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Antimicrobial Activity of Starch-based Biodegradable Antimicrobial Films Incorporated with Biosynthesized Silver Nanoparticles Against Multiple Drug-resistant *Staphylococcus aureus* Food Isolates

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Abstract

This study was conducted to determine the antimicrobial activity of starch-based biodegradable antimicrobial films incorporated with biosynthesized silver nanoparticles (Ag-NPs) against multiple drug-resistant *Staphylococcus aureus* food isolates. Herein, the *in-vitro* antimicrobial activities of *Origanum marjorana* (OM) leaf extract, OM essential oil, OM nano-emulsion, chemically synthesized Ag-NPs (chem-Ag-NPs), and OM-based biosynthesized Ag-NPs (bio-Ag-NPs) using OM extracts were determined against a cocktail of three pathogenic *Staphylococcus* (S.) aureus strains isolated from meat products, using the agar well diffusion assay (AWDA). Afterward, homemade starch-based biodegradable antimicrobial films (SBAF) were incorporated with the suitable antimicrobials, based on AWDA and preliminary experiments, and investigated for their antimicrobial properties against *S. aureus* cocktail through the disc diffusion assay (DDA). The obtained results showed that in WDA, bio-Ag-NPs (1mM) had a significantly higher antimicrobial activity than chem-Ag-NPs (1mM), with inhibition zones accounting for 23 and 19mm, respectively. Whereas both types of nanoparticles were significantly more potent in their antimicrobial properties than different concentrations of OM extract, essential oil, and nano-emulsion ($p<0.05$). In concern to SBAF incorporated with antimicrobials, SBAF incorporated with chem-Ag-NPs (SBAF/chem-Ag-NPs) showed a significantly stronger antimicrobial effect than SBAF incorporated with bio-Ag-NPs (SBAF/bio-Ag-NPs) in the DDA, while both types of films produced significantly larger zones of inhibition than other antimicrobials ($p<0.05$). These homemade biodegradable films incorporated with bio-Ag-NPs could be a good alternative to petroleum-based packaging (plastic) in food packaging applications and meanwhile improve food safety and quality. Further studies investigating the effectiveness of these films on bacterial isolates inoculated in real food samples are suggested.

KEYWORDS
Biosynthesized nanoparticles, *Staphylococcus aureus*, Biodegradable packaging

INTRODUCTION

Foodborne illness is a major threat that faces the food sector. It could be induced through foods contaminated with pesticides, heavy metals, parasites, fungi, and viruses. Bacterial food poisoning is the most reported type of foodborne illness. One of the famous pathogens that cause food poisoning is *Staphylococcus* (S.) *aureus* which contaminates food through food contact surfaces, workers' hands, or contact between raw and cooked food. Staphylococcal foodborne intoxication is one of the most common forms of foodborne illness globally, and it is caused by eating foods contaminated with one or more preformed heat-stable enterotoxins (SEs) produced due to growth of *S. aureus* in food (Hennekinne *et al*., 2012; Wang *et al*., 2013). The growth and proliferation of *S. aureus* in foods cause extreme hazards to consumer health as many serotypes can produce enterotoxins (Bennett *et al*., 2013). Misuse and extensively using of antimicrobials lead to the shifting of bacterial pathogens away from easily treated bacteria toward drug-resistant bacteria (Mahdy *et al*, 2012). Antimicrobial resistance (AMR) is one of the world's most serious problems. At least 25% of foodborne isolates have antimicrobial resistance to at least one class of antimicrobials (FAO, 2018). Consumer health protection and foodborne disease prevention need new ways that could protect foods from drug-resistant microorganisms. Additional barriers applied to restrict the growth of foodborne pathogens in foods could give a higher margin of safety for both raw and ready-to-eat (RTE) meals during long-term refrigerated storage (Mangalassary *et al*., 2008). Over the past decade, Because of public awareness and worry about the addition of synthetic chemical additives to foods, researchers and food manufacturers have been interested in using natural antimicrobials (Pattanayaiying *et al*., 2014). Many natural herbs and spices contain antimicrobial components, *Origanum marjorana* (OM) plant and its essential oil are one of them with a wide antimicrobial activity. The antimicrobial and antioxidant properties of *Origanum majorana* are attributed to the presence of many effective compounds such as phenols, terpenoids, esters, etc. Moreover, its essential oil is also being used as a flavoring agent in different types of commercially sold foodstuff. In the

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last years, its antimicrobial effect also has attracted researchers to use it as an antimicrobial agent in packaged food (Bhardwaj and Dubey 2020). Recently, nanotechnology is widely used in different applications in food manufacturing and packaging. The features of the new nanomaterials give many new opportunities to the food industry, which include more potent food flavorings, colorings, nutritional additives, and antimicrobial components for food packaging (Alfadul and Elneshwy 2010). One of the widely used nanomaterials is silver nanoparticles which have a wide range of activity against gram-positive and negative bacteria. Silver metal has been recognized as a very effective antibacterial agent, capable of killing several types of pathogens that cause various food poisoning diseases and has been used by mankind for nearly 7000 years (Chernousova and Epple 2013). Ag-NPs when compared with metallic silver it showed better antimicrobial properties due to their extremely larger surface area which provides a higher contact with the microorganisms (Toker *et al*., 2013). Proved that in *S. aureus*, Ag-NPs inhibit respiratory chain dehydrogenase by converting numerous enzymes such as glycerol-3-phosphate dehydrogenase into dihydroxyacetone, interfering with the organism's normal development and metabolism (Li *et al*., 2010). Biological approaches have been favored to overcome the difficulties of chemical synthesis of Ag-NPs and are thus well investigated as an alternate way for preparing silver nanoparticles; because biologically mediated synthesis does not involve hazardous or toxic chemicals, it has been shown to be cost-effective, easy, and ecologically benign (Katas *et al*,2018). Using green synthesized nanoparticles makes the biopolymer-nanoparticles film or composite material cheap, non-toxic, and eco-friendly (Roopan *et al*., 2013).

Therefore, the current study was carried out to synthesize silver nanoparticles using both chemical and biological methods utilizing marjoram extract as a reducing agent. Then we applied the agar well diffusion assay (AWDA) to *in-vitro* investigates their antimicrobial activities by comparing with OM ethanolic extract, essential oil, and nano-emulsions against a cocktail of three strains of coagulase-positive multidrug-resistant *S. aureus* previously isolated and identified by Saleh *et al*. (2021) from some meat products. Additionally, these antimicrobials were individually incorporated into homemade starch-based antimicrobial films, and then the antimicrobial properties of these films were examined against the same cocktail of *S. aureus* strains using the disc diffusion method.

MATERIALS AND METHODS

Materials

Local markets provided corn starch and dried marjoram leaves (*Origanum marjorana*). Sigma Aldrich (St. Louis, MO, USA) provided 70% ethanol, tri-sodium citrate, silver nitrate, glycerol, bovine skin gelatin, and xanthan gum produced from *Xanthomonas campestris*. Bacterial growth media were acquired from Oxoid, including Muller Hinton agar (MHA), Muller Hinton broth (MHB), buffered peptone water (BPW), tryptic soy broth (TSB), Baird parker's agar, and egg yolk tellurite emulsion (Hampshire, UK)

Bacterial strains

Three separate serovars of pathogenic multiple drug-resistant (MDR) *S. aureus* were employed in this investigation, which were isolated from minced beef and beef-burger from the Microbiology Culture Collection (Department of Food Safety, Faculty of Veterinary Medicine, Beni-Suef University, Egypt), identified and tested for their antibiotic resistance according to Saleh *et al*. (2021). Frozen (-80°C) bacterial strains kept in glycerol were inoculated into tryptic soy broth (TSB) and incubated at 37°C for 18 h, then streak-plated onto tryptic soy agar (TSA) plates for 18 h. Then separate colonies were recovered from TSA plates and inoculated into TSA slopes to be incubated at 37°C for 18 h. Afterward, TSA slopes were stored as stock cultures at 4°C until use in the experiments.

Preparation of Origanum majorana ethanolic extract

The percolation method was used for the preparation of marjoram ethanolic extract (Singh *et al*., 2017). One hundred grams of dried leaf powder of marjoram were mixed with 1000 mL of 70% ethanol in a clean flask with thorough shaking. Then, the herb was kept soaked in ethanol for 24 h and then percolated several times until the soaking solution was faint to clear in color. Afterward, the attained extract was filtered through a Whatman filter paper (no. 1) and concentrated in a rotary evaporator (Shimadzu, Germany) under reduced pressure to get rid of the solvent, and then stored in a dark bottle at 4°C for further use.

Chemical synthesis and biosynthesis of silver nanoparticles (Ag-NPs)

Chemically synthesized silver nanoparticles were created using the chemical reduction method described by Li *et al*. (2010). Furthermore, utilizing marjoram extract, the biosynthesis of silver nanoparticles (Bio-Ag-NPs) was carried out according to Hamelian *et al*. (2018). The generated Ag-NPs were characterized by scanning electron microscopy (SEM) imaging with a Field Emission Electron Microscope (Zeiss Sigma 500 VP Analytical FE-SEM, Carl Zeiss, Germany) (Fig. 1).

Preparation of Origanum marjorana essential oil nano-emulsion

The procedures described by Gahruie *et al*. (2017) were employed to make a marjoram nano-emulsion. The dispersed phase of the *Origanum marjorana* nano-emulsion was made with marjoram essential oil. A magnetic stirrer was used to mix 10 mL of Tween (80 nonionic surfactant) with deionized water for 30 minutes at 800 rpm at room temperature. The solution was mixed with 10 mL of 10% marjoram essential oil, and a coarse emulsion was created using a high-speed homogenizer at 12,000 rpm for 4 minutes.

In-vitro determination of antibacterial activities of different antimicrobials against S. aureus cocktail

S. aureus strains were grown overnight in TSB (37°C) and streaked onto TSA plates separately. After that, separate colonies from each strain plate were collected in test tubes containing 5 mL of sterile saline to achieve a 0.5 McFarland turbidity (1.5×10^8 CFU/mL) for bacterial culture. Then, using equal amounts of the three strains, a bacterial cocktail with an approximate concentration of 8 $\log_{10}$ CFU/mL was generated.

The antimicrobial activity of various antimicrobials developed in this study, including OM ethanolic extract, chemically synthesized (Chem-Ag-NPs), biosynthesized (Bio-Ag-NPs) silver nanoparticles utilizing OM extract, OM essential oil, and OM nano-emulsion, was assessed using the AWDA described by Jahangirian *et al*. (2013) against *S. aureus* cocktail. Under aseptic conditions, the bacterial cocktail was swabbed on the surface of

MHA plates, and 6 mm diameter wells were made and filled with 50 µl of different doses of each antibiotic, which were incubated for 24 h at 37°C. The diameter of the growth inhibition zones was evaluated after the incubation period. The inhibitory zones were measured in millimeters (mm) and reported.

Development of homemade starch-based biodegradable antimicrobial films (SBAF)

To generate homemade starch-based biodegradable control films (SBCF) and biodegradable antimicrobial films (SBAF) containing antimicrobials, 100 mL of sterile distilled water was mixed with 12.0±3.0 g corn starch, 1–3 g gelatin, 0.05–0.5 g xanthan gum, and 2–5 mL glycerol. Starch was added to distilled water with stirring using the hot plate stirrer. The mixture was then heated, and then gelatin was added with constant vigorous stirring before the xanthan gum was added to stabilize it. The temperature of the mixture was then raised to 150°C, with steady stirring, until all the ingredients had dissolved. Finally, glycerol was added to the mixture in a drop-wise manner to plasticize the biopolymer. The mixture was then autoclaved for 15 minutes at 121°C before being allowed to cool to 55°C. To make various SBAFs, the antimicrobial solutions were filter-sterilized (0.45 µm) before being added individually to the biopolymer solution. SBCF was made without the use of any antimicrobial agents from starch-based biopolymer solutions. The mixture was then spread out to a thickness of nearly 200 mm and allowed to dry for 24 to 48 h at 45 percent relative humidity. All films were maintained at 25°C in an aseptic environment until they were used in experiments.

The bactericidal activity of the SBAF was tested against a *S. aureus* cocktail using a DDA. SBAF and SBCF were aseptically cut into rounded discs (about 6 mm in diameter) and applied to the surface of MHA plates that had been streaked with the *S. aureus* bacterial cocktail and prepared in sterile saline as described previously. After 24 h of incubation at 37°C, the inhibition zones of all bacteria-inoculated MHA plates were observed. A ruler was used to measure the diameters of the inhibitory zones.

Statistical analysis

In this study, each experiment was repeated three times. To assess significant differences and comparisons between means, a one-way ANOVA test was utilized (Minitab 18 statistical software). If P < 0.05, the means were considered significantly different.

RESULTS AND DISCUSSION

Characterization of the Ag-NPs and biodegradable films by SEM analysis

SEM imaging was used to characterize the silver nanoparticles in this study (Fig. 1). Chem-Ag-NPs with a concentration of 1 mM had a diameter of 36.0±2.4 nm and were spherical. Dey *et al*. (2015) reported silver nanoparticles produced chemically with identical results. Bio-Ag-NPs synthesized at a concentration of 1 mM, were spherical with a diameter of 51.5±2.0 nm. The increased size of Bio-Ag-NPs compared to Chem-Ag-NPs could be explained by the Marjoram extract coating layer that covers the biosynthesized nanoparticles.

Additionally, the surfaces of homemade starch-based biodegradable films both control and those incorporated with Ag-NPs were observed using scanning electron microscopy (Fig. 2). It was noticeable that the surface shape was different in the control film (Fig. 2A) in comparison with antimicrobial films (Fig. 2B and 2C), as well as the surface morphology appeared homogenous. The nanoparticles incorporated in the antimicrobial films were not noticeable under this scale (1 µm).

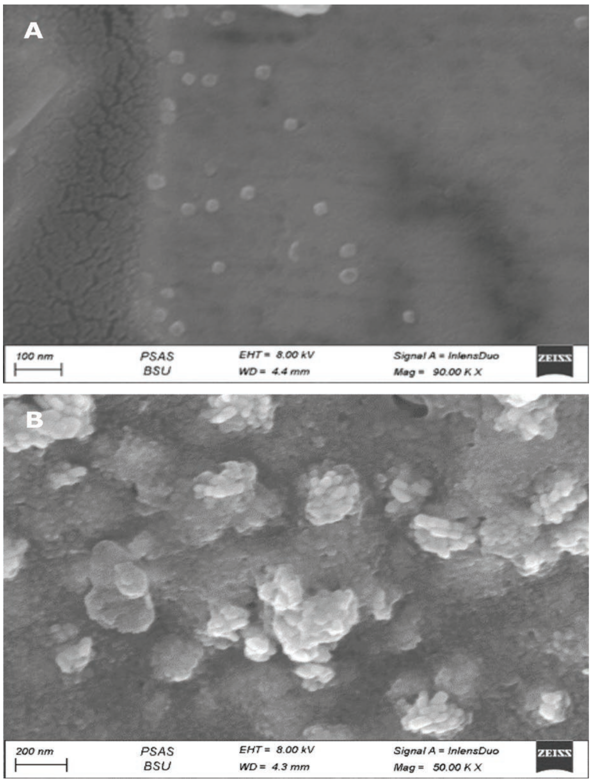

Fig. 1. SEM images of (A) chemically synthesized silver nanoparticles Chem-Ag-NPs (1 mM) and (B) biosynthesized silver nanoparticles Bio-Ag-NPs (1 mM).

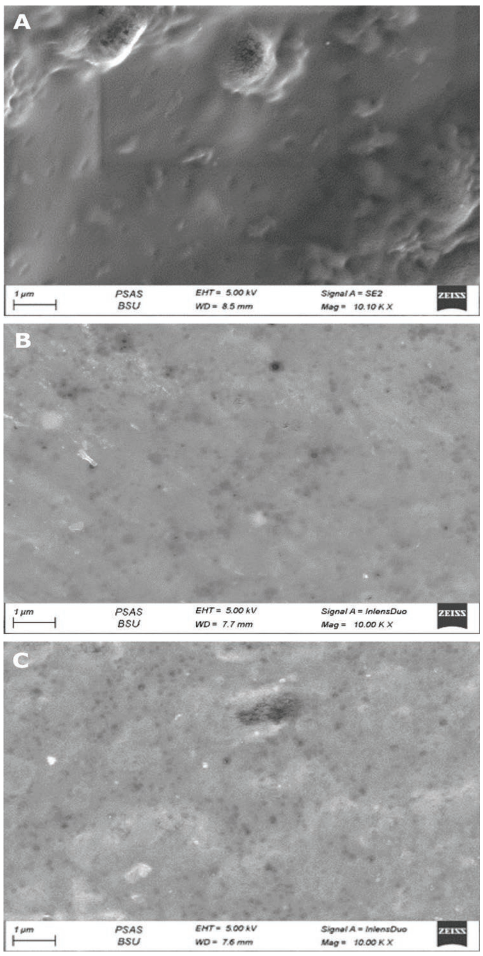

Fig. 2. SEM images of (A) homemade starch-based biodegradable control films (SBCF) and homemade starch-based biodegradable antimicrobial films incorporated with (B) Chem-Ag-NPs or (C) Bio-Ag-NPs.

The antimicrobial activity of the different used antimicrobials determined by AWDA

The AWDA is a widely used method for the detection of the antimicrobial activity of different antimicrobials. The results of the antimicrobial effect of different antimicrobials against a coagulase-positive *S. aureus* strain cocktail using AWDA are presented in Fig. 3. These strains were found MDR with MDR ratios of 0.83, 1, and 1 (Saleh *et al*., 2021).

The OM nano-emulsion 2, 5, and 10 % achieved 7, 7, and 7.6 mm of inhibition zone diameter, respectively, while the diameter of the zones created by OM crude essential oil 2, 5, 10 %, and pure oil was only 6 mm ($p<0.05$). Regarding marjoram ethanolic extract 2, 5, and 10 %, the inhibition zone diameters were 6, 6, and 7 mm, respectively. On the other hand, Chem-Ag-NPs 1 mM and Bio-Ag-NPs 1 mM achieved inhibition zone diameters of about 19 and 23 mm, respectively with a significant difference in between ($p<0.05$). Accordingly, Bio-Ag-NPs followed by Chem-Ag-NPs were the most effective antimicrobials against the *S. aureus* cocktail. Franci *et al*. (2015) proposed that because of their small size and wide surface area, Ag-NPs are well-known for having excellent antibacterial action against different pathogens such as bacteria, fungi, and viruses. Despite the lack of data on nanoparticles in vivo toxicity, Ag-NPs antibacterial properties have made it one of the nanotechnology industry's fastest-growing product categories in the last decade (Liu *et al*., 2017). According to various studies Ag-NPs can accumulate in the bacterial membrane, which make the integrity of bacteria's membranes compromised, leading to cellular death (Istiqola and Syafiuddin 2020). The obtained results confirm the synergistic effect of the marjoram extract and the silver ions in the case of Bio-Ag-NPs. Comparing these results with those reported by Qais *et al*. (2019) who prepared Ag-NPs using *Murraya koenigii* extract and reported inhibition zones of about 21 mm in diameter, the prepared Ag-NPs by marjoram in the present study had a stronger antimicrobial activity, as well as gave a stronger antimicrobial effect than the biosynthesized Ag-NPs using *Trigonella foenum graecum* extract as a capping and reducing agent prepared by Rajkumar and Patra (2019) which achieved a 9.34 mm diameter of inhibition zone. Also, Ontong *et al*. (2019) recorded inhibition zones of about 14.12 mm when prepared Ag-NPs using *Senna alata* bark extract.

Our chemically prepared Ag-NPs using tri-sodium citrate showed stronger antimicrobial activity than the chemically prepared ones by Guzman *et al*., (2012) using a mixture of hydrazine hydrate and sodium citrate (1 mM), which displayed a 12 mm diameter of inhibition zone against *S. aureus*.

Despite it was recognized that the OM essential oils are toxic to harmful fungi like *Aspergillus flavus* and have antimicrobial activity against antibiotic-resistant bacteria like methicillin-resistant *S. aureus* (Liu *et al*., 2017), this investigation did not demonstrate enough antibacterial action against MDR *S. aureus*, which could be attributed to the differences in oil sources and purities. Furthermore, it was reported that OM contains bioactive compounds, reveals an important biological activity, and is used in the medicinal and food industries, as well as the nanoparticles synthesized from it may be a bioactive material for medical and food applications (Erenler and Dag, 2022). In the current study, the ethanolic extract of OM was applied at various concentrations as a potential antibacterial agent, however, their efficacy was significantly lower than bio-Ag-NPs and chem-Ag-NPs ($p<0.05$).

Additionally, in the present study, the effects of different concentrations of OM nano-emulsion and OM crude essential oil were weaker than those previously reported by Amor *et al*. (2019), which could be due to the differences in oil sources and/or bacterial species, isolate resistance, and inoculum concentrations.

The antimicrobial activity of the different developed films through DDA.

The antibacterial activity of various SBAFs and SBCF was assessed by a DDA in order to evaluate the capability of each antimicrobial to diffuse from the biodegradable film to the media

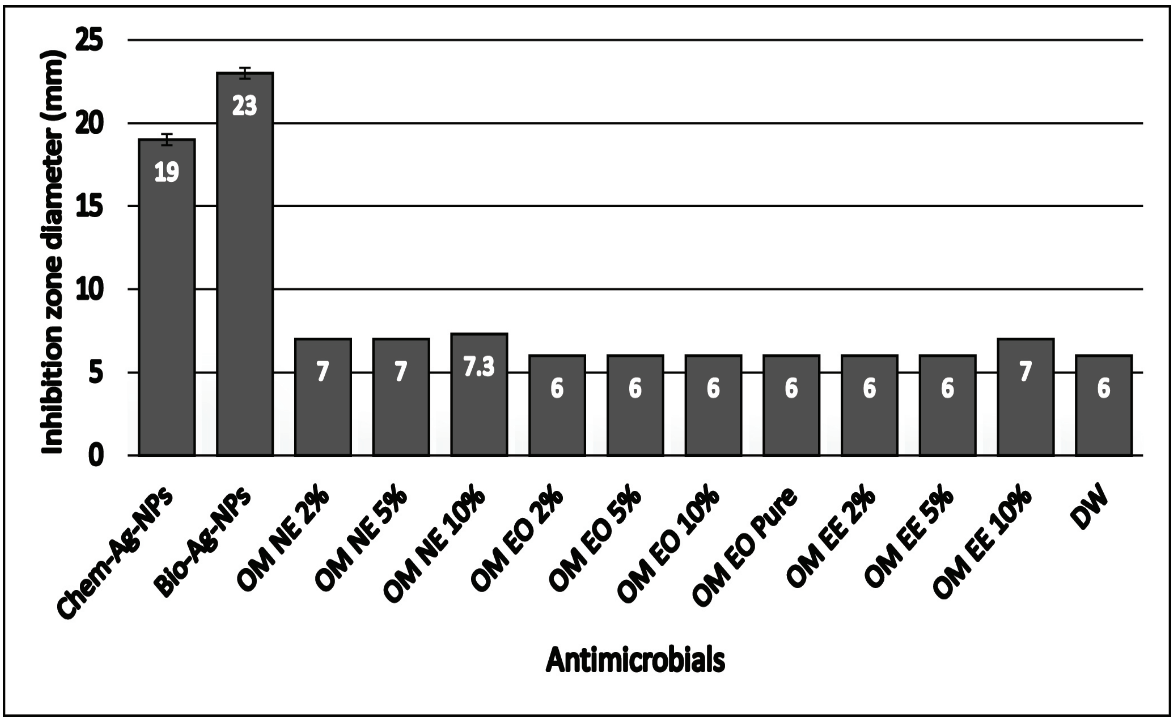

Fig. 3. The antimicrobial activity of various antimicrobial compounds, represented by the diameter of inhibition zones (mm) ± standard error, against *S. aureus* bacterial cocktail isolated from meat products, in an agar well diffusion assay (AWDA). Where, Chem-Ag-NPs= chemically synthesized silver nanoparticles, Bio-Ag-NPs= Biosynthesized silver nanoparticles, OM NE= *Origanum marjorana* nano-emulsion, OM EO= *Origanum marjorana* crude essential oil, OM EE= *Origanum marjorana* ethanolic extract, DW= sterile distilled water. Different small letters (a, b, c and d) above bars indicate significant differences between means at $p < 0.05$.

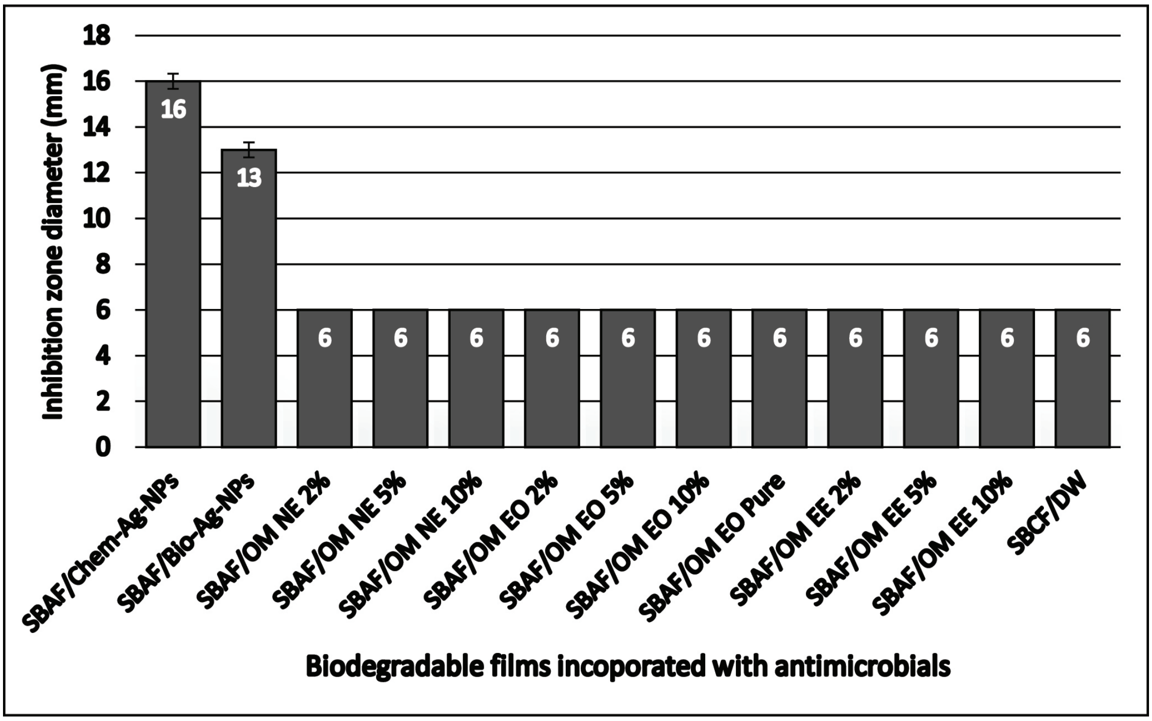

Fig. 4. The antimicrobial activity of homemade starch-based biodegradable control films (SBCF) and different starch-based biodegradable antimicrobial films (SBAF) incorporated with various antimicrobials, represented by the diameter of inhibition zones (mm) ± standard error, against *S. aureus* bacterial cocktail isolated from meat products, in a disc diffusion assay (DDA). Where, Chem-Ag-NPs= chemically synthesized silver nanoparticles, Bio-Ag-NPs= Biosynthesized silver nanoparticles, OM NE= *Origanum marjorana* nano-emulsion, OM EO= *Origanum marjorana* crude essential oil, OM EE= *Origanum marjorana* ethanolic extract, DW= sterile distilled water. Different small letters (a, b, c and d) above bars indicate significant differences between means at $p < 0.05$.

(Fig. 4). In this experiment, the films were cut into discs measuring about 6 mm in diameter and tested on MHA plates inoculated with a pathogenic three-strain *S. aureus* cocktail. It was noticeable that the DDA results matched that of the AWDA results with minor differences.

SBAF/Chem-Ag-NPs and SBAF/Bio-Ag-NPs had the largest inhibition zones against a cocktail of pathogenic *S. aureus* strains. The inhibitory zones produced by SBAF/Chem-Ag-NPs were larger than those produced by SBAF/Bio-Ag-NPs, which were 16 and 13 mm, respectively (Fig. 4). This could be due to Bio-Ag-NPs having a slower diffusion capacity from the biodegradable film to the agar than Chem-Ag-NPs. Ag-NPs have previously been shown to have antibacterial properties against pathogenic *S. aureus* when mixed into a hydroxypropyl methylcellulose matrix with used as food packaging materials (De Moura *et al*., 2012).

In this regard, many studies investigated the antibacterial activity of food packaging films containing various antimicrobials in vitro, such as Millette *et al*. (2007) who determined the antimicrobial effect of nisin-containing alginate films against a *S. aureus* strain with a clear inhibition zone was formed. Also, Hassan and Cutter (2020) examined antimicrobial films incorporated with lauric arginate and found that they had an effective inhibition against *S. aureus* cocktail. Additionally, Marzlan *et al*. (2022) recorded the antimicrobial effect of starch-based edible films when incorporated with torch ginger (*Etlingera elatior Jack)* inflorescence essential oil against *S. aureus*. Moreover, Kuorwel *et al*. (2011) reported the antimicrobial activities of starch-based film coated with carvacrol, linalool, and thymol against *S. aureus*.

In this concern, because of their antibacterial capabilities, Ag-NPs have been widely used in food packaging technology among existing inorganic nanomaterials. Ag-NPs-coated food packaging has been tried for a variety of items, including fresh meats, fresh fruits, and consumer products, especially because of the previous suggestions of Ag-NPs as safe for food packaging, with undetectable levels of silver nanoparticles that are released and migrated from impregnated containers into real food samples (Addo Ntim *et al*., 2015).

CONCLUSION

To conclude, bio-Ag-NPs has a significantly higher antimicrobial activity against *S. aureus* than chem-Ag-NPs. Furthermore, both types of nanoparticles are significantly more powerful in their antimicrobial properties than different concentrations of OM extract, essential oil, and nano-emulsion ($p<0.05$). On the other hand, SBAF incorporated with chem-Ag-NPs show a significantly stronger antimicrobial effect than that incorporated with bio-Ag-NPs, while both types of films have significantly stronger antimicrobial effects than other antimicrobials ($p<0.05$). These homemade biodegradable films incorporated with bio-Ag-NPs could be a good alternative to plastics in food packaging applications and meanwhile, reduce microbial growth and extend the shelf-life of food. Further studies for investigating the effectiveness of these films on bacterial isolates artificially inoculated in real food samples are suggested, as well as the safety of biosynthesized Ag-NPs in food packaging applications must be furtherly investigated.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in this work.

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