In Vitro Anticancer Effects of Cinnamomum verum J. Presl, Cinnamaldehyde, 4 Hydroxycinnamic Acid and Eugenol on an Oral Squamous Cell Carcinoma Cell Line

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ABSTRACT

Aim and objective: The present study was conducted to assess the in vitro anticancer effects of Cinnamomum verum J. Presl extract and its active constituents, such as cinnamaldehyde, 4 hydroxycinnamic acid, and eugenol on oral squamous cell carcinoma cell line.

Materials and methods: Aqueous, ethanolic, and hydroalcoholic extracts of C. verum J. Presl (bark) were prepared using standardized protocols. Cinnamaldehyde, 4 hydroxycinnamic acid, and eugenol were quantified in the extracts. Total saponins, tannins, and polyphenols were quantified in the selected extracts. A commercially available SCC25 cell line was cultured according to standard protocol. The anticancer effects of the extract, active compounds, and standard cisplatin were assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cytotoxicity, acridine orange/ethidium bromide staining, DNA fragmentation assay, cell cycle analysis by flow cytometry, and JC-1 staining (5,5′,6,6′-tetracyanobenzimidazolylcarbocyanine iodide).

Results: The hydroalcoholic extracts demonstrated a higher quantity of the active ingredients cinnamaldehyde, 4 hydroxycinnamic acid, and eugenol. The selected extract and active compounds demonstrated anticancer effects via apoptosis induction and S-phase arrest. Apoptosis induction was exerted by the extract via alteration in mitochondrial membrane potential.

Conclusion: Cinnamomum verum J. Presl and its active compounds exhibited in vitro anticancer effects on oral squamous cell carcinoma. Further studies in animal models have to be carried out to assess toxicity and in vivo effects.

Clinical significance: The anticancer properties of Cinnamomum verum J. Presl could be explored further for prevention and management of oral squamous cell carcinoma.

Keywords: Anticancer, C. verum J. Presl, Cinnamaldehyde, Cinnamic acid, Eugenol, Oral squamous cell carcinoma.

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INTRODUCTION

Oral squamous cell carcinoma, the sixth most common neoplasm in the head and neck region, is a major public health challenge due to the high morbidity and mortality rates. In India, it accounts for 30% of the malignancies.¹ The disease is multifactorial in etiology, however, tobacco and alcohol consumption accounts for 40% of the malignancies and 60% deaths due to cancer.²

The pathogenesis underlying cancer is a very complex and multistep process. Exposure to the carcinogen is followed by highly orchestrated pathways that operate at the cellular and molecular level. This involves two mechanisms: (a) sustaining cell proliferation in an uncontrolled manner and (b) evading or escaping immune surveillance and destruction and also the apoptotic pathways that work against proliferation. It is noteworthy to emphasize at this point that free radicals play a major role in carcinogenesis. Also, not to underestimate is the pivotal role played by bacteria such as Streptococcus mutans and fungi such as Candida albicans. Hence, an ideal cancer therapy will not only target the cancer cells per se but will also have antimicrobial, antifungal, and antioxidant activity.³⁵

As of today, surgery, chemotherapy, and radiotherapy or a combination of the above procedures is the gold standard treatment for the disease. The above-mentioned procedures are associated with a plethora of side effects that include esthetic and functional impairment to the patient in concern. Also the high cost that the patients need to bear to undergo the treatment that is often a sad scenario in developing nations like India. Despite several recent advances in the current therapeutic strategies, the 5-year survival rate stands at 62% worldwide and 35% in India.²
In addition to this, delivering an affordable and equitable care in India is a challenge.

Recently, several studies are being conducted on the use of herbs for the management of cancer. The basis of herbal therapies for cancer management lies in the fact that herbs contain rich amounts of polyphenols, flavonoids, alkaloids, terpenoids, tannins, and glucosinolates. These constituents confer anticancer, antioxidant, and antimicrobial activities to the herbs that make them play a multifaceted role in cancer prevention and management. In this regard, *Cinnamomum verum* J. Presl, commonly known as Ceylon cinnamon an evergreen tree with aromatic barks and leaves are known to contain phytochemicals such as cinnamaldehyde, cinnamic acid, and eugenol that could exert anticancer effects.6–8 This herb is widely cultivated and is easily available throughout the world. It is not costly and can be easily procured. However, there is no study to have assessed the anticancer effects of *Cinnamomum verum* (J. Presl) bark extract in oral squamous cell carcinoma. With the available information, the present study was conducted to assess the *in vitro* anticancer effects of *C. verum* J. Presl extract, cinnamaldehyde, 4 hydroxycinnamic acid, and eugenol on an oral squamous cell carcinoma cell line to find out if this herb can be used as an adjunct in oral cancer management.

**Materials and Methods**

**Collection of Plant Material and Authentication**

*Cinnamomum verum* J Presl (bark) was procured from a reputed spice market and authenticated at Plant Anatomy Research Center by macroscopic and microscopic characterization.

**Preparation of Crude Herbal Extracts**

The collected plant material was washed, weighed, shade dried, ground using a cutter miller, and powdered using mortar and pestle. Aqueous, 100% ethanolic, and hydroalcoholic (70:30) extract of the powdered plant material were prepared by maceration with the respective solvents for 72, 48, and 24 hours. The pooled extracts were collected by filtration using Whatman paper. The concentration of the extracts was done using rotary flash, water bath, and vacuum desiccator. The prepared extracts were labeled and stored at 2 to 8°C until use.

**Quantification of Cinnamaldehyde, 4 Hydroxycinnamic Acid and Eugenol by High-Performance Thin-layer Chromatography**

10 μL of the samples of cinnamaldehyde (12.5 mg/mL in chloroform), 4 hydroxycinnamic acids (9.6 mg/mL in methanol), eugenol (11.5 mg in 1 mL chloroform), ethanol extract of *Cinnamomum verum* J. Presl (bark) (10.2 mg in 9.5 mL ethanol and 0.5 mL water), hydroalcoholic extract of *Cinnamomum verum* J. Presl (bark) (70:30) (10.5 mg in 0.5 mL ethanol and 0.5 mL water), aqueous extract of *Cinnamomum verum* J. Presl (bark) (10.3 mg in 0.5 mL water and 0.5 mL ethanol) were spotted on HPTLC plates precoated with Si-gel Si60F254 (E. Merck) of band length 5 mm. Chromatograms were developed with toluene:ethyl acetate:hexane:formic acid::2:2:5:0.5 as mobile phase. The plate was dried and scanned at 254 nm. Quantification was done by using standard calculations.9–11

**Quantification of Polyphenols, Tannins, and Saponins in the Extracts**

Total phenol content in the selected extract was estimated by Folin Ciocalteu reagent using standardized protocols. A standard graph of gallic acid was used to estimate the total phenol content in the extract. To 1 mL of the extract taken from stock solution (0.1 mg/mL) of the extracts, 5 mL of Folin Ciocalteu reagent followed by 4 mL of 7.5% w/v sodium carbonate and incubated at room temperature for 90 minutes. Absorbance of blue color was read at 765 nm using a UV spectrophotometer.12

Estimation of tannins in the extract was done by using Folin Dennis reagent using suitable literature references and standard protocol using a standard graph of tannic acid. To 1 mL of the extract taken from stock solution (0.1 mg/mL) of the extract, 0.5 mL of Folin-Denis reagent, and 5 mL of 35% sodium carbonate solution were added and incubated in room temperature for 10 minutes following which absorbance at 725 nm was recorded using UV spectrophotometer.12

Saponins in the selected extracts were quantified by high-performance liquid chromatography with 25 mg of Digoxin used as standard and 20 μL of 1 mg/mL stock solution of the selected extract was used for the study. Reverse-phase C18 column was used as a stationary phase and acetonitrile:methanol:water (30:30:40) was used as a mobile phase with a flow rate of 1 mL/minute. Detection and quantification were performed using a UV detector at 220 nm.13

**Cell Culture**

SCC25 ATCC CRL1628 cell line was procured from American type culture collection, USA. SCC 25 cells were cultured with 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F12 medium containing 1.2 g/L sodium bicarbonate, 2.5 mM L-glutamine, 15 mM HEPES, and 0.5 mM sodium pyruvate and supplemented with 1000 ng/mL hydrocortisone and 15% fetal bovine serum and incubated at 37°C and 5% CO₂. The monolayer culture of SCC25 cells at 80% confluence was trypsinized and seeded at the required density for the experiments.

**Assessment of Anticancer Activity**

Cisplatin was used as the standard drug for comparison. The cells were treated with various concentrations of hydroalcoholic extract of *Cinnamomum verum* J Presl (bark), cinnamaldehyde, 4-hydroxycinnamic acid, and eugenol, and the below-mentioned assays were performed.

**MTT Cytotoxicity Assay**

The cells were seeded on to 96-well plates at a density of 1.0 × 10³ cells/well. On completion of 24 hours, the cells were treated with various concentrations of the standard and test substances and incubated for 48 hours. Following this MTT cytotoxicity assay was done to assess IC₅₀ concentration according to standard protocol.14,15

**Acridine Orange and Ethidium Bromide Staining**

The cells were seeded on to 96-well plates at a density of 1.0 × 10³ cells/well. On completion of 24 hours, the cells were treated with 100 μL of the standard and test substances and incubated for 48 hours. The cells were trypsinized, and acridine orange and ethidium bromide staining according to standard protocol was done and viewed under a fluorescent microscope for assessment of apoptosis.16

**DNA Fragmentation Assay**

The cells were seeded on to 6-well plates at a density of 4 × 10⁶ cells/well and incubated at 37°C and 5% CO₂ for 24 hours. Following this, the cells were treated with the test substances for 48 hours. DNA
fragmentation assay was performed according to standard protocol and DNA fragments were observed under UV light. Images were captured using a gel documentation system.17

Cell Cycle Analysis by Flow Cytometry
The SCC 25 cells were seeded at a density of 0.5 × 10⁶ cells/well in 6-well plates and incubated overnight and treated with the test substances and incubated for 48 hours. Following this, cell cycle analysis was done according to standard protocol, and ten thousand (10,000) events were acquired and the percentage of DNA content in each cell cycle phase was analyzed using CellQuest Pro software (Becton Dickinson, USA).18

Determination of Mitochondrial Membrane Potential
The cells were seeded on a coverslip (22 × 22 mm) placed inside a 6-well plate at the density of 0.2 × 10⁶ cells/coverslip and incubated overnight following which the cells were treated with IC₅₀ concentration of selected test substances for 48 hours. JC-1 staining was done according to standard protocol and viewed under LSM 510 Meta confocal laser scanning microscope (Carl Zeiss, Germany) at 20× magnification excitation/emission = 590/610 nm for red fluorescence and excitation/emission = 485/535 nm for green fluorescence and cell images were captured from different fields.19

Results
Quantification of Phytochemicals in Cinnamomum verum J. Presl (Bark) Extracts
Hydroalcoholic extract of Cinnamomum verum J Presl (bark) demonstrated a greater quantity of cinnamaldehyde (0.17238%), 4-hydroxycinnamic acid (1.5667), and eugenol (0.3971) in comparison with aqueous and ethanolic extracts. The results are depicted in Table 1. Hence, hydroalcoholic extract was chosen for further experiments. Total saponin content, total polyphenols content, and total tannin content in Hydroalcoholic extracts of Cinnamomum verum J Presl (bark) were 0.31%, 22.5%, and 16.88%, respectively.

Effects of Hydroalcoholic Extract of Cinnamomum verum J. Presl (bark) Extract, Cinnamaldehyde,

| Active compound | Plant material | Ethanolic extract | Hydroalcoholic extract | Aqueous extract |
|-----------------|----------------|-------------------|-----------------------|----------------|
| Cinnamaldehyde  | Cinnamomum verum J. Presl (bark) | 0.05727 | 0.17238 | Nil |
| 4 hydroxycinnamic acid | Cinnamomum verum J. Presl (bark) | 1.6959 | 1.5667 | Nil |
| Eugenol | Cinnamomum verum J. Presl (bark) | 0.238 | 0.3971 | Nil |

*Comparison of cinnamaldehyde, 4 hydroxycinnamic acid and eugenol levels in aqueous, ethanolic and hydroalcoholic extracts of Cinnamomum verum J. Presl (bark)

Hydroxycinnamic Acid, Eugenol and Cisplatin on Oral Squamous Cell Carcinoma Cell Line
Cytotoxicity Screening by MTT Assay
SCC 25 cells on treatment with Cinnamomum verum J Presl (bark) extract, cisplatin, cinnamaldehyde, 4 hydroxycinnamic acid, and eugenol at concentrations ranging between 1,000 μg/mL and 1.9 μg/mL showed IC₅₀ concentration of 16.36 μg/mL, 20.21 μM, 15.64 μM, 24.51 μM, and 24.71 μM, respectively.

Acridine Orange and Ethidium Bromide Staining
The stained images of the acridine orange and ethidium bromide assay are depicted in Figure 1. The cells on treatment with Cinnamomum verum J. Presl (bark) extract showed predominantly redish-orange cells depicting late apoptosis (Fig. 1C) (Magnification 20×), Treatment of SCC 25 cells with cisplatin (Fig. 1B), cinnamaldehyde (Fig. 1D), 4 hydroxycinnamic acid (Fig. 1E), and eugenol (Fig. 1F) showed yellow-stained cells depicting early apoptosis and orange cells depicting late apoptosis in a dose-dependent manner. Control or untreated cells showed green cells depicting viable cells (Fig. 1A).

DNA Fragmentation Assay
Figure 2 depicted Agarose gel electrophoresis images of DNA fragmentation assay. SCC cells on treatment with 16 μg/mL of Cinnamomum verum J. Presl (bark) (Fig. 2B, Lane 1), 16 μM of cinnamaldehyde (Fig. 2A, Lane 3), 15 μM of 4-hydroxycinnamic acid (Fig. 2A, Lane 4), 25 μM of eugenol (Fig. 2A, Lane 5), 20 μM of standard cisplatin (Fig. 2A, Lane 2) for 48 hours demonstrated ladder pattern depicting apoptosis in comparison with the absence of ladder pattern in negative control or untreated cells (Figs 2A and B, Lane 1).

Cell Cycle Analysis by Flow Cytometry
Figure 3 depicts the histogram representing the cell cycle distribution of the cells on treatment with the test substances and control. The cells on treatment with 8 μg/mL and 16 μg/mL of Cinnamomum verum J. Presl (bark) extract (Fig. 3C), 10 μM and 20 μM of standard cisplatin (Fig. 3B), 8 μM and 16 μM of cinnamaldehyde (Fig. 3D), 15 μM of 4-hydroxycinnamic acid (Fig. 3E), 12.25 μM and 25 μM of eugenol (Fig. 3F) showed an increased population of cells in the S phase and a corresponding decrease in the G0–G1 phase in comparison with the control in a dose-dependent manner. At higher concentrations, a marked increase in sub G0 population was observed. In the absence of test substance (control), the SCC 25 cells grew as an asynchronous population in all phases of the cell cycle (Fig. 3A).

Determination of Mitochondrial Membrane Potential
Figure 4 represents confocal microscopic images of the cells treated with the test substances and control. In the control (untreated cells Fig. 4A), SCC 25 cells depicted red fluorescence indicating high mitochondrial membrane potential. SCC 25 cells when exposed to the positive control, ionomycin depicted green fluorescence due to the presence of J monomers, indicating depolarization of the mitochondrial membrane as a result of low membrane potential. Cells on treatment with 20 μM of cisplatin (Fig. 4B) and 16 μg/mL of Cinnamomum verum J. Presl (bark) (Fig. 4C), revealed a significant loss of mitochondrial membrane potential than the control group (indicated by disappearance of red fluorescence or increase in green fluorescence in most of the cells).
Effect of Cinnamon on Oral Squamous Cell Carcinoma

To summarize the results, it was noted that all test substances demonstrated anticancer effects on oral squamous cell carcinoma cell line by apoptosis induction as depicted by Acridine orange and Ethidium bromide stain (Fig. 1), confirmed by DNA fragmentation assay (Fig. 2) and S phase arrest (Fig. 3). Apoptosis induction by Cinnamomum verum J Presl (bark) extract and cisplatin was exerted via alteration of mitochondrial membrane potential confirmed by JC-1 stain (Fig. 4).

Discussion

Oral squamous cell carcinoma, a disease with high morbidity and mortality rates, has a higher incidence rate in developing countries. Herbs contain numerous phytochemicals that could exert several medicinal properties as well as anticancer effects. Moreover, they can be procured easily and could not only aid in cancer treatment but also aid in chemoprevention due to their antioxidant and antimicrobial effects.

In the present study, Cinnamomum verum J Presl was chosen due to the easy availability, and the use of this herb being well documented in Indian cuisine. This herb is also referred to as Ceylon Cinnamon as it originated from Sri Lanka as a cash crop. However, it is widely cultivated in many tropical countries including India. It is believed and documented that cinnamon bark is a rich source of many active compounds like tannins, calcium oxalate, cinnamaldehyde, cinnamic acid, terpene hydrocarbons, and eugenol that confer medicinal properties to it. It has hence been used for the management of various disorders like nausea, vomiting, cold, diarrhea, and bronchitis. It is hence justified to use cinnamon for this study as it has proven medicinal properties. Aqueous, ethanolic, and hydroalcoholic extracts of the bark were prepared with the idea to use the extract with the maximum concentration...
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Figs 3A to F: Histogram representing control (untreated cells) and the effect of cisplatin, Cinnamomum verum J Presl (bark), cinnamaldehyde, 4 hydroxycinnamic acid, eugenol on oral squamous cell carcinoma cell line. (A) It depicts the cell cycle distribution of control cells. In the absence of the test substance, the SCC 25 cells grow as an asynchronous population in all phases of the cell cycle. Cells on treatment with (B) 20 μM cisplatin; (C) 16 μg/ml Cinnamomum verum J. Presl (bark) extract; (D) 16 μM Cinnamaldehyde; (E) 15 μM 4 hydroxycinnamic acid; (F) 25 μM Eugenol. Eugenol shows S Phase arrest and a corresponding increase in sub-G0 population

of the active principles. HPTLC results showed that hydroalcoholic extract of Cinnamomum verum J Presl (bark) had a higher quantity of the phytochemicals assessed which could be attributed to the polarity of solvent used for extract preparation. Hence, this extract was chosen and used for all the assays.

Also, other phytochemicals such as total polyphenols, saponins, and tannins that could exert anticancer effects were quantified in the above-selected extract. Cisplatin was used as standard and anticancer effects of individual compounds such as cinnamaldehyde, 4 hydroxycinnamic acid and eugenol were also assessed.

The IC_{50} concentration exhibited by Cinnamomum verum J Presl (bark) extract in the present was different from the studies reported in KB cell line and L120 cell line (64, 58 μg/mL and 24, 20 μg/mL respectively). These minor variations could be attributed to the seasonal and geographic variations and difference in the property of cell line used. Concerning anticancer effects, the present study findings are concurrent with the findings of previous studies demonstrated in hepatocellular carcinoma.

The anticancer effects of cinnamaldehyde on oral squamous cell carcinoma cell lines are different from the study reported on the Jurkat cell line, U 937 cell line, wherein transcinnamaldehyde was found to induce G2M phase arrest. Similarly, trans cinnamaldehyde was found to induce G2M phase arrest on SCC 15 and Hep 2 cell lines. These variations could be attributed to the difference in the structure of the molecule used for the study and cell line type used. It is at this point noteworthy to mention that cinnamaldehyde and its congeners such as 2 hydroxy cinnamaldehyde can cause significant tumor regression in vivo animal models. This is caused by the pro-apoptotic effects mediated through PARP and caspase 3 overexpression.

The results about the effect of 4 hydroxycinnamic acid on the cell line are concurrent with the findings reported in HT 144 melanoma cell line and contrary to the findings reported on K562 cell lines. However trans-cinnamic acid was used in the above-mentioned study. It is to be emphasized that trans-cinnamic acid can also upregulate caspase 3 expression and can cause distinct microtubule disorganization thereby causing early apoptosis. It is plausible that 4 hydroxycinnamic acid could exert the same effects.

Concerning eugenol, the present study results are concurrent with the findings reported in the melanoma cell line. A contrary finding has reported G2M phase arrest induced by eugenol, which could be attributed to the use of methoxyestradiol and eugenol in combination. It is at this point that one should understand how eugenol can exert anticancer effects. Literature available describes caspase 3 and 6 upregulation and also the cleavage of caspase substrates DFF45, PARP, and Lamin A by eugenol.
The anticancer effects exerted by crude hydroalcoholic extract of *Cinnamomum verum* J Presl (bark) could hence be attributed to the presence of cinnamaldehyde, 4 hydroxycinnamic acid and eugenol. In addition to this, the polyphenols, tannins, and saponins present in the extract could also exert anticancer effects. While saponins have known anticancer effects via apoptosis induction, polyphenols and tannins regulate carcinogen metabolism, inhibit cell cycle, cell migration, cell proliferation, DNA binding, cellular differentiation, induce cell cycle arrest and blocks signaling pathways.

The present study concludes that the hydroalcoholic extract of *Cinnamomum verum* J Presl (bark) has a higher quantity of phytochemical constituents than pure ethanol and aqueous extract. The current study reports anticancer effects of hydroalcoholic extract and active compounds such as cinnamaldehyde, 4 hydroxycinnamic acid, and eugenol on oral squamous cell carcinoma cell line. However, the study has an in vitro design and further studies in this regard have to be conducted to explore not only the anticancer effects but also the bio-chemopreventive effects of *Cinnamomum verum* J Presl (bark) that would aid in the prevention of oral carcinoma.

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