scientific reports

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Auxiliary effect of trolox on coenzyme Q_{10} restricts angiogenesis and proliferation of retinoblastoma cells via the ERK/Akt pathway

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Reactive oxygen species (ROS) are essential for cancer signalling pathways and tumour maintenance, making ROS targeting a promising anti-cancer strategy. Coenzyme Q_{10} ($CoQ10$) has been shown to be effective against various cancers, but its impact on retinoblastoma, alone or with trolox, remains unreported. Cytotoxicity of CoQ_{10} alone and with trolox was evaluated in normal human retinal pigment epithelium cells (ARPE-19) and Y79 retinoblastoma cells using CCK-8. Flow cytometry was used to assess apoptosis, cell cycle, ROS, and mitochondrial membrane potential (MMP). Anti-angiogenic potential was tested using human umbilical vein endothelial cells (HUVECs) and chick chorioallantoic membrane (CAM) assays. Mechanistic studies were conducted via RT-PCR and western blotting. CoQ_{10}, alone and with trolox, reduced Y79 cell viability, induced apoptosis through excess ROS generation, and decreased MMP significantly. Both treatments caused G2/M phase cell arrest. The CAM assay showed a significant reduction in endothelial cell proliferation, evidenced by fewer number of co-cultured HUVECs when exposed to CoQ_{10} or CoQ_{10} with trolox. The combination of CoQ_{10} and trolox significantly reduced VEGF-A, ERK, and Akt receptor levels, while CoQ_{10} alone significantly inhibited ERK and Akt phosphorylation. Together, CoQ_{10} and trolox reduced protein expression of VEGFA. CoQ_{10} alone and with trolox, induces apoptosis in Y79 retinoblastoma cells by inhibiting the ERK/Akt pathway and downregulating VEGFA. This study is the first to report the in vitro and in-ovo anti-cancer potential of CoQ_{10} alone or when combined with trolox, on human retinoblastoma Y79 cells.

Keywords Rb cells, CoQ_{10}, Trolox, Anti-cancer, ERK/Akt inhibition

Retinoblastoma (Rb), is reported to be one of the most commonly occurring intraocular malignancies in small children affecting about 1 in every 15,000 children worldwide[1]. The existing line of treatment for Rb involves treating the tumour by systemic/intravitreal chemotherapy, transpupillary thermotherapy, laser-guided photocoagulation, enucleation, and radiotherapy while also employing a multidisciplinary approach to combat the side effects of the disease[2]. Furthermore, the detection of Rb mainly depends on the symptomatic growth which if undetected could at times lead to conditions like angle invasion or iris neovascularization thereby elevating eye pressure and leading to secondary close-angle glaucoma or neovascular glaucoma[3,4] which occurs in 1–23% of the cases[5–7]. Hence in conditions where Rb becomes advanced, combinatorial treatments that can inhibit growth and size of Rb tumour and are easier to access than chemotherapy and surgery will be preferred. This type of approach will increase efficiency and reduce toxicity related to the treatment procedures.[8]. Research on antioxidants as a therapy for treating cancers has been done extensively with some showing positive outcomes,

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but certain uncertainty remains in its adaptation as a treatment[9]. Also, it is necessary to target those cancers with antioxidant therapy where ROS production is reportedly a survival mechanism adopted by cancerous cells[10].

Previously reported data on the use and significance of coenzymes in cancer treatment to decipher their biological role singled out CoQ_{10}, which is an endogenously synthesized antioxidant, whose deficiency has been stated to be a causal factor in certain cancers[11,12]. Several studies thereafter reported a higher therapeutic response rate of CoQ_{10} when used with other chemotherapeutic agents[13,14] while also improving the tolerability of cancer treatments[15–17]. The mechanism of action of CoQ_{10} can be attributed to its ability to elevate ROS and cause cell death such as in pancreatic cancer cells[18], increase prooxidant activity of doxorubicin in breast cancer patients[19], reduce markers of inflammation and matrix metalloproteinases[20] while also inducing apoptotic activity in melanoma cells[21].Even though numerous studies have indicated that coenzyme deficiency may be linked to certain cancers with some encouraging results these have been inconsistent and conflicting[11]. A supportive study indicated that palm derivatives of vitamin E worked better than CoQ_{10} on breast cancer cells[22]. Hence, another antioxidant that also possesses potent anti-tumour activity is vitamin E, with early evidence of its anti-apoptotic potential available against colorectal cancer[23]. Furthermore, its various analogues like the water soluble trolox, have also proven to possess anti-cancerous properties. In a report on the combination of trolox with Tiron N-acetyl-L-cysteine, survival of glioblastoma tumorigenic cells was inhibited by cell cycle progression[24]. Trolox was also successful in inhibiting breast cells[25], reducing growth of colon[26] as well as cervical cancer cells[27]. When combined with other agents like curcumin[28] it induced apoptosis via the oxidative mechanism[29]. The anti-apoptotic potential of trolox against human myeloma cell lines exposed to a combination of trolox with arsenic trioxide[30] was also claimed by another report on mouse embryonic fibroblasts[31]. The combination study of trolox with selenium suggested that trolox may not be self-sufficient in reducing cell viability of human keratinocytes but showed aggressive cytotoxicity when paired with selenium[32] indicating towards its action as an adjuvant.

There have been several studies in the past, illustrating the enhanced activity of CoQ_{10} when used in combination with other compounds on various pathologies and cancers[33–35]. One such study was when CoQ_{10} and vitamin E showed improved cytotoxic activity against cells of malignant glioma and melanoma when encapsulated in nano capsules[36].

Despite the growing body of evidence regarding CoQ_{10} and trolox individually, there is no study yet in our knowledge, that reports on the exposure of these compounds to cancerous cells of Rb. Also, since we have recently found that trolox acts as an adjuvant to enhance the function of CoQ_{10}[37], we wanted to investigate how trolox will influence the action of CoQ_{10} on Rb cells in culture and influence their growth in the current study.

Materials and methods

Drug

CoQ_{10} (Sigma, Cat#07386) was prepared at a concentration of 2-mM (stock) by dissolving in dimethyl sulfoxide (DMSO). Trolox (Sigma, Cat #238813) was also prepared at a concentration of 2 mM (stock) by dissolving it in ethanol (Himedia). Both these drugs were prepared fresh every time before use.

Cell lines and cell cultures

The growth media for growing ARPE-19 cells, a gift from Dr. Anil Tiwari (Dr. Shroff's Charity Eye Hospital), was Dulbecco's modified Eagle's medium F12 (DMEM-F12, Himedia) with an addition of 10% fetal bovine serum (Gibco, USA) and 1% pen-strep (Himedia, India). The ARPE-19 cells were seeded on a 96-well plate (1×10^4 cells/well) with overnight incubation at 37 °C, 5% CO_2. Rb cell line Y79 was purchased from the National Centre for Cell Science (NCCS, Pune, Maharashtra, India) at PN 30 and authenticated by the STR method. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco, USA), and 1% pen-strep (Thermo Fisher Scientific). Cells were maintained in a humid environment with 5% CO_2 at 37 °C. Human umbilical vein endothelial cells (HUVEC) were a gift from Dr Subrajit Biswas (Amity Institute of Molecular Medicine and Stem Cell Research, AUUP Noida). The cells were cultured in MCDB 131 medium supplemented with 50 µg/ml endothelial cell growth supplement, 20% fetal bovine serum, 2 mM L-glutamine, 50 µg/ml heparin and antibiotics (100U/ml Penstrep). Cell cultures were maintained at 37°C in a humidified chamber with 5% CO_2 in a CO_2 incubator. All experiments using HUVECs were conducted between 2 to 5 passages. At the time of experiments, MCDB131 medium with 2% FBS was used.

Cell viability assay

To see whether the combination of CoQ_{10} and trolox was non-cytotoxic to normal cells, ARPE-19 cells were cultured (1×10^6 cells/well) on a 96-well plate and the dosing solutions of CoQ_{10} and trolox were prepared from 2 mM stocks each, in low serum DMEM F-12. On the next day treatment of cells with CoQ_{10} (10-50 µM) and trolox (20 µM) each respectively was followed by incubation for 24 h. After incubation of 24 h, CCK-8 was added to each well and incubated for 4 h. Absorbance was then measured at 470 nm. The IC_{50} values for CoQ_{10} and trolox were estimated followed by assessing drug interaction via combination index on ARPE-19 cells.

For estimating cell cytotoxicity in Rb Y79 cells, we used CCK-8. Y79 cells (1×10^5 cells/well) were seeded on a 96-well plate. After overnight incubation of 24 h, the dosing solutions of CoQ_{10} and trolox were prepared from 2 mM stocks each respectively in RPMI medium with low serum. Rb Y79 cells were then treated with CoQ_{10} (10-90 µM) and trolox (10–70 µM) for 48 h. The treated cells were incubated with 10 µL CCK-8 reagent for 4 h and absorbance read at 470 nm on Multiskan FC microplate reader (Thermo Scientific). To evaluate the growth of cells, the ratio of the absorbance of treated cells to the absorbance of the untreated cells was taken into account. The IC_{50} values for CoQ_{10} and trolox were estimated followed by assessing drug interaction via combination index.

Colony formation assay

For estimating colony forming ability of Y79, a total of 1×10^3 cells/well were seeded on poly-D-lysine coated 12-well plates. Cells were then treated with CoQ_{10} only (30 μM) and combination of CoQ_{10} + trolox (30 μM + 30 μM) for 48 h and left to incubate for 14 days in which media was changed after every 3 days. Cells were fixed with 10% formalin and stained with a 2.5% crystal violet solution at room temperature for 30 min. Washing of cells followed by air drying readied the cells to be imaged and colony density measurements were done using ImageJ software[38].

Detection of mitochondrial membrane potential (Ψm) and release of ROS

To evaluate the alterations in Ψm, the treated cells were stained using Rhodamine123 (Rh-123; R302, Invitrogen) dye as per previously available protocol[39]. Cells were seeded in a 12-well plate and treated with CoQ_{10} only (30 μM), Trolox only (30 μM) and combination of CoQ_{10} + trolox (30 μM + 30 μM) for 48 h and collected by washing with PBS followed by staining with Rh-123 (1 μg/ml) dye in the dark for 30 min at room temperature. Estimation of cellular changes was analysed by BD FACS ARIA (BD Biosciences, USA) flow cytometer.

Analysis of ROS was done using flow cytometry. A total of 3×10^4 cells were seeded per well for estimation of ROS by using 2',7'–dichlorofluorescin diacetate (DCFDA) as per previously available protocol[40]. Cells were treated with CoQ_{10} only (30 μM), Trolox only (20 μM) and combination of CoQ_{10} + trolox (30 μM + 30 μM) for 48 h. Cells were then collected and washed with PBS. The cell pellet was then exposed to 20 mM of DCFDA for 35 min, and washed with PBS before estimating ROS using flow cytometer.

Cell cycle analysis

Y79 cells were seeded overnight in 12 well culture plates at a density of 2×10^5 cells/well. This was followed by treatment with CoQ_{10} only (30 μM) and combination of CoQ_{10} + trolox (30 μM + 30 μM) for 48 h. Afterwards, collection of cells by centrifugation, and ethanol (70%) fixation was followed by resuspension in a mix of PBS + RNase A (1 mg/ml) + PI (50 μg/ml). The pelleted cells were subjected to dark conditions for 30 min and analysed using flow cytometry (BD FACS Accuri)[1].

Apoptosis (Annexin V/PI) assay

Using the dead cell apoptosis kit (V13242, Invitrogen) as per manufacturer's instructions, Y79 cells (1×10^5 cells/well) were seeded onto 96-well plates overnight and treated with CoQ_{10} only (30 μM), Trolox only (20 μM) and combination of CoQ_{10} + Trolox (30 μM + 30 μM) for 48 h. Cells were washed with PBS and centrifuged at 400 × *g* for 5 min, followed by resuspension in 100 μL 1X annexin binding buffer. Added 5 μL Alexa Fluor™ 488 Annexin V and 1 μL 100 μg/mL PI working solution to each 100 μL of cell suspension and incubated for 15 min in dark. After incubation, cells were resuspended in 400 μL of 1X annexin binding buffer (on ice) and assayed by BD FACS Accuri C6.

Assessment of anti-angiogenic potential

To evaluate how CoQ_{10} with or without trolox would affect Y79 cells growth while observing its effects on proliferation of endothelial cells in vitro, we used HUVECs and co-cultured them with Y79 cells. HUVECs were plated onto 24-well plates (10×10^3 cells/well) and incubated for 24 h using EGM2 bullet kit supplied components. After a day of incubation, we replaced the exhausted media with a mix of RPMI and EGM2 (1:1) and also added Y79 cells (1×10^4 cells/well). The now ready cell mixture of Y79 cells, and HUVECs were subjected to CoQ_{10} only (30 μM), trolox only (30 μM) and CoQ_{10} + trolox (30 + 30 μM) for 2 days. Cells (Y79 + HUVECs) left untreated served as controls and the entire set was assessed after 48 h. This was followed by the removal of Y79 cells. HUVECs were fixed with 4% PFA for 15 min at room temperature and then stained with DAPI. The estimate of live HUVECs was obtained by counting DAPI-positive cells in four fields using the cell counter plugin of Image J.

To further establish the anti-angiogenic activity of CoQ_{10} alone or with trolox, we chose to conduct the CAM assay. Y79 cells were grown to a confluency of 80% and, CoQ_{10} and trolox were prepared at a concentration of 2 mM in DMSO and ethanol respectively. The cells were then divided into groups of control (RPMI only), Y79 cells only, Y79 + CoQ_{10}, Y79 + trolox and Y79 + CoQ_{10} + trolox.

Fertilized eggs were purchased from Keggs Farms Private Limited (Gurgaon, India) and incubated at 37 °C for 72 h. After an incubation of 7 days, under aseptic conditions we created a small window in the eggshell and following previously published protocols[41,42],100μL of Y79 cell suspension (4.3×10^5 cells) was inoculated onto the exposed area of the eggs of the four groups except the control. The window was resealed using adhesive tape and eggs were incubated. On the 12th day, eggs were dissected followed by imaging and discarding of eggs. Each group was represented by two eggs and the experiment was repeated thrice.

Quantitative RT-PCR

Cells were collected 48 h after treatment, and centrifuged at 2,000 r/min. The supernatants were discarded, followed by RNA extraction for cells in each group according to the instructions of TRIzol reagent (Thermo Fisher Scientific). For RT-qPCR analysis, cDNA was synthesized from the total RNA templates using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). cDNA templates diluted to 0.1× were used for RT-qPCR, which was performed using the SYBR Green PCR Master Mix (Thermo Fisher Scientific) and the ABI Prism 7500 (Applied Bio-systems). The PCR conditions were as follows: 95 °C for 3 min, then 30 cycles of 95 °C for 30 s followed by 52.9 °C for 30 s, and 72 °C for 30 s. The final extension was performed at 72 °C for 10 min. Beta-actin gene (ACTB) was taken as the internal control. Relative quantitation was done by comparing each sample gene's threshold cycle (Ct) values to the Ct values of ACTB. ΔCt corresponds to the difference between the Ct of the gene of interest and the Ct of ACTB. Data is presented in terms of the fold change of mRNA

expression, which was calculated from the 2-ΔΔCt method. The gene-specific primers were designed using NCBI primer designing tool (Table 1).

Western blotting

Cells were washed with ice-cold phosphate buffer solution, harvested, and lysed at 4 °C with RIPA lysis buffer, and the protein concentration was estimated by Bradford assay. Using a sodium dodecylsulfate-polyacrylamide gel, samples (50 µg) were separated and subsequently transferred onto nitrocellulose filters. A minimum sample volume of 50 µg was used for separation using SDS-PAGE. Separated proteins were then transferred onto nitrocellulose filter membrane and immunoblotted with primary antibodies against VEGFA (1:500, Cat #PA5-141103, Invitrogen, USA), Akt (1:1000; Cat#4060, Cell Signaling Technology, USA), phospho Akt (1:1000; Cat#9271, Cell Signaling Technology, USA), ERK(1:2000; Cat#9102, Cell Signaling Technology, USA), phospho ERK (1:1000; Cat#3510, Cell Signaling Technology, USA) and α-tubulin (1:1000, Cat #2144, Cell Signaling Technology, USA). To ensure equal loading of protein in each lane, the blots were stripped and reprobed with an antibody against α tubulin.

Statistical analysis

The experimental data obtained was analysed by GraphPad prism 9. To figure out the significant differences between the means as defined by $p < 0.05$ and $p < 0.01$, ANOVA was used. The results of experiments (cell growth, ROS, MMP, annexin V-PI, and cell cycle) are described as the mean and standard error of the mean. Experiments were replicated at least three times.

Results

Cell cytotoxicity

To ensure that CoQ_{10}, trolox and CoQ_{10} + trolox have no adverse effect on ARPE-19 cells, we assessed cell viability after 24 h incubation with the combination of CoQ_{10} + trolox and found that there was no effect on the cell growth. However, trolox alone tends to reduce cell growth after 30 µM (Fig. 1a). The IC_{50} of CoQ_{10} was found to be 7.17 µM and that of trolox was 3.78 µM respectively with a reported synergism value of 0.9 (Fig. 1b).

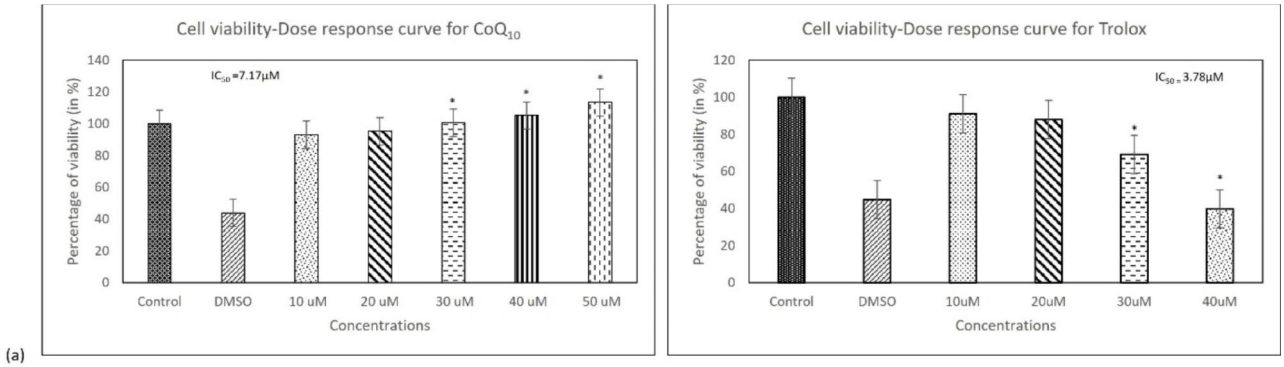
Y79 cells were treated with both CoQ_{10} and trolox individually and in combination for 48 h and assessed for cell viability morphologically (Fig. 1c) and quantitatively by CCK-8 assay. We found that CoQ_{10} + trolox could significantly inhibit the proliferation of Y79 cells ($p < 0.05$*).The concentrations of 90 µM CoQ_{10} alone and 50 µM Trolox alone for 48 h resulted in 50% reduction in cell number. TheIC_{50} of CoQ_{10} was found to be 5.04 µM and that of trolox was 3.19 µM respectively (Fig. 1d). Since the combined treatment at the concentrations of 40 µM CoQ_{10} and 30 µM of trolox resulted in a 50% decrease of Y79 cells, we went ahead with suboptimal concentrations of 30 µM + 30 µM of CoQ_{10} + trolox (marked as red) for 48 h for further experiments (Fig. 1e). The data obtained pointed towards an anti-proliferative function of CoQ_{10} when used alone as well as combined with trolox on Y79 cells.

CoQ_{10} alone and when combined with trolox could reduce colony forming ability of Y79 cells

Furthermore, the effects of CoQ_{10} and CoQ_{10} + trolox together on colony forming ability of Y79 cells were evaluated by colony formation assay. We found that even though both trolox alone and CoQ_{10} alone could reduce the number of colonies formed by Y79 in the control group from 100 to 65% ($p < 0.05$*), and 48% ($p < 0.05$*) respectively, it was the combination of CoQ_{10} + trolox which could further remarkably reduce the number of colonies formed to 40% ($p < 0.01$**) (Fig. 2). Hence, results suggest that CoQ_{10} alone and in combination with

S.No.	Gene	Primer Sequence
1	VEGFA	Forward 5′-AGGCAGCTTGAGTTAAACGAAC-3′
		Reverse 5′-TGGTGAGAGATCTGGTTCCCG-3′
2.	ERK2	Forward 5′- CGTGTTGCAGATCCAGACCA-3′
		Reverse 5′- GGACTTGGTGTAGCCCTTGG-3′
3	AKT	Forward 5′-GTGGCTATTGTGAAGGAGGGT-3′
		Reverse 5′-CGCTCCTTGTAGCCAATGAAG-3′

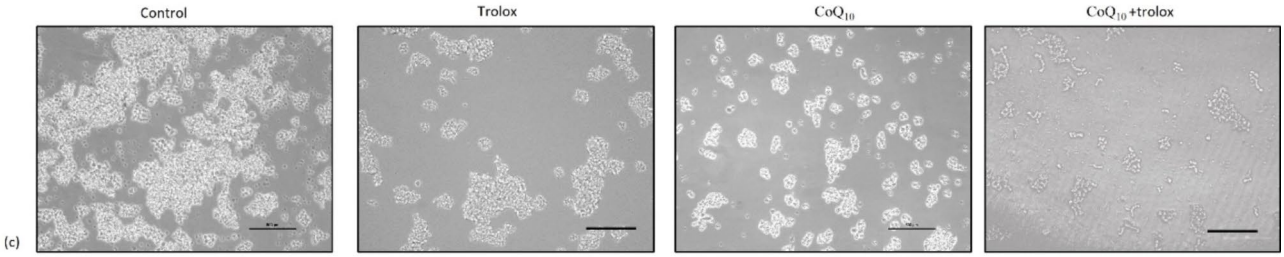
Table 1. List of primers used in the experiment.



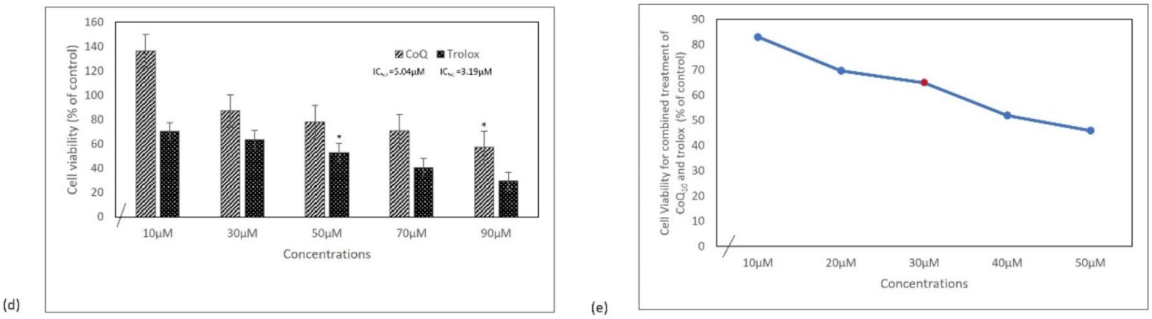
(a)

IC	CoQ_{10} (µM)	Trolox (µM)	Combination Index (CI)
30	5	1	0.6520
50	5	1	0.9619
70	5	1	1.93

(b)



(c)



(d) (e)

Fig. 1. Representative data from in-vitro assay of cell viability showing effects of CoQ_{10} alone, trolox and combination of CoQ_{10} and trolox on cell lines. (**a**) CoQ_{10} at concentrations of 50µM and 30 µM trolox were safe on ARPE-19 cells and produced no toxicity. (**b**) The combination index value was found to be 0.9 which indicates towards the synergistic activity of CoQ_{10} with trolox. (**c**) Representative images of cell death induced in Y79 cells when cultured in serum-supplemented RPMI media (control), trolox, CoQ_{10} alone and combination of CoQ_{10} with trolox. A significant decrease in cell number by both CoQ_{10} alone as well as CoQ_{10} with trolox was observed in Y79 cells. (**d**) The dose response curves of CoQ_{10} and trolox indicated that CoQ_{10} reduced Y79 population by 50% at IC_{50} of 5.04 and trolox at IC_{50} of 3.19. (**e**) The combined treatment value of CoQ_{10} and trolox was determined to be 30 µM each for Y79 cells. Each bar represents mean ± SEM where $n = 3$. $^*p < 0.05$ vs. control. DMSO = dimethylsulfoxide. Scale bar- 500 µm.

trolox could effectively reduce the cell growth while also affecting its morphology by hampering growth in terms of restricting the formation of colonies.

CoQ_{10} and CoQ_{10} + trolox increased ROS and caused breakdown of MMP in Y79 cells

Studies conducted recently have revealed that antioxidants could trigger apoptosis in cancer cells by amplifying the generation of ROS[43,44], we also wanted to estimate the levels of ROS after subjecting the Y79 cells to the treatment groups and found that CoQ_{10} alone and also in combination with trolox could significantly increase ROS levels from 64% in control to 71% and 88% respectively (Fig. 3a and b).

Mitochondria plays a key role in apoptosis. A decrease in the MMP is a critical step in the induction of apoptosis in cancer cells[45]. We assessed the levels of MMP using Rh-123 fluorescent dye and found that the

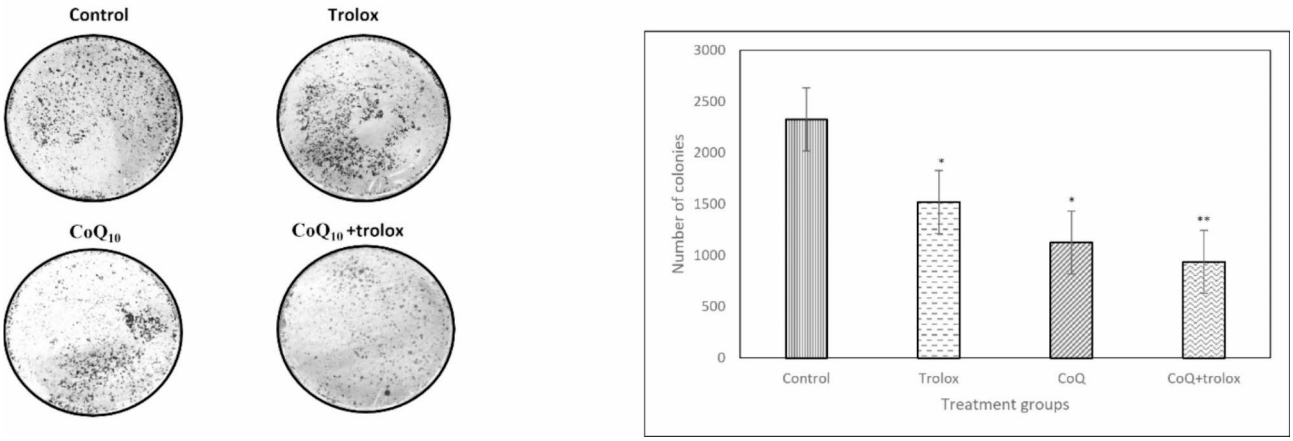


Fig. 2. Representative images showing the results of colony formation assay in Y79 cells. A significant decrease in the descending order was observed in the groups of trolox ($p < 0.05^*$), CoQ_{10} ($p < 0.05^*$) and CoQ_{10} combined with trolox ($p < 0.01^{**}$) when compared to control respectively. Each bar represents mean ± SEM where $n = 3$. Scale bar- 500 µm.

percentage of viable Y79 cells in the control group (100%) began to decrease when subjected to trolox alone (89.2%). CoQ_{10} alone (66.2%) could significantly ($p < 0.05^*$) depolarize the mitochondrial membrane and cause significant reduction ($p < 0.01^{**}$) in MMP as was also seen in the combined group of trolox and CoQ_{10} (52.25%). This in turn reduced the cellular granularity pushing cells towards death (Fig. 3c and d).

CoQ_{10} alone and when combined with trolox arrested cells in the G2/M phase

Cell proliferation is regulated by checkpoints, two of which are G1/S and G2/M and cancer cells often escape these checkpoints to ensure continuous cell division[46]. We observed that 48 h after treatment with CoQ_{10} alone, trolox alone and CoQ_{10} + trolox, DNA content decreased in the S phase and the cells accumulated in the G_2/M phase. However, significant results were observed both when CoQ_{10} was used alone and in combination with trolox (Fig. 4a). DNA content analysis describes that after treatment of Y79 cells with CoQ_{10} ,the cell cycle profile resulted in 20.55% accumulation of cells whereas when combined with trolox, it was 23.7% of the cells in the G_2/M phase (Fig. 4b).

Annexin PI results confirmed that the death induced by both CoQ_{10} and CoQ_{10} + trolox was due to apoptosis

To analyse the reasons behind the arrest of cells in the G_2/M stage, Annexin V/PI assay was conducted. On evaluating the data after the build-up of cells in the G_2/M phase after the treatment of 48 h, a considerable increase in percentage of cells undergoing apoptosis (both early and late) beginning from 8.5% in control to 21.7%, 41.8% and 56.6% was observed in trolox alone, CoQ_{10} alone and when combined with trolox respectively (Fig. 5a). In Fig. 5b the analysis of Annexin V/PI pattern clearly shows that CoQ_{10} alone and in combination with trolox might possess anti- tumour properties against Y79 cells.

Co-culture of Y79 with human umbilical vein endothelial cells (HUVECs) and the CAM assay results prove that both CoQ_{10} alone and CoQ_{10} + trolox are effective in mitigating angiogenic proliferation of cells both in vitro and in vivo

Since endothelial cells have often been used as models for angiogenesis as they promote capillary-like structure formation[47], we wanted to test the effect of CoQ_{10}, trolox and the combination of the two when co-cultured with retinoblastoma Y79 cells. Based on DAPI staining results, it was observed that the proliferation of HUVEC cells was higher when they were co-cultured with Y79 cells. However, the HUVEC population was considerably reduced by treatment with CoQ_{10} + trolox (Fig. 6a). Quantification of HUVEC populations via cell counting of DAPI-stained samples revealed a decrease in cell numbers was in the descending order starting from trolox alone (60.5 ± 1.25) to CoQ_{10} alone (37.5 ± 2.3) followed by the most significant decrease observed in the group of CoQ_{10} + trolox (20.5 ± 0.5) (Fig. 6b).

We evaluated the anti-angiogenic potential of CoQ_{10} alone or in combination with trolox through the CAM assay (Fig. 6c), a credible and often most adapted method for analyzing angiogenesis. Based on the thickness, branching and sprouting of the blood vessels we analysed the differences in the growth pattern and concluded that both CoQ_{10} alone and CoQ_{10} + trolox could attenuate the dense growth of vessels when compared to those seen in the negative control of Y79 only.

Downregulation of expression of VEGF receptor A, is driven by changes in ERK and AKT receptor levels

To understand how CoQ_{10} alone or with trolox would affect angiogenesis in Y79 cells, we studied VEGFA expression at the mRNA level (Fig. 7a). The results demonstrated that CoQ_{10} alone and trolox alone increased

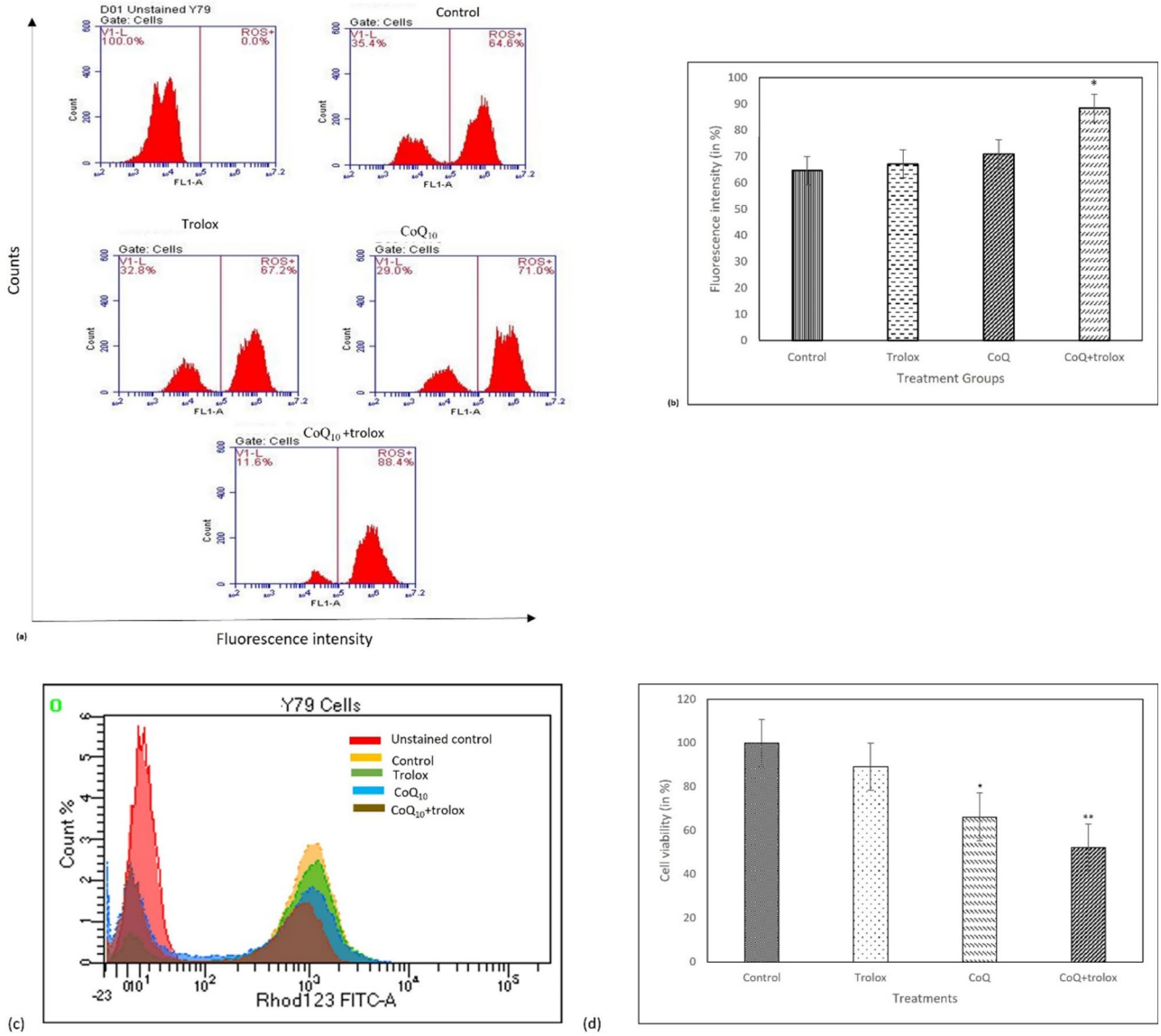


Fig. 3. Representative images of estimation of Y79 cells by flow cytometry for generation of ROS. The percent ROS generating cells were 64.6% in control and which was marginally increased to 67% cells by trolox (**a**). However, the percentage increase in ROS generating cells was significant when treated with CoQ_{10} alone (71%, $p < 0.05$*) and by CoQ_{10} with trolox (88.4%, $p < 0.01$**); x axis = fluorescence intensity and y axis = count of cells (**b**). Histogram representing changes in MMP in Y79 cells (**c**). Both the doses of CoQ_{10} alone and CoQ_{10} with trolox increased ROS generation in Y79 cells by resulting in depolarization of MMP which was evident in the cell counts. Even though MMP lowered in trolox group, significantly lowered levels could be seen only in the groups of CoQ_{10} alone ($p < 0.05$*) and CoQ_{10} combined with trolox ($p < 0.01$**) vs. control respectively (**d**). Each bar represents mean ± SEM where $n = 3$. x axis = rhodamine intensity and y axis = count percent.

the expression of VEGFA, but only the combination of CoQ_{10} and trolox could downregulate the expression of VEGFA but not significantly.

It was interesting to find that when it came to studying ERK (Fig. 7b) and Akt (Fig. 7c) modulation by CoQ_{10} alone, it showed an upregulation of both the receptor kinases as opposed to CoQ_{10} and trolox combined which could effectively downregulate each significantly ($p < 0.05$*) and almost comparable to level of control.

Cell death in Y79 occurs due to downregulation of VEGFA and inhibition of ERK/Akt pathway by both CoQ_{10} and CoQ_{10} + Trolox

The signalling molecules Akt and ERK are responsible for signalling mechanisms involved in cell growth and survival and therefore they are often targeted in cancers to suppress their aberrant expression[46,48]. Considering the potential of CoQ_{10} when used against Y79 cells either alone or in combination with trolox, it becomes necessary to study the proteins responsible for inducing apoptosis. Various protein blots were run to examine the expressions of ERK, pERK, Akt, pAkt, VEGF A and housekeeping control α tubulin (Fig. 8a). When the signal

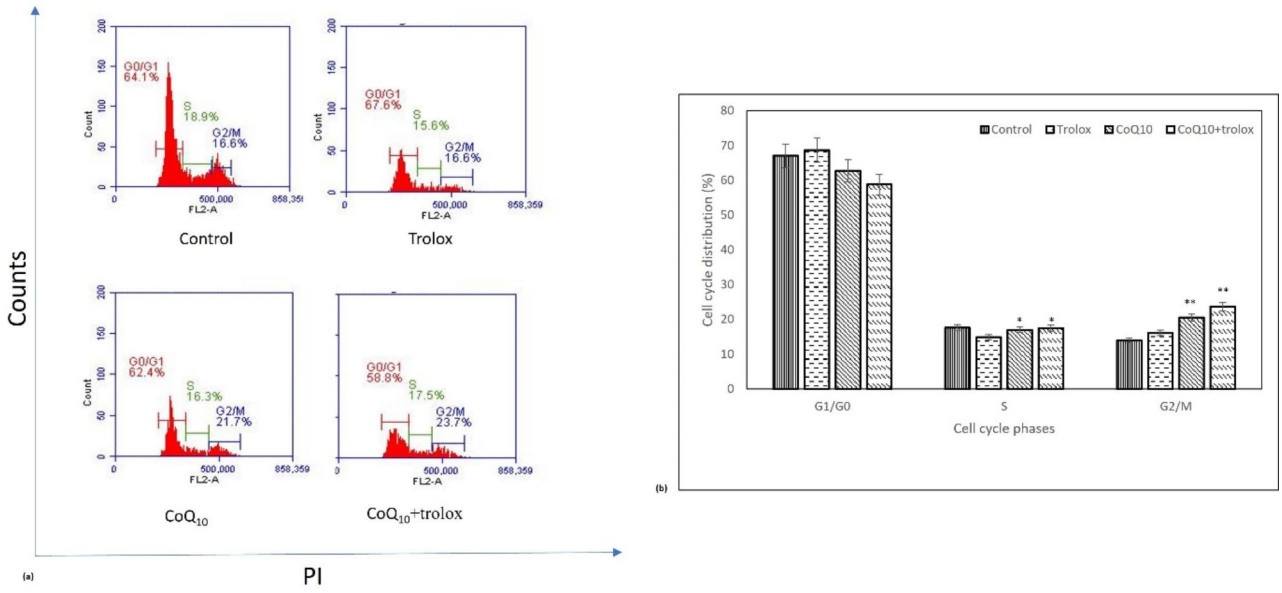


Fig. 4. A representative flow cytometric data for the analysis of cell cycle in Y79 cells. The accumulation of cells in the G2/M phase by treating with trolox (16.1%) increased marginally from the control group (13.9%). This percentage increased when cells were subjected to CoQ_{10} alone (20.5%) and CoQ_{10} combined with trolox (23.7%) after 48 h (**a**). A graphical representation of percentage of cells in the three stages of cell cycle (**b**). Each bar represents mean ± SEM where $n = 3$. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control. x axis = treatment groups; y axis = percent cell cycle distribution.

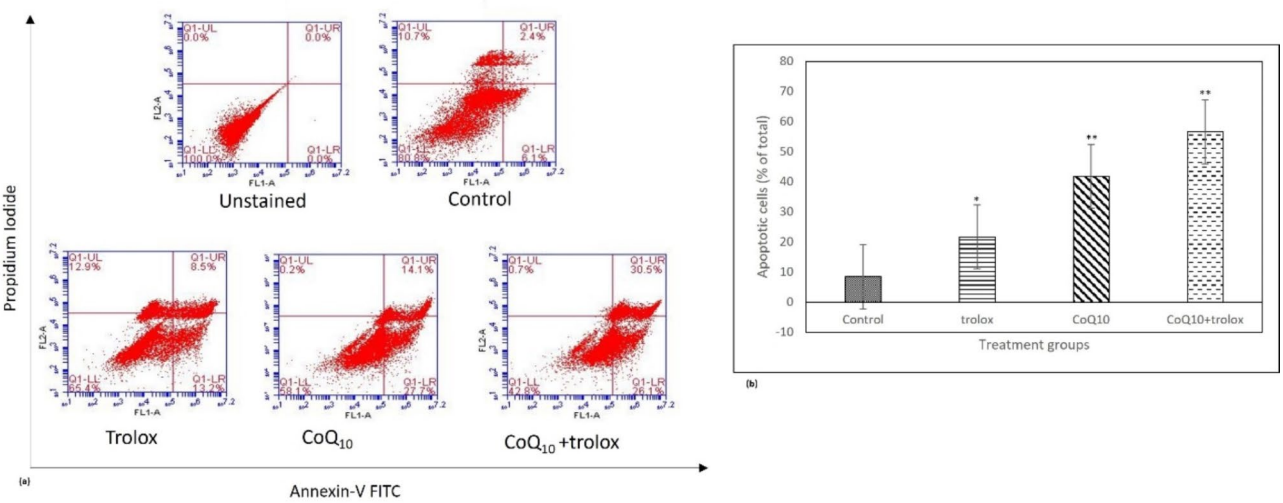


Fig. 5. The apoptotic stages of cells as demonstrated by Annexin V/PI assay in Y79 cells. The division of cells in the four apoptotic stages when exposed to treatment of trolox alone, CoQ_{10} alone and combination of CoQ_{10} with trolox (**a**). Graphical representation of quantification of cells showing significant deaths by trolox ($p < 0.05^{*}$), CoQ_{10} ($p < 0.01^{**}$) and CoQ_{10} with trolox ($p < 0.01^{**}$) (**b**). Each bar represents mean ± SEM where $n = 3$. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control. x axis = Annexin V; y axis = PI.

intensities of Akt blots were compared, it was found that the expressions of Akt was downregulated significantly by the single treatment of CoQ_{10} ($p < 0.05^{*}$) as well as that of the combination of CoQ_{10} and trolox($p < 0.05^{*}$) (Fig. 8b) but that of ERK was downregulated only by CoQ_{10} alone ($p < 0.05^{*}$) (Fig. 8d). Although, when it came to the phosphorylated forms, both pAkt and pERK were downregulated significantly by CoQ_{10} alone ($p < 0.01^{**}$, $p < 0.05^{*}$), as well as the combination of CoQ_{10} and trolox ($p < 0.05^{*}$) (Fig. 8c and e).

Since VEGF mediated signalling contributes to tumorigenesis[49] and is often targeted in Rb to reduce cell survival while targeting angiogenesis[50,51], we wanted to see whether CoQ_{10} alone or in combination with trolox

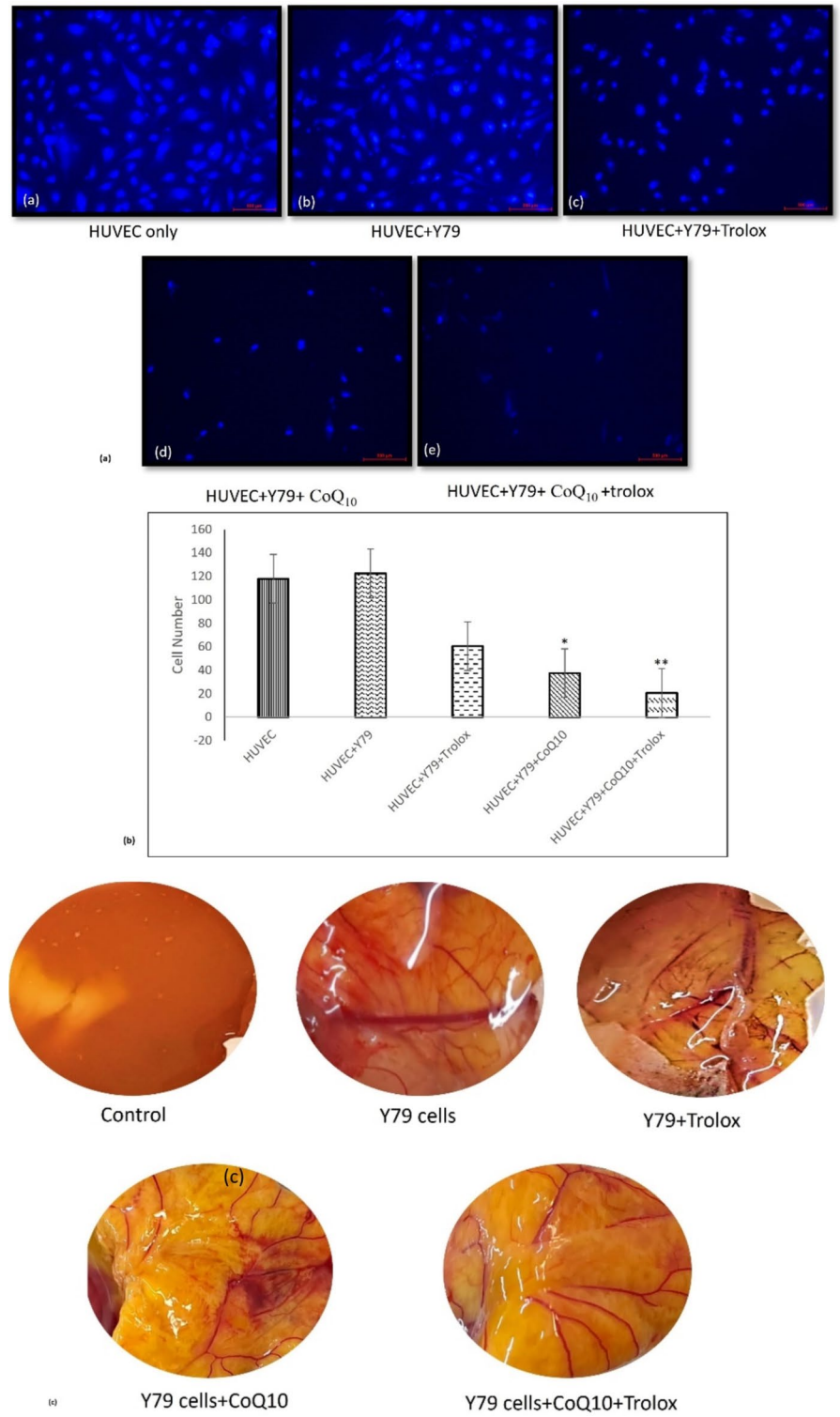


Fig. 6. Representative images of co-culture of HUVECs with Y79 cells. The cell number of HUVECs in control group of HUVECs + Y79 remained unaffected after incubation of 48 h (**a**). Quantification of cell numbers which were significantly reduced by CoQ_{10} alone and with trolox (**b**). The appearance of tumour grafts on the CAM after inoculation of Y79 cells (4.3×10^5 cells) onto the chick CAM (**c**). Tumour observation and excision was done on ED12. Each bar represents mean ± SEM where $n = 3$. $p < 0.05^*$, $p < 0.01^{**}$ vs. control. Scale bar-500 µm.

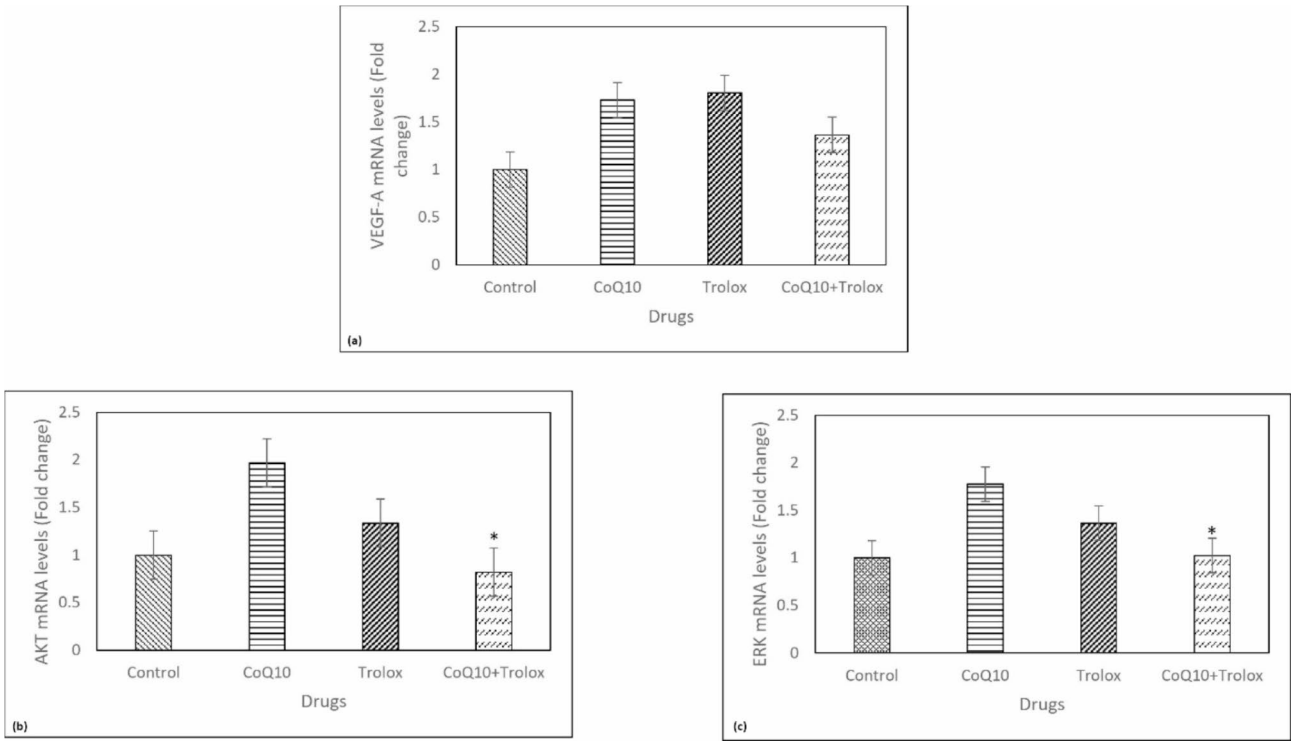


Fig. 7. Quantitative real-time RT-PCR analysis of (**a**) VEGF receptor A, (**b**) Akt, and (**c**) ERK receptor from control, CoQ_{10}, Trolox and CoQ_{10} + trolox groups ($n = 4$, each group). The mRNA levels were normalized to GAPDH housekeeping gene. Each bar represents mean ± SEM where $n = 3$. $^*p < 0.05$ vs. control.

could affect its expression levels (Fig. 8f). We found that only CoQ_{10} + trolox could bring about a significant downregulation of VEGFA levels ($p < 0.05^*$) (Fig. 8g), which substantiates the use of VEGFA as a biomarker when studying treatment modalities for Rb[52].

Discussion

Rb affects a sizeable number of children in the younger age group[53] with India seeing six times increased number of cases reported per year when compared to the cases in the USA[54]. Hence, this necessitates the need for developing therapeutics aimed at inhibiting the growth of tumour cells at its early inception or at an advanced stage by restricting the size of the tumour. Such treatment options will offer fewer side effects. Also, since time immemorial several scientists have turned to antioxidants for developing therapeutics aimed at controlling the spread of cancer[55] which have often proven to act as a beneficial adjunct to chemotherapy. The present article investigates the effects of CoQ_{10} which has proven to be successful against various cancers[17,56,57] as well as its synergistic action with trolox on Rb, since no reported account of its efficacy alone or in combination exists yet. Our study examined the effects of CoQ_{10} alone as well as in combination with trolox, testing various parameters like cell proliferation, colony formation, apoptotic activity, angiogenesis, ROS generation, cell cycle , and intracellular signalling of Rb Y79 cells.

It has been reported that antioxidants when used in combination could amend the side effects of chemotherapeutic agents[58] while leaving growth of normal cells undisturbed. We also found that when CoQ_{10} alone was tested on ARPE-19 cells in the range of 10–50 µM it did not reduce the growth of cells. However, trolox showed a decline in cell number after 30 µM which was concurrent with other studies suggesting its prooxidant activity at higher concentrations on Hela cells[27] (Fig. 1a) .The action of CoQ_{10} when combined with trolox was found to be synergistic (Fig. 1b). Earlier studies have indicated that CoQ_{10} could reduce viability of pancreatic cancer[18], melanoma cells[17], ovarian cancer[59]. The present study demonstrated that CoQ_{10} alone and when combined with trolox at 30 µM each could reduce cell number significantly (Fig. 1c,d and e).

CoQ_{10} could induce lipid response in HeLa cells and lead to its growth inhibition[60]. The effect seen on Y79 cell growth could also be explained by a similar effect observed on its colony-forming ability which was significantly inhibited by both CoQ_{10} and CoQ_{10} + trolox (Fig. 2).

It has been speculated that when the antioxidant enzyme systems of cells are downregulated or show signs of an imbalance, it increases ROS levels thereby leading to malignancies. When ROS is generated as a by-product of aerobic respiration it leads to harmful consequences like migration and invasion of cancer. Secretion of angiogenic factors takes place when oxidative stress in the tumour microenvironment is caused by increased levels of ROS[61–63]. We found that although trolox increases ROS levels the increase is not as significant as CoQ_{10} alone ($p < 0.05^*$) as compared to control, and the same result is replicated by the combination of CoQ_{10} with

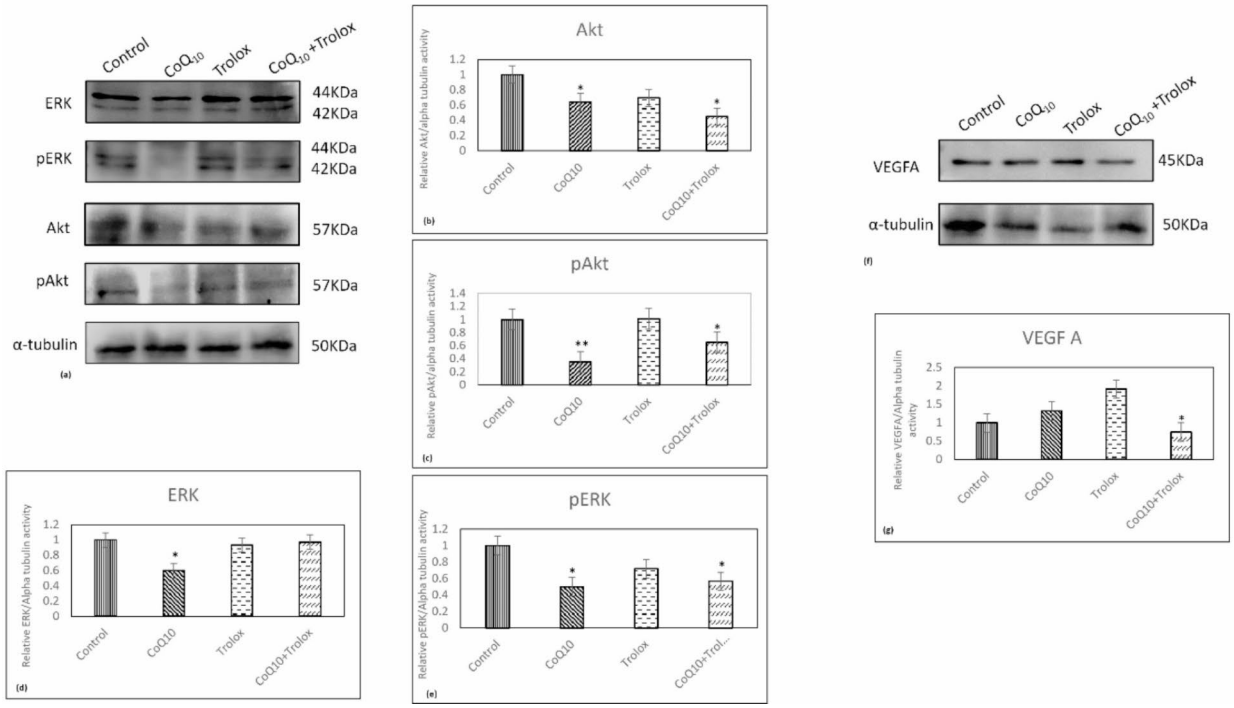


Fig. 8. The expressions of proteins produced by Y79 cells were analysed by western blotting. The blot of targeted proteins (**a**) showed that expression of targeted protein Akt was downregulated by both CoQ_{10} alone ($p < 0.05^*$) as well as when combined with trolox ($p < 0.05^*$) (**b**). A similar pattern was observed while examining the pAkt downregulation by both CoQ_{10} alone ($p < 0.01^{**}$) and CoQ_{10} with trolox ($p < 0.05^*$) (**c**). Interestingly, the expression of ERK protein was brought down by only CoQ_{10} alone ($p < 0.05^*$) (**d**), with the phosphorylated ERK expression being lowered by both CoQ_{10} alone ($p < 0.05^*$) and CoQ_{10} with trolox ($p < 0.05^*$) almost equally (**e**). Also, estimated were the variations in levels of pro-angiogenic factor VEGFA (**f**) and it was found that only the combination of CoQ_{10} with trolox ($p < 0.05^*$) could downregulate its expressions (**g**). Data is represented as mean ± SE from three different experiments. $^*p < 0.05$ and $^{**}p < 0.01$ vs. control.

trolox ($p < 0.01^{**}$) (Fig. 3a and b). Resulting cell death is speculated to have occurred due to nuclear DNA damage[64].

Mitochondria influences malignant transformation by accumulation of defects in DNA followed by subsequent activation of oncogenic signalling pathways[65]. Since changes in MMP indicate function ability and metabolism of the mitochondria[66], its loss stimulates various apoptogenic factors leading to the death of the cancer cells[45]. We found in our study that the MMP of Y79 cells could increase most significantly and incrementally when treated with both CoQ_{10} and trolox ($p < 0.01^{**}$) as compared to both CoQ_{10} ($p < 0.05^*$) and trolox alone respectively (Fig. 3c and d). Our results suggest that these observations could be explained by the potent activity of CoQ_{10} to reduce metastasis in cancer[11,67].

ROS plays a crucial role in boosting the signals required for cyclin-dependent kinases and Rb[68]. It appears to be an important mechanism for CoQ_{10} as it also acts by interrupting cell cycle progression. It has been reported earlier that CoQ_{10} administration in a conjugate form of BPM31510 markedly elevated mitochondrial O_2^- species and claimed that the elevation in mitochondrial O_2^- species was observed right before the onset of growth retardation and arrest of cells in the G2/M phase[69]. Similarly, we also observed that both CoQ_{10} alone and when combined with trolox induced Y79 cell arrest in the G2/M phase (Fig. 4).

However, the interplay of cell cycle and cell apoptosis is responsible for determining the eventual fate of cells. CoQ_{10} reportedly enhanced the cytocidal activity of doxorubicin in cancer cells both in vitro and in vivo[70], similar to trolox that enhanced toxicity of arsenic oxide towards malignant cells[71]. We also observed that the combination of CoQ_{10} and trolox along with CoQ_{10} alone could significantly cause an increase in cancer cell death pointing towards its apoptotic potential (Fig. 5).

Since angiogenesis requires proliferation and motility of endothelial cells, and their interactions with the microenvironment mediates tumour dissemination eventually causing metastasis[72] hence, it was important to study how HUVECs interacted with CoQ_{10} alone, trolox alone and CoQ_{10} combined with trolox when co-cultured with Y79 cells (Fig. 6a). We found that Y79 cells did not affect the cell number of HUVECs but when subjected to the treatment of trolox, there was visible reduction in number of cells. However, CoQ_{10} and CoQ_{10} with trolox could each visibly decrease the number of HUVECs significantly ($p < 0.05^*$, $p < 0.01^{**}$) compared to control (Fig. 6b), implying that CoQ_{10} alone or when combined with trolox possesses potential anti-angiogenic properties. Also, aside from in vitro cell cultures, it is equally necessary to study tumour formation in conditions that mimic the natural environment which is possible by carrying out in vivo experiments. The in ovo CAM

assay is a frequently used in vivo model system that offers several advantages over the standard murine model. This model provides higher reproducibility and is applicable in cancer studies, making it an excellent choice for studying angiogenesis[73–75]. Hence, when the group containing only Y79 cells was observed, it had numerous branching of blood vessels (Fig. 6c) which were reduced by treatment of trolox only. However, it was observed that when CoQ_{10} alone and CoQ_{10} with trolox were used, there seemed to be a significantly pronounced reduction in the number of blood vessels formed. Hence, these observations point towards a pre-emptive action of CoQ_{10} when used alone or in combination with trolox against the proliferation of retinoblastoma Y79 cells.

Among the different pro-angiogenic mechanisms, the VEGF signalling pathway is recognized as the main driver of tumour neovascularization. VEGF, a promising therapeutic target, initiates angiogenesis by regulating the proliferation, migration, and differentiation of endothelial cells, primarily through the activation of receptor tyrosine kinases[76]. In our study, CoQ_{10} and trolox did inhibit the rising mRNA levels of VEGFA in Y79 cells better than CoQ_{10} alone and trolox alone (Fig. 7a) but not significantly. The expression of VEGFA is reportedly very high in patients with Rb[77,78] and could not be suppressed significantly with either CoQ_{10} or trolox alone which again emphasizes towards their synergistic application as a potential way of targeting the VEGF signalling pathway. Therefore, the combination of CoQ_{10} and trolox may be used as a candidate drug to target angiogenesis in retinoblastoma by targeting VEGF/VEGFR signalling. Also, to further elucidate our findings, we investigated the role of Akt , a regulator of actin organization that interacts with and operates alongside the ERK1/2 signalling pathway[79] and found that only the combination of CoQ_{10} and trolox could significantly ($p < 0.05^*$) downregulate both ERK and Akt mRNA levels (Fig. 7b and c) leading to inhibition of the major signalling pathway for cancer cells[80,81].

Recent studies have shown that the expression of the PI3K/Akt signalling pathway-related proteins is found to be upregulated in a variety of cancers[82]. Additionally, various reports indicate that PI3K and Akt are critical for endothelial cell survival and angiogenesis, which are partly regulated by VEGF[72]. Therefore, targeted inhibition of Y79 by modulation of these pathways has become an effective therapeutic approach and was also our aim (Fig. 8). We demonstrated that both CoQ_{10} alone and CoQ_{10} and trolox in combination significantly reduced the phosphorylation of ERK and Akt (Fig. 8a–e). The obtained data is consistent with studies showing that inhibition of protein kinase B (Akt) leads to a reduction in angiogenesis and neovascularization[83,84] and that inhibition of pERK pharmacologically can inhibit tumour growth[85].

Angiogenesis is necessary for the growth and spread of tumours, with prior research indicating that patients with retinoblastoma have significantly higher levels of VEGF. A study found that the combined treatment reduced VEGF expression and ERK1/2 and Akt phosphorylation in vitro[8], with similar results observed by us when CoQ_{10} was combined with trolox which could significantly decrease the secretion of VEGF by lowering the expression of VEGF A (Fig. 8f and g) ($p < 0.05^*$). Despite the promising results, additional research is necessary to establish the optimal concentration of CoQ_{10}, both alone and in combination with trolox, for clinical treatment of Rb.

Conclusion

While coenzymes have been studied for their potential benefits alone or in combination with other treatments, little research has been done on their combined effects on retinoblastoma. Overall, we found that CoQ_{10} inhibited Rb growth and invasion, and blocked angiogenesis by targeting VEGF in Rb. This study confirmed that CoQ_{10} itself and also when combined with trolox has a stronger anti-tumour effect against advanced Rb in humans, both in vitro and in-ovo. This effect likely happens by inhibiting the PI3K/Akt and MAPK/ERK pathways. This is the first study to demonstrate the anti-tumour potential of CoQ_{10} and trolox against Rb while also illustrating the possible signalling mechanism behind their action. Therefore, the potential of this study stands to suggest that using anti-angiogenic therapy in conjunction with existing approaches like chemotherapy may aid in the treatment of retinoblastoma right at the outset as well as in advanced stages to avoid the need for surgery. While our research provides sufficient evidence suggesting the potential inhibitory effects of CoQ_{10} alone and when combined with trolox on Rb, a more comprehensive study using in vivo models is necessary to fully understand the scope of these two antioxidants in combating robust cancers like Rb.

Data availability

All data that support the findings of this study are included within this paper and its submitted files.

Received: 8 July 2024; Accepted: 10 October 2024
Published online: 09 November 2024

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Acknowledgements
The authors express their gratitude to Dr. Maryam from Amity Institute of Molecular Medicine and Stem Cell Research, Amity University, Noida, for her inputs and technical assistance in conducting western blot and FACS experiments.

Author contributions
M.P.G conceived of and designed the study. S.U and P.S collected data. S.U performed the statistical analyses. S.U , wrote the original draft, which was carefully modified by M.P.G, S.S and S.B. who further reviewed and edited. All authors reviewed and approved the manuscript before submission.

Funding
The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Declarations

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-024-76135-0.

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