Therapeutic Effects of Silymarin Gold Nanoparticles Against Thrombosis

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I am thankful to Allah Almighty, for blessing me with this opportunity, making me strong enough to start this journey at first place, opening new doors of possibilities and making my ways easier towards them. During this project and beyond that, wherever and whenever I needed HIS help, HE always guided me and made me able to accomplish my goals.

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Dedicated to my mother…

Abstract

Silybum marianum L. commonly known as milk Thistle plant (اونٹ کٹارہ) belongs to family Carduus Marianum has a 2000 years old history of medical significance. It was declared as a cure of "Melancholy diseases" by Greek physicians and has been reported as a 'safe herb' for liver and gallbladder disorder treatments as well as for protection against venoms such as snake bites, insect sting and mushroom poisoning. **Silymarin** is the major compound present in seeds and fruit of this plant. It is a complex of several flavonolignants and plays the key role in medicinal properties of milk thistle plant. Thrombosis is the condition of blood clot formation in vascular system leading to blockage in blood supply which can cause severe consequences. Free radicals in body can cause alterations in cell signaling pathways leading to multiple complications. A combined antioxidant and thrombolytic agent administration can have synergistic effect on thrombosis and complications related to it. The aim of this study was the synthesis of Silymarin Gold nanoparticles and to investigate its therapeutic potentials particularly its radical scavenging activity and thrombolytic activity as nanoparticles. Nanoparticles were synthesized by commercially purchased silymarin and gold salt by green synthesis. Antioxidant activity of silymarin gold nanoparticles was determined by DPPH assay using different concentrations of silymarin Ascorbic acid was used as a positive control. Thrombolytic activity was also determined by using these concentrations. Streptokinase was used as a positive control to check thrombolytic activity. In comparison to silymarin itself, silymarin gold nanoparticles have shown higher thrombolytic and antioxidant activity. As the a flavonolignant having both antioxidant and thrombolytic activity, silymarin in form of gold nanoparticles could be an effective treatment of thrombotic diseases with less or no side effects in comparison to commercially available thrombolytic agents.

Key words: *Silymarin, antioxidant activity, thrombolytic activity, gold nanoparticles, therapeutic potentials*

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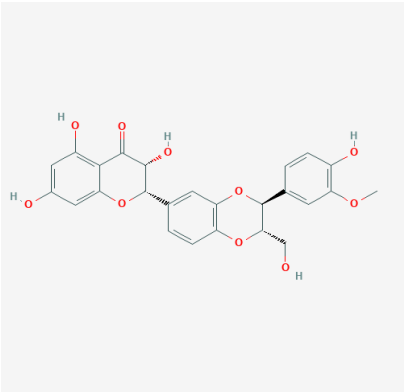
CHAPTER 1: INTRODUCTION

1.1.Background:

***Silybum marianum* L**. commonly known as **Milk Thisle** plant **(اونٹ کٹارہ)** belongs to the family of **Carduus marianum**. It has proved its significance in viedic medicine and has a 2000 years old history of being used as a curative medicine against liver disorders and inflammation. It has proven its significance against multiple gallbladder disorders as well as against venoms such as snake bite, mushroom poisoning and insect stings. This plant has can be found abundantly in North America, Mexica and Canada. In Pakistan, it can be found in Kashmir. It has a thorny appearance with large leaves and purple – reddish flowers. Seeds and fruit are considered as major medicinal part of this plant. **Dioscorides (40 – 90 AD),** a Greek physician and botanist discovered milk thistle's healing properties. It was titled as "best remedy against melancholy diseases" by **John Gerard** in **1597**. (Siegel, 2013)



The antioxidant and hepatoprotection activities of milk thistle are mainly credited to the complex of flavonolignants present in it known as Silymarin. Silymarin is the active component of milk thistle plant, comprised of flavonolignants like silybin A, silybin B, isosilybin A, isosilybin B, silychristin, neosilyhermin, silyhermin and silydianin. This component is majorly present in fruit and seeds of this plant. (Gholamreza Karimi, 2011)

Silymarin has reported as an immunomodulating, anti-inflammatory, antifibrotic and as an antioxidant agent by proving its scavenging mechanism against free radicals. Multiple studies have accepted it as a safe herbal medicine, proving its therapeutic significance without causing toxicity. (Kaur M, 2007)

Silymarin as an antioxidant have been reported to increase the activity of superoxide dismutase within RBCs and WBCs. It also can improve the membrane of hepatocyte, preventing xenobiotic from entering the cell through enterohepatic circulation. (Ramakrishnan G, 2009) Silymarin also reported promising effects against breast carcinoma, ovarian carcinoma, lung carcinoma, melanoma, prostate cancer, cervical cancer, bladder cancer and colon cancer through interaction and modulation of cell-signaling pathways like NF-kappa B, EGFR-MAPK/ERK1/2 signaling and IGF receptor signaling. (Polyak SJ, 2007) It also has shown anti angiogenic property against different tumor disorders, qualifying it as a significant medicine for basic treatment. Apart from its established role as an anticarcinogen, silymarin has also reported to boost neurotransmitter concentration in brain and also shown significant effects against Alzheimer disease by controlling protein oxidation through is antioxidation properties.

(Galhardi F, 2009)

1.2 Oxidation and Free Radicals:

Oxidation reaction in body produces free radicals causing oxidative stress. This oxidative toxicity leads to multiple mutations in cell signaling pathways and regular biochemical mechanisms of body leading to several diseases and abnormalities.

Oxidative stress is particularly higher in patients already suffering from cancer, tumor, kidney or liver cirrhosis. In such condition, free radicals damage cells to an unrepairable extent causing more complications.

1.3 Antioxidants:

Nature has given us the gift of several plants which can be used for treatment purposes. The promising effect of such plants along with minimal or no toxic side effects is the reason why researchers are keen to develop drugs derived from natural sources.

Naturally existing polyphenols and flavonoids have proved to be the best antioxidants for treatment. Not only they have exceptional action mechanism against

Thrombosis:

A blood clot is produced in result to haemostatic failure causing blockage in vascular system which ultimately leads to atherothrombotic diseases. Such diseases like cerebral infraction or myocardial infraction can cause death. (Furie B, 2008)

The term used to define such condition is called “Thrombosis”. It is classified as arterial or venous thrombosis by using the reference of location of clot formation. Drugs like streptokinase, urokinase, TPA (tissue plasminogen activator), anistreplase, alteplase are commercially available as thrombolytic agent for treatment of thrombosis. The weak substrate specificity in streptokinase and urokinase (first generation thrombolytic agents) often causes bleeding complication. (Chowdhury, 2015). Such complications and drawbacks of currently available thrombolytic agents have lead the researchers to develop thrombolytic agents which are more natural and less toxic, in an attempt to minimize the side effects of treatment. As a result of such experiments, multiple medicinal plants have shown the potential to serve as a thrombolytic agent without causing any toxicity and have been recorded as ‘safe herb’ for treatment of thrombosis. Multiple population and health-based surveys have also suggested that certain food items can also reduce the risk of thrombosis and significant results have been reported.

CHAPTER 2: LITERATURE REVIEW

2.1 Silymarin

2.1.1 Introduction:

Silybum marianum (Milk Thistle) plant is a member of family **Carduus marianum**. Commonly identified by its large leaves and thorny reddish-purple flowers, this plant can be majorly found at areas like North America, Kashmir, Canada and Mexico. Milk thistle plant has a 2000 years old history of being used as a medicinal herb. It has a significant history of being used as a medicine against liver disorders, gallbladder disorders and alcohol toxicity. It is also considered a good treatment for mushroom poisoning, insect stings and snake bites.

According to history, milk thistle plant was originally discovered by a Greek botanist and physician Dioscorides (40 – 90 AD). He explained the healing properties of this plant, which were later supported by the work of John Gerard in 1597, who titled silymarin as "the best remedy against melancholy diseases". It was used as a home remedy/folk medicine as a liver tonic. It was considered as detoxificant for liver and spleen, cleaning the obstructions within system, thus curing jaundice.

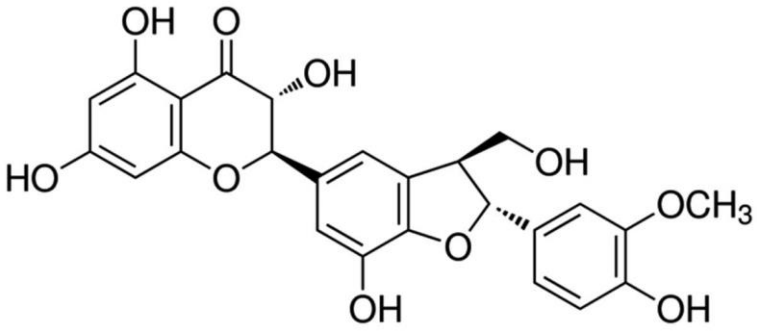
The medicinal use of milk thistle plant has a wide spectrum of possibilities and advantages. It can be used against alcohol, it has potential to save lives from mushroom poisoning, particularly from death cap mushroom. A research from Iran reported its positive effect and improvements in patients suffering from obsessive – compulsive disorder.

Multiple researches have supported the anti-viral and anti-inflammatory properties of Milk Thistle plant. It has shown great results when treated against Hepatitis C. In a study patient co-infected with both Hepatitis and HIV showed negative signs of disease after being treated with intravenous dosage of silybinin for 15 days.

Even when anti-cancer properties of Milk Thistle plant have not been clearly studied in human trials, on preclinical models it has shown a clear anti-cancer activity when treated against colon cancer cells causing apoptosis. It initiated cell senescence in breast cancer model and blocked

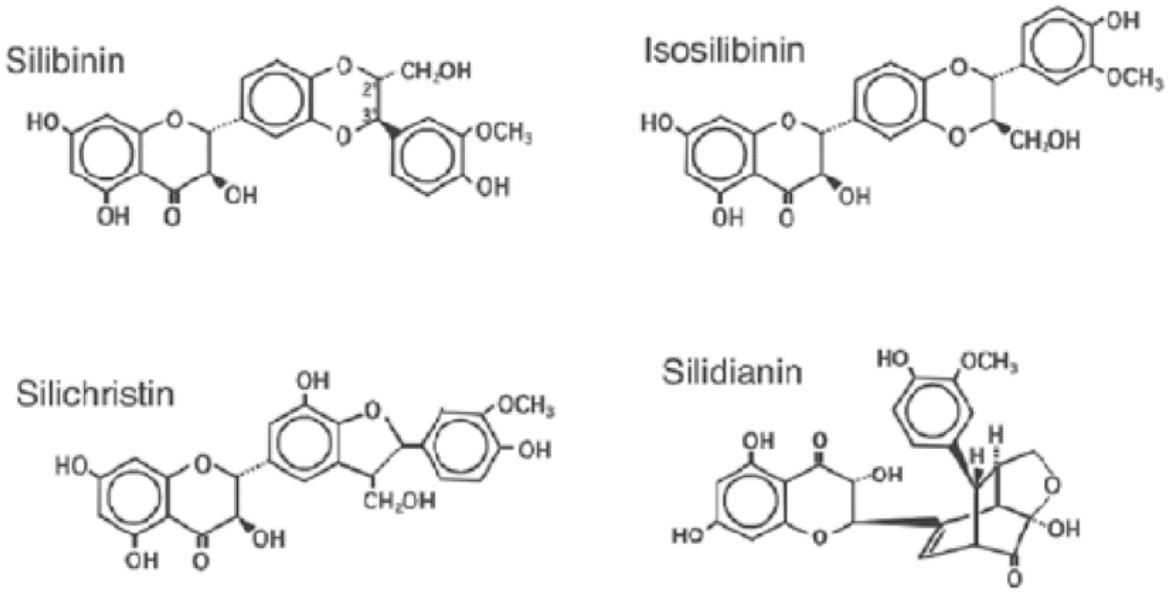
angiogenesis in prostate cancer mouse models. Milk thistle plant when used against ultra violet rays as a paint to protect from skin cancer also showed positive results.

The major flavonoid present in Milk thistle plant is Silymarin which is more of a compound and a combination of several other components. It is composed of other compounds like silybin A, silybin B, isosilybin A and isosilybin B. Also, other flavonolignants like silychristin, neosilyhermin, silyhermin and silydianin. Major concentration of components is present in fruits and seeds in comparison to other parts of plant.



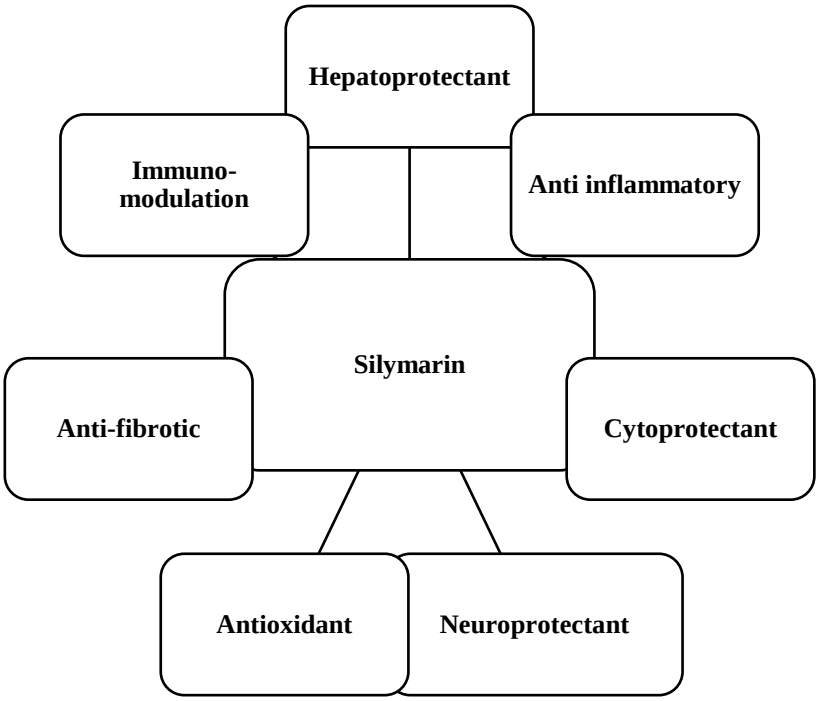
In several studies, silymarin has shown its positive effect against different organs disorder. It has shown its healing effect against CNS, kidney, liver, pancreas and liver disorders.

It has an engrained repute as an antifibrotic, immunomodulating, anti-inflammatory and antioxidant compound. It works on the principle mechanism of scavenging free radicals and increasing glutathione concentration in body. It can be an effective treatment for patients suffering from Hepatitis and liver cirrhosis.



Its pharmacological application as a herbal medicine has been supported by multiple studies, accepting it as a safe herb, provided the proper administration of therapeutic dosage.

Based on lab trials, silymarin also has the potential to be used for hepatotoxic medications for chemotherapy of tuberculosis.



2.1.2. Hepatoprotection:

Liver as the main metabolic organ of the body is continuously exposed to xenobiotics. Because of its strategic placement in body as a purifier, it has the highest potential to be affected from all the toxins absorbed by intestinal tract. Therefore, liver diseases are one of the most critical health situations. Any damage to liver cells can lead from acute hepatitis to carcinoma, apoptosis, inflammation immune response, fibrosis, ischemia, altered gene expression, regeneration and hepatocellular carcinoma.

For over centuries, silymarin is considered as a “Hepatoprotectant”. This claim is based on the free radical scavenging properties of Silymarin, which leads to the peroxidation inhibition of lipids by enhancing cellular level of glutathione. This mechanism increases the stability of hepato-membrane against xenobiotics.

Since silymarin has a steroid like structure, it increases the defense mechanism of liver cells by improving their reformative abilities elevating the DNA and RNA synthesis. This change in hepatocyte external membrane creates obstruction for xenobiotics preventing them to enter cell. TNF-α-dependent transporters, bile salt export pump, organic anion uptake transporter

peptides (OATP) and ABC transporters (P-gp) are those cell membrane receptors and transporters which can be modified by Silymarin.

2.1.3 Cytoprotection:

Modulation in cell signaling pathways like NF-kappa B, suppression of EGFR-MAPK/ERKI/1/2 and IGF-receptor and ability to interact with specific receptor makes silymarin a potential cytoprotectant herb. Research has also supported the anti-apoptotic effect of silymarin as a tumor suppressant against caused by UV radiation, by regulating gene p53 and p21C1P1

The anti-angiogenic effect of silymarin was studied through vascular endothelial growth factor and matrix metalloproteinase-2 (MMP-2) decline mechanism in human umbilical vein endothelial cells.

2.1.4 Renal Protectant:

Glomerular filtration of kidney can be affected by oxidative stress. Silymarin in assistance with Vitamin E has shown progressive improvement in serum creatinine concentrations in a preclinical study.

Renal toxicity can also cause as a side effect of anti-tumor and anti-cancer drugs. Pharmacological studies have shown that silymarin when administered with these drugs reduced their renal toxic effects, without interfering with their anti-tumor activity. Cisplatin and ifosfamide induced renal toxicity was reported to be altered by silymarin.

2.2 Thrombosis:

Thrombus – is the term known to describe the blood clot formation in vascular system. Thrombosis is the condition which results as failure of hemostasis process. Under normal conditions, hemostasis maintains the circulatory system regulating the high pressure and sustained blood flow. In case of any vascular injury on vessel wall, hemostasis by the help of circulating platelets initiates the procedure of thrombus formation by generating thrombin and fibrin. This thrombus formation is meant to be temporary and specific for the site of injury. In

case of any abnormality that may occur in regular procedure, thrombin formation exceeds the required amount leading to the condition of thrombosis.

Thrombosis is a severe condition mainly associated with arterial diseases like stroke and myocardial infarction and venous thromboembolic diseases which ultimately lead to mortality. Venous thrombosis has proven to be second leading cause of death in cancer patient. A small portion of either arterial or venous thrombus breaks off as embolus and travels down the blood circulation. This condition is known as thromboembolism. Venous thromboembolism can cause pulmonary embolism and arterial embolus can flow with arterial blood causing blockage in blood vessels.

2.2.1 Classification:

Thrombosis can be broadly classified in two main categories i.e venous and arterial thrombosis. On the basis of its location, venous thrombosis can be further divided in to i) Superficial Thrombosis, ii) Deep vein Thrombosis, iii) Renal vein thrombosis.

i) Venous Thrombosis:

Formation of blood clot in a vein is known as Venous thrombosis. Due low blood pressure in veins, the risk of thrombus formation in vein is relatively high than arterial thrombosis.

ii) Superficial Thrombosis:

Superficial thrombosis is the blood clot formation in superficial vein (i.e veins closer to the surface of body). Formation of thrombus can be witnessed by veins bulging out or redness on effected area. These kinds of thrombosis are not as serious as deep vein thrombosis but can lead to severe consequences if travel to deep veins via perforator veins.

iii) Deep Vein Thrombosis:

Deep vein thrombosis is one of the most common forms of thrombosis, occurs in deep veins mostly legs. It is more complicated since, deep veins run away from skin and receive more amount of blood flow in comparison to superficial veins. Swelling, redness and comparatively warm affected area are general symptoms of deep vein thrombosis.

iv) Renal Vein Thrombosis:

Renal vein thrombosis develops in veins taking blood away from kidneys. Renal vein thrombosis can reduce the efficiency of kidneys and their filtration mechanism. Patients

suffering from nephrotic syndrome, cancer, kidney transplant and blunt trauma to lower back and abdomen are at the higher risk of developing renal vein thrombosis. Patients suffering from RVT are generally prescribed to take anti-coagulants for the rest of their lives.

v) Arterial Thrombosis (atherothrombosis):

Arterial thrombosis has comparatively less occurrence rate than venous thrombosis. Since arteries are responsible for the transport of blood and oxygen to various parts of body, blood pressure in arteries is comparatively high than blood pressure in veins which reduces the risk of clot formation. Arterial thrombosis can cause congestion in blood flow restricting oxygen and blood supply to tissue. This situation can ultimately cause tissue necrosis, completely or partially damaging that particular area. Embolus formation in arterial thrombosis can cause heart attack, if blood supply to brain is congested due to arterial embolus patient may suffer from a stroke.

2.2.2 Pathogenesis:

Endothelium lining of vessel wall plays an important role in maintenance of its vasculature. Endothelium maintains three thromboregulators i) nitric oxide, ii) prostacyclin and iii) ectonucleotides CD93. These three thromboregulators provide defense against formation of a thrombus.

In 1856, Virchow's Triad described three contributing factors in thrombosis which are venous stasis, vascular injury and hypercoagulability. Clinically, major reasons which contribute towards thrombosis are trauma, malignancy, prolonged immobility, pregnancy, congestive heart failure, varicose veins, obesity and ageing.

Venous thrombosis occurs with mechanically altered blood flow most commonly in deep veins of legs. Even when venous valves promote the blood flow through venous circulation, they also potential sites for development of venous stasis and hypoxia. A venous thrombus is comprised of two components. Inside is a platelet rich white thrombus called the lines of Zahn covered by outer fibrin dense clot. DNA complexed histone proteins along with Fibrin form the outer scaffold, this scaffold is identified by tissue plasminogen activator which works for thrombolysis.

Arterial thrombosis is caused by injury in soft plaque cap in arterial endarterium, resulting in aggregation of platelets at the site of injury causing thrombosis. The general pathogenesis of arterial thrombosis is explained as the injury of vascular endothelial cells, changes in blood flow and increase in blood coagulability. Thrombosis can be initiated by the rupture of unstable atherosclerotic plaques and subsequent adhesion and aggregation of platelets at the sites of rupture.

2.2.3 Prevention and Control:

For deep vein thrombosis, initial parenteral anticoagulant therapy or anticoagulation is practiced. Low molecular weight heparin is also used for therapy against deep vein therapy. For patients suffering from mechanical heart valve, atrial fibrillation or thromboembolism anticoagulation is used along with vitamin K antagonist.

Drugs such as alteplase, streptokinase, urokinase and tissue plasminogen activator are used for clot lysis. These commercially available drugs are not only used for treatment of thrombosis but also for clot lysis in medical instruments like tubes and catheters during dialysis and cancer treatment. Among these drugs, urokinase and streptokinase (first generation thrombolytic agents) show weak substrate specificity. This leads to systemic fibrinolysis and complicated bleeding disorders.

2.2.4 Relation between Oxidation & Thrombosis.:

The relationship between free radicals and thrombosis can be understand by example of Photodynamic Therapy, which is used to prevent stenosis causing thrombosis in veins. This happens as a result of free radicals that are produced during hypoxia reperfusion injury of vein graft implantation. OH- radicals produced during ischemia increase at a rapid rate during early reperfusion. Naturally occurring plant antioxidants have shown potential as antibacterial, antithrombolytic, antiviral and anti-inflammatory agents.

The ability of antioxidants to reduce oxidative stress in ischemia – reperfusion injury indicates its importance for cardiac health. The administration of an antioxidant can prevent platelets activation which in result is capable of stopping thrombus formation.

Silymarin – has proved its potential as an antioxidant agent. The aggregation of platelets on endothelial cell wall of vessels caused by cell signal modulation in result to oxidative stress can be reduced by using silymarin. Thus, it can prevent thrombus formation. By interacting with extracellular DNA complexed heparin and fibrin present on outer shell of thrombus silymarin act as a thrombolytic agent.

2.3 Nanoparticles:

2.3.1 Introduction:

"Nano" is a Greek word which means dwarf. The word "nano" is used to measure one billionth part of a meter. Nanotechnology is the manipulation of matter in to small particle size range from 0.1-100 nm. The size reduction of particle to nanometers involves changes occurring at individual atom and molecules level, making it suitable for a broad range of changes

Nanotechnology is one of the most rapidly developing domains in biotechnology due to its wide area of application and benefits. The variety in application is mainly because of different methods of synthesis.

Nanomaterials and nanostructures are getting more attention in field of nanotechnology. Nanomaterials are currently being used in medicine industry, as a catalyst in multiple reactions, for water treatment and conversion of solar energy.

Nanoparticles have the potential to show tremendous amount of improvement by changing their size, morphology and distribution. Several methods are practiced for synthesis of nanoparticles. Gold, silver and copper are also used in synthesis of pure and distinct nanoparticles; however, these methods are very expensive and harmful for environment. Due to such hazardous effects of chemical synthesis, researchers are now focusing on green synthesis of nanoparticles.

2.3.2 Application in Health Care

Nanotechnology has started emerging for targeted drug delivery systems for various disease, to enhance drug administration. Successful experiments has been conducted to achieve an improve drug delivery system in diseases like diabetes, sclerosis, carcinomas, tumor, inflammation and infections.

Molecular nanotechnology in co-relation with nanomedicine is bringing a revolution in medical health care. By using this technology, early and easy detection of disease, its prevention or targeted treatment with high efficacy is now possible.

Nano medicine involves the development of therapeutic nanoparticles and nano-biosensors. Such techniques are being used for gene sequencing, DNA repair mechanism, induction of apoptosis in tumor cells and delivery of drug to the target area minimizing the cytotoxic effects to neighboring cells.

Development of lipid and polymer nanoparticles have shown improved and proper administration of drugs. The efficacy of drug delivery system depends on their capacity to adjust pharmacokinetics and plasma distribution of the drug.

2.3.3 Gold Nanoparticles:

The history of using gold nanoparticles in chemistry is dated back in Roman era, where gold nanoparticles were used to decorate utensils. Michael Faraday was the first scientist to report his observation on colloidal gold solution in comparison to bulk gold solution, stating the difference between both of their properties.

Gold nanoparticles are usually prepared in spherical and rod shape, which are used for carrying drug to cells, as gene regulators, for imaging and diagnostic purposes and as therapeutic agents. In development of a nano-medicine aimed to worked at molecular and macromolecular scale, stability, site-specificity and bioavailability are major concerns along with the delivery mechanism of drug. The surface structure of gold nano-particles is extremely adjustable and can be modified by using different methods for synthesis of nanoparticles generating excellent results as a drug carrier.

2.3.4 Green Synthesis of Nanoparticle:

There are several methods in practice for the synthesis of nanoparticles. These methods are selected on the basis of application method, purpose and structural requirement of product. However, use of chemical methods for synthesis of gold, silver, copper and other metallic nanoparticles is not only very expensive but also extremely hazardous for environment.

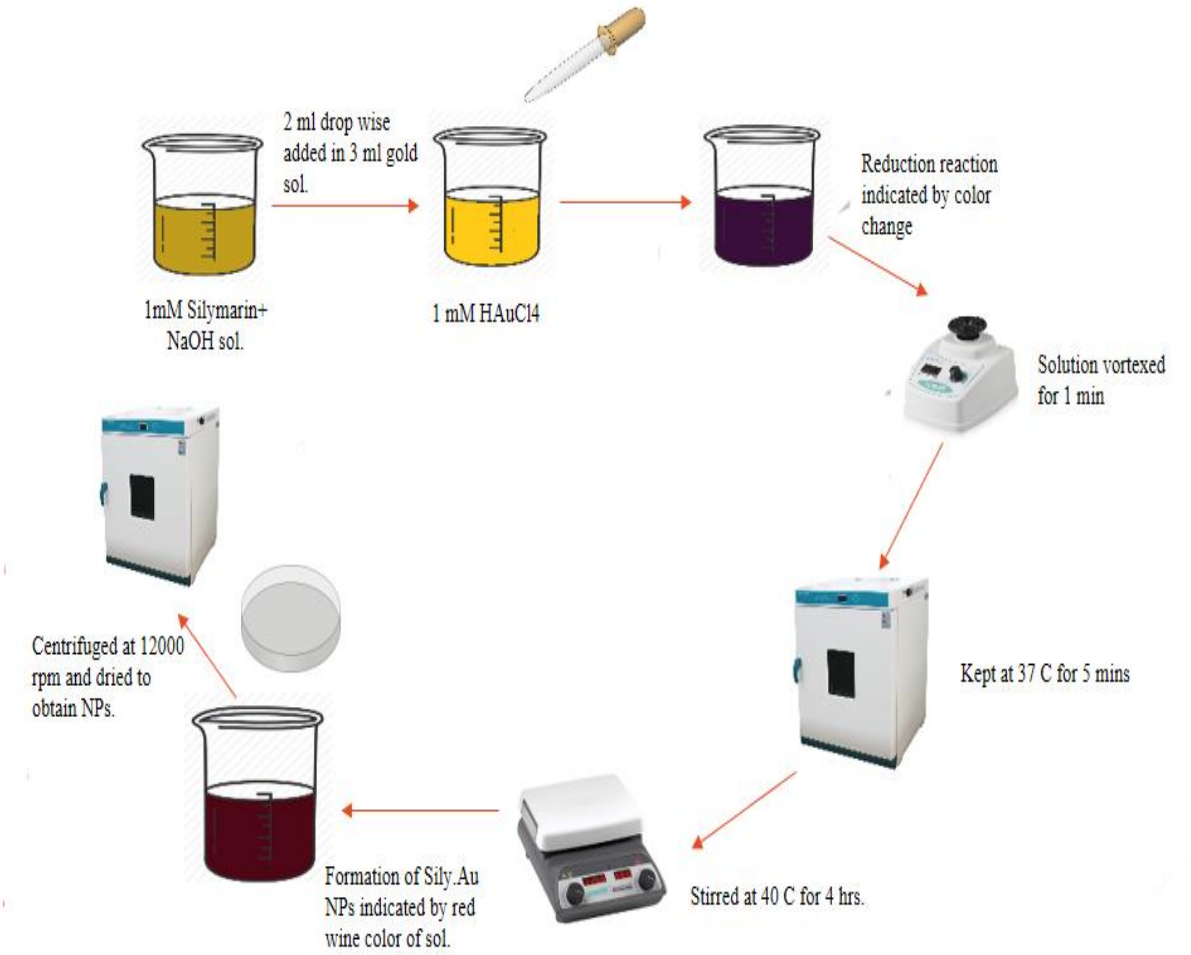
Recent advances in nanotechnology, has intrigued researchers to explore biosynthesis or green synthesis of nanoparticles using plant extracts. In green synthesis, plant extracts or flavonoids acting as reducing agent assist in formation of nanoparticles. Synthesis of nanoparticles by

using plant extracts is not only very cost effective and economical but also very helpful in large scale production of metal nanoparticles. Bio-compounds present in plants such as protein, amino acids, polysaccharides, vitamins and citrates are used for bioreduction of metals, yielding nanoparticles with significant therapeutic effects.

CHAPTER 3: MATERIAL AND METHODS

3.1) Green Synthesis of Silymarin Gold Nanoparticles:

Silymarin gold nanoparticles were prepared by green synthesis method. 1mM solution of Silymarin was prepared in 1mM aqueous solution of NaOH. NaOH was added to increase solubility of Silymarin in water. The resultant mixture was added drop wise in 1mM aqueous solution of tetrachloroauric acid. Silymarin itself works as a reducing agent. Reduction of gold salt was visible by change in colour of solution from light yellow to purple. The resultant mixture was vortexed for 3 minutes and kept in incubation at 37 C for 5 mins. Later the mixture was set for stirring at 150 rpm for 4 hrs at 41 C. A clear red wine solution was observed at the end of reaction. Formation of nanoparticles was confirmed by UV-VS spectroscopy. The optimum ratio for nanoparticles was found at 3:2 (Au:Silymarin).



3.1) Reaction Optimization:

Reaction mentioned above was optimized by performing multiple stability assays. Nanoparticles were subjected to different pH, temperature variation and by changing concentration of silymarin.

i) Temperature Analysis:

10 ml of nanoparticles solution was taken in a round bottom flask and heated up to 100 C for 30 mins. Readings were taken at 40, 50, 60, 70 and 80 C in UV-VS spectrophotometers to observe the effect of heat on nanoparticles. 5 ml of nanoparticles were kept at 0 C for 30 mins to observe the effect of extremely low temperature on their stability.

ii) pH Analysis:

Silymarin Gold nanoparticles have a 12 pH. Nanoparticles were subjected to an acidic treatment, in order to observe their pH at 2, 9 and 11 points. Effect on nanoparticles was observed by UV-VS spectrophotometers.

iii) Concentration Analysis:

The effect of Silymarin concentration on nanoparticles was observed by preparing Silymarin Gold nanoparticles on different silymarin concentrations (1- 4 mM). Results were observed and noted via UV-VS spectrophotometers.

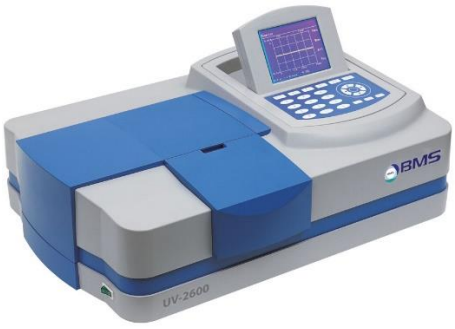
3.2) Characterization of Silymarin Gold Nanoparticles:

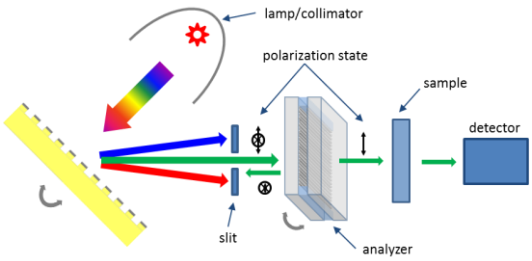
1) Ultraviolet Visible Spectroscopy Analysis:

Reduction of pure gold ions was observed by noting the UV spectrum of the reaction after 4 hours.

UV-Vis absorption spectroscopy is one of the most commonly used techniques at both medical and industrial scales. It is the measurement of the absorption capacity of a sample when it is subjected to a beam of light passing through it. Reflection of light beam indicates the absorption capacity of the sample. It works on the principle of dividing the light beam in two halves. The first half if the beam passes through the cuvette containing reference (solvent only) and the second half of light is directed towards the cuvette containing sample. Results can be observed and measured at a specific wavelength, within required range. The resultant spectrum is plotted on wavelength against absorbance, indicating absorbance of sample at a specific wavelength. Absorbance peak at a specific wavelength is called Lambda max.

Mechanism of UV-Vis spectrophotometer is followed by principle of Beer Lambert Law that states **A=EcL or E= A/cL**, where A = absorbance, c= concentration of sample, L = length of light path through cuvette in cm and E = molar absorptivity. Working on this principle, UV – Vis spectrophotometer is used to measure electronic transition of molecules. Molar concentration of sample is proportional to the absorbance od the sample. Thus, the absorption value, also known as molar absorptivity is used to compare different compounds.

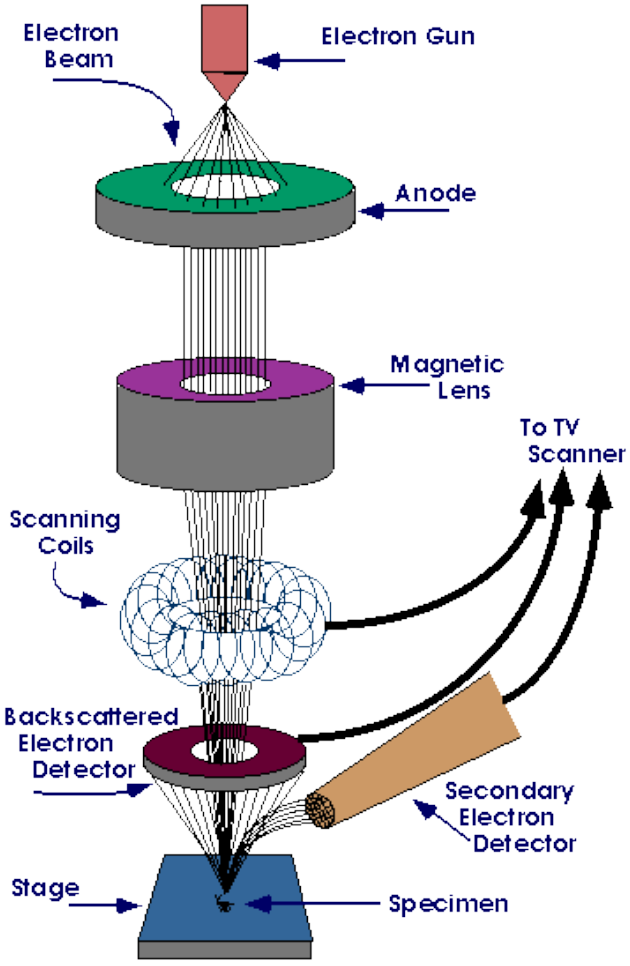




2) Scanning Electron Microscopy:

A scanning electron microscope is capable to generate results of sample in image form. The information regarding topography and surface composition of sample is generated when a beam of electrons is interacted with the surface of sample. Signals are generated when this beam of electrons is scanned upon a sample in a raster pattern. Signals mixed with the position of beam yield result as an image indicating structural and superficial information of the sample.

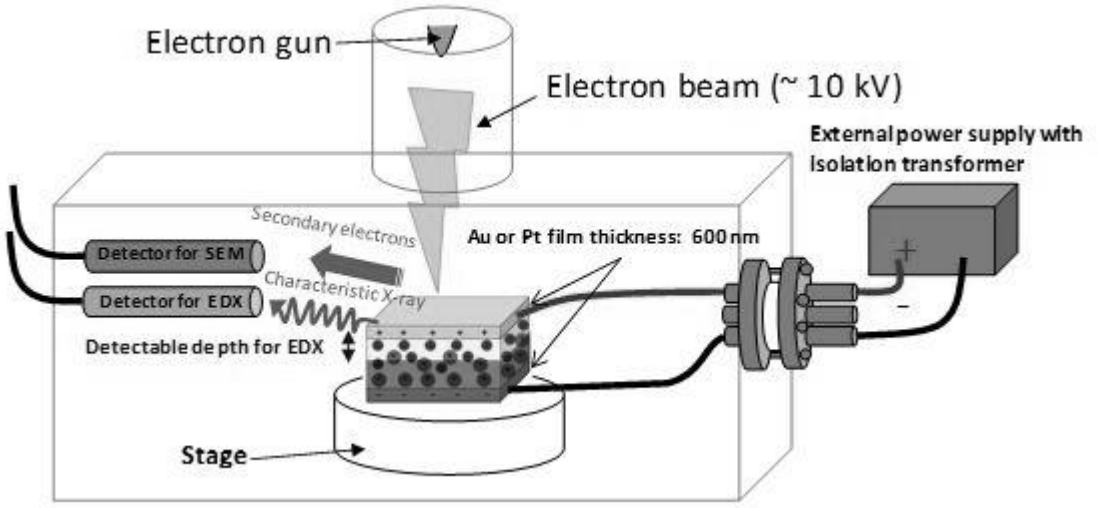
The analysis was performed on a software controlled Scanned Electron Microscope. For examination of sample the energy of electron beam was continuously adjusted between 1pA to 1µA to find a suitable beam for analysis. The sample was coated drop wise on a carbon coated copper stub and dried under mercury lamp. Each sample was analyzed by SEM.



3) Energy Dispersive X-Ray spectroscopy:

Energy dispersive X-ray spectroscopy is used for the elemental analysis and chemical characterization of sample. It works on the interaction of X-Rays with sample. Sample particles are excited by subjecting them to a beam of electrons or protons. Upon excitation an electron, which is otherwise in a grounded state bound to nucleus, ejects from the inner shell and leaves hole. Electron from the outer shell fills in the hole and the energy difference between high energy shell and low energy shell is generated in form of X-ray. This difference is measured by an energy-dispersive spectrophotometer. Since the difference of energy based on stomic structure of the emitting element, EDS allows the elemental composition of the specimen to be measured.

Nanoparticles were dried on a carbon coated copper stub and used for the EDS analysis. Since Silymarin has C, O and H as the major components group their presence and percentage weightage combined was observed to confirm presence of Silymarin and gold was observed on plot.

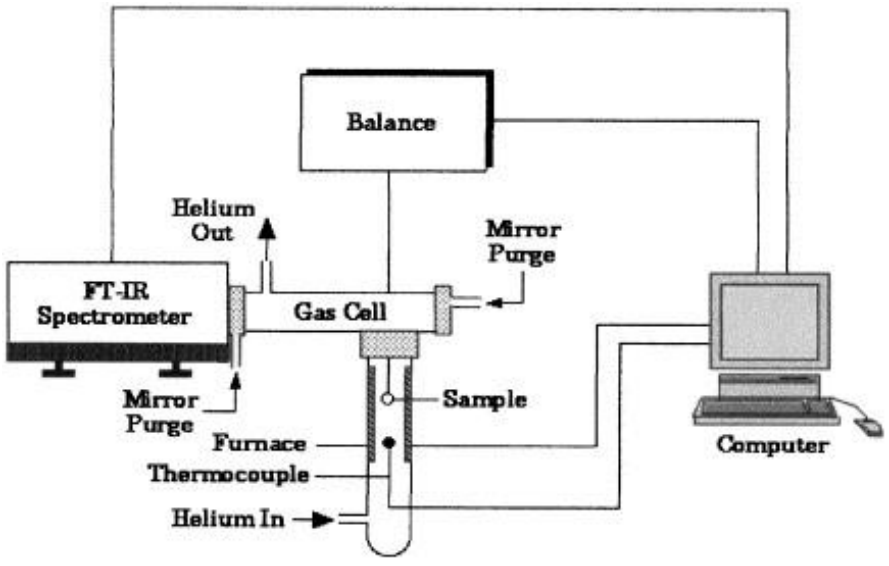


4) Fourier transform infrared spectroscopy (FTIR) Analysis:

The functional group detection, formation and chemical comparison between pure silymarin and silymarin gold nanoparticles was observed through Fourier transform infrared spectroscopy (FTIR). The spectrum was obtained between 500-4000 cm-1. It was obtained by using attenuated reflectance technique. Dried silymarin gold nanoparticles sample was placed on KBr crystals and spectrum was obtained in transmittance mode.

Fourier transform infrared spectroscopy (FTIR) is one of the most widely used characterization technique used to detect functional groups in pure compounds, as mixtures or for comparative analysis.

Spectrum of emission, absorption, photoconductivity or Raman scattering of a solid, liquid or gas compound can be recorded on FTIR. It is also very commonly used for the characterization of nanoparticles and for the surface analysis. Since there are different surface absorbents present on the surface of nanoparticles, they generate peaks in a FTIR spectrum, in comparison to the sample with no absorbents present in it. Therefore, FTIR can conveniently detect minimal surface changes. It works on a complicated electrical and mechanical system that can sense every change in energy absorbed by surface. This helps in generation of highly precise results. It can be used for the analysis of a wide range of materials even for fiber, pastes, powders, films or bulks. It can do both qualitative and quantitative analysis as indicated by size of peaks. The frequency of absorption is detected on basis of atomic mass, shape of surface and vibronic coupling associated with it.



3.2) Antioxidant Activity of Silymarin and Silymarin Gold Nanoparticles:

1) DPPH Assay:

Silymarin as a reducing agent has an excellent potential to serve as one of the naturally occurring anti-oxidant which can be used against multiple diseases caused by toxic oxides. To determine and compare the percentage anti-oxidant activity of Silymarin and its gold nanoparticles DPPH assay was used in this study. DPPH assay is one of the most famous methods for testing antioxidant capacity of natural products. Major reason for adopting this method is because its simple and sensitive. DPPH (originally known as 2,2-Diphenyl-1-picrylhydrazyl) is one of the commercially available, stable organic nitrogen radical. The method indicates reaction via change in color of DPPH solution from purple to yellow, which shows the formation of DPPH through absorption of hydrogen from an antioxidant. Since DPPH indicates strong absorption at 517 nm the antioxidant effect can be easily evaluated by using the decrease of UV absorption at 517 nm.

2) Antioxidant activity:

Following steps were performed to determine antioxidant activity of silymarin gold nanoparticles

3) Preparation of Silymarin Gold Nanoparticles

Silymarin gold nanoparticles were prepared by green synthesis method as mentioned above. Nanoparticles were prepared with silymarin concentration ranging from 1mM – 4mM. The gold to silymarin ratio remained same in all concentrations (i.e 3:2 Au:Silymarin)

4) Determination of Anti-Oxidant Activity of Gold Nanoparticles by DPPH Assay:

The antioxidant activity of silymarin gold nanoparticles was determined by the stable DPPH assay. DPPH assay is based on the reduction of an alcoholic DPPH solution in the presence of reducing agent lead to the formation of a stable non-radical form of DPPH-H molecule.

The nanoparticle samples were dissolved in methanolic solution at different concentrations (100-400 ug/ ul) then each diluted sample was added in to 500 ul of DPPH solutions in

Eppendorf's tubes. This mixture of nanoparticles and DPPH assay was incubated for 45 minutes in the dark at room temperature. Absorbance of resultant solution was taken at **517 nm** in UV-Vis spectrophotometer. Ascorbic acid was used as positive control whereas deionized water was used as negative control. The ability of nanoparticles to scavenge DPPH radical was measured by using following equation:

DPPH scavenging capacity (in percentage) = [A0 – A1/A0] x 100

Where A0 denoted the absorbance of control whereas A1 was taken as the absorbance of sample.

4. Hemolytic Assay:

Determination of hemolytic properties of nanoparticles is most important test for products which interact with blood components. For in-vitro analysis, hemolysis is evaluated by spectrophotometer by the detection of plasma free hemoglobin after the incubation of particles with blood and separation of undamaged cells through centrifugation. The results are determined by incubation time, blood conditions and wavelength used to test hemoglobin. These conditions vary from study to study. Hemolytic assay was performed through following steps:

4.1) Specimen:

12 ml of blood sample was taken from a healthy human volunteer who did not have any history of taking oral contraceptive or anti-coagulant drug.

4.2) Hemolytic Assay:

Silymarin Gold nanoparticles were prepared at different concentration of silymarin (as explained in 3.3.2). 4 ml of blood sample was taken and mixed with 8ml of phosphate buffer saline (PBS) solution. The resultant mixture was centrifuged at 10000 rpm for 10 mins. Pallet obtained was washed 3 times with PBS solution for 3 minutes at 1000 rpm. RBCs obtained were diluted with PBS solution again. 500 ul of RBC suspension was added to 500 ul of Silymarin Gold nanoparticles solution of (100-400 ug/ml) different concentrations. After 1 hour of incubation, samples were vortexed and centrifuged at 10000 rpm for 5 minutes. Supernatant obtained was collected and absorbance as taken at 550 nm. Phosphate buffer saline and 0.5% Triton X-100 were used as negative and positive control.

5. Thrombolytic Activity of Silymarin & Silymarin Gold Nanoparticles:

5.1) Thrombolytic Activity of Silymarin (*In-vivo)*

In vivo thrombolytic assay to determine thrombolytic properties of Silymarin was performed on Balb C. mice. Mice were anaesthetized and fixed in supine position. Silymarin and Silymarin Gold nanoparticles were injected through tail vein and aspirin was administered intragastrically. A cervical incision was made in the midline to expose left carotid artery and right jugular vein. 12 cm long polyethylene tube with a 6 cm long silk thread was fixed in its lumen which was filled with physiological saline. One end of the tube was inserted in to the right jugular vein. The proximal side of left carotid artery was clamped to block the blood flow. The free end of the tube was inserted in to artery and tied. Clamp was released to assure the blood flow through tube. After 20 minutes the thread was removed from the and its wet weight was immediately measured. Dry weight was measured after 20 mins at 60 C. The amount of thrombus formed was defined as the wet weight or as the dry weight minus the weight of silk thread. Streptokinase and saline water were used as positive control and negative control respectively.

5.2) Thrombolytic Activity of Silymarin and Silymarin Gold Nanoparticles (*In-vitro)*

5.2.1) Specimen:

12 ml of blood sample was collected from a healthy male and female human volunteer who had no history of taking oral contraceptive or anti-coagulant.

5.2.2) Thrombolytic Assay:

500 ul of blood sample was taken in each of previously weighed 20 Eppendorf's tubes. These tubes were set for incubation for 90 minutes. After incubation, clot formation was observed, and superficial serum was carefully removed from the tubes without disturbing clot. Tubes were weighed again to note the weight of clot. Weight of clot was determined by:

Weight of tubes with clot – weight of empty tubes

Silymarin and Silymarin gold nanoparticles were diluted (100-400 ug/ml) and stock solution was added in tubes. The resultant mixture was again set for incubation for 45

minutes. After 45 minutes serum was decanted, and tubes were weighed again. The difference in current weight of tubes in comparison to tubes weight with clots indicated the thrombolytic activity of nanoparticles. Streptokinase and distilled water was used as positive and negative control.

CHAPTER 4: RESULTS

In this study, hemolytic activity, thrombolytic activity and anti-oxidant activity of Silymarin was tested. Silymarin Gold Nanoparticles were also synthesized and tested for their thrombolytic properties, their hemolytic effect and for their antioxidant properties.

4.1 Characterization of Silymarin Gold Nanoparticles:

4.1.1 Ultraviolet Visible Spectroscopy:

UV- Spectroscopy is one of the most commonly used characterization techniques used in clinical, industrial and chemical laboratories. It works on the principle of absorption of light beam when passed through sample, reflecting the remaining beam. The reduction of pure gold ions can be detected through this technique, which can be used as a tool to predict and analyze formation of nanoparticles.

In this study, Silymarin Gold Nanoparticles were prepared through green synthesis method, in which Gold to Silymarin ratio was 3:2 respectively. Formation of nanoparticles was confirmed by UV Spectroscopy. The device used for this method was Single Beam Spectrophotometer (UV BMS – 2800). Peak confirming formation of nanoparticles was observed at 520 nm.

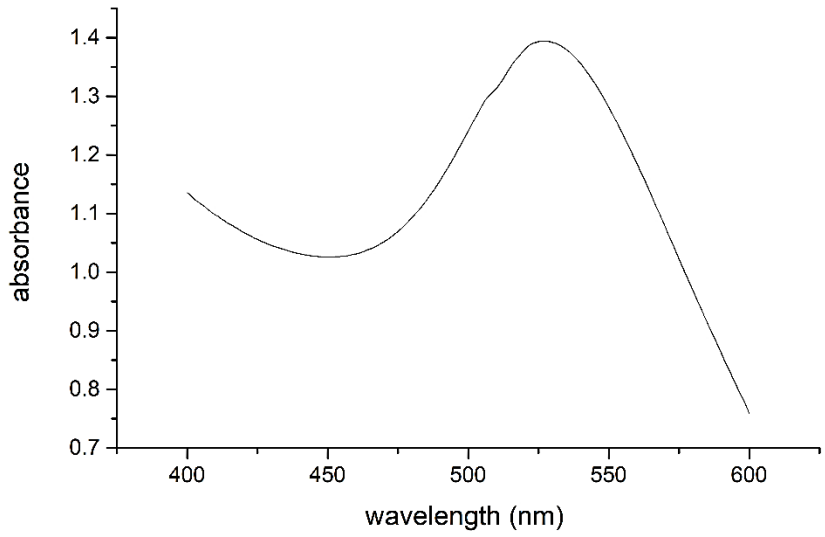


Figure 4.1 UV Spectrum of Silymarin Gold Nanoparticles

4.1.2 Scanning Electron Microscope (SEM) Analysis:

Scanning electron microscope is one of the most sophisticated characterization techniques used in nanoscience to characterize surface properties and size of particles. Scanning electron microscope generate a beam of electron which when interacts with sample generates a secondary electron beam backscattered with X- Ray characteristics. This interaction yields results in image form on screen, showing surface properties of particle.

Scanning Electron Microscope Analysis of Silymarin Gold Particles was performed at NUST US- Center for Advance Energy Systems, through TESCAN VEGA3 SEM system.

Analysis of silymarin gold nanoparticles showed conjugation when dried and treated with gold spray generating following image.

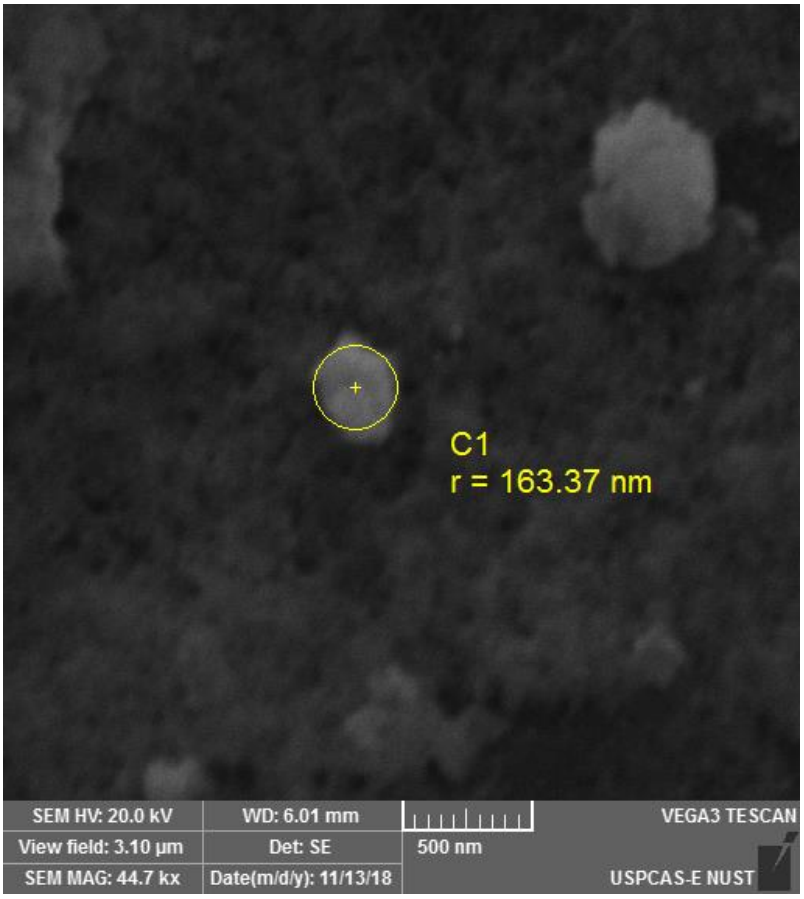


Figure 4.2 SEM Analysis of Silymarin Gold Nanoparticles

4.1.3 Fourier Transformation Infrared Spectroscopy (FTIR):

Fourier transform infrared spectroscopy is a widely used characterization technique used for pure chemical compounds to detect their functional group. An infrared spectrum of absorption, emission, photoconductivity or Raman scattering of a compound in any existing state of solid, liquid or gas is recorded.

FTIR of silymarin and silymarin gold nanoparticles have given significant peaks at 3000 cm-1 confirming presence of O-H carboxylic acid group. Another significant peak at 1450 cm-1 indicates aromatic C=C group presence. Peak at 1000 indicates presence of C=C bonding.

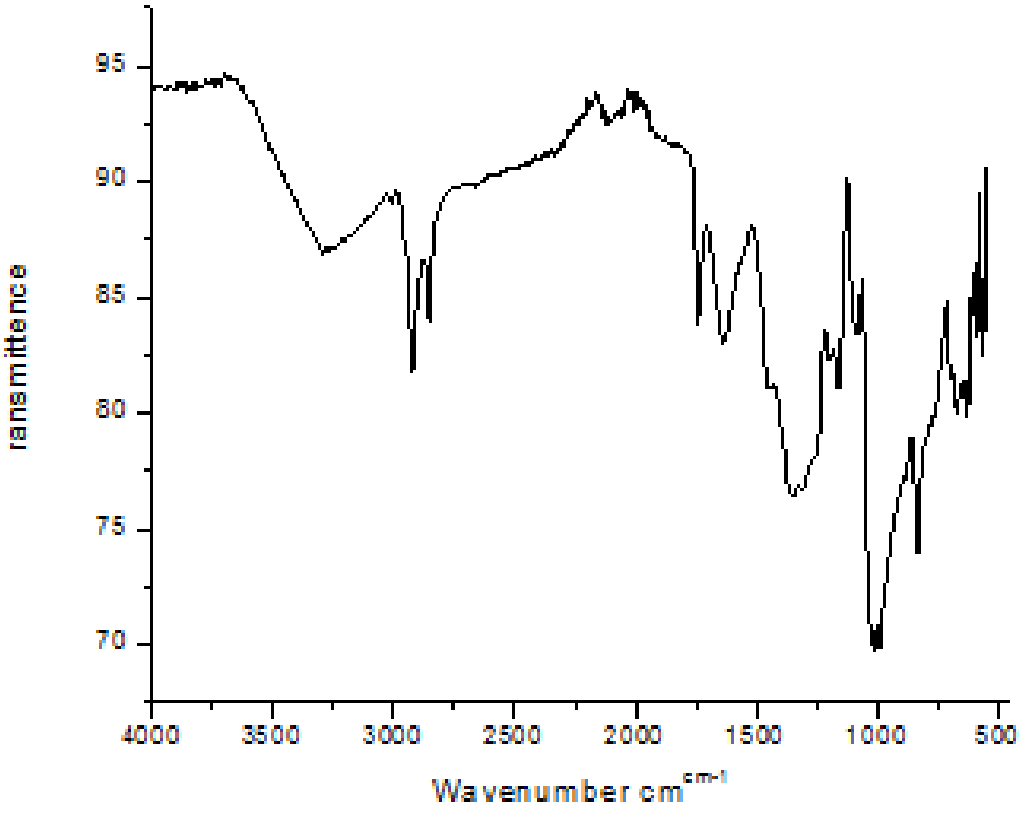


Figure 4.3 FTIR Analysis of Silymarin Gold Nanoparticles

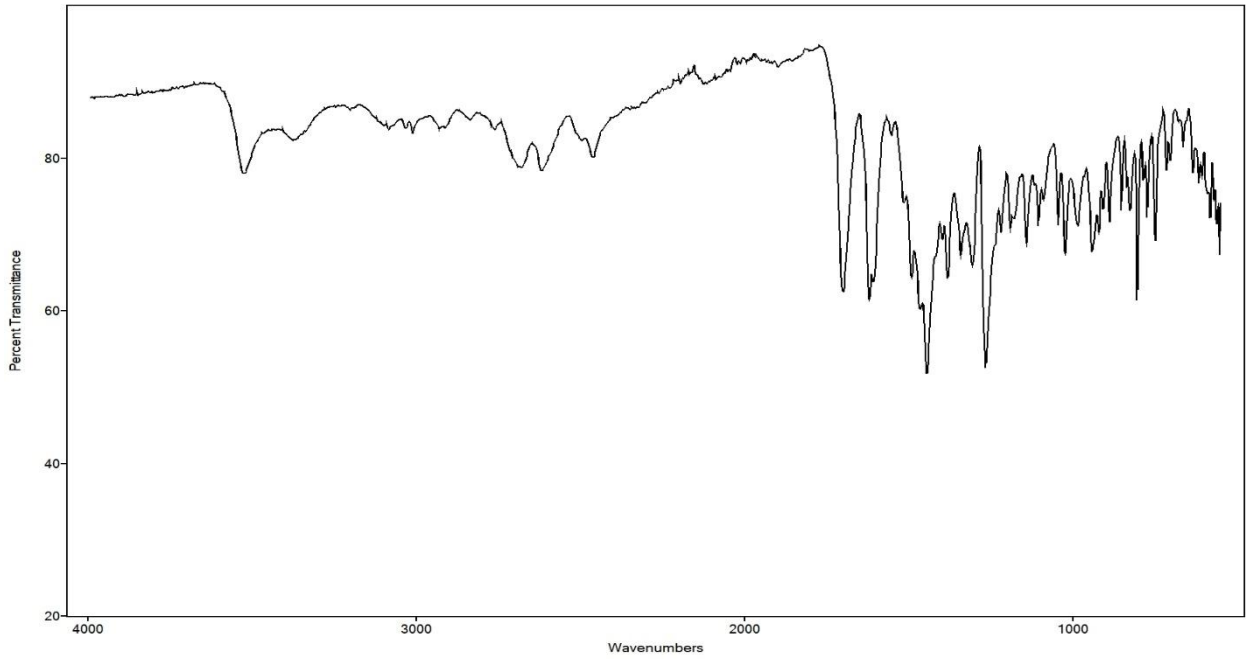


Figure 4.4 FTIR of Silymarin powder purchased commercially

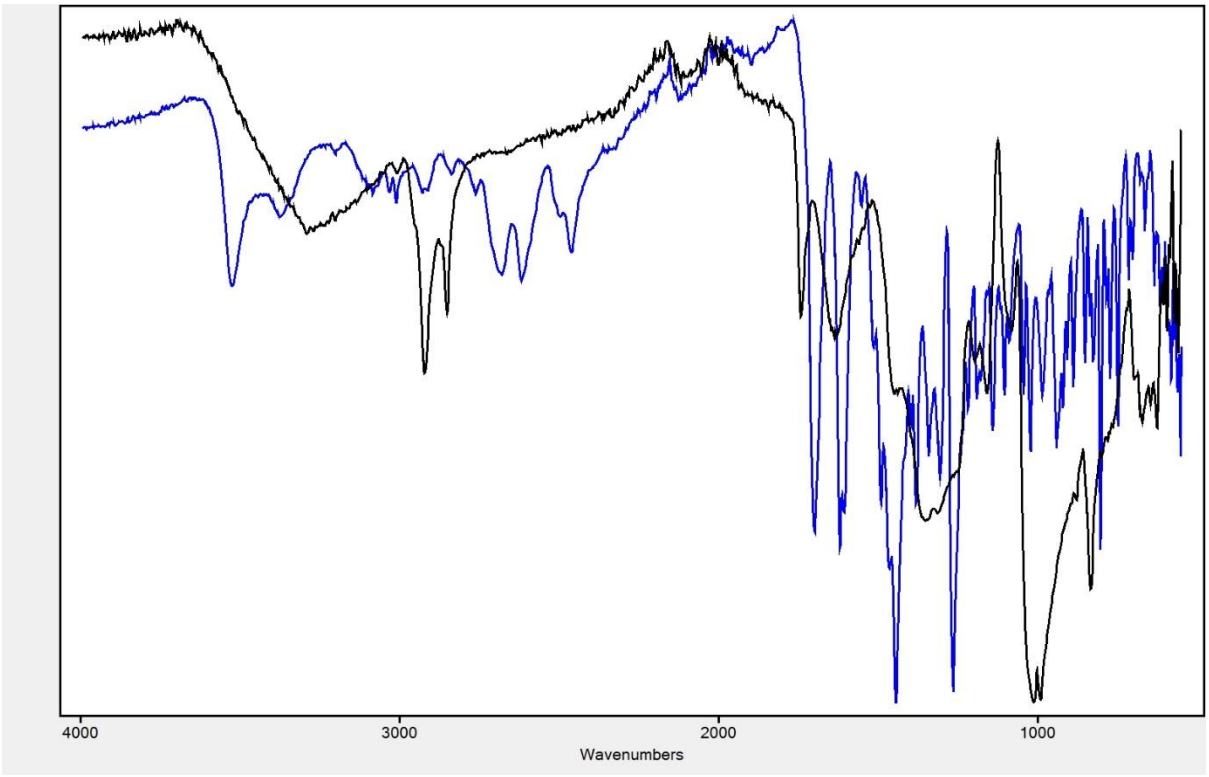


Figure 4.5 Comparison between Silymarin and Silymarin Gold Nanoparticles

4.1.4 EDS Analysis:

EDS analysis along with SEM was done to confirm and analyze elemental characteristics of Silymarin Gold Nanoparticles.

Silymarin, as a flavonoid is organic compound with carboxyl, phenols and OH groups attached with it. In image below, presence of Carbon, Oxygen and Gold was confirmed. Atomic percentage of Carbon was recorded as 33% Oxygen was recorded as 37% where as gold present in sample was recorded at 14 %.

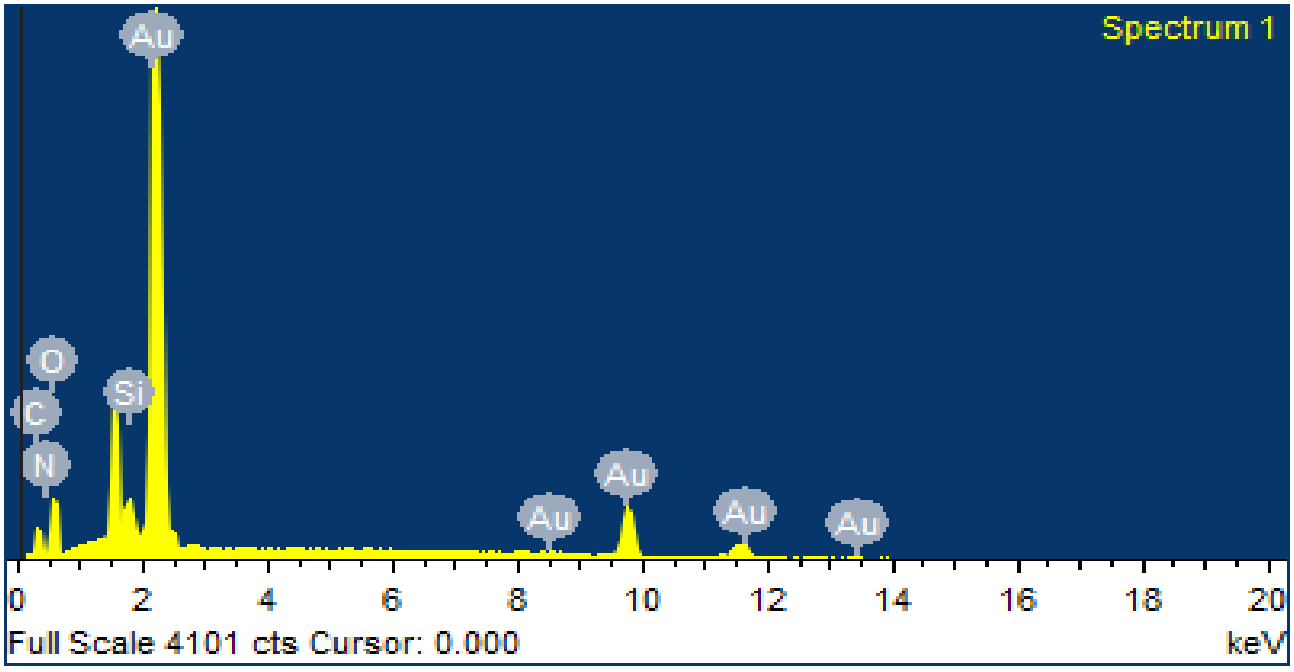


Figure 4.6 EDS of Silymarin Gold Nanoparticles

4.2 Optimization of Reaction:

Silymarin Nanoparticles were prepared by using green synthesis method. Reaction was optimized at 3:2 Gold to Silymarin ratio. Temperature, pH and silymarin concentration was tested and optimized by performing series of experiments.

4.2.1 Temperature Optimization:

Nanoparticles were prepared at different temperature settings to observe change in stability and properties to determine their response in drastic temperature changes. Nanoparticles showed increase in stability and a sharper peak at UV spectrum when treated with highest temperature.

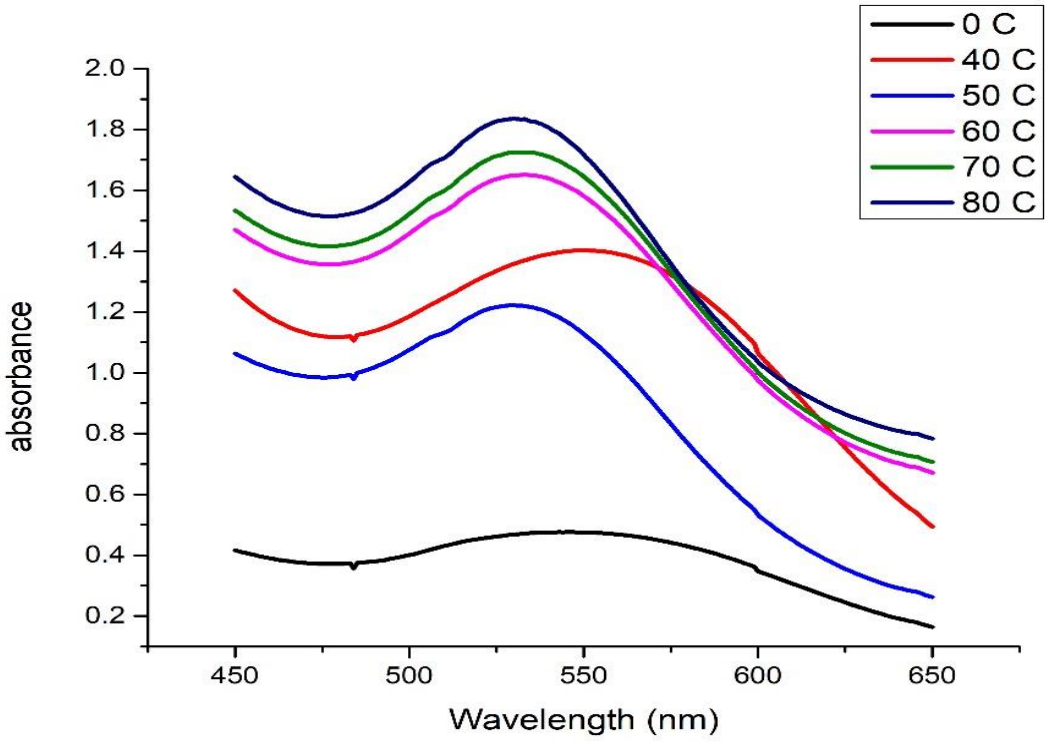


Figure 4.7 UV Analysis of Silymarin Gold Nanoparticles treated with different temperature

4.2.2 Silymarin concentration optimization:

Silymarin gold nanoparticles were prepared by changing molarity of Silymarin (1mM – 4mM) to observe the effect of change in concentration through UV analysis. A constant shift in peak from 520 to 550 was observed by increasing the concentration of silymarin. Concentration was optimized at 2mM silymarin.

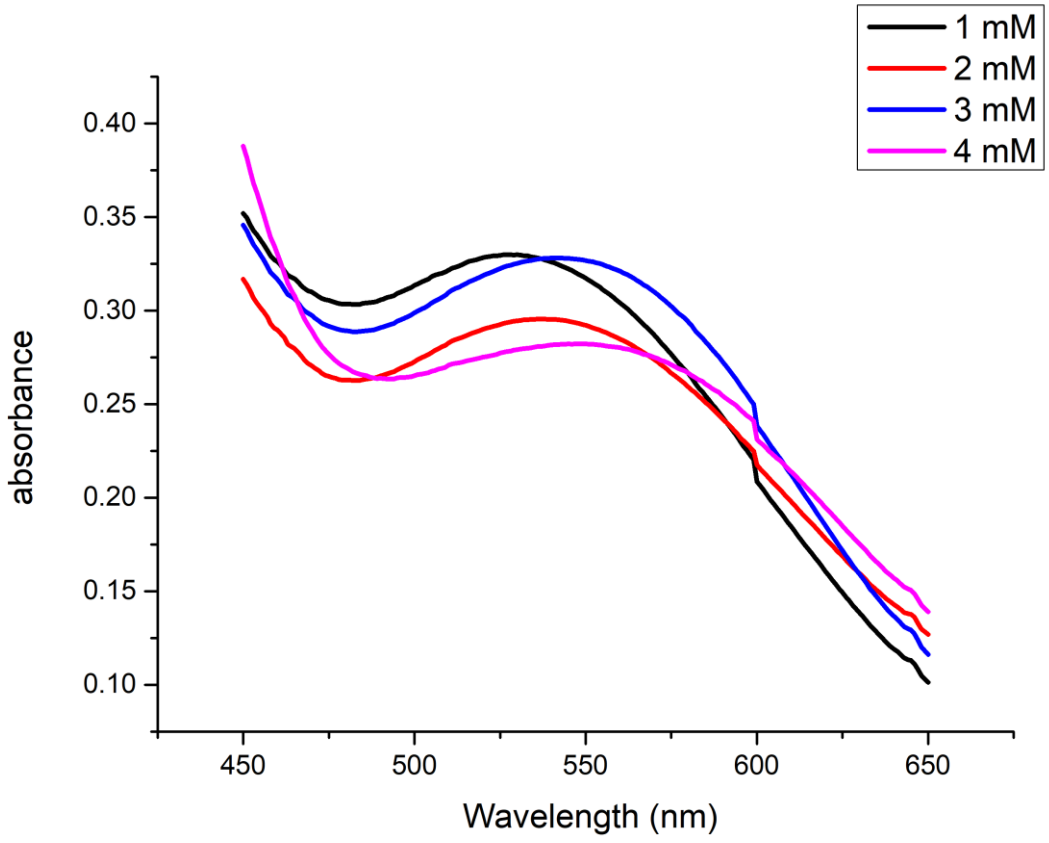


Figure 4.8 UV-Analysis of Silymarin Gold Nanoparticles prepared at different Silymarin concentration

4.2.3 Effect of change in pH on Silymarin Gold Nanoparticles:

Effect of change in pH was observed on Silymarin Gold Nanoparticles by changing pH to different acid to basic parameters. Effect on nanoparticles was observed through UV-Spectrophotometer analysis.

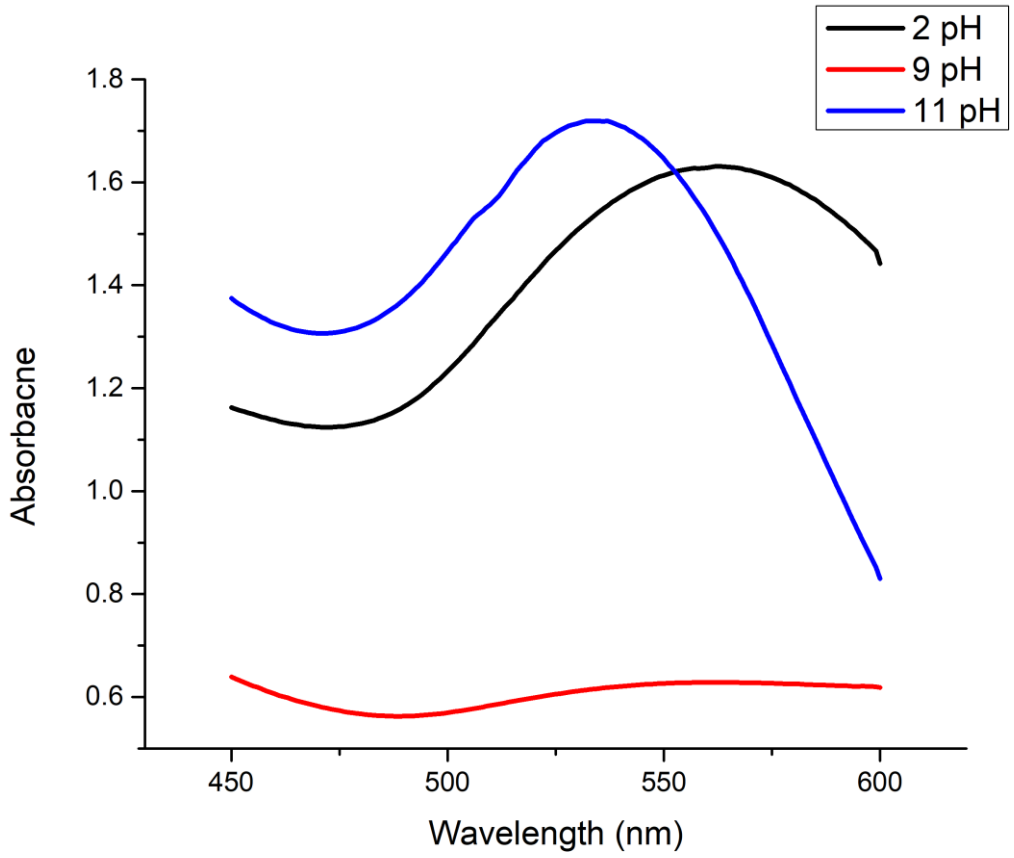


Figure 4.9 Effect of change in pH on Silymarin Gold Nanoparticles

4.3 Hemolytic Assay:

Effect of Silymarin and Silymarin gold nanoparticles on RBCs was observed through hemolytic assay to determine the cytotoxic effect of nanoparticles.

Silymarin itself showed the highest tendency of 24.5% hemolysis at 125 ug/ml concentration, whereas hemolytic effect of silymarin gold nanoparticles was comparatively lower of 9% at same ratio.

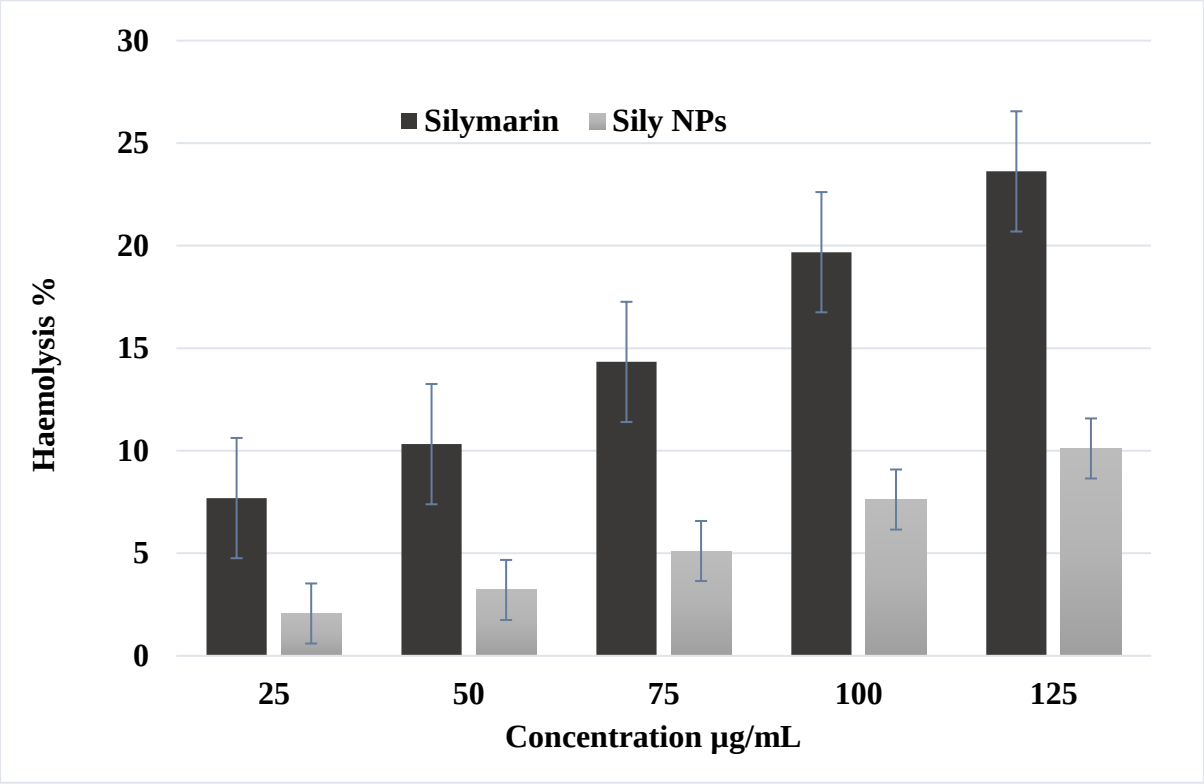


Figure 4.10 Hemolytic Effect of Silymarin and Silymarin Gold Nanoparticles at different concentrations

4.4 Antioxidant Scavenging Activity of Silymarin and Silymarin Gold Nanoparticles:

DPPH assay was used to test the antioxidant activity of Silymarin and Silymarin Gold nanoparticles.

Ascorbic acid was used as positive control and distilled water was used as negative control. Silymarin showed antioxidant activity up to 40% on average, whereas antioxidant activity of Silymarin Gold Nanoparticles was as high as 70% when added at 1 ug/ml concentration in to DPPH solution.

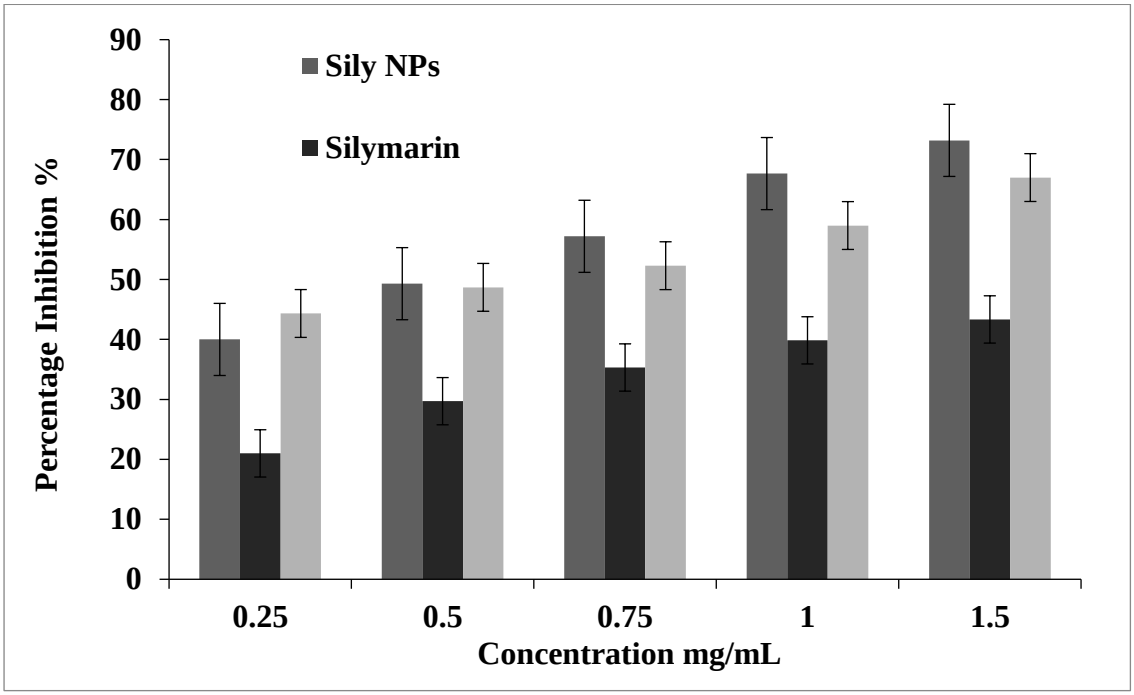


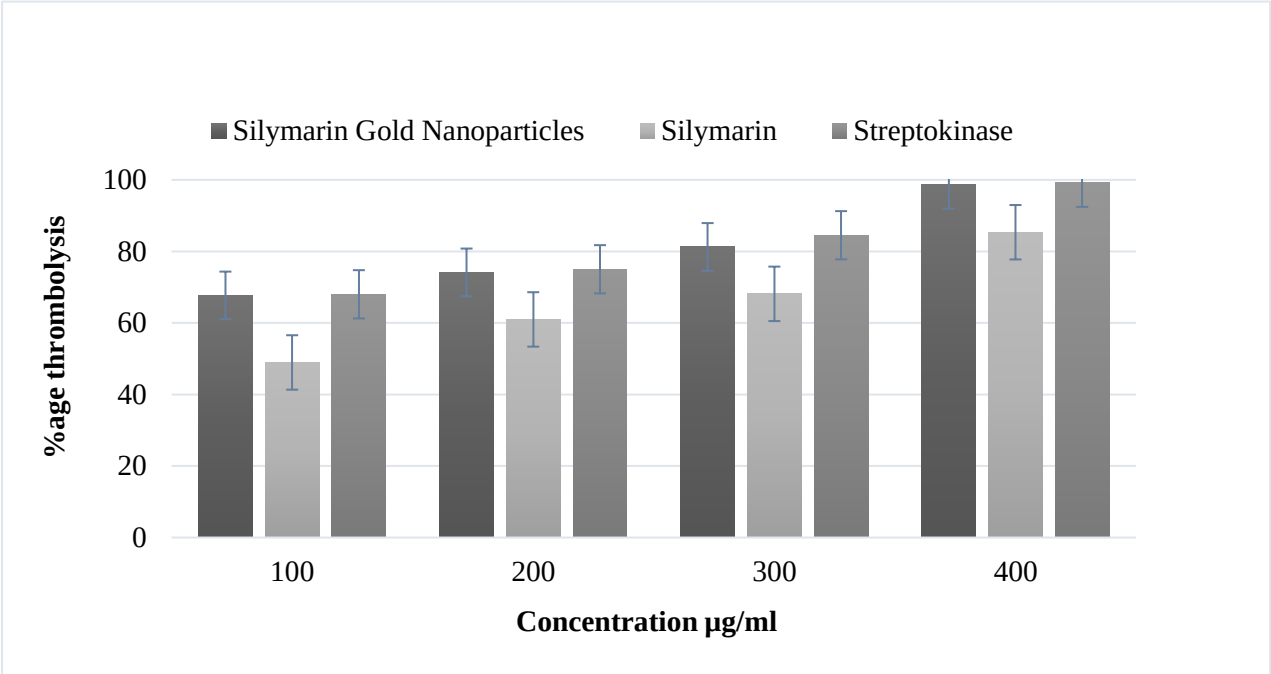
Figure 4.11 DPPH activity of Silymarin and Silymarin Gold Nanoparticles

4.5 Thrombolytic Activity of Silymarin and Silymarin Gold Nanoparticles:

Thrombolytic Activity of Silymarin and Silymarin gold nanoparticles was determined in-vitro by using clot lysis method on blood sample tested in Eppendorf's tubes. Streptokinase (150,00,000 UI) was used as positive control while distilled water was used as negative control.

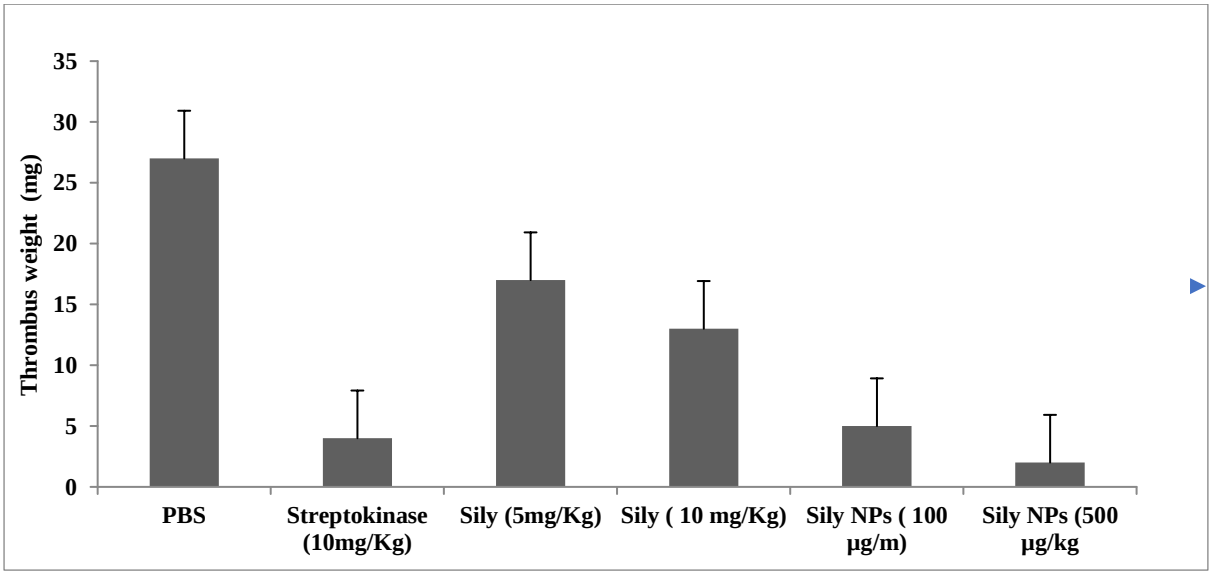
Clot lysis shown by silymarin was highest at 78.8 % when added at concentration of 400 µg/ml.

In comparison to Silymarin, Silymarin gold nanoparticles have shown impressive potential as a thrombolytic agent with clot lysis capacity up to 97%. Streptokinase showed clot lysis at up to 99%.



4.6 Arterio-venous shunt model in rats (In-vivo):

Thrombolytic activity was determined by weighing thrombous formed on silk thread. PBS showed no thrombolytic activity with highest thrombus weight. Weight of thrombus when treated with Silymarin Gold Nanoparticles was reduced to 5 mg. Confirming the effective thrombolytic ability of Silymarin Gold nanoparticles.



CHAPTER 5: CONCLUSION

Silymarin has been used as an ancient medicine since ancient times. The plant has numerous properties that incorporates cancer prevention agent and thrombolytic properties, the nanoparticles of this plant have preferred cell reinforcement and thrombolytic properties over the insignificant plant seed separates.

The antioxidant activity of Silymarin was compared with Ascorbic acid which is used as a standard for antioxidant activity. Silymarin gold nanoparticles have shown higher antioxidant activity in comparison to Silymarin itself.

UV-Vis spectroscopy of Silymarin gold nanoparticles showed SPR peak at 520 nm confirming the formation of gold nanoparticles. SEM image wasn't clear to indicate any significant results due to lack of appropriate handling, however other analysis run on nanoparticles confirmed their presence.

The FTIR spectrum of Silymarin indicated presences of phenol group, carboxylic acid and aromatic group. Significant peaks of Silymarin nanoparticles were observed at 1000 cm-1, 3000 cm-1 and 680 cm-1, which confirmed presence of primary amines and vinyl group respectively.

The hemolytic activity of Silymarin and Silymarin gold nanoparticles indicated that in comparison to direct administration of silymarin as a flavonolignants, silymarin gold nanoparticles have less toxic effect on RBCs.

Antioxidant analysis have shown a difference of 30% effectivity of silymarin gold nanoparticles with Silymarin having a radical scavenging activity at 40%, ascorbic acid at 87% and silymarin gold nanoparticles at 70%.

Thrombolytic properties of Silymarin gold nanoparticles have shown their activity as effective as Streptokinase. Silymarin itself have shown up to 60% clot lysis, indicating its potential as a thrombolytic herb.

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