บทความวิจัย

SCREENING ACTIVE NATURAL LIGANDS OF TYROSINASE FROM *Aglaonema simplex* BL. BY HPLC

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ABSTRACT

Ethanol extract of *Aglaonema simplex* rhizomes exhibited higher monophenolase inhibitory activity than arbutin, with IC_{50} values of 53.65 ± 0.80 and 138.15 ± 0.96 µg/mL respectively, while diphenolase inhibitory activity values were similar to arbutin at 6.35 ± 0.23 and 7.11 ± 0.16 µg/mL respectively. Arbutin was used as a binding ligand for screening tyrosinase inhibitors using high performance liquid chromatography with a diode-array detector (HPLC-DAD). The analytical method was validated. The calibration curve of arbutin standard generated the regression equation, y = 25.356x – 167.65, r^2 = 0.9991. The accuracy was presented in term the percentage recovery and was showed 99.70 to 102.45 %. The LOD and LOQ values were 37.74 and 114.37 µg/mL, respectively. Intra–day and inter–day precisions were presented in term the percentage of relative standard deviations and were in the range 0.07–0.13% and 0.002–0.01%, respectively. The optimized ligand screening at 37 °C and 40 min. Five unknown tyrosinase inhibitor binding ligands were identified in the ethanol extract of *A. simplex* rhizomes using HPLC-DAD with retention times of 13.95, 19.64, 21.30, 38.98 and 43.90 min, respectively. The result suggested that the extract of *A. simplex* rhizomes could be contained the marker compounds for the development of the extract in cosmeceuticals.

Keywords: *Aglaonema simplex*, arbutin, ligand, tyrosinase inhibitor, HPLC-DAD

Introduction

Tyrosinase (EC 1.14.18.1), a polyphenol oxidase, is a copper-containing enzyme found in animals, bacteria, fungi and plants (Sánchez-Ferrer et al., 1995). Tyrosinase catalyses both monophenolase activity (hydroxylation of L-tyrosine to L-DOPA) and diphenolase activity (oxidation of L-DOPA to DOPAquinone) through melanin reaction pathways (Fais et al., 2009). Overproduction

of melanin pigment may cause serious problems that manifest as age spots, freckles, melasma and senile lentigines leading to cell death.

Tyrosinase inhibitors such as arbutin, azelaic acid and kojic acid are used to ameliorate skin hyperpigmentation in cosmetic and pharmaceutical products. However, side effects of tyrosinase inhibitors can result in allergic reactions, dermatitis and erythema (Chen et al., 2016). Thus, the search for novel tyrosinase inhibitors from natural sources has recently increased to screen bioactive components (Chen et al., 2016; Chen et al., 2013).

Ultrafiltration method has been used in screening bioactive compounds from plant extracts. The ultrafiltration membrane is used to separate ligand-bound complexes from the unbound compounds. The following analysis step is commonly determined by analytical instruments such as high performance liquid chromatography or mass spectrometry. The adventages of ultrafiltration are low sample consumption, high recovery yields and reduction labor-intensive task (Wang et al., 2018; Zhang et al., 2017).

Aglaonema simplex BL., a member of the Araceae family, is native to Southeast Asia and widely cultivated in tropical rain forests (Napiroon & Vajrodaya, 2017). It is traditionally used as a Thai herb for several ethnopharmacological properties, called Wan Khan Mak in Thailand. The major constituents of the genus *Aglaonema* are polyhydroxy alkaloids and *A. simplex* exhibits glycosidase inhibitory activity (Watson et al., 2001).

Previous reports indicated that alkaloids, glycosides, phenolics, steroids and terpenoids from *A. simplex* stems and roots exhibited anti-cardiovascular effects (Ismail et al., 2017). Alkaloids, coumarins, organic compounds, phenolic compounds and terpenoids were found in lipophilic extracts of *A. simplex* rhizomes (Napiroon & Vajrodaya, 2017), while butyl stearate and β-sitosterol were isolated from *A. simplex* rhizomes. β-Sitosterol exhibited mild hyaluronidase inhibitory activity, while molecular docking of β-sitosterol showed the highest hyaluronidase inhibition (Khammee et al., 2020).

The ligand of protein is one of analytical chemistry for the qualitative and quantitative measurements in method development (Satheshkumar et al., 2021). Previous reports indicated that ligand of tyrosinase inhibitors as unbound low-mass molecules from mulberry leaves extract were analyzed using ultrafiltration and separated by high performance liquid chromatography coupled with diode array detector and mass spectrometry (HPLC-DAD-MS) (Yang et al., 2012). Ligand of biotin derivatives were immobilized on magnetic particles and analyzed by HPLC-MS via selective ion monitoring (HPLC-MS-SIM). This method was validated and showed the highest sensitivity (Yang et al., 2011). Ligand of glutathionylated derivatives in plant were immobilized using glutathione transferase and characterized by HPLC-MS (Dixon & Edwards, 2018).

Many researchers have been reported that tyrosinase inhibitors were isolated from the various plants. (+)-Dihydrokaempferol, ellagenic acid, kaempferol, myricetin, quercetin, rhamnetin-3-*O*-β-D-glucopyranoside, resveratrol exhibited antityrosinase activity (Chunhakant & Chaicharoenpong, 2019; Solimine et al., 2016; Chen et al., 2015; Sarkhail et al., 2013; Jeong & Shim, 2004). Moreover, the plant extracts have been reported on antityrosinase activity. Methanol extract of *Podocarpus elongates* stem, ethyl acetated fraction of *Cynometra cauliflora* leaves, essential oil extract from *Citrus grandis*, extract of *Rubia cordifolia* exhibited antityrosinase activity (Aumeeruddy-Elalfi et al., 2016; Biswas et al., 2015; Ado et al., 2014; Abdillahi et al., 2011). A preliminary study demonstrated that ethanol crude extract of *A. simplex* rhizomes showed strong antityrosinase activity (Rujitanapanich et al., 2018). However, scant research has been conducted concerning the tyrosinase inhibitors of *A. simplex*.

Here, the aim of this study was to evaluate an HPLC-DAD method for development of interaction between tyrosinase and ligands from extract of *A. simplex* rhizomes. In addition, the crude extract of *A. simplex* rhizomes was investigated for antityrosinase activity.

Methods

1. Chemicals

Analytical grade chemicals as arbutin, disodium hydrogen phosphate, L-DOPA, L-tyrosine, mushroom tyrosinase and sodium dihydrogen phosphate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Absolute ethanol, acetonitrile (ACN), methanol (MeOH) and water were purchased from Merck (Darmstadt, Germany). ACN, MeOH and water were of HPLC grade.

2. Instruments

UV-VIS measurements were recorded using a PerkinElmer Spectrophotometer. HPLC analysis was conducted using an Agilent Series 1100 liquid chromatography system with a quaternary pump, a vacuum degasser and a diodearray detector.

3. Plant materials

Fresh rhizomes of *A. simplex* were collected from Kanchanaburi Province, Thailand during February 2019.

4. Plant extraction

Fresh rhizomes of *A. simplex* were ground to powder and then dried in an oven at 60 °C. For extraction, the dried sample (50 g) was macerated with ethanol (0.5 L) for 24 h at room temperature for three times. The solvent extract was then filtered and evaporated under vacuum-reduced pressure to obtain ethanol crude extract of A. simplex (8.33 g).

5. Chromatographic conditions

HPLC analysis was performed on an octadecylsilyl (ODS) ThermoHypersil Keystones column of 250×4.6 mm packed with 5 µm particles (YMC Co., Kyoto, Japan) and equipped with a guard column of 20×3.0 mm with 3.5 µm particles (Phenomenex Inc., Torrance, CA, USA). The column temperature was operated at 25 °C. The injection volume was 10 µL and the flow rate was 1.0 mL/min. The DAD detection wavelength was measured at 280 nm.

The gradient-elution system was determined using a protocol modified from Zhang et al. (2017). The mobile phase consisted of 0.1% v/v formic acid in water (A) and 2:3 v/v acetonitrile : methanol (B) with the following gradient: 100% A at 0–5 min, 60% A at 6–20 min, 40% A at 21–40 min and 100% B at 41–60 min. Sample solutions were filtered before analysis through a 0.45 µm polytetrafluoroethylene (PTFE) membrane filter before analysis.

6. HPLC method validation

Ligand screening of ethanol crude extract of A. simplex was identified after validation of HPLC analysis. The method was validated to evaluate accuracy of the method, linearity, limits of detection (LOD), limits of quantification (LOQ), intra–day and inter–day precisions.

The percentage recovery was calculated to evaluate the accuracy of the method. Arbutin was prepared at concentrations of 15, 20 and 25 mg/mL in sodium phosphate buffer. Then, 1 mL of ethanol crude extract (20 mg/mL) was added to 1 mL of arbutin solution. All the data were calculated by the formula:

Where: A represents the sample contents after adding arbutin solution, B represents the sample contents before adding arbutin solution and C represents arbutin solution.

For linearity, arbutin solution for calibration curves were prepare at different concentrations. Arbutin was prepared at concentrations of 10, 15, 20, 25, 30 and 35 mg/mL in sodium phosphate buffer. Seven repetitions were determined in this method. The calibration curves were plotted using peak area versus concentrations. The calibration curve was plotted with peak area versus concentration using the method of least squares regression analysis. The values of the slope and y-intercept of the plot were computed.

LOD and LOQ were detected as the lowest concentrations of analytes in the sample. The LOD and LOQ were evaluated standard curve of arbutin solution. Each concentration were injected in triplicate. These limits were calculated by the formula:

$$\text{Recovery (\%)} = \left[\frac{(A - B)}{C}\right] \times 100 \qquad (1)$$

Where: As represents the average slope and SD represents the standard deviation of response.

$$LOD = \frac{3.3}{As} \times SD \quad (2)$$

$$LOQ = \frac{10}{As} \times SD \quad (3)$$

Intra–day precision was evaluated using three different concentrations of arbutin under the selected optimal condition. Arbutin was prepared at concentrations of 15, 20 and 25 mg/mL in sodium phosphate buffer. For inter–day precision was conducted by measuring the above concentration on five consecutive days. The percentage relative standard deviation (% RSD) was calculated to evaluate the intra–day and inter–day precisions. All the data were calculated by the formula:

$$\text{Relative standard deviation (\% RSD)} = \frac{SD}{\overline{X}} \times 100 \quad (4)$$

Where: SD represents the standard deviation and $\overline{X}$ represents the mean concentration of arbutin solution.

7. HPLC method for ligand screening

HPLC method was determined following the modified method of Zhang et al. (2017). Ligand screening of ethanol crude extract of A. simplex was evaluated to optimize ligand screening conditions, specific ligand screening conditions and ligand screening of ethanol crude extract of A. simplex. Arbutin was used as a target model compound to optimize ligand screening conditions.

Briefly, an arbutin solution and a tyrosinase solution were prepared at a concentration of 1 mg/mL in sodium phosphate buffer. The activity assay used 300 µL of the solution mixture containing 100 µL of arbutin solution, 100 µL of sodium phosphate buffer and 100 µL of tyrosinase solution. Solution mixtures were incubated at 37 °C for 40 min.

The binding mixture was then filtrated through an ultramembrane filter (Amicon® Ultra-15) and centrifuged at 10,000 rpm for 10 min. Screening conditions were optimized at different temperatures (25, 37 and 45 °C) and for different incubation times (10, 20, 40 and 60 min). All HPLC experiments were conducted in triplicate. Binding ligand affinities of the constituents to tyrosinase were calculated using the equation,

Where: A represents the chromatogram of HPLC for the control group and B represents the chromatogram of HPLC for the experimental group.

For specificity of ligand screening conditions, the denatured tyrosinase solution (1 mg/mL) was boiled for 10 min at 100 °C. An aliquot of 100 µL of the denatured tyrosinase solution

was added to 100 µL of arbutin solution (1 mg/mL). For the tyrosinase group, 100 µL of the tyrosinase solution was added to 100 µL of arbutin solution. The solution mixture was filtered and injected into the HPLC analyzer. Inactive compounds were evaluated by comparison with the tyrosinase group and the denatured tyrosinase group.

Ligand screening of A. simplex extract, binding screening of the A. simplex extract was measured using the modified method of Zhang et al. (2017). The solution mixture contained 100 µL of the crude extract of A. simplex rhizomes (1 mg/mL), 100 µL of sodium phosphate buffer and 100 µL of tyrosinase solution as the experimental group.

The control group of 100 µL of crude extract of A. simplex (1 mg/mL) was added into 200 µL of sodium phosphate buffer, and the reaction mixtures were incubated at 37 °C for 40 min. The binding mixture was then filtrated through an ultramembrane filter (10 kDa cutoff) and centrifuged for 10 min at 10,000 rpm. All experiments were conducted in triplicate.

8. *In vitro* antityrosinase activity

Antityrosinase activity was determined following the modified method of Biswas et al. (2015). L-tyrosine was used as a substrate for monophenolase inhibitory activity, while L-DOPA was used as a substrate for diphenolase inhibitory activity. Arbutin was used as a positive control. Ethanol crude extracts of A. simplex (1 mg/mL) and arbutin were dissolved in sodium phosphate buffer.

In separate reaction mixtures, 50 µL of the ethanol crude extract, 50 µL of 0.1 M substrate solution and 100 µL of 0.1 M sodium phosphate buffer (pH 6.8). The reaction mixture was mixed and incubated at 37 °C for 5 min. Then, 50 µL of tyrosinase solution (200 U/mL) was added and the reaction mixture was immediately recorded at 490 nm (t = 0 min). The reaction mixture was incubated for 40 min at 37 °C in an incubator and the absorbance was measured at 490 nm (t = 40 min).

All the experiments were determined in triplicate. Percentage antityrosinase activity was calculated using the equation,

$$\text{Antityrosinase activity (\%)} = \left[\frac{(A - B) - (C - D)}{(A - B)}\right] \times 100 \qquad (6)$$

Where: A represents the absorbance of the control, B represents the absorbance of the blank control, C represents the absorbance of the sample and the positive control and D represents the absorbance of the blank of the sample and the positive control.

9. Statistical analysis

Results were expressed as mean ± standard deviation. Statistical data analysis of variance was performed using SPSS version 24, with differences considered significant at $p < 0.05$.

Results and Discussions

1. HPLC method validation

The chromatographic method of arbutin was validated to evaluate accuracy of the method, linearity, LOD, LOQ, intra–day and inter–day precisions. The accuracy of arbutin in the spiked sample was calculated and reported in terms of percentage recovery (Table 1). The percentage recovery of arbutin ranged from 99.70 to 102.45 %. According to the International Council on Harmonization (ICH) guidelines, the acceptable range of percentage recovery were limited of 90 to 110 % (ICH guideline, 2005). Thus, the validated method presented a good accuracy.

Table 1 Accuracy results of ethanol extract of A. simplex

Concentration (mg/mL)	Recovery (%)	RSD (%)
15	99.70 ± 0.12	0.29
20	100.55 ± 0.08	0.17
25	102.45 ± 0.07	0.11

The linearity of the calibration curves between peak area and concentration was shown as Figure 1. The equation of arbutin was y = 25.356x–167.65 with regression coefficient of r^2 = 0.9991 were obtained by the calibration curves (Table 2). The LOD and LOQ values were 37.74 and 114.37 µg/mL, respectively. The percentage of relative standard deviations were reported to evaluate intra–day and inter–day precisions. The results showed that %RSD of intra–day was in the range of 0.07–0.13% and %RSD of inter–day was in the range of 0.002–0.01%.

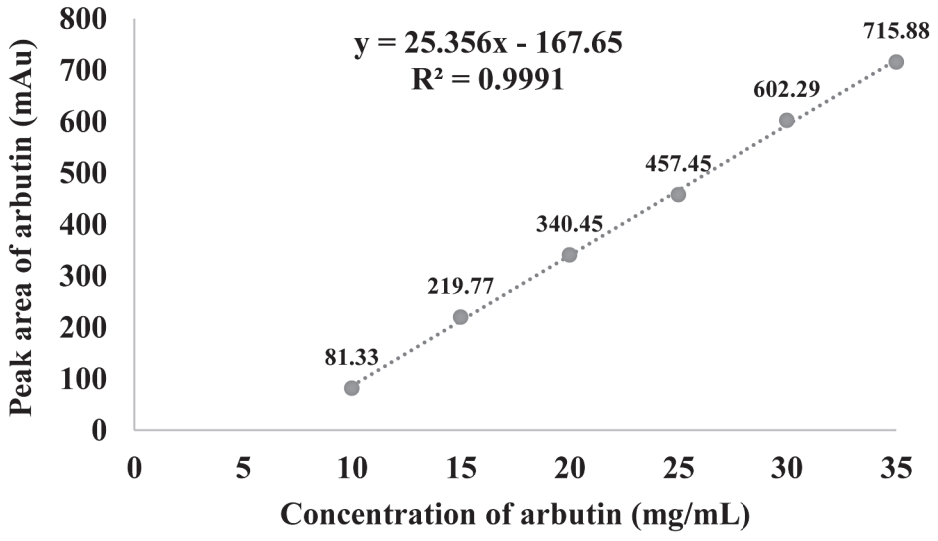

Figure 1 The calibration curves between peak area and concentration of arbutin.

Table 2 Linearity results for concentration of arbutin

Repetitions of peak area	Concentration (mg/mL)					
	10	15	20	25	30	35
1	81.15	219.98	340.51	457.86	602.53	715.85
2	81.66	219.99	340.11	457.46	602.55	715.82
3	81.51	219.6	340.81	457.19	602.11	716.52
4	81.13	219.63	340.71	457.13	602.1	715.91
5	81.38	219.31	340.12	457.57	602.18	715.91
6	81.41	219.92	340.17	457.91	602.12	715.55
7	81.06	219.95	340.69	457.02	602.46	715.62
Mean	81.33	219.77	340.45	457.45	602.29	715.88
SD	0.23	0.29	0.33	0.30	0.23	0.29
LOD (µg/mL)	37.74					
LOQ (µg/mL)	114.37					
%RSD of intra-day	0.13	0.10	0.07			

2. HPLC method for ligand screening

Arbutin was collected to optimize ligand screening conditions. Results indicated that optimal incubation temperature and incubation time for the binding ligand were 37 °C and 40 min. However, binding ligand activity decreased as time increased, as shown in Figure 2. The specificity of ligand screening with tyrosinase was investigated. Chromatographic peak area of the binding ligand with the tyrosinase group decreased when compared with the chromatographic peak area of the denatured tyrosinase group and arbutin, as shown in Figure 3.

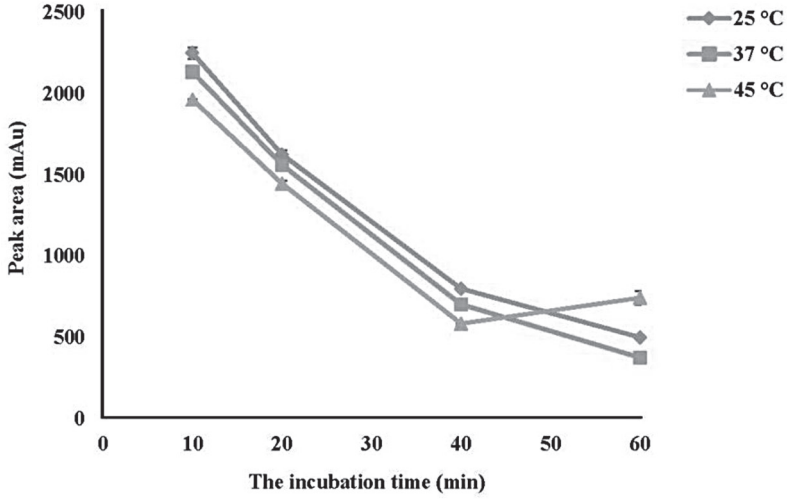

Figure 2 Effect of incubation time on ligand screening.

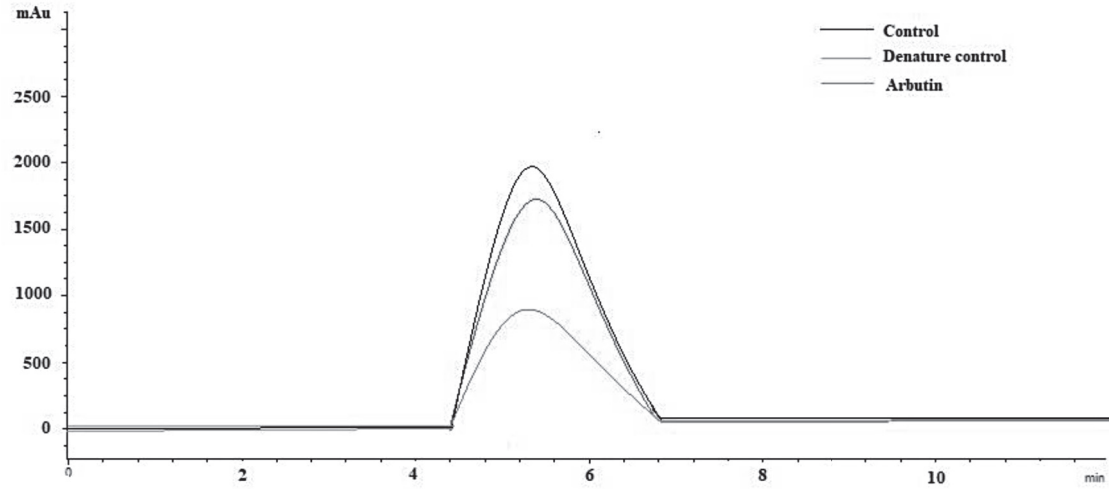

Figure 3 HPLC chromatograms for specificity of ligand screening conditions (black: control) arbutin after incubation without tyrosinase (green: arbutin) arbutin after incubation with tyrosinase (red: denatured control) denatured tyrosinase.

Duration of incubation time and incubation temperature were important for enzyme binding. Binding between arbutin and tyrosinase was achieved. The peak area of ligand binding decreased as time increased, concurring with data from the literature (Zhang et al., 2017). Moreover, the specificity of arbutin and tyrosinase binding was significant. Ligand binding depended on eluting time and organic solvent for eluting ligands (Ryu et al., 2014).

Chromatograms of the ethanol extract of *A. simplex* rhizomes for screening of tyrosinase binding are shown in Figure 4. Each peak area of unknown compounds in the chromatograms was compared. Five unknown compounds were identified as the ligand binding with tyrosinase, showing retention times of 13.95, 19.64, 21.30, 38.98 and 43.90 min.

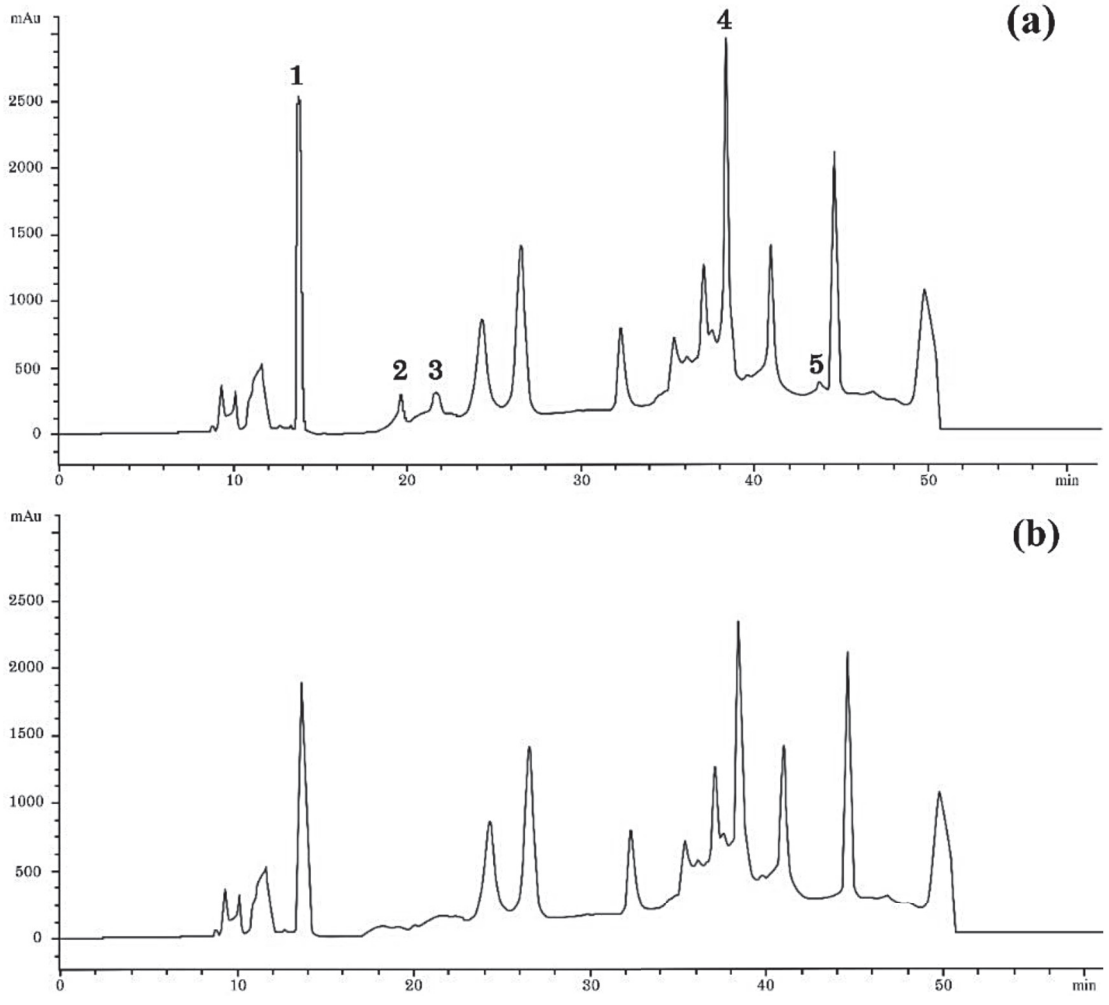

Figure 4 HPLC chromatograms of ethanol extract of *A.* simplex rhizomes (a) before and (b) after binding ligand screening with tyrosinase.

3. *In vitro* antityrosinase activity

Ethanol extract of *A. simplex* rhizomes were investigated for monophenolase and diphenolase inhibitory activities. Antityrosinase activities of A. simplex rhizomes are expressed as IC_{50} values (Table 3). Results indicated that the ethanol extract showed stronger monophenolase inhibitory activity than arbutin with IC_{50} values of 53.65 ± 0.80 and 138.15 ± 0.96 µg/mL respectively. Diphenolase inhibitory activities of the ethanol extract and arbutin were similar with IC50 values of 6.35 ± 0.23 and 7.11 ± 0.16 µg/mL respectively.

Table 3 Antityrosinase activity of ethanol extract of *A. simplex*

Material	IC_{50} (µg/mL)	
	Monophenolase inhibitory activity	Diphenolase inhibitory activity
A. simplex rhizomes	53.65 ± 0.80[a]	6.35 ± 0.23[a]
Arbutin	138.15 ± 0.96[b]	7.11 ± 0.16[a]

Findings revealed that the ethanol crude extract of *A. simplex* rhizomes significantly exhibited diphenolase inhibitory activity. Previous studies reported that phytochemicals of A. simplex fruits, leaves, roots and stems consisted of several types of alkaloids, coumarins, flavonoids, glycosides, organic compounds, phenolic compounds, steroids and terpenoids (Ismail et al., 2017; Nipiroon & Vajrodaya, 2017; Kiatsongchai 2015). Recently, β-sitosterol was isolated from *A. simplex* rhizomes and showed moderate hyaluronidase inhibitory activity (Khammee et al., 2020). Generally, β-sitosterol was one of many phytosterol from plants and was a cholesterol-like structure. β-sitosterol has been reported to exhibit lipid lowering effect, anti-inflammatory, and anti-diabetic activities (Babu & Jayaraman, 2020). However, there was no report directly determining antityrosinase activity of β-sitosterol. Several researches reported that phytochemicals have been used as tyrosinase inhibitors such as flavonoids, phenolic compounds, triterpenoids and alkaloids.

Because the inhibition mechanism based on the structure of both the substrate and inhibitor (Chen et al., 2015). The ethanol crude extract of *A. simplex* rhizomes exhibited stronger diphenolase inhibitory activity (6.35 ± 0.23 µg/mL) than monophenolase inhibitory activity (53.65 ± 0.80 µg/mL). There is a possibility that the extract inhibited the oxidation of L-DOPA.

The conventional method was isolated chemical components from plant extracts and tested their biological activities. This the conventional method was a time-consuming and labor-intensive task. Bioanalytical screening methods have been developed as alternative method for bioactive compounds. Taking advantages of bioanalytical screening method were subjected to various bioassays, simple reproducible, rapid and targeted isolation of new chemical compounds with potential activities (Wang et al., 2018; Zhuo et al., 2016). Thus, the advantages of UF-HPLC-DAD in this research were low sample consumption, high recovery yields and reduction labor-intensive task.

Results from antityrosinase activity suggested that the crude extract of *A. simplex* rhizomes consisted of several chemical constituents. However, there are a few researches that investigated the isolation of phytochemicals from *Aglaonema sp* and exhibited biological activities. It has been reported

that 2,5-dihydroxymethyl-3,4-dihydroxypyrrolidine and α-homonojirimycin have been isolated from *A. modestum* and inhibited nitric oxide synthase. α-Homonojirimycin and homonojirimycin isomers have also been isolated *A. treubii* and exhibited glycosidase inhibitory activity (Kiatsongchai, 2015). Usually, the polyphenol derivatives of flavonoids could be involved in the antityrosinase activity. The structure of chemical constituents depended on the types of chemical constituents and number of available hydroxyl groups that attacked active enzyme sites (Abdillahi et al., 2011). Therefore, *A. simplex* rhizomes could be contained the marker compounds for tyrosinase inhibitory activity.

Conclusions

In this study, the validated HPLC method was developed in term of the accuracy, linearity, LOD, LOQ, intra-day and inter-day. A UF-HPLC-DAD was applied for screening tyrosinase binding in crude extract of *A. simplex* rhizomes. This method suggested that tyrosinase could be binds unknown compounds without isolation and purification from crude extract. The advantages of bioanalytical screening method were subjected to various bioassays, less cost of solvent, rapid, and targeted isolation of marker compounds. Moreover, the ethanol crude extract of *A. simplex* rhizomes demonstrated stronger monophenolase inhibitory activity than that of arbutin. While, diphenolase inhibitory activity of the ethanol extract of *A. simplex* rhizomes was similar to that of arbutin. The results suggested that the extract of A. simplex rhizomes could be contained the marker compounds for the development of the extract in cosmeceuticals.

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