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Research Article

Investigating the antibacterial effects and modes of action of two endophytic fungal extracts from *Annona muricata* seeds against resistant *Staphylococcus aureus*

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Abstract

Introduction and objective: Antimicrobial resistance in *Staphylococcus aureus* represents a major public health concern, highlighting the need for new antibacterial agents. Endophytic fungi associated with medicinal plants are promising sources of bioactive secondary metabolites for this purpose. This study therefore aimed to evaluate the antibacterial activity and investigate the modes of action of two ethyl acetate extracts of endophytic fungi isolated from *Annona muricata* seeds, against a resistant strain *Staphylococcus aureus* NR-46003. Methods: The broth microdilution method was used to determine the Minimum Inhibitory Concentrations (MICs) while the effects of the extracts on bacterial growth were assessed by growth kinetics. Additional assays examined cellular protein content, ethidium bromide accumulation, glucose-induced extracellular acidification and antibiofilm activity. Results: Both fungal extracts exhibited pronounced antibacterial potency, with MICs of 25 µg/mL and 12.5 µg/mL for *P. citrinum* AMs3 and *Aspergillus sp.* AMs9, respectively. Growth kinetics showed concentration-dependent inhibition, with complete suppression of visible growth at the MIC and higher concentrations over 24 h. Both extracts reduced cellular protein content, increased ethidium bromide retention, and attenuated glucose-induced extracellular acidification, findings consistent with effects on protein biosynthesis, efflux activity and proton transport. Moreover, both extracts demonstrated substantial antibiofilm activity, inhibiting biofilm formation by up to 73.63% and eradicating established biofilms by up to 64.71%. Conclusion: These findings provide compelling evidence supporting the pharmacological exploitation of *P. citrinum* AMs3 and *Aspergillus* sp. AMs9 as promising lead candidates for the development of innovative therapeutic interventions against resistant *S. aureus* infections.

Keywords: Antimicrobial resistance, *Penicillium citrinum* AMs3, *Aspergillus* sp. AMs9.

INTRODUCTION

Antimicrobial resistance (AMR), an outstanding challenge for contemporary medicine and public health has emerged as a huge threat to human health[1]. In 2021, AMR was responsible for approximately 4.71 million deaths mainly in low- and middle-income countries, accounting for about 7% of the global death toll that year and ranking among the five leading causes of death worldwide. This number is estimated to increase to up to 10 million deaths per year without effective action and can cost up to 100 trillion USD by 2050[1, 2]. This is due to prolonged hospital stays, mounting healthcare expenses and lost productivity. Furthermore, the World Health Organization (WHO) has estimated that AMR could potentially push up to 24 million people into extreme poverty by 2030[3]. In this context, *Staphylococcus aureus (S. aureus)* stands out as one of the prevalent pathogens due to its great adaptability and increasing ability to develop or acquire resistance traits to antibiotic drugs over time, to the point of being included in the Bacterial Priority Pathogens List (BPPL) by the World Health Organization[4, 5]. It is a Gram-positive bacterium belonging to the Staphylococcaceae family usually acting in a commensal symbiotic relationship with humans but can result in pathogenesis, infecting through wounds or other openings in the skin[6]. *S. aureus* is the causative agent of

a wide range of infectious diseases commonly acquired in either community or hospital settings including skin infections, bacteremia, endocarditis, pneumonia and food poisoning[7]. The organism was originally a leading nosocomial pathogen and afterwards epidemiologically distinct clones emerged in community settings. *S. aureus* expresses several virulence factors which help to establish infection by facilitating tissue attachment, tissue invasion and evading the host immune response. The ability to acquire resistance to multiple antibiotics classes makes *S. aureus*, a challenging pathogen to treat[8]. The Lancet's 2019 systematic analysis of the global burden of antimicrobial resistance highlights that *S. aureus* is the second leading pathogen associated with resistance-related deaths, with methicillin-resistant *S. aureus* (MRSA) alone responsible for over 100,000 deaths worldwide. This underscores not only the high prevalence of MRSA but also its significant contribution to mortality linked to antimicrobial resistance[9]. While the number of antibacterial agents in the clinical pipeline has risen from 80 in 2021 to 97 in 2023, the demand for new groundbreaking treatments with more efficiency and low toxicity remains critical to address severe infections and replace those losing effectiveness due to overuse[3]. This quest has cut across many paths, but the most used path remains the route from a natural product among which microorganisms particularly endophytic fungi have drawn considerable attention. They are microorganisms which reside in healthy living plant tissue without causing any visible harm to the host. Endophytic fungi produce an array of structurally and chemically diverse secondary metabolites including those that are similar to their hosts and some of them have been reported to be responsible for antimicrobial properties, including antibiotic resistant *Staphylococcus aureus*[3, 10, 11]. The medicinal plant *Annona muricata (A. muricata)*, commonly known as soursop, is widespread in tropical areas of sub-Saharan Africa where the fruits, leaves, roots, seeds, bark etc, are used to cure several diseases in countries including Cameroon[12]. Although studies on endophytic fungi associated with *A. muricata* remain scarce, the available evidence highlights their promising bioactive potential. Toghueo *et al*[13] isolated 152 endophytic fungi from *A. muricata* in Cameroon, with 27 showing antiplasmodial activity and seven exhibiting strong activity with IC_{50} values below 2 µg/mL. Similarly, Yimgang *et al*[14] found that 17 of 41 endophytic fungal extracts displayed antibacterial activity against *S. aureus* ATCC 43300, including *Penicillium citrinum* AMs3 and *Aspergillus* sp. AMs9, with MICs of 9.375 and 12.5 µg/mL, respectively. Building on these findings, this study was therefore designed to evaluate the antibacterial activity and possible modes of action of two endophytic fungal extracts isolated from *Annona muricata* in Cameroon against a resistant strain of *S. aureus*.

MATERIALS AND METHODS

Preparation of extracts and reference drugs

The ethyl acetate extracts used in this work were previously obtained from two endophytic fungi (*P. citrinum and Aspergillus sp.),* both isolated from *Annona muricata* seeds and kept at 4°C[14]. Stock solutions were prepared in 100% dimethylsulfoxide at 25 mg/ml. Ciprofloxacin and vancomycin were used as reference antibiotics, prepared at concentrations of 1 mg/mL in distilled water acidified with 0.025 N HCl and 2 mg/mL in 50% DMSO, respectively.

Bacterium, culture media, and growth conditions

S. aureus NR-46003, a reference strain isolated from the trachea of a male in the United Kingdom in 1971 obtained from Bei Resources (Biodefense and Emerging Infections Research Resources Repository, https://www.beiresources.org/) was used in this work. It was deposited as resistant to methicillin, ampicillin and penicillin; positive for *mec* (subtype IV); MLST sequence type (ST) 247; eGenomic *spa* type 4, eGenomic *spa* repeats YHFGFMBQBLO; Ridom *spa* type t051. This pathogen was maintained on slant of Mueller Hinton Agar (MHA) at 4°C in the Laboratory for Phytobiochemistry and Medicinal Plants Studies, Department of Biochemistry, University of Yaoundé I. Before each experiment, the bacterium was cultured on MHA for 24 hours at 37 °C; meanwhile, MHB was used during the other assays.

Minimum Inhibitory Concentrations determination

The Minimum Inhibitory Concentrations **(**MICs) for each extract were determined according to the broth microdilution protocol number M07-A09, described by Clinical and Laboratory Standards Institute[15]. Here, 184 µL of MHB were introduced into the first wells of the microplates and 100 µL in the remaining wells. Then, 16 µL of each extract initially prepared in an intermediate plate at 2500 µg/mL were added to the wells followed by a twofold serial dilution to give a concentration range of 100 to 1.56 µg/mL. Ciprofloxacin (0.992-0.015 µg/mL) was used as the positive control. Thereafter, one hundred microliters of an intermediate inoculum (10^6 CFU/mL) prepared from the standard 0.5 McFarland corresponding to a bacterial density of 1.5×10^8 CFU/mL were then added in test and negative control wells in order to reach a final load of 5×10^5 CFU/mL. The tests were performed simultaneously for negative control (MHB + bacteria) and sterility control (MHB alone). The plates were sealed and incubated at 37°C for 24h. After this period of incubation 20 µL of resazurin at 0.15 mg/mL were added to each well and reincubated for 1 hour. The MIC was defined as the lowest concentration at which no visible bacterial growth was observed as indicated by the absence of a color change from blue to pink. Each condition was tested in three technical replicates in two independent experiments. The activity of the extracts against the tested bacterium was classified according to the following criteria: Very good activity: (MIC ≤ 25 µg/mL); Good activity: (25 < MIC ≤ 50 µg/mL); Average activity: (50 < MIC ≤ 75 µg/mL); Weak activity: (75 < MIC ≤ 100 µg/mL); Not active (MIC >100 µg/mL).

Influence of extracts on growth kinetics

The growth-kinetics assay was performed in Mueller Hinton broth as described by Babii *et al*.[16]. Briefly, 100 µL of bacterial inoculum (10^6 CFU/mL) was added to

MHB containing the extract at 4MIC, 2MIC, MIC, MIC/2, and MIC/4, alongside an untreated control, and incubated at 37°C for 24 h. Bacterial growth was monitored by measuring optical density at 620 nm at different time points (0, 2, 4, 6, 8, 10, 12, and 24 hours) using a TECAN Infinite M 200 microplate reader (Mannedorf, Switzerland). The experiment was performed in triplicate and repeated twice. Ciprofloxacin was used as the positive control, while bacteria + MHB was used as negative control. Through the assay above, the growth curve of *S. aureus* was ordered with time as the horizontal axis (0 h-24 h), and the optical densities (OD) at 620 nm as the vertical axis.

Effect on cellular protein content

The cellular protein content of *S. aureus* after exposure to the extracts was determined using the method described by Yumsu Tcham *et al.*[17]. Subculture bacterial cells (1.5×10^6 CFU/mL) suspended in 0.9% saline were incubated with MHB containing various concentrations of the extracts (4MIC, 2MIC, MIC, MIC/2 and MIC/4) in tubes. All tubes were incubated at 37°C for 24 hours, then centrifuged at 4000 g for 5 minutes at 4°C. The resulting bacterial pellet was collected and weighed, then resuspended in 5 mL of phosphate buffer (pH 7.5) supplemented with Triton X-100. Each suspension was vortexed for 1 minute centrifuged again at 4000 g at 4° C for 10 minutes. Thereafter, Bradford reagent (500 µL) was added to 500 µL sample (50 µL supernatant + 450 sterile distilled water) and optical density of the resulting solution was measured at 595 nm within 15 min of incubation in the dark. Protein quantity of each sample was determined from the equation of the best-fit linear regression obtained from the Bovine Serum Albumin (BSA) standard curve. The untreated bacterial culture served as the control. The assay was performed twice and in triplicate.

Efflux pumps inhibitory assay

Efflux-related activity was evaluated using an ethidium bromide accumulation assay as previously described by Mehta *et al.*[18]. Briefly, extracts diluted in MHB medium at five different concentrations (4MIC, 2MIC, MIC, MIC/2 and MIC/4) were distributed into wells of a sterile 96-well microplate. Next, 20 µL of ethidium bromide solution (0.1 mM) was added to each well, followed by the addition of 80 µL of bacterial suspension to yield a final bacterial load of 10^7 CFU/mL. Quercetin (10 mg/mL) was used as the positive control, whereas the negative control consisted of culture medium, ethidium bromide (EtBr) and bacterial inoculum. Afterwards, fluorescence was measured at 5 min intervals for 60 min using a TECAN Infinite M200 plate reader (Mannedorf, Switzerland) at excitation and emission wavelengths of 530 nm and 600 nm, respectively. The assays were performed in triplicate and repeated twice. Results were expressed as relative fluorescence units at each time point during incubation.

Effect on H+-ATPase pumps

The effect of the extracts on glucose-induced extracellular acidification was evaluated as an indirect measure of proton extrusion based on the previous report of Manavathu,[19] by the follow-up of glucose-induced acidification of the medium. Bacterial strains standardized to 1.5×10^6 CFU/mL were cultured in 4 mL of MHB under constant agitation at 37°C for 24 hours. The resulting biomass was pelleted via centrifugation (3000 g; 4°C) for 10 minutes, rinsed with sterile deionized water and 50 mM KCl (pH 6.5), and spun once more before being reconstituted in 100 mL of the same KCl buffer for overnight storage at 4°C to exhaust endogenous carbohydrate stores. Following this starvation period, the cells were isolated again and resuspended in 100 mL of 50 mM KCl (pH 6.5) to restore the target titer of approximately 1.5×10^6 CFU/mL. Next, 4 mL portions of this bacterial blend were treated with extracts to obtain concentrations corresponding to 4MIC, 2MIC, MIC, MIC/2 and MIC/4, with the total mixture volume equalized to 5 mL using the KCl buffer. (50 mM, pH 6.5.) After a 10-minute conditioning phase at ambient room temperature under mild agitation, metabolic activation was induced by adding 0.5 mL of a 10% glucose solution. Extracellular pH variations were measured using an electrode every 15 mins during 60 mins. Sterile distilled water was used in place of extract for the control condition. Every trial was carried out in triplicate and validated across two independent experimental runs to plot the kinetic acidification curve: pH variation = f(t). All inhibition of the acidification of the medium in the presence of an extract was attributed to the inhibition of the proton-ATPase pumps by the latter.

Effect on biofilm formation

The effect of extracts on the biofilm formation of strain *S. aureus* NR-46003 was examined, using the microdilution method as described by Cruz *et al.*[20]. Briefly, the bacterial culture was diluted to 10^8 CFU/mL in MHB supplemented with 4% of glucose and transferred to a 96-well microtiter plate containing two-fold serial dilutions of each extract at 4MIC, 2MIC, MIC, MIC/2 and MIC/4. Vancomycin served as the positive control at 1 µg/mL, while untreated bacteria served as the negative control. After incubation at 37°C for 48 h each well of microtiter plate was washed twice with sterile distilled water, fixed by absolute methanol for 30 min, and dried at room temperature also for 30 min. After that, the wells were stained with 200 µL of 0.1% crystal violet solution for 5 min. Subsequently, the excess dye was removed by washing the plates twice with sterile distilled water supplemented with 0.9% NaCl, and then the plates were dried at room temperature for 45 minutes. Stained biofilms were dissolved in 200 µL of 33% (v/v) glacial acetic acid, and the optical densities (OD) recorded at 570 nm using TECAN plate reader (Infinite M200). The percentages (%) of inhibition were calculated (inhibition% = (OD negative control − OD sample/OD negative control) × 100).

Effect on biofilm eradication

Established biofilms were cultured as defined by Saising *et al.*[21]. Two hundred microliters of growing culture (10^8 CFU/mL) were transferred to a 96-well microtiter plate and incubated at 37°C for 48 h. Then, planktonic cells

were removed and a new medium containing extract at different concentrations was added (4MIC, 2MIC, MIC, MIC/2 and MIC/4). Vancomycin was used as positive control at 1 µg/mL and the negative control consisted of untreated bacteria. After Incubation under the same conditions as before, biofilm was quantified by using crystal violet assay and optical densities read as mentioned above in biofilm inhibition assay. Optical densities obtained were used to calculate percentages (%) of eradication (eradication% = (OD negative control − OD sample/OD negative control) × 100).

Statistical analysis

All statistics have been conducted using GraphPad Prism 8.0.2 software. Data are expressed as the mean ± SD. One-way analysis of variance (ANOVA) with Tukey's multiple comparison post hoc test was utilized to calculate the significant difference between means. $p < 0.05$ was considered statistically significant.

RESULTS

Minimum Inhibitory Concentrations

The antibacterial potential of two endophytic fungal extracts isolated from *Annona muricata* seeds was quantitatively evaluated against *Staphylococcus aureus* NR-46003 using the microdilution assay. The resulting Minimum Inhibitory Concentrations (MICs), along with those of the reference antibiotic Ciprofloxacin, are summarized in Table 1.

Table 1: Minimum Inhibitory Concentrations (MICs) of endophytic fungal extracts on *Staphylococcus aureus* NR-46003

Fungal Extracts	MIC (µg/mL ± SD)
	***S. aureus* NR-46003**
Penicillium citrinum AMs*3*	25 ± 0.00
Aspergillus sp. AMs9	12.5 ± 0.00
Ciprofloxacin	0.031 ± 0.00

AMs: *A. muricata* seed; MIC: Minimum Inhibitory Concentration.

The results indicated that based on the established classification criteria in the methodology, both endophytic fungal extracts exhibit very good activity against *Staphylococcus aureus* NR-46003, with *Aspergillus* sp. AMs9 displaying the strongest inhibition (12.5 ± 0.00 µg/mL) and two times more potent than *P. citrinum* AMs3 (25 ± 0.00 µg/mL). In comparison, Ciprofloxacin, used as reference drug showed a MIC of 0.031 ± 0.00 µg/mL.

Bacterial growth curves

The growth-kinetics assay demonstrated that both endophytic fungal extracts inhibited the growth of *Staphylococcus aureus* NR46003 in a concentration and time-dependent manner over the 24 h incubation period (Figure 1).

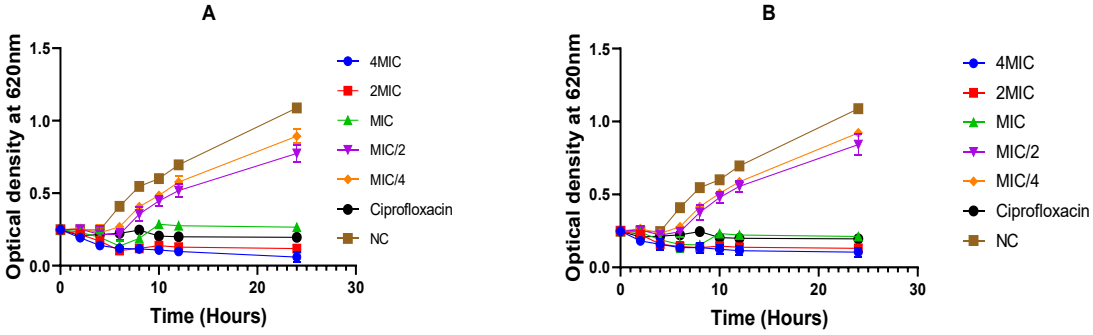


Figure 1: Effect of endophytic fungal extracts on the growth kinetics of *Staphylococcus aureus* NR-46003. (A) *Penicillium citrinum* AMs3; (B) *Aspergillus* sp. AMs9. AMs: *Annona muricata* seeds, MIC: Minimum Inhibitory Concentration, NC: Negative Control. Ciprofloxacin at MIC was used as positive control. All experiments were carried out in triplicate and data are expressed as the mean±SD.

In the untreated negative control (NC), the optical density (OD620) increased progressively from approximately 0.25 at 0 h to about 1.05 after 24 h, indicating normal bacterial adaptation, exponential growth, and entry into the stationary phase. For *P. citrinum* AMs3 (Figure 1A), treatment at the MIC (25 µg/mL), 2MIC (50 µg/mL), and 4MIC (100 µg/mL) effectively suppressed bacterial growth, with OD620 remaining nearly constant during the 24 h incubation. However, at MIC/2 (12.5 µg/mL) and MIC/4 (6.25 µg/mL), bacterial growth resumed after approximately 6–8 h, reaching OD620 values of about 0.80 and 0.95, respectively, at 24 h. A similar pattern was observed for *Aspergillus* sp. AMs9 (Figure 1B), where the MIC (12.5 µg/mL), 2MIC (25 µg/mL), and 4MIC (50 µg/mL) maintained complete growth inhibition throughout the incubation period, while MIC/2 (6.25 µg/mL) and MIC/4 (3.125 µg/mL) only delayed bacterial growth, with OD620 increasing after 4–6 h of incubation. Compared with *P. citrinum* AMs3, *Aspergillus* sp. AMs9 exhibited slightly greater inhibition at equivalent relative concentrations, particularly at the sub-MIC levels, consistent with its lower MIC value (12.5 µg/mL versus 25 µg/mL). No significant difference ($p > 0.05$) was observed between either extract and ciprofloxacin under these assay conditions at MIC, 2MIC, and 4MIC

Effect on cellular protein content

To investigate whether the antibacterial activity of the endophytic fungal extracts involves interference with protein biosynthesis in *S. aureus* NR-46003 the total cellular protein content was quantified using the bovine serum albumin (BSA) standard curve after exposition to varying concentrations of the extract as shown in Figure 2.

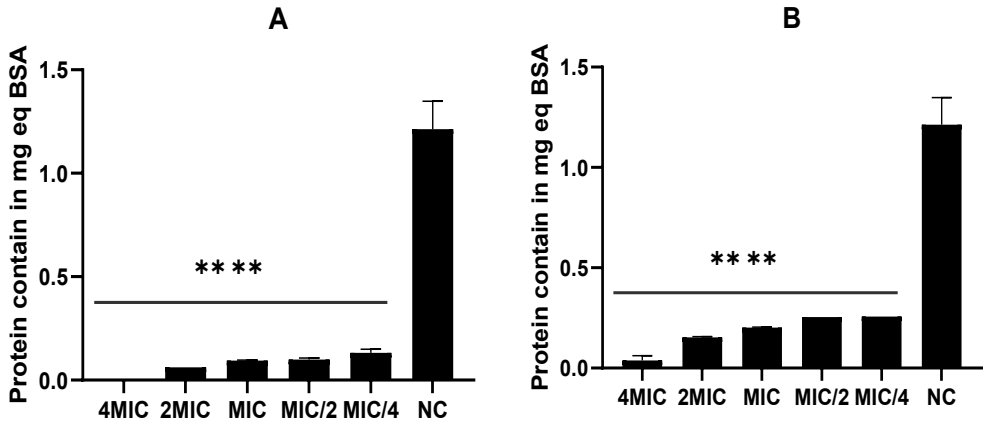


Figure 2: Effect of endophytic fungal extracts on cellular protein content of *Staphylococcus aureus* NR-46003. (A) *Penicillium citrinum* AMs3; (B) *Aspergillus* sp. AMs9. AMs: *Annona muricata* seeds, MIC: Minimum Inhibitory Concentration, NC: Negative Control. Data are expressed as the mean±SD. Significantly different from negative control **** $p < 0.0001$.

Treatment with both *Penicillium citrinum* AMs3 and *Aspergillus* sp. AMs9 markedly reduced the intracellular protein content compared with the untreated control. As the extract concentration decreased from 4MIC to MIC, protein content increased gradually, indicating that inhibition of protein synthesis is concentration dependent. The lowest measured protein content (0 mg eq BSA) was observed at 4MIC with *P. citrinum* AMs3 followed by 2MIC. Although protein levels remained lower at MIC, MIC/2, and MIC/4 than in the untreated control. Similarly, *Aspergillus* sp. AMs9 at the highest concentration tested produced the strongest inhibitory effect (0.170 mg eq BSA) with protein content significantly reduced compared with the negative control ($p < 0.0001$) but still remaining lower than that the effect of *P. citrinum* AMs3 at the same concentration. From 2MIC (25 µg/mL) to MIC/4 (3.125 µg/mL), *Aspergillus* sp. AMs9 exhibited a progressive reduction of protein content between MIC/2 and MIC/4, indicating sustained inhibition of protein production across all tested concentrations.

Effect on ethidium bacterial efflux pumps

The efflux pump inhibition activity of the extracts against *S. aureus* NR-46003 (Figure 3) was measured every 5 min as relative fluorescence over the negative control and compared to the positive control (Quercetin).

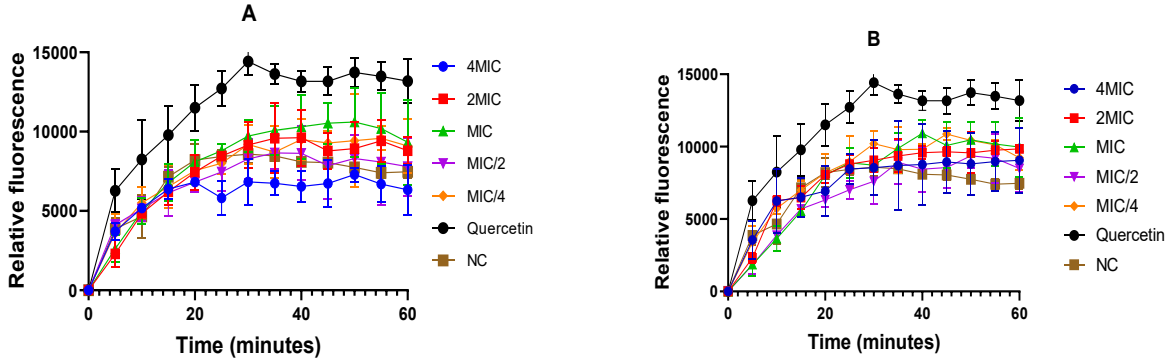


Figure 3: Effect of endophytic fungal extracts on ethidium bromide accumulation on *Staphylococcus aureus* NR-46003. (A) *Penicillium citrinum* AMs3; (B) *Aspergillus* sp. AMs9. AMs: *Annona muricata* seeds, MIC: Minimum Inhibitory Concentration, NC: Negative Control. Quercetin at 10 mg/ml was used as positive control. All experiments were carried out in triplicate and data are expressed as the mean±SD.

Ethidium bromide is a fluorophore that fluoresces weakly in aqueous media but fluoresces strongly upon intercalation into intracellular nucleic acids. In an efflux pump assay, an increase in relative fluorescence intensity directly reflects increased intracellular retention of EtBr resulting from the inhibition of active

bacterial efflux systems. Globally both extracts induced a time-dependent increase in EtBr fluorescence across MIC and subinhibitory (MIC/2 and MIC/4) tested concentrations reaching maximal accumulation near 30 minutes (10000-11000 RFU). These values were maintained higher that of the negative control suggesting that secondary metabolites in *P. citrinum* AMs3 and *Aspergillus* sp. AMs9 effectively impair active efflux mechanisms in *S. aureus* at these concentrations. However, this activity remained lower than that of the reference flavonoid Quercetin, a well-documented efflux pump inhibitor, which exhibited the most pronounced fluorescence signal, confirming effective inhibition of EtBr efflux. Conversely, at suprainhibitory concentrations (4MIC and 2 MIC), a decline in relative fluorescence was recorded for both extracts reaching peak levels of approximately 6500-7500 RFU.

Effect on proton-ATPase pumps

To evaluate the effect of endophytic fungal extracts derived from *Annona muricata* seeds (*P.citrinum AMs3* and *Aspergillus* sp. AMs9) on proton-ATPase (H^+ - ATPase) pumps in *Staphylococcus aureus* NR-46003, the extracellular acidification (measured as pH variation) was monitored over 60 minutes and results presented in Figure 4 below:

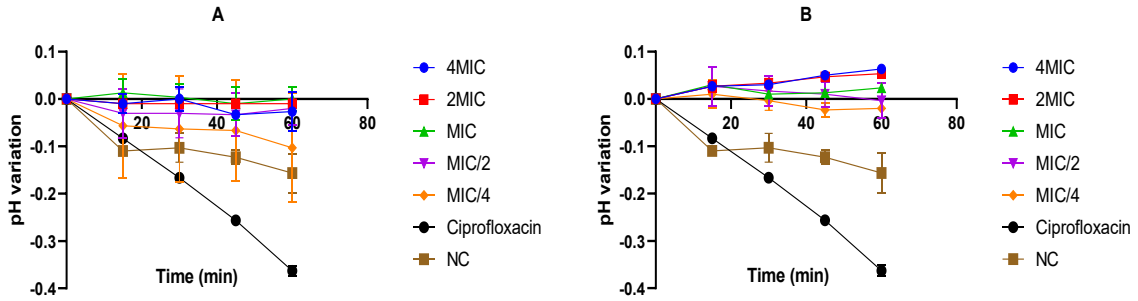


Figure 4: Effect of endophytic fungal extracts on H+-ATPase pumps of *Staphylococcus aureus* NR-46003. (A) *Penicillium citrinum* AMs3; (B) *Aspergillus* sp. AMs9. AMs: *Annona muricata* seeds, MIC: Minimum Inhibitory Concentration, NC: Negative Control. Ciprofloxacin at MIC was used as positive control. All experiments were carried out in triplicate and data are expressed as the mean±SD.

Globally, a gradual increase in the pH variation was observed over time with the two extracts reflecting a rise in the pH of the reaction medium compared to the negative control group curve which variation of pH decrease with time. Concentrations of *P. citrinum AMs3* tested on the whole allowed partial acidification materialized by almost a linear tendency of the corresponding curves. For *Aspergillus* sp. AMs9 this augmentation is greater with 4MIC, 2MIC and MIC starting at the thirtieth minute until the end of incubation. The reduced acidification observed in treated cultures is consistent with impaired proton extrusion. Overall, *Aspergillus* sp. AMs9 appeared to produce a slightly greater stabilizing effect than *P. citrinum* AMs3.

Effect on biofilm formation and eradication

The potential of the extracts to prevent biofilm and disrupt mature biofilms was evaluated using the crystal violet assay after exposure of *S. aureus* NR-46003 to five concentrations (4MIC, 2MIC, MIC, MIC/2, MIC/4) and results are presented in the figure 5 below:

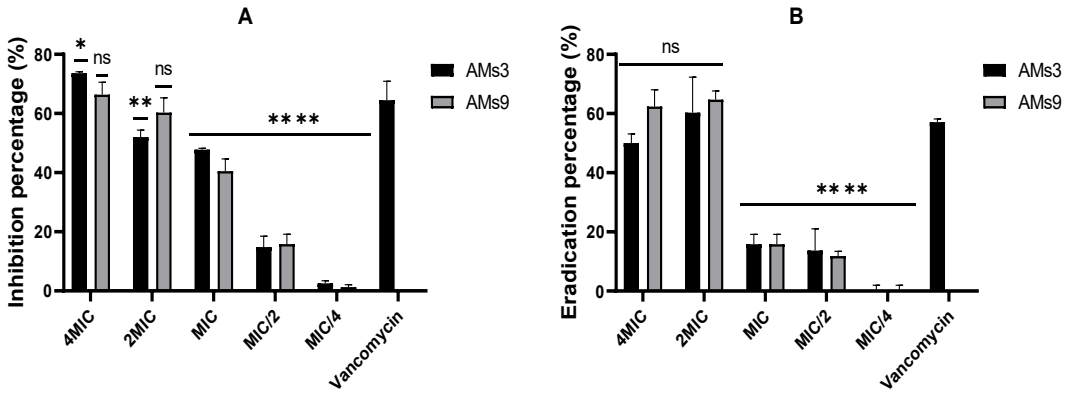


Figure 5: Inhibition of biofilm formation (A) and biofilm eradication (B) by endophytic extracts at various concentrations, MIC: Minimum Inhibitory Concentration. Vancomycin at MIC was used as positive control. All experiments were carried out in triplicate and data are expressed as the mean±SD. Significantly different from the positive control (Vancomycin at 1ug/ml), * $p < 0.05$, ** $p < 0.01$,**** $p < 0.0001$ and ns: non-significant with $p > 0.05$.

The inhibition percentages ranged from 73.63 – 1.28 % and 64.71–0.71 % for eradication. Regarding biofilm inhibition (Figure 5A), peak activity was observed at 4MIC with *P. citrinum* AMs3 (73.63 %) and this activity was statistically higher ($p < 0.05$) than that of the positive control vancomycin (64.48%). This trend reversed at 2MIC where *Aspergillus* sp. AMs9 (60.19 %) showed potent inhibition compared to *P. citrinum* AMs3 (52.02 %). However, there is no significant difference between the inhibitions at MIC, MIC/2 and MIC/4 for both extracts.

In the biofilm eradication assay (Figure 5B), eradication percentages were highest at 2MIC (60.26 and 64.71 % respectively for *P. citrinum* AMs3 and *Aspergillus* sp. AMs9) and declined progressively with decreasing concentrations for both extracts. No significant difference was observed between the extracts at 4MIC and 2MIC, whereas eradication at MIC and subinhibitory concentrations was significantly lower than at higher concentrations ($p < 0.0001$). Overall, the findings indicate that both endophytic extracts possess stronger antibiofilm formation activity than biofilm eradication activity, with efficacy increasing generally with extract concentration. Although, the response was not strict concentration-dependent at all concentrations.

DISCUSSION

The emergence and rapid dissemination of antimicrobial resistance have substantially compromised the effectiveness of conventional antibiotics, making resistant *S. aureus* one of the most challenging bacterial pathogens in clinical practice[4, 9]. The remarkable adaptability of this organism, coupled with its ability to acquire resistance determinants, regulate virulence gene expression, and establish persistent biofilms, considerably limits available therapeutic options[5]. Consequently, there is growing interest in natural products particularly metabolites produced by endophytic fungi because of their notable capacity to biosynthesize structurally diverse secondary metabolites with broad-spectrum biological activities, including antibacterial compounds with novel targets[1, 22]. Accordingly, the present investigation was designed to comprehensively assess the antibacterial potential of ethyl acetate extracts derived from the endophytic fungi *P. citrinum* AMs3 and *Aspergillus* sp. AMs9 isolated from *A. muricata* seeds with particular emphasis on elucidating the modes of action responsible for their inhibitory activity against resistant *S. aureus* NR-46003. Extracts from *P. citrinum* AMs3 and *Aspergillus* sp. AMs9 exhibited potent antibacterial activity against the bacterium used, with MICs values of 25 and 12.5 µg/mL, respectively, as determined using the broth microdilution method. The greater potency displayed by *Aspergillus* sp. AMs9 may be attributed to differences in the composition and abundance of bioactive metabolites synthesized by *Aspergillus* species, which are recognized producers of polyketides, alkaloids, terpenoids, xanthones, and other classes of compounds capable of inhibiting bacteria[10, 23]. Gomaa *et al.*[24] also reported that the crude extract of *Aspergillus* sp. associated with *Apium graveolens* seeds exhibited strong anti-MRSA activity (IC_{50} = 3.5 µg/mL), outperforming ciprofloxacin (IC_{50} = 25.7 µg/mL). Likewise, previous investigators have gotten similar results on other strains of *S. aureus* with endophytic fungi from the same plant therefore reinforcing these findings[14, 25]. Thereafter, both extracts tested for time-dependent growth exhibited bactericidal activity, as demonstrated by the reduction in optical density to near baseline levels over 24 h with increasing concentrations. Bactericidal agents eliminate bacterial cells by causing irreversible damage to essential cellular structures and processes thus playing an essential role in controlling bacterial infections[26]. The reduction in cellular protein content observed after treatment with *P. citrinum* AMs3 and *Aspergillus* sp. AMs9 extracts indicates that these fungal metabolites interfere with protein biosynthesis in *S. aureus* NR-46003 in comparison to the control without treatment. The importance of this observation is underscored by the fact that protein biosynthesis constitutes a fundamental physiological process required for bacterial growth, maintenance of cellular integrity, enzymatic activity, and adaptive responses to environmental challenges. Consequently, disruption of this process severely compromises bacterial metabolism and ultimately leads to growth inhibition and cell death[26, 27]. Similar targeting mechanisms have been described with pestalotiopyrone G a compound from endophytic fungi *Pestalotiopsis sydowiana*, that exhibit proteasome inhibitory activity, therefore interfering with the protein degradation process and contributes to the accumulation of misfolded proteins. It essentially alters the cellular protein quality-control system, thereby destabilizing pathogen viability through an indirect mechanism[28]. These observations support the fact that endophytic fungi have long been viewed as a potential source of secondary metabolites possessing diverse biological activities, such as targeting protein synthesis in pathogens[29]. Secondary metabolites from natural products can affect bacterial cells by altering membrane permeability and inhibiting efflux pumps. It constitute one of the principal determinants of multidrug resistance in *S. aureus*, actively expelling a broad spectrum of structurally unrelated antimicrobial agents from the bacterial cytoplasm, thereby reducing their intracellular concentrations below bactericidal level[30]. The ethidium bromide (EtBr) accumulation assay further demonstrated that both extracts interfere with bacterial efflux mechanisms gradually at MIC, MIC/2 and MIC/4. This inhibition may result from the direct interaction of fungal secondary metabolites with efflux pumps proteins or indirectly through disruption of the proton motive force that energizes several Major Facilitator Superfamily (MFS) transporters in *S. aureus.* Consequently, inhibition of efflux activity promotes the intracellular accumulation of toxic compounds and enhances bacterial susceptibility to antimicrobial agents[30, 31]. Endophytic fungi have been discovered as a potential source of bioactive compounds that can effectively inhibit efflux pumps, which would reverse antibiotic efficacy[32]. Specifically, Singh *et al.*[33] reported that α,β-dehydrocurvularin, a metabolite isolated from the endophytic fungus *Curvularia geniculata*, exhibited efflux-pump inhibitory activity against MRSA strains.

However, in this study, a decrease in relative fluorescence was observed at higher concentrations (2MIC and 4MIC). This reduction should not necessarily be interpreted as a loss of efflux pump inhibition. At supra-inhibitory concentrations, the strong antibacterial effect of *P. citrinum* AMs3 and *Aspergillus* sp. AMs9 may cause severe cellular damage and reduce bacterial viability. Since EtBr accumulation requires metabolically active cells, extensive physiological disruption may decrease fluorescence intensity despite effective efflux pump inhibition[34]. Proton-ATPase pumps play varying roles in bacterial cells especially in *S. aureus* where they maintain intracellular pH homeostasis by extruding excess protons and generate the proton motive force required for ATP production, nutrient transport, and other energy-dependent cellular processes[35]. Evaluation of the effect of the two extracts of endophytic fungi used on these pumps indicated that *P. citrinum* AMs3 induced a relatively moderate and almost linear reduction in acidification across the tested concentrations while *Aspergillus* sp. AMs9 exhibited a more pronounced effect, particularly at 4MIC, 2MIC, and MIC after approximately 30 minutes of incubation, indicating stronger suppression of proton extrusion. Inhibition of this enzyme suggests that the extracts may disrupt proton homeostasis and the proton motive force, reducing cellular metabolism, energy production, and ultimately bacterial survival[36]. Our results suggest that these pumps are possible targets of these extracts. Forming bacterial biofilms is one of the survival strategies of *S. aureus* to tolerate antimicrobial agents and other external stress by interfering with the penetration of antimicrobials into biofilm. They are composed of extracellular polymeric substances secreted by adherent bacterial cells, which create a protective matrix that restricts antimicrobial penetration, whereas the metabolically inactive and dormant nature of biofilm-associated bacteria further contributes to their greater resistance than planktonic cells[37]. In the study, the inhibitory activity of the biofilm formation and the disruption potential of mature biofilms of *P. citrinum* AMs3 and *Aspergillus sp*. against *S.aureus* NR-46003 were investigated. The two extracts exhibited potent antibiofilm activity by preventing biofilm formation and, to a lesser extent, disrupting established biofilms. Biofilm inhibition reached 73.63% for *P. citrinum* AMs3 and 66.38% for *Aspergillus* sp. AMs9 at 4MIC, with the activity of *P. citrinum* significantly exceeding that of the reference antibiotic vancomycin. Conversely, eradication of mature biofilms did not exceed 50.04% and 62.33% for *P. citrinum* AMs3 and *Aspergillus* sp. AMs9, respectively at the highest concentration 4MIC. This difference indicates that the fungal extracts are more effective at preventing biofilm establishment than eliminating mature biofilms, a phenomenon commonly reported by several authors[20, 21]. Moreover, the findings of the present study corroborate previous reports highlighting the antibiofilm potential of endophytic fungal extracts against *S. aureus* such as that by Fathallah *et al*.[38], in which an EtOAc extract of an endophytic fungus, *Aspergillus amstelodami*, completely eradicated *S. aureus* ATCC 25923 biofilm at concentrations > 62.5 µg/mL . Our previous investigation also demonstrated that an EtOAc extract of *Fusarium waltergamsii* AMtw3 isolated from *Annona muricata* from Cameroon had the ability to strongly inhibit *S.aureus* ATCC 43300 biofilm formation with an IC_{50} value of 0.3 µg/mL[14]. The antibiofilm activity of natural products is mainly based on suppression of cell adhesion and attachment, inhibition of polymer matrix formation, and decreasing virulence factor production, thus blocking the quorum sensing network and biofilm development[39, 40]. The extracts might have exerted their antibiofilm activity using one of these mechanisms of action.

CONCLUSION

The present investigation underscores the significance of endophytic fungi associated with *Annona muricata* seeds as a promising reservoir of bioactive metabolites exhibiting substantial antibacterial potential against resistant *S. aureus*. Extracts derived from *P. citrinum* AMs3 and *Aspergillus* sp. AMs9 demonstrated strong antibacterial activity associated with multiple cellular disturbances, including suppression of bacterial proliferation, inhibition of protein biosynthesis, disruption of efflux-mediated resistance mechanisms, interference with proton homeostasis, and reduction of biofilm formation and persistence. These findings emphasize the biotechnological relevance of *A. muricata* endophytic fungi as a valuable source of innovative antimicrobial candidates. Further investigations focusing on bioassay-guided isolation, structural elucidation, molecular target identification, and pharmacological validation are warranted to facilitate the development of these metabolites into potential therapeutic agents.

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CONFLICT OF INTEREST:

The authors declare that there are no conflicts of interest associated with this work.

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