JOURNAL OF NATURAL REMEDIES
DOI: 10.18311/jnr/2023/30525
RESEARCH ARTICLE

Assessment of Cytotoxic Effects of Latex from *Cascabela thevetia* (L.) Lippold and *Plumeria alba* L. via *In vitro* and *In silico* Approaches

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Abstract

Plant latex has been found to occur in more than 40 families and among them, Apocynaceae is one. Two plants of this family i.e., *Cascabela thevetia* (L.) Lippold and *Plumeria alba* L. had been chosen for the current experimental work. The aqueous and methanolic latex extracts were evaluated for their phytochemical constituents and cytotoxic activities. To determine the cytotoxic effects of the latex extracts, MTT [3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide] assay was performed by using cell lines A549 Human Lung Cancer and MCF-7 Human Breast Cancer. Further, for confirmation of the cytotoxic effects apoptosis assay was conducted. The richness of the latex extracts was determined by GC-MS and HPTLC methods. The preliminary phytochemical analysis of the latex extracts was conducted using GC-MS methodology. The presence of cardiac glycosides was analyzed using High-Performance Thin Layer Chromatography. Here, Digoxin was used as the standard. Studies have revealed that Na^+ K^+-ATPase can serve as a powerful target for developing anticancer drugs and cardiac glycosides have exhibited anticancer effects via inhibition of the Na^+ K^+-ATPase. Hence, molecular docking studies were conducted in which 6KPX served as the target and the compounds evaluated by the NIST library in GC-MS served as the ligands. Further, Molecular docking studies confirmed the best among the compounds based on their RF score, binding affinity, and as a binder whether these compounds were good or bad. These methodologies altogether helped in evaluating the richness of the plant latex extracts, and the potent cytotoxic molecules present in them.

Keywords: Apoptosis, GC-MS, HPTLC, Molecular Docking, MTT Assay

1. Introduction

Latex is a fluid that is produced by specialized cells known as laticifers. Latex often oozes out upon any mechanical disturbance caused to the plant[1–3]. These are mainly rich in plant defense proteins, lipids, enzymes, and secondary metabolites[4–6]. The presence of these bioactive components makes latex a great resource of pharmacological properties. There are more than 40 families in which latex is present like Apocynaceae, Asteraceae, Caricaceae, Euphorbiaceae, Moraceae, Papaveraceae etc[7,8].

In the plant kingdom, Apocynaceae is considered one of the largest families. According to the recently revised and updated classification, the family consists of 5 subfamilies- Apocynoideae, Asclepiadoideae, Periplocoideae, Rauvolfioideae, and Secamonoideae, including 424 genera and more than 4,600 species[9].

Article Received on: 27.09.2022 Revised on: 29.09.2022 Accepted on: 03.01.2023

The plants of the family are native throughout the countries like India, Bangladesh, China, Pakistan, and Sri Lanka[10]. The members of the plant family are herbaceous, shrubs, or woody plants containing milky latex. Most of the plants are ornamental and some of them are poisonous in nature[11]. In the Indian, Chinese, and Thai traditional systems of medicine, Apocynaceae plants have their place. They are also known worldwide for their medicinal and pharmacological properties[12]. In this paper two plants of this family have been utilized for the experimental purpose they are namely *Cascabela thevetia* (L.) Lippold and *Plumeria alba* L.

Cascabela thevetia (L.) Lippold (syn: *Thevetia peruviana*) is a small, ornamental, and poisonous plant. The plant is also known by other names like- yellow oleander, Mexican oleander, still tree, Currant Tree, Captain-Cook tree etc[13,14]. The plant is native to Mexico and Central America[22,16]. All the parts of the plant are cardiotoxic. Many deaths have been reported because of the intentional or accidental consumption of plant parts[22]. Throughout the plant milky latex is present[17].

Plumeria alba L. is a shrub or small tree with a height of around 4.5 m. The plant is grown occasionally in the gardens for its ornamental and fragrant flowers. The plant is greatly known for its laticiferous ducts. The leaves are lanceolate to oblanceolate. *Plumeria alba* L. is widely known for its antimicrobial, cardiotonic, diuretic, hypotensive, and purgative properties. The fruit of the plant is edible and its seeds possess hemostatic properties. The latex of the plant is used for the treatment of herpes, scabies, and ulcers[18].

To date, most research on *Cascabela thevetia* (L.) Lippold and *Plumeria alba* L. have focused on the pharmacological properties derived from the different parts of these plants. Very fewer data has been published regarding the pharmacological properties of the latex of these two plants. In this study, a focus has been made to evaluate the cytotoxic properties of latex and the possible phytochemical constituents responsible for the property. The evaluation has been carried out using *in vitro* and *in silico* methods respectively.

2. Materials and Methods

2.1 Latex Collection and Processing

The latex of the plants selected for the present study was collected from the vicinity of Gujarat University, Ahmedabad, Gujarat. The plants were authenticated by Prof. (Dr.) Hitesh Solanki, of Gujarat University. The tender branches were used for extracting fresh latex. New and completely sterile Eppendorf tubes were used for collecting the latex. The standard protocol was followed along with some modifications for preparing the respective latex extracts[19,20]. Here in the present experiment, two solvents had been used i.e., water and methanol. Therefore, in total four samples were prepared. These four samples were considered as the stock and were immediately stored at the temperature of 0˚C to -18˚C for further use.

2.2 Phytochemical Analysis by GC-MS Method

To identify the bioactive compounds that are present in the aqueous and methanolic latex extracts of *Cascabela thevetia* (L.) Lippold and *Plumeria alba* L. the extracts were subjected to GC-MS analysis (Agilent Technologies 7000 GC/MS Triple Quad). Helium was used as the carrier gas with a constant flow rate of 1mL/min. 2 µl of the sample (latex extract) was injected. The column temperature was programmed to 70˚C with an increasing temperature of 10˚C/min to 250˚C/min. The mass spectra were found through ionization energy of 70 Ev using the Electron Impact Ionization (EI) mode. Total GC-MS running time was 40 min. The bioactive compounds were identified by comparison of mass spectra with the inbuilt NIST library.

2.3 HPTLC Analysis

TLC is a chromatographic technique used for separating mixtures and for confirming the presence of bioactive compounds in a particular extract. In the present study, the solvent system that was finalized for carrying out TLC analysis was Ethyl Acetate: Toluene: Methanol in the ratio of 5:3:2 v/v. This finalization was done after carrying out many experiments using different combinations of the solvent systems. Standard drug Digoxin (CAS Number 20830-75-5) extra pure was purchased from SRL (Sisco Research Laboratory), India. The authentic standard was in powder form and light yellow in color with empirical formula- C41H64O14and molecular weight 780.94. The drug was available in a 1gm pack with Product Number 48712. This was stored at 8 to 25˚C. The standard was prepared at a concentration of 1mg/mL and all the final concentrations of plant latex samples were 5mg/mL.

The High-Performance Thin-Layer Chromatography (HPTLC) was conducted using the Camag HPTLC system. The sample applicator was Linomat V. The samples were

spotted in form of bands with a length of 8.0 mm on precoated silica gel aluminum plates 60 F 254 TLC Silica gel (Merck) of 10 cm x 10 cm size. The distance between the two tracks was 17.0 mm. An application volume of 5 µl of the standard and 10 µl of the extracts was applied. The chromatogram was developed in the development chamber consisting of a Camag twin-trough plate. The mobile phase Ethyl Acetate: Toluene: Methanol (5:3:2) was saturated for 30 min in the developing chamber at room temperature. The developed chromatogram was scanned using the Camag TLC scanner. The results (peaks) were derived using the vision CATS software. The developed plates were visualized in a UV cabinet visualizer under UV light at 254 nm and 366 nm. Images were captured respectively. The standard drug Digoxin and 4 samples were loaded simultaneously on the same plate as five tracks.

2.4 *In silico* Analysis

On studying the research and review papers it was found that the Na^+/K^+-ATPase is useful and has been considered a target in many pharmacological applications. It has also been observed that Na^+/K^+-ATPase, has been the target for chemotherapy as well[21]. Therefore, as a target 6KPX (crystal structures of Na^+/K^+-ATPase in complex with digitoxin) was retrieved from the RCSB PDB. For conducting molecular docking, the first step was the selection of the protein. Following the selection of the protein target, ligands were retrieved from the PubChem database in the .sdf format. Here, the ligands were chosen based on the compounds that were identified by the NIST library in the above mentioned GC-MS procedure.

105 ligands that were in .sdf format were converted into .pdb and further into .pdbqt format using the Open Babel software. After this .pdbqt output files were generated. Using the Open Babel software and the .pdbqt output files, 9 conformations were generated for each of the 105 ligands.

In AutoDock tools, the protein, the ligand (.pdbqt), and the 9 conformations of selected ligands were loaded to find the best confirmation coming at the binding site.

2.5 MTT Analysis

The cell lines were seeded in 96-well plates at 1×10^4 cells/well in a 100 µl culture medium and different concentrations of latex extracts i.e., 1, 10, and 100(µg/mL) were added to the microplates. These were further incubated overnight at 37˚C along with 5% of CO_2 in a humified environment (incubator). The supernatants were removed and the cell layers were washed with Phosphate-Buffered Saline (PBS) solution. To this 10 µl of MTT was added and incubated for 4 hours at 37˚C in a humified condition (for converting MTT into insoluble formazan crystals). After this, to each well, 100 µl of DMSO was added and further incubated overnight (for solubilizing formazan crystals). The absorbance of the processed MTT product was measured at 570 nm using a microplate reader (ELISA). The experiment was conducted in triplicates. Here, the untreated cells were considered as control.

For obtaining IC_{50} the following calculations were done

(a) Cell viability (%) = [(OD of treated cells-OD of blank cells)/ (OD of control-OD of blank cells)] × 100.

(b) Cytotoxicity (%) = 100 – Cell viability (%).

An online tool has been used for calculating the IC_{50} value and for plotting the graphs[22].

The formula used here is

$$Y = \text{Min} + \frac{\text{Max} - \text{Min}}{1 + (X/\ IC_{50})^{\text{Hill coefficient}}}$$

A Sigmoid graph was obtained for all the samples that had been examined for the presence of anticancer activity using the MTT assay.

2.6 Apoptosis Analysis

The apoptosis rate of cells was measured using the user's guide of Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich). A549 and MCF-7 cells (1×10^6) were treated with different concentrations of the aqueous and methanolic latex extracts of *Cascabela thevetia* (L.) Lippold and *Plumeria alba* L. for 48 hours. The cells were resuspended in the binding buffer. To this 5 µL, Annexin-V-FITC and 5 µL of Propidium Iodide were added for staining. After 10 min of incubation at room temperature and in the dark, the A549 and MCF-7 cells were analyzed using a Guava®Muse®cell analyzer. The results were obtained in the form of dot plots.

3. Results and Discussion

3.1 Phytochemical Analysis by GC-MS Method

In most cases, Gas Chromatography (GC) is coupled with mass spectrometry (MS). GC-MS plays a vital role in analyzing the unknown and known components of plant

origin. GC-MS ionizes compounds and further measures their mass numbers. Here, in the present experiment GC-MS revealed the active constituents present in the aqueous and methanolic latex extracts of *Cascabela thevetia* (L.) Lippold and *Plumeria alba* L. Presence of the compounds was confirmed using the NIST data library. The spectrum profile of the aqueous and methanolic latex extract of *Cascabela thevetia* (L.) Lippold has been presented in Tables 1 and 2 and the spectrum profile of the aqueous and methanolic latex extract of *Plumeria alba* L. has been presented in Tables 3 and 4. The tentatively identified compounds with their retention time have been presented in the tables. The chromatogram has been represented in Figure 1.

Table 1. Phytocomponents identified in *Cascabelathevetia*(L.) Lippoldaqueous latex extract

S.No	Plant sample Highest Peak	m/z	RT (min)	Hit	Name of The Compound (CTA)	Molecular Weight	Molecular Formula
1	44 999	44.8	1.773	Hit-1	Methylsilane	46	CH_6Si
2				Hit-2	2,3-Epoxybutane	72	C_4H_8O
3				Hit-3	Oxirane, 2,3-dimethyl-, cis-	72	C_4H_8O
4				Hit-4	Oxirane, 2,3-dimethyl-, trans-	72	C_4H_8O
5				Hit-5	2-Isopropoxyethylamine	103	$C_5H_{13}NO$
6	46 999	46.4	2.282	Hit-1	Methanethiol	48	CH_4S
7				Hit-2	Phosphine, methyl-	48	CH_5P
8				Hit-3	Ethane, fluoro-	48	C_2H_5F
9				Hit-4	Methyl hydrogen disulfide	80	CH_4S_2
10				Hit-5	Tetraborane (10)	54	B_4H_{10}
11	84 999	83.5	2.550	Hit-1	Krypton	84	Kr
12				Hit-2	3-Piperidinol, 1-acetyl-6-propyl-, (3R-trans)-	185	$C_{10}H_{19}NO_2$
13				Hit-3	Piperidine, 1,1-dithiobis-	232	$C_{10}H_{20}N_2S_2$
14				Hit-4	1-Silacyclo-3-pentene	84	C_4H_8Si
15	77 999	77.2	3.672	Hit-1	Arsine	78	AsH_3
16				Hit-2	Silanamine, N-phenyl-	123	C_6H_9NSi
17				Hit-3	Ethanimidamide, 2-chloro-N-(1,2-dichloroethenyl)-	186	$C_4H_5Cl_3N_2$
18				Hit-4	1-Propanesulfonyl chloride, 3-chloro-	176	$C_3H_6Cl_2O_2S$
19				Hit-5	1-Ethanol, 2-(ethylsulfinyl)-	122	$C_4H_{10}O_2S$
20	44 999	44.1	26.967	Hit-1	3-Methoxy-2,4,5-trifluorobenzoic acid, eicosyl ester	486	$C_{28}H_{45}F_3O_3$
21				Hit-2	3-Methoxy-2,4,5-trifluorobenzoic acid, nonadecyl ester	472	$C_{27}H_{43}F_3O_3$
22				Hit-3	3-Methoxy-2,4,5-trifluorobenzoic acid, octadecyl ester	458	$C_{26}H_{41}F_3O_3$
23				Hit-4	3-Methoxy-2,4,5-trifluorobenzoic acid, heptadecyl ester	444	$C_{25}H_{39}F_3O_3$
24				Hit-5	3-Methoxy-2,4,5-trifluorobenzoic acid, hexadecyl ester	430	$C_{24}H_{37}F_3O_3$
25	207 999	206.7	27.415	Hit-1	1-(4-Methoxyphenyl)imidazoline-2-thione	206	$C_{10}H_{10}N_2OS$
26				Hit-2	Ursan-16-one, 3-hydroxy-, (3.beta.,18.alpha.,19. alpha.,20.beta.)-	442	$C_{30}H_{50}O_2$
27				Hit-3	6-Quinolinamine, 4-chloro-N,N-dimethyl-	206	$C_{11}H_{11}ClN_2$
28				Hit-4	alpha-Tetralol, 2-amino-5,6-dimethoxy-	223	$C_{12}H_{17}NO_3$

Table 1. (Continued)

S.No	Plant sample Highest Peak	m/z	RT (min)	Hit	Name of The Compound (CTA)	Molecular Weight	Molecular Formula
29	207 999	207.2	28.701	Hit-1	1-(2-Acetoxyethyl)-3,6-diazahomoadamantan-9-one oxime	267	$C_{13}H_{21}N_3O_3$
30				Hit-2	Ursan-16-one, 3-hydroxy-, (3.beta.,18. alpha.,19. alpha.,20.beta.)-	442	$C_{30}H_{50}O_2$
31				Hit-3	8-Carbethoxy-1-methyl-1,4,5,6,7,8-hexahydropyrrolo[2,3-b] azepin-4-one-3-carboxylic acid	280	$C_{13}H_{16}N_2O_5$
32				Hit-4	1-(4-Methoxyphenyl)imidazoline-2-thione	206	$C_{10}H_{10}N_2OS$
33				Hit-5	(+)-1,2,3,4-Tetrahydroisoquinoline, 6,7-dimethoxy-1-phenmethanol-2-methyl-	313	$C_{19}H_{23}NO_3$
34	207 999	207.3	29.456	Hit-1	4,6-Difluoro-2-phenylaminopyrimidine	207	$C_{10}H_7F_2N_3$
35				Hit-2	7-[2-(Ethoxycarbonyl)-3alpha,5beta-dimethoxycyclopentyl-1]-heptanoic acid, ethyl ester	358	$C_{19}H_{34}O_6$
36				Hit-3	8H-Pyrano[3,4-b]pyrimido[5,4-d]furane, 5,6-dihydro-4-hydrazino-6,6-dimethyl-2-methylthio-	280	$C_{12}H_{16}N_4O_2S$
37				Hit-4	2-(6,7-Dimethoxy-1-methyl-1,2,3,4-tetrahydroisoquinolyl)propan-2-ol	265	$C_{15}H_{23}NO_3$
38				Hit-5	Calycotomine, N-methyl-	237	$C_{13}H_{19}NO_3$
39	207 999	207.3	34.299	Hit-1	Calycotomine, N-methyl-	237	$C_{13}H_{19}NO_3$
40				Hit-2	(+)-1,2,3,4-Tetrahydroisoquinoline, 6,7-dimethoxy-1-phenmethanol-2-methyl-	313	$C_{19}H_{23}NO_3$
41				Hit-3	Carnegine	221	$C_{13}H_{19}NO_2$
42				Hit-4	(6,7-dimethoxy-2-oxo-3,4-dihydro-1H-quinolin-4-yl) acetic acid	265	$C_{13}H_{15}NO_5$
43				Hit-5	Quinoline-4-carboxylic acid, 6,7-dimethoxy-2-oxo-1,2,3,4-tetrahydro-	251	$C_{12}H_{13}NO_5$
44	207 999	207.3	34.678	Hit-1	1,3-Dihydro-5-(3-nitrophenyl)-2H-1,4-benzodiazepin-2-one	281	$C_{15}H_{11}N_3O_3$
45				Hit-2	2-p-Tolyl-2,3-dihydro-1H-benzo[1,3,2]diazaborole	208	$C_{13}H_{13}BN_2$
46				Hit-3	Nitrazepam	281	$C_{15}H_{11}N_3O_3$
47				Hit-4	1H-Indole, 5-methyl-2-phenyl-	207	$C_{15}H_{13}N$
48				Hit-5	6-Quinolinamine, 4-chloro-N,N-dimethyl-	206	$C_{11}H_{11}ClN_2$
49	207 999	206.9	36.474	Hit-1	8H-Pyrano[3,4-b]pyrimido[5,4-d]furane, 5,6-dihydro-4-hydrazino-6,6-dimethyl-2-methylthio-	280	$C_{12}H_{16}N_4O_2S$
50				Hit-2	Carnegine	221	$C_{13}H_{19}NO_2$
51				Hit-3	2-(6,7-Dimethoxy-1-methyl-1,2,3,4-tetrahydroisoquinolyl)propan-2-ol	265	$C_{15}H_{23}NO_3$
52				Hit-4	4-Acetyloxyimino-6,6-dimethyl-3-methylsulfanyl-4,5,6,7-tetrahydro-benzo[c]thiophene-1-carboxylic acid methyl ester	341	$C_{15}H_{19}NO_4S_2$
53				Hit-5	Calycotomine, N-methyl-	237	$C_{13}H_{19}NO_3$

(*Continued*)

Table 1. (Continued)

S.No	Plant sample Highest Peak	m/z	RT (min)	Hit	Name of The Compound (CTA)	Molecular Weight	Molecular Formula
54	207 999	207.4	37.343	Hit-1	1H-Pyrrolo[3,4-c]quinoline-1,3,4(2H,5H)-trione, 6,7,8,9-tetrahydro-	218	$C_{11}H_{10}N_2O_3$
55				Hit-2	2-(4-Ethoxyphenyl)-1,3-thiazolidine-4-carboxylic acid	253	$C_{12}H_{15}NO_3S$
56				Hit-3	alpha-Tetralol, 2-amino-5,6-dimethoxy-	223	$C_{12}H_{17}NO_3$
57				Hit-4	Carnegine	221	$C_{13}H_{19}NO_2$
58	207 999	207.4	37.396	Hit-1	1,10,25,26-Tetraaza-4,7-dioxatetracyclo[8.7.7.1(12,16).1(19,23)]hexacos-12,14,16(25),19,21,23(26)-hexaene	354	$C_{20}H_{26}N_4O_2$
59				Hit-2	2-(4-Ethoxyphenyl)-1,3-thiazolidine-4-carboxylic acid	253	$C_{12}H_{15}NO_3S$
60				Hit-3	8H-Pyrano[3,4-b]pyrimido[5,4-d]furane, 5,6-dihydro-4-hydrazino-6,6-dimethyl-2-methylthio-	280	$C_{12}H_{16}N_4O_2S$
61				Hit-4	Carnegine	221	$C_{13}H_{19}NO_2$
62				Hit-5	alpha-Tetralol, 2-amino-5,6-dimethoxy-	223	$C_{12}H_{17}NO_3$
63	207 999	206.9	38.560	Hit-1	Carnegine	221	$C_{13}H_{19}NO_2$
64				Hit-2	2-(6,7-Dimethoxy-1-methyl-1,2,3,4-tetrahydroisoquinolyl)propan-2-ol	265	$C_{15}H_{23}NO_3$
65				Hit-3	Calycotomine, N-methyl-	237	$C_{13}H_{19}NO_3$
66				Hit-4	(+)-1,2,3,4-Tetrahydroisoquinoline, 6,7-dimethoxy-1-phenmethanol-2-methyl-	313	$C_{19}H_{23}NO_3$
67				Hit-5	2(1H)-Quinolinone, 4-hydroxy-6-methoxy-3-(phenylmethyl)-	281	$C_{17}H_{15}NO_3$

Table 2. Phytocomponents identified in *Cascabelathevetia*(L.) Lippoldmethanolic latex extract

S.No	Plant Sample Highest Peak	m/z	RT (min)	Hit	Name of The Compound (CTM)	Molecular Weight	Molecular Formula
1	44 999	44	1.619	Hit-1	Methylsilane	46	CH_6Si
2				Hit-2	2,3-Epoxybutane	72	C_4H_8O
3				Hit-3	Oxirane, 2,3-dimethyl-, cis-	72	C_4H_8O
4				Hit-4	Oxirane, 2,3-dimethyl-, trans-	72	C_4H_8O
5				Hit-5	2-Isopropoxyethanamine	103	$C_5H_{13}NO$
6	106 999	105.5	16.343	Hit-1	3,6,9-Triazatricyclo[7.3.0.0{2,6}]dodeca-1(12),2,10-triene	161	$C_9H_{11}N_3$
7				Hit-2	Benzenamine, 4-(4H-1,2,4-triazol-4-yl)-	160	$C_8H_8N_4$
8				Hit-3	Tetracyclo [5.3.1.1(2,6).0(4,9)] dodecane, 11-acetoxy-	220	$C_{14}H_{20}O_2$
9				Hit-4	3-(1,2,4-triazol-4-yl) aniline	160	$C_8H_8N_4$
10				Hit-5	s-Triazolo[1,5-a] pyridine, 2,5,7-trimethyl-	161	$C_9H_{11}N_3$

(*Continued*)

Table 2. (Continued)

S.No	Plant Sample Highest Peak	m/z	RT (min)	Hit	Name of The Compound (CTM)	Molecular Weight	Molecular Formula
11	161 999	160.8	16.519	Hit-1	2H-Cyclopenta[c]pyridine-4-carboxamide, 3,5,6,7-tetrahydro-3-oxo-	178	$C_9H_{10}N_2O_2$
12				Hit-2	Nicotinonitrile, 1-butyl-1,4-dihydro-	162	$C_{10}H_{14}N_2$
13				Hit-3	3,6,9-Triazatricyclo[7.3.0.0{2,6}]dodeca-1(13),2,10-triene	161	$C_9H_{11}N_3$
14				Hit-4	N-Phenylpiperidine	161	$C_{11}H_{15}N$
15				Hit-5	3-Pyrazolidinone, 1-phenyl-	162	$C_9H_{10}N_2O$
16	161 999	160.8	17.679	Hit-1	Germacrene D	204	$C_{15}H_{24}$
17				Hit-2	alpha-Cubebene	204	$C_{15}H_{24}$
18				Hit-3	beta-Cubebene	204	$C_{15}H_{24}$
19				Hit-4	alpha-Copaene	204	$C_{15}H_{24}$
20				Hit-5	Ylangene	204	$C_{15}H_{24}$
21	92 999	92.2	17.867	Hit-1	2-(3-Pentyl)pyridine	149	$C_{10}H_{15}N$
22				Hit-2	Bicyclogermacrene	204	$C_{15}H_{24}$
23				Hit-3	Pyridine, 2-(3,3-dimethylbutyl)-	163	$C_{11}H_{17}N$
24				Hit-4	4-Nitro-N-(2,6-xylyl)benzenesulfonamide	306	$C_{14}H_{14}N_2O_4S$
25				Hit-5	Oxalic acid, monoamide, monohydrazide, N-(2,5-dimethylphenyl)-N2-(4-methylbenzylideno)-	309	$C_{18}H_{19}N_3O_2$
26	161 999	160.8	18.154	Hit-1	1-Isopropyl-4,7-dimethyl-1,2,3,5,6,8a-hexahydronaphthalene	204	$C_{15}H_{24}$
27				Hit-2	1-Isopropyl-4,7-dimethyl-1,2,3,4,5,6-hexahydronaphthalene	204	$C_{15}H_{24}$
28				Hit-3	Naphthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, (1S-cis)-	204	$C_{15}H_{24}$
29				Hit-4	gamma-Muurolene	204	$C_{15}H_{24}$
30				Hit-5	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7-methyl-4-methylene-1-(1-methylethyl)-, (1. alpha.,4a. beta.,8a. alpha.)-	204	$C_{15}H_{24}$
31	207 999	206.6	37.530	Hit-1	Carnegine	221	$C_{13}H_{19}NO_2$
32				Hit-2	2-(4-Ethoxyphenyl)-1,3-thiazolidine-4-carboxylic acid	253	$C_{12}H_{15}NO_3S$
33				Hit-3	4-Quinolinol,5,8-dimethoxy-2-methyl-	219	$C_{12}H_{13}NO_3$
34				Hit-4	2-Chloro-N2-(3-cyano-4,5,6,7-tetrahydro-2-benzo[b]thienyl) acetamidine	253	$C_{11}H_{12}ClN_3S$
35				Hit-5	(6,7-dimethoxy-2-oxo-3,4-dihydro-1H-quinolin-4-yl) acetic acid	265	$C_{13}H_{15}NO_5$

Table 3. Phytocomponents identified in *Plumeria alba* L. aqueous latex extract

S.No	Plant Sample Highest Peak	m/z	RT (min)	Hit	Name of The Compound (PAA)	Molecular Weight	Molecular Formula
1	46 999	46.2	2.056	Hit-1	Methyl hydrogen disulfide	80	CH_4S_2
2				Hit-2	Methanethiol	48	CH_4S
3				Hit-3	Phosphine, methyl-	48	CH_5P
4				Hit-4	Tetraborane (10)	54	B_4H_{10}
5				Hit-5	Ethane, fluoro-	48	C_2H_5F
6	77 999	76.8	3.626	Hit-1	Butane, 2-chloro-2,3-dimethyl-	120	$C_6H_{13}Cl$
7				Hit-2	1-Propanesulfonyl chloride, 3-chloro-	176	$C_3H_6Cl_2O_2S$
8				Hit-3	Monopropylcarbonotrithioate	152	$C_4H_8S_3$
9				Hit-4	Arsine	78	AsH_3
10				Hit-5	3-chloropropane-1-sulfonyl chloride	176	$C_3H_6Cl_2O_2S$
11	105 999	105.3	17.764	Hit-1	1-Phenyl-imidazolidin-2,4-dione	176	$C_9H_8N_2O_2$
12				Hit-2	2,4(1H,3H)-QUINAZOLINEDIONE, 1-METHYL-	176	$C_9H_8N_2O_2$
13				Hit-3	2H-Indol-2-one, 1-acetyl-1,3-dihydro-	175	$C_{10}H_9NO_2$
14				Hit-4	Benzoic acid, 2-cyanamino-, methyl ester	176	$C_9H_8N_2O_2$
15	43 999	93.7	26.626	Hit-1	Pyrimidine, 2-amino-4-(4-fluorophenyl)-	189	$C_{10}H_8FN_3$
16				Hit-2	Propionic acid, 3-(m-aminobenzoyl)-2-methyl- (2)	207	$C_{11}H_{13}NO_3$
17				Hit-3	1,2-Dimethyl-5-vinylpyrrole	121	$C_8H_{11}N$
18				Hit-4	Pyrimidine, 2-amino-4-(3-fluorophenyl)-	189	$C_{10}H_8FN_3$
19				Hit-5	Benzenamine, 3-(1,3-dioxolan-2-yl)-	179	$C_{10}H_{13}NO_2$
20	120 999	120.3	26.979	Hit-1	Beyerene	272	$C_{20}H_{32}$
21				Hit-2	1-Benzyl-3-diethylamino-4-piperidinol	262	$C_{16}H_{26}N_2O$
22				Hit-3	1,5,5-Trimethyl-6-(3-methyl-buta-1,3-dienyl)-cyclohexene	190	$C_{14}H_{22}$
23				Hit-4	2,4-Diamino-6-(1,2-dioxo-2-phenylethyl) pteridine	294	$C_{14}H_{10}N_6O_2$
24	207 999	207.1	27.411	Hit-1	6-Quinolinamine, 4-chloro-N,N-dimethyl-	206	$C_{11}H_{11}ClN_2$
25				Hit-2	(Acridin-9-ylamino)-acetic acid	252	$C_{15}H_{12}N_2O_2$
26				Hit-3	Ursan-3-one, 16-hydroxy-, (16.beta.,18.alpha.,19. alpha.,20. beta.)-	442	$C_{30}H_{50}O_2$
27				Hit-4	1-(4-Methoxyphenyl)imidazoline-2-thione	206	$C_{10}H_{10}N_2OS$
28				Hit-5	2-(4-Methylphenyl)-2,3-dihydro-1H-1,3,2-benzodiazaborole	208	$C_{13}H_{13}BN_2$
29	207 999	207	37.297	Hit-1	Pendimethalin	281	$C_{13}H_{19}N_3O_4$
30				Hit-2	2-(4-Ethoxyphenyl)-1,3-thiazolidine-4-carboxylic acid	253	$C_{12}H_{15}NO_3S$
31				Hit-3	Carnegine	221	$C_{13}H_{19}NO_2$
32				Hit-4	4-Acetyloxyimino-6,6-dimethyl-3-methylsulfanyl-4,5,6,7-tetrahydro-benzo[c]thiophene-1-carboxylic acid methyl ester	341	$C_{15}H_{19}NO_4S_2$
33				Hit-5	alpha-Tetralol, 2-amino-5,6-dimethoxy-	223	$C_{12}H_{17}NO_3$

Table 4. Phytocomponents identified in *Plumeria alba* L. methanolic extract

S.No	Plant Sample Highest Peak	m/z	RT (min)	Hit	Name of The Compound (PAM)	Molecular Weight	Molecular Formula
1	46 999	47	2.355	Hit-1	3-Piperidinol, 1-acetyl-6-propyl-, (3R-trans)-	185	$C_{10}H_{19}NO_2$
2				Hit-2	Thiocyanic acid, methyl ester	73	$C_2H_3N_S$
3				Hit-3	Boronic acid, ethyl-, dimethyl ester	102	$C_4H_{11}BO_2$
4				Hit-4	Methyl hydrogen disulfide	80	CH_4S_2
5				Hit-5	4-[2-Dimethylaminopropyl] thiosemicarbazide	176	$C_6H_{16}N_4S$
6	105 999	105.3	16.344	Hit-1	Benzenamine, N,N-dimethyl-4-(1H-1,2,4-triazol-3-ylazo)-	216	$C_{10}H_{12}N_6$
7				Hit-2	3(2H)-Benzofuranone, 2,5-dimethyl-	162	$C_{10}H_{10}O_2$
8				Hit-3	Benzaldehyde, 4-butyl-	162	$C_{11}H_{14}O$
9				Hit-4	Aminorex	162	$C_9H_{10}N_2O$
10				Hit-5	1,2-Benzenedimethanamine, N1-acetyl-N2-(t-butoxycarbonyl)-	278	$C_{15}H_{22}N_2O_3$
11	92 999	92.1	17.079	Hit-1	2,1-Benzisoxazole	119	C_7H_5NO
12				Hit-2	6-Aminopyridine-2-carbonitrile	119	$C_6H_5N_3$
13				Hit-3	1H-Imidazo[4,5-b] pyridine	119	$C_6H_5N_3$
14				Hit-4	2-Aminohexa-2,4-dienedinitrile	119	$C_6H_5N_3$
15				Hit-5	3-Amino-2-cyanopyridine	119	$C_6H_5N_3$
16	161 999	105.5	18.162	Hit-1	N-Phenylpiperidine	161	$C_{11}H_{15}N$
17				Hit-2	2H-Cyclopenta[c]pyridine-4-carboxamide, 3,5,6,7-tetrahydro-3-oxo-	178	$C_9H_{10}N_2O_2$
18				Hit-3	3,6,9-Triazatricyclo [7.3.0.0{2,6}] dodeca-1(12),2,10-triene	161	$C_9H_{11}N_3$
19				Hit-4	2-Phenyl-5,6-dihydro-4H-1,3-oxazine	161	$C_{10}H_{11}NO$
20				Hit-5	Tert-Butyl cyclopropyl methyl sulfoxide	160	$C_8H_{16}OS$

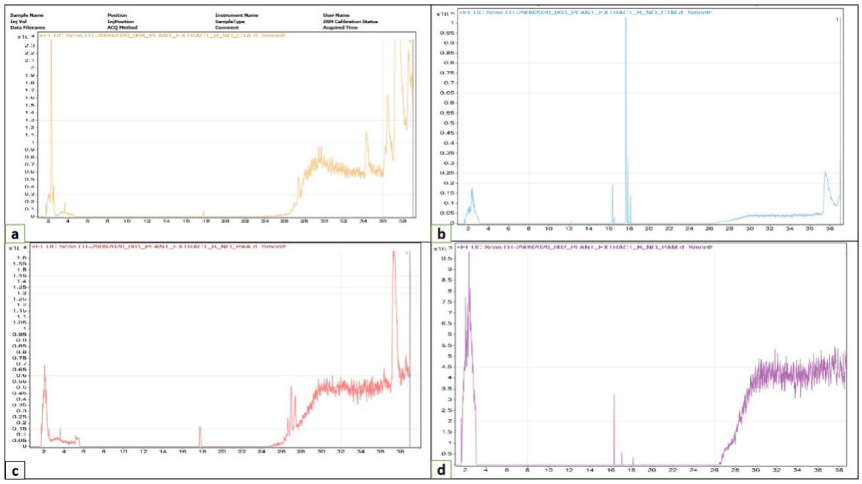

Figure 1. GC-MS Chromatogram of *Cascabelathevetia*(L.) Lippold **(a)** aqueous latex extract and **(b)** methanolic latex extract and *Plumeria alba* L. **(c)** aqueous latex extract and **(d)** methanolic latex extract.

3.2 HPTLC Analysis

The presence of Digoxin was detected by comparing the Rf value of the standard with that of the samples. Figures 2 and 3 represent the HPTLC fingerprinting of the plant latex extracts at 254 and 366 nm. Here, track 1 represents Digoxin track 2 and 3 represents aqueous and methanolic latex extract of *Cascabela thevetia* (L.) Lippold. Track 4 and 5 represent aqueous and methanolic latex extract of *Plumeria alba* L. Scanning densitometry converts the HPTLC chromatogram into a densitogram, in which the spots are observed in the form of peaks[23]. Densitogram calculates the quantity of an analyte as % area that comes amid the start Rf and end Rf[24]. Digoxin started to elute at Rf 0.576, came maximum at 0.644, and ended at 0.681. This result obtained for digoxin was comparable to that of the aqueous latex extract of *Cascabela thevetia* (L.) Lippold where the start Rf was 0.585, the maximum Rf was 0.645 and the end Rf was 0.677.

Both plants have been reported to contain glycosides[25–27]. The bands developed in the present study hence provide support that various secondary metabolites including glycosides have a probable presence in the plant latex of these two plants respectively.

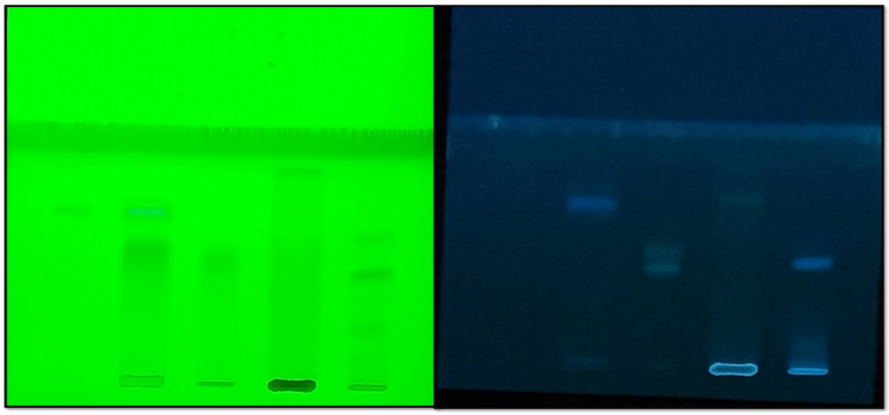

Figure 2. TLC plate exhibiting band formations at 254 nm and 366 nm respectively of the aqueous and methanolic latex extracts of *Cascabe lathevetia* (L.) Lippold and *Plumeria alba* L.

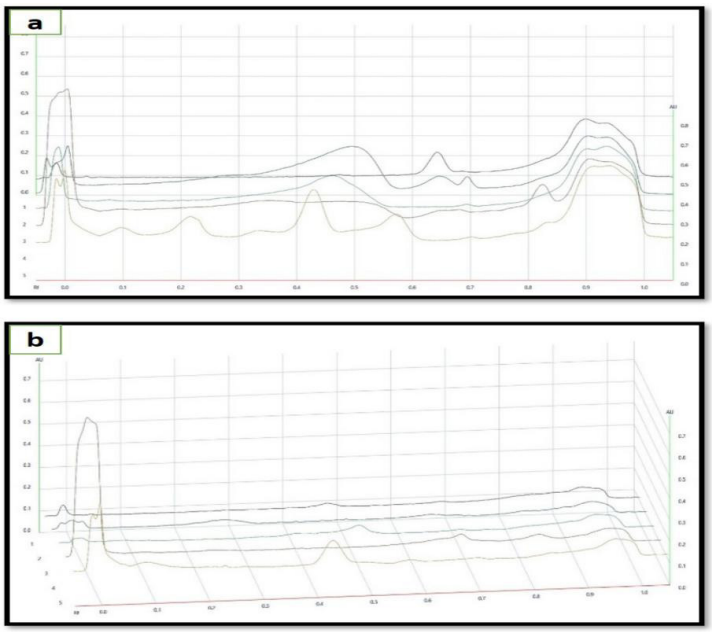

Figure 3. Graph representing HPTLC profiling at **(a)** 254 nm and **(b)** 366 nm of all the 4 latex extracts and digoxin as the standard.

3.3 *In-silico* Analysis

On performing the *In Silico* analysis, the best confirmation coming at the binding site was observed and the RF Score was calculated. Random Forest (RF) is a novel scoring function that predicts protein-ligand binding affinity[28]. RF-Score can be used for virtual screening leading to developmental purposes[29]. With the help of the Protein*Plus* server and the PoseView tool, the 2-D diagrams of the best results of the protein-ligand interaction were generated.

Table 5 represents the name of the compounds, contacting receptor residues, affinity, RF score, and the type of binder.

Figure 4 exhibits the results that were generated using the tool PoseView[30]. This automatically generated the 2D

Table 5. Represents the name of the compounds, contacting receptor residues, affinity, Rf score and the type of binder

S.No.	Name of the Compound	Contacting Receptor Residues	Affinity	RF Score	Binder
1.	1,10,25,26-Tetraaza-4,7-dioxatetracyclo [8.7.7.1(12,16).1(19,23)] hexacos-12,14,16(25),19,21,23(26)-hexene	Thr823, Glu338, Phe935	-7.9	5.967	Good
2.	1-Benzyl-3-diethylamino-4-piperidinol	Thr823, Tyr334, Tyr334	-6.8	5.957	Good
3.	8-Carbethoxy-1-methyl-1,4,5,6,7,8-hexahydropyrrolo[2,3-b]azepin-4-one-3-carboxylic acid	Arg906, Tyr334, Tyr334, Glu338	-7	5.950	Good
4.	Calycotomine, N-methyl-	Tyr334, Leu819, Tyr334	-6.3	5.954	Good
5.	Carnegine	Leu819, Tyr334, Tyr334	-6.4	5.950	Good

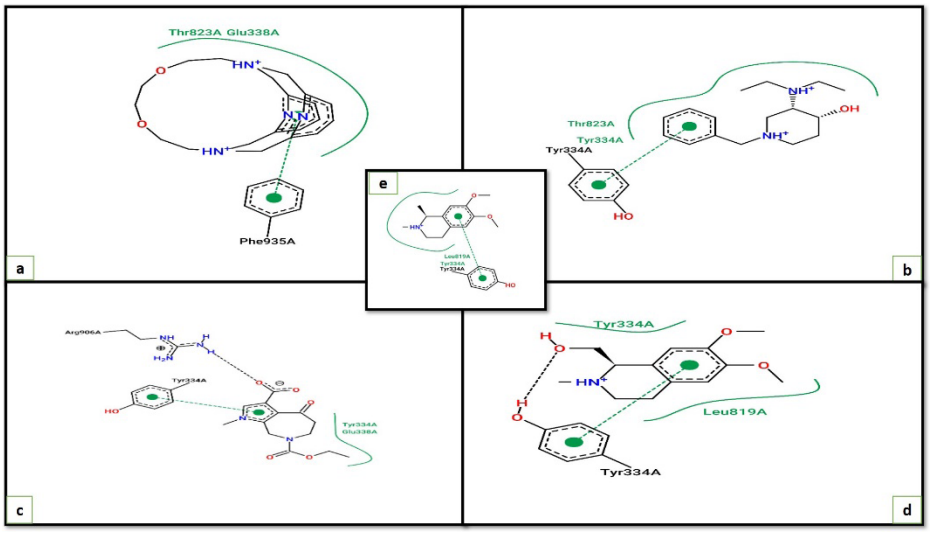

Figure 4. Interaction of ligand **(a)** 1,10,25,26-Tetraaza-4,7-dioxatetracyclo [8.7.7.1(12,16).1(19,23)] hexacos-12,14,16(25),19,21,23(26)-hexene; **(b)** 1-Benzyl-3-diethylamino-4-piperidinol; **(c)** 8-Carbethoxy-1-methyl-1,4,5,6,7,8-hexahydropyrrolo[2,3-b]azepin-4-one-3-carboxylic acid; **(d)** Calycotomine, N-methyl and **(e)** Carnegine with the protein.

diagrams of complexes with known 3D structures. In the complex diagrams of the Figure 4, the hydrogen bonds are represented as dashed lines. Both the interacting amino acids of the receptor and ligand are represented in the structure diagrams. The spline segments represent the hydrophobic contacts between the ligand and the protein.

On performing molecular docking, it was analyzed that 1,10,25,26-Tetraaza-4,7-dioxatetracyclo [8.7.7.1(12,16).1(19,23)] hexacos-12,14,16(25),19,21,23(26)-hexene has been found present in aqueous latex extract of *Cascabela thevetia* (L.) Lippold. 1-Benzyl-3-diethylamino-4-piperidinol has been found present in aqueous latex extract of *Plumeria alba* L. 8-Carbethoxy-1-methyl-1,4,5,6,7,8-hexahydropyrrolo[2,3-b] azepin-4-one-3-carboxylic acid has been found present in aqueous latex extract of *Cascabela thevetia* (L.) Lippold extract. Calycotomine, N-methyl- has been found present in aqueous latex extract of *Cascabela thevetia* (L.) Lippold and Carnegine has been found present in aqueous latex extract of *Cascabela thevetia* (L.) Lippold, methanolic latex extract of *Cascabela thevetia* (L.) Lippold and aqueous latex extract of *Plumeria alba* L. respectively. This docking interaction further supports that Na^+/K^+-ATPase is a potent anticancer target and the leading molecules mentioned above can be used in future studies for developing anticancer drugs.

3.4 MTT Analysis

3.4.1 MTT Assay for A549 Human Lung Cell Carcinoma

The cytotoxic activities were analyzed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. The two cell lines that were chosen for assessing the cytotoxic potential of the latex extracts of *Plumeria alba* L. and *Cascabela thevetia* (L.) Lippold was A549 Human Lung Cancer cell line and MCF-7 Human Breast Cancer cell line.

The cell viability was determined after the time period of 48 hours. Based on concentrations 1, 10, and 100 (µg/mL) and the cell viability percentage the IC_{50} Value was calculated using the software. The graphical representation of concentration versus % cell viability of all four latex extracts has been represented in Figure 5. The IC_{50} values have been summarized in Table 6.

It is clear from Figure 5 and Table 6 that the best result obtained against the A549 Human Lung Cancer cell line was of CTM (methanolic extract of *Cascabela thevetia* (L.) Lippold) with an IC_{50} value of 7.884 µg/mL, followed by CTA (aqueous extract of *Cascabela thevetia* (L.) Lippold) with IC_{50} value of 9.926. The IC_{50} value of PAM (methanolic extract of *Plumeria alba* L.) was 10.059 µg/mL. Among all the extracts the highest IC_{50} value was exhibited by PAA (aqueous extract of *Plumeria alba* L.)

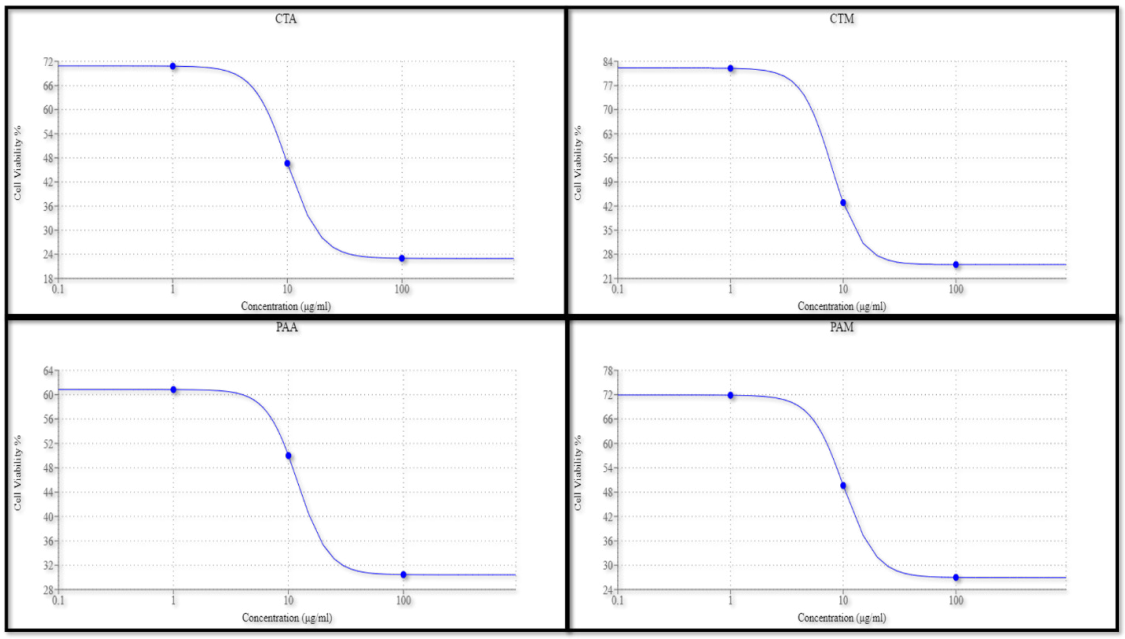

Figure 5. Graphical representation of concentration versus % cell viability of MTT assay for lung cancer cell line A549 **(a)** aqueous latex extract of *Cascabelathevetia*(L.) Lippold; **(b)** methanolic latex extract of *Cascabelathevetia*(L.) Lippold; **(c)** aqueous latex extract of *Plumeria alba* L. **(d)** methanolic latex extract of *Plumeria alba* L.

Table 6. IC_{50} values of all the latex extracts of MTT assay for lung cancer cell line

S.No. (LUNG)	Name of The Plant Extract	IC_{50} Value
1	CTA	9.926
2	CTM	7.884
3	PAA	12.011
4	PAM	10.059

with an IC_{50} value of 12.011 µg/mL. This shows that PAA extract exhibited the lowest cytotoxic potential among all the extracts.

3.4.2 MTT Assay for MCF-7 Human Breast Cell Carcinoma

After the determination of the cytotoxic activities against the A549 Human Lung Cancer cell line the second cell line considered for further evaluation was MCF-7 Human Breast Cancer cell line. Based on the concentrations 1, 10, and 100 (µg/mL) and the cell viability percentage the IC_{50} value was calculated. The graphical representation of concentration versus % cell viability of all four latex extracts has been represented in Figure 6. The IC_{50} values have been summarized in Table 7.

Among all the four extracts the best cytotoxic activity against the MCF-7 Human Breast Cancer cell line was exhibited by CTM (methanolic extract of *Cascabela thevetia* (L.) Lippold) with the IC_{50} value of 6.976 µg/ml followed by CTA (aqueous extract of *Cascabela thevetia* (L.) Lippold) with IC_{50} value of 8.259. The IC_{50} value of PAM (methanolic extract of *Plumeria alba* L.) was 9.955 µg/ml. The highest IC_{50} value was of PAA (aqueous extract of *Plumeria alba* L.) i.e., 12.586. This shows that PAA extract exhibited the lowest cytotoxic potential among all the extracts.

3.5 Apoptosis Analysis

After the MTT assay for further analysis apoptotic assay was performed. The assay was conducted using Annexin V-FITC/PI against the A549 Human Lung Cancer cell line

Table 7. IC_{50} values of all the latex extracts of MTT assay for breast cancer cell line

S.No. (BREAST)	Name of The Plant Extract	IC_{50} Value
1	CTA	8.259
2	CTM	6.976
3	PAA	12.586
4	PAM	9.955

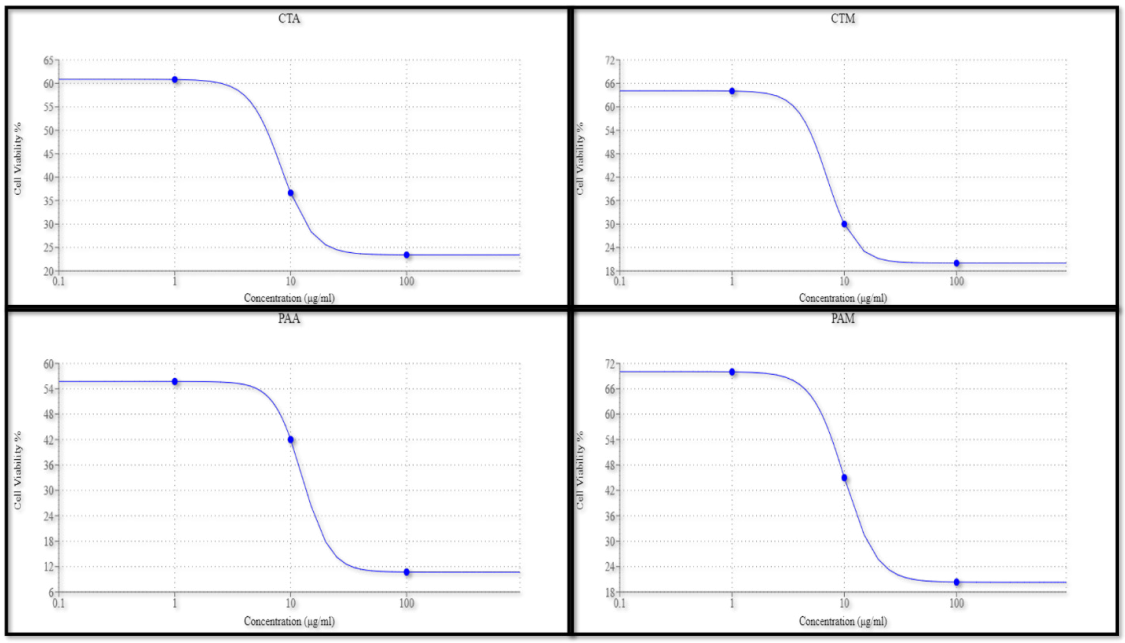

Figure 6. Graphical representation of concentration versus % cell viability of MTT assay for breast cancer cell line MCF-7 **(a)** aqueous latex extract of *Cascabelathevetia*(L.) Lippold; **(b)** methanolic latex extract of *Cascabelathevetia*(L.) Lippold; **(c)** aqueous latex extract of *Plumeria alba* L. **(d)** methanolic latex extract of *Plumeria alba* L.

and MCF 7 Breast Cancer Cell Line. Various apoptotic phases were analyzed along with the percentage of viable and dead cells. Annexin V (phospholipid-binding protein) has a high affinity for phosphatidylserine (phospholipid) expressed on the cell surface, while PI helps in characterizing the integrity of the cell membrane as PI does not enter cells with intact membranes. Here, the results generated are represented as two-dimensional dot plots.

3.5.1 Apoptosis Assay for A549 Human Lung Cell Carcinoma

Figure 7 represents the Annexin V and Dead Cell Flow cytometry analysis of all four latex extracts respectively against the A549 Human Lung Cancer cell line.

It was found that in comparison to the treated the untreated cells exhibited decrease in the percentage of apoptotic cells. As shown in Figure 7 the percentage of apoptotic cells treated with the methanolic latex extract of *Cascabela thevetia* (L.) Lippold (CTM) ranged from 87.80 % to 0.06 %. Whereas, the aqueous latex extract of *Cascabela thevetia* (L.) Lippold exhibited 81.42 % of late apoptotic cells and 4.08 % of early apoptotic cells. The results obtained for the aqueous latex extract of *Plumeria alba* L. against the A549 Lung Cancer Cell Line were 76.78% and 0.00% of late and early apoptotic cells respectively. Whereas, 81.86% of late apoptotic cells and 0.54 % of early apoptotic cells were observed in the methanolic latex extract of *Plumeria alba* L. (PAM).

Hence, among all the extracts the best result was exhibited by CTM (methanolic latex extract of *Cascabela thevetia* (L.) Lippold) followed by CTA (aqueous latex extract of *Cascabela thevetia* (L.) Lippold), PAM (methanolic latex extract of *Plumeria alba* L.) and PAA (aqueous latex extract of *Plumeria alba* L.) against the A549 Human Lung Cancer cell line.

3.5.2 Apoptosis assay for MCF-7 Human Breast Cell Carcinoma

After the apoptosis analysis of the A549 Human Lung Cancer cell line, the best results were noted. The extracts that provided the best results were further utilized for the apoptosis analysis against the MCF 7 Human Breast Cancer cell line. Figure 8 represents the annexin V and Dead cell Flow Cytometry analysis of aqueous and methanolic latex extract of *Cascabela thevetia* (L.) Lippold.

As shown in Figure 8 among both the extracts the best result against MCF 7 Human Breast Cancer Cell Line was obtained in the methanolic latex extract of *Cascabela thevetia* (L.) Lippold. (CTM) with 45.91% of late and

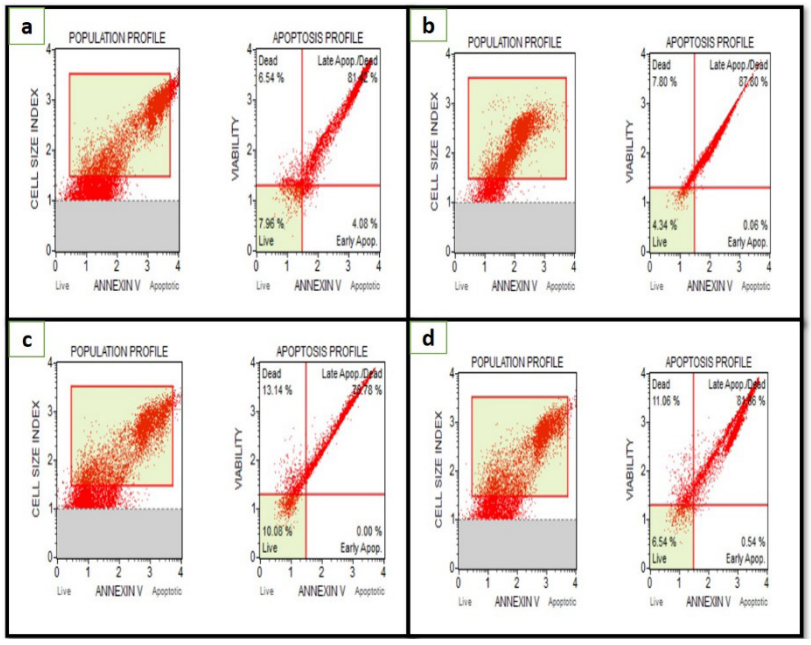

Figure 7. Annexin V and Dead cell flow cytometry analysis for breast cancer cell line MCF-7 of **(a)** aqueous latex extract of *Cascabe lathevetia* (L.) Lippold; **(b)** methanolic latex extract of *Cascabe lathevetia* (L.) Lippold; **(c)** aqueous latex extract of *Plumeria alba* L. and **(d)** methanolic latex extract of *Plumeria alba* L.

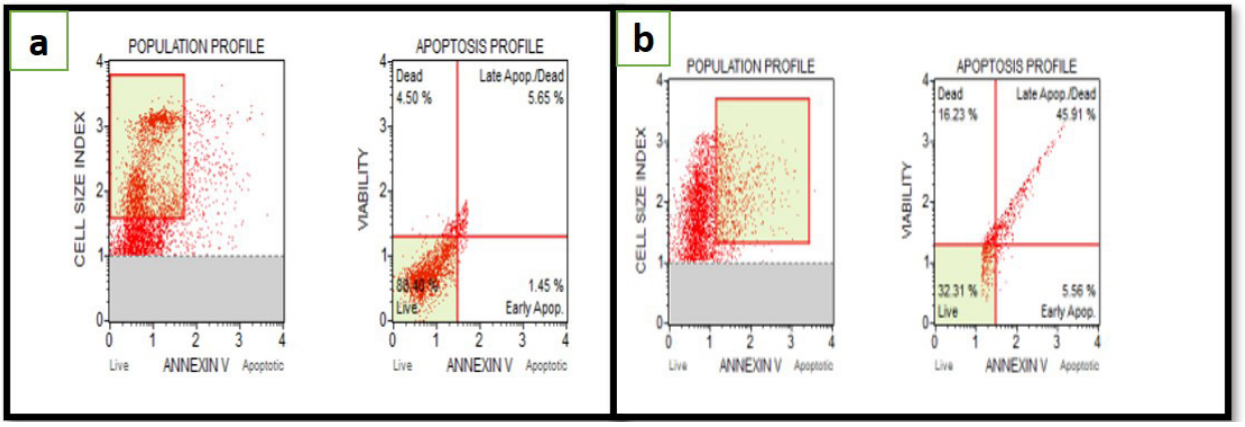

Figure 8. Annexin V and Dead cell flow cytometry analysis for breast cancer cell line MCF-7 of **(a)** aqueous latex extract of *Cascabelathevetia*(L.) Lippold; **(b)** methanolic latex extract of *Cascabelathevetia*(L.) Lippold.

5.56% of early apoptotic cells. This was followed by the aqueous latex extract of *Cascabela thevetia* (L.) Lippold. (CTA) with 5.65% of late and 1.45% of early apoptotic cells respectively. Flow cytometric analysis revealed that the latex extracts could induce apoptosis of the cancer cells. Hence, it can be said that the apoptosis assay has further confirmed the presence of the cytotoxic activities of the latex extracts.

4. Conclusion

It was observed from the MTT and apoptosis assay that the aqueous and the methanolic latex extracts of the plants *Cascabela thevetia* (L.) Lippold and *Plumeria alba* L. have potent cytotoxic properties. The phytochemical analysis using GC-MS and HPTLC methodology has supported these findings. The HPTLC method confirmed the presence of Digoxin-like cardiac glycoside in the aqueous latex extract of *Cascabela thevetia* (L.) Lippold. It was further observed that different bands appeared on the TLC plate showing the richness of various other secondary metabolites. The components that have been found using the GC-MS technique reveal that the latex extracts are rich in volatile and semi-volatile components. Further using the molecular docking technique, it was proved that these volatile and semi-volatile components can bind at the specific sites of the sodium-potassium pump.

In conclusion, it can be said that the presence of cardiac glycosides and the volatile and semi-volatile components in the latex extracts can play a very important role in developing anticancer drugs as these components bind to the sodium-potassium pump. Although here in the present study Na^+/K^+-ATPase has been supported as a potent anticancer target further mechanisms of action are still needed to be explored. The role of other secondary metabolites present in the latex extracts can also be evaluated in future studies for developing a polyherbal formulation from the latex extracts against human cancers.

5. Acknowledgment

For the financial support, the authors would like to acknowledge the department of education of the Gujarat State, for the SHODH (ScHeme of Developing High-Quality Research) scholarship.

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