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STABILITY STUDY OF *BROUSSONETIA PAPYRIFERA* LEAF EXTRACT

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INTRODUCTION

Broussonetia papyrifera, known as paper mulberry or por-sa or por-gra-sa in Thai, is a plant of the family Moraceae. The stems of por-sa were used in paper and textiles industry but the leaves were disposed as waste. Ko et al. (1) reported that the methanolic extract of por-sa leaves consisted of broussonetones A, B and C which have inhibitory activity on mushroom tyrosinase and superoxide free radical scavenging activity. For their inhibitory activity on mushroom tyrosinase, the IC_{50} values of broussonetones A, B and C were reported to be 0.322, 0.317 and 0.323 mM, respectively. Thus, por-sa leaf extract (PSE) shows high potential to be used as a natural skin lightening agent. However, there is no report on the stability of por-sa leaf extract (PSE). Therefore, this study was aimed to investigate the stability of PSE under various temperatures.

MATERIALS AND METHODS

Plant material

The leaves of *B. papyrifera* (por-sa) were collected from Sawankhalok, Sukhothai. The fresh leaves were washed and air-dried. Then, they were dried at 50 °C using hot-air oven (Memmert, Germany) for a further 48 hrs. Dried leaves were ground into powder by a milling machine (Fritsch, Germany).

Extraction process

Extraction was performed by maceration technique using 80% ethanol. The maceration was performed for 24 hrs in a shaker (Asheville, U.S.A.) at 180 rpm. Then, the extract was filtered and evaporated under reduced pressure using a rotary evaporator (Buchi Rotavapor R-114, Switzerland) at 50 °C. Crude extract was kept at -20 °C until used. Percentage yield of the crude extract was calculated by the following equation:

$$\% \text{ yield} = [\text{weight of dried crude extract (g)} / \text{weight of dried leaf powder (g)}] \times 100$$

Antityrosinase activity assay

Antityrosinase activity assay was performed by mushroom tyrosinase assay (2). Briefly, forty µl of test sample, 80 µl of 67 mM phosphate buffer (pH 6.8) and 40 µl of 25 mM L-DOPA were added to a 96-well microplate, and then mixed with 40 µl of mushroom tyrosinase (125 U/ml). After incubation at room temperature (RT) for 20 min, the optical density at 490 nm (OD_{490}) of the mixture was determined by using a microplate reader (Spectra Count®, USA). Kojic acid was used as a positive control. Inhibitory effect on the tyrosinase activity was calculated using the following equation:

$$\% \text{ Inhibition} = [1-(\text{sample } OD_{490} / \text{blank } OD_{490})] \times 100$$

Where the sample OD_{490} is the absorbance of the test sample and the blank OD_{490} is the absorbance of the blank (without test sample) at 490 nm.

Antioxidant activity assay

The antioxidant activity assay was performed by DPPH method (3). Briefly, seventy-five µl of test sample was mixed with 150 µl of 0.2 M DPPH in a 96-well microplate. After incubation at RT for 30 min, the optical density at 515 nm (OD_{515}) of the mixture was determined by using a microplate reader (Ceres W900 Bio-Tek Instruments, Inc.). Butylated hydroxytoluene (BHT) was used as a positive control. The ability to scavenge the DPPH radical was calculated using the following equation:

$$\% \text{ Radical scavenging} = [1-(\text{sample } OD_{515} / \text{blank } OD_{515})] \times 100$$

Where the sample OD_{515} is the absorbance of the test sample and the blank OD_{515} is the absorbance of the blank (without test sample) at 515 nm.

In both antityrosinase and antioxidant activity assays, the concentration of crude extract providing 50% inhibition (IC_{50}) was calculated from the graph of percentage of inhibition or percentage of radical scavenging plotted against sample concentration using GraphPad Prism Version 2.01 (GraphPad Software Inc., California, USA.).

The stability of por-sa leaf extract

The dried crude extracts were stored in well-closed glass containers kept at 75±2% relative humidity in three temperatures: refrigerator temperature (4±2 °C), room temperature (RT) and high temperature (45±2 °C). The stability of the PSE was evaluated at weeks 0, 2, 4, 6, 8, 12, 16, 20 and 24.

Determination of antityrosinase and antioxidant activities

The IC_{50} of antityrosinase and antioxidant activities was determined as described above.

Degradation analysis of por-sa leaf extract using high performance liquid chromatography (HPLC) analysis

An isocratic reversed-phase HPLC method was developed for the analysis of the marker peaks of PSE. The HPLC equipment (LC-20AT, Shimadzu, Japan) consists of a Shimadzu LC-10A pump and SPD-M10A photodiode array detector. Chromatography was performed on phenomenex luna C18 column (5 µm, 150x4.60 mm) with a mobile phase of acetonitrile: 0.02M phosphate buffer pH3 (17:83) and a flow rate of 0.8 ml/min. The volume injected is 20 µl with detection at 340 nm. The remaining percentage of PSE was calculated based on peak area of HPLC chromatogram of the marker peaks having various retention times.

RESULTS

Appearance and percentage yield

The PSE obtained was dark green sticky resin and the percentage yield was 6.86%.

Antityrosinase and antioxidant activity assays

Antityrosinase and antioxidant activities of PSE were shown as IC_{50} values and the data were shown in Table 1.

Table 1 IC_{50} values of antityrosinase and antioxidant activities of PSE extract and positive controls

Extract code	IC_{50} (µg/ml)	
	antityrosinase	antioxidant
PSE	17.68 ± 5.30	144.90 ± 9.01
Kojic acid	1.67 ± 0.33	-
BHT	-	32.07 ± 1.94

The stability of PSE

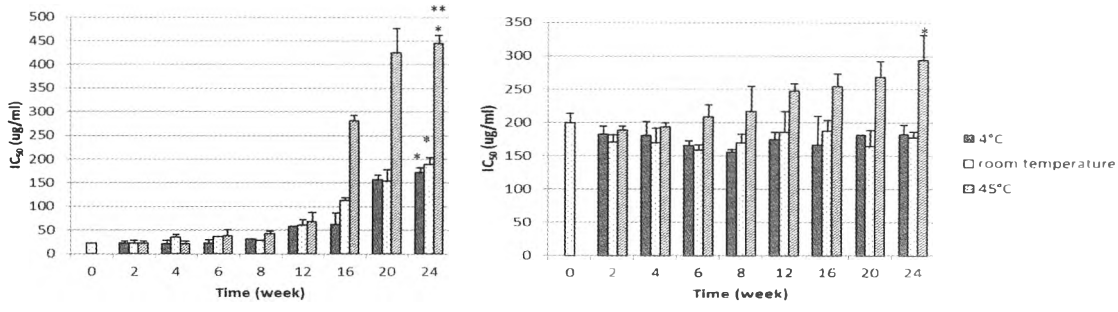
Following the storage of the extracts for 6 months at 4 °C, RT and 45 °C, it was found that the color of PSE did not change.

Determination of antityrosinase and antioxidant activities

It was found that, over 24 weeks, IC_{50} of antityrosinase activity of PSE was rather stable over 8-week storage at 4 °C and RT (Figure 1A). However, IC_{50} of antityrosinase activity of PSE increased continuously after 12 weeks, especially when stored at 45 °C, indicating the reduction in the activity (Figure 1B). Similarly, the IC_{50} of antioxidant activity of PSE stored at 4 °C and RT did not change over 24 weeks while that of PSE stored at 45 °C increased continuously.

Determination of the marker peaks of por-sa leaf extract using HPLC analysis

Using HPLC technique, the marker peaks were well-resolved under isocratic elution. The three peaks of interest were those with retention times of 8.8, 9.5 and 17.6 min (Figure 2). At this step, due to our inability to identify each peak of the HPLC chromatogram, the reduction of peak area was used to indicate the degradation of some compounds in the crude extract.



(a) (b)

Figure 1 Antityrosinase (a) and antioxidant (b) activities, shown as IC_{50} (μg/ml), of PSE after storing for 24 weeks at 4 °C, RT and 45 °C, * indicates statistically significant difference ($p<0.05$) compared to week 0, ** indicates statistically significant difference ($p<0.05$) compared to 4 °C and RT.

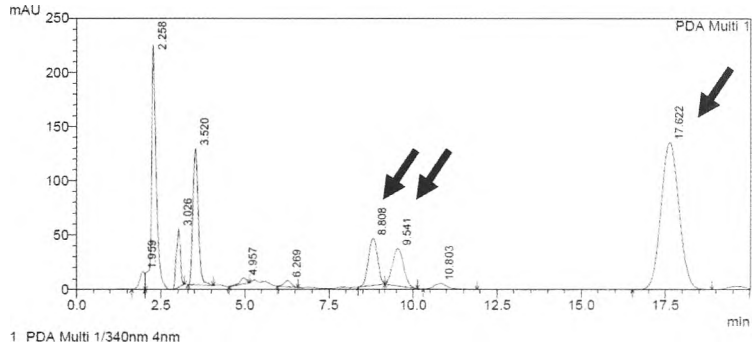
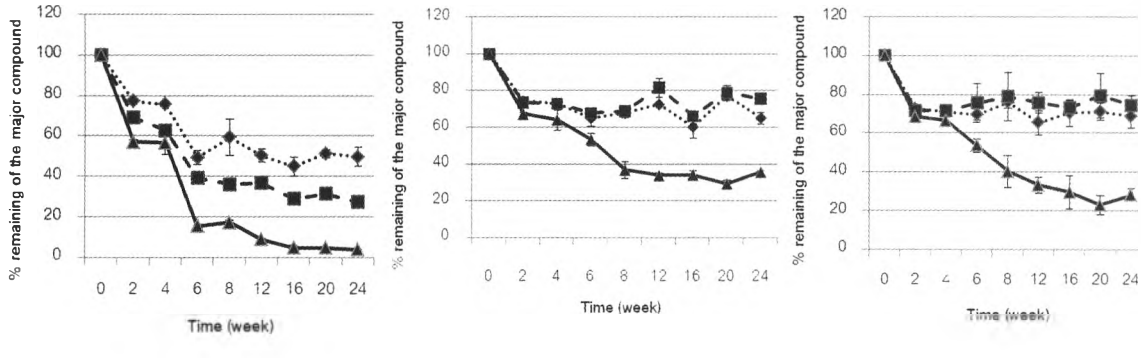


Figure 2 The HPLC chromatogram of PSE at a concentration of 4 mg/ml.

The remaining percentages of the major compounds decreased with time and temperature (Figure 3). High temperature (i.e. 45 °C) affected the stability of por-sa extract more than RT and 4 °C, respectively.



(a) (b) (c)

Figure 3 The remaining percentages of PSE calculated based on peak area of HPLC chromatogram of the marker peaks having retention time a) 8.8 min, b) 9.5 min and c) 17.6 min after storing for 24 week at 4 °C (◆), RT (■) and 45 °C (▲).

DISCUSSION

Following stability test, biological activities including antityrosinase and antioxidant activities of PSE were investigated. For antityrosinase activity, it was found that the activity decreased as a function of time and temperature. The antityrosinase activity of PSE was greatly affected when stored at 45 °C. Again, the antioxidant activity of PSE decreased when it was stored at 45 °C but slightly changed when it was stored at 4 °C and RT. The decreasing of the activities especially at high temperature (45 °C) indicates the degradation of some components in the extract might occur. Broussonetone A in the extracts before and after the stability test was also determined using TLC technique. It was found that following the stability test broussonetone A in PSE could still be detected but lesser amount was detected for PSE stored at 45 °C (data not shown). As the available marker broussonetone A could not be quantified using HPLC technique, the fingerprint of HPLC chromatogram was used to determine the change of components in the extracts. In natural extract, active compounds such as phenols, flavonoids, coumarins and other derivatives are related with hypopigmenting agent (4). Currently, approximately 4000 flavonoids have been identified and this class of plant polyphenols can be found in leaves, bark and flowers (5). Since light in the UV-Vis range of 272 and 333 nm is absorbed by flavonoids (3), the compounds in PSE were detected using HPLC analysis at 340 nm. PSE showed three major peaks in the HPLC fingerprint and thus these peaks in the HPLC chromatogram were used as markers for the quantitative analysis and the stability of the extracts was determined according to the reduction of peak area in the HPLC chromatogram. After storing PSE at different temperatures, HPLC peak areas were greatly decreased in the first four weeks, then slowly decreased. After 24 weeks, the remaining percentages of the markers of PSE stored at 4 °C and RT was about 60%, whereas the remaining percentage of the markers of PSE stored at 45 °C was about 30%.

CONCLUSION

The stability of the plant extracts is a major concern and this information is beneficial for developing products from por-sa leaves. The stability in term of antityrosinase and antioxidant activities was dependent on temperature. After storing at 45 °C, antityrosinase activity of PSE was found to be greatly decreased. Antioxidant activity of PSE was more stable at 4 °C and RT than when it was stored at 45 °C. The results of HPLC assay again confirmed that the stability of the extract was affected by temperature, especially at 45 °C

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