Original Article

Amalgamation of quercetin with anastrozole and capecitabine: A novel combination to treat breast and colon cancers – An *in vitro* study

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ABSTRACT

Context: Globally, cancer stands as the principle cause of mortality and immediate attention on its treatment options is required. Natural compounds stay at first priority in encountering novel therapeutics without adverse effects.

Aim: The aim of the study is to extract flavonol quercetin from leafy vegetables of *Anethum graveolens* L. and *Raphanus sativus* L. and find out its potential in combination with drugs used for chemotherapy to reduce the adverse effects of drugs.

Settings and Design: Observational study.

Materials And Methods: Column chromatography is used for quercetin extraction and anticancer activity of quercetin + anastrozole and quercetin + capecitabine were determined by (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay (MTT), apoptosis assay, cell cycle analysis, mitochondrial membrane potential, and caspase 3 expression.

Statistical Analysis Used: Cytotoxic assay results were assessed by mean, standard deviation and ANOVA; and results were compared for determining its significance.

Results: The results noted that quercetin at very less concentration (16 and 31 μg/ml on Michigan Cancer Foundation-7 and 43 and 46 μg/ml on COLO 320) in combination with anastrozole and capecitabine was able to control the growth of cells, increase cell death, arrest cell cycle, and induce mitochondrial depolarization and expression of caspase 3.

Conclusions: The natural compound used in the present study is effective in treating breast and colon cancer at minimal concentrations in combination with the drugs. This combinational treatment appears to be reported for the first time in the present study.

KEY WORDS: Anticancer, COLO 320, flavonoid, Michigan Cancer Foundation-7

INTRODUCTION

Cancer, being top ranked, files about 9.6 million deaths a year that triggered the drug investigators to identify novel molecules.[1] According to 2018 reports of the World Health Organization, the top ranked cancers were breast and colorectal which relies on "one size fits all" approach for treatment. During initial treatment, cancer cells easily gain drug resistance by activation of biological pathways.[2,3] These combinations were preferred majorly in chemotherapy.[4] Drugs such as anastrozole is effective in combination with other drugs for treating breast cancer patients after surgery.[5,6] Capecitabine is opted for off-label indications in colorectal cancer exclusively for monotherapy in advanced or metastatic settings. Its combination with oxaliplatin in the advanced or metastatic settings and concurrent radiations is used for the neoadjuvant treatment in colorectal cancer.[7,8] Hence, introduction of unrevealed combinations would be a promising approach to achieve impairments in minimizing side effects coupled with conventional chemotherapy that cracks on quercetin.

Flavonoids are proved anticancer agents, among which quercetin has ability over cell-signaling, pro-apoptotic, antiproliferative, and antioxidant

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effects.[9-11] Long-term treatments with quercetin result in pro-apoptotic effects that covalently link electrophilic compounds with glutathione, in particular glutathione S-transferase-P1-1 π in various cancers.[12-15] It has been appraised in Phase I clinical trials for treating acquired mutations caused by hematological menaces as a tyrosine kinase inhibitor without side effects. Experimental attempts were in progress in gauging quercetin and its combination with classical drugs for cancer treatment.[16,17] Therefore, quercetin extracted from unexplored sources (*Anethum graveolens* L. and *Raphanus sativus* L) is planned for the study. *A. graveolens* (dill weed) is an annual herb of Apiaceae family with pharmacological activities such as antimicrobial, antihyperlipidemic, and antihypercholesterolemic activities.[18] *R. sativus* is an annual root vegetable of Brassicaceae family, and the seed, root, and leaf of *R. sativus* are claimed to have several medicinal properties.[19] With this background, the study is aimed to crack the chemotherapeutic potential of quercetin extracted from leaves of *A. graveolens* and *R. sativus* with anastrozole and capecitabine combinations in treating breast and colon cancers.

MATERIALS AND METHODS

Plant materials

The plants used for quercetin extraction were *A. graveolens* L. and *Raphanus sativus* L. grown in herbal garden. The plants were identified by Dr. Madhava Shetty, Assistant Professor, Department of Botany, S. V. University, Tirupathi, with a voucher specimen numbers of 1236 *(A. graveolens*) and 2232 (*R. sativus*). The water-stressed leaf material was collected during morning hours, dried, powdered, and stored in separate containers for further analysis.

Isolation and identification of quercetin

For solvent extraction, about 25 g of leaf powder was taken in 100 ml ethanol, kept on constant shaking at 120–130 rpm for 48 h. The residue was filtered using Whatman number 1 filter paper followed by evaporating remaining solvent in an air-circulating oven at 54°C until total dryness. About 12 g of obtained dried extract was collected and stored at 4°C for further analysis.[20] Column chromatography by gradient elution methods was performed according to established methods available.[21,22] About 100 mg of extract was loaded on the column (Silica) and was run with suitable solvents such as hexane, ethanol, and ethyl acetate in various combinations such as 1:1, 1:2, and 1:5 and the eluents were separately collected with 2 min time interval. The obtained fractions were pooled, concentrated, and used for checking purity by advanced chromatography, i.e., high-performance liquid chromatography (HPLC) along with the standard quercetin.

Cells and chemicals

Breast cancer cell line, Michigan Cancer Foundation-7 (MCF-7), and Colorectal adenocarcinoma cell line, COLO 320 of adherent type, were acquired from the National Centre for Cell Science, Pune. The cell culture medium [high glucose DMEM (#AL111) and fetal bovine serum (# RM10432)] were obtained from Hi media. Anastrozole and capecitabine drugs were procured from Sigma-Aldrich and remaining solvents used were of analytical grade.

Determination of cytotoxicity

Cell lines of breast (MCF-7) and colon cancer (COLO 320) were grown individually and experiments were performed.[23] Briefly, 200 µl of cell suspension (2×10^4 cells/mL) was added per well in a 96-well plate and grown for 24 h. Initial experiments were performed with quercetin with concentrations ranging from 10 to 50 µg/ml, as the cytotoxicity of compound is found to be effective with increasing concentrations, and further experiments were planned with higher concentrations. The test compound (quercetin) extracted from both the plants and standard drugs (anastrozole and capecitabine) at 50 µg/ml, 100, 150, 200, and 250 µg/ml concentrations was made individually, added into well of 100 µl each, and incubated in a 5% CO_2 incubator for 24, 48, and 72 h. The media were removed and (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) was added to a final concentration of 0.5 mg/ml of total volume and incubated for 2 h. Later, MTT was removed followed by addition of 100 µl DMSO for solubilization and absorbance was measured at 570 nm and IC50 was determined. The same experiment was repeated for combination of plant quercetin, standard quercetin, and standard drugs at similar concentrations in 1:1 ratio. Absorbance was recorded at 570 nm and IC50 values were determined and the IC50 value obtained at 72 h time interval was considered for rest of the experiments [Table 1].

Dual stain method

Cell lines of MCF-7 and COLO 320 were cultured to a concentration of 2×10^4/ml and dispensed into a 96-well plate of 100 µl into each well separately.[24] The seeded cells were treated with the combination mentioned in Table 1 and was incubated for 72 h followed by addition of 0.25% trypsin. When cells had sloughed off, 25 µl was taken onto glass slide and added 1 µl dual fluorescent staining solutions containing 100 µl/ml acridine orange (AO) and 100 µg/ml ethidium bromide (EB)

Table 1: Concentration of test and drug combinations presented in the study

Treatment groups
Cell control: only cells
Standard control (standard): Anastrozole/capecitabine
S1 (quercetin from *A. graveolens*)
S2 (quercetin from *R. sativus*)
S1 + standard control
S2 + standard control
Concentrations
Standard control (anastrozole) at 38 µg/ml on breast cancer
Standard control (capecitabine) at 45 µg/ml on colon cancer
S1 + anastrozole at 16 µg/ml on breast cancer (MCF-7)
S2 + anastrozole at 31 µg/ml on breast cancer (MCF-7)
S1 + capecitabine at 43 µg/ml on colorectal cancer (COLO 320)
S2 + capecitabine at 46 µg/ml on colorectal cancer (COLO 320)

MCF-7=Michigan Cancer Foundation-7

and covered with coverslip. The morphology of apoptotic cells was examined and cells were counted within 20 min under a fluorescent microscope. The observations were noted after the repetition of experiments for three times.

Cell cycle assay

Cancer and control cells (free of drug) in a 6-well plate with a cell density of 2 × 10^5/2 ml were incubated for 24 h in a CO_2 incubator followed by treatment with required concentration of combinational compound [Table 1] and incubated for 72 h.[25] Later, medium left in the plates was removed, washed with phosphate-buffered saline (PBS), and decanted. Two ml culture medium was added to all the wells containing cells and harvested directly into 12 mm × 7 mm polystyrene tubes and centrifuged at 300 × *g* at 25°C for 5 min, decanted the supernatant, and sweep up with PBS. Then, cells were fixed in 1 ml of cold 70% ethanol dropwise and mixed thoroughly to avoid clumping. Further, cells were fixed for at least 30 min on ice and pelleted at high speed compared to live cells for 5 min. Aspirated the supernatant carefully and washed twice with PBS. To ensure the DNA staining, pelleted cells were treated with 50 µl ribonuclease A to get rid of RNA. To each tube, propidium iodide (PI) of 400 µl/million cells was added, mixed, and incubated for 5–10 min at room temperature. Samples were analyzed by flow cytometry in PI solution without washing.

Mitochondrial membrane potential analysis

Cancer cells were maintained in a 6-well plate with a cell density of 3 × 10^5/1 ml and incubated for 24 h at 37°C in a CO_2 incubator.[26] Cells were treated with the combination of quercetin and drug as mentioned (table 1) and incubated for 72 h. At the end of treatment, cells were transferred into 12 mm × 75 mm polystyrene tubes and centrifuged at 300 × *g*, 25°C for a period of 5 min. The upper layer was decanted and washed the cells with PBS. JC-1 dye (5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimi-dazolyl carbocyanine iodide, 0.5 ml) was supplemented to cell pellet and incubated for 10–15 min at 37°C in a CO_2 incubator.

Caspase assay

Cancer cells were taken in a 6-well plate with a cell density of 0.5 × 10^6/2 ml and incubated for a period of 24 h in a CO_2 incubator at 37°C. Media was taken out and test compound and standard [Table 1] were given and incubated for 72 h.[27] Cells were splashed with PBS, and 300 µl of trypsin-EDTA was added and incubated for 3–4 min at 37°C. Then, 2 ml of medium was supplied and cells were picked directly into 12 mm × 75 mm polystyrene tubes and centrifuged at 300 × *g*, 25°C for 5 min time period. The top layer was decanted and cells were given PBS wash twice. The PBS-free cells were fixed in 1 ml of prechilled cold 70% ethanol added dropwise and left on ice for 30 min. Cells were pelleted at high speed, supernatant was discarded followed by PBS wash. Then, 5 µl of fluorescein isothiocyanate caspase 3 antibody was added, mixed, and kept at room temperature for 30 min in dark. Cells were then washed with 1 × PBS contained 0.1% sodium azide, mixed the cells thoroughly with PBS, and analyzed by flow cytometry.

Determination of cytotoxicity on normal cell line

Mouse fibroblast cell lines, 3T3-L1, were grown in DMEM and FBS medium and 200 µl cell suspension was seeded on a 96-well plate at required cell density (2 × 10^4 cells). To these cells, 100 µl of different concentrations (50, 100, 150, 200, and 250 µg/ml) of standard drugs (anastrozole and capecitabine) and combination with quercetin were added. Plates were incubated for 72 h at 37°C in a 5% CO_2 atmosphere and followed by addition of MTT (0.5 mg/ml) and further incubated for 3 h. Later, 100 µl of solubilization solution (DMSO) was added to all the wells and gently stirred on a gyrator shaker and absorbance was read at 570 nm. The IC50 value was determined using linear regression equation.[23]

$Y = M X + C$ (Here, $Y = 50$, M and C values were derived from the viability graph).

Statistical analysis

The data obtained were calculated for mean ± standard deviation. The statistical differences of all the treated groups at different time intervals were determined by ANOVA (IBM SPSS Software) for comparison with various combinations of test compound and drug, $P < 0.05$ is considered as significant.

RESULTS

Identification and purification of compound

The fractions of column chromatography when subjected to HPLC depicted similar retention times for the quercetin extracted from *A. graveolens* and *R. sativus* (14.76, 14.78 min) and standard quercetin (14.45 min) as depicted in Figure 1 and considered as 98% pure.

Inhibitory outcome of quercetin and drugs on the proliferative activity of MCF-7 and COLO 320

Upon initial screening of cytotoxicity by MTT assay, IC50 values for all the tested cancer cell lines of breast and colon were calculated. It was found that the IC50 values of breast and colon cancer at 72 h treatment were found encouraging. Interestingly, the combination of quercetin with drugs showed less IC50 value compared to standard drug treatment alone on breast and colon cancer cell lines at same time interval [Tables 2 and 3]. The results proved that quercetin extracted from *A. graveolens* and *R. sativus* in combination with anastrozole at 250 µg/ml exhibited IC50 value of 16 and 31 µg/ml on breast cancer and 43 and 46 µg/ml on colon cancer. Hence, the resulted potential cytotoxic activity exhibited by combination of drug with quercetin at 72 h was taken forward to screen the mechanism of action on breast and colon cancer cell lines.

Quercetin prompts apoptosis with drug combination against MCF-7 and COLO 320

Cells of MCF-7 and COLO 320 were stained by AO/EB for 72 h

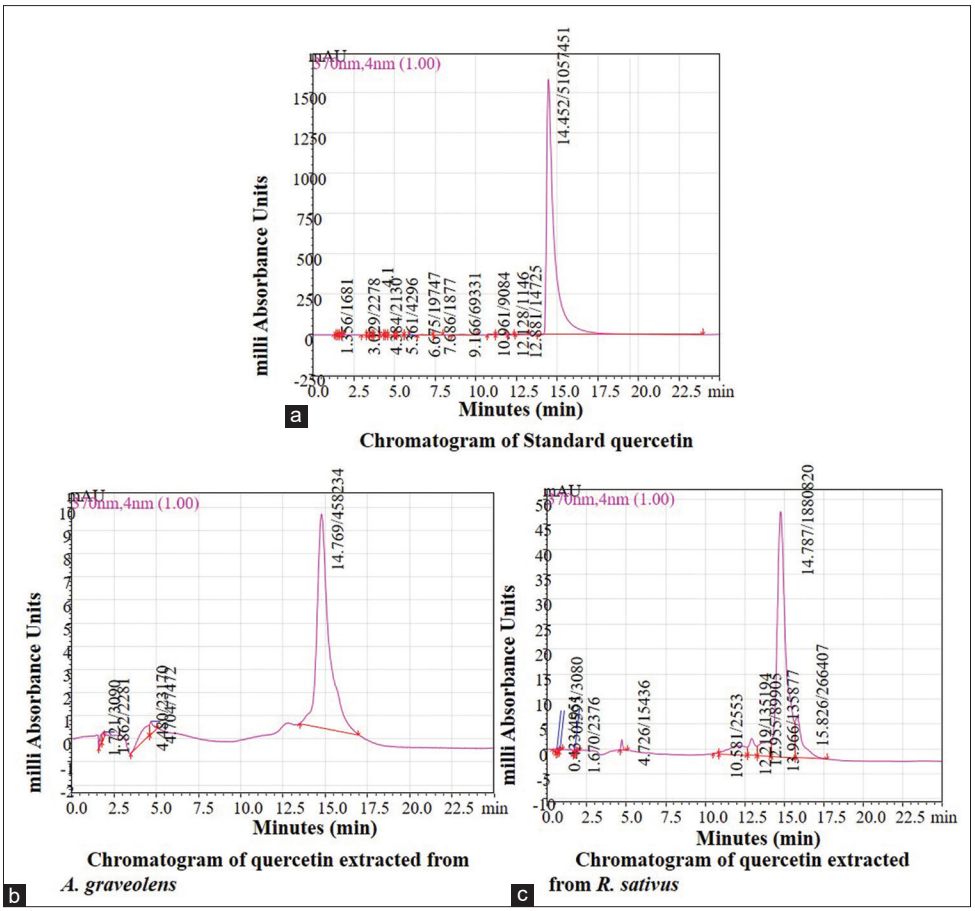

Figure 1: A: HPLC chromatogram of quercetin standard; B: HPLC chromatogram of quercetin extracted from *A. graveolens* and C: HPLC chromatogram of quercetin extracted from *R. sativus*

Table 2: Cytotoxic potential of test compounds on MCF-7 cell line at different time intervals

Test	IC50 (µg/ml)		
	For 24 h	For 48 h	For 72 h
Quercetin from *A. graveolens* (S1)	329	233	161
Quercetin from *R. sativus* (S2)	474	280	158
Anastrozole (standard)	216	134	38
S1 + standard	173	67	16*
S2 + standard	202	116	31*

ANOVA was used for comparison and *P* value was found to be significant (0.02337)

Table 3: Cytotoxic potential of test compounds on COLO 320 cell line at different time intervals

Test	IC50 (µg/ml)		
	For 24 h	For 48 h	For 72 h
Quercetin from *A. graveolens* (S1)	324	175	106
Quercetin from *R. sativus* (S2)	546	203	131
Capecitabine (standard)	194	56	45
S1 + standard	246	118	43*
S2 + standard	298	140	46*

ANOVA was used for comparison and P value was found to be significant (0.02337)

and were analyzed by fluorescent microscope. Quercetin from both the plants (*A. graveolens* and *R. sativus*) with anastrozole and capecitabine combination at 16 and 31 µg/ml on breast cancer and 43 and 46 µg/ml on colon cancer presented effective morphological changes like cell shrinkage and condensation of chromatin depicted in Figures 2 and 3 respectively.

Quercetin and standard drugs boost cell cycle arrest at various stages

Further to MTT assay (cell viability), growth arrest was explained by cell cycle analysis. Therefore, changes in cell cycle after 72 h of stimulation with combination of quercetin and drug using flow cytometry were assessed. Treatment of MCF-7 cells with quercetin (*A. graveolens*) and anastrozole (1:1 ratio) at 16 µg/ml concentration showed significant G1 phase arrest than the treatment with standard drug anastrozole of the similar concentration. Likewise, more number of cells were arrested in G2/M phase at 16 and 31 µg/ml concentrations of quercetin from *A. graveolens* and *R. sativus*, which is more significant than the G2/M arrest exhibited by standard drug, a novel identification. No significant differences were noted in SubG0/G1 (Apoptotic phase) of cell cycle in test compounds, but in standard control, it showed significant increase compared to cell control as depicted in Figure 4.

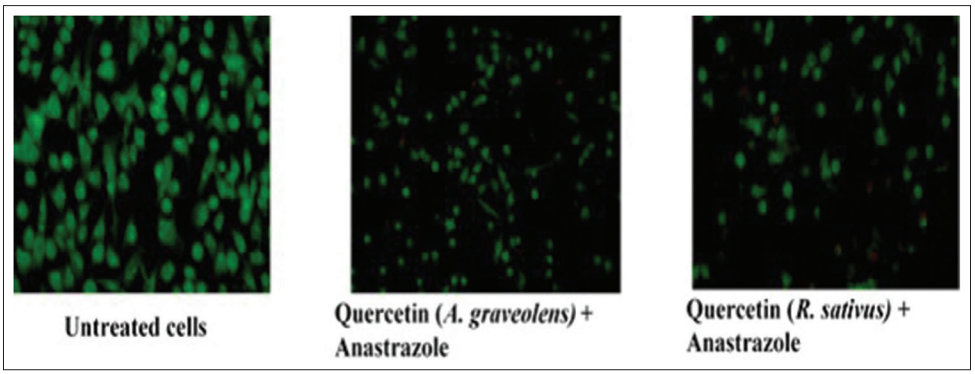

Figure 2: Acridine orange/ethidum bromide staining of *in vitro* grown cultures of MCF-7 cells in treatment with test compound and standard drug

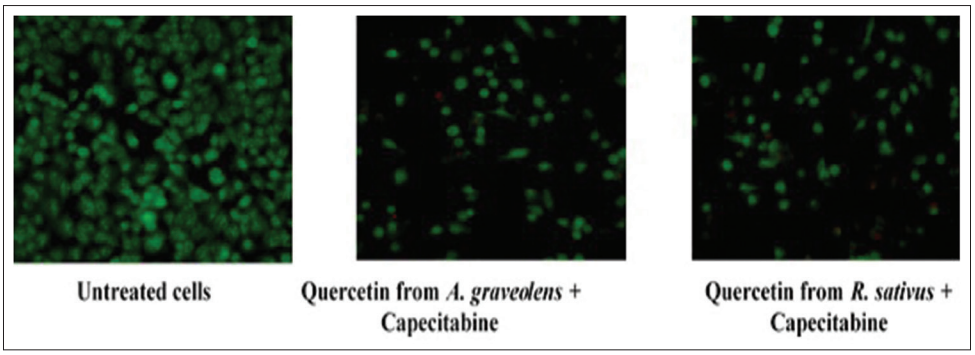

Figure 3: Acridine orange/Ethidum Bromide staining of *in vitro* grown cultures of Colo 320 cells in treatment with test compound and standard drug

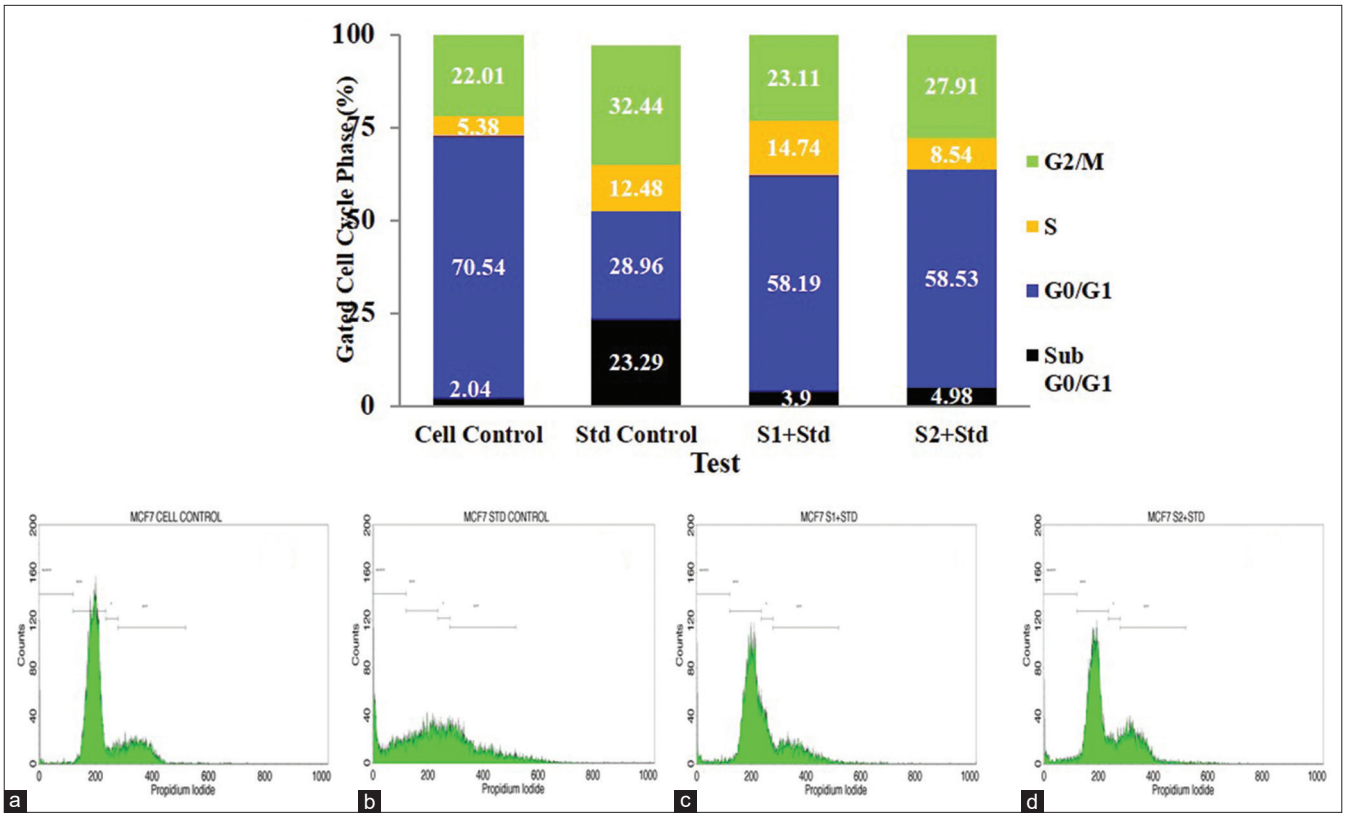

Figure 4: Propidium iodide histogram of the gated cell singlets distinguishes cells at the sub G0/G1, G0/G1, S and G2/M cycle phases and images of flow cytometric analysis showing cell control (a), standard control (anastrozole) (b), quercetin from *A. graveolens* + anastrozole (S1 + standard control) (c) and quercetin from *R. sativus* + anastrozole (S2 + standard control) (d) respectively against MCF-7 cells

The results of cell cycle by flow cytometry on COLO 320 cells suggested that compared to cell control, drug treatment of test compounds (namely quercetin from *A. graveolens* L. at 43 µg/ml concentration [S1 + standard] and quercetin from *R. sativus* L. at 46 µg/ml concentration [S2 + standard]) clearly established cell cycle arrest in Sub G0/G1, G0/G1 phase and also G2/M phase of cell cycle. There were no significant differences noticed in S (synthetic phase) of cell cycle in test compounds and standard control compared to cell control. This seems to be the first report to knock out the novel drug combinations with quercetin that effectively arrest the cell cycle at different phases at minimal concentrations as shown in Figure 5.

Quercetin induces depolarization to mitochondrial membrane potential

A sensitive cationic and lipophilic JC-10 fluorescent probe was preferred to monitor the mitochondrial membrane potential alteration in the cells. Quercetin combination with drugs showed a significant reduction of mitochondrial membrane integrity against MCF-7 and COLO 320 cells by showing the shift of cells from upper right quadrant-red (FL2) to lower right quadrant-green (FL1). The obtained results clearly suggest that the mitochondrial depolarization happened in MCF-7 and COLO 320 by the action of the compounds, led to cell death. It resulted an observation that compound S1 (quercetin from *A. graveolens*) has significant mitochondrial membrane depolarization/integrity potential compared S2 (quercetin from *R. sativus*) by conjugating with **s**tandard drugs in the ratio of 1:1 against MCF-7 and COLO 320 cell lines after incubating for 72 h as shown in Figures 6-9.

Quercetin with anastrozole and capecitabine combination induces activation of expression of caspase 3

The observations in statistical data of caspase 3 expression by a technique called flow cytometry suggest that caspase 3 level is very low in untreated MCF7 Cells (8.82 mean fluorescence intensity [MFI]) compared to anastrozole (41.33 MFI) after the incubation period of 72 h. The test compounds (S1 + anastrozole and S2 + anastrozole) showed caspase 3 expression at 37.82 and 32.10 in terms of MFI after 72 h of incubation [Table 4 and Figure 10].

The observations in statistical data of caspase 3 expression indicate that it expresses very low in untreated COLO-320 cells (8.69MFI) compared to capecitabine (61.88MFI) after the incubation period of 72 h. The test compounds (S1 + capecitabine and S2 + capecitabine) showed caspase 3 expression at 47.79 and 42.45 MFI after 72 h of incubation [Table 5 and Figure 11].

The nontoxic nature of combinational treatment on normal cell line, 3T3-L1

The cytotoxic effect of quercetin in combination with

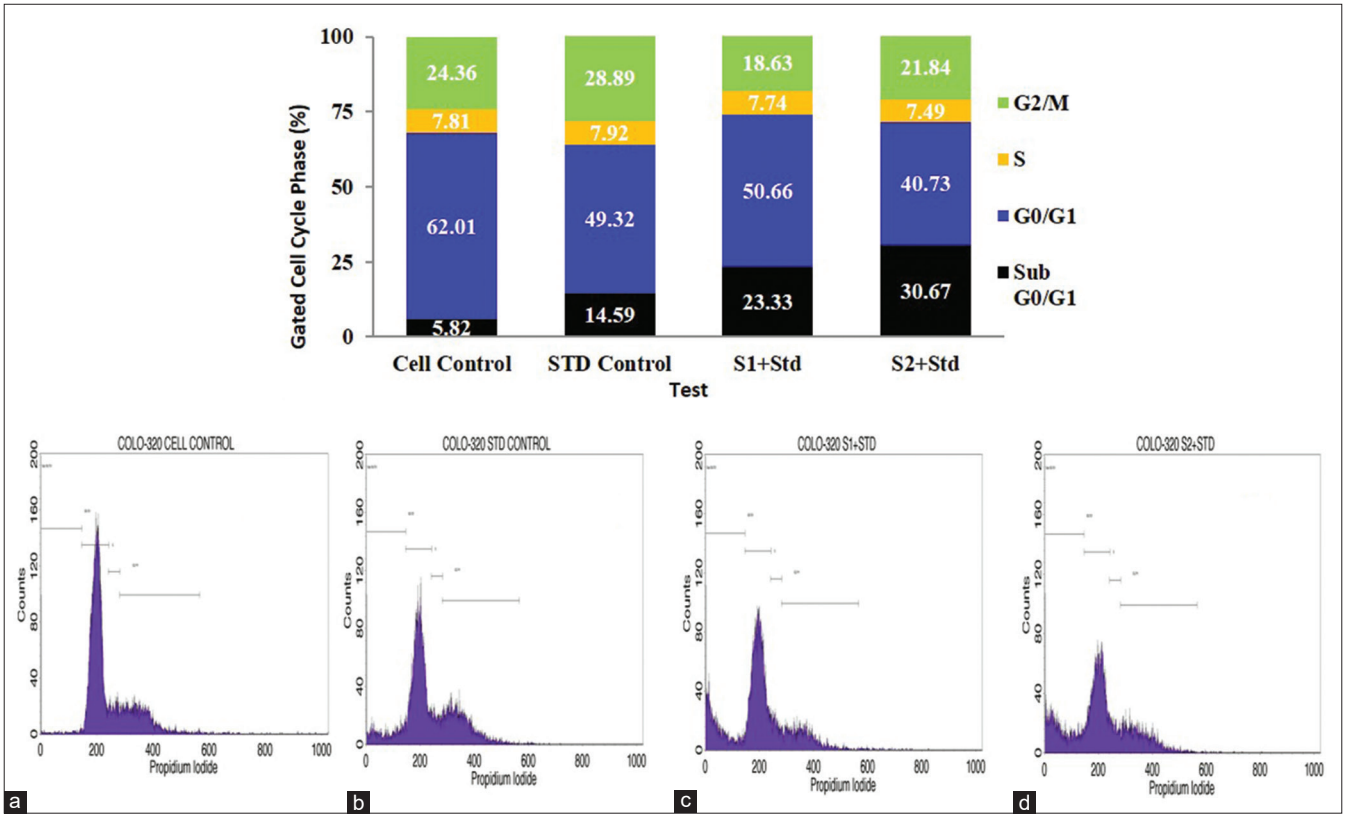

Figure 5: Propidium iodide histogram of the gated cell singlets distinguishes cells at the sub G0/G1, G0/G1, S and G2/M cycle phases and images of flow cytometric analysis showing cell control (a), standard control (capecitabine) (b), quercetin from *A. graveolens* + capecitabine (S1 + standard control) (c) and quercetin from *R. sativus* + capecitabine (S2 + standard control) (d) respectively against COLO 320 cells

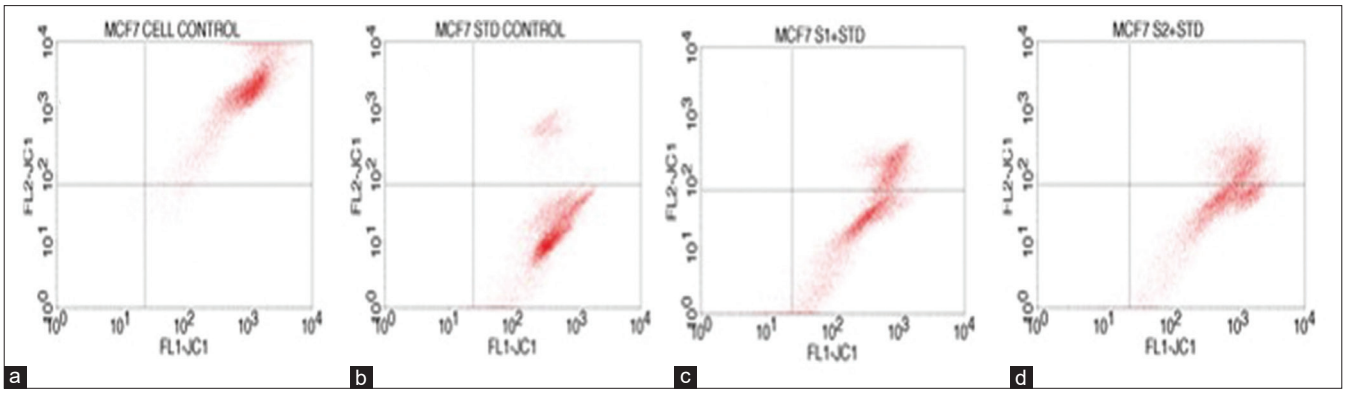

Figure 6: JC1 expression study of MCF-7 cell line by flow cytometry. The quadrants depicting mitochondrial membrane depolarization in different groups *viz.*, cell control (a), standard control (anastrozole) (b), S1 + Standard (c) and S2 + standard (d) with IC50 concentrations

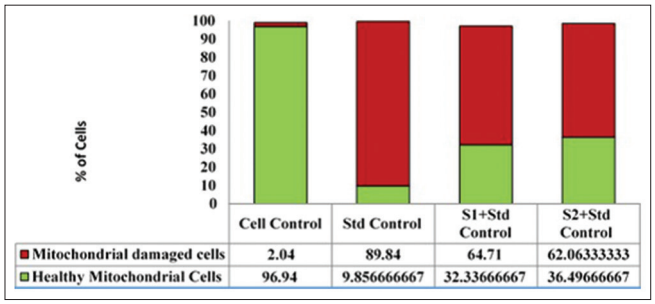

Figure 7: Histogram showing the healthy and damaged mitochondrial cells upon treatment with combination of anastrozole and quercetin from *A. graveolens* (S1) and *R. sativus* (S2) standard against Michigan Cancer Foundation-7 cells

anastrozole and capecitabine was evaluated on 3T3-L1. The results showed that the combination of quercetin and standard drugs were not cytotoxic to normal cells even after 72 h of incubation at IC50 above 100 µg/ml and up to 250 µg/ml [Figure 12]. The final conclusion drawn from the study suggests that S1(quercetin from *A. graveolens*) and S2 (quercetin from *R. sativus*) in combination with standard drugs on 3T3-L1 cell lines exhibited IC50 values greater than the IC50 values obtained on MCF-7 and COLO-320 cancer cell lines after the incubation period of 72 h. Hence, it is proved that quercetin does not have any cytotoxicity against mouse normal fibroblast cells (3T3-L1).

DISCUSSION

It is observed that only 10% of plant types are investigated for pharmacological benefits out of 250,000 species. We used the leaves for quercetin isolation as nearly 25% of modern drugs are derived from plants. The leaves can be consumed as salad and also as one of the ingredients in Indian cuisine. Hence, plant-based quercetin can be incorporated into the system directly rather than using commercial quercetin. These two plants are also added in the literature along with the other available plant sources for quercetin.[28] The phytochemicals of various parts of plant such as phenolics,

Table 4: Mean fluorescence intensity of caspase 3 against the untreated, standard control (anastrozole) and the combination of anastrozole with the test compounds, S1 and S2 with the ratio of 1:1 treated on MCF-7 cells

Caspase 3	Relative mean fluorescence intensity y.a.u (arbitrary units of fluorescence)
Cell control	8.82
Anastrozole	41.33
S1 + anastrozole	37.82
S2 + anastrozole	32.1

Table 5: Mean fluorescence intensity of caspase 3 against the untreated, standard control (capecitabine) and the combination of capecitabine with the test compounds S1 and S2 with the ratio of 1:1 treated on COLO 320 cells

Caspase 3	Relative mean fluorescence intensity y.a.u (arbitrary units of fluorescence)
Cell control	8.69
Capecitabine	61.88
S1 + capecitabine	47.79
S2 + capecitabine	42.45

alkaloids, flavonoids, glycosides, gums, resins, and oils are the prioritized constituents for anticancer potential.[29] Flavonoids are the abundant components with varied biological activities. They belong to polyphenol groups having 3-hydroxyflavone backbone with quercetin, kaempferol, and myricetin subgroups. Literature revealed 9,000 different flavonoids, of which their daily intake differs between 20 mg and 500 mg from dietary supplements.[30] Quercetin is copious in plants, fruits, and vegetables and exists in aglycone or in bounded form with carbohydrates and alcohols.[31-33] It is assumed as strong antioxidant with its free radical scavenging activity that binds to metal ions.[34,35] This oxidative damage to DNA is measured as a risk factor that amounts for cancer. Quercetin acts as a strong antioxidant in cells via limiting the reactive oxygen and nitrogen species that help in cancer development.

It was illustrious that till date, 350 quercetin glycones were reported from various plant sources.[37] Therefore,

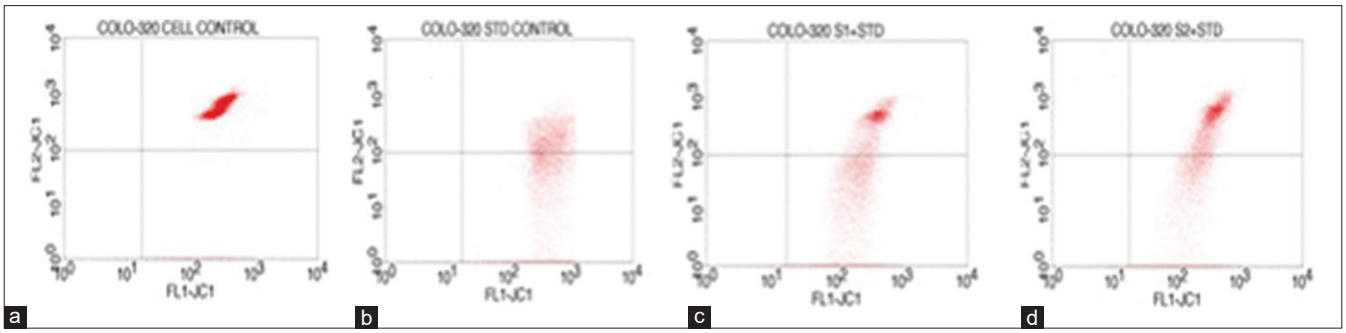

Figure 8: JC1 expression study of COLO 320 cell line by flow cytometry. The quadrants depicting mitochondrial membrane depolarization in different groups *viz*., cell control (a), standard control (capecitabine) (b), S1 + standard (c) and S2 + standard (d) with IC50 concentrations

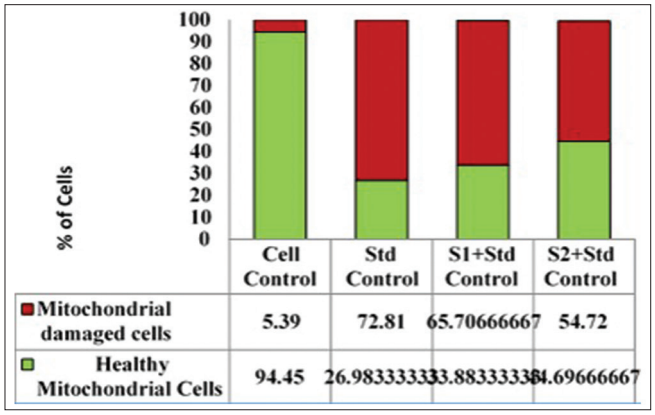

Figure 9: Histogram showing the healthy and damaged mitochondrial cells upon treatment with combination of capecitabine and quercetin from *A. graveolens* (S1) and *R. sativus* (S2) against COLO 320 cells

the identification of quercetin from diversified plant groups (*A. graveolens* L. and *Raphanus sativus* L) helps in detecting new sources of quercetin as medicine and food supplement. Leaf is considered for quercetin extraction and the portion retained from column chromatography is tested for purity by HPLC and related with standard. For the present study, leaves were used for isolation of quercetin, since leaves contain more quercetin content based on the other observations.[38] HPLC of column chromatography fractions eluted at retention time of 14.45 (standard) and 14.76 (quercetin from *A. graveolens*) and 14.78 (*R. sativus*). The HPLC elution time of quercetin fraction is in coordination with tomato-based products and *Marchantia convolute*.[39,40] As quoted earlier about the benefits of quercetin, several *in vitro* reports proved that quercetin can minimize extent of the tumor in voluminous cancer cells by its capability to induce apoptosis that makes it an important phytochemical in treating cancer. The study investigations were able to fill the existing gap of novel identified resources from edible greens, which will have additional benefit to human community upon consumption.

The American Cancer Society estimates 2.1 million newly approved breast cancers and 1.8 million colorectal cancers worldwide.[41,42] Treatment choices for cancers involve radiation and hormone therapy, surgery, and chemotherapy. Among several methods, chemotherapy is considered as the main line of treatment used before or after surgery. This chemotherapy involves only monotherapeutic agents and/or in relation with other agents to destroy cancer cells. However, chemotherapy limits its application by dose-limiting toxicity, difficulty in drug delivery of hydrophobic drugs, and resistance.[43] Cancer cells undergo complex molecular pathway; application of monotherapy brings cancer cells to use alternative pathways for recurrence.[44] This has resulted in metastasis and drawn our attention to focus on combination therapy which aids to overcome resistance and also aids to reduce dose of each agent.[45]

Multiple regimen targets are in practice for handling breast cancer and colorectal cancer. Anastrozole with trade name Arimidex is a potent nonsteroidal aromatase inhibitor employed for altered stages of breast cancer treatment since 1995.[46] The treatment with anastrozole should be long till the tumor regression ends. The anticancer therapy with anastrozole leads to adverse effects such as anemia, vaginal bleeding, skeletal complications, sexual dysfunction, and leucopenia.[47] Capecitabine a first-line medicine for colorectal cancer is commercially called as Capiibine since 1998. The adverse drug effects include chest pain, low platelet count, back pain, anemia, and jaundice.[48] Keeping in view of side effects caused by available drugs, much research is going on to minimize its effects, which is indeed presented in the study reports. For various natural sources, a precise mechanism of action is not known and being investigated. Quercetin from *A. graveolens* and *R. sativus* falls into such category where its mechanism of action and also its combination by drugs is not being reported in literature.

Food is the foremost source of bioactive components that benefit us; the combination of food components having biological efficacy with drug might reduce the toxic burden on the patient undertaking treatment. Reports in the study proved that quercetin had higher efficacy for breast and colon cancer with inhibitory concentration from 106 μg/ml to 131 μg/ml on colon cancer and 161 μg/ml to 158 μg/ml on breast

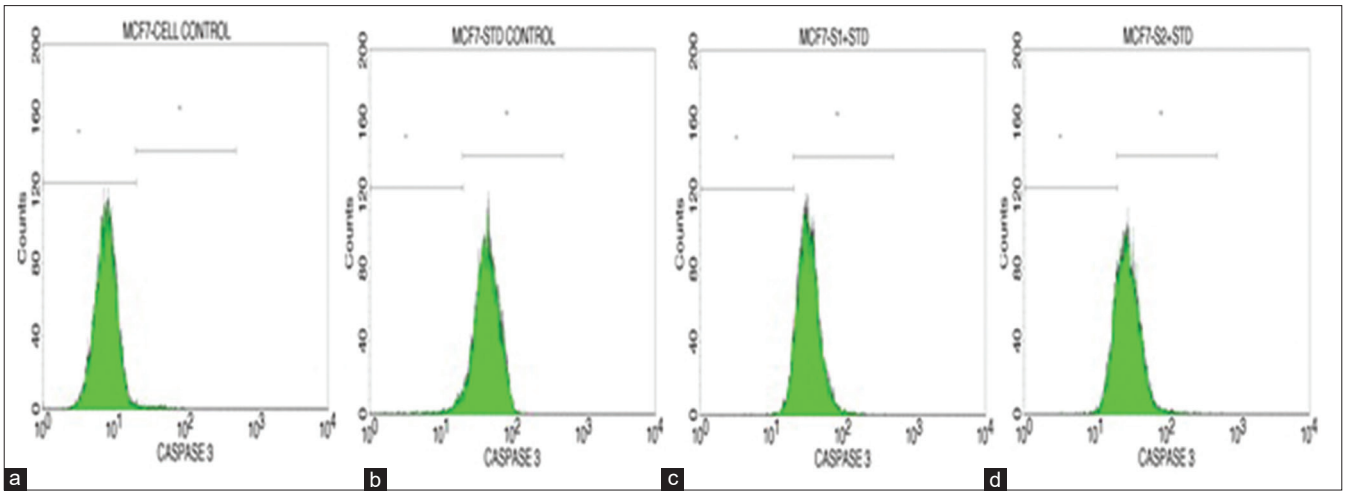

Figure 10: Caspase 3 expression study of cell control/untreated cells (a), Standard control (anastrozole) (b), quercetin from *A. graveolens* + anastrozole (S1 + standard control) (c) and quercetin from *R. sativus* + anastrozole (S2 + standard control) (d) respectively against MCF-7 cells

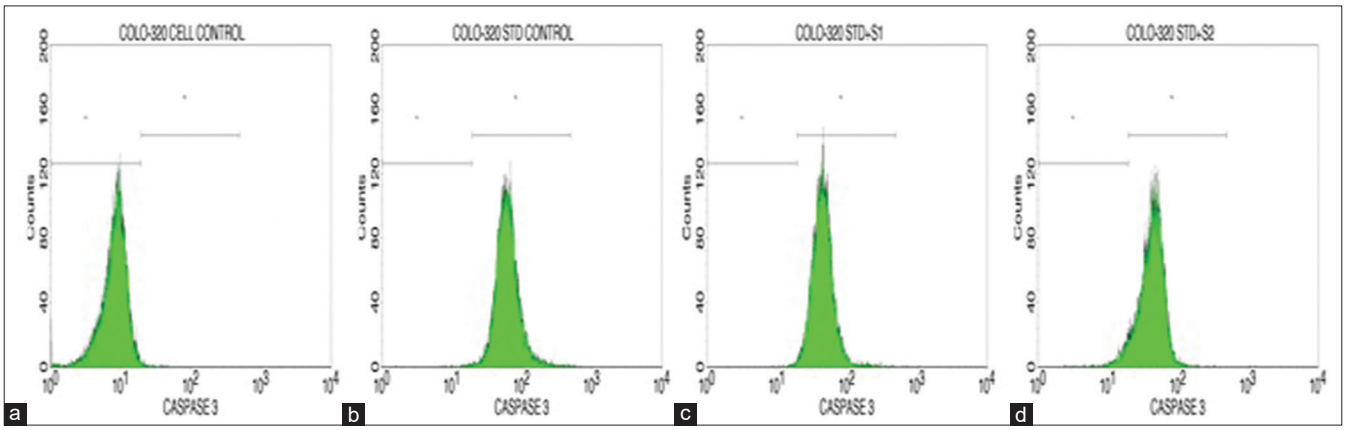

Figure 11: Caspase 3 expression study of cell control/untreated cells (a), Standard control (capecitabine) (b), quercetin from *A. graveolens* + capecitabine (S1 + standard control) (C) and quercetin from *R. sativus* + capecitabine (S2 + standard control) (d) respectively against COLO 320 cells

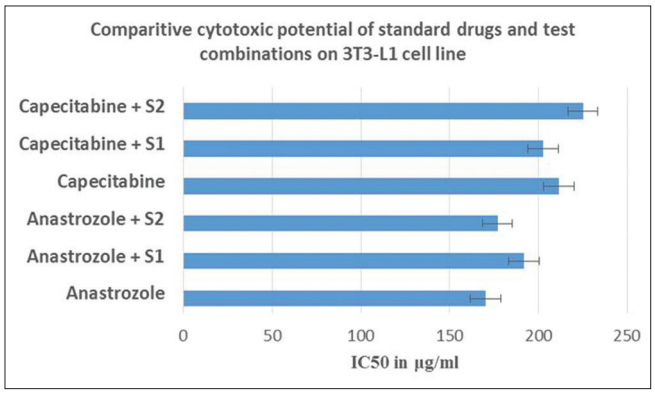

Figure 12: Cytotoxic activity of anastrozole and capecitabine in combination with plant quercetin on 3T3-L1 cells

cancer [Figure 2]. Upon combination with standard drug, quercetin significantly enhanced its efficacy with an IC50 concentration <50 µg/ml, which is an ideal concentration to be considered for formulation of any drug. This is in agreement with other studies, stating that quercetin improved its potency with drugs such as cisplatin, doxorubicin, camptothecin, 5-fluorouracil, sorafenib, oxaliplatin, and gemcitabine against various kinds of cancers.[49-53] With growing research interest, we appealed foremost to demonstrate the impression of quercetin in combination with anastrozole, a breast cancer drug, and capecitabine, a colon cancer drug in the study.

Natural compounds which control tumor cell proliferation via controlling mitosis and cell cycle are important candidates for application in basic and translational research before going to clinical trials. As uncontrolled and rapid cell division is a hallmark of cancer, untying the molecular mechanism of mitosis is key to understand how natural compounds function as antimitogenic agents.[54,55] Alkaloids and colchicine are known to block mitotic phase in cell cycle that are reported to inhibit cell proliferation in cancer of testicles and lymphocytic leukemia.[56-58] In the present study report, the combination of quercetin fraction and anastrozole and capecitabine

induced metaphase and S phase arrest in breast cancer. This observation looks to be a reason for a novel finding presented in Figures 5 and 6. The resulted findings were in agreement with similar study reports where quercetin blocked S phase arrest. However, reports derived from other studies convey that difference in the cancer types and application of compound might account for disparity in the observation of variation of cell cycle arrest.[59-65]

The combination of quercetin with ellagic acid lessened proliferation and cell cycle arrest in MOLT-4 cells of leukemia.[66-68] Quercetin with doxorubicin combination had a significant decrease in cell proliferation in MCF-7 and MDA-MB-231. However, in the present study, the combination of quercetin with anastrozole and capecitabine at low concentration revealed a significant antiproliferative activity, a unique observation than other reports. Another study also proved that quercetin narrowed T47D breast cancer cell lines sensitivity with an IC50 value at 160 μM.[69] Quercetin on ovarian cancer cells showed G2/M phase arrest at 100 μg/ml concentration.[70] In the present study, quercetin in combination with anastrozole showed G2/M and S phase arrest on breast cancer at 16 μg/ml concentration; quercetin in combination with capecitabine showed G0/G1 and G2/M phase arrest at 43 μg/ml concentration on colon cancer. This is the first report of using the lowest concentration and combination on the selected cell lines The advances in understanding the cell cycle mechanism by chemotherapeutic agents are of significant importance for improving efficacy of targeted combinational therapies with natural compounds. This helps to overcome resistance to anticancer drugs involved in cell cycle leading to cell proliferation.

Mitochondrial membrane potential is a dominant driving force for ATP synthesis. In various cancer cells, mitochondria become dysfunctional via changes in energy metabolism, elevated reactive oxygen species generation, and increased transmembrane potential. Hence, targeting mitochondria using pharmacological agents is an attractive therapeutic strategy to destroy cancer cells.[71] Quercetin cotreatment with autophagy inhibitor enhanced mitochondrial damage and autophagy inhibition in Jurkat T-cells.[72] Isorhamnetin glycoside (IGP) from *Puntia ficus-indica* L. Mill on colon cancer implied membrane permeabilization of 30% and G2/M phase arrest, which is similar to our study results as shown in Figures 7-10.[73] Quercetin contributes protection against mitochondrial impairment in an *in vitro* model of hepatocyte steatosis.[74] A study reports that quercetin acts via mitochondrial pathway in cervical cancer cells through p53 induction and NF-kB inhibition at 80 μm. However, we demonstrated enhanced effect of quercetin with anastrozole and capecitabine on lower doses on breast (16 and 31 μg/ml) and colon cancer (43 and 46 μg/ml).[75] The study investigations made on quercetin with curcumin combination presented apoptotic induction in chronic leukemia cells that lowered toxic effects on normal cells.[76] The same combination had also impact on apoptosis through mitochondrial pathway on gastric cancer MGC-803 cells, indicating that the combination has better potential.[77] These results are on par with our study results where combinations (quercetin and anastrozole combination, quercetin, and capecitabine) showed decreased cell proliferation, apoptosis through acting on mitochondrial mechanism on breast and colon cancer cells, a novel combination.

As reported in the literature, disruption in the mitochondrial potential leads to an increase in mitochondrial permeability that leads to release of pro-apoptotic proteins such as growth factors, cytokines, and caspases.[78] Caspases are crucial mediators of programmed cell death and among them caspase 3 is frequently activated death protease in different cell types. Caspase 3 also has a central role in execution-phase of apoptosis in addition to caspase 7.[79] The central role of caspase 3 makes the molecule an attractive target and attempts were continuous from decades to develop molecules to activate caspase 3. Therefore, screening the phytochemical compounds having caspase activity will have a significant role in cancer treatment. The study findings illustrate that quercetin with drugs initiates caspase 3 on MCF-7 and COLO 320 cancer cells. In another finding, tangeretin flavone displayed cell cycle arrest and caspase activation in MDA-MB-231 breast carcinoma cells.[80] By focusing on caspase 3 activation, a hallmark of apoptosis, cell death activators and inhibitors can be studied. In a study, quercetin induced apoptosis via activation of caspases in human hepatoma cell line HepG2.[81] They also reported that a continuous daily intake of polyphenols like quercetin might provoke apoptotic cell death. As caspase 3 plays a central role in execution-phase of cell apoptosis, attempts were continuous from the decade to develop molecules capable of directly activating caspase 3 for treatment use in cancer therapy.[82] The present study results were impressive where caspase activation was achieved at lower concentrations of the tested combinations on breast and colon cancers.

Even though in recent times, we find many treatment options for patients with cancer, a successful treatment with anticancer drugs remains a challenge due to nonselective cytotoxicity of the drugs. Overall, chemotherapy with cytotoxic and cytostatic drugs remains the primary treatment option. In addition, chemotherapeutic agents available today are highly cytotoxic to normal cells, resulting in a series of adverse effects.[83] A promising new compound in anticancer therapy is based not only on its ability to induce tumor cell death but also its selectivity, i.e., on its capacity to minimally affect normal cells.[84] The results of the study suggest that plant quercetin when combined with synthetic drugs is highly selective in cancer treatment by showing no toxicity toward normal cell lines when compared to cancer cell lines (3T3-L1). Hence, this combination can be a model for the development of new drugs to be used in clinical trials in the future, when tested on other models.

CONCLUSIONS

Natural flavonol fraction 'quercetin' with most potential

biological importance is identified from unexplored plant sources. Effective research on combination of natural component with available drugs on the top most cancers is useful for reducing the adverse toxic side effects related with conventional chemotherapy. The strength of the study is that it turns to be first report to rule out the combination of anastrozole and capecitabine with natural active component, quercetin, which creates an avenue for knocking new drug combinations. The limitation of the study is that we could get limited cell lines from NCCS Pune for carrying out the work.

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Conflicts of interest

There are no conflicts of interest.

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