CHAPTER III

EXPERIMENTAL

Part I : Phytochemical Study

1. Source of Plant Material

The entire plant of *Usnea siamensis* Wainio was collected from Doi Intanon National Park, Chiangmai Province, Thailand in December, 1992. The plant was identified by comparison with herbarium specimens at Royal Forest Department, Ministry of Agriculture and Cooperatives, Thailand. A voucher specimen of plant material has been deposited in the herbarium of the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.

2. General techniques

2.1 Thin-layer Chromatography (TLC)

Analytical

Technique : one way, ascending

Absorbent : silica gel 60 GF_{254} (E. Merck), 30 g/60 ml

Plate size : 2.0x7.0 cm.

Layer thickness : 0.25 mm.

Solvent system : a) silica gel 60 GF_{254} / n-hexane : benzene (2:3)

b) silica gel 60 GF_{254} / n-hexane : benzene (1:1)

c) silica gel 60 GF_{254} / n-hexane : ethyl acetate (3:2)

d) silica gel 60 GF_{254} / n-hexane : chloroform (4:1)

e) silica gel 60 GF_{254} / benzene : chloroform (3:2)

f) silica gel 60 GF_{254} / benzene : chloroform (2:1)

g) silica gel 60 GF_{254} / benzene : ethyl acetate (1:1)

h) silica gel 60 GF_{254} / benzene : acetone (2:1)

i) silica gel 60 GF_{254} / chloroform : ethyl acetate (3:2)

j) silica gel 60 GF_{254} / chloroform:acetone (9:1)

k) silica gel 60 GF_{254} / chloroform:acetone (2:3)

l) silica gel 60 GF_{254} / chloroform:methanol (4:1)

m) silica gel 60 GF_{254} / chloroform:methanol (9:1)

n) silica gel 60 GF_{254} / petroleumether:ethyl acetate (9:1)

o) silica gel 60 GF_{254} / petroleum ether:ethyl acetate (4:1)

p) silica gel 60 GF_{254} / petroleum ether:methanol (95:5)

q) silica gel 60 GF_{254} / petroleum ether:chloroform (1:1)

r) silica gel 60 GF_{254} / ethyl acetate

s) silica gel 60 GF_{254} / benzene

t) silica gel 60 GF_{254} / chloroform

Distance : 5 cm.

Temperature : laboratory temperature (30^{o}-35^{o} C)

Detection on chromatographic plate

: a) Ultraviolet light at wavelength 254 and 365 nm.

b) Chromogenic spray reagents

- Anisaldehyde / sulfuric acid (0.5% ethanolic solution of anisaldehyde with 5% sulfuric acid)
- 10% sulfuric acid in Ethanol

Plate, after spraying, was warmed gently with hot air stream from a hair dryer for 10 minutes.

2.2 Column Chromatography (CC)

Absorbent : silica gel no.9385 (E. Merck) 0.040-0.063 mm.

Packing : dry packing

Sample loading

: the portion of crude extract was dissolved in a small amount of volatile solvent, mixed with small quantity of absorbent, air dried and added onto the top of a dry column.

Solvent system

: a) petroleum ether : ethyl acetate (9:1)

b) petroleum ether : ethyl acetate (8:2)

c) petroleum ether : ethyl acetate (7:3)

d) petroleum ether : ethyl acetate (1:1)

e) ethyl acetate : n-hexane (1:1)

f) ethyl acetate : n-hexane (9:1)

g) benzene : chloroform (3:2)

h) chloroform

i) chloroform : methanol (9:1)

j) chloroform : acetone (3:2)

k) chloroform : acetone (9:1)

Collection of eluate

: fractions of 40 ml were collected.

Examination of eluate

: fractions were examined by thin-layer chromatography using the UV light and chromogenic spray reagents. Those fractions of similar pattern were combined.

2.3 Physical Constants

Optical rotation

: Optical rotations were determined by Bendix-NPL automatic polarimeter.

Melting Points

: Melting points were determined by Gallenkamp melting point apparatus. The values recorded are uncorrected.

2.4 Spectraoscopy

Ultraviolet-visible (UV) Absorption Spectra

: Ultraviolet-visible absorption spectra were obtained on Milton Roy Spectronic 3000 Array.

Infrared Absorption Spectra

: Infrared absorption spectra were obtained with a Shimadsu IR 440 Spectrometer. The materials were examined in potassium bromide disc.

Nuclear Magnetic Resonance (NMR) Spectra

: Proton (^{1}H) and Carbon-13 (^{13}C) NMR Spectra were obtained with a JEOL-GSX (500 MHz) Spectrometer in deuterochloroform ($CDCl_3$) and dimethylsulfoxide-d_6 (DMSO-d_6) using tetra-methylsilane TMS(=0) as internal standard.

Mass Spectra (MS)

: Mass Spectra were recorded on a Fisons VG Trio 2000 Mass Spectrometer for EIMS. Operating at 70 ev with inlet temperature 150°-240° C.

3. The Extraction and Isolation of Compounds

3.1 Extraction

The dried coarsely powdered plant of *Usnea siamensis* Wainio (1.5 kg) was macerated with petroleum ether (3x6L) for 3 day-period and filtered by suction. The combined filtrate was evaporated under reduced pressured to yield dry crude extract, CP (6.0 gm).

The marc after macerated with petroleum ether, had been macerated with portions of chloroform (3x6L) and filtered. The combined chloroform filtrate was evaporated in vacuum to yield dry crude chloroform extract, CC (35.0 gm).

The marc was finally macerated with portions of acetone (3x6L) and filtered. The combined filtrate was evaporated in vacuum to yield dry crude acetone extract, CA (22.0 gm).

Thin layer chromatograms of these crude extracts, shown in Figures 12-14, pp.134-136, indicated the presence of many compounds.

3.2 Isolation and Purification

Crude petroleum ether extract (CP), 6.0 gm, was divided into 2 equal portions and each one was treated in the same manner. Each portion was dissolved in chloroform (5 ml) and mixed with small amount of silica gel. The content was air dried and packed onto the top of dry silica gel column (4.5x22 cm). The column was eluted sequentially using petroleum ether : ethyl acetate (9:1), (8:2), (7:3), (1:1) and then washed with acetone until no trace of compounds could be detected. Twenty-millilitre of each fraction was collected and compared by TLC. Those fractions of similar pattern were combined and evaporated to dryness under reduced pressure. The resulted residues were purified further as follows :

a) Residue A (fractions 7-15), 0.017 gm was rechromatographed on silica gel (2.5x15 cm) column using n-hexane and chloroform as eluent to furnish mixed compounds and subsequently characterized as triterpene.

b) Residue B (fractions 16-30), 0.025 gm was rechromatographed on silica gel (2.5x22 cm) column using n-hexane : ethyl acetate(1:1) and (1:9) as eluents to furnish 0.010 gm of US-2 (0.007%) and subsequently characterized as atranorin.

c) Residue C (fractions 31-66), 0.050 gm.

d) Residue D (fractions 67-120), 0.200 gm.

e) Residue E (fractions 121-144), 3.900 gm was rechromatographed on silica gel (3.5x25 cm) column using benzene : chloroform (3:2), chloroform and chloroform : methanol (9:1) as eluents to furnish 3.293 gm of US-1 (0.22%) and subsequently characterized as usnic acid.

Crude chloroform extract (CC), 35.0 gm, was divided into 2 equal portions and each one was treated in the same manner. Each portion was dissolved in chloroform (5 ml) and mixed with small amount of silica gel. The content was air dried and packed onto the top of dry silica gel column (4.5x22 cm) was eluted with benzene : chloroform (3:2), chloroform and chloroform : methanol (9:1). Forty-millilitre fractions were collected, evaporated and compared by TLC. Those fractions of similar pattern were combined and evaporated to dryness under reduced pressure.

a) Fractions 1-5 were designated as US-2 (0.003 gm, 0.002%).

b) Fractions 6-50 were crystallized upon the addition of small volume of methanol. It was designated as US-1 (15.397 gm, 1.026%).

Crude acetone extact (CA), 22.0 gm, was divided into 2 equal portions and each one was treated in the same manner. Each portion was dissolved in acetone (5 ml) and mixed with small amounts of silica gel. The content was air dried and packed onto the top of dry silica gel column (3x22 cm). The column was eluted with chloroform, chloroform : methanol (9:1), (8:2), (7:3), (6:4), (1:1) and methanol. Forty-mililitre fractions were collected, until no trace of compounds could be detected. The volume of each eluting solvent was 500 ml. The fractions were examined by TLC and those fractions of same pattern were combined to give the following portions:

a) Fractions 1-17 were combined and crystallized upon the addition of small volume of methanol as US-1 (1.970 gm, 0.001%).

b) Fractions 18-22 were combined and designated as US-3 (0.025 gm, 0.017%) and subsequently characterized as isomeric form of stictic acid.

4. Identification of the Isolated Compounds

The isolated compounds were identified by comparison of hRf values, melting points, optical rotation, ultra-violet absorption spectra, nuclear magnetic resonance spectra and mass spectra and data published previously.

4.1 Identification of US-1 as Usnic Acid

US-1 was crystallized from methanol as yellow needle crystals. It was soluble in benzene, chloroform and ethyl acetate.

4.1.1 hRf values

The hRf values given were obtained from the following systems:

a) silica gel 60 GF_{254} / benzene : chloroform (2:1) = 45

b) silica gel 60 GF_{254} / benzene : chloroform (3:1) = 34

c) silica gel 60 GF_{254} / benzene : n-hexane(2:1) = 49

d) silica gel 60 GF_{254} / chloroform : n-hexane (1:1) = 13

e) silica gel 60 GF_{254} / chloroform : ethyl acetate (1:1) = 63

f) silica gel 60 GF_{254} / chloroform : methanol (9:1) = 55

g) silica gel 60 GF_{254} / chloroform = 74

h) silica gel 60 GF_{254} / petroleum ether : methanol (95:5) = 70

The thin layer chromatogram of US-1 was showm in Figure 12, p.134.

4.1.2 Optical Rotation (in $CHCl_3$)

$[\alpha]_D^{16}$ = +489.4 (c = 0.696 gm/100 ml in chloroform)

4.1.3 Melting Point

195°C (uncorrected)

4.1.4 Molecular Weight

344 (mass spectrometry)

4.1.5 UV Absorption Spectrum : (MeOH) (Figure 15, p.137)

λ_{max} (nm) : 232, 284

4.1.6 IR Absorption Spectrum (KBr) (Figure 16,p.138)

ν_{max} (cm^{-1}) : 1550,1640 (diketone), 1700 (carbonyl) and 3000 (hydroxyl).

4.1.7 Mass Spectra : (EI) (Figure 17,p.139)

m/z (%, relative intensity)

344 ([M+], 61.5%), 260 (62.4%), 234 (15.33%), 233 (100%), 217 (18.4%)

4.1.8 Proton NMR Spectrum ($CDCL_3$, 500 MHz) (Figure 18, p.140)

Table 8 : ^{1}H-NMR spectrum of US-1

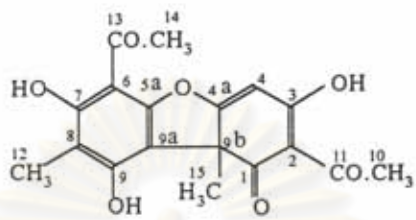
Chemical shift (ppm)	Proton	Multiplicity
1.72	C(12)-H_3	3H,s
2.04	C(15)-H_3	3H,s
2.63	CO-C(10)H_3	3H,s
2.64	CO-C(14)H_3	3H,s
5.94	C(4)-H	1H,s
10.99	C(9)-OH	1H,s
13.30	C(7)-OH	1H,s
18.80	C(3)-OH	1H,s

4.1.9 Carbon-13 NMR Spectrum ($CDCl_3$, 125 MHz) (Figures 19-20, p.141-142)

Table 9 : ^{13}C-NMR spectrum of US-1

Carbon	Chemical shift (ppm)
C(1)	197.97
C(2)	105.16
C(3)	191.64
C(4)	98.27
C(4a)	179.27
C(5a)	155.13
C(6)	101.43
C(7)	163.78
C(8)	109.20
C(9)	157.41
C(9a)	103.89
C(9b)	59.01
C(10)	32.09
C(11)	201.72
C(12)	27.87
C(13)	200.28
C(14)	31.25
C(15)	7.50

From the above data, US-1 was in complete agreement with the structure of (+)-usnic acid. It is therefore concluded that US-1 was (+)-usnic acid, the structure of which was shown below.



4.2 Identification of US-2 as atranorin

US-2 was obtained as colourless prisms from chloroform-petroleum ether. It is soluble in chloroform, hexane and ethyl acetate.

4.2.1 hRf value

The hRf value given were obtaine from the following system:

a) silica gel 60 GF_{254} / chloroform : methanol (1:1) = 14

b) silica gel 60 GF_{254} / ethyl acetate : n-hexane (4:1) = 86

c) silica gel 60 GF_{254} / chloroform = 58

d) silica gel 60 GF_{254} / benzene : chloroform (1:1) = 16

e) silica gel 60 GF_{254} / petroleum ether : ethyl acetate (9:1) = 40

4.2.2 Melting Point

196-197° C

4.2.3 Molecular Weight

374 (mass spectrometry)

4.2.4 UV Absorption Spectrum (MeOH) (Figure 21, p.143)

λ_{max} (nm) : 218, 262

4.2.5 IR Absorption Spectrum (Nujol) (Figure 22, p.144)

ν_{max} (cm^{-1}) : 1660, 1590, 1350, 1280, 1270, 1220, 1170, 1100, 820 and 810

4.2.6 Mass Spectrum : (EI) (Figure 23, p.145)

m/z (%, relative intensity)

374 ($[M]^+$,73.3%),196(96.3%),180(68.9%),179(100%),177(67.9%), 165 (88.2%), 164 (92.0%), 136 (79.7%)

4.2.7 Proton NMR Spectrum : ($CDCl_3$, 500 MHz) (Figure 24, p. 146-148)

Table 10 : ^{1}H-NMR spectrum of US-2

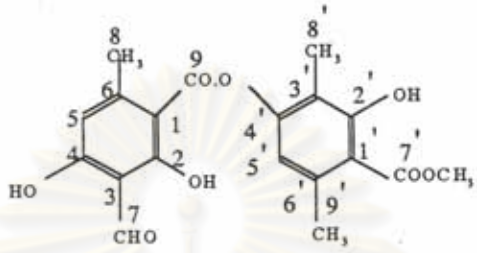
Chemical shift(ppm)	Proton	Multiplicity
2.09	C(9')-H_3	3H,s
2.55	C(8')-H_3	3H,s
2.69	C(8)-H_3	3H,s
3.98	C(7')-$OOCH_3$	3H,s
6.40	C(5)-H	1H,s
6.51	C(5')-H	1H,s
10.36	C(7)-HO	1H,s
11.94	C(2')-OH	1H,s
12.49	C(4)-OH	1H,s
12.55	C(2)-OH	1H,s

4.2.8 Carbon-13 NMR Spectrum : ($CDCl_3$, 125 MHz) (Figure 25, p.149)

Table 11 : ^{13}C-NMR spectrum of US-2

Carbon	Chemical shift(ppm)
C(1)	102.67
C(2)	169.10
C(3)	108.58
C(4)	167.51
C(5)	112.87
C(6)	152.44
C(7)	169.70
C(8)	25.57
C(9)	193.85
C(1')	116.80
C(2')	162.88
C(3')	110.29
C(4')	152.01
C(5')	116.03
C(6')	139.88
C(7')	172.20
C(8')	24.00
C(9')	9.37
$COOCH_3$	52.33

From the above data, US-2 was in complete agreement with the structure of atranorin. It is therefore concluded that US-2 was atranorin, the structure was shown below.



4.3 Identification of US-3 as isomeric form of stictic acid

US-3 was obtained as white amorphous powder from acetone part. It is soluble in DMSO, slightly soluble in acetone, and poorly soluble in ethanol.

4.3.1 hRf values

The hRf values given are obtained from the following systems:

a) silica gel 60 GF_{254} / acetone : chloroform (6:4) = 40

b) silica gel 60 GF_{254} / chloroform : methanol (9:1) = 30

c) silica gel 60 GF_{254} / chloroform : methanol(4:1) = 35

4.3.2 Melting point

270-272°C

4.3.3 Molecular weight

386 (mass spectrometry)

4.3.4 UV absorption spectrum : (EtOH) (Figure 26, p.150)

λ_{max} (nm) : 217, 262, 318

4.3.5 IR absorption spectrum : (Nujol) (Figure 27, p.151)

ν_{max} (cm^{-1}) : 3480, 3250 (hydroxyl), 1745, 1730, 1690 (depsidone carboxyl and carbonyl)

4.3.6 Mass spectrum : (EI) (Figure 28, p.152)

m/z (%, relatively intensity)

386 ($[M]^+$,36%), 368(20%), 193(42%), 191(36%), 83(100%)

4.3.7 Proton-NMR spectrum : (DMSO-d_6, 500 MHz) (Figure 29, p.153)

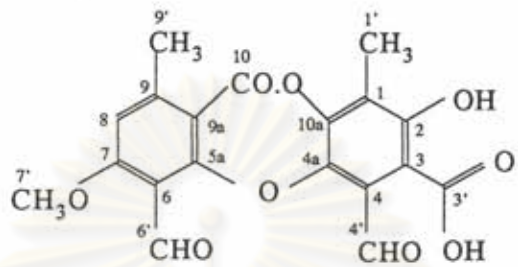
Table 12 : ^{1}H-NMR spectrum of US-3

Chemical shift (ppm)	Proton	Multiplicity
2.13	C(1)-H_3	3H,s
2.49	C(9)-H_3	3H,s
3.90	CO-C(7')H_3	3H,s
7.05	C(8)-H	1H,s
10.16	C(6)-HO	1H,s
10.24	C(4)-HO	1H,s

4.3.8 Carbon-13 NMR spectrum (DMSO-d_6, 125 MHz) (Figure 30, p. 154)

Carbon	Chemical shift(ppm)
C(1)	118.15
C(2)	159.13
C(3)	114.27
C(4)	131.50
C(4a)	136.74
C(5a)	162.00
C(6)	114.38
C(7)	161.55
C(8)	112.35
C(9)	150.49
C(9a)	113.21
C(10)	164.14
C(10a)	144.21
C(1')	9.13
C(3')	169.97
C(4')	191.09
C(6')	186.99
C(7')	56.61
C(9')	21.45

From the above data, US-3 was in agreement with the structure of isomeric form of stictic acid as shown below.



US--3

Part II : Quantitative Analysis of Usnic Acid

Experimental

1. Plant Material

The dried whole plant of *Usnea siamensis* Wainio were obtained from two sources of Thailand as follow:

Northern : Doi Intanon, Chiang Mai

North-eastern : Pu luang, Loei

2. General techniques

2.1 Reagents and Standards

1. Acetone, benzene, chloroform, ethyl acetate, hexane, (analytical grade from Merck, Damstadt, Germany)

2. Silica gel $60GF_{254}$ plates were purchased from Merck, Damstadt, Germany.

3. Pure natural usnic acid was isolated from *Usnea siamensis* Wainio by soxhlet extraction with benzene and purified by column chromatography and crystallization. It was identified as usnic acid by its polarity sequence on TLC plate and confirmed by elucidating structure from its spectroscopic analysis.

2.2 Preparation of Standard Solutions

For the preparation of standard solution, ten milligrams of usnic acid was dissolved in 100 ml ethyl acetate to give 0.1 mg/ml stock solution. A stepwise half-dilution of the stock solution was performed and the standard curve in the range of 0.025-0.100 mg/ml was constructed for usnic acid (Figure 31, p.96).

2.3 Sample Preparation

For sample preparation, one-gram fine powder of *Usnea siamensis* Wainio (passing No.40 sieve) was accurately weighed and extracted with 200 ml of various solvents including acetone, benzene and ethyl acetate under two methods, soxhlet and reflux extraction for one hour. After cooling, the extracts were filtered through Whatman no.1 in a Buchner funnel and adjusted to the volume of 250 ml with extracted solvent in a volumetric flask.

2.4 Thin Layer Chromatography Conditions for Usnic Acid

Technique	:	one way, ascending
Absorbent	:	silica gel $60GF_{254}$ (precoated, Merck)
Plate size	:	6x5 cm
Layer thickness	:	0.2 mm
Solvent system	:	chloroform : hexane (4:1)
Sample size	:	5 µl
Developing time	:	3 times

Distance : 5 cm

Temperature : 25-30 ^{o}C

hRf value : usnic acid (30)

2.5. Densitometric Analysis

After thin layer chromatography, it was quantitated by densitometric method which were described below

Model : Shimadsu Dual Wavelength Model CS-930

Lamp : deuterium

Scan mode : linear

Determination mode : absorption

Scan width : x = 10.0 mm

y = 0.2 mm

Sensitivity : medium

Slit width : 1.2x1.2 mm^2

Wavelenth : 295 nm

The content of usnic acid was calculated based on its standard curve.

Results

1. Optimization of TLC-Densitometric Conditions for Usnic Acid Determination

1.1. Extraction Conditions for Pure Usnic Acid

Initially, extraction of *Usnea siamensis* Wainio from Doi-Intanon was done by soxhlet extractor with benzene, 60-70 ^{o}C, one hour. The crude extract was then evaporated under vacuum to syrupy mass and usnic acid was isolated by column chromatography using benzene : chloroform (3:2) as eluting solvent. The yellow needles were recrystallized by chloroform and checked on TLC plates using various solvent systems which all showed the presence of only one spot.

1.2. Development of TLC Solvent System

In order to search for a suitable developing solvent system for TLC separation of usnic acid from other substances in crude extract, a number of solvent systems were tried and the resulted Rf value of usnic acid in each system was examined. As shown in Table 14, almost all the developing solvent systems gave relatively high Rf values of usnic acid except the system of chloroform : hexane (4:1) with double and triple developments. It was found that the solvent system of chloroform : hexane (4:1) with triple developments gave better results than the other system. No interference of other components was observed either under 254 or 365 nm.

1.3. Identification of Usnic Acid by Spectroscopy

Since usnic acid used for the preparation of standard solution was isolated and purified from *Usnea siamensis* Wainio, not authentic sample. It must be identified and confirmed by spectroscopic data. These spectroscopic data were corresponded with those described in the section of structure elucidation of usnic acid (pages 75-79).

1.4 TLC-Densitometric Determination of Usnic Acid

The separated usnic acid on TLC plate was quantitated by densitometry. To establish this method, an appropiate wavelength to be used for scanning was first selected. From the absorption spectrum of usnic acid, it was found that usnic acid showed maximum absorption at 295 nm (Figure 32 B). This wavelength was , therefore, chosen for performing quantitative analysis of this compound. Figure 33 shows a typical TLC-densitometric chromatogram of the crude extract which was separated on a silica gel plate.

1.5 Calibration Curve

The calibration curve of standard usnic acid which was obtained by plotting between the peak areas and usnic acid concentrations is shown in Table 15 and Figure 31. The calibration curve was linear in the range from 0.25 to 1.00 μg usnic acid per each spot (5 μl application volume). The correlation coefficient(r) was found to be 0.9967 and regression equation was :

$$y = 1001079\,x + 4127.932$$

where : y = peak area of usnic acid

x = the amount of usnic acid (μg)

r = correlation coefficient

1.6 Sample Preparation

To ensure complete extraction of usnic acid from *Usnea siamensis* Wainio, the extraction methods of soxhlet and reflux were investigated. In both methods, benzene was used as extracting solvent because of its good solubility for usnic acid. In this study, the proportion of foi-lom powder and the solvent was first optimized. It was found that the ratio of 4:1 (e.g. 1 gm sample in 250 ml solvent) gave the optimum usnic acid content in the range of calibration curve. It was also found that reflux extraction gave higher usnic acid content than the other methods (Table 16). To select an optimum extracting solvent, benzene, acetone and ethyl acetate were compared using reflux extraction. It was found that reflux extraction using ethyl acetate gave the highest content of usnic acid (Table 17), we therefore, chose ethyl acetate for the quantitative extraction of usnic acid from *Usnea siamensis* Wainio.

1.7 Usnic Acid Content in *Usnea siamensis* Wainio

The developed TLC-densitometry was used to determine the usnic acid content in foi-lom obtained from Doi-Intanon and Pu Luang. It was found that the content of usnic acid in *Usnea siamensis* Wainio from Doi-Intanon was 4.314%(w/w) and from Pu Luang was 3.426%(w/w) (Table 18).

Table 14 : The effect of various solvent systems on the hRf values of usnic acid in the crude extracts of *Usnea siamensis* Wainio

Developing solvent system used with Silica gel $60GF_{254}$	Ratio	hRf value
benzene : acetone	3:1	50
benzene : chloroform	3:1	34
chloroform	-	55
chloroform : acetone	2:1	65
chloroform : ethyl acetate	1:1	68
chloroform : n-hexane	4:1	30
n-hexane : ethyl acetate	3:2	47
petroleum ether : ethanol	95:5	70

* hRf value was calculated from the migration distance of usnic acid divided by the migration distance of the solvent

Table 15 : The calibration curve of standard usnic acid

Usnic acid (μg)	Peak area ($*10^3$)
0.00	0.00
0.25	57.45
0.50	104.93
0.75	159.59
1.00	199.20

Table 16 : Comparison between soxhlet and reflux extractions using benzene on the extractable usnic acid content in *Usnea siamensis* Wainio

	Extraction Method	
	Soxhlet	Reflux
extractable usnic acid (%w/w)	1.563±0.123	1.771±0.067

Table 17 : Effect of some organic solvents on the extractable usnic acid from *Usnea siamensis* Wainio under reflux at 60-70°C for one hour

	Extraction Solvent		
	Acetone	Benzene	Ethyl acetate
extractable usnic acid (%w/w)	3.970±0.878	1.771±0.067	4.314±0.236

Table 18 : Usnic acid content in *Usnea siamensis* Wainio obtained from Doi-Intanon and Pu luang

	Source of *Usnea siamensis* Wainio	
	Doi Intanon	Pu luang
extractable usnic acid (%w/w)	4.314±0.236	3.426±0.071

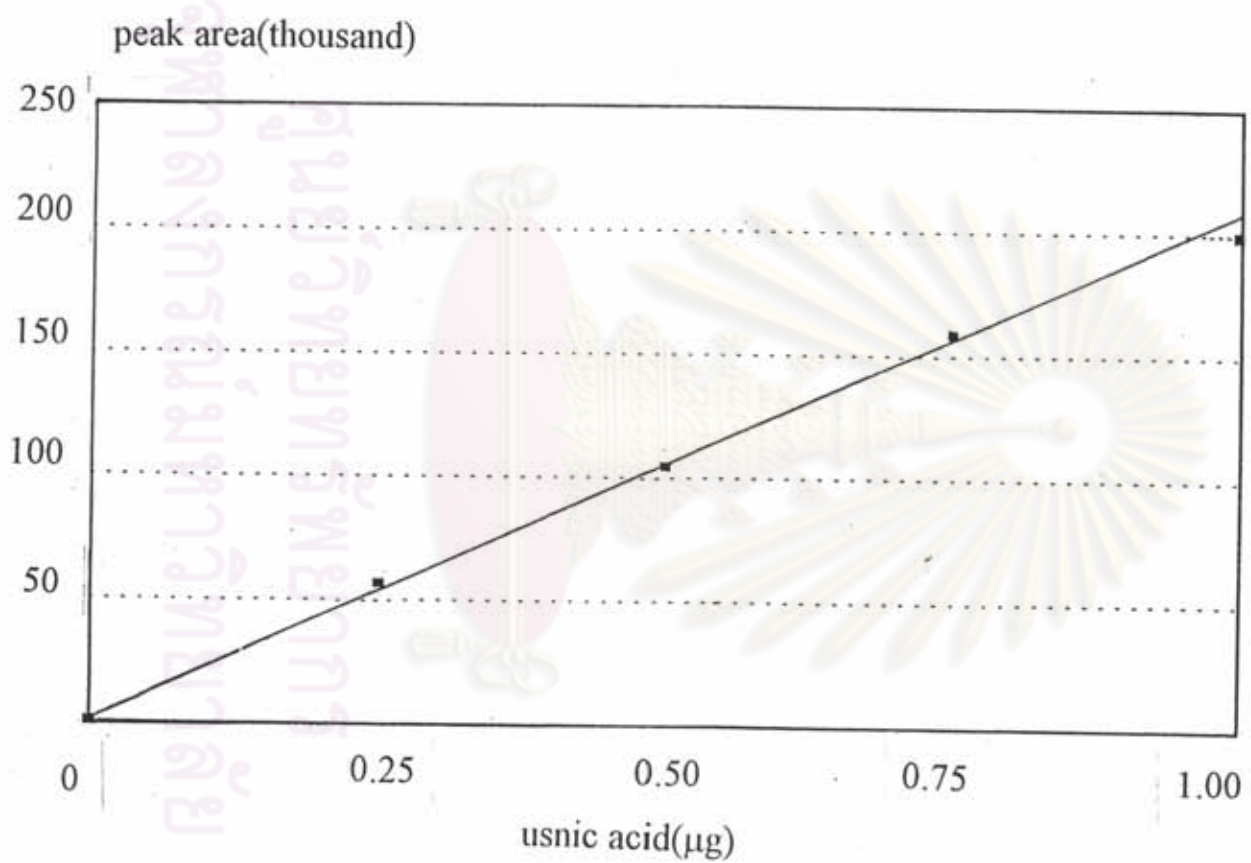


Figure 31 The calibration curve of standard usnic acid

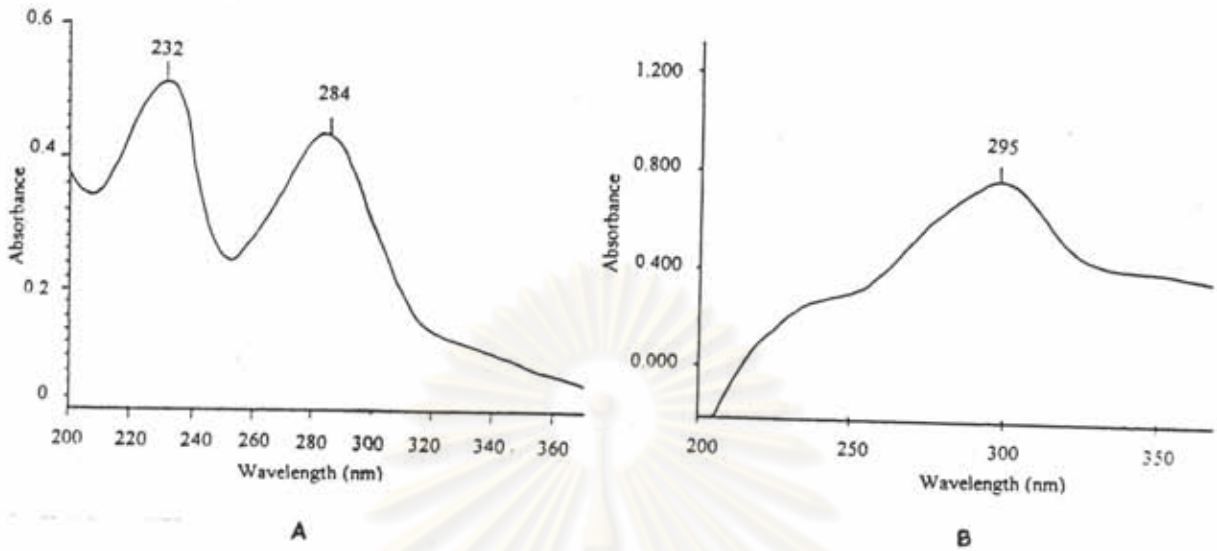


Figure 32 UV-absorption spectra of usnic acid

A) by UV- spectrophotometry

B) by TLC-densitometry

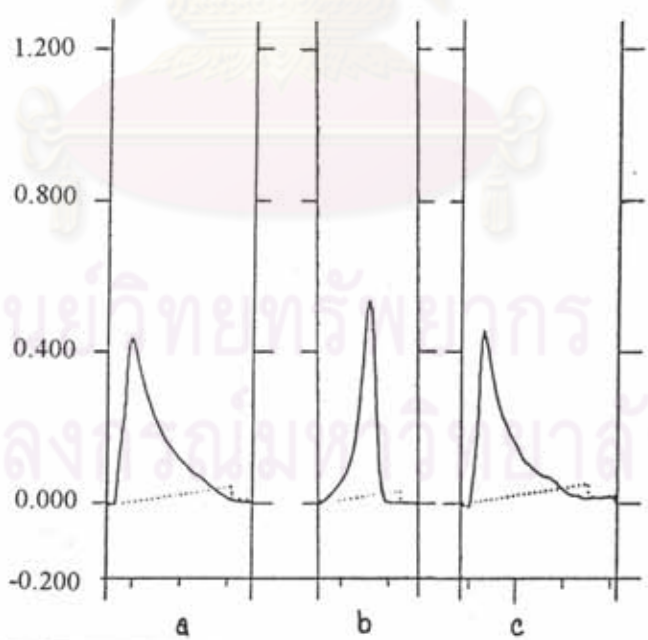


Figure 33 TLC-densitometric chromatogram of usnic acid using optimization condition a) crude acetone extract

b) crude benzene extract

c) crude ethyl acetate extract

Part III : Antibacterial activity of usnic acid

Experimental

In this study, nineteen species of two different classes of microorganisms, both gram-positive and gram-negative bacteria were investigated for their sensitivity to usnic acid.

The method this study was the microbiological agar diffusion .

1. Test Medium

Mueller Hinton Agar (Difco, control no. 0252) was popular for agar diffusion method because it was one of the complete media.

The ingredients per litre were as follows :-

Beef, Infusion form	300.0 gm
Casamino Acids Technica	117.5 gm
Starch	1.5 gm
Bacto-Agar	17.0 gm

To rehydrate the medium, 38 g of the medium was suspended in 1,000 ml of cold purified water, USP., distilled or deionized water, and heated to boiling to dissolve the medium completely. Dispended into flasks and sterilized in the autoclave for 15 minutes at 15 pound/inch^2 pressure (121^{o}C). Avoided excessive heat during rehydration or sterilization. Final pH was 7.3$\pm$0.1.

2. Preparation of samples

2.1 US-1 was dissolved to make the concentration of 100 µg/ml by 0.8% tween 80 in 95%ethanol

2.2 Dried crude extract from the chloroform part was dissolved to make the concentration of 900 mg/ml by 0.8% tween 80 in 95%ethanol

2.3 The solvent control sample was 0.8% tween 80 in 95%ethanol

The test was repeated two times.

3. Preparation of the inoculum

3.1 Test organisms; 19 microorganisms were used as following:-

Gram-positive bactria:-

- *Staphylococcus aureus* ATCC 25923
- Methicillin Resistant *Staphylococcus aureus* (MRSA)
- Methicillin Sensitive *Staphylococcus aureus* (MSSA)
- *Staphylococcus epidermidis*
- *Staphylococcus citeus*
- *Staphylococcus mirabilis*
- Coagulase negative staphylocooccus

:oxacillin resistant strains from patients at Siriraj Hospital

:penicillin and ampicillin resistant strains from patients at Siriraj Hospital

- *Sarcina lutea* ATCC 10240 (*Micrococcus flavus* ATCC 10240)
- *Bacillus cereus* ATCC 9634
- *Bacillus pumitis*

- *Bacillus subtilis* ATCC 6633

Gram-negative bacteria:-

- *Pseudomonas aeruginosa*

- *Escherichia coli*

- *Salmonella typhi*

- *Proteus vulgaris*

All test organisms were cultured overnight on Antibiotic Medium No.1 slants(difco, control no.0263) at 37° C before testing. The ingredients per litre were as following-

Bacto Beef extract	1.5	gm.
Bacto Yeast extract	3.0	gm.
Bacto Casitone	4.0	gm.
Bacto Peptone	6.0	gm.
Bacto Dextrose	1.0	gm.
Bacto Agar	15.0	gm.

To rehydrate the medium, 30.5 gm of medium was dissolved in 1,000 ml of cold distilled water. The suspension was boiled to dissolve the medium completely, allowed to cool at 45°C-50°C and then dispended in several slant tubes with the appropriate volume. The tubes were sterilized in the autoclave for 15 minutes at 15 pounds pressure (121° C). After the sterilization, allowed to cool at 45°C-50°C and then slanted the tubes. Waited until the medium became solid. Stored at 4°C-10°C. The final pH of the medium was pH 6.55±0.05.

3.2 The culture suspended with a small volume of sterile normal saline solution and adjusted the inoculum to match a 0.5 turbidity standard of Mc Farland No.1 when comparing the tubes against a white background with a contrasting black line. The inoculum suspension should not be allowed to stand longer than 15 to 20 minutes before inoculating.

4. Preparation of test plates

Mueller Hinton Agar (MHA) was melted and allowed to cool at 45°C - 50° C in a water bath. Then 25 ml of the melted agar medium was dispended aseptically into sterile glass Petri dishes, with internal diameters of 9 cm, to yield a uniform depth of 4 mm. The agar was allowed to harden on a flat level surface. If, prior to use, moisture was presented on the agar surface, the plates should be placed in an incubator at 35° C with their lid slightly ajar in order to permit the evaporation of surface moisture. Evaporation was usually complete within 30 minutes.

5. Inoculations of agar plates

A sterile cotton swab was dipped into the standardized suspension and the excess was removed by pressing and rotating the swab firmly against the inside of the tube above the fluid level. The entire surface of the MHA plates was inoculated by streaking evenly in three directions with the swab and in each direction the plate was rotated 60° subsequently to obtain a uniform inoculum.

This plate was then allowed to dry for 3 to 5 minutes, but no longer than 15 minutes, before the cup were applied.

6. Application of cups

Sterile cups was placed on the inoculated agar surface in each Petri dish aseptically. In order to ensure complete contact of the cups to the agar surface, the cups had to be pressed down with slightly pressure.

The spatial arrangement of the cups should be such that they were no closer than 15 mm to the edges of the plate and far enough apart to prevent overlapping of zones of inhibition. The cups was filled with 300 µl of three samples to each other by using 1000 µl micropipette. The plates were left at room temperature for 30 minutes, then incubated at 35-37° C for about 16 to 18 hours (overnight). Any longer delay before incubation would allow excess prediffusion of the samples.

Results

The zone diameters of inhibition were measured with a sliding calipers with an accuracy of nearest 0.1 mm. Faint growth or tiny colonies near the edge of the inhibition zones were ignored if they were presented. The results were shown in the Table 20, Figure 34-38.

Table 20 : Antibacterial Activities of Usnic Acid

Microorganisms	Inhibition zone (mm)		
	Control	Crude extract	Usnic acid
Gram-positive bacteria			
Staphylococcus aureus			
ATCC 25923	15.8	18.3	25.3
MSSA	6.2	9.6	11.5
MRSA	6.8	9.2	11.2
Staphylococcus epidermidis	24.1	25.7	27.5
Staphylococcus citeus	4.6	20.1	12.2
Staphylococcus mirabilis	6.6	8.4	11.4–
Coagulase negative staphylococcus			
C 94 PA	7.9	12.1	14.4
C 99 RO	10.6	11.6	23.6
R 48 PA	10.8	30.0	26.6
T 43 RO	5.1	9.6	12.2
Sarcina lutea ATCC 10240	14.8	22.1	23.6
Bacillus subtilis ATCC 6630	15.6	22.3	20.2
Bacillus cereus ATCC 9634	11.2	13.2	8.2
Bacillus pumitis	6.3	11.4	10.6
Gram-negative bacteria			
Pseudomonas aeruginosa	4.5	2.1	9.6
Escherichia coli	13.1	13.7	11.7
Salmonella typhi	15.7	16.0	15.0
Proteus vulgaris	8.1	4.8	6.1

*abbreviation : MRSA = methicillin sensitive *Staphylococcus aureus*

MSSA = methicillin resistant *Staphylococcus aureus*

C94 PA was isolated from CU student and resisted to penicillin and ampicillin

C 99 RO was isolated from CU student and resisted to oxacillin

R 48 PA was isolated from doctors and resisted to penicillin and ampicillin

T 43 RO was isolated from patients and resisted to oxacillin

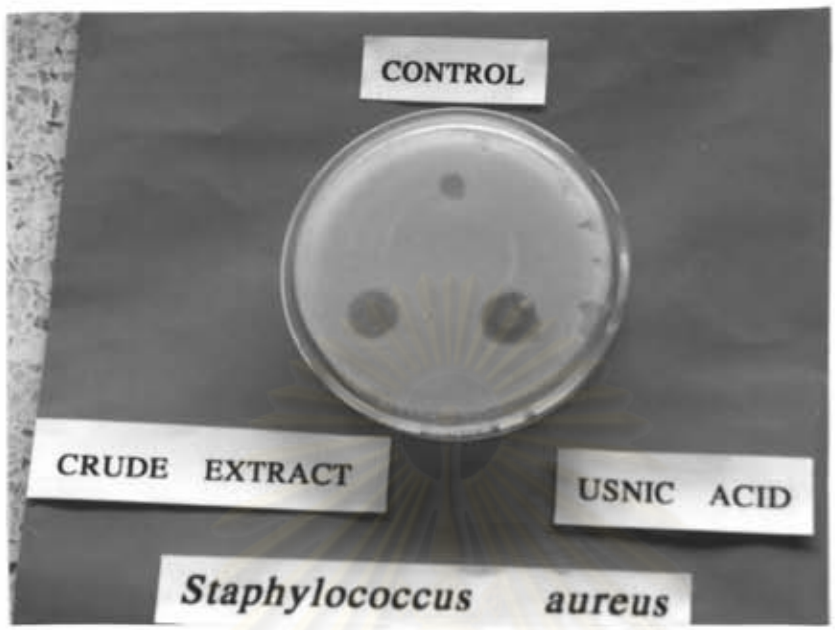


Figure 34 Activity of usnic acid against MRSA

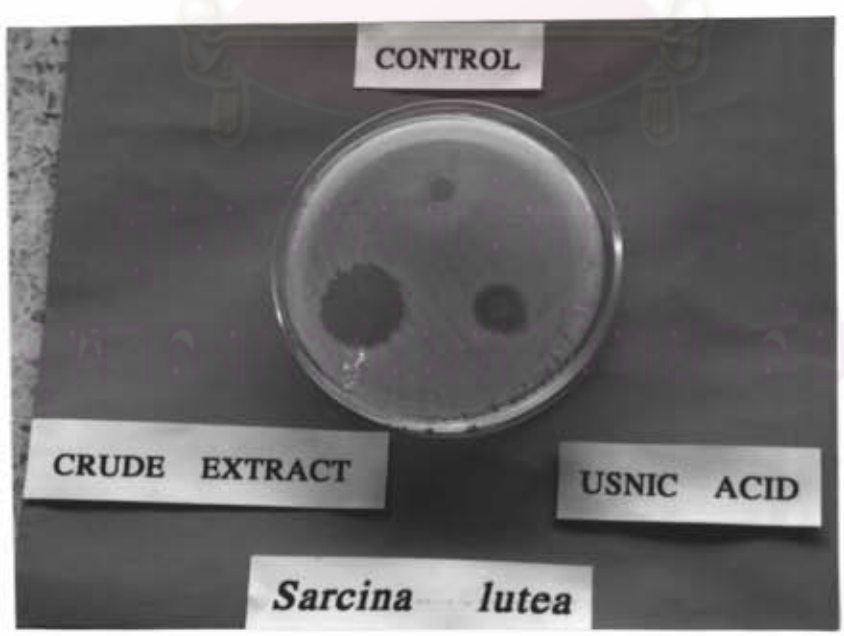


Figure 35 Activity of usnic acid against *Sarcina lutea* ATCC 10240

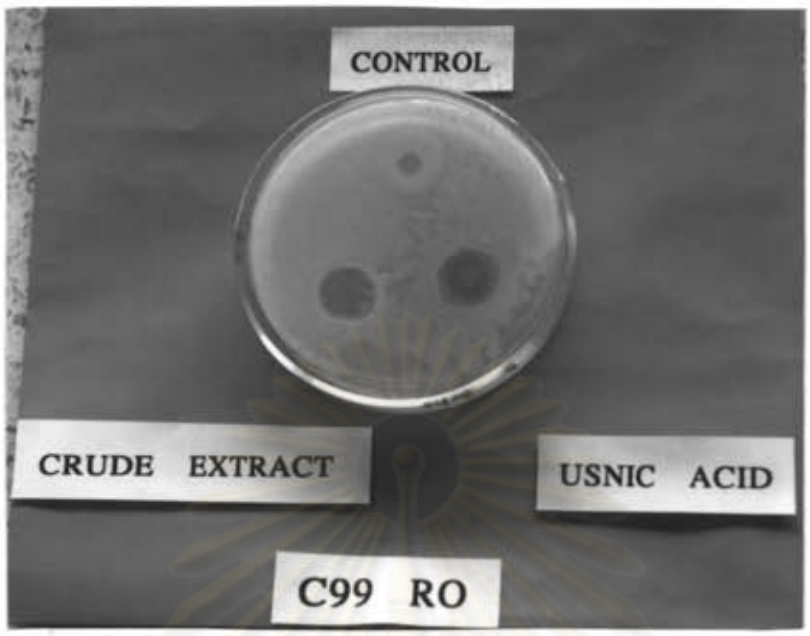


Figure 36 Activity of usnic acid against oxacillin resistant coagulase negative staphylococcus from CU student

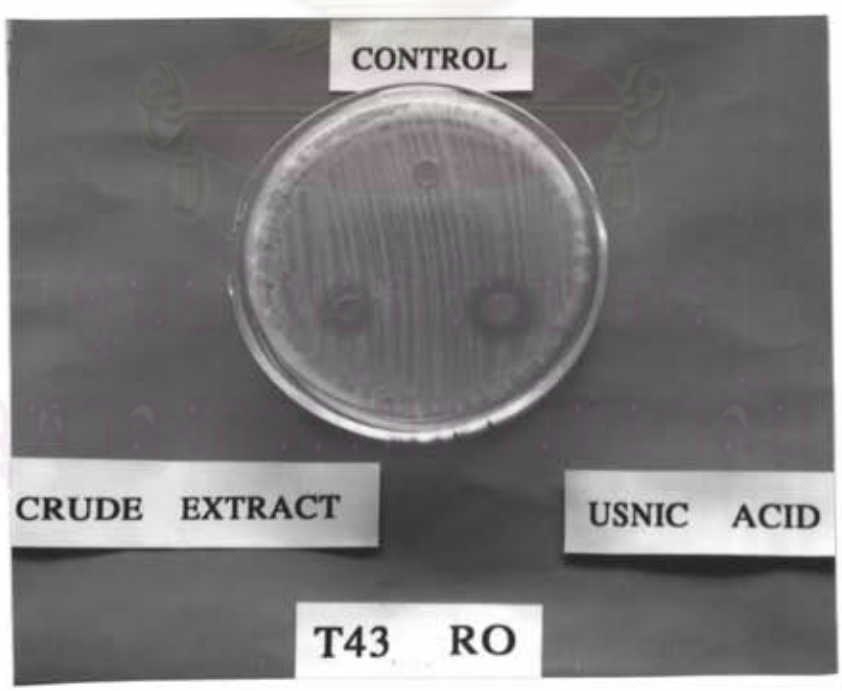


Figure 37 Activity of usnic acid against oxacillin resistant coagulase negative staphylococcus from patient

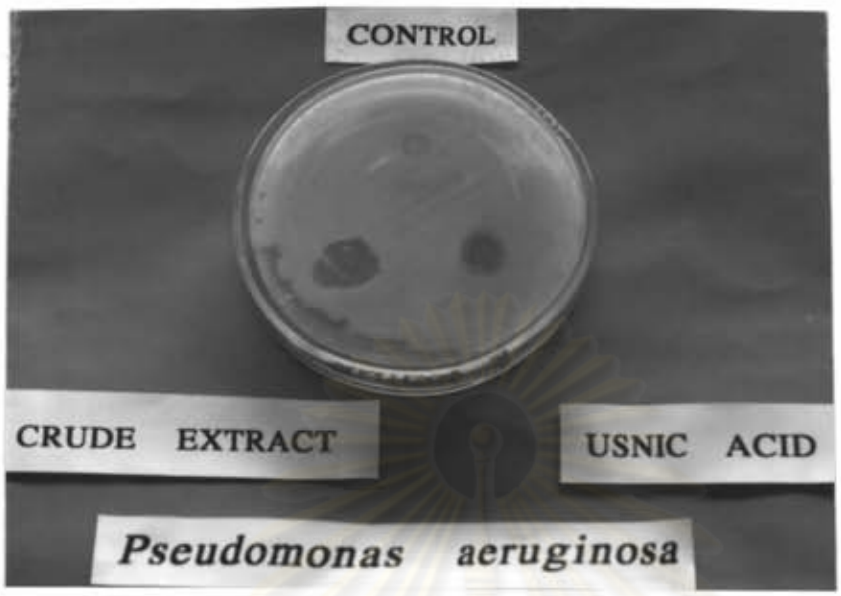


Figure 38 Activity of usnic acid against *Pseudomonas aeruginosa*