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Original Article

Total phenolic content, antioxidant and antimicrobial activities of stink bean (*Parkia speciosa* Hassk.) pod extracts♣

Suchanuch Wonghirundecha, Soottawat Benjakul, and Punnanee Sumpavapol*

Department of Food Technology, Faculty of Agro-Industry,
Prince of Songkla University, Hat Yai, Songkhla, 90112 Thailand.

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Abstract

Parkia speciosa Hassk. is a stink bean known as "Sataw". This bean is popular in the Southern part of Thailand and believed by the locals to have the medical properties. Stink bean pod, regarded as waste material, was reported to possess various biological activities. The aim of the present study was to investigate the total phenolic content, antioxidant and antimicrobial activities of Sataw-Khao and Sataw-Dan pod extracts. It was found that the extraction yield, total phenolic and total flavonoid contents of Sataw-Dan pod extract were higher than those of Sataw-Khao pod extract. For antioxidant activity, both stink bean pod extracts had potent antioxidant activity by the radical-scavenging DPPH and ABTS methods, and the metal ion chelating assay. In addition, both extracts showed antimicrobial activity against food-borne pathogenic bacteria and food spoilage bacteria. Therefore, stink bean pod extract could be effectively used as a natural preservative.

Keywords: stink bean pod, antioxidant activity, antimicrobial activity, total phenolic content, total flavonoid content

1. Introduction

The plant phenolic compounds present a wide range of biological activities including antioxidant (Prasad *et al.*, 2011; Thitilertdecha *et al.*, 2008), antimicrobial (Chanthaphon *et al.*, 2008; Kummee and Intaraksa, 2008) and antiallergic activity (Tewtraku *et al.*, 2008). They can be found in all parts of the plant (Pratt, 1992).

Stink bean (*Parkia speciosa* Hassk.) is commonly known as "Sataw" in Thailand. This bean is widely distributed in Southeast Asia including Thailand, Malaysia, Indonesia, and Singapore. Stink bean pod is regarded as waste material during the processing of commercial preserved stink bean seed. It was reported to possess the hypoglycaemic effect (Jamaluddin *et al.*, 1994), antiangiogenic property, antioxidant activity (Aisha *et al.*, 2012; Gan and Latiff, 2011) and anti-microbial activity (Musa *et al.*, 2008; Wonghirundecha and Sumpavapol, 2012).

This study focused on the possibility of using stink bean pods as a new natural preservative. The extracts from Sataw-Khao and Sataw-Dan pods were investigated for antioxidant and antimicrobial activities, including total phenolic and total flavonoid contents.

2. Materials and Methods

2.1 Chemicals

Catechin, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picryl hydrazyl (DPPH) and 3-(2-pyridyl)-5,6- bis(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Gallic acid, ethylenediaminetetraacetic acid (EDTA) and Folin-Ciocalteu were obtained from Merck (Darmstadt, Germany).

* Corresponding author.
Email address: punnanee.s@psu.ac.th
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2.2 Collection and preparation of stink bean pod

After anthesis for 70-80 days, stink beans (Sataw-Khao and Sataw-Dan) were harvested and stink bean pods were collected from small and medium enterprises (SMEs) in Songkhla province, Thailand, in April 2012. They were transported to the Department of Food Technology, Prince of Songkla University, Songkhla, Thailand, within 3 h. Stink bean pods were prepared as described by Adebowale *et al.* (2002), Tan and Kassim (2011) and Wonghirundecha and Sumpavapol (2012). Briefly, the collected stink bean pod sample was cleaned using tap water and dried at 45°C using hot air oven (Binder, Germany) to obtain a final moisture content less than 10% with drying time of approximately 12 h. Dried sample was ground into a fine powder using a blender (Microsent, Thailand) and sieved through a stainless steel sieve of 60 mesh (250 µm). The powder was placed in a polyethylene bag and stored in a freezer at -20°C until use.

2.3 Preparation of stink bean pod extract

Stink bean pod powder was mixed with 50% ethanol with a sample-solvent ratio of 1:10 (m/v) and shaking at 200 rpm in the dark at 25°C for 2 h. The extract was filtered through Whatman No. 1 filter paper. After evaporating using a rotary evaporator (Eyela, Japan) at 50°C, dried extract was kept in an air-tight amber bottle and stored in freezer at -20°C until analyses.

2.4 Analyses

2.4.1 Determination of extraction yield

The extraction yield was defined as the amount of extract recovered in mass compared with the initial amount of raw material. It was calculated as the weight (g) of crude extracts obtained from raw materials and expressed as percentage.

2.4.2 Determination of total phenolic content

Total phenolic content (TPC) in the extract was determined using Folin–Ciocalteu reagent according to the method of Singleton and Rossi (1965) with some modification as described by Tan and Kassim (2011). An amount of 1 ml of Folin reagent (10% v/v) was added to 100 µl aliquot of appropriately diluted extracts or standard solutions of gallic acid and mixed throughly. After 5 min, 800 µl of 1 M Na_2CO_3 were added and the solution was adjusted to 2.0 ml with distilled water and mixed thoroughly. After incubation for 90 min at room temperature in the dark, an absorbance was measure at 750 nm using a UV spectrophotometer (Shimadzu, Japan). The concentration of total phenolic compound was calculated from the standard curve of gallic acid with the range of 0-180 mg/l and expressed as mg gallic acid equivalent (GAE)/g extract. All samples were analysed in three replications.

2.4.3 Determination of total flavonoid content

Total flavonoid content (TFC) was determined according to the colorimetric assay of Kim *et al.* (2003). Briefly, 800 µl of distilled water was added to 200 µl of the extract followed by 60 µl of 5% (w/v) sodium nitrite solution and 60 µl of 10% (w/v) aluminium chloride solution. The mixture was allowed to stand at ambient temperature for 5 min then 400 µl of 1 M NaOH was added. Thereafter, the volume of reaction mixture was made up to 2 ml with distilled water and mixed thoroughly. Absorbance of solutions was measured spectrophotometrically at 510 nm. Total flavonoid content was calculated from the standard curve of catechin with the range of 0-250 mg/l and expressed as mg catechin equivalent (CE)/g extract. All samples were analysed in three replications.

2.4.4 Determination of antioxidant activity

Stink bean pod extracts were dissolved with 50% ethanol to obtain the final concentration of 10 mg/ml before assays for antioxidant activity.

2.4.5 ABTS radical scavenging activity

ABTS radical scavenging activity was determined by ABTS assay as per the method of Cano *et al.* (1998) and Binsan *et al.* (2008). The stock solutions included 7.4 mM ABTS solution and 2.6 mM potassium persulphate solution. The working solution was prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml of ABTS solution with 50 ml of methanol in order to obtain an absorbance of 1.1±0.02 units at 734 nm. Fresh ABTS solution was prepared for each assay. Sample (150 µl) was mixed with 2850 µl of ABTS solution and the mixture was left at room temperature for 2 h in the dark. The absorbance was then measured at 734 nm. A standard curve of Trolox was prepared. The activity was expressed as µmol trolox equivalent (TE)/g extract. All samples were analysed in three replications.

2.4.6 DPPH radical scavenging activity

DPPH radical scavenging activity was determined by DPPH assay using the method of Shimada *et al.* (1992) and Binsan *et al.* (2008). Sample (1.5 ml) was added to 1.5 ml of 0.15 to mM DPPH in 95% ethanol. The mixture was mixed vigorously and allowed to stand at room temperature in the dark for 30 min. The absorbance of the resulting solution was measured at 517 nm. The blank was prepared in the same manner, except that distilled water was used instead of the

sample. A standard curve was prepared using Trolox. The activity was expressed as μmol TE/g extract. All samples were analysed in three replications.

2.4.7 Metal ions chelating activity

The chelating activity on Fe^{2+} was determined using the method of Decker and Welch (1990). One millilitre of sample solution was mixed with 3.7 ml of distilled water. The mixture was then reacted with 0.1 ml of 2 mM $FeCl_2$ and 0.2 ml of 5 mM ferrozine for 20 min at room temperature. The absorbance was read at 562 nm. The control was prepared in the same manner except that distilled water was used instead of the sample. A standard curve was prepared using EDTA. The activity was expressed as μmol EDTA equivalent (EDTAE)/g extract. All samples were analysed in three replications.

2.4.8 Determination of antimicrobial activity

1) Bacterial cultures

Bacteria used as test organisms in this study consisted of 6 pathogenic bacteria (*Bacillus cereus* DMST 5040, *Escherichia coli* DMST 4212, *Listeria monocytogenes* DMST 1327, *Salmonella* Typhimurium DMST 562, *Staphylococcus aureus* DMST 8840 and *Vibrio cholerae* non O1/non O139 DMST 2873) and 3 spoilage bacteria (*Aeromonas hydrophila* TISTR 1321, *Pseudomonas aeruginosa* TISTR 781 and *Serratia marcescens* TISTR 1354). They were obtained from Department of Medical Sciences, Ministry of Public Health, Thailand (DMST), and Thailand Institute of Scientific and Technological Research (TISTR). The bacterial strains were sub-cultured twice in nutrient broth (NB) at 37°C for 18 h and the density of an inoculum was adjusted to the turbidity of 0.5 McFarland standard (10^5-10^6 CFU/ml) before being used (Canillac and Mourey, 2001).

2.4.9 Agar-well diffusion assay

The antimicrobial activity of stink bean pod extracts was determined by agar-well diffusion assay as described by Wonghirundecha and Sumpavapol (2012). One milliliter of freshly prepared inoculum was aseptically added to a 9 ml of sterilized Muller-Hinton soft medium (0.75% agar, w/v), immediately mixed, poured into Petri dish and left to solidify at room temperature. After being dried for 15 min in laminar air flow, 6 wells (6 mm in diameter) were made in each plate. Each well was filled with 20 μl of stink bean pod extract (40 mg/ml), 50% ethanol (negative control) and 0.5 mg/ml of tetracycline (positive control). Plates were left at room temperature for 30 min to allow diffusion of material in media and were incubated at 37°C. After 24 h of incubation, the inhibition zones in mm around wells were measured by Vernier caliper. The absence of inhibition zone was interpreted as negative (no antimicrobial activity). The extract was tested in triplicate and the experiment was repeated three times.

2.5 Statistical analysis

All experimental results were expressed in three replicates as mean value ± standard deviation. Data were determined by independent comparison T-test (SPSS 11.0 for Windows, SPSS Inc., Chicago, IL, USA). The results with $p<0.05$ were regarded as statistically significant.

3. Results and Discussion

3.1 Extraction yield, total phenolic and total flavonoid contents

Stink bean pods were extracted using 50% ethanol as extraction solvent to obtain the polyphenols from the powder. The extract obtained was brownish in colour and the extraction yield was defined as the amount of extract recovered in mass compared with the initial amount of raw material. The percentage of extraction yield of Sataw-Dan was higher than Sataw-Khao pod extracts ($p<0.05$) as shown in Table 1.

The determination of TPC was carried out by Folin-Ciocalteu's method, referring to the calibration curve using gallic acid solution as a standard solution. The content of total phenolic in two kinds of stink bean pod extract, Sataw-Dan and Sataw- Khao, were 84.24 and 71.39 mg GAE/g DW, respectively with significant differences ($p<0.05$). This result indicated that Sataw-Dan pod is richer source of phenolic compounds than Sataw-Khao pod since the percentage of extraction yield of the former was more than that of the latter ($p<0.05$) as shown in Table 1.

Table 1. Chemical compositions of Sataw-Khao and Sataw-Dan pod extracts

Chemical composition	Sataw-Khao	Sataw-Dan
Extraction yield (%)	14.85 ± 0.45^b	18.87 ± 0.34^a
Total phenolic content (mg GAE/g DW)	71.39 ± 0.08^b	84.24 ± 0.12^a
Total flavonoid content (mg CE/g DW)	5.38 ± 0.18^b	5.86 ± 0.16^a

Values are given as mean ± SD from triplicate determinations (n=9).
Different superscripts in the same row indicate significant difference ($p<0.05$).

The determination of TFC was carried out by a colorimetric assay, referring to the calibration curve using standard solution of catechin. The TFC in two kinds of stink bean pod extract, Sataw- Dan and Sataw- Khao, were 5.86 and 5.38 mg CE/g DW, respectively with significant difference ($p<0.05$).

The difference in TPC and TFC in Sataw- Dan and Sataw- Khao may be due to the different degrees of phenolic polymerisation, solubility of phenolics and interaction of phenolics with other constituents (Silva *et al.*, 2007). According to the hydrophobic structure of their molecules, flavonoids extraction may require a longer extraction time than phenolics extraction to obtain the highest yield.

3.2 Antioxidant activity

The extracts were determined for their antioxidant activities by ABTS radical scavenging, DPPH radical scavenging and metal ion chelating assay. The ABTS and DPPH radical scavenging activity has been widely used to determine the free radical scavenging capacity of various samples (Amarowicz *et al.*, 2004; Hatano *et al.*, 1988) because of its rapidity, sensitivity and reproducibility (Özcelik *et al.*, 2003). However, ABTS assay gained higher attention than DPPH assay since it is operable over a wide range of pH, inexpensive and more rapid than that of the latter one (Awika *et al.*, 2003).

The antioxidant activities of both extracts are shown in Table 2. The results showed that the free radical scavenging activity against ABTS of Sataw-Khao pod extract were higher than that of Sataw-Dan pod extract with significant difference ($p<0.05$). Bleaching of a preformed solution of the blue-green radical cation $ABTS^{\bullet+}$ has been extensively used to evaluate the antioxidant capacity of complex mixtures and individual compounds. The reaction of the preformed radical with free-radical scavengers can be easily monitored by following the decay of the sample absorbance at 734 nm (Gülçin *et al.*, 2010). The reactions with $ABTS^{\bullet+}$ radicals involve electron transfer process (Kaviarasan *et al.*, 2007). When an antioxidant is added to the radicals, there is a degree of decolourization. Antioxidants reverse the formation of the $ABTS^{\bullet+}$ radical and generate a stable form of radical as a blue-green $ABTS^{\bullet+}$ chromophore (Gülçin *et al.*, 2010; MacDonald-Wicks *et al.*, 2006).

The free radical scavenging capacity of the extracts against common free radicals ($DPPH^{\bullet}$) *in vitro* was determined as demonstrated in Table 2. The results revealed that Sataw-Khao pod extract (1218.07 μmol TE/g DW) had higher effective radical scavenging activity than Sataw-Dan pod extract (920.32 μmol TE/g DW) with significant difference ($p<0.05$). In the DPPH assay, the antioxidant was able to reduce the stable radical DPPH to the yellow coloured diphenyl-picrylhydrazine. The method is based on the reduction of alcoholic DPPH solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH–H by the reaction (Oyaizu, 1986). Measuring an absorbance at 517 nm can reveal a colour change from purple to yellow. The lower absorbance of the reaction mixture indicates higher DPPH free radical scavenging activity since the $DPPH^{\bullet}$ was scavenged by an antioxidant through donation of hydrogen to form a stable $DPPH^{\bullet}$ molecule (Matthäus, 2002; Burits and Bucar, 2000).

The chelating of ferrous ions by the extracts of Sataw-Khao and Sataw-Dan pod were estimated as shown in Table 2. The metal chelating capacity of Sataw-Khao pod extract (9.76 μmol EDTAE/g DW) showed statistically higher activity than that of Sataw-Dan pod extract (5.86 μmol EDTAE/g DW). Ferrous ions (Fe^{2+}) are the most powerful prooxidant among the various species of metal ions (Halliwell and Gutteridge, 1984). Ferrozine can quantitatively form complex with Fe^{2+}. In the presence of chelating agents, the complex formation is disrupted, resulting in a decrease in the red colour of the complex. Measurement of colour reduction therefore allows the estimation of the metal chelating activity of the coexisting chelator. Lower absorbance indicates higher metal chelating activity (Kehrer, 2000).

According to the results, there was no relationship between TPC and antioxidant activity in stink bean pod extract. This is similar to the report of Gülçın *et al.* (2003), which showed that TPC of water and ethanol extracts of *Pimpinella anisum* L. seed were 30 and 77.5 μg GAE/g DW, respectively. On the other hand, the DPPH scavenging effect of water extract was higher than that of ethanol extract. Concordantly, the percentage of metal scavenging capacity of water extract was higher than that of ethanol extract.

Table 2. Antioxidant activity of stink bean pod extracts

Antioxidant activity	Stink bean pod extract	
	Sataw-Khao	Sataw-Dan
ABTS radical scavenging (μmol TE/g DW)	1610.67 ± 11.88^{a}	1261.14 ± 17.44^{b}
DPPH radical scavenging (μmol TE/g DW)	1218.07 ± 8.72^{a}	920.32 ± 6.15^{b}
Metal ion chelating (μmol EDTAE/g DW)	9.76 ± 0.03^{a}	5.86 ± 0.02^{b}

Values are given as mean ± SD from triplicate determinations (n=9).
Different superscripts in the same row indicate significant difference ($p<0.05$).
TE: Trolox equivalents; EDTAE: ethylenediaminetetraacetic acid equivalent; DW: dried weight

Velioglu *et al.* (1998) examined the antioxidant activity of 28 plant products. They reported that the high antioxidant activity was not correlated with TPC in many cases; probably other factors played major roles as antioxidants. Thus, the phenolic compounds may not contribute directly to the antioxidative action (Duh *et al.*, 1999).

Comparison of antioxidant activity between stink bean pod extract and other sources, revealed that stink bean pod extract showed higher antioxidant activity than mangosteen peel, persimmon peel, mango seed and tamarind seed (Maisuthisakul *et al.*, 2007), rambutan peel and seed (Maisuthisakul *et al.*, 2007; Thitilertdecha *et al.*, 2008), kiwifruit peel, pink lady apple peel and core (Wijngaard *et al.*, 2009).

3.3 Antimicrobial activity

Agar-well diffusion assay is extensively used to investigate the antimicrobial activity of natural substances and plant extracts. This assay is based on the use of solutions of substances in wells to be examined. In the case of solutions with a high activity, a large inhibition zone is observed.

In this study, Sataw-Khao and Sataw-Dan pod extracts were examined for antimicrobial activity against 6 pathogenic bacteria and 3 spoilage bacteria. Tetracycline and 50% ethanol were used as positive and negative controls, respectively. The diameter of inhibition zone against 6 pathogenic bacteria and 3 spoilage bacteria was shown in Table 3 and Table 4, respectively. There were no significant differences between Sataw-Khao and Sataw-Dan pod extracts against all tested bacteria. The diameter of inhibition zone of Sataw-Khao and Sataw-Dan pod extracts against pathogenic bacteria ranged from 6.87 to 15.68 mm and 7.07 to 16.27 mm, respectively. The results indicated that stink bean pod extract exhibited broad spectrum of activity toward the bacteria tested. In addition, it can be seen that *L. monocytogenes* and *S. aureus*, which are Gram positive bacteria, were the strains

Table 3. Antimicrobial activity of stink bean pod extracts against food-borne pathogenic bacteria

Microorganisms	Inhibition zone (mm)			
	Sataw-Khao	Sataw-Dan	50% ethanol	Tetracycline
Gram positive bacteria				
Bacillus cereus	9.50 ± 0.06^{ns}	9.20 ± 0.00^{ns}	-	24.65 ± 0.08
Listeria monocytogenes	10.50 ± 0.50^{ns}	9.60 ± 0.12^{ns}	-	19.87 ± 0.33
Staphylococcus aureus	11.50 ± 0.50^{ns}	11.30 ± 0.25^{ns}	-	23.67 ± 0.19
Gram negative bacteria				
Escherichia coli	6.87 ± 0.04^{ns}	7.07 ± 0.63^{ns}	-	21.67 ± 0.19
Salmonella Typhimurium	9.15 ± 0.15^{ns}	9.10 ± 0.10^{ns}	-	25.06 ± 0.08
Vibrio cholerae non O1/non O139	15.68 ± 0.12^{ns}	16.27 ± 0.20^{ns}	-	21.98 ± 0.03

Values are given as mean ± SD from triplicate determinations (n=9).
Different superscripts in the same row indicate significant difference (p<0.05).
Twenty microliters of extract samples (40 mg/ml) were applied into each well.
Twenty microliters of tetracycline (0.5 mg/ml) and 50% ethanol were used as positive and negative controls, respectively.
-: No inhibition; ns: not significant difference.

Table 4. Antimicrobial activity of stink bean pod extracts against food spoilage bacteria

Microorganisms	Inhibition zone (mm)			
	Sataw-Khao	Sataw-Dan	50% ethanol	Tetracycline
Aeromonas hydrophila	8.90 ± 0.06^{ns}	9.07 ± 0.05^{ns}	-	20.57 ± 0.05
Pseudomonas aeruginosa	9.75 ± 0.25^{ns}	9.70 ± 0.30^{ns}	-	21.97 ± 0.35
Serratia marcescens	9.20 ± 0.15^{ns}	9.10 ± 0.06^{ns}	-	10.27 ± 0.46

Values are given as mean ± SD from triplicate determinations (n=9).
Different superscripts in the same row indicate significant difference (p<0.05).
Twenty microliters of extract samples (40 mg/ml) were applied into each well.
Twenty microliters of tetracycline (0.5 mg/ml) and 50% ethanol were used as positive and negative controls, respectively.
-: No inhibition; ns: not significant difference.

susceptible to the extract as they showed a wide range of inhibition zone. For Gram negative bacteria, the extracts exhibited a lower range of inhibition zone than Gram positive bacteria, except *V. cholerae* which was the most susceptible strain in comparison with other Gram negative bacteria tested. This is concordant with Thitilertdecha *et al.* (2008) who found that *Nephelium lappaceum* L. extract could inhibited *V. cholerae* and showed the widest inhibition zone among all tested bacteria.

All of spoilage bacteria tested are Gram negative bacteria, thereby they were less susceptible to the extract than Gram positive pathogenic bacteria. The diameter of inhibition zone of Sataw-Khao and Sataw-Dan pod extracts against spoilage bacteria ranged from 8.90 to 9.75 mm and 9.07 to 9.70 mm, respectively.

The Gram negative bacteria are less susceptible to the action of antimicrobials than the Gram positive bacteria because of the different cell wall structure. The Gram negative bacteria possess an outer membrane surrounding the cell wall, which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering. Without outer membrane, the extract is able to disrupt the cytoplasmic membrane, causing increased cell wall and cell membrane permeability. Moreover, it can disrupt the proton motive force (PMF), electron flow, active transport and coagulation of cell contents (Burt, 2004; Sánchez *et al.*, 2010). Therefore, the structural difference of bacteria plays an important role in their susceptibility. This agreed with the previous report of Musa *et al.* (2008) who observed that *P. speciosa* Hassk. extract could be against all Gram positive bacteria (*Streptococcus agalatiae, S. aeruginosa* and *S. aureus*) and some Gram negative bacteria (*A. hydrophila* and *V. parahaemolyticus*). However, the Gram negative bacteria including *Citrobacter freundii, Edwardsiella tarda, E. coli* and *V. alginolyticus* were resisted to the extract.

Stink bean pod extract could inhibit all tested pathogenic and spoilage bacteria while rambutan peel, mangosteen peel, palmyra peel and coconut husk (Wonghirundecha and Sumpavapol, 2012), rambutan peel and seed (Thitilertdecha *et al.*, 2008), pomelo peel (Suklampoo *et al.*, 2012) and banana peel (Mokbel and Hashinaga, 2005) could inhibit only some bacteria and their inhibition zones were reported to be inferior to this extract.

4. Conclusions

Both kinds of stink bean pod extracts showed strong antioxidant activities by ABTS radical scavenging, DPPH radical scavenging, and metal ion chelating assay. In addition, both extracts showed noticeable antimicrobial activity against food-borne pathogenic bacteria (Gram positive and Gram negative bacteria) and food spoilage bacteria when compared with antibiotic. Thus, *P. speciosa* Hassk. pod could be used as a natural source of antioxidant with a wide range of antimicrobial and the extract could be used as a preservative instead of synthetic preservatives.

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