CHAPTER I

Introduction

Nyctanthes arbor-tristis L. (Fig1.1) was commonly known as Night Blooming Jasmine because it was fragrant at night. A large shrub or small tree, up to 8 m. high, native of India and it was widely cultivated through out Thailand. The stems and branches were angular. Leaves were simple, opposite, 2.5-5 cm. wide and 5-10 cm. long., with surface scabrous. Inflorescence panicle, axillary, the flower jasmine-liked, with 5 white corolla tube. Fruit was flat with 2 seeds (Department of Pharmacy, Mahidol University, 2535). The plant was used as traditional medicine for the treatment of chronic fever and rheumatism. It was also used as liver tonic and neurotonic and exhibit antiviral, antifungal, and antibacterial activity (Saxena, et al, 1984). Plant tissue culture techniques were widely employed to obtain a stable supply of bioactive secondary products. Tissue culture of ligneous species is difficult. There were no reports on *in vitro* propagation of *Nyctanthes arbor-tristis* L. in Thailand.

Figure1.1 Stems and leaves of *Nyctanthes arbor-tristis* L.

Figure1.2 Flowers of *Nyctanthes arbor-tristis* L.

Plant tissue culture for regeneration (Micropropagation)

Plant tissue cultures were useful technique for mass propagation of the plant for commercial. The techniques of tissue culture were use widely for development of agriculture and other. Because of plant tissue culture will make pure line and a lot of plants in short time. The methods of micropropagation had more advantages than traditional methods in several aspects (Bajaj 1988):

- Cultures were initiated with a very small piece of plants (explants), and only a small amount of space was required to maintain a large number of plants.

- Propagation was carried out in aseptic conditions, free from pathogen.

- Plant production could be continued throughout the year and independent on seasonal changes.

- Vegetative reproduced materials could often be stored over a long period.

- No need for laborious attention between subculturing.

Micropropagation techniques have been developed and widely used in many areas. Five main stages for *in vitro* multiplication of plants has been defined (Murashige, 1974). These stages have been used widely in many commercial and institutional tissue culture laboratories. The main five steps for micropropagation were explained below:

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Stage 1: Selection and preparation of explants

Plant, a representative typical of each variety and disease free, would be selected as a mother plant for *in vitro* culture. Growth, morphogenesis and rates of *in vitro* propagation could be improved by appropriate environment and chemical pretreatment of mother plant.

Stage 2: Development of aseptic culture

The second step in the micropropagation process was to obtain an aseptic culture of the selected plant material. The explants should be aseptically transferred to the culture environment and completed a number of survived explants without contamination.

Stage 3: The production of suitable propagates

The object of this step was to bring about the multiplication of organs and structures that were able to give rise to new intact plants. This step included the prior induction of meristematic centres from which adventitious organs might develop.

Stage 4: Preparation for growth in the natural environment

Shoots or plantlets derived from stage 3 were very small and not yet capable of self-supporting for growth in soil or compost. This step was taken to grow individual plantlets that could carried out photosynthesis and survival without an artificial supply of carbohydrate. This step includes the *in vitro* rooting of shoots prior to their transfer to soil. In some species, this step was necessary to have elongated shoots ready for rooting.

Stage 5: Transfer to the natural environment

This step was to transfer plantlets from the *in vitro* growth to the extra external environment carefully. This step was very important because improper transferring methods can result in a significant loss of new plants.

Some plants in which tissue culture has been used successfully for regeneration were listed in Table 1.

Table 1.1 *In vitro* regeneration studies on some plants

Plant species	Source of explants	Morphogenesis propagation	References
Allium cepa L.	meristem segments	plantlets regenerated	Hussey 1978
Arabidopsis thaliana	immature cotyledons	shoots formation	Ratton and Meinke 1988
Capsicum annuum L.	mature embryos	plantlets regenerated	Agrawal and Chandra 1983
Centaurea junoniana	cotyledons, leaves	shoot organogenesis	Hammatt and Evans 1985
Cichorium intybus L.	mature tap roots	plantlets regenerated	Heirwegh et al 1985
Coffea arabica L.	shoot tips seedling	plantlets regenerated	Kartha et al 1981
Cucumis melo L.	cotyledons	shoots, roots organogenesis	Chee et al 1991
Digitalis thapsi L.	shoot tips	shoot multiplication, roots	Hen-era et al 1990
Gossypium arboreum L.	immature embryos	plantlets regenerated	Gill and Bajaj 1984
Helianthus annuus L.	immature embryos	shoots, roots embryogenesis	McCann et al 1988
Lotononis bainesii	cotyledons and leaf	shoots organogenesis	Bovo et al 1986
Medicago spp.	roots, hypocotyls	embryogenesis	Nagarajan et al 1986
Mentha arvensis L.	nodal segments	plantlets regenerated	Rech and Pires 1986
Papaver bracteatum	seeds	plantlets regenerated	Day et al 1986
Paspalum notatum	immature embryos	plantlets regenerated	Bovo and Mroginski 1989
Phytolacca dodecandra	shoot tip and nodal	plantlets regenerated	Demeke and Hughes 1990
Pinus ponderosa	mature embryos	buds regenerated	Bis and Bilderbach 1984
Trifolium medium L	petiole segments	plantlets regenerated	Choo1988
Triofium pratense L.	hypocotyl and petiole	plantlets regenerated	Maclean and Nowak 1989
Zea mays L.	immature embryos	embryogenesis	Songstad et al 1988
Zingiber officinale Roscoe.	rhizome	embryoids andplantlets	Hosoki and Sakawa 1977

Tissue culture of woody plants

Hansen and Lazarte (1984) cultured lateral bud and internode of *Carya illinoinensis* (Wang.) K. Koch in medium of WPM (Wood plant medium: Lioyd and McCown, 1981) with BA 3 mg/l could regenerate for 10 shoots. Tissue were induced for 93 % of root when added IBA 1mg/l in WPM for 10 days.

Lakshmi (1986) cultured lateral bud of *Eucalyptus grandis* in MS medium with BAP 1 mg/l and NAA 1 mg/l that were shown to induce for 30-50 shoots and were induced for 70% of root in white medium with IAA 0.5 mg/l.

Blomstedt et al. (1991) cultured lateral bud from seed of *Eucalyptus regnans* in MS medium with NAA 0.05 mg/l were shown to induce for shoots and for roots in (woody plant medium) WPM medium with IBA 20 mg/l after 7 day. Plantlets were transferred to medium with activated charcoal.

Shim, Ha and Lee (1992) cultured lateral bud of *Betula pendula* in WPM medium with BA 0.5 mg/l and NAA 0.01 mg/l or WPM medium with BA 0.5 mg/l and IBA 0.01 mg/l were shown to induce for 10.28, 9.8 shoots respectively and for root in GD (Gresshoff and Doy's medium) or ½ GD with IBA 0.2 mg/l.

Corchete, Diez and Valle (1993) cultured lateral bud of *Ulmus pumila* L. in MS medium with BA 0.5 mg/l were induced 5 shoots. Shoots were shown to induce roots in MS medium with NAA 0.1 mg/l or ½ MS with NAA 0.1 mg/l.

Deora and Shekhawat (1995) cultured lateral bud of *Capparis decidua* (Forsk) Edgew in MS medium with IAA 0.1 mg/l and BAP 1 mg/l were shown to induce 4-7 shoots. Shoots were induced roots in ½ MS medium with IBA 100 mg/l at night condition for 4 hours. and transferred to MS medium with activated charcoal 500 mg/l.

Sudha and Seeni (1996) cultured shoot tip and lateral bud of *Rauwolfia micrantha* in MS medium with BA 13.2 μM and NAA 2.68 μM were shown to induce 3 shoots. Shoots were induced roots in MS medium with NAA 2.6 μM.

Ajithakumar and Seeni (1998) cultured lateral bud of *Aegle marmelos* L. Corr. in MS medium with BAP 2.5 mg/l and IAA 1 mg/l were shown to induce shoots. Shoots were induced roots in ½ MS with IAA 0.5 mg/l or ½ MS with IBA 10 mg/l.

Indira et al, (1998) studied excised cotyledons, hypocotyls, roots, leaves and bases of internodes of plantlets of *Nyctanthes arbor-tristis* L. callused readily on culture. Plantlets were raised *in vitro* from isolated immature embryos. Calli from cotyledons, hypocotyls and roots exhibited faster growth compared with those from leaves and internodal bases when cultured in MS medium with 2,4-D, NAA and coconut milk. Growth of the calli was not found to decline on repeated subculture after twelve months.

Plant tissue cultures offered a number of advantages over intact plant for studies of metabolism. They were relatively easy to establish and maintain under strictly controlled nutritional and environmental conditions. In the case

of secondary matabolism, the culture of many species did not produce significant amount of the compound characteristic of intact plants. This might sometimes be due to the loss of genetic information during prolong culture, but in many cases it has been shown that long-term cultures remain totipotent (Chaleff, 1983). It was more likely that the failure of culture cell to produce the pattern or level of specific secondary metabolites from the source of plant was a consequence of the specific physiological state of cultured tissue.

Chemical composition of *Nyctanthes arbor-tristis* L.

Chauhan and Saraswat, (1978) studied phytochemical examination of the stem of *Nyctanthes arbor-tristis* L. that resulted in the isolation and identification of β-sitosterol and new glycoside (naringenin-4-o-β-glucopyranosyl- α-xylopyranoside.

Anjaneyula, et al, (1981) investigated leaves of *Nyctanthes arbor-tristis* L. reveals the presence of the friedelin, lupeol, and oleanolic acid in addition to nyctanthic acid, the only triterpene reported by earlier work. Further, the co-occurrence of oleanolic acid with the corresponding 3-4 seco acid, nycthanthic acid is of biogenetic significance.

Vandita et al, (1990) isolated three new irridoid glycoside, arborside A, B, and C from the leaves of *Nyctanthes arbor-tristis* L. The absolute structures were determined by spectral data (UV, IR, MS, NMR) and chemical correlation of the products of alkaline hydrolysis of arborside A tetraacetate, arborside B tetraacetate (on subsequence methylation and acetylation), and arborside C with known irridoids 6β-hydroxyloganin hexaacetate, loganin pentaacetate, and 6β-hydroxyloganin, respecticely.

Stuppner et al, (1993) isolated two new iridoid glycosides, 6,7 –di-O-benzoylnyctanthoside and 6-o-trans-cinnamoyl-6β-hydroxyloganin along with the previously reported iridoid 7-o-trans-cinnamoyl-6β-hydroxyloganin from leaves of *Nyctanthes arbor-tristis* L. by using UV, mass spectroscopy and NMR spectroscopy.

Kiran et al, (1993) reported re-examination of leaves of *Nyctanthes arbor-tristis* L. that led to the isolation and identification of a new minor iridoid glycosides, arborside D, as its acetyl derivative. The structure of the new compound was determined using spectral methods.

Chemical Constituents of *Nyctanthes arbor-tristis* L.

Iridoid glycosides: The Chemistry and Formation

The iridoids actually existed in plants were apparently in several forms. Iridoids, a group of glycoside and chemical structures were cyclopentanepyran ring system (aglycone). The sugar (glycone) may be attached in various positions with system.

Distribution of Iridoids Glycoside Derivative in *N. arbor-tristis* L.

Irridoids were group of natural glycoside. They were widely distributed in various plant as shown in Table 1.2 and some animal such as in Australian meat ant (irridodial, iridomymecin and iridolactone). The distribution of iridoids were mostly found in dicotyledon plants; in leaf, seed, bark and root.

Table 1.2 Distribution of iridoids in some families of plants.

Family	Botanical name	Isolated compound
Apocynaceae	*Cerbera manghas* L.	theviridoside, theveside
	Orthantha lutea	aucubin, catalpol, odontoside
	Thevetia neriifolia	theveside
Bignoniaceae	*Tecomella undulata*	6-0-veratryl catalposide
Caprifoliaceae	*Lonicera morrowii*	morroniside, kingiside sweroside loniceroside
Cornaceae	*Cornus nuttallii*	cornin, dihydrocornin
	Cornus florida	
	Cornus officinalis	morroniside
Ericaceae	*Vaccinium bracteatum*	vaccinoside monotropein orientin, isoorientin
Gentianaceae	*Gentiana spp.*	gentiopicroside
	Swertia spp.	sweroside, amaroswerin
	Gentiana triflora	gentiopicroside
	Gentiana thunbergii	morroniside
	Gentiana pannonica	deoxyamarogentin
	Gentiana purpurea	deoxyamarogentin
	Gentiana scabra	gentiopicroside

Table 1.2 Distribution of iridoids in some families of plants (cont.).

Lamiaceae	*Leonurus cardiaca*	leonuride
	Lagochilus inebrians	harpagide
	Betonica spp.	acetate
	Stachys spp.	harpagide
	Galeopsis tetrahit	gluroside
	Melittis melissophyllum	monomelitotside
Strychnaceae	*Anthocleista zambesiaca*	sweroside
	Strychnos nux-vomica L.	loganin, secologanin
Plantaginaceae	*Plantago lanceolata*	acubin
Rubiaceae	*Genipa americana*	geniposidic acid
	Ixora chinensis	ixoroside, ixoside
	Gardenia jasminoides Ellis	gardenoside, geniposide
Scrophulariaceae	*Odontites serotina*	odontoside
	Rhinanthus spp.	agnuside
	Scrophularia spp.	acetyl harpagide
	Cymbalaria muralis	antirrhinoside, linarioside
	Melampyrum silvaticum	melampuroside
	Veronica spp.	caffeoyl catalpol, isoferuloyl catalpol, protocatechuoyl catalpol, p-hydroxybenzoyl catalpol, vanilloyl catalpol, cinnamoyl
Valerianaceae	*Valeriana stolonifera*	irridoid
	Patrinia villosa	villoside, loganin
Verbenaceae	*Stilbe phylicoides*	unedoside
	Vitex megapotamica	agnuside
	Nyctanthes arbor-tristis L.	nyctanthoside

Carotenoids:

Of the various classes of pigments in nature, the carotenoid was among the most widespread and important ones, especially due to their most various functions. Noga and Lenz (1983) reported a large number of carotenoids. This marks the beginning of carotenoid research and since then continuous developments had taken place. The industrial production of carotenoids had also contributed to the knowledge in this field.

Carotenoids were group of hydrocarbons and their oxygenated derivatives (xanthophylls). They consist of eight isoprenoid units joined in such a manner that the arrangement of isoprenoid units. All carotenoids formally derived from the acyclic $C_{40}H_{56}$ structure. Chemical structure of carotenoids classified into two groups.

- Carotene-hydrocarbon such as β-carotene, α-carotene and γ-carotene

- Xanthophylls-oxygenated hydrocarbons such as cryptoxanthin, lutein and astaxanthin (Simpson et al, 1985; Latscha, 1990).

Carotenoids were found throughout the plant kingdom, animal, and marine algae. They were responsible for the beautiful colors of many fruits (pineapple, citrus fruit, tomatoes, paprika) and flowers (*Narcissus*). They protected photosynthesis organism against photodynamic destruction, acted as auxiliary light absorbers for photosynthetic and direct phototaxis, and served as provitamin A for animals (Liaaen-Jensen, 1990). Commercial synthetic carotenoids are mainly used as pigments for food (egg yolk, chickens, or farm-raised salmon) and for coloration of food products (margarine, cheese). The β-

carotene was widely used for coloring soft-drink, friuit juices, breads, cream-cakes, fishes products, ice-cream, etc. as well as a nutritional supplement (Xihai, 1990). Aravantinos-Zafiris et. al (1991) studied carotenoids were extracted from fresh orange peel with various solvents. Acetone was the most efficient of the solvent tested. Red bell peppers were good sources of carotenoids, and paprika extracts are commercially used as coloring and flavoring agents for food (Curl, 1962). Gayle et. al (1987) studied the carotenoids of redbell peppers which were analyzed without saponification by HPLC using octadecyl silica as stationary and methanol-ethyl acetate as mobile phase. The red bell peppers contained 280 μg/g total carotenoids.

Study of antimicrobial activity in *Nyctanthes arbor-tristis* L.

Nyctanthes arbor-tristis L. was extensively used as a decoction of leaves by Ayurvedic physicians for the treatment of arthritis, obstinate sciatica, malaria, intestinal worms and as a tonic, cholagogue and laxative (Kirtikar et al, 1935).

Saxena et.al (1984) studied the water soluble portion of the alcoholic leaves extract of *Nyctanthes arbor-tristis* L. (NAT) that was screened for the presence of anti-inflammatory activity. NAT inhibited the acute in flammatory oedema produced by different phlogistic agents, viz, carragreenin, formalin, histamine, 5-hydroxytryptamine and hyaluronidase in the hindpaw of rats. The acute inflammatory swelling in the knee join of rats induced by turpentine oil was also significantly reduced. In subacute models, NAT was found to check granulation tissue formation significantly in the granuloma pouch and cotton pellet test. Acute and chronic phases of formaldehyde induced arthritis were

significantly inhibited. NAT was also found to inhibit the inflammation produced by immunological method, viz. Freund's adjuvant arthritis. Thus anti-inflammatory activity in leaves of *Nyctanthes arbor-tristis* L. supported its use in various inflammatory conditions by the followers of the Ayurvedic system of medicine.

Saxena et al, (1987) gained knowledge of the leaves of *Nyctanthes arbor-tristis* L., which was used in the treatment of sciatica and arthritis. They were advocated for various kinds of fevers and painful conditions by the Ayurvedic physicians. In the present studied, the water-soluble portion of an ethanol extract of the leaves was screened for analgesic, antipyretic and ulcerogenic activity. The extract exhibited significant aspirin-like antinociceptive activity but failed to produce morphine-like analgesia. It was also found to possess antipyretic activity against brewer's yeast-induced pyrexia in rats. The extract also produced gastric ulcers following oral administration for six consecutive days in rats. Results of the present study tend to substantiate the use of this plant in fevers and painful conditions by Ayurvedic physicians.

Rai et al, (1988) screened medicinal plants of Chindwara District against *Trichophyton mentagrophytes*. Out of 19 plants/plant parts, only 7 plants, viz.. leaves of *Parthenium hysterophorus* (92.52%), followed by leaves of *Nerium indicum* (91.9%), stem bark of *Azadirachta indica* (88.18%), stem bark of *Madhuca indica* (82.36%), whole plant of *Asparagus racemosus* (81.47%), stem of *Catharanthus roseus* (77.72%), leaves of *Tridax procumbens* (76.65%) and leaves of *A. indica* (76.47%) showed comparatively

higher percentage of growth inhibition than those tested *in vitro*. The middle percentage of growth inhibition was observed in the *Nyctanthes arbor-tristis* L. (35.62%), and lowest percentage in *Tridax procumbens* (2.88%).

Talakal, et al (1995) investigated aqueous extracts of 9 indigenous plant materials which were screened *in vitro* for their activity against *Trypanosoma evansi* at concentration of 5, 50, 500, and 1,000 µg/ml. The extracts of leaves of *Achyranthus aspera*, *Caesalpinia bonducella* and *Datura alba* did not show activity at any tested concentration. The extracts of other plants, viz.. *Azadirachta indica* leaves, *Cassia occidentalis* leaves, *Cyperus rotundus* rhizome, *Hydrocotyle asiatica* leaves and *Streblus asper* leaves, exhibited moderate trypanocidal activity at different tested concentrations. However, the extract of *Nyctanthes arbor-tristis* L. at a concentration of 1,000 µg/l was highly effective.

Khan, et al (1995) reported prophylactic treatment with the ethanol (50%) extracts of the seed and root of the *Nyctanthes arbortristis* L. (p.o. once daily) provided significant ($p < 0.05$) protection of Swiss mice against *Candida albicans* systemic infection. Arbortristoside A and C (iridoid glucosides isolated from seed) at 5 mg/kg (p.o. once daily) enhanced protection. Arbortristoside C was, however, more protective (77.7%) and curative than arbortristoside A based upon reduced colony forming unit (CFU) of *Candida albicans* from kidney homogenates of treated mice compared to control. The prophylactic (-7 to -1 day) and therapeutic (+1 to +7 days post-infection) regimens (5 mg/kg) of arbortristoside A enhanced protection (71.4%), whereas, arbortristoside C was detrimental to mice. The protective

effect of these extracts or fractions may possibly be because of the strong stimulatory activity of arbortristoside A and C elicited by significant ($p < 0.001$) increase in humeral and delayed type hypersensitivity (DTH) response to sheep red blood cells (SRBC) and macrophage migration index (MMI). The combined therapy of arbortristoside A or C with ketoconazole (KTZ) did not improve protection of mice. To our knowledge this is the first report of immunomodulatory activity of *N. arbor-tristis* L. against systemic candidiasis in mice.

Objectives

This study aims at obtaining rapidly growing shoot, increasing the yield from explants of *Nyctanthes arbortristis* L. and testing the antimicrobial activity of leaf and stem extracts from mother plants and plantlets *in vitro*.