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Antibacterial Evaluation of Polygonum Multiflorum

Andy S. Demianicz
andy.demianicz@student.shu.edu

ANTIBACTERIAL EVALUATION OF *POLYGONUM MULTIFLORUM*

By

Andy S. Demianicz

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Department of Biological Sciences

APPROVED BY

_____

MENTOR

Dr. Tin-Chun Chu

_____

COMMITTEE MEMBER

Dr. Angela V. Klaus

_____

COMMITTEE MEMBER

Dr. Daniel B. Nichols

_____

DIRECTOR OF GRADUATE STUDIES

Dr. Allan D. Blake

_____

CHAIRPERSON, DEPARTMENT OF BIOLOGICAL SCIENCES

Dr. Jane L. Ko

ACKNOWLEDGMENTS

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Abstract

Polygonum multiflorum, commonly known as Chinese Knotweed, is a tonic herb primarily used to enhance bodily functions. Recently, it has been shown to contain strong antioxidant, antibacterial and antiviral activity. The extract from the roots of Chinese Knotweed was used to assess its antibacterial properties against a broad spectrum of bacterial species, including 5 Gram-positive (*B. cereus, B. megaterium, S. epidermidis, S. mutans and S. pyogenes)* and 4 Gram-negative (*E. aerogenes, E. coli, P. vulgaris* and *P. aeruginosa).* Microtiter assays were carried out to evaluate the antibacterial activity of Chinese Knotweed at 0.5%, 1%, 1.5% and 2.5% concentrations in order to determine the Minimum Inhibitory Concentration (MIC). Potential synergistic effect of Chinese Knotweed and various antibiotics was assessed using the Kirby-Bauer disk method. Possible anti-biofilm formation was studied using a Congo-red assay, and biofilm quantification was acquired through crystal violet assay. Finally, Chinese Knotweed was evaluated on the effects of inhibiting sporulation and germination of *B. megaterium.* The results suggest Chinese Knotweed contains strong antibacterial effects against all the bacteria tested in this study and the MIC was 2.5% for all. Chinese Knotweed also showed significant synergism with most of the antibiotics tested. In addition, anti-biofilm assay indicated that 1% Chinese Knotweed was sufficient to inhibit biofilm formation on most the bacteria, while 2% Chinese Knotweed was effective for inhibiting sporulation and germination of *B. megaterium* spores. Thus, Chinese Knotweed may serve as a novel compound in the treatment and management of bacterial infections and biofilm formation.

Introduction

The medicine world has changed dramatically ever since the discovery of Penicillin, the first named antibiotic by Alexander Fleming (Davies and Davies, 2010). Antibiotics marked a period of great advancement in therapeutic medicine, allowing people to live longer while successfully treating bacterial diseases (Aminov, 2010). Unfortunately, the overuse of antibiotics have caused bacterial resistant strains to arise, and the need for new therapeutics has greatly increased (Czekalski et al., 2015).

Current medications have shown limited use against resistant strains of bacteria. Thus, novel approaches to find new antibacterial therapies need to be developed. The need to combat the global spread of resistant strains of bacteria has driven researchers to look for novel agents to solve this crisis.

A possible solution to diminish the threat of resistant and multi-resistant organism may be through the use of natural products. Natural products are small molecules that are produced by a biological source (Cutler and Cutler, 2010). These compounds are of interest due to the wide therapeutic benefits they have. Once discovery of natural working products are made, new potential agents that may be able to effect the resistant species (Beghyn et al., 2008).

A potential natural product comes from the root of an herbaceous perennial vine known as *Polygonum multiflorum* or *Fallopia multiflora* or commonly referred to as Chinese Knotweed (CK). Chinese Knotweed originates from the south and central parts of China, and has been used extensively in treating different medical conditions such as

diabetes, Alzheimer's and hepatotoxicity, as well as inhibiting the proliferation of cancer cells (Wu et al., 2012; Chen et al., 2011).

Chinese Knotweed's key to its uniqueness lies in the root of the plant, often referred to as the Thunb (Wu et al., 2012). As seen in Figure 1, the root (A), the extract (B) obtained after extracting the extract from the root of the plant. Chinese Knotweed's root is composed of 8 major constituents (Figure 2), a combination of these may play a significant role in its possible antibacterial activity.

A

B

Figure 1. Photos of (A) root pieces and (B) extracts from *P. multiflorum*.

Chemical Name	Structure	Chemical Name	Structure
2,3,5,4'-tetrahydroxystilbene-2-β-D-glucoside		Emodin-8-β-D-glucoside	
6-OH-emodin		Gallic acid	
Chrysophanol		Polygonimitin B	
Emodin		Rhein	
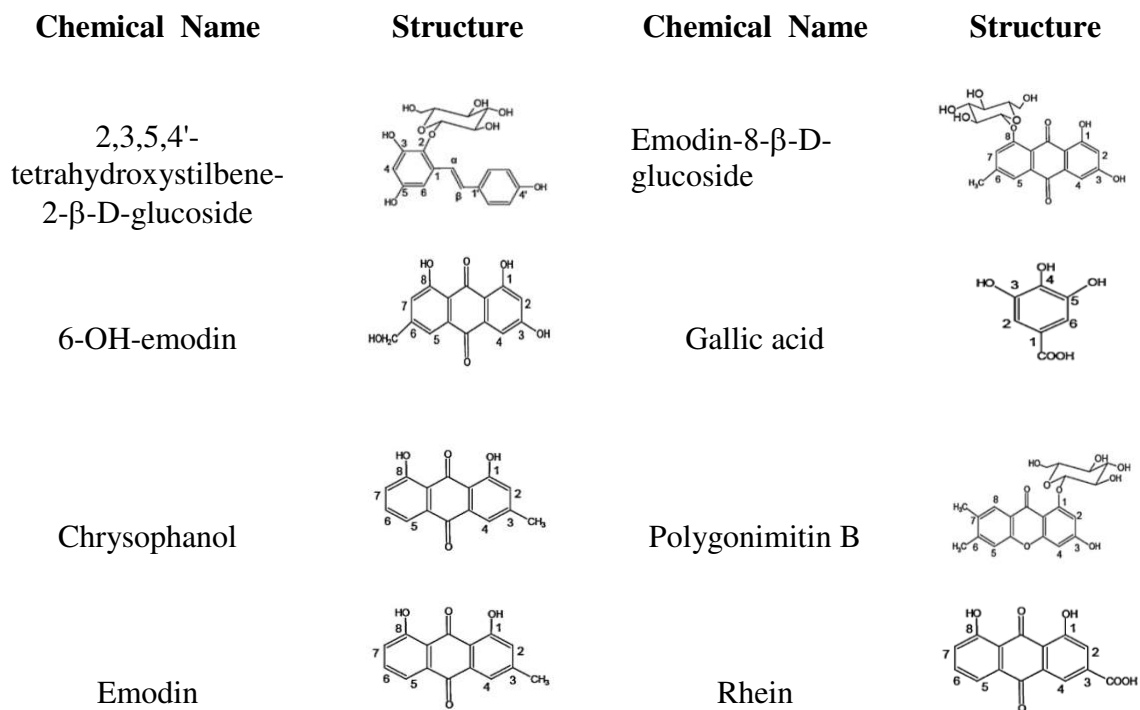
Figure 2. Chemical structures of the 8 major constituents of *Polygonum multiflorum* (Adapted from Han et al., 2012)

Chinese Knotweed extract is a mixture of 8 active constituents (Figure 2) that fall into four main chemical families: phenolic compounds, anthraquinones, alkaloid and stilbenes. Each of these molecules is what supports and provides the various unique properties of the plant. Recent literature reported that Chinese Knotweed extract has potential antimicrobial effects (Han et al., 2012).

Microorganisms play a vital role as a defensive mechanism for many mammalian species. For example, bacteria colonize the epithelial layer of the body, to act as a protective barrier against foreign and/or pathogenic organism (Davis, 1996). However, some microorganisms are invasive and pathogenic towards the human host. For instance, many *E. coli* species live in the gut flora and are harmless, but under various conditions these bacteria become opportunistic and can cause harm to their host (Fish, 2002). As a preventive method, antimicrobial drugs have been designed to combat and control the pathogenic species.

Bacteria can be categorized into two major groups, Gram-positive and Gram-negative, based on differences in their cell wall structures. Gram-negative bacteria species differentiate from others due to a very thin peptidoglycan layer and typically are surrounded by a third layer, capsular or slime layer (Bauman, 2014). Gram-negative species uniqueness is a result of their outer membrane, which acts as a barrier and produces endotoxin that can cause irritation and harm to those exposed. Besides physical protection against destruction, the outer membrane also protects against chemical agents, which makes Gram-negative bacteria a more difficult species to eliminate (Salton and Kim, 1996). A few common examples of Gram-negative bacteria include *Enterobacter*

aerogenes (E. aerogenes), Escherichia coli (E. coli), Proteus vulgaris (P. vulgaris) and *Pseudomonas aeruginosa (P. aeruginosa).*

Enterobacter aerogenes, a Gram-negative bacillus species, is typically found in soil, water and intestines of animals and humans (Carroll et al., 2012). *E. aerogenes* is known for causing nosocomial, opportunistic infections, especially in immunocompromised hosts. This species poses a major threat to human health due to the gastrointestinal tract location and its opportunistic behavior (Sanders and Sanders, 1997).

Escherichia coli is a Gram-negative bacillus species that is found in the intestine of warm-blooded organisms (Singleton, 1999). As part of the normal flora, most species do not cause harm but some strains may cause food poisoning due to food contamination. Food and water contamination is a key concern in poor and underdeveloped nations (Estrada-Garcia et al., 2013).

Proteus vulgaris is a Gram-negative rod-shape organism, mainly found in the soil and human intestines. *P. vulgaris* are related to the *Enterobacter* species and are also linked to cause opportunistic infections (Gul et al., 2013).

Pseudomonas aeruginosa is a Gram-negative facultative anaerobe bacterium that is the source of many acute and chronic infections. The bacteria can be isolated from environmental sources such as freshwater and soil. *Pseudomonas* species has a unique resistance to many microbicides, which makes these bacteria a problem in its removal from equipment and surfaces in hospitals (Lavoie et al., 2011). In addition, *P. aeruginosa* are often linked to high rates of food spoilage following harvest (Tornas, 2005).

In addition to Gram-negative bacteria, Gram-positive species also play a major role in healthcare field due to increasing occurrence of some antibiotic resistance species such as Methicillin resistant *Staphylococcus aureus* (MRSA). Gram-positive organisms are classified mainly based on the thick layer of peptidoglycan of their cell wall and lack of an outer membrane. A few common examples of Gram-positive bacteria included in this study are *Bacillus cereus* (*B. cereus*), *Bacillus megaterium* (*B. megaterium*), *Staphylococcus epidermidis* (*S. epidermidis*), *Streptococcus mutans* (*S. mutans*) and *Streptococcus pyogenes* (*S. pyogenes*).

Bacillus cereus is a Gram-positive, rod shaped microorganism that has the ability to survive harsh conditions due to their ability to form spores (Estrada-Garcia et al., 2013). They have a wide range of growth and adaptability to various environmental conditions, and have proven to cause a major problem with food contamination. Major concerns with this species include their adaptability in soil, which could lead to food spoilage, as well as meats and animal products contamination (FDA, 2012).

Bacillus megaterium, a close related species of *Bacillus cereus,* is a Gram-positive rod-shaped endospore former and an obligate aerobe. It is one of the largest known bacterial species (Bunk et al., 2010). This mesophillic bacterium has been a key player in industry due to its various benefits such as amylases (intact proteins, an exoenzyme) production, used for baking and drug production (Vary et al., 2007). *B. megaterium* is of interest due to its similarities to other bacteria from the same genus, such as *B. anthracis* (major causing agent of anthrax) which may be used as a biological weapon.

Staphylococcus epidermidis, Gram-positive cocci, is typically found as part of the normal flora of human skin. *S. epidermidis* is not usually pathogenic but those with compromised systems are at risk to develop infections (Levinson, 2010). *S. epidermidis* serves as a model for the genus *Staphylococcus* due to the similarities among the species. The concern for these bacteria is the increasing resistance to many antibiotics, which leads to the development of mutant strains such as MRSA or multi-drug resistant *Staphylococcus aureus* (MDRSA) (Otto, 2010).

Streptococcus pyogenes is a Gram-positive chain-cocci often found as part of the normal flora of human on their skins and throat regions. Although it's part of the normal flora, it has the potential to serve as an opportunist and cause both mild to severe infections in immunocompromised individuals (Gul et al., 2013). Due to their high presence in human population and the severity of infections that may occur, this bacteria is also of great concern.

Streptococcus mutans, another species in Streptococci group, is a Gram-positive facultative chain-cocci that is commonly found in the oral cavity. As one of the leading causes for dental plaque/decay, *S. mutans* is of major concern due to its predominant presence in the mouth flora (Kolenbrander, 2000).

Biofilm, bacterial communities attach to a surface, has become a significant problem in the medical field. Biofilm formation is the result of the production of bacterial exopolysaccharide, eDNA and protein that produces a slimy film, aggregate together and form a protective layer over the bacteria following the successful adhesion of the

microorganism to a surface (Merritt et al., 2007). Typically biofilm can be formed by individual species or can be a culmination of various species.

Biofilm formation begins after the successful attachment of bacterial cells. Without the initial attachment, the production of polysaccharides and the initial formation of the biofilm is impeded (Sauer et al., 2002). Bacterial motility can also affect initial biofilm formation due to the lack of aggregation and clumping that are needed in early development. It is nearly impossible to remove the biofilm once it is properly established and has fully matured. The only effectively way to remove biofilm is by physically scrapping it off the site of attachment (Hayrapetyan et al., 2015).

Furthermore, foreign materials placed into the human body such as catheters and endotracheal tubing during surgical procedures, often serve as the favorite sites of attachment for bacteria to form biofilm. Though many of these equipment are necessary, biofilm formation may worsen the patient's overall health state. (Cairns et al., 2011).

An antibiotic is any substance produced by a microorganism, i.e. bacteria or fungi, secreted outside its cell to harm or kill another microorganism. Penicillin is an antibiotic that was first discovered in 1928 through keen observation of a fungus, *Penicillium chrysogenum*. Alexander Fleming noticed that a chemical substance was produced by the species that could inhibit bacterial growth within a given area, and this was the start of the antibiotic era, chemotherapy warfare (Garrod, 1960). Before the use of antibiotics, some treatments relied on the usage of transitional metals. Unfortunately, due to their toxic nature, people were left with metal poisoning even they were cured from the bacterial infection Some of these transitional metals such as arsenic and mercury

were effective but limited on survival rate as well as effective rate in treating the patient before the patient died from poisoning. Thus, the antibiotics were thought to be the "magic bullet" when they were discovered, allowing the pathogens to be eliminated without harming the host (Gul et al., 2013).

Individual antibiotics vary widely in their effectiveness on various types of bacteria. The effectiveness differs depending on the ability of the antibiotic to reach the site of infection, and the ability of the bacteria to resist or inactivate the antibiotic. Some antibiotics can kill the bacteria (bactericidal), whereas others simply prevent the bacteria from multiplying (bacteriostatic). Antibiotics may be administered orally, topically, or intravenously (Finberg et al., 2004; Carroll et al., 2012).

In addition, some bacteria are able to adapt and/or transform themselves into a non-reproductive structure known as spores when faced with stressful or harsh conditions. Endospore formation, also known as sporulation, is the mechanism used by bacteria in the genus *Bacillus* and *Clostridium* in order to withstand unfavorable conditions. The spore contains a thick layer named spore coat which protects the bacteria until conditions resume to normal, allowing them to germinate to vegetative cells again (Cano and Bonucki, 1995).

In this study, Chinese Knotweed was used to evaluate its antibacterial effects against nine selected bacteria. Its potential synergism with antibiotics, the inhibitory effects on biofilm formation, sporulation and germination were also investigated.

Materials and Methods

Preparation of bacterial cultures

For this study, four Gram-negative species, *Enterobacter aerogenes, Escherichia coli, Proteus vulgaris* and *Pseudomonas aeruginosa* and five Gram-positive species, *Bacillus cereus, Bacillus megaterium, Staphylococcus epidermis, Streptococcus mutans and Streptococcus pyogenes,* were obtained from Biological Supply Company (Carolina, Burlington, NC) used to observe the effects of Chinese Knotweed. All bacterial cultures were maintained in Luria/Lennox Broth (LB) (Difco™, Sparks, MD).

Preparation of compound solution

The Chinese Knotweed powder was obtained from Dr. Chih-Yu Lo at the Department of Food Science, National Chiayi University in Chiayi, Taiwan. The 10% stock solution was made in Dimethyl Sulfoxide (DMSO) (Mallinckrodt Chemical, Phillipsburg, NJ). The solutions was then filtered with 0.45 μm Supor® membranes (Acrodisc®, Ann Arbor, MI). The compound was kept at 4$^{\text{o}}$C.

Microtiter plate antimicrobial inhibition assay

A microtiter plate assay was used to observe any antibacterial effects of the natural compounds. The overnight bacterial cultures and the 96-well clear microtiter plate (Corning, Corning, NY) were used in this assay. Each well contains 5 μL of bacteria and various concentration of Chinese Knotweed (final concentration of 0.5%, 1%, 1.5% and 2.5% respectively) with a total volume of 200 μL in each well. The growth of the bacteria

in the presence and absence of the natural products were then monitored by taking the optical density at 600 nm (OD_{600nm}) hourly up to 12 hours and then 24 hours with SpectraMax M5 (Molecular Devices, Sunnyvale, CA). Over the 24-hour monitoring period, the microtiter plate was maintained at 37°C with constant shaking at 250 rpm.

Kirby-Bauer assay

Mueller-Hinton II agar plates were used for the Kirby-Bauer assay to evaluate the effectiveness of selected antibiotics with or without compound. A total of 50 μL of 2.5% Chinese Knotweed was added for the antibiotic discs for the treatment. The plates were incubated inverted in a 37°C incubator for 8 hours. The zone of inhibition (ZOI) were then recorded in millimeter after incubation.

Table 1: A summary of all antibiotics used in this study with their mode of action.

Mode of Action	Antibiotic
Cell Wall Synthesis Inhibition	Ampicillin
	Bacitracin
Protein Synthesis Inhibition	Erythromycin
	Kanamycin
	Neomycin
	Streptomycin
	Tetracycline
Nucleic Acid Synthesis Inhibition	Nalidixic acid
	Nitrofurantoin
	Novobiocin
Folate Synthesis Inhibition	Triple Sulfa

Biofilm screening: Congo-red Assay

Congo-red assay was carried out to screen the biofilm formation for all bacteria. Tryptic Soy Agar (TSA) (Difco™, Sparks, MD) with 2% sucrose (Amresco, Solon, OH) and 10X Congo-red (Amresco, Solon, OH) were added to each well of a 24-well plate. A total volume of 20 μL of bacteria or bacteria with compound was added to the appropriate well and the plate was incubated for 24 hours at 37°C.

Biofilm Quantification: Crystal Violet Assay

Tryptic Soy Broth (TSB) with 2% sucrose was used in crystal violet assay to quantify the biofilm formation. Selected bacteria with or without various concentration of Chinese Knotweed (0.5%, 1% respectively) were added into each well and the plate was incubated at 37°C overnight. After the incubation, planktonic cells were removed and the wells were washed twice with Phosphate buffered saline (PBS) gently (without disturbing the mature biofilm) to remove any non-attached particulates. Following the wash, add 250 μL of 100% methanol to each well and wait 15 minutes. Aspirate the methanol and allow the wells to air dry for another 15 minutes. Once air dried, 0.1% of crystal violet solution was added and the cells are stained for 10 minutes. Aspirate the excess crystal violet and wash the wells with diH_2O until control wells appear clear. Then 95% ethanol (EtOH) was added to the wells and incubated for 30 minutes, shaken under low rpm and then the absorbance (OD_{600nm}) was recorded.

Scanning Electron Microscope (SEM)

- **Sample fixation**

Samples were grown overnight, washed twice with PBS and then the fixative was added. The fixative contains a 2.5% glutaraldehyde (GTA) working stock and 0.1 M cacodylate buffer. The samples were then stored overnight at 4°C.

- **Sample dehydration**

Samples were washed with 0.1 M cacodylate buffer 3 times. Buffer was aspirated after the 3rd wash. 2 mL of 1% Osmium tetroxide (OsO_4) solution was then added to each sample and then the samples were kept at 4°C for 45 min. Removed the OsO_4, added 0.1M cacodylate buffer to the samples for 10 minutes. Repeat this step three times and the samples were ready to be dehydrated and prepped for imaging. Dehydration is a result of a series of EtOH washes (30%, then 50%, 70%, 80%, 90% and then 100%). There were 8-10 minutes gaps between each wash.

- **Critical point drying and coating**

Followed by the dehydration is the critical point drying process for 40 minutes. This instrument uses carbon dioxide in order to completely remove any moisture in the sample and to preserve the samples. The samples are then ready to be coated in gold for 10-15 minutes. Once the samples are coated, they are ready for SEM imaging.

Sporulation inhibition assay

Sporulation inhibition assay was carried out to evaluate the effects of 1% Chinese Knotweed on a spore-forming bacteria, *B. megaterium*. The bacteria was starved with diH$_2$O or Chinese Knotweed for 72 hours. Schaeffer–Fulton stain procedure was then used to observe the endospore.

Germination inhibition assay

Bacillus megaterium was starved in diH$_2$O for 72 hours to induce the spore formation. The cultures were then split into two tubes: LB only and LB with 2% Chinese Knotweed. Both tubes were then incubated at 37 ^{o}C for 14 hours. Viable count were then used to determine the viable cells.

All images/ figures presented in this paper was taken by Andy S. Demianicz in order to evaluate the antibacterial potential of Chinese Knotweed.

Results

Antibacterial Assay

Figures 3 – 11 illustrated the growth curves of nine selected bacteria with or without Chinese Knotweed. Additional data and analyses containing proprietary information were not included in this thesis. Figure 3A showed the growth curve of *Enterobacter aerogenes* with various concentration of Chinese Knotweed. The concentrations of CK treated on *E. aerogenes* compare to no treatment was lower and demonstrated the CK antibacterial effect. Figure 3B is the bar graph indicating the percentage inhibition for 8th, 10th and 12th hours. Observations of *E. aerogenes* when treated with various concentration (0.5 – 2.5%) of CK resulted in complete inhibition, <97% inhibition at 2.5% CK, while at the other concentrations there was some inhibition such as at 1% resulted in 70% inhibited growth. Based on the results and the information provided by these two figures (Figure 3A and 3B), these figures provide information on the MIC and the half maximal inhibitory concentration or Inhibitory Concentration 50% (IC_{50}). The results indicated that the MIC for *E. aerogenes* is 2.5% and the IC_{50} is between 0.5 and 1% Chinese Knotweed.

Figures 4A and 4B showed the effect of Chinese Knotweed on *Escherichia coli* (*E. coli*). The MIC for *E. coli* is 2.5% while the IC_{50} could be a little more than 1%. Figures 5 and 6 indicated that the Chinese Knotweed may have better inhibition on both *P. vulgaris* and *P. aeruginosa*. While MIC for both bacteria stays at 2.5%, the IC_{50} could be just slightly above 0.5%
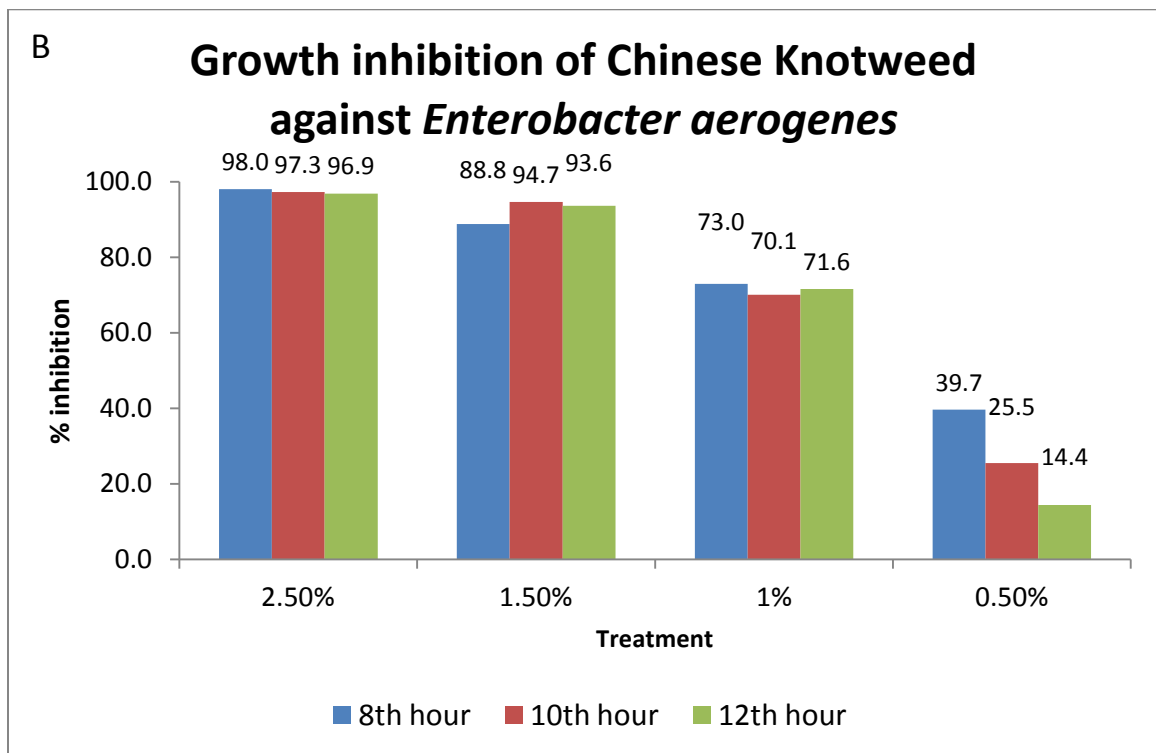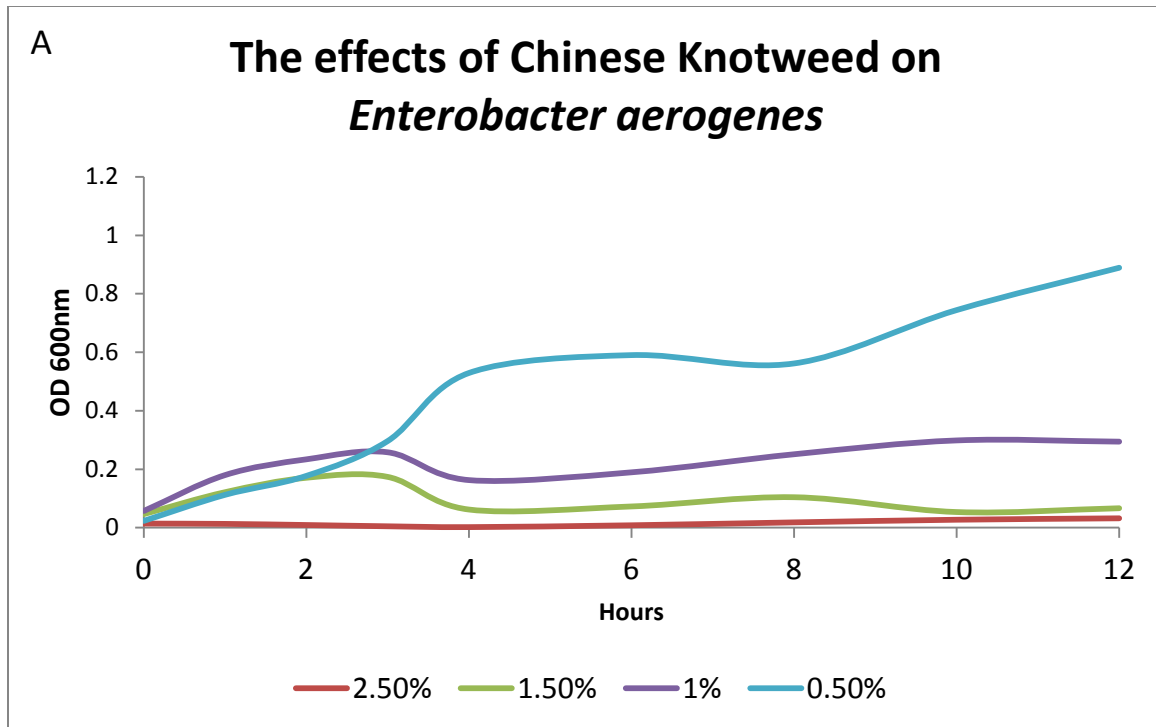
A

The effects of Chinese Knotweed on *Enterobacter aerogenes*

OD 600nm vs Hours

Legend: 2.50%, 1.50%, 1%, 0.50%

B

Growth inhibition of Chinese Knotweed against *Enterobacter aerogenes*

% inhibition vs Treatment

Treatment	8th hour	10th hour	12th hour
2.50%	98.0	97.3	96.9
1.50%	88.8	94.7	93.6
1%	73.0	70.1	71.6
0.50%	39.7	25.5	14.4

Figure 3. A) Growth curve of *Enterobacter aerogenes* treated with Chinese Knotweed. B) Percent inhibition of *Enterobacter aerogenes* treated with Chinese knotweed.
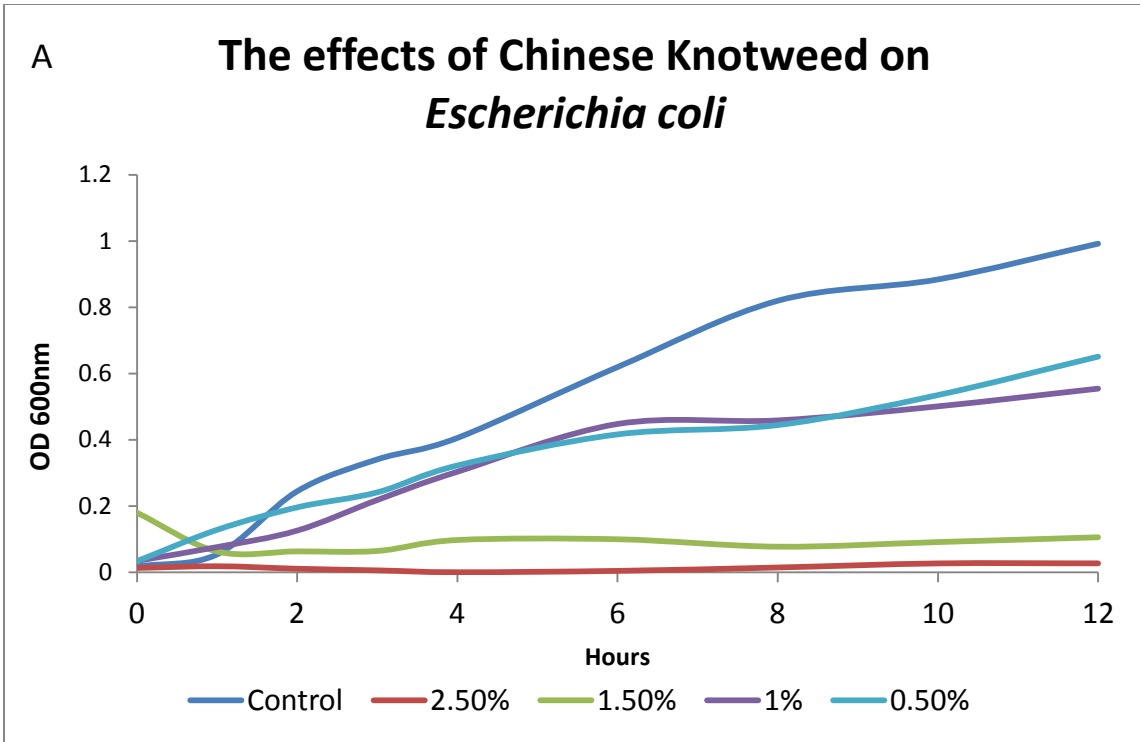
A The effects of Chinese Knotweed on *Escherichia coli*

OD 600nm vs Hours

Control — 2.50% — 1.50% — 1% — 0.50%

B Growth inhibition of Chinese Knotweed against *Escherichia coli*

% Inhibition vs Treatment

Treatment	8th hour	10th hour	12th hour
2.50%	98.3	97.0	97.3
1.50%	90.6	89.7	89.4
1%	44.1	43.4	44.1
0.50%	45.8	39.5	34.4

8th hour — 10th hour — 12th hour
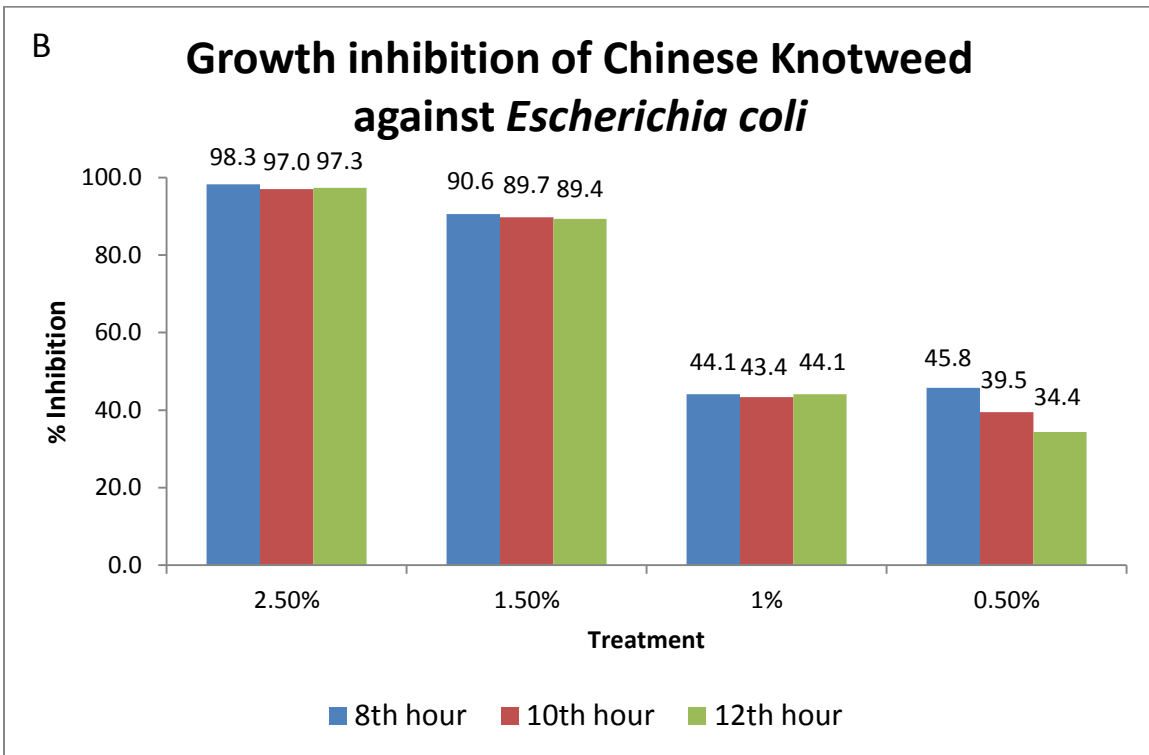
Figure 4. A) Growth curve of *Escherichia coli* treated with Chinese Knotweed. B) Growth curve of *Escherichia coli* treated with Chinese Knotweed.
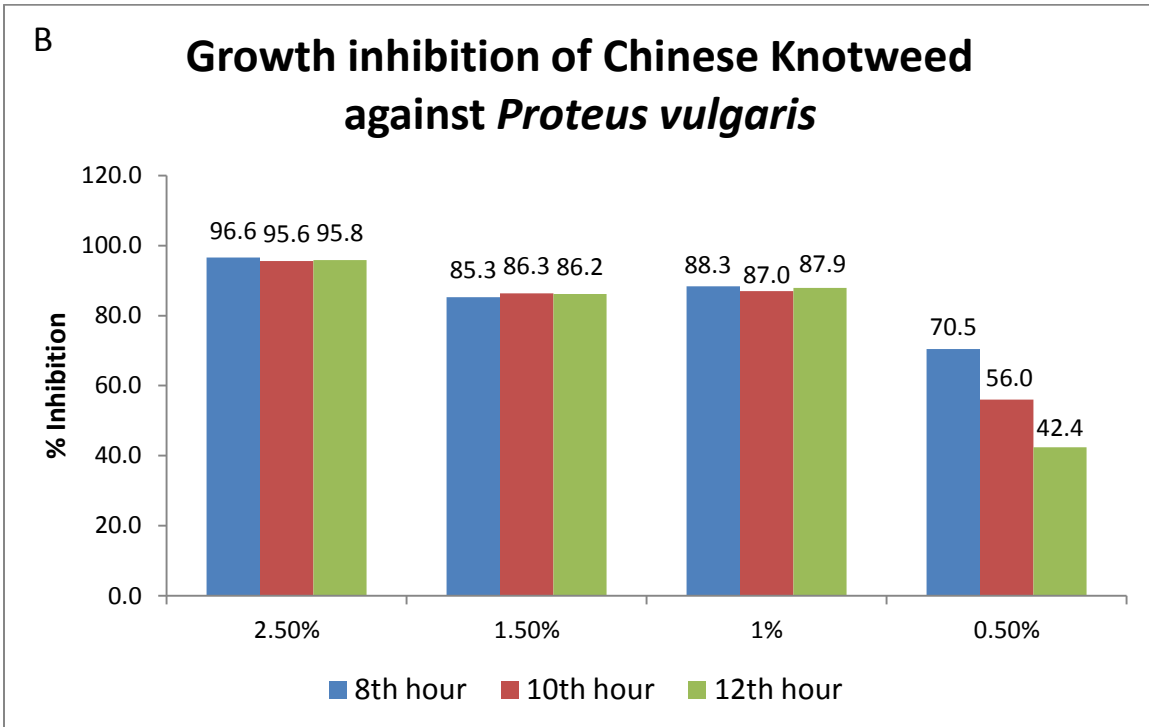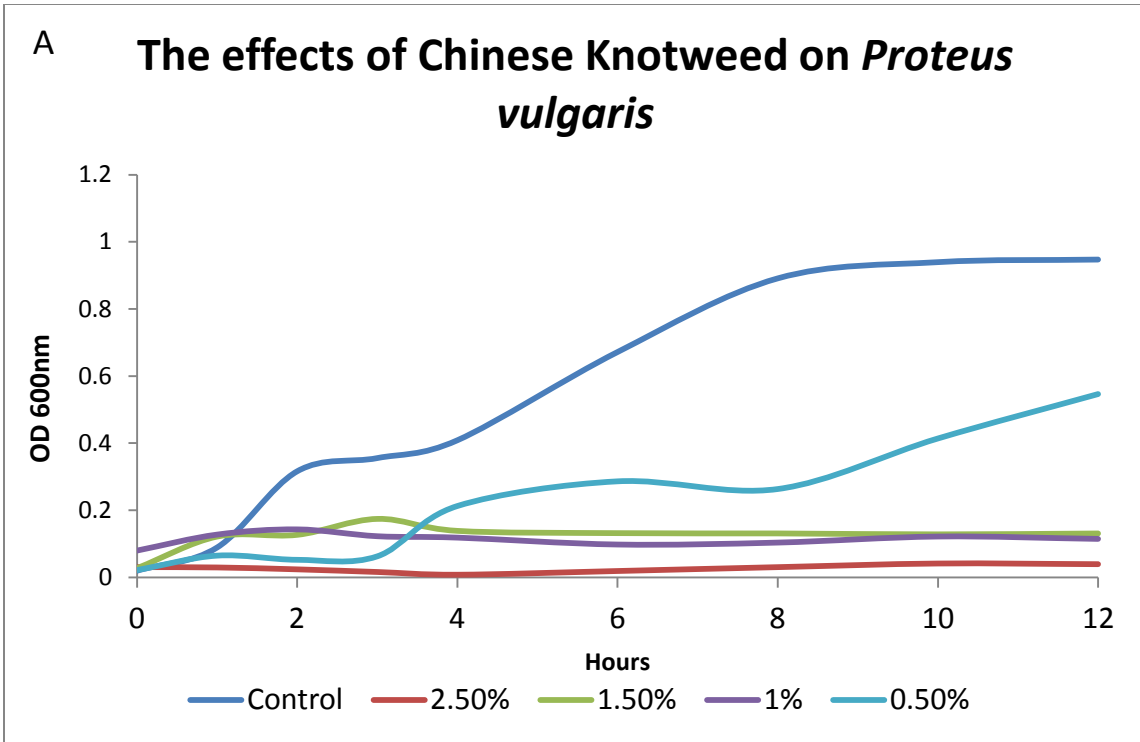
Figure 5. A) Growth curve of *Proteus vulgaris* treated with Chinese Knotweed. B) Growth curve of *Proteus vulgaris* treated with Chinese Knotweed.
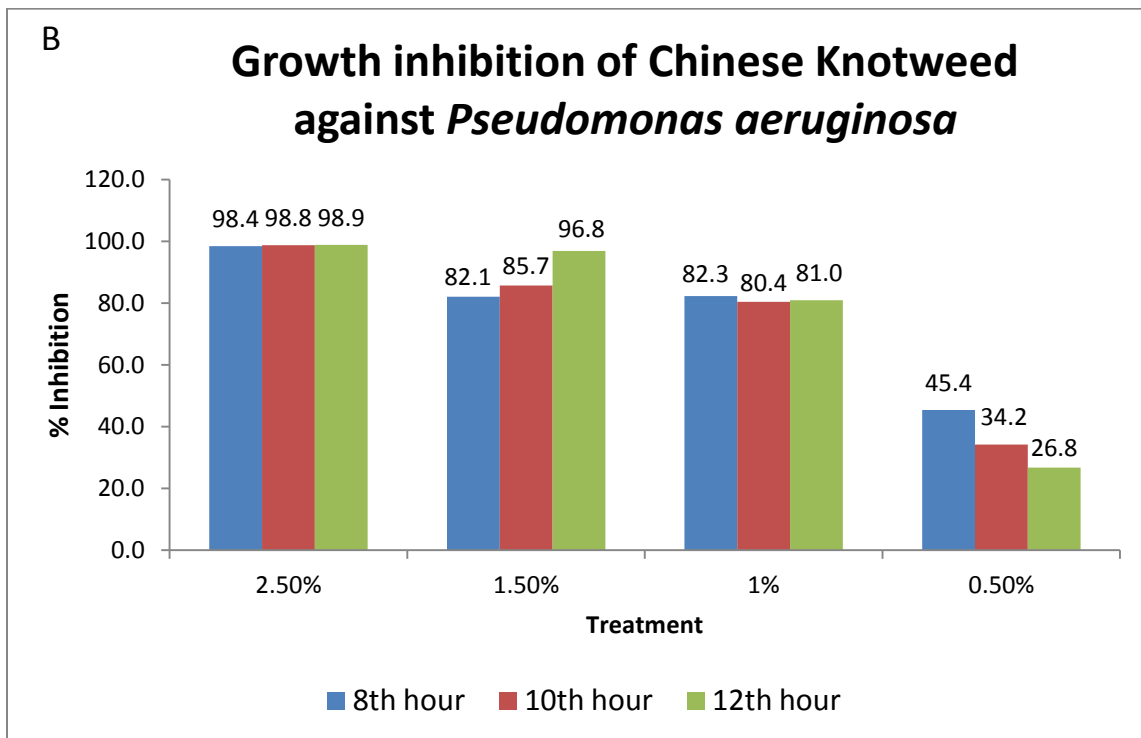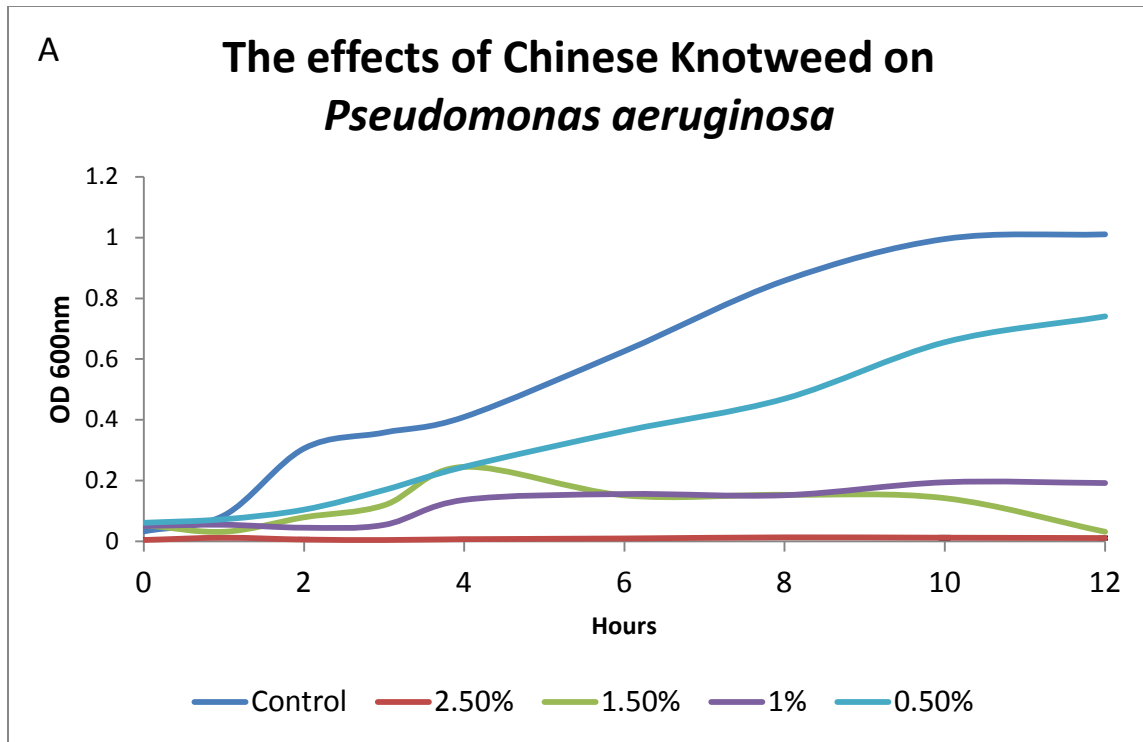
Figure 6. A) Growth curve of *Pseudomonas aeruginosa* treated with Chinese Knotweed.
B) Growth curve of *Pseudomonas aeruginosa* treated with Chinese Knotweed.

Followed by the four selected Gram negative species, same assay has been carried out for five Gram positive species. Figure 7 showed that 1, 1.5 and 2.5% CK all have great inhibition on *Bacillus cereus* with the IC_{50} falls between 0.5 – 1%. Figures 8, 9 and 10 indicated the MIC for *B. megaterium*, *S. epidermidis*, and *S. mutans* is 2.5% CK. However, Chinese Knotweed showed the strongest inhibition against *S. pyogenes*. Figures 11A and 11B suggested that 1.5% CK could be sufficient to inhibit *S. pyogenes* growth completely.

To summarize, the MIC for all bacterial species is 2.5% Chinese Knotweed (except for *S. pyogenes*: MIC 1.5%) which showed efficacy of >98%; the IC_{50} varies among species but in most cases ranges from 1% to 0.25% (Table 2).

A) The effects of Chinese Knotweed on *Bacillus cereus*

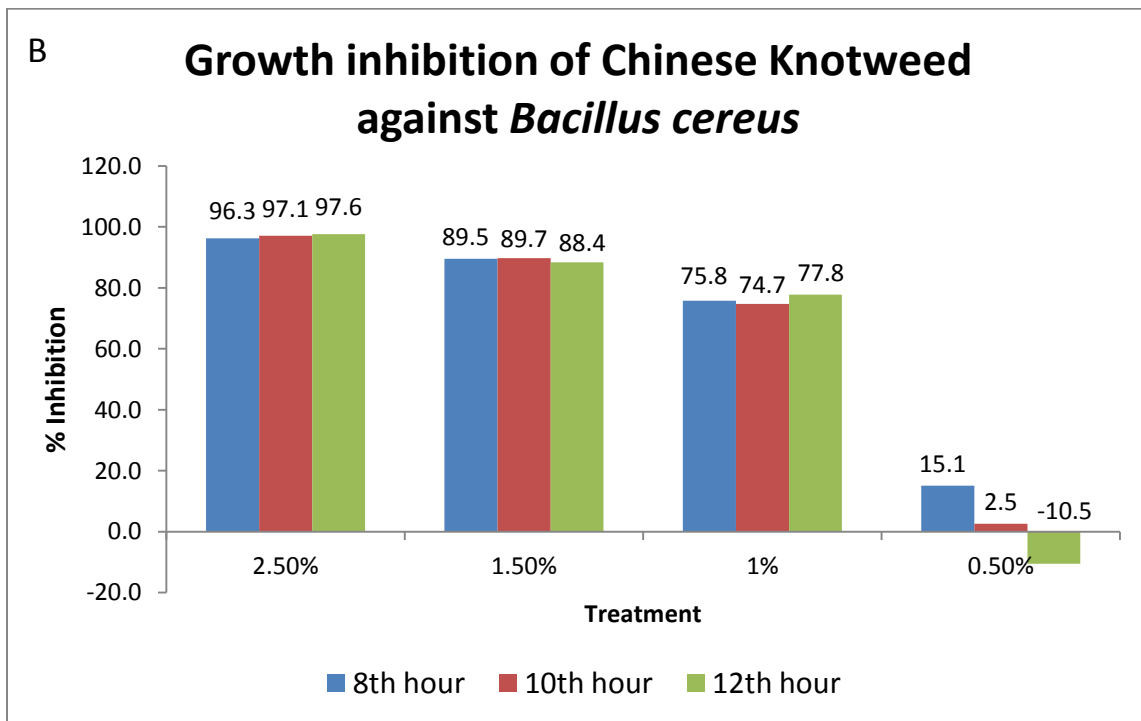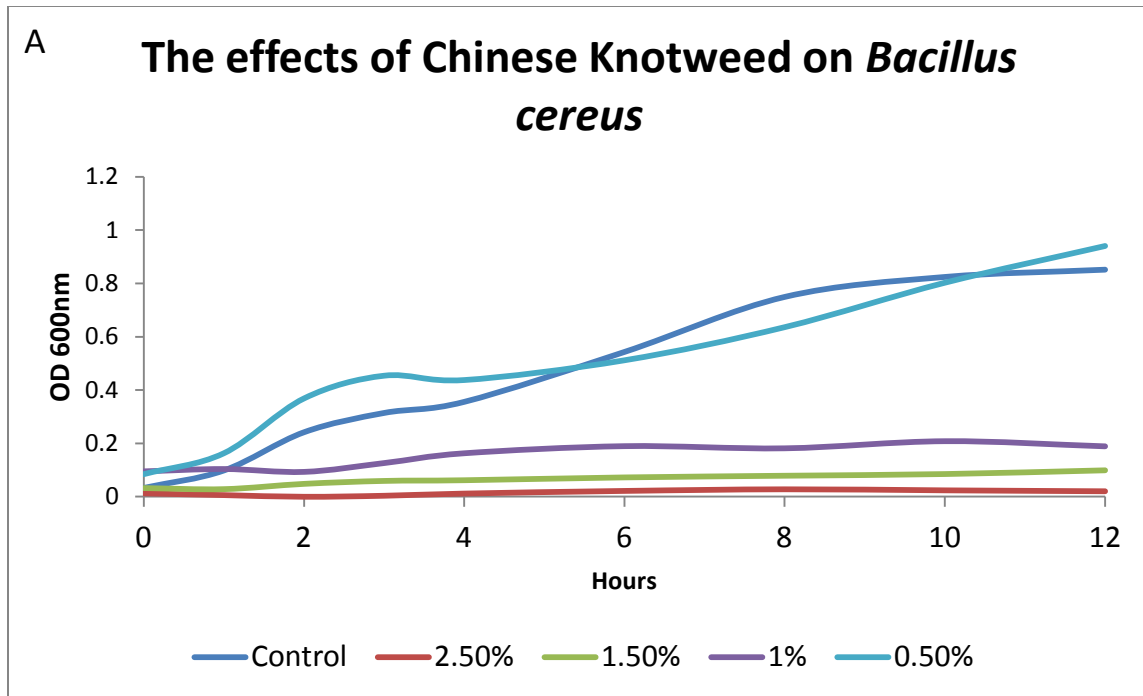B) Growth inhibition of Chinese Knotweed against *Bacillus cereus*

Figure 7. A) Growth curve of *Bacillus cereus* treated with Chinese Knotweed. B) Growth curve of *Bacillus cereus* treated with Chinese Knotweed.
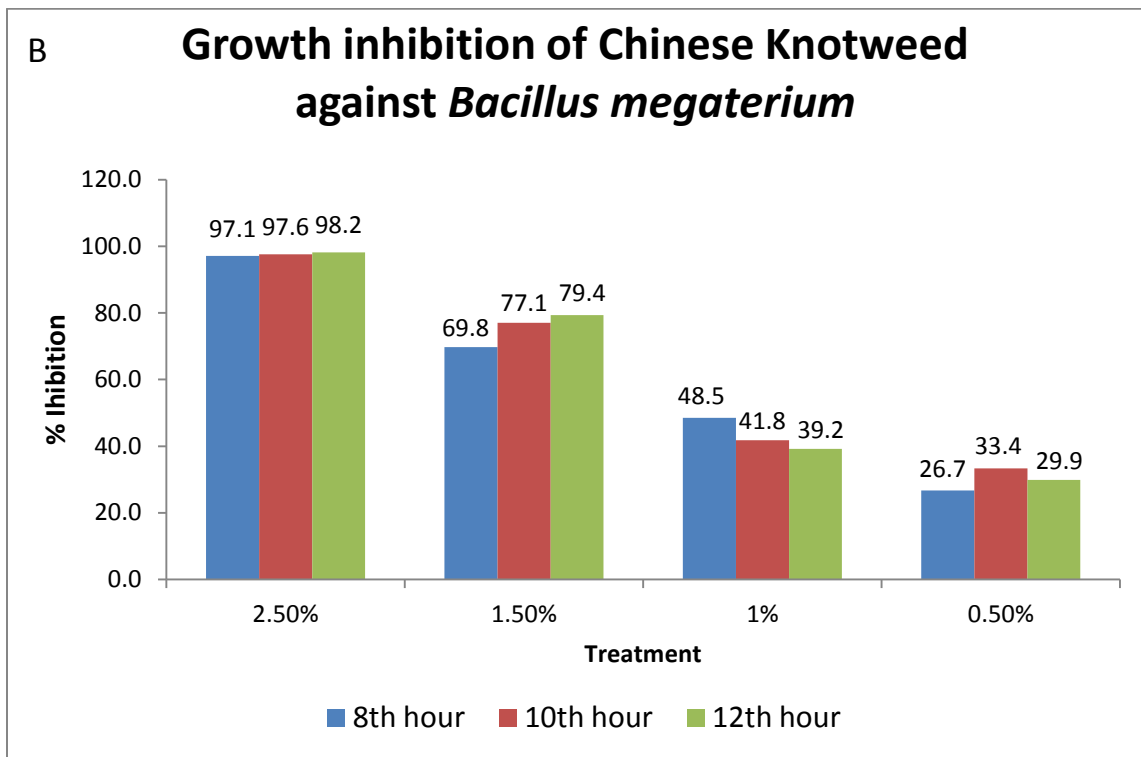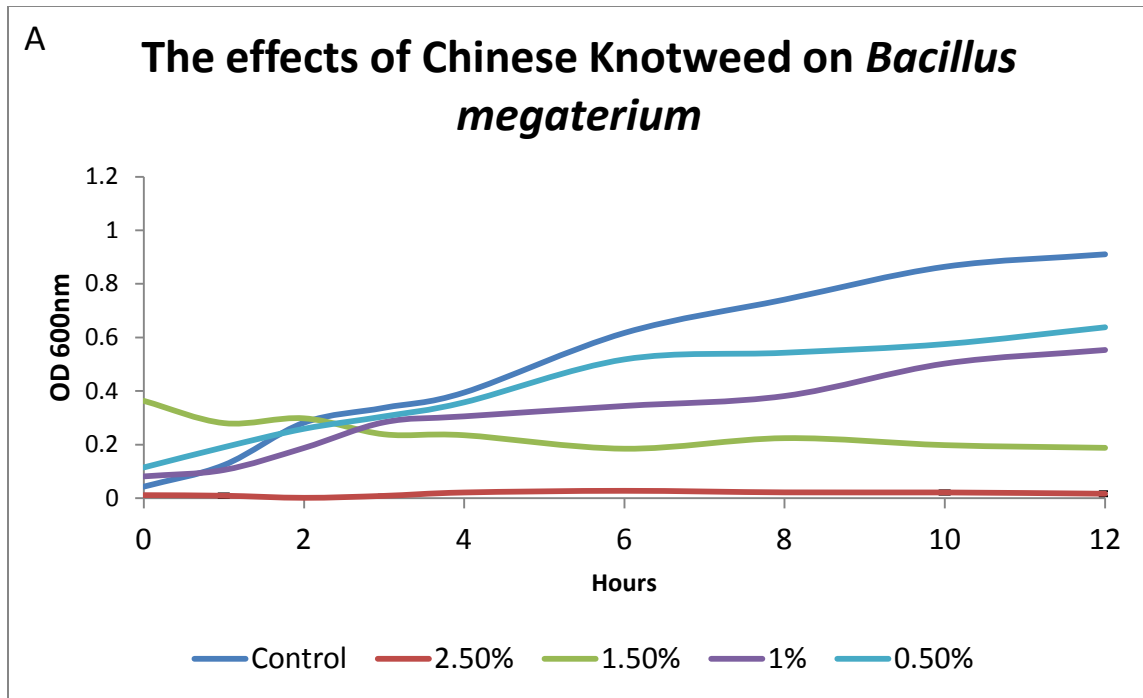
Figure 8. A) Growth curve of *Bacillus megaterium* treated with Chinese Knotweed. B) Growth curve of *Bacillus megaterium* treated with Chinese Knotweed.
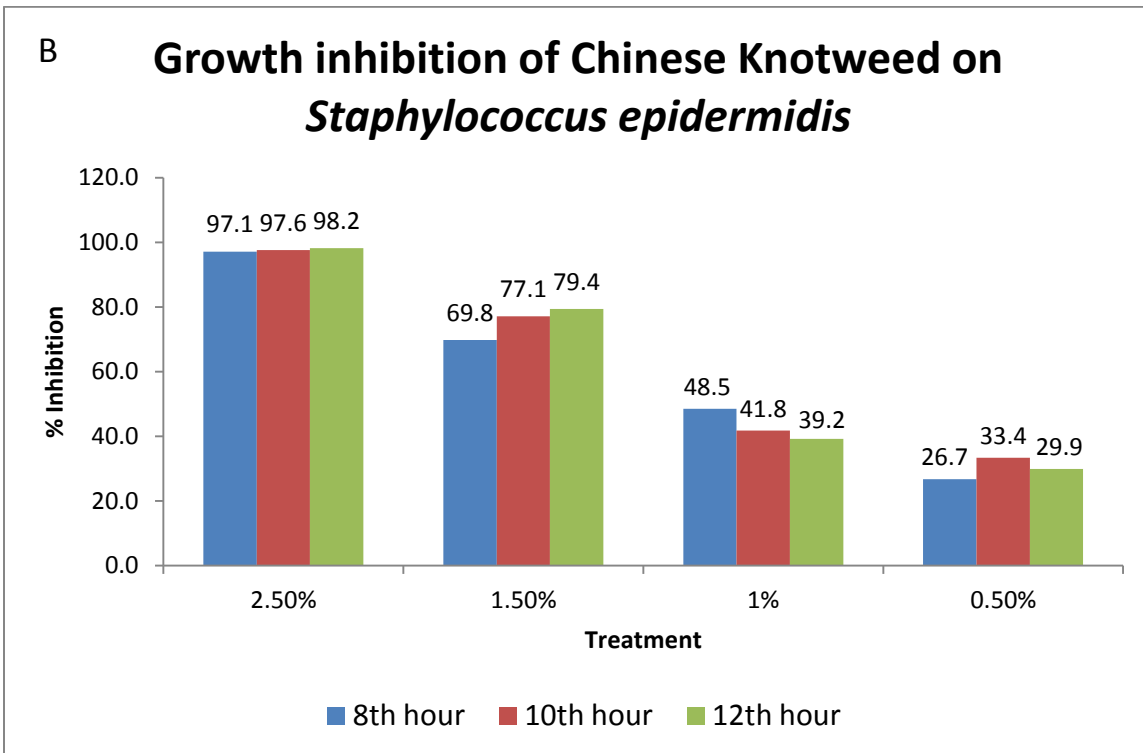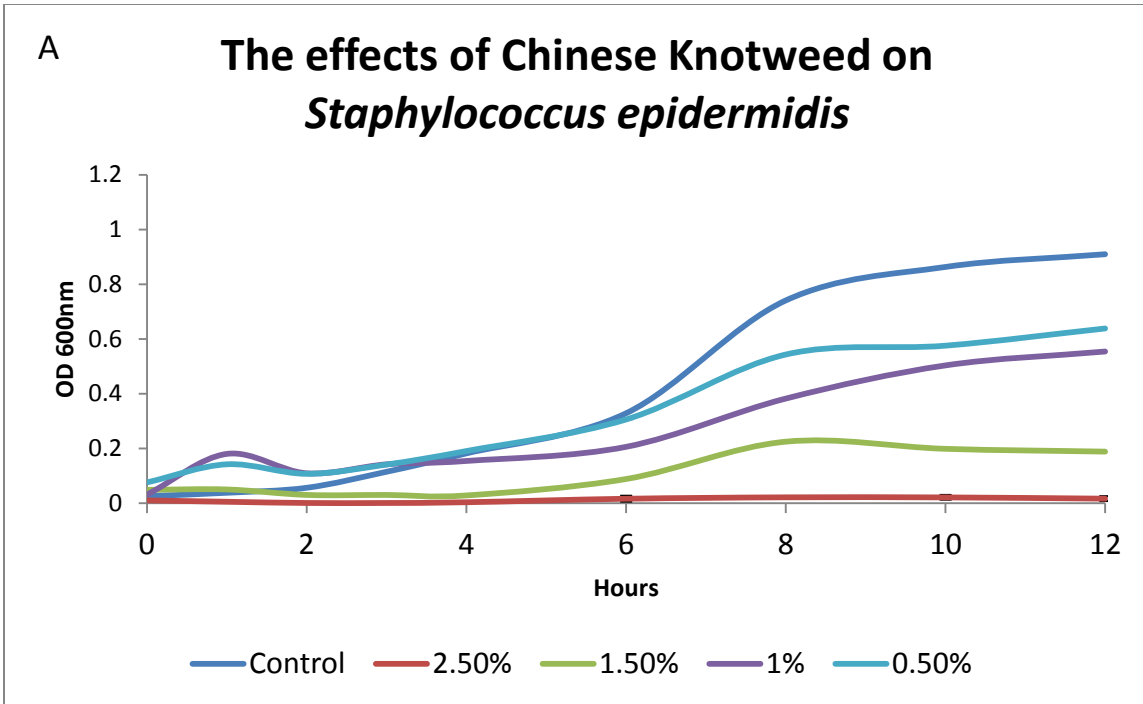
Figure 9. A) Growth curve of *Staphylococcus epidermidis* treated with Chinese Knotweed. B) Growth curve of *Staphylococcus epidermidis* treated with Chinese Knotweed.
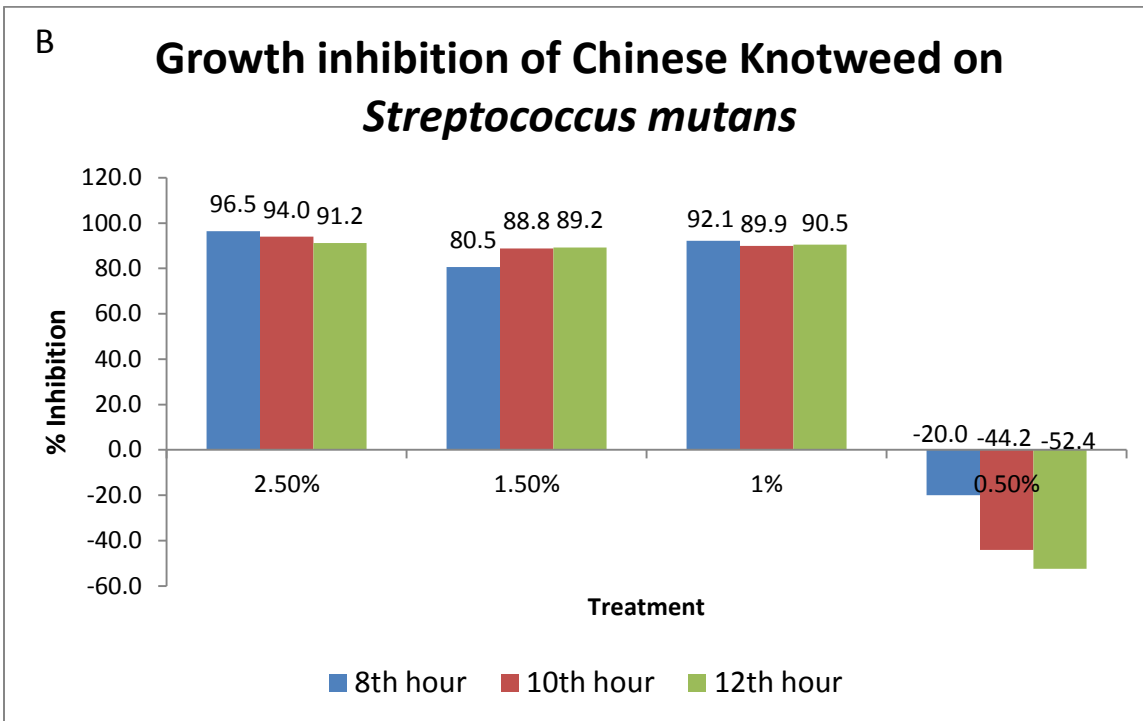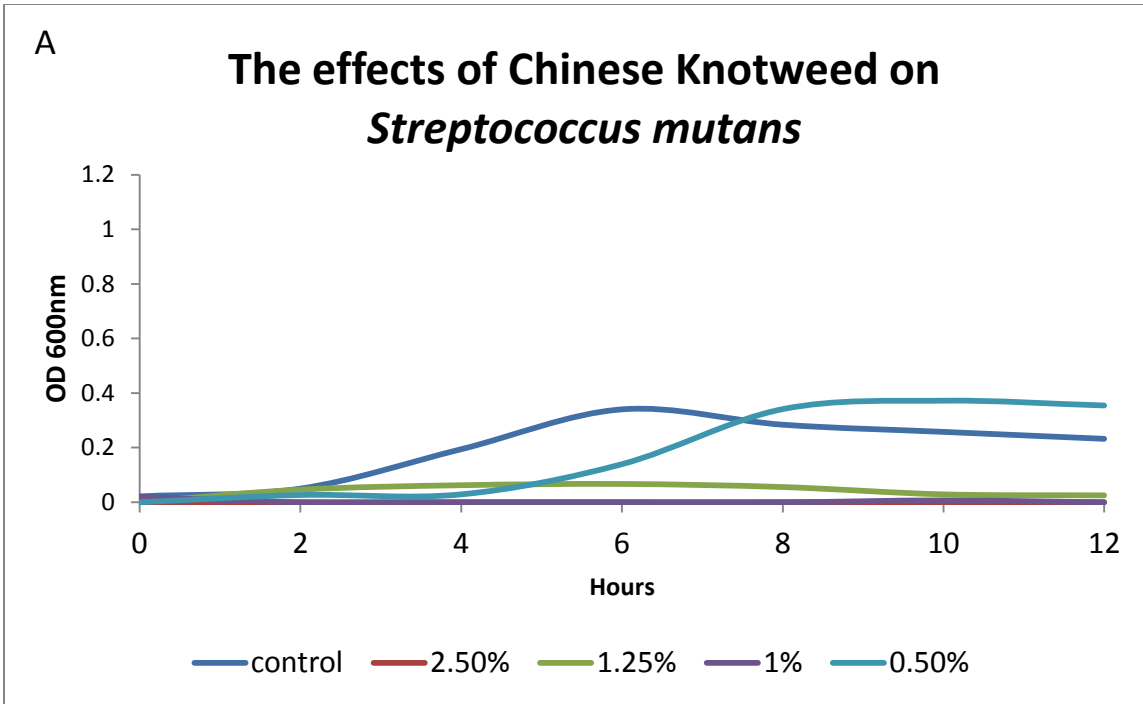
Figure 10. A) Growth curve of *Streptococcus mutans* treated with Chinese Knotweed. B) Growth curve of *Streptococcus mutans* treated with Chinese Knotweed.
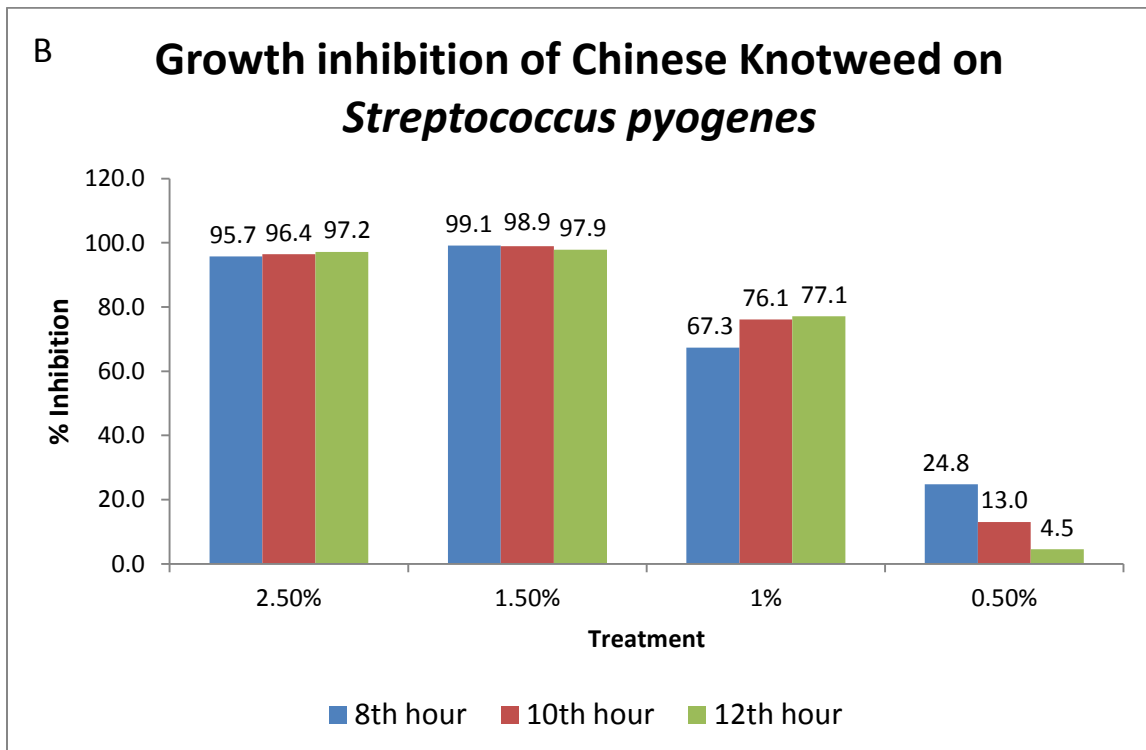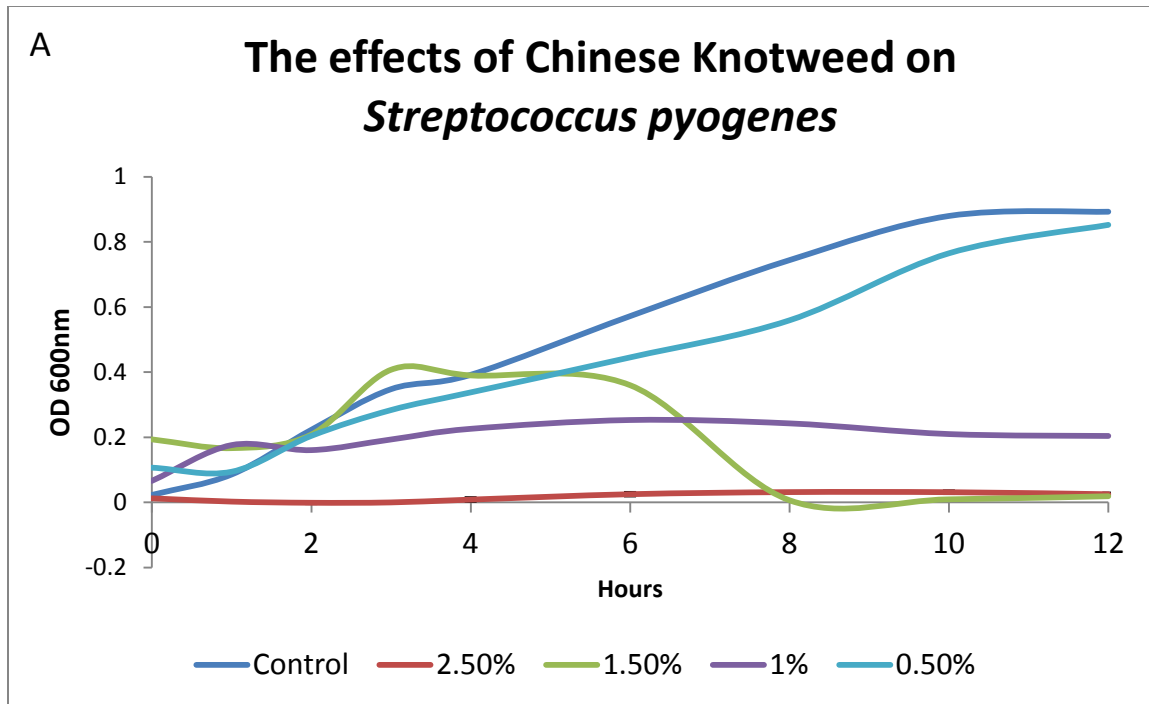
Figure 11. A) Growth curve of *Streptococcus pyogenes* treated with Chinese Knotweed.
B) Growth curve of *Streptococcus pyogenes* treated with Chinese Knotweed.

Table 2. MIC and IC_{50} of all 9 bacterial species. While MIC is 2.5% Chinese Knotweed for eight bacteria (1.5% for *S. pyogenes*), the IC_{50} is listed as follows,

Bacterial species	IC_{50}
Bacillus cereus	0.5-1%
Bacillus megaterium	1-1.5%
Staphylococcus epidermidis	1-1.5%
Streptococcus mutans	0.5-1%
Streptococcus pyogenes	0.5-1%
Enterobacter aerogenes	0.5-1%
Escherichia coli	1-1.5%
Proteus vulgaris	0.5-1%
Pseudomonas aeruginosa	0.5-1%

Antibacterial synergism of Chinese Knotweed with various antibiotics

Kirby-Bauer assays were carried out to evaluate the synergistic antibacterial activity of various antibiotics with Chinese Knotweed. A total of 11 antibiotics with different mode of action were included in this study (Table 1). Both Ampicillin and Bacitracin target bacterial cell wall synthesis inhibition. Figure 12 showed that 2.5% Chinese Knotweed was able to increase the zone of inhibition (ZOI) for 47% and 83% on *P. vulgaris* and *P. aeruginosa* when combined with Ampicillin. No significant ZOI increase was observed when combining Chinese Knotweed with Bacitracin (Figure 13).

Another common mechanism of antibiotics involves in protein synthesis inhibition. Five antibiotics, Erythromycin, Kanamycin, Neomycin, Streptomycin and Tetracycline, included in this study belong to this category. Figure 14 indicated Erythromycin had 60% ZOI increase against *E. aerogenes* when combined with CK and Kanamycin showed 44.2% synergism against the same bacteria (Figure 15). However, Kanamycin displayed better synergistic antibacterial effect than Erythromycin with CK overall. Neomycin also showed 60% ZOI increase when combined with CK against *E. aerogenes* (Figure 16). Figures 17 and 18 represent the ZOI results of Streptomycin and Tetracycline with or without CK. Both of them exhibited 60% ZOI increase when combined with the compound against *Proteus vulgaris*.

Next three antibiotics tested, Nalidixic acid, Nitrofurantoin and Novobiocin, possess the nucleic acid synthesis inhibition as their antibacterial mechanisms. Results of Nalidixic acid showed almost no synergism with Chinese Knotweed except for *S. pyogenes* (Figure 19). Similarly, Nitrofurantoin also showed limited to no synergistic

antibacterial activity when combined with CK except for *B. cereus* and *E. coli* (Figure 20). Novobiocin, showed 50% ZOI increase against *E. coli* and 36.7% against *P. aeruginosa* (Figure 21).

Triple sulfa, composed of three components; sulfathiazole, sulfacetamide and sulfabenzamide, was the last antibiotic tested in this study. This antibiotics inhibits bacterial folate production in cells. Its antibacterial activity significantly increased when combined CK against *B. cereus* (172%) and *S. pyogenes* (155%) (Figure 22).

Table 3, is a representation of all antibiotics used and bacteria used for the study and indicate the percent increase or decrease in the zone of inhibition. This is important to evaluate the statistical significance of the combination of the antibiotic and Chinese Knotweed extract. Although in many cases there are a slight increase in ZOI, but the increase is not significant or depicts a true synergism between the two chemical agents. Also many of the antibiotic in combination with Chinese Knotweed displayed an antagonistic combination which indicates that some combinations of chemicals do not go together. Although all results are not positive, the Kirby-Bauer test those show potential in determining true synergism between that of chemical agents and natural products.
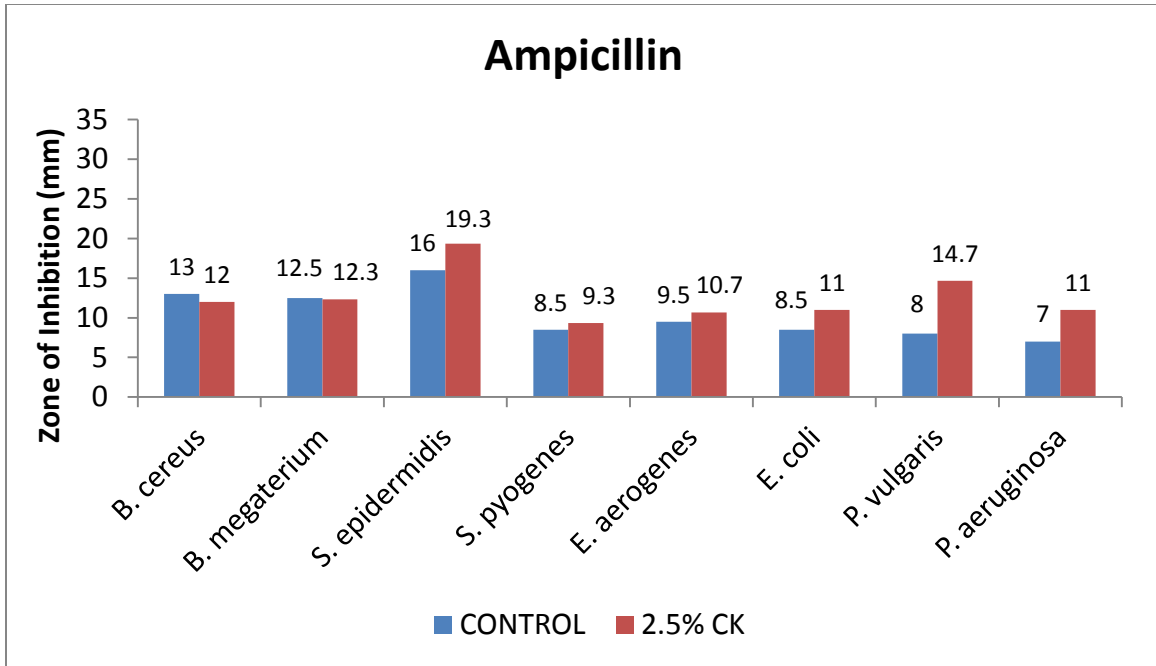
Figure 12. The antibacterial effect of Ampicillin with or without 2.5% Chinese Knotweed.
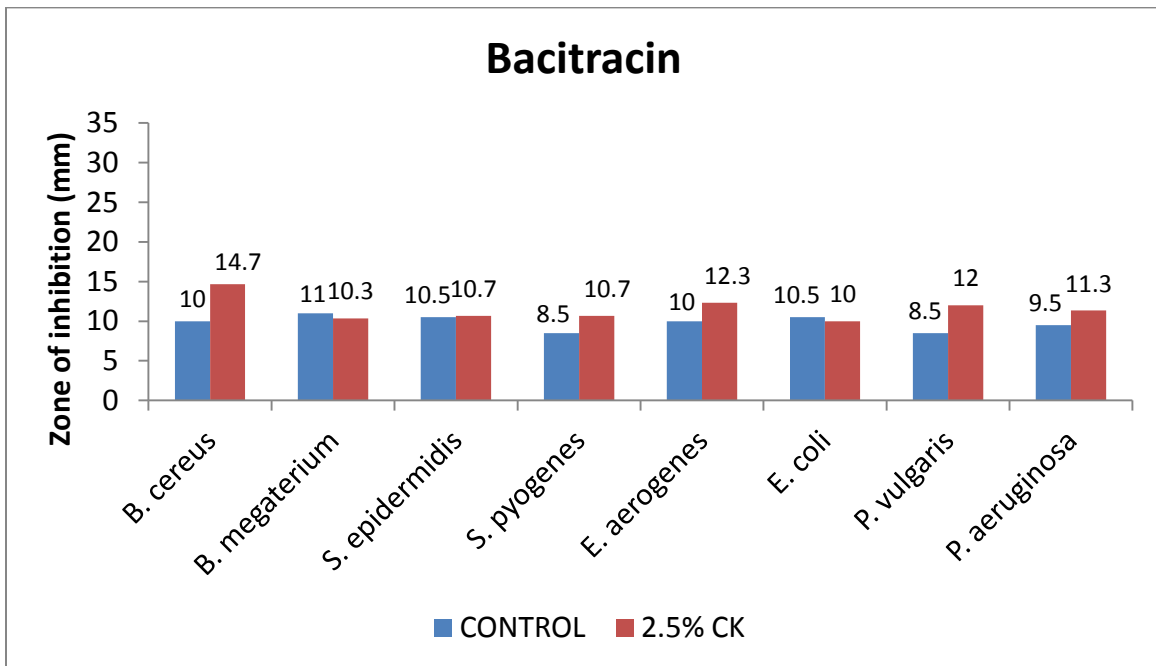

Figure 13. The antibacterial effect of Bacitracin with or without 2.5% Chinese Knotweed.
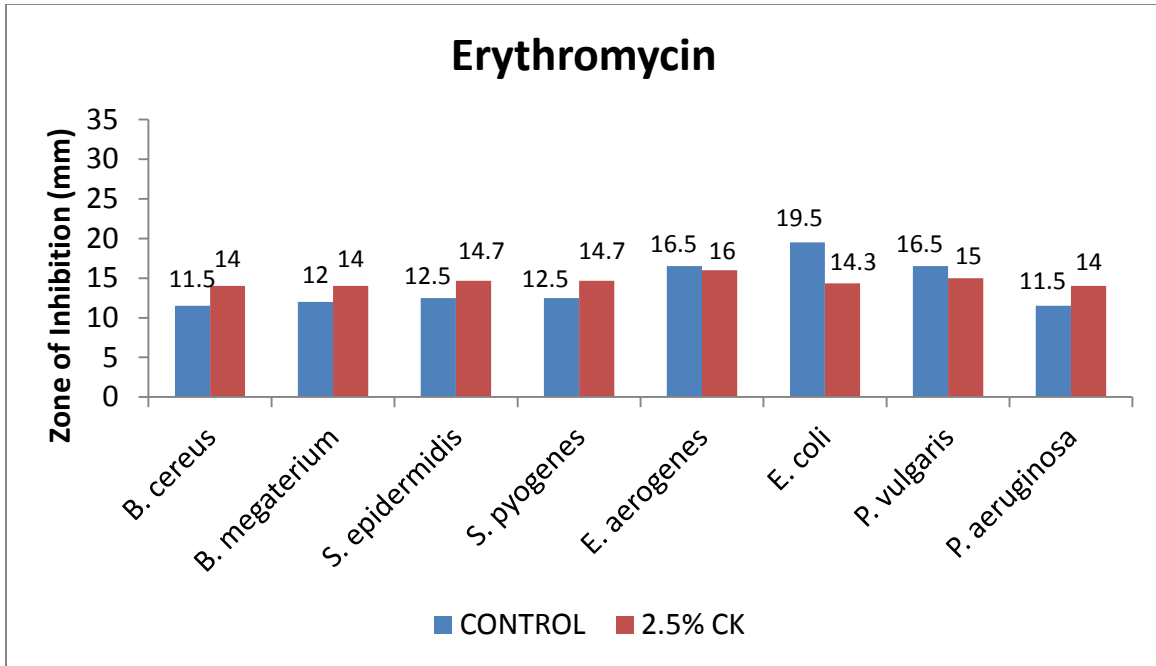
Figure 14. The antibacterial effect of Erythromycin with or without 2.5% Chinese Knotweed.
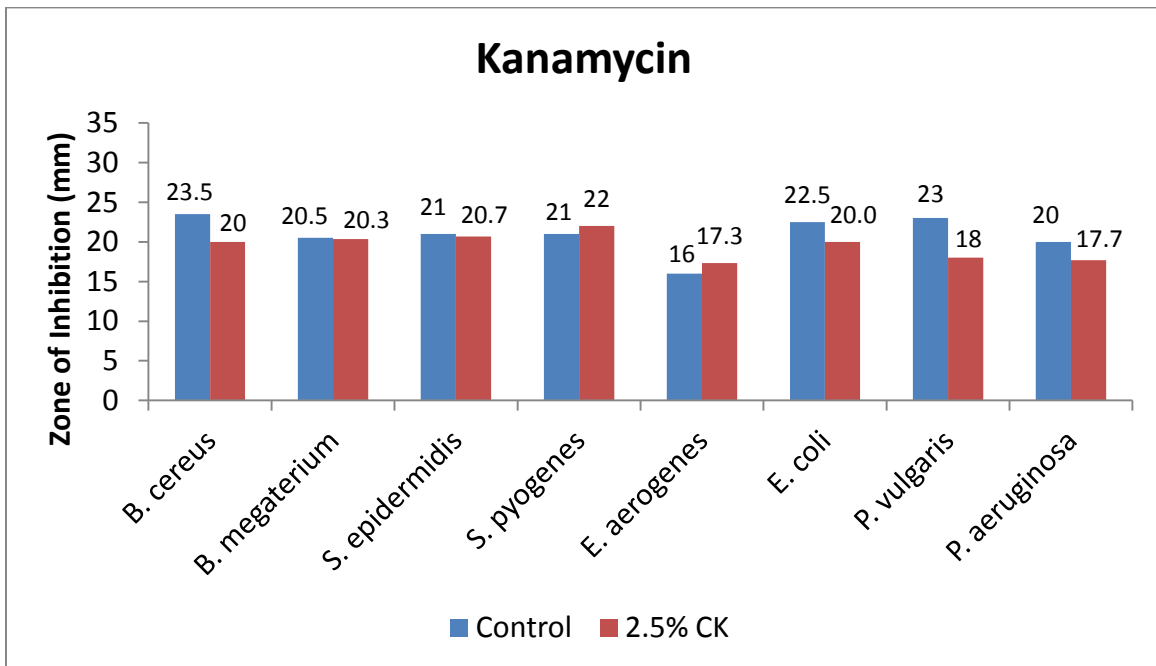

Figure 15. The antibacterial effect of Kanamycin with or without 2.5% Chinese Knotweed.
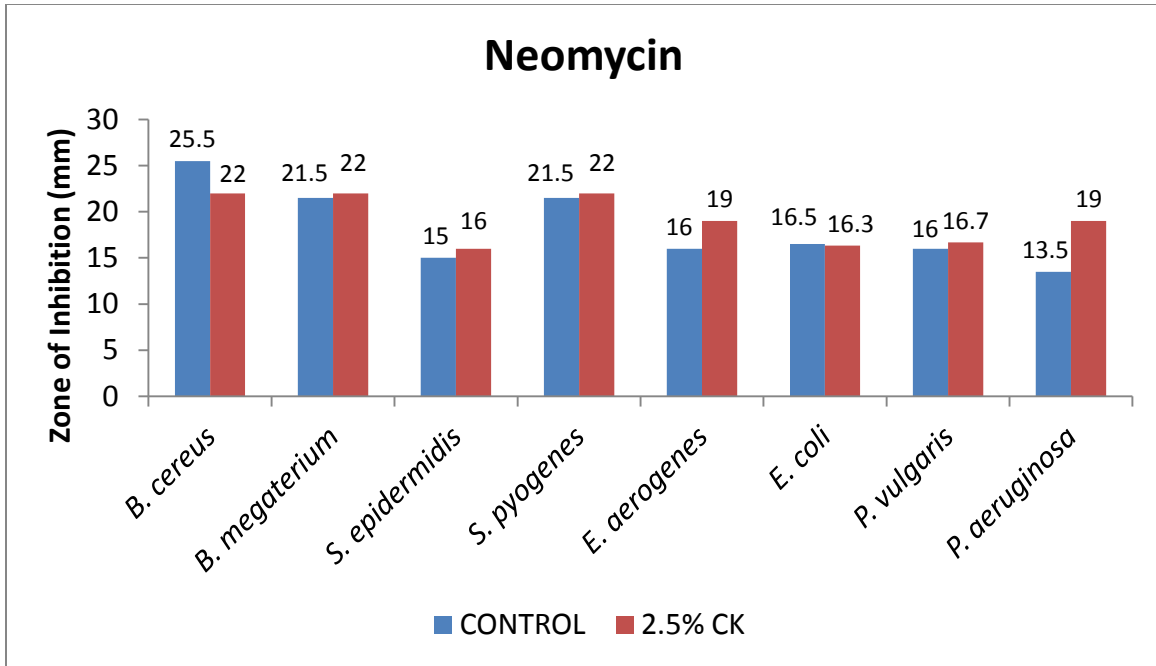
Figure 16. The antibacterial effect of Neomycin with or without 2.5% Chinese Knotweed.
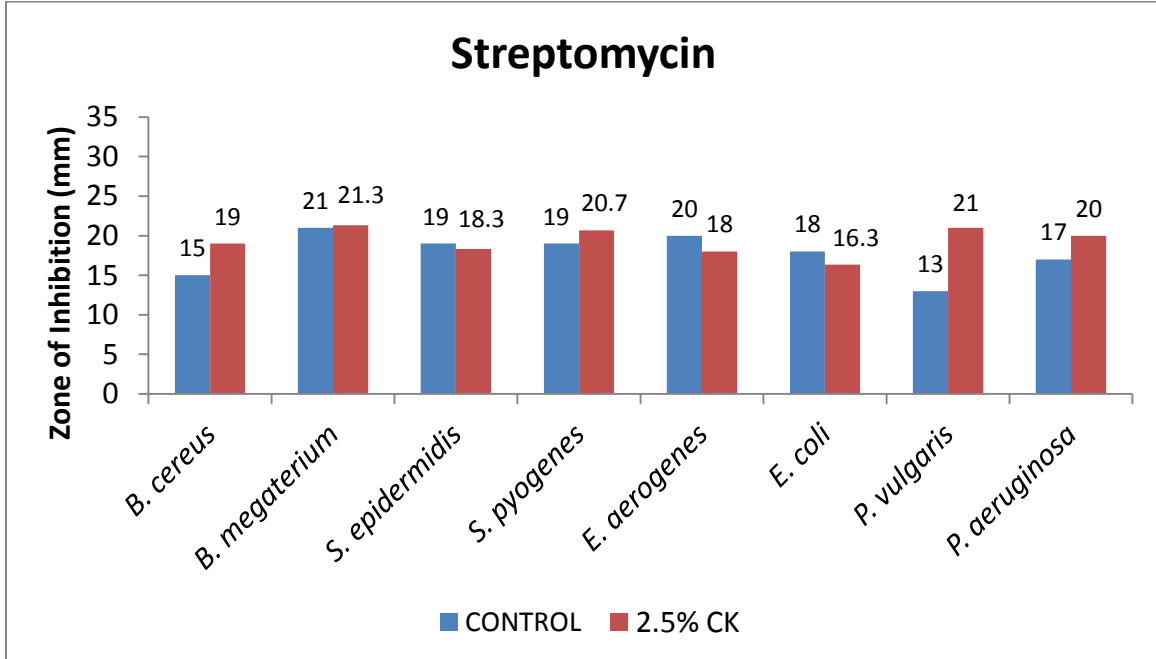
Figure 17. The antibacterial effect of Streptomycin with or without 2.5% Chinese Knotweed.
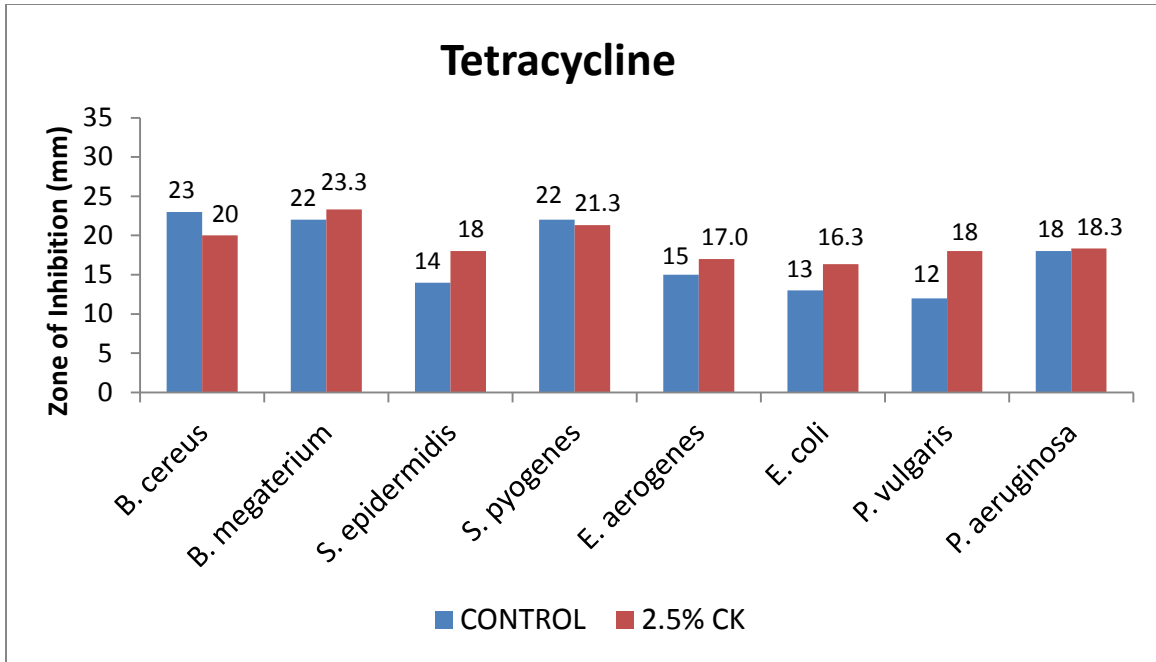
Figure 18. The antibacterial effect of Tetracycline with or without 2.5% Chinese Knotweed.
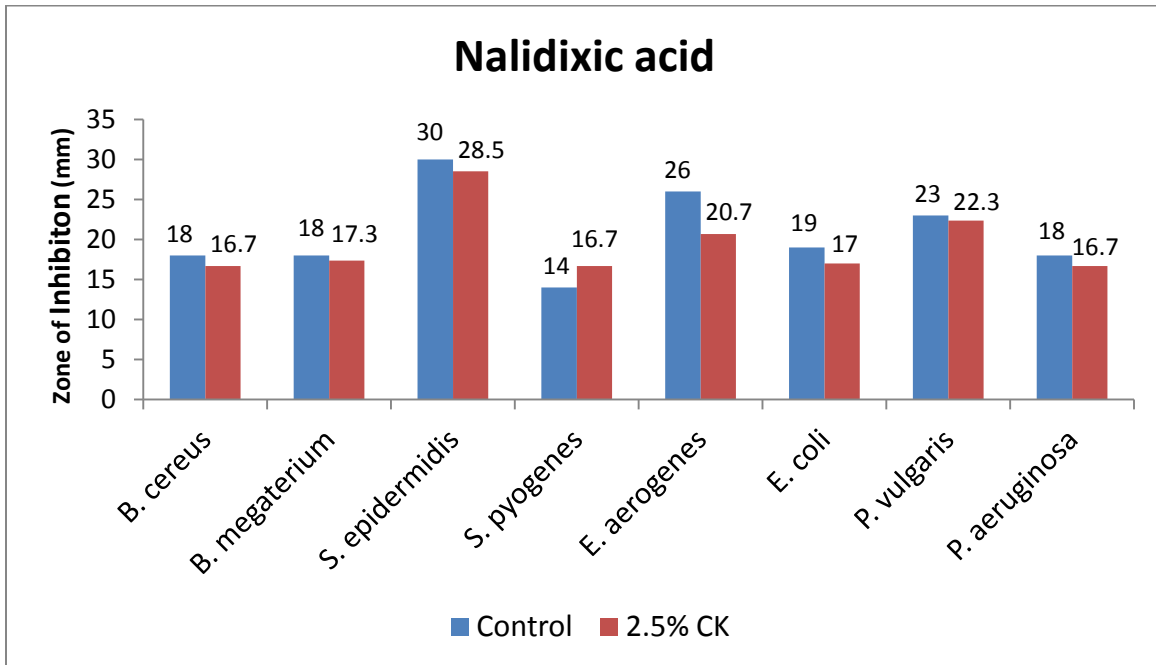
Figure 19. The antibacterial effect of Nalidixic acid with or without 2.5% Chinese Knotweed.

Figure 20. The antibacterial effect of 2.5% Chinese Knotweed with or without Nitrofurantoin.

Figure 21. The survey of microorganism against Novobiocin with and without 2.5% Chinese Knotweed.

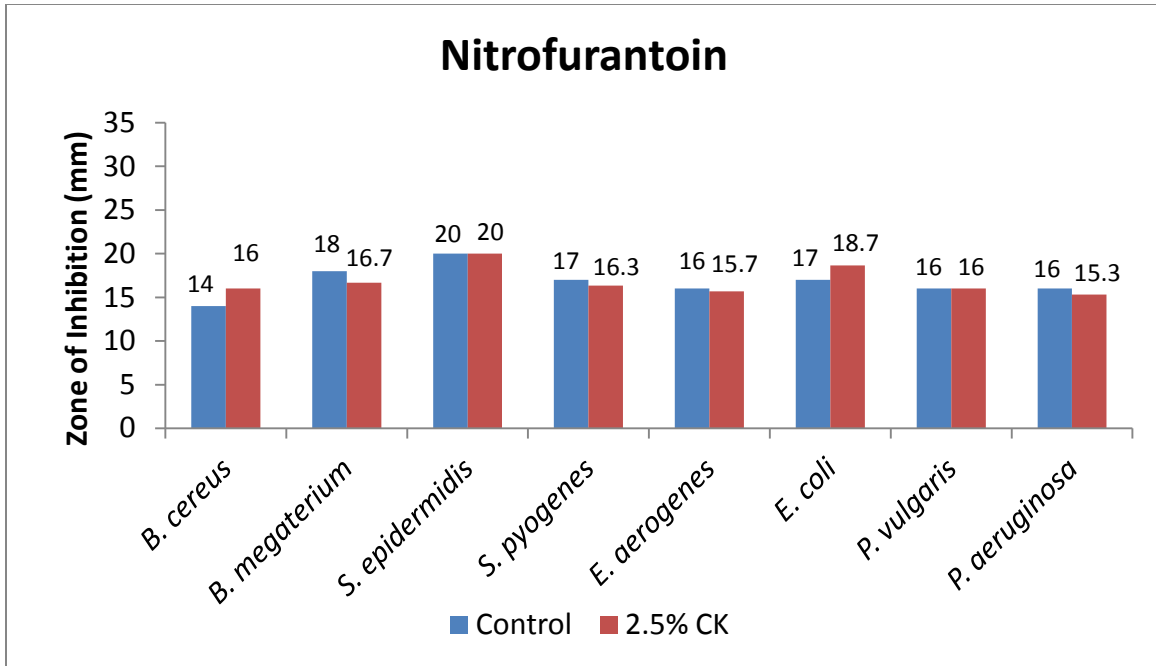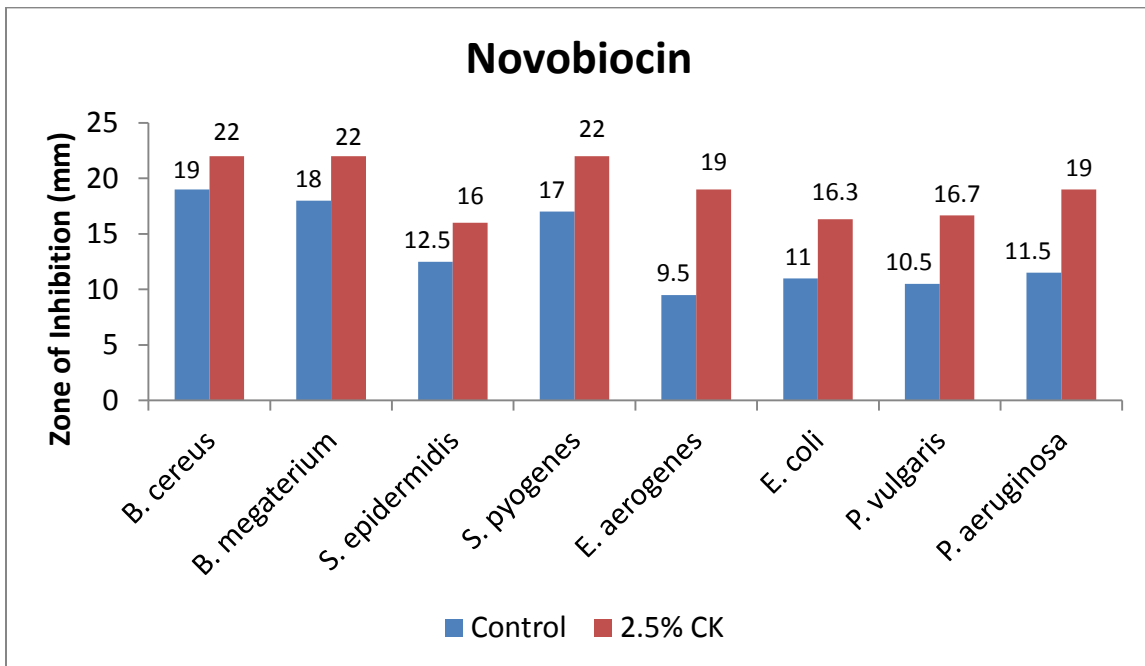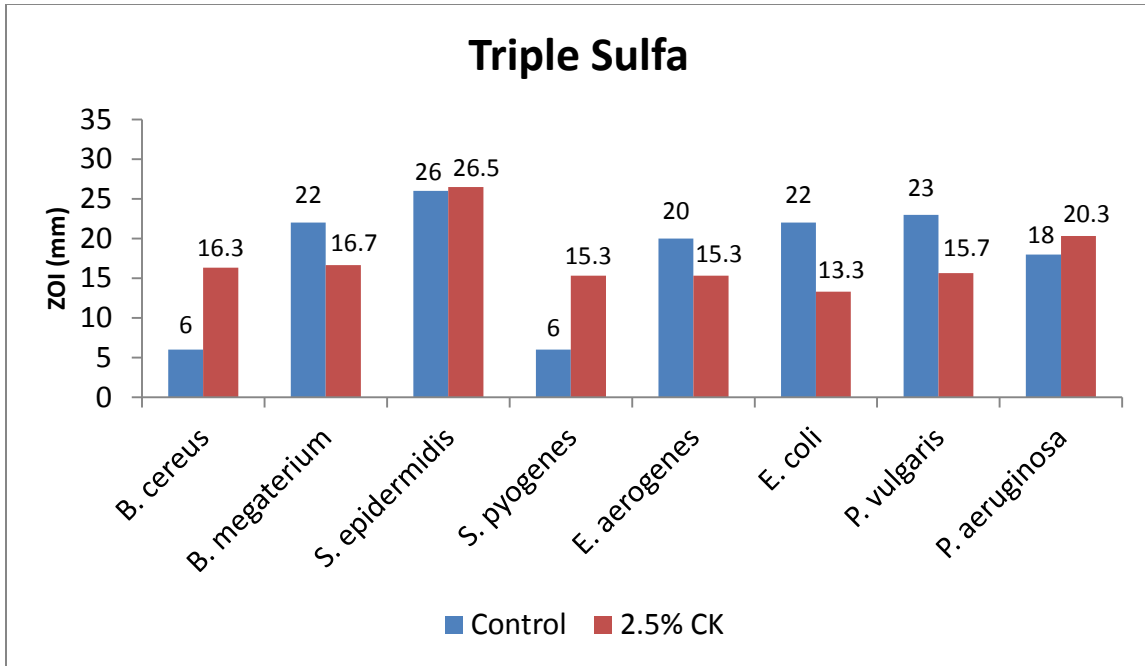Figure 22. The survey of microorganism against Triple Sulfa with and without 2.5% Chinese Knotweed.
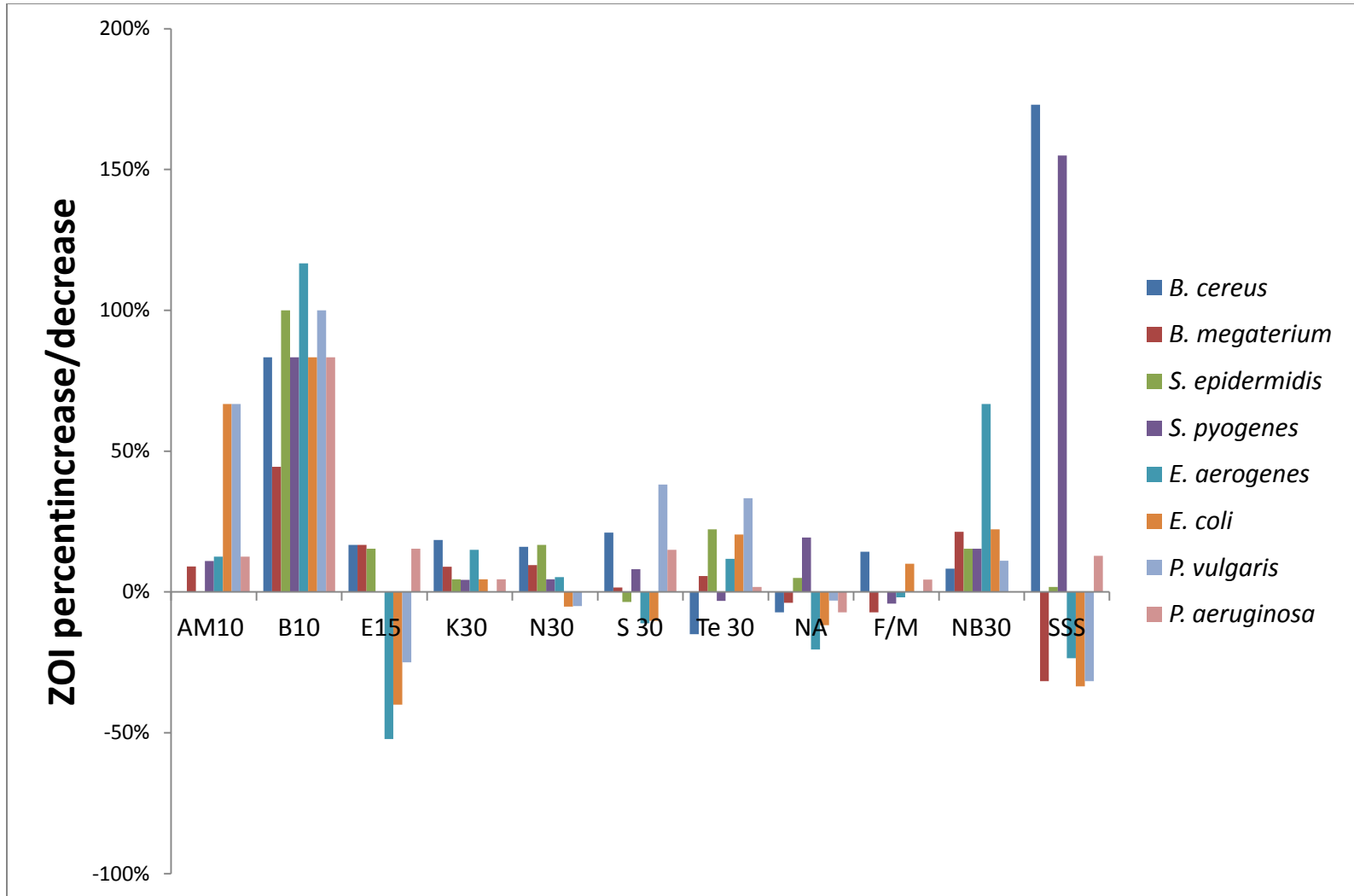
Figure 23. A summary of all the antibiotics illustrating the percent increase or decrease of the zone of inhibition for each bacteria.

Table 3. Summary of best synergistic antibacterial activity of different antibiotics with 2.5% CK.

Antibiotic	Synergism with Antibiotic	% increase
Ampicillin (AM10)	*Escherichia coli/Proteus vulgaris*	66.7%
Bacitracin (B10)	*Enterobacter aerogenes*	116.7%
Erythromycin (E15)	*Bacillus megaterium*	16.7%
Kanamycin (K30)	*Bacillus cereus*	18.5%
Nalidixic acid (N/A30)	*Streptococcus pyogenes*	19.3%
Neomycin (N30)	*Streptococcus epidermidis*	16.7%
Nitrofurantoin (F/M300)	*Bacillus cereus*	14.3%
Novobiocin (NB30)	*Enterobacter aerogenes*	66.7%
Streptomycin (S10)	*Proteus vulgaris*	38.09%
Tetracycline (TE30)	*Proteus vulgaris*	33.3%
Triple Sulfa (SSS300)	*Bacillus cereus*	173%

Biofilm

Congo-red is a rapid detection used to detect the exopolysaccharide production of the biofilm. The assay is based on a visual analysis of the agar to determine whether or not biofilm is made (Kaiser et al., 2013). If the polysaccharide is produced, it will react with the Congo-red and this will result in the media turning black (Freeman et al., 1989). The agar was also fortified with 2% Sucrose which favors the growth of Biofilm formation. Congo-red assays were carried out to screen the bacteria for potential biofilm formation. Results showed all four Gram negative bacteria, *E. aerogenes, E. coli, P. vulgaris and P. aeruginosa*, were able to form biofilm (Figure 24A) while only three out of five Gram positive bacteria, *S. epidermidis, S. pyogenes and S. mutans* formed biofilm on Congo-red plate (Figure 24B).

Figures 25A and 25B illustrated the results of anti-biofilm formation of CK on Gram negative and Gram positive bacteria, respectively. Though initial results indicated that 1% CK didn't inhibit biofilm formation for Gram positive bacteria, it showed strong anti-biofilm activity for *E. coli*, *P. vulgaris* and *P. aeruginosa*.
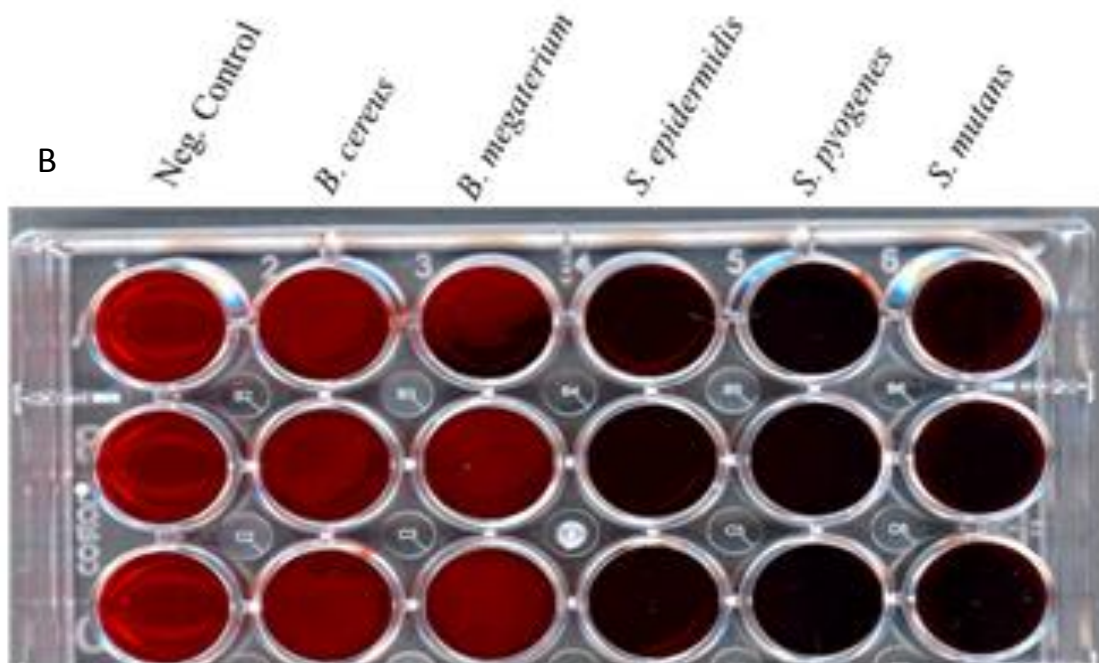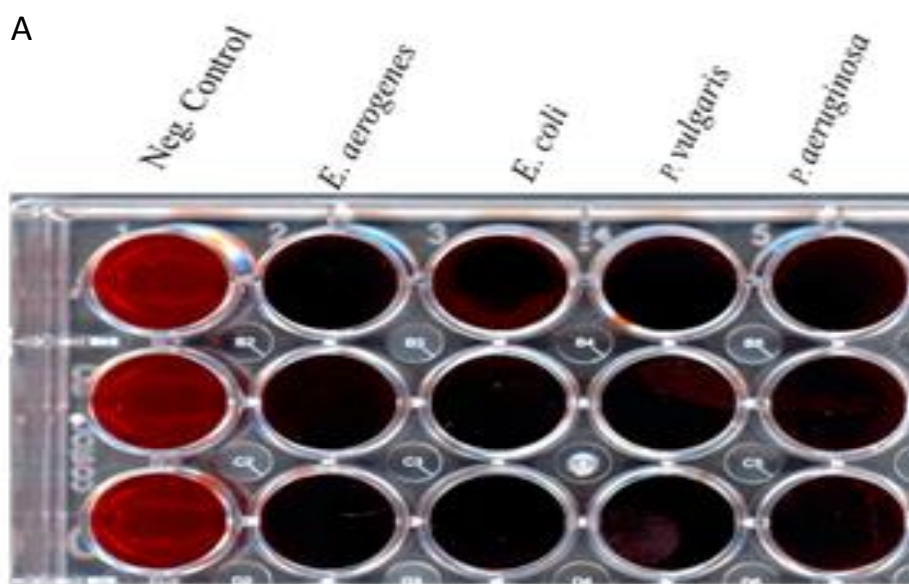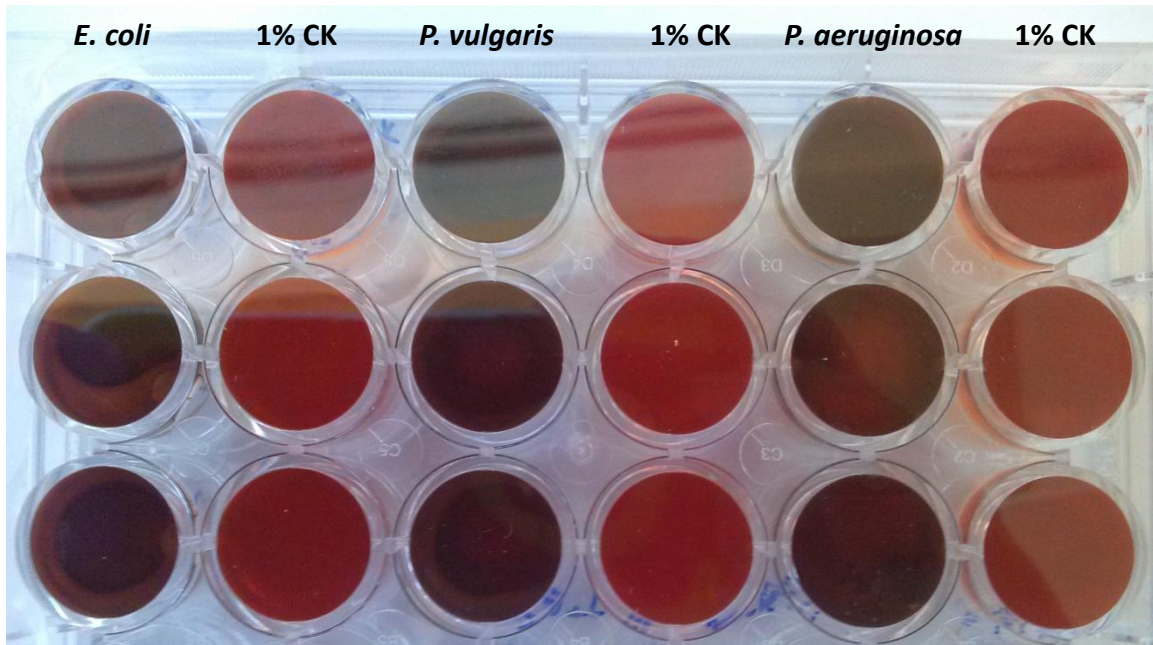
Figure 24. Congo-red assay for A) four Gram negative bacteria. B) five Gram positive bacteria.

A

B
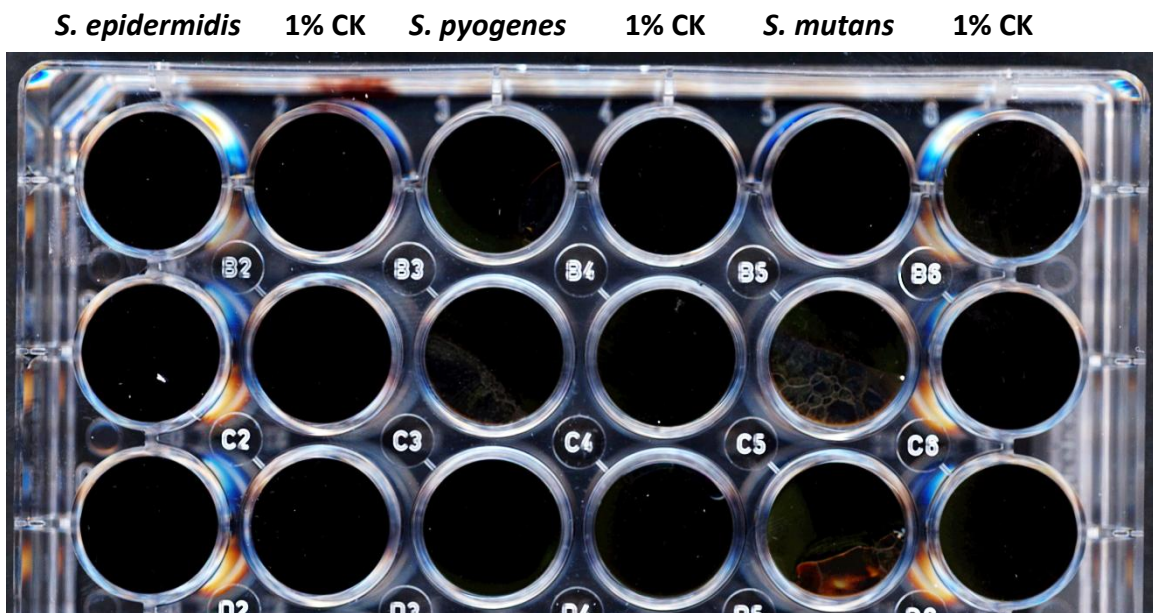
Figure 25. Anti-biofilm evaluation of 1% CK on A) three Gram negative species. B) three Gram positive species.

Crystal Violet

Result obtained from the Congo-red assay suggested that 1% Chinese Knotweed is effective to inhibit the biofilm formation especially for Gram negative bacteria. Crystal violet assay was then carried out to quantify biofilm reduction. Crystal violet is a stain that is popular to use in Gram-staining bacteria in order to differentiate Gram-positive from Gram-negative bacteria. Due to its simplicity as a simple stain it can be used to bind to the outer layer of bacteria in order to detect/ quantify the amount of biofilm formed (Peeters et al., 2008). Figure 26A showed the raw results of crystal violet for all 9 species while Figure 26B indicated the percentage reduction of biofilm formation. 1% CK was able to significantly reduce the biofilm formation for six out of nine bacteria, especially for *B. megaterium* (98.01%) and *S. mutans* (96.16%).

Figure 27 showed an example of crystal violet assay with 1.25% and 2.5% CK to illustrate that biofilm was inhibited almost completely for both concentration of CK. 1% CK was considered the MIC in regards to biofilm formation for *S. mutans* while 0.5% CK also demonstrated ~95% inhibition (Figure 28A). Microscopic observation shown in figure 28B showed significant reduction of bacteria population with 1% CK.
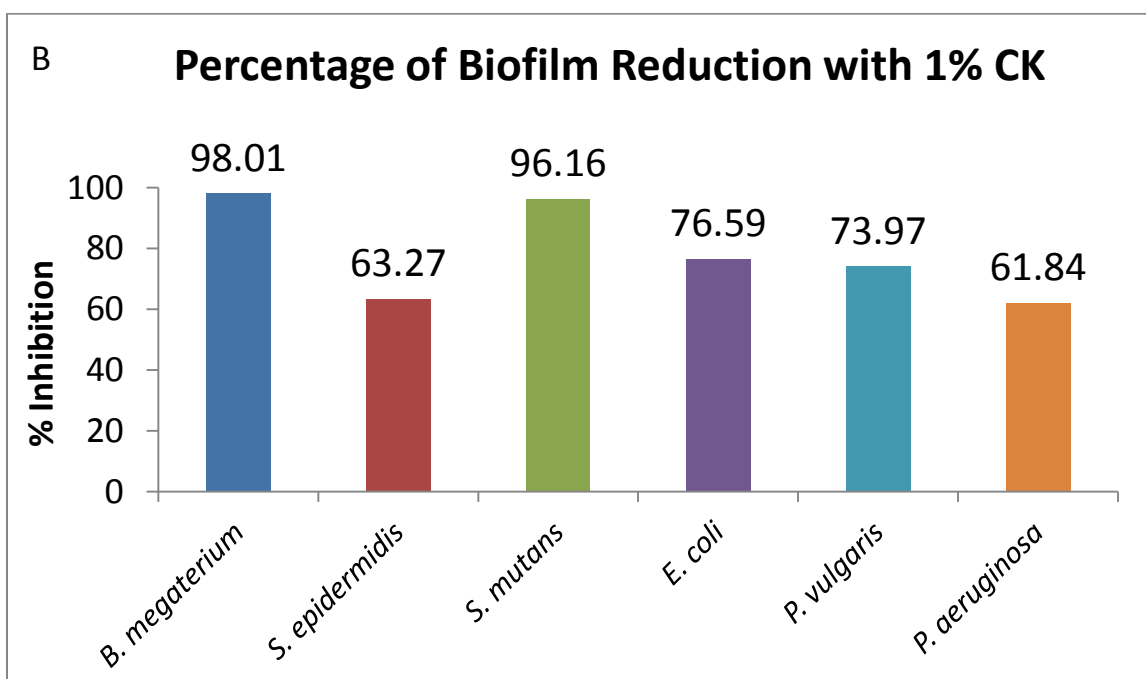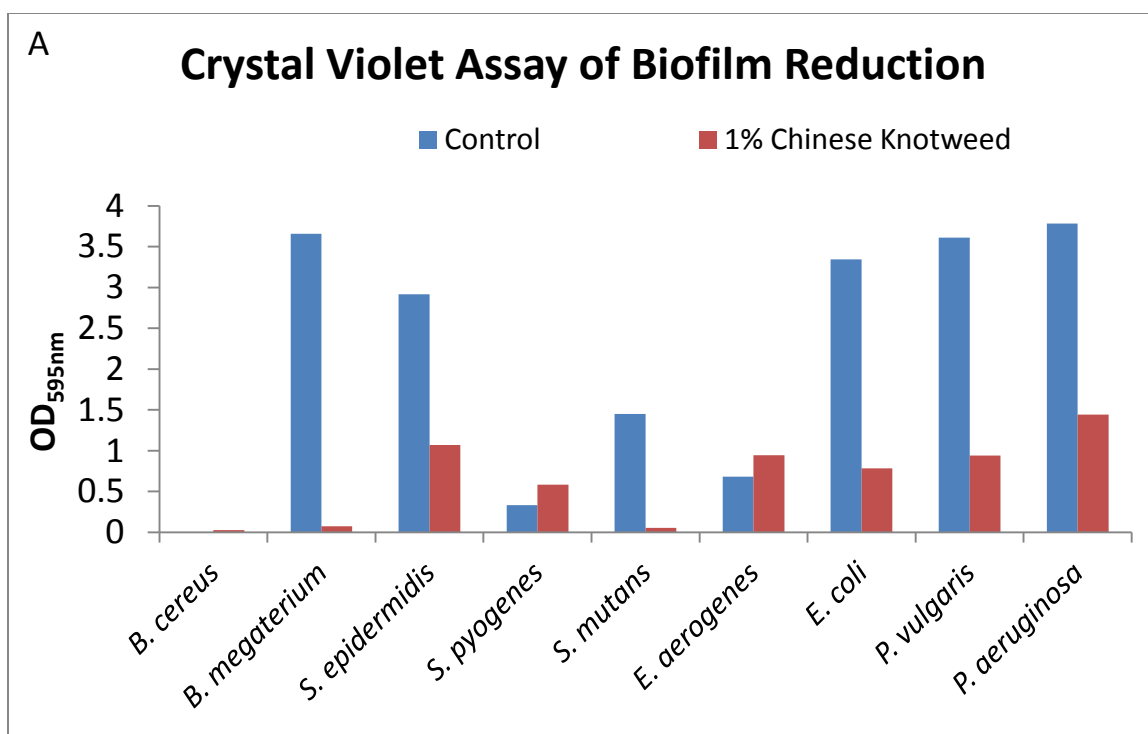
Figure 26. A) Result of crystal violet assay of Gram positive and Gram negative organisms with or without 1% Chinese Knotweed. B) Percent of biofilm reduction with 1% Chinese Knotweed treatment.

S. mutans   1.25% CK   2.5% CK
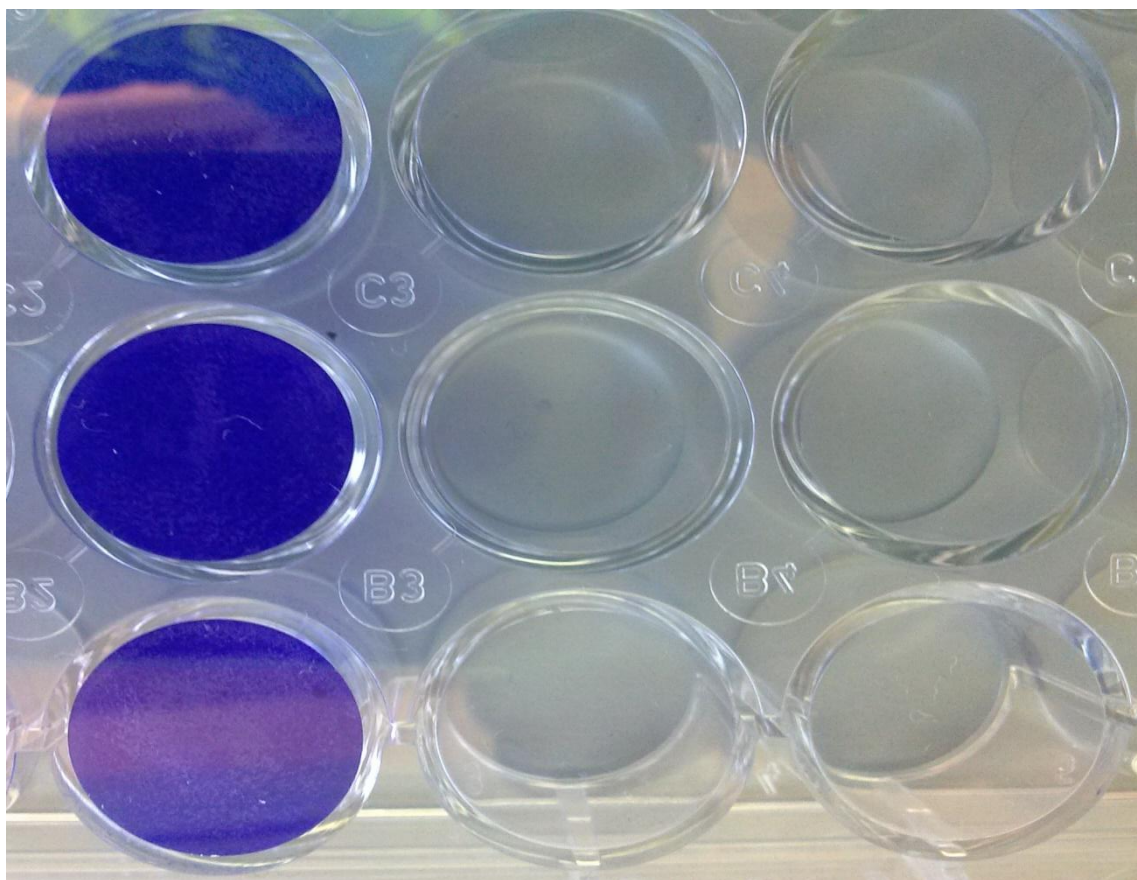
Figure 27. A sample crystal violet assay of *Streptococcus mutans* with or without Chinese Knotweed.

A

Percentage reduction of *S. mutans* biofilm

Sample	OD_{600nm}
Control	2.422
1% CK	0.023
0.5% CK	0.112
0.25% CK	0.576

B

i

ii

ii

iv
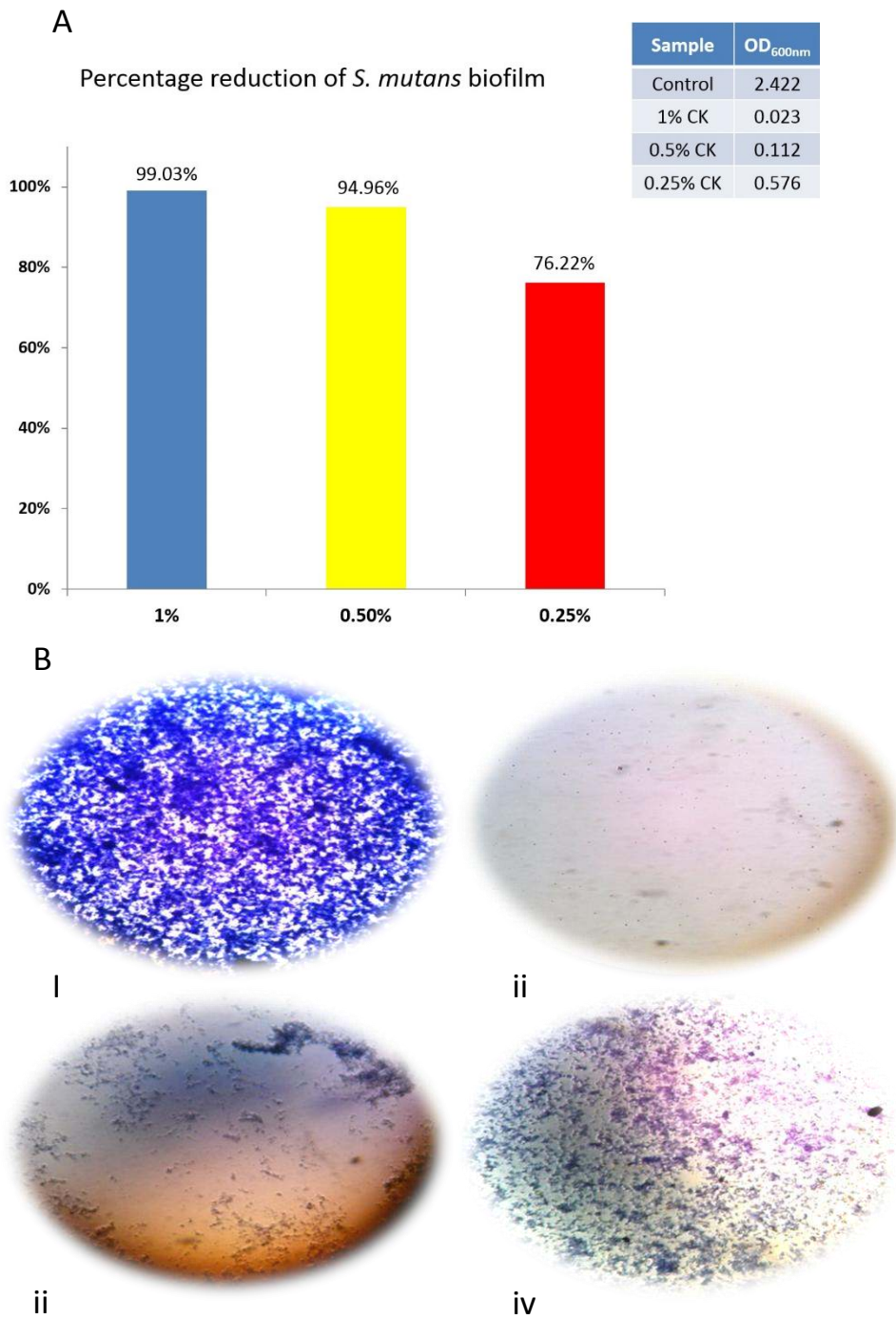
Figure 28. A) A summary of the percent reduction of biofilm inhibition based on the crystal violet assay to determine the MIC for biofilm formation with *S. mutans*. B) Microscopic observation of *S. mutans* under compound light microscope at 400x. i) Control; ii) 1% treatment; iii) 0.5% treatment; iv) 0.25% treatment.

Scanning Electron Microscope

Streptococcus mutans typically resides in the mouth and thus when biofilm form they effect the teeth and result in dental plaques. Figure 29 showed the SEM images of *S. mutans* incubated with or without 1% CK. Figure 29A illustrated *S. mutans* control cells after 6 hours incubation and Figures 29A' showed the cells incubated with 1% CK for the same amount of time. Only a few isolated cells were present in figure 28A' showing the effectiveness of 1% CK even it's only for 6 hours. Figures 29B and 29B' highlighted the substantial biofilm reduction for the treated cells after a 24-hour incubation period. When zooming in on Figure 28B', the cell surface looked impaired. All these results indicated that Chinese Knotweed may be a strong anti-biofilm agent.
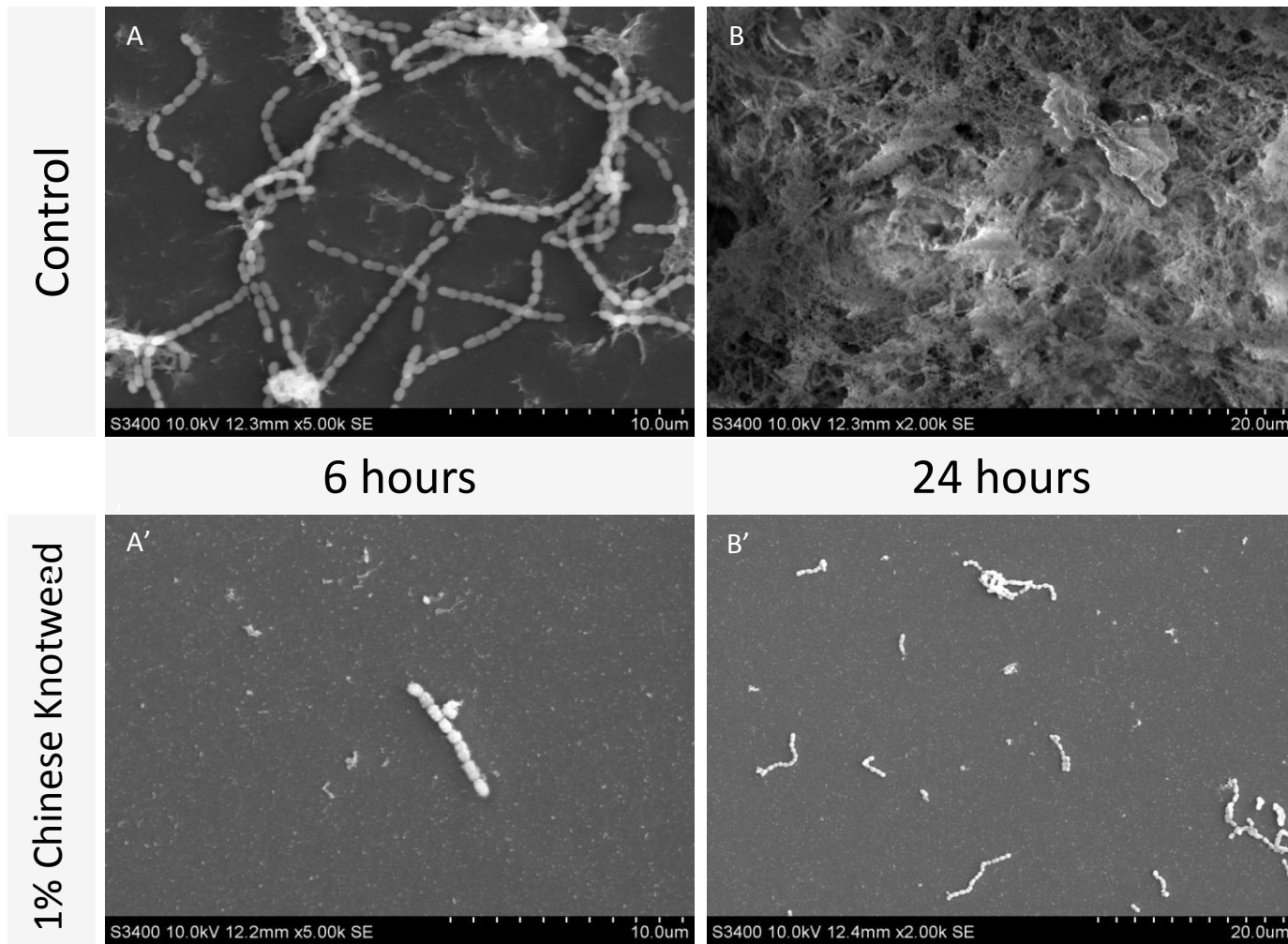
Figure 29. SEM images of *S. mutans* with or without Chinese Knotweed. A) *S. mutans* control with 6-hour incubation, 5,000x. A') *S. mutans* with 1% CK after 6-hour incubation (5,000x). B) *S. mutans* control after 24 hours incubation (2,000x). B') *S. mutans* with 1% CK after 24 hours (2,000x).

Sporulation and germination inhibition

        Sporulation is a specialized structure that is predominantly found in the Bacillus and Clostridium species. In this study, *Bacillus megaterium* was used to evaluate the sporulation and germination process. It can be seen in figure 30, there were many endospore in the control sample while almost no visible endospore observed in the 1% Chinese Knotweed sample.

        Germination inhibition with Chinese Knotweed was also examined. Figure 31A showed a *B. megaterium* control plate with a total cell population of 2.6×10^9 cells/mL while the total count from figure 31B was 9.6×10^7 cells/mL when the spores were incubated with 2% Chinese Knotweed. The results suggested that Chinese Knotweed hinder both sporulation and germination process in *B. megaterium*.
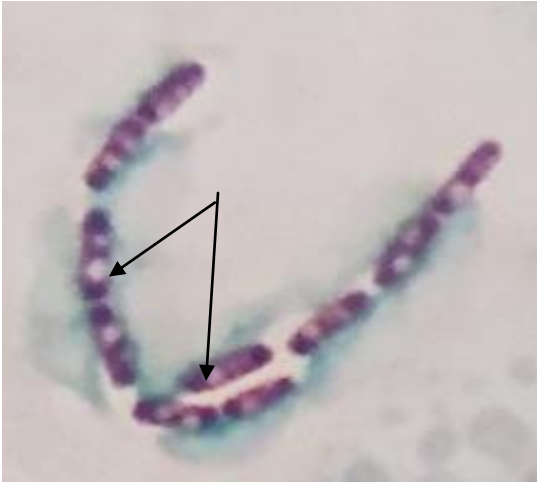
B. megaterium control   B. megaterium with 1% CK
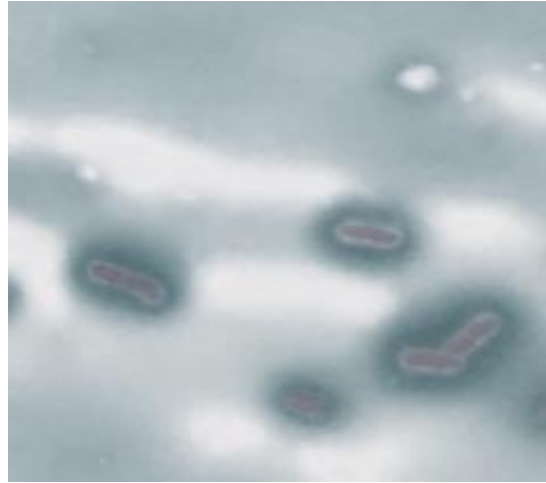
Figure 30. Schaeffer–Fulton stain (Spore stain) of *B. megaterium* with or without 1% Chinese Knotweed. Though many endospores were observed for the control, almost no spores were observed in the treated populations.
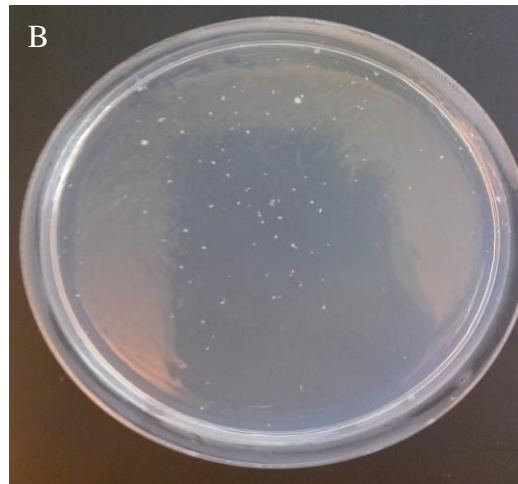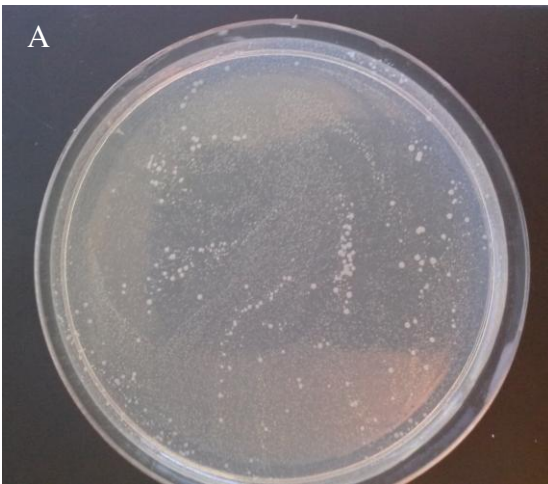
Figure 31. Viable plate count for *Bacillus megaterium*. A) Control with 10^{-8} dilution and B) 2% Chinese Knotweed treated *B. megaterium* with 10^{-6} dilution. The results indicated that the colony forming units (CFU) for the control plate was 2.6 x 10^9 cells/mL while the CFU for the treatment plate was 9.6 x 10^7 cell/mL, a ~ 100 fold reduction.

Conclusion and Discussion

In this study, evaluated the antibacterial effects of Chinese Knotweed and to observe the effectiveness of this compound against a wide array of unique bacteria as well as against special evasive structure bacteria possess. The results from the antimicrobial assay showed that at 2.5% (MIC) Chinese Knotweed effectively inhibited bacterial growth (Figure 3 - 11). Even with 1.5% Chinese Knotweed, most bacterial growth were inhibited for more than 50%. The antibacterial activity was significantly reduced when the compound concentration dropped down to 0.5%. For instance, 34% growth inhibition was shown for *Pseudomonas aeruginosa* under 0.5% Chinese Knotweed while no inhibition was observed for *Bacillus cereus*. It can also be speculated, that IC_{50} for most of the selected bacteria is in the range of 0.5-1 % of Chinese Knotweed.

As for the antibacterial synergism of Chinese Knotweed with a wide range of antibiotics, some promising results on *E. aerogenes* and *P. vulgaris* can potentially lead to alternative antibacterial agent development. It's exciting to learn that sulfa drugs showed exceptional inhibition on *B. cereus* and *S. pyogenes* when combined with Chinese Knotweed. Sulfa drugs have been one of the dominate classes of antibiotics throughout the years, but more and more antibiotic resistant bacteria have been identified. Novel antibiotics may be developed by using natural products.

One major clinical challenge is battling biofilm. Congo-red screening indicated seven out of nine bacteria tested can form biofilm. With 1% CK, biofilm formation was dramatically reduced for Gram negative bacteria. Though initial screening didn't show

inhibition on biofilm formation, crystal violet assay results were able to provide us the quantitative data on the percent reduction of biofilm for both Gram positive and Gram negative bacteria. *S. mutans*, one major causing agent for dental plaque, was significantly inhibited by Chinese Knotweed with 1.25%. SEM images also provided strong evidences of ~99% biofilm reduction. In addition to the encouraging results mentioned above, preliminary result obtained from sporulation and germination inhibition assays could potentially benefit the food industry as Chinese Knotweed may be able to serve as natural preservatives to extend the shelf life of food products.

Based on all the assays, tests and data collected, we can speculate the mode of action to target the outer membrane of bacteria. Based on the data from the SEM and antibiotic testing it shows that the bacteria outer membrane are damaged and this causes the bacteria to become more susceptible and unable to respond and react properly.

More bacteria should be included in the future studies in order to obtain a complete profile of antibacterial activity on most common pathogens. Different combination of various concentrations of antibiotics and Chinese Knotweed should be determined for the optimal antibacterial activity. Including samples from different time points would help understand the potential antibacterial mechanism of Chinese Knotweed.

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