Antioxidant properties and cytotoxic effects of *Alternanthera sissoo* and *Alternanthera bettzickiana* extracts against cancer cells

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Abstract

This work aimed to evaluate the antioxidant capacities and cytotoxic attributes of *Alternanthera sissoo* (AS) and *Alternanthera bettzickiana* Green (AB) which are commonly grown in Thailand, yet not extensively-studied. Dried leaves were extracted by maceration using ethanol for 48 h and ethanolic extract solution was used. Cytotoxic, anti-proliferative, anti-migratory capacities and apoptosis-related gene expressions of AS and AB extracts on MCF-7, HepG2, and HeLa cell lines were investigated using 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) assay, anti-colony formation and wound healing test, respectively. The antioxidant activities by DPPH assay and FRAP assay of AS (130.62±1.61 µg TE/g extract and 24.76 ± 0.54 mg Fe(II)/g extract, respectively) were significantly higher than those of AB (104.34±3.68 µg TE/g extract and 23.13± 0.90 mg Fe(II)/g extract, respectively). Likewise, total phenolic content and the total flavonoid content of AS (7.43 ±0.39 mg GAE/g and 184.22±3.20 mg RE/g extract, respectively) were significantly higher than AB (6.10±0.29 mg GAE/g extract and 168.07±7.90 mg RE/g extract, respectively). HPLC analysis showed the predominance of myricetin, rutin and ferulic acid. For MCF-7, HepG2 and HeLa, the lowest IC₅₀ values of 48.53 µg/mL, 69.94 µg/mL and 43.98 µg/mL, respectively were found in AS extract at 72 h exposure using MTT assay. Apoptotic bodies, gene expression and protein expressions related to apoptosis were more pronounced in AS treatment. Both AS and AB can be served as local foods with chemopreventive effects against breast, liver and cervical cancers. This work provided a foundation for future pharmacological research on *Alternanthera* plant extracts.

Keywords: A549; Brazilian spinach; flavonoid; HepG2; joyweed; MCF-7; phenolic acid
Introduction

Cancer is a growing worry in Thailand, as it is in the rest of the globe, as the country grows and risk factors become more prevalent. There were 190,636 new cancer cases in Thailand in 2020 alone. It is estimated that 290,000 new cancer cases would be diagnosed by 2040. According to Global Cancer Observatory (2021), five forms of cancer account for over 60% of the cancer burden: liver, lung, breast, colorectal, and prostate in Thailand. Chemotherapeutic drugs are currently the most common cancer treatment method. However, the treatment is not always effective and can result in negative side effects (Chakraborty et al., 2018). As a result, finding safe and inexpensive anticancer medications from natural sources is a hot topic of enormous interest.

Plants have an abundance of secondary metabolites, which potentially contribute to cytotoxic effects. A majority of people in underdeveloped nations use traditional herbal medicines to cure a variety of diseases and disorders, and numerous plants are used for their health advantages (Okaiyeto and Oguntibeju, 2021). Herbal medicines are often considered harmless since they are natural and do not affect the environment (Tyagi et al., 2021). Some nations continue to depend on herbal medicine as their major supply of medicament, according to the World Health Organization (WHO), whereas developing countries benefit from the therapeutic advantages of organically generated substances. Polyphenols, brassinosteroids, and taxols have been shown to be examples of anticancer chemicals isolated from terrestrial plants (Greenwell and Rahman, 2015). In cancer therapy, the usage of plant-based compounds originates from the 1950s (Raina et al., 2014). Researchers are continually looking for new information on the cytotoxicity of unexplored natural resources.

*Alternanthera sissoo* (Brazilian spinach) a.k.a Pak-Phed-Hawai (Thai) and *Alternanthera bettzickiana* Green (Joyweed) a.k.a. Pak-Phed-Kieaw (Thai) are herbaceous plants of the Amaranthaceae family, which has 64 genera and 800 species. The tropical regions of Africa, India, America, Brazil, and Thailand are home to the most members of this cosmopolitan family (Pamila et al., 2018). However, these plants are sometimes considered as weeds in some countries. The leaves of *A. sissoo* (AS) are more circular, heart-shaped and larger (Figure 1A) than those of *A. bettzickiana* (AB) which are more pointed and thinner (Figure 1B).

**Figure 1.** Leaves and stems of plants for extraction; (A) *Alternanthera sissoo* (AS); (B) *Alternanthera bettzickiana* (AB)

AS comes from Brazil and is a good source of flavonoids. It is cultivated most often in the tropical countries (Tiveron et al., 2012). The most common use of AB leaves is for medicinal purposes. The flavonol glycosides, vitamins, betacianin, and β-sitosterol are among the phytochemical compounds found in this plant (Kumar et al., 2011). The AB plant has been well-known for nourishing and cleaning the blood, as well as acting as a gentle laxative, antipyretic, galactagogue, and wound healer. In the case of AB, it was shown that its extract
might improve catalase and superoxide dismutase activity in ovariectomized mouse livers (Suphanthip et al., 2013).

AS and AB are commonly home-grown by the local people of Thailand. These plants are used to make various food recipes, such as omelettes, spicy raw tuna salad, soups, fried vegetable tempura, and fried vegetables with oyster sauce. Surprisingly, AS and AB are often mistaken as watercress (Nasturtium officinale) with chemopreventive effects by Thai local communities for unknown reasons. Watercress belongs to the Brassicaceae family, which is rich in glucosinolates and their degradation products, isothiocyanates, are extensively studied anticancer products. However, AS and AB have not been reported to contain glucosinolates or isothiocyanates, nor have they been studied for chemopreventive effects. The cost of modern medications is too high for Thai people in rural locations. Consequently, scientific confirmation of traditional medicines for any particular pharmacological activity of a previously unknown plant enables the local people (who have easy access to the plants) to obtain a targeted therapeutic benefit at considerably lower cost.

Due to the prevalence of cancer cases in Thailand, researchers have been looking for plants that may have chemopreventive properties. Both AS and AB plants are widely available and widely consumed as a vegetable by rural populations. Thus, the objectives of this work were to evaluate the antioxidant capacity and cytotoxic attributes of AS and AB. Both plants are recommended for regular consumption to exert promising anticancer properties.

**Materials and Methods**

**Chemicals and reagents**

Ethanol was purchased from RCI Lab Scan Ltd., Bangkok, Thailand. Folin-Ciocalteu reagent, sodium carbonate, aluminum trichloride, sodium acetate, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, FeCl₃, 2,4,6-Tri(2-pyridyl)-s-triazine, gallic acid, rutin, Trolox, ferrous II sulphate, formaldehyde and crystal violet were obtained from Sigma-Aldrich Co. Ltd., St. Louis, MO, USA. Materials including Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, trypsin-EDTA, 3, 4, 5-dimethylthiazol-2-yl-2-5-diphenyltetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were derived from Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA.

**Collection of plant materials**

During July 2019, fresh plants and stalks (aerial components) of AS and AB were gathered by Organic Agriculture Koh Yai Community Enterprise, Mahasarakham, Thailand. Authentication by examining mature plant features was verified by Assoc. Prof. Dr. Vijitra Luang-In from Mahasarakham University. The samples were stored in the Biotechnology Botanical Herbarium (AS voucher number 012019 and AB voucher number 022019 the Department of Biotechnology, Mahasarakham University, Thailand. Aerial parts were carefully cleaned using distilled water and dried in the shade for up to 5 days.

**Plant extraction**

Dried leaves of AS and AB were ground with a mechanical grinder to obtain fine powdered leaves. After that, plant extraction by maceration was done using 1 L of 95% ethanol per 200 g of dried leaf powder for 48 h agitated at 150 rpm. The ethanolic extract was centrifuged (10,000 g, 20 min) and the liquid portion was evaporated under vacuum at 42 °C. The extracted residues were resuspended in 95% ethanol at 20 mg/mL stock solution and kept at −20 °C.
Evaluation of antioxidant activity and bioactive compounds

According to the previous study, the ferric reducing antioxidant power (FRAP) and DPPH radical scavenging methods with slight modifications were tested (Buitrago et al., 2019). The mixtures for DPPH assay were made as follows: each solution of ethanolic plant extract (20 μL of 20 mg/mL stock solution) was mixed with 10 mM DPPH solution (180 μL). At room temperature for 30 min, the mixture was kept in the dark and then the absorption was observed at 515 nm. For FRAP, each solution of ethanolic extract (20 μL of 20 mg/mL stock solution) was mixed with a FRAP reagent (180 μL), produced from a 1:1:10 combination of 20 mM FeCl₃ solutions, 10 mM 2,4,6-Tri (2-pyridyl) s-triazine, and a 0.3 M acetate buffer at pH 3.6. The combined solution was left to react for 30 min, and then the absorption was measured at 593 nm. Triplicate was conducted. The calibration curves of Trolox and ferrous II sulfate standards were created to determine the antioxidant capacity, for both the DPPH and the FRAP tests, respectively.

The total phenolic content (TPC) and the total flavonoid content (TFC) were assessed as stated previously (Luang-In et al., 2021). For TPC, the reaction mixture consisted of 20 μL of each ethanolic plant extract solution, 80 μL of 7.35% sodium carbonate and 100 μL of 10% Folin-Ciocalteu solution. At room temperature, the combined solution was left to react in the dark, and A765 nm was recorded. For TFC, a combination of deionized water (60 μL), 10% aluminium trichloride (10 μL) and 5% sodium nitrate (10% μL) were pipetted to the plant extract (20 μL). Afterwards, the mixture was filled with 100 μL of 1 M NaOH and left to react in the dark for 30 min and its absorption was ultimately measured at 420 nm. For each determination, three replicates were assessed. Authentic standards namely gallic acid and rutin were used for TPC and TFC, respectively.

HPLC analysis of phenolics and flavonoids

Extractions of phenolic and flavonoid compounds were carried out as previously reported (Chumroenphat et al., 2021). As the samples were extracted for 12 h at 37°C in the mixture of HCl/Methanol (1:100), the shaking was used (150 rpm in the dark). The mixed filtrates were evaporated under vacuum at 40 °C. This was done in a nylon membrane filter (pore size 0.45-μm) prior to the HPLC examination of the residue in the methanol/water mixture. Polyphenolic acids and flavonoids were studied using Shimadzu LC-20AC pumps, SPD-M20A diode array detectors, and chromatographic separations on an Inertsil ODS-3, C18 column (4.6 mm x 250 mm, 5 μm). Acetic acid pH 2.74 and acetonitrile were mixed as the mobile phase at a flow rate of 0.8 mL/min (solvent B) using the elution gradients are gradients (Kubola and Siriamornpun, 2011). The column was heated to 38 °C, the injection volume was 20 μL, and the phenolic acids and flavonoids were detected using a UV diode array at a wavelength of 280 nm and 370 nm, respectively. Through external standard curves, phenolic compounds in the samples were recognized and detected through comparisons of their UV spectra to authenticated substances.

Cell cultures

The cells of MCF-7, HepG2 and HeLa (American Type Culture Collection, Manassas, VA, USA) were grown in DMEM (supplemented with 10% FBS and 1% penicillin/streptomycin) at 37°C with 5% CO₂ in the presence. Cells were trypsinized with 0.25% EDTA trypsin after each three-day medium change.

Cytotoxicity assay

Cancer cells (5×10⁴ cells/well) were put to 96-well plates at 37 °C in DMEM (100 μL) with 10% FBS for 24 h. The dissolved plant extracts (100 μL) at various concentrations (0, 12,5, 50, 100, 200, 400, and 800 μg/mL) were pipetted to microplates for 24, 48 and 72 h. Solutions were withdrawn followed by the addition of MTT (100 μL) to microplates and left for 4 h. The formazan was dissolved in DMSO (200 μL) and then an absorption at 590 nm was recorded. Triplicate testing was conducted. Both IC₅₀ and Eₘ₃ values were obtained for the cytotoxicity (%) of plant extracts to cancer cells.
**Anti-proliferation by anti-colony formation assay**

Determination of the formation of colonies after treatment with plant extracts was conducted as follows (Luang-In et al., 2021). Using a 6-well plate, cancer cells (500 cells/well) were cultivated in DMEM for 24 h. Next, plant extracts at various doses (0, 12.5, 25, 50, 100 and 200 μg/mL) were pipetted to treat cells for 24 h. The media were then withdrawn and the cells rinsed with saline phosphate buffer (PBS). Incubation took place at 37 °C for 14 days with 5% CO₂ and the media was exchanged out every two days. The cells were then cultivated for 14 days with 5% CO₂ at 37 °C and the fresh media were replaced the old one every 2 days. Cells were fixed at for 30 min with cold methanol followed by staining with Coomassie bright blue g-250 (0.5% in methanol) for 30 min. Excessive colour was removed with tap water several times. Colonies were dried at room temperature and counted. The assay was performed in triplicate.

**Cell morphology**

Cancer cells (7,500 cells/well) were seeded in the corresponding media into a 24-well plate. Cells were incubated overnight prior to getting supplemented with plant extracts (50 µg/mL) for 24 h. The changes in cancer cells were viewed under an inverted microscope (NIB-100, Xenon, China). The assay was performed in triplicate.

**Anti-migration by wound healing assay**

Cancer cells (2×10⁵ cells/well) were seeded in the corresponding media into a 24-well plate and grown to approximately 90% confluency. Next, a 200 µL pipette tip was used to scratch a wound and cell scraps were cleared using PBS. After that, cells were mixed with plant extracts (0, 12.5, 25, 50 and 100 µg/mL) for 48 h. Cell fixation was done with 4% formaldehyde followed by cell staining for 30 min with 0.5% crystal violet, rinsing with distilled water. The % relative closure of the scratch was measured in comparison to untreated cells.

Relative closure of the scratch (%) = (width of the wound at 48 h / width of the wound at 0 h) × 100

**Real-time PCR analysis**

Real-time PCR was used to examine cancer cell gene expression. Cells (2×10⁵ cells/well) were cultivated for 24 h at 37°C, then treated to AS and AB extracts (50 µg/mL) in fresh medium. One gram of total RNA per sample was transcribed to cDNA using reverse transcriptase (iScriptTM Reverse Transcription Supermix, Bio-Rad, Hercules, CA). The Fast Start Essential DNA Green Master (Roche Applied Science) was used in a QuantStudio real-time PCR (Applied Biosystems, Foster City, CA). Table 1 lists primers for Bax, Bcl-2, Caspase-3, p21, MMP-2, MMP-9, and Cyclin D1. Real-time PCR conditions were denaturation at 94 °C for 10 min, annealing at 60 °C for 10 s, and extension at 72 °C for 10 s for 45 cycles. For quantitative analysis, cDNA content was evaluated using median threshold cycle number (Ct). The expression level was normalized to that of GAPDH as a housekeeping gene.

**Table 1. Primers for real-time PCR analysis**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Size (bp)</th>
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<td>Forward</td>
<td>CACTGCCAACCTGGTGCACTGGTG</td>
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</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GTAGCCCAATGGCCCTGGAG</td>
<td></td>
</tr>
<tr>
<td>Bax</td>
<td>Forward</td>
<td>TGCTTCAGGGTTTATCACG</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGCAGGCAATCTCCCTG</td>
<td></td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Forward</td>
<td>AGGAATGGAACATTTCGGTGAC</td>
<td>149</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCTCAGTTCAGGACCAGGC</td>
<td></td>
</tr>
<tr>
<td>Caspase-3</td>
<td>Forward</td>
<td>GGCAGCTGTGGATTTTGATTAAT</td>
<td>121</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>TCCAGATTTGCTTGATTTGCC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Forward</td>
<td>AGATCGTGTCCTGGAGG</td>
<td>109</td>
</tr>
</tbody>
</table>
Reverse GCATGGTTCCTGACGGACAT

MMP-2 Forward TTGACGCTAGGACGGACTC 153
Reverse ACTTTGACGTACTCCCACATCG

MMP-9 Forward TTTGACAGCGACAAGAAGTG 208
Reverse CAGGCGGAGGACCATAAGGG

Cyclin D1 Forward ATCTCTGTACTTTGGCTTGCT 564
Reverse AGTACATGGATATTCCCCAAA

Protein extraction and Western blot analysis

Cancer cells were seeded at 3×10^5 cells/well in 6-well plates for 24 h before AB and AF extract treatment (50 µg/mL). After collecting living cells, they were washed with ice-cold phosphate buffered saline, lysed with RIPA buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% Sodium dodecyl sulfate (SDS)) for 30 min on ice, then centrifuged at 14,000g for 15 min. The protein concentration was measured using a BCA protein kit test on the supernatant (Thermo Fisher Scientific, IL, USA). Next, a protein sample (25 µg) was resolved by SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). At room temperature, the membranes were blocked for 1 h with 5% Bovine serum albumin (BSA) in Tris buffered saline plus 0.1% Tween-20 (TBST) and then treated with the primary antibody for antibodies: rabbit polyclonal anti-human Cytochrome C Ab, rabbit polyclonal anti-human Caspase-3 Ab, rabbit monoclonal anti-human p21 Ab (1:1000) and mouse monoclonal anti-human β-actin Ab (1:5000)(Boster Biological Technology, Inc., Wuhan, China) in 5% BSA at 4 °C overnight. After 3 washes with TBST, membranes were incubated with goat anti-rabbit and goat anti-mouse secondary Ab conjugated with horseradish peroxidase (1:5000) (Boster Biological Technology, Wuhan, China) for 1 h. To detect chemiluminescence, the membrane was rinsed in TBST. MageQuant TL 400 was used to record protein band densities (Amersham Pharmacia Biotech, Piscataway, NJ). The experiment was done in triplicates and β-actin was used as a house keeping protein.

Statistical analysis

Means and standard deviations were calculated from the three sets of data. Analysis of variance (ANOVA) and the Duncan multiple ranges test were performed using the SPSS software (demo version). Statistically significant differences were considered if \( p < 0.05 \).

Results and Discussion

Antioxidant activities and bioactive compounds

Antioxidant activities and bioactive contents were determined in AS and AB. The results of DPPH scavenging assay and FRAP assay (Figure 2A-B) showed that AS had significantly higher antioxidant activities (130.62±1.61 µg TE/g extract and 24.76 ± 0.54 mg Fe(II)/g extract, respectively) than those of AB (104.34±3.68 µg TE/g extract and 23.13± 0.90 mg Fe(II)/g extract, respectively). Similarly, TPC and TFC of AS (7.43±0.39 mg GAE/g and 184.22±3.20 mg RE/g extract, respectively) were significantly higher than those of AB (6.10±0.29 mg GAE/g extract and 168.07±7.90 mg RE/g extract, respectively) (Figure 2C-D). AS exhibited higher antioxidant activities and bioactive compounds.
In this study, extracts of aerial portions from AS and AB are thought to be minimally toxic sources since the plants are extensively eaten as a vegetable in several regions of the world. The plant’s secondary metabolites were extracted using the highly polar solvent ethanol, with phenolic molecules as one of the most potential compounds.

*In vitro* antioxidant activity of ethanolic AS and AB extracts was demonstrated in this study. TPC and TFC of both AS and AB ethanolic extracts were considerably lower and greater, respectively than those observed previously (Vidhya *et al*., 2015). Compared with the aqueous extract, *A. bettzickiana* collected in India when extracted in methanol had the maximum quantity of TPC (63 mg of GAE/g of extract) and TFC (38.98 mg of QE/g of extract) (Vidhya *et al*., 2015). In our work, TPC and TFC of ethanolic extract of AB were 6.10 mg GAE/g extract and 168.07 mg RE/g extract, respectively whilst those of AS extracts were 7.43 mg GAE/g and 184.22 mg RE/g extract, respectively. This suggested that the solvents used for extraction and the origin of plants may influence the bioactive contents.

The previous results of phytochemical analysis in *A. bettzickiana* collected in Pakistan showed that the extract in ethanol exhibited greater TPC (7.61 mg/g) and TFC of 70.66 mg/g (Manan *et al*., 2020). In contrast, much lower TPC of 3.09 mg GAE/g was found in *A. bettzickiana* leaves collected in India (Petrus *et al*., 2014). In comparison with our findings, TPC of AS and AB extracts (7.43 mg GAE/g and 6.10 mg GAE/g, respectively) aligned with the findings of Manan *et al.* (2020); however, our TFC of AS and AB extracts were significantly higher by more than 2-fold (184.22 mg RE/g and 168.07 mg RE/g extract, respectively). The FRAP value of *A. bettzickiana* extract was 455.24 nmol Fe(II)/mg extract (Manan *et al*., 2020). The previous work found that *A. bettzickiana* extract in ethanol contained gallic acid, sinapic acid, chlorogenic acid, quercetin, catechin, α-tocopherol, and γ-tocopherol by HPLC analysis. Gallic acid and catechin were found in greater concentrations than other substances (Manan *et al*., 2020). The Folin-Ciocalteu method assesses reducing ability by electron transfer, which is often represented in phenolic contents. As a result, the antioxidant capacity of plants appears to be caused, at least in part, if not totally, by the redox characteristics of phenolic molecules. The FRAP activity and DPPH scavenging activity of *A. bettzickiana* might be attributed to flavonoids, polyphenolics, and alkaloids, which are recognized for their antioxidant characteristics and have been linked to *A. bettzickiana's* anti-inflammatory property (Abotsi *et al*., 2011). In addition, the results of antioxidant activities and bioactive compounds were compared to the previous studies of *Alternanthera sessilis* in the same family Amaranthaceae as AS and AB. The previous work showed that hydrophilic extracts from...
edible portions of *A. sessilis* in Bangladesh exhibited TPC of 1.00 mg GAE/g FW and antioxidant capacity of 4.06 µmol TE/g FW (Tukun et al., 2014). In comparison with our results, a significantly higher TPC of 7.43 mg GAE/g DW was observed.

The HPLC examination of AS and AB extracts revealed that flavonoids were the most common phenolic components in the extracts (Figure 3). AS extracts contained significantly higher contents of phenolic compounds than AB extracts. For AS, myricetin (147.2 mg/g extract) was the most abundant compounds followed by rutin (15.2 mg/g extract) and ferulic acid (0.8 mg/g extract), respectively (Table 2). A similar trend was found in AB, but to a lesser extent.

![Figure 3. Antioxidant activities and bioactive compounds in AS and AB; (A) DPPH scavenging activity; (B) FRAP activity; (C) TPC; (D) TFC](image)

![Table 2. The phenolic acids and flavonoids identified in AS and AB extracts](table)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Flavonoid content (mg/g extract)</th>
<th>Phenolic acid content (mg/g extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rutin</td>
<td>Myricetin</td>
</tr>
<tr>
<td>AS</td>
<td>15.2±0.9 ±B</td>
<td>147.2±4.8 ±A</td>
</tr>
<tr>
<td>AB</td>
<td>7.5±0.2 ±h,B</td>
<td>40.0±5.0 ±h,A</td>
</tr>
</tbody>
</table>

When distinct lowercase and uppercase letters appear in the same columns and rows, the Duncan’s multiple range test shows a significant difference (*p* < 0.05).

Plants of the Amaranthaceae family are classified into two types based on their color: red and green. The previous research has revealed that the red variety has a superior nutritional composition, a higher level of phenolic compounds, and a stronger antioxidant capability (Othman et al., 2016). A number of factors have been shown to influence the phenolic content, including agronomic aspects, extraction techniques, and the variety used (Mohd Hazli et al., 2019; Nuez-Estevez et al., 2021). According to prior study, *A. sesillis* (red) (ASR) leaf ethanolic extract included ferulic acid of 844.29 µg/g extract, rutin of 6745.60 µg/g extract, quercetin of 394.03 µg/g extract and apigenin 2618.76 µg/g extract (Mohd Hazli et al., 2019). The first two were in greater content than our results, while the latter two were not included in our AS or AB. *A. sesillis* leaves have never before been found to contain myricetin, rutin, or ferulic acid. Previously, *Alternanthera brasiliana* was shown to contain the highest total flavonoid content (503.7 mg rutin equivalents/g) in the optimization of extraction (de Alencar Filho et al., 2020). Flavonoids were more abundant in *A. brasiliana* than phenolic acids. As a result of this new knowledge, a polyphenol database for this plant is now in place and might be used to examine its potential use as a natural source of antioxidants. Antioxidants protect the human body against cancer and the growth of tumors by quenching free radicals in the blood. It is therefore necessary to
consume foods high in antioxidants (Balasuriya and Dharmaratne, 2007). In this work, AS and AB are proven good sources for antioxidants and phenolics/flavonoids. It is possible that these bioactive compounds contribute to their chemo preventive effects. Both plant extracts might lead to cell death by inducing apoptosis pathway and proposed of possessing anti-metastasis activity through the down-regulation of certain pathways and thus rendered anti-proliferative and anti-migratory activities in cancer cells. The results help us better understand the potential health benefits of AS and AB plants which are easily grown at home and thus easily accessible by the local communities for sustainable medicinal uses.

**Cytotoxicity of plant extracts**

The AS and AB extracts exhibited dose-dependent cytotoxicity on MCF-7, HepG2, and HeLa cells assessed by MTT assay. For MCF-7, the lowest IC50 of 48.53 µg/mL with the highest Emax of 99.62% was found in AS extract at 72 h exposure time. For HepG2, the lowest IC50 of 69.94 µg/mL with the highest Emax of 94.36% was found in AS extract at 72 h exposure time. For HeLa, the lowest IC50 of 43.98 µg/mL was found in AS extract at 72 h exposure time (Table 3). Both AS and AB plant extracts exhibited higher cytotoxic activity (lower IC50 value) against HeLa > MCF-7 > HepG2, respectively (Table 3). In all cancer cells at all time intervals, AS was more effective than AB as observed by significantly lower IC50 values of AS in comparison with those of AB (Table 3). This may have been owing to the greater levels of phenolic acids and flavonoids (ferulic acid, myricetin, and rutin) in AS (Table 2).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Time (h)</th>
<th>MCF-7 (µg/mL)</th>
<th>HepG2 (µg/mL)</th>
<th>HeLa (µg/mL)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Emax (%)</td>
<td>IC50 (µg/mL)</td>
<td>Emax (%)</td>
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<tr>
<td>AS</td>
<td>24</td>
<td>94.67±1.15E</td>
<td>109.72±1.81†</td>
<td>85.28±3.37‡</td>
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<td>96.32±1.99E</td>
<td>76.44±2.35†</td>
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<tr>
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<td>98.01±0.37E</td>
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<td>98.00±1.15E</td>
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</table>

Lowercase letters indicate significant differences (p < 0.05) in the columns.

This is the first report of cytotoxicity of AS and AB against breast (MCF-7), liver (HepG2) and cervical (HeLa) cancer cell lines. The findings of this study were compared to those of earlier *A. sessilis* investigations. It was found that aqueous leaf extract of *A. sessilis* collected in China formed in gold nanoparticles caused cytotoxicity with >50% at concentrations of 10–15 µg/mL as well as apoptosis by altering intrinsic apoptotic pathways in HeLa cells (Qian et al., 2019). Hexadecanoic acid as a wound healing related phytocompound was found in a hydroethanolic extract of *A. sessilis* collected in Malaysia. After extract treatment to human dermal fibroblast (NHDF) and keratinocytes (HaCaT), a significant antioxidant, proliferative, and migratory rate was found throughout the wound healing phase (Muniandy et al., 2018). *A. sessilis* stem extracts (500 µg/ml) were also tested on cell viability of NHDF and HaCaT after incubation for 24 h by MTT assay and the data showed no discernible harm because cell viability remained over 80% (Muniandy et al., 2018). This was consistent with prior efforts that evaluated on the toxicity of *A. bettzickiana*. The whole plant of *A. bettzickiana* was extracted using water-methanol, and n-hexane displayed IC50 of 493 and 456 µg/mL, respectively in baby hamster kidney (BHK-21) cells, indicating its limited cytotoxicity to BHK-21 (Akhtar et al., 2017). Previously, the crude extract of *A. bettzickiana* did not show any toxicity at administration of a range of doses (100 – 3,000 mg of extract per kg body weight) in mice (Hossain et al., 2014). These previous reports confirmed the nontoxicity of *A. sessilis* and *A. bettzickiana* and thus they are safe for consumption. The aqueous-ethanolic extract of *A. sessilis* was discovered to have antioxidant properties. Those reported to exhibited anticancer capacity included L-glutamic acid, palmitate, 9,12-Octadecadienoic acid, methyl ester and phytol (Muniandy et al., 2018). When
compared to the previous report, *A. sessilis* leaf extract in water from China was found to exhibit >50% cytotoxicity at 10-15 µg/mL (Qian *et al*., 2019). Our results showed that AS had higher cytotoxicity (>97%) at 44-96 µg/mL suggesting possibly less effective.

**Anti-colony formation effect**

The potential of the two plant extracts to inhibit cancer cell proliferation on the long term was tested by conducting a colony formation assay. Both AS and AB extracts were shown to have a dose-dependent effect on the capacity of all three cancer cells to form colonies (Figure 4A-4C). However, AS showed more pronounced anti-proliferative effect as observed by complete inhibition of colony formation (100%) at lower concentrations of 100 µg/mL AS extract for MCF-7, 200 µg/mL AS extract for HepG2, and 100 µg/mL AS extract for HeLa than those AB extracts used (200 µg/mL, 400 µg/mL and 200 µg/mL, respectively). The lower IC$_{50}$ values of AS extracts, 37.04 µg/mL for MCF-7 was observed. The IC$_{50}$ values of AB extracts were approximately 2-fold higher (66.77±1.32 µg/mL). This suggested that AS extract was more anti-proliferative against cancer cells than AB extract. Interestingly, these IC$_{50}$ values from a colony formation assay were lower than those found in the cytotoxicity effect indicating that lower concentrations of plant extracts suffice to exert the anti-proliferative effect in a longer-term therapy (14 days) in a colony formation assay as compared to the cytotoxic impact in a shorter time (24 h, 48 h and 72 h). Both AS and AB plant extracts were shown to exhibit no drug resistance against the three cancer cells, hence hold the potential as therapeutic agents for chemoprevention.

Gothai *et al*., (2018) found that *A. sessilis* leaf extract had the most potent anti-proliferative activity against HT 29 cells, with the aerial, leaf, and stem extracts all showing a decrease in colony formation (Gothai *et al*., 2018). *A. sessilis* plant parts may also have a long-term anti-proliferative effect.

**Changes in cancer cell morphology**

Apoptosis is a vital mechanism corresponding to cancer growth inhibition. The result showed that both AS and AB extracts in this work were able to induce apoptosis as observed by characteristics of apoptotic bodies, as signs of cancer cell death over 24 h (Figure 4D). AS was more effective in inducing apoptosis than AB as observed by a higher number of apoptotic bodies. The dominant bioactive compounds in AS and AB extracts namely rutin, myricetin, and ferulic acid, which have been previously documented to exert health benefits and anti-cancer properties (Gregoriou *et al*., 2021), may contribute to apoptosis induction in MCF-7, HepG2 and HeLa cells. The genes and proteins related to apoptosis were assessed in further experiments to support the presence of apoptotic bodies.
Anti-migratory effect

The results demonstrated that both AS and AB plant extracts inhibited cancer cell migration by decreasing wound-healing capacity at 48 h exposure, in a dose-dependent effect (Figure 5). AS extract was more effective in anti-migratory activity in all cancer cells as shown by the reduced relative closure of the wound at the same concentrations as AB. For MCF-7, the lowest relative closure of the wound of 9.79% was found at AS extract of 50 µg/mL compared to that of 39.86% at AB extract of 100 µg/mL (Figure 5A). For HepG2, the lowest relative closure of the wound of 3.52% was found at AS extract of 100 µg/mL compared to that of 7.41% at AB extract of 50 µg/mL (Figure 5B). For HeLa, the lowest relative closure of the wound of 4.47% was found at AS extract of 50 µg/mL compared to that of 8.54% at AB extract of 100 µg/mL (Figure 5C).
For the first time, it was discovered that *A. sessilis* plant components effectively inhibited the migration of cells after 24 h of incubation with varied quantities of extracts (100 - 200 µg/mL) (Gothai *et al*., 2018). Leaf and aerial and stem extracts showed a significant decrease. *A. sessilis* leaf extract may have a significant impact on the reduction of metastasis. Metastasis is the outcome of extracellular matrix degradation occurs through the action of matrix metalloproteinases (MMPs). MMP-2 (called gelatinase A) and MMP-9 (aka gelatinase B), which breakdown type IV collagen and gelatin substrates, are strongly linked with tumor spread and
Invasiveness (Webb et al., 2017). To date, researchers are eager to continue exploring MMP inhibitors as new anti-metastatic treatments. The mRNA expressions of MMP-2 and MMP-9 were evaluated in the next section.

**Alterations in gene and protein expressions**

The effects of AS and AB extracts on gene and protein expression of MCF-7, HepG2 and HeLa cells were assessed for the mechanisms of their actions. Both AS and AB extracts significantly increased the gene expressions of Bax, Caspase-3, and p-21 while decreased those of Bcl-2, MMP-2, MMP-9 and Cyclin D1 in all three cancer cells (Figure 6A-C) suggesting that AS and AB extracts exerted the apoptotic intrinsic pathway was activated with more pronounced effects from AS extract. Likewise, in most cases, AS extract produced more perceivable changes in protein expressions by increasing levels of Cytochrome C, Caspase-3 and p21 proteins involved in intrinsic apoptosis (Figure 6D-F).

![Figure 6](image)

**Figure 6.** Effects of AS and AB extracts on gene and protein expression; (A-C) mRNA expressions from MCF-7, HepG2 and HeLa, respectively; (D-F) Protein expressions from MCF-7, HepG2 and HeLa, respectively

These extracts may have an effect on apoptosis in three cancer cells because of the presence of rutin, myricetin, and ferulic acid in them. The Bcl-2/Bax ratio, and cell cycle inhibition in G2 and G1 might be lowered by rutin (Satari et al., 2021). In human glioblastoma cell lines, both pro- and antiapoptotic genes (Bcl-2, Caspase-3, Bax, and TP53) were up- and down-regulated in the presence of rutin. Inhibition of cell death by antioxidants such as gallic acid, ferulic acid, and coumaric acid was discovered to be a caspase-independent mechanism (Frusciante et al., 2015). Rutin has been found to suppress prostate, breast, lung, and colon cancers and other malignancies. Drug resistance and side effects from chemotherapy can be reduced by rutin supplementation. Hence, naturally occurring rutin appears to hold promise as a potential cancer preventive supplement because of its proven efficacy, as well as its availability and the limited adverse effects it causes (Satari et al., 2021). Another study found that the expression levels of apoptosis-related genes Caspase-3, Caspase-8, Caspase-9, the Bax/Bcl-2 ratio and the genes for p53, BRCA1, and GADD45 were all considerably increased after myricetin therapy of MCF-7 breast cancer cells (Sajedi et al., 2020) indicating that myricetin was involved in apoptosis in MCF-7. Moreover, MMP-2/9 activity is down-regulated by myricetin, which inhibits breast cancer metastasis (Ci et al., 2018). Ferulic acid was found to be cytotoxic to MCF-7 and HepG2 cells (IC$_{50}$ = 75.4 and 81.38 μg/mL, respectively, after 48 h). Caspase-8 and-9 levels were increased in both cell
lines after ferulic acid treatment, indicating that it has promising therapeutic potential for the treatment of breast and liver cancers by inducing apoptosis (Khazendar et al., 2019).

To sum up, this was the first time to study the cytotoxic, anti-proliferative and anti-migratory attributes of *A. sissoo* and *A. bettzickiana* on MCF-7, HepG2 and HeLa cells in more details. Those effects may lie in the presence of rutin, myricetin and ferulic acid. These plants should be recommended as foods for anticancer benefits.

**Conclusions**

The findings, for the first time, validate the medicinal use of the underused plants, AS and AB, to exert cytotoxic, anti-proliferative and anti-migratory properties on breast, liver and cervical cancer cells. All three cancer cells were reported to be inhibited in growth and apoptosis by the ethanol-based extracts of the aerial portions. The anti-oxidant and anti-cancer properties of the AS extract were the strongest. Rutin, myricetin, and ferulic acid were discovered to be the most prevalent phenolic and flavonoid components in AS and AB extracts, and these constituents have previously been linked to chemoprevention and health benefits. The effective AS and AB extracts have been shown to activate the intrinsic route of apoptosis, reduce cell proliferation, and inhibit cell migration, according to the postulated mechanism of action.

**Authors’ Contributions**

V. Luang-In conceived, planned, and supervised the tests, processed the data, and wrote the article. W. Saengha conceived, planned, collected, and conducted the tests. T. Karirat conducted the tests. B. Buranrat and T. Katisart contributed to the reagents/materials/infrastructure and analysis tools. N. L. Ma contributed to providing supervision.

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


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