Medical Research Archives

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Published: November 30, 2022

Citation: Abachi S., Rathgeber B., et al., 2022. Inhibition and Eradication of Streptococcus Pyogenes Biofilm by Phytochemical Extracts of Cranberry and Sumac Berry, Medical Research Archives, [online] 10(11). https://doi.org/10.18103/mra.v10i11.3316

DOI: https://doi.org/10.18103/mra.v10i11.3316

ISSN: 2375-1924

RESEARCH ARTICLE

Inhibition and Eradication of Streptococcus Pyogenes Biofilm by Phytochemical Extracts of Cranberry and Sumac Berry

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ABSTRACT

Background: Diverse infections occur due to specific virulence factors of *Streptococcus pyogenes,* such as surface proteins and their biofilm formation capacity. This study aimed to assess the anti-biofilm effects of phytochemical-rich fruit extracts of cranberry (*Vaccinium macrocarpon*) and sumac berry (*Rhus typhina*).

Methods: Biofilm inhibition and eradication potential of the berries were tested by metabolic activity measurement and viable cell count technique as well as visualization using scanning electron microscopy (SEM).

Results: Minimum biofilm inhibitory concentration and minimum biofilm eradicating concentration of cranberry and sumac berry extracts ranged from 1 to >4 mg mL^{-1} and 2 to >16 mg mL^{-1}, respectively. The effects of these extracts on the pre-formed biofilms and biofilm formation were imaged. The scanning electron microscopy images were indicative that the extracts could affect the integrity of bacteria hence quenching the biofilm formation capacity of *S. pyogenes*

Conclusion: Further investigations on quorum sensing and exopolysaccharide formation can confirm the anti-biofilm potency of extracts.

Keywords: streptococcal pharyngitis, biofilm, *Rhus typhina*, *Vaccinium macrocarpon*

Introduction

There are several important steps for the initiation of group A Streptococci (GAS) infectious diseases. Among them, the capacity of bacteria to adhere to the host tissue and subsequently compete with normal microbial flora of the nasopharynx plays an important role. After successful attachment, bacteria establish interactions with salivary glycoproteins, extracellular matrix, serum components, host cells, and other microbes and then assemble in cell aggregates. Bacteria then begin to multiply, forming microcolonies that would within a short period differentiate into exopolysaccharide (EPS)-encased communities, which are called mature biofilm. EPS, among other functions, prevents the access of antibiotics to the bacterial cells embedded in the community. It has been reported that streptococci adhere in two steps: (i.) weak reversible adhesion probably mediated by hydrophobic interactions using lipoteichoic acid, and (ii.) firm irreversible adhesion step mediated by composite multivalent interactions (Almeida et al., 2008; Duarte et al., 2006; Furiga, 2008; Gregoire et al., 2007; He et al., 2006; Nobbs et al., 2009).

Pathogenesis of the bacteria is mediated by biofilm, which has experimentally been supported in several recent studies (Akiyama et al., 2003; Manetti et al., 2007; Neely et al., 2002). Bacterial activities within biofilms are regulated by the occurrence of quorum sensing, which accommodates the release of chemical signals followed by the expression of virulence genes. Per CDC report, 65% of human bacterial infections involve biofilms, and treatment of these biofilm-associated nosocomial infections costs USA more than one billion dollar annually (Frieden, 2013; Mah & O'Toole, 2001). Conventional medications like penicillin, amoxicillin, first-generation cephalosporins, and erythromycin are the recommended antibiotics for the treatment of streptococcal sore throat. Nonetheless, antibiotic treatments are not always safe and or effective on antibiotic-resistant bacteria such as erythromycin-resistant strains. To overcome the issues of antibiotics such as exposing the patient to inappropriate therapy, averting the formation of antimicrobial-resistant strains, patient non-compliance, high cost, and adverse effects, a safe alternative therapy such as the use of natural bioactive molecules is favored. In line, over the past few decades, a vast number of research studies have investigated the inhibitory potential of natural compounds and their derivatives on the bacteria, of which many have found their way to the field of discovery and design of new antimicrobials. Anti-bacterial activities of phytochemicals on *S. pyogenes* in particular have been thoroughly discussed by the authors (Abachi et al., 2016). For instance, naphthoquinones, 1,2-naphthoquinone, and 5-hydroxy-1,4-naphthoquinone, could significantly impede the growth and survival of the same strain at low concentrations (minimum inhibitory concentration (MIC): 0.39 to 6.25 μg mL^{-1} and minimum bactericidal concentration (MBC):

100 µg mL^{-1}) (Macé et al., 2017). The same phenolics imposed significant inhibitory effects on the biofilm formation of *S. pyogenes* at varying concentrations of 2.5 to 50 µg mL^{-1}. The effects are not specific to a particular class of phenolics, and many have shown anti-GAS activities (Wijesundara et al., 2021; Wijesundara & Rupasinghe, 2019; Wijesundara & Rupasinghe, 2018).

We have recently reported the antibacterial potencies of ten Atlantic Canada fruits against different strains of *S. pyogenes* (Abachi et al., 2022). We have demonstrated prominent bacteriostatic and bactericidal effects of the cranberry and the sumac berry extracts (MIC: 0.25-2 mg mL^{-1} and MBC: 4-16 mg mL^{-1}). Moreover, the same material exhibited anti-adhesion effects against different strains of *S. pyogenes*. Adhesion of the bacteria (ATCC and clinical strain of *S. pyogenes*) to protein-coated (fibronectin and bovine serum albumin) and the bare surface was significantly prevented by these extracts. Therefore, extracts affected specific and non-specific adhesion of the bacteria. Minimum concentrations at which anti-adhesion effects were observed ranged from 0.25 to 16 mg mL^{-1}. In the current study, we hypothesized that the same extracts could display anti-biofilm effects since they, in the recent study of our team, potently inverted the attachment of the bacteria. In this context, we aimed to test the phytochemical-rich aqueous and ethanolic extracts of the cranberry and the sumac berries on the *S. pyogenes* biofilms. The objectives of our study were to (1) assess the effects of extracts on biofilm formation (new biofilms), (2) test the eradicating potential of the extracts on 24-hours old biofilms, and (3) visualize anti-biofilm formation and eradication effects of the extracts (concurrent with the controls) by scanning electron microscopy. Concurrently, we aimed to compare two methods of biofilm formation and eradiation analyses (viable cell count and metabolic activity).

Materials and methods

Chemicals and reagents

Dimethyl Sulfoxide (DMSO), penicillin G sodium salt, sodium cacodylate trihydrate (98%), glutaraldehyde solution (25% in H_2O), sodium cacodylate buffer, osmium tetroxide solution (4% in H_2O), Hexamethyldisilazane (HMDS) (≥99%) and glucose were purchased from Sigma-Aldrich Ltd. (Oakville, ON, Canada). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Life Technologies (Burlington, ON, Canada). Bacteriological agar and brain heart infusion (BHI) were obtained from Oxoid Ltd. (Nepean, ON, Canada).

Bacterial strains, growth conditions, and culture preparations

We included one ATCC, as well as a clinical strain of *S. pyogenes* in our study. *S. pyogenes* ATCC 19615 and the clinical strain were acquired from American Type Culture Collection (ATCC, Manassas, VA, USA) and Dr. R. J. Davidson's laboratory (Laboratory of Bacteriology, Division of Microbiology,

Pathology & Laboratory Medicine, Nova Scotia Health Authority, Halifax, NS, Canada), respectively. Growth conditions, as well as culture preparation procedures, have been detailed out in our previous article (Abachi et al., 2022).

Preparation of fruit extracts

Aqueous and ethanolic extracts of both cranberry (*Vaccinium macrocarpon* Aiton, Cranberry Acres Ltd., Berwick, NS) and sumac berry (*Rhus typhina* L., harvested the 4th week of September 2014 at 221 Farnham Rd, Truro, NS, GPS location of 45°23'00.4"N 63°15'52.1"W) were prepared as described in our previous work (Abachi et al., 2022).

Biofilm inhibition assays

Assessment of inhibitory effects of extracts on *S. pyogenes* biofilm formation by indirect viable cell count

The assay was conducted according to a previously described method by Pettit *et al.* (2005) with modifications (Pettit et al., 2005). Biofilms were formed in the presence of extracts/compounds. Briefly, bacterial cells were seeded in 96-well tissue culture plates (flat bottom with low evaporation lid), followed by the addition of extracts at MBC and lower concentrations (including sub-MIC). The plate was incubated at 37°C for 24-hours. The density of the bacterial culture was about 5 *log* colony forming units (CFU) per milliliter. Negative and positive controls were also designed for the assay (BHI broth with and without bacterial culture). In this study, due to limited resources, only two solvent controls for the two highest concentrations of each of the extracts/compounds were tested. Solvent controls were as follows; for cranberry aqueous and ethanolic extracts (8-16 mg mL^{-1}) 6.25-12.5% of H_2O, for sumac berry aqueous and ethanolic extracts (4-8 mg mL^{-1}) 3.13-6.25% of H_2O, and for penicillin G (8-16 ng mL^{-1}) 0.01-0.02% of H_2O. After 24-hours of incubation, planktonic bacteria were discarded, and 100 µL of media (BHI broth only) was added to the plate. Subsequently, serial dilutions (10-fold) of the viable biofilm bacteria were plated onto BHI agar plates, incubated for 24-hours at 37°C, and then enumerated for CFU mL^{-1} determination. Percent biofilm formation was calculated to compensate for differences in biofilm formation of *S. pyogenes* in the presence and or the absence of various concentrations of the extracts/compounds.

Percent biofilm formation = [(*log* CFU mL^{-1} of treated well/*log* CFU mL^{-1} of untreated well) × 100]

Assessment of inhibitory effects of extracts on *S. pyogenes* biofilm formation by metabolic activity measurement

The plate was prepared as described in the preceding section. After 24-hours of incubation, planktonic bacteria were discarded, and 200 µL of media was added to the plate. BHI broth with and without bacterial culture was considered as positive and negative controls, respectively. The entire

range of solvent controls for all the tested concentrations was included in the assay. To measure the metabolic activity of the biofilms formed in the presence of extracts/compound, 20 µL of MTT (5 mg mL^{-1}, dissolved in normal sterile saline water, freshly prepared in the dark) was added to each well. The addition of MTT followed by 2- to 4-hours of incubation resulted in the formation of insoluble purple formazan by viable bacterial cells. After the incubation time, 170 µL of media was carefully discarded, not disrupting the purple color pigments. To dissolve the insoluble purple formazan, 50 µL of DMSO was added to the wells, followed by vigorous pipetting. Purple color intensity was proportional to the number of viable cells in the well. Absorbance was read at 540_{nm}. Percent biofilm formation was calculated relative to the positive control.

Percent biofilm formation = $(((A_{treatment} - A_{negative\ control}) / (A_{positive\ control} - A_{negative\ control})) \times 100)$

Where A is the absorbance reading at 540_{nm}

Biofilm eradication assays

Analysis of eradication effects of extracts on S. pyogenes biofilms by indirect cell count

The assay was conducted according to a previously described method with slight modifications (Ogawa et al., 2011). To conduct the assay, 24-hours static biofilms of *S. pyogenes* were formed in the absence of test material. Bacterial culture with the approximate density of 4.7-5 *log* CFU mL^{-1} was used to construct the biofilms. Later, free-floating bacteria were discarded, and the one-day-old pre-formed biofilm was challenged with various concentrations of different extracts/compounds (including multiple times concentrations of MBC) for 3-hours. Positive, negative, and solvent controls were included in the assay. Solvent controls were as follows; 12.5-25% of H_2O for 16-32 mg mL^{-1} of cranberry aqueous and ethanolic extracts 6.25-12.50% of H_2O for 8-16 mg mL^{-1} of sumac berry aqueous and ethanolic extracts, 0.06-0.13% of H_2O for 64-128 ng mL^{-1} of penicillin G. Following 3-hours of incubation, planktonic bacteria were discarded, and 100 µL of media was added to the plate. Thereafter, serial dilutions (10-fold) of the viable biofilm bacteria were plated onto BHI agar plates. Plates were incubated for 24-hours at 37°C and enumerated for CFU mL^{-1} determination.

Analysis of eradication effects of extracts on *S. pyogenes* biofilms by metabolic activity measurement

The preparation of the pre-formed biofilm was carried out as described in the previous section. BHI broth with and without bacterial inoculum was considered positive and negative controls. Solvent controls for the entire range of tested concentrations were included in the assay. After 3-hours of exposure to the extracts/compounds, planktonic cells were discarded, and 200 µL of media was added to the plate. Subsequently, 20 µL of freshly prepared MTT (5 mg mL^{-1}, dissolved in normal sterile saline water in the

dark) was added to each well. The addition of MTT followed by 2- to 4-hours of incubation resulted in the formation of insoluble purple formazan by viable bacterial cells. Right after, 170 µL of media was carefully discarded, not disrupting the purple color pigments, and 50 µL of DMSO was added to each well (to dissolve the insoluble purple formazan) followed by vigorous pipetting. Purple color intensity was proportional to the number of viable cells in the well. Absorbance was read at 540^{nm}. Percent biofilm eradication relative to the positive control (with 0% of eradication) was calculated. Imaging biofilms by scanning electron microscopy

In this assay, biofilms were fixed and dehydrated according to a previously described method with modifications (Austin & Bergeron, 1995). Briefly, biofilms (formed in the presence of test compounds at the bottom of 96-well plate) were fixed with 0.1 M sodium cacodylate trihydrate solution with 2% glutaraldehyde at room temperature for 2-hours. Following fixation, biofilms were washed three times with 0.1 M cacodylate buffer supplemented with 3% glucose followed by incubation with 1% osmium tetroxide solution in 0.1 M cacodylate buffer for 4-hours. Biofilms were then rinsed three times with 0.1 M cacodylate buffer and dehydrated with increasing concentrations of ethanol (35, 50, 70, 90, and 100 %). Dehydrated biofilms were chemically dried by 27:75, 50:50, 75:25 HDMS/ethanol mixture series and two times with 100% HDMS followed by air-drying under a fume hood for 2-hours. Thereafter, the bottoms of the wells (where biofilms were formed and fixed onto) were carefully cut using flamed blades and mounted on the aluminum stubs using carbon adhesive tabs, and subsequently sputter-coated with Au/Pd nanoparticles. Biofilms were imaged using scanning electron microscopy (S-4700 FE-SEM, Hitachi, Japan) at different magnification levels.

Statistical analysis

We performed three independent studies, each in triplicates, to make statistically valid conclusions. We assessed the statistical significance of means (solvent control, positive control, and the treatment) by one-way analysis of variances (ANOVA); Tukey's multiple comparisons ($P < 0.05$). To compare the results obtained by the plate count method and MTT assay, we performed a correlation analysis. The normality of our data was tested by Anderson-Darling normality test ($P > 0.05$). The analyses were conducted by Minitab (version 16).

Results

Inhibition of biofilm formation by cranberry and sumac berry extracts

Effects of the berry extracts on biofilm formation were examined by MTT assay and plate counts as well as SEM (Fig. 1A-1B and 2). Overall, the extracts could completely prevent the construction of biofilms at concentrations higher than MBC. It is noteworthy that according to the findings of our previous study, the anti-adhesion effects

of the extracts generally occurred at ½ × MBC to 1 × MBC. Overall, the minimum biofilm inhibitory concentration (MBIC) of cranberry extracts, aqueous and ethanolic, when measured by MTT was 4 mg mL^{-1}. MBIC for the same extracts, when measured by plate counts, was >4 mg mL^{-1}. MBIC of sumac berry extracts, aqueous and ethanolic ones, when measured by MTT, ranged from 1 to 4 mg mL^{-1}. MBIC for the same extracts, when measured by plate counts, was ≤4 mg mL^{-1}.

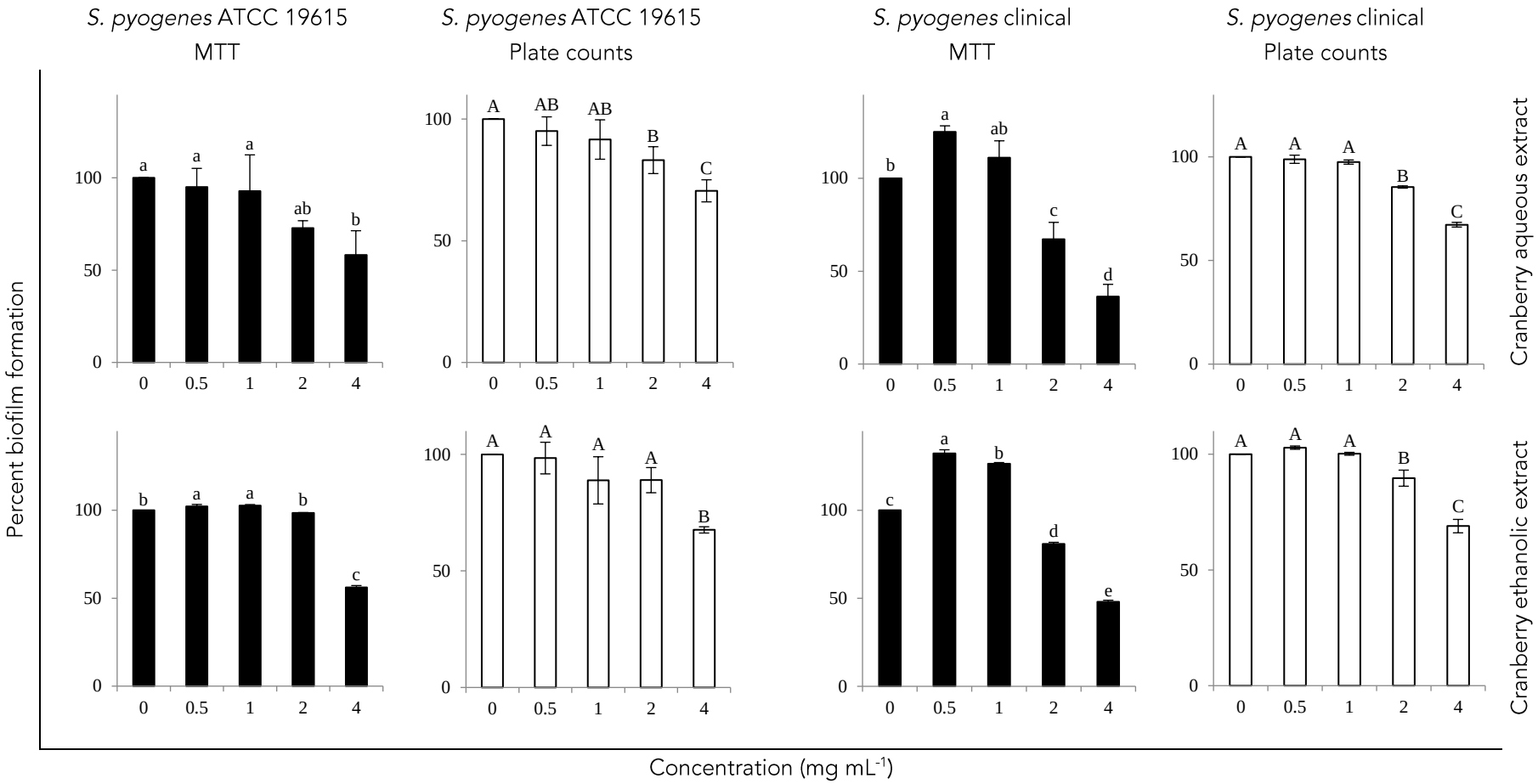

Fig. 1A: Inhibitory effects of cranberry extracts on *S. pyogenes* biofilm formation measured by quantitative and semi-quantitative methods. All panels represent the means of three independent experiments. Black and white bars respectively represent MTT measurements and plate counts. Data are expressed as percent biofilm formation relative to the positive control (control: 100%). The difference between solvent control and the positive control was insignificant. A-C and a-c letters on the bars as obtained by Tukey's multiple comparison test were used to indicate the statistical significance ($P< 0.05$) of different concentrations of the compound, and the controls (solvent and positive control). Error bars represent the standard deviation of triplicate measurements.

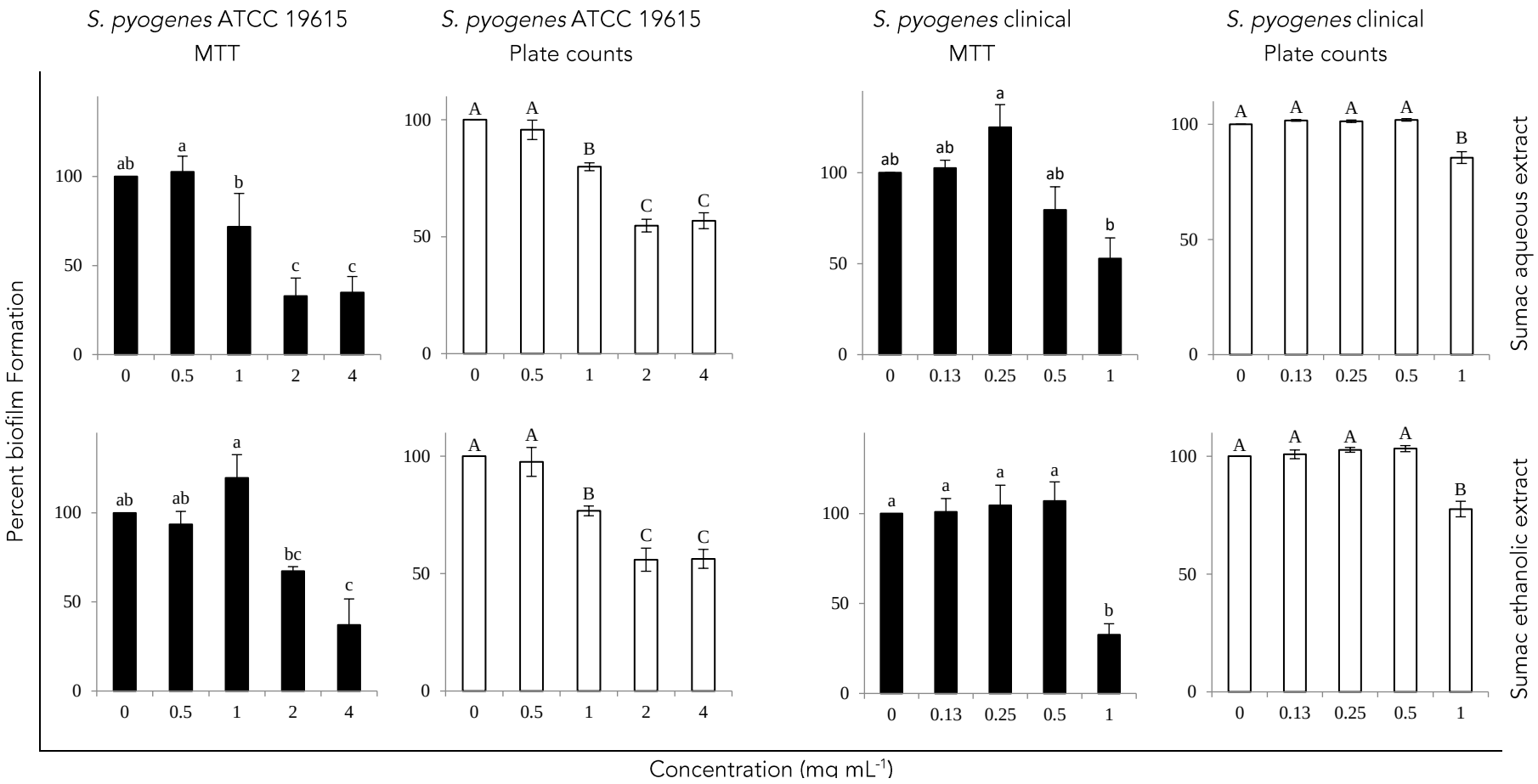

Fig. 1B: Inhibitory effects of sumac extracts on *S. pyogenes* biofilm formation measured by quantitative and semi-quantitative methods. All panels represent the means of three independent experiments. Black and white bars respectively represent MTT measurements and plate counts. Data are expressed as percent biofilm formation relative to the positive control (control: 100%). The difference between solvent and positive control was insignificant. A-C and a-c letters on bars as obtained by Tukey's multiple comparison test were used to indicate statistical significance ($P< 0.05$) of different concentrations of the compound, and the controls (solvent and positive control). Error bars represent the standard deviation of triplicate measurements.

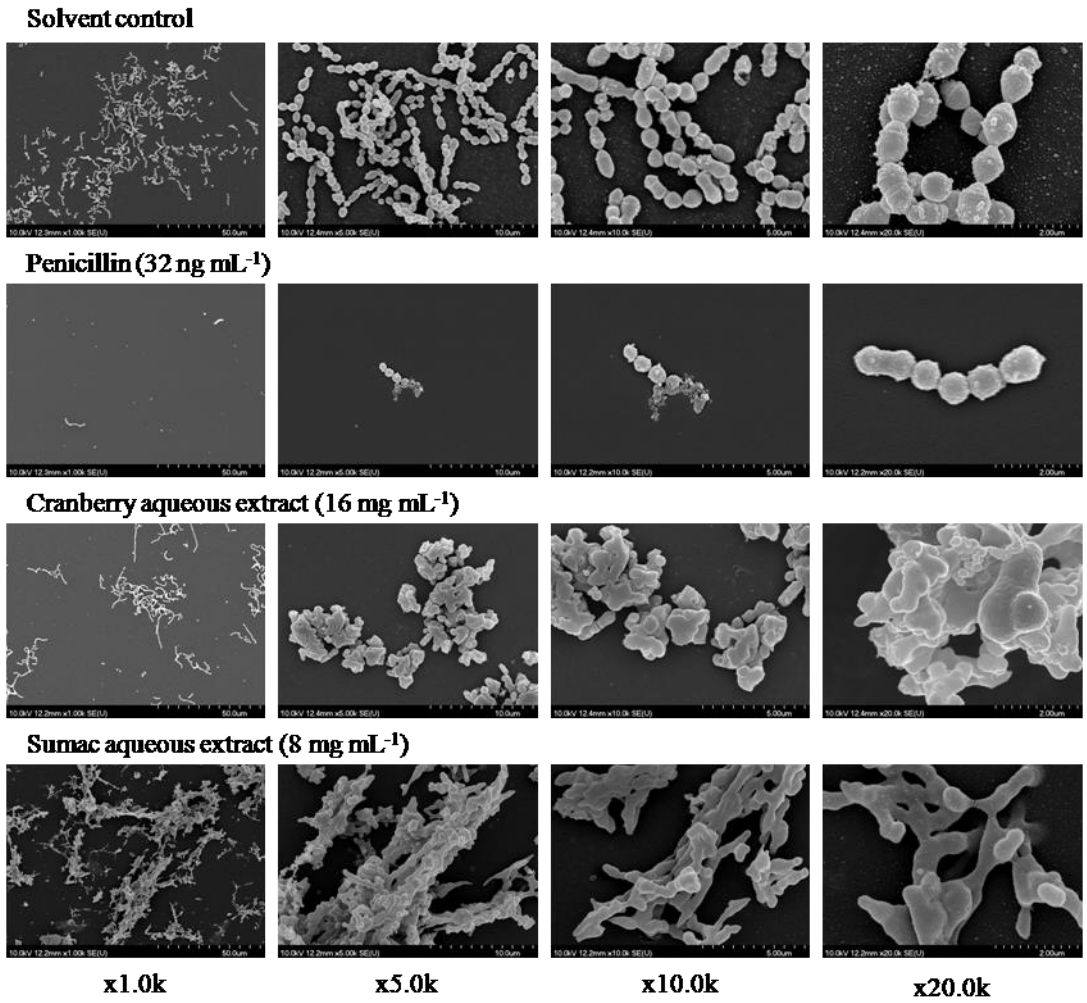

Fig. 2: SEM micrographs of biofilm inhibition of *S. pyogenes* ATCC 19615 imaged at different magnifications. The extracts and penicillin G were dissolved in sterile distilled water. Solvent control (12.5% H_2O in BHI broth) was designed for the assay. Duplicate of each sample were observed by SEM and imaged.

Eradication of one-day-old biofilms of S. pyogenes by cranberry and sumac berry extracts

The potential of the extracts for eradicating the 24-hours old biofilms of *S. pyogenes* was examined by MTT assay and plate counts as well as SEM (Fig. 3A-3B and 4). It is noteworthy that predicting the biofilm eradicating activity of the extracts based on planktonic cell susceptibility has been suggested as inappropriate (Monte et al., 2014). Therefore, the biofilm eradicating activity of the extracts must be checked with a suitable method like MTT, plate counts, and or microscopic techniques. Generally, the extracts could prevent the construction of biofilms by about 50% at MBC equivalent concentrations. Overall, the minimum biofilm eradicating concentration (MBEC) of cranberry extracts, aqueous and ethanolic, when measured by MTT ranged from 8 to 16 mg mL^{-1}. MBEC for the same extracts, when measured by plate counts, was >16 mg mL^{-1}. MBEC of sumac berry extracts, aqueous and ethanolic ones, when measured by MTT was 2 mg mL^{-1}. MBEC for the same extracts, when measured by plate counts, was ≥8 mg mL^{-1}. Moreover, we performed SEM analysis to confirm the biofilm eradicating effects of aqueous extracts of sumac berry and cranberry (Fig. 4). The findings of SEM micrographs agreed with those obtained from the plate count and the MTT assays, thus indicative of the significant biofilm eradicating attributes of cranberry and sumac berry aqueous extracts. Morphological changes of the bacteria could also be observed in the images (Fig. 4).

At 8-16 mg mL^{-1} of cranberry extracts, 28% of the pre-formed *S. pyogenes* biofilms were eradicated compared with the control (plate count measurements) (Fig. 3A). At 32 mg mL^{-1}, the cranberry extracts eradicated 42% of the *S. pyogenes* biofilms (data not shown). Results obtained from plate count showed a dose-dependent effect of cranberry extracts.

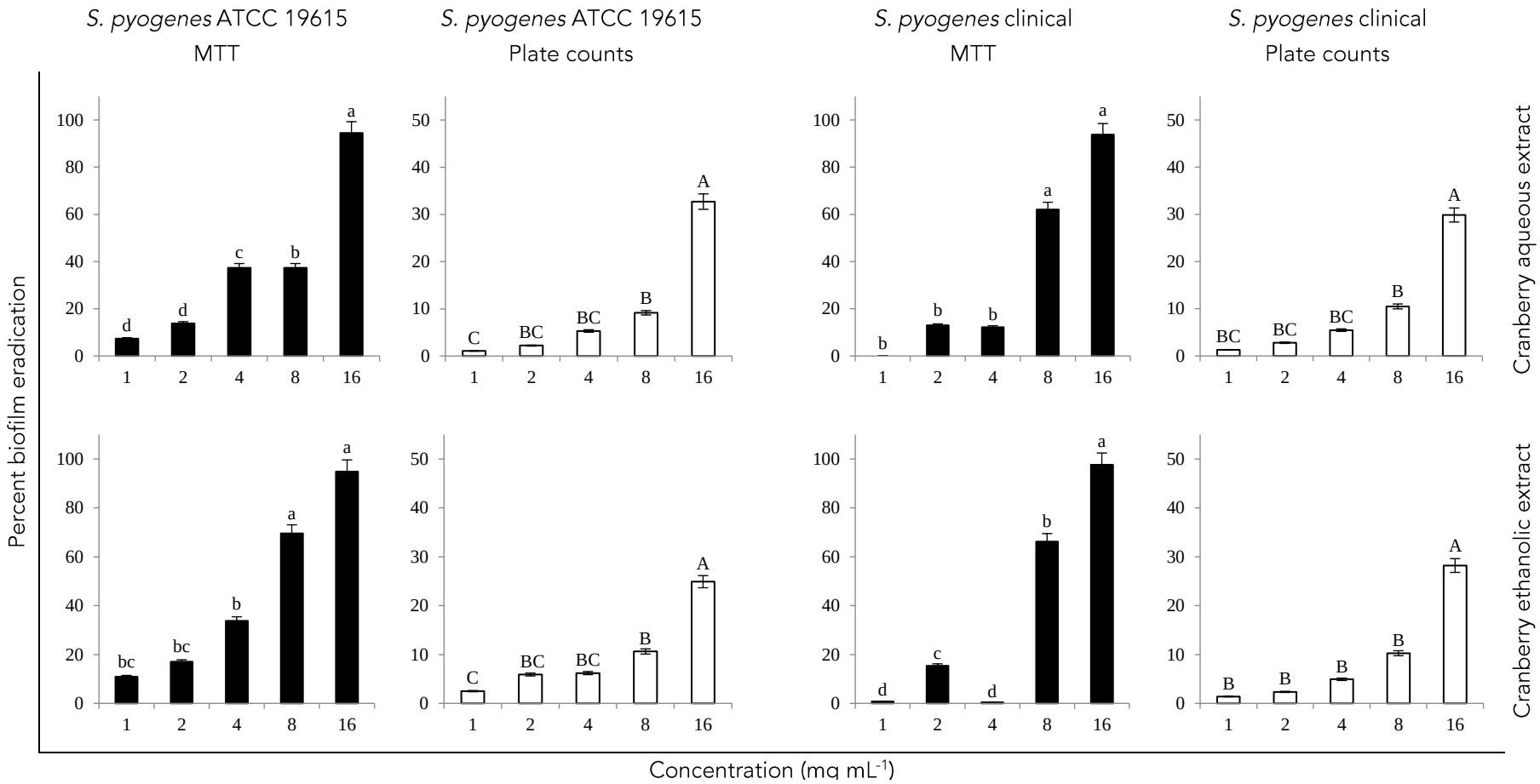

Fig. 3A: *S. pyogenes* biofilm eradication activity of cranberry extracts measured by quantitative and semi-quantitative methods. All panels represent the means of three independent experiments. Black and white bars respectively represent MTT measurements and plate counts. A-C and a-d letters on bars as obtained by Tukey's multiple comparison test were used to indicate the statistical significance ($P< 0.05$) of different concentrations of the compounds, and the controls (solvent and positive control). Error bars represent the standard deviation of triplicate measurements. Percent biofilm-eradication activity of the solvent control, 1 × MIC and ½ × MIC concentrations for all the tested compounds were not significantly different from the positive control.

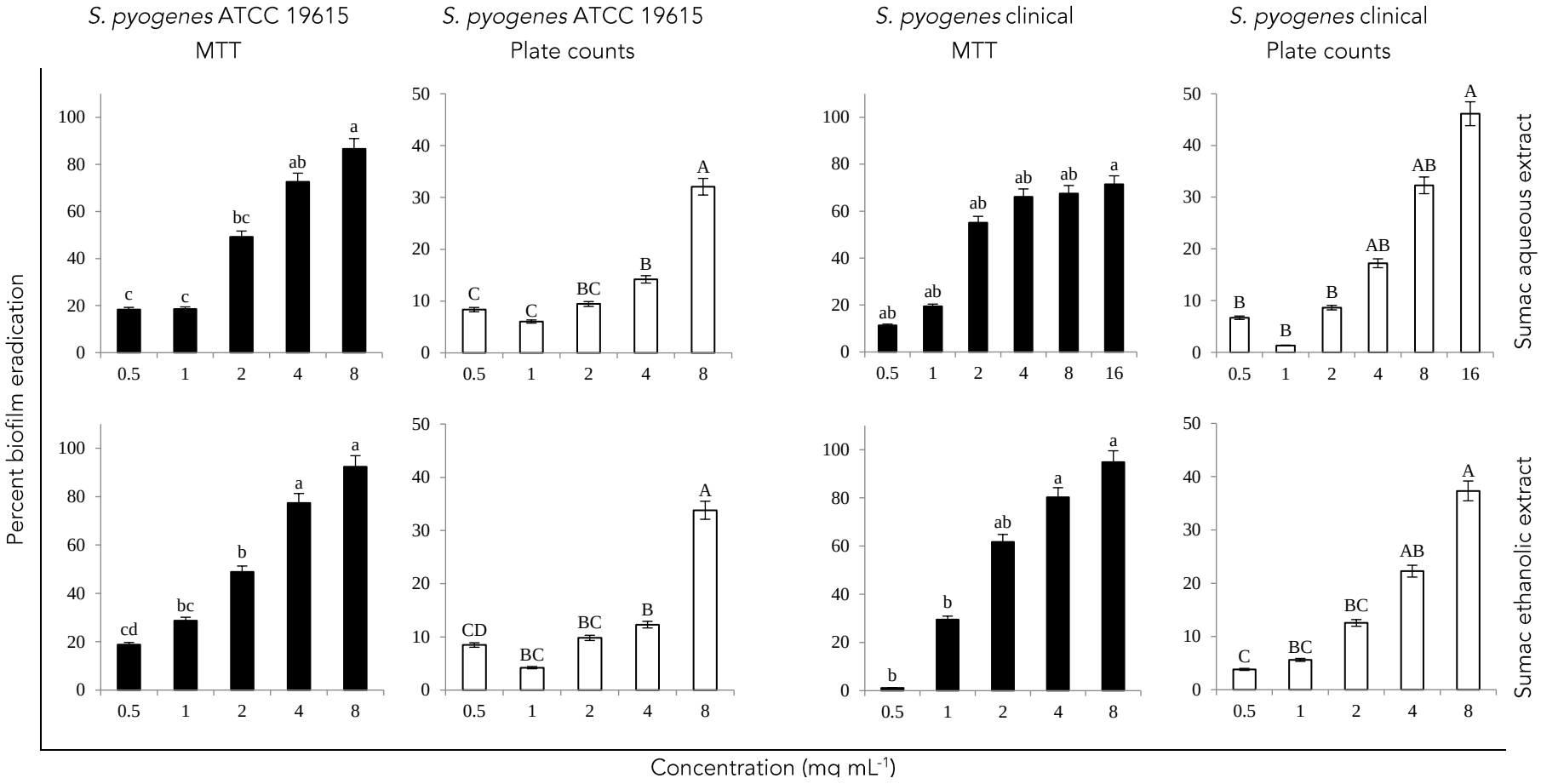

Fig. 3B: *S. pyogenes* biofilm eradication activity of sumac extracts measured by quantitative and semi-quantitative methods. All panels represent the means of three independent experiments. Black and white bars represent MTT measurements and plate counts, respectively. A-D and a-d letters on bars as obtained by Tukey's multiple comparison test were used to indicate the statistical significance ($P< 0.05$) of different concentrations of the compounds, and the controls (solvent and positive control). Error bars represent the standard deviation of triplicate measurements. Percent biofilm-eradication activity of solvent control, 1 × MIC and ½ × MIC concentrations for all the tested compounds were not significantly different from the positive control.

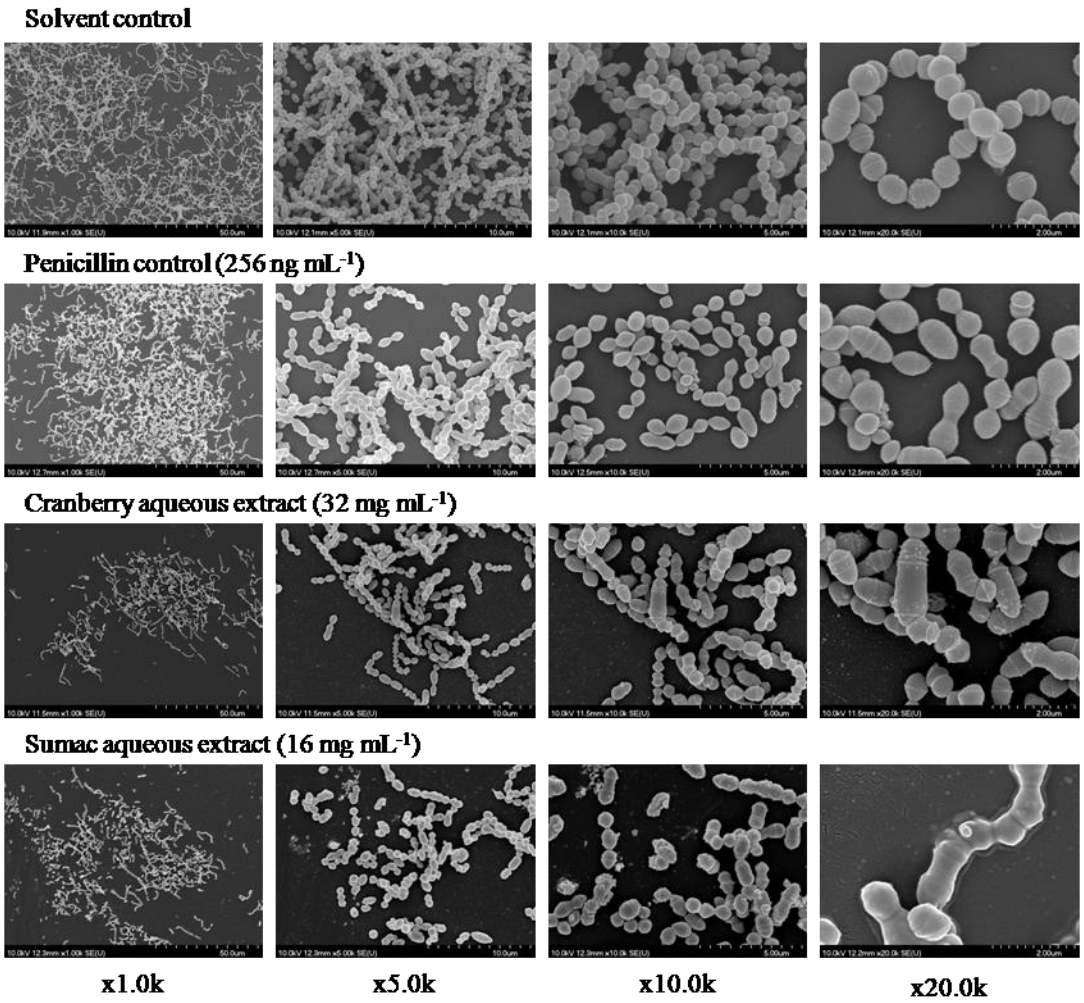

Fig. 4: SEM micrographs of eradication effect of extracts on one-day old pre-formed *S. pyogenes* ATCC 19615 biofilms after 3-hours treatment. The extracts and penicillin G were dissolved in sterile distilled water. Solvent control (12.5% H_2O in BHI broth) was designed for the assay. Duplicate of each sample were observed by SEM and imaged.

Discussion

In general, ATCC strain was less susceptible to the anti-biofilm effects of extracts compared with the biofilms of clinical strain, meaning that it could form stronger biofilm even in the presence of extracts. On the one hand, higher concentrations of extracts were required to hinder the biofilm-forming capabilities of ATCC 19615 than the clinical strain. On the other hand, the clinical strain shows more adaptation attributes to environmental conditions than the ATCC strain (although susceptible to lower concentrations). Biofilm inhibitory effects of cranberry and sumac berry aqueous extracts (including population control and penicillin G) were confirmed by SEM (Fig. 2). Changes in bacterial morphology were evident when biofilms were formed in the presence of cranberry and sumac berry extracts. This probably alters the surface characteristics of the bacteria (e.g., hydrophobicity and or its ATPase activity), thus hindering bacteria's biological activities.

Biofilm formation of *S. pyogenes* (ATCC and the clinical strain) was hindered by cranberry extracts in a dose-dependent manner. The cranberry extract at >4 mg mL^{-1}, reduced the viable cells of *S. pyogenes* to below the detection limit, indicating >3 *log* CFU mL^{-1} reduction compared with the control (~7.6-7.8 *log* CFU mL^{-1}). Cranberry (crude extract, its juice, and constituents) has repeatedly demonstrated anti-biofilm and anti-adhesion effects against attachment and biofilm formation of many types of streptococci, including *S. pyogenes* (*S. sobrinus* (6715 and B13), *S. sanguinis*, *S. mutans* (MT 8148R, JC2, Ingbritt and ATCC 10449), *S. criceti* E49, *S. oralis* ATCC 10557, *S. mitis* ATCC 9811, *S. gordonii* Challis, *S. pneumoniae* SB 53845) (Abachi et al., 2016). Antibacterial effects, in particular anti-biofilm and anti-adhesion, of this fruit are not specific to streptococci as numerous studies have reported its potency against a wide range of bacteria. For instance, cranberry-derived proanthocyanidins exhibited dose-dependent anti-biofilm activity against *Pseudomonas aeruginosa* (Ulrey et al., 2014).

Similarly, the biofilm formation inhibitory effect of sumac berry extracts against clinical and ATCC 19615 strains of *S. pyogenes* was evident. An interesting observation was that unlike cranberry extract exhibiting dose-dependent response, sumac berry extracts demonstrated rather a dose-independent effect. Recently, Wang and Zhu *et al.* (2017) have reviewed the biological activities and chemical profile of Canadian *R. typhina* known as staghorn sumac (Wang & Zhu, 2017). Rationally anti-biofilm activity of this extract may be related to the high polyphenol (anthocyanin and phenolic acid) and or organic acid content of its fruits (Table 1). Gallic acid, one of the main phenolic acids of Canadian *R. typhina*, can prevent *S. mutans* biofilm establishment up to 70% at a concentration as low as 1-4 mg mL^{-1} (Kang et al., 2008). Gallic acid, quercetin, and tannic acid have all shown anti-biofilm effects against *S. mutans,* with gallic acid being the most potent one (Sendamangalam et al., 2011). Another species of sumac, *Rhus glabra* L.

(smooth sumac) consists of mainly methyl gallate, which is also known for its antibacterial and anti-biofilm effects (Saxena et al., 1994). Methyl gallate at concentrations of 1-4 mg mL^{-1} decreased up to 90% of the biofilm formation of *S. mutans* (Kang et al., 2008). The findings of our study and other studies show that the extract of this fruit can be used in the design and discovery of antibacterial structures.

Table 1: Polyphenol profile of Canadian sumac berry

Class and group	Compound name	Quantity (mg/g, dry weight)
Flavonoid		
Anthocyanin	Peonidin-3-*O*-galloyl-hexose	270
	(449 Da)-hexose-3-*O*-galloyl-hexose	25.7
	(419 Da)-hexose-3-*O*-galloyl-hexose	21.3
	(433 Da)-hexose-3-*O*-galloyl-hexose	216
	(433 Da)-3-*O*-galloyl-hexose	15.1
	Cyanidin-3-*O*-(2″ galloyl)-galactoside	-
	7-*O*-methyl-delphinidin-3-*O*-(2″ galloyl)-galactoside	-
	7-*O*-methyl-cyanidin-3-*O*-galactoside	-
	7-*O*-methyl-cyanidin-3-*O*-(2″ galloyl)-galactoside	-
	7-*O*-methyl-delphinidin-3-*O*-(2‴galloyl)-galactoside-4-vinylcatechol-3″-*O*-glucoside	-
	7-*O*-methyl-cyanidin-3-*O*-(2‴galloyl)-galactoside-4-vinylcatechol-3″-*O*-glucoside	-
Flavonol	Quercetin	0.62
	Quercetin-3-rhamnoside	1.47
Phenolic acid		
Hydroxybenzoic acid	Gallic acid	5.97
Hydroxycinnamic acid	Caffeic acid 3-*O*-hexose	8.35
	Ellagic acid	3.18

Data are extracted from the studies of Wu *et al.* (2013) and Kirby *et al.* (2013) (Kirby et al., 2013; Wu et al., 2013).

Moreover, penicillin at concentrations of 4-8 ng mL^{-1} inhibited the biofilm formation by *S. pyogenes* up to 41% (see supplementary file). Biofilm establishment by *S. pyogenes* at the presence of MBC concentration of penicillin showed >3 *log* CFU mL^{-1} reduction; however, it could not fully prevent the activity. This may be due to the reduced sensitivity of *S. pyogenes'* biofilm to penicillin treatment. Even so, some scientists believe that biofilm

formation or, better put, micro-colony establishments could not alone lead to penicillin treatment failure and that not every GAS strain biofilm is insensitive to the antibiotic (Kuhn et al., 2001). GAS antibiotic insensitivity could be caused by various biofilm attributes such as reduced growth rate within the biofilm lifestyle, establishment of three-dimensional structure, the exopolysaccharide matrix, and altered gene expressions (Kuhn et al., 2001). In the present study, sub-MIC concentrations of penicillin G (1 ng mL^{-1}) had an insignificant effect on the biofilm formation; hence the difference between the treated biofilm compared with the control was insignificant. Others have shown that sub-MIC concentrations of penicillin may induce biofilm formation in susceptible strains of *S. pyogenes* (Baldassarri et al., 2007). It has been previously determined that penicillin-susceptible GAS forms thicker biofilm than the macrolide-resistant one (Baldassarri et al., 2007). In general, reports show that the anti-biofilm effect of penicillin against GAS is dependent on various factors of concentration as well as bacterial strain.

In line with our findings, commercial cranberry extracts at 5-10 mg mL^{-1} produced a weak eradication activity against *Staphylococcus* species (LaPlante et al., 2012). Phytochemicals frequently have been capable of eradicating microbial biofilms in experimental settings. In a systematic exploration study of natural and synthetic flavonoids for their inhibition activity against pre-established *Staphylococcus aureus* biofilms, 2% of the compounds exhibited notable eradication activity (Manner et al., 2013). The ten active compounds belonged to different groups of polyphenol classes (flavanones, chalcones, and flavans) (Manner et al., 2013).

Sumac berry extracts at 8 mg mL^{-1} disrupted *S. pyogenes* biofilms up to 37% (~2.8 *log* CFU mL^{-1} and 2.5 *log* CFU mL^{-1} compared with the untreated biofilms for ATCC and clinical strain, respectively) (Fig. 3B). Overall, *S. pyogenes* ATCC 19615 was less sensitive to sumac berry extracts than the clinical strain. Caffeic acid, a major constituent of *R. hirta* extract, decreased the *Staphylococcus aureus* (a methicillin-resistant strain (MRSA) and an ATCC strain) biofilm up to 60% whilst gallic acid showed insignificant effect against MRSA (Luís, 2014; Wu et al., 2013). Similarly, gallic and caffeic acid at the same concentrations (1-4 mg mL^{-1}) lowered the metabolic activity of *S. aureus* biofilm, although the effect was more prominent on the ATCC strain than on MRSA (Luís, 2014).

Furthermore, patterns for penicillin were somewhat different. The plate count results showed that penicillin G at 16 ng mL^{-1} reduced the clinical strain biofilm to ~9% (compared with the control), but at 128 ng mL^{-1}, we observed only ~3% of reduction (see supplementary file). Generally, the biofilm of the *S. pyogenes* clinical strain was more difficult to eradicate than the ATCC strain (ATCC 19615) by berry extracts as anticipated; however, penicillin G was more potent on *S. pyogenes* clinical pre-

established biofilm than on *S. pyogenes* ATCC 19615. Rationalizing the behavior of *S. pyogenes* biofilms to penicillin G in our study was difficult. In a study, the 48-hours biofilms of *S. pyogenes* of M serotypes 1, 2, 4, 6, 12, and 30 could only be eradicated at 400 to 800 mg L^{-1} of penicillin while MIC ranged from 0.04 to 0.08 mg L^{-1} (Shen et al., 2013). It was suggested that *S. pyogenes* in biofilm-like states could hide in their sanctuary, thus complicating its eradications by penicillin (MBEC of >400 mg L^{-1}) (Shen et al., 2013). Moreover, for comparison purposes, we compared different types of tests used in this study for biofilm inhibition and eradication assays. Accordingly, a correlation analysis of the results obtained from the plate count method and the MTT assay was performed to find out how appropriate the quantitative and or the semi-quantitative methods could be for the biofilm studies (Table 2). On the one hand, according to Pearson's correlation coefficient (*r*), the MTT assay is not a useful tool for testing the dose effects of extracts, although it could be a cost- and time-effective technique for the determination of MBIC and MBEC. On the other hand, the plate count method seemed more appropriate for the dose-effect analysis of natural compounds, nonetheless being time- and cost-inefficient. Generally, the choice of technique would depend on various factors such as the objectives of the study, resources, and type of material.

Table 2: Correlation of results obtained from plate count method and MTT cell viability assay

		Pearson's correlation coefficient (*r*) with *P*-value			
		S. pyogenes strain			
		ATCC 19615	Clinical	ATCC 19615	Clinical
Compound/Extract		Biofilm formation		Biofilm eradication	
Cranberry	Aqueous	0.988 (0.002)	0.948 (0.014)	0.977 (0.004)	0.937 (0.019)
	Ethanolic	0.916 (0.029)	0.936 (0.019)	0.926 (0.024)	0.917 (0.028)
Sumac berry	Aqueous	0.995 (0.000)	0.788 (0.113)	0.843 (0.073)	0.771 (0.127)
	Ethanolic	0.687 (0.200)	0.999 (0.000)	0.813 (0.094)	0.907 (0.034)
Antibiotic	Penicillin G	0.963 (0.008)	0.908 (0.033)	0.596 (0.289)	0.838 (0.076)

Correlation analysis was performed with Minitab version 16. *P*-values are noted in the parenthesis ().

Conclusion and future directions

In the current study, we have reported the anti-biofilm effects of cranberry and sumac berry extracts. Here we report the inhibitory effects of aqueous and ethanolic cranberry and sumac berry extracts on the new and the established biofilms of the bacteria in addition to their anti-adherence, bacteriostatic and bactericidal attributes. Nonetheless, the antibacterial activity of these fruits can further be verified and investigated by metabolomics and or well-controlled cell and/or animal studies. The antibacterial activity of our material can, in the same way, be tested

against other types of bacteria and or microbes. Active components of these materials could be isolated and purified for expanding the knowledge of the structure-activity relationship as well as for the development of potential natural health products and therapeutics.

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Authors' contributions

SA, BR, SL and VR designed the study. SA performed the research, analyzed the data, and wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

Funding

This study was funded by the Nova Scotia Graduate Entrance Scholarship, Graduate Research Training Initiative Scholarship, Natural Sciences and Engineering Research Council (NSERC) of Canada Collaborative Research and Development grant (CRDPJ 448052), and Island Abbey Foods Ltd., Charlottetown, PE, Canada.

Conflict of interest

We declare no conflict of interest. No animal and or human subjects were involved in our study.

Data availability statement

Data are available from the corresponding author on request.

Supplementary material

Supplementary material is available in the electronic version of this article.

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