RESEARCH ARTICLE

Total Phenolic Content, Antioxidant and Antiproliferative Activities of Methanolic Extract from Flowers, Twigs and Peels of *Mammea siamensis*

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Abstract

Liver cancer remains the first leading cause of cancer death in Thai patients. Nowadays, cancer chemotherapy and radiation therapy often cause side effects. The use of natural products may be an alternative for alleviating these side effects. Plants are potential sources of natural products exhibiting antioxidant and antiproliferative effects against cancer cells. *Mammea siamensis* (or Saraphi) is a Thai medicinal herb rich in coumarins and xanthones, which has been demonstrated to possess anticancer properties in many cancer cell lines. The aims of this study were to evaluate total phenolic content, antioxidant and antiproliferative activities of the methanolic extracts from flowers, twigs and peels of *M. siamensis*. The results showed that 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacities of the extracts from flowers, twigs and peels of *M. siamensis* were 21.77±0.21, 19.2±0.21 and 10.65±0.3 mg ascorbic acid equivalent (AAE)/g, which were correlated with their total phenolic contents (43.34±0.51, 43.11±1.01, and 29.22±0.83 mg gallic acid equivalent (GAE)/ g). Moreover, the methanolic extracts from flowers and twigs exhibited greater antiproliferative activity against hepatocellular carcinoma (HepG2) cells than that from peels, as shown by the IC_{50} values of 26.97±3.62, 27.99±1.04, and 187.19±4.71 µg/mL, respectively. This study concluded that there was a correlation between antioxidant activity and total phenolic content in the extracts. The methanolic extracts from flower and twigs of *M. siamensis* also possessed antiproliferative activity against HepG2 cells. The bioactive compounds from the active parts of *M. siamensis* should be identified in further studies.

Keywords: Total phenolic content, antioxidant, antiproliferative, *Mammea siamensis*, Saraphi, hepatocarcinoma cell line

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ปริมาณฟีนอลิกทั้งหมด ฤทธิ์ต้านอนุมูลอิสระ และฤทธิ์ยับยั้งการเจริญของเซลล์มะเร็งของสารสกัดเมทานอลจากดอก กิ่งและเปลือกผลของสารภี

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บทคัดย่อ

โรคมะเร็งตับเป็นสาเหตุลำดับแรกของการเสียชีวิตจากโรคมะเร็งในผู้ป่วยไทย ปัจจุบันการรักษาด้วยเคมีและรังสีบำบัดมักทำให้เกิดผลข้างเคียงแก่ผู้ป่วย การใช้ผลิตภัณฑ์ธรรมชาติอาจเป็นทางเลือกหนึ่งในการลดผลข้างเคียงดังกล่าว พืชจัดเป็นแหล่งสำคัญของผลิตภัณฑ์ธรรมชาติที่มีฤทธิ์ต้านอนุมูลอิสระและฤทธิ์ยับยั้งการเจริญของเซลล์มะเร็ง *Mammea siamensis* (หรือสารภี) เป็นพืชสมุนไพรไทยที่มีสารกลุ่มคูมารินและแซนโทนในปริมาณสูง ซึ่งเคยมีการรายงานว่าสารดังกล่าวมีฤทธิ์ต้านมะเร็งในเซลล์มะเร็งเพาะเลี้ยงหลายชนิด งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาปริมาณสารฟีนอลิกทั้งหมด ฤทธิ์ต้านอนุมูลอิสระและฤทธิ์ยับยั้งการเจริญของเซลล์มะเร็งของสารสกัดเมทานอลจากดอก กิ่งและเปลือกผลของสารภี ผลการศึกษาพบว่า ฤทธิ์ต้านอนุมูลอิสระ 2,2-diphenyl-1-picrylhydrazyl (DPPH) ของสารสกัดจากดอก กิ่ง และเปลือกผลของสารภี มีค่าเท่ากับ 21.77±0.21, 19.2±0.21 และ 10.65±0.31 มิลลิกรัม ascorbic acid equivalent (AAE) ต่อกรัม ซึ่งมีความสอดคล้องกับปริมาณของฟีนอลิกทั้งหมดในสารสกัด (43.34±0.51, 43.11±1.01 และ 29.22±0.83 มิลลิกรัม gallic acid equivalent (GAE) ต่อกรัม) นอกจากนี้ยังพบว่า สารสกัดเมทานอลจากดอกและกิ่งมีฤทธิ์ยับยั้งการเจริญของเซลล์มะเร็งตับ HepG2 ได้ดีกว่าสารสกัดจากเปลือกผล โดยมีค่า IC_{50} เท่ากับ 26.97±3.62, 27.99±1.04 และ 187.19±4.71 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ ผลการศึกษาสรุปได้ว่า ฤทธิ์ต้านอนุมูลอิสระมีความสัมพันธ์กับปริมาณสารฟีนอลิกทั้งหมดในสารสกัด และสารสกัดเมทานอลจากดอกและกิ่งสารภี มีฤทธิ์ยับยั้งการเจริญของเซลล์มะเร็งตับ HepG2 ซึ่งควรมีการศึกษาวิจัยต่อยอดเพื่อวิเคราะห์หาสารสำคัญที่มีฤทธิ์ดังกล่าวต่อไป

คำสำคัญ: ปริมาณสารฟีนอลิกทั้งหมด, ฤทธิ์ต้านอนุมูลอิสระ, ฤทธิ์ยับยั้งการเจริญของเซลล์มะเร็ง, *Mammea siamensis*, สารภี, เซลล์มะเร็งตับเพาะเลี้ยง

Introduction

The phytochemicals from plants especially phenolic compounds have been gradually discovered to be potential sources of new drugs. The antioxidant activity of phenolic compounds is mainly due to their redox properties, which can be of high importance in adsorbing and neutralizing free radicals or decomposing peroxides.[1] Currently, many plants with high phenolic contents and antioxidant activity are recommended for prevention and treatment in several human diseases.[2,3] Bioactive plant compounds could also possess anticancer activity by affecting the normal biological process of cancer cells, including initiation, promotion and progression.[4]

In 2017, data from Thai death certificates showed that liver and intrahepatic bile duct cancer was the first leading cause of cancer death in Thailand by the mortality rate of 25.1 per 100,000 population, followed by trachea, bronchus and lung cancer (21), breast cancer (12.6), cervix cancer (6.8) and prostate cancer (4.3).[5] The most common types of primary liver cancer found in Thailand are hepatocellular carcinoma (HCC) (95%) and cholangiocarcinoma (CCA) (5%).[6] The major risk factors for HCC are viral infections (chronic hepatitis B and hepatitis C) and toxicants (alcohol and aflatoxins)[7], and those for CCA are food-borne parasitic infections.[8] A variety of therapies have been used for the treatment of liver cancer, such as chemotherapy[9], radiotherapy[10], cryo-ablation[11] and transarterial chemo-embolization (TACE).[12] However, these treatments cause serious side effects, including bone marrow depression, hair loss, postembolization syndrome, and liver and renal failure. Therefore, an urgent need is arising for new active and well-tolerated treatments to improve survival among HCC patients.

Mammea siamensis is a Thai medicinal plant belonging to the family Guttiferae, locally known as "Saraphi". The flower is used as a heart tonic, fever-lowering agent and appetite enhancer in Thailand.[13] The fruit is generally considered edible and used as a vasodilator. The chemical constituents of the genus Mammea are known to be a rich source of coumarins and xanthones.[14-16] These phenolic compounds possess multiple biological properties such as antimicrobial, anti-inflammatory, antioxidant and anticancer. A part of these plants have cytotoxic activity on many cancer cell lines including colon cancer (DLD-1), breast cancer (MCF-7), cervical cancer (HeLa), lung cancer (NCI-H460)[17], liver cancer (HepG2)[18], and leukemic cells (K562).[19] However, thus far, there is no information about the total phenolic content and antioxidant activity of *M. siamensis*. Indeed, only a small number of studies reported its cytotoxicity against HepG2 cells.[13,18] This study aimed to assess the total phenolic content, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and antiproliferative activity against hepatocellular carcinoma HepG2 cells of the extracts from different parts of *M. siamensis*.

Materials and Methods

Chemicals and reagents

Folin-Ciocalteu reagent, DPPH, methanol, sodium carbonate and isopropanol were purchased from Merck (Darmstadt, Germany). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco-BRL (Gaithersburg, MD, USA). Gallic acid, ascorbic acid,

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plant material and sample preparation

The flowers, twigs and peels of *M. siamensis* were collected from Prince of Songkla University, Songkhla Province. Each part was dried in an oven at 40°C for 3 days, then ground into powder. Each dried powdered material was extracted with methanol for 7 days. The extracts were filtered using Whatman no.1 filter paper. The methanolic extract was concentrated to dryness using a rotary evaporator under vacuum at 40 °C. Extracts were stored at 25°C until use.

Determination of total phenolic content

The total phenolic content was estimated using the Folin-Ciocalteu assay according to the method developed by Velioglu et al.[20] Briefly, 10 mg of the extract was weighed and dissolved in 1 mL of methanol. Then 100 µL of each extract solution was transferred to a test tube. The 0.75 mL of a 10-fold diluted Folin-Ciocalteu reagent was added and mixed, then allowed to stand at room temperature for 5 min. Then, 0.75 mL of 6% (w/v) sodium carbonate solution was added. The mixture was allowed to stand at room temperature for 90 min. The absorbance of the mixture was measured using a spectrophotometer at wavelength of 725 nm. The total phenolic content of the extracts was calculated based on the calibration curve of gallic acid (0.02-0.10 mg/mL) and expressed as mg gallic acid equivalent (GAE)/g extract.

Antioxidant activity

The radical scavenging activity was determined by DPPH assay according to the method described by Khattak et al. with a slight modification.[21] Briefly, 10 mg of the extract was weighed and dissolved in 1 mL of methanol. The 100 µL of each extract solution was added to 900 µL of DPPH solution in methanol (150 µM) and the mixture was shaken vigorously. After incubation for 15 min at room temperature in the dark, the absorbance of each mixture was measured at 517 nm. The free radical scavenging power was calculated based on the calibration curve of ascorbic acid (0.01-0.05 mg/mL) and was expressed as ascorbic acid equivalent (AAE)/g extract.

Antiproliferative activity against HepG2 cells

Cell culture Confluent HepG2 (ATCC HB-8065) cells were seeded into 96-well plates at 1×10^4 cells/well in DMEM-low glucose supplemented with 3.7 g/L of sodium bicarbonate buffer system, 10% FBS, penicillin (100 IU/mL) and streptomycin (100 µg/mL) and then incubated for 24 h at 37°C in an atmosphere of 95% humidity and 5% CO_2.

MTT assay After incubation, the culture media were replaced with 100 µL of filtrated media mixed with plant extract solution in DMSO (<0.4% v/v in culture media) over a concentration range of 0-1,000 µg/mL or 0.4% v/v of DMSO as vehicle control. After a 24-h incubation, the medium was carefully discarded and 20 µL of MTT solution (1 mg/mL) was added into each well. The plates were left in the incubator for 3 h. Subsequently, the MTT solution was removed and 100 µL of isopropanol was added into each well. The presence of viable cells was indicated by the intensity of purple color due to the formation of formazan crystals. The absorbance

was measured at 570 nm using an automated microplate reader (Molecular Devices, CA, USA). All tests were conducted in triplicate. The percentage of cell viability was calculated as follows: $(A_{Sample}/A_{Control})\times100$. IC_{50} or the concentration of the extract required to achieve half-maximal (50%) inhibition of MTT incorporation is considered as an indicative of antiproliferative activity.

Results and discussion

Herbal medicine is one of the major traditional medicine which has gained a remarkable impact on the treatment of various diseases as well as cancer. Many Thai traditional medicines have been tested for their antiproliferative activity against cancer cell lines including *M. siamensis*. Previous studies reported that the coumarins (thetraphin C) from the bark of this plant strongly inhibited colon cancer (DLD-1), breast adenocarcinoma (MCF-7), human cervical cancer (HeLa), and human lung cancer (NCI-H460) cells with the IC_{50} values in the range of 1.6-5.7 μM.[17] Hexane fraction from the flowers demonstrated the strongest antiproliferative effect on leukemic cells (EoL-1) with an IC_{50} of 3.8±0.8 μg/mL. Furthermore, the methanolic extract from the flowers showed antiproliferative activity against human leukemia (HL-60) and stomach cancer (KATO-III) cells with the IC_{50} values in the range of 9-30 and 10-24 μM, respectively. However, it had no cytotoxicity against HepG2 cells.[13] In the present study, the antioxidant capacity, total phenolic content, and antiproliferative activity against HepG2 cell lines from flowers, twigs, and peels of *M. siamensis* were investigated. The results were summarized in Table 1.

Table 1. Antioxidant activity, total phenolic content and cytotoxicity against HepG2 cells of methanolic extracts from various parts of *M. siamensis.*

Part of plant	Antioxidant activity (DPPH) (mg AAE/g)	Total phenolic content (mg GAE/g)	Cytotoxicity (IC_{50}, μg/mL)
Flowers	21.77±0.21	43.34±0.51	26.97±3.62
Twigs	19.20±0.21	43.11±1.01	27.99±1.04
Peels	10.65±0.31	29.22±0.83	187.19±4.71

AAE, ascorbic acid equivalent; GAE, gallic acid equivalent. All values are expressed as mean± SD (n=3).

The extracts from all parts of *M. siamensis* used in this study (flowers, twigs and peels) were effective for scavenging free radicals in the DPPH assays (21.77±0.21, 19.2±0.21, and 10.65±0.31 mg AAE/g, respectively) and their total phenolic contents were 43.34±0.51, 43.11±1.01, and 29.22±0.83 mg GAE/g, respectively. Effect of the extracts on HepG2 cell viability was shown in Figure 1. The flower and twig extracts from *M. siamensis* possessed cytotoxic activity against HepG2 cells according to the criteria of cytotoxicity activity for crude extract established by the American National Cancer Institute (NCI)[22], with IC_{50} values of 26.97±3.62 and 27.99±1.04 μg/mL.

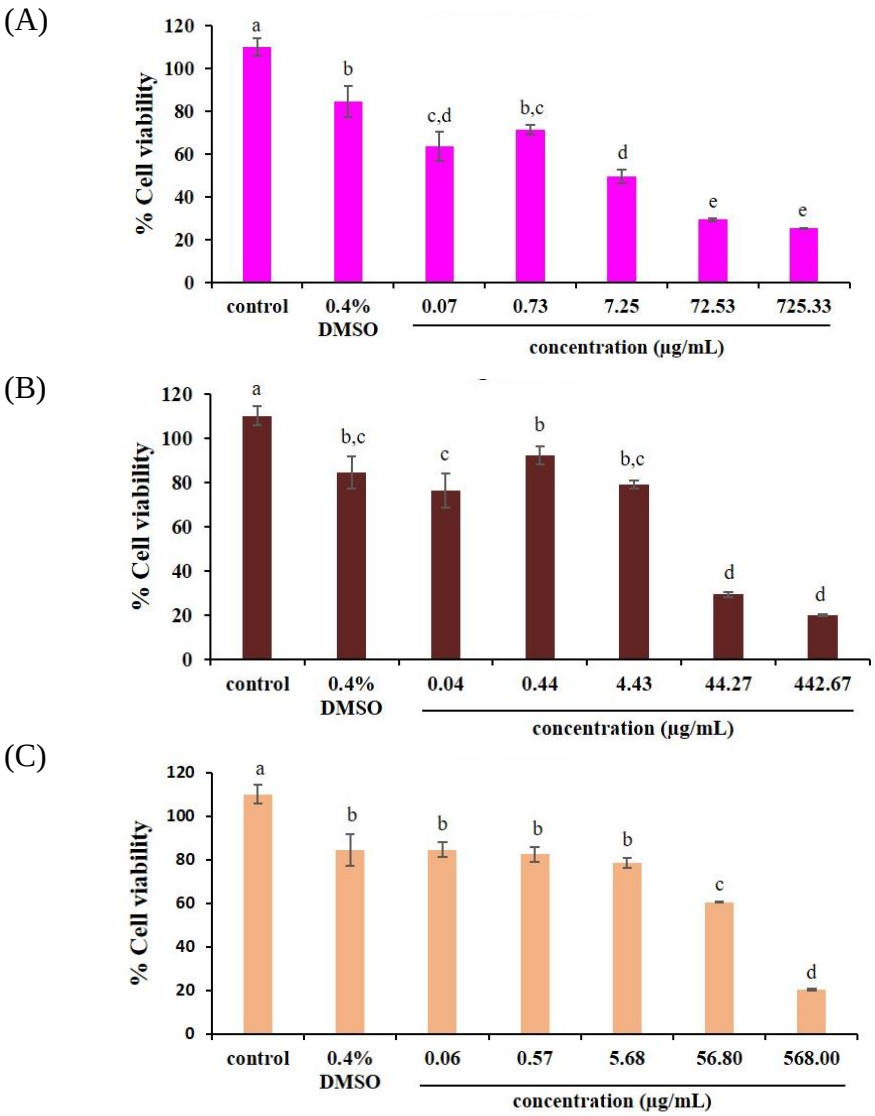

Figure 1. Effect of DMSO and methanolic extracts from (A) flowers, (B) twigs and (C) peels of *M. siamensis* on HepG2 cell viability. Results are shown as mean±SD (n=3). The different alphabet letters indicated statistically different means of each group ($p<0.05$, one-way ANOVA, Scheffe test).

The cytotoxicity on HepG2 cells might be due to the extracts or DMSO as vehicles solvent. The DMSO in culture medium above 0.1-0.5% often decreases the proliferation of cultured cells. Our results found that the 0.4% DMSO, which was the highest concentration added into culture media, had some cytotoxicity on HepG2 cells by decreasing cell viability about 15% compared to untreated control. However, there were significant differences in HepG2 cell viability between cells treated with 0.4% DMSO and the extracts at the highest concentrations (Figure 1), indicating that the cytotoxic effect was resulted from the compounds in methanolic extracts.

This study also demonstrated a positive correlation between the total phenolic contents and their antioxidant and antiproliferative activity, suggesting that phenolic compounds might have major roles in antioxidant and anticancer activities of *M. siamensis*. Phenolic compounds are known to be able to induce the cytotoxicity on various cancer cell lines and the anticancer potency has been shown to be correlated with the amount of polyphenols and antioxidant capacity.[23]

Conclusion

The methanolic extracts from the flowers and twigs of *M. siamensis* showed higher antioxidant and antiproliferative activities than those of peel extract, which may be related to their total phenolic contents. This study reports preliminary findings that were of great interest, since the bioactive compounds from the flowers and twigs of *M. siamensis* may be a promising natural source for anticancer agent. However, the chemical identification of these compounds, their biological effects and molecular mechanisms against the HepG2 cells should be further investigated.

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