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In-Silico and In-Vitro Evaluation of Annonamuricata Leaf Extract Conjugated with Doxorubicin via Liposomal Drug Delivery System against (MDA-MB-231) Triple Negative Breast Cancer Cell Line

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ABSTRACT: Breast cancer is one of the leading causes of death due to cancers in women globally. Cancer cells develop resistance to drugs and there is recurrence of cancer in patients after treatment. Multidrug resistance (MDR) accounts for more than 90% of deaths in cancer patients because of the involvement of several mechanisms such as elevated efflux of drugs, growth factors and genetic variations. In-silico evaluation consist of Drug-likeness, ADMET profiling and Molecular docking. The in-vitro cytotoxicity evaluation of MDA-MB-231 breast cancer cell line with different formulation of (AM, DOX, AM+DOX) liposomes.AM+DOX liposome shows the more significant percentage inhibition of MDA-MB-231 cell line. The AM-DOX conjugated liposomal drug delivery system significantly enhanced the delivery of Annonamuricata leaf extract and Doxorubicin to the breast cancer cells and shows the least cell viability.

KEYWORD: Breast cancer, Annonamuricata, Doxorubicin, Druglikeness, ADMET, Molecular docking, Liposome, Cytotoxicity.

I. INTRODUCTION

Breast cancer is one of the leading causes of death due to cancers in women globally. Cancer cells develop resistance to drugs and there is recurrence of cancer in patients after treatment. Multidrug resistance (MDR) accounts for more than 90% of deaths in cancer patients because of the involvement of several mechanisms such as elevated efflux of drugs, growth factors and genetic variations.[1] Female breast cancer has now surpassed lung cancer as the leading cause of global cancer incidence in 2020, with an estimated 2.3 million new cases, representing 11.7% of all cancer cases. It is the fifth leading cause of cancer mortality worldwide, with 685,000 deaths.[2] Breast cancers can start from different parts of the breast. Most breast cancers begin in the ducts that carry milk to the nipple (ductal cancers) some start in the glands that make breast milk (lobular cancers). There are also other types of breast cancer that are less common like Phyllodes tumor and angiosarcoma. Once a biopsy is done, breast cancer cells are tested for proteins called estrogen receptors, progesterone receptors and HER2. Breast cancer can spread when the cancer cells get into the blood or lymph system and are carried to other parts of the body.[3]

Annonamuricata, commonly called soursop (also known as graviola or guanabana), belongs to Annonaceae family comprising approximately 130 genera and 2300 species. It is a small evergreen tropical tree that is native to Central and tropical South America.[4] In the A. muricataspecies alone, more than 120 AGEs have been reported. Due to the special structures and extensive biological activities, AGEs have attracted significant scientific interest in recent years. Annonaceousacetogenins have been the key phytochemical agents responsible for the anticancer activity of the plant.[5]

Doxorubicin is an antineoplastic in the anthracycline class. Interaction with DNA in a variety of different ways including intercalation (squeezing between the base pairs), DNA strand breakage and inhibition with the enzyme topoisomerase II.[6] Doxorubicin has two major limiting factors, namely cardiotoxicity and emergence of multidrug resistance (MDR) to doxorubicin in cancerous cells.[7] Doxorubicin is used to produce regression in disseminated neoplastic conditions like acute lymphoblastic leukemia, acute myeloblastic leukemia, Wilms tumor, neuroblastoma, soft tissue and bone sarcomas, breast carcinoma, ovarian carcinoma, Hodgkin's disease, malignant lymphoma and bronchogenic carcinoma. . Doxorubicin is also

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indicated for use as a component of adjuvant therapy in women with evidence of axillary lymph node involvement following resection of primary breast cancer.[8]

In-silico drug design means rational design by which drugs are designed/discovered by using computational methods. Drug-likeness assesses qualitatively the chance for a molecule to become an oral drug with respect to bioavailability.[9] ADMET properties has led to their consideration in early stage of drug development, leading to a significant reduction in the number of compounds that failed in clinical trials due to poor ADMET properties.[10] Molecular docking - Protein ligand interaction is the most studied due to its varied applications in drug discovery. The ligand is generally a small molecule, which interacts with the target proteins binding sites.[11]

Liposomes are microscopic spherical vesicles varying in size from 0.025 micrometers (µm) up to 2.5 µm vesicles and composed of one or more lipid bilayers with an aqueous core. They are formed when the lipids are dispersed in an aqueous medium by stirring, in turn giving rise to population vesicles which may reach a size range. The major structural components of liposomes are phospholipids and cholesterol. The lipid bilayer is composed of phospholipids which have a hydrophilic head group and a hydrophobic tail group. The head is attracted to water, and the tail, which is made of a long hydrocarbon chain, is repelled by water. Phospholipids as the main component of the liposomes can easily get integrated with the skin lipids improving drug penetration and localization in the skin layers. The cholesterol incorporated in the lipid membrane helps in the increasing the stability of liposomes, as well as it reduces the permeability of the membranes. The properties of cholesterol lead to decrease the fluidity or increase the micro viscosity of the bilayer.[12]

The MDA-MB-231 cell line is an epithelial, human breast cancer cell line that was established from a pleural effusion of a 51-year-old caucasian female with a metastatic mammary adenocarcinoma and is one of the most commonly used breast cancer cell lines in medical research laboratories. MDA-MB-231 is a highly aggressive, invasive and poorly differentiated triple-negative breast cancer (TNBC) cell line as it lacks estrogen receptor (ER) and progesterone receptor (PR) expression, as well as HER2 (human epidermal growth factor receptor 2) amplification. Similar to other invasive cancer cell lines, the invasiveness of the MDA-MB-231 cells is mediated by proteolytic degradation of the extracellular matrix.[13]

II. MATERIALS AND METHODS

The following softwares have been used to perform the in-silico evaluations such as Drug likeness, ADMET prediction and Molecular docking are PubChem for collection of ligands, RCSB database for collection of proteins, pkCSM for ADMET prediction and Druglikeness, SPDB viewer for protein preparation, BIOVIA Discovery studio for Ligands collection and clustering and to study the protein ligand interaction, PyRx for Docking process, PyMOL for Building the protein ligand complex.

LIPOID S PC-3 (Hydrogenated Soy Phosphatidylcholine) and MPEG-2000-DSPE (1,2disteroyl-sn-glycero-3-phospho ethanolamine-N-methoxy (polyethylene glycol)-2000) was provided by LIPOID, Germany. Doxorubicin, MTT reagent (3-(4,5-Dimethylthiazol-2-yl)-2, 5-Diphenyl tetrazolium Bromide) was purchased from Sigma-Aldrich, Bengaluru. DMEM (Dulbecco's Modified Eagle Medium), FBS (Fetal Bovine Serum), PBS (Phosphate Buffer Saline) was purchased from Himedia Labs Ltd, Chennai. Cholesterol (CHO) was purchased from LobaChemie, Mumbai. Chloroform ($CHCl_3$), Ammonium sulfate ($(NH_4)_2SO_4$), Sucrose, Sodium chloride (NaCl), DMSO (Dimethyl Sulfoxide) was provided by institutional laboratory facilities. All the chemicals and reagents used in this study are analytical graded. MDA-MB-231 cell line was purchased from National Centre for Cell Science (NCCS) Pune, India.

Drug Likeness:Lipinski Rule of Five depends on five simple physiochemical parameter ranges: the molecular weight (MW), which should be less than 500 Dalton, lipophilicity (LogP) less than 5, and number of hydrogen bond donors and acceptors less than 5 and10, Topological polar surface area less than 140 respectively.

ADMET Prediction of Ligands:ADMET analysis of ligands (phytoconstituents) are predicted with the help of pkCSM software (http://biosig.unimelb.edu.au/pkcsm/) from this online platform, absorption, distribution, metabolism, excretion, toxicity profile of the drugcandidates can be calculated.

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TABLE 1: List of Ligands

S.no	Phytoconstituents	Pub Chem CID
1.	Annocatalin	10054251
2.	Annohexocin	10054746
3.	Annomuricin A	157682
4.	Annomuricin B	44575650
5.	Annomuricin C	11758463
6.	Annomuricin-D-One	44559061
7.	Annomuricin E	10371584
8.	Annomutacin	132076
9.	Annonacin A	393471
10.	Annonacin	354398
11.	Annopentocin A	5319155
12.	Annopentocin B	5319163
13.	Annopentocin C	10817542
14.	Cis-Corossolone	11093061
15.	Corossolone	4366126
16.	Doxorubicin	31703
17.	Gigantetronenin	6439484
18.	Iso-Annonacin	128014
19.	Muricatocin B	133072
20.	Muricatocin C	44584147
21.	Muricoreacin	44559047
22.	Murihexocin A	44306793
23.	Murihexocin C	10258454
24.	Murihexocin	44559048

TABLE 2: Targets for molecular docking

S.no	Targets	PDB ID
1.	Multi Drug Resistance protein 1 (MDR1)	7A65
2.	ATP Binding Cassette sub-family B member 1 (ABCB1)	6QEX
3.	Adaptor Protein 2 (AP2)	2VGL
4.	Caveolin-1 (Cav-1) Cavin protein	4QKV
5.	Transferrin	1SUV

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Plant Material and Extract Preparation: Annonamuricataleaves were collected from the locality garden of Sulur, Coimbatore, India. The plant material was identified at the Herbarium of Botanical Survey of India, Coimbatore and authenticated a specimen as BSI/SRC/5/23/2021/Tech/301.
Annonamuricataleaves were washed and shade dried. Then leaves were blended into coarse powder with the help of mixer grinder. The powdered leaves are macerated for 3 days with the hydroalcoholic mixture of water and ethanol in 1:1 ratio. Then the Iodineflasks were kept in the Gyratory shaker for 5-6 hours and kept at rest for 15-18 Hours. Then the mixture was filtered using filter paper and the solvent was evaporated by means of Rotary evaporator to obtain the hydroalcoholic extract of Annonamuricataleaves.[14]

Preparation of AM-DOX Liposome: Liposomes were prepared with Phosphatidylcholine, Cholesterol, and MPEG-DSPE. Briefly, 100 mg Phosphatidylcholine (LIPOID S PC-3), 20 mg MPEG-2000-DSPE and 30 mg Cholesterol were added in 10 mL of HPLC-grade chloroform in a 100-mL round bottom flask. The flask was rotated at 100 rpm while dipping in water bath maintaining a temperature of 55°C. Once the thin film was formed, Prepared Doxorubicin 1mg/ml solution in 0.9% NaCl and Annonamuricataleaf extract was added into the round bottom flask using the ammonium sulfate loading method. Then the flask was placed in a vacuum desiccator overnight to remove any organic solvent residue. The resulting film of the lipid polymer mixture was hydrated in 120 mM ammonium sulfate (pH 5.5). Resulting liposomes were sonicated for 15 minutes at a temperature of 55°C. And stored in a refrigerator at 2-8^{0}C.A schematic representation of the preparation of AM-DOX liposome has been shown in Figure (1).[15]

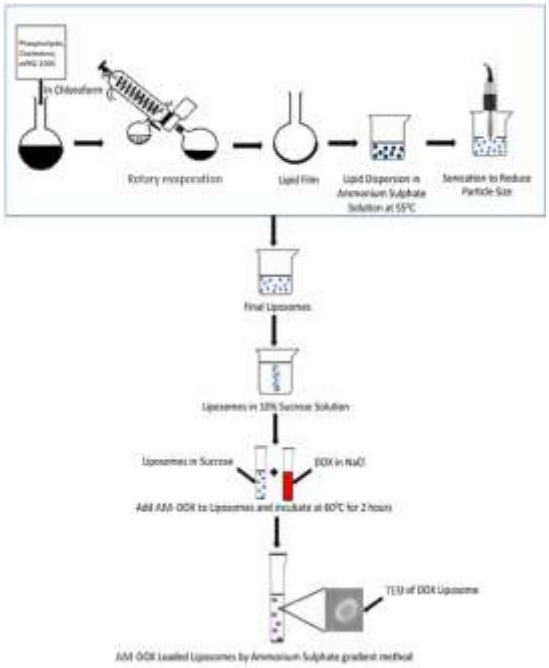

FIGURE 1: Preparation of AM-DOX loaded liposome

Preformulation Studies:
1. Physical Incompatibility studies: Physical incompatibility leads to change in color, odor, and viscosity are caused by interaction between two or more substances which shows; visible physical change, non-uniformity, unacceptable, unpalatable product formation, Insolubility, Immiscibility and Liquefaction of solids.

2. Chemical Incompatibility studies: The Compatibility of a medication with other drugs and implanted materials is an important factor impacting drug safety and efficacy. A FTIR-spectrophotometer equipped with an attenuated total reflectance (ATR) was used to investigate molecular interactions involved in complex formation between drug and excipients. Vacuum-dried samples were scanned over the infrared range (4000 to 500 cm^{-1}) and characteristic peaks were compared.

Formulation Studies:
Characterization of Liposomes:

1. Particle Size Distribution, Polydispersity Index, and Zeta Potential Measurement: The particle size distribution and surface charge of liposomes (0.5 mg/mL) in phosphate-buffered saline (PBS, pH 7.4) were measured by dynamic laser- light scattering (DLS) by Zeta sizer (Nano

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ZS, Malvern Instruments Ltd., UK). Zeta potential of liposomes was determined by electrophoretic mobility determination using Zeta sizer.

2. Morphology: Liposomes were placed on copper grid films and stained with 2% (w/v) Phosphotungstic acid for morphological observation by transmission electron microscopy (TEM) (JEM-100CX, JEOL, Japan).

3. Entrapment Efficiency: The entrapment efficiency of formulations was determined by comparing the drug:lipid ratio of the final product to initial concentrations of the lipid. The lipid bilayer of the liposomes were lysed with Triton X (1%) and Phosphate buffered saline (PBS). The mixture was centrifuged for 60 min at 14000 rpm, the supernatant containing drug was collected. The amount of entrapped drug was determined spectrophoto -metrically at 217 and 496 nm. The calibration curve was linear in the range of 10–10,000 ng/ml. The drug encapsulation efficiency was defined as the percentage of the amount of drug encapsulated in the liposomes to total amount of drug.
The absorbance was measured and the entrapment efficiency was calculated by using following formula:

%Entrapment Efficiency (EE) = (CT−CS)/CT×100

EE is the concentration of entrapped drug (ng/mL), CT is the initial concentration of drug used in formulating the liposomes (ng/mL), CS is the concentration of drug in the supernatant (ng/mL), and EE (%) is the percentage of the drug's entrapment.

4. Stability testing: The stability problem of liposomes were limited to two aspects: the physical stability against aggregation or fusion of the dispersion and the drug retention (or latency), defined as the percentage of drug that is liposome-associated. We will focus on two compounds: Annonamuricata (AM) and doxorubicin (DOX). AM is a hydrophilic compound that is assumed to have a low tendency to interact with negatively charged bilayer structures; DOX is an amphiphilic drug that interacts with the bilayer, mainly via an electrostatic interaction with the negative charge inducing phospholipids. For highly lipophilic compounds that strongly interact with the bilayer (e.g. cholesterol), leakage from the bilayer does not occur on storage. For these compounds only physical and chemical stability problems have to be addressed.

In-vitro Cytotoxicity assay
Cell Culture: MDA-MB-231 cells (epithelial human breast cancer cell line) was purchased from NCCS, Pune and were cultured in Dulbecco's Modified Eagle Medium (DMEM) is supplemented with 10% Fetal Bovine Serum (FBS), 1% L-Glutamine, 1% Sodium bicarbonate, 1% Sodium pyruvate and maintained under an atmosphere of 5% CO_2 at 37^{o}C with the humidity of 80%.

MTT assay: The sample was tested for in vitro cytotoxicity, using MDA-MB-231 cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the cultured MDA-MB-231 cells were harvested by trypsinization, pooled in a 15 ml tube. Then, the cells were plated at a density of 1×10^5 cells/ml cells/well (100 µL) into 96-well tissue culture plate in DMEM medium containing 10 % FBS, 1% L-Glutamine, 1% Sodium bicarbonate and 1% Sodium pyruvate for 24-48 hour at 37°C. The wells were washed with sterile PBS and treated with various concentrations of the sample in a serum free DMEM medium. Each sample was replicated three times and the cells were incubated at 37°C in a humidified 5% CO2 incubator for 24 h. After the incubation period, MTT (100 µL of 0.5 mg/ml) was added into each well and the cells incubated for another 2-4 h until purple precipitates were clearly visible under an inverted microscope. Finally, the medium with MTT (100 µL) were aspirated off the wells and washed with PBS (100 µl). Furthermore, to dissolve formazan crystals, DMSO (100 µL) was added and the plate was shaken for 5 min. The absorbance for each well was measured at 570 nm using a micro plate reader (Thermo Fisher Scientific, USA) and the percentage cell viability was calculated.
Statistical Analysis: The two-way analysis of variance (ANOVA) was performed for statistical analysis of the data using IBM SPSS statistical software version 25. The p-value less than ($^{*}p<0.05$) were considered as significant. The IC_{50} values are calculated using GraphPad Prism 9 software.

III. RESULTS

Evaluation of Drug-Likeness: The violation of two or more of these conditions predicts a molecule as a non-orally available drug. More over all the ligands shows two violations, but specifically five

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phytoconstituents shows four violations, they are Annohexocin, Muricoreacin, Murihexocin A, Murihexocin C, Murihexocinand Standard drug Doxorubicin is also shows four violations. Hence the violation of two or more of these rules predicts a molecule as a non-orally available drug.

ADMET Prediction of Ligands: ADMET analysis of the Phytoconstituents shows the significant results. Among the compounds Annocatalin, Annomuricin A, Annomuricin B, Annomuricin C, Annomuricin-D-one, Annomuricin E, Annomutacin, Annopentocin A, Annopentocin B, Annopentocin C, Iso-Annonacin, Muricatocin B and Muricatocin C exhibits the good ADMET profile.

Docking Interaction Analysis: The Phytoconstituents such as Annocatalin, Annohexocin, Annomuricin A, Annomuricin B, Annomuricin C, Annomuricin-D-One, Annomuricin E, Annomutacin, Annonacin A, Annonacin, Annopentocin A, Annopentocin B, Annopentocin C, Cis-Corossolone, Corossolone, Doxorubicin, Gigantetronenin, Iso-Annonacin, Muricatocin B, Muricatocin C, Muricoreacin, Murihexocin A, Murihexocin C, and Murihexocin were docked with the selective targets such as MDR 1, ABCB1, Cav-1, AP2 and Transferrin.

Interaction between MDR 1 and Ligands: The 2D and 3D structures were visualized using BIOVIA Discovery studio visualizer tool. The ligands were bonded to proteins with five van der Waals interaction such as ALA A:57, ALA A:58, HIS A:61, ILE A:59, GLY A:62 and with three conventional hydrogen bond such as LEU A:56, GLY A:64, ALA A:63 and with one alkyl bond such as ALA A:128 and with two covalent bond such as ILE A:59, HIS A:61 was shown in the Figure (2). It was observed that the compound Annopentocin A CID: 5319155 shows a better interaction with the MDR 1 protein with the binding energy of -9.2 kcal mol−1.

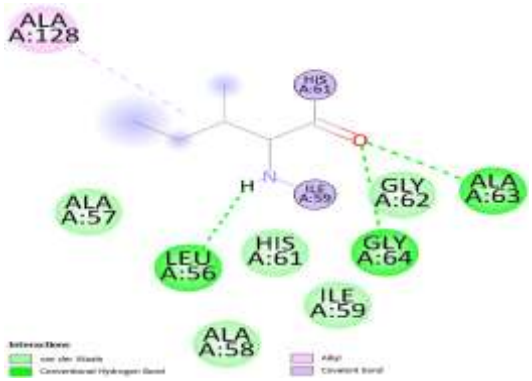

FIGURE 2: 2D complex of MDR1 and Ligands interactions

Interaction between ABCB1 and Ligands: The ligands were bonded to proteins with seven van der Waals interaction such as THR A:630, SER A:1269, GLY A:1265, ILE A:1266, TYR A:1267, MET A:1270, VAL A:1271 and with one conventional hydrogen bond such as SER A:1272 and with one pi-alkyl bond such as LYS A:1264 and with two covalent bond such as SER A:1269, TYR A:1267 was shown in the Figure (3). It was observed that the compound Annocatalin CID: 10054251 shows a better interaction with the ABCB1 protein with the binding energy of -10.5 kcal mol−1.

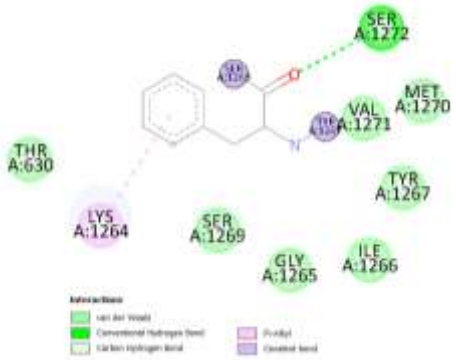

FIGURE 3: 2D complex of ABCB1 and Ligands interactions

Interaction between AP2 and Ligands: The ligands were bonded to proteins with nine van der Waals interaction such as VAL A:428, LEU A:426, LYS A:427, ILE A:424, GLU A:423, GLU A:422, LEU A:413, TYR A:456, VAL A:457 and with two conventional hydrogen bond such as ALA A:429, ARG A:421 and with two alkyl bond such as LEU A:449, ALA A:453 and with two covalent bond such as LEU A:426, ILE A:424 was shown in the Figure (4). It was observed that the compound Annocatalin CID: 10054251 shows a better

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interaction with the AP2 protein with the binding energy of -10 kcal mol−1.

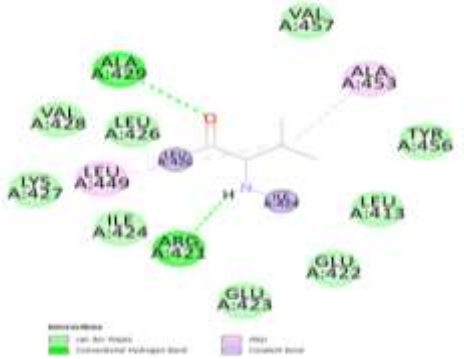

FIGURE 4: 2D complex of AP2 and Ligands interactions

Interaction between CAV-1 and Ligands: The ligands were bonded to proteins with five van der Waals interaction such as GLU A:114, LEU A:113, VAL A:116, ARG A:117, VAL A:116 and with three conventional hydrogen bond such as LEU A:112, LYS A:111, VAL A:119 and with one unfavorable positive-positive bond such as LYS A:118 and with two covalent bond such as VAL A:116, GLU A:114 was shown in the Figure (5). It was observed that the compound Annocatalin CID: 10054251 shows a better interaction with the CAV-1 protein with the binding energy of -7.6 kcal mol−1.

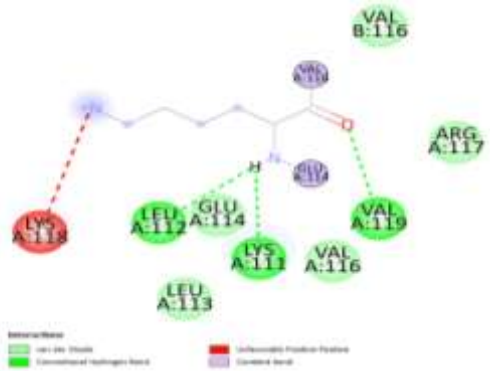

FIGURE 5: 2D complex of CAV-1 and Ligands interactions

Interaction between Transferrin and Ligands: The ligands were bonded to proteins with five van der Waals interaction such as ALA C:311, MET C:313, TYR C:314, LEU C:315, VAL C:320 and with one attractive charge such as ASP C:310 and with one conventional hydrogen bond such as GLY C:316 and with one pi-alkyl bond such as TYR C:71 and with two covalent bond such as ALA C:311, MET C:313 was shown in the Figure (6). It was observed that the compound Annomuricin B CID: 44575650 shows a better interaction with the Transferrin protein with the binding energy of -9.8 kcal mol−1.

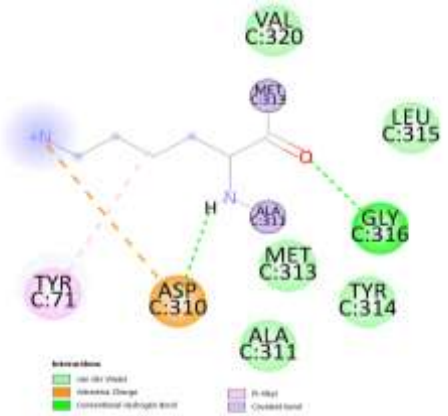

FIGURE 6: 2D complex of Transferrin and Ligands interactions

PREFORMULATION STUDIES

Physical Incompatibility studies: Physical incompatibility study reveals that there is no change in color, odor and viscosity in the single and mixture of compounds.

Compatibility studies: The FTIR spectrum shows that the functional groups in AM, DOX and Phospholipids are involved in the complex formation in AM-DOX liposome. AM showed the characteristic absorption regions at 3266.34cm^{-1}, 2928.28cm^{-1}, 1029.41cm^{-1} which are the assigned functional group –OH, C-H, C-O stretching respectively, DOX showed the characteristic absorption regions at 3315.81cm^{-1}, 3525.49cm^{-1}, 2895.31cm^{-1}, 1729.14cm^{-1}, 1412.33cm^{-1}, 1070.37cm^{-1} and 1579.11cm^{-1} which are the assigned functional group –OH, N-H,C-H, C=O, C-O, and C=C stretching respectively and Phospholipid showed the characteristic absorption regions at 2921.19cm^{-1}, 1734.05cm^{-1}, 1259cm^{-1} and 1073.90cm^{-1} which are the assigned functional group C-H, C=O, P=O and P-O-C stretching, respectively. The absorption peak of AM-DOX liposome spectrum was the sum of the characteristic AM and DOX peaks, the phenolic –OH peaks (3312.68cm^{-1}) of mixture spectrum were increased when compared to AM peak and were decreased when compared to DOX peak. In addition, the peak of C=O region of DOX and Phospholipid near 1728.82cm^{-1} shifted to little bit higher wavelength. And also their hydrogen bonding counterparts, such as P=O peak 1233.39cm^{-1}of P were increased and P-O-C peak

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signal near to 1070.14cm-1 in the AM-DOX liposome spectrum. These changes indicates the involvement of –OH, C=O, C-H, N-H, P=O and P-O-C groups in complex formation. Finally the FTIR peak of AM-DOX liposome was compactible to the individual compounds.

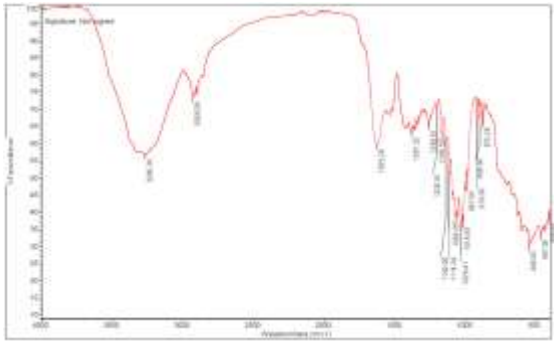

FIGURE 7: FTIR Spectrum ofAnnonamuricata

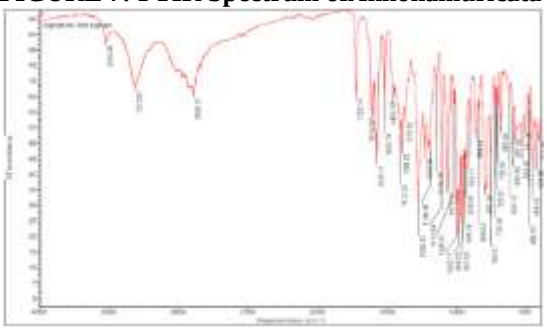

FIGURE 8: FTIR Spectrum ofDoxorubicin

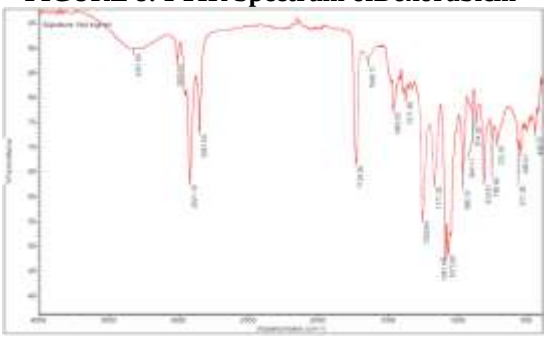

FIGURE 9: FTIR Spectrum ofPhospholipid

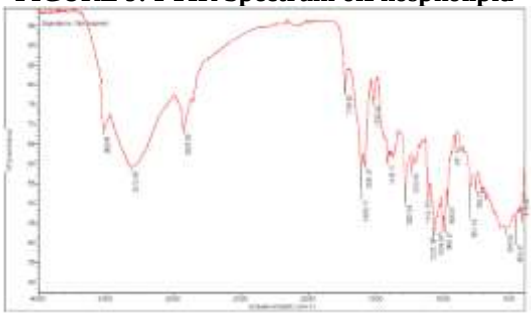

FIGURE 10: FTIR Spectrum of AM-DOX Liposome

Physiochemical Characterization: The liposomes were prepared with HSPC, DSPE-mPEG-2000 and CHO. AM, DOX, AM-DOX liposomes were made with lowest particle size and highest entrapment efficiency. The formulations AM, DOX and AM-DOX had a Particle size of 160 nm 147 nm and 181 nm; Poly dispersity index of 0.435, 0.215 and 0.625; Zeta potential of -10.7 mV, +0.774 mV and -6.87 mV respectively.

Entrapment Efficiency: The amount of entrapped drug was determined spectrophoto-metrically at 217 and 496 nm. The calibration curve was linear in the range of 10–10,000 ng/ml. The absorbance was calculated using the formula y = mx+c, and the entrapment efficiency was calculated by using following formula (EE) = (CT−CS)/CT×100. Entrapment efficiency of AM, DOX and AM-DOX Liposomes are shown in the Table (3).

TABLE 3: Entrapment Efficiency of Liposomes

S.no	Formulation	Entrapment efficiency EE (%)
1.	AM Liposome	83.1
2.	DOX Liposome	86.6
3.	AM-DOX Liposome	84.85

Morphology: Liposomes were placed on copper grid films and stained with 2% (w/v) phosphotungstic acid for morphological observation by transmission electron microscopy (TEM) (JEM-100CX, Japan). The transmission electron microscopy (TEM) images of the liposomes are shown in Figure (50). The liposomes look like vesicles, which are spherical in shape and have a diameter of about 140–200 nm.

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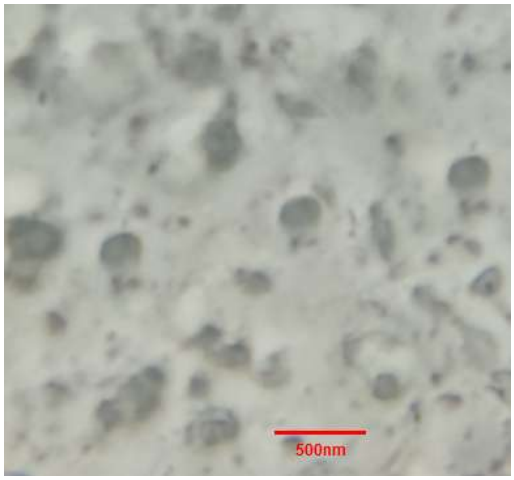

FIGURE 11: TEM Image of AM-DOX Liposome

Physical Stability of Liposomes: The stability problem of liposomes were limited to two aspects: the physical stability against aggregation or fusion of the dispersion and the drug retention (or latency), defined as the percentage of drug that is liposome-associated. The physical stability of formulations such as AM, DOX and AM-DOX liposomes at a storage of 2-8^{0}C are 2-3, 2-3, 1-2 weeks and then precipitates.

TABLE 4: Stability Study of Liposomes

S.no	Formulation	Physical Stability at 2-8^{0}C
1.	AM Liposome	Stable for 2-3 weeks, then precipitates
2.	DOX Liposome	Stable for 2-3 weeks, then precipitates
3.	AM-DOX Liposome	Stable for 1-2 weeks, then precipitates

Cytotoxicity of Liposomes on MDA-MB-231 Breast Cancer Cell line: The susceptibility of MDA-MB-231 Breast cancer cell lines were tested over the different formulations of liposomes such as AM liposome, DOX liposome and AM+DOX conjugated liposome using the standard cell viability test, the MTT assay. The assay proved that the cytotoxic effect of AM+DOX liposome was higher than that of AM liposome and DOX liposome. The formulation AM+DOX liposome shows the least cell viability of 33.1% at 100µg/ml concentration whereas AM liposome and DOX liposome shows the cell viability of 53.76% and 47.8% at 100µg/ml concentration respectively, which shows that the formulation AM+DOX liposome shows the highest percentage inhibition of MDA-MB-231 cell line. The AM+DOX liposome shows the IC_{50} value of 4.672 µM which is lower than the IC_{50} value of AM liposome (6.109 µM) and DOX liposome (5.732 µM) was shown in a Figure (). The AM-DOX liposomal drug delivery system significantly enhanced the delivery of Annonamuricata leaf extract and Doxorubicin to the breast cancer cells and shows the least cell viability.

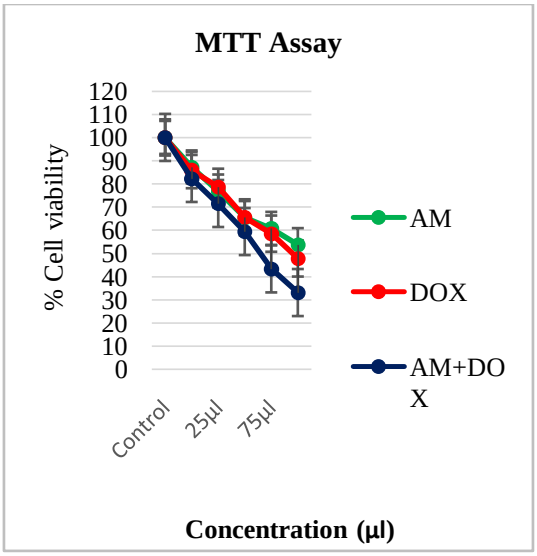

FIGURE 12: Percentage Cell viability of MTT Assay

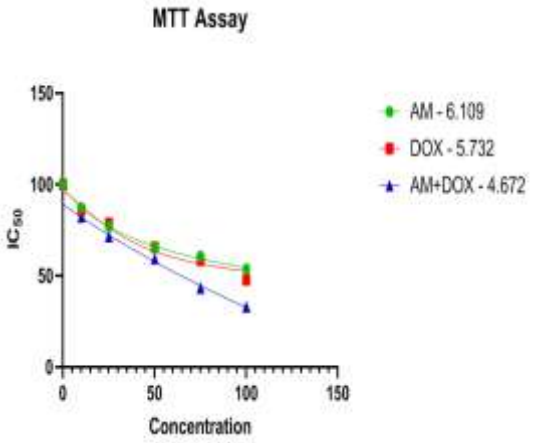

FIGURE 13: IC_{50} values of MTT assay

The p-value for the dose parameter is less than p-value 0.05, then the result is considered as significant. Hence there is a significant change in

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the viability of cells with the change in the dose. The p-value for the formulations is greater than p-value 0.05, then the result is not statistically significant. Hence the change in various formulations do not lead to a significant difference in the viability of cells. The p-value for the interaction effect of formulations and dose is less than p-value 0.05, hence we can conclude that there is a significant difference in the viability of cells with the combined effect of formulation and various dose. The estimated parameters are shown in the Table (5).

TABLE 5: Formulations and Dosage Parameter Estimation by 2-way ANOVA

Parameter	Std. Error	Hypothesis Test		
		Wald Chi-Square	Df	Sig.
(Intercept)	2.8698	1136.319	1	.000
Formulations * Dose	0.4388	21.215	1	.000
Formulations	1.3285	0.146	1	.702
Dose	.9479	52.004	1	.000

Dependent Variable: Response
Model: (Intercept), Formulations * Dose, Formulations, Dose
The p-value less than ($^{*}p<0.05$) were considered as significant.

IV. DISCUSSION

This study aims on in-silico and in-vitro evaluation of breast cancer activity. The in-silico study focused against the P-glycoprotein, which hammers the therapeutic efficacy of various chemotherapeutic agents because of P-gp efflux mechanism. In-silico study involves in theanalysis of drug-likeness, ADMET analysis and molecular docking study.

The drug-likeness study results reveals that almost all the Phytoconstituents of Annonamuricata were not suitable for oral administration due to poor bioavailability and molecular weight more than 500 Daltons and all the ligands violates the two or more violations in Lipinski rule of five. ADMET analysis of the Phytoconstituents shows the significant results. Among the compounds Annocatalin, Annomuricin A, Annomuricin B, Annomuricin C, Annomuricin-D-one, Annomuricin E, Annomutacin, Annopentocin A, Annopentocin B, Annopentocin C, Iso-Annonacin, Muricatocin B and Muricatocin C exhibits the good ADMET profile. Molecular docking of phytoconstituents reveals that Annopentocin A shows significant binding energy of (-9.2 kcal mol−1) with MDR 1 protein, Annocatalin shows significant binding energy of (-10.5 kcal mol−1), (-10 kcal mol−1) and (-7.6 kcal mol−1) with ABCB1, AP2 and CAV-1 protein and Annomuricin B shows significant binding energy of (-9.8 kcal mol−1) with Transferrin.

The Physiochemical characterization of AM-DOX liposome shows the Particle size of 181nm; Polydispersity index of 0.625; Zeta potential of -6.87mV and Entrapment efficiency of 84.85%. Further this formulation is evaluated by cytotoxicity study using MDA-MB-231 breast cancer cell line. The formulation AM-DOX liposome shows the least cell viability of 33.1% at 100µg/ml concentration compare with AM liposome and DOX liposome shows the cell viability of 53.76% and 47.8% at 100µg/ml concentration respectively, which shows that the formulation AM+DOX liposome shows the more significant percentage inhibition of MDA-MB-231 cell line. The AM-DOX conjugated liposomal drug delivery system significantly enhanced the delivery of Annonamuricata leaf extract and Doxorubicin to the breast cancer cells and shows the least cell viability.

V. CONCLUSION

The present study shows that DOX can deliver into cancer cell by the inhibition of P-gp efflux by phytoconstituents present in Annonamuricata. So, the therapeutic efficacy of DOX can be enhanced. In-silico study shows only predicted values but scientific validation of these ligands requires further in-vitro and in-vivo animal studies. Further, these studies to be carried out in future by isolation of particular above mentioned compounds from Annonamuricata and making the novel drug delivery formulation to overcome the P-gp efflux by inhibiting the multi-drug resistance proteins in the breast cancer to promote the therapeutic efficacy of the chemotherapeutic agent.

ABBREVIATIONS

ABCB1 - ATP Binding Cassette sub-family B member-1
AGEs – Acetogenins
AM - Annonamuricata
AP-2 - Adaptor Protein-2
Cav-1 - Cavin protein or Caveolin-1
DOX – Doxorubicin

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ER - Estrogen Receptor
HER2 - Human Epidermal Growth Factor Receptor 2
IC_{50} - Inhibitory Concentration
MDR 1 - Multi Drug Resistance Protein 1
PR - Progesterone Receptor
P-gp - P-Glycoprotein
TNBC - Triple-Negative Breast Cancer

VI. ACKNOWLEDGEMENT

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ANNEXURE

भारतसरकार
GOVERNMENT OF INDIA
पर्यावरण, वन और जलवायु परिवर्तन मंत्रालय
MINISTRY OF ENVIRONMENT, FOREST & CLIMATE CHANGE
भारतीय वनस्पति सर्वेक्षण
BOTANICAL SURVEY OF INDIA

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लाउलीरोड/ Lawley Road
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सं. भा.व.स./द.क्षे.कें./No.: BSI/SRC/5/23/2021/Tech. /3-1

दिनांक/Date: 6th December 2021

पौधे प्रमाणीकरण प्रमाणपत्र / PLANT AUTHENTICATION CERTIFICATE

The plant specimen brought by you for authentication is identified as *Annona muricata* L. - ANNONACEAE. The identified specimen is returned herewith for preservation in their College/ Department/ Institution Herbarium.

डॉ. एम. यु. शरीफ़/DR. M. U. SHARIEF
वैज्ञानिक 'ई' एवं कार्यालयाध्यक्ष/
SCIENTIST 'E' & HEAD OF OFFICE

सेवा में / To

Mr. PRAVEEN KUMAR K N
Final Year M.Pharm. Student
Department of Pharmacology
Karpagam College of Pharmacy
COIMBATORE – 641 032

FIGURE 13: Plant Authentication Certificate