ORIGINAL RESEARCH

Anticancer Effects of *Carica papaya* L. and Benzyl Isothiocyanate on an Oral Squamous Cell Carcinoma Cell Line: An *In Vitro* Study

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Abstract

Aim: The study aimed to assess the anticancer effects of leaves of the male and female plant and seeds *Carica papaya* L. extract and the active compound benzyl isothiocyanate on oral squamous cell carcinoma (OSCC) cell line.

Materials and methods: Extracts of CO_2 strain *C. papaya* L. seeds were prepared using water, ethanol, and ethanol:water by maceration, and benzyl isothiocyanate was quantified. Alkaloid fractions of leaves of male and female plants of *C. papaya* L. were prepared and quantified. The anticancer effects of the test substances on the SCC-25 cell line were assessed by MTT, apoptosis assay, cell cycle analysis, and determination of mitochondrial membrane potential.

Results: The ethanol:water extract of *C. papaya* L. (seeds) demonstrated the highest quantity of benzyl isothiocyanate. Male plant leaves demonstrated greater alkaloid content. The leaves of the male plant exhibited apoptosis induction and S-phase arrest, whereas the leaves of the female plant and seeds of *C. papaya* L. demonstrated G2M-phase arrest and apoptosis induction.

Conclusion: *C. papaya* L. and benzyl isothiocyanate demonstrated anticancer effects. There was a difference in the anticancer effects of leaves of male and female plants of *C. papaya* L.

Clinical significance: The anticancer effects of papaya leaves and seeds could be further explored to develop an adjunct therapy for oral cancer to improve prognosis and reduce recurrence rates.

Keywords: Apoptosis, Benzyl isothiocyanate, *Carica papaya*, Oral squamous cell carcinoma.

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Introduction

Oral squamous cell carcinoma is the most common neoplasm of the oral cavity constituting 80–90% of malignancies that occur in the oral cavity.[1] The disease has a high incidence rate of around 300,000 new cases and 145,000 deaths reported by Ferlay et al. in the year 2015.[2] It is more common in South East Asian countries, thereby increasing the burden of cancer-related socioeconomic implications.[3]

The pathogenesis of OSCC is a complex and multistep process commencing from carcinogenic exposure of the oral tissues, followed by several complex genetic and molecular mechanisms, leading to increased mitosis, evasion of apoptosis, and immune surveillance. Although several etiologic factors have been attributed and implicated as the cause of OSCC, tobacco, and alcohol are most common accounting for 40% of carcinomas and 60% of cancer-related deaths.[4] Free radicals and oxidative stress also play a vital role in carcinogenesis.[5] The role of microorganisms like *Candida albicans* in causing cancer should also not be underplayed.[6] Prompt and early diagnosis of OSCC is pivotal to treat the condition and save the life of the patient in concern. Many treatment strategies have been developed and implemented for the holistic management of OSCC.

Treatment of OSCC is a combination of surgery, chemotherapy, and radiotherapy depending upon the complexity and stage of the disease. Based on the understanding of the pathogenesis of OSCC, an agent that would target malignant cells and possess

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antimicrobial and antioxidant properties could be considered as an ideal anticancer agent and a chemopreventive agent.[5] The current treatment modalities are associated with morbidity causing impairment of esthetics and normal functions. It poses a very high burden on the socioeconomic status in developing countries, posing a challenge in delivering affordable and equitable care. Also, there has been no improvement in the five-year survival rate ranging from 62.7% (the 1980s) to 71.7% (2010–2017), as reported by Capote-Moreno et al., and 35% in India, as reported by Shukla and Shukla.[4,7]

This has led to extensive research on the use of plant and plant-based products for the management of cancer. The various phytochemical constituents such as terpenoids, alkaloids, polyphenols, flavonoids, saponins, glucosinolates, and confer medicinal properties, including antioxidant, anticancer, and antimicrobial effects to plants that could play a significant role in biochemoprevention and management of the disease. One such plant with abundant medicinal properties is *C. papaya* L. commonly known as papaya that is a soft-wooded perennial plant that originated from Southern Mexico and Costa Rica.[8] Among the various strains of papaya, CO_2 strain is a pure-line selection of local type. It is a dioecious plant with high papain content. So far, only two studies have reported minor variations in the phenol content and specific primer variations in the male and female plants.[9,10] Pharmacognostic evaluation of the same has not been reported so far. Both the leaves and seeds of *C. papaya* L. contain phytochemicals that could exert anticancer effects.[11,12] To date, there is a lack of evidence in the literature to have reported the anticancer effects of a particular strain of *C. papaya* L. on the OSCC cell line. We hence pursued the present study to determine the *in vitro* anticancer effects of leaves (of the male and female plant) and seeds of *C. papaya* L. extract and the active compound benzyl isothiocyanate on a commercially available cell line of OSCC using standardized assays.

Materials and Methods

The present study was approved by the Institutional Ethics Committee, Sri Ramachandra University. REF: IEC-NI/11/APR/22/20 dated 28.04.2011.

Procurement of Plant Material

Leaves of the male and female plant and seeds of CO_2 strain *C. papaya* L. were procured from Tamil Nadu Agricultural University, Coimbatore. Authentication by macroscopic and microscopic characterization was done at Plant Anatomy Research Center, Tambaram, Chennai, Tamil Nadu.

Preparation of Crude Herbal Extracts of *C. papaya* L. (Seeds)

Following removal of impurities, the seeds were weighed and powdered using mortar and pestle. Water, 100% ethanol, and ethanol:water (60:40) extracts of *C. papaya* L. seeds were prepared by maceration for 72, 48, and 24 hours and Whatman paper was used for the collection of the pooled extracts. The concentration of the pooled extracts was done following standard protocols with rotary flash. Further concentration of the extract was done with water bath followed by vacuum desiccator.

Quantification of Benzyl Isothiocyanate in *C. papaya* L. (Seed) Extracts

About 10 µL of benzyl isothiocyanate (11.8 mg in 1 mL dimethylsulfoxide) as standard, 100% ethanol extract of *C. papaya* L. (seeds) (9.6 mg in 1 mL of ethanol), ethanol:water extract of *C. papaya* L. (seeds) (60:40) (7.7 mg in 1 mL of dimethylsulfoxide), and aqueous extract of *C. papaya* L. (seeds) (8.7 mg in 1 mL of water) were spotted using high-performance thin-layer chromatography plates precoated with Si-gel Si60F254 with a band length of 5 mm as the stationary phase. Development of chromatograms was done using toluene:ethyl acetate:acetic acid: 5:4:1 as mobile phase. Following the drying of the plate, scanning was done at 254 nm, and benzyl isothiocyanate was quantified in the three extracts based on the peak obtained from the standard. It is a light-yellow to yellowish-orange liquid with a molecular weight of 149.21 gm/mol. The molecular formula of benzyl isothiocyanate is C_8H_7NS.[13] The extract with the highest quantity of benzyl isothiocyanate was selected for anticancer activity.

Preparation of Alkaloid Fraction of *C. papaya* L. (Leaves of the Male and Female Plant)

Following removal of impurities, the collected leaves of male and female plant of *C. papaya* L. were shade-dried, manually powdered, and macerated with methanol for 72 hours, 48 hours, and 24 hours. The obtained pooled extracts were concentrated using rotary flash and dried with a vacuum desiccator. The extract was dissolved with methanol and the pH was adjusted to 2 by adding HCl. The extract obtained was macerated with chloroform twice, and the pooled extract was placed in a separator. The chloroform nonalkaloid layer and the alkaloid layer were collected separately. The pH of the obtained alkaloid fraction was adjusted to 8 by the addition of ammonia. The extract was macerated with chloroform twice, and the pooled extract was obtained. The pooled extract was concentrated with rotary flash and dried with a vacuum desiccator. The presence of alkaloids was confirmed by using Dragendorff's reagent as indicated by formation of orange or orange–red precipitate.

Quantification of Alkaloids in *C. papaya* L. (Leaves) Extracts

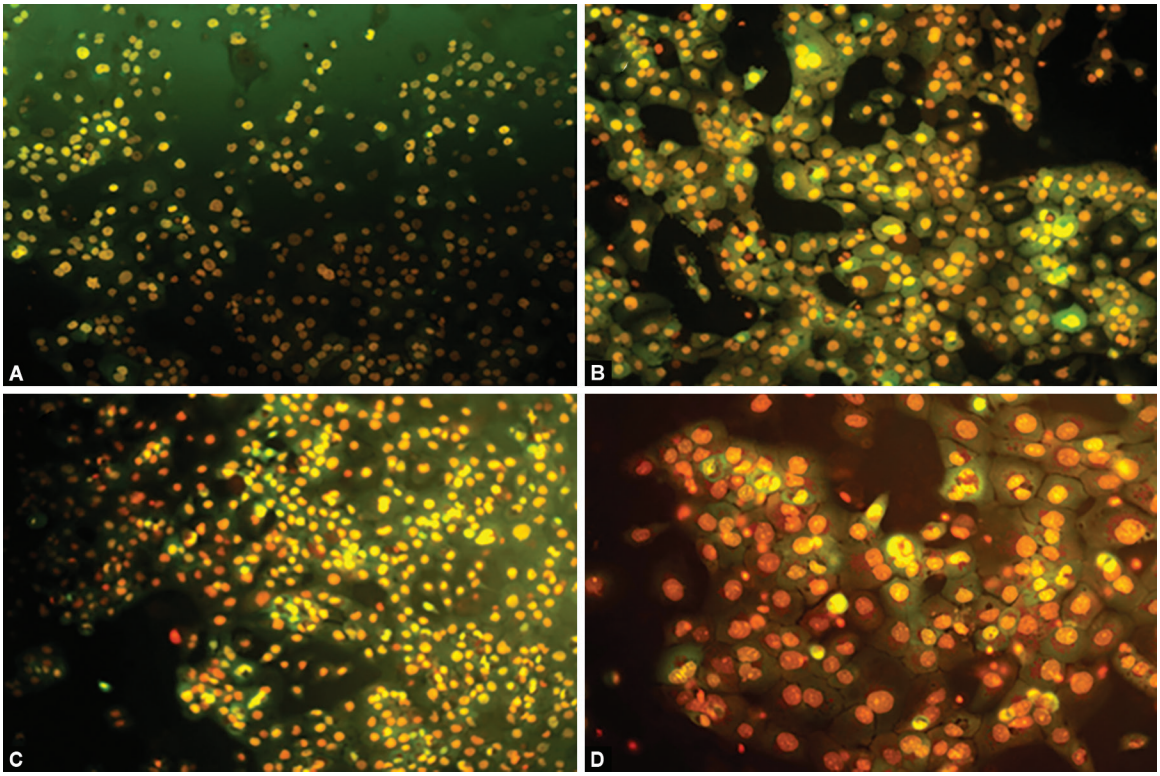
Estimation of total alkaloid content in the alkaloid fraction of leaves of male and female plant of *C. papaya* L. was done following standardized protocols using Dragendorff's reagent.[14] A standard graph of bismuth nitrate pentahydrate was used for the estimation of total alkaloid content in the extracts.

Cell Culture

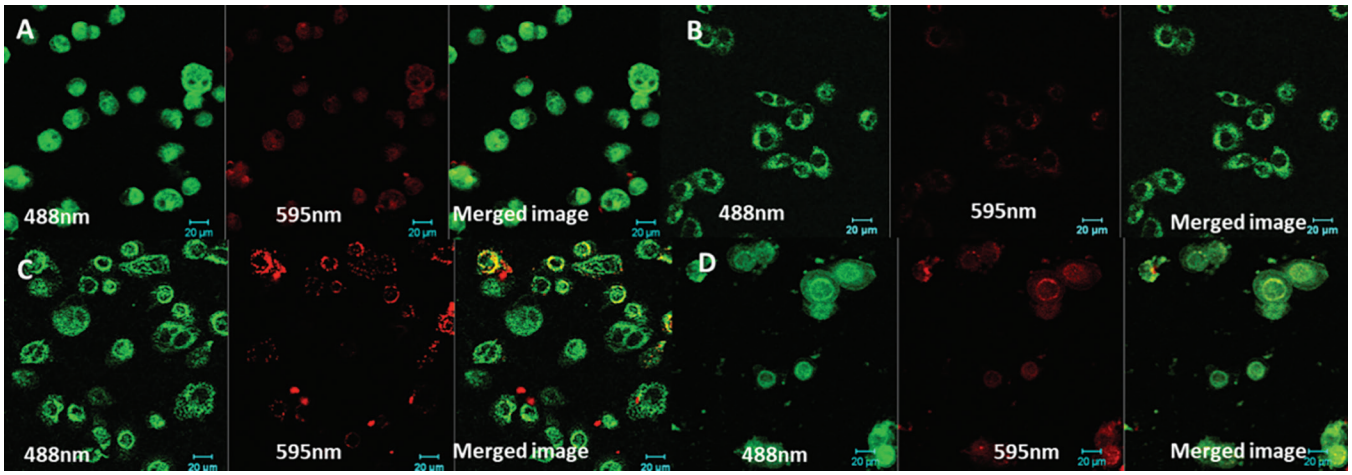
Standard protocols were followed for culturing the procured SCC25 ATCC CRL1628 cell line from ATCC. For the conduct of the experiments, the cells were trypsinized on attaining 80% confluence and the appropriate density of the cells was seeded. Briefly, 1.0×10^3 cells/well were seeded onto 96-well plates for MTT cytotoxicity and acridine orange and ethidium bromide staining assay. For DNA fragmentation assay and flow cytometry, 4×10^6 cells/well and 0.5×10^6 cells/well, respectively, were seeded onto 6-well plates. For JC-1 stain, 0.2×10^6 cells/coverslip were seeded on coverslips of size 22 × 22 mm placed inside a 6-well plate. Following this, 24 hour incubation at 37°C and 5% CO_2 was done to ensure the formation of monolayer and the following assays were performed.

Assessment of Anticancer Activity

The cells were treated with ethanol:water (60:40) extract of *C. papaya* L. (seeds) (the selected extract with the highest quantity of benzyl isothiocyanate), alkaloid fraction of *C. papaya* L. (leaves of the male and female plant) (bark), standard benzyl isothiocyanate at different concentrations, and the following experiments



Figs 1A to D: AO/EB staining for assessment of apoptosis. Cells on treatment with (A) 85 µg/mL *C. papaya* L. (leaves of the male plant); (B) 90 µg/mL *C. papaya* L. (leaves of the female plant); (C) 62.5 µg/mL *C. papaya* L. (seeds); and (D) 30 µM benzyl isothiocyanate depicting various stages of apoptosis (magnification 20×)



Figs 2A to D: Effect of benzyl isothiocyanate and *C. papaya* L. on the mitochondrial membrane potential of oral squamous cell carcinoma cells. Confocal microscopic view of stained cells after treatment with (A) 30 µM benzyl isothiocyanate; (B) 85 µg/mL *C. papaya* L. (leaves of the male plant); (C) 90 µg/mL *C. papaya* L. (leaves of the female plant); and (D) 62.5 µg/mL *C. papaya* L. (seeds) showing predominant green fluorescence (magnification 400×)

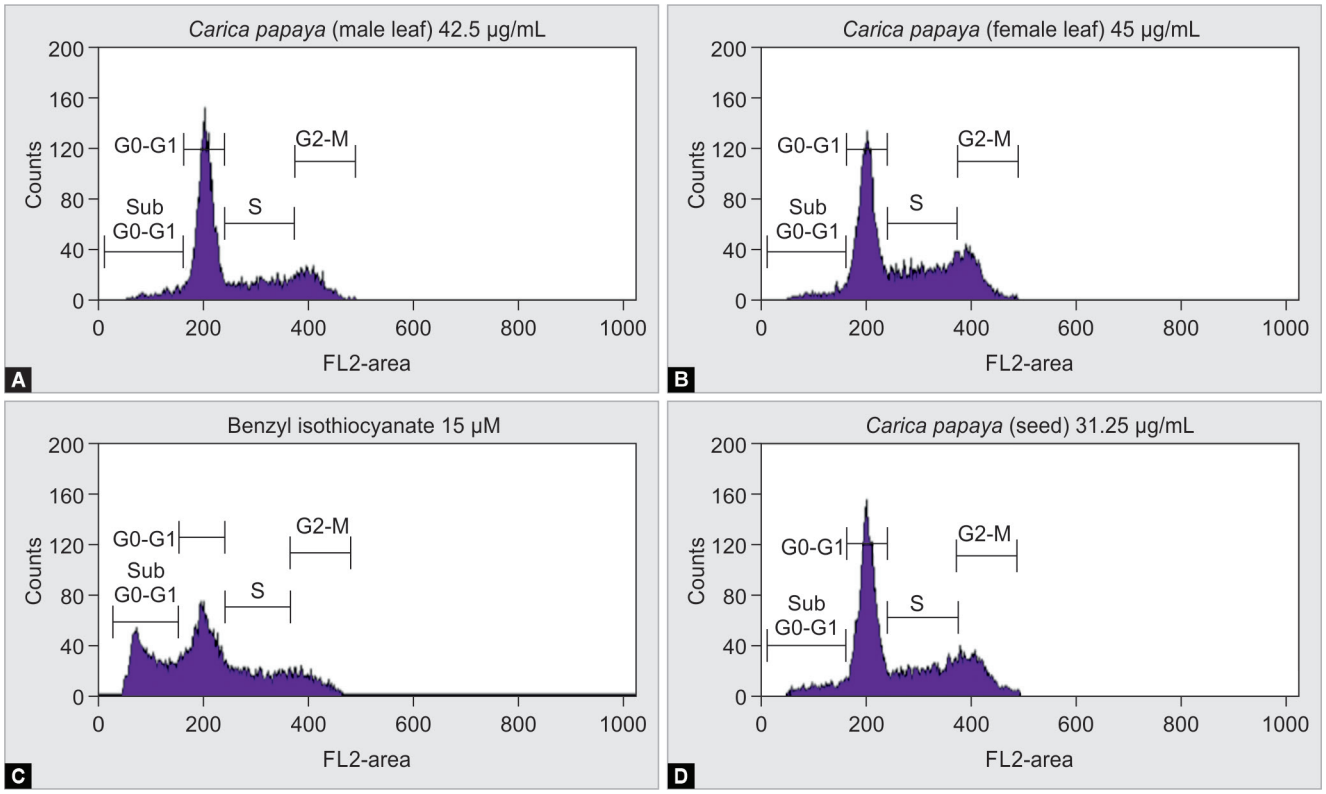
were performed (Figs 1 to 3). The activity was compared with control or untreated cells.

MTT Cytotoxicity Assay

Following treatment of the cells with the test substances and completion of the incubation period, the drug solutions were removed and 20 µL of MTT was added and incubated for 4 hours at 37 °C in a 5% CO_2 atmosphere. After removal of the supernatant, 100 µL of DMSO was added and incubated for 1 hour and absorbance was measured at 590 nm, and IC_{50} concentration was determined.[15] IC_{50} concentration represents concentration at which 50% of cells are alive, hence it was used to select nontoxic concentration of the test substances for further experiments.

Acridine Orange and Ethidium Bromide (AO/EB) Staining

The treated cells were washed with PBS and treated with 100 µL of extracts, and active compounds and the cells were observed under a microscope every 24 hours. Following 48 hours of incubation at



Figs 3A to D: Cell cycle analysis by flow cytometry. Cells on treatment with (A) 42.5 µg/mL *C. papaya* L. (leaves of the male plant) shows S-phase arrest; (B) 45 µg/mL *C. papaya* L. (leaves of the female plant); (C) 31.25 µg/mL *C. papaya* L. (seeds) showing G2M- and S-phase arrest; and (D) 15 µM benzyl isothiocyanate showing cells predominantly in sub-G0 population

37 °C in a 5% CO_2 atmosphere, the cells were trypsinized with 20 µL of trypsin to which 1 mL of AO/EB staining solution was added and mixed gently. Following placement of the cells on a microscopic slide covered with a glass coverslip, the cells were viewed under a fluorescent microscope using an excitation filter of 480/30 nm. Cells with green stain were interpreted as live cells, cells with yellow stain were undergoing early apoptosis, and orange to orange-red stained cells were interpreted as late apoptosis.[16]

DNA Fragmentation Assay

Following 48-hour incubation, the culture medium of the treated cells was removed and centrifuged at 3000×*g* for 5 minutes. Hypotonic lysis buffer was used for the removal of remaining adherent cells. Raze (0.1 mg/mL) was used for RNA digestion at 37°C for 1 hour followed by Proteinase K for 2 hours at 50°C. DNA extraction was performed by addition of phenol:chloroform:isoamyl alcohol (25:24:1). Following this, equal volume of isopropyl alcohol was added and stored overnight at 20°C to precipitate DNA. The mixture was centrifuged at 12000 × gm for 15 minutes at 4°C. The pellet obtained was air-dried and suspended in 20 µL of Tris-acetate EDTA buffer supplemented with loading buffer containing 0.25% bromophenol blue and 30% glycerol. Electrophoresis was performed in Tris-borate-EDTA buffer at 4 V/cm for about 4 hours, and observation of DNA fragments was done under ultraviolet light.[17]

Cell Cycle Analysis by Flow Cytometry

The treated cells were trypsinized, the supernatant was discarded, and the cell pellet was centrifuged for 5 minutes at a speed of 1200 rpm with PBS. Following removal of supernatant, 300 µL of 1× PBS was added and fixed with 700 µL of 100% ethanol. The cells were centrifuged at 1500 rpm for 10 minutes at room temperature, to which 5 mL of 1× PBS was added, and the procedure was repeated. Following removal of the supernatant, the cells were resuspended in 556 µL of 0.5% Triton X-100, 20 µL of RNase (1 mg/mL), and incubation at room temperature was done for 60 minutes. To this, 24 µL of PI (1 mg/mL) was added, incubated for 45 minutes, and 10,000 events were acquired in a flow cytometer (BD FACS Calibur). Data analysis was done with CellQuest Pro software.[18]

Effect of Test Substances on Mitochondrial Membrane Potential

The treated cells were washed with 1 × PBS (pH 7.4), and 100 µL of JC-1 was added to the cells. Incubation was done in the absence of light for 20 minutes. In all, 1 × PBS (pH 7.4) was used to wash the cells, and the coverslips were removed from the 6-well plate and placed over another coverslip (40 × 22 mm). The cells were viewed, and images were captured under a confocal laser scanning microscope.[19]

Results

Quantification of Phytochemicals in *C. papaya* L. Extracts

Ethanol:water extract of *C. papaya* L. (seed) demonstrated the highest quantity of benzyl isothiocyanate 0.283% in comparison with water (nil) and 100% ethanol (0.17%) extracts. Hence, ethanol:water extract was used for assessment of anticancer effects. It was observed that the leaves of the male plant contained 7.69% and the female plant contained 7.39% of total alkaloids.

Effects of *C. papaya* L. and Benzyl Isothiocyanate on OSCC Cell Line

Determination of IC_{50} Concentration

The malignant cells on treatment with *C. papaya* L. (alkaloid fraction of leaves of the male and female plant), *C. papaya* L. (seed extract), and benzyl isothiocyanate showed IC_{50} concentration of 85.16 µg/mL, 90 µg/mL, 62.49 µg/mL, and 29.80 µM. IC_{50} concentration represents the concentration at which 50% of cells were alive and aided in selecting the nontoxic concentration for further experiments.

Apoptotic Assays

Acridine orange and ethidium bromide stain: Figure 1 depict the results of the treated cells stained with AO/EB. The cells on treatment with *C. papaya* L. (leaves of a male plant, female plant, and seed) extracts and benzyl isothiocyanate showed cells undergoing early apoptosis as depicted by yellow stain and late apoptosis shown by the reddish-orange stain (magnification 20×).

DNA fragmentation assay: The cells on treatment with all the test substances showed a ladder pattern depicting apoptosis. The control or the untreated cell ladder pattern was absent.

Effect of the Test Substances on the Cell Cycle

The cells on treatment with *C. papaya* L. (leaves of the male plant) induced S-phase arrest, whereas *C. papaya* L. (leaves of the female plant and seed) induced G2M and S. The corresponding active compound and benzyl isothiocyanate demonstrated an increased proportion of cells in the sub-G0 population in a dose-dependent manner. The results were compared with the control or untreated cells. The results are depicted in Figure 3.

Effect of Test Substances on Mitochondrial Membrane Potential

The treated cells with all the test substances demonstrated loss of mitochondrial membrane potential demonstrated by increased green fluorescence and decreased red fluorescence. The results are depicted in Figure 2.

Discussion

This study was pursued with the intended aim of assessing the anticancer effects of *C. papaya* L. seeds and leaves on a commercially available OSCC cell line. It is a well-known fact that OSCC has a greater incidence rate in countries like India, which could be attributed to harmful oral habits like tobacco smoking, smokeless tobacco, and pan chewing. Also, the five-year survival rate has not seen much improvement in our country due to poor access to medical care and lack of patient education and awareness in India. In this scenario, it is worthwhile to explore the medicinal properties of herbs and the phytochemicals present in the herbs for cancer prevention and adjunctive management due to their antioxidant, antimicrobial effects, and anticancer effects that could serve as a strategy for the management of this deadly disease. Also, herbs can be procured cost-effectively, thereby aiding in conferring affordable care in developing nations.

In the present study, *C. papaya* L. was chosen as the anticancer effects of the leaves of the male and female plant have not been assessed and explored so far, and also because the medicinal properties of CO_2 strain have not been explored widely. The seeds of the plant were also chosen due to the presence of various phytochemicals that could exert anticancer effects. The common name of the herb is papaya and it is grown in several countries such as India, Hawaii, Australia, Sri Lanka, and South Africa. The major phytochemicals of papaya leaves include carpaine, pseudocarpaine, dehydrocarpaine I and II, choline, carposide, vitamin C and E flavonoids, and cyanogenic glucosides with several known medicinal properties.[8] However, the medicinal properties of the leaves of the male and female plants have not been assessed and compared to date. Also, Do et al. have reported that the alkaloid fraction of *C. papaya* L. (leaf) exhibited greater anticancer effects than the flavonoids, saponins, and other polar compounds.[11] Hence the alkaloid fraction was isolated from leaves of the male and female plants and was quantified for assessment of anticancer effects. Interestingly, the alkaloid content of leaves of the male plant was higher than that of leaves of the female plant, which is a significant finding highlighting sexual dimorphism in the papaya leaves. Also, papaya seeds contain several important phytochemicals with medicinal properties such as fatty acids, crude protein, crude fiber, papaya oil, carpaine, benzyl isothiocyanate, benzyl glucosinolate, glucotropacolin, benzyl thiourea, hentriacontane, β-sitosterolcaricin, and myrosin justifying its use in the present study.[8] Water, 100% ethanol, and ethanol:water extracts of the seeds were prepared to select the extract with the highest concentration of benzyl isothiocyanate, which is the major constituent that could exert an anticancer effect. High-performance thin-layer chromatography results demonstrated that ethanol:water extract of *C. papaya* L. (seeds) had the highest quantity of benzyl isothiocyanate due to the polarity of ethanol and water used in combination. Therefore, this extract was selected for all the experiments.

C. papaya L. (leaves of the male and female plant and seeds) showed an IC_{50} concentration of 85.16, 90 µg/mL, and 62.49 µg/mL, respectively, when subjected to assess cytotoxicity by the MTT assay. Considering the mechanism of anticancer activity, *C. papaya* L. (leaves of female plant and seeds) exhibited G2M and S-phase arrest, whereas *C. papaya* L. (leaves of the male plant) exhibited S-phase arrest. This interesting sexual dimorphism in pharmacognostic action is a first time finding. It is noteworthy that the seeds are borne by the fruits of the female plant, and both the leaves and seeds have exhibited a similar mechanism of action. Thus, future studies with regard to pharmacognostic characterization have to be carried out for confirmation of the results of the present study. The findings of the anticancer effects of alkaloid fraction of papaya leaves are concurrent with the findings of Mohan and Jeyachandran, who have reported that the alkaloids exert anticancer effects via apoptosis induction.[20] The alkaloids that have been isolated from *C. papaya* L. (leaves) that could confer anticancer effects are carpaine and pseudo-carpaine.[21,22] Considering the anticancer effect of papaya seeds, the results are concurrent with the findings of Nakamura et al., who have reported the anticancer effects of *C. papaya* L. (seed) and benzyl isothiocyanate on the HL-60 cell line via apoptosis induction.[23]

The anticancer effects of benzyl isothiocyanate via apoptosis induction on OSCC are concurrent with several studies on various cell lines other than OSCC.[12,24] Literature available describes the mechanism of apoptosis induction by increased caspase-3, -8, and -9 activities and promoted AIF and EndoG expression released from mitochondria and inhibition of STAT pathway.[25,26] On the contrary, the studies done by Huang et al. reported that benzyl isothiocyanate induced G2M arrest on A375.S2 human melanoma cancer cells.[25] This variation could partly be attributed to the cell line studied. Also, benzyl isothiocyanate has been shown to inhibit lung tumorigenesis in A/J mice.[24] It was reported that the

antitumor effect was mediated by decreased expression of PCNA, cyclin-dependent kinases, and elevated levels of apoptotic markers.

On comparison of the effect of the *C. papaya* L. (seed) extract and benzyl isothiocyanate, we have observed that the extract induced G2M and S-phase arrest, whereas the active compound demonstrated an increased proportion of cells in the sub-G0 population. The variation in results obtained with the extracts and pure compounds could be due to the cumulative effect of other phytochemicals present in the extract.

The anticancer effects exerted by crude ethanol:water extract of *C. papaya* L. (seed) could hence be attributed to the presence of benzyl isothiocyanate in combination with the other phytochemicals. The alkaloids of *C. papaya* L. (leaves of the male and female plant) have to be explored further for isolation and identification of the exact compounds with anticancer effects and the reason for sexual dimorphism in medicinal properties.

The limitations of the study include that the study had an *in vitro* design, and the *in vivo* effects and adverse effects were not assessed.

Conclusion

With the limitations of the present study, it can be inferred that leaves and seeds of *C. papaya* L. and benzyl isothiocyanate exert potent anticancer effects on OSCC cell line. Future *in vivo* and toxicity studies are the need of the hour to assess the anticancer and chemoprotective role of *C. papaya* L.

Authors' Contributions

SV – conception and design of the study, acquisition of data, and drafting the article. TMB – acquisition of data, analysis and interpretation of data, and drafting the article. MN – analysis and interpretation of data and drafting the article. DPC – analysis and interpretation of data and revising it critically for important intellectual content. DS – acquisition of data and revising it critically for important intellectual content. All authors approved the final version of the submitted manuscript.

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