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Binit Kumar Dwivedi *

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Research Article

Development and Validation of a novel High-Performance Thin-Layer Chromatography method for the Quantification of Bixin in Bixa Orellana Homoeopathic Mother Tincture

Binit Kumar Dwivedi *, Rakhi Mishra, Daya Ram

D.P. Rastogi Central Research Institute of Homoeopathy, Ministry of AYUSH, Govt. of India, Drug Standardization Unit, Department of Chemistry, A-1/1, Sector -24, Noida - 201301, Uttar Pradesh, India

***Corresponding Author:** Binit Kumar Dwivedi, D.P. Rastogi Central Research Institute of Homoeopathy, Ministry of AYUSH, Govt. of India, Drug Standardization Unit, Department of Chemistry, A-1/1, Sector -24, Noida - 201301, Uttar Pradesh, India.

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Abstract

Introduction: Bixa orellana popularly known as 'sinduri' or 'annatto' is a perennial, tall shrub of family Bixaceae. In Homeopathy, Bixa orellana seed is used for preparation of homeopathic medicine. Its seed is effective against dysentery, fever, kidney disorders and cassava poisoning. The purpose of the study to establish High Performance Thin Layer Chromatography (HPTLC) method for quantification of bixin in chloroform extract of Bixa orellana mother tincture.

Materials and methods: For HPTLC study the plate used was of pre-coated silica gel 60 F254 and mobile phase ethyl acetate: n hexane (5:5, v/v). Visualization of plate was observed at UV 476 nm.

Results and discussion: HPTLC result shows under white light, U.V 254 nm and 366 nm spot of bixin was observed at Rf. 0.28. The plate was scanned with a densitometer at UV 476 nm where bixin peak was separated and evaluated. The linear regression analysis data for the calibration plot showed linear relation with respect of peak area of bixin with correlation coefficient (r, 0.9927). The limit of detection (LOD) and limit of quantification (LOQ) were found to be 908.3419ng and 2752.5546ng.

Conclusions: The result showed the amount of bixin in Homeopathic mother tincture of Bixa orellana seed was found to be 835.5µg/milk

Kew Words: HPTLC; bixa orellana; homeopathy; mother tincture; bixin

Introduction

Bixa orellana the sindoor plant or with a common name "lipstick tree" is an ornamental plant mainly found in South America, Central America, Caribbean islands and in some parts of Asia[1]. In Asia *Bixa orellana* plant is mainly grown in India, Philippines and in Sri Lanka. In India a medium sized tree grows throughout hotter part of India and cultivated in states like Orissa, Andhra Pradesh and Maharashtra as an ornamental tree[2].In India *Bixa orellana* known as 'Sinduri' or 'latkan' and its english name is 'annatto', German name is 'orlean' while in Brazil it is popularly known as 'urucum' among indigenous communities[3]. *Bixa orellana* medicinal plant is popularly known in Ayurveda and in Brazilian and Philippines traditional medicinal system. Its seeds are slightly purgative and possess nutritive value. In West Africa, New Guinea, Guyana *Bixa orellana* seeds mainly used for the treatment of dysentery, fever and kidney disorders and used in case of cassava poisoning[4]. *Bixa orellana* showed a broad spectrum of antimicrobial activity. In traditional medicinal system it is used as a gargle for sore throats and oral hygiene. *Bixa orellana* seed serum is used as a condiment as well as laxative, cardio tonic, hypotensive, expectorant and antibiotic[10-14]. The pulp surrounding the seed is used as, mosquito repellent, haemostatic, anti-dysentric diuretic and is useful to treat epilepsy, kidney and some skin diseases. It is commonly used as aphrodisiac medicine, to treat inflammatory conditions and parasitic diseases. *Bixa orellana* is therapeutically active plant[5] and has potential against chromosomal damage induced by radiation. Also, it used as promising agent against radiations for clastogenic effects of antitumor agents. *Bixa orellana* extract act as good radio protectors for bone marrow. Its non- toxic dose suggests that it may act as promising agent for human radiation[6-9]. Bixin and norbixin are the two carotenoids present in *Bixa orellana* plant which give annatto its unique red colour[15]. Although *Bixa orellana* seeds are reported to contain carotenoids and phenolic

compounds which are responsible for its antioxidant[16] activity. The seeds of this plant produce one of the dyes frequently used in food products textile, paint and cosmetic industries[17].The orange coated pulpy layer of *Bixa orellana* seed is due to the presence of bixin[18].These pigments are valued commercially as food grade dyes. The principal pigment is bixin. It is cis-form of mono methyl ether of carotenoids. The dicarboxylic acid nor-bixin is formed by the saponification of bixin. Bixin is used all over the world as red- orange dye is a major carotenoid pigment, which comprises 70-80 % of the seed coat, is bixin (9Z-6, 6'-diapocarotene-6, 6'-dioate). Other than that annatto also contains a mixture of nor-bixin, β-carotene, cryptoxanthin, lutein, zeaxanthin, and methylbixin[19].

Materials and methods

Plant material

In this study *Bixa Orellana* seeds were collected from Homeopathic Pharmacopoeia Laboratory (HPL, Ghaziabad), Ministry of AYUSH Government of India. Authentic plant material was used to prepare the mother tincture.

Chemical and reagent

Bixin($C_{25}H_{30}O_4$), stored at -20˚C, purity by HPLC>=90 % purchased from Sigma-Aldrich, India. Solvents used were ethanol, methanol, HPLC water and chloroform of analytical grade purity (Merck Ltd., India).

Preparation of Homoeopathic Mother tincture

100 g of coarsely dried powdered *Bixa orellana* seeds were taken, in which 300 mL Purified water and 700 mL strong alcohol was added to make one thousand milliliters of the mother tincture using the percolation method (as per Homoeopathic Pharmacopoeia of India). This tincture was transferred to a tightly packed amber glass container and stored for further study[20].

Quantification of Bixin by High performance thin layer chromatography

Preparation of standard Bixin

Dissolved 1 mg of Bixin in 1mL ethanol in eppendroff tube and sonicated for 10 minutes to prepare working standard of Bixin with concentration 1mg/mL.

Preparation of chloroform extract

25 mL of Mother Tincture were taken in a 50 mL beaker. To remove the ethanol, solution was evaporated on water bath and extracted three times with 20 mL chloroform. Combined and concentrated chloroform extract upto 2 mL volume. Carried out TLC of chloroform extract of mother tincture and reference standard bixin on silica gel 60 F_{254} pre- coated plate

HPTLC Instrumentation

For HPTLC quantification study a densitometric HPTLC CAMAG Linomat 5 (Switzerland) system, was used. Analysis was performed on aluminium-backed silica gel 60 F_{254} pre-coated plate (E. Merck) 20 × 10 cm plate with an aid of sampling machine and solvent front was run up to 70 mm height. Sample was applied to the plate by CAMAG Linomat 5 automatic sample spotter with the help of Hamilton 100 µL syringe. TLC plate was developed in a saturating chamber CAMAG Twin Trough glass chamber (20 × 10cm). Densitometry was performed with a Camag TLC Scanner and software vision CATS were used.

Procedure

1µL to 7 µL standard solution and 1µL to 5µL sample solution volumes were applied on a pre-coated silica gel 60 F_{254} plate (Merck) of uniform thickness (0.2 mm). The plate was developed in chamber containing mobile phase ethyl acetate: n hexane (10:10, v/v) and TLC spots were visualized at room temperature light, 254nm and 366nmby TLC visualizer 2. (figure 1, 2 and 3). The plate was again scanned at 476 nm for bixin.

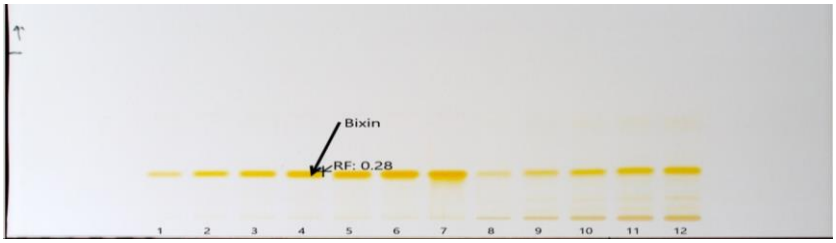

Figure 1: High-performance thin layer chromatography fingerprints of *Bixa orellana* seed at white light. Track (1-7) standard Bixin, Track (8-12) chloroform extract of mother tincture

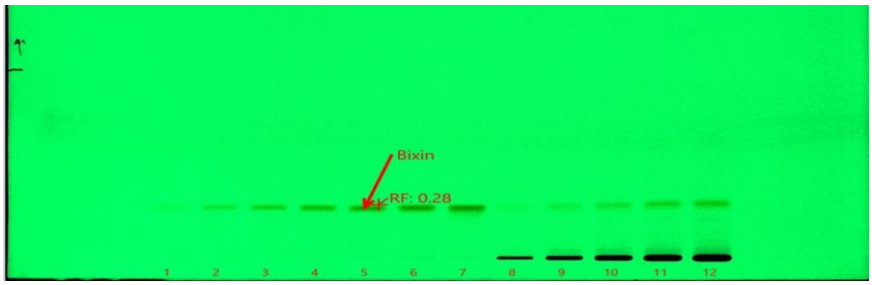

Figure 2: High-performance thin layer chromatography fingerprints of *Bixa orellana* seed at UV 254nm. Track (1-7) standard Bixin, Track (8-12) chloroform extract of mother tincture

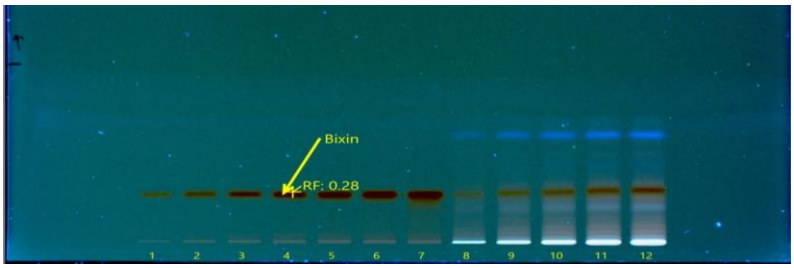

Figure 3: High-performance thin layer chromatography fingerprints of *Bixa orellana* seed at UV 366nm. Track (1-7) standard Bixin, Track (8-12) chloroform extract of mother tincture

Calibration curve for bixin

1µL to 7 µL standard solution of bixin were applied on TLC plate. The plates were developed in a solvent system of ethyl acetate: n hexane (10:10, v/v), at 25˚C up to distance 7 cm. After development, the plate was dried in air at room temperature and scanned at 476 nm for bixin. The calibration shows a linear relationship between the peak area and the concentration of bixin with a good linearity response.

The equation of linear regression curve obtained was Y= mx + C = 4.933x10-9x+5.97x10-3 with a correlation coefficient (R) = 0.99273 and relative standard deviation (RSD %) = 4.54 % (Figure 4).

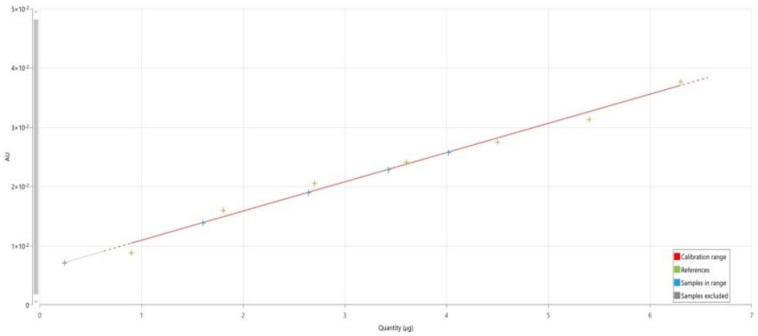

Figure 4: Calibration curve of bixin and mother tincture.

Limit of Detection and Limit of Quantification

Limit of detection (LOD) and Limit of quantification (LOQ) were determined by using the formula based on standard deviation response and the slope obtained. LOD and LOQ were calculated by using equation:

LOD=3.3xσ/slope and LOQ=10xσ/slope.

Where σ = the standard deviation of the response

S = the slope of the calibration curve

The signals to noise ratio 3:1 and 10:1 were used to measure LOD and LOQ, respectively. The LOD and LOQ of bixin were found to be 908.3419 ng and 2752.5546 ng.

Quantification of bixin in Homeopathic mother tincture

1µL to 7 µL standard solution of bixin and 1µL to 5µL sample solution volume were applied on TLC plate. The plate was developed and scanned at 476nm. According to AU value (maximum absorbance). The peaks were recorded at 476nm. The amount of bixin found in homeopathic mother tincture was 835.5µg/mL.

Results and Discussion

HPTLC Study

A single peak of standard bixin obtained in HPTLC chromatogram. The obtained HPTLC calibration curve shows a linear relationship between the peak area and the concentration of bixin with a good linearity response (Figure 4). For monitoring and selection of optimum wavelength, multi wavelength (MWL) scan mode was selected during the scanning process. At 476 nm optimum wavelength absorption maxima of bixin was observed. After development of plate when the plate was scanned at 476nm (Figure 5) the obtained 3D isometric chromatogram showed the presence of bixin in chloroform extract of *Bixa orellana* mother tincture(Figure 6).HPLTC quantification study confirms the presence of bixin in chloroform extract of *Bixa orellana* homeopathic mother tincture and the amount bixin quantified was 835.5µg/mL.

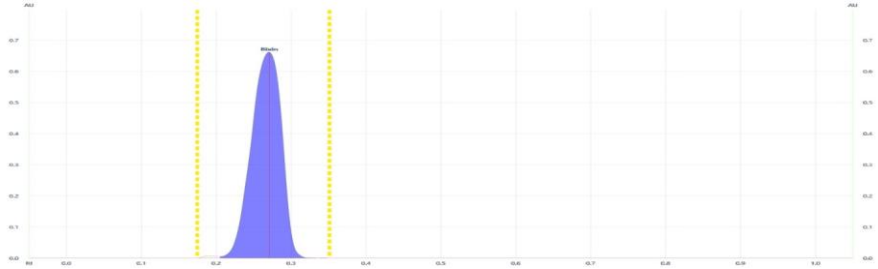

Figure 5: Densitogram of Standard Bixin. At R_f 0.28

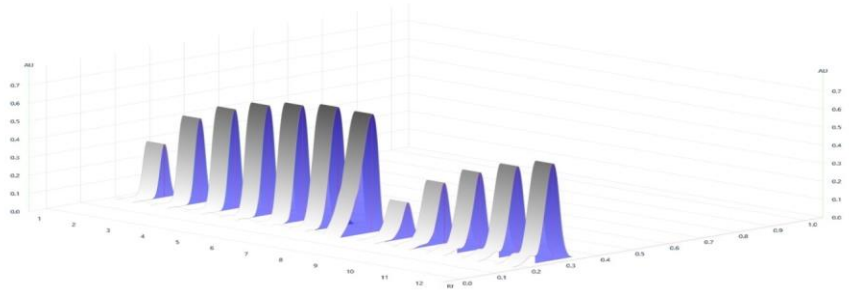

Figure 6: 3D Chromatogram of standard and in-house mother tincture.

UV spectroscopic analysis

Spectrophotometer set at 200 nm; samples and standard bixin were put in cuvette. Before analysis cuvettes were washed with ethanol and analysis were performed on win aspect software was used for the UV Analysis. Samples (in house) used for U.V analysis were prepared by mixing one part of Mother tincture and ninety nine parts of absolute alcohol (1:99) and filtered through membrane filter prior to U.V. analysis. Analysis peak were observed at λ max. 476 nm Bixin, is a natural carotenoids having a total of 11 conjugated double bonds confers efficient antioxidant properties, especially for reactive oxygen species such as singlet oxygen. With every double bond added, the bixin absorbs photons of longer wavelength (and lower energy), and the compound ranges from yellow to red in color causing the energy gap between the HOMO and LUMO, bringing the absorption band from the UV to the visible region. The HPTLC scanning of *Bixa orellana* mother tincture performed at optimum wavelength at 476 nm. The scanning report obtained after integration. After scanning, separation, identification and quantification of bixin in in-house mother tincture obtained. This quantification may lead to better quality checking of market samples which in turn will be responsible for better therapeutic efficacy.

Conclusions

In summary, the proposed method for quantification of Bixin was successfully validated. It is concluded that on the basis of HPTLC study *Bixa orellana* In-house Homoeopathic mother tincture contain Bixin of concentration 835.5µg/mL.

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