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Research Paper

CHROMATOGRAPHIC SEPARATION AND SPECTRO-ANALYTICAL CHARACTERIZATION OF FLAVONOID FROM A DYE YIELD PLANT

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Abstract

Flavoniod as the main colour component in *Phyllanthus muellerianus* (euphorbiaceare) leaves extract have traditionally been used to give colour to textile and handicrafts. Chromatographic (Thin layer chromatography (TLC) and column chromatography (CC)), spectroscopic (Ultraviolet and Visible (UV-Vs) and Fourier transform infrared (FT-IR) techniques were employed in order to quantify, purify and identify the functional groups of a fraction of flavonoid. The quantification analysis reveals that the concentration of the fraction is 80 mg/ml. The absorption spectra indicate a λ max peak at 455nm and 482nm, values associated to flavonoids. The result of functional (FT-IR) characterization revealed the presence of benzene ring (1520-1650 cm^{-1}), carbonyl group (1400-1480 cm^{-1}), methylene group 3000 cm^{-1} which are characteristic of the molecular structure of flavonoids.

Key words: Flavonoids, Dye, UV-Vis and FT-IR Spectroscopy, Chromatography.

INTRODUCTION

Phytochemicals can be obtained from parts of the plant like bark, leaves, flowers, roots,fruits and seeds [1]. Different types of phytochemicals found in plant include dyes, flavonoids, alkalokids, resins, etc [2,3]

Flavonoids are largely responsible for the colour of many leaves, fruits, herbs, and flowers that belongs to the group of plant pigments that impacts colour to natural fiber and handicrafts. Flavonoids are polyphenolic compounds mainly found in vegetables, grapes, wine, apples tea and berries [4,5,17]

The need to identify active chemical principles in plant extracts requires phytochemical and analytical techniques. Different phytochemcials have different degree of solubility in different types of solvents depending upon their polarity and structure. The first step of phytochemical survey are directed towards the discovery and structural elucidation of useful natural organic phytochemical for textile or medicinal purposes [6,7]

Flavonoids have two benzene rings separated by propane and a derivative of flavones. It is the brightly coloured conjugated compound found in plant [8,9]. Over 4000 naturally occurring flavonoids have been isolated and studied. Flavonoids are primarily responsible for the colours of leaves, fruits and herbs and are recognized as pigments [20]. Flavonoid dyes are usually mordant dyes, except for the catechins, which also can be used as direct dyes [18].

Silica gel 'GF and G' TLC were used for separation of the chemical constituents of dyes [10,11] using five different solvent system as developing media. These solvents include ethyl acetate – methanol – ammonia (40: 3:20), n-butanol - acetone – water – ammonia (5:5:1:2) benzene – acetic acid (90: 10) and ethyl acetate – methanol – formic acid – water (50:2:3-6) [12].

Different dyes have been separated by column chromatography on silica gel and alumina. The column was eluted with various solvent mixtures; these solvent mixtures washed the dyes through the adsorbent respectively [13].

The spectra of the separated dye components were measured in different solvents such as in methanol or chloroform. Flavonoid dyes absorb visible light at wave length from 400 – 700nm and show various adsorption maxima [14].

Fourier transform infrared (FT-IR) spectroscopy has become a well accepted method for the determination of plant constituents since it achieves high analysis speed and requires little or no sample preparation. Infrared spectroscopy has great importance in the identification of functional groups and stereochemistry of some positions of various compounds. The characteristic bands observed in the spectra of dyes depend on the nature and positions of functional groups and parent ring. For example, the phenolic hydroxyls are strongly absorbed at 3650-3584 cm^{-1} but the same bond of unsubstituted flavones appeared at 1650 cm^{-1} [19]

The FT-IR spectrum can be divided into four regions of the X-H stretching region, the triple-bond region, the double-bond region and the fingerprint region [20].

MATERIALS AND METHODS

Sample collection and authentication

The sample *Phyllanthus muellerianus* leaves were collected, beside the Faculty of Applied Natural Sciences of Enugu State University of Science and Technology, Agbani and authenticated by Prof. J. C. Okafor of Applied Biology and Biotechnology Department, Enugu State, University of Science and Technology, Enugu, Nigeria.

Extraction of Crude Flavonoids

About 50g of dry powdered *P. muellerianus* leaves was refluxed with 250mL of ethanol for an hour. The chlorophyll of the filtrate was absorbed with activated charcoal. The bright yellow filtrate was concentrated to a half of its volume using rotary evaporator.

Total flavonoid determination of the crude extract

Standard curve was fabricated by adding 0, 0.1, 0.2, 0.3, 0.4 and 0.5ml of 1mg/mL Rutin to six test tubes numbered 0 – 5 respectively. To test tube 7 was added 1mL of the crude extract. The volume of the Rutin in the test tubes 0-5 were made up to 1mL with distilled water (0, 10, 20, 30, 40, 50 µg/mL). Exactly 0.5mL of 5% $NaNO_2$ was added to all seven test tubes and shaken for 5 min.

Half a milititre of 10% $Al(NO_3)$ was also added, and the tubes were shaken for 5 mins. Water and 4% NaOH (4mL each) were then added, and the test tubes were shaken for 15 min. Test tube (0) was used to zero the spectrophotometer, and the absorbance was determined at 510nm [15].

Chromatographic separation

Thin layer chromatographic separation

The crude flavonoid was spotted and examined using TLC precoated silica gel F_{234} as described by Hamilton and Hamilton, 1989. The chromatogram was developed using ethyl acetate -methanol – formic acid – water (50:2:3:6) solvent system. After the development, the chromatogram was dried and the spots observed with 1% ethanolic solution of aluminium chloride.

Column chromatographic fractionation

The flavonoid dye was fractionated by column chromatography prepared with chloroform and alumina in a glass column of 60cm length with 1.5cm internal diameter. A solution of the crude flavonoid 20% in methanol was gently introduced on top of the column. The components were eluted with solvent system chloroform and methanol (8:2).

Seven fractions were collected and monitored by TLC, precoated silica F_{254} using solvent system, ethyl acetate – methanol – Formic acid – water (50:2:3:6). Spots were detected visually with 1% ethanolic solution of aluminium chloride. Identified fractions were combined after correlating their R_F values. One major fraction with distinct yellow colour was concentrated to a constant weight.

Spectroscopic analysis

UV – visible spectroscopic analysis

UV- visible investigation was carried out for the sample at NARICT, Zaria. A solution of the fraction (mg/mL) of *P. muellerianus* leaves in methanol was used. 'Peak pick method' was employed using UV 2500 PC series. For convenience spectroscopic grade methanol without added reagent was used as reference. The starting wavelength of 350nm and stop wavelength of 600nm was employed. The spectral result was scanned and printed.

Infrared functional group analysis

The IR spectra of the fraction purified with alumina and chloroform prepared column chromatography was run on IR spectrophotometer, FT-IR- 84005 using potassium bromide (KBr) disk. The result was recorded within the wave number range 4000 – 500 cm^{-1}.

Solubility test

Solubility of the flavonoid was investigated in thirteen different solvents. The solvents include deionized water, ether, methanol, ethanol, acetone, ethyl acetate, chloroform, nitrobenzene, petroleum ether, petroleum spirit (40-60^{0C}), Hexane, dil. HCl and dil. NaOH. The test was carried out by adding 10mg of the well grounded dye to 5mL of each of the above solvents in test-tube at room temperature [21].

RESULTS AND DISCUSSION

The result of the total flavonoid determination and chromatographic studies carried out on the crude flavonoids are shown in Table 1 and 2. It is of interest to note that the total flavonoids quantification of the crude dye sample gave 80 µg/ml.

Table 1: Some physical and chemical properties of isolated flavonoid.

Test	Flavonoid
Description	Pale green yellow crystals
Solubility test	Soluble in methanol, ethanol, acetone, ethyl acetate, chloroform, water
Yield (%) crude	4.85%/ 100g dry weight
Yield (%) of fraction 1-4	42%
Yield (%) of fraction 5-7	20.4%

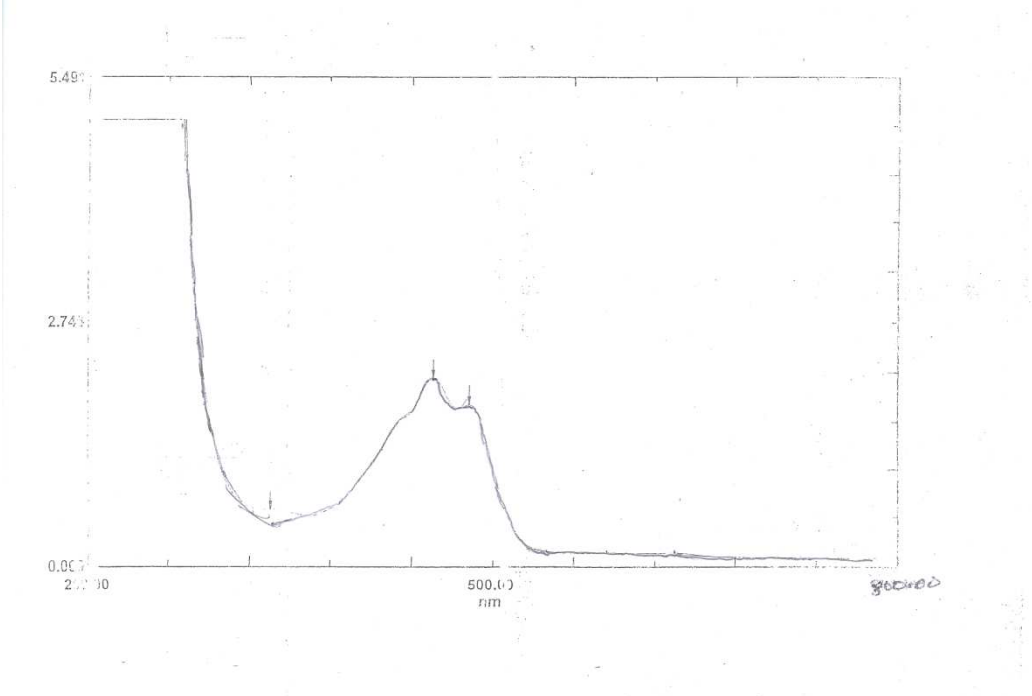
The results of the TLC – column chromatographic analysis are shown in Table 2. Two colours were separated from the original crude flavonoid sample having R_F values of 0.96 (Yellow) and 0.35 (Pale greenish brown)

Table 2: Thin layer – column chromatography results of the crude and major components and absorption parameter

Code	Fraction	Colour	R_F Values
Pm	Crude	Greenish Yellow	0.96, 0.35
PMF_1	1-4	Yellow	0.96
PMF_2	5-7	Greenish brown	0.35

The results of the fractionated pools collected on the bases of equal R_F value from column chromatography and purified on TLC reveal that major component gave 42% of the crude yield while the second component that tested negative to flavonoid test gave 20.4%.

The UV – visible spectrum (Fig. 1) shows two peaks of maximum absorption at 445nm and 482nm due to π—π* transition because of the presence of pair electrons, carbonyl group, alkene groups and keto groups generally moves the absorption maxima to longer wavelengths coupled with the presence of hydroxyl group, an auxochrome that alters both the wave-length and intensity of absorption.



Also the absorption in the visible region probably may be due to the presence of benzene ring with carbonyl functional group being part of the conjugated double bond that tend to shift absorption to longer wavelengths [16].

Table 3: The functional groups and IR-spectrum interpretation of isolated flavonoid compound.

Wave number (Cm^{-1})	Band Shape	Band	Functional growth Assignment
3650-3200	Broad	OH	Stretching of phenolic – OH
3000-2960	Sharp	C-H	C-H stretching
2500-2000	Broad	-C=C=C	Accumulated c=c double bond
2000-1500	Sharp	C=C	C=C of benzene ring
1416	Broad	CH_2, CH_3	Methylene group
1250-1050	Sharp	C-O	Carbonyl
900-850	Sharp		Substituted benzene ring
700-600	Broad	=C-H	=C-H bending vibration

Results of FT-IR spectroscopic studies have revealed the presence of various chemical (Table 3 and 4) constituents in ethanolic extract of *P. muellerianus* leaves (Fig. 2). The peaks at 3650 – 3200 cm-1 and 3000 – 2960cm-1 are corresponded to Hydroxyl and CH stretching frequency. The peak of 3000 – 2960 cm^{-1} are very useful for differentiating the saturated C-H from the unsaturated C-H A band at 2500 – 2000 cm^{-1} is corresponded to accumulated double bonds.

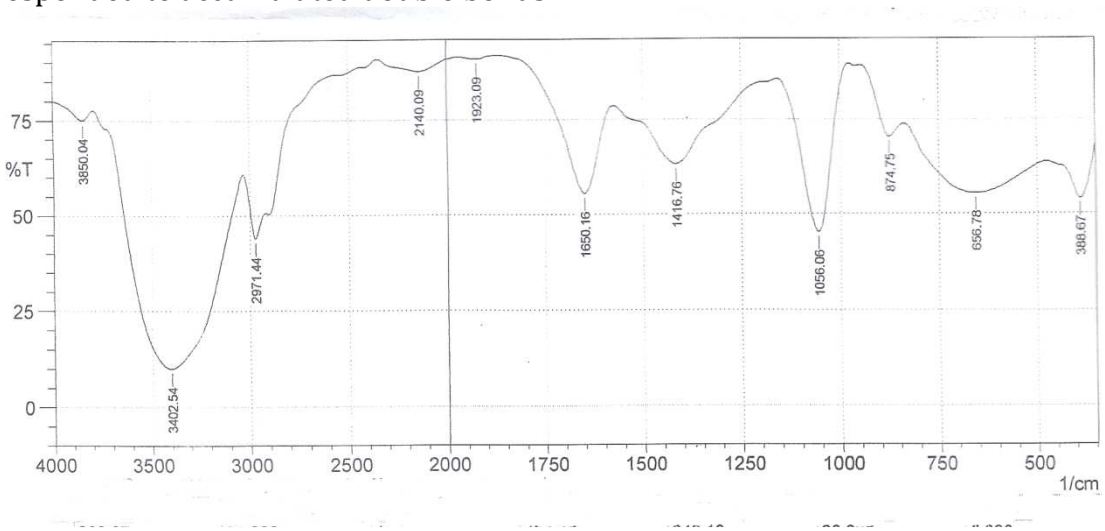


Table 4: IR spectra data (cm^{-1}) of the flavonoid

Peak	Assignment
656 (m) (b)	Bending frequency in aromatic ring =C-H
874 (s) (sh)	Substituted benzene ring with single hydrogen
1056 (w) (sh)	C-O bond
1416 (m) (b)	Methylene group – CH_2, CH_3
1650 (m) (sh)	C = O stretching
1923 (s) (b)	Region of double bond
2140 (s) (b)	Accumulated double bonds
2971 (w) (sh)	C-H stretching
3402 (w) (b)	O-H stretching

Keyword: Intensities in parenthesis; b – broad, Vs – very strong, S – Strong, m – medium, w – weak, sh – sharp.
The peak at 2000 – 1500 cm^1 is the region for double bonds which is important for the interpretation of IR spectra. A carbonyl usually shows a strong band in 1990– 1650cm^{-1}.

The absorption bands are stretching vibrations of carbon – carbon double bonds appear in this region with low intensities. Methyl group (CH_2, CH_3) always has absorption band around 1416 cm^{-1}.

Associated hydroxyl or phenol group produce IR absorption bands in the region of 1250 – 1050 cm^{-1} from the C-O band. Substituted, benzene ring with single hydrogen remaining has an IR absorption band in the region of 900 - 850 cm^{-1}
The results of the solubility study (Table 5) revealed that the dye is soluble in deionized water, methanol, ethanol, acetone, and dilute sodium hydroxide and sparingly soluble in ethyl acetate and chloroform.

Table 5: Solubility of the flvaonoid in different solvents with colour

Solvent	**Solubility**	**Colour of Solution**
Deionized water	Soluble	Pale green
Methanol	Soluble	Greenish
Ethanol	Soluble	Pale green
Acetone	Soluble	Pale green
Ethyl acetate	Sparingly soluble	Greenish
Chloroform	Sparingly soluble	Greenish
Nitrobenzene	Insoluble	-
Petroleum ether	Insoluble	-
Petroleum spirit (40-60^{0}C)	Insoluble	-
Hexane	Insoluble	-
Dil. HCL	Insoluble	-
Dil. NaOH	Soluble	Green to brown

It is also noted that the flavonoid is not soluble in nitrobenzene, petroleum ether, petroleum spirit (40-60^{o}c), n-hexane and dilute hydrochloric acid. Interestingly the flavonoid is soluble in highly polar solvents with high dielectric constants (78.30-19.1) and insoluble in non polar solvents with low dielectric constants (1.9-4.0) but sparingly soluble in slightly polar solvents with dielectric constant between 5-6.4).

CONCLUSION

It is evident from the results that the flavonoid a mixture of two coloured components. The absorption of light in the visible spectrum (400 – 700nm), the presence of chromophores such as (C=C) enthylenic and (C-O) carbonyl groups, having a conjugated system (-C=C-) and the accompanying resonance of electrons, exhibited by the chromophores, the flavonoid can be termed a dye. In conclusion, the flavonoid of *P. mulellerianus* has high potential as a dye. This finding provides an insight into the usage of the leaves of P. muellerianus in traditional dying of textiles and handicraft.

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