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Original Research Article

PHYTOCHEMICAL ANALYSIS AND EVALUATION OF BACTERIOSTATIC EFFECT OF NEEM (*Azadirachta indica*) LEAVES ON SOME CLINICAL BACTERIAL ISOLATES.

*** Shu'aibu I[1]., Hamman, J. B[2]., Goje L. J.[3], Mu'inat A. M[1]. Jauro H. A[1]. and Kabiru M. Y[4].**

1 Department of Microbiology, Gombe State University, P.M.B. 127, Gombe, Gombe State Nigeria.
2 Department of Biological Sciences, Gombe State University, P.M.B. 127, Gombe, Gombe State Nigeria.
3 Department of Biochemistry, Gombe State University, P.M.B. 127, Gombe, Gombe State Nigeria.
4. School of Health Sciences, Maryam Abacha American University of Niger, ADS Avenue, Roy Mohd VI, Du Maroc, Maradi, Republic Du Niger

Abstract: The present study was carried out to evaluate the antibacterial properties of *Azadirachta indica* leaves against certain bacterial isolates (*Staphylococcus aureus*, *Salmonella species*, *Escherichia coli* and *Shigella species*) using disc diffusion method. The leaf extracts of *Azadirachta indica* were prepared using different solvents; methanol, ethanol and petroleum ether and were screened for antimicrobial active principles. The phytochemical screening of the leaf extracts revealed the presence of alkaloids, flavonoids and saponins. Resin was only present in ethanol extract. The antibacterial susceptibility testing of petroleum ether extract, ethanol extract, and methanol extract of neem leaves were carried out by disc diffusion method using Mueller-Hinton agar. Partial inhibitions were exhibited by all the extracts; no absolute growth inhibition recorded anyway. Different control standard drugs were used on *Salmonella species*, *E. coli, S. aureus* and *Shigella species* and these were ciprofloxacin, gentamycin, streptomycin and chloramphenicol respectively. The highest concentration (100µg/disc) of petroleum ether extract recorded partial activity on all the organisms with 13.0mm, 8.0mm, 9.0mm and 9.0mm on *Shigella spp., E. coli, S. aureus,* and *Salmonella spp* respectively. Ethanol extract was only partially active on *S. aureus* and *Salmonella spp* with 7.0mm and 9.00mm zones of inhibition respectively. Methanol extract was also partially active on *S. aureus* and *Salmonella spp* with 7.0mm inhibition zone on each. The results obtained from the different standard antibiotics on different bacterial isolates indicated that only streptomycin was inactive on the *S. aureus* tested while ciprofloxacin, gentamycin and chloramphenicol showed remarkable zones of inhibitions; (40, 48.0, 46.0)mm, (19.0, 18.0, 20.0)mm and (34.0, 20.0, 20.0)mm respectively. Petroleum ether extract showed better antibacterial activity against the tested bacterial isolates.

Key words: Antibacterial, Bacteriostatic, Clinical Isolates, Phytochemical screening.

For Correspondence:
shuaibuisa2002@gmail.com

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Introduction: Medicinal plants are a source of great economic value all over the world. Nature has bestowed on us a very botanical wealth and a large number of diverse types of plants grow in different parts of the country. It is Ayurveda, the foundation of medicinal science of Hindu culture, in its eighth division that deals with

specific properties of drugs and various aspects of science of life and art of healing (Rastogi and Mehrotra, 2002).

Neem tree (*Azadirachta indica*) belongs to the family Meliaceae. *A. indica* is called Neem (English), Margosa (South-africa), Melia azaderach (Latin name), Dogonyaro, Darbejiya or Maina (Hausa), Agyena (Glavda). It is an evergreen tree that can grow up to 20m tall. *A. indica* is rarely leafless and is usually in full foliage even during months of prolonged drought. The fruits are smooth, ellipsoidal drupes, 1-2cm long, and, when matured, they become yellowish. The seeds are composed of shell and a kernel, with the kernel having high oil content. Neem will begin bearing fruits after 3-5 years; (Tewari, 1992; Chandra, 1997; Ketkar and Ketkar, 1995, 1997; Gunasena and Maramba, 1998).

There are two acknowledged varieties of neem: *A. indica* (Indian neem) and *A. indica* var. siamansis (Thai neem) (Lauridsen *et al.*, 1991). *A. indica* var. siamensis has a wide natural distribution in Thailand at altitude below 200m (Willan *et al.*, 1990). Thai peninsula neem was reported to have hybridized with Indian neem to give a progeny of intermediate characteristics. *A. indica* var. siamensis has leaves which are larger, less bitter and darker in color than the Indian neem, and is also reported to grow faster, typically with a single straight stem (Read, 1993).

Azadirachta indica is a plant native to Asia which was naturalized in West Africa for the traditional treatment of malaria and other associated diseases, in form of decoctions, in which unspecific quantities are usually consumed without due regards to toxicological and other adverse effects (Bokhari and Aslam, 1985).

Materials and Method: Tests for presence of phytochemicals (alkaloids, resins, flavonoids, saponin, carbohydrates, proteins, steroids and lipids were carried out as described by Sofowora (1978).

Preparation of Sensitivity Discs: Discs of 6mm in diameter were punched out of Whatman's No.1 filter paper with the aid of a paper punch and a hundred discs were placed in Bijou bottles. The discs were then sterilized by autoclaving at 121^0C for 15 minutes, after which they were allowed to cool.

Two different 10mg each of neem leaf extracts were dissolved in 1ml of Dimethylsulfoxide (DMSO) to form two stock solutions of each extracts. From a stock solution of each extracts; 0.1ml, 0.2ml and 0.5ml aliquots were taken to prepare three (3) different concentrations for each as 1000µg/ml, 2000µg/ml, 5000µg/ml respectively, with the addition of the other stock solution which is 10000µg/ml. This was then followed by introducing one hundred (100) sterile discs into each concentration. In the 1000µg/ml concentration, since 100 discs are put to absorb the whole solution, each disc will then have a disc potency of 1000µg/100=10µg/disc. For 2000µg/100=20µg/disc, 5000µg/100=50µg/disc and 10000µg/100=100µg/disc, and each disc is capable of absorbing 0.001ml of the prepared solution. The discs were allowed to absorb the solution and were kept in refrigerator at 4^0C for further analysis (Kirby Bauer, 1966).

Collection of Test Organisms: The bacterial isolates of *Shigella spp, Escherichia coli, Staphylococus aureus* and *Salmonella species* were collected from Federal Medical Centre, Gombe State. Biochemical tests like catalase, coagulase, urease, citrate utilization test, motility, indole production, etc as described by Cheesbrough (2000) were carried out in the Microbiology laboratory, Gombe State University, Gombe State, Nigeria to confirm their identities.

Preparation of Media: Mueller Hinton agar was used for susceptibility testing and was prepared in accordance with the manufacturer's instruction.

Standardization of Inoculum: An overnight culture of the bacterial isolates was prepared in nutrient broth. 0.1ml of the nutrient broth

containing the test organism was emulsified into 20ml of physiological saline, until the turbidity of the suspension of the test organisms matched with 0.5 MacFarland turbidity standards (Deeni and Hussain, 1994).

Bioassay Procedure: The bioassay was carried out to determine the antibacterial activity of the ethanolic, methanolic and petroleum ether extracts of neem leaves against bacterial isolates. The bioassay procedure was carried out using the procedure described by Cheesbrough (2000). Using a sterile wire loop, the test organisms were inoculated aseptically onto the sterile prepared Mueller Hinton agar medium. The inoculated plates were then allowed to stay for about 3-5 minutes for proper attachment of discs to the agar. Prepared discs of the four (4) concentrations (10µg/disc, 20µg/disc, 50µg/disc and 100µg/disc) for each of the solvents extracts of neem leaves were then aseptically placed on the inoculated Mueller Hinton agar medium. Within 30 minutes of discs application, the plates were incubated aerobically in an inverted position at 37^0C for 24 hours. Chloramphenicol, Gentamycin Streptomycin and Ciprofloxacin were used as positive controls on *Shigella species*, *Escherichia coli*, *Staphylococcus aureus* and *Salmonella species* respectively. After overnight incubation, the plates were observed for zones of inhibition. The zones of inhibition were measured in millimeters using a meter rule and recorded.

Results: Phytochemical analysis of the neem (*Azadirachta indica)* leaf extracts using ethanol, methanol and petroleum ether as solvents showed the presence and absence of the phytochemicals as shown in table 1 below. For ethanolic extract, alkaloids, resins, saponins, flavonoids were present whereas in methanolic and petroleum ether extracts only alkaloids, saponins and flavonoids were present.

Table 1: Phytochemical analysis of neem (*A. indica)* leaf extracts

S/No	Plant chemical constituent	Ethanol extract	Methanol extract	Petroleum ether extract
1	Alkaloid	+	+	+
2	Resin	+	-	-
3	Flavonoid	+	+	+
4	Saponin	+	+	+
5	Carbohydrate	_	_	_
6	Proteins	_	_	_
7	Steroid	_	_	_
8	Lipid	_	_	_

KEY: - = absent + = present

After carrying out the anti-bacterial activity of different neem leaf extracts (ethanolic, methanolic and petroleum ether) against test organisms, it was found out that all the bacterial isolates were resistant to the extracts as shown in the table 2 below. The control drugs used on different organisms were effective, except, streptomycin which was found to be inactive on *Staphylococcus aureus*.

Table 2: Antibacterial activity of ethanolic extract

	Concentration (µg/disc)				
	10	20	50	100	positive control
	Test organisms	Zones of inhibition (mm)			
Shigella species	0.00	0.00	0.00	0.00	CH (20.0)
E. coli	0.00	0.00	0.00	0.00	CN (18.0)
Staphylococcus aureus	0.00	0.00	0.00	7.00	S (0.00)
Salmonella species	0.00	0.00	0.00	9.00	CPX (48.0)

Key:- CH= Chloramphenicol (30µg), CN= Gentamycin (10µg), S= Streptomycin (30µg), CPX= Ciprofloxacin (10µg)

In table 3 below, the test organisms (bacterial isolates) showed partial resistance at 100µg/disc concentration, with complete resistance at other concentrations, except *Shigella species* which is completely resistant to all the concentrations.

Table 3: Antibacterial activities of methanolic extract

	Concentration (µg/disc)				
	10	20	50	100	positive control
	Test organisms	Zones of inhibition (mm)			
Shigella species	0.00	0.00	0.00	0.00	CH (20.0)
E.coli	0.00	0.00	0.00	0.00	CN (24.0)
Staphylococcus aureus	0.00	0.00	0.00	7.00	S (0.00)
Salmonella species	0.00	0.00	0.00	7.00	CPX (46.0)

In table 4 below, the bacterial isolates showed partial susceptibility at concentration of 100µg/disc with the exception of *Salmonella species* and all of them were resistant to all other concentrations (10µg/disc, 20µg/disc and 50µg/disc).

Table 4: Anti-bacterial activities of petroleum ether extract

	Concentration (µg/disc)				
	10	20	50	100	positive control
	Test organisms	Zones of inhibition (mm)			
Shigella species	0.00	0.00	0.00	13.0	CH (34.0)
E.coli	0.00	0.00	0.00	8.00	CN (19.0)
Staphylococcus aureus	0.00	0.00	0.00	9.00	S (0.00)
Salmonella species	0.00	0.00	0.00	9.00	CPX (40.0)

Table 5: Antibacterial activity of the leaves extract of *A. indica* leaves using Macro broth dilution technique

S/N	Bacteria	Concentrations (µg/ml)						
		MIC				MBC		
		100	50	25	12.5	100	50	25
1.	*Shigella spp.*	-	-	-	+	***	**	**
2.	*E. coli*	-	-	-	+	***	**	**
3.	*S. aureus*	-	-	-	+	***	**	**
4.	*Salmonella spp.*	-	-	-	+	***	**	**

Key: MIC=Minimum Inhibitory Concentration, MBS=Minimum Bactericidal Concentration
=Growth Observed, *= MBC above 100µg/ml,
- = Not turbid, + = Turbid

Discussion: The result obtained in table 1; showed the presence of alkaloids, flavonoids and saponins in all of the leaf extracts which corresponds with the work of Timothy *et al*., (2008). Resins were present in ethanolic extract; this explains the favorable best solvent to be used among of them (Imran *et al* 2011). According to the research carried out by Imran, *et al*., 2011; the presence of these phytochemicals identified was not realized. This may be due to the fact that, ecological or geographical differences may bring about a difference in the presence of such phytochemicals of neem trees isolated. Secondly, neem leaf collected may also vary qualitatively because of different climatic conditions in the study areas, thereby bringing variations in the chemical constituents of the neem leaves.

The presence of these phytochemicals observed, is the antimicrobial property of the neem leaf extracts. In table 2; 100µg/disc showed partial activity on the test organisms, with zones of inhibitions 7.00mm and 9.00mm on *Staphylococcus aureus* and *Salmonella species* respectively. This finding corresponds with the work of Timothy *et al*., (2008), Imran *et al*., (2011), Patel and Trivedi (1962) and Schneider (1986). Streptomycin was used as a control and its zone of inhibition was 48.0mm. While using methanolic extract 7.00mm was found to be a partial inhibition for both strains of bacteria on the concentration of 100µg/disc. 46.0mm zone of inhibition when ciprofloxacin was used as a control and there was no activity when streptomycin was used on *Salmonella typhi.*

In table 4, using petroleum ether solvent extract, 100µg/disc showed a partial inhibition on three test organisms with zones of inhibitions 13.0mm, 8.00mm and 9.00mm on *Shigella species*, *E. coli* and *Staphylococcus aureus* respectively which indicates resistance. Gentamycin was used as control drug on *E. coli* with a zone of inhibition 19.0mm. *Staphylococcus aureus* was found to be resistant to streptomycin and *Salmonella species* were inactivated with the use of Ciprofloxacin as a control drug with the zone of inhibition of 40.0mm. This correlates with the work of Thaker and Aryans, 1986; Timothy *et al*., 2008; Wendy, *et al*., 2010; Valarmathy *et al.,* 2010.

Minimum Inhibitory concentration (MIC) of the extract was determined at 25µg/ml for all the test organisms. However, the test organisms were able to grow when all tubes which showed no evidence of growth at MIC were sub-cultured on solid media for Minimum Bactericidal Concentration (MBC). This indicates that the extracts were only bacteriostatic at the concentrations (Table 5).

Conclusion: In this research, it can be concluded that *A. indica* (neem) leaf extracts using ethanol, methanol, and petroleum ether as solvents contains flavonoids, saponins, alkaloids in all the extracts with the addition of resin which was only found in ethanolic extracts. These phytochemicals are responsible for the antibacterial activities on *Shigella species, E.*

coli, Staphylococcus aureus and *Salmonella species*. Although the organisms showed resistance through partial inhibition, their purified forms of the active principles may indicate highly active inhibition.

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