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Short Communication
Biological Activities of Supercritical Carbon Dioxide Extract of *Parkia speciosa* Seeds and Pods

[1]Ka Lun Wong, [2]Chun Hoe Tan, [2]Kae Shin Sim and [3]Yun Jin Kim

[1]School of Energy and Chemical Engineering, Xiamen University Malaysia, Sepang, 43900 Selangor, Malaysia
[2]Institute of Biological Science, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
[3]School of Traditional Chinese Medicine, Xiamen University Malaysia, Sepang, 43900 Selangor, Malaysia

Abstract

Backgrounds and Objective: *Parkia speciosa* (petai) is popular as a culinary ingredient and folk medicine in treating various ailments. The extraction of novel bioactive components from plant-based natural products is crucial to meet global health needs. Nonetheless, the biological activities of plant extracts usually show a significant variation depending on the extraction methods. In this study, Supercritical Carbon Dioxide (SCD) extraction was performed on *P. speciosa* seeds and pods, and their biological activities were investigated. **Materials and Methods:** *P. speciosa* seed (SE) and pod (PE) extracts were obtained by SCD extraction method using a Waters' Supercritical Fluid Extraction System. The radical scavenging ability of the extracts was tested using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^+$) radical scavenging assays, and their cytotoxicity was screened using MTT assay inclusive of seven human cancer cell lines. The relationship between the results of the assays was measured in pairwise using Pearson's correlation. **Results:** Both PE and SE exhibited considerable DPPH and $ABTS^+$ radical scavenging activities in a dose-dependent manner. PE exhibited pronounced cytotoxicity against human colorectal carcinoma HCT 116 (IC_{50} 0.54±0.14 μg mL^{-1}) and the activity is comparable with the common chemotherapeutic cisplatin (IC_{50} 0.73±0.26 μg mL^{-1}). Pearson's correlation indicates that there is no significant correlation between the radical scavenging activity and cytotoxicity of PE. **Conclusion:** Our findings implied that PE and SE obtained using SCD extraction exhibited considerable antioxidant activity, and only PE showed potent cytotoxicity which may be developed further to contribute to the management of colorectal cancer.

Key words: Petai, supercritical fluid extraction, antioxidant, anti-cancer, HCT 116, colorectal cancer, correlation

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Corresponding Author: Yun Jin Kim, School of Traditional Chinese Medicine, Xiamen University Malaysia, Sepang, 43900 Selangor, Malaysia

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Parkia speciosa Hassk. (Fabaceae), or locally known as "petai" (Malay) is a perennial plant that is widely distributed in Malaysia, Indonesia, Thailand and Philippines[1]. The plant bears long and flat beans pod with green leguminous seeds that release the unique aroma, suggesting the presence of sulphur-containing compounds[2]. Apart from being consumed for their flavor and texture, the fresh seeds are known to exhibit various ethnopharmacological activities and are eaten raw by the locals to treat kidney disorders, diabetes, urinary bladder infection, hypertension and as laxatives[2-5]. The seeds and pods exhibited several biological and pharmacological activities, including hypoglycemic[3,6], mitogenic[7], anti-bacterial[8], anti-cancer[1] and antioxidant[9] activities, which largely attributed to the presence of bioactive compounds in the plant, such as lectin[4], stigmast-4-en-3-one[6] and thiazolidine-4-carboxylic acid[2].

Extraction is the first step in the discovery of bioactive compounds from natural products, followed by purification and isolation. Therefore, the selection of an appropriate extraction method is extremely crucial to obtain the compounds of interest from the natural products in an acceptable quality and quantity. The extraction using supercritical fluid, such as Supercritical Carbon Dioxide (SCD) has attracted much attention over time due to its comparatively high diffusivity, low viscosity and low surface tension as opposed to typical organic solvents[10]. Therefore, the application of SCD often leads to a higher extraction yield than solvent-based extraction[11,12]. Aside from being non-toxicity, SCD is easily removable from the product which allows preparation of solvent-free extracts[13]. Moreover, SCD extraction method is able to prevent the thermal degradation of labile bioactive components in the extract since the process is conducted at low temperatures and in the absence of oxygen and light[14,15]. Owing to these advantages over solvent-based extraction, the SCD extraction method is extensively applied to extract highly volatile, heat sensitive and non-polar components (such as lipids) from plant samples for their biological activity evaluation.

Although the antioxidant and anti-cancer properties of *P. speciosa* seed and pod extracts have been previously investigated, the extractions were done using organic solvents, such as methanol [8] and ethanol[16], while the biological activity evaluation of the seeds and pods SCD extracts was remained elusive. As it has been mentioned above, SCD extraction often yielded volatile components, suggesting that the phytochemical profile and biological activities could be deviated significantly from that using the solvent-based extraction method performed previously.

In this regard, we aimed to investigate the radical scavenging and cytotoxic activities of *P. speciosa* seed and pod extracts produced using SCD extraction method in the current study.

MATERIALS AND METHODS

Study area and duration: The extraction of plant materials was conducted in a biotechnology laboratory in Xiamen University Malaysia, Sepang, 43900 Selangor, Malaysia. The biological evaluation of the plant extracts was performed in the Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia. The research project was conducted from January 2018-March 2020.

Chemicals and reagents: Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), including 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) cation ($ABTS^+$), ascorbic acid, methanol, cell culture media, fetal bovine serum (FBS), accutase, trypan blue solution, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), cisplatin and phosphate-buffered saline (PBS). Potassium persulfate ($K_2S_2O_8$) was purchased from Merck (Darmstadt, Germany).

Plant materials collection and Supercritical Fluid Extraction (SFE) method: Fresh *P. speciosa* pods were collected from a local market in Sepang, Malaysia in September 2018. The pods were cleaned thoroughly with distilled water and the seeds were immediately separated from the pods. Both seeds and empty pods were freeze-dried, ground into powder ($\leq$10 mesh) using a swing-type pulverizer (stainless steel high-speed grinder) and weighed. The extraction of pods and seeds was carried out by a Waters' SFE System as previously described[17]. Briefly, the freeze-dried powder samples (200 g) were added into an extraction vessel and extracted for 60 min at 40°C under 400 bars, with a CO_2 flow rate of 40 g/min to yield the pod (PE) and seed (SE) extracts. The extracts were stored at 4°C until use.

DPPH radical scavenging assay: The assay was carried out as previously described by Navanesan *et al.*[18] with modification. Into a 96-well plate, various concentrations (100-6500 μg mL^{-1}) of solubilized extract (50 μL) and 0.3 mM of DPPH solution (150 μL) were added. Ascorbic acid and

methanol were added instead of the extract as positive and negative controls, respectively. After 30 min of incubation at room temperature in the dark, the absorbance values were measured at 515 nm against a methanol blank, using a Tecan M200 Infinite Pro microplate reader. The mean of three readings was used and the scavenging activity (%) of the extract on DPPH radical was calculated according to the Eq. 1:

$$\text{Scavenging activity (\%)} = \frac{A_{control} - A_{extract}}{A_{control}} \times 100\% \quad (1)$$

where, $A_{extract}$ is the absorbance of mixture containing the extract and $A_{control}$ is the absorbance of the negative control. The EC_{50} (mg mL^{-1}) of the extract was determined by plotting percentage of scavenging activity against the concentration of extract on a logarithmic scale using GraphPad Prism 7 software (Graphpad software Inc., CA, USA).

ABTS cationic radical scavenging assay: The assay was carried out as previously described by Chatatikun and Chiabchalard[19] with modification. The oxidized ABTS cation ($ABTS^+$) solution was prepared by mixing ABTS aqueous solution (7 mM) and $K_2S_2O_8$ (2.45 mM) in 1:1 ratio and incubated in the dark at room temperature for 24 h. Into a 96-well plate, various concentrations (100-6500 μg mL^{-1}) of extract (20 μL) and $ABTS^+$ solution (180 μL) were added. Ascorbic acid and deionized water were added instead of the extract as positive and negative controls, respectively. After 30 min of incubation at room temperature in the dark, the absorbance of each well was measured at 734 nm using a Tecan M200 Infinite Pro microplate reader. The mean of three readings was used and the scavenging activity (%) on $ABTS^+$ was calculated according to the Eq. 1. The EC_{50} values were measured using GraphPad Prism 7 software (Graphpad software Inc., CA, USA) as described in the previous section.

Cell culture: The human-derived colon carcinoma (HCT 116), colon adenocarcinoma (HT-29), breast adenocarcinoma (MCF7 and MDA-MB-231), prostate adenocarcinoma (PC-3), non-small cell lung carcinoma (A549) and nasopharyngeal epidermoid carcinoma (KB) cell lines were purchased from American Type Culture Collection (ATCC, USA). Cells were cultured using McCoy's 5A medium (HCT 116 and HT-29), DMEM (MCF7 and MDA-MB-231), RPMI-1640 medium (PC-3 and A549) and EMEM (KB), supplemented with 10% FBS and incubated at 37°C in a humidified CO_2 incubator (Esco, Singapore). The morphology of the cells was observed regularly to avoid genetic drift. The cells were detached using accutase and sub-cultured upon reaching 70-80% of confluency to maintain the exponential growth of the cells.

MTT cytotoxicity assay: The assay was conducted as previously described by Navanesan *et al.*[20]. Into a 96-well plate, the cells were seeded at optimized density and incubated overnight. The cells were treated at various concentrations (0.1-31.62 μg mL^{-1}) of extracts and cisplatin was used as a positive control. After 72 hrs of treatment, MTT was added into the mixture and incubated for another 3 hrs. The absorbance of each well was measured at 570 and 650 nm as a reference wavelength, using a Tecan M200 Infinite Pro microplate reader. The IC_{50} (mg mL^{-1}) of the extract was determined by plotting the percentage of viability against the concentration of extract on a logarithmic scale using GraphPad Prism 7 software (Graphpad software Inc., CA, USA).

Statistical analysis: All data was expressed as the Mean ± SD of three independent experiments. Statistical analysis was performed using Graphpad Prism 7.0 software (Graphpad software Inc., CA, USA). The type of statistical test that was used to evaluate the statistical significance of $p<0.05$ was indicated wherever applicable. Pearson's correlation was conducted to measure the relationship between the results of assays.

RESULTS AND DISCUSSION

In the current study, we investigated the radical scavenging and cytotoxic activities of *P. speciosa* pods and seeds extracts obtained from Supercritical Carbon Dioxide (SCD) extraction method. The pod (PE) and seed (SE) extracts are highly viscous yellowish semi-liquids that exude a unique fragrance. The previous report on the phytochemical profile of the seed SCD extract revealed that the extract is composed of mainly fatty acids, phytosterols and esters[21]. Among these, phytosterols were widely reported as potent antioxidant[22] and anti-cancer[23] agents. Consequently, we evaluated the antioxidant and anti-cancer activities of the extracts using radical scavenging and MTT cytotoxicity assays, respectively. The DPPH and $ABTS^+$ radicals scavenging assays were employed to investigate the hydrogen atom[24] and single electron transfer[25] abilities of the extracts, respectively. Both PE and SE exhibited considerable radical scavenging ability in a dose-dependent manner (Fig. 1a-b) and the DPPH and $ABTS^+$ radical scavenging abilities of the extracts are significantly and positively correlated ($p<0.05$, $r = +0.9801$) (Fig. 1c). The results were expressed in EC_{50} and summarised in Table 1. It was

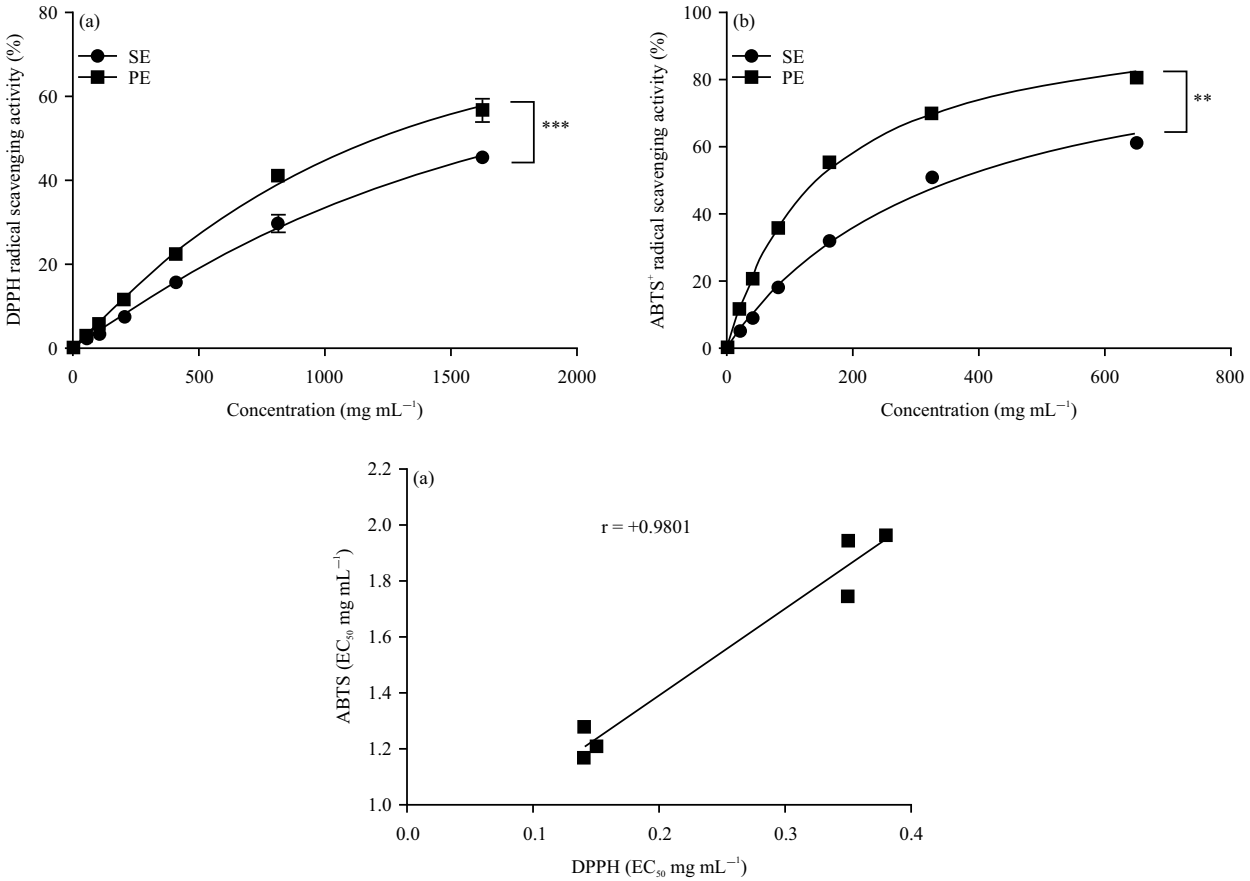

Fig. 1(a-c): Radical scavenging activity of PE and SE

The experiments were repeated thrice and expressed as Means±SD (error bars), (a) Scavenging activity by DPPH assay (***p<0.005, for PE vs. SE, Student's t-test), (b) Scavenging activity by ABTS assay (**p<0.01, for PE vs. SE, Student's t-test) and (c) Correlation analysis of ABTS^{+} and DPPH radical scavenging activities (p<0.05, ABTS^{+} vs. DPPH radical scavenging activities, Pearson's correlation analysis)

Table 1: Radical scavenging activity of PE and SE

	EC_{50} (mg mL^{-1})	
Sample	DPPH	ABTS^{+}
PE	1.22±0.05^{a,d}	0.14±0.00^{a,e}
SE	1.89±0.10^{b,d}	0.36±0.01^{b,e}
*Ascorbic acid (μg mL^{-1})	10.02±0.07^{c,d}	4.36±0.21^{c,e}

$^{a-c}$Columns with different superscripts indicate a significant difference (ANOVA, p<0.05), $^{d-e}$Rows with different superscripts indicate a significant difference (ANOVA, p<0.05), Values are the Mean±SD of three independent experiments, *Used as a positive control, PE: Pod extract, SE: Seed extract, DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS^{+}, 2,2'-azino-bis (3-ethylbenzothiazoline -6-sulphonic acid) diammonium salt

evident that PE, with an EC_{50} of 1.22±0.05 mg mL^{-1} has a significantly (p<0.05) higher DPPH radical scavenging ability than SE (EC_{50} 1.89±0.10 mg mL^{-1}), indicating that the former has a higher hydrogen transferability. Interestingly, the DPPH radical scavenging activity of PE and SE was relatively low compared to the extracts obtained using organic solvents as reported by other researchers. For instance, Gan and Latiff[8] reported that the methanolic pod extract has an EC_{50} of 0.49±0.01 mg mL^{-1}, while the ethanolic pod extract obtained by Ko *et al.*[9] has an even higher DPPH radical scavenging ability (EC_{50} 64.2±3.46 μg mL^{-1}) than PE. Similarly, SE has a lower activity than all ethanolic seed extracts of different locations in Malaysia prepared by Ghasemzadeh *et al.*[16] (EC_{50} 41.62-66.29 μg mL^{-1}). By comparison with these reported studies, it was suggested that pods and seeds may contain mostly polar antioxidants (e.g. polyphenols and flavonoids)[16] that are better extracted using organic solvents[26] rather than SCD. The extraction of *P. speciosa* seeds and pods using SCD often yields lipids and non-polar compounds[21], these compounds may have very limited radical scavenging ability compared to those polar. Therefore, co-solvents such as methanol and ethanol may be added in low quantities to enhance the efficiency of the SCD extraction[12] of *P. speciosa* seeds and pods in order to yield more polar bioactive compounds. Aside from the different methods of extraction

being used, the variation in the DPPH radical scavenging results could be attributed to seasonal, geographical and climatic factors as well[27]. Regarding $ABTS^+$ radical scavenging assay, PE (EC_{50} 0.14±0.09 mg mL^{-1}) also has a significantly ($p<0.05$) higher electron transfer ability than SE (EC_{50} 0.36±0.01 mg mL^{-1}). Moreover, both PE and SE have a significantly ($p<0.05$) higher $ABTS^+$ radical scavenging ability than their DPPH radical scavenging ability, indicative of a higher tendency in transferring electrons than hydrogen atoms by the components in the extracts.

According to the US National Cancer Institute (NCI) plant screening program, a plant extract or its pure compound is classified as cytotoxic when the IC_{50} is less than 20 μg mL^{-1} or 4 μg mL^{-1}, respectively, following 48-72 hrs of treatment[28]. In this regard, both PE and SE did not exhibit appreciable cytotoxicity against all human cancer cell lines tested (IC_{50}>20 μg mL^{-1}) upon 72 hrs of treatment, except for a pronounced growth inhibitory effect of PE against colorectal carcinoma HCT 116 (IC_{50} 0.54±0.14 μg mL^{-1}) has been observed. Particularly, PE has comparable cytotoxicity with the common chemotherapeutic cisplatin (IC_{50} 0.73±0.26 μg mL^{-1}), indicating that PE contains cytotoxic components which could be potentially developed into drug leads that are effective in the treatment of colorectal cancer. As opposed to HCT 116, PE was relatively non-toxic (IC_{50}>20 μg mL^{-1}) against colorectal adenocarcinoma HT-29, although both are human colorectal cancer cell lines. These colorectal cancer cell lines can be differentiated by the mutational status of the *p53* tumor suppressor gene, whereby the HCT 116 has intact p53 while HT-29 is a p53-mutant [29]. Although definite confirmation is beyond the scope of this study, we tentatively suggest that the cytotoxic mechanism of PE is p53-dependent since it is only cytotoxic against HCT 116 (wild-type p53). Nonetheless, more *in vitro* assays, such as microarray analysis and real time-PCR need to be carried out to validate the hypothesis. Previous study reported that the pod contains the phytosterol stigmast-4-en-3-one (β-sitostenone)[6] which can be extracted by SCD[30]. Our MTT cytotoxic result was in agreement with that reported by Jantaharn *et al.*[31], whereby stigmast-4-en-3-one was relatively cytotoxic against HCT 116 (IC_{50} 13.20±1.43 μg mL^{-1}) compared to HT-29 (IC_{50}>20 μg mL^{-1}). Hence, we suggested that stigmast-4-en-3-one could be one of the cytotoxic compounds present in PE that contributes to its cytotoxicity in HCT 116.

It is known that some of the natural antioxidants are potent anti-cancer agents[32]. Despite PE exhibited considerable radical scavenging activity and potent cytotoxicity in HCT 116, there was no significant ($p>0.05$) correlation between the results of radical scavenging and MTT cytotoxicity assays, implying that the radical scavenging ability of PE was unlikely its cytotoxicity mechanism in HCT 116.

According to US National Cancer Institute (NCI) plant screening program, plant extracts are considered *in vitro* cytotoxic when the IC_{50} values are less than 20 μg mL^{-1} or less, while it is less than 4 μg mL^{-1} for pure compounds, following incubation between 48-72 hrs[8]. Despite being a crude extract, PE exhibited specific, yet stronger cytotoxicity than cisplatin (FDA approved chemotherapeutic) in HCT 116, clearly demonstrated that PE contains cytotoxic active component(s) which could potentially be the drug leads in colorectal cancer treatment. PE demonstrated a higher growth inhibitory effect in HCT 116 (p53 intact) than HT-29 (p53 mutant), indicated that the cytotoxicity mechanism of PE is p53-dependent.

Regarding DPPH assay, though the antioxidant activity of the extracts did not in compliance with the calculated AAI values, the EC_{50} values were comparable with several reported plant extracts that showed potential antioxidant activity[9]. Similarly, with ascorbic acid, both PE and SE have a higher capability in scavenging charged free radical than neutral free radical, as observed from lower EC_{50} values in ABTS assay than DPPH assay. Taken all together, the extracts may contain phenolic compounds, notably phenolic acids, flavonoids and tannins, which are natural antioxidants in plant-based extracts[10].

Despite strong cytotoxicity and substantial radical scavenging activity exhibited by PE, there was no significant correlation between the result of MTT cytotoxicity assay and radical scavenging assays ($p>0.05$), suggested that the radical scavenging ability of PE was unlikely the cytotoxicity mechanism in HCT 116.

CONCLUSION

Our study showed that the SCD extraction method yielded PE and SE that exhibited considerable DPPH and $ABTS^+$ radical scavenging abilities and their antioxidant ability is rather weaker than those obtained using organic solvents. Hence, a modification of the SCD extraction technique needs to be conducted to enhance the extraction of bioactive components. Nonetheless, PE exhibited pronounced cytotoxicity against HCT 116, indicating that it could be a potential antineoplastic agent for the treatment of colorectal cancer and therefore, it should be further investigated. After all, these findings are not definite and conclusive yet due to the nature of the studies that used crude extracts rather than pure compounds. The crude extracts contain multiple components that may be working antagonistically against

each other which leads to a lower biological activity than their components. On that account, isolation of the pure compounds from the extracts is necessary to fully clarify the biological activity of *P. speciosa*. Moreover, more in-depth biological assays involving *in vivo* model should be conducted to ascertain the therapeutic potentials of these extracts, particularly PE.

SIGNIFICANCE STATEMENT

This study noted the radical scavenging and cytotoxic activities of the *P. speciosa* seed and pod SCD extracts and the results partly supported their ethnopharmacological application. Our study provided a preface into the feasibility of SCD extraction method to yield non-polar bioactive crude extracts from the *P. speciose* pod which show potent cytotoxicity that is comparable with cisplatin, implying that further anti-cancer activity evaluation of the pod extract is possible. Although pod is commonly disposed of during the processing of *P. speciosa*, its extract interestingly exhibited higher biological activities than the seed, which is the edible part of the plant. It is anticipated that when the extensive biological evaluation is carried out, all parts of *P. speciosa* can be fully utilized sustainably.

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