# Using *Caenorhabditis elegans* as a Model for Antimicrobial Drug Discovery

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**01-24**

## Abstract

Recently, the emergence of strains of multidrug-resistant (MDR) bacteria and well as bacteria acquiring total resistance against currently administered treatments has posed an urgent need for the scientific community to develop new antimicrobial treatments as medicine and therapy options against diseases caused by these evolved pathogens. *Caenorhabditis elegans* has presented itself as a suitable model host to uncover new antimicrobial drugs due to its genetic ortholog similarity to humans, display of extensive host immune responses and ease of use in laboratories. We therefore propose the use of *C. elegans* nematodes as an *in vivo* host platform to investigate the antimicrobial effectiveness of Traditional Chinese Medicine herbal extracts as well as domestic remedies against the pathogens *Bacillus cereus, Serratia marcescens* and *Staphylococcus epidermidis*. *In vitro* testing revealed wu wei zi (*Schisandra Chinensis)* and bai shao *(Paeonia lactiflora Pall)* extracts exhibit prominent antimicrobial effects *in vitro*, whereas *C. elegans* inoculated with extracts similarly showed a dramatic decrease in death rate upon introduction of pathogens into the cultures, suggesting a high extent of inhibition of bacteria pathogenicity against the nematodes. Hence, the antimicrobial effect of wu wei zi and bai shao are affirmed *in vivo* and it can be inferred that active compounds within these herbal extracts will exhibit similar antimicrobial activity administered in humans. It is thus encouraged that wu wei zi, bai shao and their derivatives be applied as part of new treatments and medicines targeted at these emerging MDR pathogens.

## 1. Introduction

In recent years, the evolution of bacteria against commonly administered medicines and antimicrobial compounds has led to the emergence of numerous multidrug-resistant (MDR) pathogens that exhibit resistance to many known antibiotics. The rise of MDR pathogens, as well as other high-resistance pathogens, has rendered numerous past remedies for pathogenic diseases

obsolete (Kong, Eng, Lim, & Nathan, 2016). This creates an urgent need for research and development of new compounds and treatments against these emergent strains.

*Bacillus cereus* is a Gram-positive pathogen that is the common cause of gastrointestinal infections. Its pathogenicity is intimately related with the secretion of toxins in the intestinal tract including hemolysins, phospholipases, toxins and proteases. Inhalation of *B. cereus* spores can result in onset of respiratory conditions including pneumonia, while nosocomial outbreaks involving *B. cereus* is attributed to the ability of *B. cereus* to produce dense sheathe-like biofilm structures which attaches it to catheters and prosthetic limbs (Bottone, 2010).

*Serratia marcescens* is a common opportunistic Gram-negative pathogen responsible for infections in the respiratory tract, the digestive tract and the urinary tracts. *S. marcescens* isolates tested for MDR have emerged, whereas mortality rates increase in patients infected with bloodstream diseases and with meningitis and endocarditis caused by *S. marcescens*. The underlying problem of *S. marcescens* infections, though rarely contracted, is the lack of any reliable antimicrobial therapy against it (Khanna, Khanna, Agarwal, 2012).

*Staphylococcus epidermidis* is a Gram-positive pathogen that easily colonises human skin. The high pathogenicity of *S. epidermidis*, along with its penicillin resistance, was only recently recognised. Due to its rapid growth, it is commonly behind post-burn infections surrounding wounded skin surfaces (Darby, 2005). MDR *S. epidermidis* is also associated with prolonged nosocomial infections such as prosthetic valve endocarditis, early-onset neonatal sepsis by contamination of indwelling medical devices in patients (Widerstrom, 2016).

*Caenorhabditis elegans* is a naturally occuring nematode worm measuring 1mm in length and appearing as transparent, and they are easily cultured in laboratory conditions. It is thus a fantastic and practical *in vivo* model representation on the investigation of molecular and cellular mechanisms of human diseases (Beumeister and Ge, 2002) that aids antimicrobial drug development (Couillault & Ewbanks, 2002). The complete sequencing of the C. elegans genome

in 1998 revealed that 38% of *C. elegans* genes have a human ortholog and 42% of human genes have an ortholog in the nematode, suggesting the extensive genetic similarly of *C. elegans* to humans (Shaye and Greenwald, 2011). Particularly noteworthy are *C. elegans* genes associated with host immune responses to pathogen attacks. In addition to a broad range of bacteria shown to utilise similar virulence factors in nematode and human infection, *Staphylococcus aureus* and *pseudomonas aeruginosa* have elicited disparate host responses mediated by pathogen-associated molecular patterns (Irazoqui et al, 2010). These mechanisms of pathogen detection likely have conserved roles in mammals, thus proposed extracts that are found to enhance *C. elegans* immunology will likely see similarly positive effects when administered in humans (Allen et al., 2014). Herbs that trace to Traditional Chinese Medicine were selected in this investigation as their medicinal value is largely believed to stem from improving bodily protection against disease, which potentially translates to host immunology-boosting antimicrobial action.

## 2. Objectives and Hypothesis

Our study aims to identify positive antimicrobial hits against the MDR and high-resistance bacteria strains *B. cereus*, *S. marcescens* and *S. epidermidis*. We tested a range of herbal compounds that have seen historical use in treating pathogenic diseases in the field of Traditional Chinese Medicine; namely wu wei zi (*Schisandra chinensis*), chuan xiong (*Ligusticum striatum*), and bai shao (*Paeonia lactiflora Pall)* herbs, as well as common domestic herbal remedies that are used to treat ailments but are not prescribed pharmaceutically, mint (*Menta x piperita*) and thyme (*Thymus vulgaris*). A wide range of both antimicrobial herbs and strains of bacteria were included in the study to allow us to investigate for positive hits across a larger scope of pathogen-extract permutations.

Our research hence investigates the antimicrobial effectiveness of these plant and herbal extracts in inhibiting the growth and pathogenicity of bacteria, through conducting a range of antimicrobial tests, sequentially the *in vitro* well diffusion, *in vitro* colony count and *in vivo Caenorhabditis elegans* survival assay.

It is hypothesised that all plant extracts exhibit varying degrees of antimicrobial properties against the test bacteria in the well diffusion test and colony count test. *C. elegans* infected with the bacteria would show a significant increase in survival when inoculated with the herb extracts, as compared to infected *C. elegans* which were not added with the extracts.

## 3. Methods and Materials

### Standard Preparation of Plant Extract

All plant extracts were prepared with a standard method across all tests. 0.2g of plant extracts were broken up with an electric blender in 20ml of sterile water. The contents were centrifuged at 7000rpm for 10min and the supernatant was collected and filter-sterilised to yield the final standard extract.

### In vitro Well Diffusion

The test bacteria used were *Bacillus cereus* ATCC 11778, *Staphylococcus epidermidis* ATCC 12228 and *Serratia marcescens* (Carolina). They were first inoculated into 10ml of Luria-Bertani (LB) broth and grown overnight at 30°C in a shaking incubator. 100ul bacterial culture was pipetted and spread evenly on Mueller-Hinton agar plate with a sterile swab. A sterile pipette was used to create wells in the agar. 0.080ml of standard 10% (w/v) plant extract was pipetted into the well. The positive control was 10% bleach solution and the negative control was sterile water. The plates were incubated at 30°C for 24h overnight and the diameter of the zone of inhibition was measured the next day. A greater zone of inhibition would suggest qualitatively that the extract used has greater antimicrobial potential.

### In vitro Colony Count

Test bacteria were first inoculated into 10ml of LB broth and grown overnight at 30°C in a shaking incubator. In the test setup, 0.05ml of bacterial culture was added to 0.05ml of standard 10% (w/v) plant extract and 4.9ml of LB broth. In the control setup, 0.05ml of sterile water was added in place of of plant extract. Five replicates for each setup were prepared. The absorbance of the cultures at 600nm was standardised at 0.8. The mixtures were incubated in a shaking

incubator at 30°C for 2h. Serial 10-fold dilutions were performed with 0.85% sodium chloride solution to the appropriate dilution factor, and 100ul of the diluted culture was pipetted and spread on LB agar. Plates were incubated at 30°C for 24h overnight. The number of colonies was subsequently counted. Lesser colonies present indicates a greater degree of inhibition.

**Preparation of NGM (nematode growth medium)**

1.25g NaCl, 8.5g agar, 1.25g bacto peptone were added with 500ml water. After autoclaving at 15psi for 15min, 0.3ml cholesterol (5mg/ml), 0.3ml $MgSO_4$ solution (1M), 0.3ml $CaCl_2$ solution (1M), 7.5ml $KH_2PO_4$ buffer solution pH 6.0 (1M) were added. NGM agar was poured into small experimental plates and supplemented with 25% (v/v) plant extract (test det-ups) or sterile water (control set-ups).

**In vivo *C. elegans* survival assay**

*Caenorhabditis elegans* N2 nematodes cut from laboratory plate cultures and transferred onto blank, unsupplemented NGM plates and grown for 48h. *Escherichia coli* OP50 and test bacteria were inoculated and grown in 10ml of LB broth each overnight for 24h at 30°C in shaking incubator. After incubation, 0.05 ml of *E. coli* OP50 culture or 0.05ml test bacteria culture was added to experimental NGM plates and grown for 24h overnight at 30°C, as food sources for nematodes. *C. elegans* nematodes were cut from unsupplemented NGM plates and transferred onto experimental plates previously supplemented with bacteria. The experimental plates were incubated at 30°C for 72h.

In summary, five replicates of each of the following set-ups were prepared for the assay:

*C. elegans* with test bacteria as food source on NGM agar with test plant extract *(test)*

*C. elegans* with test bacteria as food source on NGM agar with sterile water *(control 1)*

*C. elegans* with *E. coli* OP50 as food source on NGM agar added with plant extract *(control 2)*

*C. elegans* with *E. coli* OP50 as food source on NGM agar added with sterile water *(control 3)*

After 72h, the percentage survival of the worms was determined by analysis with *WormLab* software.

## 4. Results and Discussion

### In vitro Well Diffusion

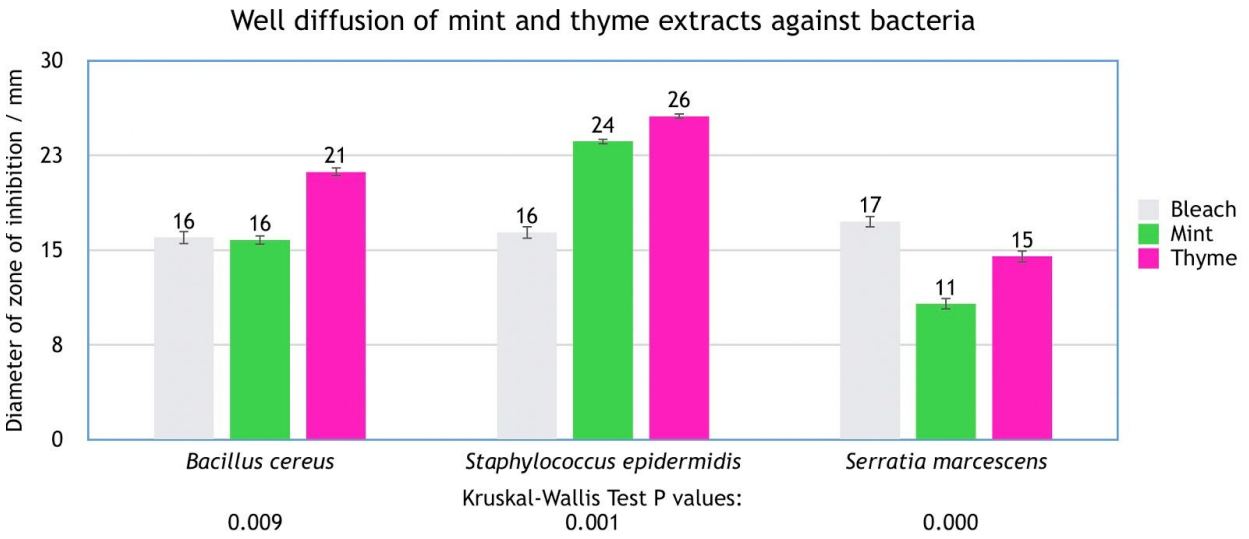



**Fig 1.1:** Well diffusion of mint, thyme against bacteria

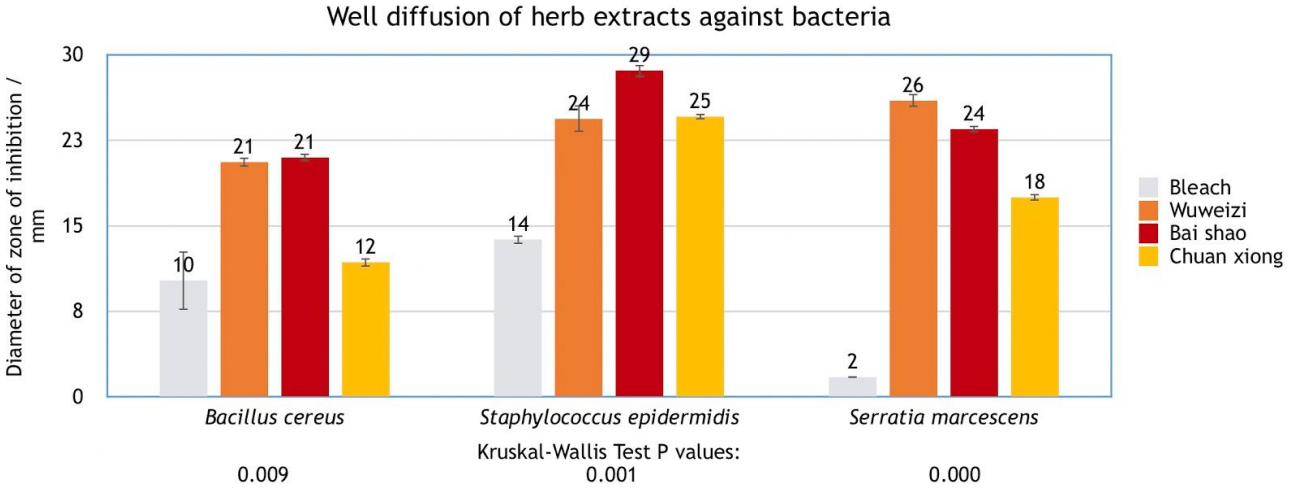



**Fig 1.2:** Well diffusion of wu wei zi, bai shao, chuan xiong against bacteria

Well diffusion revealed the high antimicrobial potential of wu wei zi and bai shao extracts. Noting the exception of mint and thyme extracts against *S. marcescens*, all extracts tested yield a

greater inhibition diameter compared to bleach control, and the antimicrobial action varies across pathogen-extract permutations. We note that all extracts used are not pathogen-specific but rather broad spectrum hits as inhibition is significant across all bacteria. The Kruskal-Wallis test p values were 0.002, 0.001 and 0.000 for *B. cereus, S. epidermidis* and *S. marcescens*, respectively, showing that wu wei zi, bai shao and chuan xiong extracts had significantly different extent of inhibition on the growth of these bacteria (Fig. 1.1 and 1.2).

## In vitro Colony Count

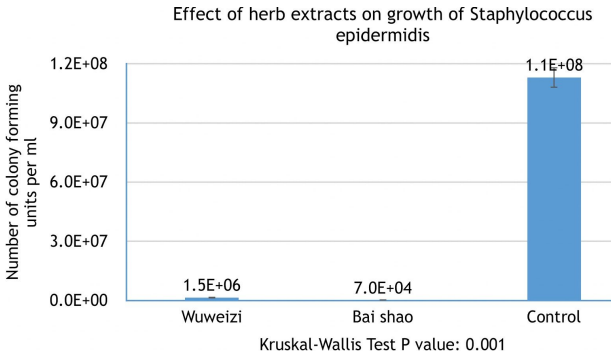



**Fig 2.1:** Effect of extracts on *S. epidermidis*

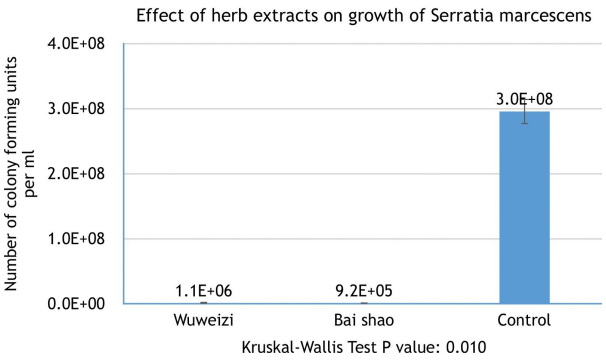



**Fig 2.2:** Effect of extracts on *S. marcescens*

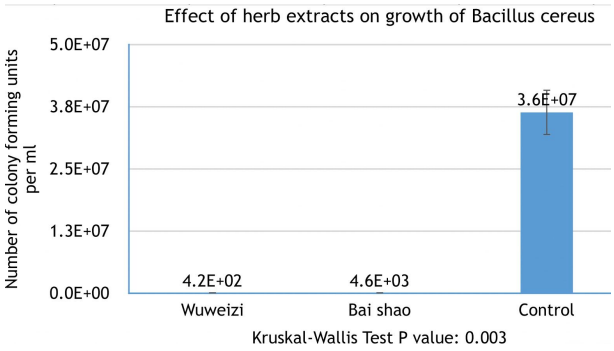
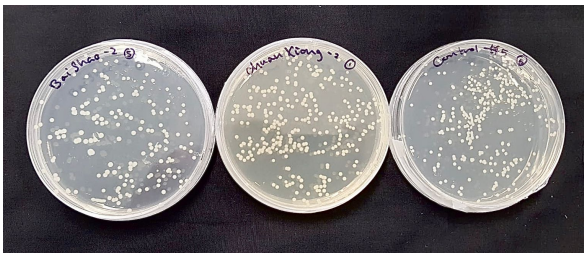



**Fig 2.3:** Effect of extracts on *B. cereus*



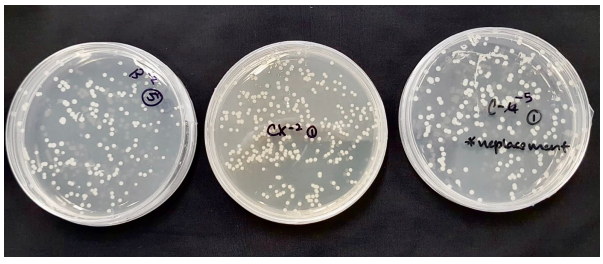


**Fig 2.4.1:** Colony Count cultures of B. cereus treated with (from left to right) bai shao extract, wu wei zi extract, and control set-up

**Fig 2.4.2:** Colony Count cultures of B. cereus treated with (from left to right) bai shao extract, wu wei zi extract, and control set-up

Colony count test was performed on strongest positive hits wu wei zi and bai shao. A lower number of colonies present indicates that the extract used has a stronger antimicrobial effect, as more colony-forming units of bacteria were inhibited or killed after inoculation compared to control set-ups. Colony count test affirmed the high antimicrobial effect of both wu wei zi and bai shao exhibited during well diffusion. The antimicrobial action of bai shao was broader, proving to be effective across all pathogens, especially *S. epidermidis* and *S. marcescens*. Wu wei zi, although less inhibitory towards *S. epidermidis* and *S. marcescens*, exhibited exceptional antimicrobial activity against *B. cereus*. The Kruskal-Wallis test showed significant differences in the number of colony forming units in the control and test setups with the two herbs used, with p values of 0.001, 0.010 and 0.003 for *S. epidermidis, S. marcescens* and *B.cereus* respectively (Fig. 2.1 to 2.3).

**In vivo *C. elegans* Survival Assay**

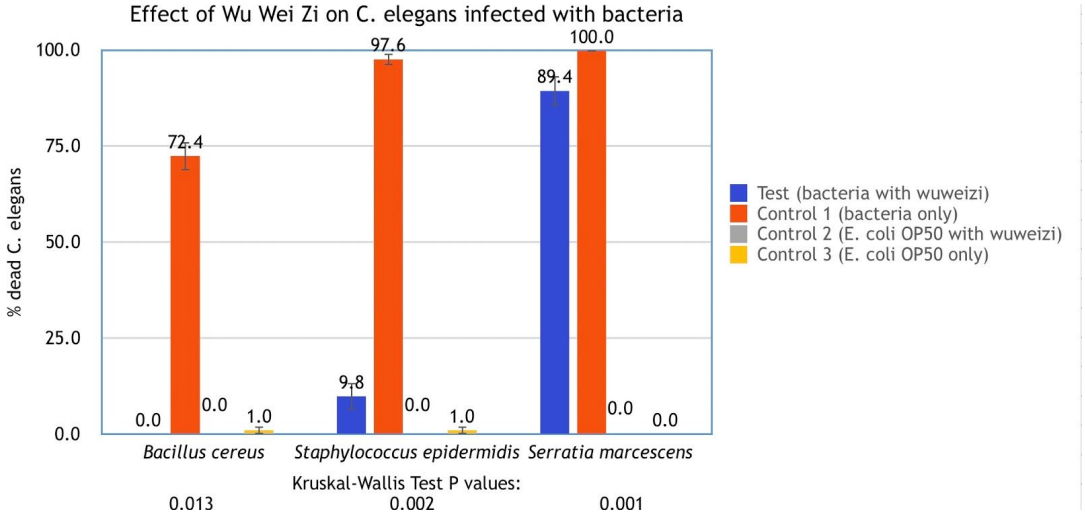



**Fig 3.1:** Effect of wu wei zi extract on *C. elegans* infected with bacteria

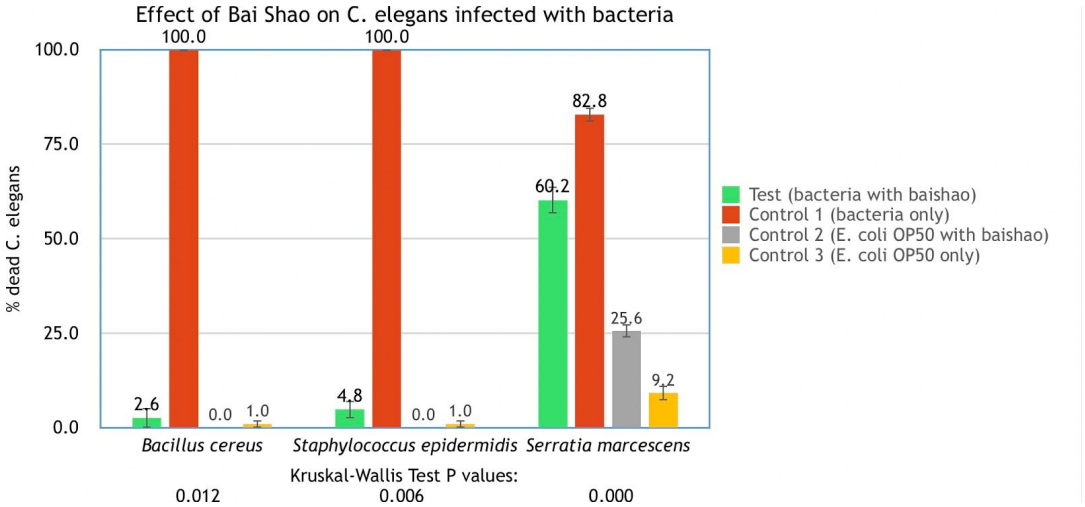



**Fig 3.2:** Effect of bai shao extract on *C. elegans* infected with bacteria

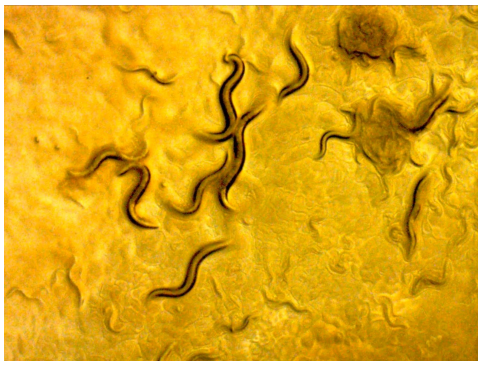


**Fig 2.4.1:** *C. elegans* inoculated with wu wei zi extract in *S. epidermidis*

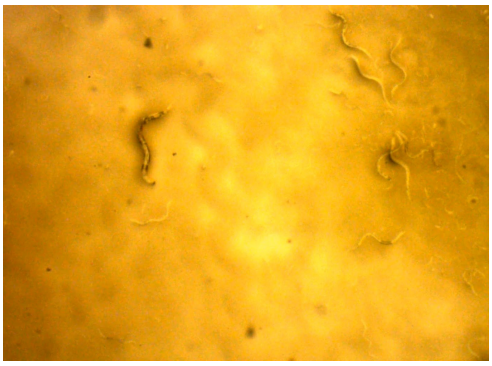


**Fig 2.4.2:** *C. elegans* in *S. epidermidis*

As hypothesised, the in vivo efficacy of both herb extracts is comparable to their in vitro performance. We generally note that both cultures of *C. elegans* nematodes inoculated separately with wu wei zi and bai shao extracts, respectively, saw a dramatic decrease in death rate or increase in survival rate across all pathogens, suggesting that wu wei zi and bai shao both drastically inhibited the pathogenicity of bacteria. In the case of *C. elegans* inoculated with wu wei zi extract and infected with *S. epidermidis*, survival rate of the nematodes increased twenty-fold, as shown from control 1 to the test setup. Interestingly, in live *C. elegans* organisms, both extracts saw a lowered antimicrobial effect against *S. marcescens*, which can plausibly be attributed to the very high potency of *S. marcescens* in the ideal growth conditions

provided by NGM in *C. elegans* cultures, as well as heightened pathogenicity of *S. marcescens* against the nematodes. As *C. elegans* have high dosage sensitivity, the dosage of 10% concentration of extracts was found to have the least toxicity against *C. elegans* while retaining strong antimicrobial activity against the bacteria. The Kruskal-Wallis test showed significant differences in the % dead *C. elegans* among the setups for wu wei zi (p values of 0.013, 0.002 and 0.001 for *B. cereus, S. epidermidis*, and *S. marcescens*, respectively) and bai shao (p values of 0.012, 0.006 and 0.000 for *B. cereus, S. epidermidis*, and *S. marcescens*, respectively).

## 5. Conclusions and Recommendations for Future Work

We summarise from our study that wu wei zi, bai shao, chuan xiong, thyme and mint extracts do exhibit some degree of antimicrobial activity *in vitro*, noting that the degree of inhibition varies with each pathogen-extract permutation, whereas all extracts tested were generally broad hits and not pathogen-specific. In particular, bai shao and wu wei zi exhibit significant antimicrobial properties both *in vitro* and *in vivo* on live *C. elegans* as medium, and further testing revealed the broader spectrum of bai shao and the targeted specificity of wu wei zi toward *B. cereus* especially *in vitro*. We therefore recommend the use of wu wei zi and its derivatives in anitmicrobial therapy amd development of medication against MDR pathogens.

In future experimentation, we can look to investigate the mechanism of action of the various herbs against the pathogens, using the enhanced specific antimicrobial activity of wu wei zi against *B. cereus* to determine the molecular or cellular conditions that results in specificity. The means to infection in *C. elegans* can be further compared to known common causes of infection in humans, such as through exposed wound surfaces or by the gastrointestinal tract, to select extracts that inhibit pathogenicity involving a mechanism seen in *C. elegans* that can be theoretically replicated in human treatment. There is also potential to chemically isolate, and subsequently synthesise, active compounds from both wu wei zi and bai shao and investigate their individual effect against the same pathogens used in this experiment.

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