Phytochemical analysis, Protein and DNA estimation and Antimicrobial Activity of Azadirachta indica (Neem)

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ABSTRACT
The present study was aimed to investigate the phytochemical screening and antimicrobial assessment of Azadirachta indica leaf extract. These plants were collected; aqueous and alcoholic extracts were prepared. The phytochemical analysis of extracts were performed, and antimicrobial activity of aqueous and alcoholic extracts of plant leaf material at dose of 100 mg/ml and 200 mg/ml concentrations against two strains were done. These extract were studied through agar diffusion method against Pseudomonas fluorescens an aerobic, gram-negative, ubiquitous organism present in agricultural soils and well adapted to grow in the rhizosphere *and Alterneria solani* fungal pathogen that produces a disease in tomato and potato plants called early blight. *The phytochemical study inferred the presence of secondary metabolites which assist their herbal properties. For a long period of time, plants have been a valuable source of medicines. Phytochemical analysis of the alcoholic extracts revealed the presence of alkaloids, glycosides, flavonoids, steroids, tannins and reducing sugars. The occurrence of these biologically active chemicals in the selected plants may justify their wide usage in traditional medicine.*

Keyword: Azadirachta Indica, Antimicrobial Activity, Pseudomonas Fluorescens, Alterneria Solani, Phytochemical Analysis.

I INTRODUCTION

Various religious supports the herbs role in health care, diseases prevention, enhancement of antioxidant activity and inhibition of bacterial growth (Zong et al. 2012). Various plants/fruits in the diseases cure Neem ingredients are applied in Ayurveda, Unani, Homeopathy, and modern medicine for the treatment of many infectious, metabolic, or cancer diseases (Bukhari et al. 1976). Neem has a wide range of uses in the control of crop and household pests, for medicinal purpose and as shade trees; also a raw material for soap, charcoal production and for production of crops and homes against pests and pathogens in an area is linked to their tradition (Govindachari et al. 1998). Azadirachta indica has complex of various constituents including nimbin, nimbidin, nimbolide, and limonoids and such types of ingredients play role in diseases management through modulation of various genetic pathways and other activities. Quercetin and ß-sitosterol were first polyphenolic flavonoids purified from fresh leaves of neem and were known to have antifungal and antibacterial activities (Girish et al., 2008). A. indica has shown the presence of biological compounds like, Alkaloids (Ameri, 1998), Flavonoids (Middleton 1998), Saponins (Desai et al. 2009), glycoside (Jimenez et al. 1994), phenols (Robards 2003). Tannins (Dixon et al. 2013)), Phlobatannins (Yadav et al. 2014), etc.

Neem (Azadirachta indica) plants parts shows antimicrobial role through inhibitory effect on microbial growth/potentiality of cell wall breakdown. Azadirachtin, a complex tetranortriterpenoid limonoid present in seeds, is the key constituent responsible for both antifeedant and toxic effects in insects. Many results suggest that the ethanol extract of neem leaves showed in vitro antibacterial activity against both Staphylococcus aureus and MRSA with greatest zones of inhibition noted at 100% concentration (Sarmiento et al. 2011). Plant proteins play various enzymatic, structural and functional roles (photosynthesis, biosynthesis, transport, immunity, etc). They also act as storage mediums to meet the growth and nutritional demands of developing seedlings (Dunn 1992). Good quality DNA is a prerequisite for all experiments of DNA manipulation. Since DNA can be extracted from various types of tissues such as seedlings, leaves, cotyledons, seeds, endosperm, tissue culture callus, roots etc., the tissue type along with the concentration of DNA finally required determine the methodology of DNA extraction to be followed by the experimenter (Dellaporta et al. 1983). The present work has been carried out with following objectives:-

(a) To assess phytochemical properties and Determination of concentration of protein in neem leaf by Lowry's method.
(b) Antimicrobial activities of neem leaf.
(c) Genomic DNA Isolation and DNA Quality Confirmation of Neem leaf.

II LITERATURE REVIEW

Bopanna et al. (1997) investigated in vivo antidiabetic activity of neem leaf extract in alloxan induced rats. Su et al. (1999) studied that Neem leaf extract are known to act on various insects in the following ways, disrupting or inhibiting the development of egg, larvae, or pupae, blocking the molting of larvae or nymphs, disrupting mating and sexual communication, repelling larvae and adults, deterring females from laying eggs. Badam et al. (1999) investigated in vitro antiviral activity of neem leaf extract against group B Coxsackieviruses Antiviral activity of methanolic extract fraction of leaves of neem (A .indica A. Juss) (NCL-11). Olexová et al. (2004) studied three types of methods for the isolation of DNA, namely, cetyltrimethylammonium bromide solubilization with liquid-phase extraction (CTAB-LPE), chaotropic solid-

phase extraction (SPE) and non-chaotropic SPE were compared on the basis of yields. Kumaran et al. (2007) studied neem leave can be used as drug for diabetes, eczema and reduce fever, and Barks of Neem can be used to make toothbrush and the roots has an ability to heal diseases and against insects. Prasad et al. (2008) studied Neem leaf aqueous extract induced changes in biomass accumulation, photosynthetic activity and status of reactive oxygen spesies, lipid peroxidation and enzymatic antioxidants in cyanobacterium plectonema boryanum. Ogbuewu et al. (2011) studied that Neem gum is used as a bulking agent and for the preparation of special purpose food. Lokanadhan et al. (2012) reported that Neem is a versatile tree having many good and useful qualities belonging to the order rutales and family mellacceae.

III MATERIALS AND METHODES

(a) Materials

Instruments (Centrifuge, Mortar Pistal, Hot Plate, Incubator, Refrigerator, Spectrophotometer, Hot Air Oven, Weighing Balance, Shaker, Water Bath, Laminar Air Flow, Agarose Gel Electrophoresis System), Glasswares (Test Tubes, Test Tube Holder, Petri Plates, Conical Flask, Beaker, Eppendorff Tube, Measuring Cylinder, Funnel, Spatula, Glass Rod, Cuvett), Chemicals (Methanol, Ethanol, Acetone, Ethyl Acetate, Wager's Reagent, Hager's Reagent, Dragondroff's Reagent, HCL, H_2SO_4, Ferric Chloride, NaOH, Na_2CO_3, Sodium Potassium Tartrate, $Cuso_4$, Folir-Ciocatteu Reagent, Al_2Cl_3 Solution, Dil. Ammonia Solution, NA Media, PDA Media, CTAB Buffer, Absolute Ethanol (Ice Cold), 70 % Ethanol (Ice Cold), Ammonium Acetate, Chloroform : Iso Amyl Alcohol (24:1), Agarose, 6x Loading Buffer, 1x TBE Solution, Ethidium Bromide Solution) and Olive oil, tap running water, double distilled water, normal distilled water, Whatman No.1 filter paper, Microfuge Tubes, Micropipette.

(b) Methodes

(i) Samples Collection The leaves of A. indica were collected from near New Gym of JIBB Lawn, SHUATS, Allahabad, Uttar Pradesh. Fresh green leaves were separated upon arrival at the laboratory molecular and cellular biology, JIBB, SHUATS. Leaves were washed with the help of tap running water. Rewash it with distilled water. After washing the leaves were put on the newspaper for air dries. The leaves were then kept in Hot Air oven at 65 ^{0}C (Extra water content removes from the leaves). After 24 hours the leaves were taken out from hot air oven. Fine grinned powder of leaves was made with the help of Mortar pestle. Then, stored the sample @4 ^{0}C.

(ii) Extract Preparation

- **Methanol Extract** In the lab of JIBB, SHUATS, Allahabad, Uttar Pradesh, methanol extract was prepared by 500 mg of plant powder sample was taken in wash and sterilized test tube. Added 5 ml 70% methanol in the sample. Kept on shaker at 80 rpm for 5 min. After that centrifuged at 3000 rpm for 10 min. The supernatant was collected in another wash and sterilized test tube. Again 5 ml 70% methanol was added in the remaining white residue and centrifuged at 3000 rpm for 10 min., and the supernatant was collected. Then 100% methanol was added in the remaining residue. Kept in the inversion for 10 min. All the supernatant were kept in one test tube. The white residue discarded. Then supernatant was kept in water bath for 10 min. at 65 ^{0}C for evaporating methanol. Storage of extract at 4^{0}C (Larson et al. 2016).
- **Water, Ethanol and Ethyl Acetate Extract** 5 g of powdered plant sample was soaked with 50 ml of water, ethanol and ethyl acetate separately. The entire mixture was incubated at 4 ^{0}C for 48 hours. After the incubation period was over, the mixture was filtered and centrifuged at 10,000 rpm at 4 ^{0}C. The extracts were concentrated to dryness and were stored at 4 ^{0}C until further used.

(c) Phytochemical Analysis Alkaloids were determined by Hager's Reagent Test, Wagner's Reagent Test, Mayer's Reagent Test and Dragendorff's Reagent Test. Flavanoids were determined on the method of Bohn and Kopcipal-Abyazan (1994). Saponins determined by Obadoni and Ochuko (2001). Taninns determined by 1 ml of extract was boiled in 20 ml of water in a test and then filtered. A few drops of 0.1% ferric chloride was added and observed green or a blue-black coloration which confirms the presence of tannin; and Phlobatannins determined by Deposition of a red precipitate when 2 ml of extract of each plant samples was boiled with 1% aqueous hydrochloric acid was taken as evidence for the presence of phlobatannins.

(d) Protein Estimation Lowry method used to determine protein concentrations of A. indica leaves, the peptide nitrogen[s] with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteay phosphomolybdic phosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids. (Dunn 1992).

(e) Genomic DNA Isolation and Quality Confirmation DNA was isolated by CTAB Method (Stefanova et al. 2013) and quality was confirmed by agarose gel electrophoresis (Voytas 1992).

(f) Antimicrobial Activity Test By the use of different A. indica leaves extract viz. Methanol, Water, Ethanol and Ethyl Acetate antimicrobial activity on Pseudomonas fluorescens and Alterneria solani were tested by Well Diffusion Method (Du et al. 2000).

IV RESULT AND DISCUSSION

(a) Phytochemical Properties Alkaloids were found in A. indica methanol leaves extract, given positive result in all tests indicated in Table No. 1 and Fig. No. 1 (a). Flavonoids were present in water and ethanol extracts where in ethyl acetate extract showed negative result. Saponins and Taninns were present in water and ethyl acetate extracts whereas they show negative result in ethanol extract. Phlobatannins was found only in water extract and showed negative results in remaining both ethyl acetate and ethanol extracts indicated in Table No. 2 and Fig. No. 1 (b), (c), (d), (e). The present study showed that a plant contains all useful phytochemicals which makes its medicinal properties.

Table 1
Assessment of Alkaloid Properties in A. indica (Neem) leaves

S. No	Test Name	Observation	Influence/Result
1	Hager's Test	Formation of Yellow precipitate	Positive
2	Wagner's Test	Formation of white precipitate	Positive
3	Mayer's Test	Formation of yellow precipitate	Positive
4	Dragondroff Test	Formation of orange precipitate	Positive

Table 2
Assessment of Flavonoids, Saponins, Taninns and Phlobatannins Properties in A. indica (Neem) leaves

Phytochemical	Extracts		
	Water	Ethyl Acetate	Ethanol
Flavonoids	Present	Absent	Present
Saponins	Present	Present	Absent
Taninns	Present	Present	Absent
Phlobatannins	Present	Absent	Absent

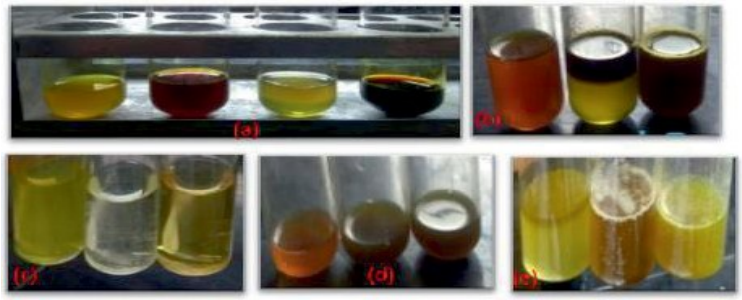

Fig. 2 Phytochemical Analysis: (a) Alkaloid Test, (b) Flavonoids Test, (c) Saponins Test, (d) Taninns Test and (e) Phlobatannins Test observation results.

(b) Protein Estimation The concentration of protein in the Azadirachta indica (Neem) plant leaves was found 3.4 mg/ml shown in fig no. 2.

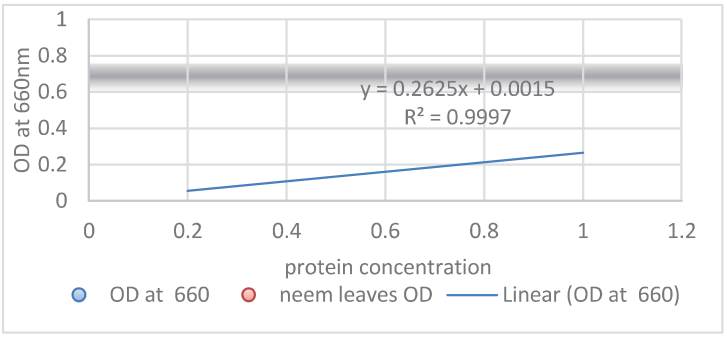

Fig. 2 Determination of Protein concentration of A. indica (Neem) by Lowry's method

(c) Genomic DNA isolation and quality confirmation The genomic DNA of A. indica (Neem) leaves isolated successfully by using CTAB method. Isolated genomic DNA shown in Fig. No. 3 (a). A very appreciable amount of DNA was isolated. The isolated genomic DNA quality was confirmed by agarose gel electrophoresis. Expose the gel to UV light and photograph (demonstration). Confirm DNA quality, presence of a highly resolved high molecular weight band indicates good quality DNA shown in Fig. No. 3 (b).

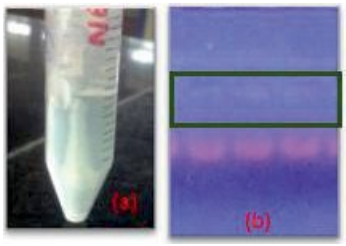

Fig. 3 Genomic DNA isolation and quality confirmation, (a) Isolated A. indica leaves genomic DNA and (b) Genomic DNA band expose in UV light highlighted in green box.

(d) Antimicrobial Activities

- **Pseudomonas fluorescens** The antibacterial activity was shown by the Acetone and Ethanol extract of A. Indica (Neem) leaves. Clear ZOI was obtained of 14 mm and 15 mm diameter respectively. The well diameter was 6 mm shown in table no. 3 and Fig. No. 4 (a).

Table 3
Antimicrobial activity test for the microorganism P. fluorescens

Solvent	Plate 1 ZOI (mm)	Plate 2 ZOI (mm)	Plate 3 ZOI (mm)	Average ZOI (mm)
Ethanol	0	15	15	15
Methanol	0	0	0	0
Acetone	0	14	14	14

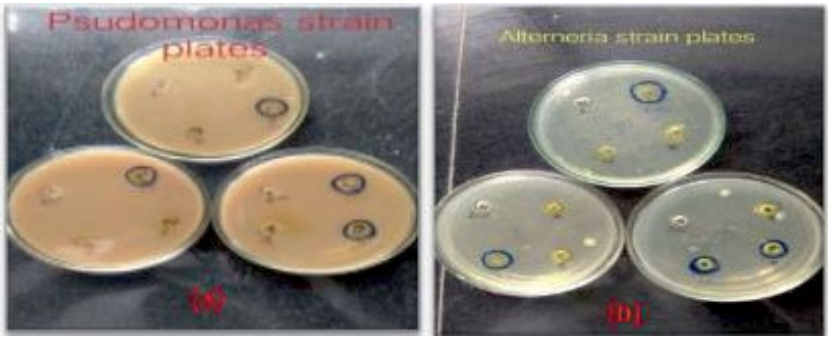

Fig. 4 Antimicrobial Activities shown by A. indica leaves, (a) Antibacterial activity in Pseudomonas fluorescens was obtained by showing Clear ZOI obtained in Acetone and Ethanol extract. (b) Antifungal activity in Alterneria solani was obtained by showing Clear ZOI obtained in all extracts viz. Acetone and Ethanol and Methanol.

- **Alterneria solani** The antimicrobial activity was shown by the Acetone and Ethanol and Methanol extract of A. Indica (Neem) leaves. Clear ZOI was obtained of 14 mm and 12 mm and 12 mm diameter respectively. The well diameter was 6 mm shown in table no. 4 and Fig. No. 4 (b).

Table 4
Antimicrobial activity test for the microorganism Alterneria solani

Solvent	Plate 1 ZOI (mm)	Plate 2 ZOI (mm)	Plate 3 ZOI (mm)	Average ZOI (mm)
Ethanol	0	12	0	12
Methanol	13	11	0	12
Acetone	0	0	14	14

V CONCLUSION

The alkaloid property in A. indica (Neem) leaves was present in the methanolic extract. In water extract flavonoid, saponin, tannins and phlobatnnins was present. In Ethanol extract flavonoid was present. In Ethyl Acetate saponin and tannins was present. The protein content was found in a very appreciable amount i.e. (approx) 3.4 mg/ml.

The Acetonic extract gives positive activity towards Pseudomonas fluorescens and Alterneria solani. The clear 14 nm diameters ZOI was obtain in both the strain. The well diameter was 6 nm in each plate. The Ethanolic extract gives positive activity towards Pseudomonas fluorescens and Alterneria solani. The clear 15 nm diameter ZOI obtain in Pseudomonas fluorescens and 12 nm diameter ZOI obtain in Alterneria solani. The Methanolic extract gives positive activity only towards Alterneria solani, 12 nm diameter clear ZOI was obtained.

Neem, the versatile medicinal plant is the unique source of various types of compounds having diverse chemical structure. Very little work has been done on the biological activity and plausible medicinal applications of these compounds and hence extensive investigation is needed to exploit their therapeutic utility to combat diseases. A drug-development programme should be undertaken to develop modern drugs with the compounds isolated from neem. As the global scenario is now changing towards the use of nontoxic plant products having traditional medicinal use, development of modern drugs from neem should be emphasized for the control of various diseases. In fact, time has come to make good use of centuries-old knowledge on neem through modern approaches of drug development.

Under the limitations of this study, it can be concluded that A. Indica has antimicrobial effects against the endodontic pathogens like Pseudomonas fluorescens and Alterneria solani. However, further preclinical and clinical trials are required to evaluate the cytotoxicity and safety issues of these plant extracts before they can be recommended as an endodontic irrigant or intracanal medicament. Furthermore, looking at the polymicrobial nature of endodontic infections these agents needs to be tested for their antimicrobial effectiveness against a wide range of microorganisms including strict anaerobes. The A. indica (Neem) contains Alkaloid, Flavonoid, Saponin, Tannins and pholbatnnins. The leaves sample contain appreciable amount of protein. The presence of a highly resolved high molecular weight band indicates good quality DNA.

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