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Determination of Digitoxin by High-performance thin layer chromatography in Digitalis purpurea

Rakhi MİSHRA[1, 2], Binit DWİVEDİ[2*], Deepshikha GUPTA[1]

1 Amity Institute of Applied Sciences, Amity University, Department of Chemistry, Sector 125, Noida, Uttar Pradesh, India

2 Dr. D.P. Rastogi Central Research Institute of Homoeopathy, Under Central Council for Research in Homoeopathy, Ministry of AYUSH, Govt. of India, Drug Standardization Unit, Department of Chemistry, Uttar Pradesh, India

ABSTRACT

A quantitative HPTLC method has been established for quantification of digitoxin in chloroform extract of *Digitalis purpurea* leaves. For HPTLC quantification method, the plate used was of pre-coated silica gel 60 F254 and mobile phase was Ethyl acetate: Methanol: Water in the ratio of volume (8.0:1.5:1.0, v/v/v) and UV detection was performed at 219nm. Four samples of mother tincture were used for the study, in-house-mother tincture (A) of *Digitalis purpurea* and market samples (B, C and D) of renowned brands. Result shows that the mother tinctures prepared by authenticated plant sample showed maximum amount of digitoxin as compared to the mother tinctures procured from the market. The present study suggested that the mother tincture of *Digitalis purpurea*, contained a cardiac glycosides (digitoxin) justifying its medicinal usage in Homeopathy. This is the reason for cure and healing property of leaves of *Digitalis purpurea*.

Keywords: *Digitalis purpurea,* HPTLC quantification, digitoxin, Homoeopathic drug

INTRODUCTION

Digitalis purpurea is commonly known as Foxglove[1] is a biennial plant belongs to the family Plantaginaceae. It is indigenous to the part of western and south-western Europe. In India, it is found in Nilgiri hills of Tamil Nadu, southern

*Corresponding author: Dr. Binit Dwivedi
E-mail: dbinit@gmail.com
ORCIDs:
Rakhi Mishra: 0000-0002-7695-1705
Binit Dwivedi: 0000-0002-9071-8432
Deepshikha Gupta: 0000-0001-7199-3618
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Sikkim and eastern Himalayan region. It is herbaceous biennial shrub grows in the colder region of Himalayan. Its leaves contain both primary and secondary glycosides. Among primary glycosides, purpurea glycoside A, purpurea glycoside B[2]and among secondary glycosides, digitoxin, gitoxin and getaloxin are most pronounced. Its leaves contain flavones, anthraquinones, saponins, degalactotigonin and F-gitonin[3]. Digitoxin is the main active constituent of *D. purpurea* plant which is used as cardiac glycosides in medicines.

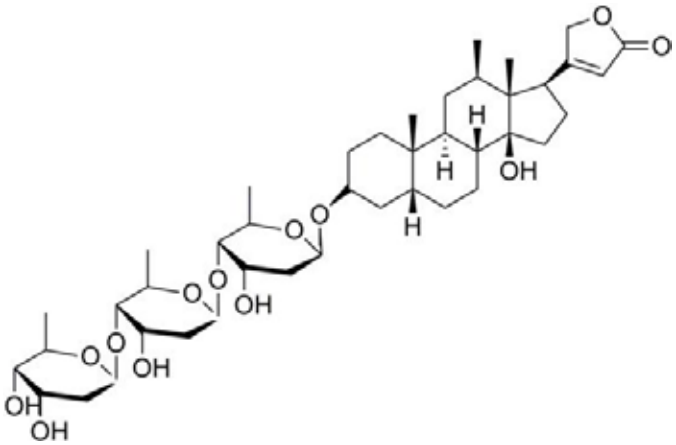


Figure 1. Structure of Digitoxin

Digitoxin (Figure 1) is generally known as highly toxic by-product[4]. In Homeopathy *D. purpurea* is mainly used for the treatment of heart related disease where the heart is primarily involved where the pulse is weak, irregular and intermittent, abnormally slow and dropsy of external and internal parts. Weakness and dilatation of the myocardium and in other symptoms of heart disease such as great weakness, sinking of strength, faintness, coldness of skin and irregular respiration, cardiac irritability, jaundice from in duration and hypertrophy of the liver. In female during labor pain in abdomen and back before menses and for uterine haemorrhage[5] *D. purpurea* homeopathic medicine is recommended in Homeopathy. As per the information given in homeopathic book Materia Medica Pura, *D. purpurea* is mainly used for disease where heart is primarily involved[6], such as in case of atrial flutter, atrial fibrillation and in case of congestive heart failure conditions. In case of congestive heart failure[7] situation, our heart is unable to pump blood effectively at the rate that meet the need of the metabolizing tissues. Our muscles weakly perform the contraction and weakly force the blood out of heart which reduces the contraction rate of heart output and increase rate of heart input leading to increase in the heart

blood volume. Because of this reason our heart feels congested. Cardiac glycosides[8] such as digitoxin present in the leaves of *D. purpurea*[9] helps to prevent congestive heart failure by increasing the force of contractions of heart in the body. In previous suggested studies digitoxin present in the leaves of *Digitalis* has highest gastrointestinal (GI) absorption 90-100% with half-life of 4-5 days[10] which is greater than other commercially available cardiac steroids such as Digoxin, Deslanoside and Ouabain11. Due to rich in active constituents *D. purpurea* shows cardiovascular, cytotoxic[12], antioxidant, anti-diabetic[13], insecticidal, immunological, cardio protective, hepatoprotective and neuroprotective effect and has greater importance in Homeopathy like alternative system of medicine[5]. The purpose of this study is to determine the concentration of digitoxin in *D. purpurea* Homeopathic mother tincture by High performance thin layer chromatography.

METHODOLOGY

Collection of Plant materials

The plant material of *D. purpurea* was collected and authenticated by staff at Center of Medicinal Plants Research in Homoeopathy (CMPRH), Emerald, Tamil Nadu. The voucher specimen was deposited in the herbarium and in the laboratory of DDPR CRI (H), Noida, Uttar Pradesh, India, for future reference with collection number 9649. Authentic plant material was used to prepare mother tincture. Digitoxin ($C_{41}H_{64}O_{13}$, Melting Point 240°C with purity by HPLC>92% w/w purchased from Sigma Aldrich, USA. Solvents used were ethanol, methanol, HPLC water and chloroform of analytical grade purity (Merck Ltd., India).

Physicochemical studies for raw drug standardization

Loss on drying

Loss on drying method used for determination of moisture content as per methods recommended in Homoeopathic Pharmacopeia of India[14].Percentage loss on drying was calculated[15].

Foreign matter determination

For foreign matter determination, 100 g of plant raw material has been taken and outspread it in thin-layer. Sample examined by 6x lens or with unaided eye, the foreign organic matter was picked manually. Ratio of total foreign matter weighed and the weight of drug taken gave the % of foreign matter[15].

Total Ash value determination

In drug, the impurity present in the form of organic matter was determined with the help of total Ash value. For its determination, 2 g of the raw drug was weighed in powdered form in a pre- weighed silica crucible. Incinerated the sample in silica crucible by gradually increasing the temperature up to 450°C for 4 hours or until it became carbon free. The crucible was cooled and weighed until constant weight was obtained[14]. Percent of total ash value was then calculated[14]by taking the ratio of loss in weight to weight of sample taken.

Acid-Insoluble ash value determination

After total ash value determination, 25 mL of 5 M hydrochloric acid was added in the dried ash and boiled on water bath for 10 minutes. Concentrated the solution till its color changed to yellow. Acid insoluble matter was filtered using Whatman paper 1 followed by washing with distilled water. The paper was again ignited in crucible at a temperature not more than 450°C for 4hours, after which crucible was kept in a desiccator, cooled and weighed[14].With reference to the originally taken air dried powdered drug, % of acid insoluble ash value calculated[15].

Water -soluble extractive value determination

For determination of water extractive value, 2 g of accurately weighed, air dried powdered drug was put in a conical flask with 100 mL water added in it. The solution was allowed to stand for 24 hours with intermittent shaking of flask after every 4 hours. The water-soluble extractive was filtered using Whatman filter paper. 25 mL of this filtrate was completed dried on a pre-weighed petri plate at 105°C. The increase in weight of petridish was noted to calculate the water-soluble extractive value determination[14].With reference to originally taken air dried powdered drug, % of water-soluble extractive value calculated[15].

Alcohol-soluble extractive value determination

For determination of alcohol soluble extractive value accurately weighed 2 g air dried powdered drug put it in a conical flask and 100 mL absolute alcohol added in it. Keep the whole solution for 24 hours. In each six hours frequently shake the solution for complete mixing and stand for 18 hours. On next day filtered the solution with the help of Whatman filter paper by taking precaution against loss of alcohol. Weighed the empty flat-bottomed Petri dish. For drying heat, the Petri dish with 25 mL of filtrate at 105°C and cooled the Petri dish in a desiccator and weighed[14]. With reference to originally taken air dried powdered % of alcohol-soluble extractive value calculated[15].

Qualitative Phytochemical screening

Phytochemical tests were performed on crude extract for qualitative estimation of cardiac glycosides with all respective testing procedures include glycosides tests, Keller Kiliani test, Raymond's test, Liebermann's test[16] as described in the text book of JB Harborne[17].

Preparation and Standardization In-house mother tinctures/crude extract

100 g of coarsely dried powdered *D. purpurea* leaves were taken, in which 532mL purified water and 468 mL alcohol was added to make one thousand milliliters of the mother tincture[18] using the percolation method[19] (as per Homoeopathic Pharmacopoeia of India). This tincture was transferred to a tightly packed amber glass container and stored for further study. Standardization of mother tincture was conducted to identify the organoleptic and physicochemical properties of mother tincture[20].

Preparation of standard Digitoxin

Dissolved 5 mg of Digitoxin in 5mL ethanol in volumetric flask, and sonicated for 10 minutes to prepare working standard of Digitoxin with concentration 1mg/mL.

Preparation of chloroform extract

25 mL of Mother Tincture (A) and three market sample B, C and D were taken in a 50mL beaker. To remove the ethanol, solution was evaporated on water bath and extracted three times with 20 mL chloroform. Combined and concentrated chloroform extract up to 2 mL volume. Carried out the TLC of chloroform extract of A, B, C and D with reference standard digitoxin on silica gel 60 F254 pre-coated plate.

HPTLC fingerprinting profile study

For HPTLC fingerprinting study a densitometric HPTLC Camag Linomat 5 (Switzerland) system, was used. In HPTLC system, Camag Linomat 5 was used as sample applicator; for development of mobile phase, a saturating chamber Camag Twin Trough glass chamber was used. Camag TLC Scanner and software vision CATS were used. HPLC grade solvents were used for all the extracts solution. Spots were made on silica gel 60 F254 pre-coated plate (Merck) 20 × 10 cm plate with an aid of sampling machine and solvent front was run up to 70mm height. Volume applied for standard 1 to 6 µL and for sample 5-10 µL. Mobile phase used was ethyl acetate: methanol: water (8:1.5:1, v/v/v) and

TLC spots were visualized after illumination at 254 and 366 nm. Anisaldehyde-sulfuric acid reagent solution was used as derivatizing agent for HPTLC profiling. Digitoxin was used as reference standard.

RESULTS and DISCUSSION

Results of Physiochemical and Phytochemical studies:

Phytochemical glycoside tests performed on the crude extract of leaves of *Digitalis purpurea* showed positive results for Keller-Kiliani test, Raymond's test and Liebermann's test. The results obtained for physiochemical studies of raw drug were tabulated in **Table1**. Organoleptic observations of the prepared in-house mother tincture indicated formation of a clear brown solution with characteristic tincture odor. The physicochemical properties of the tinctures of in-house drug sample (A) and comparison with three procured market samples (B, C and D) for parameters like Sediments, pH, total solids, alcohol content and weight per mL were analyzed and tabulated in Table 2.

Table 1. Results of Physiochemical studies of raw drug material

Name of Physiochemical parameter studied	% composition in raw drug
Foreign matter	2.00
Loss on drying	2.73
Water-soluble extractive value	40.00
Alcohol-soluble extractive value	15.00
Total ash value	5.00
Acid-insoluble ash value	0.60

Table 2. Results of physicochemical properties of the tinctures

S.No.	Parameter	In-house tincture	Market tincture samples		
		A	B	C	D
	Sediments	Nil	Nil	Nil	Nil
	pH	4.80	4.92	4.77	5.94
	Total solid	5.36 % w/v	3.36 % w/v	3.84 % w/v	2.01 % w/v
	Wt./mL	0.943 g	0.955 g	0.959 g	0.942 g
	Alcohol content	43.50 % v/v	41.70 % v/v	41.40 % v/v	44.20 % v/v

Result of HPTLC study:

Based on extensive literature reviews, various combinations of solvent systems were studied with an aim to have an appropriate mobile phase composition for best and efficient HPTLC chromatographic separation of digitoxin in *D. purpurea* chloroform extract. In mobile phase chloroform: methanol (9:1, v/v), toluene: ethyl acetate: formic acid (7:5:1, v/v/v), ethyl acetate: methanol: water (8.0:1.1:0.8, v/v/v) no appropriate resolution of band observed whereas in mobile phase ethyl acetate: methanol: water (8.0:1.5:1.0, v/v/v) efficient band resolution of digitoxin observed with improved R_f value of 0.65. Among all the mobile phase combination studied, ethyl acetate: methanol: water in the ratio of volume (8.0:1.5:1.0, v/v/v) was finalized to be ideal one for evaluation of digitoxin in *D. purpurea*. Thus, it was finalized the best appropriate mobile phase composition for entire HPTLC method development study. Table 3 recorded various mobile phase combination used for preliminary screening study for best possible separations.

Table 3. Various mobile phase combinations used for preliminary screening study for best possible chromatographic separations of Digitoxin.

Used Mobile phase combinations for evaluation of Digitoxin	R_f value	Observations
Chloroform, Methanol (9:1, v/v)	0.49	Poor resolution of band
Toluene, Ethyl acetate, Formic acid (7:5:1, v/v/v)	0.12	Poor resolution of band
Ethyl acetate, Methanol, Water (8.0:1.1:0.8, v/v/v)	0.64	No appropriate resolution of band
Ethyl acetate, Methanol, Water (8.0:1.5:1.0, v/v/v)	0.65	Efficient band resolution with improved R_f

Qualitative HPTLC study of in-house mother tincture and market samples

HPTLC study of *D. purpurea* chloroform extract of in-house mother tincture (A), three market samples (B, C and D) and standard digitoxin by using selected mobile phase ethyl acetate: methanol: water in the ratio of volume (8.0: 1.5: 1.0, v/v/v) at U.V light 254nm showed very light brown spots at R_f 0.63 (Figure 2) but no spot was visualized at the same R_f at 366 nm illumination as evident in (Figure 3). For better resolution, anisaldehyde sulfuric acid reagent was used as derivatizing agent. On spraying the plate with anisaldehyde

sulfuric acid reagent, a clear brown spot of digitoxin standard was observed at R_f 0.63 as well as in in-house mother tincture (A) and market samples (B, C and D) (Figure 4). The result of HPTLC fingerprinting profile study confirms the presence of cardiac glycoside digitoxin in in-house mother tincture (A) as well as in commercial market samples (B, C and D). However, by analyzing the separated bands observed in in-house mother tincture (A), commercial market samples (B, C and D) and quantification of bands by studying their densitogram, a quantitative picture was obtained.

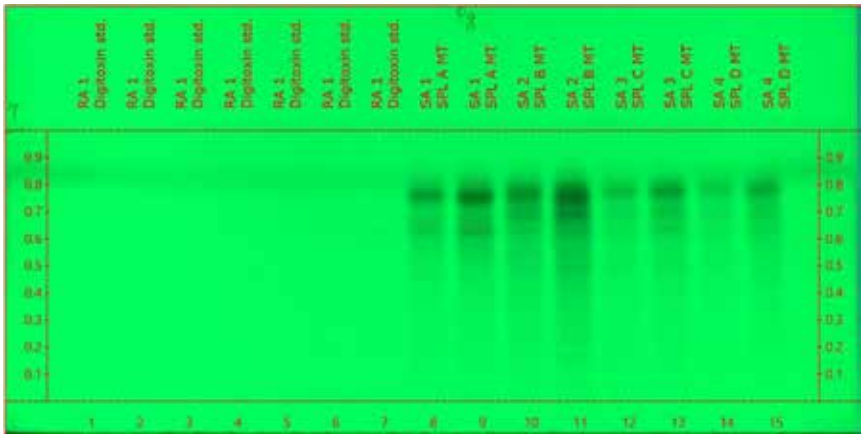


Figure 2. High-performance thin layer chromatography fingerprints of *D. purpurea* at UV 254nm. Standard Digitoxin Track (1-7), Track (8-9) in-house sample A CRI (H), Track (10-11) commercial market sample B, Track (12-13) market sample C, Track (14-15) market sample D.

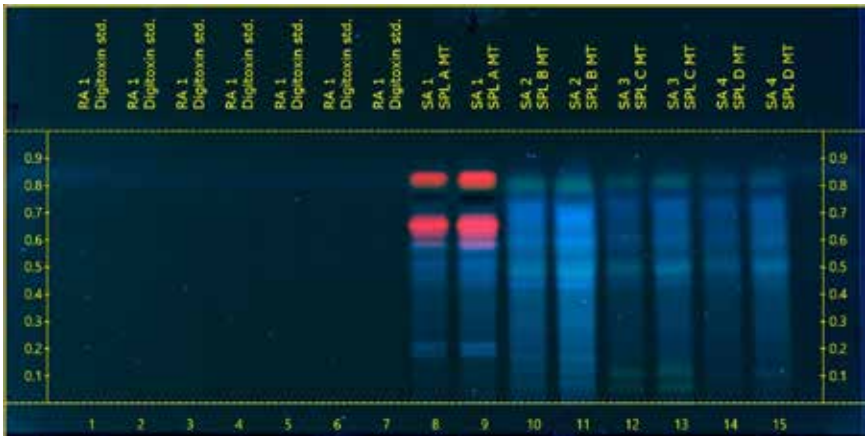


Figure 3. High-performance thin layer chromatography fingerprints of *D. purpurea* at UV 366nm. Standard Digitoxin Track (1-7), Track (8-9) in-house sample A CRI (H), Track (10-11) commercial market sample B, Track (12-13) market sample C, Track (14-15) market sample D.

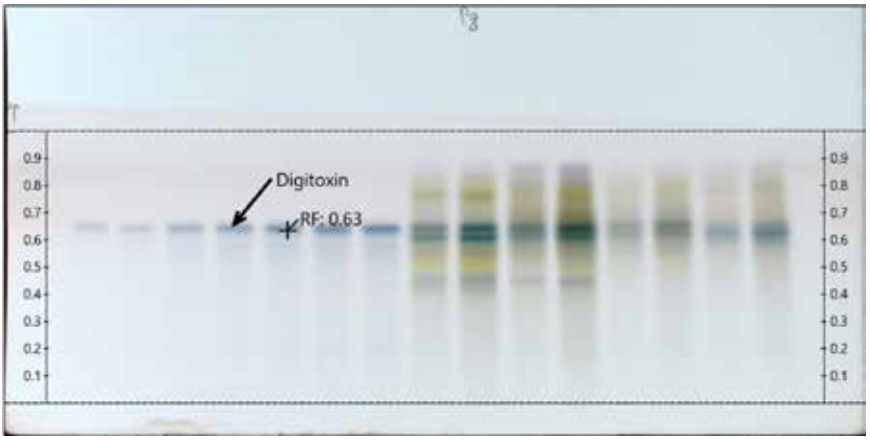


Figure 4. High-performance thin layer chromatography fingerprints of *D. purpurea* after derivatization with anisaldehyde sulfuric acid reagent. Standard Digitoxin Track (1-7), Track (8-9) in-house sample A CRI (H), Track (10-11) commercial market sample B, Track (12-13) market sample C, Track (14-15) market sample D.

HPTLC Quantitative study

To check the linearity response, the volume of the in-house sample was optimized to10 µL for quantification. Different concentrate on range 1000-6000 ng/spot of digitoxin reference standard i.e. 1 to **6µL** consecutively applied on TLC plate. The method was found to be linear with a correlation coefficient (R) = 0.995 in the concentration range 1000-6000 ng/spot. The amount of digitoxin was calculated in in-house mother tincture and available market samples and summarized in Table 4. First seven spots marked were for standard digitoxin (track 1-7), next two spots consecutively applied for chloroform extract of in-housemother tinctures (8-9) followed by three different market samples chloroform extracts (10-15). For comparative study, 10 µLin-house mother tinctures spots applied along with other three available mother tinctures on same plate(Figure 2-4). For monitoring and selection of optimum wavelength, multi wavelength (MWL) scan mode was selected during the scanning process. At 219 nm optimum wavelength showing absorption maxima of digitoxin was observed (Figure 6). For quantification and spectral match, the entire plate was further scanned and summarized. With the help of characteristic spectra, fractions of in-house mother tincture and other marketed samples were matched. In spectral scanning maximum absorbance of each fraction were observed then the plate was scanned with the selected wavelength in MWL mode. On comparison of the peaks pattern of in-house mother tincture and market samples a linearity response for various concentrations of standard digitox-

in in the range of 1000-6000ng with correlation coefficient of (R) 0.995 and relative standard deviation (RSD%) 5.21% was obtained. Thus the developed HPTLC method validation performed as per ICH Q2 R1 guidelines[21]hence the developed method once validated. The quantification of digitoxin in in-house mother tincture and market mother tincture samples was achieved. This study helped in comparison study of in-house mother tincture and market for active principles. (Figure 5-7). Results of this study helped to determine the concentration of digitoxin present in in-house mother tincture (A, track 8-9) and market samples (B, C and D, track 10-15) summarized in Table 4.

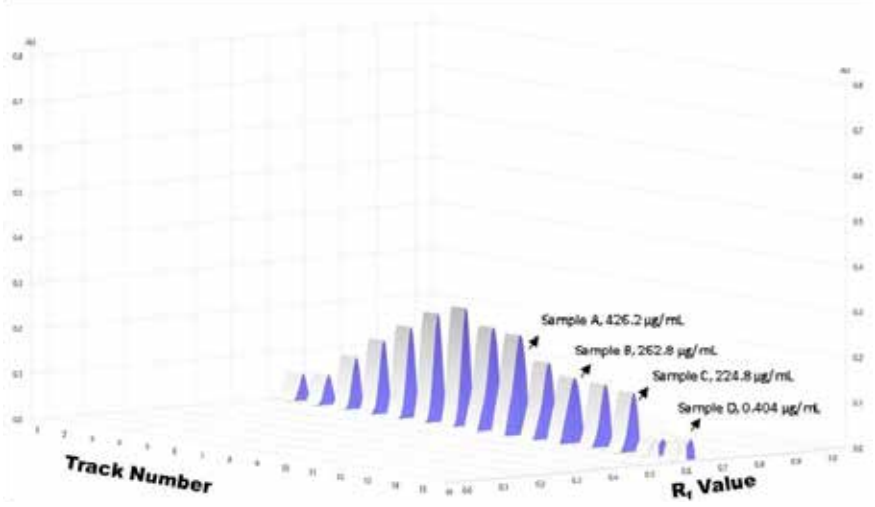


Figure 5. 3D diagram of HPTLC densitogram of chloroform extract of *D. purpurea* in-house sample and market samples with respective standard.

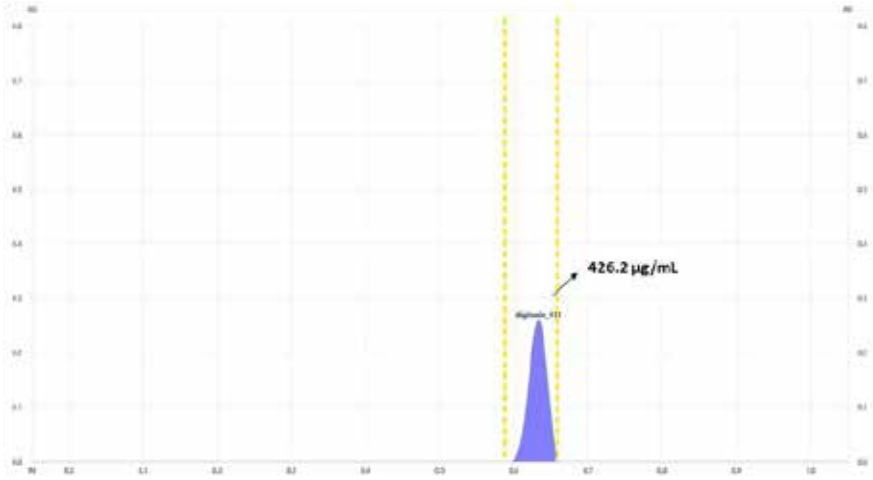


Figure 6. HPTLC peak densitogram display of standard digitoxin present in *D. purpurea* (R_f = 0.63).

Table 4. Concentration of Digitoxin found in in-house sample and market sample of *D. purpurea.*

S.No.	Sample UV detection at 219 nm	Volume applied (µL)	Concentration found in (µg/mL)
1.	Sample A	10	426.2
2.	Sample B	10	262.8
3.	Sample C	10	224.8
4.	Sample D	10	0.404

Method Validation

Linearity

For evaluation of Linearity response six different concentration of standard digitoxin were spotted in the range of 1000-6000ng. A linear relationship between the peak area and the concentration of digitoxin was observed with a good linearity response. The equation of linear regression curve obtained was $Y= mx + c = 1.472\times10^{-9}x +6.818\times 10^{-4}$, with a correlation coefficient (R) = 0.995and relative standard deviation (RSD %) = 5.21% (Table 5).

Limit of detection (LOD) and Limit of quantitation (LOQ)

Based on the standard deviation response and the slope obtained the detection limit may be expressed as:

LOD = (3.3*σ) / S

Where σ = the standard deviation of the response

S = the slope of the calibration curve

Based on the standard deviation response and the slope obtained the quantitation limit may be expressed as:

LOQ = (10*σ) / S

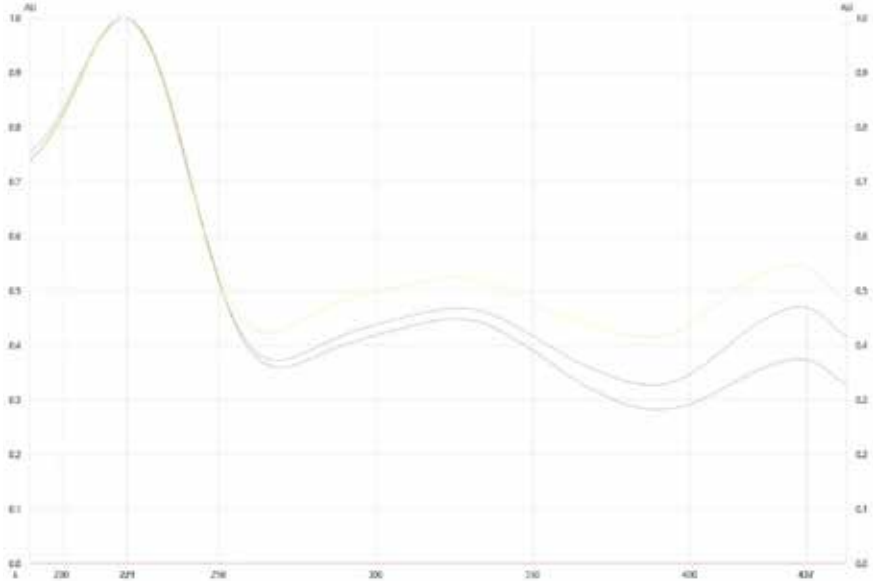
Where σ = the standard deviation of the response

S = the slope of the calibration curve

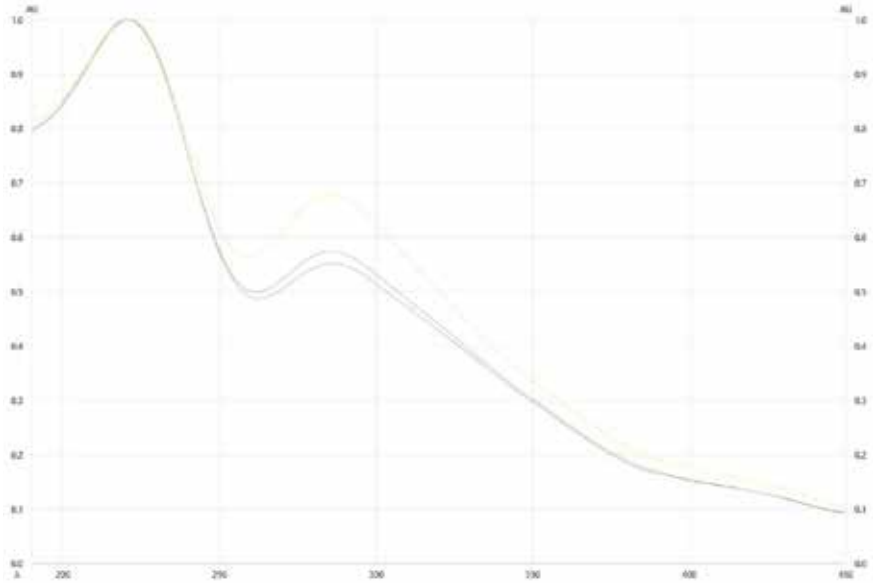
The signals to noise ratio 3:1 and 10:1 were used to measure LOQ and LOD, respectively. The LOQ and LOD of digitoxin were 1000 ng and 300ng/mL (Table 5).

Specificity

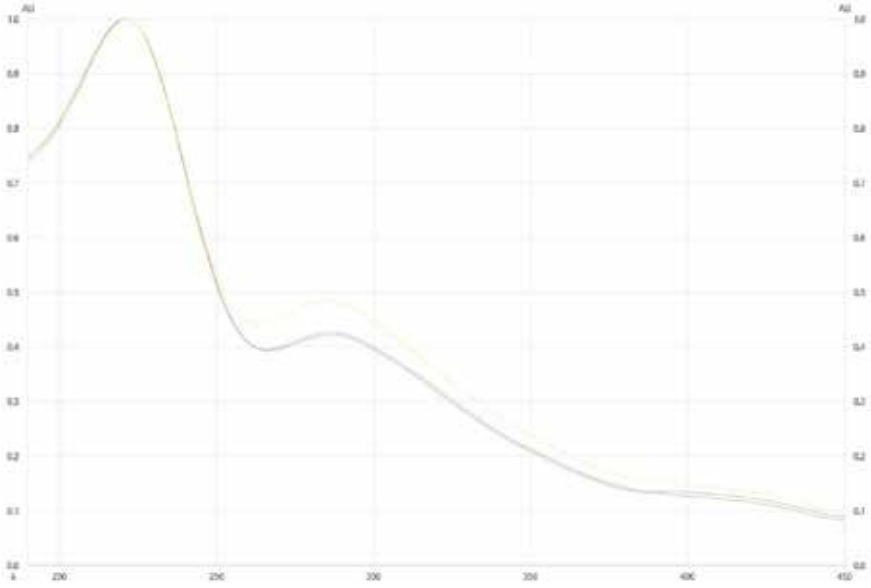
For specificity the plate was run with tracks of in-house sample A CRI (H), market sample B, C and D, standard digitoxin, solvent and mobile phase. The developed plate was scanned at lambda maximum value of standard digitoxin i.e. at 219nm. The observed peak area of standard digitoxin was integrated by using evaluation tab. The observed R_f value of separated concerned standard of digitoxin matched with pure standard R_f. The observed area under integration of standard digitoxin peak contained no any other peak in all tracks i.e. tracks of mobile phase and samples. The developed method was found to be specific as no interfering or contaminating peaks were detected and was also evidence from the peak purity data (Figure 7, Table 5)



(a)



(b)



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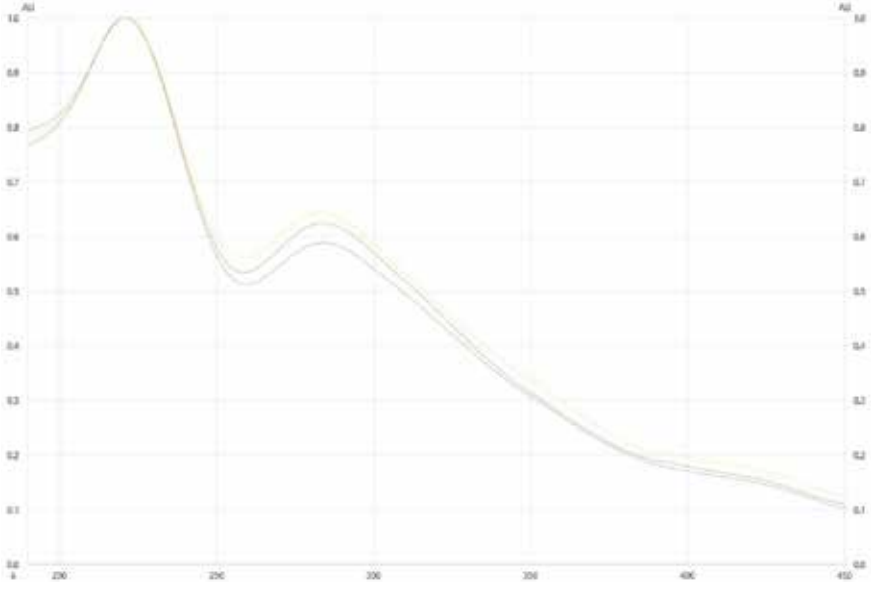
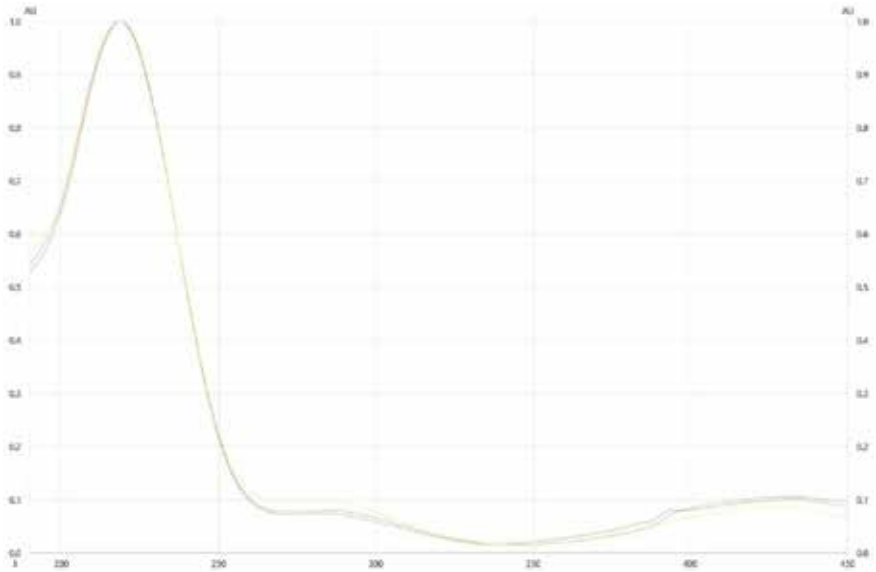


(d)



(e)

Figure 7. Overlay absorption spectra of digitoxin showing purity of up and down slopes; Max. signal @ 219 nm (a) Peak purity of test digitoxin eluted from chloroform extract of *D. purpurea* in-house sample A CRI (H); r (s;m) = 0.992,r (e;m) = 0.995; (b) Peak purity of standard digitoxin; r (s;m) = 0.999, r (e;m) = 0.998; (c) Peak purity of test digitoxin eluted from chloroform extract of *D. purpurea* market sample B; r (s;m) = 0.999, r (e;m) = 0.993; (d) Peak purity of test digitoxin eluted from chloroform extract of D. purpurea market sample C;r (s;m) = 0.999, r (e;m) = 0.998; (e) Peak purity of test digitoxin eluted from chloroform extract of *D. purpurea* market sample D; r(s;m) = 0.999, r (e;m) = 0.998.

Precision/Reproducibility

The precision was evaluated in terms of Intra-day precision and Inter-day precision. The Intra-day and Inter-day precision was used to study the variability of the method. The average coefficient for variance (CV) value observed for Intra-day and Inter-day precision were 1.77% and 1.32%. The observed coefficient for variance CV value is less than acceptable limit of coefficient for variance (CV) 2.4% (acceptable limit of CV as per ICH guidelines is CV≤ 2.4%). Hence the developed HPTLC method for quantification of digitoxin from in-house sample CRI (H) and commercial market sample B, C and D were more precise and reproducible (Table 5).

Accuracy (Recovery)

The accuracy of the developed analytical method expresses the closeness of agreement between the value either as conventional true value or an accepta-

ble reference value and the value found sometimes termed trueness as per ICH Q2 R1 guidelines[21]. To check accuracy of the method, recovery studies were carried out by addition of standard digitoxin solution to pre-analyzed in-house sample solution at three different levels 80%, 100% and 120%. The resultant solution was reanalyzed three times and the best mean recovery % observed 88.90% (Table 5).

Table 5. Summary of validation parameters:

S.No.	Validation Parameters	Results
1.	Linearity (R)	$Y= 1.472\times10^{-9}x +6.818\times 10^{-4}(R) = 0.995$
2.	LOQ	1000 ng
3.	LOD	300 ng/mL
4.	Specificity	Specific
5.	Precision (% CV)	
	Intra-day precision	1.77% i.e. CV≤ 2.4%
	Inter-day precision	1.32% i.e. CV≤ 2.4%
6.	Accuracy (mean recovery %)	88.90%

The result of HPTLC fingerprinting profile study confirms the presence of cardiac glycosides digitoxin in in-house mother tincture (A) as well as in commercial market samples B, C and D at R_f 0.63. However, by analyzing quantitatively in-house mother tincture A has highest concentration of digitoxin i.e. 426.2 µg/mL as compare to the commercial market samples B (262.8 µg/mL), C (224.8 µg/mL) and D has lowest concentration i.e. (0.404 µg/mL) (Table 4).HPTLC Results indicate screening of extraction power of *D. purpurea* in-house CRI (H) sample and commercial market sample towards digitoxin shows maximum amount of digitoxin concentration in in-house mother tincture (A) as compare to market samples B, C and D. Therefore, in-house mother tincture (A) prepared from HPI process is best choice for future development of analytical as well as industrial plant-based developments.

A validated new HPTLC method has been developed for the identification and quantification of Digitoxin from dried chloroform extract i.e. mother tincture of the leaves of *D. purpurea* in-house sample and market samples. Developed HPTLC method was more precise, accurate and specific based on validation parameters. Present study reveals that as part of pre-formulation study, the dried chloroform extract i.e. mother tincture of the leaves of *D. purpurea*

showed promising physicochemical characteristics. The study suggests that the chloroform Leaf extract of *D. purpurea* of cardiac glycosides (digitoxin) medicinal importance that justifies its medicinal usage in Homeopathy. This is the reason for cure and healing property of leaves of *D. purpurea*. For homeopathy medicine it is absolutely necessary to laid down the standards and to check the authenticity of the plant in their extract to ascertain the quality before it enters into the market and utilized for their efficacy. There is essential need for the detailed physicochemical, Phytochemical and HPTLC finger printing study to start work on quality standard of homeopathic medicines. The present study helped in further research development work which will increase the usefulness of the plant *D. purpurea* in complementary and alternative system of medicine. Quantitative estimation of rest of the compounds present in this plant responsible for its other pharmacological activity will be evaluated in future studies.

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