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Research article

Investigating Some Culinary Herbs as Potential Anti Quorum Sensing Agents in the Environment

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Abstract
Bacteria communicate via quorum-sensing (QS) before exhibiting detrimental or beneficial effects to man and the environment. This QS is indispensable for biofilm formation, which results to medical and environmental consequences. Interrupting QS using culinary herbs may address membrane biofouling and prevent biofilm-related diseases, while reducing the risks of bacterial resistance. In this study, Leaves of *Cymbopogon citratus, Rosmarinus officinalis* and *Ocimum basilicum* were extracted in organic solvents of varying polarity. Anti-quorum sensing (AQS) activities of the extracts were investigated at both qualitative and quantitative levels against a locally isolated *Chromobacterium violaceum*, using solid and liquid media bioassays. Plant extracts with peak AQS activities were characterized using chromatographic techniques. The extracts were fractionated and the promising fractions were re-isolated and delineated by Fourier-transform infrared spectroscopy (FTIR) as well as Gas chromatography-mass spectrometry (GC-MS) profiling. Solvents of higher polarity (aqueous and methanol) gave a bumper yield (5.96%, 4.77% and 4.67%) of the recovered extracts. Only two species (*R. officinalis* and *O. basilicum*) exhibited a noteworthy AQS activity in the agar well diffusion assay. However, all the extracts inhibited violacein production (3.70–60.49%) in the quantitative AQS bioassay. The FTIR and GC-MS analyses revealed the presence of alcohols and p-Cymene as the commonest functional group and most abundant compound respectively in the most active fraction (R2) of *R. officinalis*. The aqueous and methanol extracts (especially of *R. officinalis*) in both crude and isolated forms appreciably inhibited bacterial biofilms and indicated good features of AQS activities.

Key words: Quorum sensing, culinary herbs, *C. violaceum*

Introduction

Culinary herbs have received great attention due to their perceived ethnomedicinal (Kalamartzis *et al*., 2020) and antioxidant (Baldi and Bansal, 2020) advantages. Interestingly, most of these herbs are considered harmless as they are sourced from the "Generally Recognised as Safe" (GRAS) plants (Cosa *et al*., 2019).

Quorum-sensing (QS) constitutes cell-to-cell communication and regulation, which enables participating bacteria to adapt to their external environment (Deryabin *et al*., 2019). Bacteria 'communicate' via QS to develop biofilms (sessile slimy multicellular microbial communities) which can lead to several health challenges: increased resistance to antibiotics making infections harder to treat, allowing pathogens to evade the immune system leading to chronic infections as well as covering implants and medical devices causing infection complications (Sharma *et al*., 2023). Biofilms may also lead to plaque formation, dental caries and periodontal diseases (Sharma *et al*., 2023). Furthermore, biofilms are associated with environmental challenges such as corrosion and fouling of pipes and surfaces, reduced efficacy of disinfectants making sanitation efforts less effective as well as disruption of ecosystems by altering nutrient cycles, all in addition to effects on water treatment processes, leading to contamination and reduced water quality (Vinita V., 2019). Another environmental consequence of biofilms is biofouling on ship hulls and other surfaces, affecting navigation and increasing fuel consumption.

Current strategies employed to control biofilms cantered around physical cleansing with chemicals and incorporation of antimicrobial substances such as peptides and nitrofurazones (Salisu *et al*., 2021). Use of these chemicals is associated with resistance, environmental pollution and non-specificity (Lade *et al*., 2014).

Biofilm formation is directly related to and, controlled by bacterial QS. Hence, adopting the QS inhibition approach can be promising in addressing the biofilm-related health and environmental challenges (Li *et al*., 2018; Ergön-Can *et al*., 2017). In this way, life of the bacteria is not the target but their ability to form and express biofilms (Paluch *et al*., 2020).

Rosmarinus officinalis (Rosemary) is a woody, perennial shrub with fragrant, evergreen, needle-like leaves (Andrade *et al*., 2018) that is used to treat colic, dysmenorrheal, respiratory and liver disorders (Popescu *et al*., 2020; Nakavuma *et al*., 2016) probably due to its oleanolic acid (De Oliveira *et al*., 2019), rosmarinic acid, camphor, caffeic acid, ursolic acid, betulinic acid, carnosic acid, rosmarol and carnosol (Mohamed *et al*., 2017; Anna *et al*., 2014) content.

Cymbopogon citratus (Lemon grass) is a tall, monocotyledonous aromatic perennial plant with slender sharp edge, green leaves with pointed apex (Gupta *et al*., 2019). This plant has been reported for its antiparasitic (Me´abed *et al*., 2018), antioxidant (Li *et al*., 2020), antibacterial (Saddiq and Khayyat, 2010), antiseptic, anti-dyspeptic, antifever, antispasmodic, analgesic, antipyretic, diuretic (Oladeji *et al*., 2019) and anti-inflammatory activities (Costa *et al*., 2016).

Ocimum basilicum (Sweet basil) is an herbaceous shrub which is traditionally used to treat headache, cough, diarrhea, constipation, warts, worms, kidney malfunction as well as digestive problems (Purushothaman *et al*., 2018). The leaf extracts of *O. basilicum* have also revealed strong antimicrobial, anticancer, anticonvulsant and antioxidant properties (Falowo *et al*., 2019). In this research, the commonly used herbs (Meziane-Assane *et al*., 2013) of *Rosmarinus officinalis, Cymbopogon citratus* and *Ocimum basilicum* were exploited on the basis of their ethnomedicinal advantages and wide culinary applications (Kalamartzis *et al*., 2020). Anti-quorum sensing (AQS) activities of these herbs were evaluated against a common bioindicator bacterium: *Chromobacterium violaceum*.

METHODS

Collection and Identification of the Plant Samples

Following preliminary identification using relevant pictures and charts, apparently healthy plants were collected from their cultivated land at Malali Plant Gardens, Kaduna (10°32'8.7"N, 09°27'37.2" E) and their identities were authenticated as *C. citratus, R. officinalis* and *O. basilicum* at the herbarium, Department of Plant Biology, Bayero University, Kano, Nigeria.

Processing of the Plant Material

Leaves of the freshly collected plants were detached, washed with clean water (Loha *et al*., 2019) and distributed evenly to air-dry (Gahlot *et al*., 2018). The leaves were pulverised to fine powder using laboratory mortar and pestle and stored at room temperature in an air-tight dry container (Ibrahim *et al*., 2017).

Extraction of the Plant Leaves

Exactly 100 g of the leaf powder was macerated in 1000 mL each of n-hexane, acetone, ethyl acetate, methanol and distilled water (Bulugahapitiya, 2013). This set-up was allowed to stand for 72 h at room temperature with intermittent agitation (De Oliveira *et al*., 2019). The mixture was filtered first through a cheese cloth and through Whatman grade 1 filter paper. The extracts were concentrated *in vacuo* using rotary evaporator at 40 °C, air dried and weighed (Teresa-May, 2018). Percentage recovery of the extracts was calculated as follows (Ghosh *et al*., 2019):

$$\text{Percentage yield} = \frac{\text{weight of dry crude extract (g)}}{\text{dry weight of plant material before extraction (g)}} \times 100 \quad \ldots$$

Equation 1

Preparation of the Extracts' Concentrations

A quantity (0.1 g) of the crude extracts were dissolved in 10 mL of 1% Dimethyl Sulfoxide (DMSO) to achieve a concentration of 10 mg/mL (Famuyide *et al*., 2019). This stock extracts were diluted serially to 0.08, 0.16, 0.31, 0.63, 1.25, 2.50 and 5.00 mg/mL concentrations. Sterility of the extracts was verified by inoculation (via streaking) on freshly prepared nutrient agar (NA), which was incubated at 37 °C for 24 h.

Quorum-Sensing Inhibition Bioassay

The AQS activities of the extracts were evaluated against a locally isolated *C. violaceum* as follows:

Isolation and Identification of *C. violaceum* from the Water Environment

A total of 140 samples of water: 70 each from River Kaduna (10° 29' 47.13"N, 07° 25' 19.95"E) and some selected ponds around Kaduna

metropolis (10º 31' 35.73"N, 07º 23' 48.93"E) were collected into standard water sampling The samples were transported immediately to the microbiology laboratory in cold condition and screened for *C. violaceum* using enrichment method (Goh *et al*., 2014; Muharam *et al*., 2019).

Exactly 5 mL (each) of the samples were centrifuged in test tubes at 6000 rpm for 10 min. The supernatant was discarded, the pellets were suspended in 3 mL nutrient broth and incubated at 37 °C for 24 h (Goh *et al*., 2014). This cells' suspension was serially diluted to 10^{-6} using Phosphate Buffered System (PBS) and inoculated on NA and MacConkey agar plates; which were incubated at 37 °C for 18 h (Muharam *et al*., 2019). The plates were observed for appearance of purple or violate bacterial colonies. Selected isolates were purified and identified based on microscopic, biochemical and molecular techniques, using standard procedures (MacFaddin, 2000; Kodach *et al*., 2006; Cappuccino and Sherman, 2013; Goh *et al*., 2014; UK Standards for Microbiology Investigations, 2014; Julistiono *et al*., 2018; Aryal, 2019). The pure culture was stored (under freezing) in Luria Bertani (LB) broth, which was supplemented with 20% glycerol (Baloyi *et al*., 2019). Before each use, the pure culture was aerobically activated in freshly prepared LB broth under shaking at 28 ºC for 24 h.

Screening for Anti Quorum Sensing Activity

Agar well diffusion method, as described by Elmanama and Al-Refi (2017) was adopted to detect the AQS activities of the extracts by means of double layer culture plate. An existing layer of (plain) LB medium (1.5% agar) in petri dishes was overlaid with 5 mL of molten soft LB agar (0.3%), which was inoculated with 50 µL of *C. violaceum* (Baloyi *et al*., 2019). The Plates were allowed to set completely and sterile cork borer was used to bore holes into the seeded culture. These wells were filled with 50 µL of the corresponding extract's concentrations (0.63-5.00 mg/mL) and left undisturbed for 1 h, after whehey were incubated at 37 ℃ for 24 h (Baloyi *et al*., 2019). Eugenol (0.08 mg/mL) was used as positive control, while the negative control was 1% DMSO. Measurements of AQS were made (mm) from the outer edge of the wells to the edge of the activity zones.

bottles (Salisu *et al*., 2019) over the period of 6 months.

Quantitative Determination of Anti Quorum Sensing Activity

The amount of violacein produced by *C. violaceum* via QS, on exposure to the extracts was measured in accordance with the procedures described by Singh *et al*. (2009); Baloyi *et al*. (2019). Volumes (100 µL) of the extracts (0.08–10.00 mg/mL) were pipetted into correspondingly labelled test tubes containing 3 mL of LB broth. To each tube, 100 µL of *C. violaceum* culture was added and incubated under shaking (120 rpm) at 30 °C for 24 h. One milliliter (1 mL) of this culture was centrifuged at 13,000 rpm for 10 min. to precipitate the violacein. The supernatant was discarded and the pellets were resuspended in 1 mL of 100% DMSO; to which 200 µL of 10% Sodium Dodecyl Sulphate (SDS) was added to lyse the bacterial cells (Srivastava *et al*., 2020). Samples were allowed to stand for 5 min. at room temperature, followed by addition of 900 µL of water-saturated *n*-butanol. They were vortexed (to dissolve the violacein) and centrifuged at 13000 rpm for 5 min. Exactly 1 mL of the solution was transferred to cuvettes for violacein quantification at 585 nm using ultraviolet-visible light (UV-VIS) spectrophotometer. Absorbance of replicate assays was determined and the percentage violacein inhibition was calculated as: (Hossain *et al*., 2017):

$$\text{Percentage violacein inhibition (585 nm)} = \frac{\text{OD of control} - \text{OD of test}}{\text{OD of control}} \times 100 \ldots \text{Equation 2}$$

Where plain culture of *C. violaceum* was used as control

Characterization of the Plant Compounds

The plant extracts were characterized based on analytical Thin Layer Chromatography (TLC), Column Chromatography as well as preparative TLC (PTLC).

Analytical Thin Layer Chromatography

This analysis was conducted to determine the most suitable solvent system for the subsequent chromatographic techniques (Zakariya *et al*., 2015). A silica plate (5cm × 10cm) with 0.2 mm thickness of silica sorbent (stationary phase) was spotted with a dilute solution of the extract along a line drawn using a pencil, about 1cm up from the lower edge of the plate. This plate was dried

and developed in the TLC tank which contained 10 mL of the solvent system (mobile phase) to wet the lower edge of the plate without soaking the applied spot. The mobile phase was observed as the spot separates and migrates through the (distance travelled by a spot over the total distance covered by the mobile phase) (LibreTexts Libraries, 2019).

$$\text{Rf} = \frac{\text{distance traveled by the analyte}}{\text{distance traveled by the solvent}} \ldots \text{Equation 3}$$

Column Fractionation of the Extracts

The stationary phase (70 g silica gel: 60-200 mesh) was packed into half of the column's height by wet slurry (Zakariya *et al*., 2015). Analyte (2 g) was prepared with an equal quantity of the silica gel, loaded (in layers) to the stationary phase and allowed to stabilize for 30 minutes. The mobile phase was introduced gradient wise, through a separating funnel, after which elusion began. The mobile phase carried the various compounds in the extract at different rates based on their affinity to it and to the stationary phase (Bulugahapitiya, 2013). After development, separated fractions were carefully collected and allowed to concentrate at room temperature (Uraku, 2015).

Isolation of Distinct Fractions using Preparative Thin Layer Chromatography

Highly concentrated solution of the analyte was spotted along the lower edge line using a capillary tube. The set up was allowed to develop to 2/3 of the plate (8 cm × 10 cm), after which the separated bands were visualized under UV light and their respective R_f values were calculated. Similar fractions were scraped out, placed in separate flasks containing 150 mL of methanol and mixed vigorously. The silica gel was filtered off and the compounds were obtained as methanol extracts. Solvent from the extracts was evaporated at 40 ℃ *in vacuo* (Bulugahapitiya, 2013).

Bioautography of the Isolated Fractions

The isolated fractions were assayed for AQS activities against *C. violaceum* and the most active ones were identified via Gas Chromatography–Mass Spectrometry (GC-MS).

Gas Chromatography–Mass Spectrometry

The GC-MS analysis was carried out according to a procedure described by Sagbo *et al*. (2020). An Agilent 7890B GC 5977A MSD system sorbent by capillary action (Kandiyoti *et al*., 2017), which was identified by bands with different pigments under UV light (365 nm). The bands were distinguished by calculating their retention factor (Rf) values, equipped with a mass selective detector (Chemetrix [Pty] Ltd., Agilent Technologies, DE, Germany) and a Zebron-5MS (cross-linked 5% – phenyl methyl polysiloxane) column (HP-5 fused silica 30m × 0.25mm × 0.25μm film thickness) was used. Exactly 1 μL of the analyte was aspirated into the split less mode of the gas chromatograph at high injection temperature of 250 °C, ion source temperature of 280 °C and a pressure of 48.745 kpa into the InertCap 5MS/NP capillary. The oven temperature was initially started from 40 °C, held for 1 min. and increased to 240 °C at 3 °C/min. The carrier gas used was GC-grade helium at a flow rate of 1 mL/min. and a velocity of 36.262 cm/sec. The analyte was volatized by the chromatogram and its various components were separated based on size and/or polarity. These components were delivered to the mass selective detector. Mass spectra were recorded between 50–600 m/z in the electron impact (EI) ionization mode at 70eV with a scan speed of 2300. The resulting components were identified using the GC-MS library source of the National Institute of Standards and Technology (NIST) reference database 69 (NIST chemistry webbook, 2018); where the retention times of the mass spectra were compared with those of known compounds in the library database (Lulekal *et al*., 2019).

Statistical Analyses

Data were presented as mean ± standard deviation (SD) of replicate assays. The mean and standard deviation of bioassays were computed using Microsoft Excel (version 2016). Values of inhibitory activities were appraised by one-way analysis of variance (ANOVA) with the use of GraphPad Instat (version 3.10); in comparison with controls. All P-values <0.05 were regarded as significant, which were illustrated with different superscript alphabets, while those >0.05 were considered insignificant and denoted by similar superscripts.

RESULTS AND DISCUSSION

Crude Extract Yields (%w/w) after Extraction of Plants in Various Solvents

The quantity of the crude leaf extracts recovered (% w/w) following extraction in different

solvents are presented in Table 1. The aqueous extracts were found to have the highest yields of 5.96%, 4.67% and 3.74% from *Rosmarinus officinalis*, *Ocimum basilicum* and *Cymbopogon citratus*, respectively; while the lowest recovery (1.09%, 1.70% and 3.16%) for *C. citratus*, *O. basilicum* and *R. officinalis*, respectively was recorded from the n-hexane. The overall mean percentage yields of the extracts in the various solvents were determined as 4.32±1.03, 2.97±1.13 and 2.16±1.13 from *R. officinalis*, *O. basilicum* and *C. citratus* respectively. Extraction using the different solvents affected significantly (P<0.05) the yields of the extracts.

Table 1: Percentage Yield of the Crude Leaf Extracts of *O. basilicum*, *C. citratus* and *R. officinalis* Extracted in Organic Solvents of Different Polarity

Plants	*O. basilicum*	*C. citratus*	*R. officinalis*
Solvents	Yield (% w/w)		
Aqueous	4.67	3.74	5.96
Methanol	3.20	2.70	4.77
Ethyl acetate	3.05	2.20	4.62
Acetone	2.21	1.08	4.48
N-hexane	1.70	1.09	3.16
Mean ± SD	2.97±1.13[a]	2.16±1.13[b]	4.60±1.03[c]

Values are mean ±SD of percentage yield of the plant extracts. Mean values with different superscripts within the same row are significantly different (P<0.05).

Identification of *Chromobacterium violaceum*

Identity of the violacein producing bioindicator bacterial species (*Chromobacterium violaceum*) is presented in Table 2. This bacterium was isolated from a stagnant water located at Rigasa, Igabi LGA, Kaduna State.

Table 2: Cultural, Morphological and Biochemical Characteristics of *C. violaceum* Isolated from a Stagnant Water in Rigasa, Kaduna State, Nigeria

Colonial Characteristics On NA	on MacConkey	Gram's Reaction/Morphology	Biochemical Reaction	*C. violaceum*
Purple, circular, raised and smooth	Purple, large and mucoid	Gram-negative bacillus	Oxidase	+
			Catalase	+
			Citrate	-
			Glucose	+
			Sucrose	-
			H_2S	-
			Indole	-
			Motility	+

Key: +: C. *violaceum* present, - : *C. violaceum* absent.

Qualitative Anti Quorum Sensing Activity of the Extracts

Table 3 presents the mean zones of violacein inhibition indicating AQS activities of the crude leaf extracts. The methanol extracts of *Rosmarinus officinalis* recorded the highest activity (15.5±0.7 mm) at 5 mg/mL against the biosensor (*Chromobacterium violaceum*).

Percentage Violacein Inhibition by the Crude Extracts of *Rosmarinus officinalis*, *Ocimum basilicum* and *Cymbopogon citratus* at Different Concentrations

The magnitude (%) of violacein inhibition by the leaf extracts of *Rosmarinus officinalis*, *Ocimum basilicum* and *Cymbopogon citratus* at varied concentrations (0.078-10.00 mg/mL) against *Chromobacterium violaceum* is presented in Table 4.15. The highest inhibition rate (60.49%) was recorded from the methanol extract of *R. officinalis* at 10 mg/mL.

Gas Chromatography–Mass Spectrometry

Tables 5, 6 and 7 present the chemical components as identified from the active fractions of the methanol extracts of *Cymbopogon citratus* (C1), *Ocimum basilicum* (O5) and *Rosmarinus officinalis* (R2 and R5) using Gas Chromatography-Mass Spectrometry (GC-MS). From the result, an abundance of Hexadecanoic acid methyl ester, 9,12-Octadecadienoic acid (Z, Z)- methyl ester as well as p-Cymene was recorded from *C. citratus*, *O. basilicum* and *R. officinalis* respectively.

Table 3: Qualitative Anti Quorum Sensing Activities of the Crude Leaf Extracts of *R. officinalis*, *O. basilicum* and *C. citratus* Against *C. violaceum* Isolated from a Stagnant Water in Rigasa, Kaduna, Nigeria

Plant species	Zone Diameter (mm) and Associated Susceptibility Phenotype											
	Aqueous Extract				Methanol Extract				Ethyl Acetate Extract			
	Concentration (mg/mL)											
	0.625	1.250	2.500	5.000	0.625	1.250	2.500	5.000	0.625	1.250	2.500	5.000
R. officinalis	9.0±1.4	10.0±0.0	11.5±0.7	12.5±0.7	7.0±1.4	12.0±2.8	12.5±2.1	15.5±0.7	0.0±0.0	11.5±0.7	10.5±0.7	11.5±2.1
O. basilicum	7.0±1.4	0.0±0.0	8.0±2.8	10.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	9.1±0.4	0.0±0.0	0.0±0.0	9.0±0.0	7.0±1.4
C. citratus	0.0±0.0	0.0±0.0	10.0±1.4	10.5±0.7	0.0±0.0	0.0±0.0	0.0±0.0	10.5±0.7	0.0±0.0	0.0±0.0	0.0±0.0	6.0±0.0
Eug. (0.078 mg/mL)											25.5±0.0	
1% DMSO							0.0±0.0					

Concentration = 0.625–5.000mg/Ml, Values are mean (±SD) zones of AQS activities. Zones ≥15mm, 11–14mm and ≤10mm were regarded as strong, intermediate and weak AQS regions respectively (CLSI, 2019). Key: Eug. = Eugenol (positive control), DMSO = negative control.

Table 4: Percentage Violacein Inhibition of the Crude Leaf Extracts of *R. officinalis*, *O. basilicum* and *C. citratus* against *C. violaceum* Isolated from a Stagnant Water in Rigasa, Kaduna, Nigeria

Plant Extracts	Percentage Inhibition of Violacein Production (%)							
	Concentration (mg/mL)							
	0.078	0.156	0.313	0.625	1.250	2.500	5.000	10.000
Aqueous								
R. officinalis	1.85±0.000^{a}	6.79±0.021^{b}	18.52±0.000^{c}	15.43±0.004^{d}	24.07±0.002^{e}	37.04±0.000^{f}	41.98±0.006^{g}	48.15±0.004^{h}
O. basilicum	8.64±0.004^{a}	16.67±0.002^{b}	3.09±0.000^{c}	13.58±0.000^{d}	16.67±0.002^{e}	37.65±0.002^{f}	12.35±0.007^{g}	35.80±0.003^{h}
C. citratus	1.85±0.000^{a}	3.09±0.000^{b}	3.70±0.001^{c}	4.32±0.002^{d}	4.94±0.000^{e}	30.86±0.004^{f}	12.35±0.007^{g}	30.25±0.002^{h}
Methanol								
R. officinalis	0.62±0.005^{a}	26.54±0.002^{b}	18.52±0.006^{c}	15.43±0.000^{d}	26.54±0.000^{e}	38.27±0.000^{f}	45.06±0.006^{g}	60.49±0.001^{h}
O. basilicum	1.24±0.000^{a}	11.73±0.000^{b}	12.96±0.000^{c}	14.82±0.001^{d}	17.28±0.002^{e}	34.57±0.003^{f}	17.28±0.001^{g}	35.80±0.004^{h}
C. citratus	2.47±0.001^{a}	3.70±0.001^{b}	2.47±0.001^{c}	14.20±0.002^{d}	6.17±0.004^{e}	31.48±0.000^{f}	30.86±0.001^{g}	29.01±0.002^{h}
Ethyl Acetate								
R. officinalis	0.62±0.000^{a}	16.05±0.000^{b}	14.12±0.00^{c}	6.17±0.001^{d}	6.17±0.002^{e}	17.90±0.000^{f}	13.58±0.000^{g}	23.46±0.001^{h}
O. basilicum	0.62±0.000^{a}	1.24±0.001^{b}	1.24±0.000^{c}	2.47±0.001^{d}	3.09±0.000^{e}	3.09±0.000^{f}	3.70±0.000^{g}	3.70±0.001^{h}
C. citratus	1.24±0.000^{a}	3.09±0.000^{b}	3.09±0.000^{c}	0.93±0.000^{d}	0.62±0.000^{e}	3.70±0.000^{f}	3.09±0.000^{g}	4.32±0.000^{h}
Eugenol (control)	13.58±0.000^{a}	23.46±0.001^{b}	35.19±0.004^{c}	56.79±0.000^{d}	58.02±0.001^{e}	63.58±0.001^{f}	74.07±0.001^{g}	95.93±0.001^{h}

Concentration: 0.08–10.00mg/mL
Values are mean ± SD of violacein inhibition.
Mean values with different superscripts (a–h) across the various concentrations are significantly different ($P<0.05$)

Table 5: Identity of Chemical Compounds from the Methanol Leaf Extract of *C. citratus* following the GC-MS Profiling

RT (minute)	Area%	Mass Fragmentation	Chemical Class	Molecular Formula	Compound
5.735	17.03	106, 98, 91, 84, 77, 65, 58, 51, 39, 27	Hydrocarbon	C_8H_{10}	P-xylene
8.175	1.81	120, 105, 91, 77, 65, 51, 39, 27, 15	Hydrocarbon	C_9H_{12}	Benzene, 1-ethyl-4-methyl
10.991	0.23	134, 119, 105, 91, 77, 65, 51, 39, 27, 15	Hydrocarbon	$C_{10}H_{14}$	Benzene, 4-ethyl-1,2-dimethyl
12.79	0.16	134, 119, 105, 91, 77, 65, 51, 39, 27, 15	Hydrocarbon	$C_{10}H_{14}$	Benzene, 2-ethyl-1,3-dimethyl
31.338	1.60	270, 227, 199, 171, 143, 125, 101, 74, 43	Organic acid ester	$C_{16}H_{32}O_2$	Hexadecanoic acid, methyl ester
32.646	0.21	294, 262, 220, 199, 178, 150, 123, 95, 67, 41	Organic acid ester	$C_{19}H_{34}O_2$	9,12-Octadecadienoic acid, methyl ester (E, E)
32.706	1.81	296, 264, 242, 222, 200, 180, 157, 137, 110, 83, 55, 29	Organic acid ester	$C_{19}H_{34}O_2$	10-Octadecanoic acid, methyl ester
32.902	1.07	298, 255, 227, 199, 171, 143, 121, 97, 74, 43, 15	Organic acid	$C_{19}H_{38}O_2$	Methyl stearate

Key: RT = Retention Time

Table 6: Identity of Chemical Compounds from the Methanol Leaf Extract of *O. basilicum* following the GC-MS Profiling

RT (minute)	Area %	Mass Fragmentation	Chemical Class	Molecular Formula	Compound
5.780	23.64	106, 91, 77, 65, 51, 39, 27	Hydrocarbon	C_8H_{10}	P-xylene
8.175	1.42	120, 105, 91, 77, 65, 51, 39, 27, 15	Hydrocarbon	C_9H_{12}	Benzene, 1-ethyl-4-methyl
8.991	1.68	120, 105, 91, 77, 63, 51, 39, 27	Hydrocarbon	C_9H_{12}	Hemimellitene
10.698	0.19	134, 119, 105, 91, 77, 65, 51, 39, 27, 15	Hydrocarbon	$C_{11}H_{16}$	Benzene, 1-ethyl-3-propyl
10.914	0.14	134, 119, 105, 91, 77, 65, 51, 39, 27, 15	Hydrocarbon	$C_{10}H_{14}$	Benzene, 4-ethyl-1,2-dimethyl
14.320	0.04	128, 102, 87, 75, 63, 51, 39	Hydrocarbon	$C_{10}H_8$	1H- Indene, 1-methylene
26.218	0.06	224, 196, 168, 140, 112, 97, 69, 41, 26	Hydrocarbon	$C_{16}H_{32}$	3-Hexadecene, (Z)-
30.067	0.09	252, 224, 196, 168, 139, 111, 83, 57, 29	Hydrocarbon	$C_{18}H_{36}$	1-Octadecene
31.422	0.04	278, 223, 149, 123, 104, 76, 41	Organic acid	$C_{16}H_{22}O_4$	Dibutylphthalate
31.614	0.12	256, 239, 213, 185, 157, 129, 101, 83, 60, 43	Organic acid	$C_{16}H_{32}O_2$	n-Hexadecanoic acid
32.037	0.07	280, 252, 209, 167, 139, 111, 83, 63, 43	Hydrocarbon	$C_{20}H_{40}$	1-Eicosene
32.646	0.21	294, 262, 220, 199, 178, 150, 123, 95, 67, 41	Organic acid ester	$C_{19}H_{34}O_2$	9,12-Octadecadienoic acid, methyl ester (E, E)
32.703	0.08	296, 264, 222, 201, 180, 159, 138, 111, 83, 55, 29	Organic acid ester	$C_{19}H_{36}O_2$	Cis-13-Octadecenoic acid, methyl ester

Key: RT = Retention Time

Table 7: Identity of Chemical Compounds from the Methanol Leaf Extract of *R. officinalis* following the GC-MS Profiling

RT (minute)	Area%	Mass Fragmentation	Chemical Class	Molecular Formula	Compound
5.795	18.20	106, 91, 77, 65, 51, 39, 27	Hydrocarbon	C_8H_{10}	P-xylene
8.996	2.37	120, 105, 91, 77, 63, 51, 39, 27	Hydrocarbon	C_9H_{12}	Hemimellitene
9.763	2.25	120, 105, 91, 77, 65, 51, 39, 28, 14	Hydrocarbon	C_9H_{12}	Benzene, 1,2,4-trimethyl
11.695	0.07	134, 119, 103, 91, 77, 65, 51, 39, 27, 15	Hydrocarbon	$C_{10}H_{14}$	p-Cymene
14.322	0.06	128, 113, 102, 87, 74, 64, 51, 39, 27	Hydrocarbon	$C_{10}H_8$	Azulene
14.965	0.23	150, 135, 122, 107, 91, 79, 67, 55, 41	Ketone	$C_{10}H_{14}O$	Bicyclo (3.1.1) hept-3-en-2-one, 4,6,6-trimethyl-, (1S)-
26.472	0.21	224, 196, 168, 139, 111, 83, 57, 41, 15	Hydrocarbon	$C_{16}H_{32}$	Cetene
30.066	0.22	252, 224, 195, 168, 139, 111, 83, 55, 29	Hydrocarbon	$C_{18}H_{36}$	5-Octadecene, (E)-
30.190	0.14	254, 225, 197, 169, 141, 113, 85, 57, 29	Hydrocarbon	$C_{18}H_{38}$	Octadecane
31.346	3.00	270, 227, 185, 163, 143, 116, 97, 74, 43, 15	Organic acid ester	$C_{17}H_{34}O_2$	Hexadecanoic acid, methyl ester
31.423	0.44	278, 223, 149, 123, 104, 76, 41	Organic acid ester	$C_{16}H_{22}O_4$	Dibutyl phthalate
31.620	0.02	256, 239, 213, 185, 157, 129, 101, 83, 60, 43	Organic acid	$C_{16}H_{32}O_2$	n-Hexadecanoic acid
32.653	2.78	294, 263, 220, 199, 178, 150, 123, 95, 67, 41	Organic acid ester	$C_{19}H_{34}O_2$	9,12-Octadecadienoic acid (Z, Z), methyl ester
32.704	1.13	296, 264, 242, 222, 200, 180, 157, 137, 110, 83, 55, 29	Organic acid ester	$C_{19}H_{36}O_2$	10-Octadecenoic acid, methyl ester
32.910	3.04	298, 255, 227, 199, 171, 143, 121, 97, 74, 43, 15	Organic acid ester	$C_{19}H_{38}O_2$	Methyl stearate
33.115	0.21	284, 241, 213, 185, 157, 129, 97, 73, 43, 18	Organic acid	$C_{18}H_{36}O_2$	Octadecanoic acid
33.473	0.07	280, 252, 224, 196, 167, 139, 111, 83, 57, 29	Hydrocarbon	$C_{20}H_{40}$	3-Eicosene
33.533	0.04	282, 253, 225, 197, 169, 141, 113, 85, 57, 29	Hydrocarbon	$C_{20}H_{42}$	Eicosane
34.346	0.20	326, 283, 255, 227, 199, 171, 143, 97, 74, 43, 15	Organic acid ester	$C_{22}H_{42}O_2$	Eicosanoic acid, methyl ester
36.773	1.27	300, 279, 257, 231, 192, 163, 137, 115, 91, 69, 41	Alcohol	$C_{20}H_{30}O_3$	(.+/-.) –Demethylsalvicanol

Key: RT = Retention Time

DISCUSSION

Extract Yield of the Plant Materials

The highest percentage yields (5.96%, 4.67% and 3.74%) were obtained from the aqueous extracts of *R. officinalis*, *O. basilicum and C. citratus*; followed by the methanol extracts (3.20% and 2.70%) of *O. basilicum and C. citratus* respectively (Table 1). This implies that the most polar solvents yielded the biggest extracts' recovery; that is, the yield was mainly polarity dependent because polarity of the solvents seemed to be directly proportional to the yield of the extracts in this study. According to Maldonado *et al*., (2020), choice of appropriate solvent is important to recover greater extract's yield of a plant, which might contain correspondingly higher concentration of bioactive compounds. Similar to this finding, Methanol has been demonstrated (Maldonado *et al*., 2020) as an efficient solvent for the extraction of *C. citratus* collected from Guyana, South America both in terms of yield and bioactivity. Aljabri (2020) also reported the highest extraction recovery (8.91%) of *R. officinalis* obtained from Makkah, the Kingdom of Saudi Arabia in distilled water and 7.1% in ethanol; compared to only 5.84% from ethyl acetate. Generally, the extracts' recovery rate in this research varied significantly ($P<0.05$) across the plants' species. Extracts of *R. officinalis* recorded the highest yield (5.96%) while the least (1.08%) was obtained from *C. citratus*.

This variation might have resulted from the higher fibre content (with corresponding lower biomass recovery) and vice versa in leaves of the two plants respectively. This was particularly observed during the plants' extraction.

Preliminary Anti-Quorum Sensing Screening of the Plant Extracts

The qualitative AQS screening of the crude leaf extracts indicated a loss or significant reduction of the violacein pigment produced by *C. violaceum* around the agar wells. This was identified by colourless, opaque but viable halo zones around the wells. Methanol extract of *R. officinalis* inhibited the violacein in dose-dependent manner; recording (at peak) a zone spectrum of 15.5±0.7mm (Table 3). Although, this activity was only achieved at the highest used concentration (5.0mg/mL), it is still considered appreciable as zones ≥15mm are rated strong AQS regions in the present study. Violacein pigmentation controlled by QS in *C. violaceum* provides a naturally occurring and readily observable phenotype, without the need for additional substrates; which offers an easy evaluation of QS inhibition of compounds (Damte *et al*., 2013). *Rosmarinus officinalis* has similarly been reported (Vattem *et al*., 2007) to inhibit violacein pigment production (AQS activity) in *C. violaceum*. Further supporting the finding in this study, an extract of the leaves of *R. officinalis* exhibited AQS activity with an inhibition zone of 13±0.5mm, when assayed against *C. violaceum* (Al-Hussaini and Mahasneh, 2009). The present research revealed only a weak or (mostly) zero QS inhibition zones from the extracts of *O. basilicum and C. citratus*.

Quantitative Anti-Quorum Sensing Activities of the Plant Extracts

The extent of QS impediment in *C. violaceum* revealed a concentration dependent inhibition of the AHL-mediated violacein, as shown in Table 4. Among the extracts, methanolic *R. officinalis* had best (60.49%) reduced the violacein, followed by the aqueous and ethyl acetate extracts of this plant, recording 48.15% and 23.46% inhibition respectively; at 10.0mg/mL. The aqueous and methanol extracts of *O. basilicum and C. citratus* registered at peak 37.65% and 31.48% violacein inhibition respectively at similar concentration. These results indicate that the methanol extract of *R. officinalis* might be utilized as potent AQS agent possibly because of its high phytoconstituent content. Close to this finding, Vattem *et al*. (2007) reported up to 68.00% inhibition of violacein by modulation of AHL synthesis, from the methanol extract of *R. officinalis* in the United States of America (USA). Also compared to our finding, methanol extract of *O. basilicum* from the USA was found to inhibit violacein production at a slightly higher (58.00%) capacity (Vattem *et al*., 2007). Mukherji and Prabhune (2014) reported an (although) higher (50.00%) violacein inhibition from *C. citratus* in India, but that was only established at 20mg/mL, a concentration doubling ours. These differences might have arisen from a possible disparity in the plants' and/or bacterial physiology; owing to their varied geographical origins, genetic, nutritional and climatic conditions.

Identity of Active Compounds from the Plant Extracts

The GC-MS profiling of the extracts indicated an abundance of hydrocarbons (50.00%), organic acid esters (37.50%) and organic acids (12.50%) from *C. citratus* (Table 5); hydrocarbons (75.00%), organic acids (16.67%) and organic acid esters (8.33%) from *O. basilicum* (Table 6) as well as hydrocarbons (50.00%), organic acid esters (30.00%), organic acids (10.00%), ketones (5%) and alcohols (5%) from *R. officinalis* (Table 7). The major components in the GC-MS profile include Hexadecanoic acid methyl ester, 9,12-Octadecadienoic acid (Z, Z)- methyl ester and p-Cymene from *C. citratus, O. basilicum and R. officinalis* respectively. This finding indicates that all the fractions were dominated by hydrocarbons. Although the specific components from *C. citratus and O. basilicum* were mainly organic acid esters, both of these extracts recorded a much lower AQS activity (ab-initio) compared to the *R. officinalis*; from which a hydrocarbon (p-Cymene) was abundantly identified. This further depicts that p-Cymene could be responsible for the good features of AQS activity demonstrated by *R. officinalis*. Popescu *et al*. (2020) corroborate this finding as they similarly reported p-Cymene as an abundant compound found in the leaves of *R. officinalis* in Romania. The GC-MS analysis result of Tomi *et al*. (2016) also indicated that p-Cymene was predominantly identified in *R. officinalis* from Nara City of Japan. In the study

of Ababutain (2019), methanol extract of *O. basilicum* leaves from Dammam, Saudi Arabia was found to richly contain 9,12-Octadecadienoic acid (Z, Z)- methyl ester, as similarly discovered in this research. Still in concurrence to the finding in this study, Hexadecanoic acid methyl ester was maximally reported in *C. citratus* collected from the Northern State of Pulau, Malaysia, as indicated in the study of Mohamad *et al*. (2018).

CONCLUSION

The n-hexane, acetone, ethyl acetate, methanol as well as aqueous extracts of *R. officinalis, O. basilicum and C. citratus* leaves were obtained and the yield (%) of each extract was determined. Evaluation of the extracts' AQS activities revealed that the plants (especially *R. officinalis*) possessed some inhibitory features against violacein produced in *C. violaceum*. Therefore, this study identified the potential of the used plant leaves as sources of AQS compounds. The research also revealed that the plants' bioactive compounds can best be extracted using polar solvents especially methanol and distilled water. The GC-MS analysis revealed the major functional groups as p-Cymene, 9, 12-Octadecadienoic acid (Z, Z) methyl ester and Hexadecanoic acid methyl ester were the commonest constituting compounds of the most active fractions.

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