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Antibiofilm Effect of *Siegesbeckia pubescens* against *S. mutans* According to Environmental Factors

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Abstract: *Siegesbeckia pubescens* has long been used as a traditional medicine in Asia. In traditional prescriptions, it has been used mainly for its anti-inflammatory properties. Still, comparative research on its activity according to its origin has not yet been conducted. This study used estimated 30% ethanol extracts of *S. pubescens* from Yeongcheon, Chungju, and Suncheon in Korea for their antibacterial properties, glucosyltransferase (GTase) activity, organic acid production, biofilm formation, and antioxidant properties. As a result, it was determined that the minimum inhibitory concentration (MIC) against periodontitis-associated pathogens was ≥0.2 mg/mL, the antibiofilm effect was ≥80%, and the minimum IC_{50} values of the extracts were 39.64 μg/mL for DPPH and 172.1 μg/mL for the ABTS radical, and 17.73 μg/mL for SOD-like activity. The comparative analysis of climate data confirmed that the activities were inversely proportional to precipitation and humidity and directly proportional to temperature. Therefore, in light of the effect of the environment on the production of more effective and superior varieties, it is anticipated that the value of *S. pubescens* as a preventive treatment for periodontitis will increase and contribute to the development of more effective varieties.

Keywords: *Siegesbeckia pubescens*; antibacterial; antibiofilm; antioxidant; periodontitis

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1. Introduction

Periodontitis is an inflammation of periodontal tissue that can lead to tooth loss. However, the only solutions to the condition are treatment and prevention. If left untreated for an extended time, it can lead to various cardiovascular diseases and oral cancer [1]. Furthermore, if free radicals are not scavenged normally, oxidative stress builds up in cells and causes irreversible damage [2]. Consequently, it is possible to prevent and treat oral diseases by reducing oxidative stress and removing free radicals via antioxidant action.

In addition to preventing bacteria from being attacked by antimicrobial agents, biofilm formation suppresses normal immune responses to cause inflammation [3]. It protects bacteria to enable their survival in harsh environments, causing various chronic diseases and even medical device malfunctions. Furthermore, the oral cavity is home to the most commonly observed biofilm [4]. Calculus is a representative example of biofilm formation in periodontitis.

Furthermore, when sucrose is used as a substrate, glucosyltransferase (GTase) is synthesized as a water-insoluble glucan with strong adhesion. The synthesized water-insoluble glucan adheres firmly to the surface of the tooth, allowing oral microorganisms to attach and form a biofilm [5]. Oral microbes attach to biofilms and decompose sugars to produce acids as metabolites, which lower the pH of the oral cavity and cause dental caries [6]. Furthermore, bacteria that induce periodontal inflammation, including *Porphyromonas gingivalis*, *Treponema denticola*, and *Campylobacter gracilis* [7], lead to gum inflammation and later bleeding and even secondary infections.

Siegesbeckia pubescens is widely distributed throughout East Asia and has been used as a traditional medicinal herb since ancient times [8]. It is commonly found in fields or on roadsides, and its leaves and stems have been widely used as medicines. In traditional prescriptions [9], *S. pubescens* is known to have effects such as quadriplegia, arthritis, stroke, headache, high blood pressure, dizziness, jaundice, acute hepatitis, swelling, and itching of the skin [10]. Recently, additional effects such as antioxidant [11] and antitumor [12] activities have been reported. Nevertheless, research on the antibacterial and antibiofilm activities of *S. pubescens* based on the production region is insufficient.

According to a study conducted by Pacheco et al. [13], the total flavonoid content of *Stevia salicifolia* showed an increasing trend with decreasing temperature and precipitation. Additionally, Pietta [14] and Amic et al. [15] discussed the antioxidant properties of flavonoids and associated mechanisms. In contrast, Li et al. [16] confirmed that, except for a few plant species, the content of secondary metabolites increased in most plants at high temperatures and low soil moisture conditions [17]. Therefore, due to the confirmation of the antibacterial, antibiofilm, and antioxidant activities of *S. pubescens* according to the production area, it is anticipated that this study will increase the value of *S. pubescens* in treatment modalities by providing the data necessary for industrial utilization and identification to produce more efficient and excellent varieties.

2. Materials and Methods

2.1. Plant Material Preparation and Indicator Microorganisms

S. pubescens samples were harvested and dried in three different places in Korea, labeled by region as Yeongcheon (SPY), Chungju (SPC), and Suncheon (SPS). The dried *S. pubescens* was extracted with 30% ethanol (EtOH) at room temperature. The extracts were filtered with Whatman filter paper No.1, spray-dried, and then stored in the Quality Standardization Based Botanical Drug Development Center, Kyunghee University, Korea. Pathogens were obtained from the Korean Agricultural Culture Collection (KACC) and the Korean Collection for Type Cultures (KCTC). Climate-associated data were obtained from the Open Meteorological Data Portal of the Korea Meteorological Administration (KMA).

2.2. High-Performance Liquid Chromatography (HPLC) Analysis

SPY, SPC, and SPS were prepared at a concentration of 2 mg/mL in 50% methanol. Serial dilutions of the standard compound (kirenol) in methanol were prepared. HPLC was performed on a Dionex Chromeleon^{TM} chromatography data system with P580 and UVD100 detectors (Thermo Fisher Scientific Inc., Waltham, MA, USA). Chromatographic separation was conducted on an Inno C-18 column (5 μm, 4.6 × 250 nm; Young Jin). The column temperature was 25 °C, the flow rate was 1.0 mL/min, and the injected volume was 10 μL.

2.3. Inhibition of GTase Activity

GTase activity was determined by spectrophotometry of water-insoluble glucan produced using sucrose as a substrate. The *S. mutans* culture medium was centrifuged at 4000 rpm for 30 min, and then the pH of the supernatant was adjusted to 7.0. The GTase solution was prepared by adding 0.02% sodium azide and 1 mL of 2.0% sucrose, and 50 μL of *S. pubescens* extracts was added to 0.5 mL of the GTase solution, and reacted at 37 °C for 24 h. Following this step, absorbance at 600 nm was measured using a microplate reader (Molecular Devices FilterMax F5; San Francisco, CA, USA).

2.4. Inhibition of Acid Production

SPY, SPC, and SPS were added to Brain Heart Infusion broth with 1% sucrose, and *S. mutans* was inoculated at 1 × 10^6 CFU/mL. After incubation under anaerobic conditions at 37 °C and 24 h, the pH was measured using a pH meter (Hanna Instruments Ltd., Woonsocket, RI, USA) to observe the effect of inhibiting acid production, and cell

growth was measured using a UV/VIS spectrophotometer (Mecasys Co., Ltd., Daejeon, Republic of Korea).

2.5. Inhibition of Biofilm Formation

In order to confirm the inhibitory ability for biofilm formation, the experiment was conducted according to Zayed et al. [18]. SPY, SPC, and SPS and culture broth were prepared by adding 1% sucrose. Extracts were added to the *S. mutans* culture broth with 1×10^6 CFU/mL and incubated for 24 h under anaerobic conditions at 37 °C. The supernatant was removed, washed with distilled water, stained with 0.1% crystal violet for 15 min, and washed again three times with distilled water. Next, EtOH was used to dissolve and extract the biofilm, which was measured with a microplate reader at a wavelength of 595 nm.

2.6. Antibacterial Activities

The pathogens obtained from KACC and KCTC were performed using the standard disc diffusion method. Indicator bacteria (1×10^6 CFU/mL) were inoculated on Mueller–Hinton Agar plates (KisanBio Co., Ltd., Seoul, Republic of Korea). Next, 100 µL of the dissolved extract was loaded onto Whatman No.1 sterile filter paper disks (Whatman, Kansas, MO, USA) and allowed to dry for 30 min. The dried paper disks were placed onto a plate. Afterward, the plates were cultured at 30 °C for 24 h. Antibacterial activity was evaluated by measuring the radius of the inhibition zone against the tested bacteria.

The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of SPY, SPC, and SPS were estimated using the 96-well microplate dilution method. Next, 100 µL of pathogens, including *S. mutans*, was added to the sample tube so that the final concentration was 1×10^6 CFU/mL and incubated at 37 °C for 24 h. The MIC was determined to be the concentration in the test samples, where the visual growth of the indicators was observed. Additionally, MBC was determined to be the concentration in the test samples, where it did not show visual growth of pathogens.

2.7. Radical Scavenging Activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was used to measure antioxidant activity. The mixture of 0.1 mM DPPH solution in methanol solution and 31.25–1000 µg/mL of varying concentrations were processed at 37 °C for 30 min. The decrease in absorbance caused by radical scavenging capacity was measured using a 595 nm microplate reader.

The 2,2′-azino-bis-(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS) assay was determined using a reagent. The protocol was followed from the previous study with a slight modification. Next, the reaction of the mixture of ABTS solution and the diluted sample with different concentrations was processed at 37 °C for 30 min. The reduction of blue-green ABTS+ resulted in the hydrogen-donating antioxidant and was read using a microplate reader at 405 nm. The radical scavenging activity was determined using the following formula:

$$\text{DPPH and ABTS radical inhibition (\%)} = [(\text{OD0} - \text{ODx})/\text{OD0}] \times 100$$

where OD0 was the optical density measured for the negative control and ODx, which was measured for the different concentrations of SPY, SPC, and SPS or ascorbic acid concentrations.

2.8. Superoxide Dismutase (SOD)-like Activity

The SOD-like activity measurement was performed with a commercial kit (SOD kit; DoGenBio Co., Ltd., Seoul, Republic of Korea) and measured according to the manufacturer's instructions.

2.9. Statistical Analysis

Data were analyzed using a Statistical Analysis System (GraphPad Prism 5; Boston, MA, USA). All quantitative data are expressed as mean ± SDs. The significance

of the differences was determined using a one-way analysis of variance with the Student–Newman–Keuls test for multiple comparisons. Statistical significance was considered at $p < 0.05$. All experiments were performed independently in triplicate.

3. Results

3.1. Quantitative Analysis of Kirenol from SPY, SPC, and SPS

A quantitative analysis for kirenol in the SPY, SPC, and SPS revealed a peak at the same retention time of the standard kirenol (kirenol: 24.557 min, SPY: 24.567 min, SPC: 24.563 min, and SPS: 24.567 min) (Figure 1). The kirenol content of the SPY, SPC, and SPS was 10.68 ± 0.07 mg/mL, 10.52 ± 0.15 mg/mL, and 8.18 ± 0.005 mg/mL, respectively.

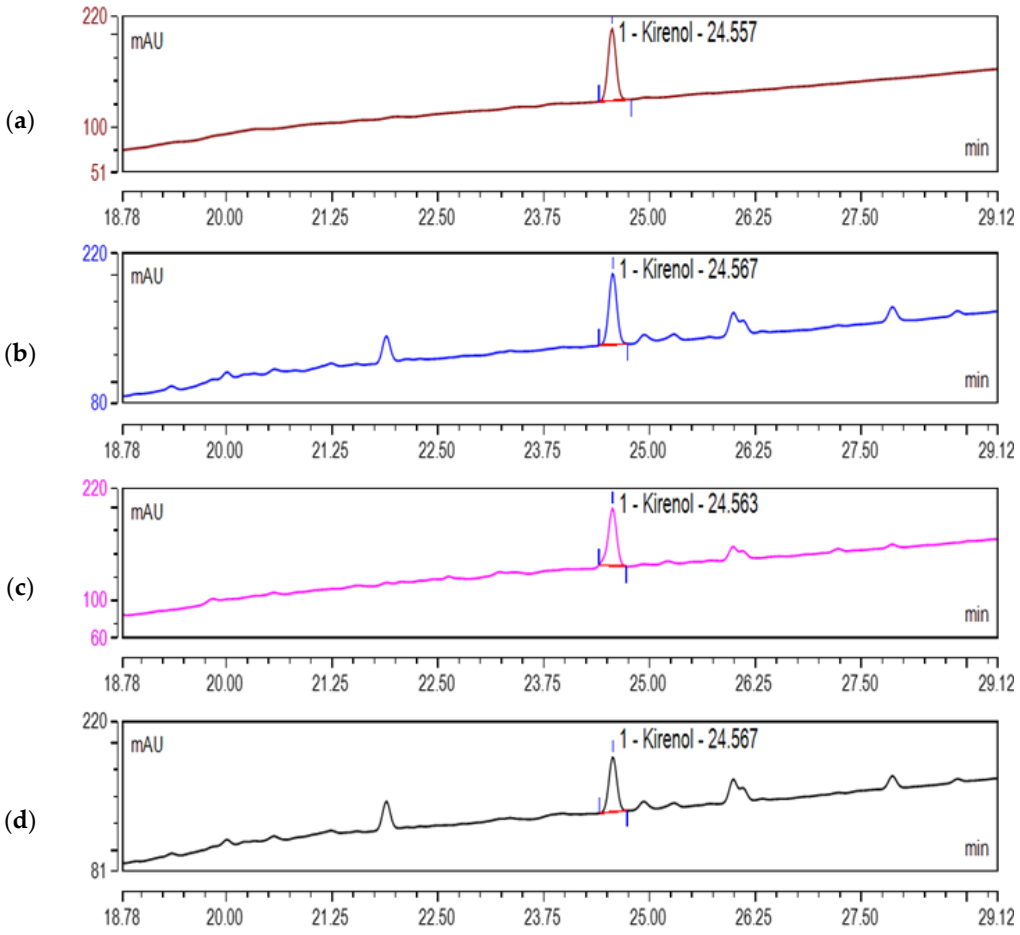

Figure 1. High-performance liquid chromatography (HPLC) analysis of kirenol standard (**a**) and the kirenol content of SPY (**b**), SPC (**c**), and SPS (**d**).

3.2. Antibacterial Activity of S. pubescens against Periodontitis-Associated Pathogens

The antibacterial activities of SPY, SPC, and SPS were tested against strains by a disc diffusion assay. All these extracts showed a clear zone with a diameter of 9.5–15.5 mm against periodontitis-associated pathogen strains (Table 1). The MIC and MBC assays were utilized to determine the required concentrations of SPY, SPC, and SPS against pathogen strains associated with periodontitis. The results indicated that the MIC of SPY, SPC, and SPS required to kill periodontitis-associated pathogens was at least 0.20 mg/mL, and the MBC required to kill periodontal pathogens was at least 0.40 mg/mL (Table 2).

Table 1. Antibacterial activity against periodontitis-associated pathogens by disc diffusion assay.

Indicators	AMX [1]	SPY	SPC	SPS
Streptococcus mutans KACC 16833	15.0 [2]	12.0	11.0	10.0
Streptococcus sanguinis KACC 11301	15.5	15.0	13.0	11.5
Streptococcus downei KACC 13827	15.0	14.0	13.0	12.0
Streptococcus gordonii KACC 13829	15.0	15.5	13.0	12.0
Streptococcus ferus KACC 13881	15.0	15.0	12.0	10.5
Streptococcus mitis KACC 16832	15.0	14.0	11.5	9.5
Porphyromonas gingivalis KCTC 5352	15.0	14.0	12.5	11.0
Treponema denticola KCTC 15104	15.5	15.5	12.0	10.5
Campylobacter gracilis KCTC 15224	15.0	13.0	11.0	10.5
Campylobacter rectus KCTC 5636	15.0	13.0	11.0	9.5
Fusobacterium nucleatum KCTC 2640	15.0	14.5	12.5	10.0
Parvimonas micra KCTC 15021	15.0	14.5	11.0	10.5
Prevotella intermedia KCTC 15693	14.5	11.0	11.0	10.5
Prevotella nigrescens KCTC 15081	15.0	14.5	12.0	11.0
Aggregatibacter actinomycetemcomitans KCTC 2581	15.5	12.0	11.0	10.0
Eikenella corrodens KCTC 15198	14.5	12.5	11.0	10.5

[1] Amoxicillin. [2] The unit of the number is millimeters.

Table 2. MIC and MBC of *S. pubescens* against periodontitis-associated strains.

Indicators	SPY		SPC		SPS	
	MIC	MBC	MIC	MBC	MIC	MBC
S. mutans KACC 16833	≥0.31 [1]	0.63	≥3.13	6.30	≥18.75	37.50
S. sanguinis KACC 11301	≥0.31	0.63	≥0.80	1.60	≥18.75	37.50
S. downei KACC 13827	≥0.20	0.40	≥0.20	0.40	≥18.75	37.50
S. gordonii KACC 13829	≥0.31	0.63	≥0.40	0.80	≥18.75	37.50
S. ferus KACC 13881	≥1.30	3.00	≥3.13	6.30	≥18.75	37.50
S. mitis KACC 16832	≥0.40	0.80	≥1.60	3.13	≥9.38	18.75
P. gingivalis KCTC 5352	≥0.20	0.40	≥0.40	0.80	≥18.75	37.50
T. denticola KCTC 15104	≥0.20	0.40	≥0.40	0.80	≥18.75	37.50
C. gracilis KCTC 15224	≥0.40	0.80	≥0.80	1.60	≥18.75	37.50
C. rectus KCTC 5636	≥0.40	0.80	≥0.40	0.80	≥18.75	37.50
F. nucleatum KCTC 2640	≥0.20	0.40	≥0.20	0.40	≥18.75	37.50
P. micra KCTC 15021	≥0.40	0.80	1.60	3.13	≥18.75	37.50
P. intermedia KCTC 15693	≥0.40	0.80	≥0.80	1.60	≥18.75	37.50
P. nigrescens KCTC 15081	≥0.40	0.80	≥0.80	1.60	≥18.75	37.50
A. actinomycetemcomitans KCTC 2581	≥0.40	0.80	≥0.20	0.40	≥18.75	37.50
E. corrodens KCTC 15198	≥0.20	0.40	≥0.40	0.80	≥9.38	18.75

[1] The unit of the number is milligrams/milliliter.

3.3. Inhibition of GTase Activity

GTase is an essential prerequisite for the formation of oral biofilms. In order to confirm the GTase-inhibiting ability of SPY, SPC, and SPS, the GTase activity was measured in three samples treated with varying concentrations of each MIC value exhibiting antibacterial activity and incubated for 24 h in the presence of 2.0% sucrose.

It was confirmed that GTase activity was inhibited in a concentration-dependent manner in SPY, SPC, and SPS. In particular, SPY inhibited approximately 82.33% of GTase activity at a concentration of 1.25 mg/mL, SPC inhibited approximately 80.75% at 3.13 mg/mL, and SPS inhibited approximately 83.22% at 9.38 mg/mL (Figure 2).

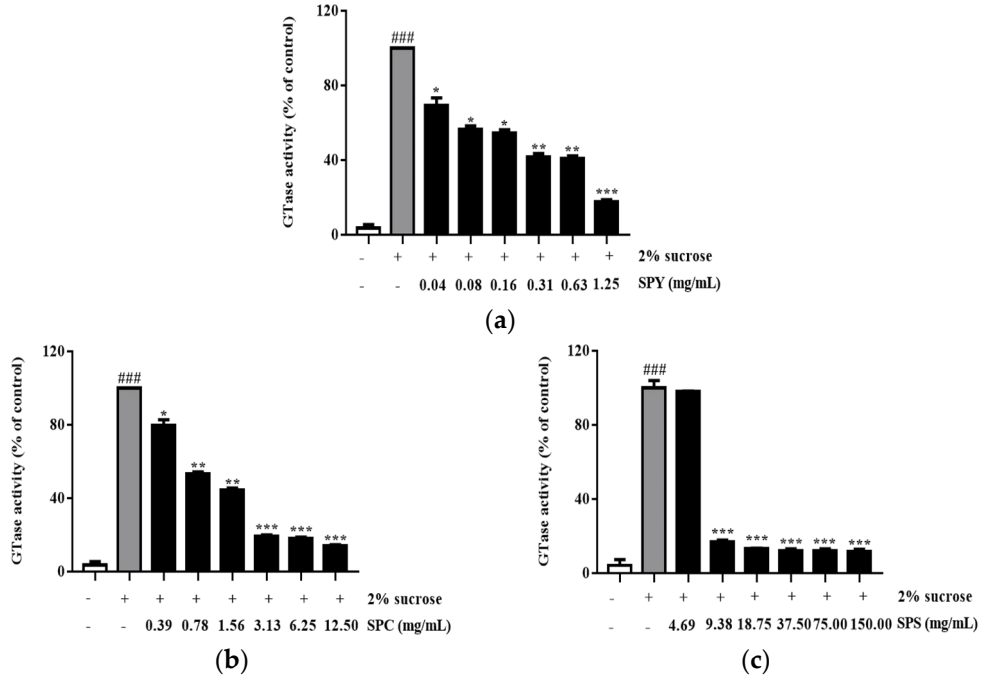

Figure 2. Effect of glucosyltransferase activity treated with extracts of *S. pubescens*: SPY (**a**), SPC (**b**), and SPS (**c**). The GTase activity was determined by spectrophotometry of water-insoluble glucan produced by using sucrose as a substrate. GTase was pretreated with the indicated concentration of SPY, SPC, and SPS for 24 h. Values are mean ± standard deviations (SD); ### $p < 0.001$ versus the non-treated group; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus the 2% sucrose-treated group.

3.4. Inhibition of Acid Production of S. mutans

The pH was measured using a pH meter and a UV spectrometer to identify the inhibited organic acids produced by *S. mutans*. As a result, SPY showed acid inhibition and inhibition of *S. mutans* growth at a concentration of 0.31 mg/mL, SPC showed this at 1.56 mg/mL, and SPS showed apparent acid inhibition effects at 18.75 mg/mL (Figure 3 and Table 3).

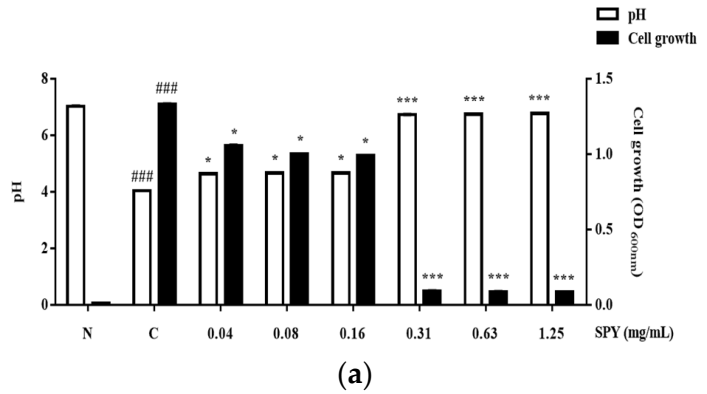

Figure 3. *Cont.*

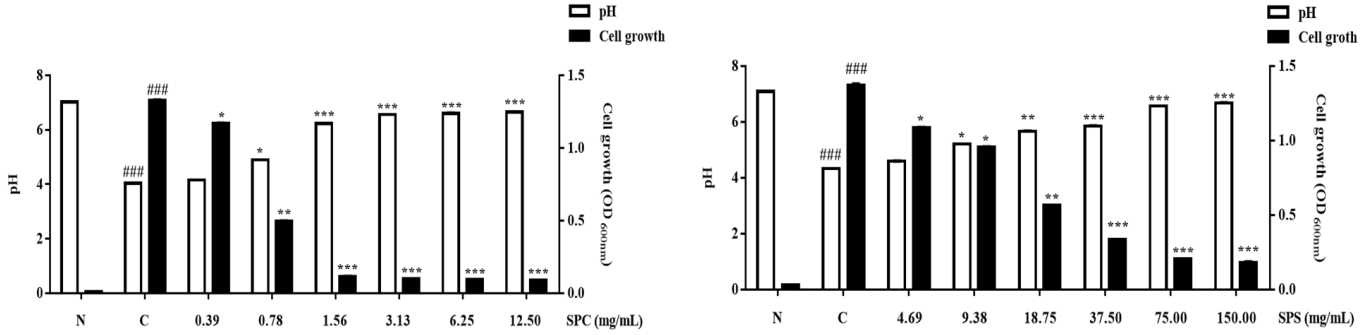

Figure 3. Measurement of pH and cell growth of *S. mutans* treated with *S. pubescens* extracts: SPY (**a**), SPC (**b**), and SPS (**c**). After treating *S. mutans* with the MIC value of SPY, SPC, and SPS for 24 h, pH and cell growth were measured. The values are mean ± standard deviations (SDs); ### $p < 0.001$ versus the non-treated group; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus the 1% sucrose-treated group.

Table 3. Rate of change regarding pH and growth.

Rate of Change	SPY	SPC	SPS
pH increase (%)	14.91	3.10	6.03
	15.42	21.44	20.35
	15.56	54.26	30.83
	66.68	62.52	35.13
	66.94	63.72	51.76
	67.73	64.98	54.20
Cell growth decrease (%)	20.60	12.14	20.92
	25.00	62.86	30.39
	25.60	91.54	58.89
	93.16	92.67	75.73
	93.53	92.93	85.13
	93.80	93.35	86.73

3.5. Antibiofilm Activity of S. pubescens

S. mutans attachment onto the surface of the tooth has been implicated as a primary causative agent of dental caries in humans. The results of the examination of whether SPY, SPC, and SPS have inhibitory effects on biofilm formation are shown in Figure 4: the result of SPY showed inhibitory effects of approximately 84.61%, 84.51%, and 86.08% at the concentrations of 0.31, 0.63, and 1.25 mg/m; SPC showed 83.48% and 88.40% at 6.25 and 12.50 mg/mL; and SPS showed about 82.98%, 87.06%, 88.26%, 90.11%, and 92.27% inhibitory effects from 9.38 to 150.00 mg/mL.

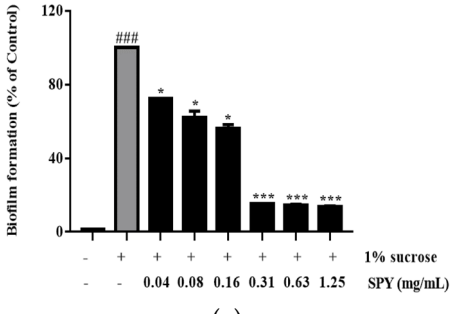

Figure 4. *Cont.*

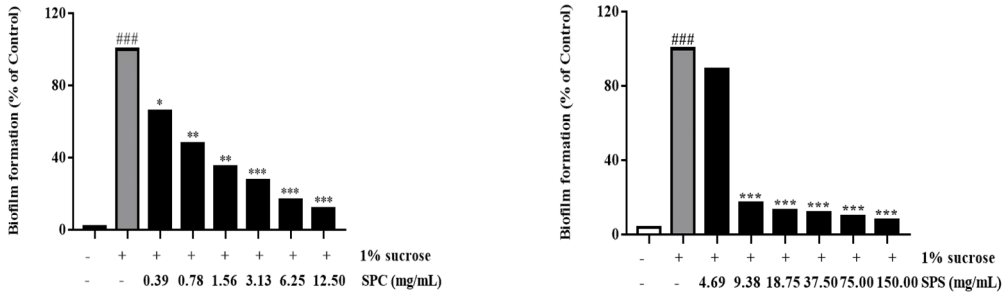

Figure 4. Effect of biofilm formation inhibition treated with extracts of *S. pubescens*: SPY (**a**), SPC (**b**), and SPS (**c**). Biofilm formation from SPY, SPC, and SPS in *S. mutans* including 1% sucrose, and incubation for 24 h; the rates of biofilm formation rates were measured with a microplate reader. Values are mean ± SD; ### $p < 0.001$ versus the non-treated group; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus the 1% sucrose-treated group.

3.6. Radical Scavenging Activities of S. pubescens Extracts

To confirm the SPY, SPC, and SPS antioxidant activities by DPPH and ABTS, radical scavenging assays were performed. As shown in Figure 5a,b, the positive control ascorbic acid showed IC_{50} scavenging activity values of 3.15 and 34.67 μg/mL. SPY, SPC, and SPS showed scavenging activities at DPPH IC_{50} values of 54.22, 71.87, and 39.64 μg/mL, and 214.1, 215.9, and 172.1 μg/mL for the ABTS radical, respectively. These findings reveal that *S. pubescens* exhibited a potent antioxidant effect.

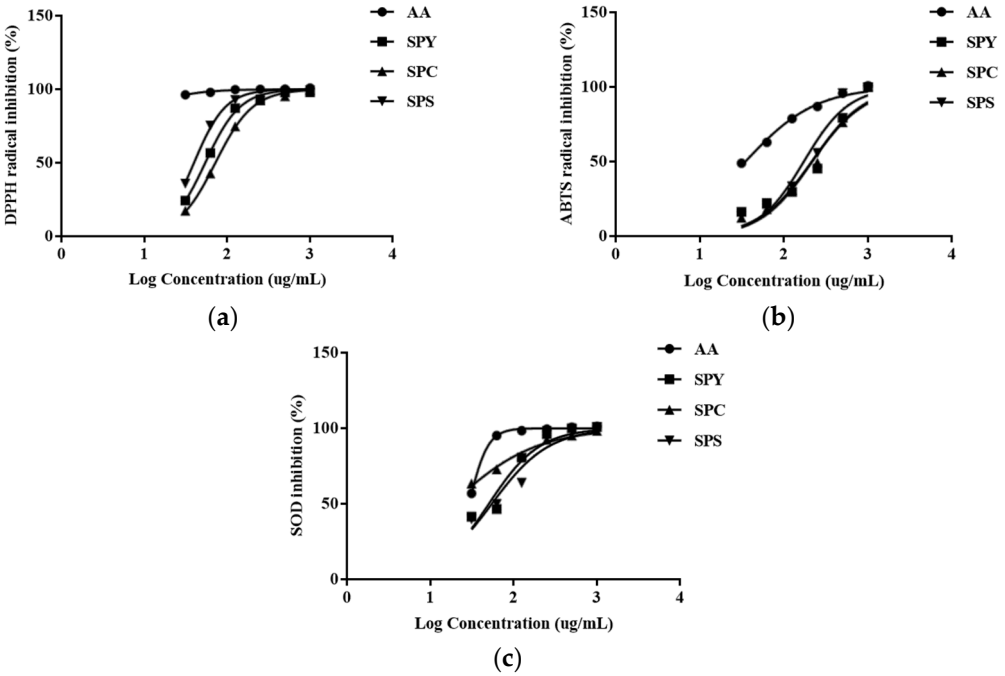

Figure 5. DPPH, ABTS, and SOD-like antioxidation activities of SPY, SPC, and SPS: DPPH radical scavenging (**a**), $ABTS^+$ cation scavenging (**b**), and SOD-like activity (**c**). Ascorbic acid was used as a positive control. Data are expressed as the mean ± SD of the results of three replicates.

3.7. SOD-Like Activity of S. pubescens Extracts

SOD was measured in SPY, SPC, and SPS using ELISA kits based on the WST-8 method. Ascorbic acid was used as a positive control because it stabilizes the enzyme activity of SOD. The findings revealed that SPY, SPC, and SPS have the SOD quench superoxide anion, which increases SOD activity in a dose-dependent manner (Figure 5c). The IC_{50} of SPY,

SPC, and SPS was 50.34, 17.73, and 54.52 μg/mL, respectively; as a positive control, the ascorbic acid IC_{50} value used was 29.04 μg/mL.

4. Discussion

S. pubescens is a plant widely distributed throughout East Asian countries and has long been used as medicine in traditional prescriptions. However, insufficient research has been conducted on the antibacterial and antibiofilm activity of *S. pubescens* in relation to its production region. In this study, the effect of environmental factors on the antibacterial and antibiofilm effects of *S. pubescens* and the consequences of antibacterial and antibiofilm activity on SPY, SPC, and SPS obtained from different production areas were investigated and compared. Additionally, the antioxidant activities of the extracts were measured by DPPH, ABTS radical scavenging, and SOD-like assays.

According to Akula and Ravishankar [19], secondary metabolites of plants are a unique source of pharmaceuticals, food additives, flavors, and biochemicals with industrial significance. Li et al. [16] have studied how environmental stress alters the amount or type of secondary metabolites produced by various plants. These studies have shown that secondary metabolites play an important role in adapting plants to the environment and overcoming stress conditions. Therefore, statistical results on the effects of environmental factors are needed, and based on the results, improvements in functionality and species can be secured. Additionally, it is precious to secure a way to improve quality in preparation for use of a raw material in medicine. According to the climate data presented in Table 4 of the KMA Open MET Data Portal, Yeongcheon has the highest average temperature, while Suncheon has the most increased precipitation and humidity. Based on these climatic results, we can predict that SPY has the best antibiofilm effect.

Table 4. Five-year climate data by region.

Region (2016–2022)	Average Temperature (°C)	Total Precipitation (mm)	Relative Humidity (%)	Total Sunshine (h)
Yeongcheon	13.51 *	1011.66	64.13	2318.27
Chungju	12.23	1201.54	67.03	2274.07
Suncheon	13.16	1401.80	72.64	2216.11

* All data mean annual average value.

Periodontal disease is a collective term for diseases caused by the proliferation of pathogenic bacteria in the mouth as a result of poor dietary practices. Dental caries is a bacterial infectious disease in which the primary causative bacteria, *S. mutans*, produce a biofilm by lowering the pH of the oral cavity with sucrose to generate an acidic environment and tooth erosion. In periodontal crevices, there are numerous pathogens, including *S. mutans*, which are primarily responsible for dental caries [20]. Inhibiting pathogen growth can prevent periodontal disease effectively. The three *S. pubescens* extracts exhibited an inhibition zone for dental caries and periodontal-associated pathogens, including *S. mutans*, as determined by the disc diffusion method. SPY exhibited a range of 11–15.5 mm, SPC exhibited a range of 11–13 mm, and SPS exhibited a range of 9.5–12 mm (Table 2). In addition, SPY, SPC, and SPS inhibited *S. mutans* growth by more than 70% at concentrations of 0.31, 1.56, and 37.50 mg/mL, respectively, to adjust the pH of the culture medium close to neutral (Figure 3).

In the initial stages of dental caries formation, plaque biofilms on tooth surfaces are constructed synergistically by the action of GTase. Exopolysaccharides produced by GTase of *S. mutans* and β-1,3-glucan from *C. albicans* contribute to forming biofilms [21,22]. Herein, we confirmed that SPY, SPC, and SPS inhibit the production of GTase, which is a crucial step in biofilm formation. SPY inhibited GTase activity by more than 80% at 1.25 mg/mL, SPC inhibited at 3.13 mg/mL, and SPS inhibited at 9.38 mg/mL, demonstrating a concentration-dependent suppression (Figure 2). In addition, 0.31 mg/mL SPY inhibits the formation of approximately 84.61% of biofilm, 6.25 mg/mL SPC inhibits 83.48%, and 9.38 mg/mL

SPS inhibits 82.98%, as determined by testing the MIC concentrations of SPY, SPC, and SPS (Figure 4).

Free radicals, which cause periodontal disease and are produced throughout its progression, can cause tissue damage and exacerbate the condition [23]. These free radicals destroy tissue cells irreversibly and can cause exacerbate disease [24]. Consequently, it can protect gum health by suppressing oxidative stress via antioxidant activity. Herein, we demonstrate that SPY, SPC, and SPS possess antioxidant properties by neutralizing DPPH, ABTS, and SOD-like radicals. SPY, SPC, and SPS inhibited DPPH and ABTS radicals with respective IC_{50} values of 54.22, 71.87, and 39.64 μg/mL and 214.1, 215.9, and 172.1 μg/mL (Figure 5a,b). In addition, SPY, SPC, and SPS exhibited SOD-like inhibition with IC_{50} values of 50.34, 17.73, and 54.52 μg/mL (Figure 5c).

Our study results indicate that SPY, SPC, and SPS positively affect antibacterial and antibiofilm activities. In addition, a crucial antioxidant effect in preventing and treating periodontitis was also confirmed. Overall, we demonstrated that, as anticipated, SPY has the highest and SPS has the lowest antibiofilm effect. Based on these findings, if the temperature is raised appropriately and the relative humidity is maintained below a certain level, it can contribute to the development of medicinally active varieties, and early treatment of periodontitis can prevent its progression.

5. Conclusions

In conclusion, *S. pubescens* generally exhibits antibacterial and antibiofilm activities against periodontitis-associated strains. However, there are certain differences in activity depending on the local environment of the production region, and more effective varieties can be improved if the factors and ranges are identified. These findings suggest that more effective preventive treatments can be developed for periodontal disease.

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