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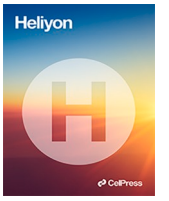

Research article

Investigating the anti-growth, anti-resistance, and anti-virulence activities of *Schoepfia schreberi* J.F.Gmel. against the superbug *Acinetobacter baumannii*

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ABSTRACT

Schoepfia schreberi has been used in Mayan folk medicine to treat diarrhea and cough. This study aimed to determine the anti-growth, anti-resistance, and/or anti-virulence activities of *S. schreberi* extracts against *Acinetobacter baumannii*, a pathogen leader that causes healthcare-associated infections with high rates of drug-resistant including carbapenems, the last line of antibiotics known as superbugs, and analyze their composition using HPLC-DAD. Ethyl acetate (SSB-3) and methanol (SSB-4) bark extracts exhibit antimicrobial and biocidal effects against drug-susceptible and drug-resistant *A. baumannii*. Chemical analysis revealed that SSB-3 and SSB-4 contained of gallic and ellagic acids derivatives. The anti-resistance activity of the extracts revealed that SSB-3 or SSB-4, combined with imipenem, exhibited potent antibiotic reversal activity against *A. baumannii* by acting as pump efflux modulators. The extracts also displayed activity against surface motility of *A. baumannii* and its capacity to survive reactive oxygen species. This study suggests that *S. schreberi* can be considered a source of antibiotics, even adjuvanted compounds, as anti-resistant or anti-virulence agents against *A. baumannii*, contributing to ethno-pharmacological knowledge and reappraisal of Mayan medicinal flora, and supporting the traditional use of the bark of the medicinal plant *S. schreberi* for the treatment of infectious diseases.

1. Introduction

Antimicrobial resistance (AMR) is a significant global public health threat, particularly with respect to the increasing prevalence of antibiotic-resistant bacterial infections, which are associated with increased morbidity and mortality rates, longer hospital stays, and higher healthcare costs [1]. Approximately 700,000 individuals lose their lives each year because of these infections, and this number is projected to reach 10 million annually by 2050 if appropriate measures are not taken to address this issue [2].

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The World Health Organization (WHO) has published a catalog identifying 12 bacterial species that pose critical, high, and medium threats to public health, owing to their ability to cause disease and resist antimicrobial agents. The purpose of this publication was to encourage research and development of new antibiotics [3]. The discovery of antibiotics during the "golden age" of antibiotic development has saved millions of lives by targeting bacterial growth. In light of the escalating threat of AMR, new strategies are being explored to develop effective anti-infectious agents that can target not only bacterial growth. But also its resistance mechanisms and virulence factors [4,5].

Acinetobacter baumannii is a Gram-negative, non-flagellated coccobacillus that is commonly found in the environment and is known to cause community- and hospital-acquired infections [6]. This pathogen is considered one of the most challenging nosocomial ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* spp.), and is at the top of the list of bacterial pathogens of critical priority because of its AMR to last-option antibiotics such as carbapenems, colistin, and tigecycline [3]. *Acinetobacter baumannii* has developed several mechanisms of resistance, including inactivation of antibiotics by β-lactamases, loss of porins, modification of its target, reduction of intracellular antibiotic concentrations through decreased permeability, and overexpression of efflux pumps [7,8]. In addition, this pathogen possesses various virulence factors, such as enzymes, immune-modulating factors, biofilm-related factors, and metabolic or nutritional factors, which contribute to therapeutic failure and complicate the control of bacterial infections [9,10].

Medicinal plants offer potential therapeutic remedies with diverse pharmacological effects and chemical structures for the identification of novel anti-*A. baumannii* agents that inhibit their growth, drug-resistance mechanisms, and virulence factors [11–13].

Mayan Medicine is a globally recognized ancient practice that is highly valued for its cultural significance in Mexico [14]. Ethnobotanical research has extensively documented the use of Mayan medicinal flora for therapeutic purposes and treatment of various illnesses, showing a remarkable diversity of biological activities, including leishmanicidal [15], antiamoebic [16], antioxidant [17], antiviral [18], antidiabetic [19], and antibacterial [20] properties as well as a broad range of phytoconstituents with unique chemical structures [21].

Schoepfia schreberi J.F.Gmel. (Schoepfiaceae) is a shrub that has been recorded in several Mexican states, including those of the Yucatan Peninsula [22], and is commonly referred to as sak beek or sip che' in Mayan folk medicine [23]. This plant has traditionally been used to treat a range of symptoms, such as diarrhea and cough, by administering its leaves and bark as decoctions [24].

Our research team is dedicated to exploring the therapeutic potential of the Mayan flora, with a particular focus on pathogenic bacteria of critical or high priority. Our previous investigations demonstrated the antimicrobial activity of *S. schreberi* extracts against methicillin-resistant *S. aureus* (MRSA) [20]. To expand our knowledge in the field of pharmacognosy, we examined the anti-*A. baumannii* properties of *S. schreberi* using three different approaches to evaluate its impact on the growth, resistance mechanisms, and virulence factors of this bacterium.

2. Materials and methods

This project received approval from the Scientific and Ethics Committees National of the Instituto Mexicano del Seguro Social (IMSS), with approval number R-785-2023-014.

2.1. Medicinal plant extracts

2.1.1. Collection

The medicinal plant *Schoepfia schreberi* was collected in April 2019 from Yucatán, Mexico (21°36′32.7″N, 88°37′21.94″W). The plant material was authenticated by Dr. José Salvador Flores-Guido and Geovani Antonio Palma-Pech of the Botany Department, Faculty of Veterinary Medicine, University Autonomous of Yucatan (UADY), and the specimen was subsequently deposited in the herbarium Alfredo Barrera Marín-UADY (voucher number 23,229). The botanical nomenclature and taxonomy of medicinal herb was authenticated using "The Plant List" [25].

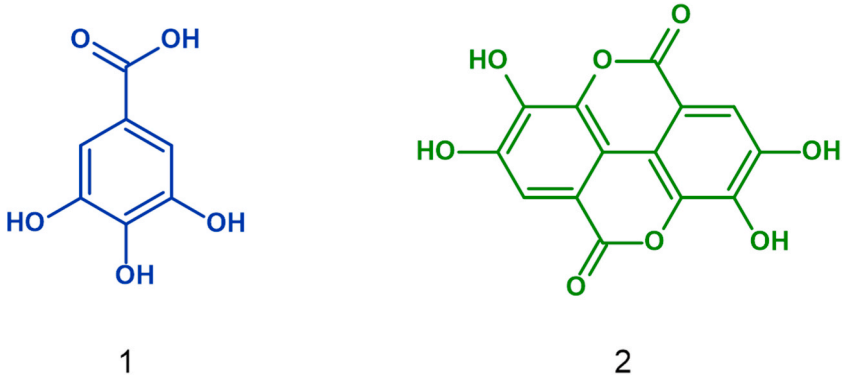

Fig. 1. Chemical structures of gallic (1) and ellagic acids (2).

2.1.2. Extracts preparation

The plant material was processed using a standardized protocol [26] that involved cutting freshly harvested leaves and bark into small pieces, oven-drying at 40 °C for 72 h and grinding using a hand mill. Aqueous extracts (AE) were obtained by decoction, following the traditional mode of preparation. The organic extracts (OE) were obtained standardized procedures previously described by our research group: the plant material was extracted sequentially using *n*-hexane (*n*-Hex), ethyl acetate (EtOAc), and methanol (MeOH) (Fermont, Monterrey, Nuevo León, Mexico) (3 ×) by soaking the plant material in an orbital shaker (Thermo Scientific, Waltham, MA, USA) at 0.106 *g* for 24 h at room temperature. The solvent was evaporated under *vacuum* by using a rotary evaporator (Büchi, Flawil, Switzerland). All crude extracts were stored at −20 °C until use. The AE was dissolved in distilled water and sterilized by filtration, while the OE was dissolved in 100 % dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA).

2.1.3. Phenolic acids

The phenolic acids gallic acid (GA) and ellagic acid (EA) were purchased commercially (Sigma-Aldrich; Fig. 1).

2.2. Bioassays

2.2.1. Acinetobacter baumannii strains

The *Acinetobacter baumannii* strains used in this study are listed in Table 1. Clinical isolates were obtained from the Microbiology Laboratory of the Unidad Médica de Alta Especialidad and were characterized in the Microbiology Laboratory Research of Unidad de Investigación Médica Yucatán, both under the Instituto Mexicano del Seguro Social in Mérida, Yucatán, Mexico. All strains were preserved at −80 °C in tryptic soy broth (TSB; Becton Dickinson, Franklin Lakes, NY, USA) supplemented with 20 % v/v glycerol (J.T. Baker, Inc, Phillipsburg, NJ, USA).

2.2.2. Anti-bacterial activity

The antibacterial activities of *S. schreberi* extracts and phenolic acids (GA and EA) against *A. baumannii* strains were evaluated using the resazurin microtiter assay (REMA) broth dilution method. First, the bacterial strains were cultured on Mueller-Hinton agar (MHA; Becton Dickinson), and then 2–3 bacterial colonies were suspended in 3 mL of Mueller Hinton broth (MHB; Becton Dickinson) and incubated at 37 °C for 2–4 h until growth was comparable to the turbidity of the 0.5 McFarland standard, diluted 1:50 and 100 μL of the suspension (10^6 CFU/mL) was incubated with 100 μL of MHB containing the eight extracts of *S. schreberi* and GA and EA at serial dilutions ranging from 1000–7.81 μg/mL. All assays included a positive control (bacterial cultures with a specific antibiotic for each strain of *A. baumannii*), a negative control (bacterial culture without any added extracts, phenolic acids, or antibiotics), and a sterility control (culture broth alone). The microplates were incubated at 37 °C for 16 h. Following incubation, 30 μL of resazurin (Sigma-Aldrich) was added to the microplates, which were then incubated at 37 °C for 2 h. The results are expressed as the minimum inhibitory concentration (MIC), which corresponded to the greatest dilution of the plant extract, where a color shift from blue to pink was not observed. The assays were independently performed three times in duplicate [20]. The minimal bactericidal concentration (MBC) of the extracts and both acids was determined through a series of experiments by reseeding 5 μL of extract-treated bacterial suspensions onto MHA. This involved treating the bacteria with the extracts at concentrations of 4, 2, 1 × , and ½ MIC. Drug-treated bacterial suspensions (positive control), untreated bacterial suspensions (growth control), and the culture medium alone (sterility control) were used. The plates were incubated for 16 h at 37 °C, after which the MBC was determined as the minimal extract concentration that prevent bacterial growth in the Petri plate. Each MBC determination was independently performed three times in duplicate.

2.2.3. Antibiotic-modulating activity

The anti-*A. baumannii* growth plant extracts of *S. schreberi* (SSB-3, SSB-4), GA and EA were tested against the UIMY-ABA-5 and

Table 1
Acinetobacter baumannii strains.

Identity	Source/Biological sample	Phenotype	Drug resistance profile
1605	ATCC	XDR, CBR	AMP, CAZ, CFZ, CIP, CRO, CTX, ETP, FEP, FOS, FOX, GEN, IPM, LVX, MEM, SAM, SXT, TZP
UIMY-ABA-88	Clinical isolate/Urine	Susceptible, CBS	–
UIMY-ABA-16	Clinical isolate/Urine	MDR, CBS	AMK, CAZ, CIP, CRO, CTX, FEP, GEN, LVX, SXT, TET, TOB
UIMY-ABA-81	Clinical isolate/Pleural liquid	MDR, CBR	AMK, CAZ, CIP, CRO, CTX, FEP, GEN, LVX, MEM, SXT, TOB
UIMY-ABA-7	Clinical isolate/Bronchial liquid	XDR, CBR	AMK, CAZ, CIP, CRO, CTX, FEP, GEN, LVX, MEM, SAM, SXT, TET, TOB
UIMY-ABA-5	Clinical isolate/Urine	XDR, CBR	AMK, CAZ, CIP, CRO, CTX, FEP, GEN, IMP, LVX, MEM, SAM, SXT, TET, TOB
UIMY-ABA-205	Clinical isolate/Blood	XDR, CBR	AMK, CAZ, CIP, FEP, GEN, IMP, LVX, MEM, SXT, TZP
UIMY-ABA-63	Clinical isolate/Bronchial	PDR, CBR	AMK, CAZ, CIP, COL, CRO, CTX, FEP, LVX, MEM, SAM, SXT, TET, TOB

CBSU: Carbapenem-susceptible; CBRE: Carbapenem-resistant; MDR: Multidrug-resistant; XDR: extensively drug-resistant; PDR: pandrug-resistant; AMK: Amikacin; AMP: Ampicillin; CAZ: Ceftazidime; CFZ: Cefazolin; CFX: Cefuroxime; CIP: Ciprofloxacin; COL: Colistin; CRO: Ceftriaxone; CTX: Cefotaxime; ETP: Ertapenem; FEP: Cefepime; FOS: Fosfomycin; FOX: Cefoxitin; GEN: Gentamycin; IPM: Imipenem; LVX: Levofloxacin; MEM: Meropenem; SAM: Ampicillin/Sulbactam; SXT: Trimethoprim/Sulfamethoxazole; TET: Tetracycline; TOB: Tobramycin; TZP: Piperacillin/Tazobactam.

UIMY-ABA-16 clinical isolates in the presence of IMP or AMK, respectively, according to the method described by Guefack et al. (2022), with modifications [27]. These clinical isolates were phenotypically characterized as possessing efflux pumps, as a mechanism of resistance to IMP and/or AMK. A 96-well microplate was used, with 50 μL of MHB in each well, followed by the addition of 50 μL of 8 × antibiotic solution and two-fold serial dilutions. Then, 50 μL of the extract at sub-inhibitory growth concentrations was added to the wells, followed by 100 μL of bacterial inoculum (10^6 CFU/mL). All assays included a positive control (cultures with the efflux pump inhibitor phenylalanine-arginine-β-naphthylamide), a negative control (culture-free wells of extracts), and sterility control (culture broth alone). The microplates were incubated, and MIC were determined using resazurin [28]. The antibiotic-modulating factor (AMF), calculated as MIC (antibiotic alone)/MIC (antibiotic + extract), was used to express the antibiotic-modulating effects of the extracts. Each assay was performed in duplicate and repeated thrice.

2.2.4. Anti-biofilm activity

The effects of SSB-3, SSB-4, GA, and EA on biofilm formation by the UIMY-ABA-81 strain were evaluated using the crystal violet (CV) staining method in flat-bottom 96-well microplates, as described by Uc-Cachón et al. (2021) with minor modifications [20]. The bacteria were first cultured on MHA, and then two bacterial colonies were grown in 3 mL of TSB (Beckton Dickinson) at 37 °C for 24 h. An aliquot of the bacteria was transferred to TSB supplemented with 1 % glucose (TSB + G) to match the turbidity of the 0.5 McFarland standard. This suspension was further diluted, and 100 μL of this suspension (10^6 CFU/mL) was incubated with 100 μL of TSB + G containing serial dilutions of SSB-3, SSB-4, GA, and EA (100–62.5 μg/mL). Ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich) solution and extract-free wells were used as the positive and negative controls, respectively. The microplates were incubated for 24 h at 37 °C, followed by gentle aspiration of the culture medium, washing with sterile distilled water to remove non-adherent cells, and drying at 60 °C for 45 min. The biofilm was stained by incubation with a 0.1 % CV solution for 30 min, followed by washing. Next, 40 % acetic acid (Fermont) was added, and absorbance was measured at 490 nm using a microplate reader (Bio-Rad, Hercules, CA, USA). The amount of biofilm formed was related to the absorbance value. The assay was repeated three times, and the concentration of extracts and phenolic acids that reduced biofilm formation by 50 % (IC_{50}) was calculated using GraphPad Prism (version 8.3.0, GraphPad software, CA, USA) [20].

2.2.5. Anti-surface motility assay

The effects of SSB-3, SSB-4, GA, and EA on the motility of *A. baumannii* (UIMY-ABA-205) were evaluated using the method described by Uškak et al. with some modifications [29]. Surface-associated motility was assessed by inoculating 3 μL (10^6 CFU/mL) of previously cultured bacteria, treated with sub-inhibitory growth concentrations of plant extracts and pure compounds, onto a Petri plate containing freshly prepared agar (1 % tryptone, 0.5 % NaCl, and 0.21 % agar). Azithromycin (Sigma-Aldrich) was used as a positive control and plant extract- or phenolic acid-free cultures were used as negative controls. The plates were then incubated for 20 h at 36.5 °C, and surface measurements were performed using ImageJ software v1.54d (National Institute of Health, MD, USA). The assay was repeated thrice.

2.2.6. H_2O_2 sensitivity assay

The present study employed the methodology outlined by Selvajad et al. with some modifications, to investigate the effects of SSB-3, SSB-4, GA, and EA on the susceptibility of *A. baumannii* to H_2O_2 [30]. Briefly, the UIMY-ABA-205 strain of *A. baumannii* was treated with SSB-3, SSB-4, GA, or EA at sub-inhibitory growth concentration for 24 h at 36.5 °C. Following treatment, 150 μL of the bacterial suspension (10^6 CFU/mL) was incubated with 150 μL of PBS (Sigma-Aldrich) containing 0.125 mM H_2O_2 for 1 h. The number of surviving colonies was determined using serial dilutions and the pour-plate method; the growth control was an extract-free culture. Each assay was performed in triplicate, and the percentage of mortality was calculated.

2.3. HPLC-DAD analysis

HPLC-DAD (high-performance liquid chromatography with diode array detection analysis) was conducted using a Waters HPLC instrument equipped with a Waters 996 (900) UV photodiode array detector and Supelcosil LC-F® packed column (25 cm × 4.6 mm, 5 μm) at a flow rate of 0.9 mL/min. The analysis involved a gradient system of 0.5 % trifluoroacetic acid:water (A:B) as the mobile phase, using the following solvent ratios: A:B, 100:0 (0–1 min), 95:5 (2–3 min), 70:30 (4–20 min), 50:50 (21–23 min), 20:80 (24–25 min), 0:100 (26–27 min), and 100:0 (28–30 min). The total execution time of the analysis was 30 min, and 10 μL of each plant extract or phenolic acid was injected. UV–Vis detection was performed from 190 to 600 nm.

2.4. Statistical analysis

Data were analyzed using GraphPad Prism software (version 8.3.0). One way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used to determinate the statistical significance of the results, with a significance level set at $P \leq 0.05$.

3. Results

3.1. Anti-bacterial activity

The antibacterial activities of the *S. schreberi* extracts (leaves: SSL1, SSL-2, SSL-3, SSL-4, SSB-1, and bark: SSB-1, SSB-2, SSB-3, and

SSB-4) were evaluated using one ATCC reference strain and seven drug-susceptible and drug-resistant clinical isolates of *A. baumannii*, including MDR, XDR, and PDR (multidrug-resistant, extensively drug-resistant, and pandrug-resistant) profiles and susceptibility or resistance to carbapenems (Table 1). The MBC/MIC (minimum inhibitory concentrations/minimum bactericidal concentration) ratio was calculated to determine the bactericidal (MBC/MIC ≤4) and bacteriostatic (MBC/MIC >4) effects of extracts and phenolic acids (Table 2).

The activity of *S. schreberi* extracts varied according to the plant part, solvent, and *A. baumannii* strain tested (Table 2). Results revealed that all extracts displayed inhibitory effects on at least one strain, with MIC values ranging from 1000 to 250 to μg/mL. SSB-3 and SSB-4 demonstrated activity against all tested strains, including susceptible and resistant strains, with MIC values ranging 500-250 μg/mL and 1000-250 μg/mL, respectively. In addition, clinical isolate UIMY-ABA-205 was susceptible to all *S. schreberi* extracts (MIC = 1000-500 μg/mL). The potential effect of SSB-3 against UIMY-ABA-63, a PDR and CBR clinical isolate (superbug), was remarkable (250 μg/mL). Our results showed that SSB-3 exhibited bactericidal activity against six of the eight *A. baumannii* strains tested, whereas SSB-4 displayed bactericidal activity only against UIMY-ABA-5.

3.2. HPLC-DAD analysis

SSB-3 and SSB-4, the most anti-bacterial extracts of *S. schreberi* against *A. baumannii*, were analyzed using HPLC-DAD to identify their chemical content. Several phytoconstituents have been identified as derivatives of EA and GA (Fig. 2a–d).

Considering that GA and EA derivatives have been identified as phytocomponents of SSB-3 and SSB-4, we assessed phenolic acids in various models to continue our investigation. Fig. 3 shows the flowchart of the methodology and results.

Notably, GA was not active against the tested strains, whereas EA exhibited strong anti-*A. baumannii* activity (MIC = 3.9 μg/mL) against UIMY-ABA-16 but was not active against the other strains (Table 1). Furthermore, EA exhibited biocidal activity against UIMY-ABA-16.

Table 2
Antibacterial activity of extracts of and phenolic acids of *Schoepfia schreberi* against *Acinetobacter baumannii* strains.

Most active — Less active

Vegetal part or phytoconstituent	ID	Solvent	MIC and MBC (μg/mL)															
			UIMY-ABA-															
			ATCC 1605 XDR CBR		88 DS CBS		81 MDR CBS		16 MDR CBR		63 PDR CBR		7 XDR CBR		205 XDR CBR		5 XDR CBR	
			MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Leave	SSL-1	Aqueous	>1000	–	>1000	–	>1000	–	>1000	–	>1000	–	>1000	–	1000	>1000	>1000	–
	SSL-2	Hex	>1000	–	>1000	–	1000	>1000	>1000	–	>1000	–	>1000	–	1000	>1000	>1000	–
	SSL-3	EtOAc	>1000	–	>1000	–	1000	>1000	>1000	–	>1000	–	>1000	–	1000	>1000	>1000	–
	SSL-4	MeOH	>1000	–	>1000	–	1000	>1000	>1000	–	>1000	–	>1000	–	1000	>1000	>1000	–
Bark	SSB-1	Aqueous	>1000	–	>1000	–	>1000	–	>1000	–	>1000	–	>1000	–	1000	>1000	>1000	–
	SSB-2	Hex	>1000	–	1000	>1000	1000	1000	>1000	–	>1000	–	>1000	–	1000	>1000	>1000	–
	SSB-3	EtOAc	500	500	250	>1000	250	250	500	500	250	>1000	500	1000	500	1000	500	1000
	SSB-4	MeOH	500	500	250	>1000	250	>1000	1000	>1000	1000	>1000	1000	>1000	500	>1000	1000	1000
Gallic acid	GA	-	>1000	–	>1000	–	>1000	–	>1000	–	>1000	–	>1000	–	>1000	–	>1000	–
Ellagic acid	EA	-	>1000	–	1000	>1000	>1000	–	3.9	3.9	>1000	–	>1000	–	>1000	–	>1000	–
Tetracycline	TET	-	–	–	0.5	–	1	–	–	–	–	–	–	–	–	–	–	–
Colistin	COL	-	1	–	–	–	–	–	2	–	4	–	2	–	2	–	1	–

ID: identity; Hex: *n*-hexane; EtOAc: ethyl acetate; MeOH: methanol; DS: drug-susceptible; MDR: multidrug-resistant; PDR: pandrug-resistant: XDR: extensively drug-resistant; CBS: Carbapenem-susceptible; CBR: Carbapenem-resistant; MIC: minimum inhibitory concentration; underline style the bactericidal extract or phytoconstituent (MBC/MIC≤4); –: Not determined.

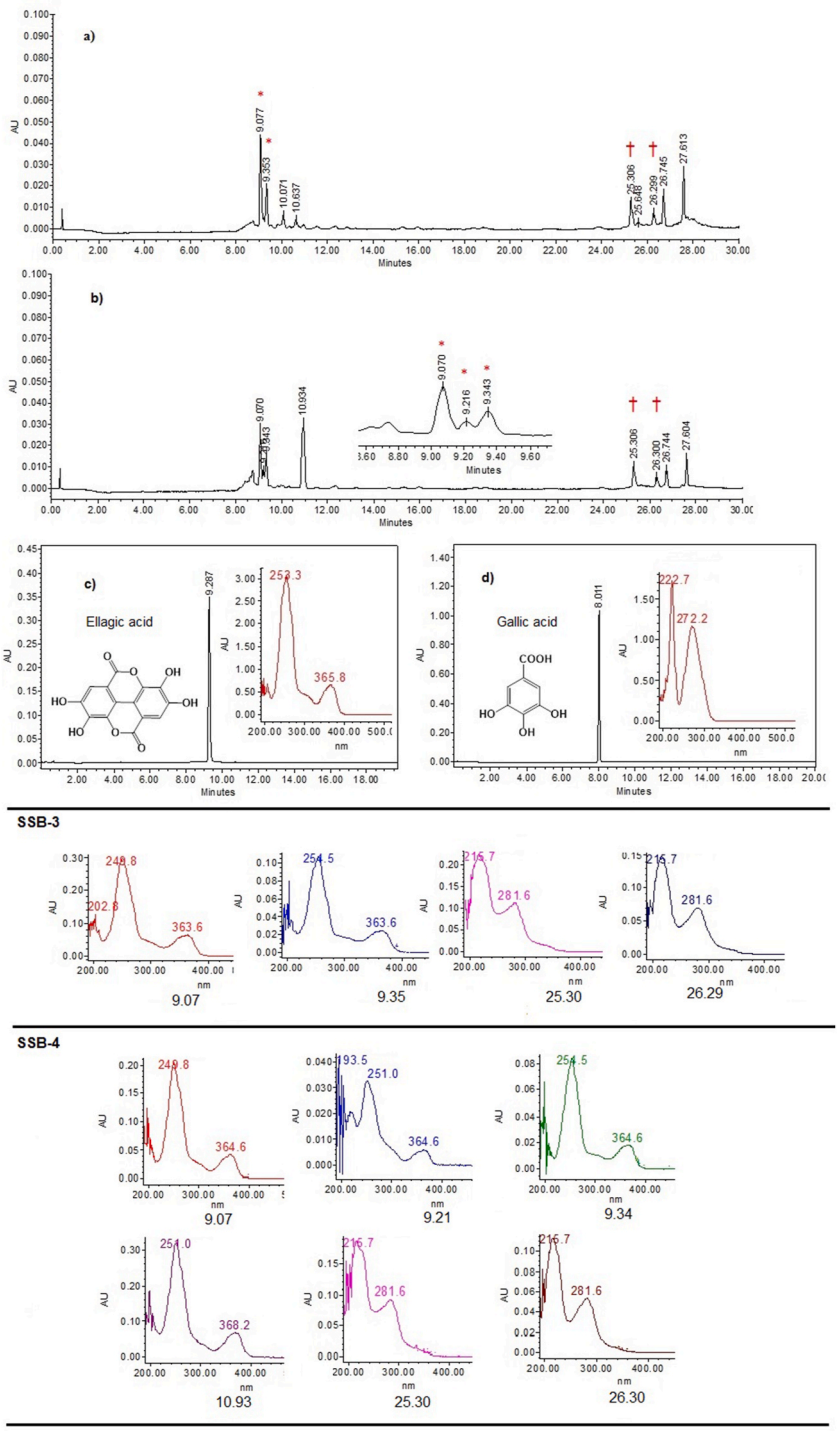

Fig. 2. HPLC-DAD chromatograms of SSB-3 and SSB-4 as well as UV spectra at 340 nm. (a) HPLC-DAD chromatogram of SSB-3. (b) HPLC-DAD chromatogram of SSB-4. (c) HPLC-DAD chromatogram of standard EA®, as well as UV spectra at 340. (d) HPLC-DAD chromatogram of standard GA®, as well as UV spectra at 340 nm. (e) UV spectra at 340 of derivatives of EA and GA from SSB-3. (f) UV spectra at 340 of derivatives of EA and GA from SSB-4. SSB-3: EtOAc *S. schreberi* bark extract, SSB-4: MeOH *S. schreberi* bark extract, GA: gallic acid, EA: ellagic acid. *Derivatives of EA. †Derivatives of GA.

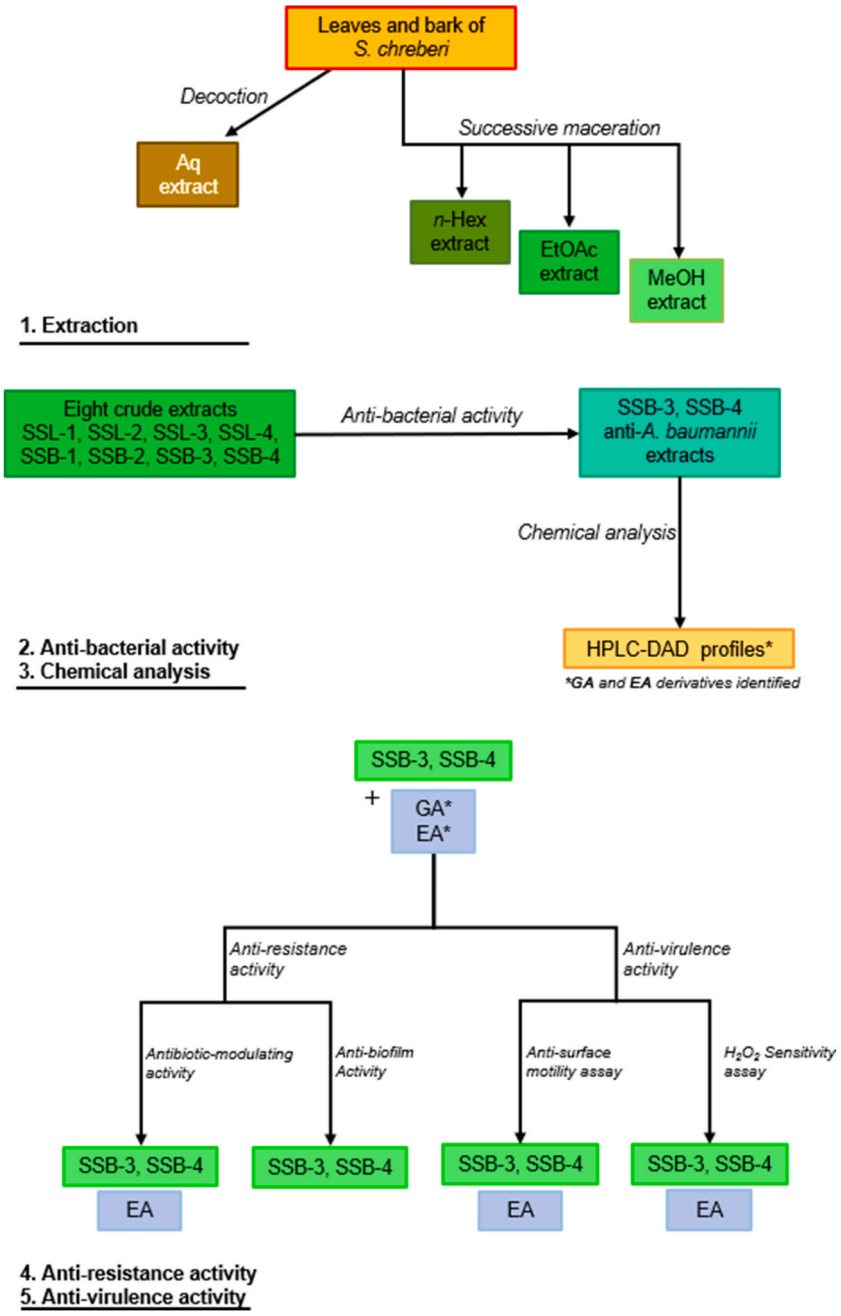

Fig. 3. Flowchart of this research. Aq: aqueous; Hex: *n*-hexane; EtOAc: ethyl acetate; MeOH: methanol; SSL: *S. schreberi* leaves extract; SSB: *S. schreberi* bark extract; GA: gallic acid; EA: ellagic acid.

3.3. Antibiotic-modulating activity

We investigated the antibiotic-modulating activity of SSB-3, SSB-4, GA, or EA in conjunction with amikacin (AMK) or imipenem (IMP) against two clinical isolates of *A. baumannii,* UIMY-ABA-5 and UIMY-ABA-16 (Tables 3 and 4). Our experiments revealed that the combination of SSB-3 or SSB-4 with the reference drugs led to a two-to four-fold reduction in MIC values, indicating antibiotic-modulating activity, except for SSB-3 at 125 mg/mL, which did not affect the MIC value of IMP in the *A. baumannii* UIMY-ABA-5 strain (Tables 3 and 4).

Among the two phenolic acids, GA (500-125 μg/mL) showed a two-to four-fold decrease in MIC values, corresponding to antibiotic-modulating factors (AMF) of 2 and 4, respectively, against UIMY-ABA-5 (plus IMP) and UIMY-ABA-16 (plus AMK) (Tables 3 and 4). However, EA (1.95–0.49 μg/mL) displayed a more significant effect, reducing the MIC values when mixed with antibiotics, being more

Table 3
MIC of IMP in combination with extracts of *Schoepfia schreberi* or phenolic acids against *Acinetobacter baumannii* UIMY-ABA-**5.**

Extract or phenolic acid	UIMY-ABA-**5 (IMP-resistant)**	
	IMP plus extracts or phenolic MIC (μg/mL)	AMF
None	128	–
SSB-3[b]	32	4
SSB-3[c]	128	0
SSB-4[b]	32	4
SSB-4[c]	64	2
GA[a]	32	4
GA[b]	64	2
GA[c]	64	2
EA[a]	16	8
EA[b]	16	8
EA[c]	32	4
PABN[d]	8	16

IMP: imipenem; AMF: antibiotic-modulating factor; SSB-3: EtOAc bark extract of *S. schreberi*; SSB-4: MeOH bark extract of *S. schreberi*; GA: gallic acid; EA: ellagic acid; PABN: phenylalanine-arginine-β-naphthylamide.
[a] 500 μg/mL.
[b] 250 μg/mL.
[c] 125 μg/mL.
[d] 50 μg/mL.

Table 4
MIC of AMK in combination with extracts of *Schoepfia schreberi* or phenolic acids against *Acinetobacter baumannii* UIMY-ABA-16.

Extract or phenolic acid	UIMY-ABA-16 **(AMK-resistant)**	
	AMK plus extracts or phenolic MIC (μg/mL)	AMF
None	64	–
SSB-3[b]	32	2
SSB-3[c]	32	2
SSB-4[b]	32	2
SSB-4[c]	32	2
GA[a]	32	2
GA[b]	32	2
GA[c]	32	2
EA[d]	2	32
EA[e]	16	4
EA[f]	32	2
PABN[g]	8	8

AMK: amikacin; AMF: antibiotic-modulating factor; SSB-3: EtOAc bark extract of *S. schreberi*; SSB-4: MeOH bark extract of *S. schreberi* bark; GA: gallic acid; EA: ellagic acid; PABN: phenylalanine-arginine-β-naphthylamide.
[a] 500 μg/mL.
[b] 250 μg/mL.
[c] 125 μg/mL.
[d] 1.95 μg/mL.
[e] 0.98 μg/mL.
[f] 0.49 μg/mL.
[g] 50 μg/mL.

potent EA (1.95 μg/mL) plus AMK (AMF = 32) against UIMY-ABA-16 (Table 4), while the mixture EA (500 and 250 μg/mL) plus IMP led an AMF = 8 against UIMY-ABA-5 (Table 3).

3.4. Anti-biofilm activity

The effects of SSB-3, SSB-4, GA, and EA on biofilm formation by the UIMY-ABA-81 strain were evaluated. As depicted in Fig. 4, both SSB-3 (23.32 % ± 1.6) and SSB-4 (23.49 % ± 5.3) displayed slight inhibition of biofilm formation at sub-growth-inhibitory concentrations of 250 μg/mL. In contrast, GA and EA stimulated biofilm formation at the same concentration (250 μg/mL).

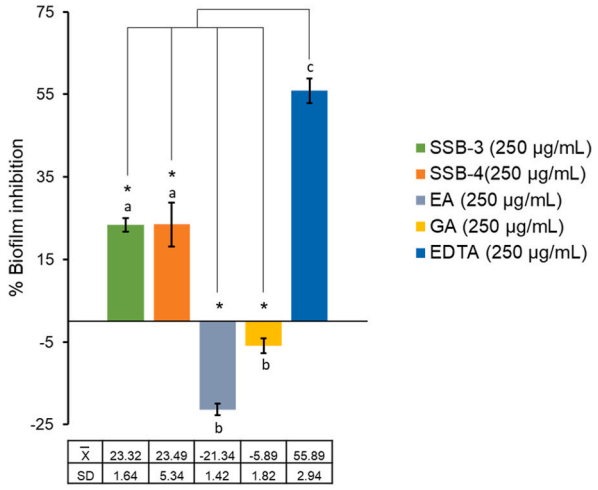

Fig. 4. Effect of extracts *of S. schreberi* and phenolic acids on the biofilm formation of UIMY-ABA-81. SSB-3: EtOAc bark extract of *S. schreberi*; SSB-4: MeOH bark extract of *S. schreberi* bark; GA: gallic acid; EA: ellagic acid. EDTA: ethylenediaminetetraacetic acid. One-way ANOVA was performed, and Tukey's post hoc test ($P \leq 0.05$) was conducted to compare the % of biofilm-formation inhibition in the different groups. Different letters indicated significant differences. In addition, the control group (EDTA) was compared to experimental groups ($^*P \leq 0.0001$).

3.5. Anti-surface motility assay

An anti-surface motility assay was conducted on the UIMY-ABA-205 strain, which displayed high motility, using semi-solid medium (Fig. 5a). Sub-growth-inhibitory concentrations (1/2 MIC) of SSB-3 and SSB-4 were employed to treat the bacteria, resulting in a decrease of up to 38.09 % ± 3.4 and 40.63 % ± 4.9, respectively, in surface motility. Both extracts, SSB-3 and SSB-4 showed significantly better activity than the positive control (66.72 % ± 4.51) with $p < 0.006$ and 0.0013, respectively. (Fig. 5b). Even SSB-3 at 1/4 MIC (125 µg/mL) was still statistically significantly superior to the positive control ($p < 0.0041$) (Fig. 5c). This decrease in the motility of *A. baumannii* due to both extracts was dose-dependent (Fig. 5c).

Conversely, EA at a sub-growth inhibitory concentration of 250 µg/mL significantly reduced surface motility by up to 62.17 % ± 7.5, similar to the positive control, without statistically significant differences between them. GA increased surface motility (Fig. 5b).

3.6. H_2O_2 sensitivity assay

UIMY-ABA-205 was subjected to sub-growth inhibitory concentration (250 µg/mL) of SSB-3 and SSB-4, as well as phenolic acids GA and EA, to evaluate its capacity to survive reactive oxygen species (ROS). As shown in Fig. 6, both SSB-3 and SSB-4 significantly reduced the survival of UIMY-ABA-205 cells in response to ROS, resulting in 49.31 % ± 3.5 and 47.53 % ± 6.2 mortality, respectively. In contrast, EA caused minimal mortality (6.65 % ± 2.5) in UIMY-ABA-205 cells following treatment with H_2O_2. Furthermore, GA prevented hydrogen peroxide-induced death in UIMY-ABA-205 bacterial cells.

4. Discussion

The increasing prevalence of AMR in bacteria is a pressing concern that requires further research and development of new antibiotic agents to effectively combat RAM [31–34]. Studies have shown that medicinal plant extracts contain potent antibacterial agents against MDR bacteria [35–38], including those used in traditional Mayan medicine [20,28,39].

One such bacterium, *A. baumannii*, has been classified by the WHO as a critical priority bacterium for which new antibiotic research and development is urgently needed, as it poses a significant threat to human health [40]. Superbug carbapenem-resistant *A. baumannii* is an emerging opportunistic pathogen in both human and veterinary medicine [41].

4.1. Approach 1: activity of Schoepfia schreberi against the growth of Acinetobacter baumannii

The antibacterial activity of the plant extracts and their phytocompounds was evaluated using the MIC values, which have been established as a standard for measuring the effectiveness of antimicrobial agents. Based on a review of the scientific literature, plant extracts were classified based on their MIC values as weak (1000-500 µg/mL), moderate (500-250 µg/mL), or strong (≤125 µg/mL) [28,42]. Based on these criteria, all the tested *S. schreberi* extracts were found to be active against at least one strain of *A. baumannii* (Table 2).

Our findings indicate that the Mayan medicinal plant *S. schreberi* contains phytochemical constituents that are capable of inhibiting the growth of *A. baumannii* strains, including the superbug carbapenem-resistant *A. baumannii*. These constituents are primarily

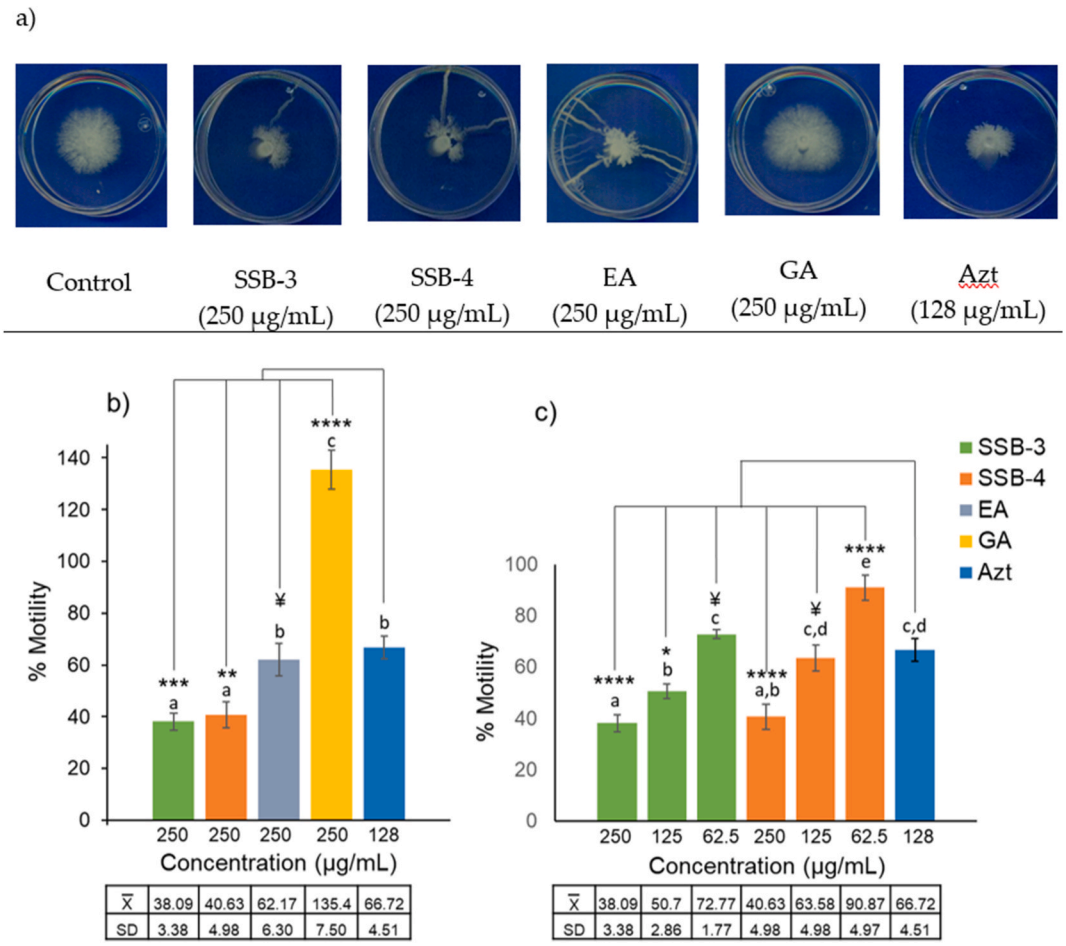

Fig. 5. (a) Images of agar plates images showing the effects of SSB-3, SSB-4, GA, and EA on the surface motility of UIMY-ABA-205 at 250 μg/mL. (b) Effect of SSB-3, SSB-4, GA, and EA on surface motility of UIMY-ABA-205 at 250 μg/mL. (c) Effect of SSB-3 and SSB-4 at three concentrations (250, 125, and 62.5 μg/mL) on surface motility of UIMY-ABA-205. SSB-3: EtOAc bark extract of *S. schreberi*; SSB-4: MeOH bark extract of *S. schreberi* bark; GA: gallic acid; EA: ellagic acid. Azt: Azithromycin. One-way ANOVA was performed, and the Tukey's post-hoc test ($P \leq 0.05$) was conducted to compare the % of motility in the different groups. Different letters indicate significant differences between groups. In addition, the control group (Azt) was compared to experimental groups (*$P < 0.0041$, **$P < 0.0013$, ***$P < 0.006$, ****$P \leq 0.0001$, ¥: not significant).

concentrated in amphiphilic (medium-polar) and hydrophilic (polar) organic extracts of the bark. Additionally, the extracts exhibited both bacteriostatic and bactericidal activity against certain strains. This phenomenon can be attributed to the presence of multiple secondary metabolites in the plant extracts, which may have different modes of action [43], as well as the manifestation of multiple resistance mechanisms and virulence factors in *A. baumannii* strains [44]. Notably, SSB-3 and SSB-4 were active against clinical isolates of *A. baumannii* with diverse drug-resistant profiles including XDR and PDR strains.

Using HPLC and UV spectroscopy, we identified GA and EA derivatives in the most active extracts (SSB-3 and SSB-4; Fig. 2a–d). EA showed a significant anti-*A. baumannii* activity against the UIMY-ABA-16 strain, according to literature [45]. Hence, EA derivatives may contribute to the activity of SSB-3 and SSB-4 against *A. baumannii* strains. Hence, the EA derivatives did not have the same effect on all *A. baumannii* strains.

Other plant extracts, such as *Rosa rugosa* containing EA, are responsible for their activity against multidrug-resistant *A. baumannii*, which inhibits 67 % of growth at 250 μg/mL [46]. Recent studies have shown that EA, is effective against *A. baumannii* and can be used into lipid nanoparticles. Results from an immunocompromised mouse model showed improved survival and reduced bacterial load in the lungs, indicating that EA liposomes may provide better treatment for *A. baumannii* infections than commonly used antibiotics [47]. Ble-González et al. (2022) reported that leaves extract of *Acalypha arvensis* which contained EA was active against *S. aureus*, *K. pneumoniae* and *P. aeruginosa* [48]. EA and pteleoelagic acid isolated from *Pteleopsis hylodendron* exhibited potent activity against *P. aeruginosa*, *K. pneumoniae*, *Streptococcus pyogenes*, *Bacillus cereus*, *S. aureus*, *Salmonella typhi,* and *Escherichia coli* [49]. Other studies have reported the anti-bacterial activity of EA against *S. aureus*, *P. aeruginosa*, *Helicobacter pylori*, *S. epidermidis*, and *K. pneumoniae* [50, 51]. The biological activity of EA may be associated with its action on the bacterial cell membranes. In addition, EA can form complexes with essential metals in bacterial cells, leading to microbial death [52].

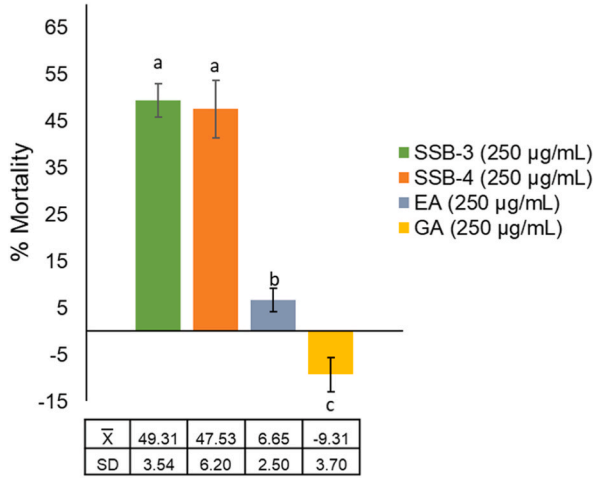

Fig. 6. Effect of SSB-3, SSB-4, GA, and EA on UIMY-ABA-205 after treatment with H_2O_2. SSB-3: EtOAc bark extract of *S. schreberi*; SSB-4: MeOH bark extract of *S. schreberi* bark; GA: gallic acid; EA: ellagic acid. One-way ANOVA was performed, and Tukey's post-hoc test ($P < 0.05$) was conducted to compare the % of mortality among the different groups. Different letters indicate significant differences.

4.2. Approach 2: activity of Schoepfia schreberi against the resistance mechanisms of Acinetobacter baumannii

Anti-resistant and anti-virulence drugs have emerged as promising new treatments for infections. Researchers have sought to identify compounds or extracts that inhibit resistance mechanisms such as biofilm formation, β-lactamase enzymes, antibiotic efflux pumps, and outer membrane permeability barriers. Combining plant extracts or phytoconstituents with conventional antibiotics has been shown to restore the effectiveness of classic antibiotics [53–55].

Our study showed that SSB-3, SSB-4, or EA in combination with IMP or AMK showed antibiotic-modulating activity on UIMY-ABA-5 or UIMY-ABA-16, respectively [56,57]. The significant antibiotic-modulation threshold was set at a decrease of more than two-fold in the MIC of the tested antimicrobial, which is referred to as AMF [58]. These clinical isolates were phenotypically characterized as possessing efflux pumps, as a mechanism of resistance to IMP and/or AMK (unpublished data). Previous studies by Chusri et al. (2009) and Jenic et al. (2021) reported that EA at 12 µg/mL and 30.2 µg/mL, respectively, acted as an efflux pump inhibitor, enhancing the *in vitro* efficacy of various antibiotics against *A. baumannii* and *E. coli* [59,60]. Similarly, Macêdo et al. (2022) reported a two-fold reduction in the MIC for tetracycline or ciprofloxacin in combination with EA (128 µg/mL) against *S. aureus* [61].

Biofilms, composed of carbohydrates, nucleic acids, proteins, and other macromolecules, enable *A. baumannii* to persist in medical environments, resist antimicrobial drugs, and cause diseases [62]. SSB-3 (23.32 %) and SSB-4 (23.49 %) showed slight anti-biofilm formation activity (Fig. 4) against UIMY-ABA-205 at sub-growth-inhibitory concentrations of 250 µg/mL. The slight anti-biofilm activities of SSB-3 and SSB-4 could be attributed to GA and EA derivatives, which stimulated biofilm formation. We previously reported that SSB-3 and SSB-4 exhibited anti-biofilm formation activity against MRSA (ATCC 43300) with IC_{50} values of 62.8 % ± 7.1, and 111.8 % ± 2.9, respectively [20]. The dissimilarity in the anti-biofilm activities of SSB-3 and SSB-4 against *A. baumannii* (Gram-negative) and *S. aureus* (Gram-positive) may be attributed to the composition of their cell walls, which are critical for adherence to surfaces and biofilm formation, or to the chemical structure of the inductors and their receptors for quorum sensing. Teichoic acid and peptides are present in Gram-positive bacteria, whereas lipopolysaccharides and acyl-homoserine lactones are present in Gram-negative bacteria [63].

Our results showed that GA and EA were inactive against biofilm formation by UIMY-ABA-81 at 250 µg/mL (Fig. 3). Sherif et al. reported that GA sub-growth-inhibitory concentrations (660–1055 µg/mL) inhibited biofilm formation (36.34–90.67 %) in a group of clinical isolates of *A. baumannii* [64]. Additionally, EA, both in its pure form and in a liposomal formulation at a concentration of 32 µg/mL, effectively inhibited biofilm formation by *A. baumannii* ATCC-19606 by 72.3 % and 60 %, respectively [47]. These discrepancies in activity may be attributed to the strain tested, as it has been previously established that the anti-biofilm activity of a compound can vary among different bacteria [64].

In light of our findings, it is possible to consider SSB-3, SSB-4, and EA as potential adjuvants in the fight against drug-resistant pathogens, including the carbapenem-resistant superbug *A. baumannii*.

4.3. Approach 3: activity of Schoepfia schreberi against the virulence factors of Acinetobacter baumannii

The third approach aims to disrupt virulent factors that allow pathogens to colonize, suppress, and evade the immune response, obtain nutrients, and damage host cells [65].

Surface-associated motility has been linked to increased virulence and lethality in the *Caenorhabditis elegans* worm model and is also associated with lipooligosaccharides, proteins related to metabolism, outer membrane, and efflux pumps [66,67]. SSB-3, SSB-4, and EA at sub-growth-inhibitory concentration of 250 µg/mL significantly reduced surface-associated motility of *A. baumannii*

UIMY-ABA-205. Previously, it was reported that EA reduced the surface-associated motility of *P. aeruginosa* PO01 [68].

Acinetobacter baumannii synthesizes antioxidant compounds and enzymes, such as catalase and superoxide dismutase, to protect itself from reactive oxygen species (ROS) generated by its own metabolism, some antibiotics, and neutrophils during phagocytosis [30,69]. Our experiments revealed that SSB-3 and SSB-4 at sub-growth-inhibitory concentration (250 μg/mL) significantly decreased the viability of UIMY-ABA-205 cells in response to ROS. It is worth noting that the inactivation of superoxide dismutase has been shown to entirely abolish cell motility [70]. This may be due to the fact that our extracts, which also inhibit motility, can also inhibit this enzyme.

Conversely, GA prevented the death of *A. baumannii* by H_2O_2, and EA slightly reduced cell survival by 6.65 %, which may be attributed to the antioxidant properties of phenolic acids that neutralize the effects of H_2O_2 [71,72]. Various studies have reported that phenolic acids possess pro-oxidant activity, and when present alongside H_2O_2, they can lead to the formation of quinones and generation of free radicals of $\cdot O_2$ [69]. In addition, the pro-oxidant effect of GA has been demonstrated in *A. baumannii* strains [69]. In contrast, our study revealed that the combination of GA and H_2O_2 did not enhance killing of the *A. baumannii* strain (UIMY-ABA-205). It is possible that the overnight contact with GA resulted in the overproduction of antioxidant enzymes by UIMY-ABA-205, which subsequently allowed their survival in an environment with H_2O_2. To the best of our knowledge, this is the first report of the anti-growth, anti-resistance, and anti-virulence activities of *S. schreberi* against *A. baumannii*.

4.4. HPLC-DAD analysis of Schoepfia schreberi bark extracts

Our analysis of the chemical compositions of SSB-3 and SSB-4 using HPLC-DAD established their profiles and identified the derivatives of EA and GA in both extracts.

5. Conclusions

The current investigation demonstrated that *S. schreberi* exhibits anti-infectious properties against diverse strains of *A. baumannii*, including carbapenem-resistant superbugs, by inhibiting bacterial growth, resistance mechanisms, such as biofilms and efflux pumps, and virulence factors including motility and catalase. These findings support the traditional use of sak beek for the treatment of infections and their related symptoms. These results suggest that the bark extracts of *S. schreberi* (SSB-3 and SSB-4) contain multi-target phytoconstituents. This study makes a significant contribution to the field of ethnopharmacology by reevaluating the medicinal flora of the Mayan culture and providing evidence for the use of *S. schreberi* bark for the treatment of infectious diseases caused by superbugs. Therefore, it is essential to conduct additional studies to isolate and identify the anti-*A. baumannii* phytoconstituent compounds from *S. schreberi* to develop novel alternative antibiotic, anti-resistance, and/or anti-virulence agents and to contribute to the phytochemical composition of this medicinal specie.

6. Strengths and limitations

The strengths of this study include the relatively complete control of the variables with precise measures. Assays were developed under controlled experimental conditions to reduce the risk of analytical errors. *In vitro* assays are very useful for mechanistic studies of biological processes and represent investigations with low cost, fast implementation, and standardization of bioassays compared to *in vivo* models. In addition, we identified that extracts from the Mayan medicinal plant *S. schreberi* could be used as alternatives to treat superbug infections. This investigation had some limitations, as the correlation between crude extracts of *S. schreberi* and antibacterial, antibiotic-modulating, anti-biofilm, anti-surface motility and anti-catalase activities seems to be significant; however, the current knowledge is mainly based on *in vitro* studies. Therefore, its applicability in the clinical setting is unknown. Additional investigations will be focused on bio-guided studies to isolate and characterize the active compounds responsible for the anti-infectious effects and to develop one phytodrug containing standardized pure compounds, enriched fractions, or crude extracts. This means that these limitations represent opportunities to continue research to answer new questions.

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Data availability statement

The original contribution presented in this study are included in the article, further inquiries can be directed to the corresponding author. Data will be made available on request.

CRediT authorship contribution statement

Andrés Humberto Uc-Cachón: Writing – review & editing, Writing – original draft, Validation, Resources, Methodology, Investigation, Formal analysis, Conceptualization. **Angel de Jesús Dzul-Beh:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis. **Manases González-Cortázar:** Writing – review & editing, Validation, Resources, Methodology, Investigation. **Alejandro Zamilpa-Álvarez:** Writing – review & editing, Resources, Investigation. **Gloria María**

Molina-Salinas: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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