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Research Article

Cytotoxic and antiproliferative effects of *Streblus asper* from northeastern Thailand on A549 lung cancer cells

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Abstract

Thailand's second-leading cause of death is lung cancer. Thai indigenous herbal plants are sought after as an alternative treatment against lung cancer. This work aimed to examine cytotoxic, antiproliferative and antimigratory capacities of different parts of *Streblus asper* (SA) from northeastern Thailand on A549 lung cancer cells. Plant leaves, twigs, bark and wood were used for ethanolic extraction by maceration. The highest cytotoxicity of 85.46% was found in twig extract (IC_{50} 57.46 µg/mL) assessed by an MTT assay. The lowest IC_{50} (18.42 µg/mL) was also found in twig extract using a clonogenic assay indicating its most antiproliferative activity in a long-term therapy. In addition, all SA extracts displayed antimigratory activity against A549 cells in a dose-dependent fashion, especially twig extract. Apoptotic characteristics were noticeable in SA extract treated cells. The maximum DPPH-scavenging activity, FRAP value, total phenolic and flavonoid content were found in twig extract. GC-MS analysis revealed that twig extract contained four prominent components namely ethyl-α-D-glucopyranoside, hexadecanoic acid, ethyl ester, lupeol and γ-sitosterol. Real-time PCR results showed that genes (*Bcl-2*, *Bax*, *p21*, and *cytochrome c*) linked to apoptosis were significantly affected by all SA extracts. The various SA components' ethanolic extracts exhibited moderate-to-high cytotoxic action towards A549 cells. This work will significantly advance the utilization of the plant as an alternative source of medicine for rural Thais, and it paves the way for future research to determine the active compound(s) and anticipate new drug candidates.

Keywords: A549; antiproliferation; apoptosis; cytotoxicity; *Streblus asper*

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Introduction

One in every 21 cases of cancer in Thailand is lung cancer, with 70% of cases discovered after disease progression (Reungwetwattana *et al.*, 2020). In 2018, the mortality rate for lung cancer in Thailand was 18.7%, the second highest after liver cancer at 20.3%. On the basis of histology, non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) are the two most prevalent kinds of lung cancer. NSCLC accounts for 85 percent of all lung cancer occurrences, making it the most common kind (Ettinger *et al.*, 2012).

Surgical removal and radiation therapy are critical components of Thailand's multimodality lung cancer treatment (Reungwetwattana *et al*., 2020). Key issues and obstacles in cancer treatment in Thailand are medication accessibility and a shortage of specialized healthcare workers. Recently, for lung cancer, more study has been undertaken on biochemicals that can be exploited as new anticancer drugs.

Secondary metabolites, which may contribute to cytotoxic effects, are abundant in plants. The majority of the population in undeveloped nations employ traditional herbal remedies to treat a wide range of ailments and conditions, and several plants are utilized for their health benefits (Okaiyeto and Oguntibeju, 2021). According to the World Health Organization (WHO), some regions still rely on medicinal herbs as their primary source of medication, whilst developing nations reap the therapeutic benefits of organically produced compounds.

Streblus asper Lour. (SA) is a genus within the Moraceae family. Plantations of this tree are widely found in Thailand, Sri Lanka, Malaysia, India, and the Philippines, among other tropical regions (Rastogi *et al*., 2006). SA extract from root to leaf and its ingredients were used to cure several diseases. Research revealed that its extract is antifilarial, antifungal, antiinflammatory, antimicrobial, antiviral, antioxidant, antihyperglycemic, antidiabetic, and anticancer (Seeni *et al*., 2012). SA has been shown to treat A549, KB, HOS, and SCC-15 cancer cells in several investigations (Vicus *et al*., 2014; Yoshida *et al*., 2018; Priaulx *et al*., 2018).

The SA tree is an evergreen that has both therapeutic and economic value. Its stem bark is used in the treatment of bleeding, tubercular adenitis, cervical lymphadenitis, and piles, as described by Ayurveda, the ancient Indian medical system (Caldecott, 2011). Trees of this species are known by a wide variety of names, including Siamese rough bush, khoi, serut, and toothbrush tree (Kumar *et al*., 2021). Its additional advantage is easy maintenance. Thai forest monks and some rural Thai people utilize SA twigs as toothbrushes since they may be chewed to clean the teeth (Kadir *et al*., 2014). Since Thai smokers have a greater chance of developing lung cancer than non-smokers (Zhang *et al*., 2022), it would be prudent to promote SA extract and encourage its usage for chemopreventive purposes among this population. The SA plant in northeastern Thailand was selected for this research so that results may be compared to those from other nations' SA plants. Variations in bioactive content of SA extracts sourced from different countries are hypothesized to arise in variations in both geography and climate.

To date, data on anticancer effects of Thai herbs grown in northeastern Thailand is still scarce in spite of increasing support from Thai government to promote northeastern Thailand as Thai Herbal Center. In this research, the capacity of SA from northeastern Thailand, with various parts of plant (leaves, twigs, bark, and wood) extracted with ethanol to inhibit human lung cancer cell growth (A549 cells) was investigated. The promising results would boost the attention to grow more SA for chemopreventive purposes around Thailand which can be more easily accessed and help reduce the cost of medical treatment for lung cancer.

Materials and Methods

Plant collection and extraction

Leaves and twigs of SA (Figure 1A and 1B) were collected from Nasinuan Community Forest, Maha Sarakham Province, Thailand in October 2021 in early morning. Wood and bark of SA (Figure 1C and 1D) were collected from Khon Kaen Province, Thailand in October 2021 in early morning. The taxonomic classification of SA plant was carried out by Asst. Prof. Dr. Chadaporn Senakun, Walai Rukhavej Botanical Research Institute, Mahasarakham University. The SA plant specimens with voucher number 012021 (Maha Sarakham) and 022021 (Khon Kaen) were stored in the Biotechnology Botanical Herbarium at the Department of Biotechnology, Mahasarakham University, Thailand.

All parts of SA were oven dried at 42 °C until dryness and once dried were ground thoroughly. Next, dried residues (100 g) were extracted in 1,000 mL of ethanol in the flask at 37 °C for 3 days at 200 rpm. Next, re-extraction was conducted twice. The extracts were collected and spun down for 30 min at 10,000 *g*. The supernatant was filtered using a no. 4 Whatman filter and the filtrate was evaporated. The plant extracts were concentrated, freeze-dried, and then kept at -20 °C. For antioxidant and bioactive compound assays, the extracts (20 mg/mL) were filtered after being re-dissolved in ethanol whilst for anticancer assays, they were re-dissolved in RPMI-1640 media Gibco (Carlsbad, CA, USA).

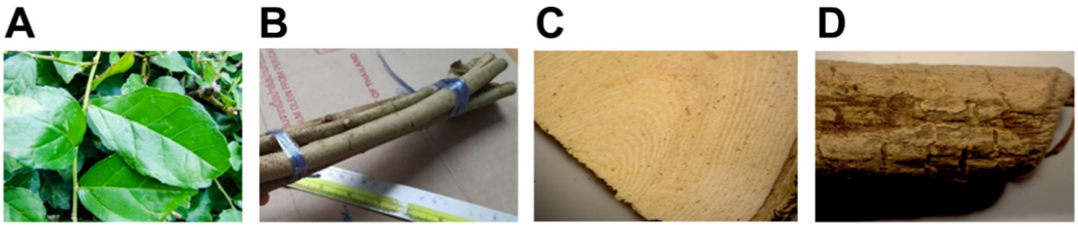

Figure 1. Parts of *Streblus asper*; (A) Leaves; (B) Twigs; (C) Wood; (D) Bark

Antioxidant activity and bioactive analysis

As previously reported, FRAP and DPPH radical scavenging techniques were employed (Saengha *et al*., 2021). Each 20 mg/mL SA extract (20 µL) was mixed with 180 µL of 10 mM DPPH (Sigma-Aldrich, St. Louis, MO, USA). A M965+ microplate reader measured 515 nm absorption after 30 min of darkness (Metertech, Taipei, Taiwan). For FRAP, plant extract (20 µL of 20 mg/mL stock solution) was mixed with 180 µL of FRAP reagent (20 mM $FeCl_3$, 10 mM 2,4,6-Tri (2-pyridyl) s-triazine, and 0.3 M acetate buffer at pH 3.6). After 30 min, 593 nm absorption was recorded. DPPH and FRAP tests used Trolox and ferrous II sulfate as antioxidant standards. All chemicals were purchased from Sigma-Aldrich, St. Louis, MO, USA.

The total phenolic content (TPC) and the total flavonoid content (TFC) were evaluated as described (Luang-In *et al*., 2021). For TPC, 20 µL of plant extract, 80 µL of 7.35% sodium carbonate, and 100 µL of 10% Folin-Ciocalteu solution were combined. A765 nm record followed 30 min dark reaction. For TFC, deionized water (60 µL), 10% aluminum trichloride (10 µL), and 5% sodium nitrate (10 µL) were pipetted into plant extract (20 µL). After adding 100 µL of 1 M NaOH (Sigma-Aldrich, St. Louis, MO, USA), the mixture reacted for 30 min before its absorbance at 420 nm was recorded. Each determination used triplicate. TPC and TFC assays employed gallic acid and rutin as standards. All chemicals were purchased from Sigma-Aldrich, St. Louis, MO, USA.

Gas chromatography-mass spectrometry (GC-MS)

The chemical analysis of the SA extracts was executed with a Shimadzu QP2010 instrument (Shimadzu, Tokyo, Japan), a fused silica HP5-MS capillary column (5% phenylmethylsiloxane, 30 m × 0.25 mm i.d.; film thickness, 0.25 µm) from Agilent, CA, USA, and a mass spectrometer as a detector. The criteria for operation

were the same as those used previously (Rawat *et al*., 2018). ChemStation was used to record and analyze the data. MS spectra were compared to reference spectra from the National Institute of Standards and Technology (NIST) library in order to positively identify the compounds. By adding up the peak areas of all the compounds in each sample, the amount of each compound in each sample as a percentage was reported.

Cell cultures

A human lung cancer cell line called A549 (ATCC® CCL-2™) was acquired from the American Type Culture Collection (ATCC, Manassas, VA, USA) and grew in RPMI-1640 media with 10% FBS, 1% Penicillin/Streptomycin, and 5% CO_2. Every three days, the medium was replaced and the cells were collected to be trypsinized with 0.25% trypsin EDTA. All chemicals were purchased from Gibco (Carlsbad, CA, USA).

Cytotoxicity assay

Cytotoxic activities of SA extracts towards A549 were assessed using 3, 4, 5-dimethylthiazol-2-yl-2-5-diphenyltetrazolium bromide (MTT) assay. A549 cells (5×10^3 cells/well) were grown in 96-well plates overnight. Various concentrations of SA extracts (0-800 µg/mL dissolved in the media) were exposed to A549 cells for 24 h. MTT reagent replaced the media and left for reaction to occur for 4 h. Afterwards, DMSO (200 µL) dissolved the formazan crystals, and A590 nm was recorded by a microplate reader. Triplicate testing was conducted. Both IC_{50} and cytotoxicity (%) of SA extracts to A549 cells were determined. All chemicals were purchased from Sigma-Aldrich, St. Louis, MO, USA.

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

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.

Colony formation assay

A549 cells were grown at 500 cells/well overnight. Cells were treated with different SA extract concentrations (0-100 µg/mL) in the media. Next, cells were washed with phosphate buffer saline (PBS) and incubated at 37 °C in 5% CO_2 for 14 days. Each day, new media was added. Cells were fixed in -20 °C methanol for 30 min. For 30 min, colonies were stained with Coomassie brilliant blue g-250 (Sigma-Aldrich, St. Louis, MO, USA). The percentage of triplicate colonies relative to untreated cells was calculated.

Cell morphology

A549 cells were seeded 7,500 cells/well in a 24-well plate. After 24 h, cells were exposed to SA extracts (0-800 µg/mL). A light microscope was used to view cells.

Wound healing assay

A549 cells (2×10^5 cells/well) confluence overnight in a 24-well plate. Wound scratching on cells was created by a 200 µL pipette tip and PBS cleaning followed. SA extracts (0-100 µg/mL) were exposed to cells for 24 h. Afterwards, cells were fixed with 4% formaldehyde, dyed with 0.5% crystal violet for 30 min, washed with distilled water, collected, and compared to untreated cells for relative scratch closure.

Real-time polymerase chain reaction (PCR) analysis

A549 cells (2×10^5 cells/well) were grown overnight in prior to plant extract treatment (50 µg/mL). Real-time PCR was employed to investigate apoptosis-related mRNA after 24 h exposure. As stated before, total

RNA was extracted using TRIzol™ (Thermo Fisher Scientific, Waltham, MA, USA), cDNA synthesized, and gene expression analyzed by RT-qPCR (Wangkahart *et al*., 2022). *Bcl-2*, *Bax*, *p21*, and *Cytochrome c* gene expressions were examined using a FX96 Touch Real-Time PCR Detection System (Bio-Rad, Watford, UK). RT-qPCR used these gene-specific primers. β-actin: F 5′ – CTGTCTGGCGGCACCACCAT – 3′, R 5′ – GCAACTAAGTCATAGTCCGC – 3, *Bax* : F 5′ – CAGCTCTGAGCAGATCATGAAGACA – 3′, R 5′ – GCCCATCTTCTTCCAGATGGTGAGC – 3′, *Bcl-2* : F 5′ – GGTGCCACCTGTGGTCCACCTG – 3′, R 5′ – CTTCACTTGTGGCCCAGATAGG – 3, *p21* : F 5′ – AGTCAGTTCCTTGTGGAGCC– 3′, R 5′ – GCATGGGTTCTGACGGACAT – 3′, *Cytochrome c* : F 5′-GGGCGAGAGCTATGTAAT GCAAG-3′, R 5′-TACAGCCAAAGCAGCAGCT CA-3′. Each gene was normalized to β-actin as an internal control. By dividing the transcription level in SA-treated groups by that of the control untreated group, gene fold changes were calculated.

Statistical analysis

One-way analysis of variance (ANOVA) and Duncan's multiple range test (SPSS, IBM, Armonk, NY, US) were performed on triplicate data to calculate means and standard deviations, with p <0.05 being significant.

Results

Antioxidant activity and bioactive contents

Twig extracts displayed the highest antioxidant capacities by DPPH and FRAP assays, TPC and TFC followed by bark and wood extracts (Table 1). However, leaf extracts showed the lowest values in all assays.

Table 1. Antioxidant activity and bioactive content assessments of SA extracts

Samples	DPPH (mg TE/g DW)	FRAP (mg Fe^{2+} /g DW)	TPC (mg GAE/g DW)	TFC (mg RE/g DW)
Wood	8.73±1.51^{b}	7.35±0.31^{c}	4.06±0.21^{b}	9.36±0.32^{b}
Bark	10.88±0.48^{a}	8.03±0.25^{b}	4.21±0.27^{b}	9.25±0.61^{b}
Twig	11.95±0.20^{a}	9.11±0.22^{a}	11.03±0.31^{a}	16.56±1.10^{a}
Leaf	1.99±0.23^{c}	5.15±0.08^{d}	3.34±0.18^{c}	6.03±0.68^{c}

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

GC-MS analysis of bioactive contents

NIST information was used to interpret GC-MS results. The discovered chemical compositions consist of triterpenes, phytosterol, fatty acids, and sugars. The results showed that different parts of SA contained mostly different compounds in varying contents (Figure 2 and Table 2). Twig, bark, wood and leaf extracts showed 19, 14, 13 and 12 compounds (Figure 2). The twig extract contained four prominent components namely ethyl-α-D-glucopyranoside (32.22%), hexadecanoic acid, ethyl ester (7.43%), lupeol (18.08%) and γ-sitosterol (7.43%)(Table 2). The bark extract also contained four major components including 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (23.73%), Methyl β-D-galactopyranoside (17.19%), α-Amyrin (14.54%), γ-Sitosterol (9.37%)(Table 2). The wood extract consisted of two main compounds namely ethyl-α-D-glucopyranoside (10.93%) and Lupeol (18.94%). The leaf extract showed three major components including sorbitol (15.29%), olean-12-en-3-ol, acetate, (3. beta.)(13.09%), and 2,6,10,15,19,23-Hexamethyl-tetracosa-2,10,14,18,2 (16.81%). Certain compounds, in spite of low abundance, found in all SA extracts exhibited anticancer activity based on previous literature (Table 2).

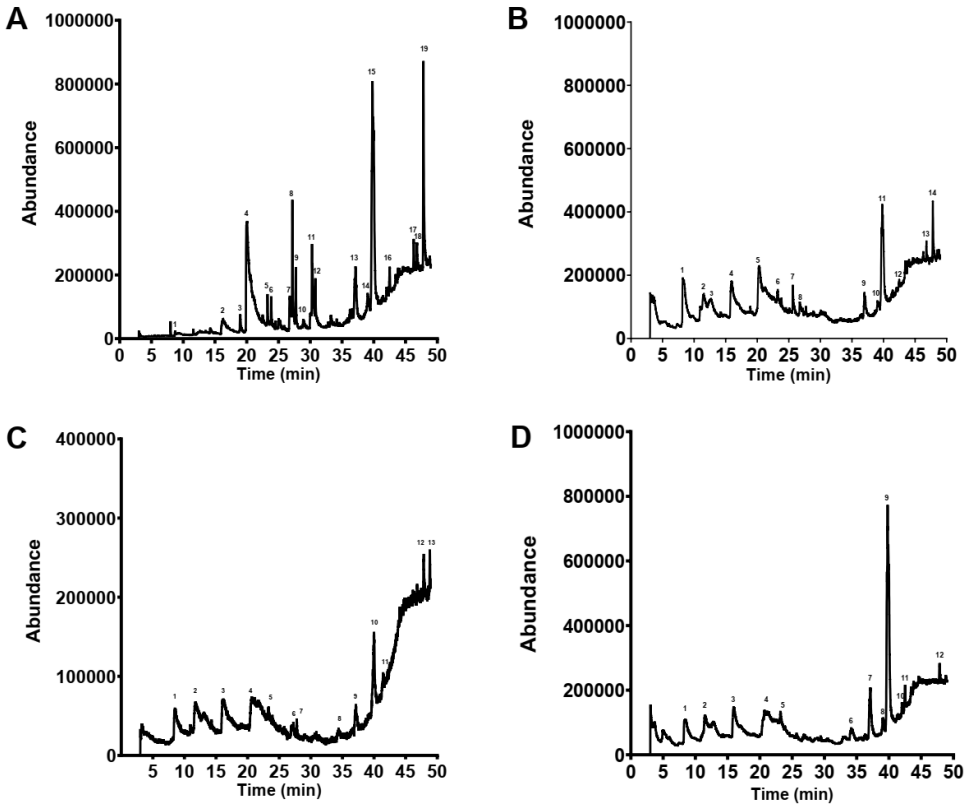

Figure 2. GC-MS chromatograms of SA extracts (A) Twig (B) Bark (C) Wood (D) Leaf

Table 2. Chemical compositions of SA extracts by GC-MS analysis

Peak no.	Retention time (min)	Area (% of total)	Constituents	Chemopreventive activities	Ref.
(A) Twig					
1	8.00	0.31	(+)-2-Bornanone	No report	No report
2	16.26	4.31	6-Amino-1-.beta.-d-ribofuranosylimidazo [4,5-c	No report	No report
3	18.96	1.17	Octadecane	Cytotoxic to HEp-2 (larynx), MCF-7 (breast) and NCI-H292 (lung), cancer cells	Bastos *et al*. (2017)
4	20.04	34.90	Ethyl-α-D-glucopyranoside	Cytotoxic against HeLa and NIH/3T3 cell	Ahmad *et al*. (2016)
5	23.27	1.08	Heneicosane	Cytotoxic to HEp-2 (larynx), MCF-7 (breast) and NCI-H292 (lung), cancer cells	Bastos *et al*. (2017)
6	23.86	1.08	Isopropyl myristate	Cytotoxic to A549 lung cancer cells	Alkhatib and Alkhayyal (2016)
7	26.78	3.63	n-Hexadecanoic acid	No report	No report

8	27.18	7.43	Hexadecanoic acid, ethyl ester	Cytotoxic to HEp-2 (larynx), MCF-7 (breast) and NCI-H292 (lung), cancer cells	Bastos *et al*. (2017)
9	27.73	2.67	Isopropyl palmitate	No report	No report
10	28.90	0.23	1-Heneicosanol	Cytotoxic to MCF-7 (breast) and HCT-116 (colon) cancer cells	Mofeed *et al*. (2021)
11	30.28	6.94	Linoleic acid ethyl ester	No report	No report
12	30.81	1.66	Octadecanoic acid, 17-methyl-, methyl ester	Cytotoxic to MCF-7 (breast) and HCT-116 (colon) cancer cells	Mofeed *et al*. (2021)
13	37.13	3.69	24-Noroleana-3,12-diene	No report	No report
14	39.80	1.69	α-Amyrin	Cytotoxic to leukemic cells	Neto *et al*. (2021)
15	40.00	18.08	Lupeol	Cytotoxic to HEp-2 (larynx), MCF-7 (breast) and NCI-H292 (lung), cancer cells	Bastos *et al*. (2017)
16	42.51	1.28	9,19-Cyclolanostan-3-ol, 24-methylene-, (3.beta.)-	Anticancer	Hasan and Kuswandi (2014)
17	46.29	1.16	Ergost-5-en-3-ol	Cytotoxic to human HCT-116 (colon) cell line	Khan *et al*. (2021)
18	46.79	1.26	Stigmasterol	Inhibit ovarian cancer by activating PI3K/Akt	Bakrim *et al*. (2022)
19	47.82	7.3	γ-Sitosterol	Cytotoxic to A549 (lung) and MCF-7 (breast)	Sundarraj *et al*. (2012)
(B) Bark					
1	8.18	23.73	2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	Cytotoxic to Ehrlich ascites carcinoma	Rajendran *et al*. (2014)
2	11.29	1.10	3-((3-Cholamidopropyl) dimethylammonio)-1-propan	No report	No report
3	11.53	1.08	Acetoxyacetic acid	No report	No report
4	15.94	17.82	Sucrose	No report	No report
5	20.32	17.19	Methyl β-D-galactopyranoside	Cytotoxic to Ehrlich ascites carcinoma	Ahmed *et al*. (2017)
6	23.25	2.49	1,54-Dibromotetrapentacontane	Antioxidant	Addai *et al*. (2022)
7	25.63	4.11	Lidocaine	No report	No report
8	26.72	0.64	(2,2,6-Trimethyl-bicyclo[4.1.0]hept-1-yl)-methanol	No report	No report
9	36.98	3.22	Olean-12-en-3-ol, acetate, (3.beta.)-	No report	No report
10	39.03	0.93	9,19-Cyclolanostan-3-ol, 24-methylene-, (3.beta.)-	Anticancer	Hasan and Kuswandi (2014)
11	39.80	14.54	α-Amyrin	Cytotoxic to leukemic cells	Neto *et al*. (2021)
12	43.47	1.47	9-Desoxy-9x-chloroingol 3,7,8,12-tetraacetate	No report	No report
13	46.79	2.29	Stigmasterol	Inhibit ovarian cancer by activating PI3K/Akt	Bakrim *et al*. (2022)

14	47.817	9.39	γ-Sitosterol	Cytotoxic to A549 (lung) and MCF-7 (breast)	Sundarraj *et al.* (2012)
(C) Wood					
1	8.48	7.6	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	Cytotoxic to Ehrlich ascites carcinoma	Rajendran *et al.* (2014)
2	11.51	8.61	d-Galactose oxime	No report	No report
3	16.37	7.63	1,3:2,5-Dimethylene-l-rhamnitol	Cytotoxic to Ehrlich ascites carcinoma	Rajendran *et al.* (2014)
4	20.04	10.93	Ethyl-α-D-glucopyranoside	Induce apoptosis to kill cancer cells	Lyantagaye (2013)
5	23.27	6.61	1,2,5-trimethyl-4-piperidinone O-(3-methylbenzoyl) oxime	No report	No report
6	26.78	3.31	n-Hexadecanoic acid	No report	No report
7	27.18	4.35	Hexadecanoic acid, ethyl ester	Cytotoxic to HEp-2 (larynx), MCF-7 (breast) and NCI-H292 (lung), cancer cells	Bastos *et al.* (2017)
8	34.17	4.89	9-Octadecenamide, (Z)-	Antimetastatic potential of BL6 skin melanoma cells	Hecht *et al.* (2015)
9	37.13	8.71	Olean-12-en-3-ol, acetate, (3. beta.)-	No report	No report
10	40.00	18.94	Lupeol	Cytotoxic to HEp-2 (larynx), MCF-7 (breast) and NCI-H292 (lung), cancer cells	Bastos *et al.* (2017)
11	42.51	5.12	9,19-Cyclolanostan-3-ol, 24-methylene-, (3.beta.)-	Anticancer	Hasan and Kuswandi (2014)
12	46.80	7.12	Stigmasterol	Inhibit ovarian cancer by activating PI3K/Akt	Bakrim *et al.* (2022)
13	47.82	6.18	γ-Sitosterol	Cytotoxic to MCF-7 (breast) and A549 (lung)	Sundarraj *et al.* (2012)
(D) Leaf					
1	8.48	5.60	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	Cytotoxic to Ehrlich ascites Carcinoma	Rajendran *et al.* (2014)
2	11.51	5.44	d-Galactose oxime	No report	No report
3	15.94	5.80	Sucrose	No report	No report
4	20.74	15.29	Sorbitol	No report	No report
5	23.18	9.68	4-(7-Methoxy-3,3,7-trimethyl-oxepan-2-yliden	No report	No report
6	34.17	3.56	9-Octadecenamide, (Z)-	Antimetastatic potential of BL6 skin melanoma cells	Hecht *et al.* (2015)
7	37.13	13.09	Olean-12-en-3-ol, acetate, (3. beta.)-	No report	No report
8	39.81	4.71	α-Amyrin	Cytotoxic to leukemic cells	Neto *et al.* (2021)
9	39.98	16.81	2,6,10,15,19,23-Hexamethyl-tetracosa-2,10,14,18,2	No report	No report

10	42.00	8.04	9,19-Cyclolanostan-3-ol, 24-methylene-, (3.beta.)-	Anticancer	Hasan and Kuswandi (2014)
11	42.49	5.86	9,19-Cyclolanostan-3-ol, 24-methylene-, acetate	No report	No report
12	47.84	6.12	γ-Sitosterol	Cytotoxic to A549 (lung) and MCF-7 (breast)	Sundarraj *et al*. (2012)

Cytotoxicity of SA extracts towards A549 cells

Cytotoxicities of SA extracts ranked the most effective against A549 cells based on IC_{50} values were as follows: twig, wood, bark and leaf with the respective *E*max% (Table 3).

Table 3. *E*max (%) and IC_{50} of SA extracts on A549 cells

SA extracts	*E*max (%) at 800 µg/mL	IC_{50} (µg/mL)
Wood	81.82±1.44 [c]	75.61±0.24 [c]
Bark	83.11±0.09 [b]	64.42±3.14 [b]
Twig	85.46±0.40 [a]	57.46±0.41 [a]
Leaf	68.02±0.90 [d]	111.53±5.55 [d]

[a, b, c ...] represent statistical significance in the columns at $p < 0.05$.

Cell morphological changes

A decrease in A549 cell density and an increase in apoptotic bodies were detected in treated A549 cells when SA twig extract concentration increased (Figure 3). These findings showed that SA extracts dose-dependently promoted apoptosis in A549 cells.

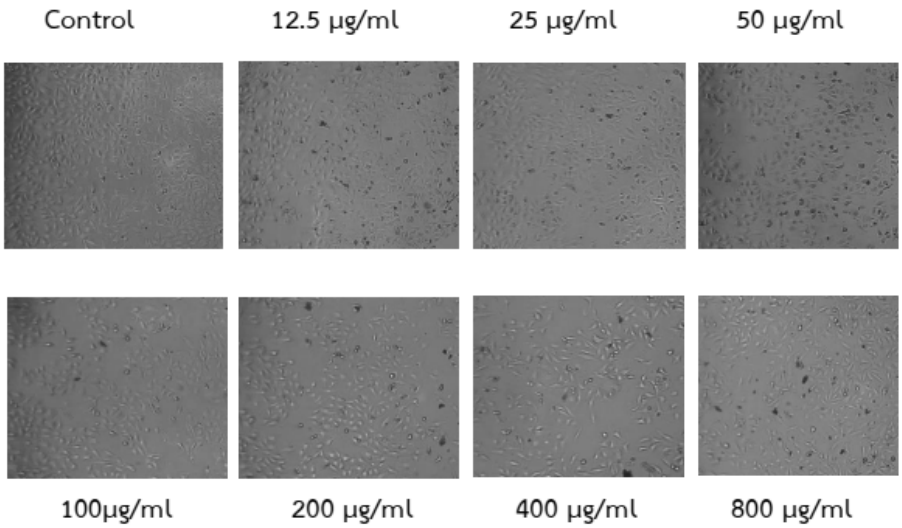

Figure 3. Effect of twig extracts in different doses on cell morphology of A549 cells

Antiproliferative activity

Results of the clonogenic assay showed that SA extracts were antiproliferative towards A549 cells in 14-day-treatment in a dose-dependent fashion (Figure 4). Twig extract with a lower IC_{50} value of 18.42 µg/mL showed significantly stronger antiproliferative activity towards A549 cells than wood (IC_{50} = 20.25 µg/mL), bark (IC_{50} = 22.45 µg/mL) and leaf (IC_{50} = 97.36 µg/mL)(Figure 4). This indicated that SA twig, wood and bark extracts exhibited a strong antiproliferative effect against A549 cells in a long-term treatment. These

results are ranked in similar order as shown in cytotoxic results based on MTT assay. However, IC_{50} values of all four SA extracts were lower than those found in the MTT assay which represented a short-term treatment.

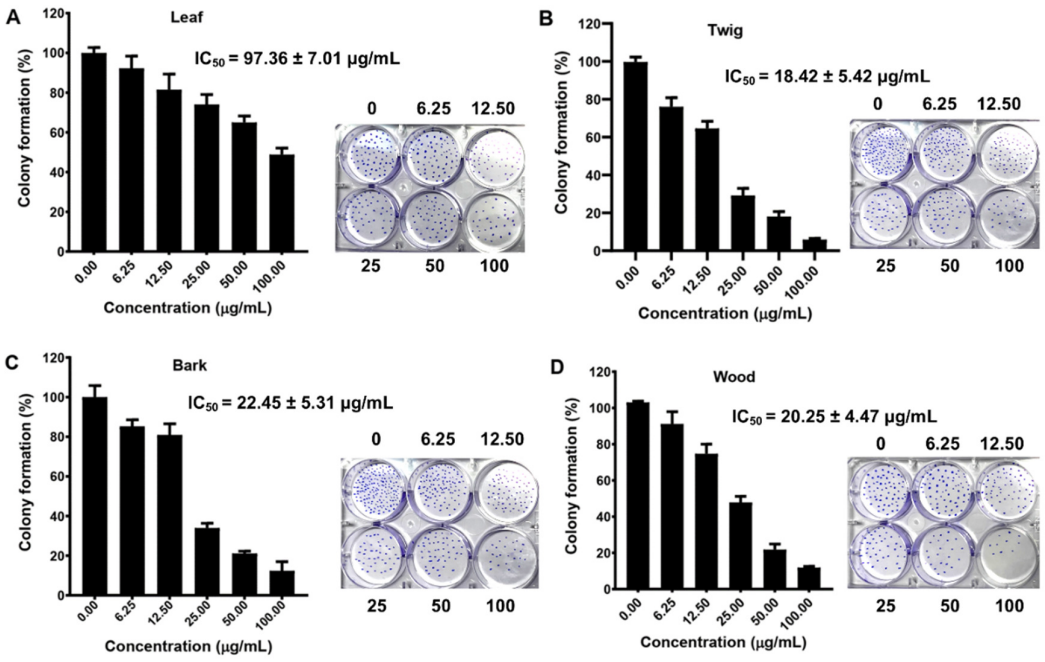

Figure 4. Effect of SA extracts on colony formation of A549 cells. (A) Leaf; (B) Twig; (C) Bark; (D) Wood

Antimigratory activity

All SA extracts substantially inhibited A549 cell migration and decreased wound coverage in a dose-dependent fashion. Overnight, the control covered almost 100% of the migratory area, whereas the SA-treated sample only covered 37-60% at 100 µg/mL (Figure 5). Cells treated with twig extract covered only 37% of the migratory region at 100 µg/mL (Figure 5B). To conclude, twig extract was the most cytotoxic, antiproliferative and antimigratory amongst all parts of SA.

Effects on apoptotic gene expressions

All SA extracts significantly decreased the gene expressions of *Bcl-2* (Figure 6A), whilst increased *Bax, p21*, and *Cytochrome C* (Figure 6B-D) suggesting that SA extracts induced intrinsic apoptosis. In most cases, twig extracts have stronger effects followed by wood, bark and leaf.

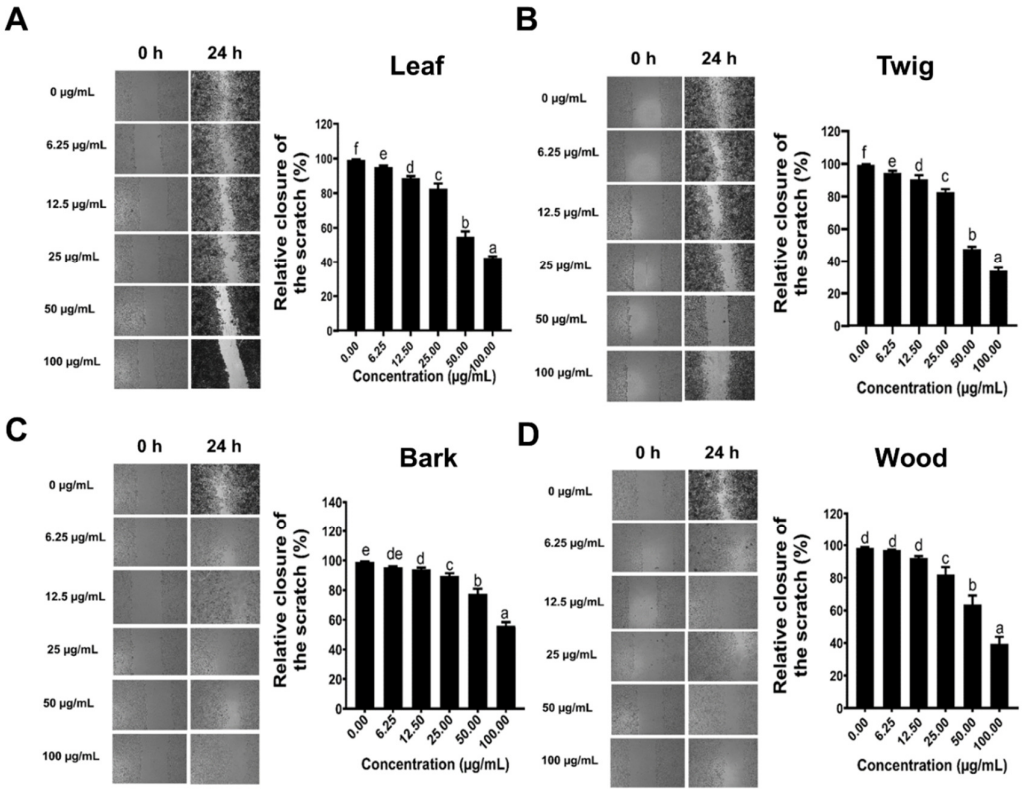

Figure 5. Antimigratory activity of RN and AE extracts using a wound healing assay
(A) Leaf; (B) Twig; (C) Bark; (D) Wood.
At $p < 0.05$, different letters in the bars denote statistical significance

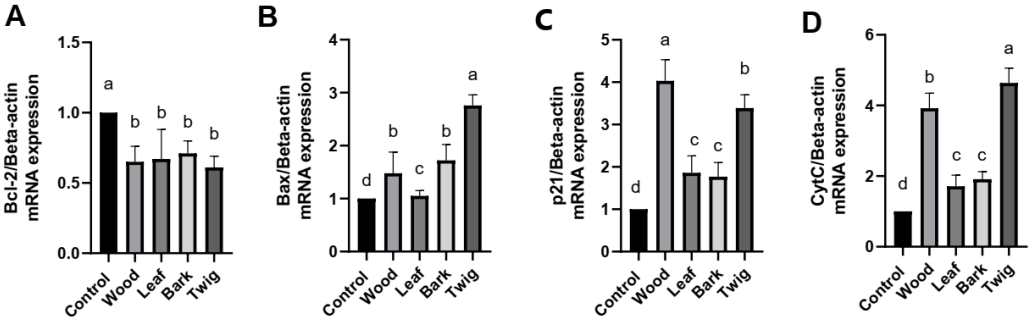

Figure 6. Effects of SA extracts on apoptotic gene expressions; (A) *Bcl-2*; (B) *Bax*; (C) *p21*; (D) *Cytochrome C*
At p < 0.05, different letters in the bars denote statistical significance

Discussion

Lung cancer, amongst other cancers, is one of a leading cause of mortality in Thailand. Smokers are at a higher risk of getting lung cancer and thus are suggested to exercise a chemopreventive regime such as taking Thai herbal medicine proven to exhibit anticancer effects against lung cancer. SA parts of plants (twig, leaf, wood and bark) from northeastern Thailand were studied for anticancer effects against A549 lung cancer cell line using MTT assay, a clonogenic assay, wound healing assay, and real-time PCR to check for apoptotic gene

expressions. In addition, antioxidant assays, TPC and TFC methods were carried out along with GC-MS analysis to determine antioxidant activity, bioactive contents and chemical compositions in SA extracts, respectively.

When compared to the previous study, SA leaf extract in this work was found to have significantly lower TPC (3.34±0.18 mg GAE/g DW)(Table 1) than SA leaf extract in 70% ethanol (302.85±0.03 mg GAE/g) originated from Sungai Petani, Kedah, Malaysia (Ibrahim *et al*., 2013). Likewise, TFC of SA leaf extract (6.03±0.68 mg RE/g DW)(Table 1) in this work was also much lower than SA leaf extract in 70% ethanol (22.70 ± 0.02 mg QE/g) (Ibrahim *et al*., 2013). The differences in bioactive content between SA leaf extract in this work and that in a previous report may lie in different solvents used for extraction (95% ethanol vs 70% ethanol) different sample preparation method (oven dry vs freeze dry), ages of plants (unknown), geographical and climate conditions (Thailand 2021 vs Malaysia 2013), to name but a few.

In this work, DPPH radical scavenging activities and FRAP values are ranked in the highest to the lowest in the following order; twig, bark, wood and leaf. These trends are in good agreement with TPC and TFC (Table 1). This data suggests that phenolic and flavonoid components may be responsible for the antioxidant properties of SA extracts. This is similar to the finding of a strong correlation between antioxidant capacity and phenolic content in SA leaf (Ibrahim *et al*., 2013).

In the previous finding, GC-MS analysis revealed the presence of fatty acids, triperpenes, sugar, and phytosterol in SA leaf fractionated extracts. Identified fatty acids are hexadecanoic acid (1.60%–18.07%), octadecanoic acid (0.61%–7.39%), phytosterols are β-stigmasterol (0.40%–1.48%) and β-sitosterol (1.27%–4.50%), triterpene is β-amyrin (1.95%) and α-amyrin (1.67%), phytol (0.52%–1.29%) is diterpene, lupenyl acetate (11.25%) and lupeol (1.78%) are triterpenoid, α-D-glucopyranoside (0.96%–10.60%) is a sugar moiety (Rawat *et al*., 2018). α-D-glucopyranoside (10.60%), glycerol (7.96%), myo-inositol (4.90%), and butanedioic acid (3.30%) are the most abundant main components found in methanolic extracts. High concentrations of hexadecanoic acid (18.07%), octadecanoic acid (7.39%), β-sitosterol (4.50%), and α-D-glucopyranoside (4.03%) are found in hexane extract. In chloroform, the extract consists mostly of lupenyl acetate (11.25%).

This list of compounds is partly similar to our GC-MS results which revealed triperpenes, phytosterol, fatty acids, and sugars in SA extracts. The twig extract with the highest cytotoxic, antiproliferative and antimigratory effects contained four prominent components namely ethyl-α-D-glucopyranoside, hexadecanoic acid, ethyl ester, lupeol, and γ-Sitosterol. Previous investigation found triterpenoids like lupeol to be very multifaceted, blocking kappa B activation (nuclear factor), signal transducer, apoptosis, transcription, and angiogenesis (Chudzik *et al*., 2015). Phytosterol-rich diets may cut cancer risk 20% due to anticancer effects. Phytosterols improve immune responses to cancer and suppress tumour invasion, cell cycle advancement, and apoptosis (Cioccoloni *et al*., 2022).

In comparison with cytotoxic results from the previous work, all SA ethanolic extracts in this work showed much lower cytotoxic effects towards A549 cells (IC_{50} of 57.46 to 111.53 μg/mL) than SA leaf extracted with methanol and fractionated sequentially with hexane, chloroform, and butanol with highly effective IC_{50} <10 μg/mL towards A549 cells (Rawat *et al*., 2018). The differences in cytotoxicity between SA leaf extract in this work and that in a previous report may lie in different solvents used for extraction (95% ethanol vs methanol, hexane, chloroform, and butanol), ages of plants (unknown), geographical and climate conditions (Thailand 2021 vs India 2015).

The results showed cytotoxic and antiproliferative activities of different parts of SA extracts by ethanol against A549 lung cancer cells. Higher cytotoxicity of 85.46% against A549 cells was detected in twig extract (IC_{50} = 57.46 μg/mL) assessed by an MTT assay (Table 3). The cells treated with all SA extracts had apoptotic features (Figure 3). Using a clonogenic test, IC_{50} = 18.42 μg/mL was calculated for twig extract, which was lower than that for wood and bark extracts (Figure 4). Also, twig exhibited a larger impact on genes (*Bax*, *Bcl-2*, *p21*, and *Cytochrome C*) involving in intrinsic apoptosis (Figure 6). The twig extract was more cytotoxic, antiproliferative and antimigratory towards A549 lung cancer cells offering better chemopreventive effects

than other parts of SA plants. This may be due to the presence of the highest antioxidant activities and bioactive contents, and most abundance of chemical compositions with potential anticancer properties in the twig extract (Table 2).

In literature, cardiac glycosides isolated from SA exhibited exceptional inhibition of cancer cell types. For instance, (+)-strebloside, a well-known cardiac glycoside, has been described as the principal cytotoxic component of SA, with the ability to inhibit A549 (human lung cancer cells), OVCAR3 (human ovarian cancer cells), and MDA-MB-231 (human breast cancer cells)(Osman Mohammed *et al*., 2022). The C-10 formyl, C-5, and C-14 hydroxy groups and the C-3 sugar unit are responsible for (+)-strebloside's cytotoxicity against cancer cells (Ren *et al*., 2017). Three novel cardiac glycosides, 5βH-16β-acetylkamaloside, strophanthidin-3-O-α-L-rhamnopyranosyl-(1→4)-6-deoxy-β-D-allopyranoside, and mansonin-19-carboxylic acid, and seven recognized steroids, including five cardiac glycosides, were extracted from SA root methanol extracts showed cytotoxicity towards HL60, A549, AZ521, and SKBR3 cells (Miao *et al*., 2018). From this, it was assumed that ethyl-α-D-glucopyranoside and methyl β-D-galactopyranoside, two glycosides found in twig, wood and bark extracts, respectively in this work may contribute to anticancer effects of SA. These two glycosides were also reported to display cytotoxic property against HeLa and NIH/3T3 cell and Ehrlich ascites carcinoma, respectively (Ahmad *et al*., 2016). Likewise, this work also showed the involvement of apoptosis in A549 cells induced by SA extracts which is similar to the report of cardiac glycosides activated apoptosis and inhibiting cancer cell growth (Zhang *et al*., 2021). In addition, other compounds found among SA extracts in this work (Table 2) including heneicosane, hexadecanoic acid, ethyl ester, lupeol were found to be cytotoxic to HEp-2 (larynx), MCF-7 (breast) and NCI-H292 (lung) cancer cells (Bastos *et al*., 2017). Isopropyl myristate was cytotoxic to A549 lung cancer cells (Alkhatib and Alkhayyal, 2016). γ-Sitosterol was cytotoxic to A549 (lung) and MCF-7 (breast)(Sundarraj *et al*., 2012). These compounds may also contribute to cytotoxic effects of SA extracts in this work.

Typically, apoptosis happens through two distinct pathways: extrinsic and intrinsic. Primarily, the intrinsic route starts with the permeabilization of the mitochondrial membrane. This mechanism induces the release of cytochrome C (Nabil *et al*., 2019). The result of increased *Cytochrome C* gene expressions by SA extracts in this work (Figure 6) indicated that SA extracts activated apoptosis. Previously, using a variety of biochemical and cellular assays, it was found that (+)-strebloside from SA root extract induced a G2/M arrest in OVCAR3 cells via increasing p21 protein expression (Chen *et al*., 2017). Similarly, this work showed increased *p21* gene expressions by SA extracts indicating apoptosis induction.

Cancer treatment in Thailand has significant challenges, including a lack of trained medical personnel and limited access to necessary medications. More research has been done recently on biochemicals that may be used to develop novel anticancer medicines for lung cancer. These findings have added further knowledge on this matter and led us to conclude that SA extracts from twigs, wood, bark and leaves originated in Thailand can suppress the growth of A549 human lung carcinoma and trigger their death via apoptosis. SA has promise as herbal cancer alternative therapy.

Conclusions

For the first time, this work revealed the anticancer activities of different parts of SA originated from northeastern Thailand extracted by ethanol against A549 human lung carcinoma. Key findings highlighted that SA twig extract displayed a moderate activity against A549 cells based on a MTT assay which represents a short-term therapy; however, it showed a strong capacity against A549 cells based on clonogenic assay which represents a long-term therapy. SA extracts displayed apoptotic-inducing properties and antiproliferative and antimigratory activities. SA extracts showed no drug resistance against A549 cells. It is possible that SA extracts will prove useful as complementary therapies alongside conventional chemotherapy for the treatment of drug-

resistant tumours. The significance of this study was that the anticancer SA extract could be obtained from SA plants commonly found and easily grown in Thailand. If the SA extract was implemented as a Thai traditional medicine for lung cancers, this would help cut down the cost of importing modern drugs and save the cost of treatment. However, the limitation of this study was that the bioactive compound in SA extract was yet to be purified and identified. The next stage should be the systematic isolation of the active chemicals in SA plants and use the purified extracts in combination of the standard chemopreventive scheme to reduce side effects.

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., T.K., T.P., and W.W. The tests were devised by S.D. and I.S. In addition, C.S. identified the plant. E.W., P.S., and S.P. analysed real-time PCR data. V.L. conceived the study, assessed the data, and composed the article. 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.

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