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Research article

High performance thin layer chromatography fingerprint and antioxidant activities of Cyclea barbata in Thailand

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Abstract

Cyclea barbata is a commonly used herbal and traditional plant, and its leaves contain a high level of pectin. However, different habitats, jelly extract and leaf age may affect the biocompounds in the leaves. Therefore, a high performance thin layer chromatography (HPTLC) technique was used for preliminary screening of some active leaf compounds from various habitats along with total phenolic analysis and antioxidant activity of this plant from different sources and leaf ages (young, middle and old). The HPTLC results of methanol leaf extract revealed various chemical patterns that were indicated by retention factors (R_f values) in the range 0.15–0.73; unique bands of two samples (P1 and P3) from Phetchabun province were detected under ultra-violet light (R_f = 0.65 at 254 nm and R_f = 0.40 at 366 nm, respectively). Additionally, alkaloid bands were not found in leaf extracts by spraying with Dragendroff's reagent. A chemical band with antioxidant activity was only found at R_f = 0.50 in old and young fresh leaves following spraying with 2,2-diphenyl-1-picrylhydrazyl. The total phenolic contents of old, fresh leaves had the highest value (525.08 mg gallic acid/g extract), whereas the 2,2'-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt radical scavenging activity of old, dry leaves was highest (97.7%). These results indicated that different R_f values of various phytochemicals and various habitats of *C. barbata* could be primarily determined using HPTLC analysis. Antioxidant activities were determined using chemical bands and leaf extracts at various ages.

Introduction

Cyclea barbata is one of the climbing shrubs in the Menispermaceae family and is commonly distributed in subtropical regions of Asia, America and East Africa (Forman, 1991). In Thailand, this plant is known as 'krueo ma noy' and is commonly consumed as a medicinal and edible plant in the Northeast region; for example, it has been reported that leaf extracts of *C. barbata* show several medicinal benefits such as antidiarrhea activity, antinociceptive activity, antiarthritic activity, anticancer activity, antioxidant activity, immunomodulatory activity and antifertility activity (Amresh et al., 2004; Amresh et al., 2007; Bafna and Mishra, 2010; Thavamani et al., 2014; Junaimuang et al., 2015). Additionally, its leaves consist of pectin providing suitable viscosity and elasticity for use as a gelling agent in developing healthy drinks (Chunthanom et al., 2013). A major component of pectin is galacturonic acid with low natural sugars (Singthong et al., 2004),

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which is useful for cooking healthy food. Therefore, the leaves of *C. barbata* are considered to be a good source of natural fiber and low-calorie foods. For example, after jelly consumption, the high fiber leaves of *C. barbata* improved ease of defecation (Siregar and Miladiyah, 2011**)** Moreover, the leaves of *C. barbata* contain various bis-benzyl-isoquinoline alkaloids, such as berbamine, chondocurine, alpha and beta cyclanoline, fangchinoline, homoaromoline, isochondocurine, isotetrandrine, lemacine, and tetrandrine (Tantisewie and Ruchirawat, 1992) and bisbenzyl isoquinoline alkaloids (Guinaudeau et al., 1993). Some alkaloids have cytotoxic and antimalarial properties (Lin et al., 1993).

Although, there have been many reports discussing its medicinal benefits, *C. barbata* shows diversity both in its genetics and leaf morphology (Silprasit et al., 2017). Genetic diversity can have an effect on the level of bioactive compounds or antioxidant activity (Thummajitsakul et al., 2017). Moreover, chromatographic fingerprint analysis between groups of fresh and dry leaves, plant sources and leaf age of *C. barbata* is still limited. High performance thin layer chromatography (HPTLC) analysis has been used in several plants for pharmaceutical and medical studies. For example, HPTLC is used to analyze antioxidant and antigout activities (Nile and Park, 2014), and to find novel bioactive compounds extensively in plant extracts both qualitatively and quantitatively (Loescher et al., 2014). Therefore, the HPTLC technique can be beneficial for the preliminary screening of some bioactive compounds from various habitats, leaf ages and leaf conditions. The major aim of the current study focused on the qualitative evaluation of bioactive compounds using HPTLC analysis and was linked to the antioxidant activities of leaf and jelly extracts based on different plant sources, leaf ages (young, middle, old leaves) and leaf conditions (fresh and dry leaves) of *C. barbata*. The basic information from this study may provide novel natural sources for new bioactive agents for the prevention and treatment of human diseases and for use in agriculture and food industries.

Materials and Methods

Chemicals

Many chemicals were purchased for this research, namely pre-coated silica gel 60-plate from Merck, linomat V applicator from CAMAG (Switzerland), Folin-Ciocalteu phenol reagent, gallic acid monohydrate and absolute ethanol (ACS grade) from Sigma-Aldrich (Steinheim, Germany) and 2,2'-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt (ABTS) and ethylenediaminetetraacetic acid from Fluka (Buchs, Switzerland).

Chemical compound screening using high performance thin layer chromatography with different sources of C. barbata.

Plant extracts preparation

Leaves of *C. barbata* in Thailand were collected from Prachinburi province (N = 11), Saraburi province (N = 4), Sa Kaeo province (N = 5), Nakhon Nayok province (N = 2), Sakon Nakhon Province (N = 6) and Phetchabun province (N = 5). The samples were cleaned with distilled water and incubated at 40 °C overnight or until dry. Dried leaf samples (each 100 g) were extracted with 20 mL of methanol using sonication at 40°C for 10 min. The methanol solvent was then evaporated until dry, dissolved with 1.5 mL methanol and sieved through a filter cloth.

High performance thin layer chromatography analysis

Each extract was spotted on a pre-coated silica gel 60 plate with the linomat V applicator. Chloroform, methanol and water (8:2:0.2), or toluene, ethylacetate and formic acid (8:2:0.1, volume per volume; v/v) were used as the mobile phase, which was performed at 7 cm distance at 75% humidity and 32°C. Finally, spots on each plate were detected under ultra-violet light at 254 nm and 366 nm and sprayed with Dragendorff's reagent and 2,2-diphenyl-1-picrylhydrazyl (DPPH; 1 g DPPH in 20 ml methanol). The ratio of (distance of spot migration above the origin) to (distance to the solvent front) was expressed as the retention factor (R_f) where the distance was measured from the center of a spot.

Chemical fingerprint and antioxidant activity of variety C. barbata leaf ages

Plants extract preparation

Young leaves or newly-expanded leaves were collected at the top of each plant whereas old leaves or fully expanded leaves were collected at the bottom of the stems of each plant and medium leaves were collected from mid-height (Kennedy et al., 1950 and Zhang et al., 2013). Samples of *C. barbata* are shown in Fig. 1. Leaves of *C. barbata* (sampled from Nakhon Nayok province) were washed with distilled water and used as fresh leaves. The washed leaves and roots were incubated at 40°C overnight or until dry and kept as dried leaves. Then, separate samples of dried and fresh leaves were ground using a homogenizer. A ground sample (approximately 1 g) was extracted with 10 mL of 95% ethanol solvent at 37°C with shaking at 100 rpm overnight, followed by filtration and drying. Each crude extract was then adjusted to a final concentration (20 mg/mL) and kept at 20°C until used.

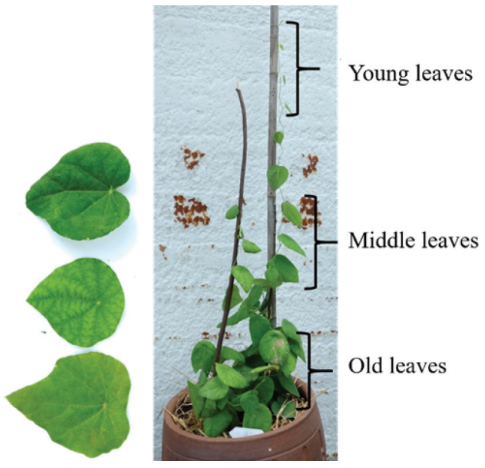

Fig. 1 *Cyclea barbata*, a climbing shrub in the Menispermaceae family, showing leaf morphology

Jelly extract preparation

The ground samples (each 30 g) of the fresh or dry leaves were added with 100 mL deionized water, then passed through a filter cloth and left at room temperature for 30 min. Then, the jelly was cut into small pieces and extracted with 10 mL of 95% ethanol solvent at 37°C with shaking at 100 rpm overnight, followed by sieving and drying, before adjusting to a final concentration (20 mg/mL).

Total phenolic contents

The total phenolic contents were determined using a Folin-Ciocalteu colorimetric method according to the protocols of Thummajitasakul et al. (2014) and Deetae et al. (2012). Briefly, each extract (300 µL) reacted with 1.5 mL of Folin-Ciocalteu reagent for 5 min and then with 1.2 mL of sodium carbonate (7.5% weight per volume) for 30 min at room temperature. Absorbance was measured at 765 nm using a spectrophotometer (Model T60UV/VIS; Beijing, China). Gallic acid was used as a standard, and the total phenolic contents were reported as milligrams of gallic acid per gram of extract.

2,2'-Azino-bis 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt radical scavenging activity

An ABTS assay was done according to Thummajitasakul et al. (2014) and Deetae et al. (2012). Briefly, the $ABTS^{\bullet+}$ radical cation was obtained from the reaction between 7 mM ABTS and 140 mM potassium persulfate at room temperature for 16 hr in the dark. Then, the radical cation solution was adjusted with distilled water until absorbance was 0.700 ± 0.050 at 734 nm. The $ABTS^{\bullet+}$ solution (3.9 mL) was thoroughly mixed with each extract (0.1 mL) and left for 6 min in the dark at room temperature, after which the absorbance was determined at 734 nm using the spectrophotometer. Each reaction was done in triplicate. The percentage of antioxidant capacity was calculated using Equation 1:

$$\% \text{ Antioxidant capacity} = (OD_{ABTS} - OD_{SAMPLE}) / OD_{ABTS} \times 100 \quad (1)$$

where OD_{ABTS} is the absorbance of the ABTS radical cation (without sample) and OD_{SAMPLE} is the absorbance of the reaction between the $ABTS^{\bullet+}$ solution and each sample.

Screening of antioxidant compounds using high performance thin layer chromatography

Each ethanol extract sample was spotted on a pre-coated silica gel 60-plate with a linomat V applicator. Chloroform, methanol and water (8:2:0.2), or toluene, ethylacetate and formic acid (8:2:0.1, v/v) was used as the mobile phase and was performed at 7 cm distance under 75% humidity at 32°C. Spots on each plate were then detected under ultra-violet light at 254 nm and 366 nm and sprayed with Dragendorff's reagent and DPPH (1 g DPPH in 20 mL methanol). The ratio of (distance of spot migration above the origin) to (distance to the solvent front) was expressed as the R_f, where the distance was measured from the center of a spot.

Statistical analysis

The total phenolic contents and the % antioxidant capacity were expressed as mean ± SD and as percentages. Analysis of variance was done using the online analysis of Soper (2015) and significance was tested at $p < 0.05$. Principle component analysis (PCA) was analyzed using the program implemented in the *Excel add-in Multibase package* (*Numerical Dynamics; Japan*).

Results and Discussion

High performance thin layer chromatography analysis

The HPTLC technique is a powerful tool that is commonly applied to identify bioactive compounds and used with chemical markers in medicinal, agricultural and food applications (Attimarad et al., 2011). The advantage of HPTLC is that it can be effectively and quickly applied in the qualitative analysis of chemical components, plant species and sources (Loescher et al., 2014). Proper solvent polarity is an important factor in separating spots using HPTLC. The amounts of antioxidants and phenolic compounds is strongly influenced by the combination and polarity of the solvents used in extraction (Alothman et al., 2009; Boeing et al., 2014). Numerous studies have reported that widely used solvents for extracting phenolics are water, ethanol, methanol and acetone (Rababah et al., 2010; Bunea et al., 2012; Boeing et al., 2014). Moreover, efficient solvents for antioxidant extraction are methanol, water, ethanol and acetone, respectively (Boeing et al., 2014).

HPTLC was used to determine bioactive compounds, antioxidant activities and for chromatographic fingerprinting in leaf and jelly extracts based on leaf age (young, middle, old), leaf condition (fresh, dry), and plant source of *C. barbata*. The results showed that no alkaloid compounds were detected in the leaf extracts whereas least 4 types were found in root extracts at R_f values of 0.15, 0.29, 0.66 and 0.73 (Fig. 2B). These results were supported by Manu et al. (2012), who reported root extracts of *Cissampelos pareira* (the family Menispermaceae) contained several alkaloids in the bisbenzylisoquinoline group, such as tetrandine, menismine, and curine, but that these compounds are not detected in leaf extracts. Alkaloids have been reported to have pharmaceutical effects such as anticancer activities and antioxidant activities (Rai et al., 2006; Zhao et al., 2016). However, alkaloids can also be potential health hazards to the human body, causing trismus and abdominal cramping (Singhapricha and Pomerleau, 2017). The current results confirmed that such alkaloid compounds were not found in the leaves of *C. barbata*. Therefore, there is no adverse health effects from alkaloids if only the leaves are consumed.

The chemical fingerprints of the leaf extracts of *C. barbata* from different sources were relatively similar; however, samples from Phetchabun province (P1 and P3) had specific light chemical bands (Fig. 2C) at $R_f = 0.65$ under UV 254 nm (Fig. 2C), and $R_f = 0.40$ under UV 366 nm (Fig. 2D) that were not found in samples from other provinces namely Nakhon Nayok (Y1, Y4, G5 and G1), and

Sakon Nakhon (S3 and S6). The pale green colour sample (Y4) showed two specific bands at R_f = 0.22 and 0.42 under UV 366 nm that differed from dark green colour samples; (G5 and G1) (Fig. 1D). Therefore, the HPTLC analysis could be used to indicate sources of *C. barbata*. Previous studies report that the HPTLC analysis has been applied for the characterization of species and bioactive compounds in plant extracts (Raman et al., 2014; Yousefi et al., 2016), chemical markers (Yadav et al., 2011), evaluation of plant different sources (Hullatti et al., 2016). The antioxidant activities of *C. barbata* leaves from different sources were found by spraying DPPH reagent. It showed several chemical bands (Fig. 3C) and implied that certain compounds on chromatography plate could hold oxidants from DPPH. Additionally, leave extracts of *C. barbata* from Phetchabun (P) and Sakon Nakhon (S) province revealed different chemical patterns at R_f = 0.65 (Fig. 2C and Fig. 3A). Interestingly, the band at R_f = 0.65 was first predicted as kaempferol (flavonoids) found in *C. barbata* leaves. However, it may be unknown a bioactive compound which related to antioxidant activity.

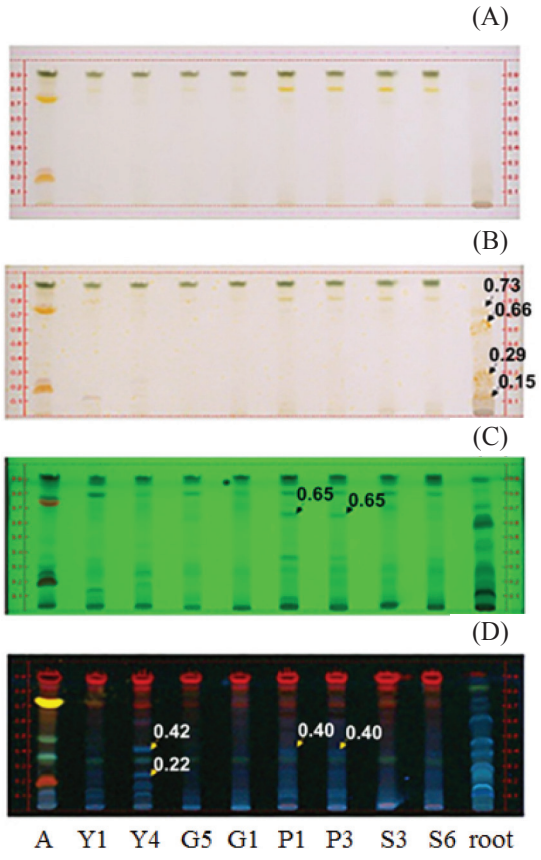

Fig. 2 Chemical fingerprints of *C. barbata* extracts from different areas of Thailand visualized using: (A) white light; (B) Dragendroff's spraying reagent; (C) ultraviolet light at 254 nm; (D) ultraviolet light at 366 nm, where samples Y1, Y4 (pale green leaf color) and samples G5, G1 (dark green leaf color) were from Nakhon Nayok province; samples P1 and P3 were from Phetchabun province; samples S3 and S6 were from Sakon Nakhon province and A was leaf extract of other plants (positive sample to confirm visualizing).

Antioxidant activities and total phenolic contents analysis

The leaves of *C. barbata* are commonly consumed by people in Thailand and contain high pectin. Many traditional foods were prepared from their jelly extract. Therefore, bioactivities in their leaf and jelly extracts were focused. Total phenolic contents and antioxidant activity were determined in 95% ethanol extracts. Since, some Menispermaceae plant extracted using methanol contained high cytotoxicity, for biological activity testing then these extracts should be highly evaporated with rotavaporation (Tungpradit et al., 2010). Ethanol was harmless solvent for application in traditional food or medicine, it was commonly selected for extracting bioactive agents and developing healthy food products. It has reported that bioassay of ethanol extracts exhibited the highest total phenolic and flavonoid contents (Das et al., 2014; Do et al., 2014).

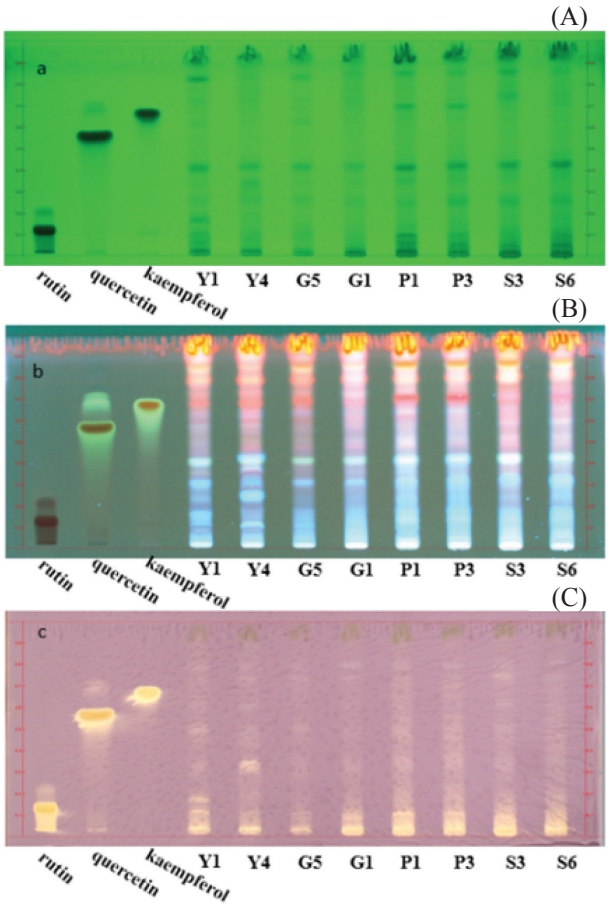

Fig. 3 Comparison of chemical components in methanol extracts of *C. barbata*, standard compounds; kaempferol, quercetin and rutin, and the tendency of the chemicals to express antioxidant activity were visualized using: (A) ultraviolet light at 254 nm; (B) ultraviolet light at 366 nm; (C) antioxidant activity with 2,2-diphenyl-1-picrylhydrazyl reagent, where mobile phase was chloroform-methanol-water (3:1:0.1, v/v) and samples Y1, Y4 (pale green leaf color) and samples G5, G1 (dark green leaf color) were from Nakhon Nayok province; samples P1 and P3 were from Phetchabun province; samples S3 and S6 were from Sakon Nakhon province.

The total phenolic contents and ABTS radical scavenging activity were measured in ethanol extracts fresh and old leaves of *C. barbata* at different ages (Table 1). Old, fresh leaf extract had the highest amount of total phenolic contents (525.08 mg gallic acid/g extract), followed by young leaves (285.51 mg gallic acid/g extract) and middle leaves (273.86 mg gallic acid/g extract), whereas the total phenolic contents in the dry leaf samples were highest for the old, (279.69 mg gallic acid/ g extract), followed by young (201.04 mg gallic acid/ g extract) and middle (193.03 mg gallic acid/ g extract). The results indicated that the total phenolic contents in the extracts of fresh leaves were greater than those of dry leave extracts corresponding to the HPTLC results. However, non-phenolic compounds such as pigments, carbohydrates, water, fat and proteins may interfere with the estimation of total phenolic contents. Therefore, they should be removed before the evaluation of total phenolic contents (Djeridane et al., 2006). For the jelly extracts, the total phenolic contents in fresh leaves at the middle, old and young ages were 180.73 mg gallic acid/g extract, 151.60 mg gallic acid/g extract and 96.26 mg gallic acid/g extract, respectively.

Table 1 Mean ± SD values of antioxidant capacity and total phenolic contents in ethanol extracts at various leaf ages of *C. barbata*

Sample extract	Total phenolic contents (mg gallic acid/g extract)	% Antioxidant capacity
Fresh leaves		
Young	285.51 ± 13.17^{aa}	74.25 ± 1.33^{aa}
Middle	273.86 ± 22.70^{ab}	54.61 ± 4.80^{ab}
Old	525.08 ± 74.18^{ac}	73.00 ± 1.75^{ac}
Dry leaves		
Young	201.04 ± 11.00^{ba}	97.45 ± 0.27^{ba}
Middle	193.03 ± 21.85^{bb}	90.23 ± 0.52^{bb}
Old	279.69 ± 22.74^{bc}	97.73 ± 0.37^{bc}
Jelly		
Young fresh leaves	96.26 ± 21.99^{ca}	13.66 ± 4.74^{ca}
Middle fresh leaves	180.73 ± 21.85^{cb}	23.14 ± 2.47^{cb}
Old fresh leaves	151.60 ± 18.19^{cc}	14.84 ± 1.88^{cc}

Means in the same column within each leaf category superscripted with different lowercase letter are significantly different ($p < 0.05$).

The ABTS results for each extract at different leaf ages showed that the young leaf extract had the highest percentage of antioxidant capacity (74.3%), followed by old leaves (73.0%) and middle leaves (54.6%). In the dry leaf extracts, the antioxidant capacity was greatest in the old leaves (97.7%), followed by the young leaves (97.4%) and middle leaves (90.2%), while the antioxidant capacity of the jelly extract from the middle leaves was the greatest (23.1%), followed by the old leaves (14.8%) and young leaves (13.7%) (Table 1). These results indicated that all dry leaf extracts from old, middle and young leaves had higher levels of antioxidant capacity than for fresh leaf extracts, so leaf age affected the total phenolic contents and antioxidant activity. These results were similar to other reports that the amounts of chemical compounds varied with leaf age and the part of the plant sampled (Achakzai et al., 2009). Nonetheless, this was inconsistent with the HPTLC results which produced a greater number of chemical bands in fresh leaf extracts than for the dry leaf extracts after DPPH spraying. However, it has been reported that drying can increase the antioxidant capacity by increasing some antioxidant agents (Chang et al., 2006). In addition, certain phenolics can act as antioxidants, and they may be involved due to their chemical structure and interaction (Djeridane et al., 2006). Furthermore, some non-phenolics can also act as antioxidants such as ascorbic acid, carotenoids, and 1-O-beta-D-glucopyranosyl-2-methoxy-3-(2-hydroxy-triaconta-3,12-dienoate)-glycerol (Golumbic and Mattill, 1941; el-Sayed et al., 2008; Mekni et al., 2013).

Chemical fingerprinting using the 95% ethanol extract was performed to screen the antioxidant compounds based on different leaf conditions and leaf ages (Fig. 4). The results showed specific chemical bands in old, fresh leaves at $R_f = 0.55$ (sample 7, Fig. 4A) and at $R_f = 0.7$ (sample 7, Fig. 4B). In addition, a chemical band at $R_f = 0.5$ involving antioxidant activity was observed only in old and young fresh leaves (samples 7 and 9, Fig. 4A and 4B, respectively). Moreover, the dried leaves at various ages had similar chemical patterns (samples 10–12). Jelly extracts from fresh leaves contained few chemical components (samples 3–5), while jelly extracts from dry leaves had higher levels of chemicals (sample 6). Nevertheless, antioxidants were not found in the jelly extracts following DPPH spraying (Fig. 4C).

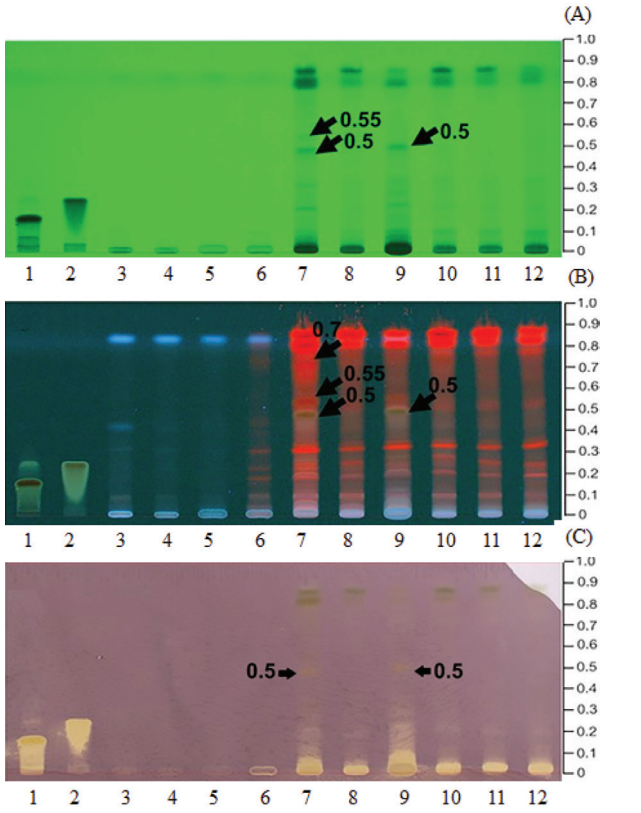

Fig. 4 Ethanol extracts and jelly extracts at various leaf ages of *C. barbata*: (A) fingerprint under ultraviolet light at 254 nm; (B) fingerprint under ultraviolet light at 366 nm; (C) antioxidant activity with 2,2-diphenyl-1-picrylhydrazyl reagent, where mobile phase was chloroform-methanol-water (4:1:0.1 volume per volume), 1 = quercetin, 2 = kaempferol, 3 = jelly from old, fresh leaves, 4 = jelly from middle, fresh leaves, 5 = jelly from young, fresh leaves, 6 = jelly from dry leaves, 7 = old, fresh leaves, 8 = middle, fresh leaves, 9 = young, fresh leaves, 10 = old, dry leaves, 11 = middle, dry leaves, 12 = young, dry leaves

The results showed that fresh leave extracts (samples 7, 8 and 9 in Figs. 4A and 4B) revealed more bands than dry leaves (samples 10, 11 and 12 in Figs. 4A and 4B), suggesting that drying the leaves may affect some bioactive compounds of the plant extracts (Zhou et al., 2011). The number of chemical bands in the extracts of fresh leaves was greater than for dry leaves after DPPH spraying (Fig. 4C), indicating the existence of higher antioxidant activity in the fresh leaves. Fourier-transform infrared spectroscopy spectral data suggested that heat treatment increased amine groups as well as the antioxidant capacity of tamarind leaves (Lavelli et al., 1999; Nantitanon et al., 2010; Siriamornpuna et al., 2012; Leng et al., 2017).

However, the antioxidant activity of bioactive compounds can be strongly affected by reactive target species and environmental factors (Abramovic et al., 2017). Moreover, certain bioactive compounds may not be soluble in reaction media where radical scavenging activity cannot occur (Shalaby and Sanaa, 2013).

Principal component analysis

The correlation patterns of the young, middle and old leaves of *C. barbata* were analyzed using PCA, which was based on the sample plot score (Fig. 5B). In addition, the total phenolic contents and % antioxidant capacities were plotted as variables (loading) (Fig. 5A). PC1 accounted for 72.4% and PC2 for 26.9% of the total variance. The PC1 loading contained total phenolic contents, and the PC2 loading contained antioxidant capacity. Pearson's correlation between the total phenolic contents and antioxidant capacity was not significant ($r = 0.455$, $p = 0.218$). Superimposing the plots of loading and score demonstrated that the extracts of old, fresh and old, dry leaves had the highest amounts of total phenolic contents (Fig. 5). In addition, the percentage of antioxidant capacity was higher in dry leaves than fresh leaves, while the jelly extracted from fresh leaves had the lowest value. Corresponding to the HPTLC results, the jelly extracts had few chemical components and no antioxidant activity following spraying with DPPH reagent. Therefore, the HPTLC analysis confirmed the existence of bioactive compounds and antioxidant capacity in fresh leaf and dry leaf extracts at different leaf ages and in the jelly extract. Moreover, this technique can be used as a chemical marker to help in the identification of plant sources. These data may be further applied for plant cultivation and the development of medicinal food products in a rural community.

In preliminary research, unknown bioactive compounds related to antioxidant activity were detected in young fresh leaves and old fresh leaves using the HPTLC fingerprint. Interestingly, the current results confirmed the existence of phenolics and antioxidant activity in the methanol and ethanol extracts of *C. barbata* leaves for different ages, leaf conditions (fresh or dry) and plant sources. The HPTLC fingerprint showed slight chemical components in jelly extracts and no antioxidant activity was not found in these extracts using DPPH spraying. However, the total phenolic contents and ABTS radical scavenging activity were slightly present in ethanol extracts of the jelly.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

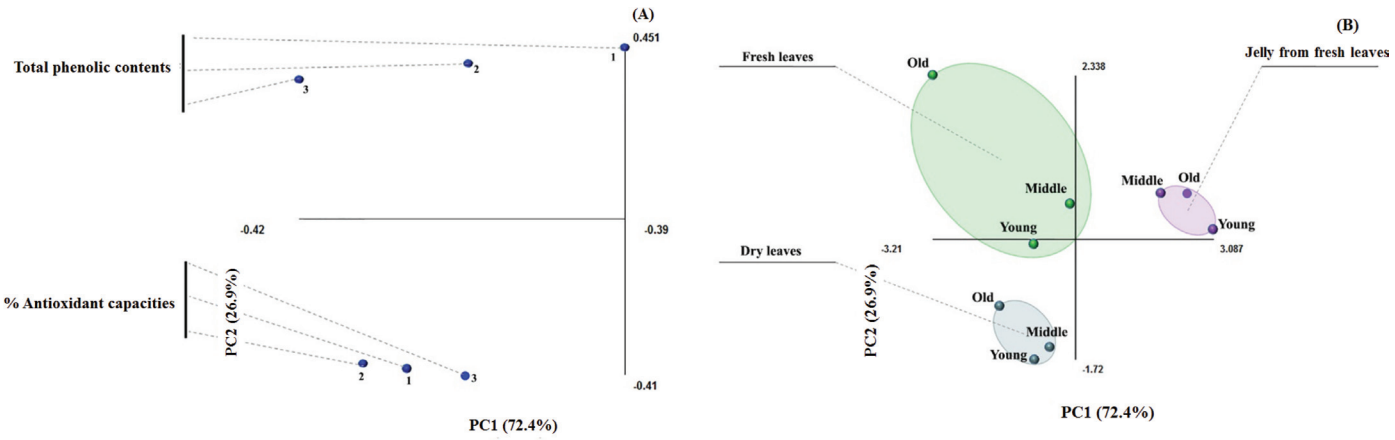

Fig. 5 Principle component analysis of ethanol extracts of *C. barbata*: (A) plot of variables (loading) for total phenolic contents and free radical scavenging activity, where labels 1, 2 and 3 show order of testing; (B) plot of samples (score) for different leaf ages (old, middle, young) of *C. barbata* dry and fresh leaves including jelly extracted from fresh leaves at different ages

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