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Research Article

Protective Effect of Gallic Acid on Doxorubicin Stressed Cardiomyocytes

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ABSTRACT

Doxorubicin a potent anti-cancerous drug agent used widely for treatment of different types of malignancies. However, its cardiotoxicity has limited its use in a dose-dependent manner majorly. Lack of prediagnosis and poor validation of cardiotoxicity have been documented in the literature as causes of death. Cardio-oncological therapies with low toxicity on cardiac cells may provide benefit as natural therapeutic interventions. Gallic acid (GA), a phenolic molecule naturally occurring in *Syzygium cumini*, has anti-inflammatory, anti-obesity, anti-cancerous effects and cardioprotective in nature. In the current study, Gallic acid, the isolated polyphenol from *Syzygium cumini* was evaluated for the anticancerous and cardioprotective efficacy in Doxorubicin-induced cardiotoxicity (DIC). To assess the anticancerous and cardiotoxic dose of Doxorubicin in MCF-7 and H9C2 cells, cell viability experiments were carried out. In addition, the anticancerous effect of gallic acid in MCF-7 cells was investigated in combination with doxorubicin. Giemsa-based microscopic analysis was used to examine the morphological changes in MCF-7 cells. We found that supplementing doxorubicin with gallic acid supported death in MCF-7 cells and efficiently protected cardiac cells stress. Also, gallic acid in cardiac cells prevented the morphological changes brought on by doxorubicin in a dose dependent manner and attenuated stress effectively. For *insilico* characterisation ProTox II and swissADME was used, to estimate toxicity and physicochemical characteristics of ligand molecule. Therapeutic potential of Gallic acid against BRCA1 and BRCA2 genes was determined *insilico* with Autodock Vina and the discovery studio visualizer. In conclusion, gallic acid protected against the cardiotoxicity caused by doxorubicin in H9C2 cardiomyocytes and has anticancerous potential against MCF-7 cells.

Keywords: Gallic acid, Doxorubicin, Cardioprotective agent, Toxicity, HPLC, MCF-7 cells, H9C2 cardiomyocytes.

1. INTRODUCTION

Globally, cancer is an illness that affects people all over the world, which results in significant morbidity and mortality, and has a remarkable financial impact on global public health[1]. With over 10 million deaths due to cancer in 2020, it is the top cause of death globally. The most prevalent cancer in 2020 was breast cancer with about 2.26 million cases[2]. Breast cancer is the most prevalent malignant illness in women and is the leading cause of death and misery around the globe[3]. About 70% of occurrences of Breast cancer are categorized as sporadic. While familial Breast cancer affecting approximate 30% of patients with high incidence of Breast cancer. High-penetrance genes includes BReast-CAncer susceptibility gene 1 (BRCA1), Breast cancer susceptibility gene 2 (BRCA2), PTEN, and TP53. DNA repair genes have been linked to a moderate chance of developing breast cancer. A number of frequently occurring low penetrance alleles linked to a slightly elevated or lowered risk of breast cancer revealed by Genome wide association studies[4]. The mutant phenotypes of the tumor suppressor genes BRCA1 and BRCA2 contribute to breast and ovarian cancers. Both genes have a role in transcriptional regulation and DNA repair. In order to maintain chromosomal integrity and safeguard the genome from harm, BRCA proteins are essential. Additionally, new research demonstrates that BRCAs control the transcription of several genes related to apoptosis DNA repair, and the cell cycle. A wide variety of cellular proteins that interact with BRCAs mediate many of these processes. Finally, it is still unclear why BRCA gene mutations cause breast and ovarian malignancies to manifest. It was recorded that understanding of both hereditary and sporadic breast carcinogenesis will have the potential to improve, by elucidating the specific molecular activities of BRCAs[5].

Chemotherapeutic drug doxorubicin is often used to treat several tumor types, including breast cancer. In addition to DNA intercalation and adduct formation, topoisomerase II (TopII) poisoning, the production of free radicals and oxidative stress, and membrane damage due to altered sphingolipid metabolism, doxorubicin is characterized by a variety of these molecular pathways[6]. Cancer treatments using doxorubicin can cause cardiotoxicity in patients with breast cancer in a dose-dependent manner. Also, the patients who would have DIC cannot currently be predicted[7-8]. In order to determine the effect of doxorubicin on breast cancer, MCF-7 cancer cell lines were used. MCF-7 cells are breast cancer cell lines, frequently employed by research teams for a variety of studies and molecular aspects[9]. The safety and efficacy of a number of molecules and their derivatives as anti-proliferative agents against the MCF-7 human breast cancer cell lines has been determined *in vitro* and *in silico*[10-12].

A naturally occurring phenolic molecule found in various plants including *Syzygium cumini* called gallic acid (GA) has a number of therapeutic effects that include anti-obesity, anti-inflammatory, and anti-cancer effects[13]. For

the prevention and treatment of cancer, Gallic Acid is a unique anti-cancer medication that is both effective and safe[14]. Through an autophagy-dependent mechanism, Gallic Acid inhibits myocardium hypertrophy and dysfunction. As a result, Gallic Acid is a viable therapeutic option for the treatment of ventricular hypertrophy and heart failure[15]. Studies have also been reported that through control of JNK2 signaling and Smad3 binding activity, Gallic Acid inhibits isoproterenol-induced heart hypertrophy and fibrosis[16].

As suggested and documented in literature, therapeutically useful natural ingredients have demonstrated a great substantial value for a long time in treating and avoiding symptoms of a wide variety of diseases and illnesses[17-20]. Di'ao Xinxuekang capsule with antioxidant and anti-inflammatory activities showed excellent health benefits in treating arrhythmia and myocardial ischemia cardiovascular disease[21]. In H9C2 cardiomyocytes, *Glycyrrhiza glabra* root extract was used to reduce doxorubicin-induced cardiotoxicity by decreasing oxidative stress and stabilizing the heart's health[22].

Previously our group performed *S. cumini* methanol seed extract (MSE) analysis using high-performance liquid chromatography where Gallic Acid was found to be most promising and widely used candidate compound. Also, cardioprotective effect of *S. cumini* against pesticide-induced toxicity was determined[23-24]. The MSE of *S. cumini* has been studied by our group for its cardioprotective effects in diabetic cardiomyopathy; however, its effects on Doxorubicin-treated H9C2 cells and against breast cancer cell lines MCF-7 cells have not been tested in conjunction with the drug medication, and their mechanism of action has not yet been evaluated entirely[25]. H9C2 cells are rat cardiomyoblast cell lines, an animal-free model system used to study cardiac hypertrophic disease and other molecular perspectives[26]. Therefore, the goal of the current investigation was to explore and assess the effects of GALLIC ACID on doxorubicin-treated MCF-7 breast cancer cell lines *in vitro* and *in silico*. Additionally, we analysed effect of GALLIC ACID in cardio protection in Drug induced cardiotoxicity.

Thus, we performed experiments on MCF-7 cells for analyzing morphological changes using Giemsa staining method. Also, for the same dose concentration of doxorubicin on H9C2 cells toxicity was determined. To optimize the dose of testing compounds, MTT, assay was performed to observe the effects of selected GALLIC ACID and doxorubicin individually and in combination. Further, to validate any alterations in cellular and nuclear morphology, microscopic Giemsa staining was performed on MCF-7 cells. Also, the same IC_{50} dose of doxorubicin was tested on H9C2 cells for its 50 % cell viability. Microscopic results provided evidence that administering an experimentally optimised dose of GA, which served as an anti-cancerous and cardioprotective agent, may mitigate the adverse effects of doxorubicin. Thereafter, molecular docking was performed for BRCA1 and BRCA2 genes with doxorubicin and Gallic Acid to study their interaction *insilico.* In Parallel with this HPLC analysis for Gallic Acid was conducted.

2. MATERIALS AND METHODS

All compounds were bought from Sigma Aldrich, USA unless otherwise stated. The study used gallic acid (Catalogue number: G7384, Sigma-Aldrich).

2.1. Maintenance of cultured cells

MCF-7 human breast cancer cell line and H9C2 cardiomyoblasts from embryonic rat cardiac tissue were grown in DMEM supplied with 10% FBS and antibiotics, respectively (NCCS, Pune, India). At 70 % confluency, cells were trypsinized and seeded in a 1:3 ratio in a humidified incubator with 5% CO_2 at 37 °C. Cells were cultivated at a density of 10^6 cells/cm^2 during this time. For 24 hours prior to the necessary treatments, cells were allowed to grow in T-25 or T-75 cultivated flasks.

2.2. MTT assay

The percentage of viable cells was calculated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay, to determine the optimal doses of doxorubicin and Gallic Acid in both cell lines. Viable cells were stained with 5 mg/ml MTT for 3 hours after the treatments. To dissolve formazan, the medium in each well was replaced using DMSO, and absorbance was recorded at 570 nm using an ELISA plate reader (Bio-Rad). Utilising the formula (Absorbance of treated cells)/ (Absorbance of untreated cells), the percentage of viable cells was determined[27].

2.3. Trypan blue dye exclusion assay

Cells from each experimental group were collected, stained with 0.4% trypan blue in a 1:1 ratio, and allowed to settle at room temperature for 5 minutes. To count the number of dead and live cells under the microscope, the cell suspension was placed onto the hemocytometer[28].

2.4. Morphological analysis of MCF-7 and H9C2 cells

Under an inverted microscope (Olympus) set at 20X magnification, cells treated with doxorubicin and Gallic Acid were examined for altered cell size and shape. Under microscope, the total number of cells in each experimental group was determined as well.

2.5. Giemsa stain

Cells were fixed with 100% methanol at −20 °C and incubated with 20% Giemsa stain diluted in 0.5% glacial acetic acid for 15 min at 27 °C to study the changes in cellular morphology upon various treatments. Morphological changes were observed under an inverted microscope at 40X magnification. According to the instructions provided, NIH image J software was also used to measure cell size.

2.6 HPLC analysis

Gallic acid was used for HPLC Analysis. Before loading on an HPLC equipment with a high-pressure binary pump and photodiode array detector (Waters 2998), Gallic acid was filtered. The concentration of the stock was fixed at 10 µg/ml. Following are the column specifications and solvent systems: C-18, particle size 15 µ, 4.6 x 250 mm. Solvent methanol: acetonitrile (90:10) was used in the mobile phase. Wavelength range 200-450 nm, total flow rate of 1 ml/min was used. Gallic acid peak was found to be comparable with literature[29].

2.7 Receptor molecule preparation

From RCSB PDB, an online protein data bank tool, 3D structures of complete proteins were downloaded in pdb format. The AutoDock Vina software 1.5.6 version was used to further prepare proteins for docking by eliminating water molecules and introducing hydrogen molecules, respectively. Each molecule's pdb file format was subsequently changed to pdbqt[30].

2.8 Ligand structures Preparation

The SDF format of the ligand molecules was retrieved from PuBchem. Doxorubicin and gallic acid were selected. Pymol was used to prepare the ligands. According to Lipinski's Rule of Five (RO5), which is based on parameters like molecular weight should be less than equal to 500 kDa, CLogP value less than equal to 5, and the value of hydrogen bond acceptor and hydrogen bond donor should be less than equal to 10 and 5 respectively, drug molecules must fulfil specific requirements in order to function as ligands. Compounds that satisfied these criteria are known to be more permeable and active as ligand molecules. [31-32]

2.9 Molecular docking

An essential step in the discovery of new drugs is molecular docking, which was used to stimulate the interaction between a protein and its ligand. Targeted protein structures were initially discovered, and tiny ligand molecules were subsequently docked to them at the atomic level. The calculated binding free energies in kcal/mol was then used to validate the interactions and reduce the expense and time associated with finding new drugs. *Invitro* and in vivo tests are required for their further validation[33].

2.10 ProTox II's assessment of safety parameters

ProTox II, a freely accessible online webserver, predicts toxicity such as immunotoxicity, hepatotoxicity, cardiotoxicity, and cytotoxicity based on pharmacophores and similarity of molecules to analyse the safety characteristics of substances. ProTox II processes input in the form of a molecule's 2D structure or PuB chem ID and provides the anticipated toxicity profile. Lethal dosage (LD50) and class toxicity profiles of compounds were established using PuB chem ID or canonical smiles of the ligand[34].

2.11 Evaluation of ADME properties

Important factors including druggability, pharmacokinetic property (absorption, distribution, metabolism, and excretion), ADME, water-solubility, physicochemical characterisation, and lipophilicity for the chosen compounds were predicted using Swiss ADME (http://www.swissadme.ch/), a publicly available tool. The boiled egg model can also be used to estimate their bioavailability[35].

3. RESULTS

For the identification and purification of polyphenols from diverse plant extracts, HPLC is reported to be a suitable method. Multiple HPLC fingerprints demonstrates the capacity for good resolution and separation of complicated mixtures as well as the ability to identify distinct enriched fractions. Gallic acid was detected using an HPLC chromatogram. Gallic acid's retention period was found to be 2.918 ±1.97 minutes. Peak intensity and area of gallic acid demonstrated the purity and concentration (**Fig.1**).

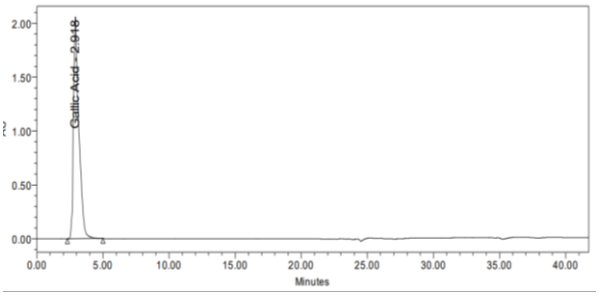


Figure 1: HPLC analysis of Gallic acid.

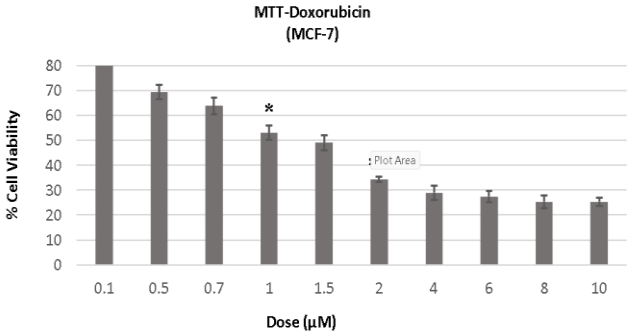


Figure 2a

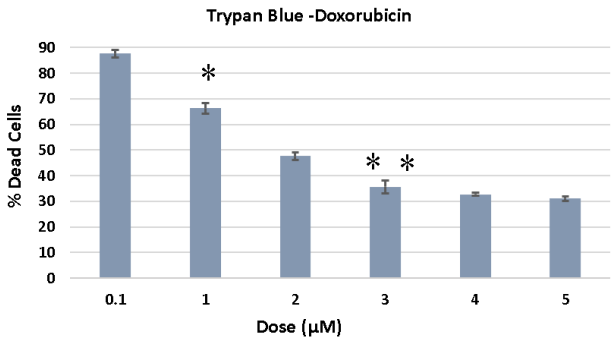


Figure 2b

Figure 2: MTT Assay for Human breast adeno-carcinoma MCF-7 cells. (a) Cell cytotoxicity assay with doxorubicin

dose ranging from 0.1 to 10 µM concentrations for 48 h. **(b)** Trypan blue dye exclusion assay for different concentrations of doxorubicin ranging from 0.1 to 5 µM.

For optimization of IC_{50} dose, cell viability assay with various concentration of doxorubicin were performed on breast cancerous cells. MCF-7 cells were induced with doxorubicin for 48 h in concentration ranging from 0.1 µM to 10 µM. Cellular death of about 50% was noticed at 1 µM doxorubicin (**Fig. 2a**). 1 µM doxorubicin concentration was further validated by Trypan blue dye exclusion assay and was chosen for following experiments (**Fig. 2b**).

Similar dose of Doxorubicin was explored on H9C2 cells and the cardiac cell viability was measured to investigate the effects of doxorubicin on cardiomyocytes in concentration dependent manner. At the concentration of 1 µM around 24 % cell death was recorded in H9C2 cells while at 10 µM it increased up to 40%. (**Fig .3**).

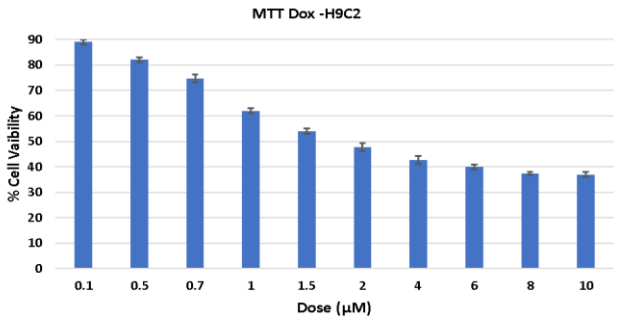


Figure 3: Cell cytotoxicity assay on H9C2 cardiomyocytes (a) MTT Assay with doxorubicin dose varying from 0.1 - 10 µM concentrations induced for 48 h.

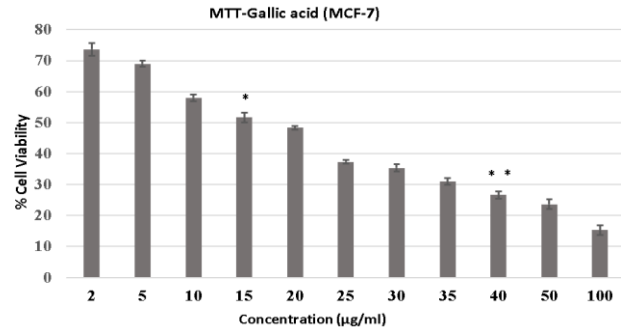


Figure 4a

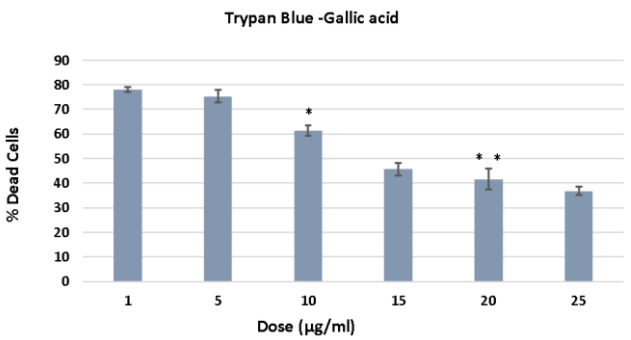


Figure 4b

Figure 4: (a) Gallic acid MTT for 48 hours with concentrations ranging from 2 to 100 µg/ml. (b) Trypan blue dye exclusion assay for different concentrations of Gallic acid ranging from 1 to 25 µM.

HPLC tested gallic acid was selected as herbal interventional study on MCF-7 and H9C2 cell lines. Initially, cell viability assay was performed to optimise IC50 dose using various doses in concentrations dependent manner. Concentration range of 2 -100 µg/ml was used on MCF-7 cells as induction for 48 h. Around 50% cell death was observed at 15 µg/ml gallic acid (**Fig. 4a**). The IC50 concentration was chosen for further experiments after being confirmed by the Trypan blue dye exclusion assay at the dose of 15 µg/ml of gallic acid (**Fig. 4b**). The effect of the optimized dose was further tested on H9C2 cells to observe the effect (**Fig. 5**).

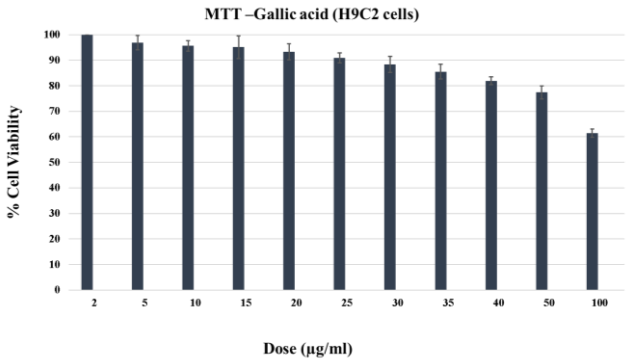


Figure 5: MTT assay on H9C2 cardiomyocytes. Cell cytotoxicity assay of gallic acid on H9C2 cells.

Further, experiments were carried out using different doses of doxorubicin and Gallic acid to investigate the synergistic effect of these compounds on the growth of cancer cells. When Gallic acid and Doxorubicin were used in combination at their optimized IC_{50} concentrations, around 72% of the cells died confirming the synergistic cytotoxic effect. Further experiments were designed using lower Doxorubicin doses. When 0.1 and 0.5 µM doxorubicin were added with 10 µg/ml of gallic acid, it was found that up to 40% and 50% of the cells died, respectively confirming that supplementing gallic acid is sufficient to reduce the Doxorubicin to kill the cancer cells. On healthy cardiomyoblasts synergistic effect of doxorubicin and Gallic acid was observed as cardiac cell death was resisted in the presence of simultaneous treatment, as demonstrated by the increase in cell viability by 14%, 20%, and 24% in the presence of 0.5, 0.7, and 1 µM Dox, with and without gallic acid respectively (**Fig. 6**). Thus, gallic acid was observed to counteract the cancer cell death effect of Doxorubicinonly at lower dose.

Gallic acid and Doxorubicineffects were further confirmed by morphological studies. Giemsa staining was used to validate the morphological alterations, which showed considerable cell death as well as distorted morphology of the cells in presence of Gallic acid and Doxorubicin(**Fig. 7a and 7b**).

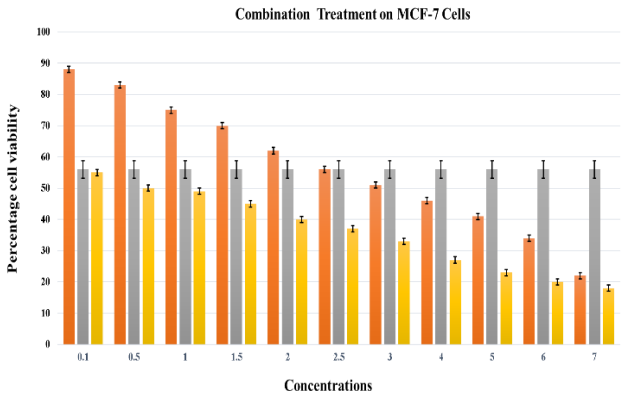


Figure 6: Combined effects of doxorubicin and curcumin on MCF-7 cells.

In order to predict the binding affinity of gallic acid and Doxorubicinmolecules with BRCA1 and BRCA2 molecular docking was performed using AutoDock vina tool. ProTox II was used to do insilico toxicity prediction in order to examine the undesired effects of phytoconstituents. The investigated compounds in the present study were the drug doxorubicin and the phytoconstituent gallic acid. Doxorubicin was classified as a class 3 molecule and gallic acid as a class 4 molecule, meaning that both are harmful when consumed (50 < LD50 ≤ 300) (**Fig. 8a-b**). Also, the Lethal dose (LD50) for gallic acid was found to be 2000 mg/kg and for Doxorubicin205 mg/kg, confirming the potential of the natural ingredients as a possible therapy and demonstrating its short-term use in order to prevent cardiotoxicity respectively.

On estimation of ADME properties molecular weight for Gallic acid and Doxorubicin was found to be 170.12 g/mol and 543.5 g/mol correspondingly. Their Pharmacokinetic characteristics, which include distribution, metabolism, excretion, and absorption, were evaluated. Based on the Swiss ADME recommendations, our molecules considered optimal when their molecular weight was below 500 g/mol, which is the lowest allowed threshold for molecules to pass through biological membranes. Also, our chosen molecule met the requirements of Lipinski's rule of five and had optimal lipophilicity. Thus, Gallic acid was found to be ideal molecule in all parameters.

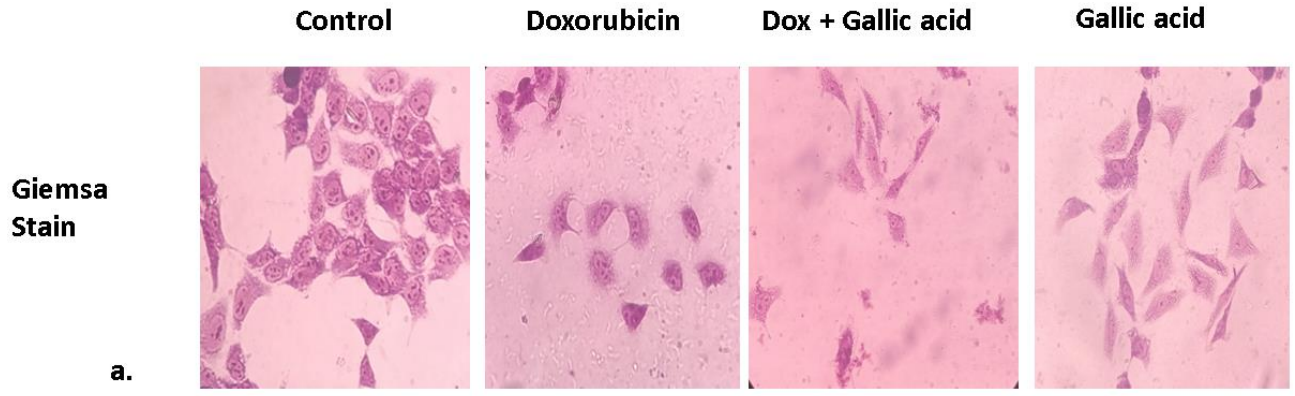


Figure 7a

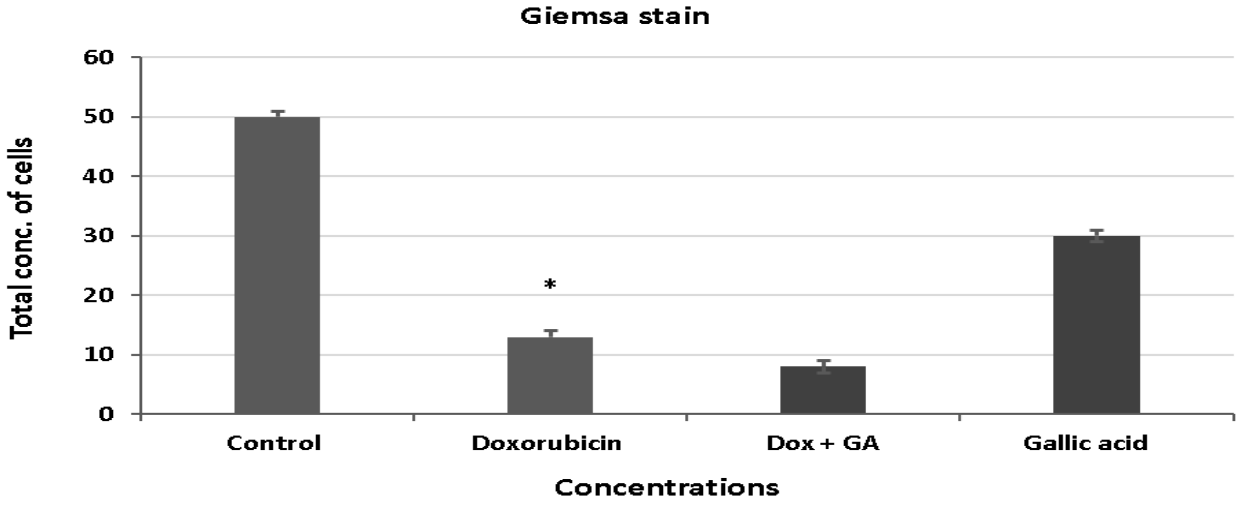
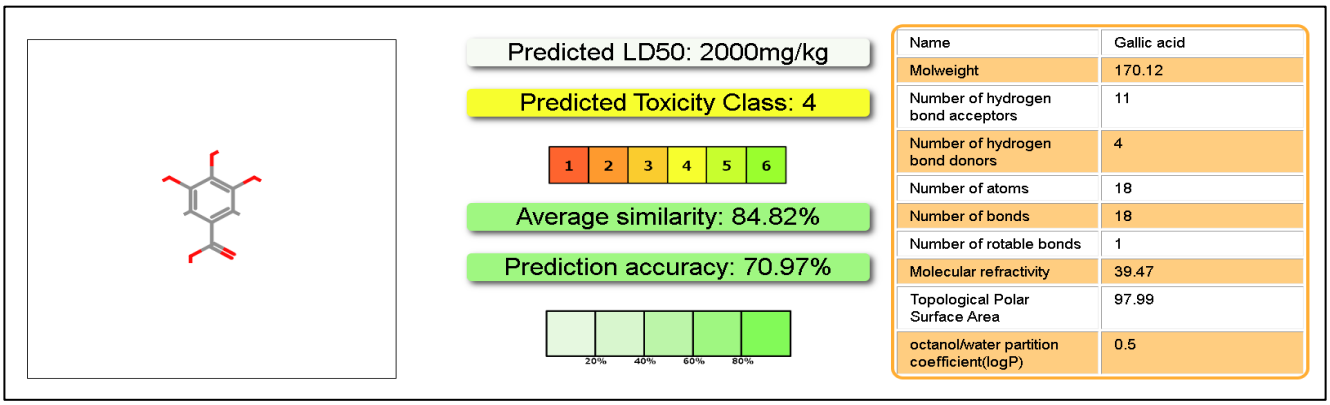
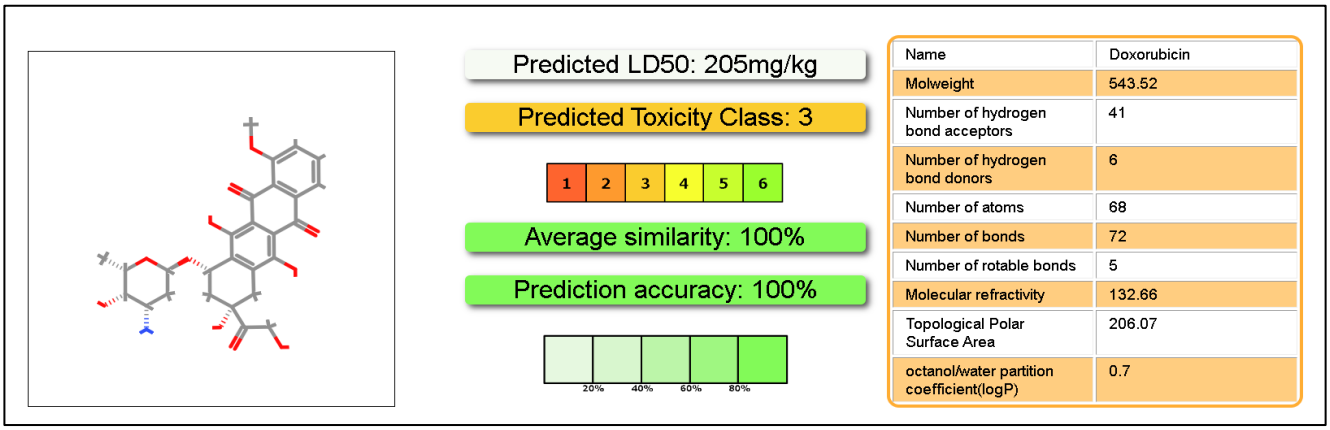


Figure 7b

Figure 7: Morphological changes of MCF-7 cells when treated with Gallic acid alone, Doxorubicin alone and in combination with Doxorubicin. (**a).** MCF-7 cells from each of the groups were observed using Giemsa staining under a microscope at x 40 magnification, to investigate how cells respond to various treatments. (**b**). Percentage of total concentrations of cells treated with Doxorubicin and Gallic acid were determined.



Name	Gallic acid
Molweight	170.12
Number of hydrogen bond acceptors	11
Number of hydrogen bond donors	4
Number of atoms	18
Number of bonds	18
Number of rotable bonds	1
Molecular refractivity	39.47
Topological Polar Surface Area	97.99
octanol/water partition coefficient(logP)	0.5



Name	Doxorubicin
Molweight	543.52
Number of hydrogen bond acceptors	41
Number of hydrogen bond donors	6
Number of atoms	68
Number of bonds	72
Number of rotable bonds	5
Molecular refractivity	132.66
Topological Polar Surface Area	206.07
octanol/water partition coefficient(logP)	0.7

Figure 8: Estimation of oral toxicity for selected compounds.

Protein Molecules 4OLE and 1MIU were downloaded from the rcsbpdb in order to estimate the binding affinity. The selected ligands were drug Doxorubicin and the phytoconstituent gallic acid. Polar hydrogen atoms and Kollman charges were added to downloaded PDB protein structures using the AutoDock Vina in order to prepare them. Grid box parameters were located by using PDBQT files that were created for the corresponding proteins and ligand molecules. The resulting log file displayed the RMSD values and the negative Gibbs free energy (ΔG) in (Kcal/mol). Following docking, these data were examined with Discovery Studio 2020 software to identify various hydrophobic interactions, hydrogen bonds, and charges. Consequently, for every ligand, the optimal energy conformations were chosen. Using the autodock tool to dock interactions, the BRCA1 receptor had the lowest binding energy (-8.3 kcal/mol) in its first pose, indicating the highest affinity with Doxorubicin, with gallic acid it was observed to be (-6.0 kcal/mol). While for BRCA2 the binding energy was (-6.0 kcal/mol) with Doxorubicin and with Gallic acid it was found to be (-5.9 kcal/mol) (**Fig. 9a-d**).

4. DISCUSSION

Since *S. cumini* has been shown to contain several polyphenols, among which gallic acid is a key component, we therefore carried out an HPLC investigation of the gallic acid (phenolic acid) concentration[36]. Without compromising its anti-cancer properties, the cardioprotective effect of gallic acid was investigated in relation to the Dox-induced cardiotoxicity. Dox-treated cancer patients have been found to be more susceptible to cardiovascular problems[37]. Gallic acid, a well-known anti-cancer polyphenol, is currently explored for its cardio-protective properties. Therefore, we hypothesized that supplementing Gallic acid and related plant such as *Digitalis lanata, Tinospora cordifolia, Terminalia arjuna* and *Atropa belladonna* cardiotoxic medicines may reduce the related toxicity[38]. To study the effects of Doxorubicin and gallic acid individually and in combination, as anticancerous agent, MCF-7 Human breast adeno-carcinoma cell lines was used. The earliest hormone-responsive cancer cell lines are called MCF-7, and they have been extensively utilised in several research throughout the world[39]. To investigate the cardiotoxic effects of Dox, H9C2 cardiomyocytes were used. H9C2 cells can be used as a model for in vitro research on cardiac disorders since they exhibit stress responses that are similar to those seen in primary cardiomyocytes[40-42].

The effects of Doxorubicin and gallic acid alone and in combination on cancer cells were examined in the current study by dose-dependent cell viability tests, followed by morphological changes analysis. In order to determine the combination of gallic acid and Dox's synergistic effects on MCF-7 cells, dose-mediated cell viability tests using the MTT assay were conducted initially. The oxido-reductase enzymes generated by the mitochondria of live cells are converted by the MTT dye into formazon in the MTT test, a calorimetric method for quantifying living cells[43-44]. By using a trypan blue dye exclusion cell viability experiment, which distinguishes between dead and living cells based on the integrity of the cell membrane, results were further validated.

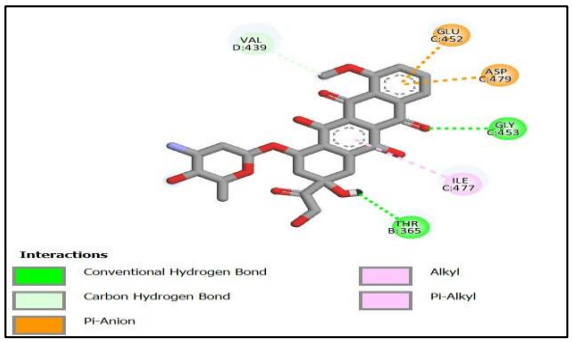
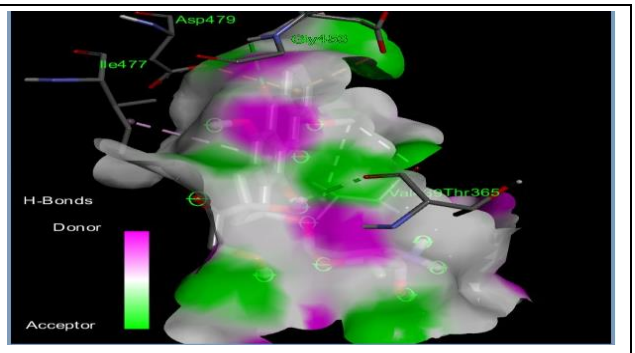
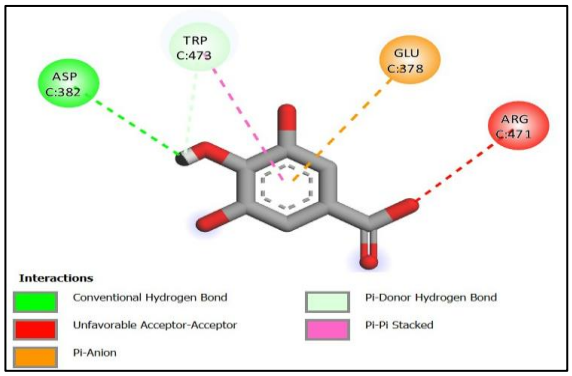
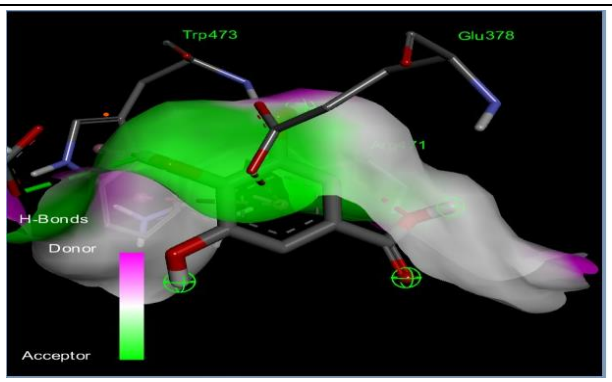
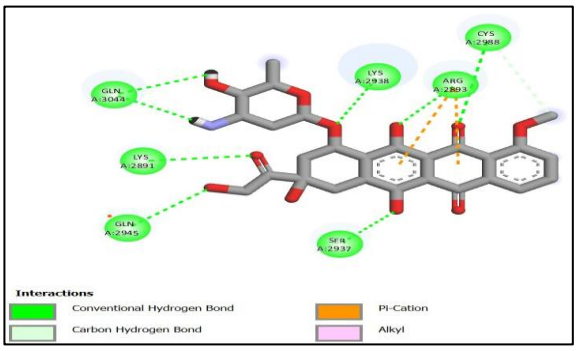
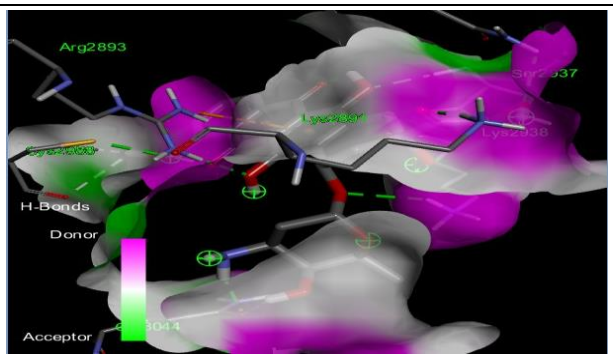
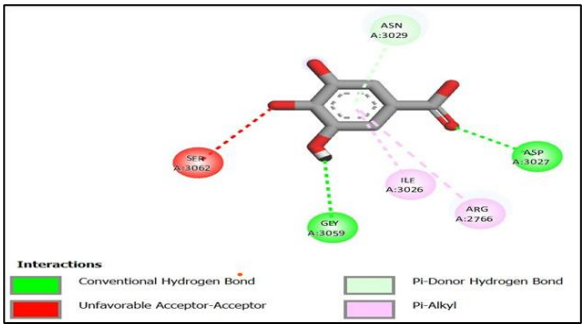
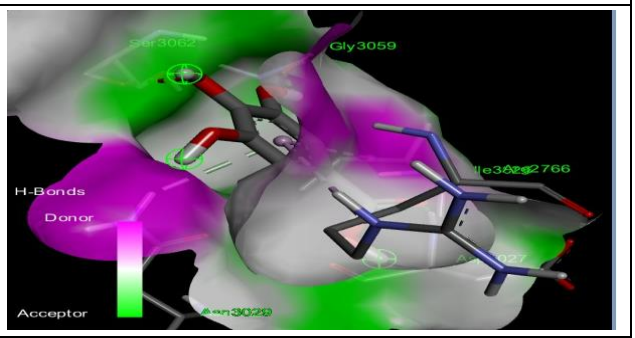
9.a		
	2D: BRCA1 : Dox	**3D: BRCA1 : Dox**
9.b		
	2D: BRCA1 : Gallic acid	**3D: BRCA1 : Gallic acid**
9.c		
	2D: BRCA2 : Dox	**3D: BRCA2 : Dox**
9.d		
	2D: BRCA2 : Gallic acid	**3D: BRCA2 : Gallic acid**

Figure 9: Molecular Docking studies

The IC50 dose optimised by MTT assay and trypan blue for Doxorubicin and gallic acid was 1 µM and 15 µg/ml, respectively. More than 72% of the cancerous cell death was visualised when these optimised IC_{50} doses were combined. While at lower dose of Doxorubicin 50 % cell death was observed treated simultaneously with gallic acid. Potential toxicity of the anti-cancerous amounts of gallic acid and Doxorubicin were also examined. Monitoring cardiotoxic side effects is extremely important since cardiac myocytes are terminally differentiated cells that do not continue to divide after any form of cellular death caused by any stress. More than 30% of the cardiac cell death was observed at Dox's anti-cancer IC50 dose showing cardiotoxicity, but gallic acid was cardioprotective. This

implies that at significantly lower doses the therapy with gallic acid enhances the anti-cancer potential of Doxorubicin and helps cardiac cells to prevent Dox-induced cardiotoxicity. There is a pressing need for novel approaches with reduced or no side-effects as cardiotoxicity is a significant barrier to emerging cancer therapies. Natural products have been investigated for their wide variety of properties in order to accomplish beneficial effects in patients for longer duration. To increase the effectiveness of current chemotherapy and minimize drug-induced cardiotoxicity, plants having anti-cancerous and cardio-protective compounds can be included

5. CONCLUSION

Our study supported that Gallic acid can serve as important formulation for cardio-oncological therapeutics interventions. However, in future, pharmacodynamic and pharmacokinetic herb-drug interactions, co-administration of prescribed drugs and herbal supplements should be thoroughly investigated.

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Conflict of Interest

There is no financial or other conflict of interest.

Availability of data and material

The paper includes in silico data as well as relevant details.

Ethics approval

No prior ethical approval is required in the study

Consent to participate

Not applicable

Consent for publication

Not applicable

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