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Antioxidant Activity, Anti-tyrosinase Activity and Fourier Transform Infrared Spectra of Extract and Lotion from Germinated Seeds of *Vigna mungo* (L.) Hepper

Sirikul Thummajitsakul[1,*], Phattiya Kriangchaikijkul[1], Supawee Hrunkham[1], Kittiraphorn Onchai[1], Kun Silprasit[2]

[1]*Faculty of Physical Therapy, Srinakharinwirot University, Nakhon-Nayok 26120, Thailand*
[2]*Faculty of Environmental Culture and Ecotourism, Srinakharinwirot University, Bangkok 10110, Thailand*

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ABSTRACT

This study demonstrated different fingerprints of ethanol extracts, aqueous extracts, and lotion samples from germinated seeds of *Vigna mungo* (L.) Hepper by Fourier transform infrared spectra (FTIR). Moreover, their total phenolic content, antioxidant activity, and tyrosinase inhibition were also determined. The results showed that ethanol and aqueous extracts of germinated *V. mungo* seeds had phenolic content, antioxidant activity, and tyrosinase inhibition. The ethanol extracts had the highest total phenolic content (40.77±7.80 mg gallic acid equivalents/g extract) and the strongest antioxidant activity ($1/EC_{50}$=0.009). The high tyrosinase inhibition was found in the lotion base ($1/EC_{50}$=3.5343), the lotion containing ethanol extracts ($1/EC_{50}$=1.4473), and aqueous extracts ($1/EC_{50}$=1.1896). A specific FTIR peak for ethanol extract and lotion samples was assigned to a range of 2853.42-2979.02 cm^{-1}, and two specific peaks for ethanol extracts were at 1741.65-1742.7 and 1539.7-1543.25 cm^{-1}. The FTIR peaks of the ethanol extract were observed in 11 wavenumber ranges higher than other samples. Interestingly, the principal component analysis and paired group clustering from the FTIR data, total phenolic content, and biological activities, were able to detect differentiation of the extracts and their lotion samples.

Keywords: Antioxidant activity; FTIR; Tyrosinase inhibition; Lotion; *Vigna mungo*

***Corresponding author:** sirikul.thum@gmail.com

1. Introduction

Vigna mungo (L.) Hepper belongs to the family Fabaceae, locally known as Black gram, and it is commonly grown in tropical Asian countries [1]. It is one of a number of native legume species in Thailand, which is conserved in the Plant Genetics Conservation Project under the Royal Initiative of Her Royal Highness Princess Maha Chakri Sirindhorn [2]. Its seed is a popular food ingredient as either main course or dessert, and can be consumed as sprouts. Moreover, several studies have reported therapeutic properties of this seed, such as anti- inflammatory, antimicrobial, antioxidant, and antidiabetic activity [3]. Furthermore, it has high nutritional value (i.e., carbohydrates, protein, vitamins, minerals, fat, and fiber) and levels of bioactive compounds (i.e., phenolic compounds, flavonoids, saponins, tannins, alkaloids, and steroids) [4]. Meanwhile, the germination process can further increase its nutritional value and the biochemical activities of its bioactive agents [5-7].

Nowadays, pigmentary disorder is a dermatological condition involving an increased amount of melanin pigments, leading to skin darkening [8]. Tyrosinase is a key enzyme involved in the synthesis of melanins within human melanocytes [9, 10]. Thus, the inhibition of tyrosinase can help to reduce overproduction of melanin pigments, which is useful for cosmetic skin whitening products [11]. It has been reported that some bioactive agents (i.e., phenolics and antioxidants) in plant extracts (i.e., mushroom and riceberry rice) are useful for controlling hyperpigmentation [12-14].

However, several studies of legume seeds focus mainly on nutritional value and bioactive compounds, and development their food products [4, 15-17]. Nowadays, knowledge of black gram sprouts is still limited in cosmetic products of skin whitening. Therefore, our major objectives were to determine the effect of extract and lotion made from germinated *Vigna mungo* (L.) Hepper seeds on fourier transform infrared spectra (FTIR) fingerprint, total phenolic content, antioxidant activity, and anti-tyrosinase activity.

2. Materials and Methods

2.1 Chemicals

Chemicals used include: Tyrosinase from mushroom (Sigma), 3,4-Dihydroxy-L-phenylalanine (Sigma), 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) (Sigma), Folin–Ciocalteu's phenol reagent (Merck), Gallic acid (Sigma-Aldrich), Absolute ethanol (Sigma-Aldrich), Potassium persulfate (Ajax Finechem), 1-Hexadecanol (Sigma-Aldrich), Potassium sorbate (Sigma-Aldrich), Emulsifying wax (Sigma-Aldrich), and Glycerol (Sigma-Aldrich).

2.2 Preparation of germinated seeds

Vigna mungo (L.) Hepper was purchased from a market and its external morphology was identified by comparing it with the samples from the E- learning of the Department of Agronomy, Kasetsart University, Kamphaengsean Campus [40]. For the germination process, the black gram was cleaned with water and then soaked in water for 24 hours. Following this, the outer layer of the germinated seeds were peeled off. After peeling, the samples were incubated at 50°C for 16 hours or until dry. Then, the dried samples were ground into fine powder and kept at 4°C until use.

2.3 Extraction of ground samples

Ground sample (10 g) was extracted with 250 ml solvent (absolute ethanol and distilled water) for 24 hours at 45 °C. The extraction process was performed twice. Each extract was sieved *via* filter cloth. Then, each filtered extract was evaporated at 45°C for either 19 mins (absolute ethanol extract) or 40 mins (aqueous extract) using a rotary evaporator (IKAa RV10) according to the protocol of Thummajitsakul et al. (2019) [18]. A gummy extract was obtained and

further diluted with its respective solvent for final concentration of 10 mg/ml.

2.4 Lotion preparation

A lotion base was prepared by mixing 3% cetyl alcohol, 3% emulsifying wax, 4% coconut oil, and 2% polysorbate 80 at 70 ºC until the mixture was clear. Then, 2% glycerol was added to the mixture and stirred until its temperature decreased to 40 ºC. For the lotion formulation, each sample of germinated *V. mungo* seed extract (10 ml) was added to the lotion base and left to cool down to room temperature.

2.5 Total phenolic contents

The total phenolic content of each extract was estimated by using the Folin-Ciocalteu method [18]. Each extract (300 µl) was thoroughly mixed with 1.5 ml of Folin-Ciocalteu reagent for 5 minutes at room temperature, followed by mixing with sodium carbonate (7.5% w/v) for a final volume of 1.2 ml, and allowed stand for 30 minutes at room temperature. The absorbance of each reaction was detected at 765 nm. Each reaction was carried out 2 times. Gallic acid (0 - 1 mg/ml) was used to generate the standard curve (R^2=1.00). The total phenolic content of each extract was expressed as mg of gallic acid equivalent per g extract.

2.6 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS)

Antioxidant activity of each sample was evaluated using the ABTS free-radical scavenging method [18]. The ABTS radical cation solution was carried out by the reaction of 7mM ABTS (10 ml) and 140 mM potassium persulfate (179 µl) under darkness at room temperature for 16 hours. Then, the ABTS radical cation solution was diluted with distilled water until its absorbance at 734 nm measured 0.700 ± 0.050. Each extract (20 µl) was then reacted in darkness with the diluted ABTS radical cation solution (3.9 ml). The absorbance of each reaction was determined after 6 minutes at 734 nm. Each reaction was performed in duplicate. The percentage of antioxidant activity was measured using the equation below:

$$\%\,Antioxidant\ capacity = \frac{(OD_{ABTS} - OD_{Sample-ABTS})}{OD_{ABTS}} \times 100\%. \quad (2.1)$$

OD_{ABTS} is the absorbance of the diluted $ABTS^{\bullet+}$ solution, while $OD_{Sample\text{-}ABTS}$ is the absorbance of a mixture of the diluted $ABTS^{\bullet+}$ solution and each sample.

The percentage of antioxidant activity of each sample was used to generate a simple linear regression (R^2=0.95-1.00) to determine the effective concentration to scavenge 50% of radicals (EC_{50}).

2.7 Tyrosinase inhibition assay

Tyrosinase inhibitory activity was determined using 3-(3,4-dihydroxyphenyl)-Lalanine (L-DOPA) as a substrate [19]. Briefly, 5 mM L-DOPA (100 µl), 0.1M sodium phosphate buffer pH 6.8 (20 µl), and seed extract (40 µl) were mixed. For the negative control, sodium phosphate buffer was used in place of extract. Then to each mixture, 40 µl of mushroom tyrosinase (200 Units/ml) was added, and then incubated at 37°C for 20 min. The absorption was determined at 450 nm. Kojic acid (10 mg/ml) was used as positive control. The percentage of tyrosinase inhibition was calculated using the equation below:

$$\%\,Tyrosinase\ inhibition = \frac{(OD_{blank} - OD_{sample})}{OD_{blank}} \times 100, \quad (2.2)$$

where OD_{blank} is the absorbance of each reaction without each sample at 450 nm, and OD_{sample} is the absorbance of each reaction with each sample at 450 nm.

The percentage of tyrosinase inhibition of each sample was used to

generate a simple linear regression (R^2= 0.70-1.00) to determine the effective concentration to inhibit 50% of tyrosinase (EC_{50}).

2.8 Fourier transform infrared spectra (FTIR) fingerprint

FTIR peaks in aqueous extracts, ethanol extracts, and lotion samples were detected in a range of 550 to 4000 cm^{-1} and a resolution of 4 cm^{-1} by FTIR spectroscopy (PerkinElmer spectrum IR version 10.6.0). Each sample was measured in duplicate [20-27].

2.9 Statistical analysis

PSPP version 0.10.5 [28] and the paleontological statistic program version 3.16 [29] were used for statistical analysis. Descriptive statistical analysis of total phenolic contents, antioxidant activity, and tyrosinase inhibitory activity is expressed as mean, standard deviation, and percentage. Moreover, the difference in total phenolic content, antioxidant activity, tyrosinase inhibitory activity, and FTIR data among sample groups were all analyzed using one-way analysis of variance (one-way ANOVA). Principal component analysis (PCA) was used to summarize the data, while paired group (UPGMA) clustering (bootstrap=10000) was applied to express differentiation of the samples.

3. Results and Discussion

The functional plant ingredients and product quality can be directly or indirectly influenced by the production process. It has been reported that legumes consist of various phytochemicals that are beneficial for human health [4, 17]. The germination process especially effects the level of bioactive compounds and antioxidants in seeds, with a germination period of 1-5 days being best for increasing the level of these compounds [30].

3.1 Fourier transform infrared spectra (FTIR) fingerprint

In current study, the FTIR fingerprints of ethanol extracts, aqueous extracts, powder sample, and lotion samples of germinated *V. mungo* seeds showed differences in the range of 550 to 4000 cm^{-1} (Fig. 1). The results demonstrated the effect of extraction solvent and lotion samples of *V. mungo* on the FTIR fingerprints. The FTIR peaks in 12 wavenumber ranges for each sample were identified and shown in Table 1. The results showed a specific peak for ethanol extract and lotion samples in the range of 2853.42-2979.02 cm^{-1}, which indicate C–H stretching in CH_2 groups (carbohydrates and fats) [20, 25, 26], while specific peaks for ethanol extract were at 1741.65-1742.7 and 1539.7-1543.25 cm^{-1}, which indicate C=O in lipids and N–H of proteins, respectively [22, 24, 26]. Interestingly, the greatest number of peaks were found in the ethanol extract for 11 wavenumber ranges, followed by aqueous extracts (8 wavenumber ranges), lotion samples (5 and 8 wavenumber ranges), and powder sample (4 wavenumber ranges) of germinated *V. mungo* seeds.

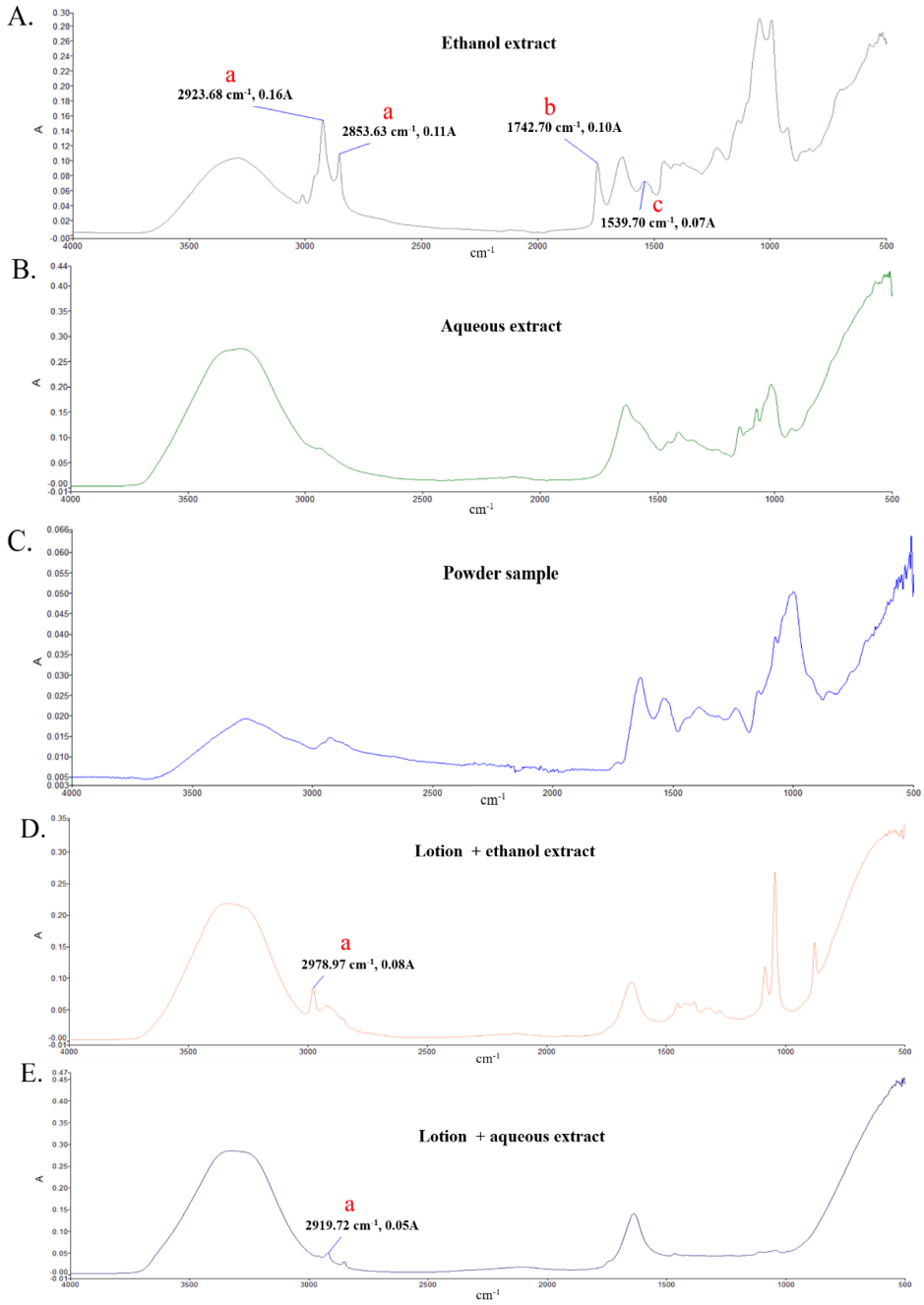

Fig. 1. Example of FTIR spectra of extracts, lotion samples, and powder sample of germinated *Vigna mungo* seeds. A: ethanol extract, B: aqueous extract, C: powder sample, D: lotion sample with ethanol extract, E: lotion sample with aqueous extract. Peak a is specific band for ethanol extract, ethanol solvent, and lotion samples, while peaks b and c are specific bands for ethanol extract.

Table 1. Wavenumber ranges of FTIR peaks and functional groups of ethanol extracts, aqueous extracts, and powder sample of germinated *V. mungo* seeds.

Peak number	Wavenumber range of extract and powder samples in this study (cm^{-1})	Wavenumber range of references (cm^{-1})	Assignment	Function groups	References
1	3011.58-3339.80	3000–3600	O-H stretch	water, alcohols, phenols, carbohydrates, peroxides	[22, 25]
2	2853.42 -2979.02	3000–2800	C–H stretching of CH2 groups	carbohydrates and fats	[20, 25, 26] [25, 26]
3	1632.44-1742.7	1600-1760	N-H bending vibrations, C=O bending vibrations	amino acids, fatty acids, ester	[26]
4	1741.65-1742.7	1700-1799	C=O of lipids	lipids	[24]
5	1632.44-1647.3	1600-1706	Amide I of proteins, C=O	proteins	[24]
6	1380.81-1456.05	1380-1465	CH3 lipids/proteins and COO− of amino acids	lipids/proteins/ amino acids	[24]
7	1539.7-1543.25	1460-1590	Amide II of proteins, N–H	proteins	[24]
8	1328.24-1411.19	1300-1450	Primary or secondary O-H bending (in-plane), and phenol or tertiary alcohol (O-H bend)	phenyl groups	[21, 22]
9	1328.24-1377.5	1300-1380	CH3 bending	lipid	[23]
10	1150.3-1229.92	1150-1270	C-O stretching vibrations	acid or ester	[22, 26]
11	1016.43-1047.52	1015-1080	glycosidic	carbohydrates	[27]
12	507.24-998.71	< 1000	C-H bending vibrations	Isoprenoids	[22, 26]

3.2 Total phenolic content

Total phenolic content, antioxidant activity, and tyrosinase inhibitory activity were determined in ethanol extracts, aqueous extracts, lotion containing either aqueous or ethanol extract, and finally lotion base. The results showed that the highest level ($p < 0.05$) of total phenolic content was significantly observed in ethanol extracts (40.77±7.80 mg gallic acid equivalents / g extract), followed by aqueous extract (17.91±2.73 mg gallic acid equivalents / g extract). For the lotion samples, the highest level of total phenolic content was found in lotion containing ethanol extract (2.18±0.74 mg gallic acid equivalents / mL lotion), followed by lotion containing aqueous extract (0.86±0.20 mg gallic acid equivalents / mL lotion), and lotion base (0.75±0.17 mg gallic acid equivalents / mL lotion) (p-value<0.05) (Table 2).

3.3 Antioxidant activity and tyrosinase inhibitory activity

Antioxidant activity of ethanol extract and aqueous extract showed low $1/EC_{50}$ values ($1/EC_{50}$= 0.009 and 0.005, respectively). Similarly, ethanol extract and aqueous extract showed tyrosinase inhibitory activity with low $1/EC_{50}$ values ($1/EC_{50}$= 0.0454 and 0.0025, respectively). However, antioxidant activity was not observed in all lotion samples; tyrosinase inhibitory activity was found at the highest level in lotion base ($1/EC_{50}$ = 3.5343), followed by lotion containing ethanol extract ($1/EC_{50}$ = 1.4473), and then lotion containing aqueous extract ($1/EC_{50}$ = 1.1896) (p-value<0.05) (Table 2).

Table 2. Total phenolic contents, antioxidant activities, tyrosinase inhibition, and the number of FTIR peaks of ethanol extracts, aqueous extracts, lotion samples and powder samples of germinated *V. mungo* seeds.

Samples	Methods of sample preparation	Total phenolic contents (mg gallic acid equivalents / g extract) **	Antioxidant activities		Tyrosinase inhibition		The number of FTIR peaks
			EC_{50} (mg/ml)	$1/EC_{50}$	EC_{50} (mg/ml)	$1/EC_{50}$	
Ethanol	extract	40.77±7.80	105.27±30.94	0.009	22.02±6.74	0.0454	11
	lotion	2.18±0.74	0	-	0.69±0.07	1.4473	8
Aqueous	extract	17.91±2.73	195.98±92.06	0.005	398.86 ±243.29	0.0025	8
	lotion	0.86±0.20	0	-	0.84±0.19	1.1896	5
Lotion base	lotion	0.75±0.17	0	-	0.28±0.05	3.5343	5
Powder samples	grinding	-	-	-	-	-	4
Kojic acid (25-100 mg/ml)					132.73±14.34	0.0076	
P-value		0.000*	0.000*		0.001*		

Note: *Differentiation of total phenolic content, antioxidant activity, and tyrosinase inhibition among ethanol extract and aqueous extract, and lotion samples of germinated *V. mungo* seeds were measured at a significance level of 0.05 (*P-value* <0.05).
** Total phenolic content of lotion samples are expressed as mg gallic/ml lotion

3.4 Principal component analysis (PCA) and paired group (UPGMA) clustering

PCA analysis was carried out on FTIR data in the 550 to 4000 cm^{-1} regions (Figure 2A). The results are shown as scatter plots of the first and second principal components, wherein PC1 contained 45.40% and PC2 contained 24.77% of the spectra variability. The PCA biplot was able to distinguish the FTIR spectrum among samples of the ethanol extracts, aqueous extracts, powder sample, and lotion samples. Additionally, the PCA results indicated that the powder sample, lotion base, and lotion containing aqueous extract had similar chemical compositions. Moreover, aqueous extract and lotion containing ethanol extract had similarity to each other, while the ethanol extract was distinguished from the other samples on the FTIR spectrum (Fig. 2A). Corresponding to the results of PCA analysis, cluster analysis confirmed differentiation of the ethanol extract from the other samples (Figs. 2A and 3A). The ethanol extracts were distributed in positive loadings plot of PC1. This plot demonstrates the major peaks found in the ethanol extract at approximately 1150.3-1229.92, 1328.24-1377.5, 1539.7-1543.25, 1741.65-1742.7, and 2918.55-2979.02 cm^{-1}. Of these bands, specific peaks for ethanol extracts were seen at 1741.65-1742.7 and 1539.7-1543.25 cm^{-1}.

Additionally, the aqueous extract and lotion containing ethanol extract were distributed in negative loadings plot of PC1. This plot demonstrates significant peaks found in the ethanol extract at approximately 1380.81-1456.05, 1328.24-1411.19, and 1016.43-1047.52 cm^{-1}. For the powder sample, lotion base, and lotion containing aqueous extract, peaks were distributed in negative loadings plot of PC2.

The results confirmed that the PCA analysis was able to detect differentiation of the FTIR spectrum of extractions and their products, which was consistent with the cluster analysis. It indicated that extraction solvent and lotion samples had an effect on FTIR spectra.

As the results in Figure 2B shows, PC1 and PC2 were 87.03% and 12.71% of the data variance of total phenolic content, antioxidant activity, and tyrosinase inhibitory activity. The PCA analysis confirmed that the highest total phenolic content and antioxidant activity was found in the ethanol extract, followed by the aqueous extract, while the highest level of tyrosinase inhibitory activity was found in lotion base, lotion containing ethanol extract, and lotion containing aqueous extract.

Similarly, cluster analysis showed differentiation between the ethanol extract and the other samples (Fig. 3B), corresponding to the cluster analysis of FTIR data (Fig. 3A). However, antioxidant activity showed the strongest positive correlation with total phenolic content (r = 0. 989, p-value<0. 05), while the correlation between antioxidant activity and tyrosinase inhibition was insignificant (r =-0.909, p-value>0.05), as were the correlations between antioxidant activity and FTIR data (r = 0. 845, p-value>0. 05), between tyrosinase inhibition and total phenolic content (r = -0. 840, p-value>0. 05), between tyrosinase inhibition and FTIR data (r =-0.571, p-value>0.05), and between FTIR data and total phenolic content (r =0.899, p-value>0.05).

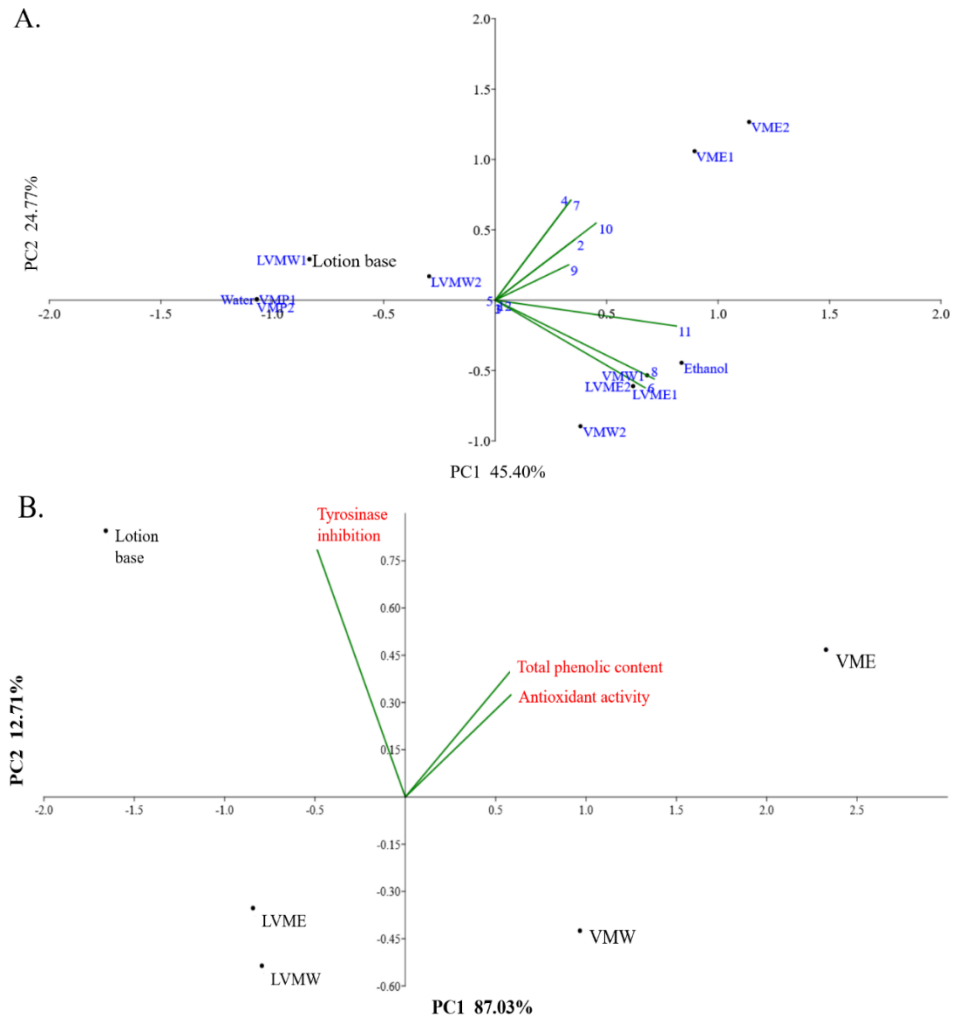

Fig. 2. Principal component analysis (PCA). A: PCA biplot from FTIR data, B: PCA biplot from total phenolic contents, antioxidant activities, and tyrosinase inhibition of ethanol extracts, aqueous extracts, and lotion samples of germinated *V. mungo* seeds. LVMW and LVME are lotion from aqueous extract and ethanol extract, respectively. VMW and VME are aqueous extract and ethanol extract, respectively. VMP is powder sample of germinated *V. mungo* seeds.

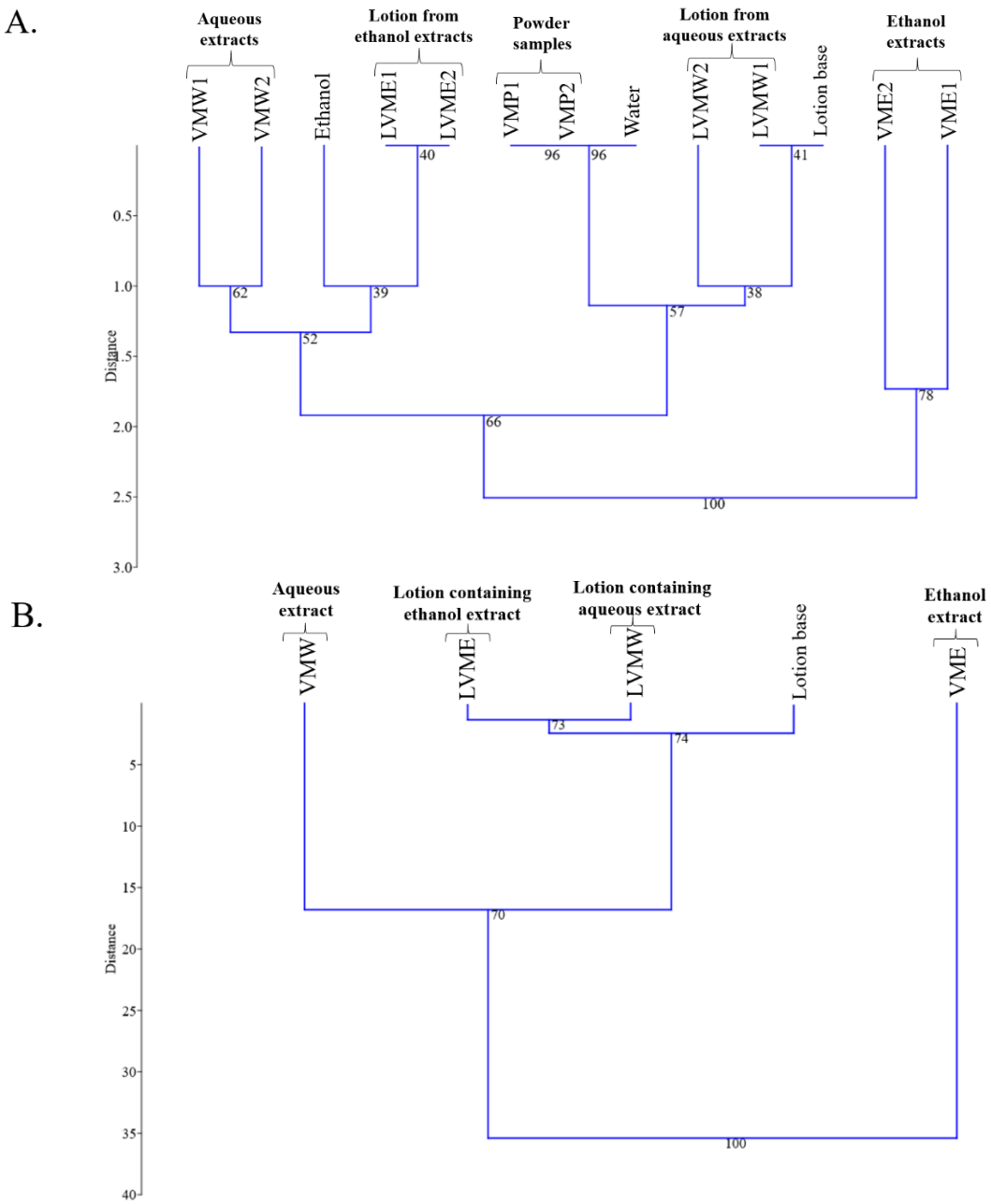

Fig. 3. Paired group (UPGMA) clustering (bootstrap=10000) of extract and powder samples of germinated seeds of *V. mungo*, and each solvent. A was UPGMA clustering from FTIR data, and B was UPGMA clustering from total phenolic contents, antioxidant activities, and tyrosinase inhibition of ethanol extracts, aqueous extracts, and lotion samples of germinated seeds of *V. mungo*.

The FTIR spectrum of germinated seed powder, all extracts, and lotion samples provided interesting results involving the presence of bioactive compounds. Several bioactive compounds provided different functional groups. These functional groups may play very important roles in enhancement of the antioxidant activity and tyrosinase inhibitory activity of the extracts and lotion samples of germinated *V. mungo* seeds. Previously, it has been reported that some bioactive compounds with a higher phenolic hydroxyl number have greater antioxidant and tyrosinase inhibitory activities (i.e., the most activity is in dihydromyricetin, followed by rosmarinic acid, baicalein, shikonin, then isoeugenol) [31]. Phenol hydroxyl compounds (i.e., flavonoids) have a similar structure to tyrosinase substrates (i.e., tyrosine and dopamine), which can affect the inhibition of tyrosinase activity [32]. A greater number of phenolic hydroxyl groups can influence the antioxidant activity by providing more H^+ to scavenge free radicals and hydrogen bonds [33]. Moreover, organic solvent may provide a greater number of phenolic compounds with active functional groups than aqueous solvent does. It has been reported that absolute ethanol extract can provide the greatest antioxidant activity, compared to those of aqueous solvent or ethanol solvents with increasing water content [34].

Previously, a study on FTIR spectroscopy in extracts of *Garcinia schomburgkiana* leaves showed that four specific functional groups are found in ethanol extract, while only one functional group is found in aqueous extract [41].

Moreover, some FTIR peaks seen in the lotion samples matched with those of the ethanol and aqueous extracts, demonstrating some FTIR peaks of extracts and powder sample being revealed in lotion samples. The results showed the presence of phenolic compounds in extracts and lotion samples of germinated *V. mungo* seeds. Phenolic compounds are responsible for various medicinal properties, such as antioxidant, anti- tyrosinase, and anti-diabetic activity [32, 33, 35]. Additionally, it has been reported that the antioxidant and tyrosinase activities of medicinal plant extracts are related to total phenolic contents [36].

However, the lotion samples showed total phenolic content and antioxidant activity lower than the ethanol and aqueous extracts, while the lotion samples showed more effective tyrosinase inhibitory activity than the extracts did. The efficiency of solvent extraction may affect solubility of multiple agents in lotion and plant extract.

However, in this study, the lotion sample contained different ingredients, namely cetyl-alcohol, emulsifying wax, coconut oil, polysorbate 80, and plant extract. Among the ingredients, coconut oil is commonly applied as an important ingredient in cosmetics such as cream and lotion and has been shown to have several biological activities such as anti-inflammatory and skin protective properties [37].

Virgin coconut oil contains several fatty acids such as lauric acid, myristic acid, palmitic acid, and linoleic acid. Among the fatty acids, linoleic acid shows a skin whitening effect [38]. The combination of various active components in the lotion base can provide greater activity than the sum of each component. For example, it has been reported that repellent cream formulations, which are prepared from different essential oils, can provide a synergistic benefit against *Culex quinquefasciatus* and *Aedes aegypti* [39]. Moreover, the anti-tyrosinase activity in the lotion base was found to be greater than the lotions containing plant extract. It indicates that the lotion base employed in this current study was not suitable since some ingredients in the lotion base may interfere with tyrosinase inhibitory activity.

4. Conclusion

In conclusion, this study confirmed that ethanol and aqueous extracts of germinated *V. mungo* seeds had phenolic content, antioxidant activity, and tyrosinase inhibitory activity. Moreover, the lotion base, lotion containing aqueous extract, and lotion containing ethanol extract showed tyrosinase inhibitory activity higher than that of the extracts. Among all lotion formulations studied, the best tyrosinase inhibitory activity was found in the lotion base. Moreover, powder germinated seeds, ethanol extracts, aqueous extracts, and lotion samples provided different FTIR fingerprints. The PCA and UPGMA clustering, obtained from the FTIR data, total phenolic content, and biological activities, were able to differentiate between extracts and their lotion samples. The FTIR data of germinated seed powder, all extracts, and lotion samples showed the presence of several bioactive compounds. The bioactive compounds with different functional groups may involve the antioxidant activity and tyrosinase inhibitory activity of the extracts of germinated *V. mungo* seeds. Thus, it can be concluded that these effective seed extracts can be applied for the formulation of skin whitening lotion.

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