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Antimicrobial Activity of Nanomaterial and Essential Oil against Foodborne Pathogen on Fish Fillets during Cryopreservation

Wael A.Saleh[1*], Ashraf M. Sharoba[2], Atef S. Osheba and[1] Ahmed E. El-Desouky[2]

[1]Meat and Fish Technology Research Dept., Food Technology Research Institute, Agricultural Research Center, Giza, Egypt.
[2]Food Technology Dept., Faculty of Agriculture, Benha University, Egypt.

THE antibacterial activity of nanoparticles and essential oil were tested against *S. typhimurium, E. coli, S. aureus* and *B.subtilis* using agar disc diffusion assays. Essential oil and nanoparticles inhibited all tested microorganisms with different concentration against *S. aureus, B. subtilis, S. typhimurium* and *E. coli* respectively. Silver and chitosan nanoparticles showed a lower inhibition for all of the tested microorganisms compared to essential oil. The present study characterized of AgNPs (~16.77nm) and ChNPs (~20.25nm) using (TEM) and GC/MS to measure chemical components of cumin and marjoram essential oils. Gamma.-Terpinene accounts for 25.54 % of total chemical components in cumin essential oil and Bis (2-ethylhexyl) phthalate accounts for 24.48 % of total chemical components in marjoram essential oil. Fish fillets inoculated were analyzed for *B. cereus* and *E. coli* during cryopreservation. Results demonstrate that cumin essential oil at 500 ppm is more active than marjoram essential oil at 500 ppm, ChNPs 1% and AgNPs 40µg/ml respectively when compared to the control. These results revealed by increased storage time were decreased microbial growth in fish fillets inoculated with *B.cereus* were more active than fish fillets inoculated with *E. coli*.Therefore, the application of nanoparticles and essential oil has a good potential to be antimicrobial activities for various food applications.

Keywords: Antimicrobial, Nanomaterial, Fish fillets.

Introduction

Every year, over two million people worldwide die from food borne illnesses, the majority of whom are children (WHO, 2010). The center for sickness control and prevention estimates that each year in the USA, contaminated foods cause about 48 million cases of disease and 3000 fatalities. In more severe cases, hemorrhagic colitis, septicemia, meningitis, articular infection, kidney failure, paralysis, miscarriage, along with diarrhoea, nausea, vomiting, abdominal pain, fever, and headache (Scallan et al., 2011).

The composition of fish meat creates a favorable situation for the growth and spread of spoilage microorganisms and common pathogens in food; therefore, spoilage of fish that occurs during storage is mainly due to microbial activity (Nair et al., 2020). Thus, it is necessary to take some measures to delay the deterioration of fish quality and prolong the shelf life of fish by inhibiting or slowing the growth of microorganisms.Fish and other seafood are extremely perishable food products and are especially susceptible to both chemical and microbiological spoilage during processing or storage. For this reason, one or more adequate preservation methods are required in order to maintain the safety and quality and extend the shelf life of such products (Ghanbari et al., 2013; Hassoun & Karoui, 2017). Fish is one of the most perishable food products, and therefore, quality deterioration of fresh fish occurs rapidly during handling and storage and limits the shelf life of

*Corresponding author : ph_wael2006@yahoo.com
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the product (Adebayo-Tayo et al., 2012). Spoilage of fish results from changes brought about by biological reactions such as oxidation of lipids, the activities of the fish muscular enzymes, and the metabolic activities of microorganisms. Due to modern trends consumers adopt towards the consumption of minimally processed foodstuffs containing no chemical preservatives, lightly preserved food products with natural additives have become popular (Khalafalla et al., 2015).

The shorter shelf-life (3–5 days) of fish and fishery products is a matter of concern due to cellular breakdown by endogenous and exogenous (phosphorylase, lipases, phospholipases, lipoxygenases, hydroperoxidases, proteases) enzymes, and also, microorganisms from both the Gram-positive and -negative class (*Shewanella, Pseudomonas, Photobacterium, Aeromonas, Bacillus, Brochothrix, Carnobacterium, Escherichia, Enterobacter, Listeria, Micrococcus, Moraxella, Proteus, Psychrobacter,* and *Vibrio*) cause degradation, (Rathod et al., 2021; Zhuang et al., 2021).

Fish is perishable during cold storage due to enzymatic and microbiological activities, and hence, innovative preservation techniques have to be applied to maintain its quality and supply for human consumption (Broekaert et al., 2011). In light of a greater emphasis on health and nutrition, natural food preservatives have been gaining much attention to extend the shelf life of food products. For instance, chitosan, soy isoflavones and various plant essential oils have been found to display antimicrobial effects and applied in the edible coatings (Dhayakaran et al.,2015;Feng et al., 2016). This important incidence of foodborne threat associated with the new social and economic implications leads to an urgent need for safer food through the development of new and nontoxic preservative agents with important antimicrobial and antioxidant properties. With this respect, the actual synthetic chemicals, commonly used to control pathogen strain, raise serious preoccupations related to human health (Falleh et al., 2019).

Nanotechnology represents an important tool and an efficient option for extending the shelf life of foods (Zambrano-Zaragoza et al., 2018). Silver nanoparticles show both unique physiochemical properties and remarkable antimicrobial activities which confer to them a major advantage for the development of alternative factors against microorganisms including multidrug-resistant bacteria, and hence silver nanoparticles are supposed to be the new generation of antimicrobials (Alavi & Rai, 2019). Chitosan and chitin have been used as building blocks in nanotechnology applications. Further more, because it binds the negatively charged residues of the bacterial cell wall, chitosan has significant antimicrobial properties. The electrostatic force between positively charged chitosan promotes a closer interaction with the negatively charged bacteria cell wall, resulting in drug penetration through the bacteria cell wall. This is due to the fact that the bacterial cell wall is composed of a layer of peptidoglycan that is rich in negatively charged carboxyl and amino groups Cava et al., (2011).

Several essential oils have antimicrobial properties and can act as biopreservers, reducing or eliminating pathogenic bacteria and increasing the microbiological quality of food products of animal and plant origin, Angane et al., (2022). Marjoram (*Origanummajorana L.*), known as an herbaceous and perennial plant, is widely used in the food, cosmetics and pharmaceuticals industries. The essential oil extracted from marjoram (MEO) has great health properties as antioxidant, antimicrobial, anticancer and anti-inflammatory due to the presence of polyphenolic compounds like terpinen-4-ol, cis-sabinene hydrate, α- and γ-terpinene, terpinolene, thymol and carvacrol(Almasi et al., 2020). Cumin (*Cuminumcyminum*) contains significant amounts of tocopherol and bioactive compounds that possess antioxidant properties (Jafarzadeh et al., 2020). Functional activities of cumin oils have been widely studied, including antimicrobial (Allahghadri et al., 2010), and there have been no studies regarding effects of essential oils and nanoparticles as natural antimicrobials against food-born pathogenic bacteria including *E. coli* and *B. cereus* in fish.

Accordingly, the present study was conducted to determine the effect of silver and chitosan nanoparticles and cumin and marjoram essential oils at 500 ppm individually and in combination to control E. coli and B. cereus in fish fillets and to ensure its microbiological safety and also to decrease the hazards of the presence of these pathogenic bacteria.

Materials and Methods

Material

Raw material

Fresh Nile tilapia (*Oreochromis niloticus*) fish were purchased from a local market, Giza, Egypt, and rapidly transported in iceboxes to the laboratory of Food Technology Research Institute, Agriculture Research Center, Giza, Egypt. Cumin (*Cuminum cyminum L.*) and marjoram (*Origanum marjorana*) essential oils were obtained from (PHATRADE) Company, Obour City, Cairo, Egypt.

Chemicals

Silver nitrate (99.8%) was obtained from Sigma Aldrich Company, Germany. Chitosan powder was obtained from ROTH Company, Germany. Sodium hydroxide was obtained from RANKEM Company, New Delhi, India. Food grade sodium tripolyphosphate (99.5% purity) was obtained from El-Gomhoria for chemicals Co., Egypt. Maize starch was supplied from Egyptian Starch and Glucose Company, Cairo, Egypt.

Bacterial strains

Bacterial strains were obtained from Cairo Microbiological Resources Center (Cairo MIRCEN), Faculty of Agriculture, Ain Shams University, Egypt. The test microorganisms were *E.coli* ATCC 25922,*B.cereus* ATCC 33221, *S.aureus* ATCC 12600, *S.typhimurium* ATCC14028 were cultivated twice on Tryptic Soy Agar at 37°C for 24 h, and keptat 4°C till using.

Microbiological media

Nutrient agar medium, trypticsoy agar medium, violet red bile agar medium (VRBA) and mannitolegg yolk polymyx in Agar medium (MYP) and *B. cereus* selective supplement was obtained from Biolife Company, Italy and the agent in Egypt Al-Badr Engineering Company.

Methods:

Fishfillets preparation and inoculation and treatment application

Fresh Nile tilapia (*Oreochromis niloticus*) fish with an average weight of 400g each were purchased from a local market, Giza, Egypt, and rapidly transported in iceboxes to the laboratory of Food Technology Research Institute, Agriculture Research Center, Giza, Egypt. Upon arrival to the laboratory, each sample was gutted, cleaned, and filleted into two pieces of about 100 g weight for each piece and rewashed with clean water sections under sterilized conditions. The standard methods of (AOAC, 2005), and the International Commission for the Microbiological Specifications of Foods (Tompkin, 2002) were adopted for preparation and microbiological analyses of seafood samples.

Then two strains of *E.coli* and *B. cereus* approximately 6 $\log_{10}$ CFU/g were inoculated using micropipettes on each side of fish fillet pieces to gain a final concentration of 4 $\log_{10}$ CFU/g. Next, samples were dipped in solutions of ChNPs 1% and AgNPs 40.0µg/ml (w/g) water for 1 min to enable attachment (ratio of 1:3) (Al-Hajj et al., 2017), and cumin and marjoram essential oil were added to fish fillets samples appropriate volumes to the surface (two sides) of each fish fillet using micropipette so as to achieve at 500 ppm oil volume per fish flesh weight (v/w) respectively, Goulas & Kontominas (2007) (Table 1), After that, all fish fillets samples were packaged in a foam plates, wrapped with polyethylene and stored at 4±1C up to 12 days. The samples were taken for analysis every 3 days periodically.

TABLE 1. Experimental treatments.

Treatments	Explanation
GA1	Fish fillet sample control with out anything.
GA2	Fish fillet sample inoculated by *E. coli.*
GA3	Fish fillet sample inoculated by *E. coli* with ChNPs 1%.
GA4	Fish fillet sample inoculated by *E. coli* with AgNPs 40.0µg/ml.
GA5	Fish fillet sample inoculated by *E. coli* with cumin essential oilat 500 ppm.
GA6	Fish fillet sample inoculated by *E. coli* with marjoram essential oilat 500 ppm.
GB1	Fish fillet sample inoculated by *B. cereus.*
GB2	Fish fillet sample inoculated by *B. cereus* with ChNPs 1%.
GB3	Fish fillet sample inoculated by *B. cereus* with AgNPs 40.0µg/ml.
GB4	Fish fillet sample inoculated by *B. cereus* with cumin essential oilat 500 ppm.
GB5	Fish fillet sample inoculated by *B. cereus* with marjoram essential oilat 500 ppm.

Transmission electron microscopeof the nanoparticles

Transmission electron microscope characterization is performed using (JEOL, JEM-1230, Japan) instrument with an acceleration voltage of 120 kV. For the TEM measurements, a drop of solution containing nanoparticles are deposited on carbon coated copper grid. After allowing the film to stand for 5 min. the extra drops were removed by means of blotting paper and the grid allows drying before the measurements, Hebeish et al. (2016).

Gas chromatography–mass spectrometry analysis (GC-MS)

The GC-MS system (Agilent Technologies) was equipped with gas chromatograph (7890B) and mass spectrometer detector (5977A) at Central Laboratories Network, National Research Centre, Cairo, Egypt (Abd El-Motaleb et al., 2021). Samples were diluted with hexane (1:19, v/v). The GC was equipped with HP-5MS column (30 m x 0.25 mm internal diameter and 0.25 µm film thickness). Analyses were carried out using helium as the carrier gas at a flow rate of 1.0 mL/min at a split ratio of 1:10, injection volume of 1 µL and the following temperature program: 40 °C for 1 min; rising at 4 °C /min to 150 °C and held for 6 min; rising at 4°C/min to 210 °C and held for 5 min. The injector and detector were held at 280°C and 220 °C, respectively. Mass spectra were obtained by electron ionization (EI) at 70 eV; using a spectral range of m/z 40-550 and solvent delay 3 min. Identification of different constituents was determined by comparing the spectrum fragmentation pattern with those stored in Wiley and NIST Mass Spectral Library data.

Microbiologicalexaminations

Antimicrobial activity of nanoparticles (well diffusion method)

The effect of different concentrations of nanoparticles (25, 50, 75 and 100 ppm) on bacteria growth was studied by using well diffusion method, according to (Hulikere & Joshi, 2019) by measuring the diameter of inhibition zone.

Antimicrobial activity of essential oils (disc diffusion method):

The effect of different concentrations of essential oils (1, 5, 10, 50 and 100%) on bacteria growth was studied using the paper-disc plate method, according to Hassanen et al. (2015) by measuring the diameter of inhibition zone.

Total bacterial count:

Ten grams of each sample was added to a culture medium (1: 10^{-1} to 1: 10^{-6} and homogenized for 2 min in a stomacher), Total bacterial count was determined using nutrient agar medium. Incubation was carried out at 37°C for 48 hrs. The counts were then calculated per gram of samples as reported by the methodology of ISO 4833-1, (2013).

*Bacillus cereus*count:

B. cereus was determined using Mannitol Egg Yolk Polymyxin Agar and *B. cereus* selective supplement. Incubate plates 18-24 h at 30°C and observe colonies surrounded by precipitate zone, which indicates that lecithinase is produced. B. cereus colonies are usually pink on MYP and may become more intense after additional incubation. If reactions are not clear, incubate plates for an additional 24 h before counting colonies, as reported by the methodology of FDA (2021).

Escherichia coli count

E. coli was determined using violet red bile glucose agar. Incubation was carried out at 37°C for 48 hrs. The counts were then calculated per gram of samples as reported by the methodology of ISO 21528-2 (2017).

Statistical analysis

The obtained results were analyzed using analysis of variance (ANOVA) and least significant different (LSD) at the 5% level of probability; as reported by Snedecor & Cochran (1994).

Results and Discussion

In recent years, there has been an increase in the hunt for innovative methods of preserving seafood that would guarantee its quality and safety. Numerous natural preservatives have drawn attention from the scientific community, consumers, business, and health sectors as broad actionantibacterial and cost-effective technique. Natural preservatives have been used frequently, and they all have a great deal of promise for usage in seafood systems. Examples include chitosan and essential oils on other hand silver metal, with a focus on maintaining quality and ensuring food safety.The current study focuses on the natural preservatives studied in seafood, with inoculated by *B. cereus* and *E. coli*.

Transmission Electron Microscope (TEM) of silver and chitosannanoparticles

Transmission electron microscope imaging

showed the morphological properties and surface appearance of AgNPs which have a nearly spherical shape and smooth surface. As illustrated in Fig. 1, it was observed that the prepared nanoparticles were an average particle size of 9.04 - 24.5 nm. Furthermore, these nanoparticles are well dispersed with no sign of aggregation. Transmission electron microscope was used for the determination of the particle size and the morphological structure of the prepared polymer matrix. It was found that chitosan/TPP has average particle size of 16.8 - 23.7 nm. Fig. (2) shows the scanning electron microscopy of chitosan nanoparticles.

GC/MS characterization of cumin and marjoram essential oils

The components of cumin and marjoram essential oils were identified by GC/MS spectrometer analysis. Their percentage compositions, calculated as the ratio of peak area to the total chromatographic area, were listed in Table 2, it could be noticed that fifteen volatile components were fractionated and identified from cumin volatile oil. The identified components represented (100%) from the cumin volatile oil. Gamma.-Terpinene, Benzaldehyde, 4-(1-methylethyl), Beta.-Pinene, O-Cymene, 1,4-P-Menthadien-7-al, 4-Isopropylcyclohexa-1,3-dienecarbaldehyde and Benzonitrile, 2-(4-methylphenyl) were the most abundant chemical compounds in cumin volatile oil which represented 995.26%) of the total identified chemical compounds. Gamma.-Terpinene (25.54%) of the total chemical compounds was the highest chemical compound in cumin volatile oil. However, I-Benzonitrile, 2-(4-methylphenyl) (7.23%) of the total chemical compounds was the lowest one among the most abundant chemical compounds in cumin volatile oil. These results are in agreement with Vieira et al. (2019). These results are on line with those obtained by Ashokkumar et al. (2021). On the other hand in Table 2, it could be noticed that twenty volatile components were isolated and identified from marjoram essential oil. The identified components represented (99.70%) from marjoram essential oil. Bis (2-ethylhexyl) phthalate, Estragole, Caryophyllene, 3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl) and Bicyclo [3.1.0] hexane, 4-methylene-1-(1 methylethyl) were the most abundant chemical compounds in marjoram essential oil which represented (69.89%) of the total identified chemical compounds. Bis (2-ethylhexyl) phthalate (24.48%) of the total chemical compounds was the highest chemical compound in marjoram essential oil. However, Bicyclo [3.1.0] hexane, 4-methylene-1-(1methylethyl) (8.67%) of the total chemical compounds was the lowest one among the most abundant chemical compounds in marjoram essential oil. These results are in agreement withElkousy et al. (2022).

Antimicrobial activity of silver and chitosan nanoparticles

Antimicrobial activity of silver (AgNPs) and chitosan (ChNPs) nanoparticles at different concentrations (25, 50, 75 and 100 ppm) against microorganisms strains, expressed as the diameters of inhibition zones (mm) are presented in Table 3, from these results it could be noticed that the effect of AgNPs on the growth of studied microorganisms was presented in Table 3. AgNPs showed various degrees of inhibition against the tested microorganisms. For the antibacterial investigation, gram-negative bacteria the inhibition zones of AgNPs were ranged from

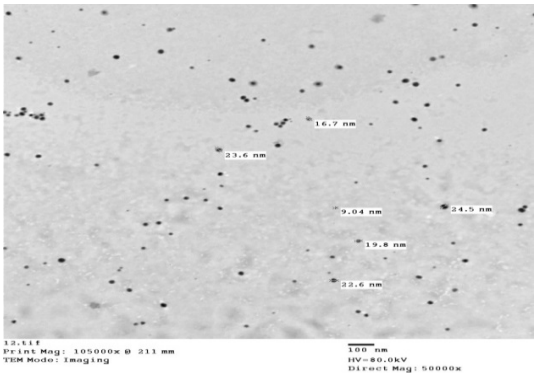

Fig. 1. Transmission electron microscopy micrograph of AgNPs

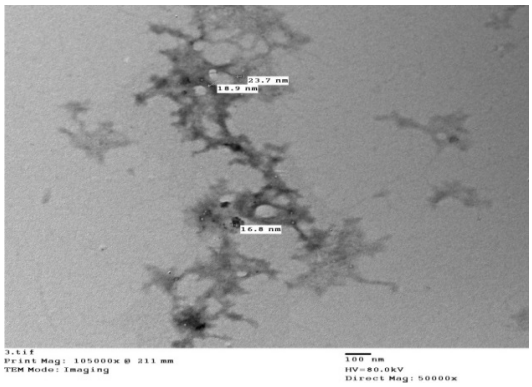

Fig. 2. Transmission electron microscopy micrograph of ChNPs

5.5 to 21.5 mm. The highest inhibition zone was obtained for *S. typhimurium* (21.5 mm), while the lowest (17.0 mm) was recorded for *E. coli*. On the other hand AgNPs showed higher antibacterial activity against gram-positive (*S. aureus and B. subtilis*) inhibition zones of AgNPs were ranged from 6.00 to 22.5 mm. The highest inhibition zone was obtained for *S. aureus*. Generally, AgNPs showed higher antibacterial activity against gram-positive bacteria (*S. aureus and B. subtilis*) than gram-negative bacteria (*E coli and S. typhimurium*). This high bactericidal activity of AgNPs is certainly due to the silver cations released from AgNPs that act as reservoirs for the Ag+ bactericidal agent (Dakal et al., 2016). These results are in agreement with those observed by Hulikere & Joshi (2019).

TABLE 2. Essential volatile components of cumin and marjoram essential oils (%) identified by GC/MS.

Compounds	Cumin essential oil		Marjoram essential oil	
	RT	Area %	RT	Area %
1-Adamantanemethylamine, .alpha.-methyl	7.995	0.29	-	-
Alpha.-pinene	8.181	0.56	-	-
Alpha.-Phellandrene	9.528	0.35	-	-
Beta.-Pinene	9.609	11.47	9.474	1.15
Beta.-Myrcene	10.181	0.73	10.04	0.56
Alpha.-Phellandrene	10.589	0.83	-	-
O-Cymene	11.311	10.8	-	-
Cyclohexane, 1-methylene-4-(1-methylethenyl)	11.451	0.84	-	-
Gamma.-Terpinene	12.553	25.54	12.488	0.88
Terpinen-4-ol	16.802	0.29	-	-
3-P-Menthen-7-al	17.396	0.85	-	-
Benzaldehyde, 4-(1-methylethyl)	18.976	21.9	-	-
4-Isopropylcyclohexa-1,3-dienecarbaldehyde	20.486	8.52	-	-
1,4-P-Menthadien-7-al	20.719	9.8	-	-
Benzonitrile, 2-(4-methylphenyl)	37.529	7.23	-	-
Bicyclo[3.1.0]hexane, 4-methylene-1-(1 methylethyl)	-	-	9.416	8.67
I-Phellandrene	-	-	10.439	0.7
Alpha. terpinene	-	-	10.926	1.2
D-Limonene	-	-	11.328	2.08
Eucalyptol	-	-	11.386	3.82
Alpha.-terpinolene	-	-	13.479	0.55
Cis-sabinene hydrate	-	-	13.962	2.27
1-Terpineol	-	-	15.361	0.65
3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)	-	-	16.853	10.03
Alpha. terpineol	-	-	17.244	4.02
Piperitolisomer I (cis)	-	-	17.366	0.4
Estragole	-	-	17.436	14.5
Cis-sabinene hydrate acetate	-	-	19.325	3.67
1,6-Octadien-3-ol, 3,7-dimethyl-, formate	-	-	19.447	3.75
Caryophyllene	-	-	24.792	12.21
Bis(2-ethylhexyl) phthalate	-	-	46.166	24.48
5A-methyl-3,8-dimethylene-2 oxododecahydrooxireno [2',3':6,7]naphtho[1,2-b]furan	-	-	47.501	4.11

RT: Retention time.

Chitosan nanoparticles on the growth of studied microorganisms were presented in Table 3 ChNPs showed various degrees of inhibition against the tested microorganisms. For the antibacterial investigation, gram-negative bacteria the inhibition zones of ChNPs were ranged from 6.0 to 15.5 mm. The highest inhibition zone was obtained for *S. typhimurium* (15.5 mm), while the lowest (13.5 mm) was recorded for *E. coli* in 100 ppm concentration, on the other hand ChNPs showed higher antibacterial activity against gram-positive (*S. aureus and B. subtilis*) inhibition zones of ChNPs were ranged from 8.00 to 18.5 mm. Chitosan exhibits higher antibacterial activity against Gram-positive bacteria than Gram-negative bacteria. These results are consistent with results Garrido-Maestu et al. (2018). Also, the high bactericidal activity of CHNPs is certainly due to change in cell permeability barrier due to interactions between the positively charged chitosan and the negatively charged microbial cell membranes as reported by Duan et al. (2019).

Antimicrobial activity of cumin and marjoram essential oils

The antimicrobial activities of cumin and marjoram essential oils against *E. coli, S. typhimurium, B. subtilis* and *S. aureus* examined in the current study and their potential was evaluated by the diameter of inhibition zones were also detected. The antibacterial activities of essential oils measured by disk diffusion methods are shown in Table (4). The antimicrobial activity of cumin essential oils at different concentrations (1, 5, 10, 50 and 100%) against above-mentioned microorganisms strains, in Table 4, these results it could be noticed that, microbial spectra were decreased by increasing the concentration of cumin essential oils for all microorganisms and the antibacterial effects of cumin essential oil were more pronounced on gram positive bacteria especially *B.subtilis* (30mm) than gram negative bacteria especially *E. coli* (23.5mm). These results are in agreement with (Palombo & Semple, 2001). The efficiency of cumin essential oil against all microbial strains is related to its components such as cumin aldehyde (35.78%) and other chemical compounds such as -terpinene (20.77%), β-pinene (14.91%) and cuminic alcohol (10.08%) and p-cymene (5.79%) (Johri, 2011).

The antimicrobial activity of marjoram essential oil in Table 3 these results it could be noticed that, microbial spectra were decreased by increasing the concentration of marjoram essential oil for all microorganisms and the antibacterial effects of marjoram essential oil were more pronounced on gram positive bacteria especially *S.typhimurium* (21mm) than gram negative bacteria especially *S.aureus* (28 mm). The efficiency of marjoram volatile oil against all microbial strains is related to its components such as limonene (17.3%), linalool (15.5%), terpinen-4-ol (7.3%), α-terpineol (13.0%) and linalyl acetate (14.1%). These results are in agreement with Badee et al. (2013) found that, microorganisms tested were susceptible to the action of marjoram essential oil, with a range of inhibition zone diameter values from 13 to 34 mm/sample. The intensity of antimicrobial efficacy was in the following order: marjoram > clove > cinnamon > coriander > caraway > cumin.

Antimicrobials effect of silver, chitosan nanoparticles and cumin, marjoram essential oil on total bacterial counts in fish fillet inoculated with E. coli during storage at 4 ±1°C for 12 days

In the last phase of this study, Ag NPs 40.0µg/mL, ChNPs 1%, cumin and marjoram essential oil at 500 were evaluated for their long-term antimicrobial effectiveness against *E. coli* when applied in tilapia fish fillet at 4 ±1°C for up to 12 days. Total bacterial count (TBC) for the culture of *E. coli* was shown in Table (5) and Fig. (3). The initial TBC of different treated were 5.681,5.484,4.854, 4.628 and 5.591 log CFU/g enumerating by GA3 sample and 5.694, 5.537, 4.951, 4.829 and 5.716 log CFU/g enumerating by GA4 sample during storage. A considerable decrease of TBC was noticed in the treated GA5 sample during cold storage. A similar incremental trend of all treatments to the end of storage periods was shown GA6 sample had the lowest increase in the total microbial load. An incremental trend in TBC during subsequent cold storage was shown in GA1sample the initial TBC was reached to 4.596, 4.872, 5.851, 6.963 and 7.633 log CFU/g. At this time, GA2 sample had the highest in TBC. Motavaf et al., (2021), studied the total viable count for rainbow trout fillets during 12 days of storage at 4°C. The highest bacterial level in the control treatment and the pure bacteria *L.monocytogenes* treatment was observed on day 12. Khaleque et al. (2016) reported that visualization of antimicrobial activity in food matrices requires twice or higher concentrations than in vitro activity to make up for losses brought on by interactions between part of the oil and the food matrix.This requires an increase in volume relative to that measured in vitro, as proteins and fats present in food bind and/or dissolve phenolic compounds, which reduce their antibacterial activity (Barbosa et al., 2009).

TABLE 3. Effect of silver and chitosan nanoparticles on inhibition growth of tested microorganisms by agar well diffusion assay.

Microbialstrains	Diameter of inhibition zones (mm)									
	Silvernanoparticles				LSD	Chitosannanoparticles				LSD
	25ppm	50ppm	75ppm	100ppm		25ppm	50ppm	75ppm	100ppm	
Escherichia coli ATCC 6933	5.50 ^{Bd} ±0.30	9.50 ^{Bc} ±0.50	12.50 ^{Bb} ±0.50	17.00 ^{Ba} ±0.60	1.683	6.00 ^{Bc} ±0.00	8.50 ^{Ab} ±0.50	12.00 ^{Ba} ±1.00	13.50 ^{Ba} ±0.50	1.697
Salmonellatyphimurium ATCC14028	7.50 ^{Ad} ±0.50	11.50 ^{Ac} ±0.50	18.50 ^{Ab} ±0.50	21.50 ^{Aa} ±0.50	1.385	7.50 ^{Ab} ±0.50	9.00 ^{Ab} ±0.20	15.50 ^{Aa} ±0.50	15.50 ^{Aa} ±0.50	1.672
LSD	1.385	1.099	1.385	1.91		0.983	1.697	1.807	1.385	
Bacillus subtilis ATCC 33221	6.00 ^{Ad} ±0.00	8.50 ^{Bc} ±0.50	17.50 ^{Ab} ±0.50	20.50 ^{Ba} ±0.50	1.200	8.00 ^{Ac} ±0.00	9.00 ^{Ac} ±0.00	12.50 ^{Bb} ±0.50	18.50 ^{Aa} ±0.50	1.797
Staphylococcus aureus ATCC 20231	6.50 ^{Ad} ±0.50	11.50 ^{Ac} ±0.50	15.00 ^{Bb} ±0.50	22.50 ^{Aa} ±0.50	1.833	9.00 ^{Ac} ±0.20	10.00 ^{Ac} ±0.50	14.00 ^{Ab} ±0.50	18.50 ^{Aa} ±0.50	1.920
LSD	0.980	1.385	1.191	1.385		1.058	1.960	1.134	1.385	

Where: A ,B,C,D in the same columns are not significantly different($p>0.05$) while a,b,c,d in the same rows are not significantly different ($p>0.05$), L.S.D: Least significant differences at ($p>0.05$). (Mean ± standard error).

TABLE 4. Effect of cumin and marjoram essential oilson inhibition growth of tested microorganisms by agar disc diffusion assay.

MicrobialStrains	Diameter of inhibition zones (mm)											
	Marjoram (%)					LSD	Cumin (%)					LSD
	1.0	5.0	10	50	100		1.0	5.0	10	50	100	
Escherichia coli ATCC 6933	9.00^{Ac} ±1.0	14.50^{Ab} ±0.5	14.50^{Ab} ±0.5	15.00^{Ab} ±0.4	20.00^{Aa} ±2.0	1.285	7.50^{Ad} ±0.5	15.50^{Ac} ±0.5	18.00^{Ab} ±1.0	19.00^{Ab} ±0.4	23.50^{Aa} ±1.5	1.859
Salmonella typhimurium ATCC14028	7.00^{Bd} ±1.0	12.00^{Bc} ±0.0	14.50^{Ab} ±0.5	15.50^{Ab} ±1.5	21.00^{Aa} ±.04	1.372	8.50^{Ad} ±0.5	14.50^{Ac} ±0.5	14.50^{Bc} ±0.5	17.00^{Bb} ±1.6	21.00^{Ba} ±1.00	1.753
LSD	0.919	0.980	1.385	1.413	1.960		1.385	1.518	1.109	1.774	1.357	
Bacillus subtilis ATCC 33221	8.00^{Ad} ±0.0	12.50^{Bc} ±1.5	13.50^{Ac} ±1.5	20.00^{Ab} ±1.00	22.50^{Ba} ±1.5	1.697	8.00^{Ad} ±0.5	14.50^{Ac} ±0.5	14.00^{Ac} ±0.5	19.00^{Ab} ±1.00	30.00^{Aa} ±1.5	1.479
Staphylococcus aureus ATCC 20231	6.50^{Bd} ±0.5	14.00^{Ac} ±0.4	14.50^{Ac} ±1.5	17.00^{Bb} ±0.5	28.00^{Aa} ±2.0	1.849	6.50^{Ad} ±0.5	12.50^{Bc} ±0.5	13.00^{Ac} ±0.4	18.50^{Ab} ±1.5	23.50^{Ba} ±1.5	1.807
LSD	1.037	1.764	1.580	1.796	1.979		1.910	1.386	1.255	1.453	1.940	

Where: A ,B,C,D in the same columns are not significantly different($p>0.05$) while a,b,c,d in the same rows are not significantly different ($p>0.05$), L.S.D: Least significant differences at ($p>0.05$). (Mean ± standard error).

TABLE 5. Effect of AgNPs, ChNPs, cumin and marjoram essential oil on totalbacterial counts (Log CFU/g) in fish fillets inoculated with *E. coli* during storage at 4 ±1°C for 12 days.

Treatments	Storage period (days)				
	Zero time	3th day	6 th day	9 th day	12 th day
GA1	4.596	4.872	5.851	6.963	7.633
GA2	5.752	6.607	7.525	8.201	8.934
GA3	5.681	5.484	4.854	4.628	5.591
GA4	5.694	5.537	4.951	4.829	5.716
GA5	5.74	4.894	3.806	3.498	4.872
GA6	5.736	4.929	3.872	3.596	4.916

Ch NPs: Chitosan nanoparticle AgNPs: Silver nanoparticle Log : Logarithm CFU: Colony Forming Unitg: Gram

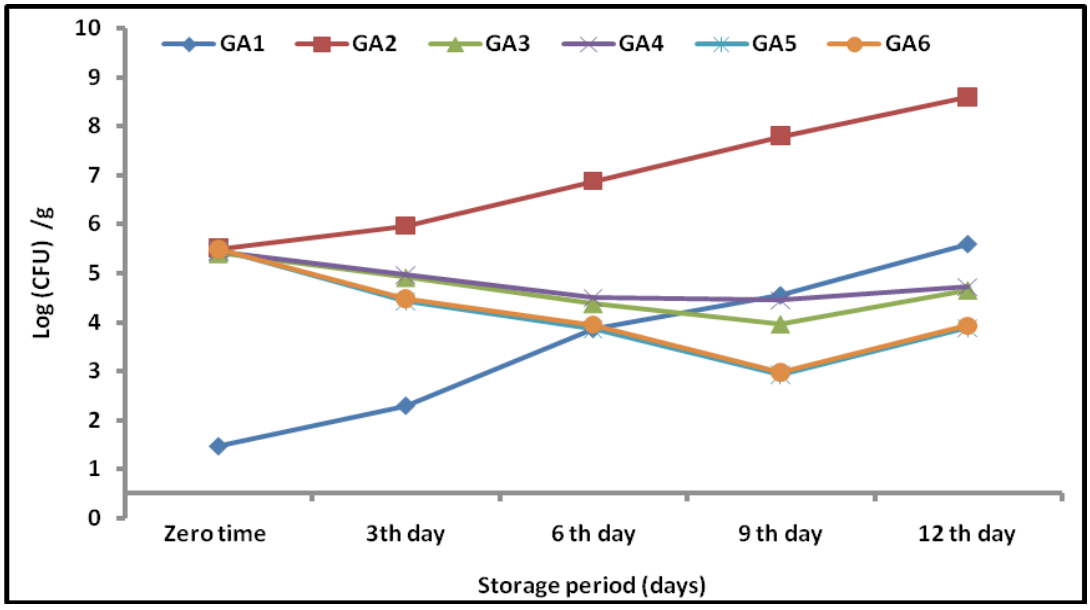

Fig. 3.Changes in total bacterial count (Log CFU /g) of fish fillets inoculated with *E. coli* during storage by using AgNPs, ChNPs, cumin and marjoram essential oil

Changes in E. colicounts by effect of silver and chitosan nanoparticles and cumin and marjoram essential oil in fish fillets during storage at 4 ±1°C for 12 days

These results clearly demonstrate that essential oils and nanoparticles inhibit the pathogen up to 12 days of refrigerated storage. Fish fillet samples during storage at 4±1°C for 12 days are shown in Table 6 and Fig. 4, at 4±1°C, the initial population of *E. coli*, in GA1 sample increased during storage period. GA4 sample treated resulted in decreased by increased during storage exhibited the lower population of *E. coli* until day 9, when compared with GA1 sample. The addition of GA3 sample treated resulted in decreased populations of *E. coli* ranging below the acceptable level of less than 3 log CFU/g from day 9 until the end of storage. The severity of the decrease of the initial *E. coli* population in fish fillets at GA5 sample. The average value of the initial microbial load of GA2 sample was 8 log CFU/g in the end of storage. The similar trend to those reported by Heydari et al. (2015). An essential aspect and their constituent parts are hydrophobicity that allows the microbes and mitochondria to divide with the fats found in the cellular membranes, making them more pliable by disrupting the cell structures. It inevitably contributes to a large degree to the destruction of microbial species owing to the leakage of vital

particles from the microbial cell membrane. Numerous substances regulate drug resistance by exploiting efflux pathways of various species of gram-negative microbes (Ferreira et al., 2021). This high bactericidal activity of AgNPs is certainly due to the silver cations released from AgNPs that act as reservoirs for the Ag+ bactericidal agent (Dakal et al., 2016). Also, the high bactericidal activity of ChNPs is certainly due to a change in cell permeability barrier due to interactions between the positively charged chitosan and the negatively charged microbial cell membranes as reported by Duan et al. (2019). The similar trend to those reported by EL-Desouky et al. (2006).

TABLE 6. Changes in *E. coli* count (Log CFU/g) in fish fillets inoculated with *E. coli* during storage at 4±1°C for 12 days by using AgNPs, ChNPs, cumin and marjoram essential oil.

Treatments	Storage period (days)				
	Zero time	3th day	6 th day	9 th day	12 th day
GA1	1.477	2.301	3.863	4.55	5.591
GA2	5.498	5.956	6.877	7.795	8.596
GA3	5.406	4.916	4.38	3.966	4.658
GA4	5.431	4.968	4.498	4.447	4.716
GA5	5.477	4.423	3.845	2.913	3.877
GA6	5.484	4.469	3.944	2.973	3.921

Ch NPs: Chitosan nanoparticle AgNPs: Silver nanoparticle Log : LogarithmCFU: Colony Forming Unitg: Gram

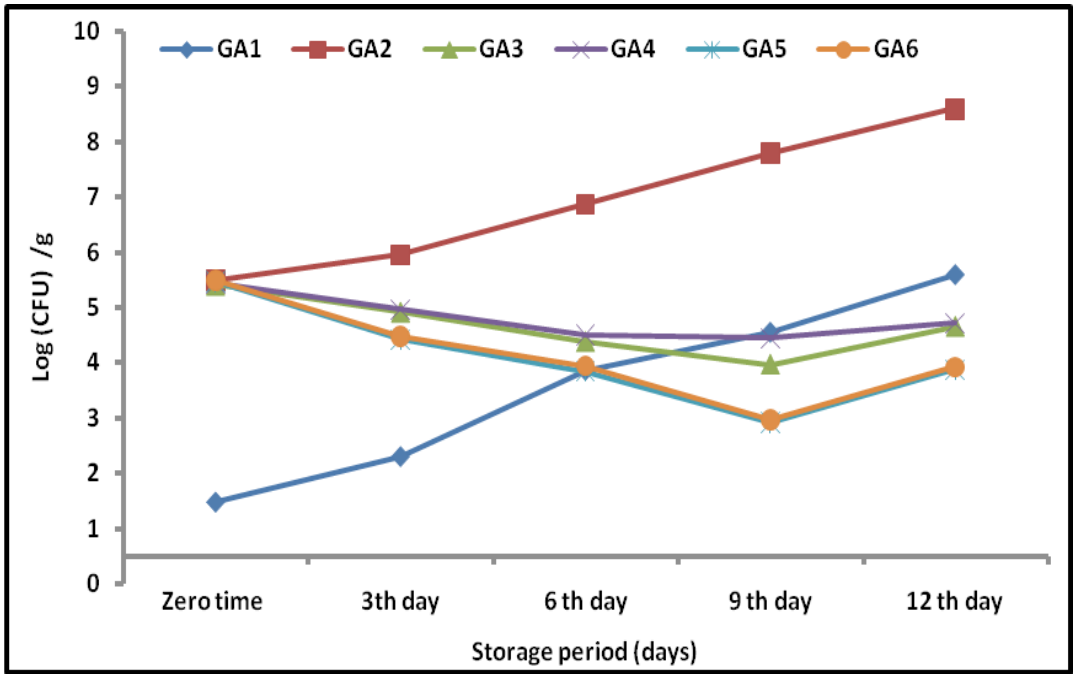

Fig. 4. Changes in *E. coli* count (Log CFU /g) of fish fillets inoculated with *E. coli* during storage by using AgNPs, ChNPs, cumin and marjoram essential oil .

Antimicrobials effect of silver, chitosan nanoparticles and cumin, marjoram essential oil on total bacterial counts in fish fillet inoculated with Bacillus cereus during storage at 4±1°C for 12 days

Total bacterial count (TBC) for the culture of *B. cereus* was evaluated for their long-term antimicrobial effectiveness when applied in tilapia fish fillets, and stored at 4 ±1°C for up to 12 days. Total bacterial count (TBC) for the culture of *B. cereus* was shown in Table 7 and Fig. (5), the initial TBC of different treated were 5.959, 5.863, 4.923.982 and 4.939 log CFU/g enumerating by GB2 sample. The similar trend to those reported by Antoniadou et al. (2019).

On the other hand 5.956, 5.9, 4.98,4.77 and 4.956 log CFU/g enumerating by GB3 sample during storage. These results are in agreement with Musa et al. (2018). AgNPs can easily uptake by the fungal cells through the disruption of fungal cell walls and AgNPs act as a reservoir for releasing of Ag+ ion, causing to cease the ATP production and to stop DNA replication by ROS and hydroxy radicals. A considerable decrease of TBC that noticed in the treated GB4 sample during cold storage. A similar incremental trend of all treatments to the end of storage periods was shown GB5 sample had the lowest increase in the total microbial load. An incremental trend in TBC during subsequent cold storage was shown in GA1 sample the initial TBC was reached to 4.596, 4.872, 5.851, 6.963 and 7.633 log CFU/g. At this time, GB1 sample had the highest TBC.

TABLE 7. Effect of AgNPs, ChNPs, cumin and marjoram essential oil on total bacterial counts (Log CFU/g) in fish fillets inoculated with *Bacillus cereus* during storage at 4±1°C for 12 days.

Treatments	Storage period (days)				
	Zero time	3th day	6 th day	9 th day	12 th day
GA1	4.596	4.872	5.851	6.963	7.633
GB1	5.993	6.724	7.648	8.72	9.469
GB2	5.959	5.863	4.924	3.982	4.939
GB3	5.956	5.9	4.98	4.77	4.956
GB4	5.989	5.491	3.838	3.525	4.462
GB5	5.991	5.518	3.905	3.685	4.562

Ch NPs: Chitosan nanoparticle AgNPs: Silver nanoparticle Log : Logarithm CFU: Colony Forming Unitg: Gram

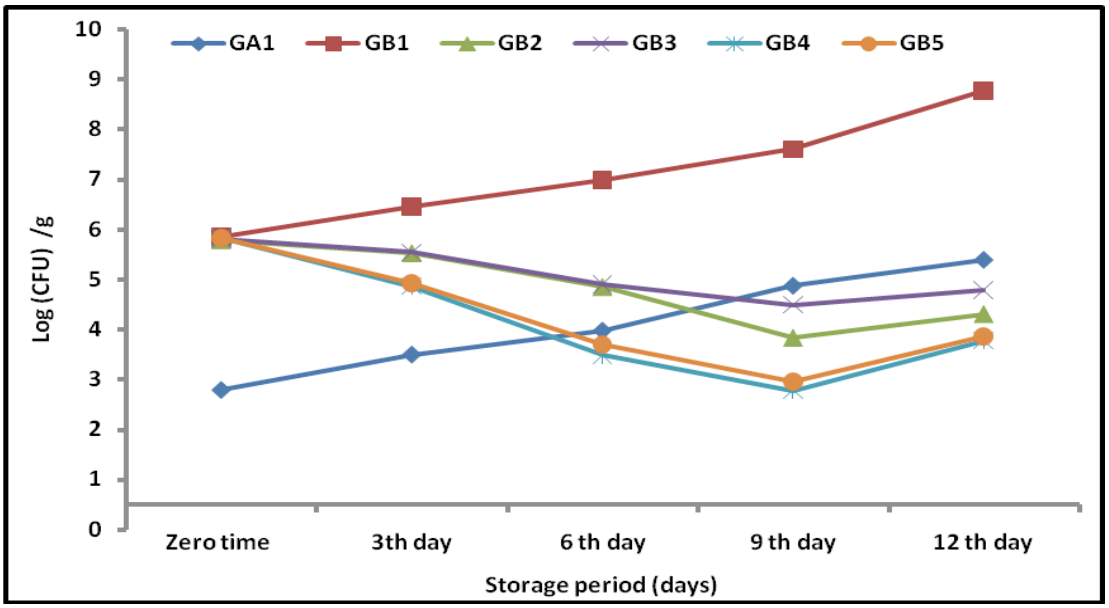

Fig. 5. Changes in bacterial count (Log CFU /g) of fish fillets inoculated with *Bacillus cereus* during storage by using AgNPs, ChNPs, cumin and marjoram essential oil

Changes in Bacillus cereus counts by effect of silver and chitosan nanoparticles and cumin and marjoram essential oil in fish fillets during storage at 4±1°C for 12 days

These results clearly demonstrate that essential oils and nanoparticles inhibit the pathogen up to 12 days of refrigerated storage with cultures by *B. cereus*. Fish fillet samples during storage at the 4 ±1°C for 12 days are shown in Table 8 and Fig. 6, the initial population of *B. cereus* in GA1 sample increased during the storage period. Treated GB3 sample resulted in decreased by increased during storage exhibited the lower population of *B. cereus* until day 9, when compared with GA1 sample. The addition of GB2 sample resulted in decreased populations of *B. cereus* ranging below the acceptable level of less than 3 log CFU/g from day 9 until the end of storage. The bacterial flora of stored smoked fish consists of *Lactobacillus spp.* and if they are predominantly present on the fish they could inhibit the growth of Listeria as a result of competition (Nilsson et al., 1997). The severity of the decrease of the initial *B. cereus* population in fish fillets GB4 sample was less than 2 log CFU/g from day 9 until the end of storage. Thus, the resistance of Gram negative bacteria to the essential oils likely lies in the protective role of their cell wall lipopolysaccharides or outer membrane proteins. Moreover, the antimicrobial activity of essential oils depends on the type of spice or herb, the chemical composition, and the content of extracts and essential oils (Castellano et al., 2008). These results are in agreement with Alboofetileh et al. (2014).The average value of the initial microbial load of GB1 sample was 8 log CFU/g in the end of storage. The reasons for the generally higher efficacy of marjoram over the other investigated essential oils are mainly attributed to marjoram's high content of phenolic compounds as well as good interaction between the constituents of the marjoram with the polymer matrix (Burt, 2004).

TABLE 8. Changes in *Bacillus cereus* count (Log CFU/g) in fish fillets inoculated with *Bacillus cereus* during storage at 4±1°C for 12 days by using AgNPs, ChNPs, cumin and marjoram essential oil.

Treatments	Storage period (days)				
	Zero time	3th day	6 th day	9 th day	12 th day
GA1	2.795	3.498	3.973	4.866	5.38
GB1	5.838	6.447	6.977	7.591	8.759
GB2	5.788	5.525	4.848	3.835	4.311
GB3	5.792	5.544	4.894	4.491	4.778
GB4	5.826	4.857	3.484	2.778	3.767
GB5	5.832	4.929	3.698	2.949	3.854

Ch NPs: Chitosan nanoparticle AgNPs: Silver nanoparticle Log : Logarithm CFU: Colony Forming Unitg: Gram

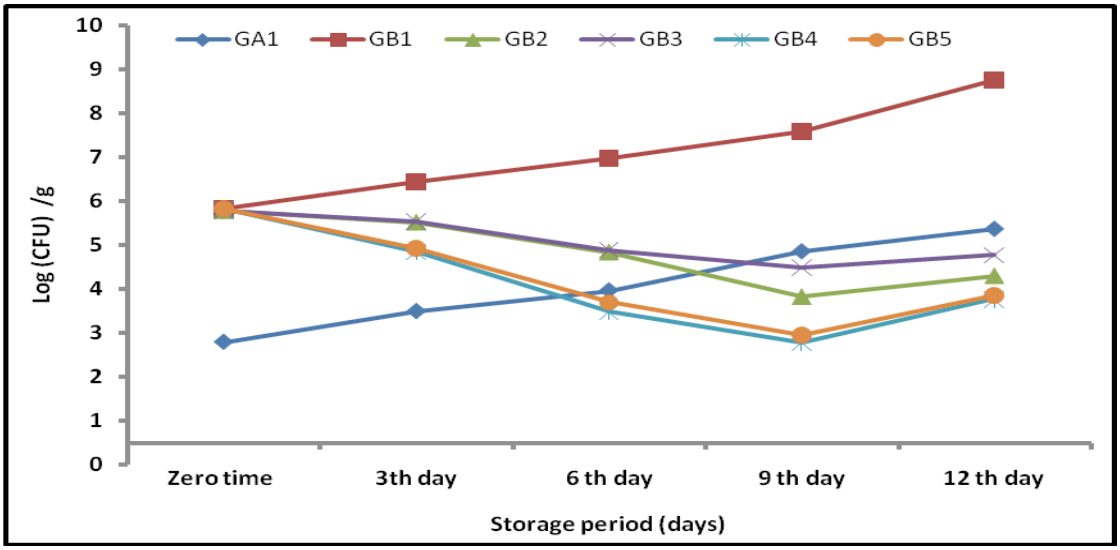

Fig.6. Changes in *Bacillus cereus* count (Log CFU /g) of fish fillets inoculated with *Bacillus cereus* during storage by using AgNPs, ChNPs, cumin and marjoram essential oil.

Conclusions

This study demonstrated the potential use of AgNPs, ChNPs, cumin, and marjoram essential oils to control *E. coli* and *B. cereus* in fish fillets at 4°C. Application of these antimicrobial agents in preserve their antibacterial activity in fish fillets during cryopreservation reduces the growth of E. coli and Bacillus cereus. Results also indicated that cumin essential oils 500 ppm had the best effect on growth inhibition of E. coli and Bacillus cereus in fish fillets when used alone, followed that marjoram essential oils 500 ppm had effect on growth the growth of these pathogenic bacteria at 4°C, followed that ChNPs1% and find AgNPs 40.0µg/ml, when compared to the control. Therefore, the producer and consumer preference for the use of natural additives in food, it is suggested that essential oils and/or nanoparticles to be practically applied in fish fillets in order to increase its safety against pathogenic bacteria; nevertheless, to highly ensure its safety, it should be applied together with other techniques.

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