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# ANTIMICROBIAL EFFECTS OF *AZADRACHITA INDICA* LEAF EXTRACTS ON *ESCHERICHIA COLI* ISOLATED FROM INFECTED DIABETIC WOUNDS

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## ABSTRACT

The purpose of this work was to evaluate the antimicrobial effect of *Azadirachta indica* leaf extracts on *Escherichia coli* isolated from infected diabetic wound. Out of 50 swab samples of infected diabetic wounds, 30 isolates were obtained of which *Escherichia coli* made up 20% of the bacterial isolates from the wound. The phytochemical analysis of the leaf's extracts showed the presence of saponin, flavonoids, steroid, alkaloids, tannins, cardiac glycoside, and phenol. The *in vitro* antimicrobial effect of the aqueous and ethanol leaf extracts was evaluated using the agar well diffusion method. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the leaf extracts were also determined. Results showed that the ethanol extract showed inhibitory effects at all concentrations used in this study, which were 125mg/ml (10.00mm), 250mg/ml (10.33mm), 500mg/ml (10.67mm) and 1000mg/ml (10.67mm) while the aqueous extract had no inhibitory effect on *E. coli*. The result of MIC was 500mg/ml and the MBC showed 1000mg/ml. The ethanol extracts possess antimicrobial activity against *E. coli* and the plants are readily available. There is need for more research on other plant with antimicrobial properties to help tackle the problem of antimicrobial resistance.

Keywords: Diabetic wound, *Azadirachta indica*, *Escherichia coli* and Antimicrobial effect.

## INTRODUCTION

The severity of diabetic foot ulcers among diabetic patents is increasing due to widespread resistance to antibiotics among the wound colonizing pathogens and this could lead to amputation of the affected foot**.** One of the serious medical, health and social global problems is drug resistance, and this problem poses additional challenges for clinicians and the pharmaceutical industry (Raja *et al*., 2013). Use of herbal medicines in the developed world continue to rise because they are available, rich source of novel drugs and their bioactive principles, form the basis in medicine, nutraceuticals, pharmaceutical intermediates, and lead compounds in synthetic drugs (Ncube *et al.*, 2008). Screening medicinal plants for biologically active compounds offers clues to develop newer antimicrobial agents. After possible chemical manipulation provide, these compounds provide new and improved drugs to treat the infectious diseases. Plant based products/ extracts are cheaper alternatives to the development of synthetic drugs (Shah *et al*., 2006).

Several plant species of medicinal plants have been identified to be naturally distributed. The traditional use of medicinal plants in addressing certain health problems has been handed down from generation to generation. Many of these plant species are known to majority as a source of medicine for treating a particular ailment, without the knowledge that two or more species could be mixed together to produce a more effective medicine. Example, *Azadirachta indica* commonly known for the treatment of malaria scourge, though it could also be used to treat ailments like hepatitis and intestinal problems when mixed with the bark and leaves of *Mangifera indica* with some fruits of *Citrus aurantifolia* (Nwachukwu *et al*., 2010).

*Azadirachta indica*, known as Neem, Maina in Hausa, Atu yabasi in Igbo, Dongoraro in Yoruba and Dongoyalo in Idoma, is a widely distributed indigenous Indian plant and it is also grown in Nigeria (Itelima *et al*., 2016). US National Assemble of Sciences recognized the importance of the neem tree and this was published in 1992 report entitled "Neem a tree for solving global problems" (Biswas *et al*., 2002). It has been reported that every part of the neem tree has been used as traditional medicine for household remedy against various human diseases from ages (Itelima *et al*., 2016). Different parts of *Azadirachta indica* are known to contain antibacterial, antifungal activities against different pathogenic microorganisms and antiviral activity against vaccinia, chikungunya, measles and coxsackie B viruses (Biswas *et al.*, 2002).

Many researchers have also studied the medicinal properties of neem leaf which include its antipyretic effect, antimalarial effect, antidiabetic effect, antifertility effect, cardiovascular effect, wound healing effect etc (Itemilia *et al*., 2016). Extracts of leaf are effective against certain human fungi (Biswas *et al*., 2002). Based on researchers' medical properties reported about the neem plant, this research work tends to determine

the antimicrobial activity of *A. indica* leaf extracts on *Eschericha coli* isolated from infected wounds of diabetic patients.

## MATERIALS AND METHODS

### Study Area

The study was carried out in Anambra State, which is the 8th most populous state in Nigeria, located in South-East geopolitical zone of Nigeria. It is located between latitude 6º20'00'' North and longitude 7º00''00'' East. Anambra state has a population of 2,061961.984. Anambra state has a tropical climate. The average temperature is 25.9℃ while the average rainfall in a year is 1386mm (Nkwoemeka *et al*., 2020)

### Study Population

The target population comprised of diabetic patients with infected wounds that are attending Chukwuemeka Odumegwu Ojukwu Teaching Hospital, Awka in Anambra state.

### Ethical Consideration

Ethical approval was gotten from ethical committee of Chukwuemeka Odumegwu Ojukwu University Teaching Hospital, Amaku, Awka with Ref: COOUTH/CMACTH.C/VOl. 1/0090.

### Isolation of Microorganisms

### Sample Collection

Fifty (50) wound swabs of patients with infected wounds and are attending different clinics in Chukwuemeka Odmegwu Ojukwu University Teaching Hospital, Amaku, Awka were collected. Wound exudates were collected using sterile cotton tipped swab before antiseptic dressing. Sterile normal saline was used to moisten the swab stick before collecting the specimen (Kavishankar *et al*., 2011)

### Preparation of Culture Media

All culture media employed in this study were prepared according to the manufacturer's instructions. They were sterilized by autoclaving at 121ºC, 15psi for 15 minutes.

### Isolation and Identification of Bacteria

Each sample was inoculated onto Nutrient agar and MacConkey agar and incubated at 37ºc for 24 hours. After the incubation, the different colonies were subcultured on Nutrient agar and MacConkey agar and incubated at 37℃ for 24 hours to get pure cultures. Then the colonies were studied using microscopic and biochemical characteristics. Microscopic examination was done after gram staining. The following biochemical tests were performed: catalase test, oxidase test, motility test, methyl red test, Voges-proskaeur test, indole test, citrate test and sugar fermentation tests (Aynalem *et al*., 2017)

### Antibiotics sensitivity of the isolates to conventional drugs

*Escherichia coli* isolates to be inoculated on the surface of Mueller Hinton agar plates were incubated into nutrient broth overnight until the turbidity is equivalent to 0.5 Mcfarland standards, allowed for few minutes at room temperature. Antimicrobial susceptibility was performed on Mueller-Hinton agar by the standard disk diffusion method recommended by the National committee for

clinical laboratory standards (NCCLS, 2002).This was done by dipping a sterile swab stick into overnight nutrient broth and carefully swabbing the entire surface of Mueller– Hinton agar plates. The antibiotics used against the test bacteria were: ofloxacin (10 µg), levofloxacin (5 µg), norfloxacin (10 µg), ciprofloxacin (5 µg) and gentamicin (30 µg).The antibiotic multi disc was then placed on the surface of the inoculated plates and gently pressed. The plates were incubated at 37℃ for 24 hours. The diameter of zone of inhibition was measured in millimeters. The experiment was carried out in triplicates and isolates were scored as sensitive or resistant by comparing with values recommended on standard.

**Plant Collection and Identification**

The leaves of *Azadirachta indica* were collected from the forest within the premises of Nnamdi Azikiwe University, Awka and were identified in Department of Botany, Nnamdi Azikiwe University, Awka with identification no: NAUH $14^{B}$. The leaves were washed with sterile water, shade dried, ground into powdered form and store in an airtight container for future use.

**Preparation and analysis of Crude Extract**

The crude extracts were prepared using the method of Kavishankar *et al*., 2011. The following constituents were analysed: saponin, flavonoid, tannin, cardiac glycerol, sterols, and alkaloid using the method of Subhashini and Kantha, 2010.

**Reconstitution of Plant Extract**

The dried extracts were reconstituted by dissolving 10 g of extract in 10 ml of dimethylsulfoxide (DMSO). These extracts were considered as 1000 mg/ml.

**Assessment of *In vitro* Antibacterial Activity of the Extracts**

Test Microorganisms: Microbial culture of *E. coli* isolated from the wounds were used for determination of antimicrobial activity.

One (1) ml of 24 hours culture of the isolate which was adjusted to 0.5 MacFarland standard corresponding to approximately $1.0 \times 10^{8}$ cfu/ml was introduced into sterile plates and then 19 ml of molten Muller Hinton agar at 45℃ was added to the plate and shaken gently so as to mix contents. Then, the molten agar and the test organism were allowed to solidify on a flat bench for 30mins. A sterile cork borer of 6mm diameter was used to drill holes 4mm deep. The wells were filled with 0.5ml of each of the extract's concentration (62.5mg/ml-1000mg/ml). Standard antibacterial solution (gentamycin) and sterile water were used as positive and negative control respectively. The plates were incubated at 37ºc for 24 hours. Antimicrobial activity was determined by measuring the zones of growth inhibition. The experiments were carried out in triplicates and results were calculated as mean ± SD.

**Minimal Inhibitory Concentration and Bactericidal Concentration of Crude Extracts**

The Minimum Inhibitory Concentration (MIC) and MBC of different extracts were determined by the tube

dilution method according to Chikezie, (2017).

**Statistical Analysis**

The data obtained were subjected to one-way ANOVA using Statistical Package for Social Sciences (SPSS) 21 for window evaluation. P-values < 0.05 was considered significant.

**RESULTS**

Out of 50 swab samples of infected diabetic wounds, 30 isolates which include 9(30%) *Staphylococcus aureus*, 7(23.3%) *Streptococcus pyrogenes*, 8(26.67%) *Pseudomonas aeruginosa* and 6(20%) *Eschericha coli*.

Table 1 show the results of the biochemical and morphological characteristics of *E. coli.*

Table 1. Microscopic and Biochemical Characteristics

| Test | Characteristics |
|---|---|
| **Gram Staining** | -ve rod |
| **Catalase** | -ve |
| **Oxidase** | -ve |
| **Urease** | -ve |
| **Methyl red** | +ve |
| **Voges-Proskaeur** | -ve |
| **Indole** | +ve |
| **Motility** | +ve |
| **Citrate** | -ve |
| **Sucrose** | +ve |
| **Glucose** | +ve |
| **Lactose** | +ve |
| **Maltose** | +ve |

KEY: +ve: positive, -ve: negative

Table 2 indicates the susceptibility pattern of the *E. coli* isolated from infected diabetic wounds. It was observed that all the isolates are sensitive to ofloxacin while all are resistant to norfloxacin. The isolate E4 is resistant to levofloxacin, ciprofloxacin and norfloxacin, therefore it is multidrug resistant.

Table 2 Antibiotics Susceptibility pattern of the *E. coli* isolates

| Isolate No. | CN | LEV | CPX | OFX | NB |
|---|---|---|---|---|---|
| **E1** | R | S | S | S | R |
| **E2** | S | S | S | S | R |
| **E3** | S | S | S | S | R |
| **E4** | S | R | R | S | R |
| **E5** | S | S | S | S | R |
| **E6** | S | S | S | S | R |

OFX: Ofloxacin (10 µg), CPX: Ciprofloxacin (5 µg), CN: Gentamicin (30 µg), NB: Norfloxacin (10 µg) and LEV: Levofloxacin (5 µg)

S: sensitive, R: resistance

Table 3 shows the percentage distribution of antibiotics susceptibility of *E. coli* isolates. It showed that there is 100% of the isolates were susceptible to Ofloxacin, 83. 7% were susceptible to gentamicin, ciprofloxacin, and levofloxacin while 100 % were resistance to norofloxacin.

Table 3: Percentage distribution of antibiotic susceptibility of *E. coli* isolated

| Antibiotics | Frequency (%) | |
|---|---|---|
| | **Resistance** | **Sensitive** |
| **Gentamicin** | 16.7 | 83.3 |
| **Ofloxacin** | 0 | 100 |
| **Ciprofloxacin** | 16.7 | 83.3 |
| **Levofloxacin** | 16.7 | 83.3 |
| **Norfloxacin** | 100 | 0 |

Table 4 shows the phytochemical constituent of *Azadirachta indica.* Both the aqueous and ethanol extracts. It was observed that both extracts contain alkaloid, phenol, tannins, flavonoids, saponins, cardiac glycoside and steroid.

Table 4: Phytochemical constituent of *Azadirachta indica* leaf extract

| Constituents | Aqueous extract | Ethanol extract |
|---|---|---|
| **Saponin** | +ve | +ve |
| **Flavonoids** | +ve | +ve |
| **Steroids** | +ve | +ve |
| **Alkaloids** | +ve | +ve |
| **Tannins** | +ve | +ve |
| **Cardiac glycoside** | +ve | +ve |
| Phenol | +ve | +ve |

KEY: +ve: positive, -ve: negative

**Antimicrobial Effects of the Crude Extracts on the Isolates**

Table 5 shows the antimicrobial effects of *Azadirachta indica* extracts (ethanol and aqueous) at different concentration on *E. coli* that is resistant to more than two (2) antibiotics using agar well diffusion method. *Azadirachta indica* ethanol extract showed inhibitory responds of 10.00mm, 10.33mm, 10.67mm and 10.67mm at 125mg/ml, 250mg/ml, 500mg/ml and 1000mg/ml concentration while *Azadirachta indica* aqueous extract showed no inhibitory response to *E. coli*

Table 5 : Antimicrobial Effect of *A. indica* Extracts on *E. coli*

| **Concentration(mg/ml)** | **Mean zone of inhibition ± SD(mm)** | |
|---|---|---|
| | **Aqueous extract** | **Ethanol extract** |
| **62.5** | 0.00±0.00 | 0.00±0.00 |
| **125** | 0.00±0.00 | 10.00±0.00 |
| **250** | 0.00±0.00 | 10.00±0.58 |
| **500** | 0.00±0.00 | 10.67±1.15 |
| **1000** | 0.00±0.00 | 10.67±1.15 |
| **+control** | 27.0±0.00 | 27.0±0.00 |
| **-control** | 0.00±0.00 | 0.00±0.00 |

Positive control: Gentamicin: 27.00±0.00mm Negative control: sterile distilled water: 0.00mm, Result is represented as Mean ± SD

The minimum inhibitory concentration and the minimum bactericidal concentration of the extracts to *E. coli* is shown in Table 6. Both extracts have MIC of 250 mg/ml and the MBC of 1000 mg/ml

Table 6: Minimum inhibitory Concentration and Minimum bactericidal concentration

| **Isolate** | **Aqueous extract** | | **Ethanol extract** | |
|---|---|---|---|---|
| | **MIC (mg/ml)** | **MBC (mg/ml)** | **MIC (mg/ml)** | **MBC (mg/ml)** |
| **E4** | 250 | 1000 | 250 | 1000 |

**DISCUSSION**

*E. coli* was isolated from wound samples this agreed with the work of Garba *et al*. (2012) which identified *E. coli* as one of the bacteria isolated from infected wound. The antimicrobial susceptibility patter showed 100 % sensitivity to ofloxacin, this result is not in concordance to that of Omololu-Aso *et al*. (2017) which reported that the *E. coli* were resistant to ofloxacin. The result also showed that 83.3% of the isolates were sensitive to ciprofloxacin and gentamicin. The result is similar to the result of Kibret and Abera (2011) which showed 74.7 % susceptibility of *E. coli* to ciprofloxacin and 81.0 % to gentamicin. The result showed 100 % resistant of *E. coli* to norfloxacin which is not in accordance with the work of Kibert and Abera (2011) that reported that *E. coli* showed 93.5 % sensitivity to norfloxacin.

The result of the phytochemical analysis of *A. indica* aqueous and ethanol extracts shows the presence of saponin,

flavonoids, steroid, alkaloids, tannins, cardiac glycoside, and phenol. This result is in concordance with that of Nwali *et al* 2012. Nwali *et al.* (2012) reported that flavonoid is an antioxidant that provides anti-inflammatory activity and that this may be the reason the extracts are used for treatment of wounds.

In this study, the *in vitro* antimicrobial activities of *A. indica* extracts (aqueous and ethanol) were done on the isolate that showed resistance to three (3) antibiotics. The result of the antimicrobial effect shows that *A. indica* aqueous extract has no inhibitory effect on *E. coli,* but *A. indica* ethanol extract shows inhibitory effect starting from 125mg/ml (10.00mm), 250mg/ml (10.33mm), 500mg/ml (10.67mm) and 1000mg/ml (10.67mm) as shown in table 3. This agreed with the work of Oscar *et al.*, 2019 for *A. indica* ethanol extract but disagrees with the work for *A. indica* aqueous extracts which have no inhibitory effect on *E. coli*. Oscar *et al.,* 2019, revealed that different parts of neem plant contain biological compounds responsible for antibacterial, antiviral, and antifungal activities. It is considered as safe medicinal plants and modulates the numerous biological processes without any adverse effect. Uwimbabaz *et al.,* 2015 revealed that *A. indica* extracts both aqueous and ethanol has no effect on *E .coli* and this is also in concordance with the result of this work but differs from the present study that showed *A. indica* ethanol had inhibitory effect on *E. coli*.

The minimal inhibitory concentration (MIC) of the extracts on *E. coli* is 250mg/ml while the MBC is 1000mg/ml. This agrees with the work of Lawrence *et al.,* (2016) which revealed that there is correlating increase in antimicrobial activity with increase in extract concentration.

## CONCLUSION

The ethanol extract possesses antimicrobial properties for *E. coli*. Since the plant is readily available, the extract can be used for the production of drugs.

## CONFLICT OF INTEREST

Authors thereby declare no conflict of interest.

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