RESEARCH ARTICLE

Evaluation of the physical characteristics of *Beta vulgaris* and *Intsia bijuga* aqueous and methanolic extracts in comparison to haematoxylin stain

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Abstract:

The haematoxylin stain is universally recognised as pathology's gold-standard nuclear staining. This study evaluated and compared the pH value, and colour concentration of *Intsia bijuga* and *Beta vulgaris* extracts to that of haematoxylin. After lyophilisation, the plant material was extracted using methanol and water. Analyses were performed on the extracts' physical colour, pH, and colour concentration, and the results were compared to haematoxylin. A pH meter was used to obtain the pH value and spectrophotometric absorbance readings were taken to determine the colour concentration. The pH range for extracts of *B. vulgaris* was between 4.18 and 5.80, whereas the pH range for extracts of *I. bijuga* was between 4.46 and 4.65. There are statistically significant discrepancies ($p < 0.001$) in the pH and colour concentration analyses, as shown by the colour concentration statistical analysis. The colour properties of *I. bijuga* and *B. vulgaris* were also discussed to further enrich the understanding of their physical characteristics. In conclusion, the pH levels of the extracts were greater than those of haematoxylin. Regarding the colour concentration, the methanolic extract of *B. vulgaris* had a lower concentration than haematoxylin, while the other extracts had more significant concentrations than haematoxylin. In conclusion, the methanolic extract of *I. bijuga* shows the most significant similarity to haematoxylin in terms of its physical qualities.

Keywords: Aqueous extract, methanol extract, *Beta vulgaris*, *Intsia bijuga*, haematoxylin

1. INTRODUCTION

Haematoxylin is one of the most excellent dyes ever created. For decades, the dye has been the gold standard for nuclei stain in pathology staining, alongside eosin in the Papanicolaou smear protocol. Due to its many advantages over any other stain, haematoxylin stains were still the primary nuclei stain. Many studies were done to find the ideal substitute for haematoxylin stain because, in the past, there have been several cases of haematoxylin shortage recorded (Dapson et al., 2010). The last case of haematoxylin shortage was reported by a few laboratories in 2008. As a result, the vendors had to offer substitutes for the nuclear stain as the availability of the stain was unpredictable. Moreover, along with its escalating price, there are also concerns that the shortage might be happening again, and there is also no way to tell how long haematoxylin production may last (Groover et al., 2009).

I. bijuga (Colebr.) Kuntze from the family *Fabaceae*, also known as the Merbau tree, is a high commercial value tree in Southeast Asia, including Malaysia. It is mainly used as materials for construction and furniture. The local communities also use Merbau for medicinal purposes to combat diseases due to its anti-microbial, anti-viral, and anti-cancer properties (Angio et al., 2022; Bradacs, 2008; Widodo et al., 2019). However, in recent years, fabric dyes have been developed from this tree. Meanwhile, *B. vulgaris L.* from the family *Amranthaceae*, also commonly known as beetroot, is a vegetable with red tapered fleshy roots. The plant is usually consumed as food and as health remedies due to its high content of fibre, nutrients, and minerals such as potassium, sodium, nitrate, vitamin C, vitamin A, and vitamin K. Research has proven that the content in beetroot possesses antioxidant and antiradical properties. It has also been shown that beetroots are helpful as natural colourant due to their high content of betalain (Alim-un-Nisa et al., 2021; Goldman & Janick, 2021; S. W. Sari et al., 2021).

Therefore, the aim of this study is to compare the physical characteristics of *I. bijuga* and *B. vulgaris* with haematoxylin as they have the potential to be developed as alternative natural nuclear staining reagent. In addition, to further enrich the understanding of the *B. vulgaris* and *I. bijuga* potential as haematoxylin alternatives, the colour properties of these two plants were discussed.

2. MATERIALS AND METHODS

2.1. Extractions

Two kg of *B. vulgaris* were peeled off. Then both peel and flesh were cut and weighed. The plants were frozen overnight under -86 ºC in an ultra-low freezer before being lyophilised using a freeze dryer (SCANVAC) for three days. The small pieces of beet peel and flesh of *B. vulgaris* (Figure 1) were then weighed again and ground into powder. *I. bijuga* were also cut into smaller pieces and ground into powder as shown in Figure 2. After that, all the powders were soaked in distilled water and methanol for aqueous and methanolic extraction, respectively, for 24 hours with stirring. The extracts were then filtered using Whatman's filter paper, and the residues from the filtration were soaked again. This process was repeated until the filtrate produced was clear. The filtrates were then concentrated with a rotary evaporator. The extracts were placed in containers and labelled as demonstrated in Table 1. The extracts were all then stored at 4ºC.

Figure 1: Small pieces of beet peel and flesh of *B. vulgaris*.

Figure 2: Ground powder of *I. bijuga*.

Table 1: Label of each plant extract.

Label	Extracts
Aqueous Extract 1 (AE1)	Aqueous extract of *B. vulgaris* peel
Aqueous Extract 2 (AE2)	Aqueous extract of *B. vulgaris* flesh
Aqueous Extract 3 (AE3)	Aqueous extract of *I. bijuga*
Methanol Extract 4 (ME1)	Methanol extract of *B. vulgaris* peel
Methanol Extract 5 (ME2)	Methanol extract of *B. vulgaris* flesh
Methanol Extract 6 (ME3)	Methanol extract of *I. bijuga*

2.2. pH and colour concentration measurement

In this research, the pH and colour concentrations of the extracts were analysed and compared with that of haematoxylin. The pH of the extracts and haematoxylin was measured using a pH meter. To determine the colour concentration, the absorbance of the extracts was measured at a specific wavelength of 630nm using PG Instruments T80+ UV/VIS Spectrophotometer. All results were then statistically analysed using Statistical Package for Social Sciences (SPSS) Software by one-way analysis of variance (ANOVA) and t-test.

3. RESULTS AND DISCUSSION

3.1. Extractions

In this study, the beetroots were lyophilised to prevent the degradation of the compounds present in the samples. This process allows moisture to be removed without destroying the compound in the samples, thus, increasing its shelf life. About 80 to 90% of the weight was lost after lyophilisation due to moisture loss. After the samples were soaked in the solvent and filtered, the extracts produced were concentrated using a rotary evaporator under vacuum condition. This approach enabled easy temperature regulation and reduced the risk of thermal degradation of the compounds in the sample. Additionally, this extraction method offered faster results compared to the boiling method.

3.2. pH Measurements

Table 2 shows the pH reading of the extracts and haematoxylin. All the extracts and haematoxylin exhibited acidic pH levels. Haematoxylin has the lowest pH at 2.55, while the aqueous extract of *B. vulgaris* peel has the highest pH at 5.94. All extracts showed higher pH values than haematoxylin. The second highest was *B. vulgaris* flesh methanolic extract, with an average value of 5.79. Meanwhile, *I. bijuga* methanolic extracts have the closest pH to haematoxylin, with an average value of 3.47. The methanolic extracts of *B. vulgaris* showed pH ranging from 4.18 to 4.56 and 5.77 to 5.95 for aqueous extract, as shown in Table 2. In

a previous study by Kale et al. (2018), beetroot juice showed a slightly higher pH at 6.5, while Madushan et al. (2021) reported a much higher pH of their extract which was at 7.5. Farghaly et al. (2019) stated that pH significantly influences the stability of betalain, one of the important pigments found in beetroot, with its optimal pH falling from 4 to 5. On the other hand, *I. bijuga* has pH values between 3.4 to 4.6. This result was slightly lower than the pH value reported by Malik et al. (2016), which ranges between 5 to 6.

Table 2: pH readings of haematoxylin and extracts.

Extracts	1st reading	2nd reading	3rd reading	Average
HX	2.55	2.57	2.53	2.55
ME1	4.18	4.20	4.20	4.29
ME2	4.54	4.54	4.56	4.55
ME3	3.46	3.48	3.46	3.47
AE1	5.94	5.92	5.95	5.94
AE2	5.80	5.77	5.80	5.79
AE3	4.63	4.60	4.65	4.63

*HX= Haematoxylin, ME1= *B. vulgaris* peel methanolic extract, ME2= *B. vulgaris* flesh methanolic extract, ME3=*I. bijuga* methanolic extract, AE1= *B. vulgaris* peel aqueous extract, AE2= *B. vulgaris* flesh aqueous extract, AE3=*I. bijuga* aqueous extract

3.3. Physical Colour and Colour Concentrations

Figure 3 shows the colour of the methanolic extracts. The physical colour of both peel and flesh *B. vulgaris* methanolic extracts seems to be retained, which is bright red, similar to the colour of the vegetable before its extractions. Meanwhile, as shown in Figure 4, the colour of the aqueous extraction of *B. vulgaris* was brown, probably due to oxidation and degradation of the compound in the extracts. On the other hand, the extraction of *I. bijuga* in both aqueous and methanol produced dark brownish colour extracts. None of the extracts resembles the physical colour of haematoxylin as seen in Figure 5. The methanolic extracts of *B. vulgaris*'s colour were much lighter than the other extracts while *I. bijuga* methanolic extracts seem to have similar concentrations with haematoxylin. However, its colour does not resemble the purple colour of that haematoxylin.

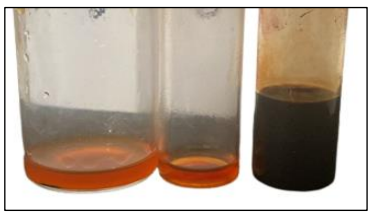

Figure 3: Physical colour of methanolic extracts
(From left; *B. vulgaris* peel, *B. vulgaris* flesh, and *I. bijuga*).

Figure 4: Physical colour of aqueous extracts
(From left; *B. vulgaris* peel, *B. vulgaris* flesh, and *I. bijuga*).

Figure 5: Physical colour of haematoxylin.

To determine their colour concentrations, absorbance were measured using the UV/Vis Spectrophotometer. The results were then compared with haematoxylin. As shown in Table 3, the average absorbance reading of haematoxylin recorded was 1.21. The average absorbance of ME1 was 0.400 and 0.421 for the flesh extracts. ME3 recorded the highest absorbance with an average of 2.994. Meanwhile, all the aqueous extract showed higher absorbance than haematoxylin. The absorbance for aqueous extracts of *B. vulgaris* peel, flesh, and *I. bijuga* were 2.256, 2.910, and 2.665, respectively.

ME1 showed the lowest absorbance reading, while ME3 recorded the highest reading. AE1 recorded the nearest absorbance reading to haematoxylin. The absorbance values were directly proportional to the concentration of the extracts; thus, methanolic extracts of *I. bijuga* have the highest concentration, while methanolic extracts of *B. vulgaris* have the lowest concentration. Except for ME1 and ME2, the other extracts have higher concentrations than haematoxylin. Since AE1 recorded the closest absorbance reading to haematoxylin, its colour concentration is the nearest to haematoxylin compared to other extracts. However, despite producing colour, these extracts might not be able to stain the nucleus if it does not possess the binding ability to the tissues (Veuthey et al., 2014). Thus, further research needs to be done to analyse their staining ability. In one study by Udonkang et al. (2021), beetroot dye was found to have a comparable staining ability to haematoxylin. The dye gave detailed features of the stained tissues making it promising as a haematoxylin alternative. Similarly, Obeta et al. (2022) also found that beetroot is suitable as a haematoxylin alternative as it possesses similar properties to haematoxylin.

Table 3: The absorbance reading for haematoxylin and extracts

Extracts	1st Absorbance	2nd Absorbance	3rd absorbance	Average
HX	1.202	1.207	1.209	1.210
ME1	0.402	0.400	0.398	0.400
ME2	0.415	0.421	0.426	0.421
ME3	2.989	2.989	3.003	2.994
AE1	2.243	2.257	2.267	2.256
AE2	2.909	2.909	2.913	2.910
AE3	2.667	2.663	2.666	2.665

*HX= Haematoxylin, ME1= *B. vulgaris* peel methanolic extract, ME2= *B. vulgaris* flesh methanolic extract, ME3=*I. bijuga* methanolic extract, AE1= *B. vulgaris* peel aqueous extract, AE2= *B. vulgaris* flesh aqueous extract, AE3=*I. bijuga* aqueous extract

3.4. Statistical Analysis

A one-way ANOVA test was performed to compare the mean between the extracts, and the results were tabulated in Table 4. The ANOVA test shows significant differences at $p<0.001$ for all the extracts. Meanwhile, an independent t-test was performed to compare the pH of the extracts to the haematoxylin. The statistical result showed that haematoxylin and ME3 have the slightest difference (t= 68.750), while AE1 has the highest difference with haematoxylin (t=233.086).

Table 4: Comparison of pH of different types of extracts.

Type of dye	N	Mean (SEM)	t(dF)	F[a]	*P* value
HX	3	2.55 (0.020)		15463.345	0.001
ME1	3	4.193(0.116)	123.250 (4)		
ME2	3	4.547 (0.116)	149.750 (4)		
ME3	3	3.467 (0.116)	68.750 (4)		
AE1	3	5.887 (0.757)	233.086 (4)		
AE2	3	5.790 (0.017)	212.108 (4)		
AE3	3	4.627 (0.025)	111.894 (4)		

*[a=]One way ANOVA test, t=t-test, df=degree of freedom, HX= Haematoxylin, ME1= *B. vulgaris* peel methanolic extract, ME2= *B. vulgaris* flesh methanolic extract, ME3=*I. bijuga* methanolic extract, AE1= *B. vulgaris* peel aqueous extract, AE2= *B. vulgaris* flesh aqueous extract, AE3=*I. bijuga* aqueous extract

One-way ANOVA analysis was also performed to obtain the mean concentration between all the extracts. The statistical test, as shown in Table 5, shows significant differences ($p<0.001$) for all extracts. An Independent t-test was done to analyse the differences between the extracts and haematoxylin. AE1 has the least difference (t=144.486), while AE2 has the most difference (t=818.095).

Table 5: Comparison of absorbance value of extract.

Type of dye	N	Mean (SEM)	t(dF)	F[a]	*P* value
HX	3	1.206 (0.004)		104300.877	0.001
ME1	3	0.400 (0.001)	338.588 (4)		
ME2	3	0.421 (0.003)	206.635 (4)		
ME3	3	2.994 (0.005)	349.844 (4)		
AE1	3	2.256 (0.007)	144.486 (4)		
AE2	3	2.909 (0.000)	818.095 (4)		
AE3	3	2.665 (0.001)	607.119 (4)		

*[a=]One way ANOVA test, t=t-test, df=degree of freedom, HX= Haematoxylin, ME1= *B. vulgaris* peel methanolic extract, ME2= *B. vulgaris* flesh methanolic extract, ME3=*I. bijuga* methanolic extract, AE1= *B. vulgaris* peel aqueous extract, AE2= *B. vulgaris* flesh aqueous extract, AE3=*I. bijuga* aqueous extract

3.5. Haematoxylin

In this study, the physical properties of the two plants mentioned were studied and compared to the properties of haematoxylin. Haematoxylin is one of the most influential and most used dyes in the world (Orchard, 2018; Avwioro, 2011). Figure 6 shows the logwood cross-section of *Haematoxylum campechianum*, the tree from which haematoxylin was originally extracted. First documented in 1502, haematoxylin was initially used as a fabric dye and as a diarrhoea treatment by the Mayan civilian (Orchard, 2018). It was also used as fabric dye to stain cotton in the 16th century and the soldiers' uniforms during the American Silver War until the Second World War (Ali et al., 2017). Haematoxylin is a colourless dye (empirical formula: C16H1406; C. I. 75290) that turns into haematein when oxidised, which is reddish brown (Titford, 2005). The molecular structures of both haematoxylin and hematein are shown in Figure 7 and Figure 8, respectively. This oxidation process can occur either naturally or by chemical means.

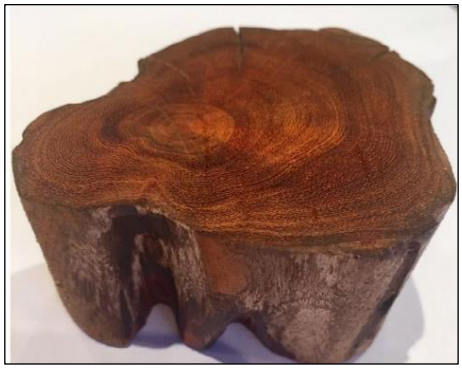

Figure 6: *Haematoxylum campechianum* logwood cross-section which is used in the production of haematoxylin (Orchard, 2018).

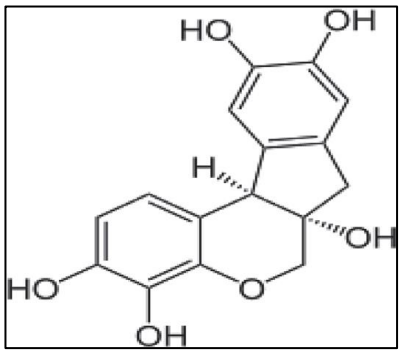

Figure 7: Haematoxylin's molecular structure before it is oxidised (Orchard, 2018).

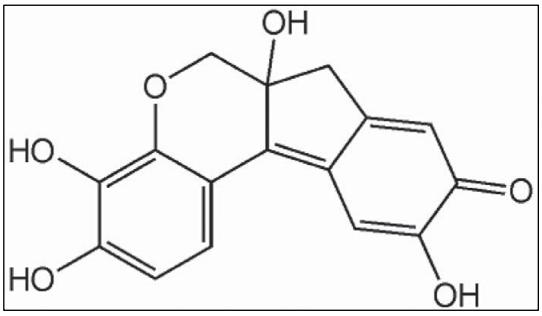

Figure 8: Hematein's molecular structure, which formed when haematoxylin oxidised (Orchard, 2018).

Historically, Bohmer was the first one to successfully make the haematoxylin stain in 1865 when he added mordant to the dye. It was Wissowzky, however, who used it first for pathology staining in 1876. Until now, haematoxylin is still the prominent nucleus stain used in cytological and histological practices.

Previous research has been done to find alternatives or substitutes for haematoxylin. One of the plant extracts that have enormous potential is roselle (*Hibiscus sabdariffa*). According to Alshamar & Dapson (2021), research on this flower as an alternative for nuclear stains has been studied since 1976. Since then, more research has been done on different extractions and mordant of *H. sabdariffa* for formulating the plant as a stain. Although it was able to stain the cells and tissue successfully, the plant was not good enough due to the stability of its components. Research by Benard et al. (2015, 2017) reported that the aqueous extract of *H. sabdariffa* was satisfactory as a nuclear stain in histological staining of brain tissue and connective skin tissue as the stain was able to give similar colour when compared to haematoxylin. Conversely, a study by Okolie et al. (2021) found that the pH of roselle extract is acidic. Thus, it is not suitable as a nuclear stain.

Other researchers studied beetroot as a haematoxylin alternative. The plant's pigment, which is responsible for the staining properties, is betalain. Obeta et al. (2022) suggested that beetroot might possess haematoxylin-like staining properties because the nucleus was stained bluish-purple when the extracts were used as a substitute in their histological staining. On the other hand, Udonkang et al. (2018) reported that beetroot could stain cytoplasm and other components of the tissue successfully but could not successfully stain the nucleus.

3.6. *B. vulgaris* and *I. bijuga* colour properties

To further understand the colour properties of these two plants, the chemical content of the plants was reviewed. Current studies unveiled the physical colour of both peel and flesh methanolic extracts of *B. vulgaris* remains bright red, resembling the colour of the vegetable itself. In contrast, the aqueous extract of *B. vulgaris* appears brown, possibly due to oxidation and degradation of compounds.

Similarly, both aqueous and methanolic extracts of *I. bijuga* yield dark brownish extracts, distinct from the colour of haematoxylin. various beneficial nutrients such as folate, potassium, nitrates, and vitamins C, A, and K. Hence, it is highly regarded as a healthy ingredient in culinary practices and traditional health remedies. Beetroots are one of the popular natural dyes in the market due to their high content of betalain pigments, which produce the red colour of the plant. Moreover, due to its health benefit and aesthetic value, the pigment gained popularity as a commercialised natural colourant, especially as a food colourant. Alim-un-Nisa et al. (2021) showed that the plant is a safe food colourant for candy, and the colour was retained until it was exposed to high temperature and pH. In addition, beetroot also can be used as a natural food preservative because betanin was found to be able to prevent the deterioration of some foods (Silva et al., 2021). Due to the aesthetic value that can be produced from beetroot extracts, the development of its fruit as beauty products has also been studied. Sari et al. (2021) studied beet root formulation as a blush that can show colour when applied to the skin without damaging them.

Table 6: Betalain, betacyanin, and betaxanthin content in beetroot in different studies.

Author	Compounds	Concentration (mg/g)	Beetroot parts	Method	Extraction method
(Mistrianu et al., 2022)	Total Betalain	1.18 ± 0.03	Not mentioned	Spectrophotometric method	Ethanolic extract
(Desseva et al., 2020)	Betacyanin Betaxanthin	2.81 ± 0.10 1.27 ± 0.00	Beetroot juice		Not mentioned
(Alonzo-Macías et al., 2020)	Betanin	30.56 ± 0.18 – 67.50 ± 1.28	Not mentioned	HPLC	Methanolic Extract
(Wang et al., 2020)	Betanin	0.26	Conventionally grown root		
		0.057			Ascorbic acid extract
		0.76	Organically grown root		Methanolic extract
		0.038			Ascorbic acid extract
(Hernández-Aguirre et al., 2021)	Betalain	3.49 ± 0.14 – 3.65 ± 0.25	Beetroot waste from peel and pulp	UV-Vis spectrophotometer	Deep eutectic solvents (DES) extract
	Betacyanin	0.23 – 0.30			
	Betaxanthin	0.09 – 0.14			
(Bárta et al., 2020)	Betalain	10.92 – 18.10 (Before boiling) 8.75 ± 1.05 – 15.04 ± 0.79 (After boiling)	Red beetroot roots	Spectrophotometric method	Ethanolic extract
	Betacyanin	4.29 ± 0.22 – 6.95 ± 0.21 (Before boiling)			
	Betaxanthin	4.29 ± 0.22 – 6.95 ± 0.21 (Before boiling) 3.17 ± 0.39 – 5.31 ± 0.28 (After boiling)			
	Betalain	1.38 – 2.07 (Before boiling) 1.16 ± 0.13 – 1.48 ± 0.79 (After boiling)	Yellow beetroot roots		
	Betacyanin	0.54 ± 0.01-0.90 ± 0.44 (Before boiling) 0.50 ± 0.50 – 0.72 ± 0.72 (After boiling)			

Betalain is one of the compounds extensively studied in beetroot and is one of the primary compounds responsible for the beetroot colour. It is the parent of two groups of pigments: betacyanin and betaxanthin. These water-soluble pigments, which exhibit red and yellow hues, can only be found in certain plant families, such as Caryophyllaceae, which

includes beetroot, pitaya, and spinach (Lembong et al., 2019; Zhao et al., 2022). The chemical structures of betaxanthin and betacyanin display a sequence of conjugation characterised by alternating double and single bonds. This conjugation is responsible for the vibrant colouration observed in these pigments (Liu, 2019).

A lot of researches has been done to study the betalain content in beetroot. Research by Mistrianu et al. (2022) found that betalain content in beetroot is 1.18 DW, while Hernández-Aguirre et al. (2021) reported slightly higher content of betalain in the *B. vulgaris* fresh weight and water extracts, which range from 3.49 to 3.99 mg/g. Similarly, Bárta et al. (2020) found almost similar betalain content in yellow beetroot powder ranging from 1.38 to 2.07 mg/g DM, while red beetroot powder showed much higher betalain content ranging from 10.92 to 18.10 mg/g DM. However, they found that the betalain content decreased by up to 30% when the powder was boiled. On the other hand, Desseva et al. (2020) studied the betalain content of beetroot juices which showed high betalain content, 2.81 mg/g of betacyanin and 1.27 mg/g betaxanthin. In addition, Wang et al. (2020) study showed that organically grown beetroot produces higher betanin content in methanolic extract (756.4 µg/g) than the conventionally grown beetroot (260.7 µg/g). The findings from these studies are summarised in Table 6.

Merbau, on the other hand, is well known for its high commercial value and mainly commercialised as construction material for house building, furnishing, panelling, and flooring. Apart from that, parts from the tree, like leaves and bark, are also utilised by local communities as traditional medicine remedies for asthma, diabetes, arthritis, liver disease, urinary diseases, and others (Widodo et al., 2019; Bradacs, 2008). Although Merbau is a well-known plant for its many uses, limited studies were found on the chemical constituent of *Intsia spp*. As mentioned by Koch et al. (2006), Hillis & Yazaki (1973) reported that the major polyphenol compound is robinetin, which was found as the yellow deposits in Merbau heartwood. Similarly, a more recent study by Sari et al. (2021) found that flavonoids are abundantly present in the plant, with robidanol and robinetin being the dominant compounds in the plant extracts.

A natural colourant from *I. bijuga* has been developed and commercialised in Indonesia, mainly used in the textile industry (Setyono, 2022). It gives a brown to reddish-brown colour when the fabric is dyed with the dye from the plant. (Rahayuningsih et al., 2022; Widyaputri, 2020). This is because the tree has reddish-brown heartwood that changes colour to dark brown or dark red when exposed to light for a period, making it attractive (Osvaldova et al., 2020). According to Rahayuningsih et al. (2022), tannins and morins are responsible for the colour of the natural dye. Other than that, flavonoids present in the plant might also be responsible for producing the colour. Some flavonoids such as robidanol, robinetin, myricetin, naringenin, and amelopsin are also found in Merbau (Angio et al., 2022; Koch et al., 2006).

4. CONCLUSION

In conclusion, an acid solution resembling haematoxylin was obtained from the aqueous and methanolic extracts of *I. bijuga* and *B. vulgaris*. The colour intensity of the *B. vulgaris* methanolic extracts was less concentrated than the haematoxylin, as measured by the absorbance value. Meanwhile, the maximum concentration of colour is seen in methanolic extracts of *I. bijuga*. While the dark colour of *I. bijuga's* methanolic extract is most similar to haematoxylin, the bright red colour of *B. vulgaris's* methanolic extract and the brown colour of its aqueous extract were not comparable. The physicochemical features of the methanolic extract from *I. bijuga* are most similar to those of haematoxylin of all the extracts tested in this work.

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