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Efficacy of food-based inhibitors from selected spices on polyphenol oxidase from Thai brinjal (*Solanum melongena* var. virginianum)

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Abstract

This study addressed the challenge of enzymatic browning in fresh produce, specifically Thai brinjal, a problem primarily caused by polyphenol oxidase (PPO). Traditional methods to control browning, involving chemical inhibitors and physical treatments have their limitations: the former pose risks to human health and the latter may alter the quality of the produce. Thus, the objective of this research was to explore the efficacy of selected spices (Cinnamon bark, clove, coriander seed, cumin, fennel seed, and star anise) as food-based inhibitors of PPO in Thai brinjal. In this study, Thai brinjal PPO showed an enzyme activity of 10293±234.48 EU/mL using pyrocatechol as a substrate. Ascorbic acid, employed as a synthetic inhibitor, demonstrated mixed inhibition, with its effectiveness ranging from 10.75±0.56% to 37.05±0.00% as the concentration increased from 0.05 mM to 0.2 mM. The spices were tested for their inhibitory effect on Thai brinjal PPO, with inhibition percentages ranging from 3.80±0.67% to 34.20±1.11%. Among the spices, cinnamon bark extract stood out as it exhibited the highest inhibition percentage (34.20±1.11%) through competitive inhibition. The findings of this study highlighted cinnamon bark as a particularly promising food-based inhibitor that potentially aids in food industrial growth by offering a healthier and more effective alternative to chemical inhibitors. This research opened avenues for utilizing spices to enhance the quality and shelf-life of Thai brinjal and other fresh-cut produce, aligning with consumer preferences for natural food preservation methods.

1. Introduction

The freshness and aesthetic appeal of fruits and vegetables used in culinary dishes significantly contribute to their overall appeal. A major challenge in maintaining this appeal is the browning phenomenon, particularly prevalent in produce like the Thai brinjal. The enzymatic browning process, primarily catalyzed by polyphenol oxidase (PPO), leads to the formation of melanin, resulting in undesirable dark pigmentation. This not only affects the visual attractiveness but also the perceived freshness and quality of the produce (Lim *et al.*, 2019; Weerawardana *et al.*, 2020; Kaya and Bağci, 2021). Understanding and controlling this enzymatic browning is crucial, especially during post-harvest handling and storage where maintaining visual and nutritional quality is important.

Traditionally, the food industry has combated this issue with physical treatments and chemical browning inhibitors. Physical methods like temperature treatments, irradiation and high-pressure processing, though effective, often alter the food's texture and color and involve high operational costs (Siew *et al.*, 2022a). Chemical inhibitors, including organic acids, chelating agents and sulfites, pose health risks, have limited effective durations and can negatively impact the sensory qualities of the food. For instance, ascorbic acid may lose its efficacy through oxidation; sulfites can cause irritation and asthma, and L-cysteine can produce off-odors (Lim *et al.*, 2019; Siew *et al.*, 2022a).

As an alternative, this study explores the potential of spices as food-based browning inhibitors, with a focus on Thai brinjal. This Southeast Asian vegetable, integral to Thai curry dishes like kaeng tai pla, green curry, red curry and salads such as som tam and phla nuea makhuea, is known for its health-promoting properties, including anti-allergy, anti-inflammatory, and antioxidant benefits, but also for its susceptibility to browning (Guldiken *et al.*, 2018; Naeem and Ugur,

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2019). This browning is particularly problematic as brinjals in these dishes must be cooked immediately after cutting to prevent browning, and a food-based browning inhibitor could allow for pre-cutting before cooking.

Food-based browning inhibitors, especially those derived from spices, are receiving increased attention as a sustainable alternative to synthetic inhibitors. Spices are rich in phytochemicals such as essential oils, fatty acids, phenolics and sulfur-containing compounds, known not only for their flavor-enhancing properties but also for their traditional medicinal uses and preservation properties, including antioxidant and antimicrobial activities (Guldiken *et al.*, 2018; Siew *et al.*, 2022b). These characteristics position spices as promising candidates for food-based browning inhibitors, offering a natural solution for fresh produce preservation.

This research investigated six spices including cinnamon bark, clove, coriander seed, cumin, fennel seed and star anise, for their potential to inhibit PPO activity in Thai brinjal. The significance of this study extends to food scientists, the food industry and consumers, all of whom are interested in natural preservation methods and fresher, more naturally preserved food options.

2. Materials and methods

2.1 Plant and spices materials

Thai brinjal (*Solanum melongena* var. virginianum) was used as the source of PPO in this study. It was purchased from a local supermarket in Kuala Lumpur, Malaysia. Six spices namely, cinnamon bark, clove, coriander seed, cumin, fennel seed and star anise were purchased from TF Value-Mart, Kuala Lumpur, Malaysia. The purchased Thai brinjal and spices were in good condition without any signs of damage or spoilage. All chemicals and reagents used in this study were of analytical grade.

2.2 Polyphenol oxidase extraction

The crude Thai brinjal PPO was extracted according to the preparation method from Ng and Wong (2015) with slight modifications. Thai brinjal (100 g) was washed with tap water to remove dirt. The stem of the brinjal was removed and the brinjal was cut into cubes (1 cm × 1 cm × 1 cm) quickly. The brinjal pieces were then homogenized with 200 mL of pre-chilled phosphate buffer (0.1 M, pH 6.8) containing 4 g of polyvinylpyrrolidone (PVP) in a pre-chilled blender (Philips, HR2021/75, Malaysia) at the maximum speed (3600 rpm) for 1 min. The homogenate was then centrifuged using a centrifuge machine (Allegra X-22R Centrifuge, Beckman Coulter, USA) at 7826×*g* (10000 rpm) at 4°C for 15 mins. The homogenate was then filtered through the Whatman No. 1 filter paper. The resulting filtrate was the crude Thai brinjal PPO extract. The crude PPO extract was stored in small aliquots at −20°C prior to analysis.

2.3 Polyphenol oxidase assay

The PPO assay was conducted according to the methods of Siew *et al.* (2022b). The substrate used was 0.1 M pyrocatechol. The reaction mixture consisted of 1.0 mL of 0.1 M pyrocatechol, 1.9 mL of 0.1 M phosphate buffer (pH 6.8) and 0.1 mL crude PPO extract. The mixture was then quickly transferred into a cuvette and the absorbance was measured at 410 nm by using a spectrophotometer (PRIM, Secoaman, France) at 15 s intervals for 5 mins. A blank solution was prepared, which consisted of 1.0 mL pyrocatechol (0.1 M) and 2.0 mL phosphate buffer (0.1 M, pH 6.8). The initial velocity was calculated from the initial slope of the absorbance against the time curve. The amount of enzyme that caused an increase in absorbance reading of 0.001 per min was corresponded to one unit (EU) of PPO activity (Siew *et al.*, 2022b).

2.4 Effects of ascorbic acid on polyphenol oxidase activities assay

The effect of ascorbic acid on Thai brinjal PPO activity was determined according to the methods used by Siew *et al.* (2022b). Ascorbic acid (0.05, 0.1 and 0.2 mM) was used as a chemical inhibitor in this study. The reaction mixture consisted of 1.0 mL pyrocatechol at several different concentrations comprising 0.025M, 0.05M, 0.075M and 0.1M, 1.0 mL of ascorbic acid, 0.9 mL of 0.1 M phosphate buffer (pH 6.8) and 0.1 mL crude PPO extract. The mixture was then transferred quickly into a cuvette and the absorbance was determined at 410 nm by using a spectrophotometer (PRIM, Secoaman, France) at 15 s intervals for 5 mins. A blank solution was prepared which consisted of 1.0 mL pyrocatechol, 1.0 mL of ascorbic acid and 1.0 mL phosphate buffer (0.1 M, pH 6.8) only. The type of inhibition, inhibition percentage, Michaelis constant (K_m), maximum velocity (V_{max}) and inhibition constant (K_i) of the chemical inhibitor were determined. The inhibition percentage of ascorbic acid was expressed as IC_{50}, which is the ascorbic acid concentration needed to inhibit the enzyme activity by 50% (Se Hoo *et al.*, 2022).

2.5 Preparation of selected spice extracts

All the selected spices (30 g) were dried in an oven (UNB 100, Memmert, Germany) at 50°C overnight, before blending (Philips, HR2021/75, Malaysia) into fine powder at maximum speed at 3600 rpm. Each of the spice powder (10 g) was extracted with 100 mL methanol via maceration for 1 hr. Subsequently, the

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extracts were filtered through Whatman No. 1 filter paper to remove the spice powders. All the spice extracts were recovered through rotary evaporation (R-200, Buchi, Switzerland) at 50°C, followed by oven-dried at 50±1°C until constant weights were obtained. The weight of the dried extracts was recorded before storage at 4°C prior to analysis.

2.6 Effects of selected spices extracts on polyphenol oxidase activities

The effect of selected spice extracts on Thai brinjal PPO activity was determined according to Siew *et al.* (2022b) with modifications. All the spice extracts were prepared at a concentration of 2.0 mg/mL by using 5.0% DMSO. The reaction mixture consisted of 1.0 mL pyrocatechol at several different concentrations comprising 0.025, 0.05, 0.075 and 0.1 M, 1.0 mL of spice extract, 0.9 mL of 0.1 M phosphate buffer (0.1 M, pH 6.8) and 0.1 mL crude PPO extract. The mixture was then transferred immediately into a cuvette and the absorbance was measured at 410 nm by using a spectrophotometer (PRIM, Secoaman, France) at 15 s intervals for 5 mins. The inhibitory effect of the selected spices was compared with 5% DMSO, which was used as the solvent control. A blank solution consisted of 1.0 mL pyrocatechol (0.1 M), 1.0 mL of spices extract and 1.0 mL of 0.1 M phosphate buffer (pH 6.8). The type of inhibition, inhibition percentage, Michaelis constant (K_m), maximum velocity (V_{max}) and inhibition constant (K_i) for each spice extract were determined.

2.7 Statistical analysis

All the experiments in this study were performed in triplicate (n = 3) and results were expressed in means±standard deviation (SD). All the statistical analysis was done by using Microsoft Office Excel 2016 and IBM SPSS Statistics 26. Turkey's Post Hoc test was used to analyse the analysis of variance (ANOVA) with significant differences at p-value < 0.05.

3. Results and discussion

3.1 Extraction and activity of polyphenol oxidase in Thai brinjal

The extraction of PPO from Thai brinjal and its subsequent activity is pivotal to understanding the enzymatic browning process in this vegetable. The PPO activity in Thai brinjal was measured to be 10293±234.48 EU/mL at pH 6.8 and room temperature using pyrocatechol as a substrate. This level of activity is significantly higher compared to round brinjal (7650±611.01 EU/mL) and is akin to that found in pearl brinjal (11200 EU/mL) (Se Hoo *et al.*, 2022; Ng and Wong, 2015).

The extraction process of PPO from Thai brinjal involved homogenization to disrupt the cells and release the intracellular enzyme. Conducted at a low temperature of 4°C, this method aimed to prevent enzyme denaturation and activity loss. The pH of the phosphate buffer, maintained at 6.8, facilitated optimal PPO activity. PVP was added to the buffer during homogenization to prevent the polymerization of phenolic substrates with PPO (Lim *et al.*, 2019; Se Hoo *et al.*, 2022). Following extraction, centrifugation separated PPO from other components and the crude extract was stored at -20°C to preserve its activity (Se Hoo *et al.*, 2022).

The observation of high PPO activity in Thai brinjal emphasizes the necessity of treatment using effective browning inhibitors, especially considering the comparable PPO levels in other brinjal varieties. This necessitates the exploration of potent inhibitors to counteract enzymatic browning.

3.2 Extraction efficiency of polyphenol oxidase inhibitors from spices

As shown in Table 1, clove had the highest extraction yield at 21.94±1.50%, followed by cinnamon bark and star anise, with yields of 12.00±1.31% and 10.12±0.45%, respectively. Cumin seed, on the other hand, demonstrated the lowest yield at 4.89±0.18%. Investigating the extraction yields of potential PPO inhibitors from spices is critical to evaluate their feasibility as food-based browning inhibitors. In this study, methanol was used as the extraction solvent for its efficiency in extracting phenolic and flavonoid compounds, known for their browning inhibitory properties.

Table 1. Extraction yield of selected spices.

Spices	Extraction yield (%)
Cumin	$4.89±0.18^a$
Cinnamon bark	$12.00±1.31^b$
Fennel seed	$5.29±0.47^a$
Coriander seed	$6.02±0.67^a$
Clove	$21.94±1.50^c$
Star Anise	$10.12±0.45^b$

Values are presented as mean±SD, n = 3. Values with different superscripts within the same column are statistically significantly different in the tested extracts (p<0.05).

Methanol's polarity makes it particularly effective in extracting a broad spectrum of phytochemicals that possess browning inhibition activity (Muzolf-Panek and Stuper-Szablewska, 2021). The variation in yields among the spices is attributed to their differing physical and chemical compositions, affecting their solubility in methanol. For example, clove showed significant (p <

0.05) highest yield is likely due to its abundant essential oils and phenolic content, both highly methanol-soluble, as supported by Trifan *et al.* (2021). Conversely, cumin showed a significant ($p < 0.05$) lower yield which may result from components less soluble in methanol or a lower phenolic content.

These extraction yields are essential to obtain PPO-inhibiting compounds at high concentrations, but also assessing the efficacy of the spice extracts as food-based browning inhibitors. For instance, clove had a high extraction yield but did not directly indicate Thai brinjal PPO inhibition efficacy reported in Table 2. This suggests the efficiency of the extraction yield is independent of the browning inhibition efficiency of the spice extracts.

3.3 Effects of ascorbic acid and selected spices on polyphenol oxidase activity

Ascorbic acid, a well-recognized antioxidant, exhibited significant inhibitory effects on PPO activity in Thai brinjal. The inhibition percentage increased notably from 10.75±0.56% to 37.05±0.00% as its concentration was raised from 0.05 mM to 0.2 mM, with the IC_{50} of 0.271mM (Table 3). This behavior indicates its function as a mixed inhibitor, influencing both the maximum reaction rate (V_{max}) and the enzyme's affinity for the substrate (K_m) of PPO.

In parallel, the study evaluated the inhibitory effects of selected spice extracts on Thai brinjal PPO. These extracts displayed varying degrees of inhibition, with cinnamon bark extract standing out for its high inhibition percentage of 34.20±1.11%, categorizing it as a competitive inhibitor (Table 2). Figure 1 shows the Lineweaver-Burk plot of the effect of cinnamon bark extract on Thai brinjal PPO.

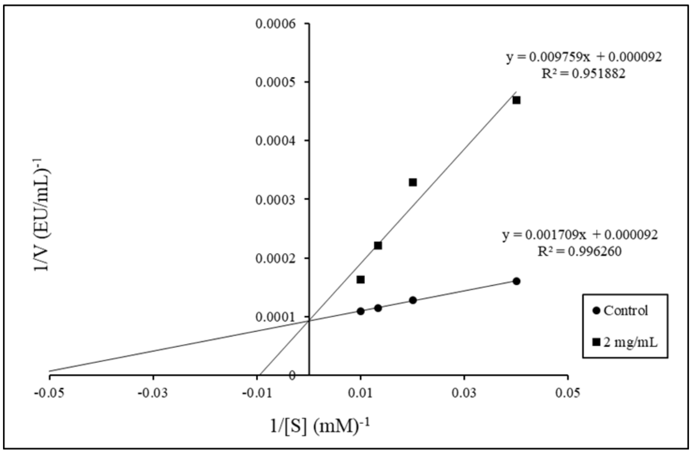

Figure 1. Lineweaver-Burk plot of the effect of cinnamon bark extract on Thai brinjal PPO.

The diverse inhibition mechanisms demonstrated by ascorbic acid are reflective of its complex interaction with different types of PPO and substrates. Ali *et al.* (2015) observed competitive inhibition in lettuce PPO at low concentrations of ascorbic acid, contrasting with its uncompetitive inhibition in sweet potato PPO and *Musa acuminata* '*Mas*' peel PPO (Lim *et al.*, 2019; Siew *et al.*, 2022). This highlights the intricate nature of PPO inhibition, which is dependent on the specific enzyme and substrate involved (Bøjer Rasmussen *et al.*, 2021; Kaya and Bağci, 2021). The mixed inhibition by ascorbic acid, binding to both free enzyme and enzyme-substrate

Table 2. Effects of selected spices extracts on Thai brinjal PPO.

Spices extracts	Inhibition (%)	V_{max} (EU/mL)	K_m (mM)	K_i (mg/mL)	K_i' (mg/mL)	Type of inhibition
Control (5% DMSO)	-	10869.57	18.58	-	-	-
Cumin	7.10±0.90^b	9900.99	19.71	12.59	20.44	Mixed inhibition
Cinnamon bark	34.20±1.11^d	10869.57	106.08	0.42	-	Competitive inhibition
Fennel seed	7.20±0.67^b	10204.08	22.35	36.80	6.39	Mixed inhibition
Coriander seed	3.80±0.67^a	10869.57	26.74	4.57	-	Competitive inhibition
Clove	4.90±0.71^{ab}	10309.28	18.90	27.50	36.80	Mixed inhibition
Star Anise	29.60±1.12^c	10309.28	18.90	25.51	36.80	Mixed inhibition

Values are presented as mean±SD, n = 3. Values with different superscripts within the same column are statistically significantly different in the tested extracts (p<0.05).

Table 3. Effects of ascorbic acid on Thai brinjal PPO.

Inhibitor	[I] (mM)	Inhibition (%)	IC_{50} (mM)	V_{max} (EU/mL)	K_m (mM)	K_i (mM)	K_i` (mM)	Type of inhibition
Control	-	-	-	11627.91	14.26	-	-	-
Ascorbic acid	0.05	10.75±0.56^a	0.271	10204.08	13.19	0.76	0.4	Mixed inhibition
	0.1	21.63±0.23^b		8928.57	12.6	0.61	0.35	
	0.2	37.05±0.00^c		7042.25	11.76	0.53	0.32	

Values are presented as mean±SD, n = 3. Values with different superscripts within the same column are statistically significantly different in the tested extracts (p<0.05).

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complexes, resulted in decreasing both K_i and K_i' values for Thai brinjal PPO, a pattern contrasting with that observed in sweet potato PPO (Lim *et al.*, 2019; Siew *et al.*, 2022b).

The spices, with their varied phytochemical compositions, exhibited different inhibitory mechanisms. Cinnamon bark extract showed the highest K_m value (106.08 mM), indicating a lower affinity for Thai brinjal PPO compared to clove extract, which had the lowest K_m value (18.90 mM) (See Hoo *et al.*, 2022). Cinnamaldehyde, the primary constituent of cinnamon bark, along with other phytochemicals such as caryophyllene, catechins, eugenol and linaloolprocyanidins, are known for their antioxidant properties, contributing to PPO inhibition (Xu *et al.*, 2020; Rao and Gan, 2014). The effectiveness of cinnamon bark as an inhibitor has been demonstrated in various fruits, such as wild banana, jujube fruits and soursop (Xing *et al.*, 2011; Weerawardana *et al.*, 2020). Their PPO inhibition activities were also supported by the high phenolic content and antioxidant capacity further support its role in PPO inhibitio (Shan *et al.*, 2005; Lu *et al.*, 2011).

Antioxidants are the major compounds of fennel (flavonoids, phenolics, reductones), cumin (Caffeic acid, citrusine, isoquercetin, myricetin-3-0-pentoside, p-coumaric acid, rosmarinic acid and vanillic acid) and clove (Eugenol, eugenyl acetate, flavonoids, phenolics, reductones, β-caryophyllene) that contribute to Thai brinjal PPO inhibition (Rani and Meena, 2014; Bouhenni *et al.*, 2021; Kumar Pandey *et al.*, 2022). These compounds have been shown to inhibit PPO activity in various studies reported previously (Chan *et al.*, 2015; Siew *et al.*, 2022; Oriani *et al.*, 2014; Chen *et al.*, 2017).

The diversity in inhibition mechanisms of ascorbic acid and the selected spices aligns with the study's objective to explore the effectiveness of spices as natural inhibitors for PPO in Thai brinjal. Cinnamon bark, with its potent inhibitory effect, emerges as a promising natural browning inhibitor, suitable for application in the food industry.

4. Conclusion

This study presented a significant advancement in the use of food-based browning inhibitors, particularly for the food industry and consumers. It investigated the inhibition of PPO activity in Thai brinjal using spices including cinnamon bark, clove, coriander seed, cumin, fennel seed and star anise. The browning inhibition percentages of these spices ranged from 9.64±0.47% to 34.20±1.11%, with cinnamon bark extract showing the most substantial inhibition via competitive inhibition. Despite ascorbic acid demonstrating greater browning inhibition at lower concentrations, the shift towards natural inhibitors is increasingly preferred for enhancing food security and reducing health risks. The study focused on extracting potential PPO inhibitors from spices, revealing that clove had the highest extraction yield followed by cinnamon bark and star anise. This finding suggests the economic viability of using these spices as natural PPO inhibitors. The varied inhibition mechanisms presented by the spices and ascorbic acid highlight the need for specific inhibitor selection based on the specific food item and its phenolic content. These findings suggest that spices are a viable source of food-based inhibitors to replace synthetic inhibitors. Future research should focus on scaling these inhibitors for industrial use and evaluating their effectiveness across a broader range of food products as part of a techno-economic assessment.

Conflict of interest

The authors declare no conflict of interests.

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