

Kinetics of Tyrosinase Inhibition, Antioxidant Activity, Total Flavonoid Content and Analysis of *Averrhoa bilimbi* L. Extracts and its Fruit Vinegar Using FTIR and Multivariate Methods

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Abstract

Melanin is a natural substance responsible for human skin pigmentation. Tyrosinase is known as a key enzyme involved in melanin production; therefore, hyperactivity of this enzyme leads to hyperpigmentation in human. In current study, leaves and fruits of *Averrhoa bilimbi* L. were extracted with ethanol and water solvents, and its fruit vinegar was produced by fermenting fresh fruits with sugar at room temperature for 10 days. The leaf and aqueous extracts of *A. bilimbi* L. and its fruit vinegar were investigated for tyrosinase inhibitory activity using L-DOPA as substrates, antioxidant activity using 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation decolorization assay and total flavonoid content (TFC) using aluminium chloride colorimetric method. Moreover, kinetic study of anti-tyrosinase activity of *A. bilimbi* extracts and its fruit vinegar, including Fourier transform infrared (FTIR) spectra and principal component analysis (PCA) were carried out. The result indicated ethanol extracts from *A. bilimbi* fruits and leaves showed the highest TFC and anti-tyrosinase activity, whereas aqueous extracts of its fruits and leaves showed the highest radical scavenging activity. Interestingly, the result revealed the existence of TFC, radical scavenging activity and anti-tyrosinase activity in the vinegar sample. The anti-tyrosinase potential of the fruit and leaf ethanol extracts of *A. bilimbi* was identified as an uncompetitive inhibitor ($K_m = 0.54$ and $0.51 \mu\text{M}$, $V_{max} = 0.0173$ and $0.0146 \text{ O.D.}_{450}/\text{min}$, respectively). Positive correlation was significantly found between radical scavenging activity and TFC, between anti-tyrosinase activity and TFC, between FTIR data and TFC, and between FTIR data and anti-tyrosinase activity, ($r = 0.83, 0.56, 0.66, 0.75$; p -value < 0.05 , respectively). Therefore, the extracts and fruit vinegar of *A. bilimbi* L. could be considered as natural source of an anti-tyrosinase compounds for cosmetics and healthy foods.

Keywords: *Averrhoa bilimbi* L., Fruit vinegar, Antioxidant activity, Flavonoids, Tyrosinase inhibition, Kinetics, FTIR, PCA, Cluster analysis

Introduction

Melanin is a natural pigment that provides coloration of human skin [1]. Tyrosinase is a major enzyme in any organism (i.e. human, animals, plants and microorganisms) that catalyzes the production of melanin pigment [2,3]. Hyperactivity of this enzyme cause to occur hyperpigmentation in human. To date, tyrosinase inhibitors from natural resources (i.e. plants and fungi) are becoming increasingly popular in pharmaceutical and cosmetic industries because of low side effects and friendly to environment. An important role in inhibition of tyrosinase enzyme, which is an oxidoreductase enzyme, is responsible for melanogenesis and browning in raw fruits, vegetables, and beverages [4,5]. Currently, several plants are investigated for their effect on tyrosinase activity. *Averrhoa bilimbi* L. is one of sour edible fruits and its fresh fruits are popularly consumed in Thailand. Previously, it has been reported that *A. bilimbi* L. has several medicinal properties such as anti-diabetic activity, antioxidant activity, anticancer activity, antihypertensive activity, thrombolytic activity, antimicrobial activity, hepatoprotective activity, and hypolipidemic activity [6,7].

It has been reported that fruit extract of *A. bilimbi* L. contain of flavonoids which show antibacterial activities [8]. Moreover, dried extract, water extract and ethanol extract of *A. bilimbi* L. leaves show antioxidant activity by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay and antibacterial activity [9]. Interestingly, functional groups from leaf powder, water extract, and ethanol extract of *A. bilimbi* L. are

identified by Fourier transform infrared (FTIR) spectroscopy which show functional groups associated with phenolic compounds which may involve with antioxidant and antibacterial activities of the extracts [9]. Moreover, *A. bilimbi* L. has high level of oxalic acid and vitamin C [10], and its leaves contain many phytochemicals such as alkaloid, tannins, flavonoids, glycosides, phenols and carbohydrates [11,12]. Additionally, there are reports of the presence of flavonoids in *A. bilimbi* L. leaves [13], and the presence of squalene and phytol compounds with anti-glucosidase activity in methanol extract of its leaves [14]. Many reports have been carried out to demonstrate phenolic and flavonoid agents as antioxidant and anti-tyrosinase activity. Flavonoids have medical properties such as antioxidant activity, anticancer activity and anti-inflammation activity, which can be applied in drug development for several diseases such as Parkinson disease and cancer diseases [15-18]. Moreover, relationship between some flavonoids and tyrosinase inhibitory activity has been reported [19]. Flavonoids can act as tyrosinase inhibitors in both *in silico* and *in vitro* models because of the presence of a hydroxyl at C7 in disturbing tyrosinase activity [20]. Structure of flavonoids on inhibitory activity of tyrosinase have been investigated. It has been reported that different functional groups of flavonoids show different inhibition effects on tyrosinase activity [21].

In this study, extracts from leaves and fruits of *A. bilimbi* L. and its fruit vinegar were prepared for determination of total flavonoid content (TFC), radical scavenging activity, and kinetic study of tyrosinase inhibitory activity. Additionally, Fourier transform infrared (FTIR) spectroscopy was used to detect functional groups of the extracts and fruit vinegar, and was then compared to standard flavonoids. FTIR is an analytical tool which is rapid and convenient in preliminary detecting bioactive compounds in plants [22]. A combination of FTIR data, principal component analysis (PCA), cluster analysis, chemical content and biological activities in this study will help to improve understanding of relationship between FTIR data, chemical content and biological activities. Thus, the knowledge obtained from this study is useful for producing healthy foods and natural cosmetics.

Materials and methods

Chemicals

Tyrosinase from mushroom, 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 3,4-dihydroxy-L-phenylalanine were purchased from Sigma. Potassium persulfate was purchased from Ajax Finechem. Rutin, apigenin, luteolin, kaempferol, quercetin, naringin, and L-ascorbic acid were purchased from Sigma-Aldrich.

Sample preparation and extraction

Leaves and fruits of *A. bilimbi* L. were collected and cleaned by water. Its leaves were incubated at 45 °C for 48 h, while its fruits were dried at 60 °C for 3 days. After that, each dried sample was homogenized into fine powder, and extracted by absolute ethanol solvent and water solvent in ratio of 1 g per 25 mL at 45 °C for 48 h. Each sample was extracted in duplicate. After that, each extract was sieved through a filter cloth, and was concentrated by a rotary evaporator (IKAa RV10). For ethanol solvent, it was evaporated at 45 °C for 20 min, while water solvent was evaporated at 45 °C for 30 min. Each concentrated sample was adjusted to final concentration (0.1 g/mL) by adding its solvent, and kept at -20 °C [22].

Preparation of fruit vinegar

The cleaned fresh fruits of *A. bilimbi* were cut into small pieces. Then, 10 g of the fresh fruits were kept in a jar for 16 h. After that, sugar (100 g) was added in the cut fresh fruits by mixing 10 g per day until 10 days. The fruit vinegar was filtrated through a filter cloth and kept at 4 °C.

Total flavonoid contents

Total flavonoid content (TFC) in all samples was determined by aluminium chloride colorimetric method [23,24]. Briefly, each sample (500 µL) was reacted with ethanol (1.5 mL), 10 % aluminium chloride (0.1 mL), 1 M potassium acetate (0.1 mL), and distilled water (2.8 mL) under room temperature for 30 min. Each extract was carried out in duplicate. The absorbance of each reaction was measured at 415 nm by a spectrophotometer (Model T60UV). Rutin (0 - 500 µg/mL) was used as positive control to produce a standard curve ($y = 0.0146x + 0.026$, $R^2 = 0.9965$). TFC was reported as µg rutin equivalent per mg extract and µg rutin equivalent per g fresh fruit.

Tyrosinase inhibitory activity

Tyrosinase inhibitory activity was performed by following the method of Liang *et al.* [25]. The mixture of each extract (40 μL), 5 mM L-DOPA (100 μL) and 0.1M sodium phosphate buffer pH 6.8 (20 μL) was prepared, following by adding 200 units/mL of mushroom tyrosinase (40 μL), incubating at 37 $^{\circ}\text{C}$ for 20 min, and determining the absorbance of each reaction at 475 nm by a spectrophotometer (Model T60UV). The percentage of tyrosinase inhibition was calculated by using below equation.

$$\% \text{ Tyrosinase inhibition} = \frac{[(A_{\text{water}} - A_{\text{waterblank}}) - (A_{\text{sample}} - A_{\text{sampleblank}})]}{(A_{\text{water}} - A_{\text{waterblank}})} \times 100$$

A_{water} and $A_{\text{waterblank}}$ were the absorbance of water with enzyme and without enzyme

A_{sample} and $A_{\text{sampleblank}}$ were the absorbance of each sample with enzyme and without enzyme

The percentage of tyrosinase inhibition was used to generate a simple linear regression for determining an effective concentration (EC_{50}), which was concentration of an extract used for 50 % inhibition of tyrosinase activity.

Kinetic study of anti-tyrosinase activity

The extracts, which showed high anti-tyrosinase activity, were used for kinetic study of anti-tyrosinase activity according of Lineweaver-Burk principle. L-dihydroxy phenylalanine (L-DOPA) was prepared at 5, 2.5, 1.25, 0.625 and 0.3125 mM concentration. After that, each concentration of L-DOPA (100 μL) was reacted with each extract (40 μL), 0.1 M sodium phosphate buffer pH 6.8 (20 μL), and 200 units/mL of tyrosinase enzyme (40 μL). The reaction rate (V) was detected by determining the raised absorbance of the reaction at 37 $^{\circ}\text{C}$ within 20 min at 475 nm by a spectrophotometer (Model T60UV). The Lineweaver-Burk curve was plotted between $1/V$ ($\text{O.D.}_{475}/\text{min}$) $^{-1}$ and $1/[S]$. K_m (Michaelis-Menten constant) and V_{max} (maximal velocity) was calculated to analyze inhibition types.

ABTS scavenging activity

The radical scavenging activity of each sample was estimated by 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical cation decolorization assay following the procedure of Thummajitsakul *et al.* [22]. Briefly, ABTS radical cation solution was prepared by the addition of 7 mM ABTS (10 μL) and 140 mM potassium persulfate (179 μL), and incubated at room temperature in the dark for about 16 h. The ABTS working solution was diluted with deionized water to obtain its absorbance of 0.700 ± 0.05 at 734 nm in a spectrophotometer (Model T60UV). Each extract (20 μL) was reacted with 3.9 mL of the ABTS working solution in dark condition for 6 min, and then its absorbance was read at 734 nm. Percentages of the ABTS scavenging activity was calculated by using equation.

$$\text{Scavenging activity (\%)} = (A_{\text{ABTS}} - A_{\text{sample}})/A_{\text{ABTS}} \times 100$$

Where A_{sample} was the absorbance of each sample and A_{ABTS} was the absorbance of the ABTS working solution.

Fourier transform infrared spectra (FTIR) technique

The standard bioactive compounds used in this study were kojic acid, luteolin, rutin, luteolin, kaempferol, naringenin and ascorbic acid. The Fourier transform infrared spectra (FTIR) analysis of the standard flavonoids and sample extracts were carried out on powder and the liquid extracts. The tool used for FTIR analysis was PerkinElmer spectrum IR version 10.6.0, duplicated scans per sample in the region of 550 to 4,000 cm^{-1} with a resolution of 4 cm^{-1} . The functional groups of each sample were compared to the previous reports in **Table 3**.

Statistical analysis

The total flavonoid contents, radical scavenging activity, anti-tyrosinase activity were subjected to descriptive statistics (i.e. mean, SD and percentage). Moreover, analytical assays of the radical scavenging activity, anti-tyrosinase activity and total flavonoid contents among sample groups were performed by using one-way analysis of variance (one-way ANOVA) and correlation analysis. Statistically significant levels were defined at p -value < 0.05 . The descriptive and analytical statistics were performed by the PSPP program version 0.10.5 [26]. Additionally, differentiation of samples was performed by principal component analysis (PCA) and cluster analysis (bootstrap = 10,000) in

paleontological statistic program version 3.16 [27]. Moreover, FTIR data was obtained by scoring the presence or absence of a peak (1/0) and used for PCA and cluster analysis. The number of FTIR peaks were used for correlation analysis.

Results and discussion

The result showed that leaves and fruits of *Averrhoa bilimbi* L. and its fruit vinegar consisted of total flavonoid content (TFC), radical scavenging activity and anti-tyrosinase activity. TFC of leaf extracts (17.04 ± 0.19 to 22.96 ± 0.46 μg rutin equivalent per mg extract) was higher than TFC of fruit extracts (0.30 ± 0.07 to 3.31 ± 0.37 μg rutin equivalent per mg extract), significantly (p -value < 0.05) (Table 1). Especially, ethanol extracts from leaves and fruits showed higher TFC than aqueous extracts. TFC result was similar with the previous report that 96 % ethanol and aqueous extracts of *A. bilimbi* L. leaves had TFC were 29.71 ± 4.66 mg QE/g, and 8.88 ± 1.14 mg QE/g, respectively [9]. In contrast to TFC result, aqueous extract of *A. bilimbi* L. leaves and fruits provided significantly potential in radical scavenging activity ($p < 0.05$) as compared to ethanol extract. However, radical scavenging activity of leaf extracts ($1/\text{EC}_{50} = 0.0193 \pm 0.0015$ to 0.0304 ± 0.0031) was higher than radical scavenging activity of fruit extracts ($1/\text{EC}_{50} = 0.0023 \pm 0.0005$ to 0.0031 ± 0.0003), significantly (p -value < 0.05).

Additionally, high anti-tyrosinase activity was found in ethanol extract from leaves ($1/\text{EC}_{50} = 0.6424 \pm 0.0202$) and fruits ($1/\text{EC}_{50} = 0.0399 \pm 0.0163$), and vinegar of *A. bilimbi* L. fruits showed high TFC (68.82 ± 8.40 μg rutin equivalent per g fresh fruit), radical scavenging activity ($\text{EC}_{50} = 5.42 \pm 0.57$ g fresh fruit /mL or $1/\text{EC}_{50} = 0.1859 \pm 0.0191$), and anti-tyrosinase activity ($\text{EC}_{50} = 1.14 \pm 0.12$ g fresh fruit /mL or $1/\text{EC}_{50} = 0.8807 \pm 0.0932$) (Table 1).

It indicated that different polarities of ethanol and water solvents can effect on solubility of bioactive compounds of plant extracts. Ethanol solvent has more ability to dissolve more phenolic compounds than water solvent [28]. While, water solvent is the best solvent for nutrients (i.e. sugar, vitamins and minerals) that some nutrients can effect on biological activity [29]. Interestingly, it has been reported that *A. bilimbi* L. is an abundant source of ascorbic acid [37], which is an important water-soluble antioxidant [38]. Moreover, the effect of ascorbic acid on melanogenesis has been reported as a depigmentation agent [39]. However, it has been reported that ascorbic acid does not affect tyrosinase inhibition activity, but it can protect melanogenesis *via* antioxidant potential and restriction of nitric oxide production [40]. Interestingly, it has been reported that ascorbic acid can activate tyrosinase activity *via* p38 mitogen-activated protein kinase [41].

Table 1 Total flavonoid contents, radical scavenging activity, and anti-tyrosinase activity of aqueous and ethanol extracts from *A. bilimbi* L. leaves and fruits, and its fruit vinegar.

Plant parts	Solvent types	Total flavonoid content (μg rutin equivalent per mg extract) ^{1*}	Radical scavenging activity		Anti-tyrosinase activity	
			EC_{50} (mg/mL) ^{2*}	$1/\text{EC}_{50}$	EC_{50} (mg/mL) ^{2*}	$1/\text{EC}_{50}$
Fruits	aqueous	0.30 ± 0.07	323.42 ± 37.98	0.0031 ± 0.0003	151.05 ± 20.51	0.0067 ± 0.0009
	ethanol	3.31 ± 0.37	445.72 ± 87.36	0.0023 ± 0.0005	31.42 ± 20.97	0.0399 ± 0.0163
Leaves	aqueous	17.04 ± 0.19	33.16 ± 3.22	0.0304 ± 0.0031	83.48 ± 7.37	0.0121 ± 0.0011
	ethanol	22.96 ± 0.46	51.91 ± 3.76	0.0193 ± 0.0015	8.66 ± 1.61	0.6424 ± 0.0202
Fruit vinegar		68.82 ± 8.40	5.42 ± 0.57	0.1859 ± 0.0191	1.14 ± 0.12	0.8807 ± 0.0932
p -value		0.000*		0.000*		0.000*
p -value		0.000**		0.000**		0.000**

* indicated that total flavonoid content of fruit vinegar was expressed as μg rutin equivalent per g fresh fruit, and

** indicated that EC_{50} for fruit vinegar was expressed as g fresh fruit /mL.

* indicated that difference of total flavonoid content, radical scavenging activity and anti-tyrosinase activity among fruit ethanol extract, leaf ethanol extract, fruit aqueous extract, leaf aqueous extract, and fruit vinegar were detected by one way ANOVA, which significant level was indicated at p -value < 0.05 .

** indicated that difference of total flavonoid content, radical scavenging activity and anti-tyrosinase activity among fruit extract, leaf extract, and fruit vinegar were detected by one way ANOVA, which significant level was indicated at p -value < 0.05 .

For kinetic study, fruit and leaf ethanol extracts were selected to study due to they showed high tyrosinase inhibition. The kinetic study was performed *via* Lineweaver-Burk plot in the with and without sample, and the Michaelis constant (K_m) and maximum velocity (V_{max}) values were determined in this study (**Figure 1**). The K_m value is the binding affinity of enzyme on its substrate, which lower K_m value indicates higher binding affinity of the enzyme on its substrate. Additionally, V_{max} is the maximum velocity of reaction in the condition of maximum saturated enzyme with its substrate. This study showed that the ethanol extracts had both K_m and V_{max} values lower in comparison with the increase of extract concentration at 1.25 mg/mL (fruit ethanol extract) and 0.25 mg/mL (leaf ethanol extract). It indicated that the rate of the reaction was decreased by inhibitors in the ethanol extracts of *A. bilimbi* L. leaves and fruits. Additionally, inhibitors in the ethanol extracts bonded with enzyme-substrate complex, which effected on the increase of enzyme affinity on its substrate (lower K_m). So, inhibition of tyrosinase by the ethanol extracts was identified as uncompetitive inhibition (**Table 2**).

Table 2 Kinetics of anti-tyrosinase activity of ethanol extracts from *A. bilimbi* L. leaves and fruits.

L-DOPA (μM)	$1/V$ (O.D. ₄₅₀ /min) ⁻¹		
	Control	Fruit ethanol extract	Leaf ethanol extract
5,000	44.2	46.9	48.0
2,500	72.7	68.0	83.0
1,250	113.6	101.5	117.6
625	100.5	116.3	141.8
312.5	238.1	150.4	166.7
K_m (μM)	1.39	0.54	0.51
V_{max} (O.D. ₄₅₀ /min)	0.0239	0.0173	0.0146
Types of inhibition	-	uncompetitive inhibitor	uncompetitive inhibitor

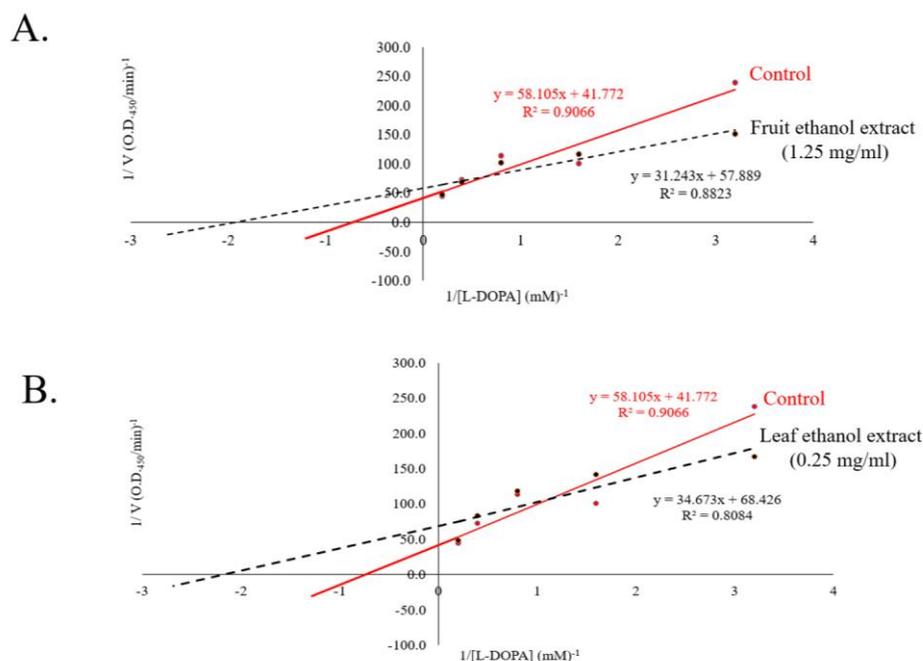


Figure 1 Lineweaver-burk double reciprocal plot in the presence of fruit ethanol extract (A) and leaf ethanol extract (B), comparing with the absence of the extracts.

Moreover, FTIR of the aqueous and ethanol extracts of *A. bilimbi* L. fruits and leaves and its fruit vinegar were determined and compared to standard flavonoids (naringenin, rutin, kojic acid, kaempferol and luteolin) and ascorbic acid. The functional groups of the extracts and its fruit vinegar were shown in **Table 3**. It showed that leaf ethanol extract showed the greatest number of functional groups corresponding to functional groups of flavonoids namely O-H, CH₂ and CH₃, C=O, C=C, C-O, -COOH and C-O-C groups (**Table 3** and **Figure 2**). Corresponding to previous study, it has been reported that ethanol extraction of *A. bilimbi* L. leaves have O-H, C-H, C=O, C=C and C-O groups [28].

Table 3 Wavenumber ranges of FTIR peaks under 550 to 4,000 cm⁻¹ found in aqueous and ethanol extracts from *A. bilimbi* L. leaves and fruits.

Wavenumber ranges of FTIR peaks found in samples (cm ⁻¹)	Function groups found in reference	Predicted phytochemicals
3,149.23 - 3,423.7	O-H stretch	[30]
2,853.33 - 2,974.99	CH ₂ and CH ₃ stretching vibrations	[31]
2,697.18	Unknown	-
1,738.77 - 1,752.68	C=O	[32]
1,634.96 - 1,659.77	Unsaturation bonds	[30]
1,603.75 - 1,659.77	Conjugation of bonds, C=O stretching vibrations	[30,33]
1,500.76 - 1,596.41	Aromaticity	[30]
1,470.69 - 1,515.62	Pyrylium ring in plan	[33]
1,436.73 - 1,455.74	Aromatic C=C stretching vibrations	[33]
1,315.63 - 1,448.6	Primary or secondary O-H bending (in-plane), and phenol or tertiary alcohol (O-H bend)	[34,35]
1,234.62 - 1,308.25	C-O stretching vibrations of aromatic ethers	[33]
1,006.74 - 1,295.44	Ester, carboxylic acid and ether	[33]
967.46 - 1,195.84	Pyranose structure	[33]
1,031.86 - 1,195.84	C-O stretching vibrations, pyranose structure	[33]
1,166.53	C-O-C stretching ring pyranose vibrations	[33]
1,097.67	Cyclic ethers, asymmetric C-O-C stretching vibrations	[33]
501.99 - 937	C-H bending vibrations	[36]
807.69 - 820.3	Cyclic ethers, symmetric C-O-C stretching vibrations	[33]

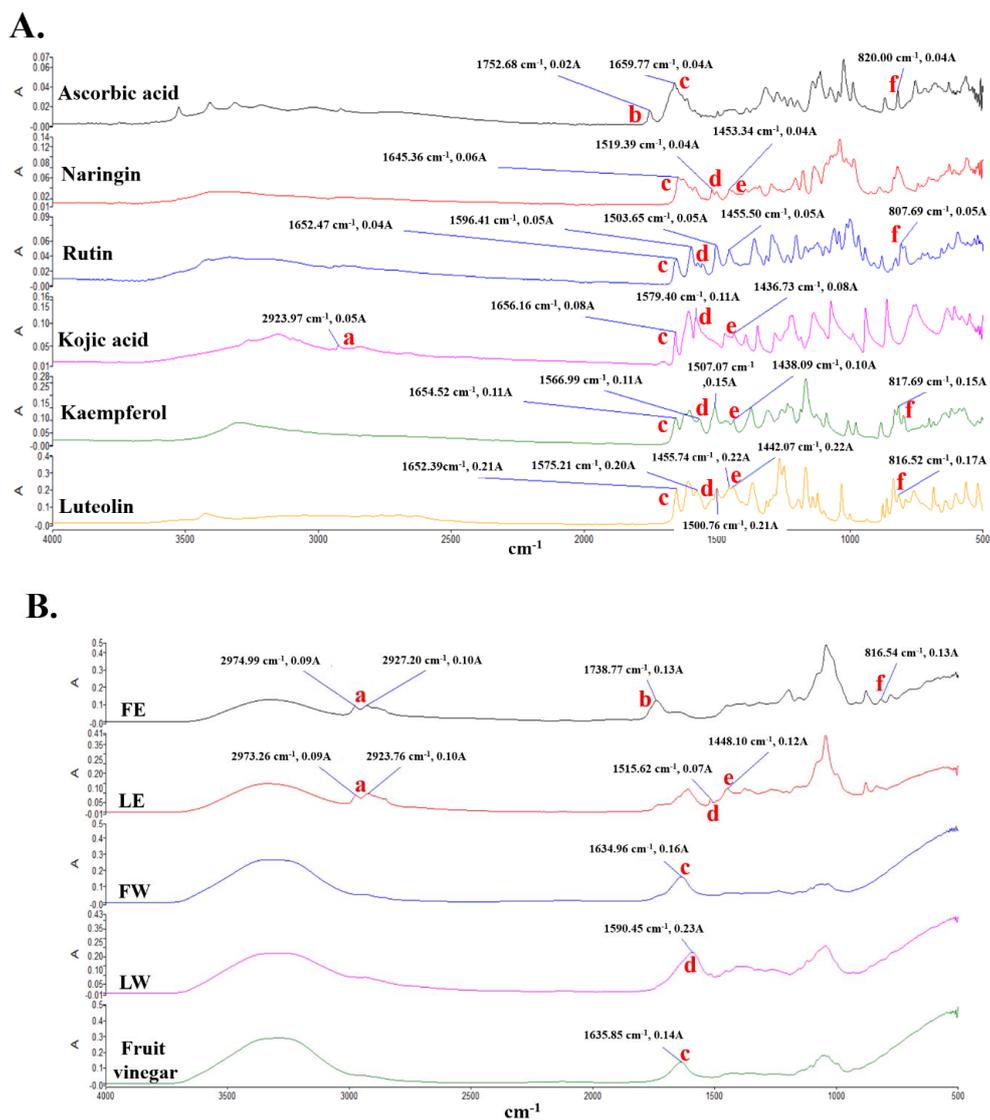


Figure 2 Comparison of FTIR spectra. (A) FTIR spectra of ascorbic acid, naringenin, rutin, kojic acid, kaempferol and luteolin, respectively. (B) FTIR spectra of fruit ethanol extract (FE), leaf ethanol extract (LE), fruit aqueous extract (FW), leaf aqueous extract (LW), and fruit vinegar, respectively. Symbol a and f were specific peaks found in ethanol extracts. Symbol b was a specific peak found in fruit ethanol extract. Symbol d was leaf ethanol extract. Symbol c was a specific peak found in fruit aqueous extract and fruit vinegar.

Moreover, the leaf ethanol extract showed 3 specific peaks corresponding to C=C group of standard flavonoids (naringenin, rutin, kojic acid, kaempferol and luteolin) and CH₂ and CH₃ group of kojic acid. The fruit ethanol extract showed 3 specific peaks corresponding to C-O-C group of ascorbic acid, rutin, kaempferol, and luteolin, C=O group of ascorbic acid and CH₂ and CH₃ group of kojic acid. While, the leaf aqueous extract showed a specific peak corresponding to aromaticity of naringenin, rutin, kojic acid, kaempferol and luteolin. The fruit aqueous extract and fruit vinegar showed a specific peak corresponding to unsaturation bonds of ascorbic acid, naringenin, rutin, kojic acid, kaempferol and luteolin.

Moreover, strongly positive correlation was significantly detected between radical scavenging activity and TFC ($r = 0.83$, p -value < 0.05), and between FTIR data and anti-tyrosinase activity ($r = 0.75$, p -value < 0.05). Moderately positive correlation was significantly detected between anti-tyrosinase activity and TFC ($r = 0.56$, p -value < 0.05), and between FTIR data and TFC ($r = 0.66$, p -value < 0.05). However, significant correlation was not found between anti-tyrosinase activity and radical scavenging activity and between FTIR data and radical scavenging activity (**Table 4**). The result indicated that

flavonoids are important roles for radical scavenging activity and anti-tyrosinase activity. Moreover, it implies that it may contain non flavonoids, which have radical scavenging activity and do not effect on tyrosinase inhibition.

Table 4 Pearson's correlation between biological activities and between biological activity and chemical content of aqueous and ethanol extracts of *A. bilimbi* L. fruits and leaves.

	Total flavonoid content	Radical scavenging activity	Anti-tyrosinase activity
Radical scavenging activity	$r = 0.83$ (p -value = 0.000)*		
Anti-tyrosinase activity	$r = 0.56$ (p -value = 0.025)*	$r = 0.21$ (p -value = 0.446)	
FTIR data	$r = 0.66$ (p -value = 0.005)*	$r = 0.14$ (p -value = 0.609)	$r = 0.75$ (p -value = 0.001)*

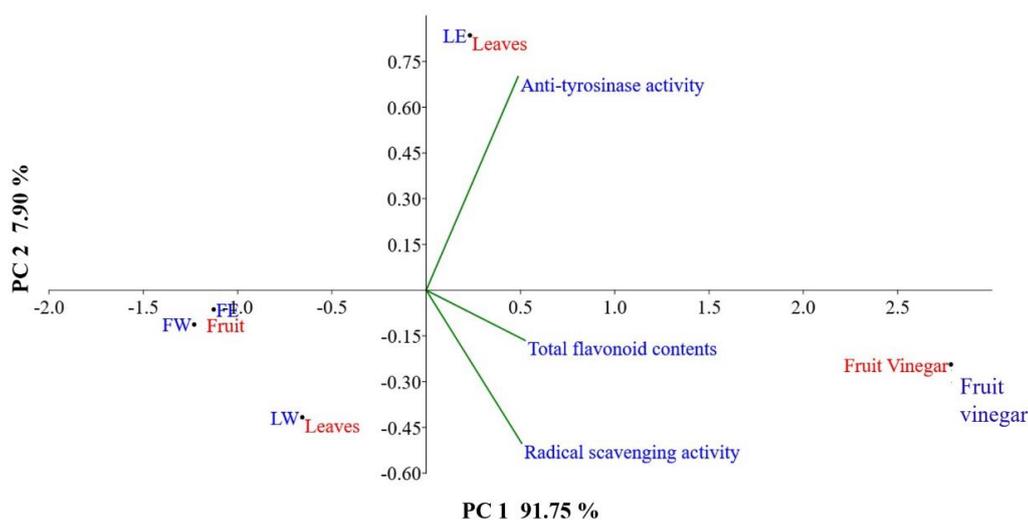
* Significant level was indicated at p -value < 0.05.

Previous studies have illustrated that several flavonoids from plants (i.e. luteolin, rutin and kaempferol) have the potential in tyrosinase inhibition. It has reported that luteolin from leaves of *Blumea balsamifera* DC has anti-tyrosinase activity [42], and kaempferol from kenaf (*Hibiscus cannabinus* L.) leaves shows anti-tyrosinase property [43]. Additionally, rutin has anti-tyrosinase potential as competitive inhibition [44]. Some flavonoids (i.e. apigenin-7-butylene glucoside and naringenin) can increase tyrosinase activity due to the enhance in the transcription and expression levels of melanogenic genes [45-47]. Flavonoids can also act as tyrosinase inhibitors by the presence of a hydroxyl at C7 in disturbing tyrosinase activity [20], and different functional groups of flavonoids can effect on inhibition of tyrosinase activity [21].

The principal component analysis (PCA) of chemical content and biological activity was analyzed. The PCA result showed 2 components. The first principal component (PC1) was 91.75 % and the second principal component (PC2) was 7.90 % of total variance (**Figure 3A**). The PCA result showed differentiation of total flavonoid content, radical scavenger activity, and anti-tyrosinase activity among samples. The result confirmed that the leaf ethanol extract showed the highest anti-tyrosinase activity and total flavonoid content, while leaf aqueous extract showed the highest radical scavenging activity. For fruit vinegar sample, it was different from extract samples due to it showed strongest total flavonoid content in μg rutin equivalent per g fresh fruit, and in unit g fresh fruit/mL for radical scavenger activity and anti-tyrosinase activity.

Moreover, the PCA analysis of FTIR data was performed. The PC1 was 34.88 % and the PC2 was 18.26 % of total variance (**Figure 3B**). It indicated that the leaf ethanol extract had several functional groups similar to the functional groups of standard flavonoids. Fruit vinegar showed functional groups similar to the functional groups of fruit aqueous extract and ascorbic acids.

A.



B.

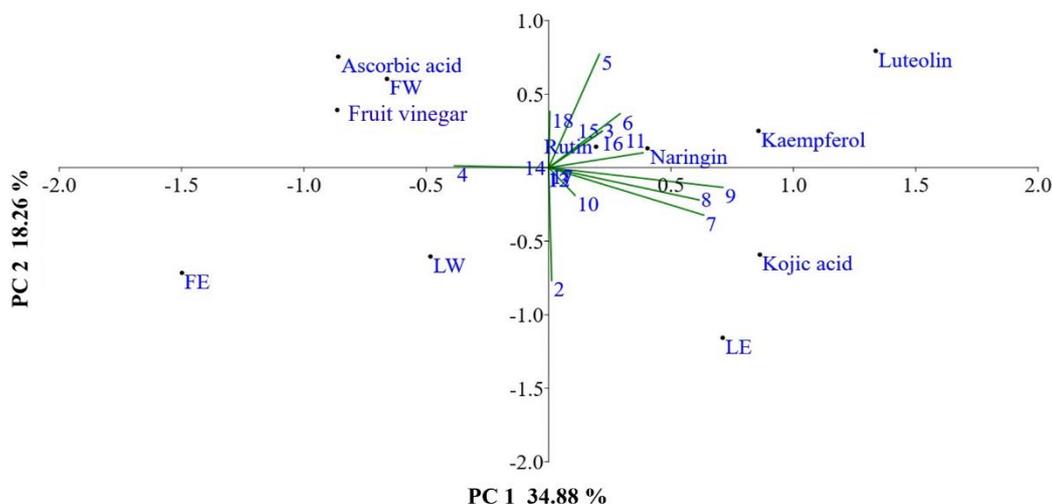
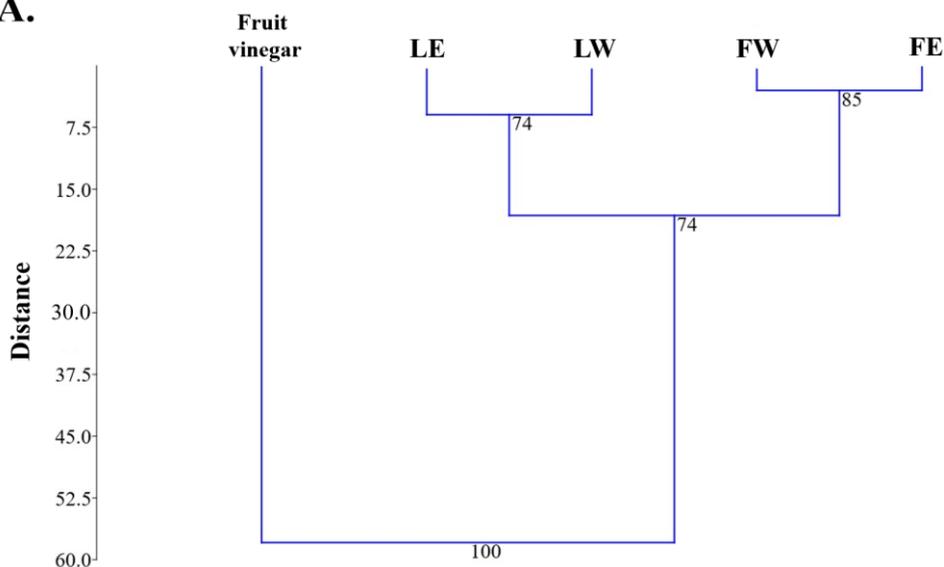


Figure 3 Principal component analysis (PCA) based on chemical contents and biological activity (A), and based on FTIR data (B). FE, LE, FW, and LW were fruit ethanol extract, leaf ethanol extract, fruit aqueous extract, leaf aqueous extract, respectively. Number 1-18 were wavenumber ranges shown in **Table 2**.

Similarity, cluster analysis of chemical content and biological activities was classified into 2 groups namely fruit vinegar and extract samples (leaf ethanol extract, leaf aqueous extract, fruit ethanol extract and fruit aqueous extract) (**Figure 4A**). Additionally, cluster analysis of FTIR data indicated that leaf ethanol extract had the most similarity with standard flavonoids (kojic acid, naringin, rutin and kaepferol), while fruit aqueous extract had the most similarity with fruit vinegar and ascorbic acid (**Figure 4B**). The result was in agreement with previous study that reported FTIR spectrum can be effected by solvent types and methods for sample preparation, and combination of FTIR, PCA and cluster analysis can help to discriminate sample groups [22].

A.



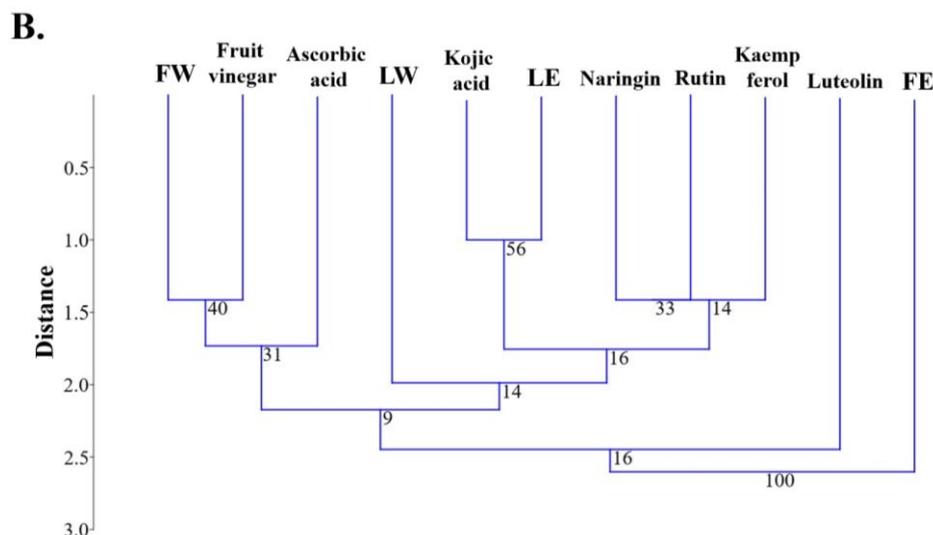


Figure 4 Cluster analysis based on chemical contents and biological activity (A), and based on FTIR data (B). FE, LE, FW, and LW was fruit ethanol extract, leaf ethanol extract, fruit aqueous extract, leaf aqueous extract, respectively.

Therefore, the PCA and cluster analysis based on FTIR data, chemical contents and biological activity could be helpful in discriminating the difference of sample groups depended on parts of plant, solvent types and sample preparatory methods. Therefore, the fruits and leaves of *A. bilimbi* L. could be used as natural source of whitening agents for skin care products and antibrowning agents for food industries.

Conclusions

According to the result above, we found that the extracts from *A. bilimbi* L. fruits and leaves, and its fruit vinegar were good source of total flavonoid content (TFC), radical scavenging activity and anti-tyrosinase activity. The ethanol extracts from *A. bilimbi* L. fruits and leaves showed the highest value of anti-tyrosinase activity, whereas aqueous extract showed the highest radical scavenging activity. Interestingly, the result revealed the existence of rich TFC, radical scavenging activity and anti-tyrosinase activity in vinegar sample, which was fermentation product from *A. bilimbi* L. fruits. The anti-tyrosinase potential of the ethanol extract of *A. bilimbi* fruits and leaves was identified as an uncompetitive inhibitor, which bonded only to the enzyme-substrate complex. For functional groups, leaf ethanol extract showed several functional groups belonging to flavonoids namely O-H, CH₂ and CH₃, C=O, C=C, C-O, -COOH and C-O-C groups. Besides, strongly positive correlation was significantly detected between radical scavenging activity and TFC, and between FTIR data and anti-tyrosinase activity. Moderately positive correlation was significantly detected between anti-tyrosinase activity and TFC, and between FTIR data and TFC. The application of PCA and cluster analysis combining with FTIR data, TFC and biological activities could differentiate extracts from *A. bilimbi* L. fruits and leaves and its fruit vinegar. However, identification of bioactive compounds in *A. bilimbi* L. fruits and leaves, and its fruit vinegar are necessary to further analyze by using several tools (i.e. gas chromatography-mass spectrometry (GC-MS), liquid chromatography- mass spectrometry (LC-MS), high performance liquid chromatography (HPLC) and nuclear magnetic resonance spectroscopy (NMR).

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