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ORIGINAL ARTICLE

Acacia catechu Ethanolic Seed Extract Triggers Apoptosis of SCC-25 Cells

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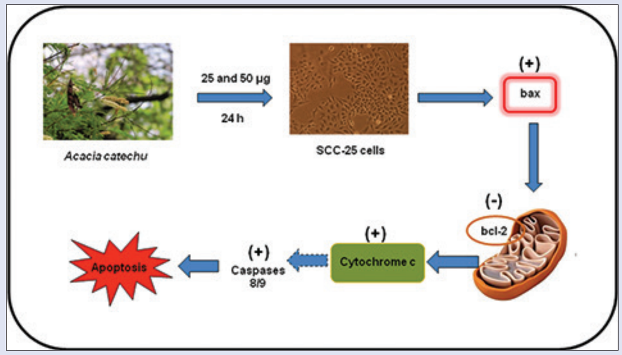
ABSTRACT

Background: *Acacia catechu* Willd (*Fabaceae*), commonly known as catechu, cachou, and black cutch, has been studied for its hepatoprotective, antipyretic, antidiarrheal, hypoglycemic, anti-inflammatory, immunomodulatory, antinociceptive, antimicrobial, free radical scavenging, and antioxidant activities. **Objective:** We evaluated the cytotoxic activity of ethanol extract of *A. catechu* seed (ACS) against SCC-25 human oral squamous carcinoma cell line. **Methods:** Cytotoxic effect of ACS extract was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, using concentrations of 0.1–1000 μg/mL for 24 h. *A. catechu* ethanol seed extract was treated SCC-25 cells with 25 and 50 μg/mL. At the end of treatment period, apoptotic marker gene expressions such as caspase 8, 9, Bcl-2, Bax, and cytochrome c were evaluated by semiquantitative reverse transcription-polymerase chain reaction. Morphological changes of ACS treated SCC-25 cells was evaluated by acridine orange/ethidium bromide (AO/EB) dual staining. Nuclear morphology and DNA fragmentation was evaluated by propidium iodide (PI) staining. **Results:** *A. catechu* ethanol seed extract treatment caused cytotoxicity in SCC-25 cells with an IC_{50} value of 100 μg/mL. Apoptotic markers caspases 8 and 9, cytochrome c, Bax gene expressions were significantly increased upon ACS extract treatment indicate the apoptosis induction in SCC-25 cells. This treatment also caused significant downregulation of Bcl-2 gene expression. Staining with AO/EB and PI shows membrane blebbing, and nuclear membrane distortion further confirms the apoptosis induction by ACS treatment in SCC-25 cells. **Conclusion:** The ethanol seed extracts of *A. catechu* was found to be cytotoxic at lower concentrations and induced apoptosis in human oral squamous carcinoma SCC-25 cells.

Key words: Apoptosis, caspases, cytotoxicity, oral cancer

SUMMARY

- *Acacia catechu* ethanolic seed extract contains phytochemicals such as epicatechin, rutin, and quercetin
- *Acacia catechu* seed (ACS) extract significantly ($P < 0.001$) inhibits the active proliferation of human oral squamous carcinoma (SCC-25) cells
- ACS extract treatment to SCC-25 cells significantly modulated the gene expressions pertaining to apoptosis and propidium iodide and acridine orange/ethidium bromide staining also confirm the apoptosis induction
- Antiproliferative and apoptosis inducing activities of ACS extract is correlated with phytochemical contents.



Abbreviations used: ACS: *Acacia catechu* seed extract; MTT: 3 (4,5 dimethylthiazol 2 yl) 2,5 diphenyltetrazolium bromide; DMSO: Dimethyl sulfoxide; AO/EO: Acridine orange/ethidium bromide; LC MS: Liquid chromatography mass spectrometry.

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INTRODUCTION

Cancer prevalence is increasing every year and it is responsible for the third most cause of death in developing countries. In particular, oral cancer is the sixth most common cancer in the world and one of the significant public health issues worldwide. In India, oral cancer is the third most common type which accounts for over 30% of all cancers. In developing countries, it affects people from lower socioeconomic groups due to a higher exposure to risk factors such as the tobacco use.[1] However, oral cancer is a preventable disease, as aforesaid its causes are directly related to behavioral and lifestyle factors, including tobacco and alcohol.[2]

Effective anticancer treatment can prolong and improve the patient's quality of life. Surgery, radiotherapy, and chemotherapy are the major treatment modalities employed in oral cancer patients depending upon the stage of cancer. Radiation therapy has been used successfully as the primary modality for treating patients with early stage of oral cancer, and it is the standard of care for use as adjuvant therapy in postoperative cases of patients with advanced stage oral cancer as well.[3] Surgery or radiation is said to effective only when the tumor is localized and small in size, and chemotherapy could be effective for a small sized tumors.[4] More than 20 oral cytotoxic drugs are available for oral cancer including 6-mercaptopurine, methotrexate, and busulfan and are widely used in oral chemotherapy. The use of these oral chemotherapy drugs has been

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reported to have narrow clinical setting in which their use is indicated and besides, they might induce certain side effects.[5] Therefore, there is a real need for new anticancer drugs with reduced side effects that could treat chemotherapy-induced adverse effects mainly nausea, vomiting, and metallic taste. To overcome these side effects caused by cancer chemotherapy, several experimental studies have concretely reported the anticancer efficacy of medicinal plants against several human *in vitro* cancer cell lines and came out with promising results.[6,7] Plant phytochemicals are diverse group of compounds that are found naturally in fruits, vegetables, spices, and medicinal plants and they have implicated for their anticancer properties.[8]

Acacia catechu Willd (*Fabaceae*), commonly known as catechu, cachou, and black cutch, is a moderate size deciduous, thorny tree widely distributed in India. Various parts of this plant have been used since ancient times in Ayurvedic medicine.[7] Numerous natural bioactive compounds for instance 4-hydroxybenzoic acid, kaempferol, quercetin, 3,4,7-trihydroxyl-3,5-dimethoxyflavone, catechin, rutin, isorhamnetin, epicatechin, afzelechin, epiafzelechin, mesquitol, ophioglonin, aromadendrin, and phenol have been isolated from heartwood, bark, roots, leaves and stem of *A. catechu* and presence of the above active compounds have been implicated for its myriad biological effects.[9-13] The phytochemicals isolated from this plant have been widely studied for their cytotoxic potentials against variety of cancer cell lines and came out with promising results.[14-16] *A. catechu* has been studied for its hepatoprotective, antipyretic, antidiarrheal, hypoglycemic, anti-inflammatory, immunomodulatory, antinociceptive, antimicrobial, free radical scavenging, and antioxidant activities.[17-22] *A. catechu* is also studied for its anticancer and cytotoxic potentials against HeLa, COLO-205, and HT-1080 cell lines *in vitro*.[23] However, the anticancer efficacy of *A. catechu* seed (ACS) extract against human oral squamous carcinoma SCC-25 cells remains unknown. Hence, in the present study, we evaluated the anticancer effect of ACS extract in human oral squamous carcinoma SCC-25 cells.

METHODS

Reagents and 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

ACS was collected during the month of December 2015 from Hosur, Tamil Nadu, India, authenticated by Green Chem Lab, Bengaluru, Karnataka, India. Seeds were washed and shade dried for a week and was milled to fine powder. This seed powder was passed through 100 mesh sieve and stored in a sealed polythene bag 2.5 kg of powdered ACS were extracted with 10 L of ethanol, at 65°C, for 1 h, in a 20 L round bottom flask with Graham condenser attached. Condenser was cooled circulating with chilled water. After 1 h of extraction, round bottom flask was cooled to room temp and the extract were filtered and collected. The marc, an insoluble residue was extracted repeatedly with 10 L of ethanol, twice. The extracts were filtered and collected. The combined extracts was evaporated to dryness under reduced pressure in a Buchi rotary evaporator (Switzerland) at 65°C, to obtain 150 g of seed powder extract. The w/w yield of the prepared extract was 6%.

Phytochemical analysis by liquid chromatography-mass spectrometry

Presence of phytochemicals such as epicatechin, rutin, and quercetin were analyzed by liquid chromatography-mass spectrometry (LC-MS) method. High performance liquid chromatography system (Shimadzu LC-20AD Prominence gradient system) was equipped with column was a Phenomenex C-18, Luna, SS column, 150 mm × 4.6 mm. Five micron particle size. The mobile phases were (A) 0.1% formic acid in water (B): acetonitrile (gradient System) at a flow rate of 1000 μL/min. The LC conditions gradient system was 10% B during 0.01 min, a linear increase from 10% to 50% during 70 min. Detection was performed at 280 nm and injection rate was 10 μL. The LC instrument was equipped with MS instrument (Shimadzu LC MS 8030; triple quadrupole mass analyzer).

Cell culture

The SCC-25 human oral squamous carcinoma cell line was procured from ATCC. Cells were maintained in Dulbecco's minimum essential media and Ham's F-12 (1:1 ratio) supplemented with 10% fetal bovine serum with 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were cultured 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%–80% confluence. Upon reaching confluence, cells were detached using 0.05% trypsin-ethylenediaminetetraacetic acid solution.

Cell treatment

A. catechu ethanol seed 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 ACS extract (25 and 50 μg/mL) or the corresponding volumes of the vehicle. The ACS extract concentration used in this study was selected based on the evaluation of IC_{50} concentration. After 24 h of treatment, cells were collected by trypsin application. Total cell number was determined by counting each sample in triplicate under inverted microscope. Viability was also evaluated by the trypan blue dye exclusion assay.

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

Cytotoxic effect of ACS extract on SCC-25 cells was assessed by MTT assay[24] using concentrations of 0.1–1000 μg/mL for 24 h. 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 ACS extract (0.1–1000 μg/well) and incubated for 24 h. Untreated cells served as control and received only 0.1% DMSO. At the end of treatment period, media from control, seed extract-treated cells was aspirated and 50 μL of MTT (5 mg/mL in phosphate-buffer saline [PBS]) was added to each well. Cells were then incubated inside the incubator. MTT was then discarded and the coloured crystals of produced formazan were dissolved in 150 μL of DMSO and mixed effectively. The purple blue formazan dye formed was measured using an ELISA reader (Bio-Rad, Hercules, CA, USA) at 570 nm.

Acridine orange/ethidium bromide (dual staining)

Acridine orange/ethidium bromide (AO/EO) orange staining was carried out by the method of Gohel *et al.*[25] SCC-25 cells were plated at a density of 1×10^4 in 48-well plates. They were allowed to grow until they are 70%–80% confluent. After 24 h the cells were treated with 25 and 50 μg/mL of ACS extract for 24 h. The culture medium was aspirated from each well and cells were gently rinsed twice with PBS at room temperature. Then equal volumes of cells from control and ACS extract treated were mixed with 100 μL of dye mixture (1:1) of EO and AO and viewed immediately under inverted fluorescence microscope (Nikon [Ti series] at ×10 magnification). A minimum of 300 cells were counted in each sample at two different fields. The percentage of apoptotic cells was determined by (% of apoptotic cells = [total number of apoptotic cells/total number of cells counted] ×100).

Assessment of nuclear morphology after propidium iodide staining

Propidium iodide (PI) staining was carried out by the method of Chandramohan *et al.*[26] SCC-25 cells were plated at a density of 1×10^4 in 48 well plates. They were allowed to grow until they are 70%–80% confluent. Then, cells were treated with 25 and 50 μg/mL of ACS extract for 24 h. After 24 h, culture medium was aspirated and 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 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. Concentration of the extracted RNA was determined and the integrity of RNA was visualized on a 1% agarose gel using a gel documentation system (Bio-Rad, Hercules, CA, USA). The first strand of cDNA was synthesized from 1 μg of total RNA by reverse transcriptase using M-MLV (Promega, Madison, WI, USA) and oligo (dT) primers (Promega, Madison, WI, USA) according to the manufacturer's protocol. Then, 2 μL of template cDNA was added to the final volume of 20 μL of reaction mixture. Reverse transcription-polymerase chain reaction (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 the specific sets of primer for Bax, Bcl-2, cytochrome c, caspase 8, caspase 9 and GAPDH used in this study were taken from literatures. Expressions of selected genes were normalized to the GAPDH gene, which was used as an internal housekeeping control. All the RT-PCR experiments were performed in triplicate.

Statistical analysis

Data were expressed as mean ± standard error mean and analyzed by Tukey's test to determine the significance of differences between groups. $P < 0.05$, 0.01, and/or 0.001 was considered statistically significant.

RESULTS

Inhibitory effects of *Acacia catechu* seed extract against human SCC-25 oral carcinoma cells

The antiproliferative effect of ACS extract in SCC-25 cells was evaluated by MTT assay. ACS extract treatment for 24 h significantly ($P < 0.001$) inhibits the proliferation of oral squamous carcinoma cells. The maximum antiproliferative effect was found to be more than 80% at a maximum concentration used in this study i.e., 1000 μg/mL of ACS extract [Figure 1].

Phytochemical analysis

As shown in the chromatograms, we were able to separate the phytochemical compounds present in the ACS extract in 30 min. The presence of phytochemicals such as epicatechin, rutin, and quercetin were analyzed and confirmed by LC-MS with their corresponding standards. The retention times of epicatechin, rutin, and quercetin were separated on 10.486, 18.408, and 30.930 min, respectively. We thus confirmed that the ACS extract contain phytochemical such as epicatechin and small amounts of rutin and quercetin [Figure 2].

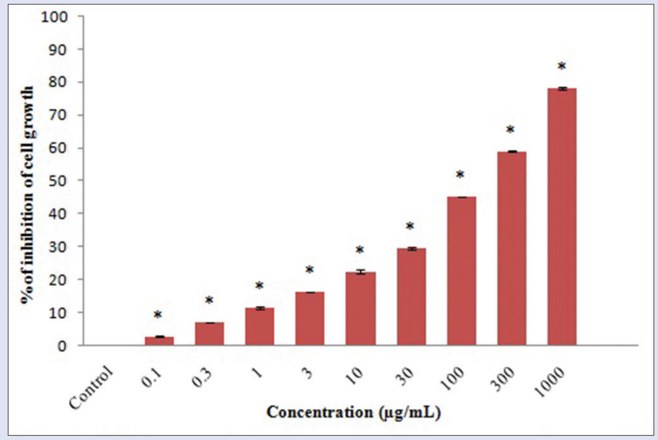

Figure 1: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay analysis of cytotoxicity. Values are expressed as mean ± standard error mean ($n = 3$). Control compared with different *Acacia catechu* seed extract treated concentrations. *$P < 0.001$

Apoptotic marker gene expressions

To examine the molecular mechanism underlying apoptosis process, we evaluated the gene expression analysis of cytochrome c, caspase 8 and caspase 9. The result shows that activation of caspase 8 and 9, and cytochrome c gene expressions in ACS extract treated groups were expressed highly than that of vehicle treated control. The data also shows that ACS extract caused significant down regulation of Bcl-2, an inhibitor of apoptosis and up regulation of Bax expression in SCC-25 cells [Figure 3a-e].

Acridine orange/ethidium bromide double-staining and fluorescent microscopy

To determine the effect of ACS treatment on the induction of apoptosis in SCC-25 cells, AO/EB immunofluorescence staining was performed to detect the number of apoptotic cells. Our result shows that no significant change and apoptosis was detected in the control group [Figure 4a]. Early-stage apoptotic cells, marked by yellow green AO nuclear staining, were detected in the experimental (25 μg/mL) ACS extract treatment [Figure 4b]. With increasing concentrations (50 μg/mL ACS), the number of early apoptotic cells increased along with late-stage apoptotic cells, orange nuclear EB staining, were also detected [Figure 4c]. The apoptotic cell number were significantly increased ($P < 0.001$) in dose dependent manner [Figure 4d].

Nuclear fragmentation analysis by propidium iodide staining

Nuclear morphology was evaluated with membrane-permeable PI staining. The DMSO treated cells did not show any significant change in the morphology of SCC-25 cells [Figure 5a]. The ACS extract treated SCC-25 cells were reported to induce apoptosis [Figure 5b and c]. The ACS extract induced apoptotic cells displayed characteristic features of reduced size, intense fluorescence of condensed nuclear chromatin, and formation of membrane blebs. The number of apoptotic nuclei noticed was significantly ($P < 0.001$) increased after treatment with 25 and 50 μg/mL of ACS extract [Figure 5d].

DISCUSSION

The plant-based drug discovery resulted primarily in the development of antioxidant, anticancer, and other anti-infectious agents and continues to contribute to the new leads in clinical trials.[27] Plant-based drug therapy is a new therapeutic modality which targets specific molecules in the cancer cell and modulates signaling pathways involved in carcinogenesis.[28] *A. catechu* has been extensively studied for its myriad beneficial effects against various etiologies.[7,21,23,29] Hence, in this study, we investigated the anticancer effect of ACS extract on human oral carcinoma cell lines. Probably, this could be the first study reporting the anticancer efficacy of ACS extract on human oral cancer cells.

In this study, ACS extract treatment significantly inhibits the proliferation of human oral squamous carcinoma cells in dose dependant manner. The profound antiproliferative efficacy of ACS extract found in this study could be due to presence of various phytochemical components in this plant as suggested by Alam *et al.* 2012.[29] Nevertheless, the data regarding phytochemicals present in ACS extract are not reported or scanty. In this study, we report that the presence of phytochemicals such as epicatechin, rutin, and quercetin in ACS extract. The cytotoxic effect of these phytochemicals isolated from different herbal plants was reported previously and was associated with their anticancer effects[30-32] and our current results are in agreement with these reports.

Apoptosis, programmed cell death plays a key role in the pathogenesis of various degenerative diseases and cancer. Induction of cancer cell apoptosis is one of the underlying principles of most current cancer therapies.[33] The apoptotic signals are complicated, and they are regulated at several levels. The tumor cells may use several molecular mechanisms to suppress apoptosis and acquire resistance to apoptotic agents, for instance, by the expression of antiapoptotic genes such as Bcl-2 or by the downregulation of proapoptotic gene expression such as Bax.[34] Bcl-2 is compartmentalized in an outer membrane of mitochondria, where it plays an important role in promoting cellular survival and inhibiting the actions of pro-apoptotic proteins (Bax). The pro-apoptotic proteins in the Bcl-2 family, including Bax, reported to interact with mitochondrial membrane to promote permeabilization, which leads to the loss in membrane potential which in turn release of cytochrome c that is one of the important signals in the apoptosis cascade.[35] These pro-apoptotic proteins (Bax) are in turn inhibited by the function of Bcl-2.[36] In light of the above reports, it is suggested that ACS treatment on SCC-25 cells could have increased the pro-apoptotic Bax gene expression and thus inturn decrease in Bcl-2 expression leading to apoptosis.

Apoptosis is characterized by biochemical changes that include caspase activation, breakdown of DNA, and protein and membrane surface modifications that allow the apoptotic cell to be recognized and engulfed by phagocytic cells as well as morphologic changes, such as chromatin condensation, nuclear fragmentation and reduction of cell volume.[37] There are two major mechanisms exist that induce apoptosis

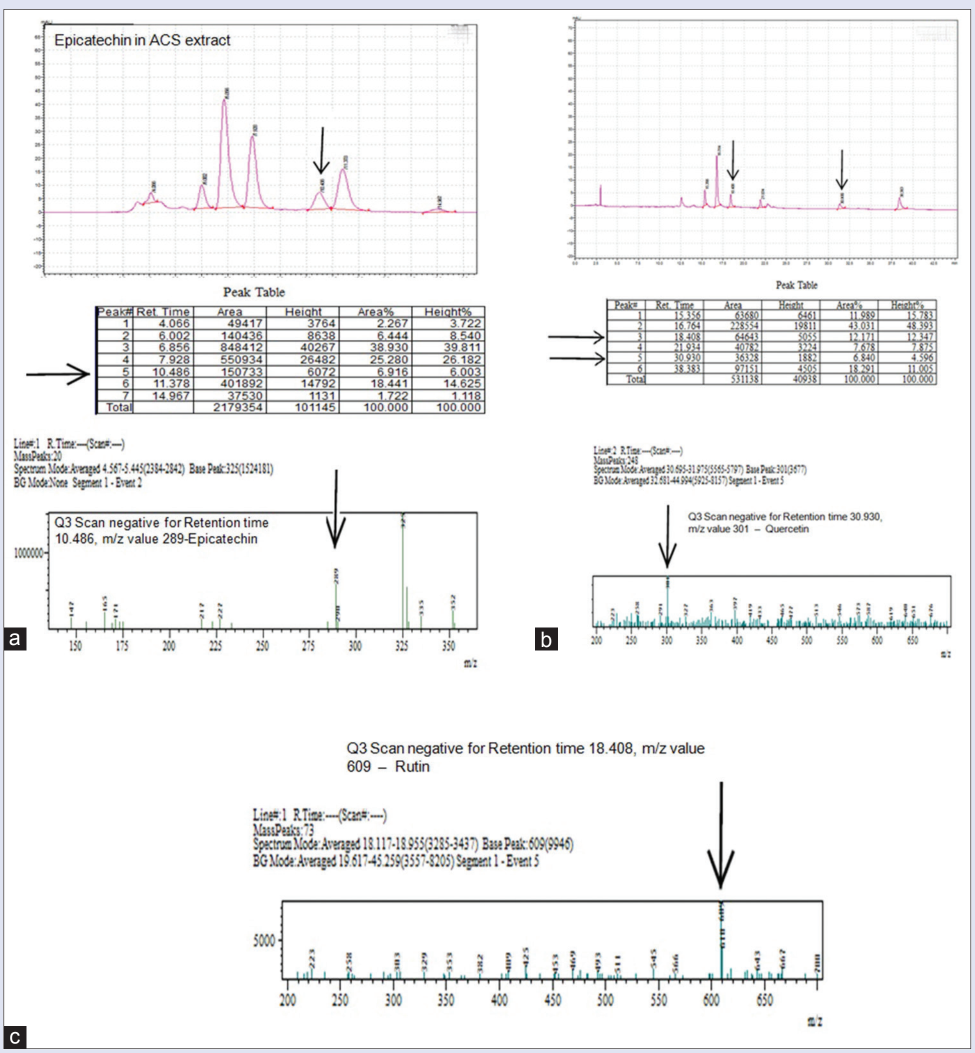

Figure 2: Liquid chromatography-mass spectrometry analysis of epicatechin (a), quercetin (b) and rutin (c)

by initiating the caspase cascade. The extrinsic pathway involves caspase 8 and the intrinsic pathway involves caspase 9 as the initiator caspase and plays a central role in the apoptosis.[38] In this study, apoptotic gene expression of caspases 8 and 9 increased significantly in ACS treated SCC-25 cells. In addition, cytochrome c expression also increased upon the above treatment. It has been reported that mitochondria, perhaps key regulators of apoptosis, have shown to be involved in integrating different pro-apoptotic pathways via release of cytochrome c into the cytosol.[39] The released cytochrome c is complexed with Apaf-1 and pro-caspase 9 in a deoxyadenosine triphosphate-dependent manner to form the "apoptosome" from which the release of activated caspase 9 further initiates the activation of caspase cascade leading to biochemical and morphological changes associated with apoptosis.[40] Therefore, release of cytochrome c from mitochondria is considered a key initial step in the apoptotic process. In light of these reports, it is suggested that the ACS treatment to SCC-25 cells could have caused the cytochrome c release in cytoplasm by altering of mitochondrial membrane potential that results in apoptosis via activation of caspase cascade.

The result of the above biochemical events may alter the morphologic appearance of dying cell through DNA damage. Hence, apoptosis was further confirmed by analyzing the nuclear morphology of ACS extract treated SCC-25 cells by PI staining. It has been reported that morphological hallmarks of apoptosis are consequence to DNA fragmentation and membrane blebbing.[41] In this study, the percentage of apoptotic nuclei after treatment with 25 and 50 μg/mL of ACS extract were increased significantly. Propidum iodide staining shows that ACS treatment could induce the nuclear morphological changes and DNA fragmentation as suggested by previous reports.[42] Further, staining of apoptotic cells with fluorescent dyes such as AO/EB is considered one of the methods for evaluating the morphology changes.[43,44] Not surprisingly, in this study, at both concentration of ACS treatments showed significant presence of early (yellow green fluorescence nucleus) and late apoptotic cells (orange colored nucleus) by AO/EB dual staining, which further confirm apoptosis induction. It has been reported previously that early apoptotic cells had fragmented DNA which exhibited intense green colored nuclei. While late apoptotic and

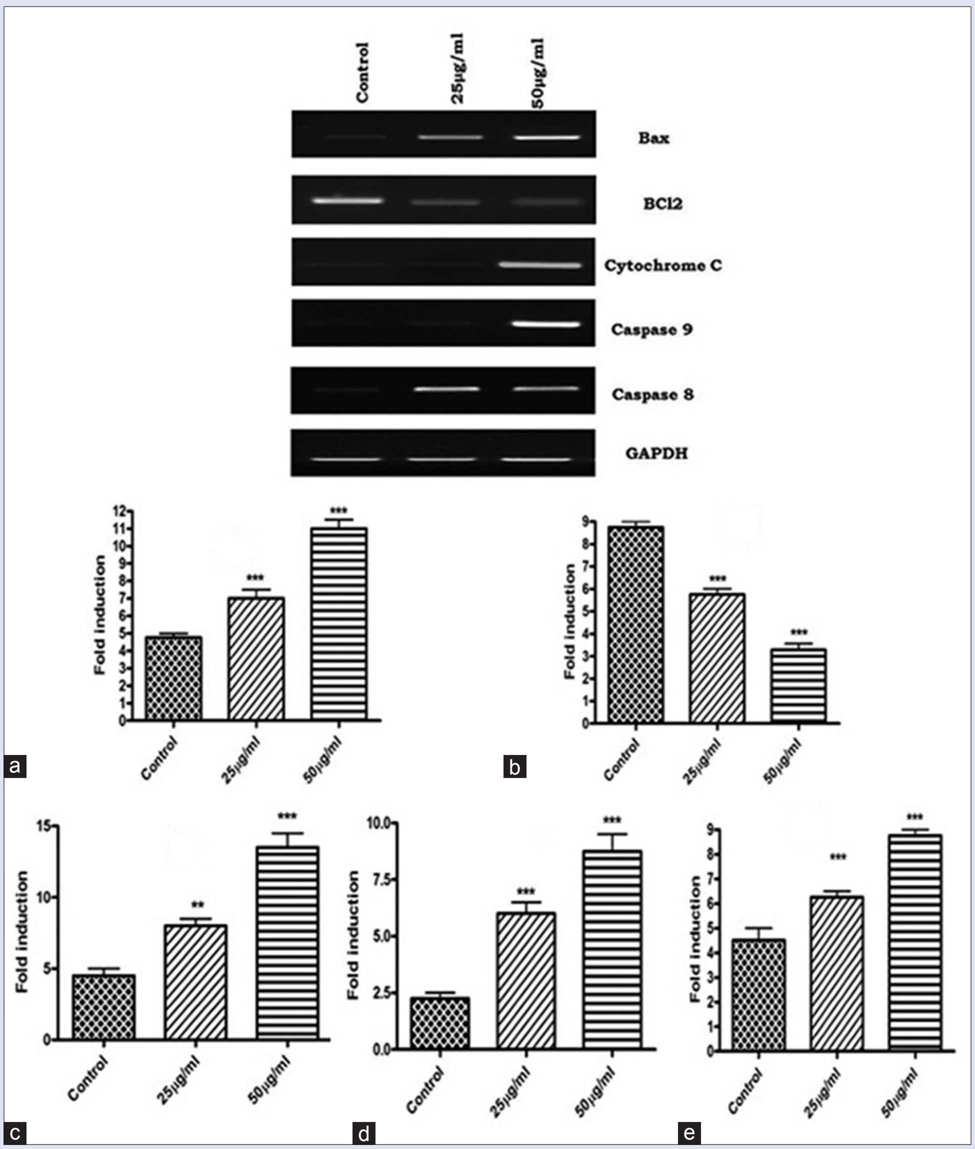

Figure 3: Apoptotic marker gene analysis. (a) Bax; (b) B-bcl2; (c) cytochrome; (d) Caspase 9; (e) Caspase 8. Quantification of apoptotic marker gene expressions. Values are expressed as mean ± standard error mean ($n = 3$). $^{**}P < 0.01$, $^{***}P < 0.001$

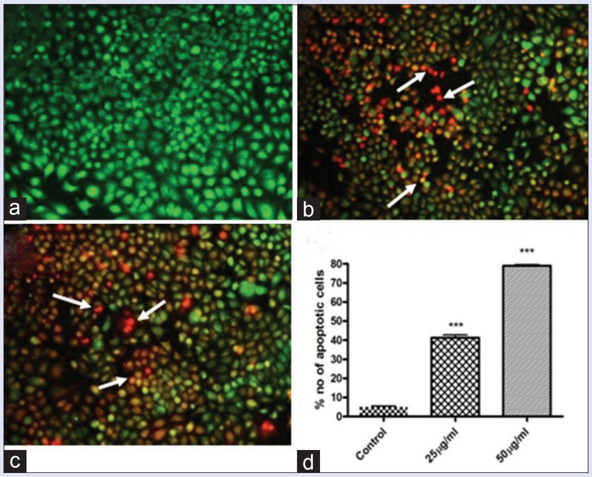

Figure 4: Apoptosis analysis by acridine orange/ethidium bromide (×10). (a) Negative control group (normal cells); (b) Nucleus showed yellow–green fluorescence by acridine orange staining (early apoptotic cells) and also showed orange fluorescence by ethidium bromide (late apoptotic cells) *Acacia catechu* seed extract 25 μg/ml treatment; (c) Nucleus showed yellow–green flurescence by acridine orange staining (early apoptotic cells) and showed orange fluorescence by ethidium bromide (late apoptotic cells) *Acacia catechu* seed extract 50 μg/ml treatment. (d) Quantification of apoptotic cells. Values are expressed as mean ± standard error mean ($n = 3$). ***$P < 0.001$

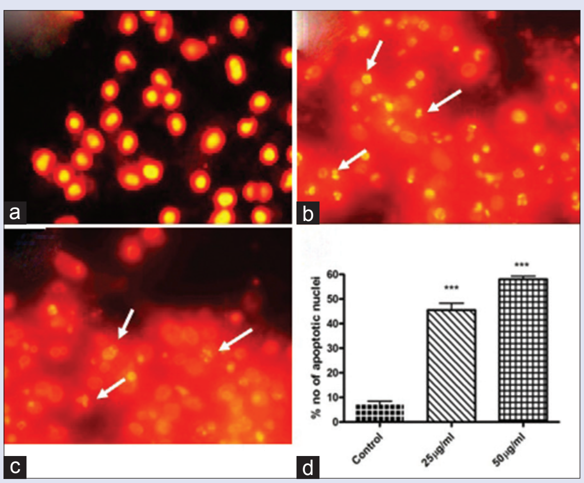

Figure 5: Nuclear morphology analysis by propidium iodide staining (×10). (a) Control; (b) *Acacia catechu* seed extract 25 μg/ml treatment; (c) *Acacia catechu* seed extract 50 μg/ml treatment. White arrow indicate the apoptotic cells with fragmented nuclei. (d) Quantification of apoptotic nuclei. Values are expressed as mean ± standard error mean ($n = 3$). ***$P < 0.001$

necrotic cells DNA were fragmented and stained orange and red[25] and our present results are in agreement with this report.

CONCLUSION

In this study, we were able to show that ACS extracts triggers apoptosis in human oral squamous carcinoma SCC-25 cells through caspases activation involving dissipation of mitochondrial cytochrome c release into the cytosol. Expression of apoptosis initiator caspases and proapoptotic molecule Bax was significantly increased and decrease in the Bcl-2 gene expression was well correlated with apoptosis induction. Our phytochemical analysis and molecular investigations were well corroborated with morphological confirmation of apoptosis was provided by AO/EB dual staining and PI nuclear staining.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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