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Determination of phytoconstituents and antimicrobial activities of aqueous and methanol extracts of neem (azadirachta indica) leaves

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Neem (Azadirachta Indica)is a plant that has been used as traditional medicine for a household remedy against various human ailments since ancient times. The objective of this study was to determine the phytoconstituents and antimicrobial activities of aqueous and methanol extracts of A. indica leaves. Qualitative and quantitative phytochemical analysis was carried out using the standard method of Association of Official Analytical Chemist and the antimicrobial activity of the concentrated extracts was evaluated by determination of the diameter of zone of inhibition against the microorganisms using the agar well diffusion method. Phytochemical screening of the leaves gave a positive result for saponins, tannins, steroids, glycoside, terpenoids, flavonoids, and alkaloids. The antimicrobial results show that both plant extracts had antimicrobial activity against the test organisms, the aqueous extracts were found to show the greater antimicrobial effect on Shigellasp (10.0±1.0 mm), Staphylococcus sp (10.0±1.0 mm), Penicilliumsp (9.2±0.8 mm) and Mould (10.2±1.3 mm) while methanol extract shows maximum efficacy on E. coli (26±1.0 mm), Vibrio sp (10.6±0.6 mm) and Yeast (9.2±0.3 mm). Therefore, these findings support the traditional knowledge of local users and it is a preliminary, scientific, validation for the use of neem leaves for the antimicrobial activity to promote proper conservation and sustainable use of the plant resources.

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Introduction

There have been cases of microbial resistance to antibiotics, mostly, in some developing countries and better understanding of medicinal potentials of plants could give great scientific and medical insights towards strategic and effective approach to combat drug resistant microbes. Plants are the richest resource of drugs of tradi-

tional systems of medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs [1]. The use of plants and plant products as medicines could be traced as far back as the beginning of human civilization. Medicinal plants are a source of great economic value all over the world. Nature has bestowed on us a very rich botanical wealth and a large number of diverse types of plants grown in different parts of the country. The ability of some plants to be used for medicinal purposes has been linked to the exis-

tence of certain chemically active substances in various parts of these plants and their extracts. These active substances are known as phytochemicals and are capable of producing specific physiological action on target organisms [2, 3]. Plants produce an extensive range of phytochemical components which are secondary metabolites [4]. These secondary metabolites work uniquely and are used directly or indirectly in the pharmaceutical industry. Phytochemicals have the ability to act as antioxidants by preventing cell damages which is usually caused by free radicals such as those associated with heart disease and cancer. Phytochemicals are also known to possess the ability to interfere with enzyme action. For instance, indoles stimulate enzymes that make estrogen less potent, and thus could lessen the risk of cancer of the breast [5]. Phytochemicals are known to possess the capability to destroy or impede the growth of bacteria. For instance, allicin is one of the phytochemicals known to possess anti-bacterial properties. Phytochemicals help in the prevention of the adhesion of pathogens to cell walls. For example, proanthocyanidins have anti-adhesion tendencies which help reduce the risk of urinary tract infections and enhance dental health. The varied phytochemical constituents and unique functioning of each phytochemical, if properly investigated and harnessed could lead to discovery of novel substances and active compounds that are effective against seemingly drug resistant microorganisms while simultaneously alleviating several of the side effects that usually accompany synthetic antimicrobials [6]. Neem (Azadirachtaindica), the versatile medicinal plant is the source of several compounds having diverse chemical structure and biological effects [7]. Some active phytoconstituents are present in neem such as steroids, glycosides, alkaloids and tannins [8]. Eczema, ringworm, acne, antihyperglycemic properties, anti inflammationare treated effectively by neem leaves. It helps to purify our blood and neutralize the free radicals. Neem leaves act as an anticancer agent [7]. Neem seed have been shown to exhibit wide pharmacological activities including; antioxidant, antimalarial, antimutagenic, anti carcinogenic, anti- inflammatory [9]. The biological activities are attributed to the presence of many bioactive compounds in different parts of the plant. Neem has a wide range of therapeutic properties such as antifungal, antiviral, anti inflammatory, antibacterial, analgesic and antioxidant [10]. Every part of the neem tree is bitter and finds the application indigenous [11].

The aim of this study is to determine the phytoconstituents and antimicrobial activities of aqueous and methanol extracts of neem (*Azadirachtaindica*) leaves.

Methodology

Collection of Neem leaf Samples

Neem leaves were obtained from the market in Yenagoa. It was sun-dried for three days and were pulverized and stored in airtight container for laboratory analysis.

Qualitative Phytochemical Screening

Phytochemical screening of the neem leave samples was carried out by a procedure that was based on those earlier reports by Odangowei*et al.* [6].

Test for saponins

The plant sample (0.5 g) was added to 5 ml of distilled water in a test tube. The solution was vortexed and observed for a stable persistent froth. The frothing was mixed with 3 drops of olive oil, vortexed and formulation of an emulsion was observed.

Test for terpenoids

Plant sample of 0.5 g was dissolved in 1 ml of chloroform and 1 ml of acetic anhydride added, with 2 ml of concentrated H₂SO₄. Formation of reddish violet colour was observed.

Test for tannins

The pulverized neem leaves (0.5 g) was boiled in 10 ml of water in a test tube and filtered. A few drops of 0.1 % ferric chloride were added and the solution observed for brownish green or a blue-black colouration.

Test for cardiac glycosides (keller-killiani test)

Neem leafsample of 0.5 g, dissolved in water (5 ml) was added 2 ml of glacial acetic acid solution containing one drop of ferric chloride solution. This was underlayed with 1ml of concentrated H₂SO₄. A brown ring at the interface indicated the presence of deoxysugar characteristics of cardenolides. A violet ring may appear below the brown ring while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

Test for flavonoids

Five (5) ml of dilute ammonia was added to a portion of an aqueous filtrate of the sample. Then, 1 ml concentrated sulphuric acid was added. A yellow colouration indicated the presence of flavonoids.

Test for alkaloids

The plant sample was dissolved in dilute HCl and filtered. Filtrates were treated with Mayer's reagent (potassium mercuric iodide). Formation of a yellow coloured precipitate indicates the presence of alkaloids.

Test for steroids

Acetic anhydride (2 ml) was added to 0.5 g of the sample and filtered. Sulphuric acid (2 ml) was added to the filtrate and observed for color change from violet to blue or green, which indicates the presence of steroid.

Quantitative Phytochemical Analysis

Total Tannin Content Determination

Total tannins were determined by slightly modified Folin and Ciocalteu method. 0.5 ml of the sample was added with 3.8 ml of distilled water and 0.25 ml of Folin Phenol reagent, 0.5 ml of 35% sodium carbonate solution was added. The absorbance was measured at 725 nm. Tannic acid dilutions (0 to 0.5 mg/ml) were used as standard solutions. The result of tannins are expressed in terms of tannic acid in mg/ml of the sample.

Total Alkaloid Content Determination

Forty (40) ml of 10% acetic acid in ethanol was added to 1 g of the powdered sample, covered and allowed to stand for 4 hours. The filtrate was then concentrated on a water bath to get 1/4th of its original volume. Concentrated ammonium hydroxide was added drop wise to the sample until the precipitation was complete. The whole solution was allowed to settle and collected precipitate was washed with dilute ammonium hydroxide and then filtered. The residue was dried and weighed.

Total Flavonoid Content Determination

Total flavonoid content of the sample was determined by following the Aluminum chloride method. The plant concentrate was mixed with distilled H₂O and NaNO₂ solution. After 6 min, AlCl₃ solution was added and enabled to stand for 6 min, NaOH solution was added to the mixture. Immediately distilled H₂O was added to bring to the final volume and then the mixture was extensively mixed and enabled to stand for another 15 min. Optical density of the mixture was recorded at 510 nm. Rutin was used as a standard compound for the evaluation of total flavonoid. The total flavonoids were calculated using the standard curve, and expressed as rutin equivalent in mg/g of the sample.

Total Saponin Content Determination

The pulverized sample were dissolved in 80 % methanol, 2 ml of Vanilin in ethanol was added, mixed well and the 2 ml of 72 % sulphuric acid solution was added, mixed well and heated on a water bath at 60°C for 10 min, absorbance was measured at 544 nm against reagent blank. Diosgenin is used as a standard material and the assay compared with Diosgenin equivalents.

were inverted, and placed in a 37°C incubator for 24 hours.

Total Terpenoid Content Determination

Neem leaves (1 g) was marcarated with 50 ml of ethanol and filtered. To the filtrate (2.5 ml), 2.5 ml of 5 % aqueous phosphomolybdic acid solution was added and 2.5 ml of concentrated H₂SO₄was gradually added and mixed. The mixture was left to stand for 30 min and then made up to 12.5 ml with ethanol. The absorbance was taken at 700 nm.

Total Steroid Content Determination

The steroid content of the plant sample was determined using the method described by Trease and Evans [12]. A portion of 2 ml was taken from a solution of 2.5 g of powdered plant material prepared in 50 ml of distilled water after vigorous shaking for 1 hour. The extract solution was washed with 3 ml of 0.1M NaOH (pH 9) and later mixed with 2 ml of chloroform and 3 ml of ice cold acetic anhydride followed by the cautious addition of two drops of concentrated H₂SO₄. The absorbance of both sample and blank were measured using a spectrophotometer at 420 nm.

Plant Sample Extraction

The extraction reagents were methanol and aqueous. About 10 g of the neem leaf sample was placed in a beaker and 25ml of methanol added and mixed by vortexing. It was centrifuged at 3000 rmp for 10 minutes. The supernatant was collected and transferred to a stoppered test tube by filtration. The resulting supernatant was evaporated to dryness with a gentle stream of nitrogen and reconstituted in 10 ml dimethyl sulphoxide and was mixed by vortexing. The same procedure was repeated for that of aqueous.

Preparation of Dried Filter Paper Discs

Whatman filter paper no. 102 was used to prepare discs. Approximately 5mm in diameter was perforated using a perforator. These were placed in a petri dish after sterilization in autoclave.

Neem leaves Extract Disc Placement

Plant disc containing 3 ml (3 μ l) concentration, as well as neem leaves were made using filter paper and then placed on the plates using sterile forcep. One sterile antibiotic disc was placed on the surface of an agar plate using a forcep. The forcep was sterilized by immersing in alcohol each time before placing another antibiotic disc. The disc was then gently pressed with the forcep to ensure complete contact with the agar surface and placed away from the edge of the plates so that it is easily measured. Once all discs were in place, the plates

Bacteria/ fungi suspension preparation

Media used: Nutrient agar, buffered peptone water, *shigella* agar, macconky agar and cetrimide agar. These media were prepared according to manufacturer's instruction. Using a sterile inoculating loop and needle for bacteria and fungi respectively, through aseptic techniques the test organisms of each colony was taken from the subculture plate. The organism was suspended in 4 ml of normal saline and vortexed for overall suspension. Mcfarland standard solution was used as a reference to adjust the turbidity of individual bacterium isolate in the suspension (1× 10⁸). And 10 fold serial dilutions was made and plated for the antimicrobial sensitivity test.

Inoculation of Isolates on the Nutrient Agar Plate Proper

A sterile swab stick was dipped into the bacterial/ fungi suspension and the test organisms were suspended in 4 ml of buffered peptone water. The swab was rotated against the side of the tube using firm pressure to remove excess fluid, but the swab was not dipped wet. The dried surface of the nutrient agar plate was inoculated by streaking the swab over the entire agar surface by rotating the plate at 60 degrees each time to ensure an even distribution of the inoculum.

Results

Phytoconstituents

Qualitative phytochemicals present are: tannin, terpenoid, saponin, flavonoid, alkaloid, steroid and glycoside. Quantitatively, tannin content was $(6.7 \pm 0.01\%)$, terpenoid (5.5 ± 0.01) , saponin $(3.2 \pm 0.01\%)$, alkaloids $(10.9 \pm 0.02\%)$, flavonoid $(3.4 \pm 0.01\%)$ and steroids (0.48 ± 0.02) (Tables 1 and 2).

Table 01: Qualitative Phytochemical Result of Neemleaf

Sample code	P h		y	t	o c		h e		m	i	С	a	1 s		
	Alkaloid		Tan	nin	Flavonoid		Saponin		Glycosides		Terpenoid		Stero	oid	
Neem leaf	+	+	+	+	+		+		+	+	+	+	+		

^{+ (}presence), ++ (abundance)

Table 02: Quantitative Phytochemical Results of neem leaf

Sample Code	P	h	y	t	0	c	h	e	m	i	c	a	1	s	%
Sample Code	A 1 1	k a l o i	d T	Гапı	nin	Flav	vonoi	d	Sapor	nin	Terp	enoi	d S	tero	i d
Neem leaf	10.	9 ± 0 .	2 6	5.7 ± 0	. 0 1	3 . 4	± 0 . 0	1 3	3.2 ± 0	. 0 1	5.5±	± 0 . 0	1 0	48 ± 0	. 0 2

Antimicrobial activity

Antibacterial activity of Gram-positive bacteria shows the diameter of inhibition zone of *Staphylococcus sp* (10.0 \pm 1.0mm, and 7.0 \pm 0.3mm) and gram-negative bacteria, such as *E. coli, Shigella sp., and Vibrio sp.,* shows the diameter of inhibition zone of 9.3 \pm 0.6 mm, 10.0 \pm 1.0 mm 7.0 \pm 1.0 mm and 26 \pm 1.0 mm, 7.0 \pm 0.2 mm, 10.6 \pm 0.6 mm for aqueous and methanol respectively. While antifungal sensitivity shows *Penicilliumsp*, Yeast and Moulds with diameter of inhibition zone for aqueous extract 9.2 \pm 0.8 mm, 7.1 \pm 0.3 mm, and 10.2 \pm 1.3 mm and methanol extract 8.5 \pm 0.3 mm, 9.2 \pm 0.3 mm, and 8.2 \pm 0.2 mm respectively (Tables 4 and 6).

Table 03: Biochemical characteristics of some bacteria

Microorganisms	SI	higel	lasp	E. coli			Staphylococcus sp.			Vibrio sp		
Cell morphology (cell shape)	R	О	d	R	О	d	Со	c c u	s C	o m m a		
Colony (cell shape)	R	o u	n d	S p	i n d	l e	Cir	c u l a	. C	u r v e d		
Gram reaction	-	v	e	-	\mathbf{v}	e	+	V	e -	v e		
Nitrate reductive	+	v	e	+	v	e	+	V	+	v e		
Oxidase	-	v	e	-	v	e	-	v	+	v e		
Catalase	+	v	e	+	v	e	+	V	9 -	v e		
Methyl red	+	v	e	+	v	e	+	V	9 -	v e		
Biochemical test $\ \ V$. $\ \ P$.	-	v	e	-	v	e	+	V	+	v e		
Indole	-	v	e	+	v	e	-	V	+	v e		
Citrate	-	v	e	-	v	e	+	V	+	v e		
H ₂ S reduction	-	v	e	-	\mathbf{v}	e	-	v		v e		
Ureas activity	-	V	e	-	V	e	+	v	<u> </u>	v e		

+ve = Positive; -ve = Negative; VP = VogesProskauer; H₂S = Hydrogen sulfide

Table 04: Antibacterial activity of Neem leaf extracts

Name	of organism	Methanol extract	Aqueous extract
S h i	gellas p	7 . 0 ± 0 . 2	1 0 . 0 ± 1 . 0
Ε .	c o l i	2 6 ± 1 . 0	9 . 3 ± 0 . 6
Staph	ylococcus sp	7 . 0 ± 0 . 3	$1 0 . 0 \pm 1 . 0$
V i b	r i o s p	$1 0 . 6 \pm 0 . 6$	7 . 0 ± 1 . 0

(Diameter of inhibition zone in mm) (Means ± SD)

Table 5. Identification of fungi with cultural morphology

О	r	g	a	n	i	i	s	m	s	Microscopic observation (Medium)	Microscopic observation (gram reaction)
Υ	е		а	s		t		S	р	White colour, creamy growth on the media surface	Pink colour large cells obtained by gram's staining, oval, budding cells obtained by LPCB staining.
P	e 1	ı i	С	i	l	l	i	и	m	Greyish-green colour colonies, smooth colonies.	Brush like conidiophores and branched mycelium spores arranged on conidiophores
M		0		и			l		d	Black huge colonial growth	Heavy mycelial growth arranged in filamentous form.

Table 06: Antifungal activity of Neem leaf extracts

N a	m e o	f or	ganis	m	M	e t h	a n	o 1	e x t	rac	t s	A	q u	e o ı	ı s	e x t	rac	t s
P e	n i c	i l l	i u m s	s p	8		5	±	0		3	9		2	±	0		8
Y	e	а	S	t	9		2	±	0		3	7		1	±	0		3
M	0	и	l	d	8		2	±	0		1	1	0		2	± 1	•	3

(Diameter of inhibition zone in mm) (Means ± SD)

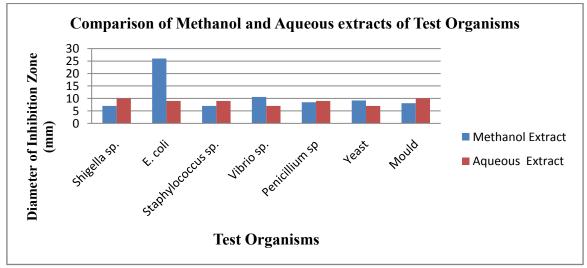


Fig 01: Comparison of Methanol and Aqueous extracts of Test Organisms

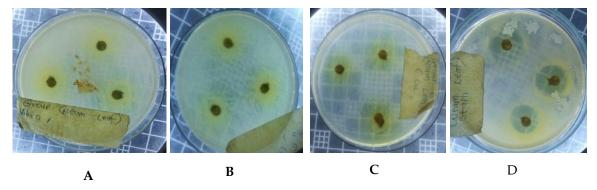


Fig 02: Antibacterial Activity of Neem leaf sample
(A) Vibrio sp (B) Shigellasp (C) E. coli (D) Staphylococcus s

Discussion

Plants have been used for many thousands of years, in food preservation, pharmaceuticals, alternative medicine and natural therapies. It is necessary to investigate those plants scientifically which have been used in traditional medicine to improve the quality of healthcare. Plant extracts are potential sources of novel antimicrobial compounds especially against bacterial pathogens. The beneficial medicinal effects of plant materials typically result from the secondary products present in the plant although, it is usually not attributed to a single compound but a combination of the metabolites. Qualitative Phytochemical screening of the leaves of A. indicain the present study revealed the presence of alkaloid, tannin, flavonoid, saponin, glycosides, terpenoids and steroid as shown in Table 01. The quantitative phytochemical of Neem leaves confirms the presence of the alkaloids with 10.9±0.2%, Tannin 6.7±0.01%, Flavonoid 3.4±0.01%, Saponin 3.2±0.01%, Terpenoid 5.5±0.01% and Steroid 0.48±0.02% (Table 02). The phytochemical results were in agreements with the findings of Aziz et al. [13], Bishnuet al. [14] and Alkaet al. [15]. These compounds were reported to be an indication of the potential of antimicrobial agents [16]. These active constituents present may justify the use of neem leaves to offer antisecretory effects and control gastroduodenal ulcers [17, 18]. The bioactivity of medicinal plants has been linked to the presence of these phytochemicals [19].

Intensive use of antibiotics often resulted in the development of resistant strains [20], these create a problem in treatment of infectious diseases, furthermore antibiotics sometimes associated with side effects [21] whereas there are some advantages of using antimicrobial compounds of medicinal plants such as often fewer side effects, better patient tolerance, relatively less expensive, acceptance due to long history of use and being renewable in nature [22]. Because of this, the search for new antibiotics continues unabated.

The present study of *Azadirachtaindica* shows the antibacterial activity in methanol and aqueous extracts. The zone of inhibition was observed against *E. coli, Staphylococcus aureus, Shigellasp Pibrio sp.* Methanol extract was having the maximum efficacy in *E. coli,* and *Vibrio sp* while aqueous extract had maximum efficacy in *Shigellasp* and *Staphylococcus aureus* as shown in Table 4 and Figures 01 and 02. These results are in agreements with the works of Alkaet al.[15] and Aziz et al. [13]. In antifungal activity shows that, the maximum zone of inhibition was observed in aqueous extract in *Penicilliumsp* and mould while yeast hadmaximum efficacy in metha-

nol extract (9.2±0.3mm) as shown in Table 6.This result is in accord with the reports of Indhumathi*et al.* [23] and Damilola*et al.* [24]. These findings support the traditional knowledge of local users and it is a preliminary, scientific, validation for the use of thisplant for antimicrobial activity to promote proper conservation and sustainable use of the plant resources. Awareness of local community should be enhanced incorporating the traditional knowledge with scientific findings.

Summary & Conclusion

In the present study, phytochemical screening of the leaves of A. indicarevealed the presence of alkaloid, tannin, flavonoid, saponin, glycosides, terpenoids and steroid. This study also shows that the plant extracts inhibited fungal and bacterial growth but their effectiveness varied. The antimicrobial activity of the plant can be attributed to the presence of the active phytochemical constituents present. This medicinal plant has being used traditionally for the treatment of inflammation, wound healing, carminative, cough, toothache, antiseptics expectorant, stomatitis and some fungal infection like candidiasis. In conclusion, these bioactive constituents and antimicrobial efficacy of Neem leaf extracts demonstrated that folk medicine can be as effective as modern medicine to combat pathogenic microorganisms. The millenarian use of this plant in folk medicine suggests that they represent an economic and safe alternative to treat infectious diseases.

Author Contribution

All authors Contributed Equally.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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