International Journal for Multidisciplinary Research (IJFMR)
E-ISSN: 2582-2160 ● Website: www.ijfmr.com ● Email: editor@ijfmr.com

A Comparative Study on Leaf Pigment Variation and Solvent Efficiency in Chromatographic Separation of Ixora Coccinea

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ABSTRACT

Ixora coccinea, commonly called Jungle Germanium, is a flowering plant species in the family Rubiaceae of Order Gentianales with native range in Western and Southern India, Sri Lanka, Bangladesh to Indochina. This paper investigates solvent efficiency for paper chromatography of Ixora coccinea leaves and provides a comparative analysis of leaf pigments in young versus old leaves. Four solvent systems were evaluated: diethyl ether, methanol:acetic acid (7:3), ethanol:water (8:2), and petroleum ether:acetone (9:1). Results demonstrate that leaf aging significantly affects photosynthetic pigment composition, with old leaves showing degraded chlorophyll and prominent anthocyanin retention. Among the tested solvents, petroleum ether:acetone (9:1) provided the most effective separation with distinct pigment bands and optimal Rf values. The study confirms that unsuitable developing solvent choice critically alters chromatographic outcomes and demonstrates the importance of solvent polarity in achieving clear pigment separation.

Keywords: Ixora coccinea, Photosynthetic pigments, Paper chromatography, Developing solvent, Rf values

1. INTRODUCTION

Plant pigments are chemical components that impart color to fruits and vegetables while playing critical roles in metabolic processes, light harvesting in photosynthesis, and defense against photo-oxidative damage. Common plant pigments include betalains, anthocyanins, chlorophylls (a and b), and carotenoids, which also offer potential health benefits when consumed. Chlorophyll a exhibits a bright blue-green color, while chlorophyll b displays a more subdued olive color. Carotenoids manifest as orange, red, and yellow pigments. Anthocyanins are flavonoid pigments found naturally in all tissues of higher plants, providing color to stems, leaves, roots, fruits, and flowers. Based on pH conditions, anthocyanins appear red, blue, purple, and other dark colors. Xanthophyll, a yellow pigment belonging to the carotenoid family, is found in plants and other organisms. Chromatography is an important biophysical technique that enables the separation, identification, and purification of mixture components for qualitative and quantitative analysis. The technique is based on the principle where molecules in a mixture applied onto a surface or into a solid and fluid stationary phase separate from each other while moving with the aid of a mobile phase. Factors affecting this separation process include molecular characteristics related to adsorption (liquid-solid), partition (liquid-solid), and affinity or differences among molecular weights. Due to these differences,

some mixture components remain longer in the stationary phase and move slowly through the chromatography system, while others pass rapidly into the mobile phase and exit the system faster Chromatography techniques based on the stationary bed include column, thin layer, and paper chromatography (Fig. 1). Column chromatography is one of the most common methods of protein purification. This study employs paper chromatography to investigate pigment separation in Ixora coccinea leaves. Various factors affect paper chromatography results, including solvent polarity, paper type, temperature, humidity, pigment overloading, and pigment solubility. Solvent polarity, defined as the measure of a solvent's ability to dissolve other substances based on the evenness of its charge distribution, is particularly critical. Polar solvents such as water and methanol attract polar molecules, while nonpolar solvents such as hexane and toluene attract nonpolar molecules, dictating how fast components move (elute) and separate. Ideal polarity balances interactions for optimal resolution.

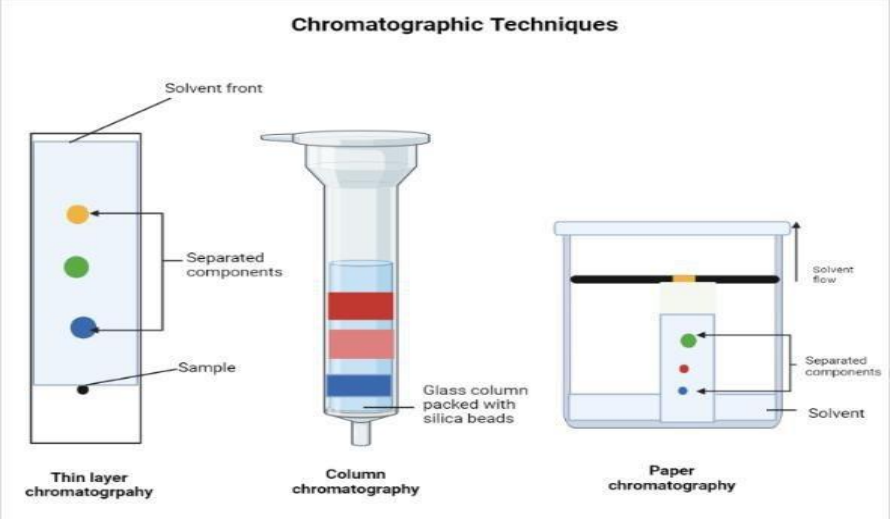


Figure. 1. Chromatographic techniques

2. MATERIALS AND METHODS

2.1. Materials

The following materials were used in this study:

- 6-7 young and old leaves of *Ixora coccinea*
- Mortar and pestle
- Whatman filter paper
- Thin capillary tubes
- 2 watch glasses
- Filter paper
- 2 test tubes
- 4 specimen jars
- Chemicals: Acetone, diethyl ether, ethanol, petroleum ether, methanol, and acetic acid

2.2. Pigment Extraction

Five to six young (light colored) and old (dark colored) leaves of Ixora coccinea were plucked and washed thoroughly with distilled water. Both leaf types were cut and ground separately with acetone using a pestle and mortar until a paste was obtained, adding acetone as required. The pastes were filtered using filter

paper to extract the pigments, which were transferred into separate watch glasses. Six Whatman paper strips (2×10 cm) were cut. A baseline was drawn 2 cm above one end of each strip using pencil. Using a capillary tube, young leaf pigment was applied to three strips and old leaf pigment to the remaining three strips. The pigment application was repeated approximately seven times, allowing drying between applications. Each strip was labeled at the top with pencil to indicate leaf type.

Figure. 2. Leaf samples used for pigment extraction: (left) young leaf showing lighter coloration; (right) old leaf showing darker coloration.

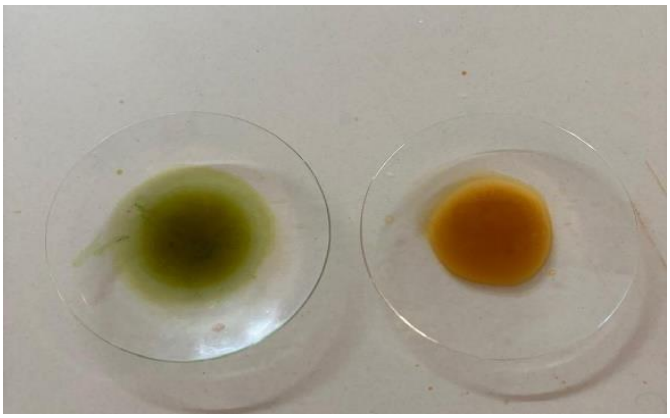


Figure. 3. Pigment extracts obtained after acetone extraction: (left) young leaf pigment extract showing bright green coloration; (right) old leaf pigment extract showing darker coloration.

The leaf samples and corresponding pigment extracts obtained after acetone extraction are shown in Figure. 2 and Figure. 3, respectively.

2.3. Preparation of Developing Solvents

Four solvent systems were prepared:

Ethanol: water (8:2) - 40 ml ethanol and 10 ml water, approximately 30 ml poured into a specimen jar Methanol: acetic acid (7:3) - 45 ml methanol and 15 ml acetic acid, poured into a specimen jar Petroleum Ether: acetone (9:1) - 45 ml petroleum ether and 5 ml acetone, approximately 30 ml poured into a specimen jar Diethyl ether - approximately 30 ml poured into a specimen jar All jars were labelled accordingly.

2.4. Chromatographic Procedure

A glass rod was used to attach one young leaf pigment strip and one old leaf pigment strip using tape. One young leaf and one old leaf pigment strip were placed in each jar such that the baseline did not touch the solvent mixture. The setup was left undisturbed for one hour. The strips were then removed and dried for analysis.

2.5. Data Analysis

The Rf (retention factor) value for each pigment band was calculated using the formula: Rf = Distance

travelled by pigment from baseline / Distance traveled by solvent front from baseline

3. RESULTS

3.1. Comparative Pigment Analysis: Young Leaves vs. Old Leaves

From the chromatograms obtained, different pigments present in the leaf extracts were separated. The Rf values of the pigments indicated the presence of chlorophyll a, chlorophyll b, xanthophyll, and anthocyanin in young leaves but only anthocyanin in old leaves.

Table 1 presents the qualitative pigment composition observed in young and old Ixora coccinea leaves.

TABLE 1 PIGMENT PRESENCE IN YOUNG AND OLD LEAVES

Pigment	Young Leaves	Old Leaves
Chlorophyll a	Present, dark green	Not observed
Chlorophyll b	Present, olive green	Not observed
Xanthophyll	Present, bright yellow	Not observed
Anthocyanin	Present, reddish brown	Present, reddish brown

3.2. Solvent System Comparison

Diethyl Ether: The chromatographic results obtained using diethyl ether are summarized in Table II. Diethyl ether exhibited poor chromatographic performance. Overlapping of chlorophyll a and chlorophyll b bands was observed, preventing clear differentiation between the pigments. Xanthophyll pigment was not clearly detected in the expected position. The corresponding chromatography strip pattern is shown in Figure. 4.

TABLE 2 RF VALUES FOR DIETHYL ETHER SOLVENT SYSTEM

Pigment	Young Leaves			Old Leaves		
	Distance (cm)	Rf Value	Band Description	Distance (cm)	Rf Value	Band Description
Chlorophyll a	7.0	0.833	Intense blue-green	–	–	Not observed
Chlorophyll b	5.0	0.595	Intense olive-green	–	–	Not observed
Xanthophyll	7.8	0.928	Intense yellow, below solvent front	–	–	Not observed
Anthocyanin	0.8	0.095	Faint reddish-brown, smudged	0.5	0.063	Intense reddish-brown

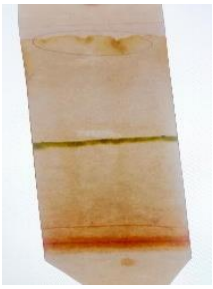


Figure. 4. Chromatography strip obtained using diethyl ether as the solvent system, showing overlapping chlorophyll bands and the absence of a distinct xanthophyll band.

Methanol: Acetic Acid (7:3): The chromatographic results obtained using the methanol:acetic acid (7:3) solvent system are summarized in Table III. The solvent system produced overlapped chlorophyll a and chlorophyll b bands appearing as a single band at the middle of the strip. Xanthophyll was barely visible on the strip edges near the solvent front. The corresponding chromatography strip patterns for young and old leaves are shown in Figure. 5.

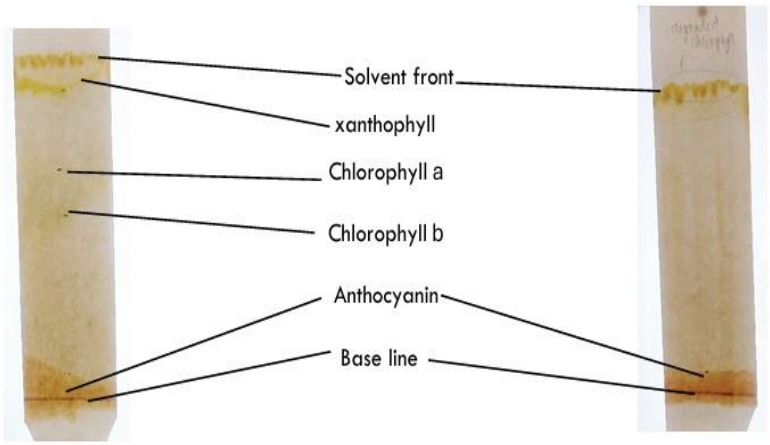


Figure. 5. Chromatography strip results for the methanol:acetic acid (7:3) solvent system: (left) young leaf chromatogram showing overlapped chlorophyll bands at the middle of the strip; (right) old leaf chromatogram showing faint and poorly resolved pigment bands.

Ethanol: Water (8:2): The chromatographic results obtained using the ethanol:water (8:2) solvent system are summarized in Table IV. The solvent system provided good separation of pigments in young leaves. However, old leaf chromatograms showed degraded chlorophyll smudged in patches at the solvent front. The corresponding chromatography strip patterns for young and old leaves are shown in Figure. 6. Degraded pigments including pheophytin, chlorophyllide, and pheophorbide were observed at the solvent front in old leaf chromatograms.

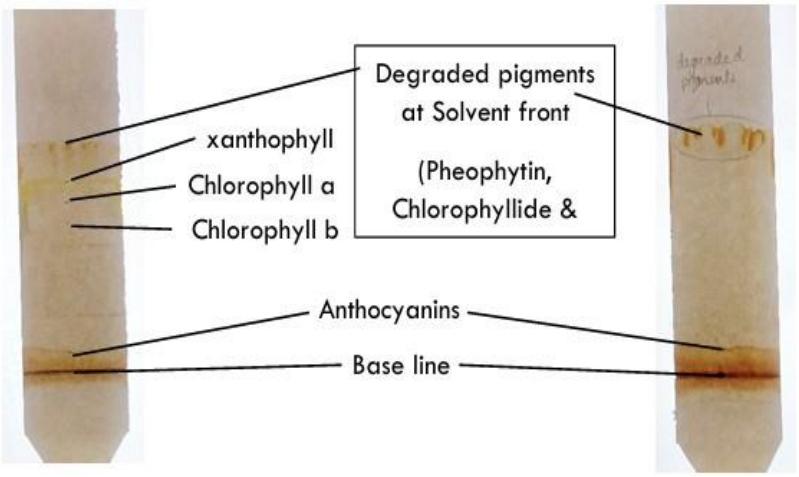


Figure. 6. Chromatography strip results for the ethanol:water (8:2) solvent system: (left) young leaf chromatogram showing well-resolved pigment bands; (right) old leaf chromatogram showing degraded chlorophyll with smudging near the solvent front.

Petroleum Ether: Acetone (9:1): The chromatographic results obtained using the petroleum ether:acetone (9:1) solvent system are summarized in Table V. The solvent system provided the most effective separation with clear, distinct bands for all pigments in young leaves. The corresponding chromatography strip patterns for young and old leaves are shown in Figure. 7.

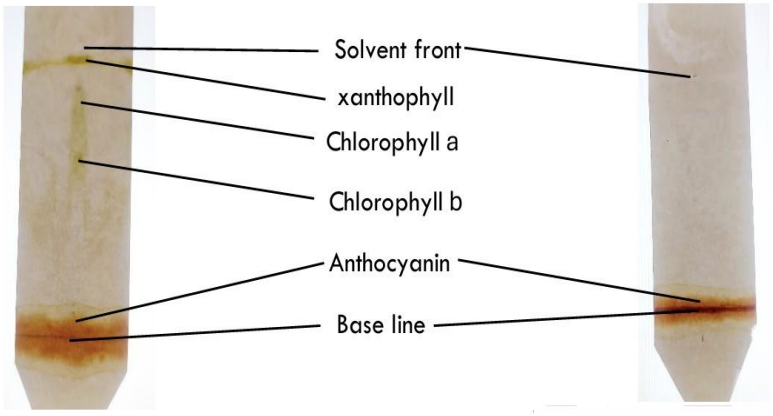


Figure. 7. Chromatography strip results for the petroleum ether:acetone (9:1) solvent system: (left) young leaf chromatogram showing clear and wellresolved pigment bands; (right) old leaf chromatogram showing distinct anthocyanin band near the baseline.

3.3. Graphical Representation of Rf Values

Figure. 8 and Figure. 9 present the comparative Rf values for young and old leaf pigments across different solvent systems. Bar graphs are plotted only for pigments detected in the chromatograms. Pigments not observed in old leaves are not represented. For diethyl ether, due to overlapping of chlorophyll a and chlorophyll b bands, Rf values could not be accurately measured.

TABLE 3
RF VALUES FOR METHANOL: ACETIC ACID SOLVENT SYSTEM

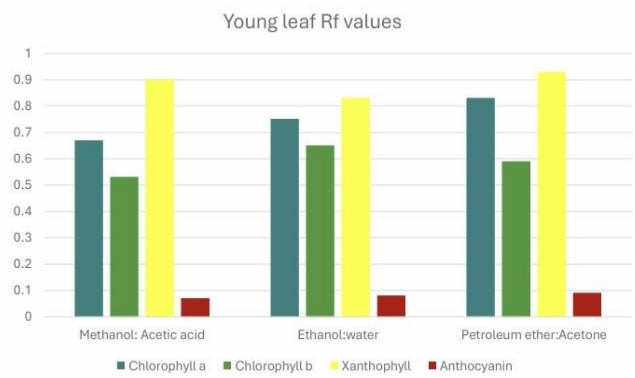
Pigment	Young Leaves			Old Leaves		
	Distance (cm)	Rf Value	Band Description	Distance (cm)	Rf Value	Band Description
Chlorophyll a & b	–	–	Overlapped, single band at middle	–	–	Overlapped, single band at middle, less quantity
Xanthophyll	–	–	Not observed	–	–	Barely visible on edges, present at top near solvent front
Anthocyanin	1.0	0.121	Higher concentration	0.8	0.070	Higher concentration, present at baseline

TABLE 4
RF VALUES FOR ETHANOL:WATER SOLVENT SYSTEM

Pigment	Young Leaves			Old Leaves		
	Distance (cm)	Rf Value	Band Description	Distance (cm)	Rf Value	Band Description
Chlorophyll a	8.0	0.667	Intense blue-green, above chlorophyll b at middle	–	–	Degraded, smudged in patches at solvent front
Chlorophyll b	6.4	0.533	Faint olive-green, below chlorophyll a at middle	–	–	–
Xanthophyll	10.8	0.900	Intense bright yellow at top	–	–	Not observed
Anthocyanin	0.9	0.075	Faint reddish-brown at baseline	0.7	0.066	Intense reddish-brown at baseline

TABLE 5
RFVALUES FOR PETROLEUM ETHER: ACETONE SOLVENT SYSTEM

Pigment	Young Leaves			Old Leaves		
	Distance (cm)	Rf Value	Band Description	Distance (cm)	Rf Value	Band Description
Chlorophyll a	5.3	0.746	Intense blue-green, above chlorophyll b	–	–	Not observed
Chlorophyll b	4.6	0.648	Intense olive-green, below chlorophyll a	–	–	Not observed
Xanthophyll	5.9	0.831	Intense yellow at top	–	–	Not observed
Anthocyanin	0.6	0.084	Faint reddish-brown at baseline	0.8	0.108	Intense reddish-brown at baseline



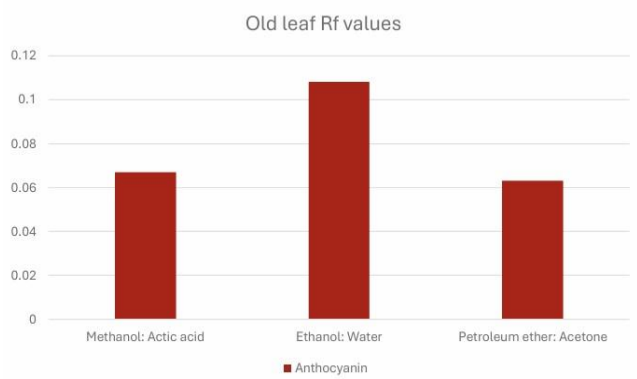


Figure. 8. Comparative Rf values of pigments in young leaves across different solvent systems
Figure. 9. Comparative Rf values of pigments in old leaves across different solvent systems.

4. DISCUSSION

Paper chromatography separates leaf pigments based on their relative solubility in the solvent (mobile phase) and their affinity for the paper (stationary phase). Pigments with greater solubility in the solvent travel farther, yielding higher Rf values.

4.1. Pigment Composition in Young Leaves

In young leaves, distinct bands of chlorophyll a, chlorophyll b, xanthophyll, and anthocyanin were observed, indicating active photosynthesis and the presence of both primary and accessory pigments. Chlorophyll a showed higher Rf values than chlorophyll b across all solvent systems, suggesting lower polarity and greater mobility. Xanthophyll exhibited the highest Rf values, reflecting its higher solubility in the solvents used. Anthocyanin showed very low Rf values and remained near the baseline, indicating strong interaction with the stationary phase and limited solubility in the tested solvent systems.

4.2. Pigment Composition in Old Leaves

In old leaves, chlorophyll pigments were reduced or degraded, while anthocyanin became more prominent, indicating chlorophyll breakdown during leaf aging and associated color changes. During senescence, chlorophyll degrades into compounds such as pheophytin, pheophorbide, and other catabolites, whereas carotenoids degrade more slowly and anthocyanins often persist or increase. Consequently, old leaves display reduced chlorophyll bands and prominent anthocyanin near the baseline. The degraded pigments, being less stable and present in lower concentrations, show irregular solubility and uneven migration during chromatography, resulting in patchy or smudged bands rather than sharp spots.

4.3. Solvent Efficiency Analysis

Diethyl ether was unsuitable for clear chromatographic separation of *Ixora coccinea* leaf pigments due to its low polarity and high volatility, resulting in poor resolution and overlapping bands. Xanthophyll migrated rapidly with the solvent front and spread laterally, accumulating near the paper edges, while rapid evaporation disrupted uniform solvent movement and produced smudged bands. Differences in Rf values among methanol:acetic acid, ethanol:water, and petroleum ether:acetone indicate that solvent polarity strongly influences pigment separation. Overall, the results confirm that both leaf age and solvent characteristics determine separation efficiency.

5. PRECAUTIONS

The following precautions should be observed to ensure accurate and reliable chromatographic results:

1. Use washed fresh leaves and extract pigments quickly to prevent degradation.

2. Only use distilled water for solvent preparation and washing purposes.
3. Draw the baseline lightly with a pencil, not pen, above the solvent level.
4. Apply small, concentrated pigment at baseline or at a spot and allow them to dry before placing in the solvent.
5. Ensure the chromatography chamber is saturated with solvent vapor and the paper strip is placed vertically.
6. Measure solvent composition accurately to maintain proper polarity.
7. Mark the solvent front and distance traveled by pigments from baseline immediately after removing the paper.
8. Minimize exposure of chromatograms and extracted pigments to light and air to reduce oxidation of pigments.
9. Measure Rf values carefully to avoid observational errors.

6. SOURCES OF ERROR

The following factors may have influenced the accuracy and clarity of the results obtained:

1. Pigment degradation due to light and air exposure.
2. Uneven solvent flow from evaporation. 3) Variation in paper thickness or texture.
3. Faint or overlapping bands causing Rf measurement errors.
4. Slightly smudged or large pigment spots.
5. Image and clarity of chromatographic strip results may vary from real outcome.

7. CONCLUSION

Among the solvent systems evaluated, petroleum ether:acetone in 9:1 ratio provided the most effective separation of Ixora coccinea leaf pigments, with clearer and more distinct bands. The obtained Rf values were found to be comparable with standard reported ranges for leaf pigments, with minor variations due to differences in solvent systems and experimental conditions. This study demonstrates that leaf senescence significantly impacts pigment composition, with old leaves showing complete degradation of chlorophylls while retaining anthocyanins. The choice of developing solvent is critical for achieving optimal chromatographic separation, with solvent polarity being a key determining factor in separation efficiency.

8. ACKNOWLEDGMENT

The authors acknowledge Adani Public School, Mundra, for providing laboratory facilities and resources to conduct this research.

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