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Full Length Research Paper

Comparative study of the major components of the indigo dye obtained from *Strobilanthes flaccidifolius* Nees. and *Indigofera tinctoria* Linn.

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Investigation on the chemical constituents including the chemical structure of major components in the water extracts from *Strobilanthes flaccidifolius* Nees. and *Indigofera tinctoria* Linn. was done. It was found that the indigo from fresh leaves of *S. flaccidifolius* Nees. fermented for three days gave the highest amount of indigo. The thin layer chromatography of the crude extract revealed four pigments; the major pigments were of red and blue having R_f values of 0.88 and 0.76, respectively. The R_f value, the maximum absorption from UV-Visible spectroscopy and infra red spectrum of blue pigment were the same as those of the standard indigo. Both blue and red pigments are highly soluble in chloroform whereas only red pigment is soluble in methanol.

Key words: Comparative study, *Strobilanthes flaccidifolius* Nees., *Indgofera tinctoria* Linn., indigo.

INTRODUCTION

In recent years, there has been a revival of the use of dyes and colors of natural origin for coloring food, pharmaceutical, cosmetic and textile products. This increasing demand for the material of natural origin is because of the toxic nature of many of the synthetic dyes. Amongst the natural dyes which are becoming widely recognized throughout the world, indigo which is one of the oldest known natural dyes (Ensley et al., 1983), is a derivative of the colorless glucosides of the enol form of indoxyl, e.g. indican (indoxyl- -D-glucoside). Indigo is formed from indican by fermentation of plant material such as *Baphicacanthus cusia* Brem., *Indigofera suffruticosa* Mill, *Polygonum tinctorium* Palette, *Isatis indigotica*, Fort etc. followed by air oxidation of indoxyl (Minami et al., 1996, 1997). *Indigofera tinctoria* (Woad) contains isatan B (indoxyl- -ketogluconate), as a major indigo precursor and indican as a minor indigo precursor (Epstein et al., 1967; Maier et al., 1990).

It was found that indole was precursor of indigo biosynthesis in plant (Lu, 1986; Xia and Zenk, 1992). In old leaves of the plant, precursors are broken down by hydrolases when the leaves are damaged and exposed to the air (Scheme 1). The liberated indoxyl is spontaneously oxidized by oxygen yielding indigo (Scheme 2). Isatin is generated from indoxyl in an oxygen-rich environment as a side product. The condensation of indoxyl with isatin produces indirubin, which is a pinky-red pigment similar to indigo blue in structure. Indirubin has been isolated from the crude extract of *B. cusia* Brem. (Ben, 1981; Tang, 1987) and from *P. tinctorium* (Maier et al., 1990; Shin and Lee, 1993). It is well known that plants contain, in addition to *trans*-indigo (indigotin, blue) and *trans*-indirubin (isoindigotin, red), certain trace compounds such as *cis*-indigo (blue), *cis*-indirubin (isoindirubin, red), indigo brown (isoindigo), indigo gluten, indigo yellow and traces of flavonoids (Perkin and Bloxam, 1907).

Herein, the extraction of the indigo dyes from fermentation of *S. flaccidifolius* Nees. and *Indigofera tinctoria* Linn. in water is reported. In the present work, the separation and chemical structure analysis of dye components was done and in addition comparison of the major components of the indigo dye obtained from *S. flaccidifolius* Nees. and *I. tinctoria* Linn., and the

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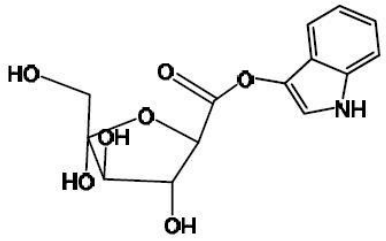
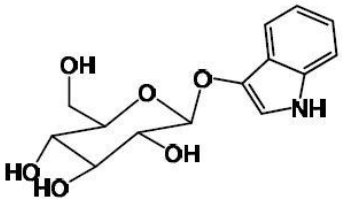
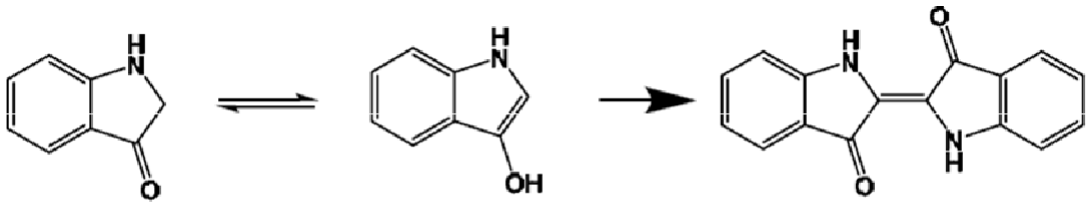


Indoxyl-5-ketogluconate = isatan B



Indoxyl-B-D-glucoside = indican

Scheme 1. The molecular for formulas of the precursors of indigo.



Indoxyl Indigo

Scheme 2. The molecular formulas of indoxyl and indigo.

preparation of the ready-to-use natural dye from these components were investigated. *S. flaccidifolius* Nees., locally known as ''kum'', has been used for dyeing purposes by the people of Manipur (North-east India). Traditionally, they extracted the dye after fermentation with the addition of oyster lime for calcination.

MATERIALS AND METHODS

Plant materials

Fresh samples of *I. tinctoria* Linn. and *S. flaccidifolius* Nees. were collected. Three different samples: Fresh, semi-dried and dried plant materials were taken from the same source. The semi- dried materials were the plants dried at room temperature (25±2°C) for 3 days. The dried materials were those that dried at room temperature (25±2°C) for 10 days.

Reference compounds

The reference compounds, indigo, indican, isatin, isatan B, oxindole and 3-acetoxy-indole (indoxyl acetate) were obtained from Sigma Chemical Co.

General experimental procedures

Melting points were determined by capillary tubes and are uncorrected. Infrared (IR) spectra were recorded on an ATI Mattson Genesis Series FTIR spectrometer. All samples were run as a thin film (produced by evaporation of a chloroform solution) on a sodium chloride plate. Absorption maxima were recorded in wave numbers (cm^{-1}). Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on Varian unity 500 (500 MHz), Bruker AC-300 and Varian XL (300 MHZ) spectrometers. ^{13}Carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on Bruker AC-300 and Varian XL (75 MHz) spectrometers. Residual non-deuterated solvent was used as an internal reference and all chemical shifts ($_{H}$ and $_{C}$) are quoted in parts per million (ppm) downfield from tetramethyl silane (TMS). All samples were run in deutero-chloroform ($CDCl_3$) as solvent unless otherwise stated. Mass spectra were recorded on a Kratas concept-IS mass spectrometer couples to a Mach 3 data system, or on a Jeol-D 300 mass spectrometer.

Extraction

Many extraction methods were tried with *S. flaccidifolius* Nees., such as,

1. Plant materials (1 Kg) were cut to small pieces, fermented in water at different periods and then added twice in volume of $Ca(OH)_2$ solution (pH, ~11), the air was blown for 15 min to precipitate indigo. The precipitated indigo was washed twice with $Ca(OH)_2$ solution and centrifuged at 9820 RPM for 10 min.
2. Extraction with water by fermentation of the fresh leaves and young buds (1 Kg) without the use of any base was performed. In this, enzyme is released to hydrolyze the glycan to indoxyl which is air oxidized to indigo blue. The extract was centrifuged at the rate of 15000 RPM for 5 min.
3. Soxhlet extraction of powdered dried leaves (2.095 Kg) with methanol
4. Cold extraction of powdered dried leaves (1.06 Kg) with methanol, and
5. Cold extraction of powdered dried leaves (1.25 Kg) with water.

The most effective method was extraction of the fresh leaves with water without use of $Ca(OH)_2$ and extraction with methanol was not so efficient. In case of extraction of the dried powdered leaves with water, there was negligible yield. The water extract was further extracted with chloroform; however, neither indigo nor indirubin was detected.

The sample *I. tinctoria* Linn. was subjected to extraction with water only.

Separation of major components by column chromatography

The water extracts *S. flaccidifolius* Nees. and *I. tinctoria* Linn as well as the methanol extracts *S. flaccidifolius* Nees. were subjected to column chromatography. The dyes were extracted from the water extracts 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 saturated with hexane was used to separate 2.0 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 thin layer chromatography (TLC) in several solvent systems. The pure pigments were analyzed for their chemical structure. If the pigment was not pure, the sub-column chromatography was done again. The fractions obtained were monitored by TLC. The mixture form of two spots was concentrated, dissolved in chloroform and centrifuged, where indigo was obtained as solid and re- crystallized. The two spots were found to be red and blue having R_f values of 0.88 and 0.76, respectively. The results were compared with indigo standard. Other pigments such as brown indigo, isoindirubin, green pigments and yellow gel were also isolated.

The methanol extracts were also separated with column chromatography same as above the yield of indigo was very less compared to water extracts.

Quantification of indigo by UV-visible spectrophotometry

Calibration curve was prepared 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) . The absorbance was recorded at 611 nm. The water extracts of the different samples were taken and was centrifuged at the rate of 15000 RPM for 5 min to get the paste. The indigo paste of both the plants was dissolved in 20 ml of H_2SO_4, diluted to 500 ml with distilled water and the absorbance was recorded at 611 nm.

The concentration of the different samples were extrapolated from the calibration curve. The following results were obtained.

Identification of indigo precursors by thin layer chromatography (TLC) analysis

The pigments were extracted by chloroform from the solid mass obtained after centrifuging the water extract. The chloroform extract was dried using a lyophilizer. This extract plated on TLC was developed and three bands/spots were found to develop at R_f values of 0.7 (brown color), 0.6 (blue color) and 0.2 (brown color) which were found to be similar to that of R_f values of the three indigo precursors, indican, isatan B and isatin, respectively.

Identification 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 to 800 nm. The infrared spectra were analyzed by film technique (spot pigment solution to film on NaCl cell) and the dried films were analyzed to find the functional group of these purified pigments. The chemical structure of the purified pigments was also analyzed by NMR and mass spectroscopy. The melting point of indigo was determined and found to be more than 300ºC which was similar to the reference standard.

The IR spectra of brown indigo and UV, IR, mass and H NMR spectra of blue indigo are shown (Figures 1 to 5)

RESULTS AND DISCUSSION

Extraction and identification of major components from *S. flaccidifolius* Nees. and *I. tinctoria* Linn.

Extraction, TLC, and column chromatography analyses were performed as described in materials and methods. The separation of the crude extract by TLC using chloroform-hexane-methanol as developing solvent gave Indican, Isatin, Isatan B, Indoxyl, and two major components of blue and red color which were the same as the major components in *B. cusia* Brem (Ben, 1981; Tang, 1987). The major components produced in *S. flaccidifolius* were identified by their TLC, UV, IR, NMR and mass spectra, and by comparing with the authentic samples (Sigma). The UV-visible spectra of the two major components ($_{max}$ 552 and 603 nm) obtained from *S. flaccidifolius* were found to correspond to *trans*-indirubin and *trans*-indigo, respectively. It is to be noted that *cis*-idirubin and *cis*-indigo had exactly the same UV-visible spectrum and mass spectrum as *trans*- indirubin and *trans*-indigo, respectively (Maugard et al., 2001). Mass spectra yielded an apparent MH^+ ion at m/z of 263 indicating molecular mass of 262 and molecular ion peak at 215 obtained after cleavage of (-CO-NH_2) in all the isomers of indigo .

The most effective way to get good yield of indigo paste was done by maceration of small cut pieces of fresh leaves and young shoots of plant material with water. Maceration of fresh *S. flaccidifolius* for 72 h gave highest amount of indigo paste with more blue color (Table 1).

The yields of the indigo dye from the fresh plant materials were highest. The semidried and dried plant material gave significantly low yields (Table 2). Moreover, the quantity of indigo precursors is also dependent on the cultivars and the period of harvest (Maugard et al., 2001). This result showed that the activities of the enzyme, ß-glucosidase (Minami et al., 1996) were more active in the leaves of fresh plants than in the leaves of semi-dried and dried plants, respectively. The activity of the enzyme ß-glucosidase decreased when exposed to heat and dryness (Kun, 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 the solution, or the dye pigments were trapped within the dried plant tissues.

The comparison of indigo in the crude indigo paste from *S. flaccidifolius* Nees. and *I. tinctoria* Linn. revealed that *S. flaccidifolius* Nees. gave more indigo than *I. tinctoria* Linn. The contents of indigo were found to

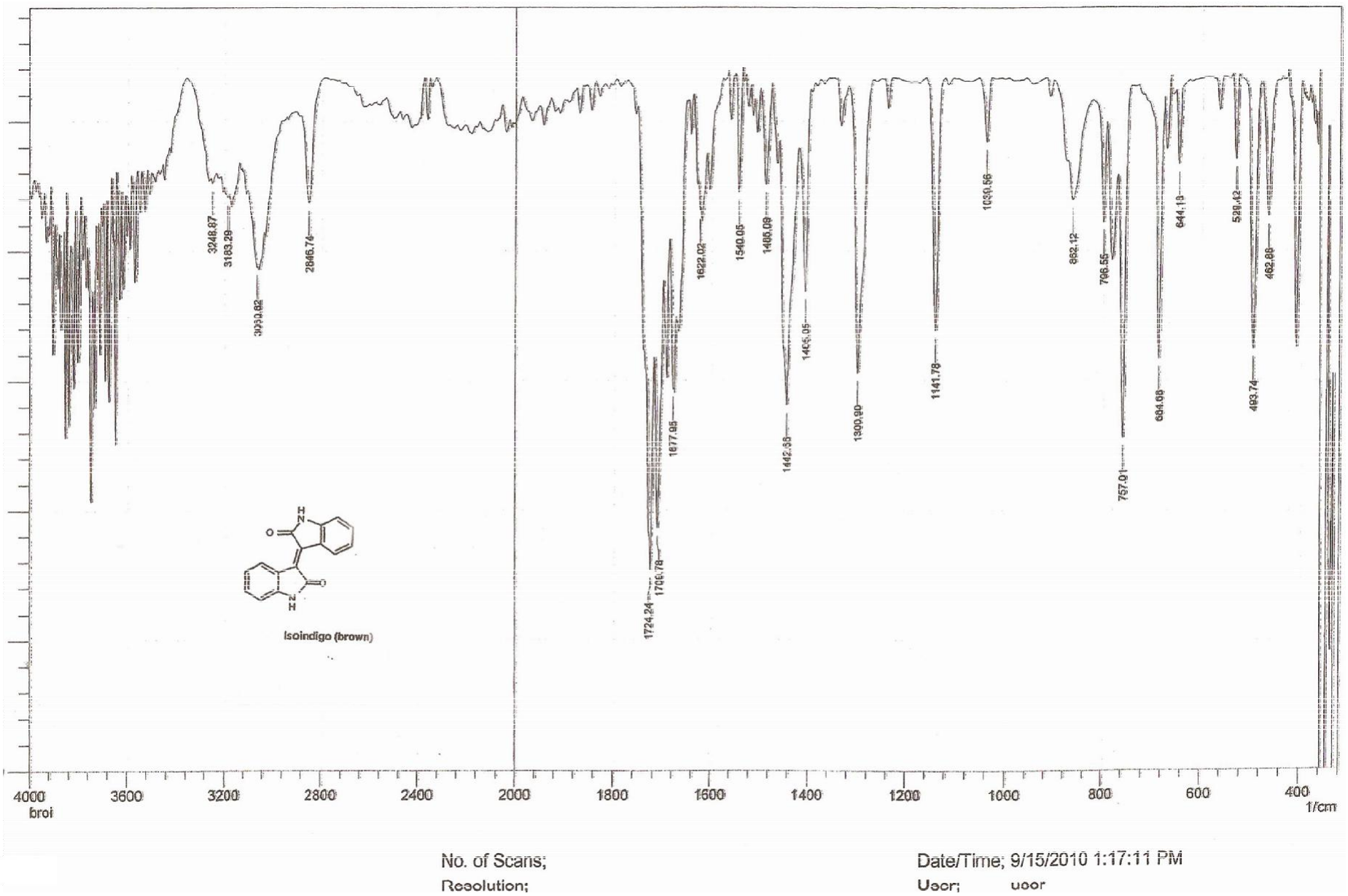


Figure 1. IR spectrum of indigo.

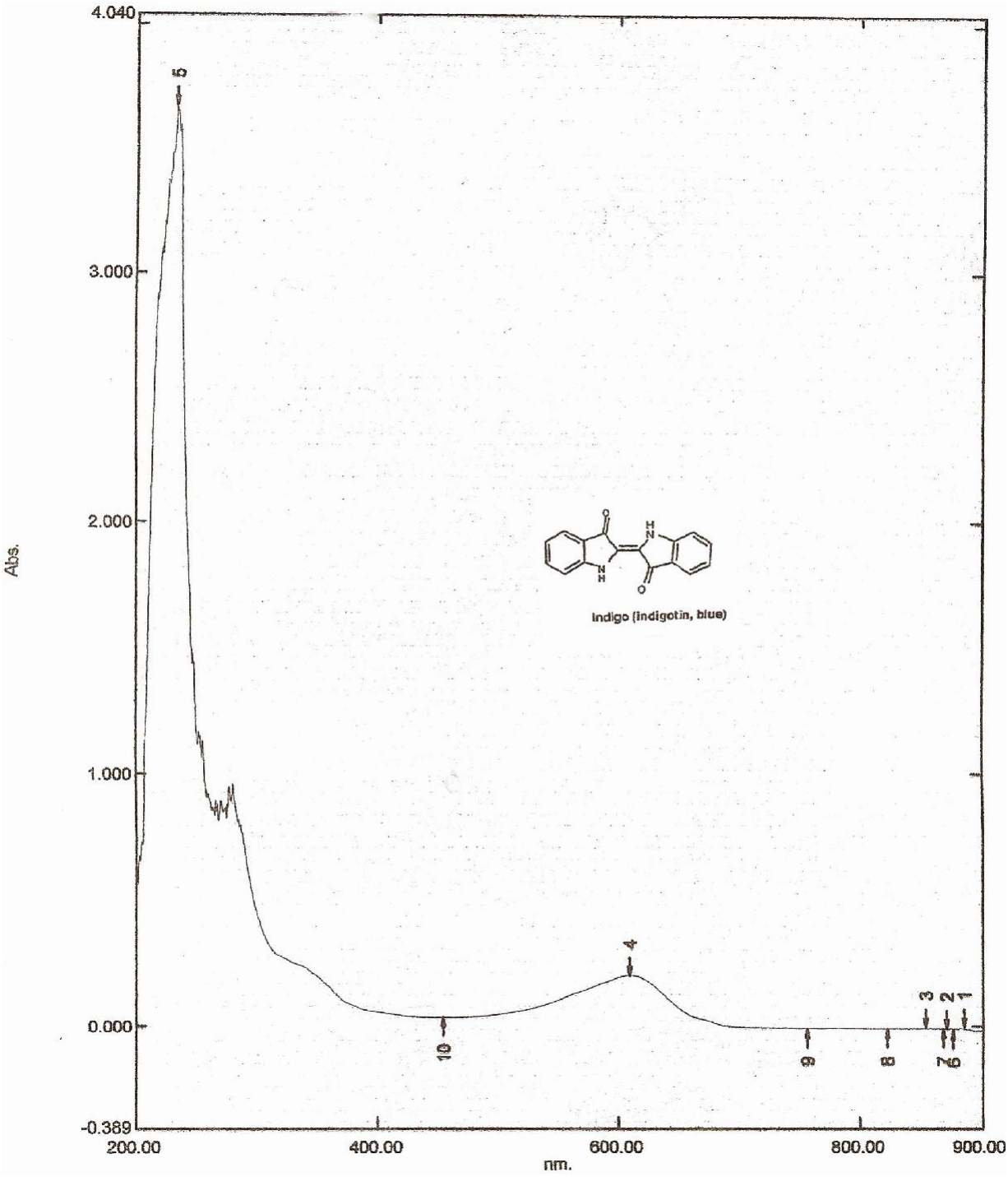


Figure 2. UV spectrum of indigo.

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. 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.

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 residual powder containing most blue pigment was finally dried. The separation of blue and red pigments from the powder of

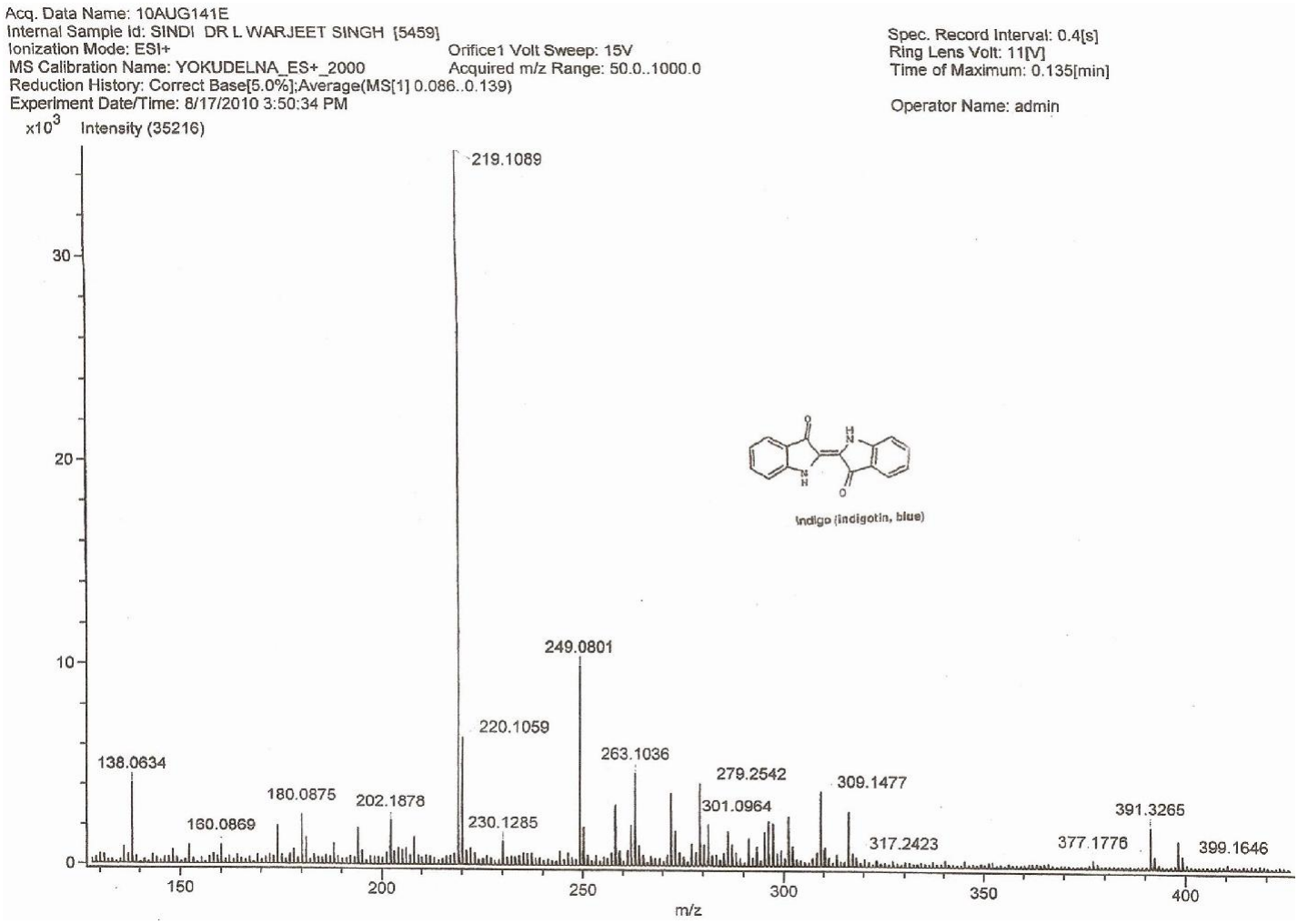


Figure 3. Mass spectrum for indigo.

the crude extract could be developed to prepare the ready-to-use natural blue and red dyes.

It is likely that the indigo plants contain glucoside indican which is hydrolyzed by enzyme to indoxyl, a highly unstable intermediate. In young leaves, plants protect indoxyl from spontaneous oxidation to indigo by glycosylation to yield isatan B. Indoxyl liberated from isatan B and indicant in old leaves is oxidized to form indigo blue by air oxidation (Kun, 1998). Isatin is generated from indoxyl in an oxygen-rich environment as a side reaction. 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 (Phutrakul et al., 2002). 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. The condensation of indoxyl with isatin produces indirubin, whereas the condensation of dioindole with isatin yields isoindirubin and indirubin, which are by-products of the biosynthsis of indigo (Maugard et al., 2001).

Conclusion

The chemical structure of major components in the water extracts from *S. flaccidifolius* Nees. was analysed. The three indigo precursors, indican, isatan B and isatin were confirmed to be present in the plants. It was found that the indigo from fresh leaves of *S. flaccidifolius* Nees. Fermented for three days gave the highest amount of indigo. Referring the theory of fermentation and the yield of indigo obtained, the best extraction method was the one with fresh leaves with water. In the case of dried

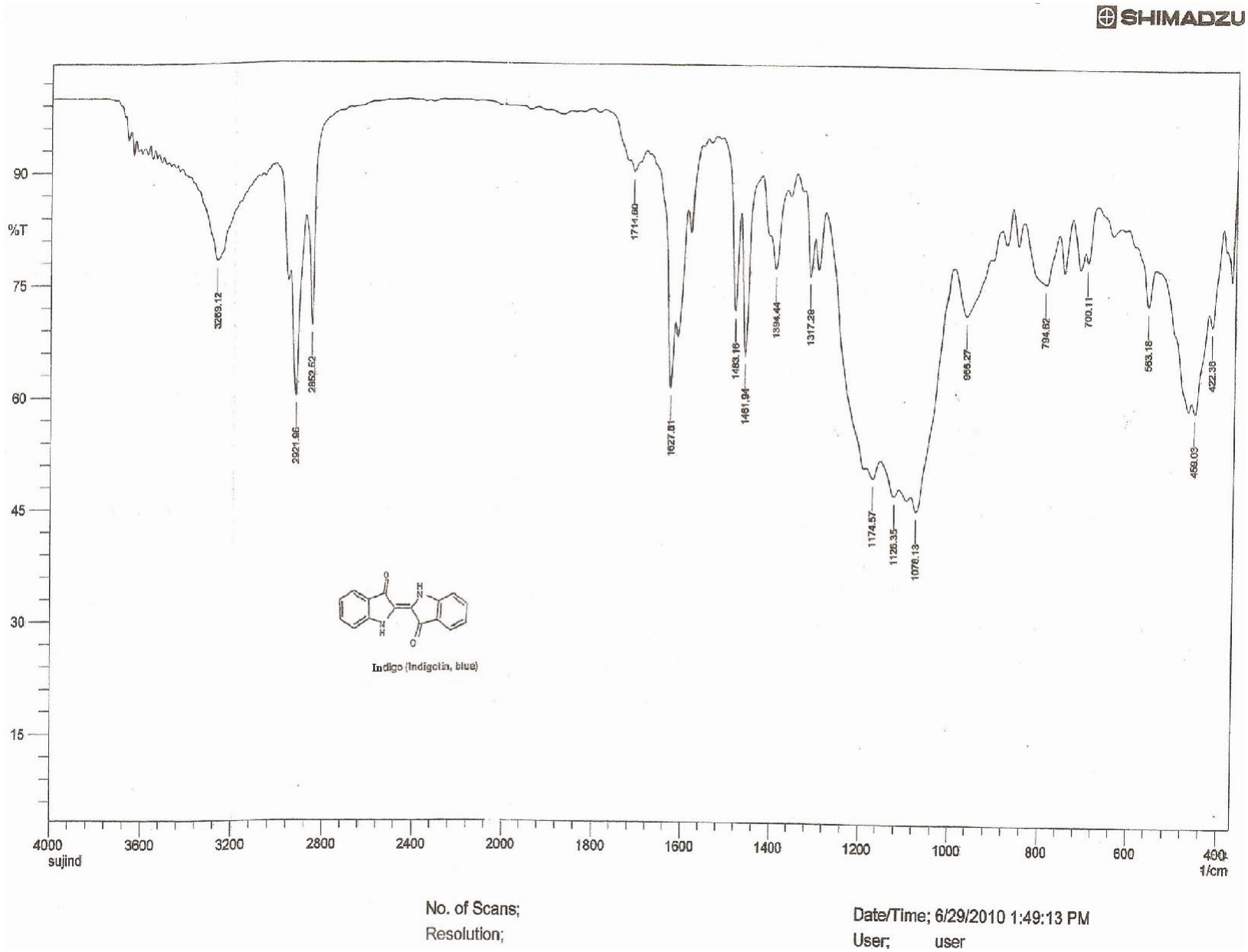


Figure 4. IR spectrum of isoindigo.

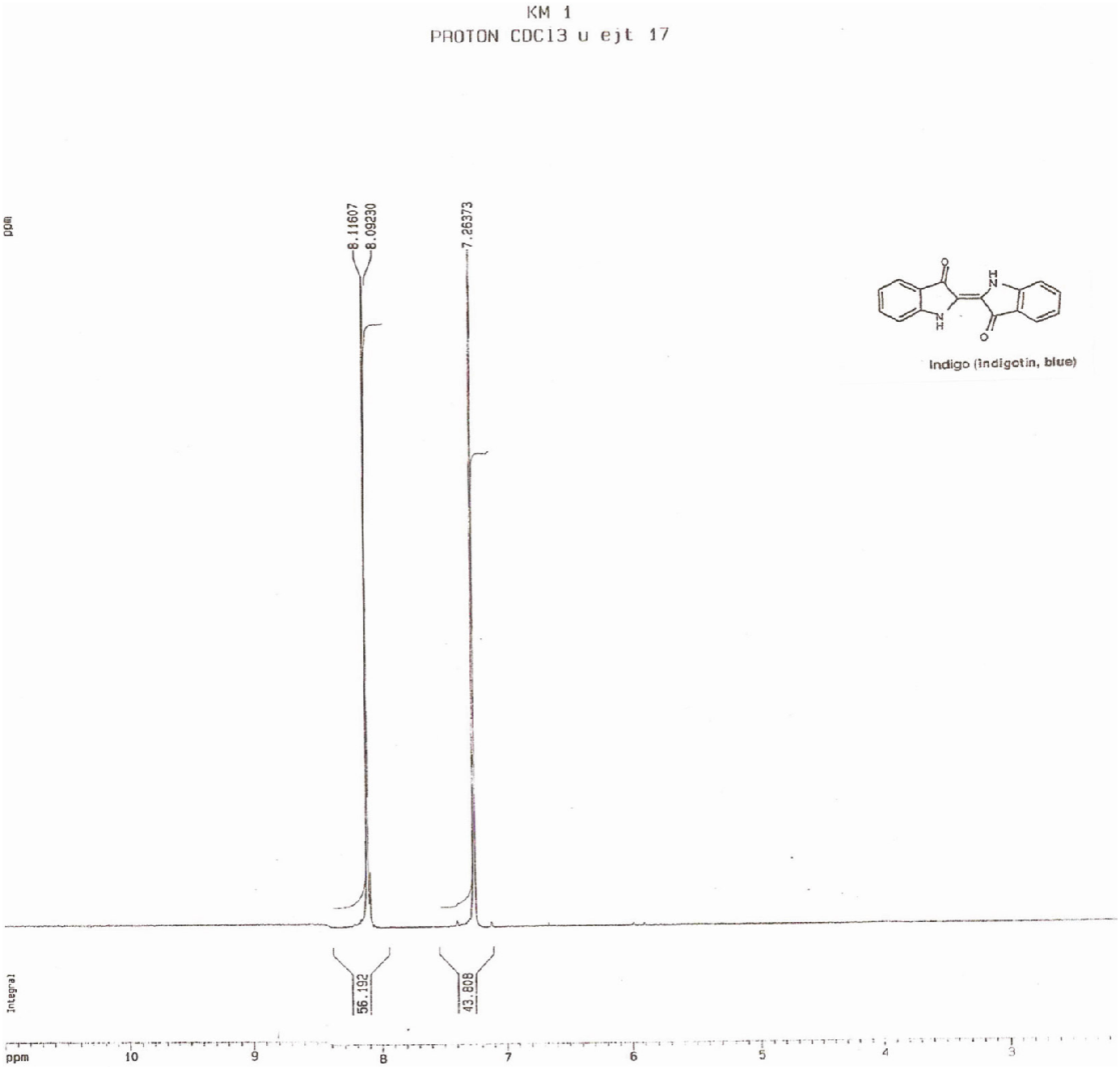


Figure 5. H^1 NMR spectrum of indigo.

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)	Color of fermented solution	pH	Color of paste
Fresh *S. flaccidifolius*	3	Yellow-green	4.8	Blue
	7	Green	4.8	Blue-green
Semi-dried *S. flaccidifolius*	3	Green-brown	5.0	Brown
	7	Brown	5.0	Brown
Dried *S. flaccidifolius*	3	Brown	6.0	Brown
Fresh *I. tinctoria* Linn.	3	Yellow-green	4.8	Blue

Table 2. Indigo dye yield from 72 h maceration of 100 g *S. flaccidifolius* Nees. and *I. tinctoria* Linn.

Type of plant	Paste (g)	Indigo dye (mg)
Fresh *S. flaccidifolius*	5.40	25.64
Semi-dried *S. flaccidifolius*	3.62	11.37
Dried *S. flaccidifolius*	1.86	7.89
Fresh *I. tinctoria*	6.83	27.33

Table 3. Indigo precursors and its Rf values.

Name of indigo precursors	Rf values
Indican	0.7
Isatan B	0.6
Isatin	0.2

leaves with water, the enzyme might have been deactivated in the drying process as there was no indigo obtained after solvent extraction with chloroform.

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REFERENCES

Maugard T, Estelle E, Patreick C, Marie DL (2001). Identification of indigo precursor from leaves of *Isatis tinctoria* (Woad). Phytochemistry, 58: 897-904.

Ensley BD, Ratzkin BJ, Osslund TD, Simon MJ, Wackett LP, Gibson DT (1983). Expression of naphthalene oxidation genes in *Escherichia coli* results in biosynthesis of indigo. Science, 222: 167-169.

Epstein E, Nabors MW, Stowe BB (1967). Origin of indigo of woad. Nature, 216: 547-549.

Perkin AG, Bloxam P (1907).Some constituents of natural indigo part . J. Chem. Soc. pp. 279-288.

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

Kun LWF (1998). Dyeing process with natural indigo: The Tradition and Technology. Revival Natural Indigo dye. Sept. pp. 20-29.

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

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

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

Phutrakul S, Lhieochaiphant S, Chanayath N (2002). Pigment extraction techniques from the leaves of *Indigofera tinctoria* Linn. and *Baphicacanthus cusia* Brem. and chemical structure analysis of their major components. CMU J., 1(2): 149-160.

Shin JH, Lee JH (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 ZQ, Zenk MH (1992). Biosynthesis of Indigo precursors in higher plants. Phytochemistry, 31(8): 2695-2697.