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Article

An *In-vitro* Study into the Inhibitory Effects of *Etlingera elatior* Flower Oil on *Acinetobacter baumannii* Biofilms

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Abstract: The current study investigates the antibiofilm properties of essential oil extracted from the Flower of a Zingiber plant used in traditional medicines. EO from *Etlingera elatior* (Jack) R. M Smith tested against one of the critical nosocomial pathogens, *Acinetobacter baumannii*. The antibiofilm studies of Flower essential oil (FEO) by crystal violet staining method exhibited maximum inhibition of 80% at a concentration of 0.7% oil. The biochemical assays and microscopic analysis showed that the FEO significantly reduced extracellular polymeric substance production. Furthermore, FEO reduced the survival rate of *A. baumannii* in human blood. The chemical composition of extracted FEO was analyzed by Gas chromatography- Mass spectrometry. Dodecanal, 1-dodecanol, and alpha-pinene were identified as the major compounds. Concerning previous research, our study is the first investigation of the antibiofilm property of *E. elatior* flower oil. More detailed studies are required to identify the compound responsible for biofilm inhibition and its mode of action against *A. baumannii* biofilms.

Keywords: *Acinetobacter baumannii*; *Etlingera elatior*; antibiofilm activity; essential oil

1. Introduction

Acinetobacter baumannii belongs to the *Moraxellaceae* family of *Proteobacteria* [1]. This Gram-negative, aerobic, non-motile, non-sporulating, non-fermentative, catalase positive, oxidase negative, pleomorphic coccobacilli has been categorized among the most alarming multi-drug resistant 'ESKAPE' pathogens: *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp. [2]. *A. baumannii* is predominantly associated with nosocomial infections with a high prevalence among immunocompromised patients. Their ability to survive in a wide range of pH and temperature, as well as in dry and moist conditions, helps them to thrive in hospital environments, especially on ventilators, surgical tools, and catheters, hence known as "nosocomial superbug" [3]. In 2017, WHO listed this bacterium among the pathogens over which novel antimicrobial agents are urgently required [4].

Over the past years, pathogenic bacteria exhibited different adaptive approaches to survive in adverse environmental conditions, the most notable of which is biofilm formation. Some bacteria form biofilms by adhering to an abiotic or biotic surface. The cells are embedded in a self-produced extracellular polymeric substance composed of polysaccharides, proteins, lipids, and nucleic acids. Bacterial cells in a biofilm exhibit elevated resistance to drugs and host defense mechanisms compared to their planktonic counterparts [5]. Nearly half (45%) of the reported strains of *A. baumannii* are multiple drug resistant [6]. The ability of *A. baumannii* to form a biofilm on the abiotic surface of the hospital environment facilitates its survival as a potent nosocomial pathogen. *A. baumannii* biofilms are the primary cause of most hospital-acquired infections such as meningitis,

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endocarditis, bloodstream infection, septicemia, wound infections, and urinary tract infections, with a mortality rate ranging from 7.8% to 43% and can reach up to 60% in vulnerable patients [7, 8].

Several factors include its quorum sensing mechanisms, presence of pili, and many proteins like outer membrane protein A (OmpA), phospholipase C, and D, and biofilm-associated proteins (bap), which contribute to the virulence of *A. baumannii*. In addition, two-component regulatory systems (TCS) regulate virulence, mainly motility, biofilm expressions, and catalase production [9]. These virulence factors are responsible for multi-drug resistance in *A. baumannii*. The widespread increase in multi-drug resistance in *A. baumannii* results in limited treatment options and a substantial economic burden, thus demands an urgent need for novel antimicrobial and antibiofilm agents.

Since the prehistoric ages, plants have been used to treat various infections, producing diverse bioactive secondary metabolites with therapeutic efficacy [10]. These have been associated with wide therapeutic applications with less toxicity. The plant family *Zingiberaceae* consists of 1400 species, including numerous medicinal plants with potent bioactive compounds [11]. Essential oils are the odiferous volatile liquids comprising various phytochemicals secreted at different plant parts as secondary metabolites and often have tremendous bioactive potential [12]. These phytocompounds are known for their antioxidant, antidiabetic, anti-inflammatory, hepatoprotective, neuroprotective, anticancer, and antimicrobial properties. However, the antibiofilm properties are least studied. *Etlingera elatior* (Jack) R.M. Smith, also known as torch ginger, native to Indonesia, is one of the significant plants in the *Zingiberaceae* family due to its traditional and commercial uses [13]. The Flower of this plant has been widely used in food, medicines, and ornaments [14]. However, the bioactivity of *E. elatior* flower oil is least investigated by researchers compared to other *Zingiberaceae* species. The current study looks into the antibiofilm properties of *E. elatior* flower oil (FEO) against the most troublesome nosocomial pathogen, *A. baumannii*.

2. Results and Discussions

2.1. Chemical Composition of FEO

The essential oil of collected *E. elatior* Flower (Figure 1) was obtained after hydro distillation using a Clevenger apparatus. The extracted oil was a colorless liquid characterized by an intense and unique odor. The GC-MS analysis revealed the presence of a total of 39 compounds. The major compounds identified were dodecanal (42.54%), 1-dodecanol (25.30%), and α-pinene (7.43%) (Table 1, Figure 2). The α- pinene exhibits antibiotic resistance modulation in *Campylobacter jejuni* [15] and inhibits its quorum sensing mechanism [16].

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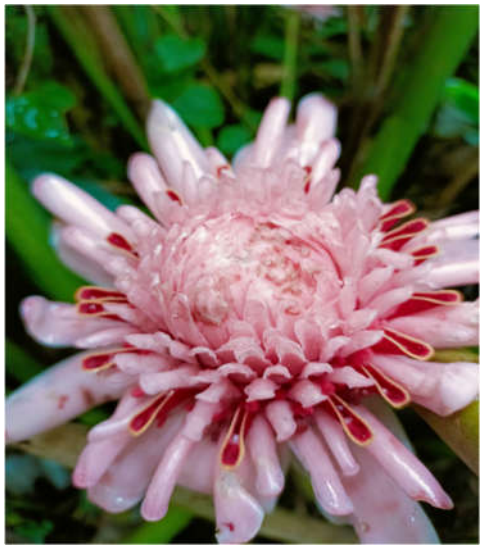

Figure 1. Collected inflorescence of *E. elatior*.

Table 1. Chemical composition of extracted essential oil.

[1]RT	COMPOUND	PEAK AREA (%)
5.203	Alpha pinene	7.43
5.617	Camphene	0.04
5.702	Bicyclo[3.1.0]hex-2-ene	0.04
6.355	Bicyclo[3.1.1]heptane	0.29
6.625	1,6-Octadiene	0.06
7.911	D-Limonene	0.23
8.467	1,3,6-Octatriene	0.05
9.898	Cyclohexene	0.14
10.064	2-Nonanone	0.06
10.422	1,6-octadien-3-ol	0.12
12.263	(+)-2-Bornanone	0.08
13.274	p-Mentha-1,5-dien-8-ol	0.11
14.257	3-Cyclohexene-1-methanol	0.71
14.748	Decanal	4.64
17.422	2-Propenal	0.25
17.587	1-Decanol	1.42
18.433	2-Undecanone	0.60
19.072	Undecanal	0.18
21.892	1-Undecanol	0.07
22.802	10-Undecenal	0.30
23.482	Dodecanal	42.54
23.694	Bicyclo[7.2.0]undec-4-ene	1.08
25.155	1,4,8-cycloundecatriene	0.17
25.546	8-Dodecen-1-ol	0.08
25.890	2-Nonen-1-ol	0.05
26.154	1-Dodecanol	25.30
26.887	2-Tridecanone	0.20
26.983	(3S,3aS,8aR)-6	0.05
30.013	Dodecanoic acid	6.50
30.958	9-Tetradecenal	0.82
31.362	Acetic acid	2.03
31.498	Tetradecanal	0.78

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33.413	cis-9-Tetradecen-1-ol	0.88
33.922	1-Tetradecanol	1.91
36.897	Benzyl Benzoate	0.27
38.135	9-Tetradecen-1-ol	0.06
38.620	1-Tetradecyl acetate	0.10
53.985	Pentacosane	0.29
59.373	Heneicosane	0.09
	Total	100

[1] RT, retention time (min)

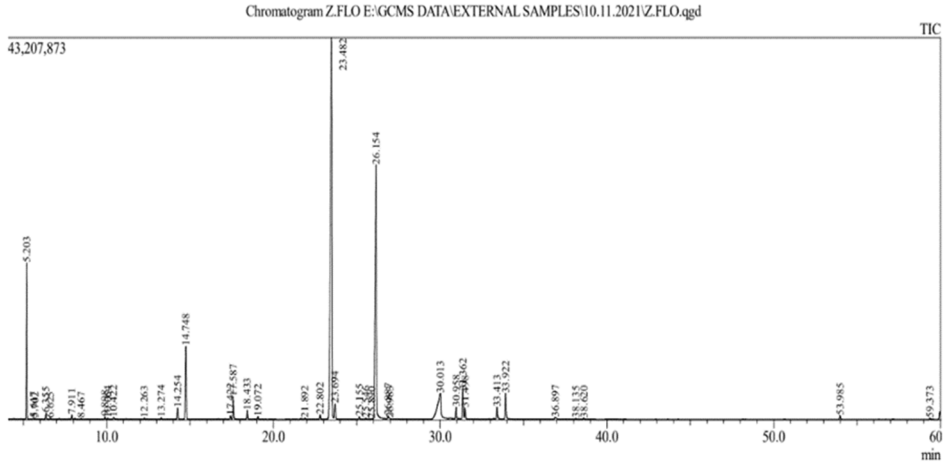

Figure 2. Gas chromatogram of extracted FEO.

2.2. Effect of FEO on A.baumannii Biofilms

The crystal violet staining method evaluated the antibiofilm potential of FEO on *A. baumannii* biofilms. FEO exhibited a concentration-dependent biofilm inhibition with 80% maximum inhibition at 0.7% (v/v) of oil concentration (Figure 3). No significant biofilm inhibition was observed above this concentration. Hence 0.7% of FEO was considered MBIC value, and further assays were performed at this concentration. The bacterial growth in control and FEO-treated samples were analyzed using spectrophotometry, confirming that biofilm inhibition was not due to growth inhibition. Hence it is confirmed that FEO exhibit an ideal antibiofilm potential.

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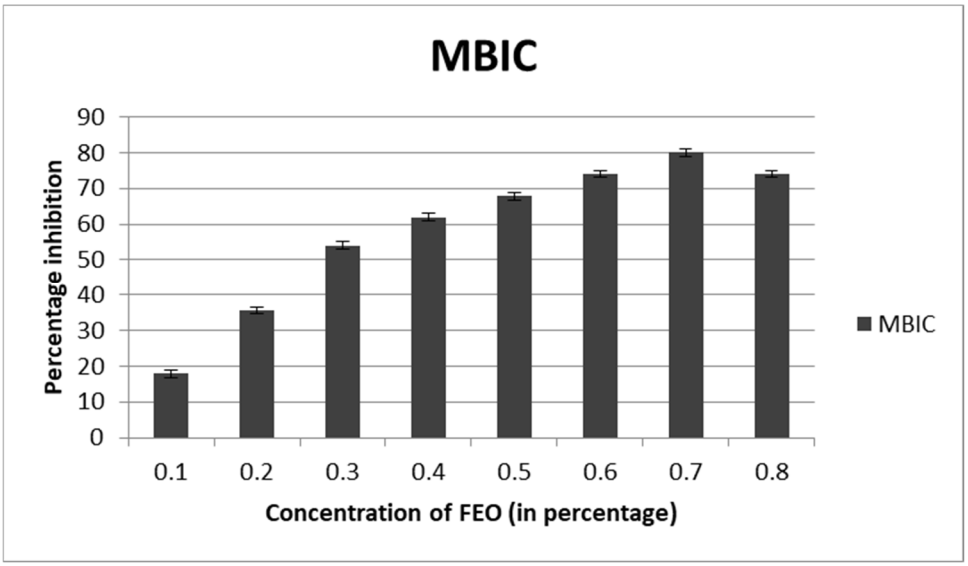
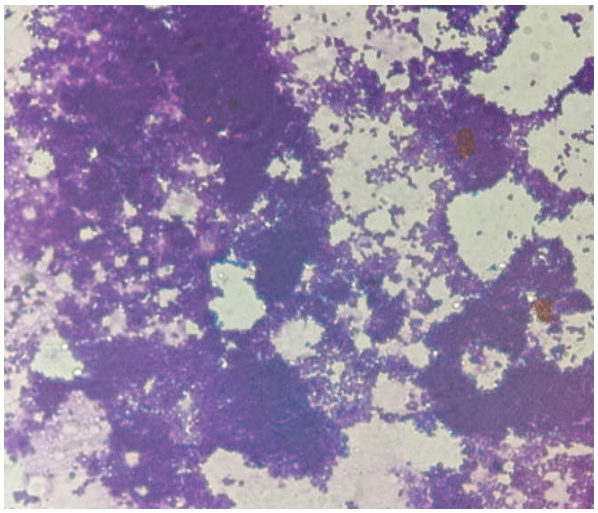


Figure 3. The graph demonstrating percentage reduction of *A.baumannii* biofilms with increasing concentration of FEO.

2.3. Microscopic Analysis

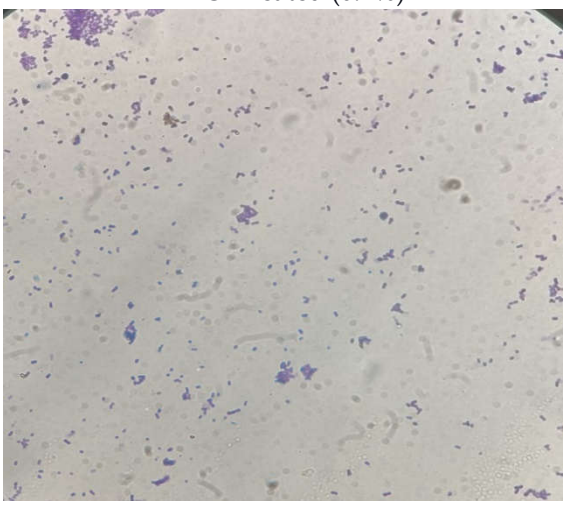
All three microscopic analyses; light, FE-SEM and CLSM displayed (Figure 4a, 4b, 4c) significant disruption of FEO-treated *A. baumannii* biofilms and microcolonies compared to the control samples. While on the contrary, untreated samples exhibited aggressive biofilm formation on glass slides. CLSM images of treated samples clearly show a substantial reduction in biofilms. FE-SEM analysis corroborated these findings. FE-SEM images of FEO treated models depicted isolated bacterial cells, whereas very densely layered *A. baumannii* cells were observed in FEO untreated glass slides. These findings confirmed the potentiality of FEO as an anti-biofilm agent.

Control



(4a)

FEO-Treated (0.7%)



(4a)

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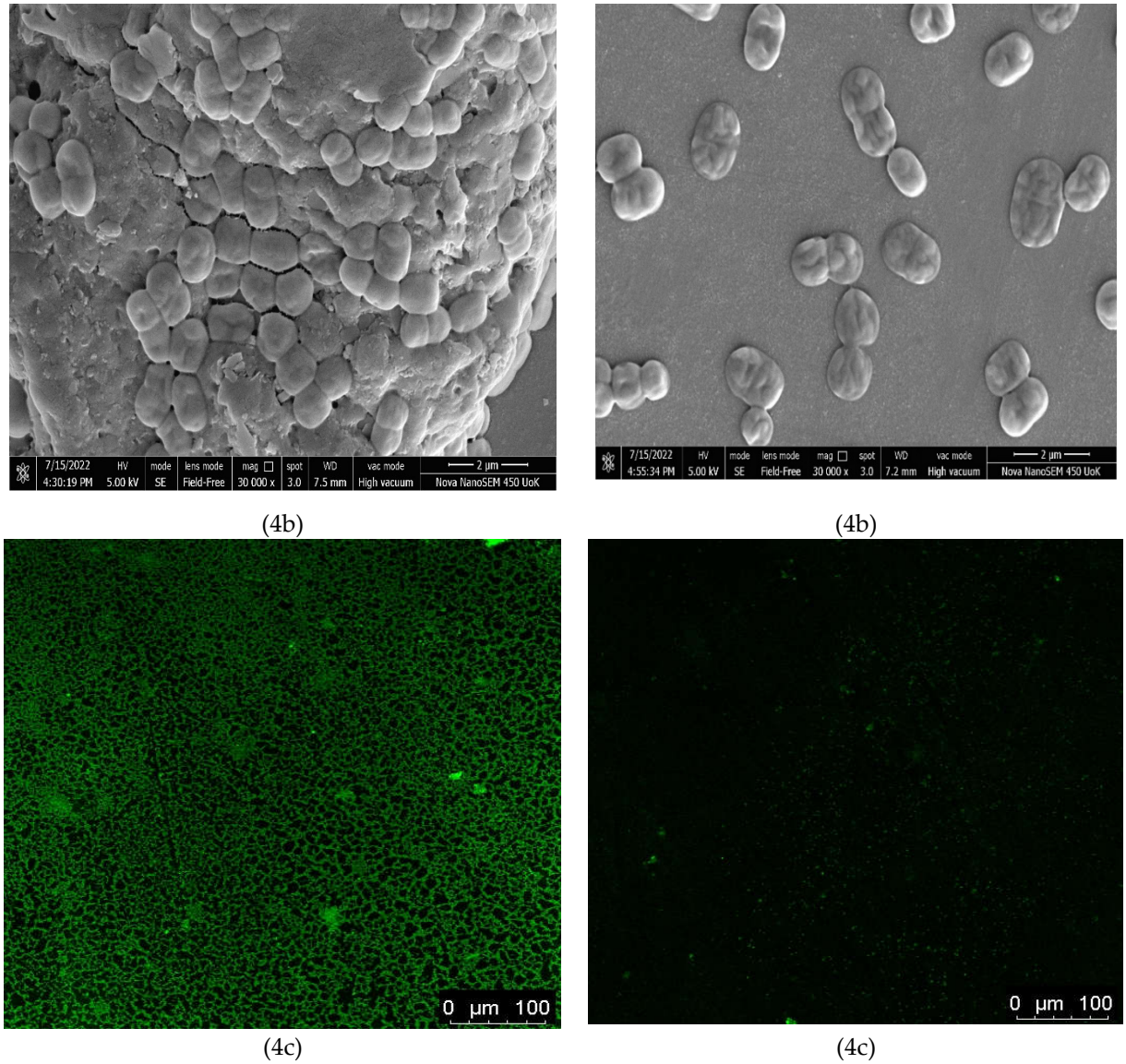

Figure 4. Microscopic visualization of *A.baumannii* biofilms showing microcolony disruption at 0.7% of oil concentration, compared to the control. (**a**) Light microscope images (magnification 100x) of CV-stained biofilms; (**b**) FE-SEM images (magnification 30,000x). (**c**) CLSM images of acridine orange-stained biofilms (scale bar= 100µm).

2.4. Effect of FEO on Cell Surface Hydrophobicity

Cell surface hydrophobicity has a significant role in biofilm formation. The initial step of biofilm formation requires cell-to-cell or cell-to-surface interaction. This interaction helps bacteria adhere to abiotic or biotic surfaces, leading to microcolony formation. Hence the effect of FEO on cell surface hydrophobicity of A. baumannii was assessed by MATH assay. The treatment of FEO considerably reduced the cell surface hydrophobicity from 89.50% to 15% (Figure 5). Reduction in cell surface hydrophobicity further inhibits biofilm formation at early stages. The current results can be compared with a previous study in which α-mangostin at its MBIC significantly inhibited the initial biofilm formation due to reduced cell surface hydrophobicity [17]. In addition. 5-hydroxymethylfurfural, a furan compound with various biological activities, including antibiofilm properties, is known to inhibit the cell surface hydrophobicity in A. baumannii [9].

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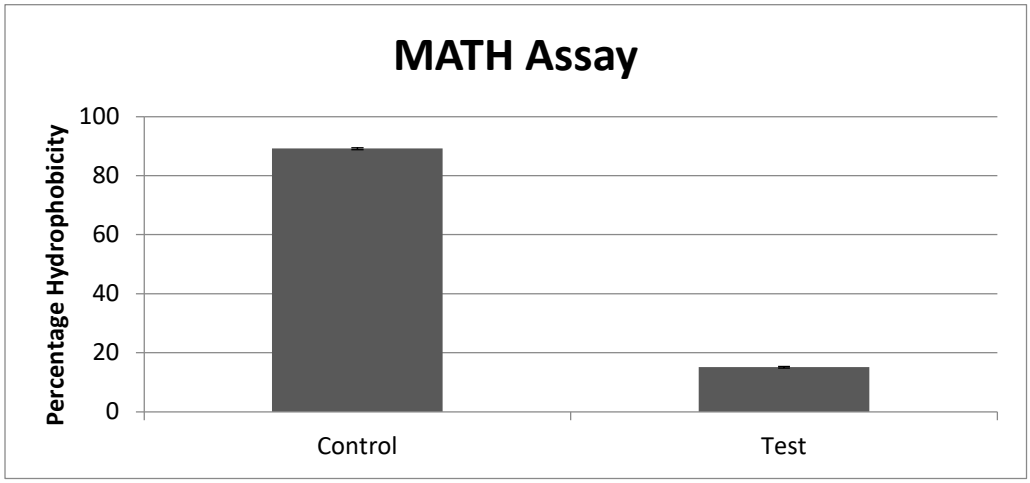

Figure 5. Graph representing the influence of FEO in the percentage production of cell surface hydrophobicity.

2.5. Effect of FEO on EPS Production

The extracellular polymeric substance or EPS is a biopolymer secreted by microorganisms in a biofilm. It is primarily composed of polysaccharides and proteins. Hence the reduction of these two components was spectrometrically analyzed to evaluate the effect of FEO on EPS production. The FEO treatment diminished the production of polysaccharides and proteins in extracted EPS solution. In brief, FEO reduced polysaccharides and protein to 70.6% and 55.1% respectively, compared to the untreated control samples (Figure 6). EPS is pivotal in maintaining biofilm architecture and mechanical stability [18]. In addition, EPS interferes with the entry of host immune cells and antimicrobial agents, leading to fewer treatment options [19]. PNAG, a polysaccharide in EPS, mediates cell-surface adhesion and protection against the host defense mechanism (encoded by pgaABCD locus) [20, 21]. The reduction of EPS production upon FEO treatment expedites biofilm inhibition and enhances the susceptibility of *A. baumannii* towards antibiotics.

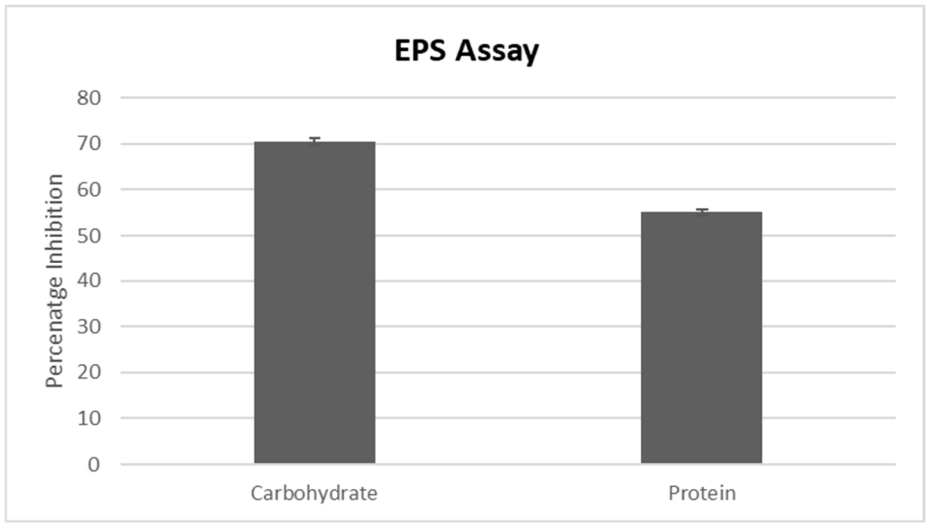

Figure 6. Inhibitory effects of FEO on the EPS components in biofilm of *A. baumannii.*

2.6. Hydrogen peroxide Sensitivity Test

An H_2O_2 sensitivity test evaluated the ability of FEO to reduce catalase production. The FEO-treated samples were more sensitive to H_2O_2 than the control. The zone of inhibition of control and test were observed as 28 mm and 43 mm respectively (Figure 7). In brief, this increased zone of inhibition in the FEO-treated sample is directly proportional to the reduced catalase production. Most antibiotics may enhance respiratory stress by

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generating reactive oxygen species (ROS) to kill bacteria [22]. The catalase (regulated by gene KatE) provides resistance to the *A. baumannii* cells to survive under H_2O_2 conditions. A recent study has demonstrated that Pyrogallol downregulates the gene responsible for catalase production in *A. baumannii* and acts as an anti-biofilm agent [23]. Furthermore, in *A. baumannii,* catalase synthesis is regulated by the quorum sensing system [24]. As anticipated, the findings of the H_2O_2 sensitivity assay revealed decreased catalase production in FEO-treated *A. baumannii* cells.

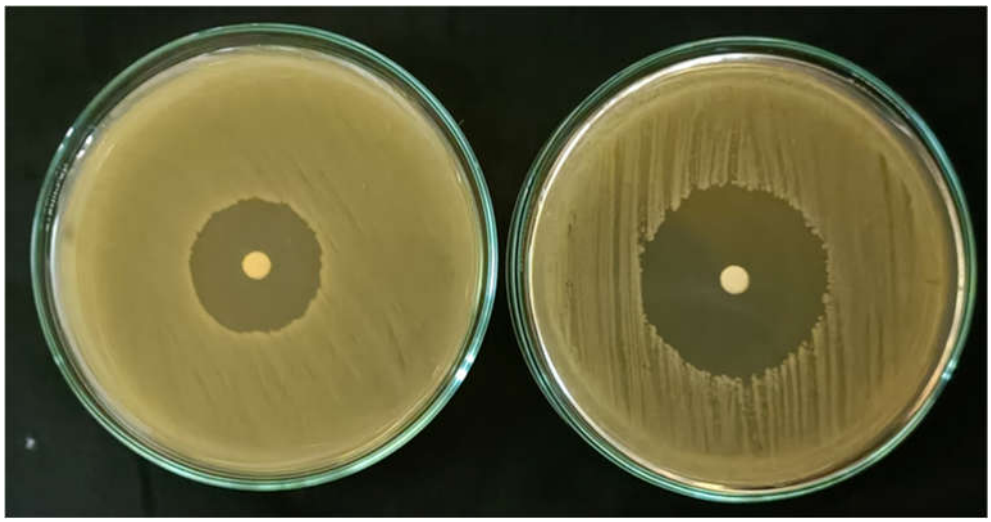

Figure 7. H_2O_2 Sensitivity assay of FEO-treated sample showing large zone of clearance compared to the control samples.

2.7. Blood Survival Assay

The ability of bacteria to evade opsonophagocytosis is directly linked to its pathogenesis. Escaping from opsonophagocytosis increases the survival rate of bacteria in the blood. We have observed that FEO treatment significantly reduced the survival of *A. baumannii* in human blood. The colonies on FEO-treated samples were less compared to control samples (Figure 8). Hence, FEO was effective in rendering *A. baumannii* vulnerable to phagocytosis. According to a study, OmpA, along with a fluid phase complement regulator factor H, interferes with complement attack by host tissue [25]. The OmpA gene is also associated with the adherence of *A. baumannii* to the host epithelial cell [26]. Previous studies revealed that compounds with antibiofilm properties could downregulate OmpA [9, 17]. Similarly, the downregulation of this gene could be responsible for the decreased survival rate of *A. baumannii* cells in human blood upon FEO treatment.

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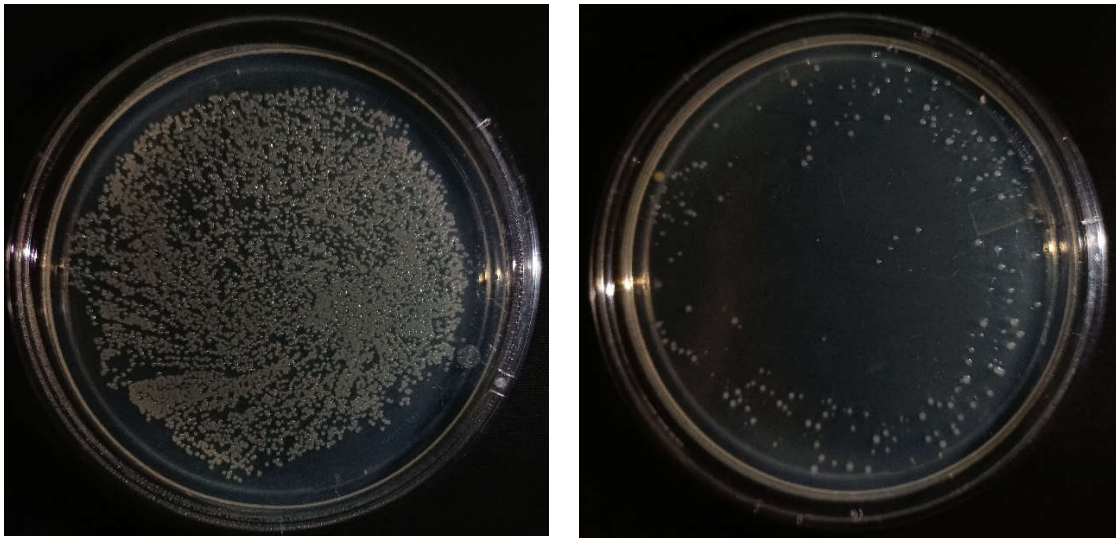

(**a**) (**b**)

Figure 8. Blood Survival Assay (**a)** Countless number of colonies observed in FEO-untreated blood samples; (**b**) FEO-treated blood samples exhibited less colonies, compared to the control . The FEO-treatment mitigates the survival rate of *A. baumannii* in human blood.

3. Materials and Methods

3.1. Bacteria Strain and Growth Condition

Acinetobacter baumannii MTCC 9829 was used in this study. The bacterial strain was grown and maintained in Luria Bertani (LB) agar. The culture was maintained in Tryptic soy broth supplemented with 1% sucrose (TSBS) at 37° C and was used for all other bio-assays. The overnight culture containing approximately ~1.6 × 107 CFU ml−1 was taken as a standard cell suspension.

3.2. Collection of Plant Material

Etlingera elatior flower was collected from the field of the Kerala University campus, Karyavattom (8.5646° N, 76.8852° E). A voucher specimen was deposited at the Herbarium of Botany Department, Kerala University, with an accession number: KUBH11149

3.3. Extraction of Essential Oil

Fresh petals of *E. elatior* were hydro distilled using a Clevenger apparatus for 4-5 hours. The extracted oil was dried over anhydrous sodium sulphate and stored at 4 °C for further analysis.

3.4. GC-MS Analysis

The essential oil was dissolved and diluted in diethyl ether and analyzed using Schimatzu GC-MS Autosampler in electron impact (EI+) ionization mode (70ev) with a mass range of 50 to 550 m/z. Helium was used as the carrier gas with a flow rate of 1.4ml/min. The temperature program; injector temperature 240 °C, oven temperature 60-250 °C (3°C/min), and interface temperature 260 °C. The components in essential oil were identified by matching recorded mass spectra in the computer library.

3.5. Determination of the Minimum Biofilm Inhibitory Concentration (MBIC)

The microdilution method was used to determine the MBIC of *E. elatior* flower essential oil (FEO) against the reference bacterium *A. baumannii* MTCC 9829. In brief, different volumes of essential oil were added individually to wells containing 200 µl of TSBS broth in a 96-well plate to make the final concentrations of 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8%. The wells were inoculated with 1% of standard cell suspension. The wells containing 1%

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DMSO are considered the negative control. After incubating at 37 °C for 24 hours, each well was washed with sterile distilled water to remove unbounded planktonic cells. The bacterial cells adhered to well were stained with 0.4% crystal violet (w/v) for 10 min. The excess stain was removed by washing the wells thrice with sterile distilled water. The bound dye was then diluted to 200µl of 20% glacial acetic acid for 10 min, and absorbance was read at 570 nm [27]. The percentage inhibition of biofilm in each well was determined using the formula:

Percentage inhibition= [(control OD570 nm – treated OD570 nm)/control OD570 nm] x100.

3.6. Microscopic Analysis

The effect of *E. elatior* flower essential oil on *A. baumannii* biofilm architecture was examined under a light microscope, field emission-scanning electron microscope (FE-SEM), and confocal laser scanning microscope (CLSM). For microscopic light analysis, 1x1cm glass slides were placed in a 24-well polystyrene plate containing 1 ml of FEO treated (0.7%) and untreated TSBS with 1% standard cell suspension. After incubating at 37 °C for 24 hours, the slides were washed with PBS and stained with 0.4% crystal violet (w/v) for 10 minutes. Subsequently, the slides were washed with sterile distilled water to remove excess stains and observed under a light microscope at 100 X magnification.

Similarly, the biofilms grown on 1x1cm glass slides in the presence and absence of FEO were washed with sterile PBS solution and fixed with 2.5% of glutaraldehyde solution for 2 hours at 4 °C. Subsequently, the slides were washed with 0.1M sodium phosphate buffer (pH 7.3) and with sterile distilled water. The biofilm-fixed slides were then dehydrated with increasing ethanol concentrations (30, 50, 60, 70, 90, 100%). The air-dried slides were then sputter coated with gold particles and observed under FE-SEM (NOVA NANOSEM 450).

The biofilms in the presence or absence of EFO were washed in sterile PBS and stained with 0.1% acridine orange for 5 min for Confocal Laser Scanning Microscope imaging. The air-dried slides were visualized under CLSM (Leica DMi8). A band emission filter of 500-640nm was used for excitation and detection.

3.7. Microbial Adhesion to Hydrocarbon (MATH) Assay

The effect of *E. elatior* flower oil on the cell surface hydrophobicity of *A. baumannii* was evaluated by MATH assay. Biofilms were grown in the presence and absence of FEO. The 24-hour-old cultures were diluted to attain OD 0.4 at 600 nm. 1 ml of toluene was added to an equal volume of diluted culture and vortexed thoroughly. After separating the aqueous/solvent phase, the cells retained in the aqueous phase were quantified by measuring absorbance at OD 600 nm. The percentage hydrophobicity was calculated using the following equation:

% hydrophobicity=[1 -(OD 600 nm after vortexing/OD 600 nm before vortexing)] x 100 [27].

3.8. Extracellular Polymeric Substance Extraction and Analysis

The EPS were extracted from FEO treated and untreated *A. baumannii* biofilms as described by Jiao *et al.* with some modifications [28]. The FEO treated and untreated *A. baumannii* were grown in TSBS at 37 °C for 24 hours. The cultures were then centrifuged at 12000 rpm for 30 min to obtain biofilm pellets. These pellets were resuspended in 30 ml of ice-cold solution of 0.2M sulfuric acid (pH 1.1). A steal bead homogenizer is used to break the biofilm matrix. Then cell suspensions were stirred continuously for 3 hours at 4 °C using a magnetic stirrer. Subsequently, the solution was centrifugated (12000 rpm, 30min) to obtain supernatant, referred to as EPS solution. The EPS solution contains total EPS of both capsular and colloidal fractions). The dry weight of EPS can be estimated by subtracting the cell pellet dry weight from the dry biofilm weight.

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The carbohydrate content of EPS was estimated using the phenol-sulphuric acid method. In brief, 100µl of extracted EPS solution were mixed with 250 µl of concentrated sulphuric acid and 50 µl of 10% phenol. The mixture was further incubated at 60 °C for 30 min, cooled, and read spectrophotometrically at 490 nm [17]. The total protein content was evaluated by treating 10 ml of EPS solution with 12% trichloroacetic acid. After the incubation in ice for 30 min, the mixture was centrifuged at 12000 rpm for 30 min. The residue was washed thrice with 10 ml of acetone [9]. Lowry's method was used to estimate the protein content.

3.9. Hydrogen peroxide Sensitivity Test

The FEO treated and untreated *A. baumannii* were grown for 24 hours at 37 °C. The standard cell suspension was then swabbed on Mueller-Hinton agar plates. Subsequently, 15µl of 30% H_2O_2 was loaded to the Whatman filter paper discs placed on MHA and incubated for 24 hours to observe the clearance zone [18].

3.10. Blood Survival Assay

The survival rate of *A. baumannii* in human blood in the presence and absence was evaluated by this assay. The 450 µl of human blood was mixed with 50 µl of FEO treated and untreated overnight cultures of *A. baumannii*. After 3 hours of incubation at 37° C, the cell viability was analysed by spread plate method on tryptic soy agar [27].

4. Conclusions

The present study highlights the antibiofilm properties of *E. elatior* flower oil against *A. baumannii* biofilms. The study demonstrated that FEO treatment inhibits biofilms and microcolony formation in reference bacterium. In addition, FEO considerably reduced cell surface hydrophobicity, EPS production, and survival rate of *A. baumannii* in the presence of H_2O_2 and in human blood, which in turn affects biofilm formation. Hence the current study proved that FEO has a great potential to inhibit *A. baumannii* biofilms. Besides, the rich phytochemical composition in FEO makes it a significant nutraceutical or bioceutical agent.

Author Contributions: Conceptualization, Sugathan S. and Türetgen I.; methodology, Naushad T. and Sugathan S.; validation, Sugathan S. and Türetgen I.; formal analysis, investigation, writing—original draft preparation, Naushad T.; resources, Sugathan S.; data curation, Naushad, T and Salim, S; writing—review and editing, Sugathan S. and Türetgen I.; supervision, Sugathan S.; project administration, funding acquisition, Türetgen I.

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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