RESEARCH PAPER

Ethanolic Extract of Adhatoda Vasica Induces Apoptosis in Kb Human Oral Cancer Cell Line Model

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ABSTRACT

The objective of this research is to examine the anticancer efficacy of Adhatoda vasica extract against oral cancer utilizing KB human oral cancer cell models. Oral cancer continues to be a significant health burden, necessitating the urgent discovery of innovative therapeutic compounds derived from plants. Adhatoda vasica, a medicinal plant, was evaluated for its cytotoxic effects on KB cells. Various quantities of the plant extract were administered to the cells, and cell viability was assessed using established assays to evaluate its efficacy in inhibiting the proliferation of malignant cells. The results demonstrated a dose-dependent reduction in cell viability, suggesting that the extract can induce cytotoxicity in oral cancer cells. The comprehensive research revealed that the cells exhibited morphological alterations typical of apoptosis, suggesting that Adhatoda vasica may induce programmed cell death pathways. Consequently, these findings suggest that the extract may serve as a complementary or alternative therapeutic agent in the treatment of oral cancer. Adathoda vasica extract regulates the expressions of p53, Bax, Bcl2, and caspase-3. The study advocates for additional research on the extraction of active compounds, clarification of the molecular pathways involved, and in vivo validation of the extract's effectiveness. This research contributes to the body of plant-based therapies in oncology and advocates for further investigation of traditional medicinal herbs for cancer treatment.

Keywords: Oral cancer, Adhatoda vasica, p53, Apoptosis, Bcl2, Bax

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INTRODUCTION

Cancer, a multifactorial illness marked by unregulated cell proliferation, invasion, and metastasis, continues to be a primary cause of mortality globally [1,2]. Oral cancer, a form of head and neck cancer, is one of the ten most common malignancies worldwide. Although laryngopharyngeal cancer is prevalent in various demographics, oral cancers are more frequent on the Indian subcontinent [3]. Asia, specifically India, constitutes 57.5% of global oral cancer cases. It constitutes 30% of all cancer cases in India, with 60–80% of patients presenting with advanced malignancies, in contrast to 40% in affluent countries [4]. This signifies an absence of knowledge and the necessity for early detection signs. Notwithstanding considerable progress in diagnosis and treatment, the overall survival rate for oral cancer has exhibited negligible enhancement over recent decades. This stalemate is primarily due to late-stage diagnosis and aggressive tumor behavior, which remain a significant clinical challenge. Consequently, oral cancer has become a critical public health concern, particularly in low- and middle-income nations [5].

Contemporary therapeutic approaches for oral cancer predominantly encompass surgical procedures, radiotherapy, and chemotherapy. A range of chemotherapy drugs, including as doxorubicin, cisplatin, carboplatin, 5-fluorouracil, paclitaxel, docetaxel, and hydroxyurea, are frequently utilized. The clinical efficacy of these medicines is frequently undermined by their systemic toxicity and significant adverse drug reactions, prompting the pursuit of safer and more effective alternatives [8]. In this context, natural compounds have garnered significant interest as prospective anticancer drugs [9,10]. Plant-derived bioactive chemicals have significant therapeutic advantages owing to their varied pharmacological properties, biocompatibility, and decreased toxicity [11]. Traditional medical systems, like Ayurveda, have

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historically employed phytotherapeutic treatments for the treatment of several illnesses, and contemporary scientific research is progressively corroborating these traditional assertions [12,13].

The principal actions of p53, a tumor suppressor, encompass DNA repair, regulation of cellular proliferation, and induction of apoptosis [14]. The tumor suppressor protein, p53 is necessary to prevent normal cells from acquiring a malignant state. The majority of human cancers exhibit somatic alterations in p53, including mutations, loss of heterozygosity, and deletions, which compromise normal p53 function. Nonetheless, alterations in p53 regulation occur in tumors that possess wild-type p53. Furthermore, p53 has been demonstrated to orchestrate cellular responses to several DNA-damaging oncological therapies, especially apoptosis [15]. Diverse responses have been associated with an individual's ability to respond to different cancer therapies. Restoring p53 functionality is a method for treating cancer associated with the TP53 mutation. This technique encompasses three categories: i) direct inhibition or degradation of mutant p53; ii) binding to mutant p53 to induce a conformational shift to a preferred form; and iii) targeting the viral enzymes responsible for p53 degradation [16-18]. Recent research has concentrated on small molecules that can interact with mutant p53 and induce conformational changes in the protein.

Adhatoda vasica Nees. Justicia adhatoda L., usually referred to as Malabar nut, is a member of the Acanthaceae family. It is extensively utilized in traditional Indian medicine, especially for addressing respiratory ailments such as asthma, bronchitis, and pulmonary infections [20]. The herb is recognized for its anti-inflammatory, antibacterial, antioxidant, expectorant, and antitubercular effects [21-23]. The ethanolic extract of *Adhatoda vasica* is recognized for its ability to suppress α-amylase activity in vitro [24]. Considering these attributes, *Adhatoda vasica* emerges as a prospective candidate for anticancer investigation. This study seeks to examine the anticancer efficacy of the ethanolic extract of *Adhatoda vasica* on the KB cell line, a model for human oral squamous carcinoma. This study aims to investigate the efficacy of *Adhatoda vasica* as a natural therapeutic agent for oral cancer, presenting an innovative, plant-derived strategy for cancer treatment.

MATERIALS AND METHODS

Extraction of plant materials

Fresh and healthy leaves of *Adhatoda vasica* were obtained and shade-dried for one week. After the leaves were thoroughly dried, they were cleaned and powdered to produce a fine powder. The ethanolic extract was made by dissolving the leaf powder in ethanol at a 1:2 ratio and allowing it to stand overnight. On the subsequent day, the mixtures were filtered utilizing Wattman filter paper, and the extracts were preserved at 4°C for future examination.

We acquired the KB (human oral cancer cell line) from NCCS in Pune, India. The cells were maintained in DMEM high glucose medium enriched with 10% FBS and a 1% antibiotic-antimycotic solution within a CO_2 incubator at 5% CO_2 and 18–20% O_2 at 37 °C. The cells were subcultured bi-daily and employed for subsequent investigations.

MTT assay

About 200µl of cell suspension was inoculated into a 96-well plate at the requisite cell density of 20,000 cells per well, devoid of the test agent. The cells were permitted to incubate for approximately 24 hours. Suitable concentrations of the aforementioned test substances were incorporated and diluted in culture media (12.5, 25, 50, 100, 200 µg/ml). The plate was incubated for 24 hours at 37°C in a 5% CO_2 atmosphere. Following the incubation period, the plates were removed from the incubator, and the spent media was discarded. The ultimate concentration of MTT reagent was 0.5 mg/mL of the whole volume. The plates were enveloped in aluminum foil to prevent light exposure. The plates underwent incubation for a duration of three hours. Subsequent to the removal of the MTT reagent, 100 microliters of solubilization solution (DMSO) were introduced. In a gyratory shaker, little agitation enhanced dissolution. Intermittently, pipetting up and down may be necessary to completely dissolve the MTT formazan crystals, particularly in dense colonies. The absorbance was measured using a spectrophotometer at a wavelength of 570 nm.

% Cell viability is calculated using below formula:

% cell viability = [Mean abs of treated cells/Mean abs of Untreated cells] x 100

The KB cell line, subjected to several concentrations of ethanolic extract from *Adhatoda vasica* leaves, exhibited a marked reduction in cell viability and demonstrated cytotoxic properties, with an IC_{50} value of 39 µg/ml. Studies on apoptosis, cell cycle, and reactive oxygen species (ROS) of the ethanolic extract of *Adhatoda vasica* on the KB cell line were conducted, yielding an IC_{50} value of 39 µg/ml.

Apoptotic study on KB cell line with ethanolic extract:

Cells were cultivated in a 6-well plate at a density of 0.5 x 10^6 cells per 2 ml and incubated in a CO_2 incubator at 37°C for 24 hours. Following a 24-hour incubation of the extract and standard substance. Approximately 2 ml of culture media was introduced, and the cells were collected straight into 12 x 75 mm polystyrene tubes. Spin the tubes in a centrifuge for five minutes at 300 x g at 25°C. The supernatant was meticulously decanted. Approximately 5µl of FITC-conjugated Annexin V was added to 100µl of binding buffer. The cells were gently vortexed and incubated for 15 minutes at room temperature (25°C) in the dark. Five microliters of propidium iodide and four hundred microliters of 1X Annexin Binding Buffer were added to each tube, gently vortexed, and subsequently analyzed using a flow cytometer.

Cell cycle study on KB cell line with ethanolic extract

Cells were cultivated in a 6-well plate at a density of 2 x 10^5 cells per 2 ml and incubated in a CO2 incubator at

37°C for 24 hours. The cells were subjected to the requisite concentration of experimental chemicals (IC_{50}) and controls, in 2 ml of culture media, and incubated for 24 hours. The cells were collected and meticulously rinsed. To guarantee that just DNA is stained (since PI stains all nucleic acids), the cell pellet was treated with 400µL of Propidium Iodide/RNase staining buffer and thoroughly mixed. Cells were cultured in the dark at room temperature for 15 to 20 minutes. Samples were examined using flow cytometry in a PI/RNase solution [25].

ROS expression of ethanolic extract treated KB cell

Cells were cultivated in a 6-well plate at a density of 0.5 x 10^6 cells per 2 ml and incubated in a CO2 incubator at 37°C for 24 hours. The wasted medium was aspirated, and the cells were treated with the necessary concentration of experimental compounds (IC50) and controls in 2 ml of culture medium, followed by a 24-hour incubation period. The H2DCFDA stock solution (4 mM) was diluted in DPBS to prepare a 10 µM working solution. The cells were suspended in the H2DCFDA working solution at a density of 1 x 10^6 cells/ml and incubated at 37 °C for 30 minutes, shielded from light. The tubes underwent centrifugation at 150 x g for 5 minutes, after which the supernatant was discarded and the cells were gently resuspended in 400 µl of pre-warmed DPBS. Analysis was conducted via flow cytometry utilizing a 488 nm laser for excitation and detection at 535 nm (FL1).

RNA isolation and quantitative polymerase chain reaction

Following treatment with ADEE, the KB cells were suspended in RNA buffer. The QIAshredder and RNeasy Kit (QIAGEN, Hilden, Germany) were utilized to extract total RNA following the manufacturer's guidelines. The QuantiFast SYBR Green PCR Master Mix (QIAGEN), primers (1 mM; Table 1), and 1 µg of complementary DNA (cDNA) were utilized in a 25-ml reaction mixture within an Eppendorf PCR apparatus to perform real-time polymerase chain reaction (PCR).

Three distinct real-time reverse transcriptase (RT)-PCR assays were conducted to analyze each target and standard β-actin cDNA in duplicate. An activation phase at 95°C for 30 seconds initiated thermal cycling, succeeded by 40 cycles of 95°C for 5 seconds and 60°C for 30 seconds. Melt curve protocols were utilized immediately following amplification to reduce primer dimers and other nonspecific byproducts.

Table 1. The primer sequence

S.No.	Gene	Primer sequences
1	P53	F-5'-TGGGACGGAACAGCTTTGAG-3' R-5'-ATTCTCTTCCTCTGTGCGCC-3'
2	Bax	F-5'-CGCATCGGAGATGAACTGGA-3' R-5'-GTAGAAGAGGGCAACGACCC-3'
3	Bcl2	F-5'- TCTCATGCCAAGGGGGAAAC-3' R-5'- CAATCCTCCCCCAGTTCACC-3'
4	Caspase3	F-5'- CTCTGGTTTTCGGTGGGTGT -3' R-5'- CGAGATGTCATTCGGTTCCA-3'
5	β-Actin	F-5'- AGACCTGTACGCCAACACAG-3' R-5'- TTCTGCATCCTGTCGGCAAT-3'

Statistical analysis

The data were processed using the statistical analysis tool SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) and are provided as means ± S.D. A P value of less than 0.05 was considered significant, and all P values were assessed using a two-tailed approach.

RESULTS

This study aimed to assess the anti-cancer efficacy of the ethanolic extract of *Adhatoda vasica* on the KB cell line. The in vitro cytotoxicity assay (Figure 1)(Microculture Tetrazolium assay, MTT) of ethanolic extract from *Adhatoda vasica* leaf was conducted on KB human oral cancer cell lines at varying concentrations of 12.5, 25, 50, 100, and 200 µg/ml. The percentage of live cells was evaluated following a treatment duration of 24 hours. The extract concentrations of 12.5 µg/ml and 25 µg/ml exhibited cell viability exceeding or approaching 50%, while the concentrations of 50 µg/ml, 100 µg/ml, and 200 µg/ml shown cell viability below 50%, indicating cytotoxicity. Ethanolic extracts of *Adhathoda vasica* exhibited considerable cytotoxicity, with an IC_{50} value of 39 µg/ml.

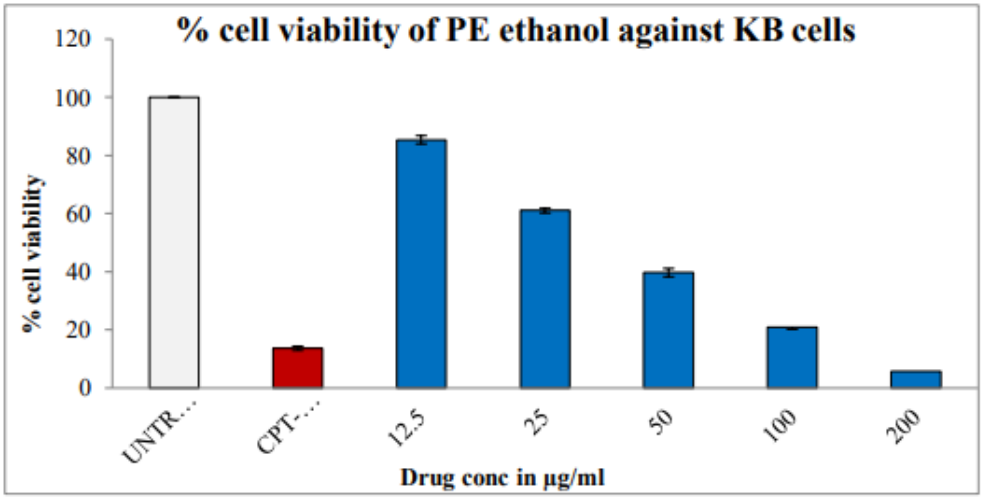
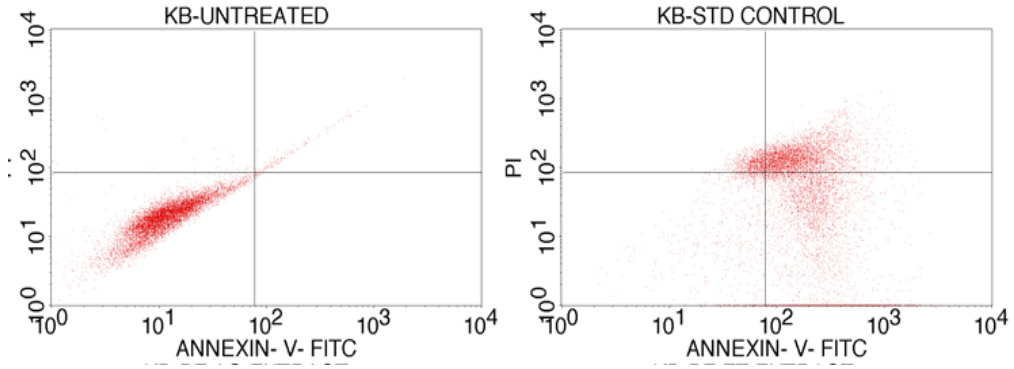


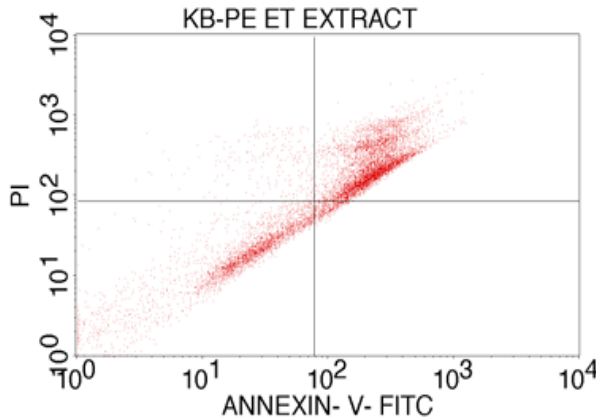
Figure 1. Effect of AVEE on the cell viability on KB cell by MTT assay. The AVEE administration inhibits cell proliferation dose-dependently at 24h treatment.

Figure 2 and Table 2 showed the effect of AVEE on Annexin V-mediated apoptosis in flow cytometer. The apoptotic investigation on the KB cell line demonstrated that the ethanol extracts resulted in a substantial increase of 64.92% in apoptosis of human oral cancer cells. The necrosis rate was 2.52 percent. The percentage of apoptosis in the ethanol extract was comparable to that of the standard control.

Table 2. showed the % cells undergone Apoptosis, Necrosis in treated and Untreated KB cells% cells undergone Apoptosis in untreated & treated KB cells by FACS.

Culture condition	% necrotic cells	%Late apoptotic cells	% viable cells	% early apoptotic cells
Untreated	0.3	1.28	98.24	0.18
Standard	9.13	39.18	6.82	44.87
AVEE	2.52	60.73	32.56	4.19





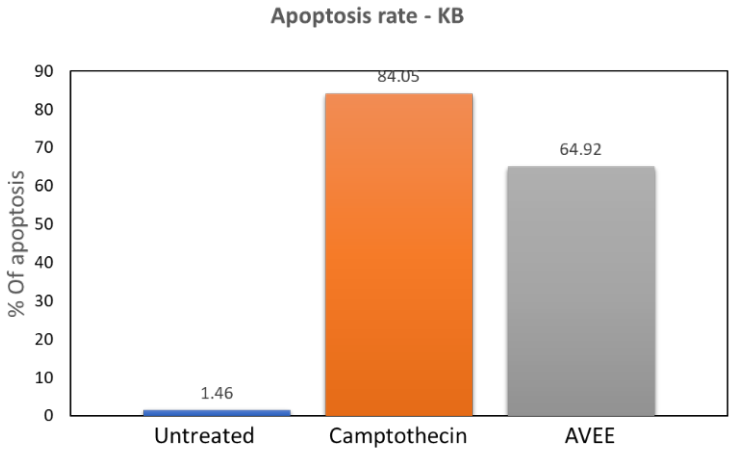


Figure 2. (A) Quadrangular plots illustrated the Annexin V/PI expression in KB cells with Camptothecin and *Adathoda vasica* ethanolic extract (AVEE), as analyzed by Flow Cytometry.

UL – Upper left: Percentage of Necrotic Cells; UR – Upper right: Percentage of Late Apoptotic Cells; LL – Lower left: Percentage of Viable Cells; LR – Lower right: Percentage of Early Apoptotic Cells – Quadrant configuration in Apoptosis/Necrosis analysis. The measurement of the percentage of apoptosis.

Figure 3. showed the cell cycle analysis of AVEE using flow cytometry. Cell cycle study results showed that Ethanol extract of *Adhatoda vasica* showed cell cycle arrest at G2/M phase and won't allow the cells to undergo cell division or proliferate in S phase and G2/M phase and may be considered as a potent anti-oral cancer drug.

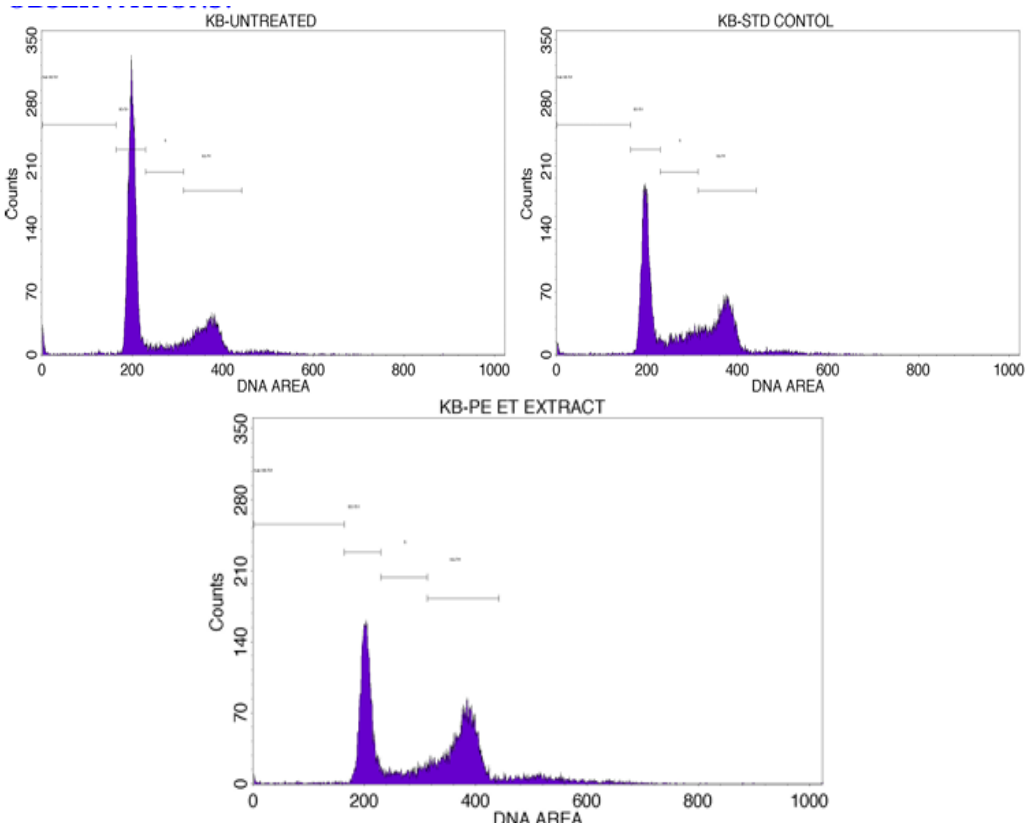


Figure 3. Flow cytometric histograms represented the phases of cell cycle distribution in the KB cell line treated by Camptothecin, AVEE with IC_{50} values compared to the control group.

The quantification of ROS using H2DCFDA on the KB cell line demonstrated in Table 3. It was inferred that the ethanol extract of *Adathoda vasica* effectively produced oxidative stress-mediated apoptosis in human oral cancer cells. The DCF intensity of the ethanol extract has exceeded that of the standard medication. Camptothecin served as the reference medication for the study. The discovered ROS expression results unequivocally validated the extract's anti-oral cancer efficacy via an oxidative ER stress-induced mechanism.

Table 3. H_2DCFDA activity in study of test compounds on KB cell line

Culture condition	% cells expressed DCF intensity
Untreated	0.03
Standard drug	59.35
AVEE	72.09

AVEE modulates p53 and apoptotic genes

Figure 4 illustrates the comparative mRNA expression levels of p53, Bax, Bcl2, and caspase 3 in the Tumour control, AVEE, and standard treatment groups of KB cells, as determined by quantitative RT-PCR analysis. The expression levels of p53, Bax, and caspase 3 were elevated twofold, whereas Bcl2 was downregulated onefold following treatment with AVEE and the standard medication substantially.

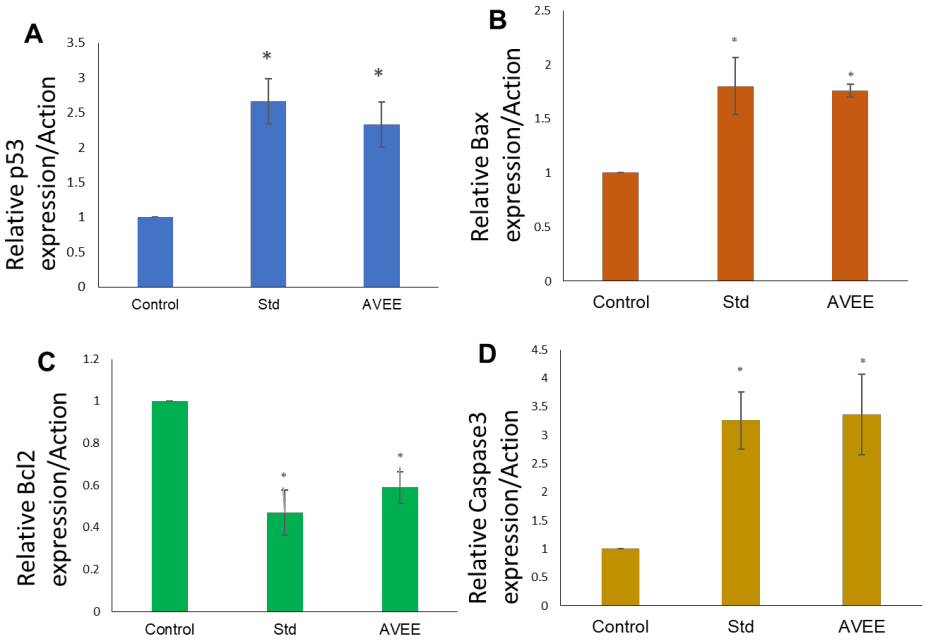


Figure 4A-C. The gene expression analysis of *p53, Bax, Bcl2* and *caspase3* upon treatment with AVEE and standard drug.

DISCUSSION

Oral squamous cell carcinoma (OSCC) constitutes over 90% of all mouth cancer instances and is among the most fatal malignancies worldwide [26,27]. The Global Cancer Observatory (GCO) estimated 377,713 cases of OSCC globally in 2020, predominantly in Asia, indicating a substantial regional burden [28]. Oral carcinogenesis is a complex and multifaceted biological process. The condition is largely caused by genetic changes in epithelial cells, which gradually result in the formation of neoplastic tumors in the oral cavity. OSCC can impact several anatomical tissues, such as the lips, tongue, upper and lower gingiva, alveolar mucosa, retromolar triangle, floor and roof of the mouth (palate), buccal mucosa, oropharynx, and salivary glands [29]. The lateral border of the tongue is the most often affected region (40%), followed by the floor of the mouth (30%) and the lower lip (30%). Besides its elevated death rate, OSCC is linked to considerable morbidity. Patients frequently endure facial deformities and functional limitations, including dysphagia, communication disorders, and altered gustatory perception. These issues significantly impact the overall quality of life in affected individuals [31]. Due to the constraints of existing therapeutic methods and the adverse effects linked to traditional therapies, there is increasing interest in utilizing medicinal plants for cancer prevention and treatment. Natural products and phytomedicines present a promising, safe, and economical alternative for managing various chronic and degenerative diseases, including cancer.

Adhatoda vasica Nees (family: Acanthaceae) is a plant extensively utilized in traditional Indian medicinal practices, especially Ayurveda. A. Vasica is recognized for its extensive range of pharmacological properties, encompassing antitubercular, cardiovascular protective, antiulcer, antiasthmatic, abortifacient, antimutagenic, hepatoprotective, antibacterial, and anticancer actions [32]. Ethanolic extracts of *Adhatoda vasica* has been examined for its bioactive components, notably alkaloids like vasicine and vasicinone, which are thought to play a role in its many biological activities [33,34]. The present work demonstrates that the injection of AVEE produces G2/M cell cycle arrest. In oral cancer, the majority of medicines were shown to block the cell cycle at the G2/M phase [35,36]. A new chemical, 2-acetyl-benzylamine, has been discovered from *Adhatoda vasica L*. leaves were recognized to elicit cell cycle arrest during the G2/M phase [37].

In cellular physiopathology, reactive oxygen species (ROS) are integral to cell growth, signaling cascades, and oxidative defense mechanisms that eliminate microbes and malignant cells. Consequently, the assessment of intracellular ROS generation may serve as a valuable metric for quantifying oxidative stress and its relevance as an anticancer agent.

Excess reactive oxygen species induce oxidative stress in cancer cells, resulting in cell death and diminished tolerance to treatment [38]. The extract's anticancer activities are likely associated with phytochemicals that alter the redox equilibrium of KB cells, essential for the survival of cancer cells.
The p53 tumor suppressor gene is crucial for orchestrating cellular responses to DNA damage and stress, including the initiation of apoptosis in compromised or aberrant cells [39]. Modifications in the expression or activity of these genes can profoundly influence the genesis, progression, and treatment response in oral cancer due to their essential roles in governing cell destiny decisions [15-17]. The RTPCR expression of p53 was elevated by AVEE therapy in the current study. Reports indicate that natural products can upregulate the expression of p53 in oral cancer [40,41].

Administration of AVEE to KB cells induces apoptosis, assessed by the Annexin V technique utilizing a flow cytometer. Apoptosis is facilitated by many proteins, including Bcl2 and Bax. The current work demonstrates that AVEE therapy results in the overexpression of Bax and the downregulation of Bcl2. The Bcl-2 family of proteins is classified into two categories: pro-apoptotic (Bax and BAD) and anti-apoptotic (Bcl-2 and Bcl-xl). An imbalance in Bcl-2 family proteins leads to a decrease in mitochondrial membrane potential, subsequently causing the release of cytochrome C into the cytosol. Kumar et al. [42] indicated that *Adathoda vasica* extract influences the expression levels of Bax, Bcl2, and caspase 3 in breast cancer cells. Conversely, vasicinone, a quinazoline alkaloid extracted from *Adathoda vasica*, causes apoptosis in lung carcinoma cells [43].

The results of this study indicate that *Justicia adhatoda* (syn. *Adhatoda vasica*) leaf extract has considerable promise as an alternate treatment for oral cancer. The bioactive components in the extract may provide anticancer advantages with less toxicity. To establish its therapeutic application, thorough preclinical research, including mechanistic studies and *in vivo* models, are necessary. Subsequent studies may ultimately facilitate the advancement of *J. Adhatoda*-derived anticancer formulations as an additional or alternative strategy in the treating of oral cancer.

Author Contribution Statement

Hameed Fathima Kamal: carried out the experiments, data analysis, drafted the manuscript, and data analysis and revised the manuscript

Jaideep Mahendra: provided research funding, supervised the experiments and revised the manuscript

Ashok Kumar Pandurangan: supervised the experiments and revised the manuscript,

Muskan Bedi: data analysis, and revised the manuscript.

Ethical approval

Not applicable

Availability of data

Data is available on reasonable request from the corresponding authors.

Conflict of interest

The authors declare that they have no conflicts of interest associated with this manuscript.

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