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Short Communication

Phytocompounds curcumin, quercetin, and resveratrol modulate lactate pyruvate levels in U87 glioma cells

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ABSTRACT

Background: Even when there is enough oxygen available, cancer cells meet their energy requirements by increasing glucose intake, glycolysis rate, and lactate generation. Otto Warburg proposed this process, which is known as the Warburg effect. A recent method of drug discovery includes developing medications that interfere with glucose consumption and aerobic glycolysis, or lactate production in cancer cells. Curcumin (C), Quercetin (Q), Ellagic acid (E), and Resveratrol (R) were examined in U87 cells for their ability to induce cytotoxicity at certain concentrations, as well as modulate lactate-pyruvate metabolism.
Results: All the tested compounds and their combinations such as C+E, C+Q, C+R and Q+R have exhibited, dose-dependent cytotoxic effects against U87 Glioma cells. Besides, the compounds (except Ellagic acid) and the combinations have modulated glucose intake, lactate-pyruvate level, and NADH/NAD ratio in U87 Glioma cells.
Conclusions: Phytocompounds those modulate cellular metabolism in cancer cells are currently unknown. Our findings imply that the phytocompounds used in the study are involved in cancer cell metabolic reprogramming and have cytotoxic properties. These substances could be used for the prevention and treatment of cancer.

1. Background

Brain and central nervous system tumours constitute 3% of the estimated cancer incidence and deaths among the total number of cancers.[1] Despite a number of therapeutic options, the median survival is only 8 months and 1-year survival is 20%.[2] Many cancers, including glioma, alter their biochemical pathways for energy metabolism, growth, survival, proliferation, and long-term maintenance.[3] Cancer cells satisfy their energy demands largely through increased glucose uptake and a high rate of glycolysis, even when there is an ample amount of oxygen. The phenomenon was initially described by Otto Warburg and the process is named after him as the "Warburg effect".[4] The uptake of glucose is the basis for diagnosis using fluoro-deoxy-glucose (FDG) PET-CT scan. The metabolic rate of glucose by aerobic glycolysis is substantially faster, with lactate being produced 10-100 times faster than glucose is completely oxidized in the mitochondria. Lactate dehydrogenase (LDH) transforms the pyruvate produced during glycolysis into lactate. LDH levels have been found to be greater in a variety of cancers, LDH is believed to be a prognostic biomarker with a higher level thought to be a predictive biomarker. The lactate helps in cell migration and angiogenesis in a hypoxic condition.[5] Besides lactate also helps in immune escape and modulating tumour microenvironment.[6] Apart from high glucose uptake and its metabolism the utilization of glutamine amino acid through glutaminolysis is another adaptive process for cancer cells in maintaining energy and other physiological demands.[7]

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A combination of surgical resection, radiotherapy or radiosurgery, and chemotherapy are the currently available treatment options for malignant glioma. To enhance overall survival time and quality of life for these patients, innovative medicines are desperately needed. Targeting cancer cell metabolism also opens up new avenues for drug discovery.

Plant-derived products have been extensively tested for different diseases including cancer. Several phytochemicals have been shown to modify glucose uptake by cancer cells.[8] The GLUT 1 enzyme which transports glucose has flavone binding sites.[9] Plant-derived products possess anticancer and chemopreventive properties.[10] Understanding the molecular mechanisms of cancer chemoprevention is important for future drug development.[11] Some of the extensively studied phytocompounds like Quercetin, Curcumin, Ellagic acid and Resveratrol show potential anticancer properties. Curcumin is the most bioactive polyphenol which is found in the spice turmeric.[12] Quercetin is a flavonoid which is found in plants and food like red wine, apple, berries and onion.[13] Ellagic acid is a polyphenol found in many fruits and vegetables.[14] Resveratrol (3, 5, 4′-trihydroxy-trans- stilbene) is a natural polyphenol that is present in red grapes, red wine, peanuts, and ground nuts.[15]

In this study, we used select plant derived compounds such as Curcumin, Quercetin, Ellagic acid and their combinations for cytotoxicity and modulation of lactate and pyruvate levels in U87 glioma cells. Our results showed that except Ellagic acid, the tested compounds and their combination modulate lactate pyruvate levels in U87 glioma cells.

2. Materials and Methods

2.1. Cultured cells and cell lines

National Centre for Cell Sciences (NCCS) Pune, India, provided the glioma cells (U87). The cells were cultured at 37°C in a 5% CO_2 humified incubator in high-glucose DMEM (Gibco, USA) with 10% foetal bovine serum and 1% penicillin streptomycin (Gibco, USA).

2.2. Drugs

Compounds such as Resveratrol, Curcumin Quercetin, and Ellagic acid were purchased from MP Biomedical. DMSO was used to dissolve the compounds and a stock concentration of 40mM was prepared and stored in -200 C until use. Ellagic acid was solubilized in water containing triethanolamine to make an initial stock solution of 40mM. Diluting the stock solution with high-glucose DMEM supplemented with 1X insulin-transferrin-selenium (Gibco, USA) and 1% Penicillin-Streptomycin resulted in the final concentrations used in the experiments.

2.3. Assay for 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium

The cell viability was assessed using the MTT test. U87 cells (2000) per well were seeded in 96-well cluster plates and incubated overnight in a humified CO_2 incubator. Curcumin, Quercetin, Resveratrol, and Ellagic acid(12.5, 25, 50, 75, and 100 μmol/L) were treated to cells for 96 h.

Each well was incubated for 4 hours at 37°C with the addition of 50μL MTT solution (Sigma-Aldrich) diluted in PBS (5 mg/mL). The formazan crystals were dissolved in 80μL of DMSO and shaken for 1 hour. On a microplate reader (Bio-Rad), optical density was determined at 595 nm. All of the tests were conducted four times. Nonlinear regression analysis, and IC_{50} calculation, were performed with the Graph Pad prism programme.

2.4. Glucose assay using DNS method

Glioma U87 cells (5×10^3) per well were seeded in a 96 well plate and allowed to adhere for 24 hours. After 24 hours the culture media was taken out and each of the phytocompound was added in a concentration range of 20 -40 μM along with the control. After a 24-hour incubation, the cells were washed twice with PBS, lysed using RIPA buffer, and centrifuged at 10000-12000 rpm. The supernatant was deproteinized with 8% perchloric acid (PCA) and centrifuged. Using DNS (2, 4 dinitrosalicylic acid), technique, the deproteinized supernatant was used to estimate the reducing sugar. In a nutshell, 80-100 μL supernatant was mixed with 1% DNS, the volume was adjusted to 1 mL with distilled water, and the mixture was heated for 15 minutes. The colour changes from light brown to dark brown indicating the presence of reducing sugar. The colour was stabilised by adding 40% potassium sodium tartatrate (PST) after a few minutes. Using a spectrophotometer, absorbance was measured at 595nm. The linearity of a standard curve made with anhydrous glucose was validated in the range of 0-1 mg/mL (r2= 0.98) and determined to be statistically significant.

2.5. Intracellular and extracellular Pyruvate and lactate concentrations

An improved approach was used to determine the concentrations of extracellular and intracellular Pyruvate & Lactate. Using the leftover lysates from the glucose experiment, pyruvate and lactate were determined. Extracellular Pyruvate and lactate were determined using the wasted culture media. For the determination of Lactate, a Glycine and Hydrazine buffer (90mM final concentration, pH 9.2) was added to a 50 μL lysate with excess NAD (2.4mM final), and the final volume was adjusted to 500L in distilled water. The reaction was catalysed by the addition of Lactate dehydrogenase

enzyme (LDH) 0.1U/l (0.1mg). Everything except the LDH enzyme was used to create a blank. With the use of a UV-Visible Spectrophotometer, the blank's absorbance was determined at 340nm. Similarly, for a total of ten minutes, the absorbances of the test samples were measured at 340nm in one-minute intervals. LDH reduces NAD to NADH, which results in a rise in absorbance at 340 nm. The formula Units/mg=(A340 x reaction volume)/(6.22 x voume (L) of sample in cuvette) was used to calculate the highest absorbance (maximum sample absorbance – blank absorbance=A340). The milimolar extinction coefficient of NADH at a concentration of 340nM is 6.22. For pyruvate measurement, 40 μL of lysate were mixed in a tris buffer (0.2 M, pH 7.3), and an excess of NADH (10 mM) was added before the final volume was adjusted to 500 μL litres with distilled water. 0.1U/l Lactate dehydrogenase enzyme was used to catalyse the process (LDH). An UV-Visible Spectrophotometer set to 340 nm was used to determine the absorbance. The formula Units/mg= (absorbance 340/min)/(6.22 x mg enzyme/mL reaction mixture) is used to calculate the decrease in absorbance at 340nm owing to the oxidation of NADH to NAD.

2.6. NADH/NAD ratio calculation

For the estimation of the NAD/NADH Ratio, Williamson et al. employed the formula Keq [pyruvate] eq [NADH]eq [H+]/([Lactate] eq [NAD] eq) =1.11610211, where pH is 7.0. The final products and reactants could be proved using the equation: Apparent Keq = Keq [H+], where Keq = [pyruvate] eq [NADH] eq/([Lactate] eq [NAD]eq) = 1.1161027 or [NAD]/[NADH] = [pyruvate] eq/(Keq [Lactate]eq) = 1.1161027.

2.7. Analysis of the data

ANOVA with a P value less than 0.05 was used to examine the significant difference between the control and tested drugs.

3. Results

Curcumin (C), Quercetin (Q), Ellagic acid (E), Resveratrol (R) as well as their combinations (C+Q, C+E C+R, Q+R) showed cytotoxic effects in glioma cells in a dose-dependent manner. Morphological alterations such as cell shrinkage, rounding, membrane blebbing, and signs of apoptosis were observed (Figure 1). The MTT-based assay was used to determine cell viability. The results were converted to % control and plotted in GraphPad prism using data from at least four experiments with triplicates. The results showed that all the four compounds and the combinations C+E, C+Q, C+R, and Q+R exhibited cytotoxic activity in U87 cells. The IC_{50} values of the compounds are indicated in the graph(Figure 1).

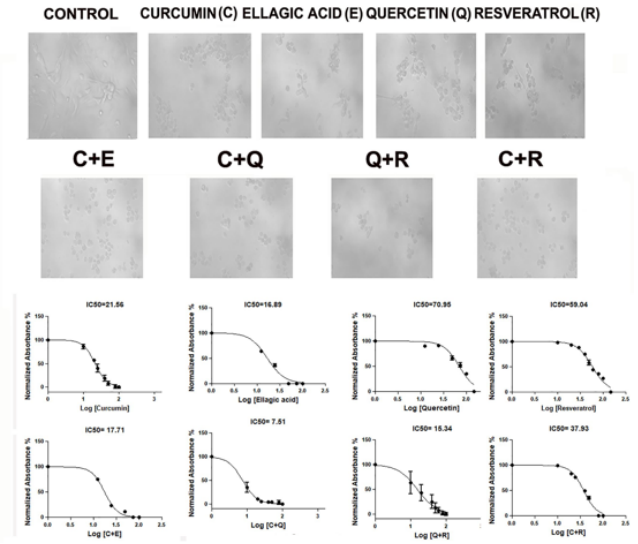

Fig. 1: Dose-dependent response of Curcumin, Ellagic acid, Quercetin, and Resveratrol and their combination on the viability of U87cells (A) Brightfield analysis of U87 cells treated with the carrier (Control) or 20μM concentrations of each of the compounds. (B) Graphs show the viability of the U87 cell as determined by tetrazolium salt (MTT) assay after 96 h of exposure of the compounds with indicated concentrations. IC_{50} values are generated using the Graphpadprism program and the value is indicated in the graph.

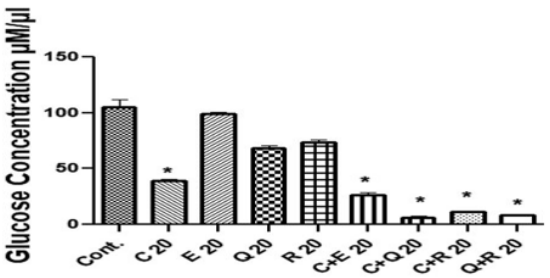

Fig. 2: Intracellular glucose concentrations of U87 cells, treated with carrier Curcumin, Quercetin, Ellagic acid, Resveratrol, and their combination for 24h. Intracellular glucose concentration significantly lower in Curcumin, Quercetin, and Resveratrol treated cells but nochange of glucose concentration was observedin ellagic acid treated cells.

Curcumin (C), Quercetin (Q), Ellagic acid (E), Resveratrol (R) as well as combinations of C+E, C+Q, C+R, and Q+R, were tested for modulation of intracellular glucose concertation in U87 cells.

A sublethal dose (20μM) of Curcumin (C) Quercetin (Q), Ellagic acid (E), Resveratrol (R), and combinations (10μM+10μM each) C+E, C+Q, C+R, Q+R were treated to U87 cells for 24 hours. The result demonstrated except Ellagic acid all other compounds and combinations decreased intracellular glucose concentration in comparison

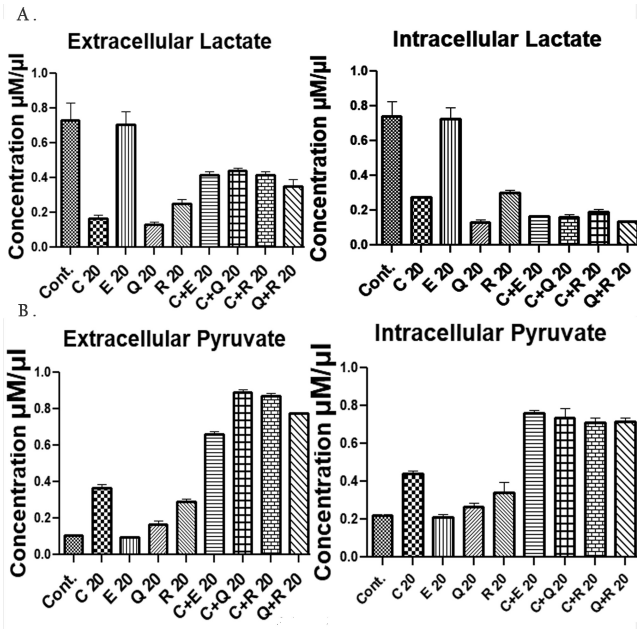

Fig. 3: A. Lactate release extracellularly and intracellularly in U87 cell line treatment with 20μM Curcumin, Quercetin, Resveratrol and Ellagic acid. B. Pyruvate release extracellularly and intracellularly in U87cell line treatment with 20μM Curcumin, Quercetin, Resveratrol, Ellagic acid and their combination.

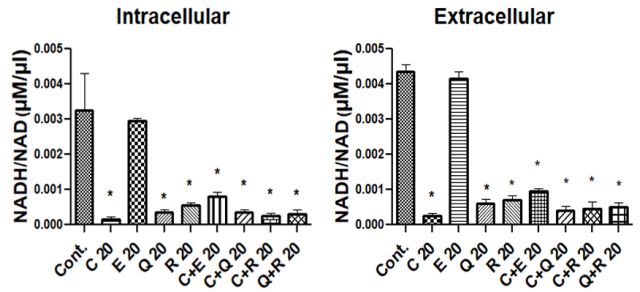

Fig. 4: NADH/NAD Ratio extracellularly and intracellularly in U87 cells treatment with 20μM Curcumin, Quercetin, Resveratrol, Ellagic acid and their Combination.

to the carrier treated cells. (Figure 2). Curcumin (C), Quercetin (Q), Resveratrol (R) and combinations C+E, C+Q, C+R, Q+R, lowered the intracellular and extracellular lactate levels while increasing pyruvate levels in U87 glioma cells.

The reversal of Warburg's effect was observed in U87 cells treated with, Curcumin (C), Quercetin (Q), and Resveratrol (R), as well as C+E, C+Q, C+R, Q+R for 24 hours, U87 cells were given a subtoxic dosage of 20M. With the exception of Ellagic acid, the data revealed a considerable drop in lactate and an increase in pyruvate (Figure 3). The information is presented in the form of Mean Standard Error. Curcumin (C), Quercetin (Q), Ellagic acid (E), Resveratrol (R) as well as C+E, C+Q, C+R, Q+R, combinations, modify the NADH/NAD ratio in U87 glioma cells.

4. Discussion

The high requirement for precursor molecules for numerous anabolic processes, such as ribose sugar for nucleotides, glycerol and citrate for lipids, various amino acids, and NADPH, is linked to cancer cell proliferation. To cope with the high energy demand and the necessity for precursor chemicals for fast cell division, cancer cells undergo metabolic reprogramming. There has been a lot of research on cancer cell metabolism in order to find new therapies for repairing disrupted metabolic pathways. The elevated lactate aids tumour development, angiogenesis, and rearrangement of tumour microenvironment. Lactate concentrations in tumours are linked to a higher likelihood of metastasis and a poor prognosis for survival in cancer patients.[16] Quercetin, Curcumin, Ellagic acid, and Resveratrol are well known for their anti-inflammatory, antioxidant, and cancer-fighting properties.[17] Curcumin has been shown to affect metabolism, resulting in lower glucose absorption and lactate generation.[18] Changes in metabolic pathways, particularly fatty acid metabolism, have been linked to phytocompounds such as resveratrol, quercetin, and epicatechin gallate. Our results show quercetin, curcumin and resveratrol, induce metabolic changes in Glioma cells. Aside from these, the compounds, with the exception of ellagic acid, reduced glucose uptake as well as lactate synthesis and increased pyruvate levels in the cells, reversing the Warburg effect. Curcumin, Resveratrol, Quercetin, modulated lactate/pyruvate metabolism at 20-40 μM concentrations, revealing cancer cells' sensitivity to these phytocompounds.

Quercetin, Curcumin Resveratrol, and Ellagic acid, appear to have a new anti-cancer mechanism, according to our findings. These compounds could be targeted for cancer therapy without destroying normal cells. One such step is metabolic restoration in cancer cells. In summary, our study demonstrates a novel anticancer effect for these selected phytocompounds, which paves the way for further research into their application in the clinical setup.

5. Conclusion

Despite considerable improvements in treatment options over the last decade, neither the incidence of cancer nor cancer-related mortality has changed in the recent 30 years. Anticancer medications now available have low efficacies, are accompanied with severe side effects, and are extremely costly. As a result, finding natural phytocompounds that do not have these drawbacks is a top focus. Natural dietary phytocompounds have been widely used in cancer prevention and therapy research in vitro, in vivo, and in preclinical models, with varying

degrees of effectiveness. Natural phytocompounds are a far more appealing approach that is fully justified in terms of understanding their potencies. It has been well established that cancer cell usually increases glucose absorption and switches to aerobic glycolysis. We demonstrated that natural phytocompounds can reverse the Warburg effect by increasing pyruvate concentration and decreasing lactate concentration. Thus, Curcumin, Resveratrol, Quercetin, and, the tested combinations of phytocompounds, are candidate anticancer compounds for further research on glioma.

6. Abbreviations

1. LDH: Lactate Dehydrogenase
2. FDG: Fluoro- Deoxy-Glucose
3. MTT: *3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium*
4. DMSO: Dimethyl Sulfoxide
5. PCA: Perchloric Acid
6. DNS: 2, 4 Dinitrosalicylic Acid
7. PST: Potassium Sodium Tartatrate
8. DMEM: Dulbecco's Modified Eagle Medium

7. Source of Funding

Department of Science and Technology, Government of Odisha.

8. Conflict of Interest

None.

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