ฤทธิ์ต้านอนุมูลอิสระ และความเป็นพิษต่อเซลล์ของสารสกัดจากพืช *Camellia nitidissima* Chi.

Hua Zhu[1], Xu Zhao[1], บังอร ศรีพานิชกุลชัย[2*], แคทรียา สุทธานุช[2], Xiao-Xun Wang[1]

บทคัดย่อ

ฤทธิ์ต้านอนุมูลอิสระ และความเป็นพิษต่อเซลล์ของสารสกัดจากพืช *Camellia nitidissima* Chi.

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บทนำ: *Camellia nitidissima* Chi. เป็นพืชสมุนไพรของจีน เรียกว่า "จินฮัวชา" ในยาแผนโบราณของจีน โดยใช้ส่วนใบในการรักษาโรคตับ, ฝีอักเสบ, ท้องเสีย, ความดันโลหิตสูง, และเนื้องอก ในการศึกษาครั้งนี้มีวัตถุประสงค์เพื่อตรวจสอบฤทธิ์ทางชีวภาพ ฤทธิ์ต้านอนุมูลอิสระ และความเป็นพิษต่อเซลล์ของสารสกัดจากใบ *Camellia nitidissima* Chi. **วัสดุและวิธีการทดลอง:** โดยเตรียมส่วนสกัดชั้นต่างๆ จากผงแห้งของใบ *Camellia nitidissima* Chi. โดยวิธีการหมักผงใบแห้งในตัวทำละลายชนิดต่างๆ ได้แก่ 70% เอทานอล (F1), 40 – 60% อีเทอร์ (F2), เอทิลอะซิเตต (F3), เอ็น-บิวทานอล (F4) และน้ำ (F5) แล้วตรวจสอบฤทธิ์ต้านอนุมูลอิสระ โดยใช้ DPPH assay และความเป็นพิษต่อเซลล์ โดยใช้ MTT assay **ผลการศึกษา:** ผลการศึกษาพบว่า ความสามารถในการออกฤทธิ์ของส่วนสกัดชั้นต่างๆ มีความแตกต่างกัน โดยส่วนสกัดชั้นน้ำ (F5) แสดงฤทธิ์ต้านมนุมูลอิสระที่ดีกว่าส่วนสกัดชั้นอื่นๆ (EC50 = 63.31 ppm) ในขณะส่วนสกัดชั้น เอ็น-บิวทานอล (F4) แสดงความเป็นพิษต่อเซลล์ ด้วยค่า IC50 เท่ากับ 184.0 µg/ml ซึ่งเป็นส่วนสกัดที่ควรทำการศึกษาเพิ่มเติมเกี่ยวกับฤทธิ์ต้านเซลล์มะเร็งต่อไป **สรุปผล:** การศึกษานี้พบว่า *Camellia nitidissima* Chi. แสดงฤทธิ์ต้านการเกิดออกซิเดชันและต้านเซลล์มะเร็งตับ HepG2 ซึ่งควรมีการศึกษาเพิ่มเติมเกี่ยวกับกลไกการออกฤทธิ์, ประสิทธิภาพในการรักษา, และความปลอดภัย

คำสำคัญ: Camellia nitidissima Chi., ฤทธิ์ต้านอนุมูลอิสระ, ฤทธิ์ความเป็นพิษต่อเซลล์, DPPH assay, MTT assay

Abstract

Antioxidative and Cytotoxic Effect of the Extract from *Camellia nitidissima* Chi.

Hua Zhu[a], Xu Zhao[a], Bungorn Sripanidkulchai[b,*], Khaetthareeya Sutthanut[b], Xiao-Xun Wang[a]

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Introduction: *Camellia nitidissima* Chi. leaves or yellow camellia is a traditional Chinese herb called "Jin Hua Cha" in Chinese medicine. It is commonly used for the treatment of liver diseases, faucitis, diarrhea, edema, hypertension, and tumor. This study aimed to investigate the antioxidative and cytotoxic effects of the extracts from

[1] Faculty of Pharmacy, Guangxi Traditional Chinese Medical University, Nanning, China.

[2] ศูนย์วิจัยและพัฒนาผลิตภัณฑ์สุขภาพจากสมุนไพร คณะเภสัชศาสตร์ มหาวิทยาลัยขอนแก่น

* ติดต่อผู้นิพนธ์: โทรศัพท์/โทรสาร 043-203149 e-mail: bungorn@kku.ac.th

[2] Center for Research and Development of Herbal Health Products. Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, Thailand.

* Corresponding author: Tel/Fax 043-203149 e-mail: bungorn@kku.ac.th

Camellia nitidissima Chi. leaves. **Materials and Method:** Dried Camellia nitidissima Chi. leaves were extracted with 70% ethanol (F1), then further extracted with 40-60% petroleum ether (F2), ethyl acetate (F3), n-Butanol (F4) and water (F5) individually to obtain 5 fractions. The plant extracts were determined for antioxidative activity by DPPH assay, and cytotoxicity effect to HepG2 hepatoma cell line by MTT assay. **Results:** The results demonstrated the different potency of action among the different extracts. The F5 (water fraction) had notable anti-oxidative potential with EC_{50} of 63.31 ppm. Based on the lowest IC_{50} at 184.0 µg/ml of F4 (n -butanol fraction) this fraction should be further studied for anti-cancer activity. **Conclusion:** The antioxidant properties and cytotoxicity to a HepG2 hepatoma cell line of *Camellia nitidissima* Chi. leave extracts were studied. However, further studies should consider identifying related mechanisms, efficacy of action, and safety for use.

Keywords: *Camellia nitidissima* Chi., Antioxidative effect, Cytotoxic effect, DPPH assay, MTT assa

Introduction

Camellia nitidissima Chi., the yellow camellia belonging to Theaceae family, is one of rare plants first discovered in 1933 (Deng *et al.*, 2000). It is distributed in a narrow region of Guangxi Province, South China, and North Vietnam, where it grows in acidic soils along shady and moist valley under evergreen forests at the latitudes of 50–650 m (Wei *et al.,* 2008; Yang *et al.*, 2008). In the early 1970s, C. *nitidissima* was honored as “the queen of camellias family” (Liang, 1993); It is one of the typical species in sect nitidissima (Ye and Xu, 1992). C. *nitidissima* has the big entomogamous flower, golden, and transparent waxy petal (Liang, 1993). It has been introduced to Japan, Australia, and North America as a useful genetic resource for commercial cultivation ofcamellias, attracting extensive attention of horticultural workers worldwide (Nishimoto *et al.*, 2004; Parks, 2000). In addition, it has been reported that the leaves, flowers, and seed oils of C. nitidissima can be of value in food and Chinese traditional medicine (Liang, 1993). In China, natural populations of camellias including C. *nitidissima* have been investigated extensively for several decades. C. *nitidissima* has been found only in two disjunctive areas of Guangxi: one is in the junction of Fushu, Longan, and Fusui near Nanning City, and the other is in Fangcheng, south of Mount Shiwan (Su and Mou, 1988). Due to deforestation and collection of seeds for horticulture, its natural population has declined dramatically in recent decades. C. *nitidissima* has been included in the checklist of State Protection Category I in China (Fu, 1992). To support the conservation and management programs for C. nitidissima, currently, a yellow Camellia public park was set up in Nanning of Guangxi and this park becomes a new sight spot in Nanning (Yan *et al.*, 2003).

In traditional Chinese medicine, C. *nitidissima* Chi. leaves have the effects of heat-clearing and detoxication, strangury-relieving, anti-cancer, inhibition of tumor growing, reducing blood lipid, preventing atherosclerosis, increasing the immune function, and draining dampness (Fu *et al.*, 2005). Normally, it uses to treat faucitis, diarrhea, edema, hypertension, and prevent liver disease and tumor (Huang, 1999). C. *nitidissima* Chi. leaves contain several of trace elements: germanium, selenium, manganese, molybdenum, vanadium, and zinc, polyphenols, and essential amino acid.

In the recent years, the extract from the plant belonging to same genus, *Camellia chrysantha* was reported to have remarkable antioxidation properties to the free radical which is produced by the Fenton reacting system and pyrogallic acid autoxidation (Qin *et al.*, 2008; Yan and Yao, 2009). Moreover, the leaves of C. *chrysantha* show inhibitory effect on the DEN-induced precancerous lesion of rat liver with dose-dependent manner (Duan *et al.*, 2006) and the human hepatoma cells BEL-7404 cultured in vitro (Li *et al.*, 2007). Therefore, this study aims to determine antioxidative and cytotoxic activity to the human hepatoma cell line (HepG2 cells) of C. nitidissima crude extracts by using DPPH and MTT assays, respectively.

Methods

1. Chemicals

95% ethanol, petroleum ether (Analytical reagent A.R.), ethyl acetate (HPLC grade) and 1-Butanol (AR grade) were purchased from Labscan Asia Co.,Ltd. (Bangkok, Thailand). Dulbecco's Modified Eagle Medium (DMEM) powder was obtained from Invitrogen corporation. All the other chemicals used were of analytical grade.

2. Preparation of herbal extract

C. nitidissima Chi. leaves were collected from Fang Cheng in Nanning, Guangxi, China. 500 g of the dried leaf powder was continuously macerated in 6 liters of 70% ethanol for 48 h, and twice repeated. Then, the supernatant was collected and filtered through Whatman no.1 filter to obtain the filtrate for drying by using rotary evaporator and freeze dryer. The dried crude extract was delivered as **'F1'**. The further fractionation was performed by dissolving 20 g of F1 in 350 ml of distilled water and successively partitioned with 350 ml of different solvents in order of petroleum ether, ethyl acetate, and n-Butanol. With % yield of 0.195-5.92, the dry extracts from each solvent was obtained as **F5** (aqueous fraction) abd **F2, F3** and **F4,** respectively (Table 1).

Table 1 Physical appearance and % yield of C. *nitidissima* leaves extracts

Fraction	Type of solvent for extraction	Physical appearance	% yield
F1	70% Ethanol	Rough and dark brown powder	5.92
F2	Petroleum ether	Rough and brown powder	0.20
F3	Ethyl acetate	Viscous and sticky dark purple mass	0.72
F4	*n*-butanol	Rough and yellow powder	2.45
F5	Distilled water	Rough and dark yellow powder	1.89

3. Determination of antioxidant activity

2,2-Diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical. When DPPH is reduced, the color of solution will change from violet to pale yellow (Milardovic and Ivekoric, 2006). It has been commonly used to screen phenolic compounds containing high free radical scavenging ability (Wettasinghe and Shahidi, 2000). Currently, DPPH method has been used as one of basic screening steps for searching new antioxidant compounds from natural resources including spices, herbs, fruits, and vegetables (Kim and Kim, 2006; Lee *et al.*, 2004; Xu *et al.*, 2005). Therefore, DPPH assay as described by Sripanidkulchai *et al.* (2008) was used in this study.

Following the method reported by Sripanid kulchai *et al.* (2008), the anti-oxidation activity was determined by using DPPH assay. Firstly, the sample and standard solutions were prepared. The sample solutions of the crude extracts (F1- F5) were separately prepared by dissolving in methanol to obtain the concentration of 150 ppm solutions. Using vitamin C as the reference standard, the standard solution concentration of 50 ppm was prepared. Then, the anti-oxidation effects of samples and vitamin C were determined which the reaction mixture contained 2,800 µl of the sample and 200 µl of 1 mM DPPH dissolved in methanol. The mixture was vigorously shaken and kept at room temperature for 15 minutes. The absorbance (A) was measured at 515 nm. The results were expressed as EC_{50} (50% inhibition concentration).

4. Determination of Cytotoxic activity

When 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetra zolium bromide (MTT) is reduced in the mitochondria of living cells resulting of the purple formazan which can be quantified by using spectro photometer. The amount of MTT formazan formed directly relates to the number of viable cells, the MTT test was commonly used to check for the cytotoxic

effect (Mossman, 1983) and it was successfully used for herbal effect on cell growth (Liu *et al.*, 2000).

4.1 Preparation of herbal test sample

The plant extract was dissolved with DMSO to make a concentration of 100 mg/ml.

4.2 Cell culture and treatment

The human hepatoma cells (HepG2) were cultured and seeded into 96-wells ($1x10^5$ cells/ml), and then incubated at 37°C, 5% CO2 for 24 h. Cells were treated with various concentrations, with triplication for each concentration, of the herbal extracts ranging between 0-400 µg/ml and incubated for 18 h. Then, MTT stock solution (5 mg/ml) was diluted to 1 mg/ml in culture medium as a working MTT solution. The treated cells were added with working MTT solution (50 µl/well) to analyze cell viability. After 3 h incubation, the culture medium was removed. The formazan was dissolved with DMSO and measured the absorbance by using microplate reader at wavelength 570 nm and 650 nm as a reference. The %inhibition and IC_{50} were calculated and expressed as Mean ± SD of IC_{50}.

Results

1. Antioxidant activity

As shown in Fig 1, standard vitamin C had strong antioxidative activity with EC50 of 6.80±0.25 ppm (N = 3). Among F1-F5 of C. nitidissima leaf, only the water fraction (F5) was shown to have notable anti-oxidative potential with EC50 of 63.32 ppm (Fig 2); other fractions showed EC50 greater than 300 ppm (results not shown).

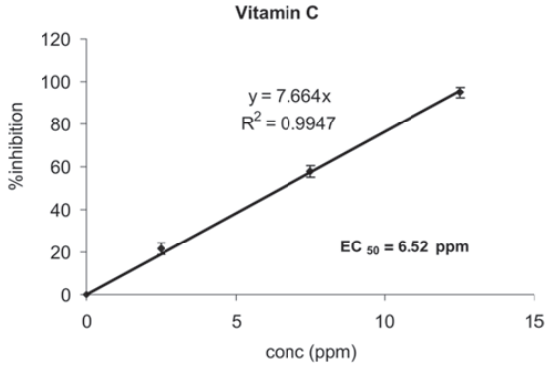

Figure 1 Inhibition curve of vitamin C from DPPH assay

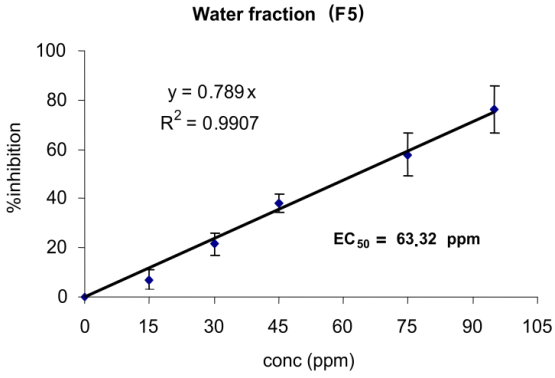

Figure 2 Inhibition curve of water fraction (F5) of C. *nitidissima* leaves from DPPH assay

2. Cytotoxic activity

The typical morphological changes of HepG2 cells were observed when treated with the plant fractions. In general, the characteristics of cell apoptosis including cytoplasmic and nuclear shrinkage, chromatin condensation, and membrane blebbing were demonstrated (Fig 3). F4 or n-butanol fraction showed the most cytotoxic effect with smallest IC50 at 183.96 ppm, whereas F5 or water fraction did not shown cytotoxic effect to HepG2 cells (Fig 4).

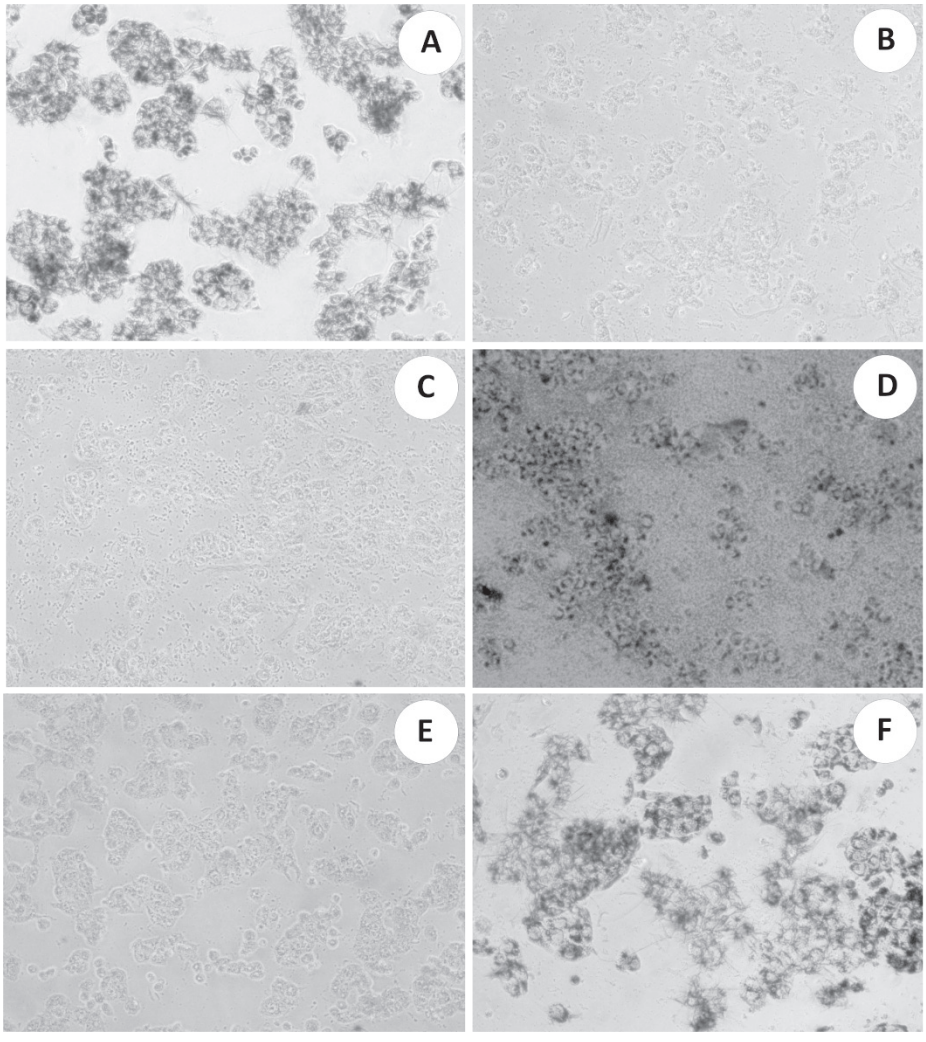

Figure 3 Morphological changes of HepG2 cells after treated with 400 μg/ml of C. nitidissima fractions (A = control, B = F1, C = F2, D = F3, E = F4, F = F5).

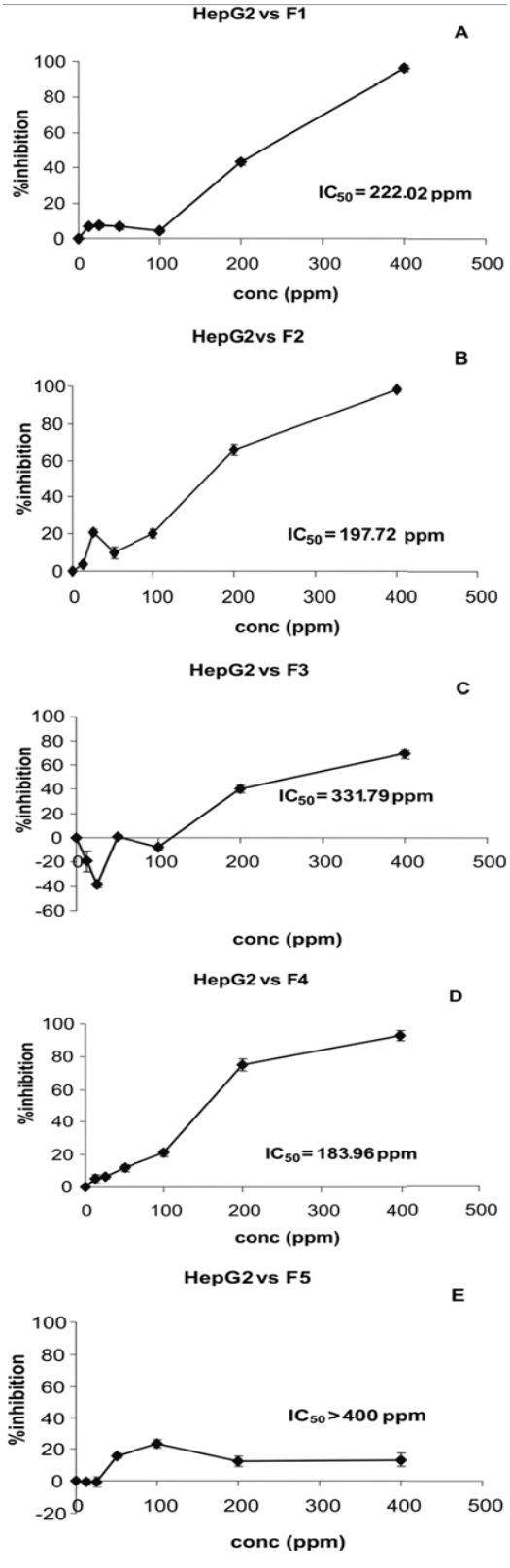

Figure 4 The inhibition curve of five fractions of C. *nitidissima* leaves on HepG2 cells.
(A = F1, B = F2, C = F3, D = F4, E = F5).

Discussion and Conclusion

This study demonstrated the pharmacological potential of C. *nitidissima* leaf extracts. By comparing EC50 of DPPH assay, an aqueous fraction (F5) showed notable antioxidative activity, however it is ten times less than standard vitamin C. This finding is concurrent with the previous report that commonly found an aqueous extractable phenolic compounds in plant leaf (Sripanidkulchai *et al.*, 2008). Under the cytotoxicity assay on hepatoma cell line (HepG2) by MTT assay, we revealed that n-butanol fraction (F4) has strongest activity. According to their IC50, the relative order of cytotoxicity was F4 > F2 > F1 > F3, whereas, the F5 showed no affect on HepG2 cells. This may be attributed to the differences of chemical composition among the fractions. Therefore, F4 would be the candidate fraction to be further isolated and purified to identify the potential active ingredients as for the anticancer agents. In conclusion, from its antioxidative effects found in water fraction (F5) and hepatoma cells cytotoxicity of other fractions (F1 - F4), C. *nitidissima* seems not to be only the ornamental but also the medicinal plant with showing of the potential on cancer protecting and treatment. However, further studies should be considered to explore the other health benefits and involving mechanisms.

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