Chemical Sensing of Benzo[a] Pyrene Using Plant Mediated Fluorescent Flavonoids

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NUST201362080MSMME62413F

MS Biomedical sciences

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Islamabad, Pakistan

2015

IN THE NAME OF ALLAH THE MOST

BENEFICENT AND THE MOST MERCIFUL

DECLARATION

It is hereby delared that this research study has been done for partial fullfillment of requirement for the degree of Master of Sciences in Biomedical Engineering. This work has not been taken from any publication. I hereby also declare that no portion of 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.

Signed: _____

Dedication

This thesis is lovingly dedicated

To my inspiring parents and Supervisor

For their endless love, support and encouragement

ACKNOWLEDGEMENTS

All praises are for supreme **ALMIGHTY ALLAH**, the omnipotent, the omnipresent, and the omniscient, who guide us in darkness and helps us in difficulties. This thesis owes its presence to assistance, support and encouragement of several people. First and foremost, First, I would like to thank my parents for letting me to recognize my own potential and supporting me in my goals and ambitions. My love and appreciation for them can hardly be communicated in words. I wish to sincerely thank my academic advisor **Dr. Nosheen Fatima Rana** for her guidance during my research. I am extremely thankful for her help, professionalism and valuable guidance throughout the project. Additionally, I would like to thank my thesis committee members **Dr. Nabeel Anwar, Dr. Adeeb Shehzad** (SMME) and **Dr. Nasir Mehmood Ahmad** (SCME) for their concern in my work.

I would like to acknowledge **Dr. Jameel.A. Rehman**, Chemistry Department, Islamia University of Bahawalpur as the second reader of my research papers and I am greatly indebted to him for his valuable comments

My sincere thanks goes to my lab colleagues for their friendship and for their supportive consultations during my research in the lab. I am greatly thankful to all of you for making such a fantastic and encouraging laboratory work environment. I extend my sincere thanks to my siblings, friends and many others within and outside the department for their all-out support and guidance in different ways throughout the period of my research work.

List of Acronyms and Abbreviations

BaP _____ Benzo[a] Pyrene

PAH _____ Polycyclic Aromatic Hydrocarbons

IARC _____ International Agency for Research on Cancer

SNPs _____Silver Nanoparticles

DMSO _____Dimethyl sulfoxide

AgNO3 _____Silver Nitrate

FTIR _____Fourier Transform Infra-Red spectroscopy

MeOH _____Methanol

LB _____ Lysogeny broth

DTT _____ Dithiothreitol

SEM _____Scanning Electron Microscopy

EDS _____ Elemental Dispersive X-Ray Spectroscopy

PBS _____Phosphate Buffer Saline

FBS _____Fetal Bovine Serum

TLC _____Thin layer chromatography

WHO _____World Health Organization

BHI _____ Brain Heart Infusion

RBC _____ Red Blood Cell

Table of Contents

LIST OF FIGURES

ABSTRACT

Chemical sensing of biological and chemical agents and isolation of high value biological Compounds by use of nanoparticles have attracted great attention in research field. Plant phytochemicals such as flavonoids are in use for the development of optical biosensor. Benzo[a]pyrene (BaP) is a pervasive environmental and dietary carcinogen that has been concerned in human cancer etiology. A fluorescent assay is developed using plant isolated flavonoid for the detection of BaP. *Corchorus depressus* plant extract was used to synthesize silver nanoparticles (SNPs). During synthesis, Saponins which are present in large amount in plant extract have been utilized in SNPs formation leaving fluorescent flavonoids in remaining mixture. SNPs and isolated plant flavonoid are analyzed using UV-VIS spectroscopy, SEM, EDS, phytochemical screening and FTIR. A unique, quick spectrofluorometric assay for the detection of BaP was developed based on the hypothesis that BaP quenches the fluorescence intensity of plant flavonoid. This relationship is linear over concentration of Bap ranging from 0.1 ppm to 200 ppm. No Mutual interference effect is observed with other carcinogens.

Chapter 1

1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAH) constitute a large group of organic adulterants. They are comprised of carbon and hydrogen forming 2-7 condensed benzene rings [1, 2]. PAHs are formed by incomplete burning of organic compounds, inadequate storage and removal of fuels, oils and wood treatment practices [3]. PAHs are highly stable hydrophobic molecules, which easily interact with cell membranes and accumulate in lipid tissues [1, 2]. International agency for research on cancer (IARC) has classified these molecules as potent carcinogens due to their proved mutagenic and carcinogenic properties. Food processing procedures such as heating, drying, flavoring etc. results in generation of these compounds. BaP is a representative member of PAH and IARC has nominated it as robust human carcinogen [4]. Acquaintance to BaP leads to adverse effects on nervous, reproductive, immunological and endocrine systems [5]. Human exposure to BaP is mainly through ingestion of food (99%), air inhalation (0.9%) and water consumption (0.1 to 0.3%) [6]. It is slightly soluble and highly toxic due to which its detection at ultra-low level is difficult to attain. Methods used for BaP analysis including enzyme linked immunosorbent assay (ELISA), gas chromatography, mass spectrometry and high pressure liquid chromatography are precise and sensitive but they need tiresome radio labelling methods and are time consuming [7-10].

Separation science and technology for the isolation and purification of different molecules and compounds is an important part of bio-oriented exploration and knowledge. Bio-nanotechnology is an interdisciplinary research area integrating the knowledge of biological doctrines with chemical and physical methods to harvest Nano-sized particles with explicit functions [11, 12]. Green synthesis of nanoparticles have gained impetus in recent years because of minimum use of harmful constituents, decreased downstream processing requirements and fast and inexpensive fabrication. Biosynthesis of nanoparticles using plant extracts, their particular properties related to uptake and distribution and exploration of practical facets of these nanoparticles are the areas which are currently under study. Another applied and recent characteristic of these nanoparticles is their role in isolation and separation of plant natural products which is termed as harvesting.

Successful application of nanoparticles for separation and purification of different molecules can lead to resolve many problems challenged during other purification strategies [13].

Antibody and aptamer based sensors have been developed for the detection of BaP. However they require multistep processing and expensive chemicals. *Corchorus depressus* belong to Tiliaceae family and is used as fold medicine [14]. Roots and branches of this plant are rich in saponins. However glycosides, alkaloids and flavonoids are also present. In present study, silver nitrate is reduced with *C. depressus* methanolic extract. The Saponins are extracted out of the extract by the formation of Silver nanoparticles (SNPs) and the flavonoids remained in the left over. The leftover found to be fluorescent, is used for chemical sensing of carcinogen BaP.

Aims and Objectives

The aim of the study was Using Bio-Nanotechnology to isolate and purify compounds and using them to detect carcinogens.

Objectives of the study were:

- To synthesize AgNPs by using *C. depressus* methanolic extract.
- To separate the nanoparticles from reaction mixture, harvesting Flavonoids. Confirming the presence of flavonoids through different characterization techniques.
- To use Fluorescent Flavonoids for chemical sensing of benzo[a] Pyrene by fluorescence quenching.
- To study the interfering effect of other carcinogens in detection of BaP.

Furthermore, Saponins involved in SNPs synthesis by using *Corchorus depresssus* were used to find their Therapeutic potential on colonization of *E. faecalis* in mice

2. LITERATURE REVIEW

2.1. Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons are produced as result of incomplete pyrolysis of organic material and are composed of two or more fused aromatic rings. These are tenacious and pervasive environmental contaminants that are found in various food, water and soil samples. [15, 16]

Distribution of PAHs in environment rest on their properties. Because of their lipophilic characteristics, PAHs can easily disperse in air and deposit in soil resulting in accumulation in sediments and biota. PAHs can enter food through the discharge from forest fires, domestic wood burning or during various geochemical progressions such as petroleum seepage or volcanism. Presence of PAHs in water samples may be due to industrial discharges, waste water managements or can be present in soil due to release from containers close to dangerous waste situates [17, 18] PAHs can enter the body as diet components, where they can diffuse into sorptive tissues together with gastrointestinal epithelium. According to several studies, the amount of PAHs found in cooked or grilled food ranges from 0.17 to 78mg/Kg,[19] while in milk and other dairy products, PAHs concentration ranges from 1.5-7.5 mg/Kg [20]. International Agency for Research on Cancer and Scientific Committee on Food Regulation grouped some PAHs as genotoxic agents, for example, Benzo[a] Anthracene, Dibenzo [A, H] Anthracene, Benzo[a]Pyrene were classified as probable carcinogens and were placed in group 2A, while 5methylchrysene, benzo[B]fluoranthene, benzo[K]fluoranthene, dibenzo[A,I]pyrene and indeno[1,2,3-CD]pyrene were classified as possible carcinogens and were placed in group 2B. Another classification based on non-carcinogenic PAHs like chrysene was entitled group 3.[21-23]

PAHs adsorption in body leads to their distribution in various tissues, particularly in lipophilic tissues because of their non-polar characteristic, through blood routes. In the body, PAHs are metabolized into various active metabolites by the action of enzymes. The most important enzymes involved in carcinogenic metabolism of PAHs are cytochrome p450 enzymes CYP1A1, 1A2, 1B1,

and 3A4. [24] Activation of PAHs to their carcinogenic metabolites diol Epoxides lead to covalent bonding with DNA. Epidemiological studies revealed a correlation between PAH exposure level and PAH-DNA adducts in blood cells.

Based on their molecular arrangement, PAHs are grouped in two classes. Molecules with up to four rings are categorized as low molecular weight (LMW) and are found in gas phase due to their high vapor pressure, while molecules with more than four rings are grouped in high molecular weight (HMW) PAHs. LMW PAHs exist partially as vapors due to their semi volatile nature and are extremely prone to atmospheric degradation procedure. Semi volatile characteristic of LMW PAHs contribute to their mobility throughout the environment by means of deposition and volatilization amongst soil, air and water bodies, therefore, referred to as regional pollutants. On the other hand, HMW PAHs are less volatile and are present as particles in atmosphere and water, hence they are less prone to degradation. Due to their particle formation property, PAHs are less oxidized in atmosphere which contribute to their transfer over long distances and hence make them ubiquitous substances in environment. Thermodynamic stability, due to their large negative resonance energies , low solubility and adsorption to soil particles result in their accumulation in environment.[25]

2.2. Benzo[a] Pyrene

Benzo[a]pyrene is a toxic PAH that is widely distributed in environment. It is made up of five carbon rings having a molecular weight 252. It is produced by incomplete combustion of organic materials but it is also found naturally in petroleum based tars. It was recognized as main tumor causing agent in coal tar in 1932. BaP is itself fluorescent molecule which exhibit strong fluorescence upon excitation in UV light.[26, 27] BaP is a lipophilic, non-polar compound having a water solubility of 1.62 ug/L AT 28C and Kow of 6.04.[28] Conferring to International Agency for research on cancer (IARC), BaP proved to be carcinogenic in mice and is probably carcinogenic in humans. It is well documented fact that local administration of BaP in body resulted in induction of tumor[29]. BaP is a pro carcinogen, metabolism of which produces the ultimate carcinogen that bind to DNA and cause cancer. Metabolism of BaP is a three step process which lead to formation of ultimate carcinogen. In first step, BaP is converted to benzo[a]pyrene 7, 8 Epoxide by the action of cytochrome p450 enzyme. Second step involves the conversion of benzo[a]pyrene 7,8 Epoxide into benzo[a]pyrene 7,8 diol catalyzed by Epoxide Hydrolase (EH) enzyme. Final step involves

the conversion of benzo[a]pyrene 7, 8 diol into four isomers of 7, 8-diol-9, 10-epoxid. Quantitatively, the most important isomers of them is Benzo (a) pyrene 7, 8 diol-9, 10 epoxide which is a crucial carcinogen. This isomers bind DNA at guanine residues form BPDE-DNA adducts which lead to mutagenesis and carcinogenesis.[30, 31]. Cytotoxicity of BaP depends upon route and place of exposure to this metabolite. The main exposure sites include inhalation, ingestion and surface exposure on skin leading to tumor development at the site of exposure. Oral exposure to BaP by mother result in sterility in progeny and developmental abnormality in decreased fetus weight and death at later stages. Long term exposure to benzo[a]pyrene have caused adverse developmental and reproductive damage in animal models.[32] Along with mutagenic and carcinogenic effects, BaP also act as endocrine disruptor and is proved to be arthrogenic. [33]

The most dominant pathway of BaP contamination is through cooking and processing of foods which include grilling, roasting and frying of meat. Kazerouni et al., revealed the presence of benzo[a]pyrene in various commonly consumed foods items from grocery stores and cafés in Maryland. The average intake was measured to be 10-160ng/d tested in 228 subjects. Largest concentration of BaP was found in grains and bread cereals (29%) and grilled/barbecued meats (21%).

According to research conducted by Kishikawa et al., concentrations of BaP in cow milk, infant formula and human milk in japan was found to be 0.03ng/g, 0.05ng/g and 0.002ng/g respectively. According to European Commission study conducted in six European countries, average dietary intake of BaP was found to be 0.05-0.29 .ug/day.

2.3. Corchorus Depressus

Corchorus depressus is a perennial herb almost 6-9 inches in length. It belongs to the family Tiliaceae and genus corchorus. It is found in Pakistan, India, Africa and Cape Verde Islands. In Pakistan, it grows to an altitude of 1000m in arid and semi-arid regions. Its roots are diffusely branched and leaves are ecliptic 2-18mm in length and 2-9 mm in width. Flowers are yellow in color and 1 mm in length. Leaves and fruits shows stunted growth in saline and rocky areas. Seed color of *C. depressus* are minute and have chocolate like color while its fruits are capsule like having length of 8-15mm. phytochemical screening of *C. depressus* revealed that this plant is rich

is phenolics, triterpenoids, cardiac glycosides, fatty acids and sterols.[34-36]. Several parts of this have been employed as folk medicines for the treatment of various diseases. Whole plant has applications in the treatment of urinary disorders, injuries, urinary associated inflammations and fever.[37] Phytochemicals possessed by this plant have distinct pharmacological properties like anti-pyretic, anti-cancer and anti-convulsive characteristics.[38] Studies revealed that therapeutic potential of this plant is due to scavenging effect of reactive oxygen species (ROS). Anti-oxidant properties of this plant is due to presence of potential bioactive compounds such as β-carotene, alkaloids, terpenoids and flavonoids.[39]. Leaves of *C. depressus* are used to prevent sexual illnesses and recover hepatic irregularities while seeds and fruits act as mucilage for treatment of gonorrhea.[40]

There are two types of luminescence depending on the nature of excited states, Fluorescence and Phosphorescence. Fluorescence is produced typically by aromatic compounds. It is characterized by the emission of excited molecule from the zeroth vibrational level of excited singlet state. Fluorescence spectroscopy is a common technique to analyze and detect benzo[a]pyrene due to its highly fluorescent nature. Fluorescence quenching technique can provide selectivity and sensitivity to detect environmental pollutants. Quenching studies are useful because it can give photo-physical and photochemical information about fluorophore, prevent fluorescence from undesired molecules having slight difference in their structure, thus making detection easier.

2.4. Detection and Monitoring of benzo [a] pyrene

Evaluations for quantitative exposure poses many challenges for intricate mixture constituents as individual exposures differ depending upon background concentrations in environment, occupation and life style aspects. Human biomonitoring of BaP depends upon the use of biomarkers to inner dosage that will serve as systematic indicators of primary biological retort after subsequent acquaintance to genotoxic agent. Generation of carcinogen exposure biomarkers takes place by uptake and metabolic processes, the consequence of which is either detoxification and excretion or bio-activation to reactive forms resulting in covalent binding to DNA and other macromolecules. Monitoring of BaP uptake after exposure to PAHs mixture can be evaluated by urinary metabolites of BaP, DNA adducts in peripheral blood cells, protein adducts in hemoglobin, serum albumin and cytogenetic impairment in lymphocytes.

Animal studies revealed that elimination of BaP and its metabolites through urine is a minor pathway as compared to elimination in feces. The hydroxylated metabolites of BaP were found to be present in urine samples of people exposed to PAHs. One of the hydroxylated metabolite, 1-oh-Py is used as surrogate marker for the internal exposure to PAHs. This is based on the assumption that presence of this metabolite in urine is correlates to exposure to high molecular weight PAHs particularly BaP. Burkley et al., observed that excretion of BaP metabolites in urine was best interrelated with incorporation of BaP from food rather than from inhalation acquaintances. Based on 24 hour personal air measurements, pulmonary uptake was estimated to be 11 and 2.3 ng/person/day, respectively for winter and summer. The average uptake of BaP from diet was assessed to be 176ng/day, while the average urinary excretion of BaP and its metabolites was estimated to be 121 and 129 ng/day, respectively in winter and summer. Gundel et al., experimented PAH exposure and urinary metabolite excretion in 19 people working in a fire proof stone factory. Based on personal and stationary air sampling, 3-OH BaP was found to be present in urine samples ranging from 0.043 to 5.41 ug/m^3 , following exposure to BaP.

2.4.1. Antibody based sensors for benzo[a]pyrene sensing

The specific interaction between antibody and antigen leads to developments of immunoassays for environmental monitoring and clinical diagnostics. Therefore, immunoassays offer greatly repeatable and precise reaction format to identify specific environmental pollutants.[41]

Miura et al. applied SPR technology to the detection of benzo[a]pyrene (BaP). An indirect inhibition assay was applied and BaP-BSA conjugate was synthesized and covalently immobilized on the sensor surface by physical adsorption. The anti-BaP-BSA antibody with a certain concentration was mixed with BaP solution, and the mixture was measured by the SPR sensor. The sensor chip was able to be reusable for more than 20 times and the LOD for BaP was determined as low as 10 ppt with a response time of approximately 15 min.

Dinh et al., used non-radioactive method, fiber optic antibody based fluoroimmunosesnors (FIS) to sense deadly chemicals such as BaP and biomarkers of environmental and human acquaintance. FIS system can be used to detect both fluorescent and non-fluorescent molecules. Monoclonal antibody used for this procedure was developed from spleen cells of mice immunized with BPDE-

Guanosine and are coupled covalently to quartz Fiber optics sensing probe of a FIR. For the excitation source, a helium cadmium laser was used. This technique proved to be useful for detection of up to 1 femtomole of BaP in 5 ul of sample.[42].

Alarie and Dinh developed antibody based submicron sized optical fibers to detect BaP DNA adducts. Advantage of using this technique was to decrease the size of fiber optic based sensor for use in submicron environment. The detection limit using this technique came out to be ≈ 300 zeptomoles (10^{-21}moles) for BaP tetrol, a product of BaP DNA adduct.[43]

Cullum et al., used fluorescence fiber optic Nano sensor tip to detect BaP metabolite in mammary carcinoma cells. Tip was coated with antibody and detection was done on the basis of fluorescence of secondary antibody. The sensor was able to detect BaP tetrol with in the cells of two different cell lines, human mammary carcinoma and rat liver epithelial cells. Detection limit of this sensor was found to be 6.4 ± 1.7 E pM of BPT. As carcinogens binds to DNA and form different BaP DNA adducts so this technique can be useful in cancer screening.[44]

2.4.2. Aptamers

An aptamer also called as chemical antibody, is either a DNA or RNA sequence selected by Systematic Evolution of Ligands and Exponential Enrichment (SELEX). Aptamers has the ability to fold into three dimensional structure during its binding to target selectively. This selective interaction between aptamer and target is through stacking of aromatic rings, electrostatic and van der waals forces, structure compatibility, hydrogen bonding or blend of all these properties.

2.4.3. High Performance Liquid Chromatography

These methods often require large sample amount and long HPLC run times. Chen et al., used HPLC fluorescent detection method to check the occurrence and level of BaP in different heat treated foods in China. Results indicated that out of 119 samples, 105 were containing BaP from 0.03 to 19.75 ug/Kg. Results clearly indicated that highest BaP levels were found in charcoal grilled and smoked foods while grains food contains trace levels of BaP. So heat treated food poses high health risk of carcinogenesis from BaP.[45]

Manchester et al., used HPLC in combination with synchronous fluorescence spectroscopy to check the occurrence of BPDE DNA adducts in placenta. In this procedure, DNA from all placenta was combined and partially digested using enzymes into oligonucleotide fragments. Polyclonal

antibodies were used to select DNA fragments containing BPDE DNA adducts by immune-affinity chromatography. Hydrolysis of column elutes under mild conditions was carried out and further extracted by using organic solvent. HPLC and Fluorescence spectroscopy was used to determine the presence of BaP tetrahydrotetrol and further confirmation was done by carrying out GC/MS analysis. 10 out of 28 samples were found to contain DNA adducts from benzo[a]pyrene [46].

Haugen et al., studied the genotoxic effects of PAHs exposure to workers in coke oven plants by using air and urine samples and analyzed by employing capillary gas chromatography and HPLC. As the ultimate carcinogen BPDE form adducts with DNA, radioimmunoassay and fluorescence spectroscopy were employed to measure BPDE DNA adducts in lymphocyte DNA. Results indicated that one third of the workers had detectable putative BPDE DNA adducts in lymphocytes. Despite of high exposure to PAHs in coke oven plants, not all samples showed the presence of BPDE DNA adducts. This may be due to variation in exposure to PAHs, balance between metabolic and deactivation , DNA repair capability and sensitivity of the detection assay[8].

Muyela et al., studied the effect of fire wood smoking and oil frying n the BaP levels in fish. Extraction of BaP was carried out with cyclohexane and dimethylformamide- water followed by clean up on silica gel column and determination by HPLC employing fluorescence detection. BaP level in smoked fish was found to be 7.46 to 18.79ug/Kg, while in fried fish, it came out to be 4.17 to 11.26 ug/Kg. Results were compared with regulatory limits and it was revealed that smoked fish samples exceed the Maximum residual limit (MRL) of 5 ug/kg , while almost 20% fried samples were under MRL. Results concluded that firewood smoking result in higher risk of BaP contamination.[47]

2.5. Fluorescence Quenching

Identification and quantification of fluorescent molecules in composite fluorescent spectra often poses serious challenges, particularly in case of low signal to noise ratio. Salmon et al., devised a method to overcome such problems in detection by using specific quenchers. It was revealed that liposoluble collisional quenchers such as n-iodo hexane and iodo benzene selectively quench the fluorescence of BaP in suspended living cells formerly incubated with BaP and BPDE. But due to the excess usage of quencher, the method didn’t worked well. So Salmon et al., suggested a computerized technique that permits the resolution of low light level multifaceted fluorescence

spectra into its constituents. Based on the integration of fluorescence intensities over full fluorescence spectra, the compounds that contribute in intricate fluorescence spectra are recognized and enumerated. Fluorescence spectra giving low signal to noise ratio can be resolved because of the integration of fluorescence intensities. The sensitivity and consistency of this technique was shown by resolution of complex intracellular fluorescence of single living cells pre-treated with BaP.[48]

Although there are methods to detect benzo[a]pyrene that have been reported in the literature, these methods are very tedious and requires multiple instruments to analyze. Hence, there is a need to develop an efficient detection method to this highly carcinogenic molecule. The disadvantages of the radioactive method include cost, radiolabeled individual metabolites are not available, and it cannot be used for the analysis of environmental samples.

2.6. Saponins

The word saponin is derived from Latin word Sapo which means soap, because of the formation of soapy foams when agitated. Soapy nature of Saponins in aqueous media is due to presence of polar and non-polar parts in their structure. Saponins contains large group of secondary metabolites in plant kingdom. They have the molecular weight of 1000Da. They are non-volatile and are known as natural detergents. Structure of saponins include one polar part characterized by at least one sugar moiety attached to a less polar sapogenin or aglycone chain that is either of triterpene or steroidal nature. Saponins possess several biological properties such as antimicrobial, antiviral, anti-inflammation and anti-cancer.

2.6.1. Classification of saponins

Although compounds have been classified previously according to their biological or phytochemical characteristics. But nowadays they can be classified on the basis of advanced knowledge of chemistry. Thus when term “saponins” come across the mind it reproduces the idea about a property of natural product classification that should be described more accurately. This becomes achievable because of the understanding of their chemical structures and their biosynthetic pathways has increased exceptionally in these days. This knowledge has encouraged the classification of natural products (e.g. saponins) on the basis of carbon skeleton biosynthesis [49-51].

2.6.1.1. Major classes

Primarily, saponins were classified into two major classes, the steroids and triterpenoids [52], both are the derivatives of a 30 carbon precursor oxidosqualene [53]. The difference is that, steroid class of saponins are 27-C atoms containing molecules but the triterpenoid class saponins have all thirty carbon atoms in their molecule. From the biosynthetic approach, this difference is not so important because triterpenoid saponins exhibit many other carbon skeletons. However, this differentiation in these two classes is rejected in a current review [54].

2.6.1.2. Subclasses

Saponins were divided into three sub classes known as 1) triterpenoid saponins, 2) spirostanol saponins and 3) furostanol saponins. Yet this classification was not based on the difference of biosynthetic pathways, but highlighted the incidental structure elements due to secondary biotransformation. There are few more compounds such as the glycol-steroid alkaloids that have been recognized as saponins [53]. Though these compounds have same biosynthetic origin as saponins and have a skeleton resemblance to steroids linked through glycosidic linkage to the monosaccharide components, but are not categorized as saponins. Glyco-steroid alkaloids have a single nitrogen atom as a natural part of glycine structure that already classify them in a separate group.

Subclasses of the major classes are arranged on additional changes of the carbon skeletons by minor rearrangements, cleavage, degradation and homologation. Oxidation causes changes in carbon skeleton and in result, the functional groups such as hydroxyl and carbonyl groups present in different positions in carbon skeleton are oxidized. These modifications lead to cause further changes, such as formation of ether bridges, spiroketals or lactons because these functional groups are usually played role in chemical reactions. Sub-classification based on these characteristics is not discussed in this literature review as these are the type of secondary biotransformation [55, 56].

Three subclasses of saponins (i.e. triterpenoid, steroid and steroidal glycoalkaloid) have different types of plant origin. Triterpenoids are usually found in dicotyledonous plants and a few monocots. Steroid saponins are present in monocotyledonous plant (e.g. Droscoracaeae and Lilliaceae) and in few dicots as well, such as Foxglove [57]. Oats exceptionally contain both triterpenoid and steroid saponins [58]. Third member of this group that is steroidal glycol alkaloids are primarily present in the plants of family Solanaceae. Saponins extracted and

purified from family Solanaceae plants and oat have been studied in detail in the reference to their potential role in plant defense mechanism against fungi [59].

2.7. Saponins Sources and Types:

2.7.1. Plant Sources:

Cortex Albiziae is a traditional medicine of South East Asia which comprises various secondary metabolites such as saponins, triterpenoids, alkaloids, tannins etc. The saponins are especially significant group of metabolites acquired from the bark, are involved in various bio-activities such as anti-tumor, anti-inflammatory and anti-sedative.

The family Ranunculaceae has a genus Cimicifuga with 25 species present all around Asia, Europe and America. This family is considered as one of the important traditional medicine in Japanese pharmaceutics. The rhizomes of Cimicifuga accompanied by other synthetic drugs are used to treat various diseases, for example, headache, pain, swelling, measles and mumps with immune-compromising characteristics. The chief components of Cimicifuga plant are 9, 19- cyclolanostane triterpenoids glycosides, steroids, alkaloids and phenolic metabolites. The significant metabolites being triterpenoids that provided the essential features to Cimicifuga genus of which more than 120 different types have been recognized.

Plant family Catunaregam spinosa is well known exceptional medicine of India and Brazil and is well-known for its extensive implications as a drug against dysentery, inflammation and fertility issues. The research on this plant have revealed the presence of diverse types of glycosides and triterpenoids Saponins. In most recent studies conducted on this plant, four new types of triterpenoids such as Catunaroside I-L, Randia-saponin-7 and Arjunetoside have been isolated from plant stem.

2.7.2. Marine Sources:

Marine environment is one of the natural foundations of bioactive molecules. Class Echinodermata, sea cucumbers, mollusks, star fish, sponges and sea urchin comprise bioactive metabolites which may be due to their feedstuff that is marine algae and phytoplankton. Sea cucumber is used for the treatment of various ailments and is well thought-out as one of the most vital marine sources of natural products. The importance of sea cucumber in medicine is usually related to the presence of Saponins in its body.

The history of studies on sea cucumbers triterpene glycosides is quite long and the ultimate reason is being the rare event of presence of saponins in animal kingdom. Glycosidic compounds were isolated from aqueous extract of sea cucumber that was named as holothurin. Therapeutic potential of saponins of group holothurin have broadly been studied over the period of last 10 years and have been considered as an essential group for treatment of various diseases. This group of saponin has been found to contain anti-oxidant properties and pro-apoptotic properties that are well studied in animal models.

Over the period of last two decades structural and taxonomic variety and biological activities of holothurin has been inspected meticulously, most important being lanostane aglycones that relays to holostane group through 18-20 lactone. They are called so because they have structural base of holostane. Lately new class of sea cucumber saponin primarily triterpene glycoside is discovered having dilerent aglycone moiety. Sea cucumber glycosides have monosaccharide group that involves D-Xylose, D-Glucose, D, 3-O-Methylglucose and D-Guinovose. These structures are quite composite and complex which may vary according to their position, number along with nature of monosaccharide moiety. Functional group may also differentiate them from one another such as unsaturated double bonds, their number and position, acetic and hydroxyl groups of aglycone group. These compounds are subfamily, genera and species specific in case of sea cucumber. These compounds are membranolytic in action due to the complex formation between glycosides and un-saturated steroids of cell membranes that may result in ion-channel formation and large number of water-pores in the target cell membrane. When glycosides are low in concentration K^+ outlet may disturb the transportation across cell membrane that may cause severe damage to the cell. Similarly when glycosides are high in concentration there is a loss of amino acid and nucleotides that may result in cell lysis. These membranolytic actions are quite important for the survival of organism as it may affect gill capillaries. Glycosides are quite important for sea cucumber in this regard as they protect them from predation due to their ichthyotoxic properties. Glycosides are also considered important due to their allelopathic properties that prevent attachment of small larvae and their food rivals. Continuous studies on sea cucumber saponins have provided the evidence that they may help in improving lipid metabolism of rats. However earlier studies did not confirm whether sea cucumber saponin improve lipid metabolism or not. This testing was done on different animal models for deep understanding of chemical and physiological changes that may occur within

the organism during NAFLD development. mRNA expression studies confirmed that sea cucumber saponins tend to increase ß-oxidation in liver cells through the pathway of PPARa-mediated gene transcription. The study showed that gene expression was up-regulated that increased the ß-oxidation process.

The triterpenes found in the marine organisms are found to have less toxicity in various in-vivo and in-vitro studies that make them an important candidate for the treatment of various disease specifically cancer but their mode of action and molecular mechanism is still not fully understood. Saponins from sea cucumber such as, cucumariosides and frondoside A have the ability to induce apoptosis and cytotoxic effects. Molecular mechanism is not known but studies have shown that Stichoposide-C and Stichoposide-D have anti-tumor effects.

2.8. Antibacterial effects of saponin:

Saponins are surface active molecules that may enter lipid bilayer membrane where it may attach to cholesterol forming saponin-cholesterol complex and in this way it may disrupt cell. However cholesterol absence does not inhibit saponins pore production. Saponins have the ability to disturb bacterial membrane permeability. Bacteria cell wall and outer membranes are bound with lipopolysaccharides in 90 % of gram negative bacteria's. Saponins interact with lipid-A to increase cell wall permeability. The complex formation of saponins with lipids may enhance anti-biotic absorbance to resist bacteria. Ampicillin in conjugation with saponins decrease the cell number in case of *Proteus miabillis* strains such as R-45 and s-1959. Main problem associated with bacterial pathogens is their elimination. Number of treatments has been proposed such as treatment with bacteriophage, prevention of adhesion and increase in cell wall permeability. Desired combination of saponins with anti-bacterial effect is to have minimum cytotoxic effect in eukaryote cells. Saponins can increase cell permeability without disrupting it due to its detergent properties. This may help in anti-biotic uptake via cell wall. *Quillaja saponaria* is used for triterpene source. Its extract is commercially available as emulsifying agent or in different drinks. Saponins show anti-bacterial effect at 12µg/ml concentration with minimum cytotoxicity. Saponins have no effect on *E. coli* bacteria in M9 Minimal media and broth made up of Luria. It shows significant effect against E.coli at 12µg/ml but this effect is not studied with MPOS media.

Bacteria are getting resistant to anti-biotics day by day due to mutational changes leading in their increased survival. It is alarming condition especially in case of nosocomial infections. One of the resistant strains of bacteria is *Enterococcus fecalis.* One of the studies carried out to check the effect of saponins on bacteria involved vancomycin resistant- enterococci. *In-vitro* testing of anti-bacterial activity of saponins was done and results showed effect of antibiotics such as gentamicin, daptomycin increase when used in conjugate with saponins. Glycyrrhizic acid saponins were also found to decrease MIC in case of gentamicin resistant VRE. But its anti-bacterial activity is weak against clinical pathogens. This study has led to discovering therapeutic potential of saponins against infectious bacteria.

2.9. Toxicology/ cytotoxicity of Saponins

Cell remains in homeostasis until there is balance between cell division and death of cell. Cell dies due to mechanisms such as necrosis and apoptosis. Apoptotic studies are important for cancer study. Cytotoxic effects of saponin are related to formation of complex between saponin and sterols present in membrane leading to ion channel formation and pore synthesis. These pores cause imbalance of osmotic pressure thus damaging the cell. When relationship of structure and cytotoxicity of saponins was studied it was found that oleanane saponins exhibit high cytotoxic effect compare to lupane saponin. In addition glycone chain length and its attachment to Carbon-3 have great impact on cytotoxic properties. But it is found that saponins have cytotoxic effect that is time and dose- dependent.

Saponins of plant origin such as ginsenoside, saiko-saponins and soya-saponins are anti-cancerous. MTT assay is used to assess the anti-cancer activity of gymnemagenol type saponins on HeLa cell lines in in-vivo studies and there was significant decrease in cell proliferation of HeLa cell line.

Sea cucumber saponins are found to be cytotoxic towards leukemia cancer and prostate cancer cell lines due to its anti-proliferative properties. Sea cucumber saponins effect against leukemia cells lines shows that these saponins have anti-apoptotic properties. The mechanism behind involve an increase in caspase-3 type protein and Bax levels with decrease in Bcl-xl level. Negative co-relation exist between c-myc, p53 and Bcl-2 when anti- apoptotic activity of saiko-saponin was observed in lymphocytes.

Plant glycosides have been studied for their anti-tumor effects. Ginsenosides Rg-s are inhibitor of cell invasion in case of different hepatomas, lung cell carcinoma and pancreatic cancer when mono-layer invasion mouse model is studied. Structural analogs to Rg-s such as Rb-2, Rg-2 and 20-R-ginsenoside Rg-2 have very less inhibitory effect. Ginsenoside Rg-3 is found to be effective against extremely metastatic cells of melanoma B16-FE7 and inhibit its metastasis to great degree.

Studies have shown that ginseng is anti-cancerous in non-specific organ cancers. White and red ginseng powders in odd ratio remarkably reduce cancer growth in case of non-organ specific cancers but fresh slices, Juice or ginseng tea was found in-effective. There is directly proportional relationship between ginseng intake and decrease in cancer risk. Ginsenosides of ginseng are reported to cause reverse-transformation in cancer cells. Rh-1 activates adenyl cyclase and increases melanin production relating it to reverse- transformational properties. Ginsenoside Rh-2 found in red ginseng inhibits different forms of cancers like liver, lungs and skin cancer in *in-vitro* studies. One of the ginsenoside Rb-2 is involved in angiogenesis suppression making it suitable candidate as an anti-cancer agent.

Saiko Saponins are triterpenoid saponins present in the *Bupleurum falactum* with steroid properties. Apoptosis is main regulator of T-cell lymphocyte function and number. Saiko-saponins are reported to have immune-modulatory effect. Its apoptotic effect against CEM lymphocyte is investigated and compared with dexamethasone. Saiko-saponins are reported to have many pharmacological applications against hepatic disorders and is known to cause apoptosis in liver, pancreas and colon carcinoma cell lines. Saiko-saponins are important for their immune-suppressant activities and anti-inflammatory properties. Saiko-saponins are involve in induction of apoptosis in immune as well as smooth muscle cells providing remedy for both restenosis and atherosclerosis. Saiko-saponins are also found to have anti-inflammatory and lipid lowering effect. Mechanism of apoptotic effect of saiko-saponins is studied in human lymphocytes. Different pathways are involved in signal transduction from membrane to nucleus. Previous work has shown that curcumin has apoptotic effect due to inhibition of expression of bcl-2 m-RNA and c-myc. Genes associated with tumor suppression like p-53 and proto-oncogenes like c-myc and bcl-2 are involved in cell proliferation phenomenon. Saiko-saponins cause decrease in bcl-2 level with an increase in proto-oncogenes levels such as p-53 and c-myc. It is found that saiko-saponins restrain DNA manufacturing and

cause apoptosis in lymphocytes namely CEM thus they can serve as template for production of anti-inflammatory drugs. When concentration of Saiko-saponins is high it causes necrosis studied by annexin- V staining techniques.

Trillium tschonoskii is a Chinese medicine used for treating different health problems such as fever, headache, blood pressure and cancer for thousands of years. Steroidal and glycosides saponins are present in it. But studies on it are done determining whether it has cytotoxic effect or not. TTB-2 is a type of steroidal saponin that is isolated in n-butanol water layer. Studies have shown that it has anti-cancerous effect against lung cancer cell lines via ROS- derived pathway. Very little is known about its mechanism of action. Most of the work is done on its analogs that are found to have medicinal properties against several disorders like edema, gastric lesions and have cytotoxic effects against different types of cancers. TTB-2 is found to have cytotoxic, anti-inflammatory properties against lung cancer. It leads to apoptosis within cancer cells via an increase in cellular ROS level with adjuvant decrease in matrix melano-proteinase. Cells aggregate during cell cycle phase G2-M representing apoptosis precursor.

Albizia julibrissin is used as folk Chinese medicine as sedative agent to cure pain, wound and ulcers. Modern *in-vitro* studies have shown that AJ has anti-cancerous effects that suggesting its use as anti-cancer drug. Crude extracts is rich in saponin content and have anti-angiogenesis properties against HMEC-1 and 3-B11 cell lines. Total saponin content of extract shows anti-angiogenesis properties along with anti-cancer properties. The mechanism behind it is yet to be disclosed. *In-vitro* and *in-vivo* studies have been conducted to study AJ anti-angiogenic properties against VEGF- mediated angiogenesis to explore it as anti-cancer drug that may target VEGF pathway of signal transduction. Angiogenesis has three main phenomenon's first is cell proliferation, second is migration and last one is endothelial cells tube development. VEGF is key factor of angiogenesis to start up this process. VEGF is found to increase cell proliferation in human endothelial cell lines such as EA.hy-926. When these cells were treated with extracts of AJ they show significant decrease in cell proliferation but this is dose dependent process. To test the effect of TS-AJ in in-vitro studies wound -healing- assay was used. After studying its effects it was found to be angiogenesis suppressor while keeping its connections with cells. TS-AJ inhibits all the primary factors of angiogenesis such as proliferation, migration and endothelial cell tube formation via acting as VEGFR-2 suppressor inhibiting

down-stream signal transduction of ERK, AKT and FAK protein receptors. It can be used orally as an antiangiogenic drug targeting signal pathways.

Saponins from Tribulus terrestris are known for their anti-tumor properties as they are involved in regulating signal transduction pathways. Mode of action of saponin is extensively studied but provides little information. Effect of saponins on breast cancer cell lines have been investigated that shows that when extract was applied, it changed the m-RNA level in three different genes namely CXCR-4, BCL-2 and CCR-7. Level of CXCR-4 expression decreases in both the cell lines MCF-7 and MCF-10A. While BCL-2 and CCR-7 decrease in just MCF-7 cell lines suggesting saponin action as cell specific. The mechanism behind it may be change in metastasis and apoptosis of cancerous cells.

Colon cancer is one of the leading causes of death in under-developed countries due to bad health conditions. It becomes malignant due to inflammation. American ginseng is potential therapeutic agent against colon cancer studied in different xeno-graft models. But these models are not specific to gut to examine colon cancer. Steroid and triterpenoid saponins isolated from *Chlorophytum borivilianum* are extremely important herb for treating diabetes, rheumatism and many types of cancers such as HCT116 and HT29 colon cancer cell lines.

Four furostanol type saponins isolated from rhizomes of Smilax scobinicaulis were found to have cytotoxic effect against cervical cancer cell lines (HeLa cell lines) and liver cancer cell lines (SMMC-7221). Some of the compounds showed high cytotoxic activity while others had no effect against cancer cell lines. Compound 7 of Smilax scobinicaulis showed highest results when compared to other compounds. This compound is spirostanol type saponin showing that spirostanol type saponins have high cytotoxic effect than that of furostanol type saponins.

Rg-3 and Rh-2 are very effective when used in combination with anti-cancer drugs. They decrease tumor growth by suppressing cell proliferation in tumors, hinder metastasis and effectively decrease reactions of therapies given to mice model. Rg-3 is an effective drug against cancer and tumors in China. In Korea cancer treatment is facilitated by BST-204 a purified form of dry ginseng extract that contains high proportion of Rh-2 and Rg-3.

Rb-2 in associated with endometrial cancer treatment as it down-regulates the activity and expression of MMP-2 without disturbing the tissue inhibitor such as metalloproteinase type 2 and type 1 expression thus making it useful against uterine endometrial cancer for inhibition of metastasis.

Modern world is facing the problem of controlling cancer spread and anti-cancer drugs currently available in market have serious side effects associated with them. So there is a need to develop herbal medicine for cancer treatment with minimum side effects. Saponins of Teucrium stocksianum are found to be anti-oxidant and anti-cancer. They were found to be cytotoxic toward brine shrimps as shrimps lethality is analogous to nasopharyngeal cancer of humans. The cytotoxicity of this plant was mainly because of high saponin content.

2.10. Bioavailability of Saponins

The fate of saponins in human or animal gut is not clearly understood. Their absorption in animal body is very poor and they are mostly expelled our unchanged or metabolized in gut. Therefore it was challenging to validate the in-vivo action of saponins and less evidence is existing for the biological activities related to saponins in-vivo.

Glycyrrhizin is a triterpenoid saponin from licorice with glycyrrhetinic acid as the aglycone part. Ishida et al., revealed that Glycyrrhizin in rat digestive system was able to hydrolyze into sugar and aglycone portions.[60] Kim et al., showed that human intestinal microflora metabolized Glycyrrhizin to 18β glycyrrhetinic acid (GA) as main product and 18β glycyrrhetinic acid-3-O-B-D-Glucuronide (GAMG) as a minor product. glycyrrhetinic acid was able to absorb in body and displayed anti-inflammatory and anti-hepatotoxic activities in-vivo. Among GA, GAMG and glycyrrhizin, GA has highest cytotoxic activity against tumor cell lines. It also showed inhibitory effect on rotavirus as well as on helicobacter growth.[61]

Odani et al., studied the absorption of steroid saponins in mice. They reported that 2% of ingested ginsenoside with a disaccharide group were absorbed from intestinal tract of rats, whereas only 0.1% of ginsenoside with trisaccharide group were absorbed. Results suggested that steroid saponins may have low availability in-vivo but these saponins were shown to be metabolized by intestinal microbes possibly due to β -glucosidase activity. They also observed that ginsenoside Rb2 was barely metabolized in gastric juice of rats and only slight oxidation has occurred. However after oral dosing in rats, six metabolites of ginsenoside Rb2 have been identified. Pattern of ginsenoside degradation in rats revealed that cleavage of terminal glucose residues of the oligosaccharide attached to C-3 or C-20 hydroxyl group of aglycone initiate the degradation process. Hydrolysis proceed further by to produce secondary products, metabolite I,II,III,IV,V and XII[62].

Sollman (1948) suggested that low concentrations of saponin in blood result in hemolysis of red blood cells.it may be due to surface action phenomenon. The glycone and aglycone parts of saponins act as surface active agents which lower the surface tension, resulting in displacement of substances or adsorbates from surfaces. This alter the membrane permeability and exert toxicity on all tissues. According to sollman, affinity of saponins for the lipids of cell envelope and stroma.

O'Dell et al. (1959) measured the quantitative index pf hemolytic property of saponins. Increase in concentration of saponins increased the amount of hemolysis [63]. Red blood cells have high cholesterol amount in their plasma membrane. Levin and Korenstein revealed that when red blood cells are treated with saponins, they transform into ghosts cells. Their biconcave structure is lost and they become spherical. This change in shape is due to destruction of membrane cytoskeleton interactions. In scanning Electron Microscopy analysis, holes were seen in red blood cells incubated with saponins along with multilamellar stacks consisted of crystallized lipids .diameter of pits was approximately 4.5 nm. [64-66]

Excessive hemolysis caused by saponins can lead to anemia and further death. The pharmacological properties of saponins can be employed if they do not enter into blood stream and do not induce hemolysis.

3. MATERIALS AND METHODS

3.1. Materials

Analytical grade Silver nitrate (AgNO3),Methanol (MeOH), Benzo[a]pyrene (BaP), dimethyl sulfonic acid (DMSO), Phosphate buffer Saline (PBS), Fetal Bovine Serum(FBS) and Brain Heart infusion (BHI) broth are purchased from Sigma-Aldrich. Diamonzide, Dithiothreitol (DTT), Croton Oil and Urethane are purchased from Santa Cruz Biotechnology. All solutions are prepared using deionized water.

3.2. Methods

3.2.1. Preparation of *C. depressus* extract in Methanol (Me OH)

Fresh *C. depressus* plant was washed with water thrice and shade dried for 1 week. 40 gm of dried plant powder was extracted in 250 ml methanol by maceration for 72 hours. After maceration, plant extract was centrifuged at 8000 rpm for 15 minutes and filtered. Crude plant extract was concentrated under vacuum using rotary evaporator.

3.2.2. Synthesis of Silver nanoparticles

To synthesize SNPs, silver nitrate (1mM) was added drop wise in plant methanolic extract. The properties of synthesized SNPs rely on different experimental conditions (temp, pH etc.). Therefore, a series of reactions are carried out at different ratios of silver nitrate to plant extract, temperature and pH to optimize the conditions for better yield of SNPs. After stable synthesis, reaction mixture was centrifuged at 14000 rpm for 15 minutes to get purified nanoparticles. After purification, it was observed that supernatant left after centrifugation was actually giving fluorescence. It was saved for further studies.

3.2.3. Characterization of Fluorescent Supernatant

Supernatant was assessed by phytochemical qualitative reactions for the presence of different phytochemicals. Screening was executed to check the presence of different phyto-constituents. The color strength and/or precipitate formation was used as analytical retorts to these tests. FTIR

analysis of supernatant was carried out to determine the functional groups present in sample leading to determination of compound. The infrared spectra were recorded using Perkin Elmer, Spectrum 100 FTIR spectrophotometer. Supernatant was further analyzed by EDS and SEM (Electron microscopy JEOL JSM-6042 A, Japan). The working distance was of 10 mm which was maintained there by using 5 kV acceleration voltage.

3.2.4. BaP detection Assay and mutual interference studies

The absorption spectra of supernatant and BaP was recorded between wavelengths of 200 to 800 nm (Fig.4). Emission spectra was recorded between 390-630nm by using Enspire multimode plate reader. To examine the selective interaction between BaP and plant flavonoid which is important for real-world applications, the fluorescence characteristics of plant flavonoid was measured in the presence of four carcinogens. Quenching assay was developed with varying concentrations of BaP to a known volume of plant flavonoid to determine limit of detection. Different ppm solutions were prepared using plant flavonoid and BaP and fluorescence spectra was recorded using Plant flavonoid as control.

To check the specificity of plant flavonoid for BaP, the complexity behavior was evaluated in the presence of different carcinogens using fixed concentration of plant flavonoid and equimolar concentrations of BaP and other carcinogens including Diamonzide, Dithiothreitol (DTT), Urethane and croton oil.

3.2.5. Preparation of *C. depressus* extract in methanol (Me OH)

Fresh *C. depressus* plant was thoroughly washed with distilled water (3 times). Cleaned plant was shade dried for 1 week and then ground to make powder. 40 grams of dried plant powder was extracted in 250 ml methanol by maceration for 48 hours. After maceration the extract was centrifuged and filtered. Filtrate was concentrated under vacuum to obtain crude plant extract.

3.2.6. Biosynthesis and Optimization of SNPs

Stock solution of plant extract was prepared by adding 2mg/ml of dried plant extract .The solution was filtered and stored for use in subsequent reactions. Nanoparticles were synthesized by mixing plant extract and silver nitrate (1mM) and kept under controlled conditions to stabilize them. *C. depressus* extract was used as stabilizing and reducing agent during the synthesis of silver nanoparticles. After synthesis the mixture was centrifuged at 8000 rpm for 15 minutes. Silver Nanoparticles were washed with water twice to get rid of unconjugated compounds. Washed nanoparticles were used for further characterization.

The shape, size, color and stability of silver nanoparticles vary depending upon the ratio of silver nitrate to plant extract, temperature and time. To obtain optimized nanoparticles, a series of different reactions were performed. Reaction mixture giving best results were chosen for further studies.

3.2.7. Characterization of Silver Nanoparticles

Synthesized silver nanoparticles were meticulously characterized by using different physico-chemical techniques. The absorbance and optical properties of the synthesized silver nanoparticles were monitored by UV-VIS Spectroscopy using UV-2800 BMS Spectrophotometer. Morphology and size of silver nanoparticles was studied by Scanning Electron Microscopy (SEM) using Electron microscopy JEOL JSM-6042 A, Japan. To determine the functional groups in biomolecules present in plant extract, silver nitrate and SNPs, Fourier Transform Infrared Spectroscopy (FTIR) analysis was carried out using Perkin Elmer, Spectrum 100 FTIR spectrophotometer. X-Ray Diffraction (XRD) was used to detect and analyze the crystallinity of silver nanoparticles using XRD model theta-theta STOE (Germany. Elemental analysis of synthesized nanoparticles was carried out by Energy Dispersive Spectroscopy (EDS).

3.2.8. Hemolysis Assay

Effect of Saponins and Saponins SNPs on red blood cells were studied by carrying out Hemolysis Assay. Blood taken from healthy donors was centrifuged at 1000g for 3 minutes at 4C. Plasma, white blood cells and platelets were removed by pipetting. Erythrocytes were washed (3 times) with 0.9% NaCl. RBCs suspension was finally diluted with freshly prepared

PBS. (150Mm, pH 7.4) to obtain suspension of RBCs. Diluted RBCs suspension (300 mm^3) was mixed with 1200 mm^3 of PBS (negative control), 1200 mm^3 of 0.5 %Triton X-10 (positive control) and 1200 mm^3 of Saponins and Saponin SNPs at concentration ranging from 1ug/ml to 150 ug/ml. The mixture was vortexed and kept for 2 hours. After two hours, samples were centrifuged and absorbance of supernatant at 550nm was recorded by using UV-2800 BMS spectrophotometer. To analyze the results further, % Hemolysis was calculated using the following formula.

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

3.2.9. In Vivo treatment of bacterial colonization

Eight weeks old female BALB/c mice were obtained from National Institute of Health Islamabad. The animals were arbitrarily apportioned into three groups of five mice each. All animals were kept under standard housing environment. Food was provided in the form of dry pellet and water. Animal experimental study was performed after the approval of Institutional Ethical Review Board, National University of Sciences and Technology.

An infectious model to test the anti-colonizing effectiveness of Saponins and Saponins SNPs was developed. *E. faecalis* grown on M17 medium (supplemented with 0.5% w/v Glucose) for 24 hours at 37°C. Overnight grown culture was used to inoculation into Brain Heart Infusion (BHI) supplemented with 40% Filter Sterilized Serum (FBS) and kept at 37°C. After that cultures were centrifuged and pellet obtained was washed with PBS. Bacterial suspension in PBS was injected intravenously in each BALB/c mice for 7 days.

Mice were divided into 4 groups. One group served as control, one group remained untreated, one group was treated with 5mg/Kg Saponins and one group was treated with 400µg/kg Saponins SNPs. Drug was administered in body intravenously for seven days. After 7 days mice were sacrificed and kidneys and liver were removed, weighed and homogenized. Serial dilutions prepared from homogenate were plated on M17 agar medium and incubated at 37°C. The effect of Saponins and Saponins SNPs was determined by $\log_{10}$CFU/gm organ. All readings were taken in replicates and analyzed by unpaired t-test.

4. RESULTS

4.1. Isolation of Flavonoids

Formation of SNPs was indicated by color change from yellow to brown. It was further confirmed by recording UV visible spectra of SNPs around 410-430 nm. The strong resonance positioned at 429 was observed which increased in intensity with time. Spectra was recorded after intervals to monitor the formation of stable SNPs. After synthesis of SNPs, UV-visible spectra of whole series of reaction was analyzed critically. The absorption intensity was maximum for 1:3 (plant extract: silver nitrate) prepared at 70ºC and pH 8 so this ratio was selected for further studies (Fig.1)

Plant contains different phyto-constituents which act as reducing and stabilizing agents for nanoparticles. It was supposed that supernatant giving yellowish green fluorescence might contain some phyto-constituents whose fluorescence was enhanced during SNPs synthesis. Following this hypothesis, supernatant was screened for different phyto constituents. Results indicated the presence of flavonoids in supernatant (Fig.1).

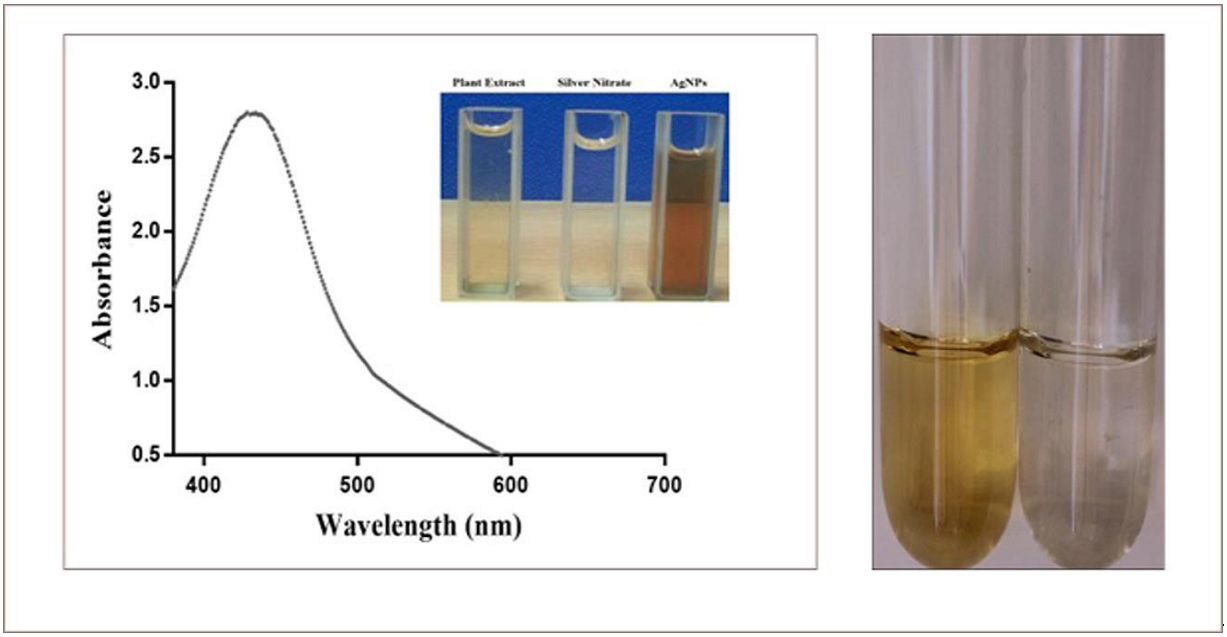


Fig.1 Synthesis of SNPs at 70C. Surface Plasmon resonance peak observed at 429 nm. Right side picture shows phytochemical screening conforming the presence of flavonoids

4.2. Characterization of Flavonoids

SEM and EDS patterns of this plant flavonoid indicated that there was no silver present in the sample. This proved that all nanoparticles are settled down during centrifugation and are separated out (Fig.2).

FTIR spectra of plant flavonoids is shown in Fig.3. The peak at 3361 cm^{-1} indicates stretching of phenolic-OH. The peak at 1649 correspond to C=C. Peaks at 2924 and 2854 correspond to C-H and CH3 respectively which indicate some alkane chains in sample. Peak at 1649 corresponds to C=O indicating the presence of ketones. The presence of peaks between 1600 and 1450 are characteristic for aromatic group, peak at 1262 shows the presence of -C-0- (polyols). Flavonoids main characteristic bonds are hydroxyl (OH), carbonyl and polyol (-C-O-) groups.

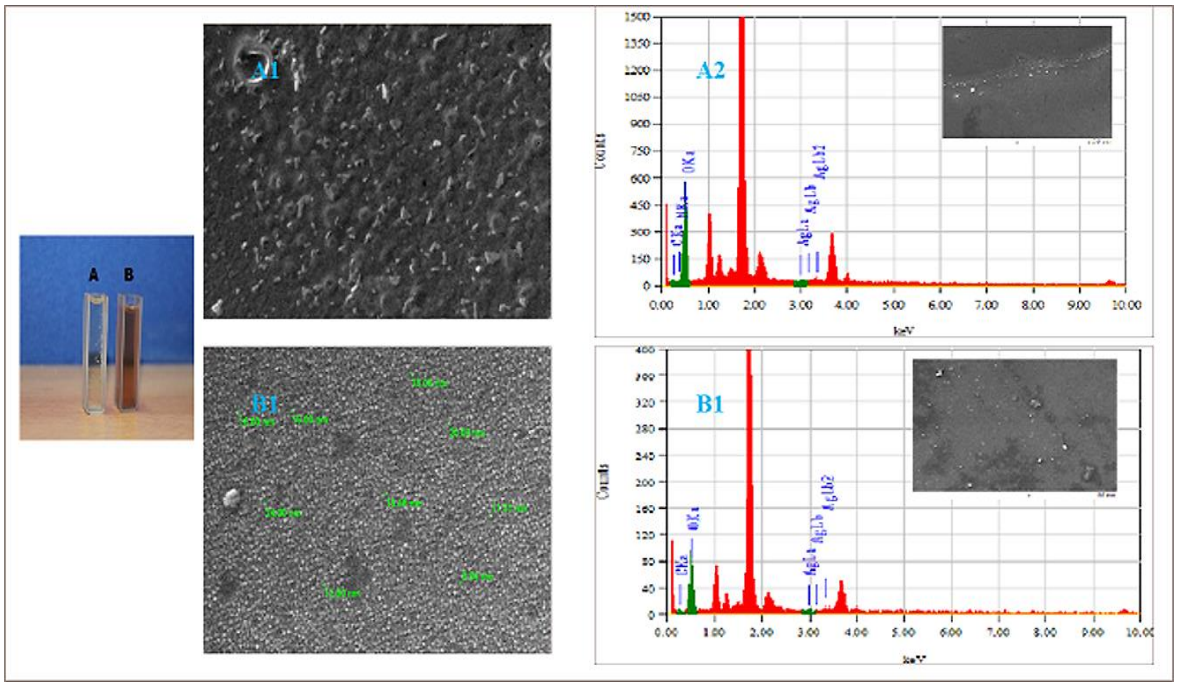


Fig.2. A1 and A2 image of plant flavonoid indicating absence of any SNPs while B1 and B2 image shows SNPs with size less than 10 nm.

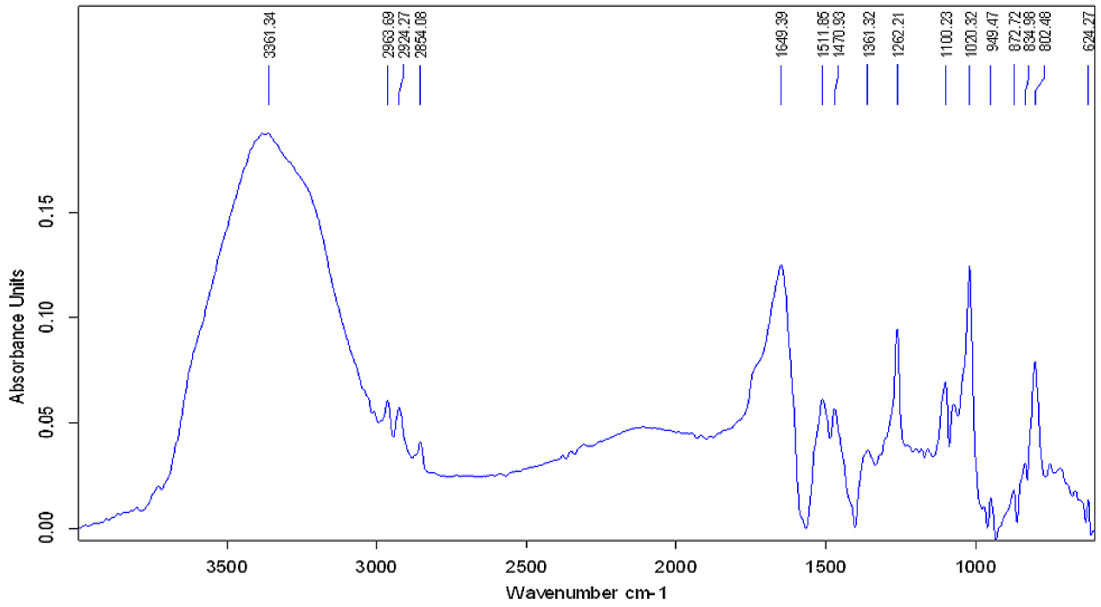


Fig. 3. FTIR ATR spectra of Flavonoids.

4.3. Spectroscopic recognition of carcinogens and interference studies

Absorption spectra of Plant flavonoid and BaP was observed. Different wavelengths for excitation were tested. After careful optimization, 230 nm was used for further analysis (Fig. 4a).

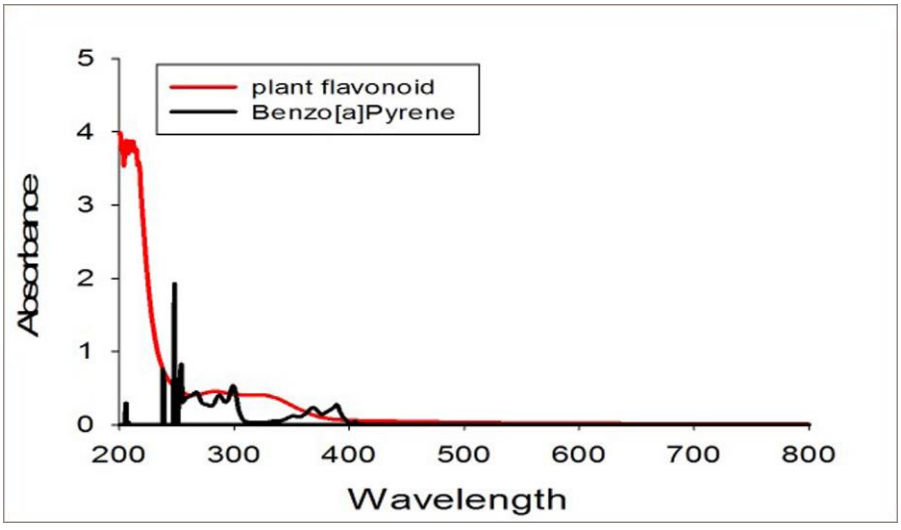


Fig.4 (a). Absorption spectra of plant flavonoid and BaP

Upon addition of BaP in plant flavonoid, a seeming change in fluorescence spectra was observed which exhibited the quenching of plant flavonoid in the presence of BaP. Maximum fluorescence intensity of plant flavonoid was recorded at 475 nm. Addition of BaP strongly quenched the fluorescence intensity of band from 60000 to 10000 approximately at 475 nm (Fig.4b).

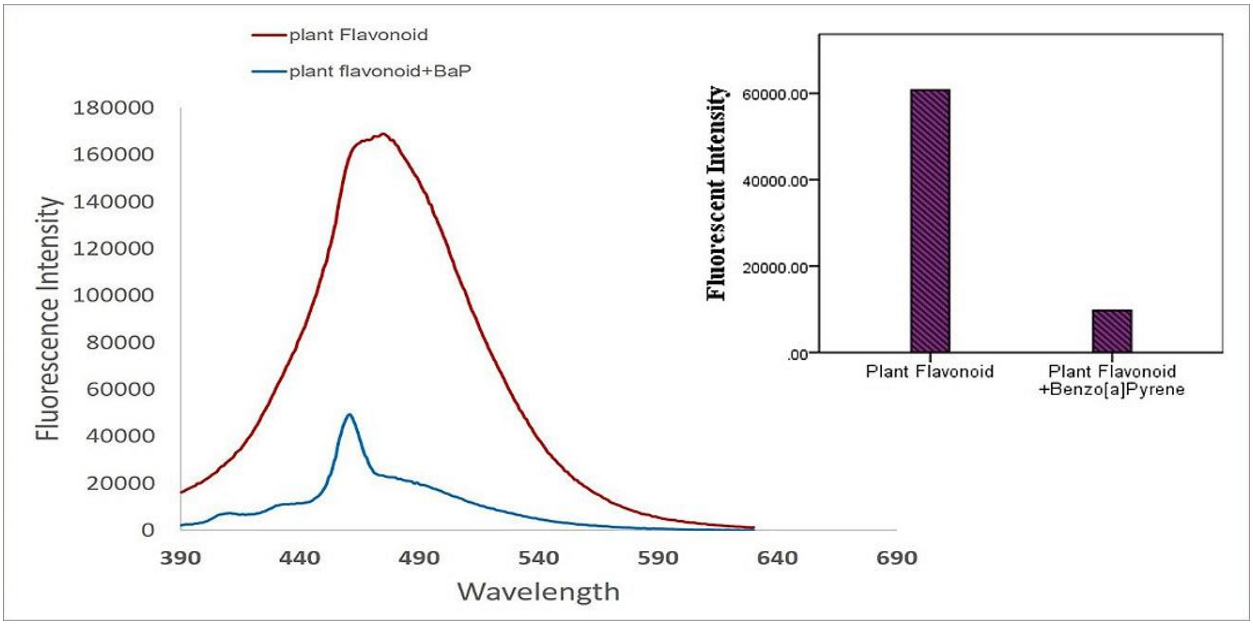


Fig.4 (b). Quenching of BaP by Plant Flavonoid

The fluorescence intensity of plant flavonoid gradually decreased by increasing BaP concentration up to 25ppm without changing emission maxima and peak shape. After this concentration, emission peaks were almost same for all concentrations. The effect of BaP on fluorescence quenching of plant flavonoid is depicted in Fig (5a).

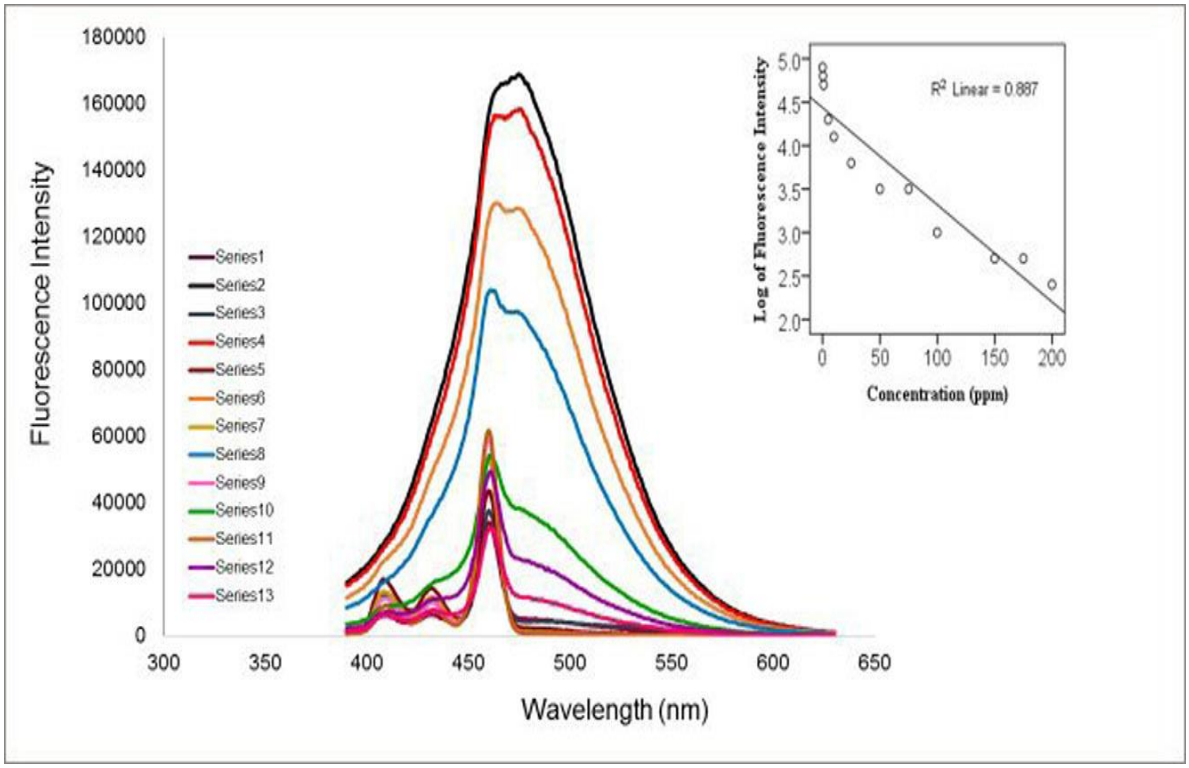


Fig.5 (a). Quenching effect of BaP on fluorescence intensity of plant flavonoid. 1= plant flavonoid 2= plant flavonoid+0.1ppm BaP 3= plant flavonoid+0.5ppm BaP 4= plant flavonoid+1ppm BaP 5= plant flavonoid+ 5ppm BaP 6= plant flavonoid+10ppm BaP 7= plant flavonoid+25 ppm BaP 8= plant flavonoid+50 ppm BaP 9= plant flavonoid+75 ppm BaP 10= plant flavonoid+100 ppm BaP 11= plant flavonoid+150 ppm BaP 12= plant flavonoid+175 ppm BaP 13= plant flavonoid+200 ppm BaP

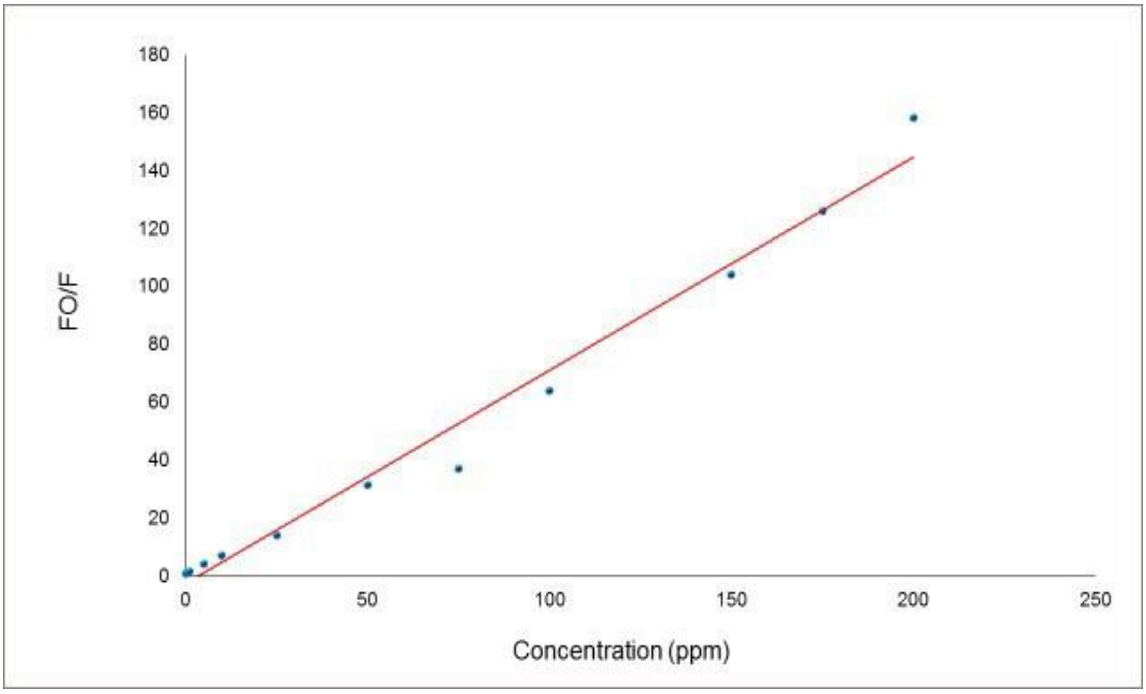


Fig.5 (b). Stern volmer plot of FO/F vs concentration of BaP. Graph shows linear relationship between Fo/F and concentration

All other carcinogens did not quench the fluorescence due to weak interaction between plant flavonoid and carcinogens (Fig.6). Mutual Interference studies results showed that presence of other carcinogens in detection media didn't interfere with quenching of BaP. Plant flavonoids were able to detect BaP even in the presence of other carcinogens. (Fig.7).

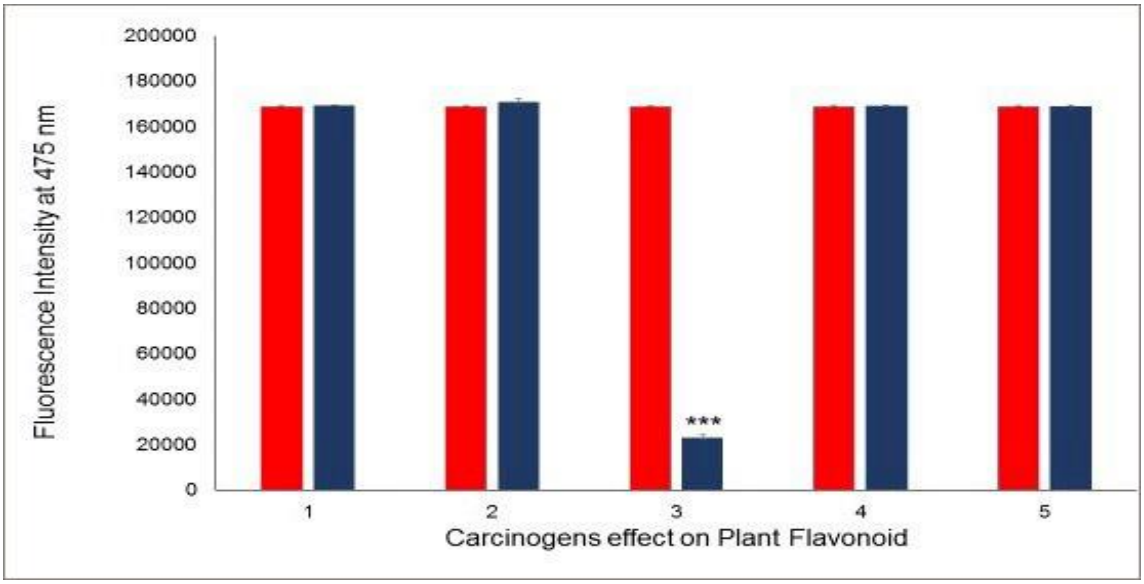


Fig.6. Complexation behavior of plant flavonoid with different drugs. 1= diaminozide, 2= Croton Oil, 3= BaP, 4= Dithiothreitol, 5= Urethane. (P< 0.001 Comparison between Gray and Orange columns) The asterisks (***) indicate the significance difference (p≤0.001). The bars represent the mean ± S.D of three replicates.

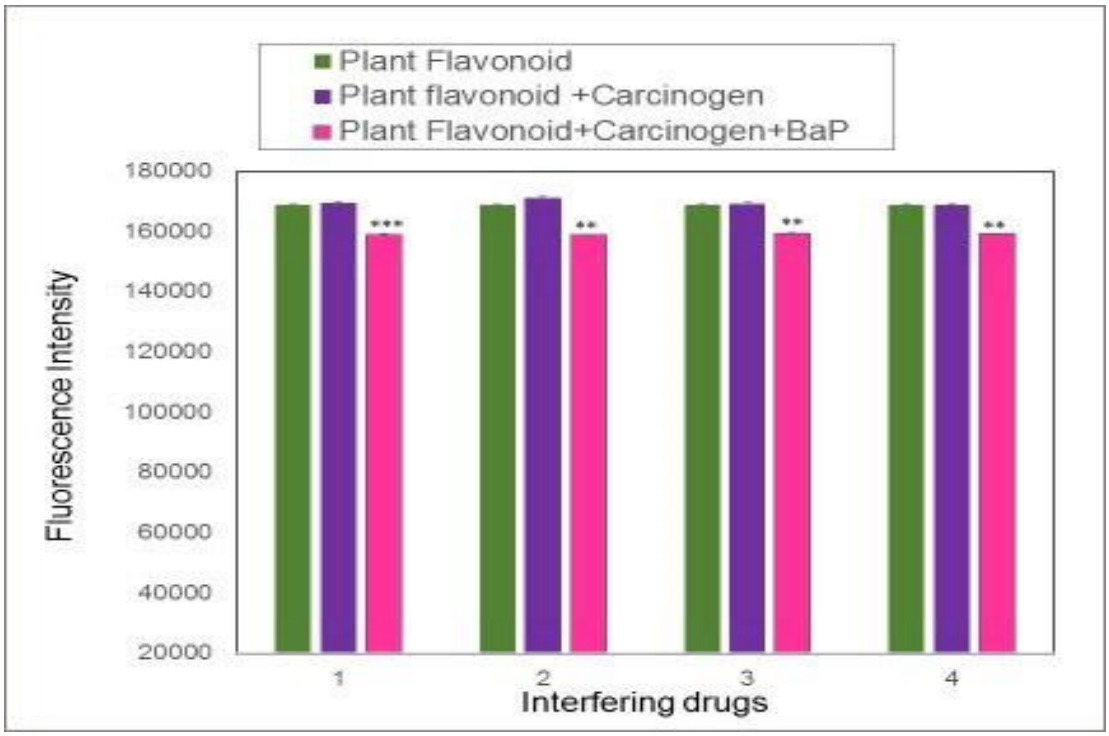


Fig.7. Mutual Interference effect of different carcinogens on Quenching of Plant flavonoid. (Purple and Pink columns are compared with green columns), 1- diaminozide, 2- Croton Oil, 3- Dithiothreitol, 4- Urethane

4.4. Synthesis and optimization of SNPs

Appearance of brown color in solution indicated the formation of silver nanoparticles. This is further confirmed by UV-VIS Spectroscopy method. Absorption spectra of plat extract and silver nitrate was recorded before synthesis of silver nanoparticles. Both of them did not show any peak in 410-430 nm range Absorption peak at 429 further confirmed the presence of silver nanoparticles. Time dependent intensity of absorption band was examined to get stable NPs. Optimization of silver nanoparticles synthesis was started with different ratios of silver nitrate to plant extract. Six different ratios (1:1 to 1:6) were tested to get optimized silver nanoparticles. First three ratios (1:1-1:3) showed absorption band around 410-430 while in rest three ratios, the absorption band was shifted above 440 nm. Silver nanoparticles were tested at three different temperatures 25ºC, 50ºC and 70ºC. Results indicated that SNPs synthesized at 70ºC showed sharp absorption peak at 429 nm. Increasing the reaction time led to increase in absorption intensity without any shift in peak position. After careful analyses of AgNPs synthesis, 1:3 ratio was selected for future studies.

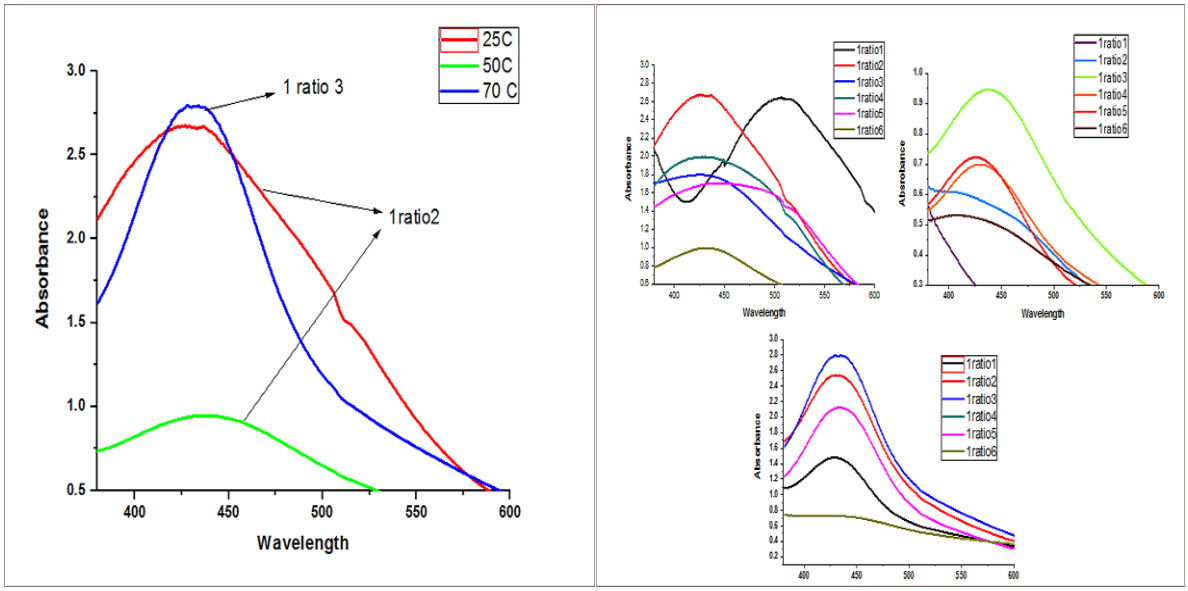
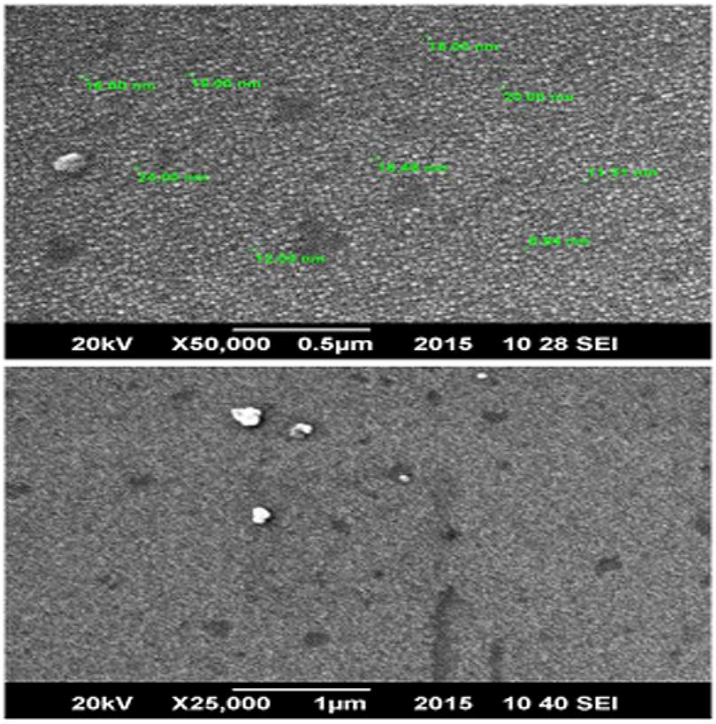


Figure 8: (a) Synthesis of SNPs. (B) Optimization Of SNPs at different ratios starting from 1:1 to 1:6. 1st graph depicts synthesis of SNPs at 25 ^{O}C , 2nd graph at 50 ^{O}C while 3rd shows synthesis at 70 ^{O}C.

4.5. Characterization of SNPs

As UV-VIS spectroscopy do not provide exact information about NPs size so morphology and size of these nanoparticles were further characterized using SEM. Results have shown that nanoparticles are almost of spherical shape and they are not agglomerated. Average nanoparticle size was determined to be 16 nm. EDS results have shown signal of elemental metal. Along with silver signal presence of carbon, oxygen and nitrogen signal may be due to phytochemicals that were involved in nanoparticles synthesis.



Element	Weight (%)
C K	4.29
O K	90.79
Ag L	4.29
Total	100

Figure 9. SEM and EDAX pattern of SNPs

FTIR analysis shows the specific peaks present in SNPs. It is shown that functional groups present in saponins have been used as reducing and stabilizing agents for the synthesis of SNPs and during this process, the peak intensities have been decreased.

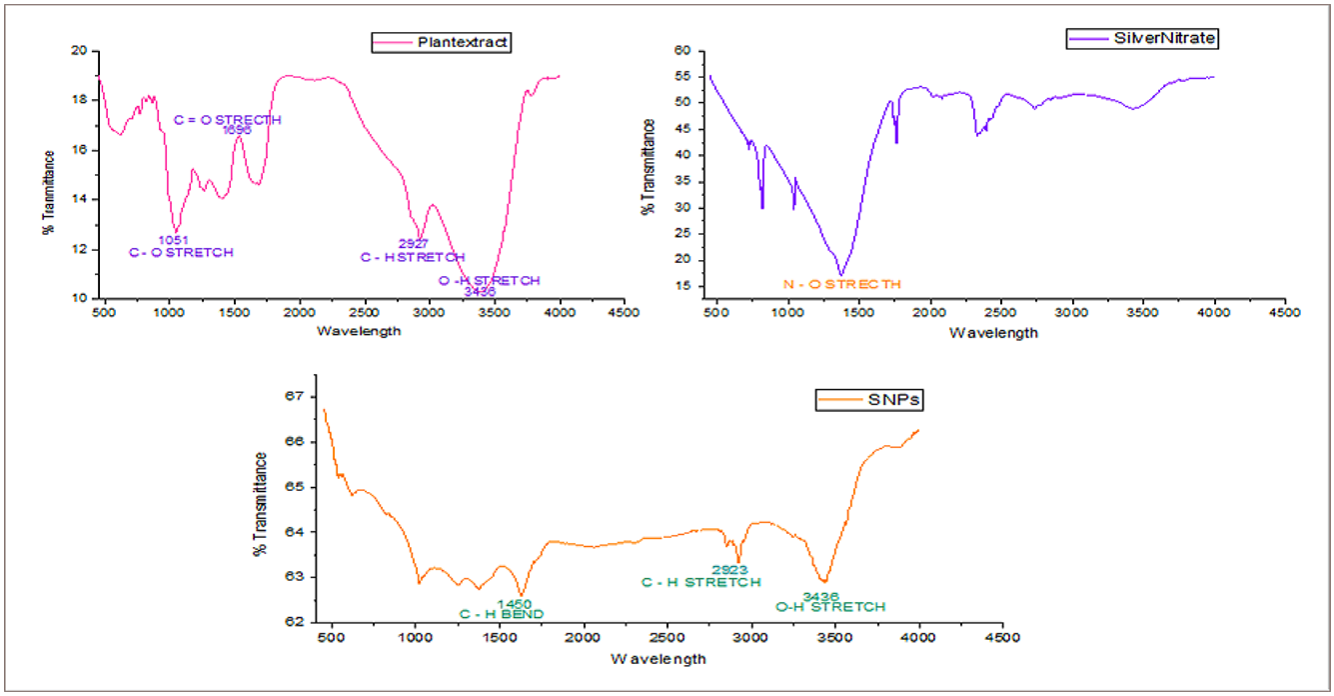


Figure 10. FTIR spectra of Saponins, AgNO3 and Saponins Silver nanoparticles.

XRD analysis of SNPs exhibited four characteristic peaks at 2θ values 37º, 44º, 64º, 77º that correspond to the 111, 200, 220, 311 planes of silver, confirming the crystalline structure of SNPs.

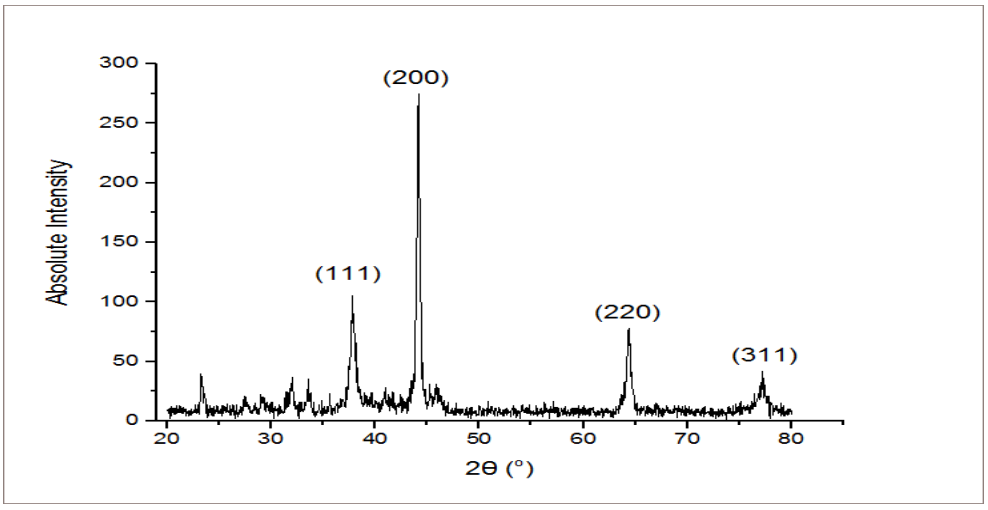


Figure 11. XRD pattern of SNPs

4.6. Hemolytic Activity

The cytotoxicity of various concentrations of saponins and saponins SNPs on red blood cells is depicted in figure 12. Results indicated that saponins NPs are more hemocompatible than saponins suggesting the successful use of saponins NPs in treatment of various diseases. An increasing trend of hemolysis by increasing concentration is depicted in figure. Hemolysis less than 5% by all concentrations shows that these nanoparticles are hemocompatible according to ISO/TR 7406 standards.

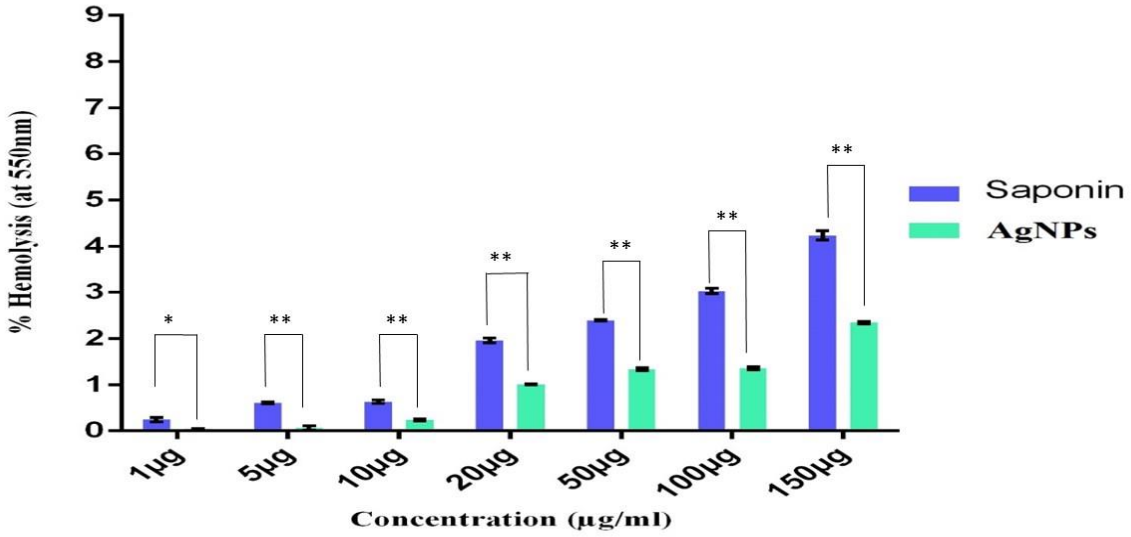


Figure 12. % Hemolysis of Red blood cells under the effect of saponins and saponins SNPs . The asterisks (**) indicate the significance difference ($p \leq 0.01$). The bars represent the mean ± S.D of three replicates.

4.7. In-Vivo anticolonization effects of Saponins and Saponin NPS on *E. Faecalis* mouse model:

An infectious model with *E. Faecalis* was designed. Treatment with saponins and saponins and SNPs was started after seven days of infection. After seven days of treatment mice were sacrificed and liver and kidneys were taken out to check the presence of viable bactreia. Results revealed that

anticolonizing activity of Saponins SNPs is much greater than Saponins. It can be concluded that bioavailability of saponins after formation of SNPs is increased the amount of dose required for effective anti-colonizing activity was decreased suggesting that saponins can be promising agents for use in therapeutics with low hemolysis and increased bioavailability.

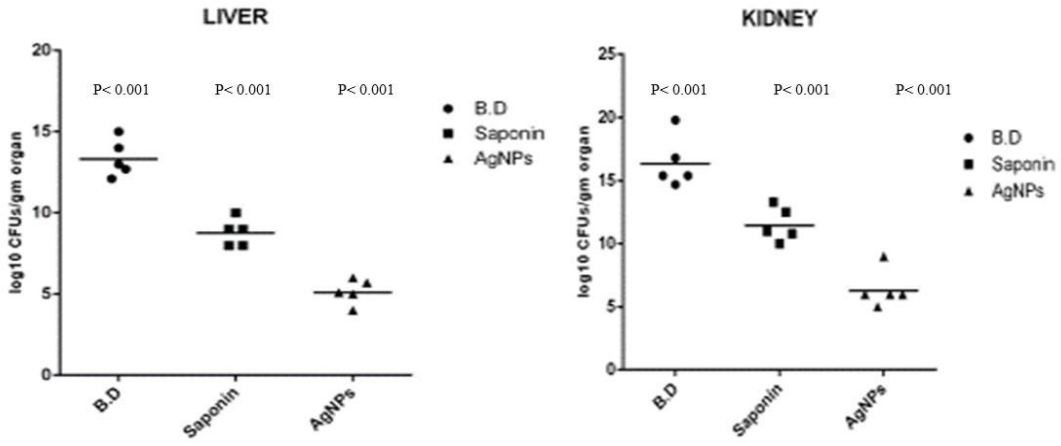


Figure 13. Effect of Saponins and Saponin SNPs within mice organ of *E. feacalis* infected mice model.

5. DISCUSSION

BaP is a globally distributed carcinogen and its interaction with DNA may cause DNA damage ultimately leading to cancer [67]. Various biosensors and techniques have been developed such as immuno-assay [68, 69] and Aptamer based biosensor [70, 71], but they all require tiresome procedures and are expensive. We are reporting an inexpensive fluorescent assay for BaP detection and quantification.

C. depressus branch extracts are rich in saponins however, other phytochemicals such as glycosides, alkaloids and flavonoids are also present. [40]. Flavonoids can give characteristic fluorescence on excitation, therefore, they can be used in chemical sensing. Methanolic extract of *C. depressus* do not exhibit any significant fluorescence on excitation However, after the synthesis of SNPs with methanolic plant extract, the reaction mixture revealed strong greenish yellow fluorescence. It was worth noted that fluorescence was retained in the solution and SNPs formed were non excitable. Optimization of SNPs synthesis was carried out at different temperatures, silver nitrate to plant ratio and pH. Maximum fluorescence was achieved with optimized SNPs. However, when these nanoparticles were separated by centrifugation, they did not exhibit any fluorescence. Rather, the supernatant retained greenish yellow fluorescence. The supernatant actually contained flavonoids and saponins got separated from mixture with SNPs. Therefore, NPs can not only be used for the extraction of desired compounds but can also be used for extraction of minor compounds. During the phytochemical screening of supernatant, formation of deep yellow color confirmed the presence of flavonoids. Flavonoids are fluorescent molecules, but due to the presence of prominent amount of saponins, whole extract do not show any fluorescence. Therefore, removal of saponins by SNPs formation resulted in isolation of flavonoids. SEM and EDS patterns show morphology and elemental composition present in sample. Evaluating plant flavonoid through SEM and EDS rejected the possibility of presence of SNPs that might be thought of possessing fluorescence.

BaP itself is a fluorescent molecule having a wide range of absorption and emission spectra as shown in Fig. 4a. Fluorescence detection of BaP is hindered by the fact that BaP is itself fluorescent

in UV-VIS region. The fluorescent detection of BaP with another fluorescence producing compounds is critical. Since BaP itself is excitable at wide range of wavelength in UV region and produces bluish green fluorescence, the absorption spectra of both these compounds was overlapped and 230 nm wavelength was found to be non-excitable for BaP. After optimizing the absorption spectra for quenching studies at 230 nm, effect of BaP on quenching of Plant flavonoid was observed. Fluorescence of Flavonoids were strongly quenched in the presence of BaP.

Assay response to various concentrations of BaP is shown in Fig.5a. Which shows that addition of varying concentration of BaP to selected solution of plant flavonoid decreased the fluorescence intensity. Linear stern Volmer plot of Fo/F vs cBaP shows that quenching process is BaP concentration dependent (Fig. 5b).

Quenching of fluorescence intensity fall in two categories, static and dynamic. Type of quenching is determined by two factors 1) fluorescence quenching spectra of substance 2) absorption spectra of fluorescence substance in the presence and absence of quencher. Stern volmer plot can analyze the fluorescence quenching spectra [72]. Linear stern volmer plot indicates the single quenching type static or dynamic while an upward curvature indicates combined quenching (static and dynamic) [73]. The absorption spectra in case of dynamic quenching remain same before and after the addition of quencher. Linear stern volmer plot of BaP quenching shows that only one type of quenching has occurred. Absorption spectra shows decrease in absorption after the addition of BaP to plant flavonoid. The absorption of plant flavonoid at 230 nm is observed to be 0.070 a.u. while after the addition of BaP, this value decreased to -0.009 a.u. This indicates that BaP and plant Flavonoid have reacted with one another resulting in complex formation.

The analytical potential of plant flavonoid was determined by quenching effect of BaP on the fluorescence intensity of plant flavonoid. BaP obeyed lambert beer's law in concentration range of 5 ppm to 200 ppm with a linear regression equation of $y = -0.0096x + 4.635$ with $R^2 = 0.88$. The positive slope of line shows that there is direct relationship between Bap quenching and plant flavonoid concentration. Complexation behavior of plant flavonoid with different carcinogens shows that all other carcinogens didn't quenched the fluorescence of plant flavonoid. But BaP quenched the fluorescence of plant flavonoid significantly (Fig. 6).

The selectivity and specific chemo sensing ability of sensing agents in the presence of different compounds in detection media leads to develop a good chemo sensor. The selectivity of BaP for

quenching plant mediated flavonoid was tested in the presence of four other carcinogens. Plant flavonoid efficiently quenched BaP even in the presence of other carcinogens which proved the sensitivity of plant flavonoid towards BaP detection (Fig.7). The selectivity of fluorescent flavonoids for BaP can lead to the development of an efficient chemo sensor.

Natural products are source of medication since the origin of humanity and are still considered as valuable materials for traditional medicine. Ethno-botanical and pharmacological traditions, modern chemical and bioinformatics provide evidences showing that secondary metabolite and other traditional drugs have many similarities regarding their chemical structures and are unparalleled source for the discovery and isolation of modern bioactive compounds with great prevalence over synthetic drugs. Over the period of time use of natural products for chronic diseases have largely expanded worldwide and is getting importance in the field of medicine day by day.

Saponins are glycoside compounds whose chemical structures are compose of fat soluble nucleus aglycone, that is either a triterpenoid(C-30) or neutral or alkaloid steroid (C-27) attached to one or more side chains of water soluble sugars glycone. Aglycone part is attached to glycone part through ether linkage at different carbon sites. It is distinguished from other glycoside by acting soap like in water and forming stable foams. Therefore, the name of this group of compounds is derived from Latin word ‘sapo’ meaning soap. These soap like properties are due to presence of hydrophilic and lipophilic functional groups. Various biological effects of saponins include Hemolytic and antibacterial activity.[74]

Modern research revealed that saponins have anti-tumor activities on several cancer cells. Several saponins impede growth of cells by cell cycle arrest and apoptosis with IC50 values up to 0.2Mm. In the meantime, saponins along with conventional tumor treatment approaches resulted in enhanced therapeutic success.

Modern research revealed that saponins have antitumor effect on several cancer cells. Several saponins impede tumor cell growth by cell cycle arrest and apoptosis with IC50 values up to 0.2 mM. In the meantime, saponins in combination with conventional tumor treatment strategies, result in improved therapeutic success. Furthermore, a much clearer understanding of how the various saponin structures are related to each other is obtained with the use of the classification presented. The objective of this review is to provide a timely update on the

sources, classification, and applications of saponins with special focus on their mechanism of antitumor effect and structure–function relationship.

Nanotechnology is gaining special attention in present era. It is a broad and interdisciplinary area of research and technology that has opened novel fundamental and functional frontiers, including the synthesis of nanoscale materials and expedition and utilization of their exotic physicochemical and optoelectronic properties. The field of nanotechnology is burgeoning day by day making an impact in all spheres of human life. Nanoparticles are particles between size of 1-100 nm that function as a whole unit with respect to their transport and properties.

Evolution of living systems to combat against metal toxicity took over millions of years. In order to reduce the toxic effects of metals, organisms release several enzymes and reducing agents to lower the oxidation states. The molecular capability of biological species has led to convert these metal ions to well dispersed nanoparticles with desired properties. The need for environmentally sustainable synthesis processes include several biomimetic approaches which refer to applying biological doctrines in materials formation.one of these biomimetic approaches is bio reduction. Green synthesis of nanoparticles is gaining impetus in recent years due to their well-defined optical, chemical, electronic properties and increased surface to volume ratio as compared to bulk materials. Employing plants to synthesize nanoparticles is favored over other biological techniques due to inherent ability of plants to accumulate metals, easy scale up, no involvement of cell culture maintenance and no requirement of aseptic environment.

Silver is a noble metal having many unique properties like chemical stability, good conductivity, catalytic activity, antimicrobial activity and increased oral bioavailability. Nano crystalline silver particles have tremendous applications in the field of high sensitivity biomolecular detection and diagnostics, antimicrobials and therapeutics, catalysis and micro-electronics.

Corchorus Depressus, commonly called as Bhauphali, is a medicinal plant. It grows in sandy and saline areas. Whole plant has applications in treatment of urinary disorders, internal injuries and fever. Leaves are used in liver disorders, as cooling and softening agent while seeds and fruits are used as fever drink and demulcent.

Conduction and valence bands in silver nanoparticles lie close to each other in which electrons can freely travel. Due to combined oscillation of electrons of AgNPs in resonance with light

waves, a rise in SPR absorption band occurs. Size shape and dielectric constant of surrounding media influence the SPR band. Sharp absorbance peak indicate small size nanoparticles. Phytochemicals present in plant extract act as reducing and stabilizing agent for silver nanoparticles. These phyto-constituents are not only involved in reduction of silver nitrate to nanoparticles but their chemical structure also wrap around nanoparticles to prevent agglomeration. [75] The color change during the formation of AgNPs is due to excitation of surface Plasmon resonance (SPR) in metal nanoparticles. Plasmon denotes combined oscillation of free electrons in metal nanoparticles which result in irradiation of nanoparticles with visible light. Resonant Plasmon peak and widths depend upon the size and shape of nanoparticle, metallic specie and surrounding reaction medium [76] Silver nanoparticles synthesized at different ratios (1:1 to 1:6) showed different absorption spectra. Increase of silver nitrate concentration in plant extract resulted in gradual increase in resonance peak up to 1:3 solutions but after that the absorption spectra showed broadening and less absorption. With the increase in reaction temperature, SPR peak become sharp and steep. This is confirmed during the synthesis in which absorption spectra of AgNPs synthesized at 70ºC showed good Plasmon resonance peak as compared to synthesis of AgNPs at 25º and 50º. Increase in temperature resulted in reduction of more silver ions to nanoparticles. Sharpness of peak also depends upon size of silver nanoparticles [77] which is confirmed by SEM analysis that nanoparticles synthesized at 70ºC are of very small size as compared to AgNPs synthesized at other reaction conditions. Synthesis of silver nanoparticles is also monitored at different pH. The optimized synthesis with small size and dispersed NPs was recorded at pH 8.

Green synthesis of NPs ensure to avoid agglomeration of NPs as phytochemicals present in plants acts as surfactants to produce well separated NPs. The mechanism behind the formation of NPs without agglomeration is impulsive nucleation and isotropic growth of NPs along with phytochemicals. As silver deposition increases resulting in chain growth (diameter), spherical particle detach from these structures and Nano-spherical particles are formed.[78] Results showed that Saponins SNPs formed are of spherical shape and not agglomerated. The average nanoparticle size was 16 nm. EDAX patterns clearly shows the presence of elemental silver along with signals of carbon and oxygen that are derived from Saponins are were involved in capping of SNPs. Absence of other elements confirmed the purity of SNPs.

FTIR spectra of SNPs shows that Saponins were involved in the reduction and coating of SNPs. Peaks at 2923 and 3436 cm^{-1} correspond to C-H and O-H stretching vibrations respectively. Peak at 1696 cm^{-1} correspond to C=C stretch. Decrease in peak intensities in FTIR spectra of SNPs showed that some functional groups present in saponins were involved in reduction of SNPs.

XRD pattern showed characteristic peaks for SNPs at 2θ values 38.1 °, 44.09 °, 64.36 ° and 77.29° which correspond to 111, 200, 220, 311 planes of silver. The crystalline structure of SNPs is confirmed by presence of these characteristic peaks. No peaks of any impurity crystalline phases were detected in XRD pattern. All the peaks can be readily indexed to face centered cubic structure of silver as available literature (JCPDS, File no.4-0783). The crystalline size of SNPs have been found by the following formula

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

Where λ is the wavelength (λ = 1.54060 Å), β shows full width at half maximum of the (200/0 diffraction, and θ depicts the angle of diffraction. The average NPs size is estimated to be 16nm.

Lipid bilayer present in biological membranes surround cell surface and proteins. Non covalent bonds in acyl groups and ionic bond among polar heads and aqua are used to stabilize this lipid bilayer. Hemolysis is caused due to lysis of lipid bilayer and cellular membrane obliteration of red blood cells. Concentration and influence of surfactants are directly related to hemolysis. [79].

The hemolytic activity of Saponins and Saponins SNPs increased in dose dependent manner. As the concentration of Saponins and Saponins SNPs increase, an increase in hemolytic behavior was observed. But saponins SNPs showed much less hemolysis as compared to Saponins suggesting that Saponins after forming SNPs lead to reduction in hemolysis.

Enterococcus Feacalis is a gram positive cocci and facultative anaerobe. Due to rough physiology possessed by *Enterococcus Feacalis*, it is able to survive oxidative stress and other strident conditions like salt concentration and pH that may contribute to risk of infection and colonization [80, 81]. It is considered a tenacious organism which is most prominent cause of nosocomial infections and is the third most prevailing pathogen isolated from blood stream infections [82]. Due to its ability to inhabit GI tract of patients for longer duration, it greatly influences the development of drug resistance. It greatly affects liver

and kidney and at chronic stage of disease, it may affect heart also. In-vivo colonizing results showed that that Saponins and Saponins nanoparticles were able to greatly reduce the *E. Faecalis* colonies in liver and kidney of mice. But the bioavailability and effectiveness of Saponins SNPs were much higher than Saponins which make them an effective choice for therapeutics. Moreover, hemolytic activity of Saponins SNPs less than 5% and low dose usage in in-vivo anti-colonization proved that Saponins are safe to use in body.

6. CONCLUSION

BaP is an abundant environmental carcinogen. Plant flavonoids based chemical sensing of BaP can be an in expensive and fast methodology for its monitoring with high selectivity and reliability. Further studies are required for device fabrication in monitoring different samples of BaP using this simple and easy technique. Furthermore, isolated flavonoids should be further analyzed.

SNPs formed with saponins were hemocompatible as compared to saponins which is important for biomedical applications of saponins. Saponins and Saponins SNPs have effective anti-colonizing activity against *E. faecalis* in liver and kidney. Using this approach, the bioavailability of saponins is increased which is important for cytotoxic effects against cancer cells.

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