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INHIBITORY EFFECT OF SELECTED SPICES ON POLYPHENOL OXIDASE FROM ICEBERG LETTUCE (*LACTUCA SATIVA* L.)

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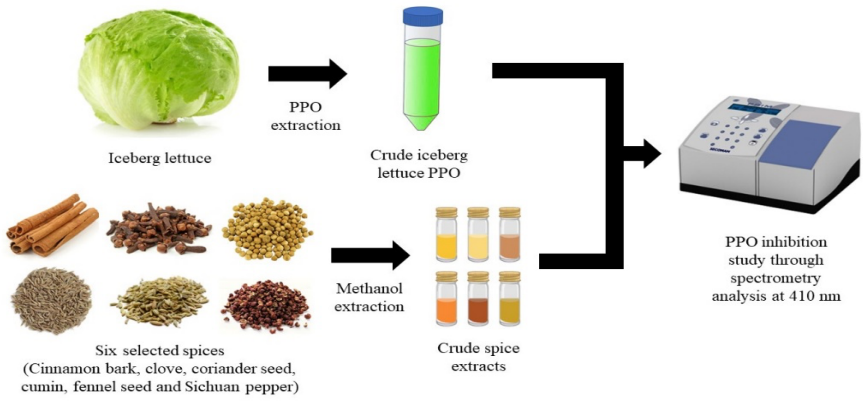
ABSTRACT

Food browning is an undesirable phenomenon that alters sensory properties and nutritional value of fresh-cut produces, which had a significant economic impact owing to decreased customer acceptability. In this study, the effect of selected spices including cinnamon bark, clove, coriander seed, cumin, fennel seed and Sichuan pepper on iceberg lettuce polyphenol oxidase (PPO) inhibition was investigated to replace synthetic browning inhibitor that often used by food manufacturers. Iceberg lettuce PPO has an enzyme activity of 13677.04 ± 21.00 EU/mL, using pyrocatechol as substrate. Ascorbic acid was used as the synthetic inhibitor of Iceberg lettuce PPO and it acted as a mixed inhibitor with the IC_{50} of 4.20 ± 0.19 mM. Among all the browning inhibition effect of the selected spices on Iceberg lettuce PPO, cinnamon bark was the best inhibitor among the six selected spice extracts, with the inhibition percentage of 32.39 ± 1.47 % inhibition was determined, followed by fennel seed (16.93 ± 1.47 %), Sichuan pepper (14.24 ± 0.83 %), cumin (13.72 ± 1.35 %), coriander seed (9.85 ± 0.75 %), and clove (9.64 ± 0.47 %) at 2.0 mg/mL. The present findings suggested the potential to expand the application of the spices to be used as food-based anti-browning inhibitors directly on the surface of iceberg lettuce as well as other fresh-cut produces or used as active ingredient of enzymatic browning-based active packaging of fresh-cut produces.



Graphical Abstract

1.Introduction

Enzymatic browning (EB) is an oxidation reaction that is caused by the catalytic reaction of the enzyme polyphenol oxidase (PPO) with the presence of phenolic compounds and oxygen (Arnold and Gramza-Michałowska, 2022). It is an undesirable reaction on fresh-cut produces as it causes darkening on their surfaces which make them unappealing to the consumers. Physical and mechanical injury such as cutting, peeling, dicing as well as extreme temperature fluctuations can destroy the tissue of the fruits and vegetables during processing or handling (Moon et al., 2020), allowing the interaction of PPO and its phenolic substrates under the presence of oxygen.

PPO (EC: 1.14.18.1) is a copper-containing enzyme from the oxidoreductase family. It can be furthered classified based on cresolase and catecholase activity, where the former catalysed monophenolase activity and the latter catalysed diphenolase activity (Biundo et al., 2020). For instance, cresolase responsible for the hydroxylation of monophenols into *o*-diphenols and catecholase responsible for the oxidase of *o*-diphenols to *o*-quinones. The formation of *o*-quinones will further undergo a nonenzymatic polymerization process, which results in the formation of high molecular-weight pigments, complexed and insoluble brown pigment known as melanin (Jiang et al., 2016).

EB is estimated to result in more than 50 % loss of global fruits and vegetables production (Moon et al., 2020). Therefore, there are various methods used by food industry to prevent the EB such as physical treatments including freezing, blanching, high-temperature short-time (HTST) method, high-pressure process and the application of synthetic anti-browning agents such as reducing agents, antioxidants, acidulants and chelating agents (Ioannou, 2013). However, physical treatments may alter the sensory properties and nutritional qualities of food, while the use of synthetic inhibitor may cause irritation and asthma that could affect the health status of the consumers (Se Hoo *et al.* 2022).

In recent times, the utilization of food and plant-based inhibitors has emerged as a viable option, as they are both safe for consumption and promote food sustainability. Spices are rich in bioactive compounds and can be utilised as food-based preservatives since they improve the nutritional value of food while also increasing its shelf life (Siew et al., 2022). Hence, it may enable food industries to use spices as food-based browning inhibitors to preserve and extend the quality and shelf life of fresh-cut fruits and vegetables, respectively (Siew et al., 2022, Sikora et al., 2021).

Iceberg lettuce (*Lactuca sativa* L.) is one of the famous *Asteraceae* family leafy vegetable. It contains numerous health benefit nutritional contents including carotenoids, polyphenols, vitamin B9, vitamin C and vitamin E that can protect against cancer, cardiovascular diseases, hyperlipidemia, metabolic syndrome, neurodegenerative diseases and osteoporosis (Lafarga *et al.* 2020). However, it is vulnerable to EB that significantly alter the organoleptic and biochemical properties of lettuce, thereby affecting its commercial value (Mai and Glomb, 2013). In this study, six selected spices including cinnamon bark, clove, coriander seed, cumin, fennel seed and Sichuan pepper were examined with their respective PPO inhibition ability on iceberg lettuce.

2. Materials and methods

2.1. Plant and spices materials

Iceberg lettuce (*Lactuca sativa* L.) originated form Cameron Highlands, Malaysia was used as the source of PPO in this study. It was purchased from TF Value-Mart, Cheras, Malaysia. The selected spices including cinnamon bark, clove, coriander seed, cumin, fennel seed and Sichuan pepper were purchased from a local market in Kuala Lumpur, Malaysia. The purchased iceberg lettuce and spices were in good condition and free from damage and spoilage. Iceberg lettuce and spices were stored in 4ºC and room temperature before usage, respectively. All the chemicals and reagents used in this study were analytical grade.

2.2. PPO extraction

The extraction of crude iceberg lettuce PPO was conducted according to Lim and Wong (2018) with slight modifications. Fresh iceberg lettuce (100 g) was washed and cut into fine strips, followed by homogenisation with 200 mL of pre-chilled (4 °C) phosphate buffer (0.1 M, pH 6.8) mixed with 4 g of polyvinylpyrrolidone (PVP) using a pre-chilled (4 °C) blender (Philips, HR2021/75, Malaysia) at maximum speed (3600 rpm) for 1 minute. The homogenate was then centrifuged (Universal 320 R, Hettich, Tuttlingen, Germany) at 7000 rpm at 4 °C for 20 minutes. The enzyme-containing supernatant obtained was proceeded with filtration with the aids of Whatman No. 1 filter paper. The resultant filtrate was the crude PPO extract of iceberg lettuce and store in small aliquots at -20 °C prior to analysis.

2.3. PPO assay

The PPO assay was adapted from the method of Lim and Wong (2018) with slight modifications. The reaction mixture consisted of 1.9 mL of 0.1 M phosphate buffer (pH 6.8) and 1.0 mL of 0.1 M pyrocatechol. The crude PPO (0.1 mL) was added into the reaction mixture and immediately transferred into a cuvette after mixing. The absorbance readings were taken at 15 second intervals over 5 minutes at 410 nm using a spectrophotometer (PRIM, Secoaman, France). The blank solution was consisted of 2.0 mL of phosphate buffer (0.1 M, pH 6.8) and 1.0 mL of pyrocatechol. The initial velocity was calculated from the initial slope of the absorbance against time curve. One unit (EU) of PPO activity is defined as the amount of the enzyme that increased the absorbance by 0.001 per min (Siew *et al.* 2022).

2.4. Effects of ascorbic acid on PPO activity

The effect of ascorbic acid on the iceberg lettuce PPO activity was determined according to the method by Lim et al. (2018) with slight modifications. Ascorbic acid was used as a synthetic inhibitor at different concentrations ranging from 0.05 mM to 0.2 mM. The reaction mixture consisted of 1.0 mL of ascorbic acid, l.0 mL of pyrocatechol at different concentrations (0.025 M, 0.05 M, 0.075 M, and 0.1 M) and 0.9 mL of phosphate buffer (0.1 M, pH 6.8). The crude PPO (0.1 mL) was then added into the reaction mixture and immediately transferred into a cuvette after mixing. The absorbance readings were taken at 15 second intervals over 5 minutes at 410 nm by using a spectrophotometer (PRIM, Secoaman, France). The blank solution was consisted of 1.0 mL ascorbic acid, l.0 mL pyrocatechol and 1.0 mL phosphate buffer (0.1 M, pH 6.8). The type of inhibition, inhibition percentage, Michaelis constant (K_m), maximum velocity (V_{max}) and inhibition constant (K_i) of the synthetic inhibitor were determined. The inhibition percentage of ascorbic acid was also expressed as IC_{50}, which is the concentration of the ascorbic acid required to inhibit 50 % of the enzyme activity (Se Hoo *et al.* 2022).

2.5. Preparation of selected spices extracts

The spices extracts were prepared according to Siew et al. (2022) with slight modifications. Each spice (50 g) was dried overnight in an oven (UNB 100, Memmert, Germany) at 50°C, before grinding (Philips, HR2021/75, Malaysia) them into fine powder at maximum speed (3600 rpm) for 1 minute. Each of the fine grinded spices (10 g) were extracted with 100 mL methanol for 1 hour via maceration. The spices extracts were then filtered by using Whatman No. 1 filter paper to remove the fine grinded spices. The extracts were recovered by using a rotary evaporator (R-200, Buchi, Switzerland) at 50 °C, followed by oven-dried (UNB 100, Memmert, Germany) 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. Effect of the selected spices extracts on PPO activities

The effect of selected spices extracts on iceberg lettuce PPO activities was conducted according to the methods reported by Lim et al. (2018) with slight modification. The reaction mixture including 0.9 mL of phosphate buffer

(0.1 M, pH 6.8), 1.0 mL of pyrocatechol (0.025 M, 0.05 M, 0.075 M and 0.1 M), and 1.0 mL of 2 mg/mL spice extract dissolved in 5 % v/v DMSO. The crude PPO solution was then added quickly into the reaction mixture at 0.1 mL and then immediately transferred into a cuvette. The absorbance readings were taken at 15 second intervals over 5 minutes at 410 nm by using a spectrophotometer (PRIM, Secoaman, France). The blank solution consisted of 1.0 mL of phosphate buffer (0.1 M, pH 6.8), 1.0 mL of substrate and 1.0 mL of 2 mg/mL spice extract dissolved in 5 % v/v DMSO. The type of inhibition, inhibition percentage, Michaelis constant (K_m), maximum velocity (V_{max}) and inhibition constant (K_i) for each spice extract was determined.

2.7. Statistical Analysis

All the experiments in this study were performed with triplicate (n=3) and statistical analysis were done by using Microsoft Office Excel 2016 and IBM SPSS statistics 26. All the data collected were expressed in means ± standard deviation (SD). Analysis of variance (ANOVA) was analysed by using the Tukey's Post Hoc test with significant difference at $p < 0.05$.

3.Results and discussions

3.1. PPO extraction

PPO is an intracellular enzyme that only can be obtained by disrupting the cellular structure of plants. The iceberg lettuce tissues were homogenised using a blender to rupture cell walls and facilitate the release of PPO into the buffer (Sabarre & Yagonia-Lobarbio, 2021).

The pre-chilled phosphate buffer was employed to solubilize the released PPO while retaining its stability (Salis et al., 2007; Sabarre and Yagonia-Lobarbio, 2021). The pH of phosphate buffer was kept at 6.8 to ensure the stability of the enzyme. It coincided with the study of Taranto et al. (2017) that the optimal pH of lettuce PPO reported were between pH 5.0 to 8.0. Apart of the buffer pH, the concentration of the buffer will also affect PPO stability, where the ability to stabilise the pH of the buffer increases with the buffer concentration. Nevertheless, most enzymes preferred the moderate ionic strength between 0.05 to 0.2 M (Papaneophytou, 2021).

The phenolic compounds naturally present in iceberg lettuce can transform into polymeric pigments by the catalytic action of PPO (Sabarre & Yagonia-Lobarbio, 2021). Inhibit phenolic oxidation and polymerization during homogenization can be done by adding polyvinylpyrrolidone (PVP) to PPO extract in order to adsorb the phenolic substrates as well as inhibit oxidation (Lim and Wong, 2018). Centrifugation was carried out to separate PPO from the suspended particle in the homogenate, where the PPO remains in the supernatant. The PPO extract was then stored at -20 °C to retain enzyme activity (Tian et al., 2014).

3.2. PPO activity

The iceberg lettuce PPO had an enzyme activity of 13677.04 ± 21.00 EU/mL at pH 6.8 and room temperature, when pyrocatechol was used as substrate. It had superior PPO activity when compared to ginger PPO (9040 EU/mL), pearl brinjal PPO (11200 EU/ml) and '*Mas*' banana peel PPO (9000 ± 43.2 EU/mL), respectively when pyrocatechol was used as the substrate (Lim & Wong 2018; Se Hoo et al., 2022; Siew et al., 2022).

Iceberg lettuce was usually stored at refrigerated temperature to maintain the freshness and its quality to a greater extent of period (Meena et al. 2022). However, storage of iceberg lettuce at low temperature will only reduce the PPO activity but not deactivate the PPO activity completely. Therefore, PPO inhibitor can be incorporated to further delay the browning process of the iceberg lettuce where they are ready to be sold on the refrigerated shelf spaces. The application of browning inhibitor may also reduce the needs of refrigerated shelf spaces to store iceberg lettuce and other fresh-cut produces.

3.3. Effect of ascorbic acid on PPO activity

Ascorbic acid is an antioxidant and acidifying-based synthetic browning inhibitor. It

suppresses EB by reducing oxidised substrates and lowering the pH of the environment (Moon et al., 2020). However, browning will still occur when ascorbic acid is completely oxidized into dehydroascorbic acid in the later stage. The inhibition percentage of the ascorbic acid increased significantly ($p < 0.05$) from 4.51 ± 0.26 to 26.40 ± 0.91 % with its concentration from 0.05 to 0.2 mM (Table 1). Similar findings were reported by Sikora et al. (2019) that increasing the concentration of ascorbic acid increased the inhibition percentage. The IC_{50} of ascorbic acid was 4.20 ± 0.19 mM, which is the concentration of ascorbic acid required to inhibit 50 % of iceberg lettuce PPO activity.

It was observed that the V_{max} decreased when increasing the concentration of ascorbic acid (Table 1). This implies that ascorbic acid lowered the maximal reaction rate by reducing PPO catalytic reaction. The reduction of PPO catalytic reaction was supported by the increase of K_m, (Table 1), which indicates the binding of ascorbic acid to the PPO reduces the binding affinity between PPO and pyrocatechol.

The decreased of V_{max} and increased of K_m also suggested that ascorbic acid acts as a mixed inhibitor on iceberg lettuce PPO. The mixed inhibition of ascorbic acid was aligned to the previous study conducted by Doğan & Salman (2007) on iceberg lettuce PPO. However, ascorbic acid was also previously reported as competitive inhibitor and non-competitive inhibitor on blackberry, lotus root and lettuce PPOs (Doğan & Salman, 2007; Wong et al., 2019; Azzouzi et al., 2022). The diverse inhibition types observed in different studies underscore the influence of different PPO origin and substrates on inhibition characteristics (Gouzi et al., 2010).

K_i is the dissociation constant of the enzyme–inhibitor complex, which represents the binding affinity of inhibitor towards PPO (Doğan & Salman, 2007). The K_i values were present in Table 1, where the K_i' values were higher than K_i. The K_i'/K_i ratio was expected to be greater than one, indicating that the inhibitor's affinity for free PPO is greater than PPO-pyrocatechol complex (Gouzi et al., 2010). This finding indicates that the inhibitor can bind to both free PPO and PPO-pyrocatechol complex with different equilibrium constants for each interaction (Gouzi et al., 2010).

Table 1. Effects of ascorbic acid on iceberg lettuce 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	-	-	-	16030.96	19.04	-	-	-
Ascorbic acid	0.05	4.51 ± 0.26[a]	4.20 ± 0.19	15715.09	23.00	2.51	0.22	Mixed
	0.10	14.26 ± 0.54[b]		14734.97	26.87	1.14	0.19	
	0.20	26.40 ± 0.91[c]		13323.54	32.96	0.99	0.20	

[a-c]Means ± standard deviations followed by different superscript letters within the same column are significant different at $p < 0.05$ according to Tukey's test.

3.4. Extraction yields of selected spices

Methanol was used as an extraction solvent in the preparation of spice extract. This is because methanol was an effective solvent to extracting potential anti-browning polyphenol and flavonoid compounds with high yields (Norsyamimi et al., 2014; Rezaei & Ghasemi Pirbalouti, 2019). The studies of Dong et al. (2004), El-Ghorab et al. (2010) and Shahwar et al. (2012) showed that methanol can obtain greater spices extracts' yield as compared to ether and hexane.

The extraction yield of the six selected spices was tabulated in Figure 1. Clove shows the highest extraction yields (21.94 ± 1.50 %) among the six selected spices, followed by Sichuan pepper (13.25 ± 0.99 %) and cinnamon bark (12.00 ± 1.31 %). There was no significant ($p > 0.05$) different between the extraction yields of coriander seed, cumin and fennel seed. These

spices show the lowest extraction yields, ranging from 4.89 ± 0.17 to 6.03 ± 0.67 %.

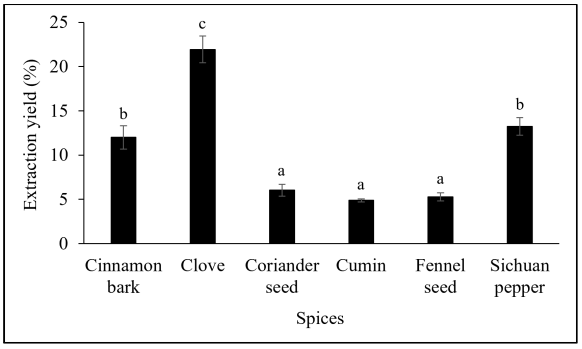


Figure 1. Extraction yield of the six selected spices.

[a-c]Different superscript letters within the same column are significant different at $p < 0.05$ according to Tukey's test.

3.5. Effects of selected spices extracts on PPO activities

The inhibition effect of the selected spices extracts on iceberg lettuce PPO were showed in Table 2. The inhibition percentage of the selected spices extracts were ranging from 9.64 ± 0.47 to 32.39 ± 1.47 %. Cinnamon bark extract (32.39 ± 1.47 %) had the highest inhibition percentage among all the selected spices extracts. It acts as a mixed inhibitor on iceberg lettuce PPO based on the Lineweaver-Burk plot plotted as shown in Figure 2.

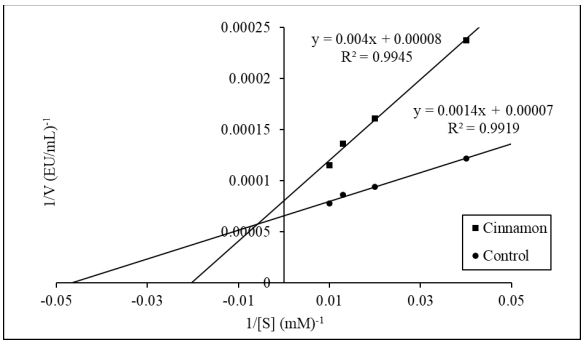


Figure 2. Lineweaver-Burk plot of the effect of cinnamon bark extract on iceberg lettuce PPO.

The mixed inhibition of cinnamon bark extract was also supported by the decrease of V_{max} and increase of K_m. Clove and Sichuan pepper extracts were also identified as mixed inhibitors based on the decreasing and increasing of the V_{max} and K_m, respectively (Table 2). Meanwhile, coriander seed, cumin and fennel seed extracts were acted as competitive inhibitors, where the V_{max} remained unchanged and the K_m increased (Table 2). Mixed inhibitor exhibits inhibition activity by binding to both free PPO and PPO-pyrocatechol complex, altering catalytic capacity, whereas competitive inhibitor exhibits inhibition activity by competing the PPO binding site with pyrocatechol (Roberts & Gibb, 2013; Ramsay & Tipton, 2017).

Cinnamon bark appeared to be the best and most effective inhibitor among the selected spices used in this study. The anti-browning activity of cinnamon bark were contributed by various bioactive compounds such as cinnamaldehyde, eugenol and cis-2-methoxycinnamic acid (Siew et al., 2022). The browning inhibition activity of cinnamon bark had also been demonstrated on banana, mushroom and soursop PPO, with the inhibition percentage ranging from 40 to 51.97 % (Marongiu et al., 2007; Weerawardana et al., 2020). It was also identified as the most effective browning inhibiting spices by Rossi et al. (2019) and Weerawardana et al. (2020).

Eugenol is also the most prevalent bioactive compound found in clove. It is responsible for its fragrant as well as the anti-browning properties. The browning inhibition of clove essential oil and eugenol emulsion had been reported by Chen et al. (2017) and Teng et al. (2020), which they were effective to inhibit the browning of fresh-cut lettuce and Chinese water chestnut.

The browning inhibition activity of cumin could be related to its major bioactive compound known as cuminaldehyde (Sunohara et al., 2021). Peng et al. (2023) reported cuminaldehyde is a strong tyrosinase inhibitor that can be found in cumin. Siew et al. (2022) had demonstrated the browning inhibitory effect of cumin and suggested that cumin could exhibited greater browning inhibition activity than the medicinal plant parts such as *Swietenia macrophylla* seeds and *Eurycoma longifolia* roots reported by Hassan et al. (2015).

Sichuan pepper contained a wide range of bioactive compounds including volatile oils, phenols and alkaloids, which phenols and alkaloids were the major antioxidant of Sichuan pepper (Sun et al., 2020). Phenolic compounds such as quercetin, hyperoside, kaempferol-3-O-rhamnoside, myricetin-3-O-β-D-galactopyranoside, rutin and dicoumarol and alkaloids such as alkylamides, tetrahydroberberine, α-sanshool, bungeanool, isobungeanool and dihydrobungeanool present in Sichuan pepper could also possessed anti-browning activity (Sun et al., 2020). According to Nirmal et al. (2015), phenolic compounds from plant extract can function as antimelanotics, owning to their metal chelating activity and structural similarity with PPO substrate (Nirmal et al., 2015).

The browning inhibition of both coriander seed and fennel seed had been reported previously. Lee & Kim (2020) observed a significant ($p < 0.05$) decrease in potato PPO activity with increasing fennel concentration. Meanwhile, coriander seed was found to have inhibitory effect on tyrosinase enzyme with IC_{50} of 13.94 ± 1.86 mg/mL (Siew et al., 2022). Their inhibition activity may be attributed to their flavonoid compounds (Mandal & Mandal 2015; Barakat et al., 2022). The flavonoid compounds might have synergistic browning inhibition effect with the presence of phenolic hydroxyl group in the fresh-cut produce, where increasing the concentration of phenolic hydroxyl group in the flavonoid compound showed positive impact on suppressing potato PPO (Jun et al., 2019). Nevertheless, the particular mechanism of driving flavonoid synergy is yet unknown and has to be researched further.

Table 2 Effects of selected spices extracts on iceberg lettuce PPO at 2 mg/mL

Spices extracts	Inhibition (%)	V_{max} (EU/mL)	K_m (mM)	K_i ' (mM)	K_i (mM)	Type of inhibition
Control (5 % v/v DMSO)	-	15215.84	21.41	-	-	-
Cinnamon bark	32.39 ± 1.47^{d}	12431.67	49.08	9.00	1.11	Mixed
Clove	9.64 ± 0.47^{a}	14098.05	24.15	25.31	9.20	Mixed
Coriander seed	9.85 ± 0.75^{a}	15221.34	35.11	-	3.13	Competitive
Cumin	13.72 ± 1.35^{b}	15210.58	39.44	-	2.38	Competitive
Fennel seed	16.93 ± 1.47^{c}	15219.01	45.70	-	1.76	Competitive
Sichuan pepper	14.24 ± 0.83^{bc}	13889.12	28.57	20.98	4.33	Mixed

$^{a-d}$Means ± standard deviations followed by different superscript letters within the same column are significant different at $p < 0.05$ according to Tukey's test.

4. Conclusion

The potential of spices of being food-based browning inhibitor had been studied on the inhibition of iceberg lettuce PPO. The browning inhibition percentage of the selected spices including cinnamon bark, clove, coriander seed, cumin, fennel seed and Sichuan pepper on iceberg lettuce PPO were ranging from 3.80 ± 0.67 to 32.39 ± 1.47 % at 2 mg/mL. Cinnamon bark extract exhibited the highest significant ($p < 0.05$) inhibition percentage (32.39 ± 1.47 %) on iceberg lettuce PPO via mixed inhibition. Although the synthetic ascorbic acid was more effective in inhibiting RB than cinnamon bark extract at lower concentration, the preference for spices being used as food-based browning inhibitor aligns with contemporary consumer demands for health, organic and sustainable options. Besides extending the shelf life of fresh-cut fruits and vegetables, the application of spices also addresses health concerns by eliminating synthetic additives. Therefore,

spices are the potential food-based browning inhibitor to replace synthetic inhibitor to inhibit EB of fresh-cut produces.

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