Preparation and characterization of Neem Bark Extract with antimicrobial activity

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Abstract

Aim and objective of this study was to prepare and characterize of Neem Bark Extract from *Azadirachata indica* (family – *Meliaceae*). In the mid of August, the stem bark was collected from the Azadirachata indica tree. After preparing neem bark methanolic extract from stem of Azadirachata Indica, extract was characterized by physical property evaluation, chemical property evaluation, antimicrobial evaluation, by determination of λ max using methanol and ethanol solvent, percentage of purity of prepared *Azadirachta indica* bark exracts using UV spectroscopic method(shimadzu-1700), doing structural elucidation by FTIR study in laboratory.

Keyword: neem, antimicrobial, chopped bark

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Introduction

Neem extract consists of a posh mixture of molecules, including normal hydrocarbons, phenolic compounds, terpenoids, alkaloids, and glycosides. Neem (*Azadirachta indica A. Juss)* is a highly beneficial plant found in a country with a tropical climate like Indonesia. [3,4,5] Neem is part of tropical plants from the *Meliaceae* family and has been used for thousands of years as medicine. Some parts of neem plants can be developed to be medicine to treat multiple diseases including acute and chronic diseases, especially in Asia and Africa. Neem plants grow in various countries in the world, including Asia, Africa, America, and Australia. The botanical name of the neem plant is *Azadirachta indica* A. Juss. The Latin name for neem is *Azadirachta indica*. Neem has been widely used by humans since prehistoric times to treat various diseases. The plant is extraordinary, and it is referred to as "21st-century trees" by the United Nations. Neem is also referred to as "a tree for solving global problems" because it has many benefits, and it can treat various diseases. [1,6,7]

Materials and methods

Collection of Plants

Azadirachta indica bark were collected from botanical garden Calcutta Institute of Pharmaceutical Technology and Allied Health Sciences, Banitabla, Uluberia , Howrah, West Bengal and then authenticated from department of botany, Vinodini PG college, Shekhawati university, Rajasthan.

Materials

Methanol, distilled water and agar were used to be analytical grade.

Preparation of crude azadirachta indica bark exract

Biological source: *Azadirachata indica (family – Meliaceae)*
Geographical source: West Bengal in India, Asia, Africa and other tropical parts of world.
Part used: Stem bark
Collection -In the mid of august the stem bark was collected from the Azadirachata indica tree. After collecting the bark was peeled from the stem with a sharp knife and chopped into pieces which were sundried for seven days. After drying the bark was cut more finely using cutter mill which was used for preparing extract.

Preparation of extract

Materials and equipments used: methanol, 250ml separating funnel, sharp knife, distillation apparatus, filter paper, hot plate, distilled water, cotton wool. Firstly the finely chopped bark was kept into a separating funnel using methanol as menstrum at room temperature for seven days after that plant extract was collected into a beaker by opening the outlet of separating funnel. [1,6,9]
This above process was repeated for four times.
After that plant extract was distillated over five hours at 80 degree centigrade for

concentrating the menstrum. After distillation the menstrum was more concentrated in hot plate around 60 degree centigrade with continuous hand stirring till the after cooling crystal was formed**.** The plant extract was filtered through filter paper. The filtrate was again concentrated till crystal was formed. The crystal was separated on filter paper. After drying, the crude extract was collected into plastic container and has been stored at room temperature till its use. [1,2,8]

Characterization of Neem Bark Extract

Physical property and Chemical property evaluation

Parameter	**Reference**	**Observed**
Physical Appearance	Brown powders	Brown powders
SOLUBILITY		
Distilled water	+	+
Methanol	+	+
Ethanol	+	+
(+) soluble, (-)insoluble		

Chemical test for terpenoid		
Procedure	Observance	Result
Salkowski test :- Powdered drug was treated with chloroform and few drops of sulphuric acid.	It was given yellow colour which was changed to red colour.	Extract may contain terpenoid which is biologically antibacterial.

In vitro Antimicrobial evaluation by zone of inhibition method

Selection of microorganism and growth medium

For testing the antimicrobial activity the *Staphylococcus aureus* ATCC 29737 (IPRS) and Escherichia coli ATCC 25992 was selected and done test for preared crude Azadirachta Indica bark extract in the laboratory.

Growth medium - General nutrient agar media

Preparation of nutrient agar media

Materials

Electronic balance, pH paper or pH meter with standard buffers. Volumetric flask: 250 ml. Beef Extract, Yeast Extract, sodium chloride, Agar, Nonabsorbent cotton and gauze to make cotton stoppers.

Composition of Nutrient Agar Media

Composition of Nutrient Agar Media	
Composition	**Percentage(%)**
Agar	2
Beef Extract	1
Yeast Extract	1
Nacl	0.5
Water	q.s

Procedure

Beef Extract, Yeast Extract, Nacl, Agar was firstly weighed to get 1%,1%,0.5%,2% in solution respectively to prepare 100 ml nutrient Agar media. It was added to 100 ml of distilled water and stirred to dissolve the yeast extract and beef extract and Nacl in volumetric flask and checked the pH between 6.0 to 7.0.Then Agar was added and after cotton plaguing the media was sterilized in autoclave and prepared the media.

Sterilization

Sterilization was done for glass apparatus in hot air oven for one hour at 160^0C.

Preparation of slant and subculture

Firstly the after sterilizing the test tube, agar nutrient media was melted at 45^0C and poured into the test tube at sixty degree angle and waited for solidification. After solidification prepared slant was kept into freeze. Prepared slant was inoculated by moving the loop gently up the surface of the agar in a snake-like fashion but was not to gouge the agar surface. Slant cultures were incubated for 24 hours and bacterial growth was observed. *[6,7,8,9]*

Preparation of inoculums

For evaluation of antibacterial activity, fresh culture of bacteria was suspended in sterile water to obtain a uniform suspension of microorganism up to 24 hours.

Determination of zone of inhibition

Antibacterial activity was checked by agar well diffusion method. In this method a previously liquefied medium was inoculated with 0.2 ml Bacterial suspension having a uniform turbidity at room temperature. 20 ml of culture medium was poured into the sterile Petri dish having an internal diameter of 8.5 cm. Care was taken for the uniform thickness of the layer of medium into plates. After complete solidification of liquefied inoculated medium, the wells were made aseptically with cork borer having 6mm diameter. In each of these plates, extract solution (1mg/1ml stock solution prepared in distilled water) was placed carefully. Plates were kept for pre diffusion for 30 minutes. After normalizing to room temperature; the plates were incubated at 37°c for 24 hrs for bacteria. After incubation period was over, the zone of inhibition was measured with

help of Hi-media. [1,2,9]

Zone of inhibition of extract

Microbial strains	Zone of Inhibition (mm)
	Azadirachta indica BARK EXRACT
Staphylococcus aureus ATCC 29737	12
Escherichia coli ATCC 25992	0

Determination of λ max

Determination of λ max in methanol &ethanol and percentage of purity of Azadirachta indica bark exract using UV spectroscopic method (shimadzu-1700). An absorption maximum of extract was determined by UV spectroscopic method using shimadzu-1700UV/Visible spectrophotometer. It had been found that the λmax for extract in methanol was 278.5 nm and in ethanol was 282 nm. [1,2,8]

Procedure

Firstly 10 mg extract was weighed and was dissolved into 10 ml methanol and ethanol respectively to prepare 1mg/ml stock solution means 1000 µg/ml stock solution. From this stock solution 5 ml solution taking was diluted to 50 ml solution to prepare 100 µg/ml solutions, from this solution several dilutions was done and λ max for extract was estimated.

Calculation

Stock solution concentration - 1000 µg/ml

1 ml stock solution of extract contains equivalent to 1000 µg extract

5 ml stock solution of extract contains equivalent to 5 * 10000 = 5000 µg extract

5 ml stock solution of extract was dissolved in 50 ml solvent, so concentration becomes diluted from 1000 µg/ml to 100 µg/ml.

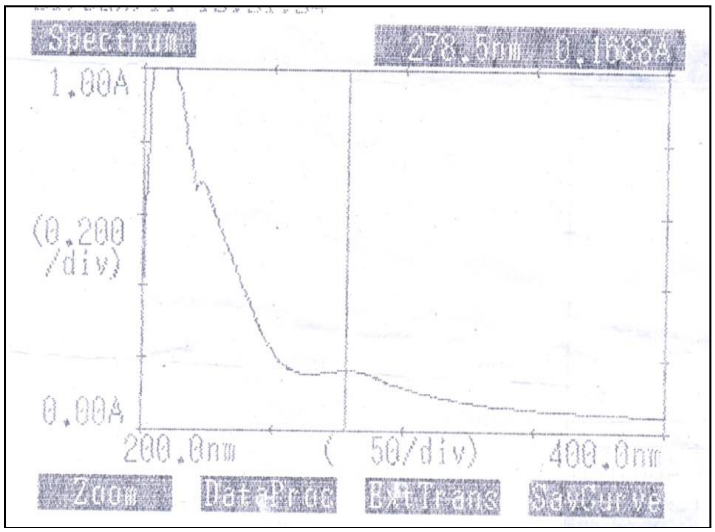
From 100 µg/ml solution following concentration was prepared to estimate the Lamda maximum (λmax) of extract –

1. 1ml to 10ml= 10 µg/ml
2. 2ml to 10ml=20 µg/ml
3. 3ml to 10ml=30 µg/ml
4. 4ml to 10ml=40 µg/ml
5. 5ml to 10ml=50 µg/ml
6. 6 ml to10ml=60 µg/ml

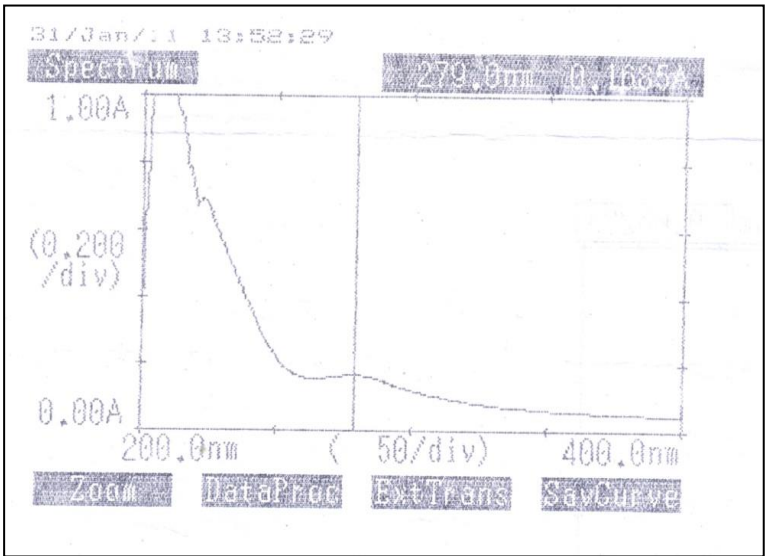
Result of UV analysis

Solvent employed	Lamda maximum (λmax) in nanometer (nm)			
	Reference	Observed		
			Concentration of extract in µg/ml	Absorbance
Methanol	279	278.5	50	0.1688
Ethanol	Not found	282	20	0.0881

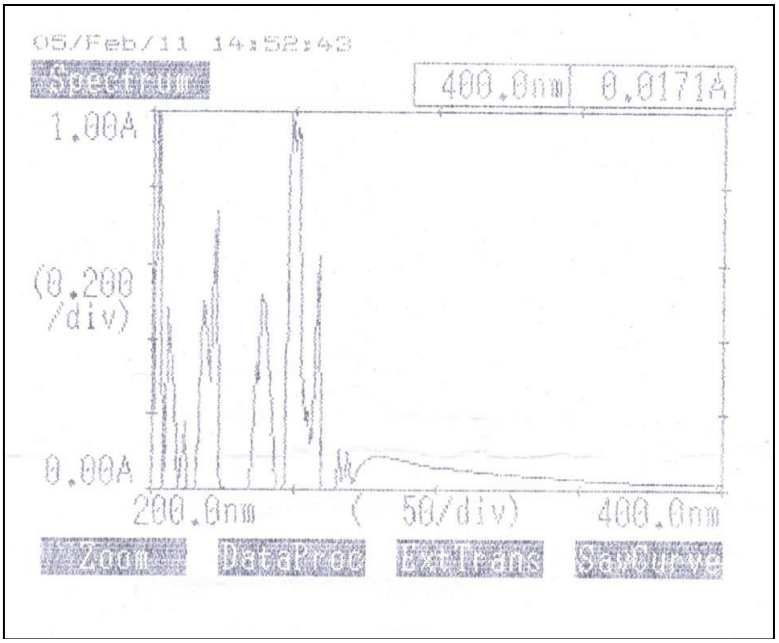
UV lamda maximum (λmax) of neem bark extract in methanol at 278.5nm.



UV absorbance of neem bark extract at 279nm in same concentration in methanol as UV lamda maximum (λmax) of extract previously found at 278.5nm.



UV lamda maximum (λmax) of neem bark extract in ethanol at 282 nm



Calculation of %purity of prepared extract

$E^{1\%}_{1cm}$ Value for prepared extract was being taken as reference data from U.S. patent (date of patent-May 7, 1985, patent no.-4515785, sheet 3of3) is 110 at 279 nm in methanol.

$E^{1\%}_{1cm}$

UV analysis for determining percentage purity

Concentration in µg/ml	Concentration in %w/v	Observed absorbance at 279 nm	$E^{1\%}_{1cm}$ value of reference at 279 nm
50	0.005	0.1685	110

Calculation

% Purity = Observed absorbance/'E' value * 100/concentration

Conversion of µg/ml to % w/v:
% w/v * 10,000 = µg/ml
So,50 µg/ml=50/10000%w/v
=0.005%w/v

%Purity=0.1685/110*100/0.005
=30.63%w/w

Structural elucidation by FTIR study

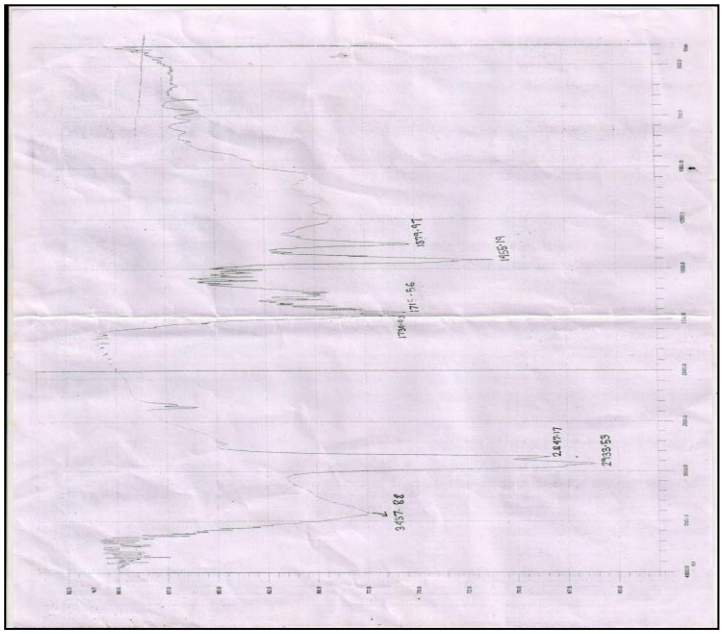
IR Spectroscopy

The IR Spectroscopy was used as to confirm the structure of extract with respect to reference FTIR data of extract. The study reveals that as identified by IR spectrum and the main peaks was found at 3437.88, 2847.17, 2933.53, 1730.03, 1715.56, 1455.19, 1379.97 cm−1 of sample which is identical to reference spectra data.

FTIR spectra of prepared neem bark extract

Compound	Stretching/Bending	Wave number (cm−1)
Prepared neem bark extract.	N-H stretching()	3437.88
	C-H stretching(alkane)	2847.17
	C-H stretching(alkane)	2933.53
	C=O stretching(aldehyde)	1730.03
	C=O stretching(acid)	1715.56
	C=C stretching(aromatic)	1455.19
	C-O stretching (phenols)	1379.97

FTIR spectra of prepared neem bark extract



Results and discussion

Prepared neem bark extract was prepared in methanol, color of this extract was brown crystalline powder, was soluble in water, methanol, ethanol as this extract was prepared in methanol, so it forms intermolecular hydrogen bonding between different molecules to be soluble, antimicrobial activity was checked against Staphylococcus aureus ATCC 29737 (IPRS) and Escherichia coli ATCC 25992, butzone of inhibition was found only against Staphylococcus aureus ATCC 29737 (IPRS) was 12 mm, solution inhibited growth of microbes around well of nutrient media . λmax and absorbance was determined using different concentration of solution for extract that in a optimized same concentration ,λmax in methanol was 278.5 nm and absorbance in methanol was found *0.1688* ,λmax in ethanol was 282 nm and absorbance was found *0.0881*. Absorbance was measured at 279nm of particular concentration of extract solution was *0.1685*to calculatepercentage

of purity of neem bark extract that was estimated at 0..005% w/v concentration, 279 nm **is 30.63% w/w** using $E^{1\%}_{1cm}$ reference value at 279nmof neem bark extract. FTIR study was done to confirm structure of neem bark extract with reference IR spectrum data and the main peaks were found at 3437.88, 2847.17, 2933.53, 1730.03, 1715.56, 1455.19, 1379.97 cm−1 of sample which was identical to reference spectra data.

Conclusions

After completing this work successfully, antimicrobial activity against *Staphylococcusaureus ATCC 29737 was found,* λmax for extract in methanol was 278.5 nm and in ethanol was 282 nm, percentage of purity of neem bark extract was estimated at o.005% w/v concentration, 279 nm is 30.63% w/w using $E^{1\%}_{1cm}$ reference value by determining absorbance at 279 nm of that concentration of extract. FTIR study was done to confirm structure of neem bark extract with reference IR spectrum data and the main peaks were found at 3437.88, 2847.17, 2933.53, 1730.03, 1715.56, 1455.19, 1379.97 cm^{-1} of sample which was identical to reference spectra data.

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