Antimicrobial, antibiofilm and cytotoxic activities of *Hakea sericea* Schrader extracts

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ABSTRACT

Background: *Hakea sericea* Schrader is an invasive shrub in Portuguese forests. **Objective:** The goal of this work was to evaluate the antimicrobial activity of *H. sericea* extracts against several strains of microorganisms, including the ability to inhibit the formation of biofilms. Additionally the cytotoxic properties of these extracts, against human cells, were assessed. **Materials and Methods:** The antimicrobial activity of the methanolic extracts of *H. sericea* was assessed by disk diffusion assay and Minimum Inhibitory Concentration (MIC) value determination. The antibiofilm activity was determined by quantification of total biofilm biomass with crystal violet. Cytotoxicity was evaluated by hemolysis assay and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. **Results:** For Gram-positive bacteria, MIC values of *H. sericea* methanolic extracts ranged between 0.040 and 0.625 mg/mL, whereas the fruits extract yielded the lowest MIC for several strains of microorganisms, namely, *S. aureus*, *B. cereus*, *L. monocytogenes* and clinical methicillin-resistant *S. aureus* (MRSA). Stems and fruits extract at 2.5 mg/mL effectively eradicated the biofilm of *S. aureus* ATCC 25923, SA 01/10 and MRSA 12/10. Regarding leaves extract, hemolysis was not observed, and in the case of stems and fruits, hemolysis was verified only for higher concentrations, suggesting its low toxicity. Fruits extract presented no toxic effect to normal human dermal fibroblasts (NHDF) cells however for concentrations of 0.017 and 0.008 mg/mL this extract was able to decrease human breast adenocarcinoma cells (MCF-7) viability in about 60%, as MTT test results had confirmed. This is a clearly demonstrator of the cytotoxicity of this extract against MCF-7 cells.

Key words: Antibiofilm, Antimicrobial, Cytotoxicity, *Hakea sericea*

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INTRODUCTION

The rise in antibiotic-resistant microorganism in recent years has led to an increasing search for new antibiotics.[1] In general, it has been possible to observe an increase in resistance of pathogenic viruses, bacteria, fungi and protozoa against known drugs.[2] Moreover, bacteria that adhere to implanted medical devices or damaged tissue can become the cause of persistent infections. These bacteria encase themselves in a hydrated matrix of polysaccharide and protein, forming a slimy layer known as a biofilm.[3] This mechanism increases the resistance to antibiotics and difficult the eradication of microorganism. To overcome the drawbacks of the current antimicrobial drugs and to obtain more efficacious drugs, an antimicrobial drug having a novel mode of action should be developed.[2] All over the world, people depended on herbs for the treatment of various ailments before the advent of modern medicine. Medicinal plants constitute an arsenal of chemicals that could be exploited by human to prevent microbial invasion.[4] Secondary metabolites produced by plants constitute a major source of bioactive substances. The scientific interest in these metabolites has increased today with the search of new therapeutic agents from plant source, due to the increasing development of the resistance pattern of microorganisms to most currently used antimicrobial drugs.[5] In a search for plant-derived biologically active compounds against infectious diseases, this work analyses the potential antimicrobial activity of *Hakea sericea* Schrader methanolic extracts. *H. sericea* is an invasive shrub in Portuguese forests. Originally from southern Australia, it was introduced in Portugal for ornamental purposes. A key characteristic of *H. sericea* is it extreme serotinous habit: all of its seeds are

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retained in pairs in tough woody follicles, which accumulate along the branches throughout the life of the plant.[6] In a previous report of our team, we have made an exhaustive study of several shrubs extracts, including *H. sericea*.[7] This shrub had demonstrated very good antioxidant properties, namely radical scavenging activity of DPPH free radicals and the inhibition of oxidation of linoleic acid molecules, which is an indicator of inhibition of lipid peroxidation.[7] These antioxidant properties are mainly due to the presence of phenolics, tannins and flavonoids.[7] In our previous work we have analyzed the phenolic composition of *H. sericea* extracts using RP-HPLC and the main compounds present in extracts were ferulic and *p*-coumaric acids.[7] Moreover, other phenolic compounds commonly found in plants were identified and quantified in *H. sericea* methanolic extracts, for example, gallic, caffeic and chlorogenic acids.[7] We have also found that *H. sericea* possesses high concentrations of some secondary metabolites, namely alkaloids, among the others above mentioned.[7] These substances are well known bioactive compounds from plants. To the best of our knowledge, no other phytochemical characterization about this plant has been published yet. In this sense, the goal of this work was to evaluate the antimicrobial activity of *H. sericea* extracts against several strains of microorganism, as well as studying the ability to inhibit the formation of biofilms. Additionally the cytotoxic properties of this extracts, against human cells, were assessed.

MATERIALS AND METHODS

Plant material

Aerial parts (stems, leaves and fruits) of *H. sericea* were collected in Serra da Estrela (GPS coordinates: N 40º20.296'; W 07º27.491'; Altitude: 730 m). Plant materials were dried at 35ºC in a ventilated oven during 48h and reduced to coarse powder (<2 mm) using a laboratory cutting mill. Harvesting, transport and storage of plant species were authorized by *Instituto da Conservação da Natureza e da Biodiversidade*, because this plant is an exotic, invasive and non-indigenous species. This vegetal species was identified by a botanist and a voucher specimen (No. LISI 13/2011) has been deposited in the Herbarium of the *Instituto Superior de Agronomia* (*Jardim Botânico d'Ajuda*, *Lisboa*).

Extraction procedure

Methanolic extracts were obtained with Soxhlet apparatus until the solvent became colorless, using approximately 100g of raw material and 1000mL of solvent. The extract solutions were filtered under vacuum using a crucible of porosity #2 and then distilled under reduced pressure until a final volume of solvent of 100mL. Prior to use, the extracts were dried using rotary evaporator under reduced pressure and then dissolved in dimethylsulphoxide (DMSO).

Determination of antimicrobial activity

Test microorganisms and culture media

The antimicrobial studies were carried out against eight bacterial strains (Gram-positive: *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 11778 and *Listeria monocytogenes* LMG 16779; Gram-negative: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Klebsiella pneumoniae* ATCC 13883; Clinical methicillin-resistant *S. aureus* MRSA 10/08 and MRSA 12/08) and two yeast strains (*Candida albicans* ATCC 90028 and *Candida tropicalis* ATCC 750). For antibiofilm activity assays, an additional four clinical strains of *S. aureus* were used (SA 01/10, SA 02/10, SA 03/10 and SA 08). Stock cultures were prepared and stored with 20% glycerol at -80ºC. The strains were sub-cultured on an appropriate agar plate 24 h prior to any antimicrobial test and when cultured from stock, they were sub-cultured before use. Brain Heart Infusion Agar (BHI) was used for the growth of bacterial species and Sabouraud Dextrose Agar (SDA) was used for yeasts. Müeller-Hinton Agar (MHA) was used for the disc diffusion assays and for Minimum Inhibitory Concentration (MIC) determination against all microorganism. Regarding the disc diffusion with yeasts, MHA with 2% glucose and 0.5 μg/mL blue methylene was used, whereas MHA with 2% glucose was used to determine the MIC.

Disc diffusion assay

Antimicrobial activity of the crude methanolic extracts of stems, leaves and fruits of *H. sericea* was determined by disk diffusion assay, using either the M2-A8 method, described by Clinical Laboratory and Standards Institute (CLSI), for bacteria or the M44-A2 method described by CLSI for yeasts.[8,9] For the preparation of inoculum, bacteria or fungi were suspended in saline solution to a cell suspension of 0.5 McFarland (about 1 to 2×10^8 colony-forming unit/mL (CFU/mL) to non-fastidious bacteria and 1 to 5×10^6 CFU/mL for yeasts). The dried plant extracts were dissolved in DMSO. The discs (6 mm diameter) were impregnated with 20 μL of the extract (4 mg/disc) at a concentration of 200 mg/mL and placed on the inoculated agar. Negative controls were prepared using DMSO as was used to dissolve the plant extracts. Positive controls were prepared using tetracycline (30 μg/disc) in the case of bacteria and amphotericin B (25 μg/disc) in the case of yeasts. The plates inoculated with non-fastidious bacteria were incubated at 37ºC for 24 h and for 48 h in the case of yeasts. After incubation, all plates were checked for inhibition zones and the diameters were measured in millimeters. All experiments were carried out in triplicate.

Determination of MIC by agar dilution method

The non-fastidious reference bacteria and isolates were tested according to the M7-A6 protocol, published by CLSI.[10] According to this protocol, each extract was diluted

and incorporated into MHA to create a series of plates with concentrations ranging from 2.5 to 0.01 mg/mL. 2 µL of 1:10 dilution of a suspension with an equivalent turbidity of 0.5 McFarland were used to inoculate the agar in order to achieve 10^4 CFU per spot. After incubation for 24 h at 37ºC, the plates were visually inspected to determine either the growth or inhibition on the surface of each dilution plate. Medium without the tested extracts were inoculated with the strain cultures and used as controls. The MIC was defined as the lowest concentration of antimicrobial agent which prevented visible growth. The experiments were repeated three times and the results were determined by the modal value. In the case of yeasts, the protocol above mentioned was slightly modified, the medium MHA was supplemented with 2% glucose and the time of incubation was increased to 48 h.

Antibiofilm activity

Antibiofilm activity was determined according to Raja *et al*., 2011 with some modifications.[11] In brief, the biofilms of seven strains of *S. aureus* (ATCC 25923, MRSA 10/08, MRSA 12/08, SA 01/10, SA 02/10, SA 03/10 and SA 08) were prepared in 96-well flat-bottom polystyrene microtiter plates. The bacterial suspensions were prepared from the overnight grown culture (37ºC, 250rpm) and the turbidity of the suspension was adjusted to 0.7 O.D. at 610nm (~1×10^9 CFU/mL). Twofold serial dilutions of *H. sericea* extracts were prepared in 100 µL volume of tryptone soya broth (TSB) supplemented with 0.5% glucose in the wells of 96-well flat bottom microtiter plate. 40 µL of fresh TSB with 0.5% glucose was added to each well, followed by the addition of 60 µL of above bacterial suspension. This resulted in final inoculums of 6×10^7 CFU/mL in each well; the final concentrations of the extracts ranged from 2.5 to 0.08 mg/mL. The plate was incubated 24 h at 37ºC. After incubation, the planktonic cells were removed from each well by washing two times with 200 µL of phosphate buffer saline (PBS). The biofilms were fixed with 200µL of methanol for 20 min, stained with 100 µL of 0.1% (wt/vol) crystal violet for 10 min and rinsed thoroughly three times with 200 µL of water. Negative control was prepared as mentioned above replacing the inoculum volume by TSB. Biofilm formation was quantified by the addition of 200 µL of 95% ethanol to the crystal violet stained cells and then 100 µL was removed for recording the absorbance at 595nm using a microplate reader.[11] This procedure was done, at least three independent times.

Cytotoxic evaluation

Hemolysis assay

Freshly obtained EDTA human blood (A, Rh+) was washed three times by centrifugation (2500 rpm for 10 min) in PBS at room temperature (20-25ºC). Buffy coat was removed of the pellet and a 10% of red blood cells (RBC) suspension was prepared in PBS (1×), aliquots of 2.5 mL of 10% RBC suspension plus 500 µL of *H. sericea* extracts at three different concentrations 0.5, 5 and 50 mg/mL were shaken in a waterbath at 37ºC. Next, 40 µL of 2.5% glutaraldehyde was added to 1mL of the mixture at 20 and 40 min to terminate the reaction. After that, they were centrifuged at 2500 rpm for 15 min to allow broken membranes and unbroken cells to settle at the bottom. For 100% hemolysis or positive control, 500 µL of 0.2% Triton (in PBS) was added to 2.5 mL of 10% RBC suspension. The supernatant was removed and the liberated hemoglobin in the supernatant was measured spectrophotometrically as absorbance at 541 nm. For negative control (0% hemolysis) only 500µL of PBS and 2.5 mL of 10% RBC suspension were used and DMSO control was done mixing 500 µL of 25% DMSO with 10% of RBC suspension. The experiment was done in triplicate and mean ± standard deviation was calculated as follows: % Hemolysis = (Absorbance sample/ Absorbance positive control) × 100.[12]

MTT test

Cells were routinely maintained at 37ºC in a humidified atmosphere containing 5% CO_2. Normal human dermal fibroblasts (NHDF) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), HEPES (0.01M), *L*-glutamine (0.02M), sodium pyruvate (0.001M) and 1% antibiotic/antimycotic (10000 units/mL penicillin, 10 mg/mL streptomycin and 25 µg/mL amphotericin B). Dulbelco's Modified Eagle's Medium (DMEM) high glucose supplemented with 10% FBS and 1% antibiotic/antimycotic was used to culture human breast adenocarcinoma cells (MCF-7). All the cells were used between passages 3 to 10 in the experiments. Cell viability in the presence of *H. sericea* extracts was studied using the method of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) according to a previous described procedure with slight modifications.[13] Briefly, 500 µL of cells were seeded in 24-well plates (2×10^4 cells/well) in the culture medium containing FBS and after 48h they were treated with 500 µL of four different concentrations (0.5, 0.167, 0.017 and 0.008 mg/mL) of *H. sericea* extracts for 48h, with untreated cells serving as control. At the end of incubation the media in the wells was removed and replaced with 300 µL of MTT solution in fresh media and incubated at 37ºC for 4h. Thereafter, media-containing MTT was removed and formazan crystals were dissolved in 300 µL of DMSO and 20 µL of glycyl-glycine buffer (for stabilizing the colour) were added. Then, 200 µL of the mixture above mentioned was removed and absorbance was recorded in microplate reader at 570 nm.[14] The experiment was carried out in triplicate and the extent of cell death was expressed as the percentage of cell viability in comparison with control cells.

RESULTS AND DISCUSSION

In this work, the antimicrobial activity of *H. sericea* methanolic extracts against several strains of human infecting microorganism was evaluated. Moreover the antibiofilm activity and cytotoxic properties of the extracts were studied. In our previous work we have analyzed the phenolic composition of *H. sericea* extracts using RP-HPLC and the main compounds present in extracts were ferulic and *p*-coumaric acids.[7] Moreover, other phenolic compounds commonly found in plants were identified and quantified in *H. sericea* methanolic extracts.[7]

The diameters of inhibition zones for *H. sericea* extracts, measured by disc diffusion assay, are presented in Table 1. The strain of *C. albicans* was not inhibited by the extracts and the extract of fruits does not inhibit the growth of *P. aeruginosa*. Generally, Gram-positive bacteria presented more susceptibility to all *H. sericea* extracts than Gram-negative and yeast strains. The fruits extract is the one which promotes less growth inhibition when compared with the extracts of the other parts of *H. sericea*. Contrariwise, all strains tested are more susceptible to the stems extract, namely *S. aureus* with an inhibition zone of 19.50 mm. The growth of *L. monocytogenes* was largely inhibited by all studied extracts. Relatively to the solvent used to dissolve the extracts, DMSO does not inhibit the growth of the microorganisms tested. In the case of conventional antibiotic and antifungic, respectively tetracycline and amphotericin B, the biggest diameters of inhibition zones were found. The results obtained by disc diffusion were promissory and indicate the potential use of these extracts as antimicrobials.

The minimum inhibitory concentrations of extracts were determined by agar dilution method and the results are presented in Table 2. The Gram-positive strains present lower MIC values than Gram-negative and yeast strains which indicates that Gram-positive bacteria were the most susceptible strains to the *H. sericea* extracts. The difference in susceptibility between the Gram-negative and Gram-positive bacteria to inhibition by plant extracts is supported by other researchers.[15,16] It is not known exactly why Gram-negative bacteria should be less susceptible but it may be related to the outer membrane of Gram-negative bacteria which endows the bacterial surface with strong hydrophilicity and acts as a strong permeability barrier.[16] The morphological differences between Gram-positive and Gram-negative bacteria could justify the different sensitivity, namely because Gram-negative bacteria have an outer phospholipidic membrane carrying the structural lipopolysaccharide components, which cannot be found in Gram-positive bacteria. This makes the cell wall impermeable to lipophilic solutes, while porins constitute a selective barrier to the hydrophilic solutes with an exclusion limit of about 600 Da.[15] The Gram-positive bacteria should be more susceptible to the bioactive compounds. Another explanation may be the inhibition of the peptidoglycan synthesis in Gram-positive bacteria, by compounds present in the extracts.[15] In the case of Gram-positive bacteria, MIC values of *H. sericea* methanolic extracts ranged between 0.040 and 0.625 mg/mL, whereas the fruits extract is the one with lowest MIC for several strains of microorganisms, namely, *S. aureus*, *B. cereus*, *L. monocytogenes* and clinical MRSA 10/08 and MRSA 12/08, which indicates a possible high antimicrobial activity. Surprisingly, this extract is the one with smaller inhibition zones; this fact can be probably due to the presence of compounds in this extract which are difficult to spread on the agar surface, but when they are diluted in agar present a high antimicrobial activity. The results now obtained for MIC values were smaller than the ones obtained by Ferreira *et al*., 2011 in their study of antimicrobial activity of extracts *Cistus ladanifer* and *Arbutus unedo*, which are also shrubs of Portuguese forests.[17] Moreover, fruits extract had a significant activity against some food-borne pathogens, *B. cereus* and *L. monocytogenes*, indicating a potential use of this plant extracts as food preservative.

In addition, since the extracts presented low MIC values for *S. aureus* strains, including clinical MRSA strains, it was decided to evaluate the antibiofilm activity of them. Biofilms are communities of surface-associated microorganisms embedded in a self-produced extracellular polymeric matrix that are notoriously difficult to eradicate and are a source of many recalcitrant infections.[11] Staphylococci are known to form biofilms on an implanted medical device or damaged tissues and these biofilms are difficult to disrupt.[11] The effect of *H. sericea* extracts on *S. aureus* biofilm formation is presented in Table 3. These extracts effectively inhibited the biofilm formation of all studied strains of *S. aureus*, except in the case of minor concentrations of fruits extract for *S. aureus* ATCC 25923, probably because in the antibiofilm assay a greater number of CFU was used than in the determination of MIC, and in this sense the extract concentration available to act on the microorganisms is reduced; or because the sub-MIC concentrations can induce stress to the bacterial cells and promote the biofilm formation. Generally, the inhibition of *S. aureus* biofilm formation corresponds to the inhibition of bacterial growth or cell death, but interestingly, for sub-MIC concentrations the formation of biofilms is also inhibited. Stems extract at 2.5 mg/mL effectively eradicated the biofilm of reference strain of *S. aureus* and SA 01/10. The total eradication of biofilm was also observed for fruits extract (2.5 mg/mL) to MRSA 12/10 which is in agreement with MIC value of this extract for that strain (0.08 mg/mL). To the best of our knowledge, this is the first report to

Table 1: Diameter of inhibition zone (mm) of *H. sericea* methanolic extracts and controls. The results are expressed in terms of mean±standard deviation

Plant part/ control	*S. aureus* ATCC 25923	*B. cereus* ATCC 11778	*L. monocytogenes* LMG 16779	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*K. pneumoniae* ATCC 13883	*Candida tropicalis* ATCC 750	*Candida albicans* ATCC 90028
Stems	19.50±0.50	13.00±0.00	15.67±0.58	8.33±0.58	10.50±0.71	12.33±0.58	15.67±0.58	6.00±0.00
Leaves	16.50±0.50	12.50±0.50	17.67±0.58	7.67±0.58	9.00±0.71	11.50±0.50	11.50±0.50	6.00±0.00
Fruits	10.00±0.00	11.00±0.00	14.67±0.58	6.50±0.50	6.00±0.00	10.67±0.58	9.50±0.50	6.00±0.00
Tetracycline	30.25±0.50	30.00±0.82	18.25±0.60	23.25±0.50	11.50±0.58	22.25±0.50	-	-
Amphotericin B	-	-	-	-	-	-	21.50±0.58	20.33±0.58
DMSO	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00	6.00±0.00

DMSO: Dimethylsulphoxide; ATCC: American type culture collection

Table 2: MIC values (mg/mL) of *H. sericea* methanolic extracts. The results are expressed as modal values

Plant part	*S. aureus* ATCC 25923	*B. cereus* ATCC 11778	*L. monocytogenes* LMG 16779	*E. coli* ATCC 25922	*P. aeruginosa* ATCC 27853	*K. pneumoniae* ATCC 13883	*Candida tropicalis* ATCC 750	*Candida albicans* ATCC 90028	MRSA 10/08	MRSA 12/08
Stems	0.315	0.625	0.315	2.500	1.250	0.625	1.250	1.250	1.250	2.500
Leaves	0.625	1.250	0.315	>2.500	2.500	1.250	2.500	2.500	1.250	>2.500
Fruits	0.155	0.040	0.040	>2.500	2.500	>2.500	>2.500	>2.500	0.315	0.080

MIC: Minimum inhibitory concentration; ATCC: American type culture collection

Table 3: Effect of *H. sericea* extracts on the biofilm formation. The results are expressed as mean±standard deviation of optical density readings for crystal violet assays

Extracts		Strains						
Plant part	Concentration (mg/mL)	*S. aureus* ATCC 25923	MRSA 10/08	MRSA 12/08	SA 01/10	SA 02/10	SA 03/10	SA 08
Stems	2.5	0.18±0.04	0.30±0.06	0.43±0.04	0.27±0.06	0.41±0.06	0.62±0.15	0.62±0.06
	1.25	0.63±0.11	0.55±0.09	0.50±0.04	0.45±0.12	0.49±0.13	0.76±0.13	0.69±0.15
	0.625	0.74±0.15	0.60±0.13	0.52±0.10	0.62±0.15	0.79±0.10	0.80±0.08	0.80±0.12
	0.315	0.78±0.10	0.77±0.09	0.62±0.12	0.85±0.08	1.07±0.08	0.86±0.15	1.03±0.19
	0.156	0.85±0.10	0.80±0.17	0.79±0.07	0.87±0.17	1.21±0.08	1.30±0.10	1.30±0.15
	0.08	0.98±0.13	0.87±0.11	0.93±0.12	1.00±0.10	1.35±0.08	1.35±0.14	1.35±0.20
	Positive control	1.27±0.15	1.19±0.15	1.01±0.18	1.70±0.19	1.50±0.13	1.50±0.15	1.50±0.16
	Negative control	0.16±0.02	0.16±0.02	0.17±0.02	0.21±0.10	0.15±0.01	0.14±0.02	0.15±0.02
Leaves	2.5	0.38±0.05	0.42±0.07	0.27±0.02	0.64±0.10	0.66±0.08	0.77±0.16	0.36±0.11
	1.25	0.47±0.02	0.52±0.07	0.29±0.05	0.70±0.10	0.72±0.10	0.80±0.04	0.42±0.16
	0.625	0.58±0.09	0.60±0.06	0.39±0.07	0.75±0.23	0.80±0.14	0.80±0.19	0.47±0.16
	0.315	0.71±0.10	1.00±0.10	0.77±0.11	1.10±0.17	1.07±0.20	1.01±0.18	0.72±0.10
	0.156	1.01±0.16	1.10±0.10	1.20±0.11	1.12±0.22	1.10±0.15	1.30±0.16	0.72±0.11
	0.08	1.08±0.15	1.16±0.12	1.30±0.10	1.24±0.20	1.20±0.15	1.35±0.21	0.72±0.06
	Positive control	1.26±0.15	1.20±0.14	1.35±0.15	1.25±0.20	1.30±0.22	1.50±0.11	0.84±0.18
	Negative control	0.15±0.02	0.16±0.02	0.16±0.01	0.15±0.02	0.14±0.02	0.16±0.02	0.14±0.01
Fruits	2.5	0.65±0.10	0.33±0.06	0.21±0.10	0.31±0.01	0.55±0.16	0.72±0.14	0.58±0.12
	1.25	0.66±0.18	0.32±0.13	0.26±0.07	0.31±0.03	0.55±0.17	0.80±0.18	0.76±0.20
	0.625	0.67±0.16	0.61±0.12	0.28±0.07	0.53±0.10	0.80±0.16	1.20±0.05	0.77±0.18
	0.315	1.05±0.16	0.74±0.10	0.87±0.10	0.96±0.12	0.92±0.20	1.35±0.07	1.07±0.17
	0.156	1.33±0.07	0.83±0.12	0.99±0.09	0.98±0.15	1.10±0.15	1.41±0.16	1.08±0.15
	0.08	1.65±0.09	0.86±0.13	1.10±0.16	0.99±0.13	1.20±0.10	1.47±0.18	1.10±0.09
	Positive control	1.23±0.19	1.19±0.15	1.20±0.17	1.10±0.20	1.30±0.10	1.55±0.11	1.20±0.09
	Negative control	0.13±0.01	0.14±0.01	0.17±0.10	0.19±0.06	0.18±0.03	0.20±0.05	0.17±0.07

ATCC: American type culture collection; MRSA: Methicillin-resistant Staphylococcus aureus; SA: S. *aureus*

provide the evidence of the antimicrobial activity of *H. sericea* and more specifically about its potential to prevent as well as to reduce the *S. aureus* biofilms formation.

Given such a good antibacterial activity of *H. sericea* extracts, namely its action against *S. aureus* biofilms, we can anticipate the use of these extracts for medical

purposes, as disinfectants, or for industrial purposes, as additives to prevent microbial growth. To be considered as such, these extracts cannot be cytotoxic, or its toxicity must be poor. The toxicity of *H. sericea* extracts was accessed by hemolysis assay and MTT test. The mechanical stability of the erythrocytic membrane is a good indicator of the effect of various *in vitro* damages levied on it by various compounds for screening of cytotoxicity and is dependent on their physical and structural properties.[12] The Figure 1a-c represents the percentage of hemolysis of extracts. In the case of stems and fruits extracts only for 50 mg/mL, high percentage of hemolysis was caused but it is much higher concentration than MIC value. There are no significant differences between the percentage of hemolysis at 20 and 40 min of the assay. The results for the hemolysis assay were similar to those previously published about another plant extracts.[18]

Cytotoxicity evaluation of extracts was completed with cell viability study with MTT test. MTT is a yellow, water-soluble tetrazolium salt. The reduction of MTT by the succinate dehydrogenase system of mitochondria in metabolically active cells yields a water-insoluble purple formazan product.[19] Fibroblasts (NHDF) and epithelial cancer cells (MCF-7) were used in the present study. Fibroblasts are mesenchymal cells with many vital functions during development and in adult organisms. They are among the most accessible normal mammalian cell types and are used as a model for cancer initiation and progression mechanisms.[20] In addition, to evaluate the potential anticarcinogenic properties of *H. sericea* extracts, a human breast adenocarcinoma cell line (MCF-7) was used. Figure 2a presents the relative cell viability of NHDF and MCF-7 cells incubated with stems extract, in concentrations ranging from 0-0.5 mg/mL. This extract displayed bioactivity on MCF-7 cells, decreasing cells viability by about 40%. For NHDF cells, stems extract presents no cytotoxic properties. In this sense, it can be said that this extract presents a selective toxicity because its bioactivity was only observed in MCF-7 cells, even in small concentrations. The leaves extract [Figure 2b] are able to promote NHDF cells viability, which is a different form of toxicity, and it can be considered a carcinogenic extract. However, for MCF-7 cells decreased viability was observed. The most surprisingly results were found in *H. sericea* fruits extract [Figure 2c]. This extract presented no toxicity towards NHDF cells, because its viability is not altered in the presence of this extract but for MCF-7 cells, 0.017 and 0.008 mg/mL, this extract was able to decrease mitochondrial dehydrogenases activity in about 60% as MTT test results had demonstrated,

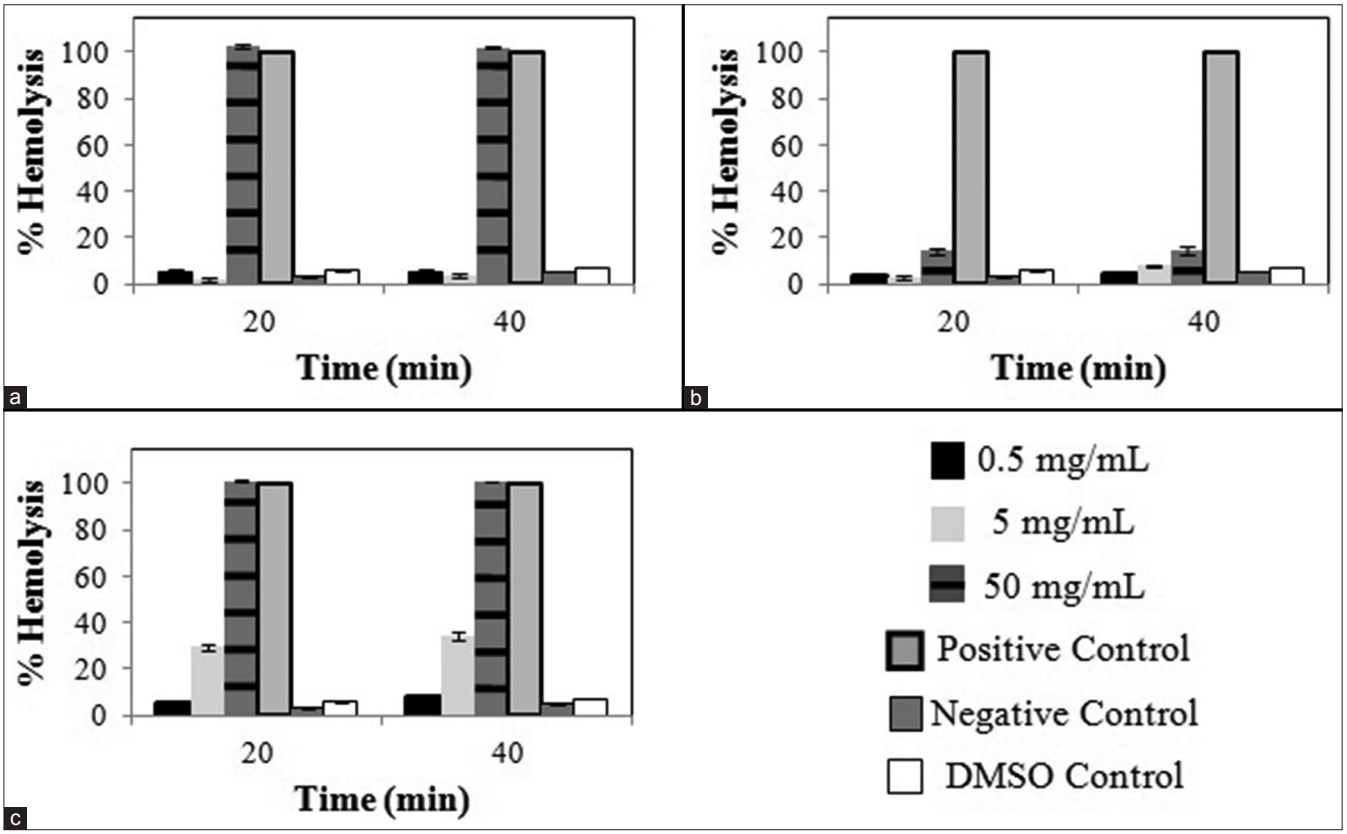

Figure 1: Percentage of hemolysis in the presence of different concentrations of extracts: (a) stems; (b) leaves; (c) fruits (mean values ± standard deviation)

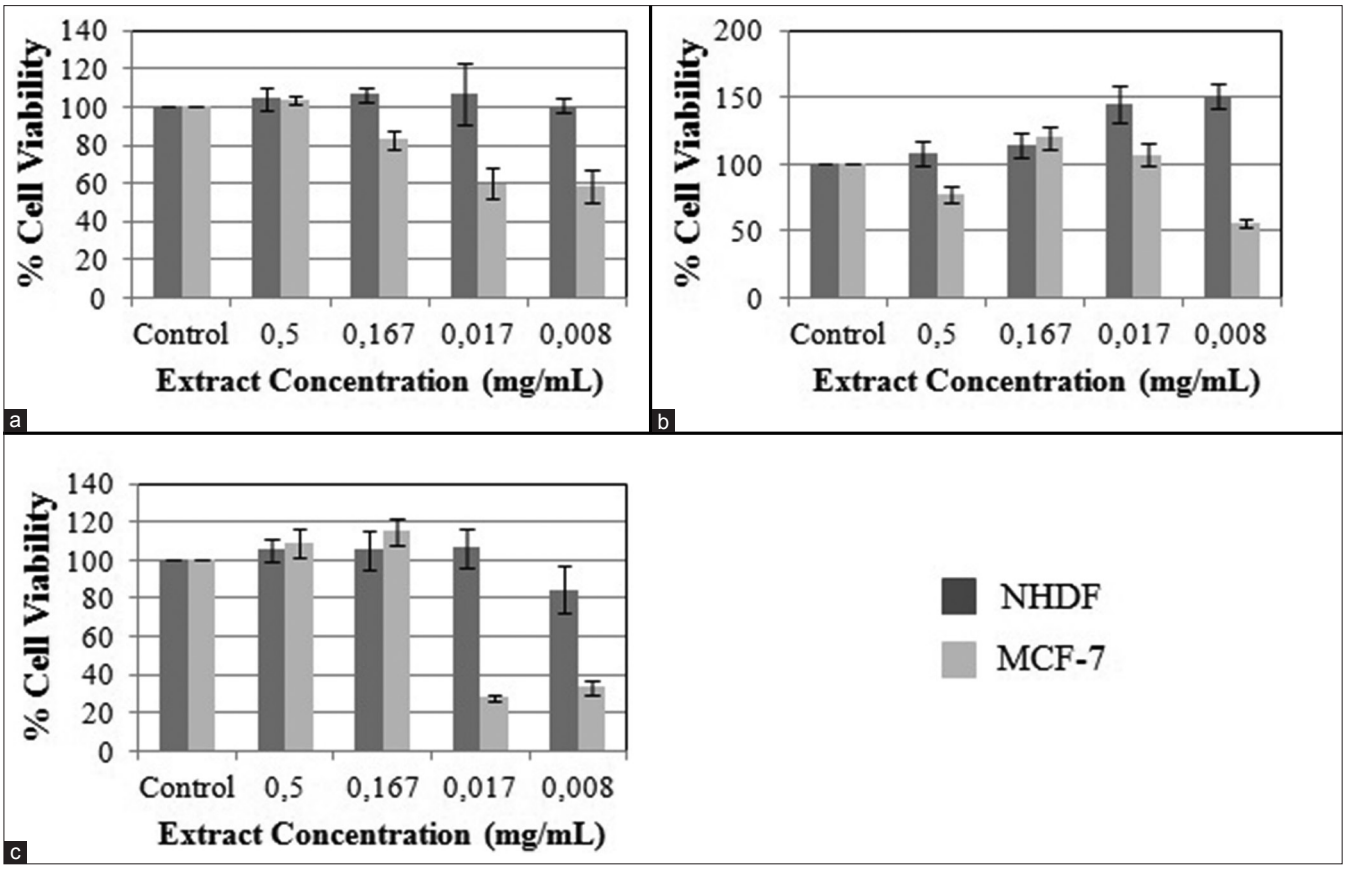

Figure 2: Relative cell viability of NHDF and MCF-7 cells incubated with extracts, in concentrations ranging from 0-0.5 mg/mL: (a) stems; (b) leaves; (c) fruits (mean values ± standard deviation)

indicating a relevant cytotoxicity towards these cancer cells. These results allow us to conclude that fruits extract probably can be used as anticarcinogenic agent. These changes in cell viability promoted by vegetal extracts were also observed by other authors.[21] In sum, *H. sericea* extracts should be considered to treat human infections because of its important biological activities and low cytotoxic effects in human cells. In future works it will be important to identify the compounds present in the extracts, responsible for the above mentioned biological activities.

CONCLUSION

In sum *H. sericea* is a potential source of bioactive compounds with antimicrobial activity, namely against several *S. aureus* strains, including clinical MRSA. The antibiofilm activity of *H. sericea* extracts in *S. aureus* biofilm formation was also demonstrated. Generally *H. sericea* presents no toxic properties, in active concentrations, against human erythrocytes. Fruits and leaves extracts presents selective toxicity decreasing MCF-7 cells viability, indicating potential anticarcinogenic properties. As far as we know this is the first report about biological activities of *H. sericea*.

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