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ORIGINAL ARTICLE

Anticancer Potential of Brucine against Oral Cancer Cells *in vitro* through Elevation of Reactive Oxygen Species

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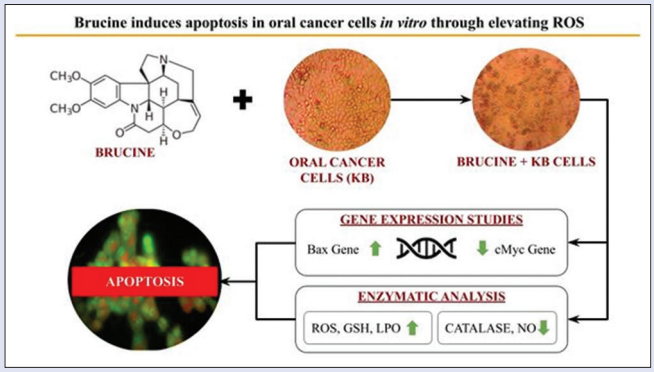
ABSTRACT

Background: Oral cancer is one of the cancers that pose a great threat to life globally. Though a number of chemotherapeutic strategies have been followed to treat oral cancer, a number of adverse effects limit the usage of chemotherapeutic agents. **Purpose:** In the current study, we evaluated the anticancer potential of brucine. **Materials and Methods:** KB cell line was treated with various concentrations of brucine (10–100 µg/ml) and the cytotoxicity and cell viability were measured. Acridine orange/ethidium bromide (AO/EB) staining was performed to assess apoptosis in cells. To evaluate the mechanism of brucine-mediated apoptosis, gene expression studies were carried out (Bax and cMyc). **Results:** Brucine caused cytotoxicity and reduced cell viability as assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay and trypan blue exclusion assay, respectively. The IC_{50} value of brucine was calculated to be 30 µg/ml. Further experiments were performed according to the IC_{50} value. Brucine-treated cells were rounded up and detached from the surface. AO/EB staining revealed apoptotic mode of cell death. Further, our experiments revealed elevated levels of reactive oxygen species (ROS) within the cells, which might have caused apoptosis. A dose-dependent rise in lipid peroxidation was evident from thiobarbituric acid reactive substance (TBARS) assay, which could be due to the oxidative stress caused by brucine. Nitric oxide levels were impaired by brucine, which correlated well with apoptosis. On the other hand, catalase activity was decreased by brucine in a dose-dependent manner, implying the reason for oxidative stress. Polymerase chain reaction (PCR) analysis revealed increased expression of Bax and impaired expression of the oncogene cMyc. **Conclusion:** Brucine triggers oxidative stress, which leads to lipid peroxidation and induces the expression of the proapoptotic gene Bax, thereby causing apoptosis. In parallel, brucine-mediated suppression of cMyc leads to impairment of metastasis.

Key words: Brucine, Apoptosis, Cancer cells, Oxidative stress, pro-apoptotic gene, lipid peroxidation

SUMMARY

- Brucine effectively inhibited proliferation of KB cell line
- Apoptosis of KB cell line was induced by brucine through elevation in ROS, reduced glutathione and Lipid peroxidation.
- Further, nitric oxide levels and catalase activity was decreased indicating oxidative stress.
- Pro apoptotic gene Bax expression was increased and apoptotic gene cMyc decreased on treatment with brucine in dose dependent manner.



Abbreviations used: 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.

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INTRODUCTION

Oral cancer remains one of the major causes of death worldwide. Various risk factors associated with oral cancer development include chewing of betel nuts, alcohol consumption, smoking, and infection by certain viruses.[1] Chewing of Khat is one of the contributing factors to oral cancer.[2] Khat is chewed for its sympathomimetic and psychotropic effects. These effects are due to ephedrine-like compounds present in Khat.[3,4] In addition, Khat contains potent carcinogens such as 4-(*N*-methyl-*N*-nitrosamino)-4-(3-pyridyl)-1-butanal and 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone.[2] A wide variety of therapeutic agents are being prescribed for the treatment of oral cancer. However, the anticancer agents affect the normal cells also, limiting their clinical applications.

Strychnos nux-vomica L. is found in tropical and subtropical regions. The seeds of this plant contain a natural alkaloid called brucine. A wide range of diseases are treated by brucine in traditional Chinese folk medicine. Brucine has also been found to have anticancer activities

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against a wide range of tumors.[5,6] It has been shown to supress hepatocellular carcinoma.[7] Brucine has also been shown to supress vascular endothelial growth factor (VEGF) during breast cancer metastasis, which is essential for the development of cancers.[8] In colorectal cancers, brucine has been shown to impair Wnt/β-catenin pathway and control the cancer progression and metastasis.[9] Similarly, brucine has been shown to inhibit hepatic cancer by employing various pathways that include c-Jun N-terminal kinase (JNK) to cause apoptosis.[10]

Even though a wide range of antineoplastic agents are currently in use for treating various types of cancers, the spectrum of adverse drug reactions limits the clinical application of the drugs. Doxorubicin, cisplatin, carboplatin, 5-fluorouracil, paclitaxel, docetaxel, hydroxy urea, and so on have been used for the treatment of oral cancers. With this background, it is necessary to find effective alternative therapeutic agents for treating cancer. Therefore, in the current work, we evaluated the effectiveness of brucine in alleviating oral cancer.

MATERIALS AND METHODS

Source of chemical and reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) – Cat. No. M6494, Dulbecco's modified Eagle medium (DMEM) – Cat. No. 11965092, fetal bovine serum (FBS) – Cat. No. A4736401, nitro blue tetrazolium (NBT) – Cat. No. N6495, potassium hydroxide (KOH) – Cat. No. R21231, dimethyl sulfoxide (DMSO) – Cat. No. D12345, KCl – Cat. No. AM9640G, 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) – Cat. No. D8451, Tris HCl – Cat. No. 15506017, and Nucleospin – Cat. No. K180001 were purchased from Thermo Fisher Scientific. *N*-(1-naphthyl)-ethylenediamine dihydrochloride (NED) – Cat. No. 222488, thiobarbituric acid reactive substance (TBARS) – Cat. No. T5500, Malondialdehyde (MDA) -Cat. No. 383538, brucine – Cat. No. 357573, hydrogen peroxide – Cat. No. 609978, and sulfanilamide – Cat. No. 63741 K180001 were purchased from Sigma Aldrich Chemicals Pvt Ltd.

MTT assay

Cytotoxic effects of brucine were measured by a modified method of Sylvester *et al.*[11] Similar protocol have been adapted in KB cells (Human epithelial carcinoma cells).[12] The KB cells with a seed density of 1×10^5 cells were grown in a 96-well plate. The cells were treated with 10 different concentrations of brucine from 10 to 100 µg/ml. After incubation for 24 h, 15 µl of MTT at a concentration of 0.5 mg/ml was added and incubated at 37°C for another 4 h. The supernatant was discarded, and formazan crystal was dissolved in 0.1 ml of DMSO. The solubilized crystal was mixed for 10 min, and the absorbance was measured at 490 and 630 nm. The effect of the compound on KB cell line and cell control was evaluated based on IC_{50} (half maximal inhibitory concentration) obtained by 50% inhibition of cells using the formula:

$$\% \text{ of inhibition} = (1- [A_{490} - A_{630} \{\text{treated}\}/A_{490} - A_{630} \{\text{control}\}] \times 100)$$

Cell viability assay

The viability of KB cell line was tested by the method of Strober *et al.*[13] About 1×10^5 cells/ml of KB cells were seeded in DMEM with 1% FBS and penicillin–streptomycin at 100 U/ml/100 µg/ml and incubated for 2 h. Different concentrations (10–100 µg) of brucine were added to the cells and further incubated again for 24 h. The viable cells were analyzed by trypan blue using hemocytometer. The viable cell density was expressed in percentage using the formula

% cell viability = (number of viable cells/total number of cells) × 100.

Morphological analysis of cells

Morphological changes of cells undergoing apoptosis were analyzed as per the protocol given in Moongkarndi *et al*[14] with some modifications. Cells were grown in six-well cell culture plates for 24 h at 37°C in the presence of 5% CO_2 atmosphere. Different concentrations of brucine (10, 30, 100 µg/ml) were added to cells and incubated for 24 h. Then, they were observed for morphological changes under a phase-contrast inverted microscope.

Measurement of apoptosis by acridine orange/ethidium bromide dual staining

The apoptosis of KB cell line was investigated by incorporating Mironova *et al.*[15] and Smith *et al.*[16] In this, 5×10^4 cells/well were seeded and incubated for 24 h at 5% CO_2. After treatment with varying concentrations of brucine, the cells were subjected to centrifugation and the pellet was washed thrice with phosphate-buffered saline (PBS). The cells were subjected to dual staining with acridine orange/ethidium bromide (AO/EB) in a ratio of 1:1 (100 µg/ml), incubated for 3–5 min, and observed under a fluorescent microscope.

Estimation of reactive oxygen species

NBT assay was performed as presented by Zhang *et al.*[17] In this, 1×10^5 cells/well were seeded in 96-well plates and incubated for 24 h. After confluent monolayer was reached, different concentrations of brucine were added and again incubated for 24 h. Ten microliters of 0.05 g/ml NBT solution was added with aspirated medium and incubated for 1 h. This was followed by addition of 60 µl of 2 M KOH with 70 µl of DMSO to each well, and the absorbance was read using a multiclan plate reader at 630 nm.

Nitric oxide assay

To estimate the nitric oxide (NO) concentration, the method described in Wahyuni *et al.*[18] was used. In this, 100 µl (100 µM) of nitrite solution was added to 96-well cell culture plate to obtain nitrite standard curve at concentrations of 100, 50, 25, 12.5, 6.25, 3.13, and 1.56 µM. To 50 µl of supernatant of the treated cells (concentration of 10, 20, 30, 40, 50 µg/ml), equilibrated sulfanilamide solution and NED solution were added. The reaction was protected from light and 50 µl of 0.1% NED solution was also added to all wells, and the absorbance was read at 520 and 550 nm within 30 min after color formation.

Lipid peroxidation assay

Lipid peroxidation was assayed by the method of Ohkawa *et al.*[19] The treated cells were homogenized with ice-cold KCl buffer and subjected to centrifugation at 5000 g for 5 min. One hundred microliters of the supernatant was mixed with 0.2 ml of 8.1% Sodium dodecyl sulphate (SDS) and 1.5 ml of 20% acetic acid and incubated for 10 min. To this reaction mixture, 1.5 ml of 0.8% TBARS buffer was added with 0.7 ml of distilled water and incubated for 1 h at 95°C. To this, 5 ml of butanol and pyridine mixture (15:1) was mixed and centrifuged at 5000 g for 15 min, and the absorbance values were measured at 532 nm. The standard curve was carried out with MDA with different concentrations and expression measured as µM.

Estimation of reduced glutathione

Glutathione (GSH) was estimated by DTNB reduction assay of Pippenger *et al.*,[20] with some modifications. Forty milligrams of DTNB was dissolved using 10 ml of DMSO and diluted 100-fold using Tris-HCl (0.1 M; pH 7.5). Aliquot 950 µl of 0.1 mM DTNB and add 50 µl ofspin medium from cells treated with different concentrations of brucine (10, 20, 30, 40, 50 µg/ml) and further vortexed and incubated for 2 min at room temperature. Absorbance was spectrophotometrically measured at 412 nm.

Catalase activity

The activity of catalase was measured by the method of Góth *et al.*[21] The cells were treated with five different concentrations of brucine ranging from 10 to 50 µg/ml. These cells were centrifuged and the supernatant was separated. To 200 µl of the supernatant, 1 ml of H_2O_2 (65 µM) and 1 ml of ammonium molybdate (32.4 mM) were added and observed for yellow complex formation, and the absorbance was measured at 405 nm. The formula used for determining catalase activity was

$$\text{Catalase activity (kU/l)} = \frac{A(\text{sample}) - A(\text{blank1})}{A(\text{blank2}) - A(\text{blank3})} \times 271$$

Blank 1 contains 1 ml substrate, 1 ml ammonium molybdate, and 0.2 ml sample; blank 2 contains 1 ml substrate, 1 ml ammonium molybdate, and 0.2 ml buffer; and blank 3 contains 1.2 ml buffer and 1 ml molybdate.

Polymerase chain reaction

DNA was extracted using DNA isolation kit (Nucleospin) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was carried out using a gradient thermal cycler machine. In this, 4.5 µl of template DNA was added to 12.5 µl of 1X PCR Emerald master mixture with 1 µl each of the specific primers and the remaining volume of 25 µl was made up with Milli Q water. Standard PCR conditions were followed. The annealing temperatures were 54°C, 59.6°C, and 54.3°C for genes β-Actin, cMyc, and Bax, respectively. The DNA obtained from PCR was analyzed in agarose gel electrophoresis (1% agarose) in 1X Tris-acetate-EDTA (TAE) buffer at 50 V for 2 h, visualized under ultraviolet (UV) and photographed.

Statistical analysis

Experiments were carried out in triplicate, and the results were analyzed by GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA). The data obtained was expressed as mean ± standard deviation with one-way analysis of variance (ANOVA) (Tukey's multiple *t*-test).

RESULTS

Cytotoxicity

Brucine is abundantly found in *S. nux-vomica*. In the current experiment, we evaluated the cytotoxicity of brucine on oral cancer cell line (KB cells). The toxicity of the compound was evaluated on cancer cells by MTT assay. Brucine was added to KB cells at 10 concentrations from 10 to 100 µg/ml [Figure 1a]. The experiment revealed a dose-dependent cytotoxicity of brucine. The IC_{50} value of brucine was calculated to be 30 µg/ml in KB cell line.

Cell viability assay

The oral cancer cells' response to brucine, in terms of viability, was estimated by trypan blue exclusion assay. Brucine was added to KB cells at 10 concentrations from 10 to 100 µg/ml and the viability of cells was observed [Figure 1b]. A dose-dependent decrease in viability of cells was observed with an increasing concentration of brucine. Beyond the concentration of 60 µg/ml, there was only 10% cell viability, indicating the strong anticancer activity of the compound. Microscopic observation of brucine-treated KB cells under phase-contrast microscope revealed a dose-dependent decline in cell viability. At 100 µg/ml, there was a considerable reduction in the number of live cells with visible morphological aberrations ($P < 0.0001$) [Figure 2a].

AO/EB dual staining

The AO/EB staining was performed to evaluate if the mode of cellular death was through apoptosis. Cells undergoing apoptosis display a variety of changes. The exposure to brucine to KB cells triggered a number of morphological changes including membrane blebbing and chromatin condensation [Figure 2b].

Estimation of reactive oxygen species

Reactive oxygen species (ROS) is a contributing factor to apoptosis, and for this reason, we estimated the level of ROS in KB cells after treatment with brucine. A dose-dependent elevation in ROS levels was observed ($P < 0.0001$) [Figure 3a].

Lipid peroxidation analysis and estimation of NO

Since there was a decline in the number of viable cells with aberrant morphological changes, cell death could have been caused by elevated ROS. Therefore, TBARS assay was carried out to quantify the extent of lipid peroxidation. The KB cells were treated with five concentrations (10–50 µg/ml) of brucine. The experiment showed a dose-dependent elevation in the quantity of malondialdehyde [Figure 3c]. With strong evidence of the involvement of lipid peroxidation, we further evaluated the involvement of NO in causing cell death. With increasing concentrations of brucine, a decline in NO levels could be witnessed [Figure 3b]. A stronger suppression of NP was seen in the KB cells treated with 50 µg/ml of brucine ($P < 0.0001$).

GSH assay

GSH is a tripeptide that exists in both oxidized and reduced forms. Treatment of KB cells with brucine showed a dose-dependent increased in the amount of GSH [Figure 3d]. Even at a concentration as low as 10 µg/ml, there was a significant increase in the GSH ($P < 0.0001$).

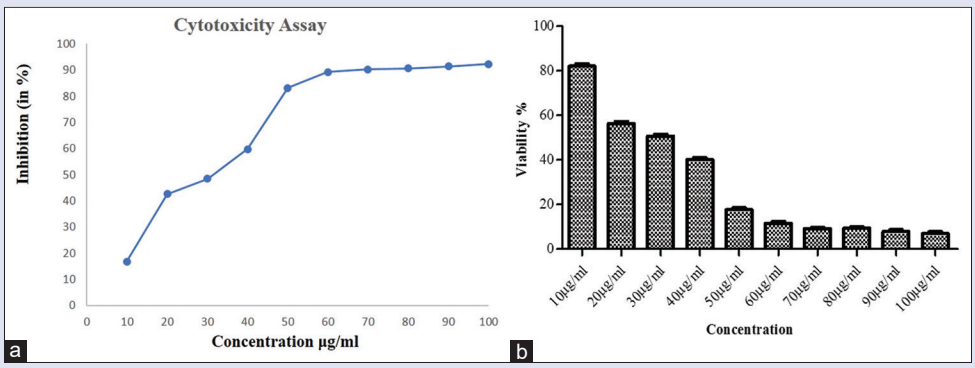

Figure 1: Effect of brucine on cell proliferation. (a) Cytotoxicity of brucine on KB cell line increased with doses. (b) Trypan blue assay shows decrease in cell viability with increase in concentration of brucine

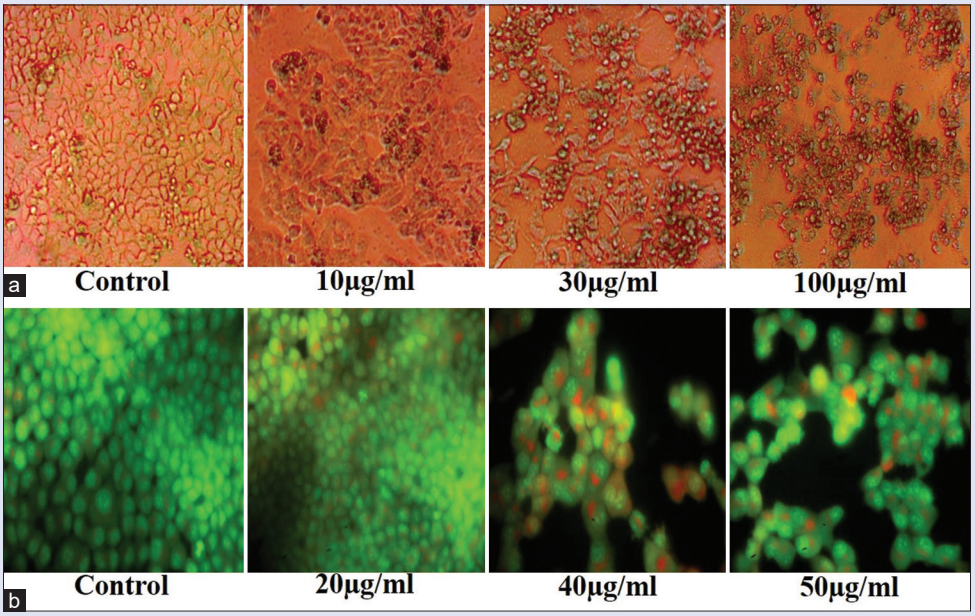

Figure 2: Antiproliferative activity of brucine. (a) Morphometric analysis by phase-contrast microscope shows morphological changes in a dose-dependent manner. (b) AO/EB dual staining shows apoptosis induction in dose-dependent manner. AO/EB = acridine orange/ethidium bromide

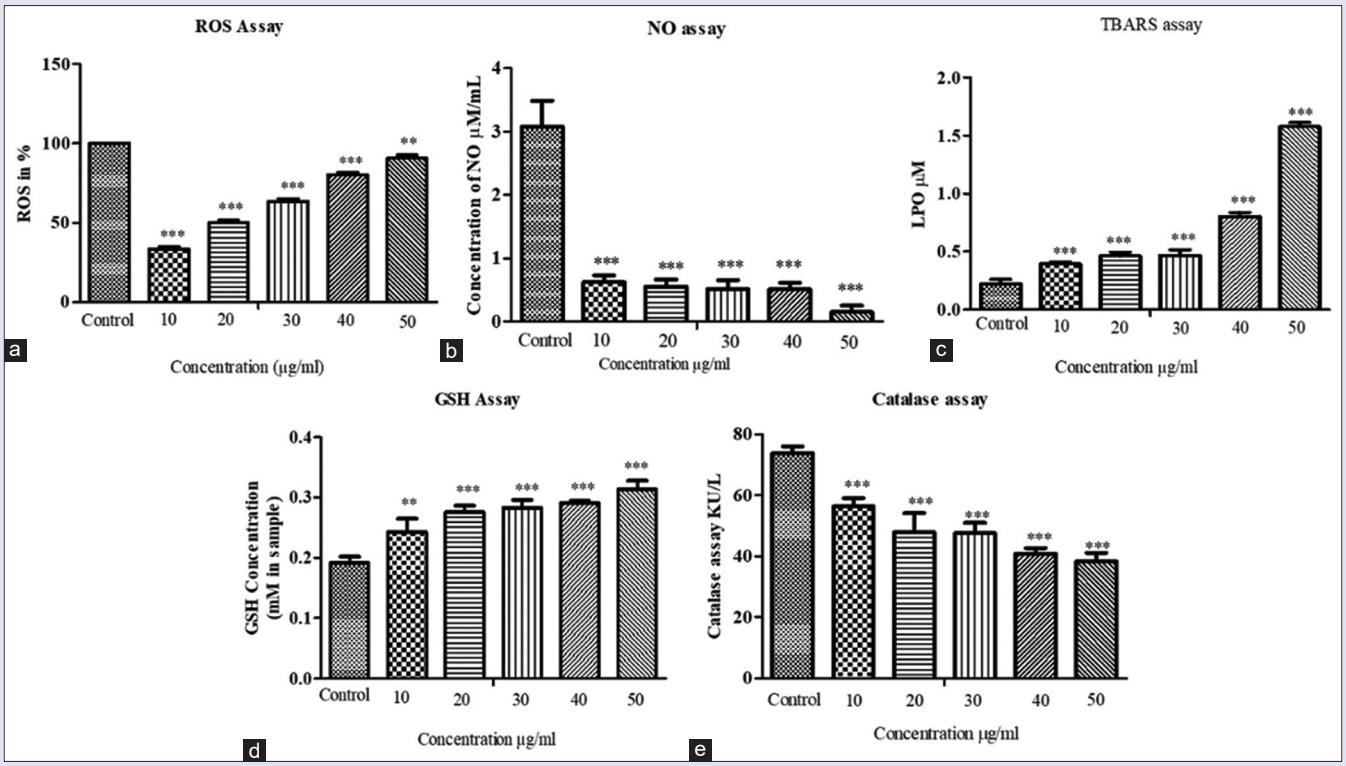

Figure 3: Effect of brucine on (a) ROS, (b) NO, (c) TBARS, (d) GSH, and (e) catalase in KB cell line. ROS, LPO, and GSH showed significant increase on treatment with brucine, whereas the NO and catalase levels were reduced. GSH = glutathione, LPO = lipid peroxidation, NO = nitric oxide, ROS = reactive oxygen species, TBARS = thiobarbituric acid reactive substance

Catalase assay

To further evaluate the status of antioxidant enzymes, we estimated the activity of catalase enzyme. It was found that in cancer cells, there was an elevated amount of catalase, whereas its activity reduced in a dose-dependent manner on treatment with brucine ($P < 0.0001$) [Figure 3e].

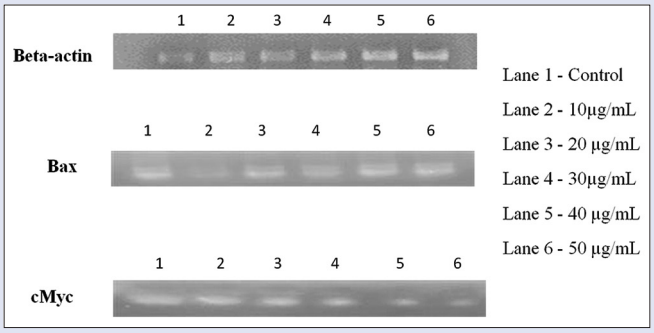

Figure 4: Effect of brucine on the gene expression of Bax and cMyc

Gene expression analysis

cMyc is an oncogene whose role in oral cancer progression has been well documented. cMyc gene products promote oral cancer cell migration and proliferation.[22] The expression pattern of cMyc gene was assessed by PCR. The expression of cMyc gene declined in a dose-dependent manner following brucine treatment in KB cells, implying that the compound has a strong inhibitory activity on the expression of cMyc gene. To further decipher the molecular mechanism of brucine-mediated apoptosis, the involvement of the proapoptotic gene Bax was assessed by PCR [Figure 4]. Brucine increased the expression of the gene increased in a dose-dependent manner.

DISCUSSION

The main source of Brucin is the seeds of *Strychnos nux-vomica* L. (Loganiaceae). In Chinese medicine, the plant has been widely utilized for treating a variety of diseases such as neuronal system disorders, dyspepsia, and chronic rheumatism.[23] In liver cancer cells, brucine has been shown to cause mitochondrial depolarization by affecting calcium homeostasis and thereby inhibit proliferation of liver cancer cells.[7] Brucine is not only effective on liver cancers but also on a variety of other cancers.[24,25] In the current work we studied the anti-cancer activity of brucine against oral cancer *in vitro*.

Since an effective anti-cancer drug is supposed to induce cell death, we first evaluated the cytotoxic effect of brucine on KB cells *in vitro* by MTT assay. Brucine displayed cytotoxicity with an IC_{50} value of 30 µg/ml [Figure 1A]. In parallel, we evaluated the viability of cells by tryphan blue assay and the pattern of viability complemented the cytotoxicity. At 30 µg/ml there was a stronger decrease in the number of viable cells. [Figure 1B]. Though there was a stronger cytotoxicity by brucine the mode of death is a point of concern. To be a better anti-cancer agent it should induce apoptosis rather than causing necrosis by triggering the genes which cause apoptosis. Therefore, we recorded the appearance of KB cells treated with brucine through phase contrast microscope. The micrographic images are another line of evidence for the elicitation of cell death in a dose-dependent manner [Figure 2A]. Significant changes in the shape were observed in KB cells treated with brucine. Microscopic images revealed that the cells were rounded up and detached from the surface which are evidences of ongoing apoptosis. It is clear that brucine has induced morphological changes indicative of apoptosis. Our results are in agreement with earlier findings that brucine causes arrest of G1 phase in cell cycle and causes apoptosis in LoVo cells.[26]

Acridine orange/ethidium bromide dual staining revealed strong signs of apoptosis. The cell death caused by apoptosis is triggered because of the genes associated with a wide range of apoptotic markers. Acridine orange has the inclination to penetrate only the intact cells and early apoptotic cells. There is a possibility that Brucine might cause apoptosis by intercalating with the DNA. Upon entering the cells acridine orange binds with DNA and emits fluorescent green colour. On the other hand, ethidium bromide shows selective affinity towards damaged cells and causes oranges red fluorescence upon binding to the DNA.[16] The fluorescence image clearly indicates that brucine induces apoptosis in KB cell lines in a dose-dependent manner. At lower concentrations of brucine, early apoptotic cells and intactcells could be visualized. Very small number of late apoptotic cells wasseen. In the medium dose, the relative proportion of preliminary and late apoptotic cells seen. By contrast, higher dose of brucine induced a large number of late apoptotic cells. In a nutshell, brucine caused apoptosis in KB cells in a dose-dependent manner.

Lipid peroxidation is one of the side effects of uncontrolled ROS. Peroxidation of lipid membrane may affect the overall architecture of the cells. Due to the peroxidation of the lipid membrane the fluidity of the membrane is lost and ultimately result in loss of cellular integrity.[27] Extensive lipid peroxidation can also induce apoptosis.[28] TBRAS assay demonstrated a dose dependent increase in lipid peroxidation in response to increasing dose of brucine. Therefore, it is compelling evidence that brucine causes lipid peroxidation because of oxidative stress which subsequently lead to apoptosis. The mechanism could be that the lipid peroxidation would have caused cytochrome C release from mitochondria through activation of caspase-3.[29]

Nitric oxide negatively regulates apoptosis.[30] Therefore, the extent of nitric oxide in KB cells in response to brucine was evaluated. Surprisingly there was a strong suppression of nitric oxide even at very low concentrations. [Figure 3B] Moreover, suppression of nitric oxide is required for Bax induction, a pro-apoptotic gene. From our experiments it is very clear that brucine inducers apoptosis in oral cancer cells through suppression of nitric oxide. On the other hand, nitric oxide is supposed to suppress lipid peroxidation.[31] However, since the nitric oxide level is decreasing it may well be correlated with elevated lipid peroxidation.

Glutathione protects the cell from oxidative damage.[32] The KB cells treated with brucine caused a dose-dependent decrease in GSH levels. This correlates well with induction of apoptosis. (Figure 3 D) Further, we evaluated the anti-oxidant activity of catalase enzyme. There was a strong decline in the catalase activity in response to treatment with brucine [Figure 3E].

Catalase activity is impaired by brucine in a dose-dependent manner and therefore, the oxidative stress was elevated in KB cells. Cancer cells develop resistance to oxidative stress through increased expression of catalase.[33,34] Otherwise, elevated amounts of oxidative stress can result in apoptosis. Suppression of catalase is one of the strategies for inducing apoptosis in tumor cells.[35] ROS can initiate apoptosis in a number of cells by a variety of mechanisms.[36] Therefore, we estimated the oxidative stress in oral cancer cells after treatment with brucine. A dose-dependent increase in ROS in response to brucine was witnessed, indicating that brucine induces oxidative stress. A wide array of evidence suggests that oxidative stress can strongly induce apoptosis.[37,38] Therefore, in the current experiment, it is convincing that suppression of catalase could be one of the reasons for the induction of apoptosis of cancer cells.

Triggering of proapoptotic genes occurs through ROS. ROS can prompt the expression of the gene Bax. Estimation of the expression of Bax gene in response to brucine was done by PCR. There was a dose-dependent increase in the expression of the gene Bax, indicating the role of ROS-mediated apoptosis.[39] In addition, we also evaluated the expression of an oncogene cMyc. Brucine strongly suppressed cMyc expression. cMyc expression is necessary for the expression of genes involved in cancer cell progression. Brucine suppresses the expression of the oncogene cMyc and inhibits cancer cell proliferation.[40]

CONCLUSION

Brucine is effective in suppressing the growth of oral cancer (KB) cells *in vitro*. Morphologically, the cells treated with brucine underwent changes such as rounding up and detachment from the surface. AO/EB staining revealed strong induction of apoptosis. Brucine also induced ROS in a dose-dependent manner, which potentially could have triggered apoptosis. In addition, lipid peroxidation was also induced by brucine, which could be another trigger for apoptosis. In parallel, the antiapoptotic signal was suppressed by decreased levels of NO. Decline of GSH concentration is another indication of increased apoptosis. Further, the antioxidant activity was decreased by impaired catalase activity, thereby increasing the chances of apoptosis. The molecular mechanism of brucine-induced apoptosis lies in increased expression of the gene Bax and suppression of the oncogene cMyc. In short, ROS induced by brucine induces the expression of Bax gene and causes apoptosis. However, the study is limited by the fact that the experiment was only done *in vitro*. Further, the current study needs to be extended *in vivo* to find the efficacy of brucine.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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