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Differential antimicrobial extract activity of Graviola (*Annona muricata*) on gram positive and gram- negative antibiotic-resistant bacteria

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Abstract

New agents are needed with the increasing prevalence of multidrug-resistant (MDR) bacteria. Identification of plants with activity against MDR bacteria is of increasing importance. The antimicrobial activity of commercially available Graviola preparations against 38 clinical and laboratory isolates, representing MDR and drug-sensitive Gram positive and Gram negative bacteria, was measured by broth microdilution. Commercially Graviola fruit, as well as leaves/stems preparations, exhibited both inhibitory and cidal activity. However, the fruit preparation showed significantly less activity than the leaves/stems preparation. The Graviola leaves/stems preparation activity titer ranged from 1:4 to 1:16 against MDR Gram positive and Gram negative bacteria; including methicillin-resistant *Staphylococcus aureus* (MRSA), extended-spectrum β-lactamase (ESBL) *Escherichia coli, Acinetobacter baumannii*, and *Klebsiella pneumoniae* carbapenemase (KPC). However, the preparation had no effect on Lactobacillus. In addition, further extractions of the leaves/stems were tested against MRSA and MSSA in an in-vitro biofilm wound infection model. The polar extracts of the stems/leaves exhibited the highest bactericidal activity in preventing and inhibiting biofilm formation. Thus, Graviola leaves/stems extracts showed differential activity for phytochemical utility against multidrug-resistant bacteria.

Keywords: Multi-drug resistance (MDR); Natural product; Antimicrobial; Biofilm; Wound management

1. Introduction

Antibiotic resistance is responsible for a significant rise in global morbidity and mortality and is one of the greatest public health challenges. In the U.S. alone, at least 2 million individuals develop community or nosocomial antibiotic-resistant infections with an associated mortality rate of ~23,000/year [1]. Furthermore, it is estimated that by 2050, the deaths due to MDR microbes will exceed that of cancer and diabetes combined [2]. The economic impact of extended hospital stays with associated high healthcare costs is coupled with the rise in MDR-associated morbidity and mortality [3, 4]. Unfortunately, the development of novel synthetic or semi-synthetic effective pharmaceuticals has yet to keep pace with the increase in multidrug-resistant (MDR) bacterial infections. One avenue for addressing the need for additional effective antimicrobial is to expand the exploration of the activity of phytomedicinal compounds. One promising natural plant is the tropical tree *Annona muricata* (Graviola, Portuguese; soursop, English). Products from the tree, e.g., fruit, leaves, and stems, have been used as part of indigenous medicines across the globe. While most phytomedicinal studies focus on defining *A. muricata* anti-cancer properties [5-7], limited early studies support traditional medicine findings that *A. muricata* also has antimicrobial activities [8-18].

In traditional medicine, the fruit and leaves of *A. muricata* are used to treat diarrhea and cutaneous infections [19-21]. However, which plant component exhibits optimal antimicrobial activity and its spectrum of activity has been poorly

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defined. This study aimed to determine the antimicrobial and anti-biofilm activity of ethanol (commercial) and polar extracts of *A. muricata* and compare their activity to that of organic extracts.

2. Material and methods

2.1. Plant preparations and extracts

Commercial preparations (ethanol extracts) of Graviola leaves/stems (GLS) and Graviola fruit (GF) were used (Rainforest Pharmacy, Miami, FL). Both formulations are reported by the manufacturer to contain a minimum dry herb potency ratio 1:3 suspended in alcohol (50-60%). To confirm the amount of material in the different preparations, the alcohol in the GLS and GF (5 ml) was evaporated (25°C) and dry weight was determined (GLS 37.28 mg ml^{-1}, GF 5.64 mg ml^{-1}). Both GLS and GF were resuspended in 60% EtOH to 5.64 mg ml^{-1} EtOH. All preparations were filtered (0.22 μm) before use and stored at 4 °C.

2.2. GLS polar and organic extractions

Polar and organic extracts were prepared from the commercial GLS preparation. All solvents used were obtained from Fisher Scientific Company. Initially, the EtOH from the commercial preparation was removed *in vacuo*. The residue was dissolved in methanol:water (4:1), then extracted with ethyl acetate. The ethyl acetate layer (organic extract) and water layer (polar extract) were separated and evaporated *in vacuo* in a pre-weighed flask. Both extractions were suspended in dimethyl sulfoxide (DMSO) to equivalent concentrations and stored at room temperature in the dark.

2.3. MBC determination

The identity of all isolates used were confirmed by standard biochemical testing then stored frozen at -80°C until use. The MDR isolates were validated and a generous gift from the lab of Paul Schreckenberger, Loyola School of Medicine. Isolates were also screened for β-lactamase production, as indicated (Table 1), by the cefinase disc test (B.D. Microbiology Systems) according to the manufacturer's instructions. For testing, the organisms were grown overnight on brain heart infusion (BHI; Difco) agar or sheep blood agar (Troy Biologicals). Standard microdilution antibiotic testing methodology was used [22]. Briefly, colonies from the BHI, or sheep blood agar cultures, were suspended in Muller-Hinton (MH) broth to the equivalent of a 0.5 McFarland Standard (0.132 Abs 600). These broth suspensions (100 μl) were added to Graviola commercial preparations or extracts (100 μl; 96 well V-bottom plates; Costar) which were serially diluted in MH Fruit and leaves/stems preparations were standardized to contain equivalent amounts of plant material. After incubation (24 h, 37°C), 5 μl of each well was placed onto MH agar and the number of colonies was counted after incubation (24 h, 37°C) (relative accuracy >200 CFU ml^{-1}). All tests were done in duplicate and repeated at least once.

2.4. Biofilm inhibition

2.4.1. In vitro wound biofilm inhibition model

To determine whether the commercial ethanol preparation or polar/organic extracts had activity in an *in vitro* model for wound infections, the model of Hammond *et al*. was used with slight modifications [23]. Briefly, a bacterial suspension (PBS; pH 7.2; 0.132 Abs 600), prepared as described above, was diluted to yield the equivalent of 100-1,000 colony forming units (CFU) in 5 μl. For each assay, CFU present was confirmed by standard spread plate enumeration. Groups of three sterile antibiotic discs (6 mm) were placed on MH agar plates and positioned so that they could be covered by the 1x1" pad of a 1x3" adhesive bandage (BandAid™) (Figure1). Various dilutions of GLS, GLS-polar, and GLS-organic extracts, or their diluent vehicle (ethanol: GLS or DMSO: GLS-polar and GLS-organic), were applied to an adhesive bandage (300 μl/ adhesive bandage) and immediately placed over a group of 3 discs. Untreated control discs consisted of adhesive bandages with added PBS (300 μl). To ensure contact between the discs and the adhesive bandage, equal weights were placed on the adhesive bandage. Plates were then incubated (24 h; 37°C), after which the adhesive bandages were removed, and each disc vortexed (12 min.; 1 ml PBS) before CFU determination by plating onto Luria agar. Experiments were performed in triplicate and repeated once.

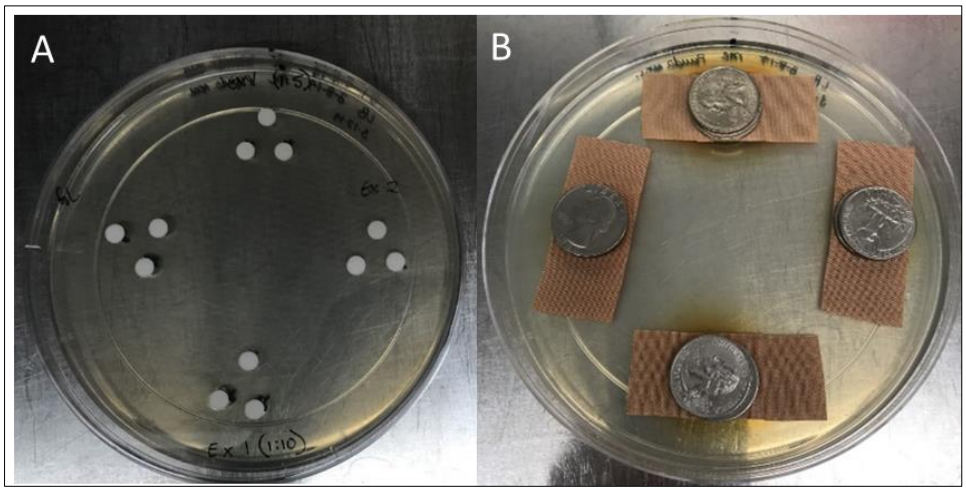

Figure 1 *In vitro* biofilm model of antimicrobial testing. (A) A Group of three replicate discs was inoculated with bacteria. (B) The discs were covered with Band-Aids and treated with Graviola, diluent, or control (PBS). Two quarters were used as a weight on top of the Band-Aids

2.4.2. In vitro wound biofilm disruption model

The biofilm disruption model mimics the ability to treat preformed biofilms in wounds. The inoculated disks were pre-incubated (24 h; 37°C) to allow the development of a mature biofilm. The disks were covered with the adhesive bandage containing the Graviola extracts as described above, followed by re-incubation (24 h; 37°C). The number of CFU $disc^{-1}$ was determined as described above for biofilm inhibition model. Experiments were performed in triplicate and repeated once.

3. Results and discussion

Early studies indicated that the annonaceous acetogenins, flavones, flavonoids, and tannins present in Graviola had potential antimicrobial activity [19-21]. The range of Graviola activity was determined by testing commercial ethanolic extract against various microbes, including multidrug-resistant organisms (Table 1). The commercial ethanol extract preparation of Graviola leaves/stems exhibited two-fold, or greater, antimicrobial activity compared to the fruit preparation. This activity included both MDR and drug-sensitive strains of Gram positive and Gram negative bacteria. The most exciting finding was that although Graviola shows broad spectrum antimicrobial activity, it did not affect the members of the microbiome tested, i.e., *Lactobacillus* and *Enterococcus.* Lactobacilli are beneficial members of the mucosal human microbiome, providing protection against colonization by pathogens. Although *E. faecalis* can cause significant problems, especially in a nosocomial environment, particularly in debilitated and/or immunocompromised individuals, they are still considered low virulence pathogens that are common colonizers of the gastrointestinal tract and other mucosal surfaces. This difference in the sensitivity of some of the Gram positive and Gram negative pathogens combined with the relative resistance of the lactobacilli and enterococci would be of advantage in adapting Graviola for treatment since a typical disadvantage in the use of antibiotics is a disruption in the homeostasis of the gut microbiome, resulting in either overgrowth of opportunistic pathogens, e.g., *Candida* and *Clostridioides difficile* or reduce protection against pathogen colonization.

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Since the relative plant concentration in each preparation was standardized, the difference in antimicrobial effects between leaves/stems and fruit extracts is most likely attributable to relative levels of active phytochemicals present. Although both Graviola fruit and leaf extracts contain annonaceous acetogenin, alkaloids, and phenols, only flavonol triglyceride and megastigmane have been found in the leaves. Because the GLS contributes to the overall better performance, the ethanolic (commercial) leaves/stems were extracted to their polar and organic extractions for further study. The antimicrobial activity GLS-polar showed significantly higher activity than the GLS-organic preparation (data not shown) and was subsequently tested against MDR microbes that span CDC list of urgent threat and significant risk

organisms (Table 2) [1]. The minimum extract concentration with cidal activity against CDC organisms of urgent (KPC) or significant risk (*Acinetobacter baumannii* and *Pseudomonas aeruginosa* ESBL) was 65 μg ml^{-1} of the polar extract. Interestingly, this concentration was below the Gram negative organisms' control (89 μg ml^{-1}). *Lactobacillus* was the most resistant to GLS-polar as determined for just the commercial ethanolic leaves/stems preparations. The MBC of *Lactobacillus* ranged from 65.6-fold above that of methicillin-resistant *Staphylococcus aureus* (MRSA) to a nadir of 1.5-fold above that measured for *K. pneumoniae* and *P. aeruginosa* controls. These findings are of particular importance since organisms in this CDC classification include MDR organisms beyond that carbapenem-resistant Gram negative bacteria, including MRSA, in which the MBC of GLS-polar is also lower by two-fold than their control, MSSA (2 μg ml^{-1} and 4.1 μg ml^{-1} respectively).

Table 1 Antimicrobial activity of commercial *Graviola* fruit and leaves/stems preparations*

		Graviola Fruit	Graviola Leaves and Stems	Ethanol Control	Cefinase Test
	Bacteria	**MBC**	**MBC**	**MBC**	
	Enterococcus. faecalis ‡	<4	<4	<4	ND
	E. faecalis VRE 16‡	<4	<4	<4	ND
	E. faecalis VRE 27‡	<4	<4	<4	ND
	E. faecalis VRE 43‡	<4	<4	<4	ND
Gram (+)	*Lactobacillus acidophilus* ATCC 4356	<4	<4	<4	ND
	L. casei ATCC 393	<4	<4	<4	ND
	Staphylococcus aureus methicillin sensitive (MSSA) ATCC 25923	<4	8	<4	-
	MSSA 35 D5 NW‡	<4	8-16	<4	-
	MSSA 12 NW‡	<4	8	<4	+
	MSSA W 66039‡	<4	8	<4	-
	MSSA 32 NW‡	<4	8	<4	-
	MRSA (methicillin resistant) ATCC 33591	<4	8	<4	+
	MRSA 2 RT‡	<4	8	<4	+
	MRSA M28035‡	<4	4-8	<4	+
	MRSA 48155‡	<4	8	<4	+
	Streptococcus mutans ATCC 35668	<4	16	<4	ND
	S. sanguis ATCC 10556	8	64	<4	ND
	S. sobrinus ATCC 27352	8	32	<4	ND
Gram (-)	*Acinetobacter baumannii* L185‡	4-8	16	<4	+
	A. baumannii L186‡	4	16	<4	+
	A. baumannii L187‡	<4	4-8	<4	+
	Acinetobacter F30656‡	4-8	16	<4	+
	Citobacter freundii ATCC 8090	<4	4	<4	-
	Citrobacter 314‡	<4	8	<4	+
	Citrobacter 21‡	<4	8-16	<4	+
	E. coli ESBL 108‡	<4	4-8	<4	+

	E. coli ESBL 109‡	<4	8	<4	+
	E. coli ESBL 5‡	<4	4-8	<4	+
	Enterobacter cloacae ESBL‡	<4	8	<4	-
	Klebsiella pneumoniae L174‡	<4	4	<4	+
	K. pneumoniae carbapenemase resistant (KPC) L133‡	<4	4-8	<4	+
	Moraxella catarrhalis S3 ‡	8	64	<4	+
	Pseudomonas aeruginosa ATCC 27853	4	8	<4	-
	P. aeruginosa ESBL ‡	4-8	8-16	<4	+
	Salmonella enterica ‡	<4	8	<4	-
	Shigella dysenteriae ATCC 11835	<4	8	<4	ND
	S. sonnei ATCC 25931	<4	4	<4	ND
	S. flexneri ATCC 12022	<4	4	<4	ND

*Numbers represent the reciprocal of dilution titer. Less than 4 (<4) indicates growth observed at the highest concentration. ND=not done, ‡Clinical Isolate.

Table 2 Minimum bactericidal concentration (MBC) of Graviola leaves/stems polar extract (GLS-polar)

	GLS-polar (µg ml-1)	**MBC Ratio Lactobacillus (µg ml-1)/ Test Microbe (µg ml-1)**
Lactobacillus acidophilus ATCC 4356	131.25	1
CDC Urgent Threat Organisms		
Klebsiella pneumoniae ATCC 27736; control	89	1.5
Klebsiella pneumoniae L133 (KPC)*	65	2
CDC Significant Risk Organisms		
Staphylococcus aureus ATCC 25923 (MSSA);control	4.1	32
Staphylococcus aureus ATCC 33591 (MRSA)	2	65.6
Acinetobacter baumannii L187*	65	2
Pseudomonas aeruginosa ATCC 27853; control	89	1.5
Pseudomonas aeruginosa ESBL*	65	2

*Clinical isolates

The potential of GLS and its extracts to be used as a topical treatment was tested using an *in vitro* wound model directed toward determining the ability of agents to prevent and/or disrupt *S. aureus* (methicillin-sensitive and methicillin-resistant) biofilm formation (Figure 1). GLS-polar, at 1:10 dilution of extract, prevented MRSA and MSSA from colony formation, i.e., biofilm development (0 colonies; n=2 in triplicate) compared to DMSO diluent controls (8.67×10^5 CFU). In contrast, MSSA and MRSA exposed to the commercial GLS (1:2 dilution of extract) were similar to the EtOH control (6.4×10^6 and 7.7×10^6, respectively). Likewise, the organic extract, GLS-organic, was similar to the DMSO diluent control for both MSSA and MRSA. In contrast, none of the preparations effectively disrupted biofilm formation, as indicated by colony formation. For the treatment of wound infections, dressings that are impregnated with antibiotic ointments or other natural antibacterial constituents like honey or silver are used because studies have shown them to be effective antimicrobial agents [24-26].

4. Conclusion

These findings on the antibacterial activity of ethanolic leaves/stems extracts of *A. muricata*, which is present in the commercial preparation, are an essential step toward the effective purification and characterization of the active compound in this extract and understanding the mechanism of anti-microbial activity of these extracts. This study showed *A. muricata* as a promising antibacterial agent against MDR bacteria. The potential for Graviola to be used as a natural alternative to topical antibiotics is an area for further studies.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest declared.

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