3 Chapter: Comparative metabolome analysis of breast cancer cells MCF-7 and MDAMB-231 treated with doxorubicin and/or coumestrol: A GC-MS-based approach

3.1 Introduction:

Breast cancer is still the most common cancer among women, causing lower survival rates even with advancements in research [165]. The current therapies for breast cancer either utilize cytotoxic medicines to eradicate cancer cells or specific biological molecule that distinguishes cancerous cells from healthy cells. A significant percentage of tumors (80%) have a hormone-dependent growth pattern, as estrogen, the female sex hormone affects the risk of breast cancer (BC) [166]. The most prevalent biomarker of breast cancer, ER, is present in nearly 70% of cases and accounts for 50% of deaths [167]. Moreover, the lack of hormone and non-hormonal receptor (HER2) expression [168], is a distinguishing feature of the aggressive form of the disease, i.e., triple-negative breast cancer (TNBC), associated with increased mortality and relapse [169]. A variety of ER-targeted drugs have been used in clinical settings to treat breast cancer [170]. Drug resistance has, however, emerged as a consequence of ongoing ER-targeted therapy [171]. Thus, finding new anti-breast cancer drugs is essential to address the problem of treatment resistance [172, 173]. Phytoestrogens, which are estrogen-like substances synthesized from plants, are structurally and physiologically comparable to endogenous estrogens [174]. This enables them to allow binding to the estrogen receptor (ER) and obstruct hormonal signaling in both humans and animals. Coumestrol, a primary component of soy, is an active phytoestrogen with numerous biological and medicinal uses [175]. According to reports, coumestrol has anticancer, osteoblastic differentiation, and neuroprotective properties [176]. It also accomplishes the role of a competitive antagonist of ER in the presence of 17--estradiol and other estrogen compounds [171]. Further, coumestrol has been recognized for its ability to interact with steroidal receptors (ER) and G-protein coupled estrogen receptors due to structural resemblance with estrogen [177]. Additionally, coumestrol impairs 17-HSD and aromatase activity, by

lowering the levels of estrogen and androgen hormones [178]. Due to its comparable affinities to both ER α and ER β, cholesterol interacts with ER subtypes in a unique way [175].]. Furthermore, by activating protein kinase CKII, it causes senescence and raises the generation of ROS (reactive oxygen species) in colon and breast cancers [179]. In particular, coumestrol inhibits hypoxia-inducible factor-1 and sphingosine kinase 1, thereby stopping hypoxia-induced PC3 prostate cancer growth by inducing apoptosis in breast cancer cells [180]. m-TOR/PI3K/AKT signaling pathways were changed, cell migration and invasion were suppressed, and cell cycle arrest was promoted. All these effects were mitochondria mediated [180] Doxorubicin has anticancer activity against breast cancer through several mechanisms, such as changes in sphingolipid metabolism, disruption of membranes, production of free radicals, inhibition of Topoisomerase-II, DNA intercalation and adduct formation, and induction of oxidative stress[181]. Although we have an overall comprehension of how coumestrol and doxorubicin work against cancer, there is still an unmet need to track cancer cells' responses to therapy and find molecular markers which can enable prompt therapeutic efficacy assessment.

Research on the altered metabolic pathways of cancerous cells has long been a major area of interest. Changes in cellular metabolism are now widely acknowledged in modern medicine as one of the main characteristics of cancer [182]. Metabolomics, a robust omics technique, is extensively applied in cancer research to accurately detect altered metabolite levels resulting from neoplastic progression within biological samples. Its versatile applications include identifying biomarkers, advancing drug development, conducting clinical toxicology assessments, exploring nutritional aspects, and achieving precise phenotyping. Among various methodologies, metabolomics has brought about a significant revolution in the area of cancer research, surpassing other methods in its impact [183]. Therefore, we examined how doxorubicin and/or coumestrol affected the

metabolic characteristics of two cell lines that reflect distinct hormonal states: MDAMB-231 (ER- PR- HER2-) and MCF-7 (ER+ PR+ HER2-) for breast cancer (Figure 14).

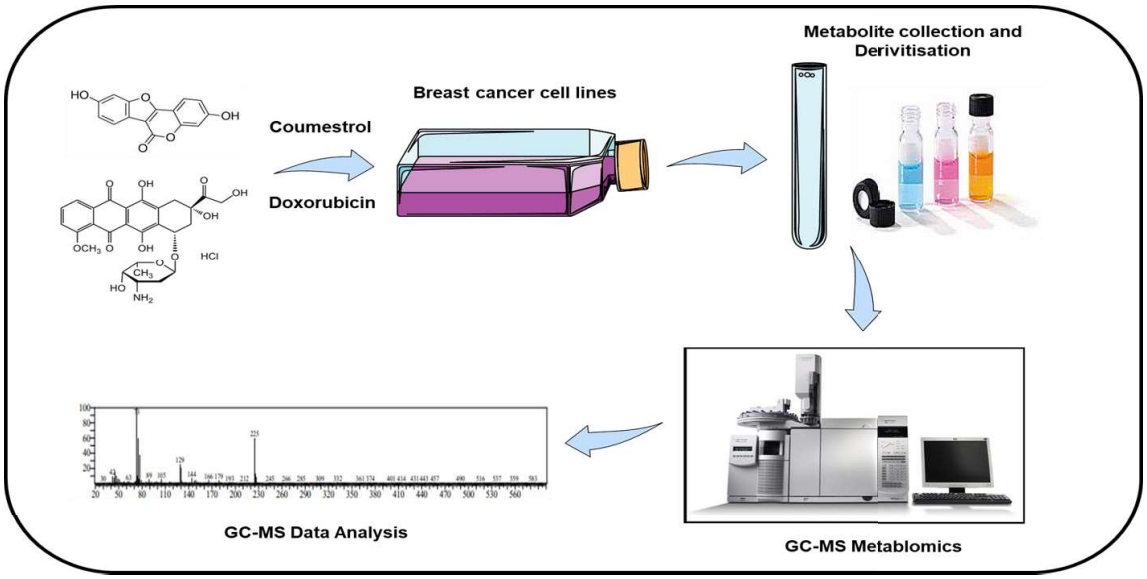

Figure 14: Graphical representation of workflow.

3.2 Materials and methods:

3.2.1 Maintaining and cultivating cells:

MCF-7 and MDAMB-231 cell lines were obtained or purchased from the National Centre for Cell Sciences (NCCS, Pune, India). The cell lines were grown in DMEM supplemented with 10% fetal bovine serum (Gibco, Invitrogen, Karlsruhe, Germany) and antibiotics (100 g/ml penicillin, 100 g/ml streptomycin sulphate) at 37 °C, 90% humidity, and 5% CO2. A commercially available detection kit was used to check for mycoplasma contamination in the cell lines. The following experiments were conducted using the cell lines that were free of mycoplasma.

3.2.2 Treatment of cells with anticancer drugs:

Coumestrol and doxorubicin were procured from Sigma Aldrich (India, catalogue no. 27885). Dimethyl sulfoxide (DMSO) was used to prepare the doxorubicin and

coumestrol solutions. Approximately, 3×10^6 cells were incubated with 5 mL of DMEM growth media for 24 h in 25 cm^2 flasks before exposing the cells to 0.5% DMSO, coumestrol (48 µM), or doxorubicin (1 µM) for 24 h in the same flasks. The experiment was conducted in triplicate and the flasks treated with DMSO (0.5 %) only were considered as vehicle control.

3.2.3 Sample Preparation for GC-MS Analysis:

After 24 h of vehicle or drug exposure, the exhausted media was decanted and phosphate-buffered saline was used to gently clean the cells to get rid of the media and other components. Further, the flasks were treated with 2 mL ice cold methanol for 1 min to halt the metabolic activity of cells. To extract the methanolic cell extract, the cell suspension was centrifuged once more for 10 minutes at 4 °C and 3000 g after the cells were scraped from the flask and collected into a 15 mL centrifuge tube. The methanolic cell extracts were preserved at −80 °C until further analysis. The frozen extracts were thawed and dried using a stream of nitrogen before derivatization. Once the dehydrated samples were combined with 50 µL of dichloromethane and 50 µL of N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA), they were vortexed thoroughly for a minute. Following this, the samples that had been derivatized were put into fresh vials and incubated in a water bath at 80°C for 30 minutes. GC-MS analysis was carried out on the samples after a final cooling to room temperature [184].

3.2.4 Gas chromatography and mass spectrometry analysis:

Mass spectrometry and gas chromatography were used to analyze derivatized cell metabolites. A GC-MS-QP2020 NX instrument (Shimadzu, Japan) was utilized, along with an SH-Rxi-5Sil MS (30.0.25.0.25) coloum A flow rate of 1 mL/min was employed for the carrier gas, helium gas. An injector set to 250 °C was used to introduce a 1 µL

injection volume. A temperature of 250 °C was also maintained for the ion source. The temperature protocol called for a 2-minute initial isothermal hold at 70 °C, a 2-minute ramp up to 250 °C, and another 2-minute hold. The temperature was then raised by 10 °C increments until it reached 300 °C, where it remained for 8 minutes. Every second, two scans of the mass spectra of the metabolites were recorded, spaced 50–600 m/z apart [185]. Based on their retention times, metabolic compounds were identified using gas chromatography, and their analysis was made easier by standard mass spectral data from the Wiley and NIST (National Institute of Standards and Technology) Libraries version 11.

3.2.5 GC-MS Data Pre-Processing:

The GC-TQ-MS raw data files were stored as Computable Document Format (CDF) files. Pre-processing was performed on these data to remove any potential biases, including background, noise, and variations in retention time (RT) between different samples. The instrumental data sets were also converted by this pre-processing into a format suitable for analysis. The findings were normalized by calculating the mean chromatogram area for each pair of triplicates and then dividing that value by the total chromatogram area.

3.2.6 Metabolite Identification:

Using the NIST spectral library version 14, retention indices (RI) and mass spectra fragmentation patterns of each chemical were compared in order to identify the metabolites in GC-MS chromatograms. Data regarding metabolites and pathways were sourced from the Human Metabolome Database (HMDB) [186].

3.2.7 Data analysis and statistical approach:

The two drug treatments were compared, and their interactions were examined, using a two-way ANOVA. Principal Component Analysis (PCA) was utilized to visualize the metabolomics data. Prior to PCA, the data were standardized and mean-centered to ensure consistent variance. Metabolite Set Enrichment Analysis (MSEA) was carried out using the online program Metaboanalyst 5.0 [187]. Furthermore, the set of unique metabolites (compound names) was employed in the pathway over-representation analysis to investigate biologically meaningful patterns. Finding the most impacted metabolic pathways and aiding in the differentiation of the mcf-7 and mdamb cell lines following their respective drug treatments was the aim of this study.

3.3 Results:

The purpose of this investigation was to examine the impact of drugs such as doxorubicin and coumestrol on the metabolism of MDA-MB-231and MCF-7, two types of breast cancer cells. The study used GC-MS based approach, to find any alterations in metabolic pathways.

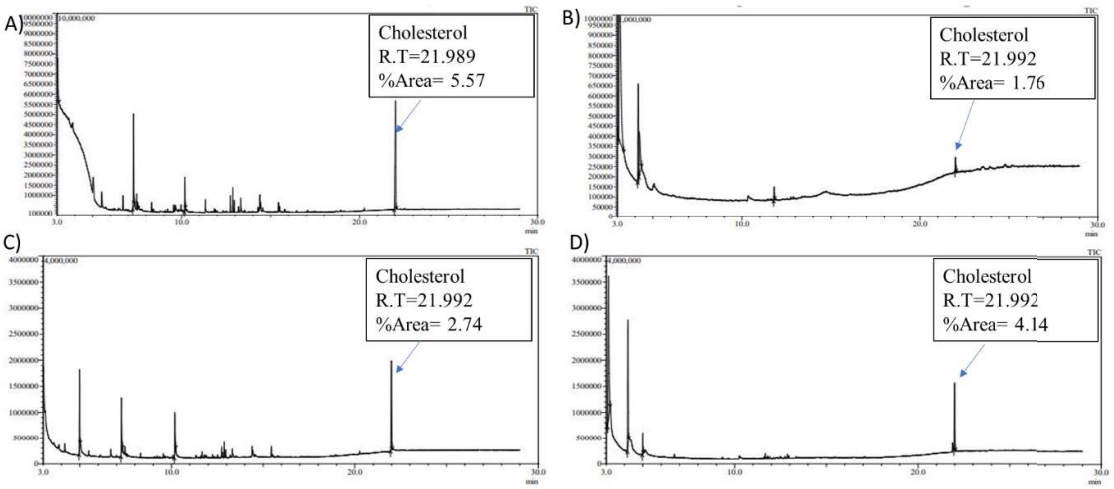

Figure 15: GC–MS TIC of metabolite extracts from MCF-7 treated with A) DMSO (control), B) Coumestrol 46 µM, C) Doxorubicin 1 µM, D) Coumestrol 46 µM + doxorubicin 1 µM.

The total ion chromatograms (TIC) for the compounds found in MCF-7 and MDA-MB-231 cells that were determined using GC-MS. We obtain a large number of dataset entries with varying retention times and chemical derivatization of 24 samples from the GC–MS spectra analysis. Ultimately, 68 metabolites were identified based on their presence in the Human Metabolome Database (HMDB) 4.0.

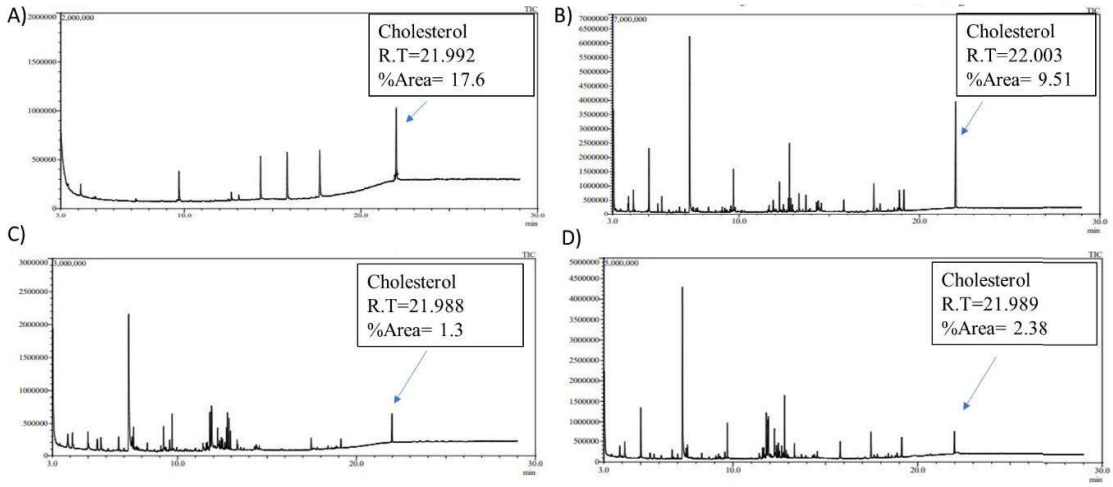

Figure 16: GC–MS TIC of metabolite extracts from MDA-MB-231 treated with A) DMSO (control), B) Coumestrol 48 µM, C) Doxorubicin 1 µM, D) Coumestrol 48 µM + doxorubicin 1 µM.

The metabolic profiles of MDA-MB-231 and control MCF-7 breast cancer cells differed significantly, as shown by PCA (Figure 17A). Notably, PCA analysis revealed that drug administration induced discernible changes in metabolic patterns within both cell lines. Particularly in the MDA-MB-231 metabolome, the impact of the drug was more pronounced compared to MCF-7.

When MCF-7 cells were the only cells being examined, the PCA showed that all drug-treated cluster groups could be distinguished from the control (Figure 17B), underscoring the effect of drug treatment on the metabolic profile of MCF-7 cells. The PCA of MDA-MB-231 showed that doxorubicin and the combination treatment clustered away from both doxorubicin and control, indicating the treatments' potent effects on the breast cancer cells (Figure 17C).

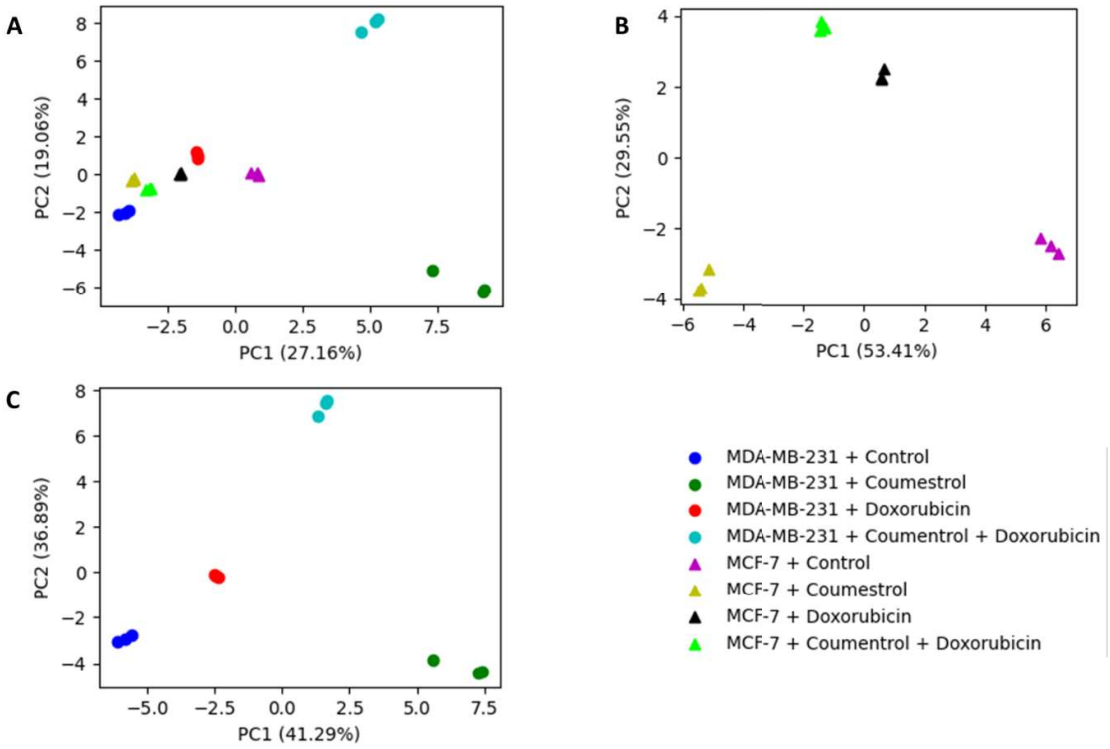

Figure 17. Principal component analysis (PCA) of the metabolomics of combined MCF-7 and MDA-MB-231 (A), MCF-7 (B), and MDA-MB-231 (C) Cells following treatment with vehicle control (0.5% DMSO), coumestrol (48 µM), doxorubicin (1 µM), or their combination. (PC1; principal component -1, PC2; principal component-2).

A two-way ANOVA was then used to assess the effect of the medication regimen on particular metabolites in MCF-7 cells (Table S1) and MDA-MB-231 breast cancer cells (Table S2). Twenty-four metabolites in total differed significantly from the control in this analysis, indicating that MCF-7's metabolome was impacted by the coumestrol and/or doxorubicin treatment (Table S1). Notably, there was a noteworthy overlap in the metabolites that exhibited changes when cells were subjected to treatment with either coumestrol or doxorubicin, as highlighted in **Error! Reference source not found.** F urther, we observed changes in the levels of palmitic acid and cholesterol occurred with all the treatments, including the combination of two drugs (Table 2). Additionally, there

were sixteen metabolites that exclusively exhibited changes when exposed to coumestrol. Furthermore, the levels of L-asparagine, D-fructose, and D-galactose only showed alterations upon doxorubicin treatment of the cells (Table 2).

Table 2: List of metabolites of MCF-7 that responsive to drug treatment:

Treatment	Total	Metabolite
Coumestrol	8	Myo-Inositol
Doxorubicin		Silicic Acid
Coumestrol and Doxorubicin		Cholesterol
		Malonic Acid
		D-Pinitol
		beta.-D-Galactofuranose
		1-Monooleoylglycerol
		Steric Acid
Coumestrol	5	Oxygen
		Hydrocinnamic Acid
		Valeric Acid
		Eicosanoic Acid
		Thiourea
Doxorubicin	1	Glucose
Coumestrol and Doxorubicin	4	L-(+)-Lactic Acid
		D-Mannitol,
		L-Threitol
		Ricinoleic Acid
Coumestrol	1	Acetic Acid
Coumestrol and doxorubicin		
Doxorubicin	4	Palmitic Acid
Coumestrol and Doxorubicin		D-(-)-Fructofuranose
		L-Alanine
		Glycine

Our statistical analysis in MDA-MB-231 cells showed alterations in 43 distinct metabolites (Table S2). We draw attention to the metabolites' overlap that manifested in this situation. varied in quantity when given coumestrol or doxorubicin treatment (Table

3). Remarkably, 11 metabolites had their levels adjusted by the combination treatment; of these, three metabolites were only affected by the combination treatment (Table 3).

Table 3. List of metabolites of MDA-MB-231 that responsive to drug treatment:

Treatment	Total	Metabolite
Coumestrol and Doxorubicin	8	Cholesterol Palmitic Acid L-Alanine Glycine L-Leucine L-Valine L-Isoleucine L-Proline
Coumestrol	16	Ribitol 2-Methylbutanoic Acid Aminomalonic Acid Glutamic Acid Xylonic Acid D-(-)-Fructofuranose, L-(-)-Arabitol Myo-Inositol Heneicosane D-Glucose D-Lactose D-(+)-Cellobiose, Squalene Eicosanoic Acid, Octadecane, Ginsenol
Doxorubicin	6	L-Asparagine D-Fructose D-Galactose Adonitol Vaccenic Acid Arsenous Acid

Coumestrol and Doxorubicin	11	D-Arabinonic Acid Mannose Oxalic Acid Heptadecanoic Acid Stearic Acid D-Lactose D-(+)-Cellobiose 2-Aminobenzoic Acid Silicic Acid, Arsenous Acid Benzene
Coumestrol Coumestrol and Doxorubicin	1	D-Ribonic Acid
Doxorubicin Coumestrol and Doxorubicin	1	D-Pinitol

The functional enrichment analysis of metabolites responsive to drug treatment in both cell lines showed that cholesterol significantly affected glucose energy metabolism, particularly in pathways like glutathione metabolism, aspartate metabolism, glucose-alanine cycle, and arginine-proline metabolism (Figure 18).

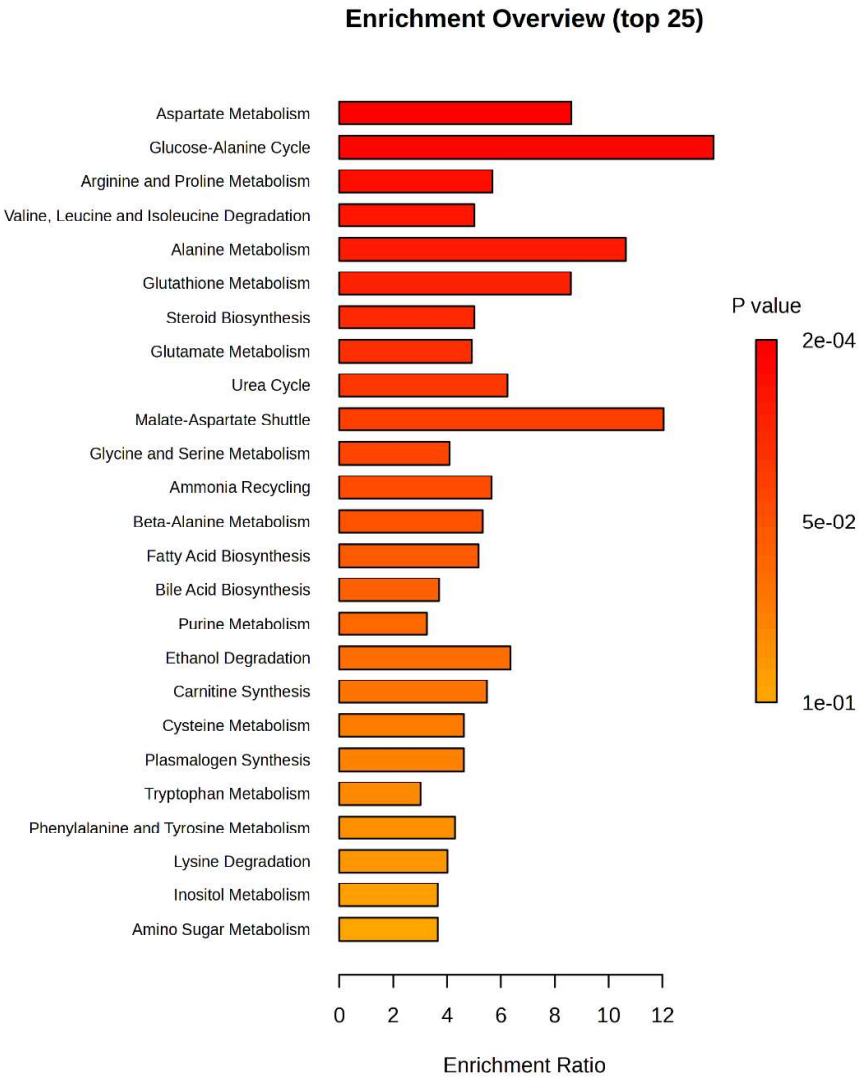

Figure 18. This Metabolomic Set Enrichment Analysis shows how BC cells' functional metabolism changed after being treated with 48µM Coumestrol. The y-axis of the graph, which was created using the MSEA online tool, shows the -log of the p-values obtained from the pathway enrichment analysis.

This analysis of the metabolic set enrichment reveals the changes in the functional metabolism of BC cells following treatment with 48µM coumestrol. The -log of the p-values derived from the pathway enrichment analysis is displayed on the y-axis of the graph, which was made using the MSEA online tool. Additionally, our findings indicate

that coumestrol affected the metabolism of glutamate, glycine and serine, alanine, isoleucine, leucine and valine, and steroid biosynthesis (Figure 18). The functional enrichment analysis for doxorubicin produced results that were similar to those found for coumestrol. Treatment with doxorubicin demonstrated a significant impact on the metabolism of glucose energy, as evidenced by the fact that seven of the top twenty enriched pathways—including those related to glutathione, lactose, and galactose metabolism—were directly linked to glucose metabolism (Figure 19).

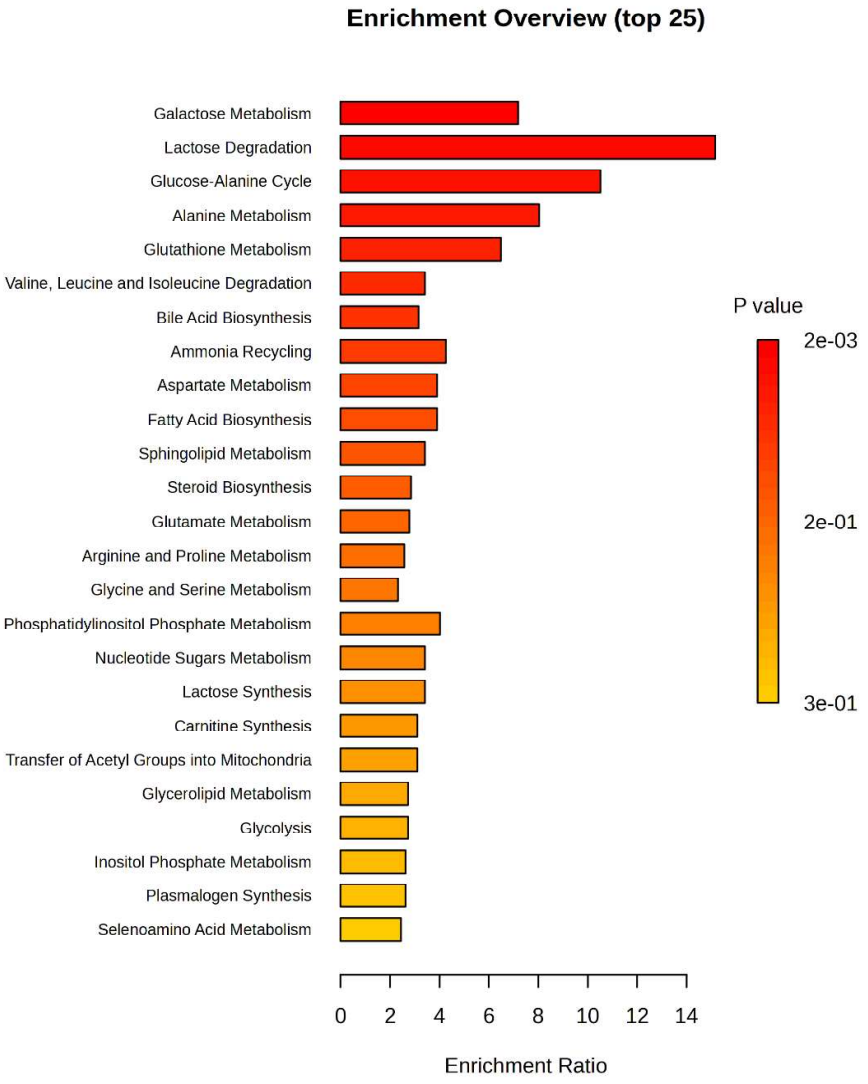

Figure 19. The Functional Metabolism of BC cells was examined using this Metabolomics Set Enrichment Analysis following treatment with 1μM doxorubicin. The -log of the p-values derived from the pathway enrichment analysis is displayed on the y-axis of the graph, which was made using the MSEA online tool.

The cholesterol and Palmitic acid levels significantly noticed in both control cell lines and upon treatment with coumestrol and doxorubicin the levels were decreased predominantly. These results were evidence that involvement of fatty acid and lipid metabolism in breast cancer.

3.4 Discussion:

Metabolomics has emerged as a promising method for the early identification of cancer by exploiting the changes in metabolite extents that are converted into biomarkers. These metabolite impressions assist in identifying the specific type of cancer cell based on their biological characteristics. This will increase the possibility of finding metabolic problems that are unique to each cancer subtype [188]. Breast cancer cell lines are functional but rough models for cancer of the same subtype, have a high mutational frequency with numerous uncertainties, and are unable to reflect breast cancer heterogeneity [189]. Prior studies using proton NMR have identified fundamental metabolic differences between MCF-7 and MDA-MB-231 cells [190, 191]. After administering coumestrol and/or doxorubicin to the cell lines MCF-7 and MDA-MB-231, we estimated the various amounts of metabolites using GC-MS. Numerous metabolites, including myo-inositol, silicic acid, cholesterol, malonic acid, d-pnitol, beta-d-galactofuranose, and 1-monooleoylglycerol, changed as a result of coumestrol and/or doxorubicin treatment of MCF-7 cells. The observed changes in 1-Monooleoylglycerol and cholesterol suggest potential implications for fatty acid and cholesterol metabolism.

This metabolic shift is often seen in cancer cells undergoing pharmacological treatment as they adapt to their heightened energy demands. Moreover, these alterations have been linked to various malignancies in recent studies [192, 193].

When MDA-MB-231 cells were treated with coumestrol and/or doxorubicin, alterations in metabolites, such as cholesterol and substances linked to fatty acids, such as palmitic acid, were seen. Specifically, palmitic acid is required for cholesterol metabolism and cell signaling. The pharmacological treatments also caused a shift in the levels of amino acids such as l-alanine, glycine, l-leucine, l-valine, l-isoleucine, and l-proline. The disruption of amino acid metabolism, which is crucial in cancerous cells in particular, may be caused by these alterations [194]. Functional enrichment analysis demonstrated that the pathways involved in energy production, including the TCA cycle, amino acid metabolism, fatty acid metabolism, glycolysis and gluconeogenesis, and amino acid metabolism, are significantly impacted by coumestrol or doxorubicin treatment [195]. It has been discovered that there is a strong correlation between the aggressiveness of the malignancy and these processes, which are crucial for managing pathways that release energy. These procedures, which are essential for controlling energy-producing pathways, have been found to be closely related to the aggressiveness of the malignancy.

In cancer cells, amino acid metabolism has a wide range of effects, including developing amino acid pools as building blocks, altering epigenetic regulation, supplying bioenergy through the production of ketoacid, detoxifying ammonia by converting it to non-toxic urea, and maintaining intracellular redox status [196]. The wide and significant functions that aberrant amino acid metabolism plays in different types of cancer are becoming increasingly evident and it is becoming progressively more important to

consider the possible effects of metabolic control and regulation in the tumor microenvironment [197]. Our findings from the functional enrichment analysis of metabolites align with the well-established understanding that a substantial number of metabolic pathways tend to be disrupted in tumor cells.

The elevated production of fatty acids (FAs) is a crucial component of lipid metabolism in carcinogenesis. Tumor cells enhance de novo FA synthesis in order to cover its requirements for membrane development, energy production, and signaling molecule production [198]. Cholesterol is an important lipid that is necessary for cellular activity, especially in cellular membranes, it plays a crucial role in controlling membrane fluidity and forming lipid rafts that support certain signal transduction pathways. Additionally, it plays vital role in the production of steroid hormones including estrogen, progesterone, and testosterone [199]. The synthesis of cholesterol, endogenous fatty acids (FAs), and cholesterol esters is increased in tumor cells and precancerous tissues and this enhanced production supports the tumor cell motility, growth, proliferation, and differentiation [200]. In our findings the cholesterol and palmitic acid metabolites levels were observed predominantly in untreated breast cancer cells characterizes require more cholesterol and fatty acids. In case of coumestrol and doxorubicin treated cells showed lower amounts of cholesterol levels. Collectively, coumestrol and doxorubicin regulate the cholesterol and fatty acid metabolism in breast cancer.

3.5 Conclusion:

Our study uncovered substantial perturbations in the major metabolic pathways of MCF-7 and MDAMB231 breast cancer cell lines. Consequently, these insights hold significant potential for enhancing our comprehension of the molecular intricacies underlying the development and advancement of cancer. Moving forward, to deepen our

insights into signaling pathways and molecular mechanisms influenced by diverse drug treatments, our forthcoming objective is to complement our existing research with integrated proteomic data. Ultimately, this strategy aims to yield predictive molecular markers that gauge the efficacy of specific treatments across different breast cancer cell lines.