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Phytochemical Composition, Antioxidant, Antimicrobial, Antibiofilm, and Antiquorum Sensing Potential of Methanol Extract and Essential Oil from *Acanthus polystachyus* Delile (Acanthaceae)

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Cite This: *ACS Omega* 2023, 8, 43024−43036

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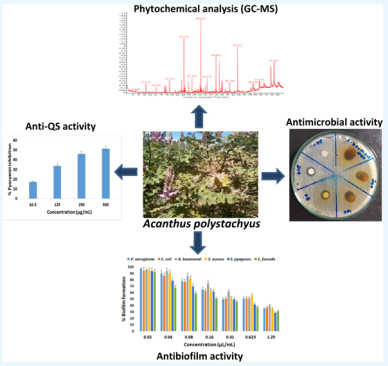
ABSTRACT: The evolution of microbes in response to conventional antimicrobials leads to antimicrobial resistance (AMR) and multidrug resistance (MDR), and it is a global threat to public health. Natural products are possible solutions to this massive challenge. In this study, the potential of *Acanthus polystachyus* extracts was investigated for phytochemical composition and biological properties as antimicrobials. Gas chromatography-mass spectra (GC-MS) analysis of methanol extract (ME) and essential oil (EO) detected 79 and 20 compounds, respectively. The major compounds identified in ME and their abundance were *β*-sitosterol acetate (16.06%), cholest-5-en-3-yl (9*Z*)-9-octadecenoate (9.54%), 1-dodecanol (7.57%), (*S*)-(*E*)-(−)-4-acetoxy-1-phenyl-2-dodecen-1-one (6.03%), neophytadiene (5.7%), (*E*)-2-nonadecene (3.9%), hexanol-4-D2 (2.92%), and decane (2.4%). Most compounds have known bioactive functions. In EO, the major compounds were stearyl alcohol (25.38%); *cis*-9-tetradecenoic acid, isobutyl ester (22.95%); butyl 9-tetradecenoate (10.62%); 11,13-dimethyl-12-tetradecen-1-ol acetate (10.14%); ginsenol (3.48%); and diisooctyl phthalate (2.54%). All compounds are known to be bioactive. The antioxidant activity of ME and EO ranged from 48.3 to 84.2% radical scavenging activity (RSA) and 45.6 to 82% RSA, respectively, with dose dependency. The disc diffusion assay for the antimicrobial activity of ME revealed high inhibition against *Acenetobacter baumannii* (130.2%), *Pseudomonas aeruginosa* (100.3%), and *Staphylococcus aureus* (87.7%). The MIC, MBC/MFC, and MBIC values for ME were 0.5−1.0, 2−4, and 0.5−1.0 mg/mL and for EO were 0.31−0.62, 1.25−2.5, and 0.31−0.62 *μ*L/mL, respectively, indicating inhibition potential as well as inhibition of biofilm formation. The tolerance test values indicated bactericidal activity against most strains and bacteriostatic/fungistatic activity against *A. baumannii*, *E. faecalis*, and *C. albicans*. The antiquorum sensing activity of ME achieved by pyocyanin inhibition assay on *P. aeruginosa* showed a 51.6% inhibition at 500 *μ*g/mL. These results suggest that ME and EO derived from *A. polystachyus* leaves are potent, valuable, cost-effective antioxidants and antimicrobials. Both extracts may effectively combat pathogenic and resistant microbes.



1. INTRODUCTION

In modern medicine, antimicrobial treatment is one of the main approaches, which is used to combat infectious diseases caused by pathogenic microorganisms.[1] However, the massive emergence and re-emergence of resistance against conventional antimicrobials pose a serious global threat to treating infections, which is of growing concern to human, animal, and environmental health.[2] In 2017, the World Health Organization (WHO) recognized antimicrobial resistance (AMR) as a major threat to global health in response to the massive increase in populations of multidrug-resistant strains.[3,4] The plausible causes of AMR include the excessive use of antibiotics in animals and humans, easy accessibility to antibiotics (over-the-counter), increased international travel, poor sanitation, and release of nonmetabolized antibiotics or their residues into the environment through manure or feces.[3] These factors contribute to genetic selection pressure for the emergence of multidrug resistance (MDR) microbes.[2] MDR and ADR give rise to infections such as hospital-acquired, urinary tract infections (UTIs), ulcerative skin, lungs, ears, eyes, and catheters leading to increased medical costs, morbidity, and mortality. Several clinically relevant bacteria such as *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA), *Acinetobacter* spp., *Enterobacter* spp., *Proteus* spp., and others cause infections, AMR, and MDR.[5,6] *S. aureus* is considered the most notorious "superbug" (e.g., MRSA, CA-MRSA). It is a nasal commensal of humans and

Received: August 22, 2023
Revised: September 20, 2023
Accepted: October 12, 2023
Published: October 31, 2023

can cause skin infections.[7] *A. baumannii* and *P. aeruginosa* are MDR opportunistic pathogens that may grow in niches with high antibiotic pressure where several other bacteria may not survive.[8] AMR and MDR patterns in Gram-negative and Gram-positive bacteria give rise to infections that are either difficult to treat or impossible to cure with conventional antimicrobials.[2,5]

The formation of biofilm and quorum sensing are the significant factors contributing to AMR and MDR.[9,10] A biofilm is a collection of microorganisms that adhere to a surface and create a matrix of extracellular macromolecular substances composed of microorganisms and extracellular polymeric substances (EPS).[10,11] EPS are organic polymers involved in bacterial interaction with their environment and mainly comprised of polysaccharides, proteins, extracellular DNA (eDNA), and lipids.[12] Both Gram-positive and Gram-negative bacteria are able to form biofilms. The formation of biofilms occurs in two stages: the planktonic stage and the adherent stage. Biofilms are highly resistant to the immune system of the host and antibacterial agents. Microbes inside biofilms can withstand 10−1000 times higher concentrations of antibiotics than cells in their plankton form.[13] Antibiotic treatment can eliminate planktonic cells; however, it is difficult to treat biofilms.[14] The increased dosage required to treat biofilm-forming microbes directly translates into an increased cost of treatment. Biofilm-related MDR significantly impacts hospital settings and the emergence of MDR. Biofilms are currently estimated to be responsible for more than 65% of nosocomial infections and 80% of all microbial infections.[15] Various mechanisms have been recognized for antimicrobial resistance, by the formation of biofilms. Most notably, reduced permeability to antibiotics by the formation of barrier, detoxification mechanism that produces enzymes to render the antibiotic inactive by disrupting or altering the antibiotic structure, reduction of intracellular concentration of antibiotics by drug efflux pumps, and drug sequestration by specific proteins that prevent the binding of antibiotics to their targets have been well documented.[10,16−18] Quorum sensing (QS), an important cell−cell communication system, plays essential roles in regulating biofilm formation, virulence gene expression, drug efflux pumps, and plasmid transfer.[9,16] The communication between cells in QS-regulated systems is carried out by the production of QS signals in the form of diffusible autoinducers (molecules), such as *N*-acylhomoserine lactone (AHL) in Gram-negative bacteria and autoinducing peptide (AIP) in Gram-positive bacteria.[9] These signal molecules function via interaction with specific enzymes and receptor−activator proteins. For example, many Gram-negative bacteria use similar LuxI-type synthases and LuxR-type activator proteins.[17,19] Therefore, developing antibiofilm agents and anti-QS inhibitors is crucial for developing antimicrobials.

The discovery and development of antibiotics during the "golden period" (1930−1960s) have saved millions of lives;[1] however, the difficulty to maintain the pace of antibiotic discovery with the emerging and re-emerging resistant pathogens has mounted the current massive challenge to contain and treat infections.[6,17] Therefore, there is an urgent need to search for and discover novel alternate treatment strategies to combat AMR and MDR. Several approaches have been developed including antibodies, vaccines, antimicrobial peptides (AMPs), probiotics, plant natural products, and nanobiotechnology.[2,20] Natural products derived from plants and microbes have received particular attention.[21] The natural products in different parts of plants are comprised of phenols, tannins, flavonoids, saponins, terpenoids, essential oils, alkaloids, steroids, lectins, polypeptides, etc. These molecules are medicinally bioactive and exert antimicrobial action via different mechanisms that target various cellular processes. Tannins are involved in the inhibition of cell wall synthesis,[22] flavonoids complex with extracellular and soluble proteins and cell wall leading to antimicrobial activity,[23] saponins are responsible for leakage of proteins and enzymes from the cell,[24] terpenoids weaken the membranous tissue leading to dissolution of the cell walls,[25] fatty acid esters in essential oils possess antibacterial properties,[26] steroids associate with membrane lipids and cause leakage from liposomes to show bactericidal action,[27] and several peptides are also known to be efficient antimicrobials.[21] Therefore, plant-derived natural products provide a wide array of compounds that may facilitate reducing and eradicating the load of pathogenic bacterial populations for treating infections.[21]

In this study, we focused on *Acanthus polystachyus* Delile to prepare leaf extracts and evaluate their biological properties. *A. polystachyus* Delile is a shrub belonging to the family Acanthaceae and native to Burundi, Ethiopia, Kenya, Rwanda, Sudan, Tanzania, and Uganda. This species grows at medium to high altitudes (1000−3200 m) in different parts of Ethiopia and is characterized by pink flowers and soft, hairy leaves.[28] The Acanthaceae family is pharmacologically important for antifungal, anti-inflammatory, antipyretic, antioxidant, antiviral, antimalarial, insecticidal, hepatoprotective, immunomodulatory, and antiplatelet activities.[29,30] *A. polystachyus* Delile has been investigated for antimalarial and wound healing activities;[28,31] however, there is only one report regarding its antimicrobial potential.[32] To the best of our knowledge, the inhibition of biofilm formation and quorum sensing by this plant species has yet to be investigated. Furthermore, a detailed analysis of the composition of extracts of this important medicinal plant has yet to be elucidated. The knowledge and understanding of the constituents of extracts are essential to elucidating the mechanisms of action. In most reported studies, solvents such as methanol, water, and acetone have been used for extraction from leaves and roots. Essential oils (EOs) have excellent therapeutic potential; however, the EO of this flowering plant species has yet to be explored. Therefore, in the present study, we prepared methanol extract (ME) and EO from the leaves of *A. polystachyus* Delile and investigated the qualitative (biochemical methods) and quantitative (gas chromatography-mass spectra (GC-MS)) phytochemical composition and evaluated the potential of the extracts for antioxidant, antibacterial, antibiofilm, and antiquorum sensing properties. We expect that the results presented here will appreciate the medicinal potential of *A. polystachyus* Delile and contribute toward combating pathogenic and resistant bacteria responsible for causing fatal diseases.

2. RESULTS AND DISCUSSION

A. polystachyus is traditionally used for various medicinal purposes and has recently been shown to be effective in treating malaria and wounds.[28,31] It also possesses antibacterial properties.[32] It has been observed and reported that the chemical constituents of plant species may vary depending on the environmental conditions, places where it grows, soil structure, and so on.[33] Therefore, the identification and analysis of phytochemical components aid in understanding of

https://doi.org/10.1021/acsomega.3c06246
ACS Omega 2023, 8, 43024−43036

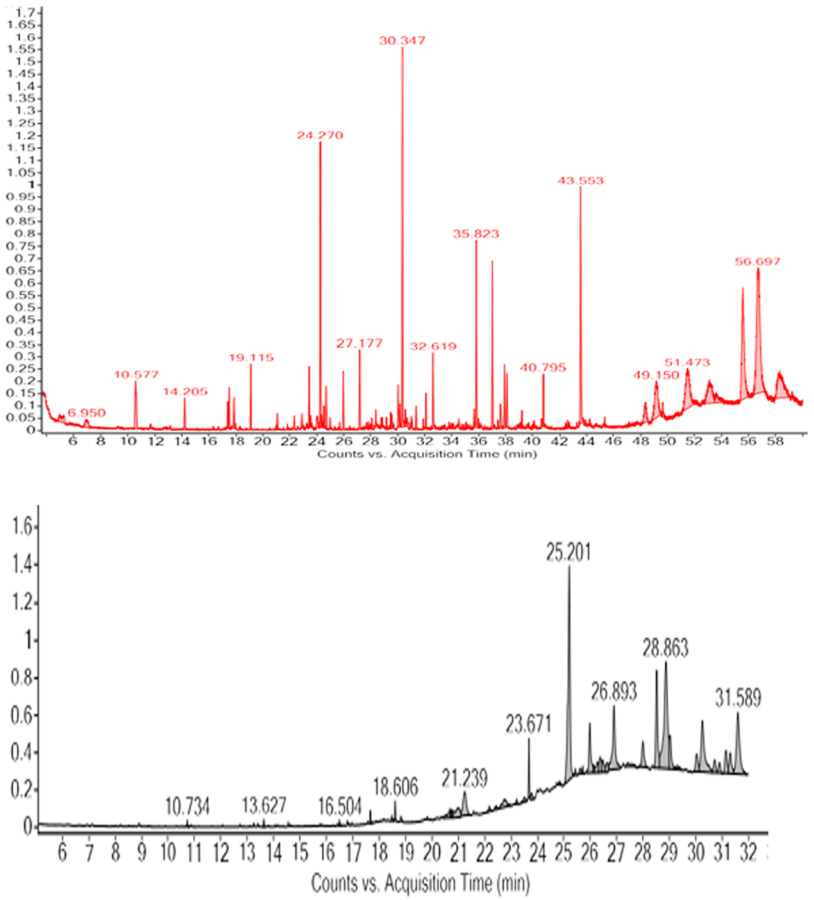


Figure 1. GC-MS chromatographic profile of ME (red) and EO (black) of *A. polystachyus*. The peaks indicate the major compounds, and the numbers above the peaks depict the retention time. The *y*-axis is for "intensity".

Table 1. Major Compounds Identified from the ME of *A. polystachyus* by GC-MS Analysis[a]

no.	formula	RT	compound name	amount (%)	chemical class
1	$C_{31}H_{52}O_2$	56.697	*β*-sitosterol acetate	16.06	sterol lipid
2	$C_{45}H_{78}O_2$	55.564	cholest-5-en-3-yl (9*Z*)-9-octadecenoate	9.54	ester
3	$C_{12}H_{26}O$	30.347	1-dodecanol	7.57	alcohol
4	$C_{20}H_{28}O_3$	51.473	(*S*)-(*E*)-(−)-4-Acetoxy-1-phenyl-2-dodecen-1-one	6.03	ketone
5	$C_{20}H_{38}$	43.553	neophytadiene	5.70	sesquiterpenoid
6	$C_{19}H_{38}$	35.823	(*E*)-2-nonadecene	3.90	alkene
7	$C_6H_{12}D2O$	3.167	hexanol-4-D2	2.92	alcohol
8	$C_{10}H_{22}$	10.577	decane	2.40	alkane

[a]RT, retention time; percent amount was calculated from the peak area.

the involvement of natural chemical constituents and their mechanisms.[34] In this study, we performed GC-MS analysis of ME and EO's phytochemical composition and various biological properties, such as antioxidation, antibacterial, antibiofilm, and antiquorum sensing.

2.1. Phytochemical Analysis (Qualitative and Quantitative). The ME yield was found to be 20.5%, and the yield from EO was recorded at 0.4%, which agrees with the yields observed from several different plant leaves. The qualitative analysis of ME based on biochemical assays indicated the presence of alkaloids, flavonoids, saponins, tannins, phenols, steroids, and terpenoids; however, glycosides were absent (Table S1). The UV spectra recorded in the range of 200−400 nm for EO indicated the presence of terpenes, phenols, flavonoids, coumarins, and alkaloids. Terpenes have a UV absorption maximum in the 210−220 nm range because of the conjugated pi systems; phenols have a UV absorption maximum in the 270−280 nm range due to the presence of aromatic rings; flavonoids have a UV absorption maximum in the 290−300 nm range due to the hydroxyl groups; coumarins have a UV absorption maximum in the 310−320 nm range due to the presence of a coumarin ring; and alkaloids have a UV absorption maximum in the 330−340 nm range due to the presence of nitrogen atoms.[35]

GC-MS is a helpful technique for the compositional analysis of phytochemicals in extracts. In the case of ME, GC-MS analysis was performed for 60 min that detected 79 compounds, while the runtime for EO was 33 min, and a total of 20 compounds was obtained. Some of the compounds appeared twice or more as multiple peaks in ME and EO, while a stationary phase was observed after 29 min in the case of EO.

https://doi.org/10.1021/acsomega.3c06246
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Table 2. Major Compounds Identified from the EO of *A. polystachyus* by GC-MS Analysis[a]

no.	formula	RT	compound name	amount (%)	chemical class
1	$C_{18}H_{38}O$	25.201	stearyl alcohol P721	25.38	saturated fatty acid
2	$C_{18}H_{34}O_2$	28.863	*cis*-9-tetradecenoic acid, isobutyl ester	22.95	fatty acid ester
3	$C_{18}H_{34}O_2$	28.511	butyl 9-tetradecenoate	10.62	ester
4	$C_{18}H_{34}O_2$	26.893	11,13-dimethyl-12-tetradecen-1-ol acetate	10.14	alcohol
5	$C_{15}H_{26}O$	27.991	ginsenol	3.48	sesquiterpenoid
6	$C_{24}H_{38}O_4$	23.671	diisooctyl phthalate @P1404	2.54	benzoic acid ester

[a]RT, retention time; percent amount was calculated from the peak area.

The chromatographic profiles for the extracts are shown in Figure 1.

The major compounds were identified based on the intensity of peaks that were generated in GC-MS chromatograms (Figure 1) and are summarized in Tables 1 (ME) and 2 (EO) (for the complete list of compounds, please see Tables S2 and S3). The peak areas were used to calculate the percent occurrence (amount) of the individual compounds. The major compounds found in ME were *β*-sitosterol acetate (16.06%), cholest-5-en-3-yl (9*Z*)-9-octadecenoate (9.54%), 1-dodecanol (7.57%), (*S*)-(*E*)-(−)-4-acetoxy-1-phenyl-2-dodecen-1-one (6.03%), neophytadiene (5.7%), (*E*)-2-nonadecene (3.9%), hexanol-4-D2 (2.92%), and decane (2.4%). Several of these compounds are potentially bioactive. *β*-Sitosterol, a phytosterol present naturally in the cells and membranes of plants, has been shown to have antibacterial and antioxidant activities with various applications;[34,36−38] cholest-5-en-3-yl (9*Z*)-9-octadecenoate was found in the extracts that showed anticancer and antibacterial activities;[26] 1-dodecanol is a long-chain fatty acid with high potential as antibacterial (MIC, 8 *μ*g/mL) and antimycobacterial agents;[39,40] neophytadiene is a diterpenoid compound that possesses antimicrobial, anti-inflammatory, antibiofilm, and antioxidant properties;[41,42] nonadecene, hexanol, and decane exhibit antibacterial activities.[43−45] Most of the major compounds were identified from ME of *A. polystachyus* (seven out of eight), which has known bioactive functions. Thus, it may be expected that ME likely has the potential to exhibit bioactivity.

The major compounds detected by GC-MS in EO were stearyl alcohol P721 (25.38%); *cis*-9-tetradecenoic acid, isobutyl ester (22.95%); butyl 9-tetradecenoate (10.62%); 11,13-dimethyl-12-tetradecen-1-ol acetate (10.14%); ginsenol (3.48%); and diisooctyl phthalate @P1404 (2.54%). Stearyl alcohol has varied uses such as pharmaceutical dispensing, cosmetic creams, perfumery, antifoam agents, resins, surface active agents, and lubricants. Stearyl alcohol possesses antioxidant and antibacterial activities.[26,40]

Cis-9-tetradecenoic acid has been reported for antibacterial, anti-inflammatory, and analgesic properties;[46] butyl 9-tetradecenoate is reported for antibacterial activity;[47] 11,13-dimethyl-12-tetradecen-1-ol acetate is reported for the antimicrobial formulation of toothpaste;[48] ginsenol is a sesquiterpenoid that shows fungistatic action;[49] and diisooctyl phthalate is reported for antimicrobial and antioxidant activities.[46,50] It is noteworthy that the chemical composition of *A. polystachyus* EO is different from the other EOs. The monoterpenes were not detected, and one sesquiterpenoid (ginsenol) was present along with saturated fatty acids, fatty acid esters, alcohols, phenols, and benzoic acid ester (Tables 2 and S3). In a previous study, the EO of *Acanthus ilicifolius* (Acanthaceae) was reported to contain alkanes, fatty acids, benzoic acid esters, alcohols, and alkenes.[51] Our result agrees with these results. However, further characterizations are required to determine the components of the EO and ME of *A. polystachyus* using techniques such as high-performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR).

2.2. Antioxidant Activity. The antioxidation potential of ME and EO was tested by using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging assay that is based on the principle of reduction of DPPH by hydrogen donors to diphenyl picrylhydrazine. Both ME and EO were found to show an effective range of radical scavenging at varying concentrations (125−1000 *μ*g/mL; *μ*L/mL for EO). The activities of ME and EO ranged from 48.3 to 84.2% radical scavenging activity (RSA) and 45.6 to 82%RSA, respectively (Table 3) with dose dependency. Ascorbic acid was used as a standard reference with the same concentrations and generated values of 90.3−96.7%RSA. The activity of ME and EO was found to be comparable with the standard ascorbic acid at higher concentrations. This may be attributed to the presence of compounds found in this study possessing antioxidation properties such as neophytadiene, *β*-sitosterol (ME) and diisooctyl phthalate (EO).[36,41,46] Several studies have reported the antioxidant property of natural products of plants.[52] The antioxidation property is beneficial in scavenging free radicals involved in several diseases and metabolic pathways and imparts immunoprotective, hepatoprotective, and neuroprotective effects.[53]

Table 3. Antioxidant Activity of ME and EO of *A. polystachyus*[a]

concentration (*μ*g/mL)	ME (%RSA)	EO (%RSA)	standard (%RSA)
125	48.3	45.6	90.5
250	65.7	61.3	91
500	76.5	71.6	92.4
1000	84.2	82	94.7

[a]Standard, ascorbic acid. For EO, the concentration is in *μ*L/mL.

2.3. Antimicrobial Activity. The antimicrobial potential of ME and EO was evaluated by using a Kirby−Bauer agar disc diffusion assay on six bacterial and one fungal species at various concentrations (50−400 mg/mL). The microorganisms were chosen based on their pathogenic relevance and AMR potential, including three Gram-negative bacteria, *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 25922), and *A. baumannii* (ATCC 19606); three Gram-positive bacteria, *S. aureus* (ATCC 25923), *E. faecalis* (ATCC 29212), and *S. pyogenes* (ATCC 12204); and one fungal strain, *C. albicans* (ATCC 10231). At the tested concentrations, both ME and EO inhibited all of the microbes with varying degrees of inhibition (Figure 2 and Tables 4 and 5). The antimicrobial assay with ME shown in Figure 2 indicates that both Gram-positive and Gram-negative bacteria were inhibited with dose dependency.

https://doi.org/10.1021/acsomega.3c06246
ACS Omega 2023, 8, 43024−43036

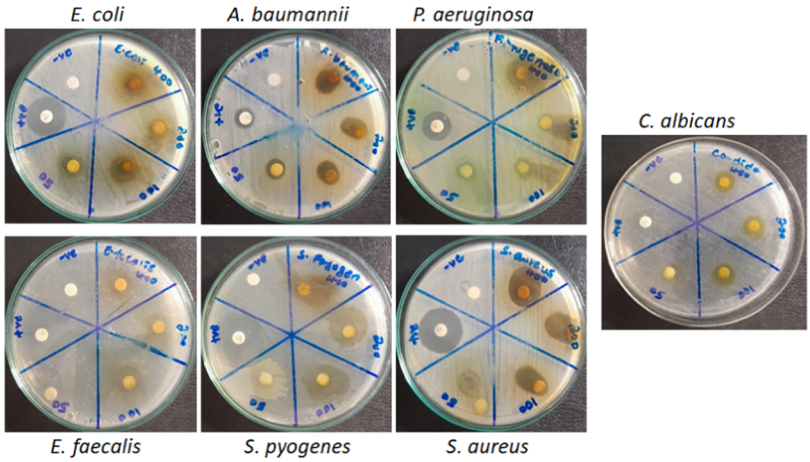

Figure 2. Antimicrobial activity of ME by the disk diffusion assay. Bacteria and fungi were treated with varying concentrations of ME (50−400 mg/mL) on discs. Gentamycin (10 μg/mL) was the positive control for bacteria and ketoconazole (30 μg/mL) for *C. albicans*. Negative control was 10% DMSO.

Table 4. Antimicrobial Activity of ME by Disc Diffusion Assay[a]

mean inhibition zone (mm) ± SD and % inhibition					
	concentration(mg/mL)				
bacteria	+ve control	50 (% RI)	100 (% RI)	200 (% RI)	400 (% RI)
P. aeruginosa	12.6 ± 0.33	8.0 ± 1.00 (63.5%)	8.5 ± 1.33 (67.46%)	9.3 ± 0.50 (74.05%)	12.8 ± 0.33 (100.6%)
E. coli	17.0 ± 0.57	6.6 ± 0.33 (39.2%)	7.3 ± 0.33 (43.12%)	7.3 ± 0.33 (43.12%)	8.3 ± 0.33 (49%)
A. baumannii	9.4 ± 0.56	7.3 ± 0.33 (77.4%)	10.3 ± 0.66 (106.3%)	10.7 ± 0.57 (111.93%)	12.3 ± 0.33 (130.20%)
S. aureus	19.3 ± 0.66	6.6 ± 0.33 (32.72%)	9.0 ± 0.57 (43.10%)	13.3 ± 0.88 (60.40%)	18.6 ± 1.20 (87.75%)
S. pyogenes	21.6 ± 1.20	8.3 ± 0.88 (38.4%)	10.6 ± 0.88 (49.24%)	13.6 ± 2.40 (63.08%)	14.6 ± 2.33 (67.7%)
E. faecalis	19.0 ± 0.57	7.6 ± 0.33 (40.3%)	8.6 ± 0.66 (45.63%)	10.0 ± 1.15 (52.63%)	12.0 ± 1.15 (63.16%)
C. albicans	25.3 ± 0.33	8.7 ± 0.33 (34.2%)	10.3 ± 0.33 (40.78%)	12.3 ± 0.33 (48.68%)	15.0 ± 0.57 (59%)

[a]% RI, % relative inhibition; + ve control, positive control.

Table 5. Antimicrobial Activity of EO by Disc Diffusion Assay[a]

mean inhibition zone (mm) ± SD and % inhibition				
	concentration(μL/mL)			
bacteria	+ve control	10 (%RI)	20 (%RI)	40 (%RI)
P. aeruginosa	13.2 ± 0.50	6.9 ± 1.2 (52.2%)	7.3 ± 1.1 (55.3%)	9.6 ± 0.20 (72.7%)
E. coli	16.4.0 ± 0.57	6.6 ± 0.66 (40.2%)	7.5 ± 0.33 (45.7%)	8.2 ± 0.50 (50.0%)
A. baumannii	9.9 ± 0.85	6.8 ± 0.33 (68.7%)	8.1 ± 0.26 (81.8%)	8.9 ± 0.33 (89.8%)
S. aureus	18.5 ± 0.33	6.3 ± 0.40 (34.0%)	9.2 ± 0.60 (49.70%)	13.7 ± 0.38 (74.0%)
S. pyogenes	22.0 ± 1.35	8.5 ± 0.70 (38.6%)	10.0 ± 0.33 (45.4%)	13.9 ± 2.60 (63.18%)
E. faecalis	19.6 ± 0.75	7.6 ± 0.20 (38.7%)	8.7 ± 0.33 (44.38%)	11.3 ± 1.00 (57.65%)
C. albicans	22.7 ± 0.46	7.5 ± 0.50 (33.0%)	9.4 ± 0.85 (41.4%)	12.2 ± 0.20 (53.7%)

[a]% RI, % relative inhibition; + ve control, positive control.

Similarly, inhibition increased with increasing concentration in the case of *C. albicans*. The zone of inhibition (ZOI) was measured and is presented in Tables 4 (ME) and 5 (EO). Due to the differential behavior of microorganisms toward antibiotics, inhibition zones were normalized with control (%RI, relative inhibition). There was no apparent difference in the inhibition patterns between the Gram-positive and Gram-positive bacteria, indicating broad-spectrum inhibitory activity of the extracts. In Gram-positive representative bacteria, inhibition was observed in the 32.72−87.75% range, while in Gram-negative representative strains, a wide range of inhibition (39.2−130.2%) was observed (Table 4). Among the Gram-positive strains, at 400 mg/mL, *S. aureus* showed higher inhibitory values (87.7%) than *E. faecalis* (63.16%) and *S. pyogenes* (67.7%). *S. aureus* is known to exhibit greater resistance to antimicrobials;[2] however, it showed higher susceptibility against ME here. An inhibition of 49% was observed in *E. coli* at 400 mg/mL; however, notably, higher inhibitory values were observed in the case of *P. aeruginosa* (100.3%) and *A. baumannii* (130.2%), which increased the inhibitory range. Gram-negative bacteria in general and *P. aeruginosa* and *A. baumannii*, in particular, have been known to exhibit higher resistance to antimicrobials and are categorized in the pathogen priority list (PPL) as "critical priority" by WHO.[54] This is also evident from the lower ZOI of PC (gentamycin) in the case of *P. aeruginosa* (12.6 ± 0.33 mm) and *A. baumannii* (9.47 ± 0.56 mm), indicating higher resistance against commercial antibiotics compared with ZOI

https://doi.org/10.1021/acsomega.3c06246
ACS Omega 2023, 8, 43024−43036

Table 6. MIC, MBC, MFC, MBC/MIC, MFC/MIC, and MBIC of ME and EO of *A. polystachyus*[a]

	Bacteria	MIC (mg/mL)	MBC (mg/mL)	MFC (mg/mL)	MBC/MIC	MFC/MIC	MBIC (mg/mL)
ME	*P. aeruginosa*	0.5	2		4		0.5
	E. coli	1	4		4		1
	A. baumannii	0.5	4		8		0.5
	S. aureus	0.5	2		4		0.5
	S. pyogenes	0.5	2		4		0.5
	E. faecalis	0.5	4		8		0.5
	C. albicans	0.5		4		8	
EO	*P. aeruginosa*	0.62	2.5		4		0.62
	E. coli	0.31	1.25		4		0.31
	A. baumannii	0.31	1.25		4		0.31
	S. aureus	0.31	1.25		4		0.31
	S. pyogenes	0.31	1.25		4		0.31
	E. faecalis	0.31	1.25		4		0.31
	C. albicans	0.62		2.5		4	

[a]ME, methanol extract; EO, essential oil; EO values are in μL/mL; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MFC, minimum fungicidal concentration; MBC/MIC, tolerance values for bacteria; MFC/MIC, tolerance values for fungi; MBIC, minimum biofilm inhibitory concentration (MBIC). The values are from three independent experiments ($P < 0.05$).

of *E. coli* (17.0 ± 0.57 mm) and other bacteria (19.0 ± 0.57−21.67 ± 1.20 mm) (Figure 2 and Table 4). However, interestingly, we found higher inhibition (susceptibility) values for ME in *A. baumannii* (130.2%) and *P. aeruginosa* (100.3%) (Table 4). This may be due to decreased resistance or higher susceptibility against ME. Thus, it emphasizes the antibacterial potency of ME as a new, valuable, yet cost-effective measure to deal with antimicrobial-resistant bacteria, thereby eliminating infections. A moderate range of inhibition (34.2−59%) was observed in the case of *C. albicans*, indicating antifungal activity of ME. The compounds listed in Table 1 from ME such as β-sitosterol, cholest-5-en-3-yl (9*Z*)-9-octadecenoate, 1-dodecanol, neophytadiene, (*E*)-2-nonadecene, hexanol-4-D2, and decane possess antimicrobial properties[36−45] that appear to be responsible for the observed inhibition of the bacterial and fungal species.

The antimicrobial activity results of EO at different concentrations (10−40 μL/mL) are presented in Table 5. The EO inhibited Gram-positive (34−74%) and Gram-negative (40.2−89.8%) representative bacteria in general without any apparent difference. However, the inhibition intensity was less than that of ME (mentioned above). Among Gram-negative strains, *E. coli* was found to be the least susceptible (40.2−50%) and among Gram-positive, *S. aureus* showed the highest susceptibility (34−74%). The "critical priority" and "high priority" resistant strains, *A. baumannii*, *P. aeruginosa*, and *S. aureus* showed lesser resistance (or higher susceptibility) in the ranges of 34−74, 68.7−89.8, and 52.2−72.7%, respectively, than other strains. These results are similar to ME and emphasizes the importance of EO as an effective antimicrobial. The major compounds detected by GC-MS in EO summarized in Table 2 indicate the presence of bioactive compounds such as stearyl alcohol, *cis*-9-tetradecenoic acid, isobutyl ester, butyl 9-tetradecenoate, 11,13-dimethyl-12-tetradecen-1-ol acetate, and diisooctyl phthalate that demonstrated antibacterial activity and may be responsible for the achieved inhibition by EO.[26,46−52] *C. albicans* exhibited an inhibition range of 33−53.7%. Ginsenol is reported for the fungistatic property.[51] Thus, ME and EO of *A. polystachyus* may be regarded as valuable for their potency against stringent and MDR bacterial strains, *S. aureus*, *A. baumannii*, and *P. aeruginosa*, in particular, and other pathogenic bacteria and fungus, in general, used in this study. In a previous report, an acetone extract of the capsule from *Eucalyptus camaldulensis* exhibited excellent antibacterial properties (140%) compared with controls against *A. baumannii* and *E. coli* and antifungal activity (96%) against *Rhizopus stolonifer*.[55]

2.4. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal or Fungicidal Concentration (MBC/MFC) of ME and EO. Determining the MIC, MBC, and MFC is necessary for evaluating the extracts for their cytotoxicity. The MIC is the lowest concentration of a substance needed to prevent the visible growth of a bacterium. At the same time, MBC/MFC is the lowest concentration required to kill the bacterium/fungus. Thus, the material's effectiveness at low concentrations is important to determine its potential as antimicrobials. A tolerance test provides information about the potency of the test extracts, where lower values indicate bactericidal potential and higher values indicate bacteriostatic activity. The MIC/MBC/MFC assays were carried out for ME and EO; the results are presented in Table 6. The MIC values of ME against the tested microbes varied from 0.5 to 1.0 mg/mL and MBCs/MFCs from 2 to 4 mg/mL. *E. coli* recorded an MIC of 1 mg/mL, which was higher than other bacteria, indicating higher resistance. Similar resistance patterns were observed in the antibacterial assay for *E. coli* (Figure 2 and Table 4). There was no other apparent difference observed among the strains, and it indicated the broad-spectrum activity of the extracts. Tolerance test values calculated as MBC/MIC (for bacteria) and MFC/MIC (for fungi) were between 4 and 8. The values of <4 indicate bactericidal activity, while a value of 8 indicates bacteriostatic action. The strains *A. baumannii*, *E. faecalis*, and *C. albicans* recorded a value of 8, indicating bacteriostatic/fungistatic activity, while all others scored 4 indicating bactericidal potential of ME and EO. In a previous report that tested ME of *A. polystachyus*, MICs ranged from 100 to 200 mg/mL (for bacteria and *C. albicans*) and MBCs from 200 to 400 mg/mL for bacteria.[32] These values are 100−400 times higher than those found in this study. This may be attributable to the cultivation, environmental, and geographical factors, including soil structure.[33] In another study that used the acetone extract of *E. camaldulensis*, MIC values of 18−20 mg/mL were reported for *A. baumannii* and *E. coli* that showed a 140%

https://doi.org/10.1021/acsomega.3c06246
ACS Omega 2023, 8, 43024−43036

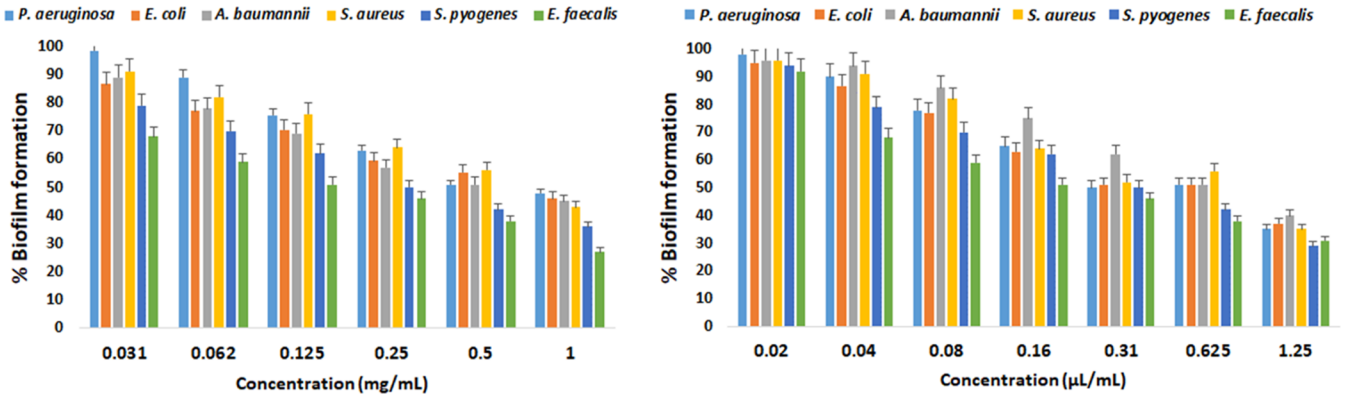

Figure 3. Antibiofilm assay of ME and EO. The biofilm formation ability of bacteria was tested by using different concentrations of ME (left) and EO (right). The %biofilm formation was inhibited by increasing the extract concentrations in a dose-dependent manner. Values are from three independent experiments ($P < 0.05$).

inhibitory activity in the antibacterial assay. The values obtained in our study are lower than in this report.[55] Several other plant species' ME have been used to explore the antibacterial activity.[56] The values observed here are either comparable to or better than those reported.

The MIC values of EO for the tested microbes varied from 0.31 to 0.62 μL/mL and MBCs/MFCs from 1.25 to 2.5 μL/mL. *P. aeruginosa* and *C. albicans* showed higher values (MIC of 0.62 μL/mL and MBC/MFC of 2.5 μL/mL), indicating higher resistance. All strains scored tolerance test values of 4, indicating EO's bactericidal/fungicidal activity. EO of several plant species have been investigated, and a wide range of MIC/MBC/MFC have been reported.[57,58] A recent report observed MICs of 50−100 μL/mL for some similar bacterial species used in this study and 6.25 μL/mL for *C. albicans*, which are much higher than found here.[57] In another study, a wide range of MICs were reported from 0.2 to 12.5 μL/mL against bacterial and fungal strains.[58] Our results are comparable to the lower range of this report. These results confirm the antimicrobial potential of the EO of *A. polystachyus* and may prove valuable against pathogenic and resistant microbial species.

2.5. Antibiofilm Activity and Minimum Biofilm Inhibition Concentration (MBIC) of ME and EO. Microbes develop resistance when challenged by conventional antimicrobials, leading to the impaired treatment of diseases. The resistance is most commonly associated with forming biofilms that protect microbes from surrounding environmental stresses, impede phagocytosis, and confer the capacity for colonization and long-term persistence. Such an ability is promoted by effective cell-to-cell communications (quorum sensing) within the microbial communities. As a result, highly structured biofilms can be formed and often identified in patients with chronic infections, such as chronic lung and wound infections. Therefore, exploring new sources with pharmaceutical properties that can interfere with, reduce, and eradicate biofilms for the effective treatment of such diseases is essential. The ME and EO of *A. polystachyus* were investigated for their ability to reduce biofilm formation. The results are shown in Figure 3 and Table 6. Both ME and EO were effective and showed dose-dependent inhibition of biofilm formation, with MBIC values in the range of 0.5−1 mg/mL (ME) and 0.31−0.62 μL/mL, respectively. The Gram-negative representative bacteria were more resistant to the biofilm inhibitory effect of ME and EO than Gram-positive strains. At the highest concentration, 1 mg/mL of ME, the biofilm inhibitory effect was in the following order: *E. faecalis* > *S. pyogenes* > *S. aureus* > *A. baumannii* > *E. coli* > *P. aeruginosa*. In the case of EO, the following order was observed: *S. pyogenes* > *E. faecalis* > *S. aureus* > *P. aeruginosa* > *E. coli* > *A. baumannii*. The reduction of the biofilm formation ability of microbes has been reported to vary across an extensive range (0.125−100 mg/mL). Our results agree with the reported MBIC values of ME of different plant species in the 0.25−1.0 mg/mL range.[59,60] Similarly, a wide range of MBICs for EO have been reported in the range of 2−8[61] and 50−100 μL/mL.[57] Our results show better efficacy of EO with lower MBIC values.

The biofilm-forming microbes are difficult to treat with conventional antimicrobials and can resist up to 1000-folds, thus increasing the cost of treatment. These microbial populations may further aggravate the AMR problem by the transfer of resistance genes to other populations.[4,5] Therefore, targeting the inhibition of biofilm formation is a plausible direction to reduce and possibly eradicate biofilm-forming pathogens. Therefore, further extensive investigations are required to establish the effectiveness of antibiofilm extracts or compounds. Possible directions would be to isolate individual components of extracts and rigorously assess the potential of single compounds or synergy combinations.

2.6. Antiquorum Sensing Activity of ME and EO. Pyocyanin is a pigment distinctive to *P. aeruginosa* and plays a crucial role in infection and virulence. There is also a favorable relationship between pyocyanin and antibiotic resistance, thus empowering *P. aeruginosa* for AMR.[62] Pyocyanin inhibition assay (PIA) is commonly employed to evaluate quorum sensing (QS) in the model bacteria, *P. aeruginosa*.[63] In this study, we evaluated the anti-QS potential of ME on *P. aeruginosa* by using PIA. The inhibition of pyocyanin production was in the range of 17.4−51.6%, indicating the effective inhibition of pyocyanin (Figure 4). The highest inhibition (51.6%) was at 500 μg/mL. Pejin et al. reported that at 0.5MIC (MIC, 19 μg/mL), phytol inhibited pyocyanin production by 51.94%.[63] Phytol was used as a purified single compound, and the 0.5MIC value (9.5 μg/mL) is much lower than our result (500 μg/mL), which contains a mixture of several compounds. However, in future studies, purification and identification of the individual compounds responsible for pyocyanin inhibition may be pursued. In another study, at 750 μg/mL, four different extracts of *Camellia nitidissima* inhibited

https://doi.org/10.1021/acsomega.3c06246
ACS Omega 2023, 8, 43024−43036

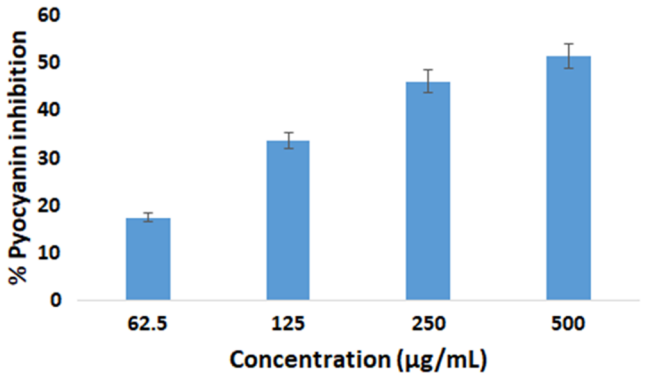

Figure 4. Pyocyanin inhibition assay of ME. *P. aeruginosa* was treated with various concentrations of ME (62.5−500 μg/mL). A dose-dependent inhibitory activity of ME was observed in the inhibition of pyocyanin production. Values are from three independent experiments ($P < 0.05$).

pyocyanin production in the 51.25−67.5% range.[62] Our results agree with those in this report. Thus, ME demonstrated pyocyanin inhibitory potential and showed promise to be further investigated.

3. CONCLUSIONS

Plant natural products rich in phytochemicals are unfolding as possible solutions to combat pathogenic and resistant microbes. In this study, we explored *A. polystachyus* for its potential as an antioxidant and antimicrobial against selected bacterial and fungal species. The phytochemical composition of extracts by GC-MS revealed bioactive compounds. The antioxidant property was found in both ME and EO by the DPPH assay. The ME exhibited high inhibition for the "critical priority" and "high priority" strains, *A. baumannii*, *P. aeruginosa*, and *S. aureus* (87.75−130.20% inhibition). The EO also showed potential inhibition for the same strains in the range of 72.7−89.8%. The MIC (0.5−1 mg/mL; 0.31−0.62 μL/mL), MBC/MFC (2−4 mg/mL; 1.25−2.5 μL/mL), and MBIC (0.5−1 mg/mL; 0.31−0.62 μL/mL) values of ME and EO confirmed the inhibition potential as well as the inhibition of biofilm formation. Pyocyanin, an important factor in antibiotic resistance, virulence, and QS, was also inhibited by ME in the anti-QS assay using *P. aeruginosa* (51.6%, 0.5 mg/mL). Thus, ME and EO extracted from *A. polystachyus* leaves have high potency and are valuable as antioxidants and antimicrobials. This study explored and established the phytochemical composition of *A. polystachyus* extracts and unveiled the potential antimicrobial properties that may prove useful to reduce the microbial population load and combat pathogenic and resistant microbes.

4. EXPERIMENTAL SECTION

4.1. Chemicals, Reagents, and Instruments. A UV spectrophotometer (Optizen 2120UV, Mecasys Co. Ltd., Daegeon, South Korea), an incubator (ThermoStable SIR-250, Daihan Scientific Co. Ltd., Gangwon-do, South Korea), a rotary evaporator (RE-501, Henan Lanphan Technology Co. Ltd., Henan, China), and a microplate reader (PLATE READER, 8 Channel ELISA Photometer, DAS Italy SRL, Rome, Italy) were used for experiments.

All chemicals and reagents were of analytical grade. Muller−Hinton, potato dextrose, agar, and ketoconazole were from HiMedia Laboratories Pvt. Ltd., Mumbai, India; LB media (Bio Basic Inc., Ontario, Canada), gentamycin (Merck, Darmstadt, Germany); anhydrous sodium sulfate, 99% methanol, chloroform, Molisch's reagent, and ferric chloride (Loba Chemie Pvt. Ltd., Mumbai, India); hydrochloric acid and sulfuric acid (CARLO ERBA Reagents S.A.S, Milano, Italy); ascorbic acid, DMSO, crystal violet (Sisco Research Laboratories Pvt. Ltd., Mumbai, India); and DPPH and Wagner reagent (Otto Chemie Pvt. Ltd., Mumbai, India).

4.2. Plant Material and Preparation of Extracts. The plant leaves of *A. polystachyus* were collected in early February (2023) from the Dilla University Botanical and Ecotourism Garden, which is located in the Southern Nations, Nationalities, and People's Region (SNNPR), Gedio Zone, Ethiopia. Dilla has a longitude and latitude of 6°24′30″N and 38°18′30″E, with an elevation of 1570 m above sea level, respectively. The plant material was authenticated by a Botanist, Mr. Ato Melaku Wondafrash, and a voucher specimen (Voucher No: HG001) was deposited at the National Herbarium, Department of Botany, Addis Ababa University, Addis Ababa, Ethiopia.

The leaves of *A. polystachyus* were washed with distilled water to remove the dirt and dried at room temperature without sunlight. The dried leaves were coarsely ground into powder by the electrical mill. Then, the powder of the plant material was mixed with 99% methanol at 1:5 (w/v) and macerated for 72 h with occasional shaking. The macerated mixture was filtered by Whatman filter paper No. 1. The residue was macerated for the second time with a fresh solvent for a total of 6 d to obtain a better yield. The filtrate of the plant material was concentrated in a rotary evaporator at 40 °C. The dried extract was stored in a refrigerator. The yield was 20.5%.

The percentage yield was calculated by

$$\%Y = \frac{W_1}{W_2} \times 100\% \quad (1)$$

W_1 is the plant extract obtained, and W_2 is the weight of the plant sample before extraction.

The EO from leaves of *A. polystachyus* was extracted by hydrodistillation in a Clevenger apparatus. A 316 g amount of powder from leaves was extracted in 1000 mL of distilled water using hydrodistillation for 3 h. The water layer at the bottom was drained to separate the oil. The oil layer was treated with anhydrous sodium sulfate (Na_2SO_4) to remove the remaining trace water. The obtained EO was protected from light and stored at 4 °C throughout the experiments.[64] The final yield was 0.4% (0.2 mL/100 g of dry matter).

4.3. Phytochemical Analysis. *4.3.1. Test for Alkaloids.* 1 mL of plant extract filtrate was treated with 2−3 drops of Wagner reagent, and a yellow color formation was observed.[65]

4.3.2. Test for Flavonoids. The plant extract (2 mL) was mixed with a few fragments of magnesium ribbon, followed by the dropwise addition of 5% concentrated HCl. The appearance of pink scarlet was observed after 2 min to indicate the presence and absence of flavonoids.[66]

4.3.3. Test for Phenols. 1 mL of the plant extract filtrate was taken in a test tube, and 1−2 drops of iron III chloride ($FeCl_3$) was added. The color change of the mixture was observed after a few min.[67]

4.3.4. Test for Saponins. The filtrate of the plant extract (1 mL) was diluted with 5 mL of distilled water in a test tube. It was shaken by hand for 15 min. A foam layer on top of the test tube indicated the presence of saponins.[66]

https://doi.org/10.1021/acsomega.3c06246
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4.3.5. Test for Steroids. The plant extract filtrate (1 mL) was dissolved in 1 mL of chloroform in a test tube. Then, 1 mL of acetic anhydride and two drops of concentrated sulfuric acid were added to the test tube by the sides. The upper layer in the test tube turned red, indicating the presence of steroids.[68]

4.3.6. Test for Tannins. The plant extract filtrate (2 mL) was taken, and then a few drops of 10% ferric chloride solution were added to the filtrate. The blue-green color appearance indicates the presence of tannins in the sample.[67]

4.3.7. Test for Terpenoids. The plant extract filtrate (1 mL) was placed in a test tube and dissolved in chloroform (1 mL). The concentrated sulfuric acid (2 drops) was placed in the test tube and shaken. The lower yellow color indicates the presence of terpenoids.[69]

4.3.8. Test for Glycosides. To 2 mL of extract were added two drops of Molisch's reagent and shaken well. Then, 2 mL of concentrated H_2SO_4 was added to the sides of the test tube. A reddish-violet ring immediately appeared at the junction of two layers, indicating the presence of carbohydrates.[67]

4.4. Phytochemical Screening of *A. polystachyus* EO. The phytochemical screening of *A. polystachyus* EO was performed by UV spectrophotometry. 100 μL of oil was diluted with 3 mL of chloroform, and UV spectra were recorded in the range of 200 nm −400 nm for determining natural compounds.[70] Terpenes have a UV absorption maximum in the 210−220 nm range. Phenols have a UV absorption maximum in the 270−280 nm range. Flavonoids have a UV absorption maximum in the 290−300 nm range. Coumarins have a UV absorption maximum in the 310−320 nm range. Alkaloids have a UV absorption maximum in the 330−340 nm range.[35]

4.5. Gas Chromatography-Mass Spectra (GC-MS) Analysis. GC-MS analysis of plant extracts was performed by a GC (7890B, Agilent Technologies) coupled with an MS (5977A Network, Agilent Technologies). The GC had an HP-5MS column (30 m × 0.25 mm internal diameter (i.d.) and 0.25 μm film thickness). Helium was used as a carrier gas with a 4 min solvent delay and a splitless injection/purge time of 1.0 min with different flow rates and runtime. In the case of methanol extract, the temperature increase was 110−330 °C, the flow rate was 1.2 mL/min, and the runtime was 60 min. For EO, the temperature increase was 100−280 °C, the flow rate was 1 mL/min, and the runtime was 33 min.

Mass spectra were recorded in an electron-impact mode, with an ionization energy of mode at 70 eV, scanning the 33−550 m/z range.[71] The volatile compounds in the oil were identified by comparing the mass spectra of the compounds in oils with those in the database of the NIST11 GC-MS libraries.[72]

4.6. Antioxidant Activities. The antioxidant activity test was performed by using the DPPH free-radical scavenging assay.[73] A 0.1 mM DPPH solution was prepared in methanol and kept in the dark for 30 min to complete the reaction. The ME dilutions were prepared at 1000, 500, 250, and 125 μg/mL. The same concentrations of ascorbic acid were used as a standard, and the sample-free DPPH solution was used as a negative control. After mixing 1 mL of DPPH solution with 3 mL of prepared samples, the mixture was incubated at room temperature in a dark place for 30 min, and the absorbance was measured at 517 nm. The assay for EO was similarly performed in microtiter plates except that the volumes were reduced. The %RSA was calculated according to the following formula

$$\%\mathrm{RSA} = \frac{A_0 - A_1}{A_0} \times 100\% \quad (2)$$

where %RSA is the percent radical scavenging activity, A_0 is the absorbance of the blank DPPH, and A_1 is the absorbance of sample.[20,73]

4.7. Antibacterial Activity. The antibacterial activity of the extracts was tested using the standard Kirby−Bauer agar disc diffusion assay and various concentrations of the extracts. The test microorganisms included six pathogenic bacterial strains (three Gram-positive and three Gram-negative) and one fungal strain known to possess antimicrobial resistance: *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 25922), *A. baumannii* (ATCC 19606), *S. aureus* (ATCC 25923), *E. faecalis* (ATCC 29212), *S. pyogenes* (ATCC 12204), and *C. albicans* (ATCC 10231). The ME dilutions were prepared at 400, 200, 100, and 50 mg/mL by dissolving in 10% DMSO. The EO was prepared in three dilutions of 40, 20, and 10 μL/mL by dissolving in 10% DMSO. First, all of the stock-cultured bacteria were activated on Muller−Hinton (MH) and *C. albicans* on potato dextrose (PD) agar plates. After activation, the cultures were subjected to inocula development by inoculating a loop full of cells from a single colony into MH/PD broth and incubated for 24 h at 37 and 48 h at 30 °C for *C. albicans*. The active pure bacterial/fungal cultures and subcultures were maintained according to the 0.5 McFarland standards to obtain 1.5 × 10^8 colony-forming units (CFUs/mL) for bacteria and 1 × 10^6 (CFU/mL) for *C. albicans*. This process was repeated every time before a new experiment. The fresh overnight cultures of each strain were swabbed uniformly over sterilized and cooled MH/PD agar medium Petri dishes. Then, 6 mm diameter sterile discs impregnated with different concentrations of ME and EO were placed onto these plates, soaked for 30 min, and incubated for 24 h at 37 °C for bacteria and 48 h at 30 °C for *C. albicans*. Discs impregnated with gentamycin solution (10 μg/mL), ketoconazole (30 μg/mL), and 10% DMSO were used as positive and negative controls. After incubation, the antimicrobial activity was determined by measuring the diameter of the zone of inhibition. The antibacterial/antifungal activity was normalized and calculated in terms of percentage relative inhibition (%RI) by the following formula: %RI = diameter of sample/diameter of control ×100.

4.8. MIC, MBC, and MFC. The bacterial/fungal growth was assessed by a broth dilution method. The ME was diluted in 10% DMSO, and the diluted extracts (100 μL) were introduced into 10 mL of LB broth (PD for *C. albicans*) to achieve the concentrations of 1 mg/mL, 500 μg/mL, 250 μg/mL, and 125 μg/mL. Inoculum (100 μL) was added from the McFarland standard calibrated broth (5 × 10^6 CFU/mL for bacteria and 1 × 10^4 for *C. albicans*) to each test tube and grown for 24 h at 37 °C and 48 h at 30 °C for *C. albicans*. Due to the presence of colored pigments, it was difficult to measure the absorbance of the culture. So, we adopted a plate assay, where 20 μL of the above mixture was spread on MH/PD agar plates and incubated overnight at 37 °C and 48 h at 30 °C for *C. albicans*. The gradual disappearance of colonies with increasing concentrations was observed visually and recorded. MIC was determined as the lowest concentration of extract that inhibited the visible growth of colonies of the organism.[42] MBCs and MFCs of the extract for different microorganisms were deduced from the lowest concentration of the total

https://doi.org/10.1021/acsomega.3c06246
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inhibition of bacteria/*C. albicans* on the respective media agar.[74]

For EO, the growth assay was performed using the broth microdilution method in a 96-well microtiter plate. A 20 μL/mL stock solution was prepared in an LB/PD broth. The concentrations were varied from 0.02 to 2.5 μL/mL in a final volume of 200 μL. Ten μL of the McFarland-calibrated (5×10^6 CFU/mL for bacteria and 1×10^4 for *C. albicans*) inoculum was added to each well and grown for 24 h at 37 °C for bacteria and 48 h at 30 °C for *C. albicans*. The plate assay for the determination of the MIC was performed as mentioned above. The determination of MBC and MFC was also as above.

4.9. Antibiofilm Assay. The antibiofilm assay was performed only for bacteria according to Raut et al. with minor modifications.[75] The varying concentrations starting from MIC to sub-MIC (500−62.5 μg/mL for methanol extract and 0.02−1.25 μg/mL for EO) were mixed with bacterial culture media at an initial turbidity of 0.05 (5×10^5 CFU/mL). The assays were performed in a 96-well microtiter plate for EO and 10 mL test tubes for ME as mentioned above. The mixtures were incubated for 48 h at 37 °C. The planktonic cells were removed by gentle washing with sterile phosphate-buffered saline (PBS), and then the adherent cells were stained with 1% crystal violet (CV) for 10 min and washed with PBS to remove the excess stain. After air drying, CV bound to biofilm was solubilized with 33% glacial acetic acid.[75] Using similar procedures, a control without any treatment was set for comparison to quantify the adherent cells, and the CV solution's absorbance was measured by a UV spectrophotometer at OD_{590}.

The percent biofilm formation was calculated by

$$\text{biofilm formation (\%)} = \frac{OD_{590}\ \text{sample}}{OD_{590}\ \text{control}} \times 100\% \quad (3)$$

Minimum biofilm inhibitory concentration (MBIC) was determined by the inhibition of a minimum of 50% biofilm formation.[75]

4.10. Anti-QS Assay: Pyocyanin Inhibition Assay. Varying dilutions (sub-MIC) of plant extracts were added to the planktonic cultures (6 mL) of *P. aeruginosa* and incubated for 24 h at 37 °C. Then, 2 mL of chloroform was added and centrifuged at 5,000 g for 5 min. The organic layer was separated and solubilized in 2 mL of 0.2 M HCl. The absorbance of each supernatant was measured at 520 nm. Control without treatment was set as 100%, and distilled water with HCl was used as a blank.[76]

The inhibition percentage was calculated as follows

$$\%\ \text{pyocyanin inhibition} = \frac{OD_{520}\ \text{of control} - OD_{520}\ \text{of treated}}{OD_{520}\ \text{of control}} \times 100\% \quad (4)$$

4.11. Statistical Analysis. Three replicates' means ± standard deviation (SD) represented the experimental results. Statistical significance was determined by P values less than 0.05. Microsoft Excel 2010 SPSS statistical software was utilized for all analyses.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c06246

Phytochemical screening of methanol extract of *A. polystachyus* (Table S1); methanol extract constituents of *A. polystachyus* by GC-MS (Table S2); and essential oil constituents of *A. polystachyus* by GC-MS (Table S3) (PDF)

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Author Contributions

[⊥]M.G. and Y.N. contributed equally. Writing—original draft and laboratory work were carried out by M.G., Y.N., and M.A. GC-MS experiments were performed by K.S. Writing—review and editing was done by S.S., N.G., and M.N. Advising was done by M.N. All authors read the final manuscript in detail and approved it.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors acknowledge the Departments of Applied Biology, Chemistry, and Institute of Pharmaceutical Science for support and Adama Science and Technology University for providing funds.

■ LIST OF ABBREVIATIONS

AMR, antimicrobial resistance; MDR, multidrug resistance; GC-MS, gas chromatography-mass spectroscopy; ME, methanol extract; EO, essential oil; RI, relative inhibition; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; MFC, minimum fungicidal concentration;

MBIC, minimum biofilm inhibitory concentration; QS, quorum sensing; EPS, extracellular polymeric substances; eDNA, extracellular DNA; AHL, *N*-acylhomoserine lactone; AIP, autoinducing peptide; AMPs, antimicrobial peptides; DPPH, diphenyl picrylhydrazine; RSA, radical scavenging activity; ZOI, zone of inhibition; PPL, pathogen priority list; PIA, pyocyanin inhibition assay; MH, Muller−Hinton; PD, potato dextrose; CFUs, colony-forming units; CV, crystal violet

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