**Antimicrobial Effect of Neem and Lemon Extract on Pathogens Associated with Urinary Tract Infection in Pregnant Women attending Antenatal Clinics in Ekpoma, Nigeria.**

**ABSTRACT**

The high incidence of urinary tract infections (UTIs) amongst pregnant women as a result of physiological and hormonal changes during pregnancy, combined with the menace of antibiotic resistance to modern antibiotics, necessitates the introduction of novel alternatives to replace conventionally used antibiotics. This study investigated the antimicrobial effect of neem and lemon extract on pathogens associated with UTIs in pregnant women attending antenatal clinic at Ujeolen and Ihumudumu Health Centres in Ekpoma, Edo State. A two-week cross-sectional study was conducted using twenty (20) urine samples collected from pregnant women attending the antenatal care clinics. Bacteria causing UTIs were isolated and enumerated to assess the incidence and distribution of UTI pathogens. Isolated UTI pathogens were identified based on their biochemical properties. The crude extract of neem and lemon leaves were tested for their antimicrobial activity against the pathogens using 25, 50, 75, and 100 mg/mL concentrations. Bacterial colony count ranged from 2.4 x 105 - 4.6 x 105 (samples from Ujeolen) and from 3.1 x 105 - 5.2 x 105 (samples from Ihumudumu). Pathogens isolated from samples collected from Ujeolen Health Centre were identified as *E. coli*, *Klebsiella* sp*., Proteus* sp. and *Enterobacter* sp., while for Ihumudumu Health Centre, they were identified *E. coli*, *Klebsiella* sp*.* and *Pseudomonas* sp. All test isolates were susceptible to the extracts at the highest concentrations ranging from 75-100 mg/ml for both ethanolic extract of neem and lemon, while lower concentrations showed intermediate antimicrobial activity. The MIC of neem crude extract was 100 mg/mL for all test isolates and 50 mg/mL for the crude lemon extract. This study has confirmed the antimicrobial activity of neem and lemon crude extract on UTIs associated pathogens. Extracts of neem and lemon could serve as alternative therapy to conventional antibiotics in the treatment of UTIs.

**Keywords**: lemon extract; neem extract; pregnant women; urinary tract infections

**INTRODUCTION**

The excessive and indiscriminate use of antibiotics as emergency contraceptive pills, combined with the hormonal and physiological changes during pregnancy, has resulted in an increased susceptibility and potential severity of urinary tract infections (UTIs) amongst pregnant women [1]. Although UTIs are perceived as common, amongst pregnant women they pose significant concern to both maternal and fetal health, and if left untreated, can lead to complications such as preterm birth, low birth weight, pyelonephritis, and even maternal sepsis [2].

The rise in antibiotic resistance has outpaced the effectiveness of the current antibiotics, thus rendering many existing antibiotics ineffective [3-5]. According to the WHO, this trend is considered the most pressing challenge in modern medicine [5]. Addressing this menace necessitates the introduction of novel approaches to tackle antibiotic resistance. Current research is exploring alternatives that utilize plant extract, specifically aimed at isolating and identifying novel bioactive chemicals with antimicrobial activity [6, 1], also given that nearly 50% of current nutraceuticals and pharmaceuticals are sourced from plants and their derivatives [6]. Although plant extracts hold promise in tackling this menace posed by antibiotic-resistant bacteria, the antimicrobial effect of many medicinal plants is largely unknown [7].

According to the World Health Organization (WHO), 80% of the developing world still relies on plant-derived traditional medicine [8-9]. With the total estimated number of 374,000 plants [10], only 28,187 species are used by humans for medicinal purposes [11]. Additionally, 20,000 plants have been cataloged by the WHO as major sources for new drug development [5, 12]. Lewis *et al*. (2013) reported 30,000 well-defined antimicrobial extracts from over 1,340 plants [13]. Among these plants, lemon and neem leaves have been discovered to be rich in bioactive phytochemicals with promising antimicrobial properties [14].

The bioactive phytochemical constituents of neem (*Azadirachta indica*) include compounds such as azadirachtin, nimbin, and quercetin, which have exhibited antimicrobial and inflammatory-modulating effects [14]. These compounds inhibit enzymatic functions and disrupt the microbial cell membrane [15]. Similarly, lemon (*Citrus limon*) contains many limonoids, flavonoids, and citric acid, all known for their antimicrobial properties [16]. Additionally, the acidic nature of lemon creates an adverse environment for bacterial growth [17]. Their accessibility, minimal side effects, and low cost represent a promising natural alternative to conventionally used antibiotics [18-19].

Pregnant women in semi-urban areas like Ekpoma, face an array notable health challenge spanning across, inadequate access to maternal healthcare services, shortage of trained maternal health personnel, and limited infrastructure, this challenge has led to an increased undiagnosed UTIs in pregnant women a subsequent complication such as preterm labor or low birth weight [2]. The risk is heightened with current studies reporting asymptomatic cases of UTIs in pregnant women [20].

This study aimed to investigate neem and lemon extract's antimicrobial effect on infections associated with UTIs isolated from pregnant women attending antenatal care at Ujeolen and Ihumudumu Health Centres, Ekpoma, Edo State.

**MATERIALS AND METHODS**

**Study Area**

This investigation was carried out at the Ujeolen and Ihumudumu Health Centres in Ekpoma, within the Esan West Local Government Area of Edo State and geographically, situated at a latitude of 6° 45' to 6° 0.1'N and a longitude of 6° 15' to 48°E.

**Sample Collection**

A total of 20 urine samples were randomly collected from pregnant women attending antenatal care at Ujeolen and Ihumudumu Health Centres in Ekpoma, Edo State. The 20 collected urine samples were collected as first morning urine specimens, which have increased test sensitivity and are typically more concentrated [21, 22]. A mid-stream catch method was employed for urine collection.

Fresh disease-free leaves of neem and lemon plants were collected from a farmland in Ekpoma in a sterile polythene bag.

Samples were transported immediately after collection to the Microbiology Laboratory at Ambrose Alli University for analysis.

**Isolation and Identification of Bacterial Isolates**

The streak plate method was used on nutrient and MacConkey agar for bacterial isolation. The plates were incubated at 37ºC for 24 hours and were subsequently subcultured to obtain a pure culture. The pure isolates obtained were identified based on their Gram staining and biochemical properties.

**Preparation of Plant Extract**

The cold percolation method was employed to prepare the plant extract. The plant leaves were processed into a fine powder and appropriately labeled. Sixty grams (60g) of the powder was soaked in 160 mL of anhydrous ethanol at 20°C for three consecutive days. Whatman No. 1 was used to filter the extract and the final filtrate was concentrated by evaporation at 50°C to obtain a crude extract via rotary evaporation. The extract was then stored in the refrigerator for subsequent evaluation [23].

**Preparation of Stock Concentration**

One gram (2g) of extract was dissolved in 10 mL of solvent to prepare a stock solution at 100 mg/mL concentration for both extracts. Varying concentrations were prepared from the main stock solution in the following proportions: 100, 75, 50 and 25 mg/mL.

**Antimicrobial Assay of Plant Extract**

The agar well diffusion method was used in Mueller-Hinton Agar (MHA) plates for the antimicrobial assay of plant extract. The test bacteria were cultured at 37°C overnight in a Nutrient broth to obtain a turbidity of 0.5 McFarland, equivalent to 1.5 × 108 CFU/mL. Additionally, a sterile 6 mm cork-borer was used to create six 5 mm wells, each filled with 50 μl of extract concentration ranging from 25-100 mg/mL plant extract. Dimethyl sulfoxide (DMSO) served as a negative/solvent control, while amikacin 30 mcg and nitrofurantoin 300 mcg acted as positive controls for isolates. After 30 minutes of diffusion at room temperature, the plates were incubated at 37°C for 18–24 hours, then checked for zones of inhibition (ZOI).

**Determination of MIC and MBC of the Plant Extracts**

The Minimum Inhibitory Concentration (MIC) was determined by the broth microdilution method following the Clinical and Laboratory Standards Institute (CLSI) guideline. A serial dilution of the plant extract in Mueller-Hinton agar was inoculated with bacteria (5 × 105 CFU/mL) and incubated at 37°C for 24 h. Amikacin was added as a positive control drug. After adding resazurin into the wells, the plates were incubated at 37°C for 30 minutes. Wells with bacterial growth turned pink; blue wells indicated inhibition. MIC is the lowest extract concentration, preventing growth [4].

**RESULTS**

**Bacteria Identification and Biochemical Characteristics**

Table 1 shows the biochemical characteristics of the bacterial isolate from urine samples of pregnant women attending antenatal care at Ujeolen Health Centre. The isolates were identified as *E. coli*, *Klebsiella* sp*., Proteus* sp. and *Enterobacter* sp. Table 2 shows the biochemical characteristics of bacteria isolated from urine samples obtained from pregnant women attending antenatal care at Ihumudumu Health Centre in Ekpoma. The isolates were identified as *E. coli*, *Pseudomonas* sp. and *Klebsiella* sp*.*

**Table 1:** Biochemical Characteristics of Bacteria Isolated from Urine Samples of Pregnant Women Attending Antenatal Care at Ujeolen Health Centre in Ekpoma

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Species  | Gram | Motility | Catalase | Coagulase | Indole | Oxidase | Urease  | Citrate  | Glucose  | Lactose  | Mannitol | Sucrose |
| *Escherichia coli* | ­- | - | - | -  | + | - | - | - | + | + | - | + |
| *Proteus* sp. |  + |  + |  + | - | - | - |  + |  + |  + | - | - | - |
| *Klebsiella* sp*.* |  + |  - |  + | - | - | - |  + |  + |  + |  + |  + |  + |
| *Enterobacter* sp*.*  |  + |  + |  + | - | - | - |  + |  + |  + |  - |  + |  + |

**Keys: + =** Positive Reaction, **− = N**egative Reaction

**Table 2:** Biochemical Characteristics of Bacteria Isolated from Urine Samples of Pregnant Women Attending Antenatal Care at Ihumudumu Health Centre in Ekpoma.

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Species  | Gram | Motility | Catalase | Coagulase | Indole | Oxidase | Urease  | Citrate  | Glucose  | Lactose  | Mannitol | Sucrose |
| *Escherichia coli* | ­- | - | - | -  | + | - | - | - | + | + | - | + |
| *Klebsiella* sp*.* |  + |  - |  + | - | - | - |  + |  + |  + |  + |  + |  + |
| *Pseudomonas* sp*.*  |  - |  + |  - | - | - | - |  + |  - |  + |  - |  + |  - |

**Keys: + =** Positive Reaction, **− = N**egative Reaction

**Microbial Load in Urine Samples**

Table 3 presents the total colony counts of bacteria isolated from urine samples collected from pregnant women attending antenatal care at Ujeolen Health Centre, which ranged from 2.4 x 105 - 4.6 x 105,CFU/ml Ihumudumu Health Centre, which ranged from 3.1 x 105 - 5.2 x 105 CFU/ml.

**Table 3:** Bacterial Colony Count of Bacteria Isolated from Urine Samples of Pregnant Women Attending Antenatal Care at Ujeolen and Ihumudumu Health Centres in Ekpoma

|  |  |  |
| --- | --- | --- |
| Health Centre  | Isolate code | CFU/ml |
| Ujeolen  | U1 | 4.1 x 105 |
|  | U2 | 3.8 x 105 |
|  | U3 | 2.6 x 105 |
|  | U4 | 3.2 x 105 |
|  | U5 | 4.3 x 105 |
|  | U6 | 3.9 x 105 |
|  | U7 | 4.1 x 105 |
|  | U8 | 4.6 x 105 |
|  | U9 | 2.4 x 105 |
|  | U10 | 3.7 x 105 |
| Ihumudumu  | I1 | 3.9 x 105 |
|  | I2 | 3.5 x 105 |
|  | I3 | 3.1 x 105 |
|  | I4 | 3.8 x 105 |
|  | I5 | 3.6 x 105 |
|  | I6 | 4.9 x 105 |
|  | I7 | 3.3 x 105 |
|  | I8 | 5.0 x 105 |
|  | I9 | 5.2 x 105 |
|  | I10 | 3.4 x 105 |

 Key: U1- U10 = Urine samples from Ujeolen Health Centre; I1- I10 = Urine samples from Ihumudumu Health Centre

**Frequency of Bacterial Occurrence**

Table 4 shows the frequency of bacteria isolated from the pregnant women. *E. coli* was the most occurring bacteria (55%) and *Proteus* sp. the least occurring (5%) bacteria isolated from the pregnant women at Ujeolen Health Centre, while *E. coli* was the most occurring (45.5%) and *Klebsiella* sp. the least occurring (18.2%) bacteria isolated from the pregnant women at Ihumudumu Health Centres.

**Table 4:** Frequency of occurrence of bacterial Isolates from urine samples of pregnant women attending antenatal care in Ujeolen and Ihumudumu Health Centres, Ekpoma

|  |  |  |  |
| --- | --- | --- | --- |
| Health centre | Isolate | Frequency | % |
| Ujeolen  | *E. coli* | 11 | 55 |
|  | *Proteus* sp. | 1 | 5 |
|  | *Klebsiella* sp*.* | 5 | 25 |
|  | *Enterobacter* sp*.*  | 3 | 15 |
| Ihumudumu  | *E. coli* | 10 | 45.5 |
|  | *Klebsiella* sp*.* | 4 | 18.2 |
|  | *Pseudomonas* sp*.*  | 8 | 36.4 |

**Antimicrobial Susceptibility of Plant Extract**

Table 5 present the antibiotic susceptibility test of ethanolic extracts of Neem and lemon. All test isolates were susceptible to the extracts at the highest concentrations, ranging from 75-100 mg/ml for both the ethanolic extract of neem and lemon.

**Table 5:** Antibiotic susceptibility test of the ethanolic extract of neem extracts against the tested bacterial strains from the urine samples

|  |  |
| --- | --- |
| Test isolate | Extract Concentration (mg/ml) |
| 100 | 75 | 50 | 25 |
| *E. coli* | 28mm | 20mm | 20mm | 8mm |
| *Proteus* sp. | 28mm | 19mm | 20mm | 5mm |
| *Klebsiella* sp*.* | 26mm | 22mm | 10mm | 8mm |
| *Enterobacter* sp*.*  | 27mm | 22mm | 10mm | 8mm |
| *Pseudomonas* sp*.*  | 25mm | 20mm | 18mm | 7mm |

Zone of inhibition: Less than 13mm=Resistance; 14 to 17mm=Intermediate; 17 and above=Sensitive

**Table 6:** Antibiotic susceptibility test of the ethanolic extract of lemon extracts against the tested bacterial strains from the urine samples

|  |  |
| --- | --- |
| Test isolate | Extract Concentration (mg/ml) |
| 100 | 75 | 50 | 25 |
| *E. coli* | 33mm | 28mm | 12mm | 11mm |
| *Proteus* sp. | 26mm | 22mm | 12mm | 7mm |
| *Klebsiella* sp*.* | 30mm | 29mm | 15mm | 10mm |
| *Enterobacter* sp*.*  | 30mm | 25mm | 10mm | 8mm |
| *Pseudomonas* sp*.*  | 31mm | 28mm | 13mm | 10mm |

Zone of inhibition: Less than 13mm=Resistance; 14 to 17mm=Intermediate; 17 and above=Sensitive

**Minimum Inhibitory Concentration (MIC) of Neem and Lemon Crude Extracts**

Tables 7 shows theMinimum Inhibitory Concentration (MIC)of neem and lemon crude extracts. The MIC of neem crude extract was 100mg/mL for all test isolates and 50 mg/ml for the crude lemon extract*.*

**Table 7:** Minimum Inhibitory Concentration of Test Isolates

|  |  |
| --- | --- |
| Test isolate | Extract Concentration (mg/ml) |
| Neem | Lemon |
| *E. coli* | 100 | 50 |
| *Proteus* sp. | 100 | 50 |
| *Klebsiella* sp*.* | 100 | 50 |
| *Enterobacter* sp*.*  | 100 | 50 |
| *Pseudomonas* sp*.*  | 100 | 50 |

**DISCUSSION**

This study investigated the antimicrobial effect of neem and lemon extract against UTIs associated bacteria isolated from pregnant women attending antenatal care clinics at Ujeolen and Ihumudumu, Ekpoma. This is in line with current research aimed at using crude plant extract as alternative therapy to conventional antibiotics.

Bacterial colony count ranged from 2.4 x 105 - 4.6 x 105 CFU/mL (samples from Ujeolen) and from 3.1 x 105 - 5.2 x 105 CFU/mL (samples from Ihumudumu), which is a significant amount (of ≥10⁴ CFU/mL), indicative of bacteriuria, indicating a potential UTI. The overall incidence of UTI amongst the pregnant women was relatively high across both hospitals. The finding is comparable to the report of studies by Okonko *et al*. [24] and Tadesse *et al*. [25] who reported a bacterial count of ≥10⁴ CFU/mL.

The bacterial isolates were *Escherichia coli, Proteus sp., Klebsiella* sp*.,* *Pseudomonas* sp., and *Enterobacter* sp*.* which are common uropathogens. *Escherichia coli* emergedas the most dominant isolate, accounting (55%) and *Proteus* sp. the least occurring (5%) bacteria isolated from the pregnant women at Ujeolen Health Centre, and same *E. coli* was the most occurring (45.5%) and *Klebsiella* sp. the least occurring (18.2%) bacteria isolated from the pregnant women at Ihumudumu Health Centres. This is consistent with the report that *E. coli* is a common uropathogen [26]. The frequency of bacterial isolates indicates the dominance of Gram-negative enteric bacteria in UTIs in the study, which is likely attributed to the hormonal and anatomical during pregnancy. Gram-negative bacteria possess a unique structure that facilitates attachment to the uroepithelium and prevents removal by urine flow, enhancing their pathogenicity [27]. Also, this unique feature enhances bacterial growth and tissue invasion, which can result in pyelonephritis occurring during pregnancy. Additionally, risk factors could be improper genital hygiene, direction of wipe after wash, lack of postcoital urination, and catheterization, as reported by Addo [28] and Arias [29].

The antibacterial activities of the crude extract of neem and lemon demonstrated high efficacy at high concentrations. The bacterial isolates showed sensitivity to crude extract of lemon at 100 mg/mL and 75 mg/mL and crude neem extract at 400 mg/mL and 300 mg/mL, however they showed reduced antibacterial activity at 50 mg/mL and complete resistance at 25 mg/mL for the crude lemon extract, similarly a reduced antibacterial activity was also observed for the crude extract of lemon at 200 mg/mL, and complete resistance at 100 mg/mL. These result support the potential use of crude extract of neem and lemon for the inhibition of *E. coli, Proteus sp., Klebsiella* sp*.,* *Pseudomonas* sp., and *Enterobacter* sp.as earlier reported [30,31].

**CONCLUSION**

This study has confirmed the inhibitory potential of neem and lemon crude extract in the management of UTIs caused by *E. coli, Proteus sp., Klebsiella* sp*.,* *Pseudomonas* sp., and *Enterobacter* sp. The study presents a potential solution, using plant extract as a suitable alternative to combat antibiotic-resistant bacteria associated with UTIs.

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