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HPTLC fingerprinting and antimicrobial activity of *Azadirachta indica* leaf extracts

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Abstract

Azadirachta indica Juss which also Known as Neem plant used from ancient times for various human ailments as a traditional medicine. To appraise the scientific study for the use of *Azadirachta indica*, ethanolic extracts of the dried leaves of the plant were subjected to HPTLC finger printing and determination of anti-microbial activity of aqueous and ethanolic extract on five different species of bacteria. The ethanolic extract of dried powdered leaves was evaluated by HPTLC finger printing using solvent system of Dichloromethane: ethanol (19:1) which exhibits the identity of different compounds present in the extracts. The antimicrobial activity of the concentrated extracts was evaluated by determination of the diameter of zone of inhibition against the microorganisms using agar well diffusion method. Both plant extracts had antimicrobial effects against the test organisms, however the aqueous extracts were found to show greater anti-microbial effect than ethanolic extract. Thus, the mean diameter zones of inhibition ranged from 0.04mm-50.00mm for aqueous extract and 0.40mm-20.00mm for ethanolic extract at the highest concentration of 50mg/ml. The finding of this study supports the use *Azadirachta indica* leaves and can be use in the treatment of various microbial infections as alternative systems of medicine

Keywords: HPTLC finger printing Antimicrobial, *Azadirachta indica*, Aqueous and Ethanolic Extract.

1. Introduction

It is well known to ancient world that plants are a rich source of a variety of chemicals with nutritive and therapeutic properties. In modern era, herbals are seen as potential medicine for a variety of medicine for a variety of diseases often viewed to supersede the pharmacological efficacy of allopathic drugs [1]. The World Health Organization (WHO) has recently defined traditional medicine (including herbal drugs) as comprising therapeutic practices that have been in existence, often for hundreds of years, before the development and spread of modern medicine and are still in use today[2].

The plant Neem (*Azadirachta indica)* family Maliaceae, has been used for a long time in agriculture and medicine [3]. Neem is a widely distributed Indian indigenous plant. The tree Neem is regarded as a "village dispensary" in India [4]. The importance of the neem tree has been recognized by US National Academy of Sciences, which published a report in 1992 entitled "Neem-a tree for solving global problems"[5]. It is established that many scientific studies that neem seeds contain chemical compounds to control more than 100 species of insects and microorganisms [6]. Every part of the tree has been used as traditional medicine for household remedy against various human ailments, from antiquity [7]. Aqueous extract of neem leaves significantly decreases blood sugar level and prevents adrenaline as well as glucose induced hyperglycaemia [8]. The excellent bioactivity of this neem products are attributed to the chemical compounds such as nimbin, nimbidin and salanin [9]. The leaves are anthelmintic, alexeferic, insecticidal, good in ophthelmia biliousness, skin disease, cough, asthma, piles and tumours

[10]. High performance thin layer chromatography (HPTLC) is an important quality assessment tool for the evaluation of herbal drugs and herbal formulations. It allows for the analysis of a broad number of compounds both efficiently and cost effectively.

In view of its varied medicinal applications, plant leaves extract is evaluated for HPTLC figure printing of chemical constituents and to investigate the antimicrobial activity of the aqueous and ethanolic extracts of the leaf of *Azadirachta indica* on some microorganisms.

2. Materials and Methods

2.1 Collection and Preparation of Plant Materials

The leaves of *Azadirachta indica* were collected from, Ganesh Nisag society Pune Maharashtra. The leaves were authenticated at the Botanical survey of India Pune Maharashtra (Ref. no. H3-15, Specimen no. RSS04. dated 4th July 2017),. The leaves of *Azadirachta indica* were dried at temperature not more than 50^{0}C and powdered. Extraction of the powdered plant material (leaves of *Azadirachta indica*) was subjected to Soxhlet extraction using solvents ethanol and water. The extract material was evaporated on water bath and *Azadirachta indica* leaves dried whole ethanolic Extract was collected. The plant material also processed for successive extraction using different solvents in the order of their increasing polarity namely petroleum ether, benzene, chloroform, acetone, ethanol and water. The photochemical analysis was carried out on each extract. The aqueous and ethanolic extracts were further used for evaluation of activities of the plant

2.2 High Performance Thin Layer Chromatography (HPTLC)

HPTLC was performed on ethanolic extract of *Azadirachta indica*. Both the extracts were subjected to qualitative evaluation of various phytoconstituents using precoated TLC silica gel G 60F254 (Merck) plates as stationary phase and most suitable mobile phase to give better chemoprofile of the constituents. Linomat 5 applicator and Hamilton 100 µL syringe was used for application. Silica gel G 60 F254 precoated HPTLC plates (E. Merck Germany) were employed for fingerprinting, in order to achieve better separation of all the components in the extract. The plates were pre-washed in methanol for removing any impurities picked up by plate during storage in the laboratory environment. After prewashing the plates were activated at 110-120°C for half an hour. Dichloromethane: ethanol (19:1) was employed as the final solvent system as it gave a good resolution of the components. The chromatographic plate was scanned at 254 nm for the purpose of obtaining a fingerprint of the extract 10 mg dried extract of *Azadirachta indica* was accurately weighed and dissolved in 10 ml of ethanol to get concentration of 1 mg/ml. Different volumes and concentration of extract of *Azadirachta indica* solution were spotted on HPTLC plate, are shown in following table.

Table No. 1 Concentration of extract of *Azadirachta indica* solution

Track no.	Concentrations (µg/ml)
1	10
2	20
3	40
4	60

2.3 Source and Preparation of Microorganisms

The microorganisms used in this study were obtained from Nisha Micro Lab Pune. The microorganisms used for the investigation included *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella* sp, *Pseudomonas aeruginosa*. The cultures of the microorganisms were on agar slopes at 40 ^{0}C and were sub-cultured into nutrient broth and incubated at 37 ^{0}C for 24 hour prior to sensitivity testing in order to obtain a more vigorous population.

2.4 Sensitivity Testing

Before carrying out the antimicrobial test, the ethanolic and aqueous plant extracts and the standard were prepared using doubling dilution method to obtain 50mg/ml, 25mg/ml and 12.5mg/ml[11]. The antimicrobial activity of the plant extracts was determined using agar well diffusion method. [12] About 0.5ml of the standardized portion of the new microbial culture was aseptically transferred into Petri dishes containing Nutrient Agar (NA) for bacterial isolates left for about 20 minutes to allow the microorganisms fix on the media. Wells where extracts were to be introduced into the plates were carefully marked using sterile cork borer (6mm diameter) and small drops of extract of various concentrations (50mg/ml, 25mg/ml and 12.5mg/ml) were added into the wells. A well was also made at the central portion of the agar medium and drops of sterile distilled water and or 95% ethanol were placed therein to serve as controls. The plates which were prepared in triplicates were incubated at 37^{0}C and the zones of inhibition were measured after 24 hours. The presence of zones of inhibition was regarded as the evidence of antimicrobial action. The zones of inhibition were measured with a ruler at right angles across the zones to find the average diameter in millimeters.

3. Result

The HPTLC figure printing of chemical constituents in ehtanolic leaves extract Chromatogram of *Azadirachta indica* extract at 254nm, Following are the chromatogram (Track 1, 2, 3 and 4) of *Azadirachta indica* leaves extract

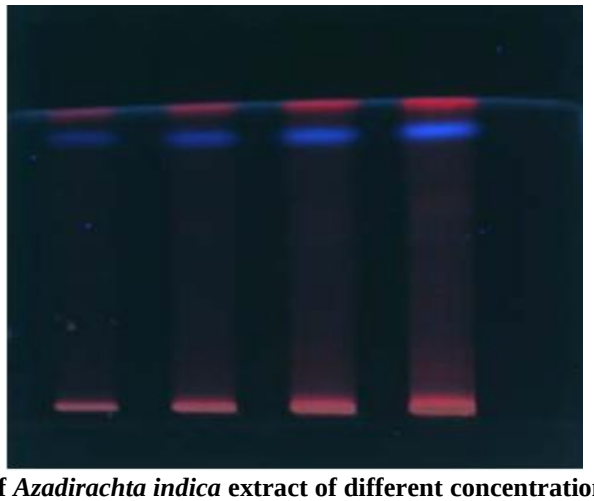
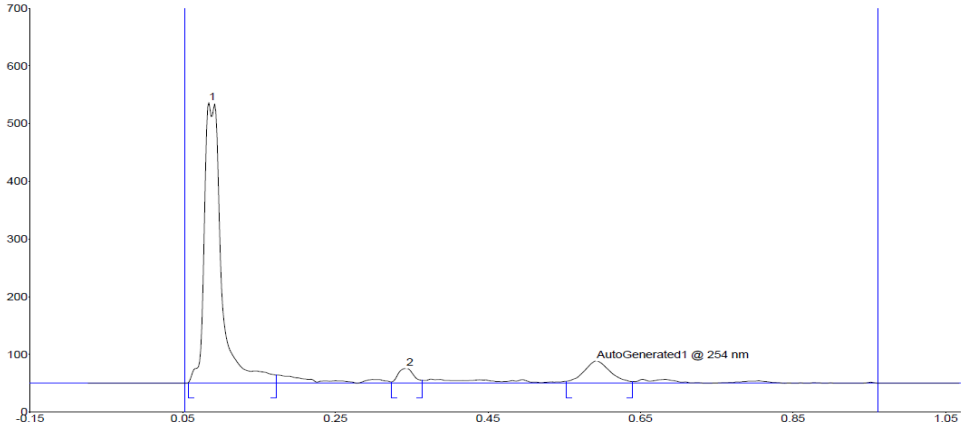
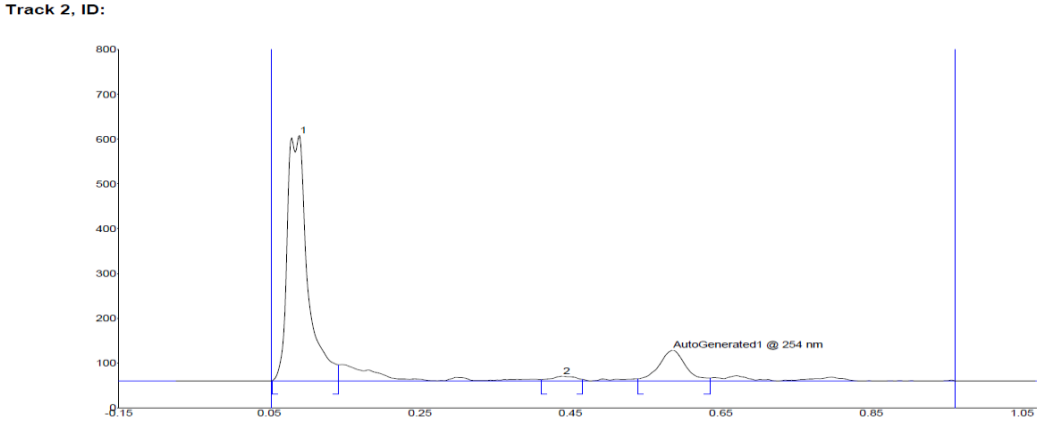


Figure 1: Chromatography of *Azadirachta indica* extract of different concentration at 254 nm (Track 1, 2, 3, 4)



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.05	0.6	0.08	486.9	88.25	0.17	14.2	8433.8	85.43	unknown *
2	0.32	2.3	0.34	26.0	4.71	0.36	5.1	406.7	4.12	unknown *
3	0.55	3.5	0.59	38.8	7.04	0.64	3.0	1032.1	10.45	AutoGenerated1

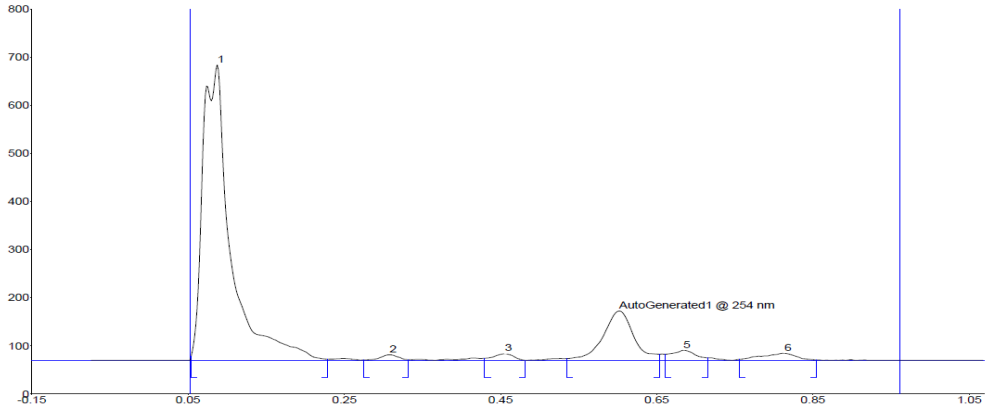
Figure 2: Track 1: Chromatogram of *Azadirachta indica* extract (10µg/ml) at 254nm



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.05	0.9	0.09	548.9	87.32	0.14	36.0	11319.7	84.26	unknown *
2	0.41	3.1	0.44	11.1	1.77	0.46	3.4	265.2	1.97	unknown *
3	0.54	5.0	0.59	68.6	10.92	0.63	6.6	1849.8	13.77	AutoGenerated1

Figure 3: Track 2: Chromatogram of *Azadirachta indica* extract (Track2 20 µg/ml) at 254nm

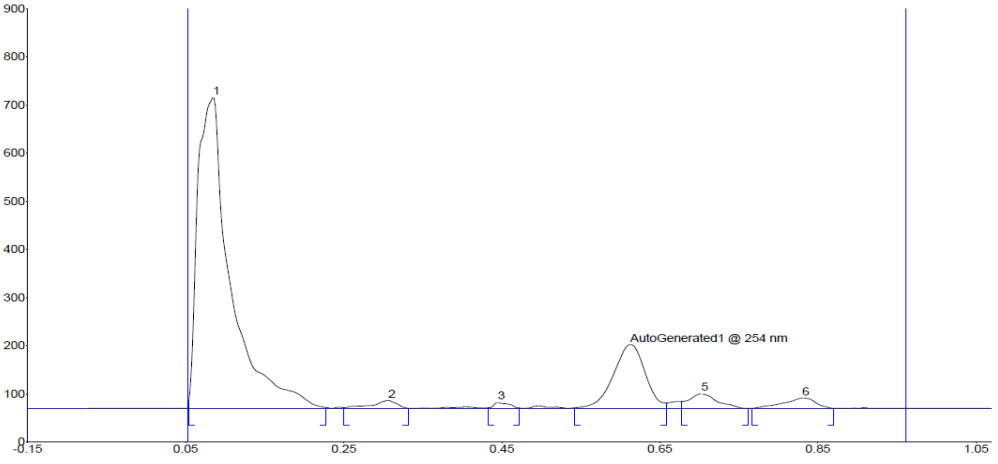
Track 3, ID:



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.05	7.9	0.09	614.4	79.00	0.23	2.2	16672.5	78.58	unknown *
2	0.27	0.6	0.31	11.5	1.48	0.33	1.3	204.7	0.97	unknown *
3	0.43	4.4	0.45	13.9	1.78	0.48	0.1	267.4	1.26	unknown *
4	0.53	3.9	0.60	102.6	13.20	0.65	12.7	3066.9	14.46	AutoGenerated1
5	0.66	12.7	0.68	20.6	2.64	0.71	5.1	500.9	2.36	unknown *
6	0.75	1.9	0.81	14.7	1.90	0.85	0.6	503.6	2.37	unknown *

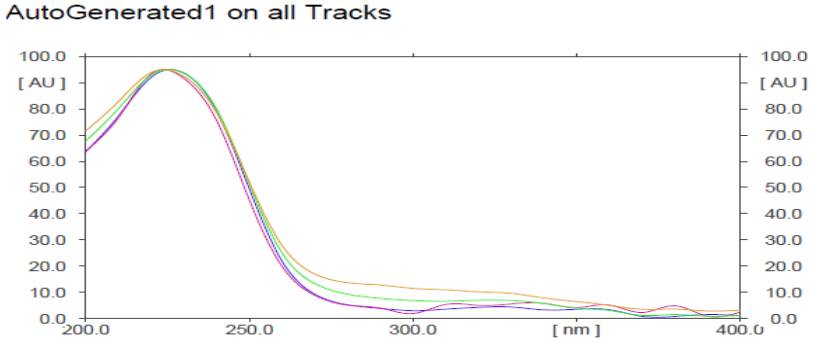
Figure 4: Track 3: Chromatogram of *Azadirachta indica* extracts (40 µg/ml) at 254nm

Track 4, ID:



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.05	14.3	0.08	646.0	75.08	0.23	2.2	20397.6	76.88	unknown *
2	0.25	1.4	0.30	16.7	1.95	0.33	0.2	395.8	1.49	unknown *
3	0.43	1.3	0.44	12.6	1.47	0.47	0.9	183.1	0.69	unknown *
4	0.54	1.3	0.61	132.5	15.40	0.66	11.2	4051.7	15.27	AutoGenerated1
5	0.68	14.7	0.70	30.8	3.57	0.76	0.0	830.0	3.13	unknown *
6	0.77	0.3	0.83	21.7	2.53	0.87	0.2	672.9	2.54	unknown *

Figure 5: Track 4: Chromatogram of *Azadirachta indica* extract (60 µg/ml) at 254nm



T	Rf	Substance	Max. @
1	0.59 Rf	AutoGenerated1	224 nm
2	0.59 Rf	AutoGenerated1	226 nm
3	0.60 Rf	AutoGenerated1	225 nm
4	0.61 Rf	AutoGenerated1	224 nm

Figure 6: Chromatogram of *Azadirachta indica* extract of all tracks

Azadirachta indica extracts showed presence of triterpens, flavonids, tannins, phenolic compounds, saponins, fatty acids and carbohydrates.

The different concentration of *Azadirachta indica* extracts showed presence of various phytoconstituents.

The overlay spectra of HPTLC chromatographs of different concentration as shown in Figure 1, exhibit R_f value of 0.59 which confirm the presence of azadirachtin and other phyto-constituents in *A .indica* plant ethanolic extract.

Table 2: Anti-microbial Effects of different Concentrations of Aqueous and Ethanolic Leaf Extract of *Azadirachta indica* on Some Microorganisms

Organism	Mean diameter of zone of inhibition (mm) at Different concentrations (mg/ml)					
	AE			EE		
Organism	**50.0**	**25.0**	**12.5**	**50.0**	**25.0**	**12.5**
Staphylococcus aureus	40.00	35.00	-	0.40	0.35	-
Bacillus subtilis	21.00	19.00	--	20.00	18.00	--
Escherichia coli	28.00	25.20	--	1.00	0.60	--
Salmonella typhi	26.00	20.50	--	3.00	2.20	--
Pseudomonas aerugenosa	0.04	0.20	--	4.00	3.00	--

AE = Aqueous Extract EE = Ethanolic Extract - = No inhibition

The aqueous and ethanolic extract of the leaf of *A. indica* have some antimicrobial activities against the test microorganisms (Table 2) at concentrations 25mg/ml and 50mg/ml; however both extracts showed no activity at concentration 12.5mg/ml. The aqueous extract on comparison with ethanolic extract, seem to exhibit higher anti-microbial effect except with *Baciilus subtilis*, which showed almost similar sensitivity to both extracts. This may be suggesting that the anti-microbial activity of A. *indica* leaf extract seemed to depend on its polar constituents with the aqueous extract being more polar than the ethanolic extract [13]. The degree of inhibition by aqueous extract was observed to be highest in *S. aureus* and lowest in *Pseudomonas aeruginosa*. Results on the effect of the ethanolic extract of the plant showed that the growth of *B. subtilis* was most strongly inhibited followed by *Candida albicans* at 50mg/ml, while activity against *S. aureus* was least at the same concentration. The crude extract of the leaf of *A. indica* was active against Gram positive and Gram negative bacteria as revealed in the study. This finding confirms the studies of Rao *et al* [9], which reported that *A. indica* possesses a wide spectrum of antibacterial activities. The inhibition of various microbial isolates used in this study by the leaf of *A. indica* can be extrapolated to explain that it could be effective in the treatment of infection caused by the organisms [14]. *Staphylococcus aureus* is found in wounds and also causes skin infections; *Bacillus subtilis* is involved in gastroenteritis, *Salmonella typhi* causes typhoid fever, *E. coli* is a common organism involved in diarrhoea of bacterial origin.

The anti-microbial property exhibited by the leaf extracts of *A. indica* may be due to the presence of individual bioactive ingredients in the plant. These chemical constituents are known to possess anti-microbial properties [15]. The presence of glycosides in the leaf of *A. indica* is important in therapeutic use of this plant

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