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Antimicrobial Activity of Compounds Isolated from *Uvaria wrayi* (King) L.L. Zhou, Y.C.F. Su & R.M.K. Saunders Leaves and Twigs

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Abstract

Antimicrobial resistance (AMR) refers to the capacity of bacteria to resist the impact of antibiotics, emerging as a significant global health concern. To discover novel antibacterial agents, the isolation and elucidation of compounds from leaf and twig extracts of *Uvaria wrayi* (King) L.L. Zhou, Y.C.F. Su & R.M.K. Saunders were investigated for the first time. 9 known compounds were performed and categorized as polyoxygenated cyclohexenes (**1**-**3**), *seco*-cyclohexenes (**4**), acrylamide derivatives (**5**,**6**), and aristolactam alkaloids (**7**-**9**). All isolated compounds were evaluated for their antimicrobial activities against 5 Gram-positive bacteria, including *Staphylococcus aureus* (TISTR 746), *Staphylococcus epidermidis* (DMST 15505), Methicillin-resistant *Staphylococcus aureus* (MRSA) (NPRC 001R), *Cutibacterium acnes* (formerly *Propionibacterium acnes*) (DMST 14916), and *Streptococcus mutans* (DMST 18777/ATCC 25175T). 2 Gram-negative bacteria, *Shigella flexneri* (DMST 4423) and *Salmonella enterica* ser. *typhimurium* (TISTR 2519) were evaluated. Most of the isolated compounds displayed moderate antimicrobial activity with a MIC value of 64 µg/mL, except compounds **1**,**9** showed antibacterial against MRSA with a MIC value of 128 µg/mL. Compounds **2**-**9** exhibited antimicrobial activity against *C. acnes* (DMST 14916) with a MIC value of 32 µg/mL. Compounds **1**-**3**, **6**, and **7** also responded to inhibit *C. acnes* with the same MBC value of 128 µg/mL. This exploration holds promise for advancing our understanding of potential antibacterial compounds within *U. wrayi* and addressing the challenges posed by antimicrobial resistance.

Keywords: *Uvaria wrayi*, Anonaceae, Antibacterial activity, Aristolactam alkaloids, Highly oxygenated cyclohexenes

Introduction

Antibiotics are antimicrobial agents that defeat infections caused by bacteria in humans and animals by either killing the bacteria or inhibiting bacteria to grow and multiply [1]. Since the first discovery of novel antibiotic classes, for instance, penicillin in 1929, synthetic sulfonamides in 1935, and streptomycin in 1944, the issue of antimicrobial resistance (AMR) has been occurring in silent and constantly appeared together with the effort to develop new generations of antibiotics [2]. AMR has become one of the main global public health problems, which directly affected 1.27 million global deaths in 2019 and contributed

to 4.95 million deaths [3]. This phenomenon has significant consequences for human health, such as treatment challenges, increased mortality rates, prolonged illness, economic implications due to increased healthcare costs, and limited treatment options caused by the decrease in the number of effective treatment options [4]. Moreover, as part of their mechanism of action, AMR can induce oxidative stress within bacterial cells by generating reactive oxygen species (ROS). Antibiotic-resistant bacteria may possess enhanced capabilities to manage oxidative stress, which is the cause of body cellular damage [5]. It is important to note that combating AMR is a complex and ongoing challenge that requires sustained efforts from all sectors of society. The way to respond to this challenge is by designing more effective preventive strategies, using existing antimicrobial drugs, and, most importantly, searching for approaches and developing new antibiotics [6].

Natural products have played a significant role in the discovery of antimicrobial drugs and have been a rich source of bioactive compounds that exhibit antimicrobial properties. The survey from 1981 to 2019 shows up to 70.6 % of antimicrobial drugs are from natural product derivatives [7]. Natural products, especially from plant sources, exhibit structural diversity and complexity, making them valuable starting points for drug development. The chemical diversity found in nature provides a wide range of potential antimicrobial agents with distinct mechanisms of action [8].

The genus *Uvaria* is a diverse genus comprising numerous flowering plants. *Uvaria* belongs to the family Annonaceae, and its species are found in tropical and subtropical regions [9]. Some studies have investigated the potential antimicrobial activities of certain *Uvaria* species or their constituents. For example, ethanolic extract of *Uvaria afzelii* root exhibited activity against *Staphylococcus aureus* strains at MIC values ranging from 0.012 - 0.048 mg/mL [10], and dichloromethane extract of *Uvaria caffra* root exhibited activity against *S. aureus* at MIC value of 0.03 mg/mL [11]. In addition, ethanol extracts of *Uvaria chamae* containing chalcone and dihydrochalcone are shown to have the ability for antimicrobial activity ranging from 25.0 - 28.3 µg/mL [12]. However, the *Uvaria* genus has not been as extensively studied. There is still a limited amount of research specifically focused on the isolation and characterization of bioactive compounds responsible for antibacterial activities. Many plant species, including those within the *Uvaria* genus, still await comprehensive study and hold the potential for discovering novel bioactive compounds with therapeutic properties.

This study will be the first report of *Uwaria wrayi* (King) L.L. Zhou, Y.C.F. Su & R.M.K. Saunders (**Figure 1**) focusing on the isolation and identification of bioactive compounds with antimicrobial activity. The identification of bioactive compounds may lead to the discovery of novel antibacterial agents. This is particularly important in the context of the global challenge of antibiotic resistance, where new therapeutic options are urgently needed.

Materials and methods

General experimental procedures

The NMR spectra were recorded on a 400 Bruker spectrometer (Bruker ® Inc.). Mass spectra (MS) were measured by thin layer chromatography-mass spectrometers model Advion expression (ESI). Silica gel 60 (5 - 40 µm, SiliCycle® Inc.), silica gel 100 (63 - 200 µm, SiliCycle® Inc.), Sephadex LH-20, and Sep-Pak's (RP-18) were used to perform column chromatography. Analytical Thin layer chromatography (TLC) was performed with precoated plates of silica gel 60 F_{254} (Merck, USA). The reagents used for antimicrobial assays included Mueller-Hinton broth (MHB), Mueller-Hinton Agar (MHA), Brain Heart Infusion (BHI) broth, Brain Heart Infusion (BHI) Agar, and Rezasurin (7-hydroxy-10-oxidophenoxazin-10-ium-3-one, sodium).

Plant materials

The twigs and leaves of *U. wrayi* (**Figure 1**) were collected in June 2021 from Narathiwat province, Thailand (N:6.158263°, E: 101.667274°). This plant was identified by Mr. Abdulromea Baka (Independent Research Group on Plant Diversity in Thailand, Sichon, Nakhon Si Thammarat, 80120, Thailand). A voucher specimen (MFU-NPR0217) was deposited at the Natural Products Research Laboratory of Mae Fah Luang University.

Figure 1 Flower, fruit, and leaves of *U. wrayi* (these pictures were taken by Mr. Abdulromea Baka).

Extraction and isolation

Air-dried twigs of *U. wrayi* (665.8 g) were extracted with EtOAc (3×5 L) at room temperature and concentrated under reduced pressure to give an EtOAc extract (16.7 g). This extract was separated by quick column chromatography (QCC) eluting with a gradient solvent system of hexanes-acetone (1:0 to 0:1, v/v) to afford 5 subfractions (UWT1-UWT5). Fraction UWT2 (4.2 g) was subjected to column chromatography (CC) over silica gel (3:7 v/v, EtOAc-CH_2Cl_2) to obtain compounds **7** (3.6 mg) and **8** (4.1 mg). Fraction UWT3 (2.9 g) was purified by silica gel CC eluting with EtOAc-CH_2Cl_2 (1:4, v/v) to give compounds **1** (6.1 mg) and **2** (2.5 mg). Compounds **3** (3.6 mg) and **6** (2.8 mg) were isolated from fraction UWT5 (958.2 mg) by silica gel CC eluting with EtOAc -CH_2Cl_2 (3:17 v/v) (**Figure 2**).

Air-dried leaves of *U. wrayi* leaves (1.9 kg) were extracted with EtOAc (3×20 L) at room temperature and concentrated under reduced pressure to yield the EtOAc extract (107.6 g). This extract was subsequently isolated by QCC over silica gel (hexanes-EtOAc, 1:0 to 0:1, v/v) to give 9 fractions (UWL1-UWL9). Fraction UWL4 (1.4 g) was further purified by CC (1:4, v/v, EtOAc- hexanes) to obtain compound **4** (2.5 mg). Fraction UWL5 (1.2 g) was isolated by CC over silica gel (1:4, v/v, EtOAc-CH_2Cl_2) to give compound **9** (2.2 mg). Meanwhile, compound **5** (2.3 mg) was obtained from fraction UWL6 (825.6 mg) by CC over silica gel (1:9 v/v, EtOAc -CH_2Cl_2) (**Figure 2**).

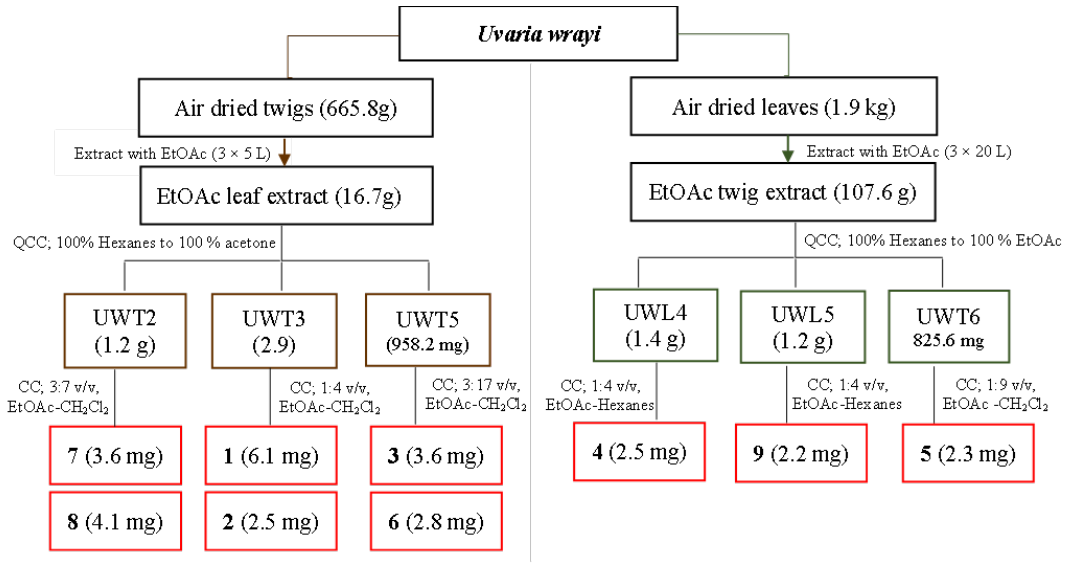

Figure 2 Scheme of the extraction and isolation of compounds **1-9**.

Antimicrobial activity

Minimal inhibitory concentration (MIC) assay

The resazurin-based MIC assay used the microdilution method, modified based on a previous report [13], to determine the minimum concentration of compounds inhibiting microorganism growth. The procedure commences with the preparation of serial dilutions of the isolated compounds in a 96-well plate, forming a concentration gradient. Then, the chosen microorganism was incubated at the appropriate temperature and time (37 °C for 24 h). After incubation, prepare a fresh 0.015 % filtered sterile resazurin solution in sterile water. Add 30 µL of the resazurin solution to each well plate and observe the color of each well compared to the control wells after 3 h. The minimum inhibitory concentration (MIC) was determined by visual inspection of resazurin-based broth microdilution assays. The lowest concentration of the test substance in wells exhibiting no color change (indicating no microbial growth) was considered the MIC. The experiment was performed in triplicate and the concentration most frequently inhibited growth was reported as the MIC. The antibacterial activity was determined against 5 Gram-positive bacteria, including *Staphylococcus aureus* (TISTR 746), *Staphylococcus epidermidis* (DMST 15505), Methicillin-resistant *Staphylococcus aureus* (MRSA) (NPRC 001R), *Cutibacterium acnes* (formerly *Propionibacterium acnes*) (DMST 14916), and *Streptococcus mutans* (DMST 18777/ATCC 25175T), along with 2 Gram-negative bacteria, *Shigella flexneri* (DMST 4423) and *Salmonella enterica* ser. *typhimurium* (TISTR 2519).

Minimum bactericidal concentration (MBC) assay

After obtaining a positive MIC result using the resazurin-based assay in a 96-well plate, the Minimum Bactericidal Concentration (MBC) can be determined using prior study methodologies [14]. Identify the MIC well by referring to the MIC assay plate and locating the well containing the lowest concentration of the test agent that inhibited visible microbial growth. Then, using a sterile pipette, transfer small aliquots (10 µL) of broth from the MIC well and 1 or 2 wells with higher concentrations (showing no growth inhibition) onto agar. Ensure even distribution of the aliquot on the agar surface. After that, incubate under appropriate conditions for the tested microorganism (37 °C for 18 - 24 h). After incubation, inspect agar plates for colony growth. The MBC is determined as the lowest concentration of the test agent, where no

visible growth is observed on the corresponding agar plate. The experiment was performed in triplicate, and the concentration most frequently associated with a lack of growth was reported as the MBC.

Results and discussion

Compounds isolated from leaves and twigs of *U. wrayi*

The leaf and twig extracts of *U. wrayi* underwent separate phytochemical investigations using various chromatographic techniques. This process led to the identification of 9 known compounds (**Figure 3**) classified as 3 polyoxygenated cyclohexenes (**1**-**3**), a *seco*-cyclohexenes (**4**), 2 acrylamide derivatives (**6,7**), and 3 aristolactam alkaloids (**8**-**9**). In the following, compounds **1**-**3** and **6**-**8** were isolated from leaf extract, while compounds **4**, **5**, and **9** were obtained from twig extract. The isolated compounds are identified as zeylenol (**1**) [15], ellipeiopsol B (**2**) [16], zeylenone (**3**) [17], microcapin B (**4**) [18], (2*E*)-3-(3,4-dihydroxyphenyl)-*N*-[2-(4-methoxyphenyl) ethyl]-2-propenamide (**5**) [19], *N*-*trans*-feruloyl tyramine (**6**) [20], oldhamactam (**7**) [21], aristolactam AIa (**8**) [22], and aristolochic acid-D methyl ether lactam (**9**) [23]. This identification was achieved by meticulously comparing their ^{1}H NMR and mass spectroscopic data with information available in the existing scientific literature.

Compounds **1**-**3** are classified as polyoxygenated cyclohexenes, indicating the presence of multiple oxygen atoms incorporated into the cycerialohexene ring [24], which displayed the double bond at C-4 and C-5. All of these structures contained 2 units of benzoyl groups at C-5 and C-7. The structures of **1** and **3** were different at C-3, where the structure of **1** was a hydroxy group and compound **3** was a carbonyl group. On the other hand, the different structures of compounds **1** and **2** were found at C-2 (hydroxy group for **1** and hydrogen atom for **2**). In a previous investigation, compound **1** had been reported from the stem of *Uvaria grandiflora* [15] and the root of *Uvaria zeylanica* [25]. Compound **2** was obtained from the aerial parts of *Ellipeiopsis cherrevensis* and leaves of *Piper betle*, while compound **3** was found in the leaves of *U. grandiflora* [26]. Compound **4** was classified as a *seco*-cyclohexene core structure. They contained 2 units of benzoyl groups, the same as compounds **1**-**3**. The main skeleton for these compounds was a 3-heptene. This compound has been reported from *Uvaria macrocarpa* [27] and *U. rufa* [18]. In the case of compounds **5** and **6**, they were acrylamide derivatives which have been reported from *Uvaria lurida* [19] and *Dasymaschalon rostratum* [28], respectively. The chemical structures of these 2 compounds displayed the *N*-substituents as *N*-(3-hydroxy-4-methoxyphenethyl) for **5** and *N*-(4-hydroxyphenethyl) for **6**, and the substituents for acryl unit were 4-hydroxyphenyl and 3-hydroxy-4-methoxyphenyl for **5** and **6**, respectively.

Compounds **7**-**9**, aristolactam alkaloids, were commonly found in various genera of the Anonaceae. Compound **7** has been isolated from the stems of *Dasymaschalon trichophorum* [29] and *Fissistigma oldhamii* [21]. Compound **8** was obtained from *Fissistigma glaucescens* [30] and *Aristolochia esperanzae* [31], while compound **9** was found in *Aristolochia indica* [32]. The structure of compound **7** contained full substituents on A-ring with 3 methoxy groups, while compound **8** was a hydroxy and a methoxy group at C-2 and C-3, respectively. In the case of compound **9**, it displayed a methylenedioxy group at C-2/C-3. The different structures of these 3 compounds were also found on the D-ring. Compounds **7** and **8** showed the hydroxy group at the same position, C-8, whereas compound **9** was the dimethoxy group at C-6 and C-8.

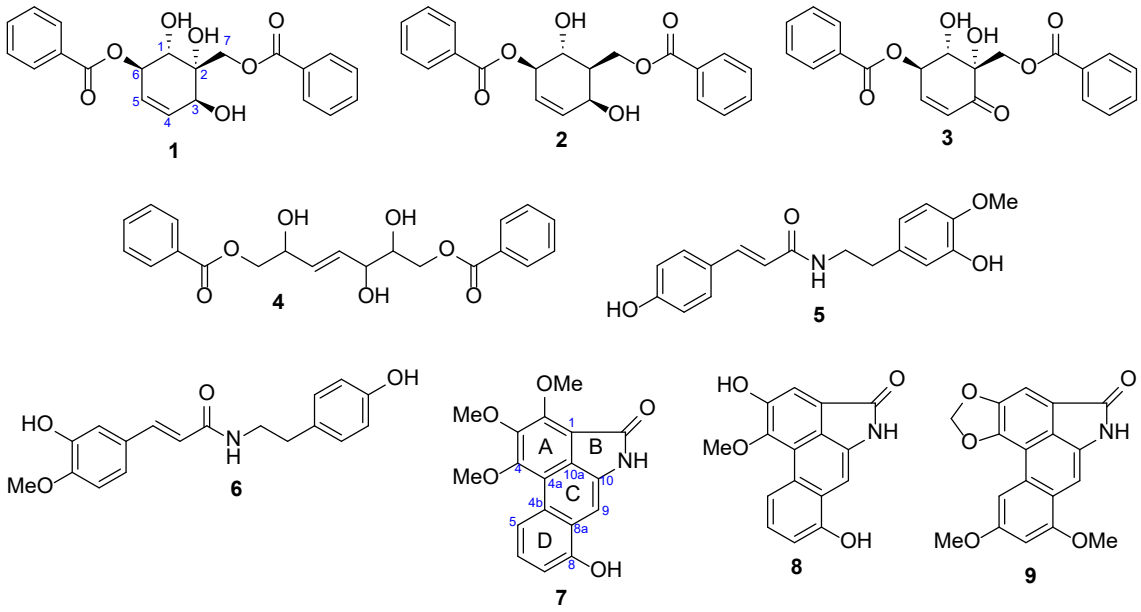

Figure 3 Structure of compounds **1**-**9**.

Antimicrobial activity of compounds isolated from *U. wrayi* leaves and twigs

All isolated compounds were evaluated for their antimicrobial activity against Gram-positive bacteria (*S. aureus*, *S. epidermidis*, MRSA, *C. acnes*, and *S. mutans*) and Gram-negative bacteria (*S. flexneri* and *S. enterica* ser. *typhimurium*) as shown in **Table 1** and **Figure 4**. Compounds **2**-**9** showed good antibacterial activity against *C. acnes* with a MIC value of 32 µg/mL. Most compounds displayed moderate antimicrobial activity against all strains of bacteria with the same MIC value of 64 µg/mL, except for **1** and **9**, which showed weak antibacterial activity against MRSA with the MIC value of 128 µg/mL. The lowest inhibitory concentration of each compound leads to the continuation of the MBC assay to determine the actual agent required to kill a particular bacterium. The outcome revealed that compounds **1-3** and **5-7** responded to inhibit *C. acnes* with the MBC value of 128 µg/mL. The results were consistent with prior studies that have been conducted on crude extracts of *Uvaria* species. For example, an ethanol root extract of *Uvaria chamae* is found to exhibit strong antibacterial activity, particularly against gram-positive multidrug-resistant species [12], extracts obtained from the stem bark and leaves of *Uvaria scheffleri*, including petroleum ether, dichloromethane, and ethanolic extracts, demonstrated antibacterial activity against *S. aureus*. Notably, the dichloromethane extract from the leaves exhibited the highest antibacterial activity against Gram-positive bacteria [33]. Based on the provided information, it is clear that *Uvaria* species have the capacity to suppress various types of microorganisms. The structure-activity relationship (SAR) of polyoxygenated cyclohexenes (**1-3**) was analyzed, focusing on their antibacterial activity against MRSA and *C. acnes*. Compound **1** demonstrated the ability to inhibit MRSA and *C. acnes* at a MIC value of 128 µg/mL and 64 µg/mL, respectively. Compound **2** was a reducing isomer of compound **1** at C-2 (H atom for compound **2** and OH for compound **1**). In contrast, compound **3** was an oxidizing isomer of compound **1** at C-3 (C=O for compound **3** and OH for compound **1**). The slightly different functional group is much more effective in antibacterial activity, in which compounds **2** and **3** have enhanced 2-fold antibacterial activity against MRSA (64 µg/mL) and *C. acnes* (32 µg/mL) compared to that of compounds **1**.

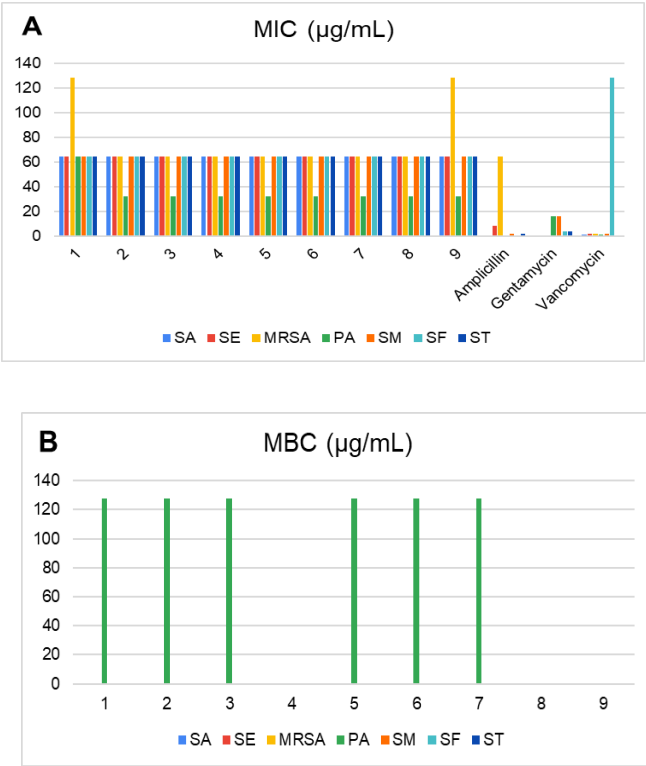

Figure 4 Antimicrobial activity diagrams display the Minimum Inhibition Concentrations (MIC) (A) and Minimum Bactericidal Concentration (MBC) (B) of compounds **1-9**. (SA = *Staphylococcus aureus* (TISTR 746); SP = *Staphylococcus epidermidis* (DMST 15505); MRSA = Methicilin Resistant *Staphylococcus aureus* (NPRC 001R); PA= *Cutibacterium acnes* (*Propionibacterium acnes*) (DMST 14916); SM= *Streptococcus mutans* (DMST 18777/ATCC 25175T); SF= *Shigella flexneri* (DMST 4423); ST= *Salmonella enterica* ser. *typhimurium* (TISTR 2519))

Table 1 Antimicrobial activity of compounds **1-9**.

Compound	MIC (µg/mL)							MBC (µg/mL)						
	Gram-positive					Gram-negative		Gram-positive					Gram-negative	
	SA	***SE***	***MRSA***	***PA***	***SM***	***SF***	***ST***	***SA***	***SE***	***MRSA***	***PA***	***SM***	***SF***	***ST***
1	64	64	128	64	64	64	64	Inactive	Inactive	Inactive	128	Inactive	Inactive	Inactive
2	64	64	64	32	64	64	64	Inactive	Inactive	Inactive	128	Inactive	Inactive	Inactive
3	64	64	64	32	64	64	64	Inactive	Inactive	Inactive	128	Inactive	Inactive	Inactive
4	64	64	64	32	64	64	64	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
5	64	64	64	32	64	64	64	Inactive	Inactive	Inactive	128	Inactive	Inactive	Inactive
6	64	64	64	32	64	64	64	Inactive	Inactive	Inactive	128	Inactive	Inactive	Inactive
7	64	64	64	32	64	64	64	Inactive	Inactive	Inactive	128	Inactive	Inactive	Inactive
8	64	64	64	32	64	64	64	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
9	64	64	128	32	64	64	64	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive	Inactive
Amplicillin	0.025	8	64	0.125	2	-	2	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Gentamycin	0.5	0.025	-	16	16	4	4	N/A	N/A	N/A	N/A	N/A	N/A	N/A
Vancomycin	1	2	2	1	2	128	-	N/A	N/A	N/A	N/A	N/A	N/A	N/A

*Inactive at >128 µg/mL. *SA* = *Staphylococcus aureus* (TISTR 746); *SP* = *Staphylococcus epidermidis* (DMST 15505); *MRSA* = Methicilin Resistant *Staphylococcus aureus* (NPRC 001R); PA= *Cutibacterium acnes* (*Propionibacterium acnes*) (DMST 14916); SM= *Streptococcus mutans* (DMST 18777/ATCC 25175T); SF= *Shigella flexneri* (DMST 4423); ST= *Salmonella enterica* ser. *typhimurium* (TISTR 2519)

Conclusions

In conclusion, the first phytochemical investigation and antimicrobial activity of their isolated compounds resulted in the isolation and identification of 9 antimicrobial compounds, including 3 polyoxygenated cyclohexenes, 1 *seco*-cyclohexene, 2 acrylamide derivatives, and 3 aristolactam alkaloids. All of these compounds displayed a broad spectrum of antimicrobial activity. In particular, they showed the most effective antibacterial activity against *C. acnes*. These results could pave the way for future research and provide a foundation for understanding the potential applications of this plant in cosmeceutical, medicine, pharmacology, or other fields. This study provides valuable insights into the potential antimicrobial activities of *U. wrayi*. However, it is important to note that further research is needed to understand their mechanisms of action and assess their safety and efficacy in various settings. As with any natural product, caution should be exercised, and additional studies are necessary to fully elucidate the therapeutic potential of *U. wrayi* in the context of antimicrobial applications.

Acknowledgments

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