CHAPTER III

EXPERIMENTAL

1. Methods for isolation of *Micromonospora*

Twenty two soil samples were collected from various areas of Thailand. Sources of soil samples were presented in Table 5. In order to isolate actinomycetes, 0.5 g of each soil sample was suspended in sterile distilled water (4.5 ml), heated at 55 °C for 5 minutes. Soil solution was diluted to 1:100, 1:1,000 and 1:10,000 dilutions. Each dilution (0.1ml) was spreaded on surface of Potato-carrot agar(PCA)and Sodium caseinate agar (SCA) plates (plus antibiotics, appendix). Plates were incubated at 30 °C for 7-14 days. The moist, pale yellow, orange, red brown, brown, black, blue green or purple colonies of *Micromonospora* species were picked up and streaked for purification on Yeast extract-malt extract agar plates (YMA, ISP-2) and were incubated at 30 °C for 7-14 days. Colonies were transferred to cultivate on YMA slants and incubated at 30 °C for 14 days. Stock cultures were kept in cold room at 4 °C.

2. Primary screening of isolates for antibiotic production

The stock cultures of *Micromonospora* strains were inoculated into a flask each containing 120 ml of Sucrose soybean medium (SS, Appendix), imcubated on a rotary shaker at 200 rpm at 28 °C for 5 to 7 days. Five milliliters of each culture broth was taken aseptically to assay for antibiotic activity against *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633 and *Escherichia coli* ATCC 25922 by using agar diffusion method (Lorian, 1980).

All test organisms were cultivated overnight on Tryptic soy agar (TSA) slant

at 37 °C. The cell cultures were washed from the agar surface with sterile normal saline solution (NSS). The culture was then standardized to match a 0.5 turbidity standard of MacFarland.

Twenty one millilitres of molten nutrient agar was poured into 9 cm diameter petridishes and allowed to solidified to form base layer while 1 ml of each test organism suspention was inoculated into 100 ml of media (45°-50°C) and was mixed to obtain seed media. Seed medium (4 ml) was then poured evenly over the surface of the base layer.

Each culture broth (5 ml) was centrifuged to give mycelial cake and filtrate. Six sterile stainless cylinder cups (6 mm internal diameter and 10 mm height) were also placed on the surface of the seed layer for each test organism. The filtrate was filled up in each cup (300 µl per cup). Plates were left out at cold room for 30 min for diffusion into the agar medium, and then they were incubated at 37 °C. The inhibition zone were measured in millimeter by vernier caliper after 16-18 hr.

3. Identification of strains

Morphological, cultural, physiological and biochemical properties of microorganisms were determined by the methods of Shirling and Gottlieb (1966) along with several supplementary tests.

3.1 Morphological and cultural characteristics

3.1.1 Determination of micromorphological characteristics

The characteristics of *Micromonospora* were detemined by using simple inclined coverslip technique (Williams and Cross, 1971). The simple inclined coverslip technique was used to dertermine the number of spores at the end of mature hyphae, the form of the spore chain and spore bearing hyphae, and other special morphological observations. For the scanning electron microscopic examination, the selected strain, *Micromonospora* sp. JSM5-1 was grown on YMA plate (ISP-2) by crosshatch streak

method (Shirling and Gottlieb, 1966) and incubated at 30 °C for 10-14 days. The culture was cut to some small cups (3-5 mm^3) and then were primarily fixed in 4 % solution of paraformaldehyde in 0.1 M phosphate buffer pH 7.2 at room temperature for 2 hr. Subsequently, they were washed 3 times with buffer and were treated with secondary fixative , a 1 % solution of osmium tetroxide in buffer, and washed in the same process before drying. The specimens were dehydrated through a gradient ethanol series (35%, 70 %, 95 % and absolute ethanol). The specimen was fixed to a stub and coated with a thin film of gold by sputter coat. An electron microscope was used to determine spore forming, spore surface, sporophore. The hyphae may be simple or complex, monopodial or sympodial. The microscopic structure of the aerial mycelium gives a clear picture of the morphology and reproductive structures of the organism.

3.1.2 Cultural characteristics

The crosshatch streak method (Shirling and Gottlieb, 1966) was used for all *Micromonospora* strains grown on YMA (ISP-2). The selected strains, JSM1-3, JSM5-1 and A-25 grew on Tyrosine agar (ISP-7), Oatmeal agar (ISP-3) on Glycerol-asparagine agar (ISP-4) They were incubated at 30 °C for 7-14 days, and examined for the color of the substrate mycelium by observing the reverse (under) side of mass growth on various media, color of spore and culture characteristics. The strain of *Micromonospora chalcea* was used as reference .

3.2 Biochemical and Physiological characteristics

3.2.1 Carbon utilization

Basal agar medium, ISP-9 (Shirling and Gottlieb, 1966) was prepared and a carbon source was added to give concentration of approximately 1 %. After autoclaved at 110 °C for 10 min, the mixture was agitated and 25 ml of this mixture was poured into 9 cm petridish .

Carbon sources and controls required for the test was repeated below :

No carbon source	(negative control)
D-glucose	(positive control)
D-melibiose	L-rhamnose
D-fructose	rhaffinose
D-manitol	D-ribose
D-xylose	D-trehalose
D-galactose	D-cellobiose
L-arabinose	

To prepare the inoculum, cell culture was washed from Yeast extract-malt extract agar slant with 5 ml of sterile distilled water, and then transferred to sterile test tube. The suspension was centrifuged at 5000 rpm for 15 min. The suspernate was decanted and the sediment was resuspended in sterile distilled water. Centrifugation and decantaton of supernate wash twice. Sterile distilled water was added to washed sediment to restore the original volume and the inoculum was used for carbon utilization tests.

The uninoculated plates were dried by leaving them at room temperature. A loopful of washed culture was inoculated on the agar surface by streaking straight across the dish. Plates were inoculated in duplicate and incubated at 28-30°C for 10-14 days.

Examination by comparing growth or a given carbon source with two controls:,growth on basal medium alone, and growth on basal medium plus glucose

Results were recorded as follows :

1. <u>Strongly positive utilization</u> (++), when growth on tested carbon in basal medium is equal to or greater than growth on basal medium plus glucose.

2. <u>Positive utilization</u> (+), when growth on tested carbon is significantly better than on the basal medium without carbon, but somewhat less than on the basal medium plus glucose.

3. Utilization doubtful (+/-), when growth on tested carbon is only slightly better than on the basal medium without carbon and significantly less than on the basal medium plus glucose.

4. Utilization negative (-), when growth is similar to or less than growth on basal medium without carbon (Utilization is always record as negative if growth is not better than no carbon control).

3.2.2 Nitrate reduction

Micromonospora strains were inoculated into Peptone KNO_3 broth (Arai,1975) and incubated at 28-30 °C for 4-6 days. On the forth day, 1 ml of the culture broth was transferred into a test tube and three drops of the sulfanilic acid reagent, and then by two drops of dimethyl -œ-napthylamine solution were added. If nitrites were present, the mixture would become pink to red .

3.2.3 Starch hydrolysis

All *Micromonospora* strains were streaked on the surface of Inorganic salts starch agar plate (ISP-4) (shirling and Gottlieb, 1966) and incubated at 28-30 °C for 10 days. After incubation was complete, Gram's iodine solution was poured on the surface of the agar plate. If starch hydrolysis was present, a dark blue color did not appear..

3.2.4 Gelatin liquefaction

All *Micromonospora* strains were inoculated into test tube of Bouillon gelatin broth (Arai, 1975) and incubated at 28-30 °C for 14 days. The inoculated tube was compared with uninoculate control when placed the both tubes at 20 °C for 30 min. If the gelatin was hydrolyzed, it became liquid, not solidify.

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3.2.5 H_2S production

All *Micromonospora* strains were was stabed into Triple sugar iron agar (Difco) and incubated at 28-30 °C for 7-14 days. If the culture produced H_2S, a black color appeared .

3.2.6 Milk coagulation and milk peptonization

Micromonospora sp. JSM1-3, JSM5-1 and A-25 were inoculated in tube of Limus milk (Arai, 1975) and incubated at 28-30 °C for 7-14 days. If milk was peptonized, milk would be converted to clear solution. If milk was coagulated , milk would precipitated.

3.2.7 Melanin production

All *Micromonospora* strains were streaked on the surface of Tyrosine agar slant (ISP-7) (Shirling and Gottlieb, 1966) and incubated at 28-30 °C for 7-14 days.

The incubated tube was compared with uninoculated control, the culture forming a greenish brown to black diffusible pigment or distinct brown pigment modified by other color was recorded as positive (+). Absence of brown to blackcolor , or total absence of diffusible pigment, was recorded as negative (-) for melanoid pigment production.

3.2.8 NaCl tolerance

All *Micromonospora* strains were streaked on YMA plates (ISP-2) to which NaCl was added to give a concentration of 0 %, 2 %, 3 %, 4 %, 5 % and 6 %. The plates were incubated at 28-30 °C for 7-14 days. Observed maximum concentration which the culture can grown were recorded.

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4. Production of antibiotics in submerged cultures

4.1 Cultivation

4.1.1 Seed medium

Micromonospora strains JSM1-3, JSM5-1 and A-25 were inoculated into 60 ml of a seed medium consisting of glucose 1.5 %, peptone 0.6 %, beef extract 0.3 %, yeast extract 0.3 %, NaCl 0.5 %, and $MgSO_4 \cdot 7H_2O$ 0.25 % in a 250 ml Erlenmeyer flask. The flask was incubated on a rotary shaker at 200 rpm at 28 °C for 3 days.

4.1.2 Production medium

The vegetative seed (2.4 ml) from 4.1.1 was transferred into a 500 ml of Erlenmeyer flask containing 120 ml of each production medium.. The culture was tested for ability to produce antibiotics in seven kinds of production medium, Sucrose soybean medium (SS), Glucose soybean medium (GS), Glucose molases medium (GM), Glycerol peptone medium (GP), Glucose NaCl medium (GN), Peptone yeast extract medium (PY) and Dextrin soybean medium.(DS) (Appendix).

4.2 Antibiotic assay

The inoculum of JSM1-3, JSM5-1 and A-25 were prepared in the same manner as described section. The previously prepared and steriled Mueller-Hinton agar medium was melt and then, cooled to 45-50 °C and 25 ml of this medium was poured into 9 cm diameter petridish. The plates was dried at 37 °C for 1 hr and the indicator strain was swabed on the surface of agar. Afterward the inoculum was swabed on the surface of agar.

The filtrate of fermentation broth was lyophilized to dry powder and dissolved with sterile distilled water to make five fold (v/v) concentration and 100 μl of the filtrate of each cuture was dropped on steriled paper disc (13 mm diameter).

Susceptibility discs were applied manually using aseptic precautions. Each paper disc was placed and gently pressed down onto the surface of the inoculated agar plate with sterile foreceps. The plates were left in cold room for 30 min and then incubated at 37 °C for 16-18 hr.

The diameters of inhibition zone from fermentation broth of each production medium were compared. The medium with the target inhibition zone was selected for fermentation study.

4.3 Fermentation conditions

4.3.1 Effect of pH and temperature on antibiotic production

The production medium selected in section 4.1.2 (120ml) were prepared in 500 ml Erlenmeyer flasks and adjusted to pH 6, 6.5, 7, 7.5 and 8, respectively. Seed culture of *Micromonospora* sp. JSM5-1 was prepared in the same manner as described in 4.1.1 and transferred to each flask of production medium. Flasks were incubated on rotary shaker at 200 rpm, 28 °C for 7 days.

The pH of the culture fluid was measured and the antibiotic production during fermentation was monitored by agar diffusion method using *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* ATCC 6633 as the test organisms. The optimum pH giving widest inhibition zone was selected for fermentation experiments.

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The seed culture was transferred to production medium which was adjusted to optimum pH, each flask was incubated on rotary shaker for 5 days at 28°, 30° and 33°C, respectively. Subsequently the antibiotic production in each flask was assayed by agar diffusion method. The optimum temperature and pH were selected for use in antibiotic production.

4.3.2 Fermentation of selected strain for antibiotic production

The cultures of *Micromonospora* sp. JSM1-3, JSM 5-1 and A-25 were inoculated into 250 ml Erlenmeyer flask containing 60 ml of seed medium (GMP) pH 7.0 and incubated on a rotary shaker at 200 rpm at 28 C for 3 days. Then 5 ml of the vegetative seed was transferred into a 500 ml Erlenmeyer flask containing 250 ml of the fermentation medium (GS medium). The fermentation was carried out at 28°C on rotary shaker at 200 rpm for 5 days.

5. General Techniques for Isolation of pure compounds

5.1 Analytical Thin layer Chromatography (TLC)

Technique	:	One dimension, ascending
Absorbent	:	Siliga gel 60 F_{254} (E. Merck) precoated plate
Layer thickness	:	0.2 mm
Distance	:	6 cm
Temperature	:	Laboratory temperature (30-35°C)
Detection	:	1. Ultraviolet light at wavelengths of 254 and 365 nm. 2. 10 % Sulfuric acid in ethanol and heated at 105 °C for 10 min.

5.2 Column Chromatography

5.2.1 <u>Flash lumn Chromatography</u>

Adsorbent	:	Silica gel 60 (No. 9385) particle size 0.040-0.063 nm (230-400 mesh ASTM) (E. Merck)
Packing method	:	Wet packing
Sampling loading	:	The sample was dissolved in a small amount of eluent, then applied gently on top of the column.
Detection	:	Fraction were examined by TLC with the plate observed under uv light at the wavelengths of 254 and 365 nm .

The TLC plate was then sprayed with 10 % sulfaric acid in ethanol and heated at 105 °C for 15 min. Fractions of similar chromatography pattern were combined .

5.2.2 <u>Gel filtration Chromatography</u>

Gel filter	:	Sephadex LH-20 (Pharmacia)
Packing method	:	Gel filter was suspended in the eluent and left standing to swell for 24 hr prior to use. It was then poured into the column and allowed to set tightly.
Sample loading	:	The sample was dissolved in a small volume of eluent and applied on top of the column
Detection	:	Fractions were examined in the same manner as described in section 5.2.1

5.3 Spectroscopy

5.3.1 <u>Mass Spectra (MS)</u>

Electron Impact Mass Spectra (EIMS) of JM1 and JM2 were performed with Micromass (VG platform II, Fisons Instrument) Spectrometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University)

5.3.2 <u>Proton and Carbon -13 Nuclear Magnetic Resonance (^{1}H and C^{13} NMR) Spectra</u>

^{1}H NMR (300 MHz) and C^{13} NMR (75 MHz) spectra were obtained with Avance DPX-300 FT -NMR Spectrometer, Bruker, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

NMR solvents used in this study were deuterated dimethylsulfoxide (DMSO-d_6) and deuterated chloroform. Chemical shifts were reported in ppm scale using the chemical shift of the solvent as reference signal.

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5.4 Solvents

Throughout this work ,all organic solvents were commercial grade and were redistilled prior to use.

6. Extraction

The culture broth from 4.3.2 (10 l) was centrifuged to give a mycelial cake and filtered. The filtrate was partitioned with ethyl acetate. Then the ethyl acetate fraction was separated and evaporated under reduced pressure to give an ethyl acetate extract

The filtrate was then partitioned with iso-butanol. The iso-butanol fraction was evaporated under reduced pressure to give an iso-butanol extract (3.72 g)

A water extract was obtained from the water fraction after removal of water by lyophilization.

The mycerial cake was macerated in methanol for 1 day and then filtered. The methanol fraction was evaporated under reduced pressure to give a methanol extract Antibacterial activity of each extract was determined by agar disc diffusion against the following organisms:

Staphylococcus aureus	ATCC 25923
S. aureus MRSA I	PCU
S. aureus MRSA II	PCU
S. epidermedis	PCU
Bacillus subtilis	ATCC 6633
Enterococcus feacalis	ATCC 25912
Escherichia coli	ATCC 25922
Pseudomonas aeruginosa	ATCC 27853
Salmonella typhi	ATCC 14028
Alcalligenes sp.	PCU

7. Isolation and purification of the extracts

The ethyl acetate fraction (1.57 g) the most active fraction, was selected for further study. It was disssolved in a small amount of chloroform and was then fractionated by silica gel-column chromatography with polarity gradient elution . Mixtures of chloroform and methanol (10:0 to 0:10) were used as eluents. Fractions of 20 ml were collected and examined by thin layer chromatography (TLC). Fractions of similar patterns were combined.

Fractions 8-11 was rechromatographyed over a silica gel column. A mixture of hexane and ethyl acetate in ratio 1:1 was used as eluent. Fractions of 10 ml were collected and examined by thin layer chromatography. Fractions (Rf 0.5, hexane and ethyl acetate in ratio 1:1) were combined and further purified by recrystallization from methanol to give JM-1 yellow needles (5 mg). It was subsequently identified as genistein.

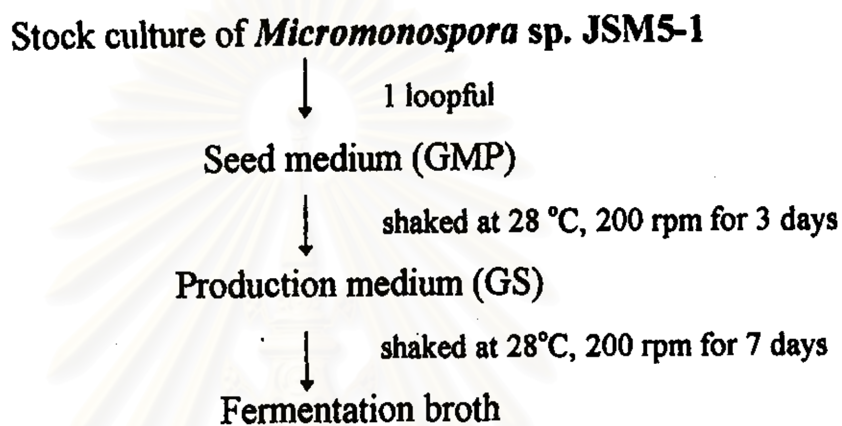
8. Spectral data of Isolated compounds

Compound JM-1

EIMS : m/z (% relative intensity)
270 (92), 153 (100), 135 (19), 124 (32),118 (52), 96 (14), 89 (25), 69 (35), 51 (17)

^{1}H nmr : δppm, 300 MHz, in DMSO-d_6
6.21 (1H, d, J=1.3, H-6), 6.39 (1H, d, J=1.3, H-8), 6.81 (2H, d, J=8.5, H-3'and H-5'), 7.37 (2H, d, J=8.5, H-2'and H-6'), 8.32 (1H, s, H-2), 12.96 (1H, s, 5-OH)

^{13}C nmr : δppm, 75 Mhz, in DMSO-d_6
94.09 (s, C-8), 99.40 (s, C-6), 104.85 (s, C-10), 115.47 (d, C-3', 5'), 121.64 (s, C-1'), 122.70 (s, C-3), 130.56 (d, C-2',6'), 154.36 (s, C-2), 157.81 (s, C-9), 158.02 (s, C-4'), 162.41 (s, C-5),164.79 (s, C-7), 180.62 (s, C-4)



Scheme 1 Fermentation of *Micromonospora* sp. JSM5-1

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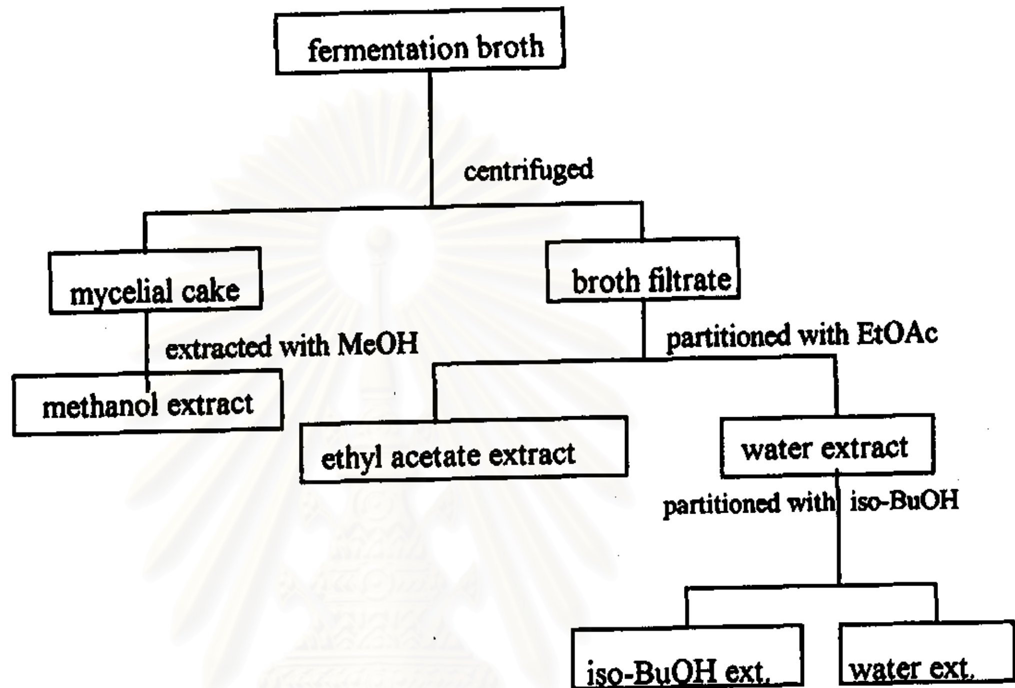

Scheme 2 Separation of *Micromonospora* sp. JSM5-1

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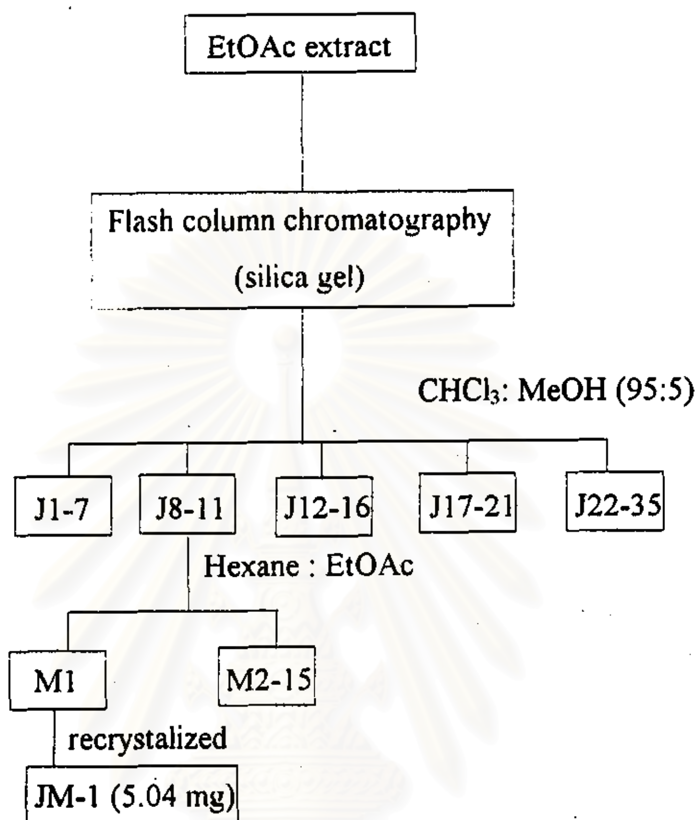

Scheme 3 Isolation and Purification of Compound JM-1

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