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Evaluation of the Antibacterial and Antibiofilm Activity of *Erythrina senegalensis* Leaf Extract Against Multidrug-Resistant Bacteria

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Abstract: Biofilms are bacterial communities on surfaces within an extracellular matrix. Targeting biofilm-specific bacteria is crucial, and natural compounds with reported antibiofilm activity have garnered significant interest. The study evaluated the antibacterial and antibiofilm activity of *Erythrina senegalensis* leaf extract against multidrug-resistant (MDR) Gram-negative bacteria, including *Salmonella* Typhimurium, *S.* Typhi, *S.* Enteritidis, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. The leaf extract was prepared using aqueous and ethanol solvents, and qualitative phytochemical screening revealed the presence of various bioactive compounds such as tannins, saponins, cardiac glycosides, flavonoids, terpenoids, alkaloids, anthraquinone, reducing sugar, and ketones. A Kirby–Bauer disc diffusion assay was performed to test the susceptibility of antibiotics, and the antibacterial efficacy of the aqueous and ethanol extracts of *E. senegalensis* was determined using the cup-plate method, while the antibiofilm activities were determined using the crystal violet titer-plate method. The aqueous and ethanol extracts of *E. senegalensis* revealed the presence of tannins, saponins, cardiac glycosides, flavonoids, terpenoids, alkaloids, anthraquinone, reducing sugar, and ketones. The study found that the Gram-negative bacteria isolates that were MDR were *S.* Typhimurium, *S.* Enteritidis, and *P. aeruginosa*, while *K. pneumoniae* was resistant to beta-lactam and fluoroquinolones, and *S.* Typhi was susceptible to all antibiotics tested. Statistically, susceptibility to antibiotics had an inverse, weak, and significant relationship with biofilm production (r = −0.453, −0.106, −0.124, −0.106, −0.018, n = 10, $p < 0.05$). The aqueous extract showed good biofilm inhibition against *K. pneumoniae* and *P. aeruginosa*, and poor biofilm inhibition against *S.* Enteritidis, while *S.* Typhimurium and *S.* Typhi exhibited no biofilm inhibition. The ethanol extract did not demonstrate any antibiofilm activity against the tested Gram-negative pathogens. The study suggests that the Gram-negative bacteria's capacity to form biofilms is negatively associated with their antibiotic resistance phenotypes, and the aqueous extract of *E. senegalensis* exhibited moderate antibiofilm activity against *K. pneumoniae*, *P. aeruginosa*, and *S.* Enteritidis.

Keywords: antibiofilm; Gram-negative bacteria; antibacterial; MDR; *E. senegalensis*

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1. Introduction

The threat posed by microbial biofilms to global public health is increasing at an alarming rate and the diseases caused by these biofilms continue to grow rapidly, increasing multidrug resistance (MDR) and death [1–3]. Universally, biofilm-associated infections are more frequent in hospital settings because of their recalcitrant nature, and difficulty

in treatment and these infections currently range from 17.9% to 100% [4]. Biofilms are estimated to be responsible for more than 65% of nosocomial infections, approximately 80% of chronic infections, and 60% of all human bacterial infections [5,6]. In recent years, Gram-negative bacteria have been among the world's most significant public health problems due to their high resistance to antibiotics, and the rates of antibiotic-resistant Gram-negative bacteria associated with biofilm-forming activity have increased worrisomely, especially among healthcare-associated pathogens [7]. Gram-negative pathogens such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Salmonella enterica* are among the main causes of hospital-acquired and chronic infections, and are associated with high antimicrobial resistance, morbidity, and mortality [8,9]. Indeed, antibiotic-resistant *P. aeruginosa* infections are estimated to be associated with over 300,000 annual deaths; *K. pneumoniae* worldwide have a nosocomial prevalence of 8.7% and 10% as reported by Mohd-Asri et al. (2021) [10] with a mortality rate of 1.2 to 5 million globally [11–14]. In addition, 17.8 million cases of typhoid fever occur in low- and middle-income countries [15], with 9 million typhoid cases leading to 110,000 mortalities in the global annual report [16]. Nigeria has estimated about 364,791 typhoid cases, leading to 4232 deaths [17]. All these nosocomial Gram-negative bacteria are found at the top of the WHO priority list for the need for research and development of new strategies for infection management [3,18,19].

Antibiotic resistance can be due to the inherent ability of a microorganism to form surface-attached communities of cells within the extracellular polymeric matrix of polysaccharides and proteins, enzymes, and nucleic acids called biofilm, and can thereby enhance anchorage to any surface irreversibly [20–22]. The matrix confers antibiotic resistance through several ways such as expression of chromosomally encoded resistant genes, multidrug efflux pumps, type IV secretion systems, restriction diffusion of antibiotics into the biofilm matrix, reduction in growth rate, and even counteracting the host immunity and the action of antibiotic modifying enzymes [22–25]. Biofilm formation by antibiotic-resistant bacteria, especially Gram-negative bacteria, can contribute to its extensive dissemination of multidrug-resistant strains and virulence by causing persistent and recurrent chronic infections that are highly resistant to antibiotics and the host immune system, leading to high morbidity and mortality, and thus, posing a serious health crisis [26,27]. The increase in biofilm resistance demonstrated by bacteria to conventional antimicrobial treatments enhances the need to develop new strategies for management of bacterial infections [27]. In this regard, knowledge of bacterial isolates' production and antibiogram profile is very important for rendering reliable empirical antibiotic therapy to patients [28].

Erythrina senegalensis (known as Ugugwa-ula in Afo) has been used to treat malaria, jaundice, infections, gastrointestinal disorders (gastric ulcer, diarrhea, constipation), amenorrhea, dysmenorrhea, sterility, onchocerciasis, and body pain (chest pain, back pain, abdominal pain, headache, and body weakness). The plant has also been reported to have wound healing and contraceptive properties [29]. It is important for the treatment of bacterial infection and the antibacterial activity of this plant to be evaluated by many researchers, but studies on the antibiofilm properties of this plant have not been evaluated. However, antibiofilm properties of plants such as *Carvacrol* sp., *Eunicea* sp., *Ammona muricata*, *Carica papaya*, *Terminalia glaucescens*, *Entada abyssinica*, *Moringa oleifera*, *Lantana camara* have been reported [30–33]. Hence, this study will focus on determining the antibacterial and antibiofilm activity of *E. senegalensis* leaf extract against multidrug-resistant Gram-negative bacteria

This study's findings will have a positive impact because it will establish a better understanding of biofilm infection, virulence, and antibiotic resistance.

2. Material and Methods

2.1. Plant Materials

Collection and Identification

The leaves of the plant were collected at Mararaba Udege of Nasarawa Local Government Area, Nasarawa State, and were identified by Adeniji Adewale (Herbarium

Department, Federal College of Forestry, Bauch Ring Road, 930105 Jos, Plateau State, Nigeria).

2.2. Processing of Plant Material

The fresh leaves of the plant sample were separated, cleaned, and washed in sterile distilled water, air-dried for two weeks, and meshed into powder finely, using a pestle and mortar. The powdered form was stored in an airtight glass container protected from sunlight until required for analysis.

Preparation of Ethanol and Aqueous Leaf Extracts of *E. senegalensis*

The extract was prepared following the method proposed by Sanchez et al. (2010) [34]. Briefly, one hundred grams (100 g) of dried leaves of *E. senegalensis* was macerated in 500 mL of ethanol (HiMedia Laboratories Pvt. Ltd., Thane, MH, India) or distilled water for 24 h at 32 °C and under occasional shaking using a magnetic stirrer. The macerate was centrifuged at 3000 rpm for 20 min and filtered through Whatman no. 1 filter paper. The filtrate was concentrated in a rotary evaporator (Napco model 630, Portland, OR, USA) under low pressure at a temperature of 45 °C until completely dry.

2.3. Qualitative Phytochemical Screening of the Plant Extract

The extracts were subjected to phytochemical tests to identify secondary metabolites such as flavonoids, saponins, tannins, cardiac glycosides, terpenoids, phlobatannins, alkaloids, anthraquinone, reducing sugars, and ketones according to standard methods [35].

2.3.1. Terpenoids

One mL of acetic anhydride and 5 drops of concentrated sulfuric acid (H_2SO_4: HiMedia Laboratories Pvt. Ltd., Thane, MH, India) were added to the extract. The formation of a blue-green ring indicated the presence of terpenoids.

2.3.2. Test for Alkaloids

The extracts (20 µL) were applied on thin layer chromatography (TLC) plates (Silica Gel 60G, 5 × 10 cm) and eluted using toluene-ethyl acetate diethylamine (70:20:10) as a solvent system. Alkaloids were detected as orange-brown spots on TLC plates after spraying them with Dragendorff's reagent.

2.3.3. Test for Ketones

Two milliliters (2 mL) of an aqueous solution of the extract were added to a few crystals of resorcinol and an equal volume of concentrated HCl, and then heated over a spirit lamp flame and observed for a rose coloration that showed the presence of ketones.

2.3.4. Test for Anthraquinone

A few drops of magnesium acetate were added to 0.2 g of the molten extract. Pink color formation indicated the presence of anthraquinone.

2.3.5. Test for Free-Reducing Sugar (Fehling's Test)

Two milliliters (2 mL) of the aqueous solution of the extract in a test tube were added into a five milliliter mixture of equal volumes of Fehling's solutions I and II and boiled in a water bath for about 2 min. The brick-red precipitate was indicative of the presence of reducing sugars.

2.3.6. Test for Cardiac Glycosides

Two milliliters (2 mL) of the aqueous solution of the extract were added into 3 drops of a strong solution of lead acetate. This was mixed thoroughly and filtered. The filtrate was shaken with 5 mL of chloroform in a separating funnel. The chloroform layer was evaporated to dryness in a small evaporating dish. The residue was dissolved in a glacial

acetic acid containing a trace of ferric chloride; this was transferred to the surface of 2 mL of concentrated sulfuric acid in a test tube. The upper layer and interface of the two layers were observed for bluish-green and reddish-brown coloration, respectively, as indicative of the presence of cardiac glycosides.

2.3.7. Test for Flavonoids

A few fragments of magnesium metal ribbon (3–4 pieces) were added to 1 mL of ethanolic extract, followed by a drop-wise addition of concentrated hydrochloric acid. The formation of pink or red color indicated the presence of flavonoids [36].

2.3.8. Saponin

Two (2) mL of distilled water was added to extracts suspended in ethanol and shaken vigorously. The formation of a copious foam layer indicated the presence of saponins [36].

2.3.9. Test for Phlobatannins (HCl Test)

Two milliliters (2 mL) of the aqueous solution of the extract were added into diluted HCl and observed for a red precipitate that was indicative of the presence of phlobatannins.

2.3.10. Test for Tannins

Extracts were treated with 1 mL of 5% ferric chloride (HiMedia Laboratories Pvt. Ltd., Thane, MH, India). The presence of tannins was indicated by the formation of a bluish-black or greenish-black precipitate [37].

2.4. Test Organism

Stock cultures of *S.* Typhimurium, *S.* Typhi, *S.* Enteritidis, *K. pneumoniae*, and *P. aeruginosa* were obtained from the National Veterinary Research Institute Vom, plateau state, Nigeria on a tryptone soy agar (TSA) slant. Discrete colonies of the bacterium were isolated on Salmonella–Shigella agar (SSA) for *Salmonella* species and MacConkey agar for *K. pneumoniae* and *P. aeruginosa* incubated at 37 °C for 18 h, and then identified using biochemical tests [38].

2.5. Preliminary Susceptibility Testing

K. pneumoniae, *P. aeruginosa*, and all *Salmonella* isolates were tested for antibiotic susceptibility using the Kirby–Bauer disk diffusion method, and the results were interpreted as susceptible (S), intermediate (I), or resistant (R) according to the standards of the Clinical and Laboratory Standard Institute [39]. The antibiotic discs containing the following antibiotics were used: beta-lactam groups: amoxicillin (20/10 µg), augmentin (30 µg); sulfonamides: cotrimoxazole (25 µg); aminoglycoside: gentamicin (10 µg), streptomycin (10 µg); fluoroquinolones: ciprofloxacin (5 µg), pefloxacin (10 µg, sparfloxacin (30 µg), and ofloxacin (10 µg). The choice of antimicrobials was based on their importance both in veterinary medicine, due to their frequent use, and in human medicine, as they are treatment options [39].

The discs were aseptically placed on the surface of Mueller–Hinton agar (MHA) plates that have already been seeded with 0.5 McFarland standards of the test isolates and will be incubated at 37 °C for 18–24 h. After incubation, the diameters of the zone of inhibitions were observed and measured in millimeters accordingly. Isolates exhibiting resistance to three or more antibiotic classes were defined as MDR.

2.6. Biofilm Production Assay

Biofilm produced by *K. pneumoniae*, *P. aeruginosa*, and all *Salmonella* species were determined by crystal violet microtiter plate method with modification. *K. pneumoniae*, *P. aeruginosa*, and all *Salmonella* species were grown overnight for 24 h at 37 °C in Brain Heart infusion broth (BHIB: Oxoid Ltd., Basingstoke, UK) supplemented with 2% glucose and 2% sucrose. The cultures were diluted (50 µL in 99.99 mL BHIB) and 150 µL of the

cell suspension (final concentration of 1×10^5 CFU/mL) was used to inoculate a sterile flat-bottomed 96-well polystyrene microtiter plate (EUROPE GmbH, Kronberg, Germany). The microtiter plate containing the cell suspension was incubated for 40 h at 37 °C; the cell suspension after incubation was then poured off and the wells were gently washed 3 times in 3 different trays of water and dried in an inverted position. The dried wells were filled with 250 µL of 0.1% crystal violet (HiMedia Laboratories Pvt. Ltd., Thane, MH, India) solution in water and allowed to stain for 20 min. The stain was poured off and the wells were washed 3 times in 3 different trays of water and allowed to dry. The presence of a layer of stained materials adhered to the inner wall of the wells was taken as a positive biofilm formation result. The biofilms to be formed were quantified by adding 250 µL of ethanol–acetic acid (95:5 *v/v*) to destain the wells. Exactly 100 µL of solubilized dye from each well was transferred to a new microtiter plate and the absorbance (A) of the solution was measured at 450 nm using a microtiter plate enzyme-linked immunosorbent assay (ELISA) reader. Each assay was performed in triplicate. The control was uninoculated media, to determine background absorbance. The mean A_{450} value from the control well was subtracted from the mean A_{450} value of the test wells which gave the amount of biofilm produced.

2.7. Determination of Antibacterial Activity

The antimicrobial activity of ethanolic and aqueous leaf extracts was evaluated using a cup-plate agar diffusion assay. Briefly, 100 µL of fresh Mueller–Hinton broth culture (approximately 10^6 CFU/mL) was spread uniformly on sterile Mueller–Hinton agar plates and allowed to air-dry. After this, 6 mm wells were made in the Mueller–Hinton agar (MHA) plates using sterilized cork borer, and the base was sealed with melted MHA. Exactly 100 µL of 50.0–200.0 mg/mL concentrations of the extract was prepared in 10% (*w/v*) dimethyl sulfoxide corresponding to 50.0 mg, 100.0 mg, 150.0 mg, and 200.0 mg of the extract, which was dispensed into the wells. The plates were allowed to stand for 1 h at 32 °C for pre-diffusion and placed in an incubator at 37 °C for 24 h. The diameter of zones of inhibition against the test bacteria was measured and recorded. Ciprofloxacin at 20 mg was used as the positive control.

2.8. Statistical Analysis

All assays were performed in triplicate and data were presented as mean ± standard deviation. A Pearson correlation coefficient test was used to establish a statistical relationship between the biofilm-forming capacity and the antibiotic resistance phenotypes among the Gram-negative pathogens (p-value < 0.05). Statistical analyses were performed using Microsoft Excel and SPSS 26.0.

3. Results and Discussion

3.1. Phytochemical Properties of Aqueous and Ethanolic Leaf Extracts of E. senegalensis

In the qualitative phytochemical analysis of aqueous and ethanolic leaf extracts of *E. senegalensis*, tannins, saponins, and flavonoids were all present, while phlobatannins were absent in both aqueous and ethanolic extracts of the leaf. Cardiac glycosides, terpenoids, alkaloids, anthraquinone, reducing sugars, and ketones were all present in the aqueous extract but absent in the ethanolic leaf extract (Table 1). Overall, the aqueous extract contained more phytochemical constituents. Phytochemical screening using aqueous leaf extract of *E. senegalensis* showed that they contained appreciable amounts of bioactive compounds such as terpenoids, reducing sugar, tannins, saponins, flavonoids, anthraquinone, cardiac glycosides, and ketones (Table 1). This agrees with the findings of Joshua et al. (2020) [40], which suggests that these bioactive compounds were consistent with the aqueous extraction of *E. senegalensis*. The ethanol leaf extract of *E. senegalensis* showed that they contained fewer bioactive components (tannins, saponins, flavonoids) in trace amounts compared to the aqueous extract. This shows that the aqueous extract extracted more bioactive constituents than its ethanolic counterpart, and this finding agrees with Kossonou et al. (2020) [41]. The

result of this phytochemical screening in this study is in contrast with previous studies that alcoholic solvents like ethanol and methanol are more suitable than other solvents such as water in extracting bioactive constituents of medicinal plants [42–45]. Solvents of high polarity such as water (Snyder Polarity Index of 9.0) have the ability and are more efficient in the extraction of plant material containing high levels of polar compounds [45–49]. Therefore, it is suggested that the effectiveness of aqueous solvent in the extraction of high yield and reasonable quantity of bioactive constituents compared to the ethanol solvent was due to the solvent polarity, solubility, and nature of the bioactive component in the plant material. Several authors have revealed that secondary metabolites are responsible for the biological activities of plant extracts [50–53]. Therefore, the presence of these secondary metabolites in this study confirms the traditional use of *E. senegalensis* as medicine [54].

Table 1. Phytochemical constituents of aqueous and ethanolic leaf extracts of *E. senegalensis*.

Phytochemicals	Aqueous	Ethanolic
Tannsins	+	+
Saponins	+	+
Cardiac glycosides	+	−
Flavonoids	+	+
Terpenoids	+	−
Alkaloids	+	−
Anthraquinone	+	−
Reducing sugar	+	−
Ketones	+	−
Phlobatannins	−	−

Present (+); absent (−).

3.2. Antibiotic Susceptibility Profile

The antibiotic susceptibility result of the tested bacterial isolates is presented in Table 2. The disk diffusion test was used to determine the zones of inhibition of the isolates and was interpreted based on the Clinical and Laboratory Standard Institute (CLSI) breakpoint values which revealed that *S.* Typhimurium was resistant to fluoroquinolones, beta-lactam, and aminoglycosides; *S.* Enteritidis was resistant to beta-lactam, fluoroquinolones, and aminoglycosides; *K. pneumoniae* was resistant to beta-lactam, and fluoroquinolones; and *P. aeruginosa* was resistant to all five classes of antibiotics: sulfonamides, chloramphenicol, beta-lactam, fluoroquinolones, and aminoglycosides. *S.* Typhi was susceptible to all the antibiotics in the classes. *S.* Typhimurium, *S.* Enteritidis, and *P. aeruginosa* were therefore considered multidrug-resistant isolates, since they were resistant to three or more classes of antibiotics (Table 2).

Table 2. Antibiotic susceptibility profile of bacterial isolates.

Isolate	Diameter of Zone of Inhibition (mm) (Mean ± S.D) of the Antimicrobials									
	SXT	CH	SP	CPX	AM	AU	CN	PEF	OFX	Streptomycin
Salmonella Typhimurium	22± 0.00 S	38 ± 0.71 S	0 ± 0.00 R	14 ± 0.00 R	0 ± 0.00 R	0 ± 0.00 R	0 ± 0.00 R	0 ± 0.00 R	6 ± 1.41 R	4 ± 0.00 R
Salmonella Typhi	28 ± 0.00 S	30 ± 0.00 S	30 ± 0.71 S	29 ± 1.41 I	30 ± 0.00 S	31 ± 1.41 S	28 ± 0.71 S	30 ± 0.00 S	30 ± 0.71 S	30 ± 0.00 S
Salmonella Enteritidis	31 ± 1.41 S	30 ± 0.71 S	18 ± 0.00 S	22 ± 0.00 I	0 ± 0.00 R	0 ± 0.00 R	24 ± 0.00 S	17 ± 1.41 R	18 ± 0.00 S	0 ± 0.00 R
Klebsiella pneumoniae	18 ± 0.00 S	29 ± 1.41 S	21 ± 1.41 S	32 ± 0.00 S	10 ± 0.71 R	8 ± 0.71 R	14 ± 0.00 I	20 ± 0.00 R	22 ± 0.00 S	14 ± 0.71 I
Pseudomonas aeruginosa	0 ± 0.00 R	0 ± 0.00 R	14 ± 0.00 I	17 ± 1.41 I	0 ± 0.00 R	0 ± 0.00 R	13 ± 1.41 I	8 ± 0.00 R	10 ± 0.71 R	4 ± 0.00 R

SD = standard deviation; R = resistant; I = intermediate; S = susceptible; SXT = cotrimoxazole; CH = chloramphenicol; SP = sparfloxacin; CPX = ciprofloxacin; AM = amoxicillin; AU = augmentin; CN = gentamicin; PEF = pefloxacin; OFX = ofloxacin.

This study revealed in Table 2 that *S.* Typhimurium, *S.* Enteritidis, and *P. aeruginosa* were multidrug-resistant Gram-negative bacterial isolates, and they exhibited resistance (100%) to beta-lactams, fluoroquinolones, and aminoglycosides classes of antibiotics. In contrast to these resistance percentage frequencies was *S.* Typhi, which was the only Gram-negative isolate interestingly susceptible (90%) to all the antibiotics except for ciprofloxacin. Ciprofloxacin is the antibiotic currently in use for the treatment of *Salmonella* infection, and in this study, it was observed that *S.* Typhimurium, intermediate against *S.* Typhi and *S.* Enteritidis, and *P. aeruginosa*, susceptible against *K. pneumoniae*, were all resistance to this antibiotic. In Nigeria, beta-lactams, cephalosporin, aminoglycosides, fluoroquinolones, and tetracycline are widely used in treating wide arrays of bacterial infections (such as urinary tract infection, enteritis, and pneumonia) and may likely lead to selective pressure, hence enhancing the evolution and development of multidrug bacterial pathogens [55]. Studies carried out by Duse (2015) [56], Eggleston et al. (2010) [57], Elsayed et al. (2017) [58], and Tansarli et al. (2013) [59] recognize improper dosage, antimicrobial use, and duration of administration as risk factors encouraging the emergence of antibiotic-resistant strains in a community. Some literature in recent years has shown the role enacted by multidrug efflux pumps in the capacity of *Salmonella* species to form a biofilm [60] Therefore, the production fitness of biofilm and resistance tends to be related [61] and obviously leads to therapeutic failure [62,63].

3.3. Biofilm Forming Potentials of the Isolates

For comparative analysis, the OD_{450nm} values were used to classify quantitatively biofilm production for the bacterial isolates according to the method described in Stepanovic et al. (2000) [64]. Briefly, the cut-off OD (ODc) was defined as three standard deviations above the mean OD of the negative control and strains were classified as follows: OD < ODc = poor biofilm producer; ODc < OD < 2 × ODc = weak biofilm producer; 2 × ODc < OD < 4 × ODc = moderate biofilm producer; and OD > 4 × ODc = high biofilm producer [65].

Based on the biofilm-forming potentials of the bacterial isolates used in this study, all isolates tested were biofilm producers, *S.* Typhimurium (0.0376), *S.* Typhi (0.0557), *S.* Enteritidis (0.0526), *K. pneumoniae* (0.0438), and *P. aeruginosa* (0.0512) (Figure 1). The ODc values were used in the standard biofilm categorization formula to determine the biofilm-forming potential. All the isolates were considered weak biofilm formers based on this categorization [65].

3.4. Antibacterial Activity of Aqueous and Ethanolic Extract of E. senegalensis

The antibacterial activity of aqueous extracts of *E. senegalensis* against Gram-negative bacterial isolates, as shown in Figure 2, revealed insignificant effects across all concentrations. Ciprofloxacin (control) showed significant inhibition zones of 36 mm ± 0.71 for *S.* Typhi and 36 mm ± 0.71 for *K. pneumoniae* and insignificant inhibition zones of 0.0 mm ± 0.0 each for *S.* Typhimurium, *S.* Enteritidis, and *P. aeruginosa* (Figure 2). Similarly, the ethanolic extracts of *E. senegalensis* demonstrated insignificant antibacterial activity against the Gram-negative bacterial isolates at all concentrations (Figure 3). Ciprofloxacin (control) again showed inhibition zones of 37 mm ± 0.71 for *S.* Typhi and 36 mm ± 1.41 for *K. pneumoniae*, and insignificant inhibition zones of 0.0 mm ± 0.0 each for *S.* Typhimurium, *S.* Enteritidis, and *P. aeruginosa* (Figure 3).

Figures 2 and 3 illustrate that the antibacterial activity of both aqueous and ethanolic extracts of *E. senegalensis* showed no inhibitory effects against the tested Gram-negative bacteria, in contrast to the broad-spectrum antibiotic ciprofloxacin, which showed inhibition zones ranging from 36 mm to 37 mm for both *S.* Typhi and *K. pneumoniae*. This result contrasts with previous studies [66], which reported the antibacterial activity of *E. senegalensis* against several bacteria including *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, *S.* Typhi, and *P. aeruginosa*, and antifungal effects against various fungi. Kone et al. (2007) [67] also reported effectiveness against resistant strains of *S. pneumoniae*, findings that contrast with the present study.

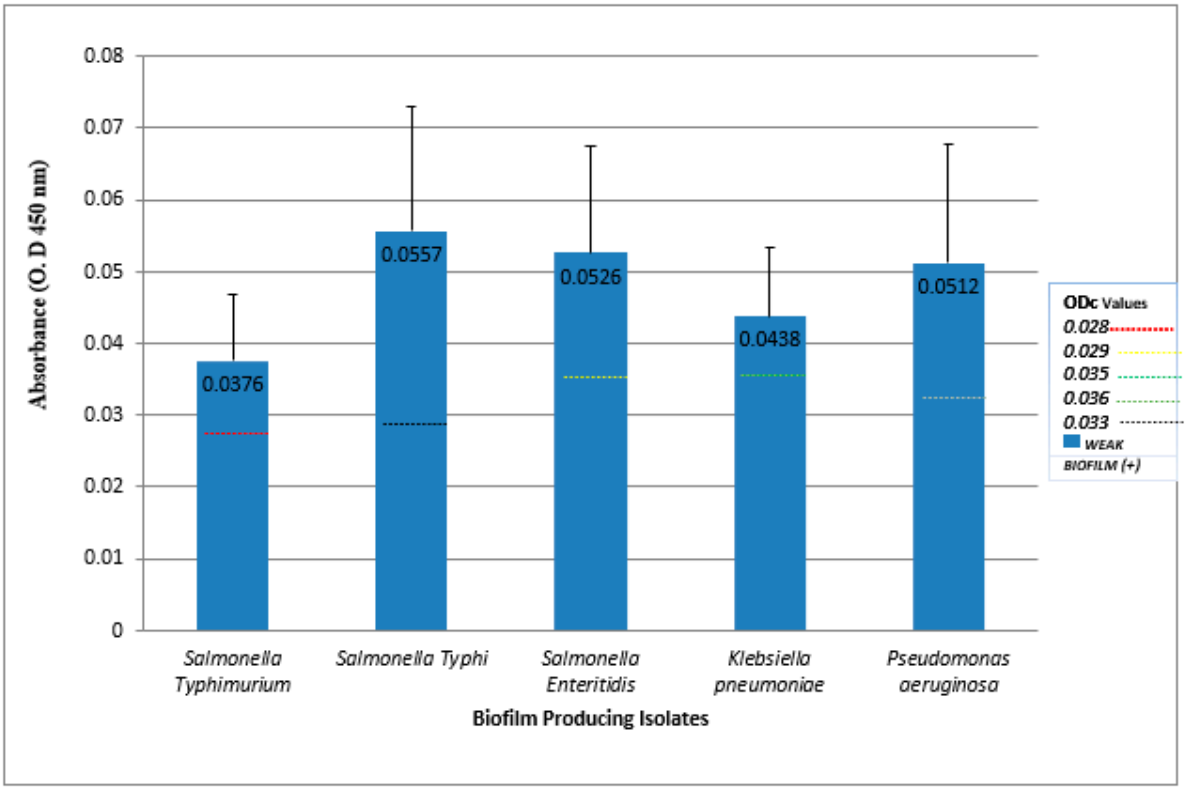

Figure 1. Biofilm-forming potential and biomass by Gram-negative bacteria isolates. ODc = Optical density cut-off values.

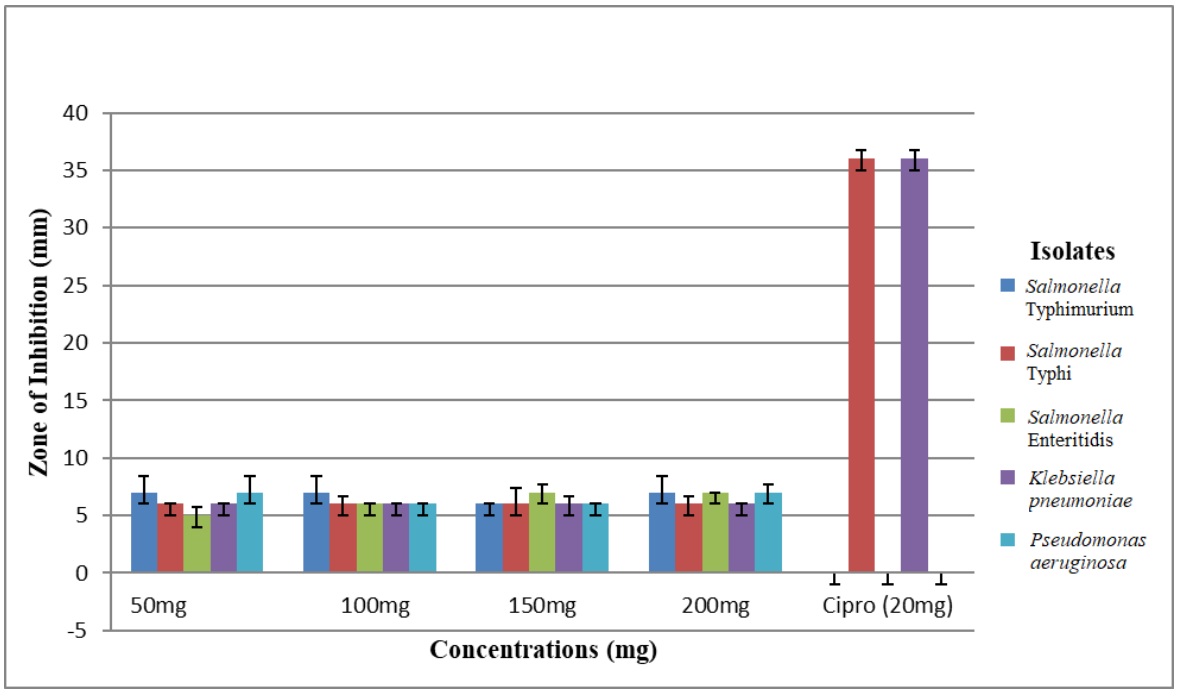

Figure 2. Antibacterial activity of *E. senegalensis* aqueous leaf extract against bacterial isolates. Cipro = ciprofloxacin.

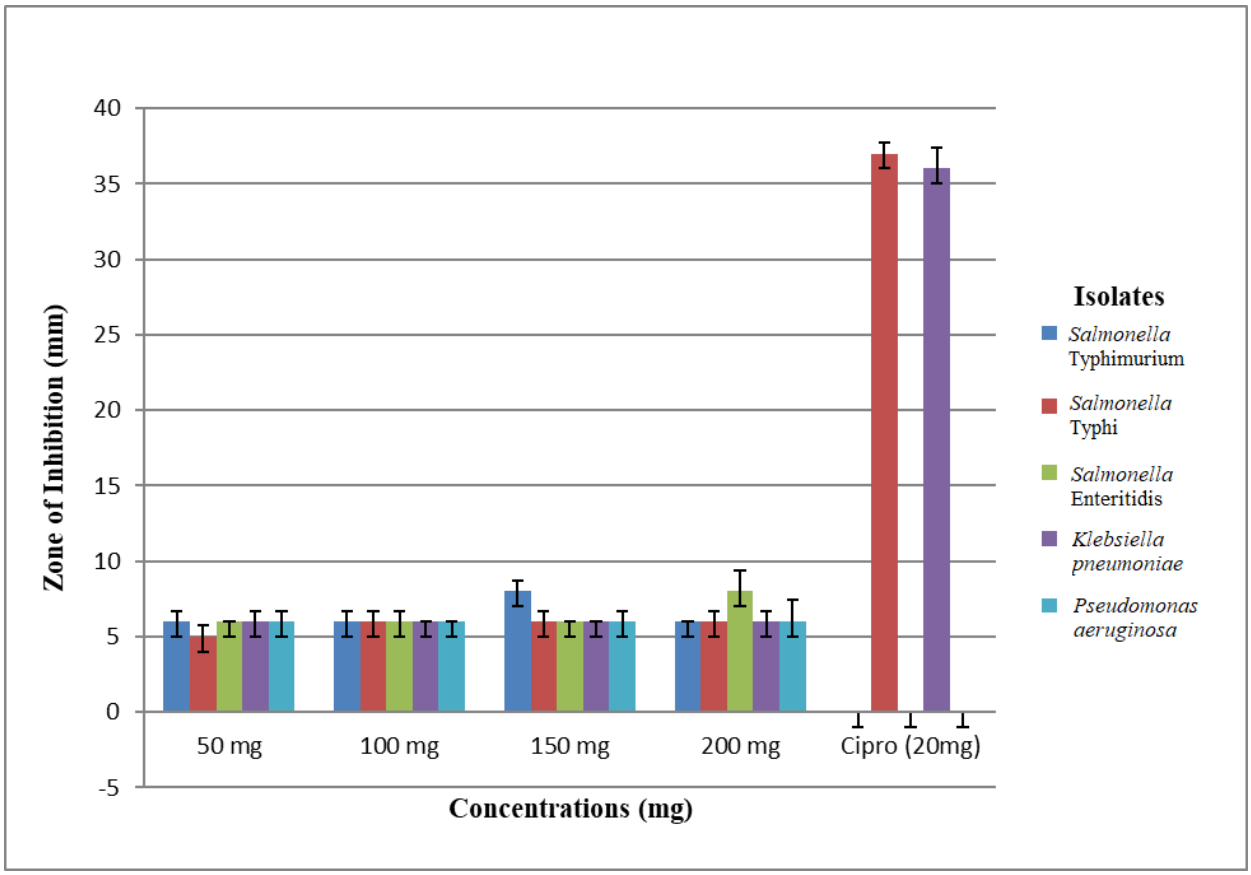

Figure 3. Antibacterial activity of *E. senegalensis* ethanolic leaf extract against bacterial isolates. Cipro = ciprofloxacin.

Although several studies have reported that bioactive components of *E. senegalensis* such as flavonoids, phenolics, terpenoids, alkaloids, and saponins—possess antimicrobial, antibacterial, and other therapeutic properties [68–73], these components were not effective against Gram-negative bacterial isolates in the current study. This lack of activity may be attributed to various reasons such as a complex structure and composition of the Gram-negative outer membrane or the insufficient concentration of the inhibitory bioactive compound against these Gram-negative bacteria. Extraction methods and solvents used to isolate the bioactive compound may not be optimal for retaining bioactive compound responsible for the antibacterial activity, and the variability in plant sources such as plant species, geographical location, and growing conditions can affect the antibacterial efficacy of the plant extract.

3.4.1. Antibiofilm Activity of Aqueous Leaf Extract of *E. senegalensis*

The biofilm percentage inhibition results of the aqueous *E. senegalensis* extract against bacterial isolates are shown in Figure 4. The extract demonstrated good antibiofilm activity against *K. pneumoniae* with inhibition percentages of 54.32% at 100 mg, 57.73% at 200 mg, 58.13% at 300 mg, and 58.53% at 400 mg. However, the extract showed poor antibiofilm activity against *S.* Enteritidis at lower concentrations (15.41% at 100 mg, 12.96% at 200 mg, 8.93% at 300 mg), and even promoted biofilm formation at 400 mg (−7.18%). For *P. aeruginosa*, the extract showed moderate antibiofilm activity at 100 mg (42.97%) and 400 mg (49.27%), but high activity at 200 mg (64.02%), and moderate activity at 300 mg (29.78%). The extract had no inhibitory effect on biofilm formation for *S.* Typhimurium and *S.* Typhi, showing negative percentages across all concentrations (Figure 4).

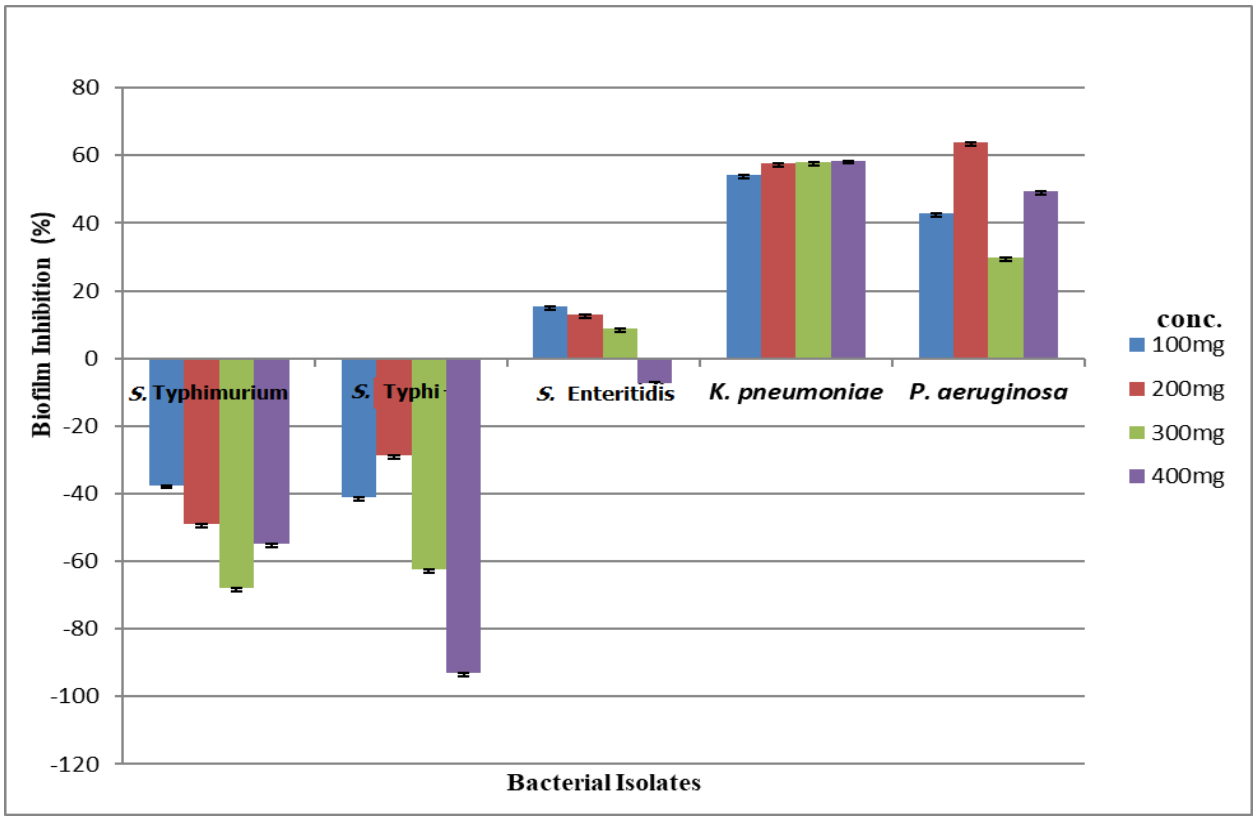

Figure 4. Inhibition of bacterial biofilm by aqueous leaf extract of *E. senegalensis*. Conc.= concentration of the plant.

The ability of the aqueous *E. senegalensis* extract to inhibit biofilm formation in *K. pneumoniae* suggests it may be a useful tool for reducing microbial colonization on surfaces and mucosal epithelia, potentially preventing serious infections [74]. The antibiofilm activity against *K. pneumoniae* is thought to be due to the extraction solvent and the presence of bioactive compounds such as phenolics, flavonoids, quinones, alkaloids, terpenoids, reducing sugars, and cardiac glycosides, which may inhibit quorum sensing and biofilm production [75–77]. It is noted that plant extracts have bioactive compounds with the following antibiofilm activity such as enzymatic or chemical properties which can degrade extracellular polymeric substance (EPS) of biofilm [78,79], interference with bacterial communication strategies (quorum sensing) [80], bacterial adhesion proteins preventing initial adherence to surfaces [81], direct killing of bacterial within the biofilm matrix [82–84], inhibit efflux pump (which is the main mechanism of resistance in biofilm formation) and thereby reducing biofilm production.

3.4.2. Antibiofilm Activity of Ethanolic Leaf Extract of *E. senegalensis*

The biofilm percentage inhibition results of the ethanolic *E. senegalensis* extract against bacterial isolates are shown in Figure 5. The extract exhibited no inhibitory effect on biofilm formation for any of the bacterial isolates tested: *S.* Typhimurium (−83.08% to −906.25%), *S.* Typhi (−590.14% to −1265.52%), *S.* Enteritidis (−118.13% to −981.95%), *K. pneumoniae* (−85.80% to −890.03%), and *P. aeruginosa* (−332.45% to −732.24%) across all concentrations (100 mg, 200 mg, 300 mg, 400 mg) (Figure 5).

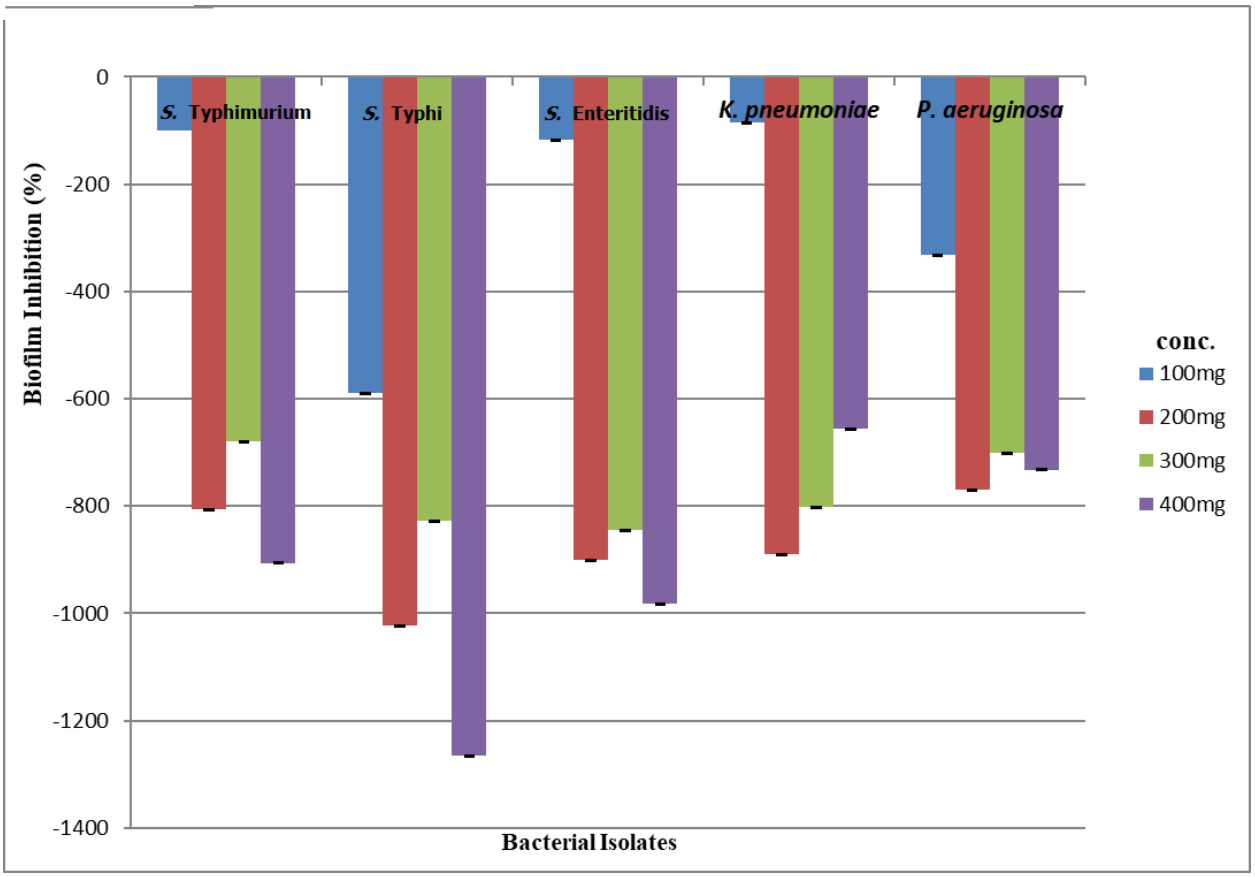

Figure 5. Inhibition of bacterial biofilm by ethanolic leaf extract of *E. senegalensis*. Conc. = concentration of the plant.

The study suggests that higher biofilm formation, despite increasing extract concentrations, may result from compounds in the extract that enhance microbial growth, thereby increasing biofilm production. This aligns with previous reports indicating that some natural compounds can enhance microbial growth and biofilm production [85–87]. The inability of the extract to inhibit biofilm formation may be attributed to the complex outer membrane structure of Gram-negative bacteria, which resists penetration by the extract, as well as the presence of certain enzymes in the periplasmic space [88–90]. Additionally, it could be attributed to the fact that ethanol is a polar solvent which extracts a broad range of bioactive compounds such as sugars, amino acids, and other nutrients that may serve as substrates that may promote bacterial growth or biofilm formation [91]. Bacteria can respond differently to extract compositions; ethanol in the extract may upregulate biofilm-related genes and trigger specific pathways that promote biofilm formation, while, in contrast, aqueous extracts may interfere with these pathways or induce stress responses that inhibit biofilm formation [92].

The study evaluated the relationship between biofilm-forming capacities and antibiotic resistance phenotypes among Gram-negative pathogens. It found a negative correlation between biofilm formation and antibiotic resistance phenotypes ($r = -0.106$ to -0.453, $n = 10$, $p < 0.05$). Non-resistant *S.* Typhi formed larger biofilm biomass compared to non-MDR *K. pneumoniae* and MDR isolates like *S.* Typhimurium, *S.* Enteritidis, and *P. aeruginosa*. This finding aligns with studies showing no significant difference in biofilm formation between multidrug-resistant (MDR) and non-multidrug-resistant isolates [93]. High biofilm production rates in *K. pneumoniae* have been reported in MDR strains, particularly those producing ESBL and harboring *bla* CTX-M genes [94]. Quinolone resistance has been linked to reduce biofilm production in both uropathogenic *E. coli* and *S.* Typhimurium [61,95], while imipenem resistance has been associated with lower biofilm production in *P. aeruginosa* isolates [96]. Dis-

crepancies in findings may be due to variations in interpretation and different methods used to assess biofilm production, such as the Congo red plate assay [97], crystal violet assay [98], and microtiter plate assay [99,100].

The study had some limitations including the use of only leaves as the plant material and only two extracting solvents other than exploring other plant parts and solvents. Additionally, using other various methods to evaluate the extract's effect on the biofilm formation would have provided better comparisons and inferences.

4. Conclusions

In this study, *E. senegalensis* leaf extract had moderate antibiofilm activity which could be attributed to the presence of different essential phytochemicals, which are present in the leaf. The plant extracts had a poor antibacterial activity which could be since the Gram-negative isolates were multidrug-resistant and thereby intrinsically possessed a structural makeup difficult for the extract to penetrate. The capacity of the Gram-negative bacteria to form biofilm in this study is negatively associated with the antibiotic resistance phenotypes.

5. Recommendations

On finding based on the study, the following recommendations were made:

i. The findings of this study suggest that *E. senegalensis* can serve as a natural source with the potential to inhibit cell attachment (biofilm) or can be explored as an antibiofilm agent against *K. pneumoniae*, *P. aeruginosa* and *S.* Enteritidis in the healthcare system.
ii. The result from the study strongly recommends aqueous solvent be the standard solvent in the extraction of bioactive components from *E. senegalensis*, since the plant contains high polar bioactive compounds that can easily be extracted using water.
iii. Since the ability of these Gram-negative bacteria to form biofilm is not associated with their antibiotic profile, routine biofilm monitoring is critical for improving the quality of treatment strategy for infection associated with biofilm.
iv. Evaluation of the synergistic effects of this plant extract with conventional antibiotics is suggested for further studies

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