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Analytico-in-vitro experimental study of aqueous extract of Neem (*Azadirachta indica* A. Juss) patra vis-a-vis anti-microbial effect

Research Article

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Abstract

Inefficiency of medical therapies used in order to cure patients with microbial infestations requires safe, effective new antimicrobial agents based on variety of parameters. Ayurveda being a holistic science uses many such safe and effective herbal and herbo-mineral interventions in the management of microbial infestations. *Nimba* (*Azadirachta indica* A. Juss) is one commonly available and well known medicinal plant used as *krimihara dravya* (antimicrobial drug). It has *tikta, rasa* (bitter taste), *Laghu guna* (light in property) *sheeta veerya* (cold in potency), *katu vipaka* (pungent of metabolism) act as *krimighna* (Antimicrobial), *kusthaghna* (alleviates Skin disease), *vranaropana* (wound healing), *raktashodhaka* (blood purifier). Also it acts as antioxidant, inhibits bacterial growth. Aim and objectives: To evaluate the physico- phyto-constituents and to assess anti-microbial effect of aqueous extract of Nimba patra. Materials and methods: The official part of the trial drug was subjected for exhaustive extraction by using distilled water and analyzed for physico-chemical, phyto-chemical analysis and antimicrobial evaluation by using aqueous extract on *Staphylococcus aureus (SA), Pseudomonas aeruginosa (PA), Proteus mirabilis (PM), E. coli (EC) and Candida albicans (CA)* with Agar well diffusion method *to asses* Minimum Inhibitory concentration, Minimal Bactericidal Concentration with a dose of Stock 10g/10ml. Observation and results: Physico-chemical study shows 4.63%w/w moisture content, 9.05%w/w Ash content, 5.16 pH value, phyto-chemical analysis indicates the presence of tannins, flavonoids, alkaloids, and anti- microbial study of aqueous extract of nimbi patra shows significant effect on bacteria and fungus in comparison with known standard drug.

Keywords: *Nimba, Azadirachta Indica* A.Juss, Phyto-Chemical, Anti-Microbial.

Introduction

Inefficiency of medical therapies used in order to cure patients with microbial infections requires not only to actively look for new therapeutic strategies but also to carefully select antimicrobial agents based on variety of parameters. Ayurveda being a holistic science uses many such remedial interventions in the management of various ailments produced by intrinsic and extrinsic factors. The plant and plant based natural products shows an important role in diseases prevention and cure through the enhancement of antioxidant activity, inhibition of bacterial growth, and modulation of genetic pathways (1).

Nimba (*Azadirachta indica* A.Juss) is known as village-doctor (2). Acharya Charaka included this in Kandüghna dashaimaneeya, Tiktaskandha (3), Acharya Susruta in Aragvadhädi, Guducyadi, Laksädi (4) and Acharya Vagbhat in Aragvadhädi, Guducydi, Laksadi gana(5). All parts are used in one or the other disease such as bark in skin diseases and boils; its decoction is anti-pyretic, like Cinchona. Internally it has wormicidal effect. Leaves act as antiseptic and applied as poultice to boils, decoction is useful in eczema and ulcers (6). It is also known as *prabhadra, picchumarda, kakaphala, hingu niryasa*(7), its patra acts as *krimighna* (Antimicrobial), *kusthaghna* (alleviates Skin disease), *vranaropana* (wound healing), *raktashodhaka* (blood purifier) by virtue of its *tikta, rasa* (bitter taste), *laghu guna* (light in property) *sheeta veerya* (cold in potency), *katu vipaka* (pungent of metabolism) (8, 9).

Nimba Leaves contains -Azadirachtin, azadirachtanin, margosine, nimbolide, Stem Bark - Nimbin, nimbidin, nimbinin, sitosterol, kulinone, margosinolide, Root Bark-Nimbin, nimbidin. Fruits-Azadirachtin, azadirachtol, azadirachnol, melianone, nimbiol, nimocin etc. Scuitt-40.49% oil Tocopherol, azadirone, azadiradione, nimbinin, salannol, nimbin, nimbidin, Flowers-Azadiradione, margosene, linoleic acid, arachidic acid etc (10).

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Aim and Objectives

To study the physico-chemical, phyto-chemical and anti microbial effect of aqueous extract of nimba (*Azadirachta indica* A.Juss) patra.

Materials and Methods

Materials and Methods of Physico-chemical study

The Physico-chemical parameters were determined as per the guidelines of API

a) Total Ash value.
b) Loss on drying or Determination of moisture content.
c) Water soluble extractive.
d) Alcohol soluble extractive.
e) p^H Value.

a) Total Ash value: Materials: Drug: Dried Nimba patra. **Equipment:** Muffle Furnace, silica crucible. **Method:** 5 gms of powdered Nimba patra was weighed and kept in the weighed crucible. Then the drug with crucible was placed in the muffle furnace at 450^{o}C for about half to one hour. (i.e. until all carbon is burnt off). After that it was subjected for self-cooling in desiccators. Then weighed the ash and calculated the percentage of total ash with reference to the air dried sample of the crude drug (11).

b) Loss on drying or Determination of moisture content. Materials: Drug: *Nimba patra.* **Equipment:** Hot air oven. **Procedure:** 2gm of powdered test sample was weighed along with china dish and dried in oven at 100- 105^{0}C. the sample was taken out, it was cooled in desiccators and loss in weight is recorded. This procedure was repeated till constant weight was obtained.

$$\text{Loss on drying (\%)} = \frac{\text{Loss of weight}}{w} \times 100$$

Where 'W' is = Weight of the drug powder in gram (12).

c) Determination of water soluble Extractive: Materials: Drug: 5gm of dried Nimba patra. **Equipment:** Beaker, Conical flask, Filter paper, Water Bath, micro weighing machine, desiccators etc. **Method:** Weighed about 5gm of the powdered drug in a beaker and transferred it to a dry 250ml conical flask. Filled a 100ml graduated cylinder to the required mark with water + chloroform was out the weighing bottle and pour the washing, together with remainder of the solvent into the conical flask. Shake the flask frequently, flask was corked and kept aside for 24hrs. Filter it into a 50ml Cylinder. When sufficient filtrate was collected transferred 25ml of the filtrate to a weighed 25ml beaker as used for the ash values determination. The residue was subjected for drying on water bath and hot air oven for 10 to 15min. Then it was subjected for self-cooling in desiccators and weighed. Calculated the percentage w/w of extractive with reference of the air dried drug (13).

$$\text{\% of water- soluble Extract for 100ml} = \frac{100 \times \text{wt.of extract.}}{\text{Wt of sample} \times 25}$$

d) Determination of Alcohol Soluble Extractive: Materials: Drug: 5gm of Nimba patra. **Equipments:** Beaker, Conical Flask, Filter Paper, Solvent, Water Bath etc. **Method:** Weighed about 5gm of the powdered drug in a beaker and transferred it to a dry 250ml conical flask. Filled a 100ml graduate cylinder to the required mark with the solvent (90% alcohol) washed out the weighing bottle and poured the washing, together with the remainder of the solvent into the conical flask. Shake the mixture frequently. Flask was corked and kept for 24hrs. Then the mixture was filtered into a 50ml cylinder. When sufficient filtrate was collected transferred 25ml of the filtrate to a weighed 25ml beaker as used for the ash values determination. Then the filtrate was subjected to dryness by subjecting it on water bath and in hot air oven at 100^{0}C about 10 - 15mins. It was subjected for self-cooling in desiccators and weighed. Calculated the percentage w/w of extractive with reference to the air dried drug (14).

$$\text{\% of water- soluble Extract for 100ml} = \frac{100 \times \text{wt. of extract.}}{\text{Wt of sample} \times 25}$$

e) Determination of p^H: Materials: Drug: Nimba patra extract. **Equipment:** Digital calibrates p^H. **Method:** 50ml of distilled water was taken in beaker; digital p^H meter was immersed up to the maximum immersion level. Allowed the reading to stabilize and using a screwdriver turned the p^H calibration trimmer to read 7.0. Then, 5gms of *aqueous* extract was added to 50ml of distilled water in a beaker and it was stirred gently with glass rod. At uniform suspension, digital p^H meter was immersed, observed for maximum immersion and reading was recorded (15).

Materials for Phyto-Chemical Study

Materials for Phytochemical Test (16)

Solubility test of Nimba patra (*Azadirachta indica* A.Juss)

a) Materials: Funnels, beaker, filter paper, test tube, fine powder of *Azadirachta indica A.Juss*.

b) Solvents: 1) Ethyl alcohol 2) Ethyl acetate 3) Petroleum Ether 4) Chloroform 5) Methane 6) Distilled water 7) Solvents ether 8) Acetone 9) Benzene 10) Toluene 11) Xylene 12) Carbon tetrachloride.

c) Methodology: The 2gm of fine powder of Nimba patra (*Azadirachta indica* A.Juss) was added to each solvent taken in a test tube and mixed well and allowed to stand for certain period. Then the mixtures were filtered through filter paper kept in different funnels. The filter paper which contains fewer residues indicates more soluble in that solvent.

Extraction: Materials: Drug: Coarse powder of Nimba patra (*Azadirachta indica* A.Juss). **Equipments required:** Flask, Beaker's of 500ml, measuring cylinder, weighing machine, filter paper, distilled water.

a) Methods: Aqueous extract was prepared by mixing 25 grams of shade dried coarse powder of Nimba leaves with 100 ml of sterile distilled water in a flask with occasional shaking. From 9 am to 5 pm every one hour shaking was done for 15 minutes. After 3 days the

extract was filtered through what's man filter paper. After filtration 90 ml aqueous extract was obtained. The obtained extracts were then subjected to water bath at 60°C and evaporated to dryness and stored in air tight bottles at 4°C for further use. Aqueous extract was further diluted to required concentration.

Preliminary phyto-chemical test: Nimba Patra (Azadirachta indica A.Juss) aqueous extract was subjected to phytochemical analysis. Presence of Sterols (Salkowski's test), proteins (Million's test), alkaloids (Mayer's test), carbohydrate **(**Molish's test**),** saponins (Foam test), tannins (Ferric chloride test) and flavonoid's (Shinoda test) were evaluated.

Methods:

i) **Test for sterols: a) Salkowski's test:** To 2ml extract added 2ml chloroform and 2 ml cone H_2So_4, shaken well.

ii) **Test for proteins: Preparation of test solution:** 0.5 gm of sample extract was added to 100ml of water and heated. This solution was used for following tests.
Million's test: To 3 ml of test solution added 5 ml of Million's reagent.

iii) **Test for Alkaloids: Preparation of test solution:** Evaporated the alcoholic extract, to residue added dilute HCl, shaken well and filtered by using the filtrate the following test are performed.
Mayer's test: To 2ml of filtrate in a test tube added few drops of Mayer's reagent.

iv) **Test for carbohydrate: Molish's test (General):** To 2ml extract in a test tube added few drops of Molish's reagent, shaken well and added few drops of H2So4 from the side of test tube.

v) **Test for Saponin's: Foam test:** The drug extract was mixed with water and shaken vigorously.

vi) **Test for Tannins: Ferric chloride test:** To 2ml extract added few drops of 5 % Fecl3 solution in a test tube.

vii) **Test for Flavonoid's: Shinoda test:** To 2ml extract in a test tube added 5ml of 95 % ethanol and few drops of Conc. HCl and 0.5gm magnesium turnings.

viii) **Chromatographic Method (17)**:

- **Instrument**: Water Alliance e2645 separation module
- **Column** Phenomenex Luna
- **Mobile Phase:** Mobile Phase A: Mobile Phase B (80:20% v/v)
- **Mobile Phase A:** Acetonitrile
- **Mobile Phase B:** 0.1% Orthophosphoric Acid in water
- **Detection wavelength:** 358nm
- **HPLC Analysis Summary:** Several HPLC trials were performed using different mobile phases composed of organic and aqueous solvents, buffers like Orthophosphoric acid, and triethyl amine, etc. Trials were also performed on different HPLC columns, among them the above-mentioned chromatographic method was chosen to quantify Azadirachtin. The HPLC analysis was performed on the Azadirachtin Biomarker as well as on the given powdered sample.
- **Preparation of Standard Stocks:** 10mg of the Azadirachtin Biomarker was dissolved in 10 ml of the Acetonitrile to obtain 1000µg/ml concentrations (Stock-I). 1ml stock-I was then pipetted out and diluted to 10ml using acetonitrile (Stock-II). Further 1ml stock-II was diluted to 10ml using acetonitrile to obtain 10µg/ml final concentration, which was then analyzed using HPLC.
- **Preparation of Sample Stocks:** 10mg of the powdered sample was dissolved in 10 ml of the Acetonitrile (Stock-I). 1ml stock-I was then pipetted out and diluted to 10ml using acetonitrile (Stock-II). Further 1ml stock-II was diluted to 10ml using acetonitrile and was analyzed using HPLC. Both the samples were analyzed in the above chromatographic system and the retention time was found to be 3.208min. Azadirachtin Biomarker exhibited an area of 175364, and the area of the powdered sample was found to be 71117 and the same area(s) were used for quantification.

- **Calculation**

  If 10µg/ml of Azadirachtin Biomarker exhibited an area of 175364
  Then (X) µg/ml of the powdered sample exhibited an area of 71117
  Therefore X = 71117*10/175364
  X = 4.06µg/ml

Laboratory Methods for assessing Anti-microbial activity:

1. **Bacterial Culture:** Staphylococcus aureus (SA), Pseudomonas aeruginosa (PA), Proteus mirabilis (PM), E.coli (EC) and Candida albicans (CA).
2. **Method used:** Minimum Inhibitory concentration, Minimal Bactericidal Concentration and Agar well diffusion method
3. **Extract concentration:** Stock 10g/10ml Working : 1000mg/ml

Determination of MIC: Minimum inhibitory concentration (MIC) was carried out as per Clinical and Laboratory Standard Institute (CLSI) guidelines. The stock solution of aqueous extract of neem patra was prepared in Distilled water (DW). Two fold serial dilutions of *Azadirachta indica* A. Juss extract (actually *Azadirachta indica* A. Juss extract was used for anti-bacterial activity by mistake *Planaxis sulcatus extract* was written) in BHI broth were carried out with concentration ranging from 1000mg/ml to 1.95mg/ml. One hundred microliters of an earlier prepared Staphylococcus aureus (SA), Pseudomonas aeruginosa (PA), Proteus mirabilis (PM), and E.coli (EC) strains was added to all the MIC tubes. After the incubation the bacterial growth was recorded visually. Further to 100µl of incubated broth 30µl of Resazurin dye and incubated for 4 hours at 37°C. Based on the color change in the well growth of bacteria was noted. The lowest concentration, at which no bacterial growth was found, was considered as the MIC value. Similarly MIC of Extract against Candida albicans was tested at 25°C for 72 hours.

Determination of MBC & MFC: Minimal Bactericidal Concentration (MBC) Clinical and Laboratory Standard Institute (CLSI) guidelines. From MIC test tubes loopful of samples were streaked on agar plates and incubated for 48 hours at 37 °C and 25 °C to check the bacterial and fungal growth respectively.

Agar well diffusion method: BHI agar Plates inoculated with the test organism and extract taken as sample and loaded into the wells of the agar. All plates were incubated (37°C) for 24hours. After incubation, the diameters of any clear zones around the antimicrobial-containing discs or wells were measured using scale (18).

Observations and Results

Physico-chemical observations

Table 01: Shows physico-chemical parameters of Nimba patra

Sl. No.	Parameters	Specification	Results	Test Protocol
1	Botanical Name	*Azadirachta indica*	Complies	....
2	Description	Brown colored powder	Complies	Visual
3	pH of1 % w/v aq. sol^{n}	4-7	5.16	IP 2014
4	Loss on drying (at105°C)	NMT 5%w/w	4.63%w/w	IP 2014
5	Ash content	NMT 20%w/w	9.05%w/w	IP 2014
6	Part Used	Leaf	Complies	....
7	Extractive value of 1% sol^{n} in Alcohol	NLT 60%w/w	60.45%w/w	IP 2014
Heavy Metals				
8	Arsenic	NMT 1ppm	Complies	'AOAC''Method
9	Lead	NMT 1ppm	Complies	'AOAC''Method
10	Mercury	NMT 1ppm	Complies	'AOAC''Method
Microbiological Tests				
11	Total Aerobic Bacteria	NMT 5000 cfu/gm	420 cfu/gm	USP
12	Yeast and Moulds	NMT 200 cfu/gm	30 cfu/gm	USP
13	Escherichia coil	Absent	Absent	USP
14	Salmonella	Absent	Absent	USP
15	Staphylococcus aureus	Absent	Absent	USP
16	Pseudomonas aeruginosa	Absent	Absent	USP

Legend: NLT= not less than, NMT=Not more than, cfu=Colony forming unit/gram

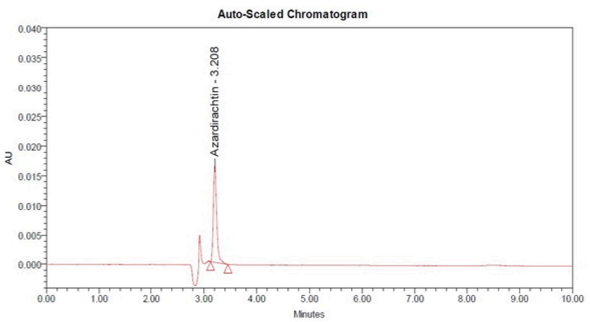
Phyto-chemical study:

Table 02: Shows phyto-chemical analysis of Nimba patra

Sl. No	Phyto-constituents of Nimba patra	Test name	Observations	Result
1	**Sterols**	Salkowski's test	No change in colour.	Absent
2	**Proteins**	Million's test	First white precipitate by warming turns into brokered and dissolves giving red colour solution.	Present
3	**Alkaloids**	Mayer's test	White colour precipitate observed.	Present
4	**Carbohydrate**	Molish's test	Violet ring formed at the junction of two liquids.	Present
5	**Saponins**	Foam test	Persistence foam observed.	Present
6	**Tannins**	Lead acetate solution test	Formation of a voluminous white ppt indicates the presence of tannins	Present
7	**Flavonoids**	Shinoda test	pink color shows the presence of flavonoids	Present

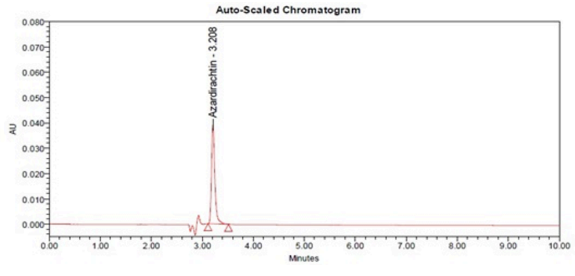
Chromatography observations and results:

Graph 1, Peak result

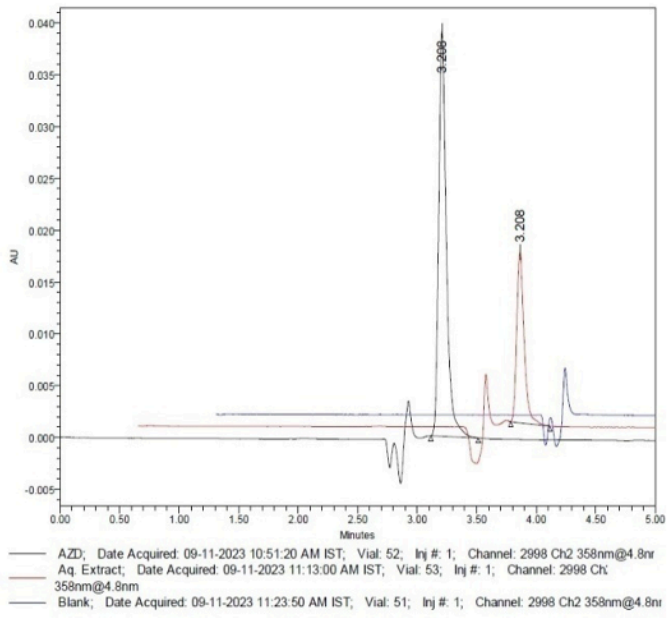
	Name	RT	Area	Height	USP plate count	USP tailing	
1	Azadirechtin	3.208	71117	16484	13664.747301	1.402727	

Graph 2, Peak result

	Name	RT	Area	Height	USP plate count	USP tailing
1	Azadirectin	3.208	175364	39110	13162.141474	1.392200



Graph 3 & 4, Overlay report



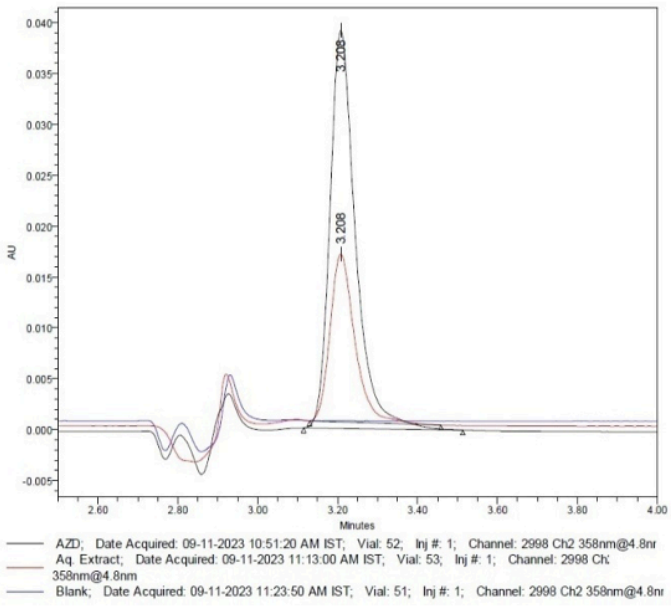


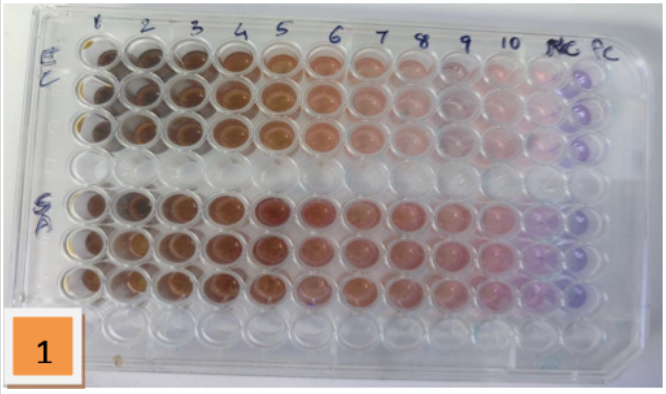
Table 04: Showing quantification of Azadirachitin in aqueous extract of Nimba Patra

Sl. No.	Particulars	Concentration	Observed Area
1	Results of API	10µg/ml	175364
2	Results of sample	Unknown	71117

∴10µg/ml API =175364, X µg/ml of Aqueous extract = 71117
Upon cross multiplication X= = 4.05 µg/ml
Conclusion: 10µg/ml of aqueous extract contains 4.05µg/ml of Azadirachitin.

Anti-microbial Activity Assessment of Aqueous extract of Nimba

Fig. No. 1 & 2, Minimum inhibitory concentrations



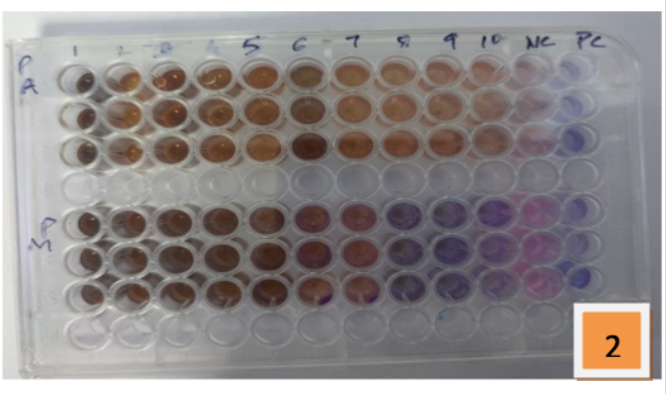


Fig. No. 3, 4, 5, & 6. Indicates MBC

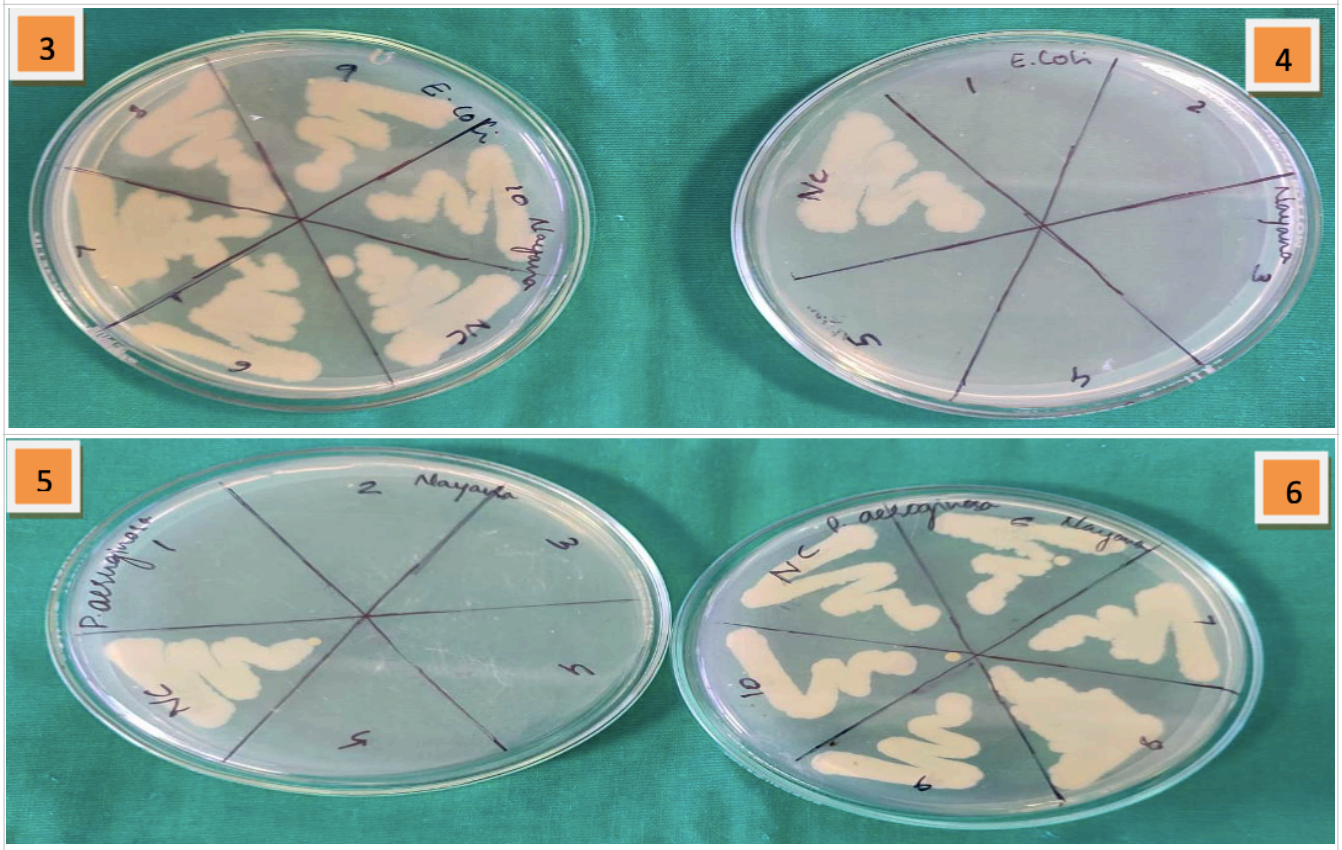
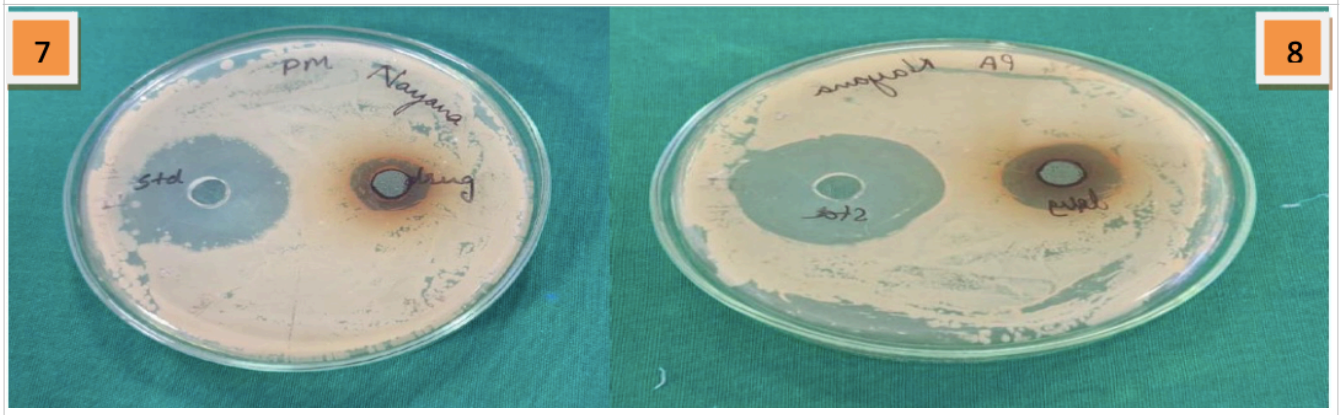


Fig. No. 7 & 8 indicates Anti bacterial Activity



Results of Anti-microbial study

Table 05: Showing results of Anti-Bacterial study

Sl. No.	Microorganisms (Bacteria)	MIC (mg/ml)	MBC (mg/ml)	Zone of Inhibition (1g/ml)	Standard (5mg/ml)
1	*Staphylococcus aureus (SA)*	500	500	12mm	34mm Ciprofloxacin
2	*Pseudomonas aeruginosa (PA)*	62.5	62.5	17mm	32mm Levofloxacin
3	*Proteus mirabilis (PM)*	62.5	62.5	15mm	25mm Levofloxacin
4	*E.coli (EC)*	62.5	62.5	16mm	5mm Levofloxacin

Table 06: Showing results of Anti-fungal study

Sl. No.	Fungi	MIC (mg/ml)	MFC (mg/ml)	Zone of Inhibition(10mg/ml)	Standard (5mg/ml)
1	*Candida albicans (CA)*	No Growth inhibition	No Growth inhibition	No Growth inhibition	34mm Fluconazole

Discussion

Nimba is a commonly available medicinal plant present mainly in the tropical regions of India. It is considered as poor man's air conditioner and medical store for the traditional healers. It is botanically identified as *Azardiracta indica* A. Juss and belongs to Meliaceae family. It is also known as *prabhadra, picchumarda, kakaphala, hingu niryasa* etc.

Acharya Charaka included it in *Kandughna dashaimaneeya* (Anti-pruritic) and *Tiktaskanda* (group of drugs predominated with bitter taste) where as *Acharya Susruta* in *Aragwadhadi*, *Guduchyadi* and *Lakshadigana*. It has *tikta rasa* (bitter taste), *laghuguna* (light property), *sheetavirya* (cold in potency), *katuvipaka* (under goes pungent metabolic changes). Leaves contain margosine. margosine, azadirachtin, azadirachtanin.

Physico-chemical analysis Nimba patra shows 4.63%w/w moisture content, 5.16 -pH of 1 % w/v aq. sol^{n}, 9.05 % w/w - Ash value, 60.45 % w/w - Extractive value, minimal inorganic compounds and microbial content. Phyto-chemical analysis shows the presence of alkaloids, glycosides, saponins, flavonoids, carbohydrate, etc. Nimba patra extract shows significant presence of Azadirectin i.e. 10µg/ml of aqueous extract contains 4.05µg/ml of Azadirachitin.

Charaka Acharya recommended three fold management of krimi. They are as;

- ***Prakriti Vighata*** (counteracting the cause of disease)
- ***Apakarshana*** (to remove the Krimi)
- ***Nidana Parivarjana*** (necessary prerequisite for cure and prevention of recurrence of disease).

Probable mode of action

Nimba patra consists *Tikta rasa, sheeta veerya* and *katu vipaka* of nimba patra. *Tikta* (*bitter* taste) rasa is ruksha gunatmaka (dry) pacifies *kapha pitta dosha*, *krimighna* (Anti-microbial), kusthaghna (cures skin diseases) and *amapachaka (moisture absorbent)*. It absorbs excessive *ardrata* (moisture) required for the growth and mortification of *krimi* (microbes).

Aqueous extract of Nimba patra possesses acidic pH (5.16) which destroys the favorable environment required for the growth of the micro-organisms. Tannins act as antimicrobial due to their ability to bind with proteins and other cellular components, leading to their inactivation leads to antibacterial, antiviral, and antifungal activities. Flavonoids, with their diverse structures, have shown antimicrobial activity against bacteria, viruses, and fungi. They may disrupt microbial membranes, inhibit enzymes essential for microbial growth, and interfere with the replication of viruses.

Hence, aqueous extract of Nimba patra shows significant minimum inhibitory effect at 500mg/ml and 62.5mg/ml on Staphylococcus aureus (SA) in comparison with Ciprofloxacin, and Pseudomonas aeruginosa (PA), Proteus mirabilis (PM), E. coli (EC*)* respectively in comparison with known standard drug Levofloxacin. Whereas it shows insignificant effect to inhibit the growth of Fungi at said concentration is compared with known standard drugs.

Conclusion

Nimba is a commonly available medicinal plant used in various diseases, aqueous extract of Nimba patra shows 5.16 pH, and less inorganic components. It also shows the presence of tannins, flavonoids, saponin, alkaloids etc. It has shown good antimicrobial effect towards both gram positive and gram negative micro-organisms in comparison with known standard drug, but it is less effective towards fungal growth.

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