https://doi.org/10.30799/jnpr.115.25100102 J. Nat. Prod. Resour. - Volume 10 Issue 1 (2025) 302–305

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ISSN: 2455-0299

Analysis of Phytochemicals in *Iresine herbstii* Leaves Collected from Nyeri County, Kenya

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ARTICLE DETAILS

Article history:
Received 28 July 2025
Accepted 22 August 2025
Available online 30 September 2025

Keywords:
Iresine herbstii
Betacyanin
Betalain
Cyclo-3,4-Dihydroxyphenylalanine
Betalamic Acid

ABSTRACT

The growing global concern over environmental pollution caused by synthetic dyes has driven increased interest in natural dyes as a sustainable alternative. Some synthetic dyes have been shown to have carcinogenic and mutagenic effects on organisms, including humans. This study focused on extracting and characterizing betalain dye from *Iresine herbstii* leaves to explore its potential as a natural dye source. Betalain dye was extracted using a solvent extraction method. The characterization was done using physical tests such as FT-IR, UV-Vis, and chemical tests. The percentage yield of betalain dye obtained after solvent extraction was 0.73±0.23 % (dw). The phytochemical test of the extract indicated the presence of betacyanin, which gives betalain a red-purple color. Chemical test results of the dye agreed with those of betalain from beetroot, which is the main source of betalain. Functional groups analysis confirmed the presence of O-H, C-H, C-O, C=C, and N-H bend. UV-Vis analysis showed a dominant absorption peak at 534 nm, indicating the presence of betacyanin. This peak was given by the uptake of conjugated double bonds contained in the extract's betalain structure, and the betacyanin content concentration was 730 ± 230 mg/100 g dry weight (dw). The findings highlight the potential of *Iresine herbstii* as a viable source of an eco-friendly dye from leaves and not tubers (such as beetroot), and contribute to efforts to reduce environmental pollution.

1. Introduction

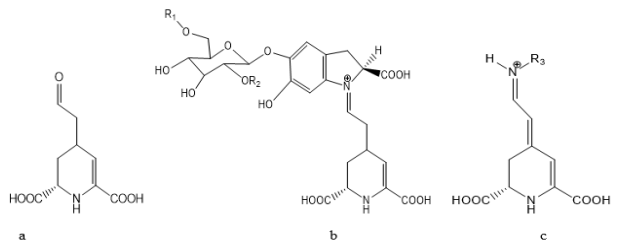
Dyes are organic compounds that give the fiber its color on the fiber's surface and inside through penetration. Dyes are categorized based on their composition, origin, hue, solubility, and application techniques [1]. They can be classified as natural and synthetic dyes based on their source. Natural dyes can be acquired from naturally occurring sources, including plants, insects, animals, and minerals [2]. Plant roots, flowers, leaves, and fruits are the parts from which natural plant dyes can be extracted [3].

Dyed material's uniformity, tone, and color are the characteristics that can either appeal to or deter customers. This makes dyeing one of the most crucial processes in the textile industry since it draws customers whereby the color of an object reflects or emits light, giving the eye a range of impressions. In the past, materials were dyed using crushed pigments from plants, beautiful hues were only available to the higher crust of society, because natural dyeing was a secret art [4].

Since the invention of synthetic dye sources, the use of natural colors has reduced significantly because they are cheaper than natural dyes and can produce vibrant colors, even though they are harmful to both the environment and humans [5]. Most synthetic dyes are directly or indirectly proven health hazards. For these reasons, natural dyes have recently gained popularity because the textile industries are looking for more sustainable and environmentally friendly dyeing techniques [6]. These natural dyes are not only safe for the environment and non-harmful, but they also give certain particularities to the substrate since many natural dyes have antimicrobial and hypoallergenic properties [7]. An example of a natural dye with these properties is betalain dye, which also has a vibrant colored pigment that is water soluble [8].

Iresine herbstii is one of many plant dyes not yet fully explored for their potential in dyeing textiles, and few studies explicitly quantify betalains in it. It is a short-lived perennial and erect herbaceous plant [9]. It can reach a height of 5' and a spread of 3' in its natural habitat [10]. They have crimson stems with oval, purple-red, 4"-long leaves with notched tips and light red veins, and need sun exposure for optimum color. It can be propagated by cutting stems in soil or water. *Iresine herbstii* has no significant pest or disease problems, and if attacked by pests like aphids, treatment can be done with the least toxic options [11]. *Iresine herbstii* comprises 80 species, but only a handful are cultivated for their attractive qualities. These include Iresine herbstii 'Brilliantissima', which has bright red leaves with pink veins; Iresine herbstii 'Aueoreticulata', which has green leaves with yellow veins; Iresine herbstii 'Blazin Rose', which has deep red–violet leaves with pink-red veins; and Iresine herbstii 'Acuminata', which has dark purple leaves with pink-red veins [12]. Iresine herbstii' Acuminata' was used in this study; the leaves of Iresine herbstii 'Acuminata' are shown in Fig. 1. Scheme 1 shows the structures of some of the compounds that comprise the betalain dye.

Fig. 1 Image of *Iresine herbstii* leaves



Scheme 1 General structures of betalains: betalamic acid (a), betacyanin (b), and betaxanthins (c). Betanin, where $R_1 = R_2 = H$; R_3 = amine or amino acid group

Given the rising demand for natural dyes and the ability of the *Iresine herbstii* plant to produce betalains in its leaves, a suitable scientific technique for the extraction of dyes and their stabilization for prolonged storage (since they are known to be unstable) needs to be devised. In this study, betalain dye was extracted from *Iresine herbstii* leaves, characterized, and its stability was tested under different conditions.

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https://doi.org/10.30799/jnpr.115.25100102

Cite this Article as: Teresia Wambere Kibicho, Benson G. Ongarora, Paul M. Sang, Peter Maina, Analysis of phytochemicals in *Iresine herbstii* leaves collected from Nyeri County, Kenya, J. Nat. Prod. Resour. 10(1) (2025) 302-305.

2. Experimental Methods

2.1 Plant Material Collection and Preparation

The *Iresine herbstii* leaves were identified and collected in Nyeri County using random sampling. Samples were put in a clean woven polypropylene bag to prevent contamination and ensure excellent moisture control. Fresh samples were sorted, unhealthy leaves were removed, and samples were washed with distilled water to remove dirt. Subsequently, they were cut into small pieces using a knife and dried in an oven at 40°C for 8 h until the sample was dehydrated and until crunchy [13] and blended into a powder using a blender [14]. Chemicals used in this study were purchased from Kobian (Kenya) Limited, which included 99.9% methanol, ascorbic acid, citric acid, ethylenediaminetetraacetic acid (EDTA), hydrochloric acid, and sodium hydroxide.

2.2 Extraction and Stability of Betalain Dye

Betalain dye was extracted from *Iresine herbstii* leaves using a solvent extraction method. Initially, 20 g of *Iresine herbstii* leaves were precisely weighed using an analytical balance, and also weighed after the removal of water content, then mixed with 100 mL of methanol. The mixture was allowed to soak for 2 hours, following a procedure adapted from literature [15] with slight modifications. The soaked *Iresine herbstii* leaves were placed in a thermostatic water bath set at 50 °C for 45 minutes to facilitate betalain extraction [16]. After extraction, the mixture was cooled for 1 hour and filtered using Whatman No. 1 filter paper (150 mm Ø × 100 circles) and a filter funnel. To precipitate chlorophyll and other non-polar compounds, 3 mL of 10% ascorbic acid was added to the filtrate, followed by a second filtration to separate the purified betalain dye from the residue. Methanol was evaporated from the filtrate at 65 °C. The procedure was carried out in triplicate, and the percentage yield of betalain dye was calculated, accounting also for the moisture content.

The percentage yield was calculated using Eqs.(1-3) according to Jin et al. [17]

$$Mc = \frac{Fresh\ weight - Dry\ weight}{Fresh\ weight} \times 100 \quad (1)$$

$$W_{dry} = wet\ weight \times (1 - \frac{Mc}{100}) \quad (2)$$

$$percentage\ yield = \frac{W_{extract}}{W_{dry}} \times 100 \quad (3)$$

where: w_{dry} is the weight of the dry matter, Mc is the moisture content, and $W_{extract}$ is the weight of the extract.

The dye stability was tested by exposing the extracts to air and light over three months and at a pH of about 3. Citric acid, EDTA, and ascorbic acid acted as stabilizing agents in this test.

2.3 Chemical Analysis of Betalain in the Dye Extract

About 5 mL extract was added to 5 mL of HCl and then heated for 5 minutes at 50 °C. Following this, 5 mL of 2 M NaOH was added dropwise, and the observed color change served as an indicator of the presence of betacyanin, as described in [18].

2.4 Functional Group Determination using Fourier Transform Infrared Spectroscopy

A Thermo Scientific Nicolet 6700 FT-IR spectrometer was used to determine the functional groups in betalain dye. Its operation was based on the interaction of infrared radiation (4000-500 cm^{-1}) with the sample, as each molecule absorbs energy characteristic of its specific intramolecular bonds. FT-IR attenuated total reflectance (ATR) spectra (32 scans and 4cm^{-1} resolution) were collected with a MIRacle, single reflection horizontal ATR accessory (PIKE instruments) having a diamond ATR crystal fixed at an incident angle of 45°. Two drops of betalain dye were mounted on an ATR crystal and pressed gently by a pre-mounted sample clamp. Air ionization in the FT-IR instrument was eliminated by purging the instrument with nitrogen gas for 2 hours. Isopropyl alcohol at a concentration of 70% was used to clean and disinfect the ATR plate. Isopropyl alcohol solvent was applied using Kimwipes to avoid scratching the ATR surface. The obtained spectrum was saved automatically in a short mathematical format (SMF) for interpretation.

2.5 Ultraviolet-Visible Spectroscopy

The compound of betalain in *Iresine herbstii* leaves was identified spectrophotometrically at 400 nm to 800 nm with a double-beam UV-Vis spectrophotometer. The scan program was used to collect a baseline spectrum, and methanol was used as a background solution for the sample without an analyte. It was put into the appropriate position to baseline the instrument. Then, betalain analysis was performed by taking 1 mL of the sample in a cuvette and running it through the UV-Vis instrument [19]. The concentration of betalain dye extracted from *Iresine herbstii* leaves was determined using a calibration graph. The data was saved in comma-separated format (CSV) to be used in Excel, and the spectra data was plotted as absorbance vs. wavelength. The quantification was expressed as mg betalain /100 g using Eq.(4) as determined by Castellar et al. [16].

$$Total\ betalain\ content(\frac{mg}{100g}) = \frac{A \times DF \times MW \times v_d}{\varepsilon l w_d} \quad (4)$$

where: A = absorbance, DF = dilution factor, MW = molecular weight of betalain (550 g/mol), volume of extract (mL), ε = Molar extinction coefficient for betalain (60000 L/mol and l = path length of the cuvette, and weight of the fresh sample (g).

3. Results and Discussion

3.1 Extraction and Stability of Betalain Dye

Iresine herbstii leaves were cut into tiny pieces and blended to reduce the heterogeneity of the sample, provide a maximum surface area for mixing with methanol solvent, and increase the yield [14]. Extraction was done at 50 °C for 45 minutes to avoid degradation of betalain, which occurs when exposed to high temperature and for an extended period, whereby it changes its red-purple color to yellow [20]. Filtration was done using filter paper to remove the solid impurities from the dye, and a measure of 3 mL of 10% ascorbic acid was added to purify the dye and make it stable [21]. Methanol was evaporated to obtain a solid dye, and the percentage yield was calculated after extracting the dye in triplicate to validate the results. The percentage yield of extracted dye obtained from *Iresine herbstii* leaves was 0.73±0.23 % dry weight (dw). These results align with 0.75% (dw) betalain yield reported for *Iresine herbstii* leaves in the literature [22]. The percentage yield of betalain extracted from wet leaves (accounting for moisture content) varies depending on the plant source, extraction method, and growing conditions. *Iresine herbstii* leaves have been reported to have lower betalain content compared to some high-yield sources like *Beta vulgaris* (Beetroots, 0.4% to 2% dw [22,23] or *Rivina humilis* (pigeon berry, 3.5% dw) [24]. Fig. 2 summarizes the steps followed in extracting and purifying the dye extract. The dye was found to be stable in air and on exposure to light for up to three months when maintained at pH 3.

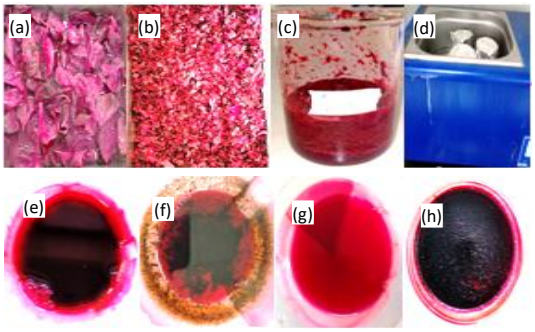


Fig. 2 Washed *I. herbstii* leaves (a), chopped *I. herbstii* leaves (b), blended *I. herbstii* leaves (c), extraction of betalain dye (d), filtration of betalain dye before addition of AA (e), filtration of betalain dye after addition of AA (f), second filtration of betalain dye after addition of AA (g), solid betalain dye after removal of methanol (h)

Iresine herbstii leaves are used as an anticancer and wound-healing agent [11], after labor tonic [25], and are applied externally to treat skin conditions like acne, pimples, and ulcers [26], and also as an antibacterial substance [27]. Moreover, *Iresine herbstii* is a haemostatic, water pill, antispasmodic, pertussis, and underlying cause of severe headache [27]. Decoction, fever, relaxation, and renal issues are all treated using leaves and flowers [28] and as a fever-lowering agent [28] reported that *Iresine herbstii* plant can reduce inflammation, can be used as an antineoplastic, systematic and intentional elimination of body cells and can lowly scavenge free radicals [29]. *Iresine herbstii* leaves are used in Brazil to formulate sunset tea, commonly known as Blood leaf tea. Research has demonstrated that Blood leaf tea exhibits significant antioxidant activity [30]. *Iresine herbstii* extract stabilized using ascorbic acid was stable for a longer period compared to the one stabilized using citric acid and ethylenediaminetetraacetic acid (EDTA). *Iresine herbstii* extract stabilized using EDTA changed color from red-purple to yellow after two days while after using citric acid it developed microorganisms after a week but after using ascorbic acid it had a shelf life of three months. This was depicted in Fig. 3.

https://doi.org/10.30799/jnpr.115.25100102

Fig. 3 *Iresine herbstii* leaves extract stabilized using ascorbic acid (a), citric acid (b), and EDTA (c)

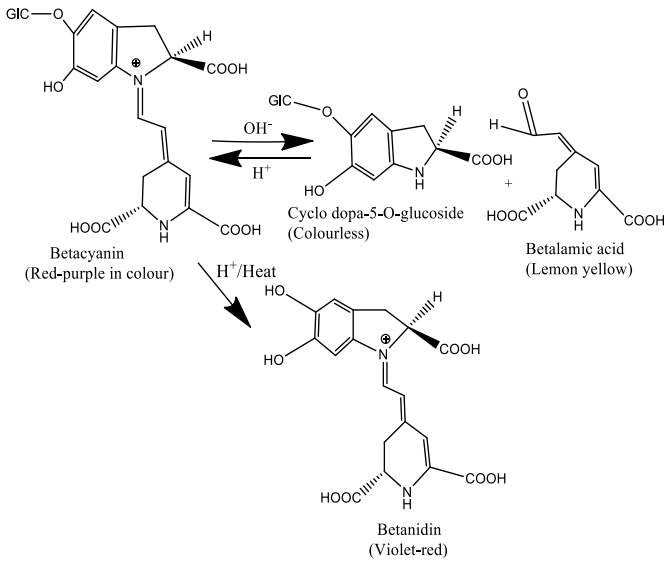
Ascorbic acid (reducing agent) helps to remove oxygen from betacyanin by donating electrons to oxygen (an oxidizing agent) and reacting with oxygen molecules, effectively preventing them from interacting with and degrading betacyanin pigment. This process helps stabilize betacyanin and prevents its degradation, especially under light, heat, or agitation conditions [26]. EDTA's influence on betacyanin color shift from red-purple to yellow over time is primarily due to its ability to act as a chelating agent, binding with metal ions that can catalyze the degradation of betacyanins. By removing these metal ions, EDTA can slow down the degrading process and extend the lifespan of the red-purple color. However, even with EDTA, betacyanin degradation can still occur, eventually forming yellow-orange products like neobetanin [31]. Citric acid, while often used as a stabilizer and preservative in food, can paradoxically promote microbial growth in betacyanin solutions after a few days of stabilization. This occurs because the initial stabilization effects of citric acid, which primarily influence the color and stability of the betacyanin, eventually wear off, leaving the solution vulnerable to microbial contamination [32].

Betacyanins are pH-sensitive, whereby their color changes depending on the acidity or alkalinity of the surrounding environment. At pH between 3 and 7, betacyanins typically exist in an anionic form, giving them a red-purple color. When the pH drops below 3, they can transform into a cationic form, resulting in a blue-violet shade [33]. Above pH 7, the color of betacyanin changed from red-purple to brown. The color change at higher pH values is often associated with the hydrolysis of aldimine bonds in the betalain structure [34].

3.2 Chemical Analysis of Betacyanin

Hydrochloric acid and sodium hydroxide were used to test the presence of betacyanin in betalain dye; the color change from red-purple to yellow indicated its presence and is depicted in Fig. 3. Betacyanin can undergo acid and base bond hydrolysis of betalain, forming colorless cyclo-DOPA-5-O-β-D-glucoside and fairly stable bright yellow betalamic acid. At pH values less than 3, the betalain structure is charged from an anionic form (red-purple) to a cationic form (violet-red) [34].

At pH values greater than 7, there is the aldimine bond hydrolysis of betalain, giving rise to betalamic acid (lemon yellow) and cyclodopa-5-O-glucoside [33]. The N-1 nitrogen atom gets protonated in the acidic media and forms a leaving group. The protonation of the N-16 nitrogen atom enhances the electrophilic reaction of C-11, C-13, and carbons and catalyzes betacyanin hydrolysis, finally contributing to the opening of a 2,6-dicarboxy-1,2,3,4-tetrahydropyridine ring of betalamic acid [22]. The formation of cyclo-DOPA and betalamic acid as the product of betacyanin degradation in the presence of acid and base is depicted in the reaction shown in Scheme 2.



Scheme 2 The chemical reaction of the betacyanin test

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3.3 Fourier Transform Infrared Analysis of Betalain Dye

The FTIR spectrum analysis of betalain dye obtained from *Iresine herbstii* leaves was done to identify the existence of major functional groups according to libraries and bibliographies. The spectrum (Fig. 4) was obtained in the spectral range of 4000-500 cm^{-1}. The spectrum has different absorption band characteristics of the functional groups of betalain. From the spectra, a strong band from 3220 cm^{-1} to 3412 cm^{-1} was attributed to the carboxylic acid OH stretching vibration. The peak at 2952 cm^{-1} and 2842 cm^{-1} was assigned to asymmetric and symmetric C-H stretching vibrations because of the aromatic ring in the extracted dye. The bending vibration observed at 1645 cm^{-1} was assigned to N-H bonding in the betalamic acid compound contained in betacyanin. The absorption band observed at 1439 cm^{-1} was assigned to C=C of the aromatic ring in the betalain dye. The peak observed at 1013 cm^{-1} was attributed to the C-OH stretch. The infrared spectrum in the Figure shows a wavenumber similar to the research results recorded by Geetha and Sumathy [3], although there is a slight difference, it is still in the same wavenumber range. Since the components of betalain have polar groups such as –COOH, –OH, and $–NH_2$ that give them a water affinity and make them soluble, they are not pigments but dyes [15].

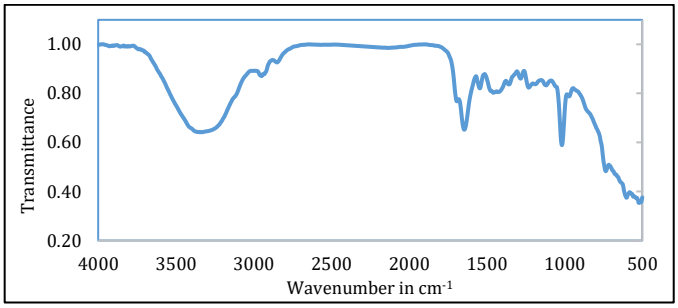

Fig. 4 FTIR spectrum of betalain dye extracted from *Iresine herbstii* leaves

3.4 Quantitative Analysis of betalain Dye Extracted from Iresine herbstii Leaves

Betalain dye extracted from *Iresine herbstii* leaves resulted in a red-purple-colored solution. The dye could absorb photons from the visible light spectrum. A maximum absorbance characterizes the betalain dye at about 535 nm (λ_{max}) for the red-purple betacyanin [35]. The absorption of conjugated double bonds in the betalain structure produced one visible signal [36]. The betacyanin content obtained from *Iresine herbstii* leaves was 730 ± 230 mg/100 g (dw). These were comparable to the findings of Cai et al. [29] wherein authors found that betacyanin content among 21 *Amaranthus* genotypes total betacyanin ranged from 460 to 1990 mg/100 g of dry weight. *Iresine herbstii* has a higher betalain concentration than *Alternanthera* species like *A. tenella,* which has 60-170 mg/100 g (dw).

4. Conclusion

Iresine herbstii leaves contain 0.73±0.23 % (dw) betalain dyes. These results align with studies reporting *I. herbstii* as a moderate betalain source compared to beetroot (0.4-2.0% dw). Chemical analysis results agreed with those previously published for other sources of betalain. Natural dyes from *Iresine herbstii*, in the spectral response to visible light, have been established. The concentration of betalain dye extracted from *Iresine herbstii* leaves was within the range as per the records in the literature, whereby betacyanin content among 21 Amaranthus genotypes, total betacyanin ranged from 460 to 1990 mg/100 g of dry weight. FT-IR results show that dye extracted from *Iresine herbstii* leaves contained C-OH, N-H, C-H, and C=C. From the FT-IR results, C-OH corresponds to the carboxylic group in betalain. The functional group that indicates the presence of betacyanin (red-violet color) compounds is the N-H group, which identifies betalamic acid. Quantitative analysis obtained from UV-Vis results delineated that *Iresine herbstii* leaves are rich sources of betalain. Hence, *Iresine herbstii* leaves extract could be recommended as a source of dye for dyeing fabrics.

Author Contributions

Teresia Wambere Kibicho: Conceptualization; methodology; formal analysis; validation; investigation; visualization; data curation; writing-original draft; writing-review; and editing. Benson G. Ongarora: Conceptualization; supervision; methodology; resources; project administration; writing-review and editing. Paul M. Sang: Conceptualization; supervision; funding acquisition; resources; writing;

review and editing; methodology. Peter Maina. Technical assistance; resources; writing-review and editing; methodology. All authors have read and agreed to the published version of the manuscript.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Competing interests

The authors declare that they have no competing interests.

Acknowledgement

The authors would like to thank Dedan Kimathi University of Technology (DeKUT) for the contribution it has done towards this study.

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https://doi.org/10.30799/jnpr.115.25100102

Cite this Article as: Teresia Wambere Kibicho, Benson G. Ongarora, Paul M. Sang, Peter Maina, Analysis of phytochemicals in *Iresine herbstii* leaves collected from Nyeri County, Kenya, J. Nat. Prod. Resour. 10(1) (2025) 302-305.