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Comparative analysis of capsaicin in twenty nine varieties of unexplored Capsicum and its antimicrobial activity against bacterial and fungal pathogens

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In this study, the capsaicin content of chile pepper extracts from 29 unexplored varieties of *Capsicum* (twenty five varieties of *Capsicum chinense* and five of *Capsicum annuum*) was quantified and correlated with the antimicrobial potential against bacterial and fungal pathogens. The capsaicin content and bactericidal activity against numerous human pathogens (*Salmonella*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus*, *Candida albicans*) was compared to identify the most effective chile pepper varieties in the treatment of bacterial and fungal pathogens. The capsaicin content of the tested varieties varied from 29 to 42,633 ppm (139 - 682,135 SHU). On average, the fruits of *C. chinense* cultivars contained much higher concentrations of capsaicin than *C. annuum* cultivars. The undiluted chile peppers extracts with capsaicin concentrations greater than 25,000 SHU demonstrated bactericidal and antifungal effects. Overall, it was determined that *L. monocytogenes* and *S. aureus* were more susceptible to the antimicrobial effects of capsaicin than *Salmonella* and *E. coli* O157:H7, while *C. albicans* was markedly more susceptible than all bacterial species examined. The extract of the sixth most pungent cultivar, *C. chinense* Bhut Jolokia Red, showed the greatest antimicrobial potency of all screened peppers. The antimicrobial activity of pepper extracts was not directly correlated with increasing capsaicin concentrations, indicating that various *Capsicum* cultivars may possess distinct capsaicin derivatives. This is the first study which showed the relationship between capsaicin contents in different *Capsicum* varieties and their antimicrobial potential, and opens avenues in the study of capsaicin derivatives and their role in health and medicines.

Key words: Capsicum, pathogen, capsaicin, peppers, antimicrobial, bactericidal.

INTRODUCTION

The emergence of antibiotic resistance has prompted scientists to explore medicinal plants, not only to ascertain claims of efficacy and safety, but also to develop phytochemical based new drugs as alternatives to antibiotics. Plants of the genus *Capsicum*, colloquially referred to as chile peppers, have been widely grown for food and medicinal purposes for centuries. It is believed that the Aztec and Tarahumara peoples used chile

peppers as herbal remedies for coughs and bronchitis. Chile peppers have traditionally been used as antiseptics in African cultures, and in gastrointestinal and anti-inflammatory applications in India (Corson and Crews, 2007; Cichewicz and Thorpe, 1996). Capsaicin, the major bioactive molecule in *Capsicum*, has garnered medical attention due to its analgesic, anticancer, cardioprotective and antimicrobial properties (Omolo et al., 2014; Dorantes et al., 2008).

Additionally, capsaicin provides a unique characteristic and pungent taste to each variety of *Capsicum*. All plants of the *Capsicum* genus produce varied amount of capsaicin, however, only a few chile pepper varieties have been scientifically examined for their antimicrobial properties against bacterial and fungal pathogens. The antimicrobial activity of chile pepper extracts have been tested on a small selection of *Capsicum annum*, *Capsicum baccatum*, *Capsicum frutescens*, *Capsicum pubescens*, and *Capsicum chinense* cultivars, representing some of the milder members of the genus *Capsicum* (Cichewicz and Thorpe, 1996; Sanatombi and Sharma, 2008; Al Othman et al., 2011; Dorantes et al., 2000; Bacon et al., 2017). Only the Habanero variety of the most pungent *Capsicum* species, *C. chinense*, has been examined for bactericidal activity. Similarly, *C. chinense* varieties with extremely high capsaicin concentrations, such as the Trinidad Moruga Scorpion, Trinidad Douglah, Trinidad 7-Pot, Trinidad Scorpion, and Bhut Jolokia ("Ghost Pepper") have never been tested for medicinal use (Omolo et al., 2014; Mills-Robertson et al., 2012).

The "hotness" of chile peppers has historically been expressed in Scoville Heat Units (SHUs), which was determined by a panel of human taste testers according to the Scoville organoleptic test. Once analyzed according to this method, chile peppers are sorted by their degree of pungency. There are five levels of pungency classification on the Scoville scale: non-pungent (0 – 700 SHU), mildly pungent (700 – 3,000 SHU), moderately pungent (3,000 – 25,000 SHU), highly pungent (25,000 – 80,000 SHU) and very highly pungent (>80,000 SHU) (Al Othman et al., 2011).

The organoleptic method is neither precise nor objective, and has led to the advancement of analytical methods to determine the capsaicin content of chile peppers. One such method is an antibody based capsaicin assay determines the amount of capsaicinoids present in peppers through comparisons to known standards in 96 micro-well plates and the use of a spectrophotometer.

Alternatively, Liquid Chromatography (LC) and High-Performance Liquid Chromatography (HPLC) have been used to determine capsaicin content in peppers (Perkins et al., 2002; Sanatombi and Sharma, 2008; Al Othman et al., 2011). These analytical methods are more time consuming and costly than antibody-based assays. The antibody based capsaicin assay was selected for this study as it is a high throughput, rapid method that has demonstrated reliability of capsaicin content determinations for whole fruits in numerous other studies (Perkins et al., 2002; Stewart et al., 2007; Rollyson et al., 2014; Kantar et al., 2016).

Monitoring cellular respiration is a common way to assess the viability of cell cultures. This practice uses biochemically active compounds that change color in the presence of electrons generated by cellular respiration. Resazurin is a deep blue, tetrazolium-based, non-toxic, oxidation-reduction dye that is reduced intracellularly to the pink compound, resorufin, by electron-generating enzymes involved in microbial respiration (Ahmed et al., 1994; O'Brien et al., 2000; Byth et al., 2001; Węsierska-Gądek et al., 2005; Fai and Grant, 2009).

The resazurin assay is non-destructive to the cell, and is useful for monitoring the viability of numerous microorganisms under environmental stressors (Byth et al., 2001; Twigg, 1945; Liu, 1989; Guerin et al., 2001; Mariscal et al., 2009). The assay is simple, sensitive, rapid, robust, reliable, relatively inexpensive, and serves to assess antibacterial properties of natural products such as plant compounds (Zhi-Jun et al., 1997; Gloeckner et al., 2001; Sarker et al., 2007; Vega-Avila and Pugsley, 2011; Rampersad, 2012).

In this study, the resazurin assay was used as an indicator of cell viability after exposure to chile pepper extracts, to test 29 previously untested chile pepper varieties for their antimicrobial properties. The 29 chile pepper extracts were tested against several pathogenic organisms: the Gram-positive bacteria *Listeria monocytogenes* and *Staphylococcus aureus*, Gram-negative bacteria *Escherichia coli* O157:H7 and *Salmonella* Typhimurium, and the pathogenic yeast *Candida albicans*.

Presently, there has been no report where the capsaicin contents of the fruits of several *Capsicum* cultivars have been correlated with antimicrobial activity against bacterial and fungal pathogens. Many of the *Capsicum* varieties regarded as the "hottest in the world" have never been examined for antimicrobial properties.

Therefore, the unique focus of the current study was to cultivate 29 varieties of *Capsicum*, including the hottest known varieties, together in a controlled environment and study the correlation of capsaicin contents with antimicrobial potential against bacterial and fungal pathogens. We identified a *Capsicum* variety with low

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capsaicin content and showed bactericidal activity, very similar to the variety with highest capsaicin content. These results of the current study provide new direction to explore derivatives of capsaicin or additional bioactive molecules with applications in the treatment of topical and digestive human diseases caused by bacterial and fungal pathogens.

MATERIALS AND METHODS

The resazurin dye powder was obtained from Difco Inc. (Corpus Christi, TX). The Capsaicin Plate Kit (Cat. # 20-0027) was purchased from Beacon Analytical Systems Inc. (Saco, ME). The capsaicin powder (99.1% pure) was purchased from Chem-Impex Int'l Inc. (Wood Dale, IL) and stored at 4°C. SSB14B stainless steel beads (0.9-2.0 mm diameter) were purchased from Thomas Scientific (Swedesboro, NJ), for tissue homogenization using a Gold Bullet Blender (Next Advance, Inc., Averill Park, NY). BBLTM tryptic soy agar (TSA), BectoTM tryptic soy broth (TSB), yeast extract peptone dextrose (YPD) media and BBLTM brain heart infusion (BHI) broth were purchased from Becton, Dickinson and Company (Sparks, MD).

Cultivation of pepper plants

Seeds of 29 pepper varieties were purchased from various seed companies across North America (Table 1) to explore their phenotypic diversity. Seed samples were procured from different geographic regions, and represent two different *Capsicum* species (*C. chinense* and *C. annuum*) (Table 1). Seed were sown 0.6 cm deep in Master Garden Premium potting mix (Premier Tech Horticulture, Ltd., Ontario, Canada) in a 32-cell tray (volume = 150 cm^3). Trays were covered with a clear plastic lid (approx. 10 cm above media) and were placed in a greenhouse maintained at 23°C air temperature. When seeds germinated and cotyledons unfolded, the lid was removed and plants were grown in the same environment for an additional 9-14 weeks under natural daylight plus 25 $\mu mol \cdot m^{-2} \cdot s^{-1}$ supplemental irradiance (0800-0200 HR; Sunblaze T5 fluorescent lights, Sunlight Supply, Inc., WA, USA; +1.62 mol m^{-2} d^{-1}). Plants fertilized with Pure Blend Pro Grow (3-2-4), Pure Blend Pro Bloom (2-3-5), and Cal-Mag Plus (all from Botanicare, AZ) for a ten-week period following the manufacturer's recommendations. All the chile pepper plants were transplanted in the University of Minnesota agricultural fields in June of 2015, developed by applying Sustane 4-6-4 Organic Plant Fertilizer (Sustane Natural Fertilizer, Inc., MN) fertilizers as recommended by Bosland et al (Paul et al., 2012). Fruits were harvested in September 2014 (Figure 1). All the ripened pepper fruits were harvested from three plants of each chile pepper cultivar variety and stored at -20°C.

Microbiological methods

L. monocytogenes Scott A, *Salmonella* Typhimurium LT2, and *Escherichia coli* O157:H7 EDL933 bacterial cultures were obtained from the Food Safety Microbiology Laboratory (Department of Food Science and Nutrition), while the *S. aureus* 6538 and *C. albicans* ATCC 10231 cultures were obtained from the Hegeman Laboratory (Department of Horticultural Science), at the University of Minnesota – Twin Cities (St. Paul, MN).

L. monocytogenes was streak plated on BHI agar media, while the other bacterial strains were plated on TSA agar medium. *Candida albicans* was grown in YPD agar medium. The plates were incubated overnight at 37°C for bacterial strains and at 30°C for *C. albicans*, and subsequently stored at 4°C. The test cultures were prepared by inoculating 5 ml of respective broth media and incubating overnight at 37°C for bacterial strains and at 30°C for *C. albicans* with shaking at 150 rpm.

Preparation of chile pepper extracts

The 29 chile pepper varieties included 24 very highly pungent varieties (samples 1 - 24) and five non-pungent varieties (samples 25 - 29) (Figure 1). For each variety, one pepper fruit was randomly selected from three different plants of each variety and treated independently, providing a total of 87 chile pepper extracts. The samples were prepared by crude methanol extraction as previously described (Beacon Analytical Systems, 2011), and this method was selected as methanol extractions of chile peppers have been shown to exhibit the greatest antimicrobial activity (Bacon et al., 2017). Whole pepper fruits were briefly washed under running tap water and dried with paper towels. 5 g of pepper fruits were cut into small pieces of approximately 5 mm and transferred to a 50 ml conical tube containing eight stainless steel beads, and homogenized in a Bullet Blender tissue homogenizer for 12 min to make a paste. The resultant pepper paste was mixed with 12.5 ml of 100% methanol and the slurry was further homogenized for 12 min. The methanolic fraction without the cell debris was centrifuged for 23 min at 4,000 rpm. The resultant pepper extracts were filter-sterilized through a 0.2 µm Acrodisc filter (Pall Corporation, NY). The methanolic pepper extracts were diluted 1:2 using sterile deionized water and stored at 20°C until used for the assays. The capsaicin contents of all the 87 extracts were determined using the Capsaicin Plate Kit, as per the manufacturer's instructions (Beacon Analytical Systems, 2011).

For the resazurin based cell viability assays, 0.02% (w/v) resazurin dye was prepared in deionized water, filter-sterilized through a 0.2 µm Acrodisc syringe filter and stored at 4°C until use (Sittampalam et al., 2014). For each test, 50, 25, 12.5, and 6.25 µl of 1:2 diluted pepper extract were added into each well of a sterile 96 well microtiter plate and diluted with BHI for *L. monocytogenes*, or TSB for all other test microbes to obtain 1:4, 1:8, 1:16 and 1:32 dilutions respectively. The resulting volume in each test well was 100 µl.

The microbial inoculum was prepared from an overnight culture by adjusting the OD_{600} to 0.08 or 0.13, representing approximately 5×10^6 or 5×10^7 CFU/ml yeast and bacteria cells respectively. 100 µl of the bacterial or yeast cell inoculum was pipetted into each test well. As a positive control, an equal amount of methanol was mixed with BHI broth for *L. monocytogenes* and TSB for all other microbes and was pipetted into the positive control well and diluted with sterile broth media to a volume of 100 µl. 100 µl of inoculum was then added for a total volume of 200 µl. 100 µl of the appropriate broth was pipetted into the growth control well, along with 100 µl of inoculum. An equivalent amount of the methanol in the samples used was pipetted into the negative control well. The methanol was diluted with the appropriate broth to a total of 200 µl. 20 µl of the 0.02% resazurin dye was added into each well. The plates were incubated for 2 h at 37°C, photographed, and the results were recorded based on change in color from deep blue to pink or purple. The changes in color were observed and scored as 1 (no change in resazurin color), 0.5 (changed to indigo/purple) and 0.0 (changed to pink). Averages < 0.5 are reported as 0. Averages <1 but ≥ 0.5 are reported as 0.5. Averages ≥ 1 are reported as 1.

Statistical analysis

A student's *t*-test analysis was used to determine the statistical

Table 1. Summary of Chile pepper cultivar and their source used in the current study.

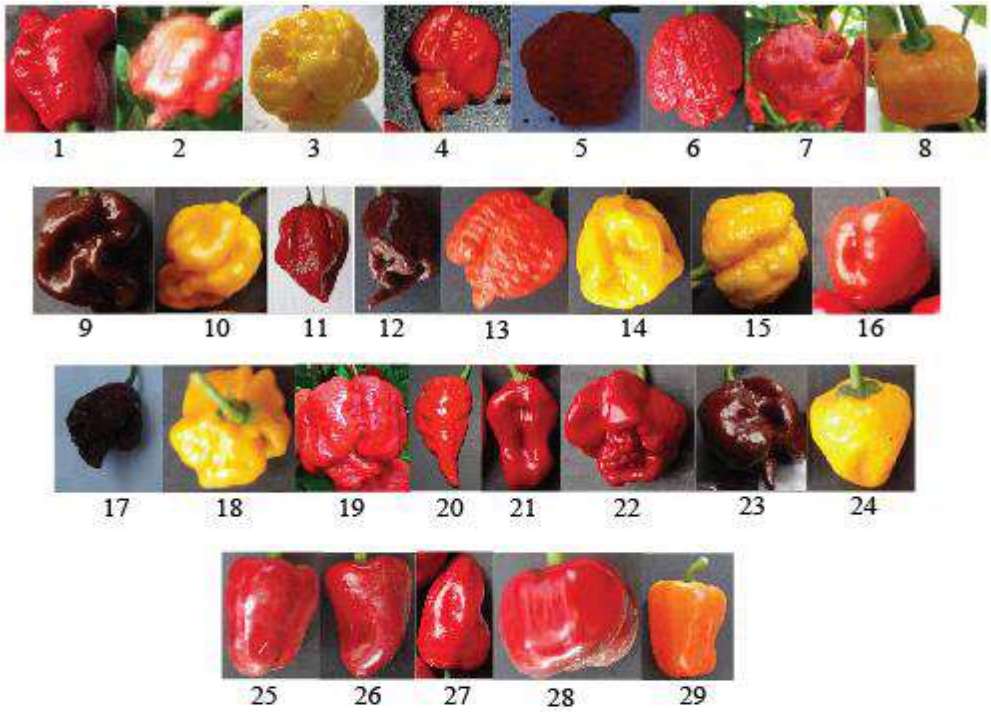
Chile pepper cultivar	Species	Seed source
Carolina Reaper	*C. chinense*	Puckerbutt Seeds, Fort Mill, SC, USA
Scotch Bonnet		Reimer Seeds, Saint Leonard, MD, USA
7-pod Congo SR Gigantic		Pepperlover.com
Brainstrain Yellow		
Trinidad 7-pot Primo		
7-pot Brown		
Trinidad 7-pot Brainstrain Red		
Yellow Moruga		
Trinidad Scorpion 7-pot		Refining Fire Chiles, San Diego, CA, USA
Trinidad Douglah		
Trinidad 7-pot		
Habanero Orange Blob		Dr. Joe Delaney, St. Paul, MN, USA
Trinidad Scorpion Chocolate		
Tobago Scotch Yellow		
Tobago Scotch Bonnet Red		
Brown Scotch Bonnet		
Trinidad Moruga Scorpion		Chile Pepper Institute, Las Cruces, NM, USA
Bhut Jolokia Red		
7-Pot Jonah		Hugo Feed Mill and Hardware, Hugo, MN, USA
Congo Trinidad		
Trinidad Large 7-pod Yellow		
Brown Trinidad Moruga Scorpion		
HHP Moruga		
Orange Blaze	*C. annuum*	Hugo Feed Mill and Hardware, Hugo, MN, USA
Red Ruffled Pimiento		
Red Majesty Sweet		
Tasty Paprika GL		
Romanian Rainbow		

significance of the difference between samples, and between samples and standards.

RESULTS

Determination of capsaicin content

The 29 varieties of *Capsicum* used in this study were harvested from the farm field and are shown in Figure 1. The mature fruits of 15 *Capsicum* were red, 8 were yellow, whereas 6 were brown in color. The details of 29 varieties of *Capsicum* and their seed source are mentioned in Table 1. The extracts of chile peppers were subjected to quantitative estimation of capsaicin and its correlation with antimicrobial activity. The extracts of 29 *Capsicum* varieties were subjected to quantitatively estimate the capsaicin content (ppm) as determined previously. The capsaicin content in 29 varieties varied from 29 to 42,633 ppm (Figure 2). In some extracts, the high standard deviation in capsaicin content is a reflection of variation in levels of capsaicin in three different fruits of the same plant, similar to variation reported in previous studies (Bacon et al., 2017). The capsaicin content (ppm) values were then multiplied by 16 to obtain Scoville Heat Unit (SHU) conversion values and summarized in Table 2. Based on SHU values, the twenty nine *Capsicum* varieties were classified into three broad categories. Nineteen Capsicum varieties were



1.7 - Pod Congo SR Gigantic
2.Carolina Reaper
3.Brainstrain Yellow
4.Trinidad Scorpion
5.7 - Pot Jonah
6.Trinidad Douglah
7.Trinidad 7–Pot Primo
8.Habanero Orange Blob
9.Trinidad Scorpion Chocolate
10.Tobago Scotch Yellow
11.Trinidad 7–Pot
12.7–Pot Brown
13.Trinidad 7- Pot Brainstrain Red
14.Trinidad Large 7– Pod Yellow
15.Yellow Moruga
16.Congo Trinidad
17.Brown Scotch Bonnet
18.7–Pot
19.Trinidad Moruga Scorpion
20.Bhut Jolokia Red
21.Tobago Scotch Bonnet Red
22.HHP Moruga
23.Brown Trinidad Moruga Scorpion
24.Scotch Bonnet
25.Red Majesty Sweet
26.Romanian Rainbow
27.Tasty Paprika GL
28.Red Ruffled Pimento
29.Orange Blaze

Figure 1. Fruits of chile peppers of 29 varieties. To prepare the chile pepper extracts, 3 fruits of chile peppers of each variety were randomly selected from each Capsicum plant.

classified as very highly pungent (>80,000 SHU), four as highly pungent (25,000-80,000 SHU) and six as non-pungent (0-700 SHU) as shown in Table 2. Trinidad 7 - Pot Brainstrain Red was the most very highly pungent variety with 682,136 SHU. Interestingly, all the five *C. annuum* varieties and one C. *chinense*, the Tobago Scotch Bonnet Red, were classified as non-pungent varieties. The six non-pungent varieties showed very trace amounts of capsaicin ranging from 139-470 SHU. Moreover, among non-pungent varieties, Tobago Scotch Bonnet Red showed more capsaicin content (470 SHU) as compared to all the five *C. annuum* varieties (139-241 SHU).

Evaluation of antimicrobial activity

The extracts of 29 varieties of *Capsicum* were tested for antimicrobial activity using a resazurin dye based assay using microwell plates. The capsaicin content in the extracts was correlated with antimicrobial activity. Different dilutions (1:4, 1:8, 1:16 and 1:32) were tested for antimicrobial activity against *L.* monocytogenes, *S. aureus*, *Salmonella*, *E. coli* O157:H7, and *C. albicans*.

Table 2. Summary of Capsaicin content of 29 chile pepper cultivars used in this study. Cultivars with similar capsaicin could be grouped together to make generalized conclusion.

Levels of pungency	Chile pepper cultivar	*SHUavg	*µg/mlavg
	Trinidad 7 - Pot Brainstrain Red	682.135 ± 308.499	42.633 ± 13.031
	Trinidad Moruga Scorpion	649.544 ± 47.816	40.596 ± 2.989
	Trinidad 7 - Pot Primo	618.371 ± 254.201	38.648 ± 15.888
	Trinidad 7 - Pot	617.228 ± 182.863	38.577 ± 11.429
	HHP Moruga	556.929 ± 5.165	34.808 ± 323
	Bhut Jolokia Red	531.873 ± 100.398	33.242 ± 6.275
	7 - Pot Jonah	520.570 ± 26.809	32.536 ± 1.676
	Trinidad Douglah	474.156 ± 124.912	29.635 ± 7.807
	Trinidad Scorpion	452.326 ± 269.854	28.270 ± 16.866
Very Highly pungent (> 80,000 SHU)	Brown Trinidad Moruga Scorpion	373.536 ± 65.883	23.346 ± 4.118
	7 - Pot	373.332 ± 36.784	23.333 ± 2.299
	7 - Pot Brown	310.395 ± 116.800	19.400 ± 7.300
	Trinidad Large 7 - Pod Yellow	181.834 ± 18.947	11.365 ± 1.184
	Yellow Moruga	171.877 ± 12.481	10.742 ± 1780
	Trinidad Scotch Yellow	159.662 ± 48.874	9.979 ± 3.055
	Trinidad Scorpion Chocolate	143.120 ± 6.044	8.945 ± 378
	Brown Scotch Bonnet	133.698 ± 74.470	8.356 ± 4.654
	Brainstrain Yellow	120.093 ± 68.353	7.506 ± 4.272
	7 - Pod Congo SR Gigantic	114.891 ± 30.554	7.181 ± 1.910
	Habanero Orange Blob	75.117 ± 16.779	4.695 ± 1.049
	Congo Trinidad	60.479 ± 10.484	3.780 ± 655
Highly pungent (25,000- 80,000 SHU)	Carolina Reaper	44.448 ± 25.544	2.778 ± 1.597
	Scotch Bonnet	27.789 ± 7.275	1.737 ± 455
	Tobago Scotch Bonnet Red	470 ± 223	29 ± 14
	Red Majesty Sweet	241 ± 16	15 ± 1
	Romanian Rainbow	231 ± 10	14 ± 0.6
Non-pungent	Orange Blaze	202 ± 14	13 ± 0.9
(0-700 SHU)	Red Ruffled Pimento	183 ± 6	11 ± 0.4
	Tasty Paprika GL	139 ± 5	9 ± 0.3

*The data represent the average capsaicin content of triplicate samples (in SHU and µg/ml). The wide standard error margins (samples 1 – 24) are reflective of the wide range of capsaicin contents observed within the same chile pepper varieties.

After resazurin dye addition, color change was observed after 2 h. The results are presented in numerical scores based on the change in original dark blue color of resazurin. A dark blue, purple and pink colors represent 1.0 (bactericidal), 0.5 (partial bactericidal) and 0.0 (bacteriostatic) scores respectively. In addition, the negative control had a score of 1, while the positive and growth controls both had a score of 0.

As a control, pure capsaicin at 200 ppm (3,200 SHU) showed partial bactericidal effects on all the tested organisms. The minimum bactericidal concentration of pure capsaicin was approximately 250 ppm (4,000 SHU) for all microorganisms tested. The extracts with low capsaicin (<700 SHU), showed no effect on the cell viability of the microorganisms tested. For some extracts even with high capsaicin content, showed partial bactericidal effect and it varied between *Capsicum* varieties as indicated in the Supplemental Table 1.

Among all the microorganisms, *C. albicans* was most sensitive to methanolic extracts of *Capsicum* and showed complete growth inhibition at 1:4 and 1:8 dilutions. Moreover, extracts of 20 *Capsicum* also showed partial growth inhibition even at 1:16 or up to 1:32 dilutions (Figure 3). At the 1:4 and 1:8 dilutions, the methanol concentrations in all the samples were high enough to kill the yeast cells as indicated by the positive control results. It was surprising to note that all the 29 extracts of *Capsicum* showed partial growth inhibition of both the Gram-negative bacteria (*S.* Typhimurium and *E. coli* O157:H7) even at 1:4 dilution. *S. aureus* and *L.*

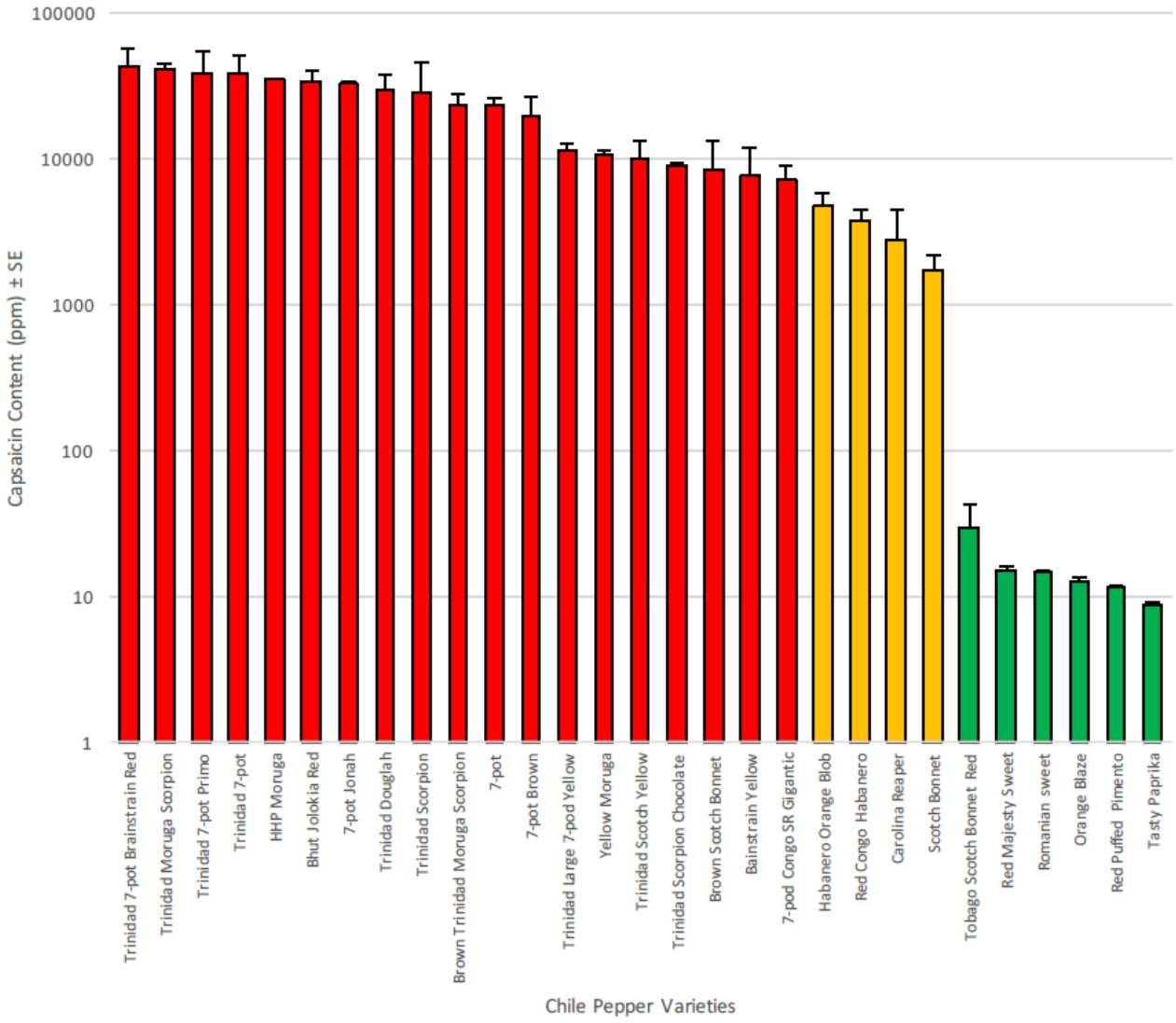


Figure 2. Capsaicin content in 29 varieties.

monocytogenes showed highly varied responses to different extracts, which would confer a bactericidal effect on one of the organisms, but a bacteriostatic effect on the other.

Out of 19 very highly pungent *Capsicum* extracts, 14 showed bactericidal effect against *S. aureus* at 1:4 dilution. Extracts of the Bhut Jolokia Red cultivar showed partial bactericidal effect even at 1:16 dilution and extracts of the Trinidad Large 7 - Pod Yellow, Yellow Moruga, Trinidad Scorpion Chocolate, and Habanero Orange Blob showed partial bactericidal effect at 1:8 dilution. It is interesting to note that Habanero Orange Blob is highly pungent variety (75,117 SHU), but showed better antimicrobial activity against *S. aureus* and *L. monocytogenes* in contrast to Trinidad 7 - Pot Primo, which is classified as very highly pungent (618,371 SHU) variety and showed only partial bactericidal effect at 1:4 dilution. Similarly, Congo Trinidad with 60,479 SHU showed bactericidal effect, but is classified as highly pungent. All the six non pungent varieties showed no antimicrobial activity against any of the microorganisms tested.

Out of 19 very highly pungent *Capsicum* extracts, only 7 showed bactericidal effect against *L. monocytogenes* at 1:4 dilutions. Similar to *S. aureus,* extracts of Bhut Jolokia Red showed partial bactericidal effect even at 1:16 dilution, thus suggesting it is uniquely potent among other *Capsicum* varieties. Extracts of three very highly pungent *Capsicum* varieties, Trinidad Scorpion, Brown Trinidad Moruga Scorpion, and Trinidad Scorpion Chocolate, also showed partial bactericidal effect at 1:8 dilution. Similarly, Congo Trinidad with 60,479 SHU showed bactericidal effect at 1:4 dilutions, but classified as highly pungent. The highly pungent Carolina Reaper and Scotch Bonnet

Table 3. Antimicrobial scores for 29 cultivars of the chile peppers (*C. chinense and C. annuum*) tested against bacterial and fungal pathogens.

Chile pepper cultivar	Capsaicin$_{avg}$ (ppm)	*S. aureus* 6538	*S.* Typhimurium LT2	*E. coli* O157:H7 EDL933	*L. monocytogenes* Scott A	*C. albicans* ATCC 10231	Total score
Bhut Jolokia Red	33.242 ± 6.275	2.0	0.5	0.5	2.0	1	6.0
Trinidad Scorpion Chocolate	8.945 ± 378	1.5	0.5	0.5	1.5	1	5.0
Yellow Moruga	10.742 ± 1.780	1.5	0.5	0.5	1.0	0.5	4.0
Trinidad Large 7 - Pod Yellow	11.365 ± 1.184	1.5	0.5	0.5	1.0	0.5	4.0
Trinidad 7 - Pot	38.577 ± 11.429	1.0	0.5	0.5	1.0	1	4.0
Habanero Orange Blob	4.695 ± 1.049	1.5	0.5	0.5	1.5	0	4.0
Brown Trinidad Moruga Scorpion	23.346 ± 4.118	0.5	0.5	0.5	1.5	1	4.0
Trinidad Scorpion	28.270 ± 16.866	1.0	0.5	0.5	1.5	0	3.5
Trinidad Moruga Scorpion	40.596 ± 2.989	1.0	0.5	0.5	0.5	1	3.5
Trinidad Douglah	29.635 ± 7.807	1.0	0.5	0.5	0.5	1	3.5
Trinidad 7 - Pot Brainstrain Red	42.633 ± 13.031	1.0	0.5	0.5	0.5	1	3.5
HHP Moruga	34.808 ± 323	1.0	0.5	0.5	0.5	1	3.5
7 - Pot Jonah	32.536 ± 1.676	1.0	0.5	0.5	0.5	1	3.5
7 - Pot Brown	19.400 ± 7.300	1.0	0.5	0.5	0.5	1	3.5
7 - Pot	23.333 ± 2.299	1.0	0.5	0.5	0.5	1	3.5
Congo Trinidad	3.780 ± 655	1.0	0.5	0.5	1.0	0	3.0
Carolina Reaper	2.778 ± 1.597	0.5	0.5	0.5	0.5	0.5	2.5
7 - Pod Congo SR Gigantic	7.181 ± 1.910	1.0	0.5	0.5	0.5	0	2.5
Trinidad Scotch Yellow	9.979 ± 3.055	0.5	0.5	0.5	0.5	0	2.0
Trinidad 7 - Pot Primo	38.648 ± 15.888	0.5	0.5	0.5	0.5	0	2.0
Brown Scotch Bonnet	8.356 ± 4.654	0.5	0.5	0.5	0.5	0	2.0
Brainstrain Yellow	7.506 ± 4.272	0.5	0.5	0.5	0.5	0	2.0
Scotch Bonnet	1.737 ± 455	0.5	0.5	0.0	0.5	0	1.5
Tobago Scotch Bonnet Red	29 ± 14	0.0	0.0	0.0	0.0	0	0.0

varieties showed partial bactericidal effect at 1:4 dilution against *S. aureus*, and *L. monocytogenes*. All the six non pungent varieties showed no antimicrobial activity against any of the microorganisms tested.

In general, the Gram-positive bacteria, *S. aureus*, and *L. monocytogenes* were more susceptible to the bactericidal effects of capsaicin than the Gram-negative bacteria, *Salmonella* and *E. coli* (Figure 3). The screened yeast, *C. albicans*, was the most susceptible to capsaicin than both the Gram-positive and Gram-negative bacteria (Figure 3).

Table 3 summarizes the scores of all the results presented in the Supplementary data Table 1. The scores in the columns represent average data from three independent experiments as previously mentioned. The last column sums up these scores to determine the chile pepper sample with the highest antimicrobial score. Bhut Jolokia had the highest score of 6 and Tobago Scotch Bonnet Red showed lowest score of zero.

Correlating capsaicin content with antimicrobial activity

Based on the student's *t*-test statistical analysis, it was surprising that that there is no significant difference between the scores from the chile peppers high in capsaicin ($p > 0.05$). In contrast, there was a significant difference between the scores from the chile peppers low in capsaicin ($p > 0.05$). However, the different in scores was statistically significant among *Capsicum* varieties with high capsaicin and those with little to no capsaicin ($p < 0.05$). The difference in scores was also statistically significant between the *Capsicum* extracts and the controls ($p < 0.05$). In addition to this, that there was a moderate correlation between the capsaicin content and the antimicrobial scores listed in Table 3 and Figure 4.

DISCUSSION

The Beacon Capsaicin Plate Kit assay is a polyclonal antibody assay specific for capsaicin determination of chile pepper fruits with reactivity to a limited number of closely related compounds (e.g. other capsaicinoids). Capsaicin is the most abundant capsaicinoid in chile peppers. The other capsaicinoids include dihydro-capsaicin, nordihydrocapsaicin, homodihydrocapsaicin and homocapsaicin. It has been established that milder

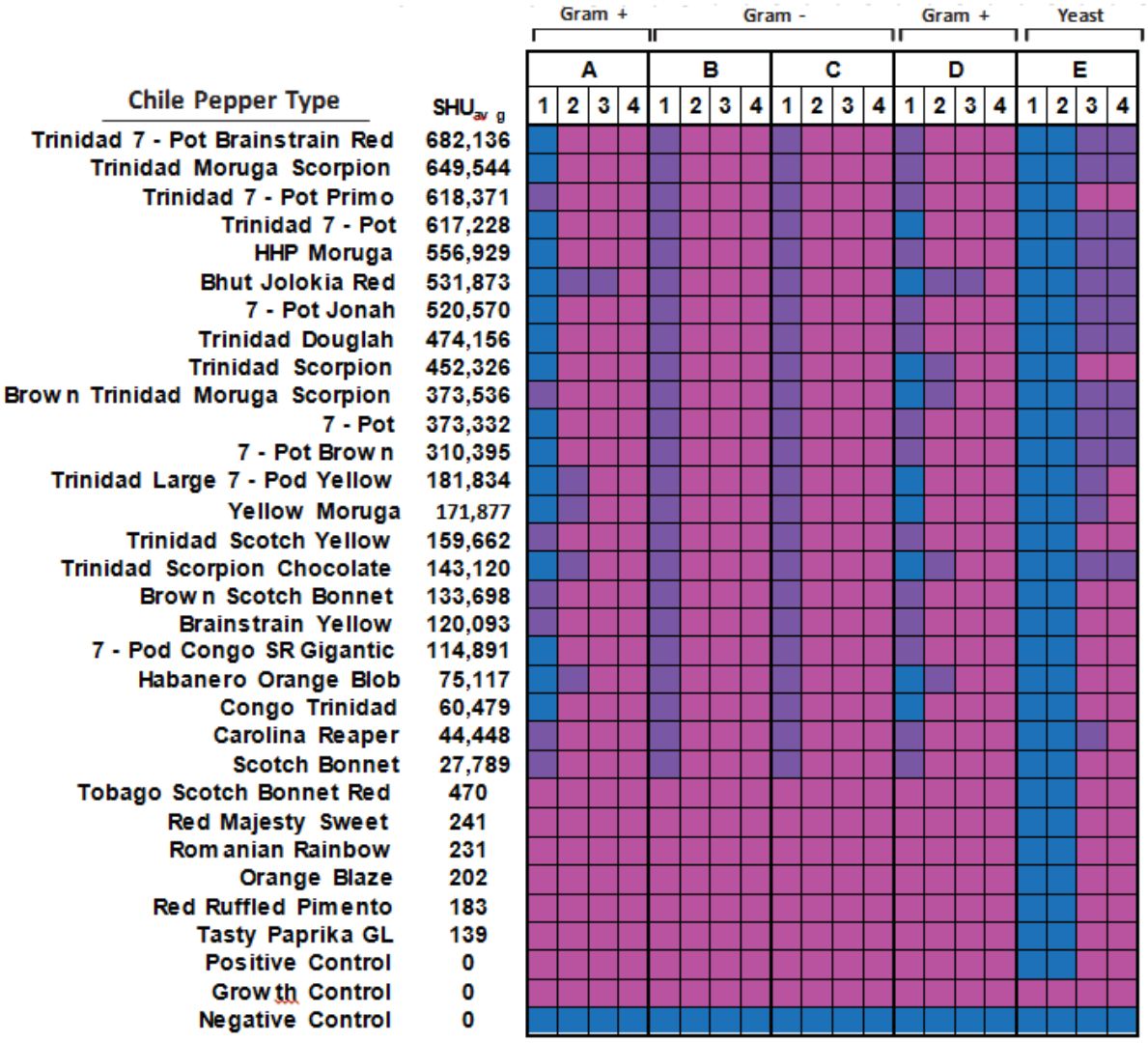


Figure 3. Extracts of 20 Capsicum.

varieties have capsaicinoid concentrations ranging from 0.003- 0.010% (w/w). Slightly hot varieties have capsaicinoids ranging from 0.01- 0.30% (w/w), while the hot varieties have a concentration range of 0.3- 1.0% (w/w) (Barbero et al., 2006). Based on this classification, the samples used herein from the *C. chinense* species were determined to be highly pungent, or very highly pungent. The samples from the *C. annuum* species were all non-pungent.

The amount of capsaicinoids in a chile pepper pod is dependent on the genetic makeup of the plant, the environment in which the plant is grown, the age of the fruit, and the position of the fruit on the plant. The heat level can vary considerably between plants of the same variety grown in a single field at the same time (Al Othman et al., 2011). When single plant heat levels of genetically identical chile pepper plants were compared with the field average, it was found that individual plants could possess heat levels as much as 78% higher than the field average, indicating that plant to plant variation of an individual variety contributes to the chile pepper heat levels (Bacon et al., 2017). Similar variations were observed in the data obtained from the capsaicin assay in this study (Figure 2).

Capsaicinoid biosynthesis is unique to the genus *Capsicum* and results from the acylation of the aromatic compound, vanillylamine, with a branched-chain fatty acid. The presence of capsaicinoids is controlled by the *Pun1* locus, which encodes a putative acyltransferase. In its homozygous recessive state, *pun1*/*pun1*, capsaicinoids are not produced by the pepper plant (Rollyson et al., 2014). The trace amounts of capsaicin in the “sweet” chile peppers were most likely because ancestral chile peppers all produced capsaicin, and the

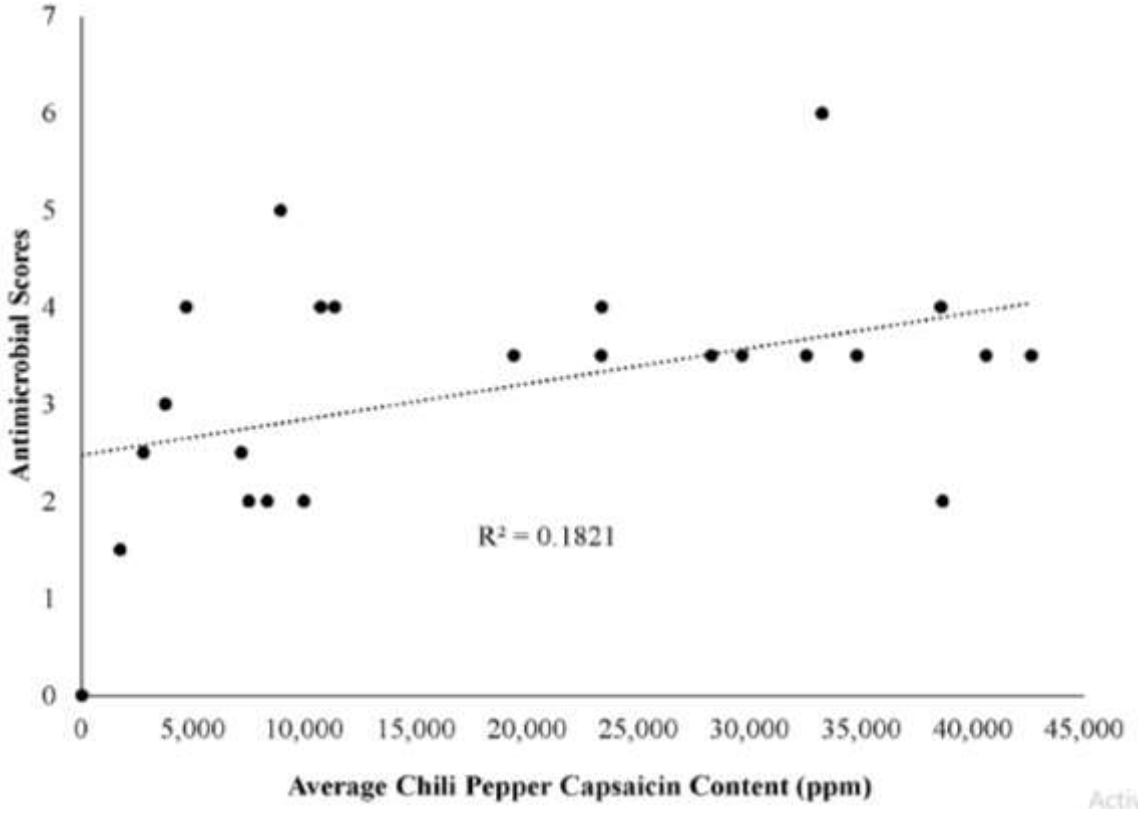


Figure 4. Antimicrobial scores for 29 cultivars of the chile peppers (*C. chinense and C. annuum*) tested against bacterial and fungal pathogens.

"sweet" chile varieties can revert to this ancestral trait.

The capsaicin data obtained in this study was used to determine whether there is a correlation between capsaicin content and bactericidal effects of the chile pepper extracts. While previous research suggests that capsaicin is the primary antimicrobial compound in chile peppers, data from this study implies that there might be other compounds or peptides with antimicrobial properties. Although Tobago Scotch Bonnet Red had very little capsaicin compared to the other samples of the *C. chinense* species, this cultivar showed some antimicrobial effects. This finding was unique because the other samples that had little capsaicin, but belonged to the *C. annuum* species, showed no antimicrobial properties. Since bactericidal activity of pure capsaicin was observed against all test organisms at >250 ppm (4,000 SHU), chile peppers lower than 4,000 SHU exhibiting partial or full bactericidal effect likely contain other antimicrobial compounds.

In this case, the Tobago Scotch Bonnet Red variety likely contains non-capsaicinoid antimicrobial compounds or peptides. Peptides isolated from the seeds of the habanero variety *C. chinense* have demonstrated antimicrobial effects against *S. aureus*, *E. coli* and other pathogenic organisms (Brito-Argaez et al., 2009). Such antimicrobial peptides may be responsible for the inhibitory effects observed in the Tobago Scotch Bonnet Red cultivar. Additional studies to isolate and determine antimicrobial properties of these chemicals are underway.

Figure 3 summarizes the average results from the resazurin assay at the various dilution levels of chile pepper extracts against all organisms. Viable cells with active metabolism can reduce resazurin dye into the resorufin product, resulting in a color change in the test wells from dark blue to purple or pink. The quantity of resorufin produced is proportional to the number of viable cells (Promega Corporation, 2016). Therefore, a purple color in a test well implies fewer viable and consequently, fewer metabolically active cells.

S. aureus was tested against four different dilution levels of the pepper samples (1:4, 1:8, 1:16, and 1:32). At the 1:4 dilutions, 16 of the samples high in capsaicin showed total bactericidal effects, while 7 varieties showed partially bactericidal effects. When the samples were diluted 1:8, five of the samples high in capsaicin were partially bactericidal, while the rest showed no observable effects. At the 1:16 dilution, only Bhut Jolokia affected cellular viability of *S. aureus*. No bactericidal effects were observed for any of the samples at the 1:32 dilution.

None of the samples showed total bactericidal effects against *Salmonella* Typhimurium at the concentrations

tested. Instead, a partial bactericidal effect was observed (at the 1:4 dilution), indicated by a resazurin change in color to purple for 23 samples. A similar effect was noted for *E. coli* O157:H7 with a partial bactericidal effect observed for the same 23 samples.

Against *L. monocytogenes*, 9 samples had a bactericidal effect on the cells at the 1:4 dilution, while 14 samples were partially bactericidal. At the 1:8 dilution, 5 samples, Bhut Jolokia, Trinidad Scorpion, Brown Trinidad Moruga Scorpion, Trinidad Scorpion Chocolate and Habanero Orange Blob, exhibited partial bactericidal effects.

C. albicans showed greatest susceptibility to the samples with 14 samples showing a partial bactericidal effect at the 1:16 dilution. 11 samples diluted as much as 1:32 were still partially bactericidal to *C. albicans* cells. At the 1:4 and 1:8 dilutions, the methanol concentrations were high enough to kill the *C. albicans* cells, so the data for these two tests were included for completion only (Figure 3). Based on the findings shown in Table 3, it was concluded that *E. coli* 0157:H7 and *Salmonella* Typhimurium were the least susceptible to the chile pepper extracts. This was expected, because both bacteria are contain an additional outer cell membrane and are generally more resistant to antimicrobials (Ibrahim et al., 2010).

The susceptibility of the microbes tested to the chile pepper samples brings into question the mechanism by which capsaicin affects fungal cells. Kurita et al. (2002) suggest that capsaicin enters the cells and functions as a toxin, possibly to the membrane structure, and/or as an osmotic stressor. Work to understand investigate the possible mechanism is currently underway in our lab. Since hospital acquired skin infections due to multidrug resistant *Candida* spp. are on the rise worldwide (Borman et al., 2016), many of the chile peppers studied in this work may hold promising treatment remedies.

As previously mentioned, the ancient Aztec and Tarahumara cultures used chile peppers to treat hundreds of human diseases, and some historians believe the ingestion of chile peppers promoted a healthier gut and could treat some diarrheal diseases, possibly due to multicellular eukaryotic parasites. There have been studies demonstrating anti-parasitic properties of chile peppers against the ectoparasite *Ichthyophthirius multifiliis* to control disease in the aquaculture industry (Ling et al., 2012), but studies with human parasites have not yet been conducted. Future studies in these areas are warranted, since chile peppers may represent a relatively inexpensive way to treat many human diseases.

Table 3 shows the total overall scores of each pepper type for each microorganism tested in this study. Bhut Jolokia Red had the highest score, meaning it had the greatest effect on microbial viability on the organisms tested. Figure 4 illustrates the linear correlation (*r*) between the antimicrobial scores and the capsaicin content of the *C. chinense* varieties. There was a moderate positive relationship between capsaicin concentration and antimicrobial scores ($r = 0.426$). Since there are over 30 classified chile pepper species, some may hold promise to treat human diseases, and studies in our lab are underway to investigate this further. Some question whether chile peppers are medically useful since they may irritate the patient. A recent study confirmed that human volunteers deemed capsaicinoid extract from the Bhut Jolokia chile pepper variety as an acceptable topical application for anti-arthritic and analgesic usage, thus overcoming an obstacle for future clinical applications of these important botanical sources (Sarwa et al., 2014, 2016). Since chile peppers are a widely accepted food item grown in every country in the world and known historically for broad-spectrum ethnopharmacological applications (Meghvansi et al., 2010), medical use of chile peppers, or isolated antimicrobial compounds, holds promise to treat many types of microbial infections and to numb pain caused by these associated diseases.

Conclusions

In this unique study, 29 cultivars of chile peppers were grown under controlled environmental conditions and correlated the capsaicin content with antimicrobial properties against fungal and Gram-negative and Gram-positive human pathogens. Prior to this study, the hottest chile pepper to be tested for antimicrobial properties was a Habanero pepper (~100,000 SHU). The presented study examines many unexplored chile pepper varieties that have recently garnered attention due to their very highly pungent flavor. Included in the examined peppers are the hottest chile pepper varieties in the world (>1,000,000 SHU). Of the 29 cultivars tested, three very highly pungent cultivars (Trinidad 7 - Pot Brainstrain Red, Trinidad Moruga Scorpion, and HHP Moruga) with very high capsaicin content displayed bactericidal activity against *S. aureus* and *C. albicans*. Furthermore, the Bhut Jolokia (Ghost pepper) displayed bactericidal activity *S. aureus*, *L. monocytogenes*, and *C. albicans* even at 1:16 dilution of the extract, making it the most effective variety of *C. chinense* against the tested microbial panel. With the rise in multidrug resistant (MDR) “superbugs”, specifically methicillin-resistant and vancomycin-resistant *St. aureus*, capsaicin and its derivatives from chile peppers should be further explored as antibiotic alternatives in the treatment of bacterial and fungal pathogens.

The results obtained in thus study also highlight that capsaicin contents can vary widely between different cultivars of same *Capsicum* variety. Additionally, peppers with the highest capsaicin concentrations did not always correlate with the greatest antimicrobial activity, as the sixth most pungent variety, Bhut Jolokia Red, showed the greatest antimicrobial activity of all screen varieties.

Therefore, we propose the notion that different capsaicinoids have different antimicrobial properties. This

opens the avenue for synthetic biology to improve the activity of capsaicin to treat multiple diseases.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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