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Research Article

# Nanoparticles Titanium Dioxide with *Thymus vulgaris* extract in preservation and prolong the shelf life of cheese

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**Abstract**
Cheese is considered a perishable food that is affected by microorganisms, and due to the properties of nanomaterials that have antimicrobial activity, they have been used synergistically with plant extracts in inhibiting the action of microorganisms that cause cheese spoilage. In this study, TiO2 (Titanium dioxide) nanoparticles were synthesized using *Thymus vulgaris* leaves extract (TVLE) . Atomic Force Microscopywas used to investigate Titanium dioxide/ TVLE nanoparticles characterize, which improved the regular spherical shape and granular distribution of nanoparticles with a particle size of 13 nm . The results showed that the minimum inhibitory concentration (MIC) of Titanium dioxide was at a concentration of 4 mg/ml and 80 mg/ml for TVLE, while it was 2 + 20 mg /ml for Titanium dioxide and TVLE .The inhibitory effect increased against *Brucella melitensis* recorded 12 mm when mixing Titanium dioxide and TVLE, compared with the inhibitory effect of Titanium dioxide, which recorded 10.5 mm and TVLE, with an inhibition diameter 8.1 mm. The effect of using titanium particles and thyme leaves' extract was studied alone at a concentration of 4 mg/ml and 80 mg/ml and also when mixed in the microbial properties and pH of white soft cheese samples, which were prepared in the laboratory and contaminated with *Brucella melitensis* at refrigerated storage conditions (5C°) for 21days. The effect of the synergism relationship between TiO2 / TVLE significantly reduced the total number of microorganisms in samples contaminated and uncontaminated with *B. melitensis*. Adding titanium dioxide and TVLE at concentrations of 4 and 80 mg/ml contributed significantly to maintaining the pH level during the storage period compared with the control group.

**Keywords:** Cheese, nanoparticles, shelf life, *Thymus vulgaris*, Titanium dioxide

## INTRODUCTION

Million deaths and diseases are caused every year worldwide by pathogenic microorganisms responsible for many cases of foodborne disease (Bintsis,2017). Cheese, specifically soft cheese, is a suitable food environment for the growth and reproduction of countless types of microorganisms because it contains the necessary nutrients present in high moisture, proteins, lactose, minerals, vitamins and salts, as these organisms play a vital role in bringing about many physiological, chemical and sensory changes in cheeses and determine the suitability if it is suitable for human consumption or not ( Pepi and Focardi,2022 ) . Cheese is contaminated with many kinds of pathological microorganisms, represented in *Staphylococcus aureus, Listeria monocytogenes, Escherichia coli, Shigella dysenteria, Salmonella typhimurium* , etc ( Munera Bedoya *et al*.,2017).

Many ways have been found to preserve food, extend its storage life for as long as possible, and make it fit for human consumption. Despite chemical preservatives being common in food preservation techniques and having a very wide inhibitory range, because of the increasing causes of resistance shown by pathological microorganisms towards these preservatives, as well as their responsibility for chronic and dangerous diseases, such as cancer, it was necessary to discover alternative materials for the possibility of using them in food preservation (Gupta and Yadav, 2021).

On the other hand, medicinal herbal plants are recognized as alternative sources of chemical materials in some food preservation processes due to their antimicrobial effect in eliminating or reducing spoilage microorganisms present in active substances (flavonoids, glycosides, phenols, alkaloids, resins, tannins, volatile oils, coumarins and fixed oils (Gonelimali *et al*.,2018).

Previous reports reveal the antimicrobial effectiveness of different Thymus parts ( flowers, branches, leaves, roots) towards bacteria responsible for food spoilage (Sikorska-Zimny *et al*., 2021 ). Despite its high inhibition efficacy, other means of preservation needed to be devised that have broader ranges in eliminating the largest number of food-contaminant pathogens (El-sayed and El-sayed, 2021).

There is a growing interest in nanotechnology. Nanotechnology, is a term given to any nanoparticle or material with dimensions of 100 nanometers or less. Possession of Titanium dioxide (TiO2) nanoparticles of electrical, voltage and reflective properties encouraged its inclusion in medicine, paper making, pharmacy, cosmetics, and environmental engineering and food industries (preservation, cultivation, processing, packaging, etc.) fields (Xiaojia *et al*., 2019). It was applied in the food sector by more than (300 companies) around the world (Jafari *et al*., 2017) . A recent study worldwide found that more than 4 million titanium dioxide nanoparticles are consumed annually (Lim *et al*., 2017).

Perhaps one of the most common examples is the inclusion of titanium dioxide nanoparticles with the symbol E171 in the pastry industry, cakes and sweets as a whitener and colorant, especially gum coated with sugar, as it is devoid of any negative side effects that may affect the health of the consumer (Close and Frewer, 2013 and Dudefoi *et al*.,2017). The use of nanoparticles in encapsulating, preserving, and protecting food components and prolonging their storage life by inhibiting and killing microorganisms present in them is one of the new methods of food preservation. There are many reports using $TiO_2$–Nanoparticle size in the biological field as antibacterial agent due to the inhibitory effectiveness towards many microorganisms (viruses, bacteria, fungi), especially bacteria-causing diseases and poisoning. The small size of $TIO_2$–NPS facilitates their entry into the organism's cells and with their vital functions (Ramanathan *et al*.,2018).

TiO2 eliminates bacteria growth via different mechanisms by forming holes in bacterial cell walls through inactivation of nucleic acids and cellular enzymes that lead to generation and activation of free hydroxyl radicals (LongLiang *et al*.,2020). Many researchers refer to the combination between nanomaterials and the extracts of medical herbal plants as synergistic relation to reducing and eliminating microorganisms growth (Saka *et al*.,2022).

The study aimed to investigate the $TIO_2$–NPS and the *Thymus vulgaris* leaves extract in microbial properties and the shelf life of soft cheese with and without contamination with *Brucella melitensis*.

## MATERIALS AND METHODS

### Collection of leaf samples

Plant samples of *Thymus vulgaris* leaves were collected from the Ministry of Agriculture /Horticulture Directorate's fields and were scientifically diagnosed in the University of Baghdad ,College of Science, Biology Department, Iraq .To remove dusty materials and suspended impurities from the leaves, they were washed well with tap water, followed by distilled water., cleaned with brushes and left in the room to provide adequate ventilation with continuous stirring for good and homogeneous drying. The leaves were ground using a Silver Crest mill SC-7880

### Preparation of *Thymus vulgaris* leaves extract (TVLE)

The alcoholic extract of the leaves was prepared by soaking 30 g of the powder + 350 ml of 70% ethyl alcohol in 500 ml volumetric flask and left at room temperature for 4 hours with a Thermomagnetic stirrer. The mixture was filtered using a three-layer medical gauze cloth, then passed through Whatman No.1 filter paper. The filtrate was concentrated, and the alcohol was discarded by a rotary evaporator, followed by preservation in clean, sterile glass containers at 4 Cº until use (Anesini and Perez , 1993).

### Preparation of Titanium dioxide / TVLE nanoparticles

A basic solution of Titanium particles (which was supplied by the American Elements company) was prepared at a concentration of (100 mg/ml) using distilled water, then 10 ml of the solution was added to 25 ml of TVLE solution and adjusted the pH to 4.5, stirring hot plate was used at 400 rpm for 60 minutes and refrigerate employed at 5 Cº till use.

### Atomic force microscopy method

To explain the properties of Titanium dioxide/ TVLE nanoparticles, which are represented in roughness,size, and distrubtion of granular, AFM scanning was achieved by sparing a thin layer of the sample on glass slides base and the image was taken (Hameed *et al.*,2019 ).

### Preparation of the bacterial suspension

In order to obtain the bacterial suspension, 3- 4 small

*Brucella melitensis* young colonies were harvested from Muller-Hinton Agar medium and transferred to 10 ml of Muller-Hinton broth by loop, and the tubes were incubated at 37 °c for 48 hours.

**Laboratory manufacturing of white cheese**

The modified method (Foda *et al*., 2010) was followed in the manufacture of soft white cheese, where the milk was pasteurized at 63°c for 30 min, chilled to 45°c, after that, inoculated with previously prepared concentrations of Titanium dioxide and TVLE , followed the addition of rennet, where the milk was left for 45 minutes until the curd formed The curd was cut into small pieces and left to get rid of the whey and placed in a layered cloth to allow the largest amount of whey to be removed, then packed into templates prepared for this purpose . Also, some samples were contaminated with *B. melitensis*, which causes food poisoning obtained from Al-Kadhimiya Teaching Hospital / Baghdad, based on turbidity equal to the turbidity of the standard McFarland tube No. 5.0.

**Determination of the MIC of TiO2 and TVLE against *Brucella melitensis***

Ascending concentrations of Titanium dioxide (1,2,4,6,8,10) mg/ml and TVLE (5,20,40,80,160,320) mg/ml were prepared, and 9 ml of nutrient broth was added to clean test tubes, followed by addition of 100 microliters of Titanium dioxide and TVLE, except for the control tube, then 100 microliters of *B. melitensis* suspension were added to all tubes and incubated at 37 °c for 24 hours. The apparent turbidity was compared with the negative control tube that contained the culture medium and bacteria without concentrations to determine MIC (Al-Amrii *et al*, 2009 ).

**Estimation of the inhibitory action of TiO2 and TVLE against *B. melitensis***

The method of agar gel diffusion to detect the antibacterial activity of Titanium dioxide and TVLE towards *B. melitensis* was based on the use of holes with a diameter of 6 mm instead of paper discs, as the medium of Muller-Hinton Agar was punctured and inoculated with 100 microliters of the bacterial culture at a cell count of 1. 5 X $10^8$ c.f.u/ml based on MacFarland's standard solution, 100 microliters of TIO2 and TVLE were placed into each hole. The results of the apparent inhibition diameters on the surface of the medium around each hole were measured in mm using a graduated measuring ruler after incubating the dishes at a temperature of 37 °c. for 24 hours (Balows *et al*., 1987).

**Statistical analysis**

The results and data of the experiment were analyzed by applying Duncan's test statistical program based on the level of significance 0.05 .

## RESULTS AND DISCUSSION

Fig. 1 shows the regular topographic distribution of Titanium dioxide / TVLE nanoparticles taken by the atomic force microscope (AFM), which shows that the largest characteristic size of the material reached 13 nanometers. The granular size of the nanoparticles ranged from 1-13 nm, while the average surface roughness (RMS) was 2.30 nm (Ragab *et al*,2022 ).

**Determination of the MIC and lethal concentration of TiO2 and TVLE against *Brucella melitensis***

Table 1 shows that the minimum inhibitory concentration (MIC) of TVLE was 80 mg/ ml , which was lower in

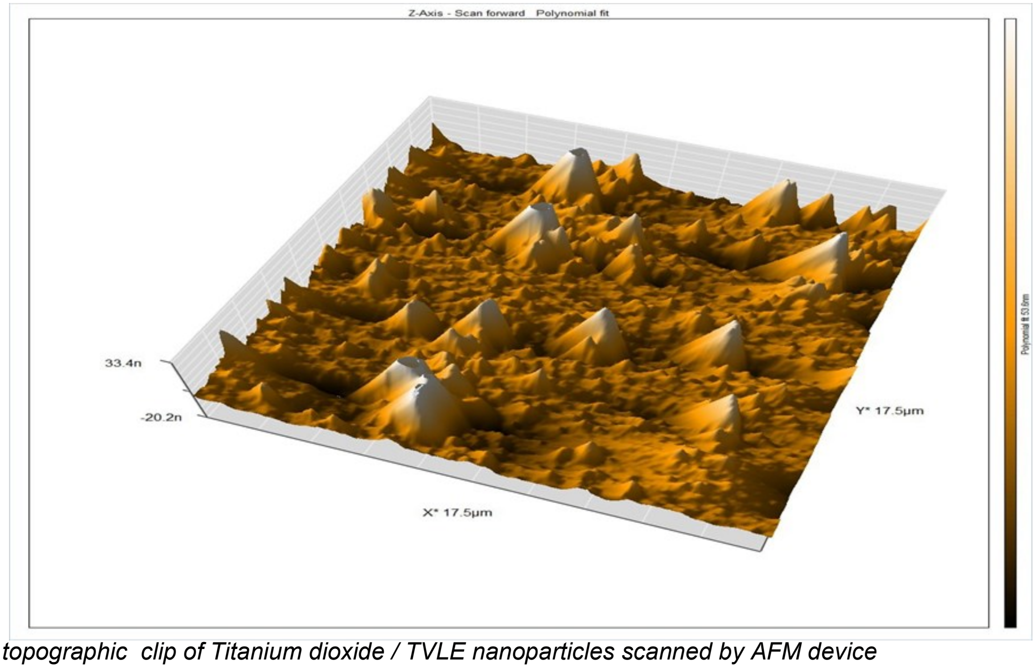


**Fig. 1.** *3D topographic clip of Titanium dioxide / TVLE nanoparticles scanned by AFM device*

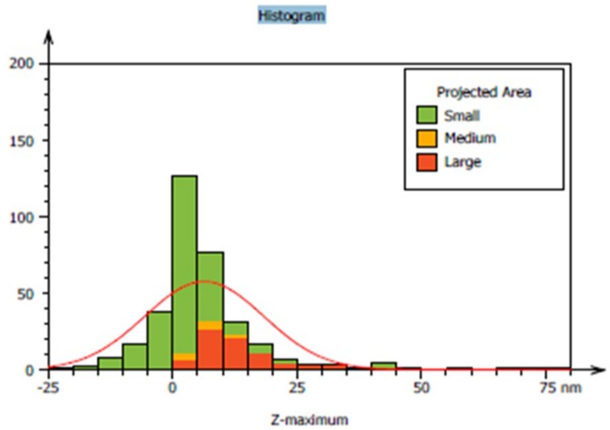


**Fig. 2.** *Particle size distribution of the nanomaterial*

inhibition activity than the concentration of TiO2 nanoparticles (4 mg/ ml). The maximum effect was observed when mix ( 2 mg/ml of TiO2 + 20 mg/ml of TVLE) with increased TiO2 and TVLE concentrations, confirming their synergistic inhibition towards *B. melitensis*.

**Estimation of the inhibitory activity of TiO2 and TVLE against *Brucella melitensis***

The results in Table 2 show the inhibitory diameters with $TiO_2$ and TVLE separately and when mixed against *B. melitensis*. The $TiO_2$ were superior, which gave an inhibitory diameter of 10.5 mm alone and 12.0 mm when mixed with TVLE compared with TVLE alone, recorded 8 mm, indicating bacteria's sensitivity towards $TiO_2$ in general.

**Estimation of the total microbial counts of cheese samples treated with TiO2 and TVLE and contaminated with *B. melitensis***

Table 3 indicates that the microorganisms numbers in the uncontaminated samples ranged between 42 $\times 10^2$ and 5 $\times 10^4$ after 1 hour of preservation, while the contamination caused an increase in the number of contaminated microorganisms of about 10 $\times$ $10^3$ and 40 $\times$ $10^4$, compared to the numbers ( 80 $\times$ $10^4$) of the control treatment at the same time. On the seventh day of preservation, the number of microorganisms decreased significantly at a probability level (P>0.05), both in uncontaminated and contaminated treatments, to which nanomaterials were added. Their numbers reached 55 $\times$ $10^2$ and 34 $\times$ $10^3$, respectively, compared to the control treatment at the same period, which recorded 58 $\times$ $10^5$.

On the 14th day of preservation, the number of microorganisms recorded a decrease in the uncontaminated and contaminated treatments, about 83 $\times$ 10 $^2$ and 46 $\times$ $10^{3,}$ respectively, to which the nanomaterial was added with the plant extract, compared with the control treatment, which amounted to 20 $\times$ $10^7$.

As for the last day, which was 21 days, the adding of nanomaterials to the contaminated treatments, either with or without TVLE, showed a decrease in the numbers of microorganisms, which were recorded to be $24\times10^4$,$13\times10^3$ for uncontaminated and $90\times10^4$, $8\times10^4$, while the number of microorganisms in the control treatment was ($30\times10^8$).

**Effect of Titanium dioxide and TVLE on the pH of soft white cheese samples contaminated with *Brucella melitensis***

Statistically, there was no significant difference (P<0.05) in the pH value of cheese samples in the first hour, seventh and fourteenth day in all treatments in which Tio2 and TVLE were added individually or together, and its value ranged between 6.5-6.7 in the first hour and from 6.1-6.3 in the seventh day and from 5.6 - 6.0 on the fourteenth day.

However, cheese samples contaminated and uncontaminated with *B. melitensis* and to which thyme plant extract TVLE was added had a significant difference in the pH value, which amounted to 4.6-4.7 with samples added to nanoparticles, which ranged between 4.9-5.2 on the 21st day of storage, but it did not differ significantly with Control group whose pH value was 4.6 .

Till now, the mechanisms of nanoparticles are undistinguished, but several potential theories have been developed and explain the ways of bacterial growth elimination represent that Biogenic nanoparticles $TIO_2$ enter the cells of the microorganism by penetrating the mem-

**Table 1.** Minimum inhibitory concentration of titanium dioxide and TVLE against *Brucella melitensis*

| | Type of active substance | Inhibitory ability of the active substance according to the concentration (mg/ml) | | | | | |
|---|---|---|---|---|---|---|---|
| | TVLE | 5 | 20 | 40 | 80 | 160 | 320 |
| | | - | - | - | + | ++ | +++ |
| *Brucella melitensis* | TiO2 | 1 | 2 | 4 | 6 | 8 | 10 |
| | | - | - | + | ++ | +++ | ++++ |
| | TiO2 + TVLE | 5 +1 | 20 +2 | 40 +4 | 80 +6 | 160 +8 | 320+10 |
| | | - | + | ++ | +++ | ++++ | ++++ |

Sensitive (+) , Insensitive (-) ; TiO2 concentrations were at 1, 2 , 4 , 6 , 8 and 10 mg/ ml TVLE concentrations were at 5, 20, 40, 80, 160 and 320 mg / ml; TVLE = *Thymus vulgaris* leave extract , TiO2 = Titanium dioxide

**Table 2.** Inhibitory activity of TiO2 and TPLE against *B. melitensis*

| | Type of active substance | Inhibition diameter (mm) |
|---|---|---|
| ***Brucella melitensis*** | TVLE | 8.1 [c] |
| | TiO2 | 10.5 [b] |
| | TiO2 / TVLE | 12.0 [b] |

*Different letters indicate the existence of significant differences in rates at a probability level 0.05; TVLE = *Thymus vulgaris* leave extract , TiO2 = Titanium dioxide

brane via surface holes, the cell membrane permeability will alter due to liberation of metal ions, besides the interaction between the membrane and nanoparticles ions leads to rise of microorganisms generation time, due to elongate the logarithmic phase during growth. On the other hand, the interaction of biogenic nanoparticles, after absorption of the particle ions inside cells, will lead to the emergence of more (ROS) and liberation of metallic ions that cause cells to drain DNA replication disorder billing up and degradation of nanoparticles in the cell membrane (Sirelkhatim *et al*.,2015 ).

Anticidal ability and mechanisms of metallic nanoparticles are based on fundamental, chemical and physical properties, the type of metal, modulation of the surface and bacteria species (Gve+ or Gve-). Usually, Gve+ is more sensitive than Gve- ( Hajipour *et al*.,2012 ). The main biocidal activity of nanoparticles revolves around the generation of ROS like superoxide anions, hydrogen peroxide, and hydroxyl radicals. As a result of interactions, those ROS stimulate oxidative reactions, the exhaust of antioxidant cellular enzymes, which leads to the devastation of cell walls and death.

This is the first study that reports the effect of $TiO_2$ nanoparticles with thyme plant extract to extend the shelf life of soft cheese. The reason for prolonged preservation of soft cheese samples may be due to the inhibitory effect of the active substances ( volatile oils, caryophyllene, Linalool, 1-8-cineole, carvacrol, thymol and others ) present in the thyme plant (Al-Sa'ady and Hussein, 2020) . It is known that phenols have anti-inflammatory properties and cause leakage of the cellular cell components due to the penetration of phenolic compounds into the bacterial cell wall and accumulation in the cytoplasm, which leads to pH decreasing, impact on energy levels, disruption of the cell mem-

**Table 3.** Total microbial counts of soft cheese samples treated with TiO2 and TVLE with and without *B. melitensis* , refrigerated at (5 °C for 21 days)

| Type of bacterial contamination | Type of treatments (4 mg /ml of TiO2 and 80 mg /ml of TVLE) | 1 hour | 7 days | 14 days | 21 days |
|---|---|---|---|---|---|
| Without *Brucella melitensis* | Control | $80×10^4$ b | $58×10^5$ b | $29 × 10^7$ b | $30× 10^8$ b |
| | TVLE | $5 × 10^4$ d | $20× 10^4$ d | $61× 10^5$ d | $35× 10^7$ b |
| | TiO2 | $10×10^3$ c | $45 × 10^3$ c | $87× 10^3$ f | $24 × 10^4$ d |
| | TiO2/ TVLE | $42× 10^2$ c | $55× 10^2$ c | $83 × 10^2$ a | $13 × 10^3$ d |
| With *Brucella melitensis* | TVLE | $40 × 10^4$ d | $81 ×10^4$ d | $11 × 10^6$ b | $77× 10^8$ b |
| | TiO2 | $60 × 10^3$ f | $67× 10^3$ f | $88 × 10^4$ e | $90 × 10^4$ e |
| | TiO2/ TVLE | $10× 10^3$ c | $34 × 10^3$ c | $46 × 10^3$ f | $76× 10^4$ d |

*Different letters indicate the existence of significant differences in rates at a probability level 0.05;TVLE = *Thymus vulgaris* leave extract, TiO2 = Titanium dioxide

**Table 4.** pH values of soft cheese samples treated with Titanium dioxide and TVLE, with and without *Brucella melitensis* refrigerated at 5 °C for 21 days

| Type of bacterial contamination | Type of treatments (4 mg /ml of TiO2 And 80 mg /ml of TVLE) | 1 hour | 7 days | 14 days | 21 days |
|---|---|---|---|---|---|
| With out Brucella melitensis | Control | 6.7 [d] | 6.1 [d] | 5.6 [d] | 4.6 [b] |
| | TVLE | 6.7 [d] | 6.1 [d] | 5.7 [d] | 4.6 [b] |
| | TiO2 | 6.5 [d] | 6.3 [d] | 5.8 [d] | 4.9 [a] |
| | TiO2/ TVLE | 6.5 [d] | 6.2 [d] | 6.0 [d] | 5.0 [a] |
| With *Brucella melitensis* | TVLE | 6.7 [d] | 6.3 [d] | 5.6 [d] | 4.7 [b] |
| | TiO2 | 6.6 [d] | 6.2 [d] | 5.8 [d] | 5.0 [a] |
| | TiO2/ TVLE | 6.6 [d] | 6.3 [d] | 5.9 [d] | 5.2 [a] |

*Different letters indicate significant differences in rates at a probability level ($P<0.05$);TVLE =*Thymus vulgaris* leave extract ; $TiO_2$ = Titanium dioxide brane and important vital functions and finally, death of the bacterial cell ( Ultee *et al*., 2002) .

The interaction of thyme plant with $TiO_2$ nanoparticles created an appropriate synergistic ratio to inhibit the microorganisms causing pollution in the prepared soft cheese samples and prolong its preservation period. This result is consistent with (Nazzaro *et al*.,2013 ), who concluded that the biological activity of thymol is attributed to the main component of thyme oil, representing the effectiveness of carvacrol and hydroxyl group on phenolic ring, as the interaction of thymol can affect on the permeability of the membrane, which in turn leads to loss of K + ions, ATP, in addition to the interactions with membrane-bound proteins.

Al-Sa'ady and Hussein (2020 ) indicated the high inhibitory efficiency of $TiO_2$ nanoparticles when used at concentrations (20, 40, 60) mg/ml against Streptococcus pneumonia, which was resistant to many antibiotics. The synergistic action between plant extracts and nanomaterials combines the active compounds' capabilities and the nanomaterials' ability to penetrate and carry the active compounds into the bodies of bacteria and then interact and destroy them . The anti-inhibitory growth of *E.coli* and *B.subtilis* was due to the penetration of TiO2 into the cell membrane after its interference with the surface of the bacterial cell (Aziz *et al*.,2020)

Boskovic *et al*., (2015 ) confirmed that the oil of the thyme plant was efficient when testing its effectiveness against G - and G+ bacteria in food. He explained this by the presence of high concentrations of biological compounds such as carvacrol, p-cymene, trans-β-caryophyllene, linalool, γ-terpinene and thymol and their ability to destroy the outer cell membrane and thus increase its permeability. The antimicrobial properties and the improvement of food properties and quality have prompted stakeholders in the food industry to add titanium dioxide particles to many processed foods ( Dorier *et al.*,2019 ).

## Conclusion

The present results revealed that the inhibitory effect of TiO2 and *T. vulgaris* leaves extract achieved potency antimicrobial against *B. melitensis* and this activity was more effective and clear when mixing Titanium dioxide and plant extract, as this contributed to reducing the numbers of bacteria-contaminated with soft cheese and adjust pH. Also there was an effect of nanocompound alone in inhibitory accounts of *B. melitensis* and superior to using plant extract and control treatment.This study also showed that the combination of TiO2 nanoparticles and T. vulgaris extracts generated a good relationship towards increasing inhibition effect to kill contaminated bacteria and prolong the shelf life of soft cheese. This provides an effective and safe way to protect food and consumer health.

## Conflict of interest

The authors declare that they have no conflict of interest.

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