Methods for determining the antibacterial activity of additives for polymeric materials

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Abstract: Four methods for assessing the antibacterial activity of natural additives with antibacterial properties intended for polymeric materials have been described: in solution, in the form of a film (agar test; bacterial culture test) and in accordance with ISO 22196. Natural additives such as berberine, quercetin, caffeic acid, curcumin and hops were tested in the form of a solution or as an additive to a polymer, i.e. poly(butylene succinate-co-adipate) (PBSA), and their activity was determined against Gram-positive and Gram-negative bacteria – *S. aureus* and *E. coli*, respectively. The best results were obtained for additives in the form of a film. On the other hand, the highest antibacterial and biocidal activity was characterized by berberine, caffeic acid and hops.

Keywords: natural antibacterial additives, antibacterial activity, *S. aureus*, *E. coli.*

Metody oznaczania aktywności przeciwbakteryjnej dodatków do materiałów polimerowych

Streszczenie: Opisano cztery metody oceny aktywności antybakteryjnej naturalnych dodatków o właściwościach antybakteryjnych przeznaczonych dla materiałów polimerowych: w roztworze, w postaci folii (test agarowy; test hodowli bakteryjnej) oraz zgodnie z normą ISO 22196. Zbadano naturalne dodatki takie, jak berberyna, kwercetyna, kwas kawowy, kurkumina oraz chmiel w postaci roztworu lub dodatku do polimeru tj. poli(bursztynianu-*co*-adypinianu butylenu) (PBSA), a ich aktywność określono wobec bakterii Gram-dodatnich i Gram-ujemnych – odpowiednio *S. aureus* i *E. coli*. Najlepsze wyniki uzyskano dla dodatków w postaci folii. Natomiast największą aktywnością antybakteryjną i biobójczą charakteryzowały się berberyna, kwas kawowy i chmiel.

Słowa kluczowe: naturalne dodatki antybakteryjne, działanie biobójcze, *S. aureus*, *E. coli.*

Natural additives as alternative antibacterial agents have been of interest to researchers in recent years, due to the drastic increase in bacterial and antimicrobial resistance (AMR) of synthetic antibiotics. It has been reported that AMR was responsible for approximately 5 million deaths worldwide in 2019 and can be considered as a global threat. The number of bacterial related deaths because of AMR will significantly increase if the issue is not resolved [1]. The significant increase in AMR can be linked to excessive use and incorrect disposal of conventional antibiotics [2]. Harmful and non-harmful bacteria often inadvertently come into contact with these overused or incorrectly disposed antibiotics and therefore are presented with opportunities to build resistance. Plant, animal, and microorganism derived additives have become more popular as potential drug candidates due to their wide range of action, with minimal risks of toxicity or resistance [3]. Plant and animal derived additives tend to contain phytochemicals with antibacterial, antifungal, antimicrobial and antiviral properties [4]. There are limitations associated with natural antimicrobials, they have low bioavailability and shorter half-lives compared to synthetic alternatives [5]. Other limitations include their lack of high temperature stability and the need to be used in higher concentrations or in combination with antibiotics [6–9]. However, when their cost is compared to synthetic agents they are deemed worthwhile alternatives.

Different researchers have investigated the mode of action for the natural additives selected as part of this

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study. Berberine is a plant-based substance found in the roots and leaves of the berberis plant. It has been suggested that its mode of action as an antimicrobial agent is the inhibition of DNA and protein synthesis [10], other studies have shown that high concentrations of berberine can damage bacteria cell walls resulting in cell death [9]. Quercetin dihydrate, a natural antioxidant has been used in packaging materials to maintain the shelf life of fresh fruit products [11]. The polyphenolic bioflavonoid found in fruit and vegetables is known to inhibit pathogen growth of harmful bacteria [12], without inhibiting "good bacteria" of the *Lactobacillaceae* family. This further supports its use as a packaging additive [13]. Caffeic acid is also a polyphenol with proven antimicrobial activity, [14] it displays antibacterial action by inhibiting RNA polymerase enzymes. In some studies, it has been known to provide synergistic action when combined with conventional antibiotics to combat methicillin resistant bacterial strains [15]. Curcumin is another natural antioxidant with antimicrobial activity. Inhibition zone tests showed that curcumin presented antibacterial activity against both gram-positive and gram-negative bacteria [16]. Beer hops are an abundant source of α- and β-acids, because of their hydrophobic nature, with the β-acids being a strong antimicrobial agent. Both α- and β-acids interact with cell membranes of gram-positive bacteria and can inhibit or reduce growth [17]. Other compounds isolated from plant-derived beer hops such as humulone and lupulone have been known to have antibiofilm properties, even allows the substances to penetrate the biofilms and reduce the number of bacteria inside it [18]. Chitosan, an animal-derived polysaccharide from chitin, has antimicrobial activity against both gram-negative and gram-positive bacteria [19]. Chitosan displays its antimicrobial effect by disrupting cell membranes leading to integrity changes in the membrane and cell death. It also interferes with microbial gene expression, by interacting with RNA and DNA resulting in growth inhibition [20].

The utilization of natural additives in active packaging is a new area of research and technology. Active packaging is the manufacturing of packaging that contains active additives that modify the conditions of the packaged goods in such a way that the shelf-life of the products and therefore quality of the goods are maintained or even extended [21, 22]. Polymers are typically used for packaging food and non-food items as their application is very versatile. They can be flat films or rigid containers, opaque or transparent, and several times cheaper than other materials like glass or metal [22].

As previously mentioned, it has become more popular for active packaging to contain biologically active natural additives. In one study, curcumin, and quercetin – both polyphenolic compounds, were used individually as additives (up to 50 wt%) in functional polypropylene membranes. Significant antibacterial activity against *S. aureus* and *E. coli* bacteria strains was observed in the functional membranes when compared to the pure polypropylene control film [23]. The authors concluded that the functional membranes had potential to be applied as packaging in the food industry. With regards to the food industry, additives must be selected carefully, due to the risk of interaction with the additives and the packaged food goods. The selection of natural additives that do not display toxicity or affect the organoleptic properties of the food is important [24]. In another study, it was found that polyethylene films coated with rosemary extracts used to package fresh meat had around 60% less lipid oxidation after 9 days of cold storage compared to meat stored in the pure polymer control [25]. The authors suggested that natural additives in packaging coating could be used as alternatives to synthetic antioxidants due to their enhanced oxidative stability. Other researchers found that edible chitosan films coated with 2% oregano essential oil had the highest inhibitory effect on spoilage microorganisms in chicken fillets. The use of oregano essential oil in combination with chitosan inhibited microbial growth and extended the shelf life of the chicken [26].

The antibacterial activity of the selected additives is an important parameter to assess while working with food/packaging additives. There are numerous laboratory methods that can be used to determine antibacterial activity [27]. One of the most popular is the disc-diffusion method, sometimes called the agar diffusion method. This method was developed in 1940 and is being used as the primary method in many clinical laboratories as a preliminary antimicrobial susceptibility test. The disc diffusion method is a qualitative assessment, the growth of bacteria will be inhibited if the substance has antibacterial activity. Therefore, the presence and size of an inhibition zone is indicative of antimicrobial efficacy [28]. The method itself relies upon the diffusion of the active substance from its support medium, during controlled incubation. If the antibacterial activity is present, the inhibition zone will be visible around the filter paper disc or polymer film carrier containing the active substance [29, 30]. The main advantages of this method are its cost-effectivity and does not require specialized equipment. It can be used to test several substances against the same microorganism, as a comparative analysis of their microbial activity. The results are acquired after a relatively short time (18-48 hours), making another reason the disc diffusion method is often used as a preliminary screening method for antimicrobial susceptibility [31]. It is important to remember that it is a qualitative test, not quantitative. Additionally, there are several factors that can affect the size of the observed inhibition zone such as humidity, pH, temperature, and diffusion rate of the substance from the support medium to the agar.

Broth dilution is a quantitative technique that can be used to determine the minimum inhibitory concentration (MIC) of the selected antimicrobial agent. It is a standardized test, recognized by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) as a reliable method to determine susceptibility. The broth

dilution method can be carried out in two ways – microdilution or macrodilution. Microdilution uses a smaller volume compared to macrodillution, requiring less materials, making this method ideal when the antimicrobial agent of interest is expensive, and researchers only have a small quantity [32].

It is a time-consuming method that requires precise serial dilution of the suspected antimicrobial agent in broth. Diluted solutions containing the additive are spiked with a standardized number of microbial cells. After incubation, the tubes are visually inspected, clear broth indicates no growth of microorganisms and turbid broth confirms growth of microorganisms. The test tube with the lowest concentration of suspected antimicrobial agent with no growth of microorganism observed is taken as the MIC [31, 32].

Several standardized normative procedures have been created to measure the activity of antibacterial agents in polymer films. ASTM E2180 evaluates antimicrobials incorporated into hydrophobic materials like plastics and resins. This method uses bacteria inoculum contained in an agar slurry. This quantitative technique compares antimicrobial activity of polymer materials between non-treated and treated with antimicrobial agents. The inoculated agar slurry is introduced to the polymer sample *via* pipette and incubated for 24h at a specific temperature and humidity. After incubation, the slurry is washed from the sample, subjected to serial dilution, and spread on agar. Following further incubation, the change in number of viable bacteria cells is observed [33].

Another popular standardized procedure is ISO 22196. It assesses the antibacterial activity of plastic or other non-porous surfaces. The sample surface is inoculated with a bacterial suspension in a liquid culture medium, and similarly to ASTM E2180 undergoes incubation. After 24h of incubation at 35°C (>90% RH), the sample is rinsed, and the rinsed liquid is further incubated to observe bacteria growth. Bacteria colonies are counted, and antibacterial activity can be reported. This method also relies on comparing the antibacterial activity of treated to untreated reference materials [35].

The aim of this study was to evaluate and select the best method for preliminary determination of antibacterial activity of natural additives. Obtained results were used to decide whether to use the assay to examine additives in active packaging for food or non-food items. The natural additives deemed suitable after antibacterial activity characterization in this investigation will be used to manufacture polymer composites. These composites, based on biodegradable polymer carriers, contained a novel mixture of natural additives. The mixture of additives was examined to display a wide range of combined antibacterial activity. Obtained composites will be examined as an active polymer food packaging.

EXPERIMENTAL PART

Materials

Poly(butylene succinate-*co*-adipate) (PBSA) granulate (FD92PM) was purchased from Mitsubishi Group Chemicals (Mortizburg, Germany). PBSA has a melting temperature of 84°C, a density of 1.24 g/cm^3, and a melt flow rate of 4 g/10 min (190°C/2.16 kg). Berberine hydrochloride ($C_{20}H_{18}ClNO_4$, M_w = 371.82 g/mol), quercetin dihydrate ($C_{15}H_{10}O_7 \cdot 2H_2O$, M_w = 338.28 g/mol), chitosan ($(C_6H_{11}NO_4)_n$, M_w = 200000 g/mol), caffeic acid ($C_9H_8O_4$, M_w = 180.16 g/mol) and curcumin ($C_{21}H_{20}O_6$, M_w = 180.16 g/mol) were supplied by Glentham Life Sciences (Planegg, Germany). Cascade beer hops were purchased from Hopsmaker (USA). Cascade beer hops contain, among others, alpha acids (4–9%), beta acids (5–8%) and essential oils (0.5–2%), i.e., myrcene, caryophyllene, humulene etc. Solvents and reagents were laboratory grade and were purchased from ChemLand (Stargard, Poland). Antibiotic control (ciprofloxacin) and blank filter paper discs were obtained from Pol-Aura (Zawroty, Poland). Mueller Hinton loose agar, plate count agar (PCA) and ready-prepared Mueller Hinton agar plates were purchased from GRASO Biotech (Owidz, Poland). The following bacterial strains were used in the study: *S. aureus* (ATCC 43300), *S. aureus* (ATCC 6538P),

T a b l e 1. Characteristics of bacterial strains used

Strain	Characteristic
S. aureus (ATCC 43300)	*Staphylococcus aureus* subsp. aureus strain F-182 is a clinical isolate from Kansas. This bacterial strain is *pvl* negative and SCCmec type II, and it is resistant to methicillin and oxacillin.
S. aureus (ATCC 6538P)	*Staphylococcus aureus* subsp. aureus strain FDA 209P is a whole-genome sequenced bacterium with applications in media testing, sterility testing, and susceptibility testing.
E. coli ATCC (43888)	*Escherichia coli* strain CDC B6914-MS1 was isolated from human feces. This whole-genome sequenced bacterial strain does not produce either Shiga-like toxin I or II and does not possess the genes for these toxins.
E. coli (ATCC 8739)	*Escherichia coli* strain Crooks is a whole genome sequenced bacterial strain that was isolated from feces. This product has applications as a quality control strain in testing antimicrobial handwashing formulations, media testing, efficacy testing, and bioresistance testing.

T a b l e 2. Antibacterial properties testing methods

Method	Description	Material	Incubation conditions	Evaluation of antibacterial activity
Method 1: Solution testing	Antibacterial activity of additives was analyzed in solution (1mg/mL). The substances were dissolved in appropriate solvents (DMSO or acetic acid) and applied to filter paper discs, which were then placed on bacteria-inoculated agar plates.	Filter paper discs	24 h, 37±1°C	Measurement of the inhibition zones around the discs
Method 2: Film form testing (agar test)	Additives (10 wt%) were incorporated into PBSA by solvent method (dichloromethane) and then placed on bacteria-inoculated agar plates.	PBSA film	24h, 37±1°C	Observation of inhibition zones around the film samples
Method 3: Film form testing (bacterial culture test)	Bacteria were cultured in suspension in the presence of PBSA films with additives (10 wt%). After incubation, bacterial counts were determined by plating on PCA medium and further incubation.	PBSA film	First incubation 24 h, 37°C Second, incubation 72 h, 30°C (on PCA)	Determination of viable bacterial count after incubation
Method 4: ISO 22196	A standardized method for evaluating the antibacterial activity of plastics and textiles. It compares the viable bacterial count on antibacterial-treated and untreated samples.	PBSA film	24 h, 35±1°C	Calculation of the difference in logarithmic bacterial cell counts

E. coli (ATCC 43888) and *E. coli* (ATCC 8739). Detailed characteristics of the strains used are presented in Table 1.

Methods

Four methods were utilized as part of this study. A brief description of the research methods used is presented in Table 2. A detailed description of the methodology is included later in the text.

In the first method, the antibacterial activity of the additives was analyzed in solutions. The additives were dissolved in suitable solvents (either 5% DMSO or 1% acetic acid) depending on their solubility. Individual stock solutions of the additives were produced at a concentration of 100 mg/mL. The stock solutions were diluted to obtain a working solution with the concentration 10 mg/mL and finally an analytical solution at a concentration of 1 mg/mL. Onto individual blank filter paper discs 20 μL of each analytical solution was pipetted. The discs were enclosed on a single use plastic petri dish and left to air dry (approximately 30 min). While the filter paper discs with additives were drying, the agar petri plates were prepared for incubation. Bacteria cultures (*S. aureus* and *E. coli*) grew 24 h before analysis, using the McFarland scale, inoculums were diluted to 1.0 McF. The bacteria culture was spread over the entirety of the pre-prepared agar plate, before adding the samples, the bacteria-loaded agar plate was also left to condition for approximately 30 min. Sample discs along with a blank control, 5% DMSO control, and an antibiotic control, were placed onto the agar plates and the agar plates were incubated for 24 h at 37±1°C. After the incubation period, if observed, the zone of inhibition for the additives and controls were measured.

In the second method, the antibacterial activity of the additives was measured in film form. Flat films using biodegradable polymer PBSA as the polymer carrier were prepared using the solvent method. An appropriate amount of polymer with 10 wt% additive was dissolved and mixed in dichloromethane (DCM) with a magnetic stirrer for 1 h at room temperature, a pure PBSA control film was also manufactured. The mixing time was to ensure that both the additive and polymer were adequately dissolved before they were poured out onto glass petri dishes with a diameter of 150 mm. The poured solution was left to evaporate overnight, leaving behind a composite film (Fig. 1). Prior to analysis, the composite films were dried at 45°C for 24 h to remove any residual solvent and cut into squares measuring 1′1cm. Readymade agar plates were streaked with bacteria inoculum in the same way first method (*S. aureus* and *E. coli*). Agar plates with film samples were incubated for 24h at 37±1°C, pure PBSA film was used as a negative control while an antibiotic was used for a positive control. After incubation, the plates were visually observed for growth inhibition zones.

The third method to determine antibacterial activity, used adapted methodology taken from Łopusiewicz *et al.* [34]. It also called for the samples to be analyzed in film form, again the concentration of the additive was 10 wt%, and squares of 1 × 1 cm were used. During this analysis, bacterial cell suspensions (0.5 on the McFarland scale)

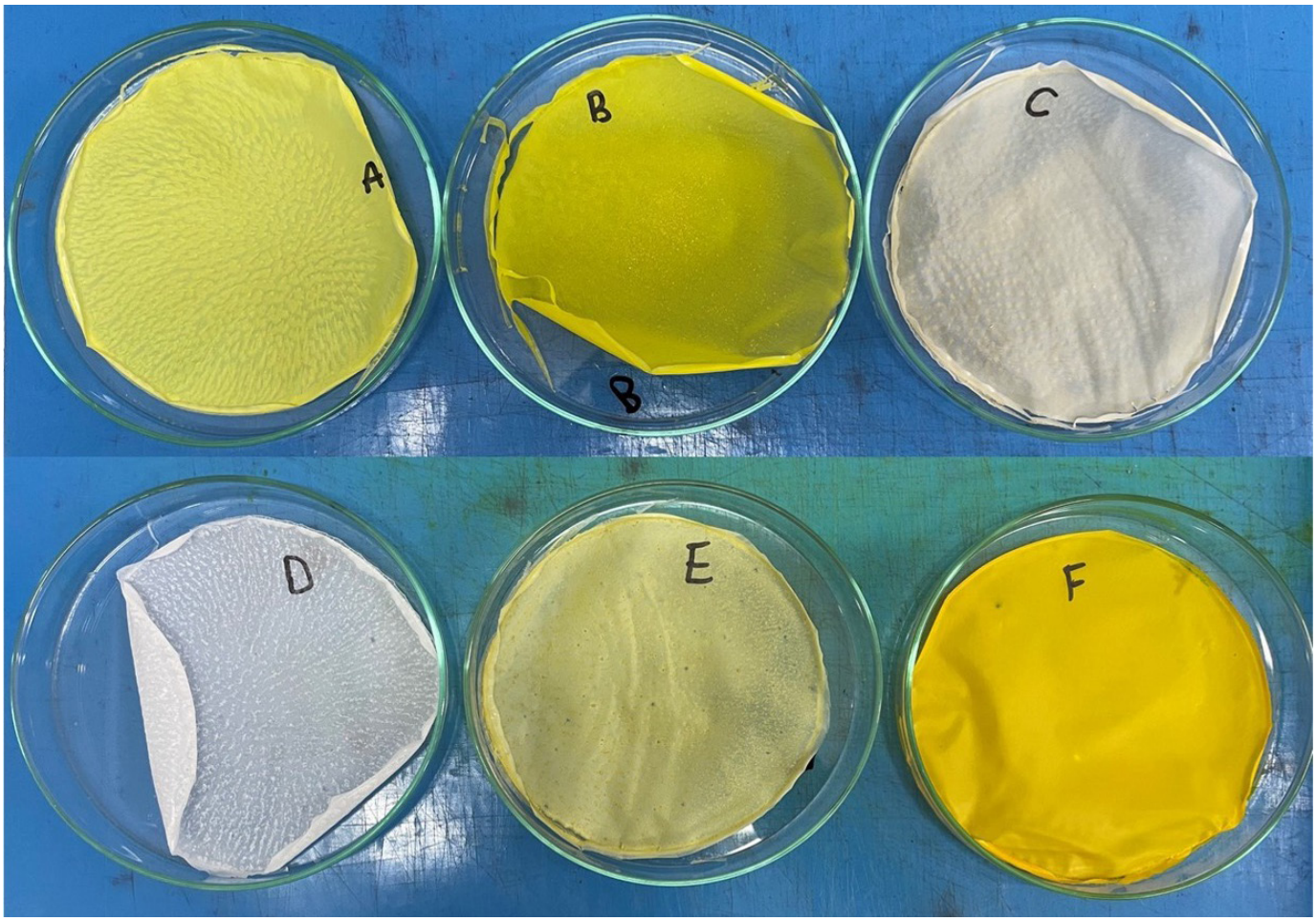

Fig. 1. PBSA films with 10 wt% additives obtained by the solvent method: A – quercetin dihydrate, B – berberine hydrochloride, C – caffeic acid, D – chitosan, E – beer hops, F – curcumin

were incubated in the presence of polymer materials containing the active ingredients. *S. aureus* and *E. coli* bacteria suspensions were produced in 100 mL agar and 1 mL was introduced to the polymer material and incubated for 24 h at 37±1°C. After incubation, the number of bacteria was determined by performing microbiological cultures on the PCA (Plate Count Agar) medium, incubation was for another 72 h at 30±1°C.

The final method, to confirm or disprove the reliability of the three previous methods, was ISO 22196. It is a method used to evaluate the antibacterial activity of antibacterial-treated plastics and textiles. This norm measures antibacterial activity by calculating the difference in the logarithm of the viable cell counts found on an antibacterial-treated product and an untreated product after inoculation with and incubation of bacteria. It was decided that a selected natural substance that consistently showed antibacterial activity in the other methods, and an additive that did not display antibacterial activity, would be analyzed in film form with 10 wt% natural additives. Similarly to other methods, pure PBSA film was used as a control. As specified in the procedure, antibacterial activity was measured against both gram-positive bacteria *S. aureus* and gram-negative bacteria *E. coli*.

RESULTS

Method 1

An example of petri plates with samples ready for incubation is presented in Fig 2. The agar plates with saturated filter paper discs were incubated for 24 h at 37°C, after incubation, the plates were visually inspected for the presence of inhibition zones. In Table 3, the observed inhibition zones against *S. aureus* and *E. coli* are presented.

It can be concluded that the antibiotic control sample displayed antibacterial activity, whereas as expected, no antibacterial activity was observed in the blank control, or the DMSO 5 wt% used to dissolve the examined substances. With regards to the potentially active natural substances, antibacterial activity was only observed in beer hops (Fig 3b).

Method 2

The thickness of the prepared composite films was measured with a micrometer screw and the results are presented in Table 4, the average thickness of 5 measurements, ranged from 0.06 to 0.13 mm. Prior to analysis, the composite films were cut into squares measuring

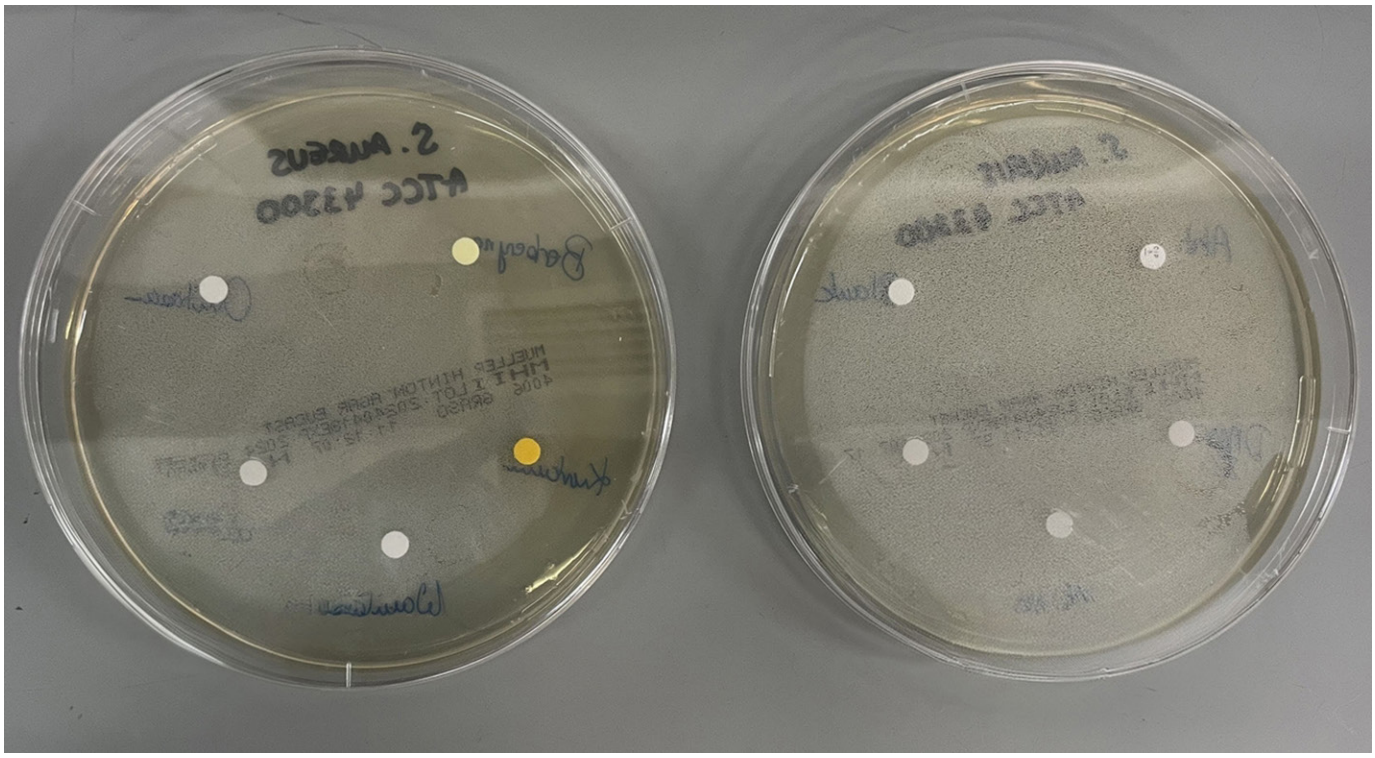

Fig. 2. Agar plate with samples ready for incubation by solution method

1 × 1 cm. These squares were sterilized under UV light for 30 min before testing. Due to the lack of antibacterial activity observed in the first method against gram-negative *E. coli* it was decided to measure activity against only gram-positive *S. aureus*. The results in Table 5 detail whether a zone of inhibition was observed.

While it can be agreed that the concentration of the additives is higher in method 2 than method 1, 10 wt% compered to 1 mg/ml, it is apparent that measuring the antibacterial activity of the natural substances whilst impregnated in a polymer film is more effective. Inhibition zones and therefore antibacterial activity were observed in film samples with berberine hydrochloride, chitosan, and beer hops. The positive antibiotic control as expected displayed antibacterial activity, moreover there was the presence of a small inhibition zone for the negative control – pure PBSA. It is suspected that some residual DCM solvent used to produce the films may be present. However, due to the absence of inhibition zones in the remaining natural substances samples (Fig. 4), it can be assumed that DCM did not influence the positive results obtained in the samples.

T a b l e 3. Observed inhibition zones in antibacterial activity tests for natural substances

Sample, 1 mg/mL	Inhibition zone, mm	
	E. coli (–)	*S. aureus* (+)
Antibiotic control	34.09	26.85
Blank disc	–	–
DMSO 5 wt%	–	–
Curcumin	–	–
Berberine hydrochloride	–	–
Caffeic acid	–	–
Quercetin dihydrate	–	–
Beer hops	–	12.77
Chitosan	–	–

Method 3

The polymer film samples with 1 mL of bacteria spiked agar were incubated individually in disposable Petri dishes. After incubation and the subsequent plate count agar method, the observed change in the number of bacteria cells was used as an indication of antibacterial activity. The two graphs presented in figures 5 and 6 show measured antibacterial activity against *S. aureus* and *E. coli*, respectively.

In the case of *S. aureus*, gram-positive bacteria, a significant reduction in microbial counts (CFU/mL) compared to that of the pure PBSA control indicating antibacterial activity was observed in sample B (berberine hydrochloride). Biocidal activity, where no bacteria was present, was observed in samples C and E (caffeic acid and beer hops).

In the case of *E. coli*, gram-negative bacteria, biocidal activity was observed in samples B and C (berberine hydrochloride and caffeic acid).

Method 4

For continuity and confirmation of suitable preliminary antibacterial activity method selection, it was

Fig. 3. Inhibition zone antibacterial activity tests against *S. aureus*.: a) inhibition zone of the antibiotic control, b) inhibition zone of beer hops

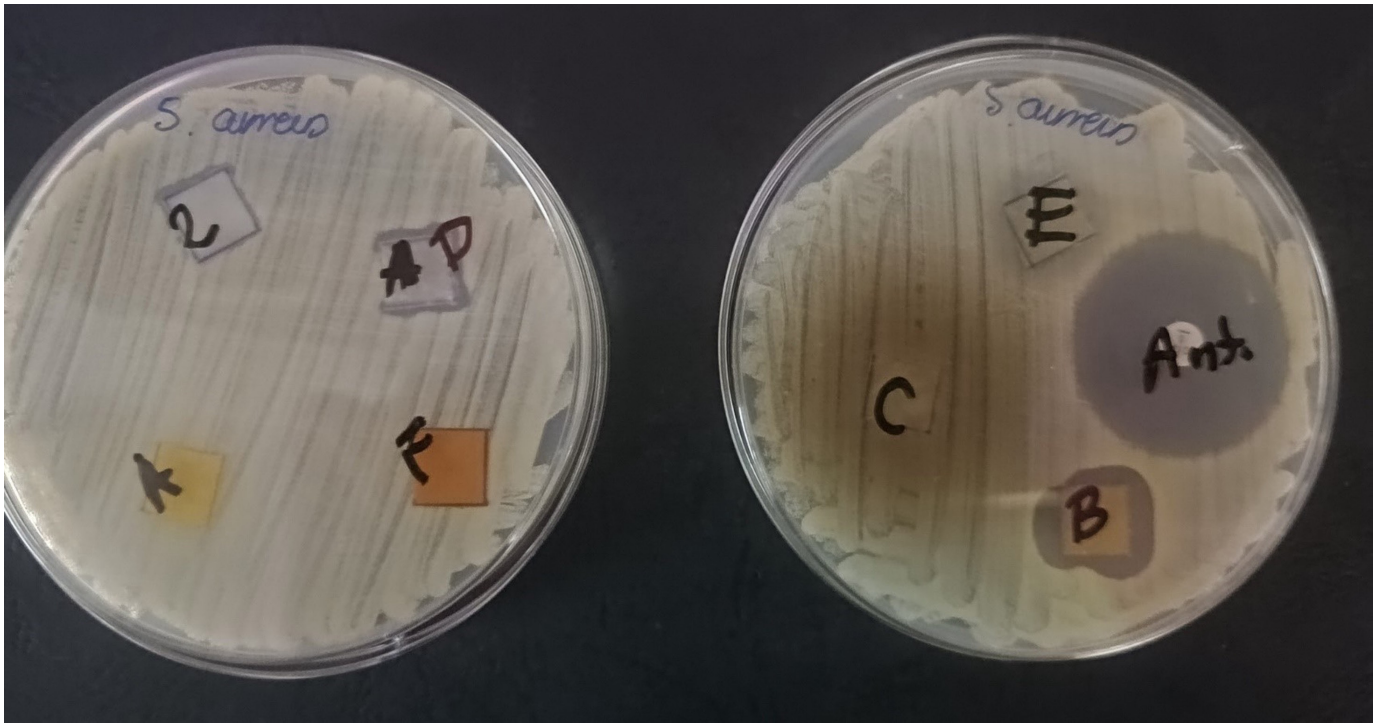

Fig. 4. Agar plates with 10 wt% additive film samples (A-F), polymer film control (2) and antibiotic control (Ant)

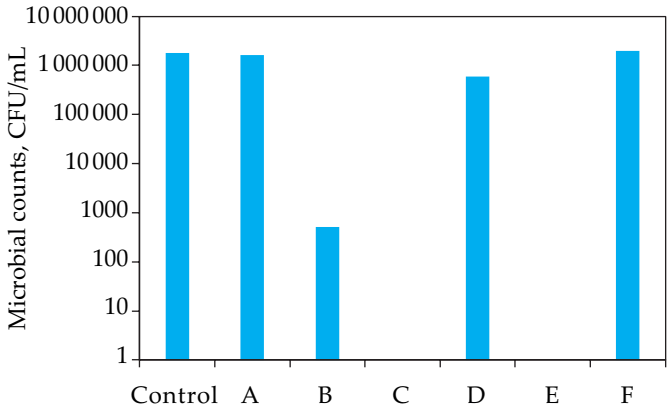

Fig. 5. Antibacterial activity for natural additive polymer films and *S. aureus*

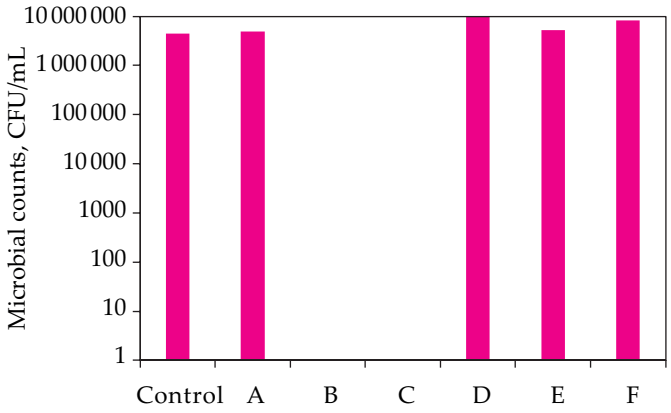

Fig. 6. Antibacterial activity in natural additive polymer films and *E. coli*

T a b l e 4. Average composite film thickness

Composite film	Sample description	Average thickness, μm
Control pure PBSA	Control	62.4±5.0
10% quercetin dihydrate	A	83.6±10.5
10% berberine hydrochloride	B	80.2±12.7
10% caffeic acid	C	92.8±14.5
10% chitosan	D	122.8±9.7
10% beer hops	E	126.0±13.9
10% curcumin	F	69.4±12.3

T a b l e 5. Presence or absences of inhibition zones for natural substances in PBSA film

Sample	Observed zone of inhibition
	S. aureus (+)
Antibiotic control	Yes
Pure PBSA control	Yes
10% quercetin dihydrate	No
10% berberine hydrochloride	Yes
10% caffeic acid	No
10% chitosan	Yes
10% beer hops	Yes
10% curcumin	No

T a b l e 6. Antibacterial activity of samples against *E. coli* and *S. aureus*

Sample	Bacteria strain	Average number of viable bacteria cells/cm^2	Calculated parameter
Control 0 h	*E. coli*	$2.5 \cdot 10^4$	$U_0 = 4.4$
	S. aureus	$2.5 \cdot 10^4$	$U_0 = 4.4$
Control 24 h	*E. coli*	$4.0 \cdot 10^5$	$U_t = 5.6$
	S. aureus	$4.4 \cdot 10^2$	$U_t = 2.6$
B – berberine hydrochloride	*E. coli*	6.3	$A_t = 0.8$
	S. aureus	6.3	$A_t = 0.8$
D – chitosan	*E. coli*	$3.8 \cdot 10^5$	$A_t = 5.6$
	S. aureus	$3.2 \cdot 10^2$	$A_t = 2.5$

decided that an additive that consistently displayed antibacterial activity in film form, an additive that has not displayed antibacterial activity in film form and the pure PBSA control would be analyzed according to ISO 22196. This method measures the antibacterial activity of treated polymers and textiles. The additives selected for this test were berberine hydrochloride and chitosan.

In accordance with the norm, the film sample sizes were 5 × 5cm and the inoculum volume was 0.4 mL. The results are presented in Table 6.

Equation 1 was used to calculate antibacterial activity (R)

$$R = (U_t - U_0) - (A_t - U_0) = U_t - A_t \quad (1)$$

Where: U_t is the average number of viable bacteria from the control sample after 24 h, U_0 is the average number of viable bacteria from the control sample at 0 h. A_t is the viable number of bacteria on the treated sample after 24 h.

For sample B (berberine hydrochloride), the suspected active natural additive, the calculated R values were R = 4.8 (*E. coli*) and R = 1.8 (*S. aureus*) this corresponds to an observed bacteria reduction of 100% and 98.59%, respectively. For sample D (chitosan), the suspected inactive natural additive, the calculated R values were

R = 0.0 (*E. coli*) and R = *0.1* (*S. aureus*) this corresponds to an observed bacteria reduction of 4.63% and 29.16%, respectively.

DISCUSSION

Some of the proposed additives proved to be effective in terms of antibacterial and biocidal properties, but their level of effectiveness depended on the characteristics of the compound, the form of its application and the research method used. The antibacterial activity of the studied compounds is likely to be mediated through multiple mechanisms that target essential cellular structures and processes. One of the primary modes of action involves the disruption of bacterial cell membrane integrity [35]. By destabilizing the lipid bilayer, these compounds can lead to increased membrane permeability, loss of cellular contents, and eventual cell lysis. Another proposed mechanism is the inhibition of vital biosynthetic pathways, such as protein synthesis or nucleic acid replication. By interfering with ribosomal function or enzymatic processes essential for bacterial growth, the compounds effectively hinder bacterial proliferation. Additionally, oxidative stress induction plays a crucial role in bacterial cell damage. The generation of reactive oxygen species (ROS) can lead to oxidative damage of proteins, lipids, and DNA, triggering apoptotic-like cell death in bacterial populations [35]. These mechanisms may act independently or synergistically, depending on the specific chemical structure and concentration of the tested compounds.

The influence of the characteristics of a given compound is clearly visible in the example of beer hoops, which was the only tested compound to demonstrate antibacterial activity against *S. aureus* in Method 1. Antibacterial activity may be the result of the multi-component nature of this substance (unlike the other compounds), where one of the components demonstrates antibacterial activity against bacteria. The observed effect indirectly confirms the appropriateness of the assumed direction of the planned research, i.e., the development of multi-component antibacterial systems with a broad spectrum of activity.

Research on antibacterial activity assessment methods has significantly evolved over the years, with many studies focusing on optimizing testing procedures to improve reliability and reproducibility. Comparative studies on different methods indicate that no single approach is universally superior; rather, the choice of method should be guided by the specific properties of the tested compounds and the experimental context. This fact was also confirmed in the presented research works.

The differences observed in the results obtained from the four antibacterial activity assessment methods can be attributed to various factors. Each method is based on distinct measurement principles, which influence their sensitivity, specificity, and overall effectiveness in detecting antibacterial properties of the tested compounds. The physical and chemical characteristics of the compounds play a crucial role in determining their behavior under different testing conditions. Factors such as solubility, stability, and interaction with the test medium can significantly affect the outcomes. Additionally, small variations in experimental conditions, including incubation time, temperature, and bacterial strain susceptibility, may contribute to inconsistencies between methods.

A decisive aspect of the effectiveness of the proposed compounds is their biocidal activity characteristics. Despite demonstrating antibacterial activity, the studied compounds exhibit certain limitations that may affect their overall effectiveness. One key limitation is the spectrum of activity, as some compounds may be more effective against specific bacterial strains while showing limited efficacy against others. Additionally, variations in bacterial resistance mechanisms, such as efflux pumps or enzymatic degradation, may reduce the potency of these compounds in certain microbial populations. Environmental factors, such as pH, ionic strength, and the presence of biological macromolecules, can also influence the bioavailability and activity of the tested compounds.

Therefore, numerous research works are undertaken trying to increase the antibacterial effectiveness of natural chemical compounds. Strategies to enhance their antibacterial potential include structural modifications aimed at increasing affinity for bacterial targets, thereby improving efficacy. Optimization of formulation and dosing strategies is also crucial for ensuring maximal therapeutic potential while minimizing toxicity and undesirable side effects. The incorporation of nanotechnology-based delivery systems, such as nanocarriers, can enhance cellular uptake and improve the stability of the compounds within biological environments. Furthermore, the combination of these compounds or combination with other antibacterial agents (synthetic compounds or antibiotics) may result in synergistic effects, reducing the likelihood of resistance development while improving overall antibacterial performance.

This last research direction in particular offers great opportunities for developing new biodegradable polymer materials with biocidal and antibacterial properties. The result of future research will therefore be finding a mixture of several natural substances that will contribute to enhancing the effect and expanding the spectrum of antimicrobial activity. As a result, a new and innovative biodegradable polymer composite with increased effectiveness of antibiological activity will be developed while maintaining the appropriate processing, mechanical and thermal properties of this material, while maintaining its ability to biodegrade in a time that does not burden the natural environment. The use of low concentrations of individual components of the system will reduce the cost of production, and the developed technology can be successfully implemented in the form of a polymer concentrate or the production of finished products, such

as films, containers or packaging, which is of interest to many companies in the polymer and medical industries.

CONCLUSIONS

The effectiveness of antibacterial additives depends on their chemical structure, application form and research method. Mechanisms of action include cell membrane destabilization, biosynthesis inhibition and oxidative stress, which can act independently or synergistically.

Methods for assessing antibacterial activity vary in their effectiveness, and their selection depends on the properties of the substances being tested and the experimental conditions. Limitations related to the narrow scope of action, mechanisms of bacterial resistance and environmental conditions affect the effectiveness of compounds.

Method 3 that utilizes additive loaded polymer films, bacterial suspensions and the plate count agar method is best for preliminary determination of antibacterial activity. Although slightly time-consuming, the method has high repeatability and reliability. Advantages of the method include easy sample preparation, with a small volume of raw materials necessary. This can be particularly beneficial when assessing the antimicrobial activity of expensive additives. This method will be ideal for testing polymeric materials containing single or complex systems of antibacterial compounds and could be used to test active food packaging. The 'success' of the method has been confirmed by Method 4 ISO 22196, a standard norm used in accredited laboratories to measure antibacterial activity of plastics and textiles. Natural additives such as berberine hydrochloride, caffeic acid and beer hops displayed antibacterial and biocidal activity. They should be selected for use as alternative antibacterial additives in further projects, i.e., the development of a new polymer material with a broad antibacterial spectrum containing a mixture of natural chemical compounds. Polymer composites with biocidal and antibacterial properties containing a mixture of these specified compounds will be effective, environmentally safe, and cost-effective.

Authors contribution

L.S. – conceptualization, methodology, investigation, writing-original draft, writing-review, and editing; K.Ł. – conceptualization, methodology, investigation, writing-original draft; A.R.K. – methodology, writing-review, and editing; M.S. – writing-review and editing; K.M. – writing-review and editing, supervision.

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Conflicts of interest

Authors declare no conflict of interests.

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