Chapter II

Materials and Methods

2.1 Equipments

- Autoclave LS-2D (Rexall industries Co. Ltd., Taiwan)
- Incubator BM-600 (Memmert GambH, Germany)
- Automatic micropipettes P100, P200 (Gilson Medical Electronic S.A., France)
- Laminar Flow
- Hot air oven
- pH meter
- Weighter
- Spectronic 20
- Rotary Evaporator
- Pennicillin caput

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2.1 Chemicals

- Absolute ethanol (Merck, Germany)
- Bacto-agar (Difco, USA)
- Bacto-yeast extract (Difco, USA)
- Bacto-tryptone (Difco, USA)
- Chloroform (Merck, Germany)
- D-Glucose (Merck, Germany)
- Sodium chloride (Merck, Germany)

2.2 Bacterial strain

- *Escherichia coli*: strain XL1 Blue
- *Bacillus substilis*: strain Ml 113

2.3 Fungus

- *Aspergillus sp.*

2.4 Yeast

- *Saccharomyces cerevisiae*

Plant Tissue Culture Techniques

Plant materials

Night Blooming Jasmine (*Nyctanthes arbor-tristis* L.)

Preparation medium for tissue culture by stock solution

Media was prepared in beaker 500 ml. from stock solution of MS , and GD as shown from appendix. 3% glucose and plant hormone were added to mixing. The volume was balanced by distilled water to 1 liter. The pH of each medium was adjusted to its desired value with 1 N Hydrochloric acid for 5.6 - 5.8, 0.8% w/v agar were added for solid media and heated on microwave. The media were sterilized by autoclave using the conditions of 121 °C, 15 lb/in^2 for 15 minutes.

Preparation and disinfection of explants

The explants were excised from internodes for 2-3 nodes per pieces. Explants were surface sterilized with distilled water 30 min and shaken in distilled water for 30 min. Clean with 10%(v/v) Clorox containing tween20 2-3 drops and shaked with magnetic stirrer 30 min for 3 times. In addition, they were disinfected in 70%(v/v) Ethyl alcohol and tween20 2-3 drops for 1-3 min. In laminar flow cabinet, they continued soaking in 10%(v/v) Clorox with tween20 2-3 drops for 30 min. Followed by rising with sterile distilled water for 5 times. Finally, cleaned explants were cut for 1-2 node per pieces.

Shoot induction and plantlet regeneration

The internodes were cultured on basal medium Murashige and Skoog (MS), Gressohff and Doy (GD) containing sucrose 3%(w/v) and 0.8%(w/v) agar. The pH of media was adjusted to 5.8 and then autoclaved at 121 °C, 15 lb/in2 for 15 min. The media was supplemented with 0.1, 0.2, 0.3, 0.4, 0.5 mg/l benzyladenine (BA) and 0.1, 0.2, 0.3, 0.4, 0.5 mg/l 1-napthalineacetic acid (NAA). The culture were incubated at 25 ± 2 °C and acquired light from white fluorescent lamps at intensity of 2,000 lux with 16/8 hr. photoperiod. Subculture were carried at intervals of four weeks.

Increasing of multiple shoots

The culture was carried out in MS medium containing BAP 0.3, 0.4mg/l and NAA 0, 0.1, 0.2, 0.3, 0.4 mg/l. Subculture were carried out at the period of four weeks and observed.

Root induction and growth of seedling

The elongated shoots culture were transferred to root induction medium which contained NAA 0.5 mg/l, BA 0.4 mg/l, 2,4-D 0.1, 0.2, 0.3 mg/l and activated charcoal.

Transfer to soil and natural environment

Complete plantlets were washed with water and transferred to mixed soil : sand : coir (1:1:1). Subsequently, They were increased initial humidity and were observed growth rate 20 days in nursery.

Extraction of crude extract from *Nyctanthes arbor-tristis* L.

Extraction of leaves from mother plants and tissue culture

Fresh leaves were completely dried in hot air oven with controlling at 50 °C for 48 hr. Followed by extracted with 50% ethanol and distillation in rotary vacuum evaporator. The total mass (10.75 g. from mother plants) and (0.227 g. from tissue culture) was fractionated with hexane 200 ml. for 3 times subsequently chloroform 200 ml. for 3 times and n-butanol 200 ml. for 3 times. An aqueous fraction was filtered with anhydrous sodium sulphate and evaporated to dryness in vacuo below 50 °C (1.03 g from mother plants and 0.018 g from tissue culture) then the extracts were solubilized in distill water 100 ml. to get 10.25 mg/ml. from mother plants and 0.18 mg/ml from tissue culture of the extract solution as shown from appendix.

Extraction of stem from mother plants and tissue culture

Fresh stems were dried in hot air oven at 50 °C for 48 hr. After that they were percolated by 50% ethanol and distilled in vacuo. The total mass (8.85 g. from mother plants) and (0.194 g. from tissue culture) was refluxed with hexane 200 ml. for 3 times, chloroform 200 ml. for 3 times and n-butanol 200 ml. for 3 times successively. The aqueous were filtrated through Whatman filter-paper No.40 and evaporated to dryness in vacuo below 50 °C (0.65 g from mother plants and 0.015 g from tissue culture) then were solubilized in distill water 100 ml. to get 6.52 mg/ml from mother plants and 0.15 mg/ml from tissue culture of extract solution as shown from appendix.

Identification of crude extract of *Nyctanthes arbor-tristis* L.

Detection of crude extract by Thin layer chromatography (TLC) was carried out on silica gel plates in the solvent systems: (I) benzene – ethyl acetate (4:1) and solvent systems: (II) toluene - ethyl acetate (93:7. The chromatograms were sprayed with H_2SO_4 50% (v/v) and Rf were recorded. After that, the spots were scrapped from TLC plates and qualitative test for sterols (Libermann-Burchard test) and iridoids were performed. Employing a concentrated solution of vanillin was added and followed by a few drop of concentrated HCl test for irridoids. The mixture was heated and the development of pink colour indicated the presence of irridoids. Subsequently Libermann burchard's test was prepared a solution of the sample (2-3mg.) in chloroform (0.5ml.) and was added a few drops of acetic anhydride, followed by one drop of concentrated sulfuric acid. If a substance was steroid, the color would gradually change from pink to deep green. In case of a substance was triterpenoid, the color would change to reddish pink.

Extraction of Carotenoids from corolla tube of *Nyctanthes arbor-tristis* L.

The fresh corolla tube of *Nyctanthes arbor-tristis* L. was ground in a blender for 2 min. The specimens were extracted with acetone for 3 times then centrifuged at 1,000 rpm for 10 min. The mixture was filtered and the residue was successively extracted. The filtrate was concentrated in a rotary evaporator at 35 °C under vaccum until 90% of the solvent was removed. They were transferred to n-hexane-diethyl ether (1:1) by addition of water in separatory funnel and added 1 ml. of sodium chloride to separate two layers. The upper layer was washed with water and dried over anhydrous sodium

sulfate. The extracted solution was evaporated at 35 °C under vaccum and stored by wrapping in aluminium foil and freezing at -20 °C.

Qualitative of carotenoids by Thin Layer Chromatography

A uniform slurry of silica gel G powder was prepared in distilled water, shaked the mixture well in a flask. The mixture was then spreaded on 20x20 cm. cleaned glass plates to a thickness of 0.25 mm. The plates were first air-dried at room temperature. After that activated in an oven at 120 °C for about 2 hr. Extraction of carotenoids and β-carotene standard was spotted on the TLC plates. The plates were placed in a chromatographic tank, lined with filter paper and containing about 100 ml of hexane:diethyl ether: acetic acid (90:10:1). Carotenoid pigments could been detected visually as orange or yellow spot.

Quantitative of carotenoids from *Nyctanthes arbor-tristis* L.

The amount of carotenoids was estimated by dissolving total carotenoids fraction in the known volume of hexane and the absorbance was measured at the wavelength 450 nm. The concentration of total carotenoids were then determined with reference to β-carotene standard as follows:

$$C = A/\varepsilon l$$

Where C = Concentration of total carotenoids in grams per 100 ml.

A = Absorbance at 450 nm

ε = Absorption coefficience of β-carotene at 450 nm. in g/100 ml is 2575

l = Thickness of solution layer in centimeter

Antibacterial acitivity of *Nyctanthes arbor-tristis* L.

- *Escherichia coli* (Gram Negative)

- *Bacillus substilis* (Gram positive)

Antibacterial acitivity in Solid medium (Agar medium)

Lactose agar medium was poured to plate meanwhile penicillin carput were placed on plate until solidified medium. Then penicillin carput were pulled out from agar medium. At that time bacteria solution were spreaded to agar medium. Afterward 0.1 ml. crude extract (leaf extract or stem extract) were dropped in a hole of penicillin carput and incubated at 37 °C for 24 hr. They were diluted 0.15 mg/ml, 0.075 mg/ml, 0.0375 mg/ml (two-fold dilution) for 3 times. Clear zone were measured with diameter around a hole of penicillin carput.

Antibacterial acitivity in liquid medium by Growth inhibition method

Bacteria were incubated in lactose broth media at 37 °C for 24 hr. Subsequently they were tranfered 5 ml with pipette in to new broth media 95 ml. and carefully shaked. They were measured absorbance at 420 nm. Similar of the previous experiment except 85 ml. broth media were used and 10 ml. of crude extract 0.15 mg/ml were added. Then they were incubated in shaker bath at 37 °C and solutions were divided every 20 min for measuring absorbance at 420 nm. for 260 min. Finally, absorbance at 420 nm. were plotted with time for incubation.

Antifungus of *Nyctanthes arbor-tristis* L.

- Fungus : *Aspergillus sp.*

Antifungus in solid medium

White agar medium was prepared (in appendix) to plate while penicillin carput were set on plate to wait for1 hr. for solidify medium. Then penicillin carput were pulled out from agar medium. At that time bacterial solution were spreaded to agar medium. Then 0.1 ml. crude extract (leaf extract or stem extract) were dropped in a hole of penicillin carput and distilled water were drop as a control. They were incubated at 28 °C for 1 week. The extracts were diluted 0.15 mg/ml, 0.075 mg/ml, 0.0375 mg/ml (two-fold dilution) for 3 times. After incubation, the inhibition zone were measured with diameter around a hole of penicillin carput.

Antifungus in liquid medium by Growth inhibition method

Fungi were incubated in white broth medium at 28 °C for 1 week. After that they were pipetted 5 ml to new broth media 95 ml. and shaked gently for 15 min. Next they were measured absorbance at 530 nm. Similar of previous experiment except 85 ml. broth media were used and 10 ml. of crude extract 0.15 mg/ml were added. They were incubated in shaker bath at 28°C and solutions were divided every 24 hr. for measuring absorbance at 530 nm. for 13 days.

Antiyeast of *Nyctanthes arbor-tristis* L.

- Yeast : *Saccharomyces cereviseae*

Antiyeast in solid medium

White agar medium was poured to plate which penicillin carput were put on plate and waited for solidify medium. Then penicillin carput were pulled out from agar medium. Subsequently bacterial solution were spreaded to agar medium. 0.1 ml. crude extract (leaf extract or stem extract) were dropped in a hole of penicillin carput and distill water were dropped too as a control. They were incubated at 28 °C for 48 hr. The extracts were diluted 0.15 mg/ml, 0.075 mg/ml, 0.0375 mg/ml (two-fold dilution) for 3 times. After incubation, clear zone were measured with diameter around a hole of penicillin carput.

Antiyeast in Liquid medium by Growth inhibition method

Yeast were incubated in White broth media at 28 °C for 48 hr. After that they were pipetted 5 ml. to new broth media 95 ml., carefully shaked and then they were measured absorbance at 530 nm. Similar previous experiment except 85 ml. broth media were used and 10 ml. of crude extract 0.15 mg/ml were added. After that they were incubated in shaker bath at 28°C and solutions were divided every 3 hr. for measuring absorbance at 530 nm for 39 hr. Finally, absorbance at 530 nm. were plotted with time for incubation.