ORIGINAL ARTICLE

Acacia catechu ethanolic bark extract induces apoptosis in human oral squamous carcinoma cells

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ABSTRACT

Oral cancer is in approximately 30% of all cancers in India. This study was conducted to evaluate the cytotoxic activity of ethanolic extract of *Acacia catechu* bark (ACB) against human squamous cell carcinoma cell line-25 (SCC-25). Cytotoxic effect of ACB extract was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide assay. *A. catechu* extract was treated SCC-25 cells with 25 and 50 μg/mL for 24 h. Apoptosis markers such as caspases-8 and 9, bcl-2, bax, and cytochrome c (Cyt-c) were done by RT-PCR. Morphological changes of ACB treated cells were evaluated using acridine orange/ethidium bromide (AO/EB) dual staining. Nuclear morphology and DNA fragmentation were evaluated using propidium iodide (PI) staining. Further, cell cycle analysis was performed using flow cytometry. *A. catechu* treatment caused cytotoxicity in SCC-25 cells with an IC_{50} of 52.09 μg/mL. Apoptotic marker gene expressions were significantly increased on ACB treatment. Staining with AO/EB and PI shows membrane blebbing and nuclear membrane distortion, respectively, and it confirms the apoptosis induction in SCC-25 cells. These results suggest that ACB extract can be used as a modulating agent in oral squamous cell carcinoma.

Key words: Caspases, cytotoxicity, nuclear membrane, squamous cell carcinoma cell line-25 cells

INTRODUCTION

Oral cancer is one of the serious and growing health problems worldwide and oropharyngeal cancer is a significant component of the global burden of cancer. The annual estimated incidence is around 275,000 for oral and 130,300 for pharyngeal cancers, two-thirds of these cases occurring in developing countries.[1,2] Oral cancer ranks in the top three of all cancers in India which account for over 30% of all cancers reported in the country.[3] Treatment modalities of oral squamous cell carcinoma (OSCC) have numerous side effects. Clinical consequences of radiotherapy include mucositis, oral candidiasis, loss of taste, and xerostomia, which may be permanent due to the detrimental effect of radiation on salivary glands.[4] Despite developments in current treatment modalities using chemotherapy, surgery and radiation, along with other palliative treatments, OSCC remains a great challenge for clinical therapy. Accordingly, new strategies are evolving to control and to treat cancer and one such strategy could be the use of medicinal plants. Recent studies have been focused on herbal medicine as potent anti-cancer drug candidates.[5]

Acacia catechu Willd (Fabaceae), commonly known as catechu, cachou, and black cutch is an important

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medicinal plant, especially in Asia.[6] Several phytochemical compounds have been isolated and characterized from *A. catechu* which include 4-hydroxybenzoic acid, kaempferol, quercetin, 3,4,7-trihydroxyl-3,5-dimethoxyflavone, catechin, rutin, isorhamnetin, epicatechin, afzelechin, epiafzelechin, mesquitol, ophioglonin, aromadendrin, and phenol and the presence of these active compounds have been implicated for its myriad biological effects.[7] *A. catechu* has been studied for its hepatoprotective, antipyretic, antidiarrheal, hypoglycemic, anti-inflammatory, immunomodulatory, antinociceptive, antimicrobial, free radical scavenging, and antioxidant activities.[6,8-12] Clinically, *A. catechu* in combination with *Scutellaria baicalensis* has tested for its safety and anti-inflammatory effects in osteoarthritis patients.[13] Previous studies have reported the anticancer efficacy of medicinal plants against several human *in vitro* cancer cell lines and came out with promising results.[14-17] However, studies regarding the anticancer potentials of *A. catechu* ethanolic bark extract on human squamous cell carcinoma cell line (SCC-25) is scanty or not available in the literature. Hence, in this study, we evaluated the anticancer potential of *A. catechu* in SCC-25 cells.

MATERIALS AND METHODS

Chemicals

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT), dimethyl sulfoxide (DMSO) was purchased from Sigma Chemical Co. India. The other chemicals used in this study were purchased locally and were of analar grade.

Plant collection and extract preparation

Acacia catechu bark (ACB) was collected during the month of December 2015 from Hosur, Tamil Nadu, India, authenticated by Green Chem Lab, Bengaluru, Karnataka, India. Barks were shade dried and was milled to fine powder. This bark powder was passed through 100 mesh sieve, and 2.5 kg of powdered ACB were extracted with 10 L of ethanolic, at 65°C, for 1 h. After 1 h of extraction, the extract were filtered and collected. The marc, an insoluble residue was extracted repeatedly with 10 L of ethanolic, twice. The extract was evaporated in a Buchi rotary evaporator (Switzerland) at 65°C, to obtain 150 g of powder extract. The w/w yield of the prepared extract was 6%.

Cell culture

The SCC-25 cell line was procured from ATCC. Cells were maintained in Dulbecco's Minimum Essential Media and Ham's F-12 supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 µg/mL streptomycin. Cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C. Cells were grown in 75 cm^2 culture flasks and after a few passages, cells were seeded for experiments. The experiments were done at 70% to 80% confluence. On reaching confluence, cells were detached using 0.05% trypsin-EDTA solution.

Cell treatment

A. catechu ethanolic bark extract was dissolved in 0.1% DMSO (v/v). SCC-25 cells were plated at 10,000 cells/cm^2. After 24 h, cells were fed with fresh expansion culture medium supplemented with different final concentrations of ACB extract (25 and 50 µg/mL) or the corresponding volumes of the vehicle. After 24 h of treatment, cells were collected by trypsin application.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide assay

Cytotoxic effect was assessed by MTT assay.[18] Cells were plated in 96-well plate at a concentration of 5×10^4 cells/well. After 24 h, cells were fed with fresh expansion culture medium supplemented with different final concentrations of ACB extract (0.1–1000 µg/well) and incubated for 24 h. After 24 h, media was discarded, and 50 µL of MTT (5 mg/mL of phosphate-buffer saline (PBS)) was added to each well. Cells were then incubated for 4 h. MTT was then discarded and the colored crystals of produced formazan were dissolved in 150 µL of DMSO. The purple-blue formazan dye formed was measured using an ELISA reader (BIORAD) at 570 nm.

Acridine orange/ethidium bromide staining

Acridine orange/ethidium bromide (AO/EB) staining was carried out by the method of Gohel *et al.*[19] After 24 h of treatment, cells were washed twice with PBS and equal volumes of cells from control and experimental group cells were mixed with 100 µL of dye mixture (1:1) of AO/EB stain and viewed immediately under inverted fluorescence microscope. A minimum of 300 cells were counted in each sample at two different fields.

Propidium iodide staining

Propidium iodide (PI) staining was carried out by the method of Mohan *et al.*[20] SCC-25 cells were plated at a density of 1×10^4 in 48 well plates. After 24 h of treatment, cells were gently rinsed twice with PBS at room temperature, before fixing in methanol:acetic acid (3:1 v/v) for 10 min, and stained with 10 µg/mL of PI for 20 min. Nuclear morphology of apoptotic cells with condensed/fragmented nuclei was examined by fluorescence microscope and at least 1×10^3 cells were counted for assessing apoptotic cell death.

Gene expression analysis

Total RNA was extracted by trizol reagent according to the standard protocol. cDNA was synthesized according to the manufacturer's protocol (Promega, Madison, WI) Then, 2 µL of template cDNA was added to the final volume of 20 µL of reaction mixture. RT-PCR cycle parameters included 10 min at 95°C followed by 40 cycles involving denaturation at 95°C for 15 s, annealing at 60°C for 20 s, and elongation at 72°C for 20 s. The sequences of primer for bax, bcl-2, Cyt-c, caspases-8 and 9, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used in this study were based on

previously published literatures. GAPDH gene was used as an internal housekeeping control.

Cell cycle arrest analysis by flow cytometry

After the 24 h of treatment, cells were harvested, washed with PBS and fixed in 75% ethanol at 4°C overnight. After washing twice with cold PBS, cells were suspended in PBS containing 40 µg/mL of PI and 0.1 mg/mL of RNase A followed by shaking at 37°C for 30 min. The stained cells were analyzed with flow cytometer (Becton-Dickinson San Jose, CA, USA) and the data were consequently calculated using WinMDI 2.9 software (TSRI, La Jolla, CA, USA).

Statistical analysis

Data were expressed as mean ± standard error of the mean and analyzed by Tukey's test to determine the significance of differences between groups. The value of $P < 0.05$ considered as statistically significant.

RESULTS

Inhibitory effects of *Acacia catechu* bark extract against human squamous cell carcinoma-25 oral squamous carcinoma cells

MTT assay shows that 24 h ACB extract treatment was able to inhibit the proliferation of SCC-25 cells. The maximum antiproliferative effect was found to be 83% at a maximum concentration used in this study, i.e., 1000 µg/mL of ACB extract. The IC_{50} of ACB extract on OSCC was calculated by linear regression analysis and was found to be 52.09 µg/mL [Figure 1]. Hence, further analyses were performed in this study with 25 and 50 µg/mL of ACB extract.

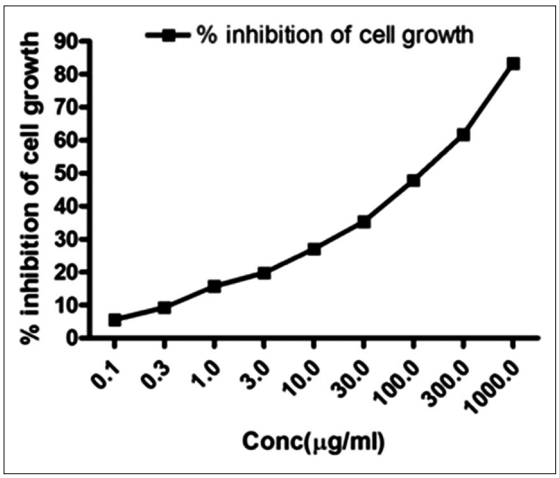

Figure 1: Cytotoxic effect of *Acacia catechu* bark extract was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide assay. Squamous cell carcinoma cell line-25 cells were treated with different concentrations of *Acacia catechu* bark extract for 24 h. Values are expressed as mean ± standard error of the mean ($n = 3$)

Dual staining assay

In this study, ACB extract-treated cells emits intense red fluorescence indicate the fact that this extract has the ability to induce apoptosis in SCC-25 cells [Figure 2a-c]. The percentage of apoptotic cells after treatment with 25 and 50 µg/mL of bark extract showed a drastic increase in dose-dependent manner ($P < 0.001$) to 58% and 84%, respectively [Figure 2d].

Nuclear fragmentation analysis by propidium iodide staining

The ACB extract treated SCC-25 cells found to be more apoptotic nuclei when compared to untreated cells [Figure 3a-c]. The ACB extract-induced apoptotic nuclei displayed characteristic features of reduced size, intense fluorescence of condensed nuclear chromatin and formation of membrane blebs. The percentage of apoptotic nuclei increased significantly ($P < 0.001$) to 48% and 65%, after treatment with 25 and 50 µg/mL of ACB extract respectively [Figure 3d].

Apoptotic marker gene expressions

To investigate the molecular mechanism underlying apoptosis process and to examine the mitochondrial damage, we evaluated the gene expression analysis of Cyt-c, caspases-8 and 9. The results showed that activation of caspases-8 and 9 and Cyt-c gene expression in ACB extract treated groups were expressed highly than that of control. The ACB extract treatment also caused significant down-regulation of bcl-2 and up regulation of bax expression in SCC-25 cells [Figure 4a-e].

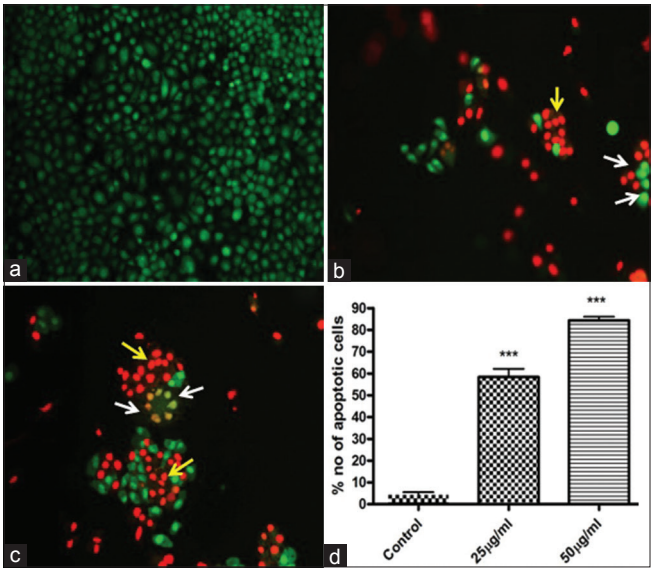

Figure 2: Apoptosis analysis by acridine orange/ethidium bromide (×10). (a) Control; (b) *Acacia catechu* bark extract 25 µg/mL treatment; (c) *Acacia catechu* bark extract 50 µg/mL treatment. White arrow shows the early apoptotic cells; Yellow arrow shows late apoptotic and DNA fragmented cells. (d) Quantification of apoptotic cells. Values are expressed as mean ± standard error of the mean ($n = 3$). ***$P < 0.001$

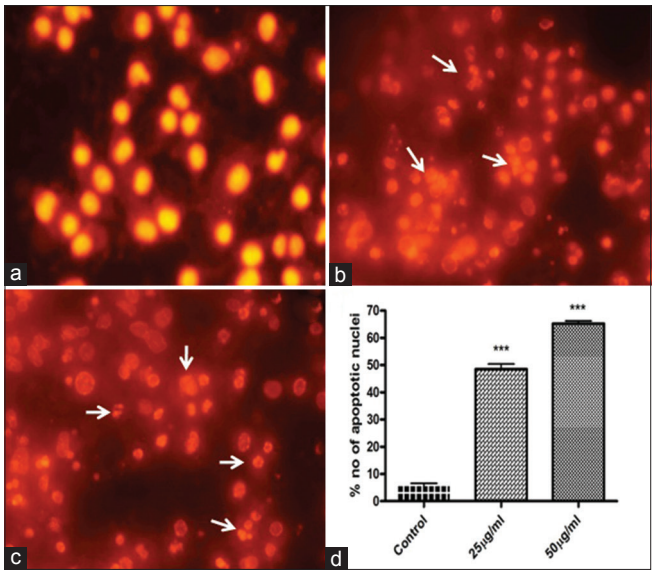

Figure 3: Nuclear morphology analysis by propidium iodide staining (×10). (a) control; (b) *Acacia catechu* bark extract 25 µg/mL treatment; (c) *Acacia. Catechu* bark extract 50 µg/mL treatment. White arrows indicate the apoptosis cells with fragmented nuclei. (d) Quantification of apoptotic nuclei. Values are expressed as mean ± standard error of the mean ($n = 3$). ***$P < 0.001$

Cell cycle distribution of squamous cell carcinoma-25 cells when treated with *Acacia catechu* bark extract

To further confirm whether the cause of cell death induced by ACB extract was apoptosis, flow cytometric analysis was performed. At lower concentration (25 µg/mL) used in this study, the ACB extract showed cell cycle arrest at S-phase with 25% cells accumulated. At higher concentration (50 µg/mL), it showed an increased to 34% at S-phase with concomitant decrease in the other phases of the cell cycle [Figure 5a-c].

DISCUSSION

Oral squamous cell carcinomas possess an important health concern worldwide. Natural products with anticancer properties could be valuable substances in cancer treatment.[5] In this study, we report that ACB extract inhibits the proliferation of SCC-25 cells. In the previous study, ACB extract has been reported to reduce the viability of human lung and oral KB cancer cell lines.[21] Further, *A. catechu* is proven for its cytotoxic potentials against various cell lines.[22] These findings suggest the fact that active phytochemical components present in *A. catechu*

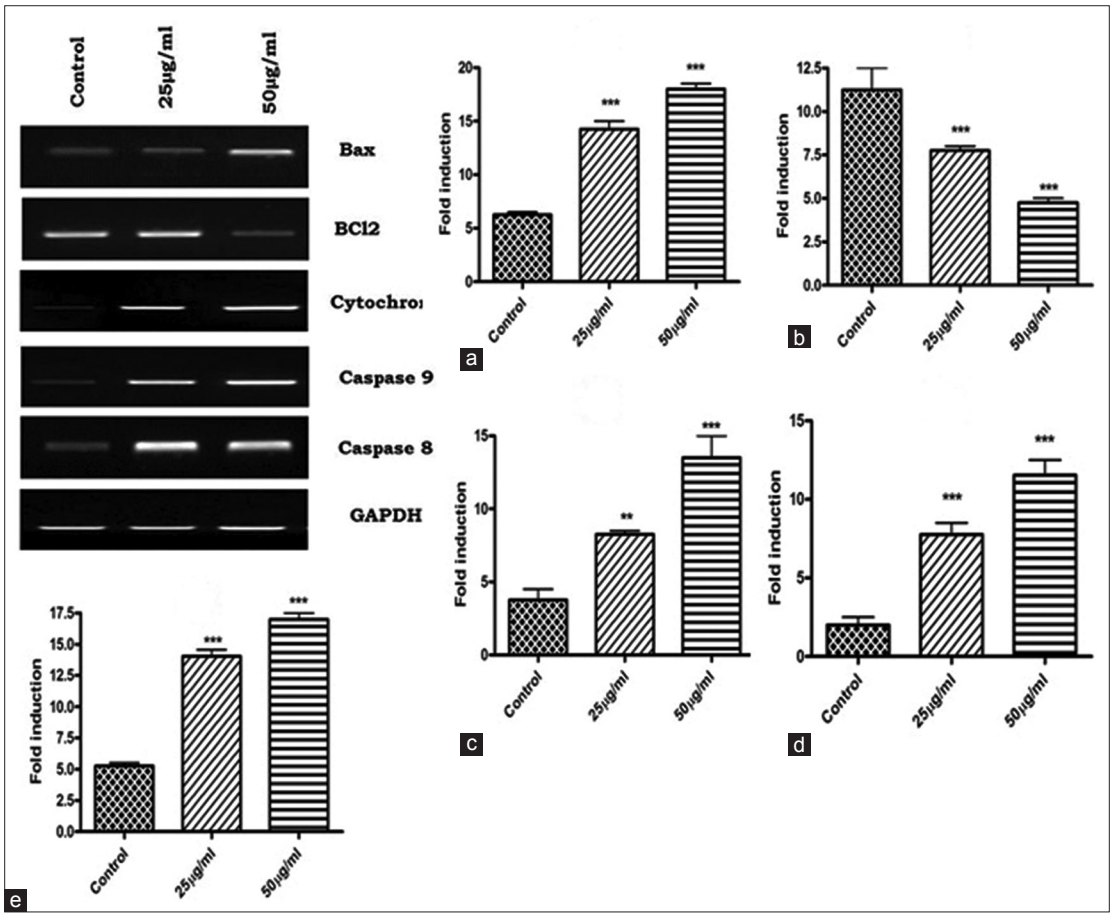

Figure 4: Apoptotic marker gene analysis. (a) Bax; (b) Bcl2; (c) Cytochrome c; (d) Caspase 9; (e) Caspase 8. GAPDH used as an internal control for optimization. Quantification of gene expression values are expressed as mean ± standard error of the mean ($n = 3$). **$P < 0.01$, ***$P < 0.001$

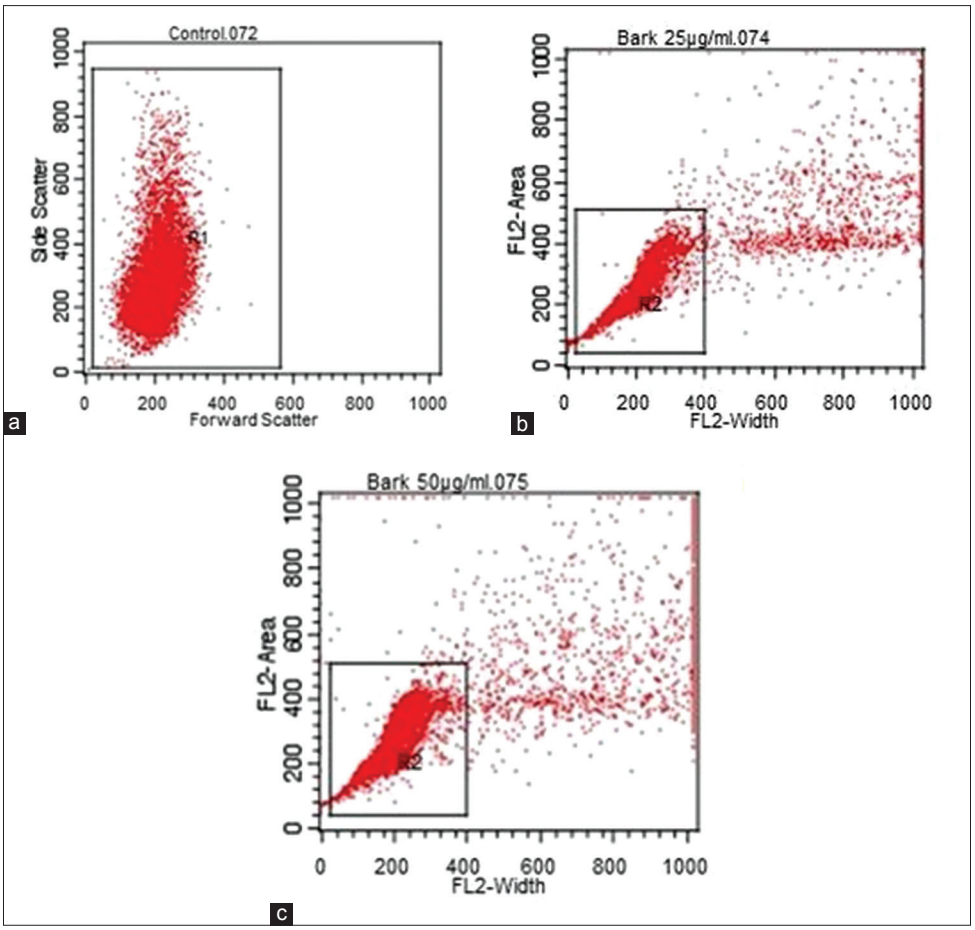

Figure 5: Cell cycle analysis by FACS. (a) control; (b) *Acacia catechu* bark extract 25 µg/mL treatment; (c) *Acacia catechu* bark extract 50 µg/mL treatment

are responsible for the mechanism by which they induce cytotoxicity and our current results are in agreement with these reports.

Staining of apoptotic cells with fluorescent dyes such as AO/EB is considered one of the methods for evaluating the nuclear morphology changes.[23-25] Previous studies have performed AO/EB staining and reported that early apoptotic cells had fragmented DNA which exhibited intense green colored nuclei. Late apoptotic and necrotic cells DNA were fragmented and stained orange and red.[19] In light of the above reports, the presence of high orange stain intensity in cells treated with high concentration of ACB extract further confirm the DNA fragmentation and apoptosis.

Mechanism of apoptosis induction in tumor cells is imperative in tumor therapy and cancer molecular biology. The caspases-8 and 9 involve extrinsic and intrinsic pathways of apoptosis.[26] Initiator caspases, i.e., 8 and 9 activate executioner caspases-3, 6 and 7 that subsequently coordinate their activities to demolish key structural proteins and activate other enzymes.[27] In the present study, caspases-8 and 9 gene expressions have significantly increased which indicate the fact that ACB extract has the ability to induce apoptosis in SCC-25 cells. Interestingly, it has been reported that once activated, caspase-8 can induce either directly or indirectly the activation of a number of executioner caspases such as caspases-3, 6 and 7 which induces apoptosis.[28,29] Cyt-c expression has been significantly increased after treatment of ACB extract in SCC-25 cells when compared to control. The caspase-9, respond to the release of Cyt-c from the mitochondria.[30-32] On entering the cytosol, Cyt-c activates caspases-3 and 9 and subsequently induction of apoptosis.[30] This could be the probable reason for the proapoptotic potential of ACB extract treatment in SCC-25 cells. The bcl-2 play pivotal role in apoptosis and this molecule is over-expressed in many types of cancer cell.[33] The protein bcl-2 interferes with the activation of caspases by preventing the release of Cyt-c.[34] In this study, we found downregulation of bcl-2 gene expression with concomitant upregulation in the expression of bax on ACB treatment which further correlates the increase in the Cyt-c expression [Figure 6]. Downregulation of bcl-2 and its related upregulation of bax expression have been reported to promote apoptosis in response to anticancer drugs[35] and the current results

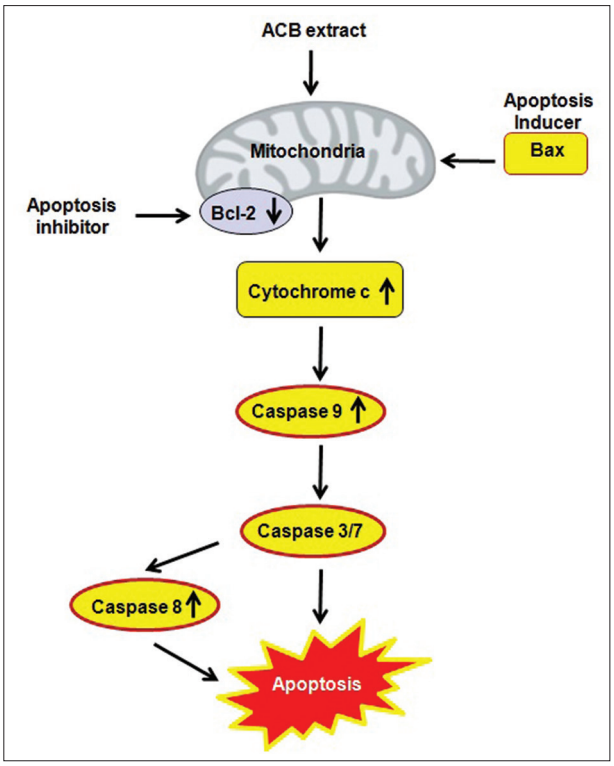

Figure 6: Probable mechanism of apoptosis induction by *Acacia catechu* bark treatment in squamous cell carcinoma cell line-25 cells

are in agreement with these reports. Similarly, cell cycle analysis also shows dose-dependent accumulation of cells in S-phase on ACB treatment implies that the cells are unable to duplicate due to DNA damage in the middle of duplicating their DNA.

CONCLUSION

A. catechu ethanolic bark extract treatment to human squamous carcinoma SCC-25 cells results in apoptosis induction possibly by two molecular mechanisms. First, earlier activation of apoptosis initiator caspases-8 and -9 gene expressions, second, this treatment also caused significant up-regulation of bax gene expression with concomitant downregulation of bcl-2 this in turn release of Cyt-c from mitochondria (intrinsic or mitochondrial apoptotic pathway) leading to apoptosis. These observations suggest that ACB extract may be useful as a therapeutic agent for the attenuation of oral squamous cell carcinoma.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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