"Effect of *Berberis lycium* flavonoids on colonization of *Enterococcus faecalis* in mice"

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A thesis submitted in partial fulfillment of the requirement for the degree of Masters of Science

In

Biomedical Sciences and Engineering

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DECLARATION

It is hereby declared that this research study has been done for partial fulfillment of requirements for the degree of Master of Sciences in Biomedical Engineering and Sciences. This work has not been taken from any publication. I hereby also declare that no portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification in this university or other institute of learning.

Sundus Riaz

CERTIFICATE OF APPROVAL TH-4

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My parents and siblings; without their encouragement and support, I would not have been able to successfully complete this research.

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PUBLISHED WORK:

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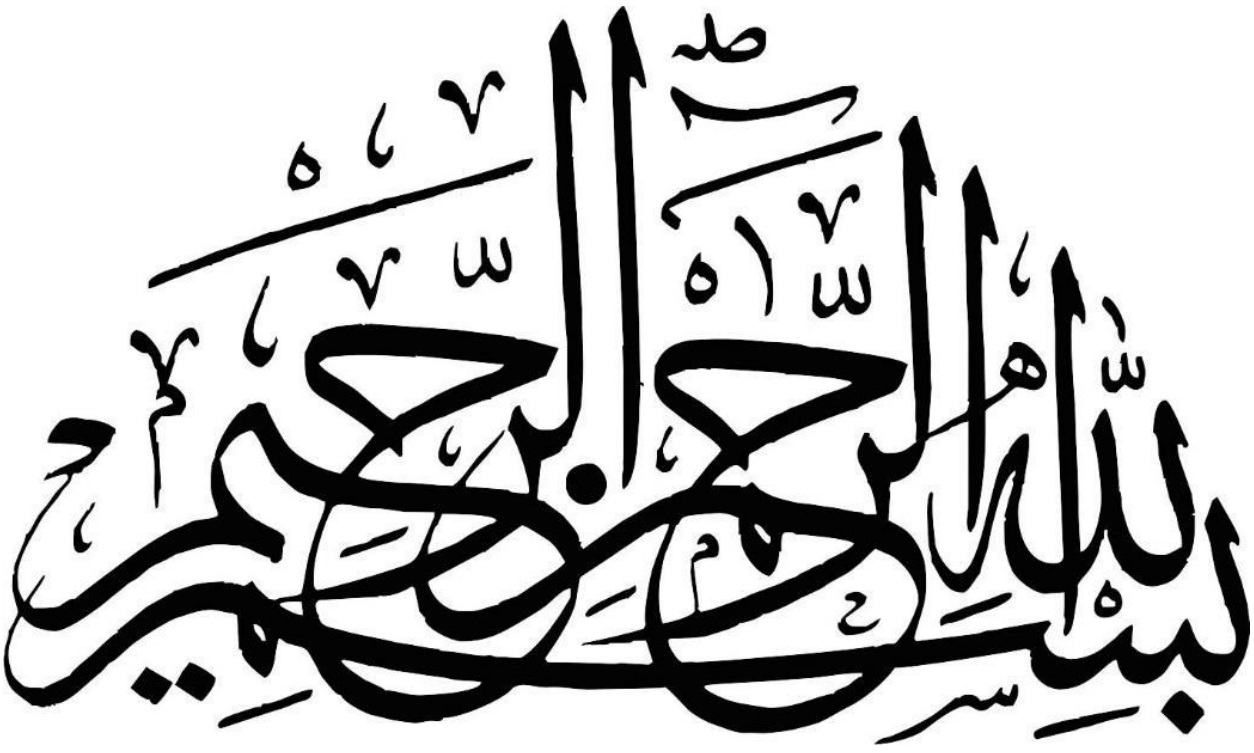
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Notation	Explanation
AuNPs	Gold Nanoparticles
FAuNPs	Flavonoids gold nanoparticles
XRD	X-ray Diffraction
FTIR	Fourier Transform Infrared Spectroscopy
UV-Vis	Ultra Violet Visible
TEM	Transmission Electron Microscopy
EDAX	Energy Dispersive Analysis of X-rays
°C	Degree Celsius
CFU	Colony Forming Unit
DMSO	Dimethylsulfoxide
MDR	Multi drug resistant

ABSTRACT

Enterococcus faecalis is a highly opportunistic multidrug resistant and common nosocomial infection causing pathogen. The study aimed to evaluate the effectiveness of *Berberis lycium* flavonoids and its gold nanoparticles on the colonization of *E. faecalis* in Liver and Kidneys of infected mice. An efficient synthesis of stable flavonoids gold nanoparticles (FAuNPs) was manifested by using ethyl acetate fraction of *Berberis lycium*. Biologically synthesized FAuNPs were characterized by UV-Vis spectroscopy, FTIR, XRD, EDAX and TEM. The potential of flavonoids and FAuNPs was investigated by *in-vitro* hemolytic activity before in-vivo testing. Characterization of FAuNPs revealed the average size of FAuNPs to be 23 nm. In vitro studies showed broad spectrum activity of flavonoids against various clinical pathogenic Gram positive and Gram negative bacteria. Hemolytic assay revealed biocompatibility and non-cytotoxicity of FAuNPs up to a concentration of 150µg/mL. FAuNPs were more potent with less effective dose (400µg/Kg) than isolated flavonoids (5mg/Kg), concluding that FAuNPs has remarkably increased the bio-efficacy, stability and bioavailability of flavonoids.

CHAPTER 1: INTRODUCTION

Nano materials used for drug delivery, as they can move inside the body to repair damaged tissues, cross blood brain barriers and can access those cells and tissues where other drugs/antibodies cannot be used as therapeutic agents **[1]**. Among nanoparticles, use of gold nanoparticles in research has a broad spectrum in the field of biomedical diagnostics, photothermal and photodynamic therapies, medicine, biosensorics, prophylaxis, and targeted drug delivery due to their small size, high surface area-to-volume ratio, inert nature, stability, non-cytotoxicity, high dispersity and biocompatibility **[2]**. Chemical synthesis of metal nanoparticles is expensive and involves toxic chemicals as reducing and capping agents, therefore, Green synthesis of nanoparticles have gained impetus in recent years because of minimum use of harmful constituents, decreased downstream processing requirements, fast and inexpensive fabrication, and environment friendly **[3]**. One step, green chemistry approach is employed to synthesize AuNPs using *Berberis lycium* leaf flavonoids due to several reasons such as (1) the plant has wide range of pharmacologically and biologically active compounds like berberine, balochistanamine, palmatine, oxyacanithine, aromoline, ß-sitosterole, punjabine, umbellatine, oxyberberine, alkaloids, saponins, cardioactive glycosides, tannins, flavonoids, polyphenols etc which act as reducing, stabilizing or capping agent for nanoparticles, (2) *Berberis lycium* is an important medicinal plant useful for the treatment of several diseases such as, piles, jaundice, internal wounds, rheumatism, diabetes, opthalmia, scabies, bone fractures, sun blindness, gingivitis, throat pain, pustules, diaphoretic, antibacterial, antihyperlipidemic, antifungal, pesticidal, anticoccidial and anticancer, (3) Conjugation of flavonoids for one pot synthesis of AuNPs have several advantages in term of water solubility, high possibility to conjugation to Au metal due to the utilization of

sequestering ability of phenolic hydroxyl groups and enhanced antibacterial activity at low concentration as compared to free flavonoids. Flavonoids are heterocyclic secondary metabolites with significant anticancer, anti-inflammatory, antioxidant, cardiovascular and antibacterial activities. The basic structure of flavonoid compounds which include two benzene rings linked by a heterocyclic pyrene ring is shown in Figure 1 **[6]**.

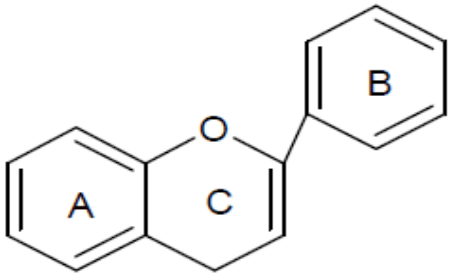


Figure 1 Basic structure of flavonoid nucleus

Enterococcus feacalis is an opportunistic pathogen, which resides in normal intestinal flora. It has high ruggedness to stress environment, hence can cause serious nosocomial infections such as, urinary tract infection, bacteremia, surgical wound infection and endocarditis. *Enterococci* infections are difficult to treat due to intrinsic and acquired ruggedness of this bacterium to major class of antibiotics such as cephalosporins, nalidixic acid, macrolides, clindamycin, trimethoprim sulfamethoxazole, and penicillin, chloramphenicol, tetracyclin, rifampin, aminoglycosides, vancomycin (a drug of last hope for multi resistant enterococci) respectively. This multi drug resistance is menifest by mutations or by horizontal exchange of foreign genetic material through transfer of plasmids and transposons **[4]**. This situation has created new challenges in the area of drug discovery that can be overcome by (1) medicinal plants, as plants are source of leads compounds which are novel and safe therapeutic agents, (2) development of novel compounds that target bacterial virulence factors rather than inhibiting cellular

components required for growth. The natural flavonoids are best choice in this regards as their mode of action on bacterial cells involves the inhibition of nucleic acid synthesis (caused by topoisomerase inhibition), damage of cytoplasmic membrane (caused by perforation or reduction in membrane fluidity), inhibition of energy metabolism (caused by NADH-cytochrome C reductase inhibition) and flavonoids can also effect biofilm formation through quorum-sensing signal inhibition **[5]**.

Phase I - Extraction, isolation and characterization of *Berberis lycium* flavonoids and FAuNPs

This research has been divided into two phases; in the first phase, our research focus is to illuminate flavonoids and FAuNPs effect on the bacterial system. For this approach, we isolated and identified bioactive flavonoids from leaves of *Berberis lycium* and utilized simple, facile and one-pot robust approach for the synthesis of thermally stable FAuNPs via classical Turkevich method.

Phase II - Effect of FAuNPs on *E. feacalis* colonization

In Phase II of the study, we present a broad spectrum effect of isolated flavonoids and biosynthesized FAuNPs by *in vitro* antibacterial activity against clinically important pathogens, such as gram positive including *Enterococcus faecalis*, *Bacillus* cereus, *Staphylococcus aureus*, whereas gram negative including *Pseudomonas aeruginosa*, *Salmonella* Typhi and *Escherichia coli*. To deduce the potential of the biosynthesized nanoparticles for *in vivo* applications, the stability of nanoconjugates was investigated against several parameters such as pH, salt concentration and temperature. The *in vitro* antibacterial activity compared with flavonoids and the commercially available antibiotic. Nanoconjugates showed more hemocompatibilty (caused less than 5 % Hemolysis) and enhanced biological activity as compared to parent drug and also

the amount of drug required for its activity was reduced. Correlative studies using flavonoids and FAuNPs is carried out to find minimum effective dose for *in vivo* studies. Effective dose was used for studying anti colonizing activity in liver and kidneys of *E. feacalis* infected mice.

Aim

To investigate the real time effects of B lycium flavonoids and its gold nanoparticles on colonization of *E. faecalis* in the liver and kidneys of mice

Objectives

- Extraction, isolation and characterization of *Berberis lycium* flavonoids.
- Synthesis and characterization of *Berberis lycium* flavonoids gold nanoparticles
- Effect of flavonoids and flavonoids gold nanoparticles on *E. feacalis* colonization.

CHAPTER 2: LITERATURE REVIEW

Antibiotics are the center part of the modern medical sciences as they have helped in decreasing the number of child mortality and thus increasing the life time, antibiotics are also integral part of invasive surgery and chemotherapies. However bacterial resistant infections are increasing worldwide. Anti-microbial resistance (AMR) is one of the major reasons that prevent the effective treatment of these ever increasing bacterial and fungal infections. Worldwide governments are taking initiatives against this increasing threat to the advancements of modern medicine and human life. Before the discovery of antibiotics minor infections and injuries could kill the individual, however due to resilient bacteria it is still possible phenomenon in 21st century.

Anti-microbial resistant is a global phenomenon prevailing all around the globe due to emerging anti-biotic resistant mechanisms. World Health Organization (WHO) released in their report in year 2012 that there is an increased resistance against HIV drugs however the level is not intense or critical. This increased resistance would lead to use of more expensive drugs. Most recent report of World-Economic Forum Global Risks has described antibiotic resistance as the greatest threat to survival of human being and their health. Bacteria has large number that is resistant to antibiotics and causes different infections of daily life such as pneumonia, urinary tract infections and some blood stream infections all over the world. Now a day's large number of nosocomial occur due to great resistance in bacteria towards antibiotics. Some of the resistant strains are Methicillin Resistant Staphylococcus Aureus (MRSA), Vancomycin Resistant Enterococci (VRE) or any gram positive resistant bacterial strain. The infections caused by these resistant bacteria are deadly and even lead to death of individuals and even require more medicinal facilities than the non-resistant bacteria.

Molecular mechanisms of antibiotic resistance:

Resistance to antibiotics by bacteria is naturally occurring process and over the years bacterial strains are evolving to develop the mechanism of resistance against antibacterial drugs. Many genes code this resistance against bacteria and these genes are transferred generation after generation. Novel mechanisms of resistance are described in new scientific researches that are producing many new vectors and genes regularly. There are two ways to acquire resistance in bacteria either it is intrinsically present in bacteria or due to chromosomal aberrations. Intrinsic bacterial resist the antibiotics by resisting their action due to genetic characteristics. However another way Gram negative intrinsic bacterial strain resists antibiotics is because of these compounds inability to pass through outer membranes of the cells: such as vancomycin bacteria resist the antibiotics by blocking the peptidoglycan cross-linking. However it is effective only in case of Gram positive bacterial strains. Vancomycin cannot pass through the outer membranes of cell and reach periplasm. Recent scientific studies have led to production of number of genes that are involved in producing resistance against antibiotics intrinsically; examples include fluoroquinolones, aminoglycosides and β- lactams **[35]** .

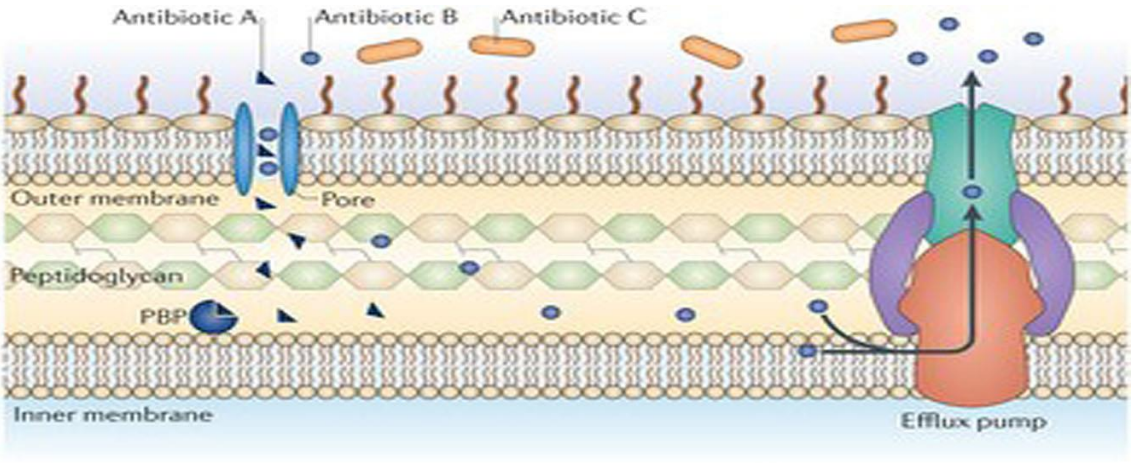


Figure 2 Intrinsic mechanisms of resistance.

The above diagram gives an overall view of intrinsic mechanism of resistance in β- lactam that target penicillin binding proteins. The mechanism of action of antibiotic A involves entering the cell via porin protein and after reaching the target area inhibits the synthesis of peptidoglycan. The mechanism for entering the cell for Antibiotic B can be same as in via porin but contrary to Antibiotic A it is eliminated by the process of efflux. However, the Antibiotic C is an exception in not able to get the access of PBP due to inability to enter cell. There are several other mechanisms in bacterial cell to acquire resistance against antibiotics. These can be divided into three major groups: firstly minimizing the concentration of antibiotics due to inability of antibiotic to reach the bacterial cell; secondly by genetic mutations or post translational modifications of antibiotics and thirdly by inactivating the antibiotics by either hydrolysis process or by any other modification.

The bacterial strains are largely appreciated for their resistance against antibiotics and in the recent years of scientific evolution our knowledge about diverse mechanisms of resistance has also increased. The recent advancements in the field of genomics, cell biology, structural

biology and system biology have further improved our understanding of these mechanisms of resistance. This knowledge if properly integrated and used can lead to great advancements and discoveries of new neutralizing agents for resistance mechanism. Indeed this increased knowledge is helpful in searching new targets that are helpful in studying the genetic mutations that are main cause of increased resistance. Before working on drug development resistance mechanism and development of resistance must be studied at early stages, such kind of studies help in inter-disciplinary coalitions between academics and industry. Now science has developed to a level to estimate the potential of bacterial resistance against the newly discovered drugs and also there are ways developed to determine the responsible mechanism of resistance. The chances to early identify the mechanism of resistance against drugs and structural modifications inside the target lead to failure of developing any target in clinical trials due to emerging resistance. There need to focus on the agents that may resist microbial effect and may drop out. Inter-disciplinary studies focusing on when and how resistance against drug develops and potential interaction between drug and targeted microbe help in determining the dose regime, thus helpful in minimizing the chances of resistance to existing or any new drug that can be later used for effective treatment.

Enterococcus feacalis:

Enterococcus feacalis is among the most common specie of enterococcus genus and is gram positive and belongs to facultative anaerobic group of species. This bacterium has different eating habits and lives in solidarity. This is the organism of harsh environmental conditions such as highly salty environment; it is alkaline pH of about 9.6 and is always present in nutrient deficient conditions. It is one of the opportunistic bacteria living in digestive tracts of organism mainly having bile salts. Other food item of these bacteria involves heavy

metals, alcohols, detergents and desiccated materials. Mostly it grows by forming biofilms. Although it is one of the common intestinal specie but it is also responsible for some serious infectious diseases in both man and animal. The most common pathogenic species of Enterococcus genera is Enterococcus feacalis causing up to 90% of the total *enterococci* infections. These infections may be fetal especially nosocomial infections are most dangerous to survive.

The numbers of urinary tract infections caused by Enterococci are 110,000 reported in United States, while the cases of wound infections, bacteremia and endocarditis are 40,000, 25,000, and 1,100 respectively in just United States. It is to be kept in mind that most of the infections are nosocomial. There is an increase in rate of infections caused by Enterococci in the recent past years. However there is a little deviation in percentage shown by the data presented in National nosocomial infections surveillance system such as surgical site infection 15% vs. 11%, urinary tract infections being 14% against 14% and enterococcus bloodstream 12% in relation to 7% in the past 20-25 years. Data of surveillance of 1980 or back is not present. It is also difficult to estimate the death caused by Enterococci because of several other medical problems simultaneously occurring, however enterococci specie is supposedly reason of around 7 to 50 % of lethal diseases.

Historical studies of common characteristics have shown that the chances of death linked with antibiotic resistant enterococci are much larger than the non-resistant strain. This trend has also led to discovering new mechanisms to combat such lethal bacteria.

Clinical importance of *Enterococcus feacalis* in nosocomial infection:

Enterococcus faecalis is one of the most stress resilient bacteria. This resilience and resistance is one of the reasons for it to colonize under highly harsh and hostile hospital

conditions and thus causing serious endemics **[35]**. The resistance posed by this organism over the years has become a challenge for medical science to combat the infections caused by it. The recent scientific data on enterococcus proves it to be the third most deadly pathogen of nosocomial infections ranging up to 12 % of the total hospital diseases and infections; the other two being the Staphylococcus aureus and Coagulase negative Staphylococcus. Its increase in resistance towards antibiotics and its prevalence has led to decrease in the options for the treatment and therapies because most of the Enterococcus strains are generally resistant to common antibiotics such as ampicillin, vancomycin and even exhibit great resistance towards aminoglycosides, these three being the common and traditional medicines used against enterococcus. New drugs such as daptomycin, tigecycline and linezolida have shown effective results against enterococcus in *in-vitro* experimentations; however the clinical trials are limited because of the limited success in the in-vivo experimentations. The other reasons for limited clinical trials are under dose of antibiotic for enterococcus and also there is an increase in resistance against antibiotic. Another agent that is used against enterococcus is oritavancin but still there is a lack for clinical practices. There is need to develop any optimum therapy for treating multi-drug resilient enterococcus diseases and infections and for that only empirical data and observations are available obtained from *in-vitro* studies. Combination therapies are the requirement to combat this deadly drug resistant bacterial strain.

The mechanism in the enterococcus species to resist antibiotics may be either intrinsic or it may be due to some genetic mutations or even due to horizontal exchange of the genes that are the effective modes for developing resistance. The genetic exchange by horizontal process is by pheromone sensitive plasmids or even by exchange of transposons. With some

exceptional cases aside these transposons and plasmids can be studied in clinical trials. Composite molecules are formed as a result of interaction between these genetic materials with either each other or even with microbial genetic machinery. Hagstead and Palmer can be reviewed for studying recent advancements in transposons and plasmids of enterococcus **[36, 37]**.

Table 1 Mechanisms of resistance to E. faecalis

Antibiotic resistance	**Mechanism of resistance**	**Associated enzyme**	**Phenotype**	**Intrinsic, sporadic or associated MGE**	**Host range**
Aminoglycosides					
	Low cell wall permeability	-	Low-level aminoglycoside resistance, synergy preserved	Intrinsic	E. faecalis
	Ribosome mutation	-	High-level aminoglycoside resistance with MIC . 128,000 mg/ml	Sporadic	E. faecalis E. faecium
	Aminoglycoside modifying enzyme (AME)	Aph(2'')-Ia-Aac(6')Ie	High-level gentamicin resistance	Tn5281	E. faecalis E. faecium
	AME	Aph(2'')-Ic	High-level gentamicin resistance	pYN134	E. faecalis E. faecium

	AME	Ant(6')-Ia	High-level streptomycin resistance	Tn1546, Inc.18, Tn5382	E. faecalis E. faecium
b-lactams and cephalosporins					
	PBP4/5 production	-	Low-level penicillin resistance; moderate to high-level cephalosporin resistance	Intrinsic	E. faecalis E. faecium
	PBP4/5 point mutation	-	High-level ampicillin and imipenem resistance	Sporadic	E. faecalis E. faecium
	Destruction of b-lactam ring	b-lactamase on bla genes	b-lactam resistance	Tn552 and others	E. faecalis E. faecium
Glycopeptides	Synthesis of alternative cell wall	VanA, VanH, VanY, VanX, VanR, VanS	Resistance to vancomycin +/- teicoplanin depending on the phenotype	Tn1546, Inc.18	E. faecalis E. faecium
Lincosamides	ABC-efflux pump	Lsa	Resistance to clindamycin, streptogrammin A and B	Intrinsic	E. faecalis
	Altered ribosome	ErmA	MLSA phenotype	Tn554	E. faecalis E. faecium
	Altered ribosome	ErmB	MLSB phenotype	Tn917, Tn1545	E. faecalis E. faecium
Linezolid	rRNA point mutations	G2576T, G2505A,	Linezolid resistance	Sporadic	E. faecalis E. faecium

		L4(F101L)			
	Methylated rRNA	Cfr	Linezolid, lincosamides, streptogramin A resistance	pEF-01	E. faecalis E. faecium
Daptomycin	Altered membranebound protein	Cardiolipin sythetase	Contributes to Daptomycin resistance through an unknown mechanism	Sporadic	E. faecalis E. faecium
	Altered membranebound Protein	GdpD	Daptomycin resistance, effect is amplified in combination liaF mutation	Sporadic	E. faecalis E. faecium
	Altered membranebound protein	LiaF	Daptomycin resistance when combined with gdpD mutation	Sporadic	E. faecalis E. faecium

In the past two or more decades there has been a rapid increase in microbial drug resistant strains of enterococcus species. The case of highly resistant gentamicin was reported in 1979 and soon after that several other cases of hospital infections were reported during the era of 1980s. Simultaneously there was a rapid outbreak of Enterococcus faecium and Enterococcus faecalis nosocomial infections due to apparent resistance towards penicillin that was because of the production of β-Lactamase. However the isolated cases were very few in number.

The question arises now that the work or strategies developed up till now to combat the resistance of these nosocomial pathogens are enough or not? The answer is no; the progress is quite slow in both public and industrial level. In order to compete in effective manner the industries are quick to response to the opportunities of the market. There is a lack in the understanding of the mechanism behind the infection caused by enterococcus faecalis that's why progress in this area is slow. The reasons behind slow public responses are as follows. (1) At the time of emergence of resistant strains of enterococci, the public was showing disinterest in the support for any infectious diseases research such as AIDS or any other such disease. (2) The mechanism of action of nosocomial pathogen was different from that of any obligate pathogen species. (3) There is lack of basic infrastructure against enterococci because it is not considered as an important agent causing diseases in man and animals, thus there was no emphasis on the basic resistance mechanism up till 1980s.

The steps that needs to be taken for this resistant strain of enterococcus are (1) there is requirement of better knowledge and understanding of the interlinked enterococcus, nosocomial infections, hospital conditions and human beings, (2) Future medicine to be used against enterococcus, (3) need of isolations and patient care facilities in the hospitals, (4) effective surveillance procedures. In addition to all these requirements there is also a need of continuous search for novel drugs along with developing new therapeutic procedures that are less harmful to the patient and also affective against drug resistant bacteria **[37]**.

Desired Antibiotic:

Antibiotic resistance is a worldwide problem prevailing rapidly thus there is an immediate need to develop new therapies and therapeutic agents to combat such problem. Ideal condition would

be development of new generation of anti-bacterial species and agent; the other option of altering the structure of drugs is not effective as bacteria have already developed resistance against such drugs. Another important way to inhibit the resistance is by producing new supplements and agents. Another effective but yet to be clinically approved way is to develop targeted therapy against the bacteria. These therapies are effective in killing the pathogens by interfering with their biological aspects e.g. host tissue attachment, rather than stopping the growth of cell components that are important for growth and survival thus decreasing the rate of infection.

Antibiotics discovery from plant sources: An integrated approach:

Natural products present great diversity of chemical compositions and thus serve as integral units of therapies against pathogenic infections. The starting point of any novel drug discovery process involves first of all the New Chemical Entities identification procedure that is either done by chemical synthesis of any product or by extracting from natural resources by biological fractionation process that is enzymatically controlled process. Most of the new antibiotics and components of novel medicines are of natural origin. In case of extracting the drug from plant source the first step involves the right specimen identification by using the wisdom of Ayurveda, mostly the traditionally documented species are used, and non-documented tribal use and extensive literature surveys are done for using any specie for any novel drug design. In depth analysis of the components of these plant species and Ayurveda are used to select the appropriate components that are to be used for drug designing. The use of Ayurvedic studies in new drug discovery has brought a shift in the process of extraction; the current system of extraction is parallel from the previous sequential procedure. Fractionation process guided by

bioassay has led to standard process of extraction. This interlinked and connected process is cost effective, cheap and time effective that is coupled with an increase in success of new drug discovery. Before the development of high tech screening process and before genomic advancements, most of the drugs designed at that point of time were of natural origin accounted for up to 80% of new drugs.

The almost more than half of the drugs designed and approved between the time period of 1981-2007 were of natural origin. During the time period of 2005 to 2007 almost 13 new drugs were approved that were also of natural origin. History is full of such examples of development of green drugs originated from plants. Morphine was one of the early drugs isolated from poppy plant pods producing opium almost 200 years back. There was a massive increase in pharmaceutical studies after the end of World War 2 that comprised various screening tests of microbes for novel drugs and antibiotics after the discovery of penicillin. There is no doubt about the revolution that natural medicines brought for example antibiotics such as pencillin, erythromycin, and tetracycline, antiparasites such as avermectin, anti-malarial drugs such as artemisinin and quinine, lipid controlling agents including analogs and lovastatin and lastly the immuno-suppressants for organ transplantations including rapamycins and cyclosporine. Plant sources also produced the anti-cancer drugs as well for example irinotecan and paclitaxel. All these medicines have revolutionized the medical sciences. These natural products have also played role in modern and contemporary medicine. This is still one of the cheap sources of medicine. Plant materials produce certain drugs that are otherwise quite impossible to synthesize synthetically. Natural sources also serve as basic components of modern medicine that can be modified and thus can be make less toxic and more effective against diseases. There are certain natural compounds that are neutral in their activity when used alone but when they

are conjugated by chemical or any other biological methods they turn into potential drugs that are difficult to obtain otherwise **[36]**.

Berberis lycium:

In the present leaves of *Berberis lycium* are used for the production of flavonoid gold nanoparticles. *Berberis lycium Royle* is the common species of Berberidaceae genus. This plant is native species of India, Pakistan and Himalaya ranges. Al-Biruni, one of the ancient plant scientists named this as Ambaribis, in Persian it was named as Zirkash named by Al-Biruni. Other names of *Berberis lycium* are Indian barberry, Sumbal, Ishkeen and Kashmal. Its different components such as leaves, shoots, fruit and roots are used in traditional medicines to cure different diseases including heart diseases **[3]**.

Traditional medicines of *Berberis lycium* are used against common diseases such as diarrhea, fever, intestinal colic, diabetes, backache, bone fractures, men gonorrhea, throat pain, and expectorant and against certain bacterial diseases, the reason for which it is known as anti-diabetic, anti-hyperlipidemic, anti-fungal, anti-bacterial and pesticidal agent. Berberis lycium has been reported vulnerable by IUCN (International Union for Conservation of Nature). The components of this plant species has large number of biologically and pharmacologically useful elements including berberine, palmatine, berbamine, punjabine, cardioactive glycosides, umbellatine, oxyacanithine, tannins, flavonoids, , anthocyanins, aromoline, alkaloids, palmatine chloroform, oxyberberine, saponins, polyphenols vitamins, carbohydrates, balochistanamine, lipids, proteins, ß-sitosterole berberine chloroform, and β crotein **[3]**. Literature studies of past have shown that berberine is the compound with broad range biological and pharmacological applications studied by both *in-vitro* and *in-vivo* examinations. Only berberine is explored in this species. Pharmacological applications of other important

component flavonoids are not completely explored up till now and due to very little or no clinical data its pharmaceutical applications are unclear to the world yet. The present study on the flavonoids of *B. lycium* was undertaken to rationalize these traditional uses and to explore mechanistic basis for these medicinal uses.

Flavonoids and their therapeutic potential as antibiotic:

Flavonoids are one of the most prominent and useful metabolites obtained from plant sources and belong to benzo-g- pyrene group of derivatives. They are present in the form of methylated, glycosides and aglycones derivatives. More than 4000 different flavonoids of plant origin are described till now. The main biological properties associated with the flavonoids are anti-cancer, anti-oxidant, anti-inflammatory and cardiovascular diseases. Flavonoids are organic compounds with heterocyclic structure. The basic structural component of flavonoid is flavane nucleus or the 2-phenyl benzo pyrene with two benzene rings bounded by heterocyclic rings. It is used in traditional medicine for various common infections of daily life like sores, acne, wound injuries and other gastrointestinal infections.

Flavonoids of natural origin are natural anti-bacterial agents against large number of pathogens. However the mechanism of this anti-microbial activity is not known yet. Three mechanisms behind the anti-microbial activity of flavonoids can be viewed in literature studies; it may damage the cell membranes either by reducing the fluidity of membranes or by perforation process. It can even inhibit the mechanism of energy by inhibiting NADH cytochromes-c reductase production. It can even inhibit nucleic acid production by inhibiting the production of topoisomerase production. Flavonoids even have synergistic properties and it can inhibit the biofilm production **[5]**.

Flavonoids Loaded In Nano Carriers:

Flavonoids are the active part of dietary components and are thus used as herbal remedies. There is an increasing trend in use of these polyphenols as a prominent ingredient of dairy products and beverages. However there are several drawbacks associated with use of flavonoids when used in oral treatments due to their properties such as stability, bio-efficacy and bio-availability **[32]**. They have very limited solubility in water even if they are in polar form as in glycosidic in nature, they have poor bio-availability and they are greatly influenced by factors such as pH, temperature and light. Due to their complex nature they are not easily absorbed in gastrointestinal tract. It is shown in scientific research that flavonoids break down by enzymes and different types of intestinal bacteria producing secondary metabolites. These metabolites are further degraded by hepatic enzymes and varying activity of organism forms more metabolites in the body.

Hydrolysis of the sugar molecules in the small intestinal track or because of some bacterial activity inside the colon aglycone molecules is produced and these are further simplified by complex metabolism. There are various factors that may limit the glucuronidated form of flavonoids thus resulting in poor permeability, bioavailability, instability and extensive low bioavailability, low water solubility, it has low stability to gastric juices and pH of colon, intestinal absorption and first pass metabolism. A new approach to combat this problem of low bioavailability is using nanosized drug carrying agents that may range between 10 to 1000 nm in size. They can enhance bio-efficacy and bio-availability of flavonoids as they have the ability to increase the solubility potential, even they can alter the pathways of absorption and thus can avoid the metabolic break down of the metabolites inside gastrointestinal tract of organism.

Nanosizer can also reduce the dose and increase the time period of the action due to gradual release of drugs. Many experimental procedures determining the pharmacokinetics of different flavonoids such as quercetin, isorhamnetin and kaempferol present in the Ginkgo biloba by orally administering the mouse have been reported. There was an increase in Cmax value of quercetin and reached 724.89 from the value of 179.21, reached the point of 323.56 from the value of 180.23 and reached the point value of 672.29 from the point of 195.96 when isorhamnetin was studied. AUC0s of these flavonoids was also found to increase by the values of 2.42, 2.35 and 1.95 respectively. The value of T-max of all these metabolites was found to decrease in term of phospholipid metabolite complex when the value of T-max is compared to the value of pure extract. The result of these activities was an increase in the bioavailability of these extracts by the use of nanoparticles **[29]**.

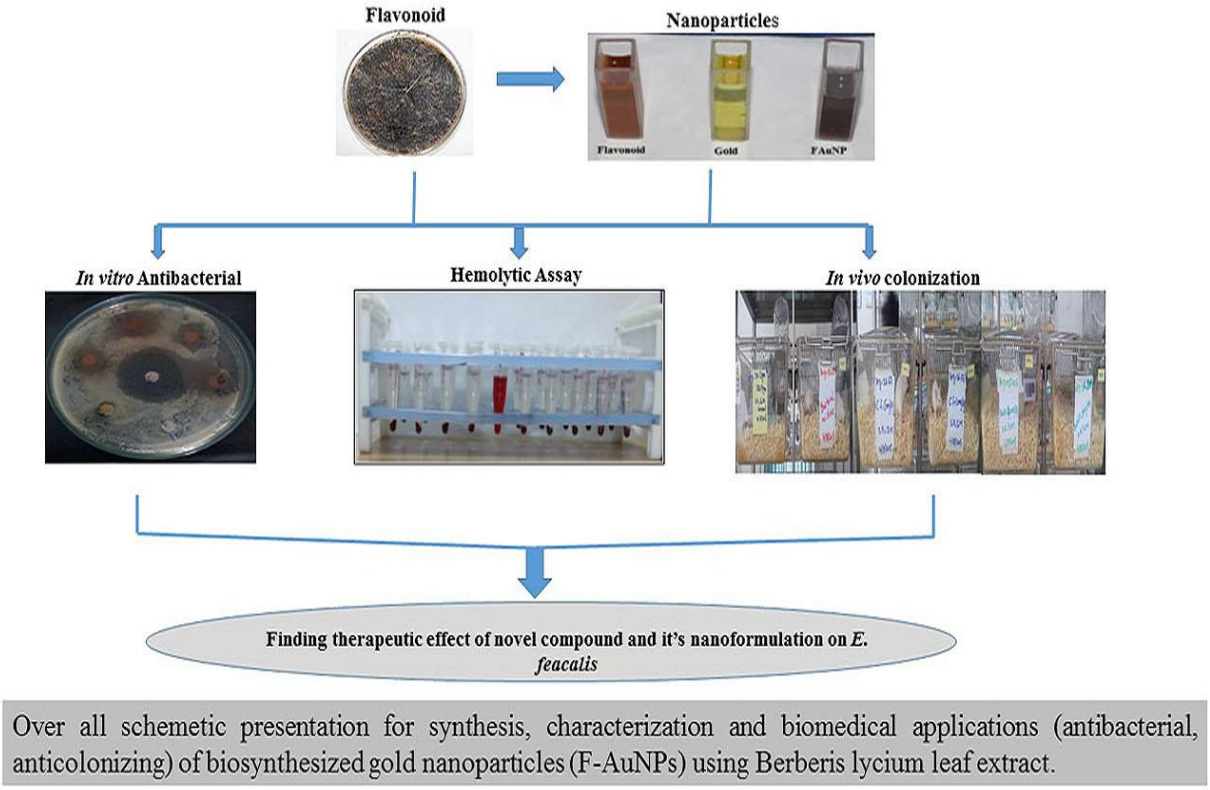
Gold nanoparticles:

In this modern nanotechnology era, Nanoparticles, Nano-films, Nano-structures and Nano-rods are the common terms used in science. Nano-sciences is a fascinating and attractive science as it has many distinguishing features such as these nanoparticles can repair damage cells and tissues in-vivo, can pass blood-brain barrier and also used in the manufacturing of super computers that are so small that can easily be placed in pockets or drawers**.** Nanotechnology has large number of applications in current modern world such as in drug delivery systems, biomedical applications, optical fibers and filters, biological detection systems involving sensors, medicinal therapies, cosmetics and food industry **[29]**. It has applications in other fields of sciences as well such as bio-chemistry, bio-technology, electronics and physics. Nanotechnology involving metallic nanoparticles is employed in various scientific fields

because of their small size and controlled shape and due to their exceptional chemical and physical properties. However the chemical process of metallic nanoparticle synthesis is expensive and hectic. It requires toxic chemicals that act as either capping or reducing agents. Hence green synthesis was integrated into Nano sciences after the emergence of medical application such as drug delivery system, tissue repair mechanism and selective imaging. Green Nano-particle is beneficial option in regard to chemical and physical synthesis process. Green nanoparticle synthesis is cheap, eco-friendly and cost effective **[28]**.

All these facts shows that plant extracts are used in nanoparticles synthesis as reducing agents. The plants have the ability to reduce the metals and thus act as enzymes due to their phytochemicals and bio-chemical properties. The current research on Nano-particles shows that gold conjugation with plant material results in increase in anti-microbial activity. Flavonoids are shown to be active in their biological process to prevent different diseases; therefore we illuminated the effect of flavonoids against bacterial system.

CHAPTER 3: Materials and Methods



Over all schemetic presentation for synthesis, characterization and biomedical applications (antibacterial, anticolonizing) of biosynthesized gold nanoparticles (F-AuNPs) using Berberis lycium leaf extract.

Figure 3 Scheme over all presentation for synthesis, characterization and biomedical applications (antibacterial, anticolonizing) of biosynthesized gold nanoparticles (F-AuNPs) using *Berberis lycium* leaf extract.

Materials and Methods (Phase I)

Materials and instruments

Leaves were sampled from Patriate, Murree, Pakistan. Methanol, petroleum ether, diethyl ether, ethyl acetate, sodium hydroxide, sulphuric acid , sodium chloride, nutrient agar, luria bertani(L.B), 3-(N-morpholino)propanesulfonic acid (MOPS), phosphate buffer saline(PBS), fetal bovine serum(FBS) and brain heart infusion broth(BHI) were purchased from Sigma Aldrich. Tetrachloroauric (III) acid trihydrate ($HAuCl_4.\ 3H_2O$) were purchased from Merck.

Deionized water was used throughout the experiment. Silica gel (SiO_2) coated TLC plates was purchased from Merk KGaA, 64271, Germany. A digital pHmeter model 510 (Oakton, Eutech) equipped with a glass working electrode and a reference Ag/AgCl electrode was used. UV–visible spectra were recorded with a UV-2800 BMS Scientific Technical Corporation (PVT) Ltd.

Flavonoid extraction

Fresh leaves of *Berberis lycium* were shade dried, crushed, weighed and used for extraction of flavonoids. Weighed sample was soxhlet extracted in 80% methanol for 24hrs and filtered. The methanol was subsequently evaporated by vacuum. The aqueous fraction was subsequently extracted with petroleum ether (40-60°C), diethyl ether and ethyl acetate by following Subramanian and Nagarajan (1969) method (Figure 4). To ensure complete extraction each step was repeated thrice **[16]**.

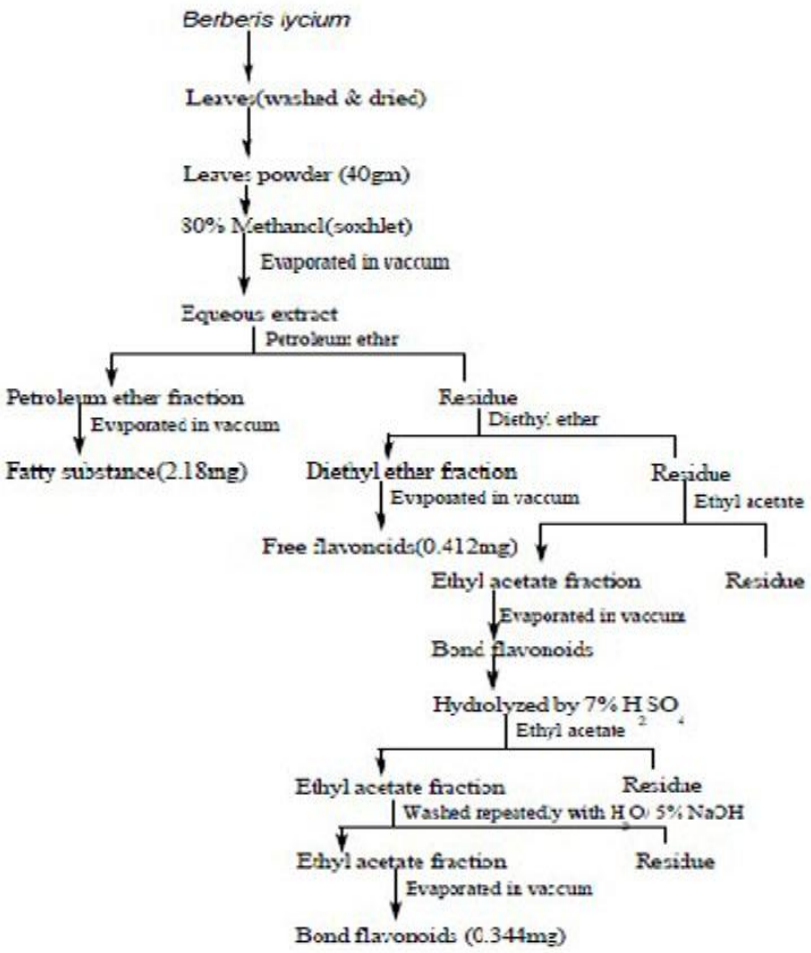


Figure 4 Scheme of flavonoids extraction

Qualitative analysis

Thin layer chromatography

The isolates were identified by TLC (Silica gel coated Aluminum plates). After application of extract (5µl) the plates were developed in ethyl acetate: formic acid: acetic acid: water (100: 11: 11: 26). Developed plates were air dried and visualized under UV light. Plates were also exposed to NH4OH bottle so as to make contact with each spot for about 5-10 seconds and fluorescent spots were marked. The developed plates were also sprayed with 5% ethanoic FeCl3 solution for further confirmation. The fluorescent bands and colored spots respectively were

noted. Several other solvent systems such as n-butanol: acetic acid: water (4:1:5, upper layer), benzene: acetic acid: water (125:72:3), ethyl acetate: formic acid: water (65:15:20), n-butanol, acetic acid, water (3:1:1) and benzene: ethyl acetate: formic acid (40: 10: 5) were also tried.

Colorimetric screening

Flavonoids screening was performed using Shinoda's Test optimized by Mir et al., 2013 and Inalegwu B, Sodipo O, 2013.

Fourier Transform Infrared (FTIR) spectroscopy analysis of flavonoids

The FTIR analysis was performed using an Agilent FTIR. 2mg flavonoids and 300mg of spectral grade potassium bromide (KBr) were compressed together in a dye to form a transparent disc. The FTIR spectrum was set at 4000-500 cm^{-1} using a Perkin Elmer FT-IR spectrometer. The internal element was a ZnSe ATR plate (50 × 20 × 2 mm) with an angle of aperture set at 45°. The sample was scanned 32 times and a spectral graph was generated using ASCII FTIR software.

Green synthesis of flavonoids coated gold nanoparticles

1Mm solution of tetrachloroauric (III) acid was prepared in deionized water and flavonoids solution (1mg/mL) was also prepared in deionized water. 2mL of flavonoids (1mg/mL) was added dropwise to 2mL of tetrachloroauric (III) acid (1 mM). During addition the color of reaction mixture changed from yellow to wine red. The resulting mixture was stirred for 4 h at room temperature, the solution was characterized by UV-VS spectroscopy. Similarly different ratio of HAuCl4, temperature and pH were tested to optimize the conditions. The optimum ratio for nanoparticles formation was found to be 1:3 (Au: flavonoids) at 70ºC and 4 pH. Flavonoids

coated gold nanoparticles were collected in the form of precipitate after centrifugation at 11,000 revolutions per minute for 10min, at room temperature. These FAuNPs were dispersed in distilled water.

***In vitro* Stability studies of flavonoids-gold nanoparticles**

Salt effect

Effect of high concentration of NaCl (2–4 M) on F-AuNPs was studied. For this experiment 2ml of flavonoids AuNPs was taken in a beaker, then 2ml of 2M NaCl solution was added. The resulting solution was kept at 37°C for 24 h. Then its spectrum was recorded by UV-vis spectrophotometer. Similarly, the effect of 3 and 4M NaCl was monitored by same protocol.

Heat effect

Effect of heat on FAuNPs was studied by taking 10mL of FAuNPs in a round bottom flask. The solution was heated up to 100°C for 30min. Then UV-vis spectrum of nanoparticle that was kept at room temperature and at 100°C was recorded.

Characterizations of Flavonoids conjugated AuNPs (FAuNPs)

Ultraviolet Visible Spectroscopy (UV-Vis)

UV-vis spectrum of FAuNPs was carried out on UV-2800 BMS Scientific Technical Corporation (PVT) Ltd. This shows the absorbance spectra of gold nanoparticles.

Fourier Transform Infrared Spectroscopy

FTIR analysis were performed to investigate any structural changes in the F-AuNPs. The F-AuNPs were smeared on KBr and compressed into a thin pellet. Infrared spectra was recorded with FTIR Spectrometer (Perkin Elmer spectrum 100 instrument) between 4000-400cm-1.

X-ray Diffraction (XRD)

XRD analysis of F-AuNPs was achieved using XRD model theta-theta STOE (Germany).

Transmission Electron Microscopy (TEM)

Microscopic image of F-AuNPs was taken with a FEI NOVA, NanoSEM 450 that is equipped with STEM detector operated at 120 keV.

Materials and Methods (Phase II)

Bacterial strains and growth media

Gram positive bacterial strains including *Enterococcus faecalis (Clinically isolated)*, *Bacillus cereus* (Soil isolated), *Staphylococcus aureus (ATCC 6538)*, whereas gram negative strains including *Pseudomonas aeruginosa(ATCC 9027)*, *Salmonella* Typhi *(ATCC 6539)* and *Escherichia coli (ATCC* 8739) were used. Nutrient Agar and Luria Bertani medium (L.B) was used as growth media for all strains except *E. faecalis* for which M17 medium with 3-(N-morpholino) propanesulfonic acid (MOPS) and for *in vivo* Brain Heart Infusion (BHI) Broth was used. The *E. faecalis* strains were cultured on M17 medium supplemented with 0.5% (wt/vol) glucose (GM17 medium) at 37°C. Overnight precultures, grown without agitation in 10 mL GM17 broth, were used to inoculate the cultures containing BHI Broth and FBS for growth analysis.

***In vitro* Antibacterial Activity**

Bacterial cultures

The tested strains were cultured twice for activation in culture tube containing 15 mL of Luria Bertani broth at 30–37°C for 24 h. Optical density (OD) was determined at 600 nm. The cultures were stopped at 0.8–1.0 OD_{600} range (equivalent to 10^8 CFU), and used for antimicrobial testing.

Antibacterial Evaluation of flavonoids Using Quantitative Method

The antibacterial activity of free and bound flavonoids was determined according to method described by Yin et al. (2010) **[9]**. Disc diffusion method is a qualitative method for the evaluation of antibacterial activity of the antimicrobial material. For this study three gram-negative and three gram positive bacterial species were selected. Different fractions of compounds were prepared in DMSO (Dimethyl sulfoxide). Chloramphenicol of concentration 1 mg/mL and DMSO were used as positive and negative controls, respectively. In sterilized petridishes, 20 mL nutrient agar was poured and bacterial inoculation was carried out by streaking to form different colonies. The disc of 5 mm was kept at the center of agar plate and 10µL of sample was poured on disk. Positive and negative control was also taken and zone of inhibition was observed after 24 hours at 37°C.

Antibacterial Evaluation of FAuNPs Using Qualitative Method

Antibacterial testing was carried out by using the method ASTM E2149. Test was conducted against the Gram-positive *E. faecalis.* 100µl of F-AuNPs with various dilution (1000 µg/mL, 800 µg/mL, 600 µg/mL, 400 µg/mL, 200 µg/mL, 100 µg/mL, 50 µg/mL, 25 µg/mL, 5 µg/mL, 1 µg/mL) was placed in an eppendorf and 100µl of bacterial inoculum (10^6 CFU/mL). The eppendorf was placed at 37°C at 150 rpm for 16 hours at shaking platform. After the incubation period the solution was serially diluted. The diluted solution was plated on nutrient agar and incubated for 24 hours at 37°C. The reduction of colonies were calculated using the following equation **[10]**:

$$I\ (\%)\ = \frac{(C - S)}{C} \times 100$$

Where S is the number of bacterial colonies survived after contacting with treated F-AuNPs and C is the number of colonies present in untreated control sample.

Antimicrobial mechanism of action study with *E. faecalis*

Bacteriostatic and Bactericidal activity:

Bactericidal and bacteriostatic activities were determined by time-kill kinetic method as described by Ooi et al. (2009), with slight modifications **[11]**. For this experiment, full growth of *E. faecalis* in M17 medium were diluted 100 times and incubated at 37°C to produce an OD_{600} of 0.8 as starting inoculum. Flavonoids and FAuNPs were added to give a final concentration of 5 × MIC (Data not shown) and incubated at 37°C with shaking, then 100µL were removed from each tube at 3 h, serially tenfold diluted and plated onto M17 medium plates, to determine the viable number of CFU/mL. Chloramphenicol and deionized water were used as positive and negative control respectively.

Hemolysis assay

Cytotoxic effect of gold nanoparticles (F-AuNPs and flavonoids) was studied by carrying out its hemolytic activity. 15 mL of the blood sample of healthy donor was collected in which 4 mL was taken and mixed with 8 mL of PBS (phosphate buffer saline solution). This mixture was then centrifuged at 10000 rpm for 10min. RBCs were collected in the pellet which was then washed three times with PBS for 3 min at 10000 rpm. The RBCs obtained so were diluted with PBS **[12]**. The test samples were prepared by making stock solution of gold nanoparticles (FAuNPs and flavonoids) using PBS as solvent. Various concentrations such as 1, 5, 10, 20, 50, 100 and 150µg/mL were used for the hemolytic study. PBS was taken as negative control and 0.5 %Triton X-100 as positive control. 1mL of each of the sample was taken and 1mL of RBC suspension was added to it. These samples were kept for incubation at 37 ^{o}C for 1 h and

4 h. After incubation the samples were vortexed and then centrifuged at 10000 g for 5 min. Supernatant was collected and poured into a cuvette. The absorbance was measured using a UV-2800 BMS Scientific Technical Corporation (PVT) Ltd spectrophotometer at 550 nm. Positive and negative controls induced 100% and 0% absorbance respectively. The % hemolysis was calculated using the following equation **[13]**:

$$\text{Hemolysis (\%)} = \frac{\text{Absorbance of sample} - \text{Absorbance of negative control}}{\text{Absorbance of positive control} - \text{Absorbance of negative control}} \times 100$$

In vivo Antibacterial Activity

Animals

The research experiments performed followed the rulings of the Institute of Laboratory Animal research, Division on Earth and life Sciences, National Institute of Health, USA (Guide for the Care and Use of Laboratory Animals: (Edition 2011). The research protocols were approved from the Ethical Review Board (EBR), Atta ur Rahman School of Applied Biosciences (ASAB), National University of Science and Technology (NUST). BALB/c mice were purchased from National Institute of Health (NIH) Islamabad. BALB/c mice (25 - 30 g) of female sex were kept in the animal house of ASAB, NUST, under controlled environment , temperature 25±2°C and the natural light and dark cycle (14hrs night and 10hrs a day) was followed. Tap water and a standard diet *ad libitum* consisting of 30% crude proteins, 9% crude fat, 4% crude fiber and 10% moisture were given to mice. A total of 30 mice were used in this study and each mice was given a unique identity. 5 mice were kept in one cage ($40 \times 20.5 \times 25cm$) and all animal have equal access to food and water. The overall timeline of the animal experiment is shown in Figure 5.

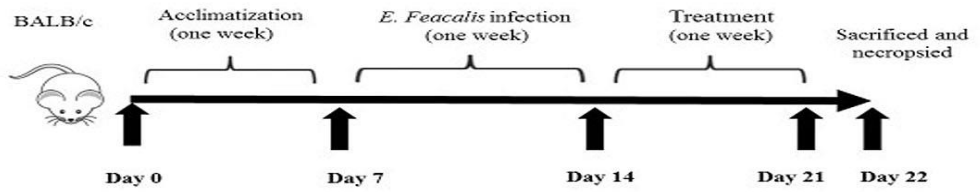


Figure 5 *In vivo* experimental design

Colonization of *E. faecalis* in BALB/c mice

To test the anticolonizing activity of flavonoids and FAuNPs, we used a well-established intravenous infection model **[14, 15]**. Briefly the *E. faecalis* strains were cultured in M17 medium supplemented with 0.5% (wt/vol) glucose (GM17 medium) at 37°C for 24hrs. Overnight precultures, grown without agitation in 10 mL GM17 broth. Brain heart infusion(BHI) medium supplemented with 40% filter-sterilized serum(FBS) was inoculated with 100µl of preculture and were incubated at 37°C with shaking; the cultured were centrifuged; the resulting pellets were washed with PBS ; and the washed pellet were resuspended in sterile PBS. These bacterial suspension contained 1×10^9 bacteria/mL. A 100µl of bacterial suspension was injected intravenously (tail vein) in each of five female BALB/c mice (9 weeks old) of each group.7 days are required for infection during this period. Sympotms of infection was observed and their intake of food and water was also observed **[24]**.

Drug treatment

Mice were divided into four groups of five each; one group served as control; one group remained untreated; one group treated with (5mg/kg) flavonoids; one group treated with

(10mg/kg) flavonoids; one group treated with (400µg/kg) FAuNPs; and one group treaed with (5mg/kg) FAuNPs. Drug was administered into the tail vein once a day for eight days starting from seventh day after infection and continuing until the day of challenge.

Anticolonizing evaluation of flavonoids and flavonoids gold nanoparticles

Mice were sacrificed after treatment and their organs (liver, kidney) were removed aseptically, weighed and homogenized. Serial homogenate dilutions were plated onto M17 agar and were incubated at 37°C for 24hrs. Finding the effect of flavonoids and FAuNPs by determining the $\log_{10}$CFU/gm organ. CFU counts resulting from three replicate experiments were analyzed by the unpaired *t* test.

Statistical analysis

Statistical analysis was performed by using Graph pad prism version 6.0. One-way analysis was applied to the data and compared by the Student t test, $p < 0.05$ was considered statistical significant. The data was represented as mean ± SD up to three significant figures. FTIR and UV graphs was done by origin 6.1, and TEM analysis was done by image j software.

Chapter 4: Results and Discussion

Results (Phase I)

Percentage yield (%)

Flavonoids have been extracted from leaves of the *Berberis lycium*. Free (diethyl ether fraction) and bound (ethyl acetate fraction) flavonoids of leaves were extracted, dried and weighed. Percentage yield of flavonoids was calculated by given formula.

$$\% \, Yield = \left[\frac{weight \; of \; crude \; extract}{weight \; of \; crude \; sapmle}\right] \times 100$$

Results revealed that maximum free flavonoids were obtained from leaves (Table 2). Measured water content in leaves was 13.83% ± 0.0038.

Table 2 Flavonoids content from leaves of *Berberis lycium*

Conc.	Free flavonoids	Conjugated flavonoids	Total flavonoids
(mg/gm. dw)	0.0103± 0.007	0.0086± 0.001	0.0189±0.009

Thin layer chromatography

Flavonoids (ethyl acetate fraction) was confirmed by its fluorescence when the chromatogram was evaluated in UV light at λ = 366nm the orange- yellow four fluorescent bands and one blue fluorescent band were appeared (Figure 6). It shows that four flavonoids and one phenolic band is present. Further confirmation is done by spraying regent, when dried developed TLC plates was sprayed with 5% ethanoic FeCl3, after air drying the bands color appeared as bluish grey. We tried six mobile phases but from these the most suitable mobile phase for separating the compounds is ethyl acetate: formic acid: acetic acid: water (100: 11: 11: 26) because it had the largest discriminating power. On the basis of Rf values which are 0.27, 0.35, 0.58, 0.63 and

0.73 we assumed that these are flavonoids and phenolics. Flavonoids were further confirmed by infrared absorption spectrum obtained by FTIR analysis.

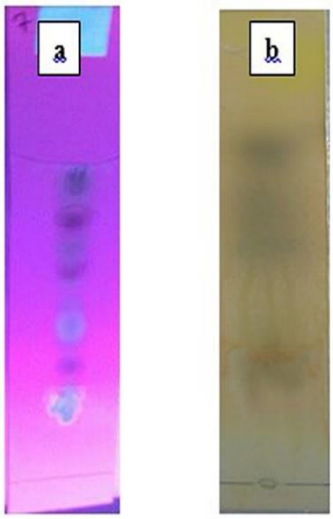


Figure 6 Chromatogram of flavonoids and phenolics obtained by TLC. (a) Fluorescence and (b) Spraying

Colorimetric screening

Flavonoids were identified by adding few drops of 1N NaOH into ethyl acetate fraction, which changed the color of extract into intense yellow. After adding dilute H_2SO_4 it turned to colorless which confirmed the presence of flavonoid (Figure 7).

Figure 7 Visual observation of flavonoid screening.

Biosynthesis and characterization of flavonoids coated gold nanoparticles

Biosynthesized AuNPs have been synthesized using flavonoids, which act as stabilizing/capping as well as reducing agent. In order to get the optimized and stable AuNPs, we have carried out a series of reactions using various concentration of flavonoids (Figure 8), different temperature (Figure 9), and pH (Figure 10).

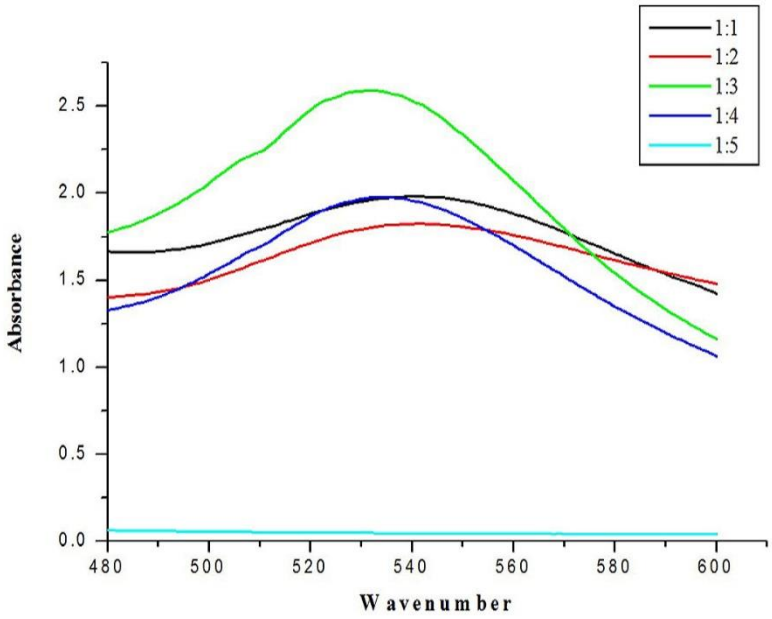


Figure 8 Optimizing the reaction by changing the amount of $HAuCl_4$

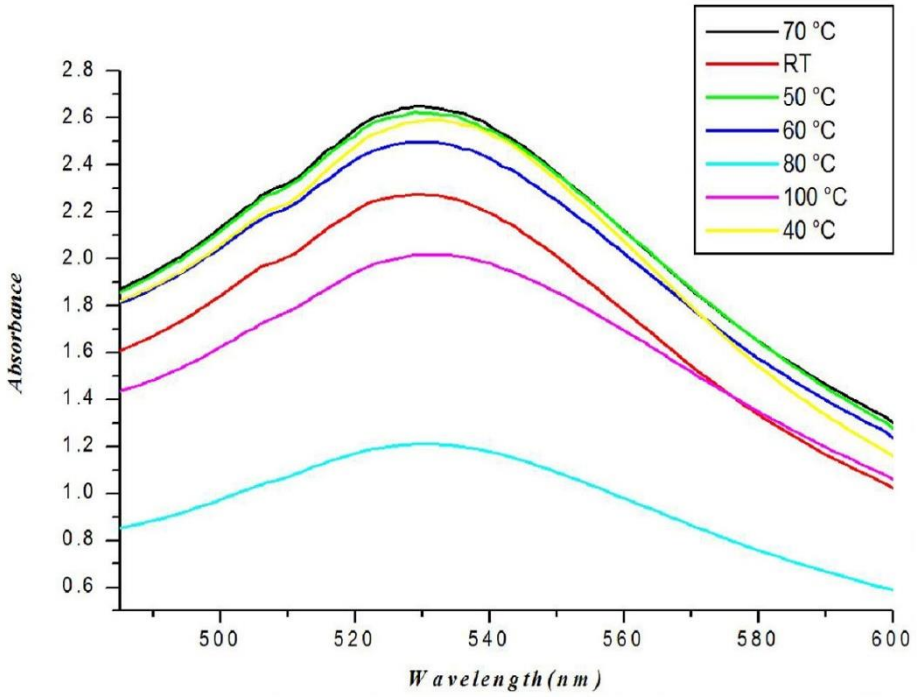


Figure 9 Optimizing the reaction by changing temperature

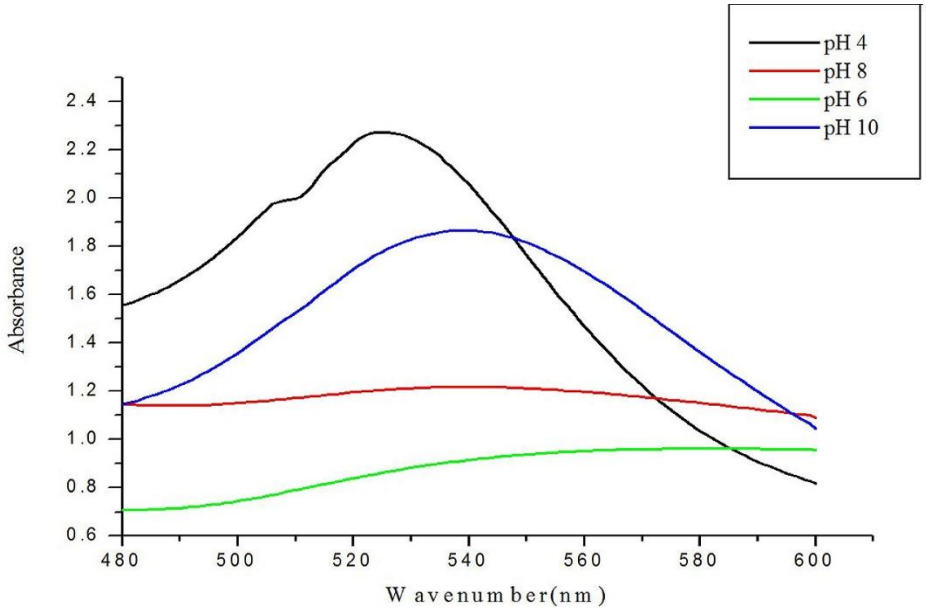


Figure 10 Optimizing the reaction by changing the pH of flavonoids

The optimized FAuNPs are highly stable and have been utilized for detailed characterization and further *in vitro* assays (antibacterial and hemolytic activity) and *in vivo* (anticolonizing) studies.

UV visible spectroscopy

The color of reaction rapidly changed from light yellow to wine red (Figure 11) after mixing flavonoids with $HAuCl_4$ solution, indicating reduction of Au^{+3} to Au^0 and synthesis of flavonoid coated gold nanoparticles. UV-vis spectra (Figure 11a) of the suspension revealed an absorption maximum at 530nm **[18]**. It may be due to the excitation of surface plasmon vibrations of AuNPs in the aqueous solution. Variation in the λ_{max} were observed when different concentration of $HAuCl_4$ were used for the synthesis of flavonoid coated gold nanoparticle. Impact of temperature and pH on the formation of F-AuNPs are presented in Figure 9 and 10, which shows that pH 7 at 70°C gives best result. However the absorption intensity was gradually increased at 1:3 (flavonoid: Au) ratio, indicating the complete reduction of gold ions and suddenly down. Increase concentration of gold was not give good result which may be due to the increase in the number of gold nanoparticles due to aggregation.

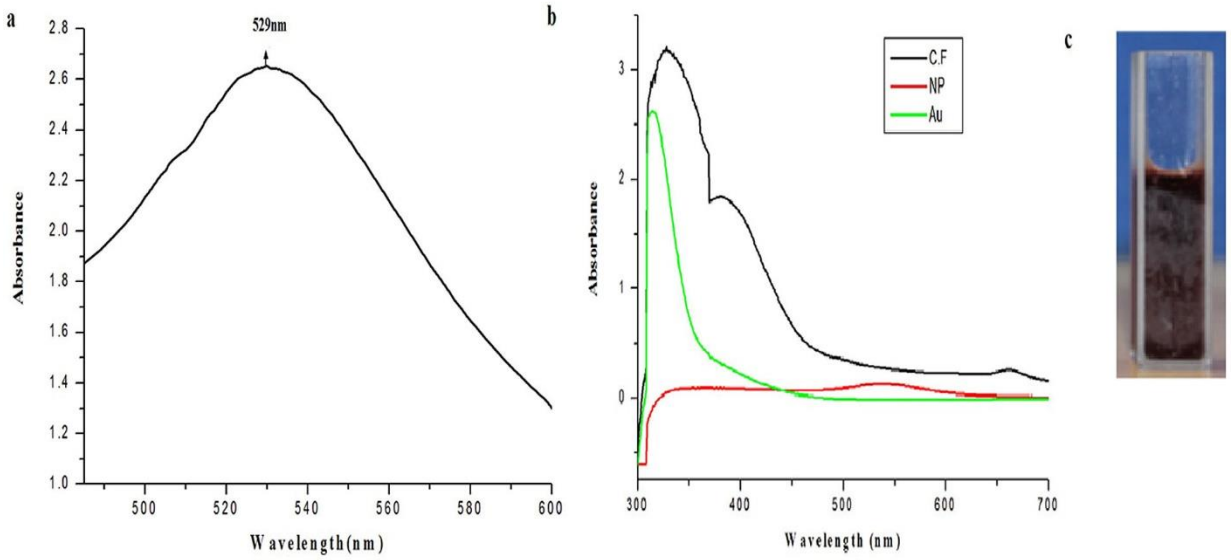


Figure 11 (a) UV-vis spectra of FAuNPs (b) comparative UV-vis spectrum of Au, C.F, FAuNPs (c) Visual detection of FAuNPs.

To check the potential of nanoparticle for *in vivo* studies the stability of biological nanoparticles was tested. For this purpose we studied salt and temperature stability. Variation of salt concentration and temperature did not affect the SPR and the stability of the suspension, as shown in Figure 12 and 13.

Figure 12 showed the effect of various concentration of NaCl (2 - 4M) on SPR peak of FAuNPs. No effect was noticed by increasing the concentration of salt from 2M to 4M NaCl solution for couple of weeks **[19]**. But for long time that higher concentration of salt decreased the λ_{max} or full width at half maximum (FWHM) also increase and that decrease the stability of nanoparticles. This decrease in absorbance of nanoparticles is due to the aggregation effect promoted by Cl^{-1} ions. From these observation it was concluded that as for as long term stability is concerned, gold nanoparticles are much more stable in water then NaCl solution.

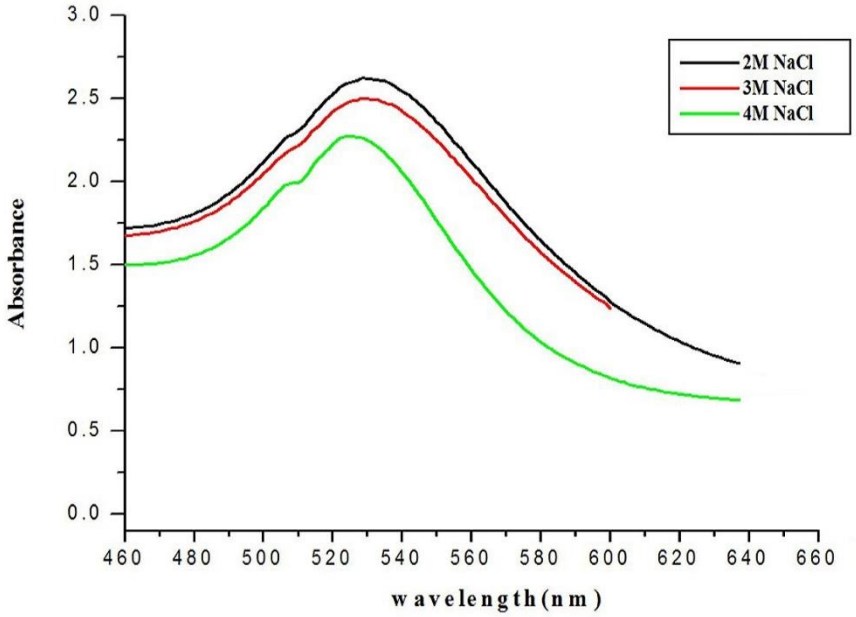


Figure 12 Effect of different concentration of NaCl on stability of flavonoids gold nanoparticles: after 24 h.

Figure 13 showed the effect of temperature on SPR peak of FAuNPs. The result indicated that the effect of temperature is negligible, minute reduction in absorbance while the plasmon peak did not shift, and no precipitation was observed.

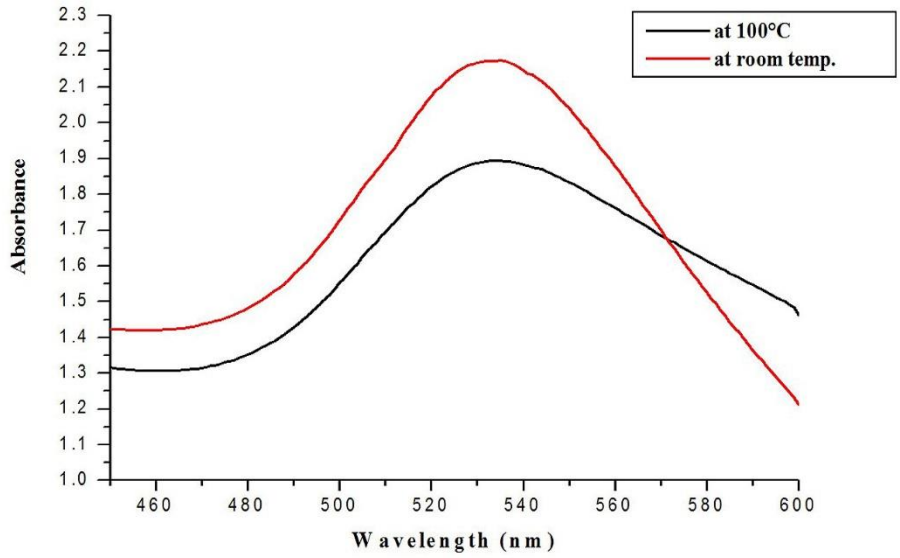


Figure 13 Effect of heat on the stability of FAuNPs stabilize with flavonoids.

Comparative FTIR analysis of flavonoid and flavonoid coated gold nanoparticles

Conjugation of flavonoids with gold is supported by FTIR spectroscopy (Figure 14), where absorbance bands related to flavonoids are observed in the region of 500 – 3500cm^{-1}. Among them, the absorbance bands at 3398 were assigned for the stretching vibration of – O– H, 2960 – 2850 (two bands) for C –H stretching vibrations(- CH_2, CH_3), 1652 for - C=O, 1600 for -C=C- (in aromatic ring) , 1554 for -C-C- (in aromatic ring), 1300 for -C-O- (ether linkage), 1210 for -C-0- (polyols), 1100 for -C-OH (stretching vibrations), 909 and 850cm^{-1} assigned for C-H bending vibrations out of plane **[20, 21]**. Comparative IR spectra indicated that the peak intensities decreased in the spectrum of FAuNPs which was a hint that some specific groups are involved in the reduction and stabilization of nanoparticles. Most probably the hydroxyl (OH), carbonyl and polyol (-C-O-) groups are responsible for reduction of Au III ion.

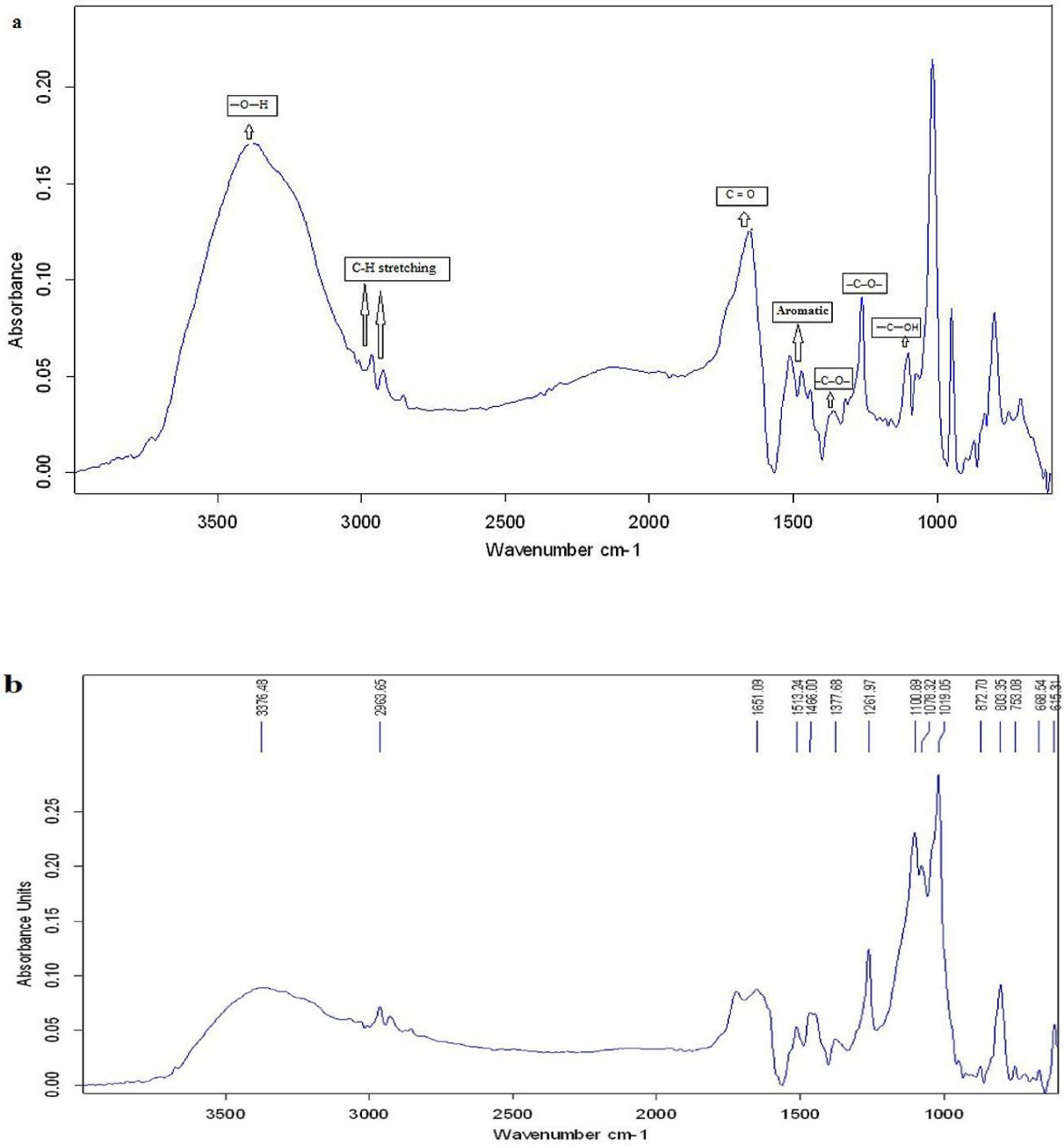


Figure 14 (a) FT-IR spectra of flavonoid (b) FT-IR spectra of FAuNPs

X-ray Diffraction (XRD) of Gold Nanoparticles

The crystal structure of the biosynthesized F-AuNPs has been investigated by X-ray diffraction patteren. The Bragg reflection indicate that AuNPs are specifically indexed to a face centered cube (FCC) crystal structure (Figure 15). In the XRD pattern of F-AuNPs, peaks were obtained at 2 theta values of 37º, 44º, 64º, 77º that correspond to the 111, 200, 220, 311 reflections of FCC gold which are in perfect agreement to the JCPDS card no. 00-002-1095 **[23]**. The (200) peak was detected in the sample, representing high intense peak of FCC material. The high degree of crystallinity of the F-AuNPs was showed by the intensity of peaks. The broad diffraction peaks were articulating the small size of crystal. FCC structure of pure gold metal was confirmed by coincided peaks.

The average crystallite size of the F-AuNPs was calculated from the XRD line broadenind using the Scherrer equation **[22]**.

$$\boldsymbol{B = \frac{0.93\lambda}{\beta cos\theta}}$$

Where λ is the wave length of the incident X-rays (λ = 1.54060 Å), β is the full width at half maximum of the (200/0 diffraction, and θ is the angle of diffraction. The diameter of F-AuNPs is 37.6nm.

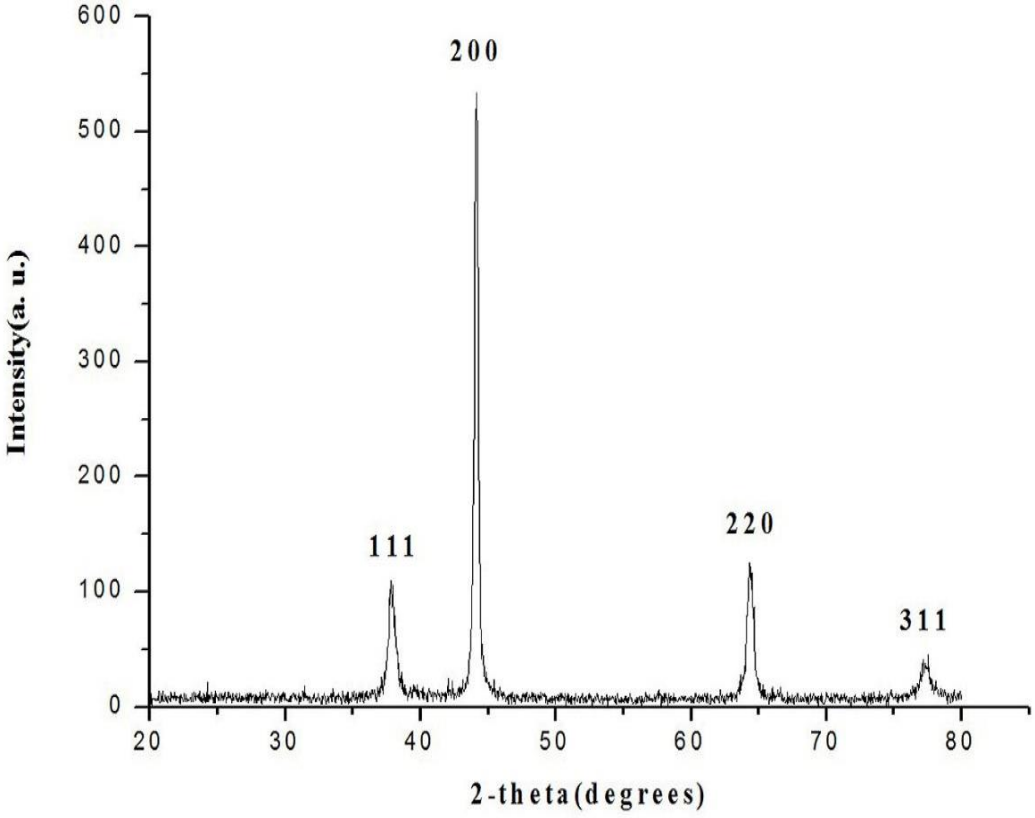


Figure 15 X-ray diffraction pattern (XRD) of F-AuNPs demonstrates the crystalline nature of FAuNPs

Transmission electron microscopy (TEM)

TEM analysis was executed to explore the size, shape and morphology of FAuNPs (Figure 16a). The FAuNPs consist of almost monodispersed spherical nanoparticles that are 3 – 50nm in range. Additionally particle size distribution calculated from TEM image fitted with a Gaussian function, by this analysis the average size of nanoparticles was found to be 23nm (Figure 16b).

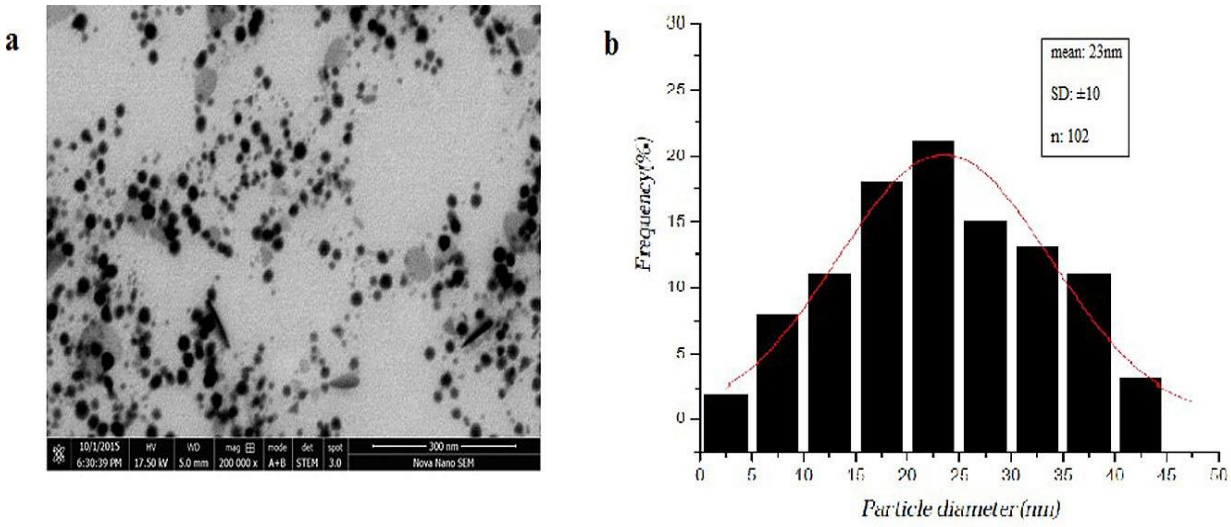


Figure 16 Nanoparticle fabrication from natural compound yields particles with totally homogeneous elemental distribution. (a) TEM micrograph of flavonoid gold nanoparticle (b) Particle size distribution of flavonoids gold nanoparticles.

The elemental composition of F-AuNPs was studied by energy dispersive analysis of X-rays (EDAX) (Figure 17). Presence of Au 4.42 wt% (Table 3) can be seen in EDAX analysis in support of XRD result. From EDAX spectrum and XRD, it was clear that F-AuNPs were composed of high purity Au.

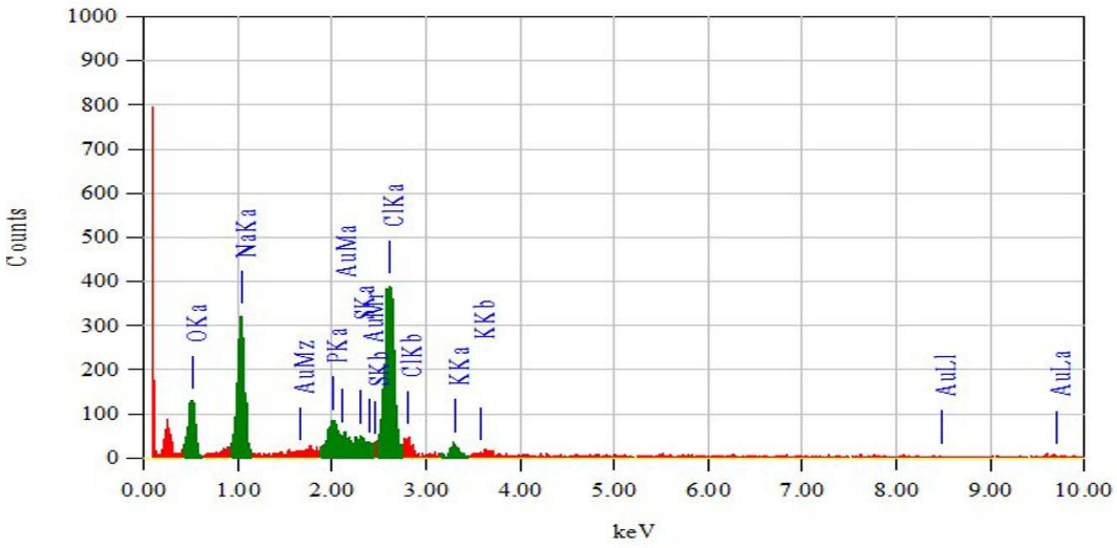


Figure 17 EDAX profile of flavonoid gold nanoparticles

Table 3 Elemental composition of flavonoids gold nanoparticles

Element	Weight (%)
O K	32.69
Na K	25.47
P K	4.38
S K	1.71
Cl K	28.71
K K	2.62
Au M*	4.42
Total	100

Results (Phase II)

In vitro Antibacterial Activity of Flavonoids

Crude extract, flavonoids (free and conjugate flavonoids) were investigated for their antibacterial activity against gram positive and gram negative pathogens and showed significant results against *S.* Typhi, *P. aeruginosa* and *E. faecalis* (Figure 18). The results obtained for conjugated flavonoids indicated that *S.* Typhi, *P. aeruginosa* and *E. feacalis* showed the maximum inhibition zone values of 24mm and 24mm and 25.5mm respectively. The free flavonoids extract expressed maximum inhibition zone against *S* Typhi and *P. aeruginosa* at 24.3 mm and 21.6 mm. The crude methanol extract expressed maximum inhibition zone against *S* Typhi and *P. aeruginosa* at 15.5 mm and 16.6 mm. These zone of inhibitions are obtained at higher concentrations of 50 mg crude compound ml^{-1} corresponding 500 µg / disc. Chloramphenicol formed an effective zone of inhibition in the range of 11 to 24 mm for Gram

positive bacteria and 20 to 27 mm against Gram negative bacteria. MIC of flavonoids and F-AuNPs against *E. feacalis* was 500µg/mL and 25µg/mL (Table 4).

Table 4 MIC of flavonoids and FAuNPs

Enterococcus faecalis	**MIC (µg/mL)**
Flavonoids	500
F-AuNPs	25

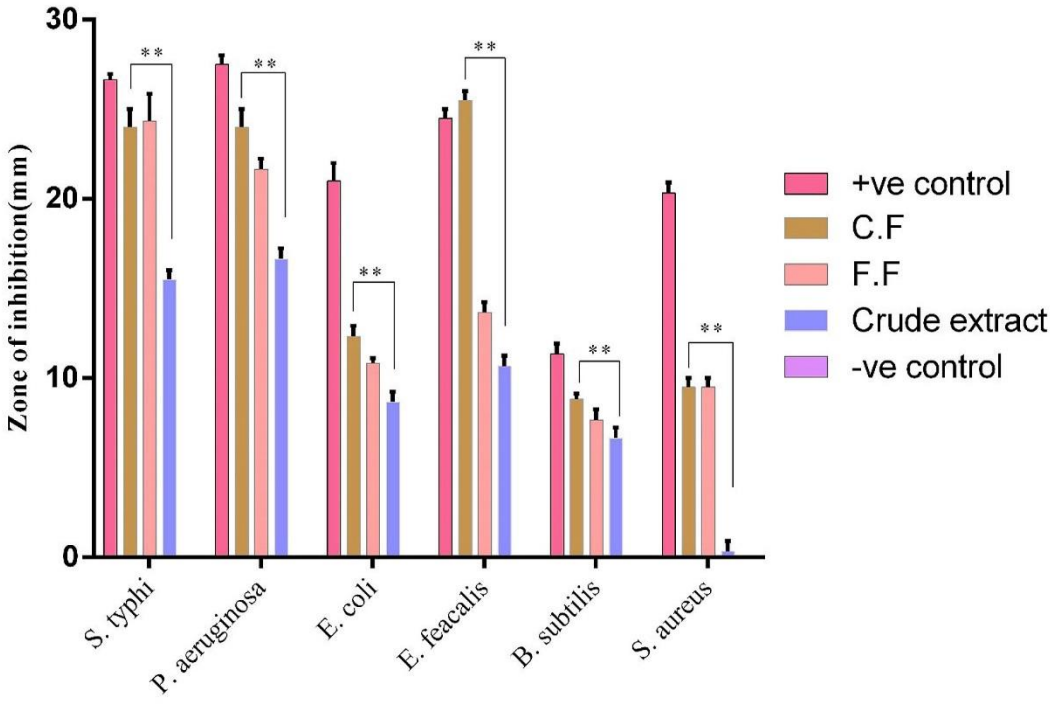


Figure 18 Antibacterial activity of flavonoids against gram negative and positive bacteria. The asterisks (**) indicate the significance difference ($p \leq 0.01$). The bars represent the mean ± S.D of three replicates. Chloramphenicol (+ve control) and DMSO (-ve control). Conjugate flavonoid (C.F); free flavonoid (F.F)

In vitro antibacterial Evaluation of FAuNPs

Quantitative microbial assessment (Table 5) showed bacterial reduction. The antibacterial activity of FAuNPs showed high activity with 99.97% as compared to control (chloramphenicol) with 98.9% bacterial reduction against *E. feacalis*. The MBC of FAuNPs for *E. faecalis* is 25µg/mL. At this concentration the viable cells reduction is almost 50% as compared to negative control.

Table 5 Antibacterial efficiency of the control and F-AuNPs

Test organism	**Flavonoids (µg/mL)**	**Control (CFU/mL)**	**Treated (CFU/mL)**	**Antibacterial reduction (%)**
E. feacalis	1000	7.67×10^{-10}	4.87×10^{-9}	93.65
	800	7.67×10^{-10}	4.3×10^{-9}	94.39
	600	7.67×10^{-10}	4.17×10^{-9}	94.56
	400	7.67×10^{-10}	2.83×10^{-8}	99.63
	200	7.67×10^{-10}	2.67×10^{-8}	99.65
	100	7.67×10^{-10}	7.7×10^{-7}	99.9
	50	7.67×10^{-10}	2.2×10^{-7}	99.97
	25	7.67×10^{-10}	2.5×10^{-7}	99.96
	10	7.67×10^{-10}	3.17×10^{-8}	99.58
	5	7.67×10^{-10}	2.13×10^{-9}	97.21
	1	7.67×10^{-10}	3.33×10^{-9}	95.65
	+ve control	7.67×10^{-10}	7.93×10^{-8}	98.9

Bacteriostatic Vs Bacteriocidal activity:

Figure 19 shows that the viable counts for *E. feacalis* were reduced in the presence of flavonoids and FAuNPs then untreated control culture between 6 and 24 h incubation. Flavonoids and FAuNPs show bacteriocidal activity as the number of viable cells after different times of incubation kill significant number of cells. The reduced cell counts did not recover to normal within 24 h.

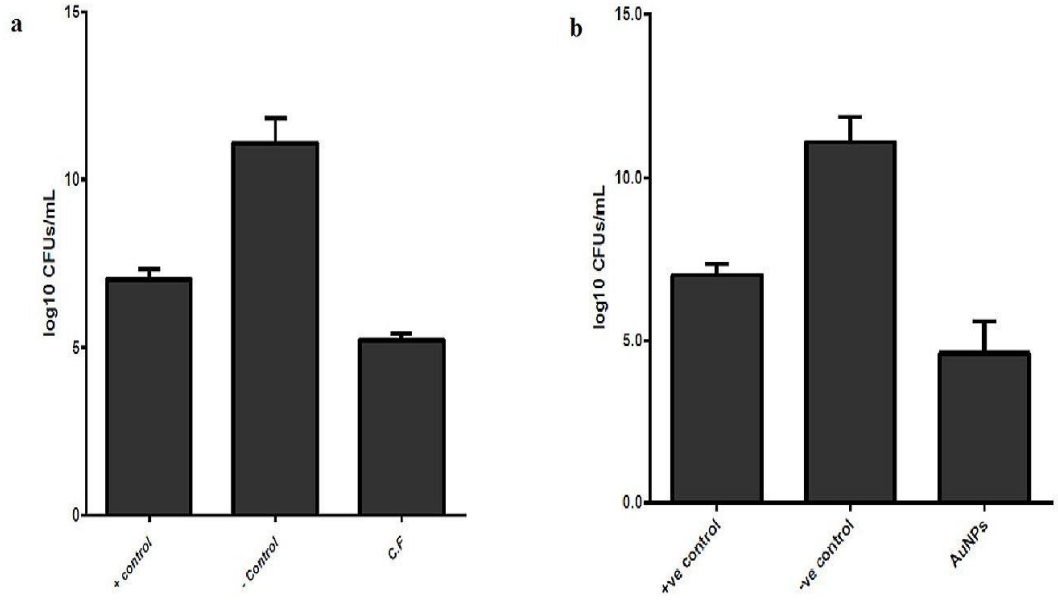


Figure 19 Antimicrobial action mechanism of (a) flavonoids (b) F-AuNPs against *Enterococcus faecalis* *All values are significant, i.e. $P < 0.05$. The bars represent the mean ± S.D of three replicates. Chloramphenicol (+ve control) and DMSO (-ve control).

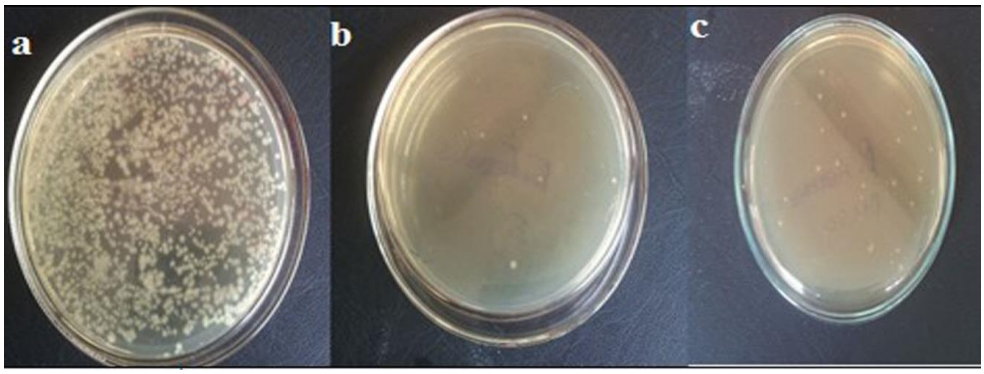


Figure 20 Antimicrobial action mechanism of (a) -ve control (b) F-AuNPs (c) +ve control against *Enterococcus faecalis*

Hemolysis assay

Hemolysis assay gave a clear indication of the extent of toxicity caused by flavonoid and FAuNPs of various concentrations (1, 5, 10, 20, 50, 100, and 150 ug/mL) on red blood cells quantitatively. The results obtained from this assay declared that FAuNPs are more hemocompatible as compared to flavonoid (Figure 21). Hemolytic behavior of FAuNPs increased gradually with increasing concentration rather then % hemolysis was less than 5 % that declares these particles to be hemocompatible and according to ISO/TR 7406 this ratio is considered as the safest value.

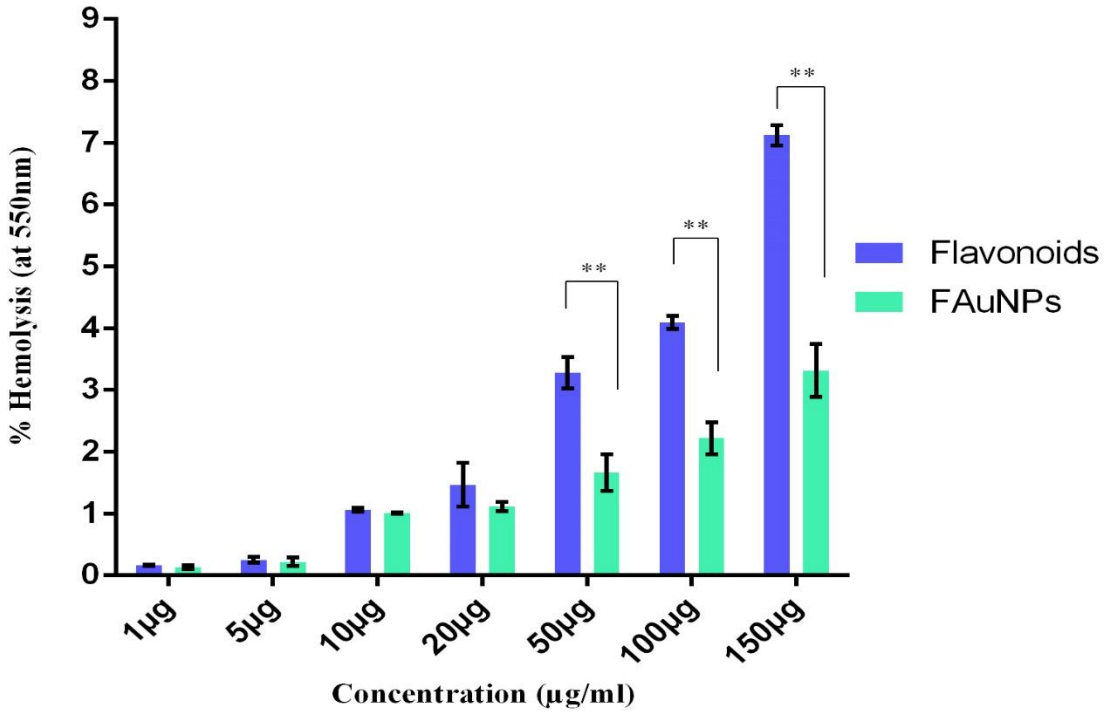


Figure 21 % Hemolysis of Red blood cells under the effect of flavonoid and FAuNPs. The asterisks (**) indicate the significance difference ($p \leq 0.01$). The bars represent the mean ± S.D of three replicates.

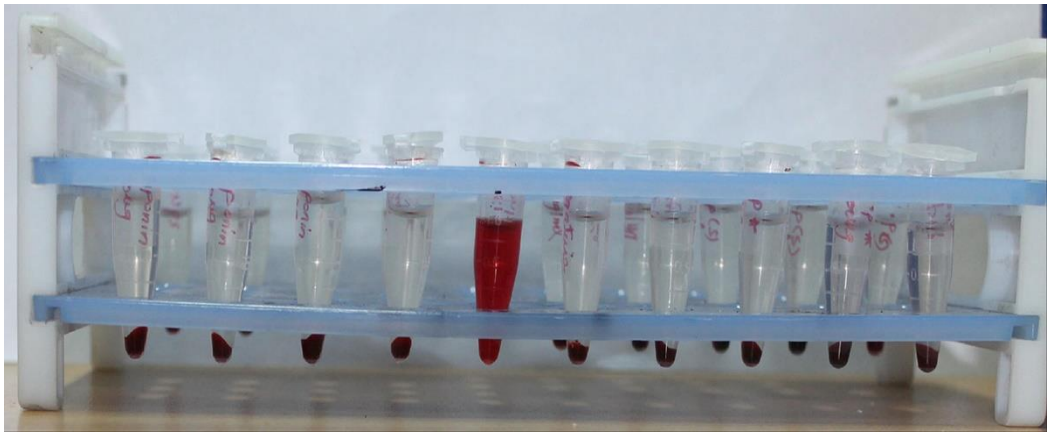


Figure 22 % Hemolysis (Visual observation) of Red blood cells under the effect of flavonoid and FAuNPs

***In vivo* Anticolonizing evaluation of flavonoids and flavonoids gold nanoparticles in infectious mice model**

The colonization of *E. feacalis* in control and treated (flavonoids and FAuNPs) were evaluated in infectious mice. The dose of flavonoids and FAuNPs were supplemented after seven days of infection. Seven days after intravenous treatment, group of mice were sacrificed, and their livers and kidneys were removed for the enumeration of viable bacteria. As shown in Figure 23, the counts (in $\log_{10}$CFU/gm of tissue) of both infected organs results revealed that, the anticolonization activity of FAuNPs (400µg/kg and 5mg/kg) was significantly greater than flavonoids (5mg/kg and 10mg/kg). From these results, it can be deduced that the nanoconjugates of flavonoids showed more anticolonizing activity and enhanced biological activity as compared to free flavonoids and also the amount of drug required for effective activity was reduced to less than 50%. All results are significant ($p< 0.001$) between control and treated group of mice.

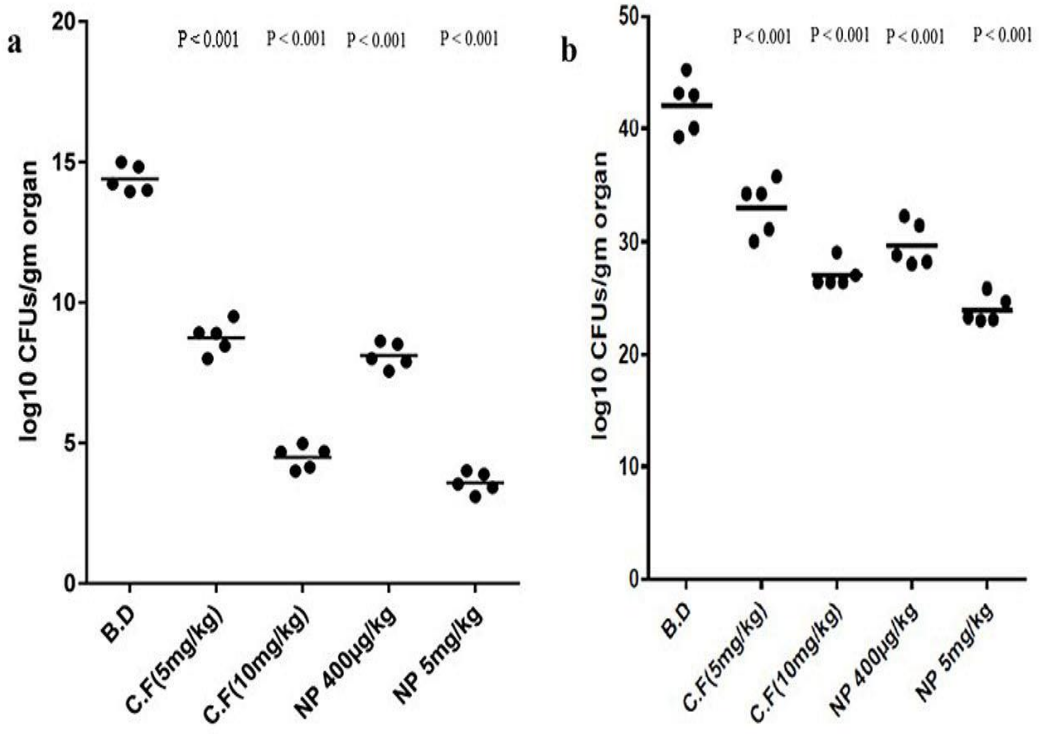


Figure 23 Effect of flavonoids and FAuNPs within mice organ of *E. feacalis* infected mice model. Groups of BALB/c mice (five per group) were injected intravenously with C.F (5mg/kg or 10mg/mg) or with the FAuNPs (400µg/kg or 5µg/kg). After 7 days of infection, the mice were sacrificed and necropsied. Organs were homogenized, and bacterial colonies on agar plates were counted. Each infection experiment was repeated 3 times. **(a)** $\log_{10}$ CFU/gram of kidney pairs; **(b)** $\log_{10}$ CFU/gram of liver. Horizontal bars represent geometric means. *P* values of < 0.05 were considered to be significant.

DISCUSSION

Enterococci are organisms with exceptional abilities and its ruggedness make them survive in harsh environment and acquire antibiotic resistance origins. The intrinsic and acquired ruggedness enable *Enterococcus faecalis* among enterococcus sp. the most persisting nosocomial causing pathogen **[24]**. The MDR evolution in these organisms poses humongous

challenges for clinicians when faced with patients having severe infection. The increased propagation and prevalence of multidrug resistant *Enterococcus faecalis* worldwide has resulted decrease in therapeutic antimicrobial options because majority of *Enterococcus faecalis* isolates are becomes resistant to vancomycin and ampicillin, and also exhibited remarkable resistance to aminoglycosides, which are the specific and most useful anti-enterococcal antibiotics **[25]**. Now, there is pressing need to develop new therapeutic agent that can target the bacterial virulence factors, that must be broad spectrum, having multiple cellular targets and which mechanism of action is difficult to control.

To intend these points we are investigating the most effective phytochemical which is not clinically explored by *Berberis lycium* leaves that is flavonoids. The *Berberis lycium* flavonoids showed phenomenal antibacterial activity against gram positive bacterial strains including *Enterococcus faecalis(*Clinically isolated*), Bacillus* cereus (Soil isolated), *Staphylococcus aureus (ATCC 6538),*and gram negative strains including *Pseudomonas aeruginosa(ATCC 9027), Salmonella* Typhi *(ATCC 6539)* and *Escherichia coli (ATCC* 8739) medicinally important species, when compared with antibiotic (Figure 18). By in vitro antibacterial activity we find that flavonoids have broad spectrum antibacterial activity, in this assay we also find that crude methanloic extract is less effective as compared to isolated flavonoids that were isolated from methanolic extract. The reason behind this is the structure-activity relationship for flavonoids antibacterial activity. Flavonoids affected the manifold range of cell functions in eukaryotic system. Published literature information indicates that flavonoids may target different components and functions of the bacterial cell **[26]**. It may be possible that individual flavonoids have multiple target site, in spite of specific site of action on cell. Perhaps these structural features may be necessary for flavonoids to gain uptake or proximity into the bacterial

cell. As the isolated flavonoids showed broad spectrum antibacterial activity were further investigated by MICs. As shown in Table 6, isolated flavonoids was the most potent inhibitor of E. feacalis with MIC value of 500µg/mL.

Table 6 MIC of flavonoids against gram positive and gram negative becteria

Bacteria	**MIC (µg/mL)**
Staphylococcus aureus	1000
Enterococcus faecalis	500
Bacillus cereus	1000
Escherichia coli	1000
Pseudomonas aeruginosa	800
Salmonella Typhi	900

O'Grady (1971) stated that, the main problem in any investigation is to distinguish the primary event in mechanism of action of an antibacterial agent **[27]**. Therefore we investigate the mode of action of isolated flavonoids, Figure 20 showed the rapid decreased in CFU and cause lysis of *Enterococcus faecalis.* This observation suggested that flavonoids mode of action could be bacteriocidal, this rapid bacteriocidal activity of compounds might be occurred by the disruption of bacterial membrane.

Antibacterial MIC studies of FAuNPs (Table 4 and 5) showed more zone of inhibition and reduced effective MIC as compared to flavonoids. Furthermore, the bacteriocidal activity of FAuNPs is reduced effectively from 50mg/ml to 25µg/ml. The bactericidal mode of action of FAuNPs (Figure 19b) is same as flavonoids, in vitro studies showed that formation of flavonoids nanoparticles enhance its antibacterial activity. Literature review reported that Au

ions react with SH groups of proteins and that mechanism play an important role in bacterial inactivation. It also interferes membrane permeability and disrupt the cellular membrane of bacteria as a result massive loss of intracellular potassium was induced. The possible targets of gold nanoparticles are protein thiol groups (respiratory enzymes) and the bacterial phospholipid portion of the membrane **[28]**.

In our study to find the real time anticolonizing effects of isolated flavonoids and FAuNPs, we designed *in vivo* model of *E. feacalis*. For this study first we find the cytotoxic effect of flavonoids and FAuNPs on blood cells. Literature reported that gold nanoparticles are assumed to be nontoxic due to their inherent nature. However, its cytotoxicity is expected at higher concentration due to generation of ROS, as reported with cell lines like MRC-5, NIH3T3. Figure 21 showed that FAuNPs are more hemocompatible as compared to flavonoid. Hemolytic behavior of FAuNPs increased gradually with increasing concentration and that the % hemolysis was less than 5 % that declares these particles to be hemocompatible and according to ISO/TR 7406 this ratio is considered as the safest value **[31]**. That safest concentration of flavonoids and FAuNPs are utilized in invivo anticolonizing study. Flavonoids have effective *in vitro* antibacterial activity but this effectiveness is reduced in *in vivo* studies, as literature survey showed that phenolics and flavonoids are absorbed and eliminated rapidly, and that their absorption efficiency is poor. As reported earlier in *in vitro* studies the effective dose of flavonoids is remarkably decreased by nanoformulation and this evidence is also investigated and verified by *in vivo* anticolonizing studied against *E. feacalis* in kidney and liver (Figure 23). However, E. feacalis is mostly residues within kidneys and livers and in chronic stage it also colonized in heart due to bacteremia. *In vivo* results showed that the bioactivity and biostability of FAuNPs is extensively effective then flavonoids. These results are strongly

supported by literature review, reported that flavonoids water solubility is poor, low bioavailability, instability to gastric and colonic pH, extensive first-pass metabolic effects, and have active efflux mechanism **[32, 33]**. We choose intravenous route, as through oral route it is believed that, flavonoids are extensively degraded by intestinal enzymes and microorganisms, producing different metabolites **[34]**. These new metabolites have varying bioactivity. So, these all consequences are may be overcome by nanosized drug carriers as FAuNPs (in size range of 5 – 50nm, Figure 16).

CHAPTER 5: CONCLUSION AND FUTURE PROSPECTIVES

Conclusion

Biosynthesized Gold nanoparticles stabilized with flavonoids of *Berberis lycium* (leaves) were synthesized and characterized by FTIR, XRD and TEM. Moreover, these highly stable nanoconjugates was not affected by high salt concentration. F-AuNPs were found active in inhibiting both gram positive and gram negative human pathogenic bacteria *in vitro*. Both flavonoids and FAuNPs were active in inhibiting *E. Feacalis* colonization in liver and kidney of BALB/c mice. This study report that leaves of *Berberis lycium* have effective antibacterial activity and it is evidenced by both *in vitro* and *in vivo* studies. Additionally, both of these flavonoids and FAuNPs serve as the lead compounds for the drug discovery, a critical requirement to counter the effects of multidrug resistant pathogenic bacteria.

Future Directions

- Over the years enterococci have demonstrated the potential to harbor and transfer resistance genes and as such have become an important clinical pathogen. A better understanding of resistance mechanisms to daptomycin and tigecycline is needed and will aid in the prediction and prevention of epidemiologic spread.
- Evaluating the metabolic pathways that are essential for the bacterial growth and are important for the bacterial colonization and infection.
- Targeting identification by *in silico* screening and docking of novel compound with specific targets.
- *In vivo* screening in Balb/c mice model, for evaluating the prospective toxicity screen.
- Optimal potency or selectivity of isolated compound.
- Determination of biodistribution and pharmacokinetic of isolated compound.

- Future directions of research must focus on development of new antimicrobial agents. Finally, efforts must continue to prevent development of antibiotic resistance and spread in the enterococci through infection control and antibiotic stewardship programs.

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