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Unraveling the Bioactive Potential of Side Dish Vegetables in Southern Thai Traditional Cuisine: Anti-inflammatory, Anticancer, Antibacterial, and Antioxidant Properties

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ABSTRACT

In Thai traditional cuisine, a variety of vegetables is frequently served as condiment dishes, offering numerous health benefits due to their richness in phytochemicals and diverse biological properties. In this study, we investigated antioxidant, anti-inflammatory, antibacterial, and anti-colorectal cancer activities of six southern Thai side dish vegetables: *Gnetum gnemon* L., *Anacardium occidentale* L., *Glochidion littorale* Blume, *Micromelum minutum* (G.Forst.) Wight & Arn., *Spondias pinnata* (L.f.) Kurz, and *Centella asiatica* (L.) Urb. Among all extracts, *A. occidentale* extracts (AOE), particularly dried leaf extract, demonstrated the most promising biological potential. The dried leaf of *A. occidentale* showed strong antioxidant activity through the DPPH assay, with an IC_{50} value of 51 µg/mL. The AOE explicitly showed anti-inflammation by suppressing nitric oxide (NO) upon induction by lipopolysaccharide (LPS) in RAW 264.7 macrophages (IC_{50} 7.14 µg/mL) and inhibiting 5-lipoxygenase activity (39.3% inhibition). The dried leaf AOE also possessed potent antibacterial activity by inhibiting food-borne pathogens, including *Staphylococcus aureus* and *Escherichia coli*, with MIC values of 0.78 and 1.56 mg/mL, respectively. Regarding anticancer activity, AOE exhibited strong cytotoxicity against HCT116 human colorectal cancer cells (IC_{50} 69.77 µg/mL). Phytochemical analysis revealed that AOE are rich in phenolic and alkaloid compounds. Taken together, the findings from the present study highlight the potential of *A. occidentale* and its diverse pharmacological properties for further development as nutraceutical health supplements.

Keywords: Southern Thai side dish vegetables, *Anacardium occidentale* L., Anti-colorectal, Anti-inflammatory, Antioxidant, Antibacterial.

Introduction

The rapid advancement of society and technology has transformed the lifestyle of people globally while simultaneously introducing health problems from external factors, such as air pollution, cigarette smoke, ultraviolet radiation, ozone, and dust. Prolonged exposure to these harmful substances can reduce antioxidant defense to neutralize detrimental free radicals, leading to oxidative stress and damage.[1]

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Free radicals contribute to various illnesses, including several non-communicable diseases (NCDs) such as inflammation, atherosclerosis, neurodegenerative diseases, cancer, and metabolic disorders, which cause over 70% of global deaths.[2]

Free radicals are unstable and highly reactive molecules that contain one or more unpaired electrons in their outer shell. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) possess high destructive potential due to their ability to readily interact with cellular biomolecules, leading to the development of numerous chronic and degenerative diseases.[3] Given their vital role in health and disease, antioxidant-rich food from plants, vegetables, and fruits should be consumed regularly to scavenge free radicals and prevent cellular oxidative damage.[4, 5] This concept has been proven to prevent risks and ameliorate NCDs.

Side dish vegetables in southern Thai cuisine have a unique characteristic with their distinctive flavor and intensely spiced compositions, creating a deeply piquant and delicious taste. A distinctive feature of Thai food is the variety of local vegetables, often served as side dishes, known locally as “Phak Noh”. The commonly used vegetables include *Gnetum gnemon* L. (Liang leaves), *Anacardium occidentale* L. (Mamuang himapan leaves), *Glochidion littorale* Blume (Mun pu leaves), *Micromelum minutum* (G. Forst.) Wight & Arn. (Mui

leaves), *Spondias pinnata* (L.f.) Kurz (Makok leaves) and *Centella asiatica* (L.) Urb. (Bua bok leaves), all of which are considered as herbal plants that offer health benefits and exhibit diverse biological activities. *G. gnemon* has antibacterial properties and inhibits the tyrosinase enzyme.[6] *A. occidentale* is rich in antioxidants like polyphenols and flavonoids[7], which help normalize glucose levels[8] and provide antibacterial and antifungal effects.[9] *G. littorale* contains antioxidants, including high amounts of β-carotene and vitamin A, and inhibits bacterial growth.[10] *M. minutum* has antimicrobial activity and exhibits cytotoxic effects against both T-lymphoblastic leukemia (CEM-SS) and promyelocytic leukemia (HL60) cell lines.[11] *S. pinnata* possesses anticancer activity and is used to treat stomach ulcers due to its anti-inflammatory and antimicrobial properties.[12] *C. asiatica* has traditionally been used to relieve internal heat, modulate blood pressure, and alleviate aches and pains.[13]

The present study investigated the pharmacological potential of six southern Thai side dish vegetables, namely *G. gnemon*, *A. occidentale*, *G. littorale*, *M. minutum*, *S. pinnata*, and *C. asiatica*, through the evaluation of their antioxidant, anti-inflammatory, anti-colorectal cancer, and antibacterial activities against foodborne pathogens such as *Staphylococcus aureus* and *Escherichia coli*, using both fresh and dried leaf extracts. Although these vegetables are widely consumed and traditionally valued for their health benefits, their biological activities have not been comprehensively or systematically characterized. This study, therefore, provides novel scientific evidence to support their potential applications in health supplements or medicinal products.

Materials and Methods

General experimental procedures

All chemicals used in this study were of analytical grade. Ethanol (95%, Sigma-Aldrich, USA) was used as the extraction solvent. Phenolic and flavonoid contents were determined using Folin–Ciocalteu reagent, sodium carbonate, gallic acid, aluminum chloride, sodium nitrite, sodium hydroxide, and catechin (Merck, Germany). Antioxidant and nitric oxide assays employed 2,2-diphenyl-1-picrylhydrazyl (DPPH), dimethyl sulfoxide (DMSO), methanol, L-ascorbic acid, sodium nitroprusside, phosphate-buffered saline (PBS), sulfanilamide, *N*-(1-naphthyl)ethylenediamine dihydrochloride (NED), Griess reagent, and lipopolysaccharide (LPS) (Sigma-Aldrich, USA). For anti-inflammatory studies, 5-lipoxygenase (5-LOX) (Cayman Chemical, USA), arachidonic acid, nordihydroguaiaretic acid (NDGA), and Bay 11-7082 (Sigma-Aldrich, USA) were used. Cell culture reagents included Dulbecco's Modified Eagle Medium (DMEM), trypsin–EDTA (0.5%), Antibiotic–Antimycotic (100×), trypan blue, and heat-inactivated fetal bovine serum (Gibco, Thermo Fisher Scientific, USA). Cytotoxicity was assessed using the MTT assay with MTT (Invitrogen, USA), PBS (Bio Basic, Canada), sodium bicarbonate (Caisson, USA), and 5-fluorouracil (Sigma-Aldrich, USA). Antimicrobial tests employed Mueller-Hinton broth (MHB) and nutrient agar (NHA) (Himedia, India), DMSO (Tokyo Chemical Industry, Japan), ampicillin, and meropenem (Sigma-Aldrich, USA). The main instruments included a UV–Visible spectrophotometer (Lambda 25, PerkinElmer, USA) and a microplate spectrophotometer (SPECTROstar Nano, BMG LABTECH, Germany).

Collection and identification of plant materials

All plant materials were collected in March 2021 from the South Market in Phitsanulok Province, Thailand (16.81278° N, 100.26045° E). The specimens were deposited in the PSRU Herbarium Room, Faculty of Science and Technology, Pibulsongkram Rajabhat University, Phitsanulok, Thailand, after being identified and authenticated by Assistant Professor Dr. Kanjana Thananoppakun, an expert botanist. The voucher specimens of each plant were as follows: *Gnetum gnemon* L. (PSRU1143), *Anacardium occidentale* L. (PSRU1144), *Glochidion littorale* Blume (PSRU1147), *Micromelum minutum* (G. Forst.) Wight & Arn (PSRU1148), *Spondias pinnata* (L.f.) Kurz (PSRU1146), and *Centella asiatica* (L.) Urb. (PSRU1149).[14]

Extraction of herbal plants

All fresh herbal leaves, comprising of *G. gnemon*, *A. occidentale*, G. *littorale*, *M. minutum*, *S. pinnata*, and *C. asiatica*, were roughly crushed using an electric blender, while the dry herbal samples were air-dried for a week before grinding. Each 100 g of fresh and dried leaves was ground and macerated in 1.5 L of 95% ethanol for four days at room temperature, with continuous shaking. The solution was filtered through cotton wool before adding fresh solvent. The maceration process was repeated until the extraction solvent became colorless. The resulting ethanolic extracts were then combined and concentrated under vacuum to obtain the corresponding extracts. Before chemical analysis and biological testing, each extractant was weighed and calculated for a percentage yield. The extracts were kept at 4 °C for further use.

Total phenolic content (TPC) determination

The TPCs of all extracts were quantified using the Folin-Ciocalteu colorimetric method, with a slightly modified procedure from the original method established by Folin and Ciocalteu in 1927.[15] Each of the 500 µL samples at a concentration of 10 mg/mL was mixed with 2.5 mL of freshly prepared Folin-Ciocalteu reagent, diluted in deionized water at a 1:10 ratio. The mixture was thoroughly stirred. After 6 minutes of incubation at room temperature, 2 mL of 7.5% w/v Na_2CO_3 was subsequently added and followed by further incubation in the dark for 90 minutes. The sample's absorbance was quantified using a UV-visible spectrophotometer at a specific wavelength of 765 nm. The TPC values were determined using gallic acid as the standard and expressed as mg of gallic acid equivalent (GAE)/ g dry weight of the extract. Each sample was analyzed in triplicate.

Total flavonoid content (TFC) determination

The TFCs of all extracts were determined using the aluminum chloride colorimetric assay, following a slightly modified procedure.[16] Each extract (250 µL) at various concentrations (0.5 mg/mL) for *G. littorale*, *A. occidentale*, *S. pinnata*, and *M. minutum*, and 1 mg/mL for *G. gnemon* and *C. asiatica* was mixed with water (1,250 µL) in the vial, followed by the addition of 5% sodium nitrite solution (75 µL). After 5 minutes of incubation at room temperature away from light, a solution of 10% aluminum chloride (150 µL) was introduced into the reaction mixture and incubated for an additional 6 minutes. Subsequently, an aliquot of 4% sodium hydroxide solution (500 µL) and water (275 µL) were sequentially added and thoroughly mixed. The absorbance was quantified in triplicate using a UV-Vis spectrophotometer at a wavelength of 510 nm. Subsequently, the TFC values were determined and expressed as mg catechin equivalent (CE)/ g dry weight of the extract.

DPPH radical scavenging activity

Each extract dissolved in dimethyl sulfoxide (DMSO) (25 µL) was placed in a 96-well plate at various concentrations (0.0049–5.000 mg/mL), followed by the addition of 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution in methanol (100 µL). The plate was subsequently incubated for 60 minutes at 37°C in the dark prior to spectrophotometrically measuring at 517 nm. L-Ascorbic acid and solvents were used as positive control and blank, respectively. The experiment was carried out in triplicate.[17] The radical scavenging percentage inhibition against DPPH was calculated using the following Equation 1:

$$\% \text{ Inhibition} = \frac{\text{Absorbance blank} - \text{Absorbance sample}}{\text{Absorbance control}} \times 100 \ldots\ldots \text{Eq. 1}$$

Nitric oxide radical scavenging activity

The Griess reaction was employed to assess the scavenging activity of the nitric oxide (NO) radical.[18] The NO radicals were produced by 10 mM sodium nitroprusside (SNP) in phosphate-buffered saline (PBS) with a pH of 7.3. Various sample concentrations (ranging from 0.625 to 5 mg/mL) were mixed with 10 mM SNP in PBS (10 µL) at pH 7.3. The reaction was allowed to take place at room temperature for 90 minutes in a 96-well plate, exposed to visible polychromatic light. Subsequently,

a solution of 1% sulfanilamide in 5% H_3PO_4 (50 µL) was introduced to the preincubated extract, further incubated in a dark environment at room temperature for 5 minutes, followed by a solution of 0.1% *N*-(1-naphthyl)ethylenediamine (NED) in distilled water (50 µL) was added into each well. The samples were then incubated at room temperature for 30 minutes under dark conditions. Sulfanilamide underwent diazotization with a nitrite ion, resulting in a coupling reaction with NED solution to form a chromophore with a magenta color. The absorbance was measured at a wavelength of 540 nm using a microplate reader. Gallic acid was used as a positive control. The nitrite level of the SNP-treated group was utilized as a reference group for determining the percentage of inhibition of the NO radical. A blank reference was established using a solvent. The % inhibition was determined using Equation 1.

In vitro anti-inflammatory activity against recombinant human 5-lipoxygenase (5-LOX)

The assessment of the inhibitory activity against 5-LOX was performed using a UV-Vis spectrophotometric approach that was adapted and modified from a previous study.[19] This study employed human recombinant 5-LOX. Different concentrations of the plant extracts, diluted in an appropriate solvent, were placed in a 96-well plate. Subsequently, a solution of 0.1 M potassium phosphate buffer, pH 6.3, was prepared and placed onto a plate. Following incubation at 25 °C for 10 minutes, 100 U of 5-LOX enzyme was added to the sample solution. The process was commenced by introducing 1 mM arachidonic acid as a substrate for the enzyme. Afterward, the reaction mixture was incubated for 10 minutes at 25°C, and the absorbance was subsequently determined at a wavelength of 234 nm using a microplate reader. The non-enzymatic control served as a baseline, whereas nordihydroguaiaretic acid (NDGA) was employed as a reference control. The experiments were conducted in triplicate. The half-maximal inhibitory concentration (IC_{50}) values were determined by measuring the percentage of inhibition (Equation 1).

Cytotoxicity on RAW 264.7 macrophage cells

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenoltetrazolium bromide (MTT) assay was used to determine the cytotoxicity of the extract on RAW 264.7 macrophage cells. RAW 264.7 cells were cultured in Dulbecco's Modified Eagle-Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin-streptomycin, and maintained at 37°C, 5% CO_2, in humidified air. The cells (1×10^4 cells/well) were cultured in a 96-well plate for 24 hours, followed by treatment with various concentrations of extracts (0–20 µg/mL). After 24 hours of treatment, MTT solution (5 mg/mL) was added with 4 hours of incubation afterwards. The formazan crystal was solubilized by adding 200 µL DMSO, and the plate was measured with a microplate reader at 570 nm. Cell viability (%) was calculated with the following Equation 2.

$$\% \text{ Cell viability} = \frac{\text{Absorbance of treated group}}{\text{Absorbance control}} \times 100 \quad \text{.....Eq. 2}$$

Suppression of nitric oxide (NO) release in RAW 264.7 macrophage cells

Briefly, 2×10^5 cells/well of RAW 264.7 macrophage cells were seeded in a 24-well plate for 24 hours. The cells were then incubated with the non-toxic concentration of tested samples (0–20 µg/mL) for 1 hour prior to stimulation with 100 ng/mL lipopolysaccharide (LPS). After 24-hour incubation, the NO levels were determined by the Griess assay. The culture medium (100 µL) was mixed with 50 µL of Griess reagent, incubated for 15 minutes, and the absorbance was measured at 540 nm. Bay 11-7082 was used as a positive control. LPS-induced macrophages without anti-inflammatory agents were the inflammation induction control. The experiments were performed in triplicate. The IC_{50} values were determined by measuring the percentage of inhibition (Equation 1).

Antimicrobial activity against S. aureus and E. coli

Antibacterial activities of southern Thai side dish vegetables were determined by a disc diffusion assay, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) against *S. aureus* ATCC 29213 and *E. coli* ATCC 25922. These bacterial strains were obtained from the American Type Culture Collection (ATCC).

For the disc diffusion assay, the crude extracts were dissolved in 100% DMSO to prepare a stock concentration of 500 mg/mL. The sterile discs were impregnated with 5, 10, and 20 µL from the stock concentration to obtain 2.5, 5, and 10 mg/disc, respectively. Ampicillin (10 µg) and meropenem (10 µg) discs were prepared by adding 10 µL from a stock concentration of 1,000 µg/mL to the sterile disc. To test antibacterial activity, an overnight culture of bacteria grown in Mueller-Hinton broth (MHB) was adjusted to McFarland No. 0.5 (equivalent 1.5×10^8 CFU/mL) before being swabbed evenly on Mueller-Hinton agar (MHA). Discs loaded with crude extracts or antibiotics were equidistantly placed on the surface of MHA. The plates were subsequently incubated at 37 °C for 18–24 hours, and the inhibition zone diameters were measured. The 100% DMSO disc was used as a negative control. All treatments were performed in triplicate. The MIC was determined by a broth microdilution assay following the standard guideline described by Clinical Laboratory Standards Institute (CLSI), with slight modification.[20] Briefly, an overnight culture of bacteria was collected by centrifugation, washed twice with normal saline, followed by adjusting the bacterial suspension to 5×10^6 CFU/mL. An aliquot (20 µL) of the aforementioned adjusted bacteria was added to 180 µL comprising of a serial dilution of crude extracts or antibiotics and MHB in a 96-well plate. Wells with and without antimicrobial agents and bacteria were used as controls. The plate was incubated at 37 °C for 18–24 hours. The lowest concentration showing no visible growth, as determined by turbidity and spectrophotometric measurement at 600 nm, was defined as the minimum inhibitory concentration (MIC). To determine the minimum bactericidal concentration (MBC), 100 µL of the solution from wells showing no growth in the MIC assay was MHA. After spreading the aliquots evenly, the plates were incubated at 37 °C for 24 hours, and the lowest concentration that produced no visible colonies was recorded as the MBC.

Anticancer activity against human colorectal carcinoma HCT116 cells

Human colorectal carcinoma HCT116 cells were cultured with DMEM supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS) and antibiotic-antimycotic, at 37°C in a humidified atmosphere of 95% air and 5% CO_2. A cytotoxicity test was performed by an MTT reduction assay.[21] HCT116 cells were plated in a 96-well plate with a density of 5×10^3 cells/well overnight. Cells were treated with different concentrations of fresh and dry leaf extracts of *A. occidentale*, *S. pinnata*, *G. littorale*, and *M. minutum*. 5-Fluorouracil (5-FU, 50 µM) was used as a positive control. After incubation for 48 hours, the media were replaced with DMEM containing 1 mg/mL MTT, and further incubation for 4 hours. Formazan crystals were dissolved using DMSO prior to being measured at 570 nm by a microplate reader, and cell viability was presented as a percentage of control, using Equation 2.

Statistical analysis

We conducted a one-way analysis of variance (ANOVA) using IBM SPSS Statistics version 29 to assess a statistically significant difference in the mean among groups ($p<0.05$). The two-tailed student's t-test was employed to determine significant differences between the two groups. A significant variation was deemed present between the cell viability of the fresh and dried leaf-treated groups at $p<0.05$.

Results and Discussion

Preparation of extracts

Ethanol was used to extract six species of culinary condiment vegetables, including *G. gnemon*, *A. occidentale*, *G. littorale*, *M. minutum*, *S. pinnata*, and *C. asiatica* from both fresh and dried leaves. Each variant of ethanol extract exhibited the properties of a thick liquid, and the extract's yield percentage per unit weight of fresh/dried leaves, as presented in Table 1. The dried leaves yielded higher extract percentages than fresh leaves for all tested plants, with *C. asiatica* showing the highest yield (30.23%) and *G. littorale* the lowest (12.51%).

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Table 1: List of selected herbal plants and percentage yields of the leaf extracts

Plant species	Weight of extract (g)		Yield (%)	
	Fresh	Dried	Fresh	Dried
G. gnemon	9.37	18.70	9.37	18.70
A. occidentale	12.75	24.79	12.74	24.79
G. littorale	9.03	12.51	9.03	12.51
M. minutum	9.58	14.18	9.58	14.18
S. pinnata	9.26	19.61	9.25	19.61
C. asiatica	8.41	30.23	8.40	30.23

TPC and TFC determination and antioxidant potentials against DPPH and NO radicals

The TPCs in the ethanolic extracts of fresh and dried leaves from six distinct southern Thai side dish vegetables are depicted in Figure 1. The ethanolic *A. occidentale* leaf extracts, particularly the dried leaves, exhibited the highest concentration of TPCs (726.414 ± 8.978 mg GAE/g crude extract), which correlated with a strong free radical inhibitory effect, followed by fresh leaf extract (646.624 ± 4.750 mg GAE/g crude extract). The TPCs of water and methanolic extracts were analyzed in accordance with the previous studies.[7, 22] The results exhibited a greater phenolic concentration in comparison to non-polar solvents.

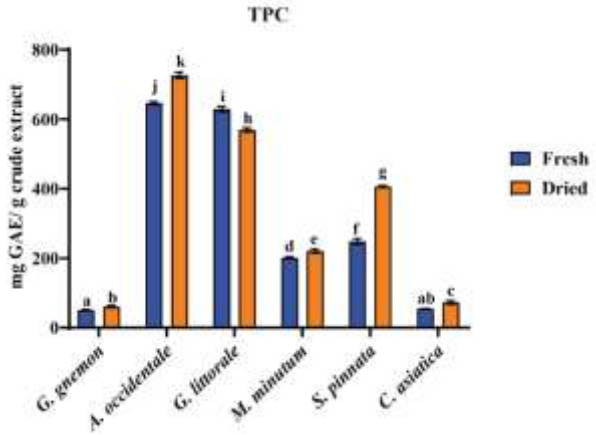

Figure 1: Total phenolic contents (TPC) of different plant extracts. Different letters indicate that there is significant difference between different plant extract conditions ($p<0.05$).

According to the analysis of flavonoid compounds (Figure 2), the study revealed that the ethanolic extracts derived from both fresh and dried *A. occidentale* leaves showed the highest TFCs (167.15 ± 0.36 and 174.91 ± 0.20 mg CE/ g crude extract for fresh and dried leaves). These findings align with a previous study on the chemical composition of *A. occidentale* leaves, which identified flavonoids, particularly quercetin, as the primary constituent.[23] Other plant leaves, such as *G. gnemon*, *G. littorale*, *M. minutum*, *S. pinnata*, and *C. asiatica* also contained different amounts of flavonoid compounds. The fresh herbal plants had their TFCs ranging from 22.97 ± 0.68 to 167.15 ± 0.36 mg CE/ g of crude extract. The dried plants, on the other hand, exhibited TFCs ranging from 37.48 ± 0.62 to 174.91 ± 0.20 mg CE/ g of the crude extract.

All ethanolic leaf extracts were determined on their antioxidant potential against DPPH radicals (Table 2). The results revealed that extracts derived from both fresh and dried *A. occidentale* leaves exhibited the most potent ability to scavenge free radicals, comparable to ascorbic acid as a positive control, with IC_{50} values of 0.060 ± 0.001 and 0.051 ± 0.001 mg/mL, respectively. These findings align with the previous study.[7] The methanol *A. occidentale* leaf extract demonstrated superior efficacy compared to the extracts from nine other vegetables, such as roselle, kaffir lime, gotu kola, coriander, wild betel, betel nut, and chilli, primarily due to its higher content of phenolic substances. The chemical constituents that function as free radical inhibitors consist of gallic acid, cinnamic acid, and quercetin.[24] The second most potent extract with the capacity to suppress free radicals was the ethanolic extract derived from *G. littorale* leaves. The IC_{50} values of *G. littorale*, *S. pinnata*, and *M. minutum* fresh leaf extracts were found to be 0.064 ± 0.003, 0.147 ± 0.008, and 0.909 ± 0.226 mg/mL, while their dried leaf extracts showed IC_{50} values of 0.069 ± 0.004, 0.104 ± 0.005, and 0.426 ± 0.201 mg/mL, respectively.

These values indicated the potency of the extracts in inhibiting DPPH radicals, and the results were specifically related to their chemical components found in fresh leaves. Regarding extracts derived from *G. gnemon* leaves, both fresh and dried leaf extracts were the least active, with IC_{50} values of 4.860 ± 0.299 and 3.868 ± 0.221 mg/mL, respectively. The predominant compounds present in *G. gnemon* leaves were primarily terpenes.[25]

Furthermore, all ethanolic extracts derived from fresh and dry leaves were tested on their antioxidant activity against NO radicals (Table 2). The results showed that only three fresh leaf extracts, *C. asiatica*, *A. occidentale*, and *G. littorale*, could inhibit NO radicals with IC_{50} values ranging from 2.646 ± 0.302 to 3.535 ± 0.563 mg/mL. It was found that all extracts derived from all dry leaves were inactive against NO radicals.

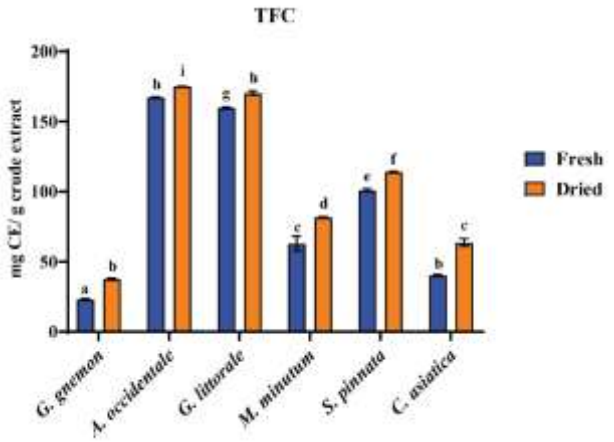

Figure 2: Total flavonoid contents (TFC) of different plant extracts. Different letters indicate that there is significant difference between different plant extract conditions ($p<0.05$).

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Table 2: *In vitro* antioxidant activities of all selected leaf extracts against DPPH and NO radicals

Plant species	DPPH (IC_{50}, mg/mL)		NO (IC_{50}, mg/mL)	
	Fresh	Dried	Fresh	Dried
G. gnemon	4.860 ± 0.299[d]	3.868 ± 0.221[d]	-	-
A. occidentale	0.060 ± 0.001[a]	0.051 ± 0.001[a]	2.646 ± 0.302[ab]	-
G. littorale	0.064 ± 0.003[a]	0.069 ± 0.004[a]	3.306 ± 0.238[ac]	-
M. minutum	0.909 ± 0.226[b]	0.426 ± 0.201[b]	-	-
S. pinnata	0.147 ± 0.008[a]	0.104 ± 0.005[a]	-	-
C. asiatica	1.838 ± 0.077[c]	1.064 ± 0.038[c]	3.535 ± 0.563[bc]	-
Ascorbic acid	0.050 ± 0.003[a]		-	
Gallic acid	-		0.11 ± 0.004	

*Inactive at concentration >5.0 mg/mL for DPPH and NO assays. One-way ANOVA ($p < 0.05$) was used to analyse the significant differences. Different superscript letters indicate significant difference among plant extracts.

Anti-inflammatory activity against 5-LOX

All ethanolic extracts exhibited 5-LOX inhibitory activity ranging from 17.27% to 29.57% for fresh leaves, whereas all dried leaf extracts showed slightly higher inhibition (27.34–41.75%). At the tested concentration (0.0625 mg/mL), dried leaf extracts of *S. pinnata*, *C. asiatica*, *A. occidentale*, and *M. minutum* demonstrated notably higher inhibition, with *S. pinnata* showing the greatest activity (41.75 ± 1.41%), followed by *C. asiatica* (40.10 ± 4.60%) and *A. occidentale* (39.23 ± 3.56%). The highest inhibition among fresh leaves was observed in *C. asiatica* (29.57 ± 1.48%). Previous studies have reported that *A. occidentale* kernel extract suppressed 5-LOX expression in a carrageenan-induced inflammatory rat model,[26] and that an aqueous leaf extract of *C. asiatica* inhibited 5-LOX activity *in vitro* with an IC_{50} value of 250 ± 2.8 µg/mL.[27] These findings further support the anti-inflammatory potential of these plant extracts.

Cytotoxic effect suppression of NO release in LPS-induced RAW 264.7 macrophages

The results from the MTT assay showed no significant difference in cell viability among the extracts at a concentration of 20 µg/mL. However, the viability of cells treated with Bay 11-7802 was significantly lower than cells treated with all extracts (Figure 3A). Further, both fresh and dried leaf extracts of *M. minutum* showed the highest NO suppression activity as compared to all species tested. Furthermore, both fresh and dried extracts of *M. minutum* and *A. occidentale* showed statistically comparable activity (Figure 3B). Furthermore, our findings suggest that *M. minutum* and *A. occidentale* exhibit promising anti-inflammatory properties by suppressing NO induced by LPS in macrophage cells. While a previous study demonstrated that *A. occidentale* kernel extract can inhibit nitrate/nitrite production in carrageenan-induced inflammation model in rats,[26] there is a lack of research specifically addressing the NO suppressive effect of both *M. minutum* and *A. occidentale* leaf extract in LPS-induced RAW 264.7 cells.

Antibacterial activity

The antibacterial activity (Table 3) determined by the disc diffusion assay showed that fresh and dried leaves of *A. occidentale*, *S. pinnata*, dried leaves of *M. minutum*, and dried leaves of *C. asiatica* particularly exhibited antibacterial activity against *S. aureus* ATCC 29213, a representative for gram-positive bacteria, while only fresh and dried leaves of *A. occidentale* inhibited the growth of *E. coli* ATCC 25922, a representative for gram-negative bacteria (Figure 4). By comparing the inhibition zone diameters among different extracts at 10 mg/disc, the result showed that fresh leaves of *S. pinnata* had the highest inhibitory activity and were significantly higher than dried leaves of *S. pinnata*, fresh and dried leaves of *A. occidentale*, dried leaves of *M. minutum*, and dried leaves of *C. asiatica* against *S. aureus* ATCC 29213 (Figure 4C). Regarding antibacterial activity on the inhibition of *E. coli* ATCC 25922, only dried and fresh leaves of *A. occidentale* possessed antibacterial activity, with no significant difference between fresh and dried leaves. The inhibitions were conducted in a concentration-dependent manner. *G. gnemon*, *G. littorale*, fresh leaves of *M. minutum*, and fresh leaves of *C. asiatica* were found to have no antibacterial activity against the *S. aureus* isolate.

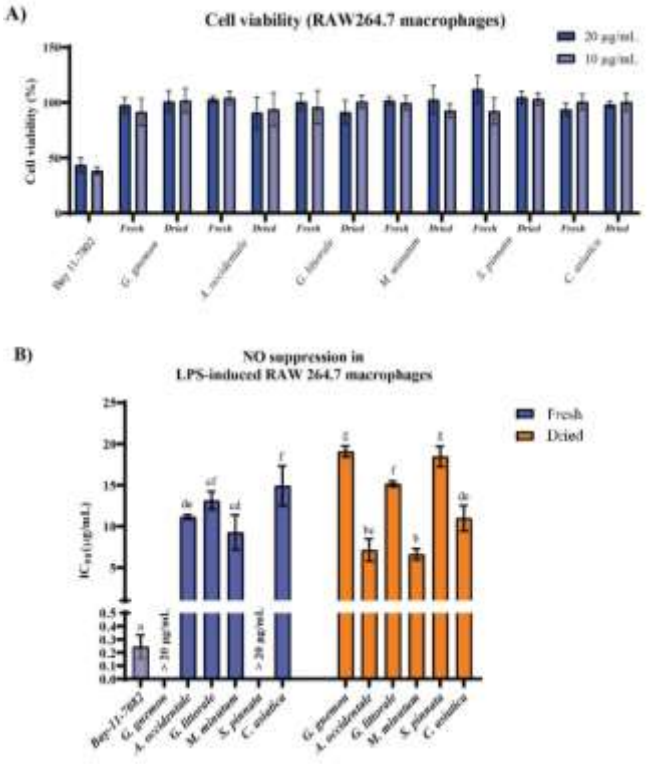

Figure 3: A). Cell viability of treated RAW264.7 macrophage cells with extract at 10–20 µg/mL, and B). NO release suppression activity of extracts in LPS-induced RAW264.7 macrophage cells. Different letters indicate statistically significant differences analyzed by one-way ANOVA ($p<0.05$).

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Table 3: Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of Thai indigenous vegetables against *S. aureus* ATCC 29213 and *E. coli* ATCC 25922

Sample	*S. aureus* ATCC 29213		*E. coli* ATCC 25922	
	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
Fresh leaves of *A. occidentale*	6.25	12.50	12.50	25
Dried leaves of *A. occidentale*	0.78	1.56	1.56	25
Fresh leaves of *G. gnemon*	12.50	>50	>50	>50
Dried leaves of *G. gnemon*	3.13	12.50	50	50
Fresh leaves of *S. pinnata*	1.56	6.25	3.13	25
Dried leaves of *S. pinnata*	1.56	6.25	3.13	50
Fresh leaves of *M. minutum*	12.50	>50	25	>50
Dried leaves of *M. minutum*	6.25	>50	50	>50
Fresh leaves of *G. littorale*	12.5	12.50	>50	>50
Dried leaves of *G. littorale*	25	25	50	>50
Fresh leaves of *C. asiatica*	50	>50	>50	>50
Dried leaves of *C. asiatica*	3.13	>50	50	>50
Ampicillin	0.002	0.008	N/D	N/D
Meropenem	N/D	N/D	<0.00013	<0.00013

N/D = Not determined. The experiment was carried out in triplicate.

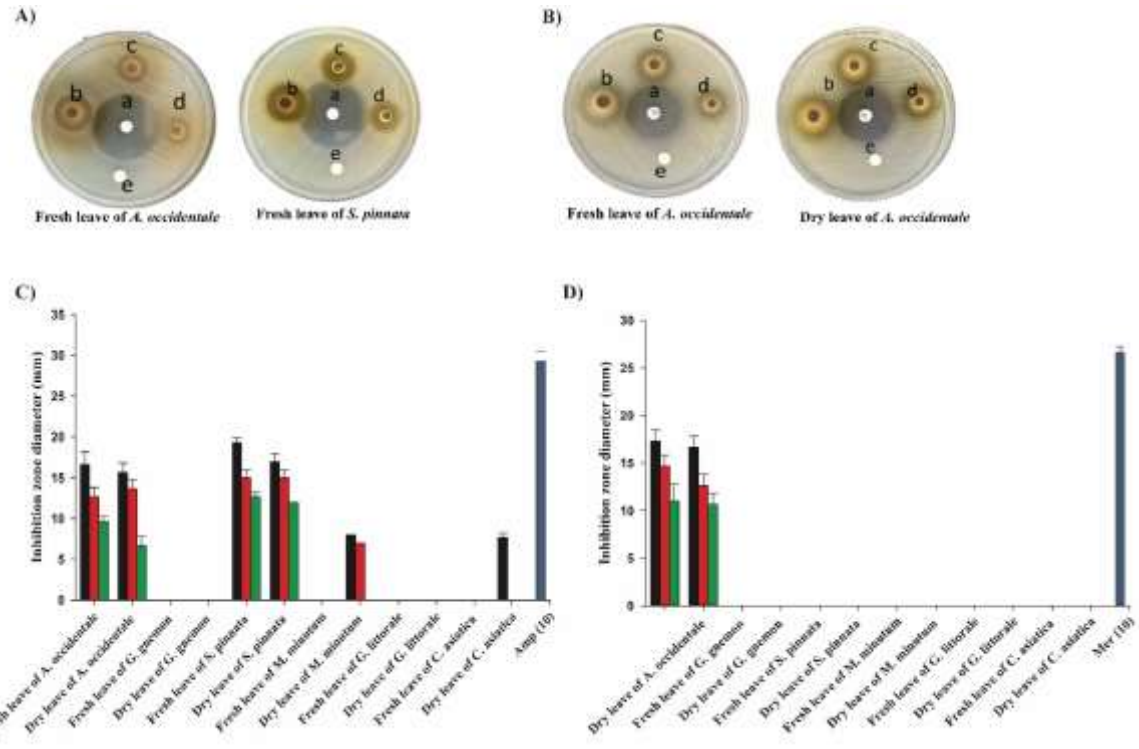

Figure 4: Representative antibacterial activity of agar well diffusion of fresh and dried leaf extracts of Thai indigenous vegetables against A) *S. aureus* ATCC 29213 and B) *E. coli* ATCC 25922. C) Inhibition zone diameters against *S. aureus* ATCC 29213, and D) toward *E. coli* 25922. a = ampicillin at 10 µg/disc for *S. aureus* or meropenem at 10 µg/disc for *E. coli*; b = extract at 10 mg/disc; c = extract at 5 mg/disc; d = extract at 2.5 mg/disc; e = 100% DMSO disc. Data from three replications were expressed as means ± S.D. Different letters and colors in C) and D) indicate statistically significant differences analyzed by one-way ANOVA and Turkey's-b ($p<0.05$).

According to the results of a previous study, *S. pinnata* leaf extract demonstrated antibacterial activity against *S. aureus*, a finding consistent with this study.[28] Based on the MBC result, the extract from the dried leaves of *A. occidentale* exhibited the most potential as a candidate against the test organisms, *S. aureus* and *E. coli*, followed by the extract from the fresh leaves of *S. pinnata.* These results are in concordance with previous studies, which reported that the extracts of *A. occidentale* [29, 30] and *S. pinnata* [28] possess high antibacterial activity.

Anticancer activity in human HCT116 cells from colorectal cancer

The investigation of the anticancer properties of all ethanolic leaf extracts revealed that *A. occidentale*, *S. pinnata*, *G. littorale*, and *M. minutum* possess this effect, with their IC_{50} values illustrated in

Figure 5. It was found that both fresh and dried *A. occidentale* leaf extracts displayed the highest cytotoxicity against HCT116 cells when treated at concentrations of 100–250 µg/mL, significantly reducing cell viability (p<0.05) with an IC_{50} value of 124.6 µg/mL and 69.8 µg/mL, respectively, compared to the other herbal extracts. Moreover, 200 µg/mL of fresh leaf and 100 µg/mL of dried leaf extracts exhibited anticancer effects that were similar to those of the chemotherapy drug 5-FU (Figure 5A). Meanwhile, *S. pinnata*, *G. littorale*, and *M. minutum* leaf extracts also exhibited cytotoxic effects on HCT116 cells. The dried leaf extract of *S. pinnata* showed more cytotoxicity than fresh leaf extracts when cells were exposed to concentrations of 150–350 µg/mL, with an IC_{50} value of 254.4 µg/mL and <150.0 µg/mL, respectively (Figure 5B). Also, *G. littorale* leaf extract significantly decreased cell viability, with an IC_{50} value of 158.4 µg/mL (Figure 5C). Furthermore, *M. minutum* leaf extracts showed cytotoxic effects only at high concentrations of 450–750 µg/mL for both fresh and dried leaf extracts, with an IC_{50} value of 399.3 µg/mL and <150.0 µg/mL, respectively (Figure 5D).

These findings evidence that AOE induced cytotoxicity on HCT116 cells, especially the dried leaf extract displaying a particularly potent anticancer effect. This is supported by previous studies that hydroethanolic *A. occidentale* leaf extract activates cytotoxic mechanisms by disrupting cell cycle progression and inducing apoptosis through caspase-3 activation.[31] Phenolic acids, flavonoids, and tannins were key bioactive components.[32] Moreover, gallic acid and galloyl hexoside, presented in ethanolic leaf extracts, have been related to cytotoxic effects against HCT116, HL60, and P815 tumor cells,[32] whilst pentagalloylglucose has been proposed as exhibiting selective cytotoxicity in cancer cells.[33]

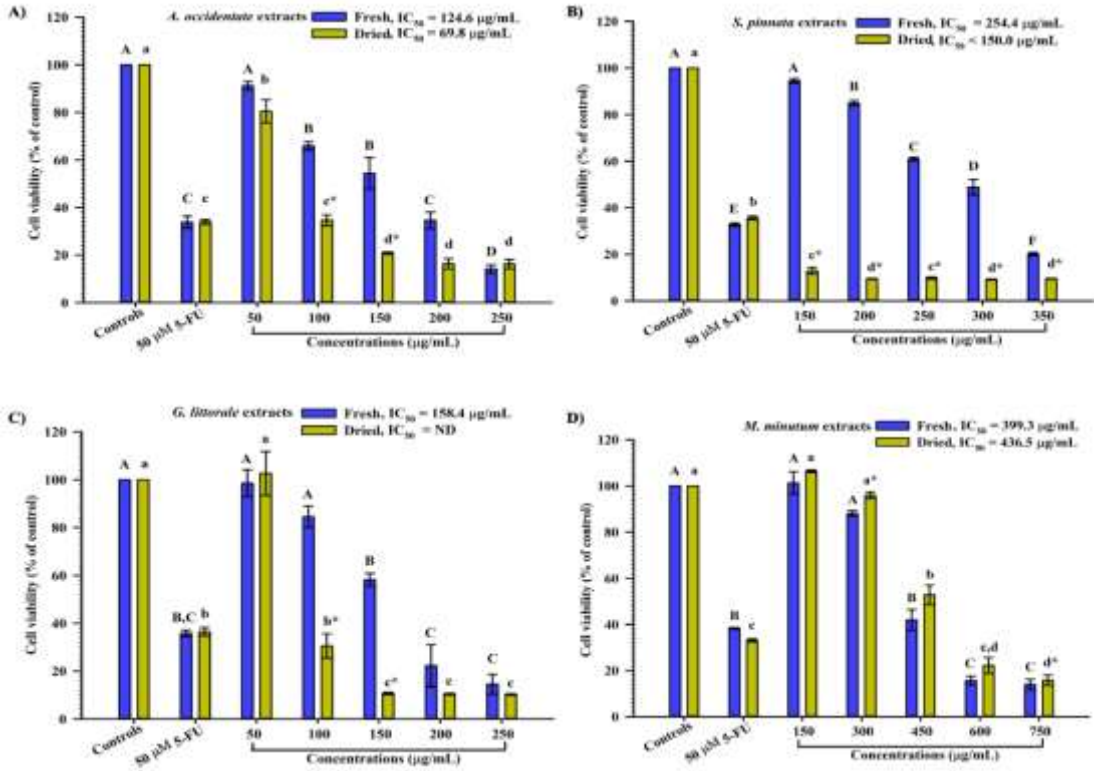

Figure 5: Effects of A) *A. occidentale*, B) *S. pinnata*, C) *G. littorale*, and D) *M. minutum* fresh and dried leaf extracts on HCT116 cells. Cells were treated with different concentrations of leaf extracts for 48 hours, and 50 µM 5-FU was used as a positive control. Cell viability was evaluated using the MTT reduction assay and presented as a percentage of control. Values represent the means ± S.D. (n=3) of independent replications. $^{A, B, C, D, E, F}p$<0.05 significance compared with the group of fresh leave-treated cells. $^{a, b, c, d, e, f}p$<0.05 significance compared within the group of dried leaf-treated cells. $^{\#}p$<0.05 significant difference was considered between the cell viability of the fresh and dried leaf-treated cells.

Conclusion

This study demonstrates that among six southern Thai side-dish vegetables, *Anacardium occidentale*, particularly its dried leaf extract, exhibits the most promising biological potential. The extract showed strong antioxidant activity, potent anti-inflammatory effects by suppressing nitric oxide production and inhibiting 5-lipoxygenase activity, as well as antibacterial activity against *S. aureus* and *E. coli*. In addition, it displayed notable cytotoxicity against HCT116 human colorectal cancer cells, supported by its high abundance of phenolic and alkaloid compounds. These findings highlight the potential of *A. occidentale* for further development as a nutraceutical or functional food ingredient with antioxidant, anti-inflammatory, antibacterial, and anticancer properties to promote health and prevent disease.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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