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Proanthocyanidin-rich Extracts from Cranberry Fruit (*Vaccinium macrocarpon* Ait.) Selectively Inhibit the Growth of Human Pathogenic Fungi *Candida* spp. and *Cryptococcus neoformans*

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ABSTRACT: Cranberry (*Vaccinium macrocarpon*) has been shown in clinical studies to reduce infections caused by *Escherichia coli* and other bacteria, and proanthocyanidins are believed to play a role. The ability of cranberry to inhibit the growth of opportunistic human fungal pathogens that cause oral, skin, respiratory, and systemic infections has not been well-studied. Fractions from whole cranberry fruit were screened for inhibition of five *Candida* species and *Cryptococcus neoformans*, a causative agent of fungal meningitis. *Candida glabrata*, *Candida lusitaniae*, *Candida krusei*, and *Cryptococcus neoformans* showed significant susceptibility to treatment with cranberry proanthocyanidin fractions in a broth microdilution assay, with minimum inhibitory concentrations as low as 1 μg/mL. MALDI-TOF MS analysis of subfractions detected epicatechin oligomers of up to 12 degrees of polymerization. Those containing larger oligomers caused the strongest inhibition. This study suggests that cranberry has potential as an antifungal agent.

KEYWORDS: cranberry, proanthocyanidins, yeast, *Candida*, *Cryptococcus*

INTRODUCTION

The American cranberry, *Vaccinium macrocarpon* Aiton (family Ericaceae), is cultivated widely in the northeastern United States, Wisconsin, the Pacific Northwest, and Atlantic Canada and is a key contributor to the economy in these regions. Cranberry production in the United States grew to a high of nearly 700 million pounds in 2008,[1] as a result of increased consumer demand, due in part to the health benefits associated with consumption of cranberry juice and products. Originally used by Native Americans to preserve dried meats and treat wounds, the consumption of cranberry juice in the prevention of urinary tract infections (UTIs) has become common practice. Within the past 25 years, the body of scientific evidence to support that use has grown[2,3] as investigations have shown that cranberry juice can prevent adherence of *Escherichia coli* bacteria to uroepithelial and other eukaryotic cells.[4−6] Since 1994, at least 15 clinical trials have evaluated the prophylactic effects of cranberry against urinary infections in various populations, showing significant reductions in incidence and recurrence of UTIs with the consumption of cranberry products.[2,3] The cranberry's status as a functional food has grown, as emerging studies suggest that cranberry may also have benefits for oral health,[7] cardiovascular diseases, and certain cancers.[8]

The biological activities of cranberry can be attributed to a diverse group of phytochemicals, including flavonoids, hydroxycinnamic acid derivatives, organic acids, and isoprenoids including ursolic acid and lutein.[9] The flavonoids fall primarily into three classes: anthocyanins, flavonols, and proanthocyanidins (PACs) or polyflavan-3-ols. Cranberry fruit PACs are primarily dimers, trimers, and larger oligomers of epicatechin, containing two types of linkages between epicatechin units: the more common 4β→8 (B-type) linkage (also found in apples, grape seed, and cacao), and a less common A-type linkage featuring both 4β→8 and 2β→O→7 interflavanoid bonds that have been associated with the antibacterial adhesion properties of cranberry.[10] Positions of the linkages may vary, so three-dimensional structures are diverse. A 100 g serving of cranberry fruit contains on average 180 mg of PAC oligomers having 10 degrees of polymerization (DP) or less, and the content of larger oligomers may be even higher.[11] Many of the reported health benefits of cranberry, particularly antibacterial activities, have been associated with the consumption of these compounds. Whereas numerous studies have addressed the antibacterial properties of cranberry fruit and juice, few have examined cranberry's ability to inhibit human fungal pathogens. Preliminary studies in our laboratory observed moderate activity of cranberry fruit extracts against *Candida albicans* and *Candida krusei* (Kondo and Neto, unpublished results), suggesting that cranberry's ability to inhibit microbes includes not only bacteria but also human fungal pathogens. *Candida* spp. are known primarily for causing oral and skin infections, particularly in immunocompromised patients. Candidiasis has been ranked among the most common nosocomial infections,[12] often developing within 48 h of hospitalization.[13] Infections caused by *Cryptococcus neoformans* can lead to cryptococcal meningitis, with the organisms entering the body through the respiratory or gastrointestinal tract and targeting the central nervous system. The reported incidence in AIDS patients is high,[14] and cryptococcal meningoencephalitis is a common incurable fungal infection in AIDS patients.[15] Increasing resistance of *Candida* and *Cryptococcus* to common antifungal agents such as amphotericin B and fluconazole deepens the need to identify alternatives. A bioassay-guided fractionation study was undertaken

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to evaluate the ability of whole cranberry and its constituents to inhibit the growth of several pathogenic *Candida* spp. and *Cryptococcus neoformans* and to identify and characterize the active constituents.

MATERIALS AND METHODS

Plant Material and Reagents. Cranberry fruit (*V. macrocarpon* Ait. var. Early Black) was harvested in September 2008 at the University of Massachuetts Cranberry Experiment Station, East Wareham, MA, flash frozen with liquid nitrogen, and stored at −20 °C until use. Reagent grade acetone, methanol, formic acid, and ethyl acetate were purchased from Pharmco Products Inc. (Brookfield, CT). HPLC grade methanol, water, and DMSO were from Fisher Scientific. Diaion HP-20 was from Supelco, Inc. (Bellefonte, PA), and Sephadex LH-20 was from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Procyanidins A2 and B2 were from Indofine Chemical Co. (Hillsborough, NJ). Quercetin-3-galactoside and cyanidin-3-galactoside were from Chromadex, Inc. (Irvine, CA). Epicatechin, epigallocatechin gallate (EGCG), and miconazole nitrate were from Sigma-Aldrich Co. (St. Louis, MO). Fungal strains were obtained from American Type Culture Collection (ATCC), Bethesda, MD: *Cryptococcus neoformans* (ATCC 14116), *Candida albicans* (ATCC 14053 and 10231), *Candida krusei* (ATCC 14243), *Candida tropicalis* (ATCC 13803), *Candida glabrata* (ATCC 2950), and *Candida lusitaniae* (ATCC 34449). RPMI 1640 medium supplemented with 165 mM MOPS and L-glutamine, without sodium bicarbonate, was from VWR International (Radnor, PA).

Preparation of Crude Cranberry Extracts. Cranberry fruit from two harvest years, 2008 (520 g) and 2009 (1044 g), was thawed and separately extracted with a solvent mixture of 40:40:19:1 acetone/methanol/water/formic acid (v/v) as described previously.[16] Fruit was macerated in 200 mL of solvent in a Waring blender for 5 min and then allowed to stand for 30 min. The slurry was filtered and the solids were blended with several additional 100 mL portions of solvent until the solids were nearly colorless. An additional extraction with 200 mL of ethyl acetate was used on the 2009 fruit. The two filtrates were concentrated by rotary evaporation and freeze-dried, then redissolved in a minimum volume of distilled water and chromatographed on Diaion HP-20 resin (5.5 × 30 cm) to remove free sugars. The extracts were loaded and allowed to adsorb to the resin for 15 min. The column was then washed with distilled water until the eluate was clear (approximately 2 L). The polyphenolics were eluted with 100% methanol until all color was gone from the resin, and the eluates were concentrated in vacuo and freeze-dried to yield 2.99 g of desugared crude extract 1 (DCE-I) from 2008 and 7.11 g of desugared crude extract (DCE-II) from 2009.

Fractionation of Crude Extracts. Fractions were prepared from each of the crude extracts by chromatography on Sephadex LH-20 using a modification of previously reported procedures developed to separate phenolic constituents by structural classes[16] as identified by HPLC analysis. DCE-I (1.025 g) was dissolved in a minimum volume of distilled water and applied to a Sephadex LH-20 column packed in 70:30 methanol/water (3 × 22 cm). Two separate elutions were performed using approximately 0.5 g each time and combining like fractions. Fractions were eluted and each band collected on the basis of visible color differences as noted, dried in vacuo, and stored at −20 °C until use. Elution began with 600 mL of 70:30 methanol/water, yielding a colorless band (fraction I-1, 32 mg); a pale yellow-orange band (I-2, 238 mg); a deep reddish band (I-3, 120 mg); and a yellow band (I-4, 185 mg). Elution with 100 mL of 70:30 acetone/water yielded a tan band (I-5, 359 mg). Separation of 0.508 g of DCE-II yielded fractions II-1 (22 mg), II-2 (87 mg), II-3 (86 mg), II-4F (29 mg), II-4SP (32 mg), and II-5 (132 mg). Additional elution with 100 mL of acetone produced a yellow fraction, II-6, of 90 mg. Fraction I-4 from DCE-I (52 mg) was further subfractionated after HPLC analysis by loading onto a Sephadex LH-20 column (3 × 18.5 cm) packed in 50:50 ethanol/water, eluting with 500 mL of 50:50 ethanol/water and collecting fractions based on visible color differences. A red band eluted first (I-4A, 3.0 mg), then a greenish yellow band (I-4F, 19 mg). Elution with 70:30 acetone/water produced an off-white solid on drying (I-4SP, 16 mg).

HPLC Analysis. HPLC analysis was performed on a Waters Millenium binary HPLC system with Waters 515 pumps, a 996 photodiode array (PDA) detector, and Millennium[32] version 32.0 software. Samples were analyzed at room temperature using one of the following programs. Absorbance was monitored at 210−600 nm, with 280 nm indicative of proanthocyanidins, 310 nm of coumaric acid derivatives, 355 nm of flavonol glycosides, and 520 nm of anthocyanins.

HPLC Method 1. Gradient elution on a Waters Symmetry C18 column (3.0 × 150 mm, 5 μm) was used, where solvent A = 2% aqueous acetic acid and solvent B = 2% acetic acid in methanol. Program: 0−5 min, linear gradient from 95 to 75% A; 5−25 min, gradient to 65% A; 25−35 min, gradient to 60% A; 35−50 min, gradient to 5% A, holding at 5% A until 55 min; 55−65 min, gradient to 0% A. Flow rate was 0.6 mL/min. Method 1 was used to analyze all fractions except I-3, II-3, I-5, and II-5.

HPLC Method 2. Gradient elution on a Waters Symmetry C18 column (4.6 × 250 mm, 5 μm) was used, where solvent A = 4% aqueous acetic acid and solvent B = 4% acetic acid in methanol. Program: 0−30 min, linear gradient from 99 to 80% A; 30−55 min, gradient to 70% A; 55−70 min, gradient to 60% A; 70−80 min, gradient to 0% A, holding until 85 min. Flow rate was 0.8 mL/min. Method 2 was used to analyze crude extracts DCE-1 and DCE-2 and fractions I-3 and II-3, which required better resolution of anthocyanins. Anthocyanins were quantified in the crudes at 520 nm as cyanidin-3-galactoside using a standard curve, and flavonols were quantified at 355 nm using a quercetin-3-galactoside standard curve.

HPLC Method 3. Gradient elution on a Waters XTerra C8 column (4.6 × 250 mm, 5 μm) was used, where solvent A = 2% aqueous acetic acid and solvent B = methanol. Program: 0−5 min, 100% A; 5−60 min, linear gradient to 0% A. Flow rate was 0.6 mL/min. Method 3 was used to qualitatively analyze proanthocyanidin fractions I-5 and II-5 and screen for the absence of other constituents.

Mass Spectrometry Analysis. electrospray ionization turbo-ion spray mass spectrometry (ESI-MS) was performed on selected fractions. A Perkin-Elmer Sciex API-150EX single-quadrupole mass spectrometer coupled to a series 200 micropump and a series 200 autosampler was employed to analyze the fragments using negative ion mode, using Analyst 1.4.2 software. Settings: declustering potential = −10 V, focusing potential = −200 V, entrance potential = −10 V. Samples were dissolved in methanol, loaded onto a Phenomenex C18 column (30 × 2 mm, 5 μm), and eluted with 100% acetonitrile at a flow rate of 300 μL/min. Fractions identified by HPLC analysis as containing proanthocyanidins, showing the presence of characteristic peaks with λ_{max} of 279.1 nm, were analyzed by matrix-assisted laser desorption/ionization−time-of-flight mass spectrometry (MALDI-TOF MS) at the University of Massachusetts—Amherst Mass Spectroscopic Facility by Dr. Stephen Eyles, using an Omniflex MALDI-TOF-mass spectrometer, Bruker Daltonics, Billerica, MA. Samples were prepared at 10 μM in methanol, mixed 1:1 with 50 mM 2,5-dihydroxybenzoic acid and 10 mM cesium trifluoroacetate solution matrix. One microliter was spotted on the target (approximately 10 pmol of analyte), and data were acquired in positive ion reflectron mode.

Broth Microdilution Antifungal Assay and Determination of Minimum Inhibitory Concentrations (MIC). Cranberry extracts, fractions, and commercial standards (procyanidin A2, procyanidin B2, epigallocatechin gallate, epicatechin) were evaluated for antifungal activity and determination of MICs against six organisms (*Candida glabrata*, *Candida lusitaniae*, *Candida krusei*, *Candida tropicalis*, *Candida albicans*, and *Cryptococcus neoformans*) by a broth microdilution method based on a modification of the Clinical Laboratory Standards

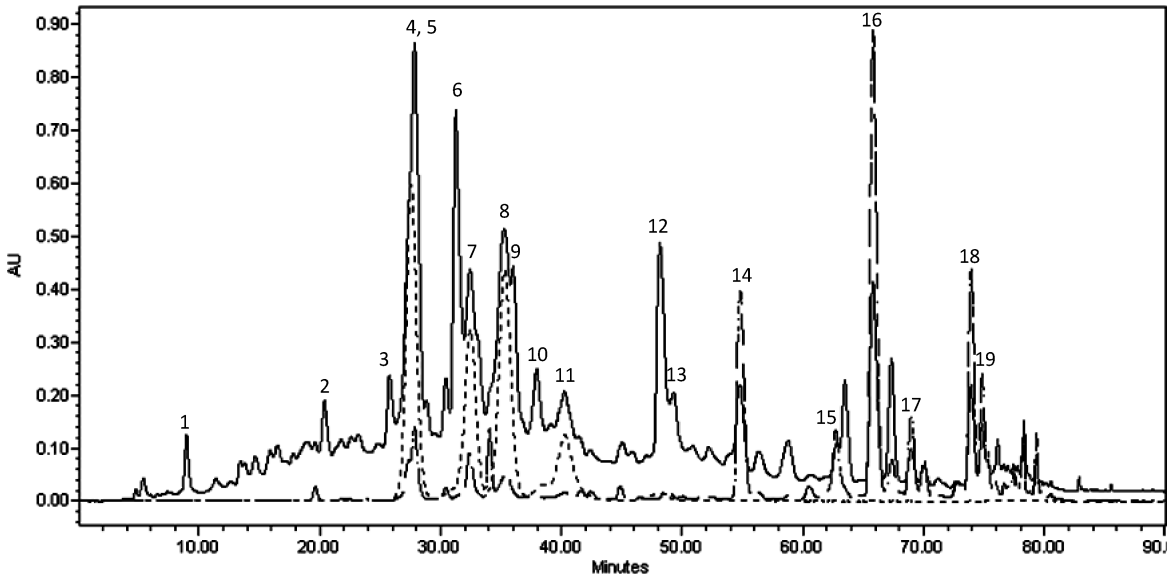

Figure 1. HPLC-DAD profile of DCE-I chromatographed on C18 using method 2. Absorbance is monitored at 280 nm (solid line) for proanthocyanidins, at 355 nm (dot-dashed line) for flavonol glycosides, and at 520 nm (dotted line) for anthocyanin glycosides. The numbered peaks are identified in Table 1.

Institute (CLSI) Broth Microdilution Antifungal Assay, M27A3.[17] Briefly, fungal strains were cultured in RPMI 1640 media supplemented with 165 mM MOPS and L-glutamine, without sodium bicarbonate. Stock cultures were prepared from isolated colonies of each freshly cultured organism standardized to a 0.5 McFarland standard at 530 nm ($1 \times 10^6 - 5 \times 10^6$ cells/mL) in 5 mL of 0.45% sterile saline. These were diluted 1:50 and then 1:20 with media to produce a standardized inoculum containing $1 \times 10^3 - 5 \times 10^3$ cells/mL. Assays were performed in sterile polystyrene 96-well flat-bottom plates, with miconazole nitrate as the positive control. Test samples were prepared from 2 mg/mL stock solutions of extracts in media, serially diluted in the wells to concentrations of 500−0.25 μg/mL (100 μL volume). Standardized inoculum (100 μL) was added to the wells containing either test samples or 100 μL of media only for growth control. Twofold serial dilutions were used to determine the MICs for active fractions. Samples and positive control were tested in triplicate alongside absorbance controls containing sample but no organism to normalize optical density (O.D.) for absorbance due to test compounds and a growth control of the organism in medium only as a reference for variability in growth rate. *Candida* species *C. glabrata*, *C. albicans*, *C. lusitaniae*, and *C. tropicalis* were incubated for 24 h at 30 °C before measuring O.D.; slower growing organisms *Candida krusei* and *Cryptococcus neoformans* were observed after 48 h of incubation. The O.D. in each well was determined using a SpectraMax M5 (Molecular Devices) plate reader. Plates were read at 530, 625, and 675 nm at room temperature (digitized using a Cooke MICROTITER System plate reading mirror and SOFTmax PRO software) and also observed visually and photographed. The data were plotted as O.D. at 625 nm versus concentration. Percent inhibition relative to growth control was determined on the basis of comparison of O.D. for treatment versus control for each organism. The mean (n = 3) and the standard error of mean (SEM) were calculated for each treatment. Statistical analysis was carried out using SigmaPlot 11.0. Treatments were compared with growth control using the Holm−Sidak test for one-way ANOVA. Treatments were considered to be significantly different if the computed P value was less than 0.001 or 0.05, indicated by "**" or "*", respectively. MIC_{50} and MIC_{90} were determined as the minimum concentrations necessary to inhibit growth relative to untreated control by 50 or 90%, respectively.

RESULTS AND DISCUSSION

Composition of Crude Cranberry Extract and Fractions. Chromatography of crude cranberry fruit extracts from 2008 (DCE-I) and 2009 (DCE-II) on Sephadex LH-20 yielded fractions that were qualitatively analyzed for composition by HPLC-DAD and MS. HPLC profiles of DCE-I crude extract and selected fractions are shown in Figures 1 and 2. The major flavonol and anthocyanin glycosides in the crude extracts were identified (Table 1) on the basis of comparison of retention time and absorbance maxima with previous data from our laboratory.[18] Peaks having an absorbance maximum at 279.1 nm characteristic of epicatechin were identified as proanthocyanidin oligomers. Procyanidin A2 was identified on the basis of commercial standards. Quantification of total flavonols as quercetin-3-galactoside and total anthocyanins as cyanidin-3-galactoside in the crude extracts by HPLC analysis with commercial standards found the crude extract composition to be approximately 9.9% (DCE-I) and 8.8% (DCE-II) flavonol glycosides and 10.2% (DCE-I) and 7.9% (DCE-II) anthocyanin glycosides by weight. Proanthocyanidin content was estimated gravimetrically at 40% (DCE-I) and 32% (DCE-II) by weight.

Chromatograms of I-1, II-1, I-2, and II-2 at 310 nm revealed predominantly phenolic acids and their derivatives with absorbance maxima between 305 and 320 nm (data not shown). Chromatograms of fractions I-3 and II-3 at 520 and 310 nm revealed predominantly anthocyanin glycosides, flavonol glycosides, and *p*-hydroxycinnamic acid present (data not shown). Further characterization of fractions I-1, I-2, I-3, II-1, II-2, and II-3 was not undertaken, as the fractions showed no inhibitory activity in the broth microdilution antifungal bioassays. Chromatograms of fractions I-4 and II-4 at 355 nm revealed predominantly flavonol glycosides exhibiting λ_{max} 350−355 nm. I-4 (Figure 2a) also contained peaks with an absorbance maximum at 279.1 nm, characteristic of epicatechin-based PACs; thus, the fraction was separated to yield I-4F containing primarily flavonol glycosides and I-4SP containing proanthocyanidins (Figure 2b). II-4SP also contained primarily proanthocyanidins, on the basis

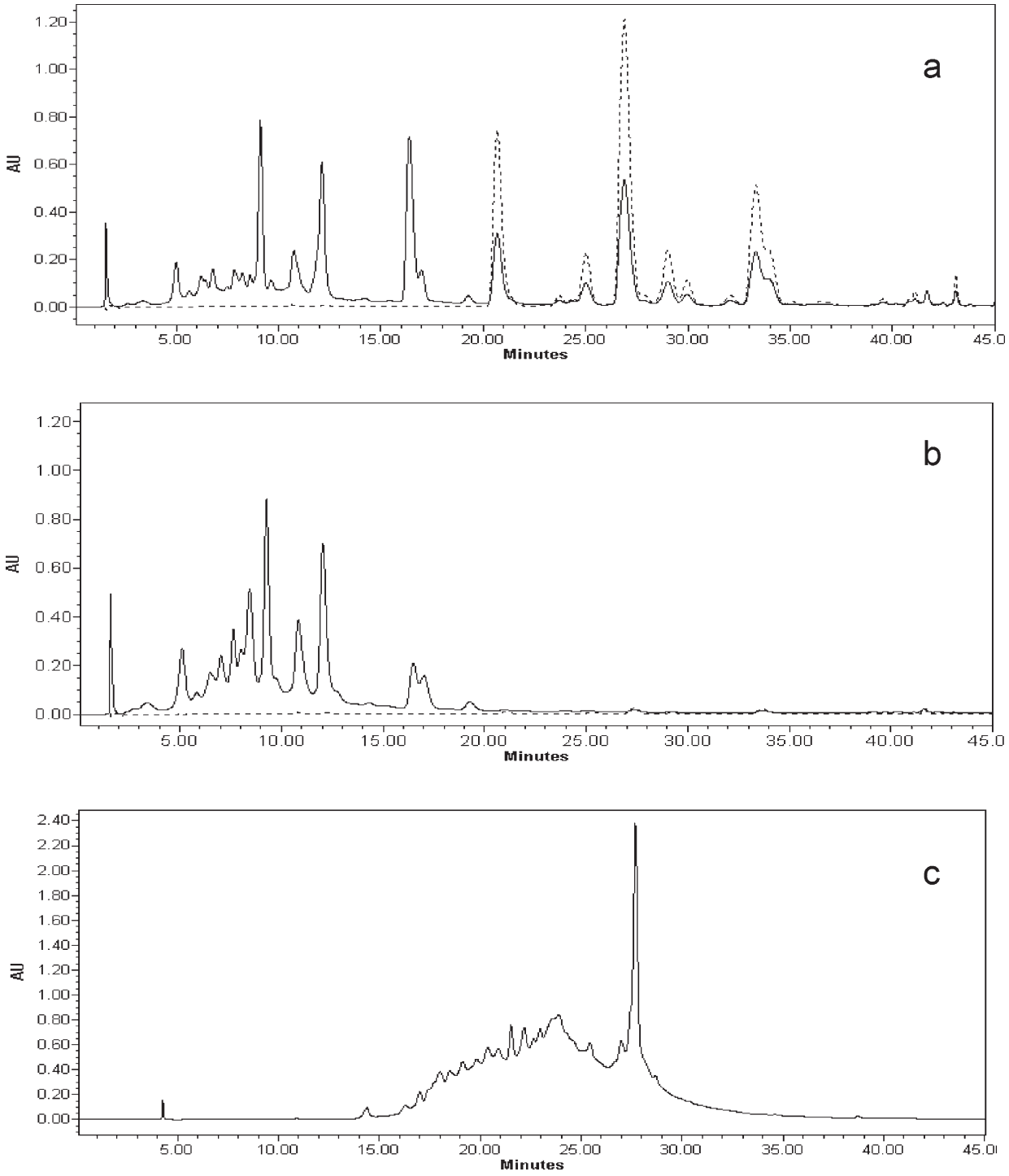

Figure 2. HPLC-DAD profiles of fractions exhibiting antifungal activity: (a) I-4 and (b) I-4SP chromatographed on C18 using method 1 (solid line = 280 nm and dashed line = 355 nm), showing the absence of flavonols in fraction I-4SP after purification; (c) I-5 chromatographed on C8 using method 3 (solid line = 280 nm) shows the poorly resolved "hump" characteristic of cranberry proanthocyanidins that results from structural diversity due to broad oligomer size range (DP = 2−12) and the presence of A- and B-type linkages. The peak at 27.5 min was identified as procyanidin A2 by comparison to commercial standard.

of the appearance of multiple peaks with λ_{max} of 279.1 nm, characteristic of epicatechin and its oligomers. Analysis of I-4SP and II-4SP at 355 and 520 nm revealed no significant content of anthocyanins or flavonols. Comparison to commercial standards confirmed the presence of procyanidins A2 and B2 in these fractions. The fractions exhibited activity in the broth microdilution assay and were therefore further characterized by MALDI-TOF MS.

HPLC analysis of fractions I-5 and II-5 using method 3, monitoring at 280 nm, showed the characteristic profile of a mixture of proanthocyanidin oligomers (I-5 is shown in Figure 2c). An indistinct hump of overlapping peaks with absorbance maximum at

Table 1. Peak Assignments for Constituents of Crude Cranberry Extract DCE-1, Appearing in the HPLC-DAD Profile (Figure 1)

peak	tentative assignment	absorbance maxima (nm)
1	unidentified proanthocyanidin[a]	235.6, 279.2
2	unidentified	235.6, 280.4
3	unidentified proanthocyanidin	235.6, 279.2
4	cyanidin-3-galactoside	236.8, 280.4, 514.1
5	*p*-coumaric acid	235.6, 314.8
6	unidentified	243.9, 276.9
7	cyanidin-3-arabinoside	236.8, 280.4, 517.7
8	peonidin-3-galactoside	236.8, 279.2, 517.7
9	procyanidin B2 dimer	235.6, 279.2
10	unidentified proanthocyanidin	235.6, 279.2
11	peonidin-3-arabinoside	236.8, 279.2, 520.2
12	procyanidin A2 dimer	236.8, 278.1
13	unidentified proanthocyanidin	235.6, 279.2
14	myricetin-3-galactoside	234.4, 262.6 sh, 357.7
15	myricetin-3-arabinoside	234.4, 263.9 sh, 355.3
16	quercetin-3-galactoside	234.5, 255.6, 355.3
17	quercetin-3-xyloside	234.4, 255.2 sh, 355.3
18	quercetin-3-arabinoside	234.4, 255.6, 354.1
19	quercetin-3-rhamnoside	234.4, 255.6, 350.6

[a] Compounds with absorbance maxima at the characteristic wavelengths for epicatechin and procyanidin B2 (235.6, 279.2 nm) are tentatively assigned as proanthocyanidins.

279 nm appears. Elution of cranberry proanthocyanidin samples containing large oligomers on a reverse-phase C8 column may be preferable to a C18, which tends to retain large oligomers more strongly, shortening column lifetime. However, peaks are not resolved due to the complexity of samples resulting from oligomers of different DPs containing both A-type and B-type linkages. This elution profile is typical for cranberry PAC oligomers on C8. Whereas normal-phase elution on silica or diol columns is effective for the B-type PACs found in cocoa,[19] we found this method to be less effective for the analysis of cranberry PAC fractions from whole fruit. The presence of procyanidin A2 in fractions I-5 and II-5 was verified by comparison to commercial standard, but larger A-type proanthocyanidin standards are not commercially available. Fractions I-5 and II-5 exhibited antifungal activity in the broth microdilution assay.

Characterization of Proanthocyanidin Fractions by MALDI-TOF MS. Characterization of proanthocyanidin fractions I-4SP, II-4SP, I-5, and II-5 was accomplished by means of MALDI-TOF MS analysis, which has been used previously to characterize PACs from cranberry fruit[20] and other plant sources including grape seed and sorghum.[21,22] MALDI-TOF MS is considered to be the mass spectrometric method of choice for analysis of polydispersed ions and tannins exhibiting large structural heterogeneity because it produces only a singly charged molecular ion for each parent molecule and allows detection of high mass with precision.[23] Other advantages include high sensitivity across a broad range of masses, allowing detection of oligomeric series of compounds, such as found in cranberry, and detection of oligomers with small differences in mass.[24] MALDI-TOF MS can distinguish molecular weight differences due to the extent of hydroxylation or the nature of interflavan bonds (A- or B-Type), but cannot assign specific stereochemistry to the molecule.

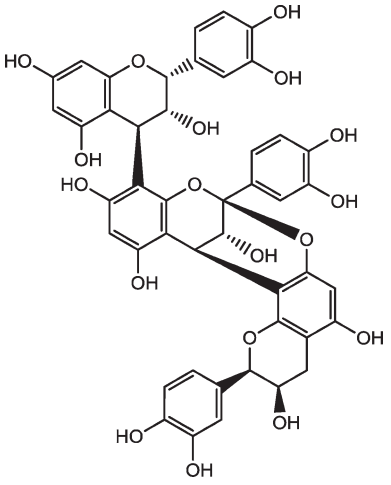

Figure 3. Structure of a proanthocyanidin trimer, previously identified in cranberry by Foo and co-workers, shows the presence of both A-type and B-type linkages between epicatechin units.

However, previous studies elucidating the structures of PAC dimers and trimers in cranberry fruit by NMR have confirmed that the major flavan-3-ol is epicatechin, rather than catechin, and that A-type linkages are present.[10] One of these structures is shown in Figure 3. It is therefore assumed that monomeric units with m/z 290 appearing in cranberry PAC oligomers are epicatechin and are assigned accordingly.

On the basis of MALDI-TOF MS data (Figure 4), fractions I-4SP and II-4SP contained primarily PAC oligomers composed of seven or fewer epicatechin units; masses are summarized in Table 2. Tentative assignments of oligomer DP and the number of A-type linkages for each ion mass in Table 2 are based on predictive mass data developed for oligomeric polyphenols containing epicatechin or galloylated catechins of various DPs containing A-type and/or B-type interflavan linkages.[24] With a mass of 290 amu for each epicatechin unit, an oligomer with simple B-type linkages between units would be given by the equation m/z 290 + 288(n), where n = the number of additional units after the terminal epicatechin. Loss of two hydrogens from the molecule to form the 2β-O-7 interflavan bond of the A-type linkage produces a mass decrease of 2 amu for each A-linkage in an otherwise B-linked oligomer, m/z 290 + 288(n) − 2(a), where a = the number of A-type linkages. Most of the oligomers appear as the cesium ion, $[M + Cs]^+$, due to the use of cesium trifluoroacetate in the matrix. Fraction I-4SP was isolated from I-4 by an additional separation step in which the flavonols (yellow in color) were removed from the fraction by elution with 50:50 ethanol/water prior to elution of the colorless oligomeric fraction. The peak with greatest intensity in the MALDI-TOF mass spectrum of I-4SP appears at $[M + Cs]^+$ = 997.4. This gives a molecular mass of 864, which corresponds to an epicatechin trimer with one A-type linkage [290 + 288(2) − 2]. A peak at 709.6 amu for $[M + Cs]^+$ indicates the A-type epicatechin dimer of mass 576 is also present; this peak also appears in the MALDI-TOF MS of commercial procyanidin A2. Figure 4a shows the MALDI-TOF MS of I-4SP for the mass range m/z 700−2500. Peaks appearing at m/z 1285.5 and 1573.9 correspond to $[M + Cs]^+$ for epicatechin tetramer and pentamer, respectively, with one A-type linkage. Peaks of lesser intensity appearing at m/z 1862.1 and 2148.5 are indicative of small amounts of hexamer and heptamer present, each with one A-type linkage. A peak of

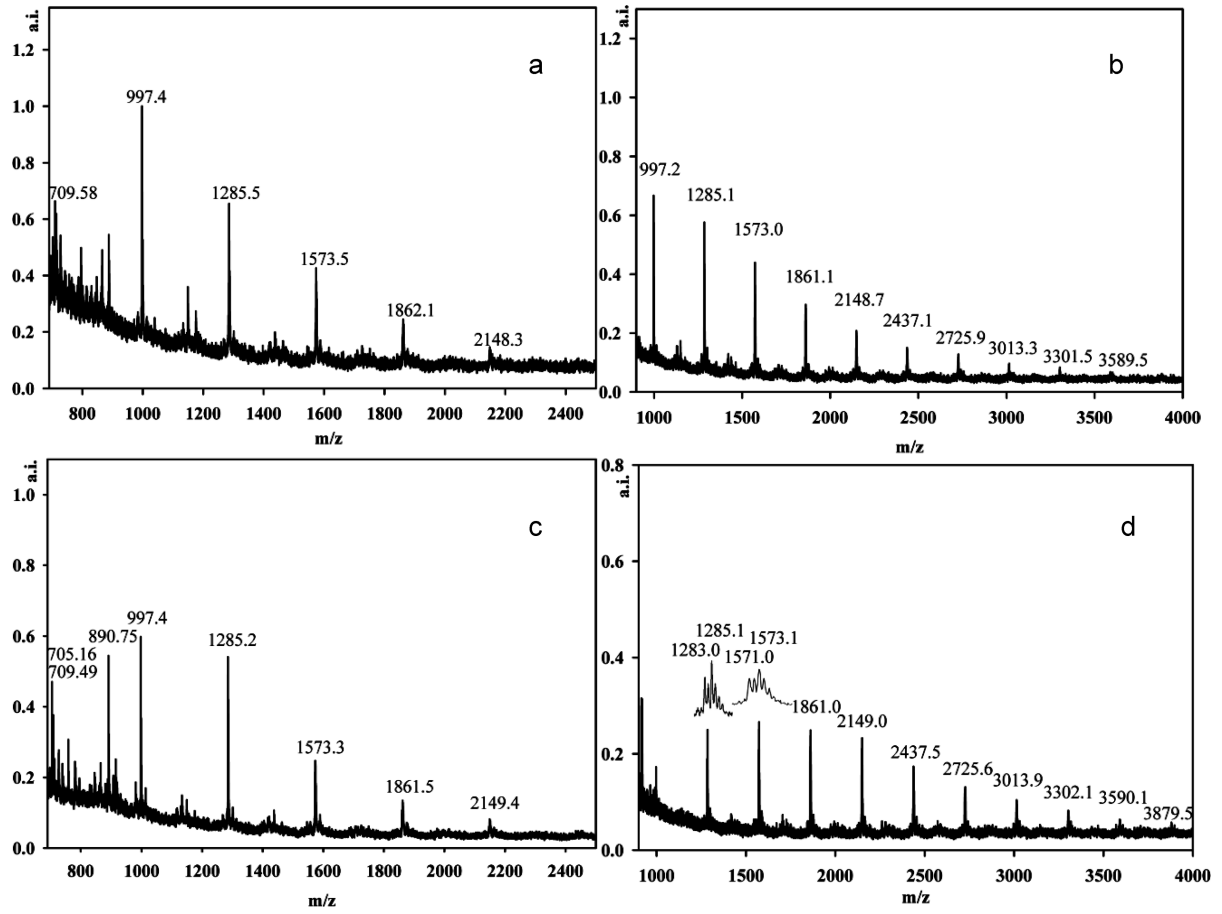

Figure 4. MALDI-TOF MS spectra of cranberry fruit fractions that inhibited the growth of selected *Candida* and *Cryptococcus* species: (a) I-4SP; (b) I-5; (c) II-4SP; (d) II-5. m/z values shown correspond to $[M + Cs]^+$ ions for oligomers, identified in Table 2.

lesser intensity appearing at m/z 1149.4 may indicate a cesium ion with a small degree of galloylation; thus, a molecular mass of 1016 could be assigned to an epicatechin trimer with a galloyl ester and one A-linkage, following the equation m/z 290 + 288(n) + 152(g) − 2(a), where g = the number of gallates. Other minor peaks appearing at intervals of 152 amu greater than the major oligomer peak suggest galloylation of larger oligomers. The distribution of masses in fraction I-4SP suggests the fraction is composed primarily of oligomers with DP = 2−5 and no detectable oligomers larger than a heptamer. The composition is similar to that previously reported by us for a cranberry PAC fraction with antiproliferative activity in tumor cell lines.[20]

II-4SP was isolated from the first separation of DCE-II by extending the elution step with 70:30 methanol/water well beyond the appearance of colored anthocyanin or flavonol bands. This nearly colorless fraction was also found to contain epicatechin oligomers by HPLC. The major ions detected by MALDI-TOF MS analysis and their tentative structural assignments are summarized in Table 2. Comparison of I-4SP and II-4SP (Figure 4a,c) suggests that the composition of the two fractions is very similar, with II-4SP having a slightly greater content of the tetramer compared to I-4SP. As with the other fraction, II-4SP appears to contain very little hexamer or heptamer, and peaks appearing at 152 mass units greater than the major oligomer masses are indicative of some galloylation.

Elution of the Sephadex LH-20 column with 70:30 acetone/water produced fractions containing larger oligomers. HPLC chromatograms of fraction I-5 from the separation of DCE-I and fraction II-5 from the separation of DCE-II (Figure 2) show a large hump of overlapping peaks with λ_{max} = 279.1 nm, indicative of epicatechin oligomers of diverse size and structure distribution. By subtracting the mass of Cs and using the predictive equation m/z 290 + 288(n) − 2(a), where n = number of nonterminal epicatechin units and a = number of A-type linkages, the major peaks in the MALDI-TOF mass spectra (Figure 4b,d) for I-5 and II-5 were assigned. Masses corresponding to epicatechin oligomers of up to DP = 13 were detected in both samples (Table 2). Whereas the intensities of individual peaks corresponding to oligomers of DP > 4 were lower than those attributed to smaller oligomers, a broader distribution of peaks in the mass range of tetramers and larger suggested greater structural heterogeneity. Masses of 1 amu greater than the major ion may be attributed to isotope effects; however, mass differences of 2 amu in proanthocyanidin oligomers are likely due to differences in the ratio of A:B type linkages.[24] Figure 4d (inset) shows an expansion of the tetramer mass region for fraction II-5 in which masses of 1283.1, 1285.1, and 1287.1 for $[M + Cs]^+$ are suggestive of epicatechin tetramers containing two, one, or zero A-type linkages, respectively. Masses 16 amu greater appearing at 1299.1 and 1301.1 suggest the presence of oligomers containing a single epigallocatechin unit in place of epicatechin. In samples I-5 and II-5, peaks also appear at m/z 152 amu greater than the epicatechin oligomer masses, suggesting galloylation is present. Similar patterns are observed at higher mass intervals, suggesting

Table 2. Observed and Calculated Masses of Oligomers in Cranberry PAC Fractions Based on MALDI-TOF MS

			linkages			
PAC fraction	DP	flavan-3-ols[a] (epi)catechin	A	B	observed mass	$[M + Cs]^+$ calcd mass
I-4SP	2	2	1	0	709.6	709.0
	3	3	1	1	997.4	997.1
	4	4	1	2	1285.5	1285.2
	5	5	1	3	1573.5	1573.2
	6	6	1	4	1862.1	1861.3
	7	7	1	5	2148.3	2149.3
II-4SP	2	2	1	0	709.5	709.0
	3	3	1	1	997.4	997.1
	4	4	1	2	1285.2	1285.2
	5	5	1	3	1573.3	1573.2
	6	6	1	4	1861.5	1861.3
	7	7	1	5	2149.4	2149.3
I-5	2	2	1	0	709.2[b]	709.0
	3	3	1	1	997.2[c]	997.1
	4	4	1	2	1285.1	1285.2
	5	5	1	3	1573.0	1573.2
	6	6	1	4	1861.1	1861.3
	7	7	1	5	2148.7	2149.3
	8	8	1	6	2437.1	2437.4
	9	9	1	7	2725.9	2725.5
	10	10	1	8	3013.3[d]	3013.5
	11	11	1	9	3301.5[d]	3301.6
	12	12	1	10	3589.5	3589.7
II-5	4	4	1	2	1285.1	1285.2
	4	4	2	1	1283.0	1283.1
	5	5	1	3	1573.1	1573.2
	5	5	2	2	1571.0	1571.1
	6	6	1	4	1861.0	1861.3
	7	7	1	5	2149.0	2149.3
	8	8	1	6	2437.5	2437.4
	9	9	1	7	2725.6[b]	2725.5
	10	10	1	8	3013.9[b]	3013.5
	11	11	1	9	3302.1[b]	3301.6
	12	12	1	10	3590.1[b]	3589.8
	13	13	1	11	3879.5	3877.9

[a] Assignments are based on predictive and observed cranberry oligomer mass relationships developed by Reed and co-workers.[24] [b] Peak appears in raw data file, but is not shown in Figure 4. [c] Highest intensity peak. [d] m/z values obtained from raw data file.

a broad distribution of oligomer structure and composition. The major difference in composition between these fractions and I-4SP and II-4SP is in the size range of the oligomers. Oligomers of DP = 6 and 7 are more plentiful than in the earlier eluting fractions, oligomers of DP = 8−11 are clearly visible, and trace amounts of higher DP are detected. The mass range above 4000 amu was not examined. Oligomers of up to DP = 23 have been detected in extracts prepared from whole cranberry presscake.[24] The similarity in composition between fractions I-5 and II-5 isolated from fruit harvested in 2008 and 2009. respectively, suggests that there was minimal season-to-season variation in PACs from the same cultivar of cranberry (Early Black) harvested from this site.

Inhibition of *Candida* spp. and *Cryptococcus neoformans* Growth by PAC Fractions. Cranberry fruit crude extract DCE-I and fractions were previously screened for activity against *Cryptococcus neoformans*, *Candida albicans* (ATCC 14053), *Candida krusei*, and *Candida tropicalis* using a disk-diffusion assay.[25] Fractions I-1 and I-2 demonstrated antifungal activity against *Cryptococcus neoformans* and *Candida albicans* with a zone of inhibition smaller than that of the positive control miconazole nitrate and only at concentrations much higher than those used

Table 3. Inhibition of Microbial Growth in Response to Treatment with Crude Cranberry Extract or Fractions Containing Proanthocyanidins (n = 3), Expressed as MIC_{50}[a] and MIC_{90}[b]

	MIC[c] (μg/mL)									
	DCE-I		fraction I-4SP		fraction II-4SP		fraction II-5		fraction I-5	
organism	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
Candida glabrata	8	8	4	16	2	8	1	2	0.5	1
Candida lusitaniae	8	125	31	63	16	63	4	16	2	8
Cryptococcus neoformans	8	125	63	125	16	>500	4	31	4	8
Candida krusei	31	125	63	125	31	>500	16	250	16	125

[a] MIC_{50} = concentration producing 50% growth inhibition as determined by O.D. of treated population relative to control. All values are significant, with $p < 0.001$. [b] MIC_{90} = concentration producing 90% growth inhibition. [c] MIC_{90} for miconazole nitrate (positive control): *Candida glabrata* (0.05 μg/mL), *Candida lusitaniae* (0.01 μg/mL), *Cryptococcus neoformans* (0.4 μg/mL).

in this study. Fractions I-3 and I-$\overline{4}$ did not inhibit growth significantly. The results from fraction I-5 were difficult to interpret. Analysis of fraction I-5 showed that it contained primarily epicatechin oligomers, and the disk diffusion assay may therefore have been negatively affected by limited solubility of the oligomers and poor diffusion through the agar. Previous disk diffusion assays had suggested that proanthocyanidin-rich fractions inhibited some *Candida* species, but the assay failed to demonstrate reproducible results over the long term (Kondo, Patel, and Neto, unpublished results). Hence a more reliable, sensitive, and reproducible standard clinical laboratory method, the broth microdilution assay, was employed to screen for inhibition and determine MIC values of cranberry phytoconstituents against the selected fungal pathogens.

Selectivity among Strains and Effect of Oligomer Size on Activity. Crude cranberry extract (DCE-I) demonstrated dose-dependent inhibition against four susceptible fungal pathogens in the broth microdilution assay: *Cryptococcus neoformans*, *Candida krusei*, *Candida glabrata*, and *Candida lusitaniae*. MIC_{50} and MIC_{90} values ranged from 8 to 125 μg/mL (Table 3). By comparison, *Candida tropicalis* and *Candida albicans* did not show significant susceptibility to the crude extract (Figure 5). Fractions from the crude extract were further tested against the four susceptible strains. Phenolic acid fractions (I-1, I-2, II-1, and II-2), anthocyanin (I-3 and II-3), and flavonol fractions did not demonstrate any significant inhibition in the broth microdilution assay. However, PAC-rich fractions (I-4SP, II-4SP, I-5, and II-5) demonstrated antifungal activity with MIC values as low as 0.5 μg/mL. Table 3 summarizes the MICs required to inhibit 50 and 90% of the microbial populations (MIC_{50} and MIC_{90} values) for the crude extract DCE-I and four proanthocyanidin-rich fractions. Among the fractions, the strongest activity was observed for fraction I-5, which inhibited growth of *Candida glabrata* by 90% at 1 μg/mL and by 50% at 0.5 μg/mL. *Candida lusitaniae* and *Cryptococcus neoformans* similarly responded more strongly to this fraction.

Dose−response data for the growth of the four organisms when treated with PAC-rich fractions (percentage growth relative to untreated control) are shown in Table 4. *Candida glabrata* and *Cryptococcus neoformans* showed significant growth inhibition by PAC fractions even at the lowest concentration tested (0.5 μg/mL). Fraction I-5 inhibited the growth of *Candida glabrata* by >50% at the lowest concentration. PAC fractions did not significantly inhibit growth of *Candida lusitaniae* and *Candida krusei* at 0.5 μg/mL. However, fractions I-5 and II-5 containing the larger oligomers significantly reduced growth of

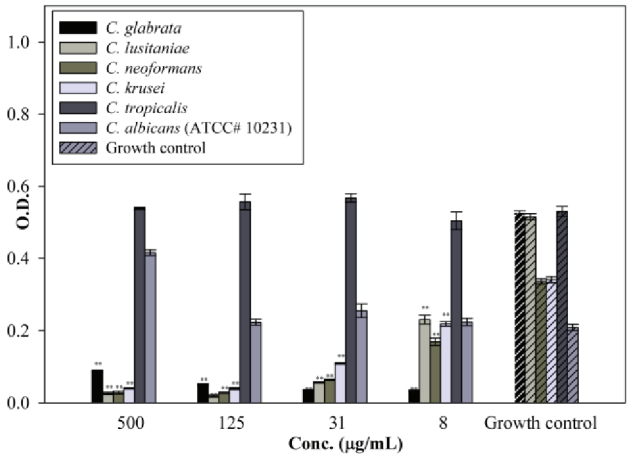

Figure 5. Growth of *Candida* and *Cryptococcus* species in response to treatment with crude cranberry extract at various concentrations, quantified by optical density measurements at 625 nm. Treatments marked with ** are significantly different from untreated control, with $p < 0.001$. *Candida albicans* and *Candida tropicalis* were not significantly inhibited by cranberry treatment.

Candida lusitaniae at 2 μg/mL. I-4SP and II-4SP were less effective. *Candida krusei* was less susceptible to treatment at these concentrations, but all fractions significantly reduced growth at 8 μg/mL. Of the four organisms, *Candida glabrata* appears to be the most sensitive to treatment with cranberry PACs.

In general, the fractions containing larger oligomers inhibited growth of all four organisms more strongly than those containing only oligomers of DP ≤ 6. On the basis of MALDI-TOF MS data, fractions I-5 and II-5 contained larger oligomers than the other fractions. PACs of up to DP = 12 were detected in I-5 and II-5, whereas the largest oligomers detected in I-4SP and II-4SP were heptamers. Although MALDI-TOF mass spectra do not provide absolute quantitative data on the composition of individual oligomers, the profiles show that the distribution of larger oligomers is higher in the fractions exhibiting stronger antifungal activity. As the DP of the oligomers increases, both changes in the three-dimensional structure and increased availability of hydrogen bonding sites may play a role in interactions with these microbes that reduce growth. MALDI-TOF MS data (Table 2) indicate that most of the oligomers contain at least one A-type linkage. The presence of the A-type linkage may influence three-dimensional structure, and its effect on antifungal activity in comparison to

Table 4. Mean Percentage Microbial Growth ($n = 3$) Relative to Control, in Response to Treatment with Cranberry Proanthocyanidin Fractions[a]

	treatment concentration			
organism and fraction	31 μg/mL	8 μg/mL	2 μg/mL	0.5 μg/mL
Candida glabrata				
I-4SP	8.2 ± 0.59	12.1 ± 0.53	59.6 ± 1.3	94.9 ± 2.1[b]
II-4SP	13.4 ± 0.97	8.8 ± 0.36	27.7 ± 0.92	87.3 ± 2.2
I-5	2.6 ± 0.63	3.2 ± 0.87	2.9 ± 0.37	37.9 ± 3.4
II-5	4.4 ± 0.68	5.7 ± 0.56	8.2 ± 0.46	60.4 ± 0.71
Candida lusitaniae				
I-4SP	24.1 ± 4.2	95.3 ± 2.9	NS[c]	NS
II-4SP	11.2 ± 0.91	79.8 ± 1.9	NS	NS
I-5	2.7 ± 0.53	7.6 ± 0.54	33.6 ± 2.4	NS
II-5	3.9 ± 0.78	16.9 ± 0.62	78.6 ± 3.0	NS
Cryptococcus neoformans				
I-4SP	53.7 ± 3.0	64.3 ± 2.8	71.9 ± 2.9	91.8 ± 5.0[b]
II-4SP	31.5 ± 0.62	58.9 ± 2.1	69.0 ± 2.3	90.7 ± 3.9
I-5	2.7 ± 0.55	9.1 ± 1.0	*d*	*d*
II-5	4.7 ± 0.62	23.9 ± 1.8	52.9 ± 1.5	78.3 ± 3.5
Candida krusei				
I-4SP	64.5 ± 1.1	85.2 ± 1.3	95.6 ± 1.9[b]	NS
II-4SP	48.0 ± 0.67	74.5 ± 1.9	90.8 ± 2.6	NS
I-5	29.6 ± 1.0	64.6 ± 6.4	NS	NS
II-5	37.3 ± 0.60	56.3 ± 1.5	77.5 ± 1.4	NS

[a] Values shown represent percentage growth relative to untreated control for each organism, based on the population of live organisms after each treatment ($n = 3$), and expressed as mean ± SEM. Treatments resulted in significant growth inhibition relative to untreated control, with $p < 0.001$. [b] Growth was significantly less than untreated control, with $p < 0.05$. [c] NS, no significant inhibition of growth observed at this concentration. [d] Data not available.

B-type oligomers is currently under investigation. Cranberry proanthocyanidin trimers containing A-type linkages have been characterized by NMR;[10] however, NMR data are lacking on larger oligomers, and thus the position of the A-type linkage in tetramers and larger is unknown. Commercially available procyanidin A2 dimer was tested using the broth microdilution assay (data not shown) and showed no significant inhibition of most organisms at concentrations of up to 125 μg/mL. Only *Candida glabrata* was inhibited by procyanidin A2 (MIC_{90} = 31 μg/mL, MIC_{50} = 8 μg/mL). Thus, the size of the oligomers appears to be an important consideration for antifungal activity. Gastrointestinal (GI) absorption and distribution throughout the body of proanthocyanidins larger than dimers has not been demonstrated, so their potential use against systemic candidiasis may be limited. However, they may be suitable for topical or oral use, or when taken orally may be present in sufficient amount in the GI tract to exert an inhibitory effect.

Antifungal activities of polyphenolic oligomers may involve interactions with proteins associated with the fungal cell wall. Proanthocyanidins from *Stryphnodendron adstringens* rich in high molecular weight (2114 Da) epigallocatechin hexamers with B-type linkages inhibited growth of *Candida albicans*.[26] The compounds were found to reduce cell surface hydrophobicity, altering cell adhesion properties and possibly inducing structural changes in the yeast cell wall. The capsule surrounding *Cryptococcus neoformans* and the cell wall of *Candida* species are rich in mannoproteins, which may act as targets for polyphenols, as may 1,3-β-glucan synthase, an enzyme that produces β-1,3-glucan in the fungal cell wall,[27] which is targeted by the antifungal cyclic peptides known as echinocandins.[28] Interaction between protein and tannins has been attributed to the presence of proline, a strong hydrogen bond acceptor,[29] and may involve face-to-face stacking of aromatic groups onto proline residues.[30] Noncovalent interactions between PACs and bovine serum albumin were found to be significantly higher in PACs with DP = 5.5−7.4 than epicatechin or procyanidin dimers.[31] Interactions of polyphenols with globular proteins are thought to be size-dependent, and studies suggest that surface-exposed aromatic residues interact more effectively with larger polyphenols.[32]

In summary, cranberry proanthocyanidin oligomers isolated from whole fruit of *V. macrocarpon* were observed to selectively inhibit the growth of several human pathogenic fungal species. In order of susceptibility from highest to lowest, *Candida glabrata*, *Candida lusitaniae*, *Cryptococcus neoformans*, and *Candida krusei* demonstrated susceptibility to treatment by cranberry fruit-derived PAC fractions at micromolar concentrations. The growth of *Candida albicans* and *Candida tropicalis*, however, was not significantly inhibited by any of the cranberry constituents tested. Increased antifungal activity was exhibited by fractions containing higher PAC oligomers of up to DP = 12. The increased efficacy of larger PACs may be a function of increased availability of free hydroxyl groups, allowing for greater interaction with proteins associated with the growth and viability of the organisms. The selective inhibition by PACs against the four sensitive species and resistance shown by *Candida albicans* and *Candida tropicalis* are areas for future investigation targeted toward better understanding of the mechanisms of action of cranberry PACs against pathogenic yeasts and their potential use as antifungals.

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