



## SHORT COMMUNICATION

### Betanin from beetroot enhanced survival of *Caenorhabditis elegans* infected with methicillin-resistant *Staphylococcus aureus*

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#### ABSTRACT

**Aims:** Betanin is a red plant pigment belonging to the group called betalain. This present study aimed at investigating the effect betanin from beetroot (*Beta vulgaris* subsp. *vulgaris*) as a potential anti-infective agent against methicillin-resistant *Staphylococcus aureus* (MRSA) using a *Caenorhabditis elegans* infection model.

**Methodology and results:** The minimum inhibitory concentration of betanin against MRSA strain ATCC33591 was determined to establish the non-inhibitory concentration. The minimum inhibitory concentration of betanin against MRSA was > 20 mg/mL. *C. elegans* were then infected with MRSA and treated with betanin at different concentrations (100, 200, 300 and 400 µg/mL). Betanin at 200 µg/mL significantly improved worm survival following infection whereby the mean time to death was extended about 76 h upon treatment. Intestinal colonization by MRSA of worms exposed to betanin extract was similar to non-betanin-treated infected worms.

**Conclusion, significance and impact of study:** The enhanced survival of MRSA-infected worms upon betanin treatment was not a result of the activation of the host antimicrobial mechanism. Betanin from beetroot can be potentially used as a natural anti-infective agent as a mean to reduce antimicrobial resistance of *S. aureus* or used in combination with established antimicrobials to increase their effectiveness.

**Keywords:** Antibacterial; anti-infective; antimicrobial; betalain; *Beta vulgaris*; MRSA

#### INTRODUCTION

Betalains are one of the most common plant pigments found in nature besides carotenoids, chlorophylls and anthocyanins. Unlike anthocyanins or chlorophylls, which are ubiquitous in the plant kingdom, betalains are found in a much smaller group of plants. *B. vulgaris* subsp. *vulgaris* or commonly known as beetroot or red beet is the major commercially exploited betalain crop. Beetroots are not only consumed as fresh or cooked vegetables but also processed to obtain desiccated or frozen products, juices and their concentrates as well as natural pigments used as food additives (Choo, 2018; Gengatharan *et al.*, 2016). Betalains possess various biological and pharmacological properties such as antioxidant, anti-cancer and anti-lipidemic (Gengatharan *et al.*, 2015). Betanin is the main betalain pigment (75-95%) in beet root (Gliszczynska-Swiglo *et al.*, 2006).

The aim of this study was to investigate the effect of betanin from beetroot (*B. vulgaris* subsp. *vulgaris*) as a

potential anti-infective agent against MRSA using a *C. elegans* infection model. The effects of betanin on worm survival and intestinal colonization by MRSA were determined.

MRSA is associated with both healthcare and community settings infection and its epidemiology is constantly changing. MRSA causes a range of illnesses, from the skin and wound infections to pneumonia and bloodstream infections that can cause sepsis and death. These are due to its versatility and enormous capacity to acquire antibiotic resistance (Medina and Pieper, 2016; Stryjewski and Corey, 2014). The rates of mortality due to MRSA infections are still high and this may be due to their intrinsic virulence and/or significant delay in the administration of appropriate antibiotics. New therapies, prevention and rapid identification are therefore urgently essential (Stryjewski and Corey, 2014).

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One compelling approach towards overcoming the problem of MRSA is the development of anti-infective agents that target virulence-associated traits only (Mühlen and Dersch, 2016) or by modulating the host natural immune defences to combat the pathogen (Hamill *et al.*, 2008). In contrast to common antibiotic therapies, anti-infective agents are not bacteriostatic (inhibit bacterial growth) or bactericidal (kill bacteria) but interfere with pathogenic properties of the bacteria which effectively disarms the pathogen and enables its clearance by the host immune system (Mühlen and Dersch, 2016). The *C. elegans* nematode model has been successfully used to investigate the host-pathogen relationship of *S. aureus* (Begun *et al.*, 2005; Irazoqui *et al.*, 2010; Jeba Mercy *et al.*, 2015; Sifri *et al.*, 2003; Thompson and Brown 2014; Wu *et al.*, 2010). These successes have led to more recent use of *C. elegans* for the discovery of potential anti-infectives against *S. aureus* (Arvanitis *et al.*, 2013; Kong *et al.*, 2014a; Rajamuthiah *et al.*, 2014). This model host innate immune system is highly conserved with that of mammals and the worm has additional low-cost advantage, rapid breeding time and no ethical constraints, thus offsetting many of the limitations on the use of murine and other mammalian models (Arvanitis *et al.*, 2013). Besides, the *C. elegans* model can simultaneously identify novel agents that target bacterial virulence and host immune response (Kong *et al.*, 2014a).

## MATERIALS AND METHODS

### Bacteria, nematode and growth conditions

The methicillin-resistant *S. aureus* (MRSA) strain ATCC33591 was obtained from the American Type Culture Collection (ATCC, Manassas, U.S.A.). The wild type *C. elegans* Bristol N2 strain and *Escherichia coli* strain OP50 were obtained from the Tan Laboratory at Stanford University, U.S.A. MRSA strain ATCC33591 was grown on Trypticase Soy (TS) agar (Pronadisa, Madrid, Spain) while *E. coli* strain OP50 was cultured in Luria Bertani (LB) broth (Pronadisa, Madrid, Spain) supplemented with 100 µg/mL streptomycin (Sigma-Aldrich, St. Louis, U.S.A.). TS agar (40 g/L) and LB broth (20 g/L) were prepared according to manufacturer's instructions. All bacterial cultures were grown aerobically in shake flasks at 37 °C overnight with shaking at 250 rpm. *C. elegans* Bristol N2 strain was routinely maintained on nematode growth medium (NGM) seeded with the standard laboratory food source, *E. coli* OP50. To eliminate the confounding effects of progeny during the scoring of surviving worms, N2 worms were made sterile through RNAi knockdown of the *pos-1* gene, which resulted in worms laying unhatched eggs (Tabara *et al.*, 1999). The worms were age-synchronized using alkaline hypochlorite and sodium hydroxide to release embryos. The embryos were then plated onto NGM agar seeded with *E. coli* expressing double-stranded RNA (dsRNA) against *pos-1* in Petri dishes and allowed to grow for 45 h at 2 °C until the worms reached young adult stage.

### Preparation of betanin

Betanin (beetroot extract) powder was purchased from Sigma-Aldrich (St. Louis, U.S.A.). Different concentrations (100, 200, 300 and 400 µg/mL) of betanin were prepared. The samples were dissolved using ultrapure water and filtering through polyethersulfone (PES) membrane filter (0.20 µm).

### Determination of minimum inhibitory concentration (MIC)

MIC of betanin was determined using the broth microdilution method described by Clinical and Laboratory Standard Institute (2012) with modifications. MRSA were grown in TS agar for 18 h at 37 °C. The overnight bacterial suspension was adjusted to 0.5 McFarland standard (0.08-0.10 absorbance at 625 nm), which was equivalent to about  $1 \times 10^8$  colony forming units (CFU). The bacterial suspension was diluted 1:1000 in TS broth. Samples were filtered through a polyethersulfone (PES) membrane filter (0.20 µm) followed by serially doubling in 96-well microtiter plates before inoculating with 100 µL of bacterial suspension. The final working concentrations were 1.25, 2.5, 5, 10 and 20 mg/mL of sample. MIC value was determined as the lowest concentration of betanin in wells that showed no turbidity indicating the inhibition of bacterial growth. The positive control was bacterial suspension with the addition of 500 µg/mL gentamicin (Mun *et al.*, 2014) whereas the negative control was bacterial suspension with ultrapure water (no betanin).

### Infection assay

Infection assays were carried out in liquid medium in a 24-well plate according to the method of Kong *et al.* (2014a). Worm M9 buffer and overnight grown MRSA culture in TS broth with 10 mg/mL of cholesterol at a ratio of 4:1 (v/v) was dispensed into a 24-well plate prior to transferring the worms. In the treatment wells, betanin was added at 100, 200, 300 and 400 µg/mL for dose-dependent tests. In control wells, MRSA were replaced with *E. coli* OP50 in the absence of betanin. Twenty worms were transferred manually into each well and the plate was incubated at 25 °C. The total number of worms amounted to 80 in four wells representing four technical replicates. The survival of the worms was monitored and scored manually every 24 h. Three independent experiments were performed. Results obtained were analyzed using the Kaplan-Meier nonparametric survival analysis in StatView® version 5.0.1 (SAS Institute, Cary, U.S.A.) to obtain mean time to death ( $TD_{mean}$ ) for worms infected with MRSA.

### Toxicity assay

Toxicity assay was carried out in liquid medium in a 24-well plate. A single colony of *E. coli* OP50 was inoculated in 100 mL LB broth supplemented with 500 µg/mL streptomycin in a shake flask and incubated overnight at

37 °C. To eliminate any possible effect of betanin on *E. coli* OP50, the overnight culture of *E. coli* OP50 was concentrated 5-fold and heat-killed at 65 °C for 30 min before adding into the M9 buffer. In the treatment wells, betanin was added at 100, 200, 300 and 400 µg/mL for dose-dependent tests. Control wells contained heat-killed *E. coli* OP50 without betanin. Twenty age-synchronized young adult worms were transferred manually into each well and the plate was incubated at 25 °C. The total number of worms amounted to 80 in four wells representing four technical replicates. Worms were scored daily as alive or dead by gentle prodding with a platinum wire for a period of 12 days. Three independent experiments were performed.

### Colony forming units (CFU) assay

*In vivo* enumeration of bacterial CFU within the *C. elegans* gut was carried out according to the method of Kong *et al.* (2014a). Briefly, the worms were infected with *S. aureus* in the presence and absence of 200 µg/mL betanin. After 72 h of exposure to *S. aureus*, 10-12 live worms were randomly picked and briefly anesthetized in 25 mM Levamisole (Sigma-Aldrich, St. Louis, U.S.A.). The worms were washed twice with 200 µL of 500 g/mL gentamicin (Merck, Darmstadt, Germany) in 25 mM Levamisole followed by incubation for 45 min to 1 h to completely kill bacterial cells present on the worm cuticle. The worms were then washed three times with 200 µL of 25 mM Levamisole to remove killed bacteria and residual antibiotic. This was followed by homogenization in 50 µL of 1% Triton X-100 (Sigma-Aldrich, St. Louis, U.S.A.) using a motorized pestle. The lysates were serially diluted and plated on TS agar. After an overnight incubation at 37 °C, colonies were counted and the CFU counts per worm were determined.

### Statistical analysis

Differences in survival of *C. elegans* between treatment group (betanin) and control group (without betanin) were assessed by the Log-rank (Mantel-Cox) significance test using StatView® version 5.0.1 (SAS Institute, Cary, U.S.A.). For other assays, data were analyzed and compared by using the unpaired, two-tailed Student's *t*-test.

## RESULTS AND DISCUSSION

The MIC of betanin against MRSA was > 20 mg/mL. Based on the MIC value from this study, four concentrations of betanin (100, 200, 300 and 400 µg/mL) were selected for the MRSA infection assay. These concentrations were much lower than the MIC and therefore should not kill the bacteria. To the authors' best knowledge, the antimicrobial activity of betanin from beetroot against MRSA has not been investigated. Using agar-well diffusion method, ethanolic extracts of beetroot pomace showed inhibitory activity towards *S. aureus* ATCC 11632 (Velićanski *et al.*, 2011; Vulic *et al.*, 2013).

Slight inhibitory activity against *S. aureus* DSM 20231 determined using cylinder diffusion method was reported using methanolic extract of beetroot containing betanin (Rauha *et al.*, 2000). Betacyanin fractions from red pitahaya and red spinach containing approximately 39% and 2% betanin, respectively, showed MIC values of 1.56-6.25 mg/mL against three MRSA strains (Yong *et al.*, 2017).

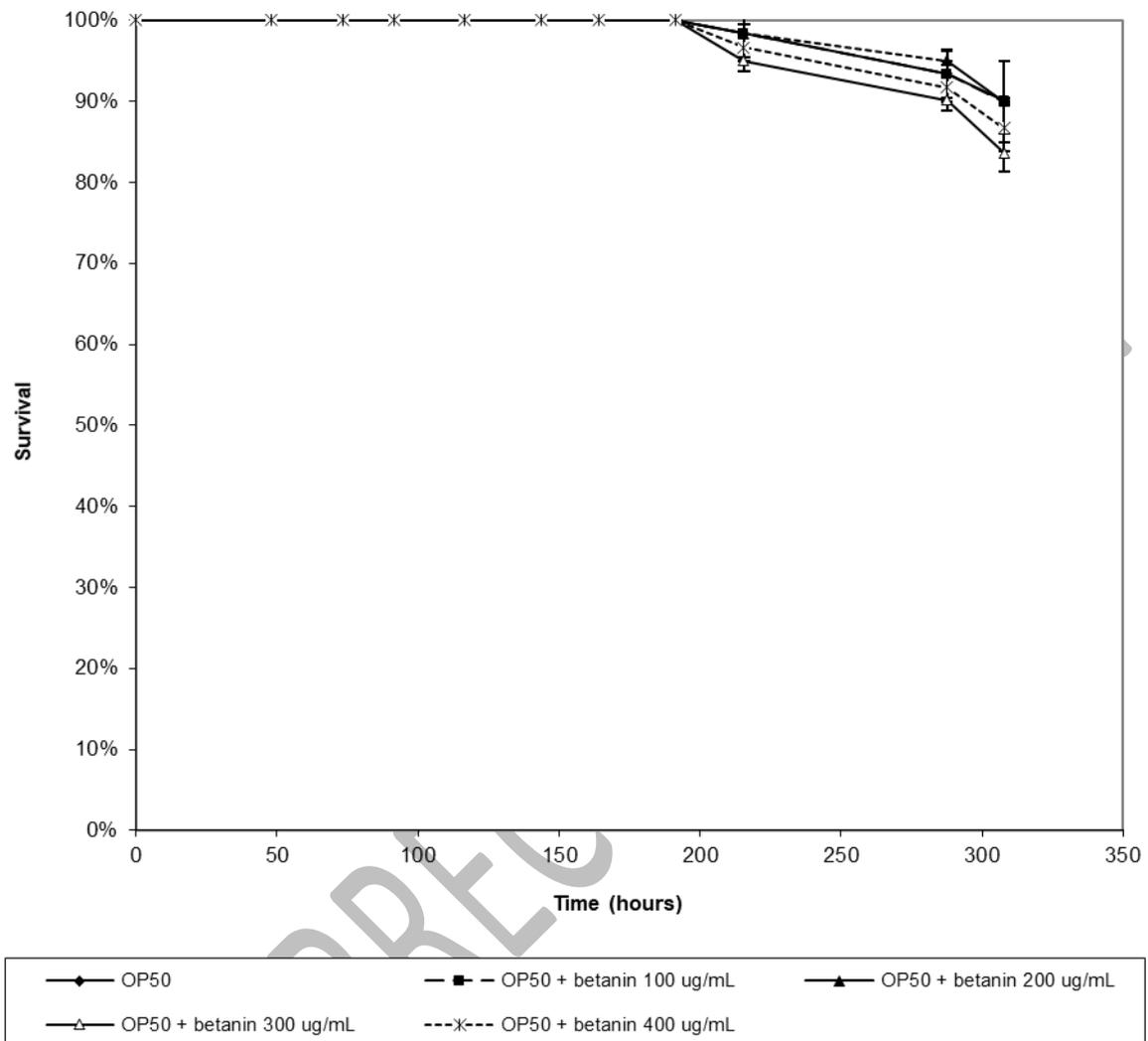
In this study MRSA required 5 to 6 days for complete killing of *C. elegans*. This is in accordance with previous studies (Kong *et al.*, 2014a; Rajamuthiah *et al.*, 2014; Sifri *et al.*, 2005). With betanin treatment at various concentrations, the TD<sub>mean</sub> of MRSA-infected worms was longer than that without betanin treatment (Table 1). The TD<sub>mean</sub> in this study was longer than the TD<sub>mean</sub> of *S. aureus* NCTC8325-4 infected worms (85.4 ± 2.8 h) (Kong *et al.*, 2014b). The difference may be attributed to the use of MRSA in this study versus *S. aureus* NCTC8325-4 as both culture conditions used for bacteria and worm (same strain) were the same. Based on the TD<sub>mean</sub>, only betanin at 200 µg/mL significantly promoted the survival of infected worms at *p* < 0.0001 (Table 1). The TD<sub>mean</sub> was extended about 76 h upon treatment with 200 µg/mL of betanin. Further study is required to determine whether betanin acted on the host defense system via modulating or stimulating the immune response towards infection. The TD<sub>mean</sub> of *C. elegans* infected with *S. aureus* NCTC8325-4 was extended by about 186 h due to treatment with aqueous extract of *Orthosiphon stamineus* leaves (Kong *et al.*, 2014b) where else the mean lifespan of *Pseudomonas aeruginosa* infected *C. elegans* was extended by 18-29 h due to treatment with whole apple extracts (2.5-10 mg/mL) (Vayndorf *et al.*, 2013). Nonetheless, the effect of betanin on the enhanced survival of worms infected with MRSA was not dose-dependent. Exposure to betanin at higher concentrations (300 µg/mL and 400 µg/mL, respectively) did not significantly extend the TD<sub>mean</sub> of MRSA-infected worms. The underlying mechanisms of how betanin exerts its protective effect on worms against MRSA infection remain to be elucidated.

**Table 1:** TD<sub>mean</sub> of MRSA-infected worms.

Sample	TD <sub>mean</sub> (h)	<i>p</i> value
MRSA	96.510 ± 7.505	-
MRSA + 100 µg/mL betanin	127.177 ± 7.232	0.0042
MRSA + 200 µg/mL betanin	173.075 ± 9.861	< 0.0001
MRSA + 300 µg/mL betanin	134.383 ± 10.685	0.0023
MRSA + 400 µg/mL betanin	126.288 ± 8.119	0.0113

Results are presented as mean ± standard error of four replicates (20 worms/replicate) from a representative of three independent experiments.

There was no significant difference between the numbers of *C. elegans* treated with and without betanin

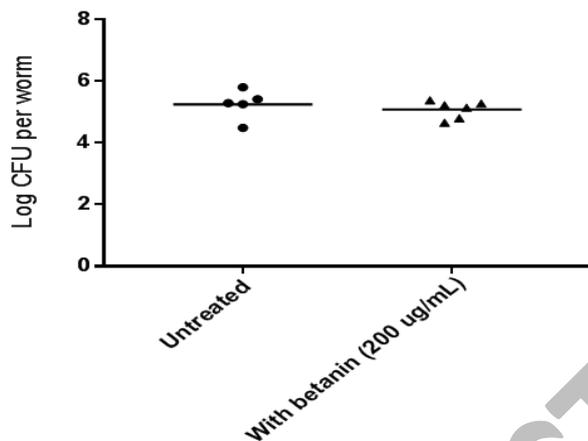


**Figure 1:** Effect of betanin on survival of *C. elegans* in lifespan assay. Graph shows the mean  $\pm$  standard deviation of four replicates (20 worms/replicate) from a representative of three independent experiments.

during 12 days of monitoring (Figure 1). These results indicate that betanin did not exert any toxic effect on *C. elegans*. There are limited *in vivo* studies on toxicity of betanin. An *in vitro* study showed no cytotoxicity after 24 h exposure of endothelial cells to betanin (1-50  $\mu$ M) from prickly pear fruit (Gentile *et al.*, 2004). Another study showed no cytotoxicity towards human embryonic kidney (HEK-293) cells and human monocytes (THP-1) after 24 h of incubation with betacyanin extracts from red pitahaya (0.39-3.13 mg/mL) (Yong *et al.*, 2018). The *in vivo* CFU counts for worms infected with MRSA in the absence of betanin after 72 h of exposure to the bacteria (Figure 2) is similar to the data reported for worms exposed to *S. aureus* NCTC8325-4 (Kong *et al.*, 2014a). This suggests that the colonization number of these two bacteria in *C. elegans* gut at 72 h was similar. The extracts from *Nypa*

*fruticans* root and *O. stamineus* leaves reduced the number of *S. aureus* NCTC8325-4 in the intestine of *C. elegans* and suggested that the mode of action of these extracts likely involved activation of host immunity to eliminate the pathogen or target the bacteria factor(s) that prevent them from accumulating in the intestine (Kong *et al.*, 2014a). However, in this study there was no significant difference in the *in vivo* CFU counts for MRSA-infected worms after 72 h of exposure to the bacteria upon treatment with 200 ug/mL betanin (Figure 2). Thus, the enhanced survival of MRSA-infected worm upon betanin treatment is not a result of reduced bacterial accumulation in the host. Betanin may attenuate MRSA virulence factors, rendering the bacteria less virulent. This, however, requires further investigation. Some plant-derived compounds have been shown to inhibit bacterial

virulence factors including biofilm formation, quorum sensing, toxins, enzymes etc. and to have lower effective concentrations than the MIC. Betacyanin fractions from red pitahaya and red spinach containing 39% and 2% betanin, respectively, showed anti-biofilm activity against five *S. aureus* strains including the MRSA strain used in this study with minimum biofilm inhibitory concentration of 0.313-1.25 mg/mL (Yong *et al.*, 2019). A genome-wide transcriptome analysis on MRSA upon exposure to betanin should provide insights not only into which MRSA virulence genes are differentially regulated upon betanin treatment, as well as into the possible molecular actions of betanin on MRSA pathogenicity.



**Figure 2:** *In vivo* CFU counts for MRSA-infected worms after 72 h of exposure to the bacteria with or without treatment with 200 µg/mL betanin. Each marker corresponds to the average bacterial CFU ( $\log_{10}$ ) extracted from ten infected worms with horizontal lines signifying the geometric mean.

## CONCLUSION

Betanin at 200 µg/mL significantly improved worm survival following MRSA infection whereby the mean time to death was extended about 76 h upon treatment. Intestinal colonization by MRSA of worms exposed to betanin was similar to non-betanin-treated infected worms. This study is the first to demonstrate that betanin from beetroot increased the survival of MRSA infected *C. elegans* most likely using a mechanism distinct from conventional antibiotics (direct bacteriostatic or bactericidal effect). This active ingredient from beetroot can potentially be used as an anti-infective agent or in combination with established or novel antimicrobials in a synergistic manner to extend the effectiveness of these drugs.

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