Green Synthesized Silver Nanoparticles Enhances Anticancer Activity of HDAC Inhibitor Panobinostat

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First and foremost, I would like to thank my greatest teacher of all: God. I know I am here and that I am able to write all of this for a reason. I will do my best to never forget what a great fortune I have had in just being here and that it comes with a lesson and a responsibility.

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Dedication

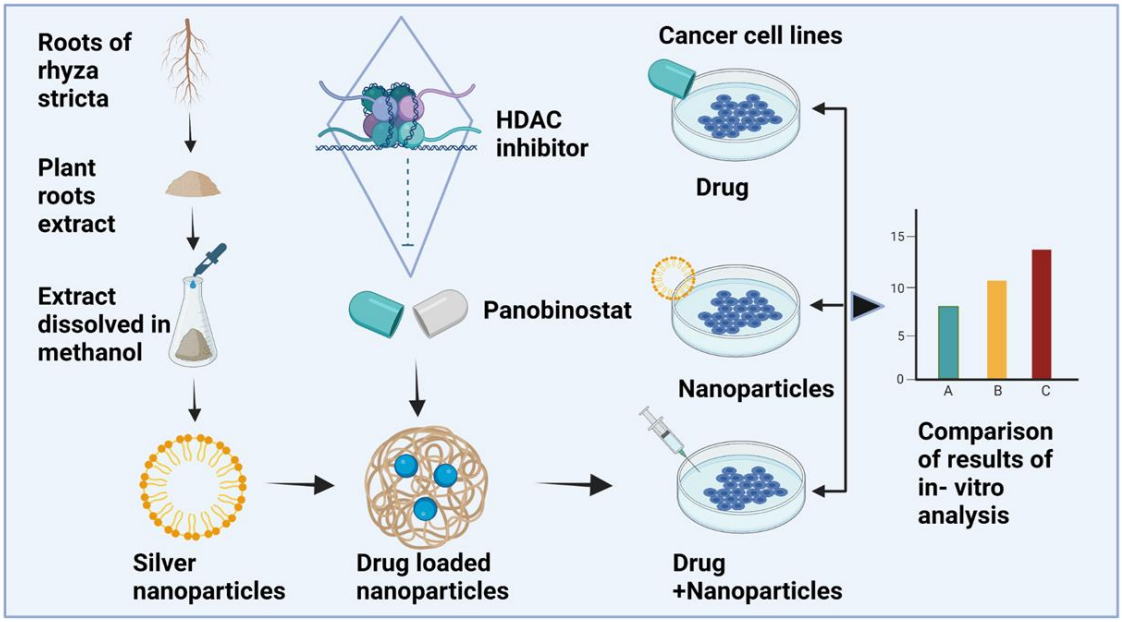
Bit usual but I would like to dedicate this thesis to myself, Tayyaba, as no one deserves this more than I do. I am proud of the person that I have become. A strong woman knows she has strength enough for the journey, but a woman of strength knows it is in the journey where she will become strong. I am the 'woman of strength'!

ABSTRACT

Breast cancer is the most common cancer among women even though numerous treatment options for breast cancer have been documented. Panobinostat, A histone deacetylase inhibitor, modifies gene expression through epigenetic pathways and prevents protein breakdown. The focus of this study is to enhance drug distribution to the tumor site and boost the efficiency of Panobinostat by using silver nanoparticles as controlled drug delivery system. Here we report the green synthesis of silver nanoparticles by using *Rhazya stricta* extract, as a nanocarriers for drug delivery. These drugs loaded nanoparticles were characterized by UV-Vis spectroscopy, XRD, FTIR, SEM, and EDX techniques. The overall findings demonstrated that AgNPs synthesized through *Rhazya stricta* has the high potential for sustained release of Panobinostat for cancer therapy. As the successfully synthesized Panobinostat-AgNPs were stable and exhibited increased in vitro anticancer activity compared with free Panobinostat, our data demonstrate that the combination of AgNPs with Panobinostat improves the drug's long-term viability, effectiveness, and active targeting as a potential targeted therapeutic molecule for the treatment of cancer. To strengthen the utilization of this combination therapy in cancer therapy trials, further research is warranted.

Keywords: Drug Delivery, Panobinostat, Silver Nanoparticles, Breast Cancer, Nanotechnology

GRAPHICAL ABSTRACT



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LIST OF ACRONYMS

FDA	Food and Drug Administration
AgNPs	Silver Nanoparticles
HDAC	Histone Deacetylase
HDACi	Histone Deacetylase Inhibitor
DNA	Deoxyribonucleic acid
EPR effect	Enhanced Permeability and Retention effect
UV-Vis spectroscopy	Ultraviolet–visible spectroscopy
XRD	X-ray Diffraction
FTIR	Fourier Transform Infrared
EDX	Energy Dispersive X-Ray Analysis
SEM	Scanning Electron Microscopy
MTT assay	(3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay

CHAPTER 1

1. INTRODUCTION

Cancer is a major public health problem globally. In 2020, 19.3 million new cases of cancer were reported, with around 10 million deaths worldwide. It is predicted that cancer cases will rise to 20 million annually in the next decade worldwide [1]. Traditional methods for cancer treatment include surgery, chemotherapy, and radiotherapy [2]. Surgery is an invasive procedure and is used only for localized tumors. Radiotherapy causes hair loss and weakens the immune system. Chemotherapy is an effective therapy that reduces symptoms and improves the quality of life. However, chemotherapeutic drugs are mostly hydrophobic, thus they need to be delivered in higher concentrations to attain the desired results. Higher concentrations lead to more toxicity and damage to healthy tissues causing adverse reactions. These are the certain obstacles to the effective treatment of this disease i.e. severe side effects of chemotherapy and multiple drug resistance. Thus effective drug delivery to the tumor cells with no side effects remains a challenge [3].

Nowadays researchers are more inclined toward nanotechnology and this field has gained attention quickly. Nanotechnology is being used for numerous applications i.e. Industrial applications, food processing, electronics, material science, and biomedical sciences. The development of nanoparticles as drug carriers proved beneficial for the therapeutic purpose i.e. targeted drug delivery, higher bioavailability, and lower toxicity. Nanotechnology has made cancer therapy

safer and more effective. This innovative approach is an encouraging strategy to deliver the anti-cancerous drug directly to the tumor cell and thus it doesn't damage the healthy cells. Other than that, nanoparticles can improve the stability of the drug as well as the accumulation of the drug in the tumor [4].

Silver nanoparticles possess anti-microbial and anti-cancerous properties such as anti-proliferation and induction of apoptosis and thus can be used effectively against cancer. Silver nanoparticles can be synthesized through physical, chemical, and biological methods [5]. In the physical method, it becomes difficult to control the size of the particle also this method is not very cost-effective. In the chemical method, chemicals may prove more toxic than effective against the cells. The biological method is an effective strategy for the synthesis of silver nanoparticles through plant extracts as it gives greater output with lesser cost.

In this study, silver nanoparticles are synthesized from the extract of *Rhazya stricta* based on low cost and easy availability. *Rhazya stricta* plant, also known as "Harmal", is located widely in South Asia. It has several medicinal advantages as it has anti-microbial, anti-oxidant, and anti-cancerous properties. It contains compounds such as alkaloids, flavonoids, and triterpenes. These compounds are responsible for the reduction of silver ions and thus the formation of silver nanoparticles. Silver nanoparticles synthesized by *Rhazya Stricta* can prove an effective therapeutic agent against cancer because of their wide range of biological activities [6].

HDAC inhibitor is a class of anti-cancerous agents that removes the acetyl group from core histones and thus relax the structure of chromatin. HDAC inhibitors

possess epigenetic roles including cell cycle arrest, apoptosis, and cell death. But, HDACi has distribution and rapid clearance problems when delivered in the free form [7]. Panobinostat is an FDA-approved HDACi that alters the transcriptional activity, damages DNA, and expresses those proteins which cause apoptosis of the cell. But it has several delivery challenges i.e. it is problematic to administer it in free form and pharmacokinetic properties show that it is rapidly cleared from tissues [8].

The ability to combine silver nanoparticles' anticancer characteristics with the pharmacological action of an HDAC inhibitor could be the key to treating cancers that have stopped responding to chemotherapy and radiotherapy. Assessing the possibility of using AgNPs to deliver Panobinostat to the tumor site, where the silver nanoparticles then release the drug and start to act against cancer cells after being up taken by the tumor cells, this is a viable cancer treatment technique [9]. This study will investigate the use of silver nanoparticles as a target-specific drug delivery vehicle for HDAC inhibitor Panobinostat with the hypothesis that entrapment within a Nano-carrier that is capable of sustained release will prove beneficial for its usage in cancer treatment.

OBJECTIVE:

- Green synthesis and characterization of silver nanoparticles.
- Encapsulation of HDAC inhibitor in silver nanoparticles.
- Application of encapsulated AgNPs on cancerous cell lines to check its treatment effect on cancer cells.

CHAPTER 2

2. LITERATURE REVIEW

2.1. Cancer: a key reason for morbidity and mortality

Cancer cells are abnormal cells that stop responding to the signals that keep them alive. After some time, they become immune to the healthy tissue's resistances, resulting in a rapid division of such cells. In a short period, cancer cells exceed the healthier cells in the environment. Even after the abnormalities of this type of cell make it a great target for apoptosis, these cells are capable of effectively avoiding death [10]. When cancer cells have progressed to a sufficient quantity in tissue, they want to expand their territory by attacking newer tissues. After undergoing several gene alterations, a normal and healthy cell becomes cancerous. Because each cell might have up to 60 mutations, identifying the specific mutation that causes cancer is a challenging feat. Cancer is the second leading reason of death worldwide. In 2018, it resulted in the deaths of 9.6 million people. According to the World Health Organization, one in every five men and one in every six women will get cancer at some point in their lives, with one in every eight men and one in every eleven women dying from it. Although several therapeutic options have been explored, there are currently no ways available that may entirely remove this life-threatening condition [1]. Cancer treatment is now confined to surgery, radiation, and chemotherapy, but each has its own set of drawbacks and frequently fails to cure the disease.

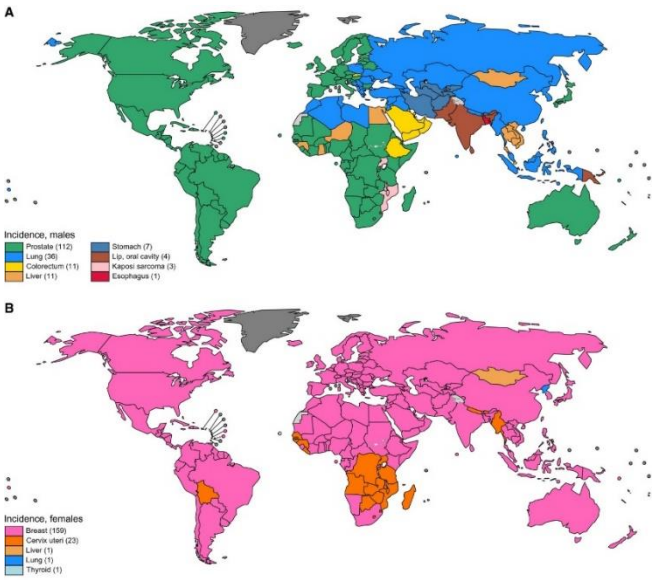

Figure 1: Global cancer incidence, (Sung et al., 2021)

The concept of the "Hallmarks of Cancer" refers to a set of functional abilities that human cells acquire as they transition from normal growth stages to malignant growth states, more especially abilities that are essential for the development of malignant tumors.

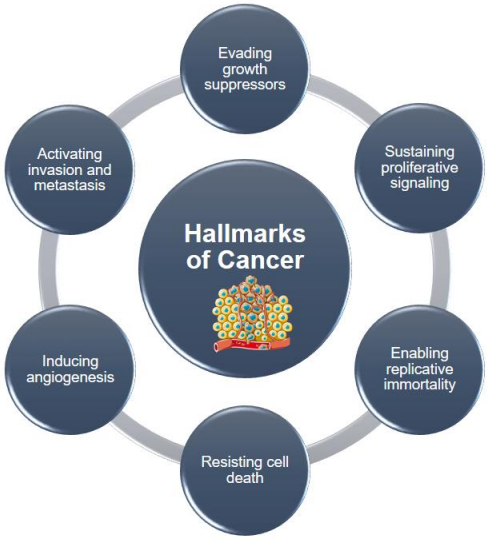

Figure 2: Hallmarks of cancer, ((Hanahan & Weinberg, 2011)

2.2. Treatment of Cancer: A Challenging Study

Even though a variety of treatments have been tested for the treatment of cancer tumors, it is well known that traditional chemotherapeutics and radiotherapy are unsuccessful due to resistance.

2.2.1. Chemotherapy

During the search for a treatment for mustard gas, scientists discovered that it permanently destroyed bone marrow, halting cell production. As cancerous cells are similar to bone marrow, research into using a mustard gas-type compound to treat cancer began. After a long period of research, viable chemotherapeutic medicines were discovered. These cytotoxic agents are now commonly utilized [11].

Cytotoxic agents are substances that are toxic to living tissue; as a result, these drugs affect the body in some way. Healthy cells are harmed, but they are managed in such a way that the cancerous drugs are the ones dying the most. Alkylating agents, one of the first forms of chemotherapy drugs, are an example of this approach [12]. When cells divide quickly, they need alkylating agents and consume huge amounts of the drugs, exposing their DNA to chemicals. The building blocks of DNA are damaged by these chemicals, and unless they are repaired, the cell will die. Because slow-dividing cells have more time to rewrite their DNA, they die less frequently than malignant cells.

Unfortunately, there is one major drawback to these drugs. In chemotherapy, hair loss, tiredness, nausea, fertility problems, and vomiting are all frequent side effects [13]. Doctors usually prescribe medications to help manage these side effects, and the unwanted effects fade away over time once the chemotherapy is completed. Chemotherapy is a very effective technique in cancer treatment; researchers are developing medications that target the desired location more precisely, trying to make chemotherapy a very powerful tool [14].

2.2.2. Radiotherapy

Radiotherapy is one of the most common cancer therapies, and its therapeutic efficacy has increased dramatically as X-ray and X-ray-related technologies have advanced. According to studies, 60 percent of patients having oncological treatment must undergo radiation therapy; yet, it may or may not be utilized in conjunction with other tumor treatment methods such as chemotherapy or surgery. In a radiotherapy procedure, high-energy X-rays or -rays are utilized to rupture nuclear material or damage DNA cells by releasing oxygen free radicals [15].

However, large doses of radiation are not practical to provide since the therapy has a high risk of causing collateral harm to nearby cells. On the other hand, giving the therapy in lower dosages may not result in effective tumor cell death, compromising the purpose of radiation therapy, which is to successfully treat cancer [16].

2.2.3. Gene Therapy

Gene therapy is a type of treatment that uses therapeutic genes to treat cancer and several other diseases. Gene therapy was created in such a way that genetic materials can be implanted into the diseased person's cells to restore the abnormal genes and distinguishable proteins. As a result, the harmful side effects associated with chemotherapy are reduced [17]. Gene therapy has been carried out using a variety of strategies, including gene amplification, inhibiting gene, and gene-mediated cell-killing therapy. In Gene therapy, tumor-specific gene delivery and strictly controlled gene release are crucial [18].

Although there are numerous benefits, there is a possibility that some damage to the body will occur. Selective targeting and transfection efficiency are two major drawbacks of this method. Not only is it hard to target tumor cells without affecting healthy cells, but only around 5% of cancer cells can be transfected once foreign genes are added [19].

2.3. HDAC inhibitors: Anticancer agents

Nucleosomes are the structural components of chromatin that are responsible for packaging eukaryotic DNA, and DNA and histones are the primary building blocks. The accessibility of DNA for transcription is affected by the changes in the structural form of chromatin to a comparatively open or condensed state. Histone deacetylase inhibitors (HDACi) stop histone deacetylases from removing the acetyl group from the lysine amino acid of histone and open the chromatin structure to permit access to transcription factors and gene transcription, hence

controlling cell proliferation [7]. The opposing actions of the enzymes histone deacetylase (HDAC) and histone acetylase (HAT) control variations in core histone acetylation, resulting in changes in gene expression and exerting anticancer mechanisms, including genes implicated in cell cycle regulation, cell cycle arrest, autophagy, anti-differentiation, angiogenesis, and apoptosis [20].

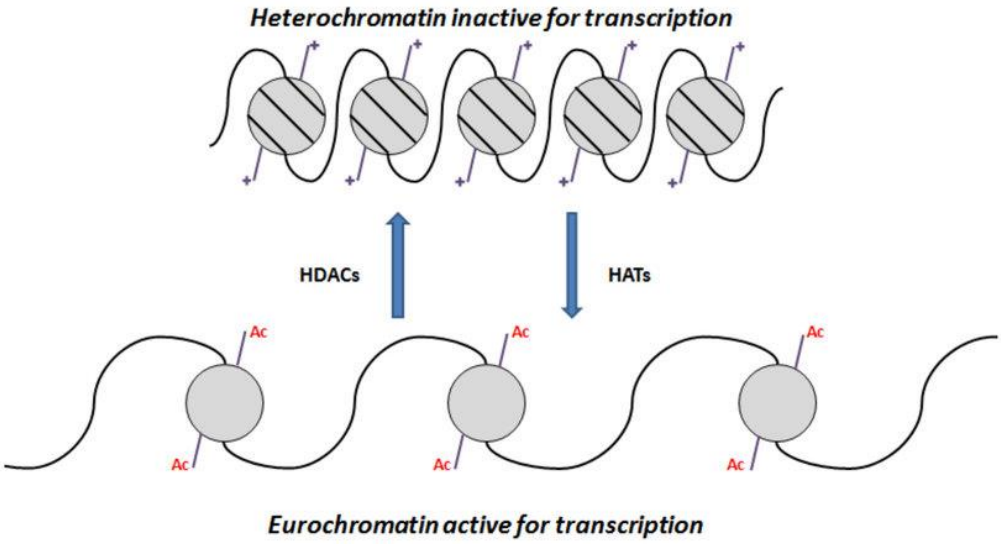

Figure 3: Transcriptional activity of Histone deacetylase, (Daśko et al., 2022)

Vorinostat (2006), Romidepsin (2009), Belinostat (2014), Chidamide (2015), and Panobinostat (2015) are five HDACi authorized by the FDA (Food and Drug Administration) for hematologic malignancies [21].

However, solid tumor treatment has limited success, which could be due to various factors. Poor pharmacokinetics, including short half-life and rapid metabolism and clearance, affect treatment efficacy; secondly, low specificity frequently leads to off-target and adverse effects; thirdly, HDACi's low

solubilization and cells permeability restrict intra-tumor delivery; lastly, drug resistance is easily developed. In HDACi-based therapy, targeted delivery and regulated drug release might be potential answers to these problems [22].

2.3.1. Panobinostat

Panobinostat belongs to a new class of drugs known as histone deacetylase inhibitors and approved by the FDA. Panobinostat displays significant inhibitory activity against all Class I, II, and IV pure recombinant HDAC enzymes at low nanomolar doses, implying full pan-DAC activity [23]. Panobinostat IC50 values against HDACs 1–9 range from 3 to 61 nM in enzymatic tests, with a somewhat higher IC50 value of 248 nM for HDAC 8 [24]. Panobinostat has shown substantial anti-proliferative and cytotoxic effects in a range of cancer cell lines while causing minimal damage in all normal cells tested, in keeping with its extremely powerful HDAC inhibition [25].

Panobinostat has a higher anti-tumor effectiveness than other deacetylase inhibitors in terms of tumor progression and viability inhibition. Panobinostat, for example, suppressed cell growth and viability in HCT116 cells, CTCL line HH, Hsp90, and BT474 cells, at nanomolar doses up to 100-fold lesser than the inhibitory values of MGCD0103 and Vorinostat. Furthermore, the doses of Panobinostat sufficient for HDAC inhibition and antitumor activity are very consistent. This could offer Panobinostat a leg up on other HDAC inhibitors when it comes to tying anti-tumor effectiveness to on-target effects [26].

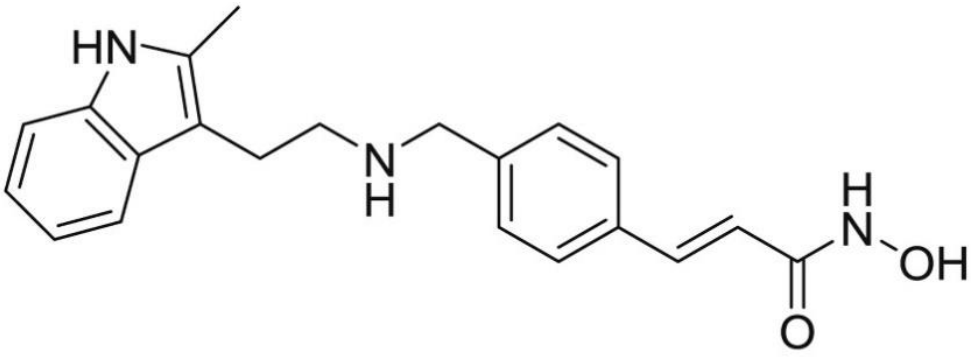

Figure 4: Chemical structure of Panobinostat, (Chua et al., 2017)

2.3.2. Mechanism of action

Panobinostat inhibits a wide variety of deacetylases (DACs), often known as histone DACs because histones were the first recognized DAC targets. DACs control the acetylation of over 1,750 proteins involved in a variety of biological activities, including DNA replication and repairing, histone modifications, transcriptional regulation, cell-cycle progression, enzyme inactivation, and cytoskeletal rearrangement. Overexpression of HDACs has been linked to poor outcomes in multiple myeloma patients [25].

Panobinostat is expected to have an anticancer effect primarily through epigenetic gene regulation and protein metabolism inhibition. Through inhibition of signals activators of transcription 3, Akt, and hypoxia-inducible factor, class I HDACs, which target histones and transcription factors like p53, it may be possible to reactivate epigenetically repressed tumor suppressor genes and change gene expression [27].

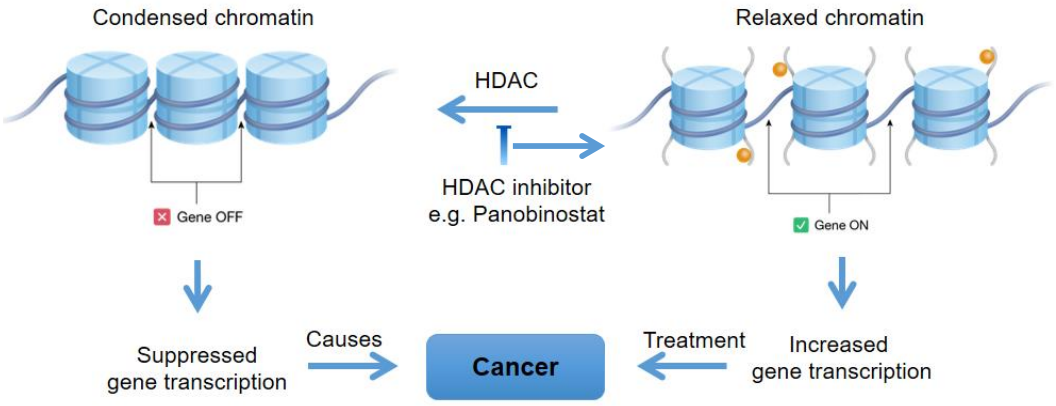

Figure 5: Mechanism of action of HDAC inhibitor, (Kulka et al., 2020)

2.3.3. Limitations of Drug

Panobinostat is an important contribution to the current therapy choices for relapsing multiple myeloma, and it paves the way for further drugs in this class with comparable modes of action to be developed. However, Panobinostat toxicity has been reported to be largely gastrointestinal and hematological. Its toxicity is very similar to other HDAC inhibitors such as Vorinostat and Romidepsin [28]. Hematologic severe adverse events, such as thrombocytopenia, anemia, and neutropenia, were the most common. For this patient group, gastrointestinal diseases and their treatment remain a major therapeutic concern. Diarrhea, which affects 76 percent of patients, is the most prevalent nonhematologic side event, followed by fatigue. In the great majority of patients, medication toxicity needed dose modification or even treatment discontinuation.

Off-target effects, drug stability, systemic toxicity, diffusion into tumor tissues, pharmacokinetic features, and oral administration are all drawbacks of HDAC inhibitors. The toxicity profile of panobinostat necessitates the development of techniques to reduce drug-induced toxicity [29].

2.4. Need for carrier

When designing a drug delivery system, the goal is to ensure that drugs are released just at the specified target location and that the drug concentration does not vary significantly over time within a therapeutic range. Regardless of how the medication is delivered, the bioavailability of the blood permits the molecules to spread and reach practically all tissues of the body, and once there is drug concentration in the blood, it penetrates the endothelial barriers. A carrier that can distribute the medicine directly toward the tumor tissues can be employed to accomplish correct targeting [14].

Most organic nanoparticles (liposomes, PLGA, carbon nano-materials, micelles, lipids), inorganic nanoparticles (quantum dots, gold, silver, polystyrene, grapheme, silica, iron), and composites were designed for use as drug carriers.

Anti-cancer drugs, like many others, have unfavorable effects on normal cells and severe side effects. Different drug delivery methods have been developed as a result of the negative effects of chemotherapy treatments. These drug delivery carriers are sometimes developed to bind to malignant cells, selectively. To achieve this, some unique ligands have been linked to the surfaces of drug delivery vehicles, allowing the drug to be given via the ligands and the malignant cells' specialized receptors. Although this process is extremely precise and

appealing, it does have some drawbacks, such as the effort and money involved in synthesizing the materials mentioned above. As a result, chemotherapeutic coupled nanoparticles are being considered as an alternate drug delivery strategy. Drug-conjugated nanoparticles have longer half-lives in the body than non-conjugated nanoparticles, and because the tumor site has less lymph drainage and more blood circulation, the drug-loaded nanoparticles concentrate in that area and are more harmful to malignant cells than normal cells [9].

Compound solubility has traditionally been a barrier to drug loading. Nanoparticle-based treatments such as nanocarriers have greatly improved the above-mentioned shortcomings. It is critical to load HDACIs into non-toxic nano-carriers to achieve targeted delivery as well as tissue-specific distribution. Most free drugs diffuse in a non-specific manner, providing a problem for directed therapies. Nano-carriers, on the other hand, can enter the tumor milieu through leaky capillaries and regulate the tumor microenvironment to accumulate in tumor tissue through the enhanced permeability retention effect (EPR). As a result, nanotechnology and material science can avoid the drawbacks of non-targeting specific molecular HDAC inhibitors. Nanomaterials as drug carriers have several advantages, including their small size (1–100 nm) and controlled release system, which allows for improved targeting techniques [30].

By addressing the drawbacks of HDAC inhibitors, nanotechnology-based delivery can enhance their anti-tumor activity. Poor solubility is a concern for several HDAC inhibitors. Because of biocompatibility and biodegradability, starch is a polysaccharide widely utilized in pharmaceutics. Using the “emulsion solvent

diffusion" approach to encapsulate CG-1521 i.e. HDAC inhibitor, into starch nanoparticles could enhance its solubility in water and cellular uptake, resulting in improved anti-tumor activity. Panobinostat has been studied as a possible therapy for glioma. However, due to its low solubility, it is not suitable for delivery in brain tumors [31].

2.5. Nanotechnology: Exciting Forefront in Biomedical Sciences

Because of their diverse physical and chemical properties, nanomaterials have recently piqued the curiosity of researchers interested in cancer therapy. The use of nanoparticles as carriers of medicinal substances has been the subject of numerous studies. Unfortunately, there are several problems in using nanoparticles as carriers of medicinal chemicals, which prevent these therapeutic approaches from being used in clinical settings. Low drug loading efficiency, low aqueous solubility, poor ability to cross barriers and enter the tumor, difficult interactions of hydrophobic therapeutic drugs with nanomaterials, in vivo instability, low target specific ability, and an inadequate drug release profile are the significant challenges [32]. Nanotechnology's application to cancer therapy could go beyond drug delivery to include the development of new therapeutics that can destroy tumors while minimizing potential damage to healthy organs and tissues, as well as the identification and eradication of cancer cells during the early stages of tumor-genesis. These small particles can also be made functional by adding ligands, peptides, or antibodies that attach to specific target molecules. Nanomaterials are attractive to use extensively for therapeutic reasons because of their specific intrinsic characteristics and biocompatibility [4].

Nanotechnology advancements in the biomedical field have greatly boosted the possibility of merging numerous therapies using adsorption and binding forces. There are numerous advantages of using nanoparticles over other materials. The NPS can persist in the tumor site due to the “Enhanced Permeability and Retention (EPR) effect”. Another factor is the huge surface-area-to-volume ratio, which allows therapeutic chemicals to be trapped proficiently and not destroyed by the microenvironment. Nanomaterials are also being employed to develop improved and more controlled drug delivery technologies. These characteristics can be employed to get around some of the drawbacks of traditional medicinal and diagnostic agents [33].

2.6. HDAC inhibitor Platforms Based on Nanotechnology for Cancer Therapy

There are two types of tumor-targeted delivery strategies: passive and active. The EPR effect, in which macromolecules or nanomaterials tend to diffuse in the tumor due to leaky vessels and poor lymphatic drainage, could allow nanomedicine to accumulate in tumors to enable passive targeting. The EPR effect is commonly found in rat tumor models, however, data in human trials is limited. Because of the variety of tumors, the EPR impact is thought to be highly varied. Another standard approach is active-targeting delivery. The overall concept is based on ligand/receptor-specific interactions, modified ligands on nanoparticles, and receptors present on cancer cells in the tumor microenvironment. Certain nanomaterials, such as albumin nanoparticles and “albumin-binding proteins”, can preferentially attach to receptors on cancer cells [34].

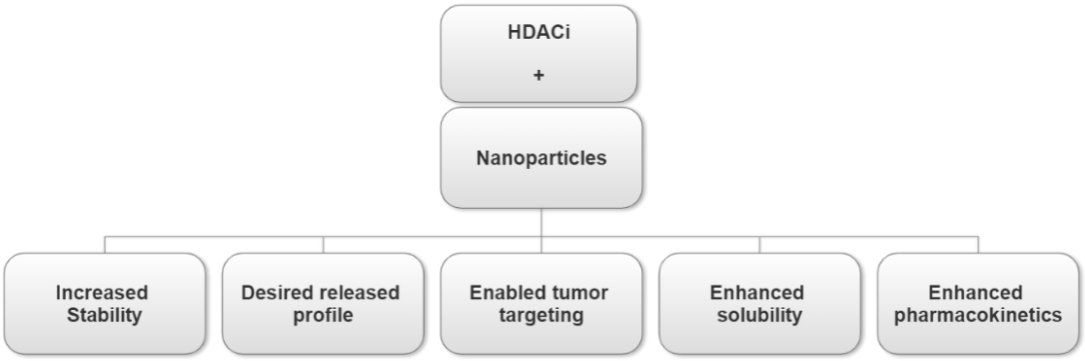

Figure 6: Combinatorial action of HDAC inhibitor and nanoparticles,

(De Souza et al., 2020)

2.6.1. Passive Targeting Using Nanotechnology

Tumor vessels have aberrant vasculatures with leaky vessels and a lack of a tight junction between epithelial cells and pericytes. When compared to normal tissues with 5-10 nm endothelial junctions, the leakage allows nanomedicine to infiltrate and concentrate in malignant tissues [35].

The tumor preferentially accumulates drugs, resulting in a lower drug dose and lesser adverse effects. Furthermore, nanomedicines can improve intracellular drug retention by reducing transporter-mediated drug efflux and reversing drug resistance due to the "size-exclusion effect."

2.6.2. Active Targeting Using Nanotechnology

For HDACi therapy, a variety of active-targeting nanoparticles have been created. Through a specific interaction between both the ligand present

on nanoparticles and the matching receptors overexpressed on tumor cells, ligand-modified nanoparticles promote tumor targeting and cell internalization. Nutrient transporters, such as albumin-binding protein, for example, are frequently overexpressed in cancerous cells because of their energy needs, and hence serve as targeted delivery receptors. Small molecules, peptides, and antibodies are the most common active targeting ligand molecules [36].

2.6.3. Combination Therapy Using Nanotechnology

Drug combinations can improve treatment efficacy while lowering side effects and reducing drug resistance. HDACi in conjunction with other anticancer drugs has been used in cancer therapy and has shown significant promise as a prospective alternative to HDACi's lack of activity in solid tumors. However, the varied in vivo fates of the combination medications provide a significant obstacle; for example, delayed distribution throughout the tumor may not result in synergistic pharmacological activities in cancer cells. It could explain the differences between in vitro and in vivo studies; many combinations perform well in cell testing but not in humans. Nanotechnology-based combination therapy, which has been investigated in HDACi therapy, can provide a synchronized pharmacokinetic property of the combined medicines and ease synchronized delivery [37].

2.7. Metal Nanoparticles: Utilization in Biomedical Sciences

Metal nanoparticles have sparked a lot of attention as a new platform for nanotechnology and biomedicine because of their bright and interesting colors. The average size of metallic nanoparticles, such as those made of silver, gold, and platinum, is 50 nm, and they have a large surface area. Due to their smaller size, they can easily pass through capillaries in tissues and cells [38]. They can carry a reasonable amount of medications due to the large surface area they offer and the ability to chemically alter their surfaces. In this way, metal Nanoparticles have been used to release medications under controlled conditions for cancer treatment [39].

Chemotherapy is a key therapeutic method used in cancer treatment. The most significant barriers to effective treatment for cancer are toxicity to healthy cells and the development of multidrug resistance to anticancer medicines. As a result, improving treatment efficacy while minimizing adverse effects requires specific amplification of anticancer drug concentrations in tumor tissues. Even though tremendous attempts have been done to overcome MDR, clinical progress has been limited [40]. Nano therapeutics is quickly developing, intending to solve the issues associated with traditional chemotherapy.

Polymeric, liposomal, metallic, and magnetic nanoparticles as well as other nano-sized carrier systems, are being studied extensively for tumor-targeted therapy, biomedical imaging, and bio sensing. Due to their specific shape, size, and surface-dependent features, metallic nanoparticles in particular have been acquiring popularity in clinical application as distinctive drug delivery vehicles.

Furthermore, due to their reported biocompatibility and lack of cytotoxicity, drug delivery has emerged as the most promising new application of metallic nanoparticles [41]. Furthermore, the convenience with which these nanoparticles' properties can be functionalized makes them appealing for this application. Metallic nanoparticles that have been properly functionalized can not only act as a drug carriers but also have a long circulation period. As a result, nanoparticles have emerged as potential choices for delivering diverse payloads to their desired targets. Small pharmacological molecules or big macromolecules could be the carriers. Amino acids, proteins, enzymes, DNA, and RNA are examples. This method does not affect the conjugated species' biological activities, such as their ease of bio conjugation and bio modification. Effective therapy requires the efficient delivery of these therapeutic substances at the targeted place [42].

2.7.1. Silver nanoparticles: Anticancer properties

Due to the distinctive chemical, physical, and biological intrinsic features, silver nanoparticles, among other metal NPs, have been used more and more in the pharmaceutical, aviation, microelectronics, food, and medical industries. They can be distinguished by their strong electrical conductivity, optical properties, and biological traits. AgNPs have proven beneficial in a variety of industrial and medicinal products, including coatings for medical equipment, biosensors, bioimaging, antibacterial, antifungal, antiparasitic, antiviral, and anticancer medicines [43].

The use of colloidal silver is not uncommon. It has been utilized for many years across numerous industries. In medicine, silver nanoparticles (AgNPs)

have been used for surgical coatings, food supplements, cosmetic products, textiles, food packaging, surgical devices, and water sterilized applications. They have also been impregnated into wound dressings and other topical treatments where antibacterial action is required. AgNPs made up 55.4% of all commercially available nanomaterial-based products in 2011. Nanoparticles are generally beneficial for imaging, medication delivery, and building cancer biomarker profiling of malignant tumors due to a number of their unique features. AgNPs have been shown to possess anticancer properties and have been utilized to treat a variety of cancers, including leukemia, breast cancer, lung carcinoma, hepatocellular carcinoma, and glioblastoma [44].

AgNPs have underlying antibacterial and anticancer characteristics through a variety of mechanisms, such as silver ions or the formation of oxygen radicals, which are released or formed in response to the uptake of AgNPs by living cells or bacteria and cause the deregulation of critical cellular mechanisms that worsen cellular damage and death [45].

A promising cancer treatment strategy would involve using AgNPs to deliver anticancer drugs to a tumor site where the AgNPs would then release the drug at the targeted site and begin acting against the cancerous cells after being taken up by the cells. In general, chemical, physical, or biological approaches can be used to synthesize silver nanoparticles [46]. For the synthesis of nanoparticles, some of these methods are thought to adhere to the majority of the Green Chemistry principles.

2.7.1.1. Chemical synthesis

Chemical reduction of Ag+ ions by organic or inorganic substances such as sodium borohydride, sodium citrate, sodium ascorbate, and polymers is the basis of these methods. The reducing agent causes metallic silver (Ag0) to form, which then aggregates to form oligomeric aggregates. These result in colloidal silver metal particles. To prevent unnecessary agglomeration and help in regulating the size of the AgNPs, capping agents and surfactants can be employed as stabilizers, along with chitosan, cellulose, polyethylene glycol (PEG), and polyvinylpyrrolidone (PVP) [47]. Anionic species, which cover AgNPs and give their surface a negative charge, are typically used to stabilize electrostatic fields. Examples of these species include citrate, halides, carboxylates, and polyanions. Polyethyleneimine (PEI), for instance, can be used to coat the surface with a positive charge. By measuring the zeta potential, it is possible to keep track of these charging-coatings in AgNPs. AgNPs can interact with large molecular groups, like organic polymers and alkylammonium cations, to achieve steric stability [48].

2.7.1.2. Physical methods

The evaporation-condensation and laser cutting procedures are the foundation of the most relevant physical methods. Both approaches avoid the use of potentially harmful chemical reagents for both the environment and human health. The equipment required for them is expensive and very specialized. The hazardous chemicals used during this type of synthesis resulted in unfriendly byproducts. This is what causes the biosynthesis of NPs to take a

green route without the use of harmful chemicals, and it also shows that there is a growing demand to create environmentally friendly processes [44].

2.7.1.3. Biological Synthesis

As a crucial area of nanotechnology, the application of green synthesis of Ag-NPs is progressing. In this field, the production of NPS from biological sources, such as plant biomass or extract, could replace harmful chemical and physical processes. In comparison to physical and chemical methods, green synthesis is more environmentally friendly, economically advantageous, and easily scaled up for the production of large quantities of nanoparticles (NPs) [49]. The green synthesis also does not require the use of hazardous chemicals, high temperatures, or high energy inputs. The majority of plant extracts used to make AgNPs have therapeutic properties such as anti-inflammatory, anticancerous, antioxidant, etc., which can be employed in conjunction with AgNPs' inherent biological action, such as its breakdown into silver ions that can disrupt a variety of cellular functions [50].

They are based on the utilization of reducing agents found in plants, fungi, bacteria, and algae that are used to decrease Ag+ ions. AgNP synthesis through plants is becoming more and more common since it is easy, affordable, accessible, and environmentally friendly. Countless natural sources can be employed, for example, certain extracts are made from plants like *Azadirachta indica*, *Eucalyptus Procera*, *Calliandra haematocephala*, and *Madhuca longifolia*. Since plant extracts can contain molecules that coat AgNPs i.e. capping agents, deliberately or accidentally, in addition to

reducing agents, their rich composition in chemical compounds has a complex action [51].

Genes involved in cell cycle advancement and apoptosis would be expressed more when AgNPs were present. Reactive oxygen species (ROS) and oxidative stress, which cause DNA damage and apoptosis, are two possible causes of toxicity. *De Matteiset al.* concluded that the release of Ag+ ions in the cytosol following the absorption of Ag nanoparticles through endocytosis and their disintegration in an acidic environment is what mostly causes the cytotoxicity in cells treated with AgNPs [52]. Therefore, the oxidative stress, DNA damage, and cell death observed in the presence of AgNPs are primarily caused by the silver ions present in the cytosol impairing natural metabolic and cell cycle mechanisms. This theory is supported by the activation of metallothioneins and the use of Ag+ chelating agents to reduce cytotoxicity. Suarez et al., on the other hand, concentrated on recognizing the physiological alterations brought on in hepatocytes by exposure to extremely low concentrations of silver nanoparticles that simulate chronic exposure [53].

AgNPs' concentration, charge, surface modification, size, and shape are some of the physicochemical factors that affect their cytotoxicity and genotoxicity. The experimental findings that have been reported up to this point are insufficient to accurately pinpoint the toxicity of AgNPs and their impacts. Toxicity, however, is a barrier to its application in vivo [54].

2.8. Plant extract: *Rhazya Stricta* for the synthesis of silver nanoparticles

Evergreen medicinal plant Rhazya stricta, often known as “Harmal,” is widely distributed in South Asia i.e. Pakistan, India, and the Middle East i.e. Saudi Arabia, Qatar, UAE, Iran, and Iraq [6]. There are two species of Rhazya, *R. stricta*, and *R. orientalis*, which are members of the Apocynaceae family, which is rich in indole alkaloids. Alkaloids, glycosides, triterpenes, and tannins are the major components of R. stricta leaf extract, which has been used for centuries to treat diseases like cancer, diabetes, arthritis, and infections. Furthermore, information on this plant's alkaloid diversity and nuclear genome has just been published. R. stricta produces several terpenoid indole alkaloids, and this can be used in biotechnology [55]. A substantial antioxidant, anti-inflammatory, and anti-cancer compound called -tocopherol is also found in this plant. Hooper was the first to identify alkaloids in R. stricta, and by 1945 it was known that the plant had a high alkaloid content. Since then, more than fifty indole alkaloids have been identified from Rhazya species, and Chatterjee and colleagues have detailed their findings.

R. stricta serves as the perfect capping agent in the current study's bio-synthesis of AgNPs, likely because the plant contains alkaloids and triterpene acids, as shown by HPLC-MS [56].

2.9. Cell lines

Cancer research and drug development frequently use cancer cell lines as useful in vitro model systems. Their remarkable ability to offer an endless amount of biological material for research purposes is the main factor influencing their

employment [57]. One of the most widely used breast cancer cell lines in biomedical research labs is the MDA-MB-231 cell line, an epithelial human breast cancer cell line that was developed from a pericardial effusion of a 51-year-old Caucasian woman with metastatic mammary adenocarcinoma1. The MDA-MB-231 cell line, which was found in a patient's pleural effusion who had invasive ductal carcinoma, was used to create a model for late-stage breast cancer [58].

CHAPTER 3

3. MATERIALS AND METHODS

3.1. Material and reagent

Rhazya Stricta plant was purchased from Islamabad Nursery Farm collected from the Southern side of Punjab. Silver Nitrate and Xylitol having ≥ 99.0% purity were purchased from Sigma Aldrich, St. Louis, USA. Xylitol is a white crystalline solid used for preventing agglomeration of nanoparticles. Methanol was also obtained from Sigma Aldrich. Whatman filter paper of 125 mm was used for filtration purposes. Breast cancer cell lines MDA-231 cell lines were used. Dimethyl Sulfoxide (DMSO) and 3-(4,5-dimethylthiazal-z-yl)-2,5-di-phenyltetrazolium (MTT) were also used for MTT assay. Phosphate Buffer Solution (PBS). Deionized water was used in reactions.

3.2. Plant extract preparation

Rhazya stricta plant was purchased from Islamabad Nursery Farm collected from the Southern part of Punjab. The roots of the plant were cut down neatly from the plant and washed with water. After washing, the roots were dried in sunlight (in the winter season) for 2 weeks. When the roots were completely dried, they were ground in powder foam. Powder of dried roots was 90 g which was soaked in 70% Methanol and kept at room temperature for a few days. After that, it was filtered using Whatman filter paper. The solvent from the extract was evaporated by using a rotary evaporator. Methanol will be evaporated. The final extract was dried in a lab oven at 40° for 4-7 days until it is completely dried.

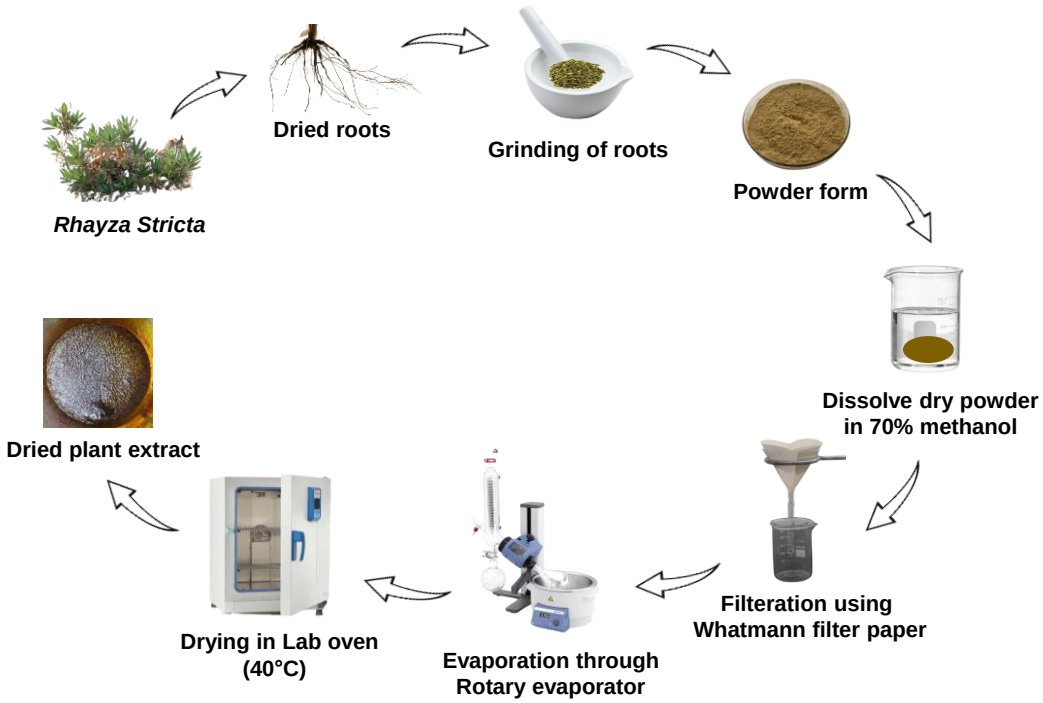

Figure 7: Schematic diagram of the preparation of plant extract

3.3. Preparation of silver nanoparticles

Silver nanoparticles were synthesized by the method of [59]. Briefly, 10g of dried plant extract was dissolved in 18ml of methanol. The solution was placed at 4°C. One millimolar solution of silver nitrate was prepared by dissolving 0.017 g of silver nitrate in 100ml of deionized water.

50ml of silver nitrate was taken and 3ml plant extract (dissolved in methanol) was added dropwise to it. 3ml of xylitol was further added to the beaker with continuous shaking. The reaction was heated on a hot plate by using a magnetic stirrer at 60° for the time of 1-2 hours until the color changed. The color change indicated the formation of silver nanoparticles.

3.4. Conjugation of Panobinostat with silver nanoparticles

The stock solution was prepared at 10 mg/ml concentration. To obtain this concentration, 2 mg of the drug (Panobinostat) was dissolved in 1 ml of Dimethyl sulfoxide (DMSO). The drug was loaded into nanoparticles through vortex mixing. They were employed for further testing.

3.5. Characterization of nanoparticles

3.5.1. UV

It is standard procedure to utilize UV-visible spectroscopy to identify the development of nanoparticles when studying nanomaterials. UV-visible spectroscopy is frequently the method of choice to measure response in an investigation employing nanoparticles when used in combination with affinity labeling. By fitting the location of the Surface Plasmon Resonance (SPR) to a straightforward wavelength function, the spectroscopic characteristics of nanoparticles can serve as a gauge of their size distribution.

The UV-Visible Spectroscopy principle is the absorption of visible and ultraviolet light by chemical substances, which generates distinctive spectra. The basis of spectroscopy is the interaction of light and matter. A spectrum is created when the substance absorbs the light through excitation and de-excitation processes. According to Beer-law, Lambert's the rate at which the intensity of a beam of monochromatic light decreases along the thickness of a solution containing a substance that absorbs monochromatic light is related to the concentration of the absorbing medium in the solution and is also directly proportional to the intensity of the incident monochromatic radiation. The

degree of radiation absorption increases with the number of absorbent molecules i.e. molecules with the capacity to absorb light of a certain wavelength.

$$\mathbf{A = \varepsilon Lc}$$

The UV Vis Spectrophotometer is a useful tool for figuring out the optical characteristics of produced Silver Nanoparticles. A double beam UV-visible spectrophotometer was used to characterize the reduction of silver ions to nano silver at various wavelengths between 300 and 700 nm. The resolution of the instrument used for this investigation, a Spectro UV-Vis Dual Beam, is 1 nm. On a Spectro UV-Vis Dual Beam PC Scanning Spectrophotometer, model number Spectro UVS-2800, the UV-Vis analysis was carried out by sampling the aqueous component at various time intervals and scanning the absorption maxima over the wavelength range of 300-800 nm.

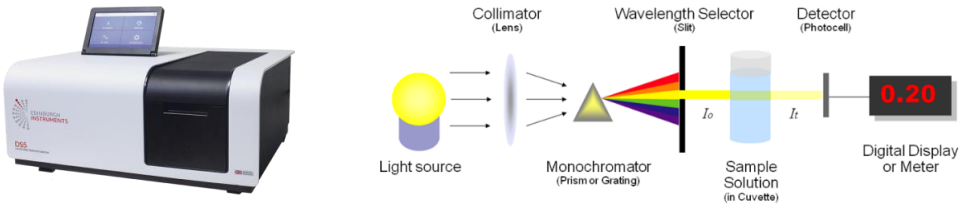

Figure 8: UV-Vis spectroscopy instrument and its working principle

3.5.2. XRD

An analytical method known as X-ray powder diffraction (XRD) is used to analyze the phase of crystalline materials. It can also tell information on unit cell dimensions.

The scattering of X-rays caused by the rotation of electrons in the atom's nucleus when the rays hit the nanoparticles, is the basis for the XRD method's operation. Atomic electrons in a crystal are thrown into vibration when a monochromatic x-ray event occurs on it. Acceleration occurs with the same frequency as the incident ray's frequency. Then, these accelerated electrons radiate in all directions at the same frequency as the incident x-rays. Interference patterns result from the scattered X-rays being reflected in different ways. Only the dispersed X-rays that interact positively cause diffraction, regardless of whether these patterns are destructive or constructive.

When two waves move in phase with one another, positive interference occurs in a nanoparticle; when they move out of phase, destructive interference occurs. Atoms with shorter periodic configurations exhibit higher diffraction angles and vice versa. The atomic-scale organization and the diffraction are significantly and inversely connected. When the circumstances are in accordance with Bragg's Law, the interaction of the incident rays with the sample results in constructive interference (and a diffracted ray).

$$\mathbf{2d \sin \theta_n = n\lambda.}$$

Both of these uses of Bragg's law are common for using Bragg's law to determine the crystal structure. It is essential that and are correctly matched. As a result, angles n produce the reflected wave's highest amplitude.

For XRD investigation, thin films of nanoparticles are created by adding a drop of nanoparticle, drug, and nanoparticles loaded drug onto a glass slide.

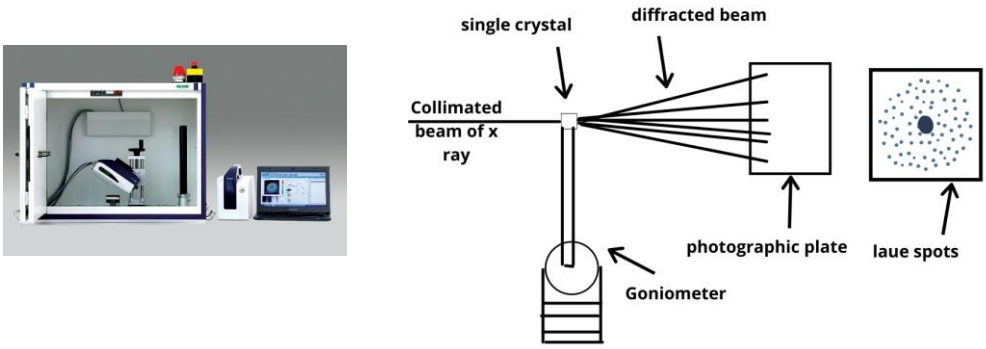

Figure 9: XRD instrument and its working principle

3.5.3. FTIR

The function of biomolecules in the reduction of silver nitrate to silver zero oxidation state can be determined using FTIR, which is a simple, quick, appropriate, non-invasive, and cost-effective approach. To explore the surface adsorption of functional groups on nanoparticles, in-situ analysis of interfaces using FTIR is possible. The ability to study a film of nanoparticles placed on the ATR element while also changing the overlaying phase is one benefit of FTIR.

By simultaneously reducing Ag+ to Ag0 and stabilizing synthesized Ag NPs by preventing coagulation, green synthesis produces biomolecules from plant extracts. Information regarding the transformation of metallic silver to the Ag0 state can be obtained from FTIR analysis. The interpretation of the synthesis of NPs can benefit from a small variation in absorbance of magnitude 10^{-3}, due to the high sensitivity of FTIR. The procedure has recently been made simpler by the attenuated total reflection (ATR) - FTIR sampling methodology, which avoids the need for any special sample preparation. The ATR-FTIR technology may directly study nanostructures in any state.

The presence of organic compounds on the surface of nanoparticles can be detected using the FTIR spectrum. In the current experiment, a phytochemical found in Rhazya Stricta extract that is responsible for reducing silver ions to silver nanoparticles was identified using an FTIR measurement. With the use of appropriate standards, FTIR analysis can provide quantitative (quantity) examination of materials in addition to qualitative (material identification) analysis. To pinpoint the potential biomolecules in charge of capping and the effective stability of the metal nanoparticles produced by plant extract, FTIR studies were made.

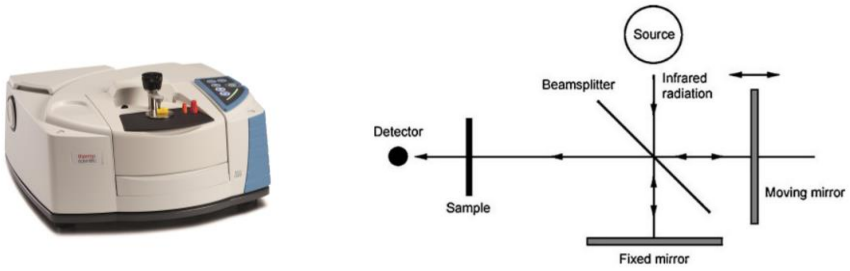

Figure 10: FTIR instrument and its working principle

3.5.4. SEM

One significant method for creating a clear visual of a particle with high clarity and spatial resolution is scanning electron microscopy (SEM). The sample is subjected in a scanning electron microscope (SEM) to a high-energy electron beam, which provides information about a material's topography, geometry, composition, chemistry, grain orientation, crystallographic information, etc. In an SEM, a small electron beam moves in parallel lines across the prepared sample. A cathode ray tube's screen can be used to detect and show the various signals that are produced as a result of the electrons' interactions with the sample.

As the investigation of structural morphology reveals how macromolecules function effectively at the atomic or molecular level, SEM offers unparalleled imaging and detecting capabilities. The success of the SEM is mainly because views of three-dimensional objects may frequently be quickly, intuitively, and interpreted by the observer. The biomedical industry can potentially use the detection of nanoparticles to enable controlled drug release from

nanoparticles. It can aid in improving drug absorption, lowering dosage requirements, and addressing the issue of non-compliance with recommended therapy.

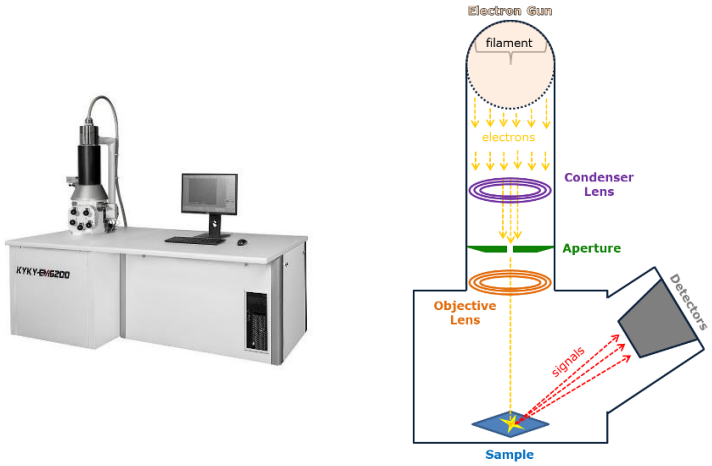

Figure 11: SEM instrument and its working principle

3.5.5. EDX

The elemental makeup of a specimen can be determined using the Energy Dispersive X-ray (EDX) technique. Biodistribution and toxicity investigations frequently encounter issues with the detection of nanoparticles in tissue. The fundamental idea behind EDAX is the creation of X-rays from a sample using an electron beam. The features and type of the elements contained in the sample are used to generate the X-rays. Therefore, this method can also be used to determine how much energy X-rays are emitting.

In the study of medicines, the EDX approach is effective for detecting nanoparticles, for example, in the investigation of medication distribution

utilized most frequently to enhance the therapeutic effectiveness of several chemotherapeutic drugs.

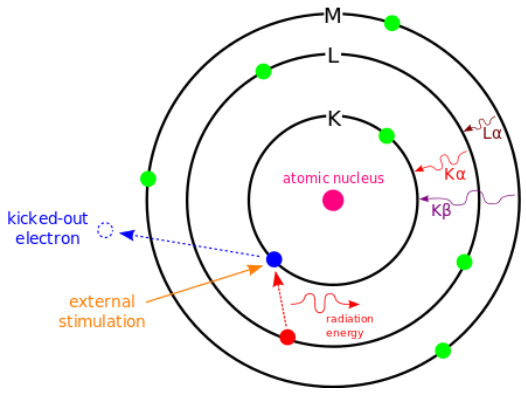

Figure 12: EDX working principle

3.6. Drug loading and Drug release

The ratio of the drug amount in the nanoparticle to the overall amount of drug used in their formulation is known as drug loading efficiency. The drug-loaded nanoparticle suspensions were centrifuged at 13,000 rpm for 1 hour, and the entrapment effectiveness of the drug-loaded nanoparticles was assessed by measuring the clear supernatant's absorption using a UV spectrophotometer. By analyzing the supernatant of blank nanoparticles, the corresponding calibration curves were created. Each sample's tests were run in triplicate. A UV-vis spectrophotometer set to the wavelength of 290 nm was used to test the drug's absorption value. The following equation gives the percentage of medication encapsulation efficiency in nanoparticles.

$$Encapsulation\ Efficience = \frac{Total\ drug\ added - free\ drug\ in\ the\ supernatant}{Total\ drug} \times 100$$

In the case of a matrix made of polymer nanoparticles, the drug is evenly dispersed or dissolved inside the matrix, and the release is brought on by diffusion or matrix erosion. Drug release occurs mostly through diffusion if drug diffusion is quicker than matrix breakdown. On a sample that had been loaded with drugs, the in vitro drug release test was conducted. Each sample was suspended in a fixed volume (10 ml) of phosphate-buffered saline (PBS) with a pH of 7.4 and a temperature of 37 C. To maintain a constant volume of the release medium, five-milliliter aliquots of the resulting suspension were obtained out of the dissolution medium at appropriate intervals (30 min) and replaced with the same volume of fresh PBS buffer. The resulting suspension was then placed in an incubated shaker at 120 rpm for a specific amount of time (1 h). UV spectrophotometer set at 290 nm was used to measure the amount of drug released.

3.7. Drug release kinetics

One or more processes that depend on the matrix's composition, geometry, preparation technique, and dissolution medium for drug release control the drug release kinetic. According to the desirable or required prediction ability and the accuracy of the model, this can be described using mathematic models. A key element in establishing a drug's therapeutic potential is how the drug is released

from the matrix. Various kinetic models have been applied to in vitro drug release to elucidate the process of drug release from AgNPs. Numerous mathematical models (zero-order, first-order, Hixon-Crowell, and Higuchi's model) were used to predict the drug release from the Panobinostat-AgNPs.

3.8. Cell culture

The breast cancer cell line (MDA-231) was bought. Cells were cultured in high glucose DMEM medium with 1% penicillin/streptomycin (W/V) and FBS (V/V). The cells were cultured in the air that was 95% humidified and contained 5% CO_2.

3.8.1. MTT assay

The MTT assay was used to determine the viability of the MDA-231 cell line, which turns live, metabolically active cells into purple formazan. Following overnight culture, the cells were exposed to various concentrations ((0, 20, 30, 50, 100, and 150 nM) of Panobinostat and Panobinostat-AgNPs in freshly produced PBS media. The plates were then incubated at 37 °C in a humidified incubator with 5% CO_2 for 72 hours. After incubation, 20 µL of MTT was put into each well, and the plates continued a further 3 hours of incubation. The excess MTT and dead cells were then removed from the solution in each well, and 100 µL of dimethyl sulfoxide reagent was added to the cells to prevent the conversion. The number of healthy cells following treatment is directly proportional to the amount of formazan produced. To calculate the percentage of cell viability, the absorbance was determined at 570 nm using a microplate reader, with 630 nm serving as the reference wavelength. The data obtained were

averaged and fitted to Equation. Control cells were grown cells that hadn't been treated. The results of the triple experiment were expressed as a percentage of cell viability compared to control cells.

$$Percentage\ cell\ viability = \frac{Value\ of\ OD\ (treatment)}{Value\ of\ OD\ (control)} \times 100$$

OD represents optical density.

CHAPTER 4

4. RESULTS

4.1. Characterization:

4.1.1. Visual Representation

Initial visual confirmation of the green synthesis of silver nanoparticles using *Rhazya stricta* extract. The extract was then subjected to a silver ion solution, resulting in the formation of silver nanoparticles. The color change of the reaction mixture from pale yellow to brown, which shows the creation of silver nanoparticles, occurred initially. The reaction mixture turned dark brown after an hour as shown in figure 13, indicating that the reduction of the silver ion process was successful. The surface Plasmon resonance excitation effect is connected to the various colors of silver nanoparticles. Similarly, produced nanoparticles remained stable for 60 days without aggregating.

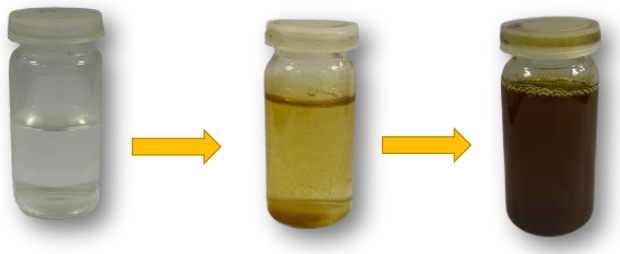

Figure 13: Visual observation of silver nanoparticles synthesis

4.1.2. UV

The primary analysis of silver nanoparticle synthesis and drug loading onto AgNPs was conducted using UV-Vis spectrophotometric analysis. Investigations were made into the solution's absorbance. The largest peak for the AgNPs peak was produced at 420 nm as shown in figure 14 (A), according to the spectral analysis. Beer's law was further demonstrated by measuring the concentrations of five different nanoparticles in methanol using a spectrophotometer; the resulting linear dependency is illustrated in Figure 14 (B). A linear curve of absorbance and concentration was drawn from these absorption spectra, demonstrating the direct proportionality between absorbance and concentration.

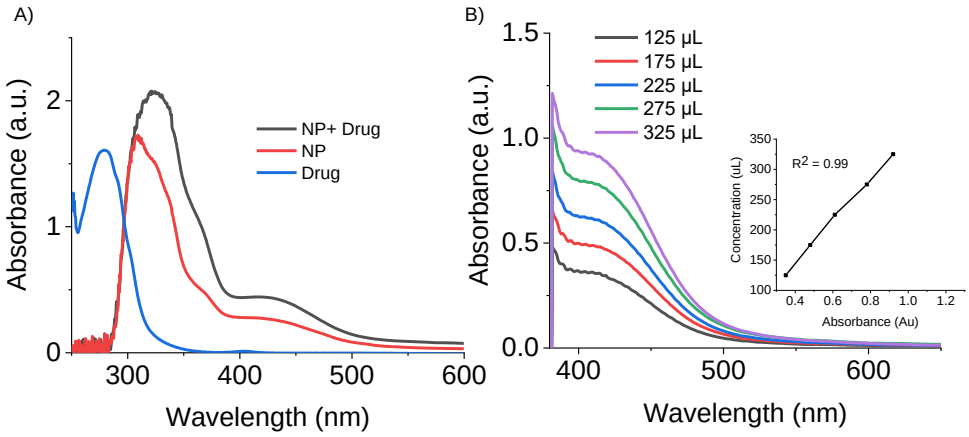

Figure 14: (A) UV spectrum of nanoparticles, drug, and drug-loaded nanoparticles, (B) UV spectrum of silver nanoparticles at different concentrations

4.1.3. XRD

X-ray diffraction (XRD) was used to assess the crystallinity of the produced silver nanoparticle, drug, and drug-loaded nanoparticles as shown in Figure 15. The Debye-Scherrer equation $D = k\lambda/\beta\cos\theta$was used to determine the nanoparticles' sizes. It was discovered that the typical crystalline size was about 20 nm.

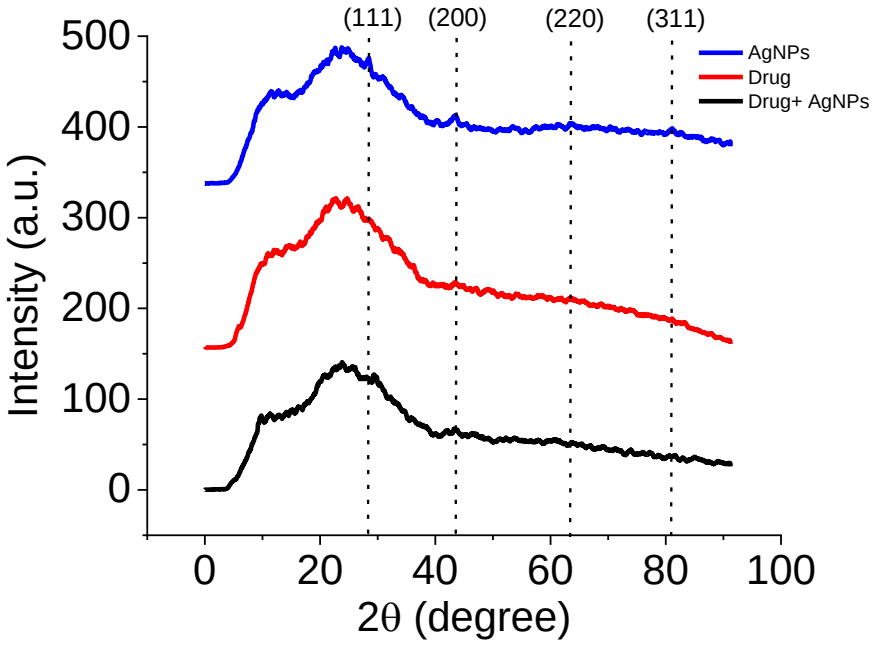

Figure 15: XRD analysis of AgNPs, Panobinostat, and Panobinostat-AgNPs

4.1.4. FTIR

The phytochemicals in Rhazya Stricta extract that are a catalyst for reducing silver ions to silver nanoparticles were found using an FTIR analysis in the current experiment. The figure shows the FTIR of the extract of Rhazya

stricta, AgNPs, free panobinostat, and panobinostat-AgNPs. The effective conjugation of Panobinostat on AgNPs was demonstrated by FTIR findings. The characteristic bands are located at 3419, 2989, 2050, 1626, 1012, and 696 on the spectrum, shown in figure 16.

The peak at 3410 in the Rhazya Stricta extract indicates the stretching of the amine group's N-H bond. Peak 2859 demonstrates the stretching of the methyl group's C-H bond, Peak 1625 the alkene group's C=C bond, Peak 1012 the C=O bond, and Peak 696 the ring of an out-of-plane aromatic band.

Silver nanoparticles demonstrated that the AgNPs' C=O symmetric stretching caused the peak at 1626 cm^{-1}.

The band at 3410 cm^{-1} demonstrated the stretching of the Panobinostat's N-H bond. The C=C and C=O vibrations are represented by the absorption bands at 1626 cm^{-1} and 1012 cm^{-1}, respectively, while the C-H stretching vibration is represented by the band at 2989 cm^{-1}. The bands in the Panobinostat-AgNPs related to the stretching vibration of the O-H group at 3350 cm^{-1}, while the band at 1626 cm^{-1} indicated strong C=C stretching. To show the conjugation of Panobinostat on AgNPs, the stretching bands at the N-H bond of the Panobinostat, which were previously at 3419 cm^{-1}, were relocated to 3442 cm^{-1}.

The drug has been linked to the surface of AgNPs as seen by the widening and shifting to higher wavelengths of the corresponding N-H peaks.

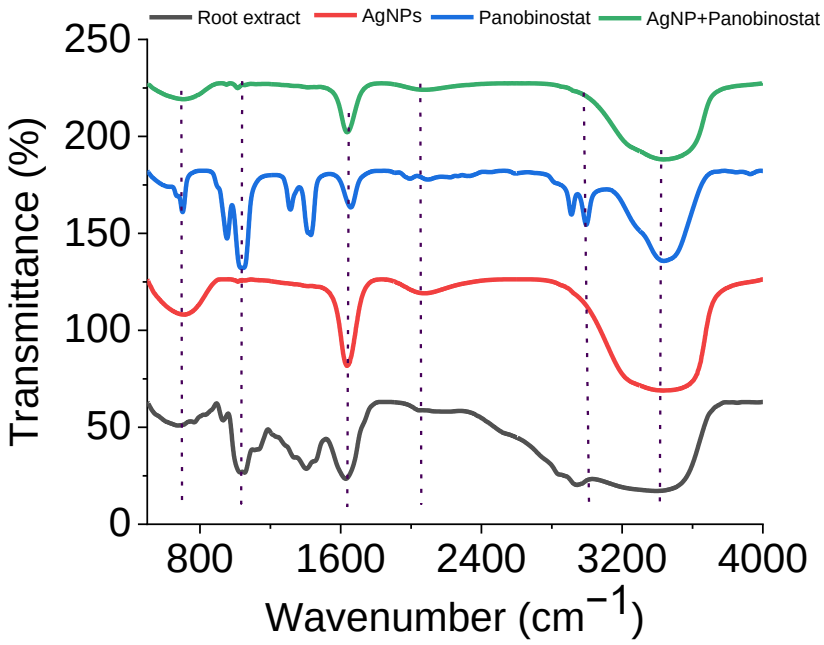

Figure 16: FTIR spectrum of plant extract, AgNPs, Panobinostat, and Panobinostat-AgNPs

Table 1: Functional groups attached with plant extract, AgNPs, Panobinostat and Panobinostat-AgNPs

Serial No.	Wavenumber (cm^{-1})	Bond	Functional Group
1.	3296	N–H stretching	Secondary amine
2.	2989	C-H stretching	Alkane
3.	2124	C≡C stretching	Alkyne
4.	1635	C=C stretching	Alkene
5.	1390	C-H bending	Aldehyde
6.	1236	C–O stretching	Aromatic ester
7.	1069	CO-O-CO stretching	Anhydride
8.	601	Ring	

4.1.5. SEM

SEM images were used to determine the morphological characteristics and size information of the synthesized Silver nanoparticle and Panobinostat-AgNPs. The SEM analysis revealed that the nanoparticles were between 20 and 30 nm in size. The presence of proteins and other molecules from the extract that were bound to the surface of the nanoparticles is what causes the size variation in the particles. Additionally, the outcome demonstrated that the produced silver nanoparticles had spherical shapes. The figure 17 shows that there is no agglomeration and that the produced silver nanoparticles are well separated.

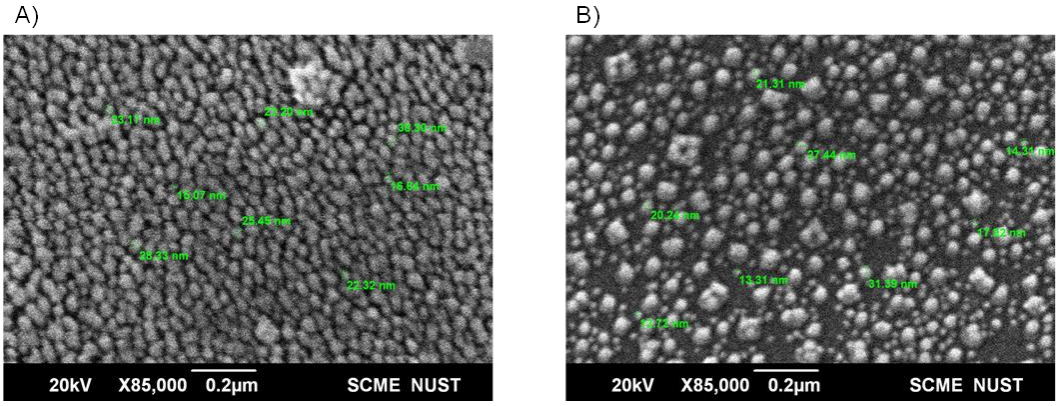

Figure 17: SEM analysis of AgNPs and Panobinostat-AgNPs

4.1.6. EDX

The silver nanoparticles' and drug-loaded nanoparticles' composition was visible in the spectrum obtained by Energy Dispersive X-ray. In the figure 18, different elemental compositions of Ag, N, and O are shown at varying binding energies. Biomolecules contained these substances, which were in charge of encapsulating nanoparticles. It was confirmed that by utilizing plant extract, silver nanoparticles were generated. At different degrees of absorption energy, additional minor peaks for Na, Mg, Si, K, and Ca were seen. Due to the ingredients of phytochemicals' molecular structure, they may result from the extract. These components play a role in the stabilization and capping agent on nanoparticles' surfaces.

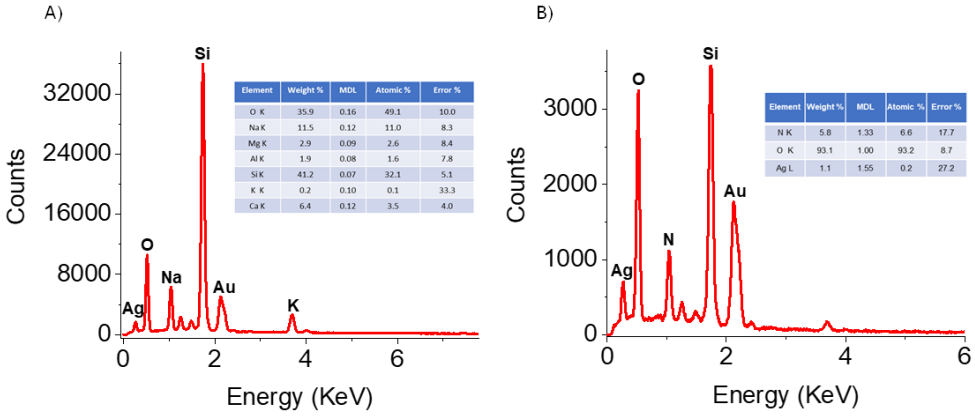

Figure 18: Elemental analysis of (A) AgNPs and (B) Panobinostat-AgNPs

4.2. Drug loading and Drug release

The fundamental principle of drug loading on silver nanoparticles is surface adsorption. The percentage of drug loading was 56% when 0.5 mg of medication

was added to AgNPs. The dialysis bag was immersed in phosphate buffer pH 7 for the drug release investigation to assess the release of panobinostat from NPs. Fig. 19 displays the percentage of cumulative drug release at intervals of 30 minutes and displays the time-dependent mean of the percentage of drug release. Since the drug is released rapidly in the early stage of drug release, it may be essential for the treatment of a condition, and the slow-release phase may solidify the therapeutic effect. The role of drugs in the therapeutic process will increase after two rounds of releasing.

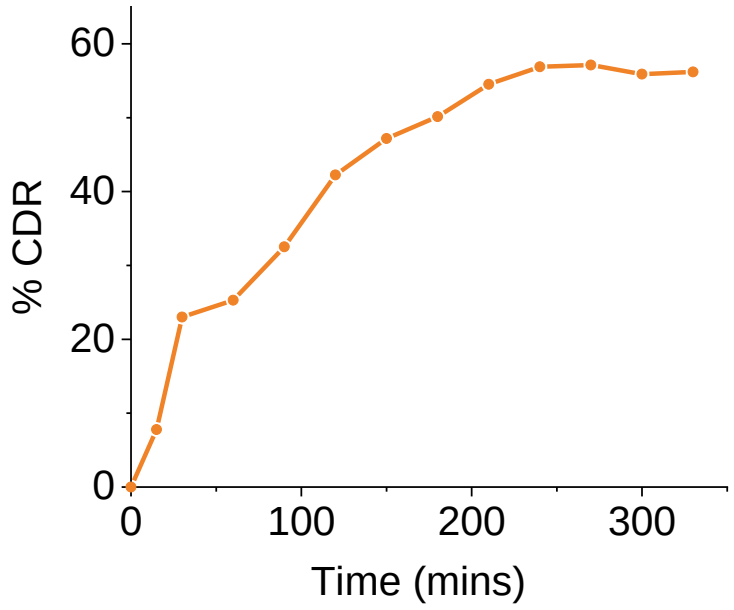

Figure 19: Percentage cumulative drug release of Panobinostat from silver nanoparticles

4.2.1. Drug release kinetics

Based on the data of the cumulative % of drug release at pH 7.4 in the specified time intervals, as shown in figure 20, various kinetic models were

used to develop the mechanism of drug release. The Higuchi square root of time equation (Diffusion model) was considered the best in comparison to all kinetic models at pH 7.4 because their R^2 values were very close to 1 and demonstrated drug release by a straightforward diffusion method. The rate of drug release (R^2) and k (slope) values calculated for all kinetic models was displayed in Table 2. Because the proposed framework of the nanoparticles is stable at this pH, the drug release was rather slow. These findings are in line with earlier studies that looked at drug release from nanoparticles.

According to first-order release kinetics, the amount of drug still inside the particles will always be proportional to the instant rate of release of the drug from the delivery system at any time t.

According to the Higuchi model, which is based on the Fickian diffusion Equation, the release of medicines from an insoluble matrix is a square root of a time-dependent process. $Q = K_H\ t^{1/2}$. The data were plotted against the square root of time as the cumulative percentage of drug release.

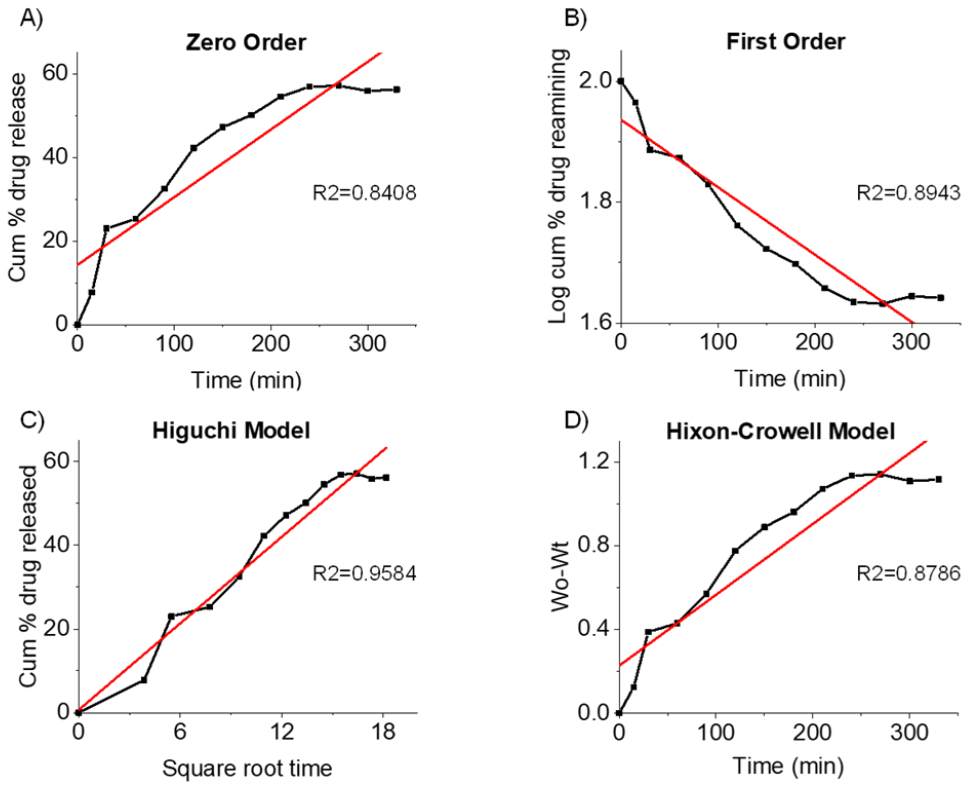

Figure 20: Drug release kinetic models (A) zero order (B) first order (C) Higuchi Model (D) Hixon-crowell method

Table 2: Drug release kinetic model equations

Model name	**Equation**	**R^2**
Zero-order	$Q = Q + k\,t$	0.8408
First-order	$\log Q = \log Q_0 - kt/2.303$	0.8943
Higuchi	$Q = k\,t_{½}$	0.9584
Hixon- Crowell	$Q_0\ 1/3 - Q_t\ 1/3 = kt$	0.8786

4.3. In-vitro Analysis

MTT assay was used to test the silver nanoparticles' cytotoxicity. It is a colorimetric assay that assesses how the enzyme mitochondrial succinate dehydrogenase reduces the yellow color of MTT to generate purple-blue formazan. Based on the ability of cancer cells to survive, the impact of silver nanoparticles was examined (breast cancer cell line). To control human breast cancer, Panobinostat-silver nanoparticles lower the viability of MDA-231 cells in a dose-dependent manner shown in figure 21.

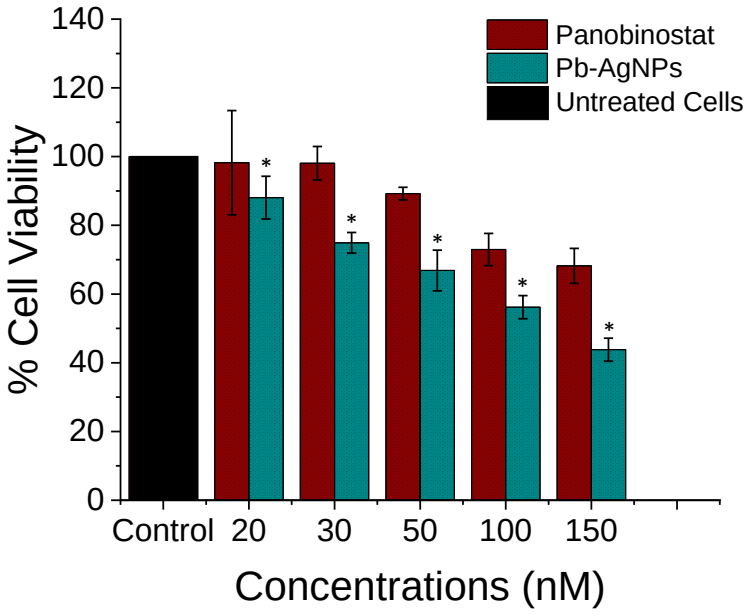

Figure 21: Cell viability of Panobinostat and Panobinostat-AgNPs against human breast cancer cell lines MDA-231 at 24h incubation.

CHAPTER 5

DISCUSSION

Histone deacetylase inhibitors (HDACi) stop histone deacetylases from removing the acetyl group from the lysine amino acid of histone and open the chromatin structure to permit access to transcription factors and gene transcription, hence controlling cell proliferation. In line with its exceptionally potent HDAC inhibition, Panobinostat, a novel class of drug called histone deacetylase inhibitors, exhibits significant antiproliferative and cytotoxic activity in a variety of cancer cell lines despite causing just minor damage to all normal cells tested. However, it has been noted that the majority of Panobinostat toxicity is gastrointestinal and hematological. The most frequent severe adverse effects are hematologic and include thrombocytopenia, anemia, and neutropenia. Due to their retention and capacity to penetrate tumor cells, silver nanoparticles are potent drug carriers that can enhance the anti-cancerous effects of nanoparticles that have been loaded as well as lower the dosage needed to achieve therapeutic benefits. AgNPs made by green synthesis could greatly solve the issue of employing chemicals that have several negative impacts. As a result, the green synthesis of nanoparticles is an environmentally beneficial method.

The antibacterial activity of R. stricta plant roots has been significantly increased by NP inclusion. The stability of the produced AgNPs was supported by the characterization methods. In experiments, xylitol was unable to reduce silver ions, but it may have contributed to the stabilization of silver nanoparticles by capping synthesized AgNPs through electrostatic attraction or hydrogen bonding with protein groups in the extract. It also prevented the NPs from clumping together. When R. stricta root extract is added, the

mixture's color changes from pale yellow to brown, signaling the beginning of the AgNPs' synthesis (fig. 13). AgNPs in aqueous solution has been observed to be yellowish brown in appearance due to surface Plasmon resonance excitation [60]. The surface Plasmon vibration proved that silver particles had biotransformed from an ionic form [61]. The color of the aqueous solution containing AgNO3 and xylitol changed from pale yellow to brown upon the addition of R. stricta root extract due to the decrease of silver ions. The peak intensity of the AgNPs increased with the addition of Panobinostat, as seen by the UV-Vis spectra of Panobinostat-AgNPs (2 mM). These alterations demonstrated panobinostat loading onto the AgNPs (fig. 14). Recent XRD results of produced AgNPs with R. stricta root extract validated the AgNPs' FCC structure and crystalline character [62]. The crystallinity of the AgNPs, Drug, and Drug loaded AgNPs samples was verified by XRD analysis. The crystalline nature of the samples is determined by measuring the XRD from 0° to 100°. The figure 15 gives the 2 angles and associated hkl planes. They were indexed to the crystalline samples with FCC structure standard planes of the “Joint Committee on Powder Diffraction Standards” (JCPDS).The (200) orientation is the one that is best for the creation of AgNPs [63]. Other peaks could be caused by the bioorganic substances employed to make Ag NPs. The sizes of the nanoparticles were calculated using the Debye-Scherrer equation (D = k/cos). The average crystalline size was found to be around 20 nm.

As seen in Figure 16, peaks were slightly shifted to a higher wavenumber following the production of AgNPs compared to plant extract. Although it was employed to cap or coat the molecules, xylitol did not diminish the silver ions in the solution. AgNPs were attracted to protein groups by electrostatic forces or formed hydrogen bonds with them,

both of which contributed to the NPs' increased stability and improved dispersion. The presence of carboxyl (-C=O), hydroxyl (-OH), and amine (N-H) groups in the R. stricta roots extract was confirmed by the FTIR spectrum, which also demonstrated their role in the reduction of silver ion to metallic AgNPs [64]. Proteins found in plant extracts have carboxyl or amino groups that cap the AgNPs [60]. It is thought that the free amino or carboxyl groups in the proteins of the root extract of R. stricta could attach to AgNPs [64]. Because of the similarity of the spectra and the minor peak position shifts, the leftover plant extract is present in the sample [65]. These findings thus demonstrate the critical role that the functional groups of compounds found in R. stricta root extract play in both the reduction and capping of AgNPs. The band at 3410 cm^{-1} demonstrated how the Panobinostat's N-H bond was stretched. The absorption band at 2989 cm^{-1} was attributed to the C-H stretching vibration, whereas the absorption bands at 1626 cm^{-1} and 1012 cm^{-1} stand for C=C and C=O, respectively. The bands in the Panobinostat-AgNPs were at 3350 cm^{-1}, which matched the strong stretching vibration of the O-H group, and at 1626 cm^{-1}, which matched the strong C=C stretching. To show conjugation of Panobinostat on AgNPs, the stretching bands at 3419 $cm^{-1}$of the Panobinostat's N-H bond migrated to 3442 cm^{-1} for the Panobinostat-AgNPs. The associated N-H peaks' broadening and shift to higher wavelengths served as evidence that the drug had bound to the surface of AgNPs [66].

SEM images acquired at 10 kV revealed the morphological properties and size details of the produced silver nanoparticles and the drug-loaded silver nanoparticles. Nanoparticle sizes in the 20-nm range were visible in SEM pictures (Figure 17). The outcome shows that the produced AgNPs are spherical. The obvious silver signal and weak oxygen and

chlorine peaks in the EDX spectrum (fig. 18) could be the result of biomolecules interacting with and adhering to the surface of AgNPs.

UV-Vis spectroscopy and FTIR were used to determine the drug's adsorption or loading on the NP. This is where the encapsulation efficiency, which measures the quantity of medication integrated into a particle at a given concentration, may be useful. The basic idea behind medication loading on silver nanoparticles is surface adsorption. Drug loading was 56% when 0.5 mg was added to silver nanoparticles. In Fig 19, which displays the cumulative % drug release in phosphate buffer pH 7.4 at intervals of 30 minutes, the time-dependent mean of percentage drug release is displayed. Since the medicine is released quickly in the first stage of drug delivery, it may be essential for the treatment of a condition, and the slow-release phase may solidify the therapeutic effect. The role of medications in the healing process will increase after two rounds of discharge [67]. As a result, increased Panobinostat loading capacity and encapsulation efficiency were seen.

The in vitro drug release in the current investigation was evaluated using a variety of kinetic models, including the zero-order, first-order, Hixon- Crowell, and Higuchi models (Fig. 20). The release of Panobinostat from the Panobinostat-AgNPs was observed as a first-order release. This demonstrates that the drug concentration directly influenced the release of Panobinostat from Panobinostat-AgNPs. Overall, the release studies showed that Panobinostat-AgNPs are dependent on particle size. Drugs are slowly released from smaller particles.

First, the ideal Panobinostat and Panobinostat-AgNPs concentrations for the best possible combined effect were chosen. Additionally, MDA-231 cells were treated with various

combinations of Panobinostat and Panobinostat-AgNPs to minimize unintended harmful effects. In MDA-231 cells, the effects of adding Panobinostat and Panobinostat-AgNPs at doses of 20, 30, 50, 100, and 150 nM were investigated. The findings demonstrate that increasing concentrations of Panobinostat or Panobinostat-AgNPs both significantly lower cell viability; however, at low concentrations of the combination of Panobinostat-AgNPs, substantially lower cell viability than higher concentrations of individual Panobinostat was observed, demonstrating an effective effect (Fig. 21).

This suggests that rather than utilizing greater quantities to cause cell death in MDA-231 cells, a lower concentration of Panobinostat and Panobinostat-AgNPs is needed to provide a synergistic effect. A physiologically tolerable concentration of Panobinostat and Panobinostat-AgNPs is attained at low concentrations. Figure 21 demonstrates that compared to Panobinostat used alone, Panobinostat-AgNPs exhibited a more substantial concentration-dependent inhibitory effect on MDA-231 cells. The combined effect was greatly increased. As a result, more combination tests were run. Our results are in line with a prior study, which showed that AgNPs might prevent the survival of a range of cancer cells, including human lung cancer (A549) and human breast cancer cells (MCF-7) [68].

This study reported the effectiveness of Panobinostat-AgNPs as an effective candidate for increasing the cell toxicity of cancerous cells when applied on breast cancer cell lines MDA-231.

CONCLUSION

It is concluded that green synthesized silver nanoparticles through *Rhazya stricta* plant extract proved beneficial as a candidate molecule for Panobinostat loading and showed effective cell viability. The characterization of blank nanoparticles and drug-loaded nanoparticles through UV, FTIR, XRD, SEM, and EDX showed the successful synthesis of nanoparticles and the conjugation of drugs with nanoparticles. The AgNPs had an average size of 20 nm and were stable over a period of time. The evaluation of drug encapsulation effectiveness and drug release capacity revealed 56% encapsulation efficiency and sustainable drug release in 6 hours. The kinetics study of drug release showed the first order reaction which means that drug concentration is proportional to drug release. The MTT assay showed that AgNPs had a potent, dose-dependent, and time-dependent anticancer activity on the cancer cell lines i.e. breast (MDA-231), which was more effective than the majority of other reported similar cases. The overall findings demonstrated that Rhazya stricta's organic compounds when used as suitable reducing and stabilizing agents, not only had a significant impact on the physicochemical characteristics of AgNPs but also facilitated the green synthesis of a potent anticancer agent with a high potential for sustained release of Panobinostat for cancer therapy. Further in vivo analysis is required.

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