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Inhibition of *Acinetobacter baumannii* Biofilm Formation by Methanolic Extract of *Nothoscordum bivalve*

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Abstract

Acinetobacter baumannii is one of the most prevalent pathogens in nosocomial infections and has been cause of concern in recent years because, it has presented multiresistance to antibiotics and besides can form biofilms on biotic and abiotic surfaces like tissues and medical devices. Therefore, the search for new alternatives of natural origin to inhibit biofilm formation is being conducted. In the present investigation, sub-lethal concentrations (5.61 mg/mL, 3.74 mg/mL and 1.87 mg/mL) of methanolic extract of *Nothoscordum bivalve* were evaluated, showing biofilm formation inhibition up to 40.8%, in one nosocomial isolated of *A. baumannii* by the microtiter biofilm formation assay using crystal violet. On the other hand, the concentrations of 5.61 mg/mL and 3.74 mg/mL, caused an overexpression (up to 15.4 times) in the genes involved in the formation of biofilm (*abaI*, *bap* and *csuE*); due to this, the interaction of the extract with the bacteria was analyzed by scanning electron microscopy (SEM) and cellular damage was observed in the structure and stability of biofilm.

Keywords

Biofilm, Sub-Lethal, *Nothoscordum bivalve*, *Acinetobacter baumannii*

1. Introduction

Acinetobacter baumannii is usually found in hospitable environments, affecting immunocompromised patients in intensive care units [1]. Is a non-fermenter, Gram negative, aerobic strict, nonhemolytic, oxidase negative and positive catalase coccobacillus [2], which has resistance to multiple antibiotics, like all beta-lactams (including carbapenems), this due to its presence in hospital environments and permanent contact with antibiotics that exert selective pressure on the microorganism [3]. Moreover, the clinical isolates of *A. baumannii* present a defense mechanism, which is the ability to produce biofilms. These microbial consortiums can resist hostile environments and increase their drug resistance, adhering to biotic or abiotic surfaces [4]. In recent years, it has been shown a correlation between the drug resistance and adhesion capacity on surfaces that are clinically relevant; among them stands out polystyrene (polymer that is used in the manufacture of medical devices) and epithelial cells [5] [6]. Several genes involved in biofilm formation have been studied; including the gene *csuE* that belongs to *csu* operon, which forms a pilus-type structure and is considered an important factor in the biofilm formation [7] [8], since the repression of this gene significantly affects the initial adhesion process [9]. The *bap* gene (biofilm-associated protein) encodes a surface protein that has the function of adhering to host cells and abiotic surfaces, as well as for the development of the biofilm [7] [10]. Finally, the gene *abaI* encodes an acyl-homoserin lactone synthetase that generates an autoinducer of the perception of quorum (*quorum sensing*), important for the formation of the biofilm and its maturation [11] [12] [13].

Several studies have been conducted in recent years using natural products and derivatives to combat microbial biofilms, this has been carried out in order to find alternatives that do not exert selective pressure as antibiotics, and thus achieve an effective inhibition of biofilms through damage adhesion, development, motility and *quorum sensing* [14].

On the other hand, around 30 species belonging to genus *Nothoscordum* (Spermatophyta: Liliales), can be found throughout the world [15]. *N. bivalve* (false garlic) can be found distributed mainly across North America (United States and Mexico) [16], although its presence has also been reported in South American countries (Uruguay, Chile and Argentina) [17] [18]. Its leaves and inflorescences originate in a bulb in a linear manner, grows about 20 inches, presents 6 to 12 white flowers in the shape of an umbel and its seeds are black [18]. Within the genus *Nothoscordum*, for the species *N. gracile* in 2016, phytochemical studies of aqueous and ethanolic extracts were carried out, as well as an analysis of phytoconstituents from leaves and bulbs by FT-IR as well as by X-ray fluorescence of dispersive wavelength [15]. On the other hand, antimicrobial activity has been reported in aqueous extracts by maceration of the *N. gracilipes* and *N. entrerianum* bulbs by inhibiting the growth of *Staphylococcus aureus*, *Aspergillus fumigatus* and *Candida albicans* [19]; in contrast to the methanolic

extract of the bulbs of *N. bivalve* which did not present activity against *Klebsiella pneumoniae* , *Enterococcus faecalis* , *Escherichia coli* , *Stenotrophomonas maltophilia and Staphylococcus aureus* [20], whereas previous laboratory studies have shown that the aerial part of the aforementioned species could contain compounds with biological activity.

The objective of the present investigation was to corroborate that the species *Nothoscordum bivalve* is a source of metabolites with anti-biofilm activity against *A. baumannii,* causing damage either to the adhesion, development or *quorum sensing.*

2. Materials and Methods

2.1. Bacterial strain

The bacterial strain of *A. baumannii* was isolated and identified by Dr. Elvira Garza González at the Hospital Universitario Dr. José Eleuterio González, Universidad Autónoma de Nuevo León, Monterrey, NL, Mexico. The strain of this study was preserved in deep freezing at −80˚C at the Laboratorio de Patología Molecular Experimental of the Universidad Autónoma de Aguascalientes, Aguascalientes, Ags., Mexico. Likewise, the samples were kept in Mueller-Hinton agar (MH) (BD Bioxon) at 4°C for conducting the tests.

2.2. Obtaining the Extract of *N. bivalve*

A sample of 100 g of the aerial part (stem and inflorescence) of *Nothoscordum bivalve* was air dried (40˚C) and crushed with the aid of a grain mill (Victoria). It was then placed in a soxhlet apparatus to obtain three different extracts, extractions were made separately and consecutively on the same plant material [21]: first an extraction with hexane (CTR Scientific) was carried out, followed by chloroform (CTR Scientific) and finally with methanol (CTR Scientific) using 1 L per extract. After each extraction, the extracts were filtered and then concentrated under reduced pressure in a rot evaporator (Yamato BM 100) to finally be placed in amber vials until use. The percentage of extraction was calculated from the initial grammage of the plant (100%) and the grammage obtained in the extract.

2.3. Phytochemical Screening of the Extract

A series of color-based tests were carried out to determine the main groups of secondary metabolites presented in the extract, briefly 5 mg of the extract were suspended in 1 mL of methanol, except for the test of saponins that distilled water was used instead of methanol. The following reagents were used for each of the tests [20]; Dragendorff for alkaloids, Shinoda for flavonoids, NaOH for coumarins, to saponins a vigorous agitation was required to see the formation of foam, concentrated H_2SO_4 for quinones, 5% ferric chloride for tannins, Liebermann-Burchard for triterpenes and sterols, Baljet for sesquiterpene-lactones y Antrone for carbohydrates.

2.4. Effect of Sub-Lethal Concentrations on the Growth of *A. baumannii*

For such purpose, sub-lethal concentrations (25%, 50% and 75% of minimum bactericidal concentrations) were used. MBC (7.5 ± 0.54 mg/mL) was obtained in previous laboratory studies which showed that the methanolic extract had the highest antimicrobial activity. The assay was carried out in 96-well microplates (Corning™), in which different concentrations of the methanolic extract corresponding to 2.5 mg/mL (25%), 3.75 mg/mL (50%) and 5.6 mg/mL (75%), were added to the previously activated strain (1×10^9 CFU/mL) in a final volume of 200 μL. The plates were incubated for 24 and 48 h at 37°C (Felisa® incubator). For the microbial count, a microdilution method was carried out [22]; for this, after incubation time, an aliquot of 20 μL from each well was taken subjected to serial decimal dilutions using sterile physiological saline solution (NaCl 0.85% w/v). Then, last 3 dilutions were seeded on agar MH plates [23], after that the plates were incubated under the conditions previously described. After the incubation time, the colonies were counted and the result was expressed as colony forming units per milliliter (CFU/mL). As growth controls, the bacteria incubated without extracts and the bacteria incubated with the volume of methanol used for the extract concentrations were used.

2.5. Anti-Biofilm Effect of the Extract against *A. baumannii*

Inhibition of biofilm formation of *A. baumannii* was performed by the methodology proposed by Hassan *et al.* (2015) with little modifications [24]; briefly, 190 μL of previously activated (1×10^9 CFU/mL approximately) *A. baumanni* strain, was placed in a 96-well microplate, plus 10 μL of sub-lethal concentrations of the extracts. As a negative control, the bacteria without extract treatment were used. Following this, the microplates were incubated for 48 h at 37°C ± 0.5°C. After that, the planktonic cells were removed, placed in a new microplate and read at 600 nm. Then, for the staining of biofilms, 220 μL of 0.1% (w/v) crystal violet (Golden Bell) were added for 30 min at room temperature. Then, the dye was removed in a single movement and the microplate was washed 3 times with distilled water, with the help of a multichannel micropipette. The residual humidity was in an oven (Riossa), for a period of 1 h at 55°C ± 1°C. Finally, 200 μl of ethanol (CTR Scientific) were added and plates were read using a microplate reader (Epoch) at a wavelength of 570 nm. The percent inhibition of the biofilm was determined using the following formula [25].

$$\% \text{ Inhibition of the biofilm formation} = \left(\frac{\left(\text{Abs of negative control} - \text{Abs control} \right) - \left(\text{Abs of treatment} - \text{Abs treatment control} \right)}{\left(\text{Abs of negative control} - \text{Abs control} \right)} \right) * 100$$

Abs of negative control: Absorbance of the broth with bacteria.

Abs control: Absorbance of only the culture medium.

Treatment Abs: Absorbance of extract and broth with bacteria.

Abs treatment control: Absorbance of the extract and broth without bacteria.

2.6. Identification of *RecA, abal, bap and csuE Genes by Conventional PCR*

From the sequenced genome of *A. baumannii* (CP015364) [26], the genes *aI, bap* and *csuE* were selected due to their importance in the formation of biofilms, and the constitutive gene *recA* (DNA recombination and repair) was also selected (LC014653, KT717635, AY241696 and EU334497 respectively) [7] [12] [27]. *Primers* were designed using Primer Quest Tool (IDT Integrated DNA Technologies, http://sg.idtdna.com/pages) with similar alignment temperatures and amplifications no higher than 150 bp (Table 1). Finally, the design was corroborated with the AmplifX tool. (Ver. 1.7.0).

To carry out the conventional PCR, DNA of the strain was extracted at a concentration using a Wizard® Genomic DNA Purification Kit (Promega), carrying out the process as suggested by the manufacturer. The *primers* were resuspended in nuclease free water at a concentration of 5 ng/μL and the DNA was adjusted to 50 ng/μL. Amplification conditions were set as follow: 1 cycle of 95˚C for 5 min, then 95˚C for 45 s, 58˚C for 45 s, 72˚C for 12 s for a total of 40 cycles and finally a cycle of 72˚C for 3 min. Agarose gel (2.5% p/v) developed at 90 V for 1.5 h, was used to appreciate the amplification of the virulence genes as well as the constitutive gene.

2.7. Quantification of Gene Expression by qPCR

To evaluate the expression of genes involved in biofilm formation of *A. baumannii* (*abaI, bap* and *csuE*), extract concentrations of 50% and 75% of MBC were evaluated, methanol and *A. baumannii* (without treatment) were used as controls. Total RNA was extracting from each treatment using the following improved procedure divided into two phases: 1) all treatments (negative control, MBC 50%, MBC 75%, solvent 50% of MBC cells and solvent of 75% of the MBC), were centrifuged for 5 min at 14,000 rpm, then were double-washed with PBS 1X pH 7.4. One hundred μL of lysozyme solution (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA and 1 mg of lysozyme), were added to each pellet, vortexed (Daigger Vortex-Genie® 2) 2 min, and after that, 0.5 μL of SDS 10% (w/v) was added and mixed for 2 min, all treatments were incubated for 5 min at room temperature. 2) Immediately after, total RNA extraction was performed using the SV Total RNA Isolation System Kit (Promega), following the manufacturer instructions.

Table 1. Sequences of *primers,* size of amplified product and alignment temperature for this study.

Gene	Forward Sequence 5' → 3'	Reverse Sequence 5' → 3'	Size of amplicon (pb)	Temperature alignment (˚C)
*abaI	CCCACCACACAACCCTATTTA	AGCCTGACTGCTAGAGGAA	144	60
*bap	GCCAGCGATGTATTGGTAGT	GGCTCAGCTGTTCCACTAAA	107	55
*csuE	TGGAAGTACGGTAATTGAGAGTG	TTCTTTGAGAGTCCTGGGTTTAG	102	55
**recA	CGTTCAGGCGCAATTGATTTA	CGCTTGTAGACCCATATGAGAG	105	55

virulence gene*, constitutive gene**.

Total RNA obtained was treated with DNAse using RQ1 RNase-Free DNase Kit (Promega). After that, the samples were analyzed with a Nanodrop (Nanodrop 2000, Spectrophotometer) to obtain of purity and concentration; finally samples were frozen at −80˚C for its subsequent use. For cDNA obtaining, 100 ng of total RNA from each sample was treated with the GoScript™ Reverse Transcription System Kit (Promega) following the procedure as indicated by the manufacturer. Finally, qPCR was performed. For this, *master mix* containing 5 μL SYBR Green/ ROX qPCR Master Mix (Thermo Scientific), 1 μL of the cDNAs (10ng), 1 μL of *primer reverse* (10 ng), 1 μL of *primer forward* (10 ng) and 2 μL of nuclease-free water to produce a final volume of 10 μL per reaction was prepared. The amplification characteristics were: maintenance stage (1 cycle) 2 min. at 50 ˚C and 5 min. 95 ˚C, cycle stage (40 cycles) 30 s at 95˚C and 40 s to 59˚C; for the Melt Curve: 15 s at 95˚C, 1 min at 60˚C and 15 s to 95˚C (+0.05˚C). All this was carried out in a Step One Real-Time PCR System (Applied Biosystems) [28] [29] [30].

2.8. Scanning Electron Microscopy (SEM)

The interaction of *A. baumannii* with the selected extract was carried out, in 24-well flat bottom plates with the sub-lethal concentrations of its MBC as described above. After 48 h of incubation, the planktonic cells were removed using a micropipette, after that the wells were washed with sterile MH culture medium 2 times. The sample was then brought to dryness [31]. Then a process, as described by Shaefiei *et al.* (2016) was carried out with some modifications [32]; glutaraldehyde was added (JT Baker) at 1.5% to each of the samples. After 4 hours, it was removed and the wells were washed with PBS 1 × pH 7.4 in 3 occasions. Then ethanol (Jalmek) was added gradually to the wells, being at 70, 80, 90 and 96% (v/v) for a period of 30 min in each of these alcohols. Finally, absolute ethanol was added for 30 min on 2 occasions. Following this, the samples were taken to the chamber of the critical point apparatus at a pressure of 900 psi where the ethanol was exchanged for liquid CO_2. Subsequently, heat was applied to the chamber of the apparatus. The critical point was reached at a temperature of 31.1˚C and a pressure of 1072 psi for 4 min. Finally, the gas was released gradually from the chamber for 12 min. The sample was obtained completely dry. For the assembly of the samples, with the help of a stereoscope, the areas to be removed were visualized with a sterile scalpel to place them on graphite tape that was adhered to a 1 cm × 1 cm aluminum cylinder. Once the sample was mounted in the cylinder, it was placed in the sample holder of the gold washer (Denton Vacuum). The introduction of current flow at an estimated time of 130 s causes gold particles to detach from the disk and deposit in the sample, providing a thickness of 100 Å. The samples were introduced into the Scanning Electron Microscope chamber (JEOL JSM-5900LV) to be observed under optimal conditions of 15 kV (voltage) of acceleration. The working distance was 10 mm at different magnifications and scales.

3. Results

3.1. Yield of the Extract of *N. bivalve*

Regarding the extractions carried out on the plant material, they showed a yield of 2.5%, 1.8% and 22.1% for the extractions of hexane, chloroform and methanol, respectively. Only the methanolic extract was used for the trials, because it showed the best performance and due to its biological activity recorded in previous laboratory studies.

3.2. Phytochemical Screening of the Extract

The phytochemical analysis of the methanolic extract of *N. bivalve* showed the presence of sterols, triterpenes, coumarins, alkaloids, tannins, flavonoids and carbohydrates.

3.3. Effect of Sub-Lethal Concentrations on the Growth of *A. baumannii*

When performing the microdilution method to quantify the viability of the strain, it was determined that in the negative control and blanks (methanol) the bacterial counts were approximately of 2.4×10^9 CFU/mL, after 24 h of incubation. Furthermore, after an incubation period of 48 h the counts were 2.4×10^9 CFU/mL. However, in the case of treatments with extracts, an average of 2.6×10^8 CFU/mL was obtained. It is important to mention that the effect of treatments or blanks on bacterial growth after 24 or 48 h, did not exhibiting a significant change in viability. It is shown that the concentration of bacteria present in the treatments within 48 h of interaction serve to corroborate that there is uniform growth during the biofilm test.

3.4. Anti-Biofilm Effect of the Extract against *A. baumannii*

Figure 1 shows the effect of sub-lethal concentrations of MBC of the extract, by inhibiting the formation of biofilms through the crystal violet microplate assay. The concentrations of 25%, 50% and 75% of the MBC (1.87, 3.74 and 5.61 mg/mL, respectively), caused biofilms inhibitions of 19.5%, 40.8% and 36.0%, respectively.
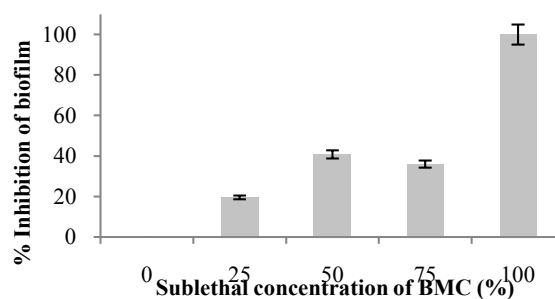

Figure 1. Effect of the concentrations of sub-lethal methanolic extract of *N. bivalve* on biofilm formation of *Acinetobacter.*

3.5 Identification of *recA, abai, bap and CsuE* Genes by Conventional PCR in the Strain of *A. baumannii*

The presence of the genes of interest in the DNA extracted from the *A. baumannii* strain of the present research was shown; confirming the similarity to the sequence CP015364 belonging to another isolate of *A. baumannii* at the Hospital Universitario Dr. José Eleuterio González. The amplification of bacterial genes *recA, bap and csuE* is shown on **Figure 2**. The amplification of genes *recA and abal* is shown on **Figure 3**. A molecular-weight size marker from 100 pb to 3 kpb was used.

The amplifications showed that the genes of interest are part of the genome of the studied strain. Likewise, with the amplifications obtained, the optimal conditions to perform the qPCR were standardized.

3.6. Quantification of Genes Expression through qPCR

When observing through the crystal violet microplate assay that the concentrations of 50% and 75% of the MBC (3.74 and 5.61 mg/mL, respectively) affected in a similar way the formation of biofilms, these interactions were considered to determine if a genetic alteration produced by the treatments, cause the decrease
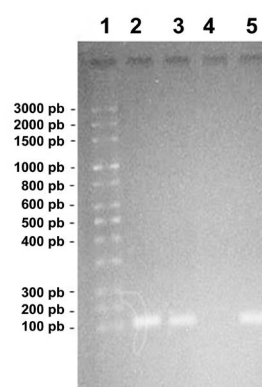

Figure 2. Amplification of genes: 1) molecular-weight size marker, 2) gene *bap* (107 pb), 3) gene *csuE* (102 pb), 4) negative control, 5) gene *recA* (105 pb).
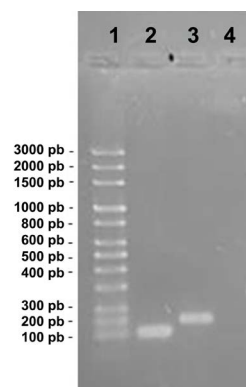

Figure 3. Amplification of genes: 1) molecular-weight size marker, 2) *recA* (105 pb), 3) gene *abal* (144 pb) and 4) negative control.

in biofilm formation. The cDNAs obtained showed the presence of constitutive gene by conventional PCR, also the concentration obtained from each of the cDNAs was >100 ng/mL and the values of its purity (ratio 260/230) contained between 1.7 and 1.9. **Figure 4** summarizes the expression of the *abaI bap* and *csuE* genes in the treatments of 50 and 75% of the MBC and blank (methanol), being observed an over expression in the target genes of cells treated with the extract in relation to the bacteria which only had treated with methanol. For *abaI,* overexpression was recorded in treatments at 50 and 75% of the extract, showing between 3.62 and 15.4 more expression compared to the case of *bap* and *csuE* genes; expression recorded in treatments at 50 and 75% of the extract was between 1.49 and 1.87 times the expression relative to control and blank (methanol).

3.7. Scanning Electron Microscopy (SEM)

Figure 5 shows the images obtained by SEM at 11,000X magnification. As it can be seen, there is damage in the structure and stability of the biofilm formed by *A. baumannii,* produced by treatments with 50 and 75% of the MBC *N. bivalve* methanolic extract with respect to the negative control and methanol evaluations of the extract (dissolvent).

4. Discussion

Various strains of *A. baumannii* have been isolated and identified in hospital
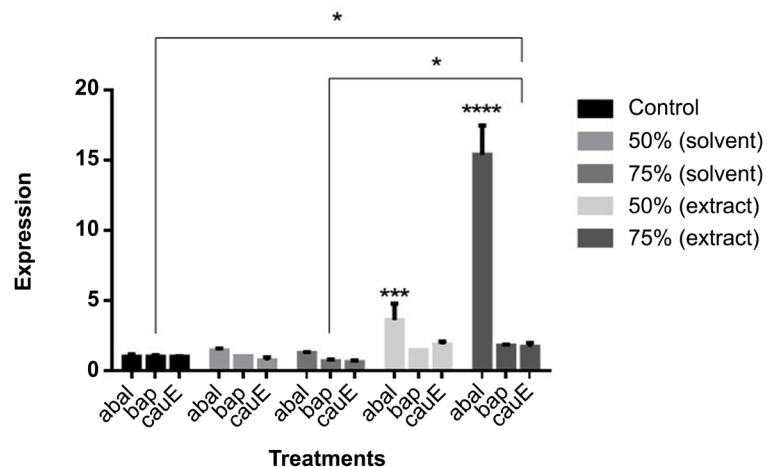

Figure 4. Expression of the *abaI*, *bap* and *csuE* genes with respect to the constitutive gene *recA*. Control: only bacteria in culture medium. The treatments with solvent (methanol) at 50% and 75% correspond to the vehicle used to place the extract, being 5% of the volume of interaction. The treatment of 50% of the MBC extract methanol of *N. bivalve* corresponds to 3.74 mg/mL, while that of 75% corresponds to 5.61 mg/mL. Each bar represents the mean ± SD standard with significant differences of $^*p < 0.05$ with t student between the control group and 75% of the extract for *bap* and *csuE*, $^{***}p < 0.001$ with comparison of means by Tukey between the control group and solvent with 50% of the extract for *abaI*, $^{****}p < 0.0001$ in comparison of means by Tukey between the control and solvent groups with 75% of the extract for *abaI*.
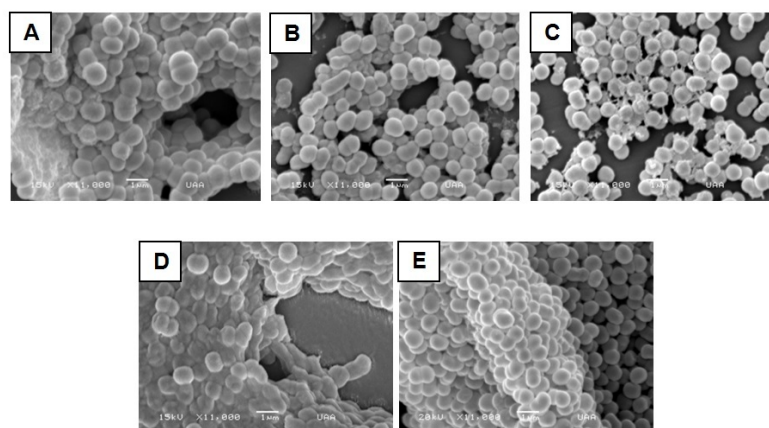
Figure 5. Analysis through scanning electron microscopy of the effect of sub-lethal concentrations of MBC extract *N. bivalve* on the microstructure of the biofilm *A. baumannii;* A) Control, only the bacteria in culture medium, B) 50% extract of the MBC (3.74 mg/mL), C) 75% extract of the MBC (5.61 mg/mL), D) and E) Solvent (methanol: vehicle used to place the extract) at 50 and 75% of the MBC, respectively.

environments worldwide in recent years, causing concern about its high resistance to antibiotics and hostile environments [33] [34]; its prevalence in Mexico has been reported in the last decade showing it as one of the main causal agents of nosocomial infections [3]. Due to the aforementioned circumstances, alternatives of natural origin are sought for their control and/or eradication. Such is the case of *Nothoscordum bivalve*, a species distributed in Mexico and which has great potential as a source of bioactive metabolites. The extraction of said metabolites in *N. bivalve* was carried out using three different solvents, being this hexane, chloroform, and methanol, in that order, through soxhlet reflux. The methanol extract presented the highest activity in previous assays; this may be due to the fact that methanol extracts the largest number of secondary metabolites [21] or due to the type of metabolites contained according to its gender. Regarding this, the group of compounds identified for *N. bivalve* in the qualitative phytochemical analysis showed the presence of sterols, triterpenes, coumarins, alkaloids, tannins, carbohydrates and flavonoids. Similar compounds have been reported in the ethanolic extract of *Nothoscordum gracile* [15] in which the presence of saponins, tannins, alkaloids, flavonoids, glycosides, and terpenoids were reported. It should be noted that, among the secondary metabolites detected in the aerial parts of both species, the flavonoids, tannins, and alkaloids stood out due to their phytochemical importance, since it has already been reported that these secondary metabolites show antibacterial activity against *A. baumannii* [35] [36] [37]. Antimicrobial activity of flavonoids can be related to the effect of membrane rigidification, by reducing membrane fluidity [38], moreover; Tang *et al.* (2012) mentions that the antibacterial effect of extracts containing flavonoids is mediated through membrane damage induced due to reactive oxygen species generation, followed by the leakage of protein molecules [39]. Meanwhile, tannins cause cell wall inhibition by forming irreversible com-

plexes with proteins [40]. On the other hand, alkaloids can intercalate into DNA, and disrupt the membrane structure by increasing the membrane permeability [41]. Finally, it is important to mention that, the biofilm formation inhibition can be explained by the presence of flavonoids which can suppress the autoinducer 2 responsible for cell to cell communication reducing biofilm synthesis [42].

The minimum bactericidal concentration (MBC) of the methanol extract of *N. bivalve* (7.50 ± 0.54 mg/mL), determined in previous studies, can be considered as an acceptable concentration to inhibit the aforementioned microorganism, since there are reports of extracts of various plants reporting MBCs from 0.32 to > 50 mg/mL against various strains of *A. baumanni* isolated from hospital environments [43] [44] [45] [46]. Due to this, their sub-lethal concentrations of 25%, 50% and 75% of their MBC (1.87, 3.74 and 5.61 mg/mL) were considered for the inhibition test in the formation of microplate biofilms using crystal violet. Before carrying out the test, it was determined if these concentrations affected the bacterial growth [47], since a decrease in viability could cause an alteration with respect to the controls used. When corroborating that the control (0% extract) and amount of dissolvent did not significantly affect the viability at 24 and 48 h of incubation, it was observed that after 48 h there was a uniform reduction in all treatments. It was proceeded then to the antibiofilm evaluation. It was found that the *N. bivalve* extract—at the assessed concentrations-showed a reduction in the amount of biofilm; both concentrations of 50 and 75% of the extract showed a similar activity rate at between 40.8 and 36.0% of inhibition (**Figure 1**). Biofilm formation is a virulence mechanism used by microorganisms to counteract antibiotics; there are reports of plant extracts which have inhibited the formation of this defense layer in *A. baumannii*. In the case of the hydro-alcoholic extracts of fungi belonging to the *Russula, Fistulina, Mycena, Leucopaxilus* and *Lepista* type, a maximum of 28.6% inhibition was found in the formation of biofilms with sub-lethal concentrations of the MBCs (<20 mg/mL) [48]. In the same way, ethanolic extracts of ginger at a concentration rate between 0.62 and 80 mg/mL had an effect significantly inhibiting biofilm formation in *A. baumannii* [49].

The amplification of the *recA* gene was used for the genetic identification of the strain under study [27] (**Figure 2** and **Figure 3**), because within the genome of *A. baumannii* this gene encodes a very important protein involved in the repair of DNA damage caused by chemical agents such as ethidium bromide, mitomycin C and UV radiation. Likewise, the amplification of the genes *abaI, bap, csuE* were indicative of the resistance and capacity of the microorganism for the formation of biofilm (**Figure 2** and **Figure 3**), since the strains that harbor these traits have high resistance to antibiotics and the ability to adhere to surfaces and biofilm formation [7] [8] [13].

After the quantification of the expression of genes *abaI, bap, csuE* regarding the constitutive gene (*recA*) by qPCR; it was observed that the control and the targets (dissolvent of 50% and 75%), did not present alterations in their level of

expression (Figure 4), demonstrating that the vehicle of the extract did not cause gene alterations in *A. baumannii* at the volumes used (5% of the total volume of interaction). Likewise, it was observed in the treatments of 50% and 75% of the MBC extract that the expression of the genes *bap* and *csuE* showed a light overexpression (between 1.49 and 1.87 times more) compared to the negative control. In the case of the *bap* gene, it has been noted that overexpression may be given due to a change in the amount of iron available in the medium [7] and for the case of *csuE*, damage is suggested by bioactive compounds of the extract directly to the membrane proteins, which could also affect the expression of the *bap* gene, since both genes encode the expression of membrane proteins [7] [8]. Therefore, more assays are suggested to confirm such theory. On the other hand, when evaluating the expression of the *abaI* gene, which encodes the enzyme that generates self-inducing acil homoserine lactone (AHL) in *A. baumanni*, molecule responsible for *quorum sensing* in biofilm formation, this showed a overexpression of 3.62 and 15.4 times more in the treatments of 50% and 75% of the MBC of the extract, respectively. He *et al.* (2016) found that the antibiotics levofloxacin and meropenem in sub-lethal concentrations from 0.03 to 0.25 µg/mL induce the overexpression of the *abaI* gene (between 1.95 and 4.60 times more) in a strain of *A. baumannii* (ABS17) isolated from a hospital in China [13]. Likewise, he described that antibiotics caused an overexpression of efflux pumps AdeFGH, causing an increase in the amount of AHL in the outer cell environment, such that the inlet of said molecule was accelerated into the cell to form AbaR-AHL complexes, causing the mentioned complex to induce synthesis for AHL and be sent to the extracellular environment so it could be detected by other cells of *A. baumannii* in the proximity, and which would result in an increase of biofilm formation. This might suggest that the compounds contained in the extract are somehow affecting the aforementioned efflux pumps in the same fashion antibiotics do. However, the high expression could indicate a direct damage to the AHL in the extracellular environment, since said molecule has been widely studied and has been confirmed as a fundamental part of the development and maturation of the biofilm in *A. baumannii* [11] [12] [13].

Finally, when observing the interaction of *A. baumannii* with the methanolic extract from *N. bivalve* under scanning electron microscopy, it was seen that 75% of its MBC (5.61 mg/mL) caused damage to the stability of its biofilm and changes in bacterial morphology (Figure 5). Tenseg *et al.* (2016) evaluated the concentration of 20 µM of the compound 5-episinuleptolide isolated from the coral *Sinularia leptoclados*, finding that it significantly affects the stability of the biofilm in a strain of *Acinetobacter baumannii* (ATCC 19606), when analyzed by scanning electron microscopy [50]; a damage very similar to that observed in this research could be noticed. On the other hand, the ethyl acetate fraction of *Pericarpium trichosanthis* extract at a concentration of 1/4 of its MBC (1.5 mg/mL) caused damage to the morphology of a strain of *A. baumannii,* suggesting that the components of this extract generate destructive damage since these can cause structural loss on the cell membrane or cause damage to membrane

proteins, which may later trigger the destruction of the cell wall and, therefore, cell death [51].

5. Conclusion

It can be concluded that the methanol extract of *N. bivalve* is a source of secondary metabolites with antibiotic activity against *A. baumannii*. The active compounds of the extract, in sub-lethal concentrations, caused damage to the stability and sustentation of its biofilm as well as to the morphology of the aforementioned bacteria. On the other hand, said compounds induced a slight overexpression in the *bap* and *csuE* genes, while causing a marked over expression of the auto inductor *abaI* (*quorum sensing*), which might suggest that the bioactive metabolites contained in the extract cause damage to the cell membrane and/or membrane proteins, affect the efflux pumps or could cause direct damage to the auto inductor (AHL) in the extracellular medium.

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Conflicts of Interest

The authors of this work do not present a conflict of interest with the publication and dissemination of the results contained in this document.

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