Characterization of non-dialyzable constituents from cranberry juice that inhibit adhesion, co-aggregation and biofilm formation by oral bacteria

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An extract prepared from cranberry juice by dialysis known as nondialyzable material (NDM) has been shown previously to possess anti-adhesion activity toward microbial species including oral bacteria, uropathogenic Escherichia coli and Helicobacter pylori. Bioassay-guided fractionation of cranberry NDM was therefore undertaken to identify the anti-adhesive constituents. An aqueous acetone-soluble fraction (NDMac) obtained from Sephadex LH-20 inhibited adhesion-linked activities by oral bacteria, including co-aggregation of oral bacteria Fusobacterium nucleatum with Streptococcus sanguinis or Porphyromonas gingivalis, and biofilm formation by Streptococcus mutans. Analysis of NDMac and subsequent subfractions by MALDI-TOF MS and 1H NMR revealed the presence of A-type proanthocyanidin oligomers (PACs) of 3–6 degrees of polymerization composed of (epi)catechin units, with some (epi)gallocatechin and anthocyanin units also present, as well as quercetin derivatives. Subfractions containing putative xyloglucans in addition to the mixed polyphenols also inhibit biofilm formation by S. mutans (MIC = 125–250 μg mL⁻¹). These studies suggest that the anti-adhesion activities of cranberry NDM on oral bacteria may arise from a combination of mixed polyphenol and non-polyphenol constituents.

Introduction

Bacterial biofilms are multiple layers of bacteria held together by and embedded in a matrix of macromolecules, usually polysaccharides, secreted by the bacteria. These biofilms play a critical role in the development of oral diseases including gingivitis and dental caries and gain a number of advantages over planktonic bacteria to promote growth and survival in a hostile environment as they become resistant to antibacterial agents. Because adhesion of pathogens to tooth surfaces and the soft tissue of the mucosa is the first step in biofilm formation and subsequent initiation of infection, the development of anti-adhesion agents has been a focus of intensive research. Such agents ideally are not bacteriocidal, but rather inhibit adhesion of bacteria to target surfaces preventing biofilm formation, and thus allow eradication of the pathogen from body surfaces without promoting the spread of emergent resistant strains.

Food-derived agents are ideal candidates in this regard as they are likely to be non-toxic, readily available and can also be delivered in supplement form. In this respect, the juice of the North American cranberry (Vaccinium macrocarpon) as well as isolated fractions and constituents including A-type proanthocyanidins have been studied extensively in vitro, in human trials for prevention of urinary tract infections, and for Helicobacter pylori eradication. The anti-adhesion activity of cranberry has largely been attributed to the presence of polyflavan-3-ols, specifically A-type proanthocyanidins. Recent studies suggest that other constituents including flavonols and oligosaccharides may also play a role in the antimicrobial activities of cranberry.

A high molecular weight fraction obtained by dialysis from concentrated cranberry juice and termed non dialyzed material (NDM) has been investigated by us for its anti-adhesion and anti-biofilm activities against a number of microbial species including oral bacteria, uropathogenic Escherichia coli,
Helicobacter pylori, and influenza virus. NDM did not exhibit any bactericidal effect in these studies, even at concentrations of 2000 μg ml⁻¹ or more. In human trials it reduced significantly the number of oral Streptococcus mutans, responsible mainly for tooth decay. Its ability to inhibit the activity of soluble and immobilized glucosyl and fructosyltransferases (GTF and FTF) and subsequent adhesion of Streptococcus sobrinus to hydroxyapatite suggest that NDM acts on oral bacteria in part by reducing synthesis of the polysaccharides that mediate adhesion. Other possible mechanisms include inhibition of host enzymes that participate in periodontal diseases, such as MMP-3, MMP-9 and elastase, as well as reduction of acid production by cariogenic bacteria.

Cranberry’s effects on oral bacteria may be particularly significant, as the oral cavity would be directly exposed whenever cranberry is consumed in the diet. However, the molecular nature of the compounds in cranberry juice NDM responsible for these activities has not been defined. Therefore, a bioassay-guided fractionation was undertaken to target the compounds in NDM responsible for anti-adhesion activities on oral bacteria. Other common pathogens were included in the study where relevant. Cranberry-derived NDM was fractionated on Sephadex LH-20 and MCI CHP-20P columns and the fractions were assessed for ability to inhibit bacterial co-aggregation and biofilm formation, and analyzed by NMR and MALDI-TOF MS in order to characterize the major constituents. This is the first study to characterize the polyphenol and non-polyphenol constituents of cranberry NDM.

Materials and methods

Chemicals and reagents

Unless otherwise indicated, all reagents employed were purchased from Sigma-Aldrich (St Louis, MO, USA). Solvents for bulk separation and HPLC analysis were from Fisher Scientific USA. Cranberry juice concentrate was provided by Ocean Spray Cranberries, Inc. (Lakeville, MA). Quercetin and myricetin standards were from Sigma-Aldrich; quercetin-3-galactoside (Cranberries, Inc. (Lakeville, MA). Quercetin and myricetin standards were from Sigma-Aldrich; quercetin-3-galactoside was from Chromadex, Inc. (Irvine, CA). DMSO-d₆ and NMR standards were purchased from Cambridge Isotope Labs. Procyanidin standards were purchased from Indoline Chemical (Hillsborough, NJ).

Bacterial strains and culture

Streptococcus mutans UA159, Staphylococcus epidermidis RP62A, as well as clinical isolates of Staphylococcus aureus and Escherichia coli (urinary tract isolate) obtained from the Clinical Microbiology Department, Hadassah Hospital, Jerusalem, Israel were employed. The bacteria were grown in Brain Heart Infusion (BHI) broth at 37 °C for 18 h followed by sedimenting the bacteria and washing the pellet with the indicated buffer. The following strains of human gingival crevice origin (a gift from Dr P. Kolenbrander, National Institutes of Dental Research) were used Fusobacterium nucleatum PK1904, Fusobacterium nucleatum PK1909, Fusobacterium nucleatum PK1594 (Fn), Streptococcus oralis J22, Streptococcus sanguinis SS34 (Ss) and Porphyromonas gingivalis ATCC 33277 (Pg). Bacteria were grown at 37 °C under anaerobic conditions (GasPack Anaerobic System; BBL) in modified Schaedler medium as described previously. Cells were harvested, washed with coaggregation buffer (CAB, 1 mM Tris, 0.1 mM CaCl₂, 0.1 mM MgCl₂, 0.15 M NaCl, 0.02% NaN₃, adjusted to pH 8.0) and stored at 4 °C until use.

Preparation of cranberry fractions

Non-dialyzable material (NDM) was obtained from the cranberry juice concentrate (50 Brix, Ocean Spray) as described previously. Briefly, the concentrated juice was dialyzed extensively against distilled water (>10× the volume of concentrated juice) and six changes for six days and the non-dialysable material was collected, lyophilized and chilled until use (about 6 g NDM from one liter concentrated juice). NDMet and NDMac fractions from NDM were obtained using a Sephadex LH-20 column as described elsewhere with minor modifications as follow. A glass column (2.5 × 60 cm) was packed with 100 g Sephadex LH-20 (Amersham –GE Cat# 17-0090-10) that was allowed to swell in 50% aqueous ethanol solution for 3 h at RT. 600 mg NDM in 50% ethanol (v/v) was applied to the column and fractions were collected at a flow rate of 2 mL min⁻¹. The resin bed was eluted consecutively with 90 ml volumes (3 times void volumes) of 50% aqueous ethanol (v/v) followed by 90 ml 75% aqueous acetone (v/v). The ethanol (NDMet, 279 mg) and acetone (NDMac, 171 mg) fractions were evaporated to dryness and the powder was stored at −20 °C until use. The procedure was scaled up to produce additional NDMet and NDMac for sub-fractionation as follows: crude NDM (1.477 g) was dissolved in 50% ethanol (10 mL) and separated on the Sephadex LH-20 (Pharmacia Biotech, Piscataway, NJ) packed column by elution first with 50% ethanol (NDMet; 1.089 g), then 75% aqueous acetone (NDMac; 0.327 g). Fractions were dried in vacuo. The NDMac fraction (0.325 g) was dissolved in 30% methanol (10 mL) and loaded onto an MCI Gel CHP20P (Supelco, Bellefonte, PA) column (30 g, 2.5 × 30 cm) preconditioned with methanol and water. A gradient elution using increasing methanol in water produced four sub-fractions, eluting with 30% methanol (NDMac-MCI1; 0.028 g), 50% methanol (NDMac-MCI2; 0.149 g), 70% methanol (NDMac-MCI3; 0.097 g), and 100% methanol (NDMac-MCI4; 0.005 g). The fractions were dried in vacuo and stored at −20 °C until use.

Quantification of total phenolics by Folin–Ciocalteu method

Determination of total content of reducing agents including polyphenols in test samples was performed using the Folin–Ciocalteu reagent as described by Ginsburg and coworkers. Briefly, 800 μL aliquots of NDM, 50% ethanol fraction NDMet or 75% acetone fraction NDMac were prepared in triplicate at 3.2 mg mL⁻¹ in normal saline (10 mM). To each, 50 μL of Folin reagent (Sigma) was added. One minute later, 150 μL of a 25% solution of sodium carbonate was added, and five minutes later, the reaction mixtures were centrifuged at 2000 rpm for 3 min and the blue color developed in the supernatant was
read at 760 nm. All tests were conducted in triplicate. A standard curve was prepared with positive control gallic acid dissolved in absolute ethanol (100 mM stock solution) and the results were expressed as gallic acid equivalents (GAE).

**Determination of oxidant-scavenging ability**

The luminol-dependent chemiluminescence assay was employed to estimate the oxidant scavenging abilities (OSA) of test samples as described previously. Briefly, 100 μL of test sample in Hanks balanced salt solution (HBSS) pH 7.4 at indicated concentration or 100 μL HBSS were added to 800 μL HBSS containing luminol (10 mM), morpholino s syndononi-mine (SIN-1) (10 mM), sodium selenite (0.1 mM) and CoCl2·6H2O. The mixture was incubated in luminometer (Lumac/3M, Biocounter M2010) for 120 s, at room temperature. The luminescence was measured every 30 s and the degree of light quenching by mixture containing test sample as compared to control was determined to estimate the OSA of the test sample. All tests were conducted in triplicate.

Using this assay, the OSA of cranberry materials bound to bacterial surface was estimated. *Escherichia coli* bacteria were grown aerobically, at 37 °C, 5% CO2, overnight in BHI medium followed by centrifugation to sediment the bacteria. The bacterial pellets were washed twice with PBS and suspended in PBS to a 1.0 OD optical density. One hundred μL of bacterial suspensions containing decreasing amounts of NDM, NDMet and NDMac in PBS were incubated at RT for 30 min. Control bacterial suspension devoid of cranberry fractions was included. The bacteria were then washed free of unbound material and the bacteria bound cranberry material were suspended in 100 μL HBSS to which 800 μL of luminol mixture as described above was added. The mixture was incubated in luminometer (Lumac/3M, Biocounter M2010) for 240 s, at room temperature. The luminescence was measured every 30 s and the degree of light quenching by mixtures containing bacteria exposed to cranberry fractions as compared to bacteria not exposed to cranberry fractions (control) was determined to estimate the OSA of the bacteria bound to materials in the cranberry fractions.

**Assay for inhibition of bacterial coaggregation**

The coaggregation inhibition assay used was as previously described. Briefly, bacterial suspensions were adjusted to an optical density of 1.5 at 400 nm (UV-Vis. Spectrophotometer, Shimadzu, Tokyo, Japan) in PBS, corresponding to 2–4 × 10⁸ cells per ml. Equal volumes of 0.05 ml of *Fusobacterium nucleatum* (Fn) suspension and 0.05 ml of the test cranberry fraction at various concentrations in PBS were mixed and after 30 minutes incubation at RT with 0.05 ml of either streptococcal suspension (*Streptococcus sanguinis* Ss or *Porphyromonas gingivalis* Pg) was added to the mixture followed by vigorous vortexing for 10 s and 30 min gentle agitation at 100 rpm (Gyrotoryshaker, New Brunswick Scientific Co., Edison, NJ) to allow formation of stable coaggregates which were visually scored. Final concentrations of cranberry fractions in the wells ranged from 5000 to 120 μg mL⁻¹. Positive controls were either lactose (Fn/Pg) or procyanidin standards (SS/Fn); negative control wells received PBS instead of cranberry fraction. All treatments were performed in triplicate. In some experiments an alternative method was employed by mixing 0.02 ml volumes of each of the bacterial strains and of PBS or cranberry fractions in the same order as above on glass slides. Coaggregation was achieved by gently mixing the mixture for 2 min at room temperature. In both assays the minimum inhibitory concentration (MIC) of cranberry fraction that inhibits coaggregation was determined; MIC was recorded as the lowest test concentration at which no co-aggregation was visible.

**Biofilm formation assay**

*S. mutans* UA159, *S. aureus* (MRSA) and *S. epidermidis* (RP62A) were employed in this assay as described elsewhere with minor modifications. Briefly, the bacteria were grown aerobically for 24 h in BHI at 37 °C and 5% CO2. The bacterial cultures were then diluted in fresh broth medium (BHI + sucrose 2% for *S. mutans*, BHI + sucrose 2% + glucose 2% for MRSA, and TS + glucose 1% for *S. epidermidis*) to obtain an optical density at 655 nm (OD₆₅₅) of 0.01. Equal volumes (100 μl) of the diluted bacteria and indicated cranberry fractions (0, 20, 40, 80, 160 and 320 μg ml⁻¹) in appropriate BHI medium were mixed into the wells of 96-well plates (Thermo Scientific, Waltham, MA, USA). Control wells with no bacteria were also prepared. All treatments were performed in triplicate. Following incubation for 24 h at 37 °C, 5% CO2, bacterial growth was recorded by measuring the OD₆₅₅ using a microplate reader. Biofilm formation was determined by first aspirating the supernatants from the wells followed by three washes with PBS to remove planktonic and loosely adherent bacteria. The bacterial mass in the biofilm adherent to the bottom of the wells was stained with 200 μl of Gram’s crystal violet for 45 min followed. The wells were then rinsed with water and dried. The amount of biofilm biomass was quantified by destaining the wells with 200 μl of 33% acetic acid followed by measurement of the absorbance of the crystal violet solution in a microplate reader set at 595 nm. MIC was determined as the lowest concentration to significantly reduce biofilm compared to untreated control (p < 0.001), based on statistical analysis using one-way ANOVA to compare absorbance of treatment vs. control.

**HPLC, NMR and MALDI-TOF MS analysis**

HPLC-DAD analysis was performed as described previously with some modifications, on a Waters binary system with two 515 pumps, a 996 photodiode array detector and Millennium 32 software (Waters Corporation, Milford, MA). Samples were prepared at 10 mg mL⁻¹ in 75:25 water/methanol containing 2% acetic acid. A mobile phase consisting of 2% aqueous acetic acid (solvent A) and 2% acetic acid in methanol (solvent B), and a reversed phase C18 column (Waters Atlantis T3, 3 μm, 4.6 mm × 150 mm) were used. The elution program proceeded as follows with a flow rate of 0.8 mL min⁻¹: 0–20 minutes, gradient from 22–40% B; 20–40 minutes, gradient from 40–100% B; 40–45 minutes, isocratic elution with 100% B. Output was monitored from 210–600 nm to detect polyphenols.
\(^1\)H NMR analysis was performed on a Bruker AVANCE III 400 MHz system (Bruker BioSpin, Billerica, MA) using Topspin 3.2 and Assure Raw Material Screen (Assure-RMS) 1.5 software for collection and processing. Samples were prepared at 25 mg mL\(^{-1}\) in DMSO-\(d_6\) with DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) (Cambridge Isotope Labs, Tewksbury, MA) as an internal standard.

Selected fractions and subfractions were characterized by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) as described previously\(^{22,23}\) to detect high molecular weight constituents. Analyses were performed at the University of Massachusetts-Amherst Mass Spectroscopic Facility, using an Omnimix MALDI-TOF-Mass Spectrometer (Bruker Daltonics, Billerica, MA). Samples were prepared in methanol (10 µg mL\(^{-1}\)), mixed 1:1 with 50 mM 2,5-dihydroxybenzoic acid and 10 mM cesium trifluoroacetate solution matrix. 1 µL was spotted on the target (approx. 10 pmol analyte) and data was acquired in positive ion reflectron mode.

**Virus-induced haemagglutination assay**

A/H1N1 California 2009 and B/Malaysia/250606/influenza viruses were employed to induce Hemagglutination (HA) of human blood group O as described previously.\(^{24}\) Briefly, hemorhinized red blood cells (RBC) were washed twice in PBS using Sorvall RC-3 in rotor HL-8, 1800 rpm, at 4 °C, 6 min and re-suspended to 0.75% suspension in PBS. The viruses were grown in tissue culture (Madine-Darby kidney cells) to obtain a viral suspension. The HA titer of the viral suspension was determined by adding 0.1 mL RBC suspension to 0.1 mL of two fold dilution of viral suspension in PBS. HA was scored after 30 minutes at room temperature. Hemagglutination units (HAU) of the viral suspension were expressed as the reciprocal of the lowest concentration dilution that induced HA (e.g. a layer of RBC visible on the bottom of the wells). In assays to determine the effect of cranberry fractions on HA, 0.1 mL viral suspension diluted to give 8 HAU was mixed with equal volumes of PBS alone (to yield 16 HAU) or PBS that contained decreasing amounts of cranberry fractions (500, 250, 125 and 62.5 µg mL\(^{-1}\)) for 60 minutes, after which 0.1 mL RBC suspension was added and the HAU of mixtures was scored after further incubation of 30 minutes. All treatments were performed in triplicate. A decrease in HAU of cranberry samples denotes inhibition of the virus-induced HA.

**Results**

**Total phenolic content and antioxidant activity of cranberry fractions**

Fractionation of NDM on Sephadex LH-20 resulted in two fractions; one from the 50% ethanol wash solution (NDMet) and the other after eluting the bound material with 75% acetone (NDMac). The NDMac fraction isolated comprised about 28.5% of the original NDM by weight; whereas about 46.5% of NDM was recovered in the NDMet fraction. The initial Folin-Ciocalteu assay found that NDM, NDMac and NDMet had a total content of reducing substances such as polyphenols, equivalent to 58 ± 6 µM, 140 ± 13 µM, and 26.5 ± 3 µM GAE per mg, respectively. When commercial procyanidin A2 and B2 were used as standards for the Folin-Ciocalteu assay, the total content of reducing substances such as polyphenols was equivalent to 32% ± 2 for NDM, 14% ± 5 for NDMet and 63% ± 11 for NDMac (Table 1). Fractionation on Sephadex LH-20 therefore was effective at concentrating reducing substances such as polyphenols in the NDMac fraction, while the content of these substances was comparatively low in NDMet. This was further confirmed by HPLC and spectroscopic analysis as discussed below. As it was previously shown that cranberry phenols are potent antioxidants,\(^{25}\) the oxidant-scavenging ability of NDM and the two major fractions was tested. The OSA of the NDMac fraction was twice that of NDM, consistent with the percentage total phenol content from Folin-Ciocalteu assay.

Previous studies have assumed that the anti-adhesive activity of the cranberry NDM is due to the ability of polyphenols to bind bacterial surface components.\(^{26}\) To test whether the cranberry phenols in the fractions bind to the bacterial surface, we examined the antioxidant activity (OSA) of cranberry fractions bound to *E. coli*. For this purpose, *E. coli* was exposed to NDM and the two major fractions, followed by washing to remove non-bound material. The OSA of the bound materials was then determined and expressed as the concentration of fraction in the pre-exposure mixture required to exhibit 50% OSA. The OSA activity by whole bacteria with bound NDMac (ED\(_{50} = 20\) µg mL\(^{-1}\)) was approximately twice that of bound NDM (ED\(_{50} = 42\) µg mL\(^{-1}\)). By contrast, there was no significant increase in OSA of bacteria pre-exposed with NDMet at 100 µg mL\(^{-1}\) or less (ED\(_{50} > 100\) µg mL\(^{-1}\)). Because a similar NDMac/NDM ratio in OSA was observed in assays using soluble fractions (no bacteria), the data suggest that most of the reducing substances such as polyphenols in both cranberry fractions was able to bind to the bacteria and exhibit antioxidant properties. These experiments indicate that NDMac fraction is about twice as rich in these substances as well as in antioxidant activity in solution or bound onto bacterial surfaces as NDMet.

**Inhibition of bacterial co-aggregation and biofilm formation by cranberry fractions**

Polyphenol bound onto bacterial surface may be responsible for blocking the surface adhesins, which enable the bacteria to adhere to each other or to inanimate surfaces and subsequently form biofilm. Further experiments, therefore, were...
aimed to determine the minimal concentration of the cranberry fractions required to inhibit the adhesion activities. Because adhesion of bacteria to each other is especially important for oral bacteria to form dental plaque, the anti-adhesion activity of the cranberry fractions was assayed in a coaggregation assay with two pairs of oral bacteria, either *Streptococcus sanguinis* (Ss) with *Fusobacterium nucleatum* (Fn) or *Porphyromonas gingivalis* (Pg) with Fn (Table 2). The crude NDM and the phenol-rich fraction NDMac were equally effective in inhibiting coaggregation of Ss and Fn (MIC = 470 μg mL\(^{-1}\)) and of Fn and Pg (MIC = 940 μg mL\(^{-1}\)). The NDMet fraction was far less effective against both pairs. Purified commercial dimers of either A-linked or B-linked catechins, Procyanidin A2 and B2 respectively, were also assessed against Ss and Fn, and were less active than the NDM or NDMac (MIC ≥ 2000 μg mL\(^{-1}\)). The data suggest that the polyphenols in NDMac fraction are the major contributors to anti-coaggregation activity by NDM. Thus, NDMac was selected for further subfractionation and assessment against the oral bacteria (Table 2). The relative efficacies of subfractions are discussed further below.

The comparative ability of NDM and NDMac to inhibit biofilm formation by three bacteria, *Streptococcus mutans*, *Streptococcus epidermidis* and a methicillin-resistant strain of *Staphylococcus aureus*, determined using crystal violet staining is shown in Fig. 1. For all three bacteria, biofilms were significantly reduced by NDMac at concentrations as low as 80 μg mL\(^{-1}\), significant inhibition was observed at 320 μg mL\(^{-1}\) for the crude NDM.

**Inhibition of influenza-induced hemagglutination by cranberry fractions**

In previous studies, it was shown that crude cranberry NDM inhibited adhesion and subsequent cytotoxicity to animal cells by influenza virus.\(^{24}\) As the NDMac fraction retained the antibacterial activities of NDM, it was also evaluated in the present study for ability to inhibit influenza-induced hemagglutination in comparison to crude NDM. Hemagglutination induced by influenza virus strain A/H1N1 California 2009 was reduced from a hemagglutinating titer of 1 : 32 to 1 : 8 by 250 μg ml\(^{-1}\) NDM, and at 62.5 μg ml\(^{-1}\) NDM there was no detectable inhibition. NDMac at 250 μg ml\(^{-1}\) reduced titer from 1 : 32 to 1 : 16 at 62.5 μg ml\(^{-1}\) from 1 : 32 to 1 : 4. However, neither NDM nor NDMac inhibited hemagglutination induced by Influenza virus strain B/Malaysia, suggesting specificity of cranberry NDM toward strain A/H1N1 California 2009.

<table>
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<tr>
<th>Fraction</th>
<th><em>S. mutans</em> biofilm</th>
<th>Ss and Fn coaggregation</th>
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<tr>
<td>NDM (crude)</td>
<td>500</td>
<td>470</td>
<td>940</td>
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<tr>
<td>NDMet</td>
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<tr>
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<td>Lactose</td>
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**Table 2** Inhibition of biofilm formation and co-aggregation of oral bacteria by fractions and subfractions of NDM

*\(^{a}\)Highest concentration tested. N/A = data not available.*

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### Table 2

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Ss: *Streptococcus sanguinis*; Fn: *Fusobacterium nucleatum*; Pg: *Porphyromonas gingivalis*. \(^{a}\)Highest concentration tested. N/A = data not available.

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Fig. 1 Dose–response data for inhibition of biofilm formation by cranberry NDM and NDMac, using crystal violet staining. Values are the mean of triplicate ± SD for (A) *S. mutans*, (B) *S. aureus*, (C) *S. epidermidis*. * denotes treatment significantly different from control, \(p < 0.001\).
Spectroscopic characterization of NDMac and NDMet

The HPLC profile of crude cranberry NDM (Fig. 2) at 280 nm shows the unresolved peaks characteristic of cranberry extracts rich in polyflavan-3-ols or proanthocyanidins (PACs), with an absorbance maximum at 279.1 nm. HPLC profiles of NDMac and NDMet (Fig. 2) indicate a substantial increase in polyphenols in NDMac but the polyphenol absorbances are greatly diminished in NDMet. This is consistent with the results of the Folin–Ciocalteu assay. 1H NMR spectra in DMSO-d$_6$ of crude NDM and the two fractions are also poorly resolved, as is typical for complex mixtures of natural products (see ESI†). However, the NMR spectrum of ND Met shows the majority of resonances occur in the 3.5–5.5 ppm region typically associated with carbohydrates with very little signal appearing downfield of 6 ppm; whereas the strongest resonances in the NDMac spectrum were broad signals occurring between 5.7–6.1 ppm and 6.5–7.2 ppm, consistent with proanthocyanidins and flavonoids previously reported in cranberry and ND Met.

MALDI-TOF MS has been demonstrated as an effective tool for analysis of cranberry proanthocyanidin mixtures. The technique has successfully detected proanthocyanidin oligomers of over 20 degrees of polymerization, and we have used it to characterize extractable proanthocyanidins in fractions derived from whole cranberry fruit with antifungal properties. A predictive mass relationship was developed for proanthocyanidins with A-type linkages that allows for tentative identification of the size, units and type of linkage for each oligomer detected. Although MS cannot distinguish between stereoisomers, previous identification of epicatechin rather than catechin in cranberry proanthocyanidins by NMR suggests that the major constituent unit present in the cranberry NDM oligomers is epicatechin (290 amu). The structure of such a trimer previously reported in cranberry is shown in Fig. 3.

The MALDI-TOF MS spectrum of NDMac (Fig. 4) exhibited masses characteristic of previously identified proanthocyanidins in cranberries. These consist of [epi]catechin units with a mixture of A and B-type linkages, as well as some mixed oligomers with anthocyanin units linked to [epi]catechin either through a B-type linkage or a CHCH$_3$ bridge likely derived from condensation with acetaldehyde. Such species are presumed to exist in cranberry and tentative identifications were made based on previously derived mass relationships (Table 3). These oligomers were: dimers of [epi]catechin with cyanidin or peonidin hexose linked by a CHCH$_3$ bridge (m/z for [M + Cs$^+$] = 896.9 and 911.9 observed); dimer of [epi]catechin with peonidin pentose either linked directly or through a CHCH$_3$ bridge (m/z for [M + Cs$^+$] = 854.8 and 880.9 observed).

Fig. 2  HPLC chromatograms of crude cranberry NDM, NDMac fraction and ND Met fraction (279 nm) show characteristic profiles of mixed proanthocyanidin oligomers, strongest in NDMac and nearly absent in ND Met.

Fig. 3  Epicatechin trimer with one A-type linkage, previously identified in cranberry by Foo and coworkers.

Fig. 4  MALDI-TOF MS spectrum of acetone fraction NDMac, positive ion mode with cesium trifluoroacetate added. The labeled m/z values correspond to [M + Cs$^+$] and are consistent with polyphenol oligomers.
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Published on 24 April 2017. Downloaded by Hebrew University of Jerusalem on 04/08/2017 09:51:07.

Table 3 MALDI-TOF MS data and tentative identification of species detected in NDM fractions

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<th>Observed ion mass</th>
<th>Tentative identification</th>
<th>NDMac</th>
<th>ND Met</th>
<th>MCI-2</th>
<th>MCI-3</th>
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<tr>
<td>786.9</td>
<td>Cyanidin hexose + (epi)catechin w/CHCH3 bridge [M + Na+]</td>
<td>x</td>
<td>x</td>
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<tr>
<td>802.0</td>
<td>Peonidin hexose + (epi)catechin w/CHCH3 bridge [M + Na+]</td>
<td>x</td>
<td>x</td>
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<tr>
<td>854.9</td>
<td>Peonidin pentose + (epi)catechin [M + Cs+]</td>
<td>x</td>
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<td>880.9</td>
<td>Peonidin pentose + (epi)catechin w/CHCH3 bridge [M + Cs+]</td>
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<td>882.8</td>
<td>Peonidin hexose + (epi)catechin [M + Cs+]</td>
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<td>1014.8</td>
<td>2 (epi)Catechin + (epi)gallocatechin, 1A linkage [M + Cs+]</td>
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respectively); dimer of (epi)catechin with peonidin hexose linked directly (m/z for [M + Cs+] = 882.8); trimer of (epi)catechin with one A-type linkage (m/z for [M + Cs+] = 997, MW = 864 amu); trimer of two (epi)catechin with one (epi)gallocatechin (m/z for [M + Cs+] = 1015 MW = 882 amu), tetramer of (epi)catechin units with one A-type linkage (m/z for [M + Cs+] = 1285, MW = 1152 amu), pentamer of (epi)catechin units with one A-type linkage (m/z for [M + Cs+] = 1573, MW = 1440 amu), hexamer of (epi)catechin units with one A-type linkage (m/z for [M + Cs+] = 1861, MW = 1728 amu). Peaks at m/z = 786.9 and 801.9 are tentatively [M + Na+] for the dimers of epicatechin with cyanidin or peonidin hexose. Due to the complexity of the sample, these assignments are tentative; further work to isolate individual compounds and obtain NMR data would be necessary to verify the structures.

The MALDI-TOF MS spectrum of NDMet exhibited few masses attributable to polyphenol oligomers while the bulk material contained regular pattern of masses consistent with increasing numbers of hexose and pentose units was observed (Fig. 5). These masses are indicative of oligosaccharide components of the cell wall known as xyloglucans, which have recently been reported in cranberry fruit.11,12 Species tentatively identified (Table 3) include xyloglucans composed of 4 hexose units + 2 pentose units (m/z for [M + Cs+] = 1063, MW = 903 amu); 4 hexose + 3 pentose (m/z for [M + Cs+] = 1195, MW = 1062 amu); 5 hexose + 2 pentose (m/z for [M + Cs+] = 1225, MW = 1092); 4 hexose + 4 pentose (m/z for [M + Cs+] = 1327, MW = 1194 amu); 5 hexose + 3 pentose (m/z for [M + Cs+] = 1357, MW = 1224 amu) and 5 hexose + 4 pentose (m/z for [M + Cs+] = 1489, MW = 1356 amu); 6 hexose + 3 pentose (m/z for [M + Cs+] = 1519, MW = 1386 amu); 6 hexose + 4 pentose (m/z for [M + Cs+] = 1651, MW = 1518 amu). Na+ ion masses (not labeled) for these xyloglucans also appear in the spectrum 110 amu lower than the Cs+ ions. Although NDM in the concentrated juice was retained in dialysis tube with 12 000–14 000 molecular weight (MW) cut off, no species of m/z above 2500 amu were detected in any of the fractions. Also, when NDM was solubilized in 50% ethanol and dialyzed against 50% ethanol solutions, all of the material was retained in the 12 000–14 000 MW dialysis tube (data not shown). In contrast, when the commercial dimers of either A-linked or B-linked catechins solubilized in water were subjected to dialysis, none were retained in the dialysis tubing, as determined by the Folin–Ciocalteu assay (see Table 1). The data suggests that NDM and its fractions may contain microaggregates of oligomers of sizes equivalent to MW ≥ 12 000 amu.

Characterization of biologically active cranberry NDM subfractions

Initial results discussed above showed that the acetone fraction (NDMac) contained active material that inhibits coaggregation of oral bacteria, influenza virus-induced hemagglutination-

Fig. 5 MALDI-TOF MS spectrum of 50% ethanol fraction NDMet, positive ion mode. The m/z values are consistent with xyloglucans previously reported in cranberry.

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tion, formation of biofilm by *S. mutans* and other bacteria; further, NDMac exhibited potent antioxidant activity while bound onto the bacterial surface. Therefore, further studies focused on subfractionation of NDMac to characterize the active components, guided by the inhibition of coaggregation and biofilm inhibition assays. The bacterial bioassay data for the subfractions is shown in Table 2. To ascertain the specificity and extent of the effect on coaggregation, a complete inhibition of Fn and Pg coaggregation was shown by lactose, a known beta-galactoside containing inhibitor and positive control. NDMac and its subfractions with MIC of 940 μg mL⁻¹ were stronger inhibitors of Fn and Pg coaggregation than lactose (MIC = 2050 μg mL⁻¹). Of the four subfractions, NDMac-MCI-3 was the most effective in reducing biofilm formation by *S. mutans* (MIC = 125 μg mL⁻¹); more effective than the parent NDMac fraction (MIC = 250 μg mL⁻¹). NDMac-MCI-3 subfraction was also more effective than the other subfractions in preventing co-aggregation of Ss and Fn, but acted at a higher MIC (940 μg mL⁻¹) than parent fraction NDMac (470 μg mL⁻¹). Subfractionation had no quantifiable effect on prevention of co-aggregation by Fn and Pg; MIC values were the same as the parent fraction. The data seems to suggest that the combination of polyphenols in NDMac is effective in preventing co-aggregation, and that their actions may be synergistic. The lack of appreciable inhibition of biofilm and coaggregation by NDMet, composed primarily of xyloglucans, supports the role of the oligomeric polyphenols identified in NDMac as the major bioactive constituents in NDM. However, some of the xyloglucans may enhance these effects, as discussed below.

Further analysis of the NDMac subfractions MCI-2 and MCI-3 by MALDI-TOF MS reveals a mixture of species present (Table 3). MCI-2 contains many of the species present in NDMac of m/z < 900 amu. These include a major constituent at m/z = 897.3, tentatively a cyanidin hexose (epi) catechin dimer with a CHCH₃ bridge [M + Cs⁺], and a lesser constituent at m/z = 881.4, tentatively a peonidin pentose (epi) catechin dimer. Species having m/z consistent with A-type (epi) catechin trimer, tetramer and pentamer also appear. MCI-3 appears to contain some of these proanthocyanidin constituents along with several m/z > 900 amu ([M + Cs⁺] = 1195, 1327, 1357) possibly attributable to xyloglucans that were also present in NDMet but only minor species in NDMac. The ¹H NMR spectra of both MCI-2 and MCI-3 (DMSO-d₆) contained broad overlapping resonances between 6.5–7.2 ppm characteristic of proanthocyanidins and appearing in the spectrum of procyanidin A2; and also resonances between 8.2–9.2 ppm, characteristic of anthocyanins and proanthocyanidins, and appearing in the spectra of cyanidin-3-galactoside and procyanidin A2 (see ES†). Also present in the spectrum of MCI-3 were sharper, more well-defined signals matching those of the quercetin (Q) and myricetin (M) standards at 6.208 (Q, M), 6.390 (M), 6.43 ppm (Q, M), 6.890–6.918 ppm (d, Q), 7.264 ppm (M), 7.554–7.570 (dd, Q) and 7.70 ppm (Q), and 12.51 ppm (Q, M). MCI-3 was the most effective in reducing *S. mutans* biofilm (MIC = 125 μg mL⁻¹) which may result from this combination of polyphenols and xyloglucans. MCI-2 was somewhat less effective than MCI-3 against biofilm (MIC = 250 μg mL⁻¹) and coaggregation of Ss and Fn (1880 μg mL⁻¹). In addition to the polyphenol resonances, the spectrum of MCI-2 contained resonances at 7.97 (d), 7.63 (t) and 7.52 (t) characteristic of benzoic acid, a known cranberry constituent or a benzoyl moiety. The identification of several different classes of compounds including polyphenols and non-polyphenol constituents in the bioactive subfractions is consistent with several recent reports on inhibition of bacterial biofilm by cranberry compounds other than proanthocyanidins, discussed below.

**Discussion**

The quest for alternative therapies for infectious diseases and other conditions caused by bacteria has been driven by the emergence of pathogens resistant to most antibiotics in common use. An important guideline for alternative antimicrobial molecules is that such molecules are not bactericidal and do not affect growth of bacteria but rather inhibit important stages of the infectious process. This approach is expected to allow the sensitive strains to dilute out the resistant strains and hence to mitigate the spread of the resistant strains. Moreover, as biofilm formation by pathogenic bacteria is responsible for most infections, it becomes a target of preference to treat/prevent serious infections and health conditions.

The purpose of the present study is to shed light on the molecular species responsible for the anti-adhesion activity in non-dialyzable materials from cranberry juice. To characterize the constituents we employed MALDI-TOF MS analysis as it produces only a singly charged molecular ion for each parent molecule, allowing detection of high mass with precision and sensitivity, making it effective for profiling a series of oligomers with small differences in mass resulting from the extent of hydroxylation and variation in interflavan bonds, such as the polyflavan-3-ols found in cranberry. Cranberry polyflavan-3-ols (PACs) have been found to be primarily composed of epi catechin units with a combination of 4β→8 (B-type) direct carbon–carbon bond linkages (also found in apples, grape seed, and cacao), and linkage featuring both 4β→8 and 2β→O→7 interflavan bonds (A-type) that has been associated with the anti-bacterial adhesion properties of cranberry. MALDI-TOF MS has been demonstrated as an effective tool for analysis of cranberry proanthocyanidin mixtures, having successfully detected proanthocyanidin oligomers of up to and over 20 degrees of polymerization. We have used it to characterize extractable proanthocyanidins in fractions derived from whole cranberry fruit with antifungal properties. A predictive mass relationship was developed for proanthocyanidins with A-type linkages that allows for tentative identification of the oligomer size, units and number of A-type linkages for each oligomer detected. Although MS cannot distinguish between stereoisomers, previous identification of epicatechin rather than catechin in cranberry proanthocyanidins by NMR.
suggests that the major constituent unit present in the cranberry NDM PAC oligomers is epicatechin (290 amu).

Several conclusions emerged from the MS analysis as well as from the HPLC and $^1$H NMR profiles of the NDM fractions and subfractions. Although both the NDMac and NDMet fractions were derived from NDM which was retained in dialysis tubes of 12 000–14 000 MW cutoff, the MALDI-TOF MS analysis failed to detect molecular species of $m/z$ above 2500 amu in either fraction. A possible explanation is that aggregation of the highly hydroxylated species prevented passage through the dialysis tubing. Because the EtOH soluble NDM or the water soluble NDM at 500 µg ml$^{-1}$ concentration are clear to the naked eye, the assembly of oligomers must be in the form of microaggregates. Once formed in water the microaggregates withstand disaggregation in 50% ethanol, suggesting stable aggregates equivalent to the size of molecules with MW of $>$12 000 amu are present; consistent with MS data showing no evidence of individual molecules of this size. Because the commercial epicatechin dimer samples were readily dialyzed through tubing of 12 000 MW pore size, it is postulated that there must be a critical number of proanthocyanidin oligomers with a degree of polymerization of three or greater present for the polyphenols to form microaggregates. This is likely to occur through hydrogen-bonding interactions of these highly hydroxylated molecular species. Other constituents including quercetin derivatives and xyloglucan oligosaccharides may be present in the microaggregates and may also enhance the anti-adhesion activity of these proanthocyanidins, particularly with respect to reduction of bacterial biofilm. The size of the epicatechin oligomers, presence of other units and position of linkages varies, hence they are difficult to resolve by HPLC.

The study data suggest that the combination of polyphenols in NDMac is effective in preventing co-aggregation of oral bacteria, and that their actions may be synergistic, as subfractionation of NDMac decreased its efficacy. The ability to inhibit formation of S. mutans biofilm on the other hand was stronger in one of the subfractions (NDMac-MCI 3) and equivalent to the parent fraction in another (NDMac-MCI 2), so the specific combination of compounds at work in this instance may differ from those disrupting co-aggregation by the other oral bacteria. The lack of appreciable inhibition of biofilm and coaggregation by NDMet, composed primarily of xyloglucans, supports the role of the oligomeric polyphenols identified in NDMac as the major bioactive constituents in NDM. However, some of the xyloglucans may enhance these effects, as they have also shown anti-adhesive properties. Previous reports of cranberry extracts that inhibited biofilm formation by S. aureus and other bacteria also suggest that the PACs were not likely to be the only constituents involved in reducing the biofilm, similarly, oligosaccharide-rich fractions isolated from cranberry fruit were found to inhibit biofilm formation by uropathogenic E. coli CFT073. A recent study using atomic force microscopy to assess the effects of cranberry juice fractions on adhesion forces in cultures of a clinical E. coli strain (B78) demonstrated that the fractions most effective at reducing adhesion force contained quercetin glycosides and moreover, that quercetin galactoside demonstrated the ability to reduce adhesion forces. Thus, these compounds may be expected to enhance the anti-biofilm and bacterial co-aggregation effects of the proanthocyanidins in cranberry. NDMac significantly inhibited biofilm formation by S. aureus and S. epidermidis, to a similar extent observed for S. mutans, suggesting that further fractionation studies with these bacteria may be warranted.

The analyses of NDMac and its most bioactive sub-fractions suggest that the observed anti-adhesion activities are due to a combination of epicatechin-containing polyphenols, flavonol derivatives, and oligosaccharides that are likely xyloglucans, and that these unique combinations of polyphenol and non-polyphenol constituents may act more specifically and effectively on some bacteria than others. Future studies targeted towards separating and characterizing the individual constituents and comparing their bioactivities with that of the complex mixture will provide some understanding of which components are necessary for the activity as well as the extent of synergistic behavior between all components.

The combination of constituents in cranberry NDMac fraction also exhibited antiviral properties superior to NDM, inhibiting hemagglutination induced by influenza virus strain A/H1N1 California 2009 more effectively than the crude NDM. These data suggest that the constituents present in the NDMac fraction may be responsible for the previously observed antiviral adhesion to erythrocytes. Cranberry NDM was shown in a 2005 study to reduce infectivity of both influenza A and B types, possibly by direct interaction with the virus preventing adsorption onto cells. Comparison of NDM in this study with purified cranberry proanthocyanidins found the NDM to be several times more effective, suggesting that other constituents present in the NDM enhance the activities of the PACs. Cranberry NDM was also shown to interfere with the neuraminidase activity of influenza A and B strains. Further studies are needed to determine whether this property can be attributed to the polyphenols and other constituents identified in NDMac.

Conclusions

In summary, analyses revealed that the anti-adhesion activity in NDM may be due to at least two types of polyphenols: epicatechin-based oligomers of molecular weight ≤ 1800 amu containing A type interflavan linkages, and derivatives of quercetin; and this activity may be enhanced by the presence of non-polyphenol constituents putatively identified as xyloglucans. In cranberry juice, these highly hydroxylated molecules may exist in aggregates of a size equivalent to molecules of molecular weight ≥12 000 amu. The results of this study suggest that mixed polyphenol-containing oligomers from cranberry juice play a role in preventing co-aggregation and biofilm formation of oral bacteria, and that these effects may be enhanced by the presence of xyloglucans. Previously observed dialysis behavior suggesting the presence of very
high molecular weight constituents in NDM may be attributed to aggregation of the mixed polyphenol oligomers and oligosaccharides identified. The specific mix of oligomers in cranberry appears to be more effective than the commercially available purified A-type or B-type dimers, supporting a therapeutic role of cranberry preparations containing this mix of compounds in maintaining oral health and preventing infection.

Acknowledgements

The authors acknowledge NSF-MRI award CHE-1229339, which provided instrumentation support, as well as the assistance of Stephen Eyles and Rinat Abzalimov at the UMass Amherst Mass Spectrometry Facility, and Xin Wang and Liang Xue at UMass Dartmouth. This work was supported by Tel Aviv University and the UMass Cranberry Health Research Center.

References


