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Abstract

Current methodologies for evaluation of antibacterial properties of traditional textiles are not applicable to foil-backed, poorly-absorbent electrospun nanofiber materials, since existing test methods require absorbent fabrics. Since electrospun nanofibers are adhered to the foil backing only by electrostatic interactions, methods used to evaluate antibacterial properties of surfaces cannot be used because these protocols cause the nanofibers to lift from the foil backing. Therefore, a novel method for measurement of the antibacterial properties of electrospun metallic foil-backed nanofiber materials was developed. This method indicated that acetate-based nanofibers manufactured to contain 5 to 30 weight percent of cold-pressed hemp seed oil or full-spectrum hemp extract inhibited the growth of *Staphylococcus aureus* in a dose-dependent manner, from 85.3% (SEM = 2.2) inhibition to 99.3% (SEM = 0.15) inhibition, respectively. This testing method represents an advanced manufacturing prototype procedure for assessment of antibacterial properties of novel electrospun, metallic foil-backed nanofiber materials.

Keywords: Antibacterial, Antimicrobial textiles, Nanofiber, Electrospinning, Hemp, *Staphylococcus aureus*

Introduction

The use of electrospinning to create novel nanoscale materials is increasing, along with potential biomedical applications for nanofibers made from natural, sustainable materials (Bhardwaj and Kundu 2010). In this process, nanofibers are deposited on a metallic foil support, and held in place by electrostatic interactions. Since the resulting material is poorly absorbent, and the nanofibers are easily disturbed and lifted from the foil backing, assessment of their antibacterial properties through use of existing methods is not possible.

The American Association of Textile Chemists and Colorists (AATCC) recommends the AATCC 100 protocol to assess antibacterial properties of fabrics. However, this protocol requires complete absorbance of a standard volume of bacterial inoculum by multiple swatches of absorbent fabric, and cannot be applied to poorly absorbent swatches of aluminum foil-backed electrospun nanofibers. The Japanese Industrial Standard (JIS) Z 2801 test assesses antimicrobial properties of nonabsorbent surfaces, but nanofibers would lift from the foil backing during the testing process and carry over to the agar plates used for enumeration of remaining bacterial load, skewing the results.

Additionally, an investigational amount of the electrospun material may not be generated in great enough quantity to provide sufficient surface area for replicate JIS Z 2801 testing.

We developed a novel testing protocol to assess the antibacterial effectiveness of acetate-based, aluminum foil-backed electrospun nanofibers containing different amounts of hemp oils extracted from different parts of the plant. This method requires only a small amount (two 2.5 cm^2 disks) of foil-backed fibers for each replicate, permitting multiple replicates to be run with limited material. This method may be useful to assess the antibacterial properties of other non-absorbent nanofiber materials created in small amounts using electrospinning technology.

Methods

Materials

Tryptic soy broth, tryptic soy agar, Bacto peptone, Bacto tryptone, and granulated agar were manufactured by Difco. Sodium chloride, dibasic potassium phosphate, and glucose were purchased from Fisher Scientific or Sigma-Aldrich. Tryptic soy agar (TSA) plates (100 mm diameter) were purchased from Teknova or prepared using TSA powder according to manufacturer's directions. Methicillin-sensitive *Staphylococcus aureus* subsp. *aureus* Rosenbach (MSSA) was purchased through American Type Culture Collection (ATCC #6538). Cold-pressed hemp seed oil and full-spectrum hemp extract were purchased through commercial vendors.

Preparation of the bacterial load

Staphylococcus aureus bacteria maintained in a glycerol stock were streaked for isolation on a TSA plate each month. Bacterial cultures (3 mL of tryptic soy broth (TSB) in sterile 13 × 100 mm borosilicate glass tubes with slip caps) were inoculated using a single colony of *S. aureus* grown on TSA, and incubated overnight at 37 °C with shaking at 175 rpm. The optical density (OD) 600 nm of the overnight culture was recorded using a Spectronic 20 Genesys spectrophotometer zeroed to air. When the culture in TSB was between 1.123 and 1.257 OD 600 nm, 60 to 90 µL of culture was added to a fresh 3 mL culture of TSB (initial OD 600 nm 0.051 to 0.075) for a final OD 600 nm of 0.097 to 0.109. Equal volumes (500 µL each) of this culture and TSA slurry were combined and vortexed before application to the samples.

Preparation and UV-sterilization of the foil-backed electrospun nanofibers

Aluminum foil-backed nanofibers were prepared using an NEU Nanofiber Electrospinning Unit manufactured by Kato Tech. The standard spinning parameters were: target speed, 1.25 m/min; sample area, 0.1 m^2; pump speed 1.5 mL/h; voltage, 18 kV. One sample of thin-fiber material was made using a slower flow rate of 1.2 mL/h, while keeping the other spinning parameters constant. In all procedures, the 18 gauge needle was positioned 15 cm from the target. The solution for preparing the nanofibers was prepared by weight, with 1 part crystalline acetate solid, 9 parts acetone solvent, and 0.1–0.3 parts full-spectrum hemp extract or cold-pressed hemp oil when incorporated. The solution was aged 48 h before use in electrospinning. Just prior to antibacterial testing, two 2.5 cm^2 disks of foil-backed nanofiber material were cut using a clean scalpel

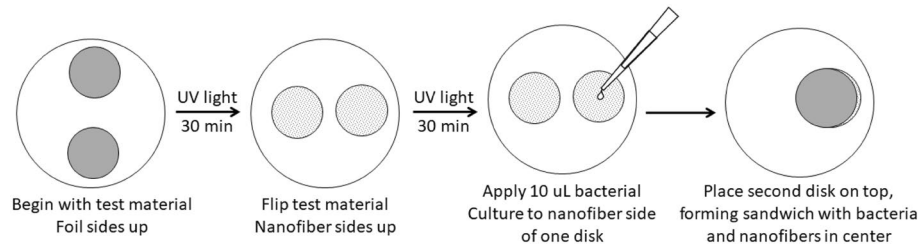

Fig. 1 Sterilization of samples with ultraviolet light and application of initial bacterial load. Inside a biological safety cabinet, disks of sample materials were placed in a plastic petri dish (without the lid) with the foil side up for the first 30 min exposure to UV light. Samples were flipped nanofiber-side-up and repositioned on the open dish before the second 30 min UV light exposure. *S. aureus* (10 µL culture) was applied to the nanofiber side of one disk, and disks were stacked as shown in the figure before incubation for 4 h at 37 °C

and a circular plastic template. Figure 1 outlines the process for UV-sterilization of the nanofiber material, along with application of the initial bacterial load. Disks were placed foil-side-up in sterile 100 mm diameter plastic culture dishes (lids removed) in a biosafety cabinet and dosed with UV light for 30 min. Then the pieces were flipped with sterile forceps, and the nanofiber-sides of the disks were treated with UV light for 30 min. Disks were repositioned on the dish so the entire inner surface of the dish was treated with UV light. Paired samples were run for each antibacterial testing trial: nanofibers containing acetate alone or nanofibers containing acetate and a percentage of either full-spectrum hemp extract or cold-pressed hemp oil. Foil disks without nanofibers were included in some trials, and run in parallel with samples of foil-backed 100% acetate nanofibers and with acetate nanofibers incorporating 30% by weight full-spectrum hemp extract, to assess plain foil for any antibacterial effects. Figure 2 shows a schematic overview of the testing process.

Application of bacteria to the nanofiber samples

Staphylococcus aureus culture in TSA slurry (Tryptone, 17 g; Peptone, 3 g; NaCl, 8.5 g; K_2HPO_4, 2.5 g; glucose, 2.5 g; granulated agar, 3 g; in 1 L; autoclave 25 min, 121 °C, 15 psi) was applied to the center of the nanofiber surface of one disk, and the second disk was placed on top, nanofiber surface down. This formed a sandwich with the bacterial culture (10 µL) contained between the nanofiber surfaces, and the foil surfaces on the top and bottom of the sandwich. A sterile disposable plastic cell spreader was used to gently smooth over the top foil surface of the sandwich, ensuring good contact between the bacterial culture and the nanofiber surfaces. If any liquid culture escaped from between the two disks, the sample was discarded. Dishes were closed and samples were placed in a 1 gallon partially-closed plastic zip-lock bag with damp paper towels to prevent dehydration of the samples. Samples were incubated at 37 °C for exactly 4 h.

Enumeration of initial load of bacteria

The initial load of bacteria was determined by serially diluting the TSA slurry culture immediately after application of bacteria to the samples. Two independent dilution series were prepared, each beginning with 100 µL of the TSA slurry culture. Dilutions (10^1, 10^2, 10^3, 10^4, 10^5) were prepared by passaging 100 µL into 1.5 mL snap cap tubes containing 900 µL sterile water. A 100 µL aliquot of the 10^3, 10^4, and 10^5 dilutions from

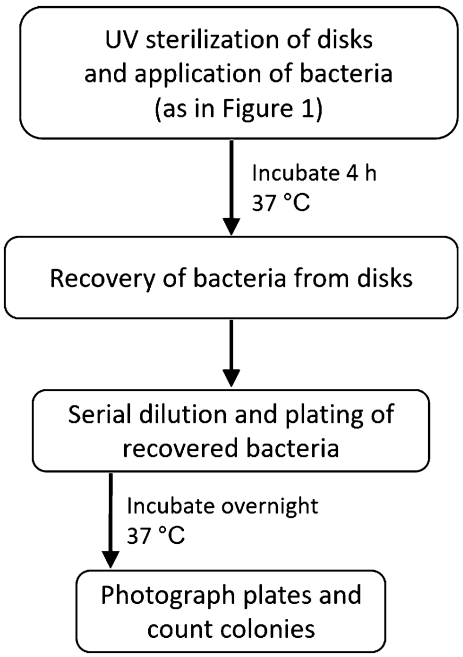

Fig. 2 Schematic overview of the test method. The test method runs over 2 days. On the morning of day 1, samples are sterilized with UV light and bacteria are applied before a 4 h incubation. In the afternoon of day 1, bacteria are recovered from samples, serially diluted, and applied to TSA plates. The TSA plates are incubated overnight, and are photographed the following day

each series was applied to TSA plates, and evenly spread using a sterile disposable plastic cell spreader. Plates were inverted, and incubated overnight (18–20 h) at 37 °C.

Enumeration of bacteria recovered from foil-backed samples

After 4 h of incubation at 37 °C, foil disks were removed from the Petri dishes, added to 10 mL of sterile water in 50 mL sterile disposable conical tubes, and vortexed for 1 min at 1200 rpm. This step recovered bacteria applied to the sample, and diluted the recovered bacteria by a factor of 10^3. Two independent serial dilutions (10^4, 10^5, 10^6) were created by passaging 100 µL of the recovered bacteria through 900 µL of sterile water. For samples of foil alone, or foil with acetate alone, 100 µL from each of the 10^4, 10^5, and 10^6 dilutions were applied to TSA plates. For samples containing full-spectrum hemp extract or cold-pressed hemp oil, 100 µL of the 10^3, 10^4, and 10^5 dilutions were plated on TSA. Plates were incubated overnight (18–20 h) at 37 °C. Images of the plates were captured using a digital camera and colonies were counted using the Colony Counter Plugin, authored by Bruno Vieira, for ImageJ v. 1.51 k (Schneider et al. 2012).

Results and discussion

A variety of methods are available for testing antibacterial properties of traditional textiles, but electrospun nanofibers are a relatively new type of material, to which existing methods for assessment of antimicrobial activities of traditional absorbent fabrics or non-absorbent surfaces are not easily applied. For a tabular overview of popular existing

methods, along with a discussion of their limitations and opportunities, see the review by Teufel and Redl (2006). Unfortunately, none of the methods outlined therein are well-suited to foil-backed nanofiber materials. Multiple factors must be considered when choosing a test method, including the chemical and physical stability of the textile material and antimicrobial agent, the type of microorganism applied or recovered from the fabric, and the ultimate use of the textile. Use of a standardized testing method facilitates comparison of antimicrobial effectiveness across textile types, since different measures of antimicrobial efficacy are obtained for the same antimicrobial textile when different testing methods are applied (Haase et al. 2017). Unfortunately, a single test method cannot be applied to all textiles, as these encompass a great variety of physical and chemical properties.

The method presented here was robust and yielded reproducible results for evaluation of antimicrobial activity of electrospun nanofibers, despite application of bacterial load in a small volume (10 µL) and multiple serial dilutions performed by different technicians and on different days (Table 1). A 10 µL inoculum has been used in other studies focusing on long-term survival of microorganisms on fabrics (Neely and Maley 2000; Koca et al. 2012). Application of a concentrated bacterial load mimics the high bacterial load in exudate associated with infected skin wounds, which can exceed 10^8 CFU/mL (Tachi et al. 2004). The initial bacterial loads applied to the materials in this study ranged from 4.9×10^6 to 2.0×10^8 CFU/mL, which are reasonable for use in evaluation of a material to be used as a dressing for infected wounds.

Table 1 Reproducibility of the test method

Antimicrobial agent	Technician(s)	Run date	% decrease of viable bacteria[a]
30% full-spectrum extract	A	06/14/2017	99.5
	A/B	06/19/2017	99.3
	B	06/22/2017	99.0
10% full-spectrum extract	A/C	06/08/2018	97.1
	A/C	06/14/2018	97.4
	C	06/20/2018	97.2
	C	07/02/2018	97.2
10% cold-pressed seed oil (thin fibers)	B/D	11/04/2017	90.1
	A	10/02/2018	90.7
	A	10/02/2018	91.2
10% cold-pressed seed oil	A/B/D	09/15/2017	91.4
	B/D	09/29/2017	84.1
	B/D	10/07/2017	90.1
	A	10/02/2018	91.2
5% cold-pressed seed oil	C	06/28/2018	89.6
	C	06/28/2018	82.3
	A	10/02/2018	85.3

The test procedure yielded reproducible data on different days and across different technicians

[a] The values shown are the percent decrease in viable bacteria recovered from the cold-pressed hemp seed oil or full-spectrum hemp extract samples, as compared to the viable bacteria recovered from the paired acetate-only samples run on the same day. All samples were on aluminum foil backing of the same type and thickness

Since the nanofibers were electrostatically associated with the aluminum foil backing, some of the nanofiber material lifted from the foil during the vortex step used to recover the applied bacteria. A control experiment was performed to determine if the antimicrobial activity was due to carryover of plant oil from the samples to the enumeration plates. Two uninoculated UV-sterilized foil disks containing 30% or 10% full-spectrum hemp extract were added to 10 mL of sterile water and vortexed the same way as in recovery of viable bacteria. Either this solution (100 µL) or sterile water (100 µL) was applied to the surface of a sterile TSA plate and allowed to be fully absorbed into the agar before application of 100 µL of 4.8×10^7 CFU/mL bacterial inoculum. Similar amounts of bacteria, less than two percent difference in the means, were recovered from both experimental and control plates (Table 2), indicating that observed antibacterial effects were not due to carry over of nanofiber material.

Development of this antibacterial testing method was driven by the desire to examine whether plant oils or extracts provided antibacterial qualities to foil-backed acetate-based electrospun nanofibers. Therefore, antibacterial efficacy of the foil-backed acetate-based electrospun nanofibers made with full-spectrum hemp extract or cold-pressed hemp seed oil was quantitated as the percent reduction of viable bacteria recovered from those samples after a 4 h incubation at 37 °C, using the amount of viable bacteria recovered from foil-backed 100% acetate nanofiber samples, run at the same time, as a baseline. Figure 3 shows representative images of *S. aureus* bacterial colonies growing on TSA plates. The same load of bacteria was applied to all samples. The number of bacterial colonies on the TSA plates reflect the load of viable bacteria initially applied to the samples, and the load of viable bacteria recovered from the samples after a 4 h incubation at 37 °C.

Aluminum foil can prevent bacterial growth by exclusion of atmosphere, nutrients, and moisture (Dogan et al. 2009). On the other hand, these items would be in rich supply on the surface of the skin, and so aluminum foil would not be able to effect suppression of bacterial growth through this mechanism if the foil-backed nanofiber material were applied in contact with skin. To determine whether aluminum foil alone, or foil-backed 100% acetate nanofibers alone, provided any antibacterial effects, initial antibacterial testing runs included samples of aluminum foil backing without nanofibers, in addition to foil-backed 100% acetate nanofibers and foil-backed acetate nanofibers made with cold-pressed hemp seed oil or full-spectrum hemp extract. No effective antibacterial qualities were observed for either the aluminum foil backing without nanofibers or for foil-backed 100% acetate nanofibers alone (Figs. 3b, c, 4). Compared to the initial bacterial load applied, aluminum foil backing without nanofibers permitted a 1153%

Table 2 Bacterial load of *S. aureus* recovered from experimental or control TSA plates

Antibacterial agent	Sample	Recovered bacterial load (CFU/mL)
30% full-spectrum hemp extract	Experimental	$3.6 \times 10^7 \pm 8.0 \times 10^5$
	Sterile water control	$3.5 \times 10^7 \pm 1.2 \times 10^6$
10% full-spectrum hemp extract	Experimental	$4.3 \times 10^7 \pm 1.5 \times 10^6$
	Sterile water control	$4.2 \times 10^7 \pm 3.6 \times 10^6$

Values reported are average and standard error of the mean of bacterial load recovered

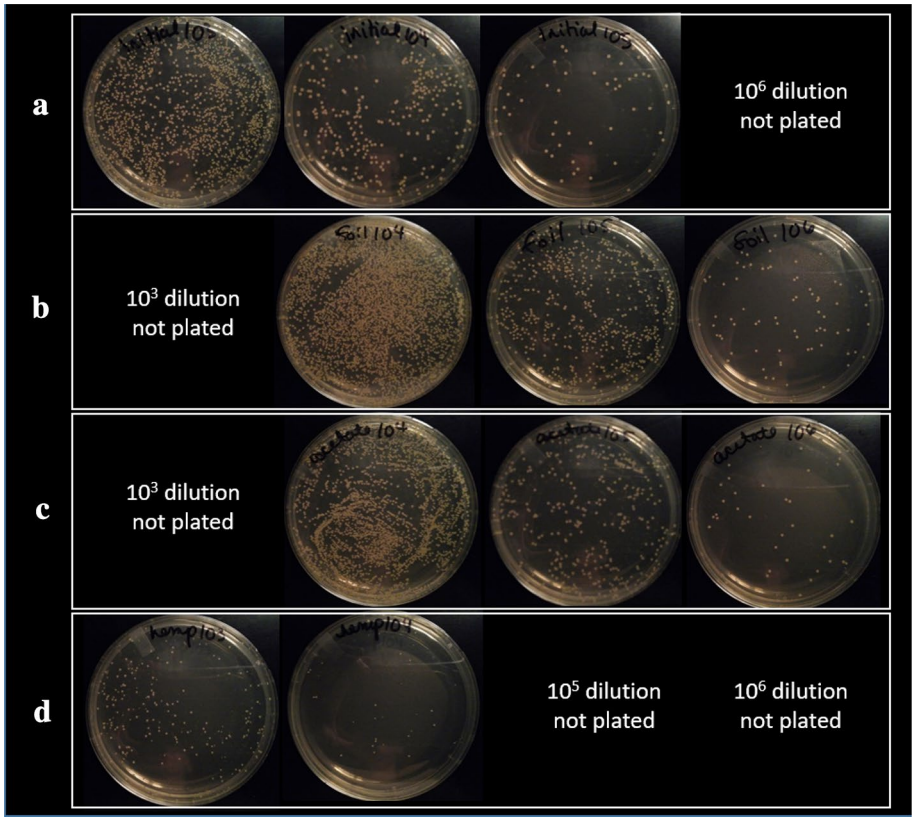

Fig. 3 Representative images of *S. aureus* colonies growing on TSA plates. The load of viable bacteria initially applied to the samples, as well as recovered from the samples, was quantitated based upon the number of bacterial colonies growing on TSA plates after overnight incubation at 37 °C. All samples were plated in duplicate, but only one plate of each duplicate is shown for simplicity of presentation. Samples from dilutions containing bacterial loads which were too high or too low were not plated, as the plates would show nearly confluent growth or no growth. For visual comparison of relative amounts of viable bacteria applied to or recovered from samples, images are aligned vertically according to the fold of serial dilution of bacteria before plating. Digital photographs were also taken of the agar surface with the lid of the plate removed, and those images were used to count the number of colonies using the Colony Counter Plugin of ImageJ (Schneider et al. 2012). **a** Bacterial colonies obtained from the TSA slurry culture from which the initial load of bacteria was applied to the sample materials. Panels **b**, **c**, and **d** show bacterial colonies recovered from sample materials after a 4 h incubation at 37 °C. Samples are as follows: **b** aluminum foil backing without nanofibers; **c** foil-backed 100% acetate nanofibers; **d** foil-backed acetate nanofibers containing 30% full-spectrum hemp extract. Note the large increase in bacterial growth permitted by the samples of aluminum foil without nanofibers (**b**) and samples of foil-backed 100% acetate nanofibers (**c**), as compared to the initial load of bacteria applied (**a**)

(SEM = 278; n = 3) increase in viable bacteria after a 4 h incubation at 37 °C, while the foil-backed 100% acetate nanofibers alone allowed a 446% (SEM = 61; n = 3) increase in viable bacteria. For comparison, foil-backed acetate nanofibers containing 30% full-spectrum hemp extract by weight, run in parallel with the aluminum foil backing alone and foil-backed 100% acetate nanofiber samples, permitted only a 3% (SEM = 1; n = 3) increase in viable bacteria.

Percent reduction of viable bacteria recovered from samples made with full-spectrum hemp extract or cold-pressed hemp oil, as compared to the acetate-only samples, differed according to the concentration and source of extract or oil incorporated into the acetate-based nanofibers (Fig. 5). As expected, greater concentrations of either

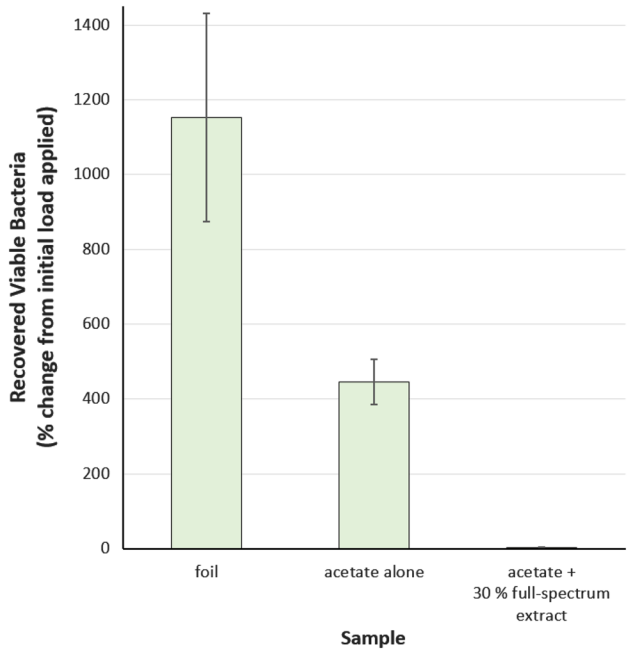

Fig. 4 Foil backing without nanofibers and foil-backed 100% acetate nanofibers lacked effective antibacterial properties. On three different days (Table 1), samples of plain aluminum foil, aluminum foil-backed 100% acetate nanofibers, and aluminum foil-backed acetate nanofibers containing 30% (by weight) full-spectrum hemp extract were run in parallel. Viable *S. aureus* were recovered from each sample after a 4 h incubation at 37 °C. The percent increase of viable bacteria, as compared to initial load applied, is displayed. Error bars show standard error of the mean

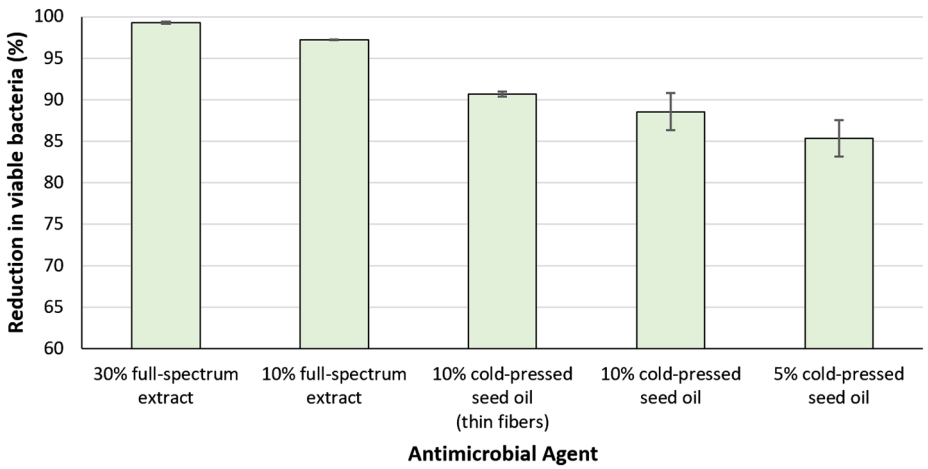

Fig. 5 Percent reduction of viable *S. aureus* recovered from foil-backed electrospun acetate nanofibers containing hemp additives. The amount of viable *S. aureus* recovered from nanofiber samples containing hemp-derived extract or oil, as compared to acetate-only nanofiber samples, was decreased in all samples after a 4 h incubation at 37 °C. Each bar represents three independent trials, except for the 10% full-spectrum hemp extract sample, which was generated from four trials. Error bars represent standard error of the mean for all trials of each sample

full-spectrum hemp extract or cold-pressed hemp seed oil showed greater antibacterial activity. Full-spectrum hemp extract provided the greatest antimicrobial activity, as compared to cold-pressed hemp seed oil. This result agrees with literature reports on the antibacterial properties of plant-derived oils. A review by Chouhan et al. (2017) summarizes evidence from multiple studies showing antimicrobial activity of various plant oils differs according to the part of the plant from which they are derived. In the case of oils derived from *Cannabis sativa*, Lelario et al. (2018) found a greater antimicrobial activity against *Bacillus* spp. when using oil extracted from flowers of hemp plants, as compared to purified cannabidiol (CBD), Δ^9-tetrahydrocannabinol (THC), or a combination of pure CBD and THC. The bioactive components of *C. sativa* were found in greater concentrations in flowers, as compared to seeds (Latta and Eaton 1975). The antibacterial properties of *C. sativa* are attributed to cannabinoids such as CBD (Appendino et al. 2008), which is present in low quantities in seed oil as compared to the rest of the plant (Leizer et al. 2000).

Acetate nanofibers prepared with 10% cold-pressed seed oil, stored in the dark at room temperature, showed no loss of antibacterial activity over 1 year's time (Table 1). Thus, preliminary stability, important for practical application, has been demonstrated.

Interestingly, the 10% cold-pressed hemp seed oil material electrospun using a slower solution feed rate had a slightly higher antibacterial activity, as compared to the 10% cold-pressed seed oil material produced with the standard flow rate. A possible explanation for this slightly increased antibacterial efficacy is that the slower feed rate yielded thinner diameter fibers on the foil backing. The smaller diameter fibers permitted a greater ratio of nanofiber surface area per material surface area, increasing the total surface available for interaction with bacterial cells. A more in-depth study which varies fiber diameter would be of interest to explore this concept and determine optimal fiber diameter, since material made with a slower feed rate uses less material and is economically advantageous.

The method proposed herein was designed to be used on foil-backed nanofibers, and is not appropriate to assess antibacterial properties of traditional woven or knit fabrics. There is great risk the small volume (10 μL) bacterial inoculum would not be sufficient to provide proper interaction with a large enough surface area of the threads or yarns of a woven or knit fabric, and could easily be lost through space between the yarns or threads. Lack of contact between the bacterial inoculum and the fibers of the fabric would lead to an underestimation of the antibacterial properties of the fabric. Additionally, reproducibility of the method from run to run would be poor.

When the test described herein is applied to foil-backed nanofiber materials, care must be taken to avoid the following pitfalls, which can lead to an over- or under-estimation of antibacterial activity of the nanofiber materials. If bacterial inoculum is accidentally lost from between the foil-backed nanofiber disks before or during the 4 h incubation step, then the test would be invalid. If bacteria are tightly adhered to the nanofibers and not released into sterile water during the recovery step after the 4 h incubation, then the antibacterial properties of the material may be overestimated. Carryover of nanofiber material to the serial dilution and plating step of the procedure may suppress bacterial growth during the overnight incubation of TSA plates, and again lead to overestimation of antibacterial properties. The initial load of applied bacteria must be carefully

quantitated, and the $OD_{600\ nm}$ values reported here are valid only for *S. aureus* ATCC 6538 in TSB media. These numbers cannot be used for different bacteria and/or different liquid medias.

Conclusions

Current methods for evaluation of antimicrobial activity of textiles are difficult to apply to electrospun nanofibers. The method described herein utilized only small amounts of electrospun material, and yielded reproducible results across multiple technicians and over different days. Electrospun acetate nanofibers incorporating full-spectrum hemp extracts or cold-pressed hemp seed oils possess antibacterial properties against *Staphylococcus aureus*, and may be useful in manufacture of novel textiles with a broad range of applications in industries where control of microbial growth is desirable.

Abbreviations

AATCC: American Association of Textile Chemists and Colorists; JIS: Japanese Industrial Standard; CBD: cannabidiol; SEM: standard error of the mean; THC: Δ^9-tetrahydrocannabinol; TSA: tryptic soy agar; TSB: tryptic soy broth.

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Authors' contributions

MAS conceptually designed hemp-containing nanofiber material for antibacterial applications; HB formulated and synthesized foil-backed nanofiber material; MAWG developed and executed antibacterial testing methodology, and oversaw on-site testing. All authors contributed to manuscript preparation and review. All authors read and approved the final manuscript.

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Availability of data and materials

Raw data (image files and colony counts) generated for this study are archived on Labarchives.com, and available upon request from the corresponding author. Residual amounts of materials tested in this project are stored on-site, and can be made using instructions provided in "Methods" section.

Competing interests

The authors declare that they have no competing interests.

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