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Screening open source traditional Chinese medicine extracts for growth-inhibiting properties towards *Acinetobacter baumannii*

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Infections caused by *Acinetobacter baumannii* and other pathogenic bacteria are a worldwide concern due to their increasingly prevalent multidrug resistance. World leaders and health organizations across the globe have voiced their concerns over antibiotic resistance and the critical need for the development of novel antibiotics. Although the number of new antibiotic drugs entering the marketplace in recent years has been limited, it is encouraging to note that there are many initiatives in academia, industry, and government focused on this issue. This study outlines the investigation of a publicly available library of Traditional Chinese Medicine extracts to discover new compounds that could potentially inhibit the growth of a multidrug-resistant *A. baumannii* strain. Within this library of over 600 natural product samples, two individual extracts were found which exhibited over 50% mean growth inhibition of this bacterial strain at an extract concentration of 10 μg/mL. Fractionation of these two extracts into more isolated compounds also resulted in inhibitory activity. The results of this study highlight the value of this natural products library as a resource for further investigation in discovering anti-infective agents, especially during this global crisis of antibiotic resistance.

Key words: Traditional Chinese Medicine, *Acinetobacter baumannii*, growth inhibition, antibiotic resistance.

INTRODUCTION

In 2014, President Barack Obama signed an executive order which effectively issued the National Action Plan

for Combating Antibiotic-Resistant Bacteria (The White House, 2015). This plan called upon the U.S. government, in partnership with foreign governments, individuals, and organizations, to reduce the threat posed by multidrug-resistant (MDR) microorganisms to public health. One essential goal of this action plan was to accelerate research and development of new antibiotics, vaccines, and other therapeutics. Hospitals throughout the U.S. have responded to these pathogen-based threats by forming antimicrobial stewardship committees in order to promote more careful prescribing of antibiotics amongst their clinicians as a measure to prevent antibiotic drug resistance.

Three years later in Geneva, Switzerland, the World Health Organization's (WHO) advisory board drafted a global priority pathogens list of antibiotic-resistant bacteria to help prioritize research and development efforts for new antibiotic treatments (WHO, 2017a). In this document, carbapenem-resistant *Acinetobacter baumannii* was named a critical priority pathogen – the highest amongst three priority categories (that is, critical, high and medium). Similarly, *A. baumannii* is considered a serious threat (level) pathogen according to the National Action Plan (The White House, 2015).

A. baumannii is an aerobic, gram-negative coccobacillus bacterium found in soil and aquatic environments and it has been reported as capable of adhering to biological and abiotic surfaces (Doughari et al., 2011; Chen et al., 2017). Infections associated with *A. baumannii* are often contracted from biofilms on Foley catheters, venous catheters, or cerebrospinal shunts (Doughari et al., 2011). It carries a public health burden of exhibiting resistance to several antibiotic drug classes while its pathogenic mechanisms of establishing infections remain unclear; however, some of its pathogenic traits may be explained by genomic studies which have identified genes involved in quorum sensing and pilus biogenesis among others (Doughari et al., 2011; Chen et al., 2017). *A. baumannii*, along with *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Enterobacter* spp., have collectively been labeled with the acronym "ESKAPE" and are known to be common and serious MDR pathogens (Howard et al., 2012).

Introduced to the world in the mid-1980s, carbapenem antibiotics belong to the beta-lactam family which act by preventing bacterial cell wall synthesis and have rapid bactericidal activity against susceptible bacteria (Deck and Winston, 2015). They are broad spectrum antibiotics which possess significant activity against both gram-positive and gram-negative bacteria and are often used as "last resort" drugs when patients with infections become extremely ill (Meletis, 2016; Papp-Wallace et al., 2011). However, resistance to carbapenems is rising. In a United Kingdom study, *A. baumannii* carbapenem resistance rate rose from 0% in 1998 to 55% in 2006,

among patients with bacteremia; while in the United States, the rate rose from 6% in 1999 to 28% in 2006 (Erdem and Leber, 2018).

Service members of the U.S. military are unique cohorts that have been affected by antibiotic-resistant *A. baumannii* infections. In a 2004 Morbidity and Mortality Weekly Report (MMWR) from the Centers for Disease and Control (CDC), there were 102 patients identified with blood cultures that grew *A. baumannii* at military medical facilities who treated individuals injured in Afghanistan, Iraq, and Kuwait (CDC, 2004). The majority of these patients had sustained traumatic injuries. It was reported that 78 of these isolates indicated widespread resistance to commonly used antimicrobial agents for *A. baumannii* (CDC, 2004). In an unrelated study, Bulens et al. (2018) reported that infections with carbapenem-resistant *A. baumannii* have been associated with death rates as high as 52%. This evidence strongly infers that *A. baumannii* is widespread, capable of lethality, resistant to many drugs in the antibiotic spectrum, and therefore requires diligent efforts to discover new agents to combat it, especially given the international concern over its pathogenicity and the general paucity of new antibiotics entering the global marketplace.

Traditional Chinese Medicine (TCM) has been used in China for over two millennia to treat infectious diseases, amongst other pathology, and offers an avenue of study which has achieved some measure of success in this area of antibiotic discovery (Zhang et al., 2013). One of the most well-known TCM extracts comes from the *Artemisia annua* plant, mentioned in *The Handbook of Prescriptions for Emergency Treatments* from the Jin Dynasty (5th to 3rd century BC), of which artemisinin was derived and has been used extensively since the late 1990s to treat malaria (Miller and Su, 2011). As a research facility dedicated to developing drugs and vaccines to protect against pathogenic threats, the infectious disease and microbiology experts at the Walter Reed Army Institute of Research (WRAIR) jointly focused on discovering novel agents capable of suppressing the growth of *A. baumannii*. In collaboration with the National Cancer Institute (NCI), efforts were directed to screening plant-based TCM extracts to identify compounds with growth-inhibitory capability towards this pathogen.

MATERIALS AND METHODS

The NCI Library of Traditional Chinese Medicinal Plant Extracts was requested from the National Products Branch, Developmental Therapeutics Program, National Cancer Institute (He et al., 2019). This publicly available library comprised 664 organic solvent and aqueous extracts of 332 samples of 132 TCM plant species in 96-well plate format. The individual crude extract samples were dissolved in 200 µL DMSO to form a final concentration of 2.5 mg/ml in each well and these resulting source plates were stored in the freezer (at -30°C) until the day of screening.

A clinical MDR strain of *A. baumannii* (AB5075) was selected for the assays which was isolated from a patient in the U.S. military health care system and catalogued at WRAIR's Multidrug-Resistant

Table 1. Growth media recipes.

Mueller-Hinton (MH) II Broth	Casein acid hydrolysate (17.5 g)
	Beef extract (3 g)
	Starch (1.5 g)
	dH_2O (1 L)
M9 (Minimal) Media	5x M9 salts [6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl] (200 ml)
	10% Casamino Acids (10 ml)
	Magnesium sulfate (2 ml)
	Calcium chloride (100 µl)
	20% Glucose (20 ml)
	dH_2O (770 ml)

Organism Repository and Surveillance Network (MRSN). This strain was prepared the day prior to the screening assay by transferring a single bacterial colony from an agar plate to a 6 mL suspension of Mueller-Hinton II (MHII) broth, followed by incubation at 37°C on a platform shaker. After 18 h of incubation, 120 µl of the bacteria-broth sample was added to 6 ml of sterile cation-adjusted Mueller-Hinton broth (CAMHB) and incubated for 4 h until the culture reached mid-log phase growth. The culture was then diluted 1:50 with fresh CAMHB and validated using a spectrophotometer to obtain an OD_{600} value of 0.02-0.03. The final concentration of this bacterial subculture was approximated at $1x10^7$ colony forming units (CFU) with this OD_{600} value.

On the day of screening, the source plates were removed from the freezer and allowed to thaw at room temperature for approximately 15 min. All TCM crude extract samples were then diluted with sterile M9 (minimal) medium to prepare six replicates of each crude extract (i.e., three at a final concentration of 10 µg/ml and three at 0.5 µg/ml). The source plates were then returned to the freezer. Each diluted TCM sample was then inoculated with 10 µl of AB5075 subculture using the Freedom EVO-200 robotic worktable (Tecan Group Ltd., Switzerland). The negative control consisted of a compound mixture of sterile 0.9% NaCl and 0.02% DMSO; while the positive control contained rifampin dissolved in 100% DMSO to a final assay concentration of 1.6 mg/mL. Both controls were inoculated with AB5075. The recipes for our stock M9 and MHII growth media are shown in Table 1.

Once inoculated with AB5075, all plates were incubated on a platform shaker for approximately 24 h at 37°C. Each plate, including the control plates, was then inserted into a spectrophotometer to obtain an OD_{600} value for each well. This value was used to calculate the percentage of AB5075 growth inhibition and served as the proxy for growth-inhibitory activity due to the TCM sample in the well. Samples with a mean growth inhibition percentage of at least 50% when compared to the controls, averaged across the results from triplicate screens at each concentration, were considered hits.

The crude extract hits were then prefractionated into seven primary fractions of decreasing polarity by an automated solid-phase extraction (SPE) based chromatographic process (Thornburg et al., 2018). These primary fractions, containing 0.5 mg of each fraction, were then reconstituted in DMSO and prepared for screening as noted above. Following identification of any active primary fractions meeting the 50% threshold, these hits were further subfractionated on semi-preparative high performance liquid chromatography (HPLC) where 1 mg of the active primary fraction was divided across 22 subfractions (that is, secondary fractions) collected into 96 deep-well plates followed by centrifugal evaporation to dry the HPLC solvent (Grkovic et al., 2020). As the secondary fractions resulting from this final step were of unknown

exact mass, only the nominal assay mass of 45 µg per well (based on an equal split of HPLC input mass) can be reported. These wells were then reconstituted in 40 µl of DMSO to provide a nominal concentration of 1.125 mg/ml, then further diluted 1:50 and 1:20 in M9 medium broth, and followed up with a 1:10 dilution (for each previous dilution) in bacteria-broth sample for a final round of screening at nominal concentrations of 2.25 µg/ml and 0.1125 µg/ml to identify a lead hit. Figure 1 features the complete screening algorithm.

RESULTS

There were two crude extract samples that demonstrated mean growth inhibition of AB5075 greater than 50% in M9 medium broth at the 10 µg/ml concentration. The first extract was sourced from the dried root tuber of the TCM plant *Polygonum multiflorum* (NSC 500111) and the second from the flowers of the TCM herb *Rosa rugosa* (NSC 500081). Each of these two crude extracts was then prefractionated into seven primary fractions for subsequent screening for antibacterial activity against AB5075 as noted above.

At concentrations of 10 and 0.5 µg/ml, the seven primary fractions from the *P. multiflorum* crude extract exhibited a mean growth inhibition range of 22.2-47% and 14.2-35.7% respectively, in M9 medium broth. A decision was made to further subfractionate the single primary fraction producing the highest mean growth inhibition (47%) to its secondary fractions due to its proximity to the 50% threshold. Collectively, the resultant 22 secondary fractions exhibited mean growth inhibition of less than 14.4 and 8.2% at the 2.25 and 0.1125 µg/ml nominal concentrations respectively, in M9 medium broth.

In contrast, the primary fractions from the *R. rugosa* crude extract exhibited a mean growth inhibition range of 26.5-73.8% and 8.6-45.4% at concentrations of 10 and 0.5 µg/ml respectively, in M9 medium broth. Four of these seven primary fractions had exhibited a mean growth inhibition greater than 50%, ranging from 59.7-73.8% in M9 medium broth at the 10 µg/ml concentration. Subfractionation of these four active primary fractions resulted in 22 secondary fractions for each which was
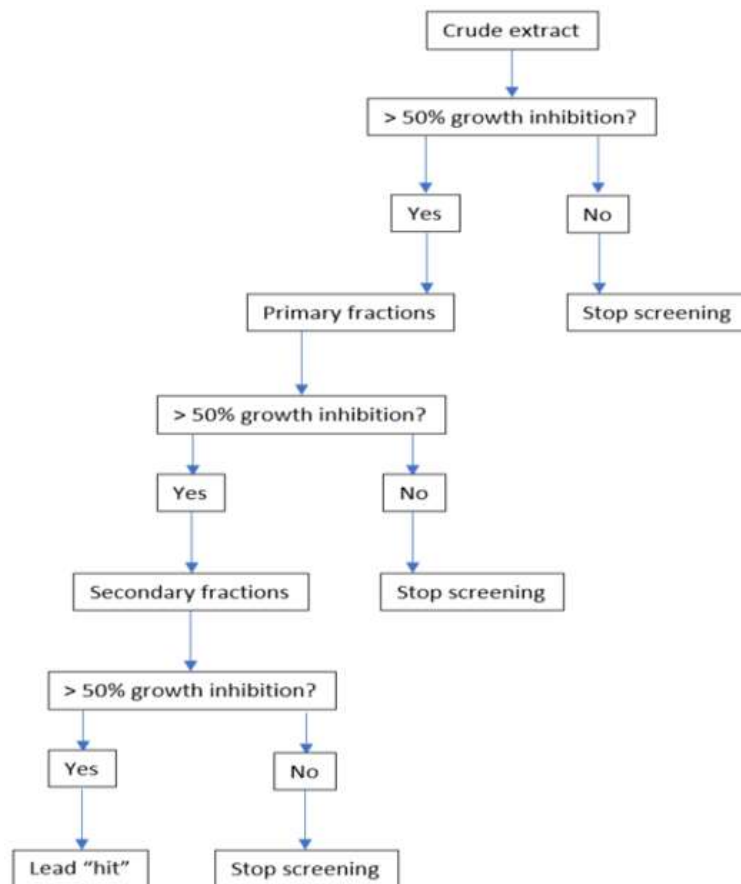
Figure 1. Screening algorithm.

tested in a final round of screening. In the M9 medium broth, 14 of 88 secondary fractions exhibited between 20-24.6% mean growth inhibition at the 2.25 µg/ml nominal concentration while the remaining had inhibition rates less than 20%. Table 2 features the mean growth inhibition rates and ranges for these compounds from both plant species at each concentration assayed.

Limitations

Designating 50% growth inhibition as the cutoff point for a hit eliminates the possibility of identifying potentially effective compounds against MDR and non-MDR *A. baumannii* strains within this library. One TCM crude extract sample exhibited 47.8% mean growth inhibition at the 10 µg/mL concentration with a range of 26.7-74% across triplicate screening in M9 medium broth. Since the threshold percentage was not reached, we did not pursue prefractionation of this crude extract sample, although some of the primary fractions may well have produced significant activity against AB5075, especially with 74% growth inhibition exhibited in one of the replicates. This process of eliminating marginally effective compounds

from further exploration is a limitation of a drug discovery program and is well characterized (Brideau et al., 2003).

Another limitation of our research was in performing each of the triplicate screens (on each crude extract sample) on consecutive days. Many of the initial 664 natural product samples exhibited steep decreases in growth inhibition percentages – at the same diluted concentration level (e.g.,10 µg/ml) - across three days which may have resulted from cycling the source plates through the freezer and room temperature on multiple days, thereby reducing the compound's biological activity. Performing the triplicate screens on a single day could have produced higher mean inhibition percentages, resulting in a greater number of extracts reaching the 50% threshold. On subsequent screenings of the fractionated compounds, we conducted the triplicate screens on a single day which generated more consistent inhibition values (e.g., smaller ranges).

Finally, the secondary fractions produced by purification of the active primary fractions were only tested at nominal concentrations based on an equal distribution of injecting 1 mg of an active primary fraction across 22 subfractions resulting from HPLC separation. It is likely that some of these secondary fractions had far

Table 2. Mean growth inhibition of AB5075.

TCM plant species	Compound form	Concentration tested (µg/ml)	Mean growth Inhibition % (range)	Notes
Polygonum multiflorum				
(n=1)*	Crude extract	10	52.2 (27.1 - 76.9)	
(n=1)*	Crude extract	0.5	41.7 (39.5 - 43.8)	
(n=7)*	Primary fractions	10	32 (22.2 - 47)	
(n=7)*	Primary fractions	0.5	29.2 (14.2 - 35.7)	
(n=22)*	Secondary fractions	2.25	-5.3 (-16.8 - 14.4)[†]	Fractionated from 1 primary fraction
(n=22)*	Secondary fractions	0.1125	-7.7 (-14.9 - 8.2)[†]	Fractionated from 1 primary fraction
Rosa rugosa				
(n=1)*	Crude extract	10	55 (32.9 - 72.6)	
(n=1)*	Crude extract	0.5	20.3 (19.3 - 21)	
(n=7)*	Primary fractions	10	51.2 (26.5 - 73.8)	
(n=7)*	Primary fractions	0.5	33.8 (8.6 - 45.4)	
(n=88)*	Secondary fractions	2.25	10.9 (-5 - 27.4)[†]	Fractionated from 4 primary fractions
(n=88)*	Secondary fractions	0.1125	10.4 (-6.5 - 26.8)[†]	Fractionated from 4 primary fractions

*Number of individual extracts or fractions tested, [†] negative percentages reflect some OD_{600} readings greater than negative control.

less than the nominal value of 45 µg per well used for the final round of screening, thus potentially contributing as a factor in having no secondary fractions reaching the 50% activity threshold for continued effort.

DISCUSSION

The objective of this research was to discover natural compounds showing efficacy against the *in vitro* growth of *A. baumannii* in growth media. We chose to use the MDR AB5075 strain in our assays to reflect real-world systemic infections. Moreover, AB5075 has been described previously as a model strain for scientific studies due to its presence in current infection isolates, virulence in multiple animal models, and antibiotic resistance profile (Jacobs et al., 2014). Doughari et al. (2011) have described that most *A. baumannii* strains are resistant to aminoglycosides, tetracyclines, cephalosporins, ampicillins, cefotaximes, chloramphenicols, gentamicins and tobramycins, where resistance operates through a plethora of mechanisms. Although testing for both drug resistance to and mode of action of our most active compounds was not emphasized in this study paradigm, understanding how these compounds may circumvent the bacteria's resistance mechanisms may be a future pursuit. Notably, some examples of antibiotic resistance amongst certain *Acinetobacter* species involve the inactivation of cephalosporins through chromosomally-encoded cephalosporinases, tetracycline resistance through bacterial efflux pumps (e.g., ATP-binding cassette type) acting against an antibiotic, and aminoglycoside resistance via aminoglycoside-modifying

enzymes and efflux pump systems (Doughari et al., 2011).

Colistin, an antibiotic discovered in 1949 but mostly abandoned in the 1980s, has seen resurgence in use and especially for treating MDR *A. baumannii* infections despite the concern of causing nephrotoxicity (Kempf and Rolain, 2012). Belonging to the polymyxin drug class, it is a natural substance produced by *Bacillus polymyxa* and acts by binding to the lipopolysaccharide molecules of the outer cell wall of gram-negative bacteria which results in cell permeability changes, cell content leakage and cell death (Florescu et al., 2012). Although colistin is generally reserved for use in infections involving highly-resistant bacteria, resistance has also been reported with this niche drug (Doughari et al., 2011). As mentioned previously, resistance to the carbapenem antibiotics is rising and is a cause for concern since they are not only used in empiric treatment of *Acinetobacter* infections, but also in combination therapy when treating resistant *Acinetobacter* isolates (Kanafani and Kanj, 2018). In fact, the WHO reports that among 27 countries reporting resistance results for more than ten *A. baumannii* isolates, 12 of these countries had percentage rates of carbapenem resistance of at least 50% (WHO, 2017b).

We were thus pleased to find compounds in the publicly available NCI Library of Traditional Chinese Medicinal Plant Extracts that demonstrated antibacterial activity against a multidrug-resistant strain of *A. baumannii*. *P. multiflorum* and its processed products have been used in Chinese medicine for centuries to moisten the intestines, alleviate insomnia, and help with anti-aging effects (Bounda and Feng, 2015; Liu et al., 2018). Moreover, *P. multiflorum* is a source organism for

the compound emodin which has been used for its anti-cancer activity and anti-inflammatory activities (Dong et al., 2016). A previously-conducted *in vitro* study reported antimicrobial activity against methicillin-resistant *S. aureus* (MRSA) at an MIC ≤ 1.43 mg/mL and that its antimicrobial effects may be due to the quinone chemical structures of the plant (Bounda and Feng, 2015; Zuo et al., 2008). Although none of the tested secondary fractions from the elected *P. multiflorum* primary fraction reached 50% threshold, it was encouraging to see modest activity against AB5075 across all seven primary fractions at the 10 µg/ml concentration with mean growth inhibition of 32% and an inhibition range of 22.2 to 47%.

R. rugosa is a perennial plant that is widely distributed in eastern Asia and is a traditional herbal medicine used to treat stomach aches, diarrhea, diabetes mellitus, and pain (Tursun et al., 2016). Its ripe fruits (hips) are high in Vitamin C and it has been reported that some animals feed on the plant as a food source, while the flower petals, traditionally known as Mei-gui hua, have been used for their anti-oxidant properties among others (Dickerson and Miller, 2002; Zhang et al., 2019). Antimicrobial activity from *R. rugosa* was demonstrated against strains of *K. pneumoniae*, *P. aeruginosa*, *Escherichia coli*, and *Proteus mirabilis* with an MIC of 1.25 mg/ml (Mármol et al., 2017). Our *R. rugosa* crude extract produced a mean growth inhibition of 55% at the 10 µg/ml concentration with an inhibition range of 32.9-72.6%. More encouraging data came from the prefractionation process where the primary fractions exhibited mean growth inhibition ranging from 26.5-73.8% at the same concentration. The top 4 of these 7 primary fractions, with mean growth inhibition values all greater than 50% (range: 59.7 to 73.8%), were selected as hits and their identifier codes were sent to the NCI for subfractionation. This final round of fractionation, generating 88 secondary fractions, did not produce any compounds exhibiting more than 50% inhibition at the nominal concentrations tested. However, there were 14 secondary fractions which exhibited mean growth inhibition between 20-24.6% at the 2.25 µg/ml nominal concentration. This suggests the possibility of additive bactericidal effects from various combinations of these secondary fractions, perhaps even present in low quantities in the individual subfractions, to produce robust values seen in some of the tested primary fractions. Indeed, testing subfractions at higher nominal concentrations may have produced greater inhibition values. The study of Miyasaki et al. (2013), which reported that an isolated fraction of *R. rugosa* displayed 67% *in vitro* growth inhibition of a different MDR *A. baumannii* strain at 250 µg/ml, supports this idea; while also corroborating the unique findings on this particular plant extract.

Conclusion

Given the worrisome reputation of *A. baumannii* as an opportunistic pathogen with growing rates of multidrug resistance, especially to carbapenem antibiotics, all existing compound libraries should be explored to find novel agents with antibacterial activity against it. The proof of concept research shows that the publicly available NCI Library of Traditional Chinese Medicinal Plant Extracts contains natural resources which are capable of suppressing the *in vitro* growth of MDR *A. baumannii*. In particular, this library's *P. multiflorum* and *R. rugosa* plant samples exhibited a unique capability of inhibiting the growth of this multidrug-resistant strain. Combinations of their secondary fractions, either alone or with other existing anti-infective agents, at higher concentrations may prove beneficial and clinically relevant in an antibiotic drug development program.

CONFLICT OF INTERESTS

FUNDING

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