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RESEARCH ARTICLE

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Usnic acid alleviates inflammatory responses and induces apoptotic signaling through inhibiting NF-κB expressions in human oral carcinoma cells

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Abstract

Usnic acid (UA) is a unique bioactive substance in lichen with potential anticancer properties. Recently, we have reported that UA can reduce 7,12-dimethylbenz[a] anthracene-induced oral carcinogenesis by inhibiting oxidative stress, inflammation, and cell proliferation in a male golden Syrian hamster in vivo model. The present study aims to explore the relevant mechanism of cell death induced by UA on human oral carcinoma (KB) cell line in an in vitro model. We found that UA can induce apoptosis (cell death) in KB cells by decreasing cell viability, increasing the production of reactive oxygen species (ROS), depolarizing mitochondrial membrane potential (MMP) levels, causing nuclear fragmentation, altering apoptotic morphology, and causing excessive DNA damage. Additionally, UA inhibits the expression of Bcl-2, a protein that promotes cell survival, while increasing the expression of p53, Bax, Cytochrome-c, Caspase-9, and 3 proteins in KB cells. UA also inhibits the expression of nuclear factor-κB (NF-κB), a protein that mediates the activation of pro-inflammatory cytokines such as TNF-α and IL-6, in KB cells. Furthermore, UA promotes apoptosis by enhancing the mitochondrial-mediated apoptotic mechanism through oxidative stress, depletion of cellular antioxidants, and an inflammatory response. Ultimately, the findings of this study suggest that UA may have potential as an anticancer therapeutic agent for oral cancer treatments.

KEYWORDS
anti-inflammatory, antioxidant, apoptosis, cytotoxicity, ROS, UA

1 | INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common type of oral cancer that affects the lining of the lips and mouth.[1] It is typically caused by risk factors such as smoking, alcohol consumption, chronic irritation, or human papillomavirus (HPV) infection.[2] Symptoms of OSCC may include a sore throat, a lump in the mouth that does not heal, loose teeth, mouth pain, or difficulty swallowing.[3] The treatment options for OSCC entail surgery, radiation, and chemotherapy.[4] However, some complications are still associated with ensuring the effectiveness and safety of chemotherapeutic agents or radiotherapy in treating malignant cells.[5] In recent times, the scientific community has been exploring using natural products to lessen the adverse

effects of chemotherapy. This can be achieved by regulating various metabolic pathways, protecting against organ damage, and improving the effectiveness of anticancer drugs.[6] Chemoprevention, which involves the use of nontoxic drugs, maybe a better approach to reducing cancer incidence. There is ample epidemiological and clinical research proving the efficacy of secondary metabolite products and phytochemicals found in various herbal medicines as potent anticancer agents.[7]

There is growing evidence that secondary metabolites found in lichens possess chemotherapeutic and chemopreventive properties for various types of cancer, especially oral cancer. This is because they act as antioxidants and scavenge free radicals. Lichen metabolites have recently gained attention as potential anticancer drugs due to their potent pharmacological activity.[8] Reactive oxygen species (ROS) are naturally occurring molecules that can cause oxidative stress. When ROS levels become too high, they damage various cellular components, including lipids, proteins, DNA, and tiny cellular organelles, leading to cell death in cancer cells.[9] Apoptosis is a process of cellular death that prevents cells damaged by DNA from transforming into cancerous cells.[10] If the damage is too severe to be repaired, p53 induces apoptosis by increasing the expression of proapoptotic markers in the Bax family.[11] The anti- and proapoptotic proteins of the bcl-2 family, cysteine proteases, and the control of the p53 tumor suppressor proteins are some of the complex processes that contribute to the induction of apoptosis.[12] The nuclear factor kappa-light chain-enhancer of activated B cells (NF-κB) is a potential transcriptional regulator that activates multiple biological pathways, including inflammation, cell proliferation, apoptosis, and angiogenesis.[13] The activation of the NF-κB signaling complex triggers downstream signaling pathways that promote the transcription of pro-inflammatory mediators, such as tumor necrosis factor-alpha (TNF-α), cyclooxygenase-2 (COX-2), interleukin-6 (IL-6), and inducible nitric oxide synthase (iNOS).[14]

Usnic acid (UA) is one of the most prevalent and prominent secondary metabolites found in lichens.[15] UA exists in two enantiomers: (−)-usnic acid and (+)-usnic acid. (+)-UA has a wide range of bioactivities including antifungal, antibacterial, antiviral, cytotoxic, antioxidant, antiherbivore, and insecticidal actions.[16–22] Moreover, several in vitro and in vivo models have shown that (+)-UA exhibits potent anticancer properties.[23] We recently reported that UA can reduce oral carcinogenesis induced by 7,12-dimethylbenz[a] anthracene in male golden Syrian hamsters in vivo by inhibiting oxidative stress, inflammation, and cell proliferation.[24] Furthermore, a study conducted by Qi et al.[25] found that UA significantly activates the ROS-dependent apoptosis of LUSC cells by interfering with the PI3K/Akt/Nrf2 pathway and the mitochondrial respiratory chain (MRC). Previous studies found that beta-caryophyllene (BCP), a natural phytochemical compound, can induce apoptosis response and inflammatory signaling in KB cell lines.[26] However, the impact of UA on anticarcinoma properties in the human oral cancer KB cell line has not yet been explored. Therefore, we aim to investigate the effects of UA-induced ROS production, DNA damage, inflammatory signaling, and apoptosis pathways on the KB cancer cell line.

Significance statement

Usnic acid (UA) has been found to inhibit the growth of cancerous cells and induce the generation of ROS (reactive oxygen species) by activating apoptotic signaling pathways in KB cells. More importantly, the present study suggests that UA Prevent cytotoxicity depletion of antioxidants, induce apoptotic molecules, and downregulation of the inflammatory response in KB cells. Due to its antioxidant effects, UA could potentially be used to develop new anticarcinoma agents, making it a promising novel therapeutic candidate for treating human oral cancer.

2 | MATERIALS AND METHODS

2.1 | Chemicals

Dulbecco's Modified Eagles Medium (DMEM), fetal Bovine serum (FBS), 0.25% trypsin EDTA, antibiotics (Streptomycin, Penicillin), phosphate buffered saline (PBS), Ethanol, Methanol, dimethyl sulfoxide (DMSO), thiobarbituric acid (TBA), phenazine methosul- phate (PMS), nitroblue tetrazolium (NBT), 5,5-dithiobis 2-nitrobenzoic acid (DTNB), Sodium dodecyl sulfate (SDS). N, N, N′, N′-tetramethylene diamine (TEMED), bovine serum albumin (BSA), and 2-mercaptoethanol were obtained from Hi-media Laboratory. Primary antibodies anti-p53, anti-Bax, anti-Bcl-2, anti-β-actin, anti-Cyt-c, anti-TNF-α, anti-Caspase-3, anti- NF-κB, anti-Caspase-9, anti-IL-6, and secondary antibodies were acquired from Santa Cruz Biotechnology (SCBT) and Cell Signaling Technology (CST). (+)-Usnic acid (UA), 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyl tetrazolium bromide (MTT), 2,7 diacetyl dichlorofluorescein (DCFH) 2, 7,-diacetyl dichlorofluorescein diacetate (DCFH-DA), Rhodamine 123, 6-diamidino-2-phenylindole (DAPI), Ethidium Bromide (EtBr, Acridine Orange (A/O), were obtained from Sigma-Aldrich).

2.2 | Cell growth

The human oral carcinoma cell line (KB) and normal human gingival fibroblast cell line (HGF-1) were purchased from the National Center for Cell Science (NCCS). The cells were developed as a monolayer in a T25 cm^2 cell culture flask maintained with DMEM containing 10% FBS, 1% glutamine, and 100 U/mL streptomycin – penicillin, 5% CO_2 at 37°C in the room atmosphere. All the experimental studies were conducted on cells in the exponential growth phase.

2.3 | Preparation of UA

UA (10 mM) was dissolved in 0.1% DMSO (dimethyl sulphoxide) and deposited at 4°C until use. DMSO was utilized for all treatments at a final concentration of 0.01% (v/v).

2.4 | Cytotoxicity assay

Cytotoxicity assay is a standard procedure used to measure cell proliferation. The reduction of MTT to formazan, which is dependent on mitochondria, reveals the mitochondrial activity of cultured cells.[27] Briefly, KB and HGF-1 cells (1.0×10^4 cells/well) were maintained white flat-bottom plated in 96-well plates. The cells were exposed to various concentrations of UA (10–100 μM). After 24 h, cells were incubated at 37°C, and 10 μL of MTT mixture (5 mg/mL) was added to each well to absorb dark light. After 3 h incubation, the MTT- reagent was detached carefully, and adding up of 100 μL DMSO to each well, a purple formazan crystal product formed. The cell viability absorbance value was calculated at 570 nm using a Multimode reader (Molecular Devices).

2.5 | Biochemical estimations of TBARS and antioxidant status

UA was treated with KB cells in different concentrations (10, 20, and 30 μM). After incubation, cells were collected by trypsinization and centrifuged to form pellets. The suspension UA-treated cells were used to investigate lipid peroxidative markers and enzymatic and nonenzymatic antioxidant activities.

Lipid peroxidation level was measured using TBA-reactive molecules or TBARS (Niehaus and Samuelsson).[28] Reduced glutathione (GSH) level was measured using Ellman's method.[29] Enzymatic antioxidant assessments such as catalase (CAT), superoxide dismutase (SOD), and glutathione peroxides (GPx) activities by the in vitro method were previously described.[30–32]

2.6 | Assessment of intracellular ROS generation

DCFH-DA (2, 7-diacetyl dichlorofluorescein-diacetate), a nonfluorescent dye method, was used to measure intracellular ROS generation.[33] Briefly, KB cells were seeded (1×10^5 cells/well) in a six-well plate; then, cells were treated with UA (10, 20, 30 μM) and exposed for 24 h. After cells were incubated, 5 μL of DCFH-DA was added to each well and kept for 30 min at 37°C in a dark place. Before measuring the ROS level, they were rinsed with cells twice in ice-cold PBS to discard the excessive staining, and measurement of intracellular ROS levels was recorded at (485 ± 10 and 530 ± 12.5 nm), using a multimode reader (Molecular Devices). Photographic images were taken using a fluorescence microscope (Floid cell imaging station; Life Technologies).

2.7 | Assessment of mitochondrial membrane potential (Δψm)

Intracellular ROS production intensively modifies membrane potential (depolarization), indicative of early apoptotic stages.[34] Rh123 staining was used to measure the alterations in MMP (Δψm). The KB Cells (2×10^5 cells/well) were seeded into six-well plates, and cells were treated with different concentrations of UA (10, 20, 30 μM) for 24 h. After the treatment, the cells were incubated with an Rh-123 staining solution in a dark place for 30 min at 37°C. A molecular device like a multimode analyzer is measured. The light emitting intensity is between 485 ± 10 and 530 ± 12 nm. Photographic images were detected under a fluorescence microscope (Floid cell imaging system; Life Technologies).

2.8 | DAPI with nuclear staining assay

The DAPI staining method was used to identify the nuclear morphology in cancer cells. The effect of UA-provoked nuclear abridgement was assessed using DAPI staining.[35] Momentarily, KB cells were introduced with different concentrations (10, 20, 30 μM) of UA and allowable to expand for 24 h at 37°C with CO_2 (5%) atmosphere condition. Later, the cells were fixed using 4% paraformaldehyde in PBS. DAPI (1 mg/1 mL) nuclear staining was used on the fixed cells, and the stained slides were kept at 37°C in a dark place. Fluorescence microscopy was used to observe the morphology of the KB cells (Floid cell imaging station; Life Technologies).

2.9 | Evaluation of apoptosis morphological changes by AO/EtBr staining

The morphologically apoptotic and necrotic cells were identified using the DNA-binding dyes AO/EtBr.[36] The morphological alterations of UA-induced apoptosis in KB cells were examined using the AO/EtBr dual staining method. UA-introduced cells were nurtured for 24 h at 37°C in 5% CO_2 condition. After that, the cells were allowed for staining for about 25 min in the dark with 3 μL of AO/EtBr at a concluding concentration of (1 mg/mL). The staining wells were washed away with PBS to discard extreme dye, and a floid cell imaging system took photographic images. The percentage of cells exhibiting apoptotic signals was calculated using an action of the total number of cells in the field.

2.10 | Comet assay

As previously (Singh et al.[37]) described, the single-cell gel electrophoresis method was utilized to assess the single-strand breakage of DNA. UA different concentrations were treated with KB cells, and 30% of the suspended cells were pipetted onto a comet slide with 100 μL of low melting point agarose. The combined solution was then kept at 4°C for 60 min. Cells were soaked in lysis solutions and incubated at 4°C for 2 h. We performed electrophoresis 30 min after using a cold alkaline buffer solution to denature the comet slides. The slide was washed with

Tris-buffer for 5 min, and then the ethidium bromide (EB) solution (40 μg/mL) was stained with the slide. Single-cell DNA damage was observed using a fluorescent inverted microscope. CASP software was used to analyze (Inverted fluorescent microscope, Eclipse Ti2E; Nikon).

2.11 | Immunoblotting analysis

The KB cells were grown in Petri dishes (60 mm) and treated with UA (10, 20, 30 μM) for 24 h. The cell lysates were kept in RIPA buffer with a protease inhibitor cocktail to separate the protein (Sigma-Aldrich). The amount of protein was measured using the Bradford method. The removed proteins were strictly detached on 10% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and then relocated into a PVDF (polyvinylidene difluoride) membrane. After transferring, the membrane was blocked with a blocking (5% BSA) solution for 2 h at 37°C. The following primary antibodies were incubated at cold conditions overnight. Later, the PVDF was twice cleaned with TBST (1X), and the membrane was preserved with HRP-coupled secondary antibody exposed to horseradish peroxidase for 2 h incubation at room temperature. Finally, the PVDF membranes were cleaned two to three times with 1X TBST. The chemiluminescence (ECL) method was then used to develop blots. Further, Image J software quantified the blotting band image strength.[38]

2.12 | Statistical analysis

The mean ± standard deviation was utilized to express all the results. The Statistical Package for Social Sciences (SPSS) 21.0 software application was used to conduct the statistical analysis. Employing analysis of variance, statistical variances were evaluated. Duncan's multiple range test (DMRT) revealed significant variations between the means ($p < .05$).

3 | RESULTS

3.1 | UA inhibits cell proliferation in KB cells

The MTT assay was utilized to examine the cytotoxic impact of UA on KB cells. As shown in (Figure 1A), UA was tested with KB cells at different concentrations (10–50 μM) for a 24 h incubation period, which demonstrated the concentration and time-dependent toxicity of UA on KB cells. Furthermore, as displayed in (Figure 1B), UA was treated with normal human gingival fibroblast cells (HGF-1) and exhibited minimal to no cytotoxicity at concentrations up to 50 μM, respectively. After 24 h of incubation, we observed a 50% cell inhibition rate at 30 μM concentration of UA in KB cells. We selected the UA concentrations of 10, 20, and 30 μM based on these findings for further study.

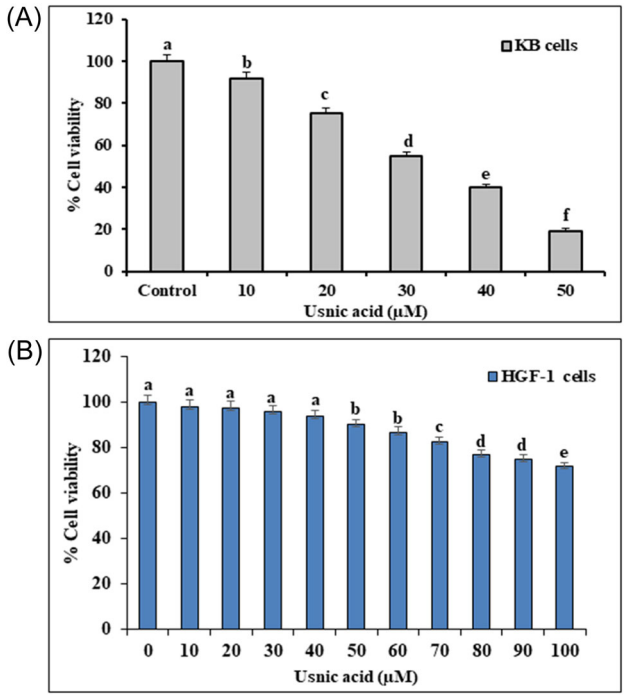

FIGURE 1 Outcome of UA on KB cells and HGF-1 cell viability. (A) KB cells were treated with UA increasing concentrations of (10–50 μM) for 24 h by MTT assay. Cytotoxicity efficacy of UA on KB cells in inhibitory concentration of 30 μM for 24 h treatment. (B) Effect of UA on cell survival study in HGF-1 cells. Cell survival significantly increased concentration up to 10–50 μM for 24 h respectively; All statistics are represented as means ± SD triplicate ($n = 3$) in each cluster. Data do not share common superscripts (a,b,c,d…) that differ significantly at $p < .05$. (DMRT).

3.2 | Effect of UA on antioxidant status and lipid-peroxidation

An elevated level of lipid peroxidation and depletion of antioxidants are well-known indicators of oxidative stress. (Figure 2) illustrates that the increased levels of lipid peroxidative by-products of TBARS and the antioxidant enzymes SOD, CAT, and GPx were significantly reduced in UA-treated KB cells compared with untreated KB cells in a concentration-dependent manner. Furthermore, our findings indicate that UA-treated KB cells exhibited reduced levels of GSH, suggesting that UA was responsible for the enhanced antioxidant level.

3.3 | UA-induced ROS generation and mitochondrial-mediated apoptosis in KB cells

It has been previously reported that UA has an anticancer effect by causing oxidative damage. To this aim, we investigated the possibility that ROS signaling factors directly relate to UA-induced cell death. Using DCFH-DA, an oxidant-sensing fluorescent probe, the Treatment of KB cells with different concentrations of UA (Figure 3) showed significantly increased levels of ROS production and green

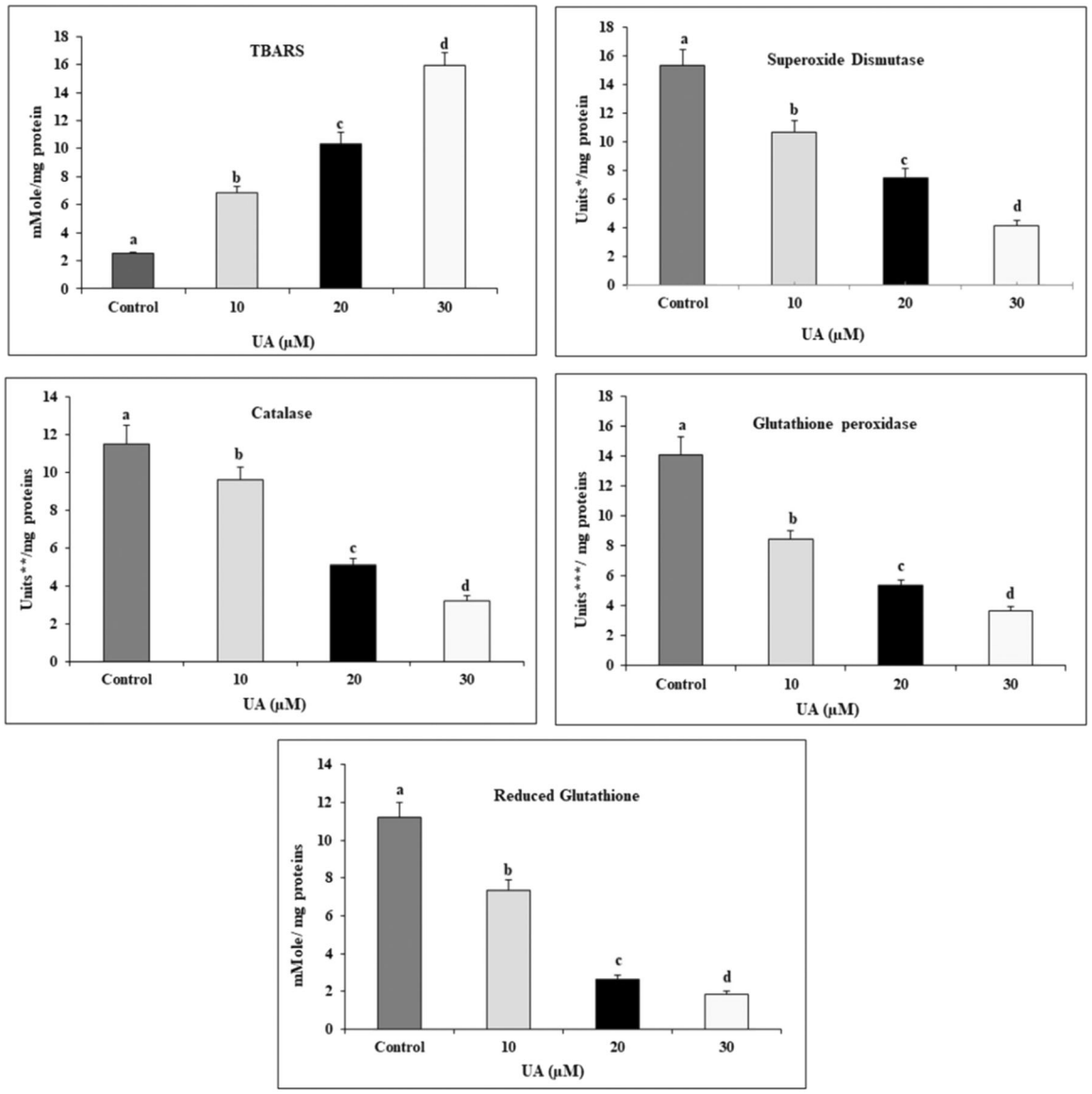

FIGURE 2 Effect of UA on lipid peroxidation and antioxidant levels in KB cells. UA treated KB cells in various concentrations (10, 20, 30, μM) for 24 h. After incubation, the UA-treated KB cells increased the level of TBARS and distorted antioxidant activities (SOD, CAT, GPx, and GSH) in KB cells. *Enzyme concentration indicated for suppressing nitroblue tetrazolium reduction by 50% in 1 min. **mmol/min of hydrogen peroxide absorption. ***mg/min of Glutathione absorption. All data are presented as means ± SD sex value ($n = 6$) per each group. Value without superscripts (a–d) differ considerably at $p < .05$. when compared with the control groups (DMRT).

fluorescence intensity when compared with the control KB cells. we found that UA could elicit a sharp dose-dependent increase in intracellular ROS in KB cells.

In addition, Rhodamine-123, a lipophilic cationic dye, was employed to measure the alteration of mitochondrial membrane potential (MMP) exhibited in the early stages of apoptosis. (Figure 4), examined the morphological alteration in the MMP level in untreated control and UA-treated KB cells using Rh123 dye. The control cells exhibit polarized mitochondrial membranes by emitting a high intensity of green fluorescence. The results showed that Rh-123 dye accumulations were decreased in different concentrations of UA-treated KB cells compared with control cells. In this study, we observed that UA-treated KB cells significantly depleted MMP, leading to mitochondrial-mediated apoptosis.

3.4 | UA induces apoptosis as evidenced by morphological changes in KB cells

To differentiate between apoptotic or nonapoptotic cells, UA's apoptotic morphological changes were analyzed using AO/EtBr dual staining. While the AO (green) only absorbed nonapoptotic cells, the red fluorescent dye of EtBr specifically entered the compacted nuclei of apoptotic cells. Our results showed that untreated KB cells had bright green fluorescence

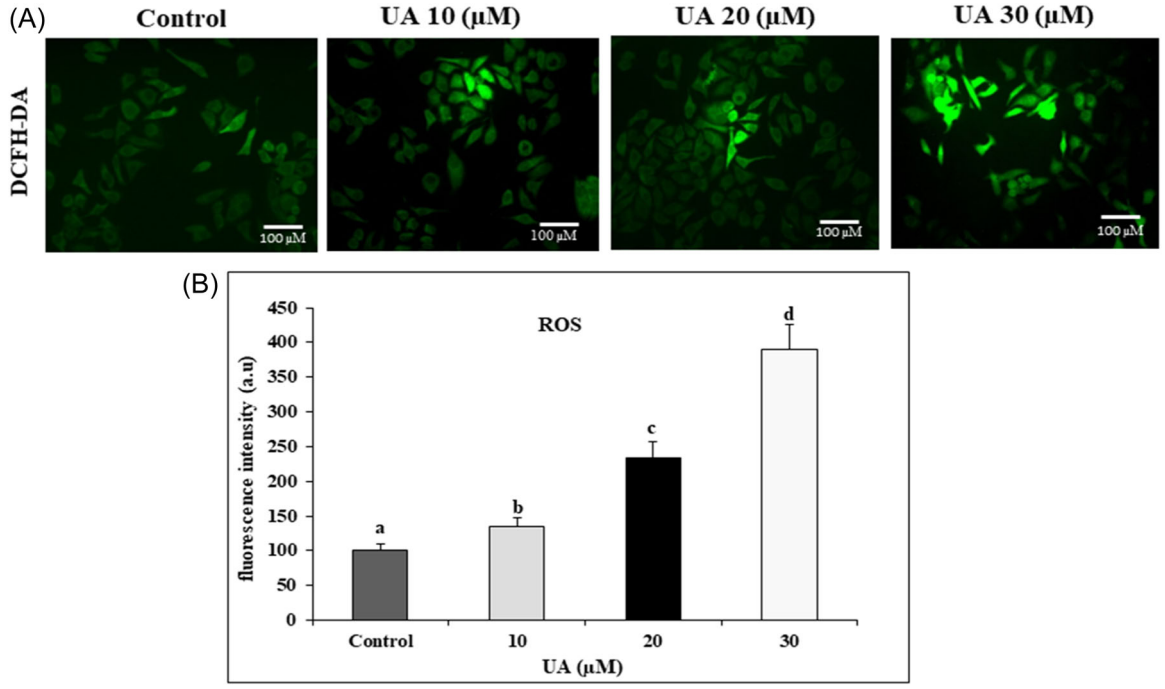

FIGURE 3 Effect of UA persuades intracellular reactive oxygen species in KB cells using the staining method (DCFH-DA). (A) The UA-treated KB cells exhibit increased green fluorescence in a photomicrograph using a green light source (×20). (B) Bar diagram percentage of fluorescence intensity under excitation and emission at 485 ± 10 and 530 ± 12.5 nm, respectively, were measured by the multimode reader (Molecular Devices). The means ± SD triplicate (n = 3) of different experiments performed in each group are displayed. The data vary suggestively at $p < .05$, and values do not share a common superscript (a, b, c, d) (DMRT).

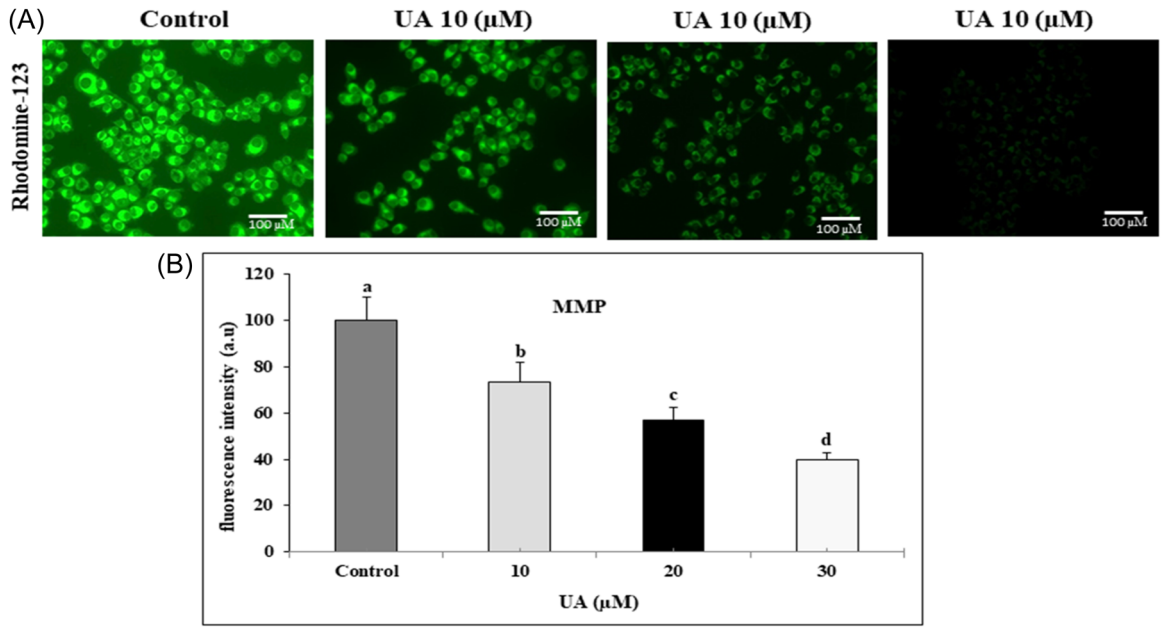

FIGURE 4 Effects of UA on MMP level by Rh-123 stain method. (A) The photomicrograph of UA-treated KB cells depicts a decrease in green fluorescence intensity in the loss of mitochondrial membrane potential (Cell Imaging Station; Life Technologies: ×20). (B) The bar graph shows the percentage of fluorescence intensity measured using a multimode reader (Molecular Devices), under excitation and emission wavelengths of 485 ± 10 and 530 ± 12.5 nm, respectively. The data from triplicate (n = 3) experiments in each group are presented as means ± SD. The data fluctuate pointedly at $p < .05$, and values do not share a common superscript (a, b, c, d) (DMRT).

nuclei, indicating living cells. However, UA-treated KB (10, 20, and 30 μM) cells exhibited orange to indicate early apoptosis and red-stained fragmented nuclei to show late apoptosis throughout 24 h (Figure 5).

3.5 | Effect of UA induces nuclear condensation in KB cell line

The DAPI nuclear staining technique was used to observe apoptotic cell death morphology. It was proposed that programmed cell death in KB cells is dose-dependent, as shown in (Figure 6). KB cells exhibited increased permeability levels of DAPI, resulting in apoptotic bodies appearing at the nuclear level and compacted chromatin. The apoptotic analysis revealed that UA-treated KB cells (10, 20, and 30 μM) increased the percentage of apoptotic cells by 14.28%, 27.21%, and 47.23%, respectively. The KB cells showed fragmented and compressed nuclei, indicating that UA-induced programmed cellular death through an apoptotic progression. Therefore, UA is recommended for inducing programmed cell death by initiating an apoptotic procedure and contracting nuclei in KB cells.

3.6 | UA prevents DNA damage by comet assay

Next, we evaluated the effectiveness of the comet assay for detecting DNA damage caused by UA-induced oxidative stress in KB cells. The results showed (Figure 7) a significant increase in DNA damage in KB cells treated with different concentrations of UA (10, 20, and 30 μM). In contrast, untreated KB cells showed no significant DNA strand breakage. The graphical representation of the data revealed an apparent increase in the percentage of tail DNA in differentiating between untreated and UA-treated KB cells.

3.7 | Effect of UA on tumor suppressor, antiapoptosis, proapoptosis, and inflammatory protein expression in KB cells

Western blot analysis was used to investigate the effect of UA treatment on apoptotic protein expressions. (Figure 8A,B) shows that treatment with UA significantly downregulation of Bcl-2 expression and the upregulation of cytochrome-c, p53, Bax, caspase-9, and 3 compared with untreated KB cells. In contrast, UA treatment significantly increased the levels of proapoptotic mediator expression. Our findings demonstrate that UA inhibited KB cell growth by mitochondrial-mediated inducing apoptosis signaling pathways. Subsequently, we investigated how UA affects the expression of inflammatory markers in KB cells. Western blot results (Figure 9A,B) show that UA-treated KB cells significantly decreased the expression of inflammatory proteins such as NF-κB, TNF-α, and IL-6 compared with untreated KB cells. The results suggest that

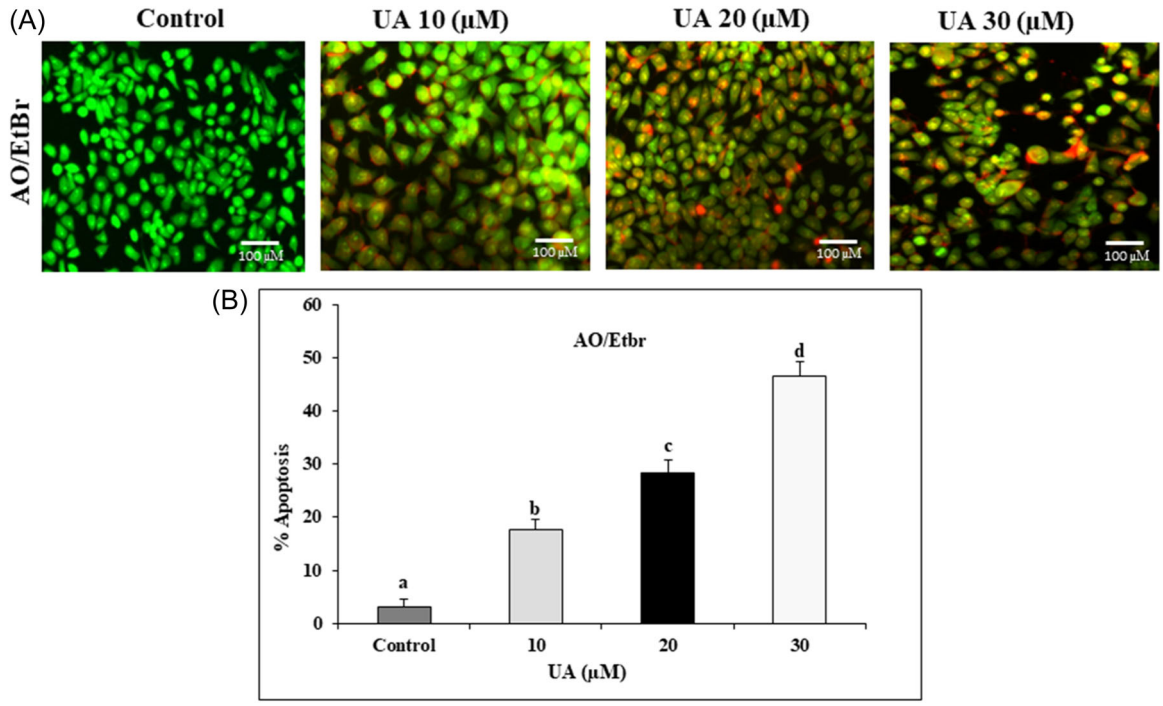

FIGURE 5 Effect of UA on apoptosis using AO/EtBr. (A) Dual staining found apoptotic morphological characteristic alterations in UA-treated KB cells. The microphotographs were taken with a fluorescence microscope (×20). (B) The percentage of apoptotic cells in UA-treated KB cells was measured. All values were expressed as means ± SD based on the findings of triplicate ($n = 3$) experiments performed in each group. The data change meaningfully at $p < .05$, and the values do not share a common superscript (a, b, c, d) (DMRT).

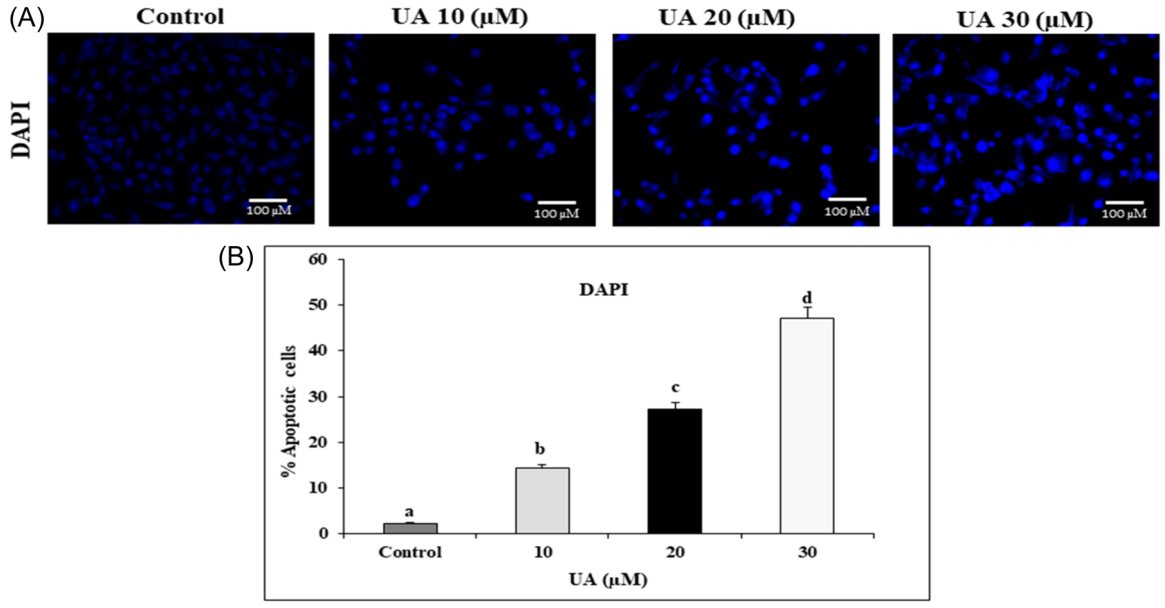

FIGURE 6 Effect of UA-treated KB-induced apoptotic cells was characterized by the DAPI staining method. (A) DAPI staining shows apoptotic cells with membrane blebbing and nuclear fragmentation in UA-treated KB cells. Apoptotic morphological characteristics were observed under a fluorescence microscope (×20). (B) Graphical representation of the percentage of apoptotic cells in UA-treated KB cells was measured. All value was expressed as mean ± SD, and all experiment data were analyzed in triplicate ($n = 3$). The results do not stake similar superscripts mentioned as (a, b, c, d), and values are not different statistically from vehicle control ($p < .05$) (DMRT).

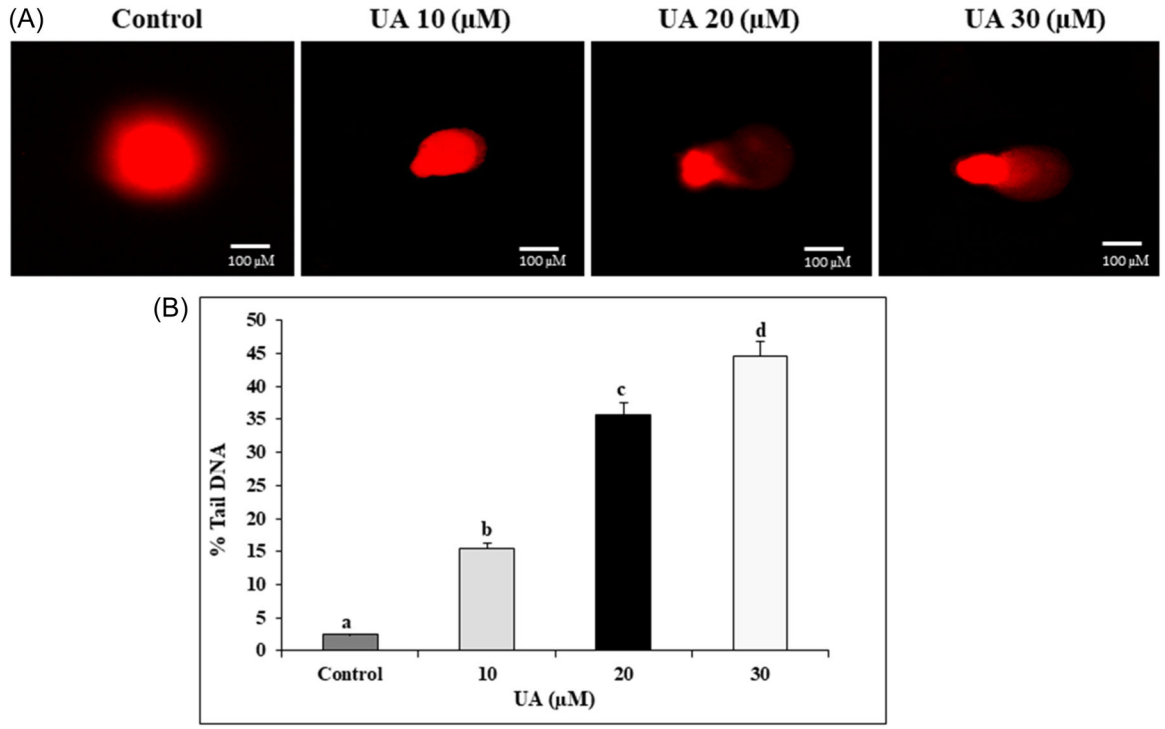

FIGURE 7 (A) The standard fluorescent microscopic image of UA-treated KB comet cells (×20) following alkaline gel electrophoresis is displayed due to DNA damage. Alkaline single-cell gel electrophoresis was used to electrophorese the cell lysate and identify DNA strand breakage using ethidium bromide (EtBr) staining. (B) The percentage of tail DNA shows DNA damage in UA-treated KB cells, was calculated by CASP software. All experiment was performed in triplicate ($n = 3$), and the data were presented as mean ± SD for each value. The values are not dissimilar statistically from the control vehicle ($p < .05$).

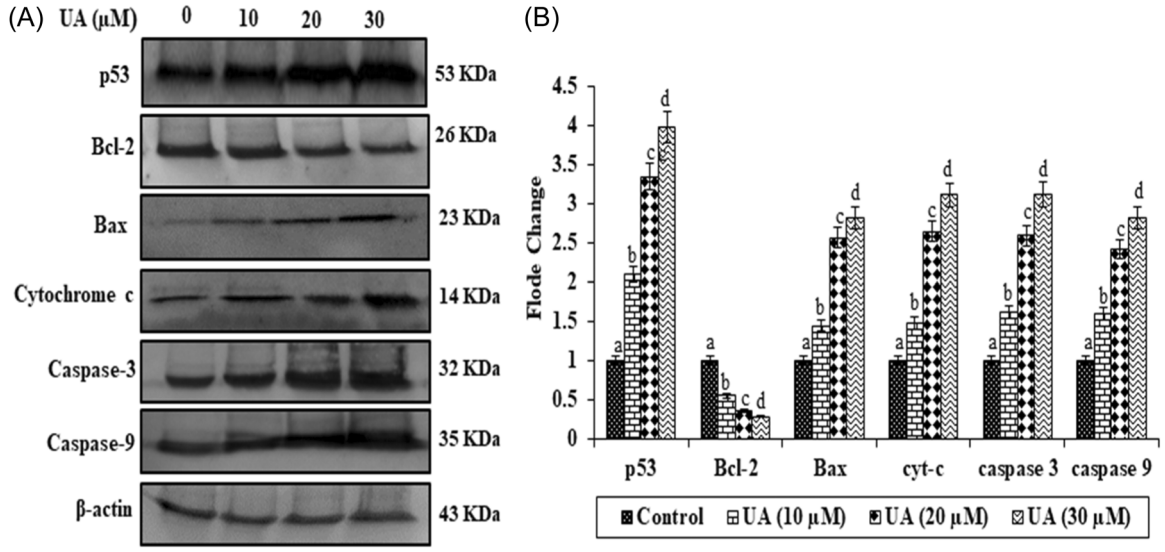

FIGURE 8 Effects of UA on p53, Bcl-2, Bax, cytochrome c, caspase-3, and caspase-9 apoptotic protein expression in KB cells. (A) The apoptotic protein band strengths were measured using densitometry and standardized to the appropriate control β-actin. (B) The comparative protein expression of fold variations in Western blots is depicted in a representative graph. All data from triplicate ($n = 3$) experiments are expressed as the ratio of the target protein to β-actin as the means ± SD. The values that change pointedly at $p < .05$ between groups do not share a common superscript letter (a–d) (DMRT).

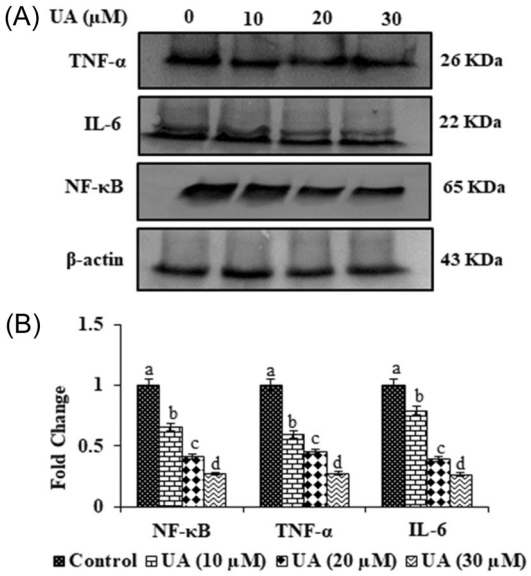

FIGURE 9 Effect of UA on inflammatory proteins IL-6, NF-κB, and TNF-α expression in KB cells. (A) Immunoblotting analysis of inflammatory proteins NF-κB, TNF-α, and IL-6, band concentrations were measured by a densitometer and regularized to the control loading β-actin. (B) A representative graph depicts the qualified expression of protein fold alterations in Western blots. All data from triplicate ($n = 3$) experiments are expressed as the ratio of the target protein to β-actin as the means ± SD. The values that diverge meaningfully at $p < .05$ between groups do not share a common superscript letter (a–d) (DMRT).

UA-treated KB cells inhibit NF-κB activation by induced regulation of pro-inflammatory cytokines response in KB cells.

4 | DISCUSSION

Chemoprevention is a promising approach for controlling cancer numbers and creating new chemotherapeutic drugs.[39] Lichens' secondary metabolites could be significant in cancer treatment since they are less toxic than regular chemotherapeutic drugs like tamoxifen and paclitaxel.[40] Many studies have reported that numerous lichens possess antitumor activity against different cancer cells, such as colon carcinoma (HT-29), hepatocellular carcinoma (Hep-G2), and breast cancer cell line (MCF-7).[41–43] Previous studies have shown that UA has numerous biological and pharmacological activities. These include exhibiting antitumor activity on various cancer cells, such as prostate cancer cells (LNCaP), breast cancer cells (SK-BR-3), hepatocarcinoma cells (SNU-449), and human gastric cancer cells (AGS).[44–47] Recently, many researchers have focused on identifying novel candidate molecules that can potentially suppress the carcinogenic process by inducing apoptosis.[48] In this study, we aimed to investigate how the KB cell line is affected by UA. We found that UA inhibited cell growth, and inflammation and induced apoptosis through the mitochondrial-mediated pathway, and inhibited the NF-κB expressions. Yurdacan et al. (2019) recently has been demonstrated that combined treatment of UA and Sorafenib (SOR), an anticancer compound, produces a synergistic anticancer effect in HCC cell lines. In addition, the combination of SOR and UA

significantly increased the cytotoxic effect on HCC cells and induced apoptotic cell death.[49] In another previous study, the authors suggested that the UA has more potential cytotoxicity effects and antitumor activities than other lichen secondary metabolites substances, including parietin and atranorin.[50] Therefore, Our present study revealed that the KB cell line exposed to UA had a strong cytoprotective effect in a dose-dependent manner. We found that the concentration of IC_{50} value (30 μM) of UA had a dose-responsive impact in KB cells. Furthermore, we also observed that normal human gingival fibroblast cells (HGF-1) exposed to UA up to 50 μM showed moderate toxicity. In addition, UA has exhibited average toxicity levels without any damage after 24 h in noncancerous breast epithelial cells (MCF-12A).[51]

Oxidative stress can cause radical oxidative damage to macromolecules by creating an imbalance in the production and/or removal of reactive oxygen radicals.[52] One of the main consequences of oxidative stress is lipid peroxidation, caused by the frequent production of free radicals.[53] Many studies have demonstrated that reduced glutathione (GSH) and glutathione peroxidase (GPx) are effective in various cancer-related physiological procedures, such as cell differentiation, proliferation, and apoptosis.[54] Previous studies have reported that UA-containing lichens of *usnea longissimi* can significantly decrease lipid peroxidation and increase the levels of SOD, GPx, and GSH, enhancing the antioxidant status and gastroprotective effects in albino-Wistar rats.[55] Furthermore, UA has been reported to down-regulate ROS generation, decrease LPO levels, and increase antioxidant markers in human lymphocyte cell lines.[56]

Similar to the present study, we found that UA-treated KB cells had an increased lipid peroxidation marker, as shown by the upregulated TBARS level and decreased antioxidant enzyme status (SOD, CAT, GPx, and GSH) compared with untreated KB cells. Therefore, we confirmed that UA's antioxidant and free radical scavenging properties may be responsible for the induced ROS and peroxidation status. Battista et al. made similar observations about UA, a chiral molecule with antioxidant properties. There are two enantiomers of UA, which differ by the position of the angular methyl group at C (9b). This difference is linked to the ability of UA, a lichenic secondary metabolite, to reduce hydroxyl radicals, scavenge peroxyl radicals, and decrease nitrite formation.[57] Several critical biological processes, including cell proliferation, transcription factor activation, gene expression, and differentiation, have been linked to the presence of ROS.[58] Some lichen's secondary metabolites like parietin, atranorin acid, gyrophoric acid, and UA have been shown to increase ROS formation to inhibit human cancer cell growth.[59] In this study, we measured the levels of intracellular ROS formation with DCFH-DA staining in both untreated and UA-treated KB cells. It is essential to determine whether UA increases ROS formation in KB cells. The results showed that UA-treated KB cells had higher ROS levels than untreated cells, indicating that UA may induce apoptosis by increasing ROS levels.

During the apoptotic pathway, several important events occur that are controlled by an activator. This includes changes in electron transport, loss or damage of MMP, and the release of caspase activators.[60] Alterations to MMP are indicative of the early stages of apoptosis. Cancer cells have lower resting membrane potentials than normal cells.[61] In this study, MMP levels were measured by observing the fluorescence of a dye called Rh-123 in KB cells treated with different concentrations of UA. The results showed that UA caused a significant depolarization of MMP, which was revealed by an increase in the fluorescence intensity of Rh-123 compared with the untreated control group. ROS production likely caused this depolarization, which promotes mitochondrial membrane permeability and ultimately leads to apoptosis. Previous reports have shown that UA can prevent apoptosis in A549 cells by depolarizing mitochondrial potential and increasing PARP cleavage, which may activate the caspase pathway.[62] During apoptosis, the nuclear morphology of cells undergoes changes that can be detected through dual staining using a fluorescent DNA-binding dye, such as AO/EtBr. Nuclear fragmentation, shrinkage, blebbing, and condensation are signs of morphological changes that occur during apoptosis.[63] Our research has shown that UA induced multiple stages of apoptosis in KB cells, as evidenced by bright green nuclei in the early stages and orange and red nuclei indicating late-stage and necrotic state apoptosis cells, respectively. Therefore, UA-induced anti-proliferation in KB cells was achieved by inducing apoptosis. A study by Yang et al. used a blue fluorescent DNA-binding dye called DAPI to distinguish between viable and dead cells in KB cells and examine the morphology of apoptotic nuclei. Ideally, the dye should involve apoptosis and necrosis.[64] Similar results were observed that UA-treated KB cells exhibited round, high nuclei free of condensation or fragmentation after 24 h of DAPI staining, while the majority of the nuclei in the UA-treated KB cells were condensed and fragmented as expected in cases of cell death.

Cancer research for developing therapeutics mainly focuses on killing cancer cells by targeting the genetic material of DNA.[65] High levels of ROS can cause severe damage to cells by inducing various metabolic changes, including oxidative DNA damage, which usually occurs as SSBs (single-strand breaks) and DSBs (double-strand breaks for purine and pyrimidine bases).[66] Many studies suggest that phytochemicals can induce programmed cell death in cancer cells by increasing ROS, which ultimately causes oxidized DNA damage. In this current study, we observed that KB cells treated with UA showed a concentration-dependent increase in nuclear fragmentation and tail DNA.

The tumor suppressor gene p53 protects cells against various biological reactions. It can either cause cell cycle arrest to encourage DNA repair and endurance or programmed cell death through apoptosis.[67] Cytosolic wild-type p53 can also enter into the mitochondria and control the transcription of apoptosis by down-regulating the expression of the antiapoptotic protein Bcl-2 and upregulating the presentation of the proapoptotic protein Bax, which induces membrane permeabilization and apoptosis.[68] Our western blot analysis results indicate that KB cells treated with UA (30 μM) modified the Bax/Bcl-2 ratio, favoring apoptosis by downregulating the expression of Bcl-2 and upregulating the expression of Bax protein.

In cancerous conditions, the production of ROS affects the permeability of the mitochondrial membrane. This causes the release of free Cytochrome-c into the cytosolic environment, which triggers the activation of caspase-3 via caspase-9 intermediation. ROS levels are reduced, which may decrease the free Cyt-c points inside the mitochondria, thereby inhibiting apoptosis.[69] Wu et al.[70] suggested that UA-induced apoptosis in HCT 116 cells inactivates mTOR and activates JNK, leading to the induction of autophagy. In addition, we also observed that UA induces apoptosis through the mitochondrial pathway as a result of an increase in the Bax/Bcl-2 ratio which leads to the release of the apoptogenic protein cytochrome c into the cytosol and the activation of caspase cascade 3 and −9.

NF-κB is a transcription factor that controls the transcription of DNA, cytokine production, and cell survival. In the context of cancer, NF-κB can promote tumor growth and protect cancer cells from apoptosis, which is the process of programmed cell death.[71] This, in turn, controls the expression of various pro-inflammatory cytokines and inflammatory proteins such as IL-6, iNOS, TNF-α, and COX-2, in all types of cancer, promoting cancer progression and chronic inflammation. Therefore, these proteins are used to estimate the prognosis of various malignancies, evaluate treatments, and assess inflammation.[72]

Previous research has demonstrated that a wide range of phytochemicals prevent inflammatory markers and cytokines in both in vitro and in vivo experiments.[73] Inflammatory markers, such as COX-2 TNF-α, IL-6, and NFκB, trigger numerous inflammatory responses by cancer cells in OSCC.[74] In this study, treatment with UA significantly reduced the pro-inflammatory cytokine marker expression of TNF-α and IL-6 in KB cells.

In addition, NF-κB regulates several genes that encode proteins implicated in inflammation and cancer progression.[75] Hence, we found that UA could attenuate NF-κB expression and therefore prevent inflammatory responses in KB cells. Similarly, Huang et al.[76] reported that UA inhibits inflammatory markers such as TNF-α, iNOS, COX-2, IL-1β, and IL-6 expression by suppressing the NF-κB in the RAW264.7 cell line. Finally, our hypothesize that UA treated with KB cells regulates NF-κB activation by scavenging ROS generation and oxidative DNA damage, resulting in the TNF-α and IL-6 downregulation, therefore which aligns with the roles attributed to it in the suppression of inflammation response and induction of apoptosis in KB cells. It's essential to explore the distinct cellular mechanism reactions to UA for each cancer cell line to investigate the molecular processes related to cell death or survival.

5 | CONCLUSION

In this study, we have provided clear evidence that UA inhibits the growth and proliferation of KB cells by generating ROS-dependent mitochondrial-mediated apoptosis. Moreover, UA activates tumor suppressor protein p53, which induces DNA damage in KB cells. UA also triggers apoptosis in KB cells by upregulating the expression of proapoptotic mediators such as Bax, Cytochrome-c, Caspase-3, and Caspase-9 activity and downregulating the protein expressions of Bcl-2. Furthermore, UA mitigates inflammatory marker expressions in KB Cells. UA effectively inhibits cell growth and inflammation and induces apoptosis by impeding the NF-κB activation by modulating pro-inflammatory cytokine marker expression in KB cells. These findings suggest that UA may be an effective therapeutic agent for managing oral cancer.

AUTHOR CONTRIBUTIONS

Study concept and design: Suresh Kathiresan. *Data acquisition*: Theerthu Azhamuthu. *Data analysis and interpretation*: Suresh Kathiresan, Theerthu Azhamuthu, Ilanchitchenni Senkuttuvan, Nihal Ahamed Abulkalam Asath, Pugazhendhi Ravichandran, and Rajeswari Vasu. *The draft manuscript preparation*: Suresh Kathiresan and Theerthu Azhamuthu. *Final version of the manuscript editing*: Suresh Kathiresan and Theerthu Azhamuthu. All authors reviewed and approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data will be available from the authors on reasonable request.

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