American Research Journal of Bio Sciences **Original Article**
ISSN-2379-7959 **Volume 1, Issue1, 2015**

Preliminary Phytochemical Evaluation and HPTLC Fingerprinting of Leaves of *Azadirachta indica*

Sunita Dalal[*], Leena Seasotiya[1], Sheema Bai[1], Pooja Bharti[1], Anupma Malik[1]

[1]Department of Biotechnology, Kurukshetra University, Kurukshetra, Haryana

Rahul Taneja[2]

[2]HSCST,DST,Panchkula,Haryana

Abstract: The fingerprint profiles of methanol extract and ethyl acetate sub fraction of Azadirachta indica leaves were conducted using preliminary phytochemical screening and high performance thin layer chromatography (HPTLC) analysis. Preliminary phytochemical screening was performed using various biochemical tests. Alkaloid, flavonoid, tannin, saponin and phenol were present in both the extract/subfraction. Toluene:Ethyl Acetate:Formic acid::5:4:1 was the finalized solvent system for both the extract and sub fraction indicating it to be the best solvent system for determination of phytochemicals of the studied plant. HPTLC finger print of methanol extract showed 16 peaks and the Rf values ranged in between 0.03 to 0.96. Similarly, ethyl acetate sub fraction confirmed 18 peaks and the Rf values ranged in between 0.03 to 0.95. HPTLC fingerprint analysis of A. indica (leaf) can be used for the exact detection of the plant. Also it is useful as a phytochemical marker and a technique to check the genetic variability in various plant species.

Keywords: Azadirachta Indica, HPTLC Fingerprinting, Solvent System, Phytochemical Analysis.

I. INTRODUCTION

Medicinal plants are traditionally used for thousands of years, in various forms (formulations, herbal preparation, decoctions, bhasmas etc.) in India. Indian medicinal plants possess various pharmacological activities. Not all the medicinal plants used in Ayurvedic preparations/formulations have been investigated in detail. Standardization of plant crude material is becoming today's necessity. Various pharmacopoeia containing plant monographs only explains the physicochemical parameters. Hence, the methods which are helpful in unfolding the correct identification of plant material and quantification of active constituents present in it may be useful in standardization of plant materials [1]. WHO have also put emphasis on ensuring the quality of medicinal plant products using current proscribed methods with suitable standards [2]. HPTLC technique gives better resolution and assessment of active components with reasonable accuracy in a shorter time [3].

Azadirachta indica is an evergreen tree growing up to 100 feet having small bright green leaves. The stem growing up to a diameter of 2.5-3 m. Its bark is rough (pale or greyish-black in color). Tree bears small star shaped flowers (white colour), with a pleasant smell. The fruits (edible) come between the age of 3 -5. *A. indica* is reported to be antibacterial, antifungal [4,5] and helpful in removing dental caries [6]. The present research will focus on the phytochemical screening and development of HPTLC fingerprints of *A. indica* leaves methanol extract and ethyl acetate sub fraction which can be utilized for identification, authentication and characterization.

II. MATERIALS AND METHODS

2.1. Plant Material

A. indica (leaves) were collected from Botanical Garden Kurukshetra University. The plant material was authenticated from Wild Life Institute of India, Dehradun bearing specimen number GS-415.

2.2. Plant Extract Preparation

The leaves were washed under running tap water followed by sterile water. The washed leaves were shade dried for 4-5 days. The dried leaves were ground to powder and stored in airtight containers. Methanol has the capability of extracting a wide range of polar and nonpolar compounds such due to its high polarity therefore it was used for

[*] *Corresponding Author: sdalal@kuk.ac.in*

extraction. 10g of powdered leaves were soaked in 100ml of methanol for 24 hrs in a conical flask. Conical flask was allowed to stand for 30 mins in a water bath (at 100°C) with occasional shaking followed by rotary shaker at 200 rpm for 24h. The preparation was filtered through a sterilized Whatman No. 1 filter paper and finally concentrated to dryness under vacuum at 40°C using a rotary evaporator. The dried extract, was sterilized by overnight UV-irradiation and stored at 4°C till further use [7].

2.2.1. Sub Fractions Preparation

In fraction based studies, methanol extract of *A. indica* was further fractionated with ethyl acetate. For fractionation the test plant methanol extract was dissolved in hot water. Thus prepared aqueous solution of methanol extract was transferred into a separating funnel and was fractionated with ethyl acetate. The sub fraction was dried in rotary evaporator and stored in refrigerator for further use.

2.3. Preliminary Phytochemical Screening

The extract/subfraction were tested for the presence of various secondary metabolites using standard methods [8].

2.3.1. Flavonoids

The sample was mixed with few pieces of magnesium turnings with dropwise addition of concentrated HCl. pink scarlet colour formation after few minutes symbolizes the presence of flavonoids.

2.3.2. Phenols and Tannins

The sample mixed with 2ml of 2% $FeCl_3$ solution. Appearance of blue-green or black colouration indicates the presence of phenols and tannins.

2.3.3. Saponins

The extract/subfraction was shaken vigorously with 5ml of distilled water in a test tube. Stable foam formation is taken as an indication for the presence of saponins.

2.3.4. Alkaloids

1% HCl (2ml) was mixed with the sample and gently heated. Added mayer's and wagner's reagent to the mixture. Turbidity of the resulting precipitate is taken as evidence for the presence of alkaloids.

2.4. Selection of a Mobile Phase

For the separarion of various phytoconstituents from the plant extract/subfraction numerous solvent system were tested as mobile phase. The best solvent system giving maximum number of spots and good separation was finalized for the extract/subfraction. The R_f values were noted. Various detecting reagents (*viz*., daylight, UV light and anisaldehyde sulphuric acid reagents) were used.

2.5. High Pressure Thin Layer Chromatography (HPTLC) Fingerprinting Analysis

HPTLC is an important analytical tool in the separation, identification and estimation of various classes of natural phytoconstituents.

2.5.1. Preparation of Extract/Subfractions

The extract/subfractions were dissolved in the respective solvents (5 mg/ml).

2.5.2. Preparation of Anisaldehyde Sulphuric Acid Reagent

Anisaldehyde (0.5ml) was mixed with glacial acetic acid (10ml); to it methanol (85ml) and conc. H_2SO_4 (5ml) was added.

2.5.3. Application of Extract/Subfractions

The extract/subfractions dissolved in respective solvents (5 mg/ml) and these solutions were applied with the help of linomat syringe using the Linomat applicator IV on the HPTLC plates (10×10 cm). 7 µl of sample was applied as a band of 5-6 mm and at a separation of 6 mm from each other.

2.5.4. Development of the Chromatogram

The principle of separation in HPTLC is same as that in case of TLC. One mobile and one stationary phase were used. Silica gel on the percoated plates acts as stationary phase. The solvent system selected was same as that used in TLC analysis. The plates were developed in CAMAG twin trough chamber. The sample travels through the stationary phase and elute the components according to the binding capabilities of components with stationary phase. Here the plates were developed up to a distance of 80 mm and after the run was completed, they were taken out of the chamber and dried in air.

2.5.5. Scanning of the Chromatogram

CAMAG HPTLC Densitometer (Scanner 3) was used as a scanner in absorbance mode at both 254 and 366 nm, the extract/subfractions were also scanned at 350-600 nm using deuterium and tungsten lamp (slit dimension 6.0 X 0.45 macro). The scanned data was subjected for integration through the software winCATS Planar Chromatography Manager. The fingerprint so developed was used for the detection of phytocomponents present in the samples and the chromatograms and R_f value were noted. Bands were resolved and their colour was noted. Spots were visible without derivatization at 254 and 366 nm wavelengths but best results were shown when TLC plates were sprayed with detection reagent (Anisaldehyde sulfuric acid reagent and plate was heated at 110°C for 5 minutes) and then visualized in visible light range 400-600nm.

III. RESULTS AND DISCUSSION

3.1. Photochemical Screening

Various phyto components like alkaloids, saponins, flavonoids, phenols and tannins were present in the methanol extract and ethyl acetate sub fraction of *A. indica* leaves (Table 1).

Table1. *Phytochemical analysis of various extract/subfraction of A. indica leaf.*

Phytoconstituents	Methanol	Ethyl Acetate sub fraction
Flavonoids	+	+
Phenols &Tannins	+	+
Saponins	+	+
Alkaloids	+	+

"+" Present

3.2. HPTLC Profile

The best results were shown using Toluene:Ethyl Acetate: Formic acid::5:4:1 as solvent system for both the extract/subfractions of *A. indica*. TLC plate of *A. indica* methanol (leaf) extract scanned at 350 nm wavelength signified the existence of sixteen phytoconstituents whose R_f values ranged from 0.03 to 0.96 (Table 2, Figure 1,2). The chromatogram showed the percentage area (Figure 2). After spraying the TLC plate with anisaldehyde sulphuric acid reagent it revealed six violet, one purple and two dark purple bands (Figure 5) showing the presence of saponins, sterols and terpenoids. Similarly ethyl acetate sub fraction showed best results when scanned at wavelength 500 nm with eighteen phytoconstituents (Table 2, Figure 3,4). R_f values were observed in the range of 0.03 to 0.95. The chromatogram clearly showed that the components with *R*f values 0.95, 0.83, 0.14 were found to be leading as the percentage area was more i.e. 12.93%, 10.24% and 10.06% respectively. Six violet, one purple, one yellow, one green and two dark purple bands (Figure 5) showed the presence of sterols, terpenoids, steroids and saponins in *A. indica* ethyl acetate sub fraction when anisaldehyde sulphuric acid reagent was sprayed.

This is the first study to account the HPTLC fingerprint of ethyl acetate sub fraction of *A. indica* leaves showing maximum number of components using Toluene: Ethyl acetate: Formic acid:: 5:4:1 solvent system at a wavelength of 500nm. Nicoletti *et al.*, 2013 report the application of HPTLC fingerprint method on analysis of different neem products (cake and oil) [9].

The chromatogram developed are particular with the finalized solvent system, R_f value and can serve as improved tool for the extract/subfraction standardization. The present study gives enough information regarding various phytoconstituents present in the ethyl acetate sub fraction and methanol extract of *A. indica* and also helps in generating basis for the quality control, correct identification and standardization of *A. indica*.

The results of preliminary phytochemical screening were further confirmed by the results of HPTLC analysis by the presence of different coloured bands symbolizing the presence of specific phytocomponents.

Table2. *HPTLC fingerprint analysis of A. indica (leaves).*

Extract/sub fraction	Finalized Solvent system	Number of peaks	R_f Value	Percent (%) Area
Methanol extract (350 nm)	Toluene:Ethyl Acetate: Formic acid 5:4:1	16	0.03, 0.07, 0.13, 0.32, 0.39, 0.43, 0.55, 0.57, 0.62, 0.65, 0.70, 0.77, 0.83, 0.88, 0.91, 0.96	2.13, 3.24, 2.82, 4.98, 5.24, 2.09, 19.95, 5.69, 9.38, 4.92, 4.86, 8.89, 8.76, 4.94, 2.73, 9.39
Ethyl acetate sub fraction (500 nm)	Toluene:Ethyl Acetate: Formic acid 5:4:1	18	0.03, 0.07, 0.14, 0.21, 0.27, 0.31, 0.37, 0.39, 0.47, 0.52, 0.56, 0.60, 0.65, 0.69, 0.76, 0.83, 0.86, 0.95	1.77, 7.75, 10.06, 0.43, 2.77, 4.05, 3.43, 1.01, 6.73, 6.64, 6.44, 6.82, 5.18, 1.96, 9.02, 10.24, 2.77, 12.93

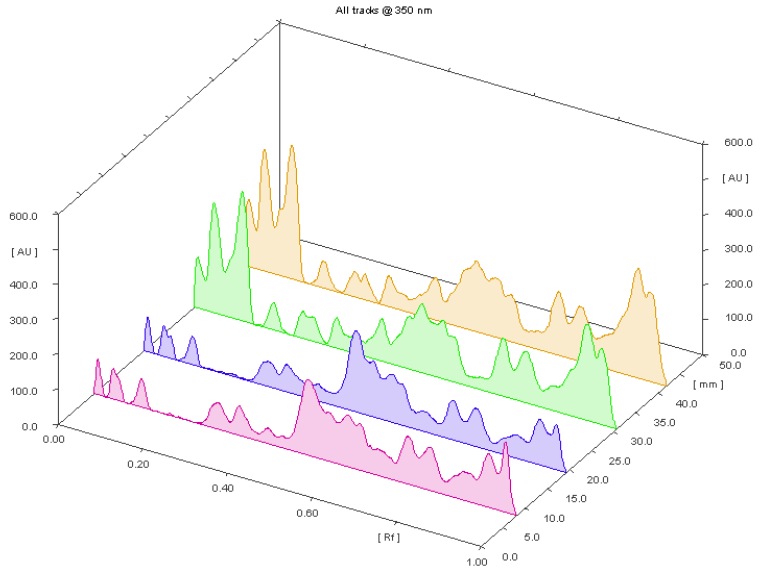

Fig1. *Three dimensional view of A. indica methanol extract chromatogram at 350 nm.*

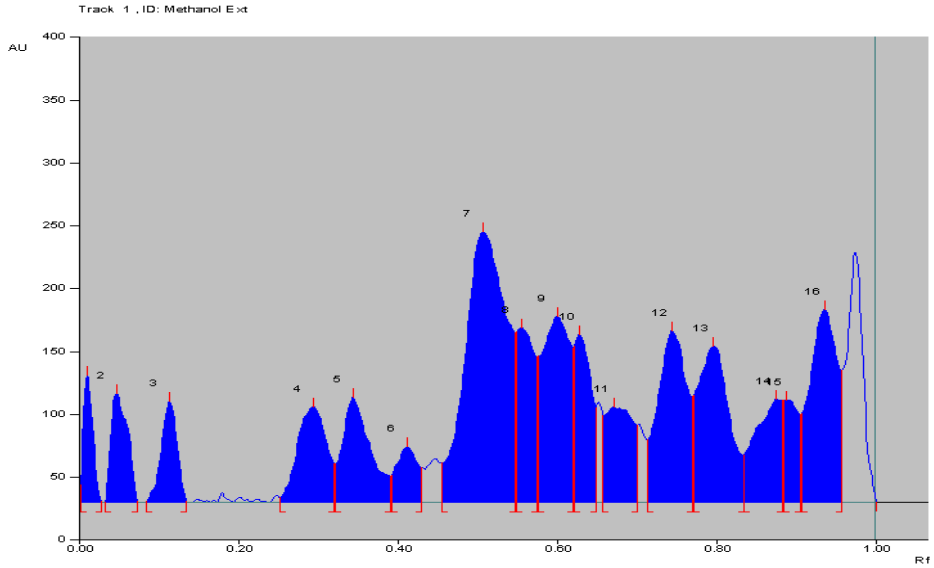

Fig2. *Chromatogram of methanol extract of A. indica (leaf) measured at 350 nm.*

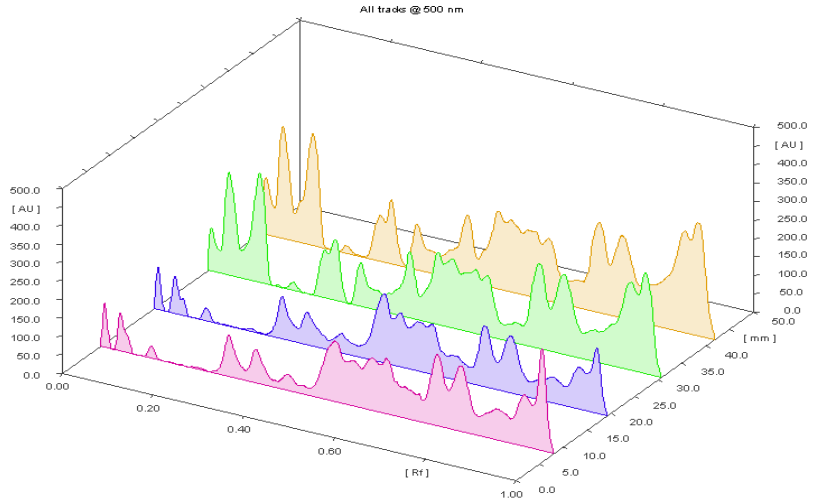

Fig3. *Three dimensional view of A. indica ethyl acetate sub fraction chromatogram at 500 nm.*

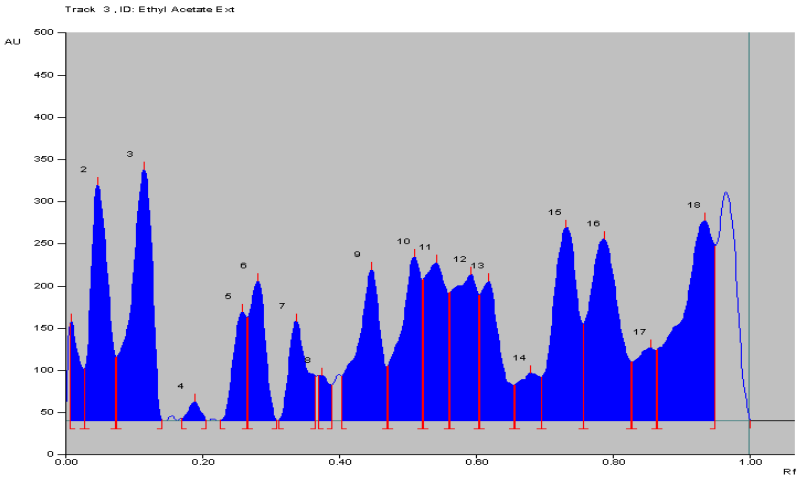

Fig4. *Chromatogram of ethyl acetate sub fraction of A. indica (leaf) measured at 500 nm.*

Fig5. *TLC plate showing different constituents of A. indica methanol extract (Track 1-2) at 350 nm and ethyl acetate sub fraction (Track 3-4) at 500 nm.*

IV. CONCLUSION

HPTLC fingerprint is a good technique to check the genetic variability present in plant species. It is a simple, renewable, linear, cost effective, defined, exact method in identifying a plant species and can also be used in standardization, characterization and authentication of medicinally significant plants. Various reports have indicated high efficacy of ethyl acetate sub fraction for various biological activities, which is in accordance with the present study. HPTLC finger printing helps in differentiating the adulterant and species. It can act as a biochemical marker for *A. indica* in the plant studies and pharmaceutical companies. Further research to characterize the phytoconstituents and to execute quantitative estimation with the help of marker compounds is also obligatory, but the HPTLC finferprint data from the present study should be considered for setting up standards to *A.indica*.

V. ACKNOWLEDGEMENT

The infrastructure facility was provided by Kurukshetra University, Kurukshetra to accomplish the research work. University Grant Commission (UGC) New Delhi is appreciatively accredited for their financial support.

Competing Interest

There is no conflict of interest.

REFERENCES

[1] Ranjit RC. Herbal Medicine for Human Health. Regional Publication. SEARO. No. 20. W.T.O. New Delhi. 1992; 1-80.

[2] Quality Control Method for Medicinal Plant Materials. W.H.O. Geneva. 1989; 1-15.

[3] Sethi P D. High Performance Thin Layer Chromatography: Quantitative Analysis of Pharmaceutical Formulations. CBS Publishers and Distributers. New Delhi. 1996; 10-60.

[4] Srinivasan D, Nathan, Sangeeta, Sursh T, Perumalsamy, Lakshman P. Antimicrobial activity of certain Indian medicinal plants used in folkoric medicine. *J. Ethnopharmacology*. 2001; 74: 217-220.

[5] Okemo PO, Mwatha WE, Chhabra SC, Fabry W. The kill kinetics of *Azadirachta indica* a. juss. (meliaceae) extracts on *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*. *African J. Sci. Technol.* 2001; 2: 113-118.

[6] Khan I, Surya RS, Surekha D, Srujana DG, Hemasundara M. Phytochemical studies and screening of leaf extracts of *Azadirachta indica* for its anti-microbial activity against dental pathogens. *Arch. App. Sci. Resear*. 2010; 2(2): 246.

[7] Aneja KR, Joshi R, Sharma C, Potency of *Barleria prionitis* L. bark extracts against oral diseases causing strains of bacteria and fungi of clinical origin. *New York Sci J*. 2010; 3: 5-12.

[8] Trease GE, Evans WC. Pharmacognosy. 11th edn.,Bailliere Tindall.1989; London. pp. 45-50.

[9] Nicoletti M, Toniolo C, Murugan K. The HPTLC approach to metabolomic determination of neem products composition. *Pharmacology online Archives*. 2013; 3: 122-127.