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Original Research Article

Crebanine Induces Apoptotic Cell Death in Human Oral Cancer KB Cells via Upregulating Pro-apoptotic Markers and Downregulating JAK2/STAT3 Pathway

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Abstract

Background: Oral squamous cell carcinoma (OSCC) signifies a major global health issue, defined by its destructive nature and often delayed diagnosis, leading to suboptimal prognoses. The global case and mortality rates of OSCC continue to increase, especially among younger populations.
Objectives: This work aims to study the anti-cancer properties of crebanine on oral cancer KB cells by inducing apoptosis via suppressing PI3K/AKT/mTOR and JAK-2/STAT-3 pathways.
Materials and Methods: Crebanine at various doses (0.5–25 μM) was evaluated for its *in vitro* free-radical scavenging properties, including 2,2-diphenyl-1-picrylhydrazyl (DPPH), peroxyl, and superoxide radicals. The impact of crebanine on the growth of oral cancer KB and normal Vero cells was evaluated with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. The mitochondrial membrane potential (MMP) level in untreated and crebanine-treated KB cells was assessed using a fluorescent staining assay. The oxidative stress markers, apoptosis-related proteins, and PI3K/AKT/mTOR and JAK-2/STAT-3 pathway proteins were evaluated in untreated and crebanine-treated KB cells.
Results: The findings of the free radical scavenging experiments demonstrated the *in vitro* antioxidant properties of crebanine. The findings of the MTT experiment revealed that crebanine considerably inhibited the viability of KB cells without significantly affecting the normal Vero cells. The crebanine treatment reduced the MMP level in KB cells, as demonstrated by the findings of the fluorescent staining assay. The crebanine-treated KB cells exhibited elevated thiobarbituric acid reactive substances (TBARS) levels, alongside decreased glutathione (GSH) and superoxide dismutase (SOD) levels. Furthermore, crebanine treatment enhanced the pro-apoptotic proteins Bax and caspase-3/9 levels, while concurrently inhibiting the PI3K/AKT/mTOR and JAK-2/STAT-3 signaling protein levels in KB cells.
Conclusion: The current study demonstrates that crebanine treatment can impede cellular proliferation, trigger oxidative stress, and facilitate apoptosis in KB cells via downregulating PI3K/AKT/mTOR and JAK-2/STAT-3 pathways.

Keywords

JAK-2/STAT-3 pathway, oral cancer, crebanine, oxidative stress, apoptosis

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Introduction

Oral cancer, also known as oral cavity cancer, refers to any cancerous growth located in the mouth, encompassing the lips, tongue, gums, inner lining of the cheeks, and the hard or soft palate. These malignant neoplasms can originate as primary lesions within the oral cavity, metastasize from distant sites, or extend from adjacent anatomical structures. Oral squamous cell carcinoma (OSCC) signifies a foremost global health issue, defined by its aggressive nature and often delayed diagnosis, leading to suboptimal prognoses (Sung et al., 2021). Globally, it stands as the sixth most common

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cancer, with significant incidence and mortality rates observed particularly in South-Central Asia. This prevalence is notably higher in developing countries, where oral and pharyngeal cancers can account for up to 25% of all malignant tumors. Despite its accessibility for visual examination, most OSCCs, which make up over 90% of oral cancers, are not diagnosed until the advanced stages, contributing to a persistently low 5-year survival rate of nearly 62% (Bray et al., 2024). This late-stage diagnosis highlights the critical need for more public awareness campaigns regarding risk factors and early signs, alongside improved clinical detection strategies. The substantial global incidence of oral cancer is predominantly influenced by lifestyle factors and environmental exposures (Miranda-Filho & Bray, 2020).

Several complex factors contribute to the etiology of oral cancer, ranging from well-established environmental exposures to genetic predispositions and viral infections. Among these, lifestyle choices, particularly the consumption of tobacco and alcohol, are widely recognized as primary etiological agents. The high morbidity and mortality connected with oral cancer further emphasize the urgency of understanding its multifaceted origins to develop effective prevention and intervention strategies (Eloranta et al., 2024). Beyond these conventional risk factors, an array of other elements, including chronic infections, poor oral hygiene, and nutritional deficiencies, also participate in the pathogenesis of OSCC. Current treatment options, encompassing surgery, radiotherapy, and chemotherapy, often present considerable challenges, including significant adverse effects and the development of resistance. While surgery is often the primary intervention, especially for early-stage lesions, radiotherapy and chemotherapy are also widely employed, either as standalone treatments or in combination, depending on the specific clinical presentation and stage of the disease (Ribeiro et al., 2022). However, these conventional therapies are frequently connected with severe side effects, such as extensive tissue damage, xerostomia, and difficulty in mastication and speech, significantly impacting the patient's quality of life. Furthermore, the systemic nature of chemotherapy agents often leads to a narrow therapeutic index, increasing the potential for off-target toxicities and limiting their long-term applicability (Pfister et al., 2020).

The arduous nature of existing treatments emphasizes the urgent need for innovative, safe, and effective alternative therapeutic agents to improve patient outcomes and minimize treatment-related morbidities. Despite the demonstrated efficacy of chemotherapy in advanced stages of cancer, its administration frequently requires high doses to achieve suitable concentrations at target tissues, leading to systemic toxicity and adverse effects that compromise patient well-being (Zittel et al., 2022). This often results in significant harm to healthy cells, leading to a compromised quality of life for patients. The continuous search for more efficient and tolerable anti-cancer treatments is thus critical, especially given that many patients develop resistance to targeted therapies over time due to tumor heterogeneity and complex mutagenesis (Kröplin & Reppenhagen, 2023).

Crebanine is a naturally occurring alkaloid compound found in the *Stephania*, a plant genus that includes several species. It has already been reported that crebanine has shown various biological effects, including anti-inflammatory and analgesic effects (Cui et al., 2022), cognition-enhancing effects (Rojsanga et al., 2012), and neuroprotective (Yang et al., 2023) activities. Furthermore, it has also been demonstrated that crebanine induced apoptosis in several cancer cells, including hepatocellular carcinoma (Tan et al., 2023), gastric cancer (Wang et al., 2020), lung cancer (Yodkeeree et al., 2014), and glioblastoma (Yeh et al., 2024). Nonetheless, its anti-cancer efficacy against oral cancer remains unexamined. Therefore, the current study aims to assess the anti-cancer effects of crebanine on oral cancer KB cells by inducing apoptosis via suppressing the PI3K/AKT/mTOR and JAK-2/STAT-3 pathways.

Materials and Methods

Chemicals

The primary chemicals and reagents, including crebanine (purity: 95%), Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), and antibiotics, were purchased from Sigma–Aldrich, USA. The biochemical marker levels were assessed using kits obtained from Abcam, MyBioSource, and Elabscience, USA, respectively.

In Vitro Antioxidant Studies

Ferric Reducing Antioxidant Power (FRAP) Analysis

The FRAP study was conducted following the previously defined protocol (Benzie & Strain, 1996). Diverse concentrations of crebanine (0.5, 1, 2.5, 5, 7.5, 10, and 25 μM) were combined with 1 mL of FRAP solution, a 300 mM acetate buffer, 10 mM TPTZ reagent, and 20 mM ferric chloride. 200 μL of the reaction mixture was deposited on a microtiter plate and incubated for 10 min. Later, the resultant product developed in the reaction medium was assessed at a wavelength of 593 nm.

2,2-Diphenyl-1-Picrylhydrazyl (DPPH) Assay

The influence of crebanine on DPPH radical scavenging was examined using the previously published technique (Kedare & Singh, 2011). A 150 μL of DPPH reagent was dissolved in ethanol (0.25 mM) and combined with different doses of crebanine (0.5–25 μM) for 30 min at 37°C. Subsequently, the absorbance was measured at a wavelength of 515 nm.

Superoxide Radical Scavenging Assay

The effects of crebanine on superoxide radical scavenging were investigated using earlier published procedures (Shimada et al., 1992). 10 μL of chemiluminescent reagent was combined with different concentrations (0.5–25 μM) of crebanine

and 80 µL of xanthine oxidase solution. The 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer was employed to prepare the control solution. Subsequently, the solution was analyzed using a luminometer following the addition of 200 µL of hypoxanthine solution (0.72 mM). The scavenging effect of crebanine was analyzed for 10 min at 10-s intervals with a luminometer.

Analysis of Oxygen Radical Absorbance Capacity (ORAC)

The ORAC level of crebanine at different concentrations (0.5–25 µM) was investigated by assessing its peroxyl radical scavenging ability. The test was conducted in triplicate utilizing the commercial kit (ab233473) and employing the manufacturer's specifications (Abcam, USA).

In Vitro Assays

Cell Culture

The oral cancer KB cells and non-malignant Vero cells were obtained from ATCC, USA, and cultured in DMEM with 10% FBS in an incubator with 5% CO_2. The cultured cells were harvested upon achieving 80% confluency and utilized for additional treatments, fluorescence staining, and biochemical analyses.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

The effects of crebanine at several doses (0.5–25 µM) on the proliferation of normal Vero and oral cancer KB cells were examined using the MTT test. Each cell type was planted separately in the 96-well plate for 24 h. Vero and KB cells were subsequently exposed to various dosages (0.5, 1, 2.5, 5, 7.5, 10, and 25 µM) of crebanine for 24 h. Subsequent to incubation, 20 µL of MTT and 100 µL of DMEM were added to each well for 4 h. 100 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan precipitates. The absorbance was ultimately taken at 570 nm.

Rhodamine-123 (Rh-123) Staining

The mitochondrial membrane potential (MMP) level in control and crebanine-treated KB cells was assessed using Rh-123 staining. Cells were grown on a 24-well plate for 24 h prior to exposure to 7.5 and 10 µM of crebanine for 24 h at 37°C. Later, the wells were treated with Rh-123 (10 µg/mL) dye for 30 min, and the MMP levels were assessed using a fluorescence microscope.

Analysis of Oxidative Stress Marker Levels

The untreated and crebanine-treated KB cells were isolated and lysed using the lysis buffer. The cell lysates were centrifuged to extract the supernatant, which was subsequently used for biochemical evaluations. The levels of glutathione (GSH; #ab239709), superoxide dismutase (SOD; #ab65354), and thiobarbituric acid reactive substances (TBARS; #ab233471) were assessed using corresponding test kits as per the manufacturer's specification (Abcam, USA).

Evaluation of Apoptotic Protein Levels

The concentrations of Bax (#E-EL-H0562), Bcl-2 (#E-EL-H0114), and caspase-3 (#E-EL-H6282)/9 (#E-EL-H0663) in the cell lysates of untreated and crebanine-treated KB cells were analyzed utilizing commercially available kits, following the manufacturer's specifications (Elabscience, USA).

Analysis of PI3K/AKT/mTOR and JAK-2/STAT-3 Protein Levels

The concentrations of PI3K (#MBS9303190), AKT (#MBS9518778), mTOR (#MBS030902), JAK-2 (#MBS0 46985), and STAT-3 (#MBS9501569) were assessed in the cell lysates of untreated and crebanine-treated KB cells utilizing the appropriate test kits, following the specifications outlined by the manufacturer (MyBioSource, USA).

Statistical Analysis

The data were presented as mean ± standard deviation (SD) of triplicates following analysis with GraphPad Prism. One-way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) were used to assess the variations in the treatment group values, employing a significant threshold of $p < .05$. In this study, the treatment groups served as the independent variable, while the measured cellular responses and biochemical parameters were considered the dependent variables.

Results

Effect of Crebanine on the In Vitro Free Radical Scavenging Activity

The various free radical scavenging tests were conducted to assess the *in vitro* antioxidant capabilities of crebanine, with the results presented in Figure 1. Crebanine treatment, at diverse dosages, significantly reduced free radical levels. Specifically, crebanine at dosages of 0.5–25 µM significantly ($p < .05$) suppressed the generation of several free radicals, including DPPH, peroxyl, and superoxide radicals. The capacity of crebanine to scavenge free radicals *in vitro* was evidenced by a reduction in the levels of DPPH, peroxyl, and superoxide radicals following treatment with elevated doses

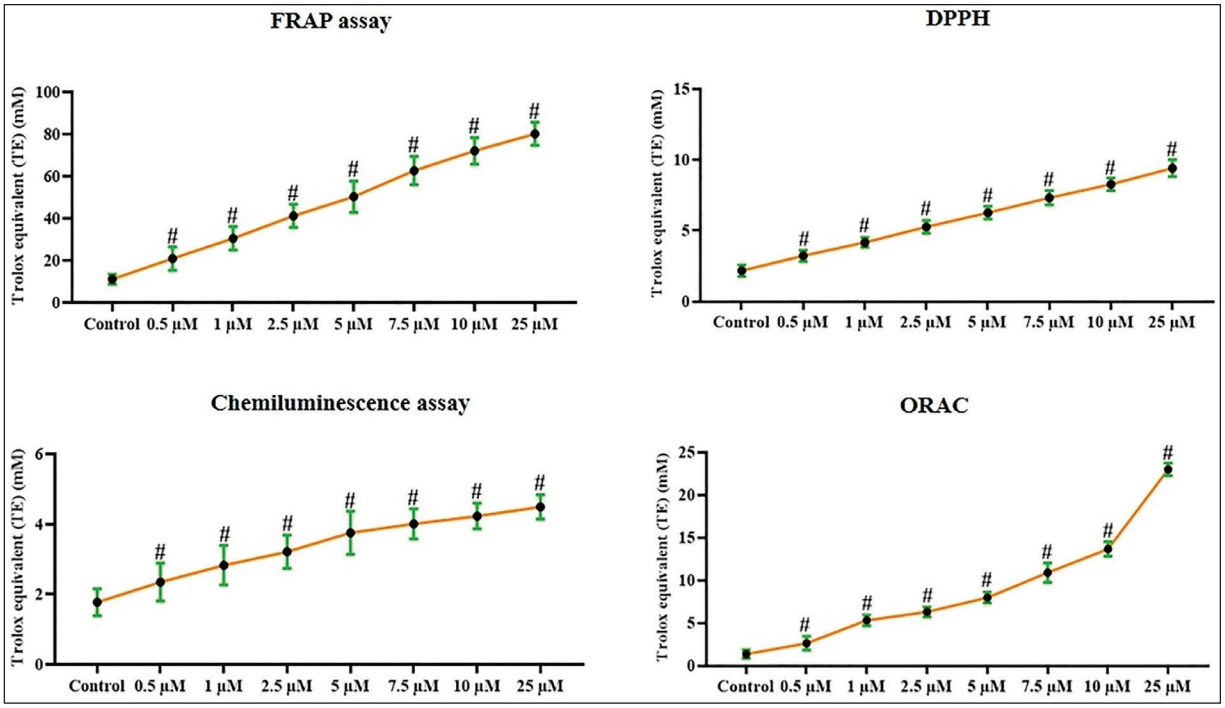


Figure 1. Effect of Crebanine on the *In Vitro* Free Radical Scavenging Activity. Data were Illustrated as Mean ± Standard Deviation (SD) of Triplicate Measurements. The Results were Analyzed Using GraphPad Prism with One-way Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT) Analyses.

Note: An asterisk (#) denotes significance at $p < .05$ from the control group.

of crebanine. These findings further indicate that crebanine possesses remarkable *in vitro* antioxidant capabilities.

Effect of Crebanine on the Growth of Vero and KB Cells

The cytotoxic level of crebanine against both Vero cells and oral cancer KB cells was assessed, with the results presented in Figure 2(A). Our results indicated that crebanine treatment at several dosages (0.5, 1, 5, 7.5, 10, and 25 µM) significantly ($p < .05$) reduced the proliferation of KB cells. The growth of Vero cells was unaltered by the similar dosages of crebanine. At elevated concentrations of crebanine, there was a slight reduction in Vero cell growth. The findings illustrate the cytotoxicity of crebanine on KB cells. The IC_{50} dosage of crebanine for KB cells was determined to be 7.5 µM; hence, the 7.5 and 10 µM were used for the remaining *in vitro* tests as the IC_{50} and high dosage, respectively.

Effect of Crebanine on the MMP Level in the KB Cells

The effect of crebanine on the MMP levels in KB cells was evaluated using Rh-123 staining. Figure 2(B) demonstrates that the control cells emit increased green fluorescence, signifying a normal and undamaged MMP. In contrast, the KB cells, following exposure to 7.5 and 10 µM of crebanine for 24 h, exhibit dull green fluorescence, which signifies that crebanine can reduce the MMP status in KB cells.

Effect of Crebanine on Oxidative Stress Markers in KB Cells

Figure 3(A) illustrates the concentrations of SOD, TBARS, and GSH in both untreated and crebanine-treated KB cells. Elevated concentrations of SOD and GSH, together with reduced TBARS levels, were seen in the untreated control cells. Concurrently, KB cells treated with 7.5 and 10 µM of crebanine exhibited significantly ($p < .05$) elevated TBARS levels and reduced GSH and SOD concentrations in comparison to the control. The data indicate that crebanine promotes oxidative stress in KB cells.

Effect of Crebanine on Apoptotic Protein Levels in KB Cells

The apoptotic protein levels of untreated and crebanine-treated KB cells were analyzed, with the results presented in Figure 3(B). The concentrations of Bax and caspase-3/9 were reduced, whereas the Bcl-2 level was elevated in the control cells. However, the administration of 7.5 and 10 µM of crebanine to KB cells significantly ($p < .05$) elevated the Bax and caspase-3/9, while concurrently diminishing the Bcl-2 level when compared with the control. Consequently, it was

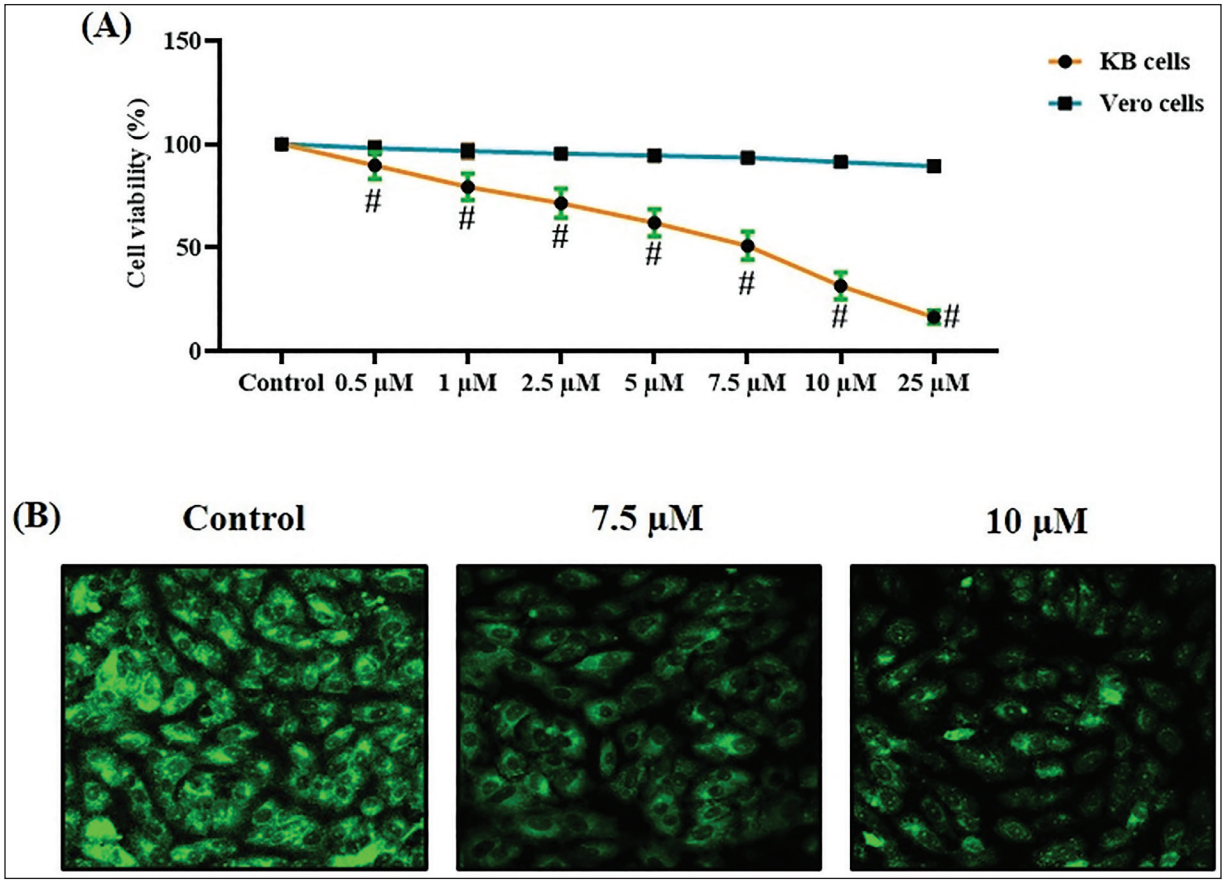


Figure 2. Effect of Crebanine on the Viability of Vero and KB Cells and the Mitochondrial Membrane Potential (MMP) Level in KB Cells. Data were Illustrated as Mean ± Standard Deviation (SD) of Triplicate Measurements. The Results were Analyzed Using GraphPad Prism with One-way Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT) Analyses.

Note: An asterisk (#) denotes significance at $p < .05$ from the control group. The KB cells had a reduced MMP level, as shown by the diminished green fluorescence compared to untreated control cells following treatment with 7.5 and 10 µM of crebanine. (A) 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay; (B) MMP analysis.

evident that crebanine can trigger apoptosis in KB cells by elevating pro-apoptotic proteins.

Effect of Crebanine on JAK-2/STAT-3 and PI3K/AKT/mTOR Pathway Proteins in the KB Cells

The impact of crebanine on the JAK-2/STAT-3 and PI3K/AKT/mTOR signaling protein levels in KB cells was analyzed, with findings presented in Figure 4. Following treatment with 7.5 and 10 µM of crebanine, the levels of AKT, mTOR, PI3K, JAK-2, and STAT-3 were significantly ($p < .05$) reduced in KB cells, which validated that crebanine downregulated the JAK-2/STAT-3 and PI3K/AKT/mTOR pathways in KB cells.

Discussion

OSCC represents a predominant global health issue, defined by high morbidity and mortality rates that seriously affect patients' quality of life. This aggressive epithelial neoplasm, predominantly OSCC, accounts for over 90% of all oral malignancies. The complex pathogenesis of oral cancer, driven by an interplay of genetic mutations, epigenetic alterations, and environmental factors, necessitates a deeper understanding to develop potential therapeutic strategies (Tranby et al., 2022). The persistent challenge of late-stage diagnosis highlights the need for reliable, non-invasive diagnostic tools and biomarkers that can facilitate early detection and improve patient outcomes. The global cases and mortality of OSCC continue to increase, especially among younger populations. Moreover, while numerous risk factors have been identified, including tobacco and alcohol consumption, human papillomavirus infection is increasingly recognized as a significant etiological agent, influencing the disease's progression and prognosis (Johnson et al., 2020). Current treatment modalities for oral cancer are often associated with significant morbidity and mortality. These conventional therapies can result in adverse effects, such as disfigurement, dysphagia, and xerostomia, compromising the patient's quality of life.

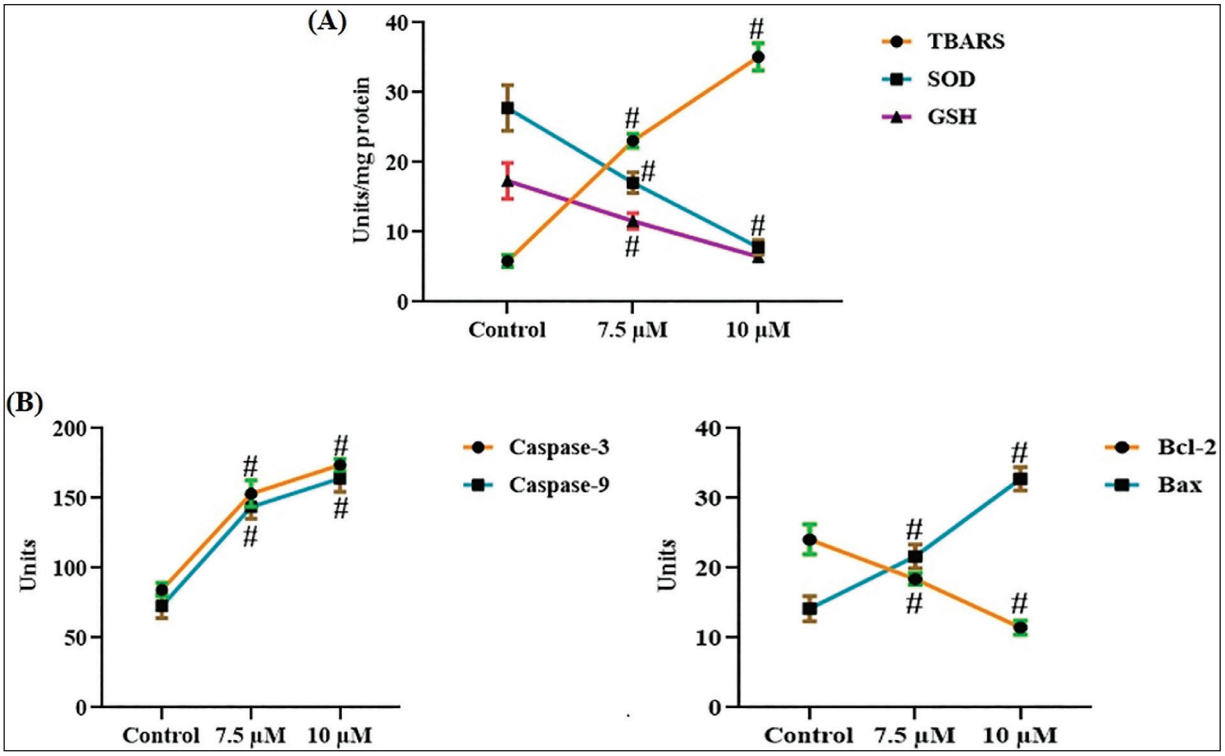


Figure 3. Effect of Crebanine on the Oxidative Stress and Apoptotic Markers in the KB Cells. Data were Illustrated as Mean ± Standard Deviation (SD) of Triplicate Measurements. The Results were Analyzed Using GraphPad Prism with One-way Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT) Analyses.

Note: An asterisk (#) denotes significance at $p < .05$ from control group. (A) Oxidative stress markers (thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD), and glutathione (GSH)); (B) Apoptotic protein levels (Caspase-3/9, Bax, and Bcl-2).

Furthermore, the onset of resistance to conventional therapies poses a major challenge to the treating of oral cancer (Dong et al., 2024). The high recurrence rate and poor prognosis of advanced-stage oral cancer also highlight the need for novel and effective therapeutic methods. Therefore, there is an urgent need to explore potential alternative therapies that can improve treatment outcomes and decrease the burden of oral cancer (Vishwani et al., 2024).

Oxidative stress-mediated apoptosis refers to a process of programmed cell death triggered by an imbalance between the reactive oxygen species (ROS) accumulation and the cell's antioxidant mechanisms. This imbalance leads to cellular damage, deoxyribonucleic acid (DNA) fragmentation, and ultimately, apoptosis. The intricate interplay between ROS and cellular antioxidant defenses plays an essential role in determining the cell fate, especially in the context of cancer therapy (Forman & Zhang, 2021). An imbalance favoring pro-oxidants can lead to oxidative stress, a condition characterized by an excess of ROS, which can damage cellular compartments, ultimately triggering apoptosis. This disturbance in redox homeostasis arises when the production of ROS overwhelms the cell's antioxidant defense mechanisms, leading to significant alterations in biomolecules and contributing to disease pathogenesis. Higher levels of these reactive species can activate signaling pathways that induce programmed cell death, while a modest increase in ROS is involved in cancer cell survival, angiogenesis, and metastasis (Aboelella et al., 2021). This delicate balance is frequently disrupted in cancer cells, which often exhibit elevated basal levels of ROS because of their altered metabolic pathways and increased proliferative demands. This makes targeting redox homeostasis a promising therapeutic strategy against cancer. Such interventions often exploit the unique susceptibility of cancer cells to further oxidative insult, which can sensitize them to damage and subsequent apoptosis (Van Loenhout et al., 2020). The continuous production of intracellular ROS by mitochondria has been implicated in regulating mechanisms such as cancer cell cycle arrest, senescence, and apoptosis. The damage caused by these reactive species can manifest as lipid peroxidation, protein oxidation, and DNA lesions, all of which contribute to the initiation of apoptotic cascades (Hayes et al., 2020).

In cancer cells, oxidative stress-mediated apoptosis can be induced by various mechanisms, including the modulation of lipid peroxidation, as measured by TBARS levels. Elevated TBARS levels indicate increased lipid peroxidation, which

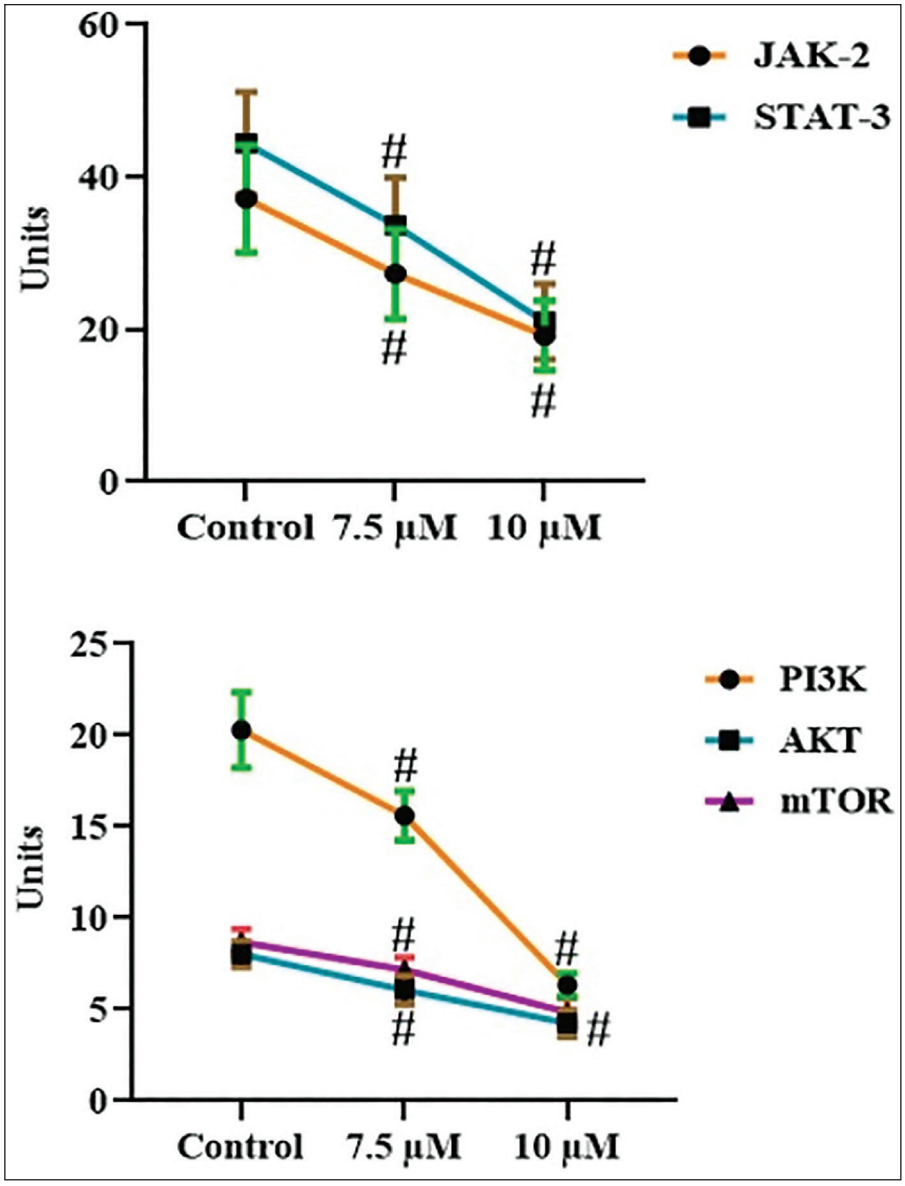


Figure 4. Effect of Crebanine on the PI3K/AKT/mTOR and JAK-2/STAT-3 Pathway Proteins in the KB Cells. Data were Illustrated as Mean ± Standard Deviation (SD) of Triplicate Measurements. The Results were Analyzed Using GraphPad Prism with One-way Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT) Analyses.

Note: An asterisk (#) denotes significance at $p < .05$ from the control group.

can disrupt cellular membranes and trigger apoptosis. Conversely, the activity of antioxidants like SOD plays a crucial role in mitigating oxidative stress. SOD converts superoxide radicals into hydrogen peroxide, thereby reducing oxidative damage (Kuang et al., 2020). GSH levels also play an imperative role in sustaining the cellular redox balance. Reduction of GSH levels can render cells more susceptible to oxidative stress-mediated apoptosis. In tumor cells, the modulation of SOD, TBARS, and GSH can be exploited to induce apoptosis, providing a potential therapeutic strategy for cancer therapy. By targeting these oxidative stress markers, it may be achievable to selectively trigger apoptosis in tumor cells while ignoring normal cells (An et al., 2024). In this work, we found elevated SOD and GSH levels, along with reduced TBARS levels, in the untreated control cells. However, KB cells treated with crebanine demonstrated enhanced TBARS levels and decreased SOD and GSH levels in comparison with control cells. These findings highlight that crebanine promotes oxidative stress, thereby facilitating apoptosis in KB cells.

Apoptosis, a tightly controlled form of programmed cell death, is essential for sustaining tissue homeostasis and for

discarding damaged or malignant cells. This intricate process is characterized by distinct morphological features and can be initiated via two primary signaling cascades: the extrinsic pathway, initiated by death receptors, and the intrinsic pathway, which is mitochondria-dependent and responsive to intracellular stimuli (Singh et al., 2019). Dysregulation of apoptosis is a hallmark of cancer, permitting cancer cells to evade cell death and multiply overwhelmingly. An understanding of the regulatory processes controlling apoptosis and survival pathways in human cancers is essential for developing novel therapeutic methods to overcome treatment resistance. Central to the intrinsic apoptotic pathway are the Bcl-2 family proteins, which include both pro-apoptotic members like Bax and anti-apoptotic members like Bcl-2, intricately balancing cellular life- and death-decisions (Kaloni et al., 2023). The balance between these opposing forces decides the cell's fate, with an overexpression of anti-apoptotic proteins often observed in various malignancies, contributing to therapeutic resistance. This imbalance leads to the evasion of programmed cell death, thereby promoting tumor growth and survival. The subsequent activation of caspases, a family of cysteine-aspartic proteases, is indispensable for the controlled demolition of the cell during apoptosis (Qian et al., 2022). These proteases execute the apoptotic program by cleaving specific substrates, resulting in the characteristic biochemical and morphological alterations related to apoptosis. Furthermore, the intrinsic pathway, pivotal in cellular quality control, culminates in the activation of initiator caspases, like caspase-9, which consequently activate executioner caspases, like caspase-3 (McIlwain et al., 2013). The dysregulation of apoptosis plays a significant role in the onset of several diseases, including cancer, where the evasion of apoptosis is considered a hallmark (de Melo Silva et al., 2024). The present findings exhibited that the levels of Bax and caspase-3/9 were reduced, whereas the Bcl-2 level was elevated in the untreated cells. However, the treatment of crebanine in KB cells resulted in enhanced Bax and caspase-3/9, while subsequently reducing the Bcl-2 levels in comparison to the control. Therefore, it can be suggested that crebanine treatment can trigger apoptosis in KB cells by elevating pro-apoptotic proteins.

The PI3K/AKT/mTOR cascade plays a pivotal role in maintaining fundamental cellular functions such as cell growth and survival, making its dysregulation a hallmark of numerous cancers. Activated PI3K signaling significantly contributes to tumorigenesis across various cancers, highlighting its central role in oncogenic transformation (Miricescu et al., 2020). This complex pathway, characterized by multiple levels of regulation and intricate feedback loops, is a subject of ongoing elucidation, with new regulatory mechanisms continually being discovered through diagnostic studies and clinical trials of targeted therapies. Moreover, the PI3K-AKT cascade is often deregulated in cancers, with genetic alterations leading to enhanced PI3K signaling, which is characteristic of a vast number of malignancies. The hyperactivity of PI3Ks, often resulting from gain-of-function mutations, leads to stimulation of mTOR through AKT, driving crucial mechanisms in tumor development (Thorpe et al., 2015). This dysregulation not only promotes uncontrolled cellular proliferation but also confers resistance to various anti-cancer therapies, highlighting the pathway's critical involvement in therapeutic failure. The aberrant activation of this pathway, particularly the PI3K/AKT axis, fuels critical metabolic reprogramming in cancer cells, shifting their energy production towards aerobic glycolysis. This metabolic shift supports rapid tumor growth and proliferation, enabling cancer cells to thrive even in nutrient-poor microenvironments (Jiang et al., 2020).

The intricate crosstalk between the PI3K/AKT/mTOR axis and the tumor microenvironment further exacerbates cancer progression and therapeutic resistance by influencing immune cell suppression, angiogenesis, and metastatic dissemination. The sustained activation of the AKT cascade, often noted in several tumors, is largely attributed to aberrant regulation by numerous oncoproteins and inactivated tumor suppressors, that intersect this critical signal transduction pathway (Liu et al., 2020). Therefore, understanding the molecular underpinnings of this pathway's dysregulation is paramount for developing effective therapeutic strategies that target its aberrant activity in cancer. This understanding extends to its significant influence on epithelial-mesenchymal transition (EMT), a critical mechanism in tumor invasion and metastasis, further solidifying its role in advanced disease (Hoxhaj & Manning, 2020). Given its pervasive dysregulation in malignancy, the PI3K/AKT/mTOR signaling pathway reveals a crucial therapeutic target for developing novel anti-cancer agents. The complex interplay between the PI3K/AKT/mTOR axis and its microenvironment creates a pro-tumorigenic milieu that facilitates continuous tumor growth and immune evasion (Krencz et al., 2021). The continuous activation of this signaling enables cancer cells to acquire a competitive growth advantage and develop resistance to various therapies, making it a critical focus for targeted interventions. Consequently, the development of PI3K/AKT/mTOR inhibitors has emerged as a hopeful method for cancer treatment, aiming to disrupt the signaling cascade and to impede tumor progression (Gao et al., 2023). The outcomes of this work indicate increased levels of PI3K, AKT, and mTOR proteins in the untreated KB cells. Whereas, the treatment with crebanine considerably decreased the PI3K, AKT, and mTOR proteins in the oral cancer KB cells. Consequently, it was evident that crebanine can downregulate the PI3K/AKT/mTOR axis in KB cells and impede tumor formation.

The JAK/STAT pathway is intricately involved in cellular proliferation, differentiation, and immune responses. This intracellular signaling cascade is crucial for regulating cellular fate and modulating phenotypic modifications, impacting processes from embryonic development to inflammatory responses. Dysregulation of this pathway,

particularly the JAK-2/STAT-3 axis, is often noted in several malignancies, participating in unchecked cell growth and therapeutic resistance (Kiu & Nicholson, 2012). Given its pivotal role in mediating cellular functions, aberrations in the JAK/STAT axis can lead to numerous hematopoietic and immune disorders. Specifically, the persistent activation of STAT3 is a hallmark in numerous cancers, often triggered by cytokines, which not only support tumor growth and survival but also influence the tumor microenvironment by regulating non-transformed cells (Thomas et al., 2015). Its hyperactivity, particularly involving STAT3 and/or STAT5, is now recognized as a defining characteristic in most solid and hematologic malignancies, often stemming from ligand-mediated receptor multimerization that brings two JAKs into proximity for activation. These activated JAKs then phosphorylate the STAT proteins, which subsequently dimerize, translocate to the nucleus, and modulate gene expression. This intricate signaling network, involving four JAKs (JAK1, JAK2, JAK3, and TYK2) and seven STAT proteins (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, and STAT6), underscores its broad impact on physiological and pathological conditions. The pathway's fundamental role extends to immune modulation, anti-viral responses, and hematopoiesis, with mutations potentially leading to severe immunodeficiencies and oncogenic transformations (Liu et al., 2018). The activation of the JAK2/STAT3 axis, often induced by signaling molecules such as IL-6, can also promote EMT and enhance the self-renewing characteristics of tumor cells, thereby contributing to metastatic potential. Its aberrant activity also contributes significantly to chemoresistance, an increasingly prevalent challenge in cancer therapy. Understanding the specific mechanisms by which JAK-2/STAT-3 contributes to oral cancer progression, including its impact on tumor cell proliferation, survival, and interaction with the tumor microenvironment, is critical for developing targeted therapies (Mengie Ayele et al., 2022). In this work, we found an increase in JAK-2 and STAT-3 proteins in the untreated KB cells. Interestingly, the crebanine treatment effectively reduced the JAK-2 and STAT-3 proteins in the KB cells, which suggests that crebanine treatment downregulated the JAK-2/STAT-3 axis in KB cells. Therefore, it was clear that crebanine can impede oral tumorigenesis via downregulating JAK-2/STAT-3 signaling in KB cells. Apart from these findings, we acknowledge that our study has several limitations, including the absence of *in vivo* validation, which is essential to confirm the therapeutic potential of crebanine in a physiological context. The dose range used was also limited, and broader concentration testing could provide more comprehensive insights into its efficacy and safety. Furthermore, we did not assess the toxicity of crebanine on normal oral cells, which is crucial to evaluate its selectivity and potential side effects. These limitations will be thoroughly addressed in our future studies to fully establish crebanine's clinical relevance and safety profile.

Conclusion

The current study highlights that crebanine treatment can impede cellular proliferation, induce oxidative stress, and facilitate apoptosis in KB cells via downregulating JAK-2/STAT-3 and PI3K/AKT/mTOR pathways. The apoptosis-inducing effect of crebanine involves the depletion of MMP levels, an increase in endogenous oxidative stress and pro-apoptotic proteins, and the inhibition of JAK-2/STAT-3 and PI3K/AKT/mTOR signaling pathways. Moreover, our work lacks comprehensive molecular assays to investigate its participation in additional molecular pathways. These limitations need to be rectified in the future to promote crebanine as a viable anti-cancer candidate for the treatment of oral cancer.

Abbreviations

DMEM: Dulbecco's modified Eagle medium; DPPH: 2,2-Diphenyl-1-picrylhydrazyl; FBS: Fetal bovine serum; FRAP: Ferric reducing antioxidant power; GSH: Glutathione; MMP: Mitochondrial membrane potential; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ORAC: Oxygen radical absorbance capacity; OSCC: Oral squamous cell carcinoma; Rh-123: Rhodamine-123; SOD: Superoxide dismutase; TBARS: Thiobarbituric acid reactive substances.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

Ethical approval was obtained from the relevant ethics committee or Institutional Review Board (IRB).

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