the result for the screening of antimicrobial activity in section 3.1.3, potent antimicrobial activity was found in the aqueous and the ethanolic extracts of the plant *C. mimosoides*, and the ethanolic extract of *P. anisum*. Therefore, for these extracts, it was of interesting to study their antimicrobial compounds, particularly the phenolic compounds.

3.2.1) Separation and partial purification of phenolic compounds by paper chromatography

The separation and partial purification of phenolic compounds from the crude extracts of the selected plants was carried out using two-dimensional paper chromatography. Their chromatograms are shown in Figure 3.2. The aqueous extract of *C. mimosoides* gave three spots on the chromatogram, while the ethanolic extract presented four spots. The ethanolic extract of *P. anisum* showed three spots on its chromatogram. Color reactions and R_f values of the spots on the chromatograms are summarized in Table 3.8. Their R_f values and color characteristics were compared with those of authentic compounds (data not shown). It was found that not all spots could be identified.

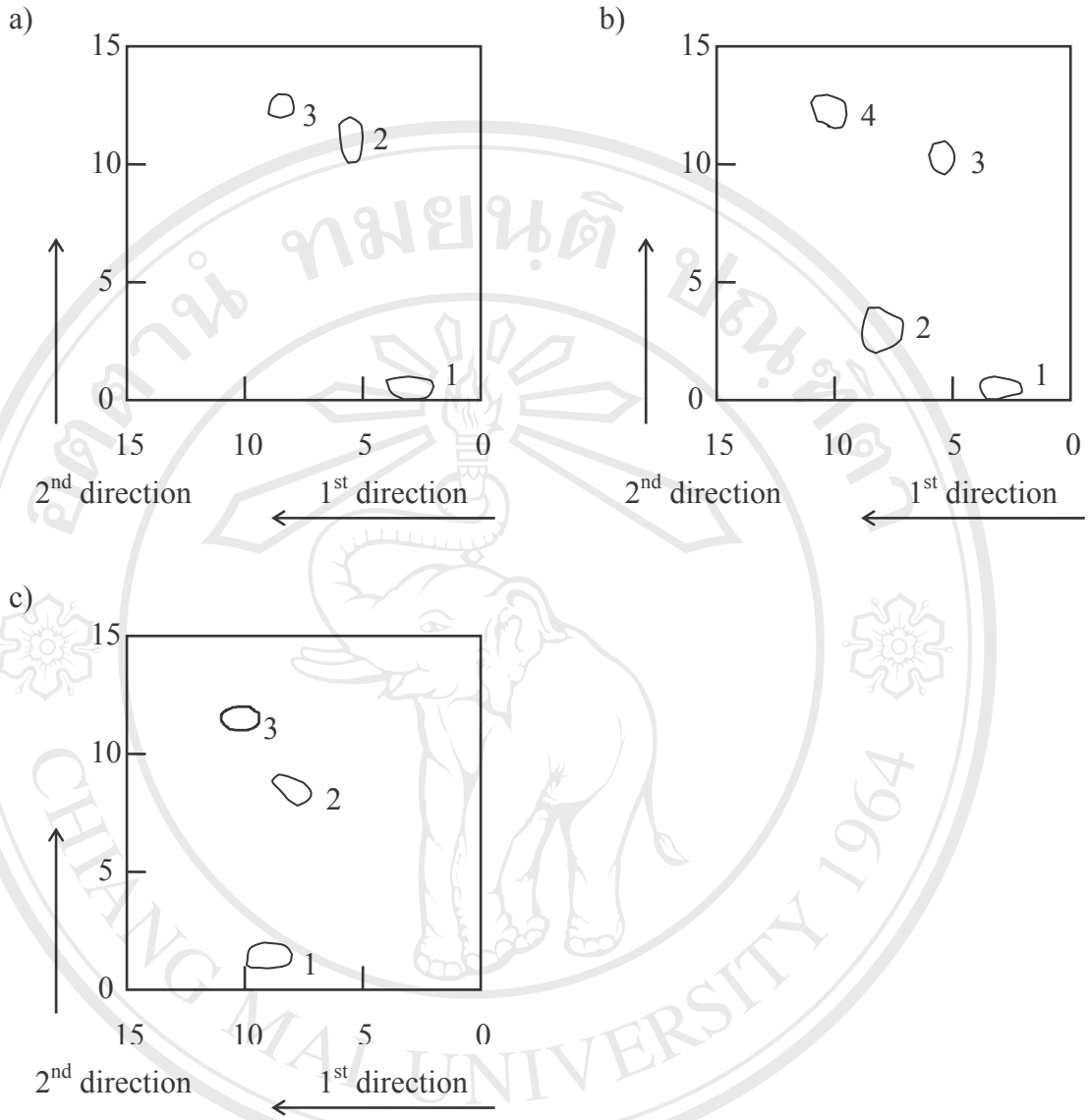


Figure 3.2 Paper chromatograms of phenolic compounds from the crude plant extracts (the chromatograms were developed with n-butanol-acetic acid-water; 6:1:2 v/v/v; in the first direction and 2% aqueous acetic acid in the second direction)

a: Aqueous extract from *Caesalpinia mimosoides*

b: Ethanolic extract from *Caesalpinia mimosoides*

c: Ethanolic extract from *Pimpinella anisum*

Table 3.8 R_f values and color reactions on paper chromatograms of phenolic compounds from the crude plant extracts

Extract	Spot No.	R_f value in solvent system*		Color** under UV light		Color with Folin-Ciocalteu's reagent
		a	b	254 nm	365 nm	
Aqueous extract from *Caesalpinia mimosoides*	1.	0.18	0.03	BB	BB	B
	2.	0.30	0.78	BB	BB	DB
	3.	0.50	0.81	BB	BB	DB
Ethanolic extract from *Caesalpinia mimosoides*	1.	0.23	0.03	BB	BB	B
	2.	0.61	0.22	BB	BB	DB
	3.	0.35	0.73	BB	BB	DB
	4.	0.66	0.89	BB	BB	DB
Ethanolic extract from *Pimpinella anisum*	1.	0.65	0.08	BB	BB	B
	2.	0.58	0.48	BB	BB	DB
	3.	0.70	0.80	BB	BB	DB

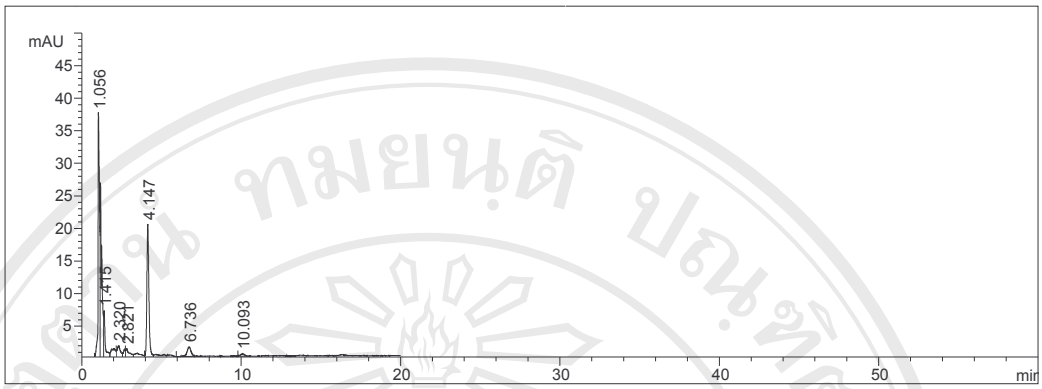
*Solvent system; a: n-butanol-acetic acid-water = 6:1:2 v/v/v; b: 2% aqueous acetic acid

**Color; B: Blue; BB: Bright blue; DB: Dark blue

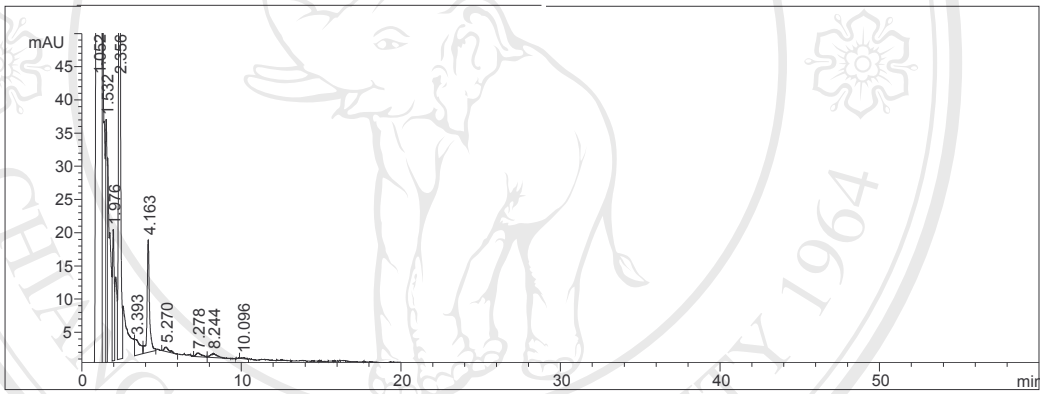
3.2.2) HPLC analysis of partial purified phenolic compounds

Phenolic compounds of *C. mimosoides* and *P. anisum* were separated by two-dimensional paper chromatography as described in section 2.4.1. Each spot on the chromatograms was cut out and eluted in a small volume of ethanol before analysis by HPLC. The HPLC chromatograms of the spots were shown in Figure 3.3-3.5. The results showed that the phenolic compounds from both plants could not be identified at this stage.

a)



b)



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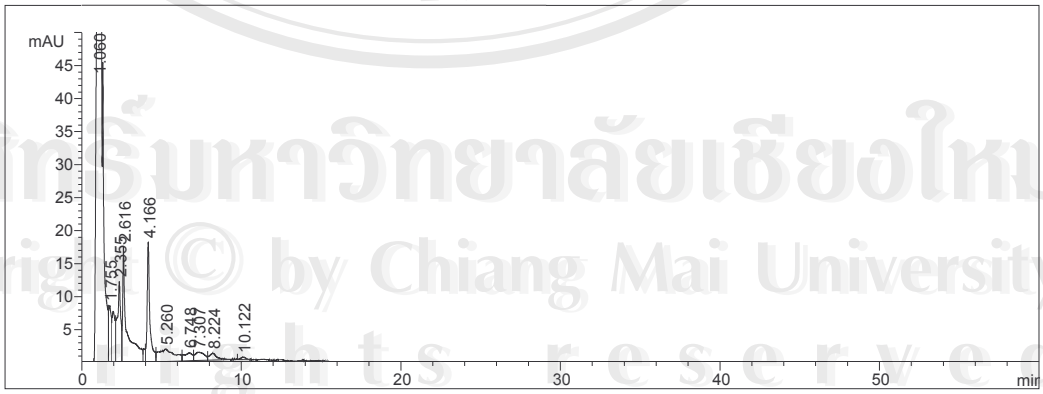
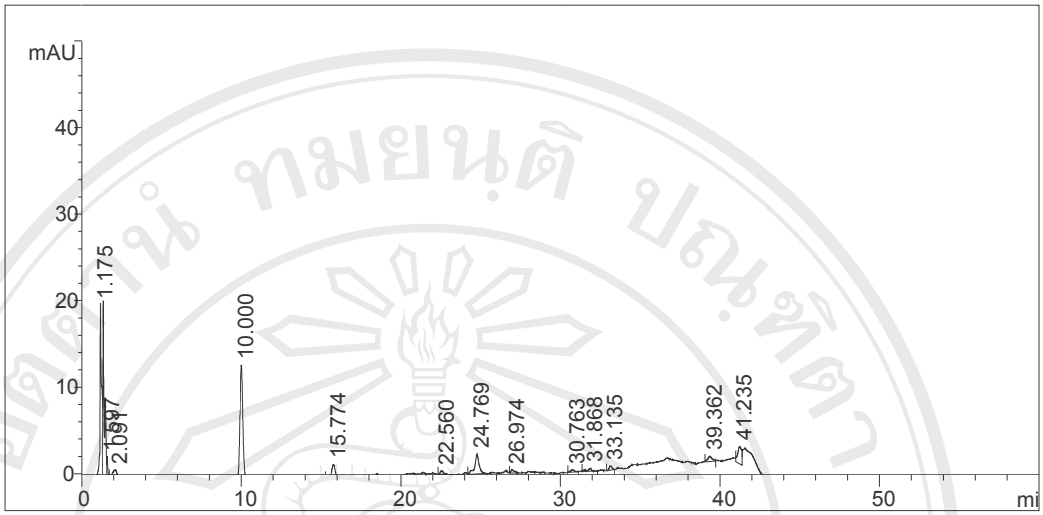


Figure 3.3 HPLC chromatograms of partial purified phenolics from the aqueous extract of *Caesalpinia mimosoides* (a: spot 1; b: spot 2; and c: spot 3 on the paper chromatogram)

a)



b)

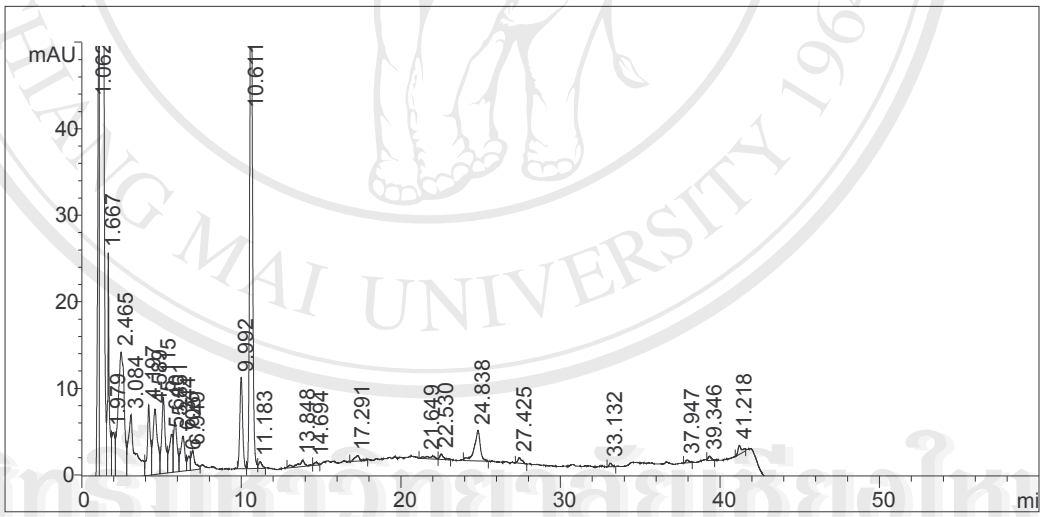
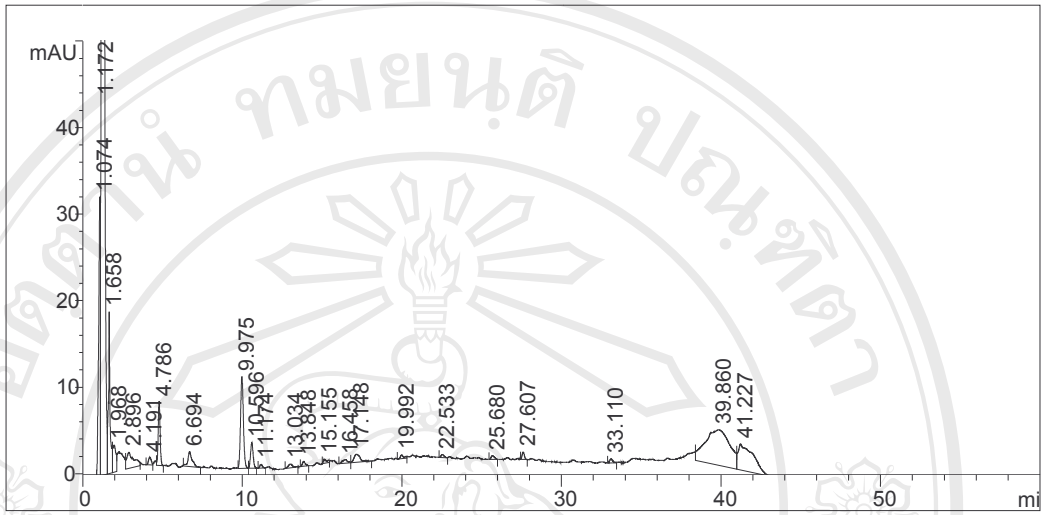


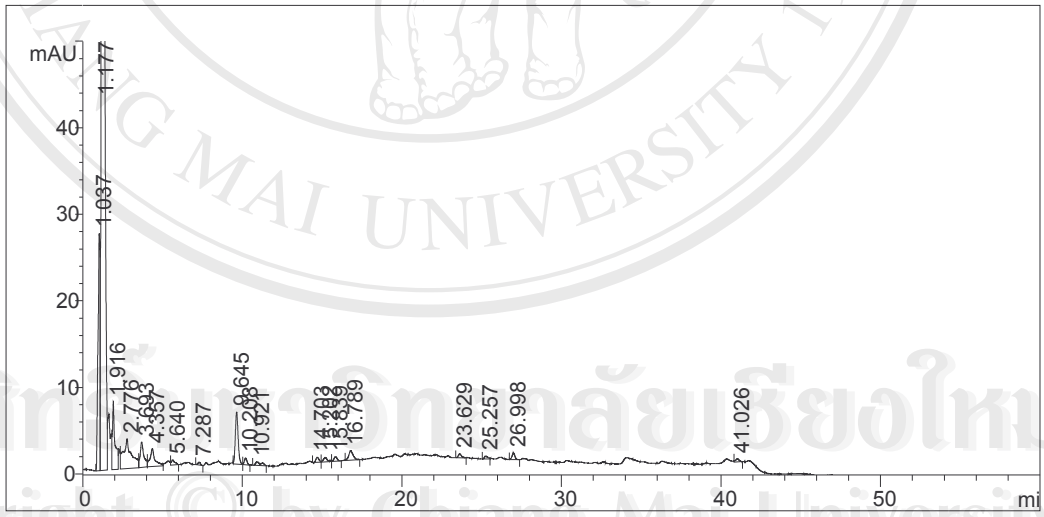
Figure 3.4 HPLC chromatograms of partial purified phenolics from the ethanolic extract of *Caesalpinia mimosoides* (a: spot 1; b: spot 2; c: spot 3; and d: spot 4 on the paper chromatogram)

Figure 3.4 (Continued)

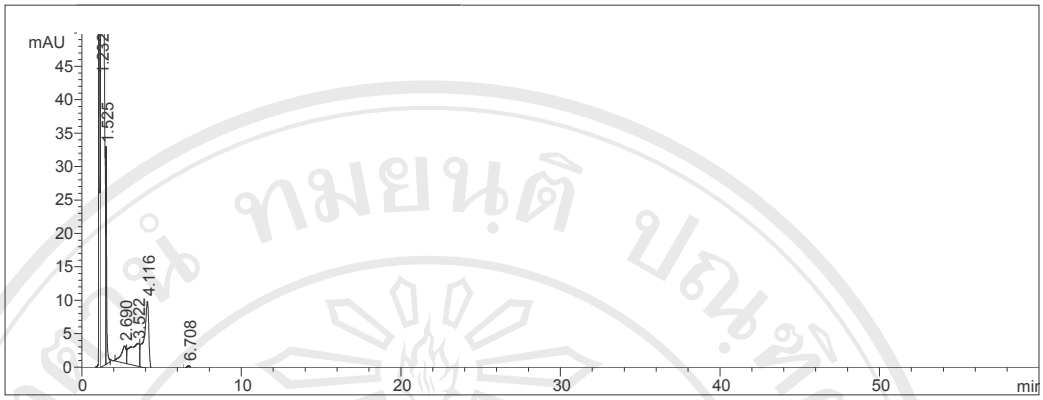
c)



d)



a)



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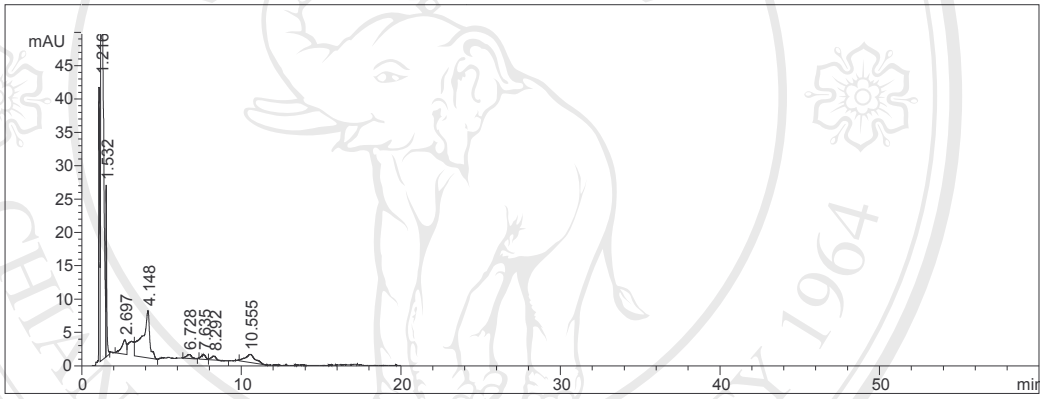


c)

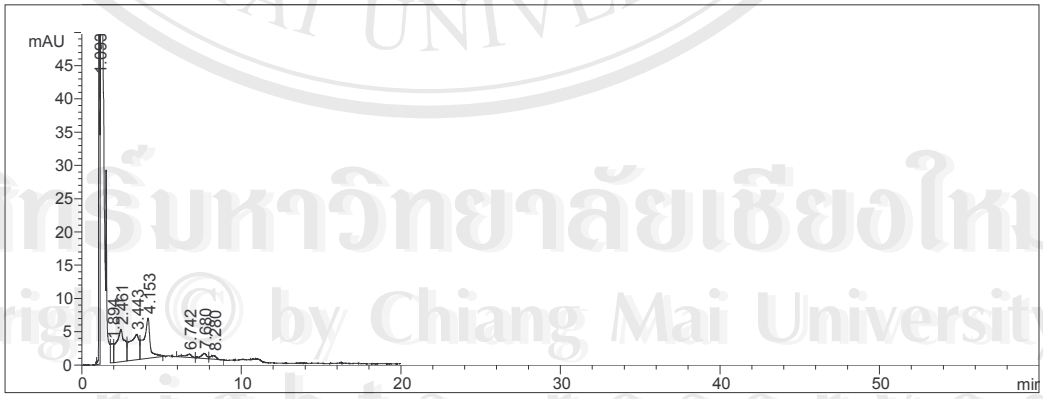


Figure 3.5 HPLC chromatograms of partial purified phenolics from the ethanolic extract of *Pimpinella anisum* (a: spot 1; b: spot 2; and c: spot 3 on the paper chromatogram)

3.2.3) Determination of antimicrobial activity by bioautography

Antimicrobial activity of the partial purified phenolics separated from *C. mimosoides* and *P. anisum* was determined by bioautographic method using two bacterial strains (*Escherichia coli* and *Staphylococcus aureus*), and two dermatophytic fungi (*Microsporum gypseum* and *Trichophyton rubrum*) as the test microoorganisms. Two-dimensional paper chromatography developed as described in section 2.4.1 were used in this assay. Bioautography of the phenolics in each spot on the chromatograms worked well with the bacterium *S. aureus*, but did not affect the other test microbes. Inhibition zones against *S. aureus* were observed around spot 2 and 3 of both chromatograms of the plant *C. mimosoides*, and spot 1 and 2 of the chromatogram of *P. anisum* (Table 3.9). The results indicated that the compounds in the spots mentioned above seem to be active compounds with antimicrobial (or antibacterial) activity.

Table 3.9 Inhibition zones of partial purified phenolic compounds from the extracts of *Caesalpinia mimosoides* and *Pimpinella anisum* against the bacterium *Staphylococcus aureus*

Extract	Inhibition zone (mm)			
	Spot 1	Spot 2	Spot 3	Spot 4
Aqueous extract of *Caesalpinia mimosoides*	-	11	13	N
Ethanolic extract of *Caesalpinia mimosoides*	-	11	11	-
Ethanolic extract of *Pimpinella anisum*	11	-	11	N

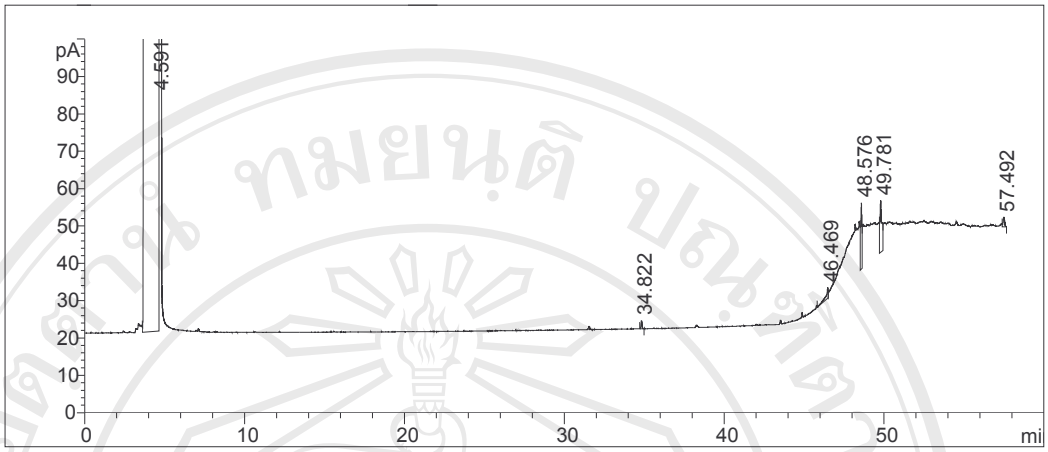
"-": No inhibition zone; N: no spot on chromatogram

3.3) PRELIMINARY IDENTIFICATION OF ANTIMICROBIAL COMPOUNDS FROM *Pimpinella anisum*

3.3.1) GC analysis of antimicrobial compounds

Based on the result of the bioautographic assay, spot 1 and 3 on the chromatogram of *P. anisum* were active compounds responsible for antimicrobial activity. The compounds in both spots were then analyzed by a Hewlett-Packard 5890 series II gas chromatograph (INNOWAX column). Identification of the compounds was based on comparison of the retention times with those of the components in the essential oil of star anise (*Illicium verum*), such as anethole, methyl chavicol, and anisaldehyde. GC chromatograms of the antimicrobial compounds of *P. anisum* and the essential oil of star anise were shown in Figure 3.6-3.7, respectively. The results showed that the retention times of the constituents of antimicrobial compounds from *P. anisum* related to those of some components in the essential oil of star anise.

a)



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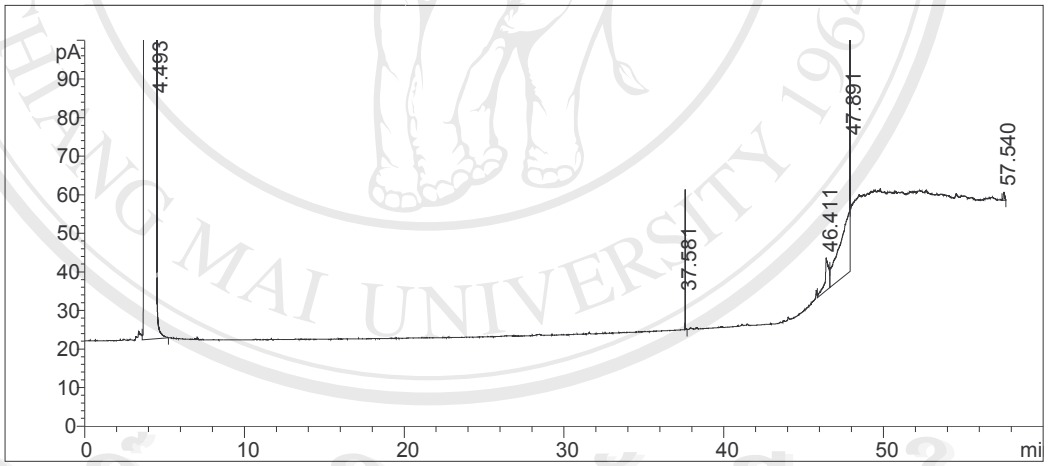


Figure 3.6 GC chromatograms of antimicrobial compounds from the ethanolic extract of *Pimpinella anisum* (a: spot 1; and b: spot 3 on paper chromatogram)

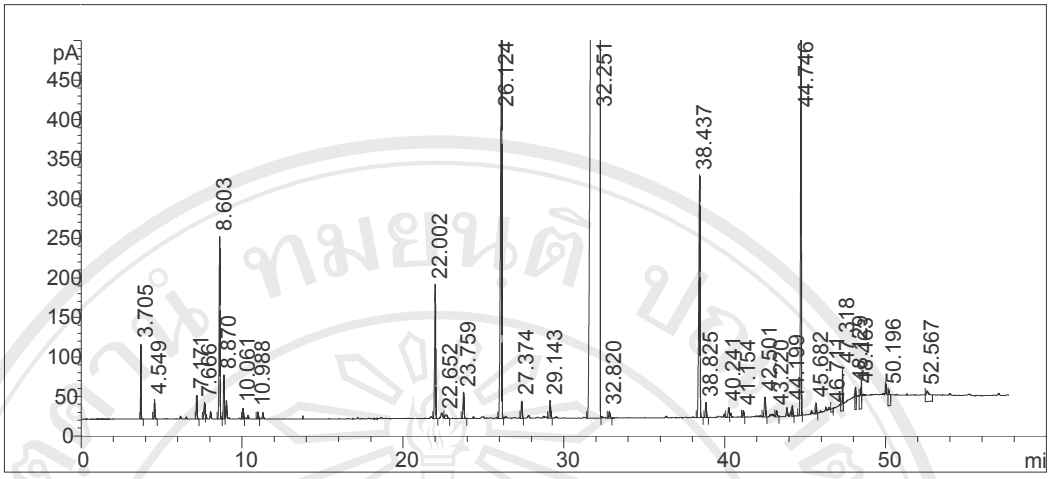


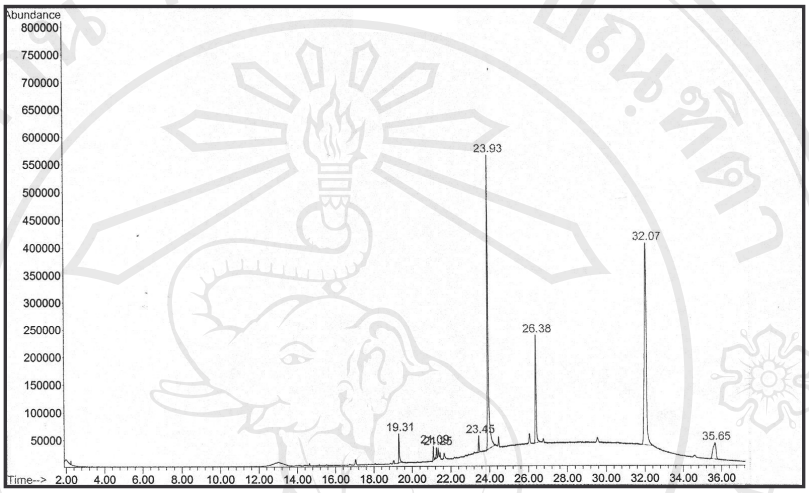
Figure 3.7 GC chromatogram of the essential oil of star anise (*Illicium verum*)

3.3.2) GS-MS analysis of antimicrobial compounds

In order to identify the antimicrobial compounds of *P. anisum*, they were also analyzed by a Hewlett-Packard 6890 series GC-MS (AT-1MS column) (Figure 3.8). Mass spectra of each constituent were analyzed by comparing with the Wiley 275 mass spectral library in which the matching quality was resulted. The GC-MS chromatogram of the antimicrobial compound in spot 1 exhibited the mass spectral pattern of hexadecanoic acid at 19.31 min (99% similarity). However, artifacts were introduced unwittingly from laboratory equipment during the experiment. The artifacts were shown at 21.25, 23.93, and 26.38 min, corresponding to the mass spectral patterns of 9,12-octadecadienoic acid (91% similarity), dioctyl hexanedioate (91% similarity), and bis (2-ethylhexyl) 1,2-benzenedicarboxylate (91% similarity), respectively. These artifacts are substances that contaminated the plant extract. Similarly, the mass spectral pattern of 1,1-diethoxyethane which was observed at 2.25

min on the GC-MS chromatogram of antimicrobial compound in spot 3 was also the artifact since this substance is relatively rare in plants.

(a)



(b)

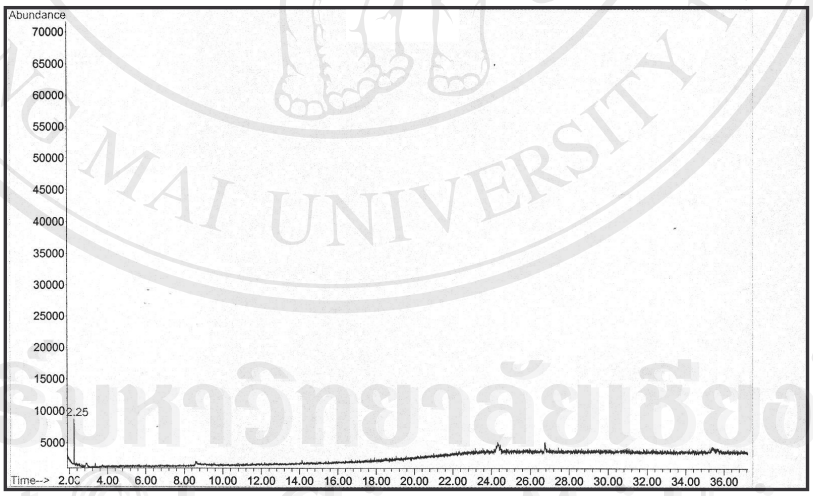


Figure 3.8 GC-MS chromatograms of antimicrobial compounds from the ethanolic extract of *Pimpinella anisum* (a: spot 1; and b: spot 3 on paper chromatogram)

The essential oil of star anise was also analyzed by GC-MS. The major constituent of the oil was exhibited at 9.58 min (Figure 3.9), corresponding to the mass spectral pattern of anethole (97% similarity). The minor components were shown at 5.57, 6.57, 8.12, 8.78, and 15.56 min, corresponding to the mass spectral patterns of limonene (98% similarity), linalool (90% similarity), methyl chavicol (98% similarity), anisaldehyde (97% similarity), and 1-(3-methyl-2-butenoxy)-4-(1-propenyl) benzene (91% similarity), respectively. These results confirmed that the chemicals such as anethole, methyl chavicol, and anisaldehyde were not the active substances responsible for antimicrobial activity in the ethanolic extract of the plant *P. anisum*.

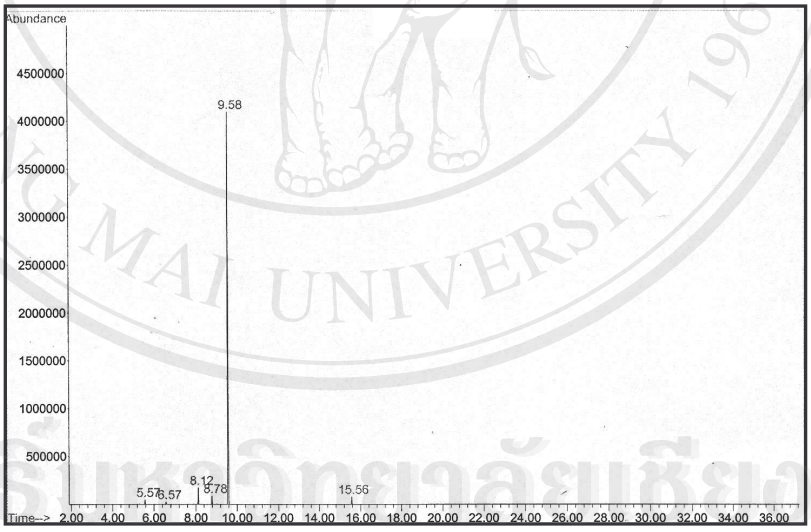


Figure 3.9 GC-MS chromatogram of the essential oil of star anise (*Illicium verum*)

3.4) SEPARATION AND PURIFICATION OF ANTIMICROBIAL SUBSTANCE FROM *Caesalpinia mimosoides*

3.4.1) Separation and purification of phenolic compounds by column chromatography

Since the phenolics of *C. mimosoides* could not be purified absolutely by two-dimensional chromatography, the other method, column chromatography was then used (Figure 3.10). The dried plant material was extracted with absolute ethanol. The concentration of the crude ethanolic extract (4.18% yields by weight from dried plant material) was then adjusted to 50 mg/ml, and a portion (10 ml) of the extract was loaded onto a Sephadex LH-20 column. The elution profile of the crude extract is shown in Figure 3.11. The chromatographic profile showed eight maxima at 280 nm (eight fractions), label F1-F8. The concentration of each fraction was then adjusted to 10 mg/ml before further study *e.g.* TLC, HPLC, and antimicrobial assays (Figure 3.12).

Figure 3.10 Apparatus for separation of phenolic compounds by column chromatography

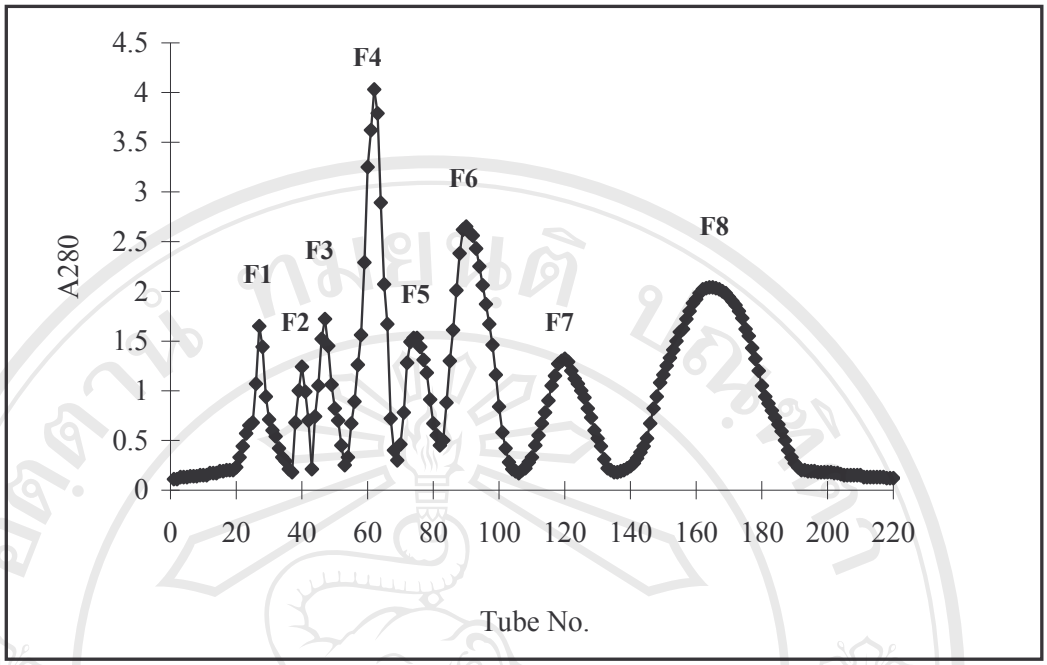


Figure 3.11 Elution profile of Sephadex LH-20 chromatography of the crude extract of *Caesalpinia mimosoides* (column size: 4x60 cm; sample volume: 10 ml (50 mg/ml); mobile phase: 95% ethanol; flow rate: 1 ml/min, fraction volume: 10 ml)

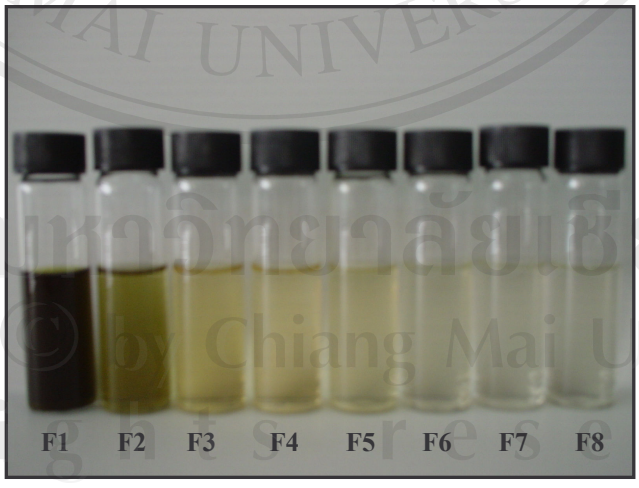


Figure 3.12 Fraction F1-F8 in ethanol (10 mg/ml) separated on the Sephadex LH-20 column

3.4.2) TLC and HPLC analyses of the fractions

In order to investigate the characteristics and the purity of the separated fractions, each fraction was analyzed by TLC and HPLC. For TLC analysis, all fractions (F1-F8) contained phenolic compounds, as they demonstrated fluorescent color under UV light (254 and 365 nm), and produced characteristic blue color with Folin-Ciocalteu's reagent (Figure 3.13). The fractions were then assayed by HPLC, and the chromatograms were shown in Figure 3.14. It was found that only fraction 4 or F4 was the purified substance, as it exhibited one peak at 2.985 min on the chromatogram. Preliminary identification of F4 was based on comparison of its retention time with those of authentic compounds, and it was identified as 3,4,5-trihydroxybenzoic acid or gallic acid which showed a peak at 2.981 min on the chromatogram (Figure 3.15).

Figure 3.13 TLC chromatogram of the fractions (F1-F8) separated on the Sephadex LH-20 column

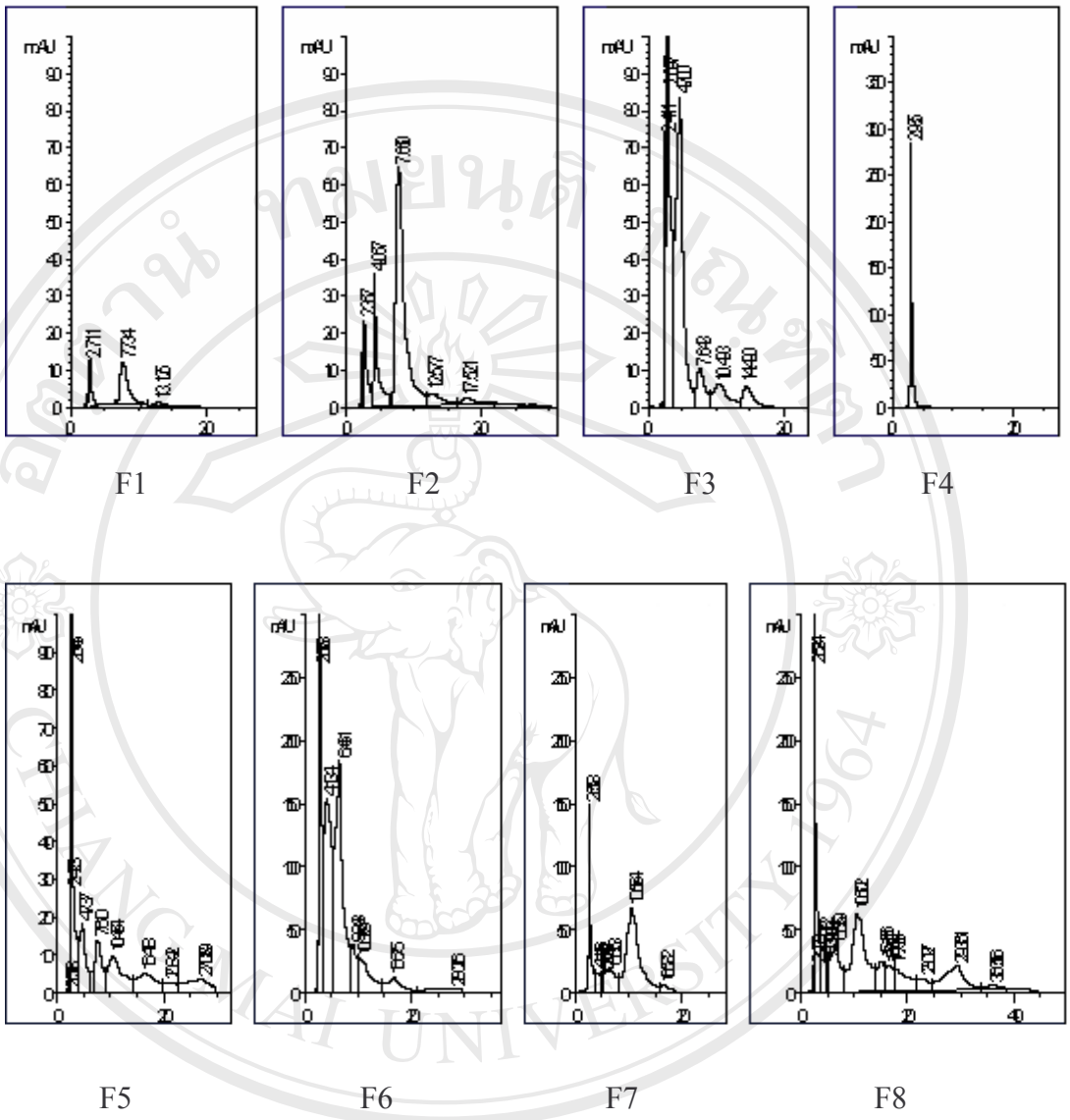


Figure 3.14 HPLC chromatograms of the fractions separated on the Sephadex LH-20 column

Figure 3.15 HPLC chromatogram of gallic acid

3.4.3) Determination of antimicrobial activity

The antimicrobial activities of the crude extract and the fractions separated on the Sephadex LH-20 column are shown in Table 3.10. The crude extract had no activity against all test microorganisms. Antimicrobial activity was observed in fraction F4 only against *Salmonella typhi* and *Staphylococcus aureus* with the same inhibition zone (7 mm). Minimum inhibitory concentration of F4 was also determined against both bacteria (Table 3.11). The bacterium *S. aureus* seems to be a sensitive strain to F4 as it showed the MIC value at 1250 μg/ml, while *S. typhi* exhibited the value at 2500 μg/ml.

Table 3.10 Inhibition zones of the crude extract and the fractions F1-F8 (200 μg/disc) against some pathogenic microorganisms

Microorganism	Inhibition zone (mm)								
	CE	F1	F2	F3	F4	F5	F6	F7	F8
Gram negative bacteria									
Escherichia coli	-	-	-	-	-	-	-	-	-
Klebsiella pneumoniae	-	-	-	-	-	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-	-	-	-	-	-
Salmonella typhi	-	-	-	-	7	-	-	-	-
Vibrio cholerae	-	-	-	-	-	-	-	-	-
Gram positive bacteria									
Enterococcus faecalis	-	-	-	-	-	-	-	-	-
Staphylococcus aureus	-	-	-	-	7	-	-	-	-
Staphylococcus epidermidis	-	-	-	-	-	-	-	-	-
Yeast									
Candida albicans	-	-	-	-	-	-	-	-	-
Filamentous fungi									
Aspergillus sp.	-	-	-	-	-	-	-	-	-
Fusarium sp.	-	-	-	-	-	-	-	-	-
*Microsporum gypseum**	-	-	-	-	-	-	-	-	-
Penicillium sp.	-	-	-	-	-	-	-	-	-
*Trichophyton rubrum**	-	-	-	-	-	-	-	-	-

CE: crude extract; *: dermatophytic fungi; and "-": no inhibition zone

Table 3.11 Minimum inhibitory concentration (MIC) values of fraction F4 against *Salmonella typhi* and *Staphylococcus aureus*

Microorganism	MIC value (μg/ml)
Salmonella typhi	2500
Staphylococcus aureus	1250

3.5) IDENTIFICATION OF ANTIMICROBIAL SUBSTANCE FROM *Caesalpinia mimosoides*

In order to confirm the result of HPLC analysis of F4 (section 3.4.2) which indicated that it was identified as gallic acid (Figure 3.16). The complete identification was carried out by spectroscopic methods as described in the section 2.7.1-2.7.4.

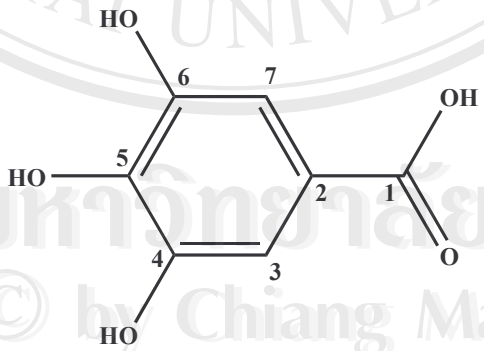


Figure 3.16 Structure of gallic acid

3.5.1) Ultraviolet-visible spectroscopy

The absorption spectrum of F4 in ethanol is shown in Figure 3.17. The spectrum showed absorption maxima at 220 and 271 nm.

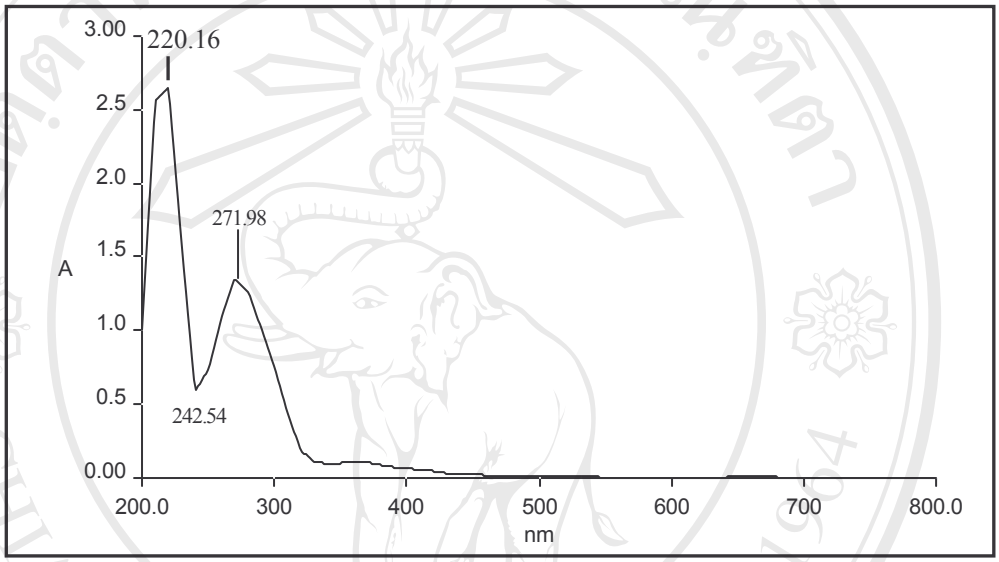


Figure 3.17 UV-visible absorption spectrum of the fraction F4 separated on the Sephadex LH-20 column

3.5.2) Infrared spectroscopy (IR)

The IR spectrum (KBr) of F4 is shown in Figure 3.18. The spectrum showed absorption peaks at 3491 and 3377 cm^{-1} (O-H stretching), 1703 cm^{-1} (C=O stretching), 1617, 1539, and 1453 cm^{-1} (C=C stretching of aromatic), and 1254 cm^{-1} (C-O stretching).

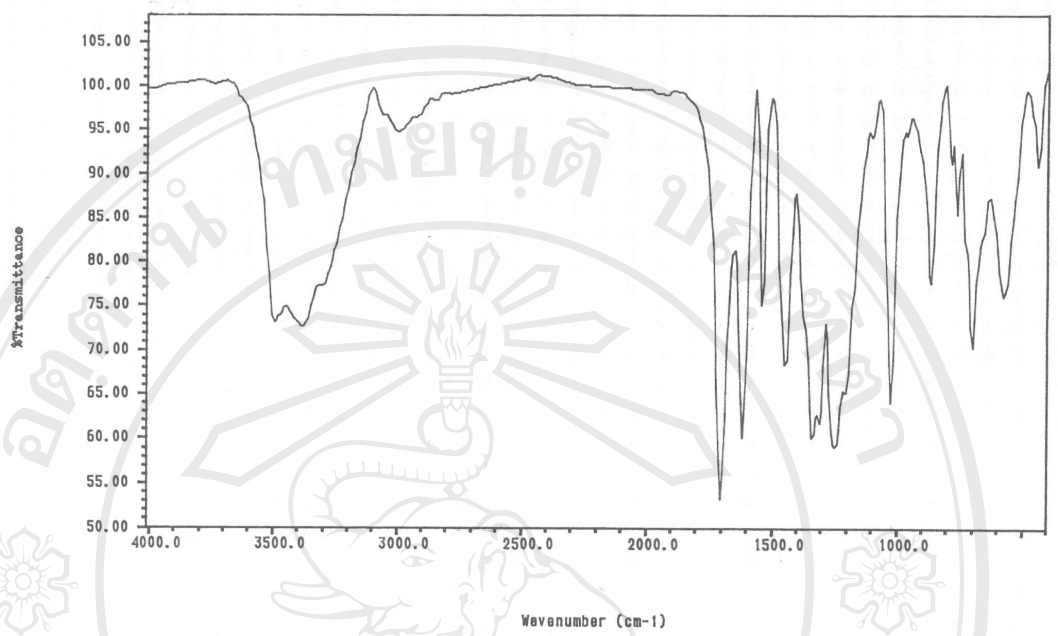


Figure 3.18 IR spectrum of the fraction F4 separated on the Sephadex LH-20 column

3.5.3) Nuclear magnetic resonance spectroscopy (NMR)

The high resolution (400 MHz) ^{1}H NMR (acetone-d_6) spectrum of F4 (Figure 3.19) showed the singlet signal of aromatic protons at δH 7.15 (2H, *s*, H-3 and H-7). The ^{13}C NMR spectrum (Figure 3.20) showed signals at δC 167.39 (carbonyl carbon, C-1), 144.94 (C-4 and C-6), 137.77 (C-5), 120.81 (C-2), and 109.14 (C-3 and C-7). The signals of carbonyl carbon (C-1) and aromatic protons (H-3 and H-7) suggested the presence of a 3,4,5-trisubstituted benzene ring. However, the signals of hydroxyl protons were not observed.

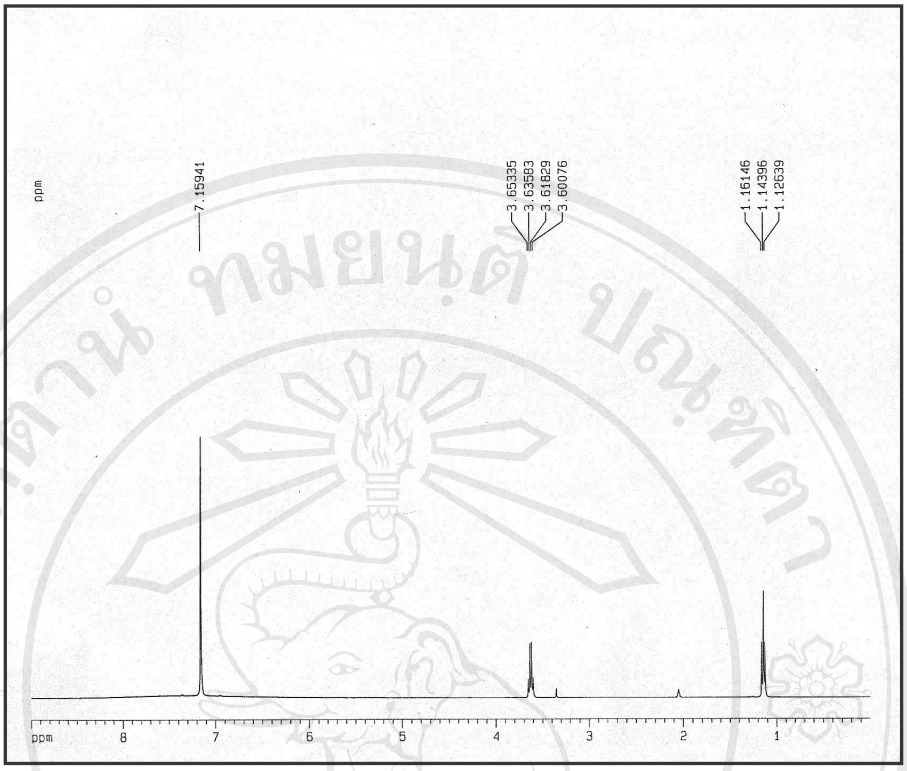


Figure 3.19 Proton (^{1}H) NMR (acetone-d_6) spectrum of the fraction F4 separated on the Sephadex LH-20 column

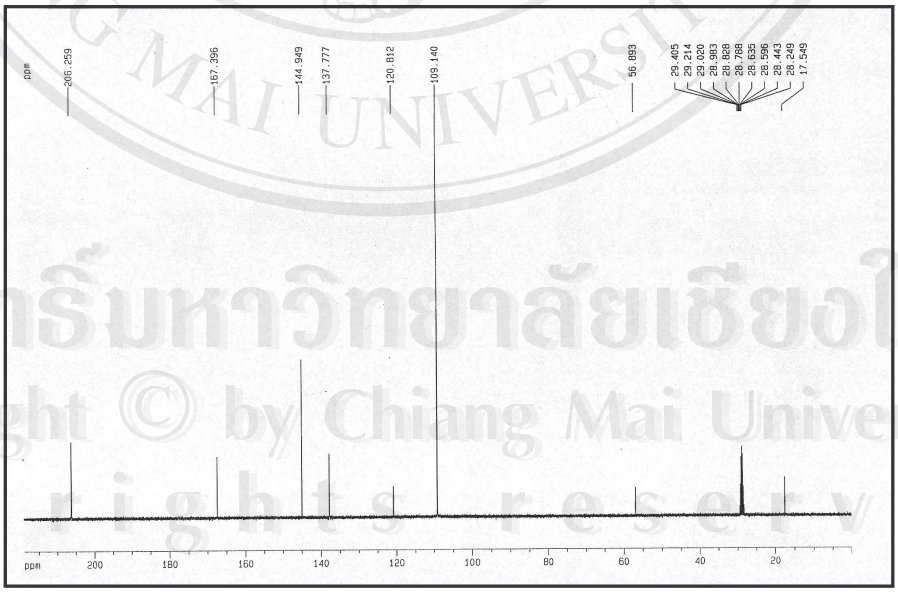


Figure 3.20 Carbon (^{13}C) NMR (acetone-d_6) spectrum of the fraction F4 separated on the Sephadex LH-20 column

As shown in Figure 3.19 and 3.20, the signals of protons at δH 3.62 (2H, *q*) and 1.14 (3H, *t*), and carbons at δC 56.89 and 17.54 were also observed in the spectra. These signals belonged to residuary solvent, ethyl group, which remained in compound F4. This presumption was proved by two-dimensional NMR technique. The HMBC spectrum of F4 showed no correlation between carbonyl carbon (C-1) and the proton of ethyl group (Figure 3.21, 3.21a, and 3.21b), confirming that the signals mentioned above did not belong to F4 structure.

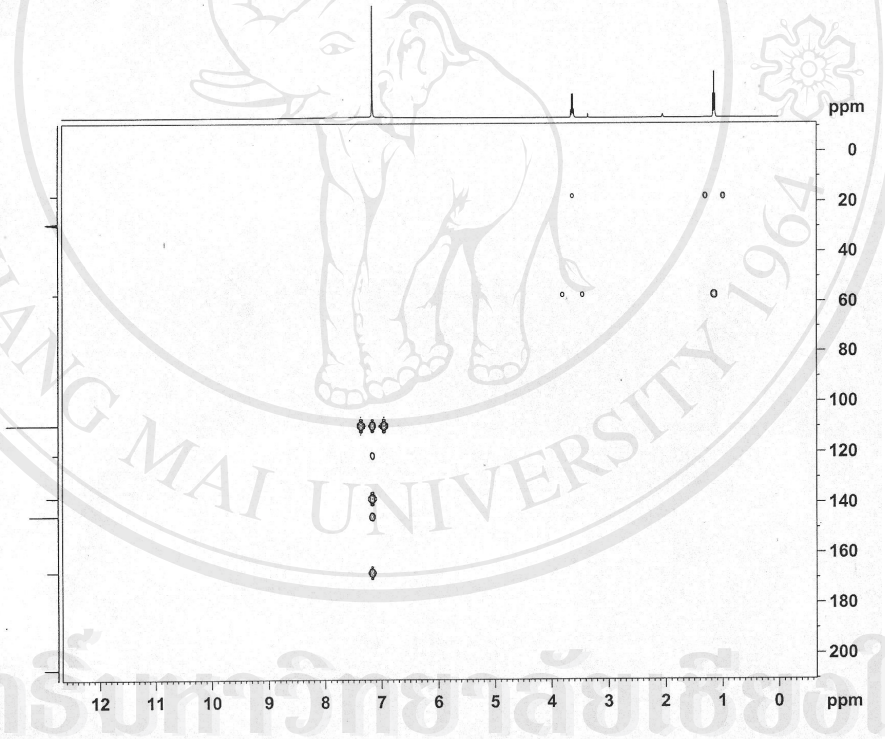


Figure 3.21 HMBC spectrum of the fraction F4 separated on the Sephadex LH-20 column

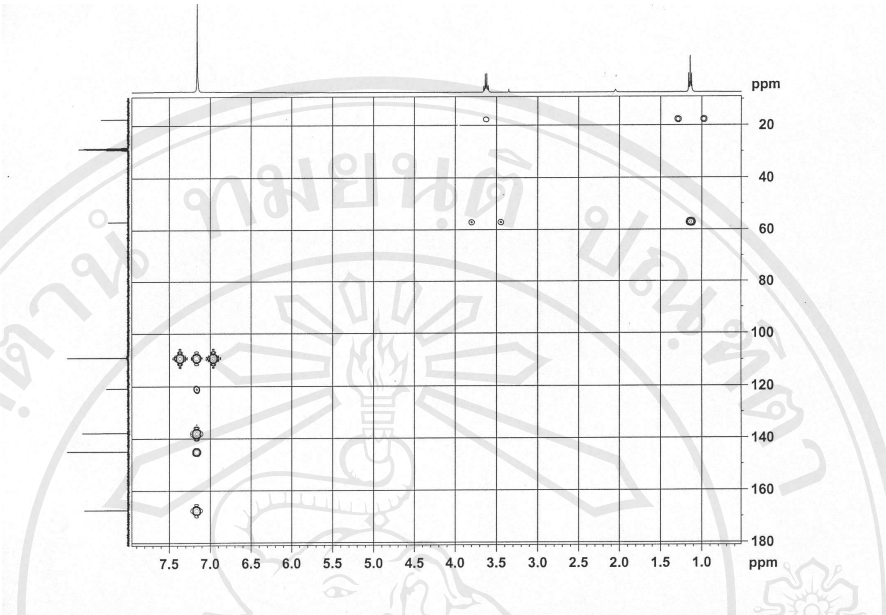


Figure 3.21a Expansion of Figure 3.21

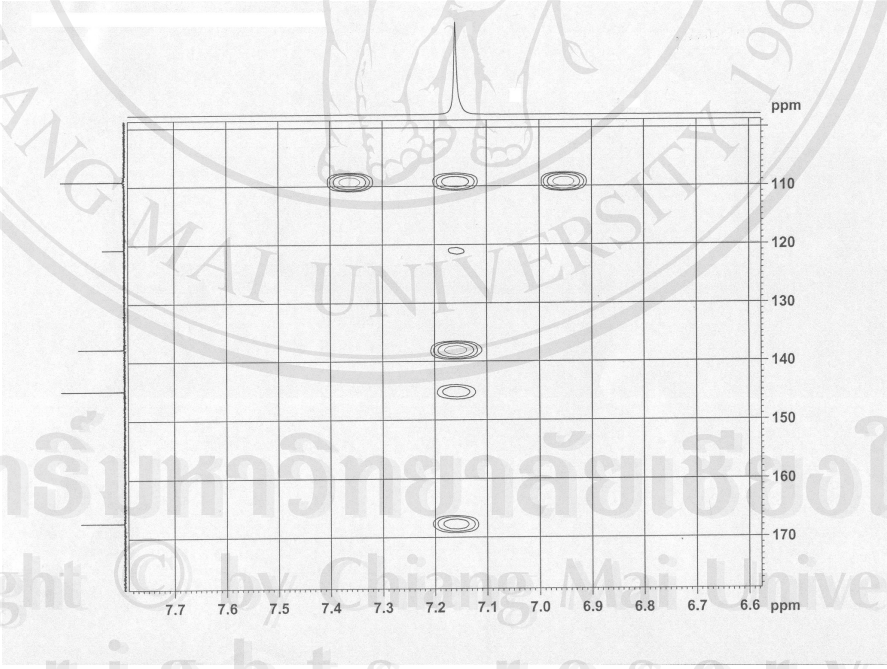


Figure 3.21b Expansion of Figure 3.21

3.5.4) Liquid chromatography-tandem mass spectrometry (LCMS/MS)

The spectrum of high resolution-mass spectrometry (electrospray) or HRMS (ESI) of F4 revealed a molecular ion peak $[M-H]^-$ at *m/z* 169.0137 (Figure 3.22), that obtained by comparison with the known compound, methyl 3,4,5-trihydroxybenzoate or methyl gallate, $C_8H_8O_5$. Thus, the molecular formula of F4 was calculated for $C_7H_6O_5$, 170.0215.

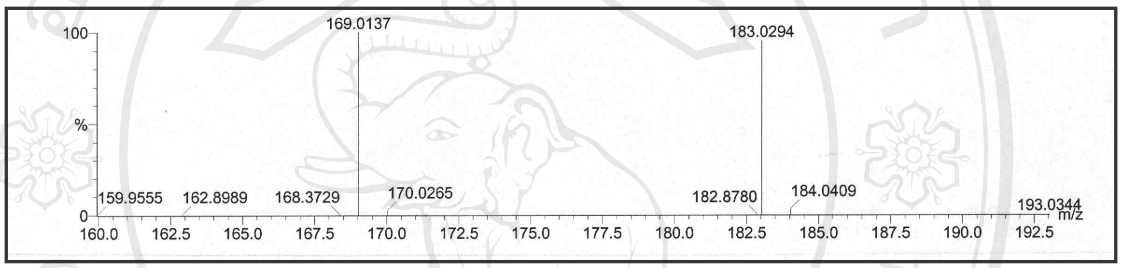


Figure 3.22 HRMS (ESI) spectrum of the fraction F4 separated on the Sephadex LH-20 column

Based on the results in this section, the compound F4 separated on the Sephadex LH-20 column was the known compound, namely gallic acid (3,4,5-trihydroxybenzoic acid). Its characteristic and spectral data are summarized below.

Gallic acid: yellow-green solid. TLC: (hexane-ethyl acetate-acetic acid, 2:1:0.3v/v/v) R_f 0.24; UV λ_{max} (ethanol) nm: 220, 271; IR (KBr) ν cm^{-1}: 3491, 3377, 1703, 1617, 1539, 1453, 1254 cm^{-1}; ^1H NMR (acetone-d_6): δH 7.15 (2H, *s*, H-3 and H-7); ^{13}C NMR (acetone-d_6): δC 167.39 (C-1), 144.94 (C-4 and C-6), 137.77 (C-5), 120.81 (C-2), 109.14 (C-3 and C-7); HRMS (ESI) *m/z* 169.0137 $[M-H]^-$ (calculated for $C_7H_5O_5$, 169.0137).

3.6) STRUCTURAL MODIFICATION OF ANTIMICROBIAL SUBSTANCE FROM *Caesalpinia mimosoides*

Since F4 or gallic acid separated from the plant *C. mimosoides* possessed a low level of antimicrobial activity, therefore, it would be interesting to modify its structure in order to enhance the activity. In this study, the structural modification was carried out by acid-catalyzed esterification with ethanol to yield yellow-green solid of ethyl gallate (ethyl 3,4,5-trihydroxybenzoate) (Figure 3.23). The modified ethyl gallate was then used for further study such as characteristic determination, structural elucidation, and antimicrobial assay.

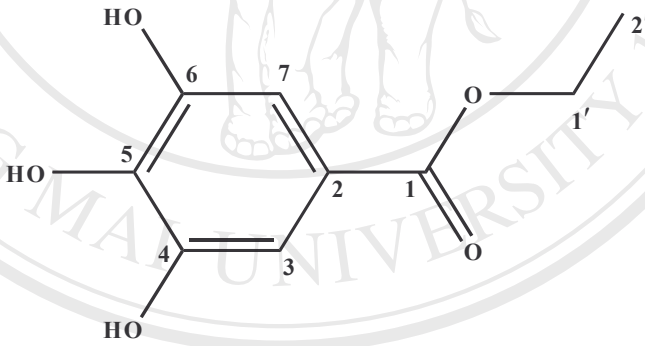


Figure 3.23 Structure of ethyl gallate

3.7) CHARACTERIZATION AND STRUCTURAL ELUCIDATION OF CHEMICALLY MODIFIED ETHYL GALLATE

3.7.1) TLC and HPLC analyses of modified ethyl gallate

TLC chromatogram of the modified ethyl gallate and its parent structure; gallic acid was shown in Figure 3.24. Both substances demonstrated fluorescent color under UV light at 254 and 365 nm, and produced a characteristic blue color with Folin-Ciocalteu's reagent. The parent structure gallic acid showed R_f value at 0.24 while the modified structure exhibited a higher value at 0.37 on the chromatogram developed with hexane-ethyl acetate-acetic acid (2:1:0.3 v/v/v).

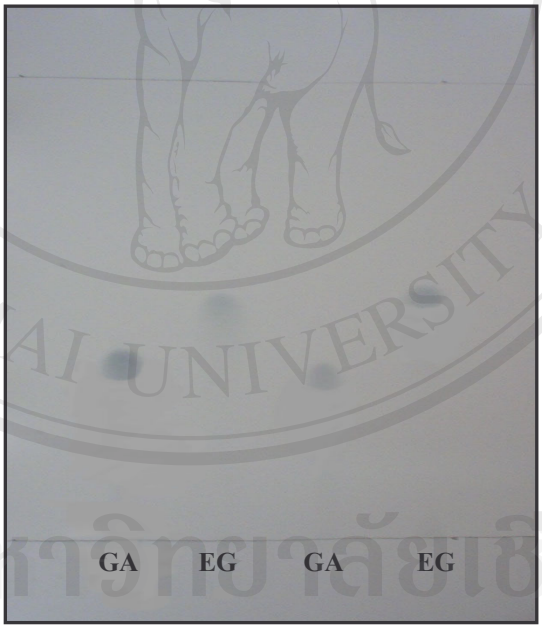


Figure 3.24 TLC chromatogram of gallic acid (GA) compared with the modified ethyl gallate (EG)

For HPLC analysis, the chromatogram gave a peak for the modified ethyl gallate at 11.256 min (Figure 3.25). Its retention time differed from that of the parent structure at 2.985 min (Figure 3.14, d).

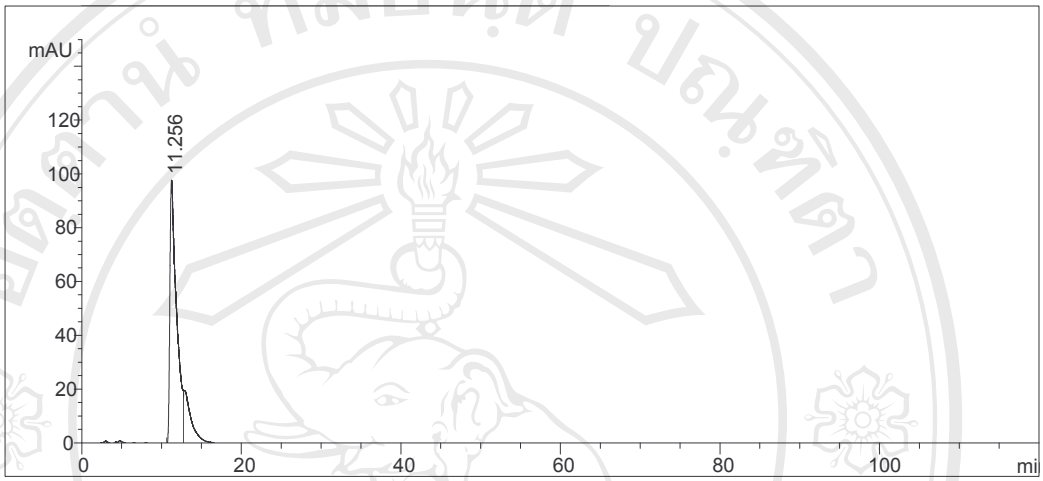


Figure 3.25 HPLC chromatogram of the modified ethyl gallate

3.7.2) Ultraviolet-visible spectroscopy

The absorption spectrum of the modified ethyl gallate in ethanol is shown in Figure 3.26. The spectrum exhibited absorption maxima at 226 and 274 nm.

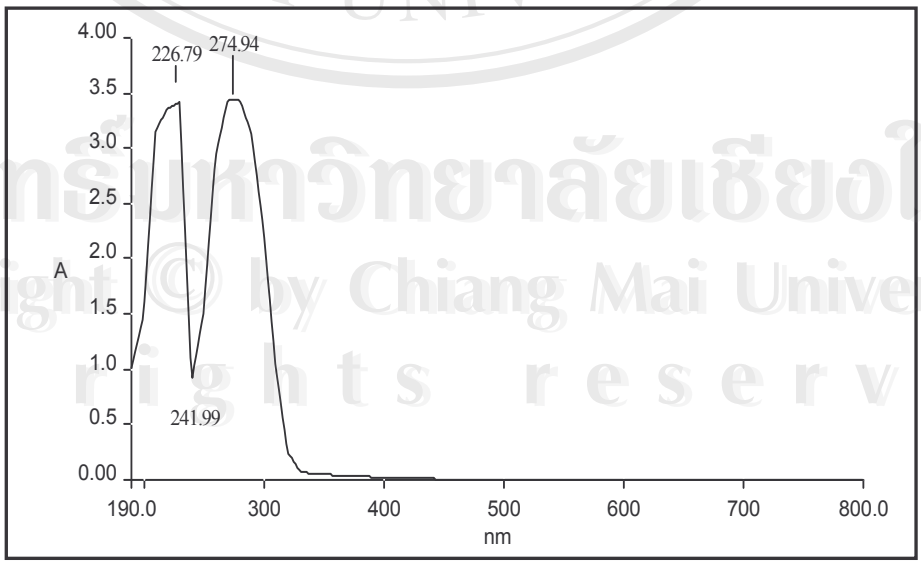


Figure 3.26 UV-visible spectrum of the modified ethyl gallate

3.7.3) Infrared spectroscopy (IR)

The IR spectrum (KBr) of the modified ethyl gallate (Figure 3.27) showed many similarities with the analogous spectrum of its parent structure (Figure 3.18). The spectrum exhibited absorption peaks at 3448 and 3306 cm^{-1} (O-H stretching), 1703 cm^{-1} (C=O stretching), 1617, 1539, and 1453 cm^{-1} (C=C stretching of aromatic), and 1247 cm^{-1} (C-O stretching). The spectrum also showed the absorption bands for alkyl group at 2971 cm^{-1} (C-H stretching of CH_2/CH_3), 1468 cm^{-1} (C-H bending of CH_2/CH_3), and 1382 cm^{-1} (C-H bending of CH_3).

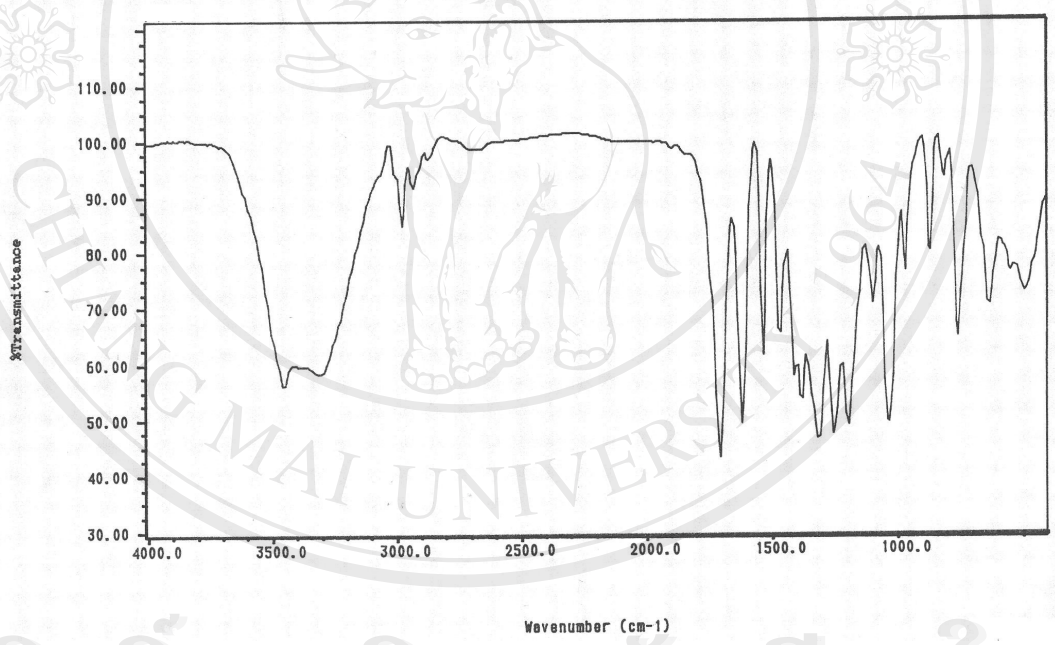


Figure 3.27 IR spectrum of the modified ethyl gallate

3.7.4) Nuclear magnetic resonance spectroscopy (NMR)

The ^1H NMR (acetone-d_6) spectrum of the modified ethyl gallate (Figure 3.28) demonstrated the signal of two aromatic protons at δH 7.12 (2H, *s*, H-3 and H-7) (Figure 3.28a). Two additional signals at δH 4.24 (2H, *q*, *J*=7.1 Hz, H_2-1′) (Figure

3.28b) and 1.30 (3H, *t*, *J*=7.1 Hz, H_3-2′) (Figure 3.28c) were also seen in the spectrum, suggesting the presence of an ethyl group. The ^{13}C NMR (acetone-d_6) spectrum (Figure 3.29) exhibited typical signals for a galloyl moiety at δC 166.44 (C-1), 145.73 (C-4 and C-6), 138.35 (C-5), 121.86 (C-2), and 109.52 (C-3 and C-7) (Figure 3.29a). In addition, two signals for the ethyl group at δC 60.63 (C-1′) and 14.33 (C-2′) were observed (Figure 3.29b), indicating that the modified ethyl gallate include the ethyl group in its structure.

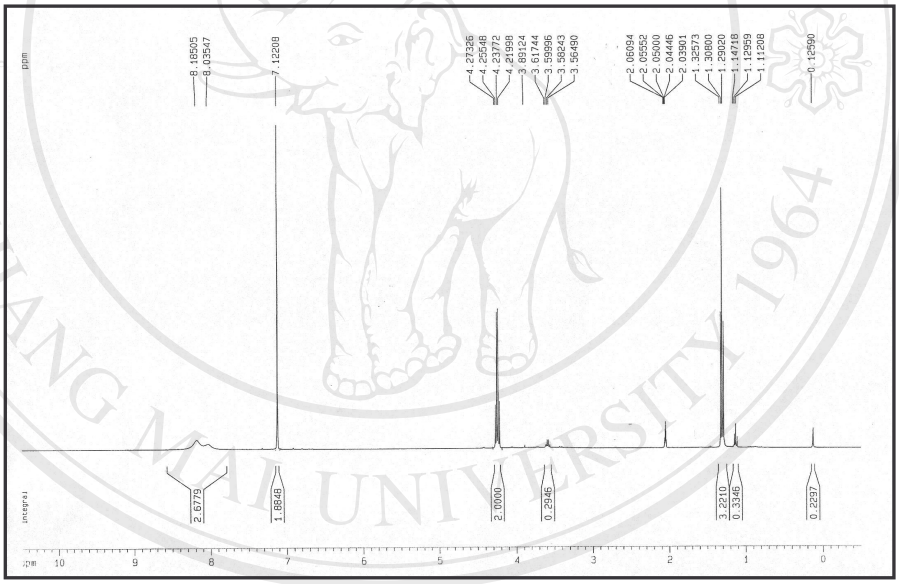


Figure 3.28 Proton (^1H) NMR (acetone-d_6) spectrum of the modified ethyl gallate

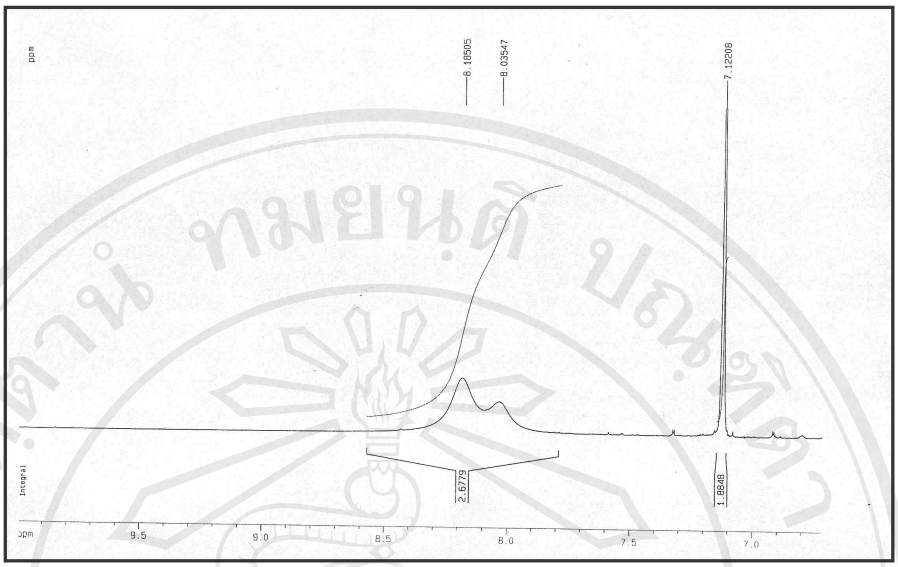


Figure 3.28a Expansion of Figure 3.28

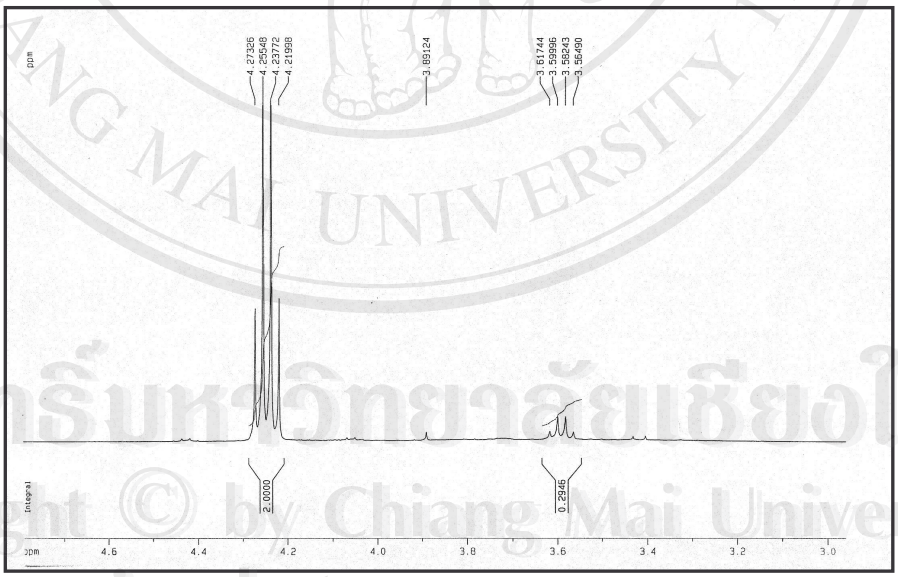


Figure 3.28b Expansion of Figure 3.28

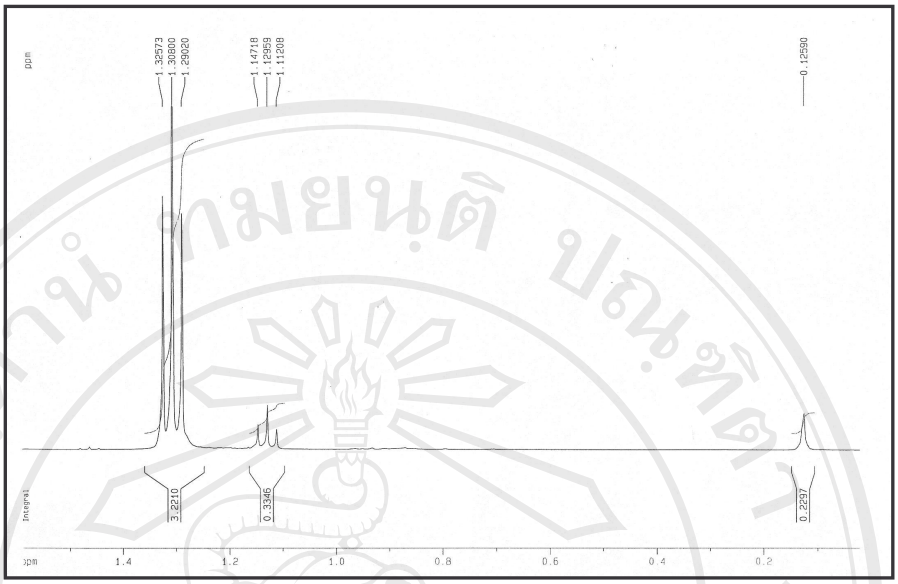


Figure 3.28c Expansion of Figure 3.28

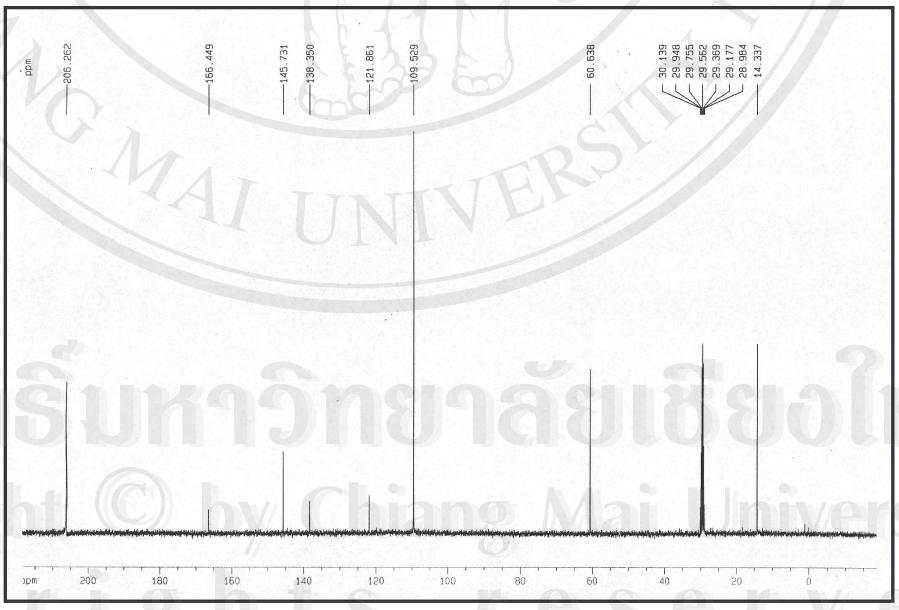


Figure 3.29 Carbon (^{13}C) NMR (acetone-d_6) spectrum of the modified ethyl gallate

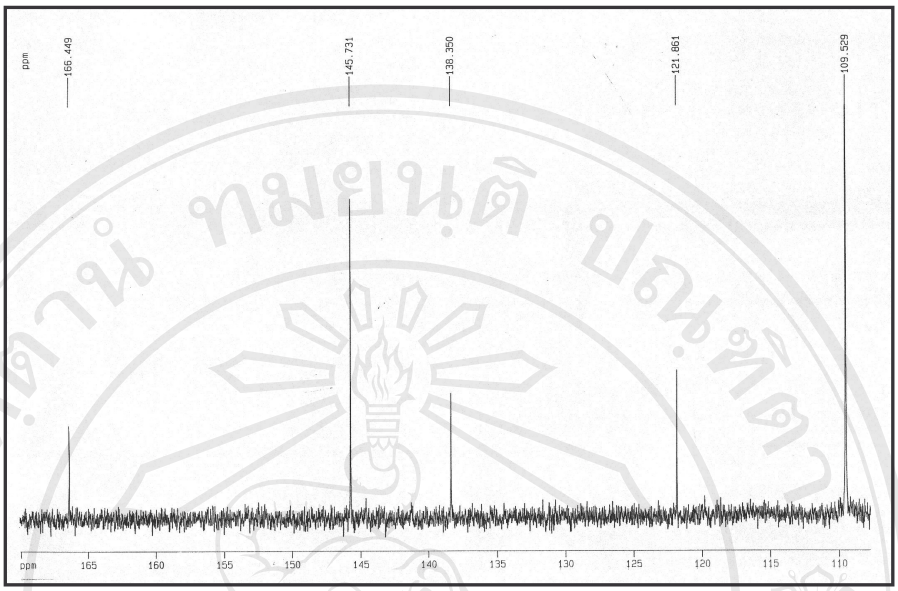


Figure 3.29a Expansion of Figure 3.29

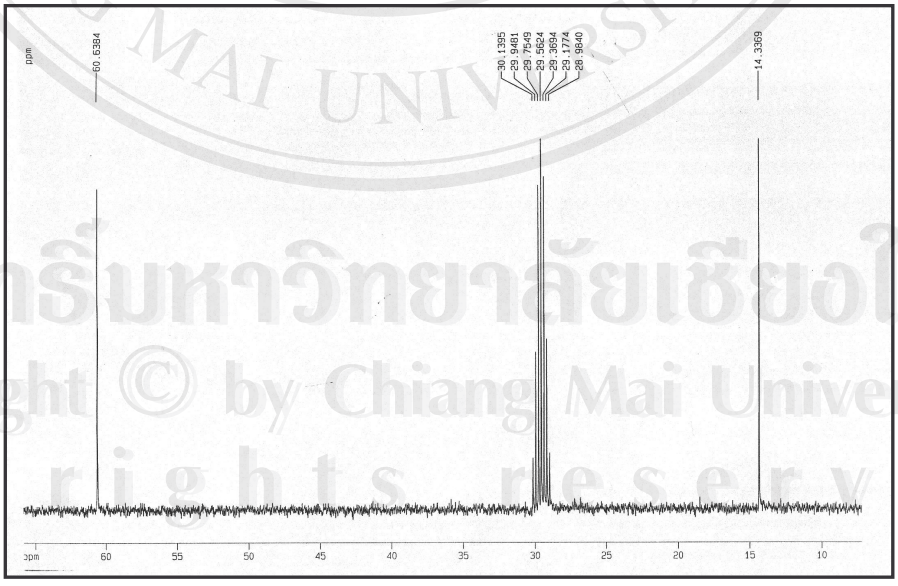


Figure 3.29b Expansion of Figure 3.29

The position of substitution of the ethyl group at the galloyl moiety was proved by two-dimensional NMR spectra. The correlation of H-1′ with C-1 was established by HMQC (Figure 3.30, and 3.30a) and HMBC (Figure 3.31, 3.31a, 3.31b, and 3.31c), confirming the presence of the ethyl group in the modified structure.

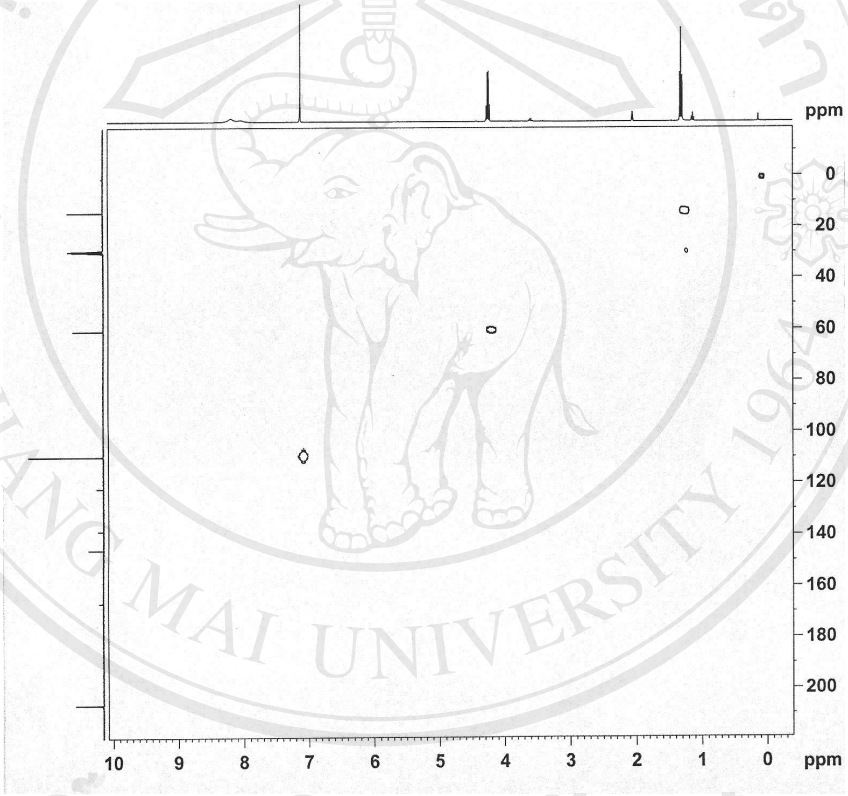


Figure 3.30 HMQC spectrum of the modified ethyl gallate

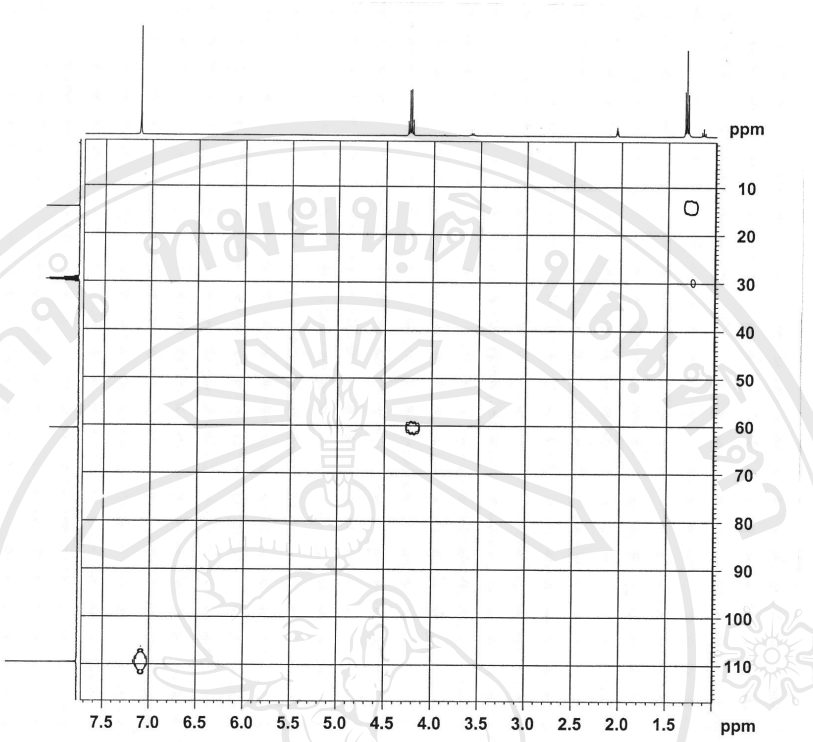


Figure 3.30a Expansion of Figure 3.30

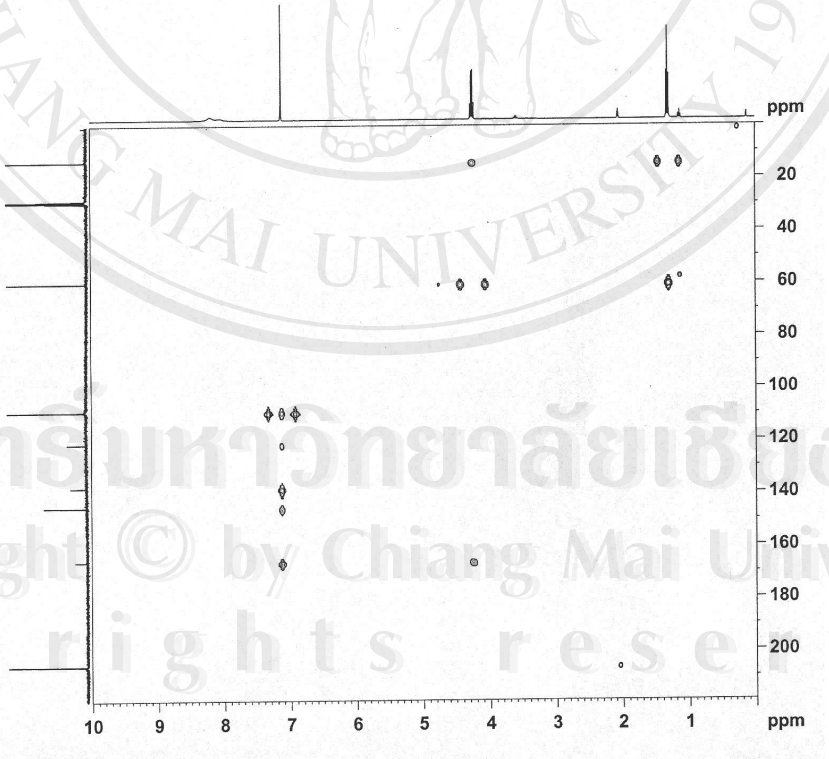


Figure 3.31 HMBC spectrum of the modified ethyl gallate

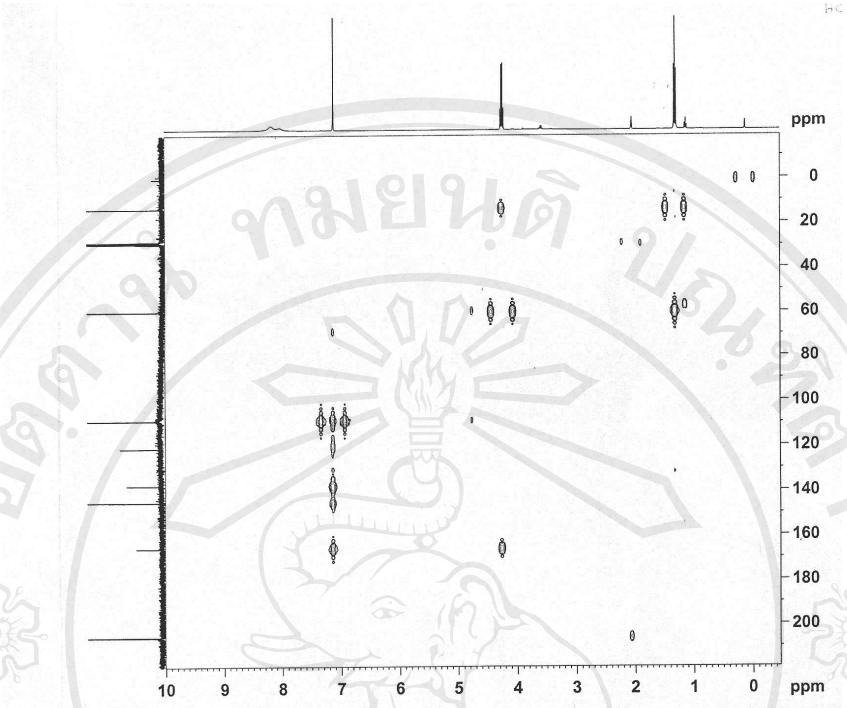


Figure 3.31a Expansion of Figure 3.31

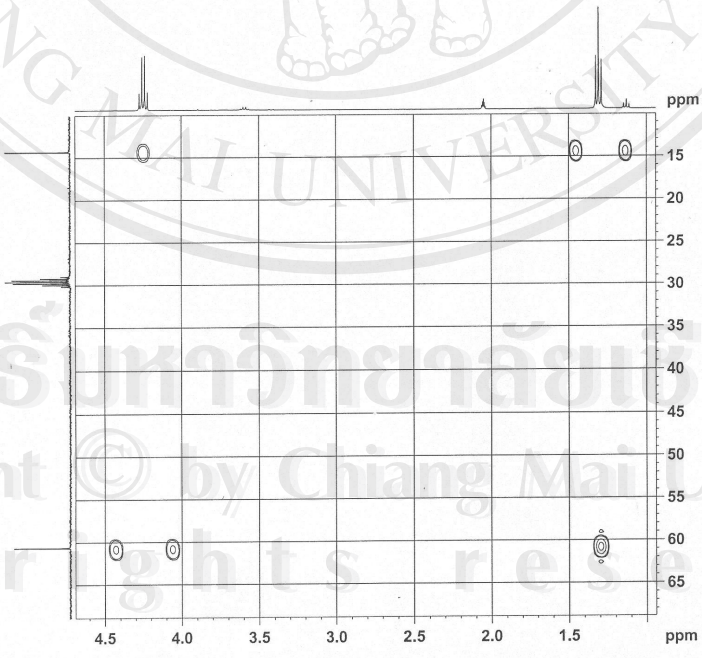


Figure 3.31b Expansion of Figure 3.31

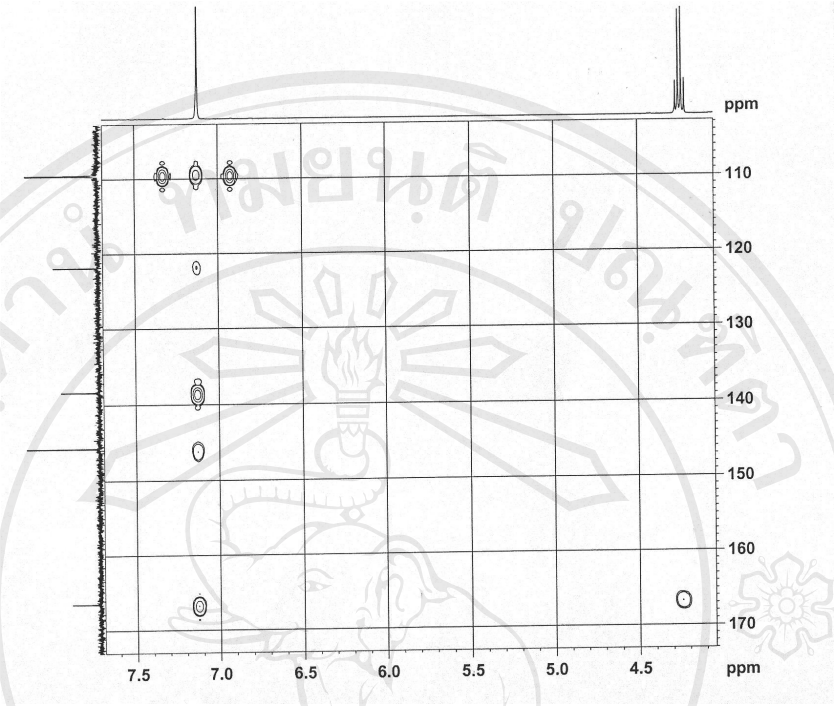


Figure 3.31c Expansion of Figure 3.31

3.7.5) Liquid chromatography-tandem mass spectrometry (LCMS/MS)

The HRMS (ESI) spectrum of the modified ethyl gallate revealed a molecular ion peak $[M-H]^-$ at *m*/*z* 197.0450 (Figure 3.32), obtained by comparison with the known compound, methyl 3,4,5-trihydroxybenzoate or methyl gallate, $C_8H_8O_5$. Thus, the molecular formula of the modified structure was calculated for $C_9H_{10}O_5$, 198.0528.

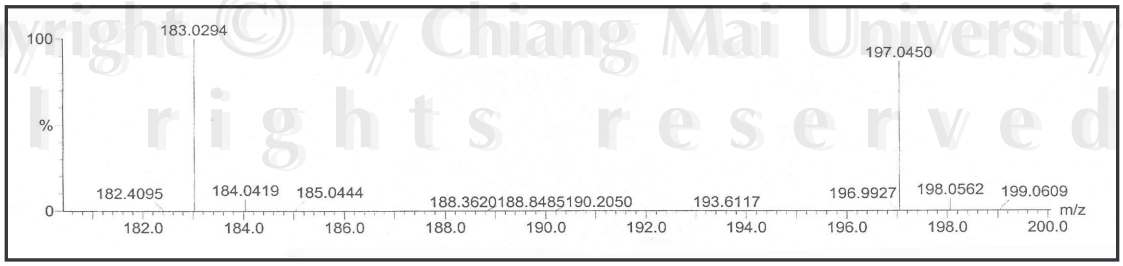


Figure 3.32 HRMS (ESI) spectrum of the modified ethyl gallate

Based on the results in this section, the spectral data confirmed that the modified structure obtained from acid-catalyzed esterification of the compound F4 was ethyl gallate. Its characteristic and spectral data are summarized below.

Ethyl gallate: yellow-green solid. TLC: (hexane-ethyl acetate-acetic acid, 2:1:0.3 v/v/v) R_f 0.37; UV λ_{max} (ethanol) nm: 226, 274; IR (KBr) ν cm^{-1}: 3448, 3306, 2971, 1703, 1617, 1539, 1453, 1247 cm^{-1}; ^1H NMR (acetone-d_6): δH 8.18 and 8.03 (3H, *br s*, OH-4, OH-5, and OH-6), 7.12 (2H, *s*, H-3 and H-7), 4.24 (2H, *q*, *J*=7.1 Hz, H_2-1′), 1.30 (3H, *t*, *J*=7.1 Hz, H_3-2′); ^{13}C NMR (acetone-d_6): δC 166.44 (C-1), 145.73 (C-4 and C-6), 138.35 (C-5), 121.86 (C-2), 109.52 (C-3 and C-7), 60.63 (C-1′), 14.33 (C-2′); HRMS (ESI) *m*/*z* 197.0450 $[M-H]^-$ (calculated for $C_9H_9O_5$, 197.0450).

3.8) DETERMINATION OF ANTIMICROBIAL ACTIVITY OF MODIFIED ETHYL GALLATE

The modified ethyl gallate was examined for its antimicrobial activity against 14 microbial strains (Table 3.12). The activity was compared with those of its parent structure (gallic acid). The antimicrobial (antibacterial) activities of the ethyl gallate were found against *Salmonella typhi*, *Staphylococcus aureus*, and *Vibrio cholerae* with the inhibition zones of 7.50, 14.75, and 7.25 mm, respectively. Whereas, the parent structure exhibited a small inhibition zone (7 mm) against only two species (*S. typhi* and *S. aureus*). It can be seen that the modified ethyl gallate showed a large inhibition zone against *S. aureus* when compared with gallic acid (Figure 3.33). Minimum inhibitory concentration values of the modified ethyl gallate were also examined comparing with gallic acid (Table 3.13). The result showed that the

bacterium *S. aureus* was the most sensitive strain tested with ethyl gallate with the lowest MIC value (156.25 μg/ml)

Table 3.12 Inhibition zones of gallic acid and the modified ethyl gallate against some pathogenic microorganisms

Microorganism	Inhibition zone (mm)	
	Gallic acid	Ethyl gallate
Gram negative bacteria		
Escherichia coli	-	-
Klebsiella pneumoniae	-	-
Pseudomonas aeruginosa	-	-
Salmonella typhi	7	7.67
Vibrio cholerae	-	7.33
Gram positive bacteria		
Enterococcus faecalis	-	-
Staphylococcus aureus	7	14.67
Staphylococcus epidermidis	-	-
Yeast		
Candida albicans	-	-

*: dermatophytic fungi; and "-": no inhibition zone

Table 3.12 (Continued)

Microorganism	Inhibition zone (mm)	
	Gallic acid	Ethyl gallate
Filamentous fungi		
Aspergillus sp.	-	-
Fusarium sp.	-	-
*Microsporum gypseum**	-	-
Penicillium sp.	-	-
*Trichophyton rubrum**	-	-

*: dermatophytic fungi; and "-": no inhibition zone

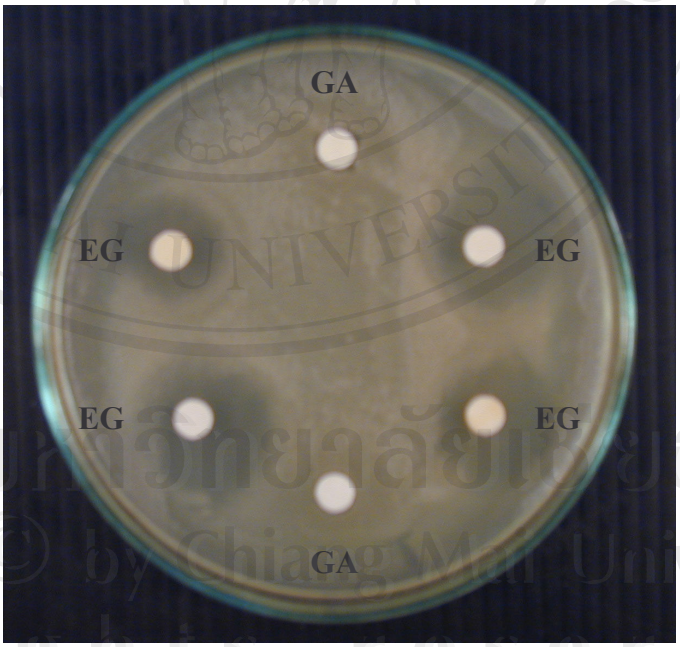


Figure 3.33 Antibacterial activity of gallic acid (GA) and the modified ethyl gallate (EG) on *Staphylococcus aureus* plate

Table 3.13 Minimum inhibitory concentration of gallic acid and the modified ethyl gallate

Substance	*Salmonella typhi*	*Staphylococcus aureus*	*Vibrio cholerae*
Gallic acid	2500	1250	NT
Ethyl gallate	1250	156.25	1250

NT: Not tested