



Concomitant Therapy of Aq. *Theobroma* Extract and Doxorubicin Reduces Stemness and Induces Ferroptosis in Therapeutic Resistant Cervical Cancer Cells

Prasanthi Chittineedi¹, Santhi Latha Pandrangi^{1*}, Gooty Jaffer Mohiddin², Juan Alejandro Neira Mosquera^{2,3}, Sungey Naynee Sánchez Llaguno²

¹Onco-Stem Cell Research Laboratory, GITAM School of Sciences, GITAM Deemed to be University, Visakhapatnam, India; ²Department of Life Sciences and Agriculture, Armed Forces University - ESPE, Santo Domingo 230101, Ecuador, South America; ³Department of Industry and Production Sciences, Quevedo State Technical University, km 11/2 via Santo Domingo, Quevedo, Ecuador, South America.

ABSTRACT

Background: Doxorubicin (Dox) is one of the potent antineoplastic drugs currently used as a chemotherapeutic agent for several cancers. Numerous studies suggested that multiple chemotherapeutic cycles with Dox might lead to development of drug resistance and cardiotoxicity in patients leading to poor prognosis, and survival outcome. Present study aims to lower the drug dosage of Dox without altering its antitumor potential. Aqueous *Theobroma* Extract (ATE) has been reported previously to have several anti-tumor properties and is known to be used as a traditional medicine in the treatment of various diseases without any adverse effects like cardiotoxicity and hepatotoxicity. In the present study we demonstrate that ATE when used in combination with Dox exhibited a synergistic antitumor effect, sensitizes therapeutic resistant cervical cancer cells through induction of ferroptosis thereby reducing both the dosage and adverse side effects imposed by this chemotherapeutic drug.

Materials and Methods: Initially, we developed carboplatin-resistant cervical cancer cells for analyzing the antitumor effects of ATE and the synergistic effects of ATE with respect to Dox. Gene expression analysis using RT-PCR and flow cytometry studies were performed to analyze the expression of target genes. Intracellular ferritin levels, lipid ROS levels were estimated to check ferroptosis induction. Lastly cell cycle analysis and apoptosis analysis were performed to confirm cell death.

Results: Previous studies demonstrated that CSCs are the root cause of cancer and are responsible for tumor recurrence and relapse. Hence it is imperative but these CSCs should be targeted to achieve complete cure for cancer. ATE showed synergistic anticancer effects with respect to Dox and potentially induced cancer cell death in drug-resistant cervical cancer cells by triggering both apoptosis and ferroptosis by downregulating Bcl-2 and degrading ferritin respectively. Also, ATE in combination with Dox reduced the expression of stem cell markers which are evaluated by RT-PCR and flow cytometry. On the other hand, cell cycle analysis and apoptosis analysis suggest that the cell has been arrested through cell death induction. Interestingly, dox when used with ATE potentially induced cancer cell death in drug-resistant cervical cancer cells at lower concentrations. Our results suggest that reduced concentration of Dox in combination with ATE induces cancer cell death through induction of ferroptosis and also reduces the risk of cardiotoxicity.

Conclusion: Based on our results, we conclude that ATE and Dox combination therapy reduces drug-resistance and enhances cervical cancer prognosis associated with reduction of Dox dosage also protecting the cells from cardiotoxicity.

Keywords: *Theobroma spp*; Doxorubicin; Ferroptosis; Cervical cancer; Therapeutic resistance; Stemness; Apoptosis

Correspondence to: Santhi Latha Pandrangi, Onco-Stem Cell Research Laboratory, GITAM School of Sciences, GITAM Deemed to be University, Visakhapatnam, India, E-mail: dpandran@gitam.edu

Received: 06-Jun-2022, Manuscript No. JCM-22-16937; **Editor assigned:** 10-Jun-2022, Pre QC No. JCM-22-16937 (PQ); **Reviewed:** 29-Jun-2022, QC No. JCM-22-16937; **Revised:** 08-Jul-2022, Manuscript No. JCM-22-16937 (R); **Published:** 18-Jul-2022, DOI: 10.35248/2157-2518.22.S32:001.

Citation: Chittineedi P, Pandrangi SL, Mohiddin GJ, Mosquera JAN, Llaguno SNS (2022) Concomitant Therapy of Aq. *Theobroma* Extract and Doxorubicin Reduces Stemness and Induces Ferroptosis in Therapeutic Resistant Cervical Cancer Cells. J Carcinog Mutagen. S32:001.

Copyright: © 2022 Chittineedi P, et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

INTRODUCTION

Cervical cancer is one of the most common malignancies with a high incidence and mortality rate among all other gynecological cancer. Current treatment strategies include surgical removal combined with radiation or chemotherapy such as Doxorubicin, paclitaxel, cisplatin, etc. However, these therapeutic strategies could not yield a better prognosis due to their adverse side effects and is also responsible for tumor relapse [1]. Previous studies demonstrate that Cancer Stem Cells (CSCs) are the crucial drivers for tumor relapse and recurrence because of their tumor initiating potential [2,3]. Administration of Dox is known to cause adverse effects such as fatigue, alopecia, nausea, oral sores, and increased the risk of secondary malignancies [4]. Dox is a microbial-derived anthracycline used to treat several malignancies such as breast, cervical, gastric, ovarian, and more. Dox acts on tumor cells in either of the two ways- it either disrupts topoisomerase-II mediated DNA repair by intercalating with the DNA leading to apoptosis or damaging the cellular membranes, proteins, and DNA by the generation of free radicals resulting in the accumulation of lipid peroxides and causing ferroptosis [5,6]. Although Dox is a potent anti-cancer drug, it has its limitations. Firstly, Dox induces cardiotoxicity [7]. Secondly, although Dox is a clinically valuable anti-cancer agent, studies showed that patients might develop therapy resistance via modulating ABCB1, ABCC1, and other genes encoding efflux pumps [8]. Figure 1 represents the hypothesis of the present study.

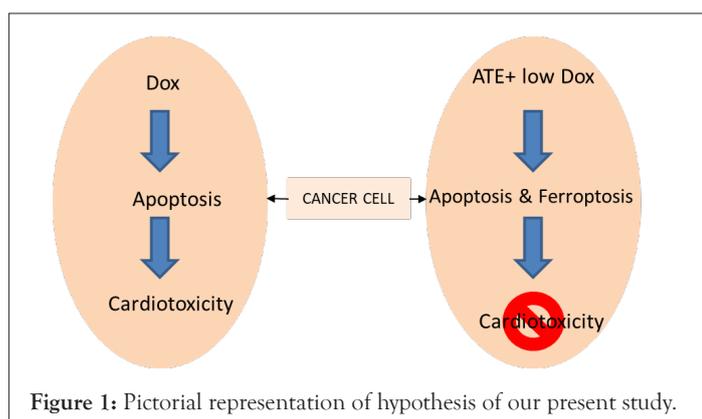


Figure 1: Pictorial representation of hypothesis of our present study.

Due to these adverse effects of these potent anti-neoplastic drugs, development of therapy resistance, incidence of cancer relapse, and recurrence, the focus was shifted towards the mode of action of nutraceuticals [9]. Nutraceuticals could be defined as active compounds that can fight against any health disorders and are derived from natural food constituents. These are often regarded as dietary supplements that are provided with carbohydrates, proteins, lipids, probiotics, phytochemicals, and enzymatic regulators [10]. Cocoa powder derived from *Theobroma cacao* is a rich source of many bioactive compounds such as epicatechin, catechin, and procyanidins which have medicinal properties and are widely being used to cure inflammation and other diseases [11,12]. Due to their high antioxidant and pro-oxidant activities, recent studies have gained attention towards procyanidins due to their anti-hypertensive, anti-aging, anti-cancer, and cardioprotective effects [13].

Interestingly, literature suggested that the active compounds present in *Theobroma* spp shows synergism to Dox in potentially inhibiting topoisomerase enzymes [14]. DNA topoisomerases are the enzymes involved in DNA repair mechanisms during their replication, recombination, transcription, and even chromatin remodeling. Individual studies performed by John L Nitiss et

al., and Gouveris et al., suggest that topoisomerase-I is a crucial target for treating various malignancies as it is upregulated in solid tumors [15–17]. Apart from suppressing the topoisomerase activity, these topoisomerase inhibitors have the potential to suppress HIF-1 α , which regulates the tumor microenvironment by maintaining hypoxic conditions [18]. Interestingly, suppression of HIF-1 α leads to inactivation of VEGF and PDGF- β , which promote cancer cell proliferation, stabilize novel blood vessels, and reduce the penetration of anti-cancer drugs, which might be the potent reason for therapy resistance and cancer relapse [19,20]. Also, studies show that HIF-1 α inhibits ferritin degradation thereby inhibiting ferroptosis [21]. The present study focuses on inducing ferroptosis in drug-resistant cervical cancer cells through reducing Dox dosage and to evaluate the synergistic effect of ATE towards Dox. Hence to study the antineoplastic effect of dox at lower concentrations and to evaluate the synergistic effect to ATE all the experiments were performed with half of the IC₅₀ value of Dox obtained from MTT assay while keeping ATE concentration constant.

MATERIALS AND METHODS

Cell line maintenance

The human cervical cancer cell lines HeLa were procured from NCCS. HeLa cells were derived from cervical tumor tissue derived from glandular tissue and is considered to be a rare adenocarcinoma. The cell line was found to be free from mycoplasma contamination. They were maintained carefully as described in our previous study [22]. Briefly, cells were grown in DMEM media (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS, 1% Penstrep, and 1% Glutamax (Invitrogen, Carlsbad, CA, USA). The media was changed every two days and incubated in a 5% CO₂ incubator. When the cells were 80% confluent, they were used for further analysis. Theobromine and Doxorubicin were procured from Sigma-Aldrich (St Louis, MO, USA).

Development of drug-resistant clones

To investigate the anti-cancer effects of ATE in combination with Dox in drug-resistant cervical cancer cell lines we developed Carboplatin resistant cervical cancer cell lines (CarbR-HeLa) as described in our previous study [23]. For this, we seeded the cells in 35 mm petri plates and grew till the cells reached 80% confluency. The cells were then treated with IC₅₀ of carboplatin for 48 hours. After incubation, the drug-containing media was replaced with fresh DMEM and the cells were allowed to grow till the cells become 80% confluent. Once the cells reached 80% confluency the cells were trypsinized and exposed to double the dose of carboplatin. This process was repeated 6 times and subsequently, MTT assay was performed.

MTT assay for validating CarbR- HeLa cell lines

To study the cell death kinetics, the cells seeded in 96-well plate were exposed to increasing concentration of carboplatin for 48 hours and evaluated cell survival by MTT assay. Briefly, cells with 80% confluency were treated with various concentrations of carboplatin and incubated for 48 hours at 37°C in a 5% CO₂ incubator. After incubation, the cells were replenished with 200 μ L DMEM, 2 mg/mL concentration of 50 μ L MTT (Sigma-Aldrich), and the plate was incubated for 3 hours. After incubation, the MTT containing media was replenished with 200 μ L DMSO to dissolve the formazine crystals and the absorbance was measured at

570 nm immediately using a plate reader.

Preparation of aqueous theobroma extract

Theobromine procured from Sigma Aldrich was used in the present study. 0.5 g of theobromine was weighed and is dissolved in 10 mL autoclaved distilled water. 50 mg/mL aqueous theobromine extract (ATE) was then placed in the shaker at room temperature for 48 hours and then centrifuged at low speed for 10 mins. The aqueous extract was collected and further filtered using a 0.4 μ syringe filter. The filter ATE was stored at 4 °C till further analysis.

MTT assay and drug treatment

The cytotoxicity effect of ATE and Dox was determined by MTT assay [23]. Briefly, 80% confluent cells placed in 96-well plate were treated with increasing concentrations of ATE and Dox respectively and incubated for 24 hours in 5% CO₂ incubator. After incubation, the cells were replenished with 200 μ L DMEM media, 50 μ L 2 mg/mL MTT (Sigma Aldrich) and plate was incubated for 3 hours at 37 °C in 5% CO₂ incubator. After incubation, the MTT containing media was replenished with 200 μ L DMSO to dissolve the formazine crystals and the absorbance was measured at 570 nm immediately using a plate reader. All the experiments were carried out in 35 mm Petri plates. When the cells reached 80% confluency, the cells were treated with obtained IC₅₀ for ATE while half of the IC₅₀ concentration was used for Dox. The plates were incubated for 24 hours in a CO₂ incubator and were used for the experiments.

Cell viability assay

To assess the cell viability, we performed trypan blue exclusion uptake test. Briefly, cells treated with ATE (1.2 mg/mL) and Dox (0.187 μ M) were washed with 1X PBS twice and 0.4% of trypan blue solution was added. The plates were incubated for 5 mins and then washed with 1X PBS to eliminate background dye. Total number of viable cells present in control were normalised to 100% viability and number cells in drug treated plates were counted.

Clonogenic assay

To evaluate the synergistic effects of ATE and Dox in inhibiting cell colony formation we performed clonogenic assay as described in our previous study with slight modifications [24]. Briefly, CarBR-HeLa was trypsinized and the 100 μ L of the single-cell suspension was seeded in 35 mm Petri plates and the cells were allowed to adhere overnight. The following day the growth media was replaced with media containing appropriate concentrations of ATE (1.2 mg/mL) and ATE along with Dox (1.2 mg/mL + 0.187 μ M), and one plate was replenished with growth media without any drugs served as control. After incubating the plates for 24 hours in 5% CO₂ incubator the drug-containing media was replenished with fresh media and pictures were taken using EVOS FL imaging system (Thermo Fischer Scientific).

Evaluation of cellular migration through scratch assay

To evaluate the cell migration ability of CarBR-HeLa cells treated with desired drugs we performed a scratch assay. To perform this assay, we seeded CarBR-HeLa cells in 35 mm Petri plates and allowed them to form a confluent layer. Once the cells reached 100% confluency the cells were washed and replenished with media, ATE (1.2 mg/mL) and ATE + Dox (1.2 mg/mL + 0.187 μ M) were added into the plates and a scratch was drawn across

the plate using a sterile tip. The cells were incubated in 5% CO₂ incubator for 24 hours. After incubation the images were captured using EVOS FL imaging system (Thermo Fischer Scientific).

Cell invasion assay

To evaluate the synergistic effect of ATE with respect to Dox on invasiveness of CarBR-HeLa cells we performed cell invasion assay. To perform this assay 80% confluent CarBR-HeLa cells were treated with ATE (1.2 mg/mL) and ATE + Dox (1.2 mg/mL + 0.187 μ M) for 24 hours. Untreated cells served as control. After incubation, cells were washed with 1X BS followed by replenishing the cells with serum free media and incubated for 24 hours. After incubation, the pore culture inserts were placed on to the matrigel pre-coated 24 well plates and 800 μ L of 10% DMEM is added. The plate was incubated with Matrigel for 30 mins at 37 °C before the pore culture inserts were placed. The serum starved cells were trypsinized and 100 μ L of the trypsinized single suspension was added to the respective wells and incubated under normal conditions for 48 hours. After incubation the inserts were removed, the cells were washed with PBS and fixed the cells with 5% 500 μ L glutaraldehyde solution for 30 mins. The cells were washed with PBS after incubation and stained with 500 μ L of 0.2% crystal violet solution for 30 mins and rinsed with distilled water. Non migrated cells were removed using cotton swab from the upper side of the insert. The membranes were dried overnight and then they were cut from the insert. The membranes were placed onto the microscopic slide and covered with a coverslip and observed under the EVOS FL imaging system (Thermo Fischer Scientific).

RNA extraction and RT-PCR gene expression analysis

To evaluate the gene expression at transcriptional level we performed RT-PCR analysis. Briefly, 80% confluent CarBR-HeLa cells were treated with ATE (1.2 mg/mL) and ATE + Dox (1.2 mg/mL + 0.187 μ M). The cells were incubated in 5% CO₂ incubator and after incubation total RNA was isolated using the TriZol reagent (Takara). The extracted RNA was converted to cDNA using a high-capacity cDNA reverse transcription kit by Applied Biosystems as per the instructions provided in the kit. The obtained cDNA was used to carry out gene expression studies using Applied Biosystems Real-Time PCR equipment with SYBER green master mix as the detector. Following genes were used to evaluate the gene expression- HIF-1 α , VEGF, CD24, and Bcl-2. GAPDH was used as an internal control. The sequence of the primers is listed in Table 1.

Stem expression analysis using flow cytometer

To evaluate the expression of stem cell marker ALDH1A1 in CarBR-HeLa cells flow cytometry analysis was carried out. Briefly the cells were treated with ATE (1.2 mg/mL) and ATE + Dox (1.2 mg/mL + 0.187 μ M) and were incubated at 37 °C in 5% CO₂ incubator for 24 hours. After incubation, the drug-treated CarBR-HeLa cell lines were trypsinized to form single cell suspension. The trypsinized cells were washed with ice-cold PBS twice. The pellet was suspended with ALDH1A1 primary antibody and incubated at 4 °C for about 30 mins. After incubation, the cells were washed and incubated subsequently with FITC conjugated secondary antibody. After incubation, the cells were washed with ice-cold PBS and the expression was analyzed using a flow cytometer.

Estimation of intracellular ferritin levels

To evaluate the intracellular ferritin levels the CarBR-HeLa cells

were treated with ATE alone (1.2 mg/mL) and in combination with Dox (1.2 mg/mL + 0.187 μ M) for 24 hours. After drug treatment the cells were washed with 1X PBS and were incubated in DMEM containing 100 mM Ferric Ammonium Citrate (FAC) for 24 hours in a 5% CO₂ incubator. To terminate iron accumulation by the cells, the cells were washed with 2 mL of ice-cold PBS twice. After washing the cells, 50 mM NaOH was added to the plate, and the cells were placed on a shaker for 2 hours. The cell lysate was collected in Eppendorf and analysed intracellular ferritin levels using COBAS auto-bioanalyzer provided along with an iron estimation kit. CarbR-HeLa cells without any treatment were used as control cells.

Cell cycle analysis

To evaluate the cell cycle inhibition potentiality of ATE and ATE with reduced levels of Dox we performed cell cycle analysis using flow cytometry. Briefly, 80% confluent cells were incubated for 24 hours with ATE (1.2 mg/mL) and ATE + Dox (1.2 mg/mL + 0.187 μ M). After incubation, CarbR- HeLa cells were gently trypsinised and washed with 1X PBS by centrifuging at 3000 rpm for 5 mins at 4°C. To the washed cells, 50 μ L of 100 μ g/mL RNase was added, followed by the addition of 200 μ L 50 μ g/mL propidium iodide. The cells were then analyzed using Flow cytometer.

Evaluation of apoptosis by FACS

This experiment was done using Annexin-V FITC Apoptosis Staining/Detection kit purchased from Abcam. Briefly, the CarbR- HeLa cells were exposed to ATE (1.2 mg/mL) and ATE + Dox (1.2 mg/mL + 0.187 μ M) for 24 hours. After incubation, the drug-treated CarbR-HeLa cells were trypsinized to obtain single cell suspension and centrifuged, and the pellet was suspended in 500 μ L 1X binding buffer, 5 μ L Annexin V-FITC, and 1 μ L SYTOX green dye. The tubes were incubated in the dark at room temperature for 10 mins. After incubation, the cells were analyzed using the flow cytometer.

Evaluation of ROS in CarbR-HeLa cell lines

Cellular lipid ROS content is evaluated in drug resistant clones and compared to parental cell lines of cervical cancer cell line. Intracellular Reactive Oxygen Species (ROS) was estimated by using the fluorescent probe, DCFH-DA as per the manufacturer's instructions. Briefly, CarbR-HeLa cell lines were exposed to ATE (1.2 mg/mL) and ATE + Dox (1.2 mg/mL + 0.187 μ M) for 24 hours. After exposing CarbR-HeLa cells to desired drugs for 24 hours the cells were treated with tertiary butyl hydrogen peroxide (100 mM) for 15 min. After incubation, the cells were washed with phosphate buffer saline (PBS, pH-7.4) followed by treating the cells with DCFH-DA (20 μ M) for 30 min. After washing with PBS, the images were recorded using EVOS FL imaging system (Thermo Fischer Scientific).

RESULTS

Analysis of drug-resistant clones and IC₅₀ of ATE and Dox

To obtain drug-resistant clones, HeLa cells were exposed to increasing concentrations of carboplatin on every cycle up to ten cycles. We observed that HeLa cells obtained significant resistant towards carboplatin after a minimum of 6 cycles. To countercheck whether HeLa cells acquired resistance, we performed an MTT

assay with IC₅₀. Surprisingly, we observed 5 times more IC₅₀ value in the drug-resistant clones when compared with the parental cell line.

ATE and Dox enhanced HeLa cell cytotoxicity

Cytotoxicity of ATE and Dox against CarbR-HeLa cell lines treated for 24 hours was measured using MTT assay. Our results suggest both ATE and Dox selectively enhanced the cytotoxicity of Carb-HeLa cells with an IC₅₀ value of 1.2 mg/mL and 0.374 μ M respectively. However, to evaluate the synergistic effects to ATE and anticancer potential of Dox at low concentration, all the experiments were performed in triplicates with half of IC₅₀ for Dox (0.187 μ M) while ATE's concentration was unchanged (1.2 mg/mL).

ATE synergistically reduced cellular viability in CarbR-HeLa cell lines in combination with Dox

To evaluate the synergistic effect of ATE in combination with Dox on cell viability we performed trypan blue staining. Trypan blue is a selective stain that could only stain dead cells as it could not penetrate into live cells due to their intact cell membrane. Interestingly, drug-treated cells were stained blue when compared with control cells suggesting that ATE alone and with Dox synergistically reduced the viability of CarbR-HeLa cell lines.

ATE in combination with Dox synergistically attenuated the colony formation in CarbR-HeLa cell lines

To evaluate the potential of the chosen drugs to inhibit colony formation thereby giving insight into the probable post-treatment behaviour of tumor cells we performed a colony formation assay. Apart from establishing the synergistic efficacy of ATE and dox, this study also hints at its potential advantage to maintain tumor-free survival. To evaluate the ability of the chosen drugs to inhibit colony formation we placed very few cells in culture plates and allowed them to form colonies. ATE shows a synergistic effect in combination with Dox to inhibit the colony formation. Figure 2 demonstrates number of colonies that are formed during efficiency of CarbR-HeLa treated with ATE and Dox.

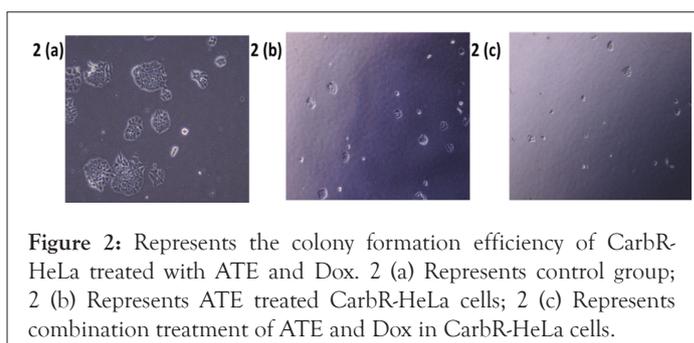


Figure 2: Represents the colony formation efficiency of CarbR-HeLa treated with ATE and Dox. 2 (a) Represents control group; 2 (b) Represents ATE treated CarbR-HeLa cells; 2 (c) Represents combination treatment of ATE and Dox in CarbR-HeLa cells.

ATE showed synergistic effects with Dox and potentially inhibited the cell migration ability

Cell migration assay or scratch assay is used to evaluate the ability of cancer cells to invade tumor surroundings leading to metastasis. To evaluate the potential of the desired drugs to inhibit metastasis we performed the scratch assay. Manually a scratch was drawn across the fully confluent cells and then the cells were allowed to fill up the scratch. However, the ability of scratch closure in treated cells is evaluated by microscopic examination. It could be observed

in Figure 3, that the chosen drugs inhibited cellular migration of Carbr-HeLa cells, outlined by rather a consistent breadth of the scratch, as compared to control cells where scratch almost completely closed at 24 h.

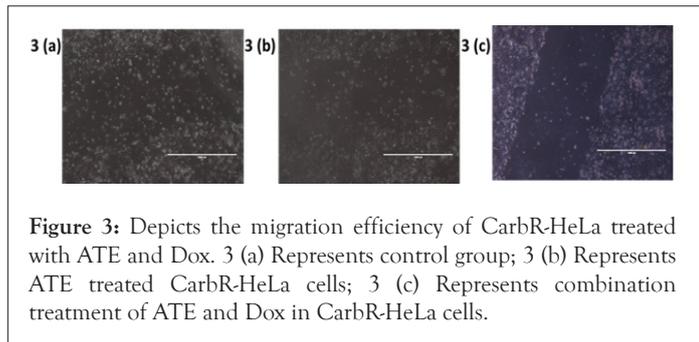


Figure 3: Depicts the migration efficiency of Carbr-HeLa treated with ATE and Dox. 3 (a) Represents control group; 3 (b) Represents ATE treated Carbr-HeLa cells; 3 (c) Represents combination treatment of ATE and Dox in Carbr-HeLa cells.

ATE in combination with Dox synergistically attenuated the invasive capacity of Carbr-HeLa cells

Invasiveness is one of the remarkable features of cancer cells that are solely responsible for metastasis and cancer recurrence. Any antineoplastic drug should not only kill the cancer cells but should also have the potential to inhibit the invasiveness of cancer cells. To evaluate whether our chosen nutraceutical compound is effective to inhibit the invasiveness property of Carbr-HeLa cells in combination with the potent chemotherapeutic drug Dox we performed an invasive assay. As expected, ATE synergistically inhibited the invasive capacity in Carbr-HeLa cells. Figure 4 represents the microscopic images representing the invasion capacity of the Carbr-HeLa cells treated with desired drugs.

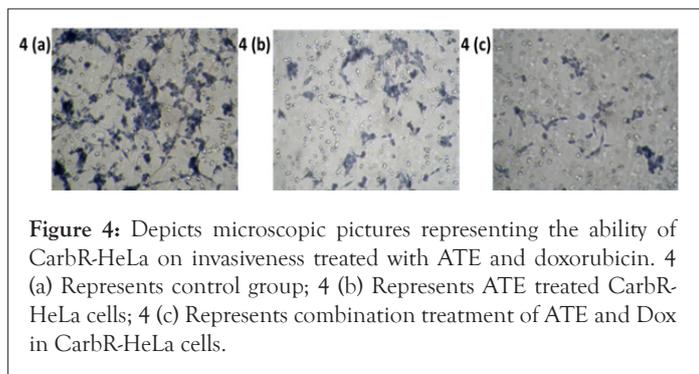


Figure 4: Depicts microscopic pictures representing the ability of Carbr-HeLa on invasiveness treated with ATE and doxorubicin. 4 (a) Represents control group; 4 (b) Represents ATE treated Carbr-HeLa cells; 4 (c) Represents combination treatment of ATE and Dox in Carbr-HeLa cells.

ATE alone as well as in combination with Dox significantly altered the expression of various markers

To evaluate the anti-cancer, anti-proliferative effects of ATE and ATE in combination with commercial drug Dox on Carbr-HeLa cells, RT-PCR and flow cytometry analysis studies were performed. Interestingly, HIF-1 α , VEGF, CD24, and Bcl-2 genes were downregulated in both groups. Figure 5 represents the relative gene expression of specific genes analyzed using RT-PCR. On the other hand, studies state that ALDH1A1 plays a crucial role in contributing drug-resistance and enhancing the cell proliferation [25-28]. Hence to visualise the expression of ALDH1A1 in ATE treated and ATE + Dox treated Carbr-HeLa cells we performed flow cytometry analysis. Surprisingly, our results demonstrated that ALDH1A1 expressions were significantly reduced in both groups when compared with the control. However, ATE and Dox

combination treatment yielded better results when compared with ATE treatment alone. Figure 6 represents the expression of specific stem cell markers in cell lysate analyzed using a flow cytometer.

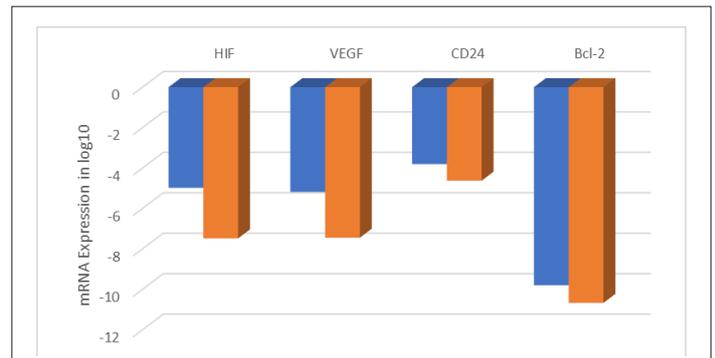


Figure 5: Graphical Representation of mRNA expression of various genes in Carbr- treated and untreated cells. Note: (■) ATE, (■) ATE + Dox

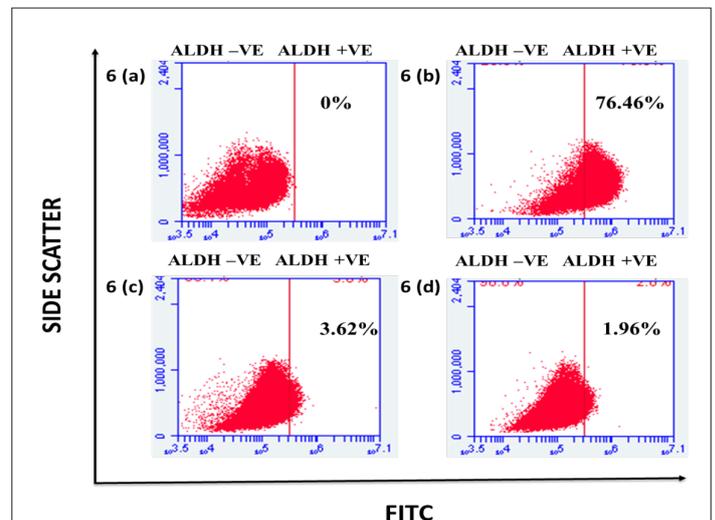


Figure 6: Flow cytometry analysis of expression of ALDH1A1 stem cell marker in Carbr- treated and untreated cells. 6 (a) represents unstained cells 6 (b) Represents control group; 6 (c) Represents ATE treated Carbr-HeLa cells; 6 (d) Represents combination treatment of ATE and Dox in Carbr-HeLa cells.

ATE in combination with Dox significantly reduced ferritin levels when compared with ATE alone

To evaluate the capability of ATE alone and in combination with Dox to induce ferroptosis in Carbr- HeLa cells, we estimated intracellular ferritin levels. We observed a drastic decrease in Intracellular ferritin levels in both groups when compared with the control. To observe whether the cells accumulated FAC, we also assessed the ferritin levels in DMEM media. To evaluate whether there is excess iron in media we determined the iron levels in FAC treated DMEM media after incubation using COBAS auto bio-analyzer. Surprisingly we did not notice any ferritin in the media, suggesting that the cells have accumulated iron from FAC. However, low ferritin levels are detected in drug-treated samples, indicating that the cells might undergo iron-mediated cell death. Table 2 represents the Intracellular ferritin levels estimated in HeLa cell lines treated with the desired drugs.

Table 1: List of primer sequences used in the present study.

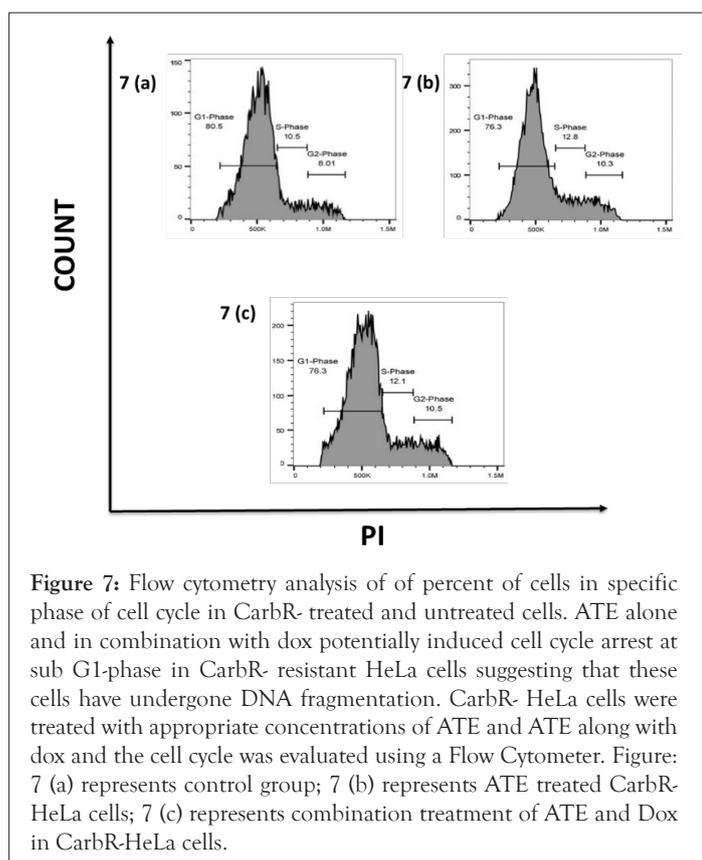
S.No	Primer	Forward Primer	Reverse Primer
1	GAPDH	ACAGTCAGCCGCATCTTCTT	GGCAACAATATCCACTTTACC
2	HIF-1alpha	TGCTCATCAGTTGCCACTTC	AAAACATTGCGACCACCTTC
3	VEGF	CTGCCTCCTGACACTTCCTC	TTTCTTGCGCTTTCGTTTTT
4	CD24	AACTAATGCCACCACCAAGG	GGACTTCCAGACGCCATTTG
5	Bcl-2	CGGCTGAAGTCTCCATTAGC	CCAGGGAAGTTCTGGTGTGT

Table 2: Representation of Intracellular ferritin levels in CarbR- HeLa cells treated with ATE and ATE in combination with Dox.

Sample	Intracellular ferritin ($\mu\text{g/mL}$)
Control	0.69
ATE	0.24
ATE and Dox	0.2

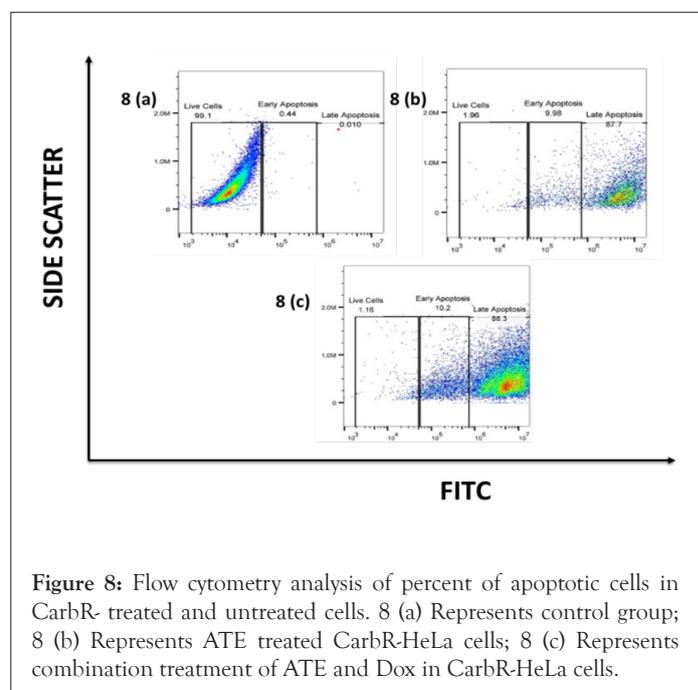
The cell cycle was arrested at sub- G_1 -phase after treating the cells with desired drug dosage

To evaluate the progression of cell cycle in CarbR- HeLa cells after their treatment with ATE alone and in combination with Dox we performed flow cytometry analysis. As depicted in Figure 7 the sub G_1 phase treated with ATE in combination with Dox increased the percent of cell population when compared with ATE alone suggests that the cells were arrested in sub G_1 phase. Our results suggest that ATE in combination with Dox induced nuclear fragmentation in drug-resistant HeLa cells and arrested the cells in the sub- G_1 -phase of the cell cycle. Figure 7 represents the cell cycle arrest induced by the desired drugs in CarbR-HeLa cells.



ATE alone and in combination with Dox potentially induced cancer cell death

We have also examined the total proportion of cells that underwent apoptosis after drug treatment by FACS and AO/EtBr staining. Interestingly, our results were correlated with cell cycle analysis. FACS analysis suggested no to very few numbers of early or late apoptotic cells in control with a high number of live cells in control. However, in drug-treated cells, the number of dead cells increased with a concomitant decrease in live cells. Among ATE and ATE in combination with dox, the latter achieved good results. Figure 8 represents the percent of apoptotic cells.



ATE in combination with Dox synergistically lowered cellular lipid ROS

Intra cellular ROS is very essential for the cell to mediate

ferroptosis because the ROS shows cytotoxic effects when present in high levels. However, little concentration of ROS is essential for cell survival which is utilized by the cancer cells and CSCs. Usually, Intracellular ROS levels triggers ferroptosis by mediating the formation of lipid peroxides. As depicted in Figure 9, the drug-treated cells showed lower cellular ROS levels when compared to untreated CarbR-HeLa cell lines.

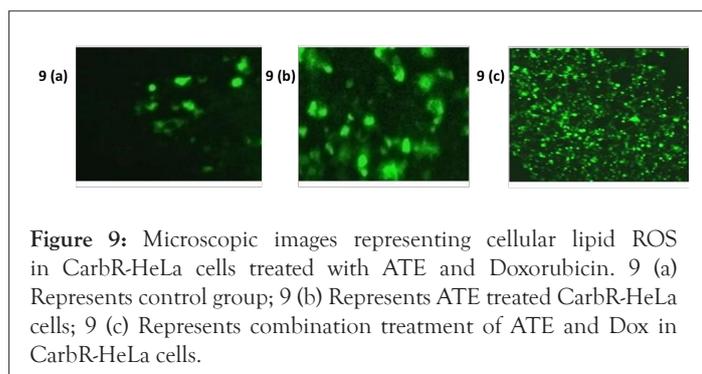


Figure 9: Microscopic images representing cellular lipid ROS in CarbR-HeLa cells treated with ATE and Doxorubicin. 9 (a) Represents control group; 9 (b) Represents ATE treated CarbR-HeLa cells; 9 (c) Represents combination treatment of ATE and Dox in CarbR-HeLa cells.

DISCUSSION

Cervical cancer is the third leading cancer with a high incidence and mortality rate. However, advanced treatment strategies were there to overcome this. Unfortunately, these treatments could not yield a better prognosis due to the adverse effects and therapy resistance from radiation and chemotherapeutic agents [29]. Present research on cancer therapeutics is focused chiefly on the anti-cancer activity of various phytochemicals derived from plants. Over the last few centuries, *Theobroma* species has been used as a medicinal plant due to its potent anti-inflammatory, anti-aging, cardioprotective, etc. [30-33]. The anti-tumour effect of cocoa powder lies in its potential to inhibit topoisomerase enzymes which are critical regulators in cancer cell proliferation [34]. Apart from their role in DNA repair mechanisms; these enzymes are also thought to modulate hypoxic conditions [35]. Yung Jung Choi and their co-workers demonstrated that HIF-1 α inhibition could be triggered by topoisomerase inhibitors [36]. Hypoxia is a critical regulator of cancer cell proliferation which inhibits various cell death pathways such as apoptosis, ferroptosis, autophagy and is also necessary for the proper regulation of VEGF and PDGF [19]. Interestingly, VEGF is responsible for the formation of new blood vessels by angiogenesis while PDGF stabilizes the newly formed blood vessels [37,38]. Other functions of PDGF include the stabilization of efflux pumps to kick out the anti-neoplastic drugs, thereby resulting in drug resistance and stemness. Therefore, inhibition of HIF-1 α mediates ferritin degradation, thereby promoting ferroptosis, inhibiting VEGF and PDGF- β expression, thereby blocking angiogenesis and reducing the stability of efflux pump resulting in sensitization of tumor cells to the anti-neoplastic drugs.

Dox which is one of the potent anti-neoplastic drugs has all these properties. However, this drug might also induce cardiotoxicity when taken in high doses [39]. On the other hand, literature suggests that hepatocellular carcinoma cells treated with Dox induced senescence which led to elevated expression of stem cell markers [1]. CSCs are a group of tumor sub-population which are believed to initiate tumors. The reason for high mortality rate due to cancer is because of CSCs which usually escape from the potent antineoplastic drugs and rests in quiescent phase (G_0) of the cell cycle [40-42]. Hence, these cells are not exposed to therapies and become more vulnerable after relapse or recurrence.

To prevent cardiotoxicity and sensitise the stem cell markers to dox, we hypothesized to target drug-resistant cervical cancer cell lines with ATE in combination with Dox. The main aim of the study is to induce ferroptosis in drug-resistant cervical cancer cells at a low dosage of Dox coupled with ATE. Hence all the experiments were performed with half of the IC₅₀ value of Dox while the IC₅₀ of the ATE was kept constant. The reason behind choosing ATE in combination with Dox is due to its potent cardioprotective effects. Additionally, literature has suggested that crude extract from this plant species is rich in flavanols and other phytochemicals, inhibits topoisomerase enzymes. Due to its resemblance with Dox in terms of its mode of action, we opted for aqueous crude extract from *Theobroma* spp to evaluate its synergistic effect towards drug resistant HeLa cancer cells.

Interestingly all our results matched our hypothesis. Initially, we assessed gene expression of HIF-1 α , VEGF, CD24, and Bcl-2. We observed a drastic decrease in both groups when compared to the control. However, when the results are compared between cells treated with crude ATE alone and in combination with Dox, HIF-1 α is significantly down regulated in cells treated with both ATE and Dox. However, VEGF expression was drastically down regulated in cells treated with the ATE treatment alone. Also, CD24 one of the potent stem cell markers was down regulated in the cells treated with both drugs. Interestingly flow cytometer analysis showed ALDH1A1 is another important stem cell marker has been down regulated [22]. Iron is an essential micro nutrient required for proper cellular homeostasis. For instance, haemoglobin is an iron containing protein that supplies oxygen to the cells. However, literature states that cancer patients become anaemic (low levels of haemoglobin) which leads to reduced supply of oxygen thereby creating hypoxic environment in which tumor cells are more sustainable [43]. It has also been stated that tumor cells need bulk amount of iron which is stored in the form of ferritin [44,45]. Ferritin is an iron storage protein that stores excess iron. Interestingly elevated levels of ferritin are one of the hallmarks of cancer cells [46]. Surprisingly, ferritin acts as a double-edged sword in cancer cells. This is because cancer cells need iron for both cell growth and cell death pathways in different oxidation states [47-49]. For cellular growth, ferric iron is essential as it acts as a co-factor for one of the major enzymes that catalyze nucleotide biosynthesis which is termed ribonucleotide reductase [50]. While coming to the cellular death pathway, ferrous iron triggers lipid peroxide generation by enhancing lipoxygenase activity [51-53]. However, cancer cells selectively inhibit the accumulation of ferrous iron.

To evaluate the ferritin degrading capacity and thereby determining the ferroptosis inducing potential of ATE alone and in combination with reduced levels of Dox, we measured the concentration of intracellular iron. Surprisingly, the ferritin estimation analysis suggested low levels of ferritin in both drug-treated groups compared with the control group, suggesting an iron-mediated cell death. Literature suggests that cancer cells and CSCs require bulk ferritin levels to sustain the harsh tumor microenvironment [47]. Degrading ferritin leads to a novel cell death pathway termed ferroptosis. As discussed in the previous paragraph, ferric iron is stored in the form of ferritin. Degradation of ferritin releases ferric iron, which is immediately converted to ferrous iron. The generated ferrous iron acts as a co-factor for the enzyme lipoxygenase, which mediates the generation of lipid peroxides which eventually leads to cellular death [54]. Hence to make our study strong we evaluated lipid ROS levels in all the groups. We observed gradual increase in

lipid ROS in cells treated with ATE and Dox more when compared with cells treated with ATE alone when compared with untreated control cells. All our results suggest that crude extract of *Theobroma* spp in combination with reduced levels of Dox potentially induced both apoptosis and ferroptosis via downregulating Bcl-2 and degrading ferritin respectively.

CONCLUSION

Based on our results, we would conclude that ATE shows synergism with Dox and could potentially induce cancer cell death, which yields better cancer prognosis thereby increasing the survival rate of cancer patients. Our present study also deciphers that Dox when used in combination with ATE could reduce the dose of Dox thereby limiting the adverse side effects caused by Dox alone. However, further in vivo and ex vivo investigations are necessary to evaluate the potentiality of the synergistic effect of ATE on drug-resistant cancer cells.

AUTHOR CONTRIBUTIONS

Wrote manuscript: SLP and PC; Performed experiments: PC; Data analysis: GJM, JANM, and SNSL; Figures and tables: PC; Conceptualised the study: SLP; Overall supervision & Funding: SLP.

ACKNOWLEDGMENTS

SLP gratefully acknowledges DBT (BT/PR30629/BIC/101/1093/2018), New Delhi; UGC (Ref No: No.F.30-456/2018 (BSR)) and SERB (Ref No: PDF/2015/000867) for the financial support. PC gratefully acknowledges DBT, New Delhi for the Junior research fellowship. We thank Dr. Akash Kalagara, Vijaya Medical Centre, Visakhapatnam for their support in performing RT PCR and FACS experiments. We also thank Dr. Karuna Kumar, Application specialist, BD Biosciences for their support in analysing the FACS data.

Disclosure of conflict of interest: None.

REFERENCES

- Karabici M, Alptekin S, Firtina Karagonlar Z, Erdal E. Doxorubicin-induced senescence promotes stemness and tumorigenicity in EpCAM-/CD133- nonstem cell population in hepatocellular carcinoma cell line, HuH-7. *Mol Oncol*. 2021;15(8):2185-2202.
- Kuşoğlu A, Avcı ÇB. Cancer stem cells: A brief review of the current status. *Gene*. 2019;681:80-85.
- Battle E, Clevers H. Cancer stem cells revisited. *Nat Med*. 2017;23(10):1124-1134.
- Sritharan S, Sivalingam N. A comprehensive review on time-tested anticancer drug doxorubicin. *Life Sci*. 2021;278:119527.
- Nitiss JL. Targeting DNA topoisomerase II in cancer chemotherapy. *Nat Rev Cancer*. 2009;9(5):338-350.
- Thorn CF, Oshiro C, Marsh S, Hernandez-Boussard T, McLeod H, Klein TE, et al. Doxorubicin pathways: Pharmacodynamics and adverse effects. *Pharmacogenet Genomics*. 2011;21(7):440.
- Zhao L, Zhang B. Doxorubicin induces cardiotoxicity through upregulation of death receptors mediated apoptosis in cardiomyocytes. *Sci Rep*. 2017;7(1):1-11.
- Micallef I, Baron B. Doxorubicin: An overview of the anti-cancer and chemoresistance mechanisms. *Ann Clin Toxicol*. 2020;3(2):1031.
- Pandurangi SL, Chalumuri SS, Garimella S. Emerging Therapeutic Efficacy of Alkaloids as Anticancer Agents. *Ann. Romanian Soc. Cell Biol*. 2022;26(01):64-74.
- Pandurangi SL, Chittineedi P, Chikati R, Mosquera JA, Llaguno SN, Mohiddin GJ, et al. Role of Lipoproteins in the Pathophysiology of Breast Cancer. *Membranes*. 2022;12(5):532.
- Fernández-Millán E, Cordero-Herrera I, Ramos S, Escrivá F, Alvarez C, Goya L, Martín MA. Cocoa-rich diet attenuates beta cell mass loss and function in young Zucker diabetic fatty rats by preventing oxidative stress and beta cell apoptosis. *Mol Nutr Food Res*. 2015;59(4):820-824.
- Ahmed S, Ahmed N, Rungtatscher A, Linardi D, Kulsoom B, Innamorati G, et al. Cocoa flavonoids reduce inflammation and oxidative stress in a myocardial ischemia-reperfusion experimental model. *Antioxidants*. 2020;9(2):167.
- Taparia S, Khanna A. Effect of procyanidin-rich extract from natural cocoa powder on cellular viability, cell cycle progression, and chemoresistance in human epithelial ovarian carcinoma cell lines. *Pharmacogn Mag*. 2016;12(2):109.
- Marinello J, Delcuratolo M, Capranico G. Anthracyclines as topoisomerase II poisons: From early studies to new perspectives. *Int J Mol Sci*. 2018;19(11):3480.
- Nitiss JL. Targeting DNA topoisomerase II in cancer chemotherapy. *Nat Rev Cancer*. 2009;9(5):338-350.
- Lakhanpal M, Singh LC, Rahman T, Sharma J, Singh MM, Katak AC, et al. Study of single nucleotide polymorphisms of tumour necrosis factors and HSP genes in nasopharyngeal carcinoma in North East India. *Tumour Biol*. 2016;37(1):271-281.
- Gouveris P, Skopelitis E, Tsavaris N. Topoisomerase I and II Expression in Recurrent Colorectal Cancer Cells: A Dubious Matter 29 Topoisomerase I and II Expression in Recurrent Colorectal Cancer Cells: A Dubious Matter. 2011.
- Rapisarda A, Uranchimeg B, Sordet O, Pommier Y, Shoemaker RH, Melillo G. Topoisomerase I-mediated inhibition of hypoxia-inducible factor 1: Mechanism and therapeutic implications. *Cancer Res*. 2004;64(4):1475-1482.
- Huang R, Zhou PK. HIF-1 signaling: A key orchestrator of cancer radioresistance. *Radiat Med Prot*. 2020;1(01):7-14.
- Lakhanpal M, Yadav DS, Devi TR, Singh LC, Singh KJ, Latha SP, et al. Association of interleukin-1 β -511 C/T polymorphism with tobacco-associated cancer in northeast India: A study on oral and gastric cancer. *Cancer Genet*. 2014;207(2):1-11.
- Shah YM, Xie L. Hypoxia-Inducible Factors Link Iron Homeostasis and Erythropoiesis. *Gastroenterology* 2014;146(3):630-642.
- Pandurangi SL, Raju Bagadi SA, Sinha NK, Kumar M, Dada R, Lakhanpal M, et al. Establishment and characterization of two primary breast cancer cell lines from young Indian breast cancer patients: Mutation analysis. *Cancer Cell Int*. 2014;14(1):1-20.
- Pandurangi SL, Chikati R, Chauhan PS, Kumar CS, Banarji A, Saxena S. Effects of ellipticine on ALDH1A1-expressing breast cancer stem cells-An in vitro and in silico study. *Tumour Biol*. 2014;35(1):723-737.
- Kumar GR, Chikati R, Pandurangi SL, Kandapal M, Sonkar K, Gupta N, et al. Molecular docking and dynamics simulations of *A. niger* RNase from *Aspergillus niger* ATCC26550: For potential prevention of human cancer. *J Mol Model*. 2013;19(2):613-621.
- Armstrong L, Stojkovic M, Dimmick I, Ahmad S, Stojkovic P, Hole N, et al. Phenotypic Characterization of Murine Primitive Hematopoietic Progenitor Cells Isolated on Basis of Aldehyde Dehydrogenase Activity. *Stem Cells*. 2004;22(7):1142-1151.
- Hess DA, Meyerrose TE, Wirthlin L, Craft TP, Herrbrich PE, Creer MH, et al. Functional characterization of highly purified human hematopoietic repopulating cells isolated according to aldehyde dehydrogenase activity. *Blood*. 2004;104(6):1648-1655.

27. Malla RR, Pandrangi S, Kumari S, Gavara MM, Badana AK. Exosomal tetraspanins as regulators of cancer progression and metastasis and novel diagnostic markers. *Asia Pac J of Clin Oncol*. 2018;14(6):383–391.
28. Hess DA, Wirthlin L, Craft TP, Herrbrich PE, Hohm SA, Lahey R, et al. Selection based on CD133 and high aldehyde dehydrogenase activity isolates long-term reconstituting human hematopoietic stem cells. *Blood*. 2006;107(5):2162-2169.
29. Gulati R, Naik Ramavath M, Satya Mahesh Kumar Metta V, Latha Pandrangi S. Exploring the CRISPR/Cas9 System in Targeting Drug Resistant Cancer Stem Cells. *Ann. Romanian Soc. Cell Biol*. 2021;25(6):20540-20255.
30. Pérez-Cano FJ, Massot-Cladera M, Franch À, Castellote C, Castell M. The effects of cocoa on the immune system. *Front Pharmacol*. 2013;4:71.
31. Decroix L, Tonoli C, Soares DD, Descat A, Drittij-Reijnders MJ, Weseler AR, et al. Acute cocoa flavanols intake has minimal effects on exercise-induced oxidative stress and nitric oxide production in healthy cyclists: a randomized controlled trial. *J Int Soc of Sports Nutr*. 2017;14(1):28.
32. Becker K, Geisler S, Ueberall F, Fuchs D, Gostner J. Immunomodulatory properties of cacao extracts–potential consequences for medical applications. *Front Pharmacol*. 2013;4:154.
33. Pandrangi SL, Chalumuri SS, Chittineedi P, Garimella SV. Therapeutic Potential of *Nyctanthes Arbor-Tristis* on Cancer and Various Diseases. *Ann Romanian Soc Cell Biol*. 2022;26(1):1690-1701.
34. Martin MA, Goya L, Ramos S. Potential for preventive effects of cocoa and cocoa polyphenols in cancer. *Food Chem Toxicol*. 2013;56:336-351.
35. Decroix L, van Schuerbeek P, Tonoli C, van Cutsem J, Soares DD, Heyman E, et al. The effect of acute cocoa flavanol intake on the BOLD response and cognitive function in Type 1 diabetes: A randomized, placebo-controlled, double-blinded cross-over pilot study. *Psychopharmacology*. 2019;236(12):3421-3428.
36. Choi YJ, Rho JK, Lee SJ, Jang WS, Lee SS, Kim CH, et al. HIF-1 α modulation by topoisomerase inhibitors in non-small cell lung cancer cell lines. *J Cancer Res Clin Oncol*. 2009;135(8):1047–1053.
37. Nieves BJ, D'Amore PA, Bryan BA. The function of vascular endothelial growth factor. *Bio Factors*. 2009;35(4):332-337.
38. Murakami M, Calvert JW. Signaling Required for Blood Vessel Maintenance: Molecular Basis and Pathological Manifestations. *Int J Vasc Med*. 2012;2012:15.
39. Chatterjee K, Zhang J, Honbo N, Karliner JS. Doxorubicin cardiomyopathy. *Cardiology*. 2010;115(2):155-162.
40. Tian J, Li X, Si M, Liu T, Li J. CD271+ Osteosarcoma Cells Display Stem-Like Properties. *PLoS One*. 2014;9(6):98549.
41. El-Ashmawy NE, Salem ML, Abd El-Fattah EE, Khedr EG. Targeting CD166+ lung cancer stem cells: Molecular study using murine dendritic cell vaccine. *Toxicol Appl Pharmacol*. 2021;429:115699.
42. Meng E, Mitra A, Tripathi K, Finan MA, Scalici J, McClellan S, et al. ALDH1A1 Maintains Ovarian Cancer Stem Cell-Like Properties by Altered Regulation of Cell Cycle Checkpoint and DNA Repair Network Signaling. *PLoS One*. 2014;9(9):107142.
43. Chapman DG, Campbell JA. Effect of bone meal in enriched flour on the utilization of iron by anaemic and normal rats. *Br J Nutr*. 1957;11(2):133–137.
44. Alkhateeb AA, Connor JR. The significance of ferritin in cancer: Antioxidation, inflammation and tumorigenesis. *Biochim Biophys Acta*. 2013;1836(2):245–254.
45. Ryu MS, Duck KA, Philpott CC. Ferritin iron regulators, PCBP1 and NCOA4, respond to cellular iron status in developing red cells. *Blood Cells Mol Dis*. 2018;69:75–81.
46. Pandrangi SL, Chittineedi P, Chikati R, Lingareddy JR. Role of dietary iron revisited : In metabolism , ferroptosis and pathophysiology of cancer. *Am J Cancer Res*. 2022;12(3):974–985.
47. Zhang C. Essential functions of iron-requiring proteins in DNA replication, repair and cell cycle control. *Protein Cell*. 2014;5(10):750–760.
48. Rambatla PK, Pandrangi SL, Rentala S, Sireesha V. A Study on the Expression of CCL5, CXCR4 and Angiogenic Factors by Prostate Cancer Stem Cells. 2021;25:1020–1028.
49. Stockwell BR, Friedmann Angeli JP, Bayir H, Bush AI, Conrad M, Dixon SJ, et al. Ferroptosis: A Regulated Cell Death Nexus Linking Metabolism, Redox Biology, and Disease. *Cell*. 2017;171(2):273–285.
50. Pandrangi SL, Chittineedi P, Chalumuri SS, Meena AS, Neira Mosquera JA, Sánchez Llaguno SN, et al. Role of Intracellular Iron in Switching Apoptosis to Ferroptosis to Target Therapy-Resistant Cancer Stem Cells. *Molecules*. 2022;27(9):3011.
51. Sui X, Zhang R, Liu S, Duan T, Zhai L, Zhang M, et al. RSL3 drives ferroptosis through GPX4 inactivation and ROS production in colorectal cancer. *Front Pharmacol*. 2018;9:1371.
52. Meng Q, Shi S, Liang C, Liang D, Hua J, Zhang B, et al. Abrogation of glutathione peroxidase-1 drives EMT and chemoresistance in pancreatic cancer by activating ROS-mediated Akt/GSK3 β /snail signaling. *Oncogene*. 2018;37(44):5843–5857.
53. Raggi C, Gammella E, Correnti M, Buratti P, Forti E, Andersen JB, et al. Dysregulation of Iron Metabolism in Cholangiocarcinoma Stem-like Cells. *Sci Rep*. 2017;7(1):1–12.
54. Chikati R, Pandrangi LS, Gundampati R, Vemuri SH, Lakhanpal M, Singh SS, et al. Molecular Studies on Evaluation of Phytol as Cytoskeleton Targeting Element in Cancer. *Int J Sci Eng Res*. 2018;9:1978-1992.