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Antibacterial and Cytotoxicity Functions of Pomegranate Peel Extracts in Fish Processing

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Abstract: Pomegranate peel extracts, both ethanolic (PPE) and aqueous (PPA), demonstrated a broad spectrum of antibacterial action against Gr+ve and Gr-ve bacteria, with bacterial isolate B2 (G^{+ve} cocci) having the maximum inhibition zone (IZ) 10.5–26.5 mm at concentrations 4.2 mg mL^{-1} to 267 mg mL^{-1}. PPE's minimum inhibitory concentration (MIC) varies among bacterial strains, with the lowest being 8.3 mg mL^{-1}. Ball-milled nano-scale pomegranate peel (NSPP) with 89.09 nm particle increased IZ 3-fold and decreased MIC significantly. MTT was used to assess the NSPPE's cytotoxicity against the Vero normal kidney and caco cell lines. At lower doses, NSPPE was safe, but increasing concentrations gradually lethal against Vero normal kidney cell line. NSPPE was highly toxic to caco cells, with an IC50 339.76±13.9 µg mL^{-1}. Fish kofta samples treated with NSPPE were completely free of *Escherichia coli* and *Staphylococcus aureus* after 8 weeks of storage. This study demonstrates that NSPPE may improve fish food safety and shelf life as a natural preservative.

1 Introduction

The pomegranate plant (*Punica granatum* L.), Punicaceae family, is considered one of the first domesticated fruits originating in Tunisia, Egypt, Turkey, Spain, Iran, Morocco, Italy and surrounding regions (Drinić et al 2020). Pomegranate fruit is famous for its delicious flavor and many health and nutritional benefits. It contains minerals, alkaloids, and phenolic substances, including tannins (ellagitannin, peduncollagen, and punicalin), and flavonoids (anthocyanins and catechins) (Mo et al 2022).

These active compounds are responsible for the well-known pomegranate anti-inflammatory, anti-cancer and antioxidant properties. They are concentrated in the crust (mesocarp and exocarp), making up approximately 50% of fresh fruit (Akhtar et al 2015). The peel of pomegranate waste accounts for 30 to 40% of its weight and is frequently discarded as biological waste materials (Peršurić et al 2020). This portion of the pomegranate, which has traditionally been regarded as a by-product in the agri-industry scale, could be classified as a co-product. It can yield numerous chemical compounds suitable for various applications, including food additives, cosmetics, pharmaceutics and nutraceuticals (Kaderides et al 2021).

Pomegranate peel extracts (PPEs) are prepared using different methods according to their biological efficiency and applications (Gigliobianco et al 2022). Wa-

ter-containing extracts, for example, have shown promise as antibacterial agents. Accordingly, pomegranate peel may serve as an eco-friendly source of natural additives in the food industry, and as an antibacterial agent to prevent foodborne diseases (Abu-Niaaj et al 2024).

Rather than relying on chemical or thermal preservation techniques, food safety and quality can be guaranteed through the usage of natural antimicrobial food preservatives, or bio-preservation (Pisoschi et al 2018). Both G^{+ve} and G^{-ve} bacteria were eliminated by the PP's phenolic compounds. Hot water PP extracts showed *in vitro* antibacterial effects against microbial cells such as *Escherichia coli*, *Bacillus subtilis*, *Salmonella* Typhimurium and *Staphylococcus aureus*. Additionally, the water extract efficiency was on the level with that of ethanol and acetone extracts (Nuamsetti et al 2012, Jacob et al 2021).

Freezing technology is essential for the long-term preservation of products, particularly seafood. Several studies have examined microbiological load, quality parameters, sensory properties and chemical composition of fish fingers made from various fish species (Tokur et al 2006). The fish processing industry is extensive and diverse, comprising a range of operations, scales and products. Plant extracts have been explored as natural antioxidants in fish products (Abou-Taleb 2022); while pomegranate polyphenolic extracts exhibited significant antimicrobial and antibiofilm effects, especially when combined with other botanical extracts or antibiotics in *in vitro* therapies against cariogenic bacteria. Their antibacterial activity is due to altering cell structure, inhibiting biofilm formation, and enhancing antibiotic effectiveness (Xiang et al 2022).

Although the health benefits and the antioxidant properties of pomegranate peels are well-documented, further scientific research is needed to enhance the peel extract's effectiveness. Improving the efficiency of pomegranate peel extracts through modern techniques in food processing, such as superfine grinding and nanotechnology, is essential (Zhong et al 2016, Kishk et al 2021). Recently, nano-technology has attracted researchers in food manufacturing, showing promising results in food preservation (Singh et al 2017). Reducing particle sizes can increase particle surface area, thereby releasing more bioactive compounds (Rosa et al 2013). This work is planned to evaluate the pomegranate peel extracts' antibacterial properties and enhance their effects through nanotechnology in addition to applying them to improve the fish kofta's shelf life, safety and quality.

2 Materials and Methods

2.1 Bacterial isolates and strains

The pathogenic bacterial strains employed in this research were obtained from the Agricultural Microbiology Department, Ain Shams University, Egypt. The pathogenic strains *Salmonella* Typhimurium As3, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus cereus* DSMZ345, *Listeria monocytogenes* DSMZ423 and *Staphylococcus aureus* As4 were obtained from Microbiological Resource Centers (MIRCEN), Egypt, in addition to bacterial isolates isolated from different frozen or fresh fish samples collected from local markets (as presented in **Table 1**). The standard inoculum was made by selecting four or five colonies of purified bacterial colonies from an agar plate, and then sub-culturing them into a test tube containing 4 mL of Müller-Hinton medium broth (OXOID, CM0405 Basingstoke, Hampshire, England). Tubes were held at 37°C for 24 h. One mL of standard inoculum contained about 3×10^7 CFU ml^{-1}.

2.2 Preparation of pomegranate peel

Pomegranate peels (PP) obtained from local markets were washed to remove any plant debris and then left for 7 days to air-dry at room temperature. Once dried, the materials were milled and sieved to obtain the finest powder and then kept at -20 °C until usage.

2.2.1 Aquatic extract preparation

In a 250 mL conical flask, 20 grams of powdered pomegranate peel (PP) were soaked in 180 mL of distilled water as part of the conservative extraction procedure. After 30 minutes of heating to 90°C, the mixture was shaken (150 rpm) overnight at 37°C (Xu et al 2008). Following filtration through filter paper (Whatman No. 1), the remaining solid was separated and concentrated at 40°C in an oven, as detailed by Yeasmen and Islam (2015).

2.2.2 Ethanolic extract preparation

In a 250 mL conical flask, about 10 g of PP or NSPP powder was mixed in a 1:9 ratio with 90 mL of 95% ethanol. The resulting mixtures were shaken at 150 rpm overnight at 37°C. After filtering to separate the unsolvable residues of liquid extracts, the mixture was concentrated at 40°C in an oven, as described by Yeasmen and Islam (2015). Dried materials were resalable in 5% DMSO (Dalir et al 2020). The crude extract was kept

Table 1. Bacterial isolates

Bacterial isolates	Source of isolation	Colony morphology	Cell morphology	Gram stain reaction	Enzymes activity		Hemolytic activity
					Protiolytic	lipolytic	
F2	*Mugilidae*	white, circular and very soft	short rods	G^-	+	-	α -hemolysis
F5	*Mugilidae*	white, not circular and like rose	short rods	G^-	+	-	α -hemolysis
B7	*Oreochromis niloticus*	creamy white, circular and soft	short rods	G^-	-	-	γ-hemolysis
Bofr2	*Mugilidae*	transparent, circular and soft	short rods	G^-	-	-	γ-hemolysis
B2	*Oreochromis niloticus*	white, circular and soft	Cocci	G^+	-	+	γ-hemolysis
Bm3	*Scomberomo-rus cavalla*	transparent, circular and soft	bacilli	G^+	-	+	γ-hemolysis
Bofr5	*Mugilidae*	transparent and circular	bacilli	G+	-	-	γ-hemolysis
F1	*Mugilidae*	white, circular and soft	bacilli in chains	G+	-	-	γ-hemolysis
Bofr1	*Mugilidae*	chalky white, not circular and rough	cocci	G+	-	-	γ-hemolysis

for further study at -20°C. The plant extract yield has been calculated using the following equation Abdelbaky and Diab (2021).

Plant extract yield= ((extract weight)/ (dried plant weight)) *100

2.3 Assay of pomegranate peel ethanolic and aquatic extracts antibacterial potential

The antibacterial effect was estimated using the agar-well diffusion assay as described by Batiha et al (2021). One milliliter of standard inoculum was used to inoculate each Petri dish (9 cm in diameter) and cultured on nutrient agar to form a lawn of bacterial growth. Only 50 µl of the PPE was transferred into a well of 10 mm in diameter. The Petri dishes were kept at 4° C for 2 h to make a pre-diffusion of plant extract into the agar then propagated at 37°C for 24 h. The plates after incubation were investigated for clear zones of inhibition surrounding the wells, and the diameters of these zones were recorded in mm. The inhibition rate (IR) was calculated as follows:

%IR = ((clear zone diameter)/ (bacteria film diameter)) X 100

2.4 Minimum inhibitory concentration (MIC) determination

The agar-well diffusion technique was used for PPE MIC determination (Batiha et al 2021). A Petri plate was inoculated with one milliliter of each examined bacterial standard inoculum. The 10 mm well was produced with a sterile metal corkborer. Concentrations of 4.2 mg mL^{-1} to 267 mg mL^{-1} PPE were prepared. On agar plates with tested bacteria, 50 µl of each concentration was added to the well and then refrigerated for 2 h at 4°C to pre-diffuse plant extract into the agar. After 24 hours at 37°C, the diameter of the clean zones around the wells was measured. Chloramphenicol was used in positive control while DMSO served in negative control. The minimal PPE extract concentration that inhibited observable bacterial growth was the MIC. The clear zones were sub-cultured into nutrient agar medium (37°C for 24 h) and the activity of pomegranate peel extract (PPE) was examined. The absence of bacterial growth on nutrient agar plates indicated a bactericidal effect (Sader et al 2014).

2.5 Preparation of nano pomegranate peel (NSPP)

First, lyophilized pomegranate peel was prepared as follows: fresh pomegranate fruits, obtained from local markets, were socked gently for 2 minutes in 200 µL

L^{-1} NaOCl solution (sodium hypochlorite) to disinfect their surface. Afterward, The edible portions were manually separated after being cleaned and peeled. Small pieces of cleaned peels, approximately 1 cm × 1 cm in size, were prepared. These pieces were freeze-dried using a freeze dryer (CHRIST) at 0.12 mbar for 72 h- at -40°C, and a condenser temperature of -85 ± 1 °C.

Lyophilized LPP was converted into nanoparticles by the method of Mekawi et al (2019). The LPP was powdered using a grinder (Moulinex, Model MC300, France) to reach fine particles with an average of 100-150 µm. It was subsequently processed using high-energy planetary ball milling (Model PM 2400, Iran) for 2 h at a rotational speediness of 320 rpm to create LPP nanoparticles. This ball-milling process was performed (25°C and 1 atm) with a mass ratio of balls to powder conserved at a 10:1 ratio. Finally, the homogeneous nanoscale pomegranate peel (NSPP) was characterized using a Zeta sizer (Nano Sight NS300, UK), which indicated an average size of (80 ± 5 nm). NSPP were stored in a dark glass bottle (oxygen-free) until used. All NSPP preparation steps were carried out under controlled conditions to reduce the collapse of antioxidants.

2.6 Antibacterial and MIC of nano-scale pomegranate peel extract (NSPPE)

The antibacterial activity of MIC and NSPPE was determined as described above.

2.7 *In vitro* cytotoxicity of NSPPE using MTT test

To assess the cytotoxic effects of NSPPE on the Vero cell line (normal kidney CCL-81) and the Caco cell line (American Type Culture Collection), the MTT 3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) (Bio Basic Inc., Toronto, Canada) assay was employed (El-Sawy et al 2024). To establish a complete monolayer, 100 µL of approximately 1 x 10^5 cells mL^{-1} were added into each well of the 96-well tissue culture plate. The plate was then incubated for 24 hours at 37°C. The growth medium was decanted from the 96-well microtiter plates once a confluent cell monolayer was formed; the cell monolayer was then washed twice with washing media. In RPMI medium, twofold dilutions of the tested sample were prepared with 2% serum (maintenance med.) as supplementary. Nearly, 100 µL of each dilution was added to each well, while three wells were left as controls, containing only the maintenance medium. The plate was kept at (37°C) and checked for any visible signs of toxicity in the cells, such as partial or complete loss of monolayer, shrinkage, rounding, or cell granulation.

The MTT solution (5 mg mL^{-1}) in PBS (BIO BASIC CANADA INC) was prepared. To each well, MTT solution (20 µL) was added, and the plate was shaken for 5min/ at 150 rpm to mix the MTT into the media. The plate was incubator for (4 h)at 37°C in a 5% CO_2 atmosphere to facilitate MTT metabolism. The formazan product (MTT metabolic product) was then re-suspended in DMSO (~200 µL). Then the mixture was kept on a shaking plate (150 rpm) for 5 min to confirm the complete mixing of the formazan into the solvent. The optical density was then measured (560 nm), with background readings taken at (620 nm). The optical density (O.D) values were expected to correlate directly with cell quantity. Morphological changes in the cells, observed during the cytotoxicity study, were examined following treatment with the PPE. These changes included partial or complete loss of the monolayer, cell granulation, rounding, or shrinkage, which indicated potential toxicity. This trial was conducted at the Science Way for Scientific Research and Consultations Company in Cairo, Egypt).

2.8 GC/MS analysis of (NSPPE)

NSPPE was analyzed at the Faculty of Pharmacy, Ain Shams University, Egypt using GC/MS (Shimadzu GCMS-QP2020, Tokyo, Japan). An Rtx-1MS fused silica column (30 m (length) × 0.25 mm (inner diameter) × 0.25 µm film thickness, (Restek, USA) was utilized. The ion source temperature was set at 200°C, and the ionization mode was adjusted to 70 eV. The temperature employed program was 45°C for two minutes, ramped up to 300°C over five minutes, and then held at 300°C for five minutes (isothermal). Helium was the carrier gas, and the injector temperature was 250°C; The flow rate was (1.41 mL min^{-1}). Samples were diluted to 1% (v/v) and injected in split mode (split ratio 1: 15). The data were searched against the Wiley and NIST Mass Spectral Data Base libraries.

2.9 Applying NSPPE as a natural preserving agent in processed fish kofta

The Nile tilapia fish samples (3 kg) were obtained from the fish farm of (the Faculty of Agriculture, Ain Shams University, Egypt), and directly transferred to the lab in an ice box. The samples were immediately prepared by removing the heads, fins, and other parts,

then gutted, cleaned and thoroughly washed under tap water; then they were skinned and filleted. The yield of flesh was 37.5%. The prepared fillets were cut and minced using a kitchen mincer (Tornado FB 1000S). The minced fish was then mixed with various ingredients to produce Nile tilapia fish kofta mince consisted of 87% Nile tilapia fillet mince, 6.5% green coriander, 1.3% fresh garlic, 1.7% fresh onion, 1.7% salt and 1.3% spices mixture (comprising: 25% cardamom, 25% black pepper, 10% red pepper, 10% cinnamon, 20% Chinese cubeb and 10% laurel leaf) (Toliba et al 2019). This mixture was divided into four batches. The first batch was supplemented by 5 mL of distilled water as the control sample (coded FK Control). The second, third and fourth batches were mixed with 0.25, 0.37 and 0.5 mg mL^{-1} of NSPPE, respectively. The samples were then stored for two months at -15 ±2°C.

2.10 Chemical analysis of manufactured fish kofta

Crude protein, fats, ash, pH, titrable acidity and moisture of manufactured fish kofta samples were chemically analyzed as described by AOAC (2023).

2.11 Sensory evaluation of fish kofta

To evaluate the consumption quality of laboratory-produced fish kofta and analyze sensory properties (taste, texture, aroma, appearance and overall acceptability), a panel of 12 evaluators (7 females and 5 males) was formed. The panel included postgraduate students, faculty members and undergraduate students at the Faculty of Agriculture at Ain Shams University, as well as their families. A 9-point hedonic scale, ranging from 0 (lowest) to 9 (highest), was utilized following the methodology outlined by Coda et al (2012). The scorecard developed by Fawzi et al (2022) was utilized for the hedonic rating test.

2.12 Statistical analysis

IBM SPSS Statistics software version 23 was utilized for analyzing the collected data, employing Duncan's Multiple Range Test at a significance level of 0.05. A one-way analysis of variance (ANOVA) was performed to assess the differences between groups.

3 Results and Discussion

3.1 Antibacterial potential of pomegranate extracts

Data in **Table 2** exhibited that pomegranate extracts had 100% spectrum effectiveness against all tested bacterial cultures. The clear zones of pomegranate peel ethanolic extract (PPE) were 10.5–26.5 mm, whereas those of PPA were 10–25.5 mm. When exposed to PPA or PPE, bacterial isolate B2 (G^{+ve} cocci) had the largest inhibition zone diameter (IZD) followed by F5 isolate (18.5 mm & 19.5mm) of PPA and PPE. In contrast, bacterial isolate No. F2 (G^{-ve} short rods) and *Bacillus cereus* DSMZ345 had the lowest IZD when exposed to PPA or PPE. The studied bacterial isolates were categorized on PPE susceptibility: B2 > F5 = B7 > Bm3> F2. Pathogenic bacteria *E. coli* 0157H7 and *Staphylococcus aureus* As4 were more susceptible to PPE and had the highest IR%s of 20 and 22.2%, respectively. PPE has a wider inhibitory spectrum than PPA throughout the studied bacterial cultures, suggesting it has considerable antibacterial capabilities. Furthermore, PPE achieved a higher extract yield of 13%, whereas the yield for PPA is only 9%. Due to its flexibility, large concentrations of ethanol may dissolve polar, semi-polar, and non-polar compounds. Rebai et al (2023) found that ethanol extracts of pomegranate peels have more total phenols and flavonoids than methanol and acetone extracts. Both microorganism type and total phenolic content (TPC) correlated with extract antibacterial activity. Pomegranate peel extracts had the highest antioxidant activity and TPC, making them essential to the best antibacterial combination (Skenderidis et al 2021). Pomegranate peel alcohol extract inhibited microbiology better than water. According to Pradeep et al (2008), alcohol extracts were more effective than raw extracts from ripe and immature fruits. Pomegranate peel alcohol extract inhibited E. coli bacteria better than water extract. Alcohol extract had a 30 mm inhibitory concentration, whereas water extract had 25 mm (Talib et al 2024).

3.2 Determination of MIC and action of PPE

The minimum inhibitory concentrations (MICs) for the pomegranate peel extract (PPE) varied among the bacterial isolates under investigation, as shown in **Fig 1**. The lowest observed MIC was for bacterial both isolates No. B2 and B7, which was 6.25 mg mL^{-1}, followed by isolate F5 (8.3 mg mL^{-1}). The greatest MICs were observed for isolate Bm3 (G^{+ve} Bacilli), at 50 mg mL^{-1}. Generally, PPE exhibited a bacteriostatic effect against all tested bacterial isolates. The presented data indicate that PPE was more active against G^{-ve} bacteria than

Table 2. Antibacterial activity of pomegranate peel extracts against bacterial isolates

Tested bacterial isolates or pathogenic strains	Pomegranate peel aquatic extract (PPA*)		Pomegranate peel ethanolic extract (PPE*)	
	IZD (m.m)	IR (%)	IZD (m.m)	IR (%)
G- short rod F2	10	11.1	10.5	11.6
G- short rod F5	18.5	20.5	19.5	21.6
G- short rod B7	18.0	20	19.5	21.6
G- Bofr2	12.0	13.3	13.0	14.4
G+ cocci B2	25.5	28.3	26.5	29.4
G+ bacilli Bm3	18.0	20	18.5	20.5
G+ Bofr5	17	18.8	17.5	19.4
G+ F1	17	18.8	18.5	20.5
G+ Bofr1	16	17.7	17.0	18.8
***Bacillus cerues* DSMZ345**	10.5	11.6	12.5	13.8
***Listeria monocytogenes* DSMZ423**	16	17.7	17	18.8
***Staphylococcus aureus* As4**	18	20	20	22.2
***Pseudomonas aeruginosa* ATCC 27853**	12.5	13.8	13.5	15
***E. coli* 0157H7**	17	18.8	18	20

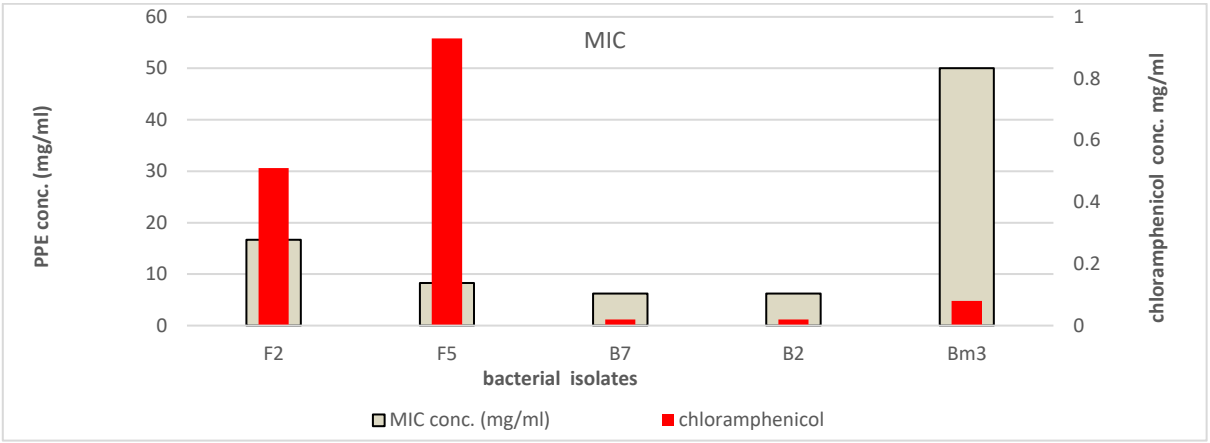


Fig 1. MIC of PPE against bacterial isolates

G^{+ve} bacteria. Other investigations stated that pomegranate also exhibited antibacterial activity at various concentrations against all tested bacteria. The most effective concentration was 4 mg mL^{-1} against *Pseudomonas aeruginosa* and 16 mg mL^{-1} against *Acinetobacter;* both *Staphylococcus aureus* and *Bacillus cereus* were inhibited at MIC (32 mg mL^{-1}) (Sharif et al 2020). The pomegranate peel extract (PPE) hydro-alcoholic compounds demonstrated a strong antibacterial activity against strains of MRSA and *St. aureus* ATCC25923, with MIC and minimum bactericidal concentration (MBC) values ranging from 4 to 12 µg $µL^{-1}$. PPE exhibited both bacteriostatic and bactericidal effects.

For other microorganisms, the minimum inhibitory concentration (MIC) values were 20 mg mL^{-1} for *Salmonella enterica* ATCC 14028, 40 mg mL^{-1} for *Escherichia coli* ATCC 25922, and 10 mg mL^{-1} for *Staphylococcus aureus* ATCC25923. For all three microbes, the minimum bactericidal concentration (MBC) has been determined to be 80 mg mL^{-1} (Forgione et al 2024).

3.3 Preparation and antibacterial potentiality of nano-scale pomegranate peel (NSPP)

To enhance the antibacterial potential of pomegranate peel, it was converted into nanoparticles for increasing extraction efficiency using a ball-mill. This conver-

sion into nanoparticles could improve the bioavailability and efficacy of the active compounds contained in the peel, potentially leading to stronger antibacterial effects. The nano-scale pomegranate peel (NSPP) was produced by ultrafine ball milling of LPP which efficiently reduced the original particle size to the nanoscale being 89.07 nm (**Fig 2**). The antibacterial potential of NSPPE was enlarged against all tested bacteria compared to that of PPE as shown in **Fig 3**.

A remarkable increase in the inhibition zone diameter (IZD) of NSPPE was observed, reaching 34.5 mm (about 3fold augmented) against the F2 isolate followed by an increase of about 26.3% in the IZD against the B7 isolate. Moreover, MIC of Bm3 decreased significantly from 50 to 0.06 mg mL^{-1} (more than 99% loss) and about 98.5% loss in MIC figures for F5 and F2 isolates. As mentioned by Muhammed et al (2021), the sol-gel method of producing the nano pomegranate peel solution at 80°C, is one of the simplest methods for preparation and AFM analysis and was investigated by Atomic Force Microscopy (AFM) shows a diameter range of 64.5 nm. The nano solution's antibacterial activity shows a diameter of 25-27 mm in *Staphylococcus aureus* and *Escherichia coli* compared to the crude solution (20 and 15 mm). The presence of these elements enhances its efficiency, making it a promising candidate for different pharmaceutical applications in the future. From the above-mentioned data, nano-technology was very efficient in amplifying the antibacterial of NSPPE.

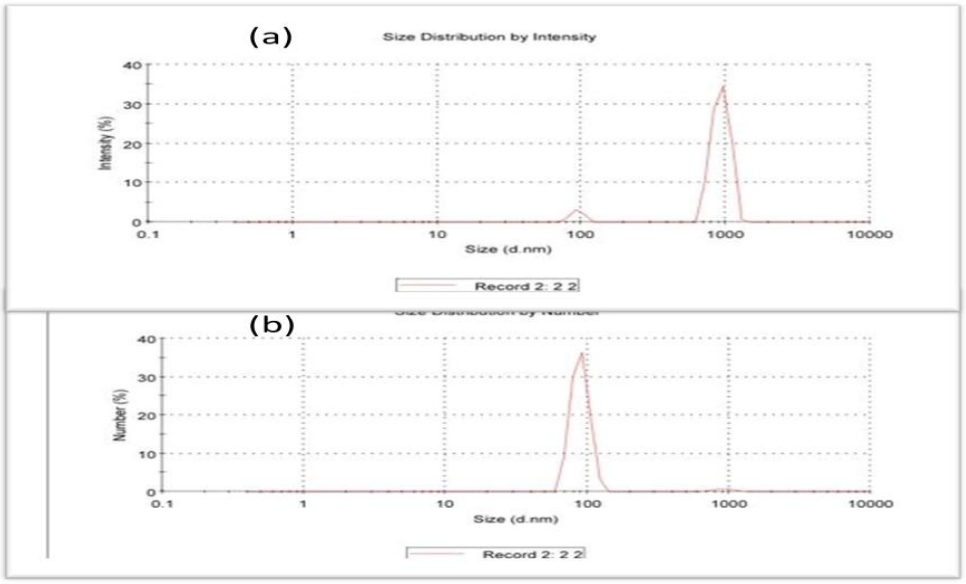


Fig 2. Size distribution plot by Number percentage of NSPP (a) distribution by intensity (b) distribution by number

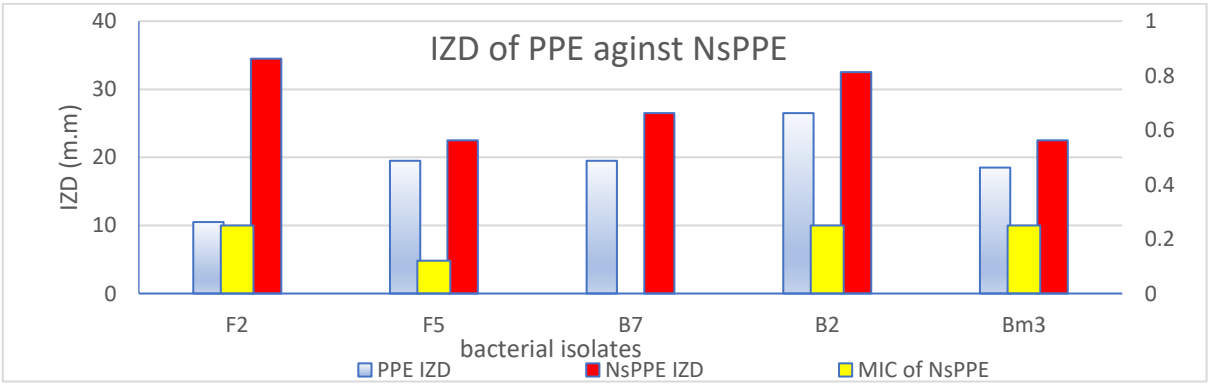


Fig 3. IZD of PPE, NSPPE and NSPPE MIC against tested bacterial isolates

3.4 *In vitro* cytotoxicity screening using MTT assay

MTT test was applied to assay the proliferation of cancer cells and the cytotoxicity of NSPPE on the Vero normal kidney cell line and Caco cell line. The Vero normal kidney cell line was exposed to NSPPE at an average concentration ranging from 31.25 to 1000 µg ml^{-1} to ensure the safety of the NSPPE. The cell viability percentage exceeded 100% when the cells were exposed to 31.25 µg ml^{-1} NSPPE. Increasing concentrations from 62.5 to 1000 µg ml^{-1} reduced the cell viability ranging from 99.9 to 98.18%, while cytotoxicity reached 1.81% at the highest NSPPE concentration. After being treated using different concentrations of NSPPE, the Vero cell lines displayed a normal morphological appearance (**Fig 4).**

According to the International Organization for Standardization (ISO 10993-5), The cell viability percentages are divided into different categories as follows: more than (80%) is considered non-cytotoxic, (80%–60%) is classified as weakly cytotoxic, (60%–40%) is classified as moderate cytotoxic, and below (40%) is considered strongly cytotoxic (López-García et al 2014). Regarding the effect of NSPPE extract in concentrations ranging from (31.25 to 1000) µg ml^{-1} on the cell cycle profile, it was observed that cytotoxicity on Caco-2 cells was low at concentrations ranging from 31.25 to 125 µg ml^{-1}. A gradual increase in cytotoxicity was observed with increasing NSPPE concentrations, reaching the highest level of 84.22% at 1000 µg ml^{-1}. To ascertain the half-maximal (50%) inhibitory concentration (IC50), GraphPad Prism version 5 was utilized. For the Caco cell line, the IC50 of NSPPE was 339.76 ± 13.9 µg ml^{-1} (**Fig 5).**

Additionally, when exposed to NSPPE lower than 125 µg ml^{-1}, the Caco cells remained viable and exhibited standard adherence. At higher concentrations (>125 µg ml^{-1}) of the ethanolic extract, significant changes in cell morphology were evident, notably apoptotic features such as cell shrinkage, presence of cell debris and substantial reductions in cell count, indicating cell death compared to control. Polyphenols in PPE have been shown in many studies to have anti-proliferative and antitumor activities in different cancer lines (Deng et al 2017). The pharmacological informed that pomegranate derivatives have established a wide variety of medical practices for the treatment of chronic inflammatory diseases and cancer prevention. In the previous few years, numerous scientific studies have suggested that pomegranate constituents possess antioxidant and anti-carcinogenic activities, proving effective against various varieties of cancer (Nasr et al 2023).

3.5 Analysis of NSPPE by GC–MS

Collected data presented in **Table 3** pointed to the phytochemical compounds identified in the NSPPE through GC-MS analysis. The results include peak area percentage (RA%), retention time (RT%), molecular weight (MW), mass spectra and name of the metabolite. The extract consists of 14 distinct phytochemical compounds. Upon comparing the mass spectra of the constituents with the NIST library the 13 phytochemical compounds were ((E)-9-octadecenoic acid ethyl ester); (di-*n*-octyl phthalate); (di-*n*-octyl phthalate); (9-octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester); (1,3-benzenedicarboxylic acid, bis (2-ethylhexyl) ester); (stigmasta-5,22-dien-3-ol,acetate,(3.beta.)); (stigmastan-3,5-diene); (dl-.alpha.-Tocopherol); (Campesterol); (beta.-Sitostero); (fucostero); (9,19-cyclolanost-23-ene-3,25-diol,3-acetate, 3.beta.,23E)) and (D:A-friedooleanan-28-al, 3-oxo-) as well as not identified compound (peak Np.3). The maximum area percentage was obtained by beta.-Sitostero (80.1%) appeared at RT 55.828 min. In addition, the main phytosterols isolated from *P. granatum* (pomegranate) seed, stem bark and skin tissues were β-sitosterol, which exhibited antibacterial properties against both *S. typhi* and *E. coli*. β-sitosterol inhibits a bacterial cell surface protein, which stops transpeptidation from happening (Nweze et al 2019). Moreover, β-sitosterol has no cytotoxic effects on non-cancerous cells and inhibits the growth of cancer cells even at minimal concentrations (Jayaprakasha et al 2007). The efficacy of β-sitosterol has been observed across a wide range of cancer types. Cell lines such as HL60 and MCF-7 are used in breast malignancy research (Tasyriq et al 2012), while cell lines COLO 320 DM and Caco 2 are employed in colon cancer (Baskar et al 2010).

3.6 Extermination of spoilage bacteria in fish kofta by NSPPE

Chemical compounds, such as chlorine dioxide, have widely been used for years to control the deterioration of fresh fish. However, due to growing concerns about food safety, there are increasing restrictions on the use of synthetic chemicals in food preservation (Viji et al 2017, Olatunde and Benjakul 2018) and using natural phytochemicals as alternatives for food preservation (Huang et al 2017, Lytou et al 2018).In the present

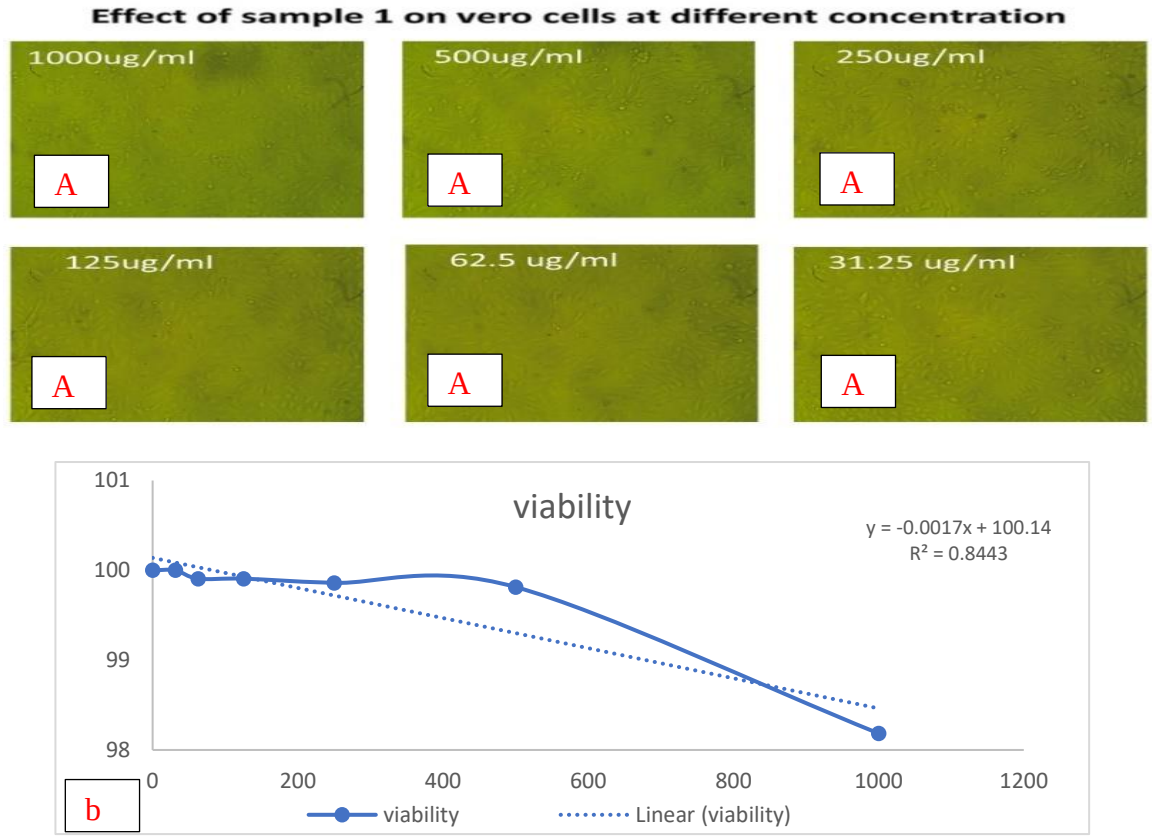


Fig 4 (A and b). The inhibitory effect of NSPPE on the proliferation of the Vero cell line (a normal kidney CCL81). A-Changes in morphology in the cell line; b- Cell line viability

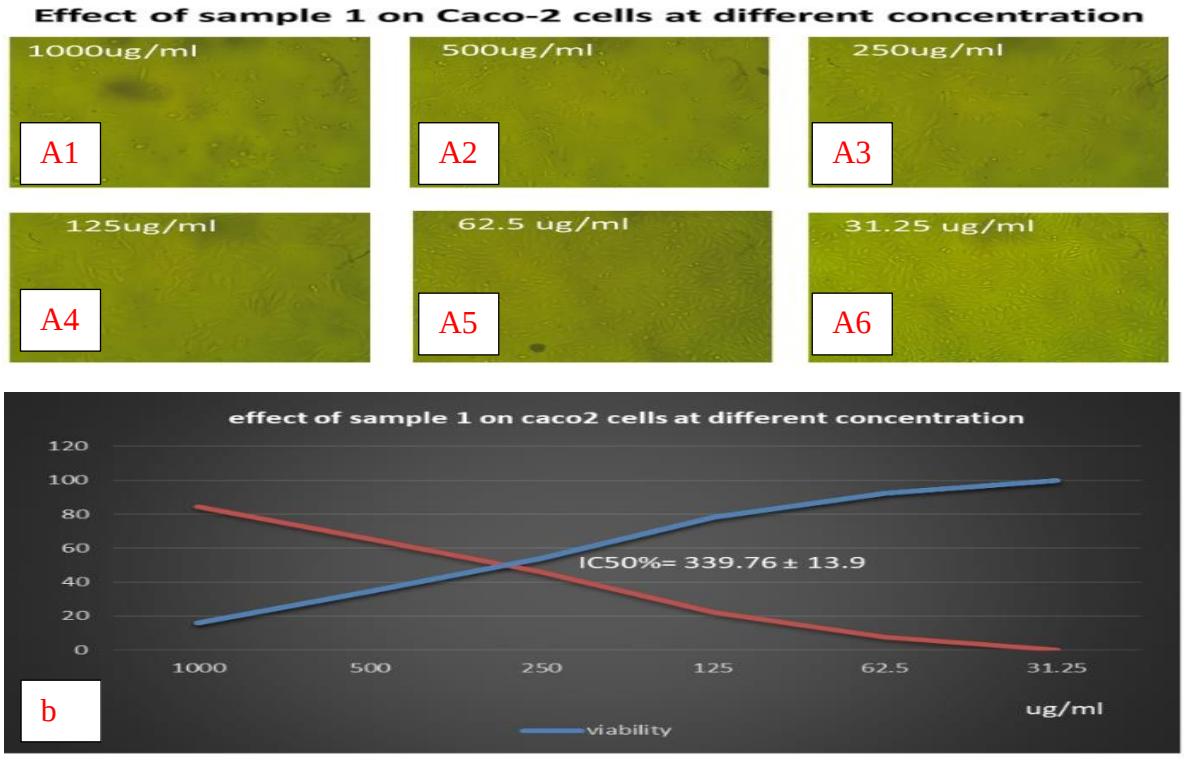
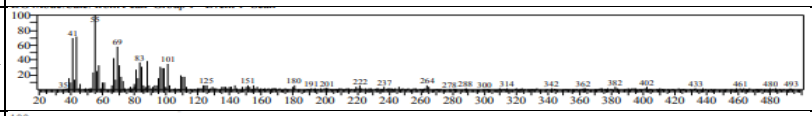
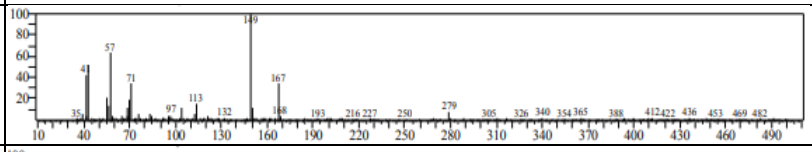
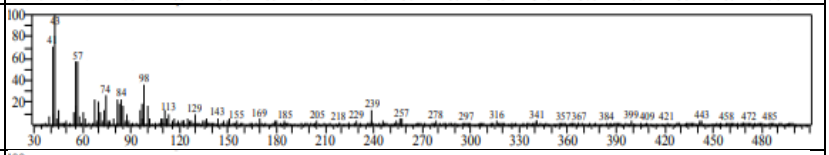
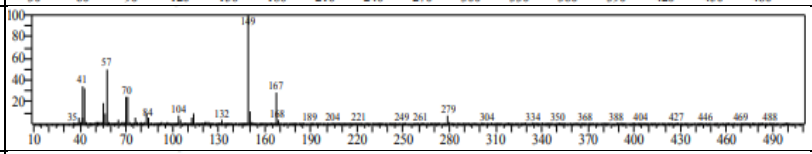
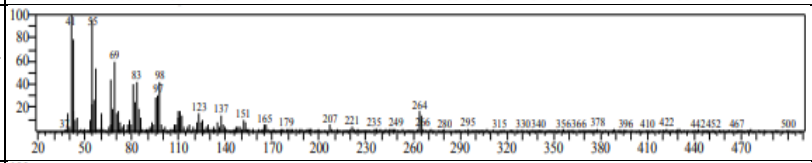
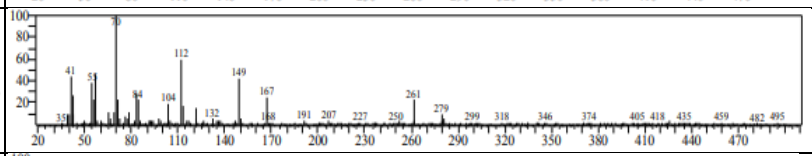
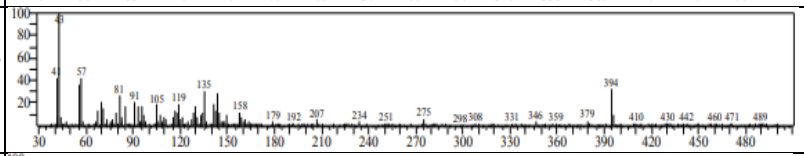
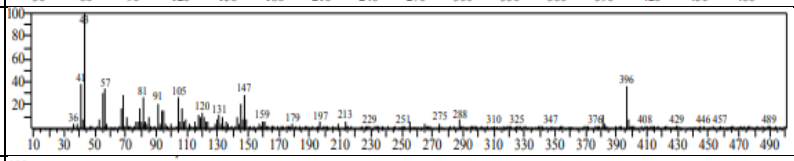
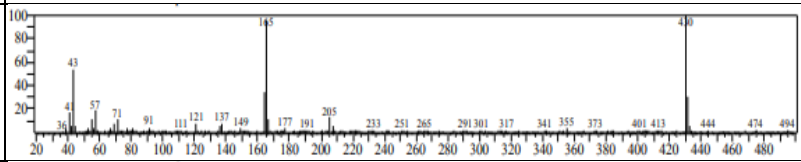
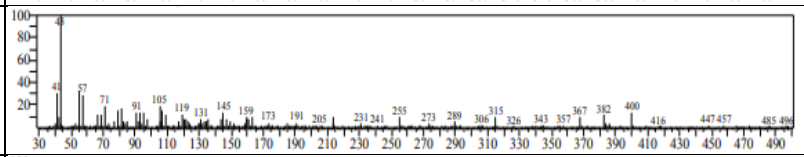
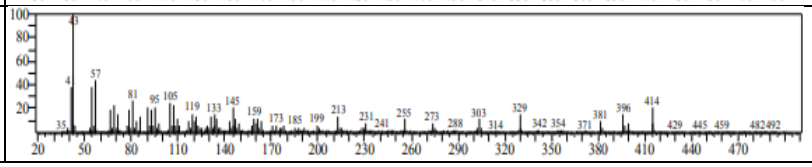


Fig 5 (A and b). The inhibitory effect of NSPPE on the proliferation of the caco cell line.
A- Changes in morphology in the cell line; b- Cell line viability

Table 3. GC-MS of nanoscale pomegranate peel ethanolic extract.

Peak	Rt (min)	RA (%)	MW	Name of the	Mass spectrum
1	37.857	0.48	310	(E)-9-Octade-cenoic acid ethyl ester	
2	43.651	0.61	390	Di-n-octyl phthalate	
3	43.779	0.39			
4	44.413	8.60	390	Di-n-octyl phthalate	
5	46.624	1.97	356	9-Octadecenoic acid (Z)-, 2,3-di-hydroxypropyl ester	
6	47.404	0.35	390	1,3-Benzenedi-carboxylic acid, bis(2-ethylhexyl) ester	
7	52.193	0.70	454	Stigmasta-5,22-dien-3-ol, ace-tate, (3.beta.)-	
8	52.485	0.63	396	Stigmastan-3,5-diene	
9	52.867	0.58	430	dl-.alpha.-To-copherol	
10	54.329	1.19	400	Campesterol	
11	55.828	80.10	414	.beta.-Sitostero	

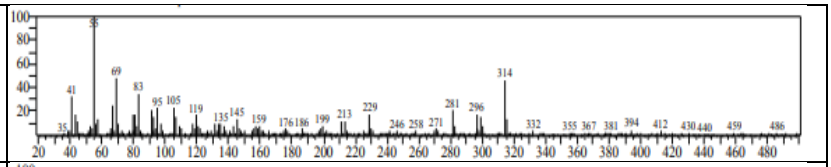
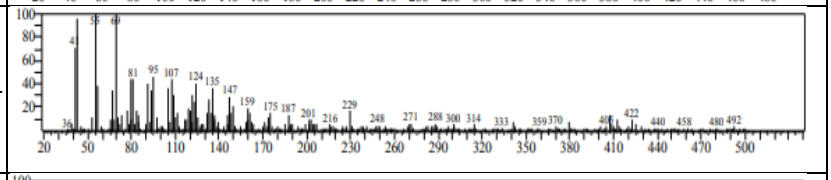
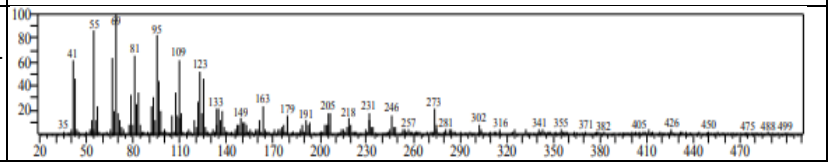
12	56.048	1.07	426	Fucostero	
13	58.221	1.44	440	9,19-Cyclolanost-23-ene-3,25-diol, 3-acetate, (3.beta., 23E)-	
14	60.059	1.90	206	D:A-Friedool-eanan-28-al, 3-oxo-	

Table 4. Microbial load (log CFU/g) of fish kofta supplemented by different conc. of NSPPE during 8 weeks of storage period under frozen condition.

Examined micro-organism	**NSPPE conc. (mg/ml)**	**Storage period (weeks)**					Mean
		0	**2**	**4**	**6**	**8**	
Total count	0	4.46	2.88	2.6	2.6	2.7	2.491[b]
	0.25		2.6	<1.5	<1.5	<1.5	
	0.37		2.6	<1.5	<1.5	<1.5	
	0.5		2.59	-	-	-	
Yeast	0	3.06	2.7	2.7	2.9	2.8	2.4525[b]
	0.25		2.7	2.6	2.6	-	
	0.37		2.6	2.6	<1.5	-	
	0.5		2.6	2.5	<1.5	-	
***Staphylococcus* Sp.**	0	2.7	2.6	2.6	2.6	2.6	2.0913[a]
	0.25		2.6	<1.5	-	-	
	0.37		2.5	<1.5	-	-	
	0.5		2.5	-	-	-	
E. coli	0	3.06	2.9	2.6	2.6	2.6	2.0368[a]
	0.25		2.6	-	-	-	
	0.37		2.6	-	-	-	
	0.5		2.5	-	-	-	
Mean		3.2913[B]	2.625[B]	1.91[A]	1.7875[A]	1.725[A]	
sources	**Df**	**F-value**	**p-value**	**R^2**			
Statistical analysis of variation (ANOVA)							
Corrected model	10	40.637	0.0001*	0.732			
Intercept	1	4629.62					
Microbial group	3	12.638					
Storage period	4	82.269					

-= no growth was observed, fecal coliform, Fungi, Spore forming bacteria, *Pseudomonas*, *Salmonella and Shigella*. a, b The values in small letters in the same column with differing superscripts or A, B The values in capital letters in the same line with differing superscripts indicate a significant difference ($p \leq 0.05$). *, significant.

work, manufactured fish kofta, supplemented with different concentrations of NSPPE at 0.25, 0.37, and 0.5 mg mL^{-1}, as well as fish kofta without NSPPE (control), were stored in frozen conditions for 8 weeks. Every two weeks, samples were microbiological, chemical and sensory inspected. Data illustrated in **Table 4** indicated that all bacterial groups were observed at zero-time in the following ascending order: *Staphylococcus aureus* (2.7 log CFU g^{-1}) < *Escherichia coli* = yeast (3.06 log CFU g^{-1}) < total count (4.46 log CFU g^{-1}). Upon extending the storage times beyond 2 weeks, the total bacterial count of *Staphylococcus aureus*, and *Escherichia coli* were eliminated in samples containing 2 MIC NSPPE. *E. coli* and *Staphylococcus aureus* showed the same trend in samples with all NSPPE concentrations when the storage period exceeded 6 weeks while fish kofta without NPPE recorded a bacterial count of 2.6 CFU g^{-1} for total count, *Escherichia coli* & *Staphylococcus aureus* and 2.9 CFU g^{-1} for yeast. The count of yeast declined with increasing storage time and was completely absent by the end of the storage period. NSPPE showed significant antibacterial activity against spoilage bacteria in fish kofta with good R2 being 0.732; the microbial groups count mean ranged from 2.036- 2.4913 CFU g-1 while the mean of storage period ranged from 1.725- 3.2913 CFU g-1. Generally, all fish kofta samples, whether treated with NSPPE or not, were free of fecal coliforms, spore-forming bacteria, *Salmonella*, *Shigella* and fungi throughout the storage period.

Chemical compositions of fish kofta at zero time and after 8 weeks of storage period under frozen conditions were illustrated in **Table 5**. Moisture, ash, fat, protein, fiber, pH and TA of fish kofta at zero time were 72.28, 3.04, 3.34, 11.59, 7.55, 6.7 and 0.12% respectively. After 8 weeks of storage period, the contents for fish kofta were in the range 72.22- 72.81, 3.01- 3.05, 3.03- 3.39, 11.19- 11.6, 7.63- 7.96 and 0.10- 0.14 % respectively.

In addition, the Sensory evaluation of fish kofta samples was executed by a panel of 12 trained evaluators to evaluate the samples' appearance, texture, aroma, taste and overall acceptability as presented in **Table 6**. Samples treated with 0.0, 0.25, 0.37 and 0.5 mg mL^{-1} NSPPE scored overall acceptability 8, 8, 7.8 and 7.8 respectively indicating fine overall product acceptability. All tested NSPPE did not show any significant difference at $p < 0.05$.

Table 5. Chemical analysis of fish kofta at zero time and after 8 weeks of storage under frozen condition.

Storage period (weeks)	NSPPE conc. (mg/ml)	Moisture %	Protein %	Fat %	Ash %	Fiber %	Carbohydrate (%)	pH	Acidity %
Zero	0	72.28	11.59	3.34	3.04	7.55	2.2	6.7	0.12
8	0	72.81	11.19	3.03	3.01	7.96	2	6.9	0.14
	0.25	72.23	11.60	3.39	3.04	7.64	2.1	6.8	0.11
	0.37	72.29	11.53	3.33	3.02	7.63	2.2	6.8	0.11
	0.5	72.26	11.58	3.33	3.05	7.58	2.2	7.1	0.10

Table 6. Sensory properties evaluation score of fish kofta treated with different NSPPE conc. (mg/ml).

properties	NSPPE conc. (mg/ml)				Mean
	0 (control)	0.25	0.37	0.50	
Appearance	9^{a}	9^{a}	8.8^{a}	8.8^{a}	8.916^{A}
Texture	8.25^{b}	8.25^{b}	7.9^{b}	7.8^{b}	8.062^{C}
Aroma	$8.5a^{b}$	8.5^{ab}	8.25^{ab}	8.25^{ab}	8.375^{B}
Taste	7.9^{b}	7.9^{b}	7.8^{b}	7.8^{b}	7.875^{C}
Overall-acceptability	8^{b}	8^{b}	7.8^{b}	7.8^{b}	7.916^{C}
mean	8.3333^{A}	8.3333^{A}	8.1333^{A}	8.1167^{A}	
sources	**df**	**F-value**	**p-value**	**R2**	
Statistical analysis of variation (ANOVA)					
Corrected model	7	9.630	0.0001*	0.225	
Intercept	1	28557.97			
NSPPE conc.	3	1.53	0.207		
properties	4	15.704	0.0001*		

a,b values with small letters in the same column having different superscripts or A, B values with capital letters in the same row with different superscripts are significant differences (at $p < 0.05$), * significant,

4 Conclusion

Pomegranate peel ethanolic (PPE) and aqueous (PPA) extracts have broad-spectrum antibacterial action, with PPE better activity. Transforming PPE into nano-scale particles (NSPP) greatly increased its antibacterial effectiveness and lowered its MIC. MTT test was applied to assay the proliferation of cancer cells and the cytotoxicity of NSPPE on the Vero normal kidney cell line and Caco cell line. NSPPE was an efficient natural preservative in fish kofta, preventing bacterial development and increasing shelf life. This research suggests that NSPPE may be a natural, effective preservative that might improve food safety.

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