Antibacterial Activity of Kalanchoe mortagei and K. fedtschenkoi Against ESKAPE Pathogens

Nicholas Richwagen, Emory University
James T. Lyles, Emory University
Brandon L. F. Dale, Emory University
Cassandra Quave, Emory University

Journal Title: Frontiers in Pharmacology
Volume: Volume 10
Publisher: Frontiers Media | 2019-02-06, Pages 67-67
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.3389/fphar.2019.00067
Permanent URL: https://pid.emory.edu/ark:/25593/tnppz

Final published version: http://dx.doi.org/10.3389/fphar.2019.00067

Copyright information:
© 2019 Richwagen, Lyles, Dale and Quave.
This is an Open Access work distributed under the terms of the Creative Commons Attribution 4.0 International License (https://creativecommons.org/licenses/by/4.0/).

Accessed September 30, 2019 5:23 AM EDT
Antibacterial Activity of Kalanchoe mortagei and K. fedtschenkoi Against ESKAPE Pathogens

Nicholas Richwagen¹, James T. Lyles¹, Brandon L. F. Dale² and Cassandra L. Quave¹,²,³,⁴*

¹ Center for the Study of Human Health, Emory College of Arts and Sciences, Atlanta, GA, United States, ² Department of Dermatology, Emory University School of Medicine, Atlanta, GA, United States, ³ Emory University Herbarium, Atlanta, GA, United States, ⁴ Antibiotic Resistance Center, Emory University, Atlanta, GA, United States

Plants in the genus Kalanchoe (Family: Crassulaceae) are used in traditional medicine throughout the tropics for treating a variety of conditions. Two species, Kalanchoe mortagei and K. fedtschenkoi, have established ethnobotanical usage but have been neglected in previous research concerning their potential bioactivity. Here, we provide a thorough review of the reported antimicrobial activities of Kalanchoe genus and evaluate the in vitro antibacterial effects of two previously unexplored species against a panel of multidrug-resistant bacteria, the ESKAPE pathogens (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter cloacae). Plant specimens were collected and voucher specimens deposited in the Emory University Herbarium. Dried plant material was ground into a powder and extracted as ethanolic macerations or as aqueous decoctions. Extracts were tested against the ESKAPE pathogens for growth inhibitory activity. Cytotoxicity to human cells was assessed via a lactate dehydrogenase assay of treated human keratinocytes (HaCaTs). K. fedtschenkoi extracts demonstrated growth inhibitory effects against two Gram-negative species, A. baumannii (strain CDC-33) and P. aeruginosa (AH-71), as well as S. aureus (UAMS-1). In these cases, growth inhibition greater than 50% (IC₅₀) was generally observed at concentrations of 256 µg mL⁻¹, though one K. fedtschenkoi extract (1465, prepared from stems) exhibited an IC₅₀ against A. baumannii at 126 µg mL⁻¹. All extracts were well tolerated by HaCaTs (LD₅₀ ≥ 256 µg mL⁻¹). Chemical characterization using HPLC and chemical standards established the presence of caffeic acid and quercetin in both plant species, as well as kaempferol in K. fedtschenkoi. These results reveal K. fedtschenkoi to be a plant of medicinal interest, and future research should aim to characterize the bioactivity of this species and its active constituents through bioassay-guide fractionation. Effects on bacterial biofilm formation and quorum-sensing are also research topics of interest for this genus.

Keywords: medicinal plants, MIC, phytochemicals, Crassulaceae, antibiotic resistance
INTRODUCTION

Ethnopharmacological Relevance of Kalanchoe Species

Plants in the genus Kalanchoe (Crassulaceae), though originating mostly in Madagascar and Southeast Africa, have a global distribution in warm climates. Frequently, Kalanchoe spp. occur as exotic or invasive species. Many members of the genus are able to self-propagate from plantlets produced on the leaf margin, making established populations hard to eradicate (Descoings, 2003; Akulova-Barlow, 2009). The presence of toxic cardiac glycosides make some Kalanchoe spp. a grazing hazard for animals in agriculture, with documented issues in Brazil, South Africa, and Australia (Botha C., 2013; Botha C.J., 2013; Mendonça et al., 2018). Nevertheless, these plants display a diverse array of stunning forms and are often grown as ornamentals for their strange beauty.

Despite their often exotic presence, Kalanchoe spp. have ethnobotanical uses wherever they are found, sometimes being called “miracle leaf” for their use in treating various ailments (Akulova-Barlow, 2009; Milad et al., 2014). In the developing world, members of this genus are used for treating myriad medical conditions. Because of its widespread distribution and ubiquitous ethnobotanical use, much research has been focused on K. pinnata, a species native to Madagascar but cultivated and distributed throughout the tropics (Descoings, 2003; Biswas et al., 2011a; Quazi Majaz et al., 2011; Pattewar, 2012; Rajsekhar et al., 2016). This species has even been the subject of bioengineering—a transgenic K. pinnata that produces an antimicrobial peptide (AMP cecropin P1) has recently been developed (Zakharchenko et al., 2016; Lebedeva et al., 2017).

Because the genus has demonstrated medicinal potential, Kalanchoe spp. neglected in research should be explored for bioactive compounds. K. mortagei and K. fedtschenkoi, two members of the section Bryophyllum within the genus, are two such species with established ethnobotanical usage, but which have been overlooked in natural products research.

Kalanchoe mortagei, also known by the synonyms K. poincarei or Bryophyllum mortagei, is a plant native to rocky/sandy soils in north Madagascar (Descoings, 2003). Compared to other members of the genus, little research has been conducted on the chemical and medicinal properties of this species (Maiti et al., 1995). Despite this, K. mortagei is grown in Mexican homegardens, and its leaves are taken orally for digestive disorders and as a local remedy for cancer in Antioquia Department, Colombia (Blanckaert et al., 2004; Vera-Marin and Sánchez-Saén, 2016). The roots of the plant are used for treating parasitic worm-related diseases in parts of Indonesia (Herawati and Husin, 2000).

Kalanchoe fedtschenkoi is a perennial native to central/southern Madagascar but is naturalized well outside its original range (Descoings, 2003). Introduced populations can be found in Florida, Texas, and Puerto Rico (USDA/NRCS, 2013). A popular garden succulent, K. fedtschenkoi is a model organism for research into Crassulacean acid metabolism (CAM) (Dittrich, 1976; Nimmo et al., 1986; Cook et al., 1995). In Brazil, this species is used as an analgesic (Cumberbatch, 2011).

Antimicrobial Resistance in the ESKAPE Pathogens

The rise of antimicrobial resistant (AMR) bacterial infections is one of the most pressing issues in medicine. Increasingly, conventional antibiotic medications are failing to stop persistent and dangerous bacterial diseases (Irenji et al., 2018; Katsuura et al., 2018). A report commissioned by the UK government notes that roughly 700,000 people die annually from AMR infections; this figure is projected to increase to 10 million deaths per year by 2050 (O’Neil, 2016) and encompasses data from across the broad spectrum of pathogenic microbes. In the face of rising morbidity and mortality due to AMR infections, the need for new drugs to address drug-resistance is clear (van der Meer et al., 2014). In 2015, the WHO launched the Global Antimicrobial Resistance Surveillance System (GLASS) to unify worldwide AMR. To date they have collected data from 42 countries and received over 500,000 AMR pathogenic strains (WHO, 2017).

Six bacterial species, the “ESKAPE” pathogens, have been highlighted by the Infectious Disease Society of America (IDSA) as being especially dangerous due to their patterns of antibiotic resistance. They are responsible for the majority of nosocomial infections worldwide (Table 1) (Boucher et al., 2009).

Kalanchoe Spp. as a Source of Antimicrobial Treatment

Plants used in traditional medicine are a potential source for novel antimicrobial compounds (Rahman et al., 2018; Salam and Quave, 2018). In the developing world, the large majority of people (75%) rely on plants for primary medical needs, including for wound healing and antimicrobial agents (Sarker et al., 2005). Historically, the bulk of manufactured drugs were derived from plant natural products, and the majority of these drugs were tied directly to their original ethnobotanical use (Chin et al., 2006; Sarker and Nahar, 2012). Even between 1982 and 2002, 79% of approved drugs worldwide had a natural product origin (Chin et al., 2006).

Secondary metabolites taken from plants used in traditional medicine have been found to inhibit microbial growth and virulence. Kalanchoe spp. have demonstrated such antimicrobial properties, and have been proven to accelerate wound-healing. For example, extracts and compounds from K. pinnata are effective against cutaneous leishmaniasis, a disease caused by trypanosome protozoa (Torres-Santos et al., 2003; Muzitano et al., 2006a,b, 2009).

In the past decade, substantial research has examined the antibacterial properties of K. pinnata and several other Kalanchoe spp. Extracts of K. blossfeldiana, K. crenata, K. laciniata, and K. pinnata have all demonstrated growth inhibitory effects on over 15 bacterial species, including four of the ESKAPE pathogens (Tables 2, 3).

In 15 studies that evaluated the antimicrobial effects of Kalanchoe spp., 12 focused solely on K. pinnata, one on...
K. laciniata (Iqbal et al., 2016) and one on K. blossfeldiana, a common household ornamental (Sarkar et al., 2015). A 2007 study compared the growth-inhibitory properties of K. crenata favorably with K. pinnata (Akinsulire et al., 2007).

Ten studies examined methanolic extracts, the most common solvent tested. Ethanol and water (five studies each) were also frequently used solvents. Research has established that methanolic crude extracts of K. pinnata outperform aqueous extracts in their growth-inhibitory effects (Akinsulire et al., 2007; Majaz et al., 2011; Nwadinigwe, 2011; Pattewar et al., 2013); this is also true for K. crenata (Akinsulire et al., 2007).

Studies also established the antibacterial effects of flavonoids extracted from K. pinnata (Okwu and Nnamdi, 2011; Tatsimo et al., 2012), as well as its leaf juice (Obasekili-Ebor, 1985; Akinsulire et al., 2007). At least one study demonstrated the effects of K. pinnata in vivo, looking at how aqueous extracts accelerate the healing of wounds infected with Staphylococcus aureus and/or Pseudomonas aeruginosa (Lebedeva et al., 2017).

Research has firmly established K. pinnata as a plant of medicinal interest, and the overall genus continues to show promise as a potential source of antimicrobial, antibacterial compounds.

The aim of this study was to evaluate the antimicrobial potential of two previously neglected species: K. mortagei and K. fedtschenkoi against a panel of clinically relevant ESKAPE pathogens.

**MATERIALS AND METHODS**

**Plant Collection and Identification**

Two plant species were used in this experiment. Kalanchoe mortagei plants were grown from a specimen collected by the first author (NR) in Bradenton, FL, United States, in May 2008 (27.468591, −82.577127). A single K. fedtschenkoi plant was procured from the University of Georgia Plant Biology Greenhouse in Athens, GA, United States, in 2015. All plant material used in this experiment came from plants propagated from these two mother specimens. Plants were grown in NR’s personal collection and at the Emory University Greenhouse. Voucher specimens of each species were deposited at the Emory University Herbarium (GEO), and species identification confirmed by Dr. Tharanga Samarakoon at GEO (Accession nos.: 22702 and 22474 for K. fedtschenkoi and K. mortagei, respectively). Specimens were digitized and are available for viewing on the SERNEC portal (SERNEC, 2018).

Bulk plant materials were harvested, dried in a dehumidification chamber, and homogenized in a Waring blender into a fine powder. Retention vouchers of dried and ground material were prepared for future reference and stored in Quave Research Group laboratories at Emory University.

**Preparation of Extracts**

A total of seven crude extracts were prepared, four from K. mortagei and three from K. fedtschenkoi (Table 4). Each extract represented a particular plant part or combination of parts, though extract creation was also guided by limitations in available plant biomass.

Dry, ground plant biomass was double macerated for 72 h each with either 80 or 95% ethanol at a 1:10 ratio (w/v). The extracts were agitated daily and then vacuum filtered. The aqueous extract (1509aq) was prepared as a decoction; the dry plant material was boiled with deionized water (dH₂O) for 20 min and then filtered. After filtration the solvent was removed by rotary evaporation at ≤40°C. Extracts were redissolved in dH₂O, shell frozen in a dry ice-acetone bath, and then lyophilized overnight on a Labconco FreeZone 2.5 Lyophilizer (Kansas City, MO, United States). Dry extracts were scraped into scintillation vials and stored at −20°C. Organic extracts were dissolved in DMSO and the aqueous extract was re-dissolved in dH₂O to yield a stock concentration of 10 mg mL⁻¹ for microbiological assays.

**Antibacterial Testing**

**Bacterial Strains and Cultures**

Seven extracts (Table 4) were tested against strains of ESKAPE pathogens (Table 5). Two species were Gram-positive, Enterococcus faecium (EU-44) and S. aureus (UAMS-1); the rest were Gram-negative: Klebsiella pneumoniae (CDC-16), Acinetobacter baumannii (CDC-33), P. aeruginosa (AH-71), and Enterobacter cloacae (CDC-08). Strains were streaked from freezer stock onto tryptic soy agar (TSA) plates and incubated at 37°C overnight. Liquid cultures in tryptic soy broth (TSB) were made from individual plate colonies in 14 mL test tubes and were...
<table>
<thead>
<tr>
<th>Kalanchoe sp.</th>
<th>Method</th>
<th>Microbes tested/Gram (+/−)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>K. pinnata (Koutcheu Mabeku et al., 2017)</td>
<td>Leaf methanol and ethyl acetate extracts were tested against Helicobacter pylori in vitro and in the guts of Swiss mice.</td>
<td>Helicobacter pylori (−)</td>
<td>Methanol extract showed a significant anti-Helicobacter activity with MIC and MBC values of 32 and 256 µg mL⁻¹, respectively. Also reduced bacterial load of gastric mucosa.</td>
</tr>
<tr>
<td>K. pinnata Transgenic and wild-type (Lebedeva et al., 2017)</td>
<td>Leaf aqueous extracts of wild-type and transgenic (cencopins producing) were applied directly to infected wounds.</td>
<td>Wounds were infected with Staphylococcus aureus (+), Pseudomonas aeruginosa (−), or a combination of both.</td>
<td>Both wild-type and transgenic extracts accelerated wound-healing and demonstrated anti-microbial effects, even in comparison to an antibiotic. Effective against both bacteria.</td>
</tr>
<tr>
<td>K. pinnata (Larasati and Wahid, 2016)</td>
<td>Leaf ethanolic extracts tested using microdilution method</td>
<td>Acinetobacter baumannii (−) and S. aureus (+)</td>
<td>In assays the crude extract was found effective against S. aureus and B. subtilis, with MIC values of 5 and 2.5 mg mL⁻¹, respectively.</td>
</tr>
<tr>
<td>K. laciniata (Io bal et al., 2016)</td>
<td>Aerial parts in a 60% methanolic extract</td>
<td>S. aureus (+) and Bacillus subtilis (+)</td>
<td>Extract reduced biofilm formation and thickness reduced secretion of virulence factors. Concentrated extract destroyed biofilms.</td>
</tr>
<tr>
<td>K. blossfeldiana (Sarkar et al., 2015)</td>
<td>Methanolic extract evaluated against biofilm production</td>
<td>P. aeruginosa (−)</td>
<td>Zones of inhibition, MICs established (30 mg for S. aureus). All extracts showed antimicrobial effects. 60% methanol extracts performed best.</td>
</tr>
<tr>
<td>K. pinnata (Pattewar et al., 2013)</td>
<td>Leaf 95% ethanolic, methanolic extracts 60% methanic, aqueous extracts</td>
<td>S. aureus (+), P. aeruginosa (−), Escherichia coli (−), and fungus Candida albicans</td>
<td>Crude extracts displayed strong antibacterial and especially antifungal effects. Ethyl acetate fractions more strongly anti-microbial. An isolated flavonoid showed particularly strong effects.</td>
</tr>
<tr>
<td>K. pinnata (Tatsimo et al., 2012)</td>
<td>Evaluation of methanolic, ethanolic crude extracts, and extract partitions (in ethyl acetate, hexane)</td>
<td>S. aureus (+), P. aeruginosa (−), Salmonella typhi (−) Fungi C. albicans, Candida parapsilosis, Cryptococcus neoformans</td>
<td>Methanolic extracts most effective against all bacteria; no extracts effective against C. albicans. Zones of inhibition, MICs established for all bacteria tested.</td>
</tr>
<tr>
<td>K. pinnata (Biswas et al., 2011b)</td>
<td>Ethanol extracts used in agar-diffusion method.</td>
<td>Bacillus megaterium (+), B. subtilis (+), S. aureus (+), E. coli (−), P. aeruginosa (−), Shigella dysenteriae (−), S. typhi (−), Vibrio cholera (−)</td>
<td>Bacterial growth was inhibited by extract, expect for, S. typh, V. cholera. Effects were strongest against E. coli, with a zone of inhibition of 8.2 ± 0.22.</td>
</tr>
<tr>
<td>K. pinnata (Majaz et al., 2011)</td>
<td>Root extracts of petroleum ether, chloroform, methanol, and water</td>
<td>S. aureus (−), E. coli (−), P. aeruginosa (−) Fungus C. albicans.</td>
<td>Methanolic extracts most effective against all bacteria; no extracts effective against C. albicans. Zones of inhibition, MICs established for all bacteria tested.</td>
</tr>
<tr>
<td>K. pinnata (Okwu and Nnamdi, 2011)</td>
<td>Two flavonoid compounds were isolated and tested directly</td>
<td>P. aeruginosa (−), Klebsiella pneumoniae (−), E. coli (−), S. aureus (−) Fungi C. albicans and Aspergillus niger</td>
<td>Bactericidal effects established against B. subtilis and S. aureus, with the methanolic extract showing strong effects. No effects against P. aeruginosa, C. albicans, and A. niger. S. aureus showed the lowest minimum inhibitory concentration (MIC) of 6.29 mg mL⁻¹ in the methanol extract, while S. typh showed the highest MIC of 9.98 mg mL⁻¹ in the aqueous extract.</td>
</tr>
<tr>
<td>K. pinnata (Nwadiningwe, 2011)</td>
<td>Stem extracts of methanol, water. Agar-diffusion</td>
<td>S. typh (−), P. aeruginosa (−), S. aureus (+), Bacillus subtilis (+), Fungi C. albicans and A. niger</td>
<td>Methanolic extracts of both species were effective against all tested, though Gram-positive bacteria were more susceptible. Aqueous extracts were less effective. K. pinnata water extracts did not affect E. coli, K. pneumoniae, S. paratyphi, Citrobacter. Aqueous for either species did not affect C. albicans. Local solvents were not effective. Leaf juice extract was effective, particularly for K. crenata, against all except C. albicans.</td>
</tr>
<tr>
<td>K. crenata/K. pinnata (Akinsulire et al., 2007)</td>
<td>Methanol, aqueous extracts. Juice from squeezed leaves. Three solvents based on local alcoholic beverages. Agar diffusion, broth dilution methods to determine MIC.</td>
<td>E. coli (−) ATCC 252922, P. aeruginosa (−), K. pneumoniae (−), Shigella flexneri (−), Salmonella paratyphi (−), Citrobacter spp. (−) S. aureus (+) ATCC 25213, Enterococcus faecalis (−), B. subtilis (+) Fungus C. albicans</td>
<td>Methanolic extracts of both species were effective against all tested, though Gram-positive bacteria were more susceptible. Aqueous extracts were less effective. K. pinnata water extracts did not affect E. coli, K. pneumoniae, S. paratyphi, Citrobacter. Aqueous for either species did not affect C. albicans. Local solvents were not effective. Leaf juice extract was effective, particularly for K. crenata, against all except C. albicans.</td>
</tr>
</tbody>
</table>
TABLE 2 | Literature review of research on the antimicrobial properties of Kalanchoe spp.

<table>
<thead>
<tr>
<th>Kalanchoe sp.</th>
<th>Method</th>
<th>Microbes tested/Gram (+/−)</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>K. pinnata</em> (Akinpelu, 2000)</td>
<td>60% methanolic extracts, tested at 25 mg mL⁻¹</td>
<td><em>S. aureus</em> (+), <em>K. pneumoniae</em> (−), <em>P. aeruginosa</em> (−), <em>E. coli</em> (−), <em>B. subtilis</em> (−), <em>S. dysenteriae</em> (−), <em>C. albicans</em></td>
<td><em>B. subtilis</em>, <em>E. coli</em>, <em>P. vulgaris</em>, <em>S. dysenteriae</em>, <em>S. aureus</em> were growth inhibited. <em>K. pneumoniae</em> and <em>P. aeruginosa</em> were not growth inhibited. Bactericidal effects against all demonstrated.</td>
</tr>
<tr>
<td><em>K. pinnata</em> (Obaseiki-Ebor, 1985)</td>
<td>Leaf juice extract 5% v/v tested</td>
<td><em>S. aureus</em> (+), <em>Streptococcus pyogenes</em> (+), <em>E. faecalis</em> (+), <em>E. coli</em> (−), <em>Proteus spp.</em> (−), <em>Klebsiella spp.</em> (−), <em>Shigella spp.</em> (−), <em>Salmonella spp.</em> (−), <em>Serratia marcescens</em> (−), <em>P. aeruginosa</em> (−)</td>
<td></td>
</tr>
</tbody>
</table>

Almost all antimicrobial work has focused on the species *K. pinnata*. Table, in part, adapted from review papers: Biswas et al. (2011a), Quazi Majaz et al. (2011), Pattewar (2012), and Rajaekar et al. (2016).

also incubated at 37°C overnight for use in growth inhibition assays.

Growth Inhibition Assays
The extracts were examined for the growth inhibitory activity following guidelines set by the Clinical and Laboratory Standards Institute for broth microdilution testing (CLSI, 2013). After incubation, TSB cultures were diluted in cation-adjusted Muller Hinton broth (CAMHB) based on their optical density (OD₅₉₀) to a confluence of 5 × 10⁵ CFU mL⁻¹, confirmed by plate counts. All assays were performed in CELLSTAR 96-well plates (Greiner Bio-One International, 655-185), and read in a Cytation-3 multimode plate reader (BioTek). An initial optical density reading was taken after bacterial cultures and extracts were added to each plate (OD₆₀₀). For *E. faecium*, *S. aureus*, *K. pneumoniae*, *P. aeruginosa*, and *E. cloacae*, assay plates were incubated for 18 h; *A. baumannii* was incubated for 22 h. After incubation, the optical density of wells was checked again (OD₆₀₀).

In the initial screen, each extract was tested at a concentration of 256 µg mL⁻¹ to determine if any growth-inhibitory effects at a level of 50% or greater were evident in comparison to the vehicle (DMSO) control. If bacterial growth was inhibited by at least 50%, microdilution assays were performed. Dose response studies were performed on bacteria-extract pairs exhibiting ≥50% growth inhibition in this initial screen. Extracts were tested by twofold serial dilution at a concentration range of 8–256 µg mL⁻¹.

Percent inhibition was calculated in order to minimize the influence of any color cast due to the plant extracts as previously described (Quave et al., 2015). The IC₅₀ values were defined as the concentration required to achieve a 50% inhibition of growth, and the MIC values (or IC₉₀) were defined as the concentration required to achieve 90% growth inhibition (as determined by OD₆₀₀ for both values). Gentamicin was used as a positive control against all strains.

Mammalian Cytotoxicity Assay
Mammalian cytotoxicity of extracts was assessed using human keratinocytes (HaCaTs) and a lactate dehydrogenase (LDH) test kit (G-Biosciences, St. Louis, MO, United States) as previously described (Quave et al., 2015). Briefly, HaCaTs were maintained in Dulbecco’s modified Eagle’s medium with L-glutamine and glucose supplemented with 10% heat-inactivated fetal bovine serum and 1× solution of penicillin and streptomycin at 37°C, 5% CO₂ in 75 mL flasks. Once 90–95% confluency was reached, the cells were detached from the flask bottom using 0.25% trypsin, 0.1% ethylenediaminetetraacetic acid (EDTA) in Hank’s balanced salt solution (HBSS) without Ca²⁺, Mg²⁺, and NaHCO₃. The culture was standardized to 4 × 10⁴ cells mL⁻¹ using a hemocytometer. Then, 200 µL of the standardized culture was added to each well in a 96-well tissue culture-treated microtiter plate (Falcon 35–3075) and the plates were incubated for 48 h in a humidified 37°C, 5% CO₂ incubator, prior to media aspiration. Either media containing extracts (4–512 µg mL⁻¹) or vehicle were serially diluted and processed 24 h later following manufacturer’s protocol for chemical induced cytotoxicity. Percent DMSO (v/v) in the wells was <2% for all tests.

Chemical Characterization
Each extract was characterized by HPLC using a method adapted from four previously published HPLC methods, one examining flavonoid compounds (Nielsen et al., 2005), and three examining bufadienolides (a type of cardiac glycoside commonly found in *Kalanchoe* plants) (Supratan et al., 2000; Huang et al., 2013; Moniuszko-Szajwaj et al., 2016). Extracts were chromatographed on an Agilent 1260 Infinity system running OpenLab CDS ChemStation (Agilent Technologies, Santa Clara, CA, United States) with an Agilent ZORBAX Eclipse XDB-C18 (250 mm × 4.6 mm, 5 µm) column with compatible guard column at 30°C. A 10 µL injection of each extract was eluted at a flow rate of 1 mL min⁻¹ using a mobile phase consisting of (A) 0.1% formic acid in H₂O and (B) 0.1% formic acid in methanol (VWR HiPerSolv CHROMANORM). The gradient profile consisted of initial conditions 98:2 A:B which were held for 20 min,
TABLE 3 | Review of Kalanchoe extracts tested against selected bacteria in previous research.

<table>
<thead>
<tr>
<th>Bacteria/plant, paper, and solvent count</th>
<th>Methanolic extract</th>
<th>Water extract</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acinetobacter baumannii</strong> (−)</td>
<td></td>
<td></td>
<td>K. pinnata</td>
</tr>
<tr>
<td>K. pinnata: 1 paper, 1 extract solvent</td>
<td></td>
<td></td>
<td>• Ethanol✓ (Larasati and Wahid, 2016)</td>
</tr>
<tr>
<td>K. laciniata ✓ (Iabal et al., 2016)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Enterobacter spp.</strong> (−)</td>
<td></td>
<td></td>
<td>K. pinnata</td>
</tr>
<tr>
<td><strong>Enterococcus faecalis</strong> (+)</td>
<td></td>
<td></td>
<td>K. pinnata</td>
</tr>
<tr>
<td>K. pinnata, K. laciniata: 2 papers, 2 solvents, and leaf juice</td>
<td>K. pinnata ✓ (Akinsulire et al., 2007)</td>
<td>K. pinnata ✓ (Akinsulire et al., 2007)</td>
<td>K. pinnata</td>
</tr>
<tr>
<td><strong>Enterococcus faecium</strong> (+)</td>
<td></td>
<td></td>
<td>K. pinnata</td>
</tr>
<tr>
<td>K. pinnata, K. crenata: 9 papers, 5 extract solvents, leaf juice, and flavonoid compounds</td>
<td>K. pinnata ✓ (Akinsulire et al., 2007; Majaz et al., 2011; Nwadinigwe, 2011; Pattewar et al., 2013)</td>
<td>✓ (Akinsulire et al., 2007)</td>
<td>K. pinnata</td>
</tr>
<tr>
<td>Escherichia coli (−) K. pinnata, K. crenata: 9 papers, 5 extract solvents, leaf juice, and flavonoid compounds</td>
<td>K. pinnata ✓ (Akinsulire et al., 2007)</td>
<td>✓ (Akinsulire et al., 2007)</td>
<td>K. pinnata</td>
</tr>
<tr>
<td><strong>Helicobacter pylori</strong> (−)</td>
<td></td>
<td></td>
<td>K. pinnata</td>
</tr>
<tr>
<td>K. pinnata: 1 paper, 2 solvents</td>
<td>K. pinnata ✓ (Koutcheu Mabeku et al., 2017)</td>
<td>✓ (Koutcheu Mabeku et al., 2017)</td>
<td>K. pinnata</td>
</tr>
<tr>
<td><strong>Klebsiella pneumoniae</strong> (−)</td>
<td></td>
<td></td>
<td>K. pinnata</td>
</tr>
<tr>
<td>K. pinnata, K. crenata: 5 papers, 2 solvents, leaf juice, and flavonoid compounds</td>
<td>K. pinnata ✓ (Akinsulire et al., 2007)</td>
<td>✓ (Akinsulire et al., 2007)</td>
<td>K. pinnata</td>
</tr>
<tr>
<td><strong>Pseudomonas aeruginosa</strong> (−)</td>
<td></td>
<td></td>
<td>K. pinnata</td>
</tr>
<tr>
<td>K. pinnata, K. crenata, K. blossfeldiana: 11 papers, 6 solvents, leaf juice, and flavonoid compounds</td>
<td>K. pinnata ✓ (Akinsulire et al., 2007; Majaz et al., 2011; Nwadinigwe, 2011; Pattewar et al., 2013)</td>
<td>✓ (Akinsulire et al., 2007)</td>
<td>K. pinnata</td>
</tr>
<tr>
<td><strong>Proteus spp.</strong> (−) K. pinnata: 1 paper testing leaf juice</td>
<td>K. pinnata ✓ (Akinsulire et al., 2007)</td>
<td>✓ (Akinsulire et al., 2007)</td>
<td>K. pinnata</td>
</tr>
<tr>
<td><strong>Salmonella typhi</strong> (−) K. pinnata: 5 papers, 5 solvents, and leaf juice</td>
<td>K. pinnata ✓ (Akinsulire et al., 2007)</td>
<td>✓ (Akinsulire et al., 2007)</td>
<td>K. pinnata</td>
</tr>
</tbody>
</table>

(Continued)
Detection of Total Phenolic Content

Total phenolic content (TPC) was determined using a Folin–Ciocalteu assay modified for 96-well plate format (Singleton et al., 1999). A 1 mg mL\(^{-1}\) gallic acid stock solution was prepared in 50% MeOH\(_{aq}\) and diluted in the same solution to yield 0–100 µg mL\(^{-1}\) gallic acid standard solutions. Extracts were prepared at 1 or 2 mg mL\(^{-1}\) in 50% MeOH\(_{aq}\) and serially diluted until their absorbance was within the range of the gallic acid standard curve. In a 96-well plate, 30 µL of gallic acid standard solution or extract was added to triplicate wells. To each well 200 µL of dH\(_2\)O was added, then 15 µL of Folin–Ciocalteu reagent. After at least 1 min, but no more than 8 min, 50 µL of 20% Na\(_2\)CO\(_3\) (w/v) was added to all wells. The plate was mixed for 30 s on an orbital shaker, incubated at 40°C for 30 min, manually mixed with a multichannel pipette, then an additional 30 s with an orbital shaker, and finally the absorbance at 760 nm was recorded using a BioTek Cytation 3 multimode plate reader. The linear range for the assay was determined as 0–100 µg mL\(^{-1}\) gallic acid equivalents (GAE), \(R^2 = 0.986\). The TPC of the extracts is expressed as mg GAE/g dry extract.
TABLE 5 | ESKAPE pathogens tested and their corresponding antibiotic resistance profiles as reported by the source provider (BEI Resources or CDC AR Bank) or as determined by antibiotic disc diffusion test (for AMC, IPM, PIP, RA, SXT, and TET) following CLSI breakpoints.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain ID</th>
<th>Alternate ID</th>
<th>Antibiotic resistance profile*</th>
<th>Other characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterococcus faecium</td>
<td>EU-44</td>
<td>HM-959; Strain 513</td>
<td>AMC, RIF, SXT, TET, TZP</td>
<td>Osteomyelitis isolate; MSSA; prototype biofilm isolate</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>UAMS-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>CDC-16</td>
<td>AR-Bank #0016</td>
<td>AMP, ATM, FOX, SAM, TET</td>
<td>Reduced susceptibility, elevated carbapenem MICs</td>
</tr>
<tr>
<td>Acinetobacter baumannii</td>
<td>CDC-33</td>
<td>AR-Bank #0033</td>
<td>CAZ, CIP, CRO, CTX, DOR, FEP, GEN, IPM, LVX, MEM, SAM, SXT, TOB, TZP</td>
<td>Reduced susceptibility, elevated carbapenem MICs</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>AH-0071</td>
<td>PAO1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>CDC-08</td>
<td>AR-Bank #0008</td>
<td>AMC, AMP, ATM, CAZ, CFZ, CIP, CRO, CTX, DOR, ETP, FOX, LVX, MEM, SAM, TET, TOB, TZP</td>
<td>Reduced susceptibility, elevated carbapenem MICs</td>
</tr>
</tbody>
</table>

*Resistance: AMC, amoxicillin–clavulanic acid; AMP, ampicillin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; CRO, ceftriaxone; CTX, cefotaxime; DOR, doripenem; ETP, ertapenem; FEP, cefepime; FOX, cefoxitin; GEN, gentamicin; IPM, imipenem; LVX, levofloxacin; MEM, meropenem; Rif, rifampicin; SAM, ampicillin–sublactam; SXT, trimethoprim–sulfamethoxazole; TET, tetracycline; TOB, tobramycin; TZP, piperacillin–tazobactam. Any antibiotics denoted with * indicates resistance.

TABLE 6 | Extracts exhibiting IC<sub>50</sub> growth inhibition (≥50%) against ESKAPE pathogens.

<table>
<thead>
<tr>
<th>Species</th>
<th>Extract ID</th>
<th>E. faecium</th>
<th>S. aureus</th>
<th>K. pneumoniae</th>
<th>A. baumannii</th>
<th>P. aeruginosa</th>
<th>E. cloacae</th>
</tr>
</thead>
<tbody>
<tr>
<td>K. mortagei</td>
<td>1420</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td></td>
<td>1468</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td></td>
<td>1508</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td></td>
<td>1508aq</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>K. fedtschenkoi</td>
<td>1421</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>128</td>
<td>128</td>
<td>&gt;256</td>
</tr>
<tr>
<td></td>
<td>1465</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td></td>
<td>1469</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Gentamicin MIC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>16</td>
<td>&gt;64</td>
<td>4</td>
<td>&lt;4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Growth inhibition is in comparison to vehicle control. All concentration values reported as µg mL<sup>-1</sup>. Extracts active at an IC<sub>50</sub> of 256 µg mL<sup>-1</sup> or less are displayed in bold.

RESULTS

**K. fedtschenkoi Exhibits Antibacterial Activity Against Three ESKAPE Pathogens**

Initial screening of extracts at 256 µg mL<sup>-1</sup> demonstrated an IC<sub>50</sub> (growth inhibition of 50% or greater) of K. fedtschenkoi extracts (1421, 1465, and 1469) against three of the ESKAPE pathogens: S. aureus, A. baumannii, and P. aeruginosa. Further testing by serial dilution assays revealed that K. fedtschenkoi extracts had IC<sub>50</sub> values ranging from 128 to 256 µg mL<sup>-1</sup> for these pathogens (Table 6).

Growth inhibition by dose response is reported in Figure 1. The only extract to exhibit >35% inhibition in E. faecium (EU-44) was 1468. No extracts inhibited growth of E. cloacae (CDC-08) by 20% or more. Extract 1465 (K. fedtschenkoi woody stems) exhibited >60% inhibition in A. baumannii (CDC-33) and an MIC of 256 µg mL<sup>-1</sup> (growth inhibition ≥90%) was observed against P. aeruginosa (AH-71). Extract 1508 was the only extract to exhibit at least 40% inhibition in growth at 256 µg mL<sup>-1</sup> against K. pneumoniae.

**Extracts Exhibit Low Toxicity to Human Keratinocytes**

Human skin keratinocytes (HaCaT) were exposed to each extract to examine possible cytotoxic effects in mammalian cells. The highest levels of cytotoxicity were observed at the 512 µg mL<sup>-1</sup> concentration, and ranged from 11 to 26% growth inhibition of human cells. All extracts at the 256 µg mL<sup>-1</sup> concentration exhibited cytotoxicity of 12% or less (Figure 2). No IC<sub>50</sub> was observed for any of the tested concentrations.

**Chemical Characterization of Extracts**

In this study, the Kalanchoe spp. extracts were screened by HPLC for the presence of several commonly occurring flavonoids: kaempferol (1) and quercetin (2), and phenolic compounds, caffeic acid (3), p-coumaric acid (4), ferulic acid (5). Both K. mortagei (extracts 1421 and 1469) and K. fedtschenkoi (extract 1468) contained 2. The extracts 1421 and 1465 of K. fedtschenkoi contained 1. The presence of 3 was also established in K. fedtschenkoi (extracts 1421 and 1465) and in K. mortagei (extract 1468) at very low levels (Figure 3).
Previous studies have shown a diverse chemistry in the genus Kalanchoe. Previous studies identified 5 in several Kalanchoe species (Gaind and Gupta, 1971; Muzitano et al., 2006a,b; Cruz et al., 2012). 1 was established in K. pinnata (Gaind and Gupta, 1971; Muzitano et al., 2006a) and K. daigremontiana (Ürményi et al., 2016). Syringic acid, 3, and 4 were identified in K. pinnata (Gaind and Gupta, 1973). A 1995 study found lupeol, lupeol acetate, β-sitosterol, and other related compounds in K. mortagei (Maiti et al., 1995).

The TFC for the Kalanchoe spp. extracts ranged from a minimum of 331 ± 33 mg GAE/g extract for 1420 to 1340 ± 116 mg GAE/g extract for 1509aq. The K. mortagei inflorescences extracts (1508 and 1509aq) had higher TFC than the other plant parts of both species, 818 mg GAE/g extract and 1340 mg GAE/g extract, respectively. The average TFC of the K. mortagei extracts with leaf and stem tissues (1420 and 1468) and the K. fedtschenkoi leaf and stem tissue extracts (1420 and 1465) were both 451 mg GAE/g extract, indicating that both species have similar TFC. However, the K. fedtschenkoi leaf and stem tissue extracts (1421, 1465, and 1469) have higher antimicrobial activities against multiple bacterial strains than the K. mortagei extracts. This suggests that the bioactivity of K. fedtschenkoi is not due to phenolic compounds.

**DISCUSSION**

In this study, K. fedtschenkoi extracts exhibited growth inhibition against two Gram-negative species, A. baumannii (CDC-33) and P. aeruginosa (AH-71), as well as Gram-positive S. aureus. All other pathogens examined, including Gram-positive E. faecium (EU-44), were largely unaffected. This contrasts with some previous work, where Kalanchoe spp. extracts tested against bacteria exhibited growth-inhibitory effects more readily against Gram-positive pathogens (Akinsulire et al., 2007). Extracts in other studies with S. aureus have always shown growth-inhibition, with the exception of the poor-performance of a hexane fraction tested (Tatsimo et al., 2012; Table 3). Tests against Gram-negative species P. aeruginosa and K. pneumoniae have had mixed results, demonstrating both positive (Akinsulire et al., 2007; Pattewar et al., 2013) and negative (Akinpelu, 2000; Nwadinigwe, 2011) results concerning growth-inhibition.

Although K. mortagei extracts 1420 and 1468 failed to inhibit growth at or above 50% (IC$_{50}$), there were differences in performance and chemical characterization of these two closely related extracts. Both 1420 and 1468 were composed of aerial parts of K. mortagei (leaves and stems), though 1420 also had immature inflorescences. Against E. faecium (EU-44) and
FIGURE 3 | HPLC chromatograms at 254 nm for each Kalanchoe extract indicating compounds identified: kaempferol (1), quercetin (2), and caffeic acid (3).
P. aeruginosa (AH-71), 1420 actually increased bacterial growth, and against all six pathogens, there were statistically different performances between these two extracts (verified with Student’s t-test). HPLC analysis revealed lower absorbance intensity in the 35–80 min region for 1420 compared to 1468, though elution peaks were similar. Caffeic acid could only be confirmed in 1420, and kaempferol was only confirmed in 1468.

It is possible that the differences are due to the harvest conditions of the K. mortagei plants used to make these extracts. Extract 1420 was prepared from a K. mortagei plant collected in December 2017, which was maintained in low-light conditions. Extract 1468, in contrast, was collected in March 2018 and was grown in bright light in a greenhouse setting. Research has shown that the chemical composition of K. pinnata is dependent on the plant’s light, growth, and harvest conditions; in bright light, the concentration of quercetin increased sevenfold, and that flavonoid compounds were more abundant during summer months (Muzitano et al., 2011). It is possible that the suboptimal growth conditions of the K. mortagei plant used for extract 1420 prevented the production of certain bioactive secondary metabolites.

CONCLUSION

Kalanchoe is an important genus with relevance to traditional medicine across the globe. We have provided a comprehensive review of the reported antibacterial activities of Kalanchoe species, in particular K. pinnata, K. crenata, K. blossfeldiana, and K. laciniata. For the first time, we have reported the antibacterial activities of two understudied species in this genus (K. fedtschenkoi and K. mortagei) against clinically relevant, multidrug-resistant (MDR) strains of Gram-positive and Gram-negative bacteria. Our counterscreens against HaCaTs demonstrated that these extracts exhibit low toxicity to mammalian cells, supporting specificity of the action of these extracts against bacterial pathogens. Extracts were also characterized by HPLC, using chemical standards for peak identification and differentiation in their composition.

We demonstrated the antibacterial potential of K. fedtschenkoi against three ESKAPE pathogens. Particularly noteworthy was the specific growth-inhibition observed for A. baumannii, a Gram-negative species with rising global incidence that currently lacks sufficient treatment options (Boucher et al., 2009). In order to fully examine the potential of K. fedtschenkoi secondary metabolites, future work should aim to characterize the bioactivity of different extracts through bioassay-guided fractionation and isolation of active fractions and/or individual compounds. Additional studies should also look to address potential biofilm-inhibitory properties and interference in bacterial quorum sensing. K. blossfeldiana extracts have been shown to reduce biofilm growth or destroy biofilms entirely (Sarkar et al., 2015), and biofilm inhibition remains one of the most likely avenues for successful implementation of an anti-bacterial agent derived from plants (Wright, 2017). Other members of the genus Kalanchoe neglected in research should also be assessed for anti-microbial potential.

AUTHOR CONTRIBUTIONS

NR grew and collected the plant specimens, prepared the extracts, and performed the antibacterial experiments. JL and NR performed the chemical analysis of the extracts. BD performed the HaCaT cytotoxicity experiments. CQ designed and directed the study. NR and CQ analyzed the data and wrote the manuscript. All authors read, revised, and approved the final manuscript.

FUNDING

This work was supported by the National Institutes of Health, National Institute of Allergy and Infectious Disease (R21 AI136563, PI: CQ). The content is solely the responsibility of the authors and does not necessarily reflect the official views of the NIH or NIAID. The funding agency had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

ACKNOWLEDGMENTS

The following reagent was obtained through BEI Resources, NIAID, NIH as part of the Human Microbiome Project: E. faecium, Strain 513, HM-959. The following reagents were obtained through the Centers for Disease Control Antibiotic/Antimicrobial Resistance Bank: A. baumannii, AR-BANK #0033; E. cloacae, AR-Bank #0008; and K. pneumoniae, AR-Bank #0016. Thanks to Dr. Alex Horswill (UC Denver) for provision of the P. aeruginosa isolate (AH-71; PA01) and Dr. Mark Smeltzer (UAMS) for provision of the S. aureus isolate (UAMS-1). Thanks to Dr. Tharanga Samarakoon (Emory Herbarium) for assistance with identification of the study species and Erik Edwards (Emory Greenhouse) for assistance with sample cultivation.

REFERENCES


 дополнительно включены данные о работе с B. aeruginosa (AH-71), показавшей увеличение бактериальной роста, и против всех шести штаммов, где наблюдается статистически значимая разница в перформансах между этими двумя экстрактами (верифицировано с помощью t-теста). HPLC анализ выявил более низкую интенсивность поглощения в области 35–80 мин для экстракта 1420 по сравнению с экстрактом 1468, хотя пик элюции был похож. Скафоидная кислота была конфirmed только в экстракте 1420, кемпферол был конфirmed только в экстракте 1468.

Есть вероятность, что эти разности обусловлены различиями в условиях уборки растений Kalanchoe mortagei, используемых для производства этих экстрактов. Экстракт 1420 был приготовлен из растения Kalanchoe mortagei, собранных в декабре 2017 года, которые были выращены в условиях пониженной освещенности. Экстракт 1468, напротив, был собран в марте 2018 года и выращен в солнечном свете в теплице. Исследования показали, что химический состав растений Kalanchoe pinnata зависит от света, условий роста и уборки; при солнечном освещении концентрация кверцетина увеличивалась в семь раз, а флавониды были более обильными в летние месяцы (Muzitano et al., 2011). Это возможно, что неприятные условия уборки растения Kalanchoe mortagei, использованного для экстракта 1420, предотвратили производство определённых биоактивных вторичных метаболитов.

ВЫВОД

Kalanchoe является важным родом с традиционным значением в медицине по всему миру. Мы выполнили обзор опубликованных данных о бактерицидных свойствах видов Kalanchoe, включая K. pinnata, K. crenata, K. blossfeldiana, и K. laciniata. За первую раз в этом роде мы сообщили о бактерицидных свойствах двух недостаточно исследованных видов в этом роде (K. fedtschenkoi и K. mortagei) против клинически значимых, мультирезистентных (MDR) штаммов Gram-положительных и Gram-отрицательных бактерий. Наша контроверсия против HaCaTs доказала, что эти экстракты имеют низкую токсичность к клеткам млекопитающих, подтверждая специфичность действия этих экстрактов против бактериальных патогенов. Экстракты были также характеризованы с помощью HPLC, используя химические стандарты для определения пиков и дифференцирования их в композиции.

Мы доказали бактерицидную способность K. fedtschenkoi против трёх ESKAPE патогенов. Особенно примечательно было обнаружение специфического ингибирования роста A. baumannii, растущего с растущим глобальным показателем, который в настоящее время не обладает достаточным режимом лечения (Boucher et al., 2009). Во втою раз было необходимо исследовать потенциал K. fedtschenkoi вторичных метаболитов, будущая работа должна стремиться к характеристике биоактивности различных экстрактов через биоассау-применимую фракционацию и изоляцию активных фракций и либо индивидуальных составляющих.
Phytochemistry. 67, 2071–2077.


Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2019 Richwagen, Lyles, Dale and Quave. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.