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RESEARCH ARTICLE

Antimicrobial Activity of Ethanolic and Aqueous Extracts of Fresh and Dried Leaves of Azadirachta indica (Neem's leaf)

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Abstract

The leaves of *Azadirachta indica* (Neem), commonly called Dogonyaro are used in parts of Nigeria right before now as antimicrobial medicine. This study was designed at investigating the antimicrobial activities of the crude ethanolic and aqueous extracts of both the fresh and dried Neem's leaf on strains of bacteria viz; *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa and Proteus vulgaris* with one of the opportunistic fungi which is *Candida albican's*, using agar well diffusion methods. The qualitative and quantitative phytochemical screening of the sample were done using the standard methods and showed a variety of phytoconstituents. The fresh Neem's leaf of aqueous and ethanolic gave zones of inhibition diameter (ZIDs), ranging from 10 – 20mm and 9 – 18mm each, while the aqueous extrac and ethanolic of from the dried neem leaves gave zone of inhibition diameter of 9 – 13mm and 9 – 14mm respectively. From the results, the organisms showed a statistically significant difference ($p<0.0001$) in ZIDs according to the concentration levels of the extract. The ethanolic extracts of fresh neems leaf when compared with the dried extracts gave a statistically significant difference ($p<0.05$) in most of the tested organisms. But when compared with the standard antibiotics discs, there was no statistically significant difference ($p>0.05$) proving that it could b used in place of the synthetic antibiotics to inhibit (Bacteriostatic) the organisms. It is recommended that the active biological ingredients in the Neem should be extracted and purified with more sophisticated techniques and used in combination with other drugs in treatment of microbial infections to avoid much resistance.

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Keywords: Antimicrobial Activity, Azadirachta indica (Neem's leaf), *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*, *Proteus vulgaris*, *Candida albican.*

1. Introduction

Since ancient time, people have been utilising herbal medicine, which has resulted in exploitation of large number of medicinal plants with effective properties to eradicating various ailments. The importance of medicinal plants becomes more patent now in developing countries. In India, it is estimated that 80% of population depends on plants for therapy and about 60% populace use medicinal plants habitually to battle certain ailments and almost 40% human use such plants in pharmaceutical industries. The World Health Organization in 2010 has outlined herbal medicine as the highest medicinal products that incorporate lively ingredients as aerial or underground accessories of plants or other plant fabric.[1] Neem's plant (*Azadirachta indica*) is one of them; others include Onion (*Alium cepa*), Ginger (*Zingiber officinale*), Guava (*Psidium guajava*), Lemon Grass, Aloe vera and Garlic.

The neems leaves, flowers, stems, root, seeds, fruit and bark are part of these herbal remedies (Figure 1). Just about each part of the tree is bitter and contains compounds with verified antiviral, anti-inflammatory, antiulcer and anti-fungal, anti-plasmodia, antiseptic, antipyretic, antibacterial and anti-diabetic activities. It has been described that aqueous crude extract of neem leaves showed significant hypoglycemic, hypolipemic, hepatoprotective, and hypertensive activities.[2] Moreover, protective effect on diabetic nephropathy in rats of neems extract on renal damage induced by malaria infection has not yet been reported.[3] However, the protective the chemical components incorporate many biologically energetic compounds that could be extracted from neems like alkaloids, flavonoids, terpenoids, phenolic compounds, carotenoids, steroids, tannins and ketones. *Azadirachtin* is authentically an amalgamation of seven isomeric compounds labelled as *azadirachtin* A-G and *azadirachtin* E is more efficacious.

Figure 1. *Neem's Plant and its Leaves*

The *Azadirachta indica* leaves are greatly used among the quite a lot of tribes of India to remedy cuts, wounds and different minor dermis illnesses.[4]

Since it is now clear that modern pharmaceuticals cannot treat every condition effectively and also for the fact that some synthetic drugs have toxic side effects. Hence there is need that scientist takes scientific approach in investigating the effectiveness of these herbal medicines or in isolation active ingredients and utilising it in development of safe drugs with standardised dosages. This is essential as studies have shown that herbal medicine is of minimal toxicity than the synthetic drugs.[5] It is also relatively accessible, available and cheap also.

The plant neem is in the mahogony family Meliacea. The scientific name of is *Azadirachta indica while* the English name is Neem.It grows mostly in tropical and sub-tropical regions. It is a fast growing tree that can reach a height of 15-20 metres. It is evergreen, but in severe drought it may shed most or nearly all its leaves. The branches are wide and spreading.[6] Neem is also known as 'arista' in Sanskrit-A word that suggests 'ultimate, entire and imperishable. In Nigeria, it is extensively and largely spread over the areas and referred to as Dogon Yaro (Hausa name), Iba shorop (Igbo).

Other Vernacular names: According to Parotta[7], Bengali: Nim, Nimgachh, Guajarati: Danujhada, Limbado, Limbra, Limdo, Hindi: Nim, Nimb, Sanskrit: Arista, Nimba, Nimbah, Picumarda, English: Indian Lilac, Margosa tree, Neem tree, Kannada: Bemu, Bevinamara, Bivu, Kaybevu, Punjabi: Bakam, Drekh, Nim. Neem is used for several biological and medicinal activities. Different parts of neem (leaf, bark and seed) have shown to fight against several diseases. *Azadirachta indica* is one among the medicinal plants and is used in ayurveda, unani and homeopathic medicine. Neem twigs are used

as toothbrushes in some tropics, but this can cause illness, neem twigs are often contaminated with fungi within two weeks of their harvest and should be avoided.[8] Neem's antiseptic properties are widely recognized now. "Neem preparations are reportedly efficacious against a variety of skin diseases, septic sores, and infected burns. The aim of this study is to investigate the Antimicrobial activities of both fresh, dried ethanolic and aqueous extract of *Azadirachta indica* leaves.

2. Materials and Methodology

2.1 Sample Collection (Neem leaves)

The work involves testing the Antimicrobial activities of both dried (under normal room temperature) and fresh (none dried) leaves of *Azadirachta indica,* using Ethanol, Aqueous as extraction solvents. Agar well diffusion method was adopted for this practical. The laboratory work was carried out in Emmanuel Research laboratory Centre, Enugu. *Azadirachta indica* leaves were collected from the fields of Botanical Garden in University of Nigeria, Enugu campus. The plant leaves collected were healthy and free from any ill features. This plant leaf was taken to the department of Plant science and biotechnology, University of Nigeria Nsukka for botanical confirmation.

2.2 Processing of the Sample

The collected Neem's plant leaves were dried under room temperature and not with direct sun. Both the dried and fresh leaves were blended with mixture blender and taken to the laboratory for further preparation. The powder is then passed from the sieve to get the equal size particles. The powder was aseptically kept in an air tight container at the moisture free place.

2.3 Preparation of the Aqueous and Ethanol Extracts

50grams of the leaf powder was weighed out and dissolved in 500ml of sterile distilled water. 50grams of the blended leaf was also dissolved in 500ml of ethanol of analar quality. The solutions were stirred at intervals for 24 hours and were sieved through whatman filter paper.

The ethanolic extract was poured on a clean round flat plates to get evaporated. The crude extract was then scrapped out and put in a sterile bottle and stored in fridge. The aqueous extract was poured into different sterile beaker. The beakers were then transferred into a regulated Water bath for the aqueous to be heated off. The crude aqueous extract was then scraped out, put in sterile bottle and stored in fridge for use.

2.4 Phytochemical Investigations

Phytochemical screening of *Azadirachta indica* leaf extract was done following the standard procedure by different inventors. Both of the prepared aqueous and ethanolic extracts were subjected to both qualitative and quantitative phytochemical screening for the presence of alkaloids, tannin flavanoids, saponins, phenolic compounds, glycosides, terpenoid and athraquinone.

2.5 Qualitative Analysis

2.5.1 Test for Alkaloids

Wagner's test

To the 5mL i.e both (aqueous and ethanol extract) of test solution, 5mL of Wagner's reagent (iodine in potassium iodide) was added. Reddish-brown coloured precipitate indicated the presence of alkaloids.[9]

Tannins Test

5 mL of freshly prepared potassium hydroxide (KOH) was added into a test tube; 1ml of the extract was added to same tube and shaken to dissolve. A dirty precipitate observed indicates the presence of tannin.[10]

2.5.2 Test for Flavonoids

Alkaline Reagent Test

About 2 ml test solution was treated with few drops of sodium hydroxide solution and observed for intense yellow coloration which disappeared on the addition of dilute Hydrogen chloride solution.[11]

2.5.3 Test for Terpenoids

Salkowski's Test

1 mL of each the extract was added to 4 mL of chloroform. 6 mL of concentrated Sulphuric acid (H_2SO_4) was carefully added to form a layer. A reddish brown coloration of the interface indicated the presence of terpenoids.[10]

2.5.4 Test for Saponins

1mL of the plant extract was shaken with water in a test tube. Frothing, which persist on warming was taking as a preliminary evidence for the presence of saponins. Few drops of olive oil was added to 1ml of the extract and vigorously shaken. Formation of soluble emulsion in the extract indicated the presence of Saponin.[10]

2.5.5 Glycosides

Keller-Kilian's Test

To 5 ml of test solution, one drop of ferric chloride solution 'was added. This was then under layer with 0.5 mL of concentrated Sulphuric acid. A brown ring

obtained at the interface indicated the presence of Cardiac glycosides.[12]

2.5.6 Test for Phenols

Ferric chloride Test

To 5 mL of alcoholic solution of extract, 1 mL of distilled water followed by few drops of 10% aqueous Ferric chloride ($FeCl_3$) solution was added. Formation of blue colour indicates the presence of phenols (Chandrashekar and Rao, 2013).

2.5.7 Isolation of organisms

The Clinical organisms, 2*Staplylococcus aureus (Nasal swab), 2Pseudomonas aeruginosa (Wound swab), 2Escherichia coli (Urine), 2Proteus vulgaris (Urine) and Candida albicans (HVS)* were isolated from clinical samples submitted to bacteriology laboratory in University of Nigeria Teaching Hospital, Ituku-Ozalla - Enugu, National Orthopaedic Hospital, Enugu and ESUT Teaching hospital Parklane, Enugu. In total, 9 isolates were used for this project study. They were identified by their Gram reactions, cultural characteristics and biochemical activities using standard Microbiological methods listed below (Chakraborty and Nishith 2008).

2.5.8 Isolation of Staphylococcus aureus

Culture

Colonies were streaked onto Mannitol salt agar and incubated at 37^0c for 24hours. The presence of cream colonies and a change in colour of the media from red to yellow was observed.

Gram Staining

Onto a clean grease free dried slide, a drop of normal saline was placed and the colonies were picked with a sterile nichrome wire loop and emulsified in it. The smear was allowed to air dried and heat fixed gently. It was then flooded with crystal violet and allowed to stain for 1mins before washing it off with distilled water. Lugol's iodine was placed for another 1mins and washed off again; Acetone was added to decolourise the smear and washed out immediately. Neutral red placed on it for 2mins and also washed off. The stained slide was allowed to air dried and examined under the microscope using oil immersion objective. Purple gram positive cocci in clusters were seen which indicate the organism *"Staphylococcus aureus"*.

2.5.9 Isolation of Escherichia coli

Culture

Colonies were streaked onto a Cystein Lactose Electrolyte Deficient (CLED) media and incubated overnight at 37^0c. The presence of opaque pinkish/ yellowish medium size colonies indicating lactose fermenters was seen.

Gram Staining

Onto a clean grease free dried slide, a drop of normal saline was placed and the colonies were picked with a sterile nichrome wire loop and emulsified in it. The smear was allowed to air dried and heat fixed gently. It was then flooded with Crystal violet and allowed to stain for 1mins before washing it off with distilled water. Lugol's iodine was placed for another 1mins and washed off again; Acetone was added to decolourise the smear and washed out immediately. Neutral red placed on it for 2mins and also washed off. The stained slide was allowed to air dried and examined under the microscope using oil immersion objective. Red rods arranged in singles were observed indicating Gram negative rods.

Indole Test

Into a peptone water broth, inoculation of the stained organisms was done and incubated at 37^0c for 48hours. 0.5ml of Kovac's reagent was added and shook gently. A red rose ring colour appeared in the surface within 10mins indicating a positive Indole test for *Escherichia coli*.

Methyl red Test

Organisms was inoculated into glucose phosphate broth and incubated at 37^oc for 24hours. 5 drops of 0.04% solution of Alcoholic methyl red solution was added mixed and read immediately. A bright red colour appeared indicating a positive test.

2.5.10 Isolation of Pseudomonas aeruginosa

Culture

Streaks of colonies were made on CLED and incubated at 37^oc for 24hours. Non Lactose fermenting flat colonies with greenish diffusible pigments were observed.

Urease Test

Using stabbing and streaking method, organism was inoculated into Urea agar slant and incubated at 35^oc for 24hours. The colour of the media remained yellow which indicated that the organism was negative for urease test.

Oxidase Test

A drop of oxidase reagent (Tetra paraphenylene diamine hydrochloride), was placed on a filter paper. A colony of the test organisms was picked with a glass rod and rubbed on the area of the filter paper

containing the oxidase reagent. Immediately, a deep purple colour was observed along the mark on the filter paper which indicated a positive result.

2.5.11 Isolation of Proteus vulgaris

Culture

Streaks of both the clinical and non-clinical gotten organisms were made on blood agar media and incubated at 37°**c** overnight. A characteristics fishy odour and swarming were perceived and observed.

Urease test

Using stabbing and streaking method, organism was inoculated into Urea agar slant and incubated at 35°c for 24hours. The colour of the media changes from yellow to brownish colour which is indicative that the organism was positive for urease test.

Oxidase test

A drop of oxidase reagent (Tetra paraphenylene diamine dihydrochloride), was placed on a filter paper. A colony of the test organisms was picked with a glass rod and rubbed on the area of the filter paper containing the Oxidase reagent. There was no deep purple colour development on the filter paper which indicated a negative Oxidase result.

2.5.12 Isolation of Candida albicans

Lactophenol Cotton Blue Test

A drop of Lacto-phenol Cotton Blue stain was placed on a clean grease free slide. With the aid of a sterile teasing needle, a part of the fungal isolate was placed on the stain and gently teased. Cover slip was placed on the teased sample and viewed using X10 and X40 objective lenses. Budding yeast and pseudo-hyphae were observed.

Germ Tube Test

The suspected colony was suspended into 0.5ml serum in a test tube and incubated at 37°C for 3hours. A drop of the suspension was placed on a clean grease free slide, and then cover slip was placed on it and viewed under the microscope using X10 and X40 0bjs lenses. Germ tubes were observed which indicated a positive test for the isolate.

Preparation of different stock solutions of the extracts

1.5g of the ethanolic extract of both fresh and dried were dissolved in 3ml of dimethyl sulphur oxide (DMSO) inside sterile bottle differently and then later top up with 4.5ml of water to make it up to 7.5ml in total.

1.5g of aqueous extract of both the fresh and dried was dissolve in 7.5ml of water inside sterile bottle differently.

Concentration of Neem's ethanolic and aqueous extract

The concentrations were made into 100%, 50%, 25%, 12.5% and 6.25%. The 100% extract was the normal ethanolic concentrated stock without dilution. 50%, 25%, 12.5% and 6.25% concentrations of the extract were obtained by passing 1ml of distilled water into 4 different clean test tubes each. Then 1ml of the extracted ethanol was mixed in the 1st tube using syringe to get a 50%conc of the extract. From the tube-1 which contained the mixture already, 1ml of the mixture was syringe out and poured into tube-2 to get the 25% conc of the extract. It was further serial diluted into the last two remaining tubes to obtained 12.5% and 6.25% diluted concentration of the ethanol extract. The same was also done in concentrating aqueous neems extract.

2.5.13 Inoculation of test organisms and antimicrobial activity

With ethanolic extract

Here, the already confirmed test organisms were picked with a sterile nichrome wire loop and streaked onto a well properly dried nutrient agar media. Using a sterile borer wells of equal diameter and reasonable distances apart from each other were made on the already streaked nutrient agar plates. Then different syringes were used to withdrew both the diluted and undiluted prepared extracts at different concentrations (i.e 100%, 50%, 25%, 12.5% and 6.25%) and injected into the respective wells labelled accordingly (N, 1,2,3 and4). The plates were left on the bench undisturbed for 1hour for proper diffusion after which they were carefully put in a canister and incubated at 37°c for 24hours. The zones of inhibition created by the action of the ethanolic extracts were observed and measured in 3dimmensional aspect and the average was taken and recorded.

With aqueous extract

The already confirmed test organisms were picked with a sterile nichrome wire loop and streaked onto a well properly dried nutrient agar media. Using a sterile borer wells of equal diameter and reasonable distances apart from each other were made on the already streaked nutrient agar plates. Then different syringes were used to withdrew both the diluted and undiluted prepared aqueous extracts at different concentrations (i.e 100%, 50%, 25%, 12.5% and 6.25%) and injected

into the respective wells labelled as follows (N, 1,2,3 and4). The plates were left on the bench undisturbed for 1hour for proper diffusion after which they were carefully put in a canister and incubated at 37°c for 24hours. The zones of inhibition created by the action of the aqueous extracts were observed and measured in 3dimmensional aspect and the average was taken and recorded.

Sensitivity testing with the standard synthetic antibiotics

Antibiogram test was performed on these organisms as a positive control using agar disc diffusion method. Bacterial colonies prepared in accordance to McFarland standard were streaked onto the nutrient agar plates. Poly antibiotics discs of both Gram positive and Gram negative were placed with the aid of a sterile forceps and plates were incubated at 37°c for 24hours. According to Clinical & Laboratory Standard Institute (CLSI), Mueller hinton media supplemented with 2% dextrose and 0.5mg/ml methylene blue improves the growth of yeast and gives a sharp zone of inhibition for the azole groups of drug. Therefore, this agar was used for fungal sensitivity testing. Colonies were prepared according to McFarland standard and streaked on Mueller hinton supplemented agar media. Fluconazole (25ug), Itraconazole(10ug), Ketoconazole(15ug) and Voriconazole(1ug) disc were place with the help of a sterile forceps and incubated at 35°c for 24hours. Zones of inhibition caused by both the antifungal/ antibiotics were observed, measured and recorded respectively.

2.6 Statistical Analyses

All statistical analyses were performed using GraphPad prism version 7.0 (GraphPad, San Diego, CA, USA). Independent student's t-test and One-way Analysis of variance (ANOVA) were used for comparison of mean differences between and among groups respectively at 95% confidence interval. P-value $\leq$ 0.05 was considered statistically significant.

3. Results

From table 1, it was shown from phytochemical analysis that both Ethanol extract of the *Azadirachta indica* contains the following compounds Viz; Alkaloids, Flavonoids, Saponins, Glycosides, Terpenoids but lacking Tannins, Phenol and Anthraqinone. While the Aqueous extract lacks only but Anthraquinone and Tannins but contains other active ingredients

Table 1. *Results of qualitative phytochemical analysis*

Parameter	Alkaloids	Flavonoids	Saponins	Tannins	Glycosides	Terpenoids	Phenols	Anthraquinone
Ethanol extract	++	++	-	+++	++	++	-	-
Aqueous extract	+++	++	++	-	+	+++	++	-

Figure 2 showed the comparison of the ZIDs for the antimicrobial effect of fresh ethanolic extract on the test organisms. From the table, the extract (at 100%) had significantly higher effect ($p<0.0001$) on *Staphylococcus aureus* (mean ZID: 13.05) compared to other organisms at the same concentration. At 50%, 25%, 12.5% and 6.25% concentrations, the extract showed a significantly higher effect on *Candida albican's* ($p<0.0001$) compared to other test organisms. At different concentrations, there was no significant difference in the effect of the extract on *Proteus vulgaris* and *Pseudomonas aeruginosa*, however with the exception of these two organisms, the extract had a significantly higher effect on other test organisms at 100% concentrations compared to other extract dilutions ($p<0.05$).

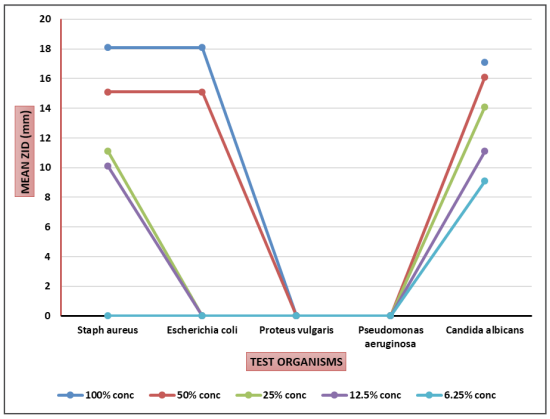

Figure 2. *Inhibitory effects of fresh ethanolic extract on the Test Organisms*

Figure 3 showed the comparison of the ZIDs for the antimicrobial effect of dried ethanolic extract on the test organisms. From the table, the extract (at 100%, 25%, 12.5% and 6.25%) had no significant effect ($p>0.05$) on the test organisms. At 50%conc, the extract showed a significantly higher effect ($p<0.0001$) on *Pseudomonas aeruginosa* (Mean ZID: 9.1mm). At 100% and 50% concentrations, the extract showed a significantly higher effect on *Staphylococcus aureus* and *Escherichia coli* ($p<0.0001$) compared to other test organisms. At different concentrations, there was no significant difference in the effect of the extract on *Proteus vulgaris, Pseudomonas aeruginosa* and *Candida albicans.*

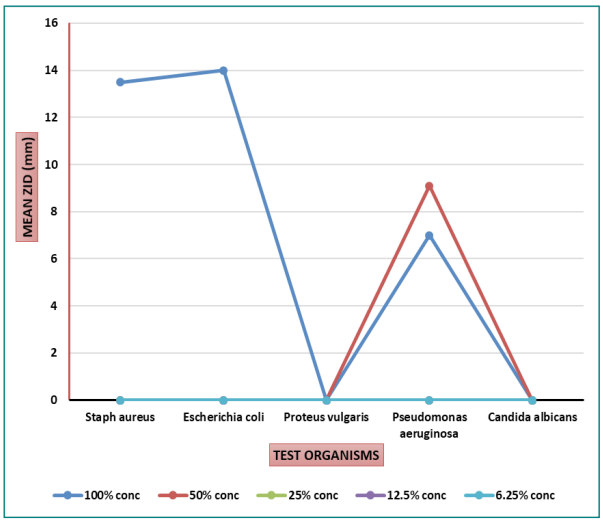

Figure 3. *Inhibitory effects of dried ethanolic extract on the Test Organisms*

Figure 4 showed the comparison of the ZIDs for the antimicrobial effect of fresh aqueous extract on the test organisms. At different concentrations, the extract showed a significant difference in antimicrobial effect on *Staphylococcus aureus, Escherichia coli* and *Candida albicans*. At 50% and 25% conc, the extract showed a significantly higher effect ($p<0.0001$) on *Staphylococcus aureus* (mean ZID: 18.05mm) and *Candida albicans* (mean ZID: 12.0mm) respectively.

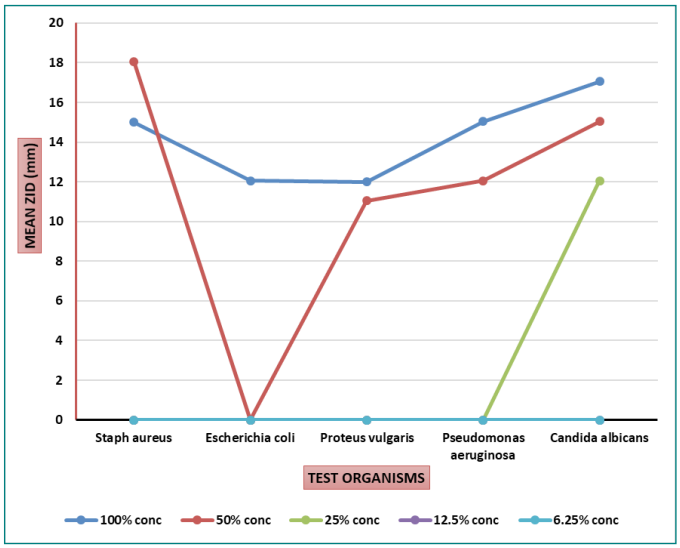

Figure 4. *Inhibitory effects of fresh aqueous extract on the Test Organisms*

Figure 5 showed the comparison of the ZIDs for the antimicrobial effect of dried aqueous extract on the test organisms. From the table, the extract (at 100%) had significantly higher effect ($p<0.0001$) on *Pseudomonas aeruginosa* (mean ZID: 13.05) compared to other bacteria at the same concentration. At 50% concentration, the extract showed a significantly higher effect on *Proteus vulgaris* ($p<0.0001$) compared to other test organisms. There was no significant difference in the antimicrobial effects of the extract on the test organisms at 25%, 12.5% and 6.25% concentrations. The extract had a significantly higher effect on all the test organisms at 100% concentrations compared to other extract dilutions ($p<0.05$).

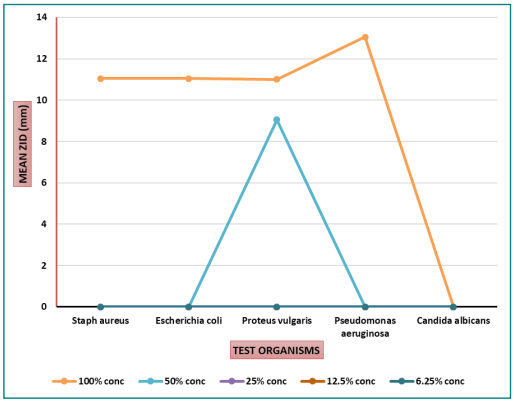

Figure 5. *Inhibitory effects of dried aqueous extract on the Test organisms*

Figure 6a-c showed the comparison of the inhibitory effect of fresh ethanolic extract and the control drugs on the test organisms. From the table, a statistically significantly higher antimicrobial effect was recorded with the standard drugs than the extract ($p<0.05$) except for *Staphylococcus aureus* ($p=0.7642$) where the 100%, 50%, 25% and 12.5% Concentrations showed almost same effect to ceftriaxone, Levofloxacin and Gentamicin. At different concentrations also there were inhibitory effect of other organisms and a statistically significant higher antifungal effect on the Candida albican's where the effect exerted were same with Fluconazole and Voriconazo but lower to Itraconazole and Ketoconazole.

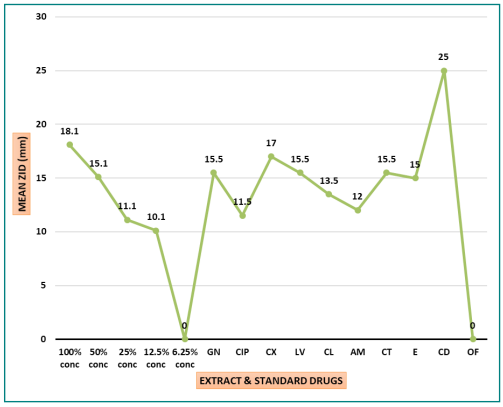

Figure 6a. *Effect of fresh ethanolic extract and standard drugs on Staphylococcus aureus*

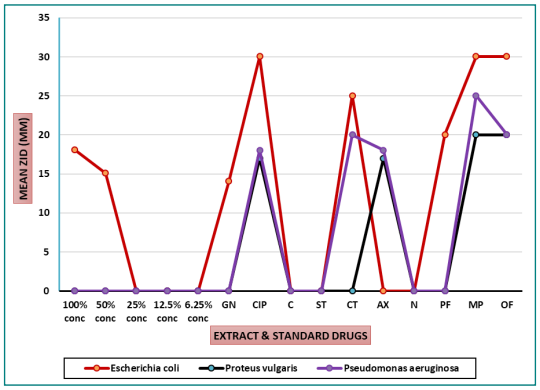

Figure 6b. *Effect of fresh ethanolic extract and standard drugs on gram negative bacteria*

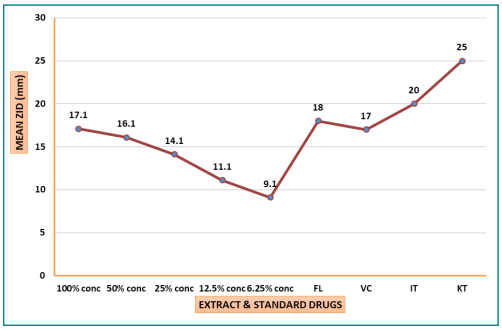

Figure 6c. *Effect of fresh ethanolic extract and standard antifungals on Candida albicans*

Figure 7a-c showed the comparison of the inhibitory effect of dried ethanolic extract and the standard drugs on the test organisms. From the table, a significantly higher antimicrobial effect was recorded with the standard drugs than the extract ($p<0.05$) both on *Staphylococcus aureus*, the Gram negative organisms and on the *Candida albicans* where the extract had no effect at all.

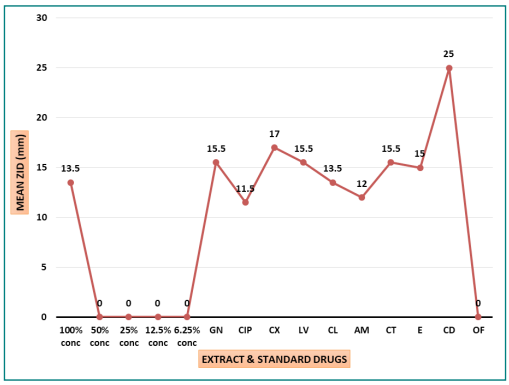

Figure 7a. *Effect of dried ethanolic extract and standard drugs on Staphyloccus aureus*

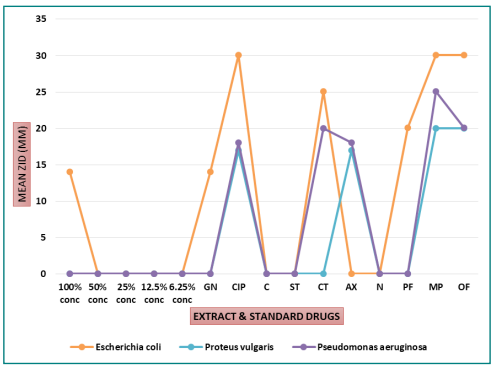

Figure 7b. *Effect of dried ethanolic extract and standard drugs on gram negative bacteria*

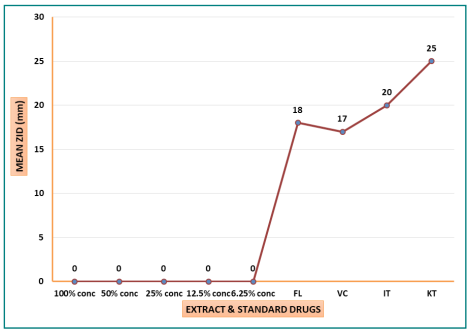

Figure 7c. *Effect of dried ethanolic extract and standard drugs on Candida albicans*

Figure 8a-c showed the comparison of the inhibitory effect of fresh aqueous extract and the control drugs on the test organisms. From the table, a significantly higher antimicrobial effect was recorded with the standard drugs than the dried aqueous extracts ($p<0.05$) except for *Staphylococcus aureus* at 100% and 50% concentrations of the extracts were same to some of the drugs and lower than others. The table illustration is shown in appendix.

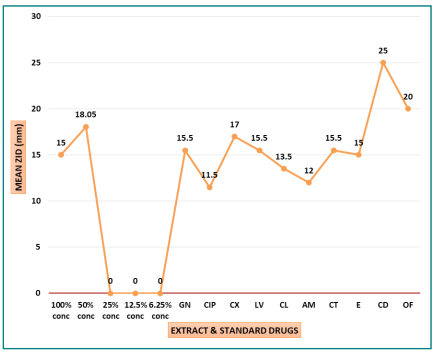

Figure 8a. *Effect of fresh aqueous extract and standard drugs on Staphylococcus aureus*

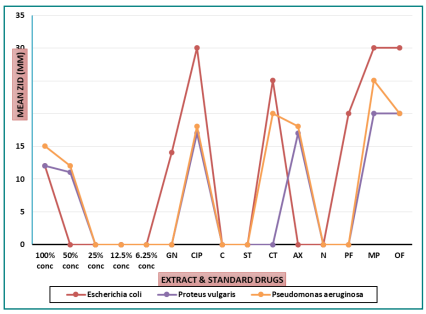

Figure 8b. *Effect of fresh aqueous extract and standard drugs on gram negative bacteria*

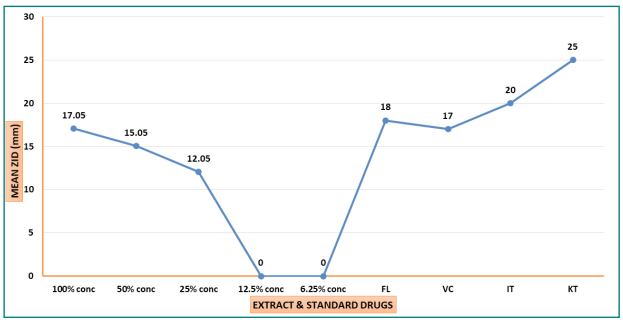

Figure 8c. *Effect of fresh aqueous extract and standard drugs on Candida albicans*

Figure 9a-c showed the comparison of the inhibitory effect of dried aqueous extract and the control drugs on the test organisms. From the table, a significantly higher antimicrobial effect was recorded with the standard drugs than the extract ($p<0.05$), being on extremely high with Ketoconazole and Itraconazole while Fluconazole and Voriconazole almost possesses same effect with the *Azadirachta indica* extracts.

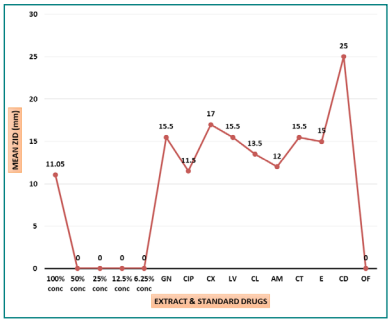

Figure 9a. *Effect of dried aqueous extract and standard drugs on Staphylococcus aureus*

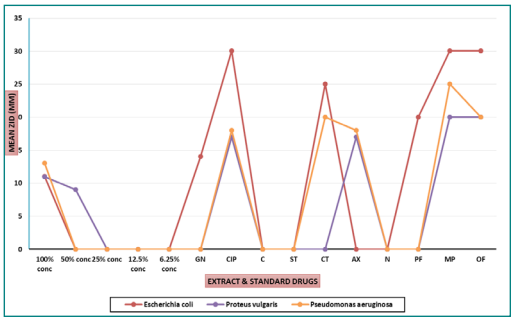

Figure 9b. *Effect of dried aqueous extract and standard drugs on gram negative bacteria*

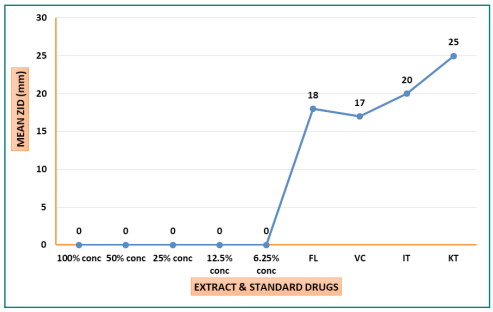

Figure 9c. *Effect of dried aqueous extract and standard drugs on Candida albicans*

Tables 2a and b showed the comparison of the inhibitory effect of ethanolic extract and aqueous extract (at 100% and 50% concentrations) on the test organisms. Fresh ethanolic extract (at 100% conc) showed significantly higher effect on *Pseudomonas aeruginosa* and *Candida albicans* than the dried ethanolic extract ($p<0.0001$). There was no significant difference in effect of fresh and dried ethanolic extract on *Staphylococcus aureus* and *Proteus vulgaris* ($p>0.05$). At 50% conc, fresh ethanolic extract showed significantly higher effect on *Staphylococcus aureus*, *Proteus vulgaris* and *Candida albicans* ($p<0.05$) while dried ethanolic showed a higher effect on *Pseudomonas aeruginosa* at 50% conc. At 100% conc, fresh aqueous extract showed a significantly higher effect on *Candida albicans* than dried aqueous extract ($p<0.0001$). Effect of fresh and dried extracts (at 100%) on other organisms was not significantly different ($p>0.05$). At 50% concentration, fresh aqueous extract showed significantly higher effect on all the organisms than dried aqueous extract ($p<0.00010$ except for *Proteus vulgaris* which showed no significant difference on the effect of fresh and dried extracts ($p>0.05$).

Table 2a: *Comparing the effects of ethanolic (fresh and dried) extracts at 100% and 50% concentrations*

Comparing fresh and dried ethanolic (at 100%)				Comparing fresh and dried ethanolic extract (50%)			
50% Conc	**Fresh (Mean ZID) Mm**	**Dried (Mean ZID) mm**	**P-value**	**50% Conc**	**Fresh (Mean ZID) Mm**	**Dried (Mean ZID) Mm**	**P-value**
Staph aureus	18.1 ± 0.58	13.5 ± 1.5	0.0938	*Staph aureus*	15.1 ± 0.58	0.0 ± 0.0	<0.0001
E. coli	18.1 ± 0.58	14.0 ± 1.0	0.0560	*E. coli*	15.1 ± 0.58	0.0 ± 0.0	<0.0001
Proteus vulgaris	0.0 ± 0.0	0.0 ± 0.0	NA	*Proteus vulgaris*	0.0 ± 0.0	9.05 ± 0.0	NA
Pseudomonas aeruginosa	0.0 ± 0.0	14.05 ± 0.05	<0.0001	*Pseudomonas aeruginosa*	0.0 ± 0.0	14.05 ± 0.05	<0.0001
Candida Albicans	17.1 ± 0.58	0.0 ± 0.0	<0.0001	*Candida albicans*	17.1 ± 0.58	0.0 ± 0.0	<0.0001

Table 2b: *Comparing the effects of aqueous (fresh and dried) extracts at 100% and 50% concentrations*

Comparing fresh and dried aqueous extract (at 100%)				Comparing fresh and dried aqueous extract (at 50%)			
100% Conc	**Fresh (Mean ZID) mm**	**Dried (Mean ZID) mm**	**P-value**	**50% Conc**	**Fresh (Mean ZID) Mm**	**Dried (Mean ZID) Mm**	**P-value**
Staph aureus	15.05 ± 0.05	11.05 ± 0.05	0.5076	*Staph aureus*	18.1 ± 0.58	0.0 ± 0.0	<0.0001
E. coli	12.05 ± 0.05	11.1 ± 0.58	0.0003	*E. coli*	0.0 ± 0.00	0.0 ± 0.0	NA
Proteus vulgaris	12.05 ± 0.05	11.0 ± 2.0	0.5425	*Proteus vulgarIs*	11.05 ± 0.05	9.05 ± 0.05	0.0012
Pseudomonas aeruginosa	15.05 ± 0.05	13.05 ± 0.05	0.5633	*Pseudomonas aeruginosa*	12.05 ± 0.05	0.0 ± 0.0	<0.0001
Candida albicans	17.1 ± 0.58	0.0 ± 0.0	<0.0001	*Candida albicans*	15.05 ± 0.05	0.0 ± 0.0	<0.0001

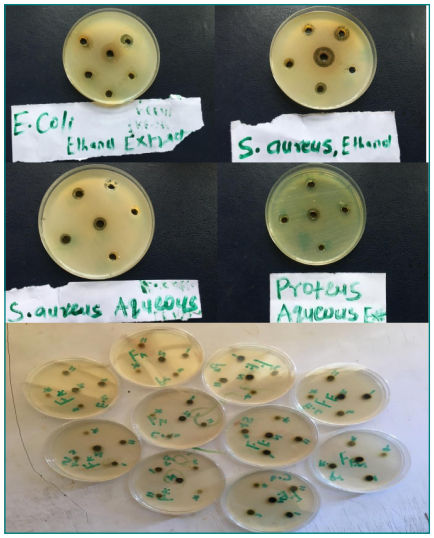

Plate 1. *Photos of plates with zones of inhibition by the extracts on different isolates with different results.*

4. Discussion

The findings of diffusion test from this research showed that both the fresh and dried ethanolic extract, including the fresh and dried aqueous extract displayed antibacterial and antifungal effect on the tested isolates. Nevertheless, it aligns with the work similarly done by Asif[13], in Institute of Management and Technology, Rajpur, india where both the ethanolic and aqueous extract has little or no effect on *Pseudomonas aeruginosa* and *Proteus vulgaris*. Though the activities presented by the aqueous extract of both fresh and dried proves statistically significant difference ($p<0.0001$) than the ethanolic extract. Probably, this is not surprising as the fresh aqueous extracts contain higher active ingredients than the ethanolic extracts.

Akin to this work as also done by Rasool *et al.*[14], shows that ethanol, aqueous and ethyl-acetate extracts were all active by inhibiting most species of *Aspergillus, Candida* and *Staphylococcus aureus.* But only the fresh neem's leaf shows inhibitory effect over the opportunistic fungi organism which is *Candida albicans* used in this research. This is related to the solubility nature of the aqueous extract over the ethanolic extracts.

This work also agrees with the work of Francine *et al.*[15], who tested the ethanolic and aqueous extract of *Azadirachta indica* leaf and bark on *Staphyloccus aureus* and *Escherichia coli*. It was deduced that the fresh extracts were more efficient that the dried extract in all cases. In same way the zone of inhibition provided by the ethanolic extract in this work were also more potent than the ones of fresh aqueous extract. Hence out of the tested bacteria, *Staphylococcus aureus* was more susceptible to all extract with statistically significant difference ($p<0.0001$) followed by *Escherichia coli* while *pseudomonas aeruginosa* and *Proteus vulgaris* poorly responded.

The research also corresponds with the analysis of Mohammed and Al Fadhil[16] where both of the extract (fresh and dried) of the ethanol and aqueous showed maximum inhibition of *Staphylococcus aureus* with moderate zone of inhibition on *Escherichia coli.*

From the research, it proves that the Neem's leaf extracts of ethanol and aqueous, be it fresh or dried leaf has only bacteriostatic effect and not bactericidal. This is in contrast with the research of El-mahmood *et al.*[2], whose work shows that the Neem seed extract is Bacteriocidal for *Staphylococcus aureus* and *Pseudomonas aeruginosa.* This might be due to the Neem seeds containing more biologically potent ingredients from the plant than the one contains by the leaf used in this analysis.

From the various activities of fresh ethanolic and dried extract, it was observed that the extracts of the fresh had almost same efficacy on *staphylococcus aureus* and *Candida albicans* were the zones of inhibition diameters acted based on the concentration levels. Also, both of the extracts did not maximally worked on *Pseudomonas aeruginosa,* Proteus vulgaris and *Escherichia coli* as when compared with their actions on Gram positive tested organism and the opportunistic fungi.

5. Conclusion

Candida albicans and *Staphylococcus aureus* were highly susceptible to both ethanolic and aqueous extract of the fresh *Azadirachta indica* leaves. *Escherichia coli* were moderately susceptible to all the extracts of both fresh and dried. *Azadirachta indica* leaves extract of both fresh and dried does not actively worked on *Proteus vulgaris* and *Pseudomonas aeruginosa. Azadirachta indica* leaves extract of fresh extract, both aqueous and ethanol has more efficacies.

6. References

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