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# Evaluation of *Alpinia officinarum* Plant Extracts as Natural Quorum Quenchers against MDR *Acinetobacter baumannii* Isolates

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**ABSTRACT**

The roots of Alpinia officinarum were extracted in different solvents, initially methanol, followed by ethyl acetate, chloroform, and petroleum ether, and tested at three concentrations (25, 50, and 75 mg/mL) to isolate the active organic constituents. This study was performed to examine the inhibitory effect of the plant extract on the biological activities of multidrug-resistant (MDR) Acinetobacter baumannii, including biofilm formation, pellicle formation, motility, gelatinase and protease production, and quorum-sensing (QS) signalling. Fifty Acinetobacter spp. isolates were recovered from blood, urine, and cerebrospinal fluid of intensive care unit patients, of which 30 were identified as A. baumannii by API 20E and PCR (16S rRNA and blaOXA-51). The isolates showed high resistance to β-lactams (62.4%), moderate resistance to carbapenems (39%), and low resistance to fluoroquinolones (26.6%), and 84% were strong biofilm producers. Nine representative MDR isolates were treated with the extract fractions. Overall, the 75 mg/mL concentration of A. officinarum extract was the most effective against all isolates, and the methanol-derived fractions (petroleum ether and ethyl acetate) showed stronger inhibitory effects than the chloroform fraction. After treatment, biofilm and pellicle formation, motility, and AHL production were markedly reduced, and most isolates became susceptible to antibiotics to which they were previously resistant. These findings indicate that A. officinarum extracts are promising natural quorum quenchers and resistance-modifying agents against drug-resistant A. baumannii, although further studies are needed to identify the active antibacterial components of the extract.

**KEYWORDS: Alpinia officinarum, Acinetobacter baumannii, quorum quenching, biofilm, multidrug resistance, plant extract.**

## 1. INTRODUCTION

*Acinetobacter baumannii* is a Gram-negative, non-motile, non-glucose-fermenting coccobacillus and an opportunistic nosocomial pathogen that exhibits multidrug resistance (MDR) to various antimicrobial agents [1]. *A. baumannii* colonizes various sites of infection, especially in immunosuppressed patients, causing urinary tract infections, skin and soft-tissue infections, pneumonia, and bacteremia [2].

Researchers have examined the quorum-quenching (QQ) effect of different materials against the biological activities of MDR *A. baumannii*, and plant extracts are generally preferred because they contain many natural compounds with fewer side effects than synthetic drugs [3]. *Alpinia officinarum*, a plant belonging to the family Zingiberaceae, is one of the main plant sources of bacterial virulence quenchers; its extracts express AHL-lactonase activity that quenches pathogen quorum-sensing (QS) signalling, inhibit efflux pumps, and significantly decrease multidrug resistance in bacterial infections [4]. *A. officinarum* is rich in a wide variety of secondary metabolites, such as alkaloids and flavonoids, which have been shown in vitro to possess antimicrobial properties [5]. QQ compounds interact with bacterial periplasmic proteins that are essential for the folding of QS signals, thereby damaging the QS signal that regulates virulence factors such as swarming, motility, and biofilm formation. Plant extracts have been developed for the treatment of several bacterial infectious diseases because plant-derived virulence quenchers inhibit QS signalling and efflux pumps and significantly reduce bacterial resistance to different classes of antibiotics [6].

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Different materials have been used as QQ agents. García-Lara *et al.* [7] used zinc oxide nanoparticles, which are able to inhibit QS-dependent virulence factors (including biofilms) in clinical strains [8]. Other researchers extracted halogenated furanones, which act on bacterial activity by mimicking the AHL signal, presumably by occupying the binding site on the putative regulatory protein, rendering it highly unstable and accelerating its turnover rate, thus resulting in the rapid disruption of QS-mediated gene regulation [9]. Saurav *et al.* [10] used modern drugs to inactivate bacterial QS through different QQ mechanisms, including enzymatic inactivation, inhibition of signal biosynthesis, and inhibition of signal detection. Pharmacologists, botanists, and microbiologists have identified several nanoparticles and chemicals, including drugs used as QS inhibitors or compounds isolated from natural extracts, for the treatment of infectious diseases [11]. The dry root and rhizome of *A. officinarum* have been used for their antioxidant, antidiabetic, antiulcer, antidiarrheal, anti-inflammatory, and anticoagulant effects [12], and different solvents are available for extracting the bioactive compounds from natural products [13]. The present study highlights the promising potential of using QS signals and efflux pumps as molecular targets for disease control, thereby broadening current approaches to the prevention of bacterial infections. This study aimed to use alternative (herbal) medicine, represented by *A. officinarum* extracts, for the treatment of dangerous pathogenic bacteria instead of drugs, since plant extracts are considered to have a harmless effect on human health compared with antibiotics.

## 2. MATERIALS AND METHODS

### Sample Collection

Clinical samples, including cerebrospinal fluid (CSF), blood, and urine, were collected from patients admitted to the Intensive Care Unit. Virulence factor tests were performed, followed by antibiotic susceptibility testing, to study the effect of the plant extract on the bacterial pathogen.

### Preparation of the Plant (*Alpinia officinarum*) Extract

*A. officinarum* extraction was carried out according to Bhargava *et al.* [14]. The dried powder of the plant material (30 g) was subjected to exhaustive extraction with methanol using a Soxhlet apparatus; after evaporation of the methanol, the residue was partitioned into three treatment fractions (petroleum ether, chloroform, and ethyl acetate), as shown in Figure (1). The virulence factor assays were performed before and after treatment with the extract to determine the QQ activity of the plant fractions at three concentrations (25, 50, and 75 mg/mL) on *A. baumannii* growth; the 75 mg/mL concentration showed the greatest QQ effect on bacterial growth.

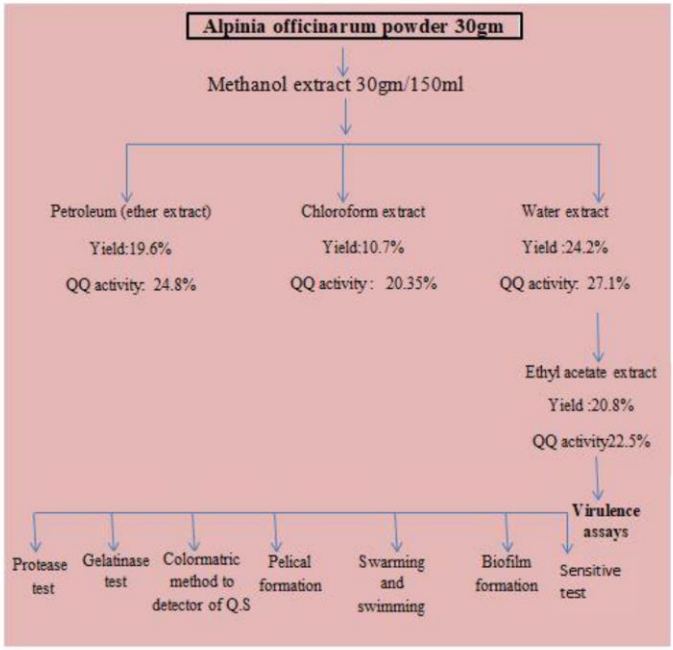



**Figure (1): Schematic representation of the quorum-quenching (QQ) study design.**

**Antibiotic Susceptibility Test**

Antibiotic susceptibility testing of the *A. baumannii* isolates was conducted by the standard disc diffusion technique using ten antibiotics. The antibiotics were classified into three groups according to class: carbapenems, including meropenem (10 µg) and imipenem (10 µg); fluoroquinolones, including levofloxacin (5 µg), ciprofloxacin (5 µg), and norfloxacin (10 µg); and β-lactams, including amoxicillin (25 µg), cefotaxime (30 µg), cefepime (30 µg), amoxicillin–clavulanate (Augmentin, 30 µg), and cefixime (5 µg). *Escherichia coli* ATCC 25922 was used as the quality-control reference strain in susceptibility determination, and isolates were categorized according to Magiorakos *et al.* [15]. For testing after plant treatment, 1 mL of bacterial broth was added to 5 mL of the plant extract suspension of each of the three treatments (chloroform, petroleum ether, and ethyl acetate) at a concentration of 3.75 mg/mL, and the assay was then completed.

**Virulence Factor Tests of *A. baumannii* Before and After Treatment with *A. officinarum***

**Biofilm Formation**

Biofilm formation was tested as described by Ghellai *et al.* [16]. Twenty microlitres of an overnight bacterial culture were used to inoculate the wells of a flat-bottom microtiter plate containing 180 µL of brain heart infusion (BHI) broth with 2% sucrose. Control wells contained only 200 µL of BHI broth with 2% sucrose. The plates were incubated at 37 °C for 24 h; after washing the wells with phosphate-buffered saline and drying at room temperature, the optical density was measured at 630 nm using an ELISA reader. For the post-treatment assay, the overnight bacterial culture was activated with the *A. officinarum* extract in the three treatments (petroleum ether, chloroform, and ethyl acetate) at a concentration of 3.75 mg/mL, and the assay was completed as above.

**Motility Test (Swarming and Swimming)**

Motility was tested using semi-solid motility medium. In the swimming assay, the bacteria were stab-inoculated with a sterile toothpick into the centre of a glass test tube, while in the swarming assay 1 µL of bacterial suspension was inoculated at the centre of the agar plate; both were incubated overnight at 37 °C [17]. For the post-treatment assay, 1 mL of *A. baumannii* culture was activated in 5 mL of the plant extract suspension at 37 °C for 24 h with the three treatments (chloroform, petroleum ether, and ethyl acetate) at a concentration of 3.75 mg/mL.

**Pellicle Assay**

The pellicle assay was performed by inoculating 5 mL of BHI broth in polystyrene tubes with a single colony of each *A. baumannii* isolate. The tubes were then incubated for 3 days at 37 °C without shaking. Pellicle formation, if any, appeared as a white layer at the surface of the broth [18]. The post-treatment pellicle assay was performed by inoculating 1 mL of *A. baumannii* broth into 5 mL of the plant extract suspension and completing the assay as above.

**Detection of AHLs by the Colorimetric Method**

Quantification of AHL molecule activity was carried out as described by Baldiris *et al.* [19]. One pure overnight colony of *A. baumannii* was cultured in 5 mL of nutrient broth at 37 °C for 24 h. From this culture, 1.5 mL was centrifuged for 15 min at 10,000 rpm; the supernatant was transferred, and this step was repeated twice. The liquid was extracted with ethyl acetate for 10 min and the organic (upper) phase was removed. The samples were then dried from residual ethyl acetate at 42 °C for 1–2 h in the incubator, 40 µL was transferred to a microtiter plate, and about 50 µL of each of two solutions was added: a 1:1 solution of 10% $FeCl_3$ in 4 M HCl : 95% ethanol, and a 1:1 solution of 2 M hydroxylamine : 3.5 M NaOH. Finally, the optical density was measured with an ELISA reader at 630 nm. For the post-treatment assay, a colony of *A. baumannii* was cultured in 5 mL of the plant extract suspension with nutrient broth, and the test was completed as above.

**Gelatinase and Protease Tests**

For the gelatin hydrolysis test, the *Acinetobacter* isolates were inoculated into tubes containing nutrient gelatin medium and incubated at 35 °C for three days. The tubes were then removed from the incubator, placed in an ice bath or refrigerator (4 °C) for 15–30 min, and observed for gelatin hydrolysis [20]. For the post-treatment test, *A. baumannii* was activated on Mueller–Hinton agar prepared with the plant extract at a concentration of 3.75 mg/mL at 37 °C for 24 h and then inoculated into the nutrient gelatin tubes. The protease test was performed according to Vijayaraghavan *et al.* [21] for screening *A. baumannii* protease: the overnight culture was streaked on skim milk agar, and the zone of casein hydrolysis around the bacterial growth was observed after incubation at 37

°C for 48 h. For the post-treatment test, the bacteria were activated in 5 mL of the plant extract suspension and the test was completed as above.

**Data Analysis**

The isolates were grouped by cluster analysis (dendrogram) according to their antibiotic resistance patterns and virulence factor results, and representative isolates were selected from each subgroup for the plant extract experiments.

## 3. RESULTS AND DISCUSSION

**Isolation and Identification**

The sources of the 50 *Acinetobacter* spp. isolates were as follows: 40 isolates from blood, 5 from urinary tract infections (UTI), and 5 from CSF. On microscopic examination, *A. baumannii* appeared as Gram-negative coccobacilli, occasionally arranged in pairs, on blood agar. In biochemical tests, *A. baumannii* gave negative results for oxidase, motility, indole, and urease, while the isolates were positive for catalase and citrate utilization. Final identification of all isolates was performed at two levels: first by the conventional API 20E system, characterized as an easy and rapid method, and second by PCR amplification of the housekeeping 16S rRNA gene to detect the genus *Acinetobacter* and of *bla*OXA-51 to identify the species *A. baumannii*. From these two steps, 30 of the 50 *Acinetobacter* spp. isolates were identified as *A. baumannii*. Both assays showed 100% specificity and sensitivity for their intended targets.

**Antibiotic Susceptibility Test**

In this study, the *A. baumannii* isolates showed high resistance to amoxicillin and amoxicillin–clavulanate. Resistance was found to three antibiotic classes: the highest resistance was against the β-lactam class (62.4%), which is in agreement with Sinha *et al.* [22], who detected a high rate of resistance to the β-lactam group in *Acinetobacter* species. The second major class was the carbapenems, to which resistance was moderate (39%). In a nationwide surveillance study conducted in the United States, 44.7% and 49.0% of isolates were resistant to imipenem and meropenem, respectively [23]. Carbapenemase production is one of the main mechanisms for the emergence of MDR and/or XDR *A. baumannii*. Although metallo-β-lactamases in general hydrolyze carbapenems poorly, so that their presence can be suggested by carbapenem resistance in the setting of imipenem and meropenem susceptibility, the frequent co-expression of the more common extended-spectrum β-lactamases in conjunction with metalloenzymes results in clinical strains that express resistance to both classes [24]. The third class was the fluoroquinolones, to which *A. baumannii* in general showed low resistance (26.6%); as a result, this group remains a drug of choice against *A. baumannii*. Resistance of *A. baumannii* to levofloxacin, ciprofloxacin, and norfloxacin results from changes in DNA gyrase and topoisomerase IV that reduce drug affinity, while chromosomally encoded influx and efflux systems mediate reduced expression of the outer-membrane proteins involved in drug influx and increased expression of efflux proteins, resulting in active drug expulsion; these mechanisms are also responsible for quinolone resistance [25].

**Virulence Factors Before Plant Extract Treatment**

**Biofilm Formation**

The microtiter plate method was adopted as the best method because it detected the largest number of biofilm-forming isolates. The fifty *Acinetobacter* spp. isolates were differentiated into three patterns (A, B, and C) according to their MDR profiles. The highest percentage of MDR isolates was observed in pattern A, 22 (44%), followed by pattern C, 15 (30%), and pattern B, 13 (26%). Out of the 50 isolates, 42 (84%) were considered strong biofilm producers, while 8 (16%) were moderate or weak biofilm producers. The percentages of MDR *A. baumannii* isolates in patterns A and C were similar, 12 (40%) each, while pattern B contained half that number, 6 (20%). All *A. baumannii* isolates in patterns A, B, and C were biofilm producers, and the isolates in patterns A and C in particular were characterized as strong biofilm formers; thus, this study demonstrated a strong correlation between MDR and biofilm formation. This result is in agreement with Babapour *et al.* [26], who found a relationship between biofilm formation in the standard microtiter plate method and MDR, noting that the highest percentage of isolates with moderate-to-strong biofilm formation were MDR isolates.

**Motility (Swimming and Swarming)**

Motility was observed in all *Acinetobacter* spp. isolates; all 50 isolates were able to swarm and swim (Figure 2). Wood *et al.* [27] reported that most, but not all, *A. baumannii* isolates are motile, and Eijkelkamp *et al.* [28] observed motility after 24 h for swimming and 72 h for swarming. In this study, all 30 *A. baumannii* isolates were motile and displayed a motility phenotype, suggesting that *A. baumannii* motility depends on type IV pili, QS autoinducer molecules, and the response to blue light, and that *A. baumannii* is capable of producing different adhesins and pilus assembly systems in response to changes in environmental conditions. It was also noticed that every *A. baumannii* isolate with the ability to move was able to form biofilm, because the type IV pili responsible for motility are also responsible for the first step of biofilm formation.



**Figure (2): Swarming and swimming tests of *A. baumannii* isolates.**

**Pellicle Assay**

*A. baumannii* can form pellicles more readily than other, less pathogenic *Acinetobacter* species. Forty-four *Acinetobacter* spp. isolates formed a white layer on the broth surface as a result of the effect of the QS AHL molecules (Figure 3), while 6 of the 50 isolates were considered weak pellicle formers. After 24 h, a thin pellicle started to form at the surface of the liquid medium; this pellicle grew in thickness, and by the end of the third day an opaque and solid structure covered the whole liquid surface. All 30 *A. baumannii* isolates were characterized as strong pellicle producers. Chabane *et al.* [29] visually analysed 86 *A. baumannii* clinical isolates for pellicle formation and separated 26 (30%) pellicle-forming isolates for further analysis, while Collet *et al.* [30] suggested differences between bacteria grown as pellicles and as biofilms at the solid–liquid interface.

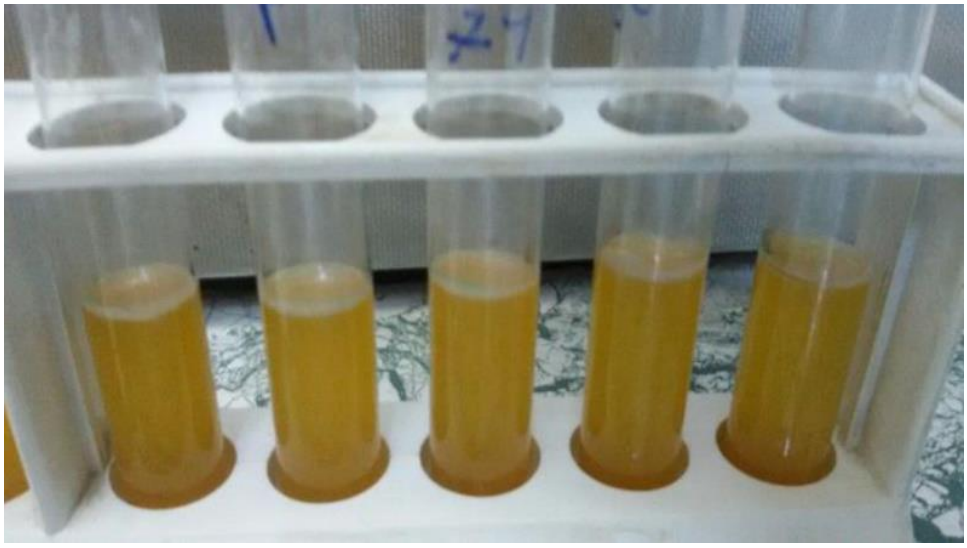


**Figure (3): *A. baumannii* forming a pellicle on the top of the liquid medium.**

## Colorimetric Detection of Quorum Sensing

All *Acinetobacter* isolates were QS producers, but at varying levels ranging from strong to weak or non-producing (Figure 4). Thirty-three *Acinetobacter* isolates were high producers of AHL molecules, while 17 isolates exhibited low AHL activity. A dark brown colour is produced in samples containing AHL molecules, but in some cases it may change to yellow depending on the concentration of the compounds. Modarresi *et al.* [31] reported that AHLs produced by different bacteria differ in the length of the R-group side chain, and that both AHL production and biofilm formation are regulated by iron concentration in a dose-dependent manner. These findings provide evidence that iron limitation plays an important regulatory role in AHL and siderophore production, resulting in strong or weak biofilm, thereby helping the organism to persist in micronutrient-limited environments.




**Figure (4): Quantification of AHLs; optical density measured with an ELISA reader at 630 nm (from left to right).**

## Gelatinase and Protease Tests

Other virulence factor tests were also conducted on the *A. baumannii* isolates. In general, 39 (78%) of the *Acinetobacter* isolates, including *A. baumannii*, were negative for gelatinase and protease, and only 11 (22%) were positive. This result is in agreement with Modarresi *et al.* [31], who found that most *A. baumannii* isolates were non-producers of gelatinase, DNase, and protease, and with Yavankar *et al.* [32], who reported that most *Acinetobacter* spp. strains were DNase- and protease-negative, with only one strain of *A. haemolyticus* being DNase- and protease-positive. According to the dendrogram obtained from the resistance patterns and virulence factor results (Figure 5), nine isolates were selected to study the effect of the plant extract: isolate 34b in subgroup A1; 10U, 48b, 46U, and 11b in subgroup A2; 27b and 13b in subgroup B1; 14C in subgroup B2; and the ninth isolate, 41b, which was considered an outgroup.

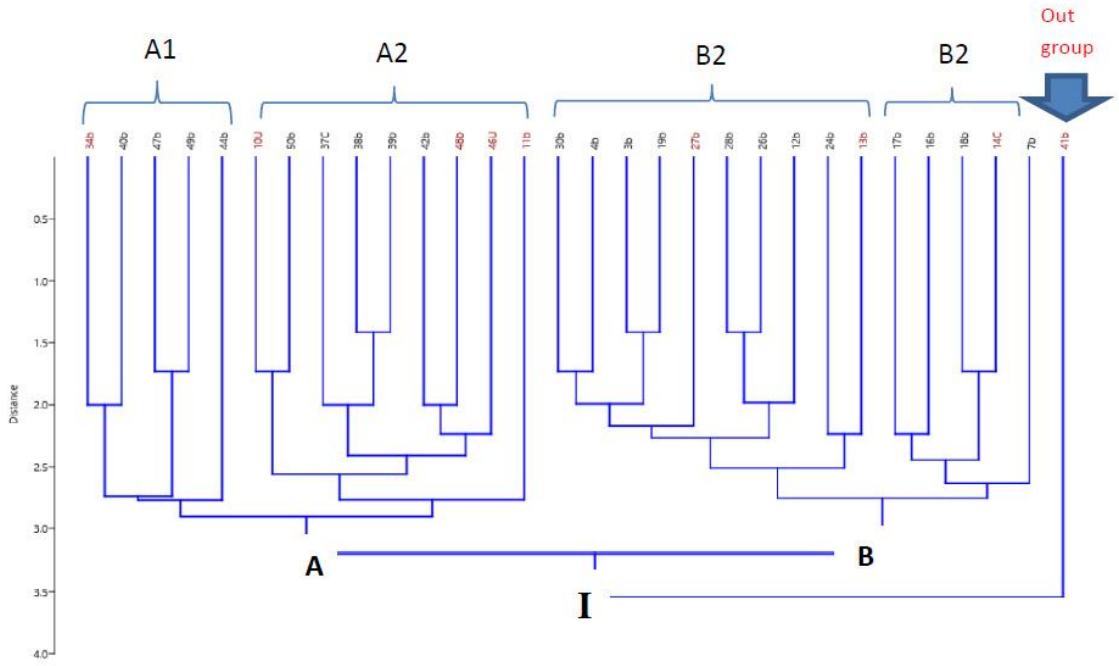



**Figure (5): Dendrogram obtained from the resistance patterns and virulence factor data.**

## Quorum Quenching by *Alpinia officinarum*

The most problematic aspect of *Acinetobacter* spp. infections is the ever-increasing antibiotic resistance and MDR of *A. baumannii*, which is putting increasing pressure on research and global health systems. As several extracts showed both antibacterial and anti-QS activities, the *A. officinarum* extract inhibited QS signalling and all other virulence-associated behaviours compared with the control grown in the absence of the extract. The extract can down-regulate the production of virulence factors and should be evaluated as a candidate for the alternative treatment of Gram-negative infections in the future. The co-administration of such efflux pump inhibitors together with an antibiotic reduces the amount of antibiotic needed for the same therapeutic effect, thereby cutting side effects. The effect of the *A. officinarum* extract on MDR *A. baumannii* was examined through the following five major tests.

## Antibiotic Susceptibility Test After Plant Extract Treatment

The *A. officinarum* extract fractions were preliminarily screened for antibacterial activity by the disc diffusion method. After activation of *A. baumannii* in the plant extract suspension with the three treatments (chloroform, petroleum ether, and ethyl acetate), the bacteria were spread on Mueller–Hinton agar and the antimicrobial discs were placed on the agar. The inhibition zones of all nine isolates changed markedly: most isolates became sensitive rather than resistant compared with their status before plant treatment. The chloroform, petroleum ether, and ethyl acetate treatments had a similar effect on the efflux pumps of the *A. baumannii* isolates, reducing their ability to release and pump antibiotics out of the bacterial cell. Therefore, this extract can be employed as a resistance-modifying agent that improves the interaction of antibiotics with target sites located inside the bacterial cell. However, some isolates remained resistant to some antibiotics (AMC, AM, CIP, and IMP). Figure (6) shows that before treatment all nine isolates were resistant to the antibiotic discs in varying percentages; isolates 11b, 48b, and 46U had the highest resistance percentages and 27b and 14C the lowest, while after treatment the percentage of resistance decreased in all isolates.

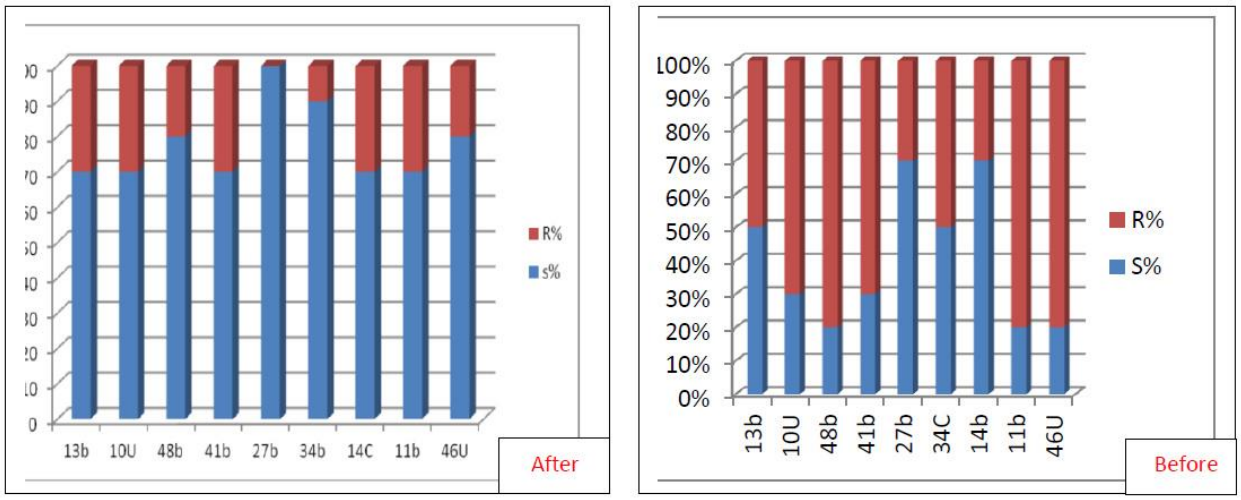



**Figure (6): Antibiotic patterns (R and S) of *A. baumannii* isolates before and after treatment.**

## Virulence Factors After Plant Extract Treatment

### Biofilm Formation

There was a marked difference in the ability of the *A. baumannii* isolates to form biofilm before and after plant treatment. Both the ethyl acetate and petroleum ether treatments (active carboxyl group, O–C=O) had a strong effect on the *A. baumannii* isolates, and all nine isolates lost the ability to form biofilm, whereas the isolates treated with chloroform (chloride group, –Cl) became weak biofilm producers, except isolate 10U, which was considered a moderate biofilm former. This may be because the QS signals were inhibited; biofilm cells treated with QS inhibitors are less tolerant than untreated biofilms, and planktonic cells dispersed from the biofilms become less tolerant to antibiotics. Another cause is that the secondary metabolites of the extract can act as anti-adhesive compounds that are effective in preventing biofilm formation and facilitate bacterial clearance.

### Motility

The ability of the *A. baumannii* isolates to swarm or swim decreased, particularly when treated with petroleum ether and ethyl acetate rather than chloroform, except for two isolates treated with chloroform, namely 41 (isolated from blood) and 46 (isolated from urine). Bhargava *et al.* [14] similarly reported inhibition of QS-mediated motility and biofilm formation of *A. baumannii* by plant-derived flavonoids. This can be explained by the effect of the plant extract on the modification of *A. baumannii* behaviours responsible for swarming and swimming, such as type IV pili and AHL molecules, which in turn led to the inhibition of biofilm formation (Figure 7).




**Figure (7): Swarming and swimming tests before and after plant extract treatment.**

**Pellicle Assay**

The white layer disappeared from the broth surface in all nine *A. baumannii* isolates; ethyl acetate, petroleum ether, and chloroform showed a similar effect, except for a single isolate, 10U from urine, which remained a pellicle producer. This is attributed to damage to the QS autoinducer molecules, which led to the inhibition of pellicle formation (Figure 8).

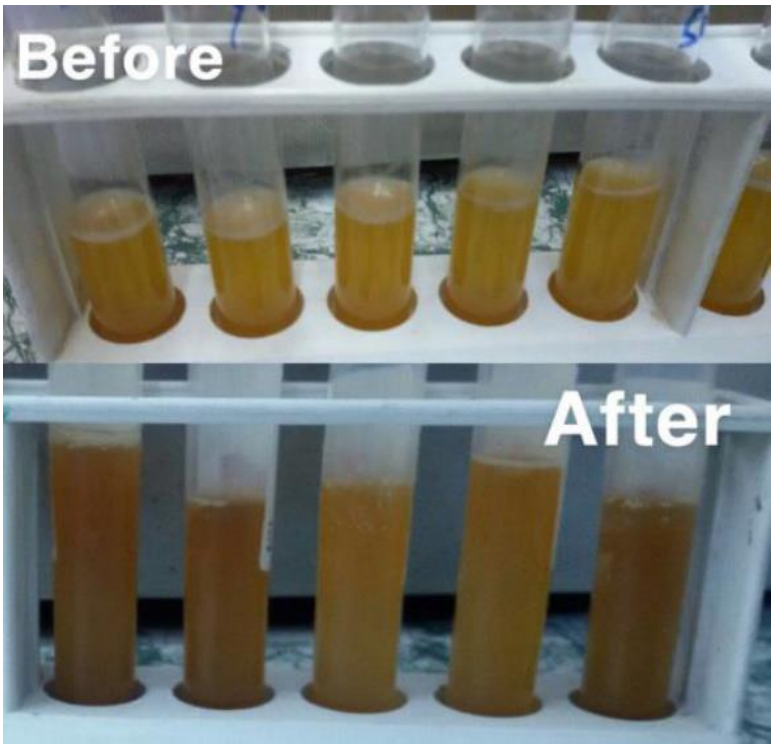



**Figure (8): Pellicle formation before and after plant treatment.**

**Colorimetric Detection of Quorum Sensing**

Figure (9) shows that the dark brown colour observed before treatment changed to yellow after the petroleum ether and ethyl acetate treatments in all nine isolates, because the bioactive group of each treatment (carboxyl, O–C=O) affected the concentration of AHL molecules, while the chloroform (chloride, –Cl) treatment also significantly affected the AHL concentration. *A. officinarum* inhibits AHL production by damaging the R-side chain of the molecule. The protease and gelatinase tests for all nine *A. baumannii* isolates remained negative before and after treatment with *A. officinarum*.

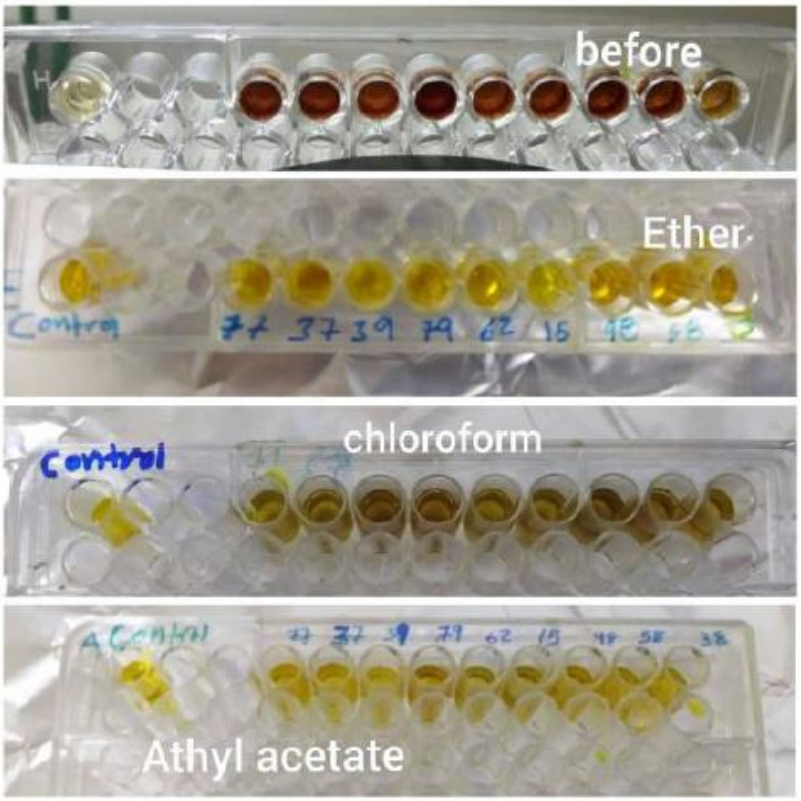



**Figure (9): Colorimetric detection of QS before and after plant treatment.**

The present study demonstrated that *A. officinarum* root extract at a concentration of 3.75 mg/mL can be used as an antibacterial agent against *A. baumannii* in vitro. These plant extracts are promising antimicrobial agents for the further treatment of human pathogens, including drug-resistant bacteria. *A. officinarum* causes both outer and inner membrane damage and coagulation of the cytoplasm.

## 4. CONCLUSION

Most first-line antibiotics have lost their therapeutic ability, and it is time to replace them with alternative agents. MDR *A. baumannii* isolates with metallo-β-lactamase activity represent a therapeutic problem in the ICU. The *A. officinarum* extract proved to have an excellent ability to inhibit and interfere with QS gene signalling, thereby reducing the virulence of the bacteria and their resistance to antibiotics, although these bacteria remain MDR isolates.

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