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Research article

Exploring the potential of some phytodyes as histological stains in wood anatomy

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Abstract: The potential of some phytodyes were examined to obtain cheap, non- toxic and eco-friendly stains for use in wood anatomy. Phytodyes from *Enantia cholorantha*, *Harungana madagascariensis*, *Hisbiscus sabdariffa*, *Sarcocephalus latifolius*, *Sphenocentrum jollyanum*, and *Sorghum bicolor* were used to stain wood sections and macerates. All the extracts had good affinity for wood fibre and other lignified tissues except *Hibiscus sabdariffa*. The results of the absorption spectrum of these dyes revealed a range of wavelength of absorption between 300.00 and 900.00 nm. These wavelengths fall within the visible region of the electromagnetic spectrum confirming the presence of colour imparting chromophores in the dyes. Each of the phytodye has minimum of two peaks, indicating that each dye had a minimum of two colour imparting chromophores. All the dyes were acidic with pH range of 2.80 to 5.90. The histochemical reactions of all the phytodyes were similar in that they all imparted their colours indiscriminately on all cells but fibre and lignified tissues took up the dyes more deeply. They were specific when used with Alican blue which have affinity for thin walled cells. Therefore, this study revealed that these dyes could be used solitarily or in combination with artificial dyes for wood histological staining.

Keywords: Phytodye - Non-toxic - Wood anatomy - Macerate - Vessel element - Fibre.

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INTRODUCTION

The use of colourants dates back thousands of years in all societies around the globe. Even before people began to spin yarn and weave cloth, they applied coloured earth, plant saps and juices directly to their skin; this was the first type of cosmetic (Maria 2009). In Nigeria, and among Yorubas, dyes from the heartwood of *Pterocarpus osun* Craib had been in use since time immemorial, as cosmetics, for painting human body during war and as medicine for treating skin diseases (Akinloye *et al*. 2010). The indigo colour from the young leaf of *Lonchocarpus cyanescens* (Schum & Thorn) Benth is used among Yoruba tribe of Nigeria to make natural textile material (adire), to paint houses floors and walls, as well as to colour young chicken so as to protect them from predators (such as hawk etc). Many other plant dyes were known among Yorubas and used for various purposes (Akinloye *et al*. 2010). Among the Hausas tribe in Nigeria, dyes from the leaf of *Lawsonia inermis* L., leaf sheath of *Sorghum bicolor* Linn and many other herbal dyes have been in use as cosmetics, medicine, curing of animal skin (leather), differentiating of animal and as household dyes. The use of vegetable dyes in India dated back to the Indua period between the 2th and the 4nd millennium BC. The historical record of indigo is patchy but references were records made by Marco Polo who saw indigo at present day Quilon in the state of Kerla in 1298 (Maria 2009). Since the use of natural Phytodyes does not cause health hazard nor environmental pollution, it is of immense importance to explore these sources of dye for use in plant histology. In Nigeria, there are few workers that have examined the potentials of Phytodyes for use in histopathology (Eom *et al*. 2001, Avwioro *et al*. 2005a, b), in plant histology (Akinloye *et al*. 2010).

The extraction of novel natural dyes from plants is in its infancy in Nigeria. Nigeria can play a vital role in this field for the development of natural herbal dyes on account of its varied climate and rich flora forest. Therefore, the present study investigated the potentials of six Phytodyes for use in Plant histology.

The following plants have been carefully selected for the extraction of Phytodyes. The selection was based

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on availability and the presence of dye in part of the plants as indicated below,

1. *Enantia chlorantha* Oliv (stem bark)
2. *Harrungana madagascariensis* Lam. ex Poir (stem bark)
3. *Hibiscus sabdariffa* L. (flower calyx)
4. *Sarcocephalus latifolius* (S.M) Bruce (root)
5. *Sphenocentrum jollyaum* Piers (root)
6. *Sorghum bicolor* L. (leaf sheath)

MATERIALS AND METHODS

Collection of Plant Materials for Pyhtodyes Extracts

The six plants used were collected in various locations (Table 1).

Table 1. Collection sites and voucher number of the plant species used for phytodyes.

Species	Collection Site	GPS Location	Voucher number	Collector
Enantia chloratha	PSP Akure Forest Reserve, Akure, Ondo State, Nigeria	+ 7′ 79′ 0′ N + 5′ 1′ 60′ E	IFE 17408	Akinloye A. J.
Harugana madagascariensis	Along Road 7, Opposite International School, Senior Staff Quarters, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria	+ 7′ 31′ 10.68′ N + 4′ 32′ 12.35′ E	IFE 17409	Akinloye A. J.
Hibiscus sabdariffa	Offa, along the way to Ipee, Kwara State, Nigeria.	+ 8′ 9′ 11′ N + 4′ 39′ 5.61′ E	IFE 17410	Akinloye A. J.
Sarcocephalus latifolius	General Hospital Road, Uromi, Edo State, Nigeria; and Along Ife-Ibadan Express way, Ikire, Osun State, Nigeria.	+ 6′ 43′ 5.53′ N + 6′ 19′ 9.29′ N + 7′ 22′ 21.47′ N + 4′ 10′ 20.66′ E	IFE 17242	Akinloye A. J.
Sphenocentrum jollyanum	Botanical Garden, Obafemi Awolowo University, Ile-Ife, Osun State, Nigeria	+ 7′ 31′ 20.47′ N + 4′ 31′ 21.79′ E	IFE 17411	Akinloye A. J.
Sorghum bicolor	Ipee, via Offa, Kwara State, Nigeria.	+ 8′ 9′ 13.73′ N + 4′ 40′ 5.62′E	IFE 17412	Akinloye A. J.

Extraction of the Dyes

Dyes were extracted from different parts of the selected six-plant species using Sohxlet extractor with ethanol as solvent according to (Akinloye *et al*. 2010). The vegetative part of each plant species used for dye extraction was collected and dried to reduce the moisture content and then pulverized. One-kilogram (1 kg) powder of plant material from each species was used. The extraction was continued until all the dyes were extracted from the sample.

Spectrophotometery

Each of the extracted dyes was concentrated to obtain the solid dye. The 1 mg of each sample was dissolved in 10 mL of absolute ethanol while 10 mL of absolute ethanol served as blank. Each of the dye samples was run on UV/ visible spectrophotometers, which automatically subtracted the effect of the blank and plotted the graph of absorbance against wavelength to determine the number of colour imparting chromophore in each dye (Popoola *et. al*. 1994, Akinloye *et al*. 2010).

Determination of pH of the Phytodyes

The pH of each extracted dye was taken using digital Mettler Toledo pH meter (Akinloye *et al*. 2010)

Staining

Wood sections and the macerates were stained in each of the extracted dyes for 5 minutes, rinsed in water, dehydrated and differentiated in series of grades of ethanol (50%, 70%, 80%, 90% and 100%). The stained sections and macerates were mounted in DPX mountant on clean slides and appropriately labelled. Another set of wood sections were counter stained with Alcian blue (Akinloye *et al*. 2010)

Microscopy

Microscopical observations of the prepared slides of the wood sections and macerates were made at 10x, 40x and 100x objective using LEICA DM500 binocular light microscope. The action of the herbal dyes on the wood sections and macerates were recorded.

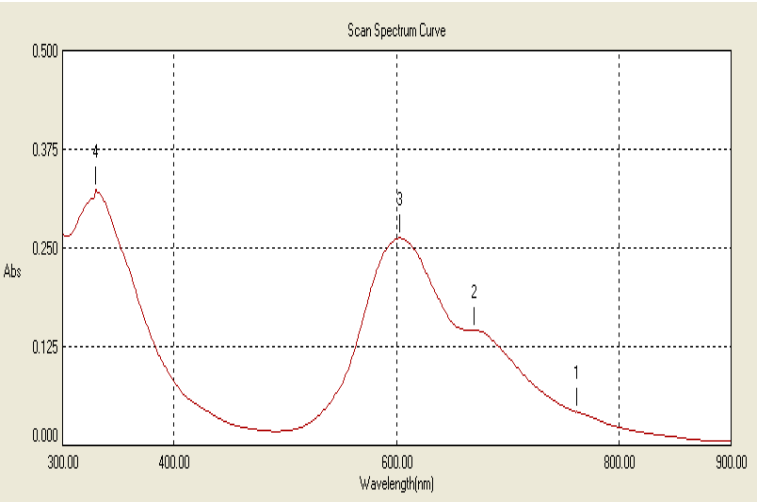
Photomicrography

Photomicrographs of the slides showing effects of the Phytodyes on the wood tissues were taken using Accu-scope trinocular microscope (ACCU-scope 33001 LED Trinocular microscope with 3.2 MP CMOS digital camera).

RESULTS

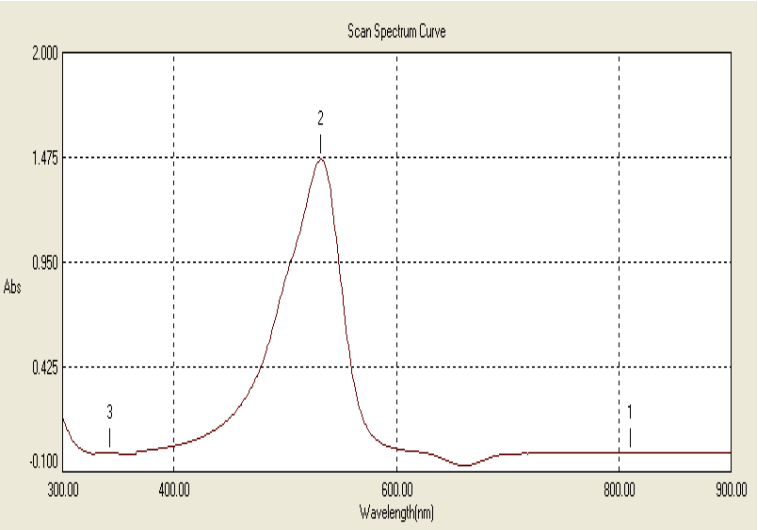
Spectrophotometery

All the extracted dyes and conventional dyes have multiple wavelengths with corresponding absorbance and multiple peaks. The recorded wavelengths of the dye extracts and the conventional stains fall within visible region of the electromagnetic spectrum and this shows the presence of colour imparting chromophore in the dye extracts (Figs. 1–9; Table 2).



No.	P/V	Wavelength (nm)	Abs
1	Peak	762.00	0.042
2	Peak	670.00	0.145
3	Peak	603.00	0.263
4	Peak	330.00	0.324

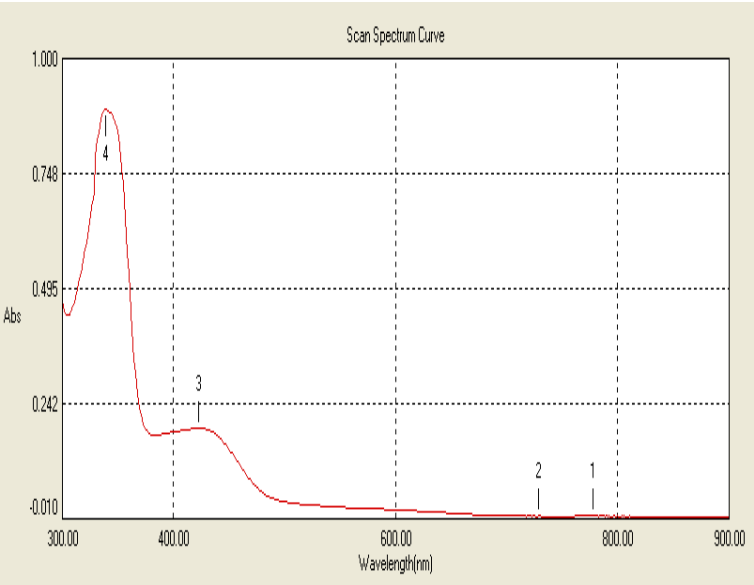
Figure 1. Electronic absorption on Alcian blue.



No.	P/V	Wavelength (nm)	Abs
1	Peak	810.00	0.008
2	Peak	532.00	1.467
3	Peak	343.00	0.009

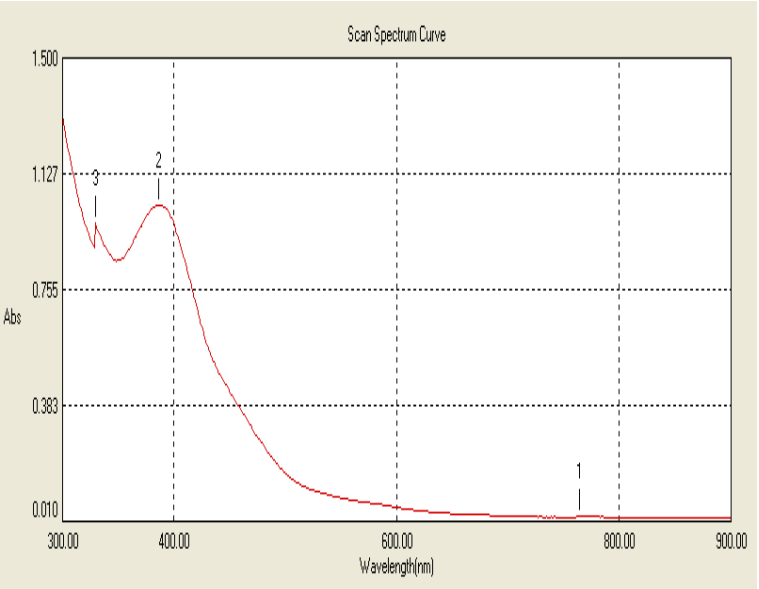
Figure 2. Electronic absorption on Safranin O.

Electronic absorption spectrum of conventional stains-Alcian blue and Safranin O are as shown in (Figs. 1 & 2). All the phytodyes used have minimum of two peaks. *Hibiscus sabdariffa* (Fig. 5) and *Sarcocephalus latifolius* (Fig. 6) had two peaks; *Harungana madagascariensis* (Fig. 4) and *Sorghum bicolor* (Fig. 7) had three peaks; *Enantia chlorantha* (Fig. 3) had four peaks: and *Sphenocentrum jollyanum* (Fig. 8) had five peaks. The number of peaks corresponds to the number of chromophores in each phytodyes. The quality or sharpness of the colour of the extract depends on the narrowness or broadness of the peak. Narrow peak tends to have sharp and



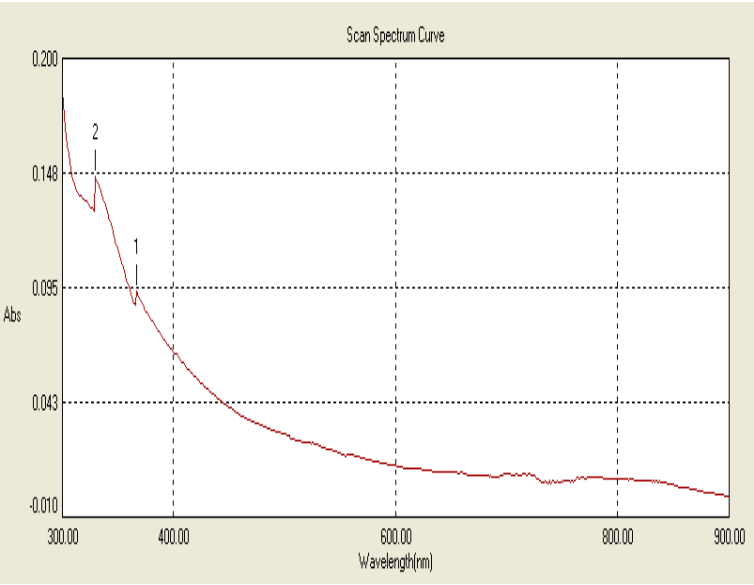
No.	P/V	Wavelength (nm)	Abs
1	Peak	778.00	0.005
2	Peak	729.00	0.006
3	Peak	423.00	0.188
4	Peak	339.00	0.890

Figure 3. Electronic absorption on *Enantia chlorantha.*



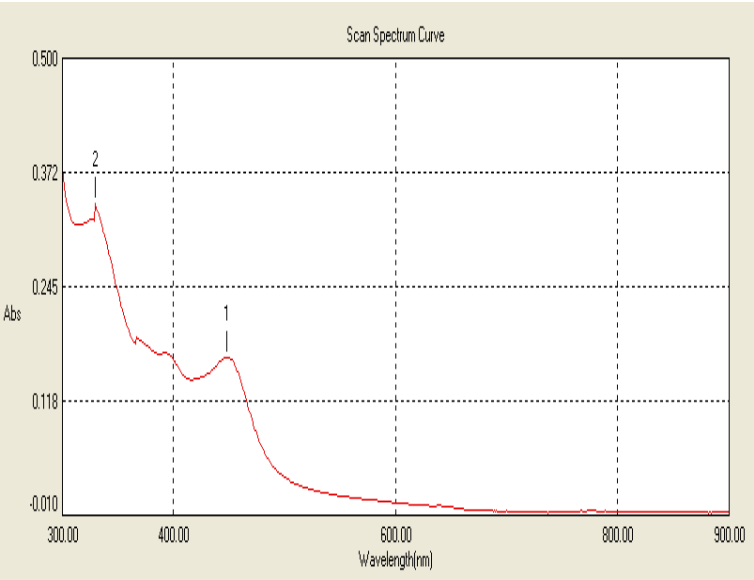
No.	P/V	Wavelength (nm)	Abs
1	Peak	764.00	0.022
2	Peak	387.00	1.028
3	Peak	330.00	0.973

Figure 4. Electronic absorption on *Harungana madagascariensis.*



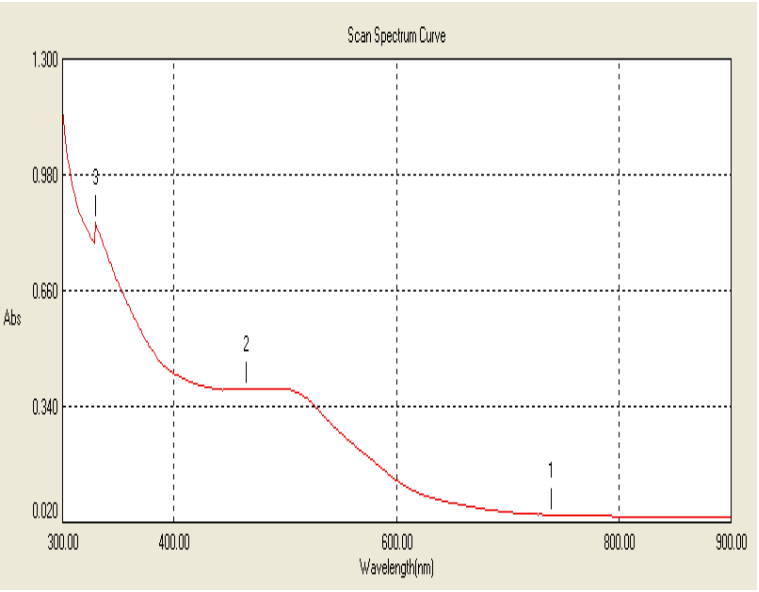
No.	P/V	Wavelength (nm)	Abs
1	Peak	764.00	0.022
2	Peak	387.00	1.028
3	Peak	330.00	0.973

Figure 5. Electronic absorption on *Hibiscus sabdariffa.*



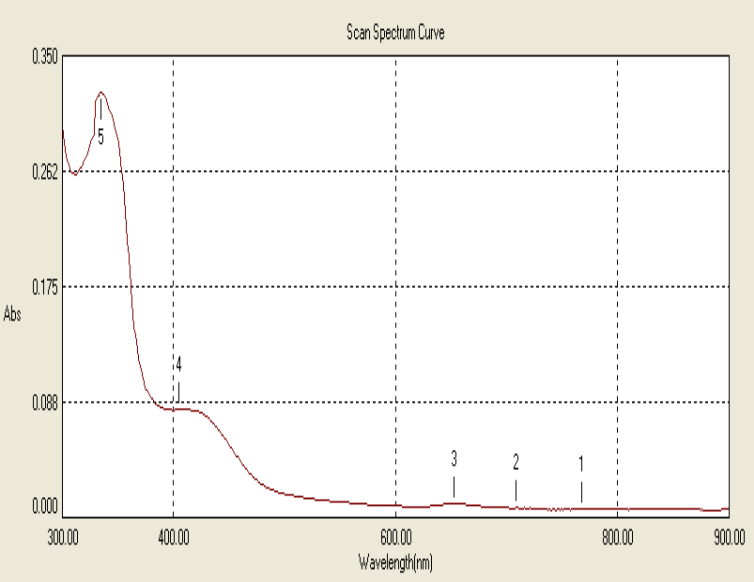
No.	P/V	Wavelength (nm)	Abs
1	Peak	448.00	0.166
2	Peak	330.00	0.338

Figure 6. Electronic absorption on *Sarcocephalus latifolus.*



No.	P/V	Wavelength (nm)	Abs
1	Peak	739.00	0.039
2	Peak	465.00	0.390
3	Peak	330.00	0.849

Figure 7. Electronic absorption on *Sorghum bicolor*.



No.	P/V	Wavelength (nm)	Abs
1	Peak	768.00	0.006
2	Peak	709.00	0.007
3	Peak	653.00	0.010
4	Peak	405.00	0.082
5	Peak	335.00	0.323

Figure 8. Electronic absorption on *Sphenocentrum jollyanum.*

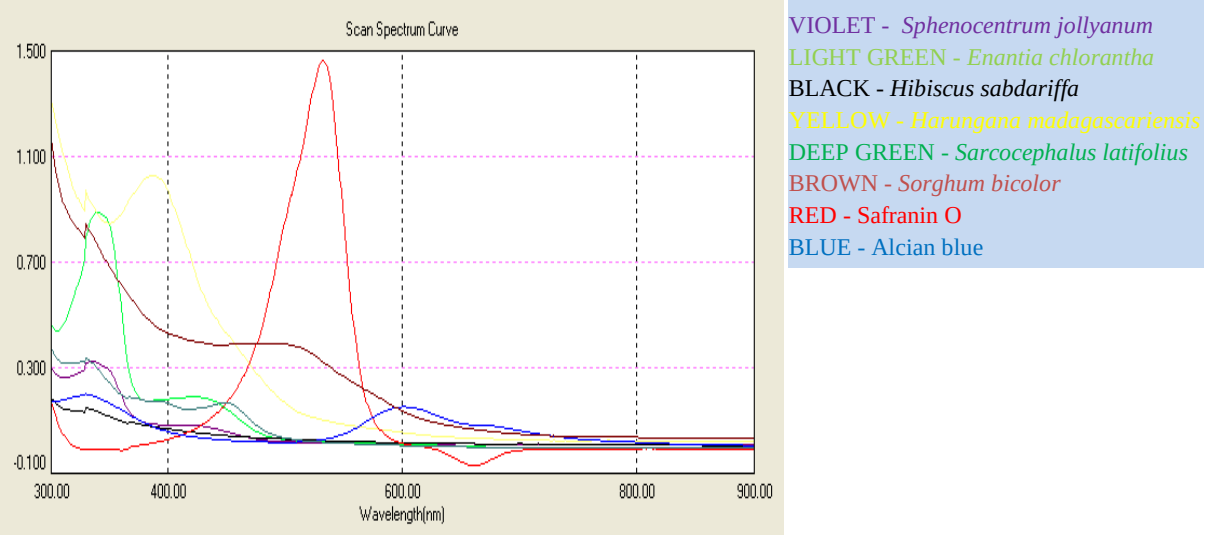


Figure 9. Electronic absorption on all the dyes used.

bright colouration while the broad peak tends to have dull colouration. Each of the Phytodye had one or more narrow peaks so this made their colouration to be bright. The combination of all the electronic absorption spectra of all the dyes used is as shown in figure 9.

Table 2. Electronic absorption spectra of the crude dye extracts and two exotic dyes used.

Dye Source	Wavelength	Absorbance
Enartia chlorantha	778.00	-0.005
	729.00	-0.006
	423.00	0.188
	339.00	0.890
Harungana madagascariensis	764.00	0.022
	387.00	1.028
	330.00	0.972
Hibiscus sabdariffa	367.00	0.094
	330.00	0.146
Sarcocephalus latifolius	448.00	0.166
	330.00	0.338
Sorghum bicolor	739.00	0.039
	465.00	0.390
	330.00	0.849
Sphenocentrum jollyanum	768.00	0.006
	709.00	0.007
	653.00	0.010
	405.00	0.082
	335.00	0.323
Alcian blue	762.00	0.042
	670.00	0.145
	603.00	0.263
	330.00	0.324
Safranin O	810.00	-0.008
	532.00	1.467
	343.00	-0.009

The action of Phytodyes on wood tissues

1. *Enartia chlorantha*: Dyes from *Enantia chlorantha* crystalized easily with brillant orange colour and indiscriminately imparted its colour on all cells but fibres and other thick-walled cells took the stain more deeply. It is a good stain for wood anatomy (Figs. 10 to 13).

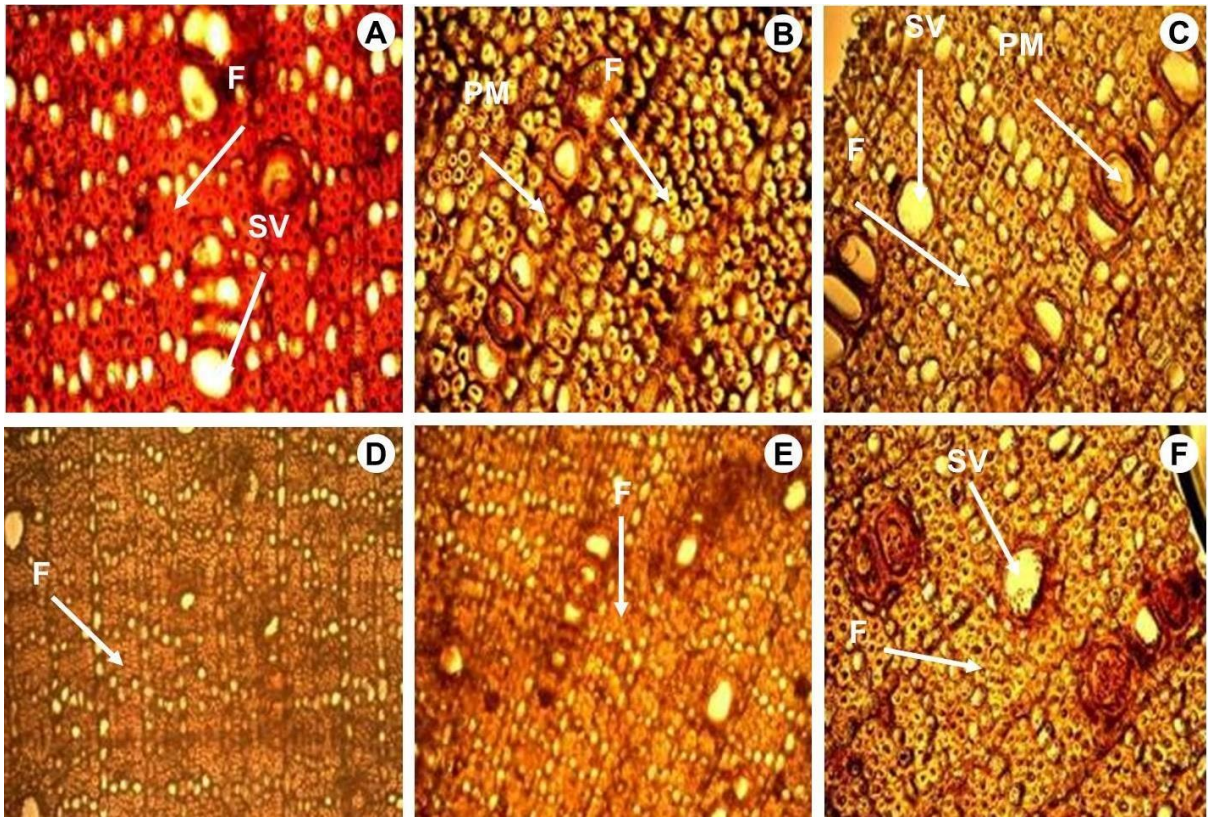


Figure 10. Single staining wood transverse sections T.S. with Phytodyes (X400): **A**, *Diospyros dendo* Welw. ex Hiern T.S. wood stained with *Sorghum bicolor*; **B**, *D. dendo* T.S. wood stained with *Enantia chlorantha*; **C**, *D. dendo* T.S. wood stained with *Hisbiscus sabdariffa*; **D**, *D. dendo* T.S wood stained with *Harungana madagascariensis*; **E**, *D. dendo* T.S. wood stained with *Sphenocentrum jollyanum*; **F**, *D. dendo* T.S. wood stained with *Sarcocephalus latifolius*. [SV= Solitary vessel, PM= Pore multiple, F= Fibre]

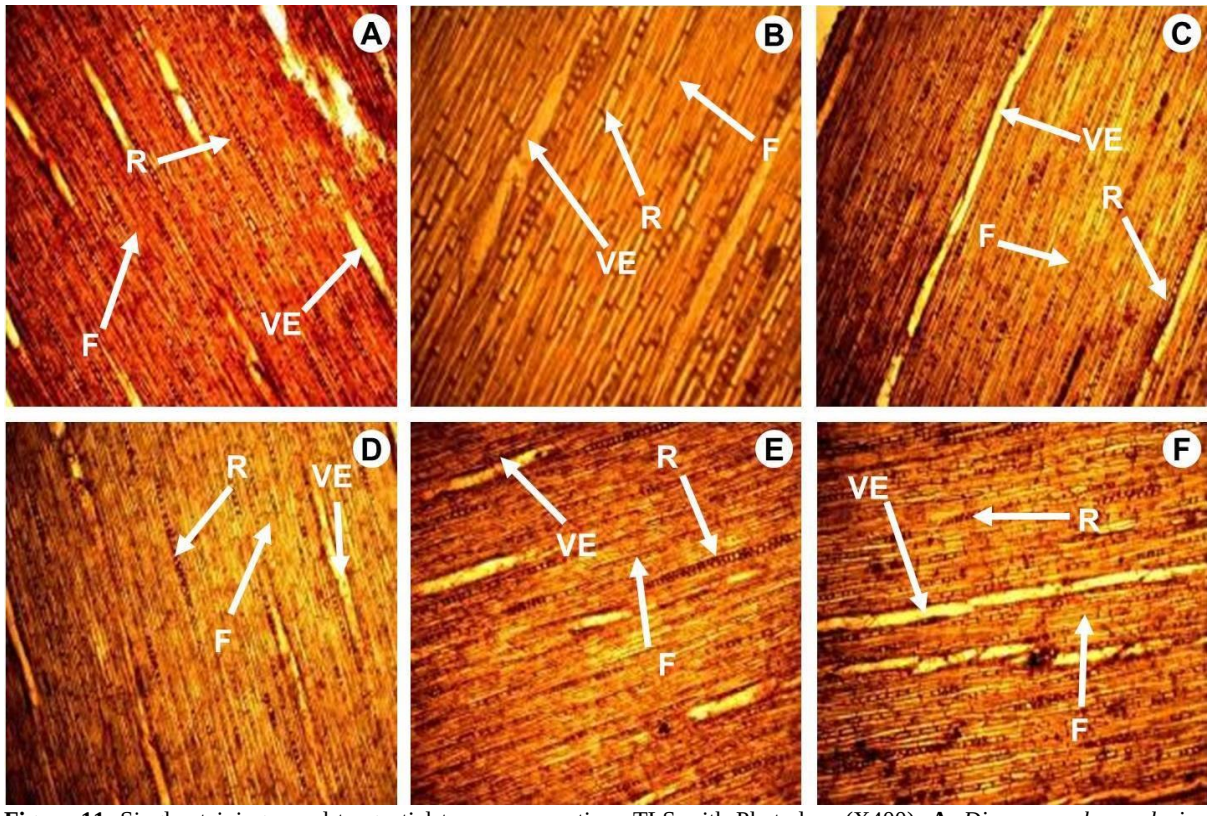


Figure 11. Single staining wood tangential transverse sections TLS with Phytodyes (X400): **A**, *Diospyros physocalycina* Gürke TLS wood stained with *Sorghum bicolor*; **B**, *D. physocalycina* TLS wood stained with *Sphenocentrum jollyanum*; **C**, *D. physocalycina* TLS wood stained with *Sarcocephalus latifolius*; **D**, *D. physocalycina* TLS wood stained with *Hisbiscus sabdariffa*; **E**, *D. physocalycina* TLS wood stained with *Harungana madagascariensis*; **F**, *D. physocalycina* TLS wood with *Enantia chlorantha*. [F= Fibre, R= Ray, VE= Vessel element]

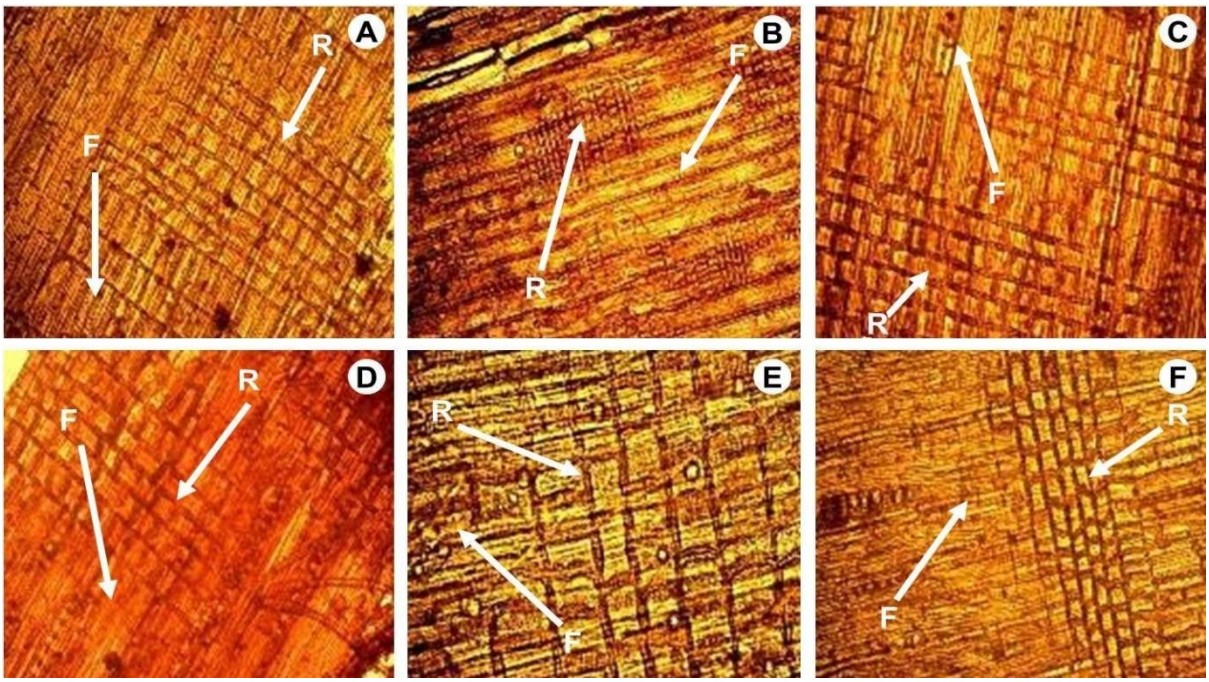


Figure 12. Staining wood radial longitudinal sections (RLS) of *Diospyros iturensis* (Gürke) Letouzey & F. White with Phytodyes (X400): **A**, Stained with *Enantia chlorantha*; **B**, Stained with *Harungana madagascariensis*; **C**, Stained with *Hisbiscus sabdariffa*; **D**, Stained with *Sorghum bicolor*; **E**, Stained with *Sphenocentrum jollyanum*; **F**, Stained with *Sarcocephalus latifolius*. [F= Fibre, R= Ray]

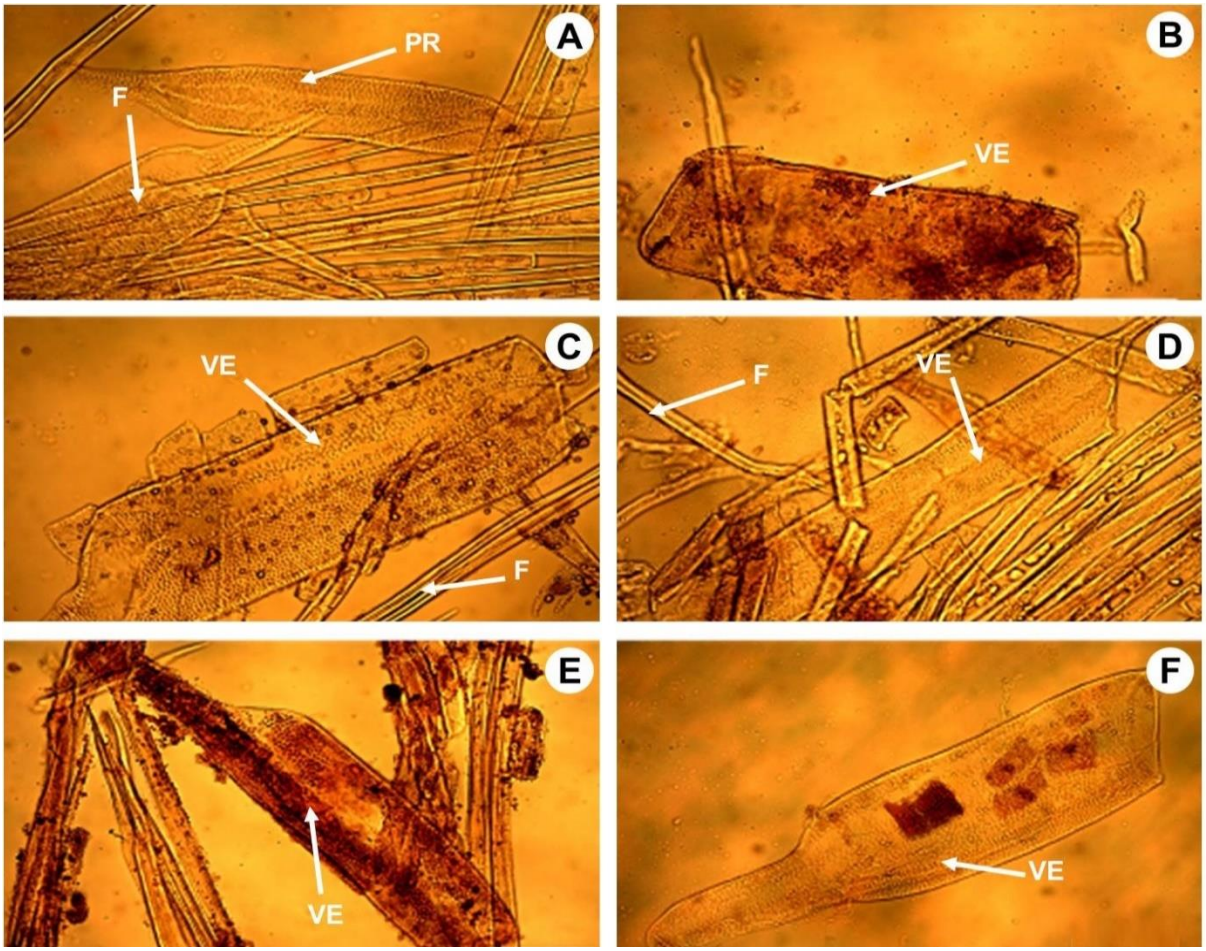


Figure 13. Wood macerates of *Diospyros piscatoria* Gürke (400x): **A**, Stained with *Hibiscus sabdariffa* showing anatomical features of fibre and vessel element with two tails; **B**, Stained with *Harungana madagascariensis* showing anatomical features of vessel element with no tail; **C**, Stained with *Enantia chlorantha* showing anatomical features of fibre and vessel element with one tail; **D**, Stained with *Sphenocentrum jollyanum* showing anatomical features of fibre and vessel element with two tails; **E**, Stained with *Sorghum bicolor* showing anatomical features of fibre and vessel element with one tail; **F**, Stained with *Sarcocephalus latifolius* showing anatomical vessel element with one tail. [F= Fibre, VE= Vessel Elements]

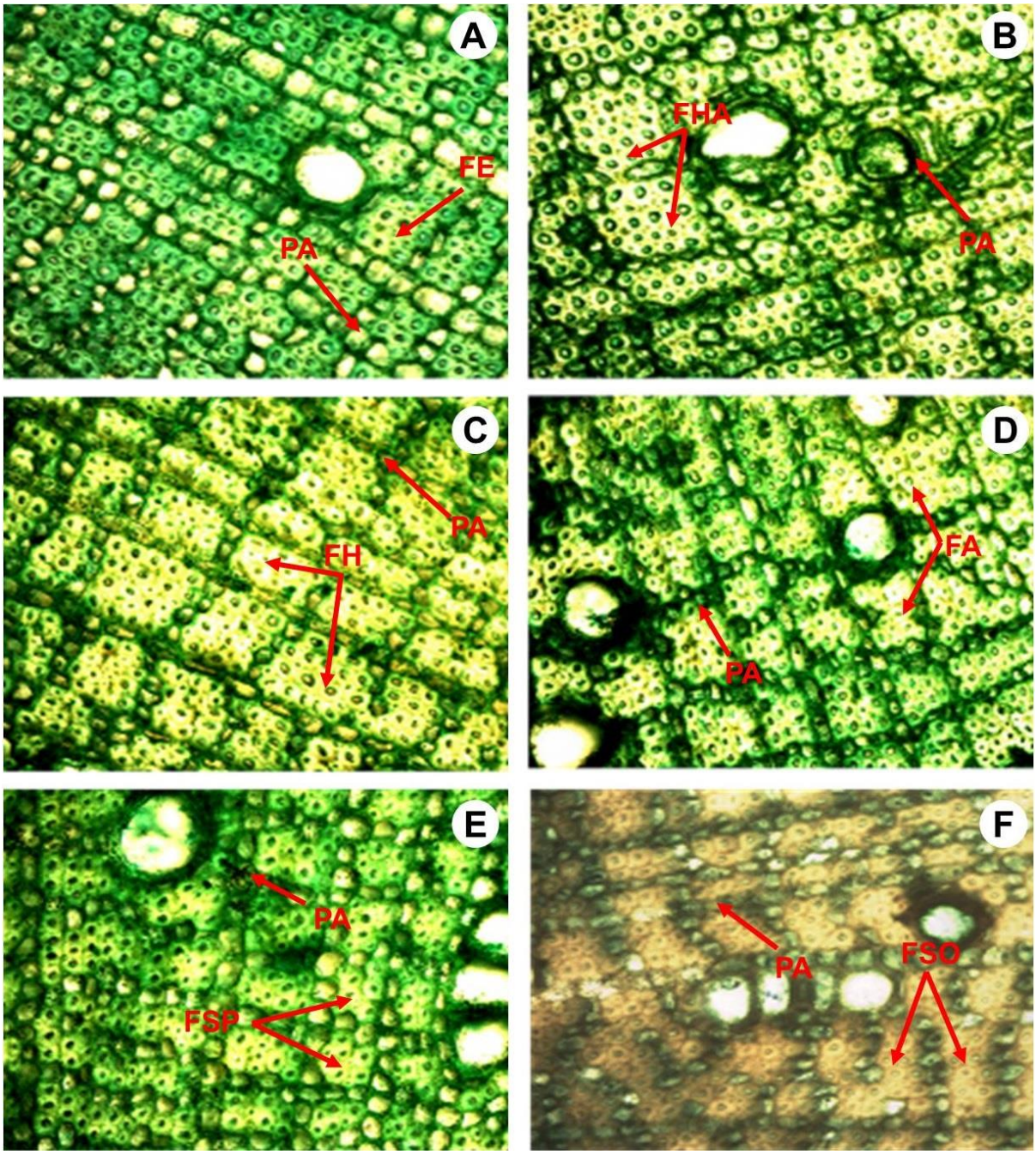


Figure 14. Counter staining extracted Phytodyes with Alcian blue (400x): **A**, *Diospyros barteri* Hiern T.S. wood stained with dye extract from *Enantia chlorantha* and counter stained with Alcian blue; **B**, *D. barteri* T.S. wood stained with dye extract from *Harungana madagascariensis* and counter stained with Alcian blue; **C**, *D. barteri* T.S. wood stained with dye extract from *Hibiscus sabdariffa* and counter stained with Alcian blue; **D**, *D. barteri* T.S. wood stained with dye extract from *Sarcocephalus latifolius* and counter stained with Alcian blue; **E**, *D. barteri* T.S. wood stained with dye extract from *Sphenocentrum jolltanum* and counter stained with Alcina blue; **F**, *D. barteri* T.S. wood stained with dye extract from *Sorghum bicolor* and counter stained with Alcian blue. [FE= Fibre took up yellow colour of *Enantia chlorantha*, FHA= Fibre took up yellow colour of *Harungana madagascariensis*, FH= Fibre took up yellow colour of *Hibiscus sabdariffa*, FA= Fibre took up yellow colour of *Sarcocephalus latifolius*, FSP= Fibre took up yellow colour of *Sphenocentrum jollyanum*, FSO= Fibre took up wine colour of *Sorghum bicolor*, PA= Parenchyma cell took up blue colour of Alcian blue]

2. *Harungana madagascariensis*: Dyes from *Harungana madagascariensis* crystalized easily and imparted its yellow colour on cells but fibres and other thick wall cells took the stain more deeply. It is a good stain for wood anatomy (Figs. 10 to 13).
3. *Hibiscus sabdariffa*: Dye from *Hibiscus sabdariffa* form slurry and does not crystalized but rather formed slurry and is highly soluble in water. It turned black in contact with water so it washed off easily when the sections were rinsed in water; the dye retained by the cells appeared faintly yellow. It is also good for wood anatomy but the other Phytodyes are better (Figs. 10 to 13).

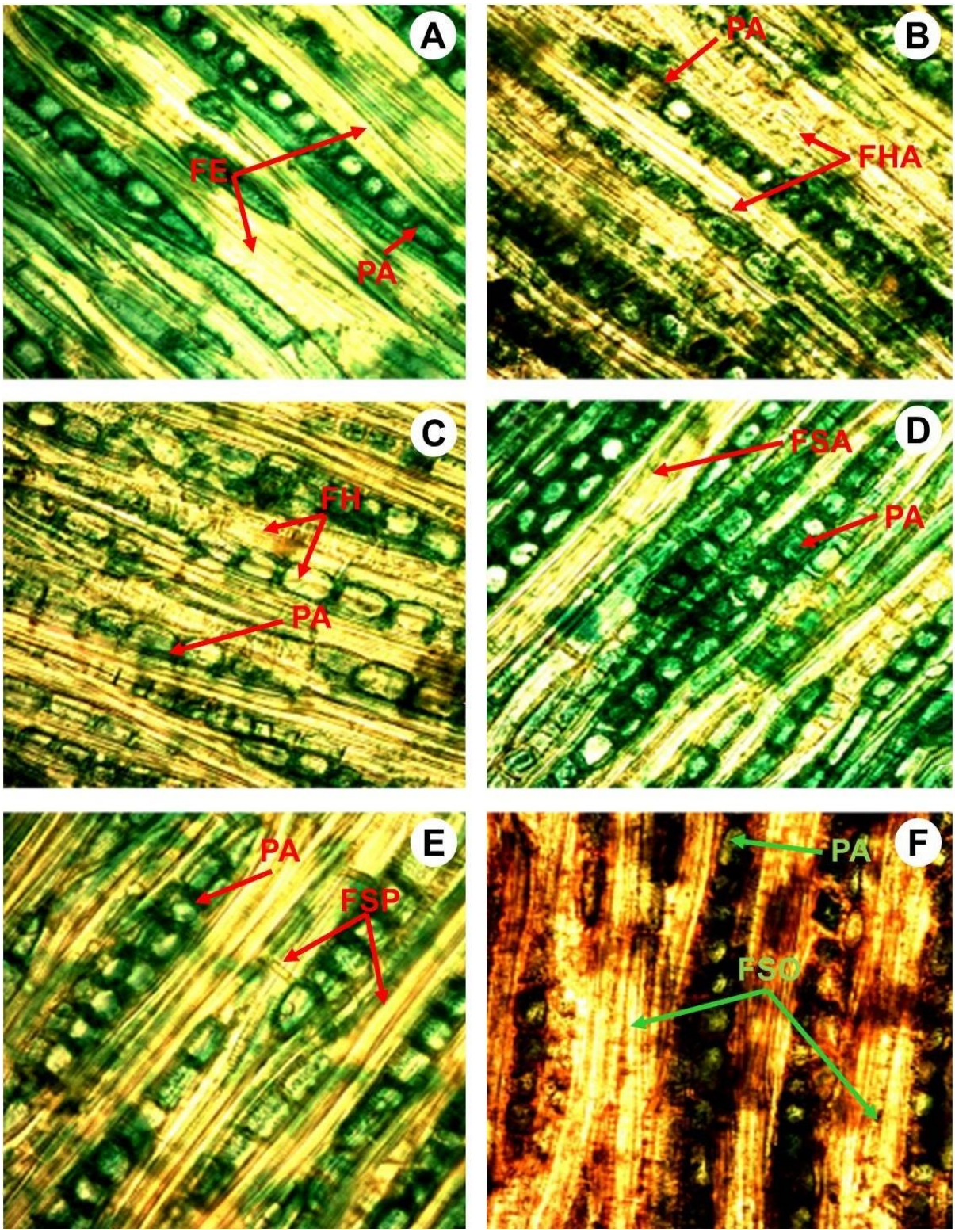


Figure 15. Counter staining extracted Phytodyes with Alcian blue (400x): **A**, *Diospyros suaveolens* Gürke T.L.S. wood stained with dye extract from *Enantia chlorantha* and counter stained with Alcian blue; **B**, *D. suaveolens* T.L.S. wood stained with dye extract from *Harungana madagascariensis* and counter stained with Alcian blue; **C**, *D. suaveolens* T.L.S. wood stained with dye extract from *Hibiscus sabdariffa* and counter stained with Alcian blue; **D**, *D. suaveolens* T.L.S. wood stained with dye extract from *Sarcocephalus latifolius* and counter stained with Alcian blue; **E**, *D. suaveolens* T.L.S. wood stained with dye extract from *Sphenocentrum jollyanum* and counter stained with Alcian blue; **F**, *D. suaveolens* T.L.S. wood stained with dye extract from *Sorghum bicolor* and counter stained with Alcian blue. [FE= Fibre took up yellow colour of *Enantia chlorantha*, FHA= Fibre took up yellow colour of *Harungana madagascariensis*, FH= Fibre took up yellow colour of *Hibiscus sabdariffa*, FA= Fibre took up yellow colour of *Sarcocephalus latifolius*, FSP= Fibre took up yellow colour of *Sphenocentrum jollyanum*, FSO= Fibre took up wine colour of *Sorghum bicolor*, PA= Parenchyma cell took up blue colour of Alcian blue]

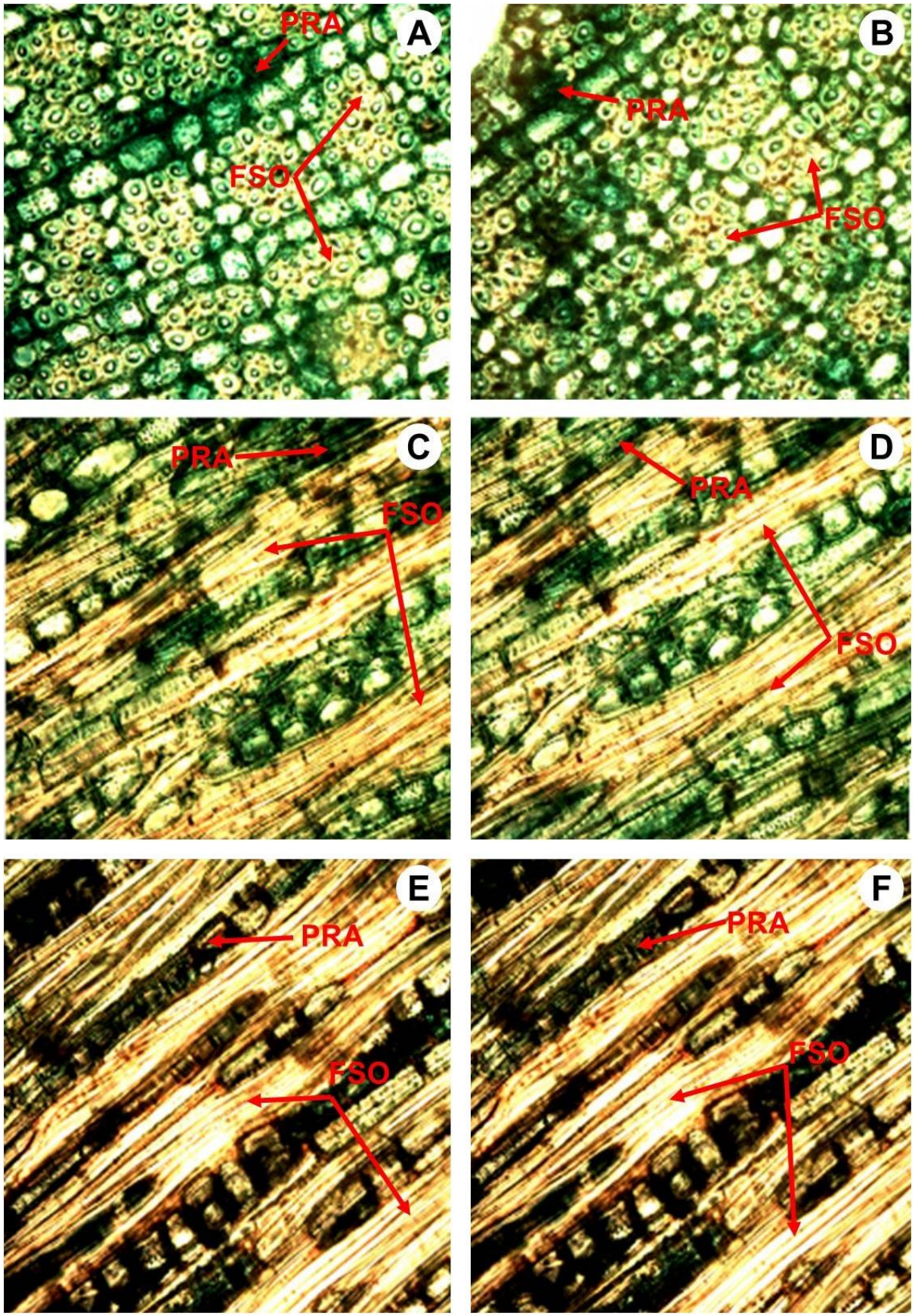


Figure 16. Counter staining Safranin O with Alcian blue (X400): **A**, *Diospyros suaveolens* Gürke T.S. wood stained with Safranin O and Alcian blue; **B**, *D. suaveolens* T.S. wood stained with Safranin O and Alcian blue; **C**, *D. suaveolens* T.L.S. wood stained with Safranin O and Alcian blue; **D**, *D. suaveolens* T.L.S. wood stained with Safranin O and Alcian blue; **E**, *D. suaveolens* T.L.S. wood stained with Safranin O and Alcian blue; **F**, *D. suaveolens* T.L.S. wood stained with Safranin O and Alcian blue. [FSO= Fibre took the yellow colour of Safranin O stain, PRA= Parenchyma and Ray cells took the blue colour of Alcian blue stain]

4. *Sarcocephalus latifolius*: Dye from *Sarcocephalus latifolius* crystalized easily and imparted its brilliant yellow colour on all cells but fibre and thick-walled cells took the stain more deeply. It is a good stain for wood anatomy (Figs. 10 to 13).
5. *Sphenocentrum jollyanum*: Dye from *Sphenocentrum jollyanum* crystalized easily and imparted its brilliant yellow colour on all cells, however, fibre and other thick-walled cells took the stain more deeply. It is a good stain for wood anatomy (Figs. 10 to 13).
6. *Sorghum bicolour*: Dye from *Sorghum bicolour* crystalized easily and imparted its brilliant wine colour on all cells but fibre and other thick-walled cells took the stain more deeply. It is a good stain for wood anatomy (Figs. 10 to 13).

Double staining Diospyros species wood sections with the extracted Phytodyes and Alcian blue

Counter staining any of the Phytodyes with Alcian blue produced brilliant contrast (Figs. 14 & 15). All the six Phytodyes were highly selective because they have affinity for fibre and other lignified cells especially when they were used together with stains that have affinity for thin wall cells (*e.g.* parenchyma cells) such as Alcian blue (Figs. 14 & 15). The results of counter staining of any of the Phytodyes with Alcian blue (Figs. 14 & 15) is similar to that of Safranin O and Alcian blue (Fig. 16).

pH of the Phytodyes

The pH of all the extracted Phytodyes fall within the acidity region ranging from 2.80 to 5.90.

Table 3. pH of the phytodye extracts and exotic dyes.

	Phytodye extract of	**pH**
1	*Enantia chloratha*	5.4
2	*Harugana madagascariensis*	5
3	*Hibiscus sabdariffa*	2.8
4	*Sarcocephalus latifolius*	5.2
5	*Sphenocentrum jollyanum*	5.1
6	*Sorghum bicolor*	5.9
	Exotic Dye	**pH**
7	Alcian blue	3.25
8	Safranin O	7.61

DISCUSSION

All the six phytodye extracts distinctively imparted their colours on all cells and tissues but more deeply on fibre and other lignified or thick wall tissues except for *Hibiscus sabdariffa* whose colour was faintly retained by these tissues when rinsed in water because it is highly soluble in water and turned black when in contact with water. The action of these Phytodyes on plant wood tissues shows that they can be used as good stains in wood anatomy (Figs. 10 & 15).

The results of the absorption spectra of the dye extract from the six Phytodyes revealed a range of wavelength of absorption between 300.00 nm and 900.00 nm which fall within the visible region of the electromagnetic spectrum confirming the presence of colour imparting chromophores in the dye extracts (Table 2, Fig. 2–9). Popoola *et al.* (1994) also discovered colour imparting chromophores in *Zingiber officinale* L. which belongs to the family *Zingeberaceae*. Akinloye *et al*. (2010) also mentioned colour imparting chromophores in the dyes from *Bixa orellana* L., *Curcuma domestica* Valeton and *Pterocarpus osun* Craib. The peak of a colour is determined by the predominant wavelength of absorption in it. Each of the Phytodye extracts has minimum of two peaks, indicating that each dye had a minimum of two colour imparting chromophores (Figs. 2–9). Popoola *et al.* (1994) made similar observation of a single peak on *Zingiber officinale*.

The quality of the colour of the extract is a product of the sharpness of its absorption spectrum which in the case of *Enantia chlorantha*, *Harungana madagascariensis* and *Sphenocentrum jollyanum* were represented by more or less narrow range of intensity profile (Figs 3, 4 & 8). This observation indicates the brightness of these dyes (Figs. 10 & 15) and support the observation of Popoola *et al* (1994) in their study of dye in *Zingiber officinale*. However, the broad absorption bands of *Hibiscus sabdariffa*, *Sarcocephalus latifolius* and *Sorghum bicolor* (Figs 5–7) do not agree with their sharpness or brightness because these three dyes imparted their colours brightly on tissues. *Hibiscus sabdariffa* imparted its bright colour on tissues better than when tissues were rinsed in water. Combining each of these Phytodyes with Alcian blue make them to behave strictly specific for fibre and vessel members just as it was observed by Akinloye *et al.* (2010) (Figs. 14 & 15). Consequently, this study has shown that Phytodyes used could be successfully employed in plant histological

studies just as noted by Gaur & Chandel (1998) in *Crocus sativus* L. and *Lawsonia inermis* L. in which they successfully utilized Phytodyes for differentiating inactive living and dead nematodes in plant tissues during bioassays and other investigations.

The results of the pH revealed that all the dye extracts are acidic (Table 3). *Hibiscus sabdariffa* dye extract was the most acidic with pH 2.8. *Sorghum bicolor* with the pH of 5.9 was the least acidic of all the dye extracts. The high acidity of *Hibiscus sabdariffa* is probably responsible for its high solubility in water. The histochemical reactions of all the Phytodyes were similar and the same with that of Safranin O in that they all imparted their colours indiscriminately on all cells except fibre and other thick walled cells and lignified tissues which took up the dyes more deeply (Figs. 10 & 13). They were specific when used with Alcian blue or any stain that has affinity for thin walled cells such as parenchyma cells (Figs. 14 & 15). Phloroglucinol is a known stain for lignin and it has pH of 4.97 which is in the acidic region. Alcian blue is a known stain for thin walled cells such as parenchyma cells and it has pH of 3.25 which is acidic. Therefore, acidity or and alkanility may not be the best parameter to determine the affinity of these Phytodyes for tissues or cells.

CONCLUSION

Dyes from the selected plants are good replacement for the exotic imported stains being used in wood anatomy. This will reduce our over dependence on imported exotic stains and thus save our country the more needed foreign exchange.

Further research work can also be done on application of the Phytodye extracts on other plant parts and their utilization in other areas of Biology such as Microbiology, Zoology and Biochemistry. Isolation and identification of prominent chromophores that are responsible for the colouration in the Phytodyes will be of immence scientific and economic interest. Further work can be carried out to check if these dyes can be used to detect the presence or absence of ergastic substances such as protein, fat and oil, starch granules and cellulose in plant tissues.

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