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Assessing the Virulence Determinants and Effect of Methanolic Extract of *Syzygium aromaticum* on *Escherichia coli* Isolated from Recreational Water in Port Harcourt Metropolis, Nigeria

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ABSTRACT

Recreational water bodies are recognized as reservoirs for pathogenic Escherichia coli, posing a public health risk to users. This concerned is heightened by the rising prevalence of multidrug-resistant strains of Escherichia coli, which has intensified the need to explore plant-derived alternatives than conventional antibiotics. The aim of this study is to assess the virulence determinant and effect of Syzygium aromaticum (Clove) on Escherichia coli isolated from recreational water. A total of six (6) samples were aseptically collected from three (3) recreational water in Eneka in Port Harcourt and were subjected to standard microbiological analysis such as enumeration, isolation, identification, virulent factors (hemolysis, motility and biofilm formation) and antimicrobial sensitivity using the disc diffusion method. The Total Heterotrophic Bacterial Count (THBC) ranged from 52.0×10^4 to 8.5x10^4CFU/ml, Total Coliform Count (TCC) ranged from 28.4×10^3 to 18.3×10^3CFU/ml, and Fecal Coliform Count (FCC) ranged from 14.5×10^3 to 5.7×10^3CFU/ml. A total of thirty (30) bacterial isolates were isolated and characterized and 33.3% were confirmed as Escherichia coli with IMViC test. All the confirmed isolates were 100% positive for beta hemolysis, biofilm formation and Motility. The isolates were subjected to different concentrations (100%, 50%, 25%, and 12.5%) of Syzygium aromaticum methanolic extract using the agar disc(impregnation) diffusion method. At 100% the methanolic extract had zone of inhibition ranging from (20mm-26mm), at 50% (16mm-23mm), at 25% (12mm-18mm) and at 12.5% (4mm-10mm). The widest zones of inhibition were recorded at concentration of 100% and 50%. The minimum inhibitory concentration (MIC) of the methanolic clove extract against the confirmed Escherichia coli was 6.25 mg/ml. These findings confirm that the recreational water harbors virulent Escherichia coli strains and Syzygium aromaticum extract can be used as a strong antibacterial agent for infections arising from Escherichia coli. Regular microbiological monitoring of recreational water sources is recommended to protect public health risks.

Keywords: *Escherichia coli; Syzygium aromaticum; recreational water; virulence determinants; biofilm; antimicrobial activity.*

Introduction

Escherichia coli is a Gram-negative, non-spore-forming, facultative anaerobic, rod-shaped bacterium belonging to the family Enterobacteriaceae. It is a natural commensal of the gastrointestinal tract of humans and warm-blooded animals, but certain pathogenic strains harbour virulence determinants that enable them cause a broad range of infections including urinary tract infections, gastroenteritis, neonatal meningitis, and septicemia [1]. These pathogenic strains are classified into intestinal and extraintestinal pathotypes on the basis of the type of disease they cause and the virulence factors they express.

Recreational water bodies such as swimming pools are widely used for swimming and other leisure activities. However, these water bodies are frequently contaminated with fecal matter originating from human settlements and activities making them important reservoirs of pathogenic microorganisms, including *Escherichia coli*. The World Health Organization [2] recognizes *Escherichia coli* as a key indicator of fecal contamination of water and a significant public health threat in recreational water environments.

Exposure to contaminated recreational water places, individuals, particularly children, the elderly and immunocompromised persons are at risk of acquiring waterborne infections.

Syzygium aromaticum (clove), a member of the family Myrtaceae, is a widely cultivated tropical plant that has been used extensively in traditional medicine systems across Africa, Asia, and South America for the treatment of various ailments including toothache, digestive disorders, respiratory infections and inflammatory conditions [3]. The plant is rich in bioactive phytochemicals, predominantly eugenol (accounting for 72–90% of its essential oil), as well as eugenol acetate, β-caryophyllene, and flavonoids, which are collectively responsible for its documented antimicrobial, antifungal, anti-inflammatory, antioxidant and analgesic properties [4].

The increasing prevalence of multidrug-resistant (MDR) strains of *Escherichia coli* has created an urgent need to identify novel and effective antimicrobial agents. Plant-derived extracts such as those from *Syzygium aromaticum* has attracted considerable scientific interest as potential alternatives or complements to conventional antibiotics [3].

However, the specific effect of *Syzygium aromaticum* extract on pathogenic *Escherichia coli* isolated from recreational water, particularly in Nigeria, remains insufficiently studied. This study therefore seeks to fill this gap by isolating *Escherichia coli* from recreational water, assessing their virulence determinants and evaluating the antibacterial effect of *Syzygium aromaticum* extract on the *Escherichia coli* isolates.

Materials and Methods

Study Area

This study was conducted in three (3) different locations in Eneka, a suburban community located in Port Harcourt, Rivers State, Nigeria. The hotels where the water samples were collected includes; Daffodil Meridian Hotel with coordinates 4.875644°N,7.052731°E, Somsot Hotels with coordinates 4.878116°N,7.029333°E and SMD hotel and Suite which had the coordinates 4.894827°N,7.024339°E. These three locations were purposively selected because their swimming pools are open to public and visitor use and receive comparatively high patronage within Eneka. This made them suitable sites for assessing the actual, currently unmonitored and microbiological risk faced by recreational swimming pool users in the area.

Collection of Water Samples

A total of six (6) water samples were aseptically collected from three different recreational swimming pools within Eneka, Port Harcourt, Rivers State, namely: Danfodil Meridian Hotels, Somsot Hotels, and SMD Hotel and Suite, using a sterile, properly labelled sample bottles. Samples were collected aseptically from the swimming pools, by dipping the bottles about 15–20 cm below the water surface before opening it to allow water to flow in. The bottles were then closed immediately while still in the water to minimize contamination. All samples were collected in the morning and transported immediately to the laboratory in the department of microbiology Rivers State university in an ice-packed cooler within 2 hours of collection for bacteriological analyses.

Bacterial Enumeration and Isolation

Bacterial enumeration and isolation were performed using the standard serial dilution and spread plate techniques. Ten-fold serial dilutions of each water sample were prepared by transferring 1 mL of the sample into 9 mL of sterile normal saline, followed by successive dilutions to obtain countable bacterial colonies. For the enumeration of total heterotrophic bacteria (THB), 0.1 mL aliquots from the 10^{-3} dilution were aseptically inoculated onto Nutrient Agar plates in duplicate using the spread plate technique. 0.1 mL aliquots from the 10^{-2} dilution were inoculated onto MacConkey Agar for the determination of total coliform counts (TCC) and Eosin Methylene Blue (EMB) Agar for the enumeration of faecal coliform counts (FCC). Nutrient Agar and MacConkey Agar plates were incubated at 37°C for 24 hours, while EMB Agar plates were incubated at 44.5°C for 24 hours to facilitate the enumeration of thermotolerant coliforms such as *Escherichia coli* with characteristics green metallic sheen [5]. Following incubation, colonies were counted and bacterial populations were expressed as colony-forming units per millilitre (CFU/mL).

Determination of Virulence Factors of *Escherichia coli* Isolates

Hemolysis Test

A pure *E. coli* colony was streaked onto 5% blood agar and incubated aerobically at 37°C for 24 hours. The plates were then examined for the type of Hemolysis produced around the colonies. Complete lysis of red blood cells with a clear, transparent zone around the colony was recorded as beta (β)-Hemolysis and considered positive for hemolytic activity. Partial lysis with a greenish discolouration around the colony was recorded as alpha (α)-Hemolysis. No lysis of red blood cells was recorded as gamma Hemolysis [5].

Biofilm Formation Test

Biofilm formation was assessed using the Congo Red Agar (CRA) method, with Brain Heart Infusion (BHI) agar as the base medium. The medium was prepared by supplementing BHI agar with sucrose and Congo red dye, with the Congo red stain prepared separately as a concentrated aqueous solution and autoclaved before being added to the sucrose-supplemented BHI agar once cooled to about 55°C. This combination allows the dye to bind directly to the exopolysaccharide slime produced by biofilm forming organisms, so that positive isolates develop black, dry, crystalline colonies on the BHI based Congo red agar, while non-biofilm forming isolates remain pink or red. Test isolates were streaked onto the prepared BHI Congo red agar plates and incubated at 35°C for 24–48 hours before colonies were examined for colour change (black) [6].

Motility Test

Using a sterile inoculating needle, a fresh, pure bacterial colony was picked and gently stabbed straight down into the centre of the semi-solid motility agar tube, about two-thirds of the way to the bottom, and incubated at 37°C for 24-48 hours. Cloudy growth extending away from the stab line throughout the medium is positive. Negative growth is confined strictly to the stab line with clear surrounding medium.

Collection of Plant

Dried flower buds of *Syzygium aromaticum* (clove) were purchased from a local market in Eneka, Port Harcourt, Rivers State, Nigeria (4.899518°N, 7.036573°E). The plant materials were visually inspected and carefully selected to ensure they were clean, properly dried and free from visible fungal growth or contamination. The selected clove buds were subsequently transferred into clean, properly labelled containers and transported to the Microbiology Laboratory, Rivers State University, Port Harcourt, for further processing and extraction.

Preparation of Methanolic Extracts of *Syzygium aromaticum*

Dried clove buds of *Syzygium aromaticum* were obtained, cleaned, and ground into a fine powder using a sterile blender and extracted using methanol in 1:1 ratio.

Antibacterial Susceptibility Testing of *Escherichia coli* Isolates Using Methanolic Clove Extract.

Prior to testing, each pure *Escherichia coli* isolate was sub-cultured from glycerol stock onto freshly prepared nutrient agar and incubated at 37°C for 24-48 hours to obtain fresh, actively growing colonies for use in the assay.

The antibacterial activity of the clove extracts against the *Escherichia coli* isolates was determined using the disc diffusion method for the methanolic extract. The turbidity of each bacterial suspension was adjusted to 0.5 McFarland turbidity standards (equivalent to approximately 1.5 x 10^8 CFU/ml). A sterile cotton swab was dipped into the standardized bacterial suspension and rotated against the inner wall of the tube to remove excess fluid, then evenly streaked over the entire surface of a Mueller-Hinton Agar plate, rotating the plate after each streak to ensure a confluent lawn of growth [7]. The inoculated plates were allowed to stand for 10-15 minutes and impregnated disk of the methanolic extract was placed on the agar and incubated. The zones of inhibition were measured and recorded.

Determination of the Minimum Inhibitory Concentration (MIC)

To determine the minimum inhibitory concentration (MIC) of the *Syzygium aromaticum* extract, Clinical Laboratory standard Institute (CLSI) [8] tube dilution procedure was followed. Seven test tubes, each containing 5 ml of Mueller-Hinton broth, were labelled 1 to 7. One millilitre of each of five extract concentrations (100 mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, and 6.25 mg/ml) was introduced into tubes 1 to 5 respectively, and mixed thoroughly. To six (6) of the test tubes, 0.1 ml of a broth culture of the test organism was added, with the sixth tube serving as a positive control (broth and culture only, without extract), while the seventh tube served as a negative control (broth only, without extract or culture). All inoculated tubes were incubated at 37°C overnight, after which they were observed for visible bacterial growth (turbidity). The MIC was recorded as the lowest concentration of the extract that inhibited visible growth of the test organism.

Data Analysis

The data generated from the study were analyzed using descriptive statistics. The prevalence, virulence determinants and the mean zones of inhibition obtained from plant extract susceptibility assays are presented in tables where appropriate. Analysis of Variance (ANOVA) was used to compare the mean inhibition zones. Across the different extract concentrations at a significance level of ($p \leq 0.05$). Statistical analysis was done using SPSS version 27 [9].

RESULTS

The highest total heterotrophic bacterial count (THBC) was recorded at SMD Hotel and Suite ($52.0 \pm 6.3 \times 10^4$ CFU/mL), followed by Somsot Hotel and Suites ($45.5 \pm 5.8 \times 10^4$ CFU/mL) and Daffodil Meridian Hotel ($8.5 \pm 0.7 \times 10^4$ CFU/mL). The reported difference among locations was statistically significant ($p = 0.0058$). Total coliform counts (TCC) were highest at SMD Hotel and Suite ($28.4 \pm 2.7 \times 10^3$ CFU/mL), followed by Somsot Hotel and Suites ($21.5 \pm 2.1 \times 10^3$ CFU/mL) and Daffodil Meridian Hotel ($18.3 \pm 1.5 \times 10^3$ CFU/mL)

Faecal coliform counts (FCC) followed the same pattern, with $14.5 \pm 2.2 \times 10^3$ CFU/mL at SMD Hotel and Suite, $11.7 \pm 2.5 \times 10^3$ CFU/mL at Somsot Hotel and Suites and $5.6 \pm 1.1 \times 10^3$ CFU/mL at Daffodil Meridian Hotel ($p = 0.046$). (Table 1)

Thirty (30) bacterial isolates were isolated across the three sampling locations, ten (10) were identified as *Escherichia coli*, corresponding to an overall prevalence of 33.3% (Table 2).

All ten (10) isolates were 100% positive for the hemolysis test producing complete clear zones of lysis around their colonies on blood agar, consistent with Beta-hemolysis. All isolates were also positive to the biofilm formation test, producing black, dry, crystalline colonies on the BHI-based Congo red agar, confirming them as biofilm positive. All confirmed *Escherichia coli Escherichia coli* isolates were motile.

The confirmed *E. coli* isolates were subjected to different concentrations of the methanolic extract of *Syzygium aromaticum.* At 100% concentration, the extract produced the widest and most consistent zones of inhibition, ranging from 20 mm to 26 mm on all isolates, with a mean of (23.4 ± 1.9 mm). Every isolate was within the susceptibility range at this concentration, indicating that undiluted methanolic clove extract was highly effective against all the *Escherichia coli* isolates tested. At 50% concentration, inhibition remained strong, ranging from 16 mm to 23 mm with a mean of (19.5 ± 2.3 mm). The 100% and 50% concentrations represent the range at which the extract was most effective against *Escherichia coli*, consistently producing susceptible and intermediate across isolates.

The methanolic extract at 100% inhibited of the isolates with high zones of inhibition (23.4 ± 1.9), while 12.5% had the lowest zone of inhibition (7.0 ± 2.2). There was a significant difference ($P \leq 0.05$) for the different concentrations of the methanolic extract.

Using the tube dilution method, the minimum inhibitory concentration (MIC) of the *Syzygium aromaticum* extract against the confirmed *Escherichia coli* isolates was determined to be 6.25 mg/ml. This was the lowest concentration tested at which no visible turbidity (bacterial growth) was observed, while the positive control tube (broth and culture without extract) showed clear turbidity, confirming active bacterial growth in the absence of the extract.

Table 1: Mean Bacterial Counts from Recreational Water

Sampling location	THBC (× 10^4 CFU/mL)	TCC (× 10^3 CFU/mL)	FCC (× 10^3 CFU/mL)
Somsot Hotel and Suites	45.5 ± 5.8^a	21.5 ± 2.1^b	11.7 ± 2.5^b
Daffodil Meridian Hotel	8.5 ± 0.7^b	18.3 ± 1.5^a	5.6 ± 1.1^a
SMD Hotel and Suite	52.0 ± 6.3^a	28.4 ± 2.7^a	14.5 ± 2.2^b
Reported p-value	0.0058	0.039	0.046

THBC = total heterotrophic bacterial count; TCC = total coliform count; FCC = faecal coliform count. Means with the alphabet along the columns shows a significant difference (p≤0.05)

Table 2: Distribution and Percentage Prevalence of Escherichia coli

Sampling location	Confirmed *E. coli* (n)	Percentage (%)
Somsot Hotel and Suites	3	10.0
Daffodil Meridian Hotel	4	13.3
SMD Hotel and Suite	3	10.0
Total	10	33.3

Table 3: Positives of Phenotypic Virulence-associated Properties of E. coli isolates

Isolate	Hemolysis Test (β-hemolysis)	Biofilm Formation	Motility Test
Escherichia coli (n=10)	10(100%)	10(100%)	10(100%)

Table 4: Antibacterial Activity of Methanolic Syzygium aromaticum Extract (mm) Against Escherichia coli Isolates from Recreational Water

Isolate	100%	50%	25%	12.5%	Control (DMSO)
1	20	16	13	6	0
2	24	19	17	8	0
3	26	23	18	10	0
4	24	20	14	7	0
5	25	21	16	9	0
6	25	22	13	6	0
7	21	17	12	5	0
8	24	21	14	10	0
9	22	18	12	4	0
10	23	18	15	5	0
Mean ± SD	23.4 ± 1.9	19.5 ± 2.3	14.4 ± 2.1	7.0 ± 2.2	0.0 ± 0.0

DISCUSSION

The recreational water recorded high bacterial loads from the study. The heterotrophic counts provide an indication of the culturable bacterial population, while coliform measurements are useful indicators of sanitary quality. This pattern is consistent with concerns about swimming-pool water quality reported by [10]. However, this could also be as a result of disinfectant residuals, filtration performance or pool usage, so these practices can cause the high bacterial loads.

The higher faecal coliform counts at SMD Hotel and Suite, followed by Somsot Hotel and Suites, warrant particular attention because faecal indicator bacteria is a signal of contamination from gastrointestinal origin and possible defecation in the water body. The World Health Organization [2] emphasizes monitoring and management of faecal contamination in recreational waters to reduce exposure risks. [11] similarly documented thermotolerant coliforms in sewage and recreational waters in coastal Ecuador, demonstrating the broader relevance of microbiological surveillance. The recovery of *E. coli* is consistent with possible faecal contamination and aligns with the Nigerian swimming-pool investigation of [12], who also recovered *E. coli* from recreational water.

All *E. coli* isolates exhibited beta-haemolysis on blood agar. Haemolysis is a phenotypic characteristic that may be associated with bacterial cytotoxicity and haemolysin production is recognized among the virulence mechanisms of pathogenic *E. coli* strains [1, 13]. The universal positivity observed in this small group of isolates raises a potential public-health concern. However, beta-haemolysis confirm the presence of the hlyA gene or establish a pathogenic *E. coli* pathotype.

The *E. coli* isolates were motile and the possession of this feature can facilitate bacterial movement and interactions with surfaces and specific to pathogenic strains [14]. Thus, haemolysis, presumptive biofilm-associated phenotype and motility indicate is a characteristic that the bacteria are pathogenic.

The methanolic extract of *Syzygium aromaticum* inhibited the recovered *E. coli* isolates in a concentration-related manner. The study reports mean inhibition zones of 23.4 ± 1.9 mm at 100%, 19.5 ± 2.3 mm at 50%, 14.4 ± 2.1 mm at 25% and 7.0 ± 2.2 mm at 12.5%. The difference across extract concentrations was statistically significant ($p \leq 0.05$). These results suggest that increasing the concentration of the preparation increased antibacterial activity under the conditions tested.

The antibacterial findings are consistent with earlier work describing clove as a source of antimicrobial constituents. [15] and [16] reported antibacterial activity of clove essential oil, while [17] demonstrated antimicrobial and antibiofilm activities of *S. aromaticum* essential oil. [18] also investigated the antimicrobial activities of clove and black pepper.

Eugenol is widely discussed as a major biologically active constituent of clove, with membrane-associated effects proposed as one mechanism [4, 3]. Present investigation used a crude methanolic extract and demonstrated high level of antibacterial potency of this extract in corroboration with other studies.

The relevance of plant-derived antimicrobial research is further illustrated by [19], evaluated flavonoid and saponin fractions from ginger and garlic against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Although those extracts and test organisms differ from those used here, both investigations demonstrate the value of experimentally assessing botanical materials rather than assuming antimicrobial activity from traditional use. The minimum inhibitory concentration (MIC) of 6.25 mg/mL for the clove extract, supporting its inhibitory effect in broth under the conditions is a significant finding in this research and indicate clinical efficacy.

CONCLUSION AND RECOMMENDATION

The study identified *Escherichia coli* among bacteria recovered from recreational swimming pools in Eneka, Port Harcourt. The study recorded high bacterial load the confirmed *E. coli* exhibited beta-haemolysis, motility indicating characteristics that merit pathogenicity. Methanolic *Syzygium aromaticum* extract inhibited the *E. coli* in vitro, with larger inhibition zones at higher concentrations. The study reported the minimal inhibitory concentration of Methanolic *Syzygium aromaticum* extract as 6.25 mg/mL suggesting potential clinical efficacy but proper monitoring, cleaning and disinfection the recreational water body is advised to prevention infections arising *E. coli*.

DECLARATIONS

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Author contributions: Barika P.N. supervised the research and corrected the manuscript while Hakam, I.O. and Dike W.S. carried out the laboratory practical and drafted the manuscript.

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