Analyzing the Antibacterial, Anticancer, and Antioxidant Qualities of the Stink Bean (*Parkia Speciosa*) by Various Extraction Techniques

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Abstract

In various Southeast Asian countries, people have long incorporated the young green seeds of *Parkia speciosa*, commonly known as stink beans, into their culinary traditions. These seeds can be prepared using various cooking methods, including boiling, microwaving, stir-frying, or even consuming them raw. While several studies have explored the pharmaceutical properties of raw stink beans, this study seeks to expand our knowledge of how different extraction methods can influence the release of bioactive compounds from these young stink bean seeds. Three distinct extraction methods, namely (1) microwave-assisted water extraction, (2) boiling water extraction and (3) 50 % ethanol extraction were employed in this research. The study focused on total phenolic content and antioxidant activities, with the 50 % ethanol extraction method yielding the most promising results. Moreover, only the extract obtained from the 50 % ethanol method exhibited antimicrobial activity against 4 gram-positive bacteria, including Methicillin-Resistant *Staphylococcus aureus*, *Staphylococcus aureus*, *Bacillus cereus*, *Micrococcus luteus* and 1 gram-negative bacterium, *Salmonella typhi*. Notably, extracts obtained from the 3 extraction methods of young stink bean seeds exhibit varying degrees of inhibition of cancer cell growth with low cell toxicity. Taken together, our findings suggest that distinct extraction methods play a key role in obtaining different sets of bioactive compounds from the young seeds of stink beans. Therefore, various cooking methods, including stir-frying, microwaving and pickling in alcohol, may offer diverse sources of bioactive compounds for promoting consumer health.

Keywords: Stink bean, *Parkia speciosa*, Microwave-assisted water extraction, Boiling water extraction, Biochemical properties

Introduction

The stink bean, locally known as "Sator" (*Parkia speciosa* Hassk.) is one of the popular vegetables in the southern part of Thailand [1]. The stink bean is a native plant in tropical rainforest regions including Indonesia, Malaysia, the Philippines and Thailand [2]. In Thailand, the stink bean has gained popularity, particularly in the southern part, reminiscent of its prevalence in neighboring Malaysia. When the stink bean pod is still young, it is characterized by an elliptical and flat shape, exhibiting a vibrant green color. Harvesting typically begins when the pod is still young and the seeds are fully developed but not yet ripened. Despite the pungent smell and distinctive taste, many individuals in the southern part of Thailand have developed a fondness for consuming stink bean seeds. These seeds are commonly eaten raw, cooked, or pickled [1,3].

Beyond being a source of protein, fat and carbohydrates [4,5], stink bean seeds offer various health benefits, including antioxidant properties [5-8], potential anticancer effects [9-11], anti-inflammatory attributes, antimicrobial [12,13] and antihypertensive [14]. A number of bioactive compounds have been identified in various parts of the stink bean. For example, the sulfur-containing compound, thiazoline-4-carboxylic acid, gives rise to the pungent smell in stink bean seeds [15]. A high quantity of total phenolic

has been determined in stink bean seeds and pods [16]. Phenols contain varying numbers of hydroxyl groups that are harmful to microorganisms. Therefore, plant extracts that are rich in compounds in phenolics also exhibit antimicrobial activity [17,18]. Also, the hydroxyl groups from phenolic compounds provide antioxidant activity [19-21].

However, the extraction methods and the length of extraction play a crucial role in determining the composition of various bioactive compounds found in plant extracts [22-24]. Some active compounds exist in a free form, while others are in the form of protein-bound or carbohydrate-bound structures [25-27]. Relying solely on solvents may not be sufficient to dissociate the bound forms of bioactive compounds, leading to an underestimation of the total bioactive compounds in the plant extract. As a result, the bioactive properties of compounds, such as antioxidant, anticancer and antibacterial activities, may also be underestimated.

Both enzymatic [28-30] and nonenzymatic methods have been employed to maximize the extraction of bioactive compounds from plant extracts. Physical methods, such as heat [31,32] microwave [33,34] and ultrasound [35,36], can accelerate the separation of bioactive compounds, such as phenolics, from protein- or carbohydrate-bound molecules. Heat, in the form of boiling and microwave treatments, has been utilized to extract various plant compounds, aiming to achieve a higher yield of bioactive compounds. Therefore, this study aims to investigate the efficiency of 3 extraction methods (1) microwave-assisted water extraction, (2) boiling water extraction and (3) 50 % ethanol extraction, in extracting bioactive compounds from young stink bean seeds, including antioxidant, antimicrobial and anticancer activities.

Material and methods

Sample preparation
Fresh stink bean seeds were harvested from Amphoe Mueang, Narathiwat province, Thailand. Stink bean seeds were collected from seed pods and subsequently rinsed with distilled water. Following this, all the fresh stink beans were thoroughly dried in an oven at 70 °C for 5 days. The dried stink bean seeds were ground into a fine powder. These ground stink beans were partitioned into 3 portions, each undergoing a distinct extraction method; microwave-assisted water extraction, (2) boiling water extraction and (3) 50 % ethanol extraction.

Microwave-assisted water extraction and boiling water extraction
A total of 20 g of ground stink bean seed was extracted in 100 mL of distilled water. For the boiling water extraction, the ground stink bean seeds were mixed with water and subjected to incubation in a water bath at 95 °C for 1 h [37]. In the case of microwave-assisted water extraction, the ground stink bean seeds were blended with water and exposed to microwave at 750 W for 2 min, followed by a cooling interval of 10 min before undergoing another 2 rounds of microwave treatment at the same power setting (750 W) [38]. The resulting extracts for each method were then filtered through the Whatman filter paper. Subsequently, the water content in the remaining portion of the extracts was removed using a freeze-drying technique. The samples were weighted and stored at –20 °C for further analyses.

Ethanol extraction
A total of 20 g of ground stink bean seed was dissolved in 100 mL of 50 % ethanol. The resulting extracts were continuously mixed using a vortex mixer for 1 h. The supernatants of the extract were separated by centrifugation at 4,500 rpm for 10 min and subsequently filtered through Whatman filter paper. The remaining supernatants were evaporated using a rotary evaporator under reduced pressure at 45 °C. The water content in the remaining extracts was further removed through freeze-drying. The resulting samples were weighted and stored at –20 °C for subsequent analyses.

Total phenolic contents
To determine phenolic contents, a 100 mg extracted sample was dissolved in 1 mL of 70 % ethanol with regular shaking for 2 h at 4 °C. Total phenolic content (TPC) was assessed using a 96-well microplate format. Each reaction consisted of 20 μL of either (1) 70 % ethanol as a blank or (2) various concentrations of gallic acid (Merck Co., Germany) as a standard or (3) the extracts. Subsequently, 100 μL of 10 % Folin-Ciocalteu reagent (Sisco Research Laboratories, India) and 80 μL of 7.5 % Na_2CO_3 were added. The reaction was incubated at room temperature for 1 h. The TPC was determined by measuring the absorbance of 765 nm [39]. The TPC was displayed as mg of Gallic acid equivalents (GAE) per g of dry weight.

Antioxidant activities
DPPH free radical scavenging activity
To assess free radical scavenging activity, a 100 mg extracted sample was dissolved in 1 mL of 70 % ethanol with regular shaking for 2 h at 4 °C. DPPH assay was conducted using a 96-well microplate format. Each well contained 190 µL of DPPH (Sisco Research Laboratories, India) and 10 µL of either (1) 70 % ethanol as a blank or (2) various concentrations of Trolox as a standard or (3) the extracts. A total volume of DPPH assay (200 µL) was incubated at room temperature for 1 h. The optical density, measured at the absorbance of 517 nm [40], was used to monitor the percentage of inhibition of the DPPH assay. The calculation is as follows:

$$\text{Inhibition of DPPH (\%)} = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100$$

The scavenging capacity of DPPH was expressed as Trolox equivalent antioxidant capacity in mg Trolox per g^{-1} DW.

ABTS free radical scavenging activity
To examine the free radical scavenging activity through the ABTS assay [41], a working solution of $ABTS^{\bullet+}$ was prepared by combining 40 mM potassium persulfate ($K_2S_2O_8$) with 7 mM ABTS. This working solution was stored at 4 °C for 16 h before use. To obtain the working solution, the $ABTS^{\bullet+}$ was diluted with 50 mM phosphate buffer (pH 7.4) until the absorbance at 734 nm approached 0.7 optical density (OD). Extracts from 100 mg were dissolved in 1 mL of 50 mM phosphate buffer (pH 7.4) with regular shaking for 2 h. The $ABTS^{\bullet+}$ assay was conducted using a 96-well microplate spectrophotometer. In each well, 180 µL of the working $ABTS^{\bullet+}$ solution was mixed with 20 µL of either (1) 50 mM phosphate buffer (pH 7.4) as a blank or (2) various concentrations of Trolox as a standard or (3) the extracts. All reactions were incubated at 30 °C for 6 min before measuring the OD at an absorbance of 734 nm. The calculation is as follows:

$$\text{Inhibition of ABTS (\%)} = \frac{Absorbance\ of\ control - Absorbance\ of\ sample}{Absorbance\ of\ control} \times 100$$

Ferric reducing antioxidant power (FRAP)
To examine the free radical scavenging activity through the FRAP assay [42], the FRAP reagent was prepared by combining 10 mL of 300 mM acetate buffer pH 3.6, 1 mL of 10 mM TPTZ in 40 mM HCl and 1 mL of 20 mM $FeCl_3$. A total of 150 µL of the FRAP reagent was added to a 96-well plate, along with 20 µL of either (1) distilled water as a blank or (2) various concentrations of Trolox as a standard or (3) the extracts. The reaction was incubated at 25 °C for 10 min in the dark before being monitored at the absorbance of 593 nm. The reducing power was calculated and reported in the form of mg Trolox Equivalent per g of dry weight.

Antibacterial activities
Bacteria growth condition
All the bacterial strains were obtained from Associate Professor Dr. Monthon Lertcanawanichakul, the School of Allied Health Sciences at Walailak University. Gram-positive bacteria, including Methicillin-Resistant *Staphylococcus aureus* (MRSA), *Staphylococcus aureus* (*S. aureus*), *Bacillus cereus* (*B. cereus*) and *Micrococcus luteus* (*M. luteus*) as well as the gram-negative bacteria; *Salmonella Typhi* (*S. Typhi*) were cultured in Luria Bertani (LB) agar at 37 °C for 18 - 24 h. Subsequently, a single colony from each bacterial strain was selected and cultured in liquid LB media at 37 °C with agitation at 200 rpm. After an overnight incubation, the turbidity of the bacterial culture was assessed by measuring the absorbance at 600 nm. The OD was adjusted by introducing 0.85 % NaCl to attain the 0.5 McFarland standard, which equated to a concentration of approximately 1.5×10^8 cfu/mL.

Agar well diffusion assay
Following the adjustment of the bacterial culture concentration, the bacteria were evenly spread across the entire LB agar plate. Small wells, each with a diameter of 6 mm, were created on LB agar. A volume of 100 µL of each extract was added to individual wells. The plates were left at 25 °C for 5 h to facilitate the absorption of the extract into the media. Subsequently, the plates were incubated at 37 °C for 18 h. The

inhibition zone was measured using a Vernier caliper. As a reference, 0.01 mg of gentamicin was used as a positive control, while sterile water served as the negative control.

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration assays

The inhibitory zone was measured through the agar well diffusion assay, where various concentrations of the extracts were introduced into the individual wells on agar plates. The concentration of extracts commenced at 300 mg/mL and was progressively diluted using distilled water, reaching a final concentration of 0.589 mg/mL. Various bacterial strains were subjected to testing and the size of the inhibitory zone was recorded. Three replicates were conducted. MIC was computed as the lowest concentration of extracts that still exhibited an inhibitory zone.

To find the minimum bactericidal concentration (MBC), the streak plate method was employed. The inoculum was taken from the inhibition zone of the minimum inhibitory concentration (MIC) with at least 2 dilutions. The inoculum was streaked onto a fresh solid LB agar medium. The agar plates were then incubated at 37 °C for 18 - 24 h. After incubation, MBC was determined by observing the results. If the crude extract can effectively kill the bacteria, the results show the absence of bacterial colonies. Therefore, the lowest dilution of the crude extract that can kill the bacteria represents the MBC. The experiment was repeated 3 times for validation.

Cell cytotoxicity
Cell culture

To evaluate the anticancer activity of young stink bean extracts using three methods (1. microwave-assisted water extraction, 2. boiling water extraction, and 3. 50 % ethanol extraction), four human cancer cell lines—A549 (lung), HT29 (colon), MCF7 (breast), and KKU-213A (bile duct)—were employed. Additionally, two normal cell lines, human dermal fibroblasts (HDF) and an immortalized human cholangiocyte cell line, MMNK-1, were used for comparison. All cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (Gibco, NY, USA) and 1 % antibiotics/antimycotics (Gibco, NY, USA), incubated at 37 °C with a humidified atmosphere and in a 5 % CO_2 incubator.

MTT assay

Healthy cells, at 70 - 80 % confluency, were trypsinized and seeded into a 96-well plate at a density of 3×10^3 cells/well, followed by overnight incubation. The following day, the old medium was replaced with a medium containing various concentrations of microwave-assisted water extraction, boiling water extraction and 50 % ethanol extraction, or with the medium containing DMSO used as controls. After a 72-hour treatment, the cells were subjected to the MTT assay. In brief, 10 µL of MTT stock solution (5 mg/mL) was added to each well and incubated for 2 h. Excess MTT solution was removed and the formazan crystal was solarized with 100 µL DMSO. The absorbance was measured at A540 on a microplate reader (Spark® multimode microplate reader, TECAN, Switzerland). The percentage of cell viability after treatment was calculated and compared.

Statistical analysis

Each experiment was conducted in 5 replicates unless otherwise specified. All data were assessed for normality using the Shapiro-Wilk test and tested for homogeneity of variance using the Bartlett test. Analysis of variance (ANOVA) and Turkey's Honestly Significant Difference (HSD) were employed for comparative analysis. Statistically significant differences were justified when the p-value was less than 0.05. The presentation of all data includes mean values ± standard deviation (SD).

Results and discussion

Total phenolic contents

Phenolics are uniquely described as a group of compounds containing an aromatic ring with one or several hydroxyl groups. When screening for bioactive compounds, researchers often prioritize the examination of total phenolic contents. This is because many phenolic compounds have been characterized and recognized for their medicinal properties, including uses as antioxidants [43], antibiotics [44], anticancer agents [45] and antidiabetics [46]. Therefore, this study examined the total phenolic contents in young stink bean seeds in various extraction methods. Three distinct extraction procedures were employed and the results indicate that 50 % ethanol extraction exhibited the highest phenolic content (75.03 ± 5.73

mg GAE g^{-1} DW) in comparison to microwave-assisted water extraction (54.38 ± 1.69 mg GAE g^{-1} DW) and boiling water extraction (48.25 ± 1.84 mg GAE g^{-1} DW) (**Figure 1**).

Our findings revealed a total phenolic content (TPC) comparable to that reported in a prior study focusing on the pods of 2 stink bean varieties: Sataw-Khao (71.39 ± 0.08) and Sataw-Dan (84.24 ± 0.12) [47]. Significantly, both studies employed 50 % ethanol extraction, aligning with the methodology utilized in our investigation. This suggests that the phenolic contents in stink bean seeds exhibit consistency, as both research groups demonstrated relatively similar results. Almost 30 % of phenolics are detected in a bound form [48], with some present in a free form. Therefore, we sought to release phenolics from bound proteins by employing heat through boiling and microwaving. In our study, however, it was shown that free phenolics were lower in the heat-extracting methods; microwave-assisted water extraction and boiling water extraction. Similar to previous studies [48,49] on heat exacting methods for phenolic contents, heat might not be a factor in releasing phenolic complex into a free form; instead, it may degrade them, and make them less available.
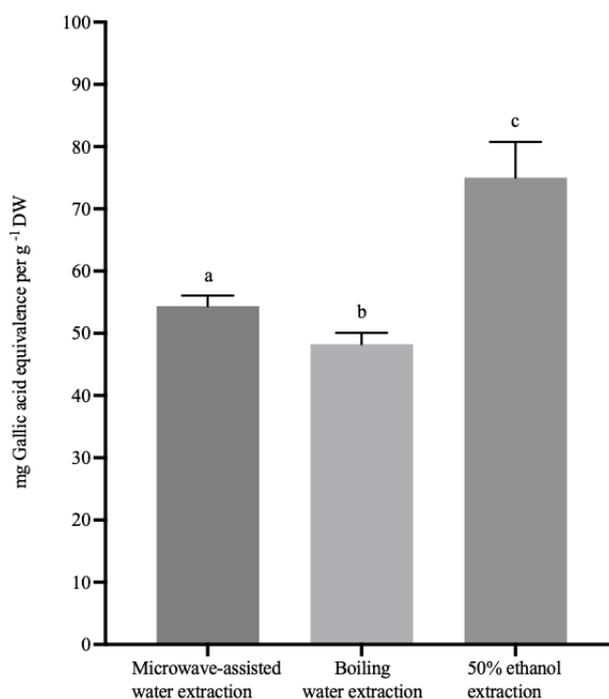

Figure 1 Total phenolic contents of young stink bean extract from three methods: Microwave-assisted water extraction, boiling water extraction and 50 % ethanol extraction. Error bars represent SD ± Mean and error bars with different letters indicate a significant difference ($p < 0.05$) from the ANOVA test with 5 replicates.

In vitro antioxidant activities.
DPPH-scavenging activity
In our studies, we employed the three most widely used colorimetric assays: DPPH, ABTS and FRAP assays for examining the antioxidant capacity.

The DPPH assay is a standard method for assessing the free radical scavenging capacity in plant extracts, favored for its simplicity. Various solvents like absolute methanol, 80 % methanol, absolute ethanol, or 70 % ethanol are commonly used. The assay relies on the conversion of stable purple DPPH to a clear yellowish color when it receives a hydrogen atom from antioxidants, leading to a reduction in absorbance at the 517 nm wavelength. The strength of the antioxidant capacity is determined by the concentration of the plant extract, compared to standard antioxidants like Trolox or L-ascorbic acid.

When three extraction criteria were compared, 50 % ethanol extraction (39.91 ± 1.94 mg TEAC g^{-1} DW) illustrated a significantly higher antioxidant capacity than microwave-assisted water extraction (25.96 ± 2.01 mg TEAC g^{-1} DW) and boiling water extraction (24.93 ± 2.05 mg TEAC g^{-1} DW) (**Figure 2(a)**). The results exhibited a similar trend to TPC, consistent with several studies [50-52] that have highlighted the correlation between the ability of phenolic compounds to act as effective hydrogen donors for free

radical molecules, such as DPPH. However, we are well aware that the DPPH assay is only limited to determining lipophilic antioxidant molecules or the less polar molecules. The results from the water-soluble assay of antioxidant capacity, namely, ABTS and FRAP would complement the limitation of the DPPH assay.
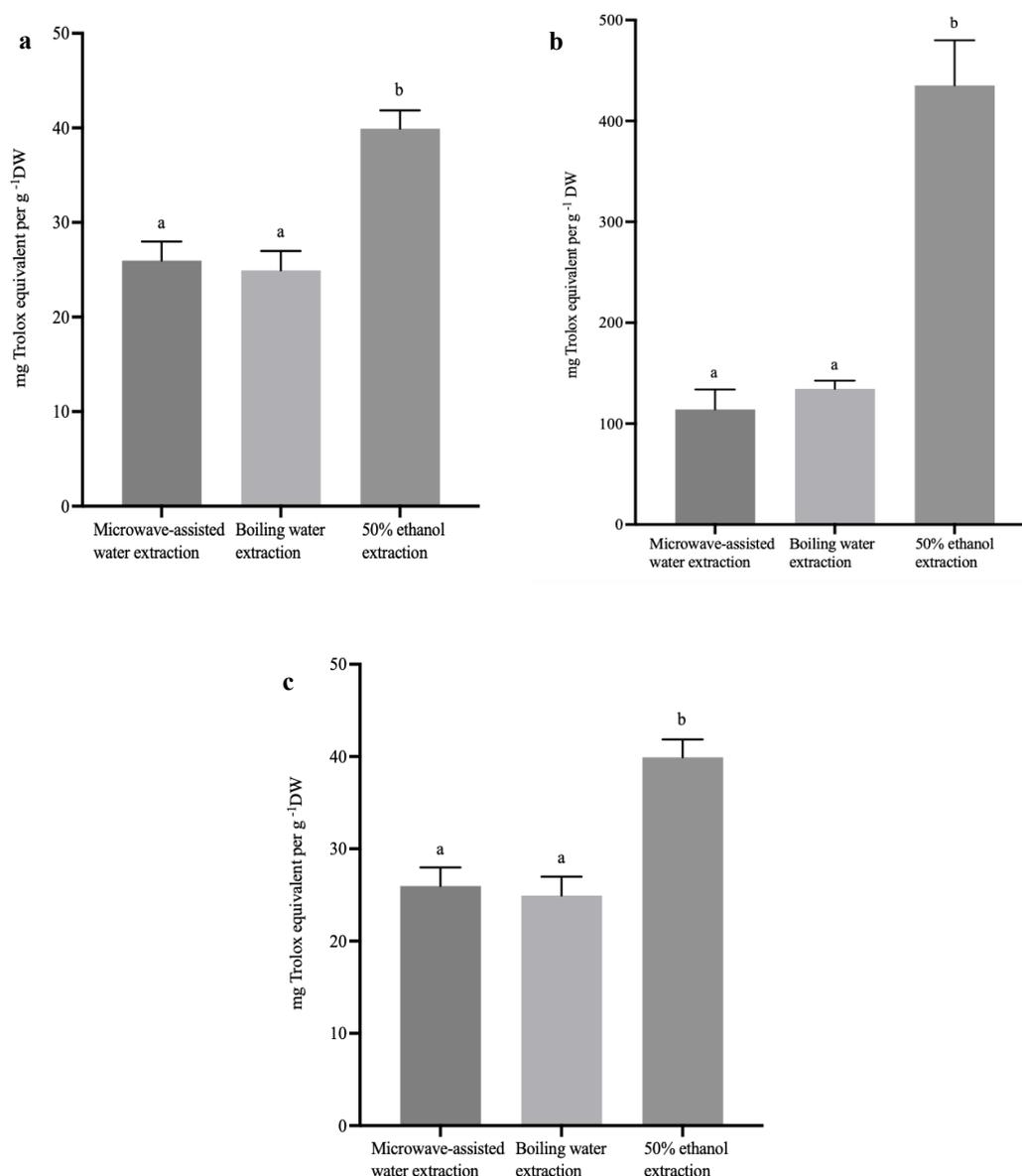

Figure 2 Antioxidant capacity of young stink bean extract from three methods: Microwave-assisted water extraction, boiling water extraction and 50 % ethanol extraction. (a) DPPH radical scavenging assay, (b) ABTS radical scavenging assay and (c) FRAP radical scavenging assay. Error bars represent SD ± Mean and error bars with different letters indicate a significant difference ($p < 0.05$) from the ANOVA test with 5 replicates.

ABTS scavenging activity

The ABTS$^{•+}$ assay is widely used for antioxidant capacity evaluation. It involves the decolorization of ABTS$^{•+}$ from dark blue to colorless when antioxidants reduce it to ABTS. The antioxidant capacity is determined by monitoring this decolorization. Similar to the DPPH assay, ABTS quantitatively measures the single electron transfer (SET) from plant extracts to convert ABTS$^{•+}$ to ABTS. This assay can be

conducted under various pH conditions; in our study, physiological pH (pH 7.4) was used to assess the antioxidant capacity of stink bean extract.

The three extraction methods of stink beans displayed the highest antioxidant capacity in 50 % ethanol extraction (435.14 ± 44.90 mg TEAC g^{-1} DW), with no significant difference between microwave-assisted water extraction (114.06 ± 19.86 mg TEAC g^{-1} DW) and boiling water extraction (134.50 ± 8.05 mg TEAC g^{-1} DW) (**Figure 2(b)**). In comparison to the DPPH assay, the antioxidant capacity determined by the ABTS assay was nearly 10-fold higher, suggesting that the majority of antioxidant compounds in stink beans are water-soluble. Similar to the DPPH assay, ABTS demonstrated a comparable trend of antioxidant capacity relative to the phenolic contents, as previously reported in stink beans [47].

Ferric reducing antioxidant power (FRAP) assay

FRAP assay was also employed in this study to determine the antioxidant capacity of stink bean seeds that are water-soluble at the acidic pH (pH 3.6). Similar to ABTS, FRAP assay is used to quantify the antioxidant capacity by measuring the appearance of blue color when ferric tripyridyltriazine complex (Fe^{3+}-TPTZ) is reduced to a ferrous (Fe^{2+}-TPTZ) form. The FRAP assay only focuses on the redox reaction with SET in the same manner as the ABTS assay. The results showed that 50 % ethanol extraction (22.38 ± 0.41 mg TEAC g^{-1} DW) produces the greatest antioxidant capacity among the 3 extraction criteria followed by the boiling water extraction (17.56 ± 0.93 mg TEAC g^{-1} DW) and the microwave-assisted water extraction (13.22 ± 0.611 mg TEAC g^{-1} DW) respectively (**Figure 2(c)**). Considering pH as a significant factor influencing the release and availability of free phenolics in extracts [53], the FRAP assay displayed a distinct pattern, showing higher antioxidant activity in microwave-assisted water extraction and boiling water extraction compared to the DPPH and ABTS assays. This variation could be attributed to the impact of lower pH in the FRAP assay on the availability of free phenolics in the extracts.

Antibacterial activity

With the emergence of numerous strains of pathogenic bacteria and the prevalence of bacteria-resistant strains, we are desperately seeking new antibiotics. A plethora of plant extracts by using different plant parts and using different criteria for extractions have been screened for antibacterial properties. In this study, we extracted bioactive compounds from stink bean seeds by employing 3 methods: (1) microwave-assisted water extraction, (2) boiling water extraction and (3) 50 % ethanol extraction. Four strains of gram-positive bacteria and 4 strains of gram-negative bacteria have been used to investigate the antibacterial properties. Gentamicin (0.1mg/mL) was used as a positive control and sterile distilled water used to dissolve stink bean seed extracts was employed as a negative control. Our results showed that only the extract of stink bean seeds that were extracted by 50 % ethanol inhibited the growth of 4 strains of gram-positive bacteria; MRSA, *S. aureus*, *B. cereus* and *M. luteus* and 1 strain of gram-negative bacteria; *S. typhi*. The minimum inhibitory concentration (MIC) assay showed that the highest inhibition zone was detected in MRSA (3.19 ± 0.09 mm), *S. aureus* (2.79 ± 0.11 mm), *B. cereus* (3.23 ± 0.16 mm), *M. luteus* (5.4 ± 0.36 mm) and *S. typhi* (1.23 ± 0.02 mm) respectively (**Table 1**). For the MBC which tests the minimum concentration that can inhibit bacterial growth, In the screening results, only stink bean seed extracts with a 50 % ethanol composition exhibited antimicrobial activity. Subsequently, we conducted MIC and minimal bactericidal concentration (MBC) tests specifically on the 50 % ethanol extracts. The findings indicated that the lowest MIC for MRSA, *S. aureus* and *B. cereus* was 18.75 mg/mL, with a consistent MBC of 900 mg/mL observed across all 3 bacterial strains. In the case of *M. luteus*, the MIC was 9.38 mg/ml, while the MBC was 150 mg/mL. For *S. Typhi*, the lowest MIC recorded was 300 mg/mL and the MBC exceeded 900 mg/mL, as presented in **Table 2**.

Similarly, 2 studies also presented the antimicrobial activity from stink bean seeds extracted by using absolute ethanol against *S. aureus*, *B. subtilis*, *E. coli*, *S. typhimurium*, *Pseudomonas aeruginosa* [54] and using 95 % against *S. aureus*, *Streptococcus agalactiae*, *Vibrio parahaemolyticus* and *Aeromonas hydrophila* [55]. Previous studies, coupled with the findings of this research, confirm that the extraction of young stink bean seeds using ethanol yields bioactive compounds capable of inhibiting the growth of a diverse spectrum of bacterial strains, encompassing both gram-positive and gram-negative types. These results serve as an impetus for future investigations aimed at extracting and purifying the specific compounds from stink bean seeds. Such endeavors hold the potential to pave the way for the development of novel antibiotics in the future.

Table 1 Antimicrobial activity of extracts from young stink beans using three different extraction methods.

Condition	Inhibition zone of extracts ± SD (mm)				
	MRSA	*S. aureus*	*B. cereus*	*M. luteus*	*S. Typhi*
Positive control (0.01 mg of gentamicin)	1.78±0.17	3.63±0.03	3.67±0.08	7.3±0.15	3.44±0.10
Negative control	-	-	-	-	-
Microwave-assisted water extraction	-	-	-	-	-
Boiling water extraction	-	-	-	-	-
50 % ethanol extraction	3.19±0.09	2.79±0.11	3.23±0.16	5.4±0.36	1.23±0.02

(-) = sensitive

Table 2 Minimum inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of 50 % ethanol extracts from young stink beans.

Strains	MIC (mg/mL)	MBC (mg/mL)
MRSA	18.75	900
S. aureus	18.75	900
B. cereus	18.75	900
M. luteus	9.38	150
S. Typhi	300	>900

Anticancer activity

The treatment of cancers encounters persistent challenges due to the disease's intricate nature, marked by variations in genetic alterations, organ involvement and prognosis among different types. Common treatments involve surgical removal, radiation, chemotherapy and targeted therapy. For patients who are ineligible for surgery, the use of chemotherapeutic drugs, which interfere with the cell cycle phases of rapidly dividing cancer cells, is a common approach [56]. However, these drugs, while effective against cancer, can induce toxicity in normal cells and are associated with multidrug resistance, causing over 90 % of cancer patient deaths during chemotherapy [57]. Targeted therapy using small molecules was believed to offer hope for cancer patients by reducing the off-target effects of chemotherapy. However, many targeted therapies face challenges such as tumor recurrence, unknown adverse effects and high costs. Ongoing efforts aim to find alternatives that balance efficacy, toxicity and resistance prevention in cancer treatment. Numerous natural products have demonstrated the ability to hinder carcinogenesis in both *in vitro* and *in vivo* models. Several clinical trials are currently underway to assess the safety and effectiveness of these natural agents in preventing or treating cancer. There is a strong possibility that natural agents could be employed for cancer prevention without notable adverse effects. These have been extensively reviewed by Wang *et al*. [58] and by Naeem *et al*. [59].

In our current study, we investigated the anticancer efficacy of extracts from stink bean seeds on various cancer cell types, along with normal cell lines. The extract of stink bean seeds from microwave-assisted water extraction demonstrated potent suppression of HT29 cell viability in a dose-dependent manner, with moderate inhibition observed in MCF7 and KKU-213A cell lines at high concentrations (**Figure 3(a)**). A549 lung cancer cells showed tolerance to the extract of stink bean seeds from microwave-assisted water extraction. Importantly, the extract of stink bean seeds from microwave-assisted water extraction exhibited low toxicity to normal cell lines, without significant impact on HDF cells and only modest suppression in MMNK-1 cells at the highest concentration. The extract of stink bean seeds from boiling water extraction inhibited HT29 and MCF7 cell growth in a dose-dependent manner, with significant suppression of HDF and MMNK-1 normal cell lines at high concentrations (**Figure 3(b)**). The other 2 cancer cell types, A549 and KKU-213A, were not affected by the extract of stink bean seeds from

boiling water extraction (**Figure 3(b)**). The extract of stink bean seeds from 50 % ethanol extraction was dose-dependently suppressed HT29 and MCF7, with mild effects on A549, KKU-213A and MMNK-1 cells and without impact on HDF cells (**Figure 3(c)**).

In summary, extracts from different extraction methods demonstrated varying degrees of anticancer activity. All extracts exhibited high activity against colon cancer (HT29) and breast cancer (MCF7) cell lines, while showing milder effects on bile duct cancer (KKU-213A) cells. Lung cancer cells (A549) displayed high resistance to the extracts. Notably, normal cell lines, especially slow-dividing HDF cells, were well-tolerated to the treatment with these extracts. While we acknowledge that the results from the *in vitro* study may not necessarily transfer directly to the *in vivo* study, these preliminary findings provide valuable guidance for the direction of anticancer research. Future studies should delve into the detailed examination of these extracts in cancer cells, exploring inhibitory effects on tumor phenotypes such as apoptosis, cell migration, cell invasion, target proteins and signaling pathways. Additionally, evaluating the anticancer efficacy and safety in mouse models is encouraged.
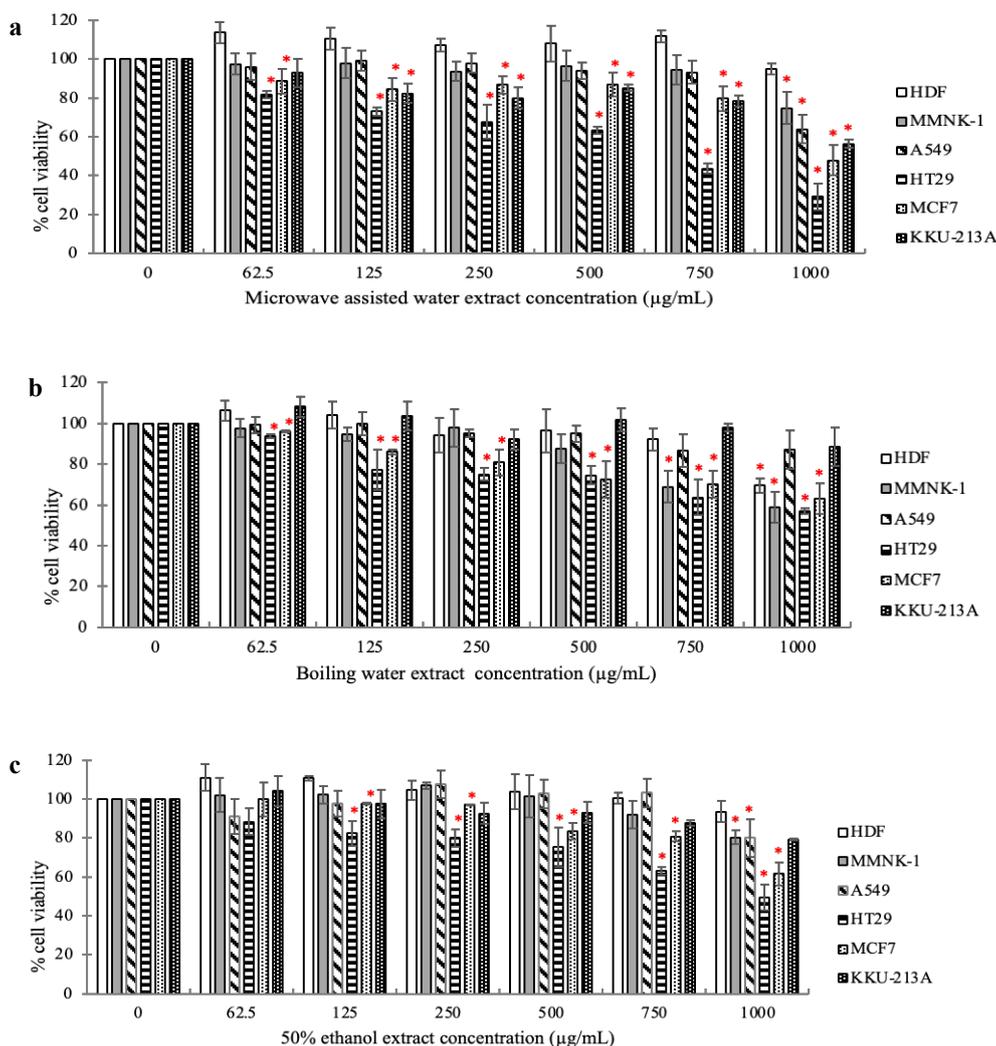

Figure 3 Cell cytotoxicity of different young stink bean extracts: (a) microwave-assisted water extraction, (b) boiling water extraction, (c) 50 % ethanol extraction against four human cancer cell lines: A549, HT29, MCF7 and KKU-213A as well as two normal cell lines: HDF and MMNK-1. Error bars represent SD ± Mean. The significant difference ($p < 0.05$) from the ANOVA test with 5 replicates was denoted with an asterisk.

Conclusions

Several studies have been conducted to investigate the bioactive compounds in stink bean seeds. We have furthered our analysis by comparing heat extraction methods, specifically microwaving, boiling and 50 % ethanol. Our findings indicate that 50 % ethanol extraction is the superior method for extracting bioactive compounds, especially phenolics. Furthermore, 50 % ethanol extraction has been verified to inhibit a spectrum of bacterial strains, encompassing both gram-positive and gram-negative species. Notably, assessments of cell cytotoxicity unveil that all three extracts exert significant inhibitory effects on various levels of cancer cell lines. The prospect of further research aimed at purifying these compounds and exploring their potential as pharmaceutical agents, including antibiotics and anticancer drugs, derived from the young seeds of stink beans, is promising.

Acknowledgments

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