Original Article

In Vitro Anticancer and Anti-inflammatory Activities of *Rauwenhoffia siamensis* Scheff Root Extracts

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Abstract

Introduction: The sixth most prevalent malignancy among women is endometrial cancer. *R. siamensis* roots were utilized to treat endometrial cancer in Thai traditional medicine. However, the scientific basis for its action is not entirely understood.

Objectives: To investigate the cytotoxicity of *R. siamensis* root extracts against uterine, ovarian, and cervical cancer cells, anti-inflammatory property and their flavonoid contents.

Methods: Cytotoxicity activity was determined by using sulforhodamine B assay. Anti-inflammatory activity was investigated by inhibition of nitric oxide production. Flavonoid content was analysed using aluminum chloride colorimetric assay.

Results: Observations showed high flavonoid contents in all *R. siamensis* root extracts. Strong cytotoxic activity was detected in the ethanolic extract of *R. siamensis* root as well as production inhibition of nitric oxide.

Conclusions: The ethanolic extract of *R. siamense* showed the significant anticancer activity. To better comprehend its mechanism, active substances should be examined for cytotoxicity, and an animal model should be investigated.

Keywords: *Rauwenhoffia siamensis,* Alternative medicine, Herb, Endometrial cancer, Gynecologic cancer

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Introduction

Rauwenhoffia siamensis Scheff belongs to the family Annonaceae. As a flavoring ingredient, its blooms are employed.[1] Phytochemicals in *R. siamensis* fruits and leaves included amides, benzylated dihydrochalcone derivatives, and flavonoids.[2,3] *R. siamensis* leaves contained chemical substances that exhibited anticancer activity against tumor cell lines KB and NCI-H187.[3] The roots of *R. siamensis* were combined with four other plants in a Thai traditional recipe of Krom Luang Chumphon Khet Udomsak scripture for treating endometrial cancer.[4] Anticancer properties and chemical composition of *R. siamensis* roots have not been reported.

In 2020, the World Health Organization reported that endometrial cancer was the sixth most common cancer in women worldwide (6.8%) between 50 and 59 years.[5] Although the incidence rate of uterine cancer was moderate, patients with endometrial cancer were reported to metastasize to the ovary and cervix.[6,7] However, the inflammatory process can promote cancer progression and enhance cancer angiogenesis.[8] Several inflammatory cytokines, including nitric oxide, IL-6, and TNF-α, accelerated cancer development.[9] *R. siamensis* roots were used for treating endometrial cancer in females by traditional Thai practitioners.[4] On the other hand, its biological activity is not fully understood. This study provided a detailed report on the cytotoxicity of *R. siamensis* root extracts against uterine, ovarian, and cervical cancer cells. Additionally, this study examined the anti-inflammatory properties and the flavonoid content of *R. siamensis* root extracts.

Methods

Plant Materials and Extraction

R. siamense roots were collected from Lampang Province, Thailand. It was defined through the identification of voucher specimens at Thai Traditional Medicine Herbarium, Thai Traditional Medicine Research Institute, Department of Thai Traditional and Alternative Medicine, Bangkok, Thailand. The voucher specimen number was TTM No.0006005. The plant materials were cleaned, cut into small pieces, and dried at 45°C in a hot air oven. The ethanolic extract was obtained by maceration with 95% ethanol. The roots of *R. Siamense* were macerated using 95% ethanol for 72 h at room temperature before being filtered through Whatman No.1 filter paper. Then, the filtrate was concentrated by the rotary vacuum evaporator and dried at 45°C to obtain the ethanolic extract (RSE). Next, the aqueous extract was performed by decoction. The plant material was boiled with water for 15 min, then filtered through Whatman No.1 filter paper. Finally, the filtrate was dried with a lyophilizer to obtain the aqueous extract (RSW). All dried extracts were stored at -20°C until used.

Cell Culture

The ovarian cancer cell line (SKOV-3) ATCC HTB-77 and the macrophage cell line (RAW264.7) ATCC TIB-71 were grown in Dulbecco's modified eagle's medium. The cervical cancer cell line (HeLa) ATCC CCL-2 was grown in Minimum Essential Media. The human endometrial adenocarcinoma cell line (HEC-1-A) ATCC HTB112 was grown in McCoy's 5a medium. All media are supplemented with 10% FBS, 100 units/mL of penicillin, and 100 µg/mL of streptomycin at 37°C in a CO_2 incubator.

Cytotoxicity Activity by Sulforhodamine B (SRB) Assay[10]

All cells were trypsinized to prepare cell suspension. The SKOV-3, Hela, and HEC-1A were seeded on a 96-well plate with concentrations of 1×10^4, 3×10^3, and 4×10^3 cells/well, respectively. Then, the plate was incubated at 37°C for 24 hours. After incubation, the various concentration of *R. siamense* root extracts was added to each well and incubated for 72 hours. Then, the supernatant was removed and washed with sterile phosphate buffer saline. Then, 200 µL/well of media was added to each well and incubated for 72 hours. Finally, cells were fixed with 40% trichloroacetic acid and stained with 0.4% SRB. The protein-bound dye in each well was dissolved by 10 mM Tris base. The optical density was measured using a microplate reader at 492 nm. The percentage of inhibition was calculated using the following equation below. The IC_{50} values were calculated using GraphPad Prism software.

$$\%\text{inhibition} = \frac{(\text{OD control} - \text{OD sample})}{\text{OD control}} \times 100$$

Anti-inflammatory Activity[11]

In vitro anti-inflammatory activity of *R. siamense* root extracts were performed in RAW264.7 cells. Cells were seeded to a 96-well plate at 1×10^5 cells/well and incubated at 37°C for 24 hours. Then, cells were stimulated with lipopolysaccharide (LPS) and treated with various concentrations of *R. siamense* root extracts for 24 hours. After that, supernate was collected to detect nitric oxide production using Griess reagent. The percentage of inhibition was calculated using the above equation. IC_{50} values were calculated using GraphPad Prism software. Cell viability was measured by MTT assay. No cytotoxicity was observed when the survival rate was more than 70%.

Total Flavonoid Content[12]

The aluminum chloride colorimetric assay was performed to determine the total flavonoid content. First, the samples or reference standard were dissolved in absolute ethanol, then 500 µL of the sample was transferred to a tube. Then, 5% $NaNO_2$ (75 µL) and 10% $AlCl_3$ (150 µL) were added to each sample. After 5 minutes of incubation, 500 µL of 1M NaOH and 275 µL of Milli-Q water were added and incubated for 30 minutes. The absorbance was determined at 510 nm. Quercetin was used as a standard for calibration curve preparation. Total flavonoid content was expressed as mg of quercetin equivalents (QE) per g of dried extract (mg QE/g of dry wt).

Statistical Analysis

The data were performed in triplicate and expressed as means ± standard error of the mean from at least three separate experiments. Statistical analysis was performed using a paired t-test. Statistical significance was indicated when the *P*-value < .05.

Results

Effect of *R. siamense* Root Extracts on Hela, HEC-1-A, and SKOV-3 Cell Lines

Cytotoxicity activity of the aqueous and ethanolic extracts of *R. siamense* root was performed on Hela, HEC-1-A, and SKOV-3 cells. Paclitaxel was used as a positive control. After treatment, the ethanolic extract of *R. siamense* root (RSE) showed growth inhibition on Hela, HEC-1-A, and SKOV-3 cells in a dose-dependent manner, as shown in Figures 1A, 1B, and 1C. However, the Hela cells were most sensitive to RSE, and their viability declined in a concentration-dependent manner up to 0.024 µg/mL. The IC_{50} value of RSE for the Hela cell line was calculated at 0.07 ± 0.00 µg/mL. Our results indicated that RSE dose-dependently reduced the survival of HEC-1-A and SKOV-3 cell lines with IC_{50} values of 30.06 ± 0.79 and 54.46 ± 0.67 µg/mL, respectively (Table 1). On the other hand, the aqueous extract of *R. siamense* root had no inhibition effect against three types of cell lines.

Table 1 IC_{50} values of *R. siamense* root extracts and paclitaxel on Hela, HEC-1-A and SKOV-3 cell lines

Sample	Hela	HEC-1-A	SKOV-3
RSE	0.07 ± 0.00 µg/mL*	30.06 ± 0.79 µg/mL*	54.46 ± 0.67 µg/mL*
RSW	> 100 µg/mL	> 100 µg/mL	> 100 µg/mL
Paclitaxel (positive control)	1.08 ± 0.04 pg/mL	0.00005 ± 0.00 pg/mL	0.008 ± 0.00 µg/mL

*P-value < .05 when compared with positive control

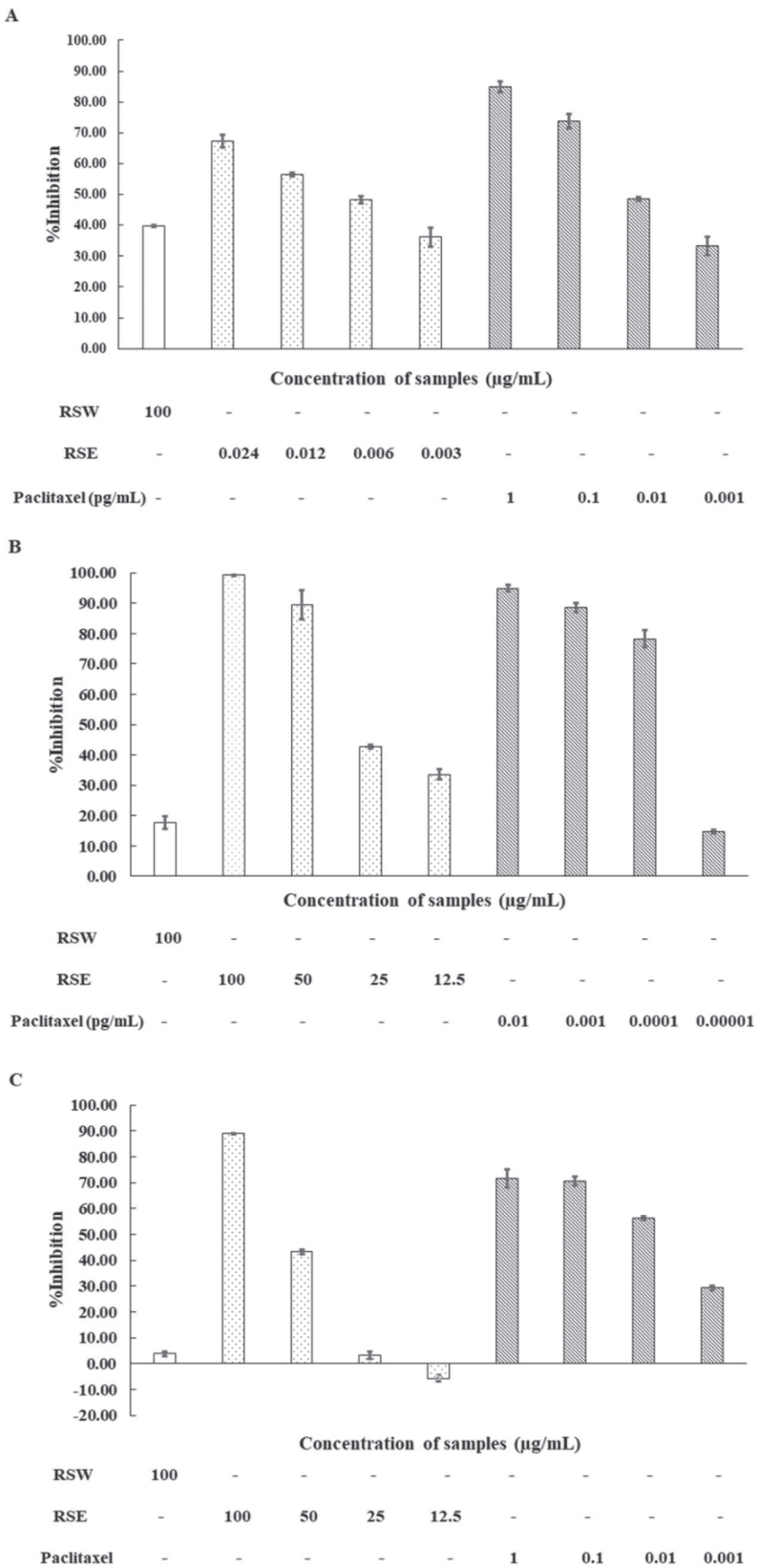

Figure 1 Cytotoxicity of *R. siamense* root extracts on (A) Hela, (B) HEC-1-A, and (C) SKOV-3 cell lines.

Anti-inflammatory Activity of *R. siamense* Root Extracts by Using Inhibition of Nitric Oxide Production in LPS-stimulated RAW264.7 Cells

The RSE treatment on LPS-stimulated RAW64.7 cells could inhibit nitric oxide production in a concentration-dependent manner, as shown in Figure 2A. In detail, a significant inhibition in nitric oxide production was shown by the IC_{50} values. The IC_{50} values of RSE and prednisolone on inhibition of nitric oxide production were 44.83 ± 2.21 and 64.16 ± 6.04 µg/mL, respectively. However, the aqueous extract showed much less inhibition effect on LPS production. In addition, all samples were incubated with RAW264.7 cells for 24 hours to study the impact of *R. siamense* root extracts on cytotoxicity on cells. The survival rate was measured by the MTT assay. The findings revealed that none of the samples had any cytotoxic effects on cells. (Figure 2B).

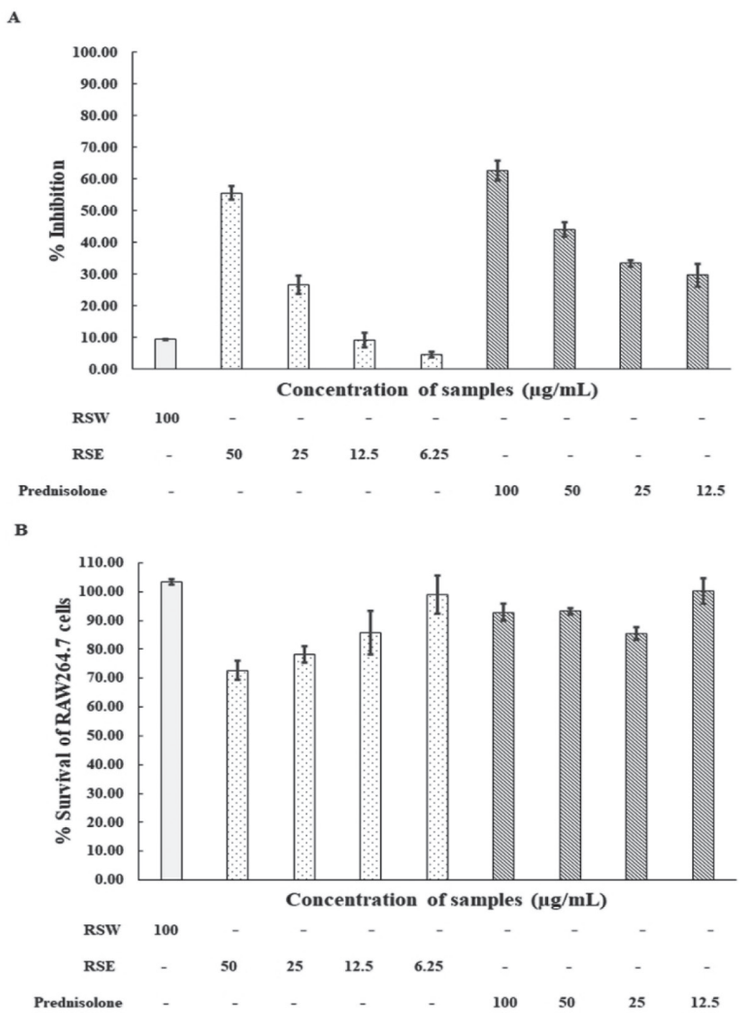

Figure 2 Effect of *R. siamense* root extracts on (A) inhibition of nitric oxide production and (B) survival rate of RAW264.7 cells.

Total Flavonoids Content in *R. siamense* Root Extracts

All examined *R. siamense* root extracts had a considerably high total content of flavonoid. Total flavonoid content was the highest in the aqueous extract of *R. siamense* root (896.67 ± 47.39 mg QE/g of dry wt), while that in the ethanolic extract was 750.83 ± 73.64 mg QE/g of dry wt.

Discussion

In recent years, increasing attention has been focused on developing plant extract to treat cancer.[13] This research might offer new knowledge for anticancer drug production, especially for women's cancer. In this study, we investigated the anticancer effect, anti-inflammatory activity, and total flavonoid content of *R. siamense* root extracts.

The anticancer activity of *R. siamense* root extracts revealed that the ethanolic extract turned out to be strongly cytotoxic toward cervical cancer cells (Hela), moderately and mildly cytotoxic toward human endometrial adenocarcinoma cells (HEC-1-A) and ovarian cancer cells (SKOV-3), respectively. Anticancer activity of *R. siamense* has been observed in human epidermoid carcinoma (KB), human breast cancer (MCF7), and human small cell lung cancer (NCI-H187) cell lines with IC_{50} values in the range of 1.7 - 6.42 μg/mL. The active compounds that inhibited KB, MCF-7, and NCI-H187 were isolated from leaves of *R. siamense.*[3] Moreover, amides, chalcones, and flavonoids have been isolated from fruits and leaves of *R. siamense.* The isolated chalcone, 2′,4,4′-trihydroxy-6′-methoxy-3′(3″-hydroxy benzyl) dihydrochalcone, has been found to have inhibition effect on NF-κB in a pancreatic β-cell line.[2]

In our study, the ethanolic extract of *R. siamense* root could inhibit the growth of cancer cells and nitric oxide production. The fact that patients with cervical cancer have been found to have a high nitric oxide levels and increasing tumor blood flow and angiogenesis were related to nitric oxide levels.[14] Therefore, the ethanolic extract of *R. siamense* root might help suppress cancer progression.

Surprisingly, the aqueous extract of *R. siamense* root showed higher flavonoid content than the ethanolic extract, but on the other hand, it had neither anticancer nor anti-inflammatory activities. Flavonoids have been revealed to have cytotoxic activity against cervical cancer cells and induced DNA fragmentation and apoptosis of cancer cells.[15] In this research, the flavonoid content of *R. siamense* root extracts based on quercetin seems unrelated to the anticancer and anti-inflammatory activities. Thus, active compounds of the ethanolic extract of *R. siamense* root might be chalcones or amides that have been shown to exert anticancer and anti-inflammatory activities in the previous studies.[2,3]

We can conclude that *R. siamense* extract, which showed the high anticancer activity, could be obtained by the maceration with 95% ethanol. The ethanolic extract of *R. siamense* roots is a potential candidate that might be used as an anticancer agent in gynecologic cancer patients. Active compounds should be tested for their cytotoxic activity against cancer cell lines. An animal model should be explored to understand the mechanism of *R. siamense* extract better.

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