

EVALUATING THE DIFFERENTIAL PATTERNS OF THE EPIGENETIC BASIS OF PHYTOCHEMICALS AND THEIR ANTI-CANCER ACTIVITY IN CERVICAL CANCER

**Thesis Submitted for the Award of the Degree of
DOCTOR OF PHILOSOPHY**

in

Molecular Biology and Genetic Engineering

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2025

CERTIFICATE

This is to certify that the work reported in the Ph. D. Thesis entitled “**Evaluating the differential patterns of the epigenetic basis of phytochemicals and theirS anti-cancer activity in cervical cancer**” submitted in fulfillment of the requirement for the award of degree of **Doctor of Philosophy (Ph.D.)** in the Department of Molecular Biology and Genetic Engineering, is a research work carried out by **Satbir Kour, Registration No.41800860**, is bonafide record of her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.



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DECLARATION

I hereby declare that the work presented in the thesis entitled “Evaluating the differential patterns of the epigenetic basis of phytochemicals and its anti-cancer activity in cervical cancer” for the degree of Doctor of Philosophy (Ph.D.) is the result of my research under the supervision of Dr. Anjuvan Singh (Administrative Supervisor), Professor in the Department of Biotechnology, School of Bio Engineering and Biosciences, Lovely Professional University, Punjab, India and Dr. Sugunakar Vure (Executive Supervisor), Senior Scientist & PI from IACRF, BIACH & RI, Hyderabad, India. In accordance with standard scientific reporting practices, due acknowledgements have been made whenever the work described is based on the findings of other researchers. This work has not been submitted wholly or partly to any other university or institute for the award of any degree.



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Dedicated to my Grand Parents
Sardarni Kaiser Kour
and
Sardar Sucha Singh ji Pardesi

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ABBREVIATIONS

ADME	Adsorption, Distribution, Metabolism, and Excretion
AgBE	Silver nitrate- Betulin
AgSH-IV	Silvernitrate-Shatavarin-IV
ASCUS	Atypical Squamous Cells of Unknown Significance
ATP	Adenosine 5'-triphosphate
BC	Betweenness Centrality
BE	Betulin
cAMP	Cyclic adenosine monophosphate (cAMP)
CC	Closeness Centrality
(CCa)	Cervical Cancer
cDNA	Complementary DNA
CESC	Cervical squamous cell carcinoma
CIN	Cervical intraepithelial neoplasia
DC	Degree Centrality
DEG's	Differentially expressed genes
DFT	Density Function Theory
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNMT3A	DNA methyltransferase
DSC	Differential scanning calorimetry
EDS	Energy Dispersive X-ray Spectroscopy
EDTA	Ethylenediaminetetraacetic acid
EMT	Epithelial-mesenchymal transition
ETBR	Ethidium bromide
FBS	Fetal bovine Serum
FDA	Food and Drug Administration
FESEM	Field Emission Scanning Electron Microscopy
FIGO	International Federation of Obstetrics and Gynecology

FTIR	Fourier-transform infrared spectroscopy
GEPIA	Gene expression profiling interactive analysis
GO	Gene ontology
GWAS	Gene-wide association studies
HAT	Histone Acetyltransferase
HDAC4	Histone Deacetylase 4
HEK	Human embryonic kidney cells
HIV	Human Immunodeficiency Virus
HPSGs	Heparan sulphate proteoglycans
HPV	Human papillomavirus
hr-HPV	high-risk Human Papillomavirus
HSIL	High squamous intraepithelial lesions
IC50	Half maximal inhibitory c
KEGG	Kyoto Encyclopedia of Genes and Genomes
KMT2C	Histone-Lysine N-Methyltransferase 2C
LCR	Long control region
Lr-HPV	low-risk Human Papillomavirus
LSIL	Low squamous intraepithelial lesions
MBNC	Metal-Betulin nano complex
MM-GBSA	Molecular Mechanics with Generalized Born and Surface Area Solvation
MSNC	Metal-Shatavarin-IV nano complex
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NC	Nitrocellulose membrane
OPLS	Optimized Potentials for Liquid Simulation
ORFs	Open reading frames
Pap	Papanicolaou
PBS	Phosphate-buffered saline
PCR	Poly Chain Reaction
PDB	Protein Data Bank
P-gp	P-glycoprotein

PPI	Protein-Protein Interaction
PVDF	Poly (vinylidene fluoride)
RMSD	Root Mean square deviation
RMSF	Root Mean square fluctuation
RNA	Ribonucleic acid
ROC	Receiver operator characteristic curve
ROS	Reactive oxygen species
RT-PCR	Reverse Transcription Poly Chain Reaction
SASA	Solvent Accessibility Surface Area
SCC	Squamous Cervical Carcinoma
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SH-IV	Shatavarin-IV
SiHa	Human Cervical Squamous Cell Carcinoma cell line
SIL	Squamous intraepithelial lesion
SNP	Single-nucleotide polymorphisms
SRB	Sulforhodamine B
TBE	Tris Borate EDTA buffer
TBS	Tris-buffered saline
TCGA	The Cancer Genome Atlas
TGS	Tumor Suppressor Genes
WHO	World Health Organization
XRD	X-ray Diffraction
ZnBE	Zinc Oxide-Betulin
ZnSH-IV	Zinc Oxide- Shatavarin-IV

Abstract:

Cervical cancer (CCa) remains a leading cause of death among women worldwide. While various treatment options exist, including agonists and antagonists, they often cause unwanted side effects. Moreover, resistance to these therapies frequently leads to disease recurrence. To address these issues, we conducted a combined computational and experimental study to evaluate the potential of triterpenoids in treating cervical cancer. Triterpenoids, naturally occurring secondary metabolites, are known for their broad biological activities, including anti-cancer effects. They have shown promise in targeting specific epigenetic marker sites, making them potential candidates for cancer therapy. Our research involved obtaining three-dimensional crystal structures of epigenetic proteins DNMT3A, HDAC4, and KMT2C from the Protein Data Bank (PDB). We sourced the structures of selected triterpenoids from PubChem and ZINC databases for analysis. Our results showed that betulin (BE) and shatavarin-IV (SH-IV) exhibited the most significant binding energy when interacting with target proteins. We applied Lipinski's Rule of Five and performed density functional theory (DFT) calculations to evaluate the pharmacokinetic properties of these triterpenoids. Additionally, 500 ns molecular dynamics simulations indicated stable binding of these compounds to the DNMT3A, HDAC4, and KMT2C sites. Incorporating nanotechnology into our research presents an innovative approach for developing drug delivery systems that reduce toxicity and enhance therapeutic efficacy. Self-assembled nanomaterials can greatly improve drug delivery and bioimaging applications. Metal nanoparticles show promise in various therapeutic areas, including cancer treatment, bacterial control, antioxidant activity, diabetes management, and inflammation reduction. The field of cancer nanotechnology is notable for its diverse applications in drug delivery, diagnostics, imaging, and advanced medical equipment. Nanoscale molecular tools can improve the specificity and effectiveness of cancer therapies while minimizing harm to healthy cells. Our research employed *in silico* and *in vitro* methods to assess anticancer properties. Molecular docking analysis revealed that BE and SH-IV effectively inhibited epigenetic targets DNMT3A, HDAC4, and KMT2C. Further evaluation of their drug-like properties, including ADME (Absorption, Distribution, Metabolism, and Excretion), indicated compliance with Lipinski's rules. Importantly, our findings showed that BE and SH-IV inhibit P-glycoprotein (P-gp), a key liver efflux pump involved in drug resistance. P-gp inhibition by SH-IV suggests an increased half-life and high bioavailability, potentially boosting therapeutic efficacy. This study also involved producing metallic nanoparticles, specifically zinc oxide and silver nitrate, using chemical methods. The

study focused on the synthesis and characterization of nanoparticles combining betulin extract (BE) and SH-IV with zinc oxide and silver. Spectroscopic analysis showed that BE-zinc nanoparticles exhibited absorption peaks linked to C-H stretching vibrations, while zinc oxide nanoparticles were identified within a specific spectral range. BE-silver nanoparticles also showed distinct spectral features. X-ray diffraction (XRD) and Field Emission Scanning Electron Microscopy (FESEM) analyses revealed that the particles had a clustered, cubic morphology.

Elemental analysis indicated the presence of zinc oxide, zinc, silver, and oxygen in varying proportions in the synthesized zinc oxide-BE nanoparticles. Fourier-transform infrared (FT-IR) spectra of both SH-zinc oxide and SH-silver nitrate nanoparticles displayed peaks corresponding to mono-substituted benzene rings and confirmed the presence of zinc oxide in the expected range. SH-silver nanoparticles exhibited multiple characteristic absorption bands, and their crystalline nature was confirmed by sharp peaks in XRD patterns.

The findings also showed that betulin was successfully encapsulated within the zinc and silver nanoparticles. Morphological studies and particle size analysis were conducted using FESEM and Energy-Dispersive X-ray (EDX) analysis. Variations in synthesis conditions—such as stirring, heating, and alkaline environments—affected particle size, with heating resulting in larger particles. Additionally, Energy-Dispersive X-ray Fluorescence (EDXRF) analysis of silver nitrate nanoparticles revealed lower levels of impurities.

To evaluate the biological effectiveness of the synthesized nanoparticles, their activity was tested on HeLa, SiHa, and HEK cell lines. Sulforhodamine B (SRB) assays demonstrated that both the pure compounds and their nanoparticle forms exhibited anticancer properties, with some formulations showing enhanced potency. Further molecular analysis using RT-PCR and Western blot techniques in treated SiHa cells assessed the effect of these treatments on HDAC4 gene expression.

Our research suggests that epigenetic modulation results in reduced HDAC4 expression. We also conducted an extensive network pharmacology analysis of triterpenoids to uncover the scientific principles underlying intelligent formulations. This investigation aimed to explore the relationships between bioactive compounds and their molecular targets, with a focus on cervical cancer. The novel AgNPs of Shatavarin-IV, which we successfully identified, may interact with and occupy the binding sites of target proteins.

Our work supports the finding that both protein and RNA levels of HDAC4 are elevated in cervical cancer cells. AgNPs effectively suppressed HDAC4 expression and inhibited the growth of SiHa cells in a dose-dependent manner. Our research suggests that triterpenoids, particularly Shatavarin-IV, show considerable potential as therapeutic agents for cervical cancer, especially when combined with innovative nanotechnology approaches. We conclude that Shatavarin-IV AgSH-IV nanoparticles may serve as promising compounds against various cancers, specifically cervical cancer. This study also provides a foundation for identifying novel molecules as emerging drugs to extend the life expectancy and improve the quality of life for cervical cancer survivors.

Chapter 1

1. Introduction

1.1 Cancer

Cancer encompasses a spectrum of disorders defined by dysregulated cellular proliferation and abnormal growth of transformed cells, which possess the capacity to invade distant tissues and, if untreated, lead to mortality. Perturbations in critical regulatory genes can disrupt normal control of the cell cycle or impair physiological mechanisms such as programmed cell death (apoptosis) (O'Connor CM et al., 2010). It represents a highly heterogeneous disease entity, originating from diverse tissue lineages and exhibiting extensive genetic and molecular variability. Cancer is often described as a "renegade" biological system of unrestrained growth arising within an organism, characterized by complex etiological and biological diversity.

Tumorigenesis is widely recognized as a multistep process in which progressive alterations in growth regulatory pathways facilitate the transformation of normal somatic cells into malignant phenotypes. This process involves the accumulation of genetic mutations, transcriptional and translational dysregulation, and aberrant post-translational modifications. These molecular aberrations not only drive neoplastic progression but also serve as critical diagnostic and prognostic biomarkers (Bruno PS et al., 2025).

Understanding these molecular mechanisms is crucial for identifying novel therapeutic targets and biomarkers, thereby enabling the development of precision medicine approaches. Advances in molecular oncology have revealed that cancer initiation and progression are closely linked to dysregulated signaling pathways, epigenetic modifications, and altered gene expression profiles, highlighting the need for integrated biomarker-driven strategies in early diagnosis and targeted therapy.

1.2 Cervical Cancer (CCa)

The development of CCa is significantly influenced by the human papillomavirus (HPV) infection (Fernandes JV et al., 2015), a DNA virus that infects the epithelial cells of the skin and mucosa, and it is primarily transmitted through sexual contact (MA. Stanley et al., 2012). The human papillomavirus (HPV) has more than 100 genotypes, which are further categorised as high-risk (Hr) and low-risk (LR). HPV strains like 16, 18, 31, 33, 35, 45, 52, 58, and 59

result in the development of cancerous tumors in the cervix, vulva, and head and neck (Paz-Zulueta, M et al., 2018). These HPV strains are regarded as high-risk. The low-risk variants of the HPV, particularly HPV 6, 11, 40, 42, 43, 44, 53, 54, 61, 72, 73, and 81, are responsible for benign mucosal lesions such as verrucae, genital warts, and condylomas (Chan PK et al., 2002).

Seven genes comprise the HPV genome: two late-transcribed genes, L1 and L2, which encode structural viral capsid proteins, and six early transcribed genes, E1, E2, E4, E5, E6, and E7 (Della Fera et al., 2021). Since the carcinogenic nature of HPV infection generally depends on the presence of viral oncogenes like E6 and E7, it is dependent on their activity. It is estimated that more than seventy-one percent of instances of CCa are caused by two strains of the virus: HPV-16 and HPV-18. Only about ten percent of women continue to be infected for longer periods (Hareza DA et al. in 2022). The enhanced expression of HPV E6 and E7 oncoproteins, as well as the deactivation of two key tumor suppressor pathways, p53 and Rb, will result in genomic instability. These precancerous stages of HPV infections can lead to invasive CCa (Chen R et al., 2021). This implies that nearly ninety percent of HPV infections become inactive or clear within two years of infection (Figure 1).

High-grade intraepithelial lesions (CIN2/CIN3) and low-grade squamous intraepithelial lesions (CIN1) are known as HSIL and LSIL, respectively. Low-grade squamous intraepithelial lesions (CIN1) and high-grade intraepithelial lesions (CIN2/CIN3) are the names given to these kinds of lesions, respectively (Mills AM et al., 2017). Squamous cervical carcinoma (SCC) that is HPV-negative is incredibly uncommon, does not spread through intercourse, and does not depend on the presence of the human papillomavirus (HPV) (Yoshida H et al., 2021).

normal cell cycle control and apoptosis, allowing for unchecked cellular proliferation (Su PH et al., 2022).

Global Hypomethylation: On a genomic scale, CCa cells exhibit a loss of DNA methylation (hypomethylation), which can lead to chromosomal instability, activation of oncogenes, and overall genomic instability, fuelling the cancer's progression (Reis AH et al., 2016).

Role of HPV in DNA Methylation: Human papillomavirus (HPV), particularly high-risk types like HPV16 and HPV18, is known to influence DNA methylation patterns in infected cells. The viral oncoproteins E6 and E7 interact with and disrupt cellular proteins, including DNA methyltransferases (DNMTs), which can drive abnormal methylation patterns and silencing of TSGs (Chen B et al., 2023).

1.2.2 Histone Modifications

Histones are proteins around which DNA is wrapped, and their modification affects chromatin structure and gene expression. The primary types of histone modifications associated with CCa include acetylation, methylation, phosphorylation, and ubiquitination (Gagnidze K et al., 2022).

Histone Methylation: Methylation of histones can either activate or repress gene expression, depending on the specific histone and lysine residue modified. In CCa, alterations in histone methylation patterns, particularly H3K27me3 (trimethylation of histone H3 at lysine 27), have been associated with poor prognosis and tumor progression. These modifications disrupt cellular processes such as apoptosis and cell cycle regulation, favouring tumor growth (Murr R et al., 2010).

1.2.3. Non-Coding RNAs (ncRNAs)

Non-coding RNAs, particularly microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), play a regulatory role in CCa by modulating gene expression at the post-transcriptional level (Srijyothi L et al., 2018).

MicroRNAs (miRNAs): Certain miRNAs act as tumor suppressors or oncogenes in CCa. For example, miR-34a is known to target HPV-E6 mRNA, leading to the reactivation of p53, a key tumor suppressor protein. However, in many CCa cases, miR-34a is downregulated, which

allows for continued E6 expression and degradation of p53, promoting cell survival and proliferation (Delman, M et al., 2017)

Long non-coding RNAs (lncRNAs): LncRNAs such as *HOTAIR* and *MALAT1* are often upregulated in CCa and have been linked to metastasis, chemoresistance, and poor outcomes (Braga EA et al, 2020). They can interact with chromatin-modifying complexes, influencing the epigenetic landscape by altering histone modifications and affecting TSG expression.

1.3 Epigenetic Therapeutics in Cervical Cancer

Given the reversible nature of epigenetic modifications, targeting these processes has become a promising therapeutic approach for CCa. Some possible strategies include: DNA Methylation Inhibitors: Drugs like 5-azacytidine and decitabine inhibit DNA methyltransferases, reactivating silenced TSGs and potentially restoring normal cell growth control. HDAC Inhibitors: HDAC inhibitors, such as valproic acid and vorinostat, have demonstrated effectiveness in reactivating TSGs and inducing apoptosis in CCa cells. These inhibitors target abnormal histone deacetylation, helping to prevent cancer progression. miRNA (Li Y et al., 2019). Therapeutics: Modulating specific miRNAs linked to (Ca), such as miR-34a, offers a strategy to counteract oncogenic effects. Delivery of synthetic miRNA mimics or inhibitors can help restore regulatory balance and inhibit cancer cell proliferation.

1.4. Phytochemicals as Therapeutic Agents:

Natural substances derived from plants have the potential to serve as healing agents, representing an innovative approach to both preventing and treating cancer. Alkaloids, terpenes, saponins, triterpenoids, and polyphenols are classified as phytochemicals (Awuchi CG et al., 2020). Phytochemicals are bioactive compounds that are further categorized based on their chemical structure (Campos-Vega R et al, 2013). These compounds are abundantly found in fruits, cereals, and vegetables that we eat regularly. Plant-based phytochemicals offer several health benefits to humans (Yahia EM et al., 2017). In addition, they possess anti-cancer, anti-inflammatory, and antioxidant properties, along with other features that help protect against various chronic diseases, including CCa. Furthermore, the primary challenges associated with CCa therapy are poor treatment outcomes with traditional medicines and the toxicity of drugs (Tiwari BK et al., 2015).

CCa cells undergo apoptosis due to the actions of phytochemicals. The process involves either the activation of the internal mitochondrial pathway or the activation of death receptor-mediated extrinsic pathways, which are the two signaling mechanisms responsible for apoptosis. Among triterpenoid glycosides are terpenoid derivatives of triterpene that belong to the saponin category of chemicals (Rufino-Palomares EE et al., 2015).

“Triterpenoid saponins are also called triterpenoid glycosides.” (Mahato SB et al., 1998). This compound comes from garlic, Java apple, ursolic acid (UA) in apples, bilberries, cranberries, lavender, moronic acid from mistletoe, and betulinic acid. (Mirjalili MH et al., 2016). Researchers have identified more than 20,000 different triterpenoids in nature (K.T. Liby et al, 2007). It has been shown that triterpenoids, which are potent phytochemicals, positively affect a wide range of human disorders, including many types of cancer (A. Bishayee et al, 2011).

1.5 Natural Therapy to Fight against Cancer:

Most natural substances exert their anti-cancer effects without directly damaging DNA and have a remarkable ability to selectively target cancer cells while sparing healthy, normal cells. They utilize multiple complementary mechanisms to prevent cancer development and progression. One key action is boosting the immune system, which enhances the body's natural defenses and helps immune cells better recognize and destroy malignant cells. Additionally, these substances inhibit angiogenesis—the process of forming new blood vessels that supply nutrients and oxygen to cancer cells. By limiting this blood supply, they effectively starve cancer cells and prevent their growth and spread.

In addition, natural compounds help in creating an internal environment that is hostile to cancer cell growth. They promote better oxygenation, improve metabolic functions, regulate body temperature, reduce sugar levels, and increase alkalinity, all of which disrupt the conditions required for cancer cells to thrive. Moreover, they play a crucial role in detoxifying the body by neutralizing and eliminating harmful toxins that could otherwise contribute to disease progression. Another vital function of these substances is their antioxidant property, which protects cells from damage caused by free radicals. By neutralizing these unstable molecules, they prevent mutational changes in DNA that could potentially initiate or accelerate cancer development. Collectively, these multifaceted actions of natural substances make them promising candidates for cancer prevention and therapy while minimizing harm to normal cells.

1.6 Phytochemical selection for study:

Suffness and Douros (1979) outlined four primary methods for selecting phytochemicals for pharmacological research: ethnomedical phytochemical selection, which involves identifying compounds based on traditional medicinal practices; random phytochemical choice, where compounds are selected without prior knowledge of their bioactivity; building on species application research, which focuses on studying plants already known for specific therapeutic uses; and chemotaxis tactics, which involve targeting plants or compounds based on their chemical characteristics.

The increasing interest in natural compounds as anticancer agents is largely attributed to their wide availability, cost-effectiveness, and reduced side effects compared to conventional chemotherapeutics. Numerous studies have demonstrated that herbal extracts can effectively target cancer progression by modulating molecular and cellular pathways. For instance, they can regulate viral oncogenes and gene expression in host cells, with notable effects observed in prostate cancer through the modulation of apoptosis and autophagy while inhibiting critical pathways such as NF- κ B and angiogenesis. Additionally, certain bioactive compounds have been shown to specifically target the HPV E6 oncoprotein, highlighting their therapeutic relevance in HPV-associated malignancies such as cervical cancer.

Furthermore, the tumor microenvironment significantly contributes to cancer progression, driven by factors such as hypoxia, nutrient deprivation, and chronic inflammation, which often lead to therapy resistance. In this context, natural compounds exhibit promise by modulating pro-inflammatory mediators associated with tumor growth. Proteins such as COX2, NF- κ B, IL-6, and VEGF are crucial markers of chronic inflammation within the tumor milieu. Among these, IL-6 is particularly significant, as it plays a central role in tumor promotion across multiple cancer types, including glioma, melanoma, and colorectal cancer. By targeting these molecular pathways and inflammatory markers, phytochemicals offer a multifaceted approach to mitigating tumor progression and enhancing cancer therapy.

Apoptosis, a critical process for cellular balance, is controlled by gene families like Bcl-2 and Bax. Calcineurin, a phosphatase involved in numerous signalling pathways, contributes to apoptosis by activating the cytochrome c/caspase pathway and regulating proteins such as Bad. Additionally, it plays a role in the cell cycle by affecting cyclin-dependent kinase 4 (cdk4), particularly at the G0/G1 checkpoint. In view of the

relationship between inflammation and cancer development, this study aims to screen natural compounds for their ability to inhibit aromatase using a structure-based pharmacophore approach. The effectiveness of these compounds will be evaluated in vitro against CCa-HeLa cells, aiding the search for new cancer treatments.

1.7 The importance of metal-based nanomedicines in CCa treatment:

We explored nano formulation to enhance the effectiveness and bioavailability of various triterpenoids. Nanotechnology refers to technology operating on a scale of one billionth of a meter. Due to their extremely small size, nanoparticles can quickly penetrate cells, interacting with the cell interior, DNA, and exerting effects on enzymes, proteins, and receptors. Thanks to their unique ability for drug loading and efficient photoluminescence, nanoparticles are valuable for targeted delivery of imaging agents and anticancer drugs (Edis Et al., 2021). An increase in arterial permeability and impaired lymphatic drainage contributed to the enhanced permeability and retention effects of nanoparticles within tumors (Nakamura Y et al., 2016, and Nehoff H et al., 2014). By facilitating drug internalization, nanoparticles can reduce drug resistance (CM Hu et al., 2009). Using nanoparticles allows for the rapid detection of cellular processes in cancer.

The metal-based bionanomaterials opened new avenues for the diagnosis and treatment of a wide range of disorders, including malignant diseases (RC Popescu et al., 2015). Metals and metal oxides treated with plant juices have been used in cancer therapy for thousands of years. However, a substantial study is necessary in this field due to the toxicity concerns associated with metals and their oxides during preparation. The current metal nanoparticles and metal triterpenoid complexes are more potent and stable than earlier versions, but they are still in the early stages of development. Research on medicinal plants is rapidly expanding worldwide, driven by renewed interest in phytochemicals and the effectiveness of traditional health systems in addressing global healthcare challenges. This study aims to explore potential alternatives to chemical agents used in treatment.

This study aimed to determine how effectively phytochemicals can assist in treating CCa. The genes DNMT3A, HDAC4, and KMT2C are involved in this process. In this study, Betulin (BE) and Shatavarin-IV (SH-IV) were selected based on

pharmacological and in-silico analyses. Both BE and SH-IV are glycosidic triterpenoid saponins with notable anti-cancer effects across various cancer types. These triterpenoids have unique structures and a wide range of bioactive compounds. Therefore, we examined the binding affinity of BE and SH-IV to DNMT3A, HDAC4, and KMT2C related to CCa using different computational techniques. The methods included molecular docking, ADMET screening, evaluation of drug-like properties, DFT calculations, and molecular dynamics simulations. Our goal was to predict the inhibitory potential of the selected triterpenoids through computational and expression analysis, aiming to explore the potential of BE and SH-IV as epigenetic modulators for DNMT3A, HDAC1, and KMT2C in CCa cells.

The results may serve as a foundation for further experimental research, which can be performed since the study was conducted via computer simulation. Additionally, identifying Betulin and Shatavarin-IV as promising lead compounds could support the development of new targeted therapies for CCa. Metal-Betulin nano complex (MBNC) and Metal-Shatavarin-IV nano complex (MSNC) were synthesized and tested for antioxidant activity and tumor targeting mechanisms in vitro against CCa. This was done to explore their possible future applications. The results provide a novel approach using triterpenoid metal nanocomplexes, which could improve therapeutic effects against cancer cells.

These medications provide patients managing this challenging condition with alternative treatment options. The process involves two steps: first, conformational changes of the ligand in the receptor's active site; second, molecular docking. Molecular docking is a two-phase process, with the initial phase being the preliminary step. The next phase assigns a score to each interaction based on the conformational energy associated with each interaction. Additionally, to evaluate the docking interaction affinity of different ligand-receptor conformations (L), tools like AutoDock and GOLD are widely used. These applications are easily accessible, allowing for the assessment of docking contact affinity across various ligand-receptor conformations. As a result, we chose GLIDE for molecular docking due to its performance, accuracy, flexibility, and thorough analysis.

In the development of new pharmaceutical substances, pharmacokinetic factors are among the most important considerations. For a product to be suitable for medicinal

use, it must demonstrate optimal biological activity and pharmacokinetic properties. P-glycoprotein, commonly known as Pgp, is a liver protein that plays a significant role in drug discovery and development. It functions as an efflux pump, influencing its pharmacokinetic characteristics. This helps prioritize and select promising compounds for further testing and evaluation. Following Lipinski's rule is essential for identifying compounds with better pharmacokinetic profiles and higher chances of successful drug development. Both outcomes are beneficial. Molecular Dynamics Simulations have been performed to study the dynamics and the effect of the solvent on the ligand-receptor complex under investigation.

1.8 Research Gap:

The altered epigenetic crosstalk highlights an important role of histone modification patterns in identifying targeted genes. The epigenetic mechanisms involved in cervical cancer are not yet fully understood. Therefore, it will be valuable to observe the epigenetic crosstalk between histone modifications and DNA methylation on the underlying genes in studying CIN1-3 of cervical cancer under in-vitro conditions. Analyzing the effects of known epigenetic modifiers, such as hypomethylating agents, on these phenomena will provide further insight into the molecular mechanisms involved. Recognizing these epigenetic mechanisms can directly contribute to a better understanding of diagnosis and clinical treatment of the disease.

1.9 Hypothesis:

Cervical cancer (CCa) remains a significant global health challenge, with incidence rates continuing to rise despite extensive research and therapeutic advancements. Conventional chemotherapy, while widely used, is often associated with severe side effects and limited selectivity, leading to damage to healthy surrounding tissues. Therefore, there is an urgent need for alternative therapeutic strategies that effectively inhibit tumor growth and induce apoptosis with minimal toxicity.

Natural compounds derived from plants have gained increasing attention for their anticancer potential. These phytochemicals are generally considered safe and have been shown to slow cancer progression with fewer adverse effects compared to standard treatments. A deeper understanding of the molecular pathways involved in CCa cell survival and proliferation, alongside the identification of effective plant-derived compounds, is essential for developing more targeted and less harmful therapies.

In this study, we employed a combination of *in silico* and *in vitro* techniques to evaluate selected triterpenoids for their potential anticancer activity. We propose that these triterpenoids exhibit chemopreventive effects against cervical cancer through both computational and experimental validation. Furthermore, their incorporation into nanoparticle-based delivery systems may enhance their therapeutic efficacy while minimizing toxicity. These findings suggest that triterpenoid-loaded nanoparticles could represent a safer and more effective alternative to conventional chemotherapy in the treatment of cervical cancer.

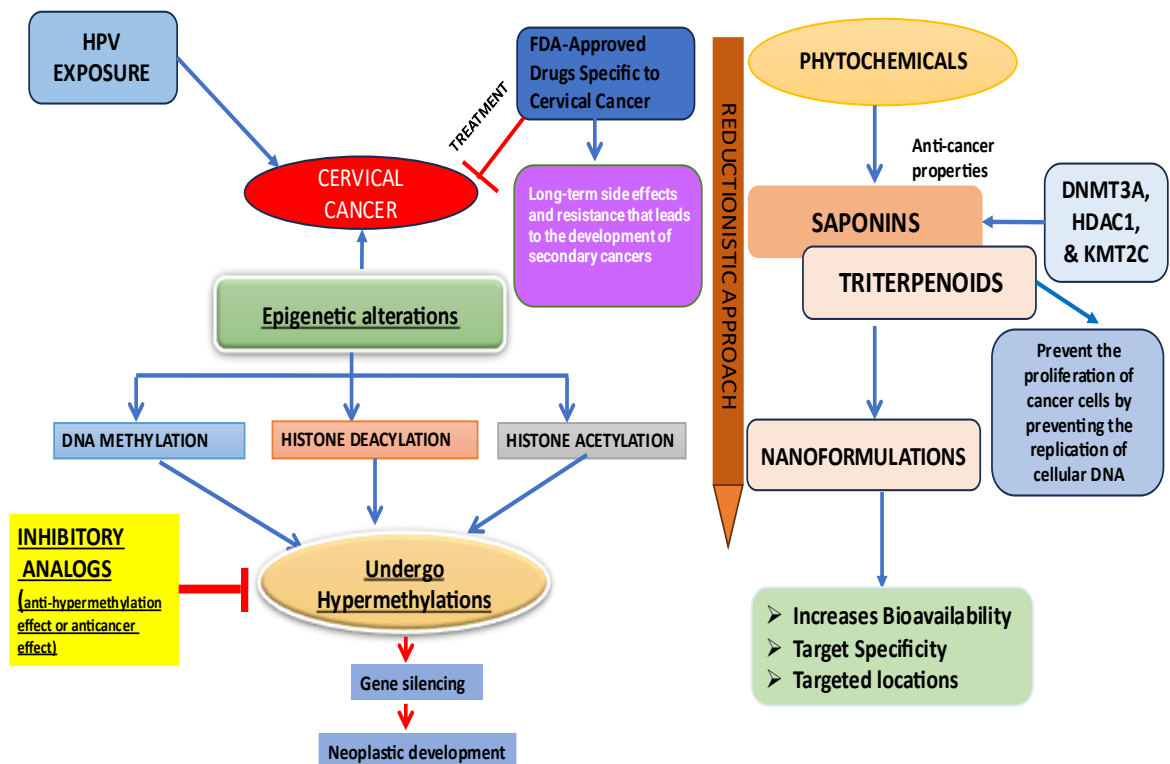


Figure 2: Graphical representations of the Hypothesis

1.10 AIMS AND OBJECTIVES

Evaluating the differential patterns of the epigenetic basis of Phytochemicals and their anti-cancer activity in cervical cancer

OBJECTIVES:

- To screen the anti-cancerous phytochemicals by using In-silico-based approaches based on the binding studies.
- To evaluate the potential of the selected molecules against Cervical Cancer (in vitro) with nano formulation-based approaches.
- To examine Qualitative and Quantitative assessment of the epigenetically altered proteins under study.
- To uncover the cervical cell lines to develop antitumor phytochemical-based drugs for enhanced efficacy using network-based analysis.

CHAPTERS 2

2. REVIEW OF LITERATURE

2.1 Cervical Cancer and Anatomical Considerations:

The cervix is the lower part of the uterus (womb) that connects the uterine cavity to the vagina through the cervical canal. While the fetus develops in the upper part of the uterus, called the uterine body, the cervix acts as a passage linking the uterus to the birth canal. Anatomically, the cervix is divided into two regions: the endocervix, located next to the uterine body, and the exocervix (also called the ectocervix) (Figure 3), which is connected to the vagina. The cervical epithelium consists of two different cell types: glandular columnar cells lining the endocervix and squamous epithelial cells covering the exocervix. These two cell types meet at the transformation zone, a region with active epithelial turnover that is highly vulnerable to cancer development and is the main site for cervical carcinogenesis.

Cervical cancer (CCa), also referred to as uterine cervix cancer, arises from DNA alterations in healthy cervical epithelial cells that progressively drive malignant transformation (Feng et al., 2023). This process typically begins within the transformation zone, where precancerous lesions gradually evolve into invasive cancer. These precancerous changes are classified as cervical intraepithelial neoplasia (CIN), squamous intraepithelial lesion (SIL), or dysplasia, reflecting varying degrees of abnormal epithelial proliferation.

Histologically, cervical cancers are categorized into two principal types:

- Squamous cell carcinoma (SCC): The most common form, originating from squamous epithelial cells in the transformation zone and extending toward the exocervix.
- Adenocarcinoma: Arising from mucus-secreting glandular cells of the endocervix.

A smaller subset exhibits features of both and is termed adenosquamous carcinoma (mixed carcinoma) (Deol et al., 2023).

The cervical epithelium also plays important physiological roles. Cells lining the endocervical canal secrete acidic and neutral mucins, a colloidal mixture of electrolytes (primarily sodium chloride) and carbohydrates (mainly glycogen) ([Eurocytology, 2023](#)). These mucosal secretions serve as a protective barrier, separating the uterine cavity from

the external vaginal environment and preventing the ascent of pathogens, thereby safeguarding reproductive health and fetal development (Lacroix et al., 2020). During the fertile phase of the menstrual cycle, cervical mucus undergoes biochemical changes that facilitate sperm migration from the vaginal canal to the uterine cavity, optimizing conditions for fertilization (Suarez et al., 2006). Conversely, if fertilization does not occur, the cervix dilates slightly to permit menstrual blood flow from the uterus (Al-Azzawi et al., 2017).

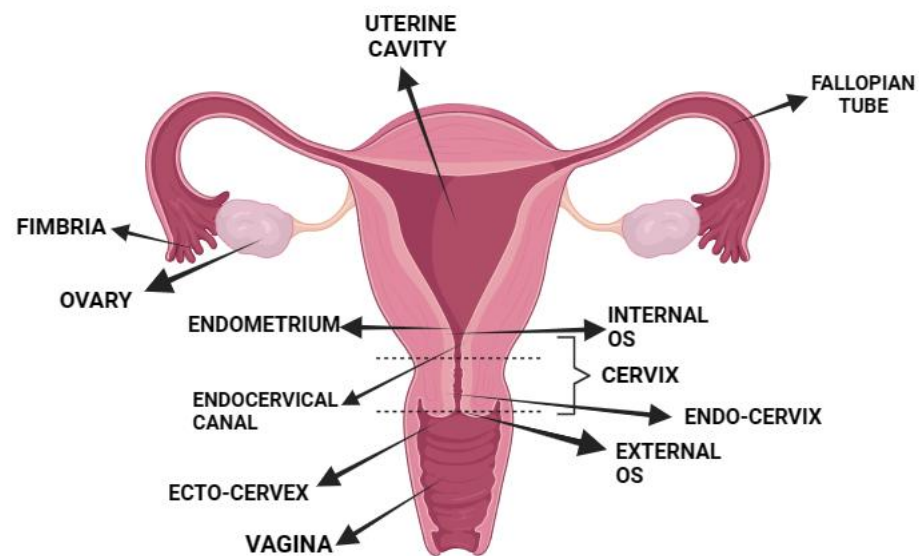


Figure 3: Female reproductive System.

2.2 Epidemiology of cervical cancer

"CCa ranks as the fourth leading cause of death and the eighth most common cancer among women." In 2022, there were 661,021 new cases and 348,189 deaths globally, resulting in a cancer mortality rate that is 57% lower than the incidence rate worldwide. (GLOBOCAN 2022). "The majority of these cases occur in Sub-Saharan Africa, Melanesia, South America, and Eastern Asia."(Sung et al., 2021) (Figure 4)

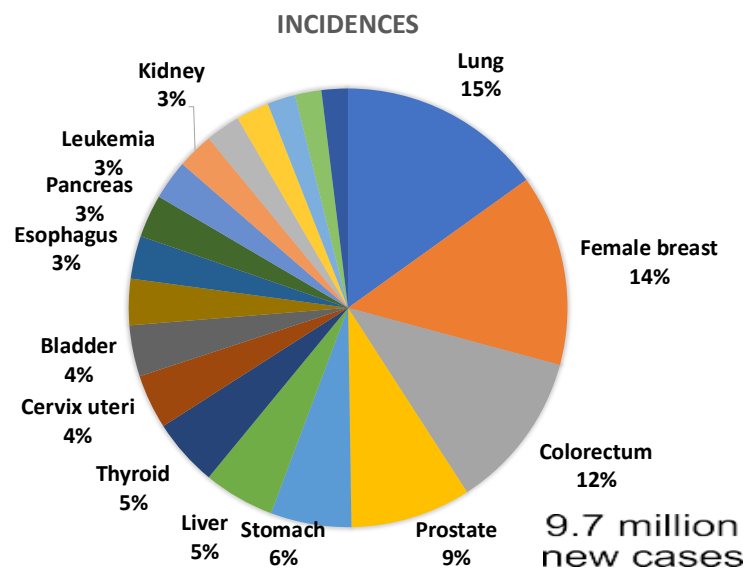


Figure 4. The graph from Globocan 2022 illustrates the number of new cases of CCa.

"Cervical cancer poses a risk to over 6714 Indian women aged 15 to 39yr, 52, 560 aged 40 to 64 years, and 19,814 aged 65 years and above." According to the most recent and accurate data, CCa annually diagnoses 127,526 women and results in 77,906 deaths. India witnessed a significant burden of CCa among young and middle-aged women, with this demographic accounting for the majority of cases (Figure 5). HPV serotypes 16 and 18 cause invasive cancer in India. India has 14.0 age-standardized CCa, 9.0 incidence, and 8.7 death rates (GLOBOCAN 2022).

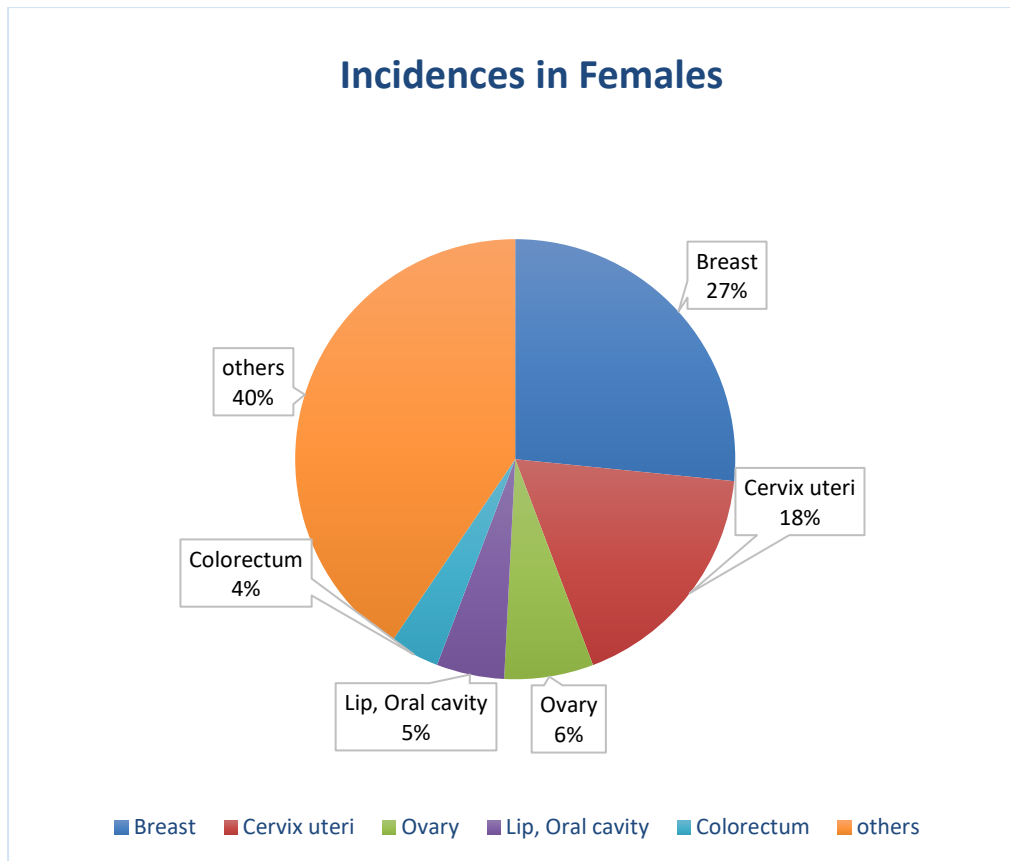


Figure 5. The graph from Globocan 2022 illustrates the number of new cases of CCa in India

2.3 Historical context of the human papillomavirus (HPV)

Human papillomavirus (HPV) is a representative of the papovaviridae family. It has been determined that there are nearly 200 different types of HPV, with more than forty of these types colonizing the genital tract. Hr-HPV and Lr-HPV infections are the two categories that are used to categorize all types of HPV infections according to the carcinogenic properties they possess. Hr-HPV types include 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 68, and 59. Others, numbered 53, 66, 70, 73, and 82, are categorized as having a potential for high risk (Haedicke et al., 2013). The most virulent high-risk genotypes are HPV-16 and HPV-18, which are responsible for seventy percent of all invasive cervical cancers worldwide. CCa can be classified into two categories: adenocarcinoma and squamous cell carcinoma. It is currently well known and proven that these genotypes are the most dangerous. According to Haedicke and Iftner (2013, adenocarcinomas are tumors that originate from the ectocervix; additionally,

2.3.1 Organization of the HPV genome

The HPV is a circular double-stranded DNA virus that is roughly 8 kilobytes in size. It is associated with cellular histone or histone-like proteins and possesses a non-enveloped icosahedral capsid. Early region (E), Late region (L), and long control region (LCR) are the three types of regions that could be functionally separated from one another in the HPV genome. Open reading frames (ORFs) are essential for the replication of the virus and transcription, and they are found in the early region of the genome. This area has been subdivided into parts E1 through E8. Late region (L1 and L2) encodes for viral capsid protein (Pinidis et al., 2016). The LCR region contains viral cis-acting regulatory sequences that activate constitutively during the process of viral replication and transcription (Stunkel and Bernard, 1999).

2.3.2 Life cycle of the HPV virus

An intraepithelial pathogen that infects primitive basal keratinocytes is known as human papillomavirus (HPV). Both the differentiation of epithelial cells and the replication of the virus are intricately connected. The first attachment of the HPV virion, known as L1, requires it to bind to the heparan sulphate proteoglycans (HSPGs) present on the surface of the squamous epithelial cell or the basement membrane. After entering the cell, the genome of the virus is transported to the nucleus, where it establishes its genome as an episome. After this, the virus divides within the host cell, producing approximately 20-100 copies of itself in each cell. During the Synthetic phase of the cell cycle, viral oncoproteins E5, E6, and E7 are responsible for viral genome replication. This replication occurs concurrently with the replication of the host cellular DNA. Following the replication of the viral genome and the division of the cell, one of the daughter cells will move into the suprabasal layer. This cell will then initiate a program of cell differentiation within the cell cycle and will proceed to the G2 phase. During the G2 phase, also called amplification, episomes are replicated to reach a total of one thousand copies per cell. Amplification, capsid synthesis, and the assembly of progeny virions are all processes that take place concurrently in highly differentiated cells. This is then followed by the release of virions to the differentiated layers of the epithelial cell surface (Graham et al., 2017; Moody & Laimins, 2010).

2.3.3 HPV infection of the cervix.

Infection with human papillomavirus (HPV) typically takes place at a squamocolumnar junction between the ectocervix and the endocervix, which is persistently present with high-risk HPV types. Heparan sulphate proteoglycans, also known as HSPGs, are found on the surface of basal layer cells, and the L1 capsid protein binds to them. When HPV binds to HSPGs, it causes conformational changes in the capsid that are mediated by cyclophilin B. These changes cause the amino terminus of the L2 component to become exposed on the surface of the virion, where it is then cleaved by Furin (Gheit et al., 2019). The L2 genome complex enters the nucleus, which is associated with the ND-10 nuclear bodies, and begins the process of early viral transcription. Following the event of infection, the viral genome is preserved in the form of a small number of episomes. The oncoproteins E6 and E7, which are required for entry into the S-S phase, are responsible for regulating the expression of early genes during the process of epithelial differentiation. During the late stages of viral replication, P670 is responsible for regulating the expression of genes that are involved in the proteins E1, E2, E3, and E5. Inducing the growth factor's signalling is the E5 protein, which specifically binds to the receptor of EGFR (growth factor). E6 is responsible for inactivating p53 and other pro-apoptotic proteins, and E7 is responsible for inactivating retinoblastoma protein (Rb), which ultimately results in the deregulation of the cell cycle (Pindis et al., 2016; McBride & Warburton et al., 2017; Conrady MC& Suarez I et al., 2020).

2.3.4 Low-risk and high-risk, and each of them is associated with cancer.

In addition to being classified as carcinogenic, the alpha HPV is further subdivided into Hr-HPV and Lr- HPV types. These types include 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59. The cutaneous and mucosal types of the alpha HPV are the two groups that make up the alpha HPV. We have determined that eight different types of human papillomavirus (HPV) are likely to cause cancer. These are HPV 26, 53, 66, 67, 68, 70, 73, and 82. High HPV types are particularly powerful etiological agents that are responsible for the development of several cancers, including those of the cervix, vulva, vagina, anus, penis, head and neck cancer (HNCs), and oropharyngeal cancer. When it comes to genital warts, HPV 6, 11, 42, 43, and 44 are examples of low-risk cutaneous HPVs (Haedicke and Iftner et al., 2013). These HPVs cause benign lesions. During the progression of cervical cancer, a premalignant transformation takes place in the squamocolumnar junction, which is closely linked with high-risk HPV16 and HPV 18 types. As a collective, these viruses are responsible for approximately half a million cases

of CCa each year across the globe, which translates to approximately 56, 9847 cases being diagnosed each year. Because of these differences, the expression of the E6 and E7 genes is altered (Table 1), which illustrates the patterns of gene expression that occur during low-risk and high-risk infections. E6 and E7 expression levels in CCa are found to increase as a result of CIN1 and CIN3 expression. p53, a gene that acts as a tumor suppressor, is rendered inactive by the high-risk HPV genome that encodes the E6 protein. Additionally, the E7 oncoprotein renders pRb inactive, which results in the abnormal progression of the cell cycle that is characteristic of cervical cancer.

Table 1: The patterns of gene expression at low-risk and high-risk infections.

	High-risk Alpha	Low-risk Alpha
E6	Encodes for E6 products	Does not encode for E6 products
	Degrade P53 and PDZ domain protein	Do not degrade the P53 and PDZ domain protein.
	Inhibition of NOTCH and the apoptotic pathway	Not known
	Activation of telomerase	No activation of telomerase
	Inhibition of interferon response	No interferon inhibition
	Destabilize pRb, P107, P130	Destabilize P130
	Suppression of STAT-1 function	NO Suppression of STST-1 function
E7	Activation of the AKT signalling pathway	Not Known
	Induction of genome instability	No Stimulation Occurs

2.4 Classification of Cervical Lesions.

According to the Bethesda III classification system 2001 (Altaf FJ et al, 2012), cervical smear samples are classified in several clinical laboratories. This system illustrates that the absence of cellular abnormalities was considered negative for intraepithelial lesions. The abnormal cells are classified according to the degree of abnormality observed in the cells.

2.4.1 The atypical squamous cells, also known as ASCs

ASC refers to a region of the cervix that contains mildly abnormal cells and may be a region of inflammation. While more than eighty percent of these cells are normalized, five to ten percent of these women have a dysplasia of CIN-II or CIN-III. It can be divided into two distinct categories.

2.4.2 Atypical cells of undetermined significance (ASC-US)

People who have ACS-US should be screened for HPV infection because the morphology of squamous cells is different from that of normal cells. It is recommended that these individuals undergo screening. Colposcopy is recommended for patients who have ACS-US that is positive for HPV.

2.4.3 Atypical squamous cells cannot exclude HSIL (ASC-H)

It is possible that the presence of atypical squamous cell lesions is a higher risk for the precancerous stage, which is referred to as CINII or CINIII. These samples are sent to a colposcopic examination for further analysis.

2.4.4 Low-grade squamous intraepithelial lesion (LSIL)

One of the mild abnormalities that can be found on a pap test and is associated with human papillomavirus infection is called LSIL. From a pathological standpoint, cervical intraepithelial neoplasia (CIN1) is considered to be an early form of dysplasia. In most cases, a pap smear or a colposcopy is used to diagnose the condition. LEEP, which stands for loop electrosurgical excision procedure, and conization are two components of the excisional treatment method that are utilized for the removal of abnormal cells. LEEP-LSIL can be eliminated through the application of either heat or electric current.

2.4.5 High-grade squamous intraepithelial lesion (HSIL)

HSIL-positive samples are considered to be moderate or severe dysplasia. These samples are primarily reported and are referred to as CIN1, CIN2/3, and CIN3. The diagnosis of CIN3 is typically made through a pap smear, colposcopy, and hysterectomy, which is among the primary treatment methods for the removal of CIN2 and CIN3 lesions. Methods of treatment, such as LEEP and conization excision, were preferred for women who were not considering becoming pregnant in the future.

2.4.6 Adenocarcinoma in situ (AIS)

At this stage, the glandular cells of the cervix are where advanced lesions are discovered. Colposcopy and biopsy are the preferred follow-up tests for endocervical screening because these lesions can develop into cancerous ones if they are not treated at an early stage.

2.5 Causes of Cervical Cancer:

2.5.1 Human Papillomavirus (HPV)

There are over one hundred different types of papillomaviruses that can cause cervical cancer, with the human papillomavirus (HPV) being the most prevalent. In 1977, Harald Zurhausen was the first person to discover that HPV DNA was involved in genital warts and cervical cancer. In 2008, he was awarded the Nobel Prize for his discovery. According to Clifford in 2003, these high-risk HPV types, which account for approximately 70 percent of all cases of CCa, are caused by the HPV16 and 18 strains. The HPV is a virus that is transmitted through sexual contact and is found in all sexually active individuals. It is more likely that a person will become infected with HPV if they engage in polyamory and sexual activity at a young age.

2.5.2 Chlamydia and the herpes virus:

Patients with invasive CCa were found to have certain Herpes viruses, such as HSV-2, HSV-6, and HSV-7 (Li and Wen et al. in 2017). Chlamydia bacteria, according to the results of the studies (Zhu et al., 2016), are associated with an increased risk of developing CCa.

2.5.3 Smoking

Because carcinogenesis in cigarettes destroys the DNA of cells in the cervix and suppresses the ability of the immune system to fight against HPV infection, smoking is associated with an elevated risk of developing CCa (Sugawara et al., 2019).

2.5.4 Diet

Several studies have demonstrated that foods high in carotenoids and vitamin A can help reduce the risk of emerging CCa. (Piyathilake et al., 2009; Pieroth et al., 2018) Research has shown that fruits and vegetables that are high in flavonoids and folic acid offer a reduction in the risk of CCa.

2.5.5 Oral contraceptives (birth control pills)

Individuals who have been using oral contraceptives for an extended period are considered to have a high risk of developing CCa (Asthana et al., 2020).

2.5.6 Multiple pregnancies:

Women who have carried more than six pregnancies are at a high risk of developing CCa due to the increased likelihood of being exposed to HIV through sexual activity (Tekalegn et al., 2021).

2.5.7 Low-socio-economic status

Because of a lack of education, a lack of awareness, and a lack of financial resources, low-income states are unable to carry out health care screening programs such as Pap smear tests. This results in a rise in the number of squamous intraepithelial lesions that occur in women (Benard et al., 2008; Akinyemiju et al., 2016).

2.5.8 Diethylstilbesterol.

The hormone diethylstilbestrol is administered to women to prevent them from having a miscarriage. A female child born to such women has a marginal chance of developing a rare form of cervical cancer (Laronda et al., 2013; Verloop et al., 2017).

2.5.9 Family History of CCa, A family history of CCa is associated with a fifteen percent increased risk of developing cancer, as indicated by several studies. The immune system modifications that contribute to these cases are most likely caused by inherited characteristics. These modifications make the individuals more susceptible to HPV infection (Bellinger et al., 2014).

2.5.10 Weakened Immune System (Natural body mechanism)

HIV inactivates the immune system, which makes the individual susceptible to HPV infection, which leads to an increase in the risk for CCa (Liu et al., 2018). Previous reports have shown that infection with HIV or taking immunosuppressive drugs after transplantation is more likely to elevate the risk of developing CCa, this is because HIV inactivates the immune system.

2.6 Characteristics of Cervical Cancer

Initial signs and symptoms are typically absent in most patients diagnosed with dysplasia or in situ carcinoma. If the abnormal epithelial cells develop into cancerous cells, they may eventually invade the surrounding tissue and cause symptoms.

The most prominent symptom that may be present is bleeding that is abnormal or out of the ordinary.

- It is more common for menstrual bleeding to be heavier than usual.
- After sexual activity, the appearance of spots
- Boost the amount of vaginal discharge
- spotting following a pelvic examination
- Other potential risk factors include smoking, polyamory, having multiple children, taking oral contraceptive pills, and other similar behaviors.

2.7 Diagnosis of Cervical Cancer:

Methods such as the following can be utilized to diagnose cervical cancer:

- a) Pap smear;
- b) Visual inspection with acetic acid (via) is the second option.
- c) The testing of DNA for HPV

In the cervix, precancerous cells as well as cancerous cells can be identified through the use of basic screening procedures such as a Pap test. A Pap smear, also known as a Papanicolaou

smear, was first introduced in 1941 and is utilized for the purpose of examining cells from the cervix or vagina. If atypical cells are discovered, they are categorized by the degree of abnormality that they exhibit. Colposcopy, cone biopsy, imaging tests such as CT scans and MRIs, DNA hybridization, and polymerase chain reaction are some of the additional tests that may be performed for further confirmation after a pap smear has identified squamous cells or neoplastic lesions in the cervix. These tests are often performed to further confirm the diagnosis.

2.8 Diagnostic procedures for the detection of infections caused by the human papillomavirus

2.8.1 Neo-Molecular Methods

To detect CCa at an earlier stage, screening methods such as visual inspection of the cervix, cytology and histology, and colposcopy are being utilized. However, it is important to note that these screening approaches are inaccurate for the detection of HPV. The accurate detection of HPV infection was accomplished through the utilization of several molecular-based methods.

2.8.2 Vaccination Against Cervical Cancer:

One of the most important preventative measures against CCa is the administration of HPV vaccines. The primary idea behind the vaccines is that they are made up of non-infectious virus-like particles (VIPs), which are similar to the immunogenicity of the native virus, and they can be administered with or without adjuvants. The next step is to induce antibodies that neutralize the HPV genotypes that are relevant. Prophylactic HPV vaccines have the potential to prevent up to 80% of CCa cases. The vaccines have been developed to prevent infection with Hr-HPV types, to reduce the number of deaths and rates of CCa. The World Health Organization has given its blessing to the development of three prophylactic HPV vaccines that have been approved for commercial use (Roden et al., 2018, and Stren et al., 2018).

2.8.2.1 Cervarix:

GlucSmithKline (GSK developed a bivalent vaccine formulated to shield against both HPV-16 and HPV-18 variants. This vaccine is administered intramuscularly in three doses, typically at the beginning of the first month, the second month, and the sixth month of the patient's life.

2.8.2.2 Gardasil:

Merck and its colleagues have developed a vaccine that is a quadrivalent HPV vaccine. This vaccine is based on an antibody that protects against both Hr-HPV and Lr-HPV types, including 16, 18, 6, and 11.

2.8.2.3 Gardasil 9:

This vaccine was developed by Merck and Company and contains live VLPs of HPV 16,18,6,11,31,33,45,52, and 58. Also, it was given in three separate doses. Before being exposed to HPV, individuals are required to receive all these vaccines, which are recommended for girls between the ages of 9 and 14 years old and females between the ages of 13 and 26 years old. For women who have been infected with HPV in the past, the vaccines that are currently available do not offer any therapeutic benefits; therefore, it is recommended that women receive their HPV vaccines before engaging in sexual activity. As of right now, phase-III prophylactic vaccines of the next generation are being tested. The purpose of these vaccines is to address the issue of cost, to ensure that they are effective for a longer period, and to prevent broad-spectrum HPV infections (Schiller et al.,2015).

2.9 Clinical Validation and Protective Impact of HPV Vaccines:

The FDA has approved the use of HPV vaccines, which have undergone extensive research confirming their safety and effectiveness for patients. The results of a meta-analysis that was carried out in 2019 by a health organization in Canada on the population-based impact of the HPV vaccine in 14 countries, which included sixty million people who had been vaccinated, demonstrated that vaccination is safe. According to the findings of a follow-up study conducted eight years after vaccination, the prevalence of HPV 16 and 18 infection had decreased by 83% in females between the ages of 15 and 19 and by 69% in females between the ages of 20 and 24 (Drolet et al., 2019). It has been demonstrated through clinical trials that the duration of protection is greater than nine years for the bivalent vaccine (Kavanaugh et al., 2017), ten years for the quadrivalent vaccine (Kjaer et al., 2018), and at least six years for the nonvalent vaccine (Hub et al., 2017).

If you are between the ages of 13 and 21, you should consider getting the HPV vaccine, Gardasil. This vaccine protects men against the majority of genital lumps and anal lumps. Symptoms such as pain, swelling at the injection site, fever, and headache are the most frequently experienced adverse reactions following an HPV vaccination. The vaccination

against human papillomavirus (HPV) has been shown to have the greatest potential for lowering the incidence of cervical cancer, according to studies.

2.10 Vaccination against HPV in India

Approximately 123,907 cases of CCa are diagnosed, and 77,348 deaths are reported in India (https://hpvcentre.net/statistics/reports/ind_fs.pdf). This indicates that the incidence of CCa is extremely high in India. 27% of all cases of CCa that are reported worldwide are reported in India; this is due to the lack of awareness, inadequate vaccination programs, and the decreased number of CCa pap smear screenings that are carried out (Zeng et al., 2018). The following is a list of strategies that significantly improve the proportion of people who survive:

- a) Vaccination should be administered to young women between the ages of nine and thirteen years.
- b) The government ought to regularly implement programs in rural areas that focus on raising awareness, screening of individuals, and vaccinations.
- c) The decision-makers in charge of health policy should make it a priority to incorporate the vaccine into the existing health screening system. It is the responsibility of the government to encourage Indian manufacturers to sell vaccinations that are approved internationally at prices that are affordable.

2.11 HPV-Targeted Treatment Vaccines

Cervarix, Gardasil, and Gardasil9 are the prophylactic vaccines that are currently available. These vaccines should be administered before the age of sexual maturity. If an individual has already been diagnosed with HPV infection, then they will not benefit from receiving these immunizations. Therefore, there is a need for a company that specializes in the development of therapeutic vaccines that have the potential to eliminate the persistent infection and reduce the overall cost of treatment.

Therapeutic vaccines are essential for the prevention of existing HPV infection because they stimulate a cell-mediated immune response (Th1 and Tc cells) and can prevent the virus from spreading. This vaccine is designed to target the oncogenic E1 and E2 proteins of the HPV, which are overexpressed during early stages of infection, before the incorporation of the viral genome into the host. Although numerous clinical trials in this field are currently being

conducted, there has not been a single therapeutic vaccination that has been approved for commercial use yet (Chadedda et al., 2018).

2.12 Epigenetics of Cervical Cancer

The structural modifications of the genome that influence gene expression without altering the DNA sequence that lies beneath are referred to as epigenetics (Kanwal et al. 2012). The number of studies that have demonstrated that epigenetic alterations play a vital role in the development of cancer. DNA methylation, changes in histones, and non-coding RNAs are all examples of epigenetic alterations that are responsible for controlling gene expression. DNA methylation, which primarily occurs in a CpG dinucleotide setting, usually results in genomic instability (Grady WM et al., 2008). Genomic instability is a common consequence of DNA methylation. Within the context of gene transcription, the process of methylation that takes place in both Constitutive Heterochromatin regions and promoter regions is typically associated with a decrease in transcription. The abnormal methylation of CpG islands has been identified as the first genomic change that occurs in certain types of human cancers. In most cases, the N-terminal tails of histones are the sites where covalent modifications like methylation, acetylation, sumoylation, and phosphorylation take place (Kanai Y et al., 2007). These modifications influence the accessibility of DNA as well as the recruitment of proteins that bind to DNA, which in turn influences the process of gene transcription. Histone deacetylases (HDACs), histone acetyltransferases (HATs), and DNA methyltransferases (DNMTs) are the enzymes that have been the subject of the most extensive research about chromatin modification (Lu D et al., 2013).

2.13. DNA Methylation-An Epigenetic Modulator of gene Expression.

The methylation of DNA is a type of chemical modification that occurs at the cytosine ring and is characterized by the presence of covalent bonds. The carbon 5 position of the molecule is modified by the addition of a methyl (CH₃) group, resulting in this modification. Given that DNA is composed of four bases and that there are, consequently, sixteen possible combinations of dinucleotides that can take place, the CpG dinucleotide ought to have a frequency of 6%, according to the facts that were mentioned earlier (Adams RL et al., 2012). In contrast, the actual presence is only five to ten percent of what was anticipated for the frequency of its occurrence. It is not the case that the methylation of the human genome is distributed in a uniform manner; rather, the human genome contains regions of segments that are not methylated, interspersed with parts that are methylated. According to (Kgatle MM et al. 2014),

CpG islands are extremely small regions of DNA that typically range from 0.5 to 5 kilobases in size and appear once roughly every 100 kilobases on average. In contrast to the rest of the genome, these smaller portions of DNA display characteristics that are distinct from those of the bigger parts (Arce C et al., 2006). Unmethylated, these regions contain a high proportion of GC (between 60 and 70 percent), and they have a ratio of CpG to GpC that is at least 0.6. Both characteristics are present. Because of this, they do not display any signs of suppressing the frequency of dinucleotide CpG.

CpG islands are present in approximately half of all human genes (Ho SM et al. in 2007). Both housekeeping genes and genes with tissue-specific expression patterns contain these islands. They can be found in both types of genes. There are at least three functioning DNA methyltransferases (DNMTs) that have been discovered; the most prevalent of them is DNMT1, which methylates hemi-methylated DNA more frequently. DNMT1 is an enzyme that localizes to replication foci and is important for regulating the amount of methylation that occurs throughout the replication process (Dhe-Paganon S et al., 2007). DNMT3a and DNMT3b are two additional pairs of methyltransferases that are known to be functional. De novo methylation is the process that these methyltransferases are responsible for during the embryonic development process (Chédin et al., 2011). Both DNMT3a and DNMT3b exhibit the same affinity for hemi-methylated DNA as well as non-methylated DNA; consequently, for this reason, they have been classified as de novo methyltransferases (El-Osta et al., 2011).

According to Jurkowska RZ et al. (2016), the machinery of methylation comprises DNMTs in addition to demethylases, methylation centers that initiate DNA methylation, and methylation protection centers. When chromatin remodelling actors are present, the effect of DNA methylation on gene transcription can be observed. This is the only circumstance in which this occurs. DNA methylation can directly interfere with the binding of transcriptional factors, which in turn inhibits replication. This is in addition to the fact that DNA methyltransferases DNMT1, DNMT3a, and DNMT3b can suppress transcription in a manner that is independent of methylation (Doerfler W et al., 2006). An additional factor that can restrict replication is DNA methylation (Marinus MG et al., 2014). Large protein complexes that contain HDACs and chromatin-remodeling activities are associated with methyl-CpG binding proteins. These proteins can recognize methylated DNA. Additionally, it has been proposed that DNA methylation could result in the silencing of genes through the recruitment of histone methyltransferases by methyl-binding domain proteins. These histone methyltransferases

methylyate lysine 9 in histone H3, which in turn represses gene transcription (Cheng X et al., 2014). Consequently, histones are deacetylated, and gene expression is generally suppressed.

2.14 Histone deacetylation:

Histones as alkaline proteins located in the nucleus of eukaryotic cells (Mueller MM et al., 2015). These proteins serve to package DNA into nucleosomes, which are structural units. (Luger et al., 2001) explain that a nucleosome consists of two copies each of histones H2A, H2B, and H3, forming the histone octamer, which is the core component of the nucleosome. Histone H1, in contrast, binds to the linker DNA connecting nucleosomes. Histones are characterized by flexible tails that extend from the nucleosome's lateral surface. These tails are susceptible to various chemical modifications on specific amino acids, including lysine (K), serine (S), threonine (T), and arginine (R) (Fuhrmann J et al., 2015). The histone code, which refers to modifications on amino acid residues, regulates chromatin structure and gene expression (Bhan et al., 2017). Histone proteins play dual roles, both structural and functional, in facilitating the transition between active and inactive chromatin states. Highly condensed chromatin, known as heterochromatin, inhibits gene transcription, while less condensed chromatin, called euchromatin, promotes transcription (Carlberg et al., 2015). Two enzyme families can covalently modify histones in their N-terminal and C-terminal tails: histone acetyltransferases (HATs), which transfer acetyl groups from coenzyme A (CoA), and histone deacetylases (HDACs), which remove acetyl groups from specific amino acid residues (Xia et al., 2020). Histone modifications regulate various biological processes, including transcription, chromosome packaging, and DNA damage response and repair.

Acetylation, methylation, phosphorylation, ubiquitination, simulation, glycosylation, and poly ADP ribosylation as the primary histone modifications (Neganova ME et al., 2017). Additionally, acetylation and methylation are the most extensively studied histone modification mechanisms, which also play crucial roles in regulating cellular proliferation, differentiation, division, and plasticity (Sun J et al., 2021).

Research has shown that specific histone modifications in cancer cells are associated with the activation or suppression of transcription pathways. Across most species, histone H3 undergoes acetylation primarily at lysines 9, 14, 18, 23, and 56. Additionally, it experiences phosphorylation at ser10, ser28, Thr3, and Thr11, as well as methylation at arginine 2 and lysines 4, 9, 27, 36, and 79. Certain modifications, such as H3K4me3, H3K9ac, and H3K20me, have been linked to gene

activation. In contrast, H3K9me3 and H3K27 epigenetic marks are involved in gene expression repression (Davison GW et al., 2023). In prostate cancer, H3K4me1 has been identified as a biomarker for tumor progression and a predictor of recurrence following radical prostatectomy, while H3K9me2 and H3Ac marks distinguish it from non-malignant tissues (Santos PB et al., 2020). The histone demethylase JMJD2B has been found to enhance hormonal responsiveness in breast cancer patients by regulating H3K4me and H3K9 modifications (Varghese B et al., 2021). A study by Qin et al. in 2020 revealed that early-stage colon cancer patients (TNM stage I and II) with low nuclear expression of H3K4me3 and high expression of H3K9me3 and H4K20me3 had a more favourable prognosis.

2.15 Phytochemicals and Their Epigenetic Regulatory Activities.

There are certain factors, both intrinsic and extrinsic, that can change the epigenetic state of genes. This state can be reversed. The epigenetic mechanism has the potential to not only regulate the physiological response to certain therapeutic interventions but also to lead to the development of abnormal phenotypes. The platform for cancer therapeutics has been revolutionized by phytochemicals, natural products (NPs), or derivatives of NPs, according to Mohapatra et al. (2022). Because of its bioavailability, low toxicity profile, and ease of production, its use in treating the majority of diseases, including cancer, has driven research interests all over the world to discover an effective and alternative treatment for cancer.

2.16 Phytochemicals as therapeutic agents:

The biochemical and pharmacological mode of action of natural anti-cancer compounds revealed that these phytochemicals exhibit bioactivities such as anti-inflammation, antioxidation, immuno-modulation, and modulating tumor-associated pathogenic signaling pathways (Kolodziej H et al., 2010). These bioactivities were found to be beneficial in the treatment of cancer. Most anti-tumor compounds cause tumor cells to undergo apoptosis, which is accompanied by a reduction in the expression of genes associated with growth and proliferation. The epigenetic pathway is significant in regulating tumorigenesis, and this is true regardless of the type of cancer that is present. On the other hand, there is a paucity of research that has been carried out to forecast the effectiveness of phytochemicals that target epigenetic proteins like DNMTs and HDACs in the context of CCa treatment (Bouyahya et al., 2022).

To protect against a variety of chronic diseases, including cardiovascular disorders, obesity, diabetes, and cancers, phytochemicals have been proposed as a possible means of doing so. It

has been determined that diets that are rich in phytochemical sources can reduce the risk of cancer by twenty percent (Forni C et al., 2019). This is a significant finding in the context of cancer prevention. Usually, the molecules that are responsible for the therapeutic properties of the medications are secondary metabolites. In recent years, the chemistry of these secondary metabolites has become an indispensable component in the manufacturing of a significant number of pharmaceuticals. According to the findings of a study that was carried out (Basso, L.A. et al., 2005), approximately 49% of the 877 secondary metabolites that were presented as new medications from 1981 and 2002 as chemical entities were either natural products, semi-synthetic analogues, or synthetic compounds based on natural product models. This information was obtained from the study.

When it comes to the treatment of cancer, plants have been utilized for a significant amount of time. Hartwell included a list of over three thousand different plant species that have supposedly been utilized in cancer treatment while he was conducting his review of plants that have been used to treat cancer (Henkin JM et al., 2019). This list was included in his review of plants that have been used to treat cancer. One of the most intriguing things to take into consideration is the fact that more than sixty percent of the anticancer drugs that are currently being used are obtained from natural sources in various ways. The bacteria, marine organisms, and plants that make up these natural sources are all included. After the synthesis and development of the Vinca alkaloids, such as Vinblastine and Vincristine, as well as the isolation of the lethal Podophyllotoxins, the search for anticancer medicines derived from plant sources began (Ravichandra VD et al., 2018). Vinblastine and Vincristine are two examples of the vinca alkaloids that were discovered and developed. The 1950s marked the beginning of this search in earnest. The National Cancer Institute (NCI) of the United States of America initiated a comprehensive plant collection effort in the 1960s as a direct result of the discoveries that were made. An extensive number of novel chemotypes that exhibited a wide range of cytotoxic activities, such as the activities of taxanes and camptothecin, were discovered.

2.17 Primary Phytochemicals as Antioxidants:

Flavonoids were extracted from the seeds of *Argemone Mexicana* (Jeffrey and Christine et al., 1983), respectively. Both of these individuals were researchers working in the phytochemical unit at Plant Science Laboratories in the United Kingdom. Additionally, the re-examination of the seed extract of *Argemone Mexicana* for the newly reported 5,7,2',6'-tetrahydroxy-flavone did not reveal the presence of any novel flavones, as stated in the study. This was the case even

though the flavone was found to be present. In addition to luteolin, which is the flavone that is found in the greatest quantity in seeds, eriodictyol, which is a flavanone that is very similar to luteolin, also exists. These findings are significant because they highlight the dangers of relying solely on spectral data for the identification of novel flavonoids. This is the reason why these findings are so important. Regarding the numerous pharmacological effects that *Asclepias curassavica* displayed, Raja and his colleagues reported their findings in their study, which was conducted in 2005. Its leaves and stems are the source of a variety of medicinal properties, including anti-inflammatory, cardiotonic, anti-cancer, and anthelmintic properties, as well as treatments for piles and gonorrhoea. These actions may be connected to an antioxidant action that takes place within this plant. This is something that needs consideration. In vitro testing was performed to determine the antioxidant properties of the hydroalcoholic extract of *Asclepias curassavica*. The radicals that were involved in these activities were the DPPH radical, the nitric oxide radical, the superoxide anion radical, the lipid peroxidation assay, the hydroxyl radical, the reducing power, and the total phenol content. With an IC₅₀ value of 8.7 µg/ml, 198.4 µg/ml, and 21.7 µg/ml for DPPH, nitric oxide, and superoxide anion radicals, respectively, the extract demonstrated its capacity to scavenge the radicals that are specific to each of these radicals. A comparison was made between the values of vitamin C, rutin, and curcumin, which served as benchmarks, and the findings revealed that the results were higher. The extract exhibited a fifty percent protection against lipid peroxidation and deoxyribose degradation when administered at a dose of 134.2 µg/ml and 41.4 µg/ml, respectively. The IC₅₀ values of the standard vitamin E are 119.2 µg/ml and 32.5 µg/ml, respectively. These values are higher than the values of the standard vitamin E.

Research was carried out by Hemadri and colleagues in 2012 on the utilization of extracts from the leaves and roots of *Asclepias curassavica* for the purpose of conducting phytochemical screening and antibacterial tests. Similarly to other medicinal substances, it is of the utmost importance because it contains important secondary metabolites (glycosides and saponins), which are necessary for the body to function properly. Plant life. We used well diffusion techniques to investigate the antibacterial activity of leaves and roots that were extracted from a number of different solvents. These solvents included petroleum spirit, hexane, chloroform, ethyl acetate, and methanol at various concentrations. To determine whether or not these extracts were effective against human pathogenic and non-pathogenic bacterial strains, the purpose of this study was to investigate the effectiveness of these bacteria. Extracts of methanol and petroleum spirit were found to be effective against the majority of the pathogenic bacteria

that were tested. This was discovered through the process of trial and error. There are some *E. Klebsiella pneumoniae*, which was one of the coliform species that was investigated, demonstrated the highest level of sensitivity to root extracts contained in methanol and petroleum spirit. The fact that this is the case demonstrates that the plant extracts have the potential to be candidates for antibiotic resistance against bacteria of this type.

A preliminary investigation into the physicochemical, qualitative, and quantitative phytochemical properties of three samples of *Woodfordia fruticosa* flowers that were collected from different geographical areas was presented by Syed in 2013. This study was a preliminary investigation. Extracts were extracted in a sequential manner using a variety of solvents that increased in polarity. After that, each of the extracted substances was put through a phytochemical screening process. The flowers that were dried were used in this process. Based on the results of the qualitative investigation, it was discovered that the substance in question contained a wide range of constituents. These constituents included carbohydrates, proteins, amino acids, glycosides, alkaloids, polyphenols, saponins, phytosterols, fixed oils, gums and mucilage, flavonoids, and resins. The quantitative studies provided additional evidence that the medication contains alkaloids, saponins, and polyphenols. This was a confirmation of the presence of these components. *Abrus precatorius*, which is also referred to as Gunja or Jequirity, is a plant that can be found in abundance all over the plants of India, ranging from the Himalayas down to the southern regions of the country.

In their 2014 study, Narendra and colleagues demonstrated that this plant possesses both ethnobotanical and phytopharmacological potential. The primary components that it possesses are alkaloids, flavonoids, triterpene glycosides, and abrin. Flavonoids are secondary components. Neuromuscular effects, neuroprotective properties, abortifacient qualities, antiepileptic properties, antiviral properties, antimalarial properties, antifertility characteristics, nephroprotective properties, immunomodulatory properties, immunostimulatory properties, anti-inflammatory activity, and antidiabetic benefits are some of the hypotheses that have been proposed regarding the abilities of the plant. As a result of the fact that this plant has the potential to be utilized as a therapeutic remedy and is considered to be a valuable source of natural products that are one of a kind, for the development of medicines in the future.

In the year 2014, Rupesh and his colleagues published a review that discussed the ethnopharmacology, phytochemistry, and bioactivity of various botanical species that are associated with the *Erythrina indica* plant. Applications of the plant that are used to treat asthma

include a vermifuge, an expectorant, and a treatment for irregular menstruation. All of these applications are used to treat asthma. There have been a great number of phytoconstituents discovered, some of which are glycosides, saponins, alkaloids, flavonoids, tannins, phenolic compounds, and fatty acids. It has been discovered that these phytoconstituents are present in large quantities.

2.18 Cytotoxicity Study:

(Johnson et al., 1960) Conducted a study that clinically validated the anticancer activity of vincalkeboblamine, which is a product of *Catharanthus roseus*. The study was carried out on DBA/2 mice that were infected with P-1534 acute lymphatic leukemia. A protein extract that was obtained from the seeds of *Abrus precatorius* L. was reported to have been discovered by Reddy and Sirsi in the year 1969. demonstrated anticancer activity as a result of their experiments. Among the various types of cancer, some examples include carcinoma, Yoshida's sarcoma, and Dalton's lymphoma ascites cancer. As stated in a paper that was published in 1981 by El-Sayed and Cordell, the alkaloid known as catharanthamine, which is derived from the plant *Catharanthus roseus*, was discovered to have a significant anticancer activity when it was tested on P-1534 leukemia.

When tested on sarcoma 180, Yoshida et al. (1990) discovered that several hydrolyzable tannins derived from *Woodfordia fruticosa*, specifically woodfordins A, B, and C, exhibited a promising anticancer potential. These findings were based on the findings of researchers. According to the findings of a study that Kapadia and his colleagues conducted in 1996 on the root extract of *Beta vulgaris*, it was found to have a significant impact on reducing the growth of tumors in both lung and skin cancer.

According to Escibano et al. (1996), a secondary metabolite known as crocin, which is derived from *Crocus sativus* extract, was discovered to possess a high level of cell growth inhibitory activity in cervical epithelioid carcinoma malignancy. Crocin, a molecule of saffron, is the one that exhibits the greatest potential as a cancer treatment agent. It is the molecule that shows the most promise.

Tinospora cordifolia was the subject of research into Ehrlich's ascites carcinoma that was carried out by Prince and Menon et al. (1999). That is in addition to Dzuor CK. In a study conducted in 2022, it was reported that a secondary compound, such as 4(α -L-rhamnosyloxy)-benzyl isothiocyanate, which is derived from the seeds of *Moringa oleifera*, has exhibited a

noteworthy antitumor-promoting activity in both skin cancer and human multiple myeloma cancer. There have been reports that plants that belong to the family Apocynaceae can inhibit the growth of cancer cells. Flavonoids are known to possess anti-mutagenic and anticarcinogenic properties, according to research that was carried out by Kuroda and Hara in the year 1999.

Research by Kamata and colleagues in 2000 demonstrated that *Asparagus racemosus* exhibits antioxidant properties, which protect rat liver mitochondria from γ -radiation damage. In the same year, Swamy and Tan conducted an in vitro cytotoxic screening of *Nigella sativa* L. extracts. The MTT assay revealed that the ethyl-acetate fraction (EAF) of the seeds displayed cytotoxicity against various cancer cell lines, including P388, Molt4, Wehi 164, LL/2, Hep G2, SW620, and J82. The chemical components were found to possess beneficial pharmacological characteristics, particularly antioxidant, immunostimulatory, and antitumor activities. These phytochemicals may activate macrophages, induce apoptosis, and prevent DNA oxidative damage, ultimately regulating carcinogenesis.

Studies on *Tragia involucrata* in carcinoma cancer and *Emblca officinalis* in liver, breast cancer, and lung fibroblast were conducted by Joshi et al. (2011; Liu et al.,2012), and Vikas et al. (2011) reported that *Woodfordia fruticosa* flowers showed in vitro cytotoxic effects on HEP-2 and SK-N-MC cancer cells. The SRB assay was employed to evaluate the in vitro cytotoxicity of *Woodfordia fruticosa* extracts against various human cancer cell lines, including lung cancer (A-549, NCI-H23), colon cancer (OLO 205, SW-620), liver cancer (HEP-2), and neuroblastoma (SK-N-MC). The ethanolic flower extract exhibited cytotoxicity against HEP-2 and SK-N-MC cell lines but showed no significant activity against the other four cancer cell lines. The 50% ethanol and hot water extracts were ineffective against all tested human cancer cell lines from the Kiranmayi strain, and the cytotoxic properties of *Argemone mexicana*'s methanolic extract using HeLa and MCF-7 cells (Kiranmayi et al.,2011). The MTT assay was used to determine the extract's anticancer properties. The study examined the effect of plant leaf extract on cancer cells, with IC₅₀ values ranging from 1.35 μ g/ μ l to 1.2 μ g/ μ l. The Hoechst test revealed that the cytotoxic activity was apoptotic rather than necrotic in nature.

The anticancer properties of the methanol extract from *Argemone mexicana* leaves may be attributed to the flavonoids present in them. This research supports the plant's therapeutic application in cancer treatment. Anubrata (2012) examined six Indian medicinal plants (*Pavonia odorata*, *Gardenia latifolia*, *Canthium dicoccum*, *Limonia monophylla*, *Bridelia*

racemosa, and *Wrightia tinctoria*) for cytotoxicity and antiproliferative characteristics. Crude methanol extraction requires initial plant extract acquisition. Each plant extract, at a 10 mg/ml concentration, undergoes an MTT assay on Calu-6, Colo-205, and HL-60 cells. The study revealed that *Pavonia odorata* and *Wrightia tinctoria* exhibited cytotoxic and antiproliferative effects on cancer cells, particularly Calu-6 cells. Both were chosen for additional pharmacokinetics and toxicological studies to support this in vitro assay before conducting in vivo or clinical trials. (Mir et al.,2013) investigated the antioxidant and cytotoxic effects of *A. precatorius* leaf extract in vitro, aiming to discover new lead structures or enhance traditional medicine. The antiproliferative effects on various human cancer cell lines and primary cell lines were studied using the MTT method, along with in vitro biochemical techniques such as DPPH, OH, NO, and SO₂- SO₂-scavenging assays, and the ability to inhibit Fe (II)-induced lipid peroxidation. The findings consistently showed that *A. precatorius* extracts inhibited the proliferation of four human tumor cell lines: human colon adenocarcinoma cells (Colo-205), human retinoblastoma cancer cells (Y79), human hepatocellular carcinoma cells (HepG2), and human leukemia cells (SupT1). The ethanol and ethyl acetate extracts of *A. precatorius* demonstrated the ability to reduce the survival rate of the tested human cancer cell lines. Moreover, the plant extracts showed selectivity against cancer cell lines, as evidenced by their inability to inhibit the proliferation of mouse peritoneal macrophages. A review by Varsha et al. (2013) indicated that *Glycyrrhiza glabra* possesses numerous therapeutic properties, including the ability to inhibit cancerous cell growth. The refined version of *G. glabra* employs water as a medium. Research conducted both in living organisms and laboratory settings has demonstrated that *glabra* suppresses the growth of Ehrlich ascites tumor cells. Additionally, studies in living subjects have revealed that *glabra* diminishes the formation of new blood vessels. The root's ethanol extract was shown to effectively induce apoptosis and halt the G1/S cell cycle in MCF-7 human breast cancer cells. In a 2014 study by Elisha Solowey, Michal Lichtenstein, and colleagues, *Urtica membranacea*, *Artemisia monosperma*, and *Origanum onites* were examined. They investigated these three whole plant extracts on human tumor cells. Each extract exhibited the ability to eliminate human-derived tumor cell lines in a manner dependent on dosage and duration. The cytotoxic effect was exclusive to tumor cells, leaving initial cultures of healthy human cells unaffected. (Mohammed et al.,2013) researched to explore the cytotoxic and pro-apoptotic properties of *Abrus precatorius* L. on the metastatic human breast cancer cell line MDA-MB-231. Cell death was assessed using the MTT assay, which showed cell inhibition exceeding 75%. Flow cytometry revealed a sub-G0/G1 peak, indicative of apoptosis. Cells treated with the extract displayed significantly increased mRNA

expression levels of apoptosis-related genes p21 and p53 compared to the control group. This study provides the sole evidence of *A. precatorius* leaf extract's growth-inhibitory effect on MDA-MB-231 cells, demonstrated through the activation of apoptosis. *Urtica membranacea*, *Artemisia monospermica*, and *Origanum dayi* post were the three plants examined in a study in 2014 by Elisha Solowey, Michal Lichtenstein, and their colleagues. Their focus was on how the extracts from these plants affected human tumor cells. Each of these extracts proved capable of killing human tumor cell lines, with the effectiveness varying based on dosage and exposure time. Notably, only tumor cells displayed cytotoxic effects, while initial cultures of healthy human cells remained completely unharmed. In a separate study by Mohammed et al. (2013), the researchers explored the cytotoxic and pro-apoptotic properties of *Abrus precatorius* L., specifically concerning the MDA-MB-231 cell line, which is known for its metastasis. The MTT assay measured cell death, revealing that more than 75% of the cells were inhibited. The presence of a sub-G0/G1 peak, indicative of apoptosis, was noted using flow cytometry. Additionally, levels of mRNA expression for the apoptosis-related genes p21 and p53 were significantly elevated in treated cells compared to the control. This study stands as the sole report demonstrating that *A. precatorius* leaf extract inhibits the growth of MDA-MB-231 cells through apoptosis activation.

In an investigation conducted by Shyam and Ishwar in 2014, HeLa cell lines were utilized to assess the efficacy of the flavonoid-rich fraction of the ethanolic extract from *Bauhinia variegata* (BBEE) in slowing cancerous cell growth. The IC₅₀ value of the extract was determined through the MTT and SRB assays, resulting in a value of 191.5 µg/ml. The application of BBEE to HeLa cell lines triggered a response leading to apoptotic cell death. Flow cytometric analysis revealed that treatment with BBEE halted the cell cycle at the G0/G1 phase, indicating the bark extract's cytotoxic effects on HeLa cells.

According to research by Tiejun Zhao and colleagues in 2015, the beneficial effects of *Phyllanthus emblica* are attributed to its polyphenol content, particularly tannins and flavonoids. This study suggested that *Phyllanthus emblica* may serve as a potent anti-cancer agent. In 2015, Marcelo Fridlender, Yoram Kapulnik, and Hinanit Koltai released a review discussing several plant-derived compounds with anticancer and chemopreventive potential, including cannabinoids, paclitaxel, and curcumin. The review also touched on the production of synthetic counterparts and their commercial applications, as well as the prospects for developing plant-based anticancer compounds in the future. Findings from a review by Jaikumar B. and Jasmine R. et al. (2016) indicated that herbal medicine is the most commonly employed complementary and

alternative therapy among cancer patients, with plants like Echinacea, St. John's Wort, and ginger green tea frequently used.

Shreya, Modi, and Jain (2016) investigated the potential of *Andrographis paniculata*, *Aegle marmelos*, *Glycyrrhiza glabra*, *Elephantopus scaber*, and *Cistanche tubulosa* to inhibit cancer cell growth. Their research utilized the MTT assay to successfully analyze the cytotoxic effects of the active components found in these plant extracts. R. Kalavathi and R. Sagayagiri reported on the anticancer activity of an ethanolic leaf extract of *Clerodendrum inerme* in 2016, employing in vitro cytotoxic tests such as MTT against the lung adenocarcinoma epithelial cell line A549. The plant exhibited anticancer properties, as shown by an IC₅₀ value of 15.6 µg/ml. Also in 2016, Sindiswa and colleagues studied the antiproliferative effects of cannabidiol, the main component of *Cannabis sativa*, and its crude extract on various cancer cell lines. The investigation employed multiple methods, including phytochemical screening, MTT assays, cell growth analysis, flow cytometry, morphological analysis, Western blot, caspase 3/7 assays, and ATP measurement. The results showed that both cannabidiol and *Cannabis sativa* extracts could inhibit cell proliferation across all tested cell lines, albeit at varying concentrations. Notably, they demonstrated that cannabidiol induced apoptosis, as confirmed by an increase in the sub-G₀/G₁ phase and apoptosis detection using annexin V. Additionally, apoptosis was supported by the overexpression of p53, caspase 3, and Bax, along with morphological changes, increased levels of Caspase 3/7, and decreased ATP levels.

2.19 Cervical Cancer cell line- HeLa

In the realm of scientific research, an immortal cell line known as a HeLa cell is utilized when conducting experiments. In addition to being the human cell line that has been around the longest, it is also the one that is used the most. Henrietta Lacks, a patient who had been diagnosed with CCa and had passed away on October 4, 1951, was the source of the cells that were utilized in the process of developing the cell line. The date of these cells' collection was February 8th, 1951. The fact that the cell line contaminated a large number of other cell lines that were used in the research led to the discovery that it was exceptionally long-lasting and prolific. This was shown by the contamination of many other cell lines. The cells from Henrietta's tumor were collected by researcher George Gey, who found that her cells exhibited unprecedented qualities: they could be kept alive and grow indefinitely. Before this discovery, cells cultured from other sources typically only lasted a few days. The researchers spent a greater amount of time attempting to maintain the cells' viability than they did researching the

cells. However, the behavior of some cells from the tumor sample taken from Lacks was distinct from that of other cells. Gey was successful in isolating a single cell, multiplying it, and establishing a starting point for a cell line. The sample was named HeLa after the initial letters of Henrietta Lacks's name. These cells were groundbreaking as the first human cells to be cultivated in a lab with immortal characteristics, meaning they could survive beyond the usual number of cell divisions.

In 1955, HeLa cells were the first to be cloned successfully without complications, leading to a significant increase in demand for them. Researchers from all over the world have been using Henrietta's cells for Research into cancer, AIDS, and other devastating diseases ever since they were put into industrial manufacturing. This has been the case ever since the cells were first used. Gene mapping, research on the effects of radiation and other harmful substances, and a wide variety of other scientific endeavors are all examples of scientific research. In order to determine whether or not humans are sensitive to a wide range of products, HeLa cells have been utilized in the testing process. These products include tape, glue, cosmetics, and many others. Over twenty tons of her cells have been cultivated by scientists, and there are almost eleven thousand patents that involve HeLa cells. In addition, there are over twenty tons of her cells that have been preserved.

2.20. Cell Signaling Pathways in Cervical Cancer and Potential Modulatory Effects of Betulin and Shatavarin

Cell signaling pathways are crucial regulators of key cellular processes such as growth, survival, migration, and metastasis. In cervical cancer, these pathways are frequently disrupted by *HPV oncoproteins* (particularly *E6* and *E7*) and various genetic mutations. This dysregulation leads to uncontrolled cellular behavior, promotion of tumorigenesis, and resistance to therapies. Several key pathways have been identified as critical in cervical cancer progression, with emerging evidence suggesting that compounds, such as BE and SH-IV, may modulate these pathways and offer potential therapeutic benefits.

Key Signaling Pathways Involved in Cervical Cancer

1. **PI3K/AKT/mTOR Pathway:** This pathway regulates crucial processes, including cell survival, growth, and metabolism. In cervical cancer, it is frequently activated through mutations in upstream receptors such as “EGFR” or by viral oncoproteins such as “HPV E6/E7”. “AKT” activation promotes cell survival, inhibits apoptosis, and enhances glucose

metabolism, leading to unchecked cell proliferation and metastasis. BE can inhibit the “PI3K/AKT/mTOR” pathway, thereby reducing cancer cell survival and proliferation, making it a potential therapeutic candidate for cervical cancer (Jia et al., 2024).

2. The Wnt pathway is essential for regulating cell proliferation, differentiation, and survival. Dysregulation in cervical cancer often leads to the accumulation of β -catenin, which translocates to the nucleus and activates genes that promote tumorigenesis and metastasis. Persistent activation of this pathway, often exacerbated by HPV oncoproteins, facilitates uncontrolled cell proliferation and metastasis. Preliminary data suggest that BE may modulate the Wnt/ β -catenin pathway, potentially reducing tumor growth and metastasis by inhibiting β -catenin accumulation (Liao et al., 2023).

3. MAPK/ERK Pathway:

The MAPK/ERK pathway regulates cell growth, differentiation, and apoptosis. HPV E6 and E7 oncoproteins can activate this pathway in cervical cancer, contributing to uncontrolled cell proliferation and resistance to apoptosis. Alterations in MAPK signalling are commonly associated with poor prognosis and treatment resistance and have been reported to inhibit the *MAPK/ERK* signaling pathway, potentially reducing cancer cell proliferation and enhancing sensitivity to apoptosis (Ahmadu AA et al., 2021).

4. NF- κ B Pathway:

The NF- κ B pathway is involved in inflammation, immune response, and cell survival. In cervical cancer, NF- κ B activation is often triggered by inflammatory signals and HPV infection, thereby promoting inflammation, immune evasion, and treatment resistance. Chronic activation of NF- κ B contributes to tumor progression and poor treatment outcomes. Betulin has been suggested to suppress the NF- κ B pathway, potentially reducing inflammation and limiting cancer cell migration and resistance to therapy (Adepoju et al., 2023).

5. JAK/STAT Pathway: This pathway is involved in immune response and cell survival. Persistent activation of JAK/STAT signaling is associated with inflammation and tumor progression in cervical cancer. The constitutive activation of this pathway promotes cancer cell survival, proliferation, and metastasis. Shatavarin may modulate JAK/STAT signaling, potentially reducing inflammation and inhibiting tumor progression (Majnooni MB et al., 2023).

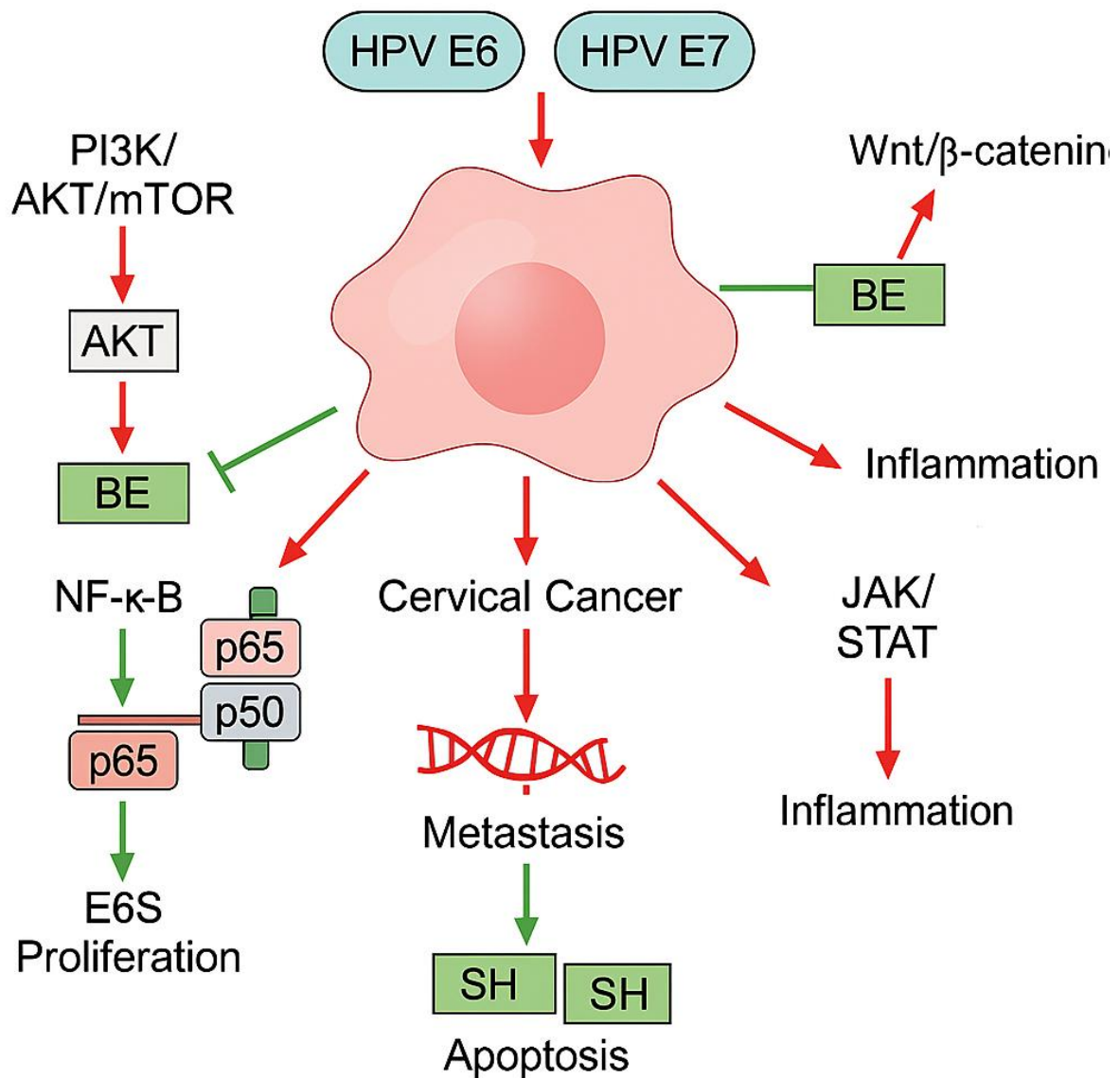


Figure 6: Schematic representation of the molecular mechanisms of Betulin (BE) and Shatavarin (SH-IV) in targeting cervical cancer pathways.

Chapter 3

3. Method and Methodology:

Cervical cancer remains a significant global health challenge despite advancements in diagnosis and treatment. The limitations of conventional therapies highlight the need for safer and more effective alternatives. Natural products have emerged as promising sources of anticancer agents due to their structural diversity, lower toxicity, and ability to modulate multiple molecular targets. Among these, triterpenoids such as betulin, shatavarin-IV exhibit apoptosis-inducing and anti-proliferative properties in various cancers, including cervical cancer. Targeting epigenetic regulators involved in gene silencing and chromatin remodeling offers a novel therapeutic strategy for this malignancy.

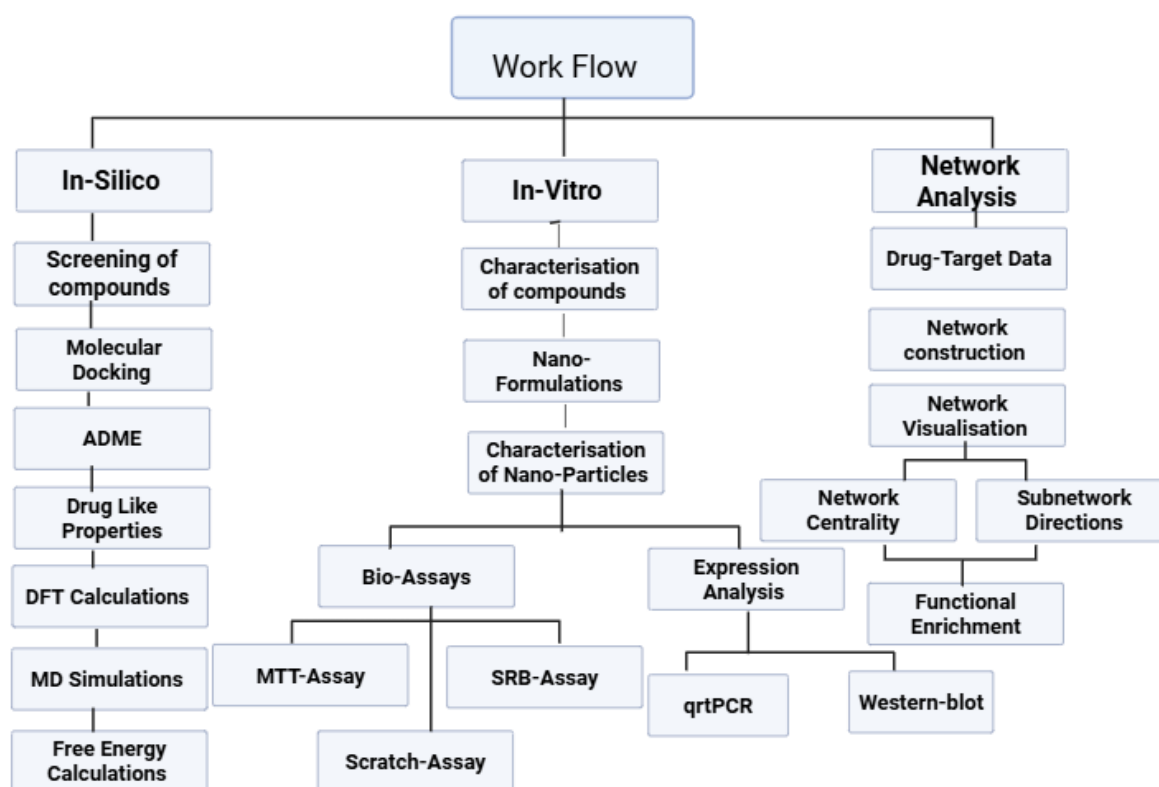


Figure 7: Workflow of research work

3.1. Objective 1: Identification and selection of the potent phytochemical using in-silico, virtual screening techniques.

3.1.1 Investigation of plant compounds:

To investigate phytochemicals with anti-cancer effects specific to CCa, we conducted a comprehensive survey of relevant literature, scientific publications, and online databases. The following actions were undertaken:

3.1.1.1 Examining the relevant material:

Searching the anti-cancer plant molecules was accomplished by hand reading of the appropriate literature. The digital repository databases were searched to a certain degree.

3.1.1.2 Extraction of Information

To identify anti-cancer molecules from plants using three separate analysis components. They were a manual review (b), a PubMed literature search (a), and a database search (c). Using PubTator, the literature search in PubMed searches abstracts. Searching the Digital repository databases allowed manual review of the literature on the full text of publications, either directly or indirectly, using online links and database searches. Two databases—Zinc database and PubChem—provided the 3D structures of the phytochemicals. These resources were utilized to identify and retrieve phytochemicals and their 3D structures. PubMed and PubTator provided literature-based evidence of anti-cancer molecules, while Zinc and PubChem offered accurate molecular structures essential for computational modeling and docking. The Open Babel program (Feliciano DN et al., 2018) helped transform the molecular forms of the molecules be transformed into various molecular formats, including mol2, sdf, etc.

3.1.2 Preparation of Ligands for Docking Studies:

The Avogadro program and Molecular Mechanics Force Field were employed to minimize the energy of these ligands. The steepest descent method was executed for 200 iterations, with RMS gradients established at 0.1. This guaranteed the ligands' stability and dependability for ensuing analyses. The minimized ligands were subsequently analysed through docking studies to forecast their binding interactions with designated target proteins (Kalirajan et al., 2017).

3.1.3 Preparation of Proteins or Docking Studies:

The three-dimensional structures of the cervical cancer-related target proteins—DNA (cytosine-5) methyltransferase 3A (DNMT3A), Histone Deacetylase 4 (HDAC4), and Histone-

Lysine N-Methyltransferase 2C (KMT2C) and their respective PDB IDs- 2QRV, 2QVJ, and 5F59 were retrieved from the Protein Data Bank. Utilizing Schrodinger software, extraneous water molecule(s) were eliminated, and polar hydrogens were incorporated to establish a biologically pertinent environment. Energy optimization was conducted to enhance structural precision and eradicate localized strain from the initial models. This optimization sought to attain a more advantageous protein conformation (Pattar SV et al.,2020). Schrödinger enabled energy minimization by producing optimized protein structures with partial atomic charges, subsequently utilized in docking analyses. The studies sought to forecast the ideal orientations of ligand-receptor complexes within the proteins' active sites and to pinpoint crucial amino acid residues implicated in ligand interactions (Cavasotto CN et al., 2003).

3.1.4 Molecular Docking using Pyrex's Autodock:

PyRx's AutoDock (V. 4.2) was employed to verify the ligands' binding capabilities with each chosen target (Macêdo et al., 2021). When it comes to understanding how the screened phytochemicals interact with proteins, docking is a great tool to have at your disposal. Using the make ligand command in PyRx, the phytochemical structures were prepared before the docking experiment. The macromolecule option was utilized to prepare the protein structures. Protein structures were found in PDB-ID 2QRV, PDB-ID 2QVJ, and PDB-ID 5F59 for DNMT3A, HDAC4, and KMT2C, respectively. Docking experiments were conducted using the AutoDock program in conjunction with the Lamarckian genetic algorithms (LGA). The analysis considers the following Lamarckian GA parameters: 30 independent runs, 150 population sizes, 25,000,000 energy evaluations, 27,000 generation numbers, a transformation rate of 0.02, and a crossover rate of 0.8. Docking of each protein was executed within its pre-defined grid size.

3.1.5 Comprehensive Molecular Docking Using Glide:

The Glide module was employed to explore the binding configurations and selectivity of target proteins interacting with chosen compounds. It aided in the assignment of hydrogens and torsions to active peptides, which were subsequently positioned into the binding sites (Desai et al., 2012). The binding site was delineated within a 5 Å radius. Following the identification of binding sites, water molecules and extraneous ligands were eliminated. A scoring function was subsequently employed to evaluate binding energy, after which a standard algorithm was executed. The docking outcomes were assessed, and the optimal conformations with the highest docking scores were chosen for additional analysis.

3.1.6 Properties Resembling Drugs and Pharmacokinetics:

Pharmacokinetic parameters are essential in the development of novel pharmaceutical compounds. Any pharmaceutical compound must demonstrate superior biological efficacy and pharmacokinetic characteristics. P-glycoprotein (Pgp), located in the liver, is crucial in drug discovery due to its role as an efflux pump. A drug molecule that inhibits Pgp is regarded as a promising candidate.

3.1.6.1. Drug-like Properties:

Lipinski's rule was applied to assess the drug-like properties of the compounds by examining key molecular characteristics. Tools like Admetlab and SwissADME were used to make predictions regarding molecular weight, partition coefficient, hydrogen bond donors and acceptors, as well as the number of rotatable bonds. The QuickPro tool allowed for accurate predictions of these properties, which helped prioritize promising compounds for further experimental evaluation. Following Lipinski's rule aids in identifying compounds likely to have favorable pharmacokinetic profiles, thereby enhancing the chances of successful drug development (Chen X et al., 2020)

3.1.6.2. Analysis of Boiled Egg Plot:

The Boiled-Egg model is a dependable predictive tool for evaluating ligand lipophilicity and polarity, especially within the brain and intestinal tract. It offers a statistical summary of the bioavailability attributes of ligand molecules, forecasting properties such as absorption in the gastrointestinal system and the permeability of the blood-brain barrier. The Swiss ADME online tool generates BOILED plots that are vital for pharmaceutical research and development.

3.1.7 Molecular Dynamics Simulation:

Molecular dynamics simulations were performed utilizing Desmond 2021-4 on an Acer workstation operating Ubuntu 22.04 to Assess the dynamic stability and conformational changes of protein-ligand complexes in a realistic environment. The topology of the complex was constructed using the OPLS-2005 force field. The system was developed utilizing a straightforward point-charge explicit water model within an orthorhombic simulation box. The solvated complex was neutralized to replicate physiological conditions, employing suitable Na⁺/Cl⁻ counter ions and a 0.15 M salt concentration. Simulations were conducted for 500 ns at 300 K and 1.0325 bar, after a 100-ps system minimization and relaxation.

3.1.8 Prime/MM-GBSA calculations

To estimate binding free energies for protein-ligand complexes and to rank their binding affinities, the docking complexes were refined and analyzed using MM-GBSA (Molecular Mechanics-Generalized Born Surface Area) calculations performed with the Schrödinger Release 2018-3 software. Prime's local optimization function was employed to minimize the docked complexes, ensuring better accuracy in receptor-ligand interactions (Walters, W. P. et al., 2012). The receptor-ligand binding energy was determined using the OPLS-AA 2005 force field and calculated using the following equation:

$$\Delta G_{\text{Bind}} = \Delta E_{\text{MM}} + \Delta G_{\text{Solv}} + \Delta G_{\text{SA}}$$

In this equation:

ΔE_{MM} represents the difference in molecular mechanics energy between the protein-ligand complex and the combined energies of the separate protein and ligand.

ΔG_{Solv} refers to the solvation energy difference between the complex and the sum of the protein and ligand solvation energies, based on the GBSA solvation model.

ΔG_{SA} accounts for the surface area energy, representing the difference in surface area energies between the complex and the unbound protein and ligand.

3.2. Objective 2: In Vitro Analysis: To Evaluate the Efficacy of Selected Molecules Against Cervical Cancer Utilizing Nanoparticle Formulation Techniques.

3.2.1 Collection of Samples:

Betulin was obtained from Otto Kemi Pvt. Shatavarin-IV from Sigma Aldrich Pvt. Ltd., Mumbai. Protein structures were obtained from the PDB database. The cell lines HeLa, SiHa, and HEK were procured from NCCS Pune.

3.2.2 Material

The research involved various standard laboratory-grade chemicals and reagents, such as chloroform (CHCl_3), methanol (CH_3OH), ethyl acetate ($\text{C}_4\text{H}_8\text{O}_2$), petroleum ether, and ethanol ($\text{C}_2\text{H}_5\text{OH}$). Additionally, Dulbecco's Modified Eagle Medium (DMEM) with high glucose, foetal bovine serum (FBS), silver nitrate (AgNO_3), and Roswell Park Memorial Institute-1640 medium (RPMI-1640) were sourced from Hi-Media, Mumbai, India. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was obtained from Thermo-

Fisher Scientific, USA, and dimethyl sulfoxide (DMSO) was acquired from Loba Chemie Pvt. Limited, Colaba, Mumbai. All other solvents and reagents used were of analytical grade.

3.2.3 Maintenance of Cell Cultures:

The SiHa and HeLa cervical carcinoma cell lines, along with HEK (normal kidney) cells, were obtained from the National Centre for Cell Sciences in Pune, India. The cell cultures were maintained in RPMI-1640 supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin/streptomycin solution (10,000 U/ml penicillin and 10,000 µg/ml streptomycin) in a humidified environment with 5% CO₂ at 37 °C.

3.2.4 Nano-particle formation:

3.2.4.1. Zinc Nanoparticle Formulation:

We adhered to the methodology delineated by (MA Saleemi et al., 2022) in Antioxidants, with certain modifications. 1.5 grams of Zinc acetate was solubilized in 90ml of distilled water and positioned on a magnetic stirrer. BE (30 mg) and SH-IV (30 mg) were dissolved in 10 ml of distilled water in a separate beaker. The pH was adjusted to 7.0 using NaOH or HCl when complete dissolution was not achieved. The two solutions were then combined and stirred at 800–1000 rpm at 50–60 °C. A change in solution color indicated the onset of nanoparticle formation. After approximately 4 h, the appearance of a white coloration signaled reaction completion, and stirring was stopped. The mixture was allowed to stand at room temperature for 1 h to promote particle sedimentation, followed by centrifugation at 2000 rpm for 10 min to remove impurities. The purified nanoparticles were collected and stored for further use.

3.2.4.2. Silver Nano-Formulation:

We adopted the method described by Zhao *et al.* (2020) in *ACS Omega*, with minor modifications. 1.5 g of silver nitrate was dissolved in 90 mL of distilled water under continuous magnetic stirring. In a separate beaker, BE (30 mg) and SH-IV (30 mg) were dissolved in 10 mL of a 1:1 ethanol–water mixture. When complete dissolution was not achieved, the pH was adjusted to 8.0 using NaOH. The two solutions were then combined and stirred at 800 rpm at 50 °C. A visible color change, observed after 10–15 min, indicated the onset of nanoparticle formation. Stirring was continued for 3–4 h until the solution developed a brick-red to brown coloration, marking reaction completion. The mixture was allowed to stand at room temperature for 1 h to promote sedimentation, followed by centrifugation at 2000 rpm for 10

min, repeated three times, to remove impurities. The resulting purified nanoparticles were collected and stored for further use.

3.2.5. Characterization of Pure Molecules and Nanoparticles:

3.2.5.1 The XRD analysis:

X-ray diffraction (XRD) is a key analytical technique used to identify phases in crystalline materials and determine the dimensions of their unit cells. The process begins with finely grinding and homogenizing the sample to ensure a consistent average composition. XRD operates on the principle of constructive interference between monochromatic X-rays and the crystalline structure of the sample. Monochromatic X-rays are generated by a cathode ray tube, filtered, and collimated to focus the beam onto the sample. Constructive interference occurs when the incoming rays satisfy Bragg's Law ($n\lambda = 2d \sin \theta$), which relates the wavelength of the X-rays, the diffraction angle, and the spacing between lattice planes. By analyzing the diffraction peaks, minerals can be identified based on their distinct d-spacings. In this study, XRD analysis was performed using the X'PERT PRO instrument at the SAIF/CIL facility at Panjab University. For the analysis, 2 g of the samples were subjected to centrifugation, grinding, and then placed in sample holders.

3.2.5.2 FTIR analysis:

FTIR is an analytical method employed to identify and characterize chemical bonds in molecules. It transforms interference patterns into spectra, elucidating specific chemical bonds via infrared absorption. Bonds demonstrate vibrational motion at diverse frequencies, which can be triggered by light absorption. FTIR analysis (Perkin Elmer Spectrum 400) was performed utilizing the KBr pellet method, scanning wavelengths from 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1} to ascertain functional groups in biosynthesized AgNP and ZnONP.

3.2.5.3 FE-SEM analysis:

The FE-SEM 450 (FEI) was employed for scanning electron microscopy (SEM) analysis, delivering high-resolution imaging for nanoscale materials. It incorporates sophisticated methods for enhancing image quality. The beam energy varies from 30 keV to 50 eV, attaining resolutions of 1.4nm at 1kV and 1nm at 15kV. Samples were prepared by extracting 30 μl aliquots of sonicated samples with micropipettes and depositing them onto glass slides or carbon tabs for SEM analysis.

3.2.5.4. Zetasizer:

The Zetasizer Nano series evaluates zeta potential in liquid media through dynamic light scattering (DLS). This method quantifies variations in light intensity caused by particle movement. The zeta potential reflects the stability of colloidal systems, where elevated values imply resistance to flocculation. The Zetasizer version 7.11 (Malvern, UK) was employed to assess zeta potential in metal nanoparticle solutions, utilizing sonicated samples placed in disposable capillary cells.

3.2.6. Cell Proliferation Inhibition Assay (MTT Assay)

The MTT assay was performed as previously outlined (Qasim et al., 2017) to quantify cytotoxic and growth inhibition of compounds on cervical cancer cell lines and to determine the IC₅₀ values of pure BE, SH-IV, and their chemically synthesized derivatives, ZnBE and AgSH-IV, against HeLa, SiHa, and HEK cells. Before the assay, HeLa, SiHa, and HEK cells were maintained in DMEM medium. The cells (8×10^3 cells per well) were seeded in a 96-well culture plate and treated with various concentrations (12.5 μ M, 25 μ M, 50 μ M, 100 μ M) for 24 hours, in a final volume of 200 μ l of DMEM medium. After treatment, the medium was removed, and 20 μ l of MTT solution (5 mg/ml in PBS) was added to fresh medium. After incubating for 3 hours at 37 °C, 100 μ l of DMSO was added to each well, and the plates were mixed for 1 minute. Absorbance was then measured at 570 nm using a multi-well plate reader. The percentage of proliferation inhibition was calculated as a fraction of the control group (without any compound).

3.2.7. Sulphorhodamine B (SRB) Assay:

In-vitro cytotoxic activity against different dose-dependent treatments of Pure BE, SH-IV, and their chemically synthesized ZnBE and AgSH-IV nanoparticles in the SiHa cancer cell line was performed using 96-well culture plates in triplicate. To each well, the 8×10^3 cells/well were seeded and allowed to grow overnight at 37°C for overnight in 5% carbon dioxide incubator. On the following day, a different concentration (doses, i.e.) of drugs was added. The plates were further incubated for 24 hours, and 100 μ L of 10% (TCA) trichloroacetic acid was added gently to cease the cell growth by thin layering of trichloroacetic acid into each well. The plates were further incubated at 37°C for 1 h to fix the cells attached to the bottom of the wells. The plates were washed five times with distilled water to remove traces of medium, trichloroacetic acid, sample, and serum proteins, and then air dried. The cell growth in air-dried plates was measured by staining with sulforhodamine B dye. The unbound dye was removed by

dissolving Tris-base buffer (100 μ L/ well, 0.01M, pH 10.4), and plates were stirred for 5 min on a mechanical shaker. The optical density was measured at 540 nm on an ELISA reader.

3.2.8 Scratch/Wound Healing Assay

To evaluate the anti-migratory effect of the nanoparticles of BE and SH-IV, SiHa cells were grown in six-well plates until they reached 70-80% confluence. A consistent wound was made by scratching across each well's width with a 10 μ L micropipette tip, which removed the attached cells. The compounds ZnO-BE and AgNO₃-SH were added to the wells at concentrations of 10 μ M and 20 μ M, respectively, with a control well remaining untreated for comparison. Images were captured at 0 hours and after 24 hours using the EVOS™ M5000 Imaging System, which features a digital inverted benchtop microscope. The width of the wound in both the untreated control and treated wells was measured at both time points, and the results were determined by comparing the change in wound area after 24 hours to the control groups.

3.3 Objective 3: Qualitative and Quantitative Evaluation of Epigenetically Modified Proteins

3.3.1 Measurement of Epigenetic Biomarkers:

The SiHa human CCa cell line was incubated overnight and then treated for 48 hours with pure Betulin (BE), pure Shatavarin (SH), ZnO-BE, and AgNO₃-SH, each at concentrations of 1.7 + 14.7/2.2 + 78 μ M. For the control group, DMSO was used at a final concentration of 0.2%. Total RNA was isolated from confluent monolayers of the SiHa cell line following the manufacturer's protocol with Tri-reagent (Sigma). All the samples were treated with RNase-free DNase to eliminate any contaminating DNA.

To synthesize complementary DNA (cDNA), (3 μ g) of RNA was transcribed using the RevertAid cDNA synthesis reagent, as per the manufacturer's instructions. SYBR Green PCR amplification was performed on a Stratagene MX3000p Real-time PCR System, with GAPDH used as the housekeeping gene. The specific primer sequences are listed in Table 2. The concentration and purity of RNA were assessed using the Nanodrop ND-1000 (Thermo Scientific, USA), measuring absorbance at 230nm, 260nm, and 280nm. The RNA samples were segmented, stored at -80 °C, and adjusted to a concentration of 250 ng/ μ L.

The iScript cDNA Synthesis Kit (BioRad) was used to convert one microgram of RNA into cDNA following the manufacturer's guidelines. The samples were diluted to 60 μ L with RNase-

free de-ionized water and stored at -20 °C. RNA was extracted from confluent SiHa cell lines grown in either standard media or SCM. The reverse transcription PCR (RT-PCR) entailed an initial denaturation step at 95 °C for 2 minutes, followed by 40 cycles of 5 seconds at 95 °C, 5 seconds at 60 °C, and a final extension at 72 °C for 5 minutes (Qayum A et al., 2022)

Table 2: Primer Specifications for Real-time PCR:

S.No.	Gene	Primer Sequence	Product Size	Tm
1.	HDAC4	F.P: CCCTCTACACATCG CCATCC	164 bp	58°C
		R.P: TCAGGTAGGAGTGAGGTGG		
2.	DNMT3A	F.P: AAGACCCCTGGAAGTCTGATAC	138 bp	58°C
		R.P: GAATGCCCAAGTCCTTCAGC		
3.	KMT2C	F.P: CAAATGCTACCAGGCTCTGC	194 bp	58°C
		R.P: CCCCCTCTCTGTCAGAAGTAA		

3.3.2 Quantification of the Epigenetic Protein via Western Blot Analysis:

To evaluate the effect of BE, SH-IV, ZnO-BE, and AGNO₃-SH on the expression of HDAC4 in SiHa cells, immunoblotting was performed. For this, SiHa cells were exposed to 10 µg/ml of each drug for 48 hours. After 48 hours, cells were trypsinized and incubated for 1 hour with RIPA lysis buffer supplemented with protease inhibitor cocktail (Promega, G6521). Then lysates were centrifuged at 12000 RPM for 20 minutes at 4°C. After centrifugation supernatant was collected and stored. Protein concentration was determined using the Coomassie protein assay (Thermo Scientific, 1856209). Protein separation was done by SDS-PAGE by loading 50 µg protein from each lysate and subsequently transferred to PVDF membrane (Millipore, IPVH00010). After blocking the PVDF membrane with 5% BSA, the membrane was incubated overnight with primary anti-HDAC (CST, cat# MA5-55207) and HRP-conjugate ACTB (Sigma, cat# 07-1570) antibody, subsequently with HRP-conjugated secondary antibody. The image was captured in a Chemidoc Chemoluminescent imaging system (Syngene Chemidoc

system) by adding HRP-substrate. Protein expression was then quantified using ImageJ software. (Kour N et al., 2024).

3.3.3. Statistical examination:

The calculated mean and standard error were determined by conducting each experiment three times. The mean intensity of each band was quantified using ImageJ software v1.51K (NIH, U.S.A.) for western blot analysis. GraphPad Prism 6.1 software (CA, USA) was employed to construct p-values between control and treated groups using the mean and standard error of the mean (SEM). A p-value of ≤ 0.05 was considered significant.

3.4 Objective 4: Evaluate the expression levels of critical epigenetic regulators (DNMT3A and HDAC4) in CCa tissues relative to normal tissues.

3.4.1. Data Sources:

UALCAN: This platform enables users to investigate cancer transcriptome data across diverse cancers and their corresponding normal tissues. It offers insights into gene expression levels, survival analysis, and correlations with clinical parameters.

GEPIA: This online tool facilitates interactive analysis of RNA sequencing expression data derived from The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) projects.

3.4.2. Methodology:

Expression Analysis: The expression levels of DNMT3A, HDAC4, and KMT2C were evaluated in CCa samples versus normal tissue samples. The databases offer visual representations and statistical information to demonstrate variations in expression.

Correlation Evaluation: Utilizing UALCAN, we assessed the relationship between the expression of HDAC4 and DNMT3A and various clinical parameters, including cancer stage, race, and age. This is essential for comprehending how these genes may influence cancer progression.

3.4.3 Target Identification for BE, SH-IV, and Cervical Cancer

3.4.3.1. Identification of Targets:

The targets for BE and SH-IV were obtained from two major databases: PharmMapper. This tool forecasts possible protein targets for small molecules based on established interactions.

Swiss-target prediction: This database offers target predictions based on chemical similarity. Targets pertinent to CCa were gathered from DisGeNET: A repository that consolidates genetic associations with diseases. The Comparative Toxicogenomics Database (CTD) offers insights into the impact of chemicals on gene and protein interactions.

3.4.3.2. Construction of Drug and Disease Target Network

A protein-protein interaction (PPI) network was established to illustrate the interactions among the targets of BE, SH-IV, and cervical cancer. This was accomplished utilizing the STRING database (version 11.0). A confidence score exceeding 0.4 was employed to ensure that only substantial interactions were taken into account. An FDR of 5% enhanced the analysis's rigor. The produced PPI networks were imported into Cytoscope (version 3.8.2) for visualization purposes. This software facilitates the development of intricate network maps, aiding in the comprehension of the complex interactions between natural compounds and cancer targets.

3.4.4. Intersected Target Network Analysis

3.4.4.1. Metrics of Centrality:

The CytoNCA plugin was employed to compute three centrality scores for the intersected network: Degree Centrality (DC), which represents the quantity of direct connections associated with a protein. Betweenness Centrality (BC): Indicates the degree to which a protein serves as a conduit between other proteins within the network. Closeness Centrality (CC): Assesses the rapidity with which a protein can engage with all other proteins within the network.

3.4.4.2. Identification of Core Targets:

A Venn diagram was employed to illustrate the intersection among the targets of BE SH-IV and cervical cancer. Core targets were identified based on their centrality scores (degree centrality, betweenness centrality, and closeness centrality) being equal to or exceeding the median.

3.4.4.3. Preparation of Subnetwork:

A subnetwork of these primary targets was constructed utilizing the CytoNCA plugin, facilitating a concentrated examination of the most pertinent proteins engaged in the interaction network.

3.4.5. Analysis of Functional and Pathway Enrichment

3.4.5.1. Analysis of Enrichment:

Gene Ontology (GO) enrichment analysis was conducted to classify the biological processes, molecular functions, and cellular components related to the core targets. This offers insights into the possible functions of these proteins in cervical cancer.

3.4.5.2. KEGG Pathway Analysis:

The DAVID bioinformatics resource facilitated the mapping of core targets to established metabolic and signalling pathways, thereby elucidating potential therapeutic pathways influenced by BE and SH-IV.

3.4.5.3. Analysis of Data:

The findings from both GO and KEGG analyses are essential for comprehending the mechanisms by which BE and SH-IV demonstrate their anti-cancer properties. Graphical depictions of these analyses

Chapter 4

4. RESULTS AND DISCUSSION

Cervical cancer is a significant health concern for women, primarily associated with human papillomaviruses (HPVs), which depend on oncogenic proteins and specific epigenetic factors for their effects. Natural compounds are being recognized as potential anticancer agents due to their accessibility and reduced side effects. Herbal extracts have proven effective against viral oncogenes and in regulating host gene expression, targeting tumor cells by modulating apoptosis, autophagy, and inhibiting NF- κ B and angiogenesis. Apoptosis is vital for normal cell function and is regulated by proteins like Bcl-2, Bax, and caspases. This study aims to identify natural compounds that inhibit aromatase using a structure-based pharmacophore approach and to evaluate their efficacy against CCa cells in vitro.

4.1 Objective 1: Identification and selection of the potent phytochemical using *in-silico* virtual screening techniques.

4.1.1 In-silico Selection of Phytochemical:

4.1.1.1 Identification of Phytochemicals:

An extensive review of the literature and phytochemical repositories uncovered multiple secondary metabolites with confirmed anti-cancer effects against Cancer and more specifically CCa. The analysis consisted of three fundamental components: A comprehensive examination of relevant scientific literature revealed specific phytochemicals with anticancer properties. A literature search on PubMed involved scanning abstracts, resulting in the identification of supplementary phytochemicals referenced in recent studies. Database Search: Queries in the Zinc database and PubChem yielded 3D structures of the identified phytochemicals, facilitating further analysis. A graphical depiction of the methodology illustrated the workflow (Figure 8) and interconnections among manual review, literature search, and database search, emphasizing the systematic identification process. The identified phytochemicals are listed in the Annexure.

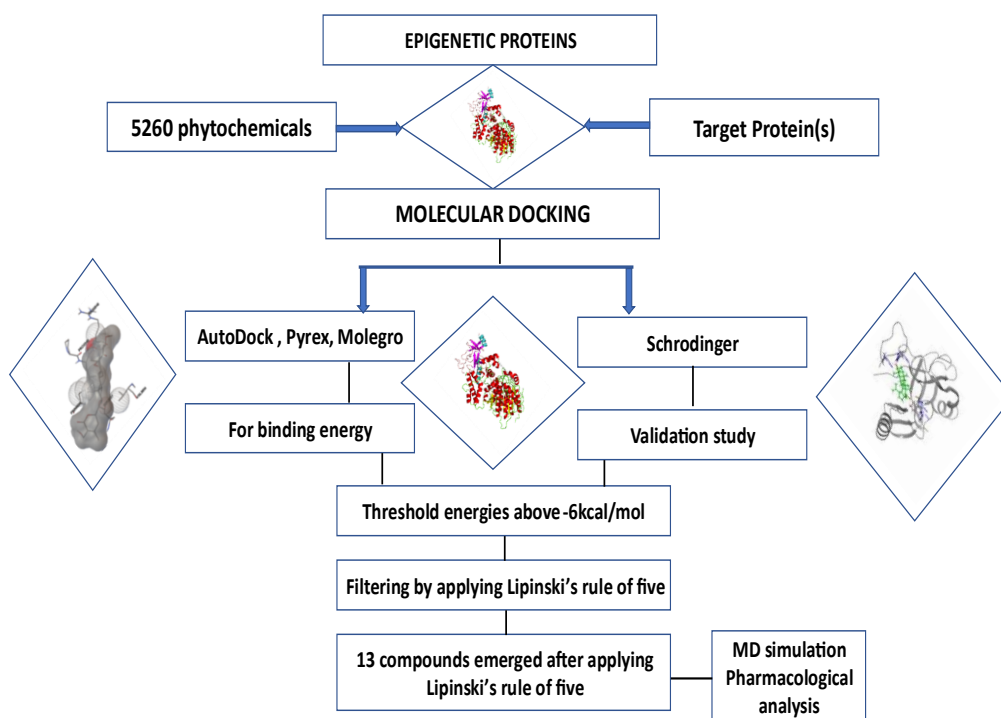


Figure.8: A graphical illustration of the workflow of identification of potent phytochemicals using an in-silico approach.

4.1.2 Screening of Phytochemicals using PyRx's Autodock

The selected phytochemicals were further evaluated for their binding affinity with the selected protein target(s) DNMT3A (2qrv), HDAC4 (2qvj), and KMT2C (5f59). The binding affinity results of the top binding molecules against the three targets were summarised in Table 3. The molecular docking results predicted about 100 top binding molecules for further analysis

Table 3: Docking Score of top binding ligands using PyRx's Autodock (4.2).

S.no	Phytochemical	Pubchem id	Binding energy (kcal/mol)			S.no	Phytochemical	Pubchem id	Binding energy (kcal/mol)		
			Dnmt3a	Hdac4	Kmt2c				Dnmt3a	Hdac4	Kmt2c
1	Betulin	72326	-9.8	-10.3	-9.4	8	Withasomnilide (102066413)	102066413	-8.1	-6.8	-6.1
2	Shatavarin iv	441896	-9.7	-10.3	-9.5	9	Albanol a	44567218	-8.1	-7	-6.4
3	2'-o- acetylsalsoloside c	23251215	-9.7	.8.5	-7.9	10	Isobutyl shikonin	479500	-8	-9.8	-8
4	3'-o- acetylsalsoloside c	23251214	-9.6	-9.7	-8.8	11	Anahygrine	12306778	-7.9	-8.5	-6.8
5	Sitoindoside ix	189586	-9.2	-8.1	-6.5	12	Withanolide q	101281365	-7.9	-6.9	-6.2
6	Honokiol	72303	-8.7	-7	-5.6	13	Saffron oil	6450930	-7.9	-7.2	-6.2
7	Scutellarein	185617	-8.6	-7.8	-6.3	14	Apigenin	5280443	-7.7	-7.4	-6.7

S.no	Phytochemical	Pubchem id	Binding energy (kcal/mol)			S.no	Phytochemical	Pubchem id	Binding energy (kcal/mol)		
			Dnmt3a	Hdac4	Kmt2c				Dnmt3a	Hdac4	Kmt2c
15	Withaferin b	11113907	-8.5	-7.8	-6.9	24	Beta-beta- dimethylacrylshikonin	479499	-7.7	-6	-5.5
16	Beta- hydroxyisovaleryl shikonin	479502	-8.4	-6.9	-6.4	25	Gingerol	442793	-7.6	-8.8	-6.9
17	Lansoprazole	3883	-8.3	-7.2	-6.2	26	Lupeol palmitate	161739	-7.5	-8.4	-6.1
18	Rutin	5280805	-8.2	-7.5	-6.8	27	Shikonin	479503	-7.5	-7.5	-7.3
19	Isovalerylshikonin	479497	-8.2	-7.7	-7.5	28	Withanolide r	101281364	-7.5	-6.8	-5.9
20	Isoliquiritigenin	638278	-8.1	-6.3	-7	29	Withanoside iv	71312551	-7.5	-9.3	-7
21	2-methyl-n-butyryl) shikonin	10429346	-8.1	-7.6	-5.6	30	<u>neferine</u>	159654	-7.4	-6.9	-5.8
22	Pseudotropine	8424	-7.4	-7.9	-6.9	31	Physagulin d	10100412	-6.9	-8.7	-6.6
23	Harmine	5280953	-7.4	-7.7	-6.7	32	Viscosalactone b	57403080	-6.9	-8.7	-6.8

S.no	Phytochemical	Pubchem id	Binding energy (kcal/mol)			S.no	Phytochemical	Pubchem id	Binding energy (kcal/mol)		
			Dnmt3a	Hdac4	Kmt2c				Dnmt3a	Hdac4	Kmt2c
33	Oblongifolin a	53364454	-7.4	-5.7	-5.7	44	Withasomniferol c	101710597	-6.9	-8.7	-6.9
34	Withasomniferanolide	102066414	-7.4	-6	-5.6	45	Dichloromethane	6344	-6.9	-8.9	-6.9
35	Withanolide o	23266146	-7.4	-6.6	-5.6	46	Medicagenic acid	65048	-6.8	-7	-6.7
36	Propionylshikonin	153984	-7.2	-7.3	-6	47	Hederagenin	73299	-6.8	-7.4	-6.9
37	Sominone	44249449	-7.2	-4.2	-3.2	48	Avenacin a1	131751579	-6.8	-8.2	-6.7
38	Butylshikonin	10089766	-7.1	-5.4	-4.8	49	Glycyrrhizin	14982	-6.8	-7.2	-6.6
39	Quercetin	5280343	-7.1	-7.9	-6.8	50	Soyasapogenol	3083637	-6.8	-8.9	-7.5
40	2,3-dihydro-3beta-methoxy withaferin a	10767792	-7.1	-7.6	-6.9	51	Ursolic acid	64945	-6.8	-8	-7.7
41	choline,	305	-7.1	-6.9	-6.6	52	Uvaol	92802	-6.8	-7.6	-6.7
42	Resveratrol	445154	-7.1	-7.6	-7	53	Alfa amylin	73170	-6.7	-8.1	-7
43	Withasomniferol a	101710595	-7.1	-2.2	-2.5	54	Erythrodiol	101761	-6.7	-7.6	-7.6

S.no	Phytochemical	Pubchem id	Binding energy (kcal/mol)			S.no	Phytochemical	Pubchem id	Binding energy (kcal/mol)		
			Dnmt3a	Hdac4	Kmt2c				Dnmt3a	Hdac4	Kmt2c
55	Crinamin	73620	-7.1	-7.2	-6	65	Beta amyrin	73145	-6.7	-8.1	-7.3
56	Scaposin	633124	-7.1	-7.1	-4.2	66	Maslinic	73659	-6.7	-8.6	-7.5
57	Viscosalactone b	57403080	-7	-7.9	-6.7	67	Betulin	72326	-6.6	-8.5	-8
58	Withalongolide a	56649343	-7	-7.9	-6.6	68	Lupeol	259846	-6.6	-7.9	-7.3
59	Acetylshikonin	32464	-7	-7.8	-7.3	69	Betulinol	236415	-6.5	-8.8	-8.3
60	Withanoside vi	91827019	-6.9	-8.2	-6.5	70	Betulinic acid	64971	-6.5	-8	-7.3
61	Digitonin	6474107	-6.4	-8.1	-7.9	71	Parkeol	12313974	-5.6	-3	-3.2
62	Baccharene	605576	-6.4	-7.8	-7.5	72	Shatavarin iv	441896	-5.2	-5.4	-4.4
63	Cholesterol	5991	-6.4	-8.2	-7.1	73	Salsoloside c	23844065	-5.2	-5	-3.8
64	Hopane	10115	-6.4	-7.6	-6.7	74	2'-o-acetylsalsoloside c	23251215	-5.2	-4.7	-4.6

S.no	Phytochemical	Pubchem id	Binding energy (kcal/mol)			S.no	Phytochemical	Pubchem id	Binding energy (kcal/mol)		
			Dnmt3a	Hdac4	Kmt2c				Dnmt3a	Hdac4	Kmt2c
75	Asiaticoside β	91618002	-6.4	-8.2	-7	85	3'-o-acetylsalsolose c	23251214	-5.1	-5.6	-3.3
76	Madecassoside	45356919	-6.3	-8.3	-6.9	86	Forskolin	47963	-4.7	-9	-8.1
77	Asiaticoside	11954171	-6.3	-7.7	-6.8	87	24-hydroxyolean-12-en-3-one	101586331	-4.6	-8.1	-7.4
78	Madecassic acid	73412	-6.3	-8.4	-6.7	88	Oleanolic acid	10494	-4.6	-9	-7.6
79	Asiatic acid	119034	-6.1	-7.9	-7	89	Sarsasapogenin	92095	-4.2	-7.9	-7.4
80	Fernenol	12305178	-6.1	-8.2	-7.2	90	Shatavarin 1	101406647	-4.2	-7.7	-6.3
81	Sorghumol	91885232	-6	-7.7	-6.9	91	Allicin	65036	-3.9	-8	-7.4
82	Tervalon	8593	-6	-8.2	-7.4	92	Somniferine	14106343	-3.5	-8	-6.8
83	Delta amyrin	12358447	-5.9	-7.9	-7.4	93	Withasomnine	442877	-3.2	-8	-7
84	Isomaltol	18898	-5.9	-5.5	-4.7	94	Hentriacontane,	12410	-3.1	-7.8	-7.2

S.no	Phytochemical	Pubchem id	Binding energy (kcal/mol)			S.no	Phytochemical	Pubchem id	Binding energy (kcal/mol)		
			Dnmt3a	Hdac4	Kmt2c				Dnmt3a	Hdac4	Kmt2c
95	Hopenol	10093995	-5.7	-4.4	-4.2	98	Garcinol	5281560	-2.1	-8	-7.7
96	Oryzalin	139597735	-5.6	-4.4	-4.2	99	Qurecitin ,	5280343	-2.3	-5.6	-7.2
97	Saponine	131757885	-2.1	-7	-6.5	100	Zeylenol	10992619	-2.1	-2.3	-7.1

4.1.3 Comprehensive Molecular Docking Using Glide:

Computer-assisted drug design (CADD) has become an essential computational tool in drug discovery and development efforts (Anwar et al., 2021). In this study, the Schrodinger-Glide software was utilized for comprehensive docking analyses to evaluate the binding scores of top phytochemicals identified using PyRx's Autodock (version 4.2) against the target proteins HDAC4, DNMT3A, and KMT2C. Ligands generally interact with target proteins through a variety of mechanisms, as revealed by docking studies. The results of the molecular docking are highly dependent on the choice of binding site residues. Residues adjacent to the co-crystallized molecule in the experimentally determined structures of epigenetic proteins were chosen as binding site residues for molecular docking analysis (Table 4) (Kour S et al., 2024). Among the top 100 phytochemicals, Betulin (BE) and Shatavarin-IV (SH-IV) demonstrated the highest binding energies with the target proteins via their active site residues

Table 4: Active site residues of the target proteins.

Sl. No.	Epigenetic Target	PDB ID	Residues forming the binding site
1.	DNA Methyltransferase (DNMT3A)	2qrv	TRP889, ILEA639, SERA888, ARG887, GLY-A638, GLY-A703, LEU-A726, PRO-A705, VAL-A661, ARG A-684, VAL A-683, ASP A-682, PHE A-636, SER A-659, CYS A-662, GLU A-660
2.	Histone Deacetylase 4 (HDAC4)	2qvj	LEU156, ASP158, GLY159, ILE167, ASN168, GLU169, ALA196, VAL198, ALA227, TRP290, PHE299, ALA330, GLY331.
3.	Histone-lysine N-methyltransferase (KMT2C)	5f59	LEU A 4783, TYR A4825, ARG A4845, GLN A 4781, TYR A 4846, GLY A 4782, ASN A 4848, TYR A 4886, CYS A 4899, ILE A 4780, HIS A 4849, MET A 4910, PRO A 4898, ILE A 4897

Table 5: Molecular docking results of top binding phytochemicals with the epigenetic proteins and their interaction profiles.

Ligands	Docking Score	Binding energy	Type of Interaction and its number	Interacting residues
	(Kcal/mol)			
DNA Methyltransferase (DNMT3A), PDB-ID-(2QRV)				
Betulin	-7.8	-50.3	Hydrogen bond (2)	PHE636, VAL661
Shatavarin iv	-6.7	-43.0	Hydrogen bond (3)	ARG483, GLY886, GLY886
Salsolside C	-6.4	-32.0	Hydrogen-bond (2)/ salt bridge (1)	CYS662, GLU660/ARG887
2'-O-Acetylsalsolside C	-5.7	-34.4	Hydrogen bond (3)	ILE639, PHE636, VAL661
3'-O-Acetylsalsolside C	-5.0	-38.5	Hydrogen bond (3)	PRO705, ILE639, CYS662
Forskolin	-2.8	-21.8	Hydrogen bond (1)	PHE636
Alpha-Amyrin	-2.3	-20.6	Hydrogen bond (1)	CYS706
Betulinic acid	-1.4	-15.5	Hydrogen bond (3)	SER704, PRO705, ILE639
24-Hydroxyolean-12-en-3-one	-1.3	-13.7	Hydrogen bond (2)	LEU726, PRO705

Oleanolic acid	-1.3	-12.4	Hydrogen bond (3)	TRP889, ARG887, VAL661
Sarsasapogenin	-0.7	-17.2	Hydrogen bond (1)	CYS662
Histone deacetylase-4 (HDAC-4) (PDB-ID-2QVJ)				
Shatavarin iv	-10.19	-53.2	Hydrogen bond	HIS 159,
Betulin	-9.7	-41.8	Hydrogen bond (2)	HIS159, PRO 298
Salsolosite C	-7.7	-33.4	Hydrogen bond (3)	HIS158, PRO156, PHE168
2'-O-Acetylsalsolosite C	-6.6	-53.3	Hydrogen bond (3)	PHE298, PHE226, PHE227
3'-O-Acetylsalsolosite C	-6.4	-45.0	Hydrogen bond (2)/ Salt bridge (1)	LEU229, PRO298/GLU329
Forskolin	-3.7	-29.0	Hydrogen bond (3)	GLY331, GLY330, PHE226
Alpha-Amyrin	-3.2	-26.9	Hydrogen bond (2)	HIS157, HIS198
Betulinic acid	-2.9	-23.3	Hydrogen bond (3)	HIS332, PRO156, ASP198
24-Hydroxyolean-12-en-3-one	-2.8	-26.0	Hydrogen bond (2)	LEU299, PHE227
Oleanolic acid	-2.8	-23.3	Hydrogen bond (4)	ASP290, HIS157, PRO156, PHE168

Sarsasapogenin	-2.6	-22.6	Hydrogen bond (2)	PRO156, LEU299
Histone-lysine N-methyltransferase (KMT2C) (PDB-ID-5F59)				
Shatavarin iv	-10.41	-55.7	Hydrogen bond (4)	MET4826, LEU4783, TRY4886, GLU4814
Betulin	-9.8	-44.6	Hydrogen bond (4)	HIS4900, PRO4898, TYR4800, ARG4845.
Salsoloxide C	-7.1	-33.4	Hydrogen bond (2)	ILE4980, GLN4781
2'-O-Acetylsalsoloxide C	-6.6	-53.3	Hydrogen bond (2)	TRY4886, TRY4884
3'-O-Acetylsalsoloxide C	-5.8	-45.0	Hydrogen bond (2)	ILE4864, GLN4885
Forskolin	-3.1	-29.0	Hydrogen bond (2)	PRO4848, HIS4900
Alpha-Amyrin	-2.7	-26.9	Hydrogen bond (4)	ARG4845, PRO4898, ILE4980, ASN4848
Betulinic acid	-2.5	-23.3	Hydrogen bond (2)	TRY 4800, PRO4898
24-Hydroxyolean-12-en-3-one	-2.3	-26.0	Hydrogen bond (3)	GLN4781, VAL4824, HIS4900
Oleanolic acid	-1.9	-23.3	Hydrogen bond (2)	PRO4898, ARG4845
Sarsasapogenin	-1.8	-22.6	Hydrogen bond (4)	GLN4781, TRY4886, ASN4848, VAL4824

The molecular interaction of phytochemicals against the target protein DNMT3A is reported with a Glide score ranging from -7.811 to -0.78 kcal/mol, as the highest and lowest binding energies of the docked ligands (Table 5). The flexible docking of BE yielded an energy of -50.305 kcal/mol and a Glide score of -7.811 kcal/mol. The ligand molecule has formed a hydrogen bond with Phe636 at a bond distance of 2.15 Å. The amino group (NH) in Val661 has formed a hydrogen bond with the OH molecule of BE with a bond distance of 2.01 Å (Figure 9a). Similarly, SH-IV binding with DNMT3A resulted in the docking energy of -43.028 kcal/mol and a Glide score of -6.735 kcal/mol. SH-IV formed three hydrogen bonds: one with Arg483 and two bonds with Gly886 at a bond distance of 2.16 Å, 2.03 Å, and 2.13 Å, respectively (Figure 10a).

The molecular interaction of phytochemicals against KMT2C resulted in a Glide score ranging from -9.83 to -1.8 kcal/mol (Table 6). The docking studies for KMT2C indicated four hydrogen bonds between BE and the protein, with the hydroxyl groups at both ends of BE forming two hydrogen bonds with the receptor (Figure 9c). One hydrogen bond was formed with Pro4898 at 1.87 Å (OH---OC), and another with His4900 at a bond distance of 2 Å (NH---OH). A hydroxyl group at the opposite end formed hydrogen bonds with Tyr4800 and Arg4845 at bond distances of 1.69 Å and 1.89 Å, respectively. His4900 was also noted to establish a hydrogen bond with the co-crystallized ligand S-adenosyl-L-homocysteine at 2.86 Å. Alongside these interactions, BE-interacting residues Arg4845 and Pro4898 exhibited hydrophobic contacts with KMT2C, similar to those observed with the co-crystallized ligand. SH-IV formed six hydrogen bonds with KMT2C, with two hydroxyl groups bonding with Met4826 at distances of 2.56 Å and 1.79 Å (HO-OC), and further hydrogen bonds with LEU4783, TRY4886, and GLU4814 at distances of 1.50 Å, 2.16 Å, and 1.67 Å, respectively (Figure 10c).

The interaction of phytochemicals with HDAC4 yielded Glide scores ranging from -9.720 to -2.698 kcal/mol (Table 5), with SH-IV and BE showing the highest binding affinities at -10.1 kcal/mol and -9.720 kcal/mol, respectively, and binding energies of -53.2 kcal/mol and -41.835 kcal/mol. The HDAC4-BE complex shows two hydrogen bonds alongside metal coordination. The hydroxyl group of BE interacted with His159 at a bond distance of 2.19 Å (OH---NC) and coordinated with the catalytic metal ion Zn1415. The histidine residue formed a hydrogen bond with the co-crystallized inhibitor TFG and coordinated with the Zn ion (Lohkamp B et al., 2003). Additionally, the oxygen in Pro298 established a hydrogen bond with a hydrogen atom of a hydroxyl group in BE at a bond distance of 2.04 Å (CN---HO) (Figure 9b).

In the HDAC4-SH-IV complex, stability was maintained through three hydrogen bonds, with two hydroxyl groups bonding with HIS159 at distances of 2.14 Å and 2.28 Å (HO-OC), and another hydrogen bond with ARG154 at 2.68 Å (Figure 10b).

Overall, the docking analysis indicates that the hydroxyl groups at both ends of SH-IV and BE target the epigenetic proteins effectively, with hydrogen bond distances ranging from 1.69 Å to 2.19 Å. These interactions contribute significantly to the stability of the complexes and are crucial for the inhibitory activity of the target proteins. However, it is important to validate the pharmacokinetic properties of these compounds before assessing their pharmacological potential.

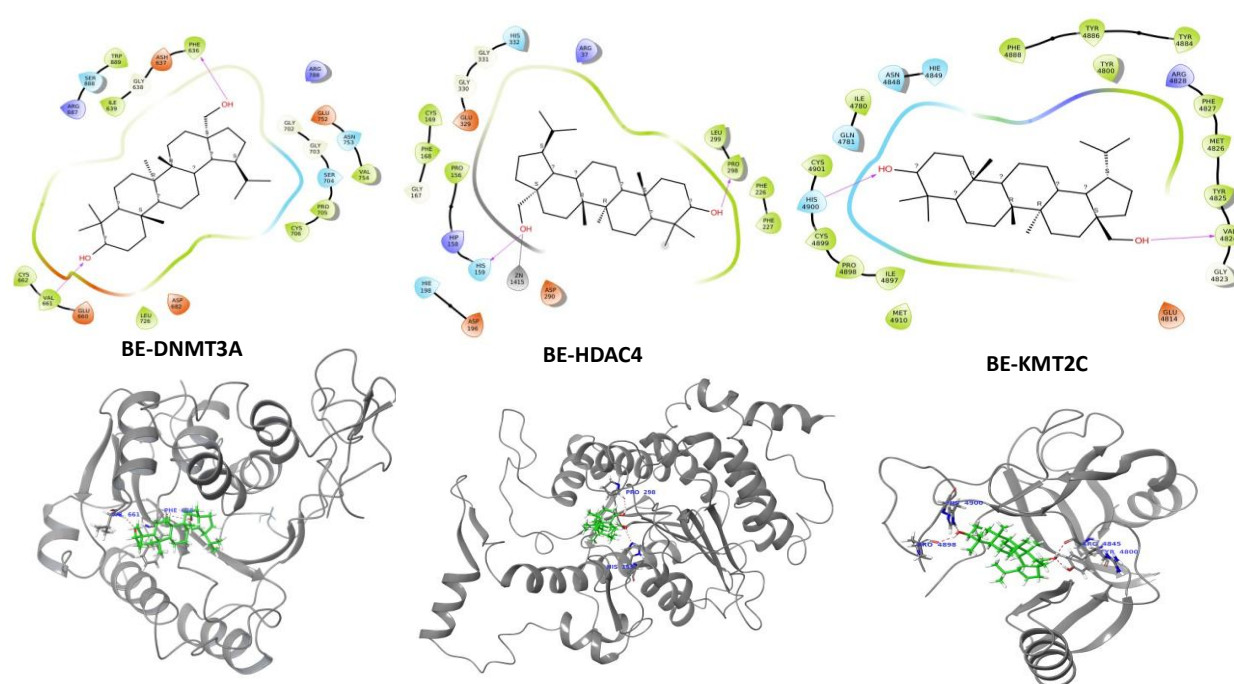


Figure 9: The two-dimensional and three-dimensional interaction profiles of Betulin (BE) with three epigenetic targets demonstrate notable binding interactions. In the BE-DNMT3A complex, two hydrogen bonds were formed with Val661 and Phe636. The BE-HDAC4 complex established two hydrogen bonds with His159 and Pro28. Furthermore, the BE-KMT2C interaction involved the formation of four hydrogen bonds with His4900, Pro4894, Tyr4800, and Arg4845.

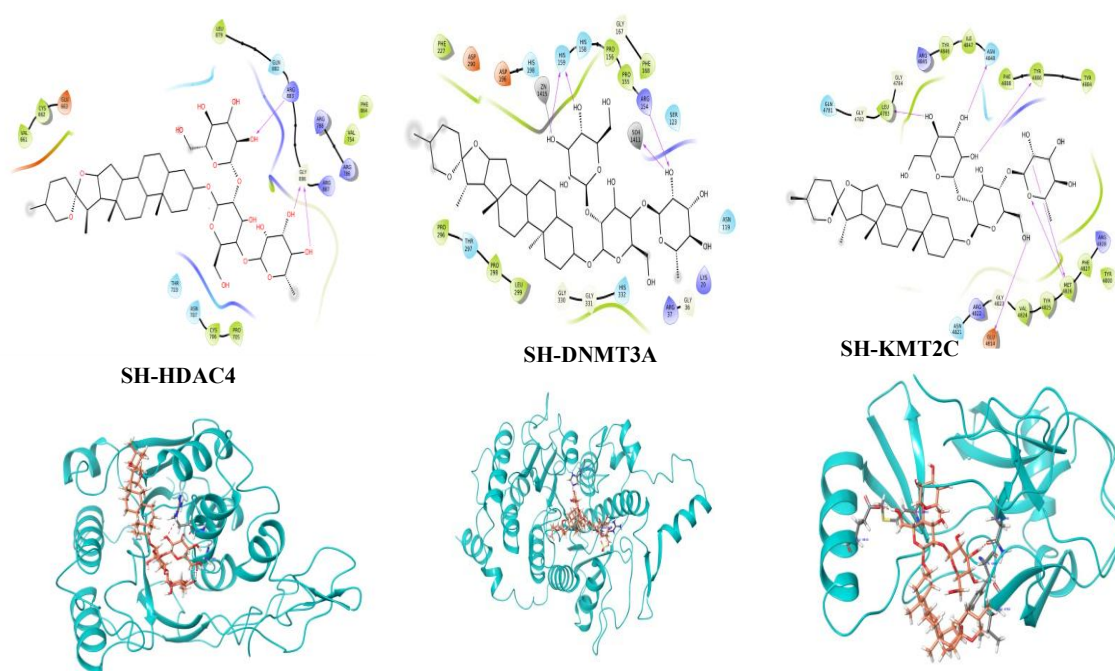


Figure 10: The two-dimensional and three-dimensional interaction profiles of Shatavarin-IV (SH-IV) with three epigenetic targets reveal significant binding characteristics. In the SH-IV-DNMT3A complex, two hydrogen bonds were formed with Val661 and Phe636. Meanwhile, the SH-IV-HDAC4 interaction established two hydrogen bonds with His159 and Pro28. Additionally, the SH-IV-KMT2C complex exhibited four hydrogen bonds, interacting with His4900, Pro4894, Tyr4800, and Arg4845

4.1.4. Physicochemical properties of ligand molecules:

Predicting the pharmacokinetics of a potential therapeutic molecule necessitates an evaluation of ADME (Absorption, Distribution, Metabolism, and Excretion), which is crucial in the realms of drug discovery and development. This evaluation typically occurs in conjunction with molecular interaction studies to assess the safety and efficacy of pharmacological agents (Pinzi L et al., 201). The quantifiable assessment of drug-likeness, known as Quantitative Estimation of Drug-likeness (QED), integrates desirability functions associated with various parameters, including molecular weight, log P, hydrogen bond donors and acceptors, aromatic rings, rotatable bonds, polar surface area, and undesirable functional group indicators. Compounds with a QED score around 0.67 are considered attractive, those with a score of approximately 0.49 are categorized as interesting, while scores around 0.34 indicate excessive complexity and lower desirability.

The primary binding phytochemicals identified through docking were analyzed for their physicochemical properties, with their SMILES representations uploaded for further evaluation. The server performed several steps for molecule curation, including calculating an accessibility score that assesses the ease of synthesizing drug-like compounds based on fragment contributions and complexity penalties. The Synthetic Accessibility Score (SAScore) for the phytochemicals was determined to be 4.936. Thirteen molecules were found to comply with Lipinski's Rule of Five, based on their physicochemical properties and insights from medicinal chemistry, as summarized in Table 6. The compounds displayed enhanced absorption and permeability according to the Pfizer Rule, categorizing them as mildly toxic candidates due to high Log P (>3) and low Total Polar Surface Area (TPSA <75). Notably, one molecule with a mass exceeding 400 g/mol exhibited Log P and Log D values of 5.328 and 5.009, respectively. Additionally, both Betulin (BE) and Shatavarin-IV (SH-IV) violated the GSK Rule and the Golden Triangle, indicating potential for a notable pharmacological profile. Their pharmacokinetic characteristics suggest they may inhibit P-glycoprotein (PgP), a significant liver efflux pump in drug discovery. Consequently, a molecule that inhibits this pump could be a strong candidate for further investigation, underscoring its potential as a therapeutic agent (Brotelli F et al., 2011).

4.1.5 ADME PROPERTIES

ADME predictions are essential for estimating the *in vivo* pharmacokinetics of potential therapeutic agents during drug development (Chandrasekaran B et al., 2018). These assessments are often conducted alongside molecular docking to evaluate the safety and efficacy of pharmaceutical compounds. The results show that the concentrations of the studied chemicals fall within an acceptable range, with the BE and SH-IV complex exhibiting high permeability. Furthermore, BE adheres to Lipinski's Rule of Five, contrasting with compounds assessed through Admetlab02. Both BE and SH-IV demonstrate high oral bioavailability and favorable pharmacokinetic properties in humans, which are critical for their role as active pharmaceutical agents. Cytochrome P450 enzymes play a pivotal role in hepatic drug metabolism, particularly CYP2D6 and CYP3A4 (Zhou SF et al., 2000). The findings suggest that BE and SH-IV are not substrates for these subtypes, although they may inhibit CYP2C9 and CYP2D6 (Table 8), indicating possible hepatic metabolism. P-glycoprotein, an energy-dependent drug efflux pump formed by a pore of 12 α -helices in the cell membrane, shows increased expression levels in normal tissues such as the liver, pancreas, kidneys, colon, and

adrenal cortex (Tandon VR et al., 2006). These findings imply a physiological role in the secretion process, while elevated expression levels in tumor tissues may correlate with specific conditions. Binding predictions for P-glycoprotein were generated using SwissADME.

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Table 6. ADME properties of phytochemicals

Compounds	Molecular weight	QP logPo/w ^b	QP logS ^c	CIQP logS ^d	QP logHER G ^e	QPP Caco ^f	QPlog BB ^g	QPP MDCK ^h	QPlogKp ⁱ	QPlog Khsa ^j	Human Oral Absorption	Pgp Inhibitor
Betulin	444.740	6.022	-7.108	-6.692	-3.818	1762.79	-0.392	912.987	-2.592	1.569	100%	Inhibitor
Salsolside C	927.091	-0.146	-4.716	-6.263	-3.86	0.237	-5.492	0.076	-7.642	-1.117	0%	Non-inhibitor
2'-O-Acetylsalsolside C	969.128	0.747	-6.135	-7.03	-4.358	0.211	-6.002	0.067	-7.742	-0.883	0%	Non-inhibitor
3'-O-Acetylsalsolside C	969.128	0.744	-5.294	-7.03	-3.915	0.368	-5.309	0.122	-7.283	-0.884	0%	Non-inhibitor
Shatavarin iv	887.069	-0.038	-4.841	-5.889	-5.652	4.727	-4.664	1.517	-6.342	-0.931	100%	Inhibitor

Forskolin	410.506	2.288	-3.69	- 4.028	-3.469	868.13	-0.676	424.588	-2.976	0.05	93%	Inhibitor
Alpha-Amyrin	426.724	6.947	-7.806	- 7.261	-3.523	4471.55	0.191	2496.99	-2.041	2.006	100%	Inhibitor
Betulinic acid	456.707	6.200	-6.712	- 6.823	-1.781	342.203	-0.403	197.425	-2.829	1.331	96%	Inhibitor
24-Hydroxyolean-12-en-3-one	440.708	6.558	-7.607	- 7.267	-3.778	2193.99 9	-0.169	1156.60 8	-2.530	1.830	100%	Inhibitor
Oleanolic acid	456.707	6.180	-6.845	- 6.947	-1.711	333.602	-0.349	192.068	-2.975	1.373	95%	Inhibitor
Sarsasapogenin	416.643	6.033	-7.471	- 6.427	-3.999	3440.95 1	0.054	1881.20 4	-2.316	1.618	100%	Inhibitor

- a. Molecular weight of the molecule (130.0 to 725.0)
- b. Estimated octanol/water partition coefficient (−2.0 to 6.5)
- c. Predicted aqueous solubility, log S in mol/dm³, which reflects the concentration of the solute in a saturated solution at equilibrium with the crystalline solid (−6.5 to 0.5)
- d. ClQPlogS: Conformation-independent predicted aqueous solubility, log S, indicating the solute concentration in a saturated solution equilibrium with the crystalline solid (−6.5 to 0.5)
- e. Estimated IC₅₀ value for blocking HERG K⁺ channels (values below −5 are concerning)
- f. QPPCaco: Estimated apparent permeability across Caco-2 cells in nm/sec (>500 is excellent; <25 is poor)
- g. QPlogBB: Estimated brain/blood partition coefficient (−3.0 to 1.2)
- h. QPPMDCK: Estimated apparent permeability across MDCK cells in nm/sec (>500 is excellent; <25 is poor)
- i. QPlogKp: Estimated skin permeability (−8.0 to −1.0)
- j. QPlogKhsa: Estimated binding affinity to human serum albumin (−1.5 to 1.5)
- k. Human Oral Absorption: Estimated human oral absorption on a scale of 0 to 100% (>80% is considered high; <25% is poor)

Table 7. Pharmacological Properties of the Selected Ligands.

S.N o.	COMPOUND NAME	CYP_2 C19	CYP_2 C9	CYP_2 D6	CYP_2 D6	CYP_3 A4	CYP_3 A4	Pgp inhibiti on
		Inhibiti on	Inhibiti on	Inhibiti on	Substra te	Inhibiti on	Substra te	
1	Betulin	No	No	No	No	No	No	Inhibit or
2	Shatavarin iv	No	No	No	No	No	No	Inhibit or
3	Salsoloside C	No	No	No	No	No	No	Inhibit or
4	2'-O-Acetylsalsoloside C	No	Inhibit or	Inhibit or	No	No	No	No
5	3'-O-Acetylsalsoloside C	No	No	No	No	No	No	Inhibit or
6	Forskolin	No	No	Inhibit or	No	Inhibit or	No	No
7	Alpha-Amyrin	No	No	No	No	Inhibit or	No	No

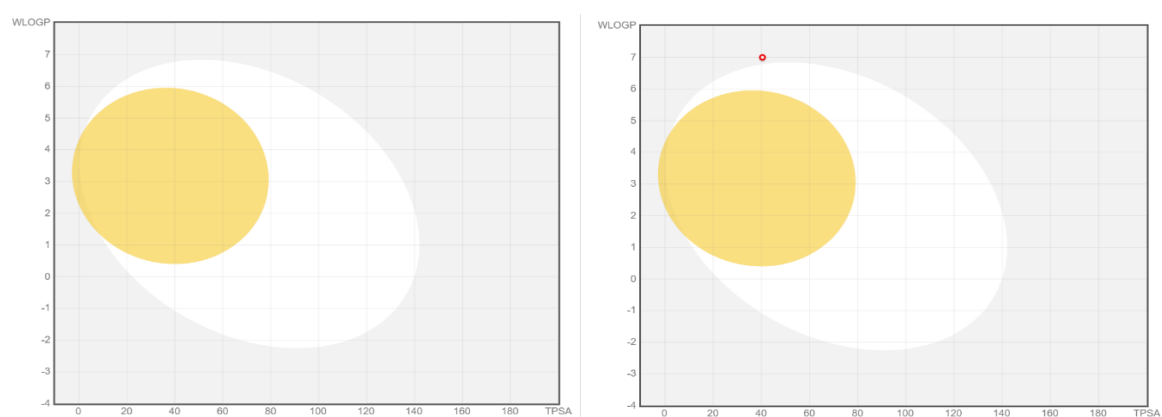
8	Betulinic acid	No	No	No	No	Inhibit or	No	Inhibit or
9	24-Hydroxyolean-12-en-3-one	No	No	No	No	No	No	Inhibit or
10	Oleanolic acid	No	Inhibit or	Inhibit or	No	Inhibit or	No	No
11	Sarsasapogenin	No	Inhibit or	Inhibit or	No	No	Inhibit or	No

Table 8. Drug-like properties of Ligands.

S.No.	Name of compound	MW	Log p	HB donor	HB accepter	No. of rotatable bonds
1	Betulin	442.72	2	2	4.31	2
2	Shatavarin iv	887.06	17	9	4.87	8
3	Salsolside C	927.08	18	10	3.59	9
4	2'-O-Acetylsalsolside C	969.12	19	9	3.72	11
5	3'-O-Acetylsalsolside C	969.12	19	9	4.48	11
6	Forskolin	410.5	7	3	2.67	3
7	Alpha-Amyrin	426.72	1	1	4.77	0
8	Betulinic acid	456.7	3	2	3.79	2
9	24-Hydroxyolean-12-en-3-one	440.7	2	1	4.31	1
10	Oleanolic acid	456.7	3	2	3.89	1
11	Sarsasapogenin	416.64	3	1	4.42	0

4.1.6 BOILED egg plot analysis:

Failures in minor molecule effectiveness and toxicity are brought on by unfavourable pharmacokinetic characteristics and ADME prediction. BE and SH-IV both have two advantageous pharmacokinetic characteristics: Gastrointestinal Absorption and the blood-brain Barrier, as illustrated in the Boiled Egg plot (Figure 11a&b). The white zone of the egg, not the yellow zone, indicates the high gastrointestinal absorption rate of this phytochemical. Therefore, the brain cannot pass through BE and SH-IV molecules. In our research, BE and SH-IV were found to be the most potent agents to treat Cervical cancer. But to confirm the results of this work, further studies are required.



A

B

Figure 11: (A) Depicts the boiled egg graph, which shows SH-IV's gastro and BBB activities. (B) Depicts the boiled egg graph, which shows BE's gastro and BBB activities

4.1.7 Minimisation and Density Functional Theory (DFT) calculations.

The DFT calculations provided insights into the primary molecular characteristics. Using the B3LYP/6-31G level of theory (Son NT et al, 2019), DFT computations were refined with the top three binding secondary metabolites. The high chemical reactivity is indicated by the HOMO-LUMO gap, which facilitates charge transport within the molecule. By examining the interactions between molecular orbitals and other species, as well as their energy differences, we can assess the chemical reactivity of structures involved in chemical processes. The electron density of a molecule can be inferred from its characteristic colour intensity. Analysis of molecular properties considered HOMO, LUMO, and MESP chemical reactivity (Maheswari G et al., 2019).

Typically, the HOMO donates electrons while the LUMO accepts them. Comparing the HOMO and LUMO energy gaps reveals the stability of the molecular structure (Shimizu A et al., 2020). The energy gap levels illustrate reactivity sensitivity due to the rapid electron transfer between the HOMO and LUMO energy levels (Table 9).

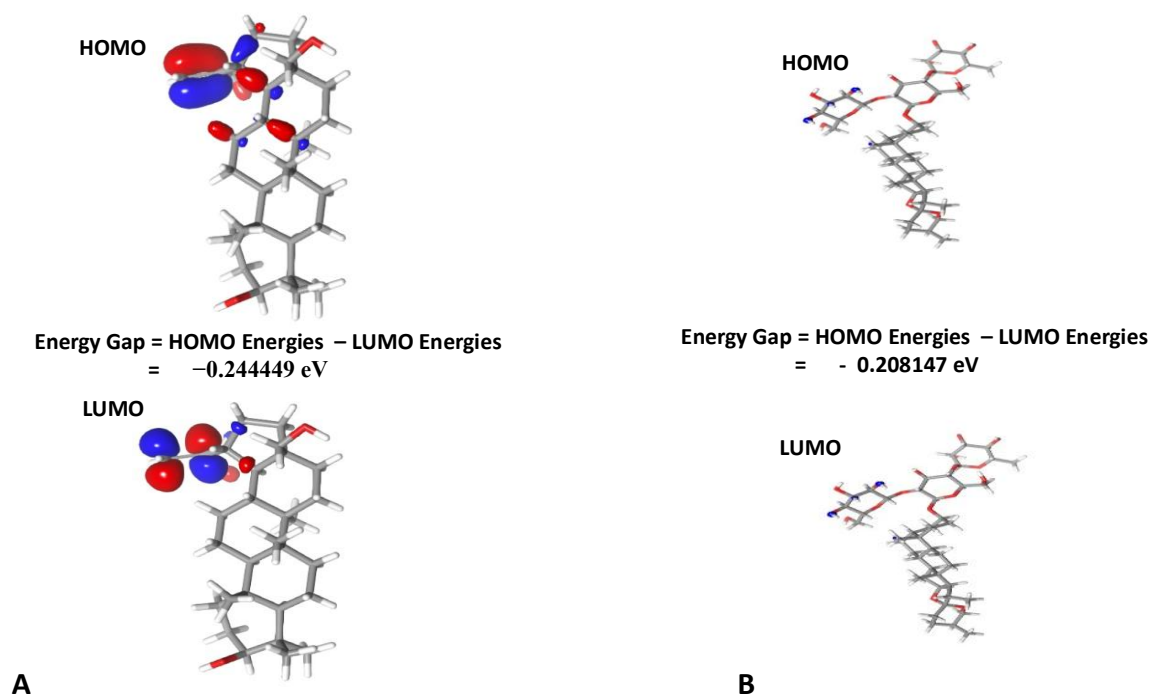


Figure 12: DFT calculation of (A) Betulin (BE), (B) Shatavarin-IV (SH-IV)

The findings highlight the remarkable chemical reactivity of the examined molecules. Factors such as total energy, dipole moment, HOMO, LUMO, and energy gap significantly influence the stability of these compounds. Figure 12 illustrates the HOMO and LUMO distributions for the inhibitors, demonstrating that the HOMO-LUMO gap plays a crucial role in facilitating charge transfer within the molecules. This relationship between the HOMO-LUMO gap and charge transfer can be analysed through molecular charge transfer assessments. When comparing the three most relevant binding compounds, the HOMO and LUMO energy gaps do not show significant differences. For instance, the Betulin (BE) molecule has HOMO and LUMO energy levels of -0.231397 eV and 0.023250 eV, respectively, resulting in an estimated energy gap of 0.208147 eV. In contrast, the Shatavarin-IV (SH-IV) molecule displays a HOMO energy of -0.260991 eV and a LUMO energy of -0.016542 eV, yielding an expected energy gap of 0.244449 eV. The energy gap between HOMO and LUMO orbitals influences the polarizability, chemical hardness, and reactivity of the lead compounds, as detailed in Table 10. Additionally, the colour coding in Figure 10 indicates regions of the molecules with the highest negative charge (red) and the highest positive charge (blue).

Table 9: DFT calculations of the top binding ligands.

S.no	Compound name	EHOMO	ELUMO	EGAP
1.	Betulin (BE)	-0.231397Ev	- 0.023250eV	0.208147eV
2.	Shatavarin-IV	-0.260991ev	- 0.016542eV	0.244449eV

4.1.8. Molecular Dynamics Simulation:

4.1.8.1 Molecular dynamic simulation for BE in complex with KMT2C using Desmond:

To confirm our results and deepen our understanding of the molecular mechanisms involved in BE binding, we performed a molecular dynamics simulation lasting 500 ns. The analysis showed that the RMSD for protein C α , backbone, and heavy atoms ranged from 1.35 to 3.0 and 2.9 to 4.8, respectively (Figure 13a). Throughout the simulation, C α atoms displayed fluctuations between 1.25 and 2.1, remaining stable during the 150 to 350 ns timeframe, with an RMSD of 4.2. Notable fluctuations in RMSD (up to 4.8) were recorded between 100 and 150 ns and at 350 ns. Persistent hydrophobic interactions were observed between Phe4826 and Tyr4884 throughout the simulation. We assessed the flexibility of residues at the ligand binding sites using root mean square fluctuations (RMSFs) metrics (Figure 13b). 24 ligand interactions were identified with various protein amino acids, ranging from Gln4781 to Lys4897 and Arg4907 to Met4910. As shown in Figure 13c, the ligand is primarily stabilized by hydrophobic interactions (5%-90% of the simulation duration) with residues such as Tyr4800, Tyr4825, Phe4827, Ile4847, Ala4857, Ile4870, Tyr4884, Tyr4886, Phe4888, and Trp4909. Between 10% and 65% of the simulation trajectory, hydrogen bonds were formed with Tyr4800, Val4824, Ala4844, Arg4845, Asp4889, Lys4896, and Met4910. The timeline of protein-ligand interactions is illustrated in Figure 13d. The hydrophobic interaction established during the docking phase with Ile4870 is maintained in the molecular dynamics trajectory, as indicated by the 2D trajectory interaction diagram (Figure 19c). The methanol group in BE contributed one hydrophobic bond to Ile4847, demonstrating an efficacy of 38%. The RMSF of the ligand (Figure 14b), relative to the initial frame, was observed to be within the range of 0.5 to 3.5 Å. Additional ligand parameters (Figure 14), such as radius of gyration, molecular surface area, solvent-accessible surface area, and polar surface area, were determined to range from 4.30 to 4.45, 388.4 to 453.42, 110.75 to 340.33, and 64.94 to 93.73, respectively.

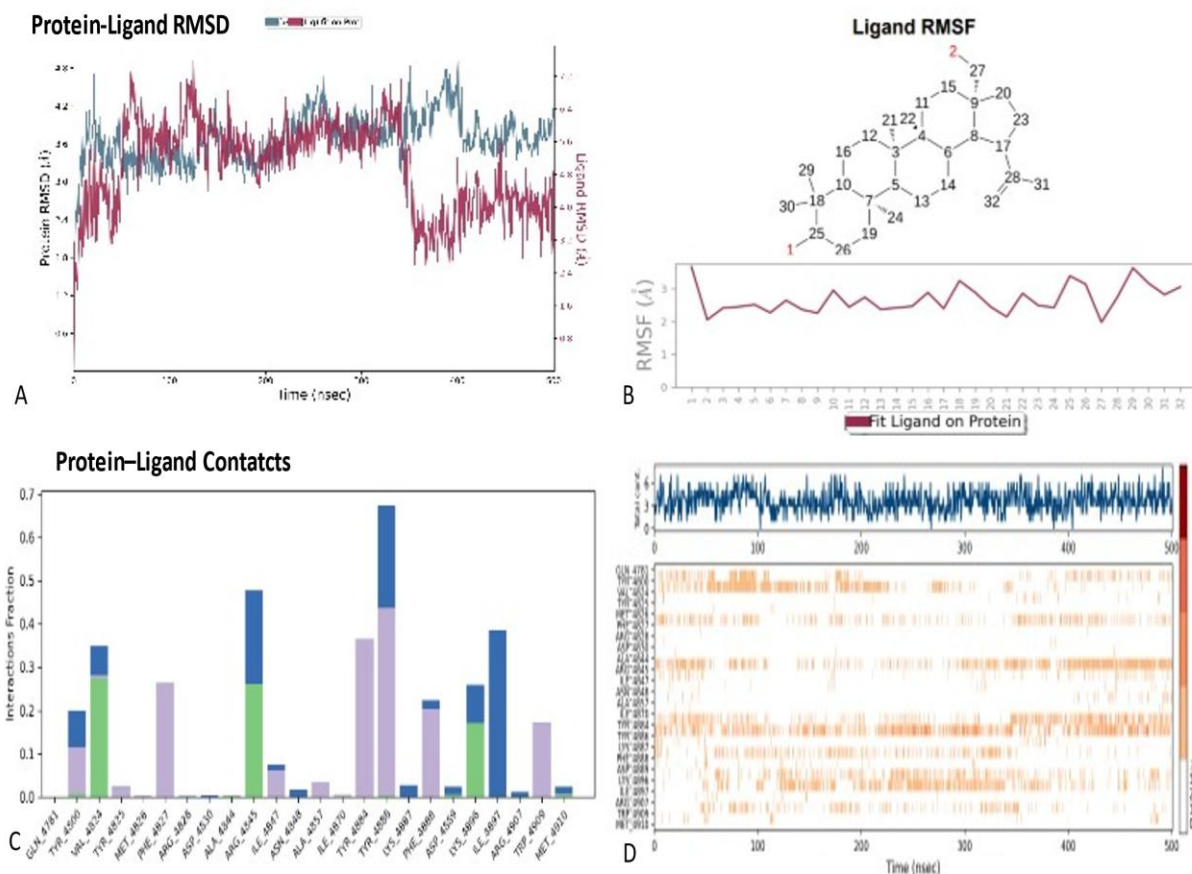


Figure13. Simulation results of the KMT2C-BE. (A) The RMSD plot of the BE-KMT2C complex. (B) depicts the RMSF of ligand fluctuations. (C) Shows the interactions between proteins and the corresponding ligand. (D) The timeline shows the interactions and contacts during the trajectory displayed in the top panel.

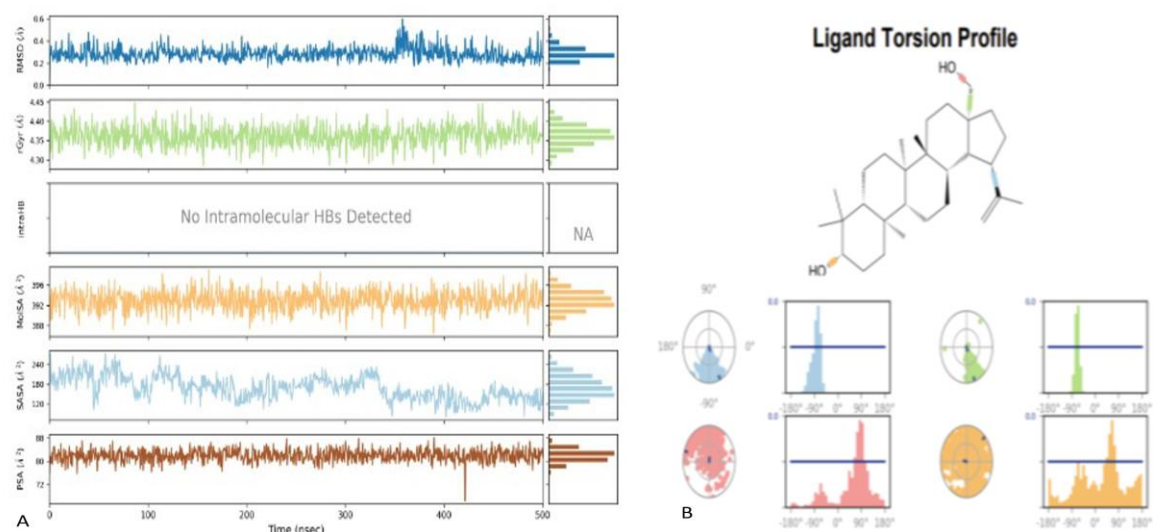


Figure 14: (A) depicts the properties of the ligand, and (B) illustrates the torsion profile of the ligand.

4.1.8.2. Molecular dynamic simulation for BE in complex with DNMT3A using Desmond:

The trajectory analysis of the DNMT3A-BE complex from 500 ns molecular dynamics simulations revealed that the root mean square deviation (RMSD) for protein C α , backbone, and heavy atoms ranged from 0.1 to 3.0 and from 2.9 to 4.8, respectively (Figure 15a). The C α atoms displayed variations between 0.25 and 2.6, remaining stable during the 350-450 ns simulation period, with a higher RMSD observed. Significant increases in RMSD (up to 3.6) occurred between 200 and 300 ns. The flexibility of residues at ligand binding sites was assessed using root mean square fluctuations (RMSFs) metrics (Figure 15b). A total of 38 ligand interactions (Figure 15c) were identified with protein amino acids, including SER634, ASP63, ILE639, THR641, GLY639, TRY656, GLU660, ASP665, ARG684, SER685, ILE701, PRO705, ASN707, ASP708, LYS717, TYR720, GLY722, ARG725, LEU72, GLU729, PHE751, GLU752, VAL754, ARG786, ASN840, and GLY886 to TRP889. As shown in Figure 15d, the ligand is stabilised through extensive hydrophobic interactions (5%-90% of the simulation duration) with residues Leu726, Phe751, and Val754. During 10%-65% of the simulation trajectory, hydrogen bonds were formed with Asp637, Asn707, Glu721, Gly722, Arg725, and Arg887. Figure 15D illustrates a timeline of protein-ligand interactions. The ligand's RMSF (Figure 15d), relative to the initial frame, ranged from 7.5 to 10.5 Å. Additional ligand parameters (Figure 16), such as radius of gyration, molecular surface area, solvent-accessible surface area, and polar surface area, were found to range from 4.20 to 5.05, 384.54 to 400.42, 110.75 to 400.53, and 55.74 to 80.73, respectively.

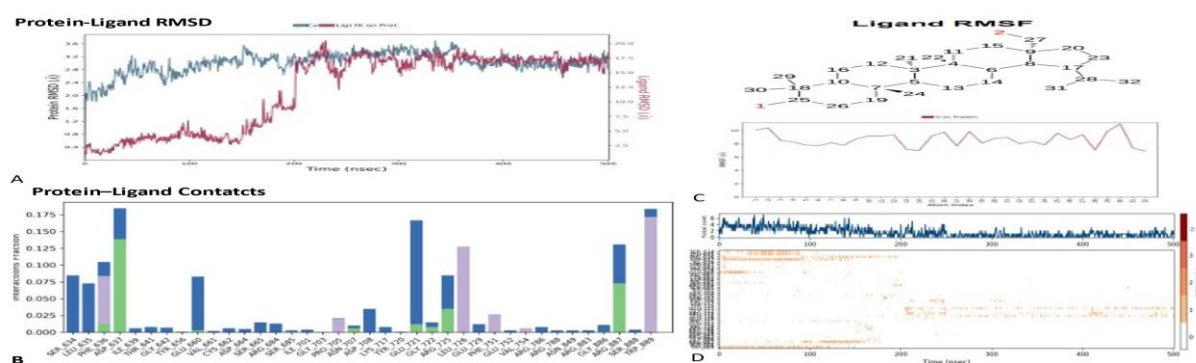


Figure 15: Simulation results of the DNMT3A-BE. (A) The RMSD plot of the BE-DNMT3A complex. (B) depicts the RMSF of ligand fluctuations. (C) Shows the interactions between proteins and the corresponding ligand. (D) The timeline shows the interactions and contacts during the trajectory displayed in the top panel.

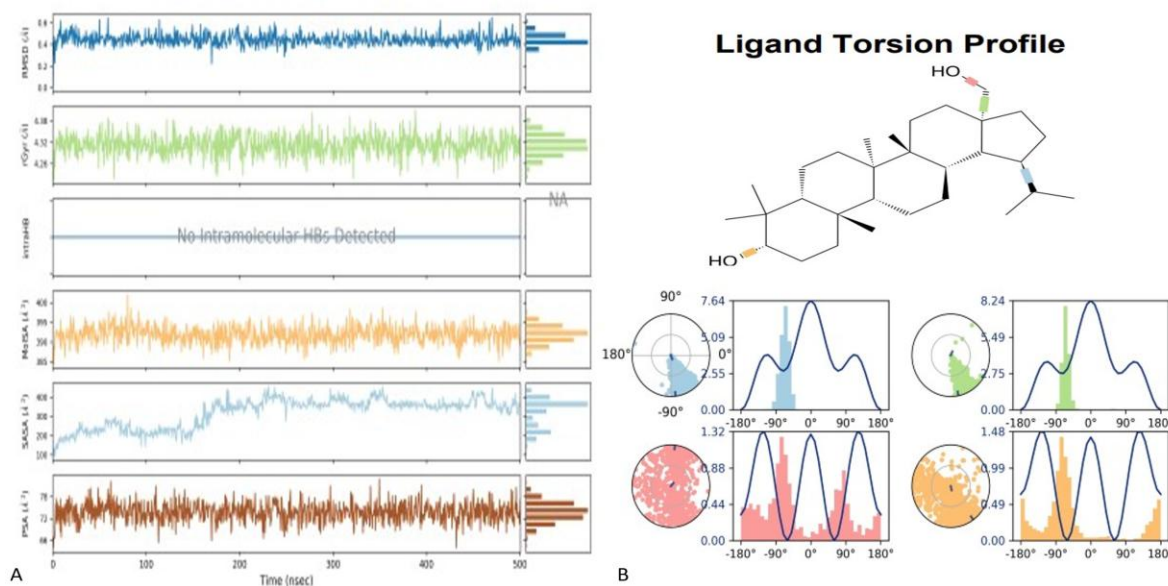


Figure. 16:(A) The properties of the ligand (B) illustrate the torsion profile of the ligand.

4.1.8.3. Molecular dynamic simulation for BE in complex with HDAC4 using Desmond:

According to the trajectory analysis, the RMSD of the heavy atoms, backbone, and protein C ranged from 0.015 to 5.85 (Figure 17a). The carbon atoms exhibited values between 0.01 and 3.98, showing stability during the simulation from 250 to 480 nanoseconds, with a root mean square deviation of 4.0. More significant variations in RMSD (up to 4.8) were observed between 100-150 ns and 250-300 ns. The flexibility of residues at the ligand binding sites was assessed using root mean square fluctuations (RMSFs) metrics (Figure 17b). Eleven ligand interactions were formed with protein amino acids, including Pro156, His159, Gly167, Phe168, Asp196, His198, Asn225, Phe227, Asp290, Pro298, and Leu299. As shown in Figure 15c, the ligand is stabilised through extensive hydrophobic interactions (5%-90% of the simulation duration) with residues Pro156, Phe227, and Leu299. It also formed hydrogen bonds with His159 during 10%-65% of the simulation trajectory. The timeline of protein-ligand interactions is illustrated in the figure. The hydrophobic interaction established by the docking conformation is maintained throughout the molecular dynamics trajectory, as indicated by the 2D trajectory interaction diagram (Figure 17a). The ligand's RMSF (Figure 19d), relative to the initial frame, ranged from 0.105 to 1.034 Å. Additional ligand parameters (Figure 20), including radius of gyration, molecular surface area, solvent-accessible surface area, and polar surface area, were found to range from 3.943 to 4.402, 381.112 to 399.601, 180.38 to 271.833, and 70.227 to 86.468, respectively.

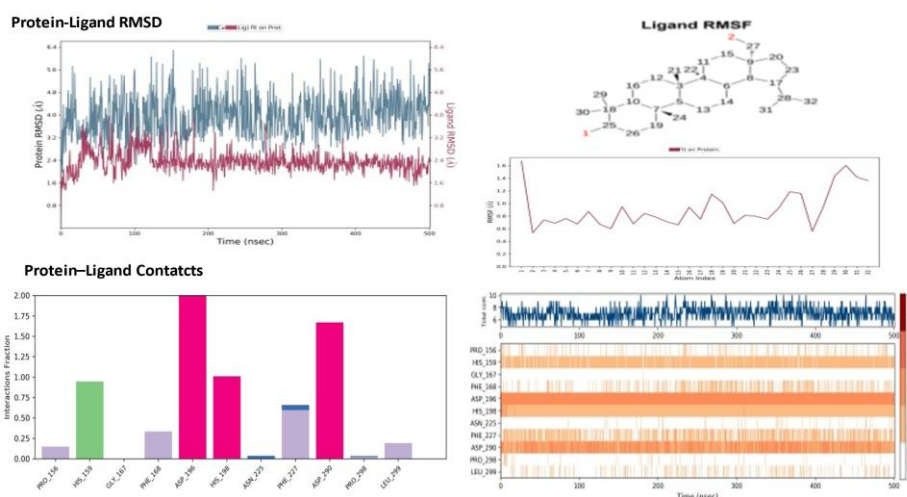


Figure. 17: The simulation results of the HDAC-BE. (A) The RMSD plot of the BE-HDAC complex. (B) depicts the RMSF of ligand fluctuations. (C) Shows the interactions between proteins and the corresponding ligand. (D) The timeline shows the interactions and contacts during the trajectory displayed in the top panel.

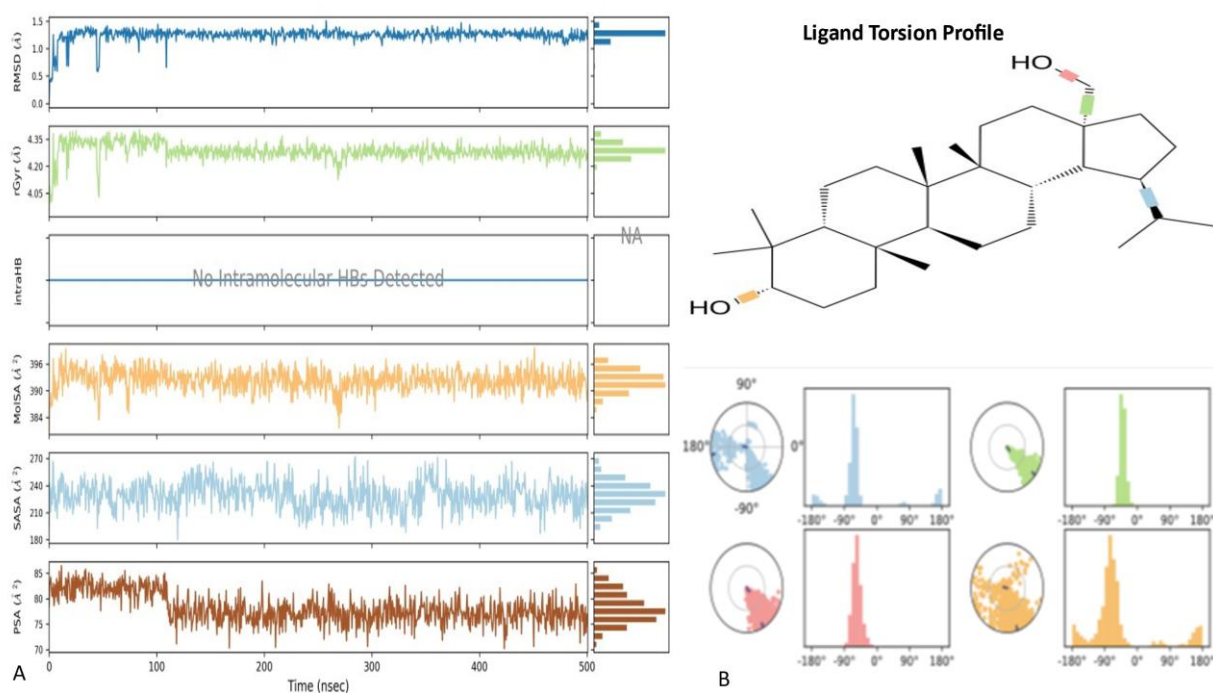


Figure 18 :(A) The properties of the ligand, and (B) illustrates the torsion profile of the ligand.

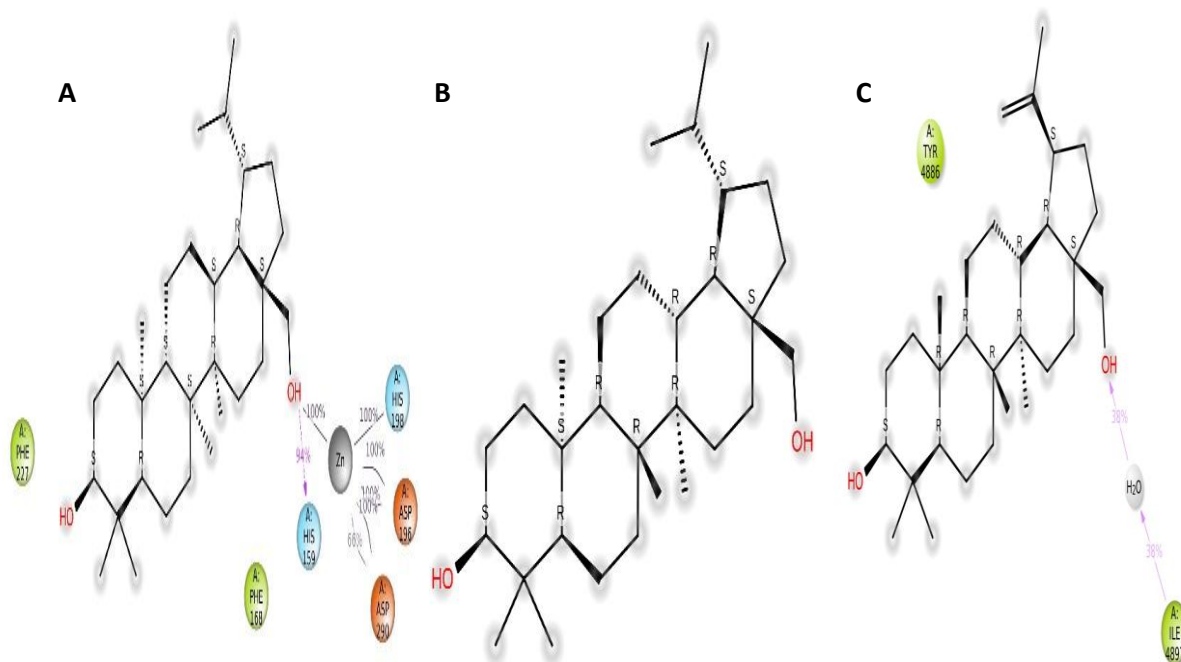


Figure 19: Two-dimensional interaction of BE showing the stable bonding at 500ns. (A). DNMT3A in complex with BE. (B) HDAC in complex with BE. (C) KMT2C in complex with BE.

4.1.8.4. Molecular dynamic simulation for SH in complex with KMT2C using Desmond:

To validate our findings and deepen our understanding of the molecular processes associated with SH binding, we performed a 500 ns molecular dynamics simulation. The RMSD of the protein C α , backbone, and heavy atoms ranged from 0.1 to 0.9 and from 1.2 to 5.8, respectively (Figure 20a). The C α atoms fluctuated between 3.6 and 4.9, remaining stable during the 400-450 ns simulation period, with a root mean square deviation (RMSD) of 3.9. More significant variations in RMSD (up to 4.2) were observed between 100 to 150 ns and at 350 ns. Consistent hydrophobic interactions were noted throughout the simulation. The flexibility of residues at ligand binding sites was assessed using root mean square fluctuations (RMSFs) metrics (Figure 20b). A total of 64 ligand interactions were formed with protein amino acids, ranging from Gln4781 to Lys4897 and Arg4907 to Met4910. Figure 18c shows that the ligand is primarily stabilized through hydrophobic interactions (5%-90% of the simulation duration) with residues Lys4764, Arg4777, Tyr4800, Glu4804, Arg4812, Glu4818, Pro4853, Arg4874, Gln4872, Arg4907, Lys4908, and Asn4905. During 10%-65% of the simulation trajectory, hydrogen bonds were established with Arg4779, Gln4781, Gly4782, Leu4783, Asn4821, Gly4823,

Val4824, Tyr4825, Met4826, Arg4874, Gln4877, Arg4907, Lys4908, and Asn4905. Figure 20C illustrates a timeline of protein-ligand interactions. The hydrophobic interaction formed by the docking conformation with Ile4870 is maintained throughout the MD trajectory, as shown in the 2D trajectory interaction diagram (Figure 29C). The ligand's RMSF (Figure 20), relative to the initial frame, ranged from 0.5 to 3.5 Å. Additional ligand parameters, as depicted in the figure, including radius of gyration, molecular surface area, solvent-accessible surface area, and polar surface area, were found to be within the ranges of 4.30-4.45, 388.4-453.42, 110.75-340.33, and 64.94-93.73, respectively (Figure 21).

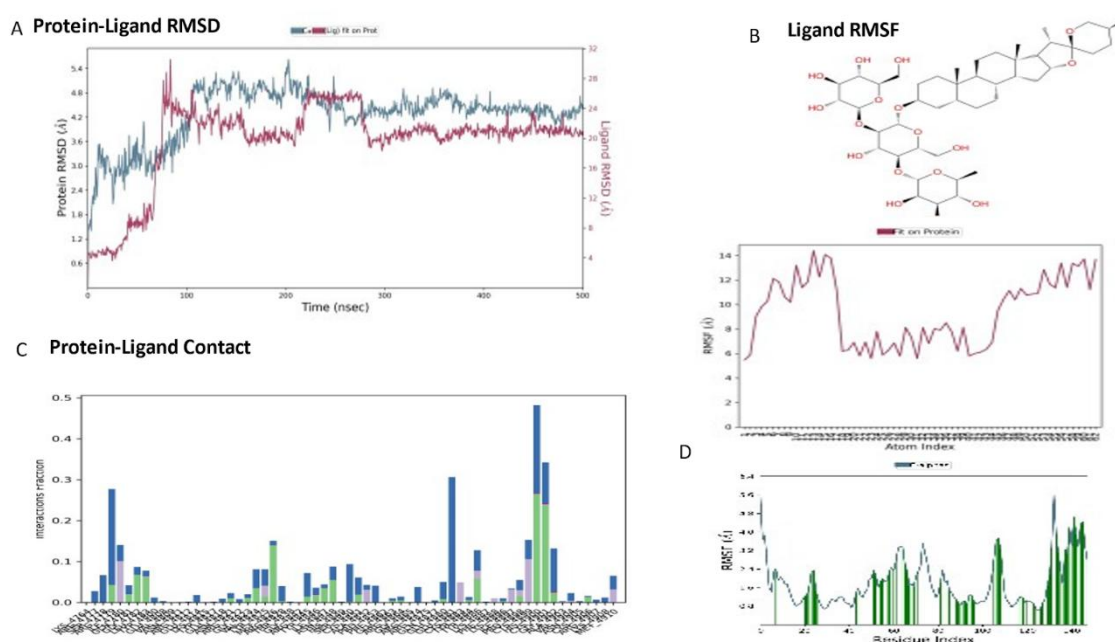


Figure 20: Simulation results of the KMT2C-SH-IV. (A) The RMSD plot of the SH-IV-KMT2C complex. (B) depicts the RMSF of ligand fluctuations. (C) Shows the interactions between proteins and the corresponding ligand. (D) Shows the RMSF of the ligands

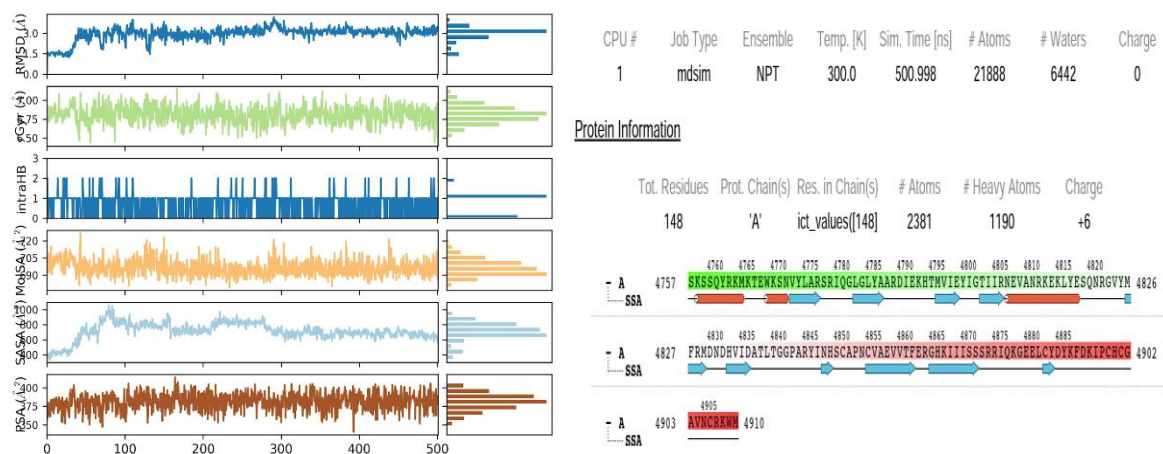


Figure. 21: (A) Demonstrates the RMSD, Intra Hydrogen bond, RGYR, MolSA, PSA, and SASA values connected to 500 for KMT2C. (B) Protein information has a total of 288 residues in chain A and a total of 4653 atoms, with heavy atoms 21888 and charges 0.

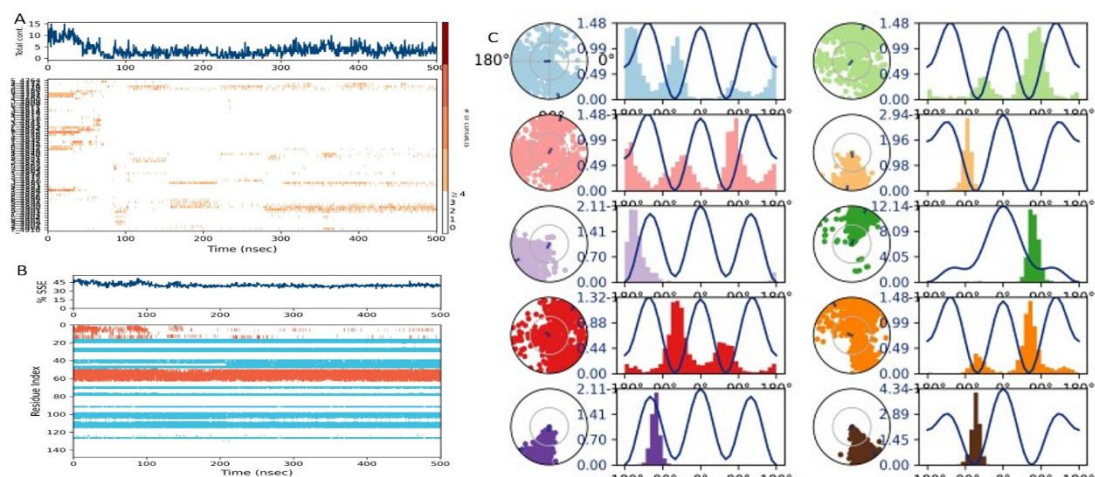


Figure 22 : (A) The timeline illustrates the interactions and contacts occurring throughout the trajectory presented in the top panel. (B) Shows the secondary structures of the protein. The upper plot represents the secondary structure elements (SSE), while the lower plot summarizes the SSE composition for each trajectory frame during the simulation. (C) Depicts the torsion profile of the ligand.

4.1.8.5 Molecular dynamics simulation for SH in complex with DNMT3A using Desmond:

The trajectory analysis of the DNMT3A-SH complex from 500 ns molecular dynamics simulations revealed that the root mean square deviation (RMSD) of the protein Cα, backbone, and heavy atoms ranged from 0.1 to 0.6 and from 0.6 to 3.2, respectively (Figure 23b). The Cα atoms fluctuated between 0.1 and 4.3, exhibiting stability during the 100-450 ns simulation period, with a higher RMSD observed. Significant variations in RMSD (up to 3.6) were noted

between 0-100 ns. The flexibility of residues at the ligand binding sites was assessed using root mean square fluctuations (RMSFs) metrics (Figure 23d). A total of 35 ligand interactions were identified with protein amino acids, including SER634, ASP63, ILE639, THR641, GLY639, TRY656, GLU660, ASP665, ARG684, SER685, ILE701, PRO705, ASN707, ASP708, LYS717, TYR720, GLY722, ARG725, LEU72, GLU729, PHE751, GLU752, VAL754, ARG786, ASN840, and GLY886 to TRP889 (Figure 23c). As shown in Figure 25, the ligand is stabilized through extensive hydrophobic interactions (5%-90% of the simulation duration) with residues Phe636, Val661, Val683, Arg684, Pro705, and Leu726. During 10%-65% of the simulation trajectory, hydrogen bonds were formed with Arg684, Cys704, Asn707, Arg883, and Arg887. The ligand's RMSF, as illustrated in the figure, ranged from 7.5 to 10.5 Å relative to the initial frame. Additional ligand parameters (Figure 26), including radius of gyration, molecular surface area, solvent-accessible surface area, and polar surface area, varied from 4.20 to 5.05, 384.54 to 400.42, 110.75 to 400.53, and 55.74 to 80.73, respectively.

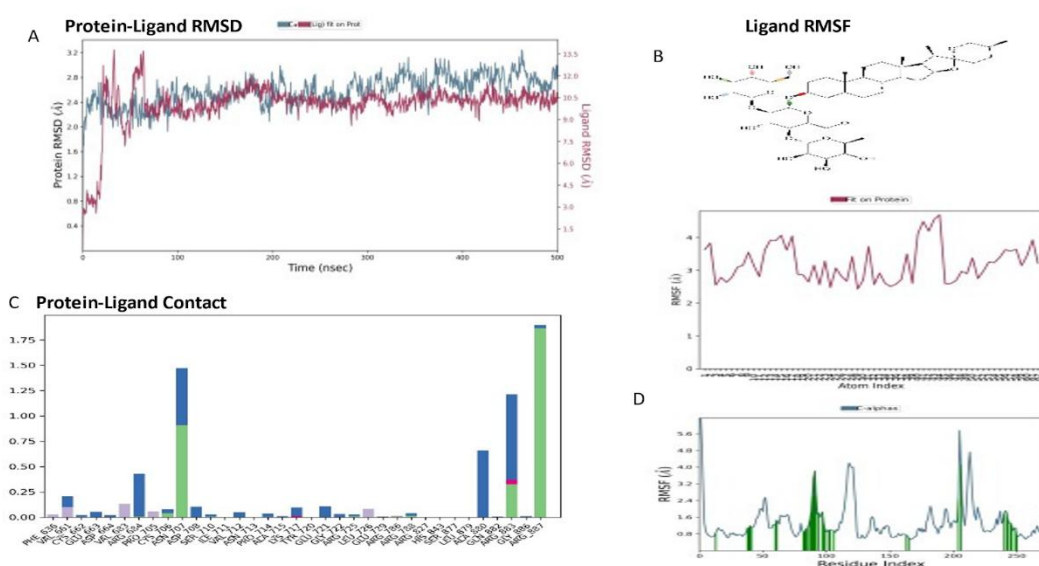


Figure 23: Simulation results of the DNMT3A-SH. (A) The RMSD plot of the BE-DNMT3A complex. (B) depicts the RMSF of ligand fluctuations. (C) Shows the interactions between proteins and the corresponding ligand. (D)

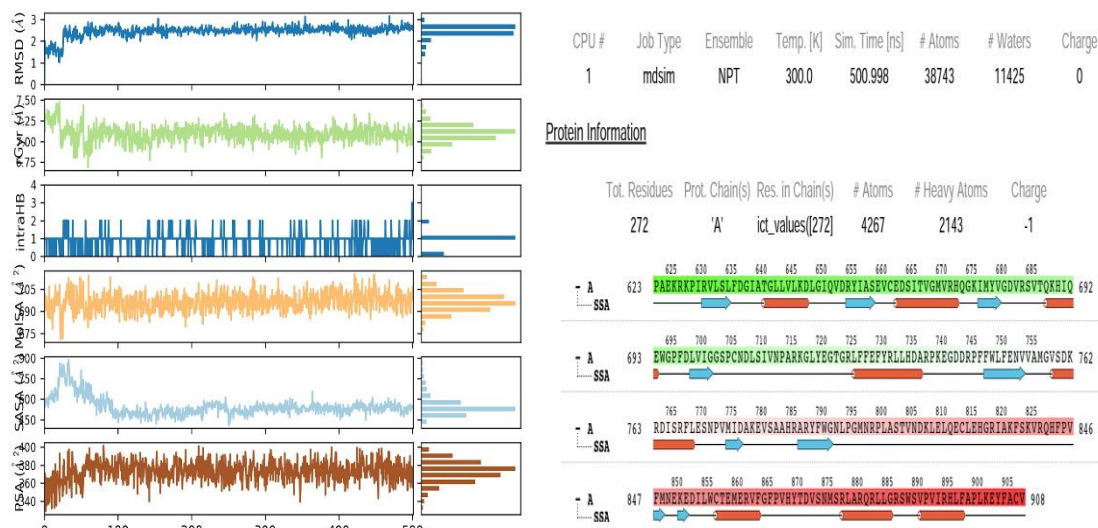


Figure 24: (A) Demonstrates the RMSD, Intra Hydrogen bond, RGYR, MolSA, PSA, and SASA values connected to 500 for DNMT3A. (B) The protein information indicates a total of 288 residues in chain A, with a total of 4,653 atoms, including 45,539 heavy atoms, and a net charge of 0.

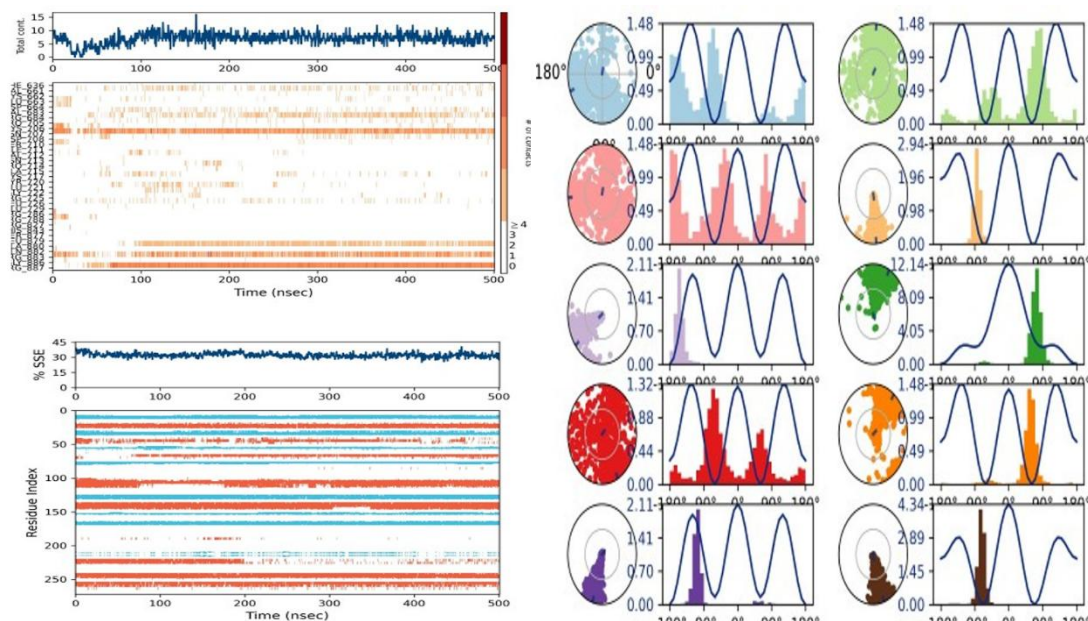


Figure 25. (A) The timeline illustrates the interactions and contacts that occurred throughout the trajectory shown in the top panel. (B) Displays the protein's secondary structures. The upper plot represents the secondary structure elements (SSE), while the lower plot summarizes the SSE composition for each frame of the trajectory over the simulation period. (C) Shows the torsion profile of the ligand.

4.1.8.6 Molecular dynamic simulation for Shatavarin in complex with HDAC4 using Desmond:

The RMSD of protein C, encompassing the backbone and heavy atoms, varied from 0.015 to 5.85 based on trajectory analysis (Figure 26). The carbon atoms showed fluctuations between 0.01 and 3.0, demonstrating stability during the initial 0-50 ns of simulation time, with a root mean square deviation of 4.0. More significant changes in RMSD (up to 4.8) were noted between 50-150 ns and 150-300 ns. The root mean square fluctuations (RMSFs) metrics were used to assess the flexibility of residues at the ligand binding sites (Figure 26b). A total of sixty-four ligand interactions were formed with protein amino acids, including Pro156, His159, Gly167, Phe168, Asp196, His198, Asn225, Phe227, Asp290, Pro298, and Leu299. As shown in Figure 26c, the ligand was stabilized predominantly through hydrophobic interactions (5%-90% of the simulation duration) with residues Pro156, Phe227, and Leu299. The ligand also formed hydrogen bonds with His159 during 10% to 65% of the simulation trajectory, as illustrated in the accompanying timeline of protein-ligand interactions. The hydrophobic interactions established by the docking conformation were maintained throughout the molecular dynamics trajectory, as indicated by the 2D interaction diagram (Figure 29). The RMSF of the ligand, relative to the initial frame, ranged from 0.105 to 1.034 Å. Additional ligand parameters (Figure 26b), such as the radius of gyration, molecular surface area, solvent accessible surface area, and polar surface area, ranged from 3.943 to 4.402, 381.112 to 399.601, 180.38 to 271.833, and 70.227 to 86.468, respectively (Figure 28).

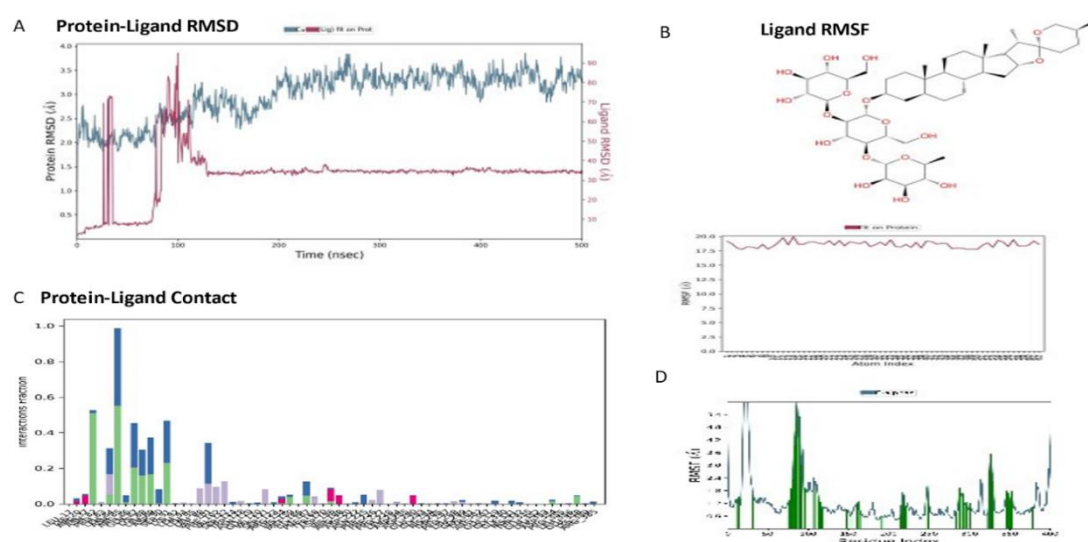


Figure 26: Simulation results of the HDAC-SH. (A) The RMSD plot of the BE-HDAC complex. (B) depicts the RMSF of ligand fluctuations. (C) Shows the interactions between proteins and the corresponding ligand. (D) The timeline shows the interactions and contacts during the trajectory displayed in the top panel.

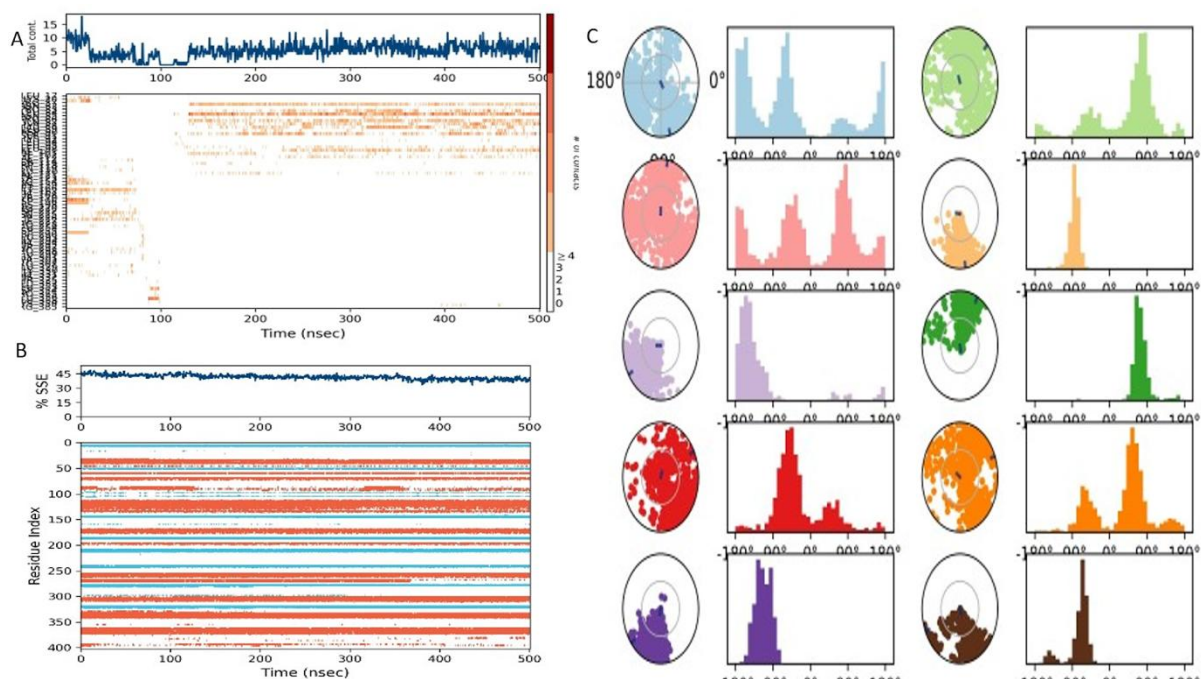


Figure. 27(A) The timeline illustrates the interactions and contacts occurring throughout the trajectory shown in the top panel. (B) Shows the secondary structures of the protein. The upper plot represents the secondary structure elements (SSE), while the lower plot summarizes the SSE composition for each frame of the trajectory during the simulation. (C) Depicts the torsion profile of the ligand.

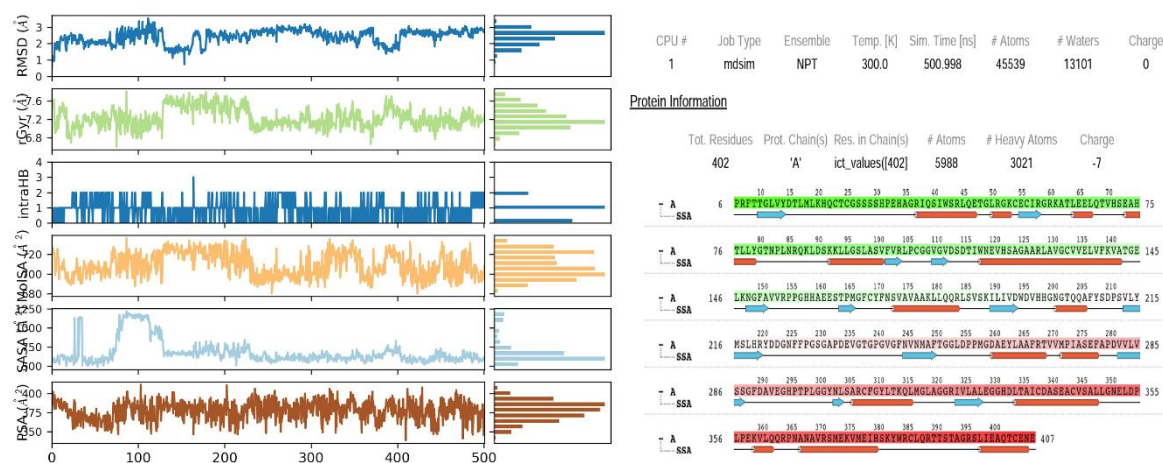


Figure 28 (A) displays the RMSD, intra-hydrogen bond, RGYR, MolSA, PSA, and SASA values associated with 500 for HDAC4. (B) The protein information indicates a total of 288 residues in chain A, with a total of 4,653 atoms, including 4,539 heavy atoms, and a net charge of 0.

Table 10: Results of binding free energy calculations for compounds associated with the protein-peptide complex.

Protein	Compound		ΔE			ΔG solv	ΔG SA	ΔG
		Time (ns)	ΔG Coulomb ^b	ΔG vdw ^c	ΔG Covalent ^d	ΔG SolGB ^e	ΔG solLipo ^f	ΔG bind ^g
DNMT3A (2qrv)	Betulin	0	-11.96124443	-18.59681244	15.73645191	20.18238191	-49.42429392	-44.65438383
	Shatavarin- iv	0	-25.2327164	-38.16497311	5.454921409	33.49471209	-32.63465379	-59.14273559
HDAC4 (2qvj)	Betulin	0	-18.605579	-13.18658538	17.65457469	23.63796887	-51.8737419	-43.05386741
	Shatavarin- iv	0	-40.86250665	-47.15158792	16.32076356	56.08043112	-62.37083436	-80.76488326
KMT2C (5f59)	Betulin	0	-22.01667915	-14.19920482	11.66557521	29.90637835	-50.04491293	-45.53037001
	Shatavarin- iv	0	-30.83810589	-47.5020928	10.44631338	39.64140032	-35.04570991	-65.37166471

All energies are in kcal/mol

^b Coulomb energy contribution to the binding free energy

^c Van der Waals energy contribution to the binding free energy

^d The covalent energy contribution to the binding free energy

^e The generalized Born electrostatic solvation energy contribution to the binding free energy

^f The surface area due to lipophilic energy contribution to the binding free energy (nonpolar contribution estimated by solvent accessible surface area).

^g Free binding energy.

The molecular docking and computational evaluation conducted in this study present compelling evidence for the potential of Betulin (BE) and Shatavarin-IV (SH-IV) as selective inhibitors of key epigenetic regulatory proteins—KMT2C, HDAC4, and DNMT3A—in CCa. Among the screened triterpenoid phytochemicals, BE and SH-IV were selected based on their high binding affinities, structural stability, and favorable drug-likeness, as determined using AutoDock, GLIDE docking, MM-GBSA calculations, and molecular dynamics (MD) simulations.

Remarkably, both compounds demonstrated strong binding interactions with KMT2C, with docking scores of -9.8 kcal/mol (BE) and -10.4 kcal/mol (SH-IV). These values reflect significant interaction strength, positioning these molecules as promising inhibitors of KMT2C, a lysine methyltransferase known to regulate gene expression through histone H3K4 methylation. Dysregulation of KMT2C has been implicated in various malignancies, including CCa, suggesting that its inhibition could restore normal chromatin states and gene transcription (Papillon-Cavanagh et al., 2017; Rao & Dou, 2015).

Compared to their moderate binding affinities with HDAC4 and DNMT3A, the selective preference for KMT2C suggests that these phytochemicals may exhibit target specificity, which is a desirable trait in epigenetic drug development to avoid systemic toxicity and off-target effects (Yoo & Jones, 2006).

The DFT (Density Functional Theory) analysis provided insights into the chemical reactivity of BE and SH-IV. The identification of electrophilic and nucleophilic regions in their molecular orbitals not only supports their potential for biological interaction but also opens possibilities for structural modification to enhance potency. The HOMO-LUMO gap and molecular electrostatic potential maps suggested that both compounds possess reactive centers suitable for chemical derivatization, a strategy often employed to improve pharmacological efficacy (Parr & Yang, 1989).

Furthermore, MD simulations over 500 ns revealed sustained binding stability, particularly of SH-IV with HDAC4, without major conformational disruption of the protein structure. This stability suggests that SH-IV can maintain functional inhibition over extended periods, a critical requirement for epigenetic therapies that often require long-term gene reactivation (Kouzarides, 2007).

ADME profiling using QikProp and BOILED-egg analysis demonstrated that both compounds exhibit high gastrointestinal absorption, low predicted toxicity, and compliance with Lipinski's Rule of Five, making them suitable for oral delivery. The finding that SH-IV is a predicted inhibitor of P-glycoprotein (P-gp) is particularly important, as P-gp overexpression is a key driver of multidrug resistance in various cancers, including cervical cancer (Sharom, 2011). By inhibiting this efflux transporter, SH-IV could potentially enhance intracellular retention and cytotoxicity of co-administered anticancer drugs.

Collectively, these computational insights suggest that BE and SH-IV are viable epigenetic modulators, capable of influencing histone methylation and deacetylation as well as DNA methylation-related pathways. Their polypharmacological potential, demonstrated by simultaneous interaction with multiple epigenetic regulators, supports their development as multi-targeted therapeutic agents for CCa (Hopkins, 2008).

However, computational predictions must be followed by experimental validation. Future studies should include *in vitro* and *in vivo* assessments to confirm the actual inhibitory effects on KMT2C, HDAC4, and DNMT3A, along with their downstream impact on tumor suppressor gene reactivation, cell proliferation, and apoptosis induction. Structure-activity relationship (SAR) studies and analog development based on the electronic and steric characteristics identified by DFT could yield more potent derivatives with improved efficacy and safety profiles.

In summary, this study provides robust computational support for the use of Betulin and Shatavarin-IV as selective, stable, and pharmacokinetically viable epigenetic regulators, laying the groundwork for further development of natural product-based epigenetic therapeutics for cervical cancer and potentially other malignancies involving epigenetic dysregulation.

4.2. Objective 2: To evaluate the potential of the selected molecules against CCa (*in vitro*) with nano formulation-based approaches.

4.2.1 Procurement of selected phytochemicals.

We procure Betulin from Otto Kemi Pvt. Ltd., Mumbai, having +98% purity, and Shatavarin was procured from Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore, having 98% purity

4.2.2. Characterisation of Pure Compounds:

4.2.2.1. Fourier Transformed Infrared Spectrophotometer (FTIR) of pure compound:

The FTIR technique was utilized to ascertain the inorganic and organic constituents of the materials by analyzing the readings of various peaks in the infrared radiation spectrum. When the chemical sample underwent FTIR analysis, the components were identified according to their peak frequencies. FTIR analysis confirmed the presence of various inorganic and organic components in the substance. Nonetheless, it possesses considerable limitations, including the inability to exhibit the metallic characteristics of any chemical. FTIR spectrum of BE (Betulin) Figure 30A. The absorption bands observed at 3395 cm^{-1} and 3748 cm^{-1} correspond to O–H stretching vibrations of hydroxyl groups, indicating the presence of alcohol functionalities. The peaks at 2921 cm^{-1} and 2846 cm^{-1} are attributed to C–H stretching vibrations of aliphatic $-\text{CH}_2$ and $-\text{CH}_3$ groups. The absorption band around 1683 cm^{-1} corresponds to C=O stretching, suggesting carbonyl functional groups, while peaks near 1444 cm^{-1} and 1370 cm^{-1} may arise from C–H bending vibrations. The strong peaks between $1100\text{--}1000\text{ cm}^{-1}$ (1089 cm^{-1}) correspond to C–O stretching of secondary alcohols or ether linkages, and peaks below 700 cm^{-1} can be attributed to skeletal vibrations of the compound. The FTIR spectrum of SH-IV (Shatavarin-IV), Figure 30B. The broadband near 3306 cm^{-1} is characteristic of O–H stretching in hydroxyl groups, while the absorption at 2918 cm^{-1} represents aliphatic C–H stretching. The peak around 1727 cm^{-1} corresponds to C=O stretching vibrations, likely from ester or glycosidic linkages. The band at 1619 cm^{-1} indicates C=C stretching from aromatic or olefinic moieties. Peaks in the range $1231\text{--}1029\text{ cm}^{-1}$ are assigned to C–O stretching vibrations, typical for glycosidic bonds in saponins. The band near 523 cm^{-1} may be due to bending vibrations in C–H or C–O groups.

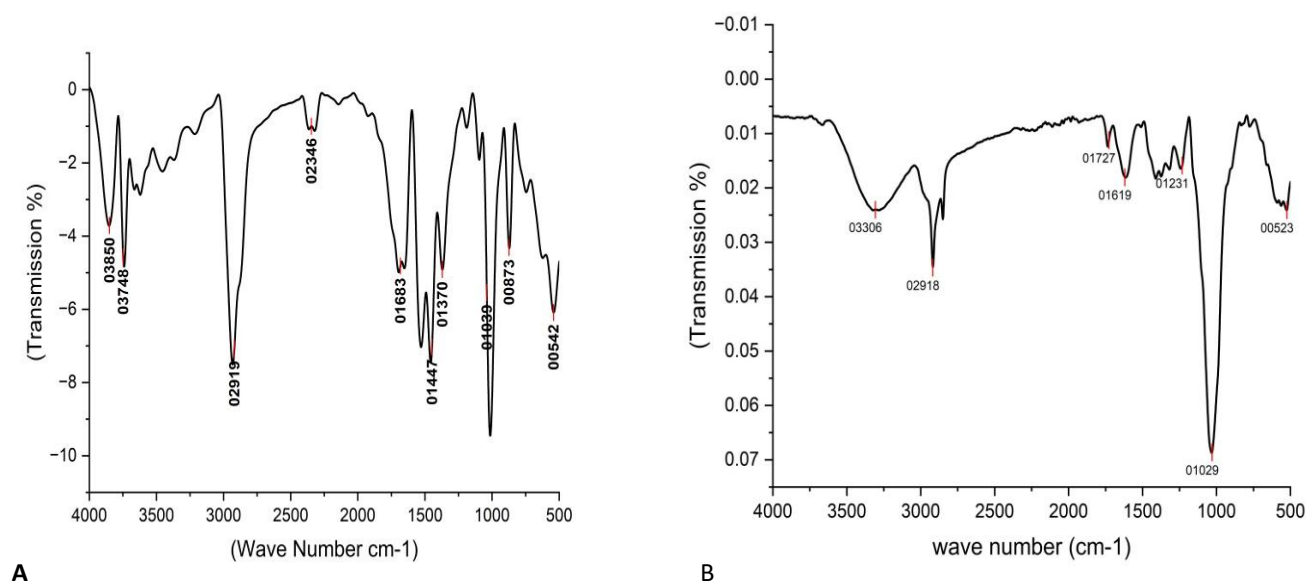


Figure 30:(a) Fourier transformed infrared spectrophotometer graph for BE, (b) Fourier transformed infrared spectrophotometer graph for SH-IV

4.2.2.2. DSC analysis of pure compound:

The DSC apparatus with thermal expansion was used to determine BE and SH. Standardization was previously performed utilizing a calibrating reagent. Subsequently, 3.3 mg of BE and 2.6 mg of SH were measured and placed in an aluminum testing crucible, which was then sealed and heated to 300 °C in a nitrogen atmosphere at rates of 5 °C/min and 10 °C/min, correspondingly. This technique is utilized to ascertain the melting range of a substance. The DSC thermogram of Betulin (BE), Figure 31A, shows a sharp endothermic peak observed at **260.71 °C**, with an onset temperature of **259.06 °C** and an end temperature of **261.52 °C**. This narrow peak suggests a well-defined melting point, indicative of the crystalline nature of BE. The enthalpy change (ΔH) of **86.3956 J/g** further supports the presence of a highly ordered crystalline lattice, which requires substantial energy for phase transition. The absence of additional broad peaks suggests thermal stability up to the melting point with no major decomposition before this temperature. The DSC thermogram of Shatavarin-IV (SH-IV), Figure 31B, shows that a major endothermic transition occurs with an onset temperature of **283.48 °C** and a peak at **333.62 °C**, ending at **381.48 °C**. The significantly broader peak and higher ΔH value (**674.3303 J/g**) compared to BE indicate a more complex thermal behavior, likely due to the breakdown of glycosidic linkages and degradation of the saponin structure. The broad nature of the endotherm suggests overlapping events, such as melting followed by partial decomposition. The high energy requirement reflects strong intermolecular interactions, possibly due to extensive hydrogen bonding from multiple hydroxyl groups in SH-IV.

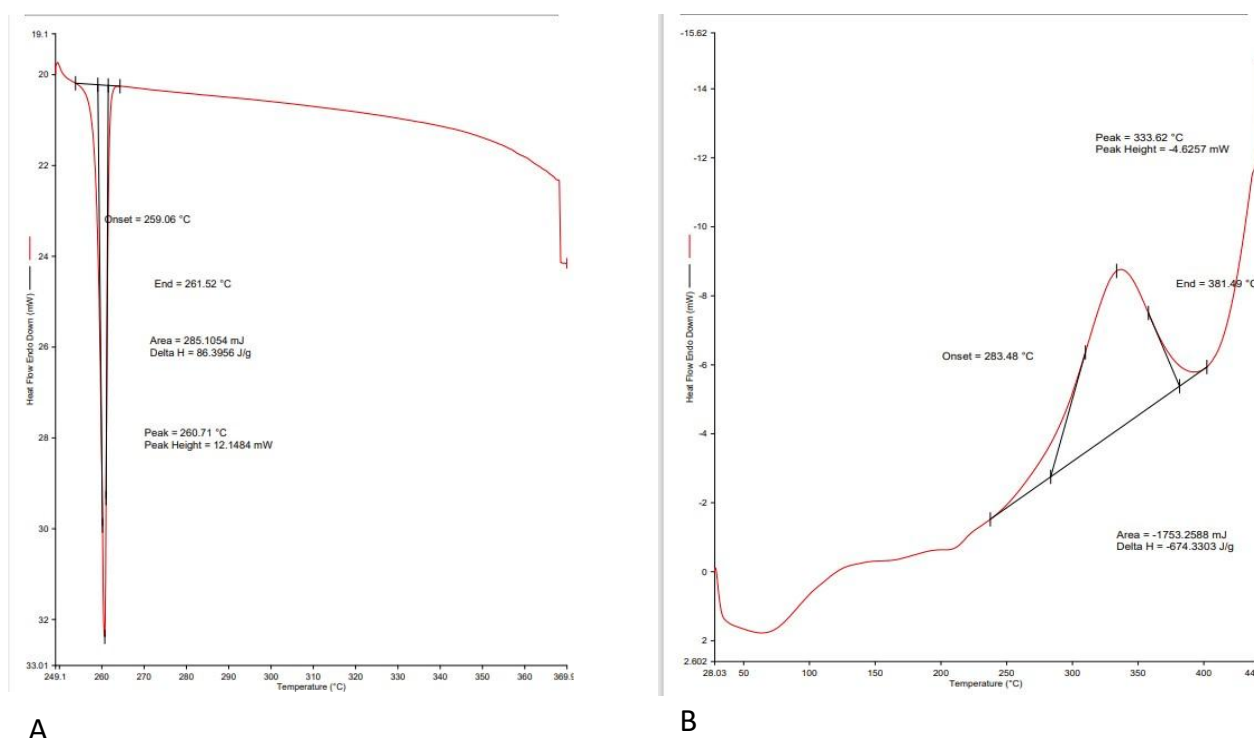


Figure 31: DSC graph (A) Betulin (B) Shatavarin

4.2.2.3. XRD analysis of Pure compounds:

This technique is utilized to ascertain the morphology or classification of the specimens, including crystalline or amorphous forms. An XRD graph (Intensity versus two theta) is utilized to determine the characteristics of the material. For pronounced peaks, the material can be considered crystalline. A broad peak indicates a polycrystalline structure, whereas the absence of a peak accompanied by a noisy pattern suggests an amorphous nature of the materials. Sharp peaks indicate that the sample exhibits a crystalline structure. The model is amorphous if the graph exhibits a flat peak. The XRD spectrum of pure Betulin (BE). The diffractogram exhibits multiple sharp and intense peaks at **2θ values** of 14.2°, 16.6°, 17.4°, 18.4°, 19.4°, 24.2°, 28.2°, 33.6°, 37.4°, and 43.6°, indicating the highly crystalline nature of BE. The strong diffraction intensity, especially around 16–18°, reflects a well-ordered molecular arrangement, consistent with its rigid pentacyclic triterpenoid structure. The presence of multiple peaks further confirms a high degree of crystallinity, which corresponds with the sharp melting point observed in DSC analysis (Figure 32A). Compared to BE, SH-IV exhibits fewer distinct peaks, with notable diffraction signals at **2θ values** of 14.9°, 22.2°, 24.3°, 26.6°, 38.2°, and 45.7°. The lower peak intensity and broader signals suggest a

predominantly amorphous or semi-crystalline structure. This is consistent with its complex saponin framework containing multiple sugar moieties, which can disrupt regular packing and limit long-range crystalline order (Figure 32B).

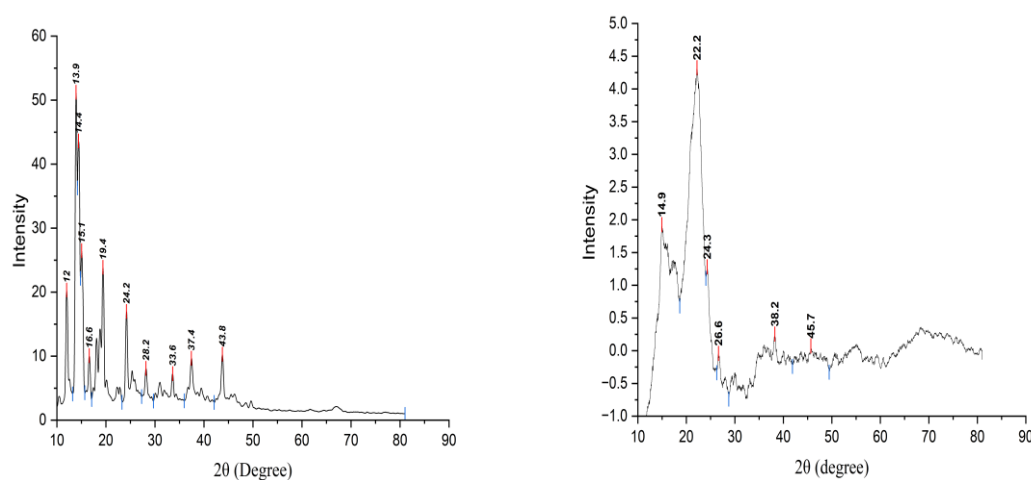


Figure 32: X-Ray diffraction graphs (A) XRD graph for BE pure compound, (B) XRD graph for SH-IV pure compound.

4.2.3. Synthesis of nanoparticles:

Two different metallic nanoparticles were synthesised: Zinc oxide and silver nitrate. The synthesised zinc oxide and silver nitrate nanoparticles by chemicals are shown in Figure 33.



Figure 33: A) ZnO-BE Nano Particles, (B) ZnO-SH Nano Particles, (C) Ag-BE Nano Particles, (D) Ag-SH Nano Particles

4.2.4. CHARACTERIZATION OF BE NANOPARTICLES:

4.2.4.1. FT-IR Spectroscopy of BE nanoparticles:

The FTIR spectra of BE–ZnO and BE–Ag nanoparticles revealed characteristic absorption bands corresponding to functional groups of Betulin along with signals indicative of metal–oxygen interactions, confirming successful nanoparticle formation. In BE–ZnO nanoparticles (Figure 34A), a broad band at 3334 cm^{-1} corresponded to O–H stretching vibrations, while peaks at 2933 and 2853 cm^{-1} were attributed to aliphatic C–H stretching. The band at 1695 cm^{-1} represented C=O stretching, and peaks in the range of $1010\text{--}607\text{ cm}^{-1}$ included contributions from C–O stretching and Zn–O vibrations, confirming the incorporation of zinc oxide. In BE–Ag nanoparticles (Figure 34B), the broad O–H stretching band appeared at $\sim 3751\text{ cm}^{-1}$, with aliphatic C–H stretching at 2929 and 2857 cm^{-1} , C=O stretching at 1657 cm^{-1} , and C–H bending at 1444 cm^{-1} . Additional peaks at 1378 and $1107\text{--}695\text{ cm}^{-1}$ corresponded to C–O stretching and C–H out-of-plane bending, respectively. In both nanoparticle formulations, noticeable shifts in peak positions and variations in intensities compared to pure BE indicated strong interactions between hydroxyl/carbonyl groups of BE and the nanoparticle surfaces, suggesting effective capping and stabilization.

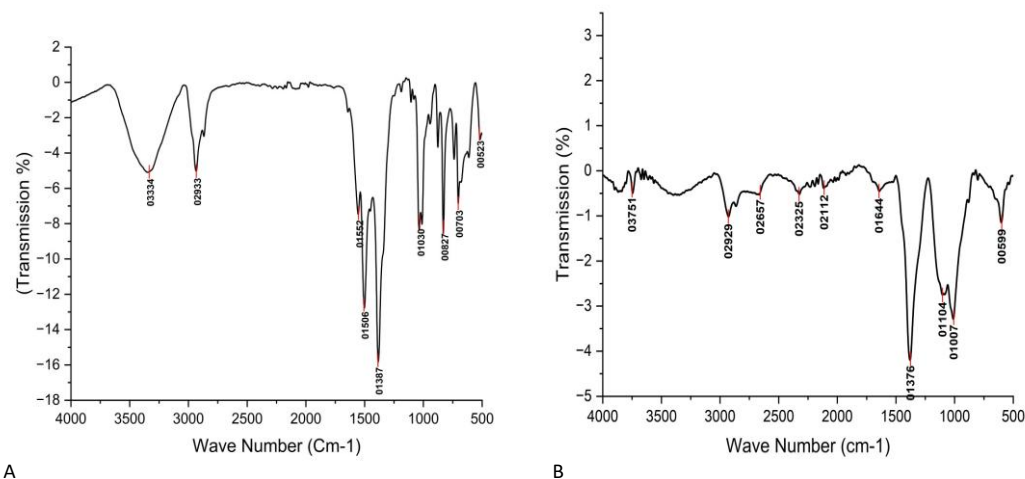


Figure 34: Fourier transformed infrared spectrum of (A) BE-Zinc oxide nanoparticles, (B) BE-Silver nanoparticles

4.2.4.2. XRD analysis of BE Nanoparticles:

The XRD patterns of BE-based nanoparticles demonstrated distinct diffraction features corresponding to the crystalline nature of the metal components and the interaction of BE with the nanoparticle surfaces. In BE–ZnO nanoparticles (Figure 35A), the diffractogram displayed broad peaks with low intensity, suggesting a predominantly amorphous or poorly crystalline structure, which could be due to the encapsulation of ZnO within the BE matrix and nanoscale particle size. The slight hump between 10° – 30° (2θ) is characteristic of organic components, while a sharper reflection near 31° indicates the presence of ZnO crystallites. In contrast, the BE–Ag nanoparticles (Figure 35B) exhibited sharp and intense diffraction peaks at characteristic 2θ values of approximately 27° , 32° , 38° , 46° , 54° , and 57° , which correspond to the (111), (200), (220), and (311) planes of face-centered cubic silver, confirming the crystalline nature of the AgNPs. The higher peak intensity in AgNPs compared to ZnO NPs indicates better crystallinity. Overall, the XRD results confirm successful nanoparticle formation, with BE–ZnO NPs displaying an amorphous to semi-crystalline profile and BE–Ag NPs exhibiting a highly crystalline structure.

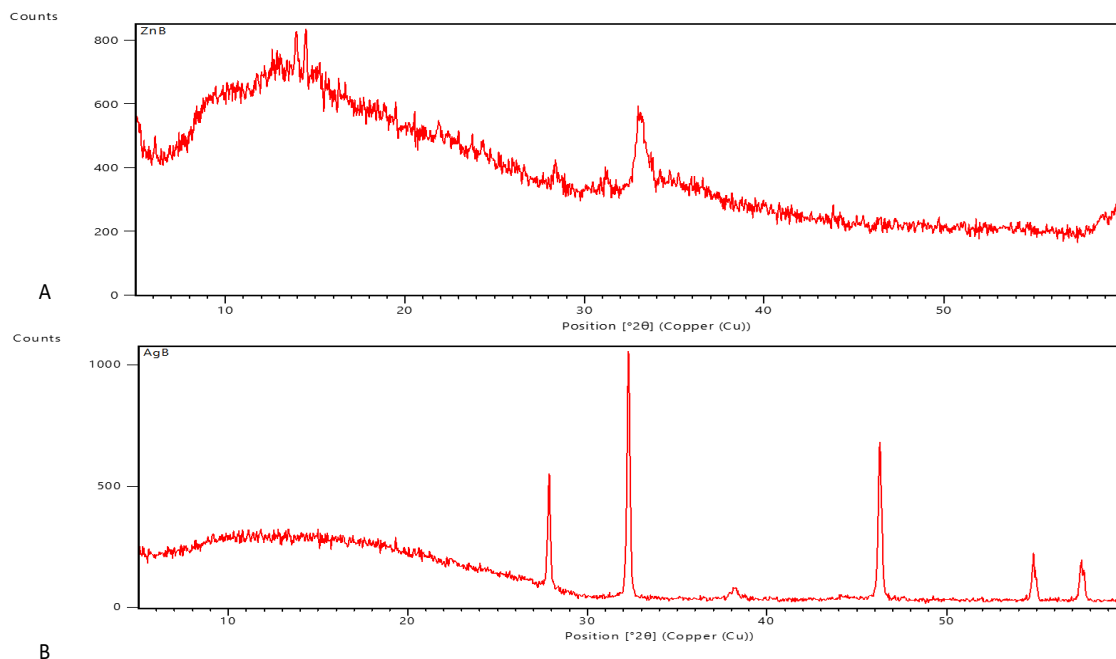


Figure 35: XRD pattern of BE Nano particles. (A) XRD pattern of BE-ZnO Nano particles. (B) BE-AgNO₃ Nano particles.

4.2.4.3 FE-SEM of BE Nanoparticles:

FE-SEM analysis revealed distinct morphological characteristics of Betulin-based nanoparticles, with ZnO–BE (Figure 36a) displaying irregular, aggregated particles embedded within a fibrous organic matrix, ranging from ~6–28 nm in size with a mean diameter of 14.03 ± 5.22 nm, whereas Ag–BE (Figure 36b) exhibited a more uniform distribution of spherical to slightly irregular particles densely deposited on the substrate surface, ranging from ~4–16 nm with a mean diameter of 8.07 ± 2.49 nm; these observations, supported by particle size distribution analysis, indicate that the synthesis process influenced both the morphology and size distribution of the nanoparticles, with Ag–BE forming comparatively smaller and more homogeneous particles than ZnO–BE.

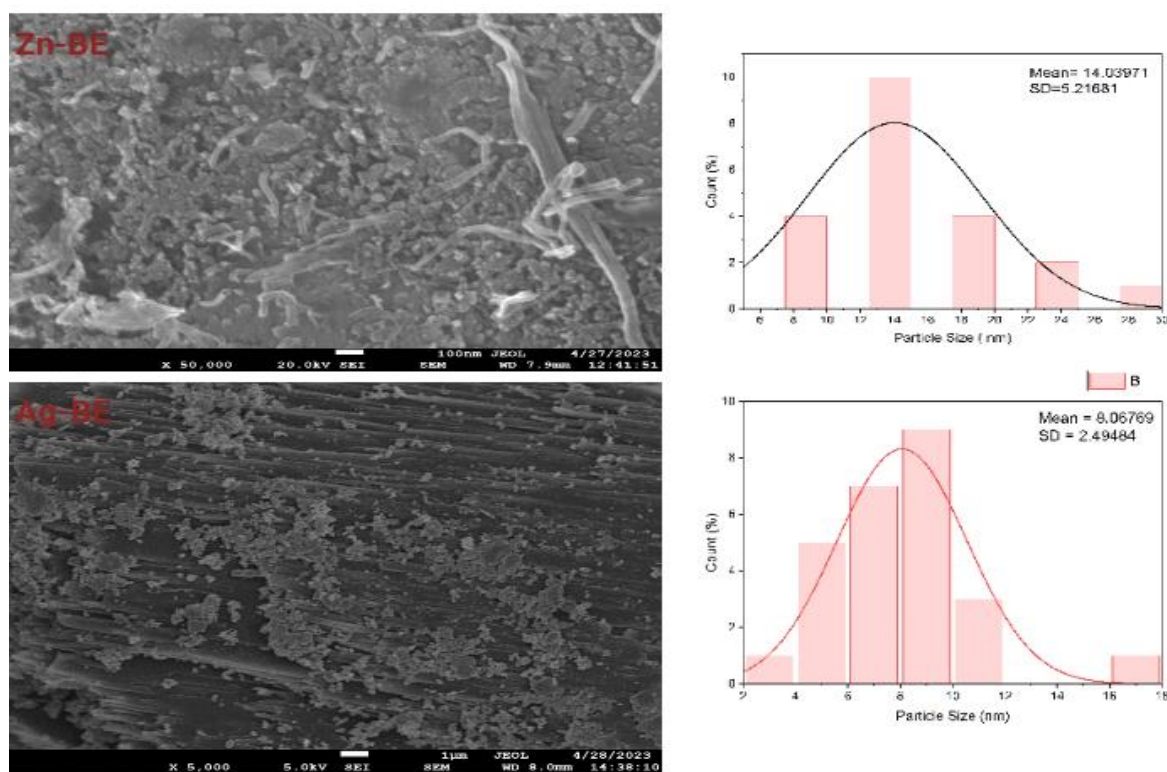


Figure 36: FE-SEM images and particle Size graph of BE Nano Particles. A) ZnO-BE Nano Particles (B) Ag-BE Nano Particles.

4.2.4.4. FESEM-EDS of BE Nanoparticles:

The composition of chemically synthesized Zinc oxide-BE nanoparticles consists of 14.99 % by weight and 18.58% by atomic in oxide (O) and 43.58% by weight and 13.28% of atomic in Zinc (Zn). The composition of Silver nitrite BE nanoparticles consists of 4.03% by weight and 6.23% by atomic in oxide (O) and 56.71% by weight and 12.99% of atomic in silver. The EDAX pattern verifies the presence of zinc oxide in the sample (Figure 37). The feeble carbon signal suggested the participation of carbon-containing biological molecules and their role in the preparation of zinc oxide and silver nitrite nanoparticles. So, based on this, we conclude that Betulin is covered with Zinc and silver, respectively.

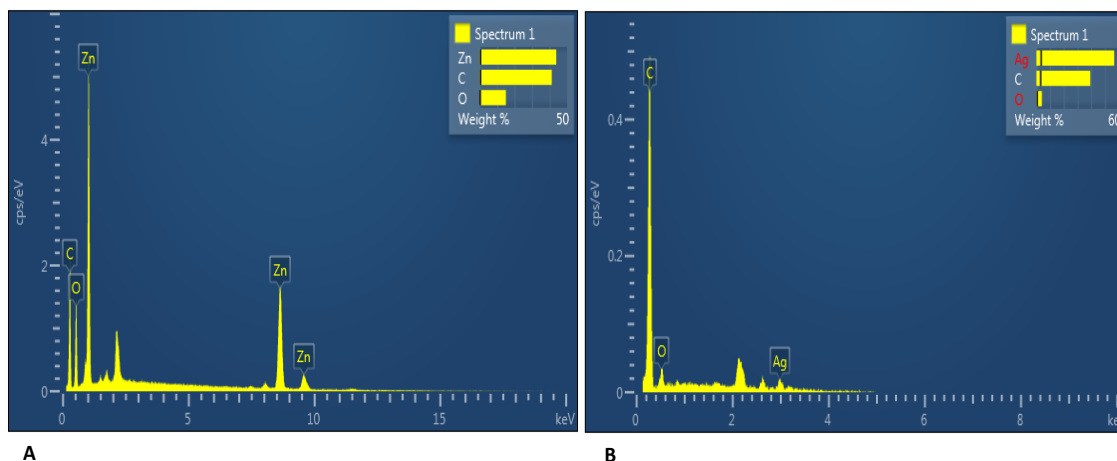


Figure 37: Depicts the FESEM EDS of BE Nano-Particles.

4.2.4.5: Zeta Potential analysis:

It is important to measure the Zeta potential (ZP) for the specification of the surface charge and stability of the synthesised nanoparticles of BE. The results of zeta potential values for ZnNPs and Ag NP's were obtained as -0.3mV and -3.0mV , respectively (Figure 38)

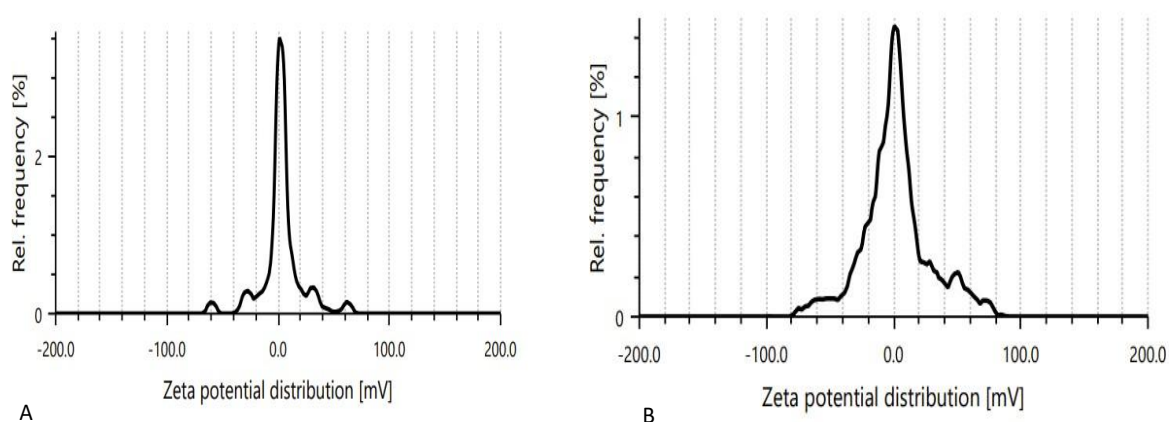


Figure 38: Zeta Potential graph of BE nano particles

4.2.5. Characteristics of SH Nano-Particles

4.2.5.1. FTIR of SH-IV Nanoparticles:

The FT-IR spectra of SH-zinc oxide nanoparticles, SH-Zinc oxide nanoparticles, and SH-Silver nitrate nanoparticles synthesised using the chemical method are shown in Figure 39a. The pattern of absorption of SH-Zinc nanoparticles at 556 and 898cm^{-1} Aromatic C-H out-of-plane bending is characteristic of a monosubstituted benzene ring and a 1,2,4-trisubstituted benzene

ring. Median absorption in the region $1170\text{--}1716\text{cm}^{-1}$ frequently denotes an aromatic ring. The weak bands near 2043 and 3024cm^{-1} represent the hydrogen O-H stretching vibrations. The presence of zinc oxide nanoparticles is confirmed at the $480\text{--}540\text{cm}^{-1}$ region. The pattern of SH-Silver nanoparticles showed prominent peaks at 2934cm^{-1} , 1763cm^{-1} , 1455cm^{-1} , 1350cm^{-1} , 1050cm^{-1} , 892cm^{-1} , and 836cm^{-1} , as shown in Figure 39b. As the AgNPs were prepared by stirring and heating, the peak of the hydroxyl group was absent; however, a low-intensity peak of the hydroxyl group was observed in an alkaline condition. Equivalent to C-C, C=C, and C-O functionalities, respectively.

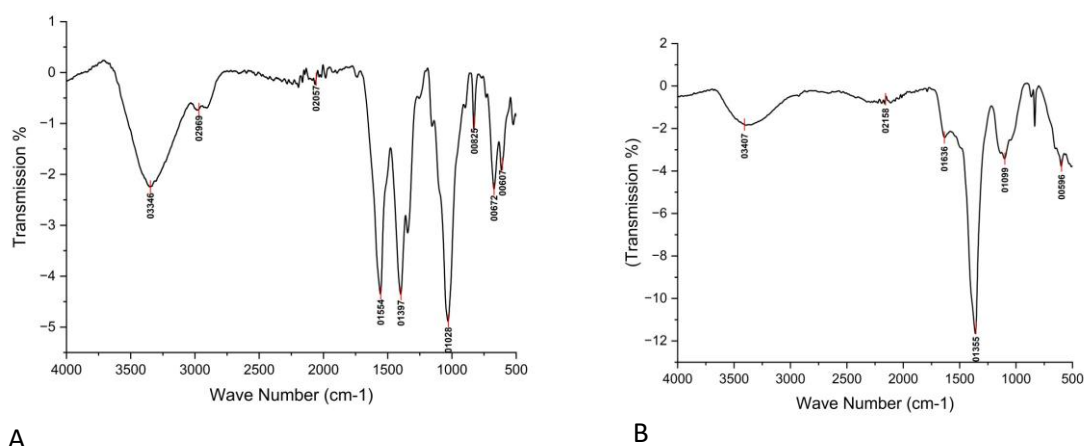


Figure 39: Fourier-transformed infrared spectrum (FTIR) of SH-Nano Particles. (A) SH- Zinc Oxide Nano Particles (B) SH-Silver nitrate Nano Particles.

4.2.5.2. XRD of SH-IV Nanoparticles:

The X-Ray diffraction pattern of SH- Zinc oxide and BE- Silver nitrite synthesised nanoparticles are shown in Figure 40. The XRD pattern exhibits sharp diffraction peaks, confirming the crystalline behaviour of the nanoparticles. The diffraction pattern of SH-ZnO showed well-defined peaks at 2θ angles of 31.6933° , 33.4402° , and 45.4449° corresponding to (101), (102), and (110). The diffraction pattern of SH-AgNO₃ also reflected the well-defined peaks at 2θ angles of 27.7658° , 32.1832° , 38.0982° , 46.1746° , 46.2498° , 54.7669° , and 57.4355° corresponding to (111), (200), (112), (222), (220), (311), and (411).

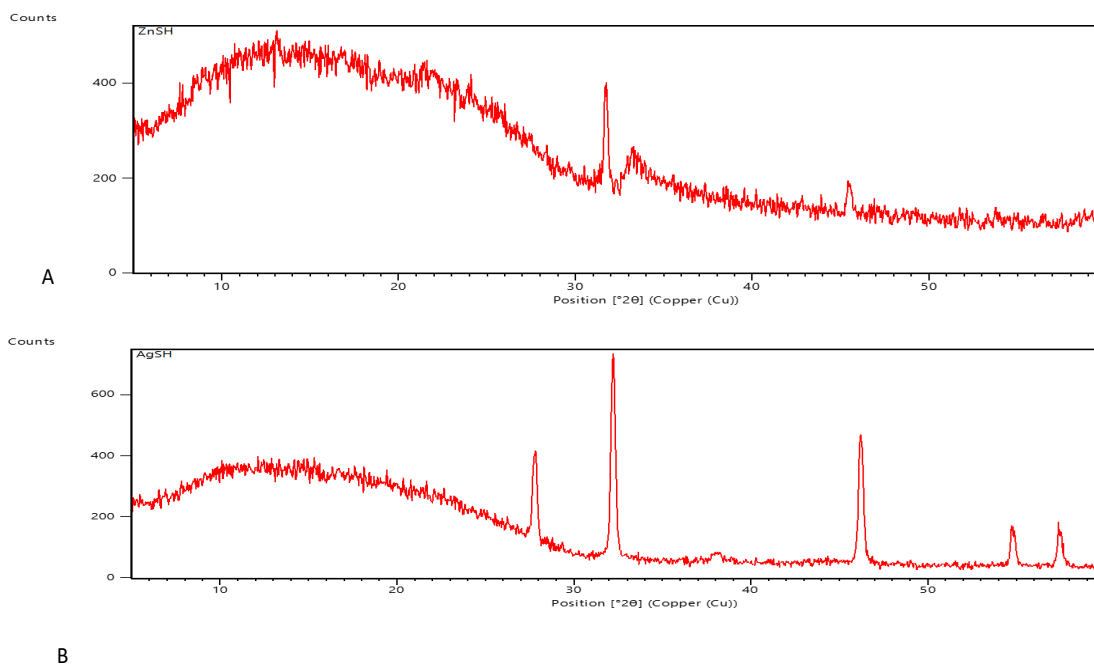


Figure 40: XRD pattern of SH Nano particles. (A) XRD pattern of SH-ZnO Nano particles. (B) SH-AgNO₃ Nano particles

4.2.5.3. FE-SEM of SH-IV Nanoparticles:

The shape and particle size of the synthesized SH-IV nano-particle samples were determined by FE- SEM analysis. The elemental analysis was carried out by using EDA. The FE-SEM images and EDA spectrum of SH nanoparticles were prepared at room temperature, by stirring, heating, and at alkaline conditions, are demonstrated in Figure 41. Comparatively, SH-NPs prepared by heating show a higher particle size. The image obtained from the FESEM and the EDS spectra of silver nitrite nanoparticles that were manufactured through the chemical method are displayed in Figure 41. The FE-SEM image that is presented here reveals that the particles are arranged in a cubic shape and are shown to be in an agglomerated state. There is a correlation between the binding energies of silver and the peak that can be found in the EDXRF spectrum around 2.4 keV. As a result, the EDXRF spectra for the Silver Nitrite that were obtained through the Chemical method demonstrated the presence of nanoparticles of silver nitrite that contained a lower concentration of impurities.

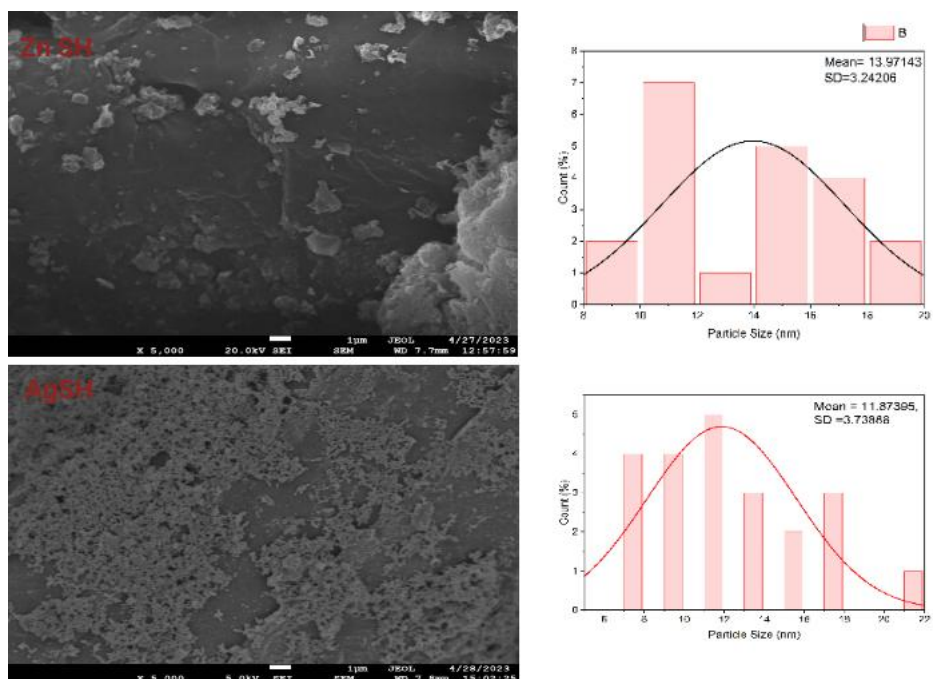


Figure 41. FE-SEM images and particle Size graph of SH Nano Particles. A) ZnO-SH Nano Particles (B) Ag-SH Nano Particles.

4.2.5.4. FESEM-EDS of SH-IV Nanoparticles:

Zinc oxide-SH nanoparticles that have been generated through chemical synthesis possess a composition that is comprised of 29.82% of weight and 48.05% of atomic in oxide (O), and 36.07% of weight and 10.68% of atomic in zinc (Zn). Silver nitrite SH nanoparticles have a composition that accounts for 435.41% of their weight and 50.34% of their atomic number in oxide (O), while silver accounts for 33.81% of their weight and 6.16 of their atomic number. Evidence exists from the EDAX pattern that reveals the existence of zinc oxide in the sample. The diminished carbon signal indicated the involvement of carbon-containing biological molecules in the production of zinc oxide and silver nitrite nanoparticles. Therefore, we can draw the conclusion that SH is covered with zinc and silver, respectively (Figure 42).

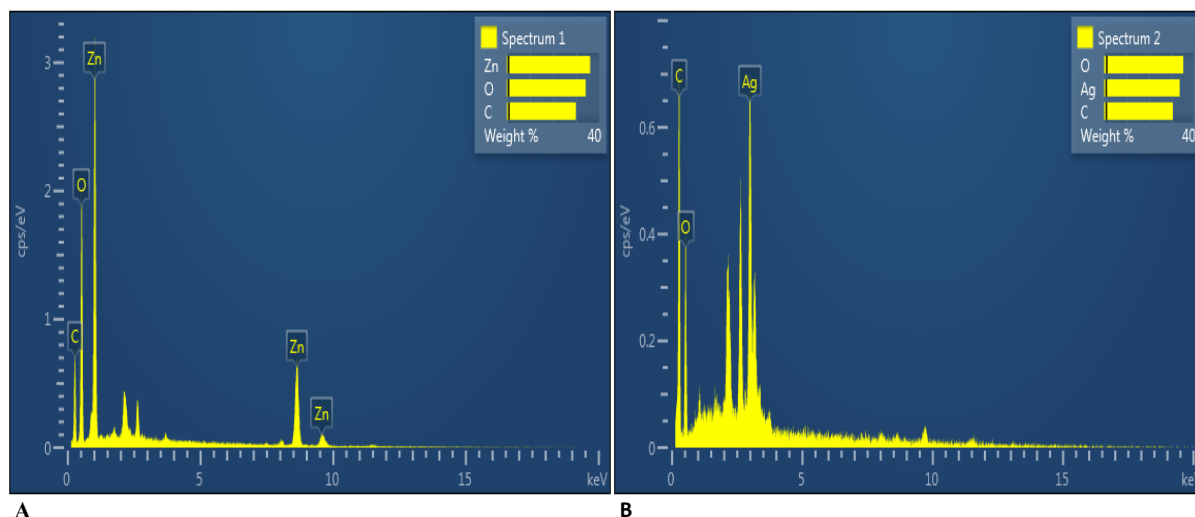


Figure 42: Figure. Depicts the FESEM EDS of SH-IV Nano-Particles.

4.2.5.5. Zeta potential analysis of SH-IV nanoparticles:

The Zeta potential of the chemically synthesised AgSH-IV and Zn -SH nanoparticles is calculated as -83.3Mv and -13.1Mv respectively. The higher negative value of AgSH-IV indicates its stability as a potent nanodrug (Figure 43)

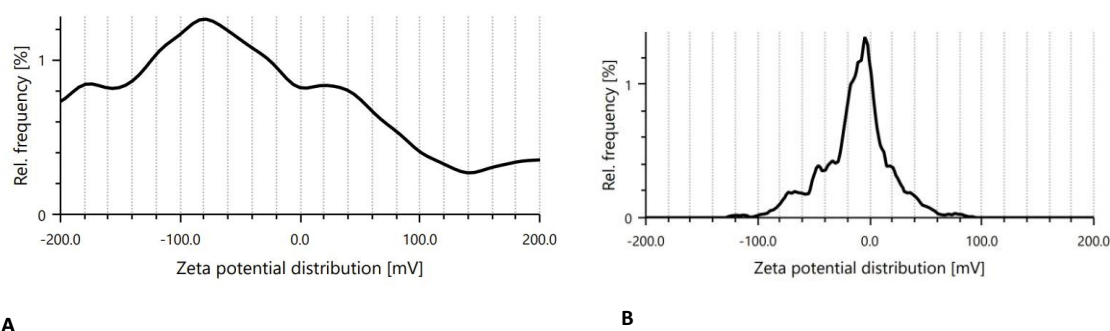


Figure 43: Zeta Potential graph of SH-IV nanoparticles.

4.2.6. In vitro Results

4.2.6.1. Cyto-toxicity

Analysis of cell viability in HeLa cells revealed a clear dose-dependent decrease in viability upon treatment with all tested compounds, with ZnBE and AgSH displaying significantly enhanced cytotoxicity and approximately two-fold lower IC₅₀ values compared to their respective pure compounds (BE and SH). ZnBE and AgSH exhibited high potency, effectively

reducing HeLa cell viability even at lower concentrations (5–10 $\mu\text{g/mL}$), whereas the pure compounds BE and SH showed comparatively higher IC₅₀ values, indicating moderate cytotoxicity that required higher concentrations for effective inhibition (Table 11). These findings demonstrate that nanoparticle formulations substantially improve drug delivery and potency against HeLa cells, likely due to enhanced cellular uptake and bioavailability. In parallel, the viability assay on HEK (normal kidney) cells indicated minimal cytotoxic effects for most compounds, with ZnBE and AgSH showing selective toxicity toward cancer cells while maintaining high viability in HEK cells, thus reflecting favorable therapeutic selectivity. Pure BE and SH also exhibited minimal impact on HEK cell viability, further reinforcing their biocompatibility; however, certain potent formulations displayed mild toxicity at higher concentrations, emphasizing the importance of dose optimization. Comparative IC₅₀ analysis confirmed that ZnBE and AgSH possess superior anticancer efficacy and higher selectivity indices (SI) compared to their pure counterparts, thereby highlighting their potential as safer and more effective therapeutic candidates for cervical cancer. Notably, ZnBE and AgSH (Samples 2 and 7) were identified as highly selective for HeLa cells, exhibiting potent cytotoxic effects against cancer cells while sparing normal HEK cells, whereas some samples (e.g., Sample 3) displayed non-selective toxicity, adversely affecting both cell types. Overall, nanoparticle formulations (ZnBE and AgSH) demonstrated significantly higher potency than pure BE and SH, with pronounced dose-dependent antiproliferative effects and lower toxicity toward normal cells, making them promising candidates for further anticancer evaluations. These findings provide a strong rationale for advancing ZnBE and AgSH to subsequent studies focused on mechanistic insights, apoptosis induction, epigenetic modulation, and in vivo efficacy testing to validate their therapeutic potential against cervical carcinoma (Figure 44).

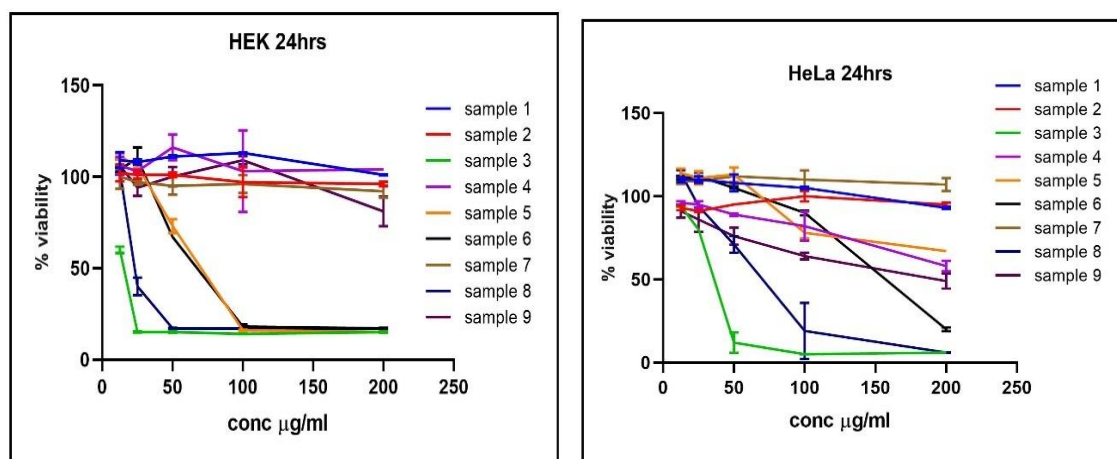


Figure 44: Cytotoxic and antiproliferative effects of pure Betulin (BE), Shatavarin-IV (SH), and their nanoparticle formulations (ZnBE and AgSH) on HeLa (cervical cancer) and HEK (normal kidney) cells after 24 hours of treatment as determined by the MTT assay. The data represent mean $\pm$ SD of three independent experiments performed in triplicate. A dose-dependent decrease in cell viability was observed in HeLa cells, with ZnBE and AgSH showing significantly enhanced cytotoxicity compared to their pure compounds. In contrast, minimal cytotoxic effects were observed in HEK cells, indicating selective toxicity of ZnBE and AgSH toward cancer cells.

Table 11: IC50 values of pure Betulin (BE), Shatavarin-IV (SH), and their nanoparticle formulations (ZnBE and AgSH) against HeLa (cervical cancer) and HEK (normal kidney) cells after 24 hours of treatment.

Samp les	Sample 1 ZnSH-IV		Sample 2 ZnBE		Sample 3 ZnBE-SH-IV		Sample 4 BETULIN		Sample 5 AgBE		Sample 6 (AgBE-SH-IV)		Sample 7 (AgSH-IV)		Sample 8 (Shatavarin-IV)		Sample 9 Control	
Cell line	HeLa	HEK	HeLa	HEK	HeLa	HEK	HeLa	HEK	HeLa	HEK	HeLa	HEK	HeLa	HEK	HeLa	HEK	HeLa	HEK
Conce ntrati on																		
200 µg/mL	90	100	20	95	0	15	90	100	70	15	90	65	20	60	20	15	40	90
100 µg/mL	100	110	30	100	10	15	95	120	100	75	100	95	30	95	60	45	0	105
50 µg/mL	105	110	90	100	20	15	95	110	110	110	110	95	50	100	90	105	80	100
12.5 µg/mL	110	110	95	100	30	15	100	110	110	110	110	100	60	100	110	110	90	105
IC ₅₀	6 µg/mL	22 µg/mL	3.5 µg/mL	3.6 µg/mL	23 µg/mL	26 µg/mL	24 µg/mL	21 µg/mL	21 µg/mL	7 µg/mL	22 µg/mL	22 µg/mL	8 µg/mL	22 µg/mL	13 µg/mL	9 µg/mL	21 µg/mL	22 µg/mL

4.2.6.2. Sulphorhodamine B (SRB) Assay:

We analyzed the impact of pure BE, SH, and their chemically synthesized ZnO₂ and AgNO₃ nanoparticles on cancer cell proliferation by conducting the SRB assay on SiHa cells. The impact of drugs was assessed by exposing the cells to specific concentrations (50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, and 3.12 µg/ml) for 24 hours, as indicated in Table 12. The treatment resulted in a significant reduction in cell proliferation in SiHa cells, with a decrease of 50-60% compared to the control group. Additionally, there were significant dose-dependent decreases in the proliferation of SiHa cells. AgSH-IV exhibited significant suppressive activity with an IC₅₀ value of 6.6 µg/ml, while ZnO-BE, Pure BE, and Pure SH-IV presented IC₅₀ values of 23.1 µg/ml, 7.5 µg/ml, and 13.2 µg/ml, respectively (Figure 45).

Table 12: Growth inhibition of BE, SH-IV, ZnBE, and AgSH against the SiHa cell line and IC₅₀ value in µg/mL of selected compounds.

Growth Inhibition against the SiHa cell line				
Compound →	Betulin	ZnB	Shatavarin-IV	AgSH
Concentration ↓				
50 µg/mL	78.63± 0.8	67.24± 6.06	80.72± 6.7	85.17± 1.8
25 µg/mL	77.27± 0.6	61.17± 13.9	71.15± 8.1	81.31± 3.7
12.5 µg/mL	73.56± 0.9	50.36± 13.7	49.21± 2.9	64.27± 8.8
6.25 µg/mL	34.98± 1.8	20.4± 3.3	11.96± 2.3	43.5± 4.8
3.12 µg/mL	24.005± 2.6	11.64± 3.9	4.175± 1.9	9.27± 1.9
IC ₅₀	7.5±1.5	23.1±8.3	13.2±1.9	6.6±1.4

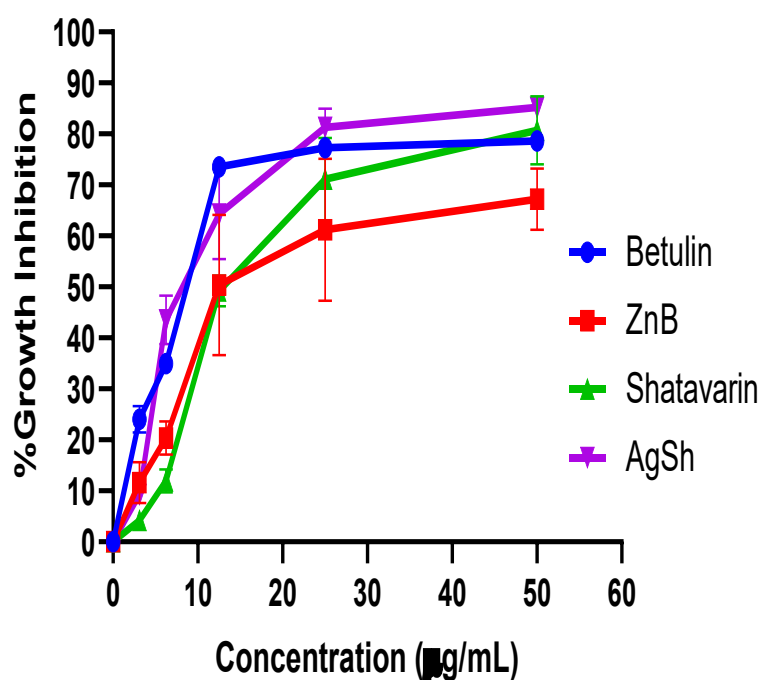


Figure 45: Percentage inhibition of growth of BE, SH-IV, ZnBE, and AgSH at different concentrations on the SiHa Cell line.

4.2.6.3 In-vitro wound healing Scratch assay

Cell migration and proliferation of CCa cells (SiHa) were inhibited by ZnBE and Ag-SH nanoparticles. To examine the compounds that may diminish the wandering behavior of SiHa cells, we conducted a scratch or wound assay. The scratch assay is a convenient, economical, and widespread method for assessing in vitro wound healing, as it simulates in vivo wound healing (C. C. Liang et al., 2007). We utilized two doses (below IC₅₀, 10 µM and above IC₅₀, 20 µM) based on the IC₅₀ of ZnBE and AgSH nanoparticles on SiHa cells. The findings demonstrated that the untreated almost completely occupied the wound within 24 hours. In contrast, the cells treated with 10 µM and 20 µM ZnBE and AgSH were unable to close the scratch, leaving the "wound" unhealed even after 24 hours. ZnBE nanoparticles at concentrations of 10µM and 20µM over 24 hours diminished SiHa cell proliferation, with relative migration percentages of 10.23% and 9.34%, respectively, compared to the untreated control of 79.0%. The AgSH nanoparticles at concentrations of 10µM and 20µM significantly diminished SiHa cell proliferation over 24 hours, resulting in relative migration percentages of 18.53% and 36.64%, respectively, compared to the untreated control of 80.1% (Figure 46a). With a statistically significant p-value of less than 0.005. The obstruction ratio of cell

propagation is quantified by assessing the vacant area after 24 hours using ImageJ Software (<https://imagej.nih.gov/ij/>). ZnBE and AgSH nanoparticles significantly inhibited the motility and propagation of SiHa cells, resulting in the scratch remaining unoccupied after 24 hours, as demonstrated in Figure 46b. These results indicate that ZnBE and AgSH nanoparticle treatment of CCa cell lines diminishes their proliferative capacity and impedes their wound healing ability.

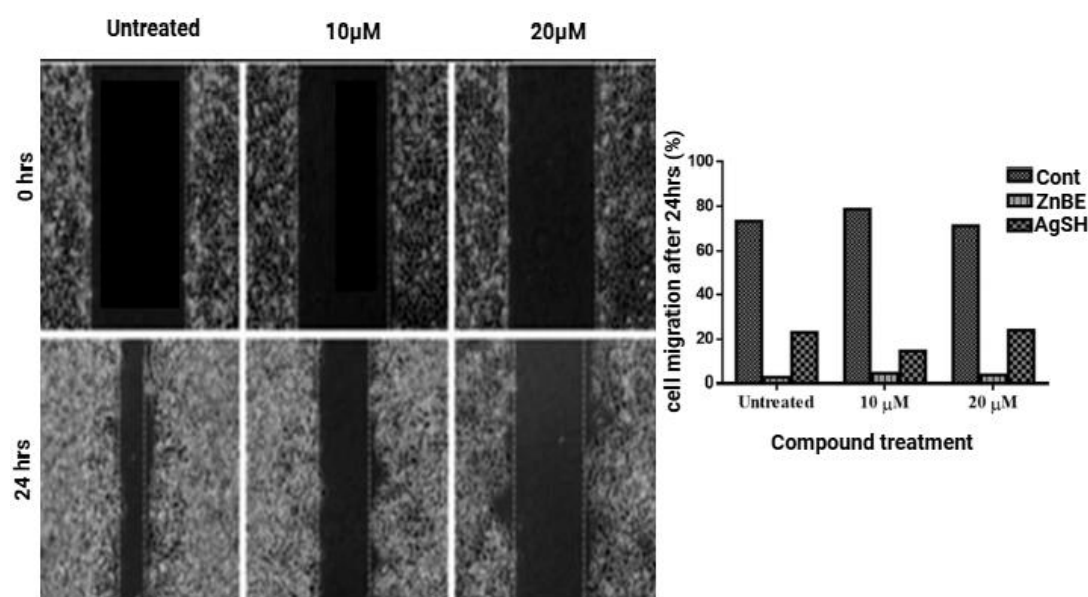


Figure 46: Compound ZnBE and AgSH impede the proliferation of SiHa cells. A) SiHa cells were grown in 6-well plates, and a scratch wound was created. The cells were treated with ZnBE and AgSH nanoparticles at concentrations of 10µM and 20µM, while a control group was left untreated. B) A bar graph displaying the quantitative measurements of the scratch area. ****indicates a p-value of less than 0.005.

The in vitro findings of the study strongly demonstrate that both Betulin (BE) and Shatavarin-IV (SH-IV) exhibit potent anticancer activity against SiHa and HeLa cervical cancer cell lines, with their nanoformulated versions (ZnO and AgNPs) displaying enhanced efficacy and therapeutic potential. Using MTT and SRB assays, a dose-dependent decrease in cell viability was observed. Among the tested formulations, AgSH-IV showed the most potent activity, with an IC₅₀ of 6.6 µg/mL, followed by pure BE (7.5 µg/mL), confirming the cytotoxic potential of both phytocompounds.

The improvement in efficacy observed in the nanoparticle formulations is attributed to enhanced solubility, stability, and cellular uptake, particularly for hydrophobic molecules like

SH-IV. Metal oxide-based nanocarriers, especially Ag and ZnO, are known to facilitate drug internalization, bypass efflux pumps, and generate reactive oxygen species that contribute to apoptosis in cancer cells (Baptista et al., 2018; Siddiqi et al., 2018). Additionally, wound healing (scratch) assays confirmed that ZnBE and AgSH-IV significantly inhibited cancer cell migration, highlighting their anti-metastatic potential, a critical factor in cervical cancer progression and recurrence.

The physicochemical characterization of the nanoparticles provided critical insights into their stability and suitability for biological applications. FTIR spectroscopy confirmed successful complexation of BE and SH-IV with ZnO and Ag, with peak shifts indicating chemical interactions between functional groups and metal ions, which are important for ensuring consistent drug release and targeting efficiency. The XRD analysis revealed nanocrystallinity in ZnO particles, enhancing reactivity due to increased surface area, while AgNPs displayed high crystallinity, contributing to colloidal stability and reduced aggregation risk in biological fluids (Iravani et al., 2014).

Field Emission Scanning Electron Microscopy (FESEM) showed uniform spherical morphology in ZnBE nanoparticles, indicating predictable surface behavior and cellular interactions. In contrast, AgSH-IV nanoparticles exhibited notable clustering, likely due to interactions between SH-IV and silver ions. While some clustering may enhance localized drug delivery, excessive aggregation can reduce bioavailability, emphasizing the importance of optimizing stabilizing agents during synthesis.

Elemental analysis (FESEM-EDS) revealed varying oxygen content and elemental distribution, suggesting differences in synthesis efficiency and compound incorporation. ZnO-SH-IV showed higher oxygen content than ZnO-BE, while AgNO₃-SH-IV had more stable elemental incorporation than its Betulin counterpart, implying that Shatavarin-IV may act as a more effective stabilizer, particularly in silver-based systems.

Perhaps the most critical physicochemical parameter influencing bioactivity is the zeta potential, which reflects nanoparticle surface charge and colloidal stability. The AgNO₃-SH-IV formulation exhibited excellent stability (-83.3 mV), which is ideal for systemic drug delivery as it reduces aggregation and ensures uniform dispersion in physiological environments. In contrast, ZnO-BE (-0.3 mV) and AgNO₃-BE (+3.0 mV) demonstrated poor

stability, which could limit their therapeutic applications unless stabilizers are optimized (Honary & Zahir, 2013).

From a therapeutic perspective, AgSH-IV combined high cytotoxicity with high colloidal stability, making it the most promising formulation in this study. Meanwhile, ZnBE's moderate cytotoxicity and lower toxicity profile may suit applications such as targeted drug delivery, where biocompatibility is a priority. These findings underscore the importance of tuning nanoparticle characteristics, not only for maximizing efficacy but also for minimizing systemic toxicity—an essential consideration in nanomedicine (Patra et al., 2018).

This study supports previous research indicating that metallic nanocarriers enhance drug bioactivity by improving intracellular accumulation, altering cell signaling, and inducing apoptosis and oxidative stress (Baptista et al., 2018; Siddiqi & Husen, 2017). However, the choice of stabilizer—BE vs. SH-IV—significantly influences particle stability, morphology, and aggregation, suggesting that formulation strategies must be tailored to the physicochemical nature of both the metal and the phytochemical.

4.3. Objective 3: To examine Qualitative and Quantitative assessment of the epigenetically altered proteins under study.

4.3.1. RT-qPCR analysis of proteins with compounds:

SiHa cells underwent treatment with pure BE, SH-IV, and their chemically synthesized Zn-BE and AgSH nanoparticles for 48 hours, followed by RT-qPCR analysis to assess the gene expression of HDAC4, DNMT3A, and KMT2C in comparison to control (untreated) samples.

RT-qPCR data have revealed that BE, SH-IV, and AgSH treatment have successfully inhibited the expression of the protein HDAC4. Figure 47, in a dose-dependent manner compared with the untreated cells. The molecules have displayed significant inhibition of the protein HDAC4 expression in CCa.

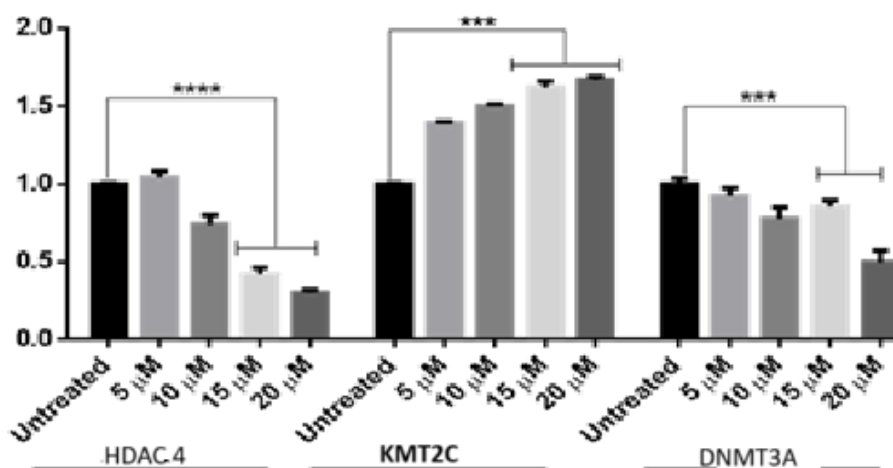


Figure 47: Expression analysis of the Target proteins

4.3.2. Expression of HDAC4 by BE, SH-IV ZnBE, and AgSH nanoparticles:

Regulation of Epigenetic Proteins. The expression of HDAC4 was assessed through western blot analysis following incubation with pure BE, SH-IV, and their chemically synthesized ZnBE and AgSH nanoparticles. We utilized the following IC₅₀ concentrations: 5 μ M, 10 μ M, 15 μ M, and 20 μ M on SiHa cells. The outcomes confirmed a dose-dependent significant decrease in the expression of HDAC4 protein in cells treated with AgSH as compared to untreated cells and the cells treated with BE, SH-IV, and ZnBE. ACTB was identified as the internal control. Data show that treatment with AgSH significantly induces epigenetic alterations in SiHa cells. According to the densitometric graph in Figure 48 B, HDAC4 expression decreases with a substantial fold change.

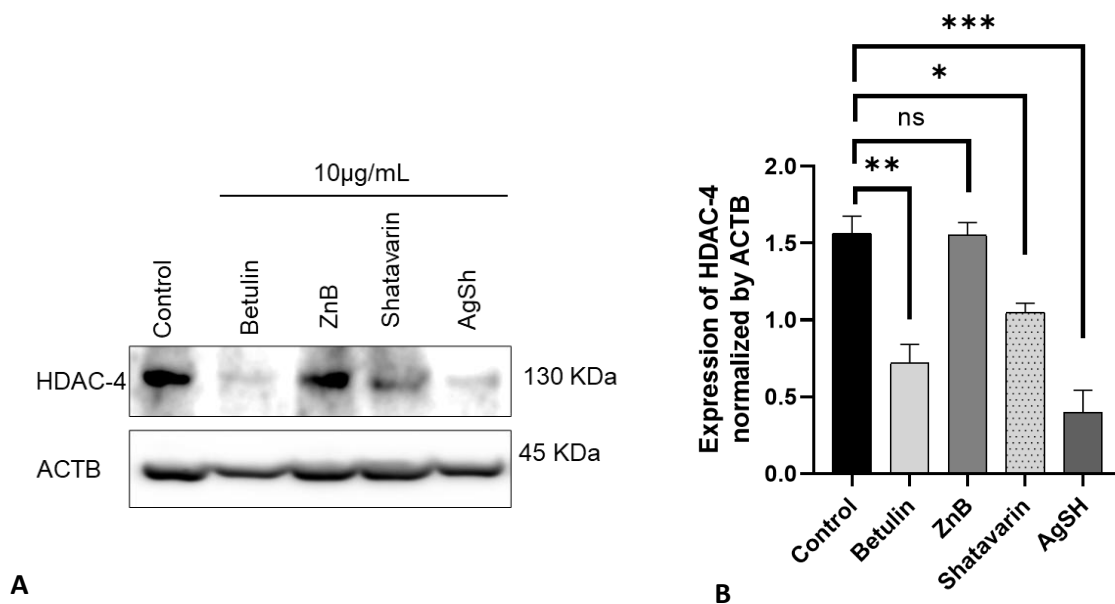


Figure 48: (A) Western blot analysis showing a decrease in HDAC4 expression in SiHa cells subjected to BE, SH-IV, ZnBE, and AgSH at specified concentrations for 48 hours, and ACTB was used as a control. (B) Significant reduction of HDAC4 was observed in response to AgSH treatment, ns-not Significant, ***significant at $p < 0.05$ level.

To investigate the molecular mechanism of action underlying the anti-cancer properties of Betulin (BE) and Shatavarin-IV (SH-IV), this study analyzed the expression of key **epigenetic regulators—DNMT3A, HDAC4, and KMT2C—**post-treatment using RT-qPCR and Western blotting in SiHa cervical cancer cells. These regulators were selected for their pivotal role in epigenetic silencing, chromatin remodeling, and tumor progression.

Treatment with BE, SH-IV, ZnBE, and AgSH-IV resulted in significant downregulation of HDAC4 and DNMT3A mRNA expression, with the most pronounced effect observed in AgSH-IV-treated cells ($p < 0.001$). Notably, AgSH-IV also upregulated KMT2C expression, suggesting a tumor-suppressive role through activation of H3K4 methylation, which is known to promote transcriptionally active chromatin states (Rao & Dou, 2015).

The dose-dependent changes in gene expression further reinforce the therapeutic relevance of AgSH-IV, indicating that higher concentrations result in stronger suppression of oncogenic epigenetic silencers (HDAC4 and DNMT3A) and greater activation of tumor suppressive

factors like KMT2C. This highlights the importance of dosage optimization for maximizing efficacy while minimizing off-target effects.

Protein-level data obtained from Western blotting closely mirrored the gene expression findings. Treatment with AgSH-IV significantly reduced HDAC4 protein expression ($p < 0.001$), confirming a strong epigenetic impact. BE also reduced HDAC4 expression, though to a lesser extent ($p < 0.01$), while SH-IV and ZnBE produced moderate suppression, indicating compound-specific activity.

The reduction of HDAC4 is particularly important, as this enzyme is associated with tumorigenesis, proliferation, and immune evasion through deacetylation of histone and non-histone proteins. HDAC4-mediated gene silencing is a hallmark of cancer epigenetics, and its inhibition has been linked to re-expression of tumor suppressor genes (Weichert, 2009; Esteller, 2008). Therefore, the marked suppression of HDAC4 by AgSH-IV supports its anti-cancer mechanism via epigenetic reprogramming.

The concurrent downregulation of DNMT3A and HDAC4, alongside upregulation of KMT2C, suggests that AgSH-IV targets multiple epigenetic layers—DNA methylation and histone deacetylation—while also enhancing histone methylation linked with active gene transcription. This trifecta of epigenetic modulation positions AgSH-IV as a potent chemoepigenetic agent, potentially superior to existing epigenetic drugs that typically target a single enzyme class (e.g., decitabine for DNMTs, vorinostat for HDACs) (Baylin & Jones, 2011; Yoo & Jones, 2006).

The nanoparticle delivery system plays a central role in enhancing the bioactivity of SH-IV. Silver nanoparticles not only improve solubility and intracellular accumulation but also facilitate targeted nuclear delivery, contributing to enhanced transcriptional effects. These results affirm previous studies indicating that metal-based nanocarriers potentiate the pharmacological efficacy of bioactive natural products (Patra et al., 2018).

Taken together, the combined results of RT-qPCR and Western blot analyses provide compelling evidence for the role of AgSH-IV in reversing oncogenic epigenetic reprogramming in cervical cancer cells. Its ability to modulate key gene regulators in a dose-dependent and statistically significant manner ($p < 0.001$) highlights its promise as a multi-targeted therapeutic. These findings pave the way for future preclinical studies, exploring in vivo efficacy, pharmacokinetics, and safety to translate this strategy into clinical applications.

4.4. Objective 4: To uncover the cervical cell lines to develop antitumor phytochemical-based drugs for enhanced efficacy using network-based analysis.

4.4.1. Epigenetic gene expression validation by GEPIA

To study the relative gene expression of Epigenetic proteins under study in cervical cancers. In comparison to normal cells, our investigation demonstrated that the expression levels of the gene we examined were substantially lower in CCa cells. Our reverse transcription polymerase chain reaction (RT-PCR) analysis confirmed this discovery, as it yielded consistent results with the data obtained from the Gene Expression Profiling Interactive Analysis (GEPIA) database.

The GEPIA database is a valuable resource that offers a diverse array of statistical analyses on gene expression in various cancer types. It provides a more comprehensive comprehension of the roles of specific genes in cancer biology by providing insights into their expression patterns. The differential expression analysis of the epigenetic gene that we concentrated on was visually represented in our study using box diagrams generated by GEPIA. The variation in gene expression levels between CCa tissues and normal tissues is further emphasized by these box plots, as illustrated in Figure 49, which supports our findings.

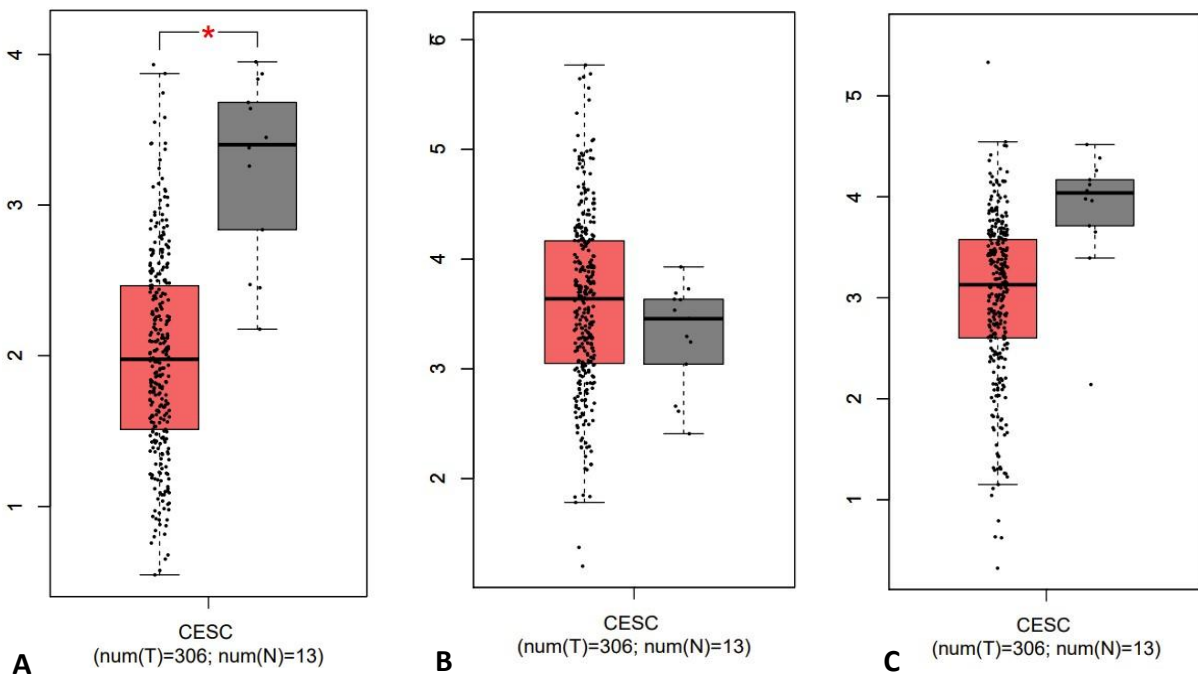


Figure 49: Expression level of HDAC4, DNMT3A, AND KMT2C genes in the GEPIA database. Epigenetic genes were downregulated in cervical cancer. The red and gray boxes represent cancer and normal tissue, respectively.

4.4.2. Expression level of HDAC4 and DNMT3A genes in ULCAN database:

The analysis using the University of Alabama Cancer (UALCAN) database indicated that the median expression levels of HDAC4 and DNMT3A transcripts were significantly ($P \leq 0.001$) downregulated in primary cervical squamous cell carcinoma. Specifically, HDAC4 expression was notably lower across all stages of the disease: Stage 1 (3.414), Stage 2 (3.263), Stage 3 (3.126), and Stage 4 (4.332) compared to normal cervical tissues (Figure 50). In contrast, DNMT3A expression was significantly higher in all stages: Stage 1 (13.405), Stage 2 (14.606), Stage 3 (11.852), and Stage 4 (9.88). Moreover, HDAC4 transcripts per million were lowest in cervical tumor samples from Asian patients (2.423), followed by African American (3.083) and Caucasian (3.352) populations. Conversely, DNMT3A transcripts per million were highest in African American patients (15.387), followed by Asians (13.726) and Caucasians (12.956) (Figure 50).

The results also showed that HDAC4 expression was significantly downregulated, while DNMT3A was significantly upregulated in tumor samples from CCa patients (Figure 51), regardless of age. The median HDAC4 transcripts in patients aged 21-40, 41-60, and 61-80 were 2.87, 3.418, and 3.704, respectively. In comparison, the median DNMT3A transcripts in the same age groups were 12.624, 13.64, and 11.92 (Figure 52).

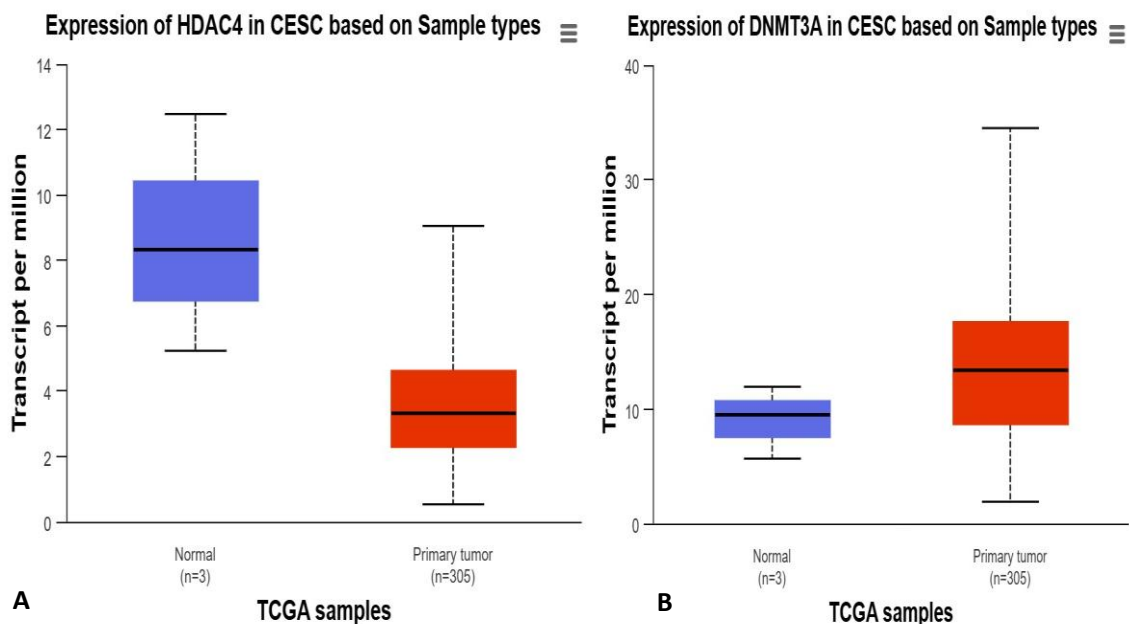


Figure 50: Differential Expression of (A) HDAC4 and (B) DNMT3A in cervical squamous carcinoma in comparison to normal tissue as assessed by UALCAN. Blue colour box represents

the expression of the gene in normal tissue, and red colour represents the expression in the tumor, respectively.

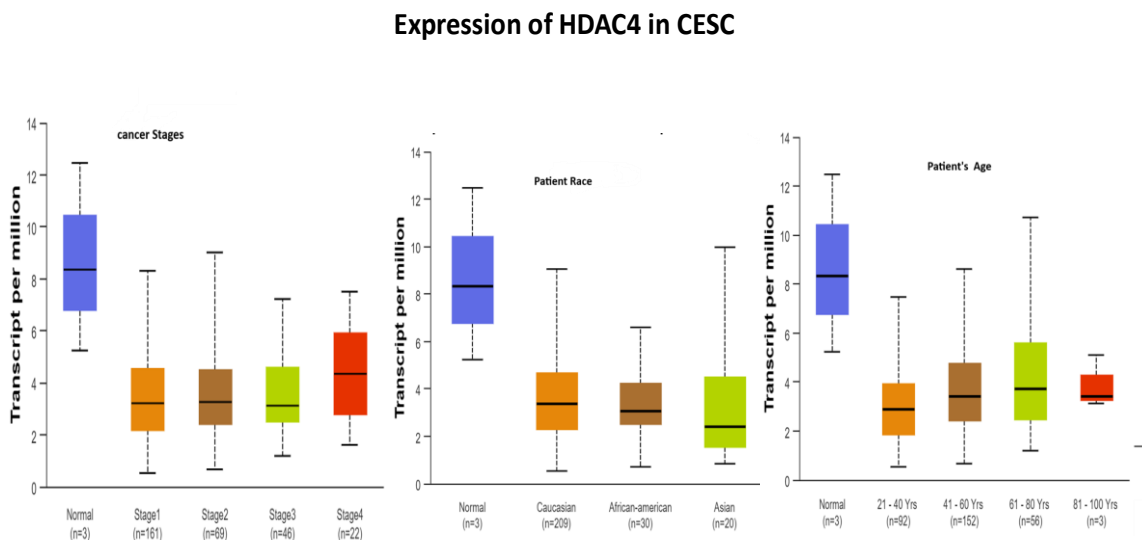


Figure 51: Analysis of HDAC4 expression in cervical squamous cell carcinoma and adjacent normal samples utilizing the UALCAN database. The images illustrate the comparison of HDAC4 expression across (a) histological subtypes, (b) cancer stage, (c) patient(s) race, and (d) patient(s) age.

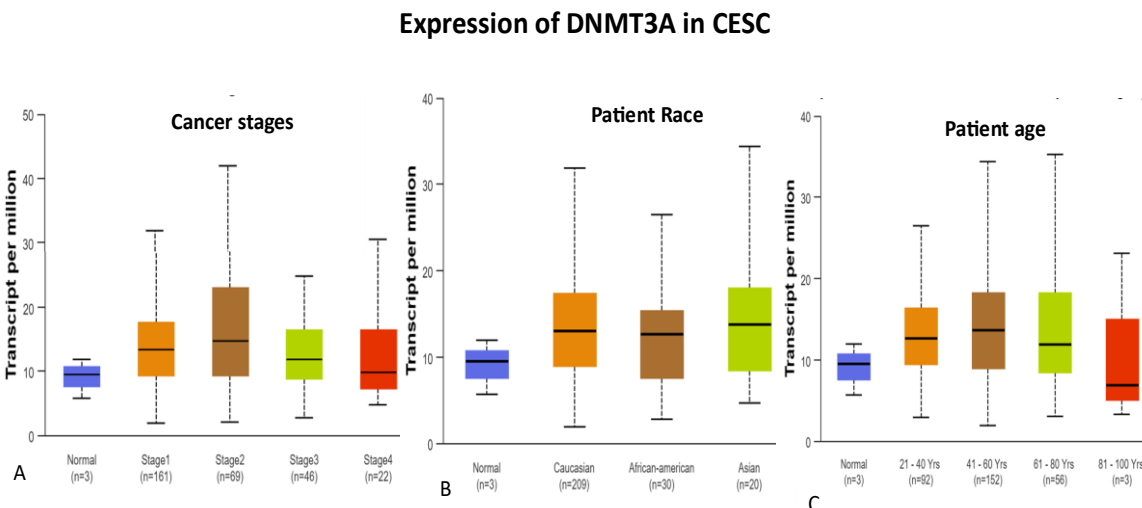


Figure 52: Analysis of DNMT3A expression in cervical squamous cell carcinoma and adjacent normal samples utilizing the UALCAN database. The images illustrate the comparison of DNMT3A expression across (a) histological subtypes, (b) Cancer stage, and (c) patients. Race, and (d) patients' age.

4.4.3. Protein-Protein interaction network of natural compounds, CCa and SiHa cell lines:

The protein-protein interaction (PPI) network of Betulin (Figure 49b) and Shatavarin-IV (Figure 53c.) consisted of 105 and 276 nodes, which further formed 524 and 2704 edges (interactions), respectively. The target network had 1347 nodes, which formed 58,522 interactions (Figure 53a), whereas the SiHa cell line had 763 nodes, forming 20518 interactions (Figure 53b).

4.4.3.1. Protein-Protein interaction network of natural compounds and SiHa cell with cervical cancer:

The intersection of the target network of Betulin, Shatavarin, and SiHa cells with CCa resulted in 4 (Figure 53d) and 13 (Figure 54d) common nodes with 1 and 260 edges, respectively. Centrality analysis (DC=BC=CC) of the common targets of Betulin (Table 15), Shatavarin-iv (Table 16), and CCa cells (Table 17) resulted in the identification of 4(Figure 53d) and 13 (Figure 54d) core targets, respectively.

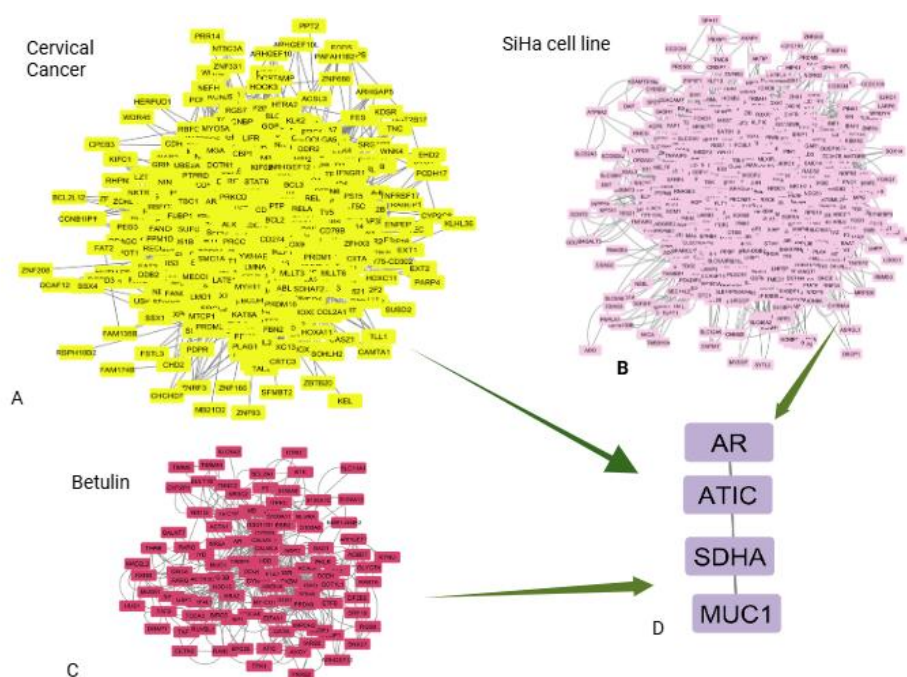


Figure 53: Identification of core targets. The images illustrate the PPI network of (a) the SiHA cell line, (b) cervical cancer, (c) Betulin, and (d) the merged network of Betulin, SiHA cells, and cervical cancer.

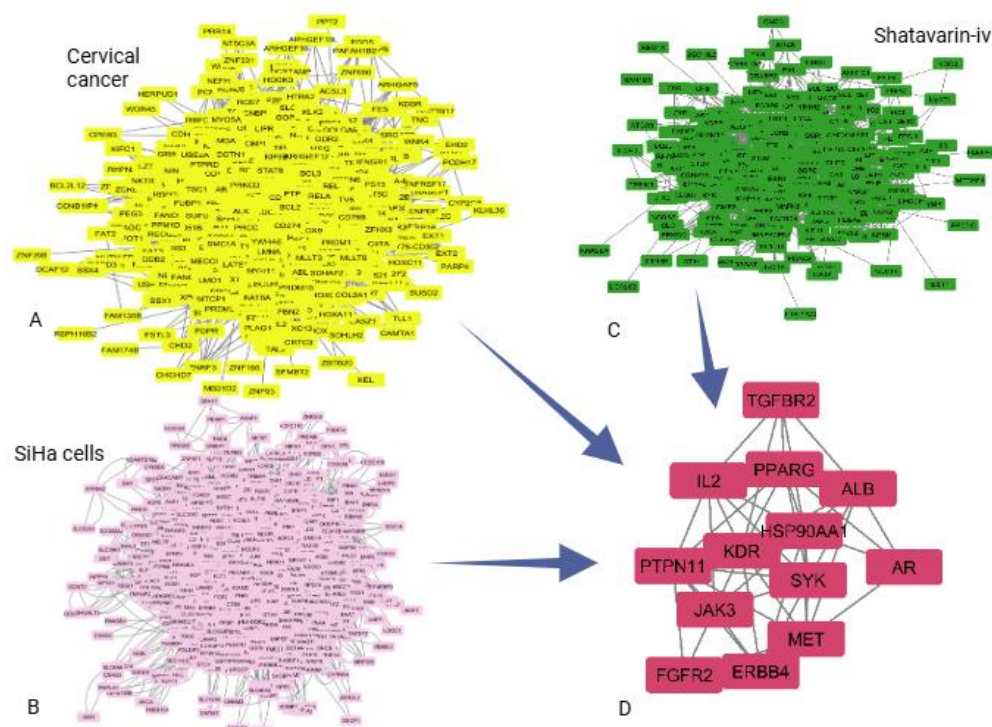


Figure 54:

Identification of core targets. The images show the PPI network for (a) the SiHA cell line, (b) cervical cancer, (c) Shatavarin-IV, and (d) the merged network of Shatavarin-IV, SiHA cells, and cervical cancer.

4.4.4: GO and KEGG pathway enrichment analysis of BE and SH-IV core targets against CCa was conducted. The enrichment analysis was carried out using the DAVID bioinformatics tool.

To investigate the mechanism of natural chemicals against CCa. The GO enrichment analysis stated that Betulin and Shatavarin's core targets against CC included signal transduction, apoptosis, drug response, negative regulation of proliferation, cell migration, and positive regulation of pri-miRNA transcription (Figure 55, $P > 0.001$). The core targets of the two natural medicines against CCa were significantly ($P < 0.001$) linked to KEGG pathways implicated in cancer progression, including PI3-Akt signalling (Figure 56).

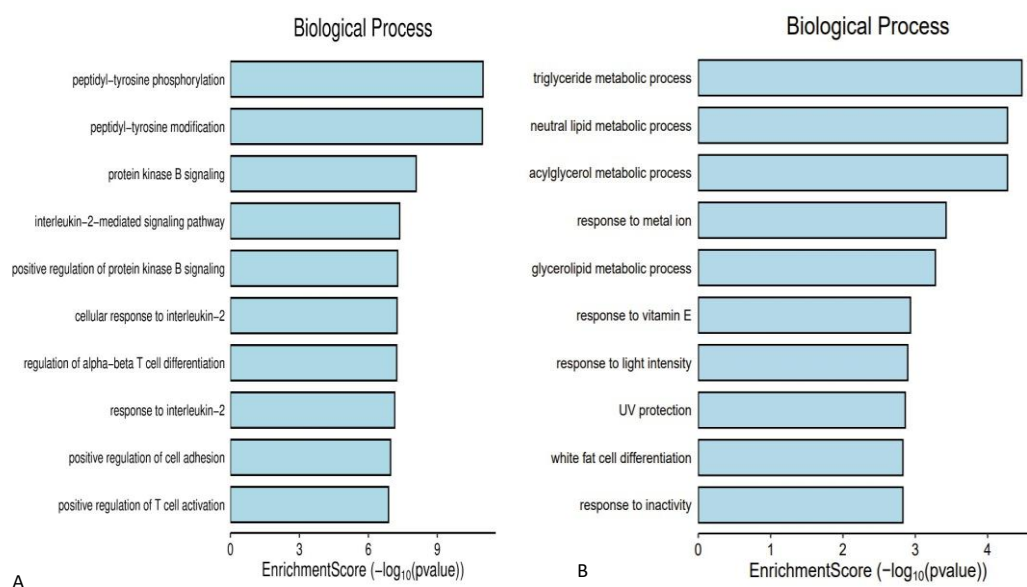


Figure 55: Gene Ontology (GO) analysis of the core targets of (A) Betulin and (B) Shatavarin-IV against cervical cancer.

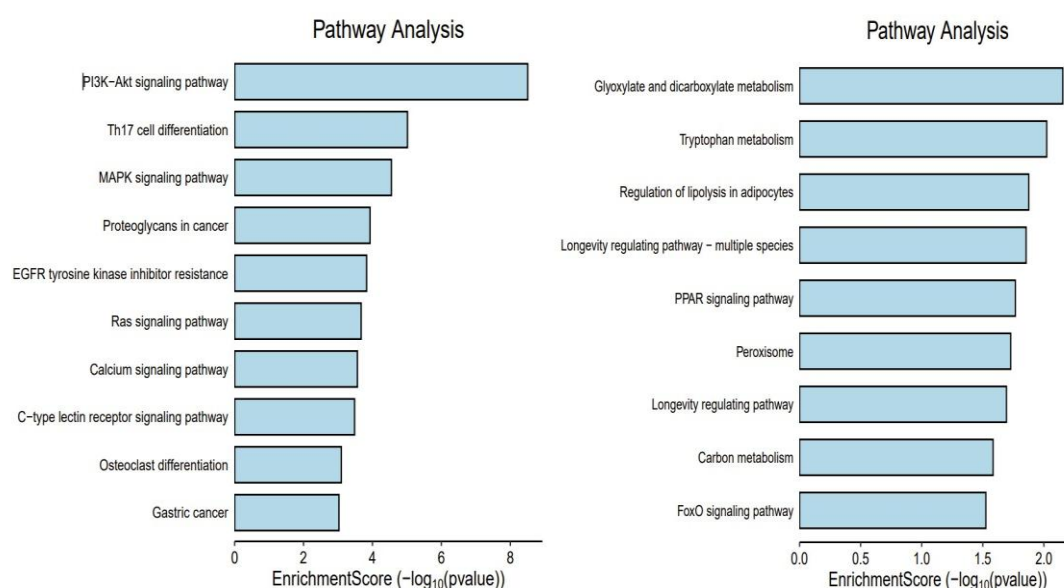


Figure 56: The KEGG pathway enrichment analysis for the core targets of (A) Betulin and (B) Shatavarin-IV about cervical cancer. The images display the top enriched KEGG pathways.

To comprehensively understand the systems-level impact of Betulin (BE) and Shatavarin-IV (SH-IV) in CCa, network pharmacology and bioinformatics tools were utilized. The integration of compound-associated targets with genes implicated in cervical cancer and the SiHa cell line

enabled the identification of shared nodes and biological pathways. These findings underscore the multi-target potential of these phytochemicals and their epigenetic modulation capabilities.

The merged protein–protein interaction (PPI) networks revealed that BE and SH-IV share key molecular targets with cervical cancer pathways. For Betulin, the interacting proteins AR, ATIC, SDHA, and MUC1 were identified as central regulators in tumor proliferation, metabolism, and immune modulation. Shatavarin-IV, on the other hand, showed strong associations with TGFBR2, PPARG, MET, JAK3, and AR, which are involved in inflammatory signaling, metabolic regulation, and tumor suppression.

These proteins have previously been identified as potential biomarkers and therapeutic targets. For example, MUC1 has been shown to mediate epithelial-mesenchymal transition (EMT) and is overexpressed in HPV-positive cervical tumors, contributing to metastasis and immune evasion (Poornima et al., 2016). Likewise, TGFBR2 and MET are part of signaling pathways frequently dysregulated in cancer, controlling cell motility, invasion, and growth.

Gene Ontology (GO) and KEGG pathway enrichment analysis confirmed the involvement of BE and SH-IV in several critical cancer-related pathways, including:

- p53 signaling
- PI3K-Akt signaling
- Cell cycle control
- Chromatin remodeling
- Apoptosis regulation

These pathways are deeply implicated in HPV-mediated oncogenesis, where viral E6/E7 proteins disrupt tumor suppressors like p53 and Rb, promoting genomic instability and epigenetic aberrations (Zhu et al., 2020; Hanahan & Weinberg, 2011). The ability of BE and SH-IV to modulate these core pathways suggests their capacity to interfere with HPV-driven carcinogenesis.

Expression data obtained from the GEPIA and UALCAN databases validated the overexpression of HDAC4 and DNMT3A and the underexpression of KMT2C in CCA tissues. These findings aligned with experimental results from qRT-PCR and Western blotting,

confirming that these targets are epigenetically dysregulated in cervical cancer and are susceptible to modulation by BE and SH-IV (Sarkar et al., 2013).

- DNMT3A, frequently upregulated in cancers, promotes DNA hypermethylation and silencing of tumor suppressor genes (Silverman et al., 2014).
- HDAC4 contributes to chromatin condensation and transcriptional repression, and its inhibition can reactivate silenced tumor-suppressive genes (Weichert, 2009).
- KMT2C, a known histone methyltransferase and tumor suppressor, when downregulated, contributes to chromatin destabilization and oncogenic transformation (Rao & Dou, 2015).

Mechanistically, Betulin was shown to enhance immune signaling, target kinase pathways, and suppress tumor progression, consistent with previous literature highlighting its anticancer and immunomodulatory properties (Wang et al., 2021). Shatavarin-IV, in contrast, modulated oxidative stress responses and metabolic regulation, consistent with its antioxidant and cytoprotective roles.

This complementarity in action—Betulin’s impact on immune and signaling axes, and SH-IV’s metabolic and redox control—supports the potential for synergistic combination therapy, where multiple cancer hallmarks are simultaneously targeted.

The systems-level analysis confirms that BE and SH-IV exhibit polypharmacological actions, interacting with critical cancer-associated proteins and modulating key pathways implicated in cervical carcinogenesis. The alignment of in-silico findings with experimental and database validation enhances the translational relevance of these compounds. Their multi-targeted nature, combined with favorable safety profiles, positions them as promising candidates for epigenetic and pathway-directed therapy in cervical cancer.

Furthermore, the study's assessment of the inhibitory effects of metal-based nanoparticles of Betulin and Shatavarin-IV on HDAC4, DNMT3A, and KMT2C demonstrated a dose-dependent inhibition in SiHa cells. These results suggest that these nanoparticles may serve as potent inhibitors of the examined epigenetic proteins, facilitating cancer therapy through mechanisms of apoptosis and cytotoxicity (Rzeski et al., 2009; Kommera et al., 2010). In silico analyses further revealed that Betulin and Shatavarin-IV effectively interact with the active sites of these targets, indicating their potential as effective therapeutic agents.

The anticancer properties of Betulin, along with its ability to elevate cellular cAMP levels, suggest a mechanism through which it may induce apoptosis in various cancer cell types (Muceniece et al., 2016). Triterpenoids like Betulin have been shown to enhance adenylyl cyclase activity, leading to increased cAMP levels that activate crucial signaling pathways for cellular proliferation and apoptosis (Hazafa et al., 2020).

Notably, the study found that ZnBE and AgSH significantly induced apoptosis in SiHa cells, altering apoptotic markers in a dose-dependent manner. The anticancer effects of Betulin and other natural compounds in cervical cancer have been documented, highlighting their potential as safe therapeutic agents due to their minimal cytotoxicity to healthy cells (Banerjee et al., 2024).

Given the complexity of cancer development, which involves numerous genes and pathways, focusing solely on single-gene therapies may overlook broader therapeutic opportunities (Assaraf et al., 2019; Nosrati et al., 2017). Natural compounds can target multiple genes and pathways, making them promising candidates for drug development (Zhang et al., 2012). Network pharmacology can elucidate the mechanisms through which these compounds interact with complex diseases, providing insights into their multifaceted roles in cancer treatment (Zhao et al., 2023).

Overall, the findings from this study indicate that Betulin and Shatavarin-IV not only inhibit key epigenetic regulators but also diminish the colony-forming capacity of SiHa cells, underscoring their suppressive impact on prolonged cell proliferation. These results support the notion that these natural compounds hold significant promise for developing effective therapeutic strategies against cervical cancer. Further research is warranted to explore these interactions in greater depth and to assess the clinical efficacy of these compounds in cancer management.

Table 13: List of targets of Betulin:

H3C13	CDCA8	RUVBL1	HBB	TRPV6	NME1-NME2	CETN2	GLYCTK
H3-3B	AR	RHO	CAT	IMPDH2	PEBP1	BLVRA	TPK1
SDHA	SIN3A	S100A11	MUC1	AHCY	MAD2L2	SPC25	GALNT7
CALML3	OXSM	BIRC5	NR3C2	ACTA1	HUS1	SNX27	DRAP1
G6PD	CYB5R3	HSD11B1	GCDH	MUS81	TAF13	RAB7A	KYNU
MDH2	S100A6	ATIC	S100A8	CSDE1	TCEA3	RGS6	ACTR3C
CALM3	GATB	EIF4A1	THRB	GAD1	USF1	ARHGEF12	
EEF1A2	HADHA	PRDX6	SIN3B	PLA2G2A	YARS2	TNNC2	
PFN1	ACADL	ETFB	NR5A2	F2	EIF2B2	ACBD7	
GSR	S100A12	FABP4	RARG	BTK	CYP2R1	TIMM9	
PKLR	MORF4L1	RXRB	CYB5B	BCL2A1	SULT1B1	S100A13	
MPO	GOT1L1	PTBP1	ITPKC	ARHGEF7	TIMM44	SLC11A1	
CYP2E1	RXRG	TAF9	RANBP2	ESR2	HBA2	SRP19	
MB	MT-CO1	NR1H3	IARS2	AKR1B1	IYD	SLC5A2	

Table 14: List of Targets of Shatavarin-IV

H3C13	CDCA8	RUVBL1	HBB	TRPV6	NME1-NME2	CETN2	GLYCTK
H3-3B	AR	RHO	CAT	IMPDH2	PEBP1	BLVRA	TPK1
SDHA	SIN3A	S100A11	MUC1	AHCY	MAD2L2	SPC25	GALNT7
CALML3	OXSM	BIRC5	NR3C2	ACTA1	HUS1	SNX27	DRAP1

G6PD	CYB5R3	HSD11B 1	GCDH	MUS81	TAF13	RAB7A	KYNU
MDH2	S100A6	ATIC	S100A8	CSDE1	TCEA3	RGS6	ACTR3 C
CALM3	GATB	EIF4A1	THRB	GAD1	USF1	ARHGEF 12	
EEF1A2	HADHA	PRDX6	SIN3B	PLA2G2 A	YARS2	TNNC2	
PFN1	ACADL	ETFB	NR5A2	F2	EIF2B2	ACBD7	
GSR	S100A12	FABP4	RARG	BTK	CYP2R1	TIMM9	
PKLR	MORF4L 1	RXRB	CYB5B	BCL2A1	SULT1B 1	S100A13	
MPO	GOT1L1	PTBP1	ITPKC	ARHGE F7	TIMM4 4	SLC11A1	
CYP2E 1	RXRG	TAF9	RANBP 2	ESR2	HBA2	SRP19	
MB	MT-CO1	NR1I3	IARS2	AKR1B1	IYD	SLC5A2	

Table 15: Targets of Cervical cancer:

CDKN3	IFI27	MICA	AMHR 2	PLAU	NAT2	CRP	GSK3 B	MIR20 4
COL11 A2	TICAM 2	KLRC4- KLRK1	GJB1	POU5F 1	MIR37 4B	MAPK1 4	ANX A1	MIR22 4
SLCO6 A1	IL2	BLACA T1	GLI3	PPARG	MIR94 4	CYP2D 6	SERPI ND1	MIR25
TAFA4	FASLG	CDK4	ANGP T2	SLC52 A1	CD24	CYP2E 1	ANX A5	MIR26 B
E2F1	KDR	NCK1- DT	SETD2	LAPT M4B	MIR28 61	CD55	HIC1	MIR29 B1
SIRT1	KRAS	CDK5	PYCA RD	DIABL O	MIR46 6	TIMM8 A	HK2	MIR29 B2

HMGB1	LGALS 3	YAP1	NOP53	SLC12 A9	LUCA T1	DHCR2 4	HLA- DOA	MIR30 A
IGF1R	LTA	KHDRB S1	STOM L2	MIR43 3	TTN- AS1	NQO1	HOX A1	EPCA M
IGF2	MIR107	CKAP4	HLA- G	MIR49 4	CCAT 1	AGER	HOX A9	SMAD 2
IL1A	MIR140	UBE2C	HMG A1	MIR50 6	SNHG 16	ATN1	HOX A11	SMAD 7
IRF1	MIR142	CHI3L1	BIRC2	SEMA6 A	SOX21 -AS1	EIF4G2	APEX 1	MCL1
GSTK1	MIR144	TMC6	IL4	RAC1	TMPO P2	EPHA3	HSPA 5	MCM 2
LGALS 1	MIR200 B	CISH	IL12B	RBBP4	KIF20 A	EPO	XIAP	MCM 4
MIR182	MIR206	CCR5	IDO1	RPE65	CDH13	ETS1	HSP9 0AA1	CD46
MIR20A	MIR22	TMC8	AR	RRM2	BCAP3 1	ALDH1 A1	HTC2	MDK
MIR203 A	MIR23 B	DNMT3 A	JUN	BDNF	TRIM2 8	FANCD 2	ICAM 3	MFAP 1
TACST D2	MIR27 B	DNMT3 B	CD82	POU5F 1P3	CDK2	PTK2B	IFNA 1	MLH1
NFE2L2	MIR29 A	JAG1	KRT17	POU5F 1P4	CCAT 2	ZEB1- AS1	IFNA 13	MMP1 1
MAPK3	MIR31	EGR1	MIR10 A	ST3GA L4	CDK6	HOXA1 1-AS	LINC 01193	MMP1 3
MIR497	MIR93	ELK1	MIR12 4-2	PMEL	CTDSP L	FEN1	IGFB P3	MIR13 5B
RAD51	SMAD4	ERBB3	MIR12 5A	STIL	MSLN	FOXC2	CCN1	MIR33 1
SOX9	MMP1	ESR2	MIR13 0A	MRPL3 6	NDRG 1	FLNB	APRT	MIR33 5

SPP1	MMP7	ALB	MIR13 2	SLC5A 5	RACK 1	FLT4	IL12A	MIR33 8
TAP2	MMP14	F3	MIR13 9	UCA1	SNHG 14	USP22	CYCS P38	MSI1
ZEB1	MPO	EFEMP 1	MIR14 8A	SLC9A 1	TACC 3	SASH1	IL18	MUC4
CCR2	MIR326	FGFR2	MIR14 9	SLN	NXF1	DICER1	CXCL 10	ATF1
CDKN2 B-AS1	MTR	FGFR4	MIR15 A	SOX1	CEBP D	SIRT3	ITGA 6	NCK1
HDAC6	MUC1	KLRK1	MIR17	STIM1	AGR2	SUZ12	JUNB	NME1
TMED7- ICAM2	NFKB1	FOXC1	MIR18 A	TAP1	CCEP R	FOSB	JUND	NOTC H3
ZNRD2	NT5E	TPX2	MIR18 3	TCF3	AHSA 1	TRIM29	KCN H1	NTRK 1
SPAG5	MIR361	TBC1D 9	MIR21 1	THBS2	TRIM3	ALPP	KNG1	MIR38 1
DCTN6	SERPIN E1	FN1	MIR22 1	TLR2	PDPN	FUS	KMT5 A	MIR38 3
HPSE	TMED7	FOLR1	MIR27 A	HSP90 B1	SPINT 2	CASC2	STMN 1	MIR42 5
CHEK1	PGR	FOS	MIR34 B	UVRA G	KLF1	TNFAIP 8	LCN2	OGG1
IL17F	ABCB1	SEC14L 2	SMAD 3	NSD2	CFTR	POU2F 3	LGAL S9	PA2G 4
H3P23	IL17D	FPR1	STS	CALC R	PPP1R 13L	RNF19 A	TUSC 8	PRKN
TWIST2	TUG1	G6PD	MBL2	RUBC NL	PGRM C1	POLDIP 2	LINC 00511	IER5
DAPK1	PRL	AMH	MET	CD276	BLCA P	DGCR5	LMX1 A	GOL M1
EIF4E	PSMD9	SLC6A8	MGM T	FOSL1	BTG3	FBXL5	HAGL R	WT1- AS

F2RL1	CIP2A	SOX4	MRC1	HMGA 2	COPS6	GREM1	MIR1 00	NOL7
FOXO1	SERPIN B4	BRCA1	MIR33 7	ACTN4	IL24	DKK3	MIR1 06B	IL23A
IL27	CCL2	STAT1	MTRR	CAT	WIF1	IL37	MIR1 0B	WVO X
GABPA	CRNDE	STC1	MYBL 2	LGR5	SLC52 A3	GLI1	MIR1 27	SERPI NF1
GATA3	ST6GA L1	AURKA	NCL	NR1I2	CTHR C1	HTRA2	MIR1 37	PEG3
GSTP1	SKP2	BSG	MIR37 8A	KLF4	ADCY AP1	SFN	MIR1 50	PGD
HGF	SLC2A 1	TCF4	PEBP1	KL	MSI2	IFNL3	MIR1 52	DCUN 1D1
HRAS	SOX2	MIR411	NDUF A13	SLC9A 3R1	TNFAI P8L1	ANGPT 1	MIR1 87	SYTL 2
HSPA4	KLF5	MIR574	PDCD 1	MUC16	COMT	GPER1	MIR1 96A1	HCFC 1R1
RIF1	TERC	MIR590	PIN1	CDK1	CRK	DLX6- AS1	MIR1 99B	ACP3
PPP2R1 B	TIMP2	MIR605	PKM	CDC42	CRKL	UHRF1	MIR2 00A	PIWIL 2
PTPA	TP53BP 1	TCF7L2	AKNA	TMPO- AS1	ADAM 10	NES	CMP K2	IDO2
FBXW7	XRCC3	TGFBR 1	ARID1 A	TNRC6 C-AS1	PCBP2 -OT1	PLK2	KLF6	ADA MTS1 8
MCM10	VTCN1	TGFBR 2	HAVC R2	CALM L3-AS1	CDK7	SRSF10	SLC3 1A1	DLX1
MIR429	CALR	TIMP3	PPM1 D	BCL2L 11	MIR68 52	ZNF268	ZFP42	DPH1
PRKAA 1	BRIP1	C1QBP	SNHG 12	TRAP	MIR68 57	SEPTIN 9	ADM	HBEG F

PRKAA 2	SOCS3	SEC62	API5	TPT1- AS1	MIR80 75	CEACA M3	COX8 A	DUSP 3
AXL	CD28	TLR3	TSLP	FRAT1	MIR68 93	LINP1	CPE	DVL1
KDM3A	CD44	TNFRS F1B	STC2	CASC1 1	CDK9	HCP5	CPOX	E2F3
GSDMB	ZEB2	NR2C2	USO1	TMEM 238L	SPRY1	CEACA M7	PIP5K L1	TYMP
MAPK8	CDH1	TRAF6	BECN 1	DEFB4 B	SPRY2	MRPS3 0	CRE M	ECM1
MYDGF	SIRT2	SNHG4	TNFR SF11A	MIR12 84	ABCC 4	LYVE1	CRH	NR0B 1
PRLR	KIN	TSG101	TNFR SF10C	MIR12 46	GHET 1	PAPOL A	FUND C1	EFNA 2
AKIP1	FOXF2	HULC	NRP1	MIR12 97	CSAG 2	SUB1	CRIP1	EGR2
PSG2	FOXD1	OIP5- AS1	GGH	MIR12 66	LILRB 2	PRDX3	DEFB 104A	EIF4A 1
PSG5	NT5C2	TWIST1	TSC22 D1	MIR66 4A	PAK4	PRSS21	CRYZ	AIF1
PSMA1	KDM2 A	UBE2B	IER3	MIR12 02	ADAR	CCNI	CSF1	ELF1
PBXIP1	CEP131	TDRG1	CCNB 1	ALL2	CDKN 2B	METAP 2	CSF1 R	ENG
MIR485	WAPL	VPS51	TIME LESS	PARP2	DLEU 1	TMSB1 5A	PIWIL 4	PRSS5 5
MIR491	FOXO3	VHL	MBD2	MIR42 62	B3GN T3	RCC1	CSF2	EPAS 1
MIR146 B	FLG	WNT10 B	MTA1	LINC0 0673	TUBA 1B	TENT4 A	OR2A G1	STOM
MIR193 B	SMC5	WNT11	LATS1	MIR37 4C	SYCP2	GPC3- AS1	CSN2	HIPK1

MIR499 A	ARL6IP 1	XIST	PTTG1	MIR39 41	NDC80	PRDM5	CSNK 2A1	EPHB 4
MIR503	FLT1	XRCC2	CD83	MIR36 66	BASP1	FGFR1 OP	LINC 00052	EPHB 6
MIR505	RRS1	XRCC5	RECQ L4	LINC0 1133	PRMT 5	KLK8	CSRP 1	EPHX 1
PCDH10	FLT3L G	ZIC2	GRAP 2	GAS5- AS1	CFDP1	RASSF 8	ILF3- DT	MTLN
SLC46A 2	FBXW1 1	MZF1	FOXQ 1	DSCA M-AS1	CDX1	ZHX1	ZNF5 82	EPS8
RAB5A	PUM2	MIR758	TP53I NP1	OCLN	IRAIN	PRRT2	CTAG 1B	NR2F 6
RAF1	SF3B1	MIR454	ABCG 2	PCAT6	GPNM B	LOC112 543491	CCN2	ERBB 4
RALA	ICMT	CA12	QKI	CASP1 2	CLEC1 0A	CYTOR	CTSB	ERCC 2
RB1	BRD4	PRDM2	AIM2	GIHCG	CEAC AM5	CMTM 7	CTSG	ERCC 4
ZNF350	CBX7	PAX8	CD34	DLG1- AS1	HOXB 13	NACC1	CTSL	ERCC 5
RFC4	SRRM2	IL1R2	ROCK 2	ABCF2	ENOX 2	PDAP1	CTSS	AKT2
ACTB	ZNF281	DEK	CD36	MIR46 36	CEBP A	TUSC2	CYBA	ESRR A
ROCK1	MAPK8 IP2	AIMP2	GDF15	MIR47 84	SEMA 4D	MGLL	CYBB	FABP 4
ROS1	LPAR3	TBL1X R1	BAG3	MIR47 78	SEMA 3C	TMEM5 4	CYP1 B1	FABP 5
MIR484	CASP1 4	TFPI2	CD40	MIR44 29	KAT5	CHRNA 4	AMO TL1	FANC A
MIR486 -1	FAM21 5A	TET1	CD47	SPRY4 -IT1	NEBL	LNCRN A-ATB	FEZF 1-AS1	FANC C

RRAS	SH3BP1	PPP1R2C	MDC1	NNT-AS1	ATG7	CKB	CYP19A1	FANCF
S100A9	NUP62	TIPARP	KEAP1	LINC00702	RNAS EH2A	H3P9	DAP	MED19
ATXN1	LDOC1	IS1	MELK	PCAT1	DDN-AS1	H3P40	BRINP1	LTO1
SERPINB3	SNHG1	CHTOP	HS3ST2	FALEC	LAMTOR5	CKS2	DCK	FBP1
CXCL5	ALK	SZRD1	ZBTB33	PTCSC3	MRPL28	ANIB1	DCN	MARCHF8
SDC1	ABCA4	PRPF31	SNORD111B	PANDAR	TXNIP	LRG1	ACE	SKA3
SFRP1	KIF4A	PTTG3P	VTRNA2-1	NPTN-IT1	POSTN	CLU	AKR1C1	LINC00518
MIR205HG	FRA11A	KAT2A	MIR744	EBI3	IGF2BP1	TMEM132D	GADD45A	FHL2
SOX17	FRK	CNNM3	MIR877	WASF2	CTCF	CMA1	DDT	DNAJC8
AKTIP	CTAG1A	GCNT2	MIR665	CASC9	POLD3	CCR6	DDX5	CPEB3
SHBG	ALPI	GFI1	MIR216B	RBM5	MASP2	ZG16B	DHX9	MLXIP
HSPB2	FRZB	LAMP3	MIR874	LINC01535	GIPC1	CYB5D2	DEFB4A	FOXG1
IAPP	FSHR	FOXP1	MIR889	C5orf66-AS1	PLAC1	OSCP1	DDX53	CARD8
APOB	FTH1P3	BBC3	LGALS7	NFKBIA	PECAM1	NBPF1	RBP1	LINC02604
ICAM1	ABL1	AGO2	LIF	TONSL	ERAP1	CHFR	RECQL	MNX1-AS1
MYPOP	MSRB3	SALL3	LIG1	NFX1	ATP8A2	CCAR1	REL	NORAD

ID1	MIR9-3HG	GPC3	LIPA	NKX6-1	GALNT7	MBD5	BCL2A1	MARCHF7
ID4	XRCC6	GLB1	ST20	NNMT	PLA2G4A	PRKCA	REN	USP46
STING1	SLC39A6	AHDC1	BCAR4	NOS1	SPA17	PRKCB	REV3L	ST3GAL3
AQP8	BRMS1	DHDH	C17orf97	NOS3	PLG	PRKCD	BCL2L2	LINC00273
IFNA17	PART1	RBMS3	TOB1-AS1	CCN3	PLK1	DCAF6	AFAP1	SIM1
IFNB1	LETMD1	UBE2S	PHOX2A	NPAT	PLOD2	HDAC8	RNAS-E3	SLC1A5
IGF2R	GAPDH	MCAT	LNPEP	NPM1	PLSCR1	DEFB103B	RNAS-E4	SLC6A6
IL1R1	MIR613	GLI2	CASC15	NPTX1	PLXNB1	KMT2E	RNAS-EL	PSG10P
IL1RAP	TCF7	GNA12	RASA4CP	YBX1	PML	MAPK6	APOBEC3G	LGALS7B
IL4R	MIR618	GNAO1	LOX	MIR377	ATP7A	MAPK10	HPSE2	PAX8-AS1
IL6R	MIR620	GOT1	LOXL2	MIR379	RIPK4	MAP2K3	CELF6	SNHG20
IL7R	MIR625	GPC1	CYP4F3	OCA2	POLR2E	MAP2K7	BCYRN1	BMP7
CXCR2	MIR630	ANG	LTBP3	BDNF-AS	WNT4	PRMT8	RPS6KB1	SLC10A2
AQP1	MIR638	SBF2-AS1	LYN	P2RX7	KRT20	LGMN	RPS12	SLC16A1
IL13	MIR641	H19	MIRLET7B	DEFB104B	ATR	MASP1	RPS19	SNAI2
IL16	TRBV200R9-2	GATC	MIRLET7D	RRM2B	POU2F1	KLK10	BDKRB2	HLTF

INPP5A	TDO2	HNF1A- AS1	MIR10 6A	SYCP3	POU3F 3	PSEN1	S100A 1	SMAR CA4
IRF3	TEK	ZSCAN 1	MIR12 2	GEMIN 4	DLL4	EIF5A2	S100A 4	SMN1
IRF6	TERF2	LYPD5	MIR12 4-1	REG3A	INO80	PSMA7	S100A 7	SMN2
ITGA2B	TFF3	LINC01 305	MIR13 0B	PAX2	POU5F 1B	PNO1	S100A 11	SMS
ITGA4	TFRC	PNPLA 1	MIR13 4	PAX3	PPAR A	PSMB4	S100B	SNAI1
ITGA5	TGFA	FAM83 H	MIR14 1	ASAP1	PPBP	SMYD2	SATB 1	SNCB
ITGAE	THBD	TMSB1 5B	MIR18 1A2	PBX3	RAB39 A	MEPE	SCN1 A	SNCG
ITGAV	THBS1	TRIM59	MIR18 6	PC	TRIM4 4	PSMB8	SCN8 A	FSCN 1
STT3A	THBS4	GPX2	MIR19 2	ASCC1	PPIA	ATXN7 L3	BHM T	BNIP3
ITPR3	THOP1	GPX3	MIR19 7	MLXIP L	AHI1	PSMB9	CCL1 6	SOAT 1
JAK3	KLF10	GRB7	MIR20 0C	ANGP TL4	TRPM 7	PSMB1 0	CCL1 9	SOX1 1
KCNA1	TIAM1	REM1	MIR21 0	ING4	APTX	BAAT	CCL2 2	SPAR C
ARF1	TIMP1	RGCC	MIR21 2	DCTN4	ESRP1	ACKR3	CXCL 6	FOXL 2
LHFPL4	TKT	MCTS1	MIR21 5	NUSAP 1	DPP8	AKR1B 10	SDHA	SRF
CA13	TM7SF 2	ATAD2	MIR21 7	MARC HF2	ST7L	PSMD2	PRD M1	SSRP1
KCNK3	TNFAI P2	NR3C1	MIR21 8-2	RXFP3	CASZ1	PSMD4	PRD M14	BRCA 2

KISS1	TOP1	NXT1	MIR22 3	TLR8	PINX1	PLAAT 1	SEL1 L	STAT 2
KLK1	CRISP2	MYLIP	MIR26 A1	ZBTB7 A	MARC HF1	NUP107	PCGE M1	STAT 5A
KPNB1	TRAF3	BRD7	MIR29 6	RHCG	ANKR D40CL	PAK5	LHPP	STAT 5B
KRT13	TRIO	PRSS50	MIR29 9	SFMBT 1	PPP1R 1A	SALL4	MAP2 K4	STAT 6
KRT14	MIR655	CXCL3	MIR30 1A	TRIAP 1	PID1	TP73- AS1	SEMA 4A	SUV3 9H1
RHOB	TTR	PDIA3	MIR30 2A	DTL	FKBP1 4	PTBP1	ROBO 3	SYK
FEZF1	MAFIP	GSN	MIR30 D	NCKIP SD	ARHG AP17	PTCH1	SFRP 2	MAP3 K7
L1CAM	DNLZ	GSTM2	MIR30 E	SIRT7	FANC L	BAG1	SFRP 4	TAPB P
CSAG3	TUFT1	GSTM3	MIR32	RAB14	CDCA 8	PTGER 3	SRSF 1	TAT
LAMB3	STXBP 5-AS1	GTF2H1	MIR32 0A	KLF13	MTPA P	CBX8	RHBD F1	TBP
ARHGA P1	TYMS	CCDC1 06	MIR7- 3	PDK1	CEP55	MIR362	CERN A2	HNF1 A
LDHA	UBB	DNMT3 L	MIR96	RBMX 2	GOLP H3L	MIR20 B	LINC 01128	MIR42 1
LEP	UBE2E 2	ERO1A	MIR99 A	RTRAF	ATAD 3A	PTGS1	ZMA T3	MIR44 9B
XRCC4	UCP2	EFEMP 2	MIR99 B	GIN52	TRIM6 2	MIR409	ARAP 3	MIR57 3
YWHA Z	UGCG	ANXA2	MAP2	CMPK 1	VPS53	MIR202	SGK1	MIR60 0
ZNF84	NR1H2	HCFC1	DEFB 103A	PIK3R3	PPP3C A	MIR492	TRIP4	FOXD 2-AS1

SYS1- DBNDD 2	VCP	HDAC2	MBNL 1	IFITM1	LARP6	MIR432	CD16 3	GEMI N2
MIR802	VDAC1	ADGRE 2	MCM5	CAV1	MIR44 9A	MIR498	SLC9 A3R2	ADO
CA11	VDR	KCNIP1	MCM7	PDLIM 4	ZNF33 1	NDRG2	SLIT2	ATAD 1
MALL	WEE1	HGFAC	MDM4	RUNX 2	YOD1	MIR519 D	PLAA	RITA1
SCLC1	WNT6	ANXA6	MECP 2	RUVB L1	OTUD 5	MIR522	COX5 A	AJUB A
USP7	WNT7 A	HLA-C	MEF2 D	EIF4EB P3	PRKA B1	MIR502	FOXP 2	SNHG 7
RHPN1- AS1	WNT2B	MNX1	MAP3 K3	AKR1C 3	NLRP2	REXO1	CD80	PIK3R 3
CENPM	XPNPE P2	HLA- DQB2	MAP3 K5	NUMB	ZNF69 2	GATAD 2B	MAP4 K4	IFITM 1
FTO	XPO1	HLA-E	MEN1	EIF3F	URGC P	PTK2	TBPL 1	CAV1
BRCC3	CDC7	HMGB2	MEST	SRSF9	ADAM 9	HECW2	CD38	PDLI M4
FSD1	FZD4	HMGB3	MELT F	B4GAL T3	RAB11 A	PTK6	GOSR 1	RUNX 2
SLC39A 7	FZD7	HMMR	SCGB 2A2	CDC7	TNFRS F6B	MIB1	ENTP D1	RUVB L1
HMBO X1	EMC6	HMOX1	KITLG	FZD4	SNAP2 3	PTK7	CD53	EIF4E BP3
NDNF	CCDC3	NR4A1	MICB	FZD7	TNFRS F10D	ARRDC 3	ISG15	AKR1 C3
RHBDF 2	SESN2	FOXA1	MKI67	EMC6	TRIM2 4	MRTFA	PPIP5 K1	NUM B
SEM1	CALN1	FOXA2	MAP3 K11	CCDC3	IL18R1	PTN	CD68	EIF3F

RABEP 2	JAM3	HNRNP A1	LHX8	SESN2	BANF 1	NCOA5	KDM 4A	SRSF9
ZNF671	LONP2	HNRNP A2B1	MMP1 5	CALN1	INPP4 B	NS2	NUP9 3	B4GA LT3
NANOG	FSD1L	HOXA5	MPG	JAM3	NRP2	PTPN6	DCAF 1	ABCC 3
GRHL2	CASP7	HOXA1 3	PSG8	LONP2	CFLA R	PTPN9	KMT2 B	NOL4
PSCA	SOX14	HOXB2	ZFAS1	FSD1L	CCN4	PTPN11	ADG RE5	URI1
LRRC8 E	SPARC L1	HOXB7	MIR14 8B	CASP7	PROM 1	PTPN13	RASS F2	CBR1
ASRGL 1	MAGT1	HOXB8	MIR32 4	SOX14	AKAP 4	PTPN14	CDC6	RIPK1
RNF34	TAGLN 2	HOXC6	MIR32 8	SPARC L1	ASAP2	PTX3	RBM1 9	TNFS F14
ITPKC	MINDY 4	HOXC8	MIR33 0	MAGT 1	ARHG EF7	BAX	CDC2 0	TNFS F9
ZKSCA N3	RPAIN	HOXC1 0	MIR34 0	TAGL N2	SPHK1	PXN	MFN2	SCAF 11
COASY	NR0B2	HOXD @	MIR34 6	MIND Y4	SQST M1	JAM2	CDC2 5A	LPAR 2
MFAP5	UTF1	HOXD9	MIR36 7	RPAIN	CCNA 2	RAD21	CDC2 5C	WDR2 0
ITIH5	ELOF1	APC	MIR37 6C	NR0B2	BCL10	RAD51 B	SLC1 2A6	AURK B
KMT2D	RECK	HP	MIR37 0	UTF1	MBD4	MOK	SCO2	MTA2
TCL1A	MAK16	HPGD	MIR37 2	ELOF1	CCND 3	RAD52	SEMA 5A	UBE2 L6
YIPF5	SPZ1	ZAR1	MIR37 3	RECK	P4HA2	BCHE	SPAG 9	PDLI M7

SPRY4	CCDC5 4	AGFG1	MIR19 6B	MAK1 6	RPS6K A4	RAP1A	CLDN 1	STK17 A
ST6GA LNAC5	AFAP1- AS1	APEH	MYO1 B	SPZ1	HSPB3	RAP2B	IL33	SOCS 6
SHARPI N	LINC00 467	HSF1	MSH2	CCDC5 4	LIMD1	PLAAT 4	USP8	NDUF S6
MIA	FOXD2 -AS1	BIRC3	MST1	AFAP1 -AS1	KLC4	RASA1	CCDC 34	NELL 1
NCOA3	GEMIN 2	HSPB1	MSX1	LINC0 0467	ACVR 1	RASGR F1	AIFM 1	NEUR OD1
NRIP1	ADO	NBN	MSX2	AXIN1	AJUB A	RBBP6	ATG1 2	NFAT C1
SMC1A	ATAD1	ATIC	MMU T	BAP1	SNHG 7	RBL1	EXO1	LINC0 0958
TKTL1	RITA1	NDUFB 8	ASTN 1	MYL5	MYOD 1			

Table 16: Centrality analysis of the merged targets of BE against CCa and Siha cell targets

Target	Degree (Weight)	Target	Betweenness (Weight)	Target	Closeness (Weight)
MUC1	1.0	MUC1	0.0	MUC1	0.3333334
AR	1.0	AR	0.0	AR	0.3333334
ATIC	1.0	ATIC	0.0	ATIC	0.25
SDHA	1.0	SDHA	0.0	SDHA	0.25

Table17: Centrality analysis of the merged targets of SH-IV against CCa and Siha cell targets

Target	Degree (Weight)	Target	Betweenness (Weight)	Target	Closeness (Weight)
KDR	10.0	KDR	13.885715	KDR	0.85714287
HSP90AA1	10.0	HSP90AA1	14.433333	HSP90AA1	0.85714287
SYK	9.0	SYK	5.7190475	SYK	0.8
ALB	8.0	ALB	4.133333	ALB	0.75
IL2	8.0	IL2	4.3190475	IL2	0.75
PTPN11	8.0	PTPN11	9.366667	PTPN11	0.75
PPARG	8.0	PPARG	5.0857143	PPARG	0.75
MET	7.0	MET	7.3523808	MET	0.7058824
ERBB4	5.0	ERBB4	0.6857143	ERBB4	0.6315789
TGFBR2	5.0	TGFBR2	0.0	TGFBR2	0.6315789
JAK3	5.0	JAK3	0.6857143	JAK3	0.6315789
AR	4.0	AR	0.33333334	AR	0.6
FGR2	3.0	FGR2	0.0	FGR2	0.5714286

Chapter 5

Summary of the Study

Cervical Cancer poses a significant public health challenge, predominantly linked to infections with human papillomaviruses (HPVs). Even though virus is thought to be the primary cause of the Cervical Cancer, these viruses can result in epigenetic alterations to cause the disease. Increasing interest has been placed on natural compounds for their potential as anticancer agents due to their widespread availability and lower associated side effects.

This study aims to discover natural compounds that inhibit aromatase using a structure-based pharmacophore approach and assess their in vitro efficacy against CCa cells. Identified phytochemicals were further investigated for their binding affinity with specific protein targets: DNMT3A (2qrv), HDAC4 (2qvj), and KMT2C (5f59). Molecular docking analyses identified approximately 100 top-binding molecules, with Betulin (BE) and Shatavarin-IV (SH-IV) showing the highest binding energy via interactions with active site residues of the target proteins.

The study highlights the interactions between selected molecules and the protein targets, where hydrogen bonding interactions stabilize and enhance inhibitory activity. Docking analysis reveals that hydroxyl groups on both ends of SH-IV and BE engage selectively with the epigenetic protein targets, indicating high-affinity binding. BE and SH-IV comply with Lipinski's Rule of Five, exhibiting high oral bioavailability and favourable pharmacokinetics in humans. Neither compound serves as a substrate for cytochrome P450s, essential for hepatic metabolism. Additionally, Boiled Egg plot analysis demonstrated that BE and SH-IV possess favourable gastrointestinal absorption and blood-brain barrier permeability.

Density functional theory (DFT) calculations provided insight into their molecular properties, with a HOMO-LUMO gap indicating high chemical reactivity. A 500 ns molecular dynamics simulation was conducted to further validate these findings and deepen our understanding of the molecular interactions between Compounds and the proteins under study.

To evaluate the efficacy of the pure Betulin (BE) and Shatavarin-IV (SH) the nanoparticles were synthesised using chemical methods. The synthesised nanoparticles were characterised for the stability and efficacy. The Fourier-transform infrared (FT-IR) spectra of metal based nanoparticles, including Zinc oxide and Silver nitrate nanoparticles, confirmed a crystalline structure with an average size of 13.9 nm for ZnSH-IV, 11.87nm for AgSH-IV, 14.03nm for

ZnBE and 8,06 for AgBE nanoparticles. X-ray diffraction (XRD) analysis further verified the crystalline properties, while field emission scanning electron microscopy (FE-SEM) and energy-dispersive X-ray spectroscopy (EDS) showed particle agglomeration and cubic shapes, with distinct binding energy peaks for Zinc and Silver.

The composition of chemically synthesized Zinc oxide-BE nanoparticles was determined to consist of 14.99% by weight and 18.58% by atomic percentage in oxide, with 43.58% weight and 13.28% atomic percentage in Zinc. Similarly, Silver nitrate-BE nanoparticles consisted of 4.03% by weight and 6.23% atomic percentage in oxide, and 56.71% weight and 12.99% atomic percentage in Silver. EDAX patterns confirmed the presence of Zinc oxide and Silver nitrite, suggesting the involvement of carbon-based biological molecules in nanoparticle synthesis. The Zeta potential analysis of the ZnNP's and AgNP's of BE were obtained as -0.3mV and -3.0mV respectively whereas the Zeta potential ZnNP's and AgNP's of SH-IV were obtained as -13.1mV and -83.3mV. The higher negative value of AgSH-IV confirms the stability of the nano particle.

In vitro studies demonstrated that both BE and SH, along with their ZnO and AgNO₃ nanoparticles, exhibited potent antiproliferative effects against CCa cells. The Sulphorhodamine B (SRB) assay showed a significant 50-60% reduction in cell proliferation. Moreover, wound healing scratch assays revealed that ZnBE and AgSH nanoparticles significantly inhibited cell motility and propagation in SiHa cells, reducing their proliferative capacity and wound healing ability.

The study also explored the regulation of epigenetic proteins, particularly HDAC4, in CCa cells. Western blot analysis demonstrated a dose-dependent decrease in HDAC4 expression in treated cells. Furthermore, GEPIA database analysis showed reduced HDAC4 and DNMT3A expression levels in CCa cells, with significant downregulation in primary cervical squamous cell carcinoma tissues.

Protein-protein interaction network analysis identified four common nodes and 13 core targets associated with Betulin and Shatavarin-IV. KEGG pathway enrichment analysis highlighted their involvement in key cancer-related pathways, including signal transduction, apoptosis, cell migration, and regulation of miRNA transcription.

Betulin and Shatavarin-IV were also found to inhibit the expression of epigenetic proteins HDAC4, DNMT3A, and KMT2C in SiHa cells. AgSH demonstrated dose-dependent

inhibition, suggesting its potential as an epigenetic inhibitor, inducing apoptosis and cytotoxicity in cancer cells. Betulin, known to elevate cAMP levels, can induce apoptosis in cancer cells through the PKA-ERK1/2-CREB signaling pathways. ZnBE and AgSH nanoparticles significantly increased apoptosis in SiHa cells, modulating apoptotic markers in a dose-dependent manner.

Overall, this study offers significant insights into the anticancer efficacy of natural compounds such as Shatavarin-IV and its chemically synthesized Silver nanoparticles (AgSH-IV) in modulating the expression of the epigenetic protein Hdac4 and modifying the tumor microenvironment to inhibit cancer progression while exhibiting minimal cytotoxicity to healthy cells, thereby establishing them as promising candidates for drug development aimed at cervical cancer. The Study also forms a platform to delve into the area of identifying new and novel molecules as emerging new drugs to extend the expectancy of the cervical cancer survivors and also to improve the quality of their life.

Significance of the study:

Cervical cancer is a significant global health concern, particularly affecting women. It ranks as one of the leading causes of cancer-related deaths among women worldwide. In light of this, exploring innovative approaches to treatment and prevention is critical, and the study findings can hold substantial promise for the fight against cervical cancer. CCa also presents a need for improved treatment options. Current treatments can be associated with adverse effects, relapse, and drug resistance. This underscores the urgency for novel therapies, and Betulin and Shatavarin-IV as explored in this study, may hold the potential to address this need.

The study's multi-faceted approach, combining in-silico and in-vitro analyses, can greatly benefit the field of CCa research. Through computational analysis, this research can provide valuable insights into the potential of Betulin and Shatavarin-IV as inhibitors of specific molecular targets relevant to cervical cancer. These insights can serve as a foundation for future experimental studies that validate and expand upon the computational findings, potentially leading to groundbreaking treatments for cervical cancer. The molecular docking approach used in this study, which examines ligand-receptor interactions, can be particularly relevant in the context of cervical cancer. It offers the opportunity to assess how Betulin and Shatavarin-IV interact with specific targets related to CCa development, potentially identifying critical pathways for intervention. Moreover, considering pharmacokinetic factors in drug development is crucial for CCa treatment. P-glycoprotein and adherence to Lipinsk's rule play

a significant role in identifying promising compounds with favourable pharmacokinetic profiles. Identifying such compounds can be a pivotal step in the development of effective CCa therapies.

Molecular dynamic simulations, as conducted in this study, can further contribute to our understanding of how Betulin and Shatavarin-IV interact with target receptors in the CCa context. This information can help in designing more precise and effective therapies. Incorporating in-vitro analysis is also of paramount importance for CCa research. Finding that Betulin and Shatavarin-IV have the potential to inhibit CCa through in-vitro experiments adds another layer of credibility and relevance to this study. In summary, this research holds great promise in advancing the field of CCa treatment and prevention. By exploring novel compounds, employing state-of-the-art computational tools, and conducting rigorous experimental investigations, this study lays the groundwork for potentially transformative therapies that could improve the lives and outcomes of women affected by cervical cancer. The findings offer hope for a future where CCa is more effectively managed, reducing its devastating impact on women's health worldwide.

Conclusion:

This study presents a thorough investigation into the pharmacological potential of Betulin (BE) and Shatavarin-IV (SH-IV), focusing on their roles as promising candidates to combat cervical cancer. The integrated approach of using ADME predictions and molecular docking studies offers a multifaceted understanding of their drug-likeness and therapeutic viability.

The pharmacokinetic analysis of BE and SH-IV reveals favourable characteristics that are essential for effective drug development. Both compounds demonstrated high bioavailability and excellent oral absorption, critical factors that enhance their potential as therapeutic agents. However, the presence of moderately toxic attributes, as indicated by their low TPSA and high Log P values, highlights the need for careful consideration of dosage and formulation strategies to mitigate toxicity. The molecular docking studies have shown that BE and SH-IV exhibit strong binding affinities to key epigenetic proteins, particularly HDAC4 and DNMT3A. The identification of significant hydrogen bonding and hydrophobic interactions supports the assertion that these compounds can effectively inhibit the regulatory role of these proteins. Such inhibition is essential since both HDAC4 and DNMT3A play pivotal roles in the epigenetic regulation of gene expression in response to cancer proliferation and survival.

The innovative formulation of BE and SH-IV into nanoparticles, specifically BE-Zinc and SH-IV-Silver, further enhances their therapeutic efficacy. The *in vitro* experiments demonstrated that these nanoparticles exhibited significantly lower IC₅₀ values against CCa cell lines, particularly SiHa cells, compared to their pure counterparts. This indicates a marked improvement in anticancer activity, likely due to enhanced solubility and bioavailability facilitated by the nanoparticle matrix.

The results from the Sulphorhodamine B (SRB) assay and the *in vitro* wound healing scratch assay reveal that the nanoparticle formulations not only inhibited cell proliferation but also significantly impaired the migration capabilities of cancer cells. These findings suggest that BE and SH-IV nanoparticles can effectively disrupt critical processes involved in cancer metastasis.

Furthermore, the study examined the impact of these nanoparticle formulations on the expression of HDAC4. Western blot analysis confirmed a dose-dependent increase in HDAC4 expression in treated cells, indicating a potential feedback mechanism where the inhibition of cancer progression may simultaneously influence epigenetic regulation. The downregulation

of HDAC4 and the upregulation of DNMT3A in CCa tissue further emphasize the importance of targeting these proteins for therapeutic intervention.

Network pharmacology analyses revealed that the interactions of BE and SH-IV with multiple molecular targets are linked to significant cancer pathways, including the PI3-Akt signaling pathway. This highlights the complexity of their mechanisms and the potential for these compounds to influence various aspects of cancer biology, including cell proliferation, apoptosis, and metastasis.

Future Directions

The encouraging results of this study provide a basis for further investigation into the clinical applications of SH-IV and its chemically synthesized AgNO₃ nanoparticles. Future research should prioritize validating these findings through clinical trials, with a focus on assessing the safety, efficacy, and optimal dosing regimens of these compounds. Moreover, exploring combination therapies that integrate BE and SH-IV with existing treatment modalities may enhance therapeutic outcomes for patients with cervical cancer.

In conclusion, this research not only confirms SH-IV and Ag-SH as promising natural compounds for the treatment of CCa but also emphasizes the potential of advanced computational techniques and nanotechnology in drug development. The comprehensive approach adopted in this study serves as a foundation for future investigations aimed at translating these findings into clinical applications, ultimately improving treatment options for patients affected by cervical cancer.

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Annexure

Table : List of Phytochemicals Selected for Screening

List of Phytochemicals Selected for Screening						
Variabilin	1,3-dihydroxy-4,7-dimethoxy xanthone	2-coumarinate	3-hydroxy-4-methoxybenzaldehyde	7,3',4'-trihydroxyflavone	Acetone	alpha-humulene
alpha-phellandrene	1,3-naphthalenediol	2-cyclopenten-1-one	3-hydroxycoumarin	7-epi-.alpha.-eudesmol	Acetylaminohexanol	alpha-humulene
Camphene	1,4-alpha-delta-glucan	2-hepten-4-one, 2-methyl-6-(4-methylphenyl)-	3-indoleacrylic acid	7-epi-alpha-eudesmol	Acetylcholine	alpha-ionone
Catechin	1,4-alpha-d-glucan	2-hydrazinoethanol	3-methoxyphenol	7-epizingiberene	acetyleneugenol_aromaticum_zdb	alpha-longipinene
Elaeocarpine	1,4-cadinadiene_longum_pc	2-hydroxy-4-methoxyacetophenone	3-methyl-1-butanol	7-hydroxy-3g4g (methylenedioxy) flavone	Acetylshikonin	alpha-murolol
b-santalol	1,4-cineole	2-hydroxy-4-	3-octanol	7-hydroxy-3,6-	Acetylcholine	alpha-phellandrene

		methoxybenzoic acid		bis(tigloyloxy)tropane		
Epicatechin	1,4-naphthoquinone	2-hydroxyadenosine	3-oxosteroid	7-hydroxy-4h-chromen-4-one_dioctia	aconitic acid	alpha-pinene
gamma-hydroxy-l-homoarginine	1,5-anhydro-1-(1,6,7-trihydroxy-3-methoxy-9-oxo-9h-xanthen-4-yl)hexitol	2-ketoglutaric acid	3-phenylpropan-1-ol	7-methyl-4-methylidene-1-propan-2-yl-2,3,4a,5,6,8a-hexahydro-1h-naphthalene	Acorenone	alpha-santalene
neolitsine	1,5-diphenyl-3-pentanone, 3-	2-methoxy-3-sec-butylpyrazine	3-phenylpropyl acetate	7-o-methyltectorigenin	Actinodaphnine	alpha-santalol
sabinene	1,6-anhydro-beta-D-glucopyranose	2-methoxyphenol	3-phenylpropyl cinnamate	8-methyl-8-azabicyclo[3.2.1]octane	actinomycin D	alpha-solamargine

				ctane- 2,6-diol		
tirucallol	1,6-bis-o- galloyl- beta-d- glucose	2-methyl- 1- propanol	3- phenylun decane_lo ngum_pc	9,10- anthrace nedione, 1,8- diethoxy -	acutoside a	alpha- terpenene
1,7,7- trimethylbi cyclo[2.2.1]heptan-2- one	1,6- digalloyl glucose	2- methylhex acosane_2 d_diocia	4 g methoxy formanili de	9,12- dioxo- 10- dodecen oic acid	acutoside b	alpha- terpinene
bornyl acetate	1,6-di-o- galloyl- beta-d- glucose	2- methylnan one_tragia _pc	4,5,7- trimethox yflavone	9'-cis- neoxanth in	acutoside d	alpha- terpineol
nona-2,6- dien-1-ol	1,8-cineol	2- methylqui noline	4-acetyl- 1,4- dimethyl- 1- cyclohexe ne	9-de-arg- bradykin in	acutoside e	alpha- terpinyl acetate
(3beta,5eta, 8eta)-24- methylenel anost- 9(11)-en-3- ol	1,8- cineole_ar omaticum _pc	2- methylnan acosane_2 d_diocia	4-acoren- 3-one	9-epi quinine	acutoside g	alpha- vetivone
(3e)-3- (aminomet hylidene)o	1,8- cineole_lo ngum_pc	2-nonanol	4alpha- methyl- 5alpha- cholest-8-	9- fluoreno ne	adamantane- 1- carboxamide,	aluminum

xane-2,4-dione			en-3beta-ol			
(3r,6e)-nerolidol	1.xi.,6.xi., 7.xi.- cadina- 4,9-diene	2-octene	4alpha-phorbol	9-hydroxy- 1,2,4,5-tetrahydr o- canthin- 6-one	Adenosine	ambolic acid
(8)-gingerol_zi ngiber_pc	1_cyclopr opyl_2, 3 diazabicyc lo[2.2.1]h ept-2- ene.mol	2-octenoic acid, methyl ester	4-amino- 1,5- pentandio ic acid	9-octadece noic acid	adhatodine_2d	ambonic acid
(c14- c18)trialkyl glyceride	1-butanol	2-pentylfura n	4-aminobut yric acid	9-o-methylco umestrol	Afzelechin	ambrinol
(e)-4,8-dimethyl- 1,3,7-nonatriene	1-desgalloyl eugeniin	2-vinylpyrid ine	4-hydroxy butyl cinnamate	10,12-peroxyca lamenene	Ajugol	amentofla vone
(e, e)-+-farnesene_ zingiber	1-hentriacon tanol	3 - o acetyl-6 - methoxy - cycloarten ol	4-hydroxy cinnamic acid	10- gingerol _zingiber _pc	Alanine	amyl cinnamat e
(e,z)-2,6-nonadienal	1-hydroxy- 1,7-dimethyl- 4-isopropyl-	3-(3,4-dihydroxy phenyl) lactic acid	4-hydroxy- 3,5-dimethox ycinnamic acid	12,15-cis- squamos atin-a	alkaloid mixture	amyrin acetate

	2,7-cyclodeca diene					
(epi)gallo catechin	1-methyl-5-(1-methylethenyl)cyclohexene	3-(4-hydroxyphenyl)propionic acid	4-hydroxyarginine	12-deoxyphorbol	alkyl benzyl dimethyl ammonium chloride	anaferine
(r)-(-)-gturmerone_longum_pc	1-octacosanol	3-(benzylthio)acrylic acid, methyl ester	4-hydroxybenzaldehyde	12-o-decanoyl phorbol-13-(2-methylbutyrate)	Allantoin	anahygrine
(sr,6e)-nerolidol	1-octadecanethiol	3,3,8,8-tetramethyltricyclo[5.1.0.0(2,4)]oct-5-ene-5-propanoic acid	4-hydroxybenzoic acid	13-hydroxygermacrone	allicin.pdb	anethole_longum_zdb
(z)-beta-ocimanol	1-o-galloyl-beta-d-glucose	3,3'-bisjuglone	4-hydroxybenzyl alcohol	18-beta-glycyrrhetic acid	alliin_allium_sativum	angustibalin
(z)-valerenyl acetate	1-o-sinapoyl-beta-d-glucose	3,4-dihydroxybenzaldehyde	4-hydroxyphenylpyruvic acid	20_beta-hydroxycdysone	allo ocimene_aromaticum_zdb	anisotine_adhatoda
(z,z)-3,6-nonadien-1-ol	1-pentanol	3,4-dihydroxy	4-methoxy	22,23-dihydro	all-trans-neoxanthin	anolignanb

		benzoic acid	lucobrassicin	rassicasterol		
(z,z)-farnesol	1-propanol	3',4'-dimethoxyacetophenone	4-methylumbelliferone	22,23-dihydrostigmasteryl	alpha bergamotene_aromaticum_pc	anthocyanin
-)-epicatechin gallate	1s)-1,7,7-trimethylbicyclo[2.2.1]heptan-2-one	3,5,7,3',4'-penta-hydroxyflavone	5-(2-aminoethyl)-2-methoxyphenol	24-methyleneecycloartanol	alpha copane_aromaticum_2d_pc	anthranilic acid
+) - epicatechin	2 - hydroxy 4 - methoxy benzaldehyde, acetate	3,5-dihydroxystilbene	5,3'-dihydroxy-6,7,4',5'-tetramethoxyflavone	24-methyleneepollinastanol	alpha humulene_aromaticum_pc	anthraniloyl-o-glucose
+) - sabinene	2-(4-hydroxyphenyl)ethanol	3,5-dioxohexanoic acid	5,6,7-trimethoxycoumarin	25-hydroxycalciferol	alpha pinene	apigenin 7-glucuronide
+)-asarone	2,3-dihydrowith	3,6-diacetoxy-1-hydroxy-7-methoxy-xanthene-9-one	5-desgalloylstachyurin	477649. mol	alpha pinene_aromaticum_pc	apigenin 7-o-beta-d-glucoside
+)-bisabolene_zingiber	2,3-dihydro-5-hydroxy-2-methyl-	3,6-di-o-galloyl-d-glucose (2d)	5-hydroxy-6,7-dimethox	477649-2.mol	alpha rotundol	apigenin

	1,4-naphthalenedione		γ-3-(4-methoxyphenyl)-4h-chromen-4-one			
+ - caryophyllene_acromaticum_pc	2,3-dimethoxycinnamic acid	3,6-di-o-galloyl-d-glucose	5-hydroxy-methylfurfural	491765. mol	alpha thujene_aromaticum_zdb	apigenin_aromaticum_zdb
+ - copaene_zingiber_2d	2,3-methylenedioxynaphthalene	3,6-ditigloyloxytropone	5-methyl-2-furylmethyl ketone	491765-2.mol	alpha.1-sitosterol	apigenin-4'-glucoside
+ - d-glucopyranoside	2,4,5,6-phenanthrenetetrol	3,7,4'-trihydroxyflavone	5-methyl-5h-dibenz[b,f]azepine	518423. mol	alpha.-amyrin	apocarpone
+ - d-lyxofuranoside	2,4,5-trimethoxybenzaldehyde	3,4'-dihydroxy-(mono-14c)-sudan	5-methylsulfonyl-4-nitroimidazole	518423-2.mol	alpha_pinen_zingiber_db.pdb	apocarpamine
+ - phellandrene_zingiber_pc	2,4-dihydroxycinnamic acid	3α,6β-eta-ditigloyloxytropone-7β-ol	6,7-dimethoxycoumarin	4941667. mol	alpha-amyrin	arachidic acid.mol
+ - sesquiphellandrene_zingiber	2,4-dimethoxycinnamic acid	3α,6β-eta-ditigloyloxytropone	6,10,14-trimethylpentadecan-2-one	4941667-2.mol	alpha-bisabolol	arachidic acid

1-(vinyl- oxy)hexane- triglyceride	2,4-dimethyl- hexanetriol	3- α - tigloyloxy- tropane	6- β - hydroxy- ipolamide	ac111ukb	α - calacorene	arcurcumin- zinger
1,3-bis(4- tert-butyl- phthalate)	2,5-dihydroxy- benzoic acid	3-azabicyclo[3.2.1]octan-2-one	6-dehydrogingerone- zingerone	ac114puz	α -carotene	aristolochic acid i
1,2,3,4,6-penta-O-galloylglucose (2d)	2,6-dimethoxy-4-(2-hydroxyethyl)-phenol-1-O- β -D-glucopyranoside (2d).mol	3- β ,20-dihydroxy-30-norlupane	6- gingerol- zingerone	ac11lavb6	α - cubebene- longum	aristolochic acid ii
1,2,4-triacetoxystyrene	2,6-dimethylheptanetriol	3- β - hydroxy- 20(29)- lupene	6-hydroxymellein	ac1n79ay	α - cyperone	arjungenin
1,2-dihydro-1,4-diphenylphthalazine	2-amino- cyclopentanemethanamine	3-carene	6-isopropenyl-4,8a-dimethyl-1,2,3,5,6,7,8,8a-octahydro- naphthalene-2-ol	ac1nqwxh	α -D- galactose	arnesyl diphosphate

1,2-dihydroxyethyl-3,4-dihydroxyfuran-2(5h)-one	2-aminohexanedioic acid	3-epioleanolic acid	6-methoxymellein	ac1nsu67	alpha-d-galacturonate	arnicolide c
1,3,5-trihydroxy-4-methoxyxanthone	2-aminopyridine	3-ethoxy-4,5-dihydroxy-benzoic acid	6-methyl-5-hepten-2-one	acacetin	alpha-elemene	arnicolide d
1,3-diaminopropane	2-aminopyrimidine	3-hexenoic acid, methyl ester	6-o-senecioplplenolin	acetic acid	alpha-farnesene	arnifolin
1,3-dibenzylthiourea	2-chloro-4-(4-chlorophenyl)phenol	3-hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-1-propanone	6-shogaol_zingiber_p c	acetaldehyde	alpha-ferulene	aromadendrene
1,3-dihydroxyacetone	2-chloro-5-(1,5-cyclohexadienyl)pyridine	3-hydroxy-4-methoxybenzoic acid	6-thioguanosine 5'-diphosphate	acetic acid	alpha-gurjunene	arsenic
delta7-avenasterol	Ecdysterone	exo-norbicycl	geraniol	heterodendrins	Isodalbergin	kobusone

		oekasantal al				
demethoxy curcumin_1 ongum_pc	edulan iv.mol	falcarindi ol	geranyl acetate	hex-3- en-1-ol	isodiprene_lo ngum_pc	l(-)- borneol
denicunine	eicosane, 2- methyl_2d_ diocia	falcarinol one	geranyl butyrate	hexadeca noic acid	Isodomesticin e	lactucin
denudatine	eicosane, 7- hexyl_2d_ diocia	fargesin_1 ongum_pc	geranyl linalool	hexahydr opyridin e _longum _zdb	Isoelaeocarpin e	lactupicri n
deoxyphor bol	elemicin_ aromaticu m_zdb	farnesene _longum_ pc	geranylac etate_zing iber_pc	hexanal	isofuranogerm acrene	l- allohydro xyproline
deoxyshiko nin	ellagic acid	fenchone	geranylge raniol, tert- butyldime thylsilyl ether.2dm ol	hexanoic acid	isohamnetin_a romaticum_pc	l-alpha- curcumen e
depositedst ructure_sid _10358178 1	ellagic acid_acro maticum_ zdb	ferruginol	germacre n a	hexocosa ne	isohexadecano ic acid	l- arabinose
depositedst ructure_sid _27339952 4	Ellagitann in	ferulic acid	germacre ne d	hexylene glycol	Isokobusone	l-ascorbic acid

desktop.ini	Elymocla vine	ferulic acid_aro maticum_z db	germacre ne d_aro maticum_zdb	himachal ol	Isolinderalact one	launobine
desmostero l	emblicani n a (2d)	ferulic_ac id.pdb	germacro ne	hinokifla vone	isomangiferoli c acid	lauric acid
dextrin	emblicani n b (2d)	fidarestat	germacycl oundecan e-6,7- dione, 1,1- diethyl-	hmdbid_ hmdb390 55	Isomultifloren ol	lawsaritol
d-galactose	Emidine	filicin	gibberelli ns	homogen tirate	Isoniazid	lawsone
d-glucose	Enerolidol	fisetin	gingereno ne- a_zingibe r_pc	hydrocin namic acid	isoniazid_adh atoda	lawsone_l ongum_z db
d-glucoside	Enicoflavi ne	flavone	gingerol_ aromaticu m_zdb	hydrocin namic acid_lon gum_zdb	Isopenniclavin e	laxanthon e i
diallyl disulfide_s ataivum	ent- zedoarone diol	flavylum perchlorat e	glibencla mide	hydrogen cyanide	Isoperezzone	l- citrulline
diallyl trisulfide_s ataivum	epi-beta- santalene	folic acid	glucose	hydrogen oxalate	Isophytol	l-cysteine
diallyl_tris ulphide.mo l	epi-b- santalene	formic acid	glucoside	hydroqui midine	Isoquercetin	ledol

diazomethane	Epibuphanamine	formononetin	glutamate	hydroquinone	Isoquercetin	lepidine
dibutylphthalate_dioxi	epicatechin_aromaticum_zdb	fraxetin	glutamic acid residue	hygrine	Isorhamnetin	letestuianin b
dihydrodehydrodiconiferyl alcohol	Epicurzerenone	friedlein	glutamic acid	hyoscyamine	Isorotundene	lettucenin a
dihydrocarveol	epieudesmin_longum_pc	fructose	glycerol 1,2-dipalmitate 3-oleate	hypaphorine	Isoscleron	leucine
dihydrocarvone	epi-friedelinol	furanodienone	glycerol	hyperoside	Isostrictinin	leucocianidol
dihydrocarvyl acetate	epigallocatechin_aromaticum_zdb	furanodienone	glyceryl behenate	hypnon_longum_zdb	Isotalatizidine	leucocyanidol
dihydropiperlonguminine_longum_pc	Episterol	furanogeromophilane	glycine betaine	i-alpha-curcuminone	Isothymusin	leucodelphinidin
dihydropyrocuzerenone	Epoxyguaiene	furanogermonone	glycine	i-ascorbic acid	Isovalencenol	leukoefdin
dihydroquercetin	Epoxylinanol	furanoid norditerpenes-diosbulbinosides d	glycitein_aromaticum_zdb	illudol	isovanillic acid	l-histidine

diosbulbin c	Eremophil ane	fusalanipy rone	glycolic acid	images.z ip	Isowillardine	l- homoseri ne
diosbulbin d	Ergosterol	galactinol	glycoprot ein	imidazol e	Jasminine	lignoceric acid
diosbulbin f	Erythroce ntaurin	galactitol	goniothal amin	indicine	jatropholone a	lignoceric acid
diosbulbin g	Erythrodi ol	galactoma nnan	gossypeti n-3- glucoside- 8- glucuroni de	indicol	Jatrorrhizine	ligsnd32
diosbulbin h	Esculetin	galactopin itol a	granatin b.mol	indole	Juglanin	limonene
diosbulbin oside f	Esculin	galactose	grandiflor enic acid	ingenol 3,20- dibenzoa te	Juglone	limonene _aromatic um_zdb
diosgenin	Ethambut ol	gallic acid	guaiacol_ aromaticu m_zdb	ingenol	Junceine	linalool
dipentene	Ethanol	gallic acid_acro maticum_ zdb	guaiane	ingenol_ zingiber_ pc	Junenol	linalool
Distearoylp hosphatidyl serin	ethinyl estradiol	galocatec hin	guaiazule ne	ingol 12- acetate	Junionone	linalool_a romaticu m_zdb
distearyl sulfide	ethyl cinnamate	galloylqui nic acid	guineensi ne_longu m_zdb	ioxynil	junipercomno side a	linalool_l ongum_z db

diterpene ii (lactone)	ethyl cinnamate _aromatic um_pc	gama terpinene_ aromaticu m_zdb	helenalin	ipolamii de	junipercomno side b	linaly acetate_ar omaticum _zdb
dl-2- aminobutyr ic acid	ethyl gallate	gamma- cadinene	hemidesci ne	iridin_tra gia_zdb	Juniperin	linalyl acetate
dl-alanine - 15n	ethyl linoleate (2d)	gamma- sitosterol	hemidesm oside a	isalon	Juniperol	linoleic acid
dl-alanine- 15n	ethyl linoleate_ allium_sat aivum	gamma- valerolact one	hemidesm oside b	isatin	kaemferol-7- o-glucoside	linolenic acid
dl-arginine	ethyl palmitate (2d)	gardenin d	hemidesm oside c	isoartemi sia_ arom atium_zd b	kaempferol 3,7- diglucoside	l- isoleucine
dl- asparagine	ethyl-p- methoxy cinnamate	gastrodin	hemidine	isobutyli sovalerat e_longu m_pc	Kaempferol	littorine
dl-aspartic acid	Eucalypto l	geijerone	heminine	isobutyls hikonin	kaempferol_a romaticum_z db	l-leucine
dl- methionine sulfoxide	Eugeniin	gemfibroz il	hemisine	isocaryo phyllene	Kasuagamyci n	l- methionin e
dl- tryptophan	eugenin _acromati cum_zdb	gemin d	hennadiol	isocaryo phyllene _longum _pc	Kaurene	l- norleucin e

dl-valine	eugenitin_ acromaticum_zdb	genistein	hentriacontanane	isocavudin	khekadaengoside k_diocia	longifolene
d-mannose	Eugenol	genistein_zingiber_db.mol	heptacosane	isocavudin	Khusimol	lophenol
docosanoic acid	eugenol_acromaticum_zdb	gentianine	heptadecene_longum_pc	isocolumbin	knapsack_c00019955	l-proline
dolichyl phosphate	Eupatin	gentisic acid_aromaticum_zdb	hernandion	isocryptomerin	knapsack_c00020215	l-serine
d-quercitol	Euphorbol	geranic acid	hesperidin	isocyperol	knapsack_c00027251	lumichrome
-ascorbic acid	beta-santalol	copaene_longum_pc	curlone	piperic acid_aromaticum_zdb	Casuarictin	chloromethyl 9-chlorododecanoate
aspartic acid	Betasdf	copper(i) phenoxide	curzerene	piperidine-2-carboxylic acid	Catechin	cholesterol
astragalin	beta-sitosterol	copper	curzerenone	piperine_longum_zdb	catechin_aromaticum_zdb	choline chloride
a-teresantalic acid	beta-solamargine	corchioside a	cuscohygrine	piperlongumine_longum_zdb	Cavanine	choline
auroxanthin	beta-terpineol	cordifolide a	c-veratroyl glycol	piperlonguminine	caviunin 7-o-glucoside	chrysanthemin

				_longum _zdb		
avicularin	Betavetivene	corilagin. mol	cyanidin	pipernon atine_longum_pc	cedryl acetate_longum_pc	chrysanthe myl alcohol.
azanide	beta- vetivone	corilagin	cyanidin- 3-(6g- malonyl)_ allium_sat aivum	piperolac tam a_longum_zdb	Celastrol	chrysetun one
zingiberene _zingiber	betcaryop hyllene oxide	cortisone	cyanocob alamin	piperonal _longum _zdb	cellulose ge	cicerin
3-carene	betulic acid	cosmosiin	cycloarten ol	piperony lic acid_longum_zdb	cellulose gel	ciceritol
camphene	Botulin	coumaperi ne_longum_zdb	cycloeuca lenol	piperund ecalidine _longum _zdb	Cerumol	
cis-carveol	betulinic acid	coumaric acid	cyclohexe ne	pisatin_l ongum_pc	Chasmanthin	
linalool	bicornin_a romaticum_2d_pc	coumarin 2	cyclolaud enol	pisol_longum_zdb	stigmasteryl glucoside	schembl 1 3324299
pipéritone	bicycloger macrene_ aromaticum_pc	coumarin	cymol_lo ngum_zdb	plateleta ctivating factor	Strophanthidin	schottenol glucoside

secoisolari ciresinol	biflorin_a chromaticu m_zdb	coumarin _aromatic um_zdb	cynarosid e	plocosid e a	Cinchonide	sciadopit ysin
methyleuge nol	biochanin a	coumaroy l-coa	cypera- 2,4(15)- diene (2d).mol	p- ocimene _longum _pc	Cinchophylla mine	scirpusin a
methylgall ate	Biochanin	crategolic acid_acro maticum_ zdb	cyperadio ne	polygala cic acid	Cinchophyllin e	scirpusin b
methyl-p- methoxy- cinnamate	biochanin a-a- 7glucosid er	crocetin	cyperene	pratol	cinnamic acid	scoparone
mi-saponin a	Biotin	croton (chalcone)	cyperenon e	proantho cyanidin	cinnamyl alcohol	scopletin. mol
mi-saponin b	bis(acetic acid)	crotofolin -a	cyperol	procurcu menol	cinnamyl cinnamate	scopolam ine
mi-saponin c	bis(acetic acid); tannins	crotonalde hyde	cyperolon e	procyani din	Cirsilineol	scopoleti n
m-m-p- trigallic acid	bisabolen ol d	crotophor bolone	cyperotun done	proline betaine	olean-12-en- 3+-yl acetate	phyllaem blicin c (2d)
molludistin	Bisabolen ol	cryptanosi de a	cystine	proline	oleanolic acid	phyllaem blicin d
momordic acid	Bisabolo	cryptocan shinone	daidzein	propyl cinnamat e	oleanolic aldehyde	phyllanthi n

moslene_lo ngum_zdb	Bisdemeth oxycurcu min	cryptodori ne	daidzein_ aromaticu m_zdb	prosapog enol	oleanolic_acro maticum_zdb	phyllantid in
multifloren ol	bisdemeth oxycurcu min_long um_pc	cryptolepi ne	dalbergen one	protobas sic acid	oleic aci	phyllemb in
mustakone	bisphenol a	cryptosin	dalbergic hromene	protocate chuic acid	oleic acid	physaguli n d
myrcene	Boldine	cucurbitac in 2-o-b- glucopyra noside_di ocia	dalbergin	protocate chuic acid_aro maticum _zdb	omega- hydroxypropi oguaiacone	physalien
myrcene_a romaticum _zdb	borneol acetate_ar omaticum _pc	cucurbitac in c	dalbergip henol	pseudoto rpine	Ononin	physostig mine
myrcene_zi ngiber_pc	Borneol	cucurbitac in k	d-alpha- pinene	psi- tectorige nin	Orientin	phytin
myricetin	borneol_a romaticu m_zdb	cucurbitac in- b_diocia	dammare nediol ii	punicaco rtein	Phytol	cirsimarit in
myricetin_ acromaticu m_zdb	bornyl acetate	cucurbitin e	daphnetin	punicafol in.mol	Phytosterols	cis ocimene_ aromaticu m_pc
myricetin- 3- arabinoside	bornyl acetate_zi ngiber_db .mol	cucurbitos ide b	daturadiol	punicala gin.mol	picrotoxin_tra gia_zdb	cis-1,2- cyclohexa nediamin e

myricitrin	bornyl cinnamate	cucurbitos ide c	daturamet elin d	punigluc onin (2d)	Picrotoxinum	cis-3- hexenal
myristic acid	brachyami de b_longum _zdb	cucurbitos ide g	daturaolo ne	punigluc onin.mol	Phytol	cirsimarit in
myrtenal	Bradykini n	cumambri n a	daturatatu rin a aglycone	putrescin e	Saponarin	columbin
n,n,n',n'- tetramethyl ethylenedia mine	Brassicast erol	cupressufl avone	daucene	pyrazina mide	saponin a	communi c acid
n-[(2e,4e)- decadienoy l]- piperidine_ longum_pc	brevifolin carboxylic acid	curcolone	daucol	pyrazina mide_ad hatoda	saponin b	condelphi ne
n-[(2e,4e)- tetradecadi enoyl]piper idine_long um_pc	Bromoxyn il	curculigos aponin c	daucoster ol	pyridine	Sarmentine	orientin_t ragia_zdb
n1,n12- bis(dihydro caffeoyl)sp ermine	b-santallic acid	curculigos aponin f	d- camphor	pyridine- 3- carboxa mide	Sarmentocym arin	oxylubim in
n-acetyl-l- hydroxypro line	b-santalol	curculigos aponin g	d- camphor_ longum_z db	pyridoxi ne	Sarmentogeni n	p_coumar ic_acid.p db
naphthsarit one	b- sitosterol	curculigos ide	d-carvone	pyrocate chol	Sarsasapogen one	palmarin

naringenin chalcone_a romaticum _zdb	Buchanani ne	curcuma longa	d- citronellol	pyrocurz erenone	Sasanquol	orientin_t ragia_zdb
naringenin _aromaticu m_zdb	butanoic acid, 2- amino- 4,4- dichloro-	curcumadi ol	dehydroc urdione	pyrogall ol	phthalamic acid	sterol (2d)
n- benzyliden e-2- azidobenzyl amine	Palmitine	curcuman olide a	dehydroc urvularin	pyrolo (3, 2 - d) pyrimidi n g 2, 4 (1h, 3h) – dione	phthalic acid, butyl undecyl ester_2d_dioci a	sterol ester
neral	Palmitodi olein	curcuman olide b	dehydropi pernonali ne_longu m_zdb	pyrrolidi ne	Phthalimide	sterol
neral_ arom aticum_zdb	Partheono lide	curcumen ol	delphinidi n_aromati cum_zdb	quercetin coumaro yl- glucosid e	Phtosterols	stigmast- 5-en- 3beta-ol
nerol	Patchoule none	curcumen one	delta-4- carene	quercetin	p- hydroxybenzo ic acid_aromatic um_zdb	stigmasta -7,25- dien- 3beta-ol
neryl acetate	p- coumaric acid_arom	shikimic acid	retrofracta mide b_longum _pc	quercetin _acromat icum_zd b	phyllaemblic acid b	stigmaste rol glucoside

	aticum_zdb					
n-formylanona	p-cresol	sinapaldehyde glucoside	rhamnetin_acromaticum_zdb	quercetin_tragia_db.pdb	phyllaemblic acid c	stigmastrol
n-heptadecane_longum_pc	p-cymene	sinapic acid	rhamnose	quercetin_zingiber_db.mol	phyllaemblicina a	stigmastrol_acromaticum_zdb
nicotinamide	p-cymol	sinapic_acid.pdb	riboflavin	quercetin e	phyllaemblicina b (2d)	stigmastrol_tragia_zdb.mol 2
nicotinate	Pectin	sinapine	riddelliine	quercitin	n-trans-feruloyl-4-o-methyldopamine	o-coumaric acid_aromaticum_zdb
n-isobutyl-2e,4e-undecadienamide_longum_pc	pedunculin (2d)	sinapinic acid_aromaticum_zdb	rosmarinic acid	quercitrin	n-trans-feruloyltyramine	octacosane_2d_diole
n-isovaleroyl glycine	Pedunculin	sissostrin	rosmarinic acid	quinamine	Nuciferol	octadecan-9,12-dienoic acid
n-methyl-2-pyrrolidone	pellitorine_longum_zdb	sitosterol	rosmarinic acid_aromaticum_zdb	quinic acid	Clerosterol	octadecanoate

n-methylactinodaphnine	Penniclavine	sitosteryl glucoside	rotundene	rabdosiin (2d)	coagulin q	octadecanoic acid, ethyl ester (cas)
nobiletin	pentadecanone_longum_pc	sodium deoxycholate	rotundeol (2d)	resorcinol	Codisterol	octan-2-one
nonacosanol-10-ol	pentadecanone_longum_pc	solanidine	rotundone	reticuline	s-1-propylenecysteine_sativum	cis-nuciferol
nonacosane	pentunidin_aromaticum_zdb	solanine	rubranine	retinol	s-2,6-diaminohexanoic acid	citric acid
nonadecane_2d_longum_pc	pentylene tetrazole_tragia_pc	solasodine	rugosin c	retrofractamide_a_longum	sabinene_aromaticum_zdb	citronellal
nonadecane_2d_longum_pc	perilla alcohol	solasonine	rutin	cis-6-nonenal	s-adenosylmethionine	citronellyl acetate_zingiber_pc
nootkatone	phenanthrene_aromaticum_zdb	solasurine	rutin_tragia_db.pdb	cis-jasmone	Salicylaldehyde	citrostadienol
nopinene_1ongum_pc	phenylacetic acid_aromaticum_zdb	solongifolene epoxide_1ongum_pc	s)-nandigerine	cis-lanceol	salicylic acid	cladospirone bisepoxide
nordalbergin	Phenylacetone nitrile	sominiiferine	phorbol acetate, caprate	stachyoside	s-allyl-cysteine_sativum	cis-3-hexenyl acetate

noreugenin	Phenylalanine	sominone	phorbol caprate, tiglate	starch	Santalol	cis-3-hexenyl-isobutyrate
norrotundene	Phloroglucinol	somniferin	phorbol	stearic acid (2d)	sapogenins glycosides	cis-4-hydroxyl-proline
norscopolamine	phloroglucinol_aromaticum_zdb	sorbamide	phosphorus	stearic acid.mol	sesquiterpene ii alcohol diglucoside (compound 1)	sesamol_longum_zdb
nortricycloekasantalic acid	phorbol 12-myristate 13-acetate	spirostan-3-ol	palmitic acid, 2-(tetradecyloxy)ethyl ester	piperanin e_longum_zdb	sesquiterpene ii alcohol diglucoside	palmitic acid
styrene	Tetrahydrofuran	tricetin	urs-12-en-28-oic acid, 3-hydroxy, methyl ester (3+)	pipercall osine_longum_zdb	piperchabamide b_longum_pc	palmitic acid_longum_zdb
sucrose	Tetrahydrojatrorrhizine	trichodesmine	urs-12-en-3beta-ol palmitate (2d)	withanolide g	Yangambin	terpinen-4-ol
sugeonol	Tetrahydropalmatine	tricyclene	ursolic acid	withanolide j	yejuhualactone	terpinolene
sugetriol	tetramethylrhodamine	tricycloekasantalol	vakerin	withanolide k	Yugenin	tert-hexadecanethiol

	thiocyanate					
sugiol	Tetramethylrhodamine	tridecane	valencene	withanolide m	z_ajoene_allium_sataivum	tetradecanoic acid
sulcatone	Thalidomide	tridecane_longum_p_c	valerenic acid	withanolide n	Zeaxanthin	withasomniferilweb
sulfurous acid, pentadecyl pentyl ester_2d_dioxia	Thannilignan	tridecanoate	valeric acid	withanolide o	Zederone	withasomniferole c
swertiamarin	thesesquiterpene alcohol-gammacostol	tridecylene_longum_pc	valoneic acid dilactone	withanolide p	Zedoarol	withasomniferole
swertisin	Thiamine	trigonelline	vanillic acid	withanolide r	Zedoarondiol	withasomnilid
sylvatine_longum_zdb	Thymol	triphenyltin hydride	vanillic_acid.pdb	withanolide s	Vetiselinol	withasomnine
syringic acid	Thymonin	triprolidine	vanillin	withanolide a	vetivenic acid	withastramonolide
syringic acid_aromaticumzdb	tiglic acid	triterpenoids	vanillin_aromaticum_zdb	withanolide b	vicenin (2d)	xanosporic acid
tangeretin	Tigloidin	tropane	vannillic acid'	withanolide c	Violaxanthin	xanthone

tannic acid	Tilianin	tropic acid	variabilin	withanos ide iv	viscosalactone b	xanthoper ol
tannin	Tinocordi side	tropine	vasicine_ adhatoda	withanos ide vi	Viscosalacton e	xanthorrh izol
tannins	Tinospono ne	tropinone	vasicinon e_adhatod a	withaoxy lacto	vitamin b5	xylan
taraxasterol	Tinospora side	tryptamin e	vasicolino ne_adhato da	withaso mniferan olide	vitamin d3	yangambi n
taraxerol	Tinospori de	tubocapse nolide f	unii- 0v56hxq8 n5	trans11- octa- decen-9- ynoic acid	vitamin e	tectorigen in
taraxerone	tinospurin a	tyrosine cation radical	unii- 7m7487z 316	trans- alpha- bergamot ene	vitamin p	tembetari ne
taraxerone- _adathoda	Tinospori none	undecane	uric acid	trans-b- santalol	Vitexin	teresantal ic acid
tea extract	Tocopher ols	undulaton e_longum _pc	urolithin a	triaconta noic acid	Withametelin	termilign an
torpine	tolunolide a	tomatiden ol	tributyltin			

Publications:

From Thesis:

1. Kour S, Prabhu D, Biswas I, Singh A, Pawar SC, Perugu S, Vuree S. Screening of DNMT3A inhibitors from phytochemicals using molecular docking and molecular dynamics simulation for their anti-cancer potential. *Molecular Simulation*. 2024 Sep 21;50(14):1001-18.
2. Kour S, Biswas I, Sheoran S, Arora S, Singh A, Prabhu D, Pawar SC, Perugu S, Vuree S. Betulin: a novel triterpenoid anti-cancerous agent targeting cervical cancer through epigenetic proteins. *Molecular Diversity*. 2024 Jul 16:1-8.

Others

1. Kour S, Biswas I, Sheoran S, Arora S, Sheela P, Duppala SK, Murthy DK, Pawar SC, Singh H, Kumar D, Prabhu D. Artificial intelligence and nanotechnology for cervical cancer treatment: Current status and future perspectives. *Journal of Drug Delivery Science and Technology*. 2023 May 1;83:104392.

Paper/Poster Presentations:

1. Pharmacoinformatic and molecular dynamics simulation-based phytochemical screening against human cervical cancer by targeting epigenetic proteins. 1st international congress on Natural sciences (ICNAS-2021) on 10-12 September 2021.
2. Phytochemicals as Noteworthy Epigenetic Therapeutics against HDAC2: A comparative docking studies at 20th International conference on bioinformatics (InCoB 2021) from Nov 6-8, 2021)
3. Molecular docking studies of phytochemicals against dnmt3a as an anti-cervical cancer during Indian conference on bioinformatics 2021 (INBIX'21) from Nov 11-13,2021
4. Phytochemicals induces modulatory effect on DNMT3B and cervical cancer. International Conference “2nd Integrative Biology & Applied Genetics” (ICIBAG – 2022) Department of Genetics & Biotechnology, Osmania University
5. Evaluation of novel DMSO dissolved compounds of *Asparagus racemosus* (Shatavari derivatives) as augmented anti-cancerous against epigenetic markers in cervical cancer in the International Conference on Bioengineering and Biosciences (ICBB2022) held on 18-19 November 2022 organized by the Department of Biotechnology, School of Bio-engineering and Biosciences in association with the Society of Bioinformatics for Experimenting Scientists (Bioclues) organized at Lovely Professional University, Punjab

Workshop/ training attended:

1. International workshop on “Tools and techniques to perform molecular modelling and computer Aided Drug Designing (MMTT) from 11-17 January 2021 organized by NIPER Guwahati.
2. Bioinformatics Industrial Training at Eminent Biosciences (EMBS), Indore from 26th October 2020- 5th feb2021.
3. International bioinformatics workshop for cancer discovery and development by decodelife.org from 8th Feb-18th Feb 2021
4. International bioinformatics workshop for cancer Genomics by decodelife.org from 1-12 March 2021
5. 07 days workshop on “fundamentals of bioinformatics” 01-07 march, 2021 by Institute of Human Genetics, University of Jammu in collaboration with Department of Zoology, University of Jammu.
6. Online Workshop on Computational Applications in Secondary Metabolite Discovery (caismd) on 08-10 March 2021.
7. Workshop on molecular docking for beginners by finoseq on 30.05.2021.
8. Elsevier Author Workshop: Fundamentals of Research Article Writing at School of Engineering and Technology, Centurion University, on Friday 04 June, 2021.
9. “Aspire to live a Healthy life”: Cancer webinar series-session#3: organized by origin life Health care Solutions and research Centre LLP, Chandigarh on 1st August 2021.

