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Antigonococcal Activity of (+)-Medicarpin

Daniel Williams, Dreyona Perry, James Carraway, Shaquwana Simpson, Pascaline Uwamariya, and Omar E. Christian*

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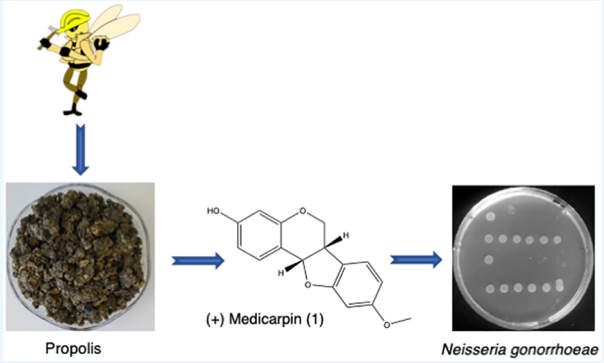
ABSTRACT: Antibiotics are the primary drugs for combating *Neisseria gonorrhoeae* infections, but with evolving antibiotic resistance of this bacterium, new druggable molecules are needed to stem the tide of this impending public health crisis. Propolis has long been recognized for its antimicrobial properties, being composed of secondary metabolites with antibacterial potential. We herein describe the evaluation of a Jamaican multifloral propolis for antibacterial activity against *N. gonorrhoeae.* The bioassay-guided evaluation of the ethyl acetate extract yielded (+)-medicarpin (**1**), whose final structure was elucidated based on spectral analysis and comparison with the known metabolites. Compound (**1**) selectively inhibited *N. gonorrhoeae* with a minimum inhibitory concentration value of 0.25 mg/mL, showing an additive effect against *N. gonorrhoeae* when combined with vancomycin.



INTRODUCTION

Neisseria gonorrhoeae (GC) is a Gram-negative bacterium that causes the third most reported sexually transmitted infection, with 800 000 cases reported in the United States and over 87 million cases estimated worldwide.[1] Due to its frequency of infection, ability to evade the host immune system, and increasing resistance to antibiotics, *N. gonorrhoeae* continues to be a public health problem. The increased number of GC infections is due in part to its increased antibiotic resistance. There is a growing concern regarding its antibiotic resistance, especially in underdeveloped countries that require expensive drugs, such as ceftriaxone and azithromycin, for effective treatment.[2] If left untreated, gonorrhea can cause pelvic inflammatory disease and sequelae such as chronic pelvic pain, infertility, and ectopic pregnancy. To combat this impending public health crisis, more effective antimicrobials are needed to effectively inhibit the proliferation of *N. gonorrhoeae.* Natural products continue to be the single most encouraging model for the discovery and development of new therapeutics.[3−5] Over the past 39 years, natural products or natural product-derived entities have accounted for 48% of the new antibacterial drugs approved for use in the Unites States.[3]

Propolis is well recognized for its natural antimicrobial properties[6−8] and, more recently, propolis has been used to treat infections and potentiate wound healing.[9] Propolis is a resin composed of beeswax, saliva, and plant exudates collected by bees (*Apis mellifera*) that serve as a waterproofing layer to protect the hive from microbial invasion and oxidative damage.[10] The phytochemical content of propolis reflects the metabolite profile of the plants from which the bees foraged.[11] Studies have shown significant chemogeographical variations in the composition and antibacterial properties of propolis from tropical regions. Central and South American collections harbor prenylated benzophenones, which are absent in North American, European, and Asian propolis.[12−14] There are over 300 known compounds from various collections of propolis.[15] Terpenes, polyphenolic compounds, flavonoids, and their derivatives isolated from various propolis collections have displayed a vast array of biological activities ranging from antibacterial to antitumor, antiviral, and immunomodulatory activity.[16−18] There have been various studies addressing the effectiveness of propolis against commonly encountered bacterial strains such as *Escherichia coli*, *Salmonella enterica*, *Staphylococcus aureus*, *Aspergillus niger*, and *Candida albicans*,[9,19] However, there are currently no data on the effectiveness of Caribbean propolis against *N. gonorrhoeae*. In our continuing efforts to expand the range of metabolites active against *N. gonorrhoeae,* we have investigated a Jamaican collection of multiflora propolis against *E. coli*, *S. aureus*, and *N. gonorrhoeae*. The initial bioautography screens of crude extracts followed by a bioassay-guided separation of the ethyl

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acetate extract resulted in the isolation of (+)-medicarpin (**1**). We herein describe the isolation and biological evaluation of (+)-medicarpin (**1**) against *N. gonorrhoeae*, *E. coli*, *and S. aureus*.

RESULTS AND DISCUSSION

The ethyl acetate extract displayed antibacterial activity in preliminary bioautography screening. The bioassay-guided fractionation of the ethyl acetate extract resulted in the isolation of the known pterocarpan (+)-medicarpin (**1**). Compound **1** displayed a $[M + H]^+$ peak of 271.09753 in the high-resolution ESI TOF consistent with molecular formula $C_{16}H_{14}O_4$. The petrocarpan core was established based on the characteristic four contiguous protons: 3.56 ppm (m, H-6*β*), 4.22 ppm (dd, 10.8, 4.8, H-6*α*), 3.51 ppm (m, H-6a), and 5.50 ppm (d, 6.7, H-11a) and ^{13}C NMR signals at 66.5 for C6, 40.0 for C6a, and 78.9 for C11a. Additionally, analysis of the 2D spectra (heteronuclear single-quantum coherence, heteronuclear multiple bond correlation, correlation spectroscopy, and total correlation spectroscopy) enabled the complete assignment of **1** (Table 1). Finally, the

Table 1. Comparison of the ^{13}C NMR Data of Medicarpin (1) with Synthetic (+)-Medicarpin (1) and (−)-Medicarpin (2)

	(+)-medicarpin (**1**)	synthetic (+)-medicarpin (**1**)	(−)-medicarpin (**2**)
1a	112.2	112.8	112.8
1	132.2	132.4	133.1
2	106.4	106.7	107.3
3	157.5	157.3	160.0
4	103.7	103.9	104.4
4a	156.6	156.8	158.8
6	66.5	66.7	67.5
6a	39.5	39.7	40.8
7a	119.2	119.3	120.8
7	124.8	125.0	125.9
8	110.0	110.0	110.7
9	160.6	160.8	162.0
10	96.9	97.1	97.5
10a	161.1	161.3	162.5
11a	78.7	78.8	80.0
12	55.9	55.7	55.9

comparison of the experimental and literature values for (+)-medicarpin (**1**) is almost identical with the only noticeable variations occurring at positions 6a and 11a of (−)-medicarpin (**2**) (Figure 1).[20]

(+)-Medicarpin (**1**) has been isolated from several sources including *Machaerium aristulatum*,[21] *Sophora japonica*,[22] and Brazilian red propolis.[23] It inhibits the proliferation of osteoclasts, indicating some potential as a treatment for estrogen-sensitive osteoporosis.[24] It also displays a wide range

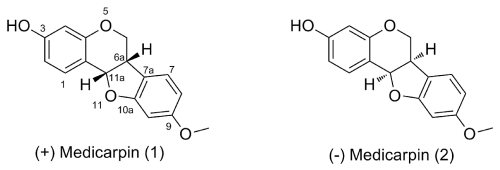

Figure 1. Structures of (+)-medicarpin (**1**) and (−)-medicarpin (**2**).

of antimicrobial activity and is responsible for much of the antifungal activity observed in red Brazilian propolis.[25]

Antibacterial Activity of Jamaican Propolis Extracts. In this study, the antibacterial activity of ethyl acetate extracts from the propolis was determined by a twofold minimum inhibitory concentration (MIC) spot dilution assay. The extract was separated into several fractions (A−I; Figure 2

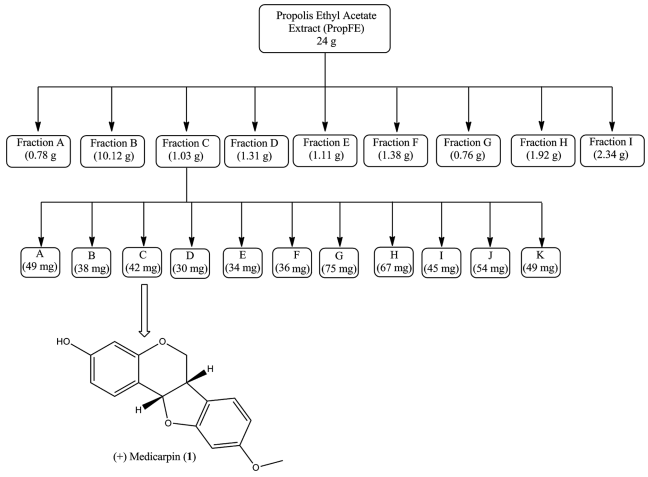

Figure 2. Schematic representation of the normal phase chromatographic isolation of (+)-medicarpin (**1**).

Table 2. Antibacterial Activity of Ethyl Acetate Extracted Fractions[a]

fractions	MIC (mg/mL)[b]		
	N. gonorrhoeae	*E. coli*	*S. aureus*
A	–	–	–
B	–	–	–
C	0.50	–	–
D	0.78	–	–
E	0.92	–	–
F	0.30	–	12.5
G	0.56	–	25
H	0.85	–	–
I	1.79	–	–

[a]Notes: –, not inhibited at the highest tested concentration (25 mg/mL). [b]Values were the averages of three or more readings.

and Table 2) that were assayed for antibacterial activity against *S. aureus*, *E. coli*, and *N. gonorrhoeae*. Fractions A and B did not demonstrate antibacterial activity against any of the tested strains. However, *S. aureus* was inhibited by fractions F and G at 12.5 and 25 mg/mL, respectively (Figure 2 and Table 2). Consequently, other studies have shown that *S. aureus* was more susceptible (0.032−1.2 mg/mL) to propolis extracts from a variety of geographical locations.[12,26] It has been suggested that geographical locations may have an enormous impact on the composition of propolis, which in turn may affect its antibacterial activity.[12] Unlike *S. aureus*, the propolis extracts (25 mg/mL) did not inhibit the growth of *E. coli*. However, *N. gonorrhoeae* was the only strain tested that was susceptible to fractions C−I over a broad concentration range (0.5−1.79 mg/mL) (Table 2). The lowest level of susceptibility determined was to fraction F for *N. gonorrhoeae* and *S.*

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aureus at 0.3 and 12.5 mg/mL, respectively. This indicates that *N. gonorrhoeae* is significantly more susceptible to the propolis extract than *S. aureus* and *E. coli* (~42-fold and 83-fold, respectively). We did not test *E. coli* above 25 mg/mL; therefore, it is possible that *E. coli* may be susceptible to the extract at a higher concentration. Therefore, *N. gonorrhoeae* is significantly more susceptible to this multifloral Jamaican propolis sample than *S. aureus* and *E. coli*.

Based on the initial selectivity, sample size, and initial metabolite profile, we prioritized fraction C for further purification to identify the compound responsible for the observed antibacterial activity. As a result, (+)-medicarpin (**1**) was isolated and the MIC was determined for *S. aureus*, *E. coli*, and *N. gonorrhoeae* in a separate assay (Table 3). Compound **1** does not display antibacterial activity against *S. aureus* and *E. coli* at the highest concentration tested (5 mg/mL) but does selectively inhibit *N. gonorrhoeae* at 0.25 mg/mL (Table 3).

Table 3. Antibacterial Activity of the Purified Metabolite, (+)-Medicarpin (1), against Gram-Positive and Gram-Negative Bacteria[a]

purified compound	MIC (mg/mL)[b]		
	N. gonorrhoeae	*E. coli*	*S. aureus*
(+)-medicarpin (1)	0.25	–	–

[a]Notes: –, not inhibited at the highest tested concentration (5 mg/mL). [b]Values were the averages of three or more readings and a standard error of the mean of 0.02.

Evaluating the Antibacterial Activity of (+)-Medicarpin (1) Combined with Vancomycin. The increasing resistance to single-dose and multidrug therapeutics suggests that bacteria are becoming a major public health issue. Gram-negative bacteria present possibly an even larger public health problem because many antibiotics are unable to penetrate their outer membrane. The use of natural products combined with conventional antibiotics may help increase the number of treatment options and improve efficacy. To this end, a checkerboard assay was performed to evaluate whether (+)-medicarpin (**1**) would reduce the concentration of vancomycin, glycopeptide antibiotic not normally used to treat Gram-negative bacteria, required to inhibit the growth of *N. gonorrhoeae*. A fractional inhibitory concentration index (FICI) was determined for (+)-medicarpin (**1**) that ranged from 0.7 to 1 mg/mL for 4 and 24 h incubation periods, respectively. As a result, (+)-medicarpin (**1**) used in combination with vancomycin was additive in inhibiting *N. gonorrhoeae* at low concentrations. Although the use of **1** and vancomycin were only additive (FICI of 0.5–4) against *N. gonorrhoeae*, the FICI value of 0.7 was close to the FICI synergistic value of <0.5, which suggests a modest impact on the efficacy of vancomycin. Wink and co-workers demonstrated that propolis collected from different European countries acted in synergy with vancomycin against several Gram-positive bacteria.[12] In the previous study, *Helicobacter pylori* were the only Gram-negative bacteria tested using propolis from European countries in combination with levofloxacin that showed susceptibility. This is not surprising given that several studies have indicated that propolis is more effective against Gram-positive than Gram-negative bacteria.[27] However, we demonstrate that *N. gonorrhoeae* is significantly more susceptible than *S. aureus* and *E. coli*, and when vancomycin is used with propolis, the MIC of vancomycin required to inhibit *N. gonorrhoeae* was reduced. Although the FICI values of combining both compounds were not synergistic, there appears to be an impact on the efficacy of vancomycin against *N. gonorrhoeae*. The additive activity observed with vancomycin is promising and could potentially lead to improved drug combinations to combat the resistance being observed in the treatment of gonorrhea worldwide.

MATERIALS AND METHODS

General Experimental Procedure. All one-dimensional and two-dimensional NMR spectra were recorded in $CDCl_3$ on a Bruker AVANCE III NMR spectrometer at 400 MHz for ^1H and 100 MHz for ^{13}C. LCMS was performed on a reversed-phase analytical column (4.6 × 250 mm^2, 5 μm) using a photodiode array detector and with an electrospray single quadrupole mass spectrometer. High-resolution mass measurements were obtained on an Agilent 6230 ESI-TOF mass spectrometer. The samples were run in positive-mode ionization with a capillary voltage of 4000 V. The drying gas (nitrogen) temperature was 325 °C delivered at 10 L/min, and the fragmentation voltage was set to 150 eV. Optical rotation was determined on a Jasco DIP 370 polarimeter. MPLC separation was performed on a Reveleris system equipped with UV and ELSD detectors. All solvents were of HPLC grade with 0.1% TFA or ACS grade.

Collection. Propolis was collected from a commercial bee farm in July 2017 from Buff Bay, Portland Jamaica (GPS coordinates 18° 13′48″ N; 76° 39′53″ W). The samples were stored at −18 °C in an airtight container until analysis. A voucher specimen (JP17A) is preserved in the Department of Chemistry and Biochemistry at North Carolina Central University, Durham.

Organic Extraction. The propolis sample (750 g) was sequentially extracted with hexane (3 × 2 L), EtOAc (3 × 2 L), and MeOH (3 × 2 L). Evaporation of the solvents yielded three crude extracts hexane (100 g), EtOAc (55 g), and MeOH (60 g). A portion of the EtOAc extract (24 g) was adsorbed onto the silica gel and subjected to flash chromatography using a EtOAc–hexane gradient (0%–100%), and nine (9) major fractions (A–I) were collected. Further purification of fraction C via normal phase chromatography eluting with mixtures of EtOAc–hexane yielded compound **1**.

Bioautography Assay. Bioautography assay was adapted from Galindo-Cuspinera and Rankin.[28] Briefly, thin-layer chromatograms (TLCs) were developed at a solvent ratio of 80/20 or 50/50 (Hex/EtOAc). The dried TLC plates were placed facing down on the agar plates inoculated with 3 × 10^8 cfu/mL of gonococci for 30 min before being incubated overnight at 5% CO_2, 37 °C. After 24 h, the TLC plates were removed and the agar plates were visualized under a dissecting scope for clear zones, which indicated bacterial inhibition.

Antibacterial Susceptibility Assay. The MIC for *N. gonorrhoeae* was determined using a broth dilution method described by the National Committee for Clinical Laboratory Standards (NCCLS). Briefly, the NCCLS method was modified for growth of *N. gonorrhoeae* as follows: the cells were grown overnight on gonococcal agar plates containing Kellogg's supplements[29] at 37 °C, 5% CO_2 for 18 h. After overnight growth, the cells were inoculated in gonococcal broth (GCB) containing Kellogg's supplements and then diluted to a 0.5 McFarland turbidity standard of 1.5 × 10^8 cfu/mL. The cell suspension (90 μL) was seeded into a 96-well

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Table 4. Antibacterial Activity of (+)-Medicarpin (1) and Vancomycin against *N. gonorrhoeae*

strain	incubation time (h)	agent	MIC (mg/mL)[a]				
			alone	combined	FIC	FICI	interpretation
N. gonorrhoeae	4	(+)Med	0.750	0.313	0.4	0.7	additive
		Van	0.060	0.021	0.3		
	24	(+)Med	0.305	0.156	0.5	1	additive
		Van	0.033	0.018	0.5		

[a]Values were the averages of three or more readings.

plate containing either the purified secondary metabolite medicarpin (1) or enriched fractions isolated from the propolis sample, which was serially diluted 2-fold (0.002−25 μg/mL). After 18 h of incubation, 2 μL from each well was spotted on GC agar plates containing Kellogg's supplements and then incubated overnight at 37 °C, 5% CO_2 for 18 h. The MIC was recorded as the lowest concentration of the propolis extract that inhibited bacterial growth. Statistical significance was calculated based on replicates from three or more independent biological assays.

Checkerboard Dilution Assay. The synergistic effect between the purified propolis compound (+)-medicarpin (1) and vancomycin (Van), a glycopeptide antibiotic not usually effective against Gram-negative bacteria, was determined using a checkerboard dilution assay (Table 4). Cell cultures were prepared as described above for the antibacterial susceptibility assay. A 96-well plate containing gonococcal cells at a 0.5 McFarland turbidity standard of 1.5×10^8 cfu/mL supplemented with 2-fold serially diluted (+)-medicarpin (1) and vancomycin. All assays were incubated at 37 °C, 5% CO_2 for 18 h and spotted on GC agar plates. The FICI was determined using the following equation: FICI = (MIC of compound 1 and Van in combination/MIC of compound 1 alone) + (MIC of Van and compound 1 in combination/Van alone). Synergistic effects were grouped as follows: antagonistic (>4), indifferent (≥1−4.0), additive (≥0.5−1), and synergistic (≤0.5) based on the FICI.[12]

AUTHOR INFORMATION

Corresponding Author

Omar E. Christian − *Department of Chemistry and Biochemistry, North Carolina Central University, Durham, North Carolina 27707, United States;* orcid.org/0000-0003-4693-6675; Phone: 919-530-5134; Email: ochristi@nccu.edu

Authors

Daniel Williams − *Department of Biological and Biomedical Science, North Carolina Central University, Durham, North Carolina 27707, United States*

Dreyona Perry − *Department of Biological and Biomedical Science, North Carolina Central University, Durham, North Carolina 27707, United States*

James Carraway − *Department of Biological and Biomedical Science, North Carolina Central University, Durham, North Carolina 27707, United States*

Shaquwana Simpson − *Department of Chemistry and Biochemistry, North Carolina Central University, Durham, North Carolina 27707, United States*

Pascaline Uwamariya − *Department of Biology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.1c01590

Author Contributions

The manuscript was written through contributions of all authors. All authors have revised and approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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