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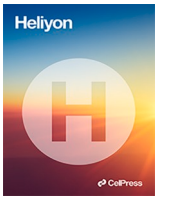

Research article

Non-competitive inhibition of acetylcholinesterase by jaboticaba (*Myrciaria cauliflora*) peel ethanolic extracts

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ABSTRACT

Specific anthocyanins and phenolic compounds exhibit acetylcholinesterase inhibitory (AChEi) activity. In this study, the AChEi activity of jaboticaba peel extracts were investigated based on their high phenol contents. Jaboticaba peel ethanolic extract (PEX) and aqueous extract (PAX) were prepared by extracting jaboticaba peel with 95% ethanol and boiling water, respectively. Through HPLC-MS/MS and HPLC-PDA analysis, gallic acid was identified in PAX with a concentration of 598.13 ± 42.43 mg/100 g extract, and ellagic acid in PEX with a concentration of 350.47 ± 8.53 mg/100 g extract. Both PEX and PAX showed dose-dependent inhibition against AChE activity, with IC_{50} values of 3.54 and 4.07 mg/mL, respectively. The mechanism of inhibition of PEX was determined to be non-competitive inhibition based on the decreasing V_{max} and relatively constant K_m with increasing PEX concentration, as determined using a Lineweaver-Burk plot.

1. Introduction

Alzheimer's disease (AD) is the most common form of dementia, a progressive neurological disorder characterized by cognitive and memory impairments, severe enough to interfere with daily life [1]. In addition to neurofibrillary tangles and neuritic plaques hallmarks, it is neurochemically characterized by a constant deficiency in cholinergic neurotransmission, affecting cholinergic neurons in the basal forebrain in particular. There are multiple physiological processes associated with AD degeneration, including the degradation of cells that synthesize and utiliz neurotransmitter acetylcholine (ACh), thus diminishing the number of neuron cells available to transmit messages to other cells [1].

ACh works in the autonomic nervous system (in the preganglionic sympathetic and parasympathetic neurons), peripheral nervous system (at the neuromuscular junction between the motor nerve and skeletal muscle), and central nervous system (in interneurons) [1]. Choline acetyltransferase synthesizes ACh from acetyl-coenzyme A (acetyl-CoA) and choline in the cholinergic neurons. The choline substrate is continuously recovered and delivered to neurons and the acetyl-CoA substrate is obtained from glycolysis. ACh is concentrated in vesicles by the vesicular ACh transporter, and by depolarization, released from the presynaptic cell. The released ACh interacts with muscarinic receptors (M1-M5) or nicotinic receptors (with 3 main CNS subtypes - IA, II and III) on post-synaptic and

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pre-synaptic cells. The post-synaptic muscarinic ACh and nicotinic receptors activations causes activation of biochemical pathways or depolarization of the target cell and, finally the transmission of the nerve impulse. Subsequently, acetylcholinesterase (AChE) rapidly inactivates ACh by breaking it down to acetate and choline, thus preventing interactions with ACh receptors [2].

AChE (EC 3.1.1.7) is a serine hydrolase found predominantly at neuromuscular junctions and cholinergic brain synapses. Its primary function is to rapidly hydrolyse the neurotransmitter ACh to acetate and choline, thus terminating the impulse transmission at cholinergic synapses. AChE has a remarkably high specificity and catalytic activity. Each molecule of AChE degrades about 25,000 molecules of ACh per second [1].

As of now, there is no drug to stop or halt AD progression, and patients with mild to moderate AD are treated with reversible AChE inhibitor (AChEi) to improve symptoms related to memory, thinking, language, decision-making, and other thought processes. As a short-term symptomatic intervention, this drug inhibits AChE activity, decreasing its ACh breakdown rate, and maintaining ACh level and duration. Therefore, the cholinergic neurotransmission is enhanced, and the brain function could be improved [1]. The prolonged ACh activity may partially improve cognitive symptoms and quality of life, diminishing caregiver burden for patients with mild to severe AD [3]. In addition to AD, AChE inhibition also serves as a strategy for the treatment of ataxia, senile dementia, myasthenia gravis and Parkinson's disease [4].

AChE inhibitors such as donepezil, rivastigmine, and galantamine are dominating the approved medications for treating AD; however, these AChE inhibitors tend to produce a dose-dependent acute gastrointestinal symptoms [5,6]. A systematic review by Takeda et al. [7] concluded that the rates of adverse effects were generally higher in the galantamine-treated than in the donepezil-treated patients, and generally higher in the rivastigmine-treated patients than in the donepezil-treated patients [7]. The notable adverse effects of donepezil, rivastigmine, and galantamine underline the interest in finding better AChE inhibitors.

Various potential AChE inhibitors has been reported in studies on AChE inhibitors. *Achillea pseudoaleppica* extract rich of quinic acid showed inhibition to AChE with IC_{50} of 2.67 mg/mL [8]. Similarly, extracts of *Thymus migricus* also showed potential AChEi activity with IC50 of 18.23 mg/mL [9]. Several metal complexes and its azo ligand were evaluated for their AChEi activity, and the Co(II) complex showed the most effective inhibition [10].

Anthocyanins possess great neuroprotective potential. Anthocyanins and their major component, cyanidin-3-O-glucoside (C3G), may act as a preventive and/or therapeutic means in various disorders in the central nervous system, including Alzheimer's disease and Parkinson's disease [11]. In AD model using scopolamine induction, anthocyanin reduced anxiety-like behavior and restored AChE activity in both cortex and hippocampus, and also prevented memory deficit [12]. Cyanidin-3-O glucoside alone does not have AChE inhibition properties [5], while reports of AChE inhibition properties of delphinidin 3-O glucoside are scarce. On the contrary, cyanidin and delphinidin, their aglycone, showed AChE inhibition with IC_{50} of 14.43 $\pm$ 0.31 and 44.67 $\pm$ 0.49 μM, respectively [6]. Protocatechuic acid, a gut microbiota-mediated metabolite of cyanidin- and delphinidin-3-O glucoside, showed *in vitro* AChE inhibition with IC_{50} of 6.50 $\pm$ 0.43 μmol/μmol AChE [13].

In this study, the AChE inhibitory potential of ethanolic extracts of Jaboticaba (*Myrciaria cauliflora*) peel was evaluated based on the high anthocyanin content in jaboticaba peels. Inada et al. [14] reported that the main compounds in jaboticaba peels, in order of concentration from high to low, are cyanidin-3-O-glucoside, ellagic acid, delphinidin-3-O-glucoside, rutin, and gallic acid (the rest are less than 1 % w/w over the total detected phenolic).

2. Materials and methods

2.1. Jaboticaba fruits (Myrciaria cauliflora)

Jaboticaba fruits were purchased from farmers in Changhua, Taiwan, washed three times in running tap water upon arrival, and stored immediately at −20 °C prior to use.

2.2. Chemicals

Aluminum chloride ($AlCl_3$) was purchased from Honeywell Riedel-de Haën (Hessen, Germany). Folin & Ciocalteu's phenol reagent, gallic acid, kaempferol, rutin, AChE, acetylthiocholine iodide (ATCI), and 5,5′-dithio-bis 2-nitrobenzoic acid (DTNB), disodium hydrogen phosphate dihydrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ellagic acid and quercetin were obtained from ChromaDex, Inc. (Irvine, CA, USA). HPLC-grade Methanol was obtained from Honeywell (Honeywell International Inc., Muskegon, Michigan, U.S.), and sodium carbonate (Na_2CO_3) was purchased from Showa Chemical Industry (Tokyo, Japan). Galantamine hydrobromide was purchased from Medchem express (Monmouth Junction, NJ, USA).

2.3. Jaboticaba peel extraction

Two kinds of extracts were prepared from the fruit peel: 95 % ethanol extract (PEX) and aqueous extract (PAX). The fruits were thawed, and the peel was manually separated from the pulp. The peel was rinsed with distilled water (dH_2O), freeze dried, and powdered with a blender (Osterizer Designer Cycle Blend 880-20 M, U.S.). For PEX, the lyophilized peel powder was immersed in 95 % ethanol with a ratio of 1:9 (w/v) and stirred with magnetic bar at 4 °C for 24 h. After centrifugation at 6000 rpm for 10 min 4 °C (Himac CR22G, Hitachi Koki, Tokyo, Japan) followed by vacuum filtration, the solid matter was discarded, and the extracted liquid was concentrated in a vacuum rotary evaporator (Eyela N-1 Tokyo Rikakikai Co. Ltd, Tokyo, Japan). Each batch of extracted liquid was stored at 4 °C and then mixed with extracts from other extraction batches to ensure the homogeneity of the extracts. After that, the

extracted liquid was freeze-dried (Eyela FD-1 Tokyo Rikakikai Co. Ltd, Tokyo, Japan) and stored at −80 °C prior to use. For PAX, the preparation process was the same as for PEX except that the freeze-dried peel was immersed in boiling double-distilled water (ddH_2O) with a ratio of 1:9 (w/v) for 2 h.

2.4. Identification of bioactive compounds

The extract and standard solution were prepared fresh separately by dissolving each standard compound with MeOH. This HPLC method was designed based on the method by Pereira et al. [15] with modifications. The analysis was performed with the Waters 600 HPLC System, which consists of a Waters 996 photodiode array detector, a 600 solvent pump, a 600 controller, a 717 autosampler, and a data processor Empower Pro Software (Marshall Scientific, Hampton, New Hampshire, U.S.). The separation was performed using Mightysil RP-18 GP (250 mm × 4.6 mm, 5 μm; Kanto Chemical Co., Inc., Tokyo, Japan). Degassed 10 mM phosphate buffer (pH 2.7) and MeOH (100 %) were used as mobile solution A and B, respectively. The separation program used a linear gradient elution as follows: 0–30 min, 100-50 % A; 30–50 min, 50-20 % A, 50–60 min, 20–100 % A; followed by column re-equilibrating with 100 % A for 10 min. The flow rate was set constantly at 0.7 mL/min. The injection volume was 10 μl, and all standards and samples were injected in 5 replicates. The wavelength for each target compound was as follows: 270 nm for gallic acid, 254 nm for ellagic acid, and 360 nm for rutin, quercetin, and kaempferol. Chromatographic peaks were identified by comparison to the retention time and UV spectra of those standards. Samples were spiked with reference compounds, and an external standard method was used for quantification.

2.5. Total anthocyanin, total phenolic, and total flavonoid content

The total phenolic content was determined with the Folin-Ciocalteu reagent [16]. All steps were performed in the dark. The total phenolic content was expressed in mg of gallic acid equivalent per g of sample. The total flavonoid assay was performed using the aluminum chloride colorimetric assay [17]. The total flavonoid content was expressed as mg rutin eq./g sample. The total anthocyanin analysis was performed according to Padmavati et al. [18].

2.6. Acetylcholinesterase inhibition assay

The protocol for measurement of acetylcholinesterase (AChE) inhibition activity was modified from Ellman et al. [19]. PEX and PAX were dissolved in 0.1 M phosphate buffer (pH 8.0) to appropriate concentrations. A substrate mixture of AChE was prepared by mixing 4 mL of 3 mM dithiobisnitrobenzoate (DTNB) in 50 mM phosphate buffer (pH 7.0), 1.0 mL of 15 mM acetylthiocholine iodide (ATCI) in ddH_2O, and 4.2 mL of 0.1 M phosphate buffer (pH8.0). The substrate mixture was then incubated in a water bath (37 °C) for 10 min. In a 96-well plate, 167 μl of substrate mixture, 50 μl of jaboticaba extract, and 20 μl of AChE (0.25 U mL^{-1} in 50 mM phosphate buffer, pH 8.0) was added to each well. It was mixed in a microplate reader (Fluostar Optima, BMG Labtech) and then the increasing absorbance was recorded using 412 nm at 37 °C for 5 min in 30 s intervals. The initial reaction rate of AChE was determined by calculating formation rate of yellow dianion of 5-thio-2-nitrobenzoic acid (TNB) using the known molar attenuation coefficient of the TNB of 13.8×10^3 M^{-1} cm^{-1} [20]. Concentrations of samples that inhibited 50 % of AChE activity under the experimental conditions were defined as the IC_{50} values. Galantamine hydrobromide (0~0.00125 mg/mL) was used as a positive control.

The mode of inhibition of jaboticaba extracts was determined according to Rogers and Gibon [21]. The Michaelis-Menten equation

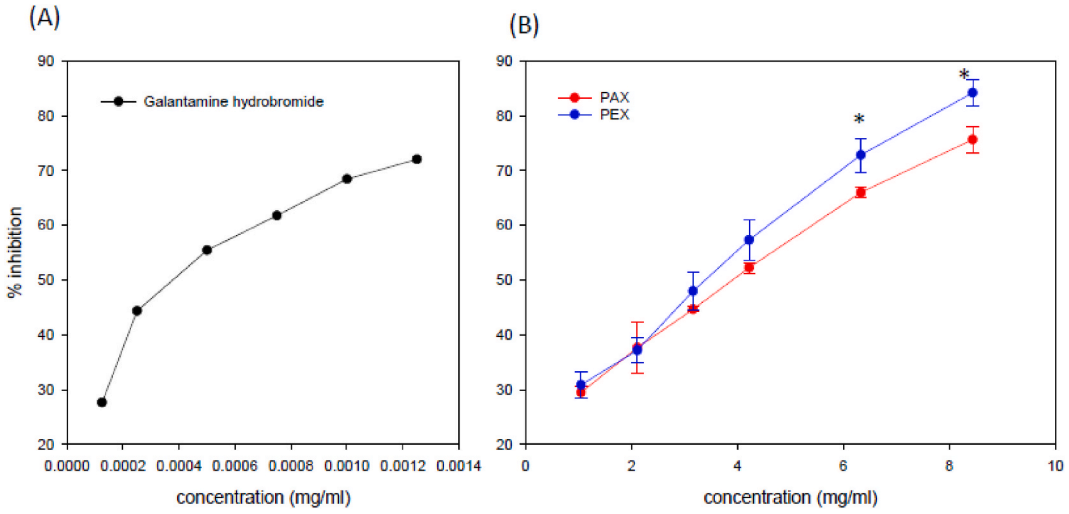

Fig. 1. (A). Galantamine hydrobromide was used as a positive control.(B). Inhibition of AChE activity by the PEX (peel ethanolic extract) and PAX (peel aqueous extract). *p ≤ 0.05, independent sample *t*-test showing significant difference between PEX and PAX.

was transformed to a double reciprocal Lineweaver-Burk plot, where the linear equations of Lineweaver-Burk plots were used to calculate the K_m and V_{max} values [22].

2.7. Statistical analysis

All data were presented as mean ± standard deviation ($n = 3$). The data were analyzed for distribution and homogeneity by the Kolmogorov-Smirnov test and the Levene's test, respectively. Since our data did not fulfill the assumptions of the parametric test, we used a non-parametric independent sample *t*-test. All statistical analyses were carried out using IBM SPSS Statistic 24 software (International Business Machines Corp., U.S.).

3. Results

In this research, both PEX and PAX demonstrated a dose-dependent inhibition against AChE activity. PEX and PAX showed linear regression of $y = 7{,}46\ x + 23.59$ ($R^2 = 0.99$) with $IC_{50} = 3.54$ mg extract mL^{-1} and $y = 6.30\ x + 24.35$ ($R^2 = 0.99$) with $IC_{50} = 4.07$ mg extract mL^{-1}, respectively (Fig. 1). However, extract concentrations exceeding 6.33 mg/mL resulted in a non-linear absorbance trend ($R^2 < 0.99$) in the initial reaction rate. This irregularity might be attributed to the intense purple color of the extract, which could've interfered with the spectrophotometry measurement.

Since PEX exhibited greater inhibition than PAX, we further investigated the possible inhibition mechanism of PEX using the Lineweaver-Burk plot as a tool. We found that PEX showed a non-competitive type of inhibition on the AChE activity (Fig. 2). This type of inhibition suggested that PEX could bind to both the enzyme alone and the substrate-enzyme complex with equal affinity. The K_m and V_{max} were determined using the initial rate of reactions for substrate concentrations ranging from 0.13 to 0.77 mM. In the absence of PEX, the V_{max} was 9.66 ± 0.89 μmol L^{-1} min^{-1}. The V_{max} value significantly decreased with the increase in PEX concentration in a dose dependent manner, reaching 4.99 ± 0.26 μmol L^{-1} min^{-1} at PEX concentration of 3.16 mg/mL (Table 1). On the other hand, the K_m values were not significantly different across all extract concentrations (approximately 0.08–0.10 mM ATCI, Table 1).

The gallic acid, ellagic acid, total phenolic content, total flavonoid content and total anthocyanin content in PAX and PEX were determined, with the results displayed in Table 2. Through HPLC analysis, gallic acid was identified in PAX at a concentration of 598 ± 42 mg/100 g extract, while ellagic acid was found in PEX at a concentration of 350 ± 8 mg/100 g extract. Notably, the total anthocyanin content in PEX is three times higher than that in PAX. Additionally, both the total flavonoid and total phenolic contents were found to be higher in PEX compared to PAX.

4. Discussions

One of the earliest theories of pathogenesis of AD is the cholinergic hypothesis, which identifies one of the main pathological features as a deficiency in the brain neurotransmitter ACh. This deficiency leads to abnormalities in the correlated cholinergic system, causing intellectual impairment, including decreasing cognitive symptoms [3]. These symptoms can be temporarily alleviated with AChEi drugs, which work by inhibiting AChE from degrading the neurotransmitter ACh. This allows for the accumulation of ACh, increasing both the level and duration of the neurotransmitter's action [1], thus allowing patients to show an alleviated AD condition. Currently approved drugs for AD treatments are mostly AChE inhibitors, such as tacrine, donezepil, rivastigmine and galantamine, all of which are limited by side effects [23,24]. Therefore, the exploration of novel potential AChEi with less toxicity and more profound activity is still required. The present study demonstrates the effectiveness of both the ethanolic and aqueous extracts of jaboticaba peel (PEX and PAX, respectively) in inhibiting AChE activity. Our results revealed a higher activity in PEX compared to PAX. Siebert et al. [25] have reported that AChEi activity of jaboticaba juice (whole fruit juice) was comparable to that of the approved AChEi drug,

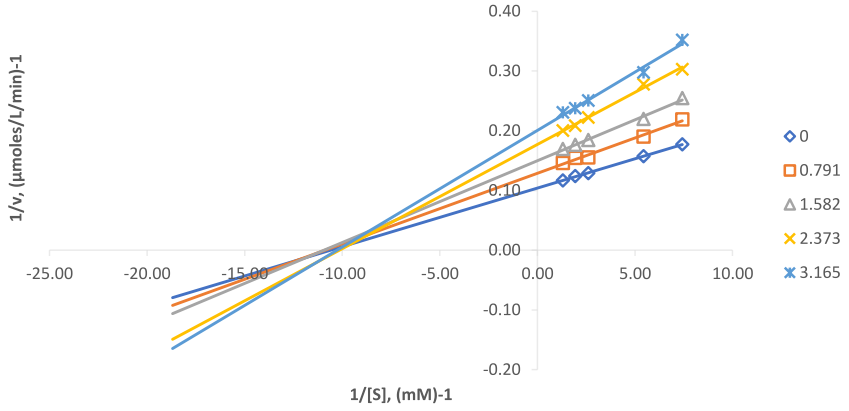

Fig. 2. Double reciprocal plot of initial rate of reaction versus the concentration of the substrate ATCI, across varying concentrations of the peel ethanolic extract (PEX) ranging from 0 to 3.16 mg/mL.

Table 1
K_m and V_{max} for the inhibitory effect of PEX on AChE activity.

PEX Concentration (mg/mL)	V_{max}	K_m
0	9.66 ± 0.89[a]	0.09 ± 0.01
0.79	7.77 ± 0.29[b]	0.09 ± 0.01
1.58	6.69 ± 0.32[bc]	0.09 ± 0.01
2.37	5.57 ± 0.30[cd]	0.08 ± 0.01
3.16	4.99 ± 0.26[d]	0.10 ± 0.00

[a-d] Different letter in the V_{max} column indicates significant difference ($p < 0.05$, One-Way ANOVA followed by Tukey Test). No significant difference among groups in K_m value ($p \geq 0.05$, One-Way ANOVA).

Table 2
Active compounds in PEX and PAX.

Assays	PEX	PAX
Gallic acid (mg/100 g extract)	N.D.	598 ± 42
Ellagic acid (mg/100 g extract)	350 ± 8	N.D.
Total phenolic content (mg gallic acid eq./g sample)	8.16 ± 0.13	3.82 ± 0.13
Total flavonoid content (mg rutin eq./g sample)	4.80 ± 0.55	3.18 ± 0.14
Total anthocyanin content (mg cyanidin-3-glucoside eq./g sample)	12.93 ± 0.40	2.66 ± 0.04

N.D.: not detected.

neostigmine. In Siebert et al. [25], jaboticaba juice (5 mg/mL) had an inhibitory activity toward AChE of 85.90 ± 1.73 %, while the present study showed PEX and PAX had an inhibitory activity toward AChE of 84.21 ± 2.45 % and 75.64 ± 2.45 % at the concentration of 8.44 mg/mL, respectively.

Jaboticaba peel contains various phenolic compounds, which may play a role in its AChEi properties. According to the report by Siebert et al. [25], both gallic acid (217.0 ± 54.2 μg/L) and ellagic acid (193.9 ± 66.0 μg/L) were detected in Jaboticaba juice. Our results showed that gallic acid and ellagic acid were identified in PAX and PEX, respectively. Gallic acid is known as a strong AChEi with IC_{50} of 5.85 μM, which is stronger than ellagic acid with an IC_{50} of 45.63 μM [26]. In comparison, galantamine, a natural USFDA-approved AChEi, showed IC_{50} of 3.2 μM, much stronger than gallic acid or ellagic acid [26]. PAX, which contains a high gallic acid content, was expected to exhibit a higher AChE inhibition activity than PEX. However, it was not observed in this study.

The higher inhibition activity of PEX was likely related to the higher total anthocyanin, total flavonoid, and total phenolic content extracted in the ethanol solvent. Another study reported that the extraction of jaboticaba peel with ethanol could yield a phenolic content up to three times higher compared to an aqueous extract [27]. In the present study, the total phenols and total anthocyanins of PEX were 2.13 times and 4.86 times that of PAX, respectively. The major anthocyanins in jaboticaba, cyanidin- and delphinidin-3-O-glucoside, possess strong AChE inhibition activity in their anthocyanidin forms, which could explain our results [6]; However, anthocyanins were not detected in our samples, which may be due to the mixing of various glucoside derivate, making them difficult to detect in the crude extract. Garcia-Oliveira et al. [28] compared different methods, including mass spectrometry (MS), nuclear magnetic resonance (NMR), and high-performance liquid chromatography (HPLC) to identify and quantify anthocyanins, and suggested that LC-MS represents a quick and efficient technique. However, Garcia-Oliveira et al. [28] pointed out that in addition to the complexity of the sample matrix, extraction protocols vary depending on the source of anthocyanins, making identification and quantification of anthocyanins difficult.

Different AChE inhibitors work through different mechanisms. For example, galantamine acts as a competitive inhibitor, donepezil as a mixed type inhibitor, and rivastigmine as a non-competitive inhibitor. The possible inhibition mechanism of PEX was investigated using a Lineweaver-Burk plot. A Lineweaver-Burk plot, a double reciprocal plot derived from the Michaelis-Menten equation, allow us to distinguish inhibition mechanism based on how inhibitor binds to the enzyme molecules. Our results showed a decreased V_{max} but a relatively constant K_m with an increasing PEX concentration. The double reciprocal graph of PEX is similar to that of a non-competitive inhibition mechanism.

The K_m value is defined as the substrate concentration needed for half of V_{max} in a steady state, and it roughly represents affinity of the substrate to the enzyme. The constant K_m in our result indicated that PEX inactivated the enzyme by binding to a site distinct from the active site, without competitively affecting the affinity of the substrate to the enzyme. PEX may inhibit AChE by binding to the ES complex (the enzyme bound with substrate) as well as to free enzyme molecules. In this type of inhibition, an increasing concentration of the substrate cannot affect the inhibition. This mechanism of inhibition differs from the competitive inhibition mechanisms of gallic acid and ellagic acid, as reported by Kim et al. [29] and Abd El-Aziz et al. [30]. This strengthens the hypothesis that other compounds, as yet undetected, may be responsible for this inhibition.

5. Conclusions

PEX and PAX, which contain considerable amounts of ellagic acid and gallic acid respectively, had shown acetylcholinesterase inhibitory activity in a non-competitive manner. The compounds responsible for this inhibition are probably the anthocyanins, which are present in higher concentrations in PEX than in PAX, or other undetected phenolic compounds.

CRediT authorship contribution statement

Katharina Ardanareswari: Formal analysis, Data curation. **Chih-Yuan Tan:** Formal analysis. **Cheng-Kuang Hsu:** Writing – review & editing, Writing – original draft, Data curation. **Yun-Chin Chung:** Writing – review & editing, Writing – original draft, Supervision, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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