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Original Research Article

Antibacterial and antivirulence effects of kale (*Brassica oleracea var. sabellica*) on *Streptococcus intermedius*

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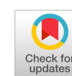

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ABSTRACT

Background: Persistent odontogenic infections caused by resistant bacterial species, such as *Streptococcus intermedius*, have consistently been associated with deep-seated infections. This in vitro study aimed to evaluate the antibacterial and antivirulence effects of kale (*Brassica oleracea var. sabellica*) on *S. intermedius*.

Materials and Methods: *S. intermedius* was freshly incubated in tryptic soy broth media. Three experiments per concentration of kale were conducted under aseptic conditions (i.e., disc diffusion, broth microdilution, and reverse-transcription polymerase chain reaction) to evaluate the antibacterial and antivirulence effects. The samples were then treated with 1000, 500, 250, 125, 65, 30, 15, 7, and 3 mg/mL kale; ampicillin (positive control); and tryptic soy broth (negative control). After 24-h incubation, the inhibition zone, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and relative gene expression of the virulence factor (intermedilysin [*ily*]) were measured. All assays were conducted in triplicate. The findings were reported and analyzed as means ± standard deviations. The agar disc diffusion and relative gene expression were statistically analyzed using one-way analysis of variance and Tukey's test, with the significance level set at $P < 0.05$.

Results: Kale showed antibacterial effects on *S. intermedius* by significantly inhibiting bacterial growth and reducing *ily* expression only at a concentration of 1000 mg/mL; it yielded an inhibition zone of 11.12 ± 1.59 mm, which was smaller than that with ampicillin. The MIC and MBC ranged from 15 to 65 mg/mL and from 500 mg/mL, respectively. Conversely, the highest concentration of kale yielded significantly less inhibition than did ampicillin.

Conclusions: The antibacterial effects of kale may be dose-dependent. Kale can inhibit bacterial growth and suppress *ily* expression under in vitro conditions of *S. intermedius*, which is mainly involved in deep-seated odontogenic infections.

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1. Introduction

Odontogenic infections are one of the most common pathologies in the oral and maxillofacial regions and a complicated problem to manage in dentistry. They are usually caused by deep-seated dental caries, periodontal disease, and pericoronitis.[1] Such infections may spread into adjacent anatomical spaces along the fascial planes in the head and neck regions, leading to the involvement of the deep spaces of the mediastinum, pleural cavities, and pericardium and consequently to serious life-threatening conditions. More than 700 bacterial species inhabit the oral

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cavity.[2] The oral pathogens responsible for the progression of dentoalveolar abscesses are polymicrobial with a mixture of aerobic, strict anaerobic, and facultative anaerobic microorganisms.[3] The most common organisms are the viridans group streptococci and *Streptococcus anginosus* initially, followed by *Fusobacterium* spp. and *Prevotella* spp.[4] *Streptococcus intermedius* is a member of the *S. anginosus* group. A part of the commensal oral flora in the oral cavity and upper respiratory tract causes minor to severe infections in the head and neck regions.[5] The pathogenic ability of *S. intermedius* is attributed to its multiple virulence factors, which help the bacteria in colonizing, forming biofilms, evading the host immune system, and damaging cell membranes.[6] A noteworthy virulence secretion is intermedilysin (ily), a member of the cholesterol-dependent cytolysin family of toxins, which aids the organism in creating pores in host cells. Previous studies have found that the *S. intermedius* strain with the *ily* gene is associated explicitly with deep-seated infections.[7] Early recognition and optimized treatment of these infections are required to improve outcomes. Most patients recover completely after adequate surgical treatment and appropriate antibiotic regimen. The prognosis of odontogenic infections has substantially improved since the advent of antibiotics. However, odontogenic infections remain prevalent worldwide.

Based on data in 2019, the World Health Organization listed antimicrobial resistance as a severe threat to public health.[8] Antimicrobial resistance causes many public health problems, such as longer treatment duration, higher treatment cost, and higher mortality rate.[9] Over the past few decades, the search for antimicrobials derived from plants has accelerated. However, as bacterial resistance to antibiotics grows, clinically useful antibiotics fail to emerge. Research has focused on screening raw materials for new natural substances to replace synthetics. To protect themselves from microbial pathogens, plants produce numerous secondary metabolites with antimicrobial properties, such as phenolic compounds, terpenoids, essential oils, alkaloids, lectins, and polypeptides.[10] The therapeutic applications of natural substances have been explored relative to antimicrobial properties.

Kale (*Brassica oleracea* var. *sabellica*) is one of the most economically significant Brassicaceae species, which has been added to the growing list of foods considered a superfood.[11] It is known to possess a diverse range of biological effects with a high content of health-promoting phytochemicals, which include antioxidant, anti-inflammatory, and antimicrobial properties. Many phytochemicals, such as polyphenolic compounds, have been shown as potential antibacterial agents.[12] Recent studies have demonstrated that kale inhibits the growth of some pathogenic bacteria, including *Staphylococcus aureus, Escherichia coli*, and *Pseudomonas aeruginosa*, and some fungi, including *Candida tropicalis* and *Candida albicans*.[13–15] Polyphenolic compounds in kale also have antimicrobial properties that prevent biofilm formation by impeding odontopathogenic bacterial adhesion and inactivating bacterial virulence factors.[16] Kale has also been found to exert a synergistic effect with antibiotics. Its structure, which renders the risk of cross-resistance highly improbable, makes it a potentially new antibiotic drug candidate or, more likely, a resistance-modifying agent in combination with conventional antibiotics.[17] In the last few years, accumulating evidence has indicated that plant-derived compounds can complement the clinical application of generally ineffective antibiotics alone.

Kale extracts are also known to enhance biological activity in the human body in association with protection against free radicals and reactive oxygen species from cardiovascular and gastrointestinal diseases.[18] Kale crudes extracted and synthesized with copper nanoparticles have shown promising findings in cytotoxicity against cancer cells.[19] However, the ability of kale to inhibit the bacterial growth and suppress the virulence factor genes of *S. intermedius* has not been thoroughly examined. Hence, the present study aimed to investigate the antibacterial and antivirulence effects of kale on *S. intermedius* using reverse-transcription polymerase chain reaction (RT-PCR) testing.

2. Materials and Methods

This study was approved by the Institutional Biosafety Committee of the Faculty of Dentistry, Chiang Mai University, Thailand (Approval number: CMUIBC A-0565007), and conducted at the Research Center and Laboratory. The methods and materials are briefly demonstrated in Chart 1.

Fresh kale (2 kg) was harvested from Ban Pa Bong Piang, Mae Chaem, Chiang Mai. It was extracted using 95% ethanol at the Faculty of Pharmacy, Chiang Mai University, and the final volume was 61.41 g.

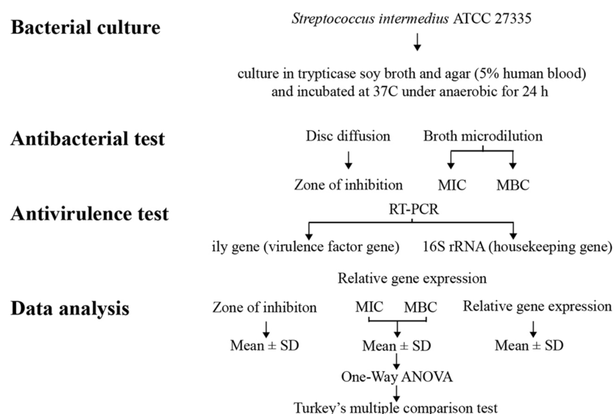

Chart 1: Study flow diagram

2.1. Microorganisms and media

Standard strains of *S. intermedius* (ATCC number: 27335) from the bacterial stock at the Research Center were used. *S. intermedius* was cultured in tryptic soy broth (Difco Laboratories, Detroit, MI, USA) and tryptic soy agar containing 5% human blood (Maharaj Nakhon Chiang Mai Hospital). The bacteria were inoculated via loop transfer from frozen tubes into 4 mL tryptic soy broth and grown at 37°C for 24 h under anaerobic conditions. The bacteria from these cultures were transferred to an appropriate solid medium and incubated overnight. Selected colonies were transferred to an appropriate liquid medium and incubated for 6 h to achieve log phase growth. The bacterial suspension was centrifuged, rinsed twice using PBS, and suspended to the proper bacterial density by comparing the OD600 (0.5 McFarland standard) of the sample with a standard curve relating OD600 to cell numbers.

2.2. Agar disc diffusion test

For the disc diffusion test, 5 mL of fresh broth agar was prepared in 90 × 15-mm petri dishes. Nine replica plates containing tryptic soy agar were spread at 5×10^5cfu with 0.1 mL of bacterial suspension using a Drigalsky's loop on the broth agar surface, and all discs were allowed to be thoroughly dried before the application on a bacterial lawn. The stock solution was then diluted to 3, 7, 15, 30, 65, 125, 250, 500, and 1000 mg/mL kale. Twenty milliliters of each dilution was impregnated into 6-mm sterile blank discs (once for each concentration) and placed at four equidistant points. The discs of tryptic soy broth and ampicillin antibiotic (250, 500, and 1000 mg/mL) were used as negative and positive controls, respectively. All plates were maintained at 25^oC for 2 h for pre-diffusion of the materials and then incubated at 37°C for 24 h under anaerobic conditions. The inhibition zone around each disc was then measured by the same operator in two perpendicular locations using a millimeter ruler (sliding calipers) with an accuracy of 0.5 mm. The size of the inhibition zone was calculated as follows: size of the inhibition zone = (diameter of the halo − diameter of the specimen) × $\frac{1}{2}$. All assays were conducted in triplicate, and the findings were recorded as the average diameter of the inhibition zone.

2.3. Antibacterial efficiency

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined in 96-well plates to assess the susceptibility of the oral pathogenic bacteria to kale extracts. For this purpose, 100 μL of 2–2.25 × 10^8 cfu of the bacterial suspension was added to 50 μL of fresh broth containing nine different concentrations of the tested compounds in each well. Tryptic soy broth and ampicillin antibiotic were again used as the negative and positive controls, respectively. The cultures were incubated at 37°C for 24 h under anaerobic conditions. After incubation, the plates were taken to a spectrophotometer to measure the OD and consequently calculate the average value. The absence of visible turbidity was considered to indicate the MIC. Thereafter, the MIC and MBC were determined via serial dilution (10^{-1} to 10^{-6}). It was subsequently inoculated (10^{-4}, 10^{-5}, and 10^{-6}) onto sterile nutrient agar plates and incubated for 24 h. These experiments were repeated three times. The lowest concentration at which no growth was observed was considered the MBC. The entire MIC and MBC tests were repeated for the third time, and the data were expressed using descriptive statistics.

2.4. Gene expression studies for antivirulence efficiency

S. intermedius ATCC 27335 was used, and the presence of the *ily* gene was confirmed via real-time polymerase chain reaction (PCR) testing. PCR testing was conducted using the synthesized cDNA as a template to compare the relative gene expression of *ily* after exposure to kale extracts. The internal control used was the 16S rRNA housekeeping gene. The primer pair was 16s-Fw (5-GGGGATAACTATTGGAAACG-3) and 16s-Bw (5-TCAGGTCGGCTATGTATCG-3). The *ily* gene was amplified using primer pair ILY-Fw (5GCAACTATCCAAAACAACAC-3) and ILY-Bw (5-GATTGTAGCCATTTCCACTC-3).[20] These were compared with nucleotide sequences on the BLAST website (www.ncbi.nlm.nih.gov/blast) to ensure the primer sequence. The strains were grown in tryptic soy broth and then incubated for 24 h with the MIC, subMIC, and MBC of kale; ampicillin, again used as the positive control; and tryptic soy broth, again used as the negative control. Thereafter, RNA was isolated using NucleoSpin® (Macherey Nagel, Duren, Germany) and quantified using NanoDrop™ 2000/200c Spectrophotometers (Thermo Fisher Scientific™, Waltham, MA, USA). Two micrograms of RNA was converted to cDNA using the QuantiTect® reverse transcription kit for 50 reactions. SYBR green real-time PCR was set up in a total of 100 reaction mixtures using the QuantiNova® SYBR green PCR kit. Primers were used to target the virulence gene (*ily*) and housekeeping gene (16S rRNA). The primers were used at 1.0 μM, and 2–5 μL of DNA template was added. The thermal cycling conditions were set up in the LightCycler® 480 II Real-Time PCR System (Roche, Basel, Switzerland), with the PCR initial activation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 20 s, and extension at 72°C for 25 s. The 16S rRNA gene was used as an internal control for data normalization. All reactions were run in triplicate.

2.5. Statistical analysis

Data were analyzed using the SPSS software (version 22.0, IBM, Chicago, IL, USA) and shown as means ± standard deviations (SDs) of three independent measurements. The statistical analysis was performed using one-way analysis of variance, followed by Tukey's multiple comparison test. Differences between the variables were considered significant at $P < 0.05$.

3. Results

In this in vitro study, the antibacterial and antivirulence effects of kale on *S. intermedius* were tested. To achieve this, we conducted agar disc diffusion and broth microdilution (MIC and MBC) tests and measured the relative gene expression of *ily* via RT-PCR testing. All assays were conducted in triplicate. Data are presented as means ± standard deviations.

3.1. Disc diffusion test

The antibacterial effect of kale on the selected oral pathogenic bacterial strains was determined by measuring the radius of the inhibition zone. This measurement showed that all concentrations of kale did not yield inhibition zones except for the concentration of 1000 mg/mL (Figure 1). Treatment with 1000 mg/mL kale yielded an inhibition zone of 11.12 ± 1.59 mm on *S. intermedius*. The inhibition zone achieved by ampicillin was significantly larger ($P < 0.05$) than that by 1000 mg/mL kale. A significant difference was noted between 1000 mg/mL and the other concentrations relative to *S. intermedius* growth. The mean ± SD values of the inhibition zones are shown in Table 1. However, the concentration of ampicillin in the present study could not predict the antibacterial efficacy of 1000 mg/mL kale, as shown in the trend line of prediction in Figure 2.

Table 1: Bacterial inhibition zones evaluated using the agar disc diffusion test

Treatment	Inhibition zone (mm)
1000 mg/mL kale	11.12 ± 1.59
500 mg/mL kale	0
250 mg/mL kale	0
125 mg/mL kale	0
65 mg/mL kale	0
30 mg/mL kale	0
15 mg/mL kale	0
7 mg/mL kale	0
3 mg/mL kale	0
Tryptic soy broth	0
250 mg/mL ampicillin	31.67 ± 0.46
500 mg/mL ampicillin	33.38 ± 0.67
1000 mg/mL ampicillin	35.76 ± 0.54

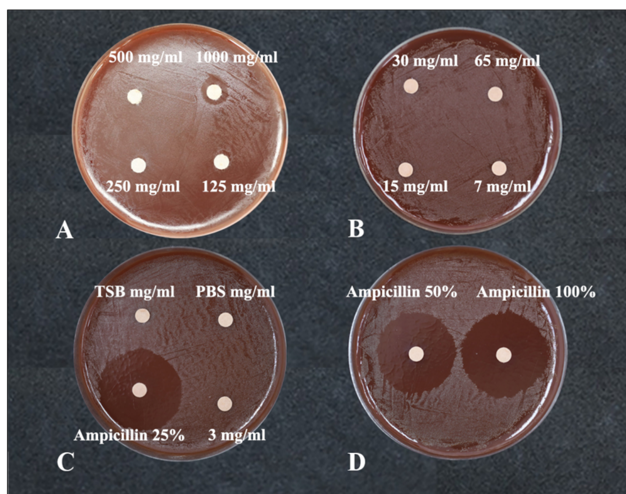

Figure 1: Antimicrobial activity of kale (*Brassica oleracea* var. *sabellica*) on *Streptococcus intermedius* evaluated using the disc diffusion test. **A**): Only 1000mg/ml kale yielded an inhibition zone; **B**): No inhibition zones present. **C and D**): Ampicillin treatment yielded inhibition zones

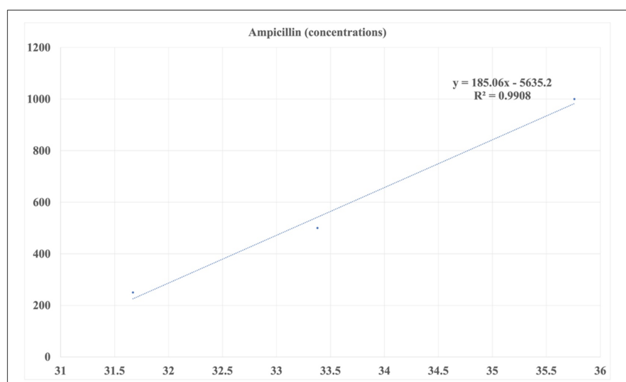

Figure 2: Trend line of prediction between 1000 mg/mL kale and ampicillin (y = 185.06x -5635.2), where x is the inhibition zone of 1000 mg/mL kale (11.12±1.59mm). The antibacterial efficacy of 1000mg/mL kale within this trend line could not be reliable predicted

3.2. Broth microdilution test

The MIC50, 70, and 90 values for kale were 15, 30, and 65 mg/mL, respectively. The MBC for 500 mg/mL kale was found to exhibit inhibitory effects on the bacterial growth and killing of *S. intermedius* (Figure 3). The difference in the antibacterial effect of each concentration of kale from 1000 to 125 mg/mL, 30 to 15 mg/mL, 7 mg/mL, and 3 mg/mL was significantly lower ($P < 0.05$), respectively. The number of colonies in each kale-treated compound for the selected bacterial strains is shown in Table 2.

Table 2: Antimicrobial activity of kale (*Brassica oleracea* var. *sabellica*) on *Streptococcus intermedius* evaluated using broth microdilution and serial dilution

Treatment	Number of colony (106)
1000 mg/mL kale	0
500 mg/mL kale	0
250 mg/mL kale	1.33 ± 2.30
125 mg/mL kale	4.67 ± 3.51
65 mg/mL kale	63.67 ± 13.32
30 mg/mL kale	279 ± 20.22
15 mg/mL kale	319.67 ± 55.20
7 mg/mL kale	660 ± 66.57
3 mg/mL kale	853.33 ± 104.33
Tryptic soy broth	1123.00 ± 68.99
250 mg/mL ampicillin	0
125 mg/mL ampicillin	0
1000 mg/mL ampicillin	0

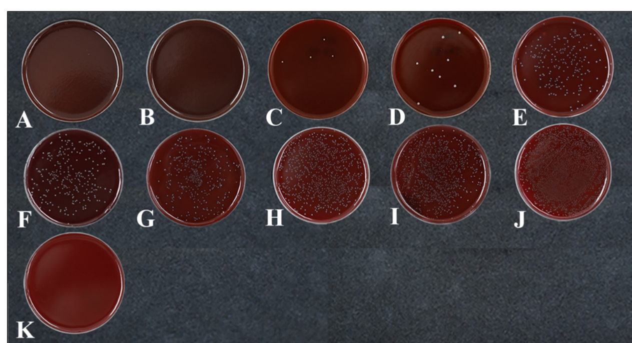

Figure 3: Bacterial colony count in serial dilution. (**A**): 1000 mg/ml kale, (**B**): 500 mg/mL kale, (**C**): 250 mg/mL kale, (**D**): 125 mg/mL kale, (**E**): 65 mg/mL kale, (F): 30 mg/mL kale, (**G**): 15 mg/mL kale, (H): 7 mg/mL kale, (**I**): 3 mg/mL kale, (**J**): Tryptic soy broth (negative control), (**K**): Ampicillin (Positive control)

3.3. Antivirulence gene expression in RT-PCR testing

The gene expression of *ily* was evaluated three times via real-time PCR testing. The SYBR green assay was performed with the *S. intermedius* strain, which is representative of the *ily* gene. The MIC, subMIC, and MBC of kale were determined against the *S. intermedius* strain. The 16S rRNA housekeeping gene was used as a control in the reaction setup. The cycle threshold was obtained from the real-time PCR analysis. The relative expressions of the types, with respect to fold increase or decrease, were determined in comparison with those of the control.

At the MIC, subMIC, and MBC of kale, the expression of the virulence factor was suppressed in a dose-dependent manner. The expression of *ily* significantly decreased ($P < 0.05$) by more than 50% at the concentration of 1000 mg/mL, as demonstrated in Table 3. The expression of *ily* was reduced at all concentrations, but the reduction was significant only at 1000 mg/mL.

Table 3: *Reverse-transcription polymerase chain reaction testing of the gene expression of the virulence factor of Streptococcus intermedius in the presence of kale*

Treatment	Gene expression ratio (2-ΔΔCt)
1000 mg/mL kale	0.41 ± 0.023
500 mg/mL kale	0.75 ± 0.1111
250 mg/mL kale	0.76 ± 0.17
125 mg/mL kale	0.92 ± 0.066
65 mg/mL kale	0.94 ± 0.06
30 mg/mL kale	0.92 ± 0.01
15 mg/mL kale	0.94 ± 0.123
7 mg/mL kale	0.91 ± 0.089
3 mg/mL kale	0.95 ± 0.16
Tryptic soy broth (control)	1.00
250 mg/mL ampicillin	0
125 mg/mL ampicillin	0
1000 mg/mL ampicillin	0

4. Discussion

Eliminating bacteria is crucial for the success of treatments for odontogenic infections, including surgical and non-surgical procedures and therapeutic medicines. Although many improvements have been achieved in recent years, the increasing antibiotic resistance has limited the use of therapeutic drugs in treating oral diseases and caused widespread bacterial infections.[21] Accordingly, the efficacy of kale in treating such conditions should be studied further to support its therapeutic application. In previous reports, kale has shown excellent antimicrobial activity against gram-positive and gram-negative bacteria. This efficacy is attributed to the potentially high content of phytochemicals and other bioactive substances. Phenolics isolated from kale leaves, which are rich in quercetin and kaempferol derivatives, have been found to substantially inhibit the growth of pathogenic bacteria, such as *Streptococcus pneumoniae, S. aureus, Enterococcus faecalis, Bacillus subtilis*, and *Moraxella catarrhalis*.[14,22] In the present study, kale demonstrated potent antibacterial activity by inhibiting the growth of *S. intermedius*. Ansar et al. reported that *B. oleracea* has potent antimicrobial activity against the gram-positive *Streptococcus* group, demonstrating an MIC of 25 μg/mL and an inhibition zone of 10.33 ± 0.57 mm. Conversely, for gram-negative microorganisms, the authors observed an MIC of <12.5 μg/mL, which is notably lower than our findings.[23] Sundaram et al. demonstrated an inhibition zone of *B. oleracea* var. *acephala* against *S. aureus* with a maximum concentration of 50 μL crude extract and copper nanoparticle of 16 and 24 mm, respectively.[19]

The *ily* gene is the most important virulence factor responsible for the invasion of cells through specific host receptors. Most studies have shown that this gene is predominantly associated with deep-seated purulent

infections.[7,24] Thus, it was crucial to test our compound against the potentially virulent strains of *S. intermedius* to check its efficacy in treating deep-seated odontogenic infections. The effects of kale on the expression of virulence factors of pathogenic organisms have been studied to support its application in medical microbiology. To our knowledge, the present study is the first to demonstrate the effects of kale on the virulence factor of *S. intermedius*, a causative agent for deep-seated odontogenic infections, in the field of dentistry. Previously, natural compounds isolated from plant naturals have shown a target bacterial virulence factor.[25] The effects of polyphenolic compounds and derivatives on strains of *S. aureus* and *S. pneumoniae* are promising. These bacteria are known to harbor toxins, such as hemolysin, which is a pore-forming toxin similar to *ily.*[26] In the present study, kale inhibited the expression of the *ily* gene dose-dependently. However, the expression of the *ily* gene was significantly reduced at the concentration of 1000 mg/mL and decreased progressively with decreasing concentrations starting from 500 mg/mL. Additionally, the gene expression was not impacted by the suppressed bacteria at each concentration. These findings suggest that kale could prevent the pathogenicity of the bacteria.

The antimicrobial activity of compounds can be affected by many factors, such as intrinsic factors (e.g., molecular weight, degree of deacetylation, and solubility), microbial factors (e.g., bacterial type, species, and age), and extrinsic factors (e.g., pH and temperature). The methods selected for testing the antimicrobial activity of kale can also influence the findings obtained.[27] The molecular weight of kale and its diffusion and solubility in tryptic soy agar influence the rate of antimicrobial diffusion through the agar. Compounds with a lower molecular weight diffuse faster than do larger molecules. A combination of these factors produces a unique breakpoint zone size for each antibiotic, which indicates susceptibility to the antimicrobial compound.[28]

Our study has some limitations, primarily stemming from the difficulty in assessing odontogenic bacteria within the Research Center of the Faculty of Dentistry, Chiang Mai University. This difficulty led to the investigation of only one species. Although collecting oral pathogens directly from patients could offer a better evaluation method, we attempted to recover some bacteria from the bacterial glycerol stock. However, only gram-positive facultative anaerobe bacteria, specifically *S. intermedius*, survived under the experimental conditions; no other relevant bacteria of interest were obtained.

In summary, our study found that kale reduced the growth of bacteria through multiple products of polyphenolic compounds. It demonstrated both bacteriostatic and bactericidal activities. The compound inhibited the expression of the major virulence factor—*ily*. By suppressing the expression of this virulence factor, kale could provide health benefits in patients with odontogenic infections. Apart from inhibiting the growth of *S. intermedius* and suppressing the expression of the virulence factor, kale could have other unknown mechanisms. Further in vivo studies are needed to examine the effect of kale and its mechanism of action.

5. Conclusion

Kale can effectively inhibit the growth of *S. intermedius* and suppress the expression of the virulence factor of the bacterium, which is mainly involved in deep-seated odontogenic infections. Kale has promising antibacterial activity, making it a potential adjunct treatment for odontogenic infections wherein antibiotic resistance is a severe problem. Further research is needed to precisely determine how this useful plant derivative exerts its effect on the mechanisms involved in the progression of diseases.

6. Source of Funding

None.

7. Conflicts of Interest

All authors declare no conflicts of interest.

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