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EFFECTIVENESS OF PLANTS FIBER IMPREGNATED WITH GREEN NANOPARTICLES FOR WATER DISINFECTION

M.Sc. Thesis By

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تفويض

نحن الموقعين أدناه، نتعهد بمنح جامعة العلوم والتكنولوجيا الأردنية حرية التصرف في نشر محتوى الرسالة الجامعية، بحيث تعود حقوق الملكية الفكرية لرسالة الماجستير الى الجامعة وفق القوانين والأنظمة والتعليمات المتعلقة بالملكية الفكرية وبراءة الاختراع.

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DEDICATION

To my beloved mother, father, brothers and sisters

To my sweetheart my son

To my husband

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In the Name of Allah, the Most Merciful, the Most Compassionate all praise be to Allah, the Lord of the worlds; and prayers and peace be upon Mohamed His servant and messenger.

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LIST OF ABBREVIATIONS

Abbreviation	Description
BOD	Biological Oxygen Demand
CCL	Contaminant Candidate List
CMNC	Ceramic Matrix Nanocomposites
DALYs	Disability Adjusted Life Years
DBPs	Disinfection ByProducts
DDW	Double Distilled Water
DIZ	Diameter Inhibition Zone
DLS	Dynamic Light Scattering
DMSO	Dimethylsulfoxide
DO	Dissolved Oxygen
E-coli	Escherichia coli
LB	Luria-Bertani
LDV	Laser Doppler Velocimetry
MBC	Minimum Bactericidal Concentration
MFC	Microbial Fuel Cell
MH	Mueller Hinton
MH	Mueller Hinton
MIC	Minimum Inhibition Concentration
MMC	Matrix Nanocomposite
MMNC	Metal Matrix Nanocomposites
NOM	Natural Organic Matter
NPs	Nanoparticles
OD	Optical Density
P.aeruginosa	*Pseudomonas aeruginosa*

Abbreviation	**Description**
PBS	Phosphate Buffer Saline
PDF	Powder Diffraction Pattern
PdI	Polydispersity Index
PMNC	Polymer Matrix Nanocomposites
PPP	Pristine Pomegranate peel extract
PPP-TiO_2	Pristine Pomegranate Peel impregnated with TiO_2-
ROS	Reactive Oxygen Species
S.aureus	*Staphylococcus aureus*
SEM	Scanning Electron Microscope
SFW	Synthetic Fresh Water
THMs	Trihalomethanes
TiO_2	Titanium Dioxide
UV	Ultraviolet light
WHO	World Health Organization
XRD	X-ray Powder Diffraction

ABSTRACT

EFFECTIVENESS OF PLANTS FIBER IMPREGNATED WITH GREEN NANOPARTICLES FOR WATER DISINFECTION

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It is well known that safe water is essential to human health and the development of the country. More than one billion in the world do not have access to safe drinking water. In order to improve the quality of water that meet communal needs, a reliable and adequate safe water supply in a cost-effective way is a vital need. In this study, Titanium Dioxide Nanoparticels (TiO_2 NP) were mixed with plant extract (pristine pomegranate peel extract (PPP)) to develop the nanocomposite (PPP-TiO_2). Throughout the research, green chemistry was applied to minimize the use or generation of potentially harmful compounds during the manufacture, application and disposal. The media were fully characterized by Scanning Electron Microscope (SEM), Dynamic Light Scattering (DLS) and X-ray Powder Diffraction (XRD). PPP-TiO_2 nanocomposite showed a randomly oriented grains with various shapes and sizes and sharp grain boundaries. Grain size ranges from 1 to 5 μm. SEM micrographs also clearly showed the damaged bacterial cells treated with PPP-TiO_2. Furthermore, the biological activity has been evaluated by well diffusion method, Microbial Inhibition Concentration (MIC), Minimum Bactericidal Concentration (MBC) and live/dead cell assay. Tests have been performed at different concentrations and for three types of bacteria (*Staphylococcus aureus*, *Pseudomonas aeruiginosa*, Escherichia coli). Higher inhibition effects have been found for PPP-TiO_2 against Gram positive than Gram negative strains indicated by lowest MIC_{90}, MIC_{50}, and maximum inhibition zone. Antimicrobial activity of PPP-TiO_2 was higher by 1.5 time compared to PPP or TiO_2 NP against same pathogenic bacteria. Furthermore, Biological Oxygen Demand (BOD_5) was used to indicate the microbial communities and organic matter in the real water samples. Lower values were found for samples containing PPP-TiO_2 indicated that the sample has lower organic matter and indirectly indicated lower microbial community. Therefore, our developed media has a potential to be used as water disinfectant.

Chapter One: Introduction

"أَوَلَمْ يَرَ الَّذِينَ كَفَرُوا أَنَّ السَّمَاوَاتِ وَالْأَرْضَ كَانَتَا رَتْقًا فَفَتَقْنَاهُمَا ۖ وَجَعَلْنَا مِنَ الْمَاءِ كُلَّ شَيْءٍ حَيٍّ ۖ أَفَلَا يُؤْمِنُونَ" (الانبياء,30)

According to the holly Quran, Allah said," Have those who disbelieved not considered that the heavens and the earth were a joined entity, and we separated them and made from water every living thing? Then will they not believe?" (Al Anbiya 21:30).

Water covers three quarters of earth surface, and is the most important resource in the life of human beings, animals, and plants. All of creatures couldn't survive without water.

In the arid Middle East, especially in Jordan there is significant future water shortage problem, water scarcity is one of the largest environmental challenge.

1.1 Water Shortage in Jordan

Jordan is suffering from water shortages due to the deficit in water resources since the early 1960s (Bureau et al., 1985). It is considered the third most water scarce country in the world, and the water scarcity will become an even greater problem due to the following:

- The continuous population growth resulted from unexpected inflow of Syrian refugees after the Syria civil war which has been started in 2011 (Hadadin et al., 2010). This unexpected immigration has led to a dramatic increase in people living on Jordanian land by more than 20% within the last four years and an increase demands on the limited water resources.

- The temperature of the earth is increasing continuously due to global warming, which will expand droughts, leads to low rainfall, and expands semi-arid and desert area (Hadadin et al., 2010).
- The major surface water resources (Jordan and Yarmouk rivers), are shared with occupied Palestinian territories and Syria who leave only a small share for Jordan.
- Water pollution is considered to be major problem in the world, which causes the death of millions of people every year (Halder et al., 2015; Unuabonah et al., 2014; Ashbolt, 2004; Cabral et al., 2010).

1.2 Water Pollution

The contamination of water sources affects all aspects of life. Water pollution is defined as any change in chemical, physical, thermal, and biological characteristics properties that considered to be detrimental to human, plant, and animal health that make it unsuitable for use. These pollutants include fertilizers and pesticides from agricultural runoff, sewage and food processing waste, lead, mercury, and other heavy metals and microbial contamination which are the most common and widespread health risk associated with drinking water. When a pollutant gets into the water, most of the time it change and makes the water unhealthy and reduces the water's beneficial uses (Manja et al., 1982; Halder et al., 2015).

Many processes has been used to treat water and make it usable for a specific purposes such as drinking, industrial, medical, irrigation, etc. The water purification process is focused on the elimination of contaminants in water. Water is purified by removing all viruses, bacteria, algae and heavy metals such as iron, manganese and sulfur.

In this regards, nanotechnology is one of the most important new technologies that has many applications in biology, chemistry, physics, and engineering. In water applications, many studies have reported that nanoparticles inhibit the growth of several microbial and can be used effectively in water purification process (Taniguchi, 1974).

In the present study we have explored the manufacturing of an eco-friendly and cost-effective, natural media impregnated with green Titanium Dioxide Nanoparticles (TiO_2 NP) using fruit peel extract of Punica granatum (Pomegranate). The media were characterized by Scanning Electron Microscope (SEM), Dynamic Light Scattering (DLS) and X-ray Powder Diffraction (XRD). Further, its efficacy to inhibit different pathogenic bacterial growth were evaluated. Antimicrobial effect against Gram positive bacteria (*Staphylococcus aureus*) and Gram negative (*Pseudomonas aeruginosa, Escherichia coli*) were evaluated for water disinfection purposes.

Chapter Two: Literature Review

2.1 Microbial Contamination

Microbial contamination in water is the growth of harmful microorganisms (pathogenic bacteria, viruses, fungi, etc…) that make it unsuitable for consumption. These microorganisms, especially bacterial pathogens, are considered to be the most common and widespread health risk associated with drinking-water (Manja et al., 1982; Cabral et al., 2010).

Bacteria are microscopic single-celled organisms among the first life forms to appear on earth, and are present in most of their habitats. They can live within soil, in the ocean and inside the human gut.

Bacteria also live in symbiotic and parasitic relationships with plants, animals and human. Humans' relationship with bacteria is complex, in one hand they are helpful and in the other hand they are destructive, causing diseases like different types of intestinal, urinary and other infections such as internal bleeding due to Escherichia coli, skin infections, food poisoning, respiratory infections such as sinusitis, pneumonia and other diseases (Epa, 1998). Bacteria classified to two type depend on its morphology and gram stain as discussed below:

2.1.1 Gram Stain and Bacterial Morphology

Bacteria can be classified depending on gram stain as either Gram positive or negative, based on their morphology and differential staining properties. Gram positive bacteria are stained blue-purple and peptidoglycan (cell wall) is large. Some Gram positive bacteria are also capable to form spores under stressful environmental conditions. These

spores allow bacteria to survive under extreme conditions and can lead to re-infection. *Staphylococcus*, *Streptococcus*, *Lactobacillus*, *Listeria*, and *Acetobacter* are some examples of Gram positive bacteria (Epa 1998; Gerba et al., 2003).

However, Gram negative bacteria are stained red and have a small peptidoglycan layer but have an additional membrane (the outer cytoplasmic membrane). This membrane creates an additional permeability barrier and results in the need for transport mechanisms across this membrane. *Escherichia E. coli*, *Enterobacter*, *Pseudomonas, Salmonella*, and *Neisseria* are examples on gram negative bacteria (Hardalo et al., 1997; Gerba et al., 2003).

2.2 Microbial Contamination in Drinking Water

Pathogenic organisms that causes several diseases like bacteria, viruses and parasites (e.g., protozoa and helminths) can be transmitted via drinking water. They are predominantly of faecal origin (and therefore known as enteric pathogens). It is well documented that microbial contaminations are the most common and widespread health risk associated with drinking-water and can lead to numerous disease outbreaks (Hardalo et al., 1997; Tallon et al., 2005; Ashbolt, 2004; WHO 2004).

2.2.1 Waterborne Infections

The greatest risk from microbes in water is associated with consumption of drinking-water that is contaminated with human and animal excreta. According to World Health Organization (WHO) (WHO 2004), about 1.1 billion people globally drink unsafe water and approximately 3.1% (1.7 million) of people dies annually. Also, 54.2 million of the annual health burden (Disability Adjusted Life Years [DALYs]) world-wide are attributable to unsafe water, lack of sanitation and inadequate hygiene. In addition, around 9 out of 10 of deaths are in children and virtually all of the deaths are in developing

countries. In general, one-sixth of the world's population suffers from difficult access to clean drinking water (Moyer, 1999; Ashbolt, 2004; WHO, 2004; Sandhya, 2016).

The better known waterborne pathogens of concern known as Contaminant Candidate List (CCL). In developing regions are listed in Table 2.1 (Epa 1998). The organisms were selected for their potential to be transmitted by water and all of these infectious agents are spread by the faecal–oral route (Epa 1998; Ashbolt, 2004; Gerba et al., 2003). In this study, three Types of strains will be tested *Escherichia coli* (*E-coli*), *Staphylococcus aureus* (*S.aureus*), and *Pseudomonas aeruginosa* (*P.aeruginosa*).

Table 2.1*:* Lists the micro-organisms of the Contaminant Candidate List (CCL) (White et al. (1972)

Waterborne pathogens of concern in developing regions		
Name of micro-organisms	Major diseases	Major reservoirs and primary sources
Bacteria		
Salmonella typhi	Typhoid fever	Human faeces
Salmonella paratyphi	Paratyphoid fever	Human faeces
Other *Salmonella*	Salmonellosis	Human and animal faeces
Shigella spp.	Bacillary dysentery	Human faeces
Vibrio cholera	Cholera	Human faeces and freshwater zooplankton
Enteropathogenic *E. coli*	Gastroenteritis	Human faeces
Yersinia enterocolitica	Gastroenteritis	Human and animal faeces
Campylobacter jejuni	Gastroenteritis	Human and animal faeces
Legionella pneumophila and related bacteria	Acute respiratory illness (legionellosis)	Thermally enriched water
Leptospira spp.	Leptospirosis	Animal and human urine
Various mycobacteria	Pulmonary illness	Soil and water
Opportunistic bacteria	Variable	Natural waters
Enteric viruses		
Enteroviruses		
Polio viruses	Poliomyelities	Human faeces
Coxsackie viruses A	Aseptic meningitis	Human faeces
Coxsackie viruses B	Aseptic meningitis	Human faeces
Echo viruses	Aseptic meningitis	Human faeces
Other enteroviruses	Encephalities	Human faeces
Rotaviruses	Gastroenteritis	Human faeces
Adenoviruses	Upper respiratory and gastrointestinal illness	Human faeces
Hepatitis A virus	Infectious hepatitis	Human faeces
Hepatitis E virus	Infectious hepatitis; miscarriage and death	Human faeces
Norovirus	Gastroenteritis	Fomites and water
Protozoa		
Acanthamocba castellani	Amoebic meningoencephalitis	Human faeces
Balantidium coli	Balantidosis (dysentery)	Human and animal faeces
Cryptosporidium homonis, C. parvum	Cryptosporidiosis (gastroenteritis)	Water, human and other mammal faeces
Entamoeba histolytica	Amoebic dysentery	Human and animal faeces
Giardia lamblia	Giardiasis (gastroenteritis)	Water and animal faeces
Naegleria fowleri	Primary amoebic meningoencephalitis	Warm water
Helminths		
Ascaris lumbricoides	ascariosis	Animal and human faeces

2.2.1.1. Infection Due to *E-coli*

E-coli is a type of bacteria that present in large numbers in the intestine and in digestive tracts of people and animals. However, some types of *E-coli* can cause intestinal infection. Water intended for drinking should contain fecal and total coli form counts of zero (Dufour, 1977; WHO, 2004; Sandhya, 2016).

E-coli infection gets by coming into contact with the feces or stool of humans or animals. This can happen when eats food that has been contaminated by feces, or any food that has been in contact with raw meat especially if the infected meat is not cooked to 71°C, and drink or swim in poor sanitation water that contain bacteria from human or animal wastes (Beuchat, 1996).

Symptoms of intestinal infection include diarrhea, abdominal pain, decreased urine output, pale skin, and fever. More severe cases can lead to bloody diarrhea, dehydration, or even kidney failure. People with weakened immune systems, pregnant women, young children, and older adults are at increased risk for developing these complications.

2.2.1.2 Infection Due to *S.aureus* (Staph infections)

Staphylococcus aureus is a Gram positive, an aerobic, non-motile bacterium, it grows between 7 to 47˚C with optimal temperature 37˚C, *S.aureus* is a group of bacteria that can cause a multitude of diseases, and it's considered to be the most important bacteria that cause disease in humans (WHO, 2004; Tatini et al., 1973).

These bacteria can be found everywhere in air, water, sewage, animals and human. More than 30-50% of humans carry *S.aureus* on skin surface and in nose. These bacteria are spread by direct contact with an infected person, by using a contaminated object, it also can spread through the bloodstream and infect distant organs.

Staph infections may cause disease due to direct infection or due to production of toxins by the bacteria. It leads to skin and soft tissue infections such as abscesses (Boils), food poisoning, bloodstream infections, pneumonia (infection of the lungs), or bone infections (osteomyelitis), and arthritis are all examples of diseases.

Most symptoms of infections caused by *S.aureus* are skin and soft tissue infections such as abscesses or cellulitis, the area surrounding the abscess is usually red, painful and swollen and the skin surrounding the abscess can feel warm to the touch (Godfree et al., 1997; Hrudey et al., 2002).

2.2.1.3 Infection Due to *P.aeruginosa*

Pseudomonas aeruginosa is a common Gram-negative, rod-shaped bacterium , aerobic, a member of the family *Pseudomonadaceae*, and unique in its ability to infect a wide range of animal, plant, and human (WHO, 2004; Cross et al., 1983)

P.aeruginosa is a resistance to antibiotics pathogen recognized for its leading to mortality. It is a common environmental organism usually found in soil, water, sewage, and skin flora, and thrives not only in normal atmospheres, but also in low-oxygen atmospheres (Hardalo et al., 1997; WHO, 2004; Hassani et al., 2015). It is responsible for an increasing proportion of infections acquired in the modern hospital setting especially in immunocompromised patients (Morrison, 1984; Cross et al., 1983).

Furthermore, it is the most important Gram-negative pathogen, because its considered to be the most predominant bacterial pathogen in some large burn centers, respiratory tract (pneumonia), cystic fibrosis, immunosuppression (especially granulocytopenia), and traumatic wounds (Morrison,1984; Hassani et al., 2015).

The symptoms of such infections are generalized inflammation and sepsis, Bloodstream (bacteremia), Ear (e.g., otitis externa and media) Eye (e.g., bacterial keratitis,

endophthalmitis), Bones and joints (e.g., osteomyelitis), diarrhea and enteritis, moreover Pseudomonal infections are complicated and can be life-threatening (Morrison, 1984). Table 2.2 summerize the classification, infection, and symptoms of these bacteria.

Table 2.2: The classification, infection, and symptoms of *E-coli, S.aureus*, and *P.aeruginosa*

Bacteria	Type	Infection	Symptoms	Ref.
E-coli	Gram negative	contact with the stool of humans or animals Eat raw meat	diarrhea, abdominal pain, decreased urine output, pale skin	Dufour 1977; Beuchat, 1996
S.aureus	Gram positive	direct contact with an infected person, by using a contaminated object	skin and soft tissue infections such as abscesses or boils	Tatini et al., 1973 ; Godfree et al., 1997
P.aeruginosa	Gram negative	predominant in some large burn centers, respiratory tract	Inflammation in Ear, Eye, Bones and joints, and diarrhea	Morrison, 1984; Cross et al., 1983).

2.3 Water Treatment

Clean and safe water is pivotal for everyday life. It is essential for health, hygiene and the productivity of our community. Drinking water sources are subject to contamination and require appropriate treatment to remove disease-causing agents. The water treatment process may use various methods to provide safe drinking water for the communities. Today, the most common steps in conventional water treatment used by community water systems include:

1. Coagulation and Flocculation: are first steps in water treatment. Traditionally, the coagulation process is described in terms of the destabilization and remove of colloids present in a water supply by adding chemicals with a positive charge to the (raw) water. The positive charge of these chemicals neutralizes the negative charge

of negative charge dissolved and non-dissolved particles in the water. This causes the tiny particles to aggregate or sticking together and form larger and heavier particles. These particles called flocs which are easier to remove by settling or filtration (Edzwald, 1993)

2. Sedimentation: is a physical water treatment process using gravity to remove suspended solids from water. As the water and the floc particles flows through the treatment process, they move into sedimentation tank where the water moves slowly, causing the heavy floc particles to settle to the bottom due to its weight (Omelia, 1998).
3. Filtration: plays a major part of most water treatment and can be compared to a sieve or micro-strainer that traps suspended material between the grains of filter media. The filters are made of varying compositions layers of (sand, gravel, and charcoal), Filtration collects the suspended impurities in water and enhances the effectiveness of disinfection by removing dissolved particles, such as dust, parasites, bacteria, viruses, and chemicals.
4. Disinfection: is of unquestionable importance in the supply of safe drinking water, Water disinfection means the removal, deactivation or killing of pathogenic microorganisms or is an effective barrier to many pathogens (especially bacteria) during drinking-water treatment to decrease the number of outbreaks of waterborne diseases. (WHO, 2004; Sandhya 2016). Figure 2.1 showed a flow chart of the main steps in conventional water treatment.

Figure 2.1: Main steps in conventional water treatment

2.4 Disinfection Types

There are several disinfectants that can be attained by means of physical or chemical methods for killing or/and deactivating pathogenic microorganisms. Examples of chemical disinfectants are chlorine and its derivatives (containing substances), peroxide, and ozone, etc. However, Ultraviolet light (UV), Electronic radiation, Gamma rays, boiling, low frequency ultrasonic irradiation, distillation, reverse osmosis, and activated carbon treatment are examples of physical disinfectants. All disinfectants have benefits and drawbacks and can be used for water disinfection**.**

The use of chemical disinfectants in water treatment usually results in the formation of chemical by-products and has limitations against the protozoan pathogens in particular cryptosporidium and some viruses.

2.4.1 Chemical Disinfectants

2.4.1.1 Chlorine

Chlorine (Cl_2) is the most commonly chemical method for disinfectant purposes. It can be used as gas or a solution, being directly introduced into water to be treated. But more frequently, it is used in the form of a hypochlorite especially in large scale treatment (Driedger et al., 2000).

Chlorine is considered the most widely disinfectant for drinking water. Unfortunately, it has several disadvantages, which are the cause of consumer and regulatory pressure on water supply companies. These include unfavorable taste and odor associated with the use of chlorine in drinking water (Dwi, 2000), ineffectiveness when it used alone against resistant microorganisms such as Cryptosporidium parvum that cause gastrointestinal problems, which in severe instances can cause death (Driedger et al., 2000). The generation of potentially toxic disinfection byproducts such trihalomethanes (THMs), is a concern due to their harmful effect to human health (Bull et al., 2001; Ric, 1985).

2.4.1.2 Chlorine Dioxide

Chlorine dioxide (ClO_2) is a member of the oxochlorine family, the first reported use of chlorine dioxide in drinking water treatment occurred in 1944, at the Niagara Falls, N.Y., water treatment plant (Synan et al., 1944; Kerwick et al., 2005; Glaze, 1987). Furthermore, it is a very strong oxidizer and it effectively kills pathogenic microorganisms such as fungi, bacteria and viruses. Chlorine dioxide is more effective than chlorine and chloramines for inactivation of viruses, Cryptosporidium

Chlorine dioxide as a disinfectant has the advantage that it directly reacts with the cell wall of microorganisms. This reaction is not dependent on reaction time or concentration. Furthermor1e, chlorine dioxide can kill the microorganisms even when they are inactive. Therefore, the chlorine dioxide concentration needed to effectively kill microorganisms is lower than non-oxidizing disinfectant concentrations so the microorganisms cannot built up any resistance against chlorine dioxide. However, chlorine dioxide process has some disadvantages like forming the specific byproducts chlorite and chlorate (Glaze, 1987).

2.4.1.3 Ozone

Ozone (O_3) came into use as a drinking water disinfectant as early as 1906 at the Eon Voyage plant in France (Ric, 1985). Ozone has been shown to be better than chlorine as a disinfectant (Prendivillc, 1986). It also, appears to form much smaller amounts of mutants than either chlorine or chlorine dioxide (Zoeteman et al., 1982). In addition, when ozone decomposes, it generates radical intermediates that have much greater oxidizing power than ozone itself does.

Because ozone is an unstable gas, it must be generated onsite. The most common technique for generating ozone is the cold plasma discharge method. In which ozone is formed by decomposition of diatomic oxygen to give radical atoms (need energy like UV-

source) which combine with diatomic oxygen to give ozone as explained in the following equations [1-4].

$$O_2 + hv \rightarrow O + O \quad \lambda < 240\ nm \tag{1}$$

$$O + O_2 \rightarrow O_3 \tag{2}$$

$$O_3 + hv \rightarrow O_2 + O \quad \lambda < 320\ nm \tag{3}$$

$$O + O_3 \rightarrow 2\ O_2 \tag{4}$$

Ozone suffers in water disinfection from two major limitations as an alternative to chlorine, First, it is unstable in water; it decomposes to oxygen at a rate proportional to the pH of the water. For example, at pH 8, which is typical of many drinking-water supplies, its half-life is less than one hour, this is too short time to ensure that the capacity of the disinfectant will remain on a large distribution system. Second, ozone reacts with natural organic substances to produce low molecular weight oxygenated byproducts that generally are more biodegradable than their precursors are. These substances will enhance biological growth in a distribution system ("regrowth"), further limiting the disinfection efficiency of ozone. The reaction of ozone with natural organic matter (NOM) in water could lead to the formation of undesirable by-products, e.g., brominated by-products among others, which are major concerns for source waters containing bromide. Other ozonation by-products such as short chain aldehydes (e.g., formaldehyde, acetaldehyde, glyoxalandmethyl glyoxal), carboxylic acids (e.g., oxalic acid, formic acid, acetic acid and soxilic acid) and ketones (e.g., pyruvic acid, glioxalic acid and ktomalonic acid) have been identified (Huang et al., 2015; Oliviero et al., 2003).

For these reasons, ozone should be used in combination with other disinfectants that maintain an active residual for longer periods, and it should be combined with some method of filtration for removing biodegradable material (Glaze, 1987).

2.4.2 Physical Disinfectants

2.4.2.1 Ultraviolet Radiation

The first application of UV irradiation in drinking water as disinfection process was in 1910 in Marseille (Henry et al., 1910). In Europe, UV has been widely applied for drinking water disinfection since the 1980s (Kruithof et al., 1992). Due to the increased information on the production of hazardous oxidation by-products during chlorination and ozonation, UV irradiation gained more attention; low-pressure UV produces almost no byproducts. UV is not broadly effective against all pathogens, bacteria, protozoa and viruses but, it is highly effective of UV irradiation against Cryptosporidium (Clancy et al., 1998) and Giardia comparing to chlorine and ozone.

Ultraviolet water purification possesses advantages over a more widespread treatment like chlorination such as their low and no chemicals requirements and provide water with no taste and color. But UV water purifier requires electricity by using electromagnetic radiation. To decontaminate water to be treated, water passes through ultraviolet rays' tube. When water exposed to ultraviolet rays the short wave radiation comes into contact with the parasite, bacteria, fungi or other microorganism and eliminates it (Hijnen et al., 2006).

The main disadvantages of ultraviolet disinfectant are that the water being treated must be in no way turbid or cloudy Any level of color present in the water will hinder the ability of the UV radiation to penetrate it and destroy the microorganisms In addition, there is no residual when using ultraviolet so it is recommended to use chlorination after water purified through the UV.

2.4.3 Non-conventional Disinfectant

Although disinfection methods currently used in drinking water treatment can effectively control microbial pathogens, but the challenge is to achieve appropriate disinfection without forming harmful Disinfection by Products (DBPs) by conventional chemical disinfectants

Therefore, there is imperative need to re-evaluate conventional disinfection methods and to consider innovative approaches that enhance the accuracy and robustness of disinfection while eliminate DBPs formation, so that nanotechnology has promoted significant interest in the environmental applications of nanomaterials. A summary of different disinfectants and their main advantages and disadvantages are listed in Table 2.3.

Table 2.3: Comparison of available disinfectants

Disinfection type	Disinfectant	Disinfection Capabilities	pH Influence on Efficiency	Residual	DBP	Reference
Chemical	Chlorine	Excellent (HOCl > OCl)	Decreases with increasing pH	Yes	THM and others	Bull et al., 2001; Ric, 1985)
Chemical	Chlorine Dioxide	Excellent	Slight increase with increasing pH	Yes	Chlorate, Chlorite	Synan et al., 1944; Kriegsheim, 1916
Chemical	Ozone	Excellent	less stable at high pH	No	Aldehyd, etc	(Glaze, 1987).
Physical	Ultraviolet	Good	Independent	No	Unknown	Clancy et al., 1998)
Novel approach	Nanoparticles	Excellent	Depend on pH reding	Yes	Unknown	Dlallo et al., 2005 Gehrke etal., 2015

2.5 Nanotechnology

Nanotechnology deals with various structures of matter having dimension of the order of a billionth of a meter and nanoparticles are normally defined as being smaller than100 nanometers.

The term nanotechnology was first defined by Professor Noria Taniguchi of Tokyo Science University in the year 1974 to describe precision manufacturing of materials at the nanometer level, he defined the nanotechnology as the processing of, separation, consolidation, and deformation of materials by one atom or by one molecule (Taniguchi, 1974).

Recently, nanotechnology is emerging rapidly growing by producing nanoproducts and nanoparticles (NPs) with its application in science and technology for the purpose of manufacturing new materials at the nanoscale for environmental applications. In particular, nanomaterials are an increasingly important product of nanotechnologies in water treatment.

The use of nanoparticles is gaining attention in the present a century, when the size of the material becomes smaller, and smaller new properties emerge such as, their novel sizes are significantly different in the physico-chemical, magnetic, optical properties, distribution and morphology compared to larger matter (Tran et al., 2013; Shanmugavadivu et al., 2014; Venkatasubbu et al., 2016; Chen et al., 2007).

Metal nanoparticles are known to have unique features including surface Plasmon absorption and catalytic activity due to their quantum size confinements, extremely large surface area to volume ratio, a high electrical and thermal conductivity, surface-enhanced Raman scattering ability to selectively mediate chemical transformations, chemical stability, and nonlinear optical behavior (Tran et al., 2013; Shanmugavadivu et al., 2014; Pelletier et al., 2010).

Presently, different metallic nanomaterials are being produced using copper (Cu), zinc (Zn), titanium (Ti), magnesium (Mg), gold (Au), calcium (Ca), and silver (Ag). The TiO_2 nano-particles are area of interest due to their unique technological properties and applications such as memory devices, sensors, photo catalysis and solar cells (Tatini ,1973) TiO_2 is suitable for applications in water treatment because it has disinfection properties, stable in water, non-toxic by ingestion and its low-cost, (Li et al., 2008).

The novel properties of NPs have been exploited in a wide range of potential applications in medicine, home water purification systems, cosmetics, electronics due to their high conductivity, renewable energies like in solar cells, textiles, sensor technology, industrial applications and commercial products, environmental remediation and biomedical devices such as fighting bacterial pathogens as an antimicrobial agent (Tran et al., 2013; Khalil et al., 2013; Venkatasubbu et al., 2016; Pelletier et al., 2010).

A number of approaches are available for the synthesis of nanoparticles chemically, physically, or biologically methods, for example thermal decomposition of silver compounds, electrochemical, laser mediated synthesis, biological reduction method, hydrothermal synthesis, sono-chemical, microwave assisted process and recently via green chemistry route (Khalil et al., 2014; Shanmugavadivu et al., 2014; Hasan, 2015).

Unfortunately, many adverse effects have been associated with nanoparticles synthesis or production methods that involve the use of hazardous chemicals, low material conversions, high energy requirements, difficult and wasteful purifications. Therefore, there is a growing need to develop environmentally and ecofriendly processes for nanoparticles synthesis without using toxic chemicals. Biosynthetic methods (Biological ways) using microorganisms, enzymes, fungus, and plants or plant extracts have emerged as a simple and viable alternative to chemical synthetic procedures and physical methods (Shanmugavadivu et al., 2014; Parveen et al., 2016)

The use of plant materials for the synthesis of nanoparticles could be more advantageous, because it does not require precise processes such as intracellular synthesis. They are synthesized using a one-step procedure and have various natures, with greater stability and appropriate dimensions (Parveen et al., 2016). So, the green synthesis method using plant extracts emerges as an alternative method for the synthesis of TiO_2 NP since it is an eco-friendly and cost effective approach. Examples of plants that have been effectively used for TiO_2 NP are: Psidium guajava (Santhoshkumar et al., 2014), Nyctanthes arbor-tristis leaves (Sundrarajan et al., 2011), Aloe Vera extract (Rao et al., 2015), Punica granatum (Dahham et al., 2010) etc. The most commonly reported biomolecules accountable for the reduction of precursor and stabilization of titanium dioxide nanoparticles are metabolites such as alkaloids, phenolic compounds, terpenoids and co-enzymes that are soluble in water (Mittal et al., 2013).

2.5.1 Nanocomposite

Nanocomposites are multiphase solid materials that incorporate nanosized particles into a matrix of standard material. A drastic improvement in the properties of media impregnated with nanoparticles will occur including mechanical strength, toughness and electrical or thermal conductivity (Camargo et al., 2009). The properties of nanocomposite materials derived by combining properties from the parent constituents into a single material. Furthermore, there is also the possibility of new properties which are unknown in the parent constituent materials.

Nanocomposite materials can be classified, according to their matrix materials, in three different categories: Ceramic Matrix Nanocomposites (CMNC), Polymer Matrix Nanocomposites (PMNC), and Metal Matrix Nanocomposites (MMNC) (Camargo et al., 2009). The MMNC is composite material with at least two constituent parts, one being a metal necessarily, the other material may be a different metal or another material, such as a ceramic or organic compound.

In the nanocomposites research area, TiO_2 has been incorporated into various matrices to provide media with photo-catalytic activities.TiO_2 has a unique photo-catalytic properties (such as photo degradation and photo-induced super hydrophilicity), stability, commercial availability, and simplicity for its preparation [Mills et al., 1997; Paz, 2010]. Because of these properties TiO_2 nanocomposite has been widely used for water splitting, water treatment, air purification, and self-cleaning of surfaces (Yin et al., 2015).

In our study we have been prepared metal matrix nanocomposite (MMC) by mixing the TiO_2 NP with pristine pomegranate (Punica granatum) peel extract (PPP).

Punica granatum is one of the oldest known edible fruits. It has been widely used in traditional medicine worldwide for the treatment of different types of diseases (Nawwar et al., 1994; Silva et al., 2010). The pomegranate cultivation in Jordan has witnessed a remarkable development and an increase in the area of land cultivated with pomegranate trees to reach about 15 thousand dunums concentrated in the north of the Jordan which about half a million pomegranate trees are scattered over large areas of agricultural land. (Nimri et al., 1999).

The chemical composition of the pomegranate peel as shown in Figure 2.2 is: Phenolic punicalagins, gallic acid and other fatty acids; catechin, quercetin, rutin, Tannins (punicalin and punicafolin), and otherflavonols; flavones, flavonones, anthocyanidins and flavones glycosides (Dahham et al., 2010; Nikfallah et al., 2014; Nimri et al., 1999).

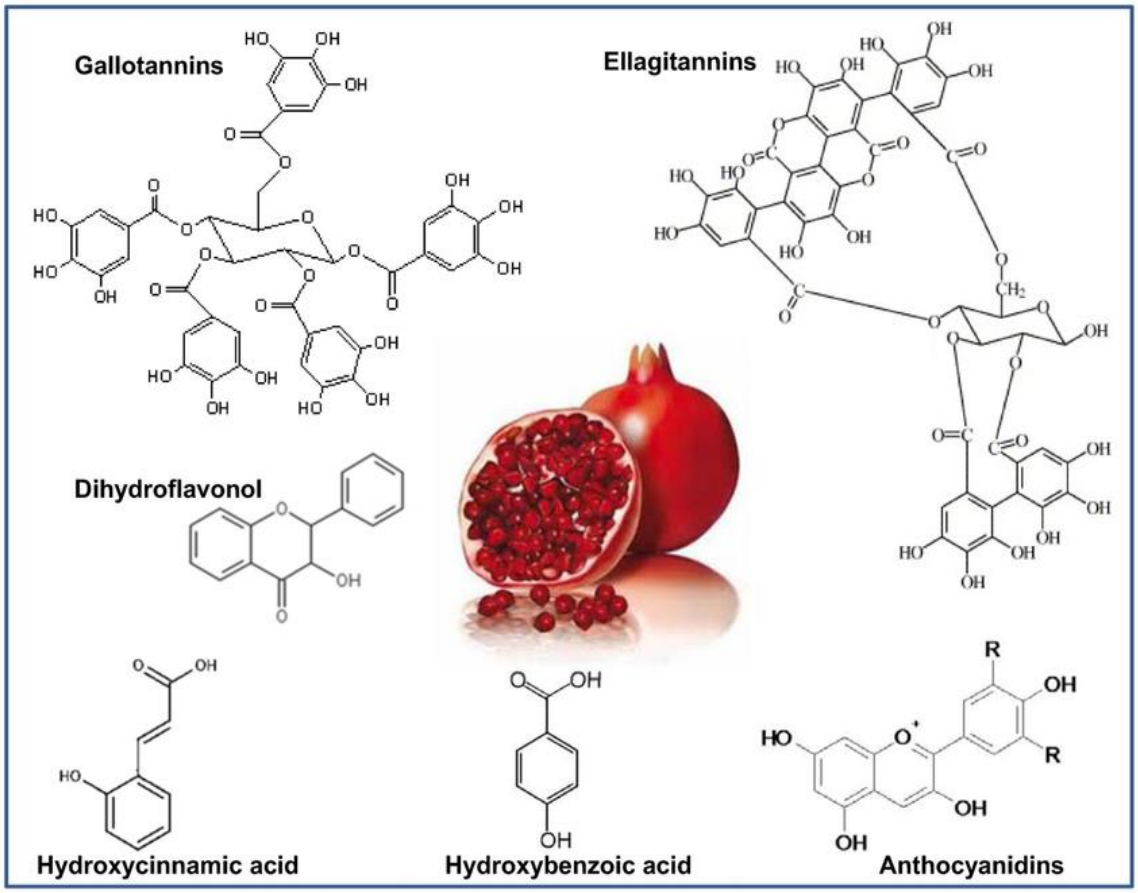


Figure 2.2: Chemical constituents of pomegranate (Nsyed et al., 2013)

Punica granatum peels were selected in our study because they are unusable part of fruit and it has the highest antibacterial activity compared to other parts on Punica granatum fruit as reported on Dahham et al., 2010.

In our study, we have explored an inventive contribution for the synthesis of copmosite of green TiO_2 NP impregnated with fruit peel extract of Punica granatum (Pomegranate). This composite was tested in bacterial removal efficacy under laboratory conditions for the purpose of water disinfection.

Chapter Three: Methods and Instrumentations

3.1 Preparations of Pristine Pomegranate Peel Extract (PPP)

Pomegranate fruits were purchased from a well-known market in Irbid city and were washed properly with deionized water. Pomegranate peel extract has been prepared using the method described by Jahir et al., 2011, where peels were separated and cut into smaller pieces, washed with tap water and followed by washing with deionized water. The peals were boiled for 30 minutes, transferred to a clean glassware and dried on oven at 102^{o}C for 6 hours. The dried peels were grounded into fine powder using an electric blender and mortar and then re-dried in the oven for another 30 minutes.

3.2 Preparation of Pristine Pomegranate Peel Impregnated With TiO_2-NP (PPP-TiO_2)

3.2.1 Preparation of PPP-TiO_2

TiO_2 NP has been synthesized using green method and tested at Prof. Albiss's laboratory in the physics department at Jordan University of Science and Technology. Peel extract was impregnated with 1% of green nanoparticles by adding 10.0 g of pomegranate fruit peel extract to 100 ml of distilled water in 250 ml Erlenmeyer flask. The solution was heated for 15 minutes to102^{o}C with stirring at 1500HZ. In a separate 250 ml erlenmeyer flask, 0.1g of green TiO_2 nanoparticles was added to 100 ml of distilled water and heated for 15 minutes at 102^{o}C with stirring at 1500HZ. The two solutions were mixed and reheated at 102^{o}C for another 15 minutes with stirring at 1500HZ, The final mixture was sonicated for 15 minutes at 50^{o}C and dried on glass petri dish, at 102^{o}C. The dried media was grounded into fine powder using an electric blender and mortar and re-dried in oven

for 30 minutes. The media was dried for another 15 minutes with nitrogen gas to remove the moisture from the powder. The step by step protocol is shown in Figure 3.1.

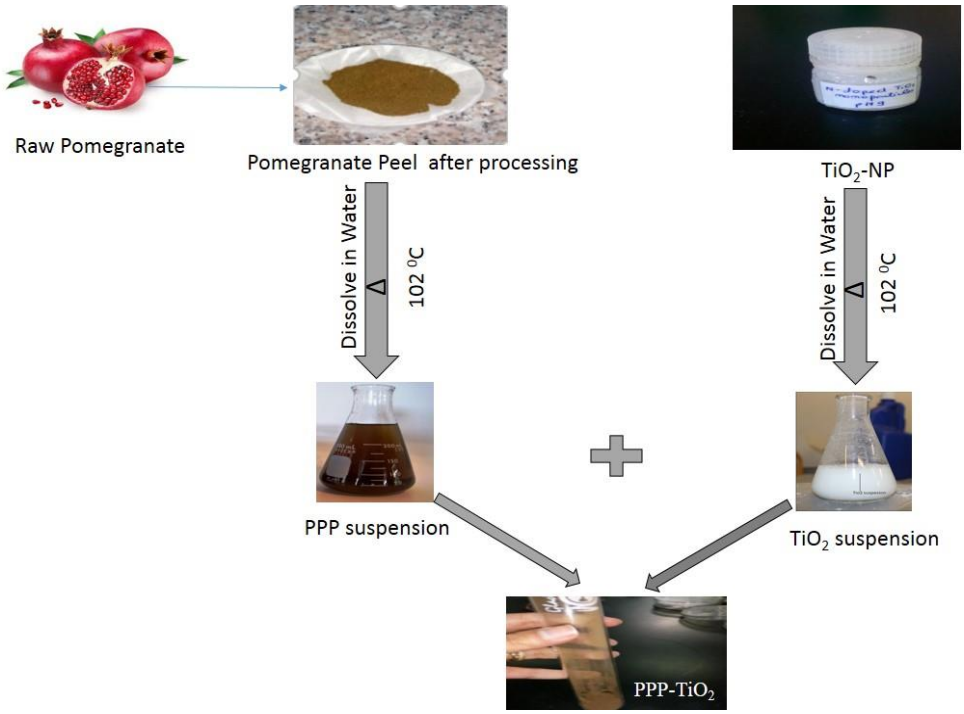


Figure 3.1: Nanocomposite (PPP-TiO_2) preparation protocol

3.3 Media Characterization

3.3.1 Scanning Electron Microscopy (SEM)

3.3.1.1 Operation Principle

The scanning electron microscope (SEM) is one of the most useful instruments available for the investigation and analysis of the microstructure morphology and chemical composition descriptions. SEM technique also used to detect surface fractures and surface contaminations, provide information in microstructures, and identify crystalline structures. The process begins when an electron gun producing a beam of energetic electrons on a series of electromagnetic lenses. The electromagnetic lenses are tubes, wrapped in coil. The coils are used to focus the incident electron beam onto the stage, where a solid sample is placed. The speed in which the electrons come in contact with the sample surface can be controlled by computer. While the incident electrons come in interact with the sample,

energetic electrons are released from the surface of the sample. The scatter patterns made by the interaction provides information on size, texture, shape, and composition of the sample. SEM instrument showed in Figure 3.2 (Reichelt, 2007).

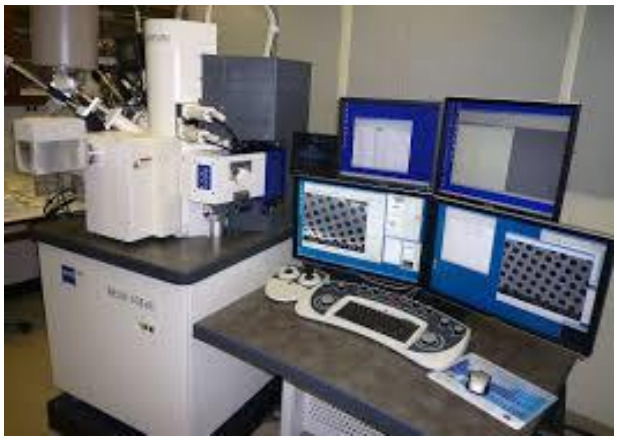


Figure 3.2: SEM instrument

3.3.1.2 Sample Preparation

The morphology, size, and shape of the media was characterized using Scanning Electron Microscope (SEM). For SEM analysis PPP, TiO_2, and PPP-TiO_2 powder was coated with gold metal (2.5 nm) and mounted on coated grid. Samples were photographed using QuantaFEI 450 SEM machine.

Scanning electron microscopy was performed to investigate the antibacterial activity of control bacteria cells (gram negative and gram positive bacteria (Escherichia coli ATCC 12900 and *Staphylococcus aureus* ATCC 700699, respectively), and (0.5 mg/mL) of PPP-TiO_2 and PPP treated cells. Each bacterial culture was centrifuged to collect the pellet. The pellets were washed with phosphate buffer saline (PBS) for three times, then 0.25% of gluteraldehyde was added, and incubated overnight at room temperature. After incubation period, the pellets were collected by centrifuge and dehydrated in an alcohol series using concentrations of 30%, 50%, 70%, 90% and 100% ethanol in distilled water. Samples were incubated in 100% ethanol for 1 hour, then attached to a stub and analyzed by SEM.

3.3.2 Dynamic Light Scattering (DLS)

3.3.2.1 Operation Principle

Dynamic Light Scattering (DLS) is one of the most widespread, useful and rapid methods used to determine the size distribution and zeta potential of particles in solution or suspension. The basic principle is simple: The sample is illuminated by a laser beam and the fluctuations of the scattered light are detected at a known scattering angle θ by a fast photon detector. DLS instrument is shown in Figure 3.3 (Goldburg, 1999)

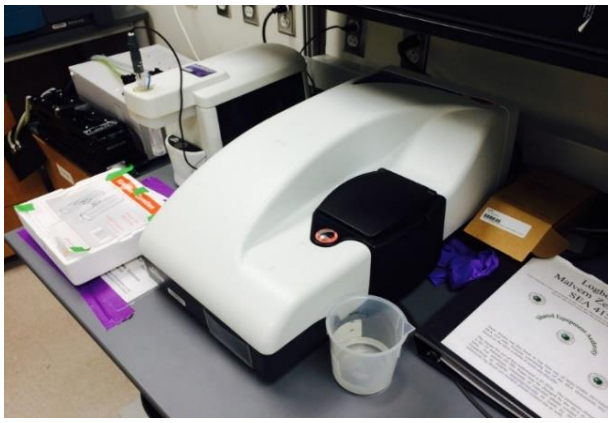


Figure 3.3: DLS instrument

3.3.2.2 Sample Preparation

The average particle size and the surface charge of TiO_2 NP, PPP, and PPP-TiO_2 were determined by particle size and zeta potential analysis (Zetasizer, Malvern Instruments Limited, ZEN3600, UK). Media was diluted in distilled water before analysis.

3.3.3 X-ray Powder Diffraction (XRD)

3.3.3.1 Operation Principle

X-ray powder diffraction (XRD) is a rapid instrumental technique mainly used for phase identification of a crystalline material, as well as minerals. The three-dimensional structure of nonamorphous materials, is defined by regular, repeating planes of atoms that form a crystal lattice. When a focused X-ray beam interacts with these planes of atoms, part is absorbed by the sample, part of the beam is transmitted, part is scattered, and part is

diffracted. X-rays are diffracted by each mineral differently, depending on the type of atoms make up the crystal lattice and the arrangements of these atoms. A diffraction pattern is indicative for a specific material, and used as a "fingerprint" for its identification. The diffraction patterns are compared against those in a computerized database to find a match. XRD instrument is shown in Figure 3.4 (Cullity et al., 1957).

Figure 3.4: XRD instrument

3.3.3.2 Sample Preparation

X-ray diffraction (XRD) analysis of the powdered TiO_2 NP, PPP, and PPP-TiO_2 was analyzed by using X-ray diffractometer (XRD; Bruker) using Cu-Kα X-rays radiation of wavelength (λ)=0.15406 nm XRD measurements were carried out in the diffraction angles (2θ) from 20 to 80 with a step of 0.1972°.

3.4 Antibacterial Activity of the Media against Gram Negative and Gram Positive Bacteria

3.4.1 Media Preparation for the Antimicrobial Assays

Stock of PPP-TiO_2 suspension was prepared at a concentration of 1µg/µl by mixing PPP-TiO_2 powder in Double Distilled Water (DDW). The solution was sonicated for 30 min. at 60 pulser by using ultrasonic processer.

3.4.2 Antibacterial Assays of the Media

3.4.2.1 Culture Preparation

Two Gram negative bacteria (*pseudomonas aeruginosa* (*P.aeruginosa*) ATCC 27853, and *Escherichia coli* (*E-coli*) ATCC 12900), and one Gram positive bacteria (*Staphylococcus aureus* (*S.aureus*) ATCC 700699) were used for testing the media's antibacterial properties. The strain was saved on refrigerator at 4^{o}C, before each experiment. Bacterial cells were pre-cultured on a nutrient agar slant and transferred to a Luria-Bertani (LB) broth. The tested bacteria was grown in an orbital shaking incubator, at a temperature of 36°C ± 1°C for 24 h. and 150 rpm. The fresh cultures were diluted in Luria-Bertani (LB) broth to give a final concentration of 0.5 McFarland standards (approximately 1 to 2 × 10^8 CFU/ml) (Khalil et al., 2013; Venkatasubbu et al., 2016).

3.4.2.2 Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The antibacterial activity of the media was determined by broth micro dilution method. It was used to determine the Minimum Inhibition Concentration (MIC) which is the lowest concentration of the nanoparticles that inhibits visible bacterial growth during 24h of incubation (Wiegand et al., 2008). The (MIC_{50}) and (MIC_{90}) are defined as the lowest concentration of antibiotic that reduced the bacterial growth to 50% and 90%, respectively during 24 h of incubation. For this purpose, a stock of suspension of natural media was prepared at a concentration of 1µg/µl (0.1 g of NPs in 100 ml of DDW). Mueller Hinton (MH) broth and media with different concentrations of PPP-TiO_2 were prepared according to Table 3.1.

Table 3.1: PPP-TiO_2 concentration prepared in MH broth and used in MIC determination

Tube No.	1	2	3	4	5
MH broth (µl)	10,000	10,000	10,000	10,000	10,000
PPP-TiO_2 stock (µl)	2000	3000	4000	6000	6500
DDW (µl)	8000	7000	6000	4000	3500
Concentration (µg/ml)	100	150	200	300	325

A broth without nanoparticles was used as positive control. 10 µl of bacterial suspension with turbidity equivalent to 0.5 Mc-Farland was added to test tubes containing each different concentration of PPP-TiO_2 (0 to 325 µg/ml). The concentration of PPP-TiO_2 that inhibits 50% and 90% of the isolates was reported as MIC_{50} and MIC_{90}.

The Minimum Bactericidal Concentration (MBC), which described the lowest concentration of antibiotic that kill 99.99% of the bacteria was also determined from batch culture studies. To test for bactericidal effect, firstly, the melted MH agar was spread onto plates. Secondly, the invisible bacterial suspensions with different concentration of samples were coated on the agar plates by spreading 0.1ml from each tube each on Mueller–Hinton agar by L-spreader. Finally, the agar plates were incubated at 37°C for 24 h. MBC were determined by counting the bacteria after the incubation period. The MBC may be defined as the minimal concentration of samples in which the number of survival bacteria was less than 5 on the agar plates (Hassani et al., 2015; Kannan,et al.,2015). All experiments were carried out three times. The MIC values were read at 610 nm in an UV/Vis spectrophotometer (BECKMAN, spectrophotometer UV05).

3.4.2.3 Well Diffusion Method

Media was also tested for antimicrobial activity by well diffusion method of Kirby Bauer using Mueller –Hinton agar. Mueller –Hinton agar was poured into sterilized petri dish and left over night at room temperature to check for any contamination before use.

20µl from 10^6 dilution of different pathogenic bacterial suspension (*S.aureus, P.aeruiginosa*, and *E-coli*) was spread on the MH agar plates. Wells of 8 mm diameter were bored using sterile borer and loaded with various concentrations of natural media. Five different PPP-TiO_2 concentrations (2%, 1.5%, 1%, 0.5%, and 0.250%) were chosen for this test. To prepare the stock solution 2g of PPP-TiO_2 were dissolved on DDW then the solution was sonicated for 30 min. to ensure good dispersion before each experiment (Jahir et al., 2011; Shanmugavadivadivu et al., 2014). For the control samples, wells were loaded with DDW. All samples were incubated at 37ºC for 24 hours.

3.4.2.4 Fluorescence Microscopic Study for Live/Dead Cell Viability

The Live/Dead BacLight TM Bacterial Viability Kit (Invitrogen-Molecular Probes, Eugene, OR, USA) was used to examine bacterial cell viability under a fluorescence microscope. The kit contains a mixture of two nucleic acid binding stains, specifically referred to as SYTO 9 and propidium iodide. These stains differ in their spectral characteristics, and in their ability to penetrate viable bacterial cells (Mo- lecular Probes, 1995). SYTO 9 stains all cells green color, while propidium iodide penetrates cells whose cell with membrane has been damaged, staining them red color.

The kit was stored at -20°C in dark, which is taken out and thawed at room temperature just prior to assay. E. coli (10 µl of 10^8 CFU) and *S.aureus* (10 µl of 10^8 CFU) cells were treated with MIC 50%, MIC90% of PPP-TiO_2, MIC90% of PPP and TiO_2 NP. Also untreated cells were taken as a control. The samples were prepared in a centrifuge tube and incubated at 37°C for 24 h. After incubation period the mixtures were centrifuged at 5000 rpm for 5 min at 4°C. Then, media was removed completely and the cells were washed and re- dispersed in 0.9 wt % NaCl. Finally, 3 µL of the BacLight dye mixture was added and incubated in dark at room temperature for 15-20 min. After incubation, 20 µL of the solution mixture was mounted over microscope slides, which

viewed under the light microscope (Nikon, Eclipse. E200) using an excitation filter of EX 450-490 nm and a band absorbance filter covering wavelength below 505 nm.

3.5 Application of PPP-TiO_2 in Real Water Samples

3.5.1 Natural Water Sample Collection and Synthetic Water Preparation

Ground water samples were collected from different wells in Irbid city, i.e. Al-Hoson, Al-Naemah, and JUST University. The selected wells provide Irbid city with drinking water in a daily basis. The purpose of this test is to evaluate the efficiency of the developed PPP-TiO_2 on disinfecting synthetic and real water samples.

The Synthetic Fresh Water (SFW) sample was prepared by adding 50 mg/l sodiumbicarbonate, 30 mg/l calcium sulfate, 30 mg/l magnesium sulfate, and 2 mg/l potassium chloride according to the procedure described by Chalew et al., 2013.

3.5.2 Antibacterial Activity of Natural Water Sample and Synthetic Water against Bacteria

Natural water and synthetic fresh water samples were tested for antimicrobial activity by well diffusion method against *S.aureus*. This method was selected because antibacterial activity can be visually identified by measuring the Diameter Inhibition Zone (DIZ).

To prepare the stock solutions for this test, 2 g of PPP-TiO_2 was dissolved in 100 ml of each natural and synthetic fresh water samples. Samples were then sonicated for 30 min. to ensure good dispersion before each experiment. Wells were loaded with five different concentrations of stock solutions (2%, 1.5%, 1%, 0.5%, and 0.25%). Natural and synthetic fresh water samples were used as a control. All samples were incubated at 37°C for 24 hours. DIZ was measured after the incubation period of each sample.

3.5.3 Biological Oxygen Demand (BOD)

Biochemical oxygen demand is a chemical procedure for determining the amount of dissolved oxygen needed by aerobic biological organisms in a body of water to break down organic material present in a given water sample at certain temperature over a specific time period. It is most commonly expressed in milligrams of oxygen consumed per liter of sample during 5 days (BOD_5) of incubation at 20°C (Association et al., 1915; Zapata et al., 2009).

In our study, BOD Measurement System BD600 was used to measure BOD for real water samples with and without natural media. 0.25% of PPP-TiO_2 was added to the water samples collected from Al-Hoson, Al-Naemah, JUST University's wells, and to the Synthetic Fresh Water (SFW). The Mueller Hinton (MH) broth mixed with 50% water was used as a blank. BOD_5 was measured and reported after five days.

The concentration of PPP-TiO_2 was determined experimentally where it was within the range of BOD reading.

Chapter Four: Result and Discussion

4.1 Characterization of the Media

4.1.1 Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) is employed to visualize the structure, average size identification and shape of the synthesized media using pristine pomegranate fruit peel extract (PPP) and green Titanium Dioxide Nanoparticles (TiO_2 NP). Figure 4.1 shows the micrographs of PPP before and after loading with TiO_2 NP at different magnification levels. Figure 4.1 (a) and (c) shows a randomly oriented grains of PPP-TiO_2 with various shapes and sizes and sharp grain boundaries. Grain size ranges from 1 to 5 μm. Moreover, considerable number of voids, micro-cracks, and clusters of TiO_2 NP can easily be seen between the grains. However, Figure 4.1 (b) and (d) illustrate quite different morphology for PPP compared to PPP-TiO_2. Large grains have been formed with smooth boundaries and almost no voids and cracks between the grains and at the grain boundaries.

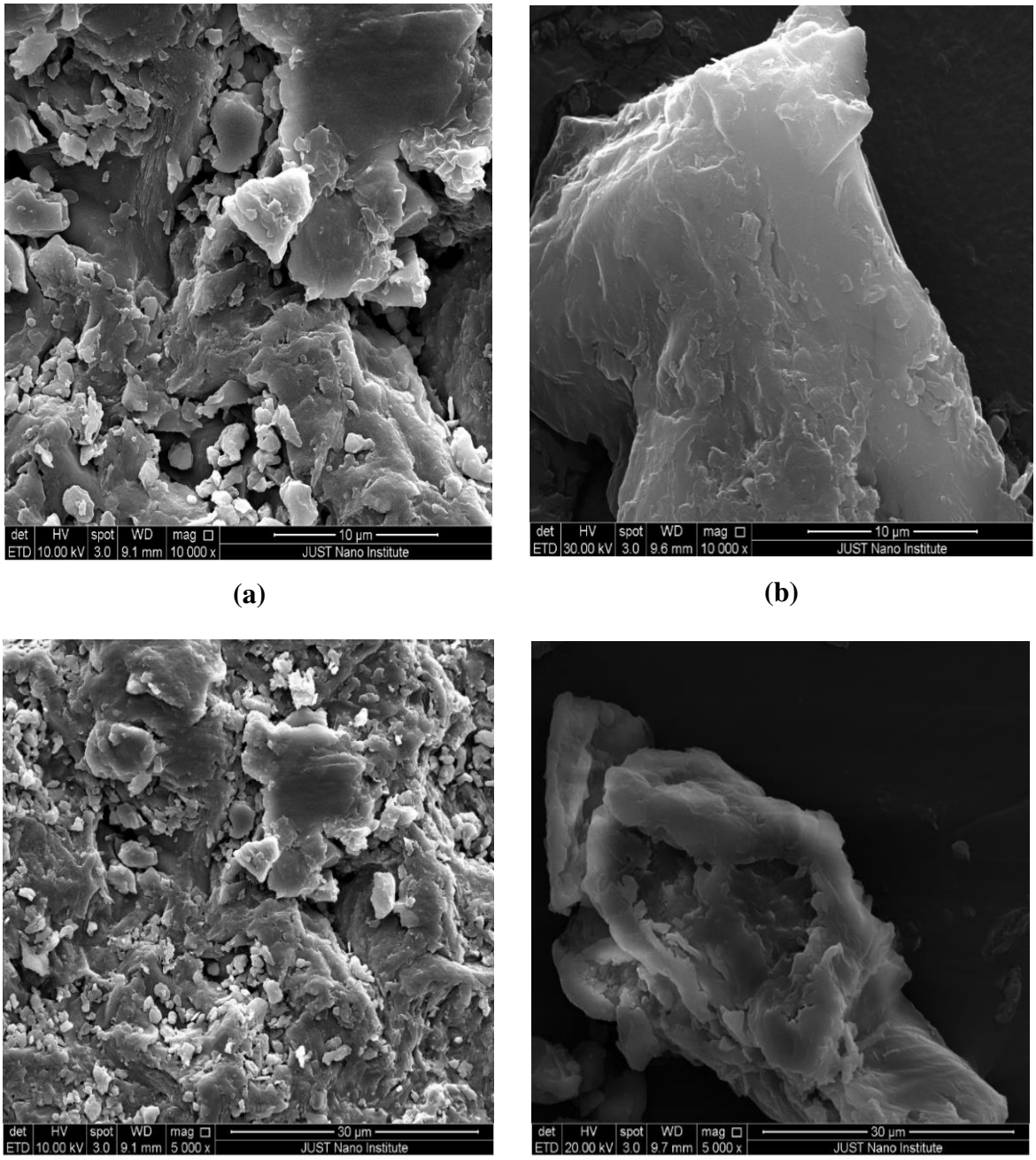
In general, synthesized TiO_2 NP by green method produce large size and sintered nanoparticles, which is consistent with our SEM results and other published studies. For example, Sundrarajan et al., 2011 studied the synthesis of TiO_2 NP using nyctanthes arbor-tristis leaves extract. The morphological dimensions of the synthesized TiO_2-NP in their SEM study demonstrated that the average size was from 100-150 nm, whereas the shapes were irregular spherical.

Furthermore, Goudarzi et al., 2016 employed the pomegranate peel extract and cochineal dye for synthesis of Ag nanoparticles. Their results clearly showed that the sample consists of highly agglomerated Ag nanoparticles, which have formed large

aggregates with diameter of 400 nm to 2.5 μm. SEM results for the developed composite consist of TiO_2-NP and organic material (pomegranate peel extract) show large and adhered particles.

In addition, Figure 4.2 shows the SEM micrographs of treated and untreated bacteria. For untreated Gram positive bacteria (*S.aureus*) (Figure 4.2 (a)), the cells are round and intact. However, for treated *S.aureus* with PPP-TiO_2 (Figure 4.2 (b)), the cells membrane is damaged and some lysed cells are found. On the other hand, the cells for untreated Gram negative bacteria (*E-coli*) Figure 4.2 (c)), are rod-shaped and intact. However, for treated (*E-coli*) with PPP-TiO_2 (Figure 4.2 (d)), the cells are dents and some deep cracks cells are observed

Our results is consistent with Ramasamy et al., 2014. They studied the antibacterial activity of gold nanorod conjugated with magnetic nanoparticle composite against *E. coli* and *E. faecalis*. Where SEM results showed the control bacteria have an intact, smooth surface. In contrast, the treated bacteria exhibited significant shape modification including wrinkling, rupture with disordered of the population, cell debris, and clearly representing serious damage to the bacterial walls resulted in loss of cellular components.



(a) **(b)**

(c) **(d)**

Figure 4.1: SEM images for PPP-TiO_2 (a and c) and for PPP (b and d) at different magnification levels

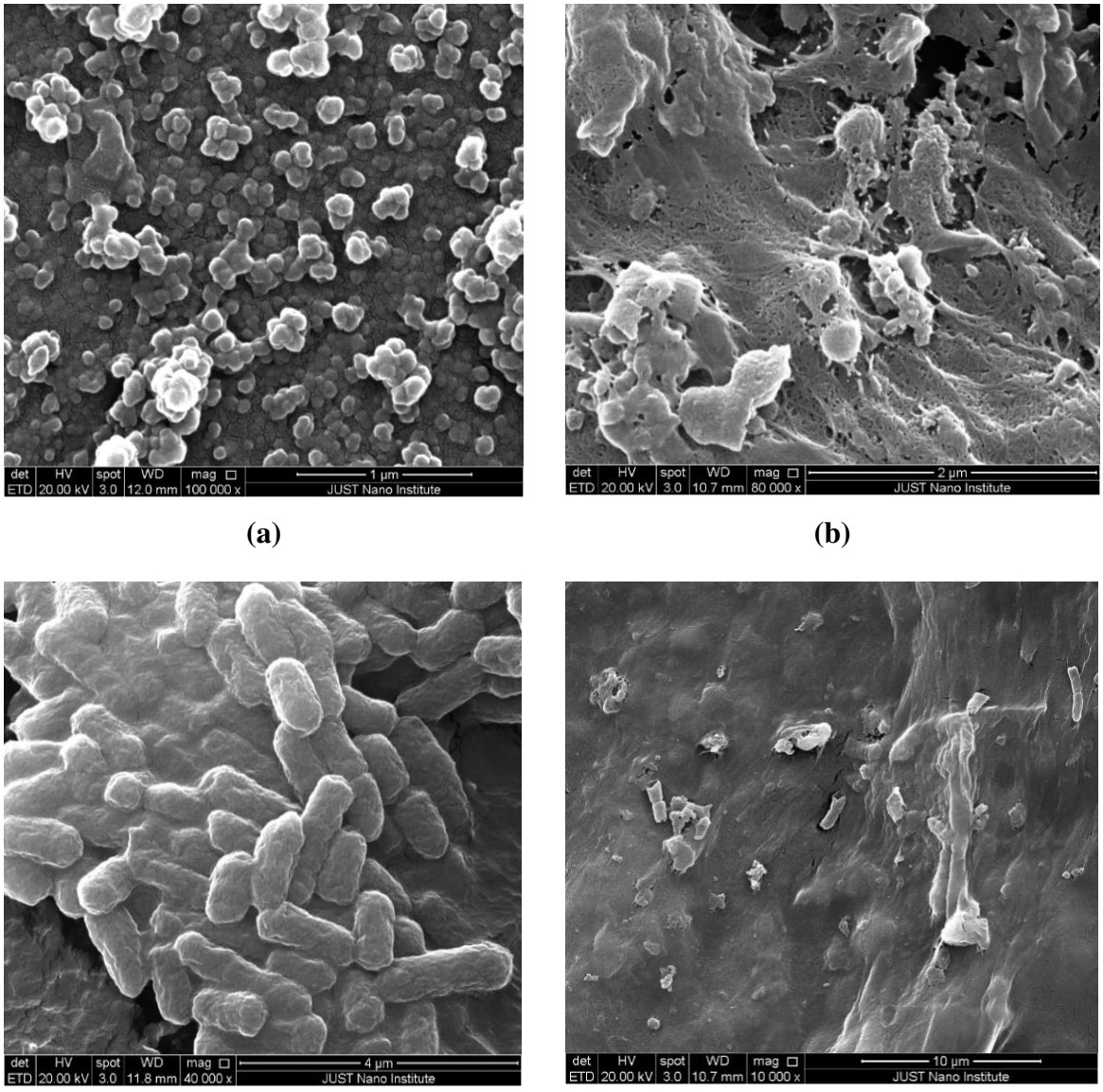


Figure 4.2: SEM micrographs of treated and untreated bacteria. (a) Untreated *S.aureus* (b) treated *S.aureus* with PPP-TiO_2. (c) Untreated *E-coli* (d) treated *E-coli* with PPP-TiO_2

4.1.2 Dynamic Light Scattering (DLS) Size Distribution Measurements

Dynamic Light Scattering (DLS) is used for characterization of size distribution of the nanoparticles and to determine the hydrodynamic diameter of the particle in solution with respects to intensity in percentage. Laser Doppler Velocimetry (LDV) is used to measure the zeta potential of the nanoparticles in solution. The measurements were performed on Zetasizer Malvern Instrument at 173^{o}of scattering angle. DLS measurements were presented in Table 4.1 for PPP, TiO_2 NP, and PPP-TiO_2. The particle size distribution

were illustrated in Figure 4.3. The results clearly show TiO_2 NP has monodisperse peak with Z-average value of 620 (in d.nm) and peak intensity of 100%. While, PPP has bimodal distribution with the peaks of the larger diameter exhibiting most of the intensity of 92.2%. And Z-average value of 1264 (d.nm). The absorption of the PPP has been shifted to a higher value, indicating a shift to a larger particle size. However, PPP-TiO_2 shows bimodal distribution peaks, confirming the presence of both TiO_2 and PPP in the composite. The Z-Average value as the mean value of the hydrodynamic diameter for PPP-TiO_2 was found to be 1230 (d.nm) with peak intensity of 87.5%. The peaks distribution of PPP and TiO_2 NP in our experiment is in quite agreement with the peaks found by Elia et al., 2014 and Murdock et al., 2008, respectively. However, our Z-average results are higher than values published by Hess, et al., 1986; QIAO et al., 2012. They reported that the Z-average value for TiO_2 NP was (486nm) and for PPP was (648 d.nm). This difference can be attributed to the agglomeration of the particles. The particles tended to form agglomerates of similar size when dispersed in either water or cell culture media (Kannan et al., 2015). It is well known that the hydrodynamic diameter of the nanoparticles is always larger than the core size because it includes surface coating materials and solvent layer attached to the surface of the particles as it moves under influence of Brownian motion (Hess, et al., 1986) or due to the media with unequal size causing nanocluster agglomeration (Qiao et al., 2012). In addition, the use of plant extracts is mostly would accompanied with higher hydrodynamic diameters.

In addition, Zeta potentials were measured for PPP-TiO_2. PPP and TiO_2. Table 4.1 shows that the PPP-TiO_2 has the highest zeta potential at -11.4 mV and was found to be -4.67 mV and -6.95 for PPP and TiO_2, respectively. The measured Poly Dispersity Index (PdI) values defined as size ranges present in the solution or width of the particle size distribution (Malvern, Instruments Ltd., 2005) were varied slightly.

The magnitude of the zeta potential gives an indication of the potential stability of the colloidal system. If all the particles in suspension have a large negative or positive zeta potential then they will tend to repel each other and there will be no tendency for the particles to come together. However, if the particles have low zeta potential values then there will be no force to prevent the aggregation (Murdock et al., 2008). This explain the particles agglomeration as shown from the SEM and Z- Average results.

Table 4.1: DLS, LDV, and PdI values for PPP-TiO_2, PPP, and TiO_2 NP

Particle	**DLS Average size distribution diameter (nm)**	**PdI**	**z-potential (mv)**	**Peak intensity (%)**	**pH**
PPP-TiO_2	1230	0.232	-11.4	Peak1=87.5 Peak2=10.7 Peak3=1.8	7.0
PPP	1264	0.376	-4.67	Peak1=92.2 Peak2=7.8	7.0
TiO_2 NP	620	0.178	-6.95	Peak1=100	7.0

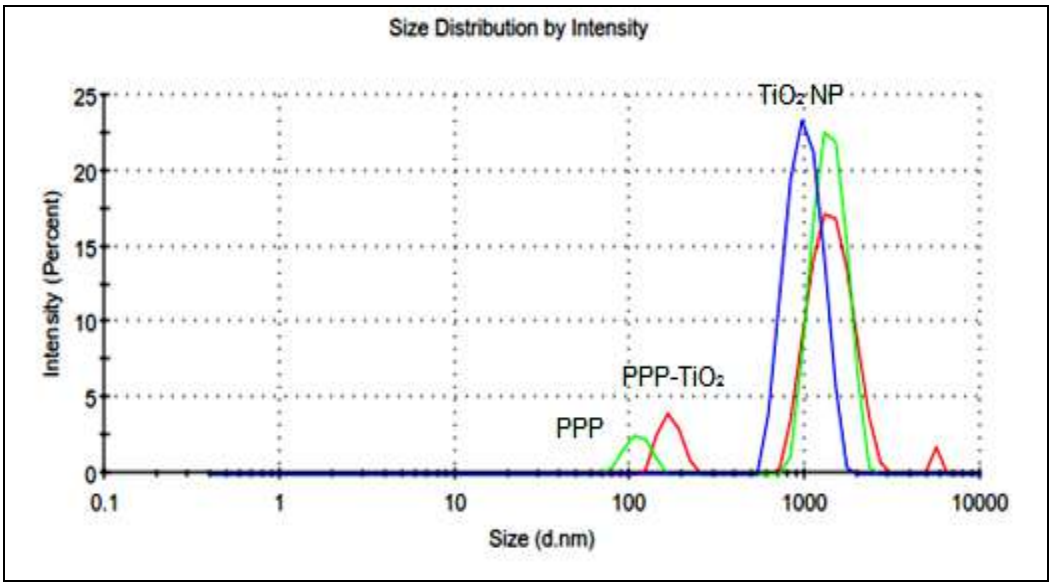


Figure 4.3: DLS analysis spectra of PPP, TiO_2 NP, and PPP-TiO_2

4.1.3 X-ray Powder Diffraction (XRD)

The X-ray diffraction patterns of the synthesized TiO_2 NP, PPP-TiO_2, and PPP are shown in Figure 4.4. The diffraction peaks correspond to TiO_2 anatase phase. The presence of the main diffraction peaks at 2θ values of 25.20◦, 37.80◦, 48.04◦, 53.89◦, 62.68◦ and 75.1◦ corresponding to the indexed planes of crystals of (101), (004), (200), (105), (211), and (204). The 2θ peaks at 25.27° and 48.01° confirms the formation of anatase TiO_2 NP. TiO_2 NP formed are crystalline in nature compared with the standard powder diffraction pattern (PDF Card No.: 00-021-1272 Quality: S). Furthermore, the lattice parameters were a = 3.7850 A°, b = 3.750 A°, c = 9.514 A°. Hence α=β=γ=90 of the cubic face centered TiO_2. The XRD pattern of PPP exhibited single broad peak signifying amorphous character. Traces of minor peaks with low intensities which may be attributed to various impurities have been observed in the PPP XRD pattern.

The intensity of XRD peaks of the sample reflects that the formed nanoparticles are crystalline and broad diffraction peaks indicate very small size crystals (Cullity 1978. However, the broad peak for PPP is related to its amorphous shape. For PPP-TiO_2, the presence of both crystalline peaks and amorphous shape at the same 2θ regions confirmed the presence of both TiO_2 and PPP in the composite.

XRD patterns of PPP-TiO_2 are in well agreement with the results found by Awad et al., 2015 and Fan et al., 2015, respectively. Awad et al., 2015 found a broad XRD peaks pattern of green AgNPs/ polystyrene nancomposite at $2\theta \sim 5–20\circ$ which corresponds to a mixture of ordered and disordered structure of the amorphous phase of polystyrene. A comparison between diffraction patterns of polystyrene and AgNPs/ polystyrene nanocomposites showed that the peaks corresponded to polystyrene became broader, suggesting the smaller AgNPs embedded in polystyrene chains. Further, Fan et al., 2015 studied the XRD and TG-FTIR of the effect of mineral matrix on the pyrolysis and

combustion of organic matter in shale char. A broad hump is observed in the XRD spectrogram of shale char organic matter. The formation of this hump at about 2θ =20° is mainly due to n-alkanes and naphthenic hydrocarbons in the organic matter.

The average crystallite sizes of the TiO_2 NP can be estimated to be about 97 nm for the higher intense peak Figure 4.4, from the X-ray peak broadening using Scherrer's formula.

$$D = \frac{K\lambda}{\beta cos\theta} \quad (5)$$

Where, D is the average particle size of crystallites, K is a constant value equal to 0.9, λ is the wavelength of the X-ray radiation, β is the full width at half maximum (FWHM) of each phase, and θ is the diffraction angle (Cullity 1978; Azaroff 1968). The data provided by the instrument's software library of TiO_2 NP is available on Appendix A. The sharp peaks and absence of unidentified peaks confirmed the crystallinity and higher purity of prepared nanoparticles (Sundrarajan et al., 2011).

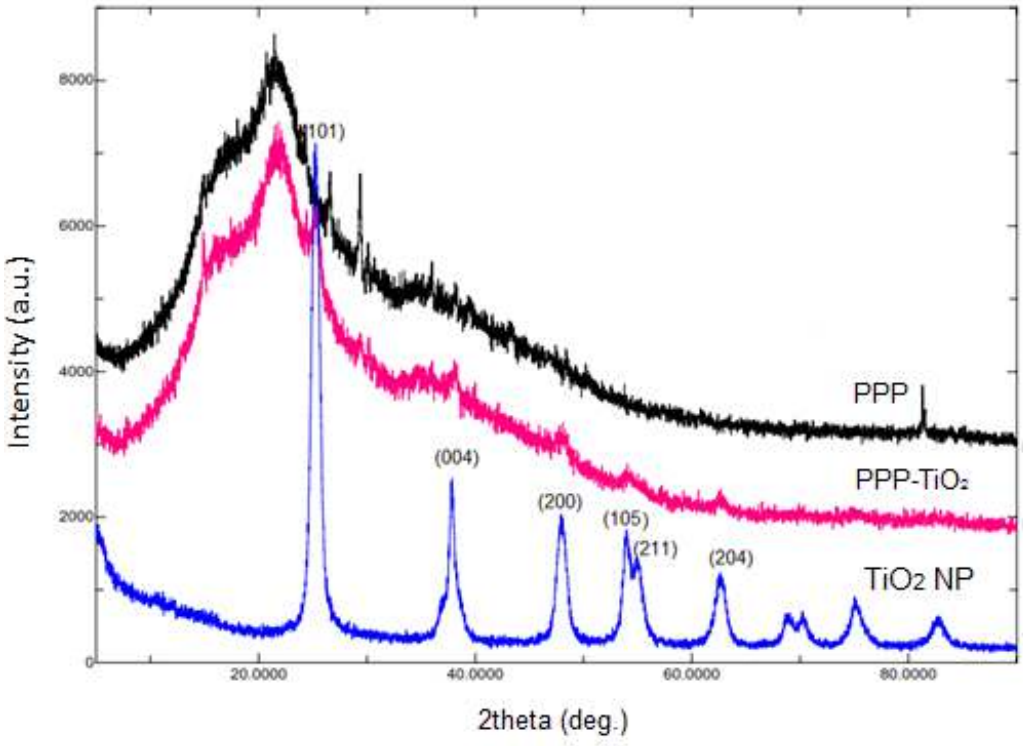


Figure 4.4: XRD patterns of the prepared samples

4.2 Antibacterial Activity of Natural Media against Gram Negative and Gram Positive Bacteria

The potential antibacterial activity of the PPP, TiO_2 NP, and PPP-TiO_2 was investigated using two Gram negative bacteria *P.aeruginosa*, and *E-coli*, and one Gram positive bacteria *S.aureus*. Several measures of bacterial growth and viability were used to evaluate their utility, Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), growth curve profiling, live dead cell, and well diffusion method assays were performed and evaluated.

4.2.1 Antibacterial Activity of PPP against Gram Negative and Gram Positive Bacteria

In our experiment the antimicrobial tests for PPP gave unclear inhibition results attributed to the poor water solubility of the active materials. Our results are in agreement with similar published papers (Dahham et al., 2010; Negi et al., 2003; Khan et al., 2011; Nikfallan et al., 2014) where they reported that pomegranate peel extracts showed clear inhibition effect if they mixed or extracted with co-solvent such as ethanol methanol, Dimethylsulfoxide (DMSO), etc.

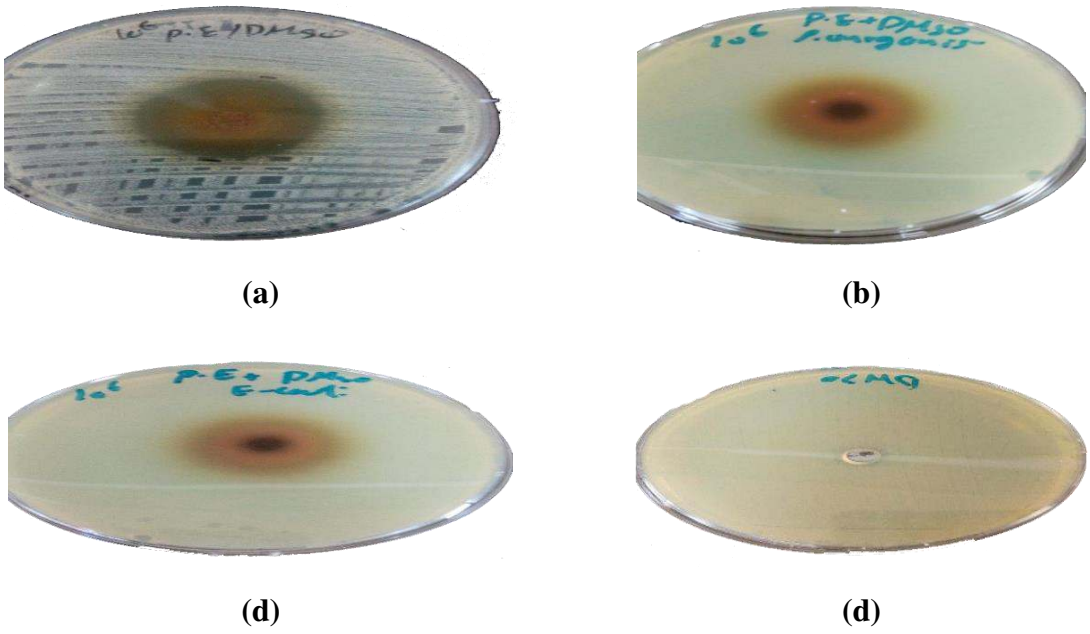
PPP was found to exhibit a fairly significant antibacterial activity against *S. aurous, P.aeruginosa*, and *E-coli* if DMSO is used as a solvent. Maximum inhibition zone of 35 mm was observed against S. aurous using 0.02 g/ml concentration of PPP. However, no inhibition zone was observed for DMSO as a control against Gram positive and Gram negative of the same selected bacteria as shown in Table 4.2 and Figure 4.5. Also, our results showed a higher inhibition effects toward gram positive strain compared to the effect on gram negative bacteria. The diameter inhibition zones (DIZ) showed maximum inhibition against *S.aureus* than *P.aeruginosa* than *E-coli* which is agreed with similar results that have been detected by other researchers (Khan et al., 2011). They reported that

the antibacterial screening of various extracts of pomegranate peel prepared in hot water, methanolic or ethanolic solvents against different pathogenic bacteria (*P.aeruginosa*, *E.coli*, and *S.aureus*) is highly dependent on the type of the used solvent. The inhibition zone was the best for extract dissolved in ethanolic solvent against *P.aeruginosa, E.coli,* and *S.aureus* respectively.

Antimicrobial activity of punica granatum (pomegranate) have been extensively studied by other researchers (Khan et al., 2011; Dahham et al., 2010). Their results indicated that this plant is ethno medically valuable. Pomegranate peel extracts are currently used for treatment of respiratory diseases and in the preparation of therapeutic formulae. The presence of therapeutic compounds in the extracts including phenols and polyphenols, tannins and hydrolysable tannins, and flavonoids as major active constituents may be responsible for the antimicrobial activity.

Table 4.2: Antibacterial activity produced by 2% wt concentration of PPP on S.*oureus, P.ouregonisa,* and *E-coli*

Bacteria	Diameter inhibition zone (DIZ)
S.oureus ATCC700699	35 mm
P.aeruginosa ATCC 27853	22 mm
E-coli ATCC12900	20 mm



(a) **(b)**

(d) **(d)**

Figure 4.5: The diameter inhibition zone on MH agar plates produced by 2% wt concentration of PPP against **a.** *S.oureus*, **b.** *P.ouregonisa*, **c.** *E-coli* and **d.** control solution (DMSO)

4.2.2 Antibacterial Activity of TiO_2 NP against Selected Pathogens

Nanoparticles used in this study had been previously synthesized and characterized in Prof. Albiss's laboratory in the physics department at Jordan University of Science and Technology using green method. Commonly well diffusion method was often used to test the antibacterial activity of TiO_2 NP against Gram negative and Gram positive bacteria. No DIZ was formed due to the poor rheology and the agglomeration or aggregation of the nanoparticles that reduce the interactions with the media components making the antibacterial activity's assessment not valid technique as shown in Figure 4.6. Our results were in consistent with results obtained by Venkatasubbu et al., 2016 and Schachtet al., 2013, where they preferred minimum inhibitory concentration (MIC) method instead of well diffusion method due to the poor rheology of ZnO and TiO_2 NPs. For this reason, the MIC method was used because of the strong dynamic contact of the nanoparticles with the tested bacterium (Venkatasubbu et al., 2016)

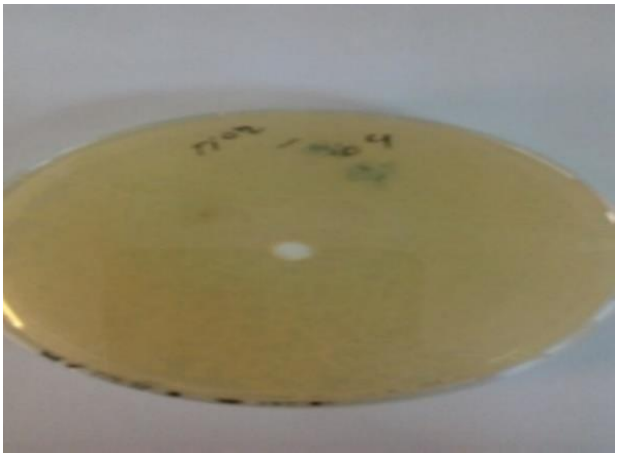


Figure 4.6: DIZ equal zero for TiO_2 NP against selected bacteria

The (MIC_{50}) and (MIC_{90}) are defined as the lowest concentration of antibiotic that reduced the bacterial growth to 50% and 90%, respectively during 24 h of incubation. Our MIC results for TiO_2 NP exhibited a fairly significant antibacterial activity against Gram positive and Gram negative (*S.aureus, P.aeruginosa,* and *E-coli*) as shown in Table 4.3. The MIC_{90} and MIC_{50} for the *S.aureus* were 203.9.1, 333.5 µg/ml respectively. While, the MIC $_{90}$ and MIC_{50} for E-coli were 271.4.1, 437.5 µg/l respectively. Gram positive species were found to be more sensitive to TiO_2 NP than Gram negative bacteria. The obtained results were in quite agreement with Azam et al., 2012 findings, where they studied the antimicrobial activity of metal oxide nanoparticles against Gram-positive and Gram-negative bacteria. They found that the antibacterial activity of the nanomaterials increases with the increase in surface-to volume ratio due to the decrease of the nanoparticles size. In addition, they indicated that nanomaterials were most effective against Gram-positive bacterial strains compared to Gram-negative bacterial strains.

Table 4.3: MIC of TiO_2 NP against two Gram negative bacteria and one Gram positive bacteria

Bacteria	MIC50 (µg/ml)	MIC90 (µg/ml)
S.aureus ATCC700699	203.9	333.5
P.aeruginosa ATCC 27853	213.9	352.4
E-coli ATCC12900	271.4	437.5

In present study, TiO_2 NP was found to inhibit the growth of Gram positive and Gram negative bacteria and the inhibition depends highly on TiO_2 NP concentrations. Figure 4.7 shows the minimum inhibitory concentration for *S.aureus, P.aeruginosa*, and *E-coli* using different concentrations of TiO_2 NP. Cell growth affected by TiO_2 NP was measured by using Optical Density (OD) at 610 nm after 24 h growth period. Our results show a reduction in OD when adding different concentrations of TiO_2 NP and similar inhibition trends were performed for all selected bacteria. The results also indicate that the efficiency of TiO_2 NP against *S.aureus* is better than *P.aeruginosa* than *E-coli*. Positive control reading referred to bacterial growth without TiO_2 NP (zero µg of PPP-TiO_2). Each sample was subtracted from its negative control (TiO_2 NP with zero µg of bacteria).Our results are in quite agreement with previous studies (Venkatasubbu et al., 2016; Hassani et al., 2015), in which nanomaterial exhibited strong antimicrobial effects towards a broadened spectrum of bacterial strains. Furthers, Haghi et al., 2012 findings. They found a reduction in OD) when adding different concentrations of TiO_2 NP against Pathogenic strain *E-coli*.

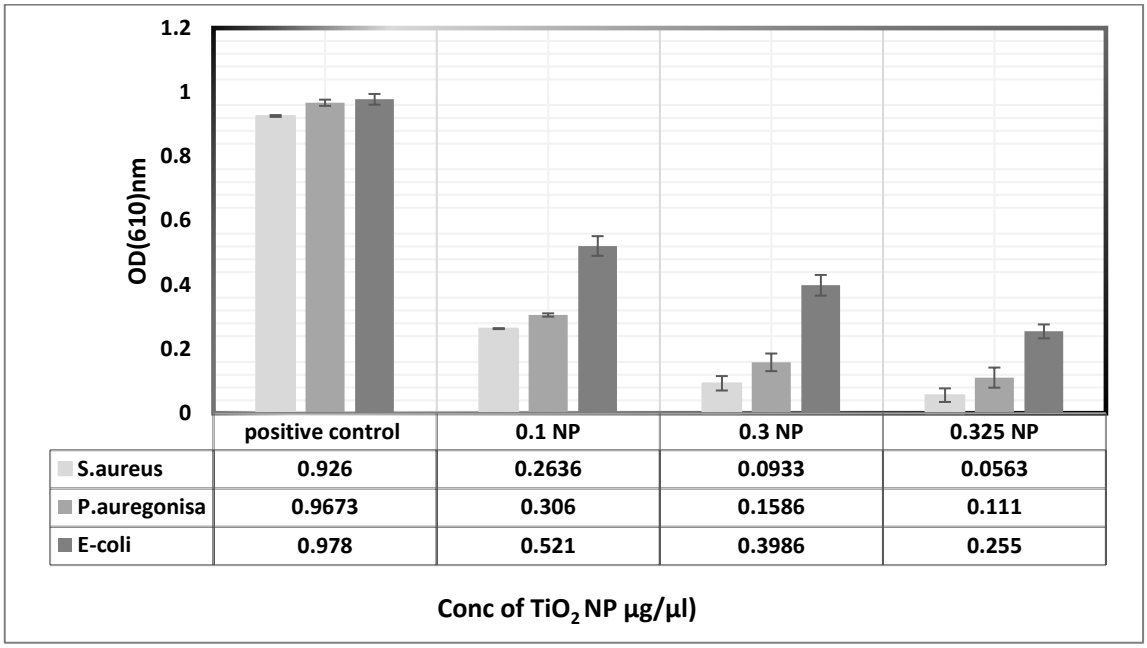


Figure 4.7: The minimum inhibitory concentration for *S.aureus, P.aeruginosa*, and *E-coli* using different concentrations of TiO_2 NP. OD was measured at 610 nm after 24 h growth period. The curves represent triplicates of three independent experiments. Error bars represent 95% confidence intervals, n=3

The antibacterial activity mechanism(s) of NPs have been perversely studied by several researchers (Venkatasubbu et al., 2016; Hassani et al., 2015; Khalil et al., 2013; Haghi et al., 2012; Emamifar, 2011)) and Figure 4.8 shows the proposed mechanism(s). They suggested that the nanoparticles can either directly interact with the microbial cells, e.g. interrupting transmembrane electron transfer, disrupting/penetrating the cell envelope, or oxidizing cell components , or produce secondary products (e.g. reactive oxygen species (ROS) or dissolved heavy metal ions). Reactive Oxygen Species (ROS) are naturally produced as byproducts of metabolism. Apoptosis is caused when these ROS molecules accumulated inside the cell. NPs may attach to the surface of the bacterial cell membrane and disrupt it integrity, disturbing permeability and respiration functions of the cell resulting in cell death. Nano materials also could deactivate the cellular enzymes and DNA by coordinating to electron-donating groups, such as: Thiols, Carbohydrates, Amides, Hydroxyls and etc. They cause pits in bacterial cell walls, leading to increased permeability and cell death.

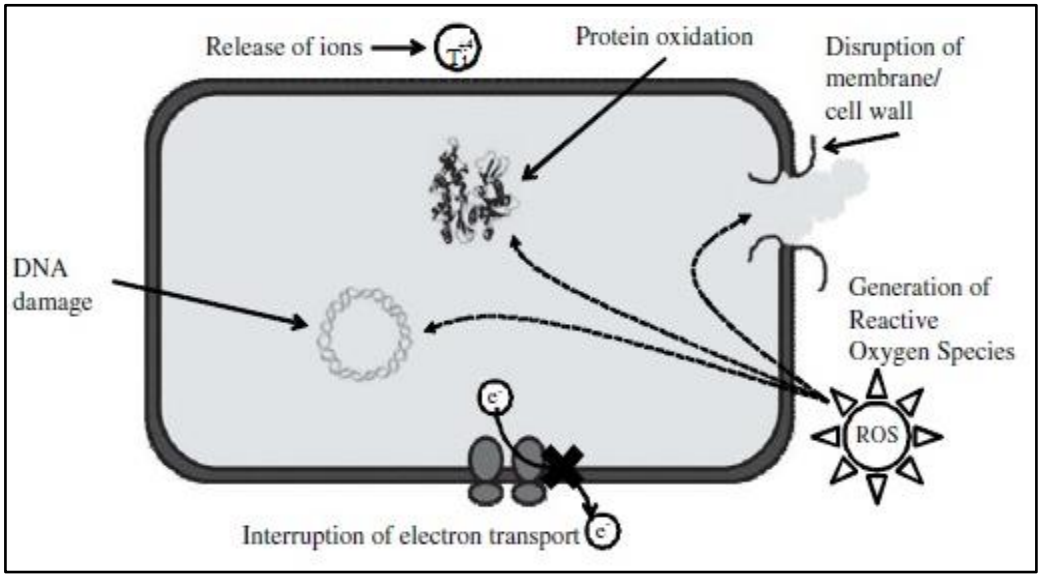


Figure 4.8: Various mechanisms of antimicrobial activities exerted by nanomaterials (Emamifar, 2011)

4.2.3 Antibacterial Activity of PPP-TiO_2 against Selected Pathogens

4.2.3.1 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

In our study, the micro broth dilution method was used to screen the antibacterial activity of PPP-TiO_2 against two Gram negative bacteria (*P.aeruginosa*, and *E-coli*), and one Gram positive bacteria (*S.aureus*). The antibacterial effect of PPP-TiO_2 was measured by determining the minimum concentration needed to inhibit the growth and/or to kill the microorganisms. The (MIC_{50}), (MIC_{90}) and (MBC) of PPP-TiO_2 values against selected bacteria was determined based on batch cultures containing varying concentration of PPP-TiO_2 in suspension as shown in Table 4.4.

Table 4.4 shows that all PPP-TiO_2 concentrations exhibited good bactericidal activity against the three tested bacterial strains after 24h of incubation. For the use of *S.aureus* bacteria led to the lowest MIC_{90}, MIC_{50}, and MBC of 189.1, 101.2, 200 µg/ml, respectively. While, the MIC_{90}, MIC_{50} and MBC against *P.aeruginosa* were 303.2, 309.7, <315 µg/ml, respectively, and against *E-coli* were 160.5, 143.6, <310 µg/ml, respectively.

For the three bacterial species that were evaluated, the observed inhibition trends were similar for all assays performed as shown in Figure 4.9. Cell growth affected by PPP-TiO_2 was measured by using OD at 610 nm after 24 h growth period. Bacterial growth rates were found to depend on PPP-TiO_2 concentrations in the range of 100 to 300 µg/ml in which bacterial growth decreases as PPP-TiO_2 concentration increases (OD decrease). The curves represent triplicates of three independent experiments. Error bars represent 95% confidence intervals, n=3.

The results also indicate that the efficiency of PPP-TiO_2 against *S.oureus* is better than Escherichia coli than *P.aeruginosa*. Positive control reading referred to bacterial growth without PPP-TiO_2 (zero µg of PPP-TiO_2). Each sample was subtracted from its negative control (PPP-TiO_2 with zero µg of bacteria). Our results is consistent with other studies where they found a reduction in OD when adding different concentrations of antibiotic against Pathogenic strain. Awad et al., 2015 studied the antibacterial activity of AgNPs/polystyrene nanocomposite against Gram positive *Staphylococcus aureus* and Gram negative bacteria E.coli, Klebsiella pneumoniae, and Salmonella. The nanocomposite that has been prepared was an effective agent against Gram positive and Gram negative bacteria.

In addition, MBC of the PPP-TiO_2 was determined on agar plates by counting the bacteria after incubation period at 37 °C for 24 h. The MBC was defined as the minimal concentration of samples in which the number of survival bacteria was less than 5 coloni on the agar plates (Hassani et al., 2015; Kannan, et al., 2015). Figure 4.10 showed the MBC produced by 200 µg/ml of PPP-TiO_2 against S.oureus (image (a)), 315 µg/ml of PPP-TiO_2 against *P.ouregonisa* (image (b)), and 310 µg/ml of PPP-TiO_2 against *E-coli* (image (c)). The survival bacteria was 5 coloni on *S.oureus* agar plate which is the lowest concentration of antibiotic that kill 99.99% of *S.oureus*. However, for *P.ouregonisa and E-coli* images, the survival bacteria was higher than 5 coloni. So the minimum bactericidal

concentration required to kill 99.99% of the *P.aeruginosa* and *E-coli* was higher than 315 and 310 µg/ml respectively.

Our Minimum Bactericidal Concentration results are in agreement with Hassani et al., 2015 results, where they studied the inhibition effect of zinc oxide nanoparticles on *P.aeruginosa*. They found that the MBC was higher than 325 µg/ml. Further Khalil et al., 2013 studied the antibacterial activity of silver nanoparticles using plant extract against *S.oureus P.aeruginosa* and *E-coli*, and the MBC was 300-700 µg/ml.

Table 4.4: MIC of PPP-TiO_2 against two gram negative bacteria and gram positive bacteria

Pathogenic bacteria	**MIC_{50} (µg/ml)**	**MIC_{90} (µg/ml)**	**MBC (µg/ml)**
S.aureus ATCC700699	102.1	189.1	200
P.aeruginosa ATCC 27853	160.5	309.2	<315
E-coli ATCC 12900	143.6	304.7	<310

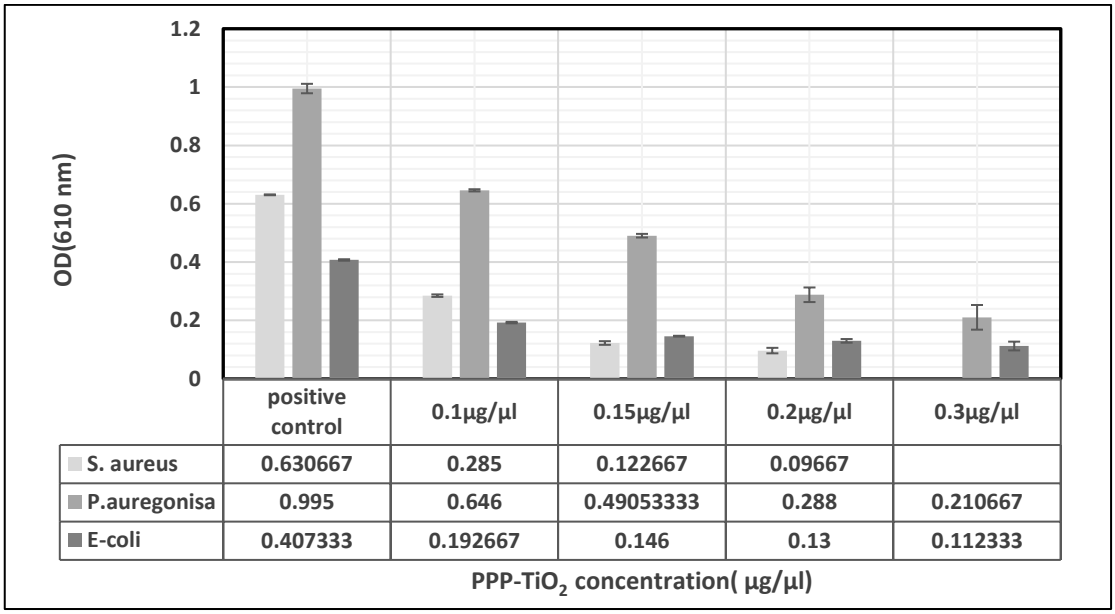
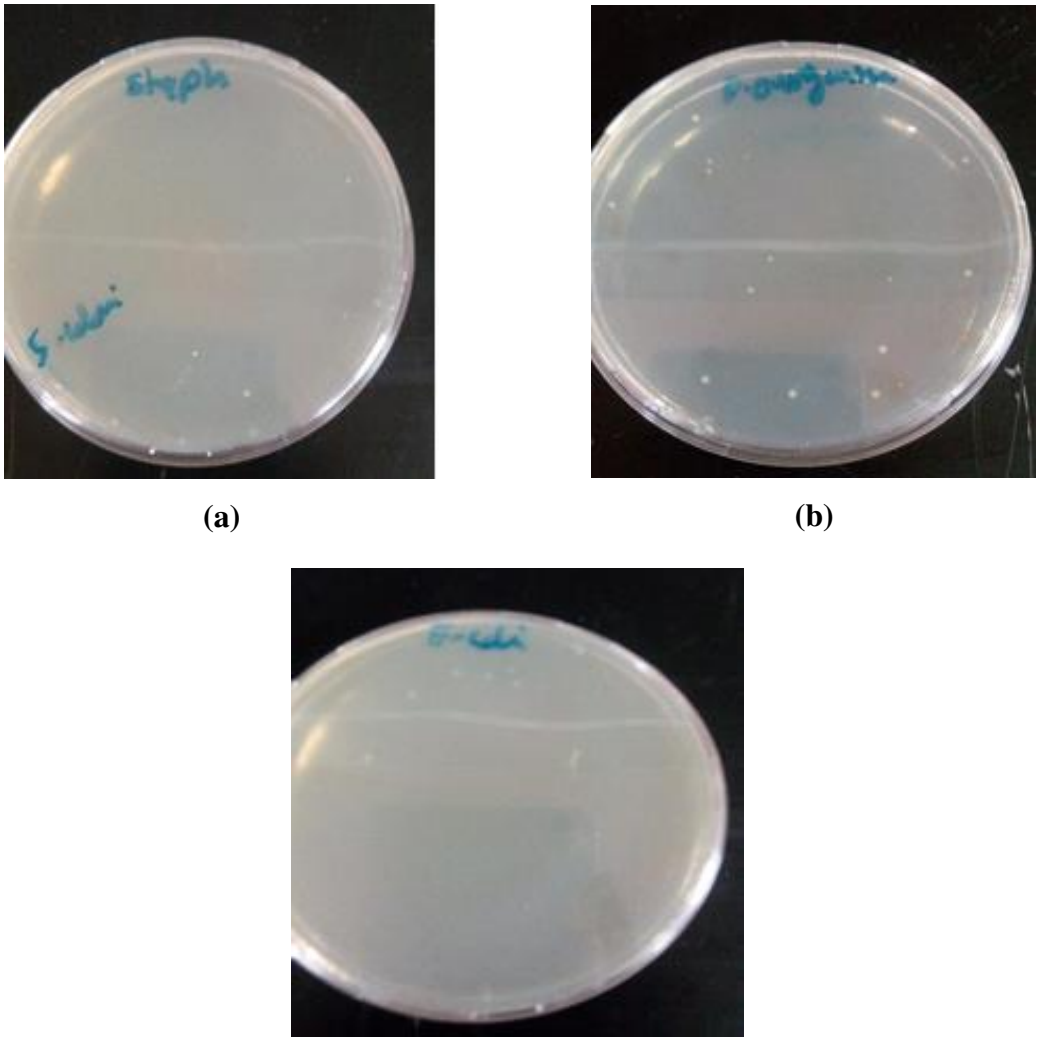


Figure 4.9: The minimum inhibitory concentration for *S.aureus*, *P.aeruginosa*, and *E-coli* on different concentrations of PPP-TiO_2. OD was measured at 610nm after 24 h growth period. The curves represent triplicates of three independent experiments. Error bars represent 95% confidence intervals, n=3



(a) **(b)**

(c)

Figure 4.10: The Minimum bactericidal concentration (MBC) on Mueller Hinton (MH) agar plates produced by different concentration of (PPP-TiO_2)) against **a.** *s.oureus*, **b.** *P.ouregonisa*, **c.** for *E-coli*

4.2.3.2 Growth Curve Profiling

In general, the growth curve of any bacteria include four phases: lag, log or exponential growth, stationary and death phase as shown in Figure 4.11. In normal condition bacterial cells reached exponential phase rapidly but under the influence of antibiotic the log or exponential phase will be shortened (Venkatasubbu et al., 2016).

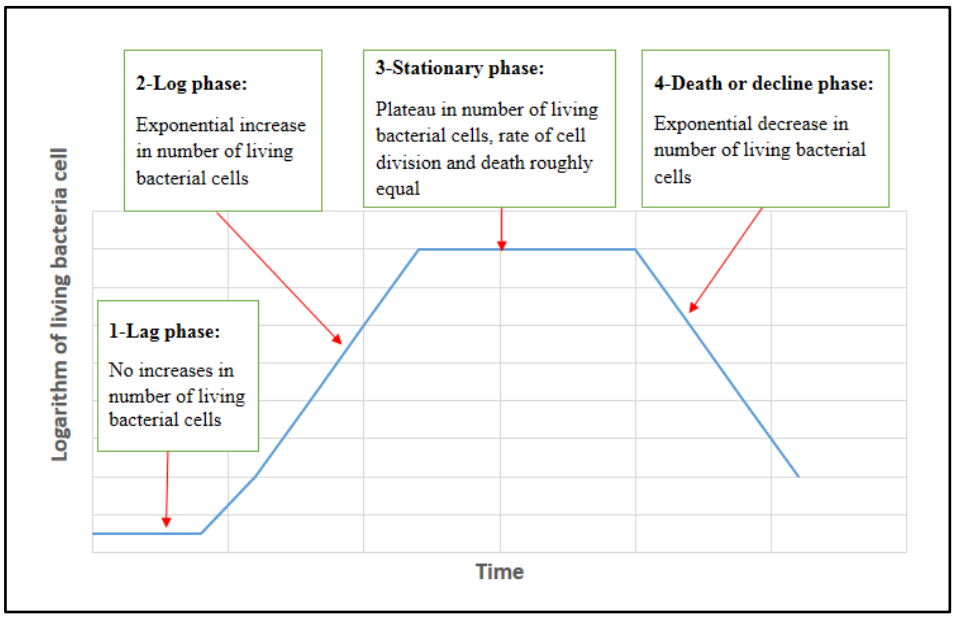


Figure 4.11: Bacteria growth curve

The antibacterial tests were also performed by monitored the changes in the growth of bacteria due to the influence of PPP-TiO_2. The time-dependent changes in the quantity of bacterial growth were monitored by measuring the OD at 610 nm at a regular interval (up to 24 h). Figures 4.16-4.18 show dynamic growth curve of *S.aureus*, *P.aeruginosa*, and *E-coli* at different concentrations of PPP-TiO_2 within 24hr.Triplicate measurements were performed and compared with positive control (zero µg of PPP-TiO_2).

Our results indicated that the PPP-TiO_2 at all tested concentrations had strong suppression of proliferation of tested bacteria compared to the positive control. Figure 4.12 shows that at time between 2 to 6 h (log phase) bacterial growth increases, then decreases until it reaches the death phase (24h). For *S.aureus*, when the concentrations of PPP-TiO_2 were above 150 mg/ml, the PPP-TiO_2 inhibited the growth of *S.aureus* completely during the whole 24h. However, at concentration of PPP-TiO_2 below 150 µg/ml, the PPP-TiO_2 inhibited 50% of the growth of *S.aureus* with short on log phase during the whole 24h. Figure 4.13 for *E-coli*, the concentrations 150, 200, 300 µg/ml of PPP-TiO_2 inhibited the growth of *E-coli* during the whole 24h. However, when the concentration of PPP-TiO_2 was below MIC, the growth of *E-coli* was not inhibited within

24h. Figure 4.14 shows the growth curve of *P.aeruginosa*, at concentrations 100,150 µg/ml of PPP-TiO_2 showed no decline in bacterial growth because the concentrations were below the MIC. When the PPP-TiO_2 concentration was above the 300 µg/ml, the growth of *P.aeruginosa* was inhibited completely.

Figures 4.12-4.14 conclude that the growth inhibition susceptibility of bacterium due to the influence of PPP-TiO_2 within 24h of incubation increases significantly with the increase in the PPP-TiO_2 concentration. The concentrations below the MIC could not affect the bacterium growth due to inadequate dosage to inhibit or kill the bacteria. The highest decline of bacterial growth was for *S.aureus* then for *E-coli* and *P.aeruginosa*, which means that the PPP-TiO_2 media has better activity against gram positive bacteria than gram negative. This agrees with published papers by Shanmugavadivu et al., 2014 in which they found that the maximum antibacterial activity of silver nanoparticles synthesized using pomegranate peel extract was more for *S.aureus* than *E-coli* than *P.aeruginosa*.

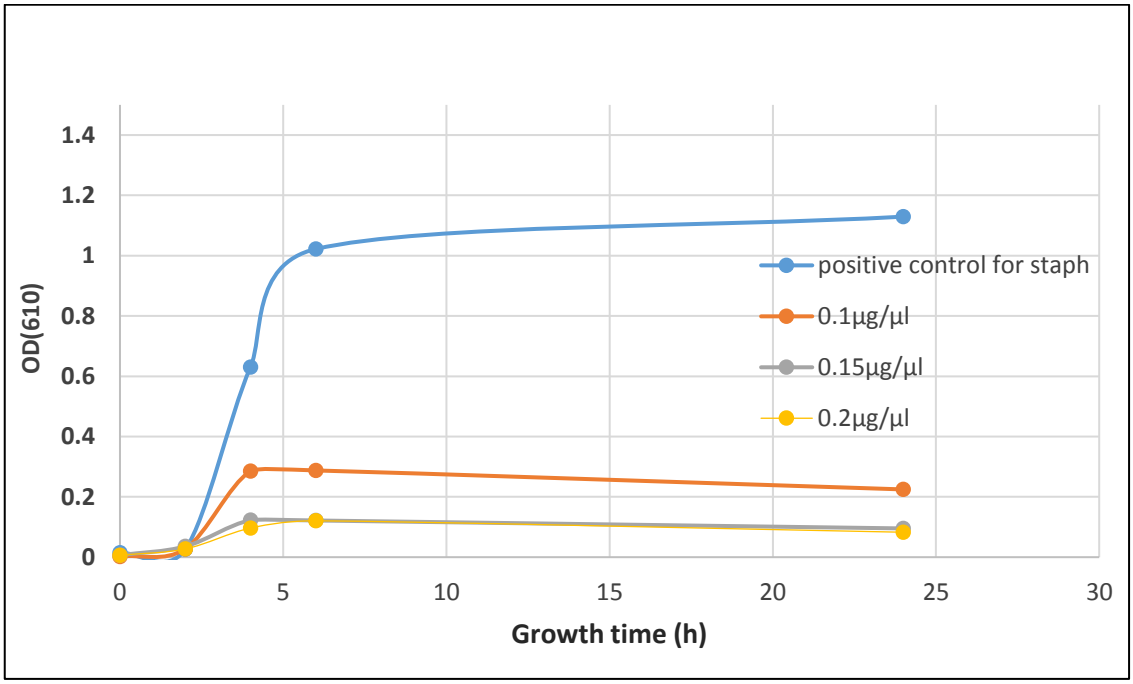


Figure 4.12: Growth curves of *S.aureus* at various PPP-TiO_2 concentrations. Cell growth was measured by optical density at 610 nm at indicated time points. The curves represent triplicates of three independent experiments

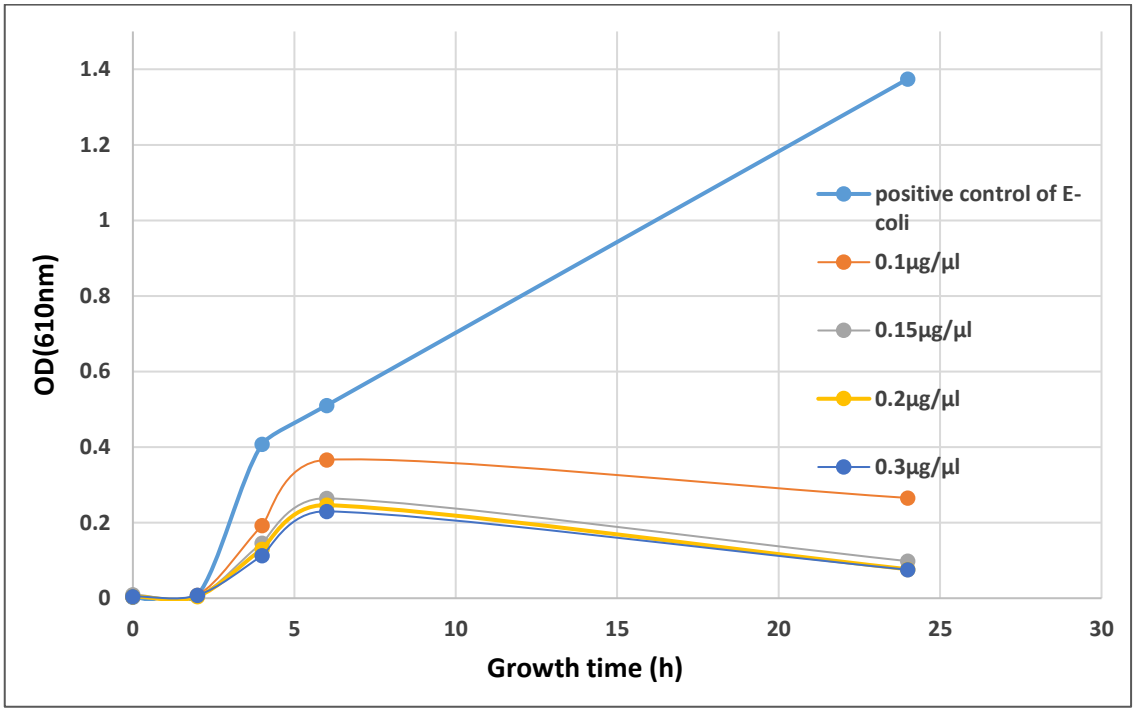


Figure 4.13: Growth curves of *E-coli* at various PPP-TiO_2 concentrations. Cell growth was measured by using optical density at 610 nm at indicated time points. The curves represent triplicates of three independent experiments

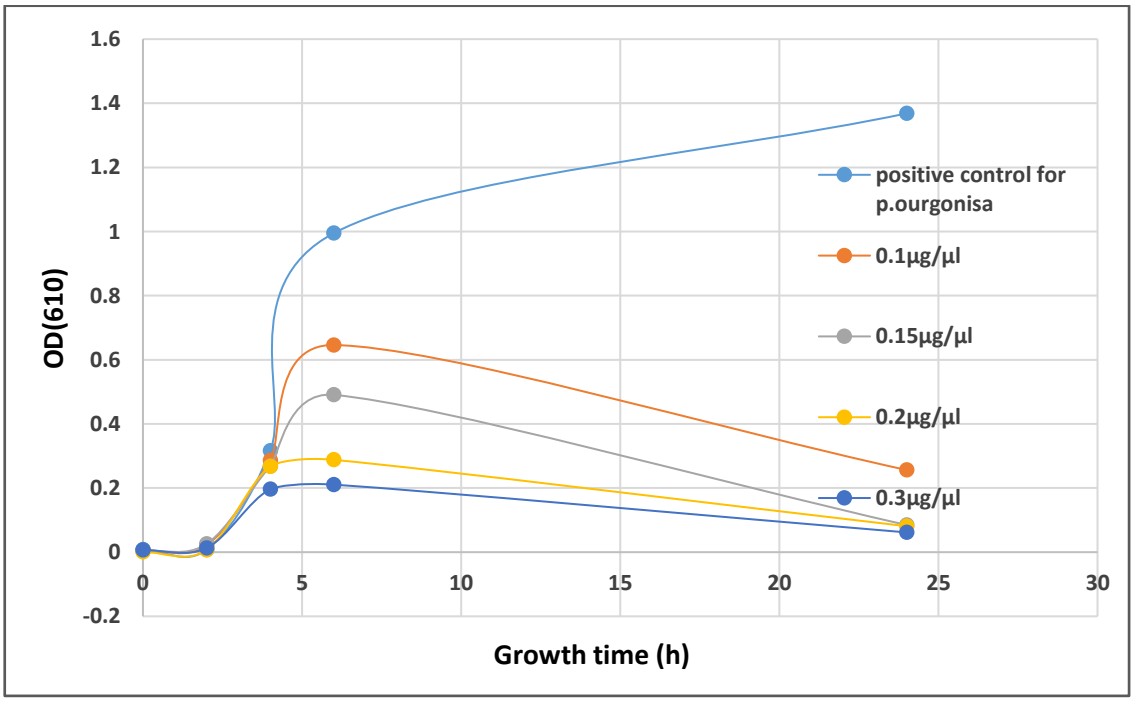


Figure 4.14: Growth curves of *P.aeruginosa* in the precence of PPP-TiO_2 at concentrations ranging from0.1-0.3 µg/µl. Positive control was used as *P.aeruginosa* in MH media Incubation period was 24hr against

4.2.3.3 Well Diffusion Method

PPP-TiO_2 has exhibited a fairly significant antibacterial activity against *S.aureus*, *P.aeruginosa*, and *E. coli*. Double distilled water (DDW) was used as a control. Our results showed a higher inhibition effects toward Gram positive bacteria compared to the effect on Gram negative bacteria which agree with our MBC and MIC results for PPP-TiO_2 testing. The DIZ showed maximum inhibition against *S.aureu* than *E-coli* than *P.aeruginosa* which also agree well with our results and other published results (Shanmugavadivu et al., 2014).

Maximum zone of inhibition of 22mm was observed against *S.aureus* when 0.02 g/ml PPP-TiO_2 was added. Table 4.5 and Figure 4.15 show the antibacterial activity of PPP-TiO_2 against selected Gram positive and Gram negative bacteria. It is clear that the added amount of PPP-TiO_2 that prevents bacterial growth was different for each type of strain. It should be noted that the diffusion assay is prone to artifacts arising from factors such as the diffusion rate, particle adsorption to the disk or well, and the hydrophobic/ hydrophilic nature of the particle (Pelletier et al., 2010).

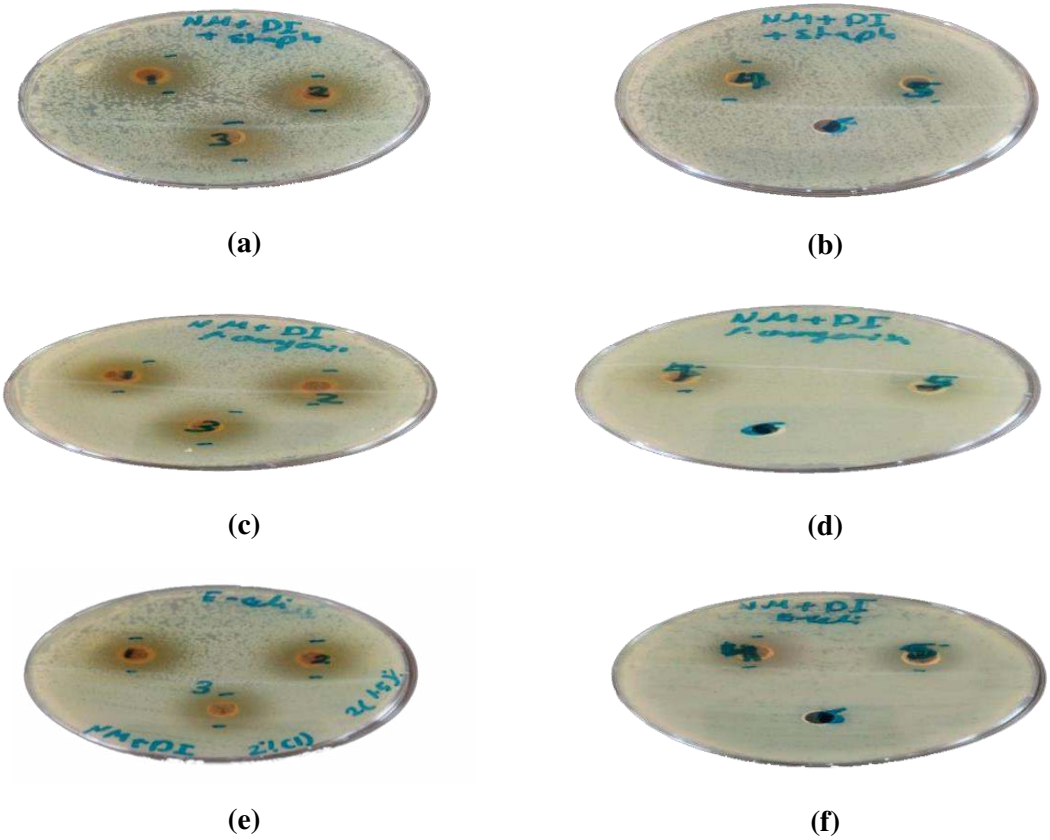
Figure 4.15 shows the diameter inhibition zone on Mueller Hinton (MH) agar plates produced when different concentrations of PPP-TiO_2 were added *against S.oureus* (a and b), *P.ouregonisa* (c and d), and *E-coli* (e and f). The numbers that were written on the plates 1, 2, 3, 4, and 5 indicate the PPP-TiO_2 concentrations of 2% wt., 1.5 wt., 1% wt., 0.5% wt. and 0.25% wt. respectively. Number 6 relate to inhibition effect of control solution which is here the double distilled water.

Well diffusion results obtained in this study are in agreement with the results found by Kannan et al., 2015 they studied the antibacterial properties of punica grantum peels and found that the DIZ for PPP against *Staphylococcus aureus*, *Seudomonas aeruginosa*, and *Escherichia coli* were 25.5, 22, and 22.5 mm respectively. Further, Haghi et al., 2012 studied the antibacterial effect of different concentrations of TiO_2 NP against Pathogenic

strain *E-coli*. The measured inhibition zone increased by increasing the added concentration of TiO_2 NP.

Table 4.5: DIZ produced by different antibicterial ratio (%) and against *S.aureus, P.aeruginosa*, and *E-coli*

Bacteria / **PPP-TiO_2Conc**	**2%**	**1.5%**	**1%**	**0.5%**	**0.250%**	**DDW (control)**
S.aureus ATCC700699	22 mm	18 mm	17 mm	15 mm	9 mm	0 mm
P.aeruginosa ATCC 27853	17 mm	16 mm	14 mm	10 mm	0 mm	0 mm
E-coli ATCC12900	19 mm	18 mm	16 mm	10 mm	0 mm	0mm



(a) **(b)**

(c) **(d)**

(e) **(f)**

Figure 4.15*:* The diameter inhibition zone on MH agar plates produced by different concentration of PPP-TiO_2 for *S.oureus* (a and b), for *P.ouregonisa*, (c and d) for *E-coli*. (e and f). The numbers that were written on the plates 1, 2, 3, 4, 5 and 6 indicate the PPP-TiO_2 concentrations of 2%wt, 1.5wt, 1%wt, 0.5%wt 0.25%wt, and control respectively

4.3 Antibacterial Activities Evaluation of PPP-TiO_2, PPP, and TiO_2 NP against Selected Gram Negative and Gram Positive Bacteria

4.3.1 Quantification Bacterial Removal Efficiency by Antimicrobial Tests

PPP-TiO_2, PPP, and TiO_2 NP showed different antibacterial activities. The developed PPP-TiO_2 exhibited the strongest antibacterial effect against different bacterial strains. To compare and assess the antimicrobial properties for PPP and TiO_2 NP, diffusion method was selected for PPP and gave a clear inhibition results. However, broth dilution method (MIC) was used for TiO_2 NP because no clear DIZ was formed due to the poor rheology and the agglomeration or aggregation of the nanoparticles that reduce the interactions with the media components.

To compare the efficiency of PPP-TiO_2 and PPP, they were dissolved in DMSO and well diffusion method was found the DIZ of PPP-TiO_2 was 32 mm, While it was 22mm for PPP, which is higher by 1.5 time for same strain (*p.ouregonisa*) as shown in Figure 4.15 (c and d) and 4.16.

Also, our results indicate that the antibacterial efficiency of PPP-TiO_2 is higher than TiO_2 NP against same pathogenic bacteria. The MIC and MBC for PPP-TIO_2 against *S.aureus* were 102.1, 200 µg/µl and for TiO_2 NP were 203.9, 350 µg/µl. However, the MIC and MBC for PPP-TiO_2 against *P.ouregonisa* were 160.5, 325µg/µl and for TiO_2 NP were 213.9, 400 µg/µl against the same pathogenic. While, for *E-coli* the MIC and MBC for PPP-TiO_2 were 143.6, 325 µg/µl and for TiO_2 NP were 217.9, 450 µg/µl against the same pathogenic. These results indicated that the PPP-TiO_2 is more efficient than TiO_2 NP by 1.5 time for the same pathogenic bacteria. The results agreed well with our hypothesis that stated that plants fiber impregnated with nanoparticles will provide a media with highly bacterial removal efficacy under laboratory conditions.

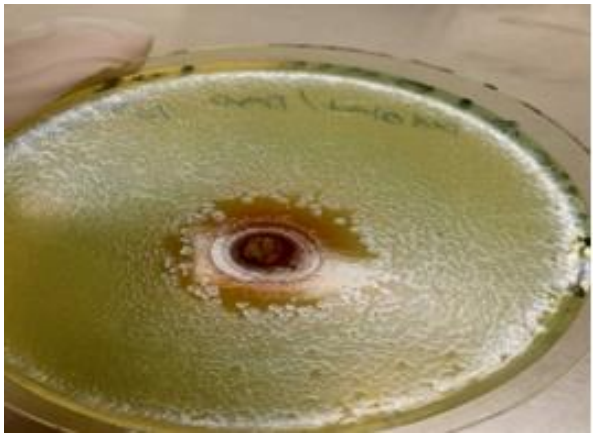


Figure 4.16: The DIZ for highest of PPP-TiO_2 concentration against P.ouregonisa with DMSO

4.3.2 Live/Dead Cell Viability Assay by Fluorescence Microscope

The Live-Dead assay provided a direct observation of the proportion of living and dead cells. Representative microscope images of PPP-TiO_2, PPP, and TiO_2NP against *S. aureus* and *E-coli* were shown in Figures 4.17, and 4.18. The combined used of SYTO-9 and propidium iodide effectively labelled both live and dead of bacteria cells under different experimental conditions. The optical observation of microscopy results revealed that PPP-TiO_2, PPP, and TiO_2NP have good performance against *S.aureus* and *E-coli*. Images (4.17 (a), and 4.18 (a) showed increasing on bacterial cells proliferation after incubation period (for 24 at 37°C). Bacteria cells exposed to PPP-TiO_2 treatment tended to be less densely celled than the control group. PPP-TiO_2 at MIC50%, and MIC90%, resulted in a large number of dead cells (stained in red) as well as a large number of black spaces, indicating the dispersion of the nanocomposite (images (4.17 (b and c)), and 4.18 (b and c)). However, PPP, and TiO_2NP exhibited lower number of dead cells and higher number of living cells (Images (4.17 (d and e)), and 4.18 (d and e)).

Live-Dead assay provided further evidence that PPP-TiO_2 has strong effective against several types of bacteria. Furthers, our Live-Dead results are well agreement with the results showed by Chaudhari et al., 2015 where they studied the antimicrobial activity of pegylated silver coated carbon nanotubes against Salmonella. They based on the

bacterial growth curve analysis to analyzed live/dead staining of bacteria. Upon exposure to various concentrations of nanocomposites, 12.5µg/mL of pegylated silver coated single walled carbon nanotubes (pSWCNTs-Ag) for 16 h, proportion of live/dead cell was approximately 7–8 fold lower when bacteria were exposed to pSWCNTs-Ag compared to non-treated controls.

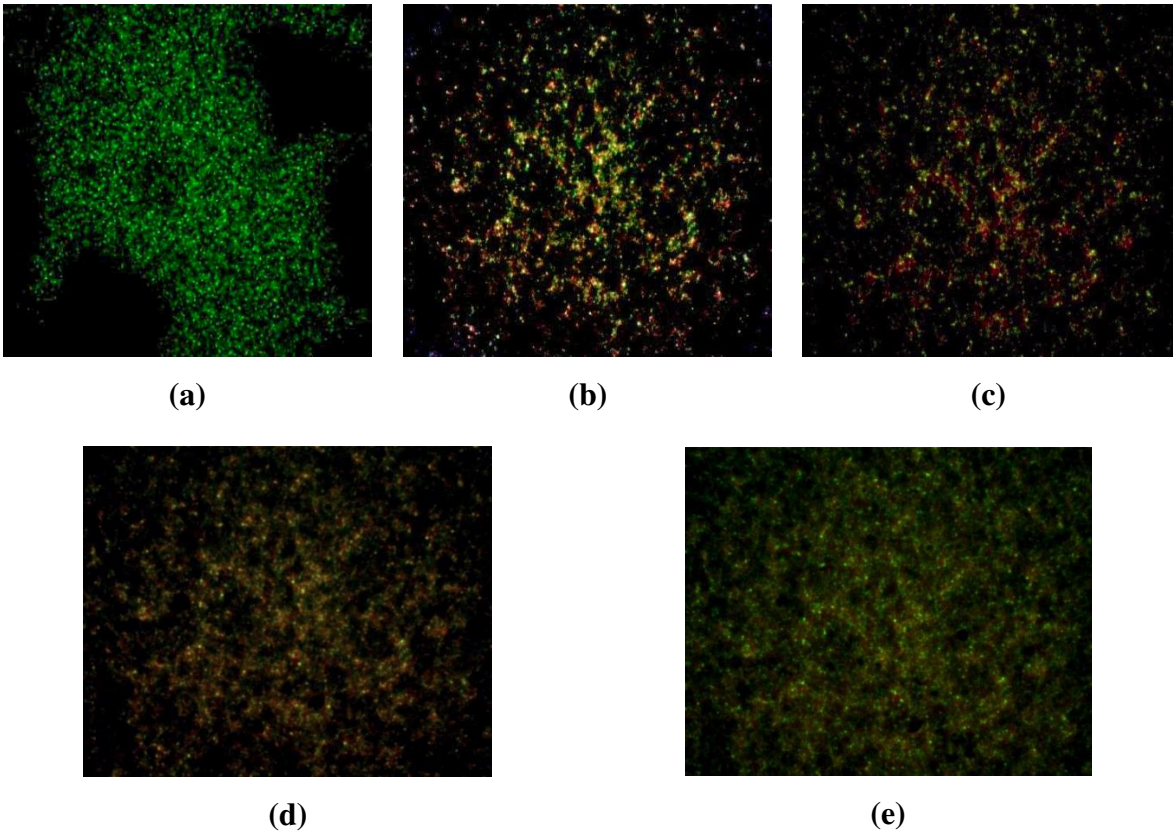


Figure 4.17: Representative microscope of (a) *S.oureus* after incubation period. Live-Dead cell staining of *S.aureus* cells after culturing with (b) and(c) for MIC50%, and MIC90% of PPP-TiO_2 respectively, (d) and (e) for PPP, and TiO_2NP respectively. Living cells were detected as green fluorescence and dead cells were detected as red fluorescence

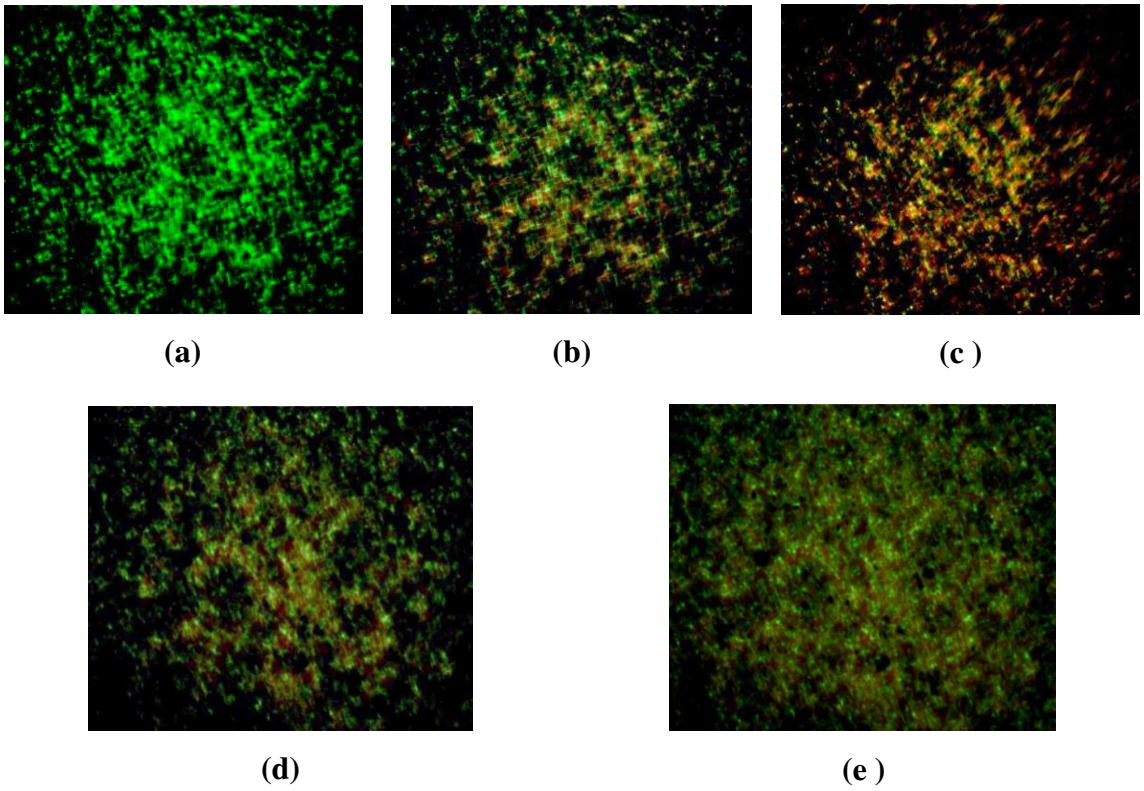


Figure 4.18: Representative microscope of (a) *E-coli* after incubation period. Live-Dead cell staining of *E-coli* cells after culturing with (b) and(c) for MIC50%, and MIC90% of PPP-TiO_2 respectively, (d) and (e) for PPP, andTiO_2NP respectively. Living cells were detected as green fluorescence and dead cells were detected as red fluorescence

.44 Antibacterial Activity of PPP-TiO_2 in Natural Water and Synthetic Water Samples against Bacteria

To test the efficiency of PPP-TiO_2 on natural water samples, different water samples were selected from vital wells that provide Irbid city. The physical water properties for the tested water are shown in Table 4.6 (From operating and maintenance unit at Jordan University of Science and Technology). Antimicrobial activity was tested by well diffusion method against *S.aureus*. Table 4.7 and Figure 4.19 show the effect of PPP-TiO_2 against selected bacteria for different water samples. Similar antimicrobial activity for PPP-TiO_2 were tested using natural water and synthetic water compared to samples tested in double distilled water samples. This indicated that the prepared media has a potential to be used effectively in treating natural water in real life applications.

Table 4.6: The physical water properties for the tested water from operating and maintenance unit at Jordan University of Science and Technology

Test	Unit	Result			Standard			Max Standard		
		JUST	Alhoson	Alnaemah	JUST	Alhoson	Alnaemah	JUST	Alhoson	Alnaemah
pH	SU	7.31	7.31	7.31	8.5	6.5	6.5-8.5	6.5	8.5	-
TDS	mg/l	491	314	305	1000	1000	1000	1000	1000	1000
TH	mg/l	190	184	168	300	300	300	500	500	500
Turbidity	NTU	NIL	NIL	NIL	1	1	1	5	5	5
Cl^-	mg/l	88	78	67	200	200	200	500	500	500

Table 4.7: DIZ after treatment of *S.aureus* with synthesized PPP-TiO_2 in natural water samples

Water Source	PPP-TiO_2 Concentration					
	2%	1.5%	1%	0.5%	0.25 %	Control
Al-Hoson	22 mm	20 mm	19 mm	15 mm	0 mm	0 mm
Al-Naemah	21 mm	20 mm	17 mm	15 mm	0 mm	0 mm
JUST	20 mm	19 mm	17 mm	12 mm	0 mm	0 mm
SFW	23 mm	20 mm	19 mm	10 mm	0 mm	0 mm

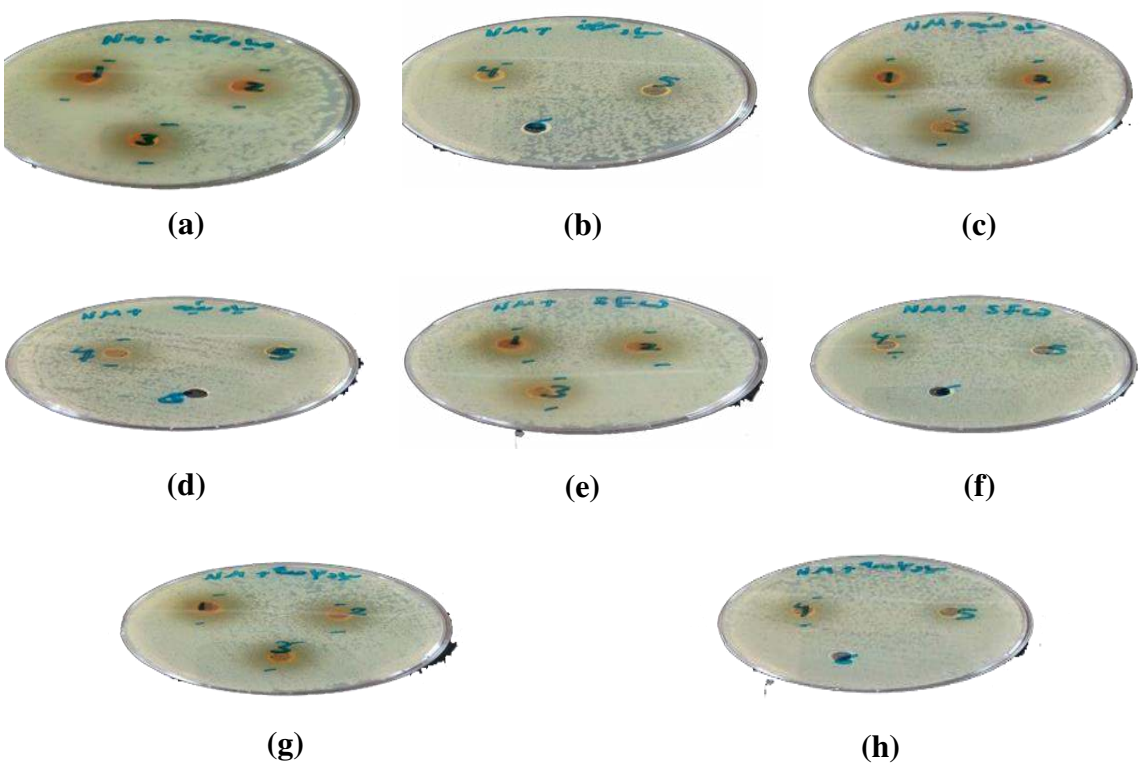


Figure 4.19: The diameter inhibition zone on Mueller Hinton (MH) agar plates produced by different concentration of PPP-TiO_2 against S.oureus for ALhoson wells sample (a and b) for ALnaemah (c and d) for SFW sample(e and f) and (g and h) for JUST weels sample. The numbers that were written on the plates 1, 2, 3, 4, 5 and 6 indicate the PPP-TiO_2 concentrations of 2% wt, 1.5%wt, 1%wt, 0.5% wt 0.25% wt, and control respectively

4.4.1 Biological Oxygen Demand (BOD)

Biological Oxygen Demand (BOD) indicates the amount of organic pollutants present in water samples. Therefore, a low BOD is an indicator of good quality water, while a high BOD indicates polluted water. Dissolved oxygen (DO) is consumed by bacteria in the process of oxidation of organic matter in water body. When BOD levels are high, dissolved oxygen (DO) levels decreases because the oxygen that is available in the water is being consumed by the bacteria and therefore, less dissolved oxygen is available for microbial growth (Dezuane, 1997).

Table 4.8 and Figure 4.20 show the measured BOD_5 for real and synthetic water samples with and without PPP-TiO_2. The MH broth mixed with 50% water was used as a blank, which is the same blank used on MIC experiment. The BOD_5 values were found

higher for water samples containing no PPP-TiO_2 and lower values for samples containing PPP-TiO_2. The high BOD_5 values in our tested water samples are due to the presence of dissolved organic materials in the media and/or tested strain (0.25% of PPP-TiO_2 and 10 µl of *E-coli*). Lower BOD_5 values were found for samples containing PPP-TiO_2 indicated that the sample has lower organic matter and indirectly indicated lower microbial community. The BOD results agree well with our antimicrobial testing results where more microbes were found on untreated sample. Furthermore, the BOD_5 result for blank was compatible with the natural water samples results. Therefore, our developed media has the potential to be used in practical applications.

Our results are in agreement with Hernández-Hernández et al., 2016 who studied the applications of polymer-clay nanocomposites for removal organic compounds of environmental interest. They showed that the nanotechnology currently enabled in water treatment technology focuses on two major areas to improve its quality: degradations of organic pollutants, and removal of pollutants by adsorption process. In addition, they confirmed that the nanocomposite has significant advantages of established adsorbents for water treatment. Furthermore, Pasternak et al., 2017 studied the Self-powered, autonomous biological oxygen demand biosensor for online water quality monitoring. They synthesized biosensor consisted from Microbial Fuel Cell (MFC) biosensors for BOD analysis have been proposed as an alternative approach for water quality monitoring. Water samples were collected from the Cotswold Water Park (UK) and they found that the lower organic load concentration test was performed after 61 days of operation, when the sensor was fed on fresh water. The sensor was able for at least 2 days and in the long run, the sensor was successfully operated for 150 days.

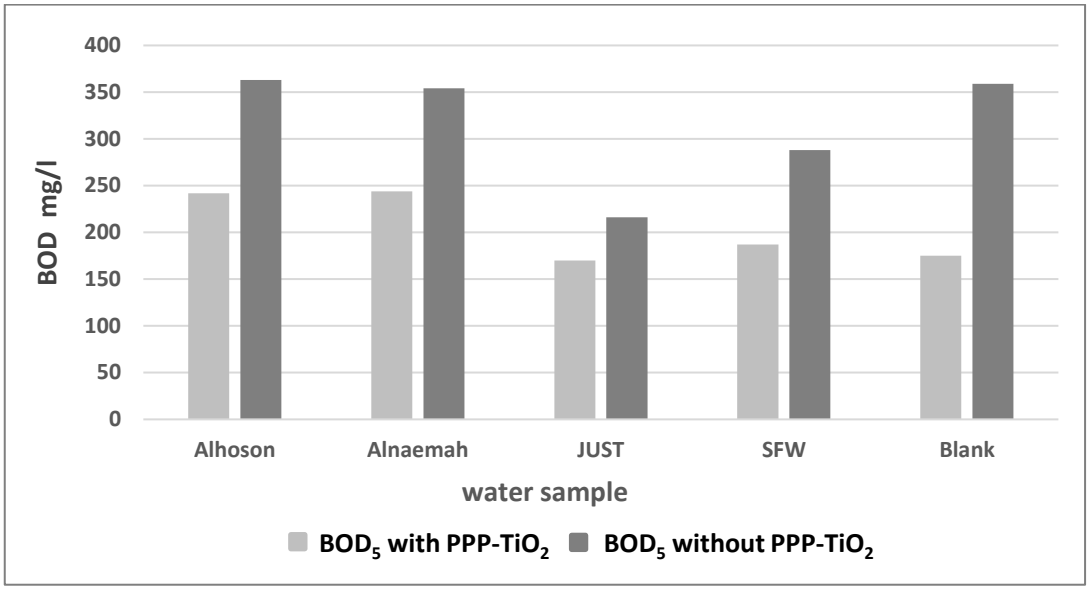


Figure 4.20: BOD value for real water sample

Table 4.8: BOD values in mg/l with and without PPP-TiO_2 in real water samples

Water sample	**BOD_5 with PPP-TiO_2**	**BOD_5 without PPP-TiO_2**
Al-Hoson	242	363
Al-Naemah	244	354
JUST	170	216
SFW	187	288
Blank	175	359

Conclusions:

PPP-TiO_2 nanocomposite has been successfully manufactured using plant meditated synthesis approach by using pristine pomegranate peel extract (PPP) impregnated with green TiO_2 nanoparticles. The approach is simple, feasible, cost-effective, and quite environmental.

The TiO_2 NPs used to prepare PPP-TiO_2 nanocomposite in the present study offers important advantageous; first, the synthesis rout is inexpensive and environmental friendly, second it involves inexpensive and non-toxic materials.

Several measures of bacterial growth and viability were used to evaluate the developed media. Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), growth curve profiling, live dead cell, and well diffusion method assays were performed and evaluated. Furthermore SEM micrographs clearly detected the damaged bacterial cells. The treated cells were showed lysed, dents, and some deep cracks cells.

Our findings confirmed the antibacterial susceptibility properties of the media (PPP-TiO_2) against Gram negative and Gram positive bacteria. Higher inhibition effects have been found against Gram-positive strains than Gram-negative strain. In addition, our results showed the DIZ had maximum inhibition against *S.aureus* than *E-coli* than *P.aeruginosa*.

Antimicrobial activity of PPP-TiO_2 was higher by 1.5 times against PPP and TiO_2 NP for same pathogenic bacteria which prove our hypothesis that assume that plants fiber impregnated with nanoparticles will provide a media with highly bacterial removal efficacy under laboratory conditions, and controlled industrial environments.

The Live/Dead BacLight TM Bacterial Viability Kit effectively labelled both live and dead of bacteria cells. The optical observation of microscopy results revealed that PPP-TiO_2, PPP, and TiO_2NP have good performance against *S.aureus* and *E-coli*. In addition, bacteril cells exposed to PPP-TiO_2 treatment tended to be less densely celled than the control group and large number of dead cells (stained in red) as well as a large number of black spaces.

In addition, the BOD_5 values were tested for real water samples with and without PPP-TiO_2. Lower BOD_5 values were observed for samples containing PPP-TiO_2, indicated that the sample has lower organic matter and indirectly indicated lower microbial community. We conclude from our results the developed PPP-TiO_2 can be used to disinfect water sources without any side effects if controlled properly.

Recommendation:

The developed media (PPP-TiO_2) by green method can be used in water disinfection applications. However, the difficulty in developing standard methodologies for synthesis, is challenge because plant extracts chemical compositions can vary within the same species when collected from different locations and seasons and can cause differences between results in different laboratories and consequently reducing the results reliability and practicality.

Future Work:

- Investigate the size effect on nanoparticles properties and nanocomposite preparation.
- Chromatographic characterization of pomegranate plant extract to indicate the major responsible compounds for the formation of TiO_2 NP and to explain the reduction and stabilization mechanisms.
- Characterization of TiO_2 NP surface functional groups by FTIR to explain the antimicrobial mechanisms.
- Investigate the effect of pH on the properties of nanocomposite.
- Apply the composite (PPP-TiO_2) in flow mode to represent practical and industrial approaches.

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Appendices

Appendix (A)

XRD Analysis Data

Table A.1: XRD analysis data for TiO_2 NP

No.	2-theta (deg)	d (ang.)	Height (cps)	FWHM (deg)	Int. I (cps deg)	Int. W(deg)	Size (ang.)	Phase name	Chemical formula	DB card number	Rel. int. I	Rel. height	Peak shape
1	25.2456	3.5248	4634.1	0.8778	5452.85	1.1767	96.87	Anatase, syn(1,0,1)	TiO_2	00-021-1272	100	100	Split pseudo-Voigt
2	36.7598	2.44289	272.9	1.1345	383.72	1.4061	77.07	Anatase, syn(1,0,3)	TiO_2	00-021-1272	7.04	5.89	Split pseudo-Voigt
3	37.8009	2.37796	1340.4	0.5698	1351.56	1.0083	153.93	Anatase, syn(0,0,4)	TiO_2	00-021-1272	24.8	28.92	Split pseudo-Voigt
4	47.959	1.89533	1537.2	0.5142	1681.51	1.0939	176.61	Anatase, syn(2,0,0)	TiO_2	00-021-1272	30.8	33.17	Split pseudo-Voigt
5	53.7977	1.70259	914.41	0.7351	1250.85	1.3679	126.57	Anatase, syn(1,0,5)	TiO_2	00-021-1272	22.9	19.73	Split pseudo-Voigt
6	55.1737	1.66334	573.8	0.9175	926.76	1.6151	102.03	Anatase, syn(2,1,1)	TiO_2	00-021-1272	17	12.38	Split pseudo-Voigt

No.	2-theta (deg)	d (ang.)	Height (cps)	FWHM (deg)	Int. I (cps deg)	Int. W(deg)	Size (ang.)	Phase name	Chemical formula	DB card number	Rel. int. I	Rel. height	Peak shape
7	62.6344	1.48195	822.35	0.6195	1083.75	1.3179	156.78	Anatase, syn(2,1,3)	TiO_2	00-021-1272	19.9	17.75	Split pseudo-Voigt
8	68.7601	1.3641	193.27	0.8276	174.71	0.904	121.48	Anatase, syn(1,1,6)	TiO_2	00-021-1272	3.2	4.17	Split pseudo-Voigt
9	70.2151	1.33936	264.58	1.3318	666.22	2.518	76.15	Anatase, syn(2,2,0)	TiO_2	00-021-1272	12.2	5.71	Split pseudo-Voigt
10	75.2111	1.2623	430.17	1.1335	829.05	1.9273	92.4	Anatase, syn(2,1,5)	TiO_2	00-021-1272	15.2	9.28	Split pseudo-Voigt
11	82.6513	1.1665	256.58	1.4544	590.67	2.3021	75.97	Anatase, syn(3,0,3)	TiO_2	00-021-1272	10.8	5.54	Split pseudo-Voigt

فعاليه الياف النباتات المخصبه بالاجسام النانويه الخضراء لتعقيم المياه

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الملخص

من المعروف أن المياه الصالحة للشرب ضرورية لصحة الإنسان وتنمية البلد، حيث يوجد أكثر من بليون شخص في العالم غير قادر على الحصول على مياه شرب صحية آمنة، من أجل تحسين نوعية المياه التي تلبي الاحتياجات المجتمعية، فإن توفير إمدادات مياه صحية، قابلة للشرب وذات تكلفة قليلة هو حاجة ضرورية.

في هذه الدراسة، تم خلط فيزيائي للجسيمات النانوية (TiO_2) مع المستخلصات النباتية (مستخلص قشر الرمان البكر (PPP) لتحضير مادة مركبة نانوية ($PPP\text{-}TiO_2$)، مع الالتزام بتطبيق الكيمياء الخضراء لتقليل استخدام أو توليد المركبات الضارة المحتملة خلال عملية التصنيع والتطبيق، وقد تم تحليل المادة المصنعة ($PPP\text{-}TiO_2$) باستخدام (SEM) (DLS) و (XRD). وأظهرت النتائج ان المادة المصنعة عشوائية الشكل والحجم ذات حواف حادة، تراوح حجم $PPP\text{-}TiO_2$ من 1 إلى 5 ميكروميتر، وكذلك أظهرت الصور الميكروغرافية SEM بوضوح الخلايا البكتيرية التالفة المعالجة بواسطة $PPP\text{-}TiO_2$، وعلاوة على ذلك، تم تقييم النشاط البيولوجي من خلال دراسة منطقة تثبيط الانتشار، وتركيز تثبيط الميكروبية (MIC)، تركيز الحد الأدنى للجراثيم (MBC) وتحديد الخلايا الحية من الميتة، وقد أجريت الاختبارات على تراكيز مختلفة ولثلاثة أنواع من البكتيريا (المكورات العنقودية الذهبية، الزائفة الزنجارية، الإشريكية القولونية) وتم الحصول على آثار تثبيط أعلى ل ($PPP\text{-}TiO_2$) ضد سلالات إيجابية الجرام أكثر من سلبية الغرام من خلال نتائج MIC_{90}، MIC_{50}،MBC ميكروغرام / مل)، وقد وجد أقصى تثبيط للمكورات العنقودية الذهبية، وكان نشاط مضادات الميكروبات من $PPP\text{-}TiO_2$ أعلى بمره ونصف من PPP اوNP TiO_2 ضد نفس البكتيريا المسببة للأمراض، وأظهرت نتائج هذه الدراسة أيضا أن تحضير المادة المركبة النانوية المصنعة لها نشاط ممتاز نسبيا لمنع الجراثيم من النمو، وأخيرا أوضحت النتائج ان قيم BOD_5 أقل للعينات المحتوية على المادة المركبة النانوية مثبتة احتواء العينة على مواد عضوية منخفضة ومجتمع ميكروبي أقل، وبالتالي لها القدرة على استخدامها كمعقم للمياه.