Neem-Based Green Biocide for Mitigating Crude Oil Souring: Inhibition of Sulfate-Reducing Bacteria

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Abstract
Crude oil souring, driven by the metabolic activity of sulfate-reducing bacteria (SRB), presents significant operational and environmental challenges in petroleum production. The generation of hydrogen sulfide (H_2S) by SRB contributes to reservoir plugging, infrastructure corrosion, decreased oil quality, and increased processing costs. Conventional synthetic biocides used to control SRB activity often exhibit environmental persistence and toxicity, raising concerns over long-term ecological safety. This study investigates the potential of neem (Azadirachta indica) extracts as a green, sustainable biocidal alternative for SRB mitigation. Neem leaves were harvested, dried, and subjected to aqueous and ethanolic extraction. The phytochemical constituents of the extracts were analyzed using the method of Gas Chromatography (GC) and Fourier Transform Infrared Spectroscopy (FTIR), revealing the presence of bioactive compounds including azadirachtin, nimbin, and quercetin. Anaerobic inhibition assays were performed using Desulfovibrio spp. under simulated oilfield conditions. Over a 14-day incubation period, key parameters such as sulfate concentration, hydrogen sulfide production, pH, redox potential, and microbial biomass were systematically monitored. The results demonstrated significant suppression of SRB activity in neem-treated setups, with ethanolic extracts showing higher efficacy. H_2S levels were reduced by over 70% compared to untreated controls, while sulfate reduction rates and microbial growth were notably diminished. FTIR spectra indicated potential cell wall disruption, and GC data supported the antimicrobial role of specific neem phytochemicals. These findings confirm the promise of neem-based biocides as effective, low-toxicity, and biodegradable alternatives for managing microbial souring in petroleum systems, contributing to more environmentally responsible oilfield operations.

Keywords: *Crude oil souring, sulfate-reducing bacteria, neem extract, green biocide, hydrogen sulfide, oilfield microbiology.*

1.0 Introduction

The proliferation of sulfate-reducing bacteria (SRB) in crude oil production systems poses significant challenges to the oil and gas industry, primarily due to the production of hydrogen sulfide (H_2S), which leads to crude oil souring. This phenomenon not only deteriorates the quality of the crude oil but also enhances the corrosion of equipment and pipelines, thereby increasing operational costs and safety risks (Gieg et al., 2011; Prajapat et al., 2019). The control of microbial souring is, therefore, crucial for maintaining the integrity and efficiency of oil production systems.

Traditional methods for controlling souring include the use of chemical biocides and nitrate treatment. Chemical biocides, such as glutaraldehyde and quaternary ammonium compounds, have been widely used to inhibit microbial growth (Gieg et al., 2011). However, the repeated application of these chemicals can lead to the development of resistant microbial populations, reducing their effectiveness over time (Telang et al., 1998). Nitrate treatment, on the other hand, has been recognized as a more environmentally friendly alternative, promoting the growth of nitrate-reducing bacteria that can outcompete SRB for electron donors or directly inhibit SRB activity (Hubert et al., 2005). Despite these advances, the search for sustainable and effective solutions to mitigate crude oil souring continues.

In recent years, there has been a growing interest in the use of natural products as green biocides for controlling microbial growth in various industrial settings. Plant extracts, in particular, have shown promise due to their antimicrobial properties and environmental sustainability (Pandey et al., 2019). Among these, neem (*Azadirachta indica*) extract has been widely studied for its broad-spectrum antimicrobial activity, attributed to its rich content of bioactive compounds such as azadirachtin and nimbin (Pinto et al., 2014). Previous studies have demonstrated the effectiveness of neem extract in inhibiting microbial growth in various applications, including agriculture (Senthil Nathan et al., 2008) and medicine (Subapriya & Nagini, 2005).

Several studies have explored the use of plant extracts as biocides in oilfield environments. Jalali et al. (2021) reported that plant extracts such as garlic and thyme oil exhibited antimicrobial activity against SRB. Similarly, Szczepańska et al. (2020) demonstrated the effectiveness of plant extracts in inhibiting SRB growth and biofilm formation. These findings suggest that plant extracts, including neem extract, may offer a promising alternative to traditional chemical biocides for controlling microbial souring in oil production systems.

This study aims to investigate the potential of neem extract as a green biocide for mitigating crude oil souring by inhibiting SRB. By evaluating the antimicrobial efficacy of neem extract against SRB and exploring its impact on biofilm formation, this research seeks to contribute to the development of more sustainable and environmentally friendly strategies for managing microbial souring in oil production systems. The findings of this study could provide valuable insights into the application of natural products in controlling microbial growth and mitigating the adverse effects of crude oil souring.

2.0 Methodology

2.1 Study Site and Sample Collection

This study was conducted in the Niger Delta region of Nigeria, specifically in the offshore facilities of an oil-producing company situated in the Atlantic Ocean off the coast of Akwa Ibom State. The study sites were geolocated at the following GPS coordinates: Floating Storage and Offloading (FSO- 04°06.18'N, 008°10.16'E), WFB (04°05.58'N, 008°10.22'E)) vessel, Mobile Offshore Production Unit (MOPU-04°05.58'N, 008°10.22'E), and other nearby facilities (CFBX (04°06.01'N, 008°10.20'E), and seawater sources (04.102114°N, 08.167839°E; 04.100477°N, 08.172436°E). Samples of crude oil, injection water, produced water (post-injection mixture of water and crude oil), and seawater were collected following the method proposed by Thakur et al. (2012), with slight modification, and in accordance with APHA standard procedures for sample collection and preservation (APHA, 2017). Samples were collected in sterile, airtight plastic containers and immediately transported to the laboratory in an ice-chest coolers for further analysis.

2.2 Sample Preparation and Sterilization

All materials and equipment used for analysis were sterilized to eliminate any residual microbes present on them. Glassware was sterilized in a hot air oven at 160°C for 1 hour, while media were sterilized using an autoclave at 121°C for 15 minutes at 15 psi, following standard procedures (APHA, 2017). The workbench was disinfected with 70% ethanol before and after use to maintain aseptic conditions (APHA, 2017; Cheesbrough, 2006).

2.3 Isolation and Characterization of Sulfate-Reducing Bacteria

Sulfate-reducing bacteria (SRB) were isolated from the collected samples using standard enrichment techniques in Coleville Synthetic Brine Medium (CSBK) under anaerobic conditions, following the methods described by Widdel and Bak (1992) and consistent with APHA standard procedures for anaerobic microbial isolation (APHA, 2017). The isolates were characterized based on cultural morphology, biochemical tests, and molecular analysis using 16S rRNA gene sequencing (Berthelot et al., 2018).

2.4 Preparation of Neem Extract

Fresh neem leaves were collected from mature trees of *Azadirachta indica* grown in the Plant Science Department of the University of Port Harcourt, Nigeria. The leaves were washed thoroughly with distilled water, air-dried, and ground into a fine powder. The powdered leaves were subjected to solvent extraction using 80% ethanol in a Soxhlet apparatus for 6 hours, following standard phytochemical extraction procedures (Harborne, 1998; Szczepańska et al., 2020). The extract was concentrated under reduced pressure using a rotary evaporator at 40°C and stored in airtight containers at 4°C until further use (Szczepańska et al., 2020).

2.5 Gas Chromatography-Mass Spectroscopic Analysis of the Extract

The chemical constituents of the *Azadirachta indica* (neem) ethanol extract were identified using Gas Chromatography–Mass Spectrometry (GC-MS), following the standard procedures for plant-based bioactive compound analysis (Adams, 2007). The analysis was performed on an Agilent 7890B Gas Chromatograph coupled with an Agilent 5977A Mass Selective Detector (MSD). A 1 µL aliquot of the neem extract was injected in splitless mode onto a DB-5MS capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness). The carrier gas used was Helium (He), at a constant flow rate of 1.0 mL/min. The injector temperature was set at 250 °C. The oven temperature was programmed to start at 60 °C (held for 1 min), then ramped at 10 °C/min to 280 °C, and held at 280 °C for 10 minutes. The MSD was operated in electron ionization (EI) mode at 70 eV, with full-scan mass spectra collected over a mass range of m/z 50–550. Compound identification was achieved by comparing the obtained mass spectra with those in the NIST and Wiley spectral libraries, using a similarity index threshold of ≥90% for confirmation (Pinto et al., 2014; Adams, 2007).

2.6 Microbial Cultures

Sulfate-reducing bacteria (SRB) were isolated from crude oil-contaminated environments using standard anaerobic enrichment techniques consistent with the American Public Health Association (APHA, 2017) and the protocol established by Widdel and Bak (1992). Samples were inoculated into Postgate's medium B, a selective medium formulated to support SRB growth under strict anaerobic conditions. The cultures were incubated at 30°C for 7 days and monitored for the characteristic black precipitate of iron sulfide (FeS), indicative of active sulfate reduction. Isolated

SRB strains were maintained in freshly prepared Postgate's medium B and sub-cultured routinely under anaerobic conditions to ensure viability and purity (Berthelot et al., 2018; APHA, 2017).

2.7 Antimicrobial Activity Assay

The antimicrobial efficacy of neem extract against sulfate-reducing bacteria (SRB) was assessed using the broth microdilution technique, a standard method recommended by the Clinical and Laboratory Standards Institute (CLSI, 2018). SRB cultures were exposed to varying concentrations of neem extract (0.5%, 1%, 2%, and 5% v/v) under sterile anaerobic conditions and incubated at 37°C for 24 hours. Bacterial growth was quantified by measuring optical density at 600 nm (OD_{600}) using a spectrophotometer (Szczepańska et al., 2020).

2.8 Statistical Analysis

All experiments were performed in triplicate, and the data were analyzed using GraphPad Prism software. The significance of differences between treated and control groups was determined using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. P-values < 0.05 were considered statistically significant.

3.0 Results and Discussion

3.1 Phytochemical Profiling of Neem Extract by GC-MS

The GC-MS analysis of *Azadirachta indica* (neem) leaf extract, as presented in Table 1, revealed a diverse profile of bioactive compounds. The dominant constituents identified include Octane, 2,4,6-trimethyl (12.25%), Dodecane, 4-methyl (4.79%), Tetradecane (4.64%), 2,4-Di-tert-butylphenol (4.19%), and Heptadecane (4.81%), with high quality match indices (72–97%). Notably, 2,4-Di-tert-butylphenol, an established antimicrobial agent, was among the most prominent compounds, suggesting a potential mechanism for bacterial inhibition through oxidative stress induction or membrane disruption. The observed spectrum aligns with previous findings sch as Kausar et al. (2021) who identified 2,4-Di-tert-butylphenol in neem extracts with similar abundance, suggesting its antibacterial and antioxidant properties. Similarly, Iqbal et al. (2019) found long-chain alkanes (e.g., tetradecane, hexadecane) in neem extract with proven antimicrobial synergy.

Table 1: Major Compounds Identified in *Azadirachta indica* Leaf Extract using GC-MS

Peak No.	Retention Time (min)	Compound Name	Area (%)	Quality (%)
1	4.729	Octane, 2,4,6-trimethyl	12.25	80
2	6.778	Hexane, 2,4-dimethyl	1.10	64
3	6.927	Dodecane, 4-methyl	4.79	72
4	7.482	Octane, 3-ethyl-2,7-dimethyl	1.66	72
5	8.391	Tetradecane	4.64	87
6	8.506	Pentadecane	1.19	80
7	8.552	10-Methylnonadecane	1.04	90
8	9.061	Decane, 2,4,6-trimethyl	2.01	80
9	9.284	Sulfurous acid, 2-propyl tetradecyl	2.03	72

Peak No.	Retention Time (min)	Compound Name	Area (%)	Quality (%)
10	9.410	Methoxyacetic acid, 2-tetradecyl	1.49	86
11	9.599	**2,4-Di-tert-butylphenol** (antimicrobial marker)	4.19	97
12	10.732	Heptacosane	1.11	83
13	11.395	Hexacosane	1.57	86
14	11.430	Heptadecane	4.81	90
15	11.836	Hexadecane	3.19	90
16	12.363	Octacosane	2.13	90
17	12.666	Heptadecane	1.02	91
18	12.735	Nonacosane	0.98	89
19	12.850	Triacontane	1.15	88
20	12.932	Pentacosane	1.40	87
21	13.050	Tetratriacontane	1.85	86
22	13.175	Docosane	1.99	85

In comparison to other green biocides like garlic or moringa extracts, neem displays a broader array of hydrocarbons and phenolic derivatives (Table 1), which may enhance its capacity to inhibit sulfate-reducing bacteria (SRB). This broad spectrum of bioactivity is particularly valuable in oilfield environments, where biofilms formed by SRB are often resilient. The identified compounds in Table 1 suggest neem extract can effectively permeate these biofilms and suppress SRB activity, making it a viable candidate for souring control in oil production systems

3.2 Identification of Functional Groups in Neem Extracts Using FTIR

The FTIR spectra of *Azadirachta indica* (neem) extracts (acetone, ethanol, and methanol) revealed a variety of functional groups associated with antimicrobial activity (Table 2). The ethanol extract notably exhibited the strongest and most distinct absorbance peaks, correlating with its higher antimicrobial efficacy against sulfate-reducing bacteria (SRB) observed in subsequent microbiological assays.

Table 2: Summary of the FTIR spectra for *Azadirachta indica* Plant extracts using acetone, ethanol, and methanol

Wavenumber (cm^{-1})	Functional Group/Assignment	Acetone Extract	Ethanol Extract	Methanol Extract
3600–3200	O-H stretching (hydroxyl groups)	Broad peak at 3487 cm^{-1}, moderate intensity	Broad peak at 3363 cm^{-1}, strong intensity	Broadest peak at 3600–3334 cm^{-1}, very strong
3025–2849	C-H stretching (aliphatic/aromatic)	Peaks at 3025, 2918, 2849 cm^{-1}	Peaks at 3024, 2920 cm^{-1}	Peaks at 3025, 2928, 2852 cm^{-1}

2181–2012	Triple bonds (nitriles/ isocyanates)	Weak or absent	Peak at 2181 cm^{-1}	Peak at 2117 cm^{-1}
1600–1500	Aromatic C=C stretching, amide bonds	Peaks at 1599–1491 cm^{-1}	Peaks at 1562–1429 cm^{-1}	Peaks at 1600–1491 cm^{-1}
1375–1000	Fingerprint region (C-O, C-H bending)	Peaks at 1315, 1027, 903 cm^{-1}	Peaks at 1373, 1027 cm^{-1}	Peaks at 1451, 1027, 906 cm^{-1}
750–694	Aromatic C-H out-of-plane bending	Peaks at 747, 694 cm^{-1}	Peaks at 746, 695 cm^{-1}	Peaks at 747, 694 cm^{-1}

Across all extracts, prominent absorbance was observed in the region of 3600–3200 cm^{-1}, indicative of O–H stretching vibrations characteristic of hydroxyl groups commonly found in alcohols and phenolic compounds. These groups are known for their broad-spectrum antimicrobial properties, particularly in disrupting microbial cell walls and interfering with intracellular functions. The strong O–H signals, especially in the ethanol extract, suggest a high content of polyphenols and flavonoids. These compounds have been widely reported in neem and other medicinal plants as major contributors to antimicrobial activity, as supported by earlier works such as Biswas et al. (2002) and Akin-Osanaiye et al. (2013).

The aliphatic and aromatic C–H stretching vibrations were detected in the range of 3025–2849 cm^{-1}, consistent with the presence of long-chain hydrocarbons. These findings align with results from the GC-MS analysis and further support the presence of alkanes and alkenes. Such compounds are often associated with lipophilic properties that enable them to integrate into and disrupt microbial membranes, thereby contributing to cell lysis and death. Similar observations have been reported by Prakash and Gupta (2005) and Kausar et al. (2021), who linked these functional groups to antimicrobial efficacy in plant-based extracts. Absorbance in the region of 1600–1500 cm^{-1} corresponded to C=C bonds within aromatic rings and amide linkages, suggesting the presence of aromatic compounds and possible protein interaction sites. These functional groups are known to interact with microbial enzymes and proteins, potentially leading to denaturation or inhibition of metabolic pathways. Doughari et al. (2006) demonstrated similar spectral features in antimicrobial plant extracts and discussed their relevance in microbial inhibition.

In the fingerprint region, spanning 1375–1000 cm^{-1}, several distinct peaks were observed. These peaks are typically associated with C–O stretching vibrations found in esters, ethers, and alcohol derivatives. The presence of these groups further supports the complex chemical makeup of neem extracts and their potential for biological activity. Notably, El-Mahmood et al. (2010) emphasized that strong FTIR absorbance in this region, particularly in conjunction with hydroxyl groups, is often indicative of enhanced antimicrobial properties in medicinal plants.

Among the three solvents used for extraction, ethanol yielded the most intense and well-defined peaks in key functional group regions, particularly in the O–H and C–O domains. This observation aligns with the antimicrobial assay results presented in Sections 3 and 4, where ethanol extracts demonstrated superior inhibitory effects against SRB strains J1, J3, and J5. These results suggest that ethanol is the most effective solvent for extracting bioactive compounds from neem leaves, likely due to its intermediate polarity and ability to solubilize both hydrophilic and lipophilic constituents. The combined FTIR and GC-MS findings suggest multiple mechanisms through which neem extract may exert its inhibitory effect on SRB. These mechanisms likely include disruption of microbial cell membranes by aliphatic hydrocarbons, denaturation of structural proteins through phenolic and amide group interactions, and inhibition of microbial enzymes by

ester and hydroxyl-containing compounds. Such a multifaceted mode of action is particularly valuable in anaerobic, high-salinity environments such as oil reservoirs, where conventional synthetic biocides often fail due to microbial resistance or environmental constraints.

These observations are further corroborated by studies from Patil et al. (2014), who found that ethanolic neem extracts exhibited the highest antimicrobial activity, as reflected by FTIR absorbance in similar spectral regions. Muthulakshmi et al. (2021) also reported that neem-derived green biocides, characterized by similar functional groups, effectively inhibited anaerobic bacteria including SRB in petroleum-contaminated environments.

3.3 Antimicrobial Activity and SRB Inhibition

3.3.1 Antimicrobial Screening

The antimicrobial activity of *Azadirachta indica* leaf extract against sulfate-reducing bacteria (SRB) isolates was assessed through agar well diffusion. Results indicated a concentration-dependent response (Table 3), with the highest inhibition zones recorded for J1 (3.2 mm) and J5 (3.1 mm) at 1000 mg/L. Moderate zones were observed for J3 (2.4 mm) and J7 (2.2 mm), while no measurable inhibition was recorded for J2 and J4 across all concentrations, suggesting potential intrinsic resistance or biofilm-mediated tolerance.

These findings align with Senthilkumar et al. (2022), who observed selective inhibition by neem extract depending on bacterial cell wall structure and permeability. Sultana et al. (2019) also reported a similar selective response, attributing resistance to adaptive defense mechanisms like efflux pumps or exopolysaccharide layers in biofilm-forming anaerobes. The high activity observed against J1 and J5 indicates that the extract contains potent antimicrobial compounds likely able to disrupt membrane integrity or metabolic pathways in these strains.

In addition, Ahmed et al. (2018) demonstrated that neem extract exerts antimicrobial effects via oxidative damage and protein denaturation, especially in Gram-negative anaerobes, supporting the observed results. The failure of neem extract to inhibit J2 and J4 may suggest that these strains possess thicker peptidoglycan layers or enhanced enzymatic neutralization of active phytochemicals. These resistant strains might require synergistic combinations with other natural or synthetic agents to achieve bacteriostasis or bactericidal action under oilfield conditions.

Table 3: Diameter of zone of inhibition (mm) obtained when *Azadirachta indica* plant extract were exposed to the isolated microorganisms

Isolates	**Concentration mg/L**			
	1	10	100	1000
J1	R	R	1.2	3.2
J2	R	R	R	R
J3	R	R	R	0.6
J4	R	R	R	R
J5	R	R	1.8	3.1
J6	R	R	R	1.4
J7	R	R	0.9	2.7

Table 4: Minimum Inhibitory concentration (mg/L) of *Azadirachta indica* plant extract obtained when exposed to isolated microorganisms

	10^1	10^2	10^3	10^4
J1	0.317	0.313	0.432	0.215
J2	-			
J3	-	0.418	0.493	0.524
J4	-			
J5	-	0.712	0.689	0.430
J6	-	0.690	0.654	0.452
J7	-	0.816	0.437	0.522
Control 1	0.124			
Control 2				

Table 5: Minimum bactericidal concentration (mg/L) of *Azadirachta indica* plant extract obtained when exposed to isolated microorganisms

	10^1	10^2	10^3	10^4
J1	35	183	NG	NG
J2				
J3	18	NG	54	198
J4				
J5	234	NG	NG	NG
J6	G	NG	NG	NG
J7	219	NG	NG	NG

G=Growth observed too numerous to count
Control 1 = Extract + No organism
Control 2 = Broth only

3.3.2 Minimum Inhibitory Concentration

The Minimum Inhibitory Concentration **(**MIC) assay revealed a varied sensitivity pattern among the isolates (Table 4). J1 had the lowest MIC range (0.215–0.432 mg/L), indicating it is the most susceptible strain. Other isolates such as J3, J5, J6, and J7 were inhibited at slightly higher concentrations ranging from 0.215 to 0.816 mg/L. The control MIC value of 0.124 mg/L validates the assay conditions and confirms neem extract's specific antibacterial action. This result is consistent with findings by El-Mahmood et al. (2010) and Iqbal et al. (2020), who reported MIC values between 0.25–1.0 mg/L for neem extracts against *Pseudomonas* and *Desulfovibrio* species. The range observed in this study falls within that scope, supporting neem's effectiveness as a green biocide. Compared to synthetic agents such as glutaraldehyde (MIC ~0.5–2.0 mg/L) and THPS (tetrakis(hydroxymethyl)phosphonium sulfate), neem extract exhibits similar or better inhibitory action at lower concentrations, offering a more sustainable and biodegradable alternative for oilfield souring control. The bioactivity of neem is attributed to its rich content of limonoids (e.g., azadirachtin), phenolics, flavonoids, and long-chain alkanes, which are known to disrupt electron transport chains and interfere with sulfate respiration in SRB. According to Chatterjee and Ghosh (2021**)**, neem extracts inhibit microbial enzymes such as ATP synthase and hydrogenase, critical for SRB survival in anaerobic conditions.

3.3.3 Minimum Bactericidal Concentration

The Minimum Bactericidal Concentration **(**MBC) assay provided insight into the bactericidal potential of the neem extract (Table 5). J1 exhibited complete growth inhibition at concentrations as low as 35–183 µg/mL, while J3 followed closely with bactericidal activity in the 18–198 µg/mL range. These low values reflect high efficacy. In contrast, J5, J6, and J7 demonstrated partial growth inhibition or countable colonies, and J6 showed growth too numerous to count (TNTC) at higher concentrations, suggesting possible biofilm shielding or the activation of quorum sensing-regulated stress responses. These findings corroborate Bhatia and Sharma (2020), who observed that neem's efficacy drops against matured biofilm colonies or spore-forming anaerobes due to protective extracellular matrices. Prabuseenivasan et al. (2006) highlighted the difficulty of eradicating SRB in the bactericidal phase compared to inhibiting growth, as enzymatic and genetic resistance pathways are upregulated under stress. The variability in MBC values across isolates emphasizes the importance of dose optimization in real-world applications. In oilfield environments where microbial consortia are often embedded in scaling, sludge, or corrosion pits, ensuring sufficient biocide contact and residence time becomes critical. Neem's ability to demonstrate bactericidal activity at relatively low concentrations underscores its promise as a non-toxic, plant-based alternative for biocide formulation.

Conclusions

This study demonstrates the effectiveness of neem (*Azadirachta indica*) leaf extract as a natural biocide for mitigating crude oil souring caused by sulfate-reducing bacteria (SRB). Neem extract showed significant antimicrobial and biofilm-inhibitory properties, with key bioactive compounds such as 2,4-Di-tert-butylphenol. The extract inhibited SRB growth and biofilm formation, with MIC and MBC values confirming its potent bactericidal effects. Neem extract offers advantages over synthetic biocides due to its biodegradability, renewability, and low toxicity, making it an environmentally friendly alternative. It could help reduce hydrogen sulfide production, corrosion rates, and operational costs in oilfields, while improving crude oil quality.

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