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MOLECULAR DOCKING STUDIES ON MEDICALLY IMPORTANT COMPOUND FROM *Azima tetracantha* AGAINST CERVICAL CANCER CELL LINE- AN *IN VITRO* AND *IN SILICO* STUDIES

B. Manimegalai

PG and Research Department of Biochemistry, Sengamala Thayaar Educational Trust Women's College (Autonomous) (Affiliated to Bharathidasan University), Sundarakkottai, Mannargudi– 614016, Tamil Nadu. India

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Abstract

This work is aimed at evaluating the bioactive compounds and *in silico* molecular docking studies on medically important compounds from *Azima tetracantha* against cervical cancer cell line. The qualitative phytochemical tests exhibited the presences of common phytocompounds including tannin, steroids, anthroquinones, saponins, terpenoids, flavonoids, polyphenol, glycosides and coumarins in both extracts while alkaloids was absences in aqueous and ethanol extracts of *Azima tetracantha* leaves. Significant amount of flavonoids and phenol were presented in *Azadirachta indica* leaves. The UV spectrum profile showed the peaks at 340, 400 and 640 nm. FTIR analysis confirmed the presence of alkanes, alcohols, alkenes, aromatics, aliphatic amines, phenols and alkyl groups in *Azima tetracantha* leaves. HPLC analysis of *Azima tetracantha* leaves showed the presence of six flavonoids compounds namely gallic acid, tannic acid, epigallocatechin, resorcinol, naringenin and myricetin malonyl glucoside. The evidence that *Azima tetracantha* leaves extract have an interesting anticancer activity, which could be due to the presence of phenolic and flavonoids compounds. *In silico* molecular docking carried out with the ligands as Gallic acid docked with protein human DNMT1 (PDB: 4WXX). Thus from the docking scores it can be inferred that the compounds binds successfully with the drug targets and the compounds may be used as anticancer drugs.

Keywords: *Azima tetracantha*, phytochemical, UV, FTIR, HPLC, anticancer and molecular docking.

Introduction

Herbal medicine is as old as a human race and is being used for the primary health care of human being since ancient period. Traditional medicinal plants contain therapeutic active substances, which is a precursor for the synthesis of herbal drugs (Morabad and Patil, 2017). Important drugs have been derived from plant resources directly or indirectly. Phytochemicals such as alkaloids, flavonoids, phenolic compounds, glycosides etc. play a significant role in pharmacological properties of plants (Pawar *et al.,* 2017). Cancer is a growing public problem the estimated worldwide new incidence of which is about 6 million cases per year. It is a leading cause of death in India and other countries. Cancer can affect any part of the human body and people at all ages, but risk for most types of cancer increases with age (Dikshit *et al.,* 2012). The term cancer is used to refer to a large group of diseases that can affect any part of the body. They are caused by uncontrolled cell proliferation that can take place in different tissues and spread into surrounding and distant organs (Jemal *et al.,* 2011).

Cancer is perceived as one of the top leading causes of death worldwide (Shokrzadeh *et al.,* 2010; Tagne *et al.,* 2014). It is a heterogenous disease characterized by aberrant cell proliferation and invasiveness of abnormal cells which spread to neighboring tissues (Bray *et al.,* 2012). Medicinal plants have been widely used as a source of natural compounds for the treatment of cancer4. Besides their antitumor activity, natural compounds have many advantages owing to their low side effects, low cost, and ease of availability (Mazher *et al.,* 2020; Khan *et al.,* 2019). Cervical cancer, the second most common gynecologic malignancy in females after breast cancer throughout the world, is continuing a serious health problem globally. It is one of the major causes of mortality in both developed and developing countries. Treatment of cervical cancer includes chemotherapy, radiotherapy, and surgery. The effectiveness of chemotherapy and radiotherapy is specific for cancer cells, and it may destroy the normal cells and have severe side effects. For treating advanced and refractory cervical cancer, effective therapies and innovations are required (Siegel and Zhu, 2009). In the present study, identification of bioactive compounds and *in silico* molecular docking studies on medically important compounds from *Azima tetracantha* against cervical cancer cell line.

MATERIALS AND METHODS

Collection of plant materials

The *Azima tetracantha* leaves powder was purchase in December 2023 from Mannargudi, Thiruvarur district, Tamil Nadu, India. The collected leaves were shade dried and make a fine powder for further analysis.

Preparation for extract

Take one gram of *Azima tetracantha* leaves powder in the extract prepared in 50 ml of ethanol and aqueous solvent, the extract shake it well for 30 minutes by free hand and wait for 24 hours. After extracts were filtered using whatman filter paper No.1 and filtrate used for further analysis.

Phytochemical screening

Chemical tests were carried out on the extract using standard procedures to identify the constituents as described by Sofowara (1993), Trease and Evans (1989) and Harborne (1973 and 1984). Determination of total

phenols by spectrophotometric method by Edeoga *et al.*, (2005). Flavonoid determine by the method of Boham and Kocipai-Abyazan (1994).

UV, FT-IR Spectroscopic and HPLC analysis

The *Azima tetracantha* leaves extracts were scanned in the wavelength ranging from 340-800nm using Perkin Elmer Spectrophotometer and the characteristic peaks were detected. FTIR analysis was performed using Perkin Elmer Spectrophotometer system, which was used to detect the characteristic peaks ranging from 400-4000 cm^{-1} corresponding to their functional groups. The peak values of the UV and FTIR were recorded. Each and every analysis was repeated twice for the spectrum confirmation. Flavonoids fractions were analyzed by using a HPLC method (Weerasak Samee *et al*., 2007).

***In vitro* cytotoxic effect determination by MTT assay**

To determine the cytotoxicity effect of test compound on HeLa cell line by MTT assay (Alley *et al.,* 1986; Mosmann, 1983).

% Cytotoxicity = 100 - [Abs (sample) /Abs (control)] x100.
% Cell Viability = [Abs (sample) /Abs (control)] x100.

***In silico* molecular docking**

Computational drug discovery technique in the recent day of Pharmaceutical research has successfully molecular modeling with different algorithm based programing software's been used. The ligand and protein binding scores according to algorithm based program thereby may use any software for protein and ligand interactions for best results (Velavan *et al.,* 2020). The grid map was centered at particular residues of the protein and was generated with grid dimension prepared (center x = -56.27, center y = 47.32 and center z = 2.57). The Lamarckian genetic algorithm and the pseudo-Solis and Wets methods were applied for minimization, using default parameters (Ghose and Crippen, 1987; Binkowski *et al.,* 2003; Vidya *et al.,* 2012; Shruthi *et al.,* 2012). Complex structures were modeled using modeling software's Pymol (1.1 version, Delano Scientific LLC, San Carlos, CA, USA), Chimera (1.10.1 version UCSF Resources for biocomputing visualization and informatics, NIH, CA, USA) and 2D pose viewed using Discovery StudioVisualizer (Trot and Olson, 2010).

RESULTS AND DISCUSSION

Herbal medicines are now widely preferred and used around the world because of their high effectiveness, ready availability, low toxicity, and environmentally friendly nature, and it reduces the time of treatment or the individual cost of anti-antioxidant, inflammatory, **anticancer** and antimicrobial drugs, resulting in lower prescription costs (Abbas *et al.,* 2021).

In the present study was carried out on the *Azima tetracantha* leaves revealed the presence of medicinally active constituents. The phytochemical characters of the *Azima tetracantha* leaves investigated and summarized in Table-1 and figure 2 and 3. The phytochemical screening *Azima tetracantha* leaves showed that the presence of tannin, steroids, anthroquinones, saponins, terpenoids, flavonoids, polyphenol, glycosides and coumarins in both extracts while alkaloids was absences in aqueous and ethanol extracts. The present study was performed to evaluate the total content of phenols and flavonoids in *Azima tetracantha* leaves. The highest amount present in the phenol followed by flavonoids.

Table 1: Qualitative phytochemical analysis of *Azadirachta indica* leaves extract

S. No	Phytochemicals	Aqueous extract	Ethanol extract
1	Tannin	++	+
2	Saponin	++	++
3	Flavonoids	++	++
4	Steroids	++	++
5	Terpenoids	++	++
6	Alkaloids	-	-
7	Antroquinone	++	++
8	Polyphenol	++	++
9	Glycosides	+	++
10	Coumarins	++	++

(+) Presence, (++) High concentrations and (–) Absences

Table 2: Quantitative analysis of *Azadirachta indica* leaves

S. No	Phytochemicals	Results (mg/gm)
1	Flavonoids	50.00±0.38
2	Total phenols	260.50±1.09

Values are expressed as mean ± SD for triplicates

Herbal medicines, which contain two or more herbal ingredients are often more effective than single drugs because of their complementary and/ or potentiating activities. The combination of two or more herbal extracts brings about increased therapeutic efficiency, enhanced pharmacological actions, faster relief, and reduced adverse effects as compared to conventional medicine due to a lower dose of administration (Nachimuthu *et al.,* 2021). The concept of herbal combination has been well established and has achieved remarkable success in allopathic medicine, providing patients with new hope. The therapeutic use of non-toxic conventional medicinal plants is gaining increasing attention as an alternative and cost-effective approach for the prevention and treatment of various diseases (Chusri *et al.,* 2017). According to the World Health Organization, about 60–80% of the population in developing countries relies primarily on plants for their primary healthcare needs. The rise of herbal medicines has significantly increased foreign trade, and the global herbal medicine market is projected to hit approximately USD 117 billion by 2024 (Yadav *et al.,* 2020).

UV Visible spectral analysis

Spectroscopy have become more effective and reliable tools used for phytochemical analysis. Ultraviolet-visible spectrophotometry (UV-Vis) is related to photon spectroscopy in the UV-visible region. The *Azima tetracantha* leaves was examined under visible UV-Visible spectrum.

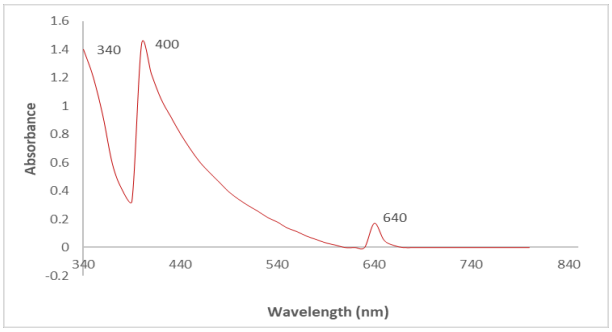


Figure 1: UV visible spectrum analysis *Azima tetracantha* leaves

Table 3: UV visible spectrum analysis *Azima tetracantha* leaves extract

S.no	Absorption maxima (Wavelength ranges) nm	Phytochemical compounds (metabolites)
1.	340	Flavonoids and their derivatives
2.	400	Tannins, Terpenoids
3.	640	Chlorophyll

The peak value of the UV-Visible was recorded. The UV spectrum The UV spectrum profile showed the peaks at 340, 400 and 640 nm and identified phytochemicals are Flavonoids and their derivatives, Tannins, Carotenoids, Terpenoids and chlorophyll respectively (Mabasa *et al*., 2021). Figure 1 shows the absorption spectrum of *Azima tetracantha* leaves extract and these are almost transparent in the wavelength region of 300-800 nm. Based on the UV-visible absorbance spectra, the flavonoid class can be predicted for each chromatographic peak separated.

FT-IR Spectroscopic analysis of *Azima tetracantha* leaves extract

The FTIR spectrum of the *Azima tetracantha* leaves extract was pronounced absorbance was recorded in the region between 4000 and 400 cm^{-1}. Figure 2 noticed the FTIR spectrum of *Azima tetracantha* leaves extract and assigned peak values. In the present study, the peak 3369.62indicates Alcohols, Phenols (O-H stretch, H-bonded), 2975.58 and 2927.61indicates Alkanes (C-H stretch), 2133.53indicates Alkynes (-C≡C- stretch), 1652.04indicates Alkenes (-C=C- stretch), 1453.34indicates Alkanes (C-H bend), 1407.34 indicates Aromatics (C-C stretch (in ring)), 1273.77indicates Aromatic amines (C-N stretch), 1087.47 and 1049.53indicates Aliphatic amines (C-N stretch), 880.81indicates Aromatics (C-H “oop”) and 665.80 indicates 1º, 2º amines.

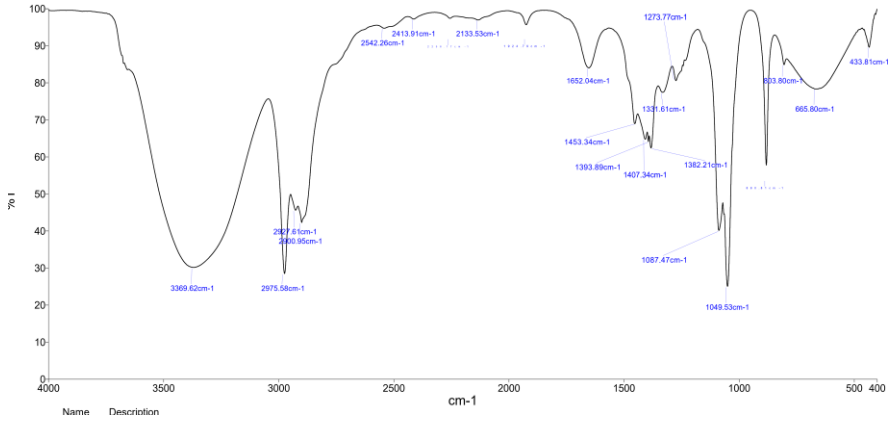


Figure 2: FTIR spectrum of *Azima tetracantha* leaves extract and assigned peak values

FT-IR has been shown to be a valuable tool for differentiating, classifying and discriminating closely related microbial strains, plants and other organisms (Kim *et al*., 2004; Janakiraman *et al*., 2015). It is one of the most widely used methods to identify the chemical constituents and elucidate the structural compounds and has been used as a requisite method to identify medicines in pharmacopoeia of many countries. FTIR spectroscopy is a physicochemical analytical technique that does not

determine concentrations of individual metabolites but provides a snapshot of the metabolic composition of a tissue at a given time (Hori and Sugiyama, 2003). The vibrations of bonds within chemical functional groups and generates a FTIR spectrum that can be regarded as a biochemical or metabolic "fingerprint" of the sample (Griffiths and Haseth, 1986).

HPLC Analysis of *Azima tetracantha* leaves extract

Phytochemical screening of *Azima tetracantha* leaves extract was carried out to check the preliminary phytoconstituents followed by HPLC analysis is performed to determine the flavonoids. HPLC profiles of *Azima tetracantha* leaves extract showed the presence of five compounds namely Gallic acid, Tannic Acid, Epigallocatechin, Resorcinol, Naringenin and Myricetin Malonyl glucoside (isomer) were identified through literature. Table 4 and figure 3 denotes the HPLC chromatogram of *Azima tetracantha* leaves extract.

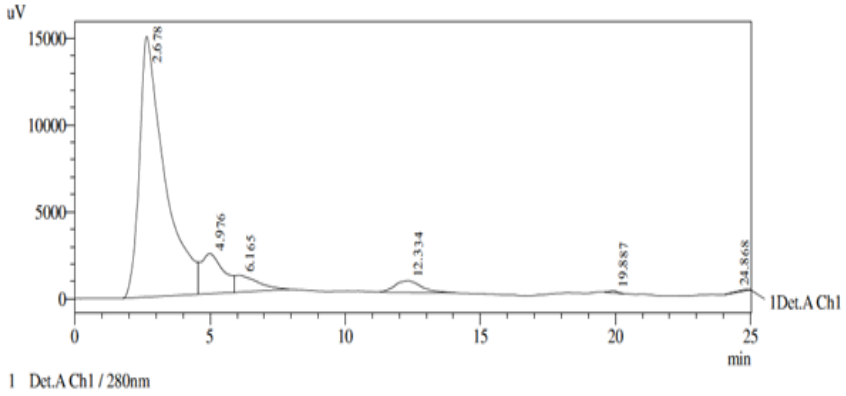


Figure 3: HPLC chromatogram of *Azima tetracantha* leaves extract

Table 4: HPLC chromatogram of *Azima tetracantha* leaves extract

Peak#	Ret. Time	Area %	Height %	Compound name
1	2.678	80.179	78.431	Gallic acid
2	4.976	11.018	12.013	Tannic Acid
3	6.165	4.631	5.003	Epigallocatechin
4	12.334	3.741	3.515	Resorcinol
5	19.887	0.205	0.665	Naringenin
6	24.868	0.226	0.373	Myricetin Malonyl glucoside (isomer)
Total	100.000	100.000		

Paranthaman *et al.* (2012) investigated the GC-MS analysis of phytochemicals and simultaneous determination of flavonoids in *Amaranthus caudatus* (Sirukeerai) by RP-HPLC. Nadia Alam *et al.* (2011) were carried out to characterize the phenolic acids and flavonoids in ethanolic extracts of *Withania somnifera* leaves by HPLC. Five phenolics (gallic, syringic, benzoic, p-coumaric and vanillic acids) and three flavonoids (catechin, kaempferol and naringenin) have been identified in *Withania somnifera* leaves.

***In vitro* anticancer activity**

The *Azima tetracantha* leaf extract was assessed for its anticancer activity on cervical cancer (HeLa) cell line. The anticancer evaluation of *Azima tetracantha* leaf was examined by MTT assay. Different concentrations (12.5, 25, 50, 100 and 200 µg/ml) of *Azima tetracantha* extract were inoculated with HeLa cell line. The test was based on the principle that the dead cell accepts the dye. As the concentration increases there is an increase in the cell growth inhibition but minimum concentration was found to be 4.65 % growth inhibition at 12.5 µg/ml and higher with 63.33% growth inhibition at 200 µg/ml. The IC_{50} value was more than 105.01µg/ml (Table 5 and Figure 4). In addition, many herbs contain a variety of phytosterols, triterpenes, flavonoids, saponins, and carotenoids, which have been shown from studies of legumes, fruit, and vegetables to be cancer chemoprotective (Jaikumar and Jasmine, 2016). Hence, the present study aims to investigate the *Azima tetracantha* leaves for their potential anticancer activity against human breast cancer cell line viz. HeLa. MTT assay is a well-established in vitro method for cytotoxicity against cancer cell lines and hence it was utilized to determine the selective activity of the methanol extract. MTT assay measures the reduction of yellow MTT by mitochondrial succinate dehydrogenase. The production of the resultant formazan appears to be proportional to the level of energy metabolism in the cells. Therefore, it is possible to measure the metabolically activated cells, even in the absence of cell proliferation. The amount of formazan produced is proportional to the amount of MTT in the incubation medium. This method was used to determine the anticancer effect of *Azima tetracantha* leaves on HeLa cells.

Table 5: Effect of different concentration of *Azima tetracantha* leaves on viability and cytotoxicity against HeLa cells cell line as determined by MTT assay

Activity	MCF7						
	Cell control	12.5 (µg/mL)	25 (µg/mL)	50 (µg/mL)	100 (µg/mL)	200 (µg/mL)	Standard (Doxorubicin)
Viability (%)	100	95.35	82.86	68.48	48.75	36.67	20.87
Cytotoxicity (%)	0	4.65	17.14	31.52	51.25	63.33	79.13
LC_{50} Value (µg/mL)	133.296						

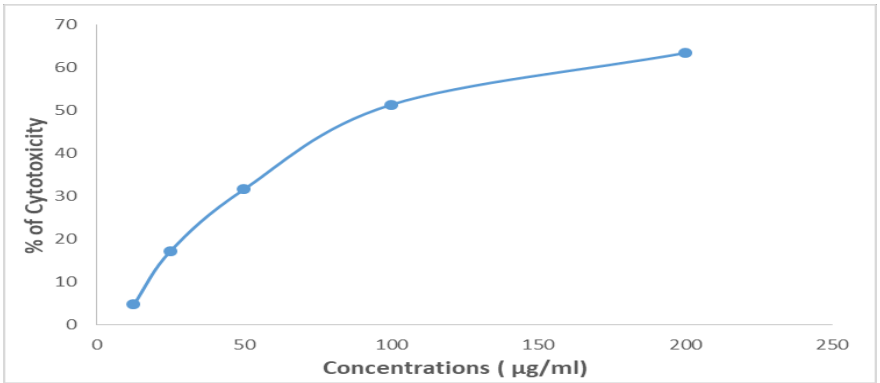


Figure 4: Effect of different concentration of *Azima tetracantha* leaves extract on cytotoxicity (Cell growth inhibition) against HeLa cells cell line

Morphological examination

Figure 5 shows the morphological changes that occurred HeLa cells upon treatment with different concentrations of plant extracts. Increasing the plant extracts concentration (upto200µg/mL) resulted in a drastic change in the morphological characteristics of the tested cell line, which was proportional to the applied concentration. Cells started to shrink and lose their capacity to adhere to the surface of the cultivation plate. Moreover, at the highest applied concentration, cells appeared rounded and were completely floated in comparison to the control morphology. The anticancer activity by inducing cell toxicity and apoptosis (morphology change) in HeLa cells.

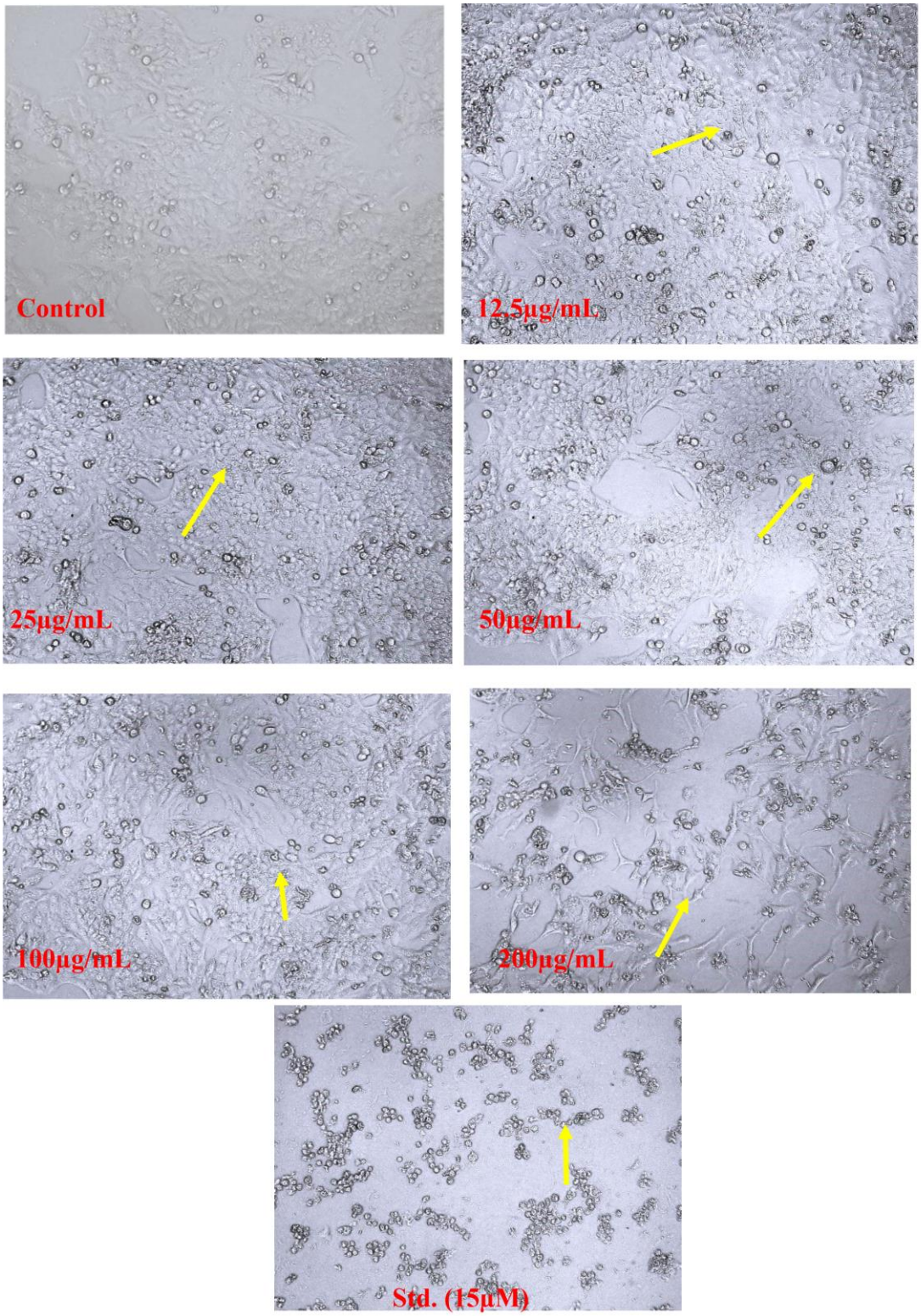


Figure 5: Effect of varying concentrations of *Azima tetracantha* leaves extract on cytotoxicity of HeLa (Cervical cancer) cell line as determined by MTT assay (Arrow mark indicate representative apoptotic cells).

In addition, many herbs contain a variety of phytosterols, triterpenes, flavonoids, saponins, and carotenoids, which have been shown from studies of legumes, fruit, and vegetables to be cancer chemoprotective (Jaikumar and Jasmine, 2016). Hence, the present study aims to investigate the *Azima tetracantha* leaves for their potential anticancer activity against human breast cancer cell line viz. HeLa. The most identifiable morphological features of apoptosis were observed by inverted light microscopy in the extract treated cells. The treated cells appeared like cells undergoing apoptosis with prominent features such as detaching from the culture plate, cytoplasmic condensation, cell shrinkage and condensation and aggregation of the nuclear chromatin, and loss of contact with neighbouring cells (Monga *et al*., 2013). However the untreated cells appeared normal and were confluent. Chemoprevention and dietary modification studies are underway to identify promising candidates for reduced cancer risk. It is concluded that the leaves extract of *Azima tetracantha* possess anticancer properties against breast carcinoma HeLa (Cervical cancer) cell line.

***In silico* study**

Cervical cancer is one of the most occurring diseases in females throughout the World. Cancer is mainly caused due to uncontrolled growth of the cell forming tumor (Waggoner, 2003). The lower negative docking score represented a high binding affinity between the receptor and ligand molecules, showing the higher efficiency of compound. The docked ligand molecules were selected based on docking energy and good interaction with the active site residues and the results are shown in Table 6. The Plate 1 to 3 represent the docking of Gallic acid (-6.60 kcal/mol) and Doxorubicin (Std.) (-9.00 kcal/mol). The binding interactions of all compounds have shown hydrogen bonding and hydrophobic interactions with the target protein. The docking studies confirmed the anticancer activity of Gallic acid and thereby inhibition of target protein as DNMT1 through the binding interactions.

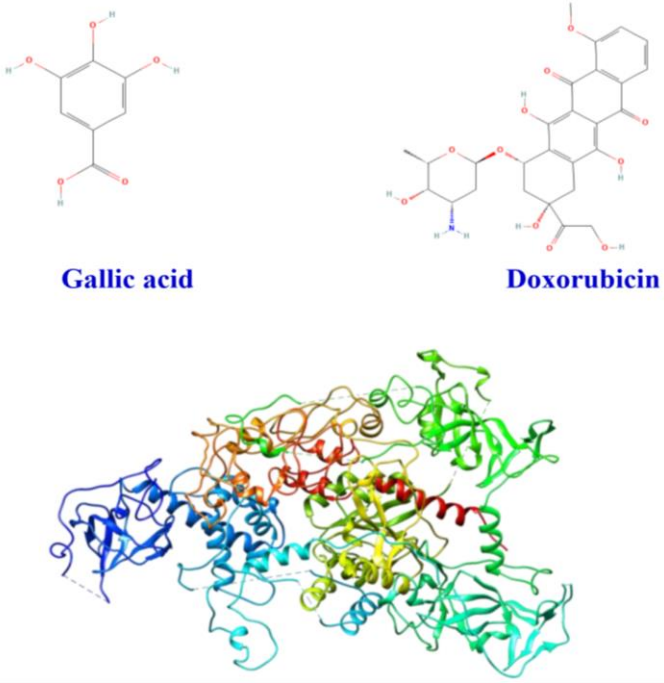


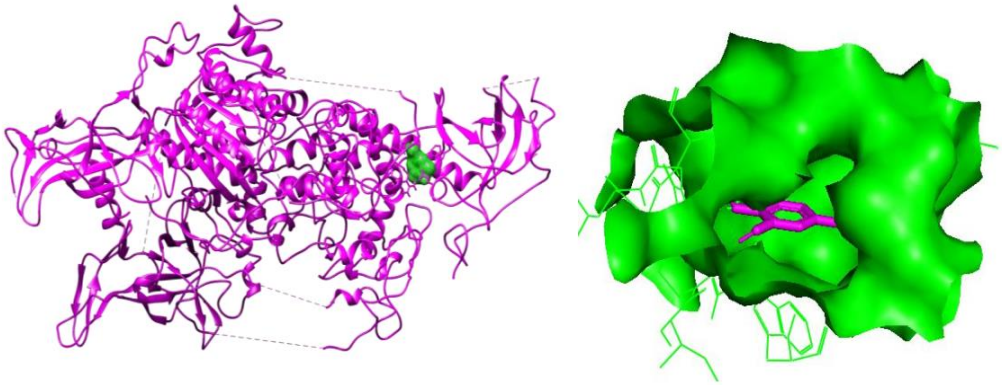
Plate 1: 2D view of ligand and 3D view of target protein

Table 6: Molecular docking results of Gallic acid against targets of cervical cancer (DNMT1)

Ligand (CID)	Molecular formula	M. weight (g/mol)	H-bond donors / acceptors	Binding Affinity (kcal/mol)	Ligand binding site of target (Protein ID: 4WXX) Amino acids
Gallic acid	$C_7H_6O_5$	170.12	4/5	-6.60	Gln 560, Ser 563, Gln 598, Arg 596, Ala 597, Trp 464, Ile 422, Ile 427, Gly 425, Thr 424, Asp 423, Glu 559, Glu 428, Phe 556, Arg 595.
*Doxorubicin	$C_{27}H_{29}NO_{11}$	543.50	6/12	-9.00	Asn 519, Asp 521, Ser 522, Asp 526, Thr 523, Thr 1528, Thr 1525, Thr 1526, Gln 1536, Gly 1223, Glu 1266, Val 1580, Arg 1310, Arg 1312, Asn 1267, Cys 1226, Asn 1578, Val 1268, Arg 1574, Gln 1227, Pro 1225.

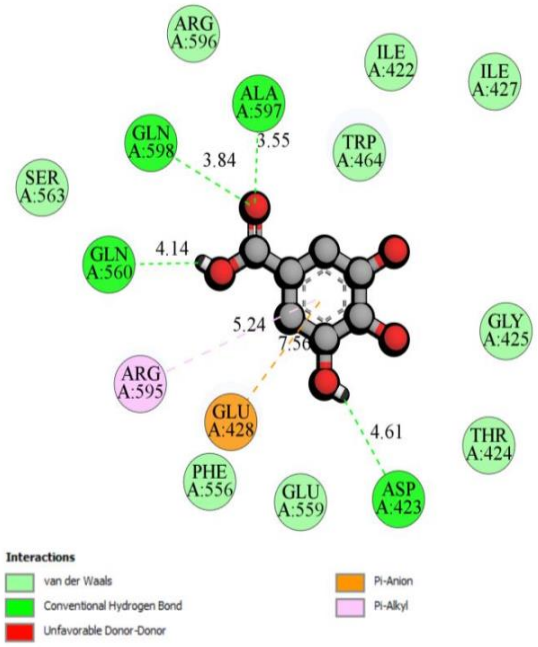
Many genetic and epigenetic alterations are responsible for cervical tumorigenesis. Among those alterations, aberrant promoter methylation of tumor-suppressor genes gives rise to its silencing functions a nd results in the significant carcinogenesis of cervical cancer (Jemal *et al.,* 2007). Currently, many types of cervical cancer-related repressor genes are known including CCNA 1, CHF, HIT, PAX1, PTEN, SFRP4, and TSC1. The genes are involved in causing cervical cancer, and performs wide variety of functions to regulate the transcription and expression, with promoter hypermethylation, which leads to the precursor lesions in cervical development and malignant transformation (Eddy, 1990). DNA methylation is mainly catalyzed by DNA methyltransferases (DNMT1). It has been reported that DNMT1 is responsible for causing cervical cancer, DNMT1 inhibits the transcription of tumor suppressor genes and facilitated the formation of tumorigenesis which finally develops into cervical cancer. The inhibition activity for DNMT1 could reduce hypermethylation of repressive genes and promote its expression, and reverse phenotype of a malignant tumor. Thus, specific inhibition of DNMT1 could be one strategy for cervical cancer therapy (Rhee *et al.,* 2002).

Traditional drugs were time and resource consuming, but now in this technical era, bioinformatics has played an important role in research to save both time and resources. Molecular docking is a technique used to screen the drugs on the basis of structure based drug designing. The interaction of the small molecules with the target protein is analyzed in docking. Structural Based Drug Designing uses molecular docking method which addresses the ligand binding sites with a protein of known three-dimensional structure. One of the computational approaches, docking, helps with screening a large set of compounds based on their free binding energies and proposes structural hypotheses of how the molecules could inhibit the target (Morris, 2008). The present study molecular docking of Gallic acid against human DNMT1 (PDB: 4WXX) protein inhibition was evaluation.



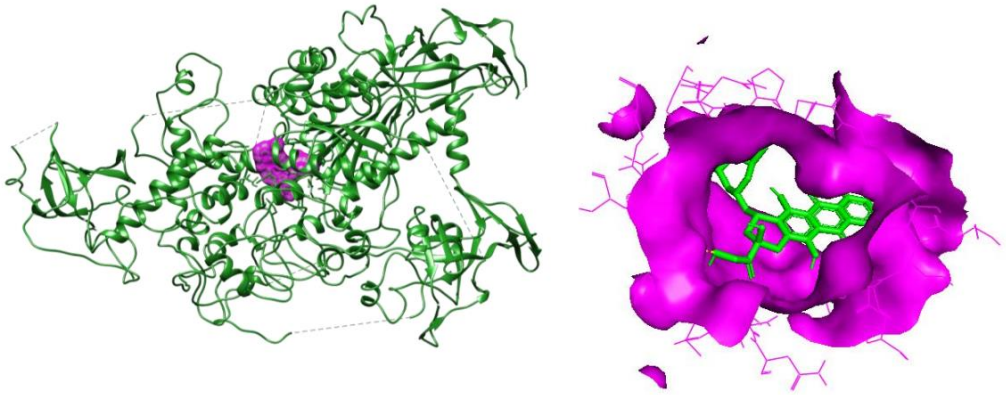
Docked complex **Ligand binding site**

3D surface view of Gallic acid against targets of cervical cancer (DNMT1; Protein ID: 4WXX)



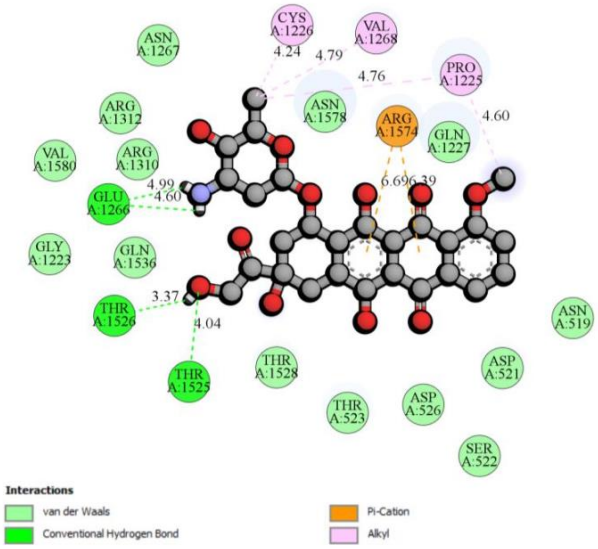
2D view of Gallic acid interaction with targets of cervical cancer (DNMT1; Protein ID: 4WXX) amino acid residues

Plate 2: Molecular docking of Gallic acid against targets of cervical cancer (DNMT1; Protein ID: 4WXX)



Docked complex **Ligand binding site**

3D surface view of Doxorubicin against targets of cervical cancer (DNMT1; Protein ID: 4WXX)



2D view of Doxorubicin interaction with targets of cervical cancer (DNMT1; Protein ID: 4WXX) amino acid residues

Plate 3: Molecular docking of Doxorubicin against targets of cervical cancer (DNMT1; Protein ID: 4WXX)

CONCLUSION

Present study concluded that *Azima tetracantha* leaves contain rich source of phytochemicals confirmed by qualitative and quantitative phytochemicals, UV spectrum profile, FTIR and HPLC analysis. The leaves extract of *Azima tetracantha* possess anticancer properties against breast carcinoma HeLa (Cervical cancer) cell line. *In silico* molecular docking carried out with the ligands as Gallic acid docked with protein human DNMT1 (PDB: 4WXX) further supported the in vivo anticancer activity. Thus from the docking scores it can be inferred that the compounds binds successfully with the drug targets and the compounds may be used as anticancer drugs. The evidence that *Azima tetracantha* leaves extract have an interesting anticancer activity, which could be due to the presence of phenolic and flavonoids compounds.The study resolved that anticancer activity of *Azima tetracantha* leaves confirmed by *in vitro* and *in silico* methods.

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