



รายงานวิจัยฉบับสมบูรณ์

โครงการ

ฤทธิ์ทางชีวภาพของสารประกอบจากพืชวงศ์

Guttiferae และ Schisandraceae

โดย รองศาสตราจารย์ ดร. จีรเดช มโนสร้อย และคณะ

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คณะผู้วิจัย

1. รองศาสตราจารย์ ดร. จีรเดช มโนสร้อย
2. ร้อยเอกหญิง รุจิดา วิไลรัตน์

มหาวิทยาลัยเชียงใหม่

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

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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 $\mu\text{g/ml}$. *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 $\mu\text{g/ml}$, 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 $\mu\text{g/ml}$ from the leaves and 9.9, 15.7 and 8.1 $\mu\text{g/ml}$ 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 $\mu\text{g/ml}$.

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-methoxyxanthone (HH2), the xanthonolignoid *trans*-kielcorin (HH3), as well as two cinnamate ester derivatives, betulinic acid-3 β -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 ^1H , ^{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-3 β -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-3 β -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-3 β -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*), มะคะหลวงหรือมะคะ (*Garcinia xanthochymus*) ตั้วขนหรือตั้วเหลือง (*Cratoxylum formosum* ssp. *Pruniflorum*) พะองหรือมะเหินคอย (*Calophyllum polyanthum*) และ *Schisandra verruculosa* ซึ่งเก็บในพื้นที่จังหวัดเชียงใหม่ ในการทดลองได้สกัดสารสกัดหยาบจากใบ เนื้อไม้ของพืชเหล่านี้และผลของมะคะหลวงโดยใช้เมธานอลและคลอโรฟอร์ม 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 ไมโครกรัมต่อมิลลิกรัม และจากส่วนของเนื้อไม้มีค่าเท่ากับ 9.9, 15.7 และ 8.1 ไมโครกรัมต่อมิลลิกรัมตามลำดับ สารสกัดส่วนคลอโรฟอร์มของสารสกัดเมธานอลจาก *H. hookerianum* และ *G. xanthochymus* แสดงฤทธิ์ในการยับยั้งการเจริญของเซลล์มะเร็งทั้งสามชนิดโดยมีค่า GI_{50} น้อยกว่า 20 ไมโครกรัมต่อมิลลิกรัม ได้คัดเลือก *H. hookerianum* และ *S. verruculosa* ในการศึกษาโครงสร้างทางเคมี เนื่องจากยังไม่มีรายงานการศึกษาในเรื่องนี้ อีกทั้ง *H. hookerianum* ยังแสดงฤทธิ์ที่ดีจากการทดสอบฤทธิ์ในการยับยั้งการอักเสบและฤทธิ์ในการยับยั้งการเจริญเติบโตของเซลล์มะเร็ง

จากการสกัดแยกเนื้อไม้ของต้น *H. hookerianum* ส่วนคลอโรฟอร์มของสารสกัดเมธานอลได้สารทั้งสิ้น 5 ตัวคือ 5-hydroxy-2-methoxyxanthone (HH1), 2-hydroxy-3-methoxyxanthone (HH2), xanthonolignoid *trans*-kielcorin (HH3), betulinic acid-3 β -yl caffeate (HH5) และสารใหม่ 4-hydroxy-3-methoxyphenyl ferulate (HH4) ส่วนสารที่ได้จากการสกัดแยกเนื้อไม้ของต้น *S. verruculosa* 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-3 β -yl caffeate (HH5) จาก *H. hookerianum* มีฤทธิ์สูงในการยับยั้งการเจริญเติบโตของเซลล์มะเร็ง โดย 4-hydroxy-3-

methoxy phenyl ferulate (HH4) มีค่า GI_{50} เท่ากับ 15.1, 18.7, 15.9 และ 21.2 ไมโครโมลาร์ และ betulinic acid-3 β -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-3 β -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) แสดงฤทธิ์ที่สูงใกล้เคียงกับวิตามินซี โดยมีค่า IC_{50} เท่ากับ 6.4 ไมโครโมลาร์ 4-hydroxy-3-methoxy phenyl ferulate (HH4) และ betulinic acid-3 β -yl caffeate (HH5) แสดงฤทธิ์ปานกลาง โดยมีค่า IC_{50} เท่ากับ 48.2 และ 15.6 ไมโครโมลาร์ ตามลำดับ จากผลการศึกษานี้ชี้ให้เห็นว่าพืชที่ทำการศึกษานี้มีศักยภาพที่จะสามารถพัฒนาเพื่อเป็นยาใหม่ได้ต่อไป

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 IC₅₀ 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 GI₅₀ 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 wildy 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.

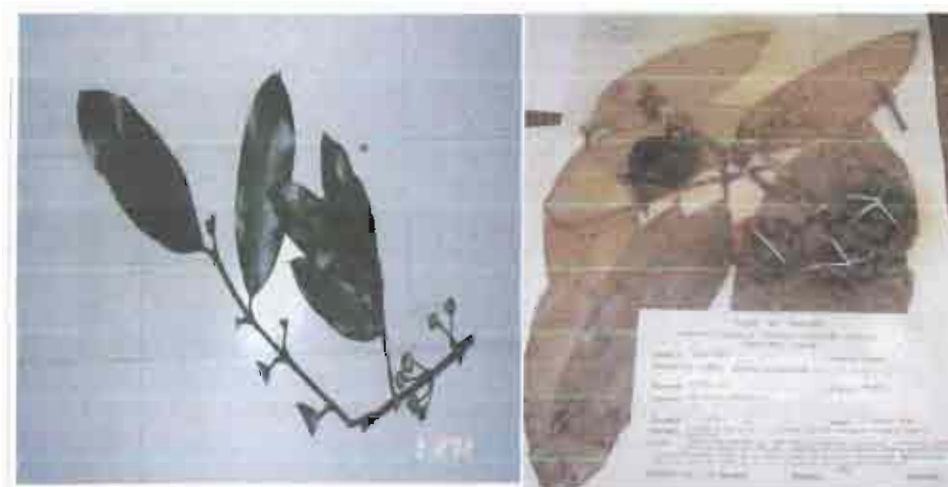


Figure 1.3 *Garcinia xanthochymus* Hook. F. ex T. Anderson

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 μ l of the methanol extracts (1 mg/ml-6.25 μ g/ml) and 75 μ l of 200 μ M 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 $\mu\text{g}/\text{ml}$ 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-CHCl₃, CHCl₃, CHCl₃-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- CHCl₃, CHCl₃, CHCl₃-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 ^1H , ^{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 ($\leq 0.25\%$) 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 $\mu\text{g}/\text{ml}$ 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 $2-3 \times 10^6$ cells/ml in RPMI-1640 supplemented with 10% FBS, 2 mM glutamine and 50 μ g/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 Guttiferae and Schisandraceae.

Plants	Moisture content (%)	Yield (%)					
		Wood		Leaf		Fruit	
		MeOH	CHCl ₃	MeOH	CHCl ₃	MeOH	CHCl ₃
Guttiferae							
<i>H. hookerianum</i>	8.65	7.36	2.01	ND	ND	ND	ND
<i>G. speciosa</i>	39.90	12.18	0.66	8.61	4.33	ND	ND
<i>G. xanthochymus</i>	41.80	19.04	1.02	12.45	1.67	23.60	9.69
<i>C. formosum</i> ssp. <i>pruniflorum</i>	38.39	3.77	1.01	14.60	4.50	ND	ND
<i>C. polyanthum</i>	48.18	4.46	2.65	11.10	8.50	ND	ND
Schisandraceae							
<i>S. verruculosa</i>	31.61	1.77	1.22	4.14	3.94	ND	ND

Note: MeOH = methanol extract; CHCl₃ = 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 IC₅₀ 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						
<i>H. hookerianum</i>	19.08	65.42	-	-	-	-
<i>G. speciosa</i>	9.75	142	65.13	168	-	-
<i>G. xanthochymus</i>	32.10	89.56	58.69	59.83	25.58	26.68
<i>C. formosum</i> ssp. <i>pruniflorum</i>	23.96	91.04	93.28	162.34	-	-
<i>C. polyanthum</i>	44.29	242.25	51.88	69.41	-	-
Schisandraceae						
<i>S. verruculosa</i>	23.34	127.34	130.00	162.18	-	-

Note: MeOH = methanol extract; CHCl₃ = chloroform fraction of the methanol extract

IC₅₀; the concentration of extract which showed 50% DPPH scavenging activity

Table 2 demonstrated the IC₅₀ of the extracts. All methanol extracts gave lower IC₅₀ 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 $\mu\text{g/ml}$ 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 $\mu\text{g/ml}$, 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 $\mu\text{g/ml}$ respectively). For *G. xanthochymus*, the IC_{50} values of methanol and chloroform fraction of the methanol extract were not significant by different in fruits (25.58 and 26.68 $\mu\text{g/ml}$) and leaves (58.69 and 59.83 $\mu\text{g/ml}$). 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 ($\leq 0.25\%$) 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 Guttiferae and Schisandraceae on the growth of human cancer cell lines.

Plant species		GI ₅₀ (µg/ml)		
		HeLa	KB	B16F10
Guttiferae				
<i>H. hookerianum</i>	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
<i>G. speciosa</i>	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
<i>G. xanthochymus</i>	M	130.0	123.3 ± 5.8	105.0 ± 7.1
	C	13.3 ± 1.5	19.0 ± 1.0	11.5 ± 0.7
<i>C. formosum</i> ssp. <i>pruniflorum</i>	M	> 250	193.3 ± 5.8	> 250
	C	41.3 ± 1.5	37.3 ± 0.6	44.5 ± 2.1
<i>C. polyanthum</i>	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				
<i>S. verruculosa</i>	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 GI₅₀ that are arithmetical means ± SD of 3 independent experiments performed in duplicate.

Doxorubicin was used as positive control (GI₅₀ HeLa = 300 ± 0.9 nM ; GI₅₀ KB = 330 ± 0.9 nM; GI₅₀ 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 Guttiferae and Schisandraceae on the growth of human cancer cell lines.

Plant species		GI ₅₀ (μg/ml)		
		HeLa	KB	B16F10
Guttiferae				
<i>H. hookerianum</i>	M	ND	ND	ND
	C	ND	ND	ND
<i>G. speciosa</i>	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
<i>G. xanthochymus</i>	M	223.3 ± 11.6	116.7 ± 5.8	160.0
	C	17.0 ± 1.0	29.3 ± 0.6	37.7 ± 4.0
<i>C. formosum</i> ssp. <i>pruniflorum</i>	M	> 250	> 250	> 250
	C	223.3 ± 20.1	180.0 ± 10	> 250
<i>C. polyanthum</i>	M	> 250	> 250	> 250
	C	19.0 ± 2.7	13.3 ± 0.6	11.0 ± 1.1
Schisandraceae				
<i>S. verruculosa</i>	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 GI₅₀ that are arithmetical means ± SD of 3 independent experiments performed in duplicate.

Doxorubicin was used as positive control (GI₅₀ HeLa = 300 ± 0.9 nM ; GI₅₀ KB = 330 ± 0.9 nM; GI₅₀ 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 $\mu\text{g/ml}$ 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 $\mu\text{g/ml}$.

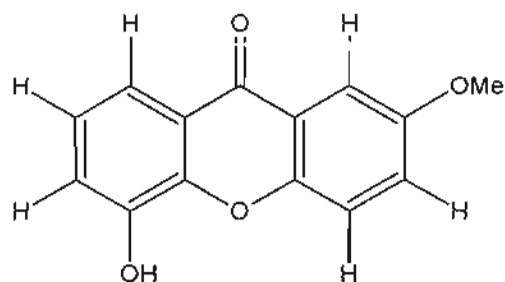
From the wood, the chloroform fraction of the methanol extract of *G. speciosa* also showed strong cell growth inhibition with the GI_{50} values of 9.9, 15.7 and 8.1 $\mu\text{g/ml}$ on B16F10, HeLa and KB cell lines, respectively. Chloroform fraction of the methanol extracts of *H. hookerianum*, and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

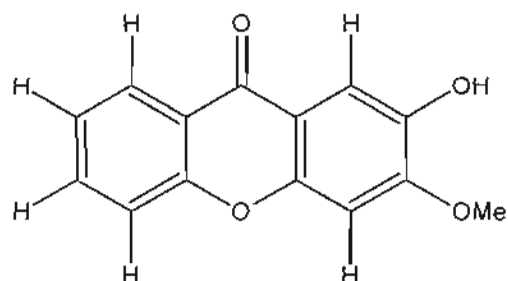
The results from this study suggested a potential for the plants with significant growth inhibitory activity for possible further study and development of pure compounds in the crude extracts to new pharmaceuticals.

3 Purification and structure elucidation of the selected plants

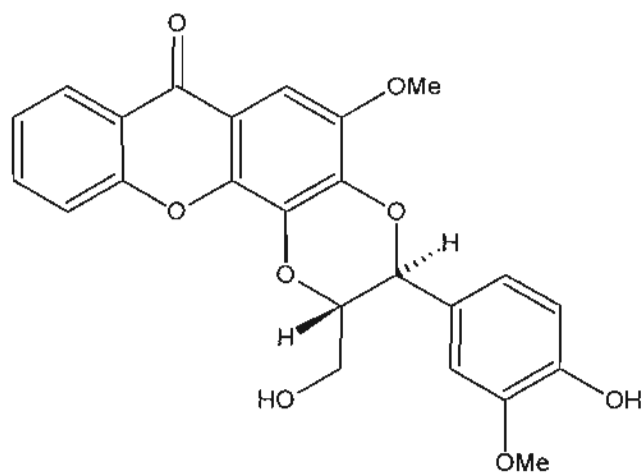
3.1 Isolated compounds from *H. hookerianum*



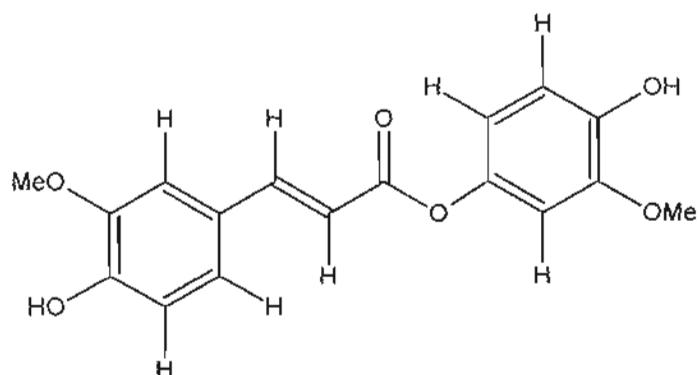
(HH1) 5-hydroxy-2-methoxyxanthone ($C_{14}H_{10}O_4$, MW= 242)



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

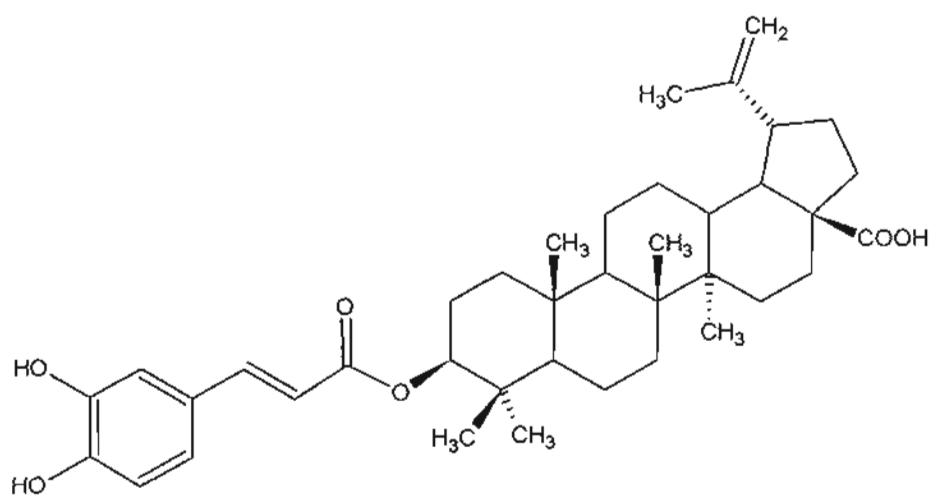


(HH3) trans-kielcorin ($C_{24}H_{20}O_8$, MW = 436)

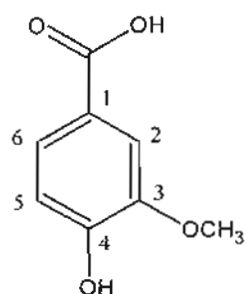


(HH4) 4-hydroxy-3-methoxy phenylferulate ($C_{17}H_{16}O_6$, MW= 316)

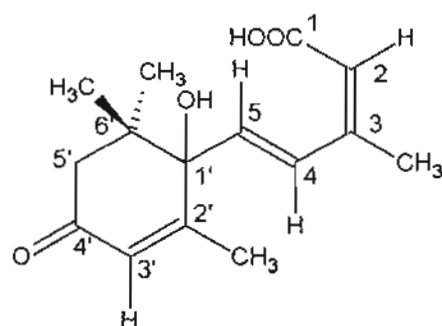
This compound has not been described previously.



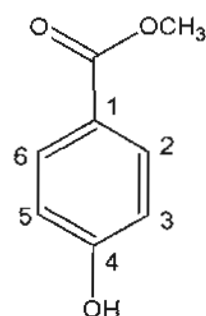
(HH5) Betulinic acid 3 β -ylcaffeate ($C_{30}H_{47}O_2$, MW= 618)

3.2 Isolated compounds from *S. verruculosa*

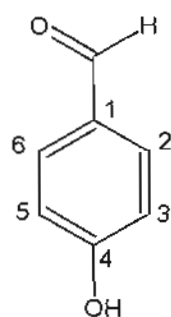
(S1) 4-Hydroxy-3-methoxybenzoic acid (Vanillic acid)



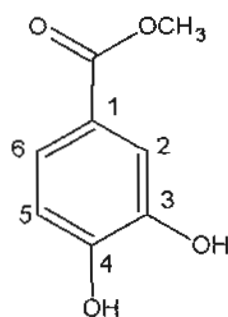
(S2) Absciscic acid



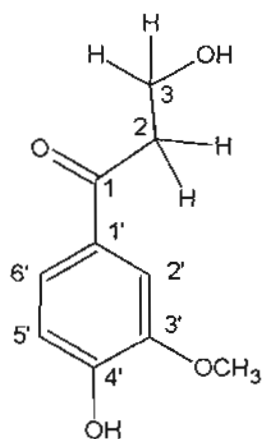
(S3) Methyl 4-hydroxybenzoate



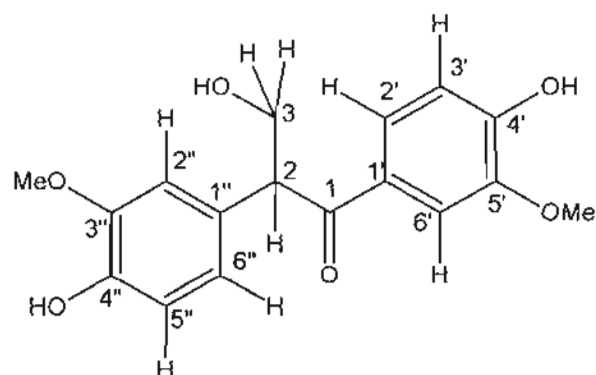
(S4) 4-Hydroxybenzaldehyde



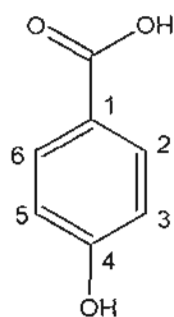
(S5) Methyl 3,4-dihydroxybenzoate



(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

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

^aResults show means $\pm$ SEM of 3-4 independent experiments performed in duplicate.

^bData from the positive control doxorubicin are expressed in nM.

Table 6 The GI₅₀ 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

^aResults show means ± SEM of 3–4 independent experiments performed in duplicate.

^bData from the positive control doxorubicin are expressed in nM.

The GI₅₀ of the compounds isolated from *S. verruculosa* 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 (IC₅₀), 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 IC₅₀ 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. verruculosa* and the results, given in concentrations that were able to cause 50% inhibition of proliferation (IC₅₀), 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} (μ M)
HH1	> 100
HH2	> 100
HH3	> 100
HH4	48.2 $\pm$ 6.1
HH5	15.6 $\pm$ 0.8
Ascorbic acid	5.3 $\pm$ 0.2

Results show means $\pm$ SEM of 3-4 independent experiments performed in triplicate.