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Antimicrobial Activity of *Azadirachta indica* (Neem) Leave Extract Against Some Clinical Isolates

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Abstract
The present study was carried out at Microbiology and Chemistry Laboratories, Federal University Dutse, Jigawa state. To investigate the activity of neem leave extract against some clinical isolates. The test organism includes Escherichia coli, Klebsiella pneumoniae, and Staphylococcus aureus. The phytochemical compounds present in the neem was extracted using ethanol as solvent, soxhlet extractor was used to get the extract phytochemical screening were carried out using the standard methods, some of the examined phytochemical are Alkaloids, Saponin, Phenol, Tannins and Flavonoids. The extract is more effective against a Gram negative E.coli and K.pneumonae as compared to S.aureus gram positive. The zone of inhibition of the extract at the 125mg/ml is 21 and 14. For gram negative and gram positive bacteria respectively. The mean of the minimum inhibitory concentration of A. indica leaf extract, the minimum inhibitory concentration (MIC) of the extract in vitro revealed to be at much lower concentration 50mg/ml against E.coli as compared to 100mg/ml against S. aureus the minimum bactericidal concentration (MBC) of A. indica leaf extract shows that the extract have a bacteriostatic effect against gram negative bacteria with static effect against gram positive bacteria
Keywords: Anti-bacteria, Escherichia coli, Klebsiella pneumoniae, neem leave extract Staphylococcus aureus.

INTRODUCTION
Plants have been utilized as medicines for thousands of years. These medicines initially took the form of crude drugs such as tinctures, teas, poultices, powders, and other herbal formulations. The specific plants to be used and the methods of application for particular ailments were passed down through oral history (Vinoth *et al.*,2012). Eventually information regarding medicinal plants was recorded in herbals (Marcy *et al.*, 2005). In more recent history, the use of plants as medicines has involved the isolation of active compounds, beginning with the isolation of morphine from

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opium in the early 19th century drug discovery from medicinal plants led to the isolation of early drugs such as cocaine, codeine, digitoxin, and quinine, in addition to morphine, of which some are still in use (Marcy *et al.*,2005). Isolation and characterization of pharmacologically active compounds from medicinal plants continue today. Natural drugs have been a part of the evolution of human, healthcare for thousands of years (Vinoth *et al.*,2012). Nowadays nearly 88% of the global populations turn to plant derived medicines as their first line of defense for maintaining health and compacting diseases. One hundred and nineteen secondary plant metabolites derived from plants are used globally as drugs, 15% of all angiosperms have been investigated chemically and of that 74% of pharmacologically active plant derived components were discovered (Vinoth *et al.*,2012).

Several hundred plants and herb species that have potential as novel antiviral agents have been studied, with surprisingly little overlap (Jassim & Naji, 2003). A wide variety of active phytochemicals, including the flavonoids, terpenoids, lignins, sulphides, polyphenolics, coumarins, saponins, furyl compounds, alkaloids, polyines, thiophenes,.
Azadirachta indica has attracted the focus of attention owing to its insecticidal and medicinal properties. *Azadirachta indica* (Neem) is a multipurpose tree with multiple health benefits (Subapriyaa & Nagini, 2003).

Different parts of the tree were shown to exhibit antimicrobial effects against a wide variety of microorganisms (Ruch *et al.,* 2014). Aqueous extract of neem leaf has a good therapeutic potential (Mossadek & Rashid, 2008; Patil *et al.,* 2013). Screening of this medicinal plant for bioactive compounds may lead to development of less expensive new antimicrobial agents with improved safety and efficacy (Hala, *et al.,* 2015). Neem elaborates a vast array of biologically active compounds that are chemically diverse and structurally complex. All parts of the neem tree-leaves, flowers, seeds, fruits, roots and bark are recognized to possess a wide range of pharmacological effects. The medicinal utilities have been described especially for neem leaf.

MATERIALS AND METHODS

Study Area

This research work was carried out at Microbiology and Chemistry Laboratories, Federal University Dutse, Jigawa state. Dutse is the capital city of Jigawa State located in the coordinate of 11.70240^{o} N, and 9.3340^{o}E.

Collection of Samples

Fresh Leaves of *Azadirachta indica* were collected in Federal University Dutse and was identified in Biological Sciences Department. It was ensured that the collected leaves was healthy and uninfected. The leaves were washed under running tap water to eliminate dust and other foreign particles. Leaves samples were air dried at room temperature in the laboratory for 15 days, and crushed using mortar and pestle, reduced to powder using warring laboratory blender for 15minutes at high speed and then stored in airtight bottles for two days prior to analysis.

Preparation of Leaf Extract

The solvent (250 ml of ethanol) was poured in to a round bottom flask, which is attached to a Soxhlet extractor and condenser on an isomantle. 50g crushed plant material was loaded into the

thimble, which is placed inside the Soxhlet extractor. The side arm was lagged with glass wool. The solvent was heated using the isomantle and began to evaporate, moving through the apparatus to the condenser. The condenser then dripped into the reservoir containing the thimble. Once the level of solvent reached the siphon, it poured back into the flask and the cycle begins again. The process was allowed to run for a total of 16 hours. The equipment was monitored due to the mix of running water and an electrical appliance (James, et *al.,* 2014). The extract was recovered using a hot air evaporator.

Biochemical Identification of the Test Organism

The test organisms were biochemically identified in the laboratory, the characterization were carried out based on some biochemical features.

Escherichia coli and Klebsiela pneumoniae

Both isolates were cultured on Mackonkey Agar and allowed for 24-48hrs. Individual isolates were subjected to the IMVIC test. *E. coli* gives Indole test positive while Klebsiella is negative, Methyl Red test positive VogesProskauer test negative and also Citrate utilization test Negative.

***Staphylococcus* aureus**

The organism was identified by culturing the isolate onmannitol salt agar. After the incubation period (24hrs), typical golden colonies of *S. aureus* was seen. Gram staining was carried out for confirmation. Sub culturing on the blood agar: *S. aureus* appears as grape-like clusters when viewed under a microscope, and has large, round, golden-yellow colonies, often with beta hemolysis, when grown on blood agar, *Staphylococcus aureus* hemolysins (alfa-hemolysin, beta-hemolysin, gamma-hemolysin and delta-hemolysis) were observed.

Determination of Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of the test organisms was determined using the tube dilution technique. Nine milliliter (9ml) of the nutrient broth was pippeted into various test tubes containing concentrations of 25mg/ml, 50mg/ml, 75mg/ml and 100mg/ml and 125mg/ml of the extract and moderate concentration of chloramphenicol, blank normal saline as a positive and Negative control respectively. The overnight culture of the test organisms aseptically obtained from the Microbiological laboratory was added to the test tubes and then incubated at 37^{o}C for 24 hours. The least concentration of the extract that did not indicate any visible growth of the incubated organisms in broth culture was taken as the minimum inhibitory concentration (MIC) Abalaka, *et al.,*(2012).

Determination of Microbial Bactericidal Concentration of the Extract

The bactericidal or bacteriostatic effect of the extract was determine by sub-culturing the well that shows no growth on a fresh medium and incubate it for 24hrs. if the growth is observed, the extract is considered to have only bacteriostatic effect, while on the other hand, if the growth is observed, the extract is said to possess bactericidal effect (Abalaka, et *al.,* 2012).

Determination of *Azadirachta indica* extract Spectrum of Action

The extracts were subjected to both gram negative and gram positive isolates. The extract is said to possess a broad spectrum if it's effective on both gram positive and gram negative organisms as well. On the other hand, the extract can be described as those possessing narrow spectrum if it's effective on only one group of organisms (Abalaka, *et al.,* 2012).

RESULTS AND DISCUSSION

The phytochemical evaluation of the ethanolic extracts of *A. indica* showed the presence of phenols, tannins, Alkaloids while flavonoid and saponins were found to be absent.

Table 1: Phytochemical composition of the leave extract of *Azadirachta indica* extract

Alkaloids	positive (+)
Phenols	positive(+)
Tannins	positive(+)
Flavonoid	negative (-)
Saponins	negative (-)

Key: + = Detected, _= Not Detected

Table 2 shows the antimicrobial susceptibly test of the Ethanolic extract of the *A. indica* leave extract against the test organisms i.e. *K. pneumoniae, E. coli and S. aureus* at varying concentration in mg/ml. The extract is more effective against the Gram Negative bacteria with even at low concentration as compared to Gram Positive bacteria. Gram positive bacteria showed some degree of resistance at lower concentration as shown in the table below:

Table 2: Susceptibility of the test extract.

Concentration (mg/ml)	25	50	75	100	125	+Ve control	–Ve control
Test organisms	Susceptibility						
Escherichia coli	S	S	S	S	S	S	R
K. pneumoniae	S	S	S	S	S	S	R
S. aureus	R	R	R	S	S	R	

Key: -S= Susceptible, R= Resistance

NB: positive control=Chloramphenicol

Negative control= distilled water

Table 3 shows the mean zone of inhibition of the extract against the test organism at difference concentration of the extract in mg/ml. The plant extract is found to be more effective against a Gram Negative bacteria (*E. coli* and *K. pneumonia)* as compared to gram negative bacteria (*Staphylococcus)*.

Table 3: Result of antibacterial screening showing the Mean Zone of Inhibition.

Test organism	25	50	75	100	125	+Ve Cont	-Ve cont.
S. aureus	4	7	11	14	16	00	
K. pneumonia	10	12	14	17	18	19	0
E. coli	11	13	16	17	21	23	0

NB: positive control=Chloramphenicol

Negative control= distilled water

Table 4 summarizes the mean of the Minimum Inhibitory Concentration of *A. indica* leaf extract. The Minimum inhibitory concentration of the extract in-vitro revealed to be at much lower concentration (50mg/ml) against *E. coli* as compared to 100mg/ml against *Staphylococcus spp.* as shown in the table below:

TABLE 4: Result of minimum inhibitory concentration (MIC) of the extract against the test organisms in mg/ml .

Test organism	25	50	75	100	125	+Ve contr.	–Ve contr.
S. aureus	+++	++	+	MIC	-	-	+++
K. pneumoniae	++	+	MIC	-	-	-	+++
E. coli	+	MIC	-	-	-	-	+++

Key: -MIC =Minimum Inhibitory Concentration, (-) =No turbidity, (+) =slightly turbid, (++) =

Moderate turbidity, (+++) =highly turbid, TTC =Tetracycline.

NB: Positive control used = chloramphenicol
Negative control used = Distilled water.

Table 5 shows the minimum bactericidal concentration (MBC) of *A. Indica* leave extract against the test organisms at different concentration in mg/ml. The leaves extract have a bacteriostatic effect against gram Negative bacteria with static effect against a Gram Positive bacteria as shown in the table below:

Table 5: Result of the minimum bactericidal concentration (MIC) of the extract. against the test organisms in mg/ml

Test organism	25	50	75	100	125	+Ve contr.	–Ve contr.
S. aureus	+++	++	+	-	-	-	+++
K. pneumoniae	++	+	-	MBC	-	-	+++
E. coli	+	-	MBC	-	-	-	++

Key:- MBC = Minimum Bactericidal Concentration, (-) No colonies growth, (+) Scanty colonies growth, (++)

Moderate colonies growth, (+++) Large colonies growth

DISCUSSION

It's noticeable that, many of the existing synthetic drugs cause various side effects. Hence, development of plant- based compounds is required to meet this demand for production of newer drugs with minimal side effects. *A.indica* leaves possess a good antibacterial activity confirming the great potential of bioactive compounds and is useful for rationalizing the use of this plant in primary health care.

The finding in this research shows that, the ethanolic extracts of *A. indica* leaves exhibit antibacterial activity against all tested organisms with concentrations depended. Several studies had been performed to investigate the antimicrobial activity of neem leaf extract and their results were almost similar to our results. One of these studies is the report of Mohammed, *et al*., (2015), who stated that crude extract of Neem plant was very effective against *Staphylococcus aureus* and *E.coli*. They found that an extract concentration of 0.5 mg/ml had significantly reduced *Staphylococcus aureus* inoculum after 24hrs, while extracts with increasing concentrations completely wiped out viable bacteria in a lesser time. On the other hand, Aslam and her co-workers were able to check the action of Neem extract on three bacterial strains: *Staphylococcus aureus, K. Pneumoniae and E.coli* and they found a 75 mg/ml concentration was very effective.

In this study, a total yield of 5.7g of the ethanolic from the original weight of 50gwas recovered from the *A. indica* leaves. The physical characteristics of the leave indicated in the Table one, shows phytochemicals composition of leave. The confirmatory test of test organism was shown in table three using appropriate identification technique as described by Ibrahim, *et al*., (2017).

The confirmatory test shows *E. coli* to be indole positive which is indicated with red ring appeared at top of inoculated peptone water and it is citrate utilization negative which is indicated on Cimon citrate agar as blue hence, maintaining the colour of the medium. However, it is methyl red positive as indicated by colour change from yellow to red after 48 hours incubation at 37°C on methyl red-Voges Proskauer broth medium. For *K. pneumoniae*, it is found to be indole negative and it is citrate utilization positive which is indicated by colour change from blue to green on Cimon citrate agar. However, it is methyl red negative which is indicated by maintaining the colour of the broth (yellow) after 48 hours incubation at 37°C on methyl red-Voges-Proskauer broth medium. *S. aureus* on the other hand, is found to be catalase and coagulase positive. Similar observations where reported somewhere else (Ibrahim, *et al.,* 2017).

The mean zone of inhibition yielded by the extract range from 11mm to 21mm against *E. coli* at 25mg/ml and 125 mg/ml concentration of the extract respectively. This indicates *E. coli* to be the most sensitive to the extract among the tested organisms which is in conformity to Ranjit *et al.,* (2014); Nwakaeze *et al.,* (2013). The least susceptibility was observed in *S aureus*, with the zone of inhibition ranges from 0mm to 14mm at the concentration of 25 and 125mg/ml, respectively which is in agreement with Nwakaeze *et al.,* (2013) report. The Minimum Inhibitory Concentration (MIC) of leaf extract of *A.indica* was found to be effective against *E. coli* at the lowest concentration (50mg/ml). Similarly, the minimum bactericidal activity (MBC) of the leaves extract was found at the concentration on the *E.coil* where 75mg/ml is the lowest concentration that killed the bacterium, on the other hand, the extact was found to be only bacteriostatic against *Staphylococcus aureus* even at higher concentration this finding is in agreement with the report of Muhammad, *et al.,* (2016).

Generally, all the tested organisms were found to be susceptible to extract with variable zones of inhibition. The observed antibacterial activity of the extract against the test organisms could be attributed to the presence of different secondary metabolites detected in the plant Ibrahim *etal*., (2017).

CONCLUSION

Based on the findings in this study, it concluded that, the leave extract of *A. indica* has a potent antibacterial activity against various strains of bacterial pathogens ranges from gram positive to gram negative bacteria with less effective against a gram positive bacteria and as such owing to its versatile characteristics neem is rightly called the 'Village pharmacy' or 'Doctor tree' or 'The bitter gem."

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