Pertanika J. Trop. Agric. Sci. 49 (5): 2323 - 2342 (2026)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Comparative Phytochemical and Biological Properties of Unripe *Spondias pinnata* Fruit Extract

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ABSTRACT

Medicinal plants are recognised as valuable sources of phytochemicals with the potential to prevent biomolecular degeneration and promote human and animal health. This in vitro study investigated and compared the biological activities and phytochemical composition of raw *Spondias pinnata* fruit extracts prepared using three solvents: 70% acetone (WRA-1), 70% ethanol (WRA-2), and 70% methanol (WRA-3). All three extracts exhibited antityrosinase, antielastase, and antioxidant activities, as well as cytotoxic effects against cholangiocarcinoma (MO55) cells. The IC_{50} values on MO55 cells were 0.58, 1, and 2.1 mg/mL for WRA-1, WRA-2, and WRA-3, respectively. WRA-3 exhibited a significantly lower cytotoxic effect on MMNK1 cells. Comparable phytochemical profiles, including phenolic compounds, flavonoids, terpenoids, tannins, and cardiac glycosides, were observed in all three extracts. The total phenolic contents of WRA-1, WRA-2, and WRA-3 were 26.35 ± 4.89, 49.77 ± 3.81, 31.91 ± 1.52 mg GAE/g extract, respectively. 5-hydroxymethylfurfural, gallic acid, verbascoside, and isorhamnetin-3-rutinoside as the major bioactive constituents in WRA-1. As this study was limited to in vitro assays and did not include in vivo or clinical validation, the biological relevance of these findings to human and animal health remains to be established. Overall, the results suggest that unripe *S. pinnata* fruit represents a promising source of bioactive compounds and warrants further investigation for potential healthcare applications.

Keywords: Anticancer, antioxidant, anti-tyrosinase, anti-elastase, wild mango fruit

ARTICLE INFO

Article history:
Received: 20 July 2025
Accepted: 28 September 2026
Published: 30 October 2026

DOI: https://doi.org/10.47836/pjtas.49.5.11

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e-ISSN: 2231-8542

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INTRODUCTION

Currently, the rapidly increasing number of elderly people has led to a demographic shift. This issue has caused many countries around the world to face the challenges of an aging society, and they are moving towards becoming super-aged societies in the next few years.

In addition, one public health problem that arises alongside entering an aging society is illnesses, disabilities, and deaths from non-communicable diseases (NCDs). According to statistics collected by the World Health Organization (WHO), NCDs are the cause of death for humans, accounting for up to 79% (World Health Organization, 2024). The top four NCDs that affect health and cause a lot of human deaths are cardiovascular diseases (19 million people), cancer (10 million people), chronic respiratory diseases (4 million people), and diabetes (>2 million people). Similarly, in Thailand, Thai people die from NCDs at a rate of up to 74% of all causes of death. The continuous increase in the number of elderly people and patients with chronic non-communicable diseases is causing healthcare systems to spend more on patient care, impacting the economy and society in every country worldwide, especially in low- and middle-income countries (Thansa, 2025). Moreover, most developing countries rely on technology and imports of healthcare products from developed countries (Alfaouri et al., 2025; Sabogal et al., 2025). The fighting around the Strait of Hormuz has affected the transport of raw materials used in making medicines, treatment drugs, and medical supplies from the United States of America (USA), Europe, and the Middle East to the Asian region. Countries in the Asian region are affected by the risk factors mentioned above, leading to shortages of many types of medicines during conflict crises, as well as higher costs for importing key ingredients. Solving the problem of essential drug shortages caused by these factors in the public health system is therefore very important.

The progression of ageing and NCDs is associated with two major factors: (1) intrinsic factors (age, sex, hormones, genes, oxidative stress, etc.) and (2) extrinsic factors (environmental, food, lifestyle, etc.). Both factors have a mechanism of co-stimulation that causes biomolecular degeneration in many forms, leading to changes in their structure and malfunction, resulting in accelerated aging and NCDs (Guo et al., 2022). Therefore, studying ways to slow down the deterioration of biomolecules related to those factors might help reduce the number of people with NCDs.

Natural products are an exciting target source for human health prevention and therapy development. The safety of treatment substances and the reduction of side effects are two primary concerns that drive the interest in natural active ingredients, leading to a continuous increase in research studies. In addition, researching medicinal herbs with therapeutic properties for use within public health systems might help solve the problem of shortages of essential drugs for treating certain diseases. Phytochemical research can

create new opportunities and treatment options, which would positively impact the public health system and help reduce the economic burden (Wang et al., 2024).

Generally, the genus *Spondias*, which belongs to the family Anacardiaceae, comprises approximately 18 species. It is widely known as wild mango or hog plum (Laksemi et al., 2019; Nitheesh et al., 2024). In Thailand, most Thai people call trees in the genus Spondias 'Makok'. Leaves are used as vegetables, while fruits are used as ingredients in a variety of dishes, especially in papaya salad, a very popular dish. This widespread culinary use makes *Spondias* trees economically valuable.

The genus *Spondias* comprises nutritious plants that contain 16.3- 23.54% total carbohydrates, 12.23-12.54% crude fat, 3.13-4.03% crude fiber, 0.9-1.23% copper, 1.3- 1.5% iron, 0.96- 1.38% sodium, and 0.15-0.93% calcium. The edible part of *S. pinnata* fruit contained 90% moisture. Protein was detected at 0.7%, with several amino acids found, such as glycine, cystine, serine, alanine, and leucine, among others. In addition, *S. pinnata* fruit contains 4.5% carbohydrates, with sugars being made up of various types, such as sucrose, glucose, and fructose, etc. Moreover, *S. pinnata* fruit was also found to contain 3.0% fat, 1% dietary fiber, and 0.5% minerals and vitamins (calcium 36.0 mg/100g; phosphorus 11 mg/100 g; iron 3.9 mg/100 g; thiamine 0.02 mg/100 g; riboflavin 0.02 mg/100 g; niacin 0.3 mg/100 g; vitamin C 21 mg/100 g; and vitamin A up to 450 IU/100 g) (Nitheesh et al., 2024; Siegel et al., 2019).

Interestingly, the *Spondias* genus, especially *S. pinnata*, has been reported to contain groups of phytochemicals that show a variety of biological and pharmacological properties. Recent reports indicated that *S. pinnata* has important pharmacological effects, including hypoglycemic, anthelmintic, anti-cancer, ulcer-protective, cytotoxic, hepatoprotective, thrombolytic, anti-microbial, anti-diarrheal, antioxidant and anti-tyrosinase activities (Kunu et al., 2025; Nitheesh et al.,2024; Patathananone et al., 2019a). The extract of the leaves and stems of *S. mombin* with 80% ethanol contains various key compounds, including tannins and pseudotannins like geraniin, galloyl geraniin, and galloyl glucose. 3-O-[α-rhamnopyranosyl-(1→2)]-α-rhamnopyranosyl-(1→6)-β-glucopyranoside is a flavonoid found in the 80% MeOH leaf extract of *S. venulosa*. The extraction of key compounds from *S. purpurea* fruit using acetone revealed various flavonoids such as rhamnetin, isorhamnetin, kaempferol, kaempferide, astragalin, isoquercetin, and quercetin dihydrate, as well as phenolic acid derivatives like gallic acid and 3-caffeoylquinic acid. (Sameh et al., 2018). Ripe *S. pinnata* fruit extracts were found to contain phytochemicals, mainly phenolics, flavonoids, and terpenoids. These phytochemicals have shown biological properties such as inhibiting tyrosinase activity, acting as antioxidants, showing cytotoxicity toward certain cancer cells, and inhibiting antibiotic-resistant mastitis pathogens (Kunu et al., 2025; Patathananone et al., 2019a; Patathananone et al., 2019b). From the information above, plants in the *Spondias* genus are sources of bioactive and pharmacological compounds that can be developed as alternatives for use in human health.

However, comparative studies on the biological properties of phytochemicals from unripe *S. pinnata* fruits remain limited, particularly with respect to tyrosinase and elastase inhibition, antioxidant activity, and cytotoxic effects against cholangiocarcinoma and normal cells.

This research aims to extract bioactive compounds from unripe *S. pinnata* fruit using a mixed solvent system that includes an organic solvent and water. It also aims to compare the efficacy of the extracts in terms of antioxidant activity and the inhibition of tyrosinase and elastase — enzymes involved in key processes such as melanin synthesis, neurotransmitter production, and skin cell strength. Moreover, this research also aims to study and compare the cytotoxicity of key compounds from *S. pinnata* fruit extracts on cholangiocarcinoma cells and normal bile duct cells to assess their selective ability.

These findings are expected to contribute to improving the precision of drug delivery in the future, which might help reduce side effects from cancer treatments or lower the production costs of drugs used to treat cholangiocarcinoma, a disease with a high incidence in Thailand.

MATERIALS AND METHODS

Unripe wild mango (*S. pinnata*) fruit, a raw plant material, was collected in Kokyai village, Ban Fang district, Khon Kaen province, Thailand, from September to October. The characteristics of the plant material are exhibited in Figure 1. The plant material was taxonomically identified based on its morphological characteristics, following the taxonomic descriptions provided by Nitheesh et al. (2024). The organic solvents were purchased from LabScan. Mushroom tyrosinase and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were bought from Sigma-Aldrich. L-Dopa and kojic acid were purchased from HiMedia company. Dulbecco's Modified Eagle Medium-high glucose (DMEM-Hg), fetal bovine serum (FBS), L-glutamine, penicillin-streptomycin, and trypsin- EDTA were ordered from Gibco B.R.L. (Grand Island, NY, USA). Additionally, 3- [4, 5-dimethylthiazol-2-yl]-2-5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide were bought from Invitrogen (Carlsbad, CA).



(A) (B) (C)

Figure 1. *Spondias* genus plant used as the research material, located in Ban Fang District, Khon Kaen Province, Thailand. (A) *S. pinnata* tree; (B) leaves of *S. pinnata*; and (C) fruits of *S. pinnata*, exhibiting morphological characteristics consistent with the species identification

Unripe *S. pinnata* Fruit Extraction Process

Unripe *S. pinnata* fruits were washed three times using water. The fruits, including pericarp that are without seeds, were collected and placed in the ice-cold chamber at 4 °C. Approximately 300 g of unripe fruits and pericarp were homogenised using the blender for 5 min. Then, the homogenate was dried using a freeze-dryer. The lyophilised powder of unripe fruits and pericarp of *S. pinnata* fruit (10 g) was placed into the extraction chamber, and then 100 mL of 70% v/v acetone, 70% v/v ethanol, and 70% v/v methanol were added to each section. These extracted chambers were stirred using a magnetic stirrer at 750 rpm at room temperature for 72 h, and then incubated at 4 °C overnight. The extracts were filtered using Whatman® No. 1 and No. 5, respectively. The clear supernatant of each extract was evaporated at 50 °C using an evaporator to remove the organic solvent and concentrate. Evaporation was continued until the supernatant in the water part remained in a small volume (20 mL). Then, the hydrated part of the extracts was freeze-dried by lyophilisation. All extracts were kept at -20 °C until used. The ID names of these extracts were shown: WRA-1 (70% v/v acetone), WRA-2 (70% v/v ethanol), and WRA-3 (70% v/v methanol).

Anti-Tyrosinase Activity Assay

According to the modified procedure, the tyrosinase-inhibitory effect of the treated samples was determined using the Dopachrome method (Long et al., 2008). The reactions were conducted in a 96-well microplate. Kojic acid was used as a positive control. Firstly, the extracted powder was dissolved in 5% v/v DMSO (10 mg/mL). Then, different concentrations (0.13 - 3 mg/mL) of treatment samples (10 µL) were mixed with 100 µL of mushroom tyrosinase (10 µg/mL mushroom tyrosinase in 20 mM phosphate buffer, pH 6.8). The banked sample was tested together without being co-incubated with the enzyme. The reaction plates were incubated at 37 °C for 10 min. Next, 20 µL of L-Dopa (20 mM L-Dopa in DMSO) was added and mixed gently; after that, it was incubated at 37 °C for 30 min. The absorbance value was measured at a microplate reader's wavelength of 495 nm (Biochrom EZ Read 2000, USA). The percentage of tyrosinase inhibitory activity of tested substances was calculated using an equation previously reported by Patathananone et al. (2022).

Anti-Elastase Activity Assay

The treatment test observed the elastase inhibitory effect as previously described by Lee and Choi (1999). The experiment was briefly modified by decreasing the volume and setting the reaction system on a 96-well plate. In the pre-incubation step, 10 µL of the different concentrations of the extracts (0.25 - 2 mg/mL) were placed into 100 µL

of porcine elastase solution (Sigma-Aldrich) (approximately 0.06 Unit/mL elastase in 20 mM Tris-HCl buffer, pH 8), and then 70 µL of buffer was added, mixed gently, and incubated at 37 °C for 10 min. The absorbance value was measured at a wavelength of 410 nm using a microplate reader. In the next step, 20 µL of substrate solution (0.8 mM succ-(Ala)-3-nitroanilide in 20 mM Tris-HCl buffer, pH 8) was added to each pre-incubated reaction. The plate was mixed and incubated at the same temperature for 30 min. The absorbance value was analysed at 410 nm. The percentage of elastase inhibition was calculated according to the formula as in Equation 1.

$$\% \text{ Inhibition} = \frac{[\text{ Absorbance 410 nm of control} - \text{Absorbance 410 nm of sample }]}{[\text{ Absorbance 410 nm of control }]} \times 100 \quad [1]$$

Antioxidant Activity Assay

The antioxidant capability of the extracts was analysed using the briefly modified DPPH method according to the report by Pientaweeratch et al. (2022). The different concentrations (0 - 80 µg/mL) of the extracts (10 µL) were added to 190 µL of DPPH (0.101 mM DPPH in methanol) solution. After that, the reactions were incubated in the dark for 30 min at 37 °C. The absorbance was measured at 515 nm using a UV- vis spectrophotometer. The percentage of inhibition was calculated following the equation previously described by Chen et al. (2010).

Determination of Cell Viability Using MTT Assay

Cholangiocarcinoma (MO55 cells) and normal bile duct cells (MMNK1 cells) were cultured in DMEM high glucose supplemented with 10% v/v fetal bovine serum and 1% v/v penicillin-streptomycin in T-25 cell culture flasks as previously described by Patathananone et al. (2019a). The culture flasks were incubated in 5% CO_2 and 95% air at 37 °C until cells grew to approximately 90% of the flask area. Cells were trypsinised, collected, and washed with 1X phosphate-buffered saline (1X PBS) 3 times. The same culture medium was used to resuspend cells to prepare for cell viability tests. The initial numbers of both cell lines were set at approximately 9 x 105 cells/mL to place in a 96-well plate at 100 µL. MO55 and MMNK1 cells were seeded in a 96-well plate according to the experiment described by Patathananone et al. (2019a). Both cell lines were treated with different concentrations (0.05 - 3.2 mg/mL) of each extract for 24 and 48 h. Then, 100 µL of 0.5 mg/mL MTT solution (0.5 mg/mL MTT in 1X phosphate-buffered saline, pH 7.4) was added to each test well and mixed gently. The plate was incubated at 37 °C for 4 h, after which the MTT

solution was carefully removed from each well. Subsequently, 100 µL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The absorbance was measured at 570 nm using a microplate reader (Varioskan Flash). Cell viability (%) was calculated following the method described by Patathananone et al. (2019b). The IC_{50} value of each treatment sample was observed and compared between MO55 cells and MMNK1 cells.

Phytochemical Analysis

Phytochemical types of each extract (WRA-1, WRA-2, and WRA-3) were analysed following the report of Patathananone et al. (2019b). The extract was evaluated with specific reagents to observe the positive reaction. The extract results were compared according to each phytochemical type's positive or negative compound reaction.

Gas Chromatography-Mass Spectroscopy (GC-MS)

The terpenes and their derivatives in WRA-1 were determined using gas chromatography-mass spectrometry (GC-MS) on an Agilent 7890B system according to previous research by Kunu et al. (2025). The samples were separated on an HP-5MS column (25 m × 250 µm × 0.25 µm) with helium as the carrier gas at a flow rate of 1.2 mL/min. The initial inlet temperature was 250 °C in splitless mode for 0.75 min, and the MSD transfer line was maintained at 250 °C. The oven temperature program was as follows. The initial temperature was 60 °C, held for 3 min [and then run for 3 min: please clarify]. The temperature was then increased to 80 °C (Ramp 1) and held for 23 min. Next, the temperature was increased to 120 °C and held until 36.33 min. Finally, the temperature was increased to 240 °C, and the run was stopped at 66.33 min. The mass spectrometer (Agilent 5977B) was operated in electron ionisation (EI) mode at a fixed electron energy of 70 eV. Data were acquired in scan mode over a mass range of m/z 45-500, with the ion source temperature at 230 °C and the quadrupole temperature at 150 °C.

Liquid Chromatography-Mass Spectroscopy (LC-MS)

The different kinds of phenolic compounds in WRA-1 were examined using the LC-MS technique. The phytochemicals were purified using ultrahigh-performance liquid chromatography (UHPLC; Thermo Scientific model Accela) using a C18 column (C18 Agilent Eclipse Plus, 4.6 × 100 mM, 3.5 µm). A regulated temperature of 35 ºC was used for the column. The mobile phases A and B are 0.1% acetic acid in water and acetonitrile, respectively. The 500 µL/min is used as the flow rate of the separating. The purification time program was adjusted to the gradient of mobile phases A and B. Electrospray Ionisation (ESI) Mass Spectrometry (Thermo Scientific Surveyor MSQ Plus) was used to assess the mass of purified peaks. The MS condition was performed with the probe temperature at

450 ºC, the needle at 4.5 kV, and the negative ESI ionisation mode. The mass/charge was then scanned at 100 - 1000 m/z. The ion mass profile was examined in the database or library (Table 1).

Table 1
The gradient conditions of liquid chromatography

No.	Times	Mobile Phase		Flow Rate (µL/min)
		A%	B%	
0	0	15	85	500
1	5	25	75	500
2	10	35	65	500
3	28	60	40	500
4	28.01	15	85	500
5	35	15	85	500

Determination of Total Phenolic Contents

Phenolics are significant phytochemicals that are used for the survival of plants. Polyphenolic compounds represent various biological activities. The Folin-Ciocalteu test was utilised to examine the total phenolic contents in accordance with the previously mentioned methodology (Kozlowskal et al., 2021; Moolwong et al., 2023). The reaction was performed by subjecting 2% w/v Na_2CO_3 solution to approximately 3.50 mL in a test tube. The extracts at different concentrations (100 µL) were added and mixed gently. The mixture was subjected to a tenfold dilution of Folin reagent (400 µL). The reactions were incubated for 30 minutes in the dark at room temperature. After that, the absorbance value was measured at 750 nm by a spectrophotometer (Thermo Science, USA). In this experiment, gallic acid was used as the standard solution. The total phenolic contents of each extract were calculated.

Statistical Analysis

The proportion of the extracts' enzymatic inhibitory effect, antioxidant activity, and nitric oxide scavenging activities were computed and presented as the mean and standard deviation (mean ± SD). The ANOVA test (IBM SPSS Statistics ver. 29.0, IBM Corp., Armonk, NY, USA) was utilised in this study to analyse the cytotoxic data. The *P values* for all data points were below 0.05 (*), signifying statistically significant differences.

RESULTS AND DISCUSSIONS

In this report, *S. pinnata* fruit extracts were investigated for biological properties in line with the development aims, such as applications in functional foods/nutraceuticals, food

supplements, and as an ingredient to prevent biomolecule degradation in health care products. The data for each biological property, including phytochemical types and total phenolic contents, are represented below.

The Inhibitory Effect of Unripe *S. pinnata* Fruit Extracts on Tyrosinase Enzyme

The antityrosinase activity results of unripe *S. pinnata* fruit extracts are shown in Figure 2. According to the results, the three unripe *S. pinnata* fruit extracts can inhibit the tyrosinase enzyme activity in vitro. The effect of antityrosinase activity was expressed as dose-dependent. The IC_{50} value of WRA-1, WRA-2, and WRA-3 was exhibited at 0.55, 0.95, and 0.65 mg/mL, respectively. The data indicated that WRA-1 showed a more substantial tyrosinase inhibitory effect than WRA-2 and WRA-3.

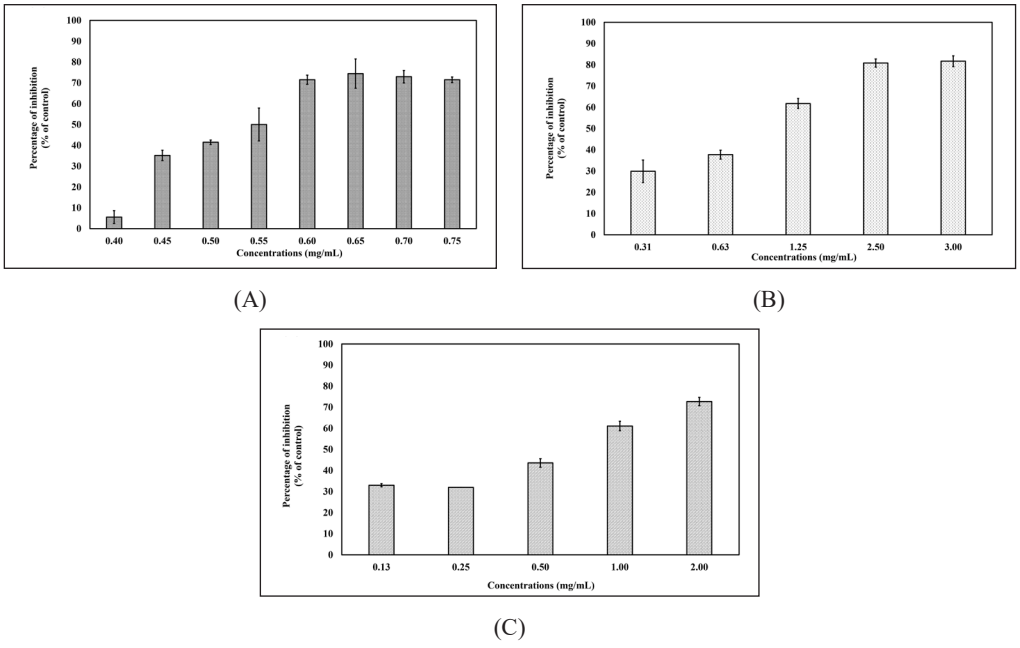


Figure 2. Antityrosinase activity of unripe *S. pinnata* fruit extracts. (A) WRA-1, (B) WRA-2, and (C) WRA-3. All samples were tested in triplicate (n = 3), and the results are presented as percentage inhibition (% inhibition) as the mean ± standard deviation (SD)

Anti-Elastase Activity

Elastase is an enzyme that can digest the protein in the extracellular matrix of cells, especially elastin protein. Therefore, elastase is the crucial enzyme associated with skin wrinkling. The elastase inhibitory effect of raw wild mango fruit extracts was expressed in Figure 3. The data indicated that the bioactive compounds of WRA-1

extract exhibited higher potential anti-elastase activity than WRA-2 and WRA-3. The IC_{50} value of WRA-1 was less than 0.25 mg/mL, while the IC_{50} dose of WRA-2 was approximately 2 mg/mL. However, WRA-3 showed lower elastase-inhibitory activity than WRA-2 and WRA-1. In addition, the results related to the tyrosinase inhibitory effect indicated that the WRA-1 extract exhibited an IC_{50} dose smaller than WRA-2 and WRA-3.

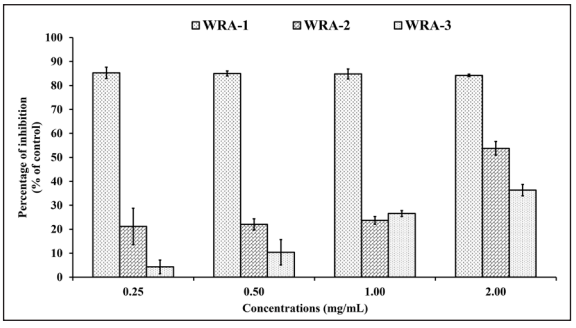


Figure 3. Elastase inhibitory activity of unripe *S. pinnata* fruit extracts. All samples were tested in triplicate (n = 3), and the results are presented as percentage inhibition (% inhibition) as the mean ± standard deviation (SD)

Antioxidant Activity of WRA-1, WRA-2, WRA-3 Extracts

The induction of oxidative stress by free radicals plays a significant role in aging. Many molecules are destroyed, which represents oxidative damage. Moreover, reactive oxygen species can activate the elastase, cellulase, and hyaluronidase activities. These enzymes can hydrolyze elastin, cellulose, and hyaluronic acid, promoting skin aging. Thus, the antioxidant activity and inhibition of these enzymes are the target goals for preventing cell aging. In this report, the antioxidant activity of extracts was analysed using a DPPH assay. Ascorbic acid and gallic acid served as reference antioxidant agents for comparison with the extracted samples. The results are expressed in Figure 4. The IC_{50} of each extract was calculated and compared with those of the standard antioxidants, namely ascorbic acid and gallic acid. WRA-1 ($y = 9.4372x + 33.55$, $R^2 = 0.9962$), WRA-2 ($y = 5.3494x + 5.822$, $R^2 = 0.9925$), and WRA-3 ($y = 10.993x + 2.447$, $R^2 = 0.9935$) exhibited IC_{50} values of 1.74, 8.26, and 4.43 µg/mL, respectively. By comparison, the IC_{50} of gallic acid ($y = 2.7307x + 4.439$, $R^2 = 0.9939$) and ascorbic acid ($y = 9.397x$, $R^2 = 0.9967$) were 16.68 and 5.32 µg/mL, respectively. These data indicate that the IC_{50} of WRA-1 was lower than that of WRA-2, WRA-3,

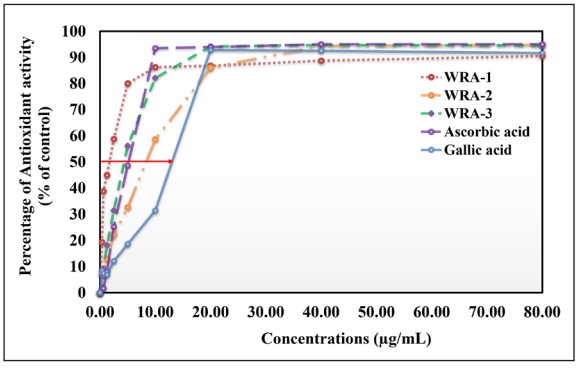


Figure 4. DPPH radical-scavenging activity of unripe *S. pinnata* fruit extracts. L-Ascorbic acid and gallic acid were used as positive controls for comparison with the extracts. All samples and positive controls were tested in triplicate (n = 3), and the results are presented as mean ± standard deviation (SD)

ascorbic acid, and gallic acid. This finding suggests that the compounds present in WRA-1 exhibited good efficacy in scavenging free radicals in vitro, with a higher capacity than the other extracts and standard antioxidant agents tested.

Cytotoxicity of Unripe *S. pinnata* Fruit Extracts

CCA is a specific localised carcinoma associated with prolonged and continuous parasite infection. Secretions and toxins produced by *O. viverrini* affect the biomolecules in the cells at the site of helminth infection. Individuals living in the freshwater basin and living an uncooked lifestyle are primary and secondary aquatic *O. viverrini* carriers. Their behavior and culture make them susceptible, and they have a higher rate of CCA detection than urban populations. CCA has few symptoms and occurs in specific locations, causing CCA to be detected in the invasion and migration stage. Reducing risk or preventing CCA is associated with various factors, especially food quality and appropriate public health systems among at-risk groups (Wasuworawong et al., 2015).

Functional foods/nutraceuticals are a factor in a relationship hypothesis that reduces risk and prevents human disease development, impacting the public health system. This report studied the *S. pinnata* fruit extracts from three solvent systems for their cytotoxic effect on cholangiocarcinoma (MO55 cells) and compared them with normal bile duct cells (MMNK1 cells). This experiment aims to analyse the feasibility of essential substances in the extracts that showed the role of selective ability between cancer cells and normal cells. The study results on the anticancer properties of raw *S. pinnata* fruit extracts on MO55 and MMNK1 cells are exhibited in Figures 5 and 6. The morphological changes of MO55 cells and the percentage of cell viability indicated the cytotoxic effect of *S. pinnata* fruit extracts. The MO55 cell density treated with unripe *S. pinnata* fruit extracts was lower than that of the control condition (untreated). This result is related to the percentage of MO55 cell viability that decreased after incubation with the tested extracts. These extracts have a cytotoxic impact that is dose- and time-dependent. The IC_{50} of WRA-1, WRA-2, and WRA-3 represented 0.58, 1, and 2.1 mg/mL, respectively. These data indicated that WRA-1 displayed a higher cytotoxic effect on MO55 cells than WRA-2 and WRA-3. In addition, the cytotoxic effect of unripe *S. pinnata* fruit extracts was compared between cholangiocarcinoma (MO55 cells) and normal bile duct cells (MMNK1 cells). The results showed that the percentage of MMNK1 cell viability similarily expressed with MO55 cells. However, the IC_{50} of WRA-2 and WRA-3 in MMNK1 is higher than what was found in MO55 cells (Table 2). The results suggest that the phytochemicals in WRA-2 and WRA-3 may be selectively cytotoxic, leading to lower cytotoxicity toward normal cells than cancer cells.

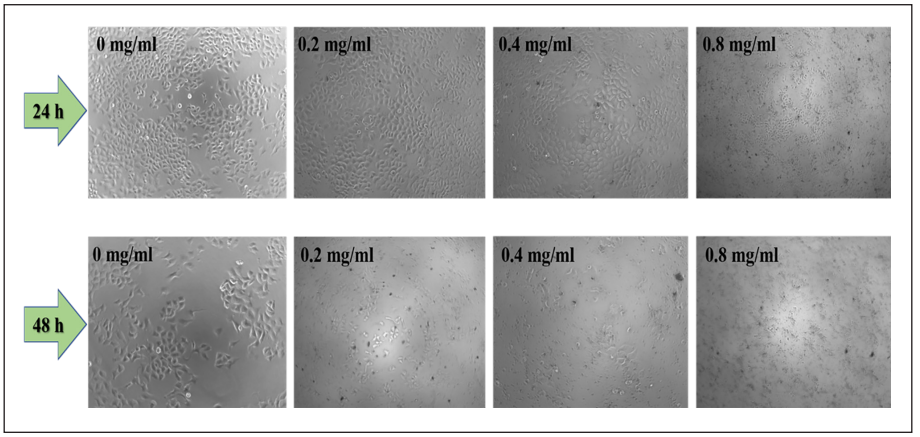
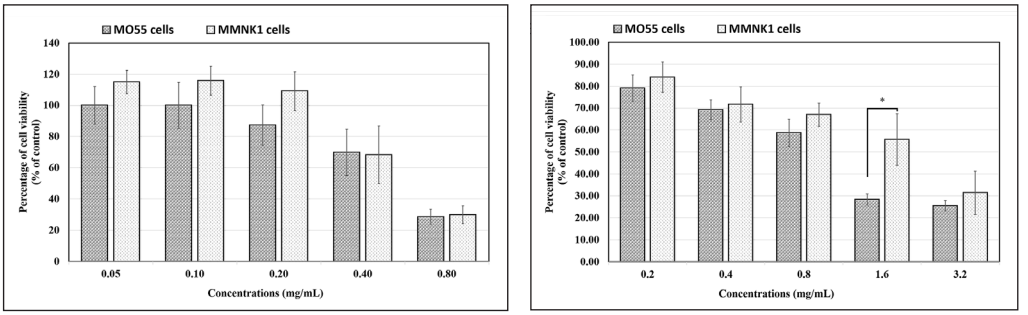


Figure 5. Morphological changes in MO55 cells following treatment with unripe *S. pinnata* fruit extract (WRA-1) for 24 and 48 h



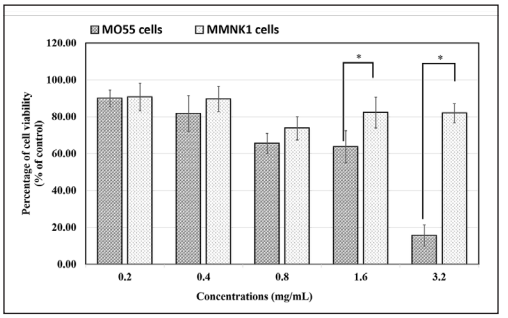


Figure 6. Comparison of cell viability (%) between MO55 and MMNK1 cells following treatment with unripe *S. pinnata* fruit extracts for 48 h. (A) WRA-1, (B) WRA-2, and (C) WRA-3. All samples were tested in triplicate (n = 3), and the results are presented as mean ± standard deviation (SD)

Table 2
The IC_{50} for cytotoxicity, antioxidant, and antityrosinase activities, including total phenolic content of all extracts (WRA-1, WRA-2, WRA-3)

Extracted ID	IC_{50} of Cytotoxicity (mg/mL)		IC_{50} of Antioxidant Activity (µg/mL)	IC_{50} of Antityrosinase Activity (mg/mL)	Total phenolic Contents (mg GAE/g extract)
	MO55 cells	MMNK1 cells			
WRA-1	0.58	0.57	1.74	0.55	26.35 ± 4.89
WRA-2	1.00	1.98	8.26	0.95	49.77 ± 3.81
WRA-3	2.10	> 3.20	4.43	0.65	31.91 ± 1.52

Total Phenolic Contents

The Folin reagent investigated the total phenolic contents of unripe *S. pinnata* fruit extract. The standard curve of gallic acid ($y = 11.202x$, $R^2 = 0.9954$) was used to calculate the total phenolic contents of the extracts. The results showed that the total phenolic contents of WRA-1, WRA-2, and WRA-3 were 26.35 ± 4.89, 49.77 ± 3.81, and 31.91 ± 1.52 mg GAE/g extract, respectively. The results indicated that the total phenolic contents of WRA-3 were higher than those of WRA-2 and WRA-1. These data showed that the high polarity of the extracted solvent can collect the phenolic compounds. Interestingly, the phenolic contents are not associated with the cytotoxicity level in MO55 cells. The potential biological activities in WRA-1 may be related to various bioactive agents. As a result, several kinds of bioactive substances in WRA-1 have been researched.

Phytochemicals in WRA-1, WRA-2, and WRA-3

The phytochemical screening results of WRA-1, WRA-2, and WRA-3 are shown in Table 3. Polyphenols/phenolic, flavonoids, flavones, terpenoids, tannins, and cardiac glycosides are phytochemical types found in these extracts. Only saponins were not detected in WRA-1. The detection of similar phytochemicals might be related to the solvent system used, which has a ratio of organic solvent mixed with water (70:30). The biological properties represented in unripe *S. pinnata* fruit extracts may be associated with these chemicals.

Table 3
Phytochemical screening data of unripe S. pinnata *fruit extracts*

Type of Phytochemical	WRA-1	WRA-2	WRA-3
Phenolic compounds	+	+	+
Flavonoids	+	+	+
Flavone	+	+	+
Leucoanthocyanidin	-	-	-
Terpenoid	+	+	+

Table 3 (*continued*)

Type of Phytochemical	WRA-1	WRA-2	WRA-3
Tannin	+	+	+
Saponin	-	+	+
Steroids	-	-	-
Cardiac glycoside	+	+	+

Moreover, WRA-1 represented a small concentration of IC_{50} in antioxidant and antityrosinase activities. Therefore, GC-MS and LC-MS were used to study the group of active compounds in WRA-1. 5-hydroxymethylfurfural (5-HMF) is represented in the GC-MS report. This compound has the molecular formula $C_6H_6O_3$. The match factor and Reverse Match Factor (RMF) presented in 891 indicate that the mass spectrum of the substance matches the database at a good to very good level. In addition, the program estimated the probability of identifying the substance at 94.9%, which supports that the identification of this substance is highly reliable. However, analysis with GC-MS and checking for similarities in these values is only a tentative identification. It's still necessary to have methods that help strengthen the data and make it more academically consistent.

LC-MS analysis of the sample revealed substance peaks at 0.4, 2.35, 2.98, 10.24, 10.43, 10.63, and 22.5 minutes. Of these, three peaks, at 2.35, 10.24, and 10.43 minutes, could be analysed further. LC-MS analysis in negative ion mode $[M-H]^-$ revealed the mass of each of these three peaks, as shown in Figure 7(B)-(D). Upon comparison with database records, the chromatographic profiles showed similarities to gallic acid, verbascoside, and isorhamnetin-3-rutinoside, respectively.

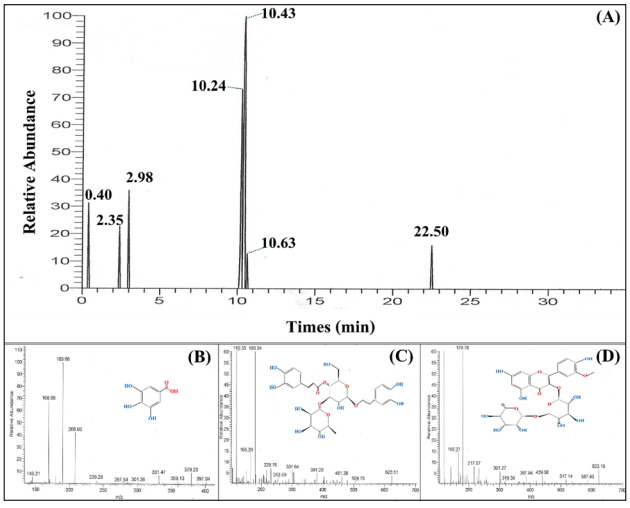


Figure 7. Purification chromatogram of WRA-1 obtained using C18-HPLC. (A) Chromatographic profile; (B- D) mass spectra of $[M-H]^{-1}$ corresponding to peaks with retention times of 2.35, 10.24, and 10.43 min, respectively

Drug discovery has long used natural products (NPs) as a source. Phytochemicals are being developed in various forms, including medicines, functional foods, and ingredients for topical products, with the primary goal of reducing human health problems (Nakadate et al., 2026).

In this study, phytochemicals were extracted using three solvent systems: (1) acetone-water, (2) ethanol-water, and (3) methanol-water, each at a 70:30 (v/v) ratio. The solvent systems (1) and (3) are similar to the study by Satpathy et al. (2011). However, this research added solvent system (2) to provide more comparative data on the efficiency of extracts from all three solvent systems. An important sample preparation technique is the combined use of mechanical and chemical extraction methods, which enhances the extraction of key compounds from raw materials. In addition, concentration involves a combination of two technologies. The first technology is evaporation, a method for removing organic solvents, followed by freeze-drying to remove the aqueous components from crude extracts. Using both technologies together helps reduce residual organic solvents in the extracts and preserves heat-sensitive compounds that cannot withstand high temperatures.

The results of this study show that the extracts WRA-1, WRA-2, and WRA-3 inhibit tyrosinase and elastase, act as antioxidants, and are cytotoxic to cancer cells. WRA-1 showed the highest overall bioactivity. Analysis of the compounds in WRA-1 identified 5-hydroxymethyl-2-furfural, gallic acid, verbascoside, and isorhamnetin-3-rutinoside.

Gallic acid and its derivatives are phenolic compounds. They are found in the extracts of various plants, including those of the genus *Spondias*. Previous research suggests that gallic acid exhibits antioxidant, antimicrobial, anti-inflammatory, and anticancer properties (Sameh et al., 2018), as well as antityrosinase activity (Patathananone et al., 2019a). The antioxidant mechanism of gallic acid involves transferring hydrogen atoms to free radicals (Hassanpour et al., 2023). Oxidative stress refers to the excessive production of free radicals within cells. These radicals damage biomolecules, causing them to degrade and lose their function. Therefore, reducing the quantity of free radicals is important for slowing the degeneration of biomolecules, thereby decreasing the risk of certain diseases, especially cancer (Kumpunya et al., 2014).

Verbascoside is a polyphenolic compound. It is a heterotrimer consisting of a catechol unit, glucose, and caffeic acid that is found in varieties of plant species. Several in vitro and in vivo studies have indicated that it exhibits neuroprotective, analgesic, antihypertensive, anti-inflammatory, and anti-tumor properties and inhibits protein kinase C (PKC) signaling pathways in the rat brain (Das et al., 2016).

Isorhamnetin and its derivatives (isorhamnetin-3-rutinoside) are secondary metabolites from various plants (Gómez-López et al., 2021). The flavonoid family, particularly the flavonol family, categorises these substances. Researchers have reported several biological activities of isorhamnetin, including antidiabetic, anti-inflammatory, antiproliferative, antioxidant, and antiobesity properties (Gong et al., 2020).

Tyrosinase (EC.1.14.18.1) is an enzyme found in many different living organisms. This enzyme is classified in class 1 of the oxidoreductases (monophenol, o-diphenol: oxygen oxidoreductase). In humans, the enzyme tyrosinase speeds up the oxidation of tyrosine to produce L-Dopa (3-(3',4'-dihydroxyphenyl)-L-alanine; L-Dopa), which then gets converted into dopaquinone. This dopaquinone goes through several reactions and eventually turns into melanin (Bae et al., 2013; Kim et al., 2016).

Although melanin helps protect DNA in skin cells from UV radiation and scavenges reactive oxygen species (ROS) generated by oxidative reactions, hyperpigmentation leading to melasma can negatively affect a person's appearance and self-confidence. Abnormal tyrosinase activity may be associated with several skin disorders, such as vitiligo, albinism, hyperpigmentation, and melanoma. Tyrosinase may also contribute to the toxicity of dopaminergic neurons and to neurodegeneration, which are implicated in Parkinson's disease (Chen, 2020). Therefore, inhibiting tyrosinase activity may be a strategy for preventing or slowing the progression of the conditions associated with excessive tyrosinase activity described above. This study shows that the phytochemicals in unripe *S. pinnata* fruit extracts (WRA-1, WRA-2, and WRA-3) inhibit tyrosinase in vitro, with WRA-1 having the lowest IC_{50} value of the three extracts and therefore the greatest inhibitory potency. However, in comparison with the positive control, kojic acid (IC_{50} = 0.03 mg/mL), all three crude extracts had significantly higher IC_{50} values, indicating lower tyrosinase-inhibitory potency. The tyrosinase inhibition activity of WRA-1 is related to the detected compounds 5-hydroxymethyl-2-furfural and gallic acid, which have been reported to have in vitro tyrosinase inhibitory properties (Patathananone et al., 2019a; Sameh et al., 2018). In addition, verbascoside and isorhamnetin-3-rutinoside may inhibit tyrosinase, and further studies will be conducted to test this hypothesis.

Elastases (EC 3.4.21.36) are a class of serine proteases with a broad range of substrate specificity that break down elastin (Famutimi et al., 2024). Abnormal expression of elastase is related to ROS free radicals (Patathananone et al., 2019a). Preventing elastin degradation may help slow skin aging (Garg, 2017). Moreover, elastase inhibition is associated with reduced cancer cell invasion and migration. Studying elastase inhibitors from plant sources may have applications in cosmetics and health care, as well as in strategies to reduce the risk of metastasis in certain cancers. Previous reports show that phenolic compounds such as catechin, epicatechin, procyanidin B2, and resveratrol, including flavonoid groups (for example, quercetin, kaempferol, and myricetin), have been reported to have high efficacy for anti-elastase activity (Pientaweeratch et al., 2016; Wasuworawong et al., 2015). The elastase inhibition results were consistent with those for tyrosinase, showing that WRA-1 has strong potential to inhibit elastase activity. The compounds detected in WRA-1 (5-hydroxymethyl-2-furfural, gallic acid, verbascoside, and isorhamnetin-3-rutinoside) may contribute to its in vitro inhibition of elastase activity. However, this study is preliminary.

Further research is needed to characterise the types and classes of compounds to elucidate the mechanism of elastase inhibition.

Free radical molecules contribute to the progression of cancer cells in the human body. Reactive oxygen species (ROS), particularly free radicals, trigger the oncogenic phenotype of cancer cells. Many factors, including an imbalance in the antioxidant system, contribute to the increasing number of free radicals. Therefore, antioxidants are important for reducing oxidative stress, which can help decrease the risk of developing various non-communicable chronic diseases, especially the risk of cancer (George et al., 2017). The results of this research indicated that phytochemicals in *Spondias* fruit exhibited good antioxidant potential. The IC_{50} of these extracts was detected at low concentrations, especially in WRA-1. The antioxidant potential present in WRA-1 may be related to the detected phytochemicals (5-hydroxymethyl-2-furfural, gallic acid, verbascoside, and isorhamnetin-3-rutinoside), which have previously been reported to show effective antioxidant properties in vitro (Das et al., 2016; Gong et al., 2020; Gómez-López et al., 2021; Hassanpour et al., 2023; Hazra et al., 2008; Sameh et al., 2018).

The cytotoxicity and selective properties of phytochemicals, which differ between cancer and normal cells, are targets for drug development research. Drugs with non-specific effects on cell groups can cause severe side effects for patients, so improving drug delivery efficiency is important for reducing these risks (Siegel et al., 2019). Interestingly, the data from this study showed that the group of phytochemicals in the WRA-3 sample displayed significantly less cytotoxicity in normal cells compared to cancer cells. This is different from WRA-1 and WRA-2, which were toxic to both cancer and normal cells at similar IC_{50} values. Based on these findings, the selective cytotoxicity of WRA-3 provides positive information for further research and development of drugs or drug delivery compounds that could be specifically targeted to certain cells in the future (Yolande et al., 2018).

CONCLUSION

Hog plum fruits in the *Spondias* genus are a source of bioactive compounds with important and diverse biological properties, like inhibiting tyrosinase and elastase enzymes, antioxidant effects, and cytotoxicity against cancer cells. Phytochemicals from *Spondias* fruits therefore have potential to be used as substitutes or alternatives in nutraceuticals and functional foods, and could also be used as ingredients in skincare products in the future.

ACKNOWLEDGEMENT

The authors would like to express their sincere gratitude to the National Research Council of Thailand (NRCT) for financial support under Grant No. 6575, which supported the initial stage of this research.

The authors also wish to thank all individuals and organisations who contributed to this study. Special appreciation is extended to the Faculty of Science and Technology, Rajamangala University of Technology Thanyaburi, Thailand, for providing the research facilities and technical support necessary to conduct this work.

The authors gratefully acknowledge the valuable assistance and constructive suggestions provided by colleagues and laboratory staff throughout the research process. Their expertise and support are sincerely appreciated.

The authors would like to express their sincere gratitude to Associate Professor Doctor Banchob Sripa (Khon Kaen University, Khon Kaen, Thailand). These cell lines were maintained and constructed as previously described by Wasuworawong et al. (2015).

Finally, the authors would like to thank the anonymous reviewers and the Editor for their insightful comments and constructive recommendations, which have significantly improved the quality of this manuscript.

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