CODEN [USA]: IAJPBB ISSN : 2349-7750

INDO AMERICAN JOURNAL OF
PHARMACEUTICAL SCIENCES

SJIF Impact Factor: 7.187
https://doi.org/10.5281/zenodo.7676825

Available online at: http://www.iajps.com *Research Article*

A COMPARATIVE ANALYSIS OF AZADIRACHTA INDICA LEAVES FROM DIFFERENT STATES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND ITS ANTIMICROBIAL PROPERTIES INVESTIGATION

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Article Received: January 2023 **Accepted:** January 2023 **Published:** February 2023

Abstract:
In the present work, Neem leaf extracts from Maharashtra and Telangana were compared by using RP-HPLC for difference in chemical concentration. The chromatographic conditions were successfully developed for the separation of Maharashtra extract by using Kromosil RPC18 4.6×150mm 5µm, flow rate was 1.0ml/min, mobile phase ratio was ACE: methanol (75:35% v/v) pH-3 (pH was adjusted with Orthophosphoric acid), detection wavelength was 260 nm. The instrument used was WATERS HPLC Auto Sampler, Separation module 2690, photo diode array detector 996, Empower-software version-2. The retention times were found to be 2.223 min and 2.324 min. The chromatographic conditions were success fully developed for the separation of Telangana extract by using Agilent C 18(4.6×150mm) 5µ, flow rate was 1.0 ml/min, mobile phase ratio was Methanol: ACN (60:40% v/v) pH 3 (pH was adjusted with orthophosphoricacid), detection wavelength was 265nm. The instrument used was WATERS HPLC Auto Sampler, Separation module 2695, photo diode array detector 996, Empower-software version-2. The retention times were found to be 2.229 min and 3.330 min. The results of the anti microbial activity studies suggest that the samples from Maharashtra and Telangana have a broad spectrum of antimicrobial activity and this effect is increased by increasing the quantity of this compound, which can be used as an alternative for antibiotics.

KEYWORDS: *Antibacterial activity, Azadirachta Indica, RP-HPLC, etc.*

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Please cite this article in press M. Upendar et al, ***A Comparative Analysis Of Azadirachta Indica Leaves From Different States By High Performance Liquid Chromatography And Its Antimicrobial Properties Investigation.,*** *Indo Am. J. P. Sci, 2023; 10 (02).*

INTRODUCTION:

Azadirachta indica, commonly known as neem, nim tree or Indian lilac, is a tree in the mahogany family Meliaceae. It is one of two species in the genus Azadirachta, and is native to the Indian subcontinent, i.e. India, Nepal, Pakistan, Bangladesh, Sri Lanka, and Maldives. It is typically grown in tropical and semi-tropical regions[1]. Neem trees also grow in islands located in the southern part of Iran. Its fruits and seeds are the source of neem oil. In India, the tree is variously known as "Heal All", "Village Pharmacy". Large sized tree grows up to 30 meters in height. Leaves compound, leaflets opposite, serrate and acuminate. Flowers yellowish white. Fruits elliptical one seeded[2].

Present work is aimed to determine and compare the analytical parameters of *Azadirachta indica* leaves obtained from Maharashtra and Telangana by RP-HPLC method and investigate their antimicrobial properties.

Analytical chemistry is a scientific discipline used to study the chemical composition, structure and behavior of matter. The purpose of chemical analysis is to interpret chemical information that will be of value to society in a wide range of contexts. The principle applications include Quality control in manufacturing industries, the monitoring of clinical and environmental samples, the assaying of geological specimens, and the support of fundamental and applied research. Analytical chemistry involves the application of a range of techniques and methodologies to obtain and assess qualitative, quantitative and structural information on the nature of matter[10].

- **Qualitative analysis** is the identification of elements, species and/or compounds present in a sample.
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- **Quantitative analysis** is the determination of the absolute or relative amounts of elements, species or compounds present in a sample.

Structural analysis is the determination of the spatial arrangement of atoms in an element or molecule or the identification of characteristic groups of atoms (functional groups). An element, species or compound that is the subject of analysis is known as analyte. The remainder of the material or sample of which the analyte(s) form(s) a part is known as the matrix[9].

The chromatography was discovered by Russian Chemist and botanist Micheal Tswett (1872-1919) who first used the term chromatography (colour writing derived from Greek for colour – Chroma and write – graphein) to describe his work on the separation of coloured plant pigments into bands on a column of chalk and other material such as polysaccharides, sucrose and insulin.

"Chromatography is a method in which the components of a mixture are separated on an adsorbent column in a flowing system".

The adsorbent material, or stationary phase, first described by Russian scientist named Tswett in 1906, has taken many forms over the years, including paper, thin layers of solids attached to glass plates, immobilized liquids, gels, and solid particles packed in columns. The flowing component of the system, or mobile phase, is either a liquid or a gas. Concurrent with development of the different adsorbent materials has been the development of methods more specific to particular classes of analytes. In general, however, the trend in development of chromatography has been faster and more efficient[11].

The acronym HPLC, coined by the Late Prof. Csaba Horvath for his 1970 Pittcon paper, originally indicated the fact that high pressure was used to generate the flow required for liquid chromatography in packed columns. In the beginning, pumps only had a pressure capability of 500 psi [35 bars]. This was called High pressure liquid chromatography, or HPLC. The early 1970s saw a tremendous leap in technology. These new HPLC instruments could develop up to 6,000 psi [400 bars] of pressure, and incorporated improved injectors, detectors, and columns. With continued advances in performance during this time [smaller particles, even higher pressure], the acronym HPLC remained the same, but the name was changed to high performance liquid chromatography[12, 13, 14].

The science dealing with the study of the prevention and treatment of diseases caused by micro-organisms is known as medical microbiology[3]. Its sub-disciplines are virology (study of viruses), bacteriology (study of bacteria), mycology (study of fungi), phycology (study of algae) and protozoology (study of protozoa). For the treatment of diseases inhibitory chemicals employed to kill micro-organisms or prevent their growth, are called antimicrobial agents. These are classified according to their application and spectrum of activity, as germicides that kill micro-organisms, whereas micro-biostatic agents inhibit the growth of pathogens and enable the leucocytes and other defense mechanism of the host to cope up with static invaders[4, 5, 6, 7].

The germicides may exhibit selective toxicity depending on their spectrum of activity. They may act as viricides (killing viruses), bactericides (killing bacteria), algicides (killing algae) or fungicides (killing fungi)[8].

Various methods have been used from time to time by several workers to evaluate the antimicrobial activity. The evaluation can be done by the following methods:

- ✓ Turbidometric method
- ✓ Agar streak dilution method
- ✓ Serial dilution method
- ✓ Agar diffusion method.

Following Techniques are used as agar diffusion method:

- ✓ Agar Cup method
- ✓ Agar Ditch method
- ✓ Paper Disc method

We have used the Agar cup Method to evaluate the antibacterial activity. It is one of the non automated in vitro bacterial susceptibility tests. This classic method yields a zone of inhibition in mm result for the amount of antimicrobial agents that is needed to inhibit growth of specific microorganisms. It is carried out in Petri plates[7, 8, 9, 10].

MATERIALS

Azadirecta indica is collected from the local regions of Maharashtra and Telangana, Methanol, Acetonitrile are bought from Yarrow chem., Mumbai. Agar, bee infusions, starch are gift samples from Teja College of pharmacy-KODAD.

METHODOLOGY:

Extraction Procedure

The collected materials are cleaned with tap water then air dried to remove excess water from the surface of the leaves, after that the materials are carefully spread on a platform under a shed for the purpose of shade drying to limit chemical constituent loss during drying. After two weeks (14days) of shade drying the materials are grounded to powder form using electronic mixer grinders. This grounded material is then soaked (500gm material in 2500ml solvent) in ethanol (cold extraction) for 8 days and kept aside and occasionally stirred to mix the materials. After 8 days the solvent is filtered out using a cotton cloth then the filtered solvent is concentrated to obtain the extract. The obtained extract is again shade dried for 7 days to remove any remaining solvent in the extract. The completely dried extract is later used for the studies[3, 8, 10].

MAHARASHTRA

Preparation of sample:

10 mg of extract is solubilized in 10ml of ethanol. Later this solution is mixed with 100ml of mobile phase solution which created the first stock solution. Later 1ml of this stock solution is injected into HPLC machine for estimation of azadirachtin.

Chromatographic trials for *Azadirachta Indica* by RP- HPLC.

Trial-5 (optimized method)

Chromatographic conditions

Column : Kromosil RPC18 4.6×150mm 5µm
Mobile phase ratio : ACE: pH 4.5 di-potassium hydrogen phosphate (75:35% v/v)
Detection wavelength : 260 nm
Flow rate : 1.0ml/min
Injection volume : 20µl
Column temperature : Ambient
Auto sampler temperature : Ambient
Run time : 7 min

Chromatographic trials for *Azadirachta Indica* by RP- HPLC.

Trial-7 (optimized method)

Column : Agilent C 18(4.6×150mm)5µ
Mobile phase ratio : Methanol: ACN (60:40% v/v)
Detection wavelength : 265nm
Flow rate : 1.0 ml/min
Injection volume : 10µl
Column temperature : Ambient
Auto sampler temperature : Ambient
Run time : 10min

DISC DIFFUSION METHOD:

The bacteriostatic property of the compounds was tested by disc diffusion method as described by Bauer Kirby's method[5].

[A] Preparation of Mueller-Hinton agar

(1) Beef infusion : 300 g
(2) Acid hydrolysate of casein: 17.5 g
(3) Starch : 1.5 g
(4) Agar : 17 g
(5) Distilled water : 1 Lit.

The above constituents were weighed and dissolved in water. The mixture was warmed on water bath till agar dissolved. This was then sterilized in an autoclave at 15 lbs. pressure and 121 ^{o}C for fifteen minutes. The sterilized medium (20 ml) was poured in sterilized Petri dishes under aseptic condition, allowing them to solidify on a plane table[5, 6].

[B] Preparation of Antibacterial Solution

All the compounds were dissolved in dimethyl Formamide (DMF). Proper drug controls were used. Compound was taken at concentration of 100 µg/ml for testing antibacterial activity. The compound diffused into the medium produced a concentration gradient. After the incubation period, the zones of inhibition were measured in mm. The tabulated results represent the actual readings control[7].

[C] Test cultures: Following common standard strains were used for screening Of Antibacterial and antifungal activities:

Escherichia coli	[Gram negative]	MTCC – 443
Aspergillus Niger	[Fungus]	MTCC – 282

[D] Inoculum's preparation

The inoculum was standardized at 1* 106 CFU/ml comparing with turbidity standard (0.5 McFarland tube).

[E] Swabs preparation

A supply of cotton wool swabs on wooden applicator sticks was prepared. They were sterilized in tins, culture tubes, or on paper, either in the autoclave or by dry heat.

[F] Experimental procedure[5, 6, 7, 8, 9]

1) The plates were inoculated by dipping a sterile swab into inoculums. Excess inoculum was removed by pressing and rotating the swab firmly against the side of the tube, above the level of the liquid.

2) The swab was streaked all over the surface of the medium three times, rotating the plate through an angle of 60 ^{o}C after each application. Finally the swab was passed round the edge of the agar surface. The inoculation was dried for a few minutes, at room temperature, with the lid closed.

3) Ditch the bore in plate. Add compounds solution in bore.

4) The plates were placed in an incubator at 37 ^{o}C within 30 minutes of preparation for bacteria and 22 ^{o}C for fungal.

5) After 48 hours, incubation for bacteria and 7-days for fungal, the diameter of zone (including the diameter disc) was measured and recorded in mm. The measurements were taken with a ruler, from the bottom of the plate, without opening the lid.

RESULTS & DISCUSSION:

Table.no.1 Chromatographic conditions

Parameter	Condition for MAIE	Condition for TAIE
Column	Kromosil RPC18 4.6×150mm 5µm	Agilent C18(4.6×150mm) 5 µm
Mobile phase ratio	ACE: methanol (75:35% v/v)	Methanol: ACN (60:40% v/v)
Detection wavelength	260 nm	265 nm
Flow rate	1.0 ml/min	1.0 ml/min
Injection volume	20µl	20µl
Column temperature	Ambient	Ambient
Auto sampler temperature	Ambient	Ambient
Run time	7 min	10min

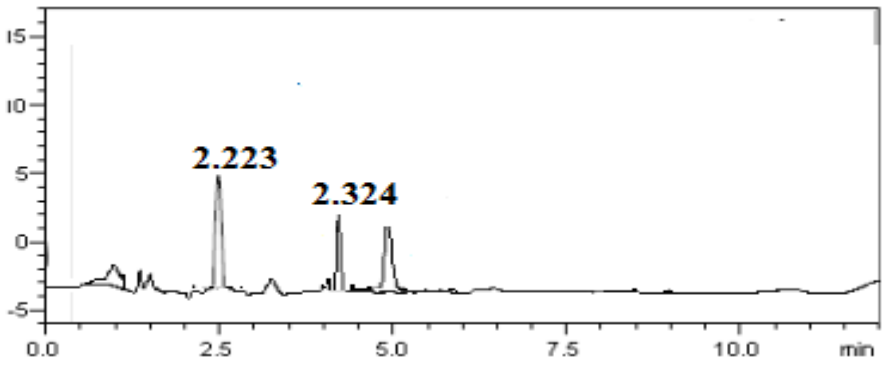

Fig.No.1. Chromatogram showing MAIE

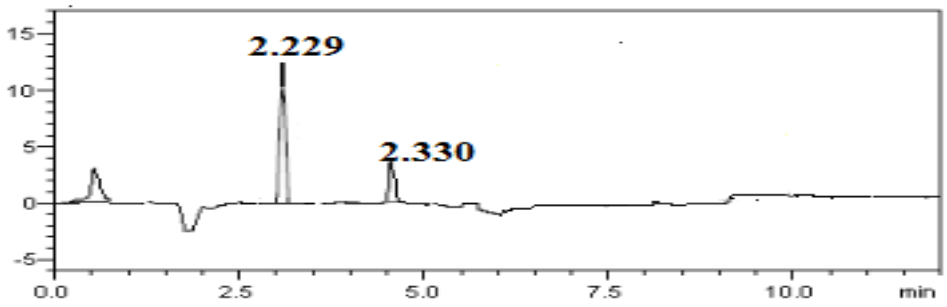

Fig.No.2. Chromatogram showing TAIE

Table.no.2. Details of MAIE, TAIE run.

Peak name	R_t	Area	Height	USP Plate count	USP Tailing	USP Resolution
MAIE-Peak-A	2.223	223649	642415	5214	1.3	1.4
TAIE-Peak-A	2.229	9147677	819856	634	2.21	1.3
MAIE-Peak -B	2.324	464121	393414	8745	1.2	1.4
TAIE-Peak -B	2.330	5362158	427568	5352	2.32	1.3

Inference: the results are compared with vergallo et al. (2019)[15] and from the analytical estimation it was clear that the material collected from the Maharashtra contained higher levels of chemical compounds (azadirachtin).

DISC DIFFUSION METHOD:

The antimicrobial activity of test compounds MAIE and TAIE was estimated by disc diffusion method. In this ciprofloxacin was taken as standard antimicrobial compound. E.coli (Gram negative bacteria) and A.niger (Fungus) were taken as test organisms. Nystatin was taken as standard molecule for antifungal activity.

Antibacterial and antifungal activities of test, standard compounds were given in below table.

ANTIBACTERIAL ACTIVITY:

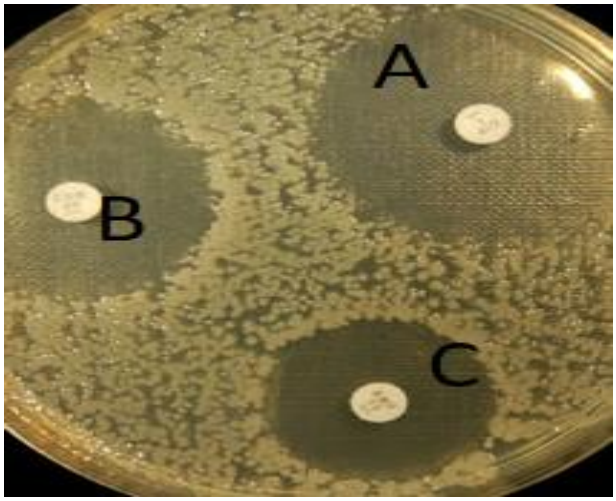

Fig 3: zone of inhibition of plant extracts

Table 3: Antibacterial Activity

S.no	Compound Name	Zone of inhibition* (mm) (activity index)std
1	Ciprofloxacin	29
2	**MAIE**	18
3	**TAIE**	17

From the above table it was confirmed that the test sample has antibacterial property.

ANTIFUNGAL ACTIVITY:

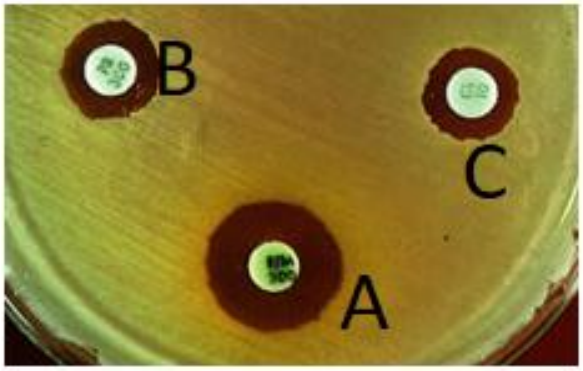

Fig 4: zone of inhibition of plant extracts

Table 4: Antifungal Activity:

S.no	Compound Name	Zone of inhibition* (mm) (activity index)std
1	Nystatin	26
2	**MAIE**	12
3	**TAIE**	13

From the above table it was confirmed that the test substance may have antifungal property also.

CONCLUSION:

The chromatographic conditions were successfully developed for the separation of chemical constituents mainly azadirachtin from TAIE and MAIE by using RP-HPLC. Analytical results were compared with the results of vergallo et al., (2019)[15]. From the analytical results it was found that MAIE has higher concentration than TAIE. The results of the anti microbial activity studies suggest that the samples MAIE, TAIE have a broad spectrum of antimicrobial activity and this effect is increased by increasing the quantity of this compound, which is not as good as synthetic compounds but can be used as an alternative for antibiotics when synthetic compounds are not available.

ACKNOWLEDGEMENTS

The authors are grateful to the management of TEJA COLLEGE OF PHARMACY-KODAD for the support in the utilization of laboratory facilities.

Conflicts of interest:

The authors express no conflicts of interest regarding the publication, all the authors worked and provided support equally and credited equally.

Abbreviations:

%-PERCENTAGE
ACN-ACETONITRILE
CM-CENTIMETER
GM-GRAM
HPLC- HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

KH2PO4- POTASSIUM DI HYDROGEN ORTHO PHOSPHATE
MAIE-MAHARASHTRA A. INDICA EXTRACT
MG-MILLIGRAM
MM-MILLIMETER
N-NUMNER OF TIMES
S.D- STANDARD DEVIATION
TAIE- TELANGANA AZADIRACHTA INDICA EXTRACT
UV-ULTRAVIOLET
µl- MICRO LITER

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