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Correlation of Morphological Characteristics with the Presence of Indicant in *Indigofera* sp. Dyestuff

(Korelasi Ciri Morfologi dengan Kehadiran Indikan dalam Bahan Pewarna *Indigofera* sp.)

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ABSTRACT

A total of 9 species of Indigofera *have been identified on the Islands of Java and Madura. Only one species has been utilized by batik makers and weavers as a natural dye, while the other 8 species are of unknown potential as natural dyes. This study seeks to demonstrate the correlation between morphological characteristics and the level and quality of indicant compound, in order to assist batik producers and weavers in determining the species that can be used as dyestuff. The potential blue colour dyestuff yield of* Indigofera *was determined by leaf immersion, followed by quantitative and qualitative examination of the indicant present. Morphological characteristics were then analysed to identify those that correlated with the presence of indicant. The indicant differences were analysed using SPSS; the relationship between the characteristics was analyzed by Pearson correlation and logistic regression. Four species, namely* I. tinctoria*,* I. arrecta*,* I. suffruticosa *and* I. longiracemosa *contained indigo. The colour produced by indigo dye obtained from these four species of* Indigofera *had a '4–5' value of colour change and colour staining in tests involving washing, sweat, bright light and heat stress. This value meets the 'good quality' standard of the Indonesian National Standards (SNI).* I. suffruticosa *has the highest indicant content at 1.4 g/kg, followed by* I. tinctoria*,* I. arrecta*, and* I. longiracemosa *with 0.414, 0.13, and 0.038 g/kg, respectively. The colour of upper and lower dried foliages were correlated to potential indigo dye producers.*

Keywords: Colouring; dried foliage; immersion

ABSTRAK

Sebanyak 9 spesies Indigofera *telah dikenal pasti di Pulau Jawa dan Madura. Hanya satu spesies digunakan oleh pembuat batik dan penenun sebagai pewarna semula jadi, manakala 8 spesies yang lain tidak diketahui potensinya sebagai pewarna semula jadi. Kajian ini bertujuan untuk menunjukkan korelasi antara ciri morfologi serta tahap dan kualiti sebatian indikan, untuk membantu pengeluar batik dan penenun dalam menentukan spesies yang boleh digunakan sebagai pewarna. Potensi pewarna warna biru hasil* Indigofera *ditentukan melalui rendaman daun, diikuti pemeriksaan kehadiran kuantitatif dan kualitatif indikan. Ciri morfologi kemudian dianalisis untuk mengenal pasti kaitan dengan kehadiran indikan. Perbezaan indikan seterusnya dianalisis dengan menggunakan SPSS; hubungan antara ciri dianalisis menggunakan korelasi Pearson dan regresi logistik. Empat spesies iaitu* I. tinctoria, I. arrecta, I. suffruticosa *dan* I. longiracemosa *mengandungi indigo. Warna yang dihasilkan oleh pewarna indigo diperoleh daripada empat spesies* Indigofera *ini mempunyai nilai '4-5' perubahan warna dan pencelupan warna dalam ujian yang melibatkan basuhan, peluh, cahaya terang dan tekanan matahari. Nilai ini memenuhi piawai kualiti baik Piawaian Nasional Indonesia (SNI).* I. suffruticosa *mempunyai nilai kandungan indikan tertinggi pada 1.4 g/kg, diikuti oleh* I. tinctoria, I. arrecta *dan* I. longiracemosa *dengan kandungan indikan masing-masing adalah 0,414, 0.13, dan 0,038 g/kg. Warna atas dan bawah dedaun kering dikaitkan dengan potensi pengeluar pewarna indigo.*

Kata kunci: Dedaun kering; pewarna; rendaman

INTRODUCTION

Indigofera, one genus of the Papilionoideae subfamily, is easily recognizable by its major characteristics: flowers in axillary racemes, clustered flowering, butterflied flowers, whole plant is covered in inbiramous trichomes and cylindrical legume types of fruit (Adema 2011). It is one of the most important genus in the natural world because it has so many purposes; inter alia, as adye (*I. tinctoria*), feed for cattle, deer and goats (*Indigo ferazollingeriana*), antioxidant (*I. tinctoria*), anticonvulsant (*I. suffruticosa*) and antimicrobial (*Indigofera glandulosa*) (Abdullah 2010; Anusuya & Manian 2013; De Almeida et al. 2013; Prabakaran et al. 2011; Schrire et al. 2009).

Indigofera shows great diversity throughout the world, having 700-750 species (Adema 2011; Schrire et al. 2009). DeKort and Thijsse (1984) reported that 39 species were found in Southeast Asia and India, and 18 species were identified in Java (Backer & Backhuizen van den Brink 1963). However, a much more recent study found only nine species of *Indigofera* in Java (Muzzazinah et al. 2013). One

species, *I. tinctoria,* has been used as a dye since 352-395 AD, while eight other species are unknown in terms of their potential as colour producers (Van Rijckkevorsel 1925). The blue colour is produced by the indicant compound contained in the leaves. Indican compound can be used as a dye after the processes of hydrolysis and oxygenation, which produce the indigo compound. The pasta form of indigo can be used as dyestuff.

Batik craftsmen and the weaving community in Indonesia are very familiar with *I. tinctoria*. They know *Indigofera* by the popular name *tom*, as a plant that produces a blue colour. Traditionally batik craftsmen use to leaves to make a blue dye in the form of a paste which is used for '*medel'* (a process of dipping batik fabric into coloured liquid repeatedly, in order to achieve the desired colour) and '*mbironi'* (covering the blue colour and filling up the dotted pattern with wax): Two procedures in the dyeing process of batik.

Indigofera diversity in Java is currently only 50%. The apparent loss of nine species of *Indigofera* from the natural habitat may be due to their not being utilized because of their unknown potential. Utilization by some communities is still limited to certain species, such as *I. tinctoria* for batik and weaving dyes and *I. zollingeriana* for substitution of cattle feed (Abdullah 2010; Schrire et al. 2009). The seven other species have not been studied. Continued exploration and conservation is needed to ensure the preservation of each species. One step in preserving the species *Indigofera* is to determine its potential as a natural dye.

So far, the morphological characteristics that can be used to determine the indicant content in the leaves are unknown. Previous studies on morphological, geographical and molecular characteristics have not been able to identify the colour-producing *Indigofera* species clearly (Schrire et al. 2009; Wilson & Rowe 2008).

Batik craftsmen and weavers in Indonesia do not have botanical knowledge about the colour-producing *Indigofera* species. In determining the *Indigofera* characteristics that can be used as a dye, it is necessary to introduce the characteristics that can be easily used to recognize *Indigofera* containing indicant as the base material of blue dye. Identification of morphological characteristics and variations of *Indigofera* must become the basic knowledge for *Indigofera* users; especially batik craftsmen, weavers and breeders. The morphological characteristics are thus important for the development of species for use by batik craftsmen and weavers in Indonesia. This study presents morphological characteristics, correlated with the content of indicant compound, in order to facilitate users' identification of the species that can be used as dyestuff. It was intended to determine the potential blue colour of *Indigofera* as dyestuff by leaf immersion, examined the quantitative indicant and to analyze the morphological characteristics that are correlated to the presence of indicant.

Materials and Methods

Plant material

Plant samples were collected from 37 locations on the islands of Java and Madura. Leaf samples of nine species of *Indigofera*, originating from Java and Madura, were used to test their dye-producing potential. Herbarium specimens from 124 individuals collected from 37 locations and representing 9 species were used to collect the morphological data (Figure 1).

Making of indigo paste

Leaf samples were taken by cutting 1-3 kg of fresh leaves from each species. A leaf-immersion method was used to determine the ability of plants to produce indigo paste. There were several steps in making indigo paste: up to 3 kg of leaves and twigs were chopped up and soaked in 5 L of water for 10-24 h of incubation; after a change of colour has been observed, the leaves and twigs were removed by filtering the water and then 1 L of water was added with 30 g of dissolved lime (CaO) and stirred vertically to get aeration; the solution was then incubated for a further 24 h and the clear liquid at the surface was disposed of to leave the sediment or paste. The blue paste, now called indigo paste, was dried to reduce the water content. This paste was made twice for each species. The indicators of indigo presence were the colour change of the immersion water to dark green, froth and pungent smells. The soaking time until the colour change and the presence of froth was recorded.

Quality test of dye on fabric

The quality test of indigo dye on fabric began by colouring some cloth. This was done by mixing 200 g of indigo paste with 125 g of cassava, 200 mL of molasses and 250 mL of lime water in 5 L of water. The mixture was stirred and incubated for 24 h. The cloth to be dyed was dipped in detergent solution to remove dirt and grease. It was then immersed in the indigo solution evenly for 5 min and then removed, rinsed in clean water and dried for 10-15 min. The last steps (dipping, rinsing and drying) were repeated 20 times. After getting the colour on to/into the fabric, this was followed by soaking the fabric in an acidic solution to 'neutralize' it. In this step, 10 mL of acetic acid, dissolved in 20 L of water was used as the 'cleaning' agent. The cloth was soaked for 30 min and then dried. The quality value of the colour on the fabric was measured by the standard colour test (Kornerup & Wanscher 1967). The quality testing of indigo dye includes tests of colour fastness against: washing at 40°C, acid and alkaline sweat exposure, day light exposure and heat stress, based on methods detailed in SNI ISO 105 C06: 2010; SNI ISO 105 – E04: 2010; SNI ISO 105 – B01: 2010, and of SNI ISO 10S – X11: 2010, respectively (BBKB 2013). Then, colour change and colour staining were evaluated by comparing each sample with a standard gray scale and staining scale (Mark 1-5: 1= poor, 2=weak, 3=average, 4 and 4-5= good, 5=excellent).

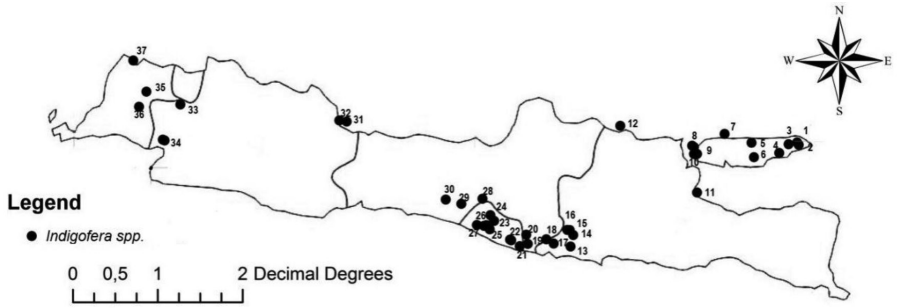

FIGURE 1. Map showing *Indigofera* sample collection sites: 1=Pakhandangan, 2=Gapura Timur, 3= Andulang, 4=Sumenep, 5= Tlanakan, 6= Jenma, 7=Jukporong, 8=Jl HPK, 9=Burneh, 10=Kalisoga, 11= Bangil, 12=Tuban, 13=Pantai Sugih, 14 =Taman, 15=JLS, 16= Wiyoro 17 =Krajan, 18= Kendal, 19= Pracimantoro, 20 = Sadeng Beach, 21= Slili Beach, 22= Krakal Beach, 23==Giriloyo, 24= Sleman, 25= Bambanglipuro, 26= Banaran, 27= Trisik Beach, 28= Purworejo, 29= Kebumen, 30= Temanggung, 31=Babatan, 32=Kejawanan, 33= IPB, 34= Cikaka, 35= Sajira, 36 =Cisimut, 37=Karangantu

The colour fastness tests were conducted in the Testing Laboratory of the Great Hall of Crafts and Batik (BBKB), Yogyakarta, Indonesia.

DETERMINATION OF INDICANT BY HPLC-DAD

Approximately 0.5 g of dry leaves were put in a glass tube containing 2 mL of H_2O/CH_3CN (75%/25%); the tube was then covered and heated at 90°C in a water bath for 2 min. The leaf material was removed and the remaining mixture was cooled to 25°C and centrifugated for 10 min at 6.000 rpm. The supernatant was put in a microtube and centrifuged for 10 min at 13.000 rpm. Then, 200 µL of supernatant was transferred to HPLC vial and 10 µL of supernatant was injected into an HPLC-DAD for identification and quantitative analysis (as suggested in Gilbert et al. (2004). Analysis of indicant was performed using an Alliance HPLC 2695 (Waters) equipped with photodiode array detector 2996 (Waters). Material separation was done on a column Symmetry C18 5 µm, 150×4.6 mm (Waters).

ANALYSIS OF CORRELATION BETWEEN MORPHOLOGICAL CHARACTERS AND INDICANT CONTENT

The samples for morphological observation were conducted exploratively. At each location, plant parts from between three and five individuals were collected. From each individual its stem, leaves, flowers and healthy fruit were taken for preparing herbarium specimens (as suggested in Rugayah 2004). The collection was stored in the Department of Biology, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University and the Biology Education Study Program of Sebelas Maret University, Surakarta.

Morphological observations were carried out in respect of 10 morphological characteristics: habitus, shoot colour, petiole length, leaf shape, fresh-leaf colour, colours of the lower and upper surface of dry leaves, length of flower bunches, pod shape and colour of flowers. For colour standardization, a comparison was made using the standard colour test (Kornerup & Wanscher 1967). Pearson correlation and logistic regression (Minitab 15) analyses were used to determine the characteristics that were positively correlated with the presence of indicant (as suggested by Bryman & Cramer 1996).

RESULTS AND DISCUSSION

INDIGOFERA POTENTIAL AS DYESTUFF

The soaking treatment of fresh leaves from nine species of *Indigofera* showed for four species, namely *I. arrecta*, *I. longiracemosa*, *I. suffruticosa* and *I. tinctoria*, the soaking water turned dark green, frothy and strong-smelling; and it turned blue after the addition of lime (Table 1). These four characteristics were an early indicator of the formation of indigo (indoxyl β-D-glucoside). Indicant is one form of secondary metabolite that serves as a precursor of indigo. Indicant degraded by the enzyme β-glucosidase will become indoxyl, which experiences dimerization and forms indigo. By contrast, the soaking water from the leaf immersion of five other species, namely *I. zollingeriana, I. trifoliata, I. galegoides, I. hirsuta* and *I. linifolia,* remained clear, with no foam or sediment.

The colour change of the soaking water of the *Indigofera* leaves was affected by the β-glucosidase enzyme, changing β-D-glucoside into indoxyl. The activity of the β-glucosidase enzyme was affected by the ambient (room) temperature and the temperature of the soaking water. This experiment used water and an environmental room temperature which were both at 29°C. Several methods have been used for extraction of indicant from other species (*Isatis tinctoria* and *Isatis indigotica*) including: fermentation, steeping leaves in water in round tanks for a certain period of time and steeping leaves in hot water (80-90°C) (Beijerinck 1899; Fortune 1846; Stanfield 1971). Treatment by soaking the leaves in hot water was intended to remove the wax coating on the leaves' surface, to accelerate the process of leaf tissue lysis (Rawson 1899) and to stimulate the release of isatan B and indicant contained in the leaves (Stoker & Cooke 2001).

TABLE 1. Indicators of indican presence in nine species of *Indigofera*

Species names	Colour of soaking water	Froth	Colour after addition lime water	Sharp smell	Average time of leaf soaking water to become dark green	Sediment after 24 h of incubation
I. arrecta	Dark green	Yes	blue	Yes	12.6	Blue sediment
I. galegoides	clear	No	Clear	No	*	No
I. hirsuta	clear	No	Clear	No	*	No
I. linifolia	clear	No	Clear	No	*	No
I. longiracemosa	Dark green	Yes	Biru	Yes	20.2	Blue sediment
I. suffruticosa	Dark green	Yes	Biru	Yes	16.6	Blue sediment
I. tinctoria	Dark green	Yes	Biru	Yes	15	Blue sediment
I. trifoliata	clear	No	Clear	No	*	No
I. zollingeriana	clear	No	Clear	No	*	No

*Colour of soaking water remains clear for 24 h after immersion; average length time of immersion could not be recorded due to no changes (within the observation period)

The addition of active lime to the soaked leaves changed it to an alkaline condition, in which indicant actively degraded into indoxil and glucose. Treatment in alkaline conditions to produce indigo has previously been used on *Isatis tinctoria* in China (Fortune 1846). However, the treatment does not appear efficacious with all species of plants; for example, in the UK, attempts at making indigo paste from *Polygonum tinctorium* was not successful even though it was already in an alkaline condition (Hill 1992; Vourema 2008).

Indigo compounds derived from indicant were separated from water and become in the form of blue sediment, a refined extract that was ready to be used as cloth dye. Indigo paste which has been drained can be used as a fabric dye. Such indigo paste is insoluble in water, but soluble in alkaline solvents as lime water and lasses.

Among four colour-producing *Indigofera*, the most efficient species, when using the process of leaf soaking, was *I. arrecta*. It took 12.6 h for the leaves to change the colour of the soaking water. In contrast, *I. longiracemosa* needed 20.2 h. From these experiments, it was further found that the faster the process of leaf soaking, the more efficient was the fabric dyeing, because the lysis process of leaf tissue occurred more quickly, resulting in a faster release of the collected indicant in the vacuoles. Treatment to accelerate the leaf lysis had been done by Fortune (1846) in a different way: by soaking the leaves of the indigo-producing *Polygonum tinctorium* from subtropical regions (Europe) in water with a temperature of 80-90°C for 30 min.

COLOUR VALUE

Five species of *Indigofera*, namely *I. galegoides, I. hirsuta, I. trifoliata, I. zollingeriana* and *I. linifolia*, did not produce indigo paste; consequently, neither did the colour test on the cloth-through-water-immersion test.

Each species produced different degrees or shades of blue colours (Figure 2). Colouring with *I. tinctoria* paste produced a grayish blue (21E6, 22E6) to dark blue (22F5, 22F7, 22F8); colouring with *I. suffruticosa* paste produced a faded blue (22E5) to grayish blue (21E6, 22E6); colouring with *I. longiracemosa* resulted in a grayish blue (22E6); and colouring with *I. arrecta* resulted in a faded blue colour (22E5).

Colouring with indigo paste of cotton fabric is done by immersion. Dyeing with natural indigo dye does not require fixation by immersion, but it needs oxidation during the process of dyeing and drying. When the cloth is dry, a blue colour will appear. Cloth dyeing should be perfect if it achieved a balance between the fabric and dye, which

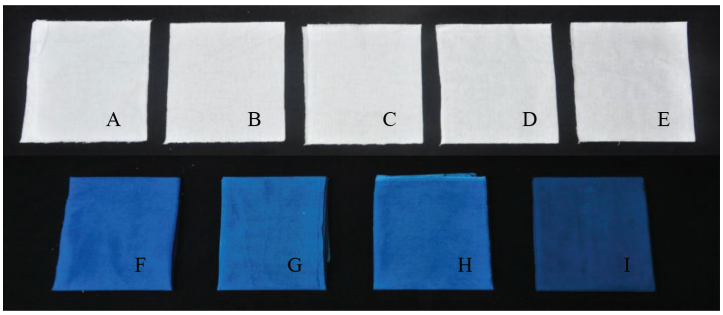

FIGURE 2. Colour of cloth after soaking in indigo from *Indigofera*. A-E. Cloth still white colour after staining with *I. zollingeriana*, *I. trifoliata, I. galegoides, I. hirsuta* dan *I. linifolia*. F. Faded blue colour (22E5) produced by *I. arrecta*, G. Greyish blue colour (22E6) by *I. longiracemosa*, H. Greyish blue (21E6, 22E6) to dark blue (22F5, 22F7, 22F8) by *I. suffruticosa* and I. Dark blue (22F5, 22F7, 22F8) produced by *I. tinctoria*

means that the dye absorbed into the fabric has reached the maximum point. A colour balance depends on the temperature of the soaking liquid, the dyeing treatment, the type of material, the solution concentration, the dye affinity and the solution's pH (Susanto 1980).

QUALITY OF INDIGO DYESTUFF

The fastness test of colour against washing at 40°C for the four species resulted in an average value of 4–5 (good). *I. arrecta* and *I. longiracemosa* produced a 'colour changed' value of 4 (good), while *I. suffruticosa* and *I. tinctoria* both gave a value of 4–5 (Table 2). These values meet the colour quality requirement set by SNI; a minimum requirement of 3–4 (moderate - good). This suggests that the bond between indigo dye and the fabric has reached the maximum balance, preventing the colour from fading. The colour 'fastness test against washing at 40°C', produced an average 'fading value' of 4–5. Four species of *Indigofera* had good fading values, with no dehydrated pigment resulting from colour staining by washing at this temperature.

The values of colour change and stain in the 'fastness test against acid and alkaline sweating' resulted in an average value of 4-5 (Table 2). Four species of *Indigofera* have a high fading resistance to acid and alkaline sweating. The value of 4-5 already meets the requirement of dye quality, which is 'at least 3' ('moderate') as set up by the SNI. At this higher value, the indigo compound has permeated into the fabric fibers, so it cannot be removed by acid or alkaline solutions.

The discolouring values in the 'fastness test of colour against light brightness' for the four species of *Indigofera* resulted in an average value of 4 ('good') (Table 2), which meets the requirement of dye quality established by the SNI. At this value, the bond between indigo dye from *Indigofera* and the fabric fibers was strong and stable, such that the molecular chains were not broken when the fabric was exposed to ultraviolet rays from the sun. The stability of the molecular chain of indigo dye in the fiber could be caused by the indigo characteristic of not requiring afixation step in colouring process; instead, the presence of oxygen makes indigotin hold tightly to the fabric and makes this fabrichighly resistant to discolouring by sunlight. Light fastness was also influenced by various other factors, such as the chemical and physical properties of dye, the concentration of dye and the chemical structure (Cristea & Vilarem 2006; Padfield & Landi 1996; Samanta & Agarwal 2009).

The discolouring test against heat stress was conducted to determine the material's resistance to physical stress. Under such pressures, the fabric should exhibit good resistance; i.e. it should not easily get stained or discoloured. The discolouring values on the fastness test against heat for the four species of *Indigofera* were on average 4-5 ('good'). Similarly, the discolouring values when using either wet or dry cotton were on average of 4-5 (Tables 2 & 3). At these values, the bond between indigotin and fabric fibers has reached the maximum balance so that when the fiber was exposed to high heat in both wet and dry conditions, it did not become dehydrated. The discolouring values resulting from staining on both wet and dry cotton, for the four species of *Indigofera* comply with the requirement set up by the SNI.

INDICANT CONTENT OF THE FOUR INDIGOFERA SPECIES

Quantitative analysis of nine *Indigofera* species showed that only four species produced indicant. The indicant content of *I. suffruticosa, I. tinctoria, I. arrecta* and *I. longiracemosa* were 1.417, 0.491, 0.130 & 0.038 g/kg, respectively. This result supported earlier observations by John and Angelini (2009). Species *I. Suffruticosa* has the highest indicant content, while *I. longiracemosa* has the lowest (Figure 3). Previous research by John and Angelini (2009) reported that *I. tinctoria* growing in India had indican content varying from 0.2 to 0.7% of the dry leaf weight. The indigo content of *I. suffruticosa* in our study

TABLE 2. Values of discolouring and staining on the colour fastness test against washing at 40°C and sweat for four species of *Indigofera* from the Islands of Java and Madura

Fastness	Washing at 40° C				Acid sweat				Alkaline sweat			
Species name[a]	A	B	C	D	A	B	C	D	A	B	C	D
Colour change	4[b]	4	4–5	4–5	4–5	4–5	4–5	4–5	4–5	4–5	4–5	4–5
Colour staining:												
Acetate	4	4–5	4–5	4	4–5	4–5	4–5	4–5	4–5	4–5	4–5	4–5
Cotton	4–5	4–5	4–5	4–5	4–5	4–5	4–5	4–5	4–5	4–5	4–5	4–5
Polyamide	4	4	4–5	4	4–5	4–5	4–5	4–5	4–5	4–5	4–5	4–5
Polyester	4–5	4–5	4–5	4–5	4–5	4–5	4–5	4–5	4–5	4–5	4–5	4–5
Acrylic	4–5	4–5	4–5	4–5	4–5	4–5	4–5	4–5	4–5	4–5	4–5	4–5
Wool	4–5	4–5	4–5	4–5	4–5	4–5	4–5	4–5	4–5	4–5	4–5	4–5

[a]A=*I. arrecta*, B=*I.longiracemosa*, C=*I. suffruticosa*, D=*I. tinctoria*. [b]4=good 4–5=good 5=excellent

TABLE 3. Values of discolouring and staining on the colour fastness test against heat stress and daylight for four species of *Indigofera* from the Islands of Java and Madura

Fastness	Heat stress				Day light			
Species name[a]	1	2	3	4	1	2	3	4
Colour change	4–5[b]	4–5	4–5	4–5	4	4	4	4
Colour staining:								
Dry cotton	4–5	4–5	4–5	4–5				
Wet cotton	4–5	4–5	4–5	4–5				

[a]A=*I. arrecta*, B=*I.longiracemosa*, C=*I. suffruticosa*, D=*I. tinctoria*. [b]4=good 4–5=good 5=excellent

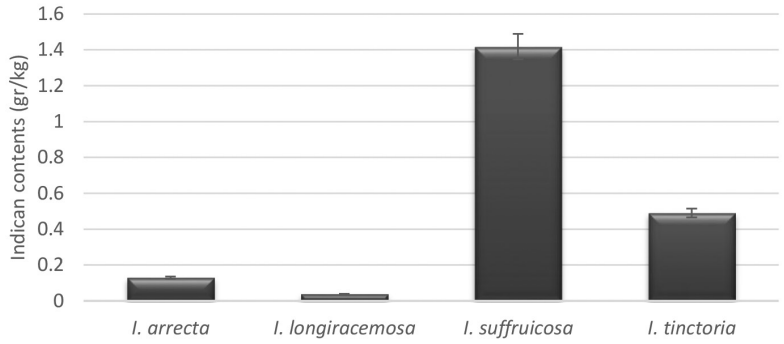

FIGURE 3. Indican levels in the leaves of four species of *Indigofera* . The content levels of indican were calculated for dried plant material obtainedfrom many locations on Java and Madura islands, Indonesia

was 1% of the dry leaf weight. The plant's origin, time to harvest, age of the plant, (high) temperature, light intensity, (in adequate) rainfall - all had significant effects on leaf yield and indigo amount (John & Angelini 2009; Lu 1986; Sales et al. 2006; Stoker et al. 1998).

MORPHOLOGICAL CHARACTERISTICS INDICATING INDIGOFERA SPECIES AS POTENTIAL COLOUR PRODUCER

Of the 10 morphological characteristics tested, two were found to be highly positively correlated with the presence of indigo: the colour of the upper surface of dry leaves and the colour of the lower surface of dry leaves. Of the other characteristics examined, habitus, petiole length, flower colour and racemus length were highly negatively correlated; while shoot colour, fresh leaf colour and pod shape showed no correlation with the presence of indigo. The colour of the upper surface of dry leaves had a positive correlation with the presence of indigo in leaves with a coefficient of 0.913. This means that the darker the dry leaf colour of *Indigofera*, the higher the indican content. Direct observation of the colours of the upper and lower surfaces of dry leaves found some colour variation between the nine species. Four species had grayish to dark gray leaves, while the other five species did not produce colour (Figure 4). The colour of the upper surface of *I. arrecta* dry leaves varies from greenish gray (27D2), through grayish green (29D5, 30E6) to grayish colour (27D5). *I. longiracemosa* has a specific gray colour (29F1) on the upper surface of its dry leaves. The upper surface of the dry leaves on *I. suffruticosa* is dark green (25F3, 29F5, 29F3), whereas that of *I. tinctoria* is greenish gray (26E2, 30F2). Thus, colours ranging from grayish to dark gray, were indicators of indican presence in the dry leaves of *Indigofera* species exhibiting those colour characteristics.

The colour of the lower surface of dry leaves also has a high correlation with the presence of indigo in leaves, giving a coefficient of 0.77. The colour of the lower surface of dry leaves was less varied than that of the upper surface of dry leaves. The colour of the lower surface of dry leaves on *I. arrecta* originating from Yogyakarta was dark green to faded green (30D8-30E3), whereas *I. arrecta* from Temanggung showed a variety of leaf colours, from dark green (30D8), through faded green (30E3) to grayish green (29C4,29D4, 29D5) and greenish gray (29F1, 29F2, 30F2). Whilst, the lower surface of dry leaves on the three other species, *I. suffruticosa, I. tinctoria* and *I. longiracemosa,* was greenish gray (29F1, 29F2, 30F2).

Logistic regression analysis of the colour of the upper and lower surfaces of dry leaves against the presence of indican, showed a goodness of fit test result of $p > 0.5$. This result suggested that the derived model can adequately explain the data (Figure 5(a) and 5(b)). Measurement of the association between the response variable and predicted probabilities of indican content, showed 99.5% for the upper surface colour of dry leaves and 88.5% for the lower surface colour. It was concluded therefore, that the colour of upper and lower surfaces of dry leaves in *Indigofera* spp. can be used to predict the presence of indican in leaves.

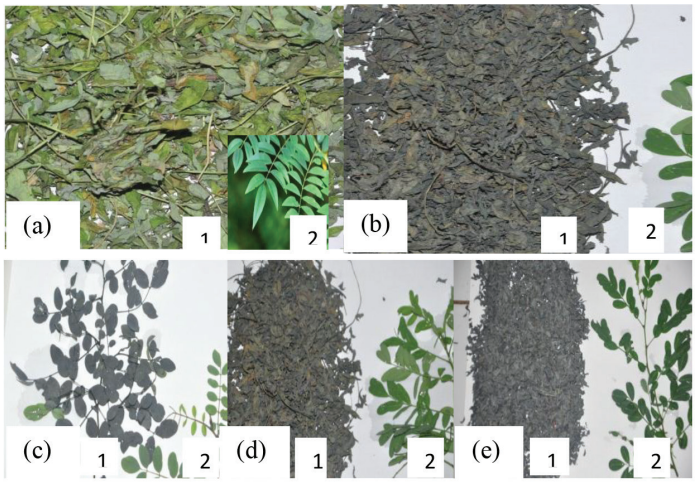

FIGURE 4. The colour of dried leaf indicateswhether they contain indican (B, C, D, E), or not (A). A=*I. zollingeriana,* B=*I. arrecta*, C=*I. longiracemosa*, D=*I. suffruticosa*, and E=*I. tinctoria*. 1=Dried leaf, 2=Fresh leaf

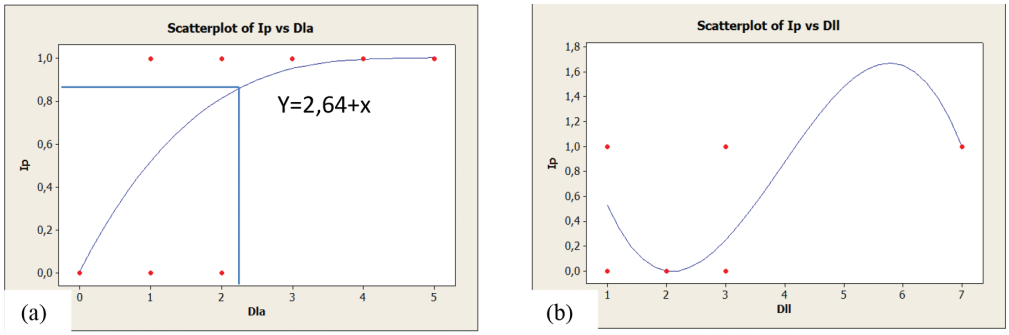

FIGURE 5. Morphological correlation with the indigo content. A. The correlation of upper surface of dry leaf colour with presence of indican, B. The correlation of lower surface of dry leaf colour with presence of indican

The colour characteristics of the upper and lower surfaces of dry leaves has never previously been used as indicators for identifying *Indigofera* in Asia or Australia (Wilson & Rowe 2008). However, this study found that these two newly-highlighted characteristics are important as indicators of the presence of indican in *Indigofera* species.

CONCLUSION

Four species of *Indigofera* from the islands of Java and Madura, namely *I. arrecta, I. longiracemosa, I. suffruticosa* and *I. tinctoria* can be used as natural blue dye stuff plants and produce good colour quality. The colour fastness against washing, sweat, light brightness and heat suppression of the product from the four species was classified overall as good (4-5) and met colour fastness requirements according to ISO standards. The indican content levels in each of the four species varied. The indican content levels in *I. suffruticosa*, *I. tinctoria*, *I. arrecta* and *I. longiracemosa,* were 1.417, 0.491, 0.130 and 0.038 g/kg, respectively. The four species were similar in respect of colours of the upper and lower surfaces of dry leaves. The colour of upper and lower dried foliages can therefore be used as an identifying characteristic of colour-producing species.

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