Evaluation of Residues and Antibacterial Effect of *AgNPs* Containing Packages of Fish Fillet

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Abstract

This study evaluated the microbial load of fish fillet packaged with silver nanoparticles (AgNPs) containing coat and refrigerated at 4∘C/12 days. Estimate AgNPs migration from package material to the fish fillet. 12.5 kg fish fillet divided and packaged by (0.5%, 1.0%, 1.5% & 2.0%) AgNPs contents, refrigerated at 4∘C/12 days and evaluated periodically; microbiologically (TBC, TSC, Escherichia coli, Staphylococcus aureus & Bacillus cereus) and AgNPs residues in fish fillet. Results showed the clear potent effect of addition of AgNPsto packages which inhibit the growth different bacterial counts and increased by prolonged storage. The highest bacterial counts were on control samples and increased by time whoever, AgNPs packaging containing material inhibit the different bacterial counts with positive relationship by prolonged storage period and high packages AgNPs contents. The highest residues of Ag increased with higher AgNPs samples packages contents and prolonged storage period. More studies are needed on AgNPs to determine how to use it in food preservation safely.

Keywords: AgNPs migration, antimicrobial activity, food packaging, Nano toxicity, silver nanoparticles.

1. Introduction

Fish meat is one of highly rich in; protein, vitamins, lipids, essential minerals. In addition to it's highly palatability to all family member with wide average price according to the fish species for the consumers. Furthermore, fish is one of rapid perishable foodstuff and difficulty of storage and rapid spoilage which may appear as changes in sensory characters which leading to unacceptability of fish. Fish shelf life affected by; storage temperature, initial microbial load, fish species and packaging methods. A fish fillet is one of fish packaging method which defined as; fishmeal sliced parallel from the backbone and removed all fish scales performed from any type of fish but avoid the fish species which have small spine in between its muscles (**Aliza, 2010 and Mizielin´ska***, et. al.,* **2018**).

Fish packaging aimed to protect different fish products from damage or contaminated and delays their deterioration which extend the products shelf-life in addition to providing the consumers awareness with all the product nutritional information and ingredients. Advanced fish production techniques concerning to improving fish packaging not only to protect the product from outside contamination but to destroy the internal pathogens and/or any spoilage microorganisms and extend the shelf life of the final products by safe preservatives specially on highly perishable fish such as different fish types (**Draughon, 2004; Medina,** *et. al.,* **2007; Shinn, 2012; Sharma,** *et. al.,* **2015 and Kuuliala,** *et. al.,* **2018**).

Nano materials is manipulation of specific natural or manufactured atomic molecules up to 100 nanometres (nm) of substances on the package materials which uses for different types of food which has antimicrobial properties which perform an impact worldwide food production. Silver nanoparticles is one of the most recent antimicrobial packaging films which has abroad antimicrobial effect. Silver ions (Ag^+) nanoparticles has broad spectrum of antimicrobial properties with highly heat stability for refrigerated at 4∘C/12 days (**Šimon,** *et. al.,* **2008; European Commission 2011/696/EU; Mustăţea & Călinescu, 2014 and Singh,** *et. al.,* **2018**).

Escherichia coli, *Staphyl ococcus aureus* and *Bacillus cerus* are food poisoning microorganisms which can found in different types of meat as fish fillet by multiplication of microorganisms or through their toxins which appears as gastrointestinal symptoms as; nausea, abdominal pain, vomiting, diarrhea **(Cushen,** *et. al.,* **2013)**.

Although these benefit but we have shortage in studies which study the side effect of silver nanoparticles residues on the products and consumers (**Cushen,** *et. al.,* **2013 and Călinescu,** *et. al.,* **2014)**.This study objected to evaluate the microbial load of fish fillet which stored by package with silver nanoparticles which refrigerated at 4∘C/12 days and comparing it with other fish fillet store by package without silver nanoparticles during the same storage conditions. Estimate if the silver nanoparticles transferred from package material to the fish fillet and lift silver residues in fish meat and if this residues within daily acceptable intake.

2. Material and Methods

2.1. Preparation of Packages with AgNPs

Packaging with monomer of polyethylene glycol 600 diacrylate (SR610) with 2hydroxy-1-[4-(2-hydroxyetoxy)phenyl]-2-methyl-1-propanonephoto-initiator (Irgacure 2959) were used as received of silver nanoparticles which synthesis by silver nitrate AgNO3 (Aldrich) with different concentrations content of AgNO3(0.5, 1, 1.5 and 2%, % wt) by irradiation of coated fish fillet samples, the film thickness about ~ 6 microns (Lightning cure L8333 with a Hamamatsu L8253 Xe-Hg 100 W lamp). About 12.5kg of fish fillet divided into 125 portion (100 gm/each) and cover 25 portions by 0.5% silver nanoparticle polyethylene packages, 25 portions by 1.0% silver nanoparticle polyethylene packages, 25 portions by 1.5% silver nanoparticle polyethylene packages, 25 portions by 2.0% silver nanoparticle polyethylene packages and 25 portions covered by polyethylene packages without nanoparticle as control samples. All samples stored in refrigerator at 4∘C/12 days and test 5 samples from each packaging type at (zero, 3, 6, 9, 12) day of storage (**Noshirvani,** *et. al.,* **2017)**.

2.2. Determination of Antibacterial Activity on Fish Fillet Coated with Package Containing AgNPs

weighing about 10 gm fish fillet from each packages types samples aseptically homogenized with 90 mL of 0.1% sterile peptone water in a Seward stomacher (400R/UK) and serial dilutions (10^{-1}) to evaluate the bacterial load of each silver nanoparticles packaged fish fillet content and control samples. Total viable bacteria plated on plate count agar (PCA) at 30 ℃/48 hrs, psychrotrophic bacteria were pour plated in plate count agar (PCA) and incubated at 7℃ for 10 days, *Escherichia coli* on violet red bile agar at 37 ℃/24 hrs (VRBA), *Staphylococcus aureus* the enriched broth was plated on Baird-Parker Agar (Oxoid, CM 275) supplemented with Egg Yolk-Tellurite Emulsion (Oxoid, SR 54) for *Staphylococcus areus* incubated at 37℃/24 hr & 48 hr. (**Valls,** *et al.***, 2000),**and *Bacillus cereus* inoculated on blood agar and incubated at 30℃/24 hours (**Markin***et al.,* **2003)**.

2.3. Determination of AgNPs Residues on Fish Fillet Meat

Digested samples performed by mixing sample with 10 ml of nitric / perchloric/ sulfuric acids (Oxoid) (8: 1: 1). Then left at room temperature for 4 hr. followed by heated to 40-45°C/1 hr. in water bath, followed by raising the temperature to 75°C until the digestion completed. The next step was lift the mixture to cool at room temperature then diluted to 20 ml. with deionized water which followed by filtration through 0.45 µl Whitman filter paper. Then refrigerate the filtrated sample to avoid evaporation. Determination of silver content by Atomic Absorption Spectrophotometer (Sens AA; GBC scientific equipment Spectrophotometer).

4. Statistical Analysis (GraphPad Instant, 2009): The statistical program, GraphPad Instant version 3 for window, was used for determination of means, the analysis of variance between the different data and treatment in this study were determined using standard error and analysis of variance (*P<0.05*).

3. Results

3.1. Effect of Different (AgNPs) Packages Contents on Some food Spoilage and Food poisoning Microorganisms on Fish Fillet during 12 day of Storage Refrigerated Period:

Figure (1) & table (1) declared the different inhibition effect of AgNPs against total bacterial count (TBC) and total psychrophilic count (TSC) which may cause fish fillet spoilage and some food pathogenic microorganisms; *Escherichia coli* as one of gram negative, *Staphylococcus aureus* and *Bacillus cerus*as gram positive food poisoning microorganism.

Results showed that there is clear potent effect of adding of AgNPs packaging of fish fillet and this effect were not inhibit the growth but also it were decrease the count of examined microorganisms appear potently with in

prolonged storage period. The highest mean TBC and TSC reported in control samples which were increased by time as following; $3.5 \times 10^3, 3.7 \times 10^3, 3.9 \times 10^3, 4.2 \times 10^3, 4.5 \times 10^3$ CFU/gm of mean TBC at zero day, 3rd day, 6th day and 12th day of refrigerated fish fillet samples respectively. While, mean TSC of control fish fillet samples were; $3.3 \times 10^3, 3.4 \times 10^3, 3.7 \times 10^3, 4.0 \times 10^3, 4.1 \times 10^3$ CFU/gm of zero day, 3rd day, 6th day, & 12th day refrigerated samples respectively. Whoever using of AgNPs packaging material inhibit the count of TBC and TSC with positive relationship by prolonged storage period and high AgNPs contents in packages. The mean TBC & TSC begin from 3.4×10^3 & 3.1×10^3 respectively on samples packaged by 0.5% AgNPs which decreased gradually until reach to 2.9×10^3 & 1.5×10^3 respectively on the same type of samples after 12 day of refrigeration, while using of packages containing 1% AgNPs revealed about 3.0×10^3 & 2.8×10^3 of TBC & TSC respectively at zero day of storage and inhibit TBC & TSC load gradually by time to became about; $2.2 \times 10^3, 1.2 \times 10^3$ respectively. The degradation of mean TBC & TSC developed in samples packaged by AgNPs contained 1.5% to record about; 2.8×10^3 & 2.6×10^3 respectively at zero day and about 1.9×10^2 & 9×10^2 respectively at 12th day of refrigeration. The more potent inhibition effect reported on samples packaged by 2% AgNPs as following; 2.2×10^3 & 2.0×10^3 of TBC & TSC respectively at zero day while it reached to; 1×10^2 & 6×10 of TBC & TSC respectively at 12th refrigeration day.

Figure (1): Effect of Different AgNPs Packages Contents on TBC and TSC during the Storage Refrigerated Period
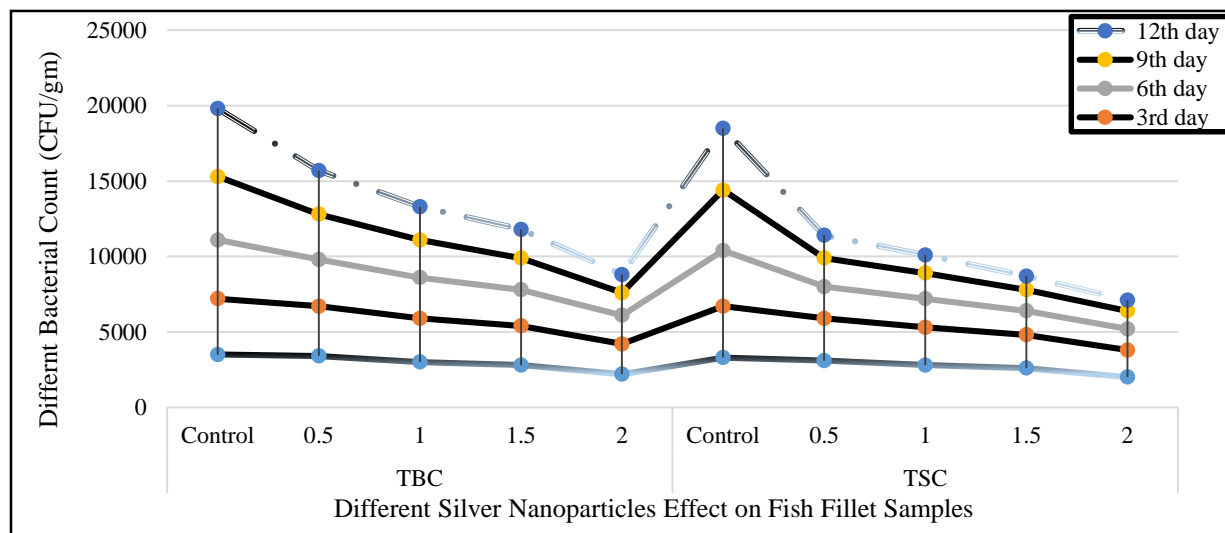

Table (1) revealed the effect of AgNPs different packages content on the load of *Escherichia coli*, *Staphylococcus are us* and *Bacillus cereus* as food pathogens. The recorded results declared the potent inhibition effect of AgNPs against all tested food poisoning pathogens specially with extend storage period and higher content of AgNPs as following; the mean *Escherichia coli* content of control samples were about ($1 \times 10^3, 3 \times 10^3, 4 \times 10^5, 5 \times 10^6$ & 8×10^7) CFU/gm of samples stored at zero day, 3rd day, 6th day, 9th day & 12th day. *Escherichia coli* count inhibited to about; $<10^3$ FCU/gm on all samples packaged by 0.5% AgNPs along all storage period and there were not any detection of *Escherichia coli* on any samples packed with package contained (1.0%, 1.5% & 2.0%) of AgNPs. Less inhibition potency were against *Staphylococcus areus* and *Bacillus cereus* as following; *Staphylococcus areus* mean count were about; ($3.3 \times 10, 3.1 \times 10^2, 3 \times 10^3, 4 \times 10^4$ & 5×10^6) CFU/gm of samples stored at zero day, 3rd day, 6th day, 9th day & 12th day. The count reduced to about $<10^3$ FCU/gm on all samples packaged by 0.5% AgNPs along all storage period and same resulted reported on samples packaged by 1.0% AgNPs at (zero day & 3rd day) of refrigerated samples while, at 6th day, 9th day & 12th day refrigerated packaged samples were not detected. There were not also any detection of *Staphylococcus areus* on any samples packed with package contained (1.5% & 2.0%) of AgNPs. On the other hand, *Bacillus cereus* on control samples recorded about; $<10^3$, $10^3, 2 \times 10^3, 3.8 \times 10^3$ & 5.1×10^3) CFU/gm of samples at (zero day, 3rd day, 6th day, 9th day & 12th day) of refrigeration. Samples stored in packages contained 0.5% AgNPs recorded about; $<10^3$ CFU/gm at zero day, 3rd day & 6th day of refrigeration while packages contained 1.0%, 1.5% & 2.0% of AgNPs were not detect *Bacillus cereus* completely.

Table (1): Effect of Different Content of AgNPs on Some Pathogenic Microorganisms in Fish Fillet Samples during Refrigerated Storage Period

(Ag) Nano particles/ storage period	*Escherichia coli*					*Staphylococcus areus*					*Bacillus cereus*				
	Control	0.5 %	1.0 %	1.5 %	2.0 %	Control	0.5 %	1.0 %	1.5 %	2.0 %	Control	0.5 %	1.0 %	1.5 %	2.0 %
Zero day	10^3	<10^3	ND	ND	ND	3.3×10	<10^3	<10^3	ND	ND	<10^3	<10^3	ND	ND	ND
3rd day	3×10^3	<10^3	ND	ND	ND	3.1×10^2	<10^3	<10^3	ND	ND	10^3	<10^3	ND	ND	ND
6th day	4×10^5	<10^3	ND	ND	ND	3×10^3	<10^3	ND	ND	ND	2×10^3	<10^3	ND	ND	ND
9th day	5×10^6	<10^3	ND	ND	ND	4×10^4	<10^3	ND	ND	ND	3.8×10^3	ND	ND	ND	ND
12th day	8×10^7	<10^3	ND	ND	ND	5×10^6	<10^3	ND	ND	ND	5.1×10^3	ND	ND	ND	ND

3.2. Estimation Different AgNPs Contents immigration to Fish Fillet along The Storage Refrigerated Period were viewed in figure (2) which declared that all control samples were clear and free from any AgNPs during all over the storage refrigerated period. However the highest residues of Ag increased with higher AgNPs samples packages contents with prolonged storage period as follow; 0.1 ± 0.008, 0.14 ± 0.012, 0.14 ± 0.012, 0.17 ± 0.014, 0.2 ± 0.016 µg/gm on (zero day, 3rd day, 6th day, 9th day & 12th day) of samples packaged in packages contained 0.5% AgNPs respectively. The Ag residues recorded about; (0.14 ± 0.013, 0.16 ± 0.015, 0.2 ± 0.018, 0.23 ± 0.019 & 0.25 ± 0.02)µg/gm on (zero day, 3rd day, 6th day, 9th day & 12th day) of samples packaged with 1.0% of AgNPs contents respectively. Estimated Ag residues in samples packaged in case content 1.5% of AgNPs were (0.17 ± 0.014, 0.2 ± 0.018, 0.24 ± 0.021, 0.26 ± 0.024 & 0.28 ± 0.028)µg/gm on zero day, 3rd day, 6th day, 9th day & 12th day respectively. The highest Ag residues concentrated on samples packaged on cases contained 2.0% of AgNPs as following; (0.2 ± 0.018, 0.22 ± 0.021, 0.25 ± 0.023, 0.3 ± 0.028 & 0.39 ± 0.032)µg/gm on zero day, 3rd day, 6th day, 9th day & 12th day respectively. There were a significant difference of Ag levels in fish fillet samples which refrigerated on cases contained different contents of AgNPs within 12 day of storage at (*P>0.05*).

Figure (2): Estimation Different AgNPs Contents immigration to Fish Fillet along The Storage Refrigerated Period

Means followed by a different letter in the line are significantly different (p>0.05)
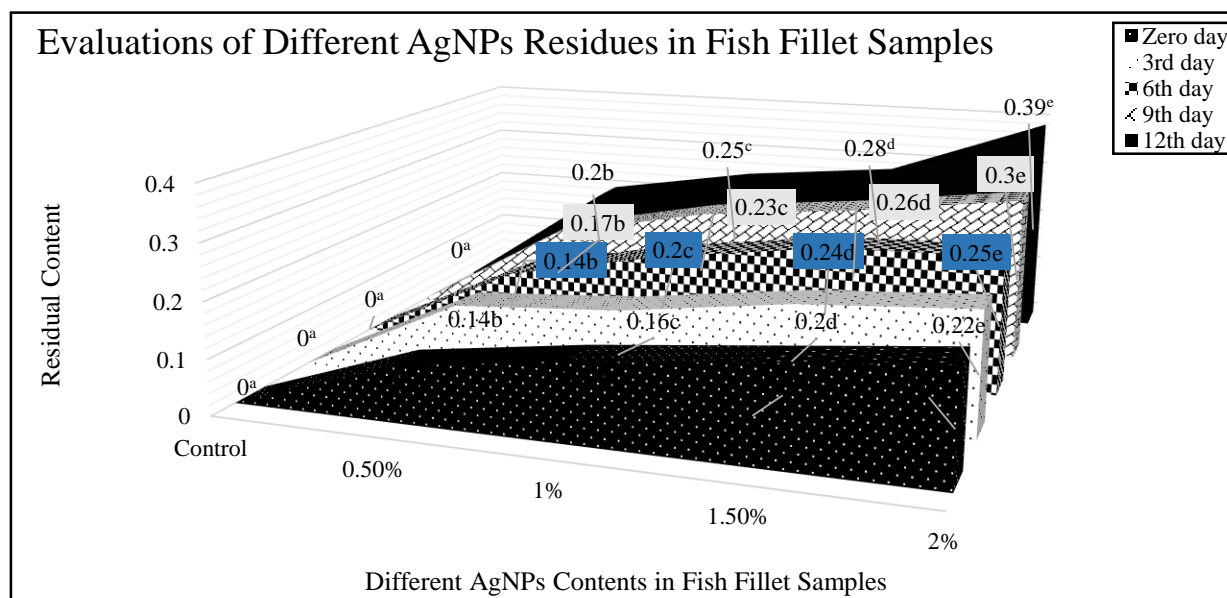
4. Discussion

Nanotechnology benefits has promising future on food industry to prolonged the shelf life of different food products safely and avoid or decrease adding of chemical preservatives and avoid their hazardous using some nanomaterials which has unique characters that, will increase the exposure of the nanomaterials residues on the consumers with time which may accumulated in different organs of the consumers body and may resulting on adverse effects. Therefore we need studies concern nanomaterials public health impact **(Berekaa, *et. al.,* 2015)**. Various silver salts derivatives has antimicrobial potency through its interaction with bacteria phosphorus compounds in bacterial DNA which leading to prevent replication of DNA. Furthermore, silver nanoparticle react with sulfur-containing proteins which causing inactivation of bacterial cell membranes enzymes functions and increasing the permeability of bacterial cell membrane which leading to bacteria death**(Morones, *et. al.,* 2005; Petica, *et. al.,* 2008 and Rai, *et. al.,* 2009)**.

Different inhibition effect of AgNPs against total bacterial count (TBC) and total psychrophilic count (TSC) which may cause fish fillet spoilage and some food pathogenic microorganisms; *Escherichia coli* as one of gram negative, *Staphylococcus aureus* and *Bacillus cerus* as gram positive food poisoning microorganism. Results showed that there is clear potent effect of adding of AgNPs packaging of fish fillet and this effect were not inhibit the growth but also it were decrease the count of examined microorganisms appear potently with in prolonged storage period. The highest mean TBC and TSC reported in control samples which were increased by time whoever, using of AgNPs packaging material inhibit the count of TBC and TSC with positive relationship by prolonged storage period and high AgNPs contents in packages. While, the effect of AgNPs different packages content on the load of *Escherichia coli*, *Staphylococcus areus* and *Bacillus cereus* as food pathogens. The recorded results declared the potent inhibition effect of AgNPs against all tested food poisoning pathogens especially with extend storage period and higher content of AgNPs. Similar results reported by **(Jung, *et. al.,* 2008)** whom found the AgNPs on *Escherichia coli* and *Staphylococcus aureus* cell morphology while higher results reported by (**Feng, *et. al.,* 2000**) who mentioned that AgNPs has similar effect in both the Gram negative and Gram positive bacteria. **(Cho, *et. al.,* 2005 and Martínez-Castañón, *et. al.,* 2008)** mentioned that the potency of AgNPs which able to penetrate the bacterial cell and resulting from antimicrobial activity.**(Jung, *et. al.,* 2008 and Ratyakshi& Chauhan, 2009)**found that AgNPs has strong antibacterial activity against wide range of Gram positive and Gram negative microorganisms. **Li, *et. al.,* (2010)** declared the potent effect of AgNPs against *Escherichia coli*. **Ahangaran, *et. al.,* (2012)** reported that 5% of AgNPs inhibited about 70% of *Escherichia coli* and *Staphylococcus aureus*. **Shahrokh, &mtiazi, (2009)** evaluated the inhibition effect of AgNPs against *Bacillus cereus*. **Panea, *et. al.,* (2013)** stated that AgNPs has broad spectrum antimicrobial activity on meat packaged on AgNPs during 0, 7, 10, 15 or 21 days of storage. **Mustăţea and Călinescu, (2014)** determined the antimicrobial characters of AgNPs on packages food containing AgNPs, results declared increase of inhibition zone with higher AgNO3 amount which were more pronounced in case of *S. aureus* and *E. coli*. **Gallocchio, *et. al.,* (2016)** evaluated antimicrobial activities of AgNPs on a commercially food coated by packages of chicken meat and its effect on proliferation of spoilage bacteria and found that no significant difference in the (TBC, Enterobacteriaceae and Pseudomonas spp.) on meatballs packaged on plastic bags AgNPs and control bags. **Hosseini, *et. al.,* (2017)** measured AgNPs inhibition zone against *Escherichia coli* and *Staphylococcus aureus* on 48 food refrigerated packages after 0, 1, 2, 3, 4, 5 and 6 days and comparing the results with 24 food packages free from AgNPs. The inhibition zone were about 6 time than penicillin and phosphomycin which were significantly larger than control samples and tested antibiotics discs ($P < 0.01$).

Recent studies concerns only the positive side of using and applications of nanotechnology with a dearth of concernment which study the side effects of exposure to nanoparticles for long period on consumers' health and environment. Identification of emerging hazards from nanoparticles depending on 4 factors; identification of hazard, identification of hazard exposure route, detection the characteristics of hazards and evaluation of toxicity degree (**Linkov, *et. al.,* 2008**).

Estimation of Different AgNPs Contents immigration to Fish Fillet along The Storage Refrigerated Period declared that the highest residues of Ag increased with higher AgNPs samples packages contents with prolonged storage period. Higher results reported by **Metak, *et. al.,* (2015)** estimated the AgNPs migration from different coated food film to the food content during 10 days of storage at 40 °C. which wasn't significant as the measured level was; 5.66 ± 0.02 μgL^{-1}oncontrol samples while it reached to 0.41 ± 0.02 μgL^{-1} on AgNPs coated samples after 10 days of storage.

Lower results reported by **Gallocchio, *et. al.,* (2016)** evaluated Ag migration on a commercially food coated by packages containing AgNPs into chicken meat as 0.010 mg kg^{-1}. **Cushen *et al.* (2013)** concluded that the highest nanoparticles metal able to migrate from packages food to the food content is silver while the lowest ability of nanoparticles metal migration recorded in copper. **Loghman, *et. al.,* (2012)** reported that AgNPs penetrate the chickens hepatic cells causing a lot of hepatocytes changes such as; hyperaemic, hepatic cells swollen, hepatocytes focal necrosis. **Koohi*et al.* (2018)** investigated AgNPs toxicity on rabbits and showed their adverse effect on; liver, heart, kidney, brain and skin causing hyperkeratosis, epidermal warts, edema, fibrosis, erythema, swelling, redness and cell death. **Stoermer, (2017)** examined different nanoparticle models film and concluded that AgNPs not the best film model due to it's highly capability to migrate inside the food. **Hosseini, *et. al.,* (2017)** measured AgNPs residues on 48 food refrigerated packages after 0, 1, 2, 3, 4, 5 and 6 days and comparing the results with 24 food packages free from AgNPs the Ag migration finding were significantly higher than control samples ($P < 0.05$). According European regulation EC no. 450/2009authorizednanomaterialsmigrations to packaging food to about 0.01 mg/kg and stated that AgNPs toxicity began after repeated intake of food contains Ag for about 90-day **(Cushen, *et. al.,* 2013)**. According to FDA (Food and Drug Association) standards Ag is quite stable element that is not pose any major biological hazards if consumed within limits **(Zhao, *et. al.,* 2008 and FAO, 2015)**.

5. *Conclusion*

Results indicated that AgNPs is a stable element that is not pose any major biological hazards if consumed within limits with high antimicrobial activity which increased with prolong refrigeration period and higher content of AgNPs. Ag can migrate from the packaged materials containing AgNPs against wide range of Gram-negative and Gram-positive microorganisms which resulting in extending the shelf life of the fish fillet. Ag particles migrate from packages containing AgNPs to the fish fillet meat although that the fish meat consider safe to consumers when utilize within limited content of AgNPs. The study recommended to perform further studies to establish more applications and more studies on AgNPs and other nanoparticles to determine their antimicrobial properties and their side effects and how to use them safely to gain their benefits to extend food shelf life with avoid any side effects of them and/or other chemicals preservatives.

6. *Acknowledgements*

7. *Referances*

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