Original research article

Tyrosinase inhibition, antioxidant activity and total phenolic content of selected Mimosaceae pericarps ethanolic extracts

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Abstract

The pericarp is the outer layer of a fruit. Often, pericarps are not used and are usually discarded. However, they may have untapped pharmacological potential. This study explored the tyrosinase inhibition, antioxidant activities, and total phenolic content of five pericarp extracts from Mimosacea family, namely *Adenanthera pavonina*, *Archidendron jiringa*, *Leucaena glauca*, *Parkia speciosa* and *Pithecellobium dulce*. Ethanolic extract was obtained after continuous extraction of dried pericarp with petroleum ether, dichloromethane and ethanol using Soxhlet apparatus. L-Tyrosine and L-Dopa are the substrates of tyrosinase in the monophenolase and diphenolase reaction, respectively. Both reactions are crucial in melanin production. Thus, tyrosinase inhibitor has broad potential in the fields of cosmetics and medicine. The antioxidant activity was evaluated by reducing power assay and metal chelating assay. Extracts inhibited both diphenolase and monophenolase reactions were analyzed by HPLC-PDA. From the results, at 500 µg/ml *A. jiringa* and *P. speciosa* extracts exhibited both monophenolase and diphenolase reactions. HPLC-PDA analysis detected catechin and epicatechin from the extract, respectively. Inhibition activity of *A. jiringa* and *A. pavonina* were not significantly different with kojic acid in monophenolase and diphenolase reaction, respectively. It can be implied that the inhibition of monophenolase reaction was attributed by the high total phenolic content, presence of flavanols and high reducing power. However, reducing power appeared irrelevant to the inhibition of diphenolase reaction, due to the lowest activity presented by the *A. pavonina* extract. This study showed *A. jiringa* and *A. pavonina* extracts inhibited tyrosinase enzyme in different reactions during melanin production.

Keywords: flavanol; tyrosinase inhibitor; reducing power, mimosaceae, pericarp

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1.0 Introduction

Tyrosinase is the enzyme that catalyzes reactions during the formation of melanin. The two important reactions are the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (Dopa), and the oxidation of dopa to dopaquinone. Tyrosinase has becomes the most prominent and successful target to inhibit melanogenesis. Tyrosinase inhibitors such as kojic acid and arbutin are commonly used as fairness enhancer in skin whitening products. Tyrosinase inhibitors have also been suggested as hyperpigmentation treatment. Despite the limited evidence-based research and clinical trials that evaluated the treatment of hyperpigmentation with natural ingredients, several natural ingredients such as, extract of licorice (*Glycyrrhiza glabra*), soy (*Glycine max*), and mulberry (*Morus alba*) showed efficacy as natural treatments for patients with hyperpigmentation disorders by acting as tyrosinase inhibitors (1). In addition, the ability of a tyrosinase inhibitor to scavenge free radical is an equally valuable characteristic in managing hyperpigmentation. For instance, kojic acid possessed antioxidant attributes such as capability of chelating iron (2) and scavenge reactive oxygen species (3).

Hence, there is a great need to identify efficient tyrosinase inhibitors. Exploring natural tyrosinase inhibitor particularly from plants is of interest because undoubtedly parts of plants are rich sources of bioactive phytochemicals (4). In this study, five pericarp ethanolic extracts of Mimosacea family namely *Adenanthera pavonina* Linn., *A*rchidendron *jiringa* I.C Nielsen, *Leucaena glauca* Benth, *Parkia speciosa* Hassk and *Pithecellobium dulce* Bent. were investigated. Pericarps are the outer layer of fruits. The function of pericarp is mainly to provide protection during dispersing of the seeds; thus, pericarps contain chemical compounds that benefits the embryo for the germination of plants (5). Pericarps of *A. pavonine* and *A. jiringa* are woody whereas *P. dulce* are unpalatable. However, the young pericarps of *Leucaena glauca* and *P. speciosa* are sometimes cooked or eaten as salad. The latter is occasionally pickled in brine together with the seed and served as traditional delicacy in Malaysia.

Fruit pericarps are valuable sources of phytochemicals such as phenolics, triterpenoids and saponins with pharma-cological activities include cytotoxic, antioxidant and anti-inflammatory (6). For instance, *A. jiringa* pericarp extract exhibited a better antioxidant activity than butylated hydroxytoluene (BHT); a recognized anti-oxidant in food and cosmetic industry (7). This study explores the tyrosinase inhibitory activity, total phenolic content and antioxidant

property include the reducing power and metal chelating activity of pericarp extracts.

2.0 Materials and Methods

2.1 Plant material

Pericarps were collected from botanical garden in Rayong, Thailand. Samples were deposited at the Faculty of Pharmaceutical Sciences Herbarium, Chulalongkorn University prior to the identification and authentication process of each species by Associate Prof. Nijsiri Ruangrungsi. All samples were air dried before grounded and were stored at room temperature.

2.2 Extraction

Each sample was extracted consecutively using petroleum ether, dichloromethane and ethanol. Sample weighed 30 g was extracted using Soxhlet apparatus for 8 hours with 500 ml solvent according to the boiling point of solvent used. Three extracts of different solvent polarity were obtained, but only the activity of ethanol extract was investigated. The solvent in the extract was removed by rotary evaporator. The extract yield was weighed, recorded and stored at 4˚C until it was analyzed.

2.3 Tyrosinase inhibition assay

The assay was carried out according to Tian *et al.* (8) with modification. Into a 96 well microplate, 100µl of extract (concentration 500 µg/ml) was pipetted. Next, 50 µl of L-tyrosine solution or L-dopa solution (4 mM in 0.1 M NaH_2PO-K_2HPO_4 buffer, pH 6.8) was added and followed by 50 µl of 30 U/ml mushroom tyrosinase (EC 1.1418.1) in the same buffer. The reaction was measured at the absorbance at 475 nm using microplate reader for every 20 minutes for 100 minutes. Control (without extract) was the mixture of all reagents with 2.5% DMSO in 0.1 M NaH_2PO-K_2HPO_4 buffer. Kojic acid, as a positive control, was tested at concentration 20 µg/ml. All determination was carried out in triplicate. The percentage inhibition of enzyme by extract was calculated as; Inhibition (%) = ($A_{control}$- A_{sample})/$A_{control}$ x 100%.

2.4 Antioxidant assays and HPLC analysis

The assays were carried out according to Ramli *et al.* (9).

2.4.1 Reducing power assay

A 148µl of extract (concentration ranged from 1 to 200 µg/ml) was pipetted into a 96-well microplate, followed by 50 µl of 1% potassium ferricyanide (w/v). The mixture was incubated at 50 °C for 20 min. Next, 50 µl of 10% tricloroacetic acid (w/v) and 2 µl of 1% of ferric chloride (w/v) was added into each well. The mixture was mixed until homogenized before being measured spectrophotometrically at 700 nm. Quercetin was used as positive control. The assay was carried out in triplicate. The graph of absorbance at 700 nm against the correspondent extract concentration was plotted. The EC_{50} was arbitrarily defined as the concentration of extract that exhibited absorbance of 0.5 at 700nm obtained from a line of best fit from the plotted graph.

2.4.2 Iron chelating assay

In the 96 well microplate, 15 µl of $FeCl_2$ in ultrapure water (2 mM) was added into 110 µl of extract (5 mg/ml). The reaction started by adding 75 µl of aqueous ferrozine (5 mM). The mixture was left for 10 minutes, before the absorbance of reaction was measured at 562 nm. EDTA was used as positive control. Control is the reaction mixture with ethanol substituting the extract. Fe^{2+} chelating activity of test compound was calculated as:

Chelating activity (%) = (Absorbance control –Absorbance sample)/ Absorbance control x 100.

2.5 Total phenolic content

A 100µl of extract in methanol was pipette into 96 well microplate, followed by 100 µl of 15% Folin Ciocalteau. The mixture was left for 5 minutes before addition of 100 µl Na_2CO_3 aqueous (0.105 g/ml). The absorbance of extract was measured at 756nm after incubation at 30˚C for 60 minutes. All determinations were performed in triplicate. A standard curve consisted of different concentrations of gallic acid (0.03, 0.06, 0.12, 0.25, 0.5 and 1 mg/ml) were prepared. Total phenolic content was expressed as mg of gallic acid equivalents per g dry weight of extract using the linear equation from gallic acid standard curve (9).

2.6 HPLC analysis.

One mg/ml sample was filtered through the Ultrafree-MC membrane centrifuge-filtration unit (hydrophilic PTFE,0.20 µm, Milipore), and 5µl of the filtrate was loaded into a TSK-gel Super ODS column (2.0 µm, 2.0 × 100 mm, TOSOH, Tokyo, Japan) at 40 °C. The mobile phase consisted of water (A) and acetonitrile (B) both containing 0.1% formic acid with the flow rate of 200 µl/min. The separation and detection were performed by an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA) with gradient condition of 5%-60%B for 35 minutes. The HPLC system was consisted of a pair of LC 10AD VP pumps, a DGU 12A degasser, a CTO 6A column oven, an SPD 10A VP photodiode array detector (PDA), and a SCL 10A VP system controller (Shimadzu, Kyoto, Japan) (9).

2.7 Statistical analysis

All experiments were carried out in triplicate. Data were reported as mean ± standard deviation (SD) for triplicate determinations. The statistical analysis was performed with one-way ANOVA followed by Tukey post hoc test. Differences were considered significant when $p \leq 0.05$.

3.0 Results

3.1 Tyrosinase inhibition activity.

From the % inhibition activity of extracts, monophenolase inhibition activity reached plateau after 20 minutes (Figure 1), whereas there are diphenolase inhibition activities after 40 minutes until 100 minutes. All extracts exhibited inhibition to diphenolase activity of tyrosinase (Figure 2). Inhibition percentage of tyrosinase enzyme at 100 min is depicted in Figure 3. The *A. iiringa* and *P. speciosa* extracts at concentration of 500 µg/ml inhibited 68.74% and 50.35% of the hydroxylation of tyrosine to 3,4-dihydroxy-phenylalanine (Dopa), respectively. All pericarp extracts inhibited the oxidation of dopa to dopaquinone, with *A. pavonina* extract exhibited 30.95% which is the highest inhibition percentage of diphenolase activity. Analysis by one-way ANOVA showed that the tyrosinase inhibition activity of extracts are significantly different in both substrate ($p<0.05$). It was noted that, statistical analysis results showed monophenolase inhibition effect of *A. jiringa* and diphenolase inhibition effect of *A.pavonina* was not significantly different compared to 20 µg/ml kojic acid ($p > 0.05$).

3.2 Antioxidant Activity

Quercetin and EDTA were employed as positive control for reducing power and metal chelating assay, respectively. From Table 1, *P. speciosa* pericarp extract showed the highest reducing power with EC_{50} about 14±3.62 µg/ml, this implied the good electron donation ability of the extract that

able to reduce Fe^{3+} into Fe^{2+}, whereas the lowest activity was showed by *A. pavonina* pericarp extract with EC_{50} of 104±12 µg/ml. However, the activity was lower than quercetin that showed EC_{50} of 3.75 ±0.4 µg/ml. The reducing power of all extracts were significantly different ($p<0.05$). At the concentration of 5 mg/ml, only the extract of *Leucaena glauca* pericap showed metal chelating activity. The extract was able to chelate about 3.9 ± 0.022 % of available iron, however the activity was lower compared to EDTA that chelated 50% of iron at concentration of 0.052 mg/ml.

3.3 Total phenolic content and HPLC analysis of extract

The total phenolic content of all extracts was significantly different. *P. speciosa* extract exhibited the highest total phenolic content followed by *A. jiringa* extract (343.18 and 320.94 mg of gallic acid equivalents (GAE) per gram of extract, respectively), whereas *P. dulce* displayed the lowest total phenolic content (Figure 4).

Therefore, based on tyrosinase inhibition, antioxidant and total phenolic content results, both *A. jiringa* and *P. speciosa* extracts were analyzed using HPLC with photodiode array detector (PDA) at wavelength 254 nm. The effectiveness of HPLC method was evaluated with standards; gallic acid, catechin, epicatechin, epigallocatechin-gallate and quercetin. All standards were detected at wavelength 254 nm. The chromatogram of both extracts is shown in Figure 5.

Compound (a) and (b) were tentatively identified as catechin and epicatechin, respectively (Table 2). The identification of the compounds was performed on the basis of their retention times and UV-visible spectrum compared with standards.

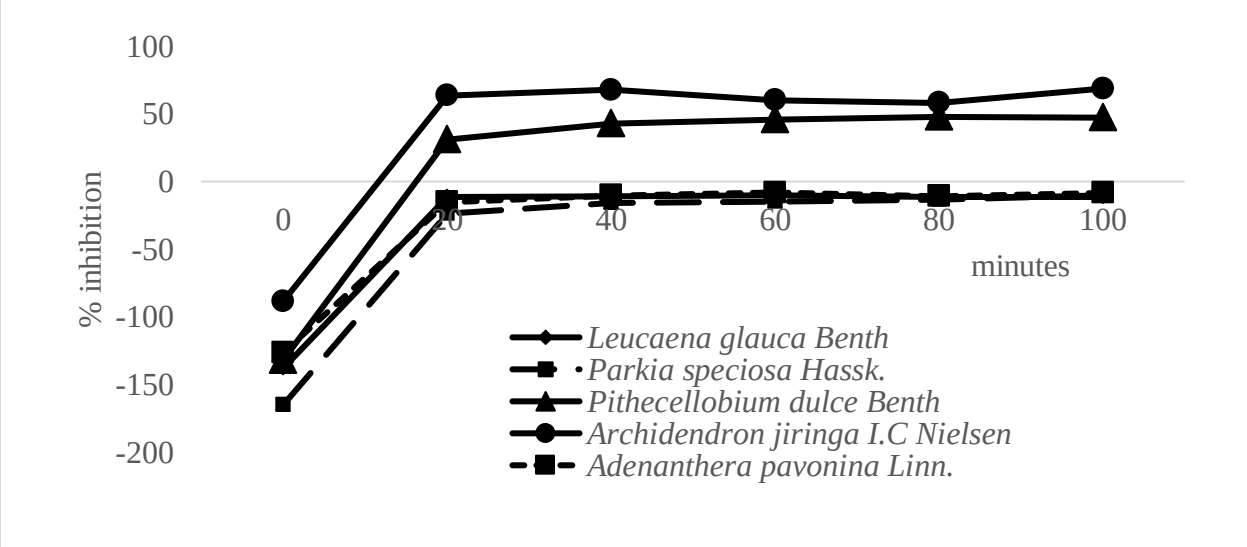

Figure 1: Monophenolase inhibition activity of pericarp extracts at 0 to 100 minutes. The monophenolase inhibition activity reached plateau after 20 minutes.

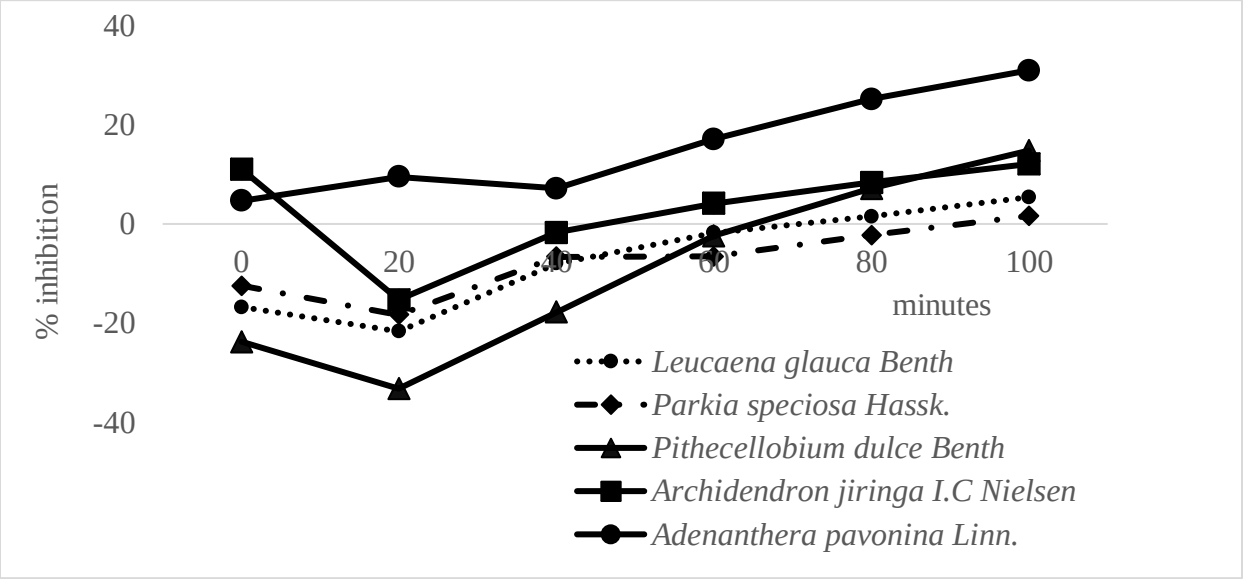

Figure 2: Diphenolase inhibition activity of pericarp extracts at 0 to 100 minutes. There was increasing diphenolase inhibition activities from 40 minutes until 100 minutes.

Table 1: EC_{50} values of pericarp extracts in reducing power assay and metal chelating activity. All data are presented as mean ± standard deviation (n =3). The best reducing power activity was demonstrated by *P. speciosa* extract while only *L. glauca* exhibited metal chelating activity. NA=no activity. The superscript alphabets in the table were obtained by one-way ANOVA followed by Tukey test indicated significant difference ($p<0.05$).

	Reducing power (µg/ml)	**Metal chelating (mg/ml)**
Adenanthera pavonina Linn.	104.26 ± 11.86 [a]	NA
Pithecellobium dulce Benth	61.88± 5.44 [b]	NA
Leucaena glauca Benth	57.68 ± 5.96 [c]	3.9 ± 0.02
Archidendron jiringa I.C Nielsen	21.52±1.64 [d]	NA
Parkia speciosa Hassk.	14± 3.62 [e]	NA
Quercetin	3.85±0.14 [f]	-
EDTA	-	0.052 ± 0.003

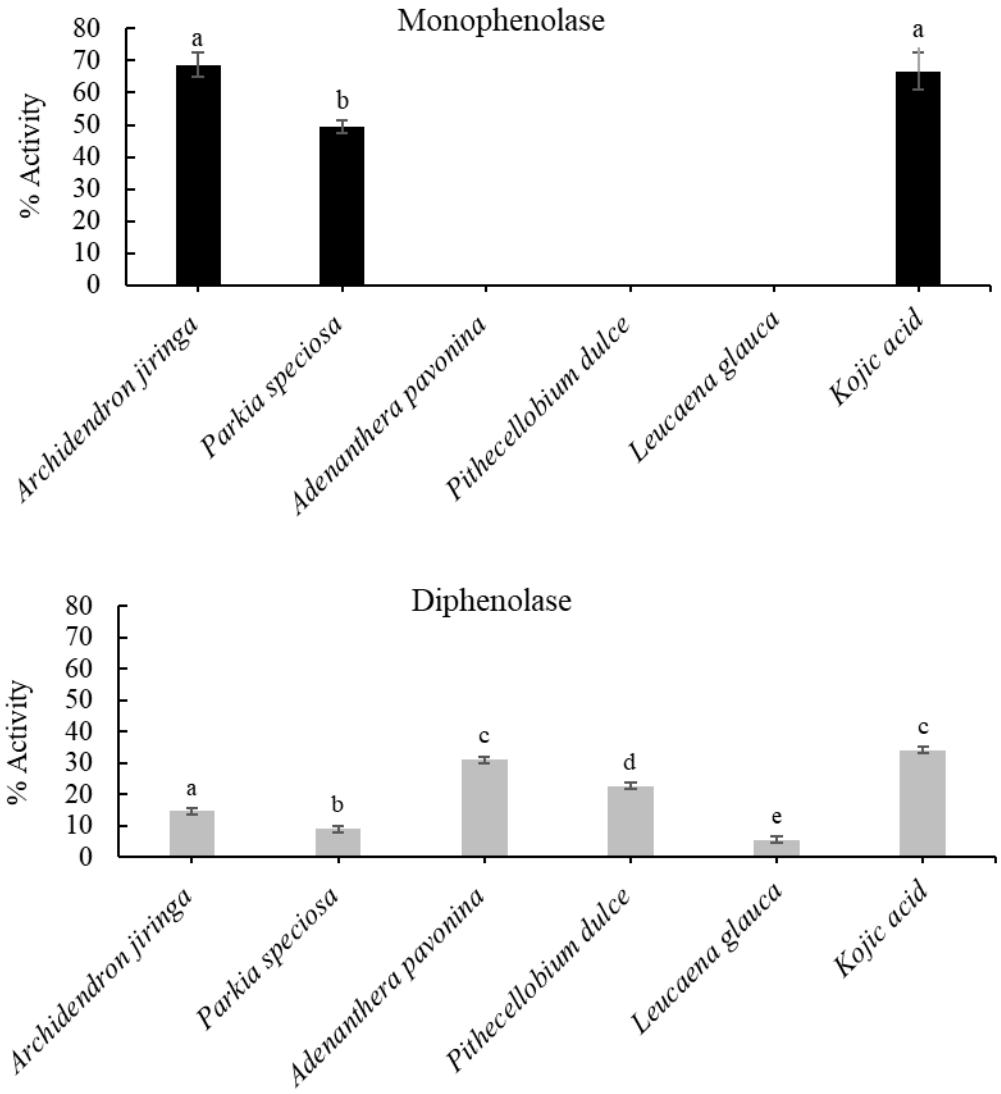

Figure 3: Tyrosinase inhibition activity of Mimosaceous plant pericarp extracts (500 µg/ml) and kojic acid (20 µg/ml) at 100 min. Monophenolase and diphenolase activity of the extract was compared with Kojic acid. The superscript alphabets in the graph were obtained by one-way ANOVA followed by Tukey test indicated significant difference ($p<0.05$).

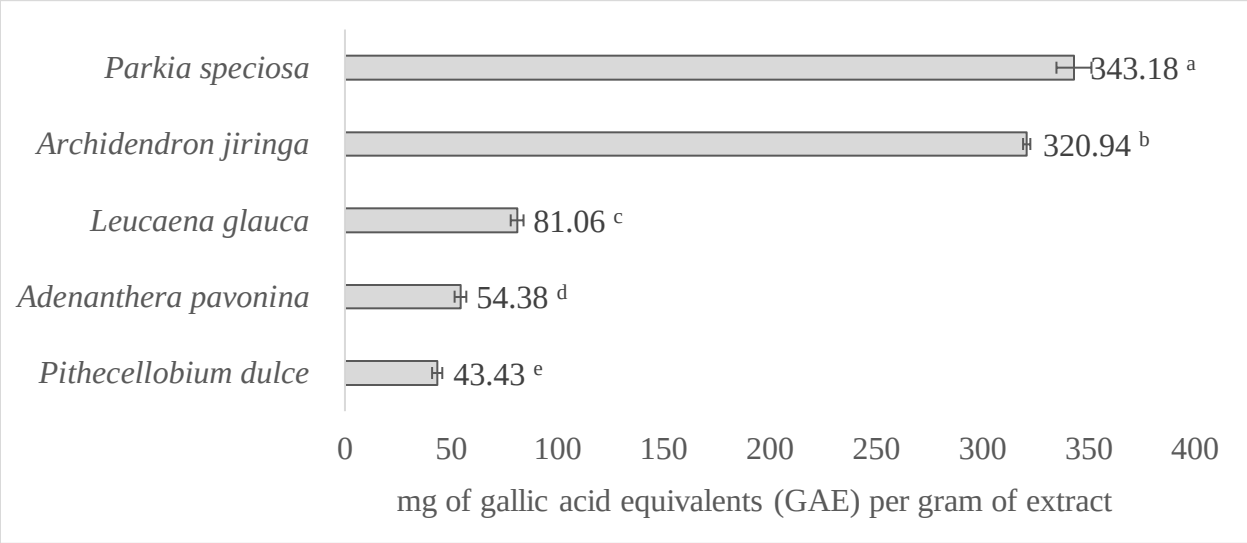

Figure 4: Total phenolic content of pericarp extract. The superscript alphabets in the table were obtained by one-way ANOVA followed by Tukey test indicated significant difference ($p<0.05$).

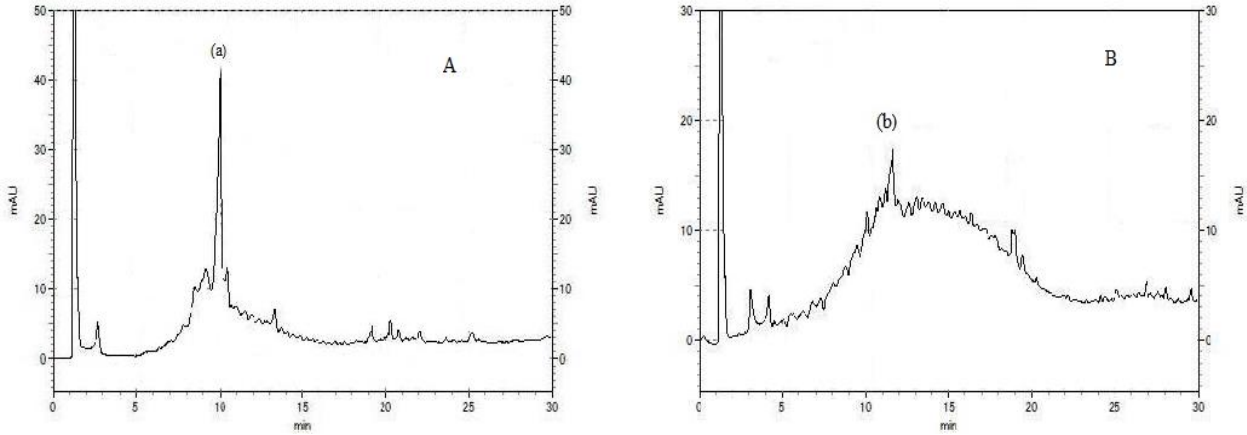

Figure 5: HPLC chromatograms A) *A. jiringa* extract) *P. speciosa* extract

Table 2: Tentative identification of compound in extract *A. jiringa* and *P. speciosa*.

Sample	Peak	Retention time (min)	Λ max (nm)	Tentative identification
A. jiringa	(a)	10.01	216, 272	Catechin
P. speciosa	(b)	11.68	219, 281	Epicatechin

4.0 Discussion

A total of 5 pericarp extracts were studied. Only *A. jiringa* and *P.speciosa* extracts inhibited both monophenolase and diphenolase activity of tyrosinase. Catechin and epicatechin are common compounds of black catechu or cutch. It is a solid extract obtained from the heartwood of *Acacia catechu* which is also a Mimosaceous plant. Both compounds are flavonoids from subclass flavanols. The percentage of chelating activity of 100 µl/ml of catechu extracts containing catechin and epicatechin were found in the range of 0.65-16.12% compared to EDTA (10). Although, HPLC-PDA analysis resulted in catechin and epicatechin detected in extracts, however, in this study both *A. jiringa* and *P. speciosa* showed no metal chelating activity. At concentration 5 mg/ml, only *L. glauca* exhibited percentage of chelating activity within the range of the previously mentioned study.

Catechin, and epicatechin are also commonly found compounds in tea (*Camilla sinensis*). Previously, the inhibitory effect of mushroom tyrosinase by green tea components including catechin and epicatechin (40 µM), was measured using L-tyrosine as substrate. Both epicatechin and catechin showed low tyrosinase inhibition activity (<10%) compared to gallocatechin-3-O-gallate because both compounds lack of flavan-3-o1 skeleton with galloyl moiety at 3 position, that allows the gallocatechin-3-O-gallate to be a competitive tyrosinase inhibitor thus exhibited more than 70% inhibition of monophenolase and diphenolase activities of tyrosinase. Epicatechin was found to inhibit monophenolase activity better than catechin (11). Therefore, the significant difference between *A. jiringa* and *P. speciosa* monophenolase inhibition can be discerned from the presence of other compounds than catechin and epicatechin in both extracts.

Phenolic structural group is a structural analogue to the tyrosine or dopa substrate. Phenolic compound would compete with the substrate for the active site of tyrosinase, thus inhibiting its activity. However, a study showed there are structural requirements for flavonoids to inhibit tyrosinase. The study reported, hydroxylation at C3′ on ring B of flavones significantly weakened the inhibitory activity although it slightly enhanced the binding affinities, whereas increase in the size due to glycosylation of the flavonoids may prevent flavonoids from entering the active center of tyrosinase. Therefore, even though flavonoids might be inserted into the hydrophobic cavity of tyrosinase or bind near the cavity and hinder the entry of substrate tyrosine or dopa, the ultimate inhibition was caused by the direct interaction of flavonoids with the active site. From the study, the mode of inhibition of flavonols, isoflavones, chalcones and dihydroflavols was indicated as competitive and mixed–type inhibitors of tyrosinase (12). Furthermore, phenolic compounds such as cinnamic acid, ferulic acid, quercetin, isorhamnetin, gallic acid have also been studied individually and as combination to elucidate their tyrosinase inhibition property (13).

The tyrosinase inhibition assay has been commonly employed to examine many putative inhibitors. However, extract or compound with good reducing power is able to reduce back o-dopaquinone to dopa, thus avoiding dopachrome formations without truly inhibiting tyrosinase enzyme (14). In this study, the experimental observation of tyrosinase inhibitory was accomplished, but the assay is unable to regard the extracts as true inhibitor. For instance, a study observing oolong tea extract (0–500 mg/ml) was less effective in inhibiting tyrosinase activity using L-Dopa substrate compared to L-ascorbic acid, which is antioxidant. But the extract showed inhibitory effect on cellular tyrosinase activity (15).

From the results, *A. jiringa* and *P. speciosa* extracts that inhibited monophenolase activity also showed high total phenolic content and reducing activity. This is anticipated as plant extracts with good antioxidant activity are often associated with its rich phenolic compounds (16). Additionally, same extract showed different response to antioxidant assays. For instance, an extract exhibited good results in free radical scavenging activity assay, but poor in iron chelating assay (9). With regards to the extraction method in this study, sequential solvents with different polarity and heating procedure in the Soxhlet extraction were applied. This is to simplify the extract and lead to liberation of some bounded phenolics compounds polysaccharides that can react with Folin reagent thus resulting in high amount of total phenolic content (17).

A. pavonina extract exhibited highest diphenolic inhibition activity but showed low reducing power and total phenolic content. Nevertheless, the overall results from this pericarps study are in accordance with the results from a previous study. A hundred of plant extracts (50 µg/mL) were screened for the inhibition of diphenolase activity of tyrosinase. In the reported study, from 100 extracts, 17 extracts enriched in flavonoids and phenolics were found promising as enzymatic inhibitor. A positive correlation was established between increasing total phenolic and flavonoid content of plant extracts with tyrosinase enzymatic inhibitory. Nevertheless, the found correlations were not strong, although the highest positive correlation ($r=0.3535$ and $p=0.0003$) was found between tyrosinase inhibition and total phenolic content, other compounds than flavonoids and polyphenols might be responsible for the activity against tyrosinase (18).

5.0 Conclusion

A. jiringa and *P.speciosa* pericarp extracts inhibited both monophenolase and diphenolase activity of tyrosinase at concentration of 500 µg/ml. Both extracts showed a considerably high total phenolic content, antioxidant activity and diphenolase inhibition activity, Catechin was detected in *A.jiringa* while epicatechin was detected in *P.speciosa*. The compounds are inferred as contributing to the observed diphenolase inhibition activity. This study inferred *A. jiringa* and A. pavonina extract inhibited tyrosinase enzyme in different reactions during melanin production. Further study such as measuring tyrosinase activity using cellular model is recommended to confirm both extracts as true tyrosinase inhibitor that is valuable for cosmetic or medicine purposes.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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