Development of Novel Biodegradable Polymer-based Drug Composite for the application of Cardiovascular Implants

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December, 2021

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A thesis submitted in partial fulfillment of the requirements for the degree of

MS Biomedical Sciences

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DEPARTMENT OF BIOMEDICAL ENGINEERING AND SCIENCES

SCHOOL OF MECHANICAL & MANUFACTURING ENGINEERING

NATIONAL UNIVERSITY OF SCIENCES AND TECHNOLOGY,

ISLAMABAD, PAKISTAN

DECEMBER, 2021

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Dedicated to

My Amma & Abu

for their utmost support and encouragement

Acknowledgements

All praises to Allah almighty who is the most beneficent and the most merciful. I am grateful to Allah Almighty for giving me the strength to complete my master's degree and for the strength that kept me standing in the most difficult circumstances and completing the degree.

I feel great pleasure and privilege to have this opportunity to express my gratitude to my supervisor Dr. Murtaza Najabat Ali. I feel honored to work and learn under his kind supervision. I am thankful to my supervisor who gave me enthusiasm, esteemed inspiration, and guidance throughout my research work.

I would like to pay special thanks to Sadia Hassan for her tremendous support and cooperation. Without her assistance I wouldn't have been able to complete my thesis. I appreciate her kind help and guidance throughout the whole thesis. I also wish to express my thanks to Bakhtawar Ghafoor for guiding me, teaching me how to properly work in lab and also for being so supportive.

I am profusely thankful to my beloved parents Mr. Qaim Javaid Ali Khan and Mrs. Amina Javaid for providing me this opportunity to study at one of the best institutes. Without their support this journey was impossible.

I would also like to thank my friends and lab fellows Ramsha Munir, Sania, Namra Bilal, Aimen Arshad and Aroosa Younis for their constant support, help and cooperation. Finally, I would like to express my gratitude to all the individuals who have rendered valuable assistance to my study.

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CVD	Cardiovascular disease
WHO	World health organization
VAD	Ventricular assisted device
CAD	Coronary artery disease
AHV	Artificial heart valve
BHV	Bioprosthetic Heart Val
MHV	Mechanical heart valve
Al_2O_3	Aluminum Oxide
ZrO_2	Zirconium Oxide
DLC	Diamond like carbon
Ti	Titanium
Cu	Copper
V	Vanadium
PE	Polyethylene
PP	Polypropylene
PTFE	Polytetrafluoroethylene
PU	Polyurethane
PMMA	Polymethylmethacrylate
PA	Polyamines
MAA	Methyl acrylic acid
EGDA	Ethylene glycol diacrylate

LMWPLA	Low molecular weight Poly lactic acid
HMWPLA	High molecular weight poly lactic acid
DMF	Dimethylformamide
PBS	Phosphate buffer saline
DPPH	2,2-diphenyl 1-1-picrylhydrazyl
DCM	Dichloromethane
SEM	Scanning electron microscopy
FTIR	Fourier transform infrared region
EDX	Energy dispersive X-rays
PT	Prothrombin Time
a-PTT	Activated partial thromboplastin Time
RBCs	Red blood cells

Abstract

Biodegradable polymer-based drug loaded films and composite were prepared as potential anti-coagulant coating for the application of cardiovascular implants for the treatment of thrombosis. Cinnamon Cassia, Ocimum tenuiflorm and Panax Ginseng are pharmaceutically active in many ways, having properties such as anti-coagulation, anti-oxidative activity and can be used to treat thrombosis after implantation of cardiovascular implants. For this purpose, Drug loaded films were fabricated using solvent casting method and PLA (Poly lactic acid) was used as drug carrier and above-mentioned drugs were used as anti-coagulant drugs. The surface morphology and chemical compositions of coatings were analyzed by Scanning electron microscopy (SEM) and Fourier transform infrared region (FTIR). In this study, *In vitro* anticoagulation activity, drug release studies, degradation studies, hemocompatibility and anti-oxidative studies of coatings were investigated. *In vitro* anti-coagulation activity of coating films was checked by a-PTT and PT test and drug composite indicated anti-coagulation activity equals to commercially available anticoagulant drug and anti-coagulation behavior of coating films was improved as the concentration of drug was increased. Drug release and degradation behavior of drug composite were almost same to commercially available anti-coagulant drug. In addition, drug composite exhibited high anti-oxidative and non-hemolytic activity. To conclude, the results of the present study showed that drug composite has a potential application as anti-coagulant coating for cardiovascular implants.

Key Words: *Thrombosis, Implant coatings, Drug carrier, Composite, Biomaterials, Cardiovascular implants, Surface modification*

Chapter 1

INTRODUCTION

A disease related to the circulatory system in humans is known as a cardiovascular disease (CVD). CVDs are a major health problem in Pakistan and are also leading cause of deaths globally. Cardiovascular diseases include the problems of valves, muscles and coronary arteries which effect the functions of heart and reduce the ability of heart to circulate blood through the arteries and vessels. Coronary artery diseases are common heart diseases, in which plaque builds up within the arteries and disturbs the normal flow of blood in them. The disturbance of blood flow in the arteries by plaque accumulation is called atherosclerosis (Borhani, Hassanajili, Tafti, & Rabbani, 2018).

According to World Health Organization (WHO), 17.9 million people died from cardiovascular diseases in 2019, indicating 32% of global deaths and 85% of them were due to heart attack and strokes. Three quarter of deaths from cardiovascular diseases happened in low and middle- income countries. Out of 17.9 million deaths, 17 million deaths were premature deaths due to non-communicable diseases and 38% of them were because of cardiovascular diseases.

According to centers for disease control and prevention, heart diseases are leading cause of deaths in United States including men, women, people of racial and ethnicity groups. In united states, one person dies of cardiovascular diseases in 36 seconds and total 6,59,000 people die from heart diseases annually. In 2016 to 2017, total cost of heart diseases in United States was about $363 billion annually which includes all the health care services and medicines (Virani et al., 2021). Out of all heart diseases coronary heart diseases are the common type of heart diseases which have killed 360,900 people in 2019 and 2 in 10 deaths happened in adults less than 65 years of age (Centers for Disease Control and Prevention, National Center for Health Statistics. About multiple

cause of deaths 1999-2019). In united states, an estimated 18.2 million people age 20 and more have coronary artery diseases (CAD) and 805,000 people had heart attack and out of them, 605,000 were the ones who had heart attack first time and 200,000 are the ones, who already had heart attack (Fryar, Chen, & Li, 2012).

Every year, deaths due to cardiovascular diseases are increasing significantly and primary cause of coronary diseases is atherosclerosis that develop due to high cholesterol levels in blood, smoking and high blood pressure that cause dysfunction of endothelium layer of vessels. Due to dysfunction of endothelium, low density lipids enter the intimal layer and start immune responses. In the intimal layer, low density lipids are engulfed by macrophages resulting in foam cells formation which are main reason of arthrosclerosis. Foam cells cause the proliferation of smooth muscle cells, and that proliferation of smooth muscles cells form lipid containing fibrous cap which is known as plaque that block the blood flow and result in heart attack and in most cases end up the death of patients (Bentzon, Otsuka, Virmani, & Falk, 2014; Otsuka et al., 2015)

For the treatment of cardiovascular diseases, cardiovascular implants are being employed extensively but these implants possess some problems such as thrombosis, inflammation, cytotoxicity, and corrosion that lead to their failure due to limited endothelialization of implants surface. Therefore, to solve these problems implant surfaces are modified with biomaterials(Zou et al., 2021).

Cardiovascular implants with surface modification exhibit antiproliferative, antithrombotic, non-toxic, non-corrosive and anti-inflammatory properties by releasing drugs from polymeric matrix. Thus, it is desirable to fabricated coatings which are not only biocompatible but also have biodegradative properties in order to prevent undesirable effects. In addition, drugs that are used

for loading purposes in drug delivery systems should have properties such as re-endothelialization, anti-inflammatory response and should have anti-proliferative properties. Polylactic acid is widely being used in drug delivery applications because of its characteristic features such as biocompatibility, biodegradability, has outstanding film fabrication properties. It also has excellent mechanical strength and flexibility as well as it is cost effective and easily available (Lee et al., 2019).

Many plants based anti-coagulant agents are being used as alternative to synthetic drugs due to their bioactive chemicals such as extracts of Cinnamon cassia, Ocimum tenuiflorum, and Panax ginseng have anticoagulation properties and can be used as an alternative to drugs that are currently in use for the treatment of blood coagulation. Phenolic chemical constituents of the cinnamon cassia, ocimum tenuiflorum and panax ginseng are responsible for their antioxidant, anti-inflammatory properties (Gunendren, Nordin, Ramachandran, Samad, & Sciences, 2017; Gupta, Garg, Uniyal, & Kumari, 2008; H. Liu, Lu, Hu, & Fan, 2020; Singh, Srivastava, Singh, & Srivastava, 1995; Triveni et al., 2013).

Therefore, the goal of our study was to develop a novel herbal drug composite based on biodegradable polymer for the application of cardiovascular implant, characterization of drug composite and evaluation of its *in vitro* anticoagulation activity, antioxidant activity and hemolytic activity. Additional objective of this research was to check cumulative drug release, degradation rate of individual drug loaded films and drug composite and further compare them with commercially available anticoagulant synthetic drug. To achieve those objectives, the best suited methods were employed and the best candidate with respect to anticoagulation activity, hemolytic activity, antioxidant activity as well as drug release and degradation studies was selected for the coating of cardiovascular implants.

Chapter 2

LITERATURE REVIEW

2.1 Cardiovascular material and implants

Cardiovascular diseases (CVDs) bring large financial burden and are major reason of deaths all around the world. Traditional medical treatments are not preferred to treat these diseases because they are not effective enough. However, the improvements in the field of blood contacting devices could make them to be used in surgeries for saving the life of patients. FDA has approved millions of implants that are commercially available for the treatment cardiovascular diseases and these implants are being implanted in millions of patients but still there is a need to improve these devices to prevent the risks of cardiovascular diseases and help patients to recover completely (De Mel, Bolvin, Edirisinghe, Hamilton, & Seifalian, 2008).

One example of Cardiovascular implants is stents. Two types of stents are being used in dilating blood vessels: Bare metal stents and Drug eluting stents. Bare metal stents possess limited hemocompatibility due to the release of noxious ions in the biological environment such as Cr ions, Ni ions and Co ions etc. Thus, they induce inflammatory reactions as a result in stent restenosis occurs which becomes the reason of deaths in patients having percutaneous transluminal coronary angioplasty (Garg & Serruys, 2010). Development of stents, loaded with drugs was considered the revolution in stent layout, but clinical studies have proved that their performance is not so well in long term as they could cause late thrombosis because of the drug release from their surface, resulting delay in re-endothelialization (Joner et al., 2006).

Another example of vascular implants is vascular grafts, used for the treatment of blocked arteries by replacing and bypassing them. Vascular grafts with 5mm and smaller diameter induce restenosis and thrombosis because of poor biocompatibility of their surface. Consequently, there is a need to develop grafts that are biocompatible, mechanically favorable with smaller diameter that will help to prevent thrombosis and hyperplasia as both are fatal hazards after implantation of synthesis vascular grafts and cardiovascular stents (De Mel et al., 2008)

Ventricular assist device (VAD) is another traditional example of cardiovascular implant that act as blood pump and support blood circulation. Even though, no cell damage and hyperplasia were reported in clinical studies but as these devices are blood contacting medical devices so the interaction between blood and surface can cause common complications such as infections and thromboembolism. To prevent the risks of blood coagulation and infections, blood contacting devices should be designed with biomaterials having outstanding hemocompatibility (Sin, Kei, & Miao, 2012).

Prosthetic heart valves are commonly used cardiovascular implants, used to treat two types of valvular heart diseases; congenital and acquired. For congenital heart diseases bioprosthetic heart valves are employed and for acquired heart diseases mechanical heart valves are used (Sacks, Merryman, & Schmidt, 2009). Calcification of artificial heart valves(AHV), cracks at the surface of bioprosthetic heart valves (BHV) and blood clotting around mechanical heart valves (MHV) surface have been reported in many studies and patients with heart valves need to take anticoagulant and immunosuppressive drug therapy whole life to prevent blood coagulation and calcification of implants (Zilla, Brink, Human, & Bezuidenhout, 2008). Furthermore, cardiovascular devices including blood support devices, hemodialyzers, oxygenators, catheters

and extracorporeal tubings are also used for the treatment of cardiovascular diseases and these devices also cause similar problems which are reported in multiple studies.

2.2 Biomaterials used on cardiovascular implants

According to reports of market data forecast, in 2021, the estimated global market size for biomaterials is 110.6 billion $US and it has been estimated, it will increase 217.6 billion $US with a CAGR of 14.5% by 2026 because of the need of people for biomaterial based medical products.

Different biomaterials are used for the different biomedical applications depending upon their function and requirement. They are used for the development of implants as well as for their coating purposes. The basic criteria to select biomaterials for implant fabrication are dependent upon applications their application. Corrosion, abrasion, degradation properties of biomaterials, their bioavailability, their physicochemical and mechanical properties are also main considerations for their selection. Commonly used biomaterials for cardiovascular implants include: bio-ceramics, polymers, composites based on polymers and drugs as well as metals and metal alloys (Stanisławska, 2014).

Nowadays, biomaterials are used in biomedical applications; as carrier of drug delivery, as scaffolds in tissue engineering, in development of synthetic blood vessels and artificial heart valves, in pacemaker fabrication and as artificial joints etc. (Kara, 2012) According to the report of research and markets, biomaterials used in cardiovascular applications are dominating the market and it is expected that cardiovascular biomaterials market would rise up in year 2021 to 2031. Major use of biomaterials in cardiovascular applications is due to their biocompatibility. As cardiovascular implants are blood contacting medical devices and have direct contact with blood

cells, endothelial cells, myocardium, and many other cells therefore, their biocompatibility make them to be used widely in cardiovascular applications.

2.2.1 Bio-ceramics

Hemocompatibility of biomaterials play important role for the application of bio-ceramics in implant fabrication. Bio-ceramics are inert, in-organic, non-metallic and have excellent mechanical properties, commonly used for the hard tissues replacements such as joints, bones, and dental implants. Despite these applications, bio-ceramics have been used for cardiovascular applications as a matrix or coating of implants because of its inert nature and anti-thrombogenic properties (Parlak et al., 2019). They are being used in hermetic seals on pacemakers, insulation of radio-ablation catheters, single crystal sapphire leaflets for heart valves and for coating purposes by chemical vapor deposition technique to improve wear properties of implants (Stanisławska, 2014).

2.2.1.1 Al_2O_3 and ZrO_3

In past few year, scientists have investigated some of the bio-ceramic materials to check their hemocompatibility and some studies have reported controversial results of inert oxide bio-ceramics such as Al_2O_3 and ZrO_2 (Fischer, Luk, Oedekoven, Telle, & Mottaghy, 2007). However, some studies have indicated their hemocompatibility for cardiovascular applications(Lim et al., 2014). Hemocompatibility of Al_2O_3 has been reported in various studies.(Dion et al., 1993; Takami et al., 1997; Yuhta et al., 1994). In another study, stents coated with ZrO_2, and Titanium monoxide showed hemocompatibility in *in vitro* and *in vivo* studies. Additionally, coatings of ZrO_2 and titanium monoxide induce low inflammatory responses and exhibit improved endothelialization in comparison with other materials (Mikhalovska et al., 2011)

2.2.1.2 Diamond-like carbon

Another well know example of ceramics is diamond like carbon (DLC) that are used for the coating of medical devices because of their excellent chemical resistance, temperature stability and biocompatibility. Diamond like carbon (DLC) can be alloyed with other biomaterials by Co-deposition method such as titanium (Ti), copper (Cu), vanadium (V). These DLC coatings show high resistance to platelet aggregation and blood clotting (Okrój et al., 2006). Coatings of Diamond-like carbon have been effectively employed for the applications of cardiovascular implants such as artificial heart valves, stents, and scalpels etc (Stanisławska, 2014).

2.2.1.3 LTI carbon

As carbon is an essential bio-ceramic due to its excellent biocompatibility, and inertness. It has been used in biomedical applications widely in different forms and as low temperature isotropic carbon (LTI carbon) it has been used to fabricate synthetic heart valves. In 1969, for the first time LTI carbon was used for the fabrication of synthetic heart valves and currently Silicon low temperature isotropic (LTI) carbon alloy are being used for artificial heart valves synthesis to increase their mechanical strength (Davis, 2003).

2.2.2 Polymers

Polymers are also used for biomedical applications due their minimal density, significant flexibility, sufficient mechanical strength, and biocompatibility. Though, two types of polymeric biomaterials are being used: synthetic and natural. Synthetic polymers include silicon (Si), polyethylene (PE), polypropylene (PP), polytetrafluoroethylene (PTFE), polyurethanes (PU), polymethyl methacrylate (PMMA), and polyamides (PA) etc. Source of natural polymers are living organisms, as they are fundamental elements of their tissues. Natural polymers are mostly

proteins which include collagen, silk, and fibrinogen as well as polysaccharides which are chitin, cellulose, and starch. Synthetic polymer PTFE is used for the fabrication of vascular grafts and PU are used in prosthetic heart valve synthesis .(Helmus, 1991; Smith & Lamprou, 2014)

2.2.3 Metals and metal alloys

Metal and metal alloys are used for biomedical applications from many years as biomaterials which include 316 stainless steels, Cobalt Chromium alloys as well as titanium and titanium alloys. These metal and metal alloys have advantages as well as some disadvantages. They possess high mechanical strength, but they have poor resistance to corrosion. Most commonly used metal for implants is austenitic stainless steels but it has less resistance to corrosion, and can get damage easily when heavy load is applied upon them (Talonen, Hänninen, Nenonen, Pape, & A, 2005).

Currently in most of the applications, 316L stainless steels is used due to its outstanding resistance to corrosion, antioxidative nature and excellent processability but they have some limitations such as their mechanical strength is low as compared to other metals and cause friction. From past few years, problem of poor mechanical strength has drawn attention and scientist are trying to develop approaches to strengthen them. In cardiovascular applications, 316L stainless steels are used for stent fabrication. Nitinol metal is also used for carotid stents fabrication. Titanium and titanium alloys have excellent mechanical strength and relatively have high resistance to corrosion. Moreover, they could facilitate the in-growth of bone and is commonly used biomaterial in commercial implants. For example, Titanium and Titanium alloys are used in artificial bones for the replacement of hard tissues, in joint replacements as well as in dental implants. In cardiovascular application, they are being used widely for prosthetic heart valves synthesis (Elias, Lima, Valiev, & Meyers, 2008). Implants made of Titanium and Titanium alloy can stay within the body more than 25 years (Stanisławska, 2014).

2.2.4 Composites

Composites are the materials in which more than one type of materials are combined and have wide range of applications in the field biomedical. At present many composite materials are used such as polymer- polymer, polymer- metal, ceramic-polymer, ceramic- metal etc. As composite, mechanical strength of biomaterials improves and they become more resistant to high temperatures (Blazewicz, 2001). Furthermore, carbon fiber reinforced in polymeric matrix are also being used as composite in bone fixation application as a screw. As they possess good mechanical strength, they are also used in the synthesis of prosthetic heart valves (Nowacki, Dobrzański, & Gustavo, 2012). Composite of metal with ceramics are used for implant fabrication as combination, these two biomaterials increases the strength of ceramic material due to the addition of plastic metal into it (Stanisławska, 2014).

All the biomaterials used in cardiovascular applications including metals and metal alloys, bio-ceramics, polymers, and composites do not show significant biocompatibility and hemocompatibility which is essential for their practical applications. Therefore, to prevent the undesirable interaction between implants and tissues, their surfaces are modified with different biomolecules to alter their surface properties (Qi, Maitz, Huang, & Technology, 2013).

2.3 Problems of cardiovascular implants

After implantation of medical device into the host, blood/foreign material and tissue/foreign material reactions are initiated due to vascular injuries. Response of host begin immediately after implantation and continue over time based on the structure and chemical properties of biomaterials. After implantation, adsorption of proteins onto biomaterial's surface leads to cell

adhesion and due to the presence of microbes, they can lead to infections and sepsis problems (I. H. Jaffer & Weitz, 2019; L. Liu, Shi, Yu, Yan, & Luan, 2020).

Titanium and Titanium alloys are most commonly used biomaterials for implants because of their outstanding biocompatibility, mechanical strength, corrosion resistance and reduced ion formation ability. Despite these advantages, they can cause endoluminal injury which lead to overgrowth of smooth muscle cells (SMCs) and induce inflammatory reactions and extracellular matrix (ECM) deposition in lumen of vessels and a major limitation for their long-term success. This occurs in 20-30% patients within 6 months after stent implantation.(Jang et al., 2019)

Excellent mechanical properties are the reasons of implants prevalence, but implants possess some limitations such as corrosion which plays important role on the implant's success. Corrosion of implants release toxic ions into host body which changes the environment around the implants by promoting the overgrowth of endothelial cells which activate the immune cells and consequently give rise the inflammatory response.(Okazaki & Gotoh, 2008; Sojitra et al., 2010). Corrosion of implants surface also play important role in its functionality. Fractures in the stent due to corrosion have also been detected in 1-3% of patients after stent implantation (Scheinert et al., 2005) and fractured stents have also been associated with platelet activation ,stent thrombosis and neointimal growth due to overgrowth of proinflammatory factors (Chakravarty et al., 2010). Implantation of medical devices illicit a foreign body response that is dependent upon the surface topography and chemistry of biomaterial. Thus, implant design is important factor for their successful application. For example, the sharp edges promote greater inflammatory responses which as a result induce a place for the bacterial infections (Rochford et al., 2012).

2.3.1 Thrombosis (blood coagulation)

As we know that vascular implants are blood contacting medical devices so when implants come in-contact with blood they induce thrombosis (blood coagulation) by forming obstructive thrombus and in turn result in thrombus embolization induced stroke and lead to device failure.(Hilal, Mudd, DeLoughery, thrombosis, & haemostasis, 2019).

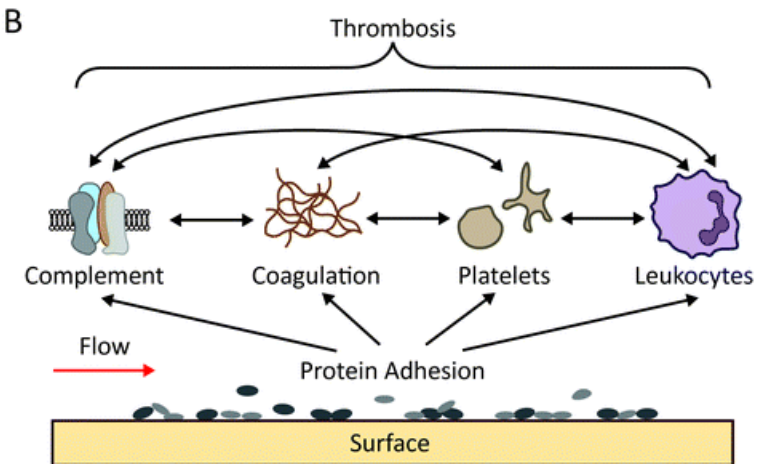


Figure 2.1 : Illustration of vascular implant induced thrombosis adapted from ref.(Hong et al., 2020)

When implants come in contact with blood, plasma proteins adsorb to the implant surface which subsequently activate clotting factors that cause aggregation of platelets in blood, white blood cell and cascade proteins which in turn results thrombosis.

2.3.3 Coagulation Cascade

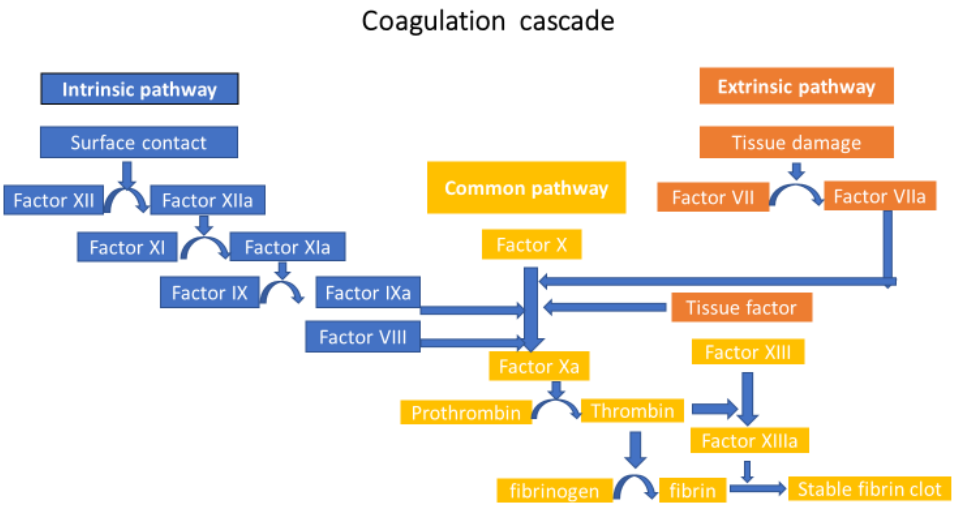


Figure 2.2: Illustrates of coagulation pathway

There are two ways by which coagulation process is initiated; intrinsic and extrinsic pathways. Intrinsic pathway is initiated when blood comes in contact with implants it activates plasma protein called factor XII which then activate factor XI. Activation of factor XI initiate series of proteolytic reactions and cause activation of factor IX and factor VIII which subsequently activate factor X. Activated factor X then convert prothrombin into thrombin which then convert fibrinogen into fibrin. Thrombin not only activate fibrinogen but also promote platelet aggregation thus create a platelet-fibrin thrombus (blood clots) that thrombus leads to device failure (I. Jaffer, Fredenburgh, Hirsh, Weitz, & Haemostasis, 2015).

Extrinsic pathway is initiated by tissue factors activation. When endothelial cells are damaged by injury or implantation, tissue factor VII activate. Activated factor VII facilitate the activation of factor X. Alternatively, factor VII can activate the factor IX which in turn can activate the factor

X. Once the factor X is activated, it proceeds to activate prothrombin into thrombin. Thrombin then convert fibrinogen into fibrin(Chaudhry, Usama, & Babiker, 2018).

2.3.3 Anticoagulant Coatings

Over the last few decades, to prevent blood clotting heparin is used as it has outstanding anticoagulation properties. For the coating of implants, heparin either implanted into the polymeric matrix or it is ionically bind with the surface of polymeric films as it can reduce 70% thrombus formation. But heparin has some limitations, as it is an anticoagulant drug, therefore, it thins the blood and increases the risk of bleeding. Another limitation of heparin is heparin induced thrombocytopenia in which platelet count in blood becomes very low and this increases the risk of thrombosis around implants.(Ramkumar et al., 2018; Wang et al., 2018)

Due to these problems of heparin, other biomolecules, polymers, and coatings have begun to be explored. Synthetic polymers containing abundant amount of carboxyl group and sulfur containing functional groups can be used as anticoagulant coatings because heparin contains these functions which are responsible for its anticoagulation property. In one study heparin mimicking coating was fabricating using MAA and EGDA and it is found to have low platelet aggregation and has increased the blood clotting time (Wang et al., 2018).

Another polymer called polyurethanes exhibit anticoagulation activity when modified it with methacryloyloxyethyl phosphorylcholine and it has showed reduced platelet adhesion even after repeating test multiple times, but their mechanism of anticoagulation activity was unidentified(Chi et al., 2018). Along with polymeric coatings, non-polymeric coatings are also being for anticoagulation(X. Li et al., 2018) A combination of hyaluronic acid and polydopamine have showed excellent anticoagulation and anti-inflammatory activities but this coating also has some

limitations such as nitrous oxide are responsible for its anticoagulation activity, but it does not stay for long period of time(Wu et al., 2016). Recently, another non-polymeric coating was explored which exhibited excellent anticoagulation activity. For this coating, ultra-nanocrystalline diamonds were used which are apparently very difficult to synthesize. The only limitation of this type of coating this the fabrication as they are very difficult to synthesize. They have smooth surface and are very cost-effective and when results of anticoagulation activity of diamond coating were compared with pyrolytic carbon and they both exhibited same results, but diamond coating exhibited better mechanical strength than pyrolytic carbon(Zeng, Jarvik, et al., 2016; Zeng, Yin, et al., 2016).

Coatings	Anti-coagulation mechanism	Pros	Limitations	Ref.
Copper bearing coating	Copper	Anti-coagulation, anti-infection properties	Not as effective as heparin	(Wang et al., 2020)
Heparin coatings	Heparin	Very effective	Chances of developing HIT High cost	(Le et al., 2019)
Metal phenolic /catecholamine coating	Nitrous oxide	Anti-coagulant, anti-inflammatory and anti-bacterial properties	Nitrous oxide does not stay for long time	(Li et al., 2018)
Polyurethane films	Surface properties	Very effective, highly modifiable	Not enough research for this type yet	(Chi et al., 2018)
Artificial endothelium	Nitrous oxide	Very Effective	Nitrous oxide does not stay for long time	(Amoako et al., 2016)
Heparin/ graphene coating	Heparin	Effectiveness of heparin and anti-bacterial properties of graphene	Becomes cytotoxic at 4%wt of graphene.	(Pan et al., 2016)

Table 2.1: Already available anticoagulant coatings of biomedical implants

2.4 Polylactic acid (PLA)

A Swedish chemist named Scheele first time discovered PLA in 1700 and for the first time, in medical applications, it was used for the repairment of mandibular fractures in dogs (Tan, Yu, Wan, Yang, & Technology, 2013). PLA is basically a thermoplastic, biodegradable, inexpensive and stable polymer derived lactic acid by fermentation process of corns and cereals having low melting temperature in the range of 180-220^{o}C(P Pawar, U Tekale, U Shisodia, T Totre, & J Domb, 2014).It has been widely used for biomedical applications such as tissue engineering scaffolds, drug delivery carriers, implants coatings and various bioresorbable medical implants because of its biocompatible nature, ability to biodegrade, significant mechanical strength and easy processability. PLA was first approved by FDA for blood contacting medical devices in 1970 (MS Singhvi, Zinjarde, & Gokhale, 2019).

2.4.1 Market size of PLA

According to marketsandmarkets report, the estimated market size of lactic acid (LA) in 2020 was 1.1 billion USD and by 2025 it would reach to 2.1 billion USD with CAGR of 12.8%. The annual PLA (polylactic acid) market size was 786 million in 2020 and is expected to reach 1756 million USD by 2025 with CAGR of 17.4% due to significant increase in the use of lactic acid in biodegradable polymers for food and biomedical applications as it is environmentally friendly, easily processible, compostable and non- toxic.

2.4.2 PLA synthesis

Synthesis of PLA occurs in three steps; first lactic acid is synthesized and then polymerization of lactic acid form lactides, after that condensation polymerization of lactic acid form LMWPLA or

ring opening polymerization form HMWPLA (Mamata Singhvi, Zendo, Sonomoto, & biotechnology, 2018).

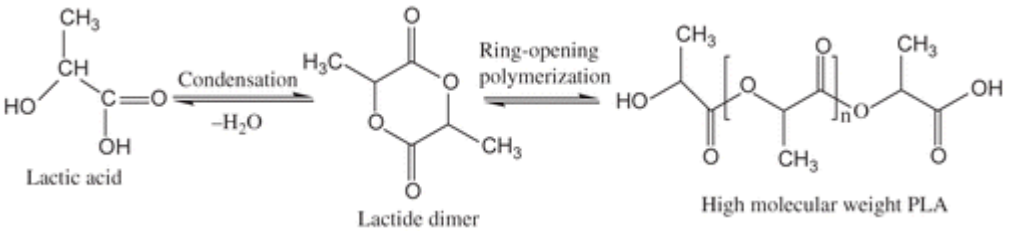


Figure 2.3: Polymerization of lactic acid. Adapted from ref.(Ahmed & Varshney, 2011)

2.4.3 Degradation of PLA

In different environmental conditions, PLA may result in undesired degradation that leads to their property loss. PLA degrade naturally by moisture, oxygen, and microorganisms by hydrolysis of ester bonds and PLA degrade into water, CO_2, and some non-toxic byproducts. Degradation of PLA is influenced by variety of factors including temperature, hydrolysis, pH moisture content, shape, and size of device etc.

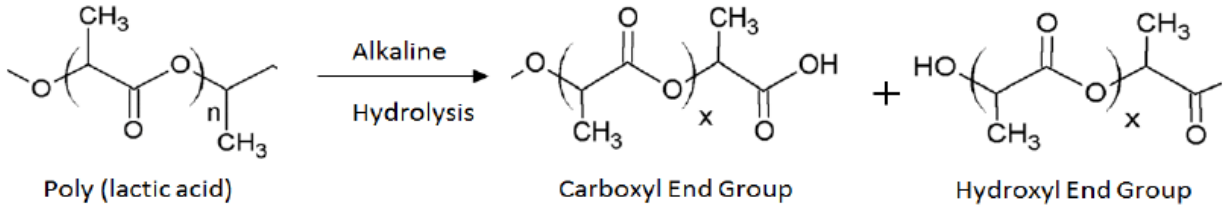


Figure2.4: Hydrolysis of PLA

Hydrolysis of ester bond of PLA cleaves the polymer backbone chain and lessens the molecular weight of PLA, subsequently results in the formation of soluble oligomer and monomer molecules

(Castro-Aguirre, Iniguez-Franco, Samsudin, Fang, & Auras, 2016). Hydrolysis of backbone chain reduces the pH and hydrogen ion concentration that speeds up the reaction. In addition, hydrolysis mostly occurs at the amorphous region of PLA and subsequently increases its crystallinity(Valentina, Haroutioun, Fabrice, Vincent, & Roberto, 2018).

There are four common parameters that control the hydrolytic degradation of PLA such as absorption of H_2O, rate of diffusion, solubility of degradation byproducts and fragments of PLA chain and two degradation mechanisms are involved in PLA degradation such as surface erosion also known as heterogenous degradation and bulk erosion or homogenous degradation. Surface erosion occurs when the hydrolysis rate is higher than the water absorption rate within PLA matrix as a result shrinkage of PLA occurs while, for bulk erosion the shape of PLA remains same, but weight decreases over time (Casalini, Rossi, Castrovinci, Perale, & biotechnology, 2019).

2.4.4 Drug release of PLA

Polymers used as drug delivery carriers, are usually biodegradable that eradicates the need of surgical procedures for the removal of implants after their need and biocompatibility of such polymers should be considered carefully in order to avoid complications such as toxicity and inflammation. PLA has physicochemical properties that help PLA to retain drug within matrix for long period of time (Harting, Johnston, & Petersen, 2019).

Different types of drug release mechanisms are reported in multiple studies and based on these mechanisms, polymers control the release of drug entrapped into their matrices by diffusion mechanism, degradation, or erosion of PLA, swelling or water uptake etc. Diffusion of drug particles occur when drug loaded films are submerged into the aqueous solution and the entrapped drug particles release form the matrix due to penetration of release medium into the center of the

films this subsequently result in the cleavage of polymer chain and sustained release of drug (Harting et al., 2019; Sonawane, More, Pandey, Patil, & Deshmukh, 2017).

2.5 Biomedical applications of PLA

Patients who are suffering for coronary artery diseases, they need implants to bypass and repair blockages. Implants can cause complication such as infections and toxicity which lead to implant failure, but implants made of PLA have suitable mechanical properties and its biodegradability make it promising for biomedical applications. *In vitro* Studies have reported synthetic PLA grafts reduces 0.77% weight after 25 days and 1.93% weight in 50 days. Biocompatibility is required for arterial grafting and multiple studies showed, PLA grafts exhibit endothelial cell viability which confirms their biocompatibility (Kabirian, Ditkowski, Zamanian, Heying, & Mozafari, 2018)

PLA is also being used for drug delivery systems and multiple studies have showed that drug loaded in PLA have improved their bioavailability. PLA and tamoxifen composite showed improve anti-tumor activity (Maji, Dey, Satapathy, Mukherjee, & Mondal, 2014). Similar findings were observed in the delivery of Docetaxel loaded on PLA as they had greater drug at targeted tumor site (Hrkach et al., 2012).PLA is also being used widely in implant coatings as with controlled degradation rate of PLA anti-biotics can be delivered at the sites of implantation to limit the risks of infections after implantations and implant failure can be avoided efficiently (P Pawar et al., 2014).

2.6 Anti-coagulant drugs

According to World health organization (WHO), an estimated 80% of the total population use herbal medicines to the cure diseases. Due to chemical diversity of herbal plants, they are considered important source of medicines from which therapeutic drugs can be obtained. Drugs

obtained from herbal plants are safe to use and inexpensive in comparison with synthetic drugs that have side effects as well as have wide therapeutic window. Herbal plants have antioxidant, antimicrobial and anticoagulant activities. Therefore, herbal drugs can be alternative of synthetic anticoagulant drugs to overcome their limitations such as bleeding problems of warfarin and heparin that have been reported in many studies (Sasidharan et al., 2011).

Cinnamon Cassia

Panax Ginseng

Ocimum Tenuiflorum

2.6.1 Cinnamon Cassia

Cinnamon cassia belongs to genus Cinnamomum, and family Lauraceace commonly known as Cassia. It is commonly found in Asia and found in China, Laos and Vietnam, consist of more than 250 aromatic compounds and have been used for the aromatic spices worldwide. Cinnamon Cassia is employed as herbal medicine for the treatment of various diseases including Bronchitis, inflammation, fever, and improve circulation of blood. It comprises cinnamaldehyde, coumarin, polyphenol, cinnamic acid and diterpenoids which are responsible for its antifungal activities(Singh et al., 1995), antioxidant activities(Yang, Li, & Chuang, 2012) and anti-microbial activities. essential oil of cinnamon and an important component of oil cinnamaldehyde are responsible for anti-platelet aggregation activity. Ethanolic extract of Cinnamon Cassia has anti-

platelet and anticoagulation activity and been reported in multiple studies (Kim et al., 2010)

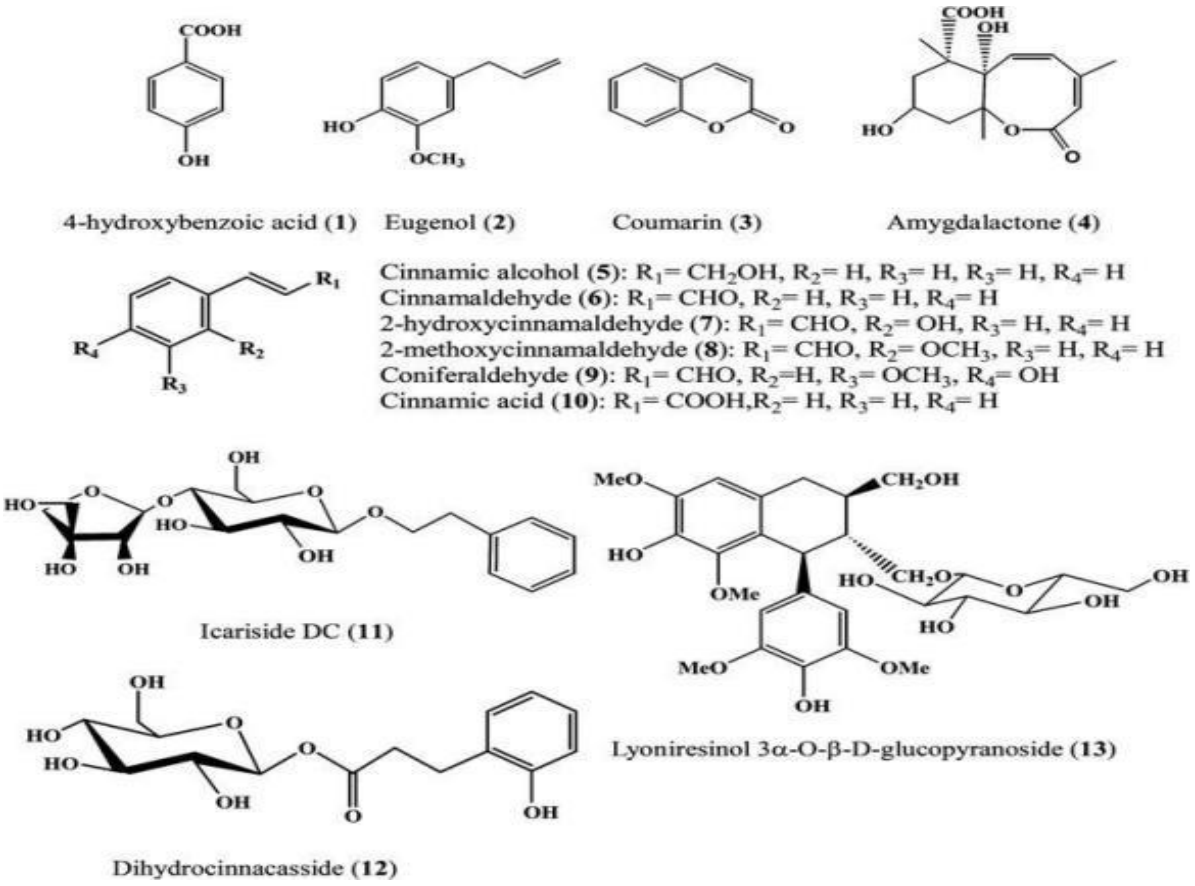


Figure 2.5: Structure of chemical constituents of Cinnamon Cassia. Adapted from ref.(Kim et al., 2010)

2.6.2 Ocimum tenuiflorum (Tulsi)

Ocimum tenuiflorum belongs to the genus Ocimum and the family is Lamiaceae, commonly known as Holy basil and Tulsi plant. It is mostly found in sub-continent region (Triveni et al., 2013) and has been widely used to cure various diseases including asthma, bronchitis, flu, malaria, cough, sore throat ulcers, and asthma (Jung, Je, Kim, & Kim, 2002).It has also been used for wound healing, as anti-coagulant drug, anticancer, antioxidant as well as anti-inflammatory drug. Aqueous extract of ocimum tenuiflorum has anticoagulant activity that is reported in literature and there was no toxicity observed in human blood plasma in in-vitro studies(Gunendren et al., 2017).

2.6.3 Panax Ginseng

Panax ginseng belong to family Araliaceae and used as herbal medicine worldwide. Panax ginseng also called blood medicine as it helps to stop bleeding, reduces inflammation or act as anti-inflammatory drug as well as relieves pain(C. T. Li, Wang, & Xu, 2013)

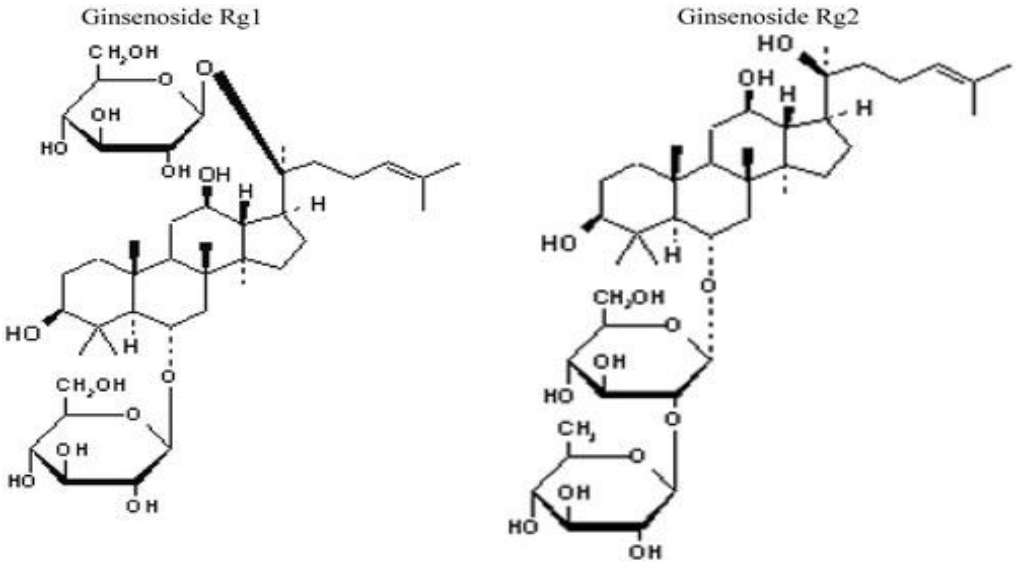


Figure2.6: Chemical structure of ginsenosides (Rg1 and Rg2), active constituents of panax ginseng which act as anticoagulants

Panax ginseng is widely used in Asia, mainly in China not as a medicine but people of those areas use it as tea and in wine making. In 2018, estimated marketed production value was 55.2 billion in China and total production was more than 3800 tons and it was expected that production value of ginseng will reach till 80 billion by 2020, due to its high demand for medicinal purposes (Al-Ani, 2019; H. Liu et al., 2020). Antiplatelet activity and anticoagulation activity of panax ginseng has been reported in multiple studies (Lau et al., 2009). Thus, it could be used as anticoagulant drug for the treatment of blood clotting.

Drugs	Chemical constituents	Ref.
Cinnamon Cassia	Consist of 10 phenolic compounds—4-hydroxybenzoic acid, eugenol, cinnamic alcohol, cinnamaldehyde , 2-hydroxycinnamaldehyde, 2-methoxycinnamaldehyde, **coniferaldehyde**, cinnamic acid, **dihydrocinnacasside**, and lyoniresinol 3α-*O*-β-D-glucopyranoside—along with **coumarin**, **Icariside DC**, amygdalactone	(Kim et al., 2010)
Panax Ginseng	**Ginsenosides(Rg1 and Rg2),** polysaccharides, amino acids, volatile oils, and polyacetylenes	(C. T. Li et al., 2013) (H. Liu et al., 2020)
Tulsi	**Fixed oil**(Linoleic acid, Linolenic acid, Oleic acid, Palmitric acid, Stearic acid) Eugenol, Carvacrol, Linalool, and β-caryophyllene	Panchal P, Parvez N (2019)

Table2.2: Chemical constituents of drugs responsible for their anticoagulation activity

Chapter 3

MATERIALS AND METHODS

3.1 Materials

Poly-lactic acid (PLA), N, N-dimethylformamide (DMF), Herbal anti-coagulant drugs (Panax Ginseng, Oscimum tenuiflorm (Tulsi), Cinnamon Cassia (Cassia)), PBS, DPPH and Ethanol.

3.2 Selection criteria

For film fabrication, PLA was selected as polymeric matrix for drug as it has widely been used in biomedical application, tissue engineering and pharmaceutical applications due to its biocompatibility, biodegradability. DMF was selected as solvent due to excellent solubility of PLA in it. As purpose of our study was to develop anti-coagulant coating so three herbal drugs were used in this study based on their reported anti-coagulant, antioxidant properties.

3.3 Optimization of solvent and PLA concentration

PLA concentration was optimized by using different concentrations in different solvents. For this purpose, 1%,2%,3%,4% PLA concentrations were dissolved in different solvents separately such as Dichloromethane, Chloroform, Acetone and Dimethylformamide. At these concentrations of PLA, films were not peeled off from the petri plate as the films were too thin. Then PLA concentration was increased to 5% and again dissolved it in Chloroform, Acetone, Dichloromethane (DCM) and Dimethylformamide (DMF). It was observed, dissolution of PLA in Chloroform, Acetone, Dichloromethane (DCM) was slow as compared to DMF and the films were brittle and had rough surface with visible pores. The films of PLA/DMF were flexible, had smooth surface with less visible pores. Based on these observation, Dimethylformamide (DMF) was used further for film fabrication in this study.

Optimization of solvent and PLA conc.		
Polymer Conc.	**Solvents**	**Outcome**
1,2,3 and 4% PLA	Chloroform, Dichloromethane, Acetone and DMF	Unable to peel them off from petri plate
5% PLA	Chloroform	• Dissolution was slow • Brittle • Rough surface with visible pores
	Dichloromethane	
	Acetone	
	DMF	• Fast dissolution • Flexible film • Smooth surface with less visible pores

3.4 Film fabrication

To fabricate PLA thin film using solvent casting method,(Byun et al., 2012)first 5% PLA was mixed in 35ml DMF at 65^{o}C for 45mins under constant stirring at hotplate to get a clear and uniform PLA/DMF solution(Xu, Wang, Gao, & Wang, 2019). After dissolving PLA, the homogenous solution was poured into petri plate and kept in oven at 80 ^{o} C for 90mins to get thin film by evaporating solvent. After evaporation of solvent the film was peeled off from petri dish.

Figure 3.1: Pure PLA film

3.5 Extract preparation

Extracts of drugs were prepared by following the methods reported in literature. For Cinnamon Cassia extract preparation, we used the method reported by (Gupta et al., 2008). Panax ginseng extract was prepared using method of (C. T. Li et al., 2013) and for Ocimum Tenuiflorm (tulsi),we followed the method reported by (Gunendren et al., 2017)

3.5.1 Extraction of Cinnamon Cassia (Cassia)

Cinnamon cassia bark was grounded using pestle mortar in order to obtain a fine powder. Cinnamon powder was weighed using weighing balance and (1g powder in 5ml solvent) soaked in 5% ethanol solution in media bottle for 48 hours at room temperature with frequent shaking. After 48hrs, mixture was centrifuged at 3500rpm for 20mins and then filtered through whatmann filter paper no.1. Then filtrate was evaporated using rotary evaporated under reduced pressure until a semi solid substance is obtained. Extract was then stored at 4^{o}c for further use.

3.5.2 Extraction of Panax ginseng

Panax ginseng roots were grounded using pestle mortar to get a powder. Powdered roots were extracted by boiling them in distilled for 30 mins. Extracts were then centrifuged at 3500rpm for 20 mins and supernatant was collected. Residues were re-extracted by boiling them again with distilled water and then combined all the extracts and rotary evaporated to get concentrated material.

3.5.3 Extraction of Oscimum Tenuiflorm (Tulsi)

For the extract preparation of oscimum tenuiflorm, first Tulsi leaves were grounded into fine powder using grinder. Finely grounded powder was then mixed with distilled water and boiled for

30 mins. Extract was then filtered through Whatmann filter paper no.1. and filtrate was then stored in refrigerator for further use.

3.6 Optimization of drug concentration for drug loaded film fabrications

As PLA has not been used with these drugs previously so, there was a need to optimize the drug concentration in PLA films. For optimization purpose, 5%, 10%, and 15% concentration of drugs were added into homogenous PLA/DMF solution on hot plate and dissolved in this solution for 20 mins using magnetic stir. Rest followed the same method that was used for plain PLA film fabrication. Same temperature and time were used for solvent evaporation in drying oven that were used for pure PLA film fabrication. Results showed that at 10% and 15% concentrations of drugs could not get integrated well with PLA and we could not be able to get films at 10% and 15% concentrations. Based on this observation, in this study 5% and below 5% concentration of drugs were used for drug loaded films fabrications.

Figure 3.2: 10% P. Ginsneg

Figure 3.3 : 10% P.Ginseng

Figure 3.4: 10% Tulsi

Figure 3.5: 7% P. ginseng

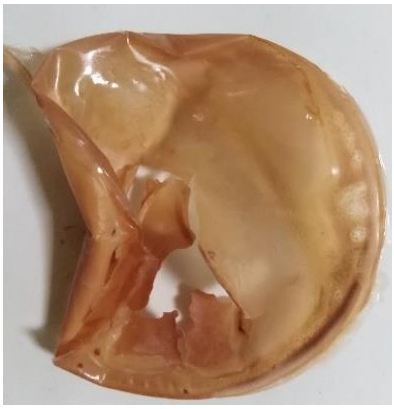


Figure 3.6: 10% C.Cassia

3.6.1 Drug loaded films fabrications

For the fabrication of drug loaded films, we used 1%, 2.5%, 5% drugs concentrations and kept PLA concentration constant that was 5%. All Drugs were dissolved in PLA solution separately under constant stirring and homogenous solution of PLA with drug was poured on petri plate and dried in heating oven at 70-80^{o}C for 90mins.For composite preparation, lowest concentration (1%) of all drugs were added into PLA solution. For heparin loaded film, 1% of heparin was added into PLA solution. Heparin loaded film in this study was used a positive control for comparative study.

Figure 3.7: 1X1cm size 1%,2.5% and 5% Oscimum Tenuiflorum films

Figure 3.8: 1x1cm size 1%,2.5%and 5% Panax Ginseng loaded films

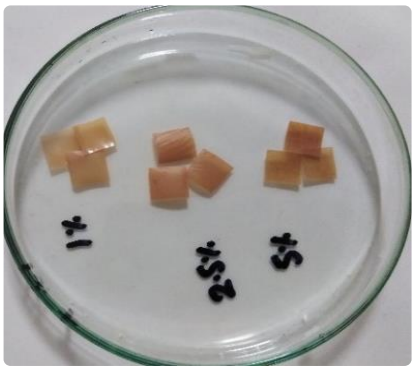


Figure 3.9: 1x1cm size 1%,2.5%,5% Cinnamon Cassia loaded films

Figure 3.10: 1x1cm size 1% drug composite film

3.7 Compositions

Compositions	Cinnamon Cassia			Oscimum tenuiflorm			Panax Ginseng			Heparin (Control)	PLA
	1%	2.5%	5%	1%	2.5%	5%	1%	2.5%	5%	1%	
C1	✓	-	-	-	-	-	-	-	-	-	5%
C2.5	-	✓	-	-	-	-	-	-	-	-	
C5	-	-	✓	-	-	-	-	-	-	-	
T1	-	-	-	✓	-	-	-	-	-	-	
T2.5	-	-	-	-	✓	-	-	-	-	-	
T5	-	-	-	-	-	✓	-	-	-	-	
G5	-	-	-	-	-	-	✓	-	-	-	
G10	-	-	-	-	-	-	-	✓	-	-	
G15	-	-	-	-	-	-	-	-	✓	-	
Composite	✓	-	-	✓	-	-	✓	-	-	-	
Heparin	-	-	-	-	-	-	-	-	-	✓	

Table 3.1: Illustrates the compositions of drug loaded films

3.8 Characterization

To characterize drug loaded films, three characterization approaches were used.

- **SEM**
- **EDX**
- **FTIR**

3.8.1 Scanning electron microscopy (SEM) analysis

Surface morphology and dispersion of drugs on film surface were evaluated using JSM-6490A analytic scanning electron microscope (SEM), operated at 10kv acceleration voltage. Samples were sputtered with Au prior to imaging using sputter coater. Images were taken at magnification x200micron (Arbeiter et al., 2021)

3.8.2 Fourier transform infrared spectrophotometer (FTIR) analysis

Elemental composition of drug loaded PLA films and pure PLA films as well as interaction of drugs with PLA were measured at room temperature using Fourier transform infrared spectrophotometer (PerkinElmer; spectrum 100 FTIR spectrophotometer) at wavenumber range 400 - 4000cm^{-1} (X. Ma, Wang, Wang, & Xu, 2017).

3.8.3 Energy dispersive X-rays (EDX) analysis

Elemental concentration and distribution on the film was determined by energy dispersive X-rays analysis (EDX) at randomly selected areas of film(H. Li, Wang, Zhang, & Pan, 2018).

3.9 Anti-coagulation studies

- Prothrombin time test (PT Test)
- Activated-partial thromboplastin time test (a-PTT Test)

3.9.1 PT Test

In vitro anticoagulation activity of drug loaded films, heparin loaded films and pure PLA films was examined using pro-thrombin time test (PT) that indicate the bioactivity of extrinsic blood coagulation factors. For PT test, all drug loaded films, pure PLA films and Heparin loaded films were cut into 1x1 cm size. Fresh human blood was centrifuged at 1500rpm for 15mins, and plasma was separated from RBCs and RBCs were discarded. 1x1 size sample films were submerged into the separated plasma and then PT reagent (Neo plastin-Cl plus) was added into tubes containing sample films and incubated for 3mins and clotting time was measured using Coagulation analyzer from Behnk Elektroik GmbH and Co. Germany and this test is performed using the method reported by Boonkong et al in their study. (Boonkong, Petsom, & Thongchul, 2013).

3.9.2 a-PTT Test

a-PTT test is more sensitive than PT, in vitro anticoagulation activity of individual drugs was cross checked by a-PTT test. a-PTT test shows the bioactivity of intrinsic blood coagulation factors. This test was performed by following the method reported by (Allu et al., 2014). For a-PTT test, three compositions of each drug loaded films were cut into 1x1cm size and immersed in human blood plasma isolated from fresh human blood by centrifuge it at 1500rpm for 15 mins. a-PTT reagent was added into the tube containing sample films with plasma and incubated at room temperature for 3 mins and then clotting time was measured using Coagulation analyzer from Behnk Elektroik GmbH and Co. Germany(Yakub et al., 2014).

3.10 *In-vitro* Studies

- Drug release studies
- Degradation studies
- Hemolysis studies
- Antioxidant studies

3.10.1 Drug release studies

In-vitro drug release profile was evaluated in order to determine the amount of drug being released into the release medium from polymeric matrix as well as to compare the percentage (%) drug release of drug composite film with Heparin loaded film. Drug release from individual drug loaded films, drug composite and heparin loaded films were measured using UV-spectrophotometer by taking absorbance values at specific wavelengths. The absorbance wavelengths selected were 320nm,286nm, 544nm and 631nm for Ocimum tenuiflorum, Cinnamon Cassia and Panax ginseng and heparin, respectively. For the release studies, each drug loaded film was cut into 1x1 cm size and immersed into 3ml PBS solution in glass vials and after incubating for specific time intervals, complete PBS solution was withdrawn to analyze the amount of drug released by checking the absorption of each sample through UV- spectrophotometry and refilled the glass vials with fresh PBS solution.(Allu et al., 2014; Puttipipatkhachorn, Nunthanid, Yamamoto, & Peck, 2001)

3.10.2 Degradation studies

In- vitro degradation of drug loaded PLA films is the weight loss of film over time at room temperature. For degradation studies, each drug loaded film was cut into 1x1 cm size and weighed the films (w_o) After weighing them, placed those films into 5ml of PBS solution in glass vials. At selected time intervals, removed the film from PBS solution and dried them and weighed them(w_t).

Percentage degradation at each interval was determined by using a formula written below.(GÜMÜŞDERELİOĞLU & Deniz, 1999; Yu, Cui, Wang, Yang, & Li, 2020)

Degradation rate (%) = [W_O-W_t / W_O] x 100

3.10.3 Hemolysis test

Hemocompatibility of drug loaded PLA films, pure PLA and heparin loaded films (positive control) was checked by hemolysis test. As these drugs loaded films are fabricated to coat biomedical implants, they should have hemocompatibility due to its direct contact with body fluids and blood regularly.

For Hemolysis test, fresh human blood was taken in blood bag having anti-coagulant drugs. To isolate red blood cells (RBCs), blood was centrifuged with PBS at 5000rpm for 10mins. RBCs were washed three time with PBS and then isolated RBCs were diluted with PBS solution for the immersion of drug loaded films. In this procedure, TritonX-100 and PBS solution were employed as positive and negative control. In diluted RBCs drug loaded films were immersed and kept at room temperature for 2hrs and then centrifuged for 5 mins at 5000rpm.RBCs were discarded, and supernatant was collected for the measurement of absorbance through UV spectrophotometry at 540nm and then percentage hemolysis of each sample measured using the formula written below. Where, A_s is the absorbance of sample films and A_n is the absorbance of Negative control and A_p is the absorbance of positive control (Yu et al., 2020).

Hemolysis (%) = [(A_s – A_n)/ (A_p)- (A_n)] x 100

3.10.4 Antioxidant studies

As novel coating for vascular implants, this should have a significant antioxidant activity. Across implants, there is a considerable oxidative stress due to the presence of reactive oxygen species (ROS) induced by blood clotting after injury or injury caused by implantation. Therefore, coating with antioxidant activity on implant's surface would be significantly favorable for success of implant.

Antioxidant activity of drug composite, pure PLA and heparin loaded film was determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. Film samples were cut into 1x1cm size and submerged into ethanolic solution of DPPH and kept at room temperature in dark for 1hr. Absorbance was measured using UV spectrophotometry at 517nm. Positive control and reference for this study were ascorbic acid and ethanol solution. Antioxidant activity of films was represented in percentage antioxidant activity and following equation was used to measure antioxidant activity. Where Ao is absorbance of control and As is absorbance of sample films (Q. Ma, Ren, & Wang, 2017)

Percentage antioxidant activity = [(Ao – As) / Ao] x100

Chapter 4

RESULTS AND DISCUSSION

4.1 Scanning Electron microscopy

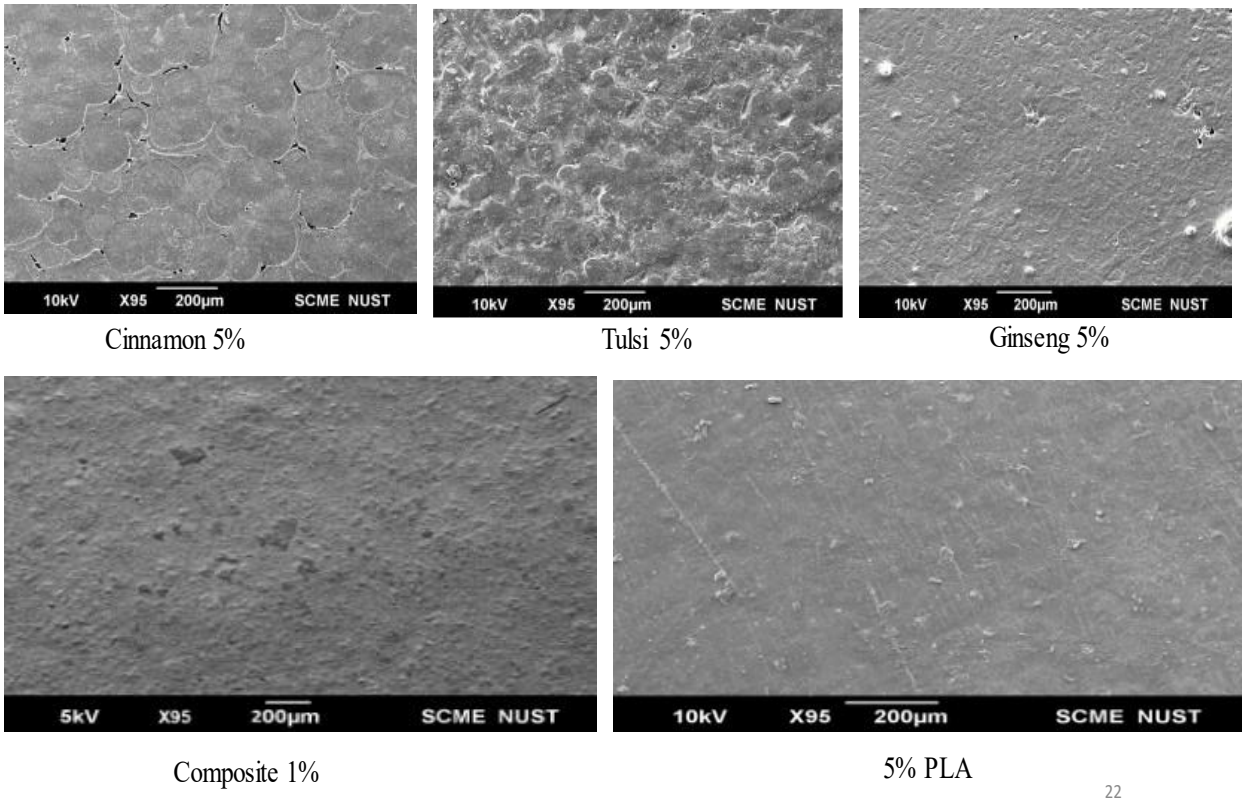


Figure 4.1: SEM micrographs of pure PLA film and different drug loaded films

SEM images showed that, the surface of pure PLA showed smooth, continuous, and homogenous microstructure without grainy and porous structure. Drug loaded films had quite rough surfaces as compared to pure PLA film. Though, pores, cavities and small particles were observed in drug loaded films due to the dispersion of the drug into the polymeric matrices and presence of high concentration of drug leads to increase in their roughness because of the essential oils present in the drug extracts which have extended over the surface of polymeric matrices and as a result reduced their regularities. SEM images of Cinnamon Cassia (Cassia) and Panax ginseng also

exhibited microcrack because of the brittleness of the film. Generally, surface roughness increases with drug incorporation in drug loaded films due unequal distribution of hydrophobic molecules during process of their formation that cause agglomeration of drug particles.

4.2 EDX analysis

Energy dispersive X-ray microscopy was used to determine the elemental concentration and distribution on the film surface. It was evident from the table that pure PLA film and drug loaded films had same elemental compositions and no additional elements were observed in the polymeric films after drug loading.

FILMS	C (Weight %)	O (Weight%)
PLA	56.5	43.5
Composite	51.4	48.6
Cinnamon Cassia	53.4	46.6
Panax Ginseng	49.6	50.4
Tulsi	53.7	46.3

Table4.1: Illustrates the percentage weight of elemental composition in Pure PLA Films and Drug loaded PLA films

Table of EDX results showed that percentage weight of carbon in PLA film was more than drug loaded films. However, with drug loading the elemental composition remained same but percentage weight of oxygen increased, and percentage weight of carbon decreased in drug loaded films which confirmed the uniform distribution of drug particles on PLA matrix.

4.3 FTIR

The interaction of drugs with PLA was examined by FTIR analysis. All the spectra displayed peaks at 2882cm^{-1}, 2947cm^{-1}, 2997cm^{-1} and 1754cm^{-1} corresponding to CH_3 and -C=O-stretching, 1381 is representing CH, 1266 is C=O,1180 is -C-O-C ,1127,1080 are representing -C-O-H and 1043 is -C-C-. It can be observed from the spectra, with drugs in PLA there is no significant change in peaks positions which indicated the presence of chemical interactions and compatibility between drugs and polymer. However, Changes in peak intensities were observed. All drug loaded compositions had decreased peaks intensity corresponding to C=O stretching at 1754 cm^{-1} as compared to pure PLA films. Peaks at 2882,2947 and 2997 were more noticeable in neat PLA whereas in drug loaded PLA peak intensity had decreased. In addition, the disappearance of stretching vibration at 1720cm^{-1} which may also indicate the possible interaction between drugs and PLA. This interaction might be the hydrogen-bonding between OH group of drug and CH of PLA. It is generally accepted that there is no interaction between drugs and PLA if the spectra remain unchanged upon addition but a minute change in peak position and change in peak intensity tells the possible interaction between drugs and polymer.

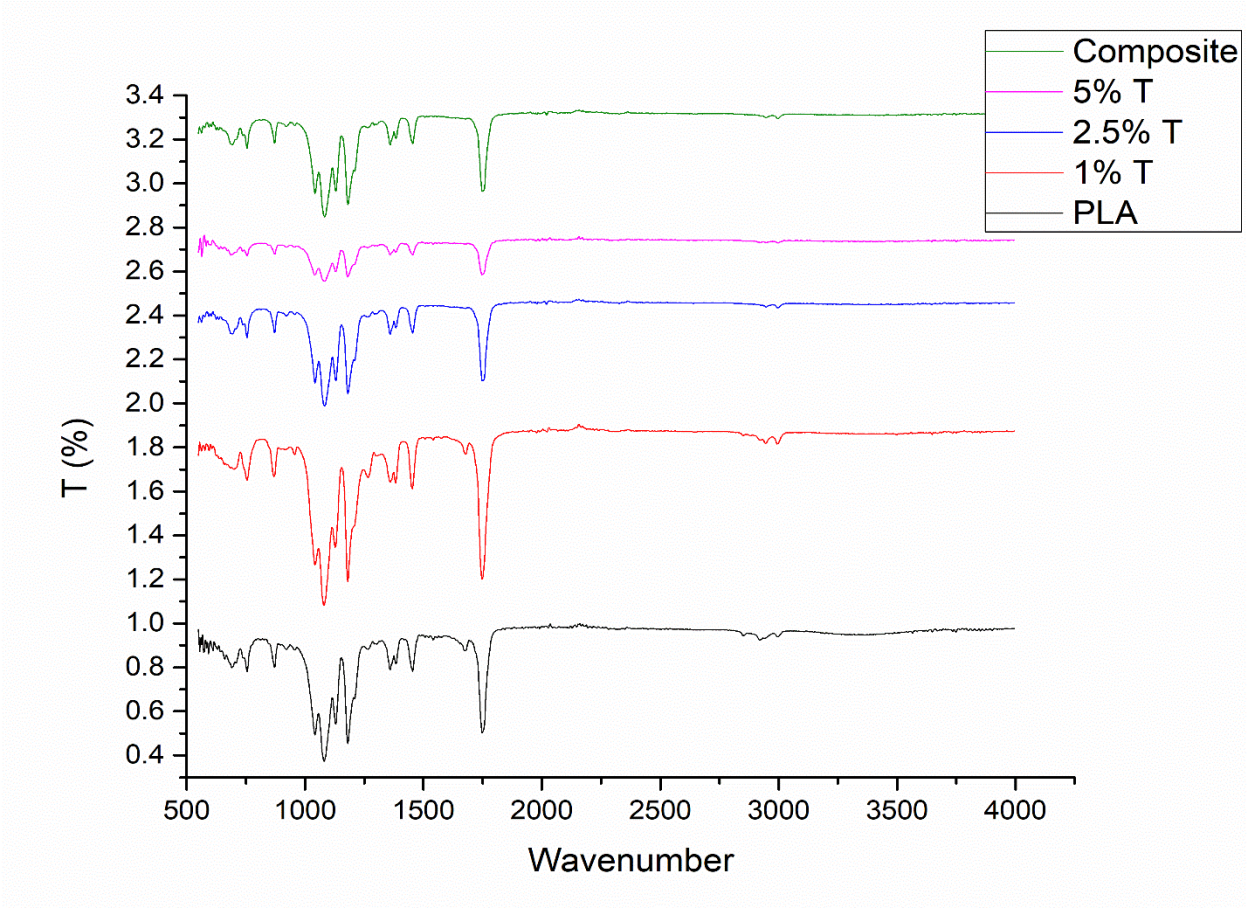


Figure 4.2: Illustrates the FTIR spectrum of all compositions of Oscimum Tenuiflorum (Tulsi) along with PLA and composite films.

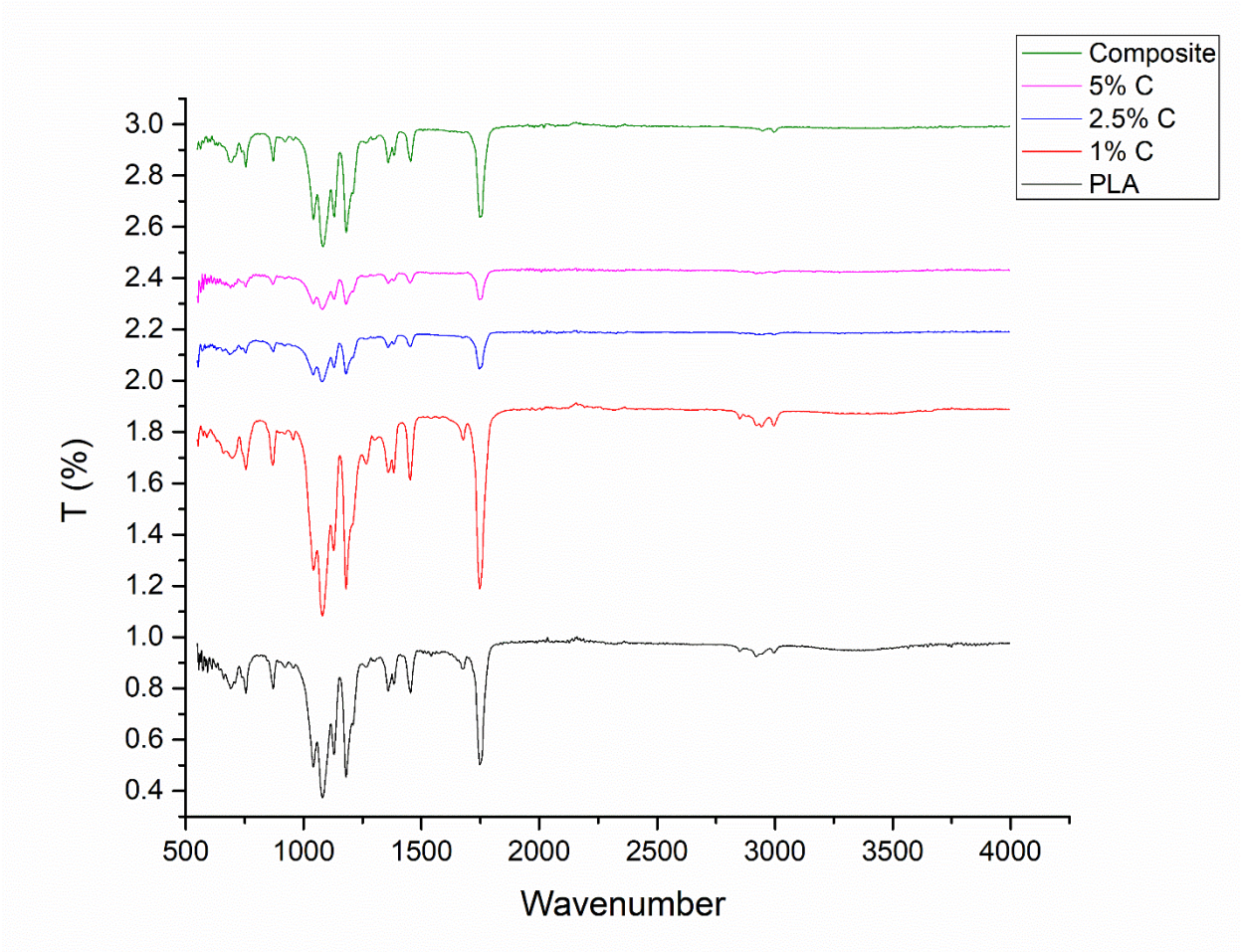


Figure 4.3: Illustrates the spectrum of all compositions of Cinnamon Cassia (Cassia) along with PLA and composite films

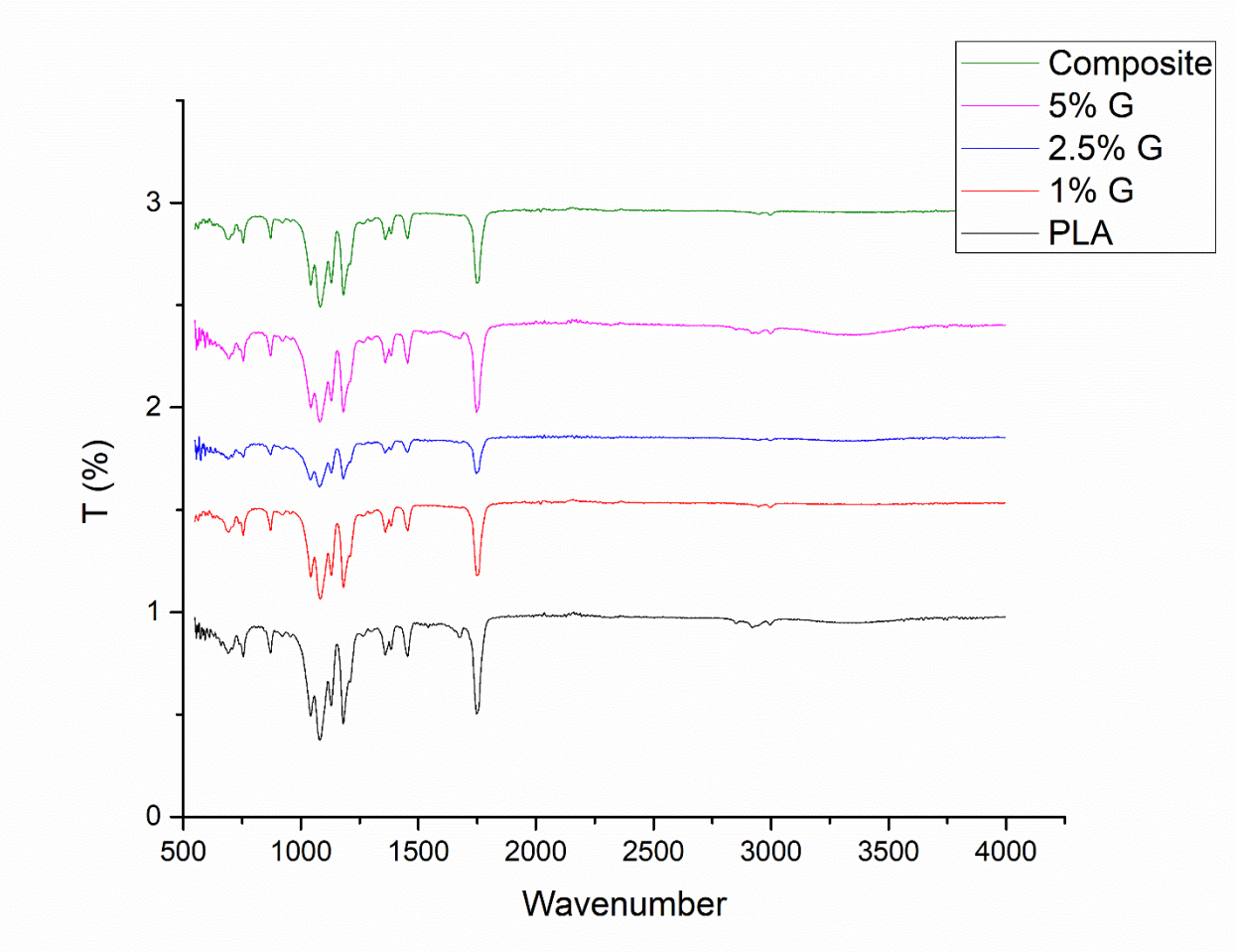


Figure4.4: Illustrates the FTIR spectrum of all compositions of Panax Ginseng along with PLA and Composite films.

4.4 *In-vitro* anticoagulation studies

As the anticoagulation activity of extract of individual drugs have reported in literature and it has been proved that their anti-coagulation activity increases with the increase in concentration of drugs(Gunendren et al., 2017; C. T. Li et al., 2013) and similar behavior was expressed by our drug compositions when were incubated with human blood plasma and results of anticoagulation studies proved that with polymer matrix drugs still show anticoagulation activity but the drug release rate is dependent on the release of drug into the release medium as drugs are incorporated into the polymeric matrix. Drug release and degradation polymer is interrelated mechanisms as the polymer degrade, drug is released from the polymer and with slow degradation of polymer drug release slowly.

4.4.1 Prothrombin time test (PT)

In vitro anticoagulation activity of drug composite, heparin loaded films and pure PLA was examined using pro-thrombin time test (PT) that indicate the bioactivity of extrinsic blood coagulation factors. In this study, blood plasma was used a negative control and heparin loaded films were used positive control. When the blood plasma was incubated with pure PLA films, PT values did not change and when human blood plasma was incubated with drug loaded films, small increase in the PT value of plasma was observed which indicate the anticoagulation property of drug loaded films. While the heparin loaded films and drug composite films enabled the PT value to become significantly high. Moreover, the coagulation activity of heparin loaded films and drug composite films were same which indicate the activation of extrinsic blood coagulation factors because of drug release from drug composite and heparin loaded films as a result coagulation of blood was suppressed due to the release of drugs from drug loaded films. With the release of drugs from drug loaded films the prothrombin time (PT) values also prolonged. Thus, we assumed that

drug composite films prolonged the PT values due to presence of all chemical constituents of drugs (Cinnamon Cassia (Cassia), Panax Ginseng, and Ocimum Tenuiflorm (Tulsi)) responsible for their anticoagulation activity.

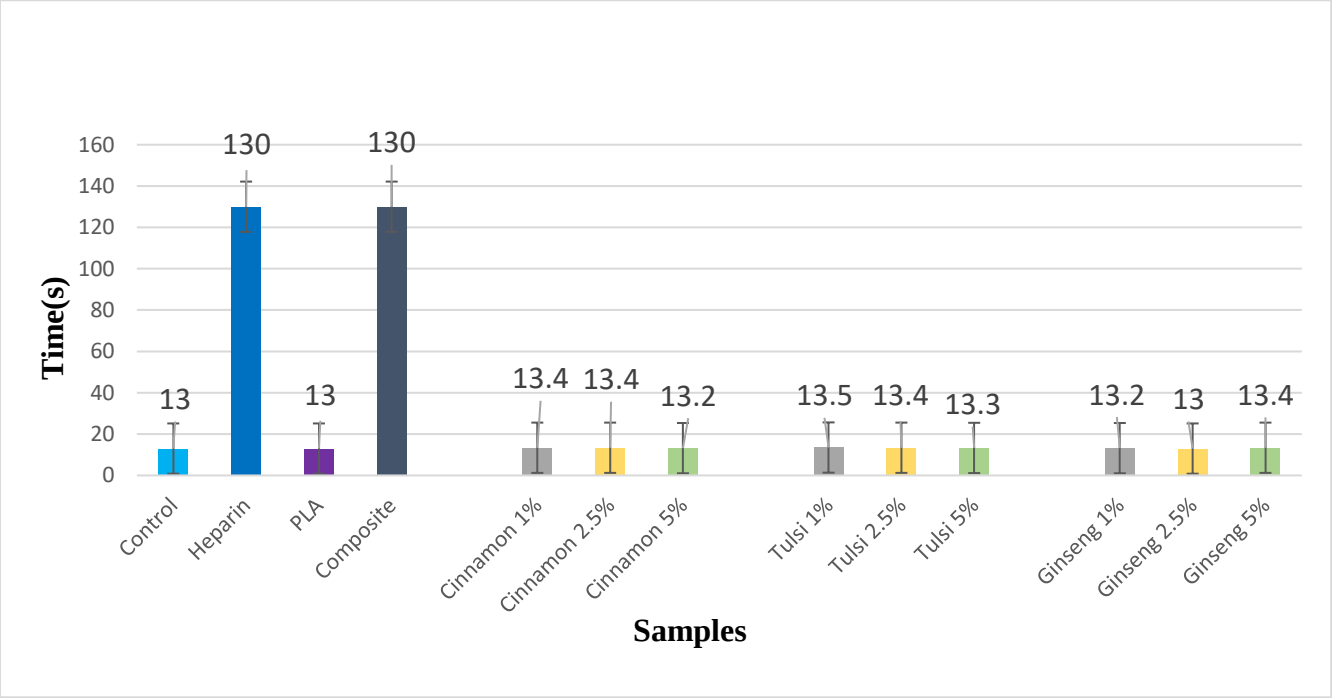


Figure 4.5: Illustrates PT results of pure PLA films, Cinnamon cassia, Tulsi, Panax Ginseng and heparin loaded films.

4.4.2 Activated partial thromboplastin time (a-PTT)

Activated partial thromboplastin time (a-PTT) Test is more sensitive than prothrombin time test (PT) and coagulation activity of drug loaded films was cross checked by a-PTT test.

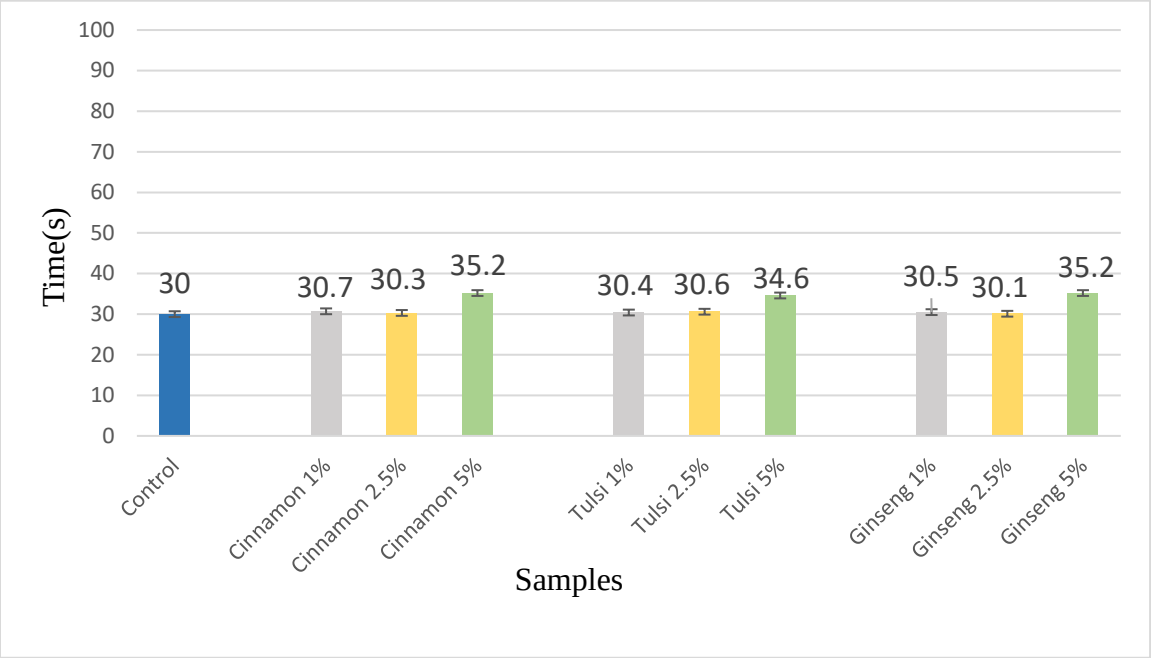


Figure 4.6: Illustrates a-PTT results of Cinnamon, Tulsi, Panax Ginseng loaded films

In a-PTT, all drugs displayed anti-coagulation activity and trend of their anticoagulation activity was, with increase in the drug concentration their a-PTT value also increased due to the presence of more anti-coagulant chemical constituents in the films having high concentration of drug. Normal value of a-PTT was 30s and after incubation of drug loaded films in blood plasma the value of a-PTT increased. Cinnamon 1%, 2.5% and 5% displayed prolonged coagulation activity such as 30.7s, 30.3s and 35.2s a-PTT values. Ocimum tenuiflorum (Tulsi) also exhibited increased a-PTT value such as 30.4s, 30.6s, and 34.6s and Panax ginseng displayed 30.5s,30.1s and 35.2s. Prolonged a-PTT values were due to the inactivation of coagulation factors in intrinsic pathway

by drug release from drug loaded films. Results of a-PTT test confirms anti-coagulation properties of individuals drugs, and this may prevent blood coagulation after coating on implants surface.

4.5 *In-vitro* studies

4.5.1 Drug release studies

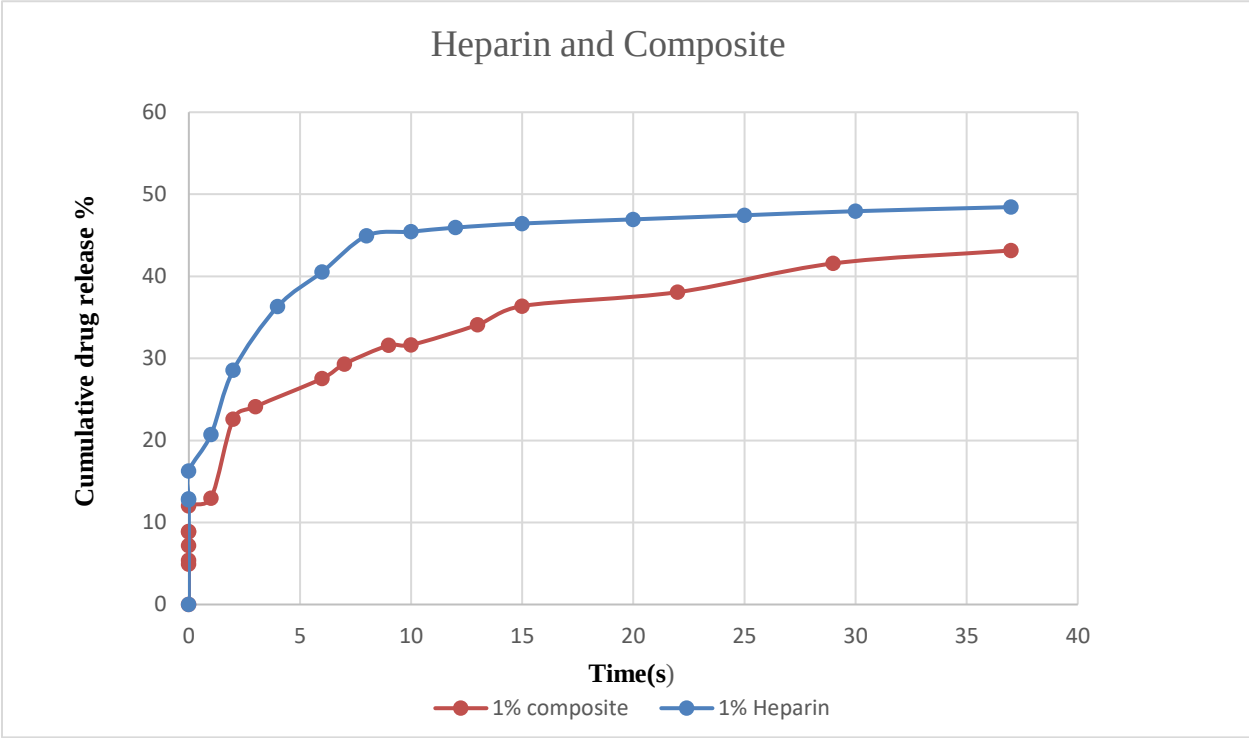
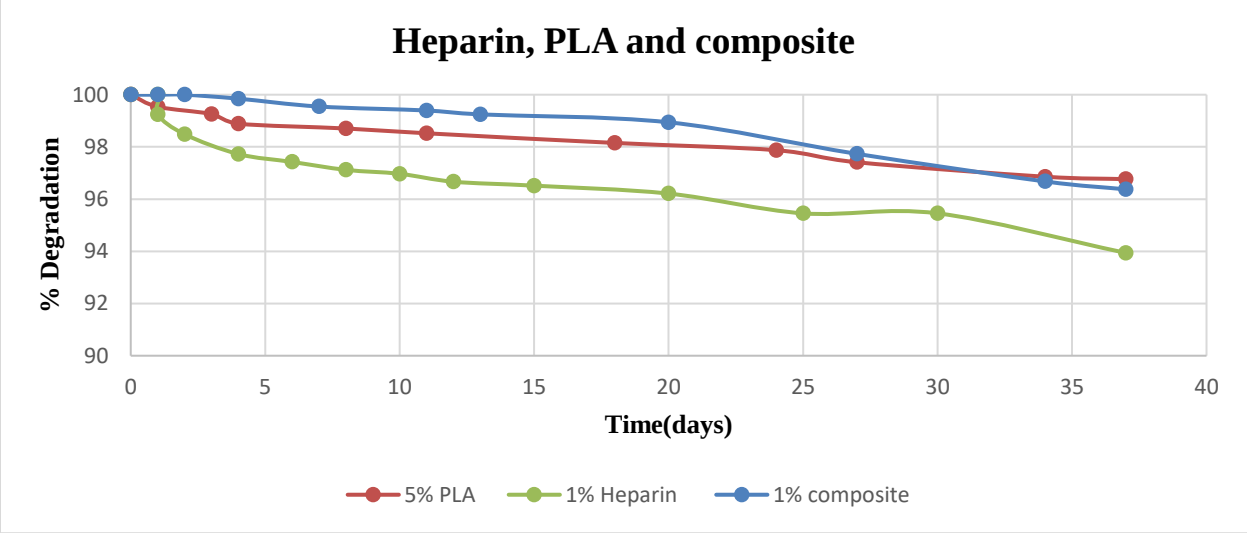


Figure 4.7: Drug release studies of Drug composite film and Heparin loaded film

Drug release of composite film and heparin loaded film are shown in Figure 4.7. From the graph, it was observed that heparin loaded film and drug composite showed initial burst release of 18% and 13% respectively in the first day of release and drug release continued steadily thereafter. It can also be seen the drug release rate of heparin loaded film was higher as compared to heparin loaded film. In 38 days, drug release of heparin loaded film was 48% and drug release of drug composite was 43%. The phenomenon of initial burst release and sustained release are attributed to the diffusion of drug through the pre-existing pores on the surface of films, that form during

film formation process. The remaining of drug release slowly as the PLA degrades into the release medium. For drug release studies, sample films were immersed into the PBS solution which penetrates from surface of the film to the center of film which in turn result in the hydrolytic cleavage of the PLA chain and diffusion of drug from PLA matrix to the release medium. Lactic acid is a hydrophobic part of PLA that control the release of drug from polymeric matrix. Presence of lactic acid group make PLA less hydrophilic and hence make it degrade more slowly.

4.5.2 Degradation studies



Graph 4.8: Degradation studies of Pure PLA, Composite and Heparin loaded films

For degradation studies, composite film, heparin loaded film and pure PLA films were submerged into PBS solution. Percentage weight loss of films represent their degradation rate and prolongation of degradation time effect films qualitatively and quantitatively by significantly changing the film morphology and weight. Percentage degradation of pure PLA film and composite films were almost same in 37 days which was 3% while heparin loaded film was degraded little fast having the degradation rate of 6% in 37 days. Degradation of PLA films is

based on microscopic dissolution process in which the structure of PLA is destroyed by the hydrolysis of backbone ester chain and split into small fragments which then completely dissolved by changing into water soluble by products such as CO_2 and water. In case of drug loaded PLA films, drug particles that adsorb on the film surface are released into the release medium after their immersion into the PBS, leaving behind pores on film surface. The porous structure of PLA and hydrophilicity, speedup the permeation of water into the matrix which increase the contact area between water and film, leaving more degradation sites which in turn increase the hydrolysis rate. This could be the reason that heparin loaded film had fast degradation rate than composite film because some of the drug particles were adsorbed on the film surface which then released into the release medium after immersion of film into the release medium and made the film surface porous and porous film surface subsequently increased its degradation rate.

4.5.3 Hemolysis test

As the composite is to be coated on implants thus, it should have hemocompatibility which was analyzed by two approaches: Qualitative analysis and Quantitative analysis using UV spectrophotometry.

4.5.3.1 Qualitative analysis

Color of supernatant shows hemolytic and non-hemolytic nature of films. From qualitative analysis of hemolysis test, it was observed that positive control (TritonX-100) showed 100% hemolysis whereas negative control did not exhibited hemolysis. Same was the case with our samples they did not show any color which means they are non-hemolytic

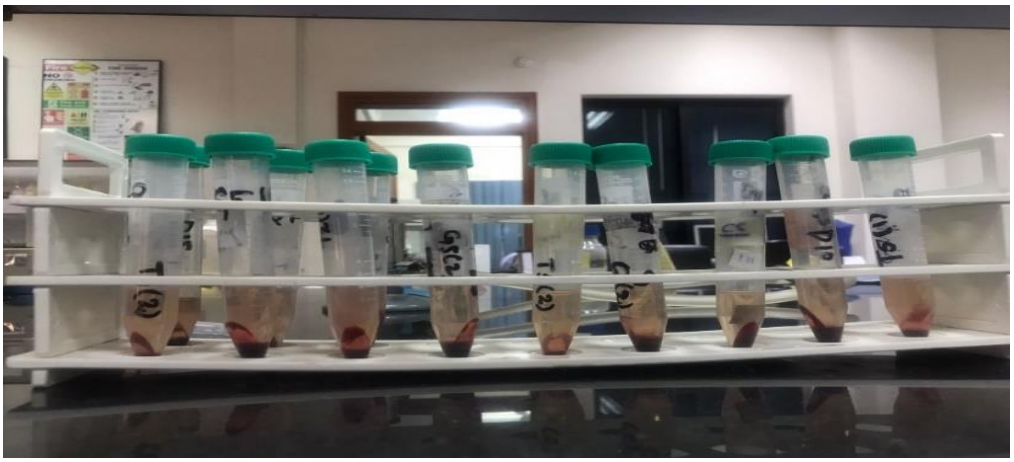


Figure4.9: Qualitative analysis of hemolysis test

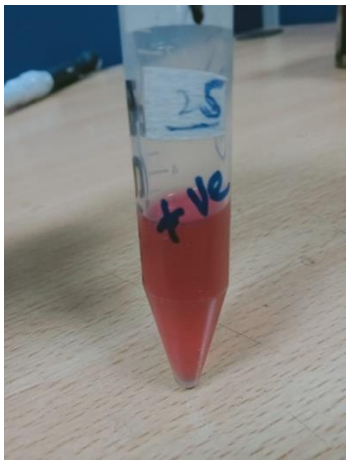


Figure 2: Positive control

4.5.3.2 Quantitative analysis

Results of qualitative analysis was further confirmed by calculating their percentage hemolysis (%). To calculate the percentage hemolysis of drug loaded films, UV spectrophotometry was used, and it was found out that all samples were non-hemolytic as their percentage hemolysis was in the range of non-hemolytic materials defined by ASTM F756 standard. Heparin loaded films showed 1.7% hemolysis while pure PLA and drug composite showed 1.22% and 1.29% hemolysis, respectively.

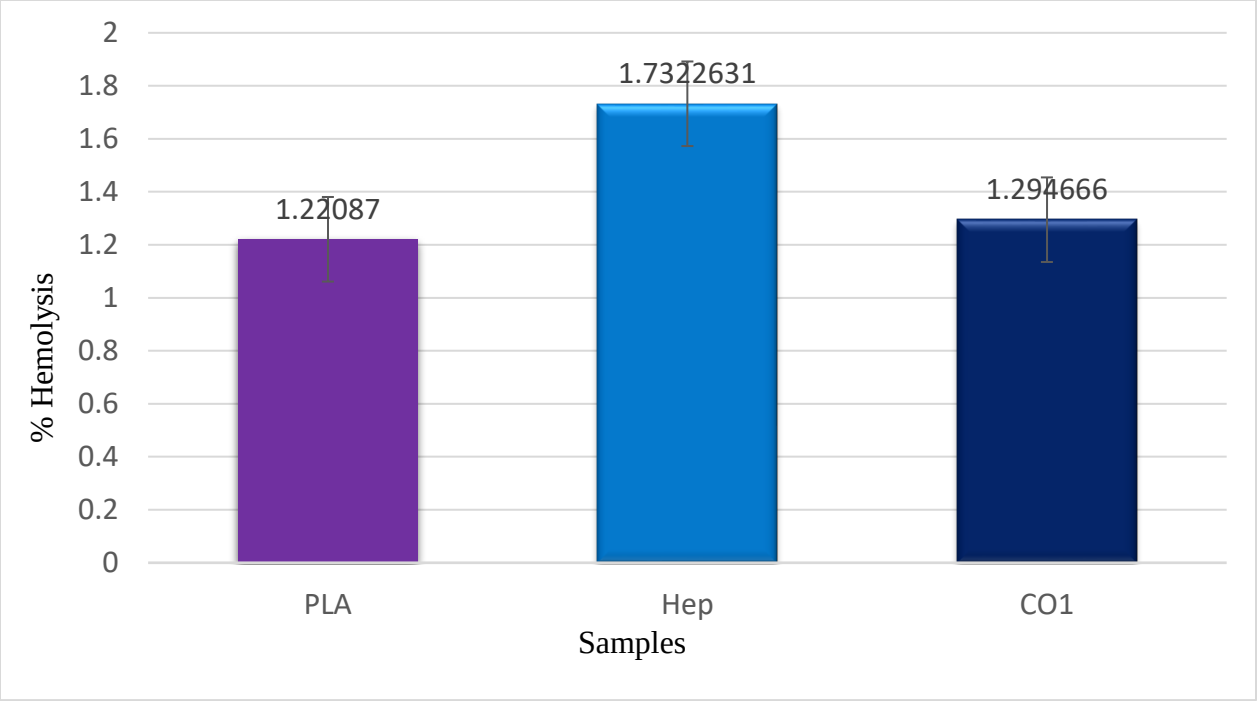


Figure 4.10: Hemolytic activity of PLA films, Heparin loaded films and drug composite film

6.1 Antioxidant studies

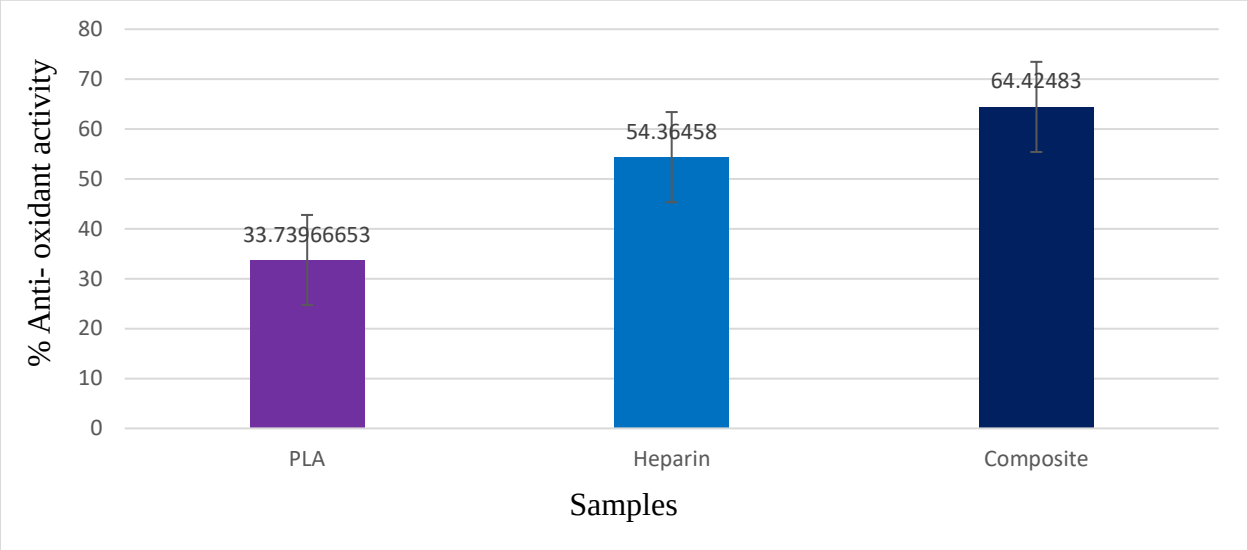


Figure 4.11: Antioxidant activity of PLA films, Heparin loaded films and drug composite films

The objective of this study was to check the antioxidant activity of novel drug composite. Although antioxidant activity of all drugs has been reported in literature but as composite based on biodegradable polymer its antioxidant activity needs to be checked.

Graph of antioxidant activity showed that pure PLA films had 32% antioxidant activity while heparin loaded films and drug composite films showed 54% and 64% antioxidant activity, respectively. Phenolic compounds of drugs are responsible for their antioxidant activity and when the concentration drug increases antioxidant also increases because of presence of phenolic compounds and it was evident from the graph that antioxidant activity of drug composite is enhanced by addition of drugs. Similar results were found in multiple studies as the concentration drug increases, antioxidant activity also increases.(Kajaria et al., 2012; Yakub et al., 2014; Yang et al., 2012)

Chapter 5

CONCLUSION

Based on the results, it was concluded that drug composite is proposed composition as a coating material for the drug eluting cardiovascular implants for the treatment of thrombosis because drug composite showed anticoagulant activity which was equal to commercially available anticoagulant drug (Heparin). Hemolysis assay showed that all individual drug loaded films and drug composite had anti- hemolytic activity which were in the range of non-hemolytic material defined by ASTM 756 standard. Furthermore, antioxidant activity was greater than heparin loaded film while drug release and degradation rate of drug composite were almost same in heparin loaded film and composite.

Chapter 6

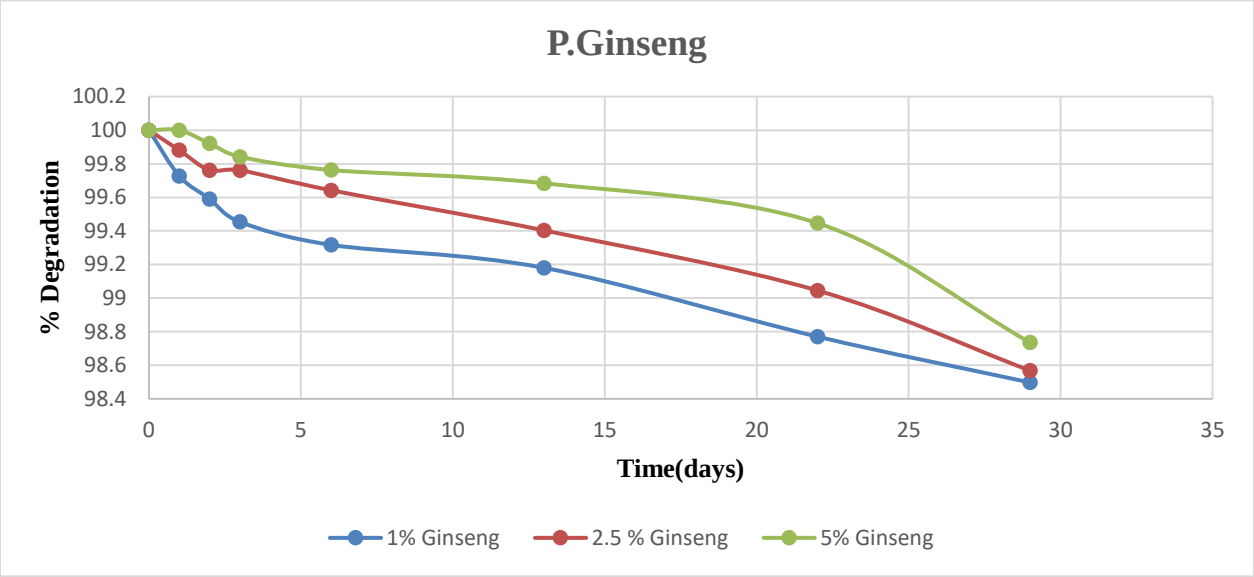
FUTURE PROSPECTS

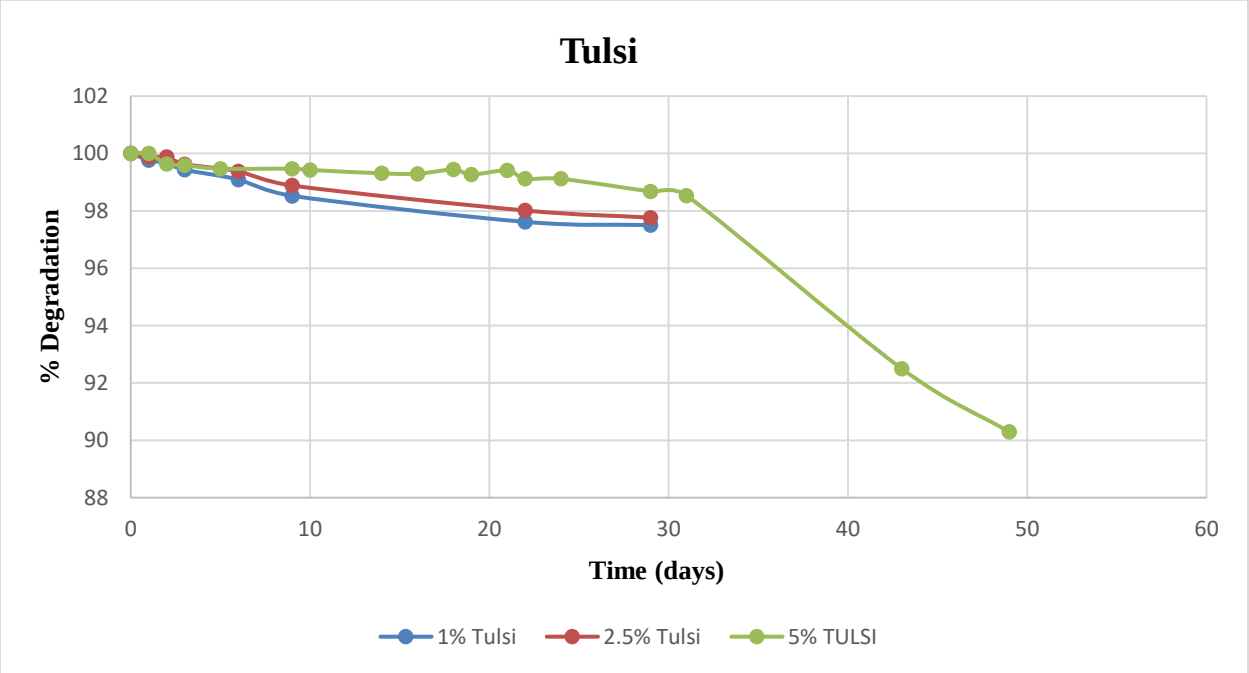
In future, we will coat the developed drug composite onto the Implants specifically stents to modify their surface properties and their biocompatibility, hemocompatibility and anticoagulation activity will be tested further on animals before taking it to humans. Further, the drug release and degradation rate of coating can be increased or decrease according to the need of application by using different molecular weight of polymer. As high molecular weight polymer degrades slowly because of its highly crystalline structure resulting in slow hydrolysis of its backbone chain. Additional optimization of drug concentration can also help to increase or decrease the anticoagulation activity of coating significantly. Thus, after conducting further biocompatibility tests on the drug composite coated on the implants surface, it could be used as antithrombic coating for the treatment of cardiovascular diseases.

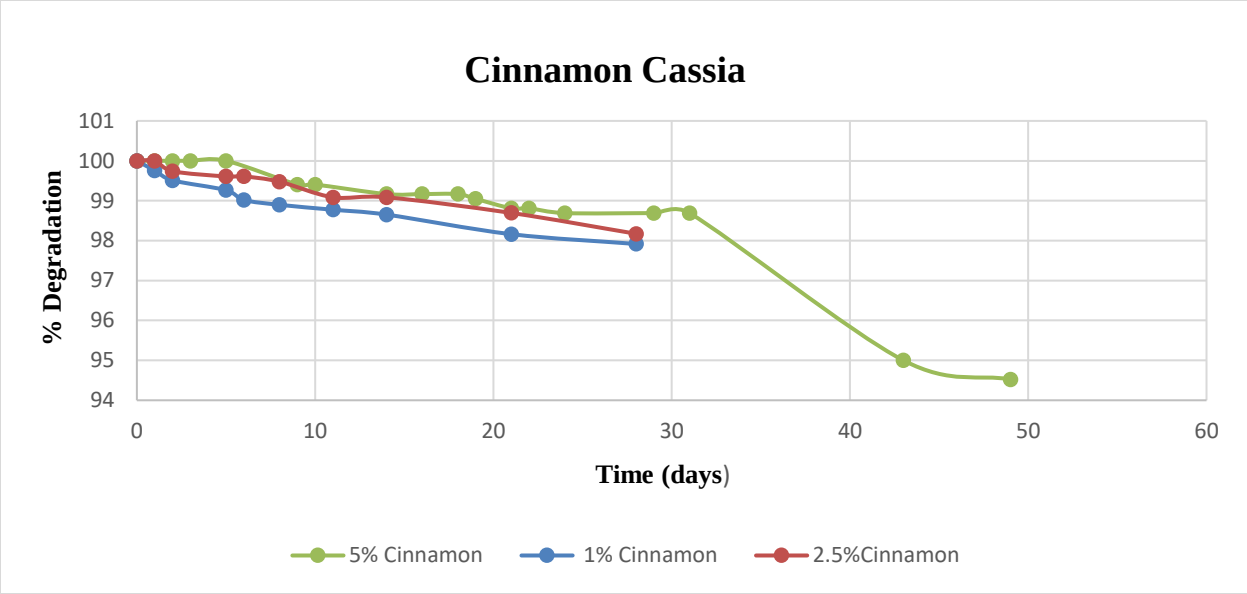
Chapter 7

Supplementary data

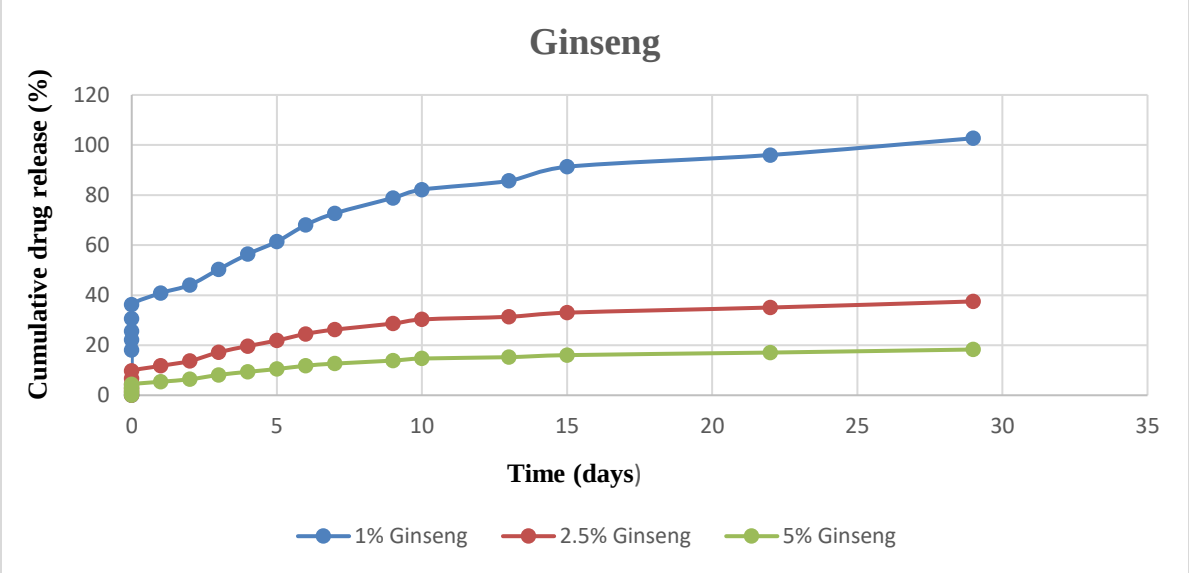
7.1 Degradation studies of P.ginseng, O.tenuiflorum and C.cassia

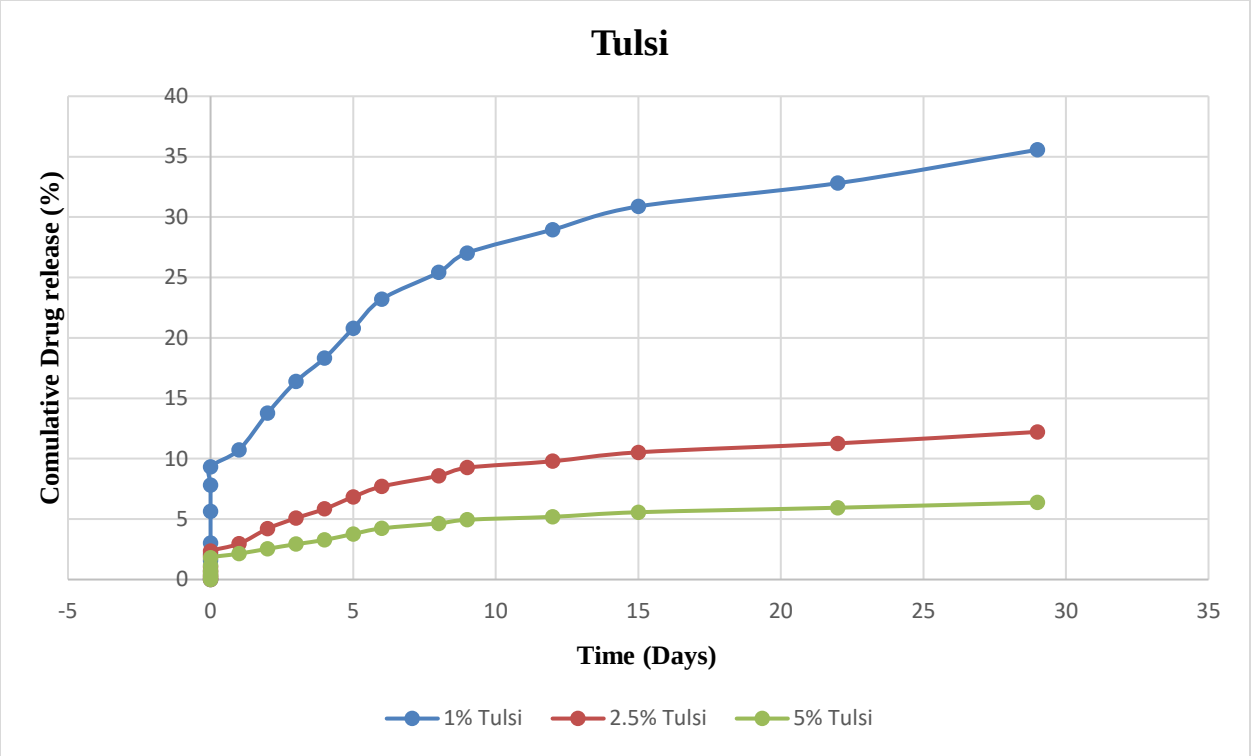


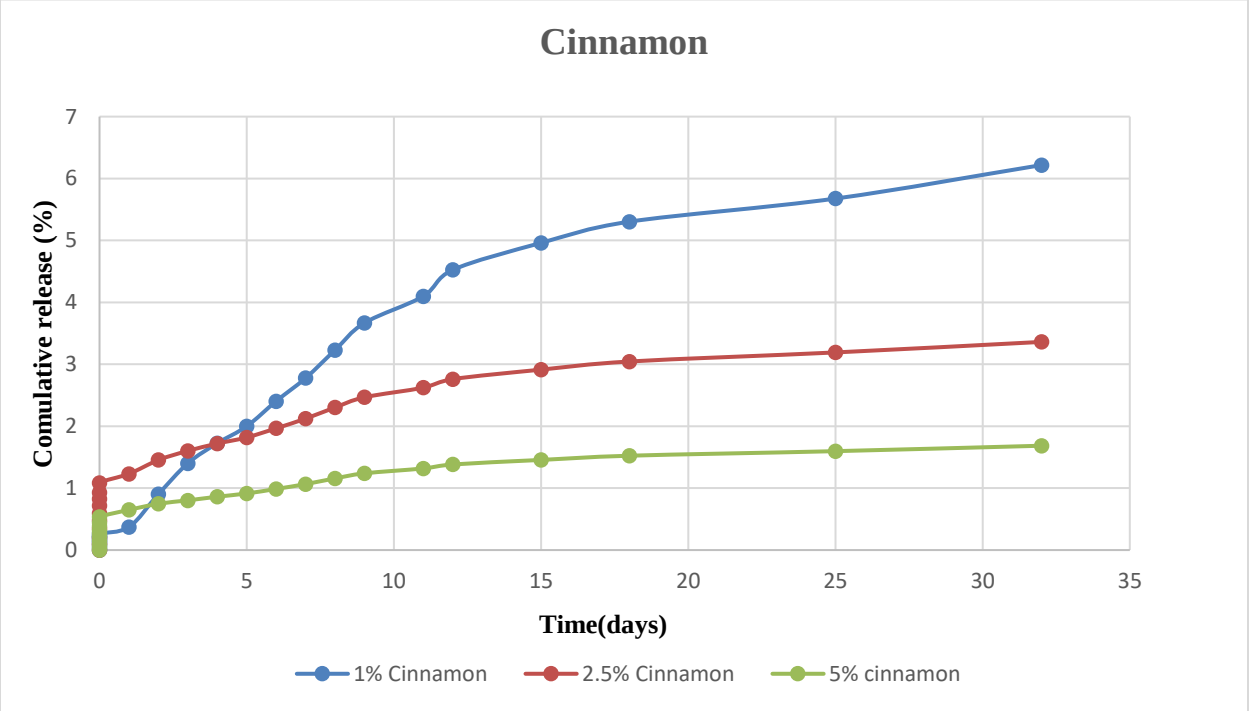




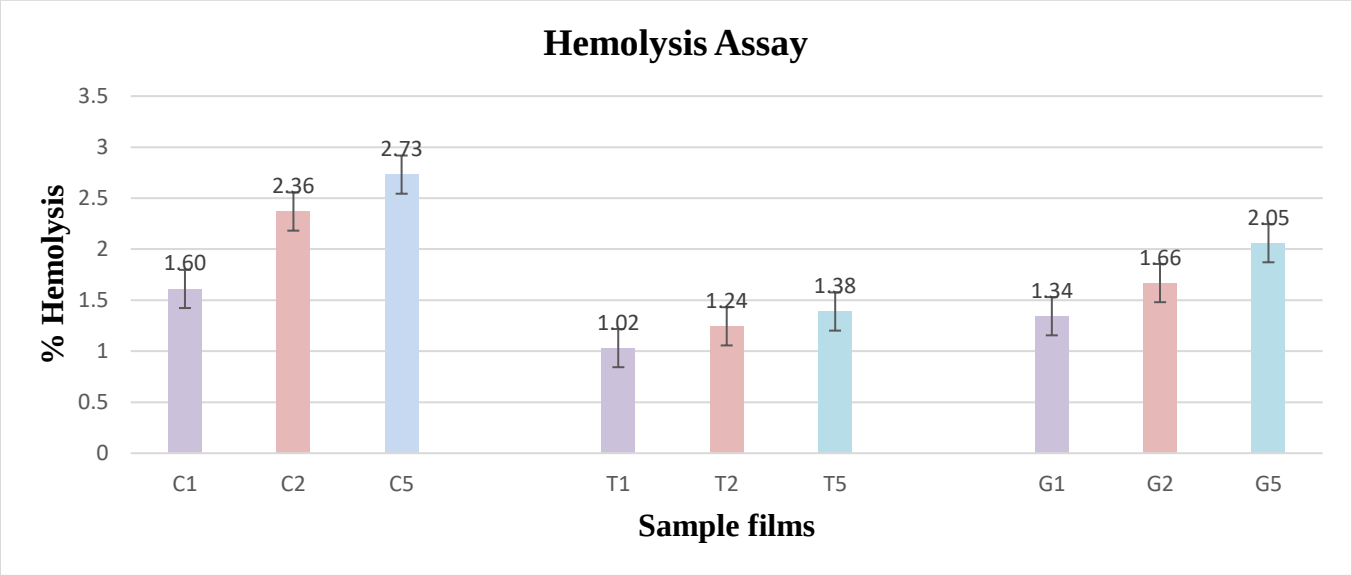
7.2 Drug release studies of P.ginseng, O.tenuiflorum, C.Cassia



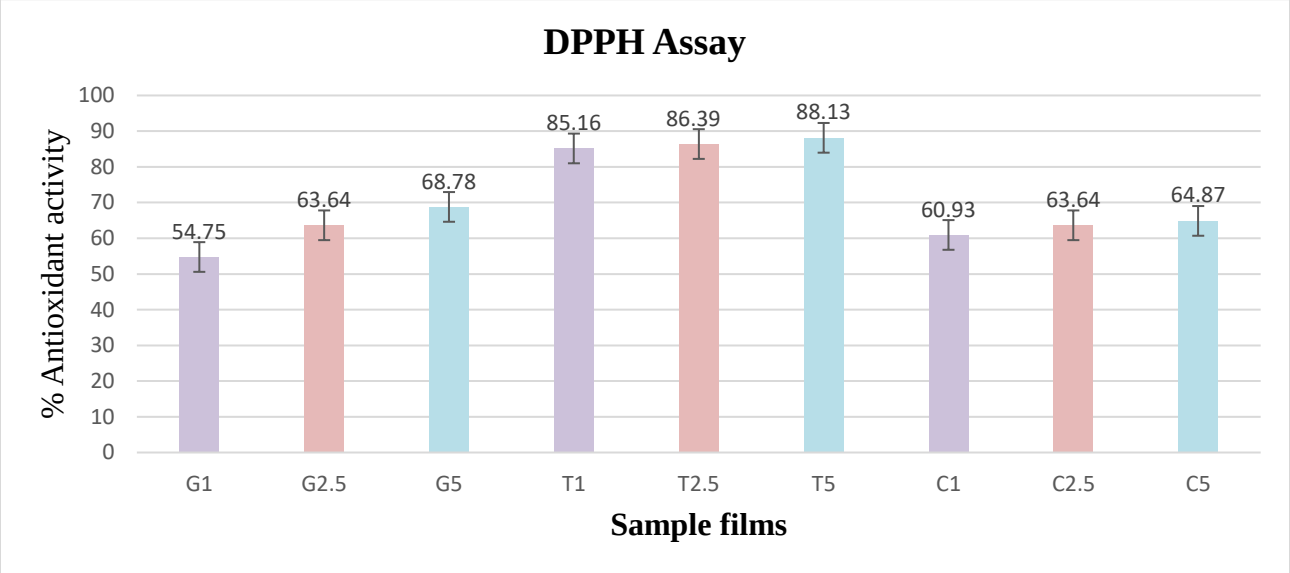

Tulsi
Comulative Drug release (%)
40
35
30
25
20
15
10
5
0
-5
0
5
10
15
20
25
30
35
Time (Days)
1% Tulsi
2.5% Tulsi
5% Tulsi


Cinnamon
Comulative release (%)
7
6
5
4
3
2
1
0
0
5
10
15
20
25
30
35
Time(days)
1% Cinnamon
2.5% Cinnamon
5% cinnamon

7.3 Hemolysis studies of individual drugs



7.4 Antioxidant studies of individual drugs



Chapter 8

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