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Article

Natural Antimicrobials from Cloudberry (*Rubus chamaemorus*) Seeds by Sanding and Hydrothermal Extraction

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ABSTRACT: We have developed an organic solvent-free process to enrich natural antimicrobials from the important Nordic *Rubus* berry species, especially from cloudberry. The process utilizes industrial berry byproducts as raw-material, and it is based on seed sanding technology and water-based extraction. The extracts showed strong antimicrobial activity against Gram-positive *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA). A concentration of 5 mg/mL totally eliminated these bacteria in 24 h. Effects against Gram-negative bacteria were modest. Polyphenol analysis of the hydrothermal extracts showed that the antimicrobial activity is correlated to ellagitannins, mainly dimeric sanguiin H-6 and sanguiin H-10 isomers. Sanguiin H-10 is not commonly found in intact cloudberry seeds, but it is formed from the dimeric and trimeric ellagitannins, during the extraction. Surprisingly hydrothermal extracts had no or minor effects on beneficial bacteria, *Lactobacillus rhamnosus*. This interesting finding might offer an application potential in controlling skin microbial pathogens, for example, in wound healing.

KEYWORDS: *Rubus species, berries, byproducts, seeds, antimicrobial activity, MRSA*

INTRODUCTION

Cloudberry (*Rubus chamaemorus*) is a perennial herb with boreal distribution. In Finland, cloudberries grow especially in the undrained wetlands in Northern Finland, where they also produce the highest yield. When the fruit ripens, it turns into an amber-colored, juicy, flavourful, and fragrant berry. The annual crop of cloudberries in Finland is estimated to be a few million kilograms. Cloudberry fruit is of economic importance in Nordic countries, and it is a desired raw material for the food, beverage, and cosmetic industries. The juice is used by the food and beverage industry for liqueurs, jams, and jellies. After juice pressing the remaining press cake, cloudberry pomace, which largely contains seeds, is mainly used for seed oil extraction for the cosmetic industry. We recently developed a dry fractionation method for cloudberry pomace based on dry fractionation using milling and sieving technologies in order to obtain bioactive fine and coarse fractions.[1]

Cloudberries are rich in polyphenols called ellagitannins, which are known to possess many beneficial and health-promoting properties, such as strong antimicrobial activity against many harmful and pathogenic human bacteria,[2] antiadhesion activity against uro-pathogenic *E. coli*, anti-inflammatory activity in activated macrophages,[1] and anti-fungal activity.[3]

Chemically, ellagitannins are derivatives of more simple galloylglucoses such as pentagalloylglucose, as their adjacent galloyls are linked together to form the characteristic hexahydroxydiphenoyl (HHDP) moiety found as such or as further modified in most of the natural ellagitannins. Ellagitannins exhibit very high structural variability because of the different positions in which the HHDP and galloyl groups are linked with the core glucose moiety, the different ways the HHDP group is modified, and the strong tendency of ellagitannins to form oligomeric structures.[4] Oligomeric forms of ellagitannins are typical for wild *Rubus* species such as wild blackberries.[5] Altogether 26 ellagitannins have been identified in cloudberry fruit, in which trimeric lambertianin C and dimeric sanguiin H-6 are the main ones.[6] Ellagitannins are also the main phenolic compounds in *Rubus* seeds.[7] Cloudberry seed ellagitannins have been recently studied in detail. The seeds contain a complex mixture of ellagitannins, including lambertianin C and sanguiin H-6[1].

In industrial juice processing, berries are usually mashed, heated to 40−50 °C, and treated with depectinizing enzymes to degrade the viscous gels that are formed during mashing so that extraction is more effective.[8] After the juice is separated, the pomace is quickly dried to avoid microbial contamination. Water-soluble antioxidants that are accumulated in the cell vacuoles are released to the juice during pressing, whereas compounds that are attached with the cell wall remain in the pomace.[9] Holtung et al.[10] reported 1.3−1.7 -fold higher dry matter related to polyphenol content of chokeberry and blackcurrant press residues compared with that of the respective berries.

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There have been several approaches for the valorization of the berry byproducts. Hidalgo and Almajano[11] recently reviewed recovery processes of bioactive polyphenols from the byproducts of berries, such as raspberries, chokeberries, blueberries, and sea buckthorn berries. Various alternative processes to classical solvent extraction methods to concentrate polyphenols from plant material include enzyme-assisted extraction,[12] ultrasound extraction,[13] microwave extraction,[14] and supercritical carbon dioxide or pressurized solvent extraction.[15]

The aim of this study was to develop an eco-friendly and solvent-free green process to recover polyphenols from the cloudberry pomace. We describe here the seed sanding technology, in which antimicrobially active ellagitannins can be recovered from the outer layer of the cloudberry seeds by sanding technology, followed by gentle extraction. In this study we show that the cloudberry seed fractions, rich in ellagitannins, possess strong antimicrobial activity against *Staphylococcus aureus* and clear growth inhibition of *Pseudomonas aeruginosa* and *Escherichia coli* without affecting the human beneficial bacteria. The potential use of the extracts in various health care applications is discussed.

MATERIALS AND METHODS

Cloudberry Material. Dry cloudberry (*R. chamaemorus*) press cake material originating from commercial juice pressing processes of Finnish origin were obtained from Aromtech (www.aromtech.com), berry ingredient producer (Tornio, Finland), and from Kiantama (www.kiantama.fi), wild berry processing specialist (Suomussalmi, Finland). The dry press cake material was stored at +15 °C until processed.

Microbes Used in the Study. Bacterial strains *Staphylococcus aureus* VTT E-70045 (ATCC 6538), *Staphylococcus aureus* MRSA VTT E-183582 (DSM 11822), *Pseudomonas aeruginosa* VTT E-84219 (ATCC 15692), and *Escherichia coli* VTT E-94564^T (ATCC 11775) were cultured aerobically at 37 °C on Nutrient Agar (NA, Oxoid) or in Nutrient Broth (NB, Oxoid) with agitation (150 rpm). *Lactobacillus rhamnosus* VTT E-96666 (ATCC 53103) was cultured in aerobic atmosphere at 37 °C on De Man, Rogosa and Sharpe agar (MRSA, Oxoid) or in equivalent broth (MRSB, Oxoid). The bacterial strains were stored frozen (−80 °C) as stock cultures and revived on their corresponding media described above (NA or MRSA).

Seed Sanding. A dried pressed cake of cloudberry from commercial juice pressing process was first sieved as a pretreatment for sanding. The seeds were separated from the skin fraction by using a vibratory sieve shaker (Retsch AS 200 control, Retsch GmbH, Haan, Germany) with a 1.6 mm screen at settings of 5−10 min sieving time and 1.5 mm amplitude. The seeds were then sanded by using an abrasive machine (barley pearling machine, tailor-made for VTT) for 5, 15, and 40 min sanding time. The resulting light yellow powder was named the seed coat fraction. The remaining seeds were still whole after the sanding process and suitable for further processing. We have described the sanding process in WO 2016/097488 A1.[16]

Basic Hydrothermal Extraction. Hydrothermal extracts were prepared from the seed coat fraction as described in WO2017/216426.[17] Briefly, 1 g of the seed coat fraction was extracted with 20 mL of water for 1 h at 80 °C. Extraction was carried out using magnetic stirring (500 rpm). The mixture was filtered using 4-fold Miracloth filter material with 22−25 μm pore size. The filtrate was freeze-dried (Christ Alpha 1−4 LDplus, Rotational-Vacuum-Concentrator, Germany) and stored at −20 °C prior to antimicrobial or polyphenol analyses.

Modification of the Hydrothermal Extraction Method. In order to identify best extraction conditions for further process development longer extraction time, enzyme-aided and autoclave-aided extraction were evaluated.

Extended Extraction Time. Longer extraction in room temperature before elevating temperature was evaluated. In these experiments, 2.5 g of the seed coat fraction was first mixed with 50 mL of water (no shaking) and incubated in room temperature for 2, 4, 6, or 18 (o/n) hours followed by normal hydrothermal extraction (80 °C, 500 rpm, 1 h). The mixture was filtered using Miracloth filter material, freeze-dried, and stored as described above.

Autoclave-Aided Extraction. Autoclave extraction was studied using Finn-Aqua 696 E autoclave (Steris Finn-Aqua, Finland). In these experiments 2.5 g of the seed coat fraction was suspended in 50 mL of water. In this process extraction temperature was 120 °C, pressure 2 bar, and extraction time 20 min. The heating time to reach the temperature was 17 min, and the cooling time was 35 min to reach 67 °C. After the process, the samples were cooled for 10 min and then filtered using Miracloth, freeze-dried , and stored at −20 °C prior to analyses.

Enzyme-Aided Extraction. Prior to the hydrothermal extraction, the cloudberry seed coat fraction was treated with a combination of cell wall degrading enzymes to find out whether this treatment could release more antimicrobially active compounds from the cell wall matrix. Seed coat powder was treated with a combination of cellulose, pectinase, and xylanase. In these experiments, 10 g of the seed coat powder was first suspended in 200 mL of water. Enzymes were added according to the main activity of each enzyme preparation: 1500 nkat/g of Econase CE (lot. 200103001), Pectinex Ultra SP-L (KRN 05402) and Depol 740L (Batch: 3323906). Enzyme treatment was carried out in a 45 °C water bath for 2 h (300 rpm mixing) followed by normal hydrothermal extraction (80 °C, 500 rpm, 1 h). A control treatment was carried out under the same conditions, without enzymes. After extraction, the mixtures were filtered and freeze-dried as described above.

Drying of the Hydrothermal Extracts. In order to identify the best drying method for the hydrothermal extracts, the standard freeze-drying process was compared to oven drying and spray drying.

Oven Drying. In the oven drying process, different drying times and temperatures were studied. In these experiments, 2.5 g of the seed coat powder was suspended in 50 mL of water followed by normal hydrothermal extraction (80 °C, 500 rpm, 1 h). The following oven conditions were used when drying the extracts: 2 h, 85 °C; 4 h, 85 °C; 2 h, 95 °C; 4 h, 95 °C; 1.45 h, 110 °C; and 3.5 h, 110 °C (Salvislab Incucenter IC40-400).

Spray Drying. Pilot scale Niro Mobile Minor (Denmark) equipment was used in the spray drying experiments. Seven liters of the hydrothermal extract was spray dried using the following parameters: drying time 4 h; temperature in 178.8−180.2 °C; temperature out 70.7−82.1 °C; feed 27−32 mL/min ; and evaporation capacity 1.6−1.9 kg/h. No carrier material was used.

Analyses of the Polyphenol Content of the Seed Coat Fraction and Hydrothermal Extracts. For the seed coat fraction and selected hydrothermal extracts, detailed quantitative analyses of phenolic compounds were carried out using UHPLC-DAD-3Q-MS analyses according to Suvanto et al.[18] with the methods created by Engström et al.[19,20] Briefly, 20 mg of freeze-dried and ground sample material was extracted twice on a planar shaker for 3 h using acetone/water (1400 μL, 4:1, v/v) after overnight maceration in the same solvent. The supernatants achieved via centrifugation (7800*g*) were combined. Acetone was evaporated in vacuo, and the resulting water extract was freeze-dried and stored at −20 °C until analysis. All samples were extracted in duplicates. First sample was dissolved in 1 mL water, vortexed for 10 min, filtered using a 0.2 μm PTFE filter and diluted 1:4 (v/v) with water prior to injection. The second sample was treated similarly but dissolved and diluted with methanol instead of water. The instrument consisted of a Waters Acquity UPLC (Waters Corporation, Milford, MA, USA) equipped with an Acquity UPLC BEH Phenyl column (1.7 μm i.d.; 2.1 × 100 mm, Waters Corporation) and a photodiode array detector coupled to a Xevo TQ triple quadrupole mass spectrometer (Waters Corporation) using electrospray ionization. All instrument and data processing parameters were identical to Suvanto et al.[18]

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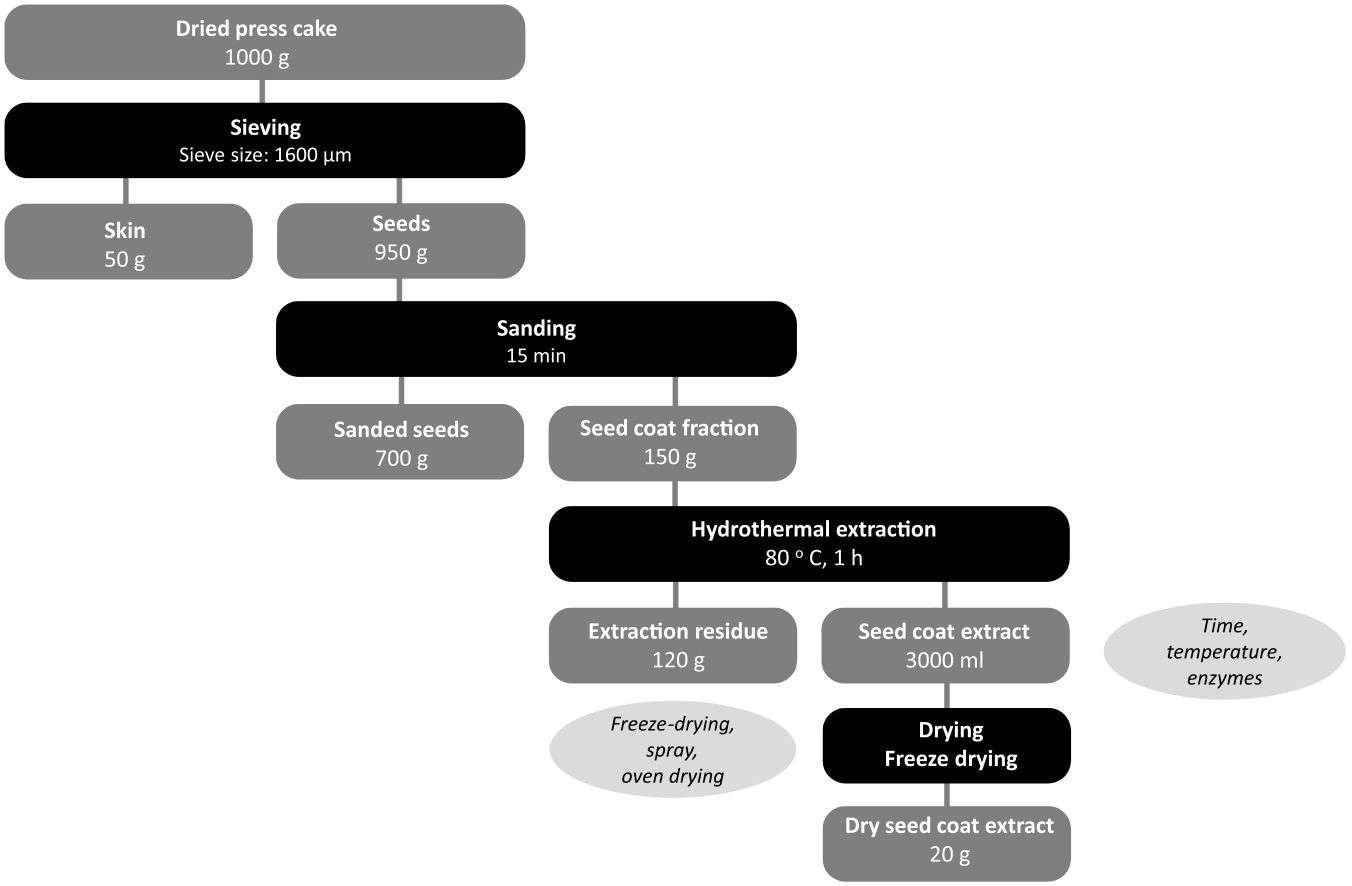

Figure 1. Seed sanding and hydrothermal extraction of dried cloudberry press cake (1 kg). One kg of dried cloudberry press cake corresponds to 20 kg of fresh berries. Estimated yields are marked in gray boxes. Further steps in process development are indicated in the figure by circles.

To further identify the main ellagitannins, the same water and methanol extracts were analyzed using an Acquity UPLC (Waters Corporation) equipped with an Acquity UPLC BEH Phenyl column (1.7 μm i.d.; 2.1 × 100 mm, Waters Corporation) and a photodiode array detector coupled to a hybrid quadrupole-Orbitrap mass spectrometer (Q Exactive, Thermo Fisher Scientific GmbH, Bremen, Germany) using heated electrospray ionization. All instrument and data processing parameters were identical to Suvanto et al.[18]

Antimicrobial Activity. Antimicrobial activities of the hydrothermal extracts were measured as described by Puupponen-Pimiä et al.[1] A few microbial colonies of *S. aureus*, *S. aureus MRSA*, *E. coli*, and *P. aeruginosa* were each suspended into Nutrient broth, and colonies of *L. rhamnosus* were suspended into De Man, Rogosa and Sharpe broth. These cultures were incubated for 18−24 h and used to prepare the inoculum for antimicrobial test cultures. Each microbial strain was analyzed individually in a 1.0 mL culture with the selected extracts. Cultures with no extract were used as controls for bacterial growth, and the antibiotic chloramphenicol (50 or 25 μg mL^{-1}) was used as a reference compound for inhibition of bacterial growth in the culture. The culture growth in the Eppendorf tubes was sampled 3 to 4 times during the incubation period of 24 h, the samples were diluted in peptone saline (Maximal Recovery Diluent, Lab M), and the proper dilutions were plated on NA or MRSA for the evaluation of colony counts at each time point. The growth curves of each sample were drawn on the basis of the colony counts of two parallel samples, and the inhibitory effects of the extracts on the bacterial cultures were evaluated by comparing the control growth curves with those obtained with the extracts.

RESULTS AND DISCUSSION

A solvent-free extraction system based on dry fractionation and gentle hydrothermal extraction was developed for the seeds of unique Nordic berry species, cloudberry (*Rubus chamaemorus*). The process diagram of seed sanding and hydrothermal extraction of cloudberry press cake is presented in Figure 1. The basic hydrothermal extraction was further studied regarding extraction time, temperature, and use of enzymes as well as different drying processes. Our criteria in the process development was increased antimicrobial activity of the resulting extract against harmful bacteria.

Sieving and Sanding of the Press Cake. At first, the dried press cake of the cloudberry fruits was sieved to separate berry skins from the seeds. The skin fraction was 4−7% of the whole press cake. Berry skins are also very rich in bioactive compounds, such as polyphenols. This fraction could be easily used in foods (e.g., in bakery products, as a healthy ingredient). Table 1 shows yields obtained in various steps of our process

Table 1. Yields Obtained by the Different Process Steps and Distribution of Fractions by Sieving and Sanding[a]

process	sample	yields
-	dried cloudberry press cake	100%
separation of seeds from press cake by sieving	sieved seeds	93−96%
	skin fraction	4−7%
sanding of sieved seeds	sanded seeds	69−74%
(sanding time 15 min)	**seed coat fraction**	**11−18%**
	loss	4−13%

[a]Sanding time was 15 min.

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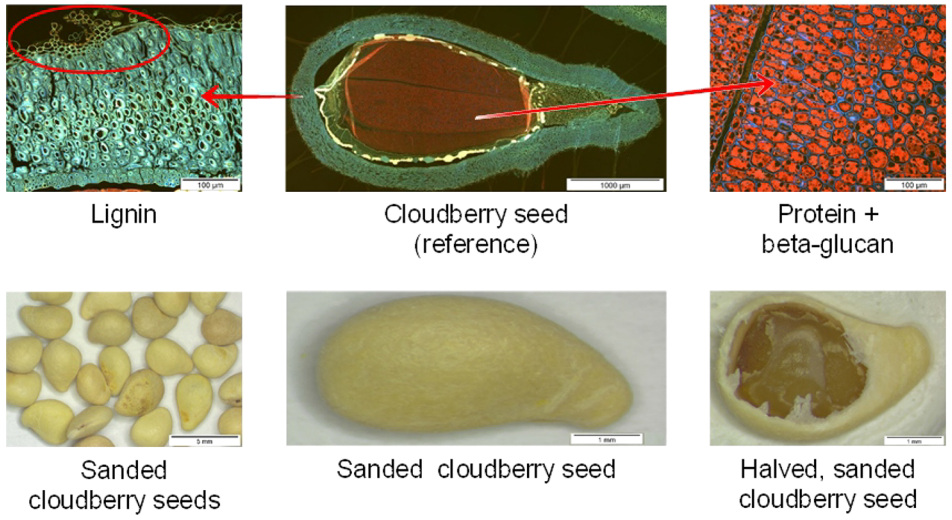

Figure 2. Fluorescence microscopy pictures (upper line) of cloudberry seed and stereo microscopy pictures (below) of sanded cloudberry seeds (sanding time 15 min). The surface of the seed coat recovered by sanding is indicated by a circle in the upper picture.

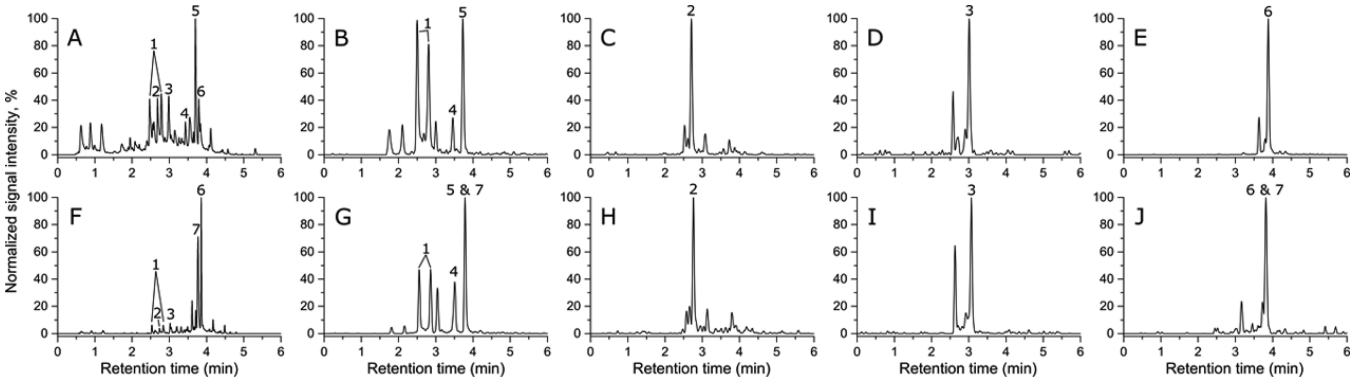

Figure 3. UV and extracted ion chromatograms (EICs) of cloudberry seed coat hydrothermal extract (A–E) and cloudberry seed coat (F–J), both extracted with acetone–water. (A,F) UV chromatograms (λ = 280 nm); (B,G) EICs m/z 783; (C,H) EICs m/z 633; (D,I) EICs m/z 708; (E,J) m/z 934. Main ellagitannins are numbered as follows: 1, pedunculagin; 2, strictinin isomer; 3, unknown dimeric ellagitannin; 4, sanguiin H-10 isomer 1; 5, sanguiin H-10 isomer 2; 6, sanguiin H-6; and 7, lambertianin C.

described in Figure 1. Different sanding times of 5, 15, and 40 min of the seeds were tested. Our aim was not to break the seed and thus to save the valuable seed oil, located mostly in inner parts of the seed (Figure 2). When we used a 15 min sanding time, the majority of the seeds remained whole, and the yield and the quality of seed oil, extracted by supercritical CO_2 process, was even better when sanded seeds were compared with control seed material without any treatment (data not shown).

Seed sanding proved to be suitable not only for cloudberry but also for all berries and fruits with relatively large seeds. Especially, all *Rubus* berries such as raspberries (*Rubus idaeus* L.), arctic brambles (*Rubus arcticus*) and boysenberries (*Rubus fruticosus*) were ideal for the process. In addition, seeds of sea buckthorn (*Hippophae rhamnoides*), rosehip (*Rosa canina*) as well as apple (*Malus domestica*) and grape (*Vitis vinifera*) were excellent material for the process (data not shown). Especially, red raspberry is interesting as it is a globally and economically important berry with remarkable pomace production, which is usually considered as waste. Annual pomace production of raspberry is almost 500 000 tons (http://www.fao.org/faostat/en/#data). Raspberry pomace is underexplored and rich in bioactive compounds.[12]

Hydrothermal Extraction. The average yield of the dried hydrothermal extract derived from the seed coat powder was 13% (varied between 7.5%–18.8% in different extraction batches). This means that about 1 kg of dry cloudberry seeds is needed to produce about 20 g of hydrothermal extract (Figure 1). These extracts were water-soluble and free of any solvent residues and thus suitable ingredients for food, cosmetic, and health care purposes.

Polyphenol Composition of the Hydrothermal Extract. Prior to the detailed analyses of the polyphenol content, the two cloudberry press cake batches provided by different suppliers were compared. Their polyphenol content and composition were alike (data not shown). The main polyphenols in seed coat fraction, extracted with acetone–water, were ellagitannins. Ellagitannin content was 18.6 mg/g d.w. (dry weight). Content of ellagitannins and galloyl derivatives of the hydrothermal extracts of different seed batches varied between 12.8 and 14.4 mg/g d.w. The extracts also contained 0.4–0.5 mg/g d.w. of procyanidins. The mean degree of polymerization for procyanidins was 2. In addition to ellagitannins, hydrothermal extracts contained other seed-specific water-soluble compounds, but they were not analyzed in this study.

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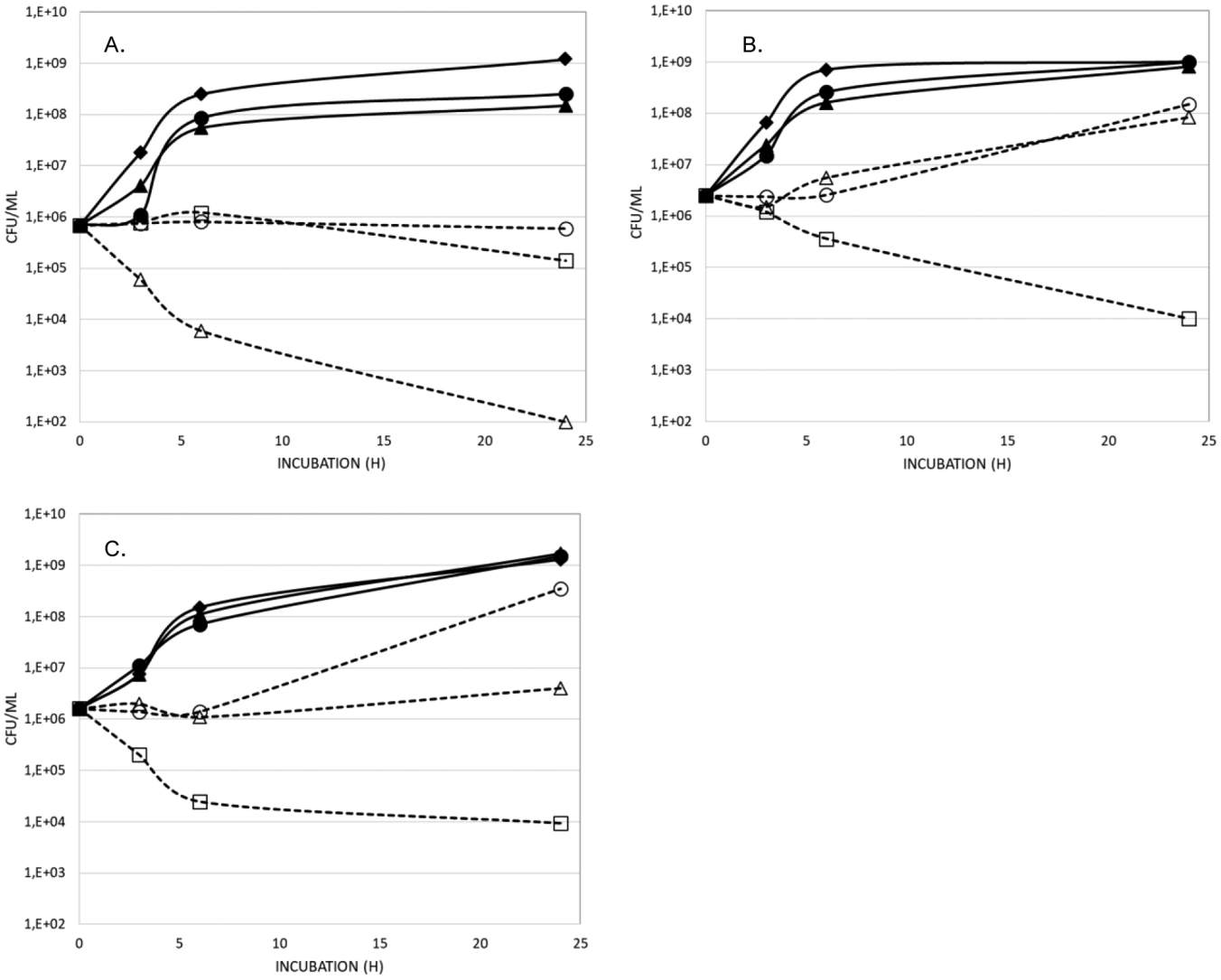

Figure 4. Antimicrobial activity of hydrothermal (HT) extracts of cloudberry seed coat powder and whole seeds against *Staphylococcus aureus* (A), *Escherichia coli* (B), and *Pseudomonas aeruginosa* (C). Control (♦); chloramphenicol 50 μg/mL (□); seed coat fraction, HT extract 1 mg/mL (▲); seed coat fraction, HT extract 5 mg/mL (△); whole seed, HT extract 1 mg/mL (●) whole seed, HT extract 5 mg/mL (◯).

The UV chromatogram at 280 nm along with extracted ion chromatograms of the main ellagitannin signals in cloudberry seed coat powder and the hydrothermal extract are presented in Figure 3. The main peaks in seed coat powder were sanguiin H-6 and lambertianin C, which are the main ellagitannins of both cloudberries and raspberries identified by ESI-Orbitrap-MS.[6] Interestingly, the main peak in our hydrothermal extract was an unidentified sanguiin H-10 isomer. In addition, our seed coat extract contained very small amounts of lambertianin C compared with acetone extract. Sójka et al.[21] recently studied the structure and stability of raspberry ellagitannins, lambertianin C, and sanguiin H-6 in aqueous solutions. Studied parameters were temperature (varied between 20–80 °C), medium pH (varied between pH 2–8), and incubation time (varied between 0–24 h). Lambertianin C and sanguiin H-6 were stable in acidic conditions, but they rapidly degraded in neutral and mildly basic media at elevated temperature (60–80 °C). In mildly acidic conditions (pH 6), ellagitannins hydrolyze to intermediate products, such as sanguiin H-10. In our study, hydrothermal extraction conditions were acidic, as pH was between 3.7 and 4. The main ellagitannins in our hydrothermal extracts were sanguiin H-10 isomers. The results indicate that in our specific extraction conditions, the trimeric lambertianin C is largely hydrolyzed to dimeric structures, especially to sanguin H-10 isomers. In addition, pedunculagin and strictinin isomers, identified in hydrothermal extracts, can be originated from both sanguiin H-6 and lambertianin C. In addition, according to the chromatograms our hydrothermal extract contained many other small degradation products of natural ellagitannins present in cloudberry seed coat, but which were not found in acetone–water extract.

The extraction residue turned out to be very interesting as free ellagic acid, 80 mg/g d.w., was concentrated in it. Ellagic acid is known for many health-promoting properties, such as cancer-preventive properties.[22] The extraction residue was also rich in fiber and healthy fatty acids such as linoleic acid and alpha-linolenic acid. The yield of the extraction residue was also remarkable after the hydrothermal extraction process, as production of 20 g of the dry hydrothermal extract generated about 120 g of the dry extraction residue (Figure 1). The chemical composition of the extraction residue suggests that further studies are needed to show its potential as a healthy food ingredient.

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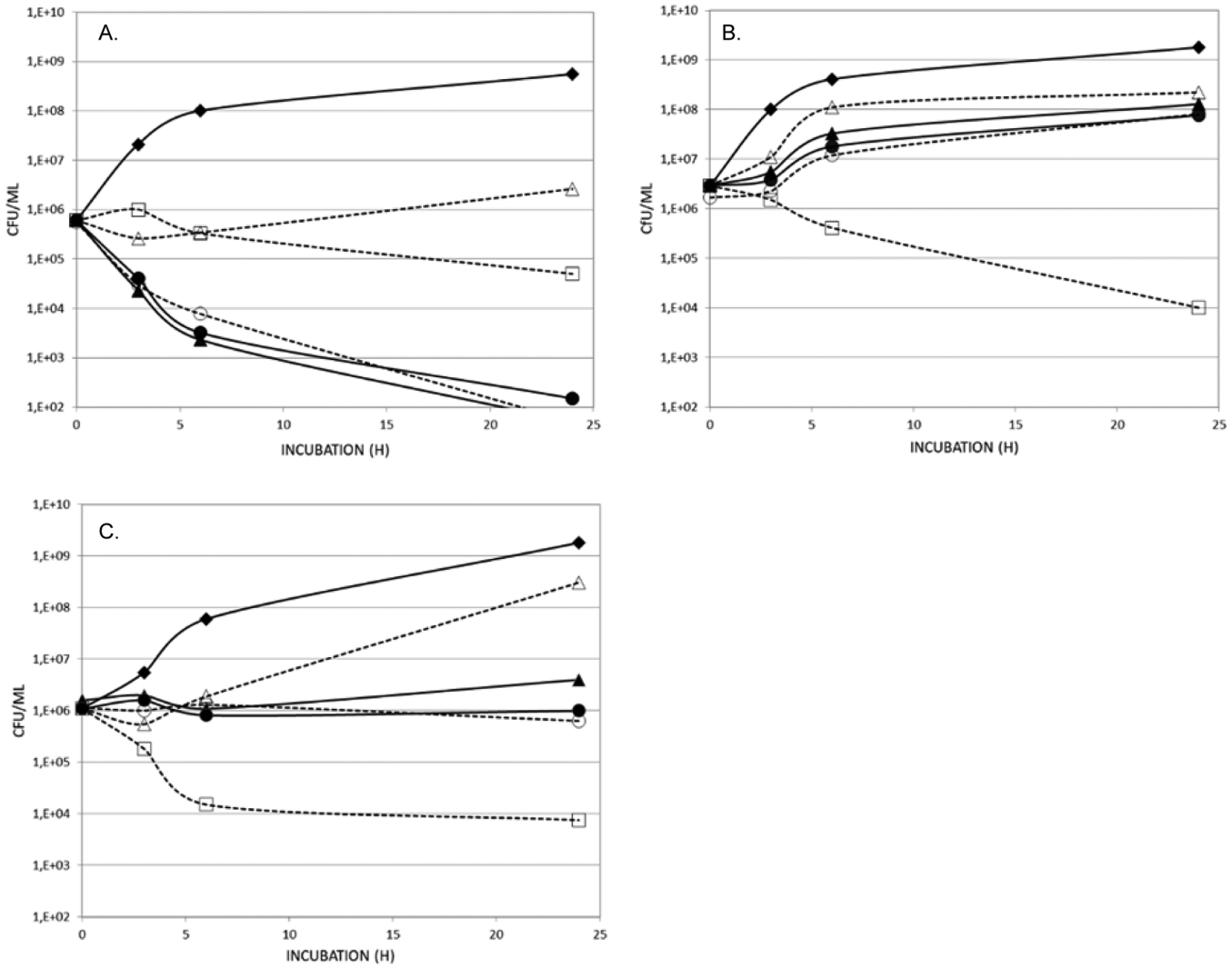

Figure 5. Effects of enzyme treatment, incubation in elevated extraction temperature without enzymes and autoclaving on antimicrobial activity of cloudberry seed coat powder against *Staphylococcus aureus* (A), *Escherichia coli* (B), and *Pseudomonas aeruginosa* (C). Control (♦); chloramphenicol 50 μg/mL (□); seed coat fraction, HT extract 5 mg/mL (▲); seed coat fraction, enzymes HT extract 5 mg/mL (△); seed coat fraction, no enzymes, HT extract 5 mg/mL (●); seed coat fraction, autoclave extract 5 mg/mL (◯).

Antimicrobial Activity of Cloudberry Seed Extracts. Antimicrobial effects of the hydrothermal extracts prepared of cloudberry seeds and seed coat powder were evaluated against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* in liquid cultures. Prior to the detailed antimicrobial study, extracts of the two cloudberry press cake batches provided by different suppliers were compared. Their antimicrobial activities were alike, showing good repeatability of our sanding and extraction system (data not shown).

Basic Hydrothermal Extraction. Hydrothermal extracts of cloudberry seed coat fractions were very effective against Gram-positive bacteria *S. aureus*. A bactericidal effect was demonstrated with 5 mg/mL of this extract by decrease of the colony forming unit (cfu) below a detection limit of 100 cfu after a 24 h incubation. The lower concentration (1 mg/mL) showed growth-preventing properties of the *S. aureus* cultures (Figure 4A). For a comparison, the hydrothermal extract was prepared from the whole grounded seed. The seed coat fraction extract was clearly more effective against *S. aureus* than the whole seed fraction (Figure 4A).

Antimicrobial effects of the extract against Gram-negative bacteria *E. coli* and *P. aeruginosa* were not as strong (Figure 4B,C) as those of *S. aureus* and are in line with our earlier results.[1] The antimicrobial activities of all the studied extracts against *E. coli* were growth retarding, and hydrothermal extracts (5 mg/mL) from the seed coat and the whole seed were equally effective (Figure 4B). However, the hydrothermal extract with high concentration (5 mg/mL) had bacteriostatic activity against *P. aeruginosa*, while the effect of whole seed extract (5 mg/mL) was weaker, retarding the growth of the culture (Figure 4C).

The pH values of the microbial cultures were measured at the beginning and at the end of the cultivation period of 24 h. It varied between pH 5.5−7. Especially *Pseudomonas aeruginosa* is known to be sensitive to low pH.[23] However, pH seemed to have no major changes in antimicrobial activity.

In our previous study, we demonstrated that purified acetone−water extracts of cloudberry fruit and seeds were antimicrobially very effective, as they showed bactericidal and growth retarding effects against the same studied bacteria already in low concentration, 1 mg/mL. These two extract

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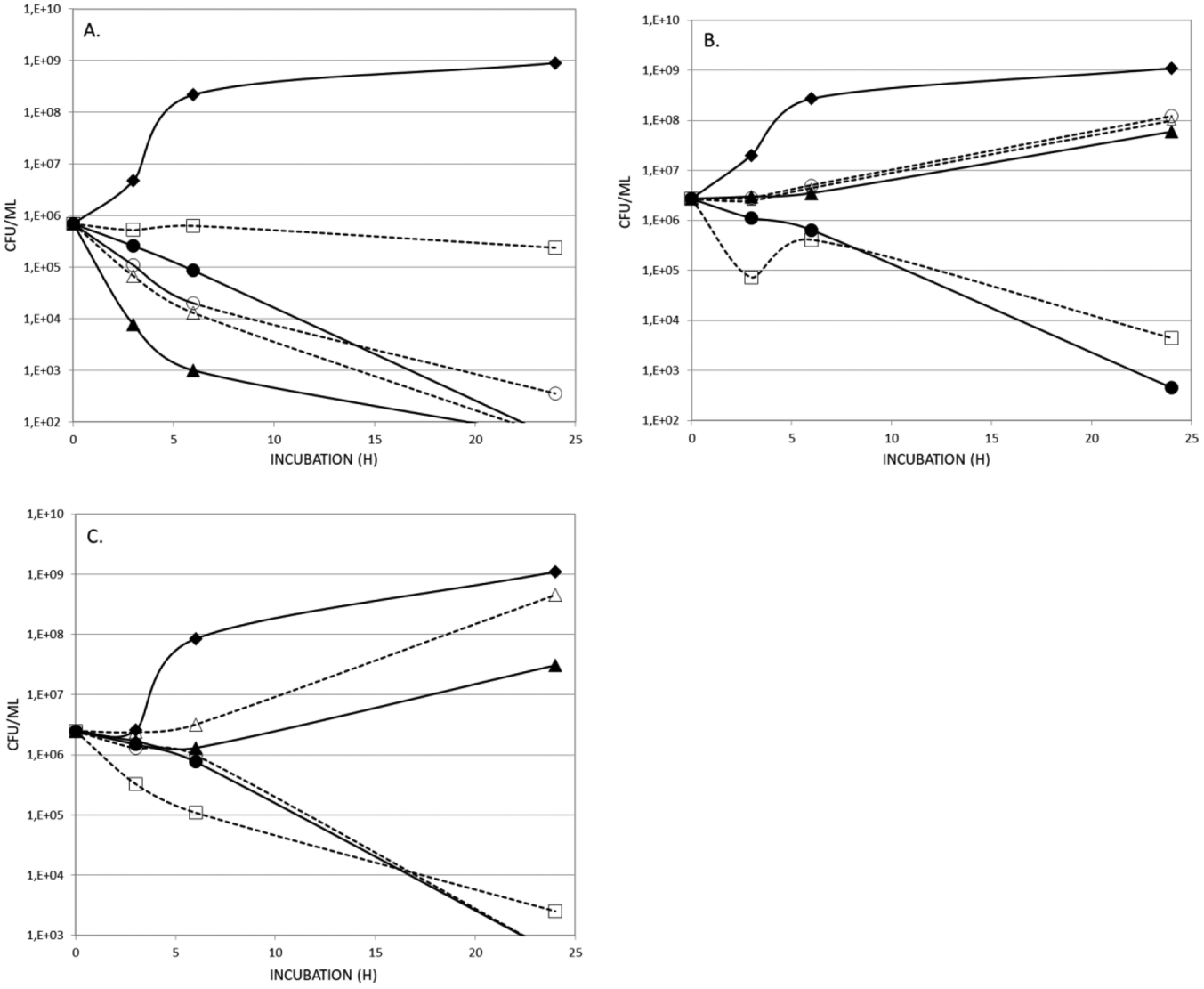

Figure 6. Effects of different drying methods on antimicrobial activity of hydrothermal extracts against *Staphylococcus aureus* (A), *Escherichia coli* (B), and *Pseudomonas aeruginosa* (C). Control (♦); chloramphenicol 50 μg/mL (□); seed coat fraction, freeze-drying HT extract 5 mg/mL (▲); seed coat fraction, oven drying 2 h, 95 °C, HT extract 5 mg/mL (△); seed coat fraction, oven drying 1.45 h, 110 °C HT extract 5 mg/mL (●); seed coat fraction, oven drying 3.5 h, 110 °C HT extract 5 mg/mL (○).

types are different, as the concentration of ellagitannins in purified acetone−water extract was much higher (around 200 mg/g d.w.) compared with the hydrothermal extract (around 12 mg/g d.w.). This is due to better extractability of ellagitannins in acetone−water compared with water and further concentration of ellagitannins by reverse phase chromatography (C_{18} cartridge) in these acetone−water extracts. In addition, composition is different as lambertianin C and sanguiin H-6 were the main compounds in acetone−water extracts, and sanguine H-10 was only the minor compound.[1]

Puljula et al.[24] have recently studied antimicrobial effects of 22 individual ellagitannins against several bacterial species including *S. aureus* and *E. coli*. All of them were active against *S. aureus*, and almost all of them were active against *E. coli*. Stronger effects were detected against *S. aureus* than *E. coli*. Structural features of ellagitannins that affected antimicrobial activity were type of oligometric linkage for *S. aureus* and molecular size for *E. coli*. They also found that lambertianin C and sanguiin H-6 were very active against both studied bacteria. In addition, pedunculagin and strictinin, which we also identified in our extract, had moderate activity against the two studied bacteria. However, sanguiin H-10 was not included in their study.

De Silva et al.[25] analyzed the antimicrobial activity of ethanol−water extracts of *Rubus almiflius* Schott fruit, commonly known as a wild blackberry. They identified sanguiin H-10 among potential polyphenols giving the extracts bacteriostatic effects against several Gram-positive and -negative bacteria, including *S. aureus*, *E. coli*, and *P. aeruginosa*.

These results indicate that dimeric and monomeric ellagitannins, particularly, sanguiin H-6 and sanguiin H-10 isomers, which were the main ellagitannin coumpounds in our hydrothermal extracts, were responsible for the antimicrobial activity. Most probably sanguiin H-6 is the most active compound in the extract; however, sanguiin H-10 seems to have at least moderate activity, and synergistic effects between these two ellagitannins may be very potential. In addition, other smaller ellagitannins formed as degradation products during extraction may have increased the activity.

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Extended Extraction Time. In order to see the effects of longer extraction time in room temperature on antimicrobial activity, cloudberry seed coat fraction samples were incubated for 2, 4, 6, or 18 (o/n) hours at room temperature (RT) before elevating the temperature to normal hydrothermal extraction (1 h, 80 °C). The results in antimicrobial activity showed that normal hydrothermal extraction alone gave the best result, because the antimicrobial effects of the samples preincubated in RT were somewhat lower against all three studied bacteria when compared with normal hydrothermal extraction (1 h, 80 °C) (data not shown).

Enzyme-Aided Extraction. Enzyme treatment (2 h at 45 °C) of the seed coat fraction prior to hydrothermal extraction diminished the antimicrobial activity against all studied bacteria, which was especially strongly as shown with *S. aureus*. However, the incubation for 2 h at 45 °C without enzymes, followed by normal hydrothermal extraction, had slight positive effects on antimicrobial activity of Gram-negative bacteria (Figure 5). We have earlier shown that enzyme-aided extraction of polyphenols from bilberries using a combination of cell-wall-degrading pectinases clearly increased polyphenol content of the berry mash and increased its antimicrobial activity against *Salmonella* and *Staphylococcus* species.[26] However, bilberry polyphenols are mostly anthocyanins, and ellagitannins are not commonly detected in *Vaccinium* berries. The results indicate that a berry-specific extraction system is needed for optimal recovery of polyphenols. Our seed sanding and hydrothermal extraction system was developed for *Rubus* species, which are particularly rich in ellagitannins. It can be speculated that ellagitannins, which are known to precipitate proteins,[27] inactivate the enzyme function, which also resulted in decreased antimicrobial efficacy of the ellagitannins.

Autoclave-Aided Extraction. Extraction in an autoclave at high temperature and elevated pressure (120 °C, pressure 2 bar) was also tested. Autoclave extracts had similar antimicrobial activity as normal hydrothermal extracts against all three bacteria species. (Figure 5). However, the autoclaved extract is sterile, which is an extra advantage in further product development. Autoclave extraction has been recently introduced among various extraction methods. However, there are no reports concerning any berry species. Suh et al.[28] compared autoclave, water, and ethanol extraction to enrich polyphenols from *Opunyia ficus-indica* fruit. They found that in autoclaved extracts, total polyphenol and flavonoid content were higher than those of water extract but slightly lower than those of ethanol extract. The results indicate that polyphenols in general are rather stable in autoclave conditions. Our results are in line with these observations.

Drying of the Hydrothermal Extracts. Freeze-drying of the hydrothermal extract (5 mg/mL), which was the standard method used in our extract preparation, was the best method to prepare the most antimicrobial extract against *S. aureus* (Figure 6A). This is in good line with other reports, as freeze-drying is known to be a gentle method to recover polyphenols.[29] In our process, development freeze-drying was compared to oven drying and spray drying.

In oven drying experiments, three different drying temperatures (85, 95, and 110 °C) were studied in more detail using two treatment times in each temperature. All oven-dried samples gave good antimicrobial activity. Interestingly, strong oven heating (110 °C) was very effective in preparing extracts with antimicrobial activity against Gram-negative bacteria *E. coli* and *P. aeruginosa*. Using these conditions, the antimicrobial activity of the extracts was clearly better compared with freeze-drying. The temperature of 110 °C and heating time of 1.45 h was bactericidal against *E. coli* (Figure 6B), and both times of 1.45 and 3.5 h were bactericidal against *P. aeruginosa* (Figure 6C).

These results indicate that oven drying could be a method of choice when developing a large-scale production of the extract, as it is more economic than freeze-drying. Mphahlele et al.[30] compared the antimicrobial activity of methanol extracts of freeze and oven-dried (40, 50, and 60 °C) pomegranate peels against selected Gram-positive and -negative bacteria. They concluded that the freeze-dried extracts had highest concentration of total phenolics, tannins, and flavonoids, and therefore, freeze-drying can be further studied as a feasible method for processing pomegranate peel. In our experiments, a high temperature (110 °C) in oven drying was associated with a strong increase in antimicrobial activity against *P. aeruginosa*. Molecular changes in ellagitannins in high temperature should be studied in more detail in order to evaluate the potency of this technology.

Spray dried extracts did not have significant antimicrobial activity against the studied bacteria. Spray drying is a commonly used technique for producing dry polyphenol extract.[31] Fang and Bhandani[32] reported 94−96% retention of polyphenols and anthocyanins during spray drying of bayberry extract with maltodextrin carrier. We did not use any carrier in our experiments, which might have affected antimicrobial activity of our extracts. More experiments are needed to evaluate full potency of this technology.

Antimicrobial Activity of Hydrothermal Extract against MRSA. The antimicrobial activity of the basic hydrothermal extract of cloudberry seed coat fraction, which proved to be most effective against *S. aureus*, was finally tested against methicillin resistant *Staphylococcus aureus* (MRSA). It was very effective as 5 mg/mL concentration causing the decrease of the number of MRSA colonies below detection limit of 100 cfu in 20 h of incubation (Figure 7). This is a very important result, as antibiotic resistance is among the greatest challenges of the

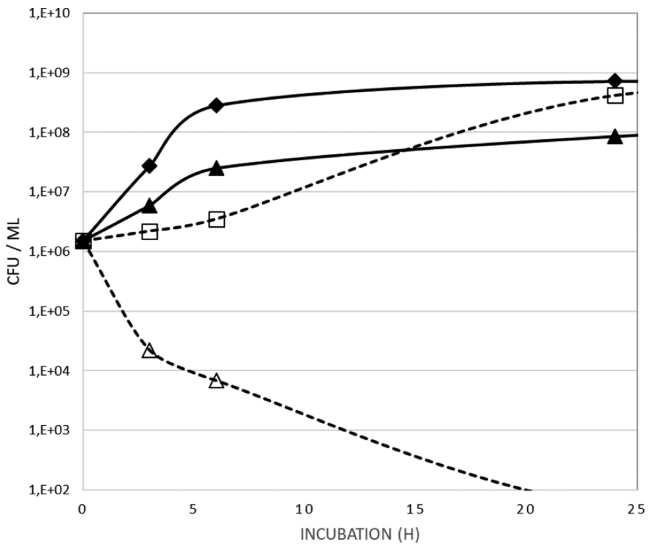

Figure 7. Antimicrobial activity of hydrothermal extract of cloudberry seed coat powder against *Staphylococcus aureus* MRSA. Control (♦); chloramphenicol 50 μg/mL (□); seed coat fraction, HT extract 1 mg/mL (▲); seed coat fraction, HT extract 5 mg/mL (△).

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new millennium in global health care (WHO, 2014). Of particular concern are the pathogenic bacteria showing multiple drug resistant phenotype to all classical antibiotics, such as MRSA. Our study shows also here the resistance of the current MRSA-strain against Chloramphenicol (50 μg/mL) (Figure 7), which was bacteriostatic against *S. aureus* (Figures 4−6). Salaheen et al.[33] evaluated the effects of blackberry (*Rubus fruticosus*) pomace against MRSA. They found that a phenolic extract completely inhibited the growth of vegetative MRSA *in vitro* and phenolic extract + methicillin significantly reduced MRSA biofilm formation on plastic surface. Al-Habib et al.[34] evaluated the efficacy of grape seed extract, rich in proanthocyanidins, against several MRSA strains. They were all sensitive, and 3 mg/mL completely inhibited the growth. However, ellagitannins were not the main phenolic compounds in either of these two studies. Reddy et al.[35] found that the tannin-rich extracts of pomegranate, containing, for example, punicalins and punicalagins, were active against several pathogenic human bacteria, including MRSA. Recently, de Silva et al.[25] demonstrated the potential of sanguiin H-10-containing hydromethanolic extract of wild blackberry against clinical isolate of MRSA, as the extract exhibited bacteriostatic effects against MRSA. The minimum inhibitory concentration (MIC) was 10 mg/mL. All these reports are very interesting and in line with our results, and they may open new application areas for *Rubus* byproducts in health care, especially in preventing and treating infections caused by antibiotic-resistant bacteria in topical applications. Our further studies with clinical MRSA strains as well as *in vivo* experiments are ongoing.

Antimicrobial Activity against Human Beneficial Bacteria. The effects of the cloudberry seed coat hydrothermal extract were also tested against human beneficial bacteria using *Lactobacillus rhamnosus* as an example. The results showed that hydrothermal extracts (2.5 mg/mL) were not affecting the growth of this bacteria (Figure 8). Bioactive phenolic extracts from berry pomace have been reported even to stimulate the growth of beneficial microbes including *Lactobacillus casei*.[36] Puljula et al.[24] detected no or very weak antimicrobial effects of pure ellagitannins against *Lactobacillus plantarum*. Our results indicate that hydrothermal extracts are particularly effective when selective controlling of harmful and pathogenic bacteria is needed. Interestingly, our extracts were not effective against lactic acid bacteria. We have recently isolated and stored an indigenous lactic acid bacteria, *Lactococcus lactis*, from red raspberry (data not shown). Natural occurrence of lactic acid bacteria in *Rubus* berry material can be one reason why these bacteria are resistant to phenolic berry compounds. They are also able to metabolize phenolic compounds in plant materials.[37] Lactic acid bacteria have several mechanisms to protect themselves from oxidative stress[38] and it can be postulated that they can thus benefit from the strong antioxidants present in the berry materials.

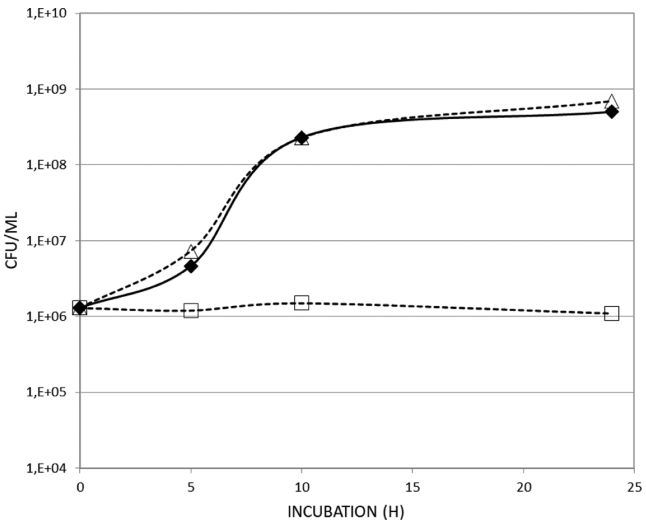

Figure 8. Effects of hydrothermal extract of cloudberry seed coat powder on *Lactobacillus rhamnosus*. Control (♦); Chloramphenicol 20 μg/mL (□); Seed coat fraction, HT extract 2.5 mg/mL (△).

Mechanisms behind Inhibitory Effects. There are several possibilities for what kind of mechanisms are behind these strong antimicrobial activities of the phenolic extracts. Our earlier studies on gastrointestinal microbes indicate that the cloudberry extract not only killed Gram-positive staphylococcal cells but also immobilized the cells to the berry matrix. By contrast, Gram-negative *Salmonella* cells, which were not detectable by a plate count technique, were adhered to cloudberry extract and were mostly still viable.[2] Another well-known mechanism is the ability of phenolic compounds to influence the permeability of the bacterial cell membranes, both cytoplasmic (CM) and outer membranes (OM), as well as electrochemical changes (membrane potential) in CM.[39] We were also able to show that phenolic extracts of cloudberry (1 mg/mL) destabilized OM of *Salmonella* strains as indicated by NPN uptake increase and analysis of liberation of [14C]Gal-LPS. In addition, this effective permeabilization phenomenon was associated with gallic acid, a structural component of ellagitannins (data not shown).

Furthermore, we have been able to show that phenolic cloudberry extracts are able to block quorum sensing (QS) in a bacterial community, thus acting as QS inhibitors and inhibiting biofilm formation.[40] QS is involved in both *S. aureus* and *P. aeruginosa* related skin infections, and the great majority of clinical isolates implicated in human infections have been shown to possess active QS systems.[41] Cloudberry and raspberry phenolics inhibit AHL (N-acyl homoserine lactones) mediated QS signaling without affecting growth and inhibit biofilm formation (unpublished results). The inherent resistance of biofilm communities to antibiotics is partly due to difficulties of antibiotics to penetrate the matrix and slow metabolic activity of the cells. The biofilm mode of growth also promotes the spread of antibiotic resistance genes within community.

As a conclusion, our results clearly showed that antimicrobially active ellagitannins are accumulated in the outer layer of the seed coat. The simple, mechanical sanding system developed in this study was very effective in recovering these compounds. In addition, a gentle hydrothermal extraction system modified composition and structure of these ellagitannins in an unexpected way, resulting in a compound profile not typical to classical solvent extracts. However, the resulting mixture of ellagitannins in the extract had strong antimicrobial activity against Gram-positive *Staphylococcus aureus*, including also MRSA. Further process development indicated that Gram-negative bacteria can also be inhibited, especially when modifying drying conditions of the extract. In general, the results showed that highly active extracts can be prepared from the berry byproducts without using organic solvents, such as acetone or methanol.

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Our earlier results indicate that the antimicrobial activity of cloudberry extracts is likely caused by multiple mechanisms, such as immobilization of bacterial cells to phenolic extracts, changes in cellular membranes, and inhibition of QS and biofilm formation. Synergistic effects are also obvious, as these extracts contain various compounds, for example, weak organic acids, phenolic acids, tannins, and their mixtures of different chemical forms, that could interfere with several cellular targets.

These results indicate the potential of cloudberry seeds and *Rubus* seeds in more general, pharma, and health care applications. Thus, the side streams of these berries should be advantaged in a more efficient manner, which means re-evaluation of the berry value chain in order to advantage all its potential.

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Notes

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ABBREVIATIONS USED

HHDP, hexahydroxydiphenoyl; MRSA, methicillin resistant *Staphylococcus aureus*; cfu, colony forming unit; d.w, dry weight; HT, hydrothermal; OM, outer membrane; CM, cytoplasmic membrane; QS, quorum sensing

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