AN INVESTIGATION INTO THE VIABILITY OF PHYTOALEXINS AS ALTERNATIVE ACNE TREATMENTS IN COMPARISON TO OVER-THE-COUNTER ACNE TREATMENTS

Do phytoalexins such as resveratrol, gossypol and xanthotoxin possess comparable antibacterial effects against *Bacillus subtilis* bacteria to over-the-counter acne treatments such as benzoyl peroxide and salicylic acid?

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1. INTRODUCTION

1.1. Research question

Do phytoalexins such as resveratrol, gossypol and xanthotoxin possess comparable antibacterial effects against *Bacillus subtilis* bacteria to over-the-counter acne treatments such as benzoyl peroxide and salicylic acid?

1.2. Context

Phytoalexins are "antibacterial compounds synthesized de novo by plants in response to pathogenic attack"[1]. With many of them demonstrating antioxidative activity[2], phytoalexins are found commercially as dietary supplements to improve health, particularly in the gut where phytoalexins' antioxidative activity may "promote probiotic growth and strengthen the gut's epithelial lining"[3]. These antioxidative actions relate to the bacterial species *Propionibacterium acnes (P. acnes)* found as part of the microflora in the gut[4] as well as on the skin[5], where antioxidants play a major role in the treatment of inflammatory conditions such as acne[6] by, for example, neutralizing free radicals.

The "relationship between the gut and the skin has been explored in the gut-skin axis theory"[7], hypothesizing how changes to the microflora in the gut may stimulate skin inflammations such as acne. With this intricate relationship in place, it's only fair to question whether phytoalexins, used to improve gut health, may also be used to treat skin inflammations such as acne. Such a relationship may strengthen the feasibility of the gut-skin axis theory and provide insight into the interconnectedness of the human body, as will be explored in this investigation.

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[1] Liu, Hung-Wen. Comprehensive Natural Products II. Elsevier Science, 2010.
[2] Malavolta, Marco, and Eugenio Mocchegiani, eds. *Molecular Basis of Nutrition and Aging*. Molecular Nutrition Series. Amsterdam ; Boston: Elsevier, AP, 2016.
[3] Westwood Wellness. 2018. *Do Antioxidants Improve Gut Health?* . November 1. Accessed August 4, 2019. https://westwoodwellness.com/antioxidants-improve-gut-health/.
[4] Dréno, B., S. Pécastaings, S. Corvec, S. Veraldi, A. Khammari, and C. Roques. "Cutibacterium Acnes (Propionibacterium Acnes) and Acne Vulgaris: A Brief Look at the Latest Updates." *Journal of the European Academy of Dermatology and Venereology* 32 (June 2018): 5–14. https://doi.org/10.1111/jdv.15043.
[5] "Propionibacterium Acnes." Micropia. Accessed August 4, 2019. https://www.micropia.nl/en/discover/microbiology/propionibacterium-acnes/.
[6] Addor, Flavia Alvim Sant'anna. "Antioxidants in Dermatology." *Anais Brasileiros De Dermatologia* 92, no. 3 (June 2017): 356–62. https://doi.org/10.1590/abd1806-4841.20175697.
[7] Bowe, Whitney P., and Alan C. Logan. "Acne Vulgaris, Probiotics and the Gut-Brain-Skin Axis - Back to the Future?" *Gut Pathogens* 3, no. 1 (January 31, 2011): 1. https://doi.org/10.1186/1757-4749-3-1.

2. BACKGROUND

2.1. The epidemiology and pathogenesis of acne vulgaris

Acne vulgaris, known simply as acne, is a "chronic skin disease characterized by the blockage and/or inflammation of hair follicles and their accompanying sebaceous glands"[8]. It's estimated that acne affects "50 million people in the United States alone"[9], approximately 85% of which are between the ages of 12 and 25[10]. This age group is particularly affected due to the factors which are thought to trigger acne such as hormonal changes (e.g. in puberty), dietary factors, and genetic history[11].

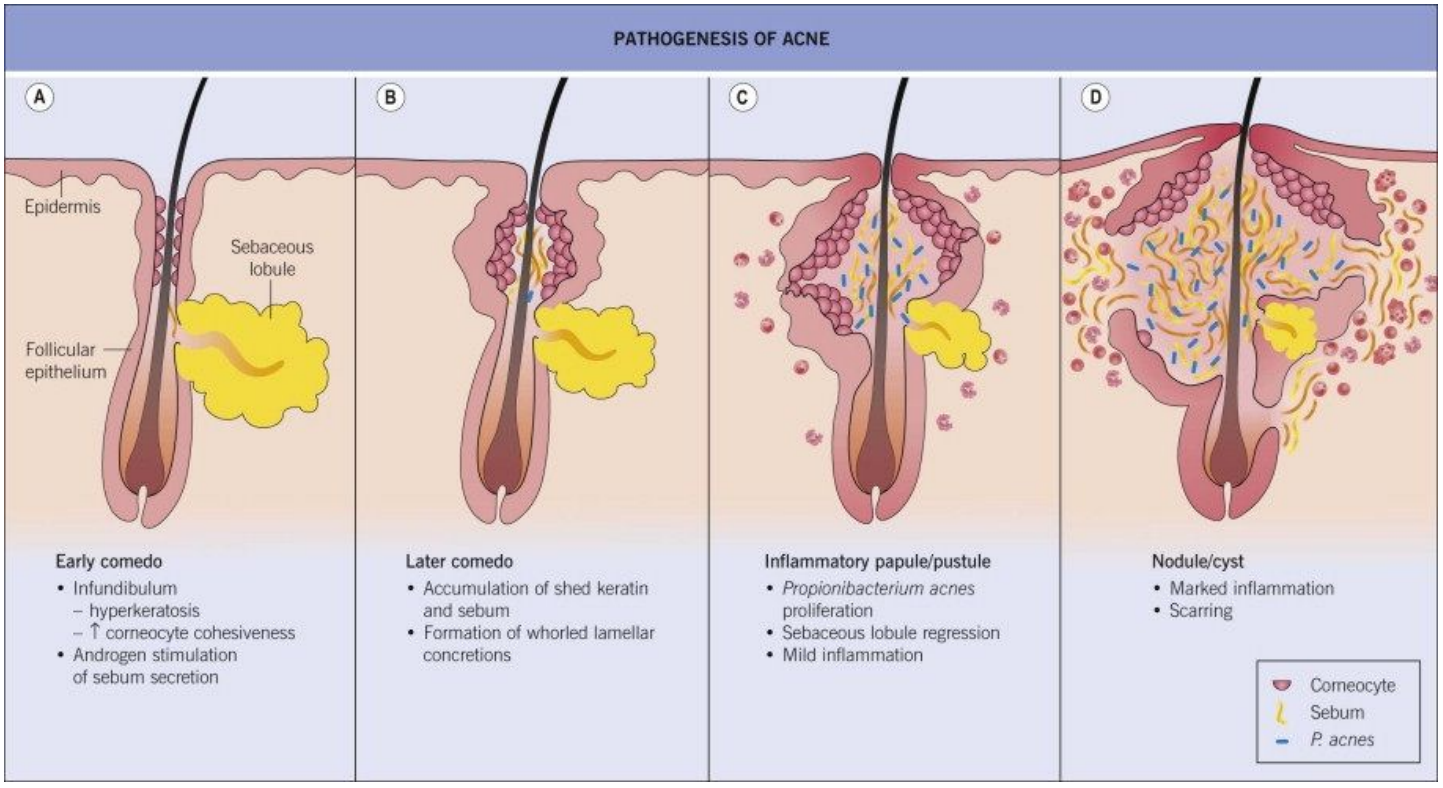
Although the cause of acne is not completely understood, its pathogenesis is heavily studied. In acne, excess sebum production in the sebaceous glands is stimulated by, for example, increased production of local androgens[12]. This is thought to induce follicular hyperkeratinization[13], a disorder preventing the normal shedding of keratinocytes (cells lining the inside of hair follicles)[14] which causes cellular debris to accumulate in follicles and result in follicular obstruction[15]. This causes the formation of a comedo, as seen on panel B in Figure 1. The obstructed follicle is an ideal environment for *P. acnes* bacteria to proliferate as they feed on the excess sebaceous matter[16]. Despite taking part in the maintenance of skin health, *P. acnes* is also an opportunistic pathogen[17] which, in large numbers, secretes chemotactic factors that bring about an immune response[18]. This leads to inflammation in the

[8] Rao, Jaggi. 2019. *Acne Vulgaris* . March 26. Accessed August 4, 2019. https://emedicine.medscape.com/article/1069804-overview.
[9] American Academy of Dermatology. 2018. *Skin conditions by the numbers* . Accessed August 4, 2019. https://www.aad.org/media/stats/conditions/skin-conditions-by-the-numbers.
[10] Ibid.
[11] Mayo Clinic. n.d. *Acne.* Accessed August 4, 2019. https://www.mayoclinic.org/diseases-conditions/acne/symptoms-causes/syc-20368047.
[12] "Androgen deficiency in women." 2018. Accessed August 4, 2019. https://www.betterhealth.vic.gov.au/health/conditionsandtreatments/androgen-deficiency-in-women.
[13] Joyce, A. COMPOSITIONS FOR TREATMENT AND PREVENTION OF ACNE, METHODS OF MAKING THE COMPOSITIONS, AND METHODS OF USE THEREOF. WO 2010/087964 A2, n.d.
[14] Lall, Namrita, ed. *Medicinal Plants for Holistic Health and Well-Being*. London, United Kingdom: Academic Press, an imprint of Elsevier, 2018.
[15] Ibid.
[16] "Propionibacterium Acnes." Micropia. Accessed August 4, 2019. https://www.micropia.nl/en/discover/microbiology/propionibacterium-acnes/.
[17] Dréno, B., S. Pécastaings, S. Corvec, S. Veraldi, A. Khammari, and C. Roques. "Cutibacterium Acnes (Propionibacterium Acnes) and Acne Vulgaris: A Brief Look at the Latest Updates." *Journal of the European Academy of Dermatology and Venereology* 32 (June 2018): 5–14. https://doi.org/10.1111/jdv.15043.
[18] Dréno, B., S. Pécastaings, S. Corvec, S. Veraldi, A. Khammari, and C. Roques. "Cutibacterium Acnes (Propionibacterium Acnes) and Acne Vulgaris: A Brief Look at the Latest Updates." *Journal of the European Academy of Dermatology and Venereology* 32 (June 2018): 5–14. https://doi.org/10.1111/jdv.1504

obstructed follicles and causes the formation of papules and pustules, as seen in image C in Figure 1, or inflammatory cysts and nodules[19], as seen in image D in Figure 1.

Ultimately, antibacterial treatments are able to inhibit the growth of and kill *P. acnes* bacteria, effectively clearing the obstructed follicles and reducing inflammation[20], and are thus essential in the treatment of acne.

Figure 1: *an illustration of the pathogenesis of acne vulgaris*[21]



[19] Bajaj, Lalit, and Stephen Berman. *Berman's Pediatric Decision Making Rev. Ed. of: Pediatric Decision Making*. Philadelphia, PA: Elsevier/Mosby, 2011.
[20] Healthline. n.d. *How to Treat Acne with Benzoyl Peroxide* . Accessed September 10, 2019. https://www.healthline.com/health/benzoyl-peroxide-for-acne#benefits.
[21] "Pathogenesis of acne." 2016. Accessed August 5, 2019. https://plasticsurgerykey.com/acne-vulgaris/.

2.2. Bacillus subtilis as a prokaryotic model organism

"Given its opportunistic nature and harmful host-dependent side-effects"[22], *P. acnes* can't be used in this investigation as it's considered a biohazard. As a result, I needed to choose which suitable model organism to use in this investigation.

After necessary research, the chosen model organism was *Bacillus subtilis (B. subtilis),* a "non-pathogenic bacterium frequently considered a gram-positive equivalent to the commonly used *Escherichia coli* bacterium"[23]. To justify this choice, a comparison chart between *P. acnes* and *B. subtilis* was created, as illustrated in Table 1.

Table 1: *a comparison table of Propionibacterium acnes and Bacillus subtilis*

Characteristic	Propionibacterium acnes	Bacillus subtilis
Gram stain	Gram-positive[24]	Gram-positive[25]
Genus	Propionibacterium	Bacillus
Oxygen preference	Facultative anaerobe[26]	Facultative anaerobe[27]
Pathogenicity	Most strains are pathogenic	Most strains are not pathogenic[28]
Found in…	the skin, in hair follicles[29] and throughout the gastrointestinal tract of humans[30]	"soil and throughout the gastrointestinal tract of humans"[31]

P. acnes and *B. subtilis* are both gram-positive, a classification given due to the thick peptidoglycan layer which both bacterial species possess. This similarity is crucial when considering

[22] Dréno, B., S. Pécastaings, S. Corvec, S. Veraldi, A. Khammari, and C. Roques. "Cutibacterium Acnes (Propionibacterium Acnes) and Acne Vulgaris: A Brief Look at the Latest Updates." *Journal of the European Academy of Dermatology and Venereology* 32 (June 2018): 5–14. https://doi.org/10.1111/jdv.15043.
[23] "Bacillus subtilis." 2015. June 7. Accessed August 4, 2019. https://microbewiki.kenyon.edu/index.php/Bacillus_subtilis.
[24] Achermann, Y., E. J. C. Goldstein, T. Coenye, and M. E. Shirtliff. "Propionibacterium Acnes: From Commensal to Opportunistic Biofilm-Associated Implant Pathogen." *Clinical Microbiology Reviews* 27, no. 3 (July 1, 2014): 419–40. https://doi.org/10.1128/CMR.00092-13.
[25] Nakano, Michiko M., and Peter Zuber. "ANAEROBIC GROWTH OF A 'STRICT AEROBE' (BACILLUS SUBTILIS)." *Annual Review of Microbiology* 52, no. 1 (October 1998): 165–90. https://doi.org/10.1146/annurev.micro.52.1.165.
[26] Ibid.
[27] Ibid.
[28] Harwood, C. R. "Bacillus Subtilis and Its Relatives: Molecular Biological and Industrial Workhorses." *Trends in Biotechnology* 10, no. 7 (July 1992): 247–56.
[29] "Propionibacterium Acnes." Micropia. Accessed August 4, 2019. https://www.micropia.nl/en/discover/microbiology/propionibacterium-acnes/.
[30] Dréno, B., S. Pécastaings, S. Corvec, S. Veraldi, A. Khammari, and C. Roques. "Cutibacterium Acnes (Propionibacterium Acnes) and Acne Vulgaris: A Brief Look at the Latest Updates." *Journal of the European Academy of Dermatology and Venereology* 32 (June 2018): 5–14. https://doi.org/10.1111/jdv.15043.
[31] Ibid.

B. subtilis as a model organism, given that the cell wall structure of bacteria is the target of many antibacterial agents.[32]

Antibacterial agents also target bacterial enzymes to disrupt cell metabolism[33], making it important for a model organism for *P. acnes* to possess similar metabolic enzymes to it. The fact that both *B. subtilis* and *P. acnes* are facultative anaerobes means they possess similar respiratory enzymes, further suggesting that the two also possess similar metabolic enzymes.

Conclusively, due to the similarities between *P. acnes* and *B. subtilis*, *B. subtilis* was chosen as a suitable model organism for this investigation.

2.3. Phytoalexins

Phytoalexins are antibacterial defence compounds which have different mechanisms of action[34]. These mechanisms can be classified as being bacteriostatic or bactericidal; bacteriostatic phytoalexins inhibit bacterial growth and reproduction whereas bactericidal phytoalexins kill bacteria[35]. All the phytoalexins used in this investigation are insoluble in water but highly soluble in organic solvents such as ethanol[36,37,38].

2.3.1. Resveratrol

Resveratrol is a phytoalexin produced by knotweeds (*Polygonum*)[39] which demonstrates antibacterial, antioxidant[40], and anti-inflammatory qualities[41]. Studies suggest that resveratrol "affects bacterial

[32] "Bacillus subtilis." June 7, 2015. Accessed August 4, 2019. https://microbewiki.kenyon.edu/index.php/Bacillus_subtilis.
[33] Silver, Lynn L. "Appropriate Targets for Antibacterial Drugs." *Cold Spring Harbor Perspectives in Medicine* 6, no. 12 (December 2016): a030239. https://doi.org/10.1101/cshperspect.a030239.
[34] Khachatourians, George G., and Dilip K. Arora, eds. *Applied Mycology and Biotechnology*. 1st ed. Amsterdam ; New York: Elsevier, 2001.
[35] lumen. n.d. *Overview of Antimicrobial Therapy* . Accessed August 4, 2019. https://courses.lumenlearning.com/boundless-microbiology/chapter/overview-of-antimicrobial-therapy/.
[36] PubChem. n.d. *Resveratrol.* Accessed August 4, 2019. https://pubchem.ncbi.nlm.nih.gov/compound/Resveratrol#section=Computed-Properties.
[37] "Xanthotoxin." TGSC Information System. Accessed August 4, 2019. http://www.thegoodscentscompany.com/data/rw1037841.html.
[38] Enzo Life Sciences. n.d. *Gossypol.* Accessed August 4, 2019. http://www.enzolifesciences.com/BML-EI130/gossypol/.
[39] Oregon State University. n.d. *Resveratrol.* Accessed August 4, 2019. https://lpi.oregonstate.edu/mic/dietary-factors/phytochemicals/resveratrol.
[40] Gupta, Ramesh C., ed. *Nutraceuticals: Efficacy, Safety, and Toxicity*. Amsterdam: Elsevier, Academic Press, 2016.

binary fission, inhibits ATP synthesis and hydrolysis in facultative anaerobes"[42], and alters plasma membrane permeability[43], all of which contribute to its antibacterial activity. At different concentrations, resveratrol demonstrates the ability to be either a bacteriostatic or bactericidal agent[44].

2.3.2. Xanthotoxin

Xanthotoxin is a phytoalexin produced by parsnip plants (*Pastinaca sativa*)[45]. A study by Gnonlonfin *et al.* (2012) observed that xanthotoxin forms covalent cross-links with DNA in bacterial cells[46], preventing DNA replication and transcription and thus inhibiting bacterial growth and reproduction. However, bacterial species such as *E. coli* have demonstrated an ability to repair these crosslinks after some time; making xanthotoxin's mechanism of action purely bacteriostatic[47].

2.3.3. Gossypol

Gossypol is a phytoalexin produced by cotton planta (*Gossypium*)[48] which demonstrates antioxidant, antifungal and antibacterial qualities[49]. Although the mechanism(s) of gossypol's toxicity remain

[41] Donnelly, Louise E., Robert Newton, Gina E. Kennedy, Peter S. Fenwick, Rachel H. F. Leung, Kazuhiro Ito, Richard E. K. Russell, and Peter J. Barnes. "Anti-Inflammatory Effects of Resveratrol in Lung Epithelial Cells: Molecular Mechanisms." American Journal of Physiology. Lung Cellular and Molecular Physiology 287, no. 4 (October 2004): L774-783. https://doi.org/10.1152/ajplung.00110.2004.

[42] Vestergaard, Martin, and Hanne Ingmer. "Antibacterial and Antifungal Properties of Resveratrol." *International Journal of Antimicrobial Agents* 53, no. 6 (June 2019): 716–23. https://doi.org/10.1016/j.ijantimicag.2019.02.015.

[43] Milardi, G. L., A. Stringaro, M. Colone, A. Bonincontro, and G. Risuleo. "The Cell Membrane Is the Main Target of Resveratrol as Shown by Interdisciplinary Biomolecular/Cellular and Biophysical Approaches." *The Journal of Membrane Biology* 247, no. 1 (January 2014): 1–8. https://doi.org/10.1007/s00232-013-9604-1.

[44] Docherty, John J., Heather A. McEwen, Thomas J. Sweet, Erin Bailey, and Tristan D. Booth. "Resveratrol Inhibition of Propionibacterium Acnes." *Journal of Antimicrobial Chemotherapy* 59, no. 6 (June 1, 2007): 1182–84. https://doi.org/10.1093/jac/dkm099.

[45] Al-Barwani, Fatma M., and Elsadig A. Eltayeb. "Xanthotoxin and Other Furanocoumarins as Phytoalexins in Pastinaca Sativa L. Roots." *Sultan Qaboos University Journal for Science [SQUJS]* 9 (June 1, 2004): 7. https://doi.org/10.24200/squjs.vol9iss0pp7-17.

[46] PubChem. n.d. *Methoxsalen.* Accessed August 4, 2019. https://pubchem.ncbi.nlm.nih.gov/compound/methoxsalen

[47] Cole, R. S., and R. R. Sinden. "Repair of Cross-Linked DNA in Escherichia Coli." *Basic Life Sciences* 5B (1975): 487–95.

[48] Dalefield, Rosalind. *Veterinary Toxicology for Australia and New Zealand*. Amsterdam, Netherlands ; Kidlington, Oxford ; Cambridge, MA: Elsevier, 2017.

[49] Enzo Life Sciences. n.d. *Gossypol.* Accessed August 4, 2019. http://www.enzolifesciences.com/BML-EI130/gossypol/.

unclear[50], studies have suggested that gossypol targets respiratory enzymes in prokaryotes, thus disrupting bacterial metabolism and acting as a bactericidal agent[51].

2.4. OTC acne treatments

Similarly to phytoalexins, the mechanism of over-the-counter (OTC) acne treatments can be classified as either bacteriostatic or bactericidal.

2.4.1. Benzoyl peroxide

The topical application of benzoyl peroxide "causes it to decompose into reactive oxygen radicals"[52], acting as a bactericidal agent against *P. acnes* by a process called lipid peroxidation[53]. In this process, the oxygen radicals damage the plasma membrane of bacteria by reacting with intermembrane fatty acids[54], altering membrane fluidity and thus causing cell death by the release of "intracellular metabolites"[55]. *P. acnes* has not developed resistance to this process, thus making treatment using benzoyl peroxide favourable to maintain bacterial sensitivity. Despite this, topical treatment with benzoyl peroxide often leads to negative side-effects such as redness, itching and skin dryness[56].

2.4.2. Salicylic acid

Salicylic acid is a lipid-soluble acid[57] that can penetrate sebaceous follicles in the skin[58]. This causes keratinocytes to shed more readily, thus unblocking obstructed follicles[59], while also lowering the skin's pH, which denatures prokaryotic proteins and thus inhibits bacterial growth and reproduction. It

[50] Dalefield, Rosalind. *Veterinary Toxicology for Australia and New Zealand*. Amsterdam, Netherlands ; Kidlington, Oxford ; Cambridge, MA: Elsevier, 2017.
[51] Margalith, P. "Inhibitory Effect of Gossypol on Microorganisms." *Applied Microbiology* 15, no. 4 (July 1967): 952–53.
[52] Drugbank. n.d. *Benzoyl Peroxide.* Accessed August 4, 2019. https://www.drugbank.ca/drugs/DB09096.
[53] Maddison, Jill E., Stephen W. Page, and David Church, eds. *Small Animal Clinical Pharmacology*. 2nd ed. Edinburgh ; New York: Saunders/Elsevier, 2008.
[54] Ibid.
[55] Roy, Hervé, Kiley Dare, and Michael Ibba. "Adaptation of the Bacterial Membrane to Changing Environments Using Aminoacylated Phospholipids." *Molecular Microbiology* 71, no. 3 (February 2009): 547–50. https://doi.org/10.1111/j.1365-2958.2008.06563.x.
[56] RxList. 2019. *BENZAGEL* . July 16. Accessed September 8, 2019. https://www.rxlist.com/benzagel-drug/patient-images-side-effects.htm#info.
[57] Jaques, Renee, and Rebecca Dancer. 2019. *Here's Exactly What Salicylic Acid Does to Your Skin.* June 7. Accessed August 4, 2019. https://www.allure.com/story/what-does-salicylic-acid-do.
[58] Moghimipour, Eskandar. "Hydroxy Acids, the Most Widely Used Anti-Aging Agents." *Jundishapur Journal of Natural Pharmaceutical Products* 7, no. 1 (2012): 9–10.
[59] Rakel, David, ed. *Integrative Medicine*. Fourth edition. Philadelphia, PA: Elsevier, 2018.

is, therefore, a bacteriostatic agent. Topical salicylic acid use also has negative side-effects such as skin irritation, rashes and peeling[60].

2.5. Relevance of investigation

We are entering an age of antibiotic resistance, with an increasing frequency of antibiotic-resistant infections demonstrating the need for alternative treatments to common diseases. This is relevant to acne treatment as "more than 50% of *P. acnes* strains have become resistant to common topical acne antibiotics"[61].

Benzoyl peroxide and salicylic acid are both routinely prescribed together with acne-treating antibiotics such as erythromycin in order to prevent the development of antibiotic resistance[62]. However, given their frequent overuse, the negative side-effects of benzoyl peroxide and salicylic acid become increasingly apparent, rendering treatment with these substances far from ideal.

Phytoalexins may be a solution to this impending development of antibiotic resistance in acne treatment as they might be able to deliver the antibacterial properties necessary for acne treatment while avoiding antibiotic resistance. Although the scope of this investigation is too limited to reach a conclusive answer to this, the comparison of certain phytoalexins with OTC acne treatments will give specific insight into the antibacterial activity of phytoalexins, and thus their general viability as alternative acne treatments.

2.6. Preliminary testing

Preliminary testing revealed the limited availability of the chosen phytoalexins, with only milligrams of each available for use. After thorough calculations, this guided me to choose the appropriate concentrations of the phytoalexin solutions, 2% and 4%, which I would be able to reconstitute with this limited quantity.

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[60] EverydayHealth. 2018. *What Is Salicylic Acid Topical?* . September 11. Accessed September 10, 2019. https://www.everydayhealth.com/drugs/salicylic-acid-topical.

[61] Walsh, Timothy R, John Efthimiou, and Brigitte Dréno. "Systematic Review of Antibiotic Resistance in Acne: An Increasing Topical and Oral Threat." *The Lancet Infectious Diseases* 16, no. 3 (March 2016): e23–33. https://doi.org/10.1016/S1473-3099(15)00527-7.

[62] Taylor, Gina A., and Alan R. Shalita. "Benzoyl Peroxide-Based Combination Therapies for Acne Vulgaris: A Comparative Review." *American Journal of Clinical Dermatology* 5, no. 4 (2004): 261–65. https://doi.org/10.2165/00128071-200405040-00005.

Furthermore, by placing only one filter paper disk per Petri dish, the preliminary testing shed light on how many divisions each solution would require per Petri dish. Solutions which formed smaller zones of inhibition (ZoI) (e.g. 2% resveratrol solution) could be tested with 6 division per Petri dishes whereas solutions which formed larger ZoI (e.g. 4% benzoyl peroxide solution) required 3 division per Petri dish in order to prevent overlapping ZoI.

2.7. Hypotheses

Hypothesis 1:

Given their accepted use as acne treatments, the OTC acne treatments will demonstrate a greater inhibitory effect than the phytoalexins after 24 hours of incubation.

Hypothesis 2:

Higher concentrations of phytoalexins will demonstrate a greater inhibitory effect than lower concentrations.

Hypothesis 3:

Given their mechanisms of action, bacteriostatic agents will demonstrate decreased antibacterial activity over the 48-hour incubation whereas bactericidal agents will maintain the same antibacterial activity over the 48-hour incubation period.

3. METHODOLOGY

3.1. Rationale for chosen practical experiment

The Kirby-Bauer test[63] aims to "determine the sensitivity or resistance of facultative anaerobic bacteria to various antimicrobial compounds"[64], which aligns with the aim of this investigation. The test provides a commensurate measure of the antibacterial activity of chosen substances, in contrast to

[63] Antimicrobial Susceptibility Testing EUCAST Disk Diffusion Method. EUCAST, January 2019.
[64] Jan Hudzicki. "Kirby-Bauer Disk Diffusion Susceptibility Test Protocol." American Society for Microbiology, December 8, 2009.

other procedures, such as the broth dilution assay, where the antibacterial effects of certain substances is "qualitatively analyzed by changes in turbidity of a solution"[65].

A suitable practical experiment for this investigation needs to be feasible with small quantities of antibacterial substances given the minimal quantity of phytoalexins available. While the Kirby-Bauer test requires small volumes of solution to impregnate filter paper disks, other procedures, such as the broth dilution assay, require the use of increasingly more concentrated solutions which would require large quantities of phytoalexins.

3.2. Variables

3.2.1. Independent variable

This investigation utilizes two independent variables; substance type and concentration. The substances and concentrations tested in this investigation are 2% and 4% resveratrol, 2% and 4% xanthotoxin, 2% and 4% gossypol, 4% salicylic acid and 4% benzoyl peroxide. Given that these solutions were created using ethanol as a solvent, ethanol was used as a control in the experiment.

3.2.2. Dependent variable

The dependent variable in this experiment is the diameter of the zone of inhibition in millimetres (mm). This will be measured using a Vernier calliper (±0.05 mm).

[65] Dwivedi, Charu, Ishan Pandey, Himanshu Pandey, Pramod W. Ramteke, Avinash C. Pandey, Shanti Bhushan Mishra, and Sandip Patil. "Electrospun Nanofibrous Scaffold as a Potential Carrier of Antimicrobial Therapeutics for Diabetic Wound Healing and Tissue Regeneration." In *Nano- and Microscale Drug Delivery Systems*, 147–64. Elsevier, 2017. https://doi.org/10.1016/B978-0-323-52727-9.00009-1.

3.2.3. Controlled variables

Table 2: *table of controlled variables, their effect on the experiment's result and their method for control*

Controlled variable	Effect on the result	Method for control
Time of incubation	The doubling time of *B. subtilis* (the time for the bacterial population to double under ideal growing conditions) is 30 minutes[66]. Given this, the size of the *B. subtilis* population will increase exponentially as it's incubated for longer, forming a thicker bacterial lawn which would not allow for a fair comparison of the phytoalexins and OTC acne treatments in this investigation.	All Petri dishes were incubated for 48 hours in total. The ZoI was measured at two separate time periods; after 24 and 48 hours of incubation.
Volume of nutrient agar poured into each Petri dish	The thickness of the agar layer can affect both the rates of diffusion of the antibacterial solutions[67] as well as the rate of growth of the *B. subtilis* bacteria (given that thinner agar layers will have less nutrients for the bacteria to grow), both of which may affect the sizes of the ZoI formed by the phytoalexins and OTC acne treatments.	Identical 100mm x 15mm sterilized polystyrene Petri dishes were used throughout the experiment. The same volume of nutrient agar solution (25 cm^3) will be poured into each Petri dish using a 25 cm^3 volumetric pipette, thus maintaining a uniform thickness of nutrient agar across all Petri dishes.
Strain of *Bacillus subtilis* used	Different strains of *B. subtilis* may be more or less resistant to certain substances. Therefore, all substances should be tested on the same strain of *B. subtilis*.	The same strain of *B. subtilis*, QB928, was used to inoculate all Petri dishes in the experiment.
Temperature of incubation	The optimum growth temperature of a bacterial species depends on its enzymes[68]. "At temperatures that are too low, enzyme activity diminishes, while temperatures that are too high may denature enzymes and thus also diminish enzyme activity"[69]. Bacteria incubated at different temperatures will therefore grow at different rates due to their different rates of metabolic activity, thus not allowing for a fair comparison in the investigation.	*B. subtilis* is a mesophilic organism and therefore grows optimally in a temperature range of 20 to 45 degrees Celsius[70]. Following International Baccalaureate guidelines, the incubation temperature of the Petri dishes was kept at 27 degrees Celsius.
Diameter of filter paper disk	Larger filter paper disks will absorb larger volumes of antibacterial solution, thus producing larger ZoI. Given this, if the effects of substance type and concentration are to be investigated, the diameter of the filter paper disks should be kept constant.	Filter paper disks which were 6 mm in diameter were used throughout the experiment. These were created using a 6 mm hole puncher.
Sterilization techniques	Contamination of all apparatus used must be minimized as much as possible throughout the investigation. If contamination occurs, the results of the experiment may be jeopardized as other contaminant bacteria and fungi are introduced into the sterile environment of the Petri dishes.	All apparatus used, including the filter paper disks, were sterilized regularly and appropriately by following sterilization guidelines (Appendix A)

[66] iGem. 2012. *Bacillus subtilis.* Munich.
[67] Jan Hudzicki. "Kirby-Bauer Disk Diffusion Susceptibility Test Protocol." American Society for Microbiology, December 8, 2009.
[68] "PHYSICAL FACTORS THAT AFFECT MICROBIAL GROWTH." Hawaii. Accessed August 5, 2019. https://www2.hawaii.edu/~johnb/micro/m140/syllabus/week/handouts/m140.9.1.html.
[69] Worthington. 2019. *Introduction to Enzymes* . Accessed August 4, 2019. http://www.worthington-biochem.com/introbiochem/tempEffects.html.
[70]"PHYSICAL FACTORS THAT AFFECT MICROBIAL GROWTH." Hawaii. Accessed August 5, 2019. https://www2.hawaii.edu/~johnb/micro/m140/syllabus/week/handouts/m140.9.1.html.

3.3. Materials

Table 3: *the quantities of specific measurement equipment, general apparatus and chemicals required for the experiment*

Measurement equipment	General apparatus	Chemicals
(1) Vernier calliper ±0.05 mm	(1) *B. subtilis* culture	40 mg of resveratrol powder
(6) 0.5 cm^3 graduated pipettes ± 0.005cm^3	(31) 100mm x 15mm sterilized	24 mg of xanthotoxin powder
(1) electronic weighing scale ±0.0001g	polystyrene Petri dishes	32 mg of gossypol powder
(1) electronic weighing scale ±0.01g	(112) 6mm filter paper disks	1 cm^3 of benzoyl peroxide 4% solution
(1) 1000 cm^3 graduated cylinder ± 5cm^3	(9) 20 cm^3 plastic containers with lids	1 cm^3 of salicylic acid 4% solution
(1) 25cm^3 volumetric pipette ±0.03 cm^3	(1) McCartney bottle	500 cm^3 of 97% ethanol
	(1) Bunsen burner	17.8g of nutrient agar powder[71]
	(1) cell spreader	700 cm^3 of distilled water
	(1) inoculation loop	
	(1) forceps	
	(1) micro spatula	
	(1) pressure cooker	
	(1) 27ºC incubator	
	(1) magnetic stirrer	
	(1) 1000 cm^3 conical flask	
	(1) plastic weighing boat	
	(1) pair of heat resistant gloves	
	(1) permanent marker	

3.4. Aseptic technique guidelines

A key element of this investigation is adherence to guidelines regarding aseptic practices. The sterilization guidelines proposed by the University of Utah's Genetic Science Learning Center[72,73,74] (Appendix A) were followed in this investigation due to the University of Utah's credibility and expertise in this field.

[71] Sourced from: PASCO. n.d. *Agar Nutrient.* Accessed August 4, 2019. https://education.scichem.com/Catalogue/ProductDetail/agar-nutrient?productID=9a2abf64-dc3c-48bb-afc6-50f43e65432f&catalogueLevelItemID=00000000-0000-0000-0000-000000000000.
[72] "Sterile Technique." 2015. Accessed August 4, 2019. https://teach.genetics.utah.edu/content/microbiology/steriletechnique/.
[73] "Sterilizing Liquids." 2015. Accessed August 4, 2019. https://teach.genetics.utah.edu/content/microbiology/liquids/.
[74] "Sterilizing Solid Objects." 2015. Accessed August 4, 2019. https://teach.genetics.utah.edu/content/microbiology/solids/.

3.5. Procedure

3.5.1. Part A: Experimental preparations

The first preparatory step is the producing and pouring of the nutrient agar solution into the Petri dishes, the procedure of which is an adaption of the University of Utah's[75] method (as seen in Appendix B) utilizing the recipe of the nutrient agar powder sourced from SciChem[76]. As outlined in Table 2, a total of 31 Petri dishes are required. It is proposed that 25 cm^3 of nutrient agar solution[77] should be poured into each Petri dish and thus a total of 775 cm^3 of nutrient agar solution needs to be produced.

The second preparatory step is creating a stock plate of the *B. subtilis* bacteria. This was done in a sterilized workspace while working around a Bunsen burner. The Bunsen burner's flame was used to sterilize equipment as well as to "create an updraft in the local area, thus preventing airborne contamination"[78]. The stock plate of *B.subtilis* bacteria was made following the Kirby Bauer method[79] (Appendix C), labelled as a stock plate, sealed with Sellotape and incubated upside-down at 27ºC overnight. In this stock plate, no antibacterial filter disks need to be applied.

3.5.2. Part B: Plating bacteria and filter paper disks

On the following day, the phytoalexin solutions which would impregnate the filter paper disks were created. The OTC treatments were found as a commercial solution with 4% concentration and thus required no further adjustments while the phytoalexins were in powder form and could be diluted into solutions of 2% and 4% concentrations. Given that all three phytoalexins used were insoluble in water they were reconstituted in an organic solvent; ethanol. The phytoalexin solutions were produced according to the scheme outlined in Table 4:

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[75] "Making Agar Plates." 2015. Accessed August 4, 2019. https://teach.genetics.utah.edu/content/microbiology/plates/.

[76] PASCO. n.d. *Agar Nutrient.* Accessed August 4, 2019. https://education.scichem.com/Catalogue/ProductDetail/agar-nutrient?productID=9a2abf64-dc3c-48bb-afc6-50f43e65432f&catalogueLevelItemID=00000000-0000-0000-0000-000000000000.

[77] "Making Agar Plates." 2015. Accessed August 4, 2019. https://teach.genetics.utah.edu/content/microbiology/plates/.

[78] "How Good Is Your Sterile Technique?." 2017. Accessed August 4, 2019. https://bitesizebio.com/6630/how-good-is-your-sterile-technique/.

[79] Jan Hudzicki. "Kirby-Bauer Disk Diffusion Susceptibility Test Protocol." American Society for Microbiology, December 8, 2009.

Table 4: *solution scheme*

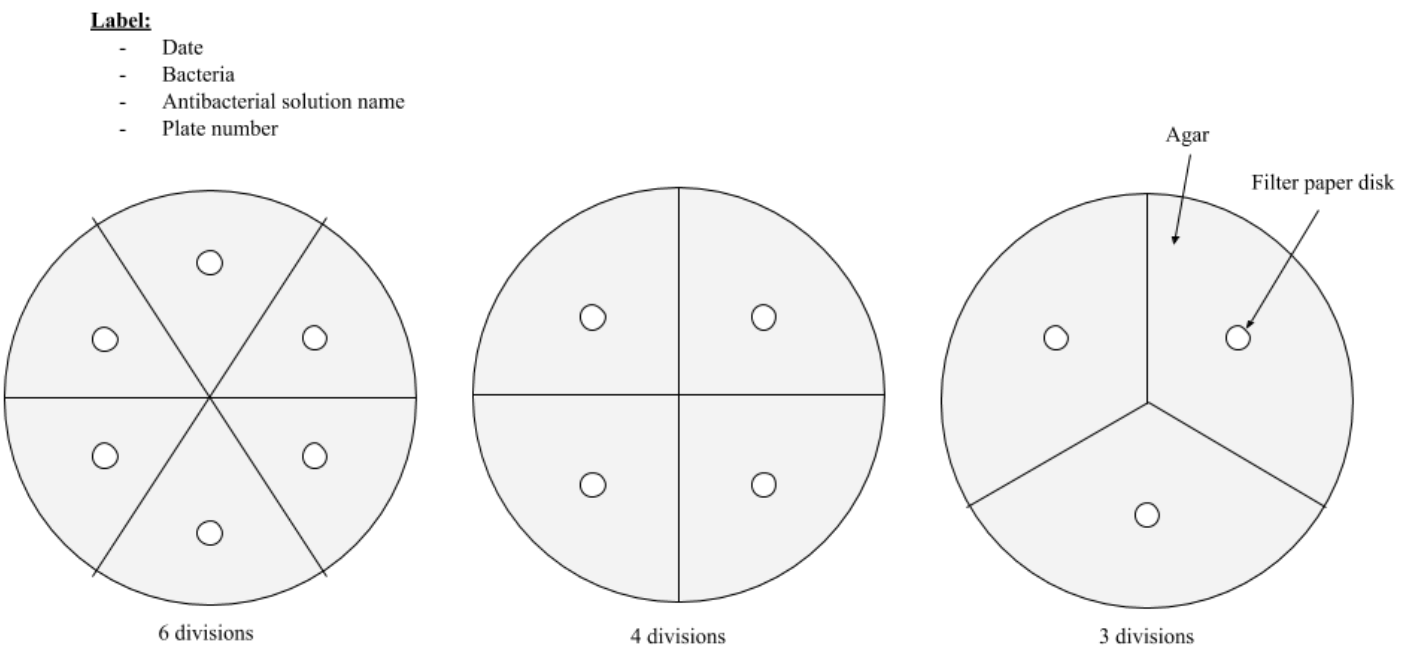
Solution name	Solution description	
RV 4%	40 mg of resveratrol mixed with 1.0 cm^3 of ethanol	**Key:** RV = resveratrol XA = xanthotoxin GO = gossypol
RV 2%	0.5 cm^3 of "RV 4%" solution mixed with 0.5 cm^3 ethanol	
XA 4%	24 mg of xanthotoxin mixed with 0.6 cm^3 of ethanol	
XA 2%	0.3 cm^3 of "XA 4%" solution mixed with 0.3 cm^3 of ethanol	
GO 4%	32 mg of gossypol mixed with 0.8 cm^3 of ethanol	
GO 2%	0.4 cm^3 of "GO 4%" solution mixed with 0.4 cm^3 of ethanol	

Hereafter, 100 6mm disks of filter paper were punched out using a hole punch, placed in a McCartney bottle and sterilized in a pressure cooker. Subsequently, the stock plate was taken out of the incubator and the nutrient agar Petri dishes were taken out of the fridge. The workspace was sterilized using ethanol, a Bunsen burner was set up and the Petri dishes were organized and labelled according to the plating scheme in Table 5. For example, 4 Petri dishes with 3 divisions were required for the SA 4% solution. The labels and divisions were made as illustrated in Figure 2. The label included the date, bacterium inoculated, the antibacterial solution used and the plate number.

Table 5: *plating scheme*

Solution name	Number of Petri dishes	Number of divisions per Petri dish	Total number of readings	
RV 2%	2	6	12	**Key:** RV = resveratrol XA = xanthotoxin GO = gossypol SA = salicylic acid BP = benzoyl peroxide
RV 4%	4	3	12	
XA 2%	2	6	12	
XA 4%	3	4	12	
GO 2%	3	4	12	
GO 4%	4	3	12	
SA 4%	4	3	12	
BP 4%	4	3	12	
Ethanol 97% (control)	4	4	12	

Figure 2: *labels and divisions for Petri dishes (self-made diagram)*



Once all Petri dishes were labelled, each was inoculated using bacteria from the *B. subtilis* stock plate and had the appropriate filter paper disks applied following the Kirby Bauer method (outlined in Appendix C). They were then incubated upside-down at 27ºC for 48 hours.

3.5.3. Part C: Collecting results

The diameter of the ZoI surrounding the filter paper disk was measured using a Vernier calliper at two time periods; after 24 and 48 hours of incubation.

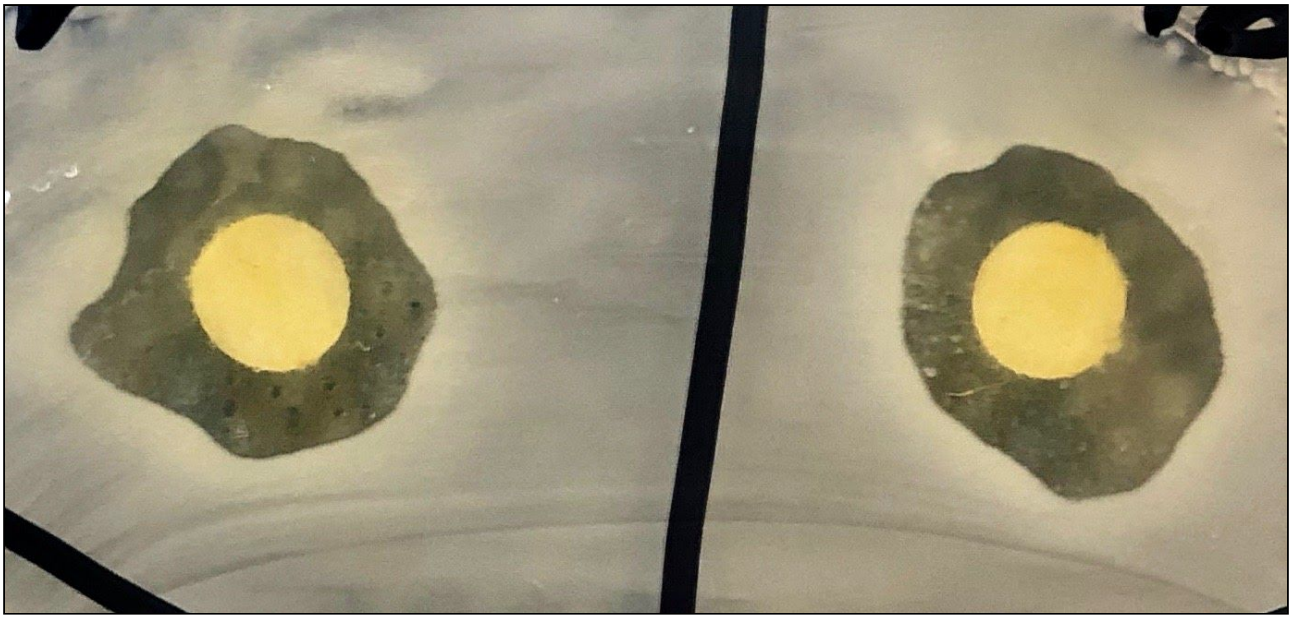
4. DATA COLLECTION AND PROCESSING

4.1. Qualitative data

A first observation made was the over-saturation of some filter paper disks. Once some disks were dipped in an antibacterial solution, they absorbed excess fluid, causing a spillover of fluid onto the surface of the nutrient agar as the disks were placed on it.

Furthermore, during the data collection it was observed that some ZoI were non-circular in nature, as seen in Figure 3.

Figure 3: *non-circular zones of inhibition (self-made photograph)*[80]



4.2. Quantitative data

While the diameters of the circular ZoI were measured normally using a Vernier calliper, the diameters of the non-circular ZoI seen in Figure 3 were measured by averaging the smallest and largest diameters of the ZoI shape. A sample calculation of this for XA 4% is shown below:

$$Diameter\ of\ zone\ of\ inhibition\ = \frac{maximum\ diameter + minimum\ diameter}{2} = \frac{12.30 + 10.60}{2} = 11.45\ mm$$

Using this calculation when necessary, the diameters of each ZoI was measured after 24 hours of incubation and recorded in Table 6.

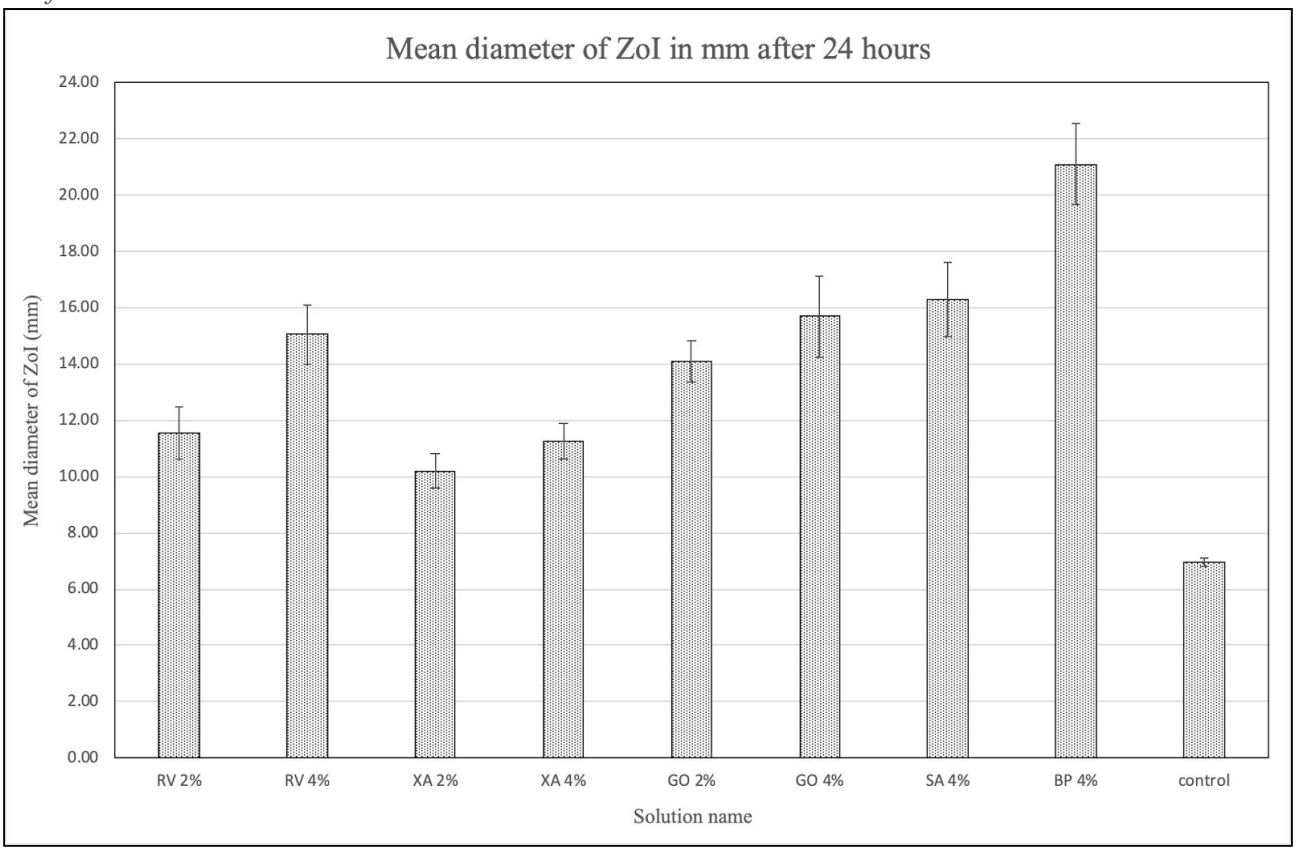
[80] *Non-Circular Zones of Inhibition*. Photograph. Maastricht, June 12, 2019.

Table 6: *raw and processed data showing the diameter of the ZoI for each trial, the mean diameter of the ZoI and the standard deviation for each solution after 24 hours of incubation*

Solution name	**Diameter of ZoI** millimeters / mm ±0.05 mm												**Mean diameter of ZoI** millimeters / mm ±0.05 mm	**Standard deviation** millimeters / mm
	Trials													
	1	2	3	4	5	6	7	8	9	10	11	12		
RV 2%	12.30	11.50	12.35	11.75	9.57	11.20	11.15	12.68	12.70	11.25	10.40	11.95	11.57	0.93
RV 4%	15.40	13.80	14.70	13.40	14.88	16.10	16.80	14.80	15.60	14.35	14.53	14.10	14.87	0.97
XA 2%	9.68	9.50	10.50	10.45	10.70	10.00	9.40	11.25	10.30	10.45	10.15	9.10	10.20	0.61
XA 4%	11.45	11.35	11.22	10.50	13.68	10.70	11.60	11.55	10.35	11.30	11.20	11.77	11.26	0.62
GO 2%	13.65	13.95	14.50	13.10	14.53	14.75	13.50	13.65	15.08	13.45	14.05	15.53	14.08	0.74
GO 4%	15.40	16.50	13.30	14.60	15.78	14.65	16.90	16.75	17.35	17.25	17.80	17.73	15.69	1.43
SA 4%	15.50	16.20	16.60	17.05	19.45	17.85	15.45	15.50	14.40	16.30	15.55	15.75	16.30	1.33
BP 4%	22.35	19.60	21.05	20.00	21.45	20.80	19.85	21.20	23.67	21.60	23.70	19.60	21.11	1.43
Ethanol 97% (control)	7.10	6.80	7.10	6.90	6.90	7.20	7.25	6.95	6.80	7.05	7.00	6.85	6.99	0.15

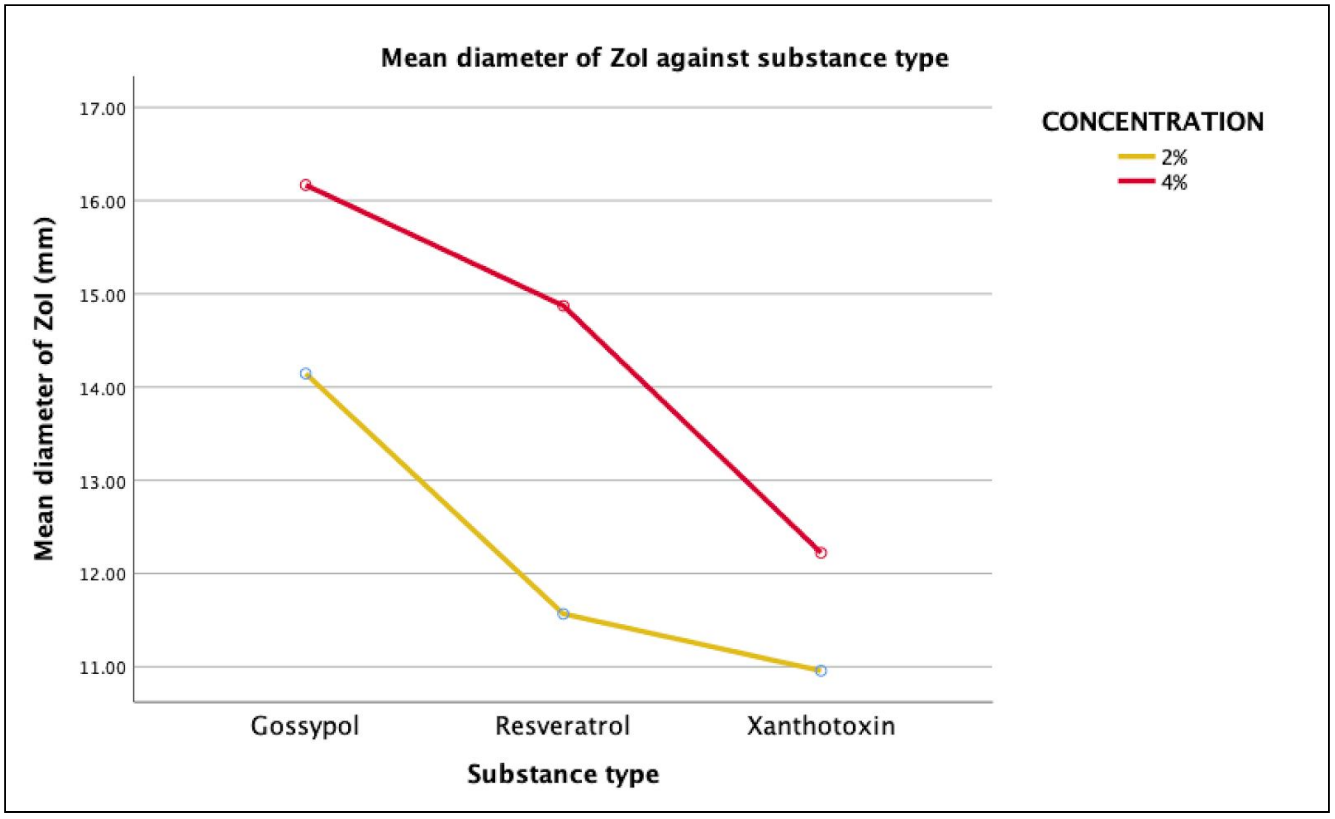
The mean and standard deviation were calculated using Microsoft Excel. Graph 1 visualizes the relationship between the ZoI produced by the different solutions.

Graph 1: *a bar chart illustrating the mean diameter of the ZoI produced by each solution after 24 hours of incubation with standard deviation as error bars*



Graph 2 was constructed to highlight the relationship between the size of the ZoI produced by different concentrations of the different phytoalexin solutions.

Graph 2: *a line graph comparing the antibacterial activity of different phytoalexins at 2% and 4% concentration*



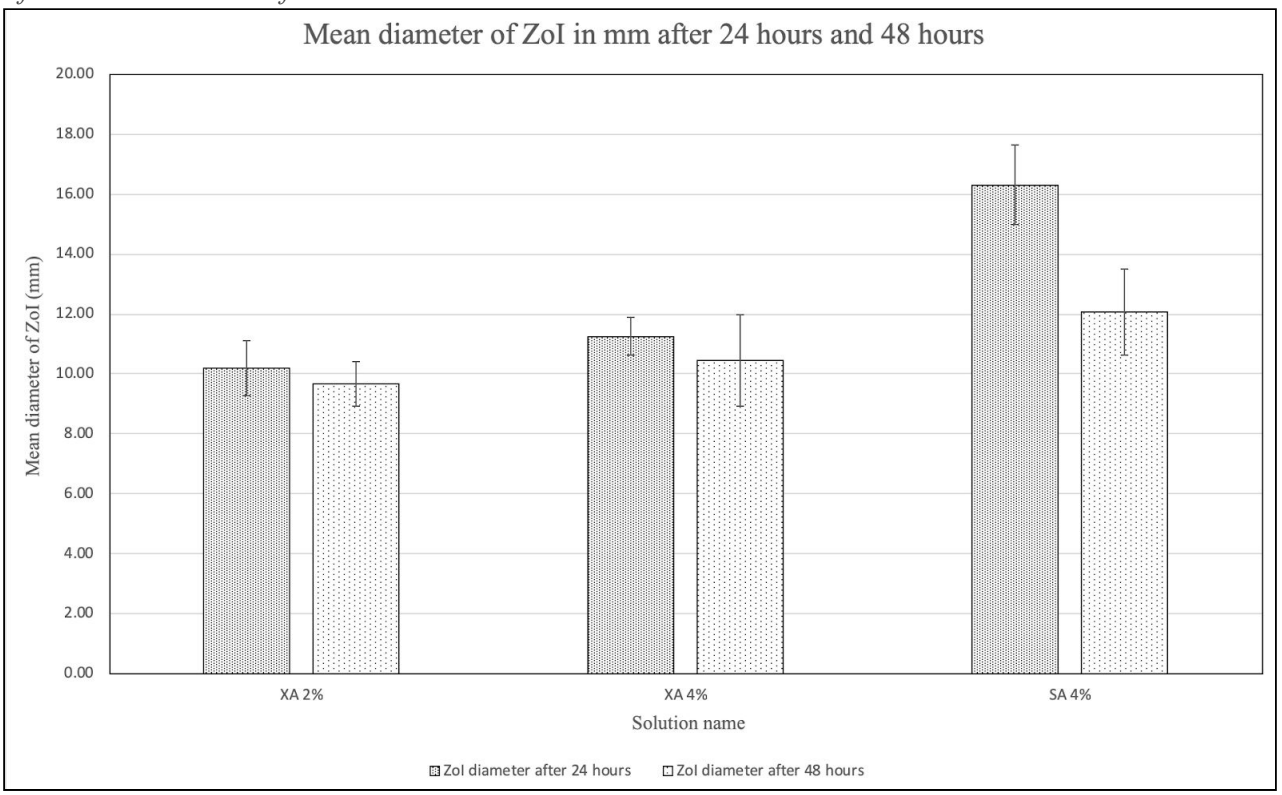
The diameters of the ZoI formed by each solution were also measured after 48 hours of incubation. Only three solutions; XA 2%, XA 4% and SA 4%, demonstrated a decrease in the size of the ZoI they produced between the 24- and 48-hour period of measurement, as shown in Table 7.

Table 7: *raw and processed data showing the diameter of the ZoI for each trial, the mean diameter of the ZoI and the standard deviation for chosen solutions after 48 hours of incubation*

Solution name	**Diameter of ZoI** millimeters / mm ±0.05 mm												**Mean diameter of ZoI** millimeters / mm ±0.05 mm	**Standard deviation** millimeters / mm
	Trials													
	1	2	3	4	5	6	7	8	9	10	11	12		
XA 2%	10.03	9.50	9.85	9.35	10.00	9.95	8.40	10.15	10.20	10.45	9.95	8.00	9.65	0.74
XA 4%	11.10	8.70	8.75	10.50	10.83	9.70	10.45	10.25	13.80	10.70	10.85	11.77	10.45	1.51
SA 4%	14.00	13.50	13.63	12.00	10.10	11.80	10.10	11.80	9.70	12.50	12.70	12.90	12.06	1.45

Graph 3 was created in order to illustrate the change in diameter of the ZoI produced by these three solutions after 24 hours and 48 hours of incubation:

Graph 3: *a bar chart illustrating the mean diameter of the ZoI produced by XA 2%, XA 4%, and SA 4% after 24 and 48 hours of incubation with standard deviation as error bars*



In order to put these changes into perspective, the mean percentage change in size was calculated. This utilized the percentage change formula, as illustrated in the sample calculation below for XA 2%:

$$Percentage\ change = \left(\frac{diameter\ after\ 48\ hours - diameter\ after\ 24\ hours}{diameter\ after\ 24\ hours}\right) \times 100 = \left(\frac{9.65 - 10.20}{10.20}\right) \times 100 = -5.39\%$$

Ultimately, the percentage changes were; -5.39% for XA 2%, -7.19% for XA 4%, and -26.01% for SA 4%.

4.3. Statistical Analysis

4.3.1. ANOVA[81]

A one-way ANOVA is used on data with one independent and one dependent variable which, in this investigation, will be valuable in determining if there's a statistically significant difference in

[81] "Are the means equal?." October 30, 2013. Accessed August 4, 2019. https://www.itl.nist.gov/div898/handbook/prc/section4/prc43.htm.

the ZoI produced by the different antibacterial solutions. There are two hypotheses in a one-way ANOVA, as shown in Table 8:

Table 8: *hypotheses of a one-way ANOVA*

Hypothesis	Explanation
Null hypothesis (H_0)	"There is no difference in the means of the different groups" (the ZoI are the same for the different phytoalexins and OTC treatments)
Alternate hypothesis (H_a)	"The means of the different groups are not all equal" (the ZoI aren't all the same for the different phytoalexins and OTC treatments)

Using the ZoI data from the 4% concentration solutions, the one-way ANOVA was conducted. Its results are displayed in Table 9.

Table 9: *results of one-way ANOVA*

ANOVA					
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*
Between Groups	515.78	4	128.94	89.42	0.000
Within Groups	79.31	55	1.44		
Total	595.09	59			

To determine if the results are significant, the p-value from the ANOVA results is compared to the α-value (0.050). If the p-value is smaller than the α-value, one can reject H_0 and accept H_a. This is the case in this one-way ANOVA, as the p-value (0.000) is smaller than the α-value.

A two-way ANOVA was also conducted for the phytoalexin solutions, which is similar to a one-way ANOVA but considers two independent variables instead of one, which in this case are 'phytoalexin type' and 'concentration'. There are 3 pairs of hypotheses in a two-way ANOVA, as shown in Table 10:

Table 10: *hypotheses of a two-way ANOVA*

Hypothesis	**Explanation**
INTERACTION	
Null hypothesis (H_0)	"There is no interaction between factors A and B"
Alternate hypothesis (H_a)	"There is an interaction between factors A and B"
INDEPENDENT VARIABLE A: PHYTOALEXIN TYPE	
Null hypothesis (H_0)	"There is no difference in the means of independent variable A"
Alternate hypothesis (H_a)	"There is at least one difference in the means of independent variable A; not all means are equal"
INDEPENDENT VARIABLE B: CONCENTRATION	
Null hypothesis (H_0)	"There is no difference in the means of independent variable B"
Alternate hypothesis (H_a)	"There is at least one difference in the means of independent variable B; not all means are equal"

The results of the two-way ANOVA are displayed in Table 9:

Table 9: *results of the two-way ANOVA test*

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F-critical*
Concentration	86.944	1	86.944	92.105	0.000	3.986
Phytoalexin type	153.032	2	76.516	81.058	0.000	3.136
Interaction	12.751	2	6.376	6.754	0.002	3.136
Within (error)	62.302	66	0.944			
Total	86.944	1	86.944	92.105	0.000	3.986

An α-value of 0.050 is used again to test the significance of the results. The results of the hypothesis testing are illustrated in Table 11 below.

Table 10: *hypothesis testing from the two-way ANOVA*

Hypothesis	Conclusion
INTERACTION	
H_0	reject H_0 and accept H_a as the p-value (0.002) < α-value (0.050)
H_a	
INDEPENDENT VARIABLE A: PHYTOALEXIN TYPE	
H_0	reject H_0 and accept H_a as the p-value (0.000) < α-value (0.050)
H_a	
INDEPENDENT VARIABLE B: CONCENTRATION	
H_0	reject H_0 and accept H_a as the p-value (0.000) < α-value (0.050)
H_a	

4.3.2. Tukey HSD test

The ANOVA tests determined that there are significant differences between the ZoI formed from the different substances as a whole[82], but don't specify where these differences lie. This is why the post-hoc Tukey HSD test is required.

The Tukey HSD test identifies which differences between means are statistically significant. If the significance value is less than the α-value (0.050), the antibacterial activity of two substances is statistically different. The results of the Tukey HSD test are displayed in Table 12.

[82] Laerd Statistics. 2018. *One-way ANOVA in SPSS Statistics (cont...)* . Accessed August 4, 2019. https://statistics.laerd.com/spss-tutorials/one-way-anova-using-spss-statistics-2.php.

Table 12: *results of Tukey HSD test*

Multiple Comparisons

Dependent Variable: ZoI

Tukey HSD

(I) substance	(J) substance	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Benzoyl Peroxide	Control (ethanol)	14.2642*	.44810	.000	12.9489	15.5794
	Gossypol	5.0717*	.44810	.000	3.7564	6.3869
	Resveratrol	6.3675*	.44810	.000	5.0523	7.6827
	Salicylic Acid	4.9392*	.44810	.000	3.6239	6.2544
	Xanthotoxin	9.0167*	.44810	.000	7.7014	10.3319
Control (ethanol)	Benzoyl Peroxide	-14.2642*	.44810	.000	-15.5794	-12.9489
	Gossypol	-9.1925*	.44810	.000	-10.5077	-7.8773
	Resveratrol	-7.8967*	.44810	.000	-9.2119	-6.5814
	Salicylic Acid	-9.3250*	.44810	.000	-10.6402	-8.0098
	Xanthotoxin	-5.2475*	.44810	.000	-6.5627	-3.9323
Gossypol	Benzoyl Peroxide	-5.0717*	.44810	.000	-6.3869	-3.7564
	Control (ethanol)	9.1925*	.44810	.000	7.8773	10.5077
	Resveratrol	1.2958	.44810	.096	-.0194	2.6111
	Salicylic Acid	-.1325	.44810	1.000	-1.4477	1.1827
	Xanthotoxin	3.9450*	.44810	.000	2.6298	5.2602
Resveratrol	Benzoyl Peroxide	-6.3675*	.44810	.000	-7.6827	-5.0523
	Control (ethanol)	7.8967*	.44810	.000	6.5814	9.2119
	Gossypol	-1.2958	.44810	.096	-2.6111	.0194
	Salicylic Acid	-1.4283*	.44810	.056	-2.7436	-.1131
	Xanthotoxin	2.6492*	.44810	.000	1.3339	3.9644
Salicylic Acid	Benzoyl Peroxide	-4.9392*	.44810	.000	-6.2544	-3.6239
	Control (ethanol)	9.3250*	.44810	.000	8.0098	10.6402
	Gossypol	.1325	.44810	1.000	-1.1827	1.4477
	Resveratrol	1.4283*	.44810	.056	.1131	2.7436
	Xanthotoxin	4.0775*	.44810	.000	2.7623	5.3927
Xanthotoxin	Benzoyl Peroxide	-9.0167*	.44810	.000	-10.3319	-7.7014
	Control (ethanol)	5.2475*	.44810	.000	3.9323	6.5627
	Gossypol	-3.9450*	.44810	.000	-5.2602	-2.6298
	Resveratrol	-2.6492*	.44810	.000	-3.9644	-1.3339
	Salicylic Acid	-4.0775*	.44810	.000	-5.3927	-2.7623

5. DISCUSSION AND EVALUATION

5.1. Evaluation of results

Graph 1 shows the difference between the size of the ZoI produced by the phytoalexins and OTC acne treatments. Evidently, the benzoyl peroxide solution produced the largest ZoI while the xanthotoxin solutions produced the smallest ZoI. Evaluating these differences in a one-way ANOVA suggested that some significant differences exist between the five substances tested. Tukey HSD testing identified that the sizes of the ZoI produced by the xanthotoxin and benzoyl peroxide solutions were significantly different from all other solutions (sig.values = 0.000). However, the sizes of the ZoI

formed by the gossypol, resveratrol and salicylic acid solutions were not significantly different from one other (sig.-values = 0.056, 0.096, 1.000). The Tukey HSD test also showed that the sizes of the ZoI formed by all substances were significantly different from the control group (all sig.-values = 0.000), confirming the reliability of the results. Ultimately, these results lead us to reject hypothesis 1.

The inhibitory effect of different concentrations of phytoalexin solution was also evaluated, following the statement made in hypothesis 2. This difference is outlined in Graph 2 which suggested that 4% phytoalexin solutions had a consistently greater inhibitory effect than 2% solutions. The two-way ANOVA revealed that an interaction exists between the two independent variables, phytoalexin type and concentration, thus suggesting that the antibacterial activity of all phytoalexins is dependent on their concentration. Furthermore, the two-way ANOVA revealed that a statistically significant difference exists between the antibacterial activity of the 2% and 4% phytoalexin solutions, leading us to accept hypothesis 2.

Lastly, the antibacterial activity of the substances was evaluated over the 48-hour incubation period, following the statement made in hypothesis 3. Of the three solutions which demonstrated a decrease in ZoI size between the 24- and 48-hour period of measurement, SA 4% demonstrated the strongest initial antibacterial activity, with a mean diameter of 16.30 millimetres for the ZoI after 24 hours of incubation. However, this antibacterial activity diminished the most over time, with an average 26.01% decrease in the size of the ZoI after 48 hours of incubation. This contrasts the ZoI produced by XA 2% and XA 4% which decreased by only 5.39% and 7.19% respectively. These results support hypothesis 3 given that both xanthotoxin and salicylic acid are known to be bacteriostatic agents, while the substances whose antibacterial activity remained the same are all bactericidal agents.

Conclusively, the low values for standard deviation in the experiment, with the lowest value being 0.15 and the highest value being 1.51, suggest that the collected data was precise and consistently around the respective mean values.

5.2. Evaluation of methodology

Qualitative observations revealed a first flaw in the methodology used. Although the diameter of the filter paper disks was kept constant, it was observed that some filter disks became over-saturated with solution, causing there to be excess fluid where the filter paper disk was placed on the nutrient agar. A greater volume of the solution would contain more particles of the antibacterial substance, causing more bacteria to be inhibited and therefore forming a larger ZoI. This is a variable which should've been controlled as it directly affects the results. Therefore, an improvement would be to impregnate each disk with an equal, appropriate volume of 15 µl of solution using a micropipette, as suggested by Nostro *et al.* (2000)[83].

Although 12 trials were conducted for each solution, some solutions (e.g. RV 2%) only required two Petri dishes given the small size of the ZoI which they formed. Due to this, any factor that would affect the results in one of the Petri dishes (e.g. airborne contamination) would automatically affect 6 trials, making the reliability of the results questionable. Conclusively, in order to improve the reliability and precision of the results more trials should be conducted (e.g. 40 trials per solution), spread over a larger number of Petri dishes.

Improvements can also be made to the methodology to allow for a more thorough analysis of the results. An example of this would be to incubate the plates for a longer period of time (e.g. 72 hours) and take measurements of the ZoI more frequently (e.g. every 8 hours). This would further highlight the changes in ZoI size over time, possibly shedding light on substances which demonstrate decreased antibacterial activity after 48 hours of incubation.

Lastly, the analysis of the results would greatly benefit by experimenting on a larger range of concentrations of each substance (e.g. 2%, 4%, 6%, 8%, 10%), thus enriching the comparison of the phytoalexins and OTC acne treatments at different concentrations (which is essentially the essence of the research question). A range of 2 to 10 percent is "considered safe for handling benzoyl peroxide"[84]

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[83] Nostro, A., M.P. Germano, V. D'Angelo, A. Marino, and M.A. Cannatelli. "Extraction Methods and Bioautography for Evaluation of Medicinal Plant Antimicrobial Activity." *Letters in Applied Microbiology* 30, no. 5 (May 2000): 379–84. https://doi.org/10.1046/j.1472-765x.2000.00731.x.

[84] American Academy of Dermatology. 2014. *Dermatologists advise patients that over-the-counter acne products can have benefits and a place on their medicine shelf* . January 30. Accessed September 10, 2019. https://www.aad.org/media/news-releases/dermatologists-advise-patients-that-over-the-counter-acne-products-can-have-benefits-and-a-place-on-their-medicine-shelf.

and, given the results of this investigation, should thus also be safe for the handling of the other, less potent substances.

5.3. Literature survey

The sources used in this investigation are scientific journals published on NCBI, a government organization whose standards of submission guarantee that the journals published are on par with scientific standards for research and also free of bias, thus providing a degree of objectivity and credibility in the sources. The remainder of the sources used either came from academic websites or non-government science databases (e.g. ScienceDirect).

Despite this, research into phytoalexins is very novel and thus ever-changing, meaning that articles from recent years may already contain information that is no longer accurate. It would, therefore, be an improvement to commit to using only recent sources in order to be utilizing the most accurate information available on the subject matter.

6. CONCLUSION

In conclusion, this investigation provides an answer to the research question. Ultimately, the benzoyl peroxide solution demonstrated the strongest initial antibacterial activity as well as sustained antibacterial activity over the measuring period; suggesting that it is superior to the other substances for the treatment of acne. Conversely, the xanthotoxin solutions rendered the least suitable for the treatment of acne, demonstrating weak and diminished antibacterial activity over time.

While hypotheses 2 and 3 were accepted, hypothesis 1 was rejected due to results proposing that no statistically significant difference exists between the antibacterial activity of gossypol, salicylic acid and resveratrol. This suggests that the antibacterial activity of these phytoalexins is, in fact, comparable to that of salicylic acid.

However, in contrast to gossypol and resveratrol, the antibacterial activity of salicylic acid was greatly diminished over time, with the ZoI size decreasing by 26.01% between the 24- and 48-hour measuring period. This observation is significant in the context of wider academic reading on the

subject. A study by Taylor *et al.* (2014)[85] suggested that salicylic acid demonstrates a strong yet temporary bacteriostatic effect on *P. acnes* while resveratrol, "despite not having as strong of a killing capability, inhibited bacterial growth for a longer period of time"[86].

The results of this study and my own investigation suggest that, while salicylic acid may not possess a prolonged bacteriostatic effect against *P.acnes*, its use in combination with phytoalexins such as resveratrol and gossypol may produce an antibacterial effect that is both strong and sustained over time.

Exploring the antibacterial effects of different ratios of resveratrol/gossypol and salicylic acid solutions may thus be an interesting extension to this investigation.

7. LITERATURE

Achermann, Y., E. J. C. Goldstein, T. Coenye, and M. E. Shirtliff. "Propionibacterium Acnes: From Commensal to Opportunistic Biofilm-Associated Implant Pathogen." *Clinical Microbiology Reviews* 27, no. 3 (July 1, 2014): 419–40. https://doi.org/10.1128/CMR.00092-13.

Addor, Flavia Alvim Sant'anna. "Antioxidants in Dermatology." *Anais Brasileiros De Dermatologia* 92, no. 3 (June 2017): 356–62. https://doi.org/10.1590/abd1806-4841.20175697.

Al-Barwani, Fatma M., and Elsadig A. Eltayeb. "Xanthotoxin and Other Furanocoumarins as Phytoalexins in Pastinaca Sativa L. Roots." *Sultan Qaboos University Journal for Science [SQUJS]* 9 (June 1, 2004): 7. https://doi.org/10.24200/squjs.vol9iss0pp7-17.

American Academy of Dermatology. 2014. *Dermatologists advise patients that over-the-counter acne products can have benefits and a place on their medicine shelf* . January 30. Accessed September 10, 2019. https://www.aad.org/media/news-releases/dermatologists-advise-patients-that-over-the-counter-acne-products-can-have-benefits-and-a-place-on-their-medicine-shelf.

American Academy of Dermatology. 2018. *Skin conditions by the numbers* . Accessed August 4, 2019. https://www.aad.org/media/stats/conditions/skin-conditions-by-the-numbers.

"Androgen deficiency in women." 2018. Accessed August 4, 2019. https://www.betterhealth.vic.gov.au/health/conditionsandtreatments/androgen-deficiency-in-women.

Antimicrobial Susceptibility Testing EUCAST Disk Diffusion Method. EUCAST, January 2019.

"Are the means equal?." October 30, 2013. Accessed August 4, 2019. https://www.itl.nist.gov/div898/handbook/prc/section4/prc43.htm.

[85] Taylor, Emma J. M., Yang Yu, Jackson Champer, and Jenny Kim. "Resveratrol Demonstrates Antimicrobial Effects Against Propionibacterium Acnes In Vitro." *Dermatology and Therapy* 4, no. 2 (December 2014): 249–57. https://doi.org/10.1007/s13555-014-0063-0.

[86] Ibid.

"Bacillus subtilis." June 7, 2015. Accessed August 4, 2019. https://microbewiki.kenyon.edu/index.php/Bacillus_subtilis.

Bajaj, Lalit, and Stephen Berman. *Berman's Pediatric Decision Making Rev. Ed. of: Pediatric Decision Making*. Philadelphia, PA: Elsevier/Mosby, 2011.

Bowe, Whitney P., and Alan C. Logan. "Acne Vulgaris, Probiotics and the Gut-Brain-Skin Axis - Back to the Future?" *Gut Pathogens* 3, no. 1 (January 31, 2011): 1. https://doi.org/10.1186/1757-4749-3-1.

Bristol, James A, and David W Robertson. *Annual Reports in Medicinal Chemistry.* San Diego: Academic Press, 1996.

Cole, R. S., and R. R. Sinden. "Repair of Cross-Linked DNA in Escherichia Coli." *Basic Life Sciences* 5B (1975): 487–95.

Cotterill, J. A. "Benzoyl Peroxide." *Acta Dermato-Venereologica. Supplementum* Suppl 89 (1980): 57–63.

Cunliffe, W J. "Acne Vulgaris: Pathogenesis and Treatment." *BMJ* 280, no. 6229 (June 14, 1980): 1394–96. https://doi.org/10.1136/bmj.280.6229.1394.

Dalefield, Rosalind. *Veterinary Toxicology for Australia and New Zealand*. Amsterdam, Netherlands ; Kidlington, Oxford ; Cambridge, MA: Elsevier, 2017.

Dayan, Nava, ed. *Skin Aging Handbook: An Integrated Approach to Biochemistry and Product Development*. Personal Care and Cosmetic Technology. Norwich, NY: William Andrew, 2008.

Decker, Ashley, and Emmy M. Graber. "Over-the-Counter Acne Treatments: A Review." *The Journal of Clinical and Aesthetic Dermatology* 5, no. 5 (May 2012): 32–40.

Docherty, John J., Heather A. McEwen, Thomas J. Sweet, Erin Bailey, and Tristan D. Booth. "Resveratrol Inhibition of Propionibacterium Acnes." *Journal of Antimicrobial Chemotherapy* 59, no. 6 (June 1, 2007): 1182–84. https://doi.org/10.1093/jac/dkm099.

Donnelly, Louise E., Robert Newton, Gina E. Kennedy, Peter S. Fenwick, Rachel H. F. Leung, Kazuhiro Ito, Richard E. K. Russell, and Peter J. Barnes. "Anti-Inflammatory Effects of Resveratrol in Lung Epithelial Cells: Molecular Mechanisms." *American Journal of Physiology. Lung Cellular and Molecular Physiology* 287, no. 4 (October 2004): L774-783. https://doi.org/10.1152/ajplung.00110.2004.

Dréno, B., S. Pécastaings, S. Corvec, S. Veraldi, A. Khammari, and C. Roques. "Cutibacterium Acnes (Propionibacterium Acnes) and Acne Vulgaris: A Brief Look at the Latest Updates." *Journal of the European Academy of Dermatology and Venereology* 32 (June 2018): 5–14. https://doi.org/10.1111/jdv.15043.

Drugbank. n.d. *Benzoyl Peroxide.* Accessed August 4, 2019. https://www.drugbank.ca/drugs/DB09096.

Dwivedi, Charu, Ishan Pandey, Himanshu Pandey, Pramod W. Ramteke, Avinash C. Pandey, Shanti Bhushan Mishra, and Sandip Patil. "Electrospun Nanofibrous Scaffold as a Potential Carrier of Antimicrobial Therapeutics for Diabetic Wound Healing and Tissue Regeneration." In *Nano- and Microscale Drug Delivery Systems*, 147–64. Elsevier, 2017. https://doi.org/10.1016/B978-0-323-52727-9.00009-1.

Eady, E. Anne, and Eileen Ingham. "Propionibacterium Acnes - Friend or Foe?:" *Reviews in Medical Microbiology* 5, no. 3 (July 1994): 163–73. https://doi.org/10.1097/00013542-199407000-00003.

Enzo Life Sciences. n.d. *Gossypol.* Accessed August 4, 2019. http://www.enzolifesciences.com/BML-EI130/gossypol/.

EverydayHealth. 2018. *What Is Salicylic Acid Topical?* . September 11. Accessed September 10, 2019. https://www.everydayhealth.com/drugs/salicylic-acid-topical.

Gnonlonfin, G. J. Benoit, Ambaliou Sanni, and Leon Brimer. "Review Scopoletin – A Coumarin Phytoalexin with Medicinal Properties." *Critical Reviews in Plant Sciences* 31, no. 1 (January 2012): 47–56. https://doi.org/10.1080/07352689.2011.616039.

Gupta, Ramesh C., ed. *Nutraceuticals: Efficacy, Safety, and Toxicity*. Amsterdam: Elsevier, Academic Press, 2016.

Harwood, C. R. "Bacillus Subtilis and Its Relatives: Molecular Biological and Industrial Workhorses." *Trends in Biotechnology* 10, no. 7 (July 1992): 247–56.

Healthline. n.d. *How to Treat Acne with Benzoyl Peroxide* . Accessed September 10, 2019. https://www.healthline.com/health/benzoyl-peroxide-for-acne#benefits.

Hoffmann, T., B. Troup, A. Szabo, C. Hungerer, and D. Jahn. "The Anaerobic Life of Bacillus Subtilis: Cloning of the Genes Encoding the Respiratory Nitrate Reductase System." *FEMS Microbiology Letters* 131, no. 2 (September 1, 1995): 219–25. https://doi.org/10.1111/j.1574-6968.1995.tb07780.x.

"How Good Is Your Sterile Technique?." 2017. Accessed August 4, 2019. https://bitesizebio.com/6630/how-good-is-your-sterile-technique/.

iGem. 2012. *Bacillus subtilis.* Munich.

"Interpret the key results for One-Way ANOVA." 2019. Accessed August 4, 2019. https://support.minitab.com/en-us/minitab-express/1/help-and-how-to/modeling-statistics/anova/how-to/one-way-anova/interpret-the-results/key-results/.

Jan Hudzicki. "Kirby-Bauer Disk Diffusion Susceptibility Test Protocol." American Society for Microbiology, December 8, 2009.

Jaques, Renee, and Rebecca Dancer. 2019. *Here's Exactly What Salicylic Acid Does to Your Skin.* June 7. Accessed August 4, 2019. https://www.allure.com/story/what-does-salicylic-acid-do.

Jeandet, Philippe. *Phytoalexins: Current Progress and Future Prospects*. Basel, Switzerland: MDPI, 2015.

Jończyk-Matysiak, Ewa, Beata Weber-Dąbrowska, Maciej Żaczek, Ryszard Międzybrodzki, Sławomir Letkiewicz, Marzanna Łusiak-Szelchowska, and Andrzej Górski. "Prospects of Phage Application in the Treatment of Acne Caused by Propionibacterium Acnes." *Frontiers in Microbiology* 8 (February 8, 2017). https://doi.org/10.3389/fmicb.2017.00164.

Joyce, A. COMPOSITIONS FOR TREATMENT AND PREVENTION OF ACNE, METHODS OF MAKING THE COMPOSITIONS, AND METHODS OF USE THEREOF. WO 2010/087964 A2, n.d.

Kawashima, Makoto, Toshitaka Nagare, and Masaharu Doi. "Clinical Efficacy and Safety of Benzoyl Peroxide for Acne Vulgaris: Comparison between Japanese and Western Patients." *The Journal of Dermatology* 44, no. 11 (November 2017): 1212–18. https://doi.org/10.1111/1346-8138.13996.

Khachatourians, George G., and Dilip K. Arora, eds. *Applied Mycology and Biotechnology*. 1st ed. Amsterdam ; New York: Elsevier, 2001.

Laerd Statistics. 2018. *One-way ANOVA in SPSS Statistics (cont...)* . Accessed August 4, 2019. https://statistics.laerd.com/spss-tutorials/one-way-anova-using-spss-statistics-2.php.

Lall, Namrita, ed. *Medicinal Plants for Holistic Health and Well-Being*. London, United Kingdom: Academic Press, an imprint of Elsevier, 2018.

"Laminar Flow Cabinets." Types of Laminar Flow Cabinets – Uses and Benefits – Information Guide. Accessed August 4, 2019. https://www.laminarflows.co.uk/.

Liu, Hung-Wen. *Comprehensive Natural Products II*. Elsevier Science, 2010.

lumen. n.d. *Overview of Antimicrobial Therapy* . Accessed August 4, 2019. https://courses.lumenlearning.com/boundless-microbiology/chapter/overview-of-antimicrobial-therapy/.

Maddison, Jill E., Stephen W. Page, and David Church, eds. *Small Animal Clinical Pharmacology*. 2nd ed. Edinburgh ; New York: Saunders/Elsevier, 2008.

"Making Agar Plates." 2015. Accessed August 4, 2019. https://teach.genetics.utah.edu/content/microbiology/plates/.

Malavolta, Marco, and Eugenio Mocchegiani, eds. *Molecular Basis of Nutrition and Aging*. Molecular Nutrition Series. Amsterdam ; Boston: Elsevier, AP, 2016.

Malesky, Mallory. 2017. *Why Are Agar Plates Kept Inverted Whenever Possible?* . April 24. Accessed August 4, 2019. https://sciencing.com/agar-kept-inverted-whenever-possible-6311918.html.

Margalith, P. "Inhibitory Effect of Gossypol on Microorganisms." *Applied Microbiology* 15, no. 4 (July 1967): 952–53.

Mayo Clinic. n.d. *Acne.* Accessed August 4, 2019. https://www.mayoclinic.org/diseases-conditions/acne/symptoms-causes/syc-20368047.

Milardi, G. L., A. Stringaro, M. Colone, A. Bonincontro, and G. Risuleo. "The Cell Membrane Is the Main Target of Resveratrol as Shown by Interdisciplinary Biomolecular/Cellular and Biophysical Approaches." *The Journal of Membrane Biology* 247, no. 1 (January 2014): 1–8. https://doi.org/10.1007/s00232-013-9604-1.

Mills, Otto H., Maressa C. Criscito, Todd E. Schlesinger, Robert Verdicchio, and Ernest Szoke. "Addressing Free Radical Oxidation in Acne Vulgaris." *The Journal of Clinical and Aesthetic Dermatology* 9, no. 1 (January 2016): 25–30.

Moghimipour, Eskandar. "Hydroxy Acids, the Most Widely Used Anti-Aging Agents." *Jundishapur Journal of Natural Pharmaceutical Products* 7, no. 1 (2012): 9–10.

Murphy, Chris. 2019. *Over-The-Counter – OTC* . April 17. Accessed August 4, 2019. https://www.investopedia.com/terms/o/otc.asp.

Nakano, Michiko M., and Peter Zuber. "ANAEROBIC GROWTH OF A 'STRICT AEROBE' (BACILLUS SUBTILIS)." *Annual Review of Microbiology* 52, no. 1 (October 1998): 165–90. https://doi.org/10.1146/annurev.micro.52.1.165.

Non-Circular Zones of Inhibition. Photograph. Maastricht, June 12, 2019.

Nostro, A., M.P. Germano, V. D'Angelo, A. Marino, and M.A. Cannatelli. "Extraction Methods and Bioautography for Evaluation of Medicinal Plant Antimicrobial Activity." *Letters in Applied Microbiology* 30, no. 5 (May 2000): 379–84. https://doi.org/10.1046/j.1472-765x.2000.00731.x.

Oregon State University. n.d. *Resveratrol.* Accessed August 4, 2019. https://lpi.oregonstate.edu/mic/dietary-factors/phytochemicals/resveratrol.

PASCO. n.d. *Agar Nutrient.* Accessed August 4, 2019. https://education.scichem.com/Catalogue/ProductDetail/agar-nutrient?productID=9a2abf64-dc3c-48bb-afc6-50f43e65432f&catalogueLevelItemID=00000000-0000-0000-0000-000000000000.

"Pathogenesis of acne." 2016. Accessed August 5, 2019. https://plasticsurgerykey.com/acne-vulgaris/.

Perry, Alexandra, and Peter Lambert. "Propionibacterium Acnes: Infection beyond the Skin." *Expert Review of Anti-Infective Therapy* 9, no. 12 (December 2011): 1149–56. https://doi.org/10.1586/eri.11.137.

"PHYSICAL FACTORS THAT AFFECT MICROBIAL GROWTH." Hawaii. Accessed August 5, 2019. https://www2.hawaii.edu/~johnb/micro/m140/syllabus/week/handouts/m140.9.1.html.

"Propionibacterium acnes." July 1, 2011. Accessed August 4, 2019. https://microbewiki.kenyon.edu/index.php/Propionibacterium_acnes.

"Propionibacterium Acnes." Micropia. Accessed August 4, 2019. https://www.micropia.nl/en/discover/microbiology/propionibacterium-acnes/.

PubChem. n.d. *Methoxsalen.* Accessed August 4, 2019. https://pubchem.ncbi.nlm.nih.gov/compound/methoxsalen.

PubChem. n.d. *Resveratrol.* Accessed August 4, 2019. https://pubchem.ncbi.nlm.nih.gov/compound/Resveratrol#section=Computed-Properties.

Rakel, David, ed. *Integrative Medicine*. Fourth edition. Philadelphia, PA: Elsevier, 2018.

Rao, Jaggi. 2019. *Acne Vulgaris* . March 26. Accessed August 4, 2019. https://emedicine.medscape.com/article/1069804-overview.

Roy, Hervé, Kiley Dare, and Michael Ibba. "Adaptation of the Bacterial Membrane to Changing Environments Using Aminoacylated Phospholipids." *Molecular Microbiology* 71, no. 3 (February 2009): 547–50. https://doi.org/10.1111/j.1365-2958.2008.06563.x.

RxList. 2019. *BENZAGEL* . July 16. Accessed September 8, 2019. https://www.rxlist.com/benzagel-drug/patient-images-side-effects.htm#info.

ScienceDirect. n.d. *Phytoalexins.* Accessed August 4, 2019. https://www.sciencedirect.com/topics/biochemistry-genetics-and-molecular-biology/phytoalexins.

Silver, Lynn L. "Appropriate Targets for Antibacterial Drugs." *Cold Spring Harbor Perspectives in Medicine* 6, no. 12 (December 2016): a030239. https://doi.org/10.1101/cshperspect.a030239.

"Sterile Technique." 2015. Accessed August 4, 2019. https://teach.genetics.utah.edu/content/microbiology/steriletechnique/.

"Sterilizing Liquids." 2015. Accessed August 4, 2019. https://teach.genetics.utah.edu/content/microbiology/liquids/.

"Sterilizing Solid Objects." 2015. Accessed August 4, 2019. https://teach.genetics.utah.edu/content/microbiology/solids/.

Taylor, Emma J. M., Yang Yu, Jackson Champer, and Jenny Kim. "Resveratrol Demonstrates Antimicrobial Effects Against Propionibacterium Acnes In Vitro." *Dermatology and Therapy* 4, no. 2 (December 2014): 249–57. https://doi.org/10.1007/s13555-014-0063-0.

Taylor, Gina A., and Alan R. Shalita. "Benzoyl Peroxide-Based Combination Therapies for Acne Vulgaris: A Comparative Review." *American Journal of Clinical Dermatology* 5, no. 4 (2004): 261–65. https://doi.org/10.2165/00128071-200405040-00005.

Tuttnauer. 2015. *Autoclave Sterilization Process Guide* . November 2. Accessed August 4, 2019. https://tuttnauer.com/blog/autoclave.

Vestergaard, Martin, and Hanne Ingmer. "Antibacterial and Antifungal Properties of Resveratrol." *International Journal of Antimicrobial Agents* 53, no. 6 (June 2019): 716–23. https://doi.org/10.1016/j.ijantimicag.2019.02.015.

Walsh, Timothy R, John Efthimiou, and Brigitte Dréno. "Systematic Review of Antibiotic Resistance in Acne: An Increasing Topical and Oral Threat." *The Lancet Infectious Diseases* 16, no. 3 (March 2016): e23–33. https://doi.org/10.1016/S1473-3099(15)00527-7.

WebMD. n.d. *How to Choose Your Best Acne Treatment* . Accessed August 4, 2019. https://www.webmd.com/skin-problems-and-treatments/acne/features/acne-right-treatment#2.

Westwood Wellness. 2018. *Do Antioxidants Improve Gut Health?* . November 1. Accessed August 4, 2019. https://westwoodwellness.com/antioxidants-improve-gut-health/.

WickhamLaboratories. n.d. *Fact Sheet: Bacillus subtilis.* Accessed August 4, 2019. https://wickhamlabs.co.uk/technical-resource-centre/fact-sheet-bacillus-subtilis/.

Worthington. 2019. *Introduction to Enzymes* . Accessed August 4, 2019. http://www.worthington-biochem.com/introbiochem/tempEffects.html.

"Xanthotoxin." TGSC Information System. Accessed August 4, 2019. http://www.thegoodscentscompany.com/data/rw1037841.html.

8. APPENDICES

APPENDIX A: University of Utah's sterilization guidelines

"General sterile practices include disinfecting all workspaces and hands with ethanol prior to conducting experimentation, must be followed throughout the entirety of an investigation. Furthermore, guidelines for both wet heat (steam) and dry heat (flame) sterilization must be used throughout the entirety of an investigation when applicable.

Wet heat sterilization includes the sterilization of nutrient agar solution and filter paper disks in a pressure cooker (15 psi for 30 minutes). Steam acts as a "sterilizing agent" in a pressure cooker, destroying microorganisms and thus sterilizing whatever is placed inside it.

Dry heat sterilization includes the sterilization of apparatus used for bacterial plating, e.g. inoculation loop, cell spreader and forceps, by dipping them in 97% ethanol and momentarily exposing them to a Bunsen burner flame. This method utilized heat to kill all microorganisms, thus disinfecting the apparatus. "

APPENDIX B: University of Utah's nutrient agar preparation

1. "Choosing a recipe

The nutrient agar recipe used in this investigation instructs to add 23 g of powder per 1000 cm^3 of distilled water. Most recipes make 1000 cm^3 of solution, so make sure to scale them up or down for the number of Petri dishes requires. Given that this investigation uses 31 Petri dishes, with each requiring 25 cm^3 of solution, 775 cm^3 of nutrient agar solution needs to be created which will require 17.8 g of the nutrient agar powder.

2. Gathering supplies

Preparing the nutrient agar requires 31 100mm x 15mm sterilized polystyrene Petri dishes, a 1000 cm^3 graduated cylinder, a pressure cooker, heat-resistant gloves, a magnetic stirrer, an electronic weighing scale and a 1000 cm^3 conical flask and cotton.

3. Preparing nutrient agar solution

To prepare the solution, measure 775 cm^3 of distilled water in a 1000 cm^3 graduated cylinder and pour it into a 1000 cm^3 conical flask and place it on the magnetic stirrer. Weigh 17.8 grams of the nutrient agar powder on an electronic weighing scale and gradually add into the conical flask, allowing the powder to dissolve completely.

4. Sterilization

Roll out some cotton and place it in the mouth of the conical flask, acting as a stopper which allows steam through. Then place the conical flask in the pressure cooker for sterilization at 15 psi for 30 minutes.

5. Pouring

After sterilization, measure out 25cm^3 of the sterilized nutrient agar solution using a 25cm^3 volumetric pipette and pour into each individual Petri dishes using heat-resistant gloves. Leave these Petri dishes undisturbed for 15 minutes until the nutrient agar solidifies. Hereafter seal the Petri dishes and store them upside-down in a refrigerator at 4ºC in order to prevent any condensed water from the lid from dripping onto the agar and interfering with future bacterial growth."

APPENDIX C: Kirby-Bauer method

1. "Disinfect the workspace with ethanol and set up a Bunsen burner. Make sure to work in close proximity to the Bunsen burner in order to minimize the risk of contamination.
2. Using a sterilized inoculation loop, inoculate bacteria from the *B. subtilis* stock plate in one, fluid motion and gently deposit it onto the nutrient agar. Make sure to not tear the surface of the nutrient agar.
3. Sterilize a cell spreader and use it to spread the bacteria across the surface of the nutrient agar, forming a bacterial lawn. Make sure to make smooth movements across the Petri dish while slowly rotating the Petri dish in order to evenly spread the bacteria.
4. After the Petri dish is inoculated comes time to apply the antibacterial filter paper disks. Sterilize forceps and use them to pick up a filter paper disk and dip it into the required solution. Hereafter place the filter paper disk on the surface of the nutrient agar and tap it gently so that it lies flat. Make sure that all the disks are evenly spaced on the surface of the nutrient agar of the Petri dish (depending on the number of divisions per Petri dish). Once the appropriate number of filter paper disks are placed, close and seal the Petri dish with Sellotape.
5. Repeat steps 2 to 4 for all the required Petri dishes."