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Research Article

Anticancer effects of *Rhinacanthus nasutus* and *Acanthus ebracteatus* extracts against human cervical cancer cells

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Abstract

Cervical cancer is second only to breast cancer in terms of incidence; however, it is the most lethal form of cancer among Thai women due to the asymptomatic nature of its early stages. This work aimed to examine cytotoxic and antiproliferative capacities of *Rhinacanthus nasutus* (RN) and *Acanthus ebracteatus* (AE) extracts against human cervical cancer cells (HeLa). Plant leaves were used for ethyl acetate extraction. The antioxidant assays, HPLC analysis, a cytotoxic MTT assay, a clonogenic assay and real-time PCR were conducted. Both RN and AE displayed similar DPPH scavenging activity (3.97 and 4.05 mg TE/g DW) and ferric reducing antioxidant power (4.79 and 4.35 mg Fe^{2+}/g DW). However, AE was richer in total phenolic content than RN (13.30 and 10.84 mg GAE/g DW, respectively). Rutin, catechin, chlorogenic acid, and cinnamic acid were found in AE, whilst only cinnamic acid with much higher content was found in RN. Higher cytotoxicity of 91.73% against HeLa cells was found in RN (IC_{50} value of 62.06 µg/mL). RN showed higher antiproliferative effect (IC_{50} of 25.24 µg/mL) than AE (34.35 µg/mL). Genes (*Bcl-2*, *Bax*, *MMP-2, MMP-9*, *caspase-3*, *p21*, and *cyclin D1*) and proteins (cytochrome c, caspase-3 and p21) linked to apoptosis and migration were substantially more affected by RN. To conclude, both RN and AE hold promise as anticancer herbal plants against human cervical cancer; however, RN was more cytotoxic and antiproliferative in HeLa cells. RN offers a better alternative herbal medicine or complementary remedy to the standard drug for human cervical cancer treatment.

Keywords: *Acanthus ebracteatus*; apoptosis; cytotoxic activity; HeLa cells; *Rhinacanthus nasutus*

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Introduction

According to the ICO/IARC HPV Information Centre (2021), cervical cancer ranks as the third most common disease among Thai women and the second most prevalent cancer affecting Thai women aged 15–44. Cervical cancer often has no noticeable symptoms in its early stages (Bouvard *et al*., 2021). The social stigmas that exist in Thailand regarding women's health also play a role. Due to a lack of education, patients typically wait until the condition has progressed significantly before seeking treatment, increasing the risk of permanent disability or death. Chemotherapy is an important means to treat cervical cancer. Tumor cells, however, are notorious for developing resistance to many chemotherapeutic agents due to their structural and mechanistic differences. Chemotherapy failure, tumor recurrence, and even patient mortality can all be attributed to tumor cells' multidrug resistance (MDR) (Wang *et al*., 2019). Thus, increased research has been conducted on natural compounds that can be used as alternative anticancer medicines against cervical cancer.

All around Thailand, people are increasingly turning to traditional medicine. Herbal medicines and store-bought pharmaceuticals are both employed. Natural products and plant-derived substances inhibit a wide variety of human malignancies and can activate apoptosis with minimal impact on normal cells, making them a promising alternative cancer treatment (Banjerdpongchai *et al*., 2014; Nourazarian *et al*., 2015). Flavonoids, carotenoids, terpenoids, vitamins, and polyphenoids are common phytochemicals (Chinembiri *et al*., 2014).

Certain plants of the Acanthaceae family endemic to Thailand and other regions of Southeast Asia are well-known for treating numerous diseases. In particular, *Rhinacanthus nasutus* (L.) Kurz (RN), its name, Snake Jasmine, comes from the shape of its blossoms and the fact that the plant's root has long been used as a remedy for the effects of snake venom (Brimson *et al*., 2012). The healing herb, which may be consumed as tea or transformed into an ointment, has long been used to cure conditions including ringworm and inflammation. Previous works showed the cytotoxicity of root extracts of RN against HeLa cervical cancer cells (Siripong *et al*., 2006; Siripong *et al*., 2009). Antioxidant properties have also been shown in ethanol and water extracts of RN leaves (Thephinlap *et al*., 2013). The main bioactives are rhinacanthin-C, -D, and -N. Variation in plant genetics, climate, and harvesting method can all affect the amounts of phytochemicals. The largest concentration of total rhinacanthins was found in the roots, and then the leaves, across all harvest times. In September, both the roots and the leaves (4.91% and 4.42% w/w, respectively) had the maximum level of total rhinacanthins, while in March, they had the lowest concentrations (3.73% and 3.18% w/w, respectively) (Suksawat and Panichayupakaranant, 2022). However, the extract of aerial parts (stem and leaves) of RN originated from north-eastern Thailand in ethyl acetate solvent was rarely studied. Aerial parts of RN are more easily accessed with greater amounts to be collected for extraction than its roots. Thus, aerial parts were chosen for investigation in this work.

In addition, the shrubby herb *Acanthus ebracteatus* (AE) also belongs to the Acanthaceae family. The extract of *A. ebracteatus* using hot water halted the development and cancers of cell-implanted nude mice with cervical cancer (Mahasiripanth *et al*., 2012). The recent study showed that AE leaf ethanolic extract with chlorophyll removal using sedimentation process contained dihydroferulic acid 4-O-glucuronide, caffeic acid, 4-glucogallic acid, chlorogenic acid, kelampayoside A and hydrojuglone glucoside (Olatunji *et al*., 2022). These bioactive compounds are thought to contribute to its antioxidant activities (the ferric reducing antioxidant power (FRAP): 1113.2 μmol Trolox Equivalent (TE)/g and and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging 498.8 μmol TE/g) (Olatunji *et al*., 2022). Previously, AE protein hydrolysates were shown to exhibit anticancer activity towards skin cancer cell A431 by inducing apoptosis through increased RelA (p65) and Cyclin D1 protein levels (Khamwut *et al*., 2019). However, no report is documented about the cytotoxic and antiproliferative properties of AE against human cervical cancer cells based on literature search on Pubmed. Thus, this work aimed to study anticancer activity of AE extract from aerial parts against HeLa cells. This study used MTT, clonogenic, and wound healing assays to assess whether ethyl acetate-extracted leaves of RN and AE from Thailand exhibited cytotoxic and antiproliferative properties against HeLa cervical

cells. Both the transcriptional regulation of apoptosis and the protein expression in cells were studied in relation to plant extracts.

Materials and Methods

Plant collection and extraction

The selected Thai herbs, RN (Figure 1A and 1B) and AE (Figure 1C and 1D) at their vegetative stages were harvested in early morning (7.00 am on 22th October 2019) in Maha Sarakham Province, Thailand. The taxonomic classification of these plants was carried out by Assoc. Prof. Dr. Vijitra Luang-In, Department of Biotechnology, Faculty of Technology, Mahasarakham University.

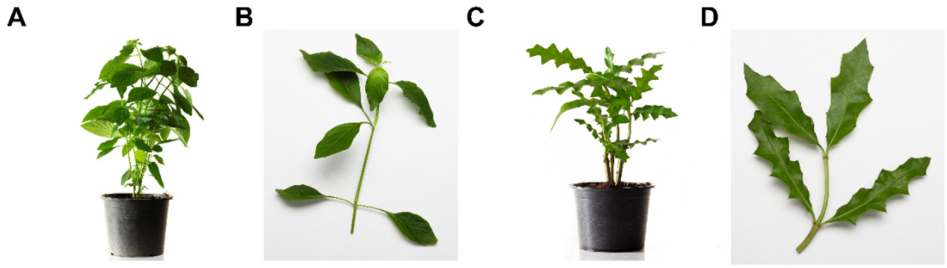

Figure 1. Herbal plants; (A) Aerial parts of RN; (B) Leaves of RN; (C) Aerial parts of AE; (D) Leaves of AE

The leaves of the herbs were shade dried for several days and once dried were ground thoroughly. Next, dried residues were extracted in 1,000 mL of ethyl acetate in the flask at 37 °C for 3 days with agitation at 200 rpm. After that, re-extraction was conducted twice. The extracts were collected and spun down at 10,000g for 30 min. The supernatant was filtered through a no. 4 Whatman filter and evaporated in a Rotavapor® R-100 (Buchi, Flawil, Switzerland). The plant extracts were concentrated, freeze-dried, and then kept at -20 °C. For bioassays, the extracts (20 mg/mL) were filtered in dimethylsulfoxide (DMSO)(Sigma-Aldrich, St. Louis, MO, USA).

Evaluation of antioxidant activity and bioactive compounds

The ferric reducing antioxidant power (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging strategies with minimal alterations were examined as previously reported (Luang-In *et al.*, 2021). Each 20 mg/mL plant extract solution (20 µL) was combined with 180 µL of 10 mM DPPH (Sigma-Aldrich, St. Louis, MO, USA). Thirty minutes of dark reaction was followed by 515 nm absorption reading using a M965+ microplate reader (Metertech, Taipei, Taiwan). For FRAP, plant extract (20 µL of 20 mg/mL stock solution) was subjected to a FRAP reagent (180 µL) made from 20 mM $FeCl_3$ (Sigma-Aldrich, St. Louis, MO, USA), 10 mM 2,4,6-Tri (2-pyridyl) s-triazine, and a 0.3 M acetate buffer at pH 3.6. After 30 min, the M965+microplate reader registered 593 nm absorption (Metertech, Taipei, Taiwan) in triplicate. Trolox and ferrous II sulfate standards (Sigma-Aldrich, St. Louis, MO, USA) were used to measure antioxidant capability for DPPH and FRAP experiments, respectively.

The total phenolic content (TPC) and the total flavonoid content (TFC) were measured as specified (Luang-In *et al.*, 2021). The 20 µL solution of each plant extract, 80 µL of 7.35% sodium carbonate (Sigma-Aldrich, St. Louis, MO, USA), and 100 µL of 10% Folin-Ciocalteu solution (Sigma-Aldrich, St. Louis, MO, USA) were mixed for TPC. The dark reaction of 30 min was followed by A765 nm record. For TFC, deionized water (60 µL), 10% aluminum trichloride (10 µL) and 5% sodium nitrate (10 µL) were pipetted into the plant extract (20 µL). After adding 100 µL of 1 M NaOH (Sigma-Aldrich, St. Louis, MO, USA), the mixture was

left for 30 min to react before its absorbance at 420 nm was measured. Each determination required three replicates. Authentic gallic acid and rutin (Sigma-Aldrich, St. Louis, MO, USA) were used in TPC and TFC tests, respectively.

HPLC analysis of phenolic acids and flavonoids

Protocol-based phenolic and flavonoid extractions were done (Chumroenphat *et al*., 2021). Shaking was utilized for the 12-hour HCl/Methanol (1:100) extraction at 37 °C (150 rpm in the dark). The extracts were evaporated and filtered. Polyphenolic acids and flavonoids were analysed using Shimadzu LC-20AC pumps (Shimadzu, Kyoto, Japan), SPD–M20A diode array detectors, and an Inertsil ODS-3, C18 column (4.6 mm x 250 mm, 5 µm) (Sigma-Aldrich, St. Louis, MO, USA). As the mobile phase (with a flow rate of 0.8 mL/min), acetic acid pH 2.74 (Solvent A) and acetonitrile (Solvent B) (Sigma-Aldrich, St. Louis, MO, USA) were used (Phuseerit *et al*., 2021). The sample of 20 µL was fed into a 38 °C column, and phenolic acids and flavonoids were identified at 280 nm and 370 nm, respectively. UV spectra of samples were compared to external curves of legitimate standards (Sigma-Aldrich, St. Louis, MO, USA) to identify phenolic and flavonoid chemicals.

Cell cultures

HeLa (ATCC® CCL-2™), a human cervical cancer cell line, was purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI-1640 media supplemented with 10% fetal bovine albumin and 1% Penicillin/Streptomycin at 37 °C with 5% CO_2. Every three days, the media was changed and the cells were trypsinized. All chemicals were purchased from Thermo Fisher Scientific, Waltham, MA, USA.

Cytotoxicity assay

Cytotoxicity of plant extracts towards HeLa was detected using 3, 4, 5-dimethylthiazol-2-yl-2-5-diphenyltetrazolium bromide (MTT) assay. HeLa cells (5×10^3 cells/well) were added to 96-well plates for 24 h at 37 °C. Various concentrations of plant extracts (0-250 µg/mL dissolved in the media) were exposed to HeLa cells for 24 h. MTT reagent (Sigma-Aldrich, St. Louis, MO, USA) replaced the media. The samples were incubated for 4 h. Next, DMSO (200 µL) dissolved the formazan crystals, and A590 nm was recorded by a M965+microplate reader (Metertech, Taipei, Taiwan). Triplicate testing was conducted. Both IC_{50} and cytotoxicity (%) of plant extracts to HeLa cells were determined.

$$\text{Cytotoxicity (\%)} = \frac{(\text{Acontrol} - \text{Asample})}{(\text{Acontrol})} \times 100$$

An IC_{50} result of less than 50 µg/mL indicates a high level of cytotoxicity. Furthermore, the IC_{50} range from 50-100 µg/mL indicates moderate cytotoxicity, while 100-200 µg/mL indicates mild cytotoxicity, and 200-300 µg/mL indicates extremely poor cytotoxicity.

Crystal violet assay

The survival of HeLa cells after receiving the plant extracts was observed by crystal violet staining. A 96-well plate was planted with 5×10^3 cells/well. The cells were exposed to extracts (0-250 µg/mL) for 24 h. The media culture was then discarded, fixed for 30 min with 4% formaldehyde (Sigma-Aldrich, St. Louis, MO, USA), stained for 30 min with 0.5% crystal violet (Sigma-Aldrich, St. Louis, MO, USA), and air-dried. Pictures were collected under an NIB-100 inverted microscope (Xenon Industrial, Shenzhen, China) observation.

Colony formation assay

HeLa cells were plated at 500 cells/well for 24 h. Cells were exposed to extracts in culture media and the medium was withdrawn. Cells were washed with phosphate buffer saline (PBS) and incubated at 37 °C in 5%

CO_2 for 14 days. Every three days, new media was added. Cells were fixed in cold methanol (Sigma-Aldrich, St. Louis, MO, USA) for 30 min. Coomassie brilliant blue g-250 (Sigma-Aldrich, St. Louis, MO, USA) was used to stain colonies for 30 min. The percentage of triplicate colonies relative to untreated cells was calculated.

Cell morphology

HeLa cells were seeded 7,500/well in a 24-well plate. After overnight incubation, cancer cells were exposed to plant extracts (0-250 µg/mL). An NIB-100 inverted microscope (Xenon Industrial, Shenzhen, China) was used to view cells.

Wound healing assay

HeLa cells (2×10^5 cells/well) were planted in a 24-well plate and allowed to confluence overnight. The wound was then scratched with a 200 µL pipette tip and cleaned with PBS. The cells were exposed to plant extracts for 24 h. Next, cells were fixed with 4% formaldehyde, stained with 0.5% crystal violet for 30 min, rinsed with distilled water, collected, and examined for % relative closure of the scratch, compared with the untreated cells.

Real-time polymerase chain reaction (PCR) analysis

HeLa cells (2×10^5 cells/well) were cultured at 37°C for 24 h before being exposed to plant extracts (50 µg/mL) for 24 h. After that, apoptotic and migration-related mRNA components were examined by real-time PCR. Total RNA was extracted using TRIzol™ (Thermo Fisher Scientific, Waltham, MA, USA) and transcribed to cDNA with reverse transcriptase (iScript™ Reverse Transcription Supermix, Bio-Rad, Hercules, CA, USA). QuantStudio real-time PCR (Applied Biosystems, Foster City, CA, USA) was used for quantitative RT-PCR with Fast Start Essential DNA Green Master (Roche Applied Science, Penzberg, Germany). Real-time PCR was conducted. The expressions were normalized to *GAPDH* expression levels. The DNA templates were denatured at 94 °C for 10 min, annealed at 60 °C for 10 s, and extended at 72 °C for 10 s for 45 cycles.

Protein extraction and Western blot analysis

Western blot was utilized to investigate apoptotic and migration-related protein changes. In 6-well plates, HeLa cancer cells were treated to plant extracts (50 µg/mL) for 24 h. The cells were lysed in RIPA buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) (Thermo Fisher Scientific, Waltham, MA, USA) for 30 min on ice and centrifuged at 14,000g for 15 min at 4 °C to remove debris. BCA protein kit test (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the total protein concentration. Protein (25 µg) was resolved by SDS-PAGE using 12% polyacrylamide gel electrophoresis (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Tris buffered saline with 5% BSA containing 0.1% Tween-20 (TBST) (Sigma-Aldrich, St. Louis, MO, USA) was used to block membranes for 1 h before primary antibody incubation (Boster Biological Technology, Wuhan, China). The membranes were washed three times with TBST and incubated with secondary Ab conjugated with horseradish peroxidase (1:5000) for 1 h at room temperature. Membranes were rinsed in TBST and treated with Amersham ECL TM Prime for chemiluminescent detection. IMageQuant TL 400 (Cytiva, Marlborough, MA, USA) was used to visualize protein band densities. β-actin expression served as a housekeeping protein loading control in all experiments.

Statistical analysis

One-way analysis of variance (ANOVA) and Duncan's multiple range test (SPSS, IBM, Armonk, NY, US) were used on data collected in triplicate to determine means and standard deviations, with differences at $p<0.05$ being considered significant.

Results

Antioxidant activity and bioactive contents

The antioxidant capacities by DPPH and FRAP assays of RN and AE were no different. DPPH values were in the range of 3.97-4.05 mg TE/g DW, while FRAP values were 4.35-4.79 mg Fe^{2+}/g DW. Likewise, TFC values of RN and AE were similar (22.51-22.74 mg RE/g DW). However, TPC value of AE (13.30 mg GAE/g DW) is statistically greater than that of RN (10.84 mg GAE/g DW).

Table 1. Antioxidant activity and bioactive content assessments

Samples	DPPH (mg TE/g DW)	FRAP (mg Fe^{2+} /g DW)	TPC (mg GAE/g DW)	TFC (mg RE/g DW)
RN	3.97±0.07^{a}	4.79±0.08^{a}	10.84±0.55^{b}	22.51±0.35^{a}
AE	4.05±0.02^{a}	4.35±0.03^{a}	13.30±0.05^{a}	22.74±0.12^{a}

Statistical significance ($p < 0.05$) is shown by different letters in the same columns.

HPLC analysis of bioactive contents

Results showed that RN contained much higher cinnamic acid than AE and there is a peak at 58 min in high abundance representing an unknown compound (Table 2 and Figure 2A). However, flavonoids detected in AE included rutin, catechin and phenolic acids (chlorogenic acid and cinnamic acid)(Table 2 and Figure 2B).

Table 2. Flavonoid and phenolic acid content identified in RN and AE

Sample	Flavonoid content (mg/g DW)		Phenolic acid content (mg/g DW)	
	Rutin	Catechin	Chlorogenic acid	Cinnamic acid
RN	ND	ND	ND	33.4±2.2^{aA}
AE	18.7±2.6^{aA}	17.6±1.6^{aA}	1.6±0.4^{aA}	11.7±1.5^{bB}

^{a,b} represents statistical significance in rows at $p < 0.05$. ^{A,B} represent statistical significance in columns at $p < 0.05$.
The results of three separate tests are presented as the mean±SD. ND = not detected.

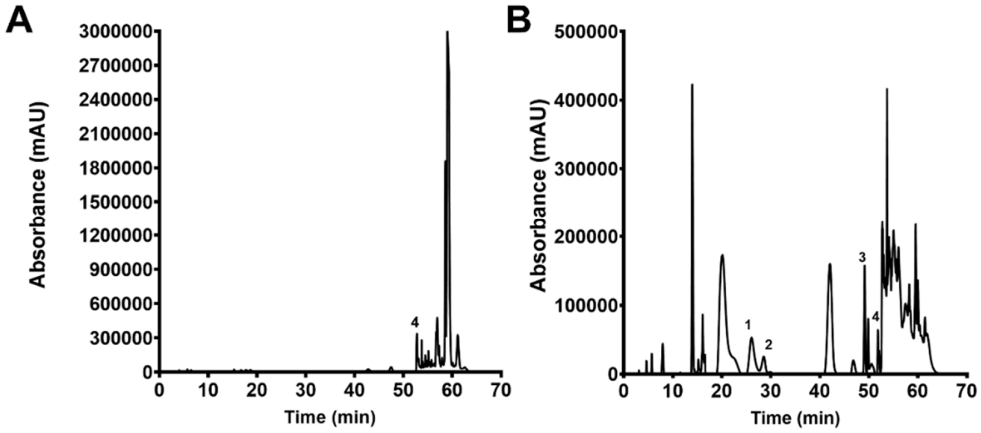

Figure 2. HPLC chromatogram of RN (A) and AE (B)
Peaks 1 = catechin; 2 = chlorogenic acid; 3 = rutin, and 4 = cinnamic acid

Cytotoxicity and cell viability

Cytotoxicity by MTT assay results showed that RN had significantly higher %cytotoxicity of 91.73% at 250 µg/mL, with lower IC_{50} value of 62.06 µg/mL (Figure 3A) than AE (%cytotoxicity of 52.50% at 250 µg/mL and IC_{50} value of 115.36 µg/mL)(Figure 3B).

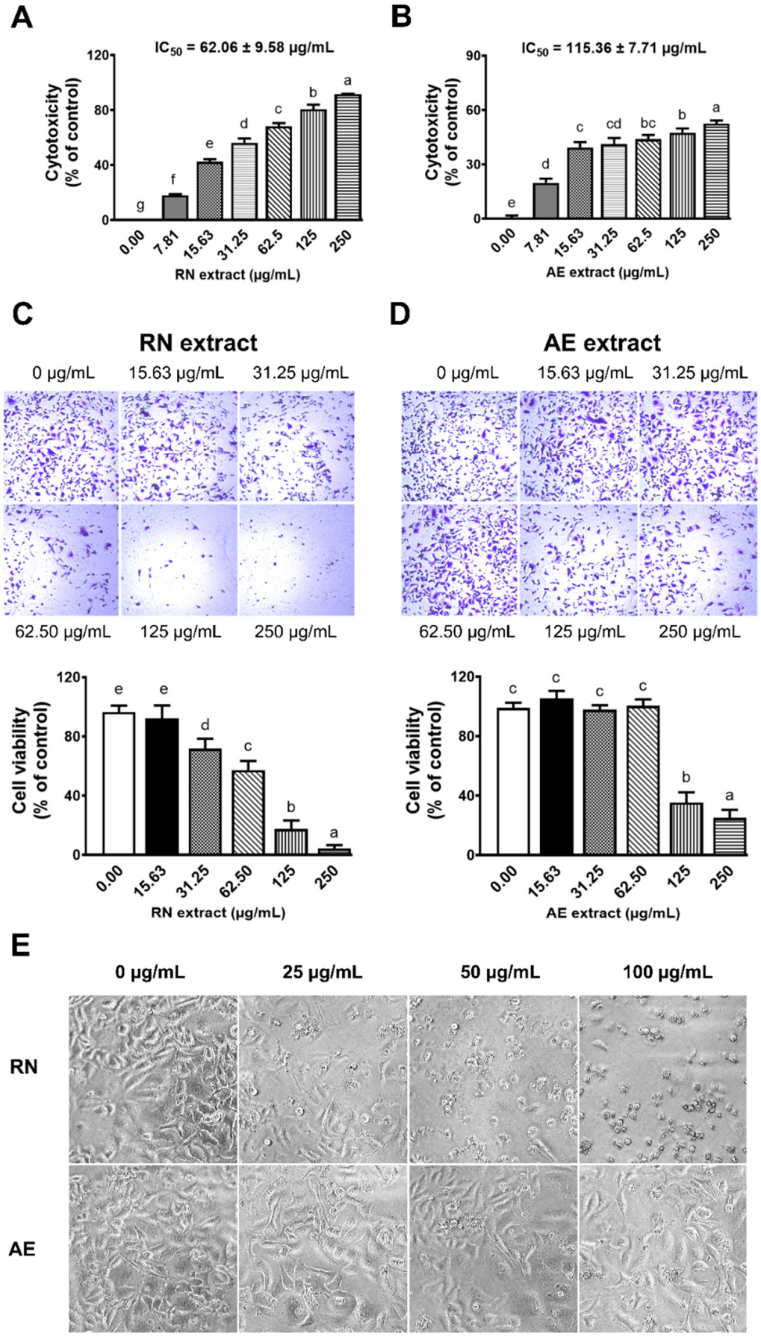

Figure 3. Cytotoxicity of RN and AE and cell viability of HeLa cells; (A) Cytotoxicity of RN; (B) Cytotoxicity of AE; (C) Cell viability of RN-treated HeLa cells; (D) Cell viability of AE-treated HeLa cells; (E) Cell morphology in treatments with varying doses

Cytotoxic effects of both RN and AE were clearly dose-dependent. Likewise, cell viability test by crystal violet staining showed that RN had significantly lower % cell viability of 8.27% at 250 µg/mL (Figure 3C) than that of AE at 30.32% (Figure 3D). As the plant extract doses increased, the viable cell density decreased, especially at 125 and 250 µg/mL, in both RN (with more pronounced effect) and AE (Figure 3C and 3D). Morphological alterations such as decrease in HeLa cell density and increase in apoptotic bodies were detected under an inverted microscope when plant extract concentrations increased (Figure 3E).

These results demonstrated a dose-dependent sensitivity of HeLa cells to apoptotic induction by RN and AE extracts. Shrunken cells, membrane blebbing, and apoptotic bodies, all indicators of cell death via apoptosis, were first observed at 50 µg/mL RN concentrations in HeLa cells and were most pronounced at 100 µg/mL RN concentrations (Figure 3E), whilst at the same concentrations AE did not show clear signs of apoptosis yet.

Antiproliferative and antimigratory activities

Results of clonogenic assay showed that both RN and AE were antiproliferative towards HeLa cells in14-day-treatment in a dose-dependent fashion (Figure 4A). RN with a lower IC_{50} value of 25.24 µg/mL showed significantly stronger antiproliferative activity towards HeLa cells than AE with IC_{50} value of 34.35 µg/mL (Figure 4B).

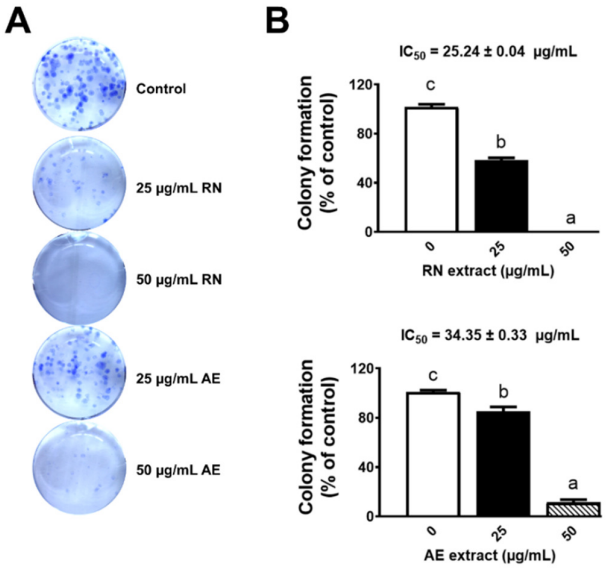

Figure 4. Effect of RN and AE extracts on colony formation of HeLa cells; (A) Colony formation of treated HeLa cells; (B) IC_{50} values of RN and AE on colony formation of HeLa cells at 14-day treatment.

Both RN and AE extracts substantially inhibited HeLa cell migration and decreased wound coverage in a dose-dependent fashion. Overnight, the control covered 59% of the migratory area, whereas the RN-treated sample only covered 15% at 50 µg/mL (Figure 5A and 5B). Cells treated with AE extract covered 29% of the migratory region at 50 µg/mL (Figure 5A and 5C). RN gave a higher antimigratory impact than that of AE. When compared to untreated cells, percentages of migratory regions of RN and AE-induced HeLa cells were considerably lower.

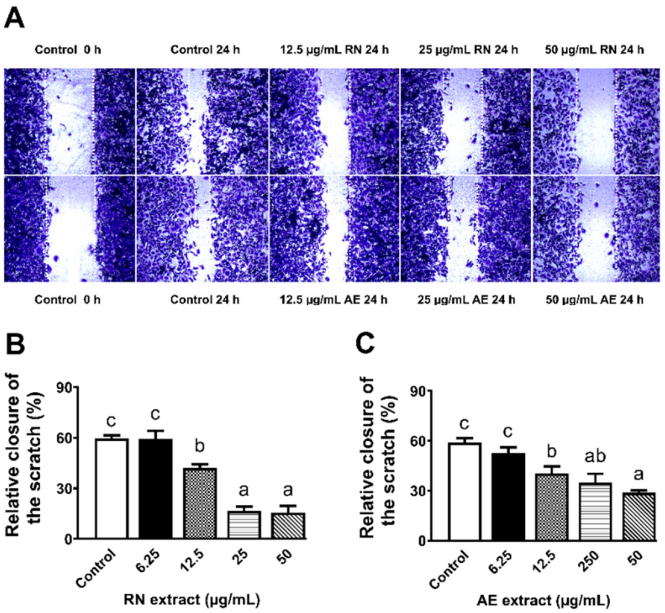

Figure 5. Antimigratory activity of RN and AE extracts using a wound healing assay
At $p < 0.05$, different letters in the bars denote statistical significance

Apoptosis and migration-associated gene and protein expressions

Real-time PCR findings revealed that RN substantially affected all examined genes *Bax, Bcl-2, p21, caspase-3, MMP-2*, *MMP-9* and *cyclin D1* compared to the control (untreated cells) (Figure 6A).

In most cases, AE also affected gene expressions, except for *p21* and *MMP-2* (Figure 6A). *Bax, caspase-3* and *p21* mRNA levels significantly increased, indicative of apoptosis induction and activation of the caspase-cascade response, while *Bcl-2*, *MMP-9, MMP-2* and *cyclin D1* levels decreased, indicative of apoptosis induction, antimigratory activity due to downregulation of MMP-2 and MMP-9 and cell cycle arrest at G1/S transition. RN and AE exhibited potential as antitumor drugs.

RN significantly influenced cytochrome c, caspase-3 and p21 protein expression whilst, AE only affected caspase-3 and p21 compared with untreated cells (Figure 6B). However, RN resulted in more pronounced changes (more fold increase/decrease) in gene and protein expressions than AE in accordance with lower IC_{50} values of RN in MTT assay (Figure 3A) and clonogenic assays (Figure 4B).

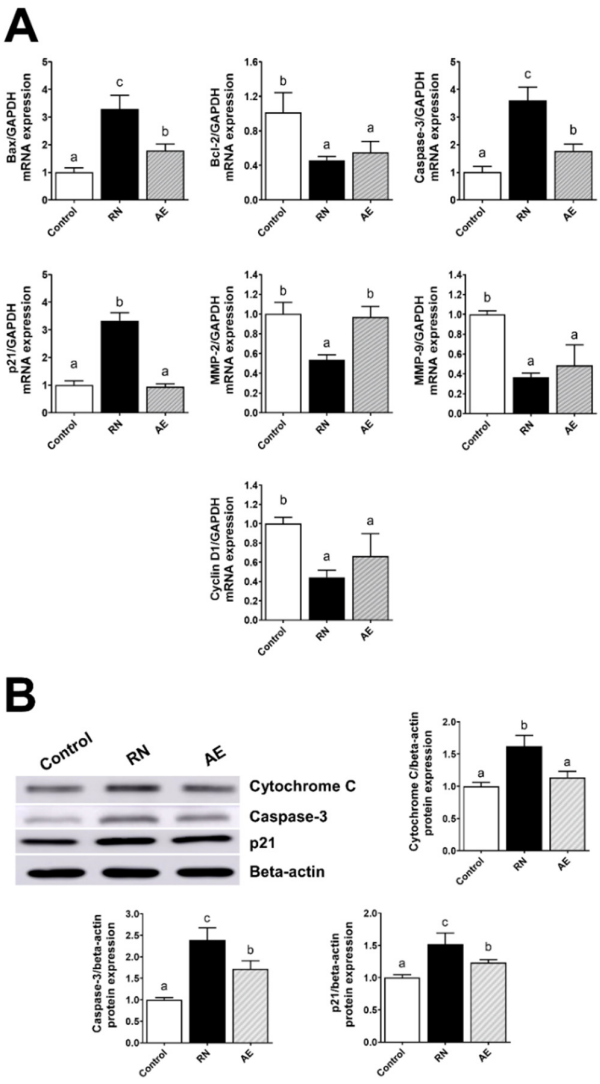

Figure 6. Effect of RN and AE extracts on gene and protein expressions; (A) Gene expressions from RN and AE treatments; (B) Protein expressions from RN and AE treatments
At $p < 0.05$, different letters in the bars denote statistical significance

Discussion

This work reported the cytotoxic and antiproliferative activities of RN and AE leaf extracts by ethyl acetate against HeLa cervical cancer cells. The HPLC results found rutin, catechin, chlorogenic acid, and cinnamic acid in AE, while only cinnamic acid with much higher content was found in RN. Higher cytotoxicity of 91.73% against HeLa cells was found in RN with IC_{50} value of 62.06 µg/mL assessed by an MTT assay. Using a clonogenic test, the IC_{50} for RN was reported to be 25.24 µg/mL, which was lower than that for AE (34.35 µg/mL). The cells treated with RN had stronger apoptotic features. Also, RN exhibited far larger impact on the expression of genes (*Bax*, *Bcl-2*, *p21*, *caspase-3*, *MMP-2*, *MMP-9*, and *cyclin D1*) and proteins expressions (caspase-3, cytochrome c and p21) involving in apoptosis and cell migration. Both RN and AE hold promise as anticancer herbal plants against cervical cancer; however, RN was more cytotoxic and antiproliferative in HeLa cells offering better chemopreventive effects.

The antioxidant activities of RN and AE leaf ethyl acetate extracts were similar based on DPPH and FRAP assays (Table 1). DPPH value (4.05 mg TE/g DW) of AE was much lower than that of leaves of AE (448.10 μmol TE/g dry extract) collected from Surat Thani Province, Thailand (southern part of the country) and extracted by 70% ethanol using a maceration method (Olatunji *et al*., 2022). Likewise, TPC (13.30 mg GAE/g DW) and TFC (22.74 mg RE/g DW) values of AE were markedly lower than those in a previous work, TPC of 138.20 mg GAE/g and TFC of 107.60 mg CE/g dry extract (Olatunji *et al*., 2022). The differences in antioxidant capacity and bioactive content between AE in this work and AE in a recent report may lie in different solvents used for extraction, ages of plants, growth conditions and also their origins.

Based on their HPLC results, caffeic acid, dihydroferulic acid 4-O-glucuronide, 4-glucogallic acid, chlorogenic acid, kelampayoside A and hydrojuglone glucoside were tentatively identified in AE leaf ethanolic extract (Olatunji *et al*., 2022). In addition, aliphatic alcohol, phenolic glycosides, aliphatic glycosides, terpenes, flavonoids, megastigmane glycosides, and lignan glycosides have all been documented to be present in AE in the prior investigation (Prasansuklab and Tencomnao, 2018). Compared to our results, the only phenolic compound of AE in common with the previous report was chlorogenic acid (Table 2). The phyto-constituents found in the plant are mostly responsible for the bioactivity of herbal plant extract. Plant extracts and isolated bioactive components are among the many natural products that have shown multiple pharmacological actions related to their ability to control oxidative stress and display antioxidant capabilities (Song *et al*., 2021). Therefore, the extract's antioxidant activity is crucial to its pharmacological benefits.

In this work, AE extract contained at least rutin, catechin and phenolic acids (chlorogenic acid and cinnamic acid). Richer content of cinnamic acid in RN (Table 2) and the unknown compound in high abundance at the retention time of 58 min (Figure 2A) may account for higher bioactivity of RN than AE. In terms of anticancer activity, RN was more effective in cytotoxicity (Figure 3), antiproliferation (Figure 4) antimigration (Figure 5) and induction of apoptosis-related gene/protein changes (Figure 6) of HeLa cells than AE.

Cinnamic acid found in both AE and RN was known to induce apoptosis. Growth of cinnamic acid - treated HeLa cells was paused at the G1 phase, indicating a blockage in the shift from G1 to S phase, as shown by cell cycle analysis (Sali *et al*., 2016). HeLa cell lines treated with rutin showed reduced cell viability and a rise in the proportion of cells in the G0/G1 transition state. There was also a drop in *Bcl-2* expression and a surge in *Bax* expression, both of which led to the cleavage of caspase-3, caspase-8, and caspase-9, and hence the upregulation of the caspase cascade (Pandey *et al*., 2021). The proliferation of HeLa cells was observed to be inhibited by catechin metabolites, as compared to a control group (Hara-Terawaki *et al*., 2017). Chlorogenic acid was able to inhibit HeLa cell viability by 31.13%, at 200 μM (Ryu *et al*., 2019).

The sea cucumber *Holothuria tubulosa* extract from Muğla (Turkey) displayed IC_{50} value of 21.01 μg/mL at 72 h in the HeLa cell line. Epicatechin and dihydroxybenzoic acid were the most abundant components of the extract; they dramatically enhanced the apoptotic cues in HeLa cells, leading to DNA breakage and activating caspase-3 (Alper and Gunes, 2020). Thus, catechin found in this work may contribute to anticancer activity towards HeLa cells.

Previous studies demonstrated that rhinacanthins-C, -N, and -Q found in RN root extract had potent antiproliferative activity against HeLaS3 cells (Siripong *et al*., 2006). When tested against HeLa cells, ethyl acetate extracts of RN leaves grown in north-eastern Thailand, primarily in Buriram Province between September and October 2006, showed the highest cytotoxicity (IC_{50} = 3.63 μg/ml) (Siriwatanametanon *et al*., 2010). The greatest levels of β-sitosterol and lupeol were found in RN leaf ethanolic extract. Nonetheless, the maximum concentration of stigmasterol was found in the RN root ethanol extract (Brimson *et al*., 2012).

Root methanolic extract of RN contained rhinacanthone, a main bioactive naphthoquinone, that inhibited HeLa cell proliferation with IC_{50} values of 1.2 - 5.5 μM for 2-24 h (Siripong *et al*., 2009). Chromatin condensation, DNA breakage, and a rise in the percentage of sub G(1) apoptotic cells were all seen in HeLa cells treated with rhinacanthone (Siripong *et al*., 2009). An upregulation of pro-apoptotic protein Bax and a

downregulation of anti-apoptotic proteins Bcl-2 and survivin, along with the upregulation of caspase-9 and caspase-3, characterize the apoptotic processes induced by rhinacanthone therapy.

Previously, nude mice were implanted with human papillomavirus (HPV) 16–containing cervical cancer cells as a tumor model, and the impact of AE extract on cancer development and angiogenesis were studied. Mice treated with 14 or 28 days with AE extract (3,000 mg extract/kg body weight) showed considerable suppression of tumor angiogenesis and VEGF expression increased (Mahasiripanth *et al*., 2012). In addition, aqueous extract of AE leaves from Japan displayed IC_{50} value of 1,499 μg/mL against HeLa cells; however, the ethanol extract of AE root showed IC_{50} value of 1,239 μg/mL against HeLa cells (Gotoh *et al*., 2004). In comparison with our results, AE leaf ethyl acetate extract from Thailand showed a lower IC_{50} value of 62 μg/mL against HeLa cells indicating a higher anticancer activity. The differences in anticancer capacity among AE extracts may lie in different solvents used for extraction, ages of plants, growth conditions and also their origins.

Our findings indicate that RN and AE can suppress the proliferation of HeLa human cervical cancer cells and trigger their death via apoptosis. Expressions of *Bcl-2*, *MMP-2*, *MMP-9*, and *cyclin D1* were suppressed while those of *Bax*, *caspase-3*, and *p21* were stimulated when apoptosis was induced. The results of this study's experiments reveal that RN and AE have considerable promise as adjuvant therapy for the prevention and treatment of chemotherapy-induced side effects in the management of cervical cancer. Plant extracts like this could be used in conjunction with standard anticancer medications to reduce the side effects of chemotherapy and make cancer treatments more effective.

Conclusions

Here, we found that RN and AE could serve as natural compounds as anticancer products against cervical cancer cells. This is the first finding detailing the anticancer activities of the ethyl acetate extracts of RN and AE leaves originated from Thailand against HeLa cervical cancer cells. Key findings highlighted that RN and AE displayed moderate activities against HeLa cells based on MTT assay which represents a short-term therapy whilst both plants showed strong capacities against HeLa cells based on clonogenic assay which represents a long-term therapy. RN exhibited higher impacts on genes (*Bcl-2, Bax, MMP-2, MMP-9, caspase-3, p21* and *cyclin D1*) and proteins (cytochrome c, caspase-3, and p21) relating to apoptosis. RN and AE plant extracts displayed apoptotic-inducing properties antiproliferative and antimigratory activities. Both plants showed no drug resistance against HeLa cells. It is possible that the RN and AE plant extracts will prove useful as complementary therapies alongside conventional chemotherapy for the treatment of drug-resistant and refractory tumors. The next stage, however, should be the systematic isolation of the active chemicals in RN and AE plants.

Authors' Contributions

The authors are responsible for any claims arising from the content of this article and will be held liable for any damages. Testing was carried out by W.S. and T.K. The tests were devised by B.B. and A.A.A. In addition, T.K. and P.L. collected and sorted the cancer cells. V.L. conceptualized the research, evaluated the data and produced the manuscript.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

References

Alper M, Gunes M (2020). Evaluation of cytotoxic, apoptotic effects and phenolic compounds of sea cucumber *Holothuria tubulosa* (Gmelin, 1791) extracts. Turkish Journal of Veterinary and Animal Sciences 44(3):21. *https://doi.org/10.3906/vet-1909-80*

Banjerdpongchai R, Wudtiwai B, Sringarm K (2014). Cytotoxic and apoptotic-inducing effects of purple rice extracts and chemotherapeutic drugs on human cancer cell lines. Asian Pacific Journal of Cancer Prevention 14:6541-6548. *https://doi.org/10.7314/APJCP.2013.14.11.6541*

Bouvard V, Wentzensen N, Mackie A, Berkhof J, Brotherton J, Giorgi-Rossi P, ... Lauby-Secretan B (2021). The IARC perspective on cervical cancer screening. The New England Journal of Medicine 385(20):1908-1918. *https://doi.org/10.1056/NEJMsr2030640*

Brimson JM, Brimson SJ, Brimson CA, Rakkhitawatthana V, Tencomnao T (2012). *Rhinacanthus nasutus* extracts prevent glutamate and amyloid-β neurotoxicity in HT-22 mouse hippocampal cells: possible active compounds include lupeol, stigmasterol and β-sitosterol. International Journal of Molecular Sciences 13(4):5074-5097. *https://doi.org/10.3390/ijms13045074*

Chinembiri TN, du Plessis LH, Gerber M, Hamman JH, du Plessis J (2014). Review of natural compounds for potential skin cancer treatment. Molecules 19:11679-11721. *https://doi.org/10.3390/molecules190811679*

Chumroenphat T, Somboonwatthanakul I, Saensouk S, Siriamornpun S (2021). Changes in curcuminoids and chemical components of turmeric (*Curcuma longa* L.) under freeze-drying and low-temperature drying methods. Food Chemistry 339:128121. *https://doi.org/10.1016/j.foodchem.2020.128121*

Gotoh A, Sakaeda T, Kimura T, Shirakawa T, Wada Y, Wada A, ... Okumura K. (2004). Antiproliferative activity of *Rhinacanthus nasutus* (L.) Kurz extracts and the active moiety, Rhinacanthin C. Biological and Pharmaceutical Bulletin 27(7):1070-1074. *https://doi.org/10.1248/bpb.27.1070*

Hara-Terawaki A, Takagaki A, Kobayashi H, Nanjo F (2017). Inhibitory activity of catechin metabolites produced by intestinal microbiota on proliferation of HeLa cells. Biological and Pharmaceutical Bulletin 40(8):1331-1335. *https://doi.org/10.1248/bpb.b17-00127*

Khamwut A, Jevapatarakul D, Reamtong O, T-Thienprasert NP (2019). *In vitro* evaluation of anti-epidermoid cancer activity of *Acanthus ebracteatus* protein hydrolysate and their effects on apoptosis and cellular proteins. Oncology Letters 18(3):3128-3136. *https://doi.org/10.3892/ ol.2019.10647*

Luang-In V, Saengha W, Karirat T, Buranrat B, Matra K, Deeseenthum S, ... Katisart T (2021). Effect of cold plasma and elicitors on bioactive contents, antioxidant activity and cytotoxicity of Thai rat-tailed radish microgreens. Journal of the Science of Food and Agriculture 101:1685-1698. *https://doi.org/10.1002/jsfa.10985*

Mahasiripanth T, Hokputsa S, Niruthisard S, Bhattarakosol P, Patumraj S (2012). Effects of *Acanthus ebracteatus* Vahl on tumor angiogenesis and on tumor growth in nude mice implanted with cervical cancer. Cancer Management and Research 4:269-279. *https://doi.org/10.2147/CMAR.S33596*

Nourazarian SM, Nourazarian A, Majidinia M, Roshaniasl E. (2015). Effect of root extracts of medicinal herb glycyrrhiza glabra on HSP90 gene expression and apoptosis in the HT-29 colon cancer cell line. Asian Pacific Journal of Cancer Prevention 16:8563-8566. *https://doi.org/10.7314/APJCP.2015.16.18.8563*

Olatunji OJ, Olatunde OO, Jayeoye TJ, Singh S, Nalinbenjapun S, Sripetthong S, ... Ovatlarnporn C (2022). New insights on *Acanthus ebracteatus* Vahl: UPLC-ESI-QTOF-MS profile, antioxidant, antimicrobial and anticancer activities. Molecules (Basel, Switzerland) 27(6):1981. *https://doi.org/10.3390/molecules27061981*

Pandey P, Khan F, Farhan M, Jafri A (2021). Elucidation of rutin's role in inducing caspase-dependent apoptosis via HPV-E6 and E7 down-regulation in cervical cancer HeLa cells. Bioscience Reports 41(6):BSR20210670. *https://doi.org/10.1042/BSR20210670*

Phuseerit O, Wrigley C, Siriamornpun S (2021). Bioactives and antioxidant activities of marigold flower juices and the effect of different storage times. Science Technology and Engineering Journal 7(2):58-73.

Prasansuklab A, Tencomnao T (2018). *Acanthus ebracteatus* leaf extract provides neuronal cell protection against oxidative stress injury induced by glutamate. BMC Complementary and Alternative Medicine 18(1):278. *https://doi.org/10.1186/s12906-018-2340-4*

Ryu JY, Kang HR, Cho SK (2019). Changes over the fermentation period in phenolic compounds and antioxidant and anticancer activities of blueberries fermented by *Lactobacillus plantarum*. Journal of Food Science 84:2347-2356. *https://doi.org/10.1111/1750-3841.14731*

Sali VK, Mansingh DP, Vasanthi HR (2016). Relative apoptotic potential and specific G1 arrest of stigmasterol and cinnamic acid isolated from the brown algae *Padina gymnospora* in HeLa and A549 cells. Medicinal Chemistry Communication 7:1429-1435.

Siripong P, Hahnvajanawong C, Yahuafai J, Piyaviriyakul S, Kanokmedhakul K, Kongkathip N, ... Oku N (2009). Induction of apoptosis by rhinacanthone isolated from *Rhinacanthus nasutus* roots in human cervical carcinoma cells. Biological and Pharmaceutical Bulletin 32(7):1251-1260. *https://doi.org/10.1248/bpb.32.1251*

Siripong P, Yahuafai J, Shimizu K, Ichikawa K, Yonezawa S, Asai T, ... Oku N (2006). Induction of apoptosis in tumor cells by three naphthoquinone esters isolated from Thai medicinal plant: *Rhinacanthus nasutus* KURZ. Biological and Pharmaceutical Bulletin 29(10):2070-2076. *https://doi.org/10.1248/bpb.29.2070*

Siriwatanametanon N, Fiebich BL, Efferth T, Prieto JM, Heinrich M (2010). Traditionally used Thai medicinal plants: *In vitro* anti-inflammatory, anticancer and antioxidant activities. Journal of Ethnopharmacology 130(2):196-207. *https://doi.org/10.1016/j.jep.2010.04.036*

Song P, Sun C, Li J, Long T, Yan Y, Qin H, ... Olatunji OJ (2021). *Tiliacora triandra* extract and its major constituent attenuates diabetic kidney and testicular impairment by modulating redox imbalance and pro-inflammatory responses in rats. Journal of the Science of Food and Agriculture 101:1598-1608. *https://doi.org/10.1002/jsfa.10779*

Suksawat T, Panichayupakaranant P (2022). Variation of rhinacanthin content in *Rhinacanthus nasutus* and its health products. Journal of Pharmaceutical and Biomedical Analysis 224:115177. *https://doi.org/10.1016/j.jpba.2022.115177*

Thephinlap C, Pangjit K, Suttajit M, Srichairatanakool S (2013). Anti-oxidant properties and anti-hemolytic activity of *Psidium guajava*, *Pandanous odorus* and *Rhinacanthus nasutus*. Journal of Medicinal Plants Research 7:2001-9. *https://doi.org/10.5897/JMPR12.800*

Wang H, Zhu Y, Hao C, Fan J, Liu Y, Wang Y (2019). Establishment of a drug-resistant human cervical cancer cell line and research on its drug-resistance. Journal of Cancer Research and Therapeutics 15:1221-1225. *https://doi.org/10.4103/0973-1482.204900*

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