A solution to combat Erythromycin-resistant bacteria isolated from returned activated sludge in Salt Lake City, Utah

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ABSTRACT

Treatments offered in clinics are now ineffective due to antibiotic resistance in particular bacterial strains. Plant-based antibiotics are in high demand in developing and developed countries; they are common medications that are simple to use, pose no environmental risks, have no adverse side effects, and are competitively priced. This study aimed to screen plant-based medicine against Erythromycin-resistant bacteria such as *E. coli*, *Enterobacter*, *Enterococcus*, and *E. Faecalis*. The disk diffusion method and the agar well diffusion method were used to determine the zone of inhibition of *Coriandrum Sativum L.* (coriander), *Zingiber officinale* (ginger), and *Spinacia oleracea* (spinach). Minimum inhibitory concentration was evaluated via UV-visible Spectrophotometry at 600nm, while Polymerase Chain Reaction and Electrophoresis identified genomic activity for ErmB. Coriander was found to be the most effective against Erythromycin-resistant bacteria, and ErmB genes were found in almost all of the isolates.

Keywords: *Coriandrum Sativum*, *Zingiber officinale*, Spinacia *oleracea*, Antimicrobial activity, and Erythromycin

1 INTRODUCTION

Antibiotic resistance is a severe problem among bacterial strains. Due to genetic changes, the efficacy of popular antibiotics might be lost in as little as five years (Chandra et., al. 2017). Antibiotic pollution and resistance genes in the environment have become a rising threat to human health. Antibiotics and antimicrobial medicines that are ineffective against superbugs are bypassed and returned to the water treatment facilities. They then return to our bodies and the biosphere. Between 2000 and 2015, antibiotic usage in 76 countries was analyzed based on sales. (Klein EY, et., al. 2018). Antibiotics' extensive usage has increased their prevalence in water and wastewater, raising worries about antimicrobial resistance. Antimicrobial activity reduction during water and wastewater treatment procedures has been measured using clinical antibiotic susceptibility testing (Hain E, et., al. 2021). For a variety of reasons discussed elsewhere, present antibiotic discovery/development strategies are unlikely to be as successful in the future. Antimicrobial medication research has much potential in traditional medicine. Despite this, only a few antibiotic agents from plants are widely used (Ilanko A, Cock IE. 2019). Antibiotics have proven to help treat microbiological illnesses, but medication resistance has become a problem. There is a pressing need to find and develop novel and valuable plants that can be used to manufacture natural antibiotics with high biological potential and few adverse effects (Alamholo M. 2020).

Currently, the medical practitioner recommends several synthetic antibiotics to treat nearly all minor infectious diseases and major diseases (Dash, P., & Ghosh, G. 2018). Natural products have achieved incredible results following the new antibacterial medication disclosure requirements. Antibiotics obtained following the recommendations are

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also natural. Bioactive plant extracts are prospective sources of the main component of pharmaceuticals, as can be shown (Bibi, Y., et., al. 2011). Because of their low toxicity, pharmacological activity, and economic feasibility, the therapeutic qualities of plants have attracted much attention because of scientific advances (Chouhan S. et al., 2017).

The rhizome of the monocotyledonous perennial plant ginger (*Zingiber officinale*) is native to Asia and is widely used as a food and nutritional supplement and in traditional medicine in various countries. Ginger is said to have analgesic, antipyretic, antiviral, antidiabetic, anti-inflammatory, anti-helminthic, anticancer, and antioxidant effects and treat various gastrointestinal and respiratory problems. Traditional dishes and beverages are primarily flavoured with ginger. However, because of its numerous advantages, its antibacterial activity has been investigated in numerous research (Beristain-Bauza SDC et al. 2019). For millennia, coriander (*Coriandrum sativum L.*) has been utilized in Chinese cookery and medicine. It, like anise, is a member of the parsley family. Coriander has a variety of tastes and is thus used in various ways. Coriander is a herb that is used in cooking. It is an annual herb n Mediterranean western Europe and in Asia (Laribi B. et al. 2015). Coriander has a vast range of biological activities, such as antimicrobial, antioxidant, antidiabetic, anxiolytic, anti-epileptic, anti-depressant, anti-mutagenic, anti-inflammatory, anti-dyslipidemia, anti-hypertensive, neuroprotective, and diuretic (Maroufi K. et al., 2010). Spinach (*Spinacia oleracea*) is an essential leafy green vegetable high in vitamins and minerals. It is extensively grown all over the world. According to modern nutritionists, spinach is the most acceptable source of iron and other elements. (Munir M. et al., 2019). It is high in folic acid and other vitamins (A, E, K, C, and B complex) and minerals (iron, P, Ca, Mg, Na, K, Cu, Zn, Mn). It also has diuretic, detoxifying, relaxing, coagulant, soothing, demulcent, and laxative qualities. This green vegetable may be used to make a variety of dishes and a highly healthy spinach juice (Galla NR et al. 2017). This study aimed to evaluate some plant sources with antibacterial properties that could be used in drugs against Erythromycin-resistant bacteria (ERB); therefore, there is a pressing need to search for new and novel antimicrobials.

2 METHODOLOGY

2.1 Sample collection:

The samples were collected and processed in Salt Lake City (SLC), Utah, from SLC's returned activated sludge. Erythromycin-resistant bacteria (*Escherichia coli, Enterobacter, Enterococcus, and E. faecalis*) were isolated and identified via microbial culture methods.

2.2 ERB isolation using microdilution method:

Erythromycin was measured up to 24.10 g and placed in an Eppendorf tube as a screening medication. To synthesize the antibiotic solution, milli-Q water was autoclaved to prepare de-ionized sterile water. The antibiotic concentration (in g/ml) was labelled as 512, 256, 128, 64, 32, 16, 8, 4, 2, 1 in ten different Eppendorf tubes. The powder was made by suspending 16.5g/500ml in filtered water and shaking well to dissolve adequately, following the typical technique of ChromAgar manufacture. All solutions and media were boiled at 100 ^{o}C and autoclaved at 121 ^{o}C for 15 minutes before cooling to 45-50 ^{o}C and dispensed. In ChromAgar, two-fold dilutions of the antibiotic solution were prepared, and 19ml of ChromAgar was placed into Petri plates with each erythromycin dilution to allow them to settle. For

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incubation, the plates were held at 37°C for 24 hours. Various colonies were discovered on the plates after 24 hours; more significant colonies of varied bacteria were identified in low concentrations of antibiotics, but a low number of colonies were detected at high concentrations.

2.3 ERB colonies isolation:

Muller Hinton broth was synthesized by suspending 21 grams in 1,000 mL of distilled water; after heating dissolved medium was transferred and dispensed into tubes as needed. All media were autoclave sterilized for 15 minutes at 121°C and cooled to 45-50°C. Using a disposable wire loop, an appropriate proportion of each microbe, such as *E. coli, Enterobacter, Enterococcus, and E. faecalis,* was inoculated in 20ml Muller Hinton broth in four different sets of the test tubes. All test tubes were incubated at 37°C for a 24-hours.

2.4 Plants extraction:

Coriander, ginger, and spinach were bought from a local market in Salt Lake City, Utah, USA. After washing using tap water, then vegetables were washed using distilled water. To make plant extracts, these plants were mashed, and a paste was made in a mortar and pestle. Crude aqueous coriander, ginger, and spinach extracts were prepared in Milli-Q water. Whatman filter paper discs (6mm) were sterilized, and the crude extracts were loaded onto them and left to soak until the extracts were saturated entirely on the discs.

2.5 Time-dependent estimation of tested extracts against ERB:

Fresh re-cultures of ERBs were produced in 10 mL of Mueller Hinton broth. The ERBs were streaked in Muller Hinton agar medium plates using a sterilized disposable wire loop. Plant discs were produced and dispensed on plates. They were incubated for 24 hours at 37°C. Zones of inhibition were evaluated after 24 hours of incubation. A UV-spectrophotometer with an optical density of 600nm was used to determine the minimum inhibitory concentration (MIC).

2.6 DNA Extraction and Quantification:

DNA was extracted from ERB isolates using the DNeasy PowerSoil Kit (QIAGEN cat. No. 12888-50). Thermo Scientific's NANODROP 2000c was used to measure the concentration of DNA in the plant treated samples.

2.7 Polymerase Chain Reaction and Electrophoresis:

The forward and reverse primers for each ErmA, ErmB, and ErmC gene were used in the reaction mixture. The Polymerase Chain Reaction (PCR) method took less than two and a half hours. PCR approaches found aberrant erythromycin protein binding genes; 1 mL of the ladder (control) and 1 mL of dye were combined in a separate tube and were fixed on the colour black, which constituted a negative charge. The substance was carefully loaded into gel blocks in the BIO-RAD Company's Molecular Imager Gel Doc^{TM} XR+ imaging system. The gel was run at 80V for 1

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hour. After an hour, the electronic images of the bands were observed and connected serially to a computer for image processing, which identified the genes present in ERB.

3 RESULTS AND DISCUSSION

3.1 Micro dilution analysis of ERB:

The least inhibitory concentration was detected at varied erythromycin concentrations for *E. coli*, *Enterobacter*, *Enterococcus*, and *E. faecalis*. As shown in figure 1, ten dilutions of 2 fold were prepared to start from 512 mg/L of erythromycin. The highest number of colonies were formed at 64 mg/L of erythromycin concentration from returned activated sludge samples at ChromAgar cultured media. The most delicate colonies were formed on a 64g/ml plate and were detected as *E. coli*, *Enterobacter*, *Enterococcus*, and *E. faecalis*.

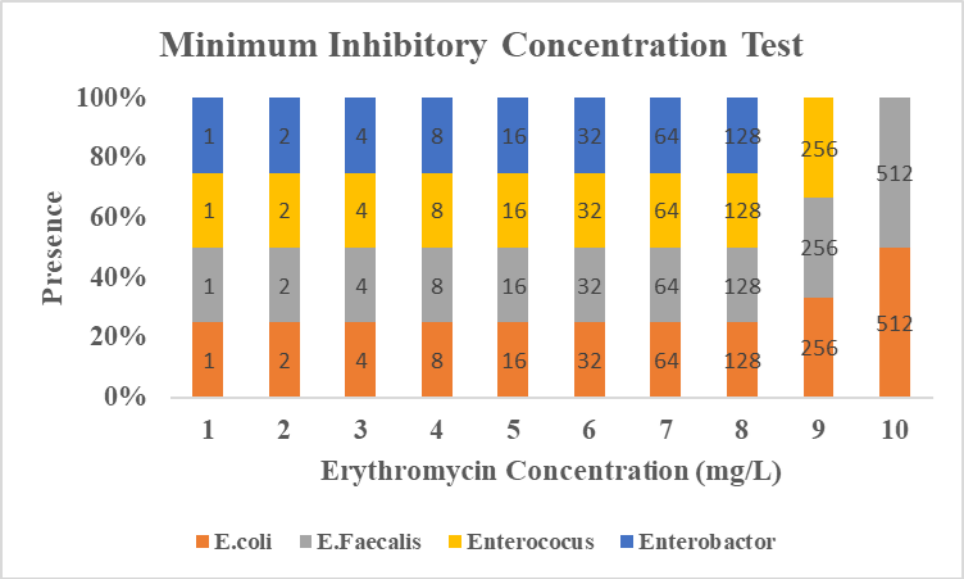

Figure 1: MIC test by microdilution analysis

3.2 Estimation of plant extracts activities against ERB:

In terms of zone of inhibitions, as shown in table 1, ginger demonstrated the best activity against *E. coli* (11±0.6 mm) and the lowest activity against *Enterobacter* (7.6±0.8 mm) when tested using the disc diffusion technique. On the other hand, coriander and spinach demonstrated good effectiveness against *Enterococcus*, with inhibition zones of 12.6±0.6 and 11±0.6 mm, respectively. Coriander and spinach had intermediate efficacy against *Enterobacter* 8±0.6 and 7±0.6 mm, respectively.

According to the agar well diffusion technique, as shown in table 2, ginger had shown the highest activity against *Enterococcus* (10±1.2 mm) and no activity against *Enterobacter*. Coriander had the highest activity against *E. coli* (13.3±0.3) and the lowest activity against *Enterococcus* (8±1.2), whereas spinach had potent activity against *E. faecalis* (10.3±0.8) and moderate activity against *E. coli* (9.3±0.6).

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Table 1: Antimicrobail activity of ainger, coriander and spinach using the disk diffusion method

Categories	Disk diffusion (Zone of inhibition in mm)		
	Spinach	**Coriander**	**Ginger**
E.coli	8±1.1	9±1.5	11±0.6
Enterobacter	7±0.6	8±0.6	7.6±0.8
Enterococcus	11±0.6	12.6±0.6	10±1.2
E. Faecalis	10.3±0.8	11±0.6	9.6±1.2

Table 2: Antimicrobail activity of coriander, ginger and spinach using the agar well diffusion method

Categories	Agar well Diffusion (Zone of Inhibition in mm)		
	Spinach	**Coriander**	**Ginger**
E.coli	9.3±0.6	13.3±0.3	9.6±0.6
Enterobacter	10±1.2	9.6±1.2	0
Enterococcus	10±1.2	8±1.2	10±1.2
E. Faecalis	10.3±0.8	11±0.6	9±0.5

3.3 Time-dependent estimation of the growth of ERB:

Erythromycin (control), ginger, coriander, and spinach (test) was used to see if they affected *E. coli* growth. The optical density was measured at 600nm every 15 minutes for nearly 2 and a half hours, and a relationship between growth and time was revealed. Erythromycin inhibited the growth of *E. coli* when incubated for a specific time, as illustrated in figure 2. After 60 minutes, the best activity was seen for *E. coli* at 0.685nm; it reached its maximum level at roughly 0.096nm after 150 minutes; however, the wavelength (λ) value as in equation mx + c (-0.0062x + 0.9051) revealed Regression Coefficient (R^2) 0.9404.

After 90 minutes, the noticeable activity, as shown in figure 3 for *Enterobacter*, was measured at 0.27nm and went to its maximum level at about 0.33nm at 105 minutes; however, the λ value in equation mx + c (-0.0058x + 0.8554) and the regression coefficient (R^2) was 0.942. As shown in figure 4, the best activity for Enterococcus was at 0.213nm after 90 minutes, and it was at its maximum level at roughly 0.416nm after 105 minutes; however, the λ value as in equation mx + c (-0.0059x + 0.849) revealed Regression Coefficient (R2) 0.942. After 90 minutes, the best activity

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for *E. faecalis* was measured at 0.189nm, as illustrated in figure 5. It reached its maximum at 0.673nm after 105 minutes; however, the λ value as in equation mx + c (-0.0053x + 0.789) revealed Regression Coefficient (R2) 0.9719.

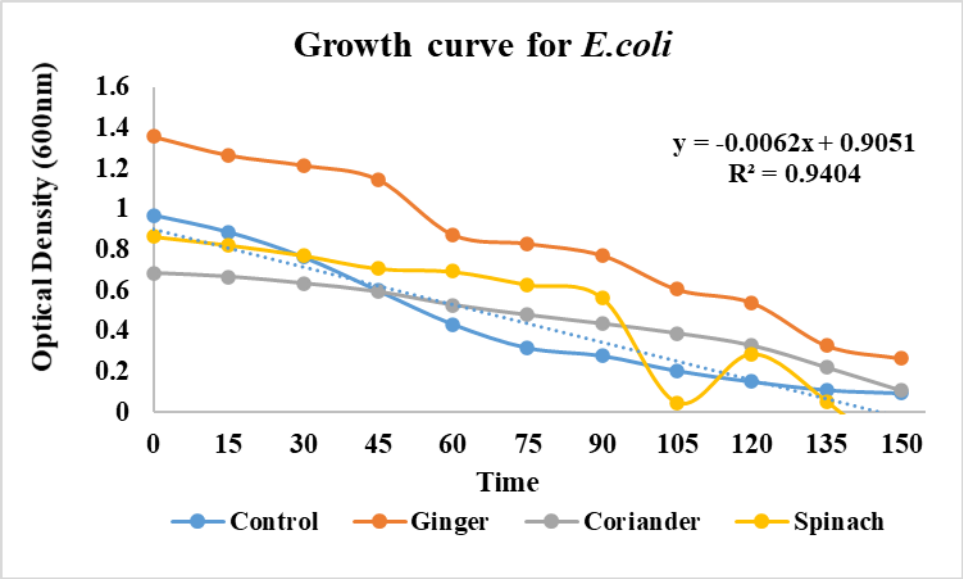

Figure 2: Growth curve for *E. coli*

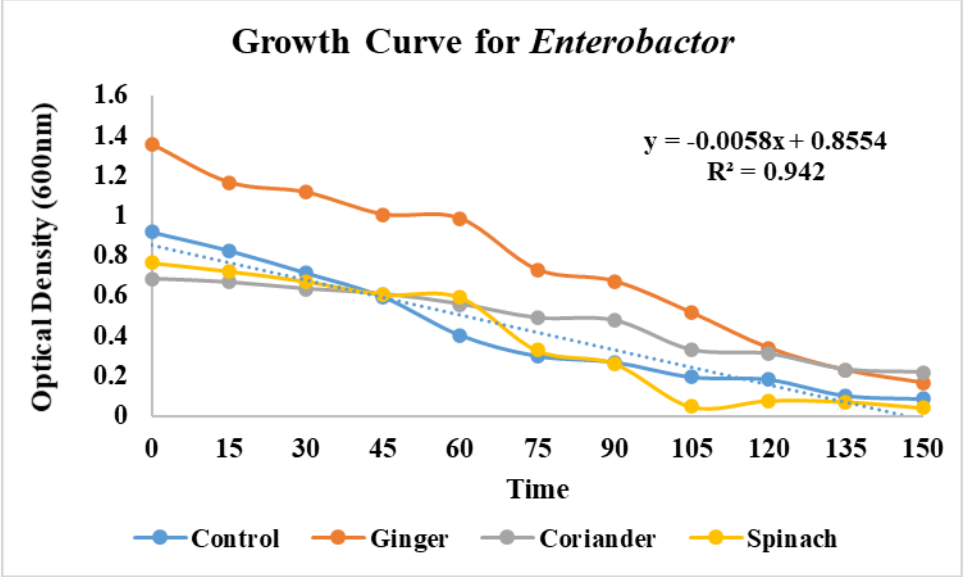

Figure 3: Growth curve for *Enterobacter*

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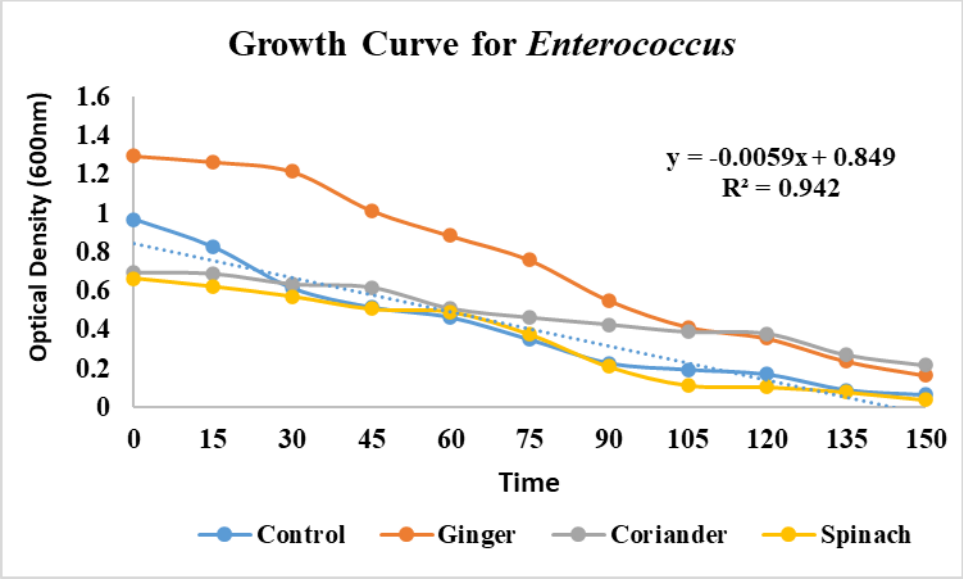

Figure 4: Growth curve for *Enterococcus*

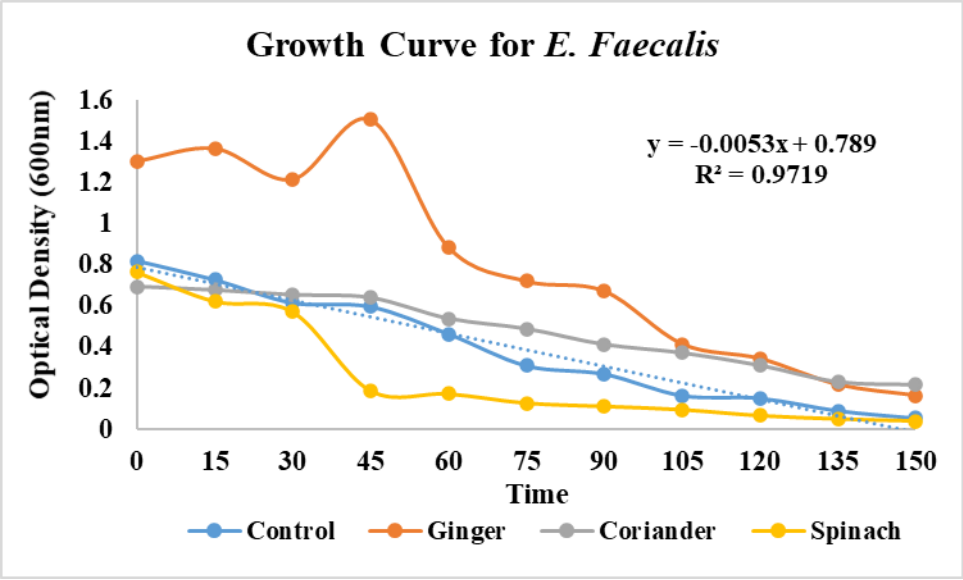

Figure 5: Growth curve for *E. faecalis*

3.4 Quantification of ERB resistant gene:

The DNA of the ERB isolates (*E. coli*, *Enterobacter*, *Enterococcus, and E. faecalis*) was extracted to discover genomics. As illustrated in figure 6, UV absorbance was utilized to determine the purity of the isolated DNA. The absorbance ratio at 260 nm to 280 nm (A260/A280) for *E. coli* was greater than 2.0, which indicated that it was

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extracted without using any protein or organic solvent. At the same absorbance ratio, *Enterobacter* was more than 1.3, which showed that it was not removed as pure as it might have been. It might have a small quantity of protein or any organic solvent. Whereas the absorbance ratio for *Enterococcus* was more than 2.2, indicating that it was also extracted without using any protein or organic solvent. The absorbance ratio for *E. faecalis* was more than 6.0, which was also indicating that it was extracted without the use of any protein or organic solvent. The weight in grams of isolated DNA from all ERB was measured using NANODROP 2000/2000c, such as *E. coli* (0.04g), *Enterobacter* (0.071g), *Enterococcus* (0.314g), and *E. Faecalis* (0.230g).

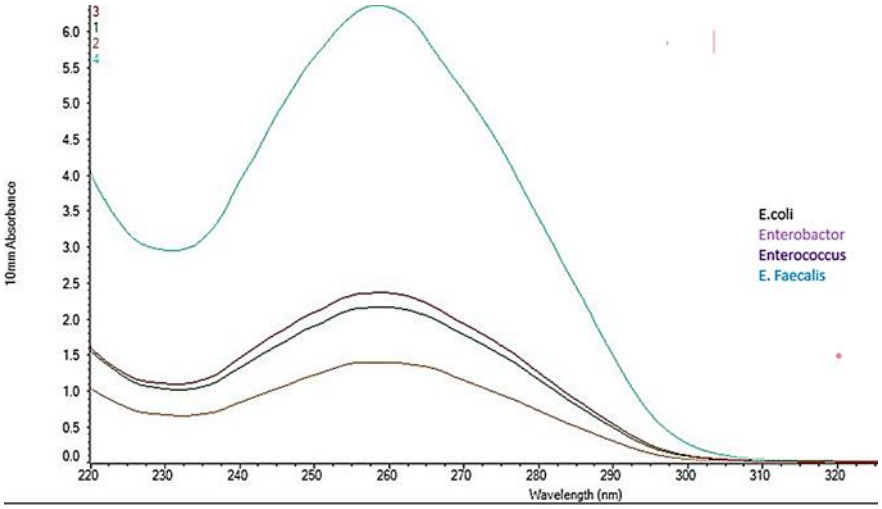

Figure 6: DNA extraction absorbance

3.5 ErmB gene identification through PCR and electrophoresis:

To discover the gene among *E. coli, Enterobacter, Enterococcus, and E. faecalis* extracted DNA, gel electrophoresis was used to construct the gel, which was then loaded with extracted DNA. PCR and electrophoresis were used to identify the ErmB gene in all isolates by keeping this gel in a Molecular Imager Gel Doc™ with an XR+ imaging system.

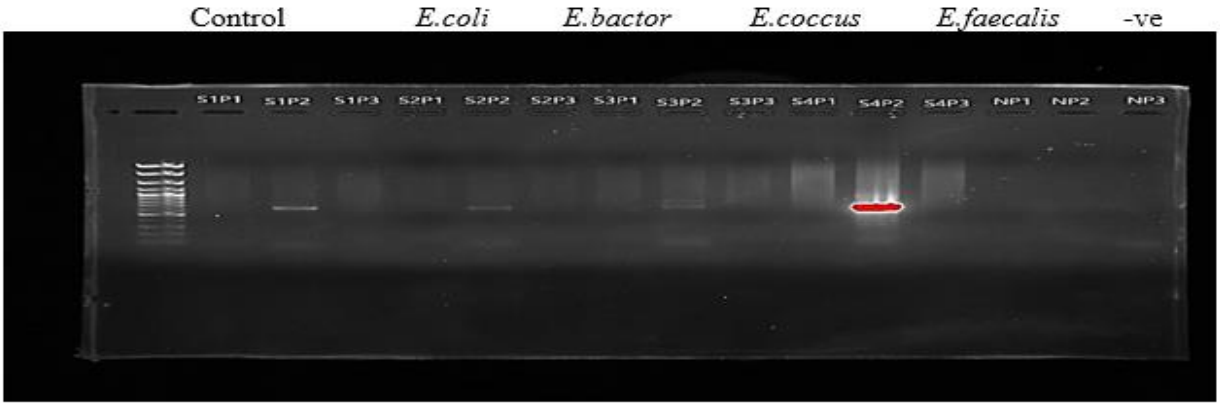

Figure 7: Gel electrophoresis for ErmB gene in ERBs

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In figure 7, all ERBs have the ErmB gene. At the same time, the base pairs were measured as 640/Kbp. Compared to *E. coli*, *Enterococcus, and Enterobacter*, the ErmB gene band was most dominant in *E. faecalis* strains.

4. CONCLUSION

Natural remedies have been shown to have suppressive effects on the ERB. At a 64 mg/mL concentration of erythromycin, the best colonies of ERBs (*E. coli, Enterobacter, Enterococcus, and E. Faecalis*) were identified. Ginger, coriander, and spinach were highly active against *Enterococcus*. Coriander showed a reduction in bacterial activity, while spinach had a lower activity level than ginger. In comparison to *E. faecalis* (0.230g), *Enterobacter* (0.071g), and *E. coli, Enterococcus* was isolated in an excellent amount of 0.314g four ERB (0.04g). The ErmB gene was found in all the tested bacterial isolates. The suppressive properties of natural products were noticed in this study; however, further research for exploring the mode of action of the potential compound in their natural products against antibiotic-resistant bacteria is required.

REFERENCES

Aelenei, P., Rimbu, C. M., Guguianu, E., Dimitriu, G., Aprotosoaie, A. C., Brebu, M., ... & Miron, A. (2019). Coriander essential oil and linalool–interactions with antibiotics against Gram-positive and Gram-negative bacteria. *Letters in applied microbiology*, *68*(2), 156-164.

Alamholo, M. (2020). Investigation of chemical composition, antibacterial and antioxidant activity of Thymus daenensis and Thymus eriocalyx essential oils against human pathogenic bacteria. *J Med Microbiol*, *8*(4), 148-154.

Babaeekhou, L., & Ghane, M. (2021). Antimicrobial activity of ginger on cariogenic bacteria: molecular networking and molecular docking analyses. *Journal of Biomolecular Structure and Dynamics*, *39*(6), 2164-2175.

Beristain-Bauza, S. D. C., Hernández-Carranza, P., Cid-Pérez, T. S., Ávila-Sosa, R., Ruiz-López, I. I., & Ochoa-Velasco, C. E. (2019). Antimicrobial activity of ginger (Zingiber officinale) and its application in food products. *Food Reviews International*, *35*(5), 407-426.

Bibi, Y., Nisa, S., Chaudhary, F. M., & Zia, M. (2011). Antibacterial activity of some selected medicinal plants of Pakistan. *BMC complementary and alternative medicine*, *11*(1), 1-7.

Chandra, H., Bishnoi, P., Yadav, A., Patni, B., Mishra, A. P., & Nautiyal, A. R. (2017). Antimicrobial resistance and the alternative resources with special emphasis on plant-based antimicrobials—a review. *Plants*, *6*(2), 16.

Chouhan, S., Sharma, K., & Guleria, S. (2017). Antimicrobial activity of some essential oils—present status and future perspectives. *Medicines*, *4*(3), 58.

Dash, P., & Ghosh, G. (2018). Amino acid profiling and antimicrobial activity of Cucurbita moschata and Lagenaria siceraria seed protein hydrolysates. *Natural product research*, *32*(17), 2050-2053.

Galla, N. R., Pamidighantam, P. R., Karakala, B., Gurusiddaiah, M. R., & Akula, S. (2017). Nutritional, textural and sensory quality of biscuits supplemented with spinach (Spinacia oleracea L.). *International Journal of Gastronomy and Food Science*, *7*, 20-26.

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Hain, E., Adejumo, H., Anger, B., Orenstein, J., & Blaney, L. (2021). Advances in antimicrobial activity analysis of fluoroquinolone, macrolide, sulfonamide, and tetracycline antibiotics for environmental applications through improved bacteria selection. *Journal of Hazardous Materials*, *415*, 125686.

Ilanko, A., & Cock, I. E. (2019). The interactive antimicrobial activity of conventional antibiotics and Petalostigma spp. extracts against bacterial triggers of some autoimmune inflammatory diseases. *Pharmacognosy Journal*, *11*(2).

Klein, E. Y., Van Boeckel, T. P., Martinez, E. M., Pant, S., Gandra, S., Levin, S. A., ... & Laxminarayan, R. (2018). Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. *Proceedings of the National Academy of Sciences*, *115*(15), E3463-E3470.

Laribi, B., Kouki, K., M'Hamdi, M., & Bettaieb, T. (2015). Coriander (Coriandrum sativum L.) and its bioactive constituents. *Fitoterapia*, *103*, 9-26.

Maroufi, K., Farahani, H. A., & Darvishi, H. H. (2010). Importance of coriander (Coriandrum sativum L.) between the medicinal and aromatic plants. *Advances in Environmental Biology*, *4*(3), 433-436.

Munir, M., Ahad, A., Gull, A., Qayyum, A., Siddique, N. R., Mumtaz, A., ... & Qureshi, T. M. (2019). Addition of Spinach Enhanced the Nutritional Profile of Apricot Based Snack Bars. *Pakistan Journal of Agricultural Research*, *32*(3).

Noshad, M. (2021). Investigation of chemical properties and antimicrobial activity of Rumex alveollatus methanolic extract on Enterobacter aerogenes, Staphylococcus aureus, Salmonella typhi, and Streptococcus pyogenes: An "in vitro" study. *Food Science and Technology*, *18*(117), 109-117.

Wang, X., Shen, Y., Thakur, K., Han, J., Zhang, J. G., Hu, F., & Wei, Z. J. (2020). Antibacterial activity and mechanism of ginger essential oil against Escherichia coli and Staphylococcus aureus. *Molecules*, *25*(17), 3955.

Yadufashije, C., Niyonkuru, A., Munyeshyaka, E., Madjidi, S., & Mucumbitsi, J. (2020). Antibacterial activity of ginger extracts on bacteria isolated from digestive tract infection patients attended Muhoza Health Center. *Asian Journal of Medical Sciences*, *11*(2), 35-41.

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