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Original Article

Troxerutin, a Bioflavonoid Exhibits Oxidative Stress-induced Cytotoxic Effects on KB Cells

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ABSTRACT

Background: The prevalence of oral cancer is high and it is the sixth most occurring cancer in the world. Some of the drugs used to treat oral cancer cause adverse effects and are resistant to treatment. **Objectives:** This study was designed and performed to evaluate the cytotoxic and anti-cancerous effects of troxerutin. **Materials and Methods:** Cell proliferation, cell viability, and morphological analysis were performed with different doses of troxerutin. Once the IC_{50} value was obtained, doses were fixed and nitric oxide and lipid peroxidation were measured. Qualitative analysis of ROS and mitochondrial membrane potential was performed using 2′,7′-dichlorofluorescein methods and rhodamine 123 fluorescent staining respectively. **Results:** Troxerutin caused cytotoxicity and reduced cell viability. The IC_{50} value was 50 µg/ml. Morphological analysis revealed KB cells suffered after troxerutin treatment. Nitric oxide and TBARS levels were elevated. ROS concentration increased and mitochondrial membrane potential diminished with a higher concentration of the drug. **Conclusion:** Troxerutin exhibits an anti-cancer effect and can act as a NO-donating and ROS-promoting compound aiding in chemotherapy.

Keywords: Oral cancer, Troxerutin, Nitric oxide, Oxidative stress, Anti-cancer, Chemotherapy.

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INTRODUCTION

Oral Cancer (OC) is one of the most prevalent forms of cancer in both sexes and it is the sixth most frequently occurring malignancy worldwide.[1,2] The development of OC is attributed to several processes that mainly include the accumulation of genetic and epigenetic changes that are modulated by risk factors and genetic predisposition.[3] Several oral cancer risk factors are gender, age, consumption of alcohol, use of tobacco, lack of oral hygiene, radiation, infection by the human papillomavirus, exposure to asbestos, and inflammation.[4] Alcohol consumption and smoking are majorly considered to be risk factors as they are present in 90% of oral cancer patients.[5]

The treatment option for OC includes radiation, surgical removal of tumours, monoclonal antibodies, chemotherapy, or a combination of the treatment options.[6] These options have significant limitations. The current chemotherapeutic options for OC are anticancer drugs like cetuximab, cisplatin, docetaxel, methotrexate, paclitaxel, and fluorouracil, either used separately or in different combinations. Some of these drugs cause systemic toxicity and have acquired resistance.[7,8] The administration route for most of these drugs is intravenous and this causes damage to healthy tissue and an increase in adverse reactions is seen. To overcome the shortcomings in the treatment of OC, different research has been focused on developing new alternatives that are more effective and less toxic.

Flavonoids are naturally occurring polyphenols that are found in some fruits and vegetables. Most flavonoids are known to induce apoptosis in cancerous cells and inhibit angiogenesis.[9,10] Flavonoids are an emerging candidate for anti-cancerous and chemotherapeutic drugs. Troxerutin, a flavonoid commonly found in the flower buds of the Japanese pagoda tree has been reported to have anticancer, neuroprotective, radioprotective, anti-diabetics, and anti-inflammatory properties.[11,12] Furthermore, it had no side effects when administered to pregnant women.[13] The activity of troxerutin hasn't been well studied in oral cancer. Therefore, the current study aimed to investigate the potential of troxerutin as an anti-cancer agent in oral cancer KB cells.

MATERIALS AND METHODS

Cell Line

Human oral epidermal carcinoma cell line KB was used in this study. These cancer cells were cultured using DMEM culture media supplemented with 5% heat-inactivated FBS and 1% Penicillin-Streptomycin at 37°C and 5% CO_2 atmosphere. After

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reaching confluency of 70-80% the cells were passaged. In the present study, cells with passage numbers 3-5 were used.

MTT-Cell Proliferation Assay

Troxerutin effects on the cytotoxicity of KB cells were investigated by MTT assay.[14] 1.2×10^4 KB cells were seeded per well in a 96-well plate and incubated for 24 hr at the conditions mentioned above. After incubation, troxerutin was dissolved in sterilized distilled water and added to the wells at 10 different concentrations from 10 - 100 µg/ml. Cells were maintained with troxerutin for 48 hr. After that, the media was removed and MTT substrate was dissolved in PBS (0.5 mg/ml) and 10 µl was added to the well and incubated in a dark environment at 37°C for 4 hr. In the next step, the MTT solution is removed and 100 µl of 1% DMSO was added to each well to dissolve the formazan crystal. Following this, the absorbance was measured at 490 nm and 630 nm. The cellular cytotoxicity is expressed in % and the control group cells were considered to be 100% viable.

$$\text{Inhibition percentage} = 1 - \left[\frac{\text{Absorance}_{\text{Treated}}}{\text{Absorbance}_{\text{Control}}}\right] \times 100$$

Absorbance $_{\text{Treated}} = A_{490} - A_{630}$ of treated cells

Absorbance $_{\text{Control}} - A_{490} - A_{630}$ of control group cells

Trypan Blue Exclusion Test

To assess the viability of troxerutin on KB cells trypan blue exclusion test was performed.[15] KB cells with a density of 1.2×10^4 KB cells were seeded per well in a 96-well plate and incubated for 24 hr. After that troxerutin at 10 different concentrations (10 - 100 µg/ml) was added and incubated for 48 hr. After the 48-hr incubation, the cells were stained in trypan blue. The live and dead cells were counted using a hemocytometer and the viability was calculated by dividing the number of viable cells (unstained) by the number of dead cells (dark blue stained).

Morphological assessment of KB cells

To visualize the morphological changes of KB cells because of troxerutin. 2.2×10^5 KB cells/well were seeded and grown in a 6-well plate. Different concentrations of troxerutin were supplemented to these cells and incubated for 24 hr. After that, the changes in the morphology of the cells were visualized using inverted microscopy at a magnification of 200X.

Determination of Nitric Oxide levels

Nitric oxide measurement was performed by the protocol previously described in Dirsch *et al.*[16] 2% sulphanilamide - Griess A reagent and 0.2% NED - Griess B reagent were prepared using 5% phosphoric acid. 100 µl supernatant of KB cells cultured with different concentrations of TEX was combined with 90 µl of Griess A and 90 µl of Griess B in a 96-well plate. The mixture was mixed well and incubated in a dark environment for 5 min. After a color change (dark blue) is seen, absorbance is measured at 540 nm. The concentration of NO was measured in µMol.

Thiobarbituric acid-reactive substance (TBARS) assay

TBARS assays was performed by the method given in Ohkawa *et al.*[17] to measure lipid peroxidation. Troxerutin-treated KB cells were homogenized using ice-cold KCl buffer. This homogenate was centrifuged at 7000 rpm for 5 min. The resulting pellet was suspended in a mixture containing 8.1% SDS - 0.2 ml, 20% ethanoic acid - 1.5 ml, and incubated for 10 min. To this mixture, 0.8% TBARS - 1.5 ml and 0.7 ml distilled water was added and incubated for 1 hr at 95°C. After the temperature reached 25°C, 5 ml of Butanol: Pyridine (15:1) was added and centrifuged. The supernatant was read at 532 nm and the LPO was expressed in µM.

Reactive Oxygen Species (ROS) Detection

To investigate the generation of intracellular ROS levels, fluorescent oxidizable dyes such as 2′,7′-dichlorofluorescein (DCFH-DA) was used. This dye easily diffuses into the cells and the hydroperoxides that are produced oxidize DCFH to 2′,7′-dichlorofluorescein (DCF) which is highly fluorescent. The amount of peroxide inside the cells is proportional to the intensity of fluorescence. Briefly, KB cells were treated with different concentrations of troxerutin and incubated for 48 hr and after that, they were treated with 500 µL of DCFH-DA (10 µM) for 30 min. Finally, the cells were washed with PBS twice and 500 µL 1X PBS/well was added and images were taken using a fluorescence microscope.

Mitochondrial Membrane Potential (Δψm)

To evaluate the effect of troxerutin on mitochondrial membrane potential, KB cells that were incubated for 24 hr with troxerutin were washed with PBS twice. 1.7×10^7 KB cells were combined with rhodamine 123, a cationic lipophilic dye (final concentration, 1µM). The mixture was incubated at RT for 10 min and then photographed using a confocal microscope.

Statistical Analysis

The results obtained were expressed in mean ± standard deviation (SD). Statistical analysis was performed using the turkey multiple comparison test (one-way ANOVA) and GraphPad Prism 5 software was used for plotting graphs. $p < 0.05$ was considered to be significant.

RESULTS

Troxerutin effect on cell proliferation, viability, and morphology

Troxerutin effect on KB cells was examined by MTT assay and is shown in Figure 1. Cell proliferation was decreased in a

dose-dependent fashion. The IC_{50} of troxerutin on KB cells was 50 µg/ml. The viability of KB cells decreased with an increase in troxerutin concentration as seen in Figure 2. At 60 µg/ml the percentage of viable cells was 44% and at 100 µg/ml it was 27% indicating troxerutin has anti-cancer activity. In Figure 3, the morphological changes to different concentrations of troxerutin are observed. With higher concentration, the morphological aberrations increase. Changes such as shrinkage, detachment, shape disorientation, and membrane blebbing were induced by troxerutin when compared to the cells in the control group.

Nitric Oxide Levels

The concentration of nitric oxide in KB cells treated with various concentrations of troxerutin is shown in Figure 4. Treating cells with troxerutin increased the NO levels in a dose-dependent manner in comparison with cells in the control group (1.23 ± 0.084 µMol). The mean values of NO were 1.67 ± 0.056, 1.875 ± 0.120, and 2.27 ± 0.0282 µMol for troxerutin doses 40, 50 and 60 µg/ml.

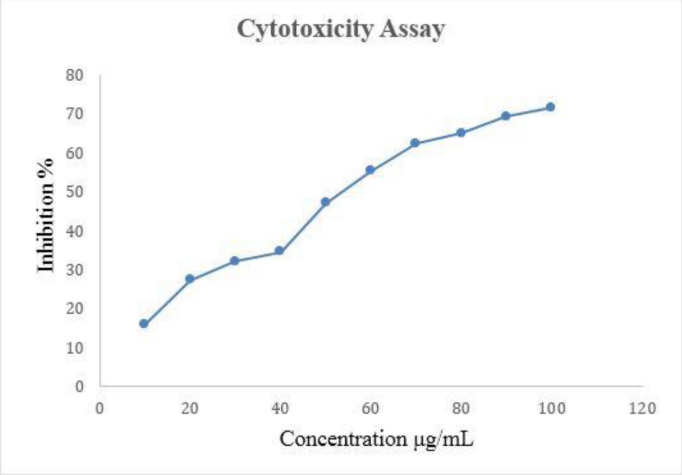

Figure 1: cytotoxic effect of Troxerutin on KB cell line.

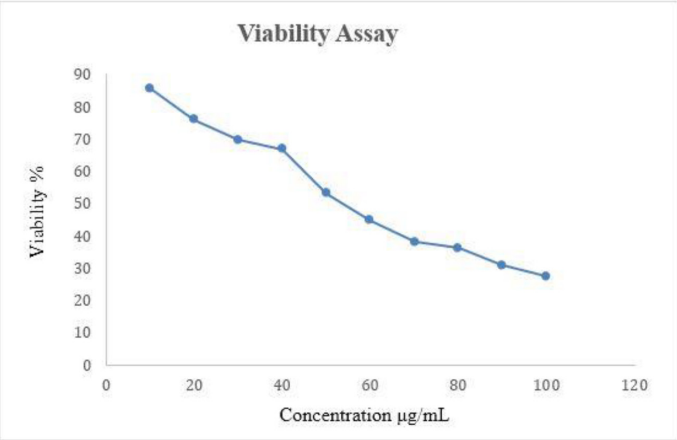

Figure 2: Effect of Troxerutin on cell proliferation.

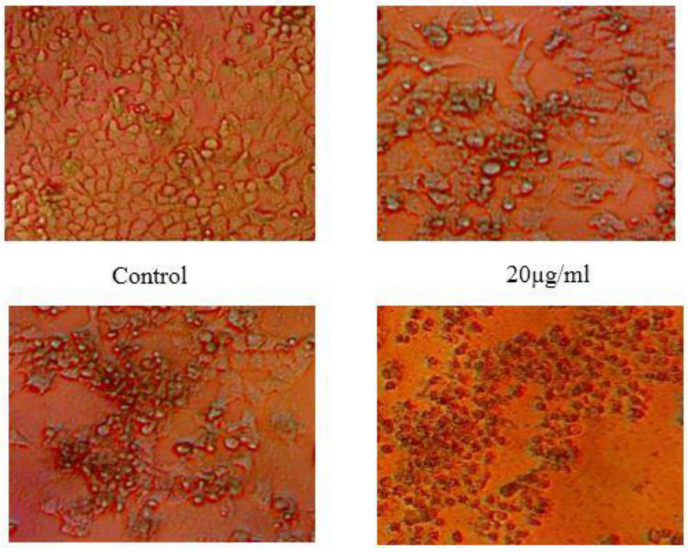

Figure 3: Morphological changes in Troxerutin treated oral cancer KB cells.

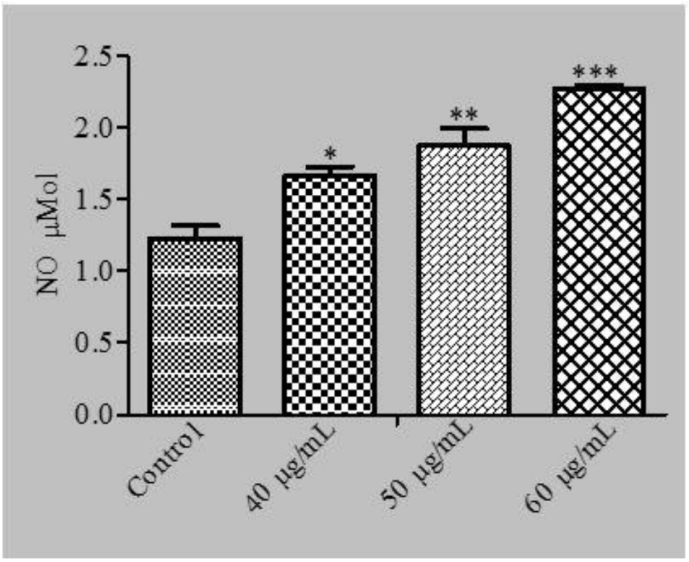

Figure 4: Effect of troxerutin on nitric oxide production in KB cell line.

Thiobarbituric acid reactive substance (TBARS) activity

The TBARS activity in the control group KB cells is 0.305 ± 0.007 µM. Similar to the nitric oxidase, when the cells are treated with troxerutin at different concentrations, the TBARS activity increases. A dose of troxerutin at 40, 50, and 60 µg/ml causes an increase in TBARS activity (0.345 ± 0.007, 0.515 ± 0.021, and 0.63 ± 0.014 µM, respectively). This is illustrated in Figure 5.

Troxerutin Effect on ROS

The effect of troxerutin on the ROS levels of KB cells was detected using the fluorescent method by DCFH-DA staining and it is illustrated in Figure 6. KB cells were treated with 30, 40, and 50 µg/ml of troxerutin for 48 hr. The staining of the treated cells revealed an increase in the peroxides inside the cells in a dose-dependent manner.

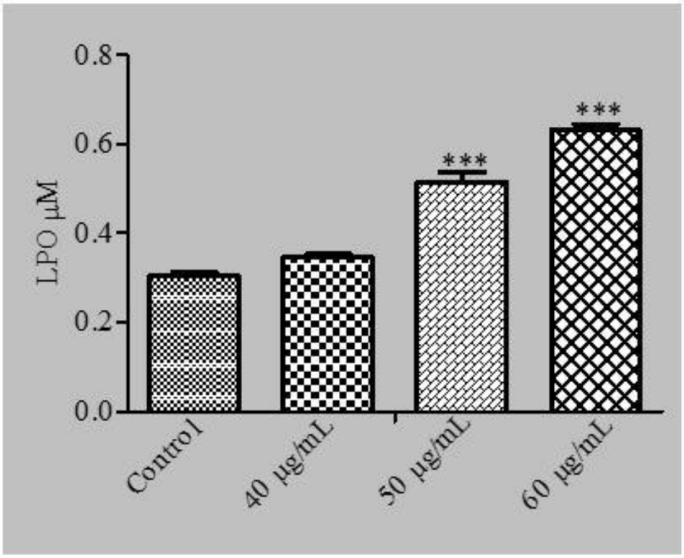

Figure 5: Effect of Troxerutin on lipid peroxidation in KB cell line.

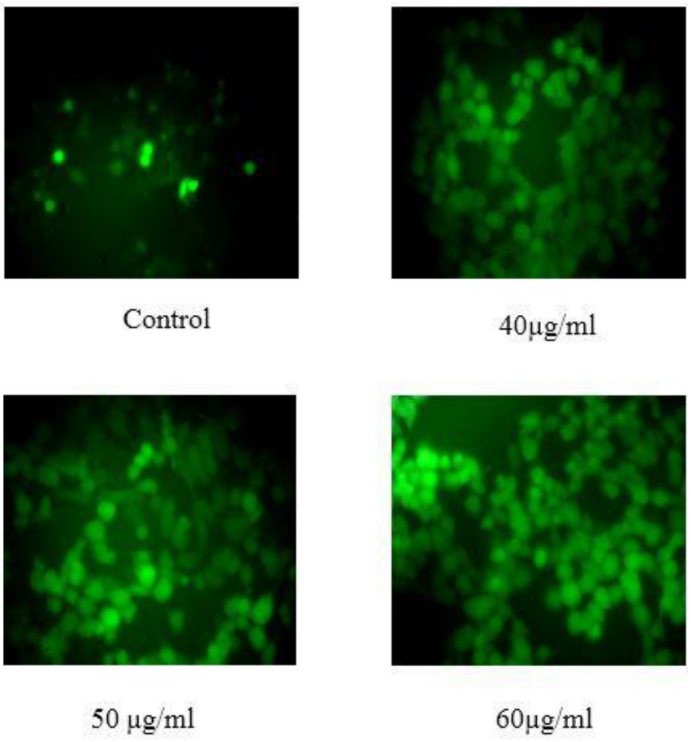

Figure 6: Effect of Troxerutin on the intracellular ROS generation in oral cancer (KB) cells by DCFH-DA staining assay.

Effect of Troxerutin on Mitochondrial Membrane Potential (MMP)

MMP was measured using the fluorescent method by Rhodamine staining. The effects of troxerutin on the MMP of KB cells are depicted in Figure 7. The cells in the control group show high fluorescence but with the increase in troxerutin dose, the green fluorescence decreases gradually indicating that the activity of mitochondria is decreased.

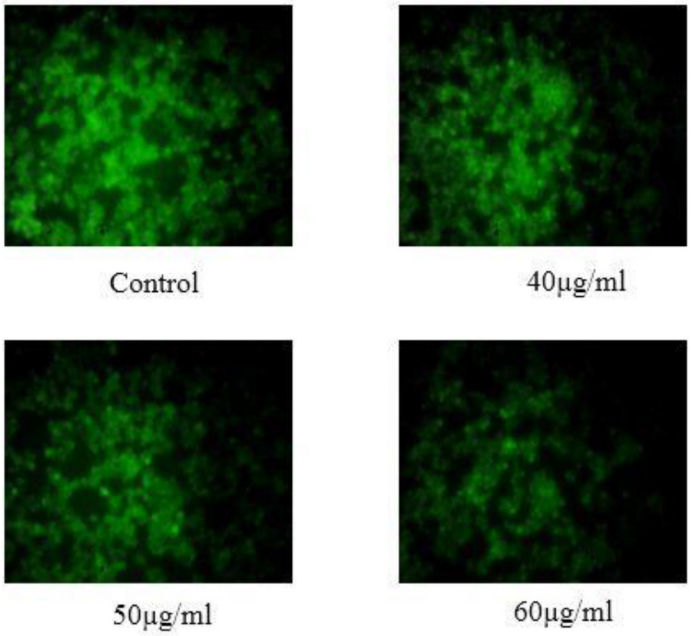

Figure 7: Effects of Troxerutin on the mitochondrial membrane potential of oral cancer (KB) cells.

DISCUSSION

Treatment of options for OC with several anticancer and chemotherapeutic drugs comes with side effects like systemic toxicity, problems with the specificity of the drugs, and many more.[18] Identifying and developing drugs against oral cancer with lower side effects is critical. Troxerutin is a bioflavonoid that is a rutin derivative. It preferentially binds to the minor groove of DNA and this characteristic may induce damage to DNA that will cause the death of cells.[19] Troxerutin inhibited the migration of cells and induced apoptosis in hepatocarcinoma by activation of the Nrf2 pathway.[20] In colon cancer cells, it showed chemo preventive effects.[21] Considering the pharmacological and anti-cancer properties of this bioflavonoid, in the current work, the anti-cancer ability of troxerutin against oral cancer KB cell line was studied.

After treating the oral cancer cells with troxerutin for 48 hr, the effect was analysed by MTT assay and trypan blue dye exclusion. Both these assays showed a dose-dependent reduction. An inhibition in cell proliferation with IC_{50} being 50 µg/ml was seen in MTT assay results. Like the MTT assay, the dye exclusion assay result showed only 27.37% viability at the dose of 100 µg/ml. This establishes that troxerutin can exert cellular inhibition. To further substantiate the cytotoxic effect, membrane blebbing was seen when treated with higher concentrations. Membrane blebbing is an indicator and a defining feature of apoptosis.[22,23]

Nitric oxide is a free radical gas that is produced endogenously and is a signalling molecule. Several cancer-related processes have been regulated by nitric oxide.[24] NO has a dichotomous role in cancer.[25] Based on the location, timing, and concentration,

NO can have tumour-promoting properties as well as tumoricidal properties. At lower concentrations, NO is known to promote the proliferation of tumour cells, have antiapoptotic effects, and induce angiogenesis. Treating the KB cells with troxerutin increased NO levels. At a higher concentration, NO has cytotoxic effects and is more prone to trigger apoptosis and cell cycle arrest.[26,27] From our results, we can infer that troxerutin may act as a NO-donating agent and it can be a promising chemotherapeutic candidate.

A state of oxidative stress occurs when there is an imbalance in the production of free radicals and the antioxidant capacity to scavenge the produced free radicals.[28] ROS is one of the most produced free radicals produced as a result of many cellular processes. ROS can influence and interfere in many signalling pathways and is the basis of cancer and many other metabolic and neurodegenerative diseases.[29] Similar to nitric oxide, several clinical studies have shown ROS to have a dual role. It can facilitate cancer development. Contrariwise, it promotes the death of cancer cells via several mechanisms.[30] In the cancerous environment, ROS induced hypoxia which enhances cancer cell survival and tumor angiogenesis.[31] However, at a higher concentration, senescence, cell cycle arrest, and death of cancer cells occur.[32] Survival, proliferation, and metastasis are inhibited when there is an oxidizing milieu.[33] The results from this study show, that when troxerutin is given to KB cells the ROS concentration increases. Along with ROS-scavenging inhibitors, troxerutin can be combined and used as a ROS-producing agent to promote oxidative stress-induced death of cancer cells and also reduce the resistance to chemotherapeutics.[34] Oxidative stress results in lipid peroxidation which in our study is measured by TBARS assay. Acrolein is one of many lipid peroxidation products, it can induce cytotoxic effects and death of human cancer cells.[35,36] Besides that many lipid peroxidation products have been used along with anticancer drugs to enhance antitumor effects.[35] The current works show an increase in lipid peroxidation and this Increase in lipid peroxidation can cause apoptosis.[37]

Mitochondria is pivotal for the proper functioning of cells and its roles range from the production of energy to apoptosis regulation. Mitochondrial membrane potential indicates the functioning and health of mitochondria.[38,39] The dissipation of this potential is an early indicator of programmed cell death.[40] By targeting various components in the mitochondria, increased ROS can collapse the mitochondrial membrane potential. This causes the release of several mitochondrial components into the cytosol that in turn forms apoptosome complex, activates caspase, and lead to apoptosis.[41] The results obtained show an increase in ROS and a decrease in the mitochondrial membrane potential indicating apoptotic prompting effects of troxerutin.

CONCLUSION

The study findings show that treating oral cancer KB cells with troxerutin for 48 hr at a concentration 50 µg/ml shows anti-cancer effects. Morphologically, after treating the cells, they had different changes like blebbing and detachment. Troxerutin significantly increased Nitric oxide levels, reactive oxygen species, and lipid peroxidation causing the death of cancer cells. Mitochondrial membrane potential was also reduced, indicating that apoptosis was triggered in the cancer cells. Overall, it can be concluded that troxerutin can inhibit the progression of oral cancer and induce cell death. Although to understand how it performs these, further in-depth analysis is required.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

OC: Oral cancer; **ROS:** Reactive oxygen species; **NO:** Nitric oxide; **TBARS:** Thiobarbituric acid; **DMEM:** Dulbecco's Modified Eagle Medium; **FBS:** Fetal bovine serum; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **DMSO:** Dimethyl sulfoxide; **NED:** N-(1-Naphthyl)ethylenediamine; **SDS:** Sodium dodecyl sulphate; **DCFH-DA:** Dichloro dihydro fluorescein diacetate; **PBS:** Phosphate buffered saline **MMP:** Mitochondrial membrane potential; **RT:** Room temperature.

SUMMARY

- Troxerutin inhibits the proliferation of KB cells after being treated for 48 hr.
- Troxerutin causes induction of Nitric oxide levels and increases lipid peroxidation.
- ROS generation and Mitochondrial membrane potential diminishing are induced by troxerutin.
- Troxerutin has anti-cancerous activity and can be used to aid chemotherapeutics.

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