A COMPARATIVE STUDY OF QUANTITATIVE ANALYSIS OF SPECIFIC BIOACTIVE CONSTITUENT I.E. WITHANOLIDE-D IN DIFFERENT PLANT PARTS OF *WITHANIA SOMNIFERA* (L) DUNAL BY USING HPLC-UV AND HPTLC.

ABHAY PRAKASH MISHRA[a*], SARLA SAKLANI[a] AND MUKESH KUMAR[b]

[a*]Department of Pharmaceutical Chemistry, H.N.B.Garhwal (Central) University, Srinagar, Uttarakhand (INDIA).

[b]Department of Chemistry, Uttaranchal College of Science and Technology, Dehradun, Uttarakhand (INDIA).

ABSTRACT

Withania somnifera is an herb commonly used as an ayurvedic medicine. The present study emphasized on the identification and quantitation of withanolide-D on different parts of *Withania somnifera* and compared the presence of withanolide-D compound by using HPLC and HPTLC.All types of chemicals were of analytical and HPLC grade. Extraction process was done by solvent extraction method. Withanolide-D was identified by separation and quantified through HPLC and HPTLC on different parts of *Withania somnifera*. The amount of withanolide-D estimated in leaves was 0.22%, 0.21%; in roots was 0.05%, 0.04%; in branches was 0.12%, 0.10%; in fruits was 0.02%, 0.05% and in fruit covers was 0.22%, 0.17% by HPLC and HPTLC analysis respectively. Hence the current study states that both the instruments were very effective in quantifying withanolide-D which gave at least similar percentage of compound in the different parts of plant and also in HPLC and HPTLC chromatogram.

KEY WORDS: *Withania somnifera* (WS), Withanolides, Withanolide-D, HPLC, HPTLC.

1. INTRODUCTION

Withania somnifera (L.) Dunal (family, Solanaceae), known as *Ashwagandha* is an erect, evergreen, perennial shrub of Asiatic and Southern Mediterranean origin and is widely dispersed throughout the drier tract of country (Gupta, 2007). *W. somnifera* consists of 23 species of the *W. genus* including *W. coagulens* and *W. simonii*. The plant is cultivated in waste places and on bunds. It has been extensively domesticated from the wild form (Winters, 2006). *W. somnifera* has traditionally been grown in South Africa and commercially cultivated in a large scale in India (particularly in M.P.). It is one of the most valued medicinal plant having a potential property of pacifying 'Vata' in herbal drugs (Dhuley, 2001). *W. somnifera* has a wide range of medicinal, pharmacological and therapeutic applications. It is extensively used in Ayurveda, Unani and Sidha medicines as home remedy for various diseases in Indian traditional system. It exhibits immunomodulatory, neurological, adaptogenic, abortifacient, anti-pyretic, analgesic and tonic properties (Ali, 1997) and is used as an important ingredient in different medicinal formulation in national and international markets.

The chemical constituents of this plant have been the targets of many investigations and the structures of many constituents have been characterized (Matsuda, 2001). The major active molecules reported from *W. somnifera* are withanolides. These compounds are structurally diverse steroidal compounds with an ergosterol skeleton in which C-22 and C-26 are oxidized to form δ-lactone (Gupta, 2007). The therapeutic potential of *W. somnifera* has been attributed to the presence of withanolides which inhibit tumor cell proliferation and angiogenesis and also induce phase-II enzymes (Nair, 2006). A large number of withanolides have been identified in *Withania* roots and leaves. Some of these, like withaferin A which have been associated with anti-inflammatory and immunosuppressive properties, sitoindoside IX and X are immunostimulatroy, sitoindoside VII and VIII are anti-oxidants and withanolide-D has anti-tumor activity (Dhar, 2006).

*** Address for Correspondence**
E-mail: abhay_cimap@yahoo.com,
abhaycimap@gmail.com

2. MATERIALS AND METHODS

Plant material

Plant of *W. somnifera* was collected from the garden of National Botanical Research Institute (N.B.R.I.), Lucknow, India and was also identified by B. S. I. (Botanical Survey of India, Dehradun, INDIA).

Chemicals and Instruments

All the chemicals used for the study were of analytical grade purchased from Merck Co. USA and methanol used was of HPLC grade purchased from Ranbaxy chemicals ltd. (Rankem). Water was prepared with Milli-Q water purification and was filtered through a 0.45 μ m filter before use in HPLC (Shimadzu) and HPTLC (Cammag) system. HPTLC plates used were purchased from Merck (Darmstadt, Germany).

Extraction Process

In order to extract the withanolide, the oven dried (40^{0}C) powdered leaves, roots, branches, fruits and fruit covers of *Withania somnifera* (4.0g) were extracted overnight in 20 ml of methanol water (25:75 v/v) at room temperature on shaker and filtered. The filtrate was collected and the residue extracted twice more at 4 h intervals with the same amount of extractants. The filtrates were pooled and extracted with n-hexane (3 × 60 ml). The n-hexane fraction was discarded and methanol-water fraction was further extracted with chloroform (3 × 60 ml). The chloroform fraction was pooled and concentrated upto dryness. The residue was dissolved into HPLC grade methanol (2ml), filtered through 0.45 μ m nylon filter. The solution was further diluted to 10 folds and injected into HPLC and HPTLC.

Quantification of bioactive constituent by HPLC-UV analysis

Details of HPLC condition

Analytical Conditions

Instrument	:	Shimadzu
Software	:	Shimadzu Class VP
Detector	:	UV-VIS
Wavelength	:	227 nm
Column	:	Phenomenex RP-18, 250 x 4.6mm ID, 5ìm
Injection volume:		20ìl
Reference standard:		Withanolide-D
Flow rate	:	0.6 ml/min
Mobile phase	:	A (H_2O, 0.1% acetic acid) / B(MeOH, 0.1% acetic acid).

Gradient : Solvent system was carried out at room temperature (25^{0}C) and was initially at 60% A, changed to 40% A in 30.0min, maintained for the next 2.0min, changed to 25% A in 45.0min, and then to 5% A in 54.0min at a flow rate of 0.6 ml/min changed to 0% A in 55min and this solvent composition was maintained until the run time reached 60min.

Before use the components were filtered through 0.45 μ m nylon filters and de-aerated in an ultrasonic bath. Data was integrated by Shimadzu Class VP series software and results were obtained on comparison with standard. Results are the mean values of three replicate of the same sample. All samples and solutions were filtered through 0.45 μ m nylon filters (Millipore) before analysis by HPLC. To identify the peaks present in the blank mobile phase was treated in the same way.

Quantification of bioactive constituent by HPTLC analysis

Chromatographic Conditions: Chromatography was performed on pre-activated (at 110^{0}C) silica gel 60 F_{254} HPTLC plate (20 ×10 cm; 0.25mm layer thickness). Sample and standard compounds were applied to the layer as 6mm wide bands, positioned 10mm from the bottom of the plate, using an automated TLC applicator Linomat V (CAMAG, Muttenx, Switzerland) with nitrogen flow providing delivery from the syringe at a speed of 10s/ μ l. These critical parameters were maintained for all analyses performed.

Detection of compounds: The development of the TLC layer was performed using a CAMAG twin trough glass tank which had been presaturated with mobile phase for 1h and the solvent front was allowed to run to a height of 8cm. the composition of the mobile phase was optimized by testing different solvent compositions of varying polarities. TLC analysis was made under laboratory conditions of 20±5^{0}C and 50% relative humidity. After development, the layer was dried and the components visualized by immersing the plate in a freshly prepared visualizing agent using an automatic immersion device (CAMAG) followed by heating of the layer at 110^{0}C for 15min with the aid of a CAMAG TLC plate heater.

Mobile Phase :	Chloroform: ethyl acetate: methanol: toluene (70:04:08:24).
Stationary phase:	Silica gel.
Visualizing reagent:	500¼l anisaldehyde + 20ml acetone + 80ml distill water + 10ml (60% perchloric acid).

3. RESULTS AND DISCUSSION

Simple reversed-phase HPLC-UV method, with gradient elution, was used to qualitatively and quantitatively for the estimation of withanolide-D in plant extracts of *W. somnifera*. A typical HPLC-UV chromatogram was obtained using gradient elution of a crude extract, which exhibited a clean and smooth baseline with excellent resolution where all the marker peaks could be identified and quantified. The amount of withanolide-D in different parts of plant is shown in table-1 and table-2.

Hence from ongoing discussion, it was observed that both the analytical tools (HPLC and HPTLC) effectively resolve the peaks for identification and quantification [Fig: 01-03] of a bioactive constituent in different parts of *Withania somnifera*.

4. CONCLUSIONS

This study will be useful to enhance the analysis and cost effective for rapid isolation, separation, identification and quantification of withanolide-D using analytical tools HPLC and HPTLC from *W. somnifera*. The isolated compound has been found in least amount in roots (0.05%) and highest amount in leaves and fruit covers (0.22%) by HPLC technique while by HPTLC it has again been found lowest in roots (0.04%) and higher in leaves (0.21%). Hence HPLC is more effective than HPTLC for separation of withanolide-D from *W. somnifera*.

5. ACKNOWLEDGEMENT

The authors would like to thanks National Botanical Research Institute, Lucknow, (U.P), INDIA, for providing plant samples and recording HPLC and HPTLC data and Head of Department, HNB Garhwal(Central) University,(UK) INDIA.

Table.1. Amount of withanolide-D in various plant parts of *W. somnifera* analyzed by HPLC

Plant Parts	Estimation of withanolide-D (%) using HPLC
Leaves	0.22
Roots	0.05
Branches	0.12
Fruits	0.02
Fruit Covers	0.22

Table.2. Amount of withanolide-D in various plant parts of *W. somnifera* analyzed by HPTLC

Plant Parts	Estimation of withanolide-D (%) using HPTLC
Leaves	0.21
Roots	0.04
Branches	0.10
Fruits	0.05
Fruit Covers	0.17

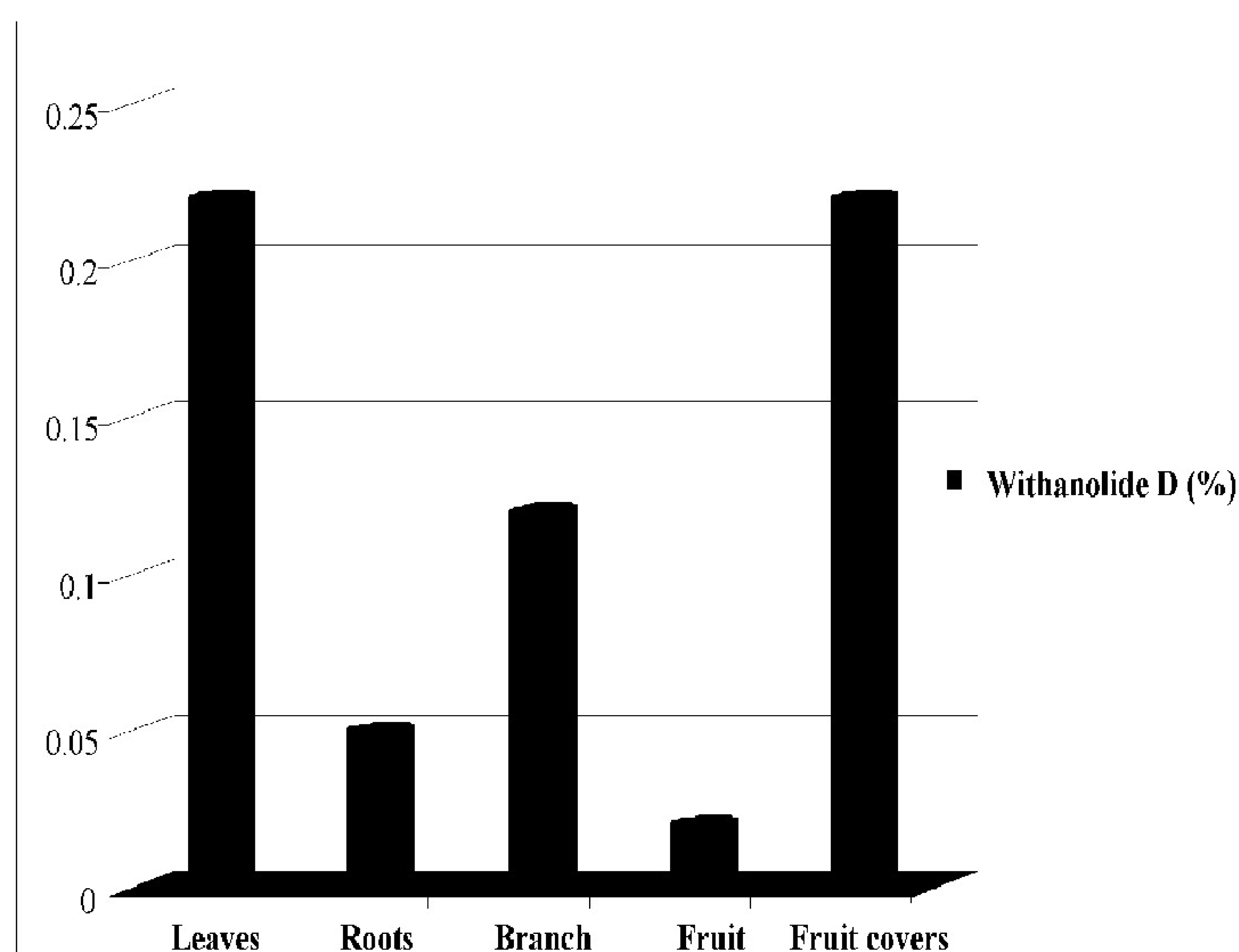


Fig. 01: Amount of Withanolide D (%) in various parts of Withania somnifera by HPLC

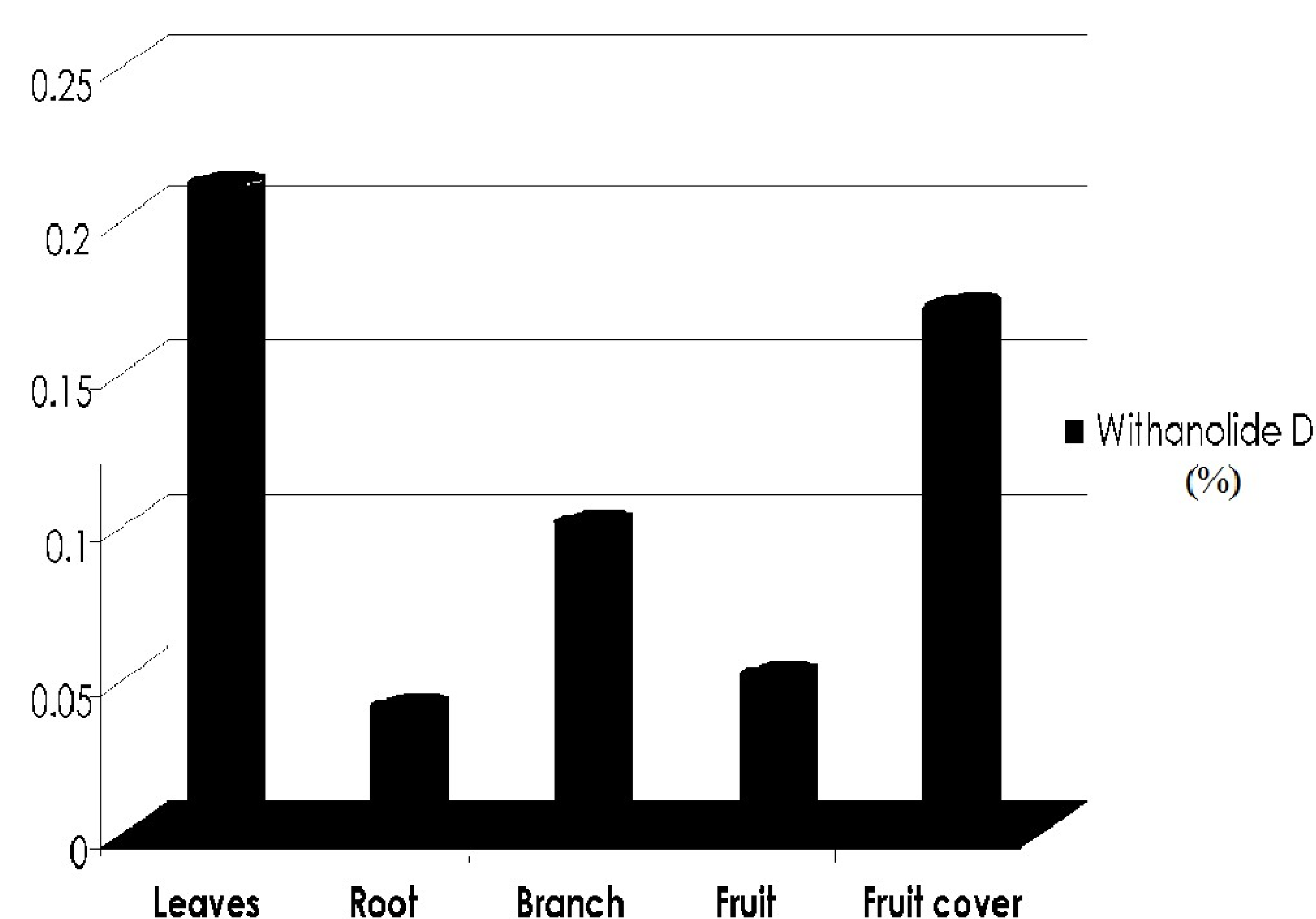
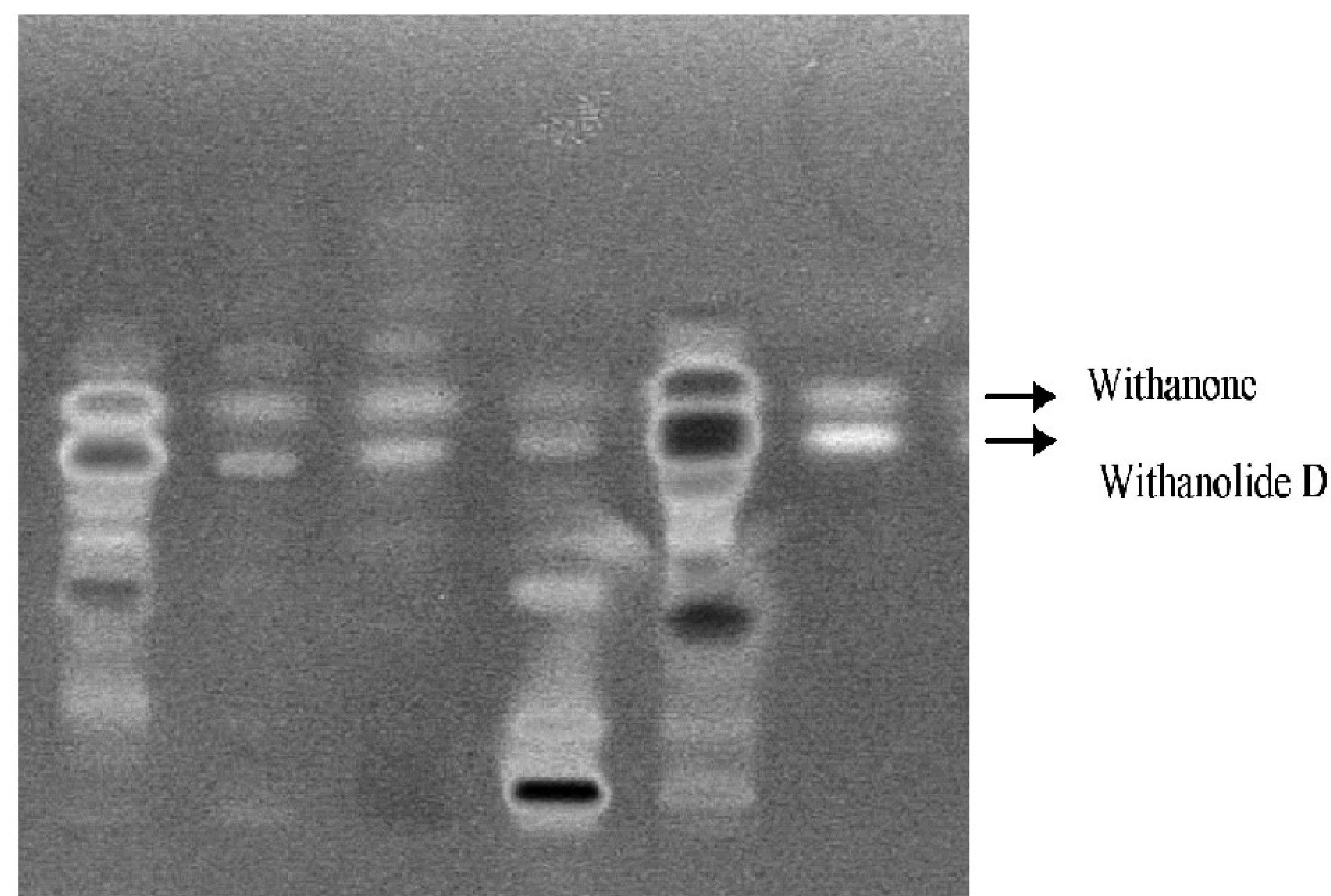


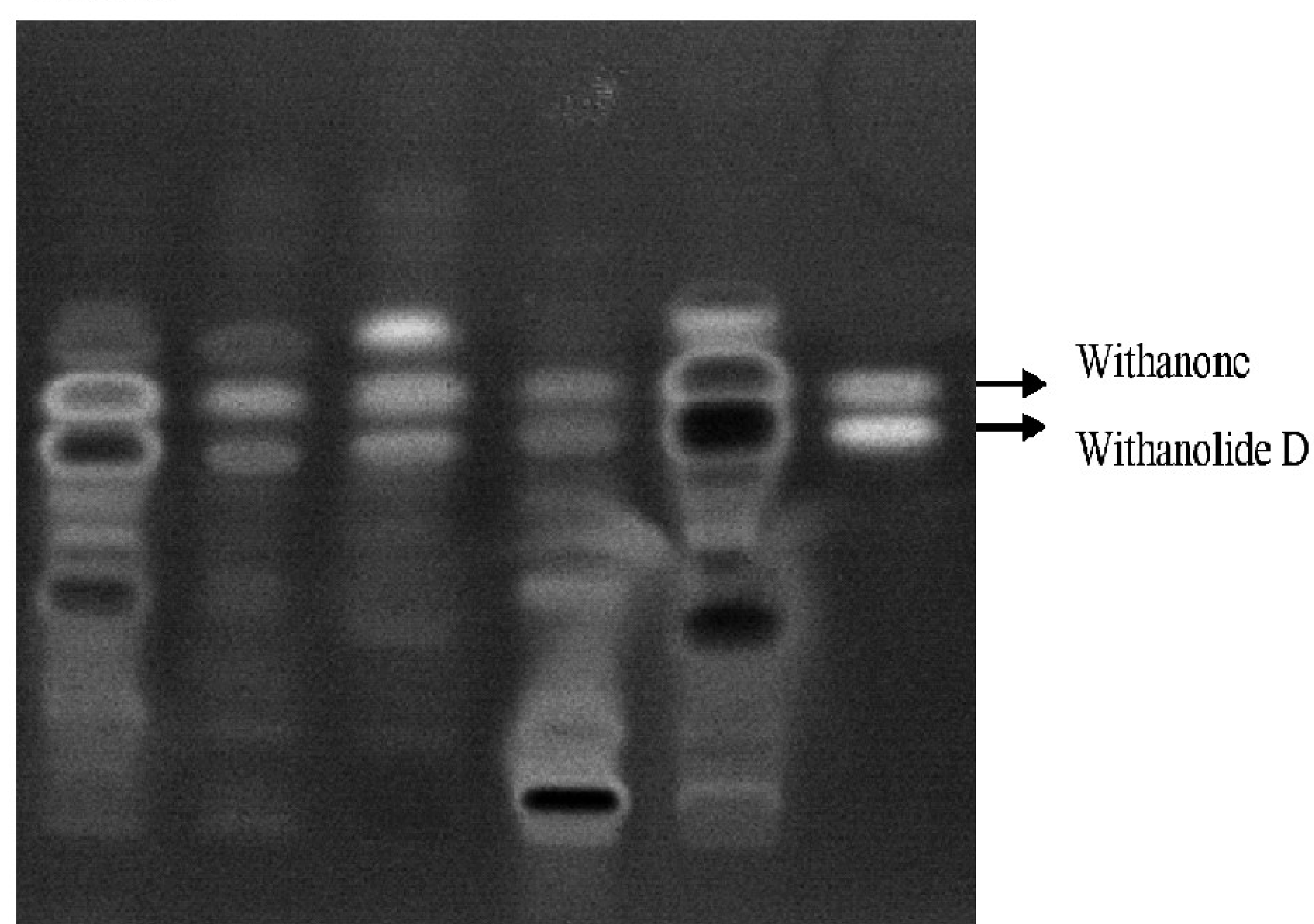
Fig.02: Amount of Withanolide D (%) in various parts of Withania somnifera by HPTLC method.

Fig. 03: Photo documentation of various parts of *W. somnifera* through HPTLC

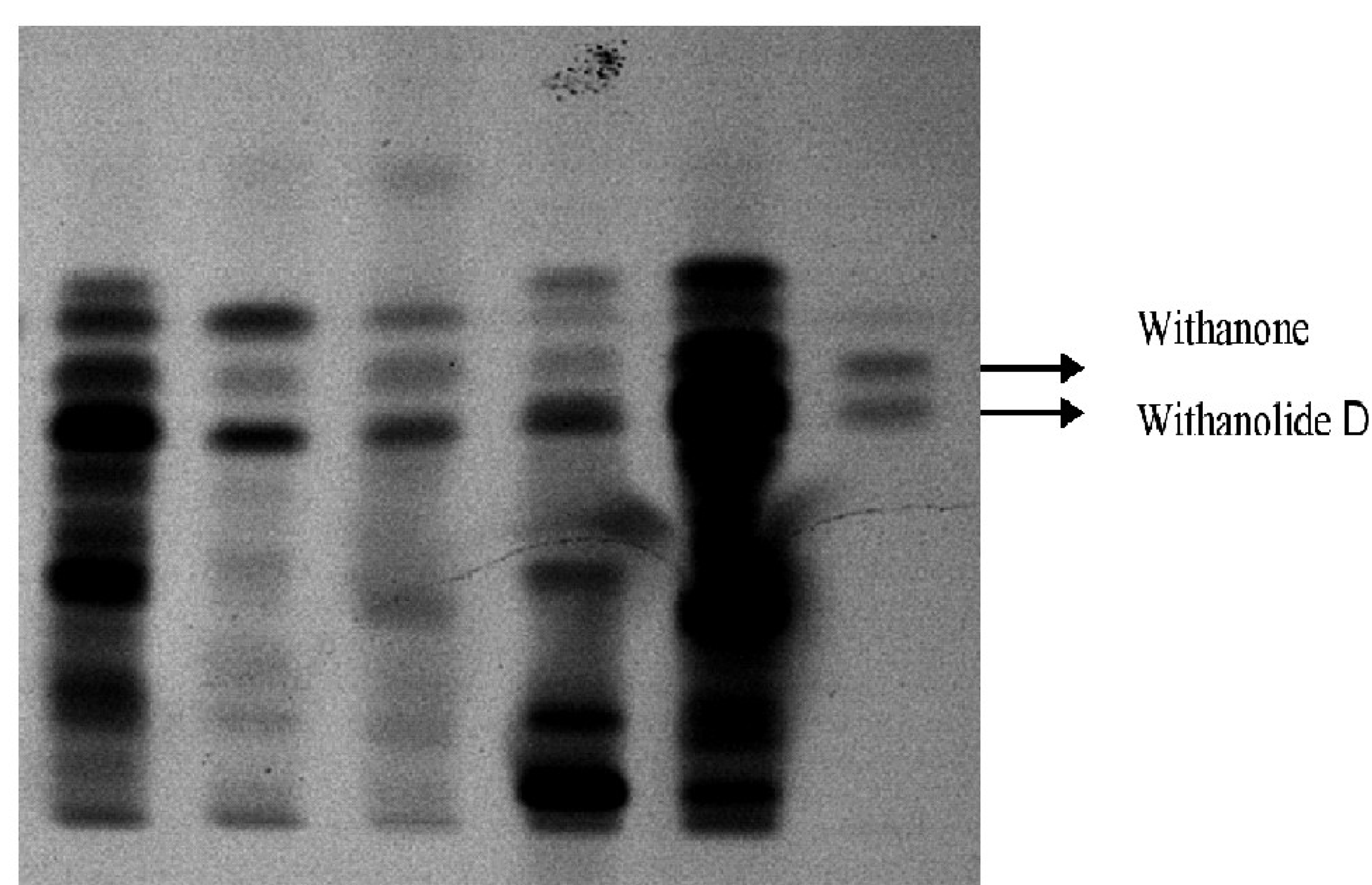
λ max = 254nm



λ max = 366nm



Visible after derivatization



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