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Original Article

Investigating the Pharmacognostic and Pharmacological Activities of *Azadirachta indica* L. through Biochemical Assays

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ABSTRACT

Background: *Azadirachta indica* L., commonly known as 'neem' is a member of the Mahagony family, widely available in Indian sub-continents. Neem tree is popularly known for its vast therapeutic properties such as antimicrobial, antiparasitic, antidiabetic, insecticides and pesticides. The plant leaves are commonly used in several Ayurvedic formulations such as '*kwath*' and '*churna*.' Due to its high therapeutic value, it is important to standardize and document the quality parameters of the plant leaves. The *A. indica* leaves were collected, shade dried and pharmacognostic parameters were performed using techniques such as microscopy, physicochemical tests, viz., extractive values, total ash, acid insoluble ash. Apart from this, pharmacological assays for antioxidant, antidiabetic and antimicrobial activities were performed. Based on the assays and preliminary phytochemical analysis, best extract was selected for further compound(s) detection by HPLC and HPTLC. **Results:** Neem *methanolic* extract was found to possess various phytochemicals compared to *hexane* extract, the *methanolic* extract of neem leaves also exhibited excellent antioxidant and antidiabetic activity along with good antimicrobial against gram-positive bacteria and fungi. Through HPLC and HPTLC analysis, presence of rutin hydrate, ellagic acid, and quercetin was observed in the neem leaf methanolic extract. The pharmacognostic tests values were found similar to that of stated in IP-2022. **Conclusion:** This is one of the first kind of research to provide a comprehensive pharmacognostic profile of leaves of *A. indica* L. as well as its pharmacological evaluation along with compounds detection by high throughput analysis.

Keywords: *Azadirachta indica*, Physicochemical analysis, Antioxidant assay, α-amylase enzyme inhibition assay, HPLC, HPTLC.

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INTRODUCTION

Since the ancient times, human civilization is dependent on nature to fulfil their needs. To overcome various illness and ameliorate diseases, humans are using natural products, especially plant derived products as medicines. The importance of plants can be discerned by the popularity of Ayurveda across the world. Today, where synthetic medicines are failing to cure the disease, moreover causing the rapid increase of resistance in the micro-organisms against the existing medicines, plants are emerging as a new reservoir for the potential lead compounds.[1-3] Plants are known for their vast resource of secondary metabolites which are constitutively produced. These include variety of phytochemicals broadly characterized as alkaloids, phenols, terpenoids, steroids, glycosides, and their derivatives. Secondary metabolites are known to possess pharmacological and therapeutic activities and hence explored to find potent chemical moiety.[4] Phytochemicals research has been greatly facilitated by the use of modern analytical techniques for isolation, identification, and structure elucidation of the isolated phytochemical compounds.

Azadirachta indica L., (Fam. Meliaceae) also known as 'Neem', is commonly found in Indian sub-continents and most of the African countries as they can easily be grown in tropical and sub-tropical forests. Neem tree is known for its several therapeutic properties and known for curing diseases such as acne, stomach infection, fever, and more. Ayurveda states that neem balances *kapha* and *pitta* out of the three *doshas* in the human body. Neem oil is known to cure toothache, and also used as mosquito repellant. In Tamil Nadu, tender shoots of neem tree are used to cook a soup like dish "*Veppampoo charu.*" Neem flowers are used to prepare a famous Bengali dish "*Begun bhaja.*" Powdered neem leaves are used to prepare various *churna* and *kwath* in

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Ayurveda. Recent research study proved that *A. indica* leaves are known to have antifungal and antibacterial activities along with anti-inflammatory, antiarthritic, antipyretic, hypoglycemic, anti-gastric ulcer, antitumor activities.[5-8]

Despite the high therapeutic efficacy of neem leaves, there is a very little knowledge about the requirements for its standardization. Since the standardization of the raw material quality for the extraction and product development is required, the present study has documented the required pharmacognostic parameters such as extractive values, loss on drying, total ash, acid insoluble ash, and assay by HPTLC, of dried neem leaves along with their microscopic analysis. Apart from this, we conducted the preliminary phytochemical screening for antioxidant, anti-diabetic and anti-microbial activities of neem leaf extract. HPLC analysis along with some analytical standards were also performed to surmise the presence of bioactive compounds and their pharmacological activities, validating the therapeutic activity of the plant.

MATERIALS AND METHODS

Plant Material Collection and Authentication

Leaves of *Azadirachta indica* were collected locally and submitted to CSIR-National Institute for Science Communication and Policy Research for authentication (Authentication no. – NISCAIR/RHMD/Consult/2021/4160-61-1). Leaves were shade dried, finely powdered, and stored in airtight container for further use.

Chemicals and Reagents

Solvents used for experimentation such as ethanol, methanol, ethyl acetate, dimethylsulfoxide (DMSO) and chloroform were of analytical grade, purchased from Finar Ltd. And Merck. HPLC grade solvents were purchased by Standards for HPTLC analysis such as Rutin hydrate, Quercetin, Gallic acid, and Ellagic acid were purchased from Sigma Aldrich. DNS reagent, Anisaldehyde reagent, Alpha-amylase enzyme, maltose, and starch was purchased from SRL Chemicals. Bacterial and fungal media nutrient broth, nutrient agar, and potato dextrose agar were purchased from Himedia Ltd.

Bacterial and fungal samples procurement

For anti-microbial activity determination, two gram-positive bacteria, viz., *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 6535, two gram-negative bacteria, viz., *Escherichia coli* ATCC 10536 and *Salmonella typhi* ATCC 14025, and one fungi (yeast) *Candida albicans* ATCC 10231 were procured from Microbiology division, Indian Pharmacopoeia Commission, Ghaziabad.

Microscopical Evaluation

The fresh leaves of *Azadirachta indica* were washed with distilled water and thin sections were cut using a sharp razor blade to obtain a very thin, almost transparent slice, placed on a glass slide and covered with a coverslip. Some leaves were also crushed using mortar and pestle and a drop of crushed leaves was placed on a clean glass slide and covered with a coverslip, making sure that no air bubble remains in the coverslip. The slides were observed under the microscope (Nikon eclipse Ts4) at different magnifications and the best images were clicked with the help of camera attached with it.[9]

Determination of physicochemical parameters

Physicochemical standards like extractive value, total ash, loss on drying were determined as per the standard procedures stated in IP-2022 (Indian Pharmacopoeia 2018, Volume 2).[10-12] The procedures of the tests performed are explained below.

Loss on Drying

5g of plant material was accurately weighed in a crucible and dried at 105°C for 3hr in a hot air oven. Weight was taken after drying to observe the loss. The dried sample was continued dried at 1 hr intervals until we get the constant weight. Constant weight was reached when two consecutive weight difference is not more than 0.05mg. For weighing, the crucibles were cooled under the desiccator so as not to catch any moisture during the cooling process from the atmosphere. % Loss on Drying was calculated by the formula given below: -

% Loss on Drying = (Difference in the weight of air-dried sample and oven dried sample/weight of air-dried sample) X100

Determination of water-soluble extractive value

5g of air-dried coarsely powdered neem was macerated with 100ml of milli-Q-water in a closed flask for 24 hr, shaken occasionally during the first 6 hr, and allowed to stand for 18 hr. Thereafter filtered through Whatman filter paper no.41. 25ml of the filtrate was evaporated to dryness in a preweighed flat-bottom petri dish, dried at 105°C (for moisture removal) and weighed. % w/w water-soluble extractive value was calculated by the given formula: -

% Water soluble extractive = [{weight of the dried 25ml extractive/weight of sample (5g)} X dilution factor X 100]

Weight of sample taken – 5g; Dilution factor – 4; extractive weight can be calculated by subtracting the initial petri plate weight from the dried plate with extractive

Determination of Alcohol soluble extractive value

5g of air-dried coarsely powdered neem was macerated with 100ml of ethanol in a closed flask for 24 hr, shaken occasionally during the first 6 hr, and allowed to stand for 18 hr. The mixture was filtered through Whatman filter paper No.41 and 25ml of the filtrate was evaporated in a preweighed flat-bottom petri dish

dried at 105°C and weighed accordingly. % w/w alcohol soluble extractive value was calculated by the given formula: -

% Alcohol soluble extractive = [{weight of the dried 25ml extractive/weight of sample (5g)} X dilution factor X 100]

Weight of sample taken – 5g; Dilution factor – 4; extractive weight can be calculated by subtracting the initial petri plate weight from the dried plate with extractive

Determination of Total Ash

About 2g of air-dried drugs was accurately weighed in a previously ignited and tarred silica crucible (empty crucible weight was taken). The sample was ignited by gradually increasing the heat from 500 to 600±25°C until it formed white ash in a muffle furnace for 4 hr. The furnace and crucibles were allowed to cool and carefully removed from the furnace using tongs and placed in the desiccator until it cools off to room temperature. The % w/w total ash concerning the air-dried material was calculated by the given formula: -

Total ash % = {Total ash formed in the crucible/weight of sample (2g)} X 100

Crucibles in the furnace should be handled with great care as it may cause severe burn.

Determination of Acid Insoluble Ash

The ash formed in the above experiment was subjected to 25ml of 2M HCl, and boiled for 5 min. The solution was filtered through ashless filter paper, and placed in the same crucible. The crucibles with filter paper were reignited at 600±25°C. until it formed white ash. The furnace and crucibles were allowed to cool and carefully removed from the furnace using tongs and placed in the desiccator until it cools off to room temperature. The % w/w acid insoluble ash concerning the air-dried material was calculated by the given formula: -

Acid insoluble ash % = {Total ash formed in the crucible with ashless filter paper/weight of sample (2g)} X 100

Crucibles in the furnace should be handled with great care as it may cause severe burn.

Extract preparation

Two types of extracts of *A. indica* leaves were prepared; viz., methanol and hexane. 5g each of coarsely powdered leaves was refluxed with 100ml hexane and methanol for 2 hr in separate round bottom flask via Soxhlet apparatus. The filtrate obtained were concentrated under vacuum rotary evaporator. Yields were calculated on the basis of percentage w/w by the given formula: -

% Yield = (weight of dried extract/total weight of sample) X 100

Preliminary Phytochemical Screening

Preliminary phytochemical analysis of various extracts of *Azadirachta indica* (leaves), was carried out by using standard procedure with slight modifications.[13–20]

Test for alkaloids: (Wagner test) few drop of Wagner reagent was added with 2ml of leaves to extract reddish brown precipitate indicating the presence of alkaloids.

Test for flavonoids: (Alkaline reagent) 2ml of NaOH was added with 2ml of plant extract yellow color formed which become colorless when dil. acid was added to indicate flavonoid present.

Test for tannins: 2ml plant extract with a few drops of ferric chloride was added violet color appearance that indicate tannins were present.

Test for terpenoids: (Salkowski Test) 5ml of the extract was mixed with 2ml of chloroform and concentrated sulphuric acid was added to form a layer. A reddish-brown color indicates the presence of terpenoids.

Test for glycoside: 2ml of acetic acid and 2ml of chloroform were added with the plant extract to allow cool down of the sample add a few drops of H_2SO_4 green color to indicate the presence of glycoside.

Test for anthraquinone: 5ml of extract, added dilute H_2SO_4, and 1ml of diluted ammonia. The appearance of pink Color indicates the presence of anthraquinone.

Test for phytosterols: 2ml of chloroform was added with the plant extract then 2ml of H_2SO_4 was added to the red color form in the chloroform layer.

Quantitative analysis of neem extracts' phytochemicals

***Total alkaloids content*:** Bromocresol green solution was prepared by dissolving 69.8 mg bromocresol green in with 3ml of 2N NaOH solution and 5ml of distilled water. Heat was applied for complete dissolution. The solution was further diluted to 1000ml with distilled water for experimentation. To 1ml of of 10mg/ml prepared extract, 5ml Phosphate buffer solution (pH 4.7) and 5ml bromocresol green was added and shaked vigorously. Then, 5ml Chloroform was added to the mixture and chloroform layer was separated by using separating funnel. The chloroform layer was subjected to UV-visible spectrophotometer at 470nm. The reading was noted and alkaloid content was determined by the standard curve plotted by Caffiene (standard), subjected to the same process at series of concentration. The total alkaloid content was expressed as Caffiene Equivalent.[21,22]

***Total phenols content*:** 1.5 ml Folin Ciocalteu's reagent was added to 1ml of 10mg/ml extract, and allowed to incubate at room temperature for 5 min. 4 ml of 20% (w/w) Na_2CO_3 was added, adjusted with distilled water up to the mark of 10 ml, agitated and

left to stand for 30 min at room temperature. Absorbance of the sample was measured at 765 nm against blank. The reading was noted and total phenolic content was determined by the standard curve plotted by Gallic acid (standard), subjected to the same process at series of concentration. The total phenols content was expressed as Gallic acid Equivalent.[16,23,24]

***Total flavonoids content*:** 0.5ml of 10mg/ml extract was mixed with 1.5 ml of methanol, 0.1 ml of aluminium chloride (10% w/v in water), and 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm with UV/VIS spectrophotometer. The reading was noted and total flavonoids content was determined by the standard curve plotted by Rutin (standard), subjected to the same process at series of concentration. The total flavonoids content was expressed as Rutin Equivalent.[24]

***Total terpenoids content*:**100mg of plant extract was dissolved in 9mL of ethanol and incubated for 24hr. The extract was filtered and extracted with 10mL of petroleum ether, and concentrated to obtain terpenoid. The contents were measured in terms of per gram of dry extract.

***Total steroids/phytosterols content*:** A measured portion of extract was dissolved in 90% ethanol in water. It was partitioned with n-hexane, filtered and concentrated. The content was expressed in per gram of extract sample.

Pharmacological assays

To assess the therapeutic actions of *A. indica* leaf extract, *in-vitro* antioxidant, anti-diabetic, and anti-microbial activities were studied. The method of each test is explained below.

Antioxidant assay – Phosphomolybdenum assay

Phosphomolybdenum assay, also known as reducing power assay, is commonly used to determine the antioxidant potential of samples. For the assay, 0.1 mM solution of Phosphomolybdenum reagent was prepared by mixing equal volumes of 0.6M sulfuric acid, 28mM sodium phosphate, and 4mM ammonium molybdate. 1 ml prepared reagent was added to 1 ml of the solution of the extract in methanol at different concentrations (0, 20, 40, 60, 80, and 100 µg/ml) and incubated 95°C in water bath for 90 min. The absorbance was measured at 517 nm accordingly.[25,26]

In-vitro Anti-diabetic assay – α-amylase enzyme inhibition assay

To assess anti-diabetic activity of *A. indica* leaf extract, *α*-amylase enzyme inhibition assay was performed. For this, 1mg/ml stock solution of extract was prepared along with stock solution of α-amylase enzyme (10 units/ml) and starch solution (1% w/v) in water. The maltose formation due to enzyme activity was detected by adding DNS reagent, which imparts color with mono and oligo-saccharides. Enzyme activity was calculated with the help of standard curve of maltose, made by its serial dilution (Table 1). Metformin was used as standard drug for comparison. Whole reaction was performed in 1X Phosphate Buffer Saline (PBS).[6,27] The assay process is explained below in Table 2.

In-vitro Anti-microbial activity

Bacterial culture preparation – Stocks of above-mentioned bacterial strains were revived by inoculating in nutrient broth and incubated for 24 to 48 hr at 37°C. When turbidity reached 0.6 OD (600nm), the cultures were inoculated on nutrient agar plates.

Fungal culture preparation – Stock of *C. albicans* were revived by inoculating in nutrient broth and incubated for 24 to 48 hr at 37°C. When turbidity reached 0.6 OD (600nm), the cultures were inoculated on potato dextrose agar.

Well diffusion assay for antimicrobial activity assessment – Fresh Nutrient agar plates for bacteria and Potato dextrose agar plates were prepared by dissolving measured amount of media in distilled water and subsequently autoclaved at 120°C, 15 Psi pressure for 15 min. Serial dilution of bacterial and fungal cultures was made so as to obtain 3×10^6 CFU/ml bacterial/fungal cultures (based on McFarland's constant). Each diluted cultures were spread on the agar plates and left for 1-2 min so that cultures get absorbed on agar surface. Four wells were bored on the agar plates at equal distances and 1ml of extracts (stock solution 10mg/ml and 100mg/ml in 4% DMSO) were added inside the well. 4% DMSO solution was used as negative control. The plates were incubated for 24 to 48 hr or until full plate growth was observed. The diameter zone of inhibition was measured by using Vernier Calliper and recorded in diameter.[20,26,28]

High performance thin layer chromatography (HPTLC) *A. indica* leaf extract

Reference solution: Stock solutions of 1mg/ml was prepared for standards viz., Rutin hydrate, Quercetin, Gallic acid, and Ellagic acid in methanol.

Test solution: Stock solution of 1mg/ml of *A. indica* methanolic leaf extract was prepared.

Mobile phase: 25 volumes of ethyl acetate, 15 volumes of n-butanol. 5 volumes of formic acid, and 5 volumes of water was dissolved in 10 ml volumetric flask, sonicated and cooled.

10 µl of each solution was applied to a 10×10cm silica plate with aluminum coating (Silica 60 F_{254}) as bands of 10 mm by 2 mm. Allow the mobile phase to rise 8 cm. Dry the plate in air and examine under ultraviolet light at 254 nm and 366 nm. The plate was post-derivatized by spraying with *anisaldehyde sulphuric acid reagent*. Heat the plate at 105°C for 5 min and examine in day light. The pictures of the developed and derivatized plates were documented by *CAMAG* detector.[11,12]

Table 1: Procedure for standard curve plotting of maltose.

Maltose(µl)	PBS (ml)	DNS reagent	Incubate for 15 min at boiling temperature and add water 2ml if needed
0	2.0	1ml	10
1.9	20		1.8
30	1.7		40
1.6	50		1.5
60	1.4		70
1.3	80		1.2
90	1.1		100
1.0			

Table 2: Procedure for α-amylase enzyme inhibition assay.

Extract (µl)	1X PBS (ml)	Enzyme (ml)	Incubate at room temperature for 10 min	Starch (ml)	DNS reagent	Incubate for 15 min at boiling temperature, cool, read at 517nm
0	2.0	0.5	0.5	1ml	20	1.8
40	1.6				60	1.4
80	1.2				100	1.0
0	2.0				0	

High throughput screening and detection of *A. indica* leaf extract by HPLC analysis

HPLC analysis of neem methanolic extract was done by using Agilent infinity 1260. Extract separation and detection was done on column C_{18}; Orbit, 150mm×4.6mm; 3.5µm. the sample injection volume was 20 µl, while mobile phase used was ACN: 0.5% formic acid solution in MiliQ water (30:50). Elution was isocratic and run time was 30 min. Before sample injection, column was washed for 45 min with the above-mentioned mobile phase. The peak detection was done at 340nm.[11,12]

Statistical analysis

All analysis was done in triplicate manner. The results were represented in Mean ± Standard deviation wherever required. The HPLC software used was EZchrome elite, while HPTLC software used was WinCats. For calculation and graph layout, MS excel was used. Negative and positive controls were used in biochemical assays and best was done to minimize zero error.

RESULTS

Microscopy

The leaves are dark green, and the petioles are short. The shape of mature leaflets are more asymmetrical and margins are dentate except the base of their basiscopal half, which is very strongly reduced, and cuneate or wedge-shaped leaves. Upper and lower epidermis exhibit two layers of palisade cells below the upper epidermis. The spongy parenchyma with intercellular spaces is abundant on the borderline of palisade cells. The midrib shows numerous collenchyma cells below the upper and lower epidermis. A characteristic zone of vascular bundles is present. Stomata can also be observed. (Figure 1).

Physio-chemical tests

Azadirachta indica leaf powder was found to have 9.72% moisture content as observed % LOD. The % water soluble extract was found to be 11.25%, while ethanol soluble extractive was 7.69%. It was observed that neem leaf contains Σ9.89% total ash, out of which only 1.45% ash in insoluble in acid. The physicochemical results obtained were found to be correlated to that mentioned in neem monograph of IP-2022 (Table 3).

Extract yield and Phytochemical screening

Percentage yield of *methanolic* extract of *A. indica* leaves were found higher than *hexane* extract, showing 10% yield in methanol, while only 3% yield was recorded in hexane. From phytochemical screening, it was observed that *hexane* extract was devoid of several class of compounds such as alkaloids, flavonoids, glycosides, etc. *Hexane* extract was found to be rich in phenols, terpenoids and phytosterols. While, *methanolic* extract was found to possess almost all phytoconstituents, hence further pursued for pharmacological activities. (Table 4).

Quantitative analysis of neem extracts' phytochemicals

From the quantative analysis, it was found out that the ethanolic extract consisted phenols in highest amount (429.73±1.89mg/G extract) followed by flavonoids (317.08±7.09mg/G extract). While, alkaloids, terpenoids, and phytosterols were found at low quantity. Whereas, hexane extract was found to possess phytosterols at highest quantity (356.09±5.03mg/G extract), followed by terpenoids (126.58±1.02mg/G extract) and phenols (54.66±0.3mg/G extract). The hexane extract of *A. indica* was found to have alkaloids and flavonoids in negligible amount (Figure 2).

Pharmacological assays

Antioxidant activity

The methanolic extract of *Azadirachta indica* was found to have excellent antioxidant activity. The amount of the presence of antioxidants was measured relative to ascorbic acid. It was found that 1 gram of extract contains approximately 267.46±48.42 mg of Ascorbic acid equivalent (AAE) antioxidants. The results are described in detail in Tables 5 and 6, and Figures 3 and 4.

Anti-diabetic activity

The methanolic extract of *A. indica* was found to exhibit excellent anti-diabetic activity, as it was found to inhibit α-amylase enzyme more than 90%, even at lower concentration. The enzyme activity inhibition potential of neem extract was found to be comparable of the standard drug Metformin. While metformin showed 99% inhibition at 11mg/ml concentration, neem extract showed 92.67% inhibition. It is to be observed that standard drug showed ~70% inhibition at 20mg/ml concentration, while neem extract showed 90.95% inhibition at the same concentration. It is to be noted that metformin is a pure compound, while neem extract contains several compounds, hence the inhibition showed by the extract becomes significant. Result description can be assessed from Tables 7-10; Figures 5, 6 and 7.

Antimicrobial activity

The methanolic leaf extract of *Azadirachta indica* showed good antimicrobial activity against gram-positive bacteria – *S. aureus* and *B. subtilis*. The extract was found to be more active against S. aureus, exhibiting inhibition at 10mg/ml, while it showed no effect on gram-negative bacteria. It is to be noted that the extract was also found to be effective on *Candida albicans* (fungi). Both *S. aureus* and *C. albicans* are known for skin disease, hence it is inferred that *Azadirachta indica* leaves may be used to treat skin diseases (Table 11; Figure 8).

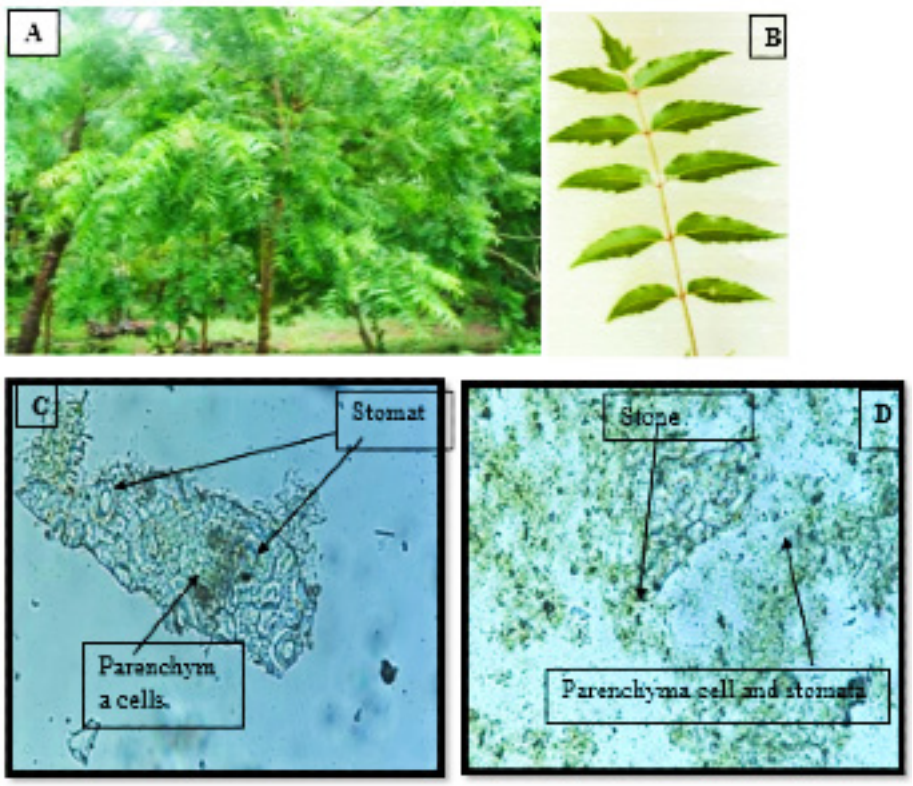

Figure 1: A) Neem tree, B) neem leaf, C) Microscopy of neem leaf by section cutting, and D) Microscopy of neem leaf by crushing.

Table 3: Results of physicochemical parameters of *Azadirachta indica* leaf.

Parameters	Results (%)	Limits given in IP 2022	Complies/Not Complies
Loss on Drying	9.72±0.5	NMT 12.0%	Complies
Alcohol Soluble Extractive	7.69±0.25	NLT 6.0%	Complies
Water Soluble Extractive	11.25±0.33	NLT 19.0%	Complies
Total ash	9.89±0.23	NMT 10.0%	Complies
Acid insoluble ash	1.48±0.01	NMT 2.0%	Complies

NMT: Not more than; NLT: Not less than; Values were represented as Mean±SD

Table 4: Phytochemical screening of neem extracts.

Phytochemicals	Methanol extract	Hexane extract
Alkaloids	+	-
Flavonoids	+	-
Phenols	+	+
Tannins	-	-
Terpenoids	+	+
Glycosides	+	-
Anthraquinones	-	-
Saponins	-	-
Phytosterols	+	+

(+) - Indicates presence(-) – Indicates absence

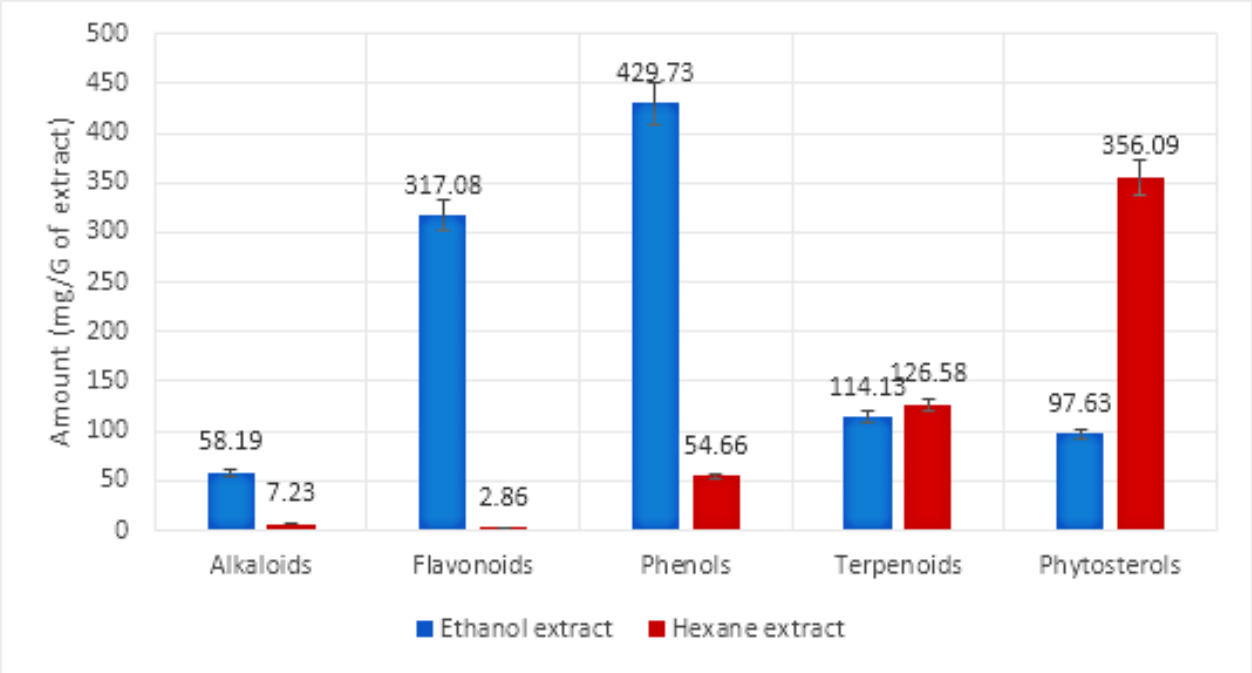

Figure 2: Graph representing the quantitative assessment of the phytoconstituents present in neem extracts.

Table 5: Ascorbic acid concentration for standard curve plot.

Ascorbic acid Concentration	Absorbance at 695 (1)	Absorbance at 695 (2)	Mean Absorbance	Standard Deviation
0	0	0	0	0
10	0.1196	0.1225	0.12105	0.00205061
20	0.1458	0.1727	0.15925	0.019021172
30	0.1743	0.2112	0.19275	0.02609224
40	0.2356	0.2629	0.24925	0.019304015
50	0.2865	0.3291	0.3078	0.030122749
60	0.3455	0.3169	0.3312	0.020223254
70	0.3987	0.3869	0.3928	0.00834386
80	0.4985	0.4922	0.49535	0.004454773
90	0.5112	0.5236	0.5174	0.008768124
100	0.5826	0.583	0.5828	0.000282843

Table 6: Antioxidant activity assay of *A. indica* methanolic leaf extract.

Sample concentration	OD at 695nm	Reducing power AAE	Antioxidants mg/g of extract
0	0	0	0.00
20	0.027	4.576271186	228.81
40	0.077	13.05084746	326.27
60	0.111	18.81355932	313.56
80	0.114	19.3220339	241.53
100	0.134	22.71186441	227.12
		Mean	267.46
		SD	48.42

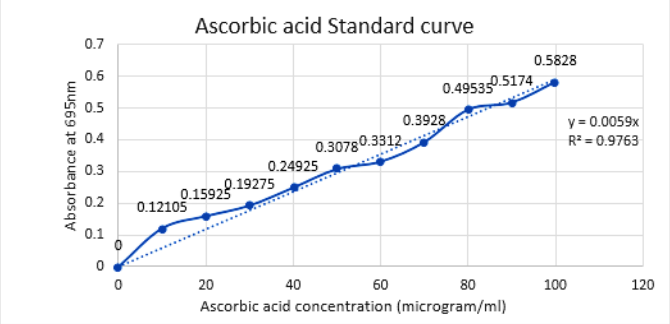

Figure 3: Standard curve plot of ascorbic acid.

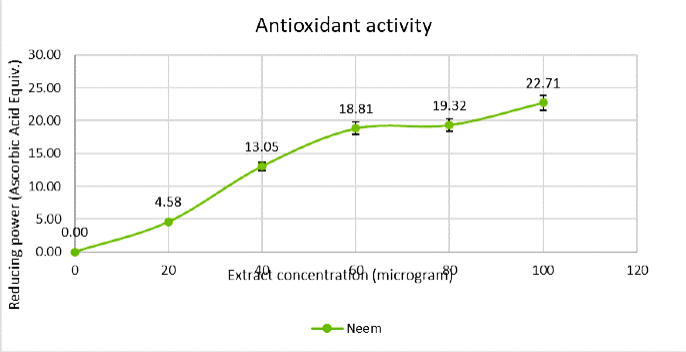

Figure 4: Graphical representation of antioxidant activity *A. indica* leaf extract.

High performance thin layer chromatography (HPTLC) *A. indica* leaf extract

To assess the phytoconstituents of *A. indica* methanolic leaf extract, TLC was performed using several analytical standards. Out of many, rutin, gallic acid, ellagic acid, and quercetin were found in the extract., having rutin and quercetin in higher quantity. Rutin was detected at R_f 0.44, while quercetin and gallic acid were detected at 0.79, 0.75. Ellagic acid, although present (R_f 0.5), did not formed uniform band, hence not counted. After derivatization with Anisaldehyde sulfuric acid, all bands were visible under daylight (Figure 9).

High throughput screening and detection of *A, indica* leaf extract by HPLC analysis

The quantification of bioactive compounds by HPLC showed that *A. indica* leaf extract contains gallic acid, rutin, ellagic acid, and quercetin by comparing the chromatogram of the reference standards. Retention time of the standards were found to match the resolved peaks of neem extract. Gallic acid peak was observed at 2.20 min, which exactly matches to a small peak present in the extract. Rutin hydrate was eluted at 4.533 min, while in the extract it was found at 4.653 min with 0.12 min delay. Ellagic acid was detected at 4.947 min, while in the extract it was detected at 5.08 min with 0.133 min delay. Quercetin was detected

Table 7: Maltose standard curve reading.

Maltose (mM)	Abs at 517 nm
0	0
10	0.024
20	0.063
30	0.098
40	0.114
50	0.162
60	0.186
70	0.228
80	0.279
90	0.314
100	0.32

Table 8: Percentage α-amylase enzyme inhibition.

Sample Concentration (mg/ml)	% Inhibition (Mean±SD)	
	Metformin	Neem
20	71.32±0.09	90.95±0.98
40	84.11±0.04	91.81±0.23
60	89.92±0.57	91.81±1.08
80	90.31±1.06	91.81±0.54
100	99.22±0.87	92.67±0.37

Table 9: Enzyme activity in the presence of extracts.

Sample concentration	Absorbance	Maltose formation (mM)	Enzyme activity (Unit/ml)	% Enzyme inhibition	Enzyme activity/mg of extract
0	0.232	70.30	10.55	0.00	
20	0.021	6.36	0.95	90.95	452.27
40	0.019	5.76	0.86	91.81	228.41
60	0.019	5.76	0.86	91.81	152.27
80	0.019	5.76	0.86	91.81	114.20
100	0.017	5.15	0.77	92.67	92.27

Table 10: Percentage enzyme activity of neem extract and metformin.

Sample Concentration	Metformin	Neem extract
0	11.73±0.07	10.55±0.08
20	3.36±0.04	0.95±0.03
40	1.86±0.01	0.86±0.07
60	1.18±0.00	0.86±0.05
80	1.14±0.02	0.86±0.08
100	0.09±0.03	0.77±0.03

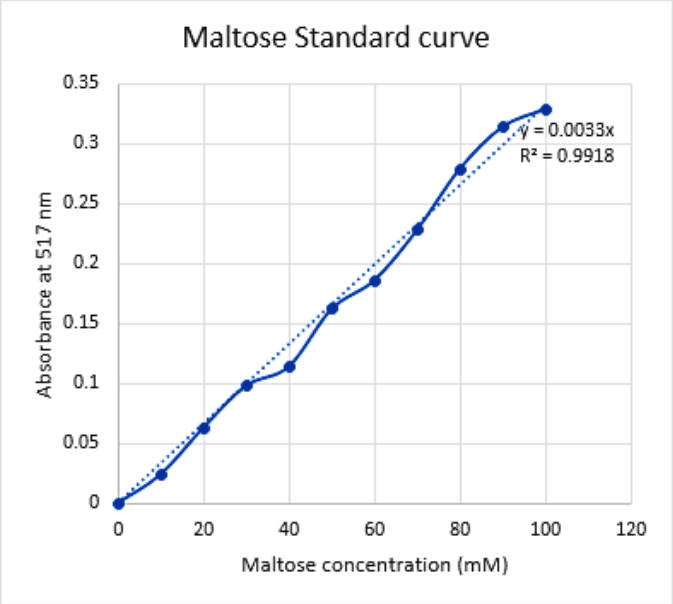

Figure 5: Maltose Standard Curve.

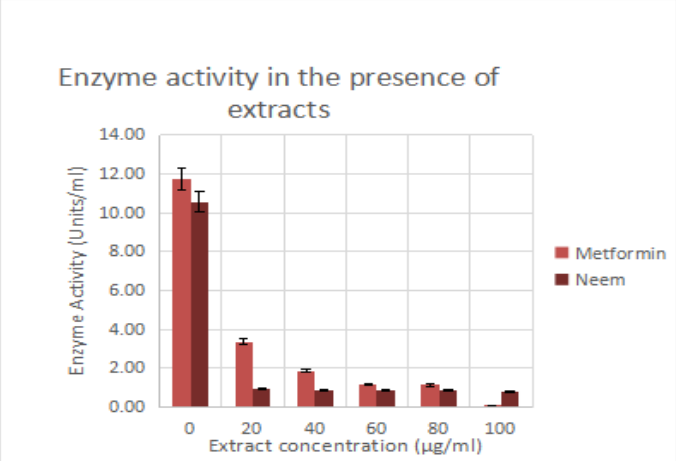

Figure 6: Graphical representation of Alpha-Amylase enzyme inhibition.

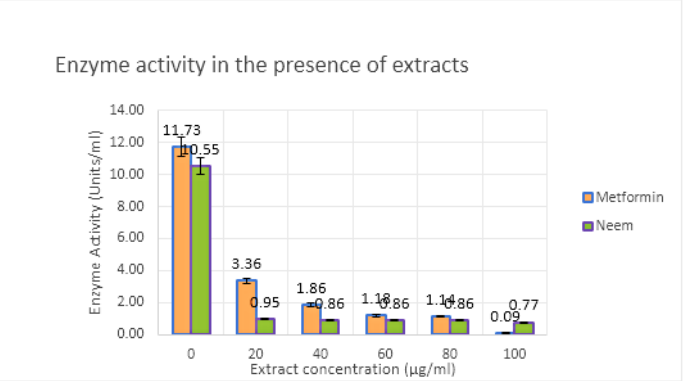

Figure 7: Graphical representation of enzyme activity of neem extract and metformin.

Table 11: Anti- microbial activity of *Azadirachta indica* leaf methanolic extract.

Microorganism	Extract concentration		Negative control
	10mg/ml	100mg/ml	4% DMSO
B. subtilis ATCC 6633	ND	16.5 mm	ND
S. aureus ATCC 6535	12.4 mm	15.5 mm	ND
E. coli ATCC 10536	ND	ND	ND
S. typhi ATCC14025	ND	ND	ND
C. albicans ATCC10231	ND	13.0 mm	ND

ND – Zone of inhibition Not Detected

Table 12: Retention time of standards separately and within the extracts detected in HPLC.

Standard	Standard peak RT	Extract peak RT
Gallic acid	2.220	2.227
Rutin hydrate	4.533	4.653
Ellagic acid	4.947	5.080
Quercetin	13.767	13.947

at 13.767 min, while in the extract it was detected at 13.947 min with 0.18 min delay (Table 12; Figure 10).

DISCUSSION

Azadirachta indica, also known as neem, is very commonly used in Indian household remedies. People put neem leaves in water during bath as a natural cleanser. Neem leaves paste is used for curing skin diseases, healing wounds, reducing diabetes etc. All these therapeutic properties and its extensive use in Ayurvedic medicines make *A. indica* a highly valued medicinal plant for pharmaceutical and research industries. Hence, it is very important to standardize its physicochemical parameters. Through our study, it was found that the microscopic analysis of neem leaves was found matching to that described in the literatures. Physicochemical parameters of neem leaves were studied and found out to be correlated to the limits defined in the herbal monograph of Neem Leaf in Indian Pharmacopoeia 2022, Volume 4.

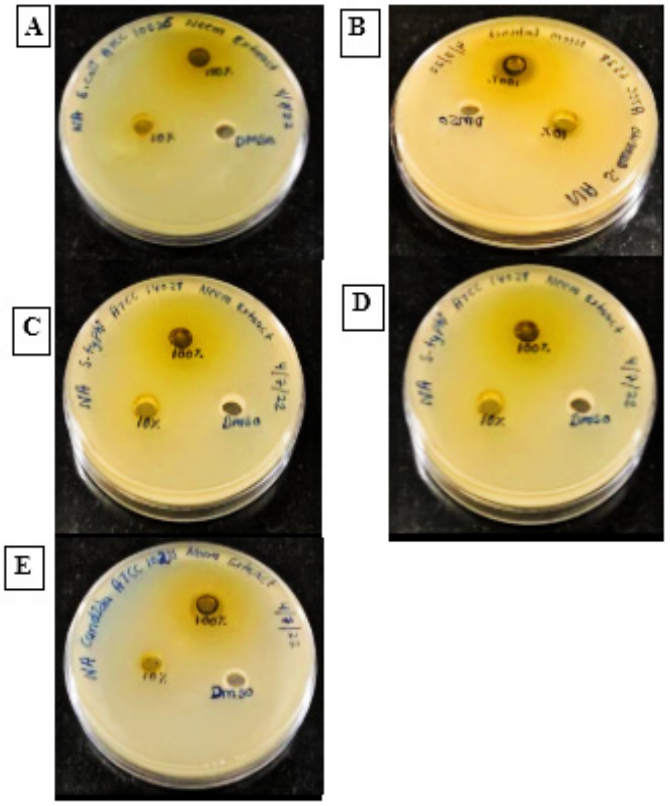

Figure 8: Pictures of well diffusion plates showing zone of inhibitions in A) *E. coli*, B) *S. aureus*, C) *B. subtilis*, D) *S. typhi*, and E) *C. albicans.*

Apart from that, *A. indica* leaf methanolic extract was found to be rich in several types of phytoconstituents such as alkaloids, flavonoids, steroids, tannins, and others. The methanolic extract of neem leaf was also found to be rich in antioxidants, and also showed *in-vitro* antidiabetic and antimicrobial activities. The extract was found to be active against gram-positive bacteria but not against gram-negative one. As the extract was found to be antioxidant rich, the extract was tested with some known antioxidant compounds by TLC analysis. Finally, through HPTLC analysis and high throughput analysis by HPLC, the presence of Rutin hydrate, Ellagic acid, Gallic acid, and Quercetin was detected in the extract.

CONCLUSION

The pharmacognostic study of *Azadirachta indica* leaf was established successfully. The morphological, microscopical, physicochemical, and preliminary phytochemical analysis would definitely support the plant identification and prevent the adulteration of the raw materials. This study will be highly useful for pharmaceutical industries. It is also helpful in determining its purity and quality standards during the practice of Ayurvedic formulations. The pharmacological studies and

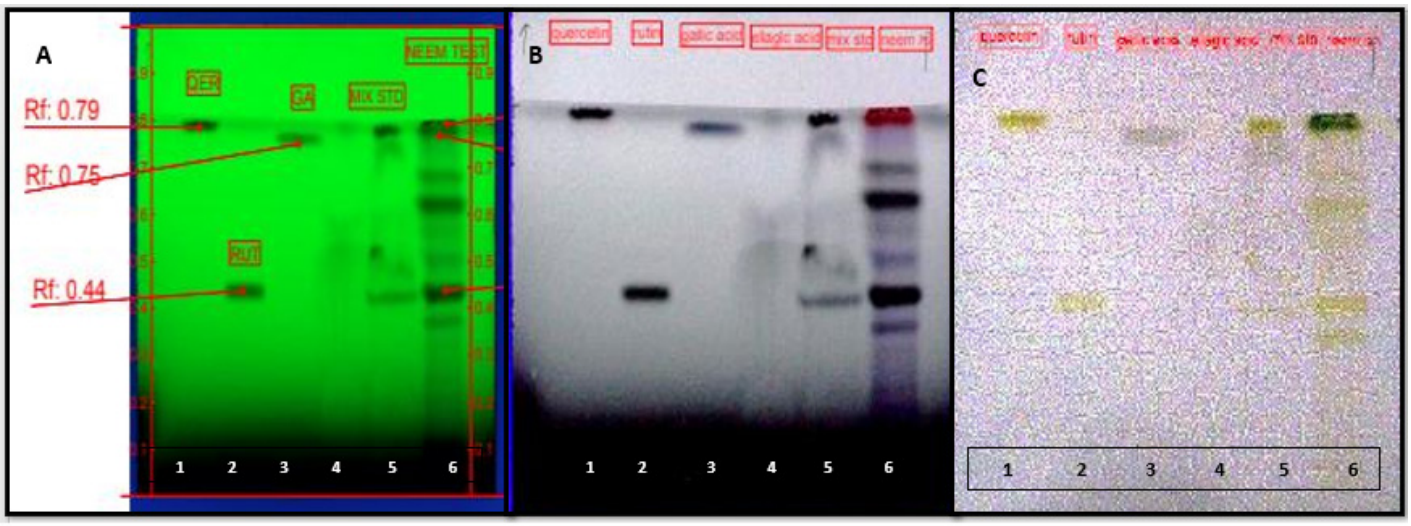

Figure 9: HPTLC analysis of neem methanolic extract with standards. Picture A shows plate at 254 nm, B shows plate at 366nm, C shows plate under daylight after derivatization. Track 1 - Quercetin, Track 2 – Rutin, Track 3 – Gallic acid, Track 4 – Ellagic acid, Track 5 – Standard mix, Track 6 – Neem extract.

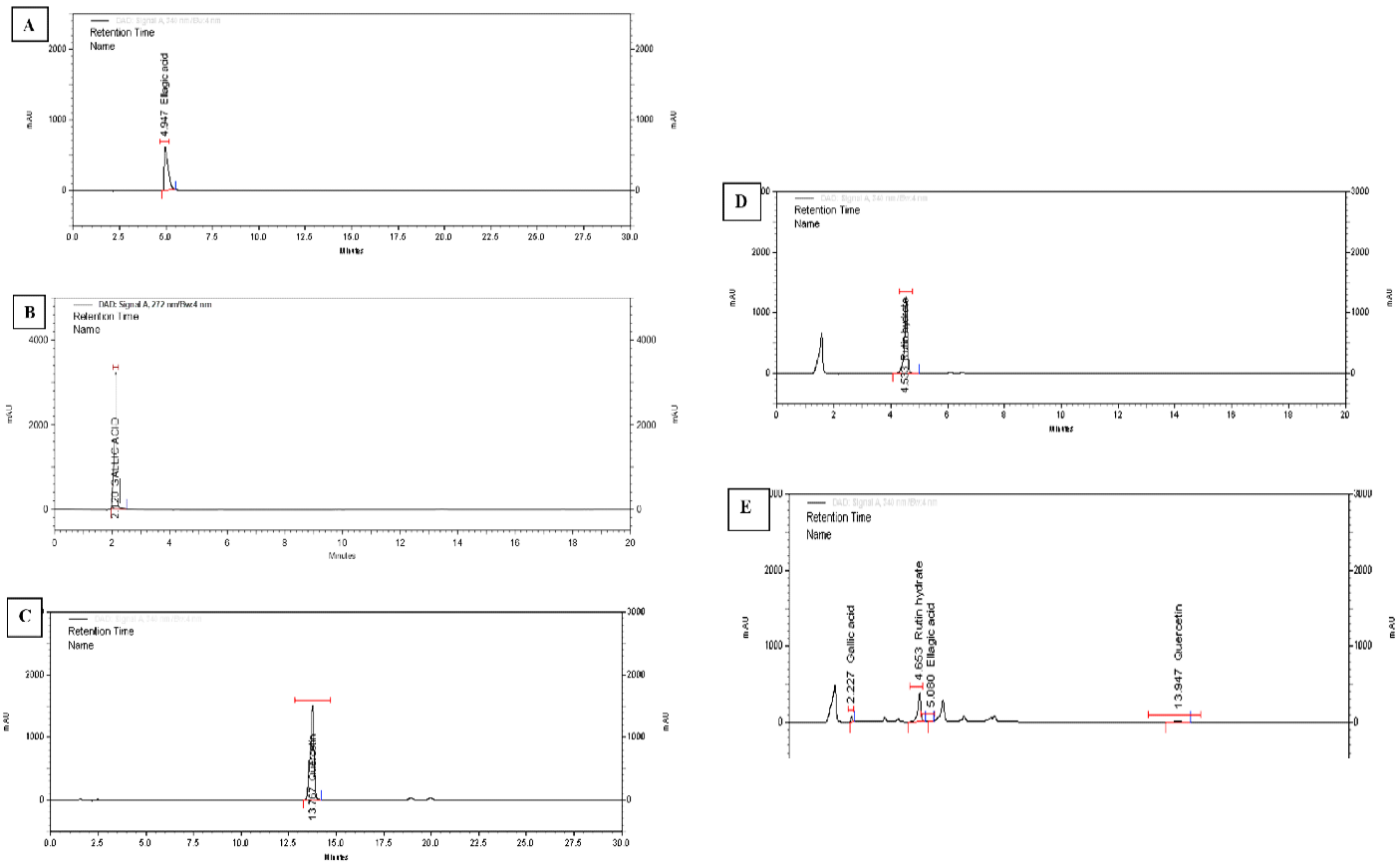

Figure 10: HPLC Chromatograms of A) Ellagic acid, B) Gallic acid, C) Quercetin, D) Rutin hydrate, and E) Methanolic leaf extract of *Azadirachta indica*.

HPLC and HPTLC chromatographic analysis will also add value to the research community. Through high throughput screening, it can be concluded that *Azadirachta indica* leaves are rich in antioxidants and have potent antimicrobial and antihyperglycemic activity. The leaves were also found rich in Rutin hydrate, along with Ellagic acid, Gallic acid, and Quercetin. Although the compounds pursued are of analytical standards and not the biomarkers, these standards are of highly valued in the phytopharmaceutical market. This data may work as a scaffold for Phytopharmaceutical Ingredient (PPI) monograph development. Further analysis, HPLC and HPTLC method validation and quantitation is underway and will be in public soon. *In-silico* study on *A. indica* compounds such as Nimbin, Azadirachtin, and Nimbolides are underway. This study paves the way for the advanced monograph development of herbal products and formulations.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

Authors Contribution

The experiments were conducted by the research trainee, Ms. Mamta. HPLC analysis was conducted by Ms. Smita Mishra. HPTLC part was performed by Mr. Mayank Shukla. Anti-microbial study was conducted by Ms. Bhawna, under the supervision of Dr. Anil K Teotia. The study was jointly supervised by Mrs. Ritu Tiwari, Dr. V. Kalaiselvan, and Dr. Varsha Mehra. Whole team was involved in manuscript designing, writing and reviewing.

ABBREVIATIONS

IPC: Indian Pharmacopoeia Commission; **HPTLC:** High Performance Thin Layer Chromatography; **HPLC:** High Performance Liquid Chromatography; ***A. indica*:** *Azadirachta indica*; **LOD:** Loss on Drying.

SUMMARY

Azadirachta indica is highly valuable medicinal plant, hence the present study has documented the required pharmacognostic parameters such as extractive values, loss on drying, total ash, acid insoluble ash, and assay by HPTLC, of dried neem leaves along with their microscopic analysis. The preliminary phytochemical screening revealed the presence of various phytochemical compounds, and the extract showed excellent antioxidant, anti-diabetic and anti-microbial activity. HPLC and HPTLC analysis along with some analytical standards were also performed to surmise the presence of bioactive compounds, which showed the presence of rutin, ellagic acid, gallic acid, and quercetin. The study can be extrapolated for the HPLC method development of PPI monograph of *Azadirachta indica*.

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