Study of antimicrobial activity of *Azadirachta indica* (Neem) bark extract on bacteria found on Indian paper currency

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Introduction

Microorganisms are found almost everywhere around us in our environment and are known to spread through contaminated water, food, air and vectors such as fleas, mites, rats, dogs and also through inanimate objects like clothes, utensils, furniture, hand railings or a door knob which we are generally unaware of. Paper currency notes are one of the most commonly exchanged objects and its potential to transmit pathogenic organisms has been well recognized (Khin etal.1989 ; Abrams, 1972 ; Goktas, 1992; Basavarajappa etal.2005) .Earlier studies in many countries have shown that circulating currency bills are most common fomite as it is handled by a large number of people under a variety of personal & environmental conditions (Uneke & Ogbu, 2007;El Dar 2005 ; Kalita etal.2013)

Currency bills are exchanged for goods & services by one & all and a few seconds of money transaction can bring along a whole lot of pathogens. Numerous studies have well documented that paper currency acts as a vehicle to spread bacteria and other microorganisms and can accommodate a variety of contaminants for long periods (Lamichhane etal 2009 ;Angelakis etal 2014 ; Wamae 2009).

The antimicrobial properties of the neem plant have been reported earlier by several workers been the primary source of medicine (Ketkar etal 2004; Biswas etal.2002). Neem, also referred to as *Azadirachta indica* is one such plant that has been so named because it provides freedom from all diseases, and used for thousands of years in Indian and African continents (Gupta,etal 2017). Neem (*Azadirachta indica*) belongs to family, Meliacea and has been known as the wonder tree for centuries in the Indian subcontinent with proven therapeutical & ethnomedicinal values for mankind well known for its antibacterial , antiviral, and antifungal properties (Sarmiento etal,2011) .Different parts of the plant including flowers, leaves, seeds and bark have been used to treat both acute and chronic human diseases.

Nimbidin, Azadirachtin and nimbinin are active compounds present in Neem which are responsible for antibacterial activity. Neem bark is used as an active ingredient in a number of toothpastes and toothpowders. Neem bark has anti-bacterial properties (Laxmi etal. 2015; Krüzselyi D,2016)

Various phytochemicals have been isolated from neem leaves using different methods of extraction and their therapeutic use in preventing several diseases (Yadav etal 2016)

The methanol extract of neem leaf was tested for its antibacterial, antisecretory and antihemorrhagic activity against *Vibrio cholerae*. *Azadirachta indica* extract had significant antibacterial activity against the multi-drug-resistant *Vibrio cholerae* (Thakurta etal,2007). The methanolic extract of *Azadirachta indica* have an antibacterial effect against *Enterococcus faecalis* strains during the first 24 and 48 hours (Arévalo-Híjar, etal 2018). Neem (*Azadirachta indica A. Juss*) oil was assayed against forty-eight isolates of *Escherichia coli* by standardised disc diffusion test and microdilution test. The oil showed biological activity against all isolates (Del Serrone,2015)

In present study the effect of the increasing concentrations of methanolic neem bark extract (0.0078x 0.0156x ,0.0312x ,0.0625x ,0.125x ,0.25x ,0.5x ,1.0x) was studied on the growth of pure culture isolates of microbes swabbed from the Indian paper currency in circulation measured in terms of Zone of Inhibition(in mm) on Muller Hinton Agar (MHA) plates. (Kirby-Bauer disc diffusion method)

Hypothesis

Null Hypothesis : The decreasing concentrations of the neem bark extract (X times) will not exhibit antimicrobial properties & will show no Zone of Inhibition (NZ) in mm.

Alternate Hypothesis : The bacterial growth will become higher as the concentration of the neem bark extract(dilutions of 100 µL) is reduced in the discs and the Zone of inhibition becomes smaller (in mm)

Variables

Independent Variable: Increasing concentration of *Azadirachta indica* bark extract (0.0078x ,0.0156x 0.0312x ,0.0625x ,0.125x ,0.25x ,0.5x ,1.0x)
Dependent Variable: Diameter of Zone of Inhibition (in mm)

Fixed Variables:

Fixed Variable	Why to Fix?	How to Fix?
Temperature (°C)	Every bacterial species has specific growth temperature requirements which is largely determined by the temperature requirements of its enzymes. The growth rates are the highest at the optimum growth temperatures. And it is important to give same environmental conditions to all the microbial isolates so that growth patterns are not affected by the difference in temperatures. Hence it is important to set the temperature of the incubator at a constant temperature while conducting the experiment.	All agar plates were kept in incubator at 37°**C** for culture of the bacteria
Agar media	Many different types of culture media containing nutrients & physical growth factors are needed to facilitate microbial growth .Selective medium is designed to suppress growth of some microorganisms while allowing the growth of others. Uniformity in type of media during entire course of experiment is important for uniform growth patterns in different bacterial isolates. Mueller-Hinton agar (MHA) and MHA supplemented with 5% sheep blood (MHS) were used in the disk-diffusion assay (Joyanes etal ,1997)	Muller Hinton Agar (MHA) plates were used for pure culture isolates & neem extract susceptibility test as it is non-selective & non-differential media known to absorb toxins released from bacteria & mediates rate of diffusion of antimicrobial components/antibiotics through agar.MacConkey agar was used raise the isolated microflora from the currency.
Time Duration	Time duration is a very important factor as bacteria multiply very fast & continuously and if the cultures are kept for a longer period of time the bacteria may start dying as the concentration of bacterial toxins in the media increases with time & will inhibit the further growth of bacteria. So it is important to fix the parameter of time duration.	All the culture plates were ideally incubated for 24 hours in the incubator.
Amount of powdered neem bark used to make concentrations	Neem bark has an active pharmaceutical ingredient which has shown anti bacterial activity against various gram positive organisms (Laxmi etal ,2015) Variation in the amount of the bark powder used for the extraction of the active ingredient will lead to difference in the amount of active ingredients and hence effect the results on Zone of Inhibition. Also the solvent used for extraction	In this experiment 10 gm of powdered neem bark was used for active ingredient extraction in 250 ml of solvent
Solvent used for extraction of the active ingredient from neem bark	Results showing inhibition of the microbial growth using neem extracts in different solvents like water, ethanol & methanol has shown variations. Hence it is important to use the same solvent for extraction of active ingredient from the neem bark. The methanolic extract of *Azadirachta indica* have shown antibacterial effect against *Enterococcus faecalis* strains during the first 24 and 48 hours[28] The methanol extract of neem leaf was tested for its antibacterial, antisecretory and antihemorrhagic activity against *Vibrio cholerae*[29]	In preliminary investigations carried out it was seen that neem extract in methanol showed very good results. Hence for this experiment methanolic neem extract were used .

Methodology

Step1: Isolation of microbes from Indian paper currency and macroscopic and microscopic characterization of isolates

Step 2: Pure culture Isolation

Step 3: Processing of neem bark samples using Soxhlet apparatus.

Step 4: Antimicrobial Activity Testing by Kirby-Bauer disc diffusion method

Step 1: Isolation of microbes from Indian paper currency

Indian currency of Rs.10 and Rs. 20 were collected from the medical stores around SMS hospital in Jaipur. These currency were kept in polyethylene bags and brought to the microbiology lab. Sterile cotton swab were soaked in the sterile Peptone water and then gently swabbed onto the currency .This cotton swab was then transferred into the tube of fresh sterile peptone water and incubated at 37˚C/15min. After 15 minutes of incubation they were plated onto sterile MacConkey agar plates and again placed in the incubator for 24-36 hours. Eight different types of colonies were observed on day 2.Gram stained slides of these 8 different types of bacteria were prepared .Macro and Microscopic examination of these cultured colonies were done and a observations were recorded in Table 1

Gram staining protocol

Preparation of slide smear: With the help of nichrome loop wire little amount of culture isolate was transferred on a clean slide with a drop of water on it and a thin smear was prepared & heat fixed.

Gram Staining :

i. *Crystal violet stain was added over the fixed culture and let stand for 10 to 60 seconds.The excess stain was poured off & the slide was gently rinsed with water.*
ii. *Subsequently Gram's iodine solution was poured on the smear, enough to cover the fixed culture & let stand for 10 to 60 seconds. Excess iodine solution was poured off and the slide was rinsed with running water. Excess water from the surface was shaken off.*
iii. *Few drops of decolorizer was added on the slide and quickly rinsed off with water after 5 seconds.*
iv. *Finally the slide was counterstained with basic fuchsin solution for 40 to 60 seconds and solution was washed off with water.*
v. *The slide was shaken to remove most of the water and air-dried. Alternatively bibulous paper can be used lightly to dab the excess water.*

Observe under compound microscope in Oil-immersion lens.

Microscopic (Gram staining) images of all eight isolates

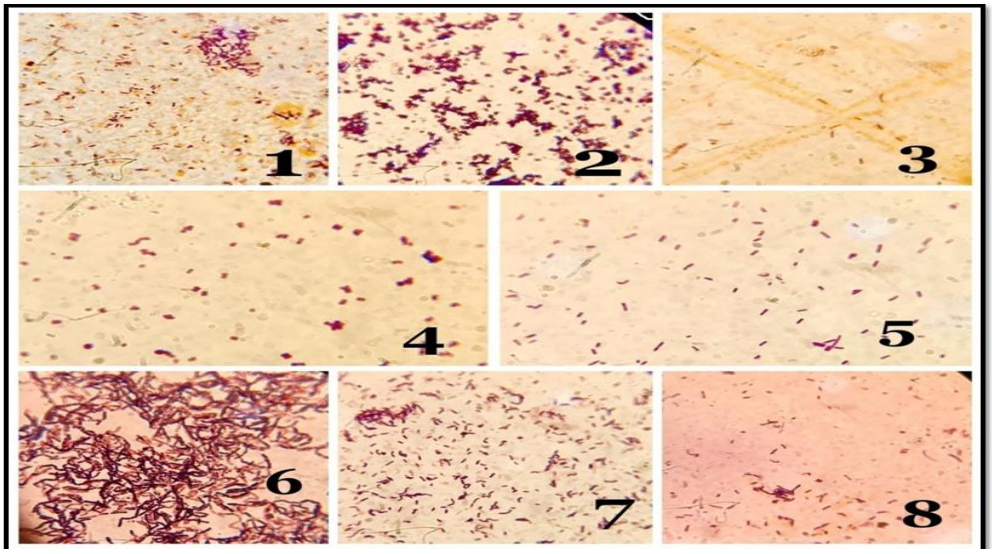


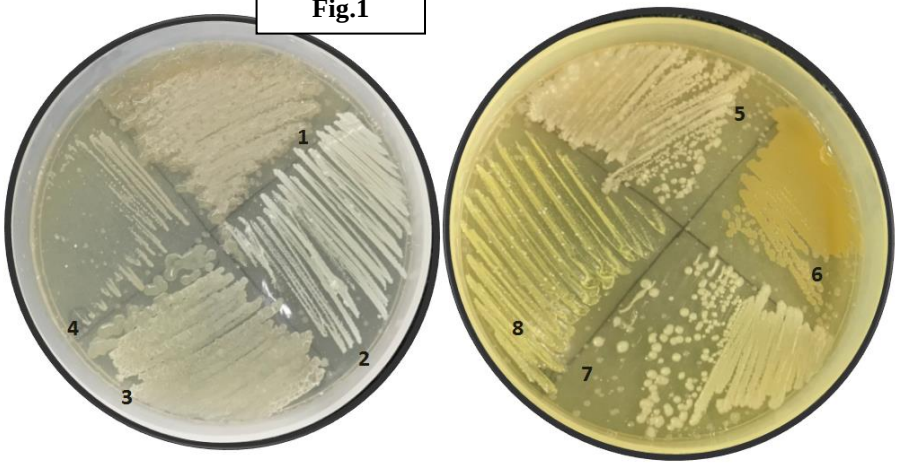
Table 1 :Colony (Macroscopic) & Microscopic Characteristics

Sr. No.	Size	Shape	Color	Margin	Elevation	Opacity	Gram's nature
1	18-20mm	Irregular	Off white	Undulated	Raised	Opaque	Gram Positive bacilli
2	2-3mm	Regular	Off white	Entire	Raised	Opaque	Gram Positive cocci
3	9mm	Irregular	Off white	Undulated	Raised	Opaque	Gram negative bacilli
4	2-3mm	Regular	Off white	Entire	Flat	Translucent	Gram Positive bacilli
5	1mm	Regular	Yellow	Entire	Flat	Opaque	Gram Positive bacilli in cluster
6	1cm	Irregular	Off white	Entire	Flat	Opaque	Gram positive coccobacilli
7	1cm	Regular	yellow	Entire	flat	Opaque	Gram Positive bacilli
8	1.8-2cm	Irregular	Off white	Undulated	Raised	Opaque	Gram Positive bacilli

Step 2: Pure culture Isolation

- A small amount of colony from each of the eight different types of colonies isolated from the paper currency were picked using the nichrome loop wire and streaked on fresh sterile Muller Hinton Agar (MHA) plates
- The plates were incubated at 37˚C for 24hours.
- Eight different types of pure colonies were observed on the plates (Fig.1)

Fig.1



Eight different types of colonies were streaked on the Mueller-Hinton agar Plate.

Result & Discussion

A preliminary experiment was performed in which the antimicrobial activity of ethanolic, methanolic & aqueous extracts of neem bark was assessed & observed on the 8 different Gram positive & Gram negative bacteria isolated from the Indian paper currency collected from the vicinity of SMS medical hospital .The antimicrobial activity was measured in terms of Zone of Inhibition (in mm) on Muller Hinton Agar (MHA) plates using Kirby-Bauer disc diffusion method & microdilution test. The extracts showed biological activity against all the isolates.(Table 1)

TABLE 1

Isolates	Positive Control	Negative Control	Methanol	Ethanol	Water
Isolate 1	25 mm	NZ	18 mm	12 mm	16 mm
Isolate 2	26 mm	NZ	20 mm	19 mm	21 mm
Isolate 3	26 mm	NZ	16 mm	15 mm	NZ
Isolate 4	33 mm	NZ	22 mm	20 mm	20 mm
Isolate 5	23 mm	NZ	19 mm	18 mm	13 mm
Isolate 6	30 mm	NZ	20 mm	18 mm	17 mm
Isolate 7	30 mm	NZ	19 mm	19 mm	19 mm
Isolate 8	29 mm	NZ	18 mm	14 mm	NZ

Key: NZ: No zone of inhibition.

Three Different concentration Trials :

From the above result of antimicrobial activity, 3 Isolates were chosen based on the activity.
Isolate 4
Isolate 6
Isolate7
Antimicrobial activity of three different concentration (1X,0.5X and 0.25X) of neem bark extract in different solvents was tested on these three isolates (4,6,7). The observations made are in the Table 2 below

TABLE 2

Isolates	Solvents	1X	0.5X	0.25X
Isolate 4	**Methanol**	**23 mm**	**22 mm**	**21 mm**
	Ethanol	19 mm	18 mm	18 mm
	Water	20 mm	18 mm	16mm
Isolate 6	**Methanol**	**24 mm**	**21 mm**	**20 mm**
	Ethanol	24 mm	23 mm	21 mm
	Water	20 mm	18 mm	16mm
Isolate 7	**Methanol**	**23 mm**	**20 mm**	**18 mm**
	Ethanol	18 mm	16 mm	15mm
	Water	21mm	16mm	14mm

From the observation & results of the preliminary experiments the methanolic neem extract showed better results *(Table3 Fig.1)* Hence for the main experiment five different more concentrations (0.0078x ,0.0156x ,0.0312x ,0.0625x ,0.125x) of methanolic neem bark extract were assayed against the bacterial isolate no.4 from the Indian paper currency*(Table 4 Fig.2)*

Fig. 1

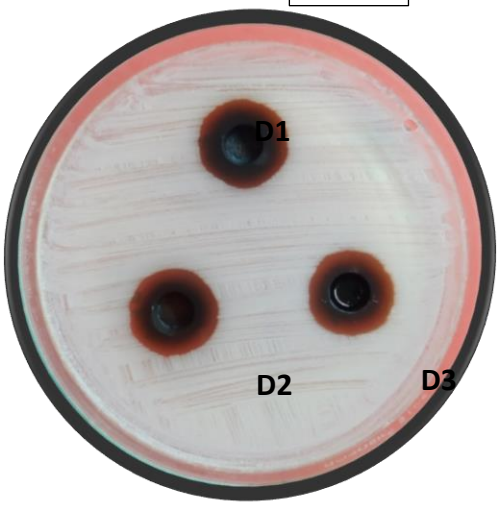


Fig.2

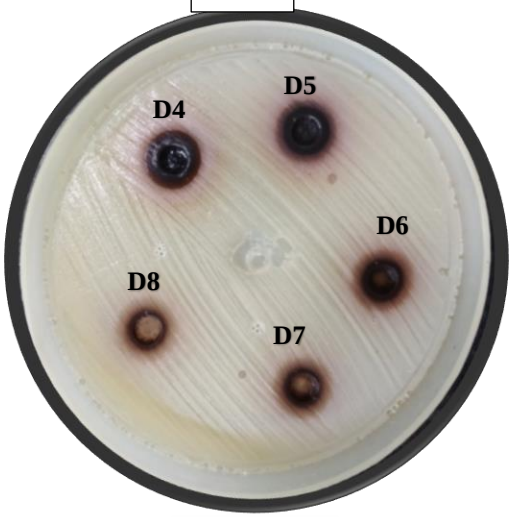


TABLE 3

Isolate 4	Solvents	1X (D1)	0.5X (D2)	0.25X (D3)
	Methanol	23 mm	22 mm	21 mm

TABLE 4

Bacteria	Methanolic Bark Extract				
Isolate-4	0.125 X (D4)	0.0625 X (D5)	0.0312 X (D6)	0.0156 X (D6)	0.0078 X (D7)
	16 mm	15 mm	13 mm	NZ	NZ

Conclusion

The methanolic neem extract showed biological activity against all isolates. The bacterial growth inhibition zone by disc diffusion method (100 µL) ranged between 13.00 ± 0.70 and 23.00 ± 1.00 mm. The antibacterial activity was furthermore determined at lower concentrations. Minimum inhibitory concentration was 0.0312 X (D6).

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