รายงานวิจัยฉบับสมบูรณ์

โครงการ

ฤทธิ์ทางชีวภาพของสารประกอบจากพืชวงศ์ Guttiferae และ Schisandraceae

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คณะผู้วิจัย

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Table of contents

Abstract

The objectives of this study were 1) to screen the bioactivities of the crude extracts from the selected Guttiferae and Schisandraceae plants, 2) to purify and elucidate the structures of the isolated compounds, and 3) to determine the bioactivities of the isolated compounds. Leaves, wood of *Hypericum hookerianum*, *Garcinia speciosa*, *Garcinia xanthochymus*, *Cratoxylum formosum* ssp. *pruniflorum*, *Calophyllum polyanthum* and *Schisandra verruculosa* and the fruit of *G. xanthochymus* collected from Chiang Mai Province, Thailand were carried out to extract and screen for determining the free radical scavenging and antitumor activities. DPPH assay and SRB assay towards human cancer cell lines were performed. The methanol wood extract of *G. speciosa* exhibited the highest scavenging activity with an IC_{50} value of 9.75 μg/ml. *H. hookerianum*, *S. verruculosa*, *C. formosum* ssp. *Pruniflorum*, *G. xanthochymus* and *C. polyanthum* showed the IC_{50} value of 19.08, 23.34, 23.96, 32.10 and 44.29 μg/ml, respectively. For the fruit of *G. xanthochymus*, methanol extract and chloroform fraction of the methanol extract showed no significant IC_{50}.

Chloroform fraction of the methanol extract of *G. speciosa* showed also the potent inhibitory effect with the GI_{50} value of 4.0, 6.6 and 3.7 μg/ml from the leaves and 9.9, 15.7 and 8.1 μg/ml from the wood against HeLa (cervical), KB (epidermoid) and B16F10 (melanoma) tumor cell lines, respectively. The chloroform fraction of the methanol extracts of *H. hookerianum* and *G. xanthochymus* showed the inhibitory effect on cell growth with GI_{50} value less than 20 μg/ml.

H. hookerianum and *S. verruculosa* were selected for the study of phytochemistry since the chemical constituents have not been reported and *H.*

hookerianum showed also good results both of free radical scavenging activity and antitumor activity. The chloroform fraction of the methanol wood extract of *H. hookerianum* furnished 5-hydroxy-2-methoxyxanthone (HH1), 2-hydroxy-3-methoxy xanthone (HH2), the xanthonolignoid *trans*-kielcorin (HH3), as well as two cinnamate ester derivatives, betulinic acid-3β-yl caffeate (HH5) and the new compound 4-hydroxy-3-methoxyphenyl ferulate (HH4). The chloroform fraction of the methanol wood extract of *S. verruculosa* gave vanillic acid (S1), abscisic acid (S2), methyl 4-hydroxybenzoate (S3), 4-hydroxy benzaldehyde (S4), methyl 3,4-dihydroxybenzoate (S5), 1-(4-hydroxy-3-methoxyphenyl)-3-hydroxy-propan-1-one (S6), 1,2-bis-(4-hydroxy-3-methoxyphenyl)-3-hydroxy-propan-1-one (S7) and 4-hydroxybenzoic acid (S8). Spectroscopic methods, especially ^{1}H, ^{13}C NMR, COSY, NOESY, HMBC, HSQC and HRMS were used to elucidate the structures of these compounds.

In vitro effect of these compounds on the growth of human cancer cell lines: MCF-7 (breast), NCI-H 460 (lung), SF-268 (CNS) and UACC-62 (melanoma) and the effect of these compounds on the proliferation of human lymphocyte from the mitogenic effect of phytohemagglutinin (PHA) were evaluated. Cinnamate ester derivatives from *H. hookerianum* showed the strong inhibitory effects against MCF-7, NCI-H460, SF-268 and UACC-62 with the GI_{50} value of 15.1, 18.7, 15.9 and 21.2 μM for the new compound 4-hydroxy-3-methoxyphenyl ferulate (HH4) and 12.2, 19.6, 24.3 and 31.8 μM for betulinic acid-3β-yl caffeate (HH5), respectively, while from *S. verruculosa*, only methyl 3,4-dihydroxybenzoate (S5) gave the moderate activity with the GI_{50} value of 78.9, 38.8 and 93.8 μM toward MCF-7, NCI-H460 and SF-268, respectively. Compound 4-hydroxy-3-methoxyphenyl ferulate (HH4), betulinic acid-3β-yl caffeate (HH5) and methyl 3,4-dihydroxybenzoate (S5) showed

antiproliferative activity with IC_{50} value of 26.1, 40.8 and 58.58 μM, respectively. All of the isolated compounds were also determined for the free radical scavenging activity. Methyl 3,4-dihydroxy benzoate (S5) from *S. verruculosa* showed a strong activity with the IC_{50} value of 6.4 μM, while both of cinnamate ester derivatives from *H. hookerianum* gave the moderate activities with the IC_{50} value of 48.2 and 15.6 μM for 4-hydroxy-3-methoxyphenyl ferulate (HH4) and betulinic acid-3β-yl caffeate (HH5), respectively. The results from this study suggested a potential of the selected plants with significant biological activities for further study and development to new drugs.

บทคัดย่อ

การทดลองนี้มีวัตถุประสงค์เพื่อ 1) ศึกษาฤทธิ์ทางชีวภาพของสารสกัดหยาบจากพืชวงศ์ Guttiferae และ Schisandraceae 2) ทำให้สารบริสุทธิ์และศึกษาโครงสร้างของสารประกอบที่สกัดแยก และ 3) ประเมินฤทธิ์ทางชีวภาพของสารที่สกัดแยกได้ พืชที่เลือกนำมาศึกษาคือ บัวทอง (*Hypericum hookerianum*) พะว้าหรือสารภีป่า (*Garcinia speciosa*), มะดะหลวงหรือมะดะ (*Garcinia xanthochymus*) ติ้วขนหรือติ้วเหลือง (*Cratoxylum formosum* ssp. *Pruniflorum*) พะองหรือมะแหนดอย (*Calophyllum polyanthum*) และ *Schisandra verruculosa* ซึ่งเก็บในพื้นที่จังหวัดเชียงใหม่ ในการทดลองได้สกัดสารสกัดหยาบจากใบ เนื้อไม้ของพืชเหล่านี้และผลของมะดะหลวงโดยใช้เมธานอลและคลอโรฟอร์ม จากนั้นนำสารสกัดหยาบที่ได้มาทดสอบฤทธิ์ในการจับอนุมูลอิสระและฤทธิ์ในการยับยั้งการเจริญเติบโตของเซลล์มะเร็ง ผลการศึกษาพบว่าสารสกัดจากเนื้อไม้โดยเมธานอลของ *G. speciosa* แสดงฤทธิ์สูงสุดในการจับอนุมูลอิสระโดยมีค่าความเข้มข้นที่สามารถจับอนุมูลอิสระได้ 50 เปอร์เซ็นต์ (IC_{50}) เท่ากับ 9.75 ไมโครกรัมต่อมิลลิลิตร *H. hookerianum*, *S. verruculosa*, *C. formosum* ssp. *Pruniflorum*, *G. xanthochymus* และ *C. polyanthum* แสดงค่า IC_{50} เท่ากับ 19.08, 23.34, 23.96, 32.10 และ 44.29 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ สารสกัดหยาบส่วนคลอโรฟอร์มของสารสกัดเมธานอลผลของ *G. xanthochymus* แสดงค่า IC_{50} ไม่แตกต่างกัน นอกจากนั้นสารสกัดหยาบส่วนคลอโรฟอร์มของสารสกัดเมธานอลของ *G. speciosa* ยังออกฤทธิ์สูงสุดในการยับยั้งการเจริญเติบโตของเซลล์มะเร็งปากมดลูก (HeLa) เซลล์มะเร็งในช่องปาก (KB) และเซลล์มะเร็งผิวหนัง (B16F10) โดยจากส่วนของใบมีค่าความเข้มข้นที่สามารถยับยั้งการเจริญเติบโตของเซลล์ มะเร็ง

ได้ 50 เปอร์เซ็นต์ (GI_{50}) เท่ากับ 4.0. 6.6 และ 3.7 ไมโครกรัมต่อมิลลิลิตร และจากส่วนของเนื้อไม้มีค่าเท่ากับ 9.9, 15.7 และ 8.1 ไมโครกรัมต่อมิลลิลิตรตามลำดับ สารสกัดส่วนคลอโรฟอร์มของสารสกัดเมธานอลจาก *H. hookerianum* และ *G. xanthochymus* แสดงฤทธิ์ในการยับยั้งการเจริญของเซลล์มะเร็งทั้งสามชนิดโดยมีค่า GI_{50} น้อยกว่า 20 ไมโครกรัมต่อมิลลิลิตร ได้คัดเลือก *H. hookerianum* และ *S. verruculosa* ในการศึกษาโครงสร้างทางเคมี เนื่องจากยังไม่มีรายงานการศึกษาในเรื่องนี้ อีกทั้ง *H. hookerianum* ยังแสดงฤทธิ์ที่ดีจากการทดสอบฤทธิ์ในการจับอนุมูลอิสระและฤทธิ์ในการยับยั้งการเจริญเติบโตของเซลล์มะเร็ง

จากการสกัดแยกเนื้อไม้ของต้น *H. hookerianum* ส่วนคลอโรฟอร์มของสารสกัดเมธานอลได้สารทั้งสิ้น 5 ตัวคือ 5-hydroxy-2-methoxyxanthone (HH1), 2-hydroxy-3-methoxy xanthone (HH2), xanthonolignoid *trans*-kielcorin (HH3), betulinic acid-3β-yl caffeate (HH5) และสาร ใหม่ 4-hydroxy-3-methoxyphenyl ferulate (HH4) สวนสารที่ได้จากการสกัดแยกเนื้อไม้ของต้น *S. verru- culosa* มีจำนวนทั้งสิ้น 8 ตัวคือ vanillic acid (S1), abscisic acid (S2), methyl 4-hydroxy benzoate (S3), 4-hydroxybenzaldehyde (S4), methyl 3, 4-dihydroxybenzoate (S5), 1-(4-hydroxy-3-methoxy-phenyl) -3- hydroxy-propan-1-one (S6), 1, 2- bis-(4-hydroxy-3-methoxyphenyl)-3-hydroxypropan-1-one (S7) และ 4-hydroxybenzoic acid (S8)

ในการศึกษาฤทธิ์ของสารที่แยกได้ต่อการเจริญของเซลล์มะเร็งเต้านม (MCF-7) เซลล์มะเร็งปอด (NCI-460) เซลล์มะเร็งระบบประสาทส่วนกลาง (SF-268) และเซลล์มะเร็งผิวหนัง (UACC-62) รวมถึงฤทธิ์ต่อการเพิ่มจำนวนของลิมโฟซัยท์ จากผลการทดลองพบว่าสารใหม่ 4-hydroxy-3-methoxyphenyl ferulate (HH4) และ betulinic acid-3β-yl caffeate (HH5) จาก *H. hookerianum* มีฤทธิ์สูงในการยับยั้งการเจริญเติบโตของเซลล์มะเร็ง โดย4-hydroxy-3-

methoxy phenyl ferulate (HH4) มีค่า GI_{50} เท่ากับ 15.1, 18.7, 15.9 และ 21.2 ไมโครโมลาร์ และ betulinic acid-3β-yl caffeate (HH5) มีค่า GI_{50} เท่ากับ 12.2, 19.6, 24.3 และ 31.8 ไมโครโมลาร์ ในระหว่างที่ methyl 3,4-dihydroxybenzoate (S5) จาก *S. verruculosa* แสดงฤทธิ์ปานกลางในการยับยั้งการเจริญเติบโตของเซลล์มะเร็งเซลล์มะเร็งเต้านม (MCF-7) มะเร็งปอด (NCI-460) และมะเร็งระบบประสาทส่วนกลาง (SF-268) โดยมีค่า GI_{50} เท่ากับ 78.9, 38.8 และ 93.8 ไมโครโมลาร์ ในการยับยั้งการเพิ่มจำนวนของลิมโฟซัยท์ 4-hydroxy-3-methoxyphenyl ferulate (HH4) betulinic acid-3β-yl caffeate (HH5) และ methyl 3,4-dihydroxy benzoate (S5) มีค่าความเข้มข้นที่สามารถยับยั้งการเพิ่มจำนวนลิมโฟซัยท์ได้ 50 เปอร์เซ็นต์ (IC_{50}) เท่ากับ 26.1, 40.8 และ 58.58 ไมโครโมลาร์ ตามลำดับ ในการทดสอบฤทธิ์ในการจับอนุมูลอิสระ methyl 3,4-dihydroxy benzoate (S5) แสดงฤทธิ์ที่สูงใกล้เคียงกับวิตามินซีโดยมีค่า IC_{50} เท่ากับ 6.4 ไมโครโมลาร์ 4-hydroxy-3-methoxy phenyl ferulate (HH4)และ betulinic acid-3β-yl caffeate (HH5) แสดงฤทธิ์ปานกลางโดยมีค่า IC_{50} เท่ากับ 48.2 และ 15.6 ไมโครโมลาร์ ตามลำดับ จากผลการศึกษาครั้งนี้ชี้ให้เห็นว่าพืชที่ทำการศึกษานี้มีศักยภาพที่จะสามารถพัฒนาเพื่อเป็นยาใหม่ได้ต่อไป

Executive Summary

Six Thai Guttiferae and Schisandraceae plants (*Hypericum hookerianum, Garcinia speciosa, G. xanthochymus, Cratoxylum formosum* ssp. *Pruniflorum, Calophyllum polyanthum* and *Schisandra verruculosa*) were collected from Chiang Mai, Thailand and extracted by methanol and chloroform. The extracts were screened for free radical scavenging activity using DPPH assay and the effect on the growth of B16F10, HeLa and KB human tumor cell lines using SRB assay. All extracts showed free radical scavenging activity with a dose dependent activity relationship. The lowest IC_{50} value was observed in the methanol extracts from wood of *G. speciosa*. The chloroform fraction of methanol extract from leave of *G. speciosa* gave the most potent inhibition of cancer cell growth with the GI_{50} value of 4, 6.6 and 3.7 μg/ml in HeLa (cervical), KB (epidermoid) and B16F10 (melanoma) cell lines, respectively.

Purification of *H. hookerianum* furnished 5-hydroxy-2-methoxyxanthone (HH1), 2-hydroxy-3-methoxyxanthone (HH2), *trans*-kielcorin (HH3), betulinic acid 3β-yl caffeate (HH5) and the new compound 4-hydroxy-3-methoxyphenyl ferulate (HH4). Compounds HH1-HH5 were evaluated for their effect on the *in vitro* growth of three human cancer cell lines: MCF-7 (breast), NCI-H460 (lung) and SF-268 (CNS). Cinnamate esters HH4 and HH5 exhibited strong inhibitory effect against all three cell lines; that of *trans*-kielcorin (HH3) was moderate while the inhibitory effect of xanthones HH1 and HH2 were only weak. The effect of compounds HH1-HH5 on the mitogenic response of human lymphocytes to PHA was also evaluated. Xanthones HH1 and HH2 exhibited weaker antiproliferative effects than cinnamate esters HH4 and HH5 while *trans*-kielcorin (HH3) was devoid of activity.

Vanillic acid (S1), abscisic acid (S2), methyl 4-hydroxybenzoate (S3), 4-hydroxy benzaldehyde (S4), methyl 3,4-dihydroxybenzoate (S5), 1-(4-hydroxy-3-methoxyphenyl)-3-hydroxy-propan-1-one (S6), 1,2-bis-(4-hydroxy-3-methoxyphenyl) 3-hydroxy-propan-1-one (S7) and 4-hydroxybenzoic acid (S8) were isolated from *S. verruculosa*. All the compounds were evaluated for their antitumor, antiproliferative and antioxidant activities. Only compound S5 exhibited moderate activity against three human cancer cell lines and human lymphocyte proliferation as well as strong inhibitory activity for DPPH free radicals, only slightly less than ascorbic acid. This study demonstrated the potential in the development of these plants to new drugs.

"Bioactive Compounds from Family Guttiferae and Schisandraceae Plant"

Introduction

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.

Research Methodology

1. Sample selection

Five plants (*Hypericum hookerianum* Wight et Arn, *Garcenia speciosa* Wall, *Garcinia xanthochymus* Hook. f. ex. T. Anderson, *Cratoxylum formosum* ssp. pruniflorum (Kurz) Gogel, *Calophyllum polyanthum* Wall ex Choisy) from Guttiferae and the one from Schisandraceae families (*Schisandra verruculosa* Gagnap) were selected focusing on the evidence of cytotoxicity and antioxidant activity. The plants were collected from Chiang Mai Province, Thailand in November and December 2002. The plant samples were authenticated by the Department of Biology, Faculty of Science and Faculty of Pharmacy, Chiang Mai University, Thailand, and the voucher specimens were deposited at the herbarium of the department. The details are as follows.

1) *Hypericum hookerianum* Wight et 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

2. Preparation of the crude extracts

Wood, leaves from the six selected plants and the fruit of *G. xanthochymus* were separately reduced to small pieces, dried at 40°C in a hot oven and comminuted to powder. The dried powder samples (100-300 g) were macerated in methanol for 48 h. The solvent was evaporated under reduced pressure by a rotary evaporator. The residues were re-extracted with chloroform and concentrated by partial evaporation under reduced pressure. Twenty-four extracts were obtained, and the percentage yields were calculated.

3. Bioactivities screening of the crude extracts

3.1 Free radical scavenging activity (DPPH assay)

The free radical scavenging activities of all extracts and the standards (ascorbic acid and α-tocopherol) were determined by a modified DPPH assay of Tachibana *et al.* (2001). DPPH was used as a stable free radical. Briefly, 75 µl of the methanol extracts (1 mg/ml-6.25 µg/ml) and 75 µl of 200 µM ethanol solution of DPPH were put into each well of a 96-well microplate. The reaction mixtures were allowed to stand for 30 min at room temperature, and the absorbance was measured at 570 nm by a Well Reader against a blank (ethanol without DPPH). The experiments were done in triplicates. The DPPH free radical scavenging activity was calculated according to the following equation:

DPPH free radical scavenging activity (%)

$$= \left(\frac{(\text{absorbance of the control} - \text{absorbance of the sample})}{\text{absorbance of the control}} \right) \times 100$$

The scavenging activity was plotted against concentrations. The concentration which showed 50% DPPH scavenging activity (IC_{50}) was determined.

3.2 An antitumor activity (SRB assay)

Stock solutions of extracts were prepared in DMSO and stored at –20 °C. The frozen samples were diluted with cell culture medium prior to the assay. The concentration ranges of the extracts were 3 μg to 250 μg.

The effect of extracts on the growth of human cancer cell lines were evaluated according to the procedure of the NCI for the *in vitro* anticancer drug screening using the protein-binding dye, SRB to assess cell growth (Skehan *et al.*, 1990). Three human cancer cell lines, B16F10, HeLa and KB, were used. Cells were routinely maintained as adherent cell cultures in DMEM medium supplemented with 10% heat-inactivated FCS and 50 μg/ml of gentamicin at 37°C in a humidified air incubator containing 5% CO_2. Each cell line was plated at a density of 1.0×10^5 cells/ml for B16F10 and KB; 2.0×10^5 cells/ml for HeLa in 96-well plates and allowed to attach overnight. Cells were then exposed to five serial concentrations of extracts for 48 hours. After incubation, the adherant cells were fixed *in situ*, washed and dyed with SRB. The bound dye was solubilized and the absorbance was measured at 492 nm in a microplate reader. The dose-response curves were generated and the GI_{50}, corresponding to the concentration of compounds that inhibit 50% of the cell growth was determined as described (Monks *et al.*, 1991). Doxorubicin hydrochloride was used as positive control.

4. Isolation and purification of the compounds from the crude extracts

4.1. *Hypericum hookerianum*

The chloroform fraction of the methanol extract from woody stem of *H. hookerianum* was prepared according to the process of the preparation of the crude extracts to keep the adequate quantity for the isolation. The crude extract was loaded on the silica gel G60 (0.2-0.5 mm), in a column, and eluted with the different polarity of solvent mixture (Petrol-$CHCl_3$, $CHCl_3$, $CHCl_3$-acetone) with the flow rate 1 ml/min. Fractions of 100-300 ml of were collected for each fraction. All fractions were monitored by analytical TLC and combined, according to their composition. Fractions which showed complicated spots under UV light were refractionated in the small column and subfractions were also collected. The interesting subfractions were purified by PTLC to give the isolated compounds.

4.2 *Schisandra verruculosa*

Dried and powdered stem wood of *S. verruculosa* was also prepared for the adequate quantity of crude chloroform extract.

One part of the crude chloroform fraction of the methanol extract was dechlorophyllated following the method described by Herz and Gregor (1962) before fractionation. Another part of the crude extract was applied to Silica gel 60 column and eluted with the different polarity of solvent mixture (Petrol- $CHCl_3$, $CHCl_3$, $CHCl_3$-acetone) with the flow rate 1 ml/min. Fractions of 100-500 ml were collected for each fraction. All fractions were combined according to their composition as revealed by analytical TLC. Fractions which showed complicated spots under UV

light were refractionated in the small column. Subfractions were collected and purified by PTLC to give the isolated compounds.

5. Structure elucidation of isolated compounds

Spectroscopic techniques ^{1}H, ^{13}C NMR , COSY, HSQC, HMBC and NOESY and High Resolution Mass Spectrometry (HRMS) were used to elucidate the structure of the isolated compounds.

6. Bioactivities studies of the isolated compounds

6.1 Tumor cell growth assay

Stock solutions of compounds prepared in DMSO were freshly diluted with the different culture medium prior the assays. Final concentration of DMSO (≤ 0.25%) did not interfere with the biological activities tested. Four human cell lines, MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), SF-268 (CNS cancer), UACC-62 (melanoma), were used. Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium supplemented with 5% heat-inactivated FBS, 2 mM glutamine and 50 μg/ml of gentamicin at 37°C in a humidified air incubator containing 5% CO_2. Each cell line was plated at a density that ensured exponential growth throughout the experimental period according to their growth profiles (7.5×10^4 cells/ml for NCI-H460, 1.0×10^5 cells/ml for UACC-62, and 1.5×10^5 cells/ml for MCF-7 and SF-268) in 96-well plates and allowed to attach overnight. Cells were then exposed for 48 hours to five serial concentrations of compounds. Following this incubation period, the adherant cells were fixed *in situ* washed and dyed with SRB. The bound stain was solubilized and the absorbance was measured at 492 nm in a microplate reader. For each test compound and for each cell line a dose-

response curve is generated and the growth inhibition of 50% (GI_{50}), corresponding to the concentration of compound that inhibits 50% of the net cell growth was determined as described (Monks *et al.*, 1991). Doxorubicin was used as positive control.

6.2 Human lymphocytes proliferation assay

The effect of compounds on the mitogenic response of human lymphocytes to PHA were evaluated using a modified colorimetric MTT assay (Mosman, 1983) previously described by Gonzalez *et al* (1999). Human mononuclear cells were isolated from heparinized peripheral blood of healthy volunteers by Histopaque-1077 density centrifugation and were adjusted to 2-3 x 10^6 cells/ml in RPMI-1640 supplemented with 10% FBS, 2 mM glutamine and 50 μg/ml of gentamicin. Mononuclear cells in 96-well plates were exposed for 4 days to seven serial concentrations of compounds. Following this period MTT solution (1 mg/ml) was added and plates were incubated for more 4 h. The water insoluble formazan dye was solubilized overnight at 37 °C. The absorbance of the colored solution was then measured in a microplate reader at 550 nm. The concentration giving 50% inhibition in the test system (IC_{50}) was calculated. Cyclosporin A was used as positive control.

6.3 Free radical scavenging activity

The isolated compounds were evaluated for the free radical scavenging activity using DPPH assay. The concentration which showed 50% DPPH scavenging activity (IC_{50}) was determined.

Results

1. Preparation of the crude extracts

Table 1 The percentage yields and moisture contents of methanol and chloroform fraction of the methanol extracts from various parts of the selected Thai plants in family Guttiferrae and Schisandraceae.

		Yield (%)					
		Wood		Leaf		Fruit	
Plants	Moisture content (%)	MeOH	$CHCl_3$	MeOH	$CHCl_3$	MeOH	$CHCl_3$
Guttiferae							
H. hookerianum	8.65	7.36	2.01	ND	ND	ND	ND
G. speciosa	39.90	12.18	0.66	8.61	4.33	ND	ND
G. xanthochymus	41.80	19.04	1.02	12.45	1.67	23.60	9.69
C. formosum ssp. *pruniflorum*	38.39	3.77	1.01	14.60	4.50	ND	ND
C. polyanthum	48.18	4.46	2.65	11.10	8.50	ND	ND
Schisandraceae							
S. verruculosa	31.61	1.77	1.22	4.14	3.94	ND	ND

Note: MeOH = methanol extract; $CHCl_3$ = chloroform fraction of the methanol extract; ND = not determined

The percentage yields and moisture contents of the methanol and chloroform fraction of the methanol extracts from different parts of each plant were shown in Table 1. Methanol extracts of all plants showed higher percentage yield than the chloroform-fractioned methanol extracts. This might be due to the presence of more polar compounds in the plants which are more soluble in methanol than in chloroform.

2. Bioactivities screening of the crude extracts

2.1 Free radical scavenging activity study of crude extracts (DPPH assay)

Table 2 The IC_{50} values of the selected Thai plant extracts.

Plant species	IC_{50} (μg/ml)					
	Wood		Leaf		Fruit	
	MeOH	$CHCl_3$	MeOH	$CHCl_3$	MeOH	$CHCl_3$
Guttiferae						
H. hookerianum	19.08	65.42	-	-	-	-
G. speciosa	9.75	142	65 13	168	-	-
G. xanthochymus	32.10	89.56	58.69	59.83	25.58	26.68
C. formosum ssp. *pruniflorum*	23.96	91.04	93.28	162.34	-	-
C. polyanthum	44.29	242.25	51.88	69.41	-	-
Schisandraceae						
S. verruculosa	23.34	127.34	130.00	162.18	-	-

Note: MeOH = methanol extract; $CHCl_3$ = chloroform fraction of the methanol extract

IC_{50}; the concentration of extract which showed 50% DPPH scavenging activity

Table 2 demonstrated the IC_{50} of the extracts. All methanol extracts gave lower IC_{50} values than the chloroform fraction of the methanol extracts. In comparing the extract from wood and leaf of each plant, the scavenging activity of methanol wood extract of all plants exhibited higher scavenging activity than their leaves. This might be due to the higher content of the total polyphenolic compounds in the wood than in leaves. The highest scavenging activity was found in the methanol wood

extract of *G. speciosa* with an IC_{50} value of 9.75 μg/ml which were 2.5 and 5.3 folds more potents than the standard antioxidant, ascorbic acid and α-tocopherol, respectively. *H. hookerianum*, S. *verruculosa, C. formosum* ssp. *pruniflorum, G. xanthochymus, and C. polyanthum* gave the IC_{50} values of 19 08, 23.34, 23.96, 32.10 and 44.29 μg/ml, respectively. In fact, some of these values were less than those obtained from standard antioxidants, ascorbic acid and α-tocopherol (the IC_{50} values of ascorbic acid and α-tocopherol were found to be 24.01 and 52.04 μg/ml respectively). For *G. xanthochymus*, the IC_{50} values of methanol and chloroform fraction of the methanol extract were not significant by differrent in fruits (25.58 and 26.68 μg/ml) and leaves (58.69 and 59.83 μg/ml). The extracts from *G. xanthochymus* using polar and non-polar solvents appeared to give equi-potency of the free radical scavenging activity.

2.2 An antitumor activity study of crude extracts (SRB assay)

The effect of extracts on the growth of human cancer cell lines using SRB assay were evaluated. The GI_{50} (the concentrations of extracts that cause 50% inhibition of cancer cell growth) of extracts on HeLa, KB and B16F10 cell lines, were shown in Table 3 - 4 (Calculation data are in appendix B). Final concentration of DMSO (≤ 0.25%) did not interfere with the biological activities tested. Extracts exhibited a dose dependent growth inhibitory effect on all the cancer cell lines and each extract gave the GI_{50} values which were not significant different in three cell lines.

Table 3 Effect of methanol and chloroform fraction of the methanol extracts from wood of the selected Thai plants in family Guttiferrae and Schisandraceae on the growth of human cancer cell lines.

		GI_{50} (μg/ml)		
Plant species		HeLa	KB	B16F10
Guttiferae				
H. hookerianum	M	42.3 ± 1.5	46.3 ± 1.5	51.0 ± 8.5
	C	19.7 ± 1.2	19.3 ± 1.5	14.5± 0.7
G. speciosa	M	67.3 ± 2.5	75.0 ± 0.6	82.0 ± 4.2
	C	9.9 ± 1.2	15.7 ± 0.6	8.1 ± 0.1
G. xanthochymus	M	130.0	123.3 ± 5.8	105.0 ± 7.1
	C	13.3 ± 1.5	19.0 ± 1.0	11.5 ± 0.7
C. formosum ssp. *pruniflorum*	M	> 250	193.3 ± 5.8	> 250
	C	41.3 ± 1.5	37.3 ± 0.6	44.5 ± 2.1
C. polyanthum	M	216.7 ± 5.8	156.7 ± 25.2	160.0 ± 28.3
	C	90.3 ± 3.1	74.7 ± 3.2	52.5 ± 3.5
Schisandraceae				
S. verruculosa	M	170.0 ± 10	70.7 ± 6.4	200.0 ± 14.1
	C	136.7 ± 5.7	98.0 ± 2.7	70.0 ± 1.4

Note: M = methanol extract; C = chloroform fraction of the methanol extract; ND = not determined

Results are expressed as GI_{50} that are arithmetical means ± SD of 3 independent experiments performed in duplicate.

Doxorubicin was used as positive control (GI_{50} Hela = 300 ± 0.9 nM ; GI_{50} KB = 330 ± 0.9 nM; GI_{50} B16F10 = 26 ± 0.2 nM)

Table 4 Effect of methanol and chloroform fraction of the methanol extracts from leaves of the selected Thai plants in family Guttiferrae and Schisandraceae on the growth of human cancer cell lines.

		GI_{50} (μg/ml)		
Plant species		HeLa	KB	B16F10
Guttiferae				
H. hookerianum	M	ND	ND	ND
	C	ND	ND	ND
G. speciosa	M	34.7 ± 2.3	23.7 ± 0.6	25.7 ± 0.6
	C	4.0 ± 0.3	6.6 ± 0.2	3.7 ± 0.4
G. xanthochymus	M	223.3 ± 11.6	116.7 ± 5.8	160.0
	C	17.0 ± 1.0	29.3 ± 0.6	37.7 ± 4.0
C. formosum ssp. *pruniflorum*	M	> 250	> 250	> 250
	C	223.3 ± 20.1	180.0 ± 10	> 250
C. polyanthum	M	> 250	> 250	> 250
	C	19.0 ± 2.7	13.3 ± 0.6	11.0 ± 1.1
Schisandraceae				
S. verruculosa	M	> 250	> 250	> 250
	C	160.0 ± 10	> 250	183.3 ± 11

Note: M = methanol extract; C = chloroform fraction of the methanol extract; ND = not determined

Results are expressed as GI_{50} that are arithmetical means ± SD of 3 independent experiments performed in duplicate.

Doxorubicin was used as positive control (GI_{50} Hela = 300 ± 0.9 nM ; GI_{50} KB = 330 ± 0.9 nM; GI_{50} B16F10 = 26 ± 0.2 nM)

The chloroform fraction of the methanol leaves extract of *G. speciosa* showed the most potent inhibitory effect with GI_{50} values of 4.0, 6.6 and 3.7 μg/ml on HeLa, KB and B16F10 cell lines, respectively. These values were 13, 20 and 142 folds less potent than doxorubicin, the positive control, which gave the GI_{50} values of 300 nM, 330 nM and 26 on HeLa, KB and B16F10 cell lines, respectively. The strong growth inhibitory effects were also detected in the chloroform fraction of the methanol leaves extract of *C. polyanthum* with GI_{50} value of 13.3, 19.0 and 11.0 μg/ml.

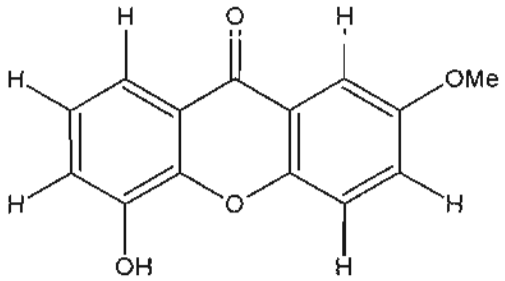
From the wood, the chloroform fraction of the methanol extract of *G. speciosa* also showed strong cell growth inhibition with the GI_{50} values of 9.9, 15.7 and 8.1 μg/ml on B16F10, HeLa and KB cell lines, respectively. Chloroform fraction of the methanol extracts of *H. hookerianum*, and *G. xanthochymus* were also exhibited the inhibitory effect on cell growth with GI_{50} value less than 20 μg/ml. The stronger inhibitory effects of most of the chloroform fraction of the methanol extracts comparing to the methanol extracts might be due to the presence of more active compounds, non polar compounds which are more soluble in chloroform.

Moderate inhibitory effect were found in the methanol leaves extract of *G. speciosa*, the chloroform fraction of the methanol leaves extract of *G. xanthochymus*, the chloroform fraction of the methanol wood extract of *C. formosum* ssp. *Pruniflorum* and the methanol wood extract of *H. hookerianum*. Both of the methanol and chloroform-fractioned methanol leaves extracts of *C. formosum* ssp. *Pruniflorum*, and *S. verruculosa* showed inhibitory activity with GI_{50} value more than 100 μg/ml.

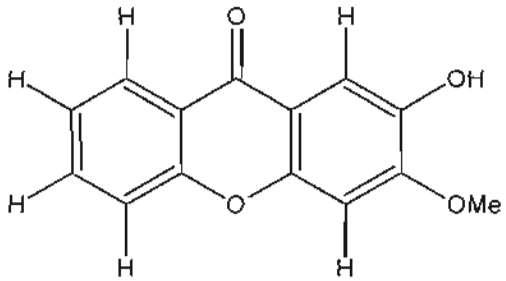
The results from this study suggested a potential for the plants with significant growth inhibitory activity for possible further study and development of pure compounds in the crude extracts to new pharmaceuticals.

3 Purification and structure elucidation of the selected plants

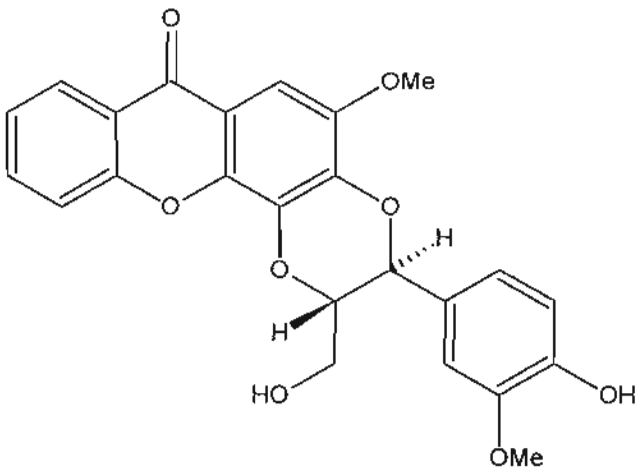
3.1 Isolated compounds from *H. hookerianum*



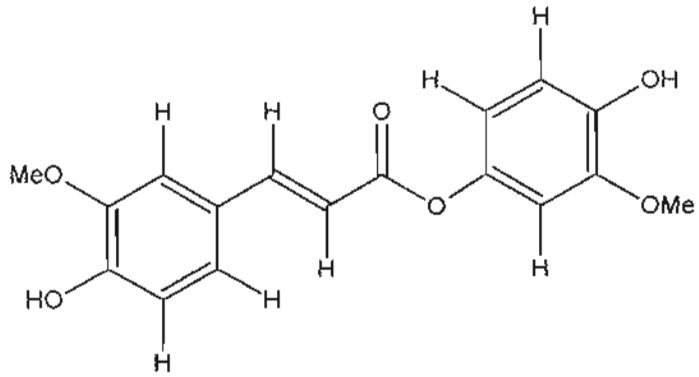
(HH1) 5-hydroxy-2-methoxyxanthone ($C_{14}H_{10}O_4$, MW= 242)



(HH2) 2-hydroxy-3-methoxyxanthone ($C_{14}H_{10}O_4$, MW= 242)

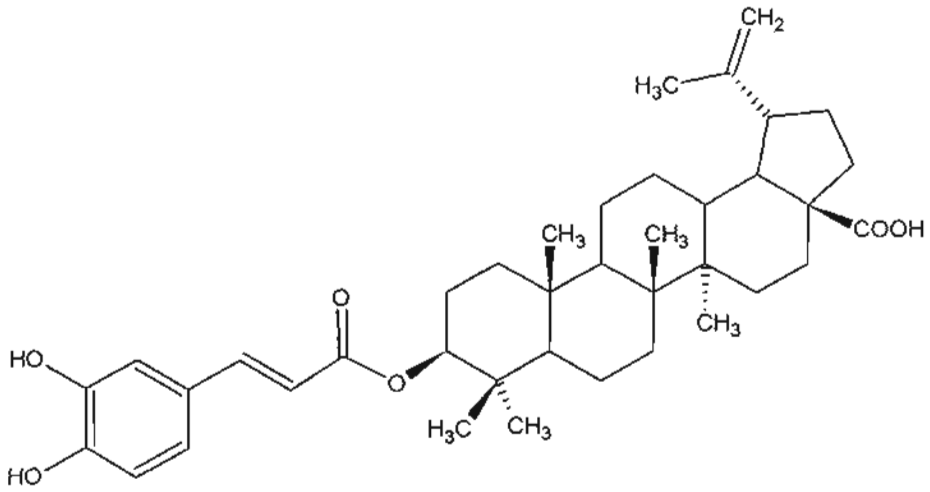


(HH3) trans-kielcorin ($C_{24}H_{20}O_8$, MW = 436)



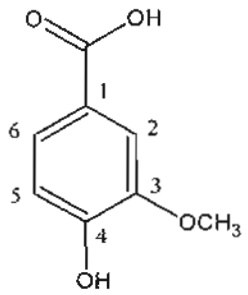
(HH4) 4-hydroxy-3-methoxy phenylferulate ($C_{17}H_{16}O_6$, MW= 316)

This compound has not been described previously.

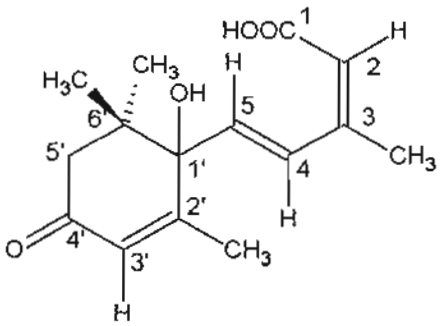


(HH5) Betulinic acid 3β-yl caffeate ($C_{30}H_{47}O_2$, MW= 618)

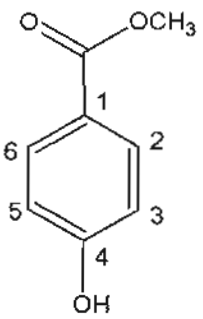
3.2 Isolated compounds from *S. verruculosa*



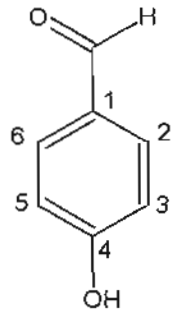
(S1) 4 Hydroxy-3-methoxybenzoic acid (Vanillic acid)



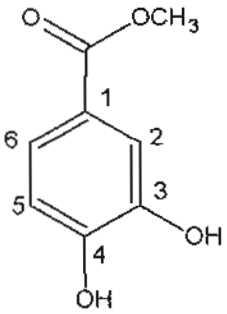
(S2) Abscisic acid



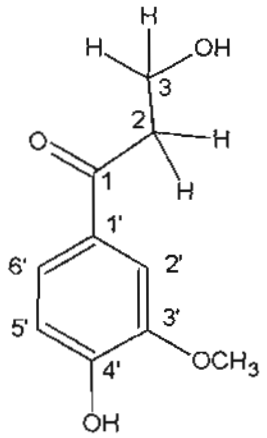
(S3) Methyl 4-hydroxybenzoate



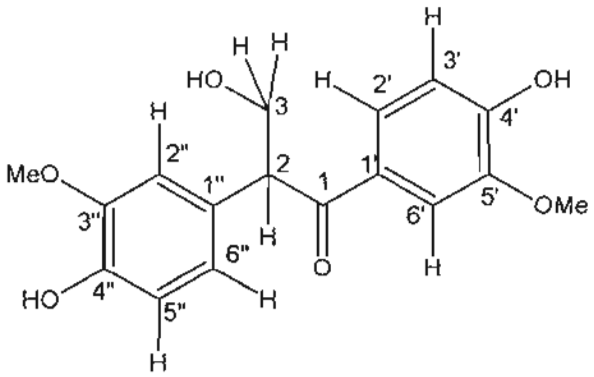
(S4) 4-Hydroxybenzaldehyde



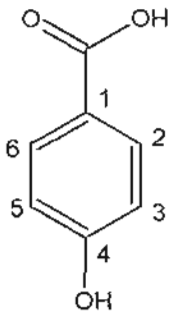
(S5) Methyl 3,4-dihydroxybenzoate



(S6) 1-(4-Hydroxy-3-methoxyphenyl)-3-hydroxy-propan-1-one



(S7) 1,2-bis- (4-hydroxy-3-methoxyphenyl)-3-hydroxy- propan-1-one



(S8) 4-Hydroxybenzoic acid

4. Bioactivities of the isolated compounds

4.1 Tumor cell growth assay

The effect of compounds from *H. hookerianum* on the *in vitro* growth of MCF-7, NCI-H460, SF-268, and UACC-62 cell lines, given in concentration that were able to cause 50% of cell growth inhibition (GI_{50}) after a continuous exposure of 48 h, is shown in Table 5. All the compounds exhibited a dose dependent growth inhibitory effect against all the tumor cell lines tested. Compounds **HH4** and **HH5** exhibited stronger growth inhibitory effects than compounds **HH1** and **HH2** While the formers exhibited activities of the same magnitude, compounds **HH1** and **HH2** showed to be more active against UACC-62.

Table 5 The GI_{50} of compounds from *Hypericum hookerianum* on the growth of human cancer cell lines

	GI_{50} (μM)[a]			
Compounds	MCF-7	NCI-H460	SF-268	UACC-62
HH1	98.1 ± 8.5	108.5 ± 15.3	134.3 ± 9.9	49.6 ± 0
HH2	100 ± 17.5	178.7 ± 17.2	144.6± 25.8	67.5 ± 1.4
HH3	55.1 ± 2.3	49.7 ± 3.0	40.5 ± 1.5	ND
HH4	15.1 ± 1.6	18.7 ± 2.3	15.9 ± 2.7	21.2 ± 0.7
HH5	12.2 ± 2.4	19.6 ± 2.3	24.3 ± 2.5	31.8 ± 0.5
Doxorubicin[b]	42.8 ± 8.2	94.0 ± 8.7	93.0 ± 7.0	94.0 ± 0.9

[a]Results show means ± SEM of 3-4 independent experiments performed in duplicate.

[b]Data from the positive control doxorubicin are expressed in nM.

Table 6 The GI_{50} of compounds from *Shisandra verruculosa* on the growth of human cancer cell lines

Compounds	GI_{50} (μM)[a]		
	MCF-7	NCI-H460	SF-268
S1	174.6 ± 8.5	> 200	> 200
S2	> 189.4	> 189.4	> 189.4
S3	172.7 ± 8.2	176.3 ± 4.4	180.9 ± 5.7
S4	> 200	> 200	> 200
S5	78.9 ± 6.1	38.8 ± 3.3	93.8 ± 7.9
S6	> 200	> 200	> 200
S7	> 157.2	> 157.2	> 157.2
S8	> 200	> 200	> 200
Doxorubicin[b]	42.8 ± 8.2	94.0 ± 8.7	93.0 ± 7.0

[a]Results show means ± SEM of 3-4 independent experiments performed in duplicate.

[a]Data from the positive control doxorubicin are expressed in nM.

The GI_{50} of the compounds isolated from *S. verrucolosa* on MCF-7, NCI-H460 and SF-268 were shown in Table 6. From the results, only compounds **S5** exhibited the moderate growth inhibitory effect against three cell lines and to be more active against NCI-H460.

4.2 Human lymphocytes proliferation assay

Table 7 Effect of compounds from *Hypericum hookerianum* on proliferation of human lymphocytes

Compounds	IC_{50} (μM)[a]
HH1	168.8 ± 4.1
HH2	171.6 ± 11.7
HH3	> 114.7
HH4	26.1 ± 3.6
HH5	40.8 ± 4.9
Cyclosporin A	0.34 ± 0.04

Results show means ±SEM of 3-4 independent experiments performed in duplicate.

The effect of compounds from *H. hookerianum* on the mitogenic response of human lymphocytes to PHA, was also studied and the results, given in concentrations that were able to cause 50% inhibition of proliferation (IC_{50}), are shown in Table 7. All compounds inhibited in a dose dependent manner the proliferation of lymphocytes. Compounds HH1 and HH2 showed once again to be weaker inhibition than compounds HH4 and HH5. HH3 showed no antiproliferative activity even at the maximum concentration tested.

Table 8 The IC_{50} of compounds from *Shisandra verruculosa* on proliferation of human lymphocytes assay

Compounds	IC_{50} (μM)[a]
S1	> 200
S2	> 189.4
S3	> 200
S4	> 200
S5	58.58 ± 5.6
S6	> 200
S7	> 157.2
S8	> 200
Cyclosporin A	0.34 ± 0.04

Results show means ±SEM of 3-4 independent experiments performed in duplicate.

The effect of compounds on the mitogenic response of human lymphocytes to PHA, was also studied with the compound isolated from *S. verrucolosa* and the results, given in concentrations that were able to cause 50% inhibition of proliferation (IC_{50}), are shown in Table 8. Compound S5 showed once again a moderate antiproliferative activity while the other compounds were devoid of activity even at the maximum concentration tested.

4.3 Free radical scavenging activity

The concentration of isolated compounds from *H. hookerianum* and *S. verruculosa*, which showed 50% DPPH scavenging activity (IC_{50}) were reported in table 9 and table 10, respectively.

The results showed that compound S5 has a strong scavenging activity for DPPH free radical with IC_{50} value close to the positive control, ascorbic acid. Compounds HH4 and HH5 showed also moderate scavenging activity.

Table 9 The IC_{50} of compounds from *Hypericum hookerianum* on DPPH free radical scavenging activity assay

Compounds	IC_{50} (μM)
HH1	> 100
HH2	> 100
HH3	> 100
HH4	48.2 ± 6.1
HH5	15.6 ± 0.8
Ascorbic acid	5.3 ± 0.2

Results show means ±SEM of 3-4 independent experiments performed in triplicate.

Table 10 The IC_{50} of compounds from *Shisandra verruculosa* on DPPH free radical scavenging activity assay

Compounds	IC_{50} (μM)
S1	> 100
S2	> 100
S3	> 100
S4	> 100
S5	6.4 ± 0.2
S6	> 100
S7	> 100
S8	> 100
Ascorbic acid	5.3 ± 0.2

Results show means ±SEM of 3-4 independent experiments performed in triplicate.

Conclusion

The Guttiferae species (*Hypericum hookerianum*, *Garcinia speciosa*, *Garcinia xanthochymus*, *Cratoxylum formosum* ssp. *pruniflorum*, *Calophyllum polyanthum*) and the Schisandraceae specie (*Schisandra verruculosa*) from the northern part of Thailand were studied. The crude extracts were screened for free radical scavenging and antitumor activity. No previous reports on chemical constituents of *H. hookerianum* and *S. verruculosa*. *H. hookerianum* exhibited high activities on the screening test. So, both of them were selected for isolation, purification and tested for the bioactivities. The results from this study can be concluded as the following:

1) *H. hookerianum*, *G. speciosa*, *G. xanthochymus*, *C. formosum* ssp. *pruniflorum*, *C. polyanthum* and *S. verruculosa* were extracted by methanol and chloroform. The extracts were screened for free radical scavenging activity using DPPH assay. All extracts showed a dose dependent antioxidant activity. The most potent with the lowest IC_{50} values were observed in the methanol extracts from the wood of *G. speciosa* which were 2.5 and 5.3 folds more potent than the two standard antioxidants, ascorbic acid and α-tocopherol, respectively. Free radical scavenging activities ranging from moderate to high were observed in both methanol and chloroform fraction of the methanol extracts from *H. hookerianum*, *C. formosum* ssp. *pruniflorum*, *G. xanthochymus*, *S. verruculosa and C. polyanthum.*

2) The extracts were tested for antitumor activity on HeLa, KB and B16F10 human cancer cell lines using SRB assay. All extracts showed the effect on the growth of human cancer cell lines with a dose response relationship. The chloroform fraction of the methanol extracts from leave of *G. speciosa* gave the most potent with

the lowest concentration that cause 50% inhibition of cancer cell growth (GI_{50}) of 4, 6.6 and 3.7 μg/ml on HeLa, KB and B16F10 cell lines respectively.

3) The chloroform fraction of the methanol extraction of the woody stems of *H. hookerianum* furnished 5-hydroxy-2-methoxyxanthone (HH1), 2-hydroxy-3-methoxyxanthone (HH2), the xanthonolignoid *trans*-kielcorin (HH3) and two cinnamate ester derivatives, 4-hydroxy-3-methoxyphenyl ferulate (HH4) and betulinic acid-3β-yl caffeate (HH5). 4-Hydroxy-3-methoxyphenyl ferulate (HH4) have not been reported for the chemical constituents previously.

4) *Schisandra verrucolosa* also has not been studied for the chemical constituents earlier. One part of the chloroform fraction of the methanol wood extract was eliminated of chlorophyll, fractionated and purified to give vanillic acid (S1) and abscisic acid (S2). Another part of the crude extract was isolated and purified to give methyl 4-hydroxybenzoate (S3), 4-hydroxybenzaldehyde (S4), methyl 3,4-dihydroxy benzoate (S5), 1-(4-hydroxy-3-methoxyphenyl)-3-hydroxy-propan-1-one (S6), 1,2-bis-(4-hydroxy-3-methoxyphenyl)-3-hydroxy-propan-1-one (S7) and 4-hydroxy benzoic acid (S8).

5) The effect of compounds HH1-HH5 from *H. hookerianum* was investigated against the growth of human cancer cell lines. The results showed that cinnamate esters HH4 and HH5 exhibited strong inhibitory effects (GI_{50}<20 μM) against four cancer cell lines; that of *trans*-kielcorin HH3 was moderate while the inhibitory effects of xanthones HH1 and HH2 were only weak (GI_{50}>100 μM).

6) The effect of compounds HH1-HH5 on the mitogenic response of human lymphocytes to phytohemagglutinin (PHA) was also evaluated and the concentrations able to cause 50% inhibition of proliferation were calculated. Again xanthones HH1

and HH2 exhibited weaker antiproliferative effects than cinnamate esters HH4 and HH5 while *trans*-kielcorin was devoid of activity.

7) The compounds S1-S8 from *S. verruculosa* were also evaluated for their capacity to inhibit growth of human tumor cell lines as well as on the proliferation of human lymphocyte. The results showed that only compound S5 exibited moderated inhibitory activity on three cell lines and on human lymphocyte proliferation.

8) All of the isolated compounds were determined for the free radical scavenging activity. Only compound S5 from *S. verruculosa* was showed a strong activity while compound HH4 and HH5 gave moderate activity.

The results from this study suggested a potential of the selected plants with significant biological activities for further study and development to new pharmaceuticals.

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Appendix

1. Publication

1.1 Jiradej Manosroi, Rujida Wilairat, Anake Kijjoa, Aranya Manosroi. Free radical scavenging activity of extracts from Thai plants in Guttiferae and Schisandraceae families. *Pharm Bio.* 2005; 43: 324-329.

1.2 Rujida Wilairat, Jiradej Manosroi, Aranya Manosroi, Anake Kijjoa, Maria São José Nascimento, Madalena Pinto, Artur M.S. Silva, Graham Eaton and Werner Herz. Cytotoxixities of xanthones and cinnamate esters from *Hypericum hookerianum*. *Planta Med.* 2005; 71: 680-682.

1.3 Jiradej Manosroi, Rujida Wilairat, Aranya Manosroi. *In vitro* antitumor activity of extracts from Thai plants in Guttiferae and Schisandraceae families on human cancer cell lines. *RTA. Med. J.* 2006; 59 (inpress).

2. Presentation

2.1 Jiradej Manosroi, Rujida Wilairat, Anake Kijjoa and Aranya Manosroi. The screening of free radical scavenging activity of extracts from Thai medicinal plants in Guttiferae and Schisandraceae family. 30th Congress on Science and Technology of Thailand, Impact Exhibition and Convention Center, Muang Thong Thani, Bangkok, Thailand, October 19-21, 2004. (Poster presentation, H0021)

2.2 Rujida Wilairat, Jiradej Manosroi, Aranya Manosroi, Madalena Pinto, Maria São José Nascimento and Anake Kijjoa. Cytotoxicity and inhibition of lymphocyte proliferation of xanthones and cinnamate esters from *Hypericum hookerianum*. 53rd Annual Meeting of the society for Medicinal Plants Research. Florence, Italy, August 21-25, 2005. (Poster presentation, P 457)

2.3 Jiradej Manosroi, Rujida Wilairat, Anake Kijjoa and Aranya Manosroi. *In vitro* antitumor activity of extracts from Thai plants in Guttiferae and Schisandraceae families on human tumor cell lines. RGJ-Ph.D. Congress VII. Pattaya, Chonburi, April 20-22, 2006. (Poster presentation, S3-P42)

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Free Radical Scavenging Activity of Extracts from Thai Plants in Guttiferae and Schisandraceae Families

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Abstract

Five Thai plants from the Guttiferae (*Hypericum hookerianum* Wight & Arn, *Garcinia speciosa* Wall, *Garcinia xanthochymus* Hook f. ex. T. Anderson, *Cratoxylum formosum* ssp. *pruniflorum* (Kurz) Gogel, and *Calophyllum polyanthum* Wall ex Choisy) and one from the Schisandraceae (*Schisandra verruculosa*) were extracted by methanol and chloroform. The extracts were screened for free radical scavenging activity using the DPPH assay. All extracts showed a dose-dependent antioxidant activity. The most potent with the lowest IC_{50} values were observed in the methanol extracts from the wood of *G. speciosa*, which were 2.5- and 5.3-fold more potent than the two standard antioxidants, ascorbic acid and α-tocopherol, respectively. Free radical scavenging activities ranging from moderate to high were observed in both methanol and chloroform extracts from *H. hookerianum*, *C. formosum* ssp. *pruniflorum*, *G. xanthochymus*, *S. verruculosa* and *C. polyanthum*. The information from this study can explain the traditional use and the further development of these extracts into new pharmaceuticals.

Keywords: DPPH, free radical scavenging activity, Guttiferae, Schisandraceae.

Introduction

Free radicals play an important role in the development of tissue damage and other pathological events such as cancer, aging, inflammation, and some degenerative diseases. The antioxidative properties of plant extracts and their isolated compounds which have free radical scavenging activity receive considerable attention for possible use in protection of cells and organs against oxidative damage (Siddhuraju et al., 2002; Velazquez et al., 2003). Natural antioxidants can be found in different plant tissues, including wood, bark, stem, leaf, root, flowers, and seeds. Many plant-derived antioxidants of free radical scavenging agents are phenolic or polyphenolic compounds (Zin et al., 2002). Several methods such as the ferric thiocyanate method and the thiobarbituric acid test are available for evaluating antioxidative activity. The free radical scavenging activity assay using DPPH (1,1-diphenyl-2-picrylhydracyl), a stable free radical, has been widely used to assess the free radical scavenging activity of antioxidants (Choi et al., 2002), as it is a simple and less time consuming method.

Compounds in plants from various parts of Southeast Asian have been screened and have exhibited significant biological activities. *Garcinia* and *Hypericum* species (Guttiferae) were found to be rich in secondary metabolites such as xanthonoids, biflavonoids, and triterpenoids (Xu et al., 1998). Some of these plants have been used in traditional medicines. For example, *H. geminiflorum* (Hemsl), an endemic plant in Taiwan, is a Chinese folk medicine for the treatment of several bacterial infections, infectious hepatitis, gastrointestinal disorder, and tumor. Two new oxygenated xanthones and constituents with antiplatelet and anti-inflammatory activity have been found in these plants (Chung et al., 2002). For plants in the Schisandraceae family, more than 19 species are widely use in traditional Chinese medicine. These plants have proved to be rich in lignans and triterpenoids with various biological activities (Li et al., 2004). Some triterpenoids

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Table I. The percentage yield of methanol and chloroform extracts from various parts of the selected Thai plants in the Guttiferae and Schisandraceae.

Plant family	Local name	Yield (%)					
		Wood		Leaf		Fruit	
		MeOH	$CHCl_3$	MeOH	$CHCl_3$	MeOH	$CHCl_3$
Guttiferae							
Hypericum hookerianum Wight & Arn	Bua Thong	7.36	2.01	ND	ND	ND	ND
Garcinia speciosa Wall	Phawa, Saraphi Pa	12.18	0.66	8.61	4.33	ND	ND
Garcinia xanthochymus Hook. f. ex. T. Anderson	Mada Luang, Mada	19.04	1.02	12.45	1.67	23.60	9.69
Crotoxylum formosum ssp. *pruniflorum* (Kurz) Gogel	Tiew Khon, Tiew Leung	3.77	1.01	14.60	4.50	ND	ND
Calophyllum polyanthum Wall ex Choisy	Pha Ong, Ma Nhae Doi	4.46	2.65	11.10	8.50	ND	ND
Schisandraceae							
Schisandra verruculosa Gagnap	—	1.77	1.22	4.14	3.94	ND	ND

MeOH, methanol extract; $CHCl_3$, chloroform extract; ND, not determined.

showed anti-HIV, antitumor, antihepatitis, and antioxidant activity (Hancke et al., 1999; Li et al., 2003). The antioxidative activity of polyphenols and phloroglucinol derivatives isolated from some *Hypericum* species and dibenzocyclooctene lignans isolated from Schisandraceae have been studied (Lu & Liu, 1992; Couladis et al., 2002; Heilmann et al., 2003). The six selected plants in this study have never been previously investigated for antioxidative activity. The methanol and chloroform extracts from the wood, leaves, and fruit of six Thai plants (*Hypericum hookerianum* Wight & Arn, *Garcinia speciosa* Wall, *G. xanthochymus* Hook. f. ex. T. Anderson, *Crotoxylum formosum* ssp. *pruniflorum* (Kurz) Gogel, *Calophyllum polyanthum* Wall ex Choisy and *Schisandra verruculosa* Gagnap) were screened for free radical scavenging activity and their potential to be developed into pharmaceutical products.

Materials and Methods

Chemicals

DPPH (1,1-diphenyl-2-picrylhydrazyl), ascorbic acid, and α-tocopherol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ethanol and chloroform were obtained from Merck Ltd. (Darmstadt, Germany) and Labscan Asia Co., Ltd. (Bangkok, Thailand), respectively.

Plant samples

Six plants were collected from Chiang Mai Province, Thailand, in November and December 2002 (Table 1). The plant samples were authenticated by the Department of Biology, Faculty of Science and Faculty of Pharmacy, Chiang Mai University, Thailand, and the voucher specimens were deposited at the herbarium of the department.

Preparation of the extracts

Wood, leaves, and fruits from the plants were separately reduced to small pieces, dried at 40°C in a hot air oven and comminuted to powder. The dried powder samples (100–300 g) were macerated in methanol for 48 h. The solvent was evaporated under reduced pressure by a rotary evaporator. The residues were re-extracted with chloroform and concentrated by partial evaporation under reduced pressure. Twenty-four extracts were obtained, and the percentage yields were calculated.

DPPH free radical scavenging assay

The free radical scavenging activities of all extracts and the standards (ascorbic acid and α-tocopherol) were determined by a modified DPPH assay of Tachibana et al. (2001). DPPH was used as a stable free radical. Briefly, 75 µl of the methanol extracts (6.25–1 mg/ml) and 75 µl of 200 µM ethanol solution of DPPH were put into each well of a 96-well microplate (Nalge Nunc International, NY, USA). The reaction mixtures were allowed to stand for 30 min at room temperature, and the absorbance was measured at 570 nm by a Well Reader (Seikagaku Corporation, Tokyo, Japan) against a blank (ethanol without DPPH). The experiments were done in triplicate. The DPPH free radical scavenging activity was calculated according to the following equation.

$$\text{DPPH free radical scavenging activity (\%)} = \left(\frac{(\text{absorbance of the control} - \text{absorbance of the sample})}{\text{absorbance of the control}} \right) \times 100$$

The scavenging activity was plotted against concentrations. The concentration that showed 50% DPPH scavenging activity (IC_{50}) was determined.

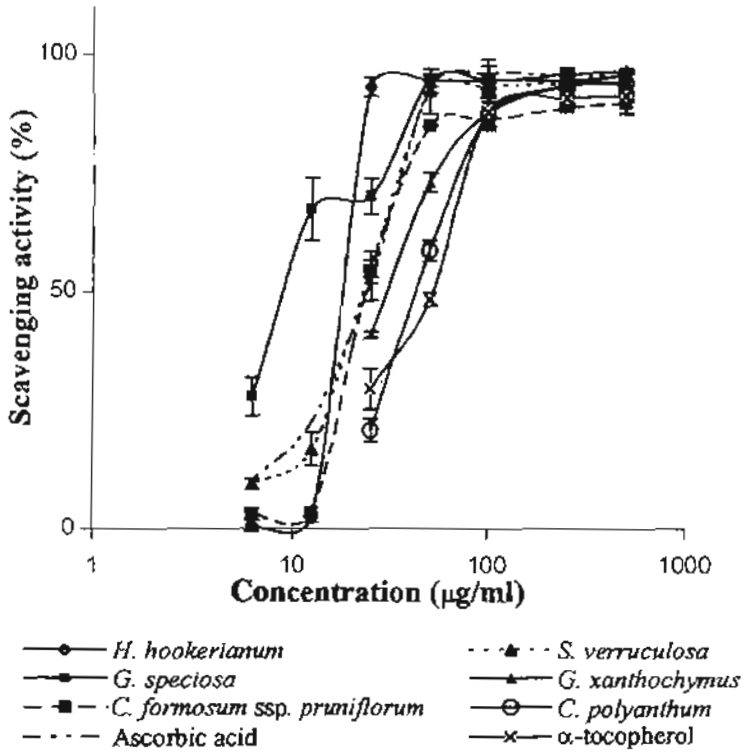

Figure 1. Comparison of free radical scavenging activity (%) of the methanol wood extracts of the six selected Thai plants and the standard antioxidants. Vertical bars represent the standard deviation of three replicates.

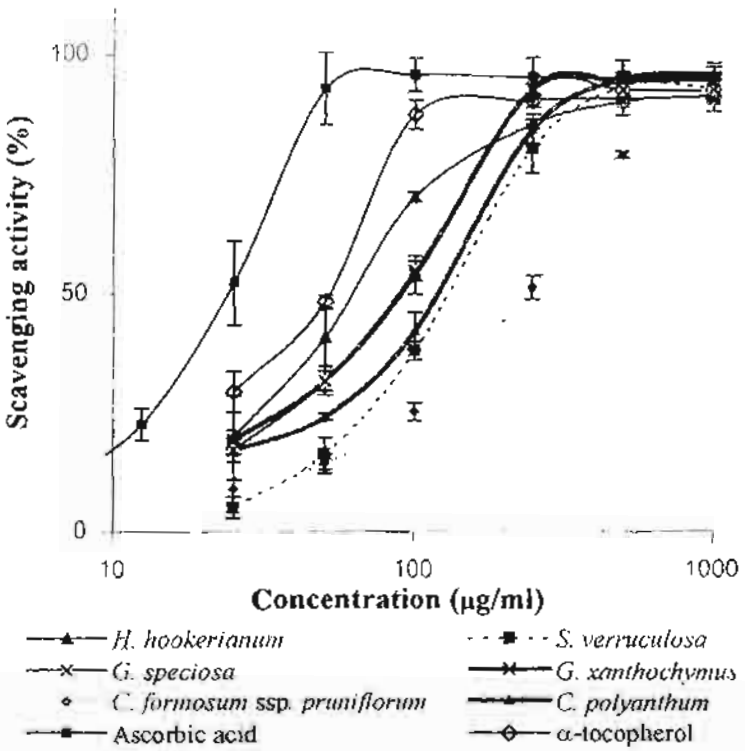

Figure 2. Comparison of free radical scavenging activity of the chloroform wood extracts of the six selected Thai plants and the standard antioxidants. Vertical bars represent the standard deviation of three replicates.

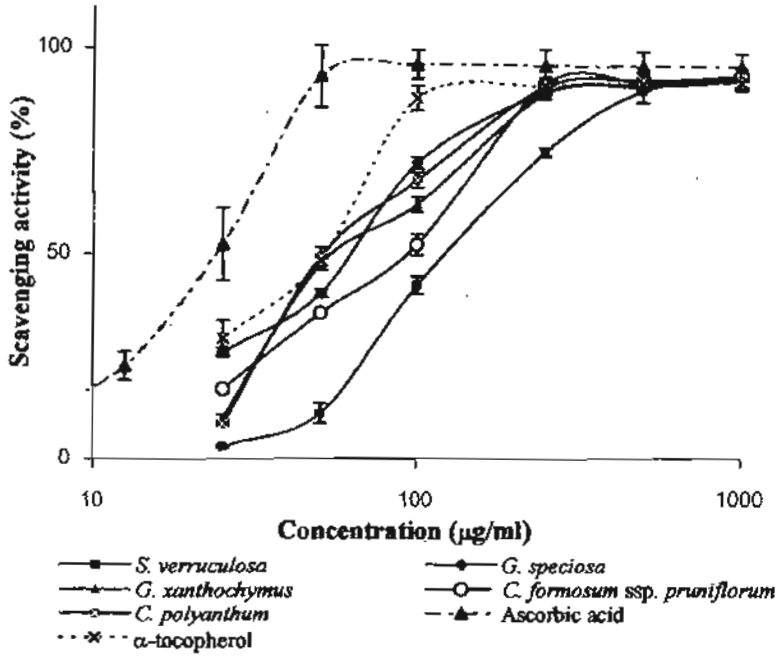

Figure 3. Comparison of free radical scavenging activity (%) of the methanol leaves extracts of the six selected Thai plants and the standard antioxidants. Vertical bars represent the standard deviation of three replicates.

Results and Discussion

The percentage yields of the methanol and chloroform extracts from different parts of each plant are shown in Table 1. Methanol extracts of all plants showed higher percentage yield than the chloroform extracts. This might be due to the presence of more polar compounds in the plants, which are more soluble in methanol than in chloroform.

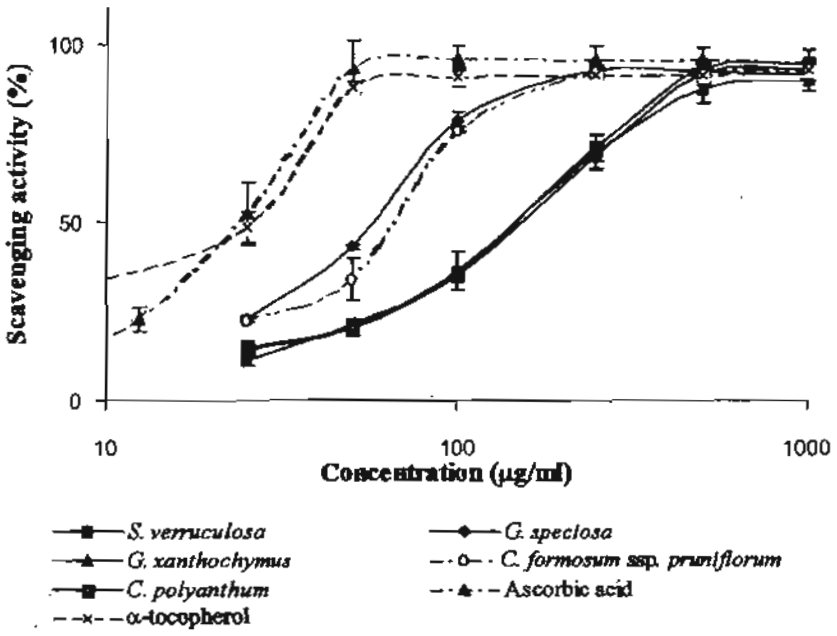

Figure 4. Comparison of free radical scavenging activity (%) of the chloroform leaves extracts of the six selected Thai plants and the standard antioxidants. Vertical bars represent the standard deviation of three replicates.

Table 2. The IC_{50} values of the selected Thai plant extracts.

	IC_{50} (μg/ml)					
	Wood		Leaf		Fruit	
Plant species	MeOH	$CHCl_3$	MeOH	$CHCl_3$	MeOH	$CHCl_3$
Guttiferae						
H. hookerianum	19.08	65.42	—	—	—	—
G. speciosa	9.75	142	65.13	168	—	—
G. xanthochymus	32.10	89.56	58.69	59.83	25.58	26.68
C. formosum ssp. *pruniflorum*	23.96	91.04	93.28	162.34	—	—
C. polyanthum	44.29	242.25	51.88	69.41	—	—
Schisandraceae						
S. verruculosa	23.34	127.34	130	162.18	—	—

MeOH, methanol extract; $CHCl_3$, chloroform extract; IC_{50}, the concentration of extract that showed 50% DPPH scavenging activity. The IC_{50} values of ascorbic acid and α-tocopherol was 24.01 and 52.04 μg/ml, respectively.

For scavenging activity, hydrogen donating ability of the extract to the free radical (DPPH) was determined. When DPPH is scavenged, the deep violet color turns to pale yellow, which can be determined spectrophotometrically. All extracts showed scavenging activity in a concentration-dependent pattern (Figs. 1–4). Table 2 demonstrates the IC_{50} of the extract. All methanol extracts gave lower IC_{50} values than the chloroform extracts. These results agree with the previous study of Moure et al. (2000) indicating the dependence of antioxidant activity of the plant extracts on the polarity of extracting solvents.

In comparing the extract from wood and leaf of each plant, the scavenging activity of methanol wood extract of all plants exhibited higher scavenging activity than their leaves. This might be due to the higher content of the total polyphenolic compounds in the wood than in leaves. These polyphenolic compounds include flavonoids, anthraquinones, anthocyanidins, xanthones, and tannins. These compounds have been reported to scavenge free radicals, superoxide and hydroxyl radical by single electron transfer (Ho et al., 1999; Choi et al., 2002). The highest scavenging activity was found in the methanol wood extract of *G. speciosa* with an IC_{50} value of 9.75 μg/ml, which was 2.5- and 5.3-fold more potent than the standard antioxidants, ascorbic acid and α-tocopherol, respectively. *H. hookerianum*, *S. verruculosa*, *C. formosum* ssp. *pruniflorian*, *G. xanthochymus*, and *C. polyanthum* gave IC_{50} values of 19.08, 23.34, 23.96, 32.10, and 44.29 μg/ml, respectively. In fact, some of these values were less than those obtained from the standard antioxidants ascorbic acid and α-tocopherol (the IC_{50} values of ascorbic acid and α-tocopherol were found to be 24.01 and 52.04 μg/ml, respectively). For *G. xanthochymus*, the IC_{50} values of methanol and chloroform extract were not significantly different in fruits (25.58 and 26.68 μg/ml) and leaves (58.69 and 59.83 μg/ml). The extracts from *G. xanthochymus* using polar and nonpolar solvents appeared to give equipotency of the free radical scavenging activity. The results from this study suggest not only antioxidant activity use but also the potential of these selected plants to be developed into new pharmaceuticals. Further studies on other biological activities and elucidation of the active compounds of the extracts are warranted.

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1.2 Cytotoxixities of xanthones and cinnamate esters from *Hypericum hookerianum*. *Planta Med.* 2005; 71: 680-682

Cytotoxicities of Xanthones and Cinnamate Esters from *Hypericum hookerianum*

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Abstract

5-Hydroxy-2-methoxyxanthone (**1**), 2-hydroxy-3-methoxyxanthone (**2**), *trans*-kielcorin (**3**), 4-hydroxy-3-methoxyphenyl ferulate (**4**) and 3β-O-caffeoylbetulinic acid (**5**) were isolated from *Hypericum hookerianum*. Compounds **1**–**5** were tested against the growth of three human tumor cell lines, MCF-7, NCI-H460 and SF-268. Compounds **4** and **5** exhibited significant inhibitory activity effects against all three; GI_{50} values for **4** were 15.1 ± 1.6, 18.7 ± 2.3 and 15.9 ± 2.7 and for **5** 12.2 ± 2.4, 19.6 ± 2.3 and 24.3 ± 2.5. Compound **3** was less active with GI_{50} values of 55.1 ± 2.3, 49.7 ± 3.0 and 40.5 ± 1.5, while **1** and **2** exhibited only weak effects. Compounds **4** and **5** were moderately effective in influencing the mitogenic response to human lymphocytes to hemoagglutinin, with IC values of 26.1 ± 3.6 and 40.8 ± 4.9, respectively.

The literature contains numerous articles on the chemistry of members of the large genus *Hypericum* (Clusiaceae), in part because of their use in traditional systems of medicine. Common constituents are xanthones, phloroglucinols and flavonoids. *Hypericum hookerianum* Wight et Arn., one of approximately 20 species occurring in India where it is said to be a traditional tribal wound-healing agent [1], is the only species occurring in Thailand and has not been examined chemically although Mukherjee et al. [2] have reported on the antibacterial activity of extracts from various parts of the plant.

In this letter we describe our investigation of the chemical constituents of *H. hookerianum* from the high mountain area of Northern Thailand and their effect on the growth of three human tumor cell lines and on the proliferation of lymphocytes. Chloroform extraction of the woody stems furnished 5-hydroxy-2-methoxyxanthones (**1**), 2-hydroxy-3-methoxyxanthone (**2**), the xanthonolignoid *trans*-kielcorin (**3**) and two cinnamate ester derivatives, 3β-O-caffeoylbetulinic acid (**5**) and 4-hydroxy-3-methoxyphenyl ferulate (**4**). Xanthone **1** has been reported previously from *Hypericum androsaemum* [3], *H. inodorum* [4], and *H. roeperanum* [5] and xanthone **2** from *H. mysorense* [6] while **3** [7], [8], [9], first reported from *Kielmeyera rubriflora* [8], has subsequently been isolated from a number of *Hypericum* species, inter al. *H. ericoides* [10], *H. reflexum* [11], and *H. canariensis* [12]. Information on melting point and rotation of caffeate **5** from *Betula* species [13], [14] was not readily accessible; however, its ^{1}H- and ^{13}C-NMR spectra have appeared in the more recent literature [15], [16]. The remaining constituent 4-hydroxy-3-methoxyphenyl ferulate (**4**) has not been described previously.

The *in vitro* cytotoxicity of compounds **1**–**5** was investigated against three human cancer cell lines. The results are shown in Table 1. Cinnamate esters **4** and **5** exhibited strong inhibitory effects (GI_{50} < 20 μM) against three cancer cell lines; that of *trans*-kielcorin (**3**) was moderate while the inhibitory effects of xanthones **1** and **2** were only weak (GI_{50} > 100 μM). The effect of compounds **1**–**5** on the mitogenic response of human lymphocytes to phytohemagglutinin (PHA) was also evaluated and is depicted in Table 2 which shows the concentrations able to cause 50% inhibition of proliferation. Again xanthones **1** and **2** exhibited weaker antiproliferative effects than cinnamate esters **4** and **5** while **3** was devoid of activity.

Material and Methods

Plant material: Aerial parts of *Hypericum hookerianum* Wight et Arn., a woody shrub, were collected at 2500 m altitude at Doi Inthanon, Chiang Mai, Thailand in June 2003 and were identified by Dr. J. F. Maxwell. A voucher specimen (N° 91–776) was deposited in the CMU Herbarium, Faculty of Pharmacy, Chiang Mai University, Thailand. Leaves and green branches were removed and the remaining woody stems were air dried in the shade prior to extraction.

General experimental procedures: ^{1}H- and ^{13}C-NMR spectra were recorded at ambient temperature in $CDCl_3$ on a Bruker AMC instrument operating at 300.13 and 75.47 MHz, respectively, or a Bruker DRX instrument operating at 500 and 125 MHz, respectively. EI mass spectra were measured on a Hitachi Perkin-Elmer RMV-GM instrument. HR mass spectra were measured on a Kratos Concept II 2 sector mass spectrometer. Silica gel for chromatography was silica gel 60 (0.2–0.5 mm Merck), for analytical and for preparative TLC silica gel 60 GF 254 Merck.

Extraction and isolation: Dried and powdered woody stems of *Hypericum hookerianum* (5.5 kg) were percolated by MeOH (3×10 L) at room temperature. The solution was evaporated at reduced pressure and the crude residue (81 g) was dissolved in $CHCl_3$ (3×500 mL) under sonication. The $CHCl_3$ extracts were combined and evaporated at reduced pressure. The residue (62 g) was applied to a silica gel column (300 g) and eluted with pet-

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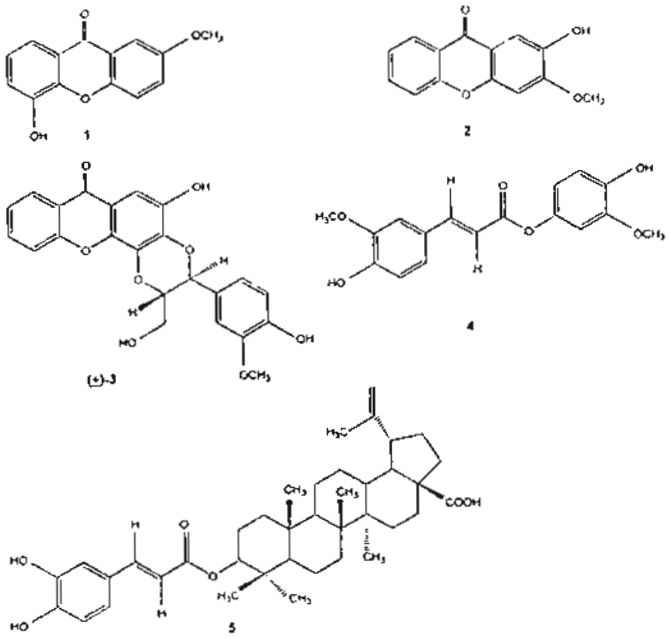

rol-$CHCl_3$, $CHCl_3$, and $CHCl_3$-acetone, 250 mL fraction being collected as follows: Frs. 1 – 133 (petrol-$CHCl_3$, 7 : 3), 134 – 263 (petrol-$CHCl_3$, 1 : 1), 264 – 365 (petrol-$CHCl_3$, 1 : 4), 366 – 464 ($CHCl_3$-acetone, 9 : 1), 465 – 487 ($CHCl_3$-acetone, 7 : 3). Frs. 101 – 138 (1.3 g) were combined, placed on a silica gel 60 column (30 g) and eluted with petrol-$CHCl_3$, 300 mL subfractions being collected as follows: Subfrs. 1 – 40 (petrol-$CHCl_3$, 7 : 3), and 41 – 55 (petrol-$CHCl_3$, 1 : 1). Purification of Subfrs. 6 – 12 (163 mg) by TLC (silica gel, $CHCl_3$-petrol-HCO_2H, 95 : 5 : 0.1) gave yellowish crystals of **1** (79 mg). Sbfrs. 13 – 23 (87 mg) were combined and purified by TLC (silica gel, $CHCl_3$-acetone-HCO_2H, 95 : 5 : 0.1) to give yellow needles of **2** (38 mg). Frs. 139 – 172 (2.1 g) were combined, placed on a silica gel 60 column (40 g) and eluted with petrol-$CHCl_3$, 250 mL subfractions being collected as follows: Sbfrs. 1 – 33 (petrol-$CHCl_3$, 1 : 1), 34 – 80 (petrol-$CHCl_3$, 3 : 7) and 81 – 95 (petrol-$CHCl_3$, 1 : 9). Sbfrs. 4 – 11 (134 mg) were combined and purified by TLC (silica gel, $CHCl_3$-acetone-HCO_2H, 90 : 10 : 0.1) to give **2** (41 mg). Sbfrs. 14 – 21 (39 mg) were combined and similarly purified by TLC to give **5** (35 mg). Purification of Sbfrs. 46 – 50 (189 mg) by TLC (silica gel, $CHCl_3$-acetone-HCO_2H, 85 : 15 : 0.1) afforded **4** (44 mg). Frs. 265 – 272 (89 mg) were also combined, placed on a silica gel 60 column (15 g) and eluted with petrol-$CHCl_3$, 100 mL subfractions being collected as follows: Sbfrs. 1 – 41 (petrol-$CHCl_3$, 1 : 1), and Sbfrs. 42 – 60 (petrol-$CHCl_3$, 1 : 4). Purification of Sbfrs. 26 – 45 (125 mg) by TLC (silica gel, $CHCl_3$-acetone-HCO_2H, 95 : 5 : 0.1) afforded **3** (35 mg). Frs. 316 – 330 (100 mg) were purified by TLC (silica gel, $CHCl_3$-EtOAC-acetone-HCO_2H, 85 : 10 : 5 : 0.1) to give **5** (10 mg) while purification of Frs. 331 – 350 (556 mg) by TLC (silica gel, $CHCl_3$-acetone-HCO_2H, 95 : 5 : 0.1) afforded **3** (52 mg) and **5** (72 mg).

The structures of the known compounds **1** – **3** and **5** and the previously unreported **4** were established by mass, ^{1}H- and ^{13}C-NMR spectrometry, COSY, HMBC and in the case of **4**, also NOESY.

Compound **1** had m.p. 247 – 249 °C ($CHCl_3$), lit. [5] m.p. 245 – 248 °C; EI-MS (electrospray): m/z = 243 [M + H$^+$].

Compound **2** had m.p. 173 – 175 °C ($CHCl_3$), lit. [6] m.p. 174 – 175 °C; EI-MS (electrospray): m/z = 243 [M + H$^+$].

Compound **3** was optically inactive and had m.p. 248 – 250 °C ($CHCl_3$), lit. [7] m.p. 250 – 251 °C; FAB-HR-MS: m/z = 437.12370 [M + H$^+$] (calcd. for $C_{24}H_{21}O_8$: 437.12364).

Compound **5** was a colorless solid, m.p. 262 – 264 °C, m.p. and rotation not reported in the available literature; EI-MS (electrospray): m/z = 617 [M – H$^+$]; $[\alpha]_D^{20}$: 138 ($CHCl_3$, c 0.145 g/100 mL); ^{1}H- and ^{13}C-NMR spectra were as reported [15], [16].

4-Hydroxy-3-methoxyphenyl ferulate (**4**): Yellowish gum; HRMS: m/z = 315.08691 [M – H$^+$] (calcd. for $C_{17}H_{16}O_6$ – H$^+$: 315.08686). The loss of a proton from a hydroxyl-containing compound in EI spectrometry is relatively common. ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 3.77 (3H, s, 3′-OMe), 3.92 (3H, s, 3-OMe), 5.52 (1H, brs, 4-OH), 5.93 (1H, brs, 4′-OH), 6.29 (1H, d, J = 15.9 Hz, H-8), 6.54 (1H, s, H-2′), 6.61 (1H, d, J = 8, H-6′), 6.81 (1H, d, J = 8 Hz, H-5′), 6.91 (1H, d, J = 8.2 Hz, H-5), 7.01 (1H, s, H-2), 7.07 (1H, d, J = 8.2 Hz, H-6), 7.59 (1H, d, J = 15.9 Hz, H-7); ^{13}C NMR (125.77 MHz, $CDCl_3$): δ = 167.28 (C-9), 148.05 (C-4), 146.76 (C-3), 146.43 (C-3′), 145.17 (C-7), 143.87 (C-4′), 131.68 (C-1′), 126.81 (C-1), 123.09 (C-6), 121.71 (C-6′), 115.15 (C-8), 114.72 (C-5), 114.13 (C-5′), 111.22 (C-2′), 109.42 (C-2), 55.96 (3-OMe), 55.73 (3′-OMe). Assignments are based on COSY, HSQC and HMBC experiments.

Table 1 Effect of compounds from *Hypericum hookerianum* on the growth of human cancer cell lines

Compounds	GI_{50} (μM)[a]		
	MCF-7 (breast)	*NCI-H460 (lung)*	*SF-268 (CNS)*
1	98.1 ± 8.5	108.5 ± 15.3	134.3 ± 9.9
2	100 ± 17.5	178.7 ± 17.2	144.6 ± 25.8
3	55.1 ± 2.3	49.7 ± 3.0	40.5 ± 1.5
4	15.1 ± 1.6	18.7 ± 2.3	15.9 ± 2.7
5	12.2 ± 2.4	19.6 ± 2.3	24.3 ± 2.5
Doxorubicin[b]	42.8 ± 8.2	94.0 ± 8.7	93.0 ± 7.0

[a] Results show means ± SEM of 3 – 4 independent experiments performed in duplicate.

[b] Data from the positive control doxorubicin are expressed in nM.

Table 2 Effect of compounds 1–5 on proliferation of human lymphocytes

Compounds	IC_{50} (μM)*
1	168.8 ± 4.1
2	171.6 ± 11.7
3	> 114.7
4	26.1 ± 3.6
5	40.8 ± 4.9
Cyclosporin A	0.34 ± 0.04

* Results show means ± SEM of 3–4 independent experiments performed in duplicate.

Cytotoxicity assays: Reagents and procedures were those described earlier [17]. Doxorubicin hydrochloride of approximately 98% purity by TLC was purchased from Sigma-Aldrich Co.

Human lymphocytes proliferation assays: The effect of compounds 1–5 on the mitogenic response of human lymphocytes to PHA was evaluated using reagents and procedures described earlier [18]. Cyclosporin (minimum purity 95%) was purchased from Sigma-Aldrich Co.

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1.3 *In vitro* antitumor activity of extracts from Thai plants in Guttiferae and Schisandraceae families on human cancer cell lines. *RTA. Med. J.* 2006; 59 (inpress)

In vitro Antitumor Activity of Extracts from Thai Plants in Guttiferae and Schisandraceae Families on Human Cancer Cell Lines.

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Abstract

Purpose: To screen the in vitro antitumor activity of extracts from the selected Guttiferae and Schisandraceae plants. **Materials and Methods:** Twenty-two methanol and chloroform extracts from Guttiferae and Schisandraceae families collected from the northern region of Thailand were tested for antitumor activity on HeLa (cervical carcinoma), KB (epidermoid carcinoma) and B16F10 (melanoma) human tumor cell lines using the sulforhodamine B (SRB) binding assay. **Results:** All extracts showed an antitumor activity with a dose response relationship. The chloroform extract of *G. speciosa* showed the potent inhibitory effect with the 50% growth inhibition (GI_{50}) value of 4.0, 6.6 and 3.7 μg/ml from the leaves and 9.9, 15.7 and 8.1 μg/ml from the wood against HeLa , KB and B16F10 tumor cell lines, respectively. Chloroform extracts of *H. hookerianum* and *G. xanthochymus* showed the inhibitory effect on cell growth with GI_{50} value less than 20 μg/ml. **Conclusion:** The information from this study can support the use of these plants in Thai traditional medicine and the further development of these extracts to new pharmaceuticals.

Keywords: Guttiferae; Schisandraceae; SRB assay; Antitumor activity; Thai plants; Extracts

Introduction

The Guttiferae, mainly found in tropical and northern temperate regions and well known to be rich in secondary metabolites such as xanthonoid, biflavonoid and triterpenoid[1] are widely used in traditional medicine. These plants have been screened and found to exhibit significant pharmaceutical activity. *Hypericum hookerianum*, a traditional tribal wound healing agent, have been evaluated for antibacterial activity[2] and cinnamate esters isolated by our group exhibited significant inhibitory effect against MCF-7, NCI-H460 and SF-268 tumor cell lines, and were moderately effective in influencing the mitogenic response of human lymphocytes to phytohemagglutinin[3]. *Garcinia speciosa* have been studied for antiviral activity and effects on apoptosis[4-5]. Compounds isolated from wood of *G. xanthochymus* which had NGF-potentiating activity also have been reported[6]. Wood of *C. formosum* ssp. *Pruniflorum* was found to contain quercetin, hyperoside, xanthones, mangiferin and isomangifin[7] but the biological activities of these compounds were not known.

For plants in the Schisandraceae family, the winding stem twist around the trunks of trees and climb to their top, over 19 species have been widely used in Chinese traditional medicine. These plants have been proved to be rich in lignans and triterpenoids with various biological activities[8]. Isolated compounds from *Schisandra propinqua* fruit, seed and stem, exhibited the antihepatotoxic, antioxidant and antitumor activities[9].

In the framework of our chemical and biological investigations on plant species, the methanol and chloroform extracts from wood and leaves of the six Thai plant species in the Guttiferae (*Hypericum hookerianum* Wight & Arn, *Garcinia speciosa* Wall, *G. xanthochymus* Hook.f ex T.Anderson, *Cratoxylum formosum* ssp. *pruniflorum* (Kurz) Gogel and *Calophyllum polyanthum* Wall ex Choisy) and Schisandraceae (*Schisandra verruculosa* Gagnap) were studied for free radical scavenging activity[10]. In the present work, these

extracts were found to possess *in vitro* antitumor properties against three human tumor cell lines, HeLa (cervical carcinoma), KB (epidermoid carcinoma) and B16F10 (melanoma).

Materials and Methods

Plant material.

The plants materials in the present investigation were collected from Chiang Mai Province, Thailand in November-December 2002. Voucher specimens were authenticated and deposited at the herbarium of Biology Department, Faculty of Science and Faculty of Pharmacy, Chiang Mai University, Thailand.

Preparation of the extracts and stock solution.

The extracts and stock solutions were prepared by a modified method of Wilairat et al.[3]. Wood and leaves from the plants were separately chopped to small pieces, dried at 40°C in a hot air oven and ground to powder. The dried powder samples (100 – 300 g) were macerated in methanol for 48 h. The solution was evaporated under reduced pressure by a rotary evaporator. The residues were re-extracted with chloroform and concentrated by partial evaporation under reduced pressure.

Stock solutions of methanol and chloroform extracts were prepared in DMSO (Sigma Chemical Co., MO, USA) and stored at –20 °C. The frozen samples were diluted with cell culture medium prior to the assay. The concentration ranges of the extracts were 3 to 250 μg/ml.

Cell lines and culture medium.

HeLa, KB and B16F10 cell lines used in the experiments were kindly provided by National Cancer Institute, Bangkok, Thailand. Cells were routinely maintained as adherent cell cultures in DMEM medium (Sigma Chemical Co., MO, USA) supplemented with 10%

heat-inactivated FCS (Gibco BRL, Canada) and 50 µg/ml of gentamicin (Sigma Chemical Co.. MO, USA) at 37°C in a humidified air incubator containing 5% CO_2 .

Treatment of cells with extracts.

When the cultures reached approximately 80% to 90% confluency they were sub-cultured by treating with 0.25% trypsin, and cell viability was tested by the tryphan blue dye exclusion method. Cell counts were performed in quadruplicated on a haemocytometer. The cell viability was always found to be greater than 98%. Each cell line was plated at a density of 2.0×10^5 cells/ml for HeLa and 1.0×10^5 cells/ml for KB and B16F10 in 96-well plates and allowed to attach overnight. The following day, cells were exposed to five serial concentrations of extracts. Doxorubicin hydrochloride (Dabur Pharma Ltd, UK) was used as positive control. The plates were incubated at 37°C for 48 h.

SRB assay.

The effect of extracts on the growth of human tumor cell lines were evaluated according to the procedure of the National Cancer Institute (NCI, USA) for the *in vitro* anticancer drug screening using the protein-binding dye, SRB to assess cell growth[11]. After incubation period, the adherant cells were fixed *in situ*, washed and dyed with SRB (Sigma Chemical Co., MO, USA). The bound dye was solubilized and the absorbance was measured at 492 nm in a microplate reader. The dose-response curves were generated for each extract tested and for each cell line, and the GI_{50}, corresponding to the concentration of compounds that inhibit 50% of the cell growth was determined as described by Monks et al.[12].

Results

The effect of the methanol and chloroform wood and leaves extracts on HeLa, KB and B16F10 cell lines were shown in Table 1 and 2, respectively. Final concentration of DMSO (≤ 0.25%) did not interfere with the biological activities tested. All extracts exhibited

a dose dependent growth inhibitory effect with no significant difference in GI_{50} in the three cell lines understudied.

The chloroform leaves extract of *G. speciosa* showed the most potent inhibitory effect with GI_{50} values of 4.0, 6.6 and 3.7 μg/ml on HeLa, KB and B16F10 cell lines, respectively. These values were 13, 20 and 142 folds less potent than doxorubicin, the positive control, which gave the GI_{50} values of 300 nM, 330 nM and 26 nM on HeLa, KB and B16F10 cell lines, respectively. The strong growth inhibitory effects were also detected in the chloroform leaves extract of *C. polyanthum* with GI_{50} value of 13.3, 19.0 and 11.0 μg/ml.

From the wood, the chloroform extract of *G. speciosa* showed the strong cell growth inhibition with the GI_{50} values of 9.9, 15.7 and 8.1 μg/ml on HeLa, KB and B16F10 cell lines, respectively. Chloroform extracts of *H. hookerianum*, and *G. xanthochymus* also exhibited the inhibitory effect on cell growth with GI_{50} value less than 20 μg/ml.

Moderate inhibitory effect were found in the methanol leaves extract of *G. speciosa*, the chloroform leaves extract of *G. xanthochymus*, the chloroform wood extract of *C. formosum* ssp. *Pruniflorum* and the methanol wood extract of *H. hookerianum*. Both of the methanol and chloroform leaves extracts of *C. formosum* ssp. *Pruniflorum*, and *S. verruculosa* showed no inhibitory activity at any concentration ($GI_{50} > 100$ μg/ml).

Discussion

For the *in vitro* primary screening of anticancer drug, an assay using the protein-binding dye sulforhodamine B (SRB) is used by the NCI. The SRB binds to the basic amino acids of cellular macromolecules and the solubilized stain is measured spectrometrically to determine relative cell growth in treated and untreated cells[11].

In Guttifeare family, 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 the 50% cell killed or 50% lethal concentration (LC50) values ranging from 25 to 46 mg/ml and moderate activities on brine shrimps, ranging from 22 to 150 mg/ml[13]. Griffipavixanthone, a novel bixanthone with cyclized prenyl groups providing the xanthone-xanthone linkage isolated from bark of Malaysian plants, *G. griffithii* and *G. pavifolia* showed high *in vitro* cytotoxicity against mouse leukemia (P388), mouse Lewis lung carcinoma (LL/2) and mouse fibrosarcoma (Wehil 64) cell lines with the 50% effective dose (ED_{50}) of 3.40, 6.80 and 4.60 μg/ml, respectively[14]. Coumarins isolated from bark of Myanmar plant, *Kayea assamica* (Clusiaceae), have been evaluated for their cytotoxicity on human cancer cell lines using SRB assay. They exhibited strong cytotoxicity activity againts Col2 (colon), KB (epidermoid) and LNCaP (lung) human cancer cell lines with the 50% inhibition concentration (IC_{50}) values in the range 3.5-13.1 μM[15]. Ethanol extract of the stems of Taiwanese plants, *Schisandra arisanensis* which was useful as an antirheumatic, exhibited cytotoxicity against 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[15].

In our study, all of plant extracts which were in the genus related to the aforementioned studies also showed antitumor activity on HeLa, KB and B16F10 cell lines. The chloroform extracts from wood and leaves of all plants showed stronger inhibition than the methanol extracts. This might be due to the presence of more active non polar compounds which are more soluble in chloroform.

The results from this study supported that the selected plants from Guttiferae and Schisandraceae family had significant growth inhibitory activity. Further study on the mechanism of action and isolation of pure compounds in the extracts for potential uses as new pharmaceuticals should be encouraged.

Acknowledgements

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Table 1 Effect of methanol and chloroform extracts from wood of the selected Thai plants in family Guttiferrae and Schisandraceae on the growth of cell lines.

		GI_{50} (μg/ml)		
Plant species		HeLa	KB	B16F10
Guttiferae				
H. hookerianum	M	42.3 ± 1.5	46.3 ± 1 5	51.0 ± 8 5
	C	19.7 ± 1.2	19.3 ± 1.5	14.5± 0.7
G. speciosa	M	67.3 ± 2.5	75.0 ± 0.6	82.0 ± 4.2
	C	9.9 ± 1.2	15.7 ± 0.6	8.1 ± 0.1
G. xanthochymus	M	> 100	> 100	> 100
	C	13.3 ± 1.5	19.0 ± 1.0	11.5 ± 0.7
C. formosum ssp. *pruniflorum*	M	> 100	> 100	> 100
	C	41.3 ± 1.5	37.3 ± 0.6	44.5 ± 2.1
C. polyanthum	M	> 100	> 100	> 100
	C	90.3 ± 3.1	74.7 ± 3.2	52.5 ± 3.5
Schisandraceae				
S. verruculosa	M	> 100	70.7 ± 6.4	> 100
	C	> 100	> 100	70.0 ± 1.4

Note: M = methanol extract; C = chloroform extract

Results are expressed as GI_{50} that are arithmetical means ± SD of 3 independent experiments performed in duplicate.

Doxorubicin was used as positive control (GI_{50} Hela = 300 ± 0.9 nM ; GI_{50} KB = 330 ± 0.9 nM; GI_{50} B16F10 = 26 ± 0.2 nM)

Table 2 Effect of methanol and chloroform extracts from leaves of the selected Thai plants in family Guttiferrae and Schisandraceae on the growth of cell lines.

Plant species		GI_{50} (μg/ml) HeLa	KB	B16F10
Guttiferae				
H. hookerianum	M	ND	ND	ND
	C	ND	ND	ND
G. speciosa	M	34.7 ± 2.3	23.7 ± 0.6	25.7 ± 0.6
	C	4.0 ± 0.3	6.6 ± 0.2	3.7 ± 0.4
G. xanthochymus	M	> 100	> 100	> 100
	C	17.0 ± 1.0	29.3 ± 0.6	37.7 ± 4.0
C. formosum ssp. *pruniflorum*	M	> 100	> 100	> 100
	C	> 100	> 100	> 100
C. polyanthum	M	> 100	> 100	> 100
	C	19.0 ± 2.7	13.3 ± 0.6	11.0 ± 1.1
Schisandraceae				
S. verruculosa	M	> 100	> 100	> 100
	C	> 100	> 100	> 100

Note: M = methanol extract; C = chloroform extract ; ND = not determined

Results are expressed as GI_{50} that are arithmetical means ± SD of 3 independent experiments performed in duplicate.

Doxorubicin was used as positive control (GI_{50} Hela = 300 ± 0.9 nM ; GI_{50} KB = 330 ± 0.9 nM; GI_{50} B16F10 = 26 ± 0.2 nM)

2.1 The screening of free radical scavenging activity of extracts from Thai medicinal plants in Guttiferae and Schisandraceae family. 30th Congress on Science and Technology of Thailand, Impact Exhibition and Convention Center, Muang Thong Thani, Bangkok, Thailand, October 19-21, 2004. (Poster presentation, H0021)

THE SCREENING OF FREE RADICAL SCAVENGING ACTIVITY OF EXTRACTS FROM THAI MEDICINAL PLANTS IN GUTTIFERAE AND SCHISANDRACEAE FAMILY

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Abstract: The objective of this study was to screen free radical scavenging activity of extracts from Thai medicinal plants in family Guttiferae and Schisandraceae. Six Thai medicinal plants (*Hypericum hookerianum*, *Garcinia speciosa*, *G. xanthochymus*, *Cratoxylum formosum* ssp. *Pruniflorum*, *C. polyanthum* and *Schisandra verruculosa*) were extracted by methanol and chloroform. The extracts were screened for free radical scavenging activity using DPPH (1,1-diphenyl-2-picrylhydrazyl) as a stable radical. All extracts showed a dose dependence activity relationship with the lowest IC_{50} value of the methanol extracts from wood of *G. speciosa*. This IC_{50} value was less than those of the standard antioxidants (ascorbic acid and α-tocopherol) of 2.5 and 5.3 folds, respectively. Similar free radical scavenging activities were also observed for extracts from other plants. This study indicated the potential of these plants for further development as pharmaceuticals. We are now in the process of isolation of bioactive compounds from these extracts.

Methodology: Five plants from family Guttiferae and one plant from family Schisandraceae were selected and collected from Chiang Mai Province, Thailand. Their wood, leaves and fruits were extracted by methanol and chloroform. The extracts were tested for free radical scavenging activity using DPPH assay. Vitamin C and α-tocopherol were used as standard references. The scavenging activity was calculated and plotted against concentrations of the extracts. The concentrations which showed 50% DPPH scavenging activity (IC_{50}) were determined. IC_{50} values were compared. The experiment was done in triplicate.

Result, Discussion and Conclusion: Among the wood and leaf parts of the plants, the scavenging activity of methanol wood extract of all plants exhibited higher scavenging activity than leaves. The highest scavenging activity was found in the methanol extract of wood from *G. speciosa* with an IC_{50} value of 9.75μg/ml. For other plants, the IC_{50} values were in the range of 19.08 to 44.29 μg/ml. Some of these values were less than those obtained from the standard antioxidants, ascorbic acid and α-tocopherol. The IC_{50} values of ascorbic acid and α-tocopherol were 24.01 and 52.04 μg/ml respectively. For *G. xanthochymus*, IC_{50} values of methanol and chloroform extract were similar in fruits (25.58 and 26.68 μg/ml) and leaf (58.69 and 59.83 μg/ml). This study suggested the potential for the application of these plants to be developed as new drugs.

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Keywords: Guttiferae; Schisandraceae; Free radical scavenging activity; DPPH

2.2 Cytotoxicity and inhibition of lymphocyte proliferation of xanthones and cinnamate esters from *Hypericum hookerianum*. 53rd Annual Meeting of the society for Medicinal Plants Research. Florence, Italy, August 21-25, 2005. (Poster presentation, P 457)

53rd Annual Meeting of the society for Medicinal Plants Research. Florence, Italy.

August 21-25, 2005

Cytotoxicity and Inhibition of Lymphocyte Proliferation of Xanthones and Cinnamate Esters from *Hypericum hookerianum*

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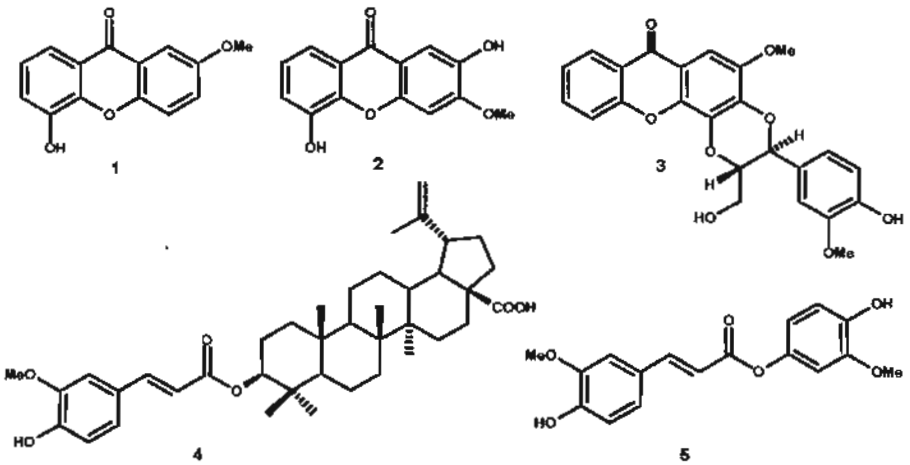
Hypericum hookerianum Wight et Arn., one of approximately 20 species occurring in India where it is said to be a traditional tribal wound-healing agent (1) is the only species occurring in Thailand. Though its extract was claimed to possess the antibacterial activity (2), it has not been previously investigated chemically. Now, we want to report the isolation of 5-hydroxy-2-methoxyxanthone (**1**), 2-hydroxy-3-methoxyxanthone (**2**), *trans*-kielcorin (**3**), betulinic acid 3β-yl caffeate (**4**) and 4-hydroxy-3-methoxyphenyl ferulate (**5**) from the chloroform extract of the stem wood of *Hypericum hookerianum* collected from the North of Thailand. Compounds **1-5** were evaluated for their effect on the *in vitro* growth of three human cancer cell lines: MCF-7 (breast), NCI-H460 (lung) and SF-268 (CNS). The results showed that cinnamate esters **4** and **5** exhibited strong inhibitory effect against all three cell lines; that of *trans*-kielcorin (**3**) was moderate while the inhibitory effect of xanthones **1** and **2** were only weak. The effect of compounds **1-5** on the mitogenic response of human lymphocytes to PHA was also evaluated. Again, xanthones **1** and **2** exhibited weaker antiproliferative effects than cinnamate esters **4** and **5** while *trans*-kielcorin (**3**) was devoid of activity.



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2.3 *In vitro* antitumor activity of extracts from Thai plants in Guttiferae and Schisandraceae families on human tumor cell lines. RGJ-Ph.D. Congress VII. Pattaya, Chonburi, April 20-22, 2006. (Poster presentation, S3-P42)

In *vitro* Antitumor Activity of Extracts from Thai Plants in Guttiferae and Schisandraceae Families on Human Tumor Cell Lines

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Objective

To screen the in *vitro* antitumor activity of the extracts from Thai plants in Guttiferae and Schisandraceae Families on human tumor cell lines using SRB assay.

Methods

The plants from Guttiferae (*Hypericum hookerianum, Garcinia speciosa, G. xanthochymus, Cratoxylum formosum* ssp. *pruniflorum* and *Calophyllum polyanthum* and Schisandraceae (*Schisandra verruculosa*) were extracts with methanol and chloroform. The effect of extracts on the growth of human tumor cell lines were evaluated according to the procedure of the NCI for the *in vitro* anticancer drug screening using the protein-binding dye, SRB to assess cell growth. The GI_{50}, the concentration of the extracts that inhibit 50% of the cell growth was determined. The three human tumor cell lines were used; HeLa (cervical carcinoma), KB (epidermoid carcinoma) and B16F10 (melanoma).

Results

All extracts showed an antitumor activity with a dose response relationship. The chloroform extracts from leaves of *G. speciosa* gave the most potent with the lowest concentration that caused 50% inhibition of cancer cell growth (GI_{50}) of 4.0, 6.6 and 3.7 μg/ml in HeLa, KB and B16F10 cell lines, respectively. The strong growth inhibitory effects were also detected in the chloroform leave extract of *C. polyanthum* and the chloroform wood extracts of *H. hookerianum*, *G. speciosa* and *G. xanthochymus* with GI_{50} value less than 20 μg/ml.

Conclusion

Methanol and chloroform extracts from Thai plants in Guttiferae and Schisandraceae Families collected from the northern region of Thailand showed an antitumor activity. The information from this study can confirm the use of these plants in Thai traditional medicine and the further development of these extracts to new pharmaceuticals.

Keywords: Guttiferae, Schisandraceae, SRB assay, Antitumor activity

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