Pigment Extraction Techniques from the Leaves of *Indigofera tinctoria* Linn. and *Baphicacanthus cusia* Brem. and Chemical Structure Analysis of Their Major Components

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ABSTRACT

Indigofera tinctoria Linn. and Baphicacanthus cusia Brem. are the plants which have been used as natural source of indigo dye and are available in Northern part of Thailand. In this study we carried out methods to find the optimum condition for effective extraction of indigo from the leaves of Indigofera tinctoria Linn. and Baphicacanthus cusia Brem. and investigated chemical constituents including the chemical structure of major components in the water extract from both kinds of plant to develop the extraction method for preparation of ready-to-use natural dyes. It was found that by cutting raw material to small pieces and put into cotton bag before soaking in water for 24 hrs could decrease unpleasant smell and it was easy to get rid of waste. The indigo from fresh Baphicacanthus cusia Brem. fermented for 24 hrs gave the highest amount of indigo (0.005 g/g fresh leaves). Separation of the crude extract by Thin Layer Chromatrography (TLC) using chloroform-hexane-methanol (7:4:1 v/v/v) as solvent system gave two major pigments of blue and red colour which had Rf values of 0.69 and 0.49, respectively. The Rf value, the maximum absorption from UV-Visible spectroscopy and infrared spectrum of blue colour pigment were the same as those of the indigo standard. Moreover, the red pigment extracted from Baphicacanthus cusia Brem. was purified and analysed by UV-Visible spectroscopy, mass spectrum 13C NMR and 1H NMR and showed that it had chemical formula of C16H10N2O2 and chemical structure as indirubin and it was used as indirubin standard. The red pigment from Indigofera tinctoria Linn. was also purified and gave the same Rf value, UV-Visible absorption and IR spectrum as the indirubin standard. Both blue and red pigment could dissolve in chloroform whereas only red pigment could dissolve in methanol. Separation method of the blue and red pigment from the crude extract would be developed to get the powder of the red and blue pigments which could be used to prepare the ready-to-use natural dyes.

Key words : Indigo, Indirubin, Extraction, Structure analysis, *Indigofera tinctoria* Linn., *Baphicacanthus cusia* Brem.

INTRODUCTION

Indigo, after which this group of carbonyl dyes is named, is one of the oldest known natural dyes. It is a derivative of the colorless glucosides of the enol form of indoxyl, e.g. indican. Indigo is formed from indican by fermentation of plant material such as *Baphicacanthus cusia* Brem., *Indigofera suffruticosa, Polygonum tinctorium, Isatis indigotica* followed by air oxidation of indoxyl (Minami et al.,1996,1997).

Many studies have investigated the biosynthesis of indigo precursors and it was found that indole was precursor of indigo biosynthesis in plant (Lu,1986;Xia and Zenk,1992). Indirubin, which is a pinky-red pigment similar to indigo blue in structure was produced from *Polygonum tinctorium* (Maier et al.,1990;Shin and Lee,1993). Moreover, indirubin has been isolated from the crude extract of *Baphicacanthus cusia* Brem. (Ben,1981;Tang,1987). Indirubin inhibited Lewis lung carcinoma in mice and Walker carcinosarcoma 256 in rats (Ji et al.,1981). In a clinical study, treatment with indirubin of patients with chronic myelocytic leukemia at doses of 300-450 mg daily showed 26% complete remissions and 33.4% partial remissions (Indirubin Cooperative Group,1980).

In this study the indigo dye was obtained from fermentation of *Indigofera tinctoria* Linn. and *Baphicacanthus cusia* Brem. in water. The objective of this work is to develop the method of indigo extraction. Moreover, the separation and chemical structure analysis of dye components, comparision of the major components of the indigo dye obtained from *Indigofera tinctoria* Linn. and *Baphicacanthus cusia* Brem. and the preparation of the ready-to-use natural dye from these components are investigated.

MATERIALS AND METHODS

Plant Materials Fresh materials of *Indigofera tinctoria* Linn. and *Baphicacanthus cusia* Brem. were collected from the Upper North of Thailand. Three different samples : fresh, semi-dried and dried plant materials were taken from the same source. The semi-dried materials were the plants that dried at room temperature (30±2 ºC) for 3 days. The dried materials were those that dried at room temperature (30±2 ºC) for 7days. Standard indigo was obtained from Fluka.

Extraction of Indigo Dye Plant materials were cut to small pieces, fermented in water at different periods and then added twice in volume of $Ca(OH)_2$ solution (pH~11), blew the air for 15 mins to precipitate indigo. The precipitated indigo was washed twice with $Ca(OH)_2$ solution and centrifuged at 9820xg for 10 mins.

Indigo Determination Calibration curve was done by using various amount of standard indigo, obtained by dissolving 8 mg of the standard indigo in 20 ml of H_2SO_4 and diluted to 500 ml with distilled water. The solution was then diluted to different concentration with the H_2SO_4 solution (H_2SO_4 : distilled water ; 1:24)and measured the absorbance at 611 nm.The indigo paste was dissolved in 20 ml of H_2SO_4, diluted to 500 ml with distilled water and measured the absorbance at 611 nm.

Separation of Major Components by Thin Layer Chromatography The crude extracts were dissolved in chloroform and spotted on TLC plate that had silica gel $60F_{254}$ as

absorbent. The developing solvent system was found by varying solvents. The most suitable one was used to separate the major components by Thin Layer Chromatography and Column Chromatography. The results were compared with indigo standard.

Separation of Major Components by Column Chromatography The dyes were extracted by chloroform and evaporated under reduced pressure to dryness. The red pigment was separated by extracting with methanol after which the blue pigment was extracted with chloroform. Both pigments were evaporated to dryness and separated by column chromatography. A glass column 45x3 containing 60 g of silica gel $60F_{254}$ saturated with hexane was used to separate 2 g of dye pigment and eluted with chloroform-hexane (4:1 v/v) and chloroform-hexane-methanol (7:4:1 v/v/v), respectively. The eluates were concentrated. The purity of the pigments was then tested by TLC in several solvent systems. The pure pigments were analysed for their chemical structure. If the pigment was not pure, the subcolumn chromatography was done again.

Structural Analysis of the Major Components The blue pigment was dissolved in chloroform and the red pigment in methanol to study by the UV-Visible absorption and infrared spectroscopy, respectively. The UV-Visible spectra were recorded in the range of 200-800 nm. The infrared spectra were analyzed by film technique (spot pigment solution to film on NaCl cell) and the dried films were analysed to find the functional group of these purified pigments. Moreover, the chemical structure of the purified pigments were also analyzed by Mass spectroscopy and ^{13}C Nuclear magnetic resonance and ^{1}H Nuclear magnetic resonance spectroscopy.

RESULTS AND DISCUSSION

Extraction of Indigo Dye from *Baphicacanthus cusia* Brem. and *Indigofera tinctoria* Linn.

The most effective way to get good yield of indigo paste was done by maceration of small cut pieces of plant material with water in cotton bag and covered the container to reduce unpleasant smell and it was easy to get rid of waste. Maceration of fresh *Baphicacanthus cusia* Brem. for 24 hrs gave highest amount of indigo paste with more blue colour (Table 1).

Table 1. The characteristic of crude indigo paste in different freshness of plant materials and different periods of maceration in water

Type of plant	Period of fermentation (Days)	Colour of fermented solution	pH	Colour of paste
Fresh *Baphicacanthus cusia* Brem.	1	Yellow-green	4.8	Blue
	3	Green	4.8	Blue-green
Semi-dried *Baphicacanthus cusia* Brem.	1	Green-brown	5.0	Brown
	3	Brown	5.0	Brown
Dried *Baphicacanthus cusia* Brem.	1	Brown	6.0	Brown
Fresh *Indigofera tinctoria* Linn.	1	Yellow- green	4.8	Blue

The indigo plants contain glucoside indican which is hydrolyzed by enzyme to indoxyl and then oxidized to form indigo blue by air oxidation (Kun Lestari,1998). Indigo blue is water-insoluble pigment. So, in the dyeing process, the indigo blue must be base-oxidized to leuco indigo which is colorless and dissolves in water before dyeing. After that the leuco indigo is oxidized by the air and turns to be indigo blue again (Fig. 1). The enzyme could come from the indigo plants and maceration of fresh indigo plants would release the glycolytic enzyme from the plant cells to hydrolyze glycan and gave indoxyl which was air oxidized to indigo blue. The enzyme in dried and semi-dried indigo plants might be inactivated during drying process, therefore the yield of the indigo dye from such plant materials was very low.

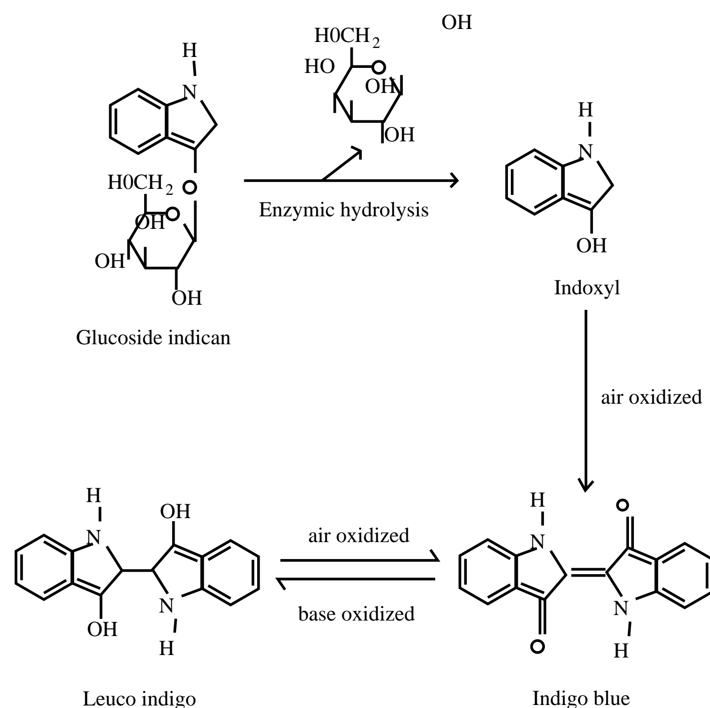

Figure 1. Diagram showing the chemical changing of indigo from plant material

Table 2. Indigo dye yield from 24 hrs maceration of 100g *Baphicacanthus cusia* Brem. and *Indigofera tinctoria* Linn.

Type of plant	Paste (g)	Indigo dye (mg)
Fresh *Baphicacanthus cusia* Brem.	15.69	472.27
Semi-dried *Baphicacanthus cusia* Brem.	11.62	31.37
Dried *Baphicacanthus cusia* Brem.	9.86	7.89
Fresh *Indigofera tinctoria* Linn.	26.83	327.33

The yields of the indigo dye from the fresh plant materials were highest. The semi-dried and dried plant material gave significantly low yields (Table 2). This result showed that the activities of enzyme ß-glucosidase (Minami et al.,1996) in the plants' leaves was more active in the fresh plants than in the semi-dried and dried plants, respectively. The activity of the enzyme ß-glucosidase was decreased when exposed to heat and dryness (Kun Lestari,1998). However, the lower yield from semi-dried and dried materials could also be due to other

factors. It could be that the dye molecules aggregated and did not come out in to the solution, or the dye pigments were trapped within the dried plant tissues.

The comparision of indigo in the crude indigo paste from *Baphicacanthus cusia* Brem. and Indigofera tinctoria Linn. revealed that *Baphicacanthus cusia* Brem. gave more indigo than *Indigofera tinctoria* Linn. in the ratio of 4:3. The contents of indigo were found to depend on the plant origin and the age of plant (Lu,1986). Moreover, addition of bacteria to the fermentation process did not affect the indigo yield and addition of some acids decreased the indigo yield (data not shown). It could be concluded that the natural enzyme and bacteria of the plant material were effective enough for fermentation but the addition of acid might destroy the enzyme and bacteria instead.

Purification and Analysis of Major Components from *Baphicacanthus cusia* Brem. and *Indigofera tinctoria* Linn.

The separation of the crude extract by TLC using chloroform-hexane-methanol (7:4:1 v/v/v) as developing solvent gave two major components of blue and red colour which was the same as the major components in qingdai (Ben,1981;Tang,1987) which had Rf value 0.69 and 0.49, respectively (Fig. 2).

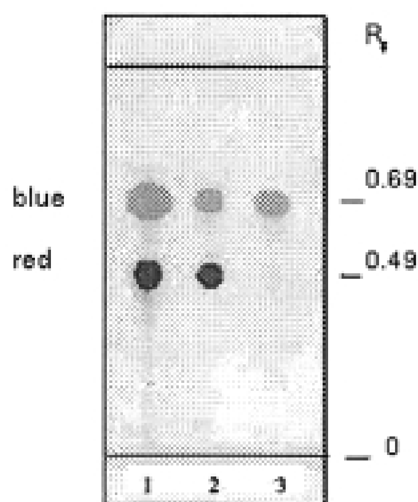

Figure 2. TLC of indigo dye and the crude indigo extract from *Baphicacanthus cusia* Brem. and *Indigofera tinctoria* Linn.
Spot 1 crude indigo paste from *Baphicacanthus cusia* Brem.
Spot 2 crude indigo paste from *Indigofera tinctoria* Linn.
Spot 3 indigo standard

The purified blue pigment obtained from the crude extract of *Baphicacanthus cusia* Brem. and *Indigofera tinctoria* Linn. had exactly the same Rf value, UV-Visible spectrum (Fig. 3) and finger print of IR spectra (Fig. 4) as the indigo standard. Table 3 showed the functional groups of purified blue pigment as N-H primary and secondary amines, C=C aromatic, C-N amine and C-H out of plane bending of aromatic.

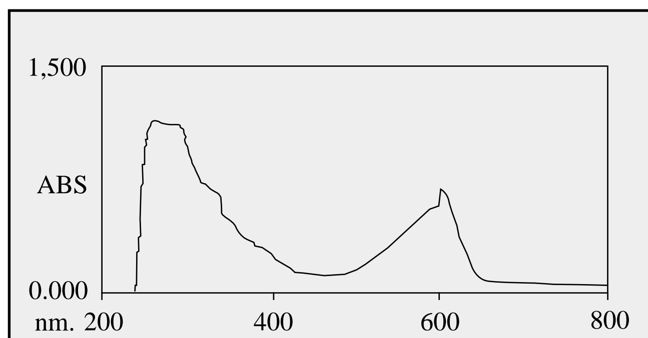

Figure 3. UV-Visible spectrum of the purified blue pigment from *Baphicacanthus cusia* Brem.

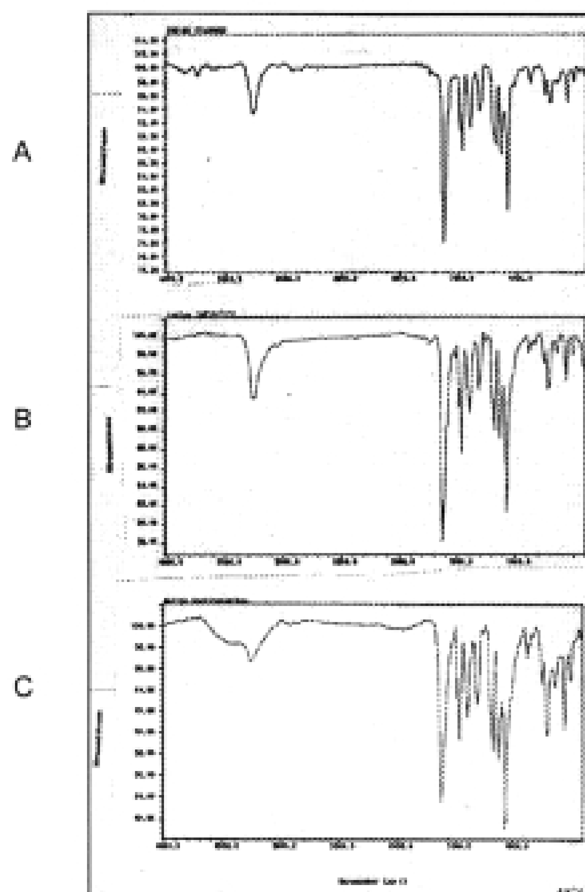

Figure 4. Infrared spectra of standard indigo (A), the purified blue pigment from *Indigofera tinctoria* Linn. (B) and the purified blue pigment from *Baphicacanthus cusia* Brem. (C)

Table 3. The functional groups of the purified blue pigment from IR spectra

Wavenumber (cm^{-1})	Functional group
3500-3100	N-H Primary and Secondary Amines
1600	C=C Aromatic
1350-1000	C-N Amines
900-690	C-H Aromatic (Out-of plane bending)

The red pigment extracted from *Baphicacanthus cusia* Brem. was purified and analysed by mass spectrum and ^{13}C NMR and ^{1}H NMR. Mass spectrum analysis showed that the red pigment had molecular ion at m/e equal to 262 (Fig. 5). The assignment of ^{13}C-NMR spectrum is shown in Fig. 6 and Table 4 and the assignment of 1H NMR spectra in Fig. 7 and Table 5. It could be concluded from these analysis results that the red pigment had chemical formula of $C_{16}H_{10}N_2O_2$ and chemical structure as indirubin (Fig. 8). The indirubin from *Baphicacanthus cusia* Brem. was used as indirubin standard. The red pigment from *Indigofera tinctoria* Linn. was also purified and gave finger print of IR spectra of indirubin (Fig. 9) and showed the functional groups as N-H Primary and Secondary Amines, O-H Bonding, C-HStretching of Alkyl, C=C Aromatic, C-N Amines and C-H Aromatic (Out-of plane bending) (Table 6). Moreover, its UV-Visible absorption spectrum (Fig. 10) was also the same as the indirubin standard.

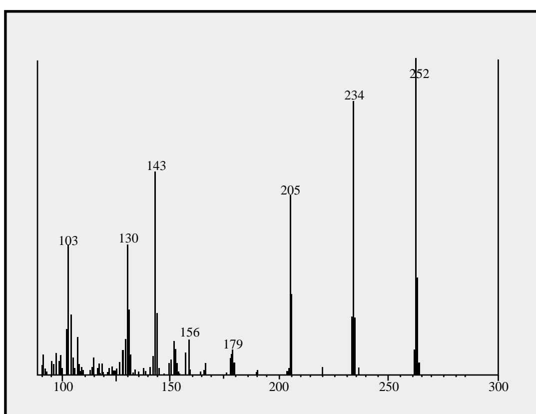

Figure 5. Mass spectrum of the purified red pigment

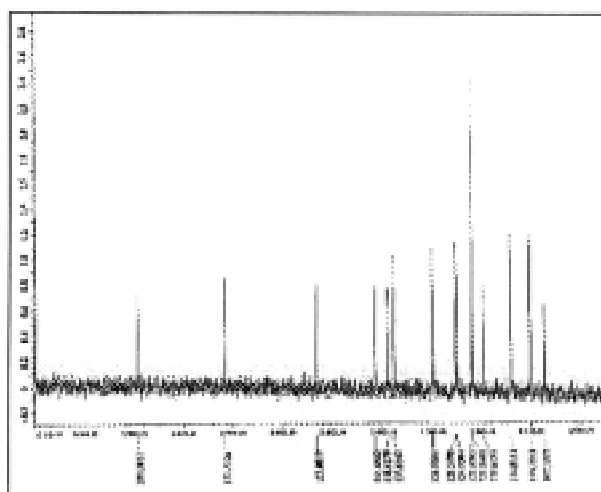

Figure 6. ^{13}C NMR spectrum of the purified red pigment

Table 4. ^{13}C-NMR chemical shift (ppm) of the purified red pigment

Peak No.	Position of ^{13}C	Chemical shift
1	C-3’	189.2032
2	C-2	171.5126
3	C-2’, C-9’	153.0819
4	C-5	141.4820
5	C-7’	138.9279
6	C-5’	137.6842
7	C-7	129.8595
8	C-4	125.2470
9	C-9	124.9304
10	C-8	122.0385
11	C-4’	121.8440
12	C-6	119.6198
13	C-3	114.0154
14	C-6’	110.1583
15	C-8’	107.1597

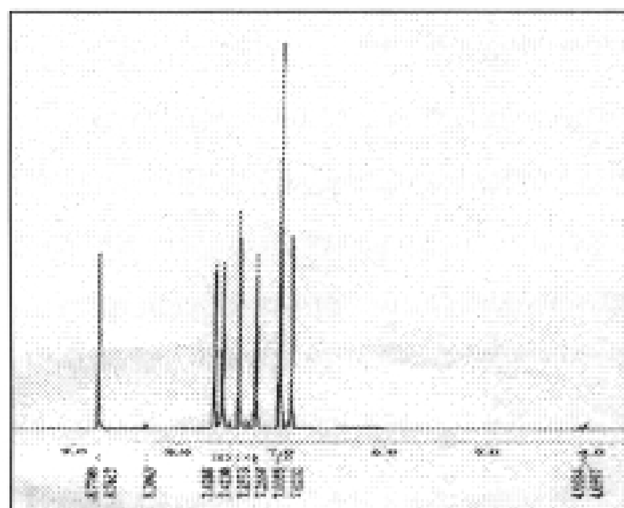

Figure 7. ^{1}H NMR spectrum of the purified red pigment

Table 5. ^{1}H-NMR chemical shift (ppm) of the purified red pigment

Peak No.	Position of ^{1}H	Chemical shift
1	H-1	8.7740
2	H-6	8.7612
3	H-5’	8.3063
4	H-7’	7.4268
5	H-9	7.4130
6	H-7	7.2573
7	H-8	7.2554
8	H-6’	7.0355
9	H-8’	7.0232
10	H-1’	4.0684

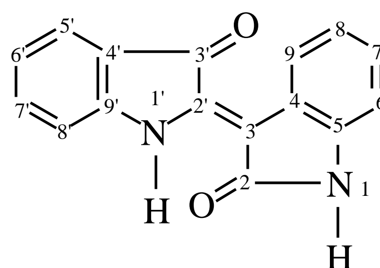

Figure 8. Chemical structure of indirubin

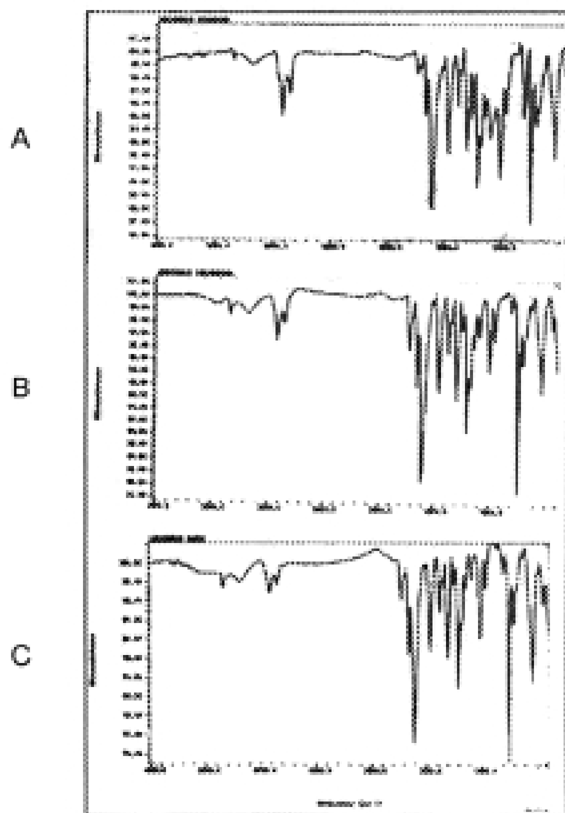

Figure 9. Infrared spectra of standard indirubin (A), the purified red pigment from Indigofera tinctoria Linn. (B) and the purified red pigment from Baphicacanthus cusia Brem. (C)

Table 6. The functional groups of the purified red pigment from IR spectra

Wavenumber (cm^{-1})	Functional group
3500-3100	N-H Primary and Secondary Amines
3200	O-H Bonding
2950-2880	C-H Stretching of Alkyl
1600	C=C Aromatic
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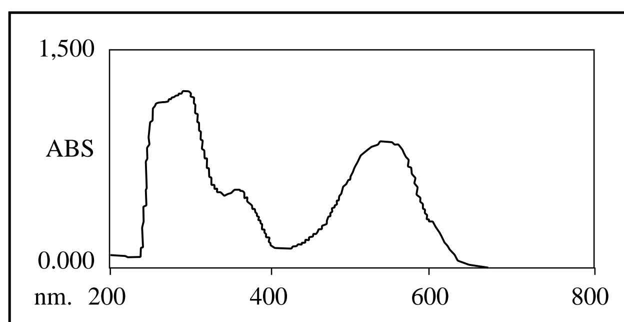

Figure 10. UV- spectrum of the purified red pigment from *Baphicacanthus cusia* Brem.

Both blue and red pigments are highly soluble in chloroform but only red pigment is soluble in methanol. The red pigment could be separated from the blue pigment by dissolving the crude indigo powder in chloroform and evaporating to dryness. The methanol was then added to extract the red pigment. The residual powder containing most blue pigment was finally dried. The separation of blue and red pigments from the powder of the crude extract could be developed to prepare the ready-to-use natural blue and red dyes.

CONCLUSIONS

Maceration of the fresh leaves of *Baphicacanthus cusia* Brem. and *Indigofera tinctoria* Linn. for 24 hrs was the optimum condition for effective extraction of indigo dye from these plants. The major components of the crude indigo extract from both kinds of plants were blue and red pigments. The blue pigment was indigo and the red pigment was its isomer indirubin. Separation of these two components could be done to prepare ready-to-use natural red and blue dyes.

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REFERENCES

Ben, B.L. 1981. Column Chromatrography- spectrophotometric determination of indigo and indirubin in Qingdai, a traditinal Chinese medicine. Chin. Trad. Herb. Drugs. 12 :11-15.

Indirubin Cooperative Group. 1980. Clinical study of indirubin in the treatment of 314 patients with chronic granulocytic leukemia. Chin. J. Hematol. 1:132.

Ji, X.J., F.R. Zhang, J.L. Lei, and Y.T. Xu. 1981. Studies on the antineoplastic effect and toxicity of synthetic indirubin. Acta. Pharm. Sin. 16 :146-148.

Kun Lestari, W.F. 1998. Dyeing process with natural indigo : The Tradition and Technology. Revival Natural Indigo dye. Sept. 20-29.

Lu, R.G. 1986. Determination of indirubin and indigo in natural indigo (Qingdai) with dual wavelength spectrometry. Chin. Pharm. Bull. 21 :72-74.

Maier, W., B. Schumann, and D. Groger. 1990. Biosynthesis of indoxyl in *Isatis tinctoria* and *Polygonum tinctorium.* Phytochemistry 29 : 817-819.

Minami, Y., T. Kanafuji, and K. Miura. 1996. Purification and characterization of a ß-glucosidase from *Polygonum tinctorium* which catalyzes preferentially the hydrolysis of indican. Biosci.Biotech.Biochem. 60 :147-149.

Minami, Y., H. Takao, T. Kanafuji, K. Miura, M. Kondo, I. Hara-Nishimura, M. Nishimura, and H. Matsubara. 1997. Glucosidase in the indigo plant : intracellular localization and tissue specific expression in leaves. Plant Cell Physiol.38 :1069-1074.

Shin, J.H., and J.H. Lee. 1993. Cultural conditions and growth characteristics of indigo (*Polygonum tinctorium*) cells in an air-lift bioreactor. Kor. J. Biotechnol. Bioeng. 8 : 193-199.

Tang, Y. 1987. Determination of indirubin in Qingdai (*Baphicacanthus cusia Bremk.*) and Chinese medicines containing it. Chin. J. Pharm. Anal. 7: 40-42.

Xia, Z.Q., and M.H. Zenk. 1992. Biosynthesis of Indigo precursors in higher plants. Phytochemistry 31(8) : 2695-2697.