A Review on Separation of Phytoconstituents Present in Aqueous Extract of Azadirachta Indica by TLC/HPLC/Column Chromatography Method.

Dr. Balkrishna Tiwari[1], Dr. Yogesh Thorat[*], Mr. Prachet Bagewadikar[2], Ms. Shruti Narayankar[3], Ms. Pornima Parshetti[4]

[1]Principal, [2]Associate professor, [3]Assistant professor
Department of Pharmaceutics
(Amepurva Forum's Nirant Institute of Pharmacy, Solapur, Maharashtra, India.)

Abstract: Azadirachta indica. L(Meliaceae) is smart in distribution and generally used in several native medical practices. In India, varied vegetative parts of A. indica, which includes leaves, flowers, fruits, seeds, and bark are extensively used in Indian traditional drug for treating varied human conditions and diseases. It's also called as storage of phytochemicals. The chemical composition is relatively complex. Numerous medicinal plants studied for their phytochemical contents. Azadirachta indica is one of these plants. This research, primarily aimed to carry out a primary phytochemical screening to determine the major classes of bioactive composites presented in Azadirachta indica dry leaves. Numerous solvent (Methanol, Ethanol, Ethyl acetate, Acetone, acetic acid and Distilled water) were used to determine the stylish solvent that can be used for extraction and to perform Thin Layer Chromatography profiling of all successional extracts. TLC was carried out in Silica gel plates using mobile phase protocol, Chloroform Ethanol Methanol (111) and using six mentioned solvents. Thin Layer Chromatographic studies of the Azadirachta indica leaf extract (Methanol, Ethanol, Ethyl acetate, Acetone, Acetic acid and Distilled water) constituted different coloured phytochemical composites with different Rf values. Methanol extract is the stylish which shows seven bands with Rf lower than 1 for 24 hrs. After seven days among all the extracts, methanol extract was set up to contain the outside number of bioactive composites which showed seven bands with Rf lower than 1. High Performance Liquid Chromatography (HPLC) point of the constituents of acetone extracts of A. indica was farther investigated. Highest change yield (10.20) was attained for acetone extract of A. indica. Varying ingredients glycosides, protein, anthraquinone, flavonoid, tannin and terpenoid were identify. An array of mixes was separated at different peak heights corresponding to concentration of the mixes Myricetin was detected in topmost chance (39.6) in acetone extracts of A.indica while alpha funebren detected in lowest chance(1.5).Column chromatographic studies of methanolic extract using the solvent system Chloroform ethanol methanol, shows the better separation with methanol solvent. From this research it concluded that the methanol extract for Azadirachta indica leaves contain a advanced content of bioactive composites, which can be used for farther research on this plant.

Keywords: Azadirachta indica, TLC, HPLC, Column chromatography.

INTRODUTION

Neem is an evergreen, temperature, tolerant, flowering plant, native to the whole Indian key, which can be set up growing in a lot of tropical and semi-tropical countries located in the tropical belt. Botanically it's classified as a member of the family Meliaceae, under the Latinised name Azadirachta indica A. Juss (A. indica). This name driven from the Persian words "azad"(free), "darakht" (tree) and "i- hind"(of Indian origin) ,which literally mean the free tree of India. (1)

The various vegetative parts A. indica, which include, leaves, flower, fruits, seeds, roots, bark and oil will encompass of variety of phytochemicals with significant biological and pharmacological activities. Few biomolecules are reported with anticancer, antimalarial, antibacterial ,antifungal, anti-viral and anti-inflammatory properties and sometimes even used as insecticidal, larvicidal and spermicidal.(2) Azadirachtin is a triterpenoid of the class of limonoids set up in the

trees of A indica.[3] In Ayurveda and other societies, the condiment is generally used as a natural antiseptic, antifungal, and crack healing agent to treat skin conditions ranging from ringworm to abscesses and snakebites[4]. Maximum herbal drugs and their secondary products were frequently prepared from the crude factory excerpts, which comprise a complex compound of different phytochemical ingredients (factory secondary metabolites) [5]

Thus, the quality control of the herbal drugs and their concluded product is delicate, newly, the chromatographic characteristics of the factors, especially by high performance liquid chromatography-diode array discovery (HPLC-pater),is a important and extensively used method to dissect plant extract because this method could totally outline the composition of samples and it focuses on the identification and ingredients.[5] In the present study, coherent system TLC and LC-MC was accepted for relating phytoconstituent. Thin Layer Chromatography (TLC) is a veritably generally used method for associating composites is a system of analysis in which the stationary phase a finely divided solid is spread as a thin layer on a rigid supporting plate; the mobile phase a liquid is allowed to travel across the face of the plate (Gennaro,2000). This logical tool is used because of its simplicity, speed of separation, cost effectiveness and high perceptivity. [6]

Ayurveda states that neem balances kapha and pitta out of the three doshas in the the mortal body. [7] The phytochemicals which are active against different pathogens, nimbolinin, nimbin, nimbidin, nimbidol, sodium nimbinate, gedunin, salannin and quercetin. Leaves contain ingredients such as nimbin, nimbanene, nimbandiol, nimbolide, ascorbic acid, n-hexacosanol 6-desacetylnimbinene and amino acid,7-desacetyl-7-benzoylazadiradione,7-desacetyl-7-benzoyl-gedunin,17 hydroxyazadiradione and nimbiol. Fresh leaves extract of neem gives the following active biological compounds i.e Quercetin and sitosterol, polyphenolic flavonoids, they have antibacterial and antifungal properties and the neem seeds contains constituents including gedunin and azadirachtin in it.[8]

METHODS

- Thin Layer Chromatography (TLC)
- High Performance Liquid Chromatography (HPLC)
- Column Chromatography

THIN LAYER CHROMATOGRAPHY(TLC)

TLC was carried out to separate the principle constituents that were present in utmost effective extracts of plants. The TLC was performed by using the solvent system is chloroform Methanol Ethanol (1:1:1).The leaves of Azadirachta indica were collected from the circling areas. The collected plant material is washed completely with water, dried under shade at room temperature and crushed using a hand shop to make a coarse powder, also they're stored in well-closed light resistant holders for farther use.

Extraction (Maceration)

Fresh leaves of Azadirachta indica were collected, cleaned, dried and crushed. 50g of the powder was dissolved in 500ml of Methanol, Ethanol, Acetic acid, Acetone, Ethyl acetate and Distilled water singly for overnight i.e 24 hours. After 24 hours, the solvent was strained by using whatman Filter paper.[9]

Table No.1 Maceration of Azadirachta indica leaf powder by different solvents.[9

Solvent used	Quantity of Neem Powder	Quantity of Solvent
Methanol	50 g	500 ml
Ethanol	50 g	500 ml

Acetic Acid	50 g	500 ml
Acetone	50 g	500 ml
Ethyl Acetate	50 g	500 ml
Distilled Water	50 g	500 ml

Method

The plant extracts were with 6 different solvents (Methanol, Ethanol, Acetic acid, Acetone, Ethyl acetate and Distilled water).Each extract is applied on-pre covered TLC plates by using Capillary tubes. Drawing a Light line on the end of the plate and spots to know the place of each extract applied on the plate. After using the mobile phase the TLC plates were air dried and observed under the ultraviolet light. Latterly the development of the separated bands movement was expressed by its retention factor (Rf) values being calculated for different sample extracts. [9]

$$Rf = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

Fig No. 1 Performing the TLC of different solvent extractions. [9]

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY(HPLC)

Extraction

After authentication, extraction was done following medication of the leaves therefore; the leaves were washed, under a running tap and air dried for five days at room temperature as described by Akerele etal.(2008).The dried plant materials were crushed independently to a fine powder with an electric miller(Master Chef Blender, Mode MC-BL1980,China). The powdered material of A. indica was counted(10g) into two separate beakers and was extracted independently by cold percolation system using 100 ml of acetone and aqueous as the menstruum. The extraction were done for 48 hours with constant shaking at intervals. The homogenate was filtered through Whatman purifier paper(Number 1) to yield the crude extract (Nenaah and Ahmed,2011), which was latterly evaporated to dryness using a rotary evaporator(Model shaft Zhengzhou, Henan China).The crude extracts were counted, stored in labelled sterile airtight containers and stored at 4℃ for farther use. The yield of the extract was determined using the formula Yield= (Dry weight of extract÷Dry weight of factory material) × 100.

Method

The HPLC characteristic of each solvent extract was carried out following therefore; powdered dried leaves(1.0g) was macerated with acetontrite /water(11;v/ v,10.0 mL), also centrifuged for 10 minutes at 3000 rpm and filtered. The crude extract from the filtrate was assayed directly by HPLC-UV; a modular Shimadzu (Nexeramx) LC-10 system comprised of an LC-10AD pump, a CTO-10A column roaster, an SPD-10 A UV-DAD sensor, a CBM-10A interface, and an LC-10 Workstation was used. An LC-18 column(250mm ×4.6mm ID ×5mm) from(Ubondapak,Bellefonte,USA)was employed at 30℃.Separations were done in the isocratic mode, using acetonitrile water(4060;v/v) at a flow rate of 1.0mL per minute with an injection volume (“loop”)of 10µL,UV detection was at 254nm. [10]

COLUMN CHROMATOGRAPHY

Extraction

Fresh leaves of Azadirachta indica were collected, cleaned, dried and crushed. 50g of the powder was dissolved in 500ml of Methanol, Ethanol, Acetic acid, Acetone, Ethyl acetate and Distilled water singly for overnight i.e 24 hours. After 24 hours, the solvent was strained by using whatman Filter paper.

Preparation of plant extract for column chromatography

The methanolic leaf extract solvent was removed by evaporation under the room temperature. The residue was semi solid dark green in colour, thick in consistence. The acquired extract was stored at 4℃.

Method

Methanol extract(15g) was subordinated to column chromatography on silica gel(80-120 mesh-Merck) packed and eluted with a compound of chloroform, ethanol, methanol of adding polarity to obtain fractions.15g of the methanol extract was Chromatographed over a silica gel column(80-120 mesh).The compound was packed on a silica gel column(Merck, India) and elution started with 100 chloroform and increased with solvent polarity ethanol and methanol. Chloroform gave a colourless compound and followed by farther purification with ethanol and methanol for the isolation of bioactive mixes. [9]

RESULT

Chromatographic Purification (24 hrs extract) Thin Layer Chromatography (TLC)

Chloroform Methanol Ethanol (111) TLC of Methanol extract of Azadirachta indica leaves revealed the presence of 7 composites having Rf values of 0.66, 0.72, 0.77, 0.80, 0.86, 0.96 independently. With Ethanol extract of some solvent showed 5 bands having Rf values of 0.81,0.85,0.91,0.94,0.98 independently. With Acetone extract shows 5 bands having Rf values of 0.81,0.87,0.93,0.95,0.98 independently. With Acetic acid extract shows 4 bands having Rf values of 0.87,0.90,0.93,0.95 independently. With Ethyl acetate extract shows 3 bands having Rf values of 0.80,0.93,0.98 independently. Still with water extract shows no bands.

Chromatographic Purification (7 days extract) Thin Layer Chromatography (TLC)

Chloroform Methanol Ethanol(111) TLC of Methanol extract of Azadirachta indica leaves revealed the presence of 7 composites having Rf values of 0.65,0.68,0.72,0.73,0.76,0.89 independently. With ethanol extract of the same solvent showed 5 bands having Rf values 0.62,0.66,0.70,0.73,0.77 independently. With acetone extract shows 5 bands having Rf values 0.63,0.68,0.71,0.74,0.81 independently. Ethyl acetate extract shows 5 bands having Rf values of 0.62,0.67,0.71,0.73,0.85 independently. And with acetic acid extract shows 2 bands having Rf values of 0.71,0.84 independently. As the methanol extract shows the presence of all the factors like alkaloids, glycosides, flavonoides, tannins, phenols, steroids and saponins indeed after 7 days of maceration, it was farther subjected to column chromatography foe the effective separation of those factors.

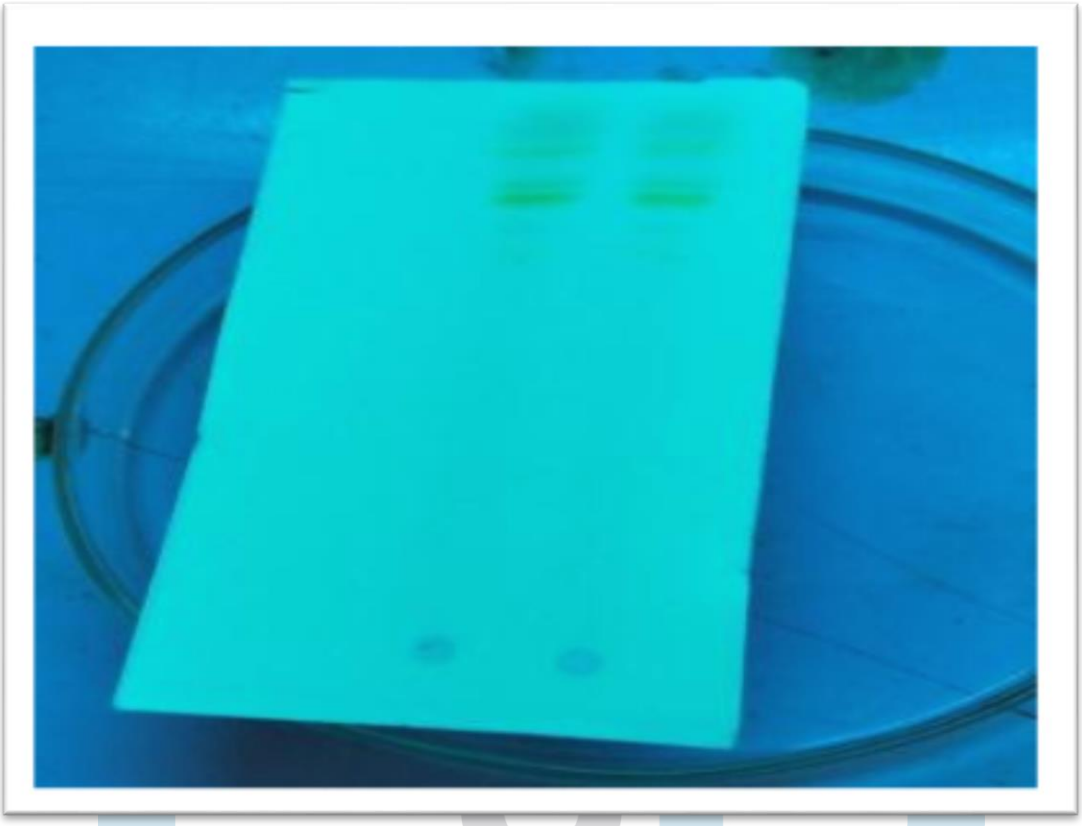

Fig No. 2 Observation of TLC under UV light

HPLC ANALYSIS

The concentration of components represented as peak heights, separated in leaves of A. indica sample is shown in the chromatograms profile given in Figures .Out of the seven fractions of compounds separated in A.indica, myricetin(39.6%) was detected in the highest quantity while alpha funebren (1.5%) was detected in the least quantity.

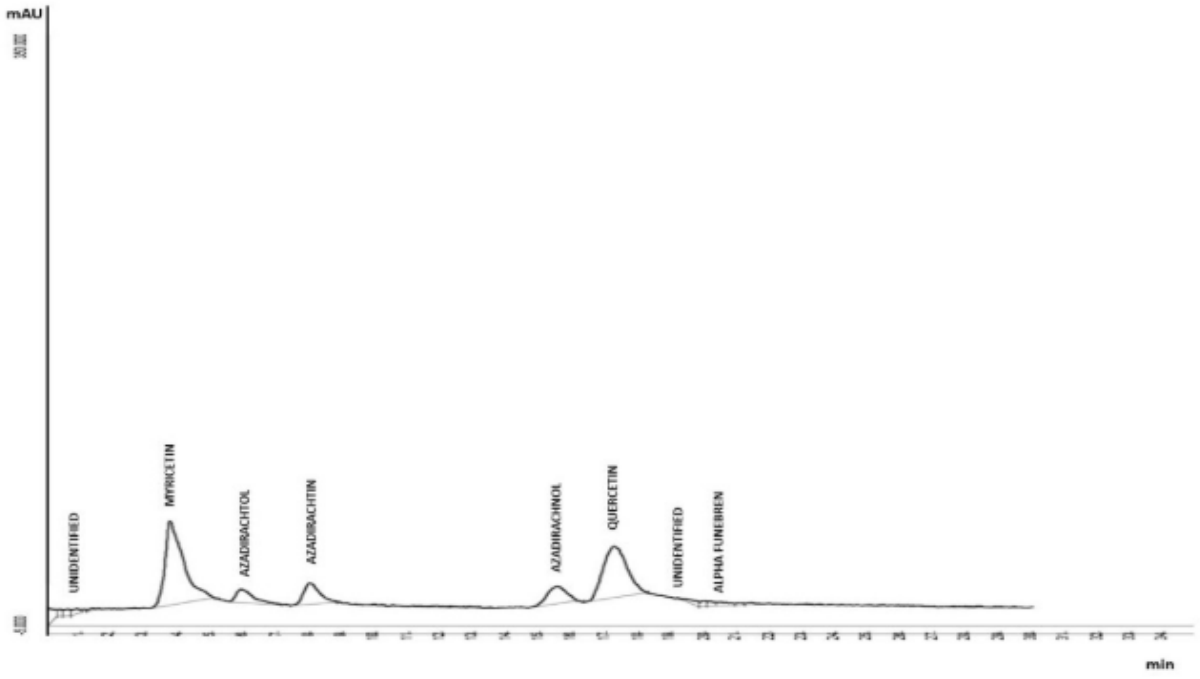

Fig N0.3 Chromatogram profile of Leaves of A.indica by HPLC

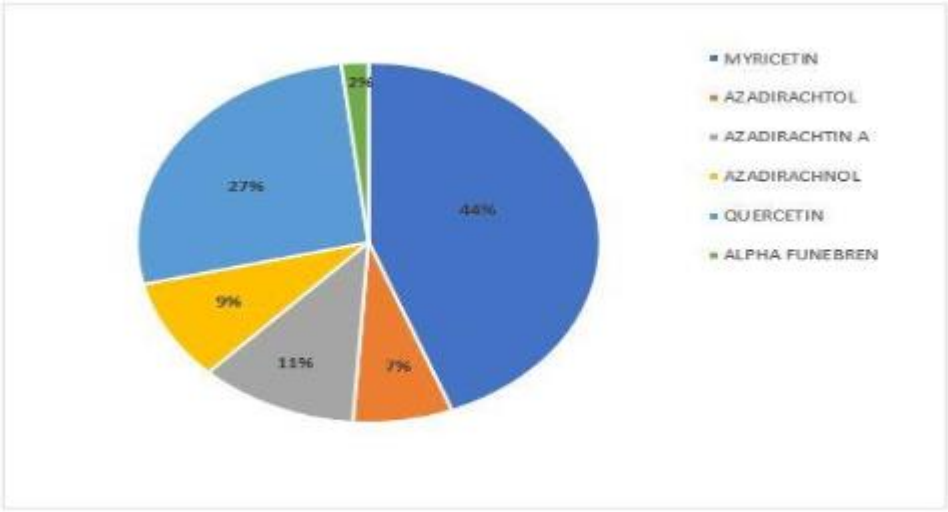

Fig No.4 Percentage occurrence of compounds in detected in A.indica

CHROMATOGRAPHIC PURIFICATION; COLUMN CHROMATOGRAPY

Isolation of compound was performed by column chromatography by using colourful solvents (chloroform, ethanol, methanol) of adding order of polarity and 15 fragments were collected and phytochemical screening was performed for each fraction. The methanol fraction showed the better separation of bioactive compounds than chloroform and ethanol. The result of the column chromatography is presented in the table.

Table No.2: Phytochemical analysis of column fraction of Azadirachta indica Juss.

Solvents used	**Alkaloids**	**Glycosides**	**Flavonoids**	**Tannins**	**Phenols**	**Saponins**	**Steroids**
Chloroform	+	++	-	-	-	-	-
Methanol	+++	+++	+	+++	+	+	+
Ethanol	-	+	+	-	+	++	-

CONCLUSION

The methanol extract yielded alkaloids, glycosides, tannins at high quantities and which formerly have places in antioxidant, anti-inflammatory, antimicrobial and anticancer conditioning. The saponins, flavonoids, phenols, steroids were also set up to be present in the extract at lower amount. The present study is a modernized interpretation and commercially exploitable system for separating the most important remedial natural products; from Azadirachta indica by applying RP-HPLC system. The system has been set up to be precise, accurate, direct, robust, profitable, sensitive and can fleetly isolate both the crucial phytoconstituents from the extracted natural materials. Therefore, the study opens avenues for the operation of this system in industry based functions as well as quality control measure at varied operability scale.

REFERENCE

Cristian vergallo, Elisa Panzarini and Luciana Dini(et.al) High Performance Liquid Chromatographic profiling of antioxidant and antidiabetic flavonoids purified from Azadirachta indica (neem) leaf ethanolic extract .Pure Appl.chem.2019;91(10);1631-1640.

1. Pasupuleti Sreenivasa Rao, Gangapatnam Subramanayam, Perali Ramu Sridhar(et.al). Flavonol glycosides from Azadirachta indica.L. Drug Invention Today/ Volume,10,Issue 8.2018,page No 1.
2. T.R.J. Jeba Malar(et.al) In-vitro phytochemical and pharmacological bio-efficacy studies on Azadirachta indica A.Juss and Melia azedarch Linn for anticancer activity. National Library of Medicine, National centre for Biotechnology information.
3. Muhammad Ihtisham Umar(et.al) Multi-constituent synergism is responsible for anti-inflammatory effect of Azadirachta indica leaf extract. Pharmaceutical Biology.page No.3
4. Min Yang (et.al) Phytochemical analysis of traditional chinese medicine using liquid chromatography coupled with mass spectrometry. Journal of chromatography A.
5. R.Raissa, A.Safitri, MAG Beltron and A. Aulanniam(et.al) Phytoconstituents Investigation on the Ethanolic Extract of Azadirachta indica var. Indonesian and Philippines.Journal of physics: Conference series.page No. 2
6. Mamta Yadav, Smita Mishra(et.al) Investigating the Pharmacognostic and Pharmacological Activities of Azadirachta indica L. Through Biochemical Assay. Pharmacognosy Research Volume 15, Issue 2, page No.1
7. Santosh Kumar Srivastava (et.al) Phytochemicals of Azadirachta indica Source of Active Medicinal Constituent Used for Cure of Various Disease: A Review. Journal of Scientific Research. Volume 64, Issue1,2020, page No. 2
8. T.Anju, A.Divya (et.al). Phtochemical Screening and Sepration of Bioactive compounds from Azadirachta indica leaves by TLC and Column chromatographic techniques. World Journal of Pharmaceutical research. Volume 12, Issue 4, 2080-2090.2023
9. Quadri O. Nurudeen, Mansurat B, Falana(et.al) Identification and Quantification of Secondary Metabolites and The Antimicrobial Efficacy of Leaves Extracts of Some Medicinal Plants. ZANCO Journal of Pune and Applied Sciences.