CHAPTER 1

INTRODUCTION

1.1 Statement and significance of the problem

Plants are important sources of lead compounds for research and development of new drugs. Numbers of substances from plants can be used as alternatives for the treatment of several life threatening diseases especially for cancer and HIV, for example paclitaxel (Taxol®) for the treatment of cancer and calanolides, coumarin derivatives from *Calophyllum lanigerum*, which possess anti-HIV activity (Kashman *et al.*, 1992). These successes have spurred an effort in many areas of biological and therapeutic interest to continue the discovery of novel natural products with a higher level of activity or reduced toxicity (Grzybek *et al.*, 1997). In the plant kingdom, only few numbers of plants were investigated. The rest which is a large number of plants wait for further studies with high potential to be used as therapeutic agent.

Phytochemical study of South-East Asian plants as a source of bioactive natural products led to the isolation and structural elucidation of novel compounds. Compounds from various parts of the world have been screened and exhibited significant activities. The Guttiferae, mainly found in tropical and northern temperate regions, is well known to be rich in secondary metabolites such as xanthonoid, biflavonoid and triterpenoid (Xu *et al.*, 1998). Some have been used as traditional medicine. Novel bioactive compounds from these plants with cytotoxic activity have been reported (Cao *et al.*, 1998; Kosela *et al.*, 1999).

Plants in the Schisandraceae family grow wild mainly in China, Japan, the

Himalayas and Jawa. Over 19 species are wildly use in Chinese traditional medicine. Much attention has been focused on the family Schisandraceae because the lignans isolated from this family show various biological activities. In recent years, several species have been reported to contain triterpenoids. Some triterpenoids showed anti-HIV, hepatotoxicity and antioxidant activities (Hancke *et al.*, 1999; Li *et al.*, 2003).

In this study, some Thai Guttiferae and Schisandraceae plants were selected for the extraction, purification, elucidation and investigation of their biological activities which may be further developed to pharmaceutical products.

1.2 Objectives

The objectives of this study are:

1) To screen the bioactivities of crude extracts from selected Guttiferae and Schisandraceae plants.
2) To purify and elucidate the structure of the isolated compounds.
3) To determine bioactivities of the isolated compounds

1.3 Scope of study

1) Sample selection

Plant species were collected focusing on the plants of the family Guttiferae and Schisandraceae showing the evidence of cytotoxicity and antioxidant activity. The plants are from Chiang Mai Province, Thailand.

2) Preparation of the crude extracts

The selected Guttiferae and Schisandraceae plants were extracted and identified for the bioactive compounds using proper organic solvents of different polarity and chromatographic techniques.

3) Bioactivities screening of the crude extracts

The crude extracts were screened for their bioactivities for free radical scavenging activity by DPPH assay, cytotoxicity in tumor cell lines using SRB assay.

4) Isolation and purification of the bioactive compounds from the crude extracts

Column chromatography, TLC and Preparative TLC were used for this process.

5) Structure elucidation of the compounds.

The isolated compounds were elucidated for their structures by appropriate techniques, for examples, NMR, COSY, HSQC, HMBC and HRMS.

6) Bioactivity studies of the isolated compounds.

The isolated compounds were evaluated for the free radical scavenging activity by DPPH assay, tumor cell cytotoxicity assay using SRB and human lymphocyte proliferation using a modified MTT assay.

1.4 Literature review

1.4.1 Plants

1.4.1.1 Guttiferae

The Guttiferae, also known as the Clusiaceae, comprises 1,350 species in 47 Genera. They are trees, shrubs and herbs with yellow or otherwise brightly colored resinous juice. A number of useful timbers, drugs, dyes, gum, pigments and resins are derived from members of the family (Watson & Dallwitz, 1992).

1.4.1.1.1 Hypericum

Hypericum is a large genus comprising 400 species, wide spread on temperate region and tropical mountains of the world. Some taxonomists classify this genus in a separate family, the Hypericaceae. Numerous articles are available on the chemistry of this genus, in part because of their use in traditional systems of medicine. Common constituents are xanthones, phloroglucinols and flavonoids. The recent widespread interest in the antidepressant activity of *H. perforatum* (St. John's Wort) has encouraged the investigation of secondary metabolites from Hypericum species, many of which are biologically active compounds with an acylphloroglucinol moiety.

H. ascyron, a Chinese herbal medicine, is used in the treatment of numerous disorders such as abscesses, boil, headache, nausea, and stomachache. Isolation and structure elucidation of aerial parts of *H. ascyron* gave eight xanthones, including a new 3, 6-dihydroxy-1,7-dimethoxyxanthone, together with friedelin (Hu *et al*., 1999).

H. annulatum, a species endemic to the southern part of the Balkan Peninsula, contains hypertomarin, a prenylated phloroglucinol compound, occurring in two tautomeric form exhibiting antibacterial activity (Fodulovic *et al*., 2003). Two new benzophenone O-glucoside, annulatophenone and hypericophenoside, which were the

second benzophenone O-glucoside found in nature, were isolated from the methanol extract. Moreover, two benzophenone O-arabinosides, annulatophenonoside and acetylannulatophenone, were also isolated from the methanol extract, and a chromone, 5-, 7- dihydroxy-3-methychromone was isolated from the chloroform extract of this herb (Kitanov & Nedialkov, 2001; Nedialkov & Kitanov, 2002).

H. androsaenum grows wild in shadowy sites in the northern region of Portugal. The leaves are used in folk medicine to prepare teas with diuretic and antihepatotoxic activities. It's pharmacological properties have been attributed to phenolic compounds which have scavenging activity against superoxide radical, hydroxyl radical and hypochlorous acid (Patricia *et al.*, 2002). The major phenolics produced by *in vitro* culture of *H. androsaenum* are xanthone and xanthone C-glycosides (Dias *et al.*, 2000).

H. brasiliense is a species growing in south and south-east Brazil. Four new phloriglucinols, hyperbrasilol A-C and isohyperbrasilol B which exhibited the strong antibacterial activity have been isolated from a chloroform extract of leaves and flowers while a new γ-pyrone, hyperbrasilone and xanthones which showed antifungal activity and found to be inhibitors of monoamine oxidases (MOA) have been isolated from a dichloromethane extract of stems and roots (Rocha *et al.*, 1996). *H. caprifoliatum*, another species from South Brazil, was evaluated for the antidepressant activity according to the forced swimming test, a classical animal model for antidepressant drug screening. The chemical analyses showed it to be rich in phenolic compounds, mainly of the phroroglucinol type (Daudt *et al.*, 2000).

The aerial parts of *H. calycinum* furnished a new phloroglucinol derivative, which was fungicidal against *Cladosporium cucumerinum* in a TLC bioassay and was

also found to exert an interesting antimalarial activity in an *in vitro* test system, and new cell growth inhibitory cyclohexadienone derivatives, hypercalin A and B which inhibited significantly the growth of Co-115 colon carcinoma cell line (Decosterd *et al.*, 1991). Novel spiro compounds, hyperolactones A-D together with the antiviral acylphloroglucinols, chinesin I and II were isolated from stems and leaves of *H. chinense* (Aramaki *et al.*, 1995).

Antimicrobial and cytotoxic activity of rottlerin-type compounds, drummodins A, B, C and F were isolated from hexane extract of the roots of *H. drummondii* while the hexane extract of the stems and leaves have led to the isolation of four additional new antimicrobial filicinic acid derivatives, drummodin D, iso drummodin D, drummodin E and F (Jayasuriya *et al.*, 1991).

H. erectum is a herb in Chinese medicine as antihemorrhagic agent, astringent and antibiotic agent, which has been reported to contain some antiviral prenylated phloroglucinol derivatives and two anti-hemorrhagic compounds, otogirin and otogirone (Tada *et al.*, 1991). Three new polyprenylated phloroglucinol derivatives, erectquinone A-C also have been reported from the whole plant (An *et al.*, 2002).

Three quercetin glycosides, quercetin 3-glucuronide-3'-sulphate and a new flavonol sulphate, quercetin 3'-sulphate were isolated from Portugal plant, *H. elodes* (Seabra & Alves, 1991).

The aerial part of *H. empetrifolium* that was collected from different locations in Greece were tested on brine shrimps, human colon carcinoma (Caco-2) and human hepatoma cell lines (HepG2) for cytotoxic activities. The results showed that methanol extract of *H. empetrifolium* exhibited high activities on cell lines with LC50

value ranging from 25 to 46 mg/ml and moderate activities on brine shrimps, ranging from 22 to 150 mg/ml (Couladis *et al.*, 2002).

H. geminiflorum, an endemic plant in Taiwan, is a Chinese folk medicine used for the treatment of several bacterial disease, infectious hepatitis, gastrointestinal disorder and tumor. Five new phenolic constituents were isolated from the heartwood and root. A novel compound containing an oxepane ring, hypertricone, two new pentaoxygenerated xanthones and the constituents which showed antiplatelet and anti-inflammatory activities were isolated from the leaves (Chung *et al.*, 2002).

H. japonicum has been used in Chinese herbal medicine for the treatment of some bacterial disease, infectious hepatitis and tumor. Seven antimicrobially active phloroglucinols, two new phloroglucinol derivatives, sarothralen C and D, a lactone compound, flavonoids and 2-pyrone were isolated from methanol extracts and new bisxanthones, jacarelhyperols A and B which showed significant inhibitory effects against PAF-induced hypotension by an *in vivo* evaluation method were isolated from the chloroform soluble part of the methanol extract (Ishiguro *et al.*, 2002). Wu *et al.* (1998) also reported the isolation of a fatty acid, eleven flavonoids, two chromene glycosides and six xanthones, two of which showed positive activity in a coagulant test in vitro.

H. patulum has been used in Chinese herbal medicine for treatment of hepatitis, bacterial diseases and nasal haemorrhage. The *in vitro* cytotoxicity and antitumor properties of the extracts against the malignant rhabdomyosarcoma (RD) cells, Caucasian male larynx epithelium carcinoma (Hep-2) cells, Dolton's lymphoma ascites (DLA) cells and normal African green monkey (Vero) cell have been studied (Vijayan *et al.*, 2003). The isolation and structure of a new phloroglucinol derivative,

Paglucinol, 13 prenylated xanthones and epicatechin from cell suspension have been reported by Ishiguro *et al.* (1998).

Studies of the plants that are employed in the traditional medicine of Papua New Guinea, *H. papuanum* which leave and woody herb are used for the treatment of sores, five new prenylated tricyclic, papuaforin A-E and three new acylphloroglucinol derivatives, hyperguinones A and B and hyperpapuanone have been isolated. Furthermore, the cytotoxicity toward KB nasopharyngeal carcinoma cells and antibacterial activity of the isolated compound were determined (Winkelmann *et al.*, 2000; Winkelmann *et al.*, 2001).

H. perforatum is used for the treatment of mild to moderate forms of depression. The antidepressant efficacy of the extracts has been confirmed in several clinical studies. Reports about the mechanism of antidepressant action of extract and their constituent both in vivo and in vitro have also been published. Antidepressant activity was reported for the phloroglucinol derivative hyperforin, the naphthodianthrones hypericin and pseudohypericin and for several flovonoids (Simbrey *et al.*, 2004). Current lead natural products for the chemotherapy of HIV infection, hypericin and pseudohypericin could block HIV-1 infection through a variety of mechanisms (direct virucidal effect, inhibition of secondary virus spread, inhibition of virus budding, and inactivation of the preintegration complex) (Clercq, 2000).

H. polyanthenum is another species from south Brazil. Three new benzopyrans were isolated from the aerial part (Ferraz *et al.*, 2001). *H. roeperanum*, a small tree growing in tropical Africa used to cure female sterility in folklore. Four new antifungal xanthones were isolated from the root (Rath *et al.*, 1996). The aerial

parts of *H. reflexum* contain xanthones, pyranoxanthones, xanthonolignoid a biphenyl and two new spiropenoids hyperireflexosides A and B (Cardona *et al.*, 1990).

H. sampsonii is a Chinese herbal medicinal used in the treatment of numerous disorders such as backache, burns, diarrhoea, snakebites, swelling and also has been used to decrease blood stasis, relieve swelling and as a detoxifying agent, especially as one of the antihepatitis and antihepatoma herbs in Taiwan. Xanthones and polyprenylated benzophenone derivatives, sampsoniones A-M and six new polyprenylated derivatives, Hypersampsone A-F, have been isolated from the aerial part (Hu & Sim, 2000; Lin & Wu, 2003).

H. scabrum is one of the most popular medicinal herbs in Uzbekisan and used in the treatment of numerous disorders such as liver, gall bladder, intestinal and heart disease, rheumatism and cystitis. Nine new polynylated benzoylphloroglucinol derivatives, hyperibones A-I have been reported. Some showed antibacterial activity against *S. aureus* (Matsuhisa *et al.*, 2002).

H. triquetrifolium is native to Eastern Europe and the Mediterranean area. It has been traditionally used for its sedative, antihelminthic, anti-inflammatory, antiseptic effect, treatment of burns and gastrointestinal disease. The methanolic extract of the aerial part possesses significant antioxidant activity. This activity is related to the presence of flavonoids and particularly to the presence of I3, II 8-biapigenine (Conforti *et al.*, 2002). Furthermore, *H. triquetrifolium* showed the high activity on the brine shrimps lethality test (Couladis *et al.*, 2003).

1.4.1.1.2 Calophyllum

The genus Calophyllum which comprises 200 species is widely distributed in the tropical rain forest where several species are used in folk medicine. This genus is well known as a source of biologically active compounds such as coumarins, xanthones and triperpenoids. Xanthone are prominent constituents of the wood of Calophyllum species. Recent interest has been focused on several coumarin derivatives which are reported to inhibit *in vitro* replication and cytopathicity of HIV-1. The Phytochemical study of *C. dispar*, six coumarins have been isolated from fruits and stem bark. Some of these coumarins exhibited a significant cytotoxicity against KB cells (Guilet *et al.*, 2001).

The two new coumarins, teysmanones A and B were isolated from the bark of Malaysian plant, *C. teysmanni var. inophylloide* (Coa *et al.*, 1998). The study on the wood of *C. teysmanni var. inophylloide* from Thailand revealed the novel xanthones which have been tested for immunomodulator activity and some showed significantly suppression of mitogenic response of lymphocyte to phytohaemagglutinin (Gonzalez *et al.*, 1999; Kijjoa *et al.*, 2000).

Investigation of the heart wood of *C. inophyllum* from Malaysia showed the presence of a new xanthone, 2-(3-hydroxy-3-methylbutyl)-1,3,5,6-tetrahydroxy xanthone and triterpenoids (Kumar *et al.*, 1976; Goh & Jantan, 1991). Current lead natural products for the chemotherapy of HIV infection, calanolides from the tropical rainforest tree, *C. lanigerum* and inophyllum from the Malasian tree, *C. inophyllum* have been reported to interact with the reverse transcriptase (Clercq, 2000).

1.4.1.1.3 Cratoxylum

Cratoxylum, a small genus belonging to the Guttiferae family, is found mainly in Southeast Asia and has been used in traditional medicine. The occurrences of flavonoids, triterpenoids and xanthones have been reported.

Xanthones were isolated from the wood of *C. maingayi* from Thailand (Kijjoa *et al.*, 1998). Isolation and structure elucidation of the root of *C. formosanum* reveals two new xanthone, 2,7-dihydroxy-1,8-dimethoxyxanthone and 1,4,7,-trihydroxy-8-methoxyxanthone (Iinuma *et al.*, 1996).

The leaves and stem of *C. formosum* ssp. *pruniflorum* contained quercetin, hyperoside, 1,3,6,7-tetra- hydroxyxanthone, mangiferin and isomangifin, whereas celebixanthone was isolated from the bark of *C. sumatranum* ssp. *Sumatranum*. Phytochemical study of the constituents of the bark of *C. cochinchinense* revealed as a bicyclic triterpenoid, friedelin, tocotrienols, xanthones, xanthonoid and bisxanthone (Nguyen & Harison *et al.*, 1998).

1.4.1.1.4 Garcinia

Searching for secondary metabolite of Garcinia species in Asia showed that many of them were rich in biologically active compounds. *G. cambogia* is seen abundantly in the evergreen forests of South India where many traditional recipes use it for its distinct flavour. Administration of flavonoids from *G. cambogia* significantly lowered lipid levels in rats fed with normal and cholesterol-containing diets. The hypolipidemic activity of these flovonoids may be due to a lower rate of lipogenesis and higher rate of degradation (Koshy *et al.*, 2001).

Isolation and structure elucidation of extracts from of *G. fusca* collected in

Thailand furnished eight new xanthones, fuscaxanthone A-H and eight known compounds from the stem bark. Furthermore, it was in a primary screening test for novel cancer chemopreventive agents (antitumor promoters), found that several xanthones and depsidones showed potent inhibitory effects of Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-O-tetradecanoylphorbol-13-acetate (TPA) in Raji cells (Ito *et al.*, 2003).

Leaves of the Malasian plant, *G. gaudichaudii* yielded gaudichaudione A-H which are pentacyclic tetra-isoprenylated xanthonoids, gaudichaudione H, a novel bridgehead methoxylated triprenylated xanthonoid and gaudichaudiic acids A-E. All of these compounds were found to exhibit significant cytotoxicity against several cancer cell lines (Cao *et al.*, 1998).

Griffipavixanthone, a novel bixanthone with cyclized prenyl groups providing the xanthone-xanthone linkage from bark of Malaysian plants, *G. griffithii* and *G. pavifolia* showed the high *in vitro* cytotoxicity against mouse leukemia (P388), mouse Lewis lung carcinomar (LL/2) and mouse fibrosarcoma (Wehil 64) cell line with ED_{50} of 3.40, 6.80 and 4.60 μg/ml, respectively (Xu *et al.*, 1998).

Potent cytotoxicity against HeLa and HEL cells was also observed in a crude extract of the gamboge resin of *G. hanburyi*. Gamboge is used as pigment and folk medicines. Eleven new and four known xanthones were isolated (Asano *et al.*, 1996).

The dried fruit rind of *G. indica* which is used as a garnish for curry and in some of the folklore medicine in India contain 2-3% garcinol, a polyisoprenylated benzophenone. The structure of which contains both phenolic hydroxyl groups and a β-diketone moiety. Garcinol has been reported to possess antibiotic activities, antiulcer activity, induction of apoptosis through cytochrome c released and activation

of caspase in human leukemia HL-60 cells. It also showed nearly three times greater DPPH free radical scavenging activity than α-tocopherol by weight (Sang *et al.*, 2001).

Lateriforone, a spiroxalactone with a novel skeleton from the bark of *Garcinia lateriflora Bl.*, exhibited activity against P388 cancer cell line with ED_{50} value of 5.4 μg/ml (Kosela *et al.*, 1999).

A preliminary study of the leaves and trunk bark of *G. valersiana* from Vietnam reported that the leaves contained flavanones, whereas the trunk bark contained xanthones and triterpenoids (Nguyen & Harrison, 2000).

1.4.1.2 Schisandraceae

The Schisandraceae comprises 47 species in 2 Genera, *Kadsura* and *Schisandra*. The winding stems twist around the trunks of trees and climb to their top (Watson & Dallwitz, 1992).

In the investigation of the antitumor potential of Taiwanese plants, an ethanol extract of the stems of *Schisandra arisanensis* was useful as an antirheumatic and exhibited cytotoxicity against nasopharynx carcinama cells (KB) *in vitro*. Bioassay-directed fraction of this extract led to the isolation and characterization of four unique C19 homo lignans with a 5,4'-butano-2, 4-cyclohexadienone-6-spiro-3'(2;-3'-dihydrobenzo[*b*]furan) skeleton : schiarisanrin A-C, and the biological evaluation of them demonstrated cytotoxicity against KB, colon carcinoma (COLO-205), hepatoma (HEPA) and cervix (HELA) cancer cells (Kuo *et al.*, 1997). Kuo *et al.* (1999) also reported the isolation and characterization of a new C18 dibenzocyclooctadiene ignan, kadsumarin A, a new anti HBeAg lignan from *Kadsura matsudai* and *S. arisanensis*.

S. chinensis has been used in traditional Chinese medicine for thousands of years. In the last decades, the pharmacology and chemistry of this plant has been extensively studied. Much evidence shows that *S. chinensis* and its dibenzocyclo-octene lignans may act on the function of the liver. The findings are useful for further understanding of the pharmacological basis of *S. chinensis* as an antioxidant, anticancer, tonic, sedative and anti-aging drugs. *Schisandra* lignans seem to be also a potential source of new synthetic drugs. In recent years, it has been clinically used for anti-HIV and antihepatotoxic effect (Hancke *et al.*, 1999).

Study on the fruit of *Schisandra henryi* var *yunnanensis* from China resulted in dibenzocyclo octadien lignan compounds, schisanhenol, schisanhenrin, schisanhenric acid and kadsuric acid being isolated and recently, four novel nortriterpenoids, henridilactones A-D were isolated from the leaves and stems (Li *et al.*, 2004).

A novel bisnortriterpenoid, lancifosilactone A, and four new nortriterpine, lancifosilactone B-E have been isolated from the leaves and stems of *Schisandra lancifolia* which was commonly used in Chinese traditional medicine to staunch, treat fracture and eliminate stasis to reduce swelling (Li *et al.*, 2003; Li *et al.*, 2004).

A herbal medicine preparation in which the stems and roots of *S. propinqua* are a major component has been used for treatment of lung carcinoma in several hospitals in Yunnan. Nigranoic acid, manwuweizic acid, triterpenoid acids isolated from stem of *S. propinqua*, showed significant cytotoxic effect against human decidual cells and luteal cells in vitro (Chen *et al.*, 2001).

Schisanhenol, a dibenzocyclooctene lignan isolated from *S. rubriflora*, was the most active one from six dibenzocyclooctene lignans which can inhibit iron/cyteine-

induced lipid peroxidation of rat liver microsomes as well as superoxide anion production in the xanthine/xanthine oxidase system. The antioxidant activities of them were much more potent than vitamin E at the same concentration of 1 mM (Lu & Liu, 1992).

Schisandra sphaerandra, distributed in southern China, is a traditional medicinal plant that has been used for treatment of stomach disorder. Nigranoic acid, an anti-HIV RT triterpenoid, was isolated from stem (Sun et al., 1996) and schisanol, a new triterpenoid and two new lignans, benzoylgomisin Q and benzoylgomisin P were isolated from the fruit (Yue *et al*., 1994; Ikeya *et al*., 1990).

1.4.1.3 Plants under study

1) *Hypericum hookerianum* Wight &Arn (Fig. 1.1)

Local name: Bua Thong

Location: Doi Inthanon National Park, Jom Thong, Chiang Mai

Note: Shrub; branchlets light green and turning brown; pedicels sepals green; petals 5, anthers, filaments yellow; entire pistil light green; blades dull green, light green underneath.

Use in traditional medicine: The tribal people of the Shola forest (Tamilnadu, India) use the aerial parts for treating burns and wounds (Mukherjee et al, 2001).

Figure 1.1 *Hypericum hookerianum* Wight &Arn

2) *Garcinia speciosa* Wall (Fig. 1.2)

Local name: Phawa, Saraphi Pa

Location: Doi Suthep, Muang, Chiang Mai

Note: tree; bark thin, roughly cracked and flaking, black; sap yellow; elder branchlets gray-brown, younger parts green: fruits hard, green, blades dark glossy green above, pale light greenish underneath

Figure 1.2 *Garcinia speciosa* Wall

3) *Garcinia xanthochymus* Hook. F. ex T. Anderson (Fig. 1.3)

Local name: Mada Luang, Mada

Location: Doi Suthep, Muang, Chiang Mai

Note: tree; bark thick, slightly roughened, brown, sap light yellow; fruiting pedicels, sepals, immature fruits green, mature fruits light yellowish, soft, juicy, with yellow sap; aril yellow. Slightly sour and edible; blades dark green above, green underneath

Use in traditional medicine: fruit has been used widely for bilious condition, diarrhea and dysentery in Thailand.

Figure 1.3 *Garcinia xanthochymus* Hook. F. ex T. Anderson

4) *Cratoxylum formosum* ssp. *pruniflorum* (Kurz) Gogel (Fig. 1.4)

Local name: Tiew Khon, Tiew Leung

Location: Doi Suthep, Muang, Chiang Mai

Note: tree, bark thin, roughly flaking, trunk with spin-like short branches; pedicels and fruits calyx gray-light greenish; capsules greenish to maroon-brown; blades dark green above, gray-greenish underneath

Figure 1.4 *Cratoxylum formosum* ssp. *pruniflorum* (Kurz) Gogel

5) *Calophyllum polyanthum* Wall ex Choisy (Fig. 1.5)

Local name: Pha Ong, Ma Nhae Doi

Location: Doi Suthep, Muang, Chiang Mai

Note: tree, scattered in disturb area in gallery montane forest, by roadside, leaves shin dark green above, young fruits light green sap yellow

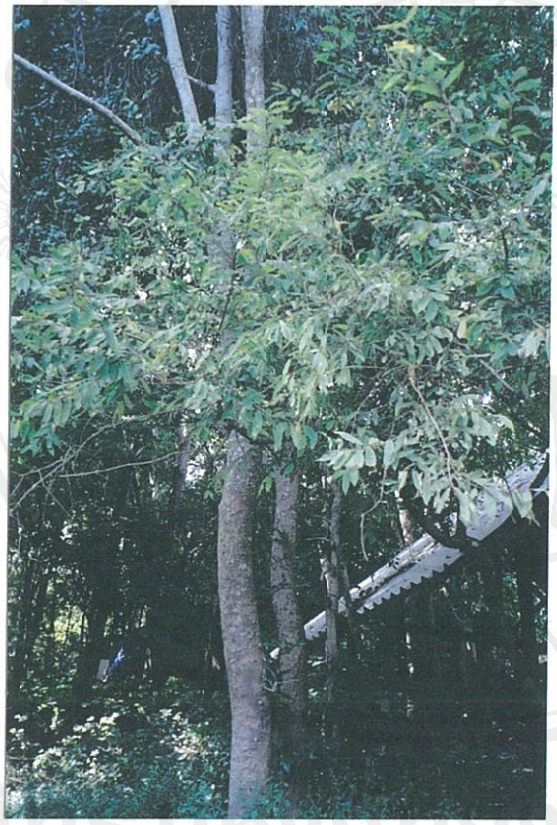

Figure 1.5 *Calophyllum polyanthum* Wall ex Choisy

6) *Schisandra verruculosa* Gagnap (Fig. 1.6)

Local name: -

Location: Doi Mawn Ngaw, Mae Tang, Chiang Mai

Note: everygreen woody climber, basal diameter 5-6 cm. deeply and roughly cracked, brown; branchlets, peduncles brown, individual fruits light green, dull dark green above, dull light green underneath

Figure 1.6 *Schisandra verruculosa* Gagnap

The chemical and biological studies of *H. hookerianum, G. speciosa, G. xanthochymus, C. formosum ssp. pruniflorum, C. polyanthum* and *S. verruculosa* were summarized as the following:

Table 1.1 Summary of the selected Thai plants in family Guttiferrae and Schisandraceae.

Plant sample	Biological Activity	Isolated constituents	References
Guttiferae			
H. hookerianum	Wound healing property, antibacterial CNS active potential	No report	Mukherjee & Suresh (2000) Mukherjee *et al.* (2001)
G. speciosa	Antiviral activity, cytotoxicity, apoptosis, inhibition of acidic-sphingomyelinase	bark and stem - triterpenes, benzophenone, lanostanes, friedolanostanes, friedelin, stigmasterol, β-sitosterol, alpha-mangostin, cowanin, cowanol	Rukachaisirikul et al. (2003); Viera *et al.* (2004) Okudaira *et al.* (2000)
G. xanthochymus	NGF-potentialing activity cytotoxicity, apoptosis DPPH scavenging activity	leaves - flavones, vitexin, friedelin, betulin, β-sitosterol, canophyllol, fruits-xanthones, flavones, benzophenone wood - xanthones	Chanmahasathien *et al.* (2003) Baggett *et al.* (2005)
C. formosum ssp. *pruniflorum*	No reports	wood - quercetin, hyperoside, xanthones, mangiferin, isomangifin	Nguyen & Harison (1998)
C. polyanthum	No reports	seed-coumarins, flavones, β-sitosterol, β-dauosterol	Ma *et al.* (2004)
Schisandraceae			
S. verruculosa	No report	No reports	

1.4.2 Biological activity assay

1.4.2.1 DPPH free radical scavenging activity

Currently, there has been an increasing evaluation of the antioxidant properties of plant extracts or isolated compounds from plant origin for protection of cell and organ against oxidative damage caused by superoxide, hydroxyl and peroxyl radicals (Siddhuraju *et al.*, 2002). The antioxidative activity of natural sources is due to the active compounds present in the plant. Natural antioxidant can be found in different parts of plant including wood, bark, stem, leaf, root, flower and seeds. Many plant derived antioxidants or free radical scavenging agents are phenolic or polyphenolic compounds (Zin *et al.*, 2002). The *in vitro* antioxidants or free radical scavenging activities of flavonoids have been demonstrated by many researchers (Kim *et al.*, 2003).

Several methods such as ferric thiocyanate method and thiobarbituric acid test are available for evaluating antioxidative activity. The free radical scavenging activity assay using DPPH (1,1-diphenyl-2-picrylhydracyl) (Fig. 1.7), a stable free radical, has been widely used to monitor the free radical scavenging abilities (the ability of a compound to donate an electron) of various antioxidants (Choi *et al.*, 2002) since it is a simple and less time consuming method.

DPPH, a radical generating substance, has a deep violet color due to its unpaired electron and free radical scavenging can be followed spectrophotometrically by the loss of absorbance at 517 nm as the pale yellow non-radical form is produced. After DPPH methanolic solution reacted with samples, the absorbance of the resulting solutions were measured and compared with the absorbance of DPPH in the absence of sample solution. The lower the absorbance represents the higher activity.

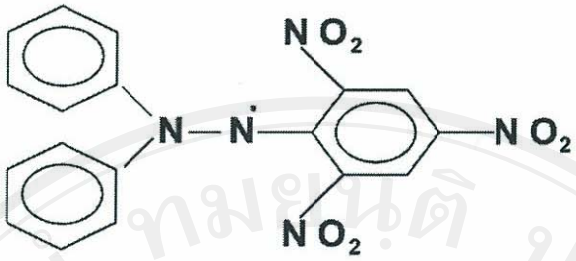

Figure 1.7 Structure of DPPH

Fifty-one tannins and forty-one flavonoids isolated from Oriental medicinal herbs were evaluated for their antioxidant ability by DPPH assay. The results showed that tannins and certain flavonoids are potential free radical scavengers, and that their activity against the DPPH radical is closely associated with their chemical structure. A comparison of the two classes of compounds showed that tannins are more potent than flavonoids because almost all tannins demonstrated significant scavenging action within a low concentration range, whereas the number and position of hydroxyl groups were important features for the scavenging of free radicals by flavonoids. Moreover, it appeared that when the free hydroxyl group was methoxylated or glycosylated, the inhibitory activity was obviously decreased or even abolished (Yokozawa *et al.*, 1998).

Cakir *et al.* (2003) studied the antioxidant potential of *H. hyssopifolium* and its phenolic methabolites. The ethyl acetate fraction showed the highest DPPH radical-scavenging activity and the amount of phenolic compounds was highest in it. Five flavonoids and one napthodianthrone were isolated from this fraction and all isolated compounds showed antioxidant and DPPH radical scavening activities.

Artemisia apiacea, a herb of Compositae family which is wildly distributed in Korea, Japan and China, has been used traditionally as an effective ingredient to dermatomycosis, jaundice, debubitus and alopecia. From n-buthanol fraction of *A. apiacea*, Kim *et al.* (2003) reported the isolation of coumarins, steroids and two flovonoids (apigenin and cacticin) which were found to cause significant free radical scavenging effects on DPPH.

The antioxidant activity of the medicinal herbs used in the traditional Paraguay medicine was studied using free radical generating systems. The methanol extracts of *Cecropia pachystachya*, *Eugenia uniflora* and *Schinus terebinthifolia* showed the high similar activity in the reduction of NBT and DPPH radical (Velazquez *et al.*, 2003).

The preliminary studies on the antioxidant activity of Indian Laburnum, *Cassia fistula*, showed that the antioxidant activity was in the decreasing order of stem bark, leaves, flowers and pulp, and this was correlated with the total polyphenolic content of the extracts. The reason for low antioxidant activity in the flower and pulp fractions could be the presence of some prooxidants, such as chrysophanol and reducing sugar which dominate the antioxidant compounds present in the extracts. Thus, the stem bark had more antioxidant activity in terms of reducing power, inhibition of peroxidation, and DPPH radical scavenging ability (Siddhuraju *et al.*, 2002).

Free radical scavenging effect against DPPH radical of the methanol extracts of *Diospyros kaki*, *Laminaria japonica* and *Undaria pinnatifida* which have been used traditionally in Korea to promote maternal health were investigated. The extract of *D. kaki* was found to be the most potent, with an IC_{50} value of 0.11 mg/ml, while an IC_{50}

value of ascorbic acid, a reference standard, was 0.17 nM (Han *et al.*, 2002).

Murraya koengii leaves are well known as curry leaves and have been used as a folk medicine to increase digestive secretion and relieve nausea and vomiting. Phytochemical studies on leaves, stem bark and the root have resulted in the isolation of carbazole alkaloids which have been evaluated for their radical scavenging ability against DPPH (Tachibana *et al.*, 2001).

Several Korean medicinal plants were selected to evaluate for free radical scavenging capacities and antioxidant activities using standard assays. Flavonoids such as morin, naringenin, quercetin and rutin were included and used as standards in this study. Each sample under assay condition showed a dose-dependent free radical scavenging effect of DPPH. The root bark of *Morus alba* and the leaf of *Saururus chinensis* showed stronger SC_{50} values than other plant extracts (Choi *et al.*, 2002).

Olea europea is cultivated for its edible fruits to obtain olive oil. The material, which is left over after compression of the fruits, is known as olive cake. The antioxidative activity of different butanol fractions of olive cake was investigated. Fractions tested showed good hydrogen donating abilities, indicating that they had effective activities as radical scavengers. The presence of the phenolic compounds, coumaric acid, protocatechuic acid, caffeic acid acid , ferulic acid, oleuropein and cinnamic acid in the olive cake explains their activity and makes it useful for application in cosmetics, especially in anti-aging and anti-wrinkle products (Amro *et al.*, 2002).

The ethanolic extract of *Striga orobanchioides* (Scrophulariaceae), which have been reported to have antiandrogenic, antibacterial, antihistaminic, mast cell stabilizing and antifertility properties, was also screened for *in vitro* and *in vivo*

antioxidant activities using standard procedures. The ethanol extract exhibited IC_{50} value of 8.65 ±1.46 μg/ml in DPPH assay. These values were less than those obtained for ascorbic acid and rutin, used as standards (Badami *et al.*, 2003).

1.4.1.3 Sulforhodamine assay

In vitro cytotoxicity assay in human cancer cell has been recognized as a primary tool for the screening of anticancer agents. Mosmann (1983) established the (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay and subsequently, modified tetrazolium salts like XTT,WST-1 have become available. The advantage of these new compounds is that viable cells convert them to a water soluble formazan. Thus, a methabolic assay with any of these compounds requires one less step (solubilization of product) than an assay with MTT. Using these assays a large number of tests can be carried out in a rapid, reproducible and sensitive fashion.

The development of the sulforhodamine B (SRB) protein binding assay for the *in vitro* measurement of cellular protein content of adherent and suspension cultures for evaluation of cytotoxicity and cell proliferation in microplate was established by Skehan *et al.*, (1990); subsequently it was adopted for routine use in the National Cancer Institute (NCI, USA) *in vitro* antitumor screening.

SRB is a bright pink aminoxanthene dye. It posseses 2 charged SO^{-}_{3} groups which are capable of electrostatically binding to positive counterions. Under mildly acidic conditions, SRB binds to the positive fixed charges of biological molecules. In TCA fixed cells, these binding sites are primarily the amino groups of proteins. The SRB binds to the basic amino acids of cellular macromolecules and the colorimetric

evaluation provides an estimate of total protein mass which is related to cell growth or viability in treated and untreated cells. SRB behaves like a bromphenol blue, naphthol yellow S, and Coomassie Blue, which are also used widely as protein stains. With TCA fixed cultures, SRB gives a higher OD and better signal to noise ratio at low cell density than do these other dyes. The method has replaced the tetrazolium based assays by exhibiting a number of advantages including better linearity, higher sensitivity, a stable end point that does not require time-sensitive measurement and lower cost (Papazisis *et al.*, 1997).

According to NCI, the 50% cell growth inhibition values are considered active when the value is less than 20 μg/ml for extract and less than 4 μg/ml for pure compound (Codell, 1993). Recently, cytotoxicity assay of anticancer agent was performed on a panel of human cancer cell lines which comprised about 60 cell lines from nine organs (see in Table 1.2). Three human cell lines, MCF7 (breast), NCI-H460 (lung), SF-268 (CNS), were used in the prescreen test for cytotoxic activity at NCI-Frederick. Additional lines evaluated for use in the screen and currently available are listed separately in Table 1.3.

Table 1.2 A list of 60 human cell lines and inoculation density used in the screening and maintenance at NCI (Monks *et al.*, 1991)

Cell line	Cells/well	Cell line	Cells/well	Cell line	Cells/well
Leukemia		**Colon**		**Lung**	
CCRF-CEM	40000	COLO 205	15000	A549/ATCC	7500
HL-60(TB)	40000	HCC-2998	15000	EKVX	20000
K-562	5000	HCT-116	5000	HOP-62	10000
MOLT-4	30000	HCT-15	10000	HOP-92	20000
RPMI-8226	20000	HT29	5000	NCI-H226	20000
SR	20000	KM12	15000	NCI-H23	20000
Melanoma		SW-620	10000	NCI-H322M	20000
LOX IMVI	7500	**CNS**		NCI-H460	7500
MALME-3M	20000	SF-268	15000	NCI-H522	20000
M14	15000	SF-295	10000	**Ovarian**	
SK-MEL-2	20000	SF-539	15000	IGR-OV1 Restricted Use	10000
SK-MEL-28	10000	SNB-19	15000	OVCAR-3	10000
SK-MEL-5	10000	SNB-75	20000	OVCAR-4	15000
UACC-257	20000	U251	7500	OVCAR-5	20000
UACC-62	10000	**Breast**		OVCAR-8	10000
Renal		MCF7	10000	SK-OV-3	20000
786-0	10000	NCI/ADR-RES	15000	**Prostate**	
A498	25000	MDA-MB-231	20000	PC-3	7500
ACHN	10000	HS 578T	20000	DU-145	10000
CAKI-1	10000	MDA-MB-435	15000		
RXF 393 Restricted Use	15000	MDA-N Not Available	15000		
SN12C	15000	BT-549	20000		
TK-10 Restricted Use	15000	T-47D Restricted Use	20000		
UO-31	15000				

Table 1.3 List of additional cell lines use in the screening of cytotoxicity

Cell line	Panel name	Cells/well	Cell line	Panel name	Cells/well
LXFL 529 Restricted Use	Lung	10000	RPMI-7951	Melanoma	20000
DMS 114	Lung	20000	M19-MEL	Melanoma	10000
SHP-77	Lung	40000	RXF-631 Restricted Use	Renal	10000
DLD-1	Colon	50000	SN12K1	Renal	10000
KM20L2	Colon	10000	MDA-MB-468	Breast	20000
SNB-78	CNS	20000	P388	Leukemia	50000
XF 498 Restricted Use	CNS	20000	P388/ADR	Leukemia	50000

Pedro *et al.* (2002) have evaluated twenty-seven oxygenated xanthones for their capacity to inhibit the growth of three human cancer cell lines, MCF-7, TK-10 and UACC-62 according to the protocol adopted by NCI. Differences on their potency towards the effect on the growth of the human cancer cell lines can be described to the nature and positions of the substituents on the xanthonic nucleus.

Triterpenes isolated from the bark of *G. speciosa* were evaluated for their ability to inhibit the *in vitro* growth of MCF-7, NCI-H460 and SF-268 human cancer cell lines. Most were moderately active (Vieira *et al.*, 2004).

An inhibition of the growth of human cancer cell lines was also observed with isomeric xanthonolignoids, kielcorins and dihydroxyxanthones on MCF-7, TK-10 and UACC-62 cell lines. The growth inhibitory effect was, in general, moderate but was

shown to be dose dependent and due to growth arrest and not to cell death, as inferred from the SRB assay (Sousa *et al.*, 2002)

Coumarin derivatives, theraphin A-D isolated from the bark of a Myanmar medicinal plant, *Kayea assamica* were examined for cytotoxicity based on a panel of human cancer cell lines using the standard SRB assay. Theraphin A, B and C exhibited good cytotoxicity against Col2, KB and LNcaP with IC_{50} values in the range 7.5-42.8 μM (Lee *et al.*, 2003).

1.4.2.3 Lymphocyte proliferation Assay

When lymphocytes are exposed to certain test substance, a few small resting lymphocytes would response by changing into blast cells over 3 days. This process is lymphocyte transformation. Stimulating substances are widely divided into three types. There are antigen, mitogen and allogenic lymphocytes. Phytohaemagglutinin (PHA) is a kind of mitogen that can activate human lymphocyte which related specifically with T cells.

Many assay protocols have been used to measure cell viability and proliferation by e.g. counting cells that include/exclude a dye, measuring released Cr-labeled protein after cell lysis and measuring incorporation of radioactive nucleotides during cell proliferation. The most convenient method assays have been developed in a microplate format (96-well plates) which can reduce the amount of culture medium and cells required as well as cost of plasticware. Colorimetric assays allow samples to be measured directly in the microplate with an ELISA plate reader. One parameter used as the basis for colorimetric assays is the metabolic activity of viable cells. For

example, a microtiter plate assay which uses the tetrazolium salt MTT is now widely used to quantitate cell proliferation.

Colorimetric MTT (tetrazolium) assay which was described by Mosmann (1983) has been to develop a quantitative colorimetric assay for mammalian cell survival and proliferation. The assay detects living, but not dead cells and the signal generated is dependent on the degree of activation of the cell. This method can therefore be used to measure cytotoxicity, proliferation or activation. The main advantages of the colorimetric assay are its rapidity, precision and lack of any radioiotope. The principle of MTT assay is that the yellow tetrazolium MTT is reduced by metabolically active cells in the part by the action of dehydrogenase enzymes. The tetrazolium ring is cleaved in active mitochondria, and so the reaction occurs only in living cells. The resulting intracellular dark blue formazan crystal can be solubilized and quantified by multiwell spectrophotometer (microplate reader). The structure of MTT and their corresponding reaction products are shown in Figure 1.8.

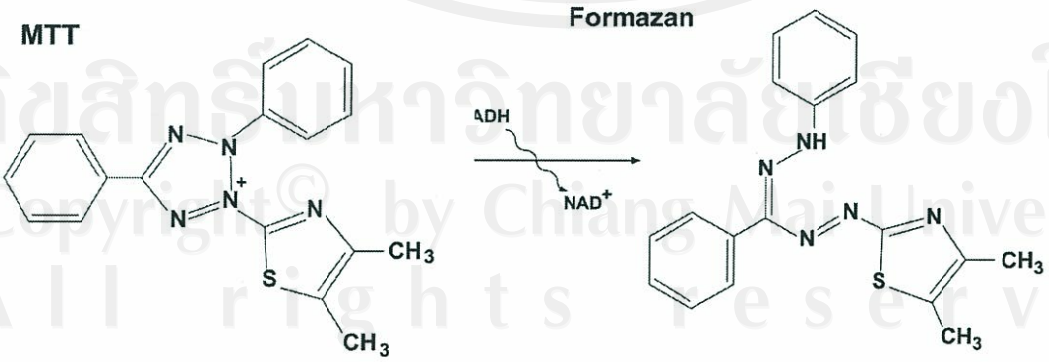

Figure 1.8 Structure of MTT and their corresponding reaction products

The effect of xanthones on the proliferation of human T-lymphocytes to PHA was also evaluated by Pedro *et al.* (2002) using the modified MTT assay. The deoxygenated derivatives 2-hydroxy-1-methoxyxanthone and 3,4-dihydroxyxanthone showed the most interesting results for the inhibitory response.

Inhibition of human lymphocyte proliferation induced by PHA was detected with Kielcorins. The antiproliferative acivity detected was dose dependent and could not be attributed to a toxic effect on lymphocytes as demonstrated by the lymphocytotoxicity assay (viability of exposed lymphocytes > 70%). The (±)-trans-Kielcorin showed no inhibitory effect even when tested at 100 μM (Sousa *et al.*, 2002).