Silymarin silver nanoparticles loaded biomimetic coating for titanium implants

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Master of Science in

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Supervisor: Dr. Nosheen Fatima Rana

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Dedicated to my grandparents

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

NPs	Nanoparticles
Ag	Silver
TSB	Tryptic Soy Broth
XRD	X ray diffraction analysis
FTIR	Fourier transform infrared
UV	Ultra violet
SEM	Scanning electron microscopy
CoCr	Cobalt Chromium
SS	Stainless steel
Ti	Titanium

Abstract

Titanium is most frequently utilized for making orthopedic and orthodontic implants. Titanium implants are most extensively used because of their anti-corrosive properties, having high success rate. Despite high success rate, there still are number of cases in which implant rejection is observed. Surface modification techniques for improving surface properties, surface roughness and their interaction with biological environment has been explored to overcome the risk of implant associated infections. This research aims at designing a silymarin silver nanoparticles loaded biomimetic coating for titanium implants which results in implants having improved anti-biofilm activity, improved osseointegration and biocompatibility. Green synthesis approach is employed for silver nanoparticles synthesis using *Silybum marianum L*. plant extract, which is rich in free hydroxyl groups which can reduce Ag+ ions into Ag nanoparticles. Titanium implants are bio-inert they cannot interact with the bone to form a bond that limits their osseointegration. Bone like apatite coating on the titanium surface is being explored for improving osseointegration potential of the surface. The potential of silymarin as a reducing agent is confirmed by the UV spectrometry, XRD, FTIR, SEM and zeta potential analysis of silver nanoparticle. Anti-biofilm crystal violet assay confirmed the anti-biofilm potential of the coated titanium discs. SEM analysis showed uniform coating of calcium phosphate and silver nanoparticles on the titanium surface. Decrease in contact angle of the coated titanium disc confirmed increase in the hydrophilicity of the titanium disc, which denotes increase in the osseointegration of titanium discs.

Key words: *Silymarin extract, silver nanoparticles, simulated body fluid, green synthesis, biomimetic coating*

CHAPTER 1: INTRODUCTION

1.1. Metallic Implants used in body

For many years, metals and alloys has been employed to replace, repair, or enhance tissues and structures of human body. The way that metals behave when they are used in the body is a major consideration in view of their interaction with the host environment. In orthopedic, dental, and cardiovascular medical devices, titanium alloys, CoCrMo alloys and stainless steel alloys make up the majority of metals utilized in a various applications. The environment implants encounter include the mechanical environment and the electrochemical environment produced by the biological environment of the living system. Titanium implants are used extensively as it possess great physical properties. They are highly anticorrosive and have load bearing abilities thus providing us with long term implants. Titanium implants are bio inert; they do not interact with the biological system in the body thus raising a concern of reduced osseointegration. Surface modification is done to improve the implant surface to make it more prepared for osseointegration with bone. In few titanium implant cases peri-implantitis is observed caused by the bacterial colonization. To improve the anti-biofilm activity, the implant surface is coated with various anti-microbial materials.

1.2 Surface modification of Titanium implant

The optimal implant need to possess both antibacterial and osseointegration capabilities. Many coatings that have been created over the years to improve antimicrobial qualities have shown promise. These coatings can be modified chemically or physically, or even both. Various types of coatings such as polymer coatings, antiseptics, antibiotics, nanomaterials have been explored for their potential as antimicrobial coating materials. Silver nanoparticles have proved to be an excellent choice for their use as antimicrobial agent.

Calcium phosphate coating on the implant surface gives promising resuts for reduction in the contact angle and improved potential of osseointegration. Biomimetic CaP coating on the implant by dip coating the implant in SBF solution deposits a coating of calcium phosphate. This bone like apatite coating improves the biocompatibility and osseointegration of the titanium implant.

1.3 Green synthesis of silver nanoparticles

The most adopted method used for silver nanoparticles synthesis is chemical reduction method using chemical reagents for reduction of Ag+ ions to Ag nanoparticles. Chemical reagents also add to the toxicity of the nanoparticles. Biosynthesis of silver nanoparticles using *Silybum marianum* plant extract can provide us with a clean, economic and environment safe approach for nanoparticles synthesis. Flavonoids, which are phytochemicals produced by the plant to defend against fungi, bacteria, algae, viruses, and other organisms, are present in high concentration in the seeds of *Silybum marianum*. By scavenging and anti-oxidizing free radical metabolites, flavonoids also prevent lipid peroxidation. Hepatoprotection, anti-oxidation, anti-inflammatory, and heat protection are only a few of the qualities of silymarin. Polyphenolic groups, comprising seven different types of flavonolignans and one flavonoid, make up silymarin. One of the primary components of this complex of flavonolignans is silybin, which has a significant reducing activity due to its many hydroxyl groups. Silyamrin can be used as a reducing agent for silver nanoparticles synthesis.

Improvement in the osseointegration potential by biomimetic calcium phosphate coating and increased biofilm potential by silver nanoparticles coating can be observed.

CHAPTER 2: LITERATURE REVIEW

2.1 Usage of Implants for restoration of function

The use of metallic materials for making implants started as early as the 19th century [1]. The need for methods of bone repair, fixing of long bones led to the fabrication of metallic implants [2]. The majority of orthopedic devices, including both permanent implants for example total knee replacement and temporary implants for example pins, and screws, are made of metallic materials [3]. Metals were additionally utilized in orthodontics and dentistry at the same time for tooth fillings and roots [4]. The hard tissue reconstructive surgery has drawn increased attention in metallic biomaterials research recently. Examples of such applications comprise the fabrication of novel alloys for bone tissue engineering [6] and NiTi alloys as vascular stents [5].

Only a small percentage of implants used are compatible with the human body. Major groups of metallic materials most commonly used for making are stainless steel, cobalt , titanium and their variations [7]. Below is a summary of the present state of the major types of metallic biomaterials and their clinical uses [8].

Table 2. 1: Major types of materials and their application as implants

Category	Application	Usage	Reference
Stainless steel 316L	1) Temporary implants i.e. plates, nails, screws 2) Total hip replacement implants	Routinely used	[9]
Co based alloys	1) total hip replacement 2) dentistry	Routinely used	[9]
Ti based alloys	1) cup of total hip replacement 2) nails, pacemakers	Routinely used	[9]

2.2 Major types of Implants used in human body

Devices or tissues that are inserted into or applied to the body are termed as medical implants. A significant percentage of implants are prostheses meant to replace lost parts of the body. Some implants maintain organs and tissues, monitor physiological processes, or provide medication. Skin, bone, and different tissues in the body are used to make some implants. Others are constructed out of ceramics, metal, plastic, or other materials. Implants can be inserted in place permanently or removed when no longer required. Orthopedic and orthodontic implants are two types of implants [7].

2.2.1 Classification of Orthopedic Implants

Orthopedic implants are either permanent implant or temporary implants. Permanent orthopedic implants are implanted into body for lifetime, while temporary implants are supposed to be there only until the bones heal.

2.2.1.1 Permanent Orthopedic Implants

Clinically, replacements for a variety of complete joints including elbow, finger, knee, ankle, shoulder, and hip, are employed [10].Metals, polymers, and ceramics are frequently utilized. There has been a rapid development and clinical acceptability of hip and knee joint prostheses. However, designing such prostheses is extremely challenging due to the intricate load transmission and sensitive articulation. The components of a typical complete hip replacement are shown in (Figure.2.1A). The stem is used to mount the femoral head, which is then inserted into the liner and secured with a cup. The host bone is shielded by the cup and the liner guards against wear and tear. The components usually included in a total knee replacement are depicted in (Figure 2.1B).

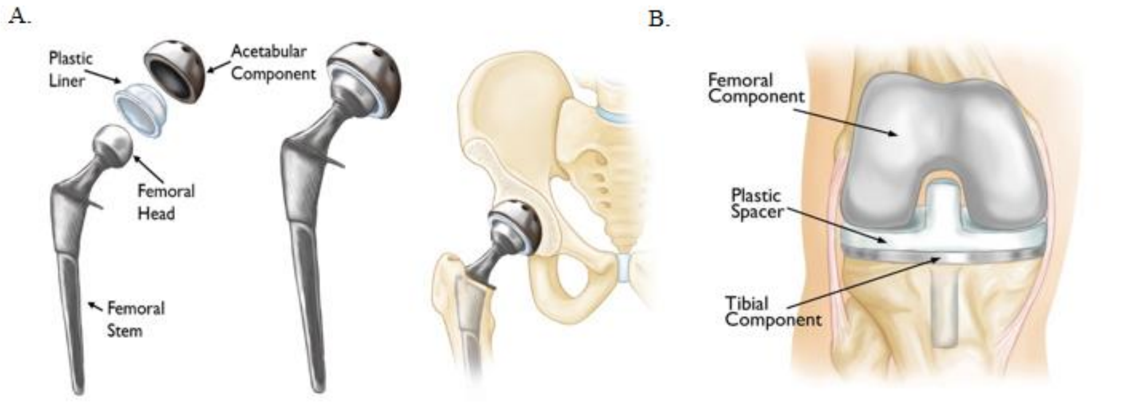

Figure 2. 1: Examples of orthopedic implants. Total hip replacement implant (A), Total knee replacement implant (B)

2.2.1.2 Temporary Orthopedic Implants

Temporary orthopedic implants are another kind that are used to stabilize shattered or fractured bones while they heal. The purpose of these implants, such as intramedullary nails, wires, pins and plates [2] (Figure 2.2), is to allow bones to mend over a brief period of time. Plates are used to squeeze the bones together during the healing time [11].

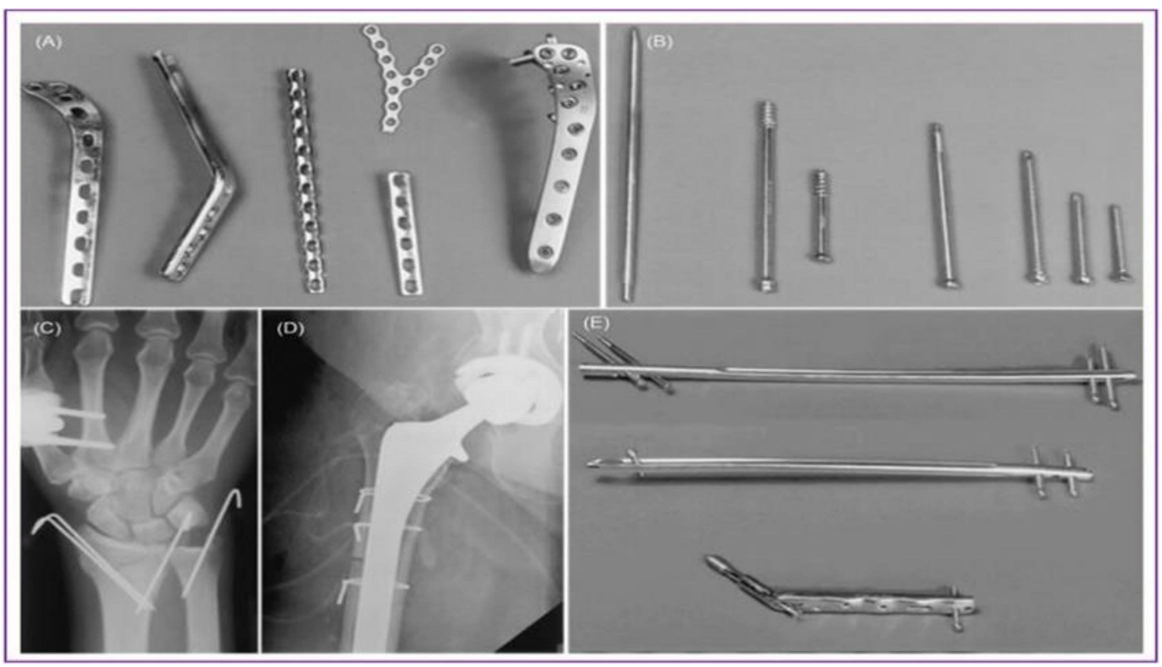

Figure 2. 2: Plates (A), screws (B), pins (C), wires (D) and nails for fracture fixing (E)

2.2.2 Classification of orthodontic implants

Orthodontic implants are classified into three major types depending upon the technique employed for insertion of implants in the jawbone. The bone mass of jawbone is an important consideration for selection of technique for inserting a dental implant. The 3 major classification of orthodontic implants are;

- Endosteal implant
- Periosteal implant
- Zygomatic implant

Endosteal implants are inserted in jawbone, periosteal implants have a frame inserted over the jawbone and zygomatic implants are inserted in the cheekbone because of extremely low bone mass of jawbone.

2.2.2.1 Endosteal Implants

Endosteal implants, also referred to as root form implants, are the most prevalent type of implant. These implants, which resemble screws, are surgically placed right into the mandible. Constructed from biocompatible materials like titanium, they provide as a strong basis for dental restorations like crowns, bridges, and dentures by simulating the structure of a natural tooth root. Endosteal implants are adaptable and can be used to replace one, many, or all of the teeth in a sufficiently dense jawbone.

2.2.2.2 Subperiosteal Implants

For individuals with low height or density of jawbone, subperiosteal implants represent an additional option. Rather than being buried within the jawbone, these implant frames rest on top of it. The dental restoration is attached to a metal framework that has posts that poke through the gum line. Subperiosteal implants are less common than endosteal implants, although they are nevertheless a good option in some circumstances.

2.2.2.3 Zygomatic Implants

A specific kind of implant called a zygomatic implant is utilized when there is significant atrophy of the jawbone, especially in the upper jaw. Because there is not enough bone in the jaw itself, these lengthier implants are fixed in the sturdy zygomatic bone of the cheek. In difficult situations where conventional implants would not be practical, zygomatic implants present a special option.

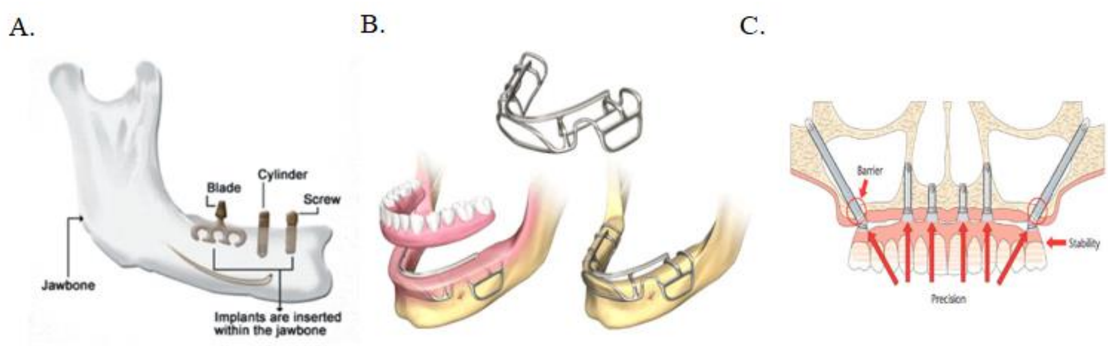

Figure 2. 3: Different types of orthodontic implants. Endosteal implants (A), subperiosteal implants (B) and zygomatic implants (C)

2.3 Use of biomaterials in human body

The usage of biomaterials in close proximity to biological organisms is a typical characteristic. The definition of a biomaterial in materials science is a biomaterial is any naturally occurring or artificially created biocompatible material that is utilized in close proximity to an organ or tissue to replace or support a portion of it. It should be noted that "biocompatible" is what is meant to be implied by the word "bio" in biomaterials.

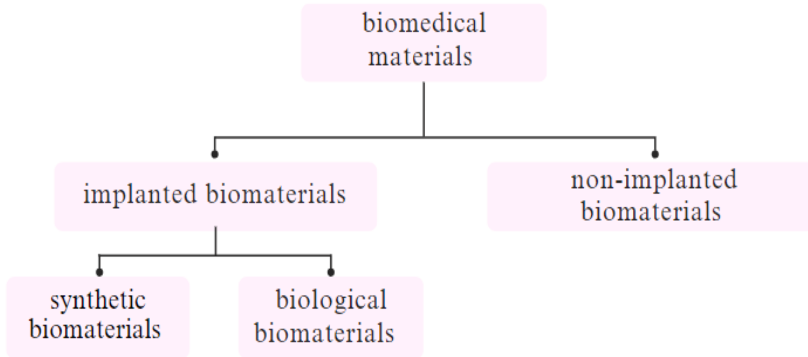

Figure 2. 4: flow diagram showing classification of biomedical materials

2.3.1 Concept of biocompatibility

The implanted material must not have any negative consequences because biomaterials are intended to be employed in close proximity to living tissue. According to Williams, biocompatibility encompasses every facet of bio-device operation, encompassing the interplay between implanted biomaterials and cells and tissues [12], [13]. The stringent and intricate standards for this biocompatibility change depending on the particular medical use. For example, an orthopedic implant that is rejected because of the hazardous metallic ion discharge will eventually fail. Therefore, it is anticipated that a metallic implant will be composed of non-toxic materials and won't result in any detectable allergic or inflammatory responses in body. A medical device's biocompatibility encompasses both the material compatibility and the device's design (such as its shape, mechanics, and electrical control).

2.3.2 Essential properties for design of metallic biomaterials

A biomaterial's specialized medical application determines its design and selection. For a metallic implant to perform its function well and safely over a large time period without getting rejected, it is supposed to possess mentioned below fundamental properties, among others:

- Biocompatibility
- Corrosion resistance
- Good mechanical properties
- Wear resistance
- Osseointegration

2.3.3 Selection of alloying elements and its effect on biocompatibility

Since no substance is totally inert in a live organism for an extended length of time, naturally occurring elements may be the first thing to consider when choosing an alloying element. Human cells contain 65–90 weight percent water, or H2O. It follows that the rest of mass is carbon and oxygen. Table 2.2 enlists all the elements. The elements 96% make up to 96% of the weight. The remaining (about 4%) mass is mostly composed of key electrolytes and bone minerals.

Table 2. 2: Table showing the concentration of elements present in human body and their function

Element	Wt%	Function in body
O	65.0	Makes structures of proteins
C	18.5	
H	9.5	
N	3.3	
Ca	1.5	Structure of bone and teeth
P	1.0	
S	0.3	Elements of amino acids
K	0.4	

Na	0.2	electrolyte maintaining pH and osmotic balance.
Cl	0.2	
Mg	0.1	Important in bone structure
Trace elements	<0.01	physiology

2.3.4 Implant corrosion in host body

Environment in human body differs from the outside world in both physical and chemical aspects. As a result, a metal that functions well in the atmosphere can experience major internal corrosion. In actuality, stainless steels having high anti-corrosion frequently result in long-term allergic and poisonous effects, which can be identified when a suitable amount of time has passed after implantation [14]. The success of metallic implants in long term is determined by resistance to corrosion; however, the pH and oxygen content of different bodily parts vary.

2.3.5 Mechanical properties of implants

Biomaterials should have good mechanical properties to work under the same conditions as bone. Human bones are very strong and durable. The mechanical characteristics of toughness, UTS and Young's modulus are generally significant for the development of biomaterials [10]. Table 3 shows mechanical characteristics for the three main metallic biomaterials. The main reasons these three metals are still in use are because, they can support heavy loads and experience plastic deformation before failing.

Table 2. 3: List of Mechanical properties of metallic implant materials

Material types	Young modulus (GPa)	UTS (MPa)	Fracture resistance (MPam)
CoCrMo	230	1540	100
stainless steel 316L	190	1000	100
Titanium	125	800	80

Since the implant has a higher modulus of elasticity, it may be able to support almost all of the load. Bone that experiences less mechanical pressure may atrophy or other biological reactions, especially in the area surrounding the implant; this means that additional revision surgery may be necessary [15].

2.3.6 Osseo-integration

Osseo-integration, a word used to describe process of integration of new bone, it is a basic necessity in orthopedics [16]. Fibrous tissue will grow around an implant if its surface is unable to adhere to surrounding bone and other tissues as a result of micro motions, which will make the prosthesis to become looser. As such, an implant's surface needs to be suitable in order for it to blend in effectively with the bone. For optimal osseointegration, surface properties must be taken into account [3], [16].

An implant must have modulus comparable to that of bone, good bone bonding ability, and outstanding fatigue resistance, anti-corrosive and wear resistance. The creation of such an alloy would reflect a current unfulfilled goal in tissue engineering, the creation of a synthetic entity having 100% success rate for period of 30 years that would replace injured bone at load-bearing places [17]. The major concern for the risk free metallic implants is the management of material issues, such as biocompatibility and corrosion resistance [10]. The mechanical performance of these implants under biological working conditions is crucial for orthopedics at load-bearing sites.

2.4 Major classes of implant materials

The ascending order of metal ions toxicity is as;

Iron<titanium<chromium<nickel<vanadium<cobalt

2.4.1 316L Stainless steel

Stainless steel is major class of metals utilized for making implants. These applications in human body frequently call for a material that can be easily shaped into intricate designs. But for implants, only austenitic stainless steels are utilized [18].

2.4.1.1 Biomedical applications of stainless steels

Given below is tabular representation of applications of stainless steel in human body.

Table 2. 4: Tabular representation of medical applications of stainless steels [19].

Types	**Implants/Devices**	**Usage**
stainless steel 316L	screws, pins, plates, nail, wires	Internal fixation
	Housing of Cardiac pacemaker	packaging of electronics and power source
Types 304, 316, and 316L SS	Sutures	Wound closure
	Electrodes	Anode and cathode electrodes
Types 302, 303, 304 and 316 SS	Retention pins, endodontic, post and core, dental crowns	Dental restorations

2.4.2 Cobalt and its alloys

Haynes originally created the cobalt molybdenum superalloy, known as stellite, for use in aviation engines [20]. In comparison to other super alloys, it demonstrated superior corrosion resistance and increased strength at greater temperatures. CoCr alloys have superior mechanical qualities and a corrosion resistance that is more than ten times higher than that of stainless steels [19]. These alloys have been created into various compositions by altering vitallium.

Table 2. 5 : CoCr alloys and their use in surgical implants [19]

Compositions	**Implant type**	**Medical application**
CoCrMo	Permanent implant	Components of joints, bone plates
CoCrWNi	Temporary implant	Fixation wires, vascular stents, heart valves
CoNiCrMo	Permanent implant	Conductor wires, catheters, orthopedic cables

2.4.3 Titanium alloys in orthopedic implants

Titanium variants are divided into four classes namely α alloy, near-α alloy, α–β alloy and β alloy based on their microstructure.

Titanium has better specific strength (strength/density) than cobalt-chromium alloys and stainless steels. Titanium more anti corrosive than other classes of metallic biomaterials [19].

2.4.3.1 Classification of titanium alloys

The two main factors used to grade unalloyed titanium are its iron and oxygen contents. Higher purity grades are more formable and have lower transformation temperatures, strengths, and hardness than higher interstitial content grades. With almost 45% of all titanium produced, the extensively utilized titanium variant is titanium grade 5 also called titanium grade 5. Roughly one-third of the production is made up of unalloyed grades, with the remaining 25% being made up of all the other alloys combined. Titanium grade 5 is the most commonly utilized in biomedical applications, however during the last ten years, there has been a surge in the usage of β-titanium alloys.

2.4.3.2 Biocompatibility of titanium

Titanium is highly non-toxic even in when administered in high doses. It also has no biological function in body [21]. The majority of titanium was observed to be eliminated when individuals consumed up to 0.8 mg of titanium per day. Titanium implants typically make strong anatomical bonds with the host bone and not rejected by the body. In vivo, white blood cells are subject to size-specific biological impacts from titanium particles. Because of their exceptional corrosion resistance, titanium alloys have shown to be more biocompatible than cobalt and stainless steel alloys [22]. Certain elements that stabilize β, such Ta, Zr, and Mo, are employed as alloying elements. When compared to aluminum and vanadium, they are seen to be reasonably safe [22].

2.4.3.3 Wear & fatigue resistance of titanium implants

At room temperature, grade 5 Ti offers good tensile characteristics and a useful creep resistance up to 300°C. Excellent resistance to fatigue and crack propagation. Similar to the majority of titanium alloys, Grade 5 exhibits exceptional resistance to corrosion in a wide range of industrial and natural process conditions. This alloy is the most commonly utilized specification across all product types, mainly for its resistance to corrosion. The alloy has exceptional strength to weight ratio, great strength at cryogenic temperatures, highly anti-corrosive and have good strength.

2.4.3.4 Mechanical attributes of titanium grade 5

Mechanical properties of the widely applied titanium grade 5 are given below;

Table 2. 6 : Mechanical attributes of Ti grade 5

Alloy	Young modulus (GPa)	Yield strength (MPa)	UTS (MPa)	Elongation Factor (%)	Fatigue Limit (MPa)
Ti–6Al-4V (grade 5)	110	860	930	10–15	500

2.4.3.5 Medical applications of titanium alloys

Grade 5 and αβ micro structured titanium alloys are the most often utilized in biomedical applications. The titanium alloys that are most commonly used in biomedical applications are enlisted in the table below.

Table 2. 7: Medical applications of titanium alloys

Alloy type	**Attributes**	**Limitations**	**Applications**
Commercially pure Ti	1.anti-corrosion 2.Biocompatibility 3. durability	1. Low strength at ambient temperature	1. Pace-maker housing 2. Drug pump 3. Orthodontic implants 4. Screws and staple
α–β microstructure	strengthened by heat treatment	-	1. Total joint replacement arthroplasty 2. Spinal components 4. Plates, fasteners 6. Tubing and intramedullary nails

2.4.3.6 Use of grade 5 titanium in dental implants

A dental implant is a surgical component that supports and permits the mounting of replacement teeth. The most common type of implant is a titanium post. The implant osseointegrates with the human bone once it is placed in the jaw, offering a strong foundation.

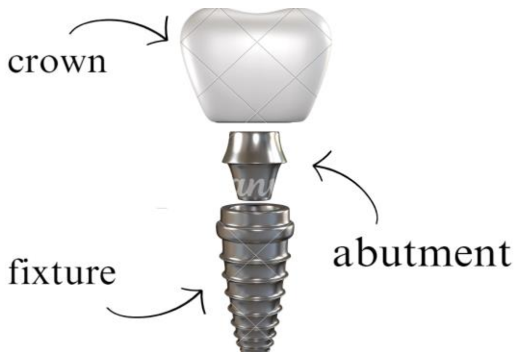

Figure 2. 5*:* A dental implant composed of fixture, abutment and crown

Orthopedics uses a lot of grade 5 titanium [26]. It is observed that using this alloy is biologically acceptable and is also utilized in dental applications [26]. This alloy also releases which can have negative biological effects [26].

Research has demonstrated that this alloy will osseointegrate satisfactorily [25], particularly when surface-treated to improve the oxide layer [27], [28]. A patient must be carefully chosen before receiving an implant, in part because of its design. For the implant to be stable and supported, there must be sufficient bone in the afflicted mandible or maxilla [29], and a healthy blood supply must be present at the location. This implies that in addition to not smoking, the patient must not have any circulatory issues. This latter aspect is crucial because tobacco smoke causes blood capillaries to constrict, which lowers the amount of blood that reaches the soft tissues. Finally, it is imperative that patients practice appropriate dental hygiene. This will lessen the chance that the tissues next to the implant may become infected.

Dental implants are being used much more frequently to replace missing teeth in patients since titanium alloys were first made available for usage in 1981 [23]. Adult tooth loss is most frequently caused by periodontal disease, while it can also result from trauma and developmental anomalies [23]. It is important to handle implants cautiously while using them in dentistry to prevent contamination. It is recommended to take precautions in implant handling and avoiding contact with any surface, to maintain the surfaces' meticulous cleanliness. Even with these safety measures, surfaces are found to retain significant levels of contamination when handled [30]. Usually organic in nature, this contamination exhibits significant carbon and trace amounts of oxygen.

Reports of survival rates spanning a decade have reached a minimum of 89%; however, actual numbers have frequently surpassed this threshold by significant margins. Several hundred implants have been studied in these research, and the survival rates fall between 97% and 99%.

Table 2. 8: Table showing success rate of Ti implants in long term studies

Follow up years	Pretreatment of Ti implant	Survival rate %	Reference
10	Sandblasting	98.8	[29]
10	Sandblasting & acid etching	99.7	[31]
20	Plasma spraying with titanium	89.5	[32]

Dental implants are one of the most popular and successful treatment alternatives for lost teeth. Nevertheless, infections like peri-implant mucositis, an inflammation brought on by biofilms that can cause bone loss and peri-implant mucositis, still cause them to fail a good deal of the time [26].

2.4.3.7 Titanium Implant related Infections

Since a significant portion of implant failure is attributable to infections connected to the implants, the implantation process necessitates both resistance against bacterial colonization and excellent osseointegration [33].

Research shows that approximately 29.48% of dental implants and 46.83% of subjects have peri-implant mucositis, and around 9.25% of implants and 19.83% of subjects experience peri-implant mucositis [27]. Without any indication of accompanying bone loss, peri-implant mucositis is an inflammation caused by biofilm that is restricted to the soft peri-implant mucosa (Figure 2.6) [34].

After bacterial biofilms have accumulated, it emerges from the healthy peri-implant mucosa surrounding osseointegrated dental implants. Bleeding on probing (BOP) is the most common clinical sign of peri-implant mucositis. Research on patients revealed that peri-implantitis can be recovered with at least three weeks of improved biofilm control and oral hygiene. However, in the event that treatment is not received, the inflammatory process can worsen and cause the implant's surrounding bone to gradually deteriorate, leading to peri-implantitis [34].

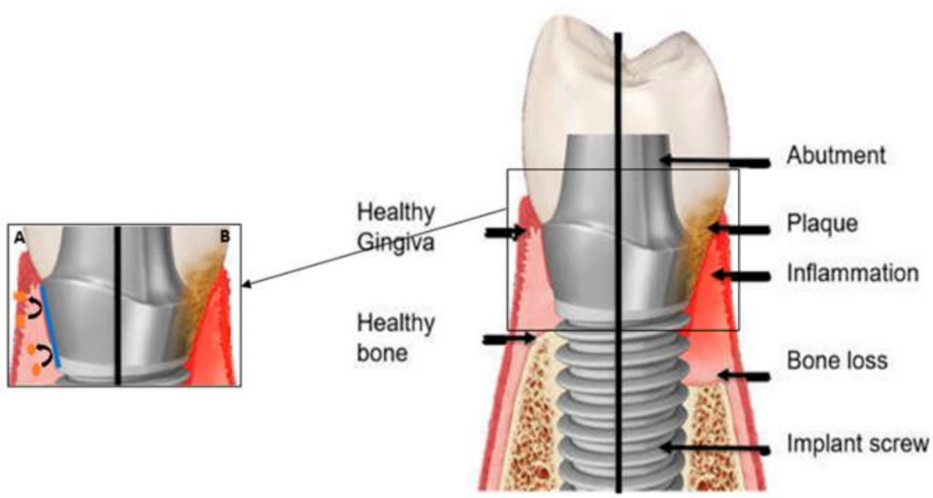

Figure 2. 6*:* peri-implant mucositis in tissue around implant

If left untreated, these kinds of infections cause implants to loosen and need to be removed. Excellent osseointegration characteristics and resistance to the germs that cause peri-implant mucositis are essential for the perfect dental implant. Because of a number of qualities, including excellent corrosion resistance, biocompatibility, and strong biological environment tolerance, titanium and its alloys are preferred.

Studies and proposals have been made on surface modifications to implants to increase success rates. Enhancing osseointegration was made possible by modifying the surface's physicochemical properties including wettability, surface roughness and free energy. The next stage was to manage and stop the bacterial growth surrounding implant because it results in formation of biofilms and bacterial adherence that happens right after implantation. Additionally, once developed, biofilm is difficult to treat since it is resistant to many antimicrobial treatments. Coatings having antimicrobial qualities were thought to be a dependable answer to this issue in order to stop bacterial infections.

A biofilm is a thin coating that microorganisms produce on a material's surface or another interaction. They typically form on surface of material, indicating that it has an effect on biofilm formation. The material type influences the development and growth of biofilms. Eighty percent of biofilm is made up of bacteria, extracellular polymeric materials, and water. Biofilm has a slippery exterior. Bacteria have a slimy surface due to a mechanism called quorum sensing, which allows them to cling to surfaces and multiply while expelling polysaccharides from their colony. In addition to polysaccharides, proteins, lipids, and nucleic acids—specifically, DNA and RNA—are also synthesized in biofilm. When combined, these ingredients form extracellular polymeric materials.

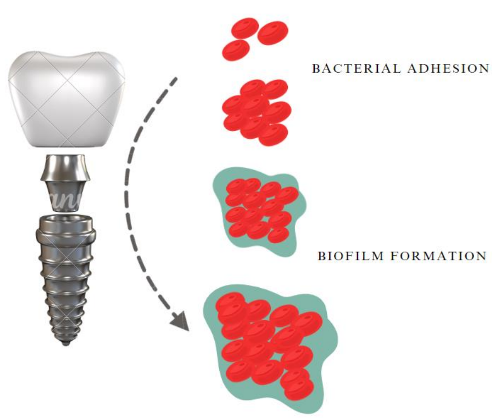

Figure 2. 7: Slimy biofilm formation on the dental restoration by bacterial colonization

Depending on the properties of surface of implant, bacteria may adhere to titanium implants. Coatings can be deposited on implant surface to stop bacterial colonization [24].

2.4.3.8 Surface modifications of titanium implants

Because of their antibacterial properties, coating materials including silver, copper, zinc, chlorhexidine, and some medicines looked like a viable approach to stop bacterial colonization. However, the techniques needed to alter and apply coatings to the implant surface are costly and time-consuming. Furthermore, biocompatibility and osseointegration qualities may be compromised in the pursuit of maximal antibacterial capabilities. The balance will always be crucial in assessing a coating's potential. The optimal implant need to possess both antibacterial and osseointegration capabilities. Many coatings that have been created over the years to improve antimicrobial qualities have shown promise. These coatings can be modified chemically or physically, or even both.

Based on a 1951 study by Branemark on the biocompatibility of titanium, titanium alloys have taken center stage as the primary component of dental implants. Surface modification has the potential to promote integrity between implant surface and bone tissue as well as favorable bone regeneration. This method enhances the clinical efficacy of dental implants while maintaining the original biocompatibility of titanium alloys.

2.5 Nanotechnology in Implant surface modifications

Particles having an elevated surface to volume ratio that lie in the size range of nanometers are known as nanoparticles. Their enormous surface area and tiny size enable them to function as designed and interact with the biological environment efficiently. In the realm of nano-biotechnology, significant strides have been achieved lately toward the creation of various nanomaterial types with a broad variety of uses. Noble metal nanoparticles have shown promise for use in biomedical applications among metal nanoparticles. Nanoparticles' small size makes it easy for them to interact with biomolecules on the outside as well as inside of cells, improving signaling and target specificity for treatments and diagnostics. The researchers were motivated by noble metal nanoparticles because of their amazing ability to identify and treat terrible diseases.

The production of items at the nanoscale, such as materials, electronics, and/or systems, is the focus of nanotechnology. With regard to improving veterinary and human health as well as combating the deadliest diseases of the future, the swift advancement of nanotechnology has given rise to fresh optimism. As of right now, it is widely accepted despite serious concerns about possible health hazards. Exposure to nanoparticles (NPs) results in lung inflammation, oxidative stress, apoptosis, toxicity, and inflammation, all of which can contribute to pulmonary illnesses.

There are two types of nanoparticles (NPs): non-engineered NPs, which are naturally occurring and originate from erosion, and engineered NPs (ENPs), which are created by humans using various materials. The strength, flexibility, performance, and durability of ENPs, together with their physico-chemical and biological characteristics, have attracted interest from a variety of sectors, including engineering, biology, and medicine.

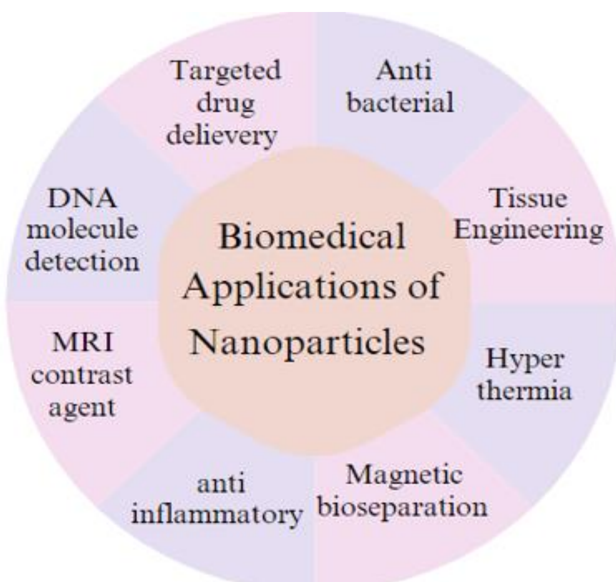

Figure 2. 8*:* Graphical representation of biomedical applications of nanoparticles

Size, shape, and particle diameter are some of the characteristics of NPs that influence their physical stability and in vivo performance. As NPs get smaller, their surface area grows, and their higher antibacterial activity is connected with an increasing surface to volume ratio. Various ENP types with sizes between 0.5 and 1000 nm have been created and examined to determine their suitability for use in biomedical domains. Additionally, surface characteristics like hydrophobicity and surface charge affect the efficiency of ENPs. Surface charge and surface hydrophobicity of NPs is ascertained using their surface Zeta potential values. The charge, intensity, and electrostatic contact of the NPs and the bioactive chemicals determine how they interact. The surface characteristics of NPs is necessary for the application of ENPs in medication administration, phototherapy, and imaging. Furthermore, problems with size control and stability still arise during the synthesis of ENPs.

2.5.1 Biomedical applications of Silver nanoparticles

While some bacteria, viruses, algae, and fungus are poisonous to silver ions and compounds, humans are not highly toxicated by them. Numerous microbiological organisms can be killed by its germicidal properties. As a result, both the metal and its salts have contributed significantly to the advancement of medicine. Silver is regaining popularity because it may stop disease-causing germs accumulation. Silver releases Ag^+ ions, which have antibacterial characteristics.

Additionally, metallic silver finds use in several surgical applications, including prosthetics (such as silicon-silver penile implants) and structural devices (such as aneurysm clips, support plates and suture wire).

2.5.1.1 Anti-bacterial activity of Silver Nanoparticles

A number of inorganic NPs, such as silver NPs (AgNPs), have demonstrated antibacterial action against a variety of microorganisms. The greater affinity that silver ions have for phosphate and sulfur groups may play a major role in its antibacterial activity. When silver ions (Ag^+) are liberated from nanoparticles, they interact with the cell membrane. Ag+ is major reason for cell death because it generates ROS inside the cell.The primary determinant of nanoparticles' effectiveness against microorganisms is their size. Less than 10 nm in size results in cell membrane gaps or pores, which finally cause microbial cell death. Different pathogenic microorganisms have different minimum inhibitory concentrations (MIC) for AgNPs based on size and shape of nanoparticles [28].

AgNPs that have been biosynthesized and have demonstrated improved inhibitory potential have been tested against a number of diseases. Higher antibacterial activity was demonstrated by the AgNPs made using Andrographis paniculata leaf extract against strains of Gram-positive Enterococcus faecalis [35]. Furthermore, through ROS-mediated cell surface damage, the AgNPs made using Ocimum gratissimum showed anti-biofilm activity [21].

AgNPs/GO demonstrated antibacterial potential among other various nanocomposites that have been synthesized and studied for antibacterial activity.AgNPs embellished on thiol (-SH) grafted GO layers had a suppressive impact on bacterial colonization [35].

2.5.12 Biocompatibility of silver

There are no recognized biological functions for silver, and its potential health implications are up for debate. Silver by itself is non-toxic, but the majority of its salts are, and some of them may even cause cancer. Certain biomolecules have intermolecular connections where sulphur groups can be bound by silver ions.

2.6. Green synthesis approach for silver nanoparticles synthesis

The most popular technique for creating silver nanoparticles is chemical reduction, which uses a variety of chemical reagents including polyethylene glycol, sodium hydroxide, and sodium borohydride. Synthesis of silver nanoparticles using plant extract have gained extreme popularity in recent era.

The utilisation of chemical processes to synthesize silver nanoparticles (Ag NPs) has some drawbacks, including environmental and human toxicity and potential injury to various normal cells.

2.6.1 Silybum marianum L. extract as a reducing agent

Silybum marianum L. which is most commonly known as milk thistle plant belonging to the family of *Carduus marianum*. The plant extract obtained from its seeds is called silymarin which has obtained great popularity because of its availability, low cost and properties.

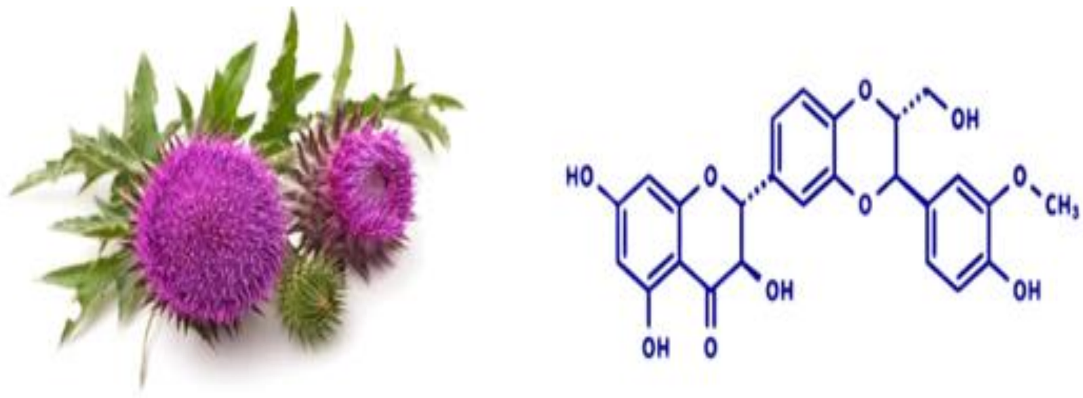

Figure 2. 9*:* Silybum marianum L. flower and its structural formula

Silybum marianum seeds are composed of high concentration of flavonoids, which are the phytochemicals produced by the plant to protect against fungi, bacteria, algae, viruses and other microbes. Flavonoids also inhibit lipid peroxidation, which is done by anti-oxidation and scavenging of free radical metabolites. Silymarin has properties such as hepatoprotection, anti-oxidation, anti-inflammatory and heat protection. Silymarin is composed of polyphenolic groups which include seven types of flavonolignans and one flavonoid.

Out of this complex of flavonolignans one of the main component of is silybin which is composed of many hydroxyl groups thus can have high reducing activity. It is capable of removing both free radicals and reactive oxygen species and thus it can protect the cells from free radicals. Silybin acting as an anti-oxidant protects the cells from these free radicals and reaction oxygen species. Silymarin also show great results in anti-diabetic models. Silymarin has been proved safe to be used in animal models and have not shown any adverse reactions in human models.

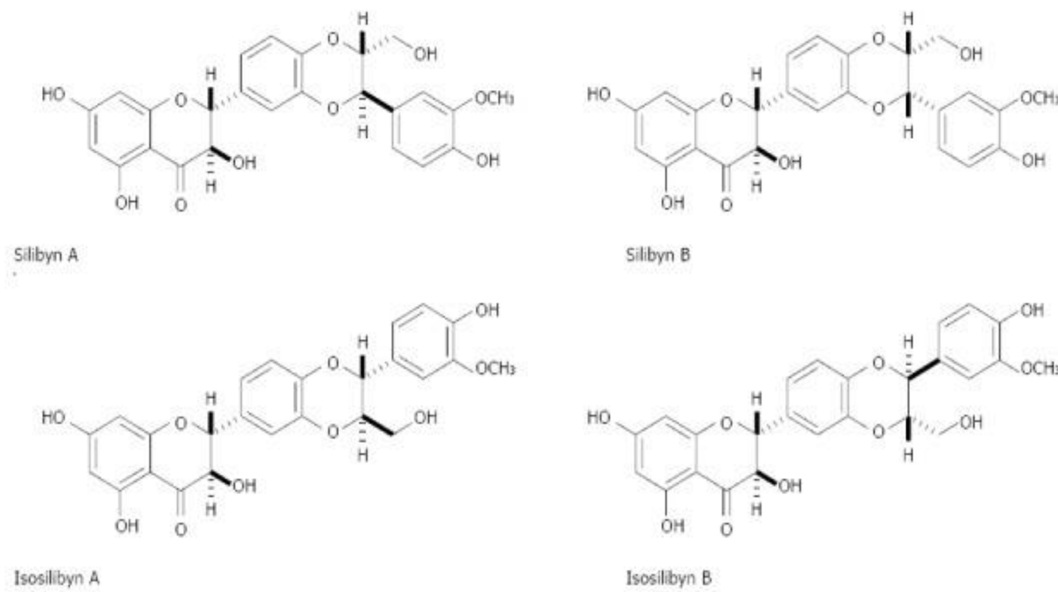

Figure 2. 10: Structural formula of flavonolignans namely silybin A, silybin B, isosilybin A, isosilybin B

The primary ingredients that facilitate the environmentally friendly production of nanoparticles from plant extracts are phytochemicals. The substances called phytochemicals are what cause ions to be reduced to nanoparticle size. The endogenous enzymes produced by silymarin extract play role in preserving the tissue homeostasis of the kidney, gastrointestinal system, different malignancies, and platelets. Silymarin uses the NNF-Kb pathway to demonstrate its anti-inflammatory effects.

Phytochemicals are the primary agents that mediate the formation of nanoparticles. The primary phytochemicals that cause the ions to reduce spontaneously include amides, quinones, aldehydes, ketones, terpenoids, and flavonoids. [36]

2.6.2 Silybum marianum L. extract Applications in medicine

Silybum marianum L. medicinal applications are summarized in graphical representation.

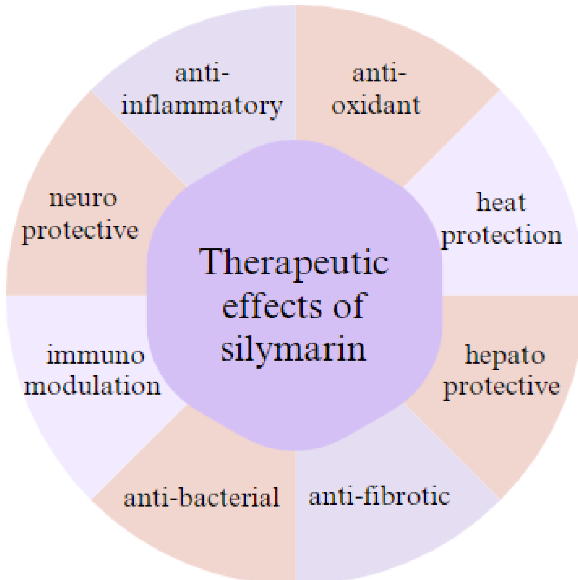

Figure 2. 11: Graphical representation of therapeutic effects of silymarin

2.7 Biomimetic calcium phosphate coating of titanium implants

Because of great bio-corrosion resistance, mechanical characteristics, biocompatibility, and light weight, titanium and titanium alloys have become particularly appealing biomaterials. Currently, dentistry uses the comparatively weak commercially pure titanium, while a number of stress-bearing orthopedic applications use the stronger Ti-6Al-4V alloy. Despite these fantastic qualities, titanium and alloys based on titanium are regarded as bio-inert, meaning that when they are put into the human body, fibrous tissue usually encases them and prevents them from chemically bonding with bone.

It is widely acknowledged, therefore, that compared to untreated titanium implants, coating of calcium phosphate produces long term implants. Metal implants can now be coated using a variety of techniques. Because of this, numerous authors have conducted research to enhance the Osseo-integration of these materials [39], [40].

The chemical and physical attributes of surface of implant can all be altered by surface treatment [41]. The surface of metallic implants can be coated with hydroxyapatite ($Ca10 (PO4)6OH$), to improve tissue integration. At the moment, hydroxyapatite coatings for metallic implants are applied by plasma deposition, a process that results in non-uniform coatings. The biomimetic method is a excellent approaches to produce coating of calcium phosphate because it emulates the mineralization process of bone.

By using aqueous solutions having ionic composition akin to human blood plasma, the biomimetic approach enables the apatite coating on metal implants.

2.7.1 Alkaline & heat treatment of surface prior to coating

Alkaline and heat treatment of titanium disc prior to soaking in SBF prepares the surface for the coating and proved to have a positive effect on the surface modification. Alkaline treatment id done by soaking the disc in sodium hydroxide solution.

2.7.2 Effect of wettability on osseointegration

Hydrophobicity/hydrophilic surface wettability is one of the key factors effecting the reaction to implant biomaterial. Considering how highly hydrophilic surfaces interact with biological fluids, cells, and tissues, they appear to be preferable over hydrophobic ones. For titanium implant surfaces, Values obtained from contact tangle measurements range from 0 denoting hydrophilic to 140 denoting hydrophobic. Because of its strong affinity for biological fluids, biomimetic calcium phosphate greatly increases wettability when compared to the initial substrates. On the same surface, the relationship between wetness and contact angle is inverse. Consequently, a reduction in this angle raises the surface wettability capacity. Biomimetic CaP coating improves hydrophilicity of titanium surface thus improving osseointegration.

CHAPTER 3: MATERIAL AND METHODS

3.1 Green Synthesis of Silver Nanoparticles:

Silver nanoparticles are synthesized by green synthesis method using silymarin extract for reducing Ag+ ions. 1mM solution of silymarin powder was made in 1mM aqueous solution of sodium hydroxide (NaOH); which increases the solubility of silymarin powder. The reaction confirmed by the color change from light yellow to deep yellow. 1Mm aqueous solution of $AgNO_3$ was prepared in distilled water. Silymarin solution was added dropwise into the silver nitrate solution. Silymarin reduces Ag ions into Ag nanoparticles. The solution changes from deep yellow into wine red color which is an indication for the reduction reaction. The resultant solution was then vortexed for 3 minutes and then incubated at 37°C for 5 minutes. The solution was stirred at 40°C at speed of 150rpm for 4 hours to give the solution enough time to happen. The optimum ratio for the reagents was found to be at 2:3 (silymarin: Ag). Formation of silver nanoparticles was confirmed by the UV-VS spectroscopy.

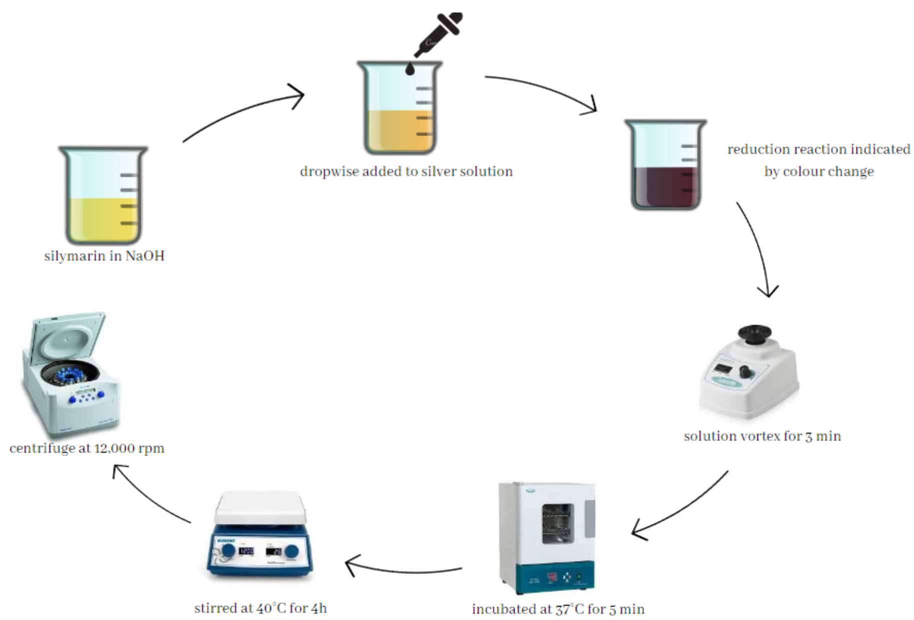

Figure 3. 1: Flow diagram showing the stepwise protocol for silver nanoparticles synthesis

3.2 Coating of Titanium Discs:

Titanium discs of grade 5 are used for coating purpose. Ti discs are divided into three groups including group I for uncoated Ti disc, group II for Ti disc coated with Simulated Body Fluid (SBF) and solution of silymarin silver nanoparticles for 7 days and group III for Ti disc coated with SBF and solution of silymarin silver nanoparticles for 14 days.

3.2.1 Alkaline treatment

Titanium discs are soaked in 5M NaOH solution for 72h. After completion of 72h disc are dried at 40°C for 24h. Alkaline treatment of disc is done to prepare the surface of disc for coating of SBF and nanoparticles.

3.3.2 Heat treatment

Heat treatment of titanium disc was done at 600°C for 1h in an electrical furnace. Heat treatment of discs was done at SCME (school of chemical & materials engineering), NUST. Heat treatment of disc is done to prepare the surface of Ti disc for coating of SBF and nanoparticles.

3.2.3 Dip coating

Simulated body fluid is a fluid having the ion concentration same as human blood plasma. It is prepared in laboratory by dissolving chemical reagents including NaCl, MgCl26H2O, CaCl22H2O, Na2HPO4H2O and NaHCO3 in pure distilled water. SBF solution deposits a coating of CaP on the titanium surface. The bone like apatite coating improves the biocompatibility of the titanium discs. Calcium phosphate coating enhances wettability of Ti surface which holds importance in view of the interaction of the titanium surface with the biological fluid, tissues and cells. Hydrophilicity is required. In this study, 5*SBF ion concentrations are used. The more ion concentrations improves the rate of CaP coating. Ionic oncentrations of SBF and human blood plasma are given below.

Table 3. 1: Table showing ionic concentrations of human blood plasma and SBF

Ion concentration	Na^{+}	K^{+}	Mg^{2+}	Ca^{2+}	Cl^{-}	$(HPO4)^{-2}$	$(HCO3)^{-}$	$(SO4)^{-2}$
Human Blood Plasma	142	5	1.5	2.5	103	1	27	0.5
SBF	142	5	1.5	2.5	148.8	1	4.2	0.5
5* SBF (1000ml)	710	25	7.5	12.5	744	5	21	2.5

Each disc is soaked in 30ml of SBF solution, and solution of 0.01g silymarin silver nanoparticles in 10ml distilled water. One Ti disc is dip coated in SBF and nanoparticles solution for 7 days and one Ti disc is soaked in SBF and nanoparticles solution for 14 days. SBF and nanoparticles solution is refreshed every 48h. After the completion of 14 days, Ti disc is coated with a uniform coting of silymarin silver nanoparticles and bone like apatite coating.

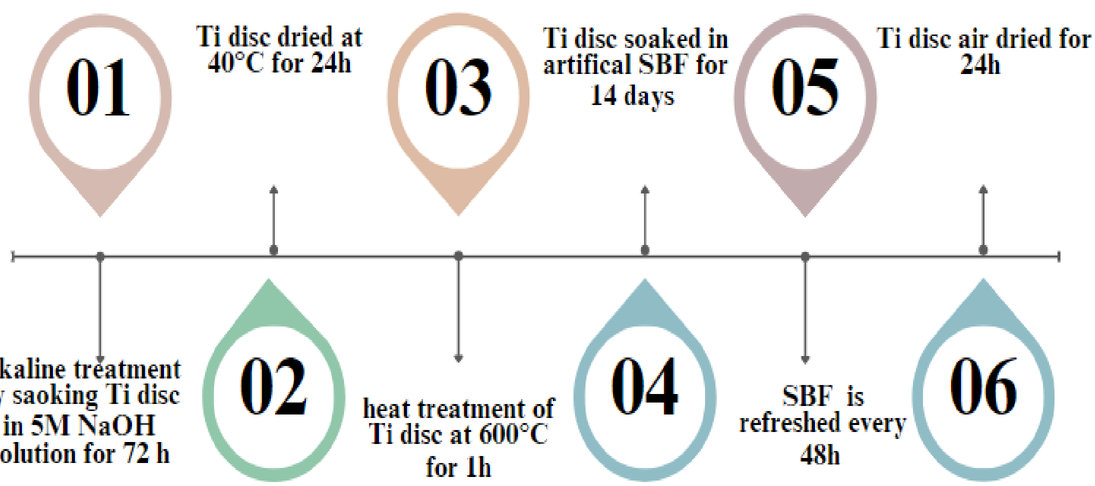

Figure 3. 2: Flow diagram showing the process of Ti disc coating with SBF and nanoparticles

3.3. Characterization of Silymarin Silver Nanoparticles

Characterization of silymarin silver nanoparticles is done through UV spectrum, SEM, FTIR, XRD, Zeta potential analysis and laser diffraction particle size distribution to ensure the synthesis of silver nanoparticles.

3.3.1 Ultraviolet Visible Spectroscopy

Silver nanoparticles synthesis was confirmed from the results for UV spectrum of the reaction after 4 hours.

UV-Vis absorption spectroscopy is widely employed methods for the characterization at medical and also industrial scale. It works by measuring light absorbed by sample when a beam of light goes through it. It operates on the idea of splitting the light beam in half. The second half of the light is directed towards the cuvette holding the sample, whereas the first half of the beam travels through the cuvette holding the reference (solvent). Within the necessary range, results can be observed and measured at a certain wavelength. The resulting spectrum, which shows the sample's absorbance at a particular wavelength, is plotted on wavelength against absorbance. Every material gives maximum absorbance at a specific wavelength range only which can confirm synthesis of a specific material. Lambda max is the absorbance peak at specific wavelength.

Beer Lambert Law is working principle of UV spectrometer, which states that;

$$A=EcL$$

Where, A stands for absorbance, c for sample concentration, L for the length of the light path through the cuvette in centimeters, and E for molar absorptivity.

The UV-Vis spectrophotometer measures the electronic transition of molecules based on this principle. The sample's absorbance is directly proportional to its molar concentration. Hence, to compare various chemicals, the absorption value also referred to as molar absorptivity is employed.

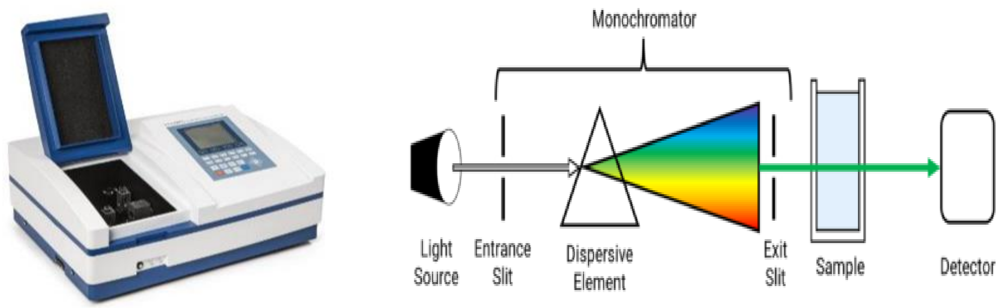

Figure 3. 3: UV spectrophotometer and its working principle

3.3.2 Scanning Electron Microscopy

SEM can provide us with the magnified images of a sample. It uses beam of electron to generate images of a sample. The electrons generated collimated electron beam has parallel rays thus they spread minimally as they propagate. When an electron beam falls on the sample's surface, information on the sample's topography and surface composition are produced. When this electron beam is directed on sample, signals are produced. Signals combined with the beam's location produce an image that shows the sample's surface and structural characteristics.

Using a software-controlled scanning electron microscope, images were obtained. The electron beam's energy was continually varied between 1pA and 1µA to examine the sample and select an appropriate beam for analysis. The sample was applied dropwise on a copper stub coated with carbon, and it was then dried under a mercury lamp.

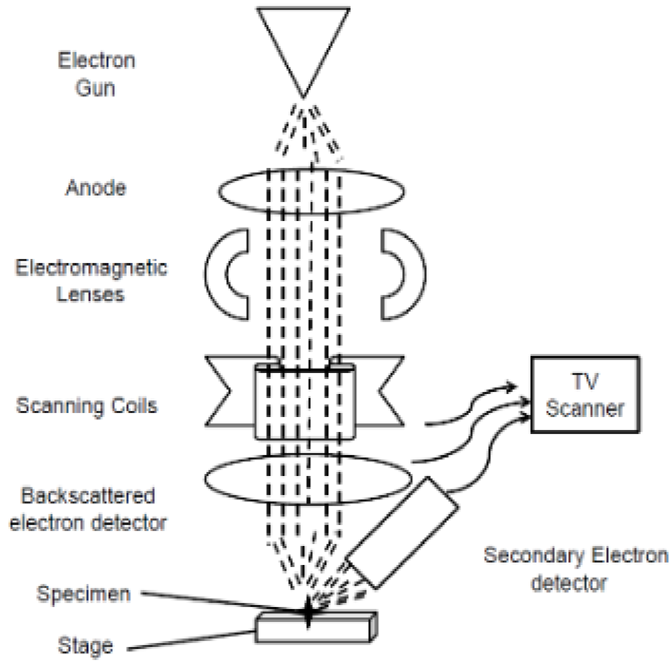

Figure 3. 4: Working principle of scanning electron microscope

3.3.3 Fourier transform infrared spectroscopy (FTIR) Analysis

FTIR is characterization technique to detect functional groups, formation and chemical analysis of compounds and used to do the comparative analysis. It is done to detect functional groups, formation and chemical comparison of pure silymarin and silymarin silver nanoparticles. The spectrum is obtained ranging 500 -4000 cm-1. After placing a sample of dried silymarin silver nanoparticles on KBr crystals, transmittance mode spectrum was obtained. FTIR can record the emission, absorption and photoconductivity of a solid, liquid, or gaseous chemical. It is also widely employed in surface analysis and nanoparticle characterization. When compared to a sample lacking absorbents, nanoparticles produce peaks in an FTIR spectrum due to the many surface absorbents that are present on their surface.

Smallest surface alterations can be easily detected through FTIR. It operates on mechanical and electrical systems that are able to detect any degree of variation in the amount of energy absorbed by the surface which can generate very precise results. It can be used for analysis of fibers, pastes, films, powders and materials in bulk. The absorption frequency depending on the atomic size, shape of surface and associated vibronic coupling.

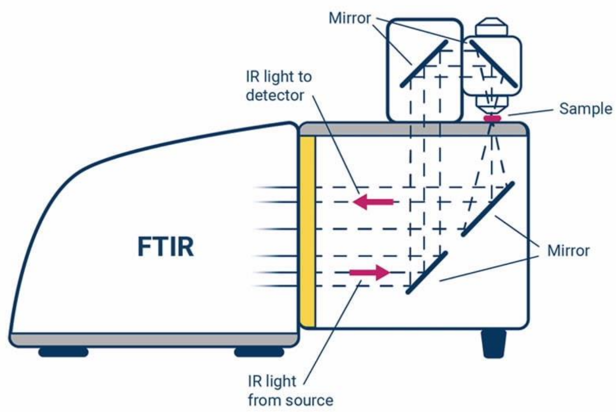

Figure 3. 5: FTIR apparatus

3.3.4 X-ray diffraction Analysis (XRD)

XRD is done to obtain information about the crystalline structure of the sample. It can work on liquid, powder and solid samples.

Material may have single phase or can be having multiple phases. Material can also contain crystalline material or non-crystalline material. X-ray diffractometer analyze material on the basis of their diffraction patterns. Different crystalline phase have different diffraction patterns. International center of diffraction data has a defined list of diffraction pattern of all materials which we use as a reference. The relative strength of various peaks of diffraction give data on the full composition of a multiphase compound.

XRD is a common analysis done for phase analysis. The data for phase orientation, size of crystallite (diameter) and residual stress all together provide information about the microstructure of polycrystalline material. XRD can be used to do qualitative and quantitative analysis of pure materials and mixtures.

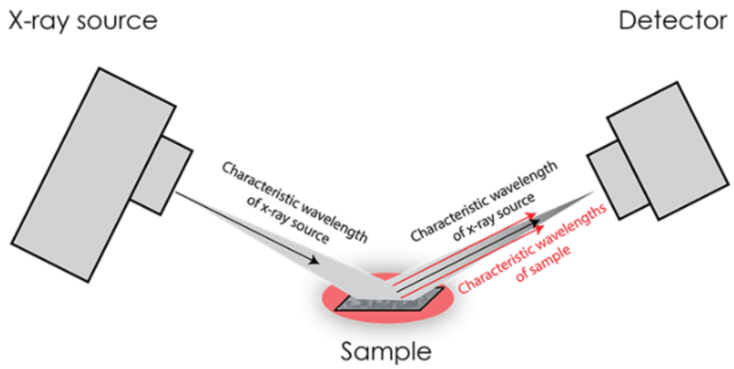

Figure 3. 6: Representation of XRD working principle

3.3.5 Laser particle size distribution Analyzer

Laser diffraction particle size distribution analysis is a common analysis for the estimation of percentage of particle size. it is an essential control factor which effects both the production process and properties of the product. In nanotechnology, the size of nanoparticle is the factor governing its efficiency and its ability to perform its function in the body.

Laser diffraction is an important and easy tool for estimation of particle size ranging from nanometers to millimeters. It provides us a graphical plot for the distribution of particle in each size area and also provides us with average, smallest and largest particle size. Light diffraction works on the principle of determination of size of obstacle, the light rays is falling on. It is based on the statement that angle of diffraction of light and size of the particle it is diffracting from are inversely related. Laser is used as a light source thus referring to the technique as laser diffraction.

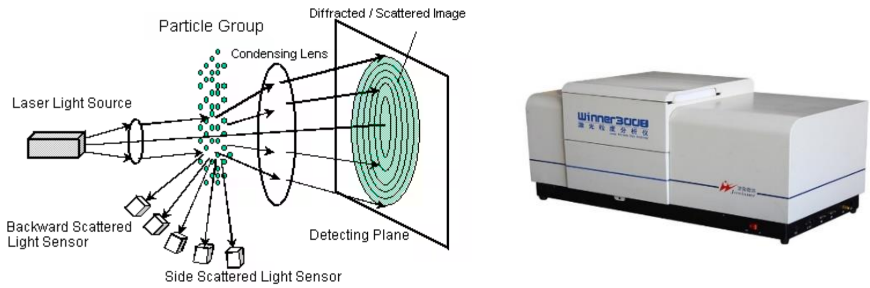

Figure 3. 7: Laser diffraction particle size distribution analyzer

3.3.6 Zeta potential Analysis

Zeta potential is most widely tool used for the estimation of the stability of the dispersed particles. It is measures the forces of attraction and repulsion. The measurement of attractive and repulsive forces can gives as insight into stability of the particles, their aggregation and dispersion, the knowledge of which can be applied to bring changes to the formulation process.

A charged particle attracts particle sf opposite charge thus becoming away from the particle system. The amount of these opposite charges determines the zeta potential. Zeta potential is property of particle system (particles and the solvent they are dispersed in) not a single particle, that's why estimation of the pH is essential for the estimation of the zeta potential.

Table 3. 2: Zeta potential range and respective stability

± Zeta Potential (mV)	Stability
0-10	High instability
10-20	Limited stability
20 to 30	Moderate stability
> 30	Good stability

From zeta potential we can get prediction and control of the stability and performance of the solutions that can be applied to enhance product quality, shelf life and efficiency of the overall process.

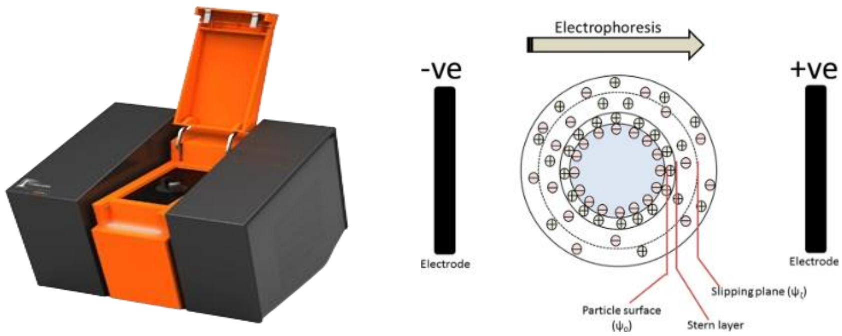

Figure 3. 8: Zeta potential analyzer

3.3.7 Optical Microscope

Light microscopy studies the topography of samples, Light microscope creates magnified images of small samples using one or more lenses. It uses visible light as a light source. To examine the image in greater detail, the magnifying lenses are placed between the sample and eyepiece. To allow the light to pass through the tube of microscope, objective lens is brought closer to the sample. As a result, enlarged, inverted images of the sample are created that can be viewed through the eyepiece of the microscope.

The most common application of optical microscopy is in microbiology, microelectronics, nano-biotechnology, nanophysics and pharmaceutical research. It is also used to view biological samples in laboratory and diagnostic centers for medical diagnosis.

Figure 3. 9: Optical Microscope

3.3.8 Angle of contact measurement

Measurement of Contact angle is done to evaluate the wettability of the implant surface; the degree of its hydrophilicity and hydrophobicity. Hydrophilicity is more appreciated than hydrophobicity. Considering how the implant surface interacts with the biological environment in the body wettability is an important factor. There is a direct relation between wettability and osseointegration potential.

3.4 Anti-biofilm crystal violet Assay

For evaluation of anti-biofilm potential of the uncoated and coated titanium disc, anti-biofilm crystal violet assay is performed.

A fresh culture of S. aureus isolates was prepared in 5ml of TSB and incubated overnight at 37°C. A dilution in ratio 1:100 of the prepare culture is made in TSB. Each well of the 6 well micro titer plate is poured with 100µl of prepared dilution using a micropipette. Positive control, blank and disc coated for 7 days and 14 days are placed in the wells and incubated at 37°C, overnight.

After 24h, the wells of plate are emptied through aspiration and gently washed with autoclaved distilled water. Crystal violet 0.1% w/v solution is prepared and 250µl of solution is poured in each well using a micropipette, and incubated for 15 minutes at 37°C.

After 15 minutes, the wells are emptied and washed with autoclaved distilled water and placed to air dry. After the wells are completely dried, 300µl 95% ethanol is added to each well and plate is placed for 15 minutes with lid closed. Then, the contents are gently mixed using micropipette and transferred to fresh wells of 96 well microtiter plate to check the absorbance at 630nm using Multiskanlt spectrophotomter. The values for absorbance are noted and the percentage inhibition is calculated using the equation;

$$\{(C-B)-(T-B)/(C-B)\}*100\%$$

Where,

B= absorbance of the blank (TSB)

C= absorbance of the control

T= absorbance of the test

CHAPTER 4: RESULTS

In this study, synthesis and characterization of silymarin silver nanoparticles is done. Titanium discs are heat and alkaline treated and coated with nanoparticles and biomimetic calcium phosphate coating. Characterization of titanium discs is done after coating. Coated titanium disc are tested for their antibacterial properties.

4.1 Characterization of Silymarin Silver Nanoparticles

4.1.1 Ultraviolet Visible Spectroscopy

UV spectroscopy is the most common laboratory characterization technique which can be used for the prediction and analyzing the synthesis of nanoparticles. The incident light beam on the sample and its reflection back is the working principle of UV spectroscopy. The nanoparticles formation using silymarin as a reducing agent can be detected through this characterization technique.

Silymarin silver nanoparticles were prepared by green synthesis method using silymarin as a reducing agent, keeping a ratio of 2:3 for silymarin and silver. The formation of nanoparticles is confirmed by the absorption spectrum of the nanoparticles using UV spectrophotometer. UV spectrophotometry was done at SNS (school of natural sciences), NUST. Device used for detection was single beam spectrophotometer (UV BMS – 2800). Peak at 424 nm at absorption of 1.2 confirmed synthesis of silver nanoparticles.

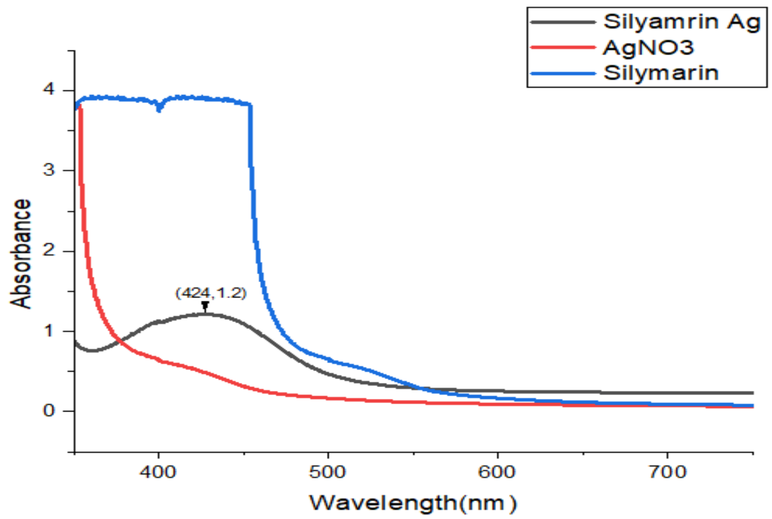

Figure 4. 1: UV Spectrum of Silymarin Silver Nanoparticles

4.1.2 Scanning Electron Microscope Analysis

SEM is an important characterization tool for analysis of surface properties and particle size. Electron beam is used for the image generation. Electron beam falls on the sample and scatters back with data of the surface properties. The result obtained in image form which contains information about morphology and size of nanoparticles. Particle shape can also be observed using this technique. Scanning electron microscope can generate images on different magnification levels.

Scanning electron microscope analysis was done at SCME (school and chemical and materials engineering), NUST using SEM model JSM-6490-LA.

Analysis of silymarin silver nanoparticles showed spherical nanoparticles of size ranging from to 48 nm to 88.09 nm with average size at 72.50nm.

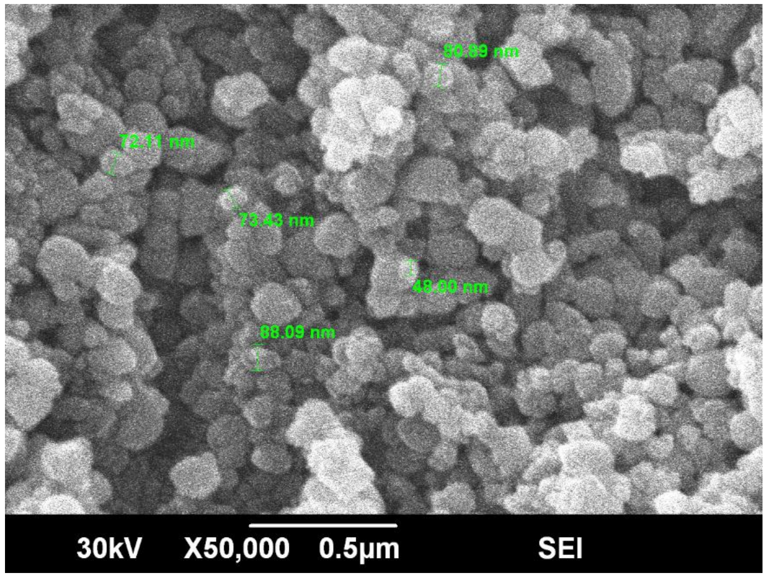

Figure 4. 2: SEM Analysis of Silymarin Silver Nanoparticles at scale bar of 0.5μm

4.1.3 Fourier Transformation Infrared Spectroscopy (FTIR)

FTIR analysis is done for the detection of the functional groups in a chemical compounds. FTIR graph is plotted against wavenumber (cm^{-1}) which is the amount of energy of infrared light absorbed by the sample and absorbance which indicates amount of infrared light absorbed at each wavenumber. It plots graph in range from 500 cm^{-1} to 4500 cm^{-1}, which contains the peaks for most of the essential functional groups.

FTIR of pure silymarin powder and silymarin silver nanoparticles was done at USPCASE, NUST in range of 500 cm^{-1} to 4500 cm^{-1}, giving strong peaks at around 3200 cm-1, peaks at <600 cm-1 shows presence of Ag-O bond, which confirms synthesis of silymarin silver nanoparticles.

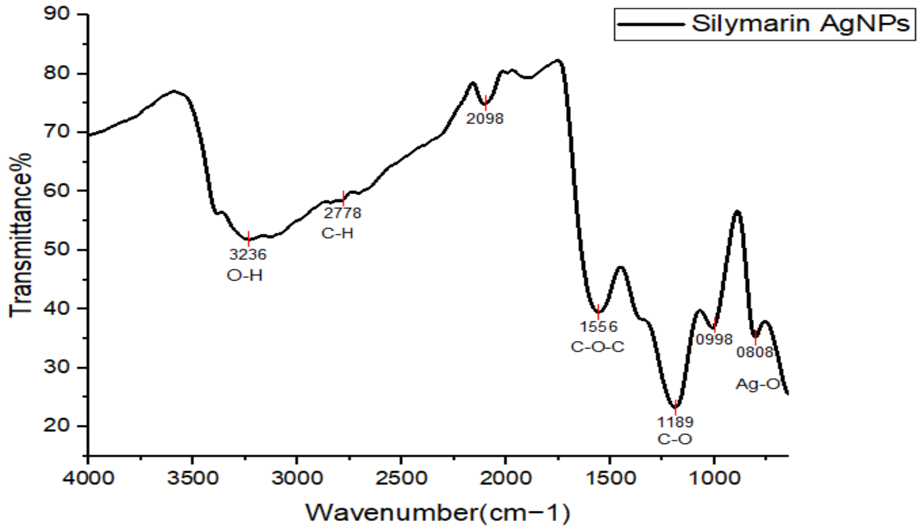

Figure 4. 3: FTIR Analysis of Silymarin Silver Nanoparticles

Peaks at around 2800 and 1700 cm-1 show the presence of linear C-H bonding and C-H bending. Stretching at around 1000 cm-1 confirm the presence of C=C bonds, all these bonds confirm the phenolic structure of silymarin.

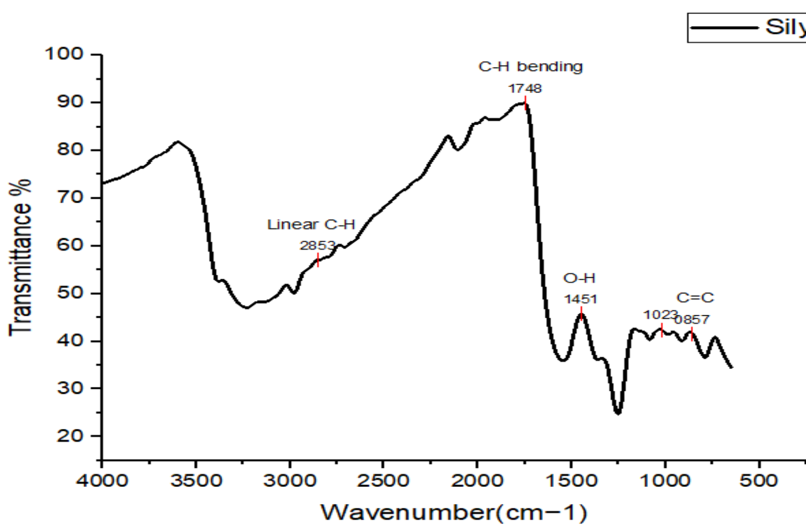

Figure 4. 4: FTIR of Silymarin powder purchased commercially

The comparison of FTIR for silymarin Ag and silymarin powder confirm the synthesis of Ag nanoparticles using silymarin as a reducing agent.

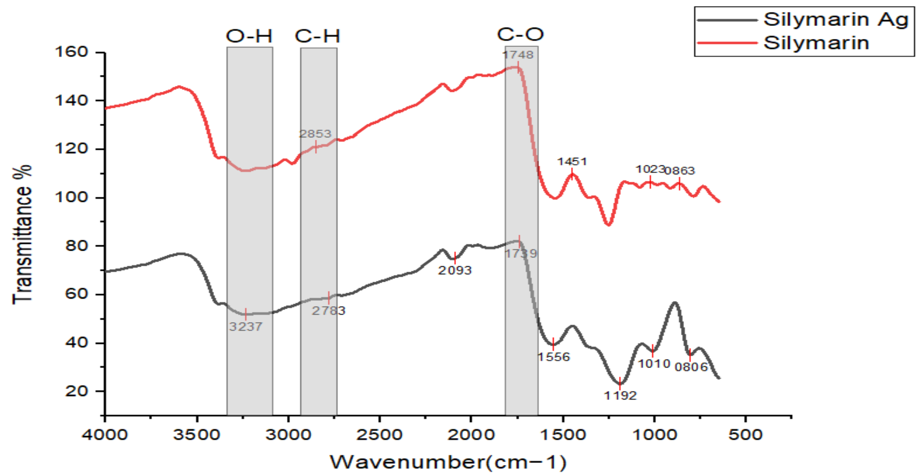

Figure 4. 5: Comparison between Silymarin and Silymarin Silver Nanoparticles

4.1.4 X ray diffraction Analysis

XRD analysis is done to get information about the crystalline structure of a material, its phase orientation. The graph is plotted against intensity of scattered x-ray and angle of x-rays incidence denoted by theta 2θ. It works on principle of Bragg's equation. The angle at which peaks occur gives information about the crystal lattice parameters. The height of peaks tells how intense the scattered x-rays are. The sharper the peaks, the more crystalline the materials is. Broder humps show the presence of smaller crystallite or non-ordered structures i.e. amorphous structure.

X-ray diffraction analysis of silymarin powder and silymarin silver nanoparticles was done at SCME (school of chemical & materials engineering), NUST.

XRD graph for silver nanoparticles shows strong peak at 38° which corresponds to the plane (111) for Face Centered Cubic structure of silver nanoparticles. Additional peaks present at 44°, 64° and 77° attributes to the plane (200), (220) and (311) for silver nanoparticles, are also present, confirming the crystalline structure of silver nanoparticles.

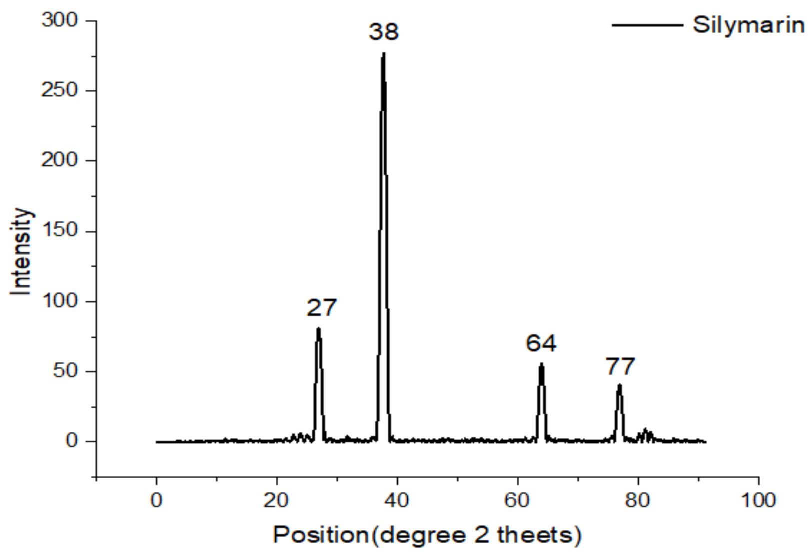

Figure 4. 6: XRD Analysis of Silymarin Silver Nanoparticles

XRD graph for silymarin powder shows strong peak at 27° and 38° which corresponds to the crystalline structure of silymarin powder. Additional peaks present at 64° and 77° are also present confirming the crystalline structure of silymarin.

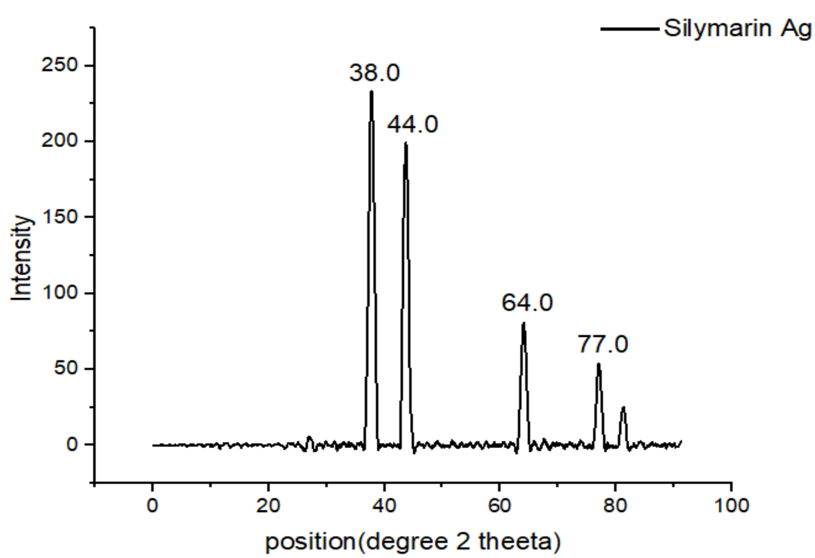

Figure 4. 7: XRD of Silymarin powder purchased commercially

The comparison of XRD graph for silymarin and silymarin silver nanoparticles shows the successful silver nanoparticles synthesis using silymarin as a reducing agent.

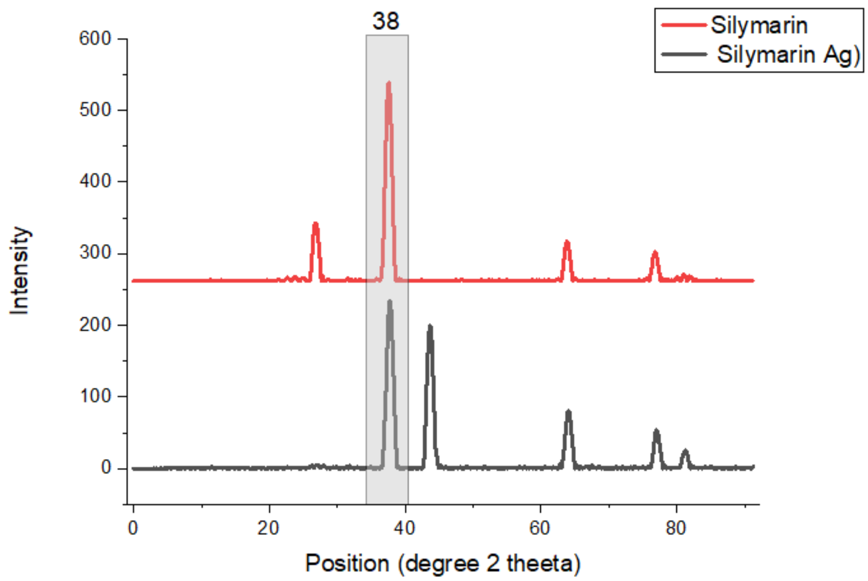

Figure 4. 8: Comparison of XRD spectra for Silymarin & Silymarin Silver Nanoparticles

4.1.5 Zeta Potential Analysis

Zeta potential is the technique for the estimation of the stability of the material. It measures the attractive and repulsive forces between particles in a solution. The more the zeta potential moves away from the zero either positive or negative, the more is the stability of the particles. It is a characteristic of the particles and the also the solvent. Measuring pH of solution before analysis is important. Repulsive forces between the particles make them freely dispersed in the solution and thus increasing the stability of the solution.

Zeta potential for silymarin silver nanoparticles was done at SCME (school of chemical & materials engineering), NUST using Wallis zeta potential analyzer having high resolution up to 0.1 mV. The analyzer measures the potential in 3 sequences and then gives average value of the potential.

The potential was found to be ranging from -34.91mV to -44.88 mV and mean zeta potential at -39.06 mV that shows highly stable silver nanoparticles.

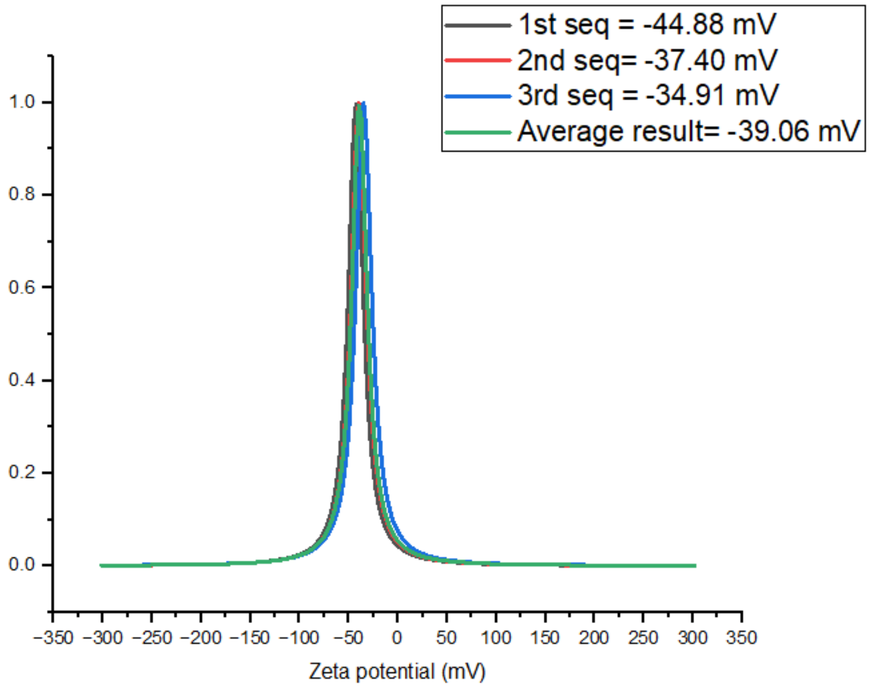

Figure 4. 9: Zeta Potential of Silymarin Silver Nanoparticles

4.1.6 Laser diffraction size distribution analysis

Particle size analysis gives us data on distribution of particle size. It uses laser beam to estimate the particle size. The laser beam falls on the particles and reflects back. The degree of reflected beam is evaluated for the estimation of the size of the obstacle (particle) it collided with. The system plots a graph for the distribution of the particle size in various ranges. It can measure particle size in range from millimeter (mm) to nanometer (nm). The graph is plotted for q (%) and diameter (µm).

Laser diffraction particle size distribution for silymarin silver nanoparticles was done at SCME (school of chemical & materials engineering), NUST using HORIBA Laser Scattering Particle Size Distribution Analyzer LA-920. The graph for size distribution is displayed below which shows that nanoparticles lie in the size range of nanoparticles.

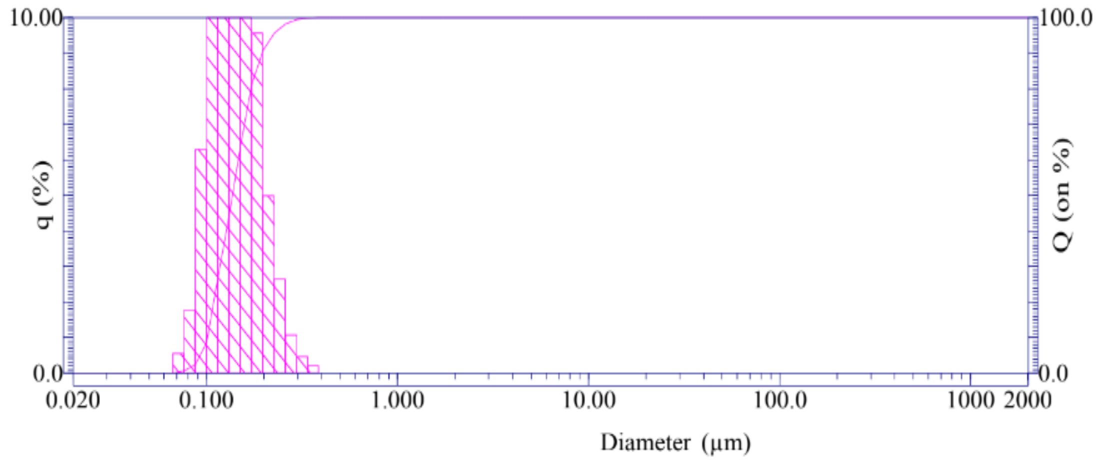

Figure 4. 10: Particle size distribution of Silymarin Silver Nanoparticles

4.2. Characterization of Titanium Disc

Titanium disc of grade 5 are used for the coating purpose. The disc are divided to 3 groups. Group I is control which include untreated Ti disc, group II is Ti disc coated in SBF+ nanoparticles solution coated for 7 days and group III is Ti disc coated in SBF+ nanoparticles solution for 14 days.

Figure 4. 11: Images of uncoated titanium disc, disc coated for 7 and 14 days

4.2.1 Optical Microscope

Optical Microscope is a common laboratory equipment which can produce magnified images of a sample. It uses visible light as a source of illumination. When the light falls on the sample, objective lens produces magnified, inverted image of the sample which can be seen through the eyepiece and can also be saved in the computer. Ti disc (untreated) and Ti disc (treated) after alkaline and heat treatment were observed under the optical microscope.

Optical microscope images were taken at SCME (school of chemical & materials engineering), NUST using LM PLAN OPTIKA optical microscope.

Image of Ti disc untreated is shown below taken at 5x magnification and numerical aperture of 0.15. Image of Ti disc after alkaline treatment by soaking discs in 5M NaOH and heat treatment by heating the disc up to 600 °C for 1h are shown below taken at 5x magnification and numerical aperture of 0.15.

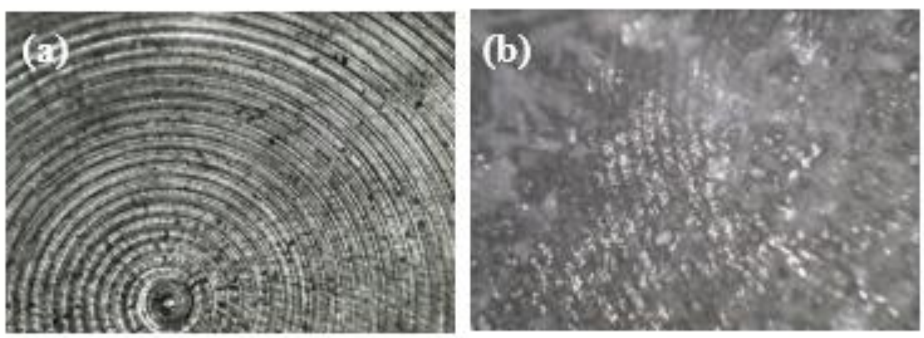

Figure 4. 12: Images of untreated Ti disc under optical microscope at 5x magnification. Image of uncoated titanium disc (a), image of titanium disc after alkaline treatment (b)

4.2.2 Scanning Electron Microscopy

SEM is a more sophisticated microscopy technique for generating magnified images of an object. It uses electron beam as a source of light. Electron produced inside electron gun are aligned in parallel rays to form an electron beam which falls on the sample surface. The backscattered electrons can be evaluated to generate images of sample.

SEM of the treated and untreated Ti discs was done at SCME (school and chemical and materials engineering), NUST using SEM model JSM-6490-LA. Shown below are SEM images of Ti disc coated with SBP + nanoparticles solution for 7 and 14 days at scale bar of 500, 100, 10 and 1 μm shows a uniform coating on Ti disc. Spherical shapes shows silymarin silver nanoparticles and rod shapes show CaP formation. An increase in the uniformity of coating is observed for disc coated for 14 days.

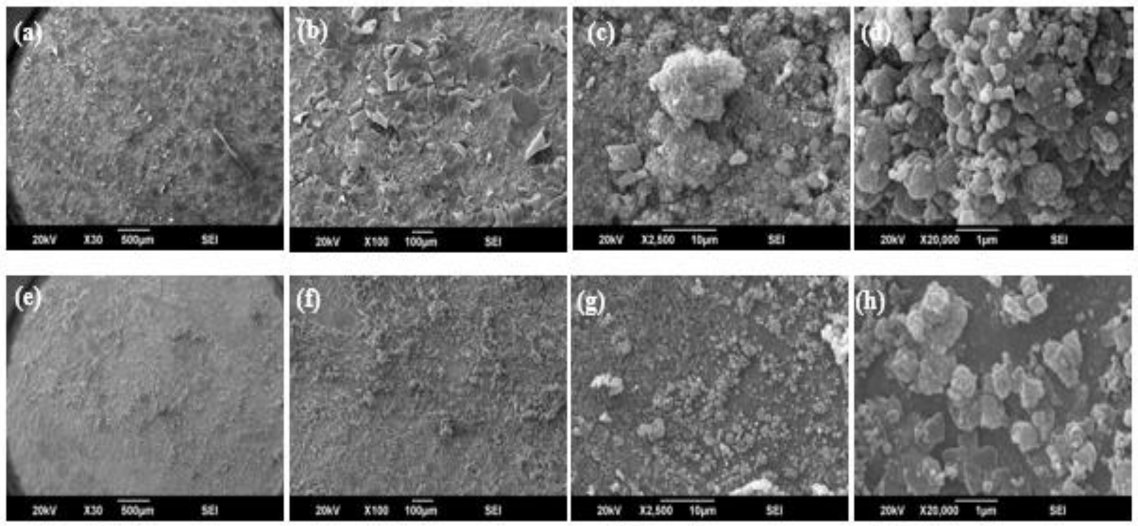

Figure 4. 13: SEM Images of 7 and 14 days coated Ti disc at scale bar 500, 100, 10 & 1μm. 7 days coated disc at scale bar 500μm (a), at scale bar 100μm (b), at scale bar 10μm (c), at scale bar 1μm (d), 14 days coated disc at scale bar 500μm (e), at scale bar 100μm (f), at scale bar 10μm (g), at scale bar 1μm (h)

SEM images for uncoated titanium disc, disc coated for 7 days and 14 days at scale bar of 500 and 100μm are presented below.

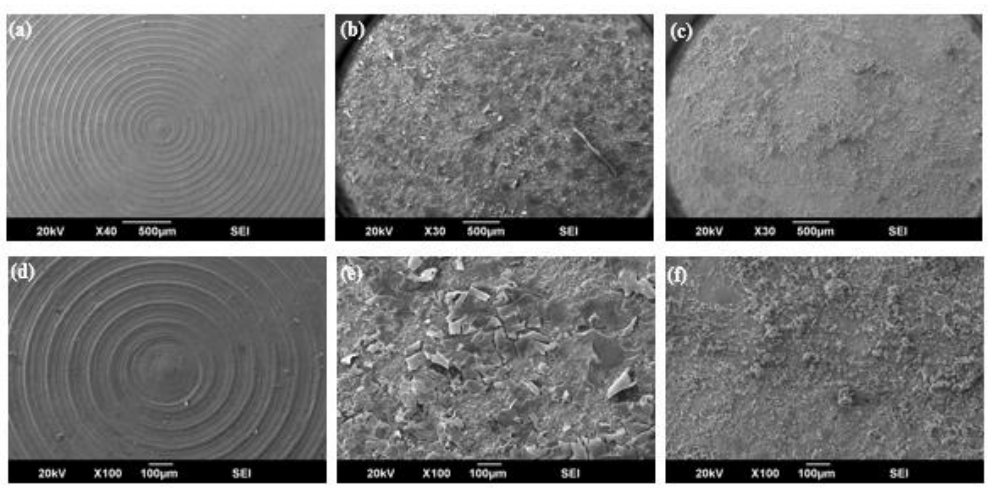

Figure 4. 14: SEM Images of uncoated Ti disc, Ti disc coated for 7 days and Ti disc coated for 14 days at scale bar 500 and 100μm. Uncoated disc at scale bar 500μm (a), 7 days coated disc at scale bar 500μm (b), 14 days coated disc at scale bar 500μm (c), uncoated disc at scale bar 100μm (d), 7 days coated disc at scale bar 100μm (e), 14 days coated disc at scale bar 100μm (f)

4.2.3. Contact Angle measurement

Contact angle measurement showed a significant decrease in the contact angle for titanium disc coated for 7 days and 14 days. Decrease in contact angle shows improved wettability which means more hydrophilicity of the titanium disc. There is an inverse relationship between the wettability and contact angle. Hydrophilicity is an important factor in view of the interaction of the titanium disc with the surrounding biological environment. Decrease in contact angle shows significant increase in the osseointegration potential of the titanium disc by biomimetic calcium phosphate coating.

Contact angle measurement was done at USPCASE, NUST. Contact angle decreased was observed to be at 67.5° for untreated Ti disc (untreated), 58.9° for Ti disc coated for 7 days and 34.35° for Ti disc coated for 14 days.

Image showing the contact angle for control group, untreated Ti disc with left contact angle at 68.60° and right contact angle at 66.40°.

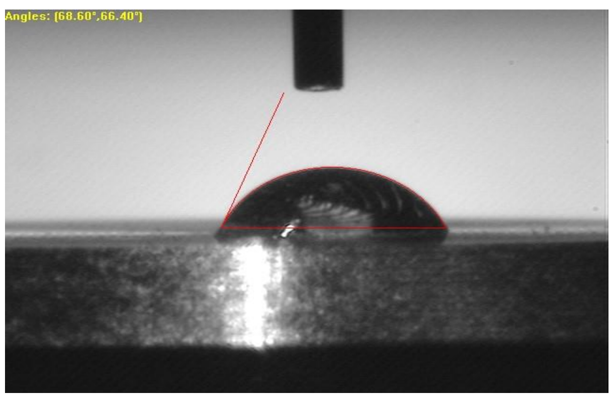

Figure 4. 15: left and right contact angle for untreated Ti disc

Image showing the contact angle for control group, untreated Ti disc with left contact angle at 68.60° and right contact angle at 66.40°.

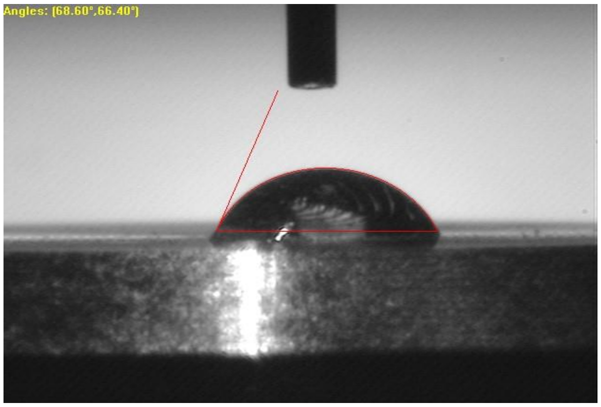

Figure 4. 16: Left and right contact angle for Ti disc treated for 7 days

Image showing the contact angle for control group, untreated Ti disc with left contact angle at 28.8° and right contact angle at 39.9°.

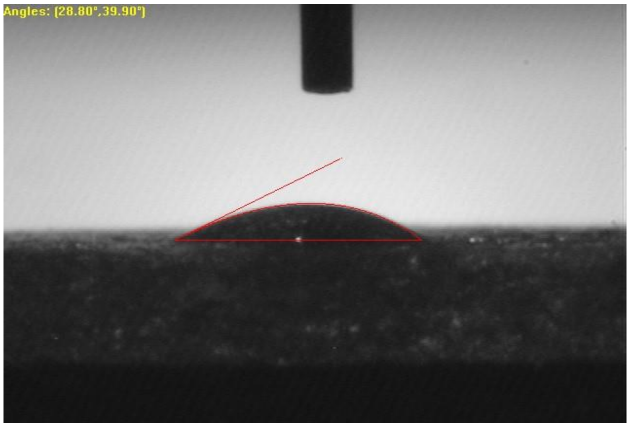

Figure 4. 17: Left and right contact angle for Ti disc coated for 14 days

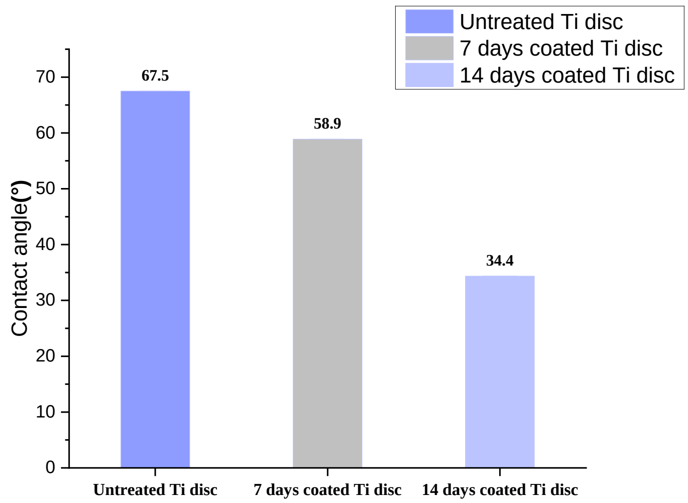

Figure 4. 18: Graphical representation of comparison of contact angle for untreated Ti disc, 7 days and 14 days coated Ti discs

4.3 Anti-biofilm crystal violet assay

Anti-biofilm crystal violet assay of coated and uncoated Ti disc was done to draw a comparison between the anti-biofilm activities of respective discs. Anti-biofilm assay showed significant decrease in the anti-biofilm activity of coated titanium discs. A significant difference was observed in the anti-biofilm activity of the disc coated for 7 days and 14 days.

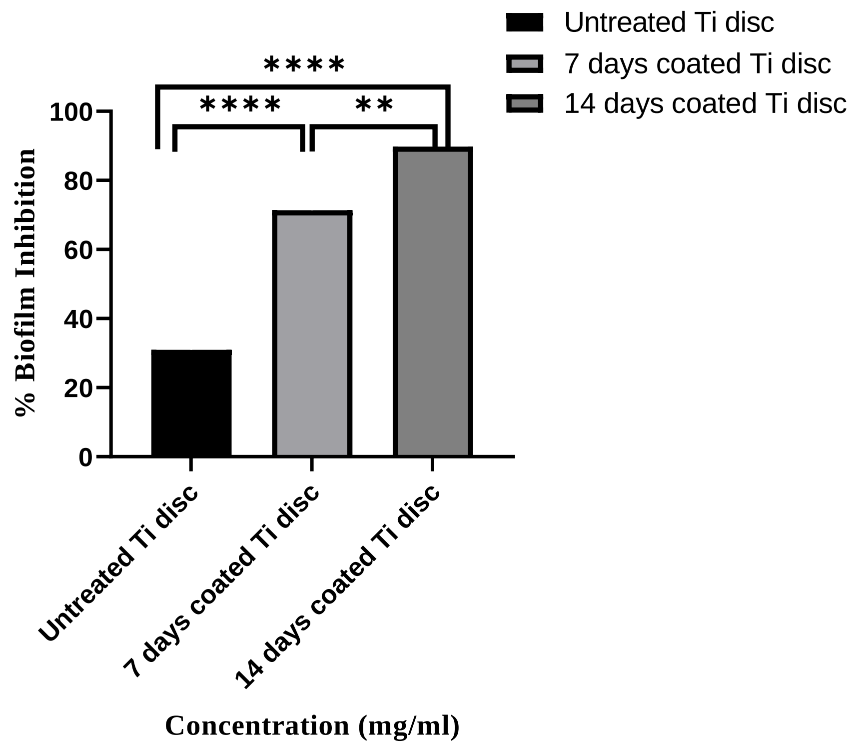

Figure 4. 19: Comparison for anti-biofilm activity of untreated Ti disc, disc coated for 7 days and disc coated for 14 days at p value<0.0001

CHAPTER 5: CONCLUSION

Anti-biofilm activity and osseointegration of titanium implant has been significantly improved by the designed silymarin silver nanoparticles loaded biomimetic coating. Green synthesis approach is applied for preparing silver nanoparticles in a clean, economical and environment friendly way. The potential of silybum marianum L. plant extract has been explored and proved as a reducing agent. The successful synthesis of silver nanoparticles is confirmed using various characterization techniques. UV spectrophotometer peak observed at 424nm confirmed the synthesis of silver nanoparticles. SEM imaging showed synthesis of spherical, stable nanoparticles ranging in size of 32.26nm to 40nm with mean size at 35.67nm. Zeta potential was observed at an average of -39.06 mV that confirms the synthesis of stability of nanoparticles. The particles are present in dispersed state in the solution that confirms the stability of silymarin silver nanoparticles. FTIR spectra showed the stretching for O-H, C-O, and Ag-O bonds that also confirmed synthesis of silver nanoparticles. Presence of linear C-H bonding, C-H bending and C=C confirm the phenolic structure of silymarin powder. XRD spectra was evaluated to show peaks at 38°, 44°, 64° and 77° confirming the crystalline structure of silver nanoparticles.

Anti- biofilm activity of silver nanoparticle synthesized using silymarin has been proven to increase the anti-biofilm potential of titanium surface thus inhibiting the risk of implant associated infections. The anti-biofilm potential was observed to be significantly increased for the disc coated for 14 days as compared to disc coated for 7 days. Highly significant results are obtained for comparison of untreated titanium disc with titanium disc coated for 7 days and 14 days. Successful deposition of biomimetic calcium phosphate coating using simulated body fluid is confirmed from the SEM images of Ti discs. Bone like apatite coating improved the osseointegration potential of the disc as confirmed from the values of contact angle. Contact angle deceased from 67.5° to 34.4° for disc coated for 14 days. The decrease in angle of contact of the coated Ti disc showed improved wettability which shows the improved osseointegration potential of coated titanium discs.

CHAPTER 6: FUTURE PERSPECTIVES

In this research, in vitro analysis is done by observing the anti-biofilm activity of coated titanium disc by anti-biofilm crystal violet assay. Coating of silver nanoparticles significantly increased the anti-biofilm activity of the titanium surface. Research on coating of different types and morphology of nanoparticles can pave the way to discovery of nanoparticles and biomaterials that can significantly enhance anti-biofilm activity of titanium surfaces. In future research can be conducted on the in-vivo analysis of coated titanium disc in animal models to check the anti-biofilm activity in the animal study models. The in-vitro analysis in the animal study models can give us estimation whether these titanium discs can be implanted in the human body as orthopedic or orthodontic implants. These analysis can give us estimation about the implant stability in the human body and success of coated titanium implants for long term implantation. Analysis can be done to estimate changes in the healing time for coated and uncoated titanium implants. Further analysis can be to test the biocompatibility of titanium implants in human body. In this study, anti-biofilm activity is tested only against strains of *staphylococcus aureus,* which is bacterial strains that accounts for around 15% of the cases in which implant rejection is observed because of bacterial colonization. The anti-biofilm activity can be tested against various bacterial strains to test whether silymarin silver nanoparticles can show biofilm activity against different bacterial strains that cause implant rejection in orthopedic and orthodontic implants. Further research can be conducted on testing the effect of biomimetic calcium phosphate coating on biocompatibility of the titanium implants.

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