RESEARCH PAPER

Assessment of phytochemical composition and cytotoxic activity of Zingiber officinale rhizome extracts in T-DOK oral keratinocytes: An in vitro observational study.

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ABSTRACT

Background: Precancerous lesions often occur before oral cancers, making chemoprevention possible and Zingiber officinale, with its phytochemical bioactives, may be a promising option.
Objectives: To analyze the biologically active compounds in stem extracts of Zingiber officinale (ginger) and determine how cytotoxic they are to T-DOK oral keratinocyte cell lines.
Materials and Methods: This observational in vitro study was conducted over a period of more than 1 year (July 2020 to August 2021) in which Zingiber officinale samples were sourced from Vellayani agricultural institute, Trivandrum Kerala along with certification. Methanolic extract of Zingiber officinale was analyzed for phytochemicals, and dysplastic oral keratinocyte (DOK) cells were transformed into proliferative transformed-DOK (T-DOK) cells.
Results: Phytochemical analysis of the methanolic extract of Zingiber officinale revealed the presence of alkaloids, phenolic compounds terpenoids, flavonoids steroids and phlobatannins in varying percentages of 11.2mg/ml, 0.12mg/ml, 0.052mg/ml, 10.6mg/ml respectively. Benzo[a]pyrene and 7,12-dimethylbenz[a]anthracene (B(a)P/DMBA) treatment increased DOK cell proliferation by 1.3–1.98 fold, confirming transformation into dysplastic T-DOK cells. Ginger extract showed significant cytotoxicity with half maximal inhibitory concentration (IC50) values of 207.35, 181.82, and 145.53 µg/mL at 24, 48, and 72 hours, respectively, and induced apoptosis in T-DOK cells after 72 hours.
Conclusion: The methanolic extract of Zingiber officinale exhibited significant cytotoxic and apoptotic effects on transformed premalignant T-DOK cells, indicating its potential chemopreventive role against oral premalignant lesion progression.
Keywords: Inhibitory Concentration 50, MeSH Terms Ginger extract, Phytochemicals, Precancerous Conditions, Zingiber officinale
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INTRODUCTION

Cancers of the lip and oral cavity rank as the 16th most common malignancies worldwide, and the overall incidence of head and neck cancers is projected to increase by nearly 30% by 2030.[1] In India, oral cancer represents a significant health burden, ranking first among males and second overall, and remains among the top three cancers for both incidence and mortality, with Rajasthan reporting a

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32.18% prevalence of head and neck and oral cancers.[2] Oral Potentially Malignant Disorders (OPMDs), previously termed precancerous lesions or conditions, were redefined by the WHO in 2005 as any oral mucosal abnormality associated with an increased risk of progression to oral cancer. OPMDs represent a heterogeneous group of lesions with varying malignant transformation potential, and their global prevalence is estimated to be approximately 4.5%, with considerable geographic variation.[2,3]

Ginger, derived from the rhizome of *Zingiber officinale* Roscoe belonging to the family *Zingiberaceae*, is among the most widely used spices and culinary ingredients worldwide. In addition to its culinary value, ginger has long been utilized in phytotherapy, a branch of medicine that employs plant-based substances for the treatment of diseases and promotion of health.[4,5]

Ginger and its bioactive constituents—including 6-gingerol, 6-shogaol, 6-paradol, zingerone, and zerumbone exhibit significant anticancer activity through induction of apoptosis, activation of tumor-suppressor pathways, and inhibition of angiogenesis via VEGF suppression. Ginger extracts have also demonstrated inhibitory effects on oral cancer cells, partly through ER stress-mediated autophagy and apoptosis. In particular, 6-shogaol, a key active compound of dried *Zingiber officinale*, regulates signaling molecules such as AKT, MAPK, STAT3, cyclin D1, Bcl-2, and caspases, thereby influencing cell proliferation, apoptosis, and tumor progression.[6-8] However, it is still uncertain how ginger extract and its active ingredients affect oral pre-cancer in pre cancer cell lines or human studies. No published studies till date are available globally and in India to the best of our knowledge. Thus, the present study aims to demonstrate a clear relationship between Zingiber's anti-cancer cytotoxicity mechanism in oral pre-cancer cell lines.

Materials and Methods:

Study Design and Ethics:

This observational in vitro study was conducted over a period of more than 1 year (July 2020 to August 2021) in which *Zingiber officinale* samples were sourced from Vellayani agricultural institute, Trivandrum Kerala along with certification. Institutional ethical clearance was obtained (IEC NO.-2021/RS/95).

Study Population: Invitro study with DOK cell lines

Study Procedure: Dysplastic oral keratinocyte (DOK) cell lines derived from premalignant human tongue epithelium were obtained and further transformed into more dysplastic T-DOK cell lines using the carcinogens benzo[a]pyrene and 7,12-dimethylbenz[a]anthracene (B[a]P/DMBA). Previous studies reported transformation of DOK cells into OSCC lines after three months of exposure to the same carcinogens; however, in this study, the exposure period was limited to six weeks to achieve a premalignant transformation. Fresh ginger (*Zingiber officinale*) extract was prepared using the Soxhlet extraction method. The ginger sample was washed with distilled water, sliced, and 20 g of fresh rhizome was ground, followed by the addition of 100 mL absolute methanol as solvent. Extraction was carried out at 78.4°C for 12 hours, with 5–6 heating cycles using a heating mantle.[9] The resulting extract underwent qualitative phytochemical screening to identify major constituents including alkaloids, carbohydrates, phytosterols, terpenoids, tannins, saponins, phenolic compounds, flavonoids, and glycosides, and their concentrations were expressed in mg/mL. DOK cells and B[a]P/DMBA were procured from Sigma-Aldrich Laboratories. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Himedia) until reaching 80% confluency, followed by serum starvation for 24 hours. Cells were then seeded at a density of 0.5×10^6 cells in T25 flasks and incubated at 37°C in a humidified atmosphere containing 5% CO_2. To induce dysplastic transformation, cells were treated every alternate day with 70 µM B[a]P/DMBA for 90 minutes. Every fifth day, cells were passage into new T25 flasks at the same seeding density, and exposure to B[a]P/DMBA was continued for six weeks. To confirm cellular proliferation and transformation, cells were incubated for 1–5 days, and a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed daily in duplicate. A total of 0.5 mg/mL MTT reagent (Himedia) was added to each well and incubated for 3 hours, after which absorbance was measured at 570 nm using a spectrophotometer. For cytotoxicity assessment, 200 µL of T-DOK cell suspension was seeded into 96-well plates and incubated overnight. Cells were then treated with five concentrations of *Zingiber officinale* extract (31.25, 62.5, 125, 250, and 500 µg/mL). After 24, 48, and 72 hours of incubation, cell viability and cytotoxic effects were evaluated using the MTT assay. Absorbance was measured at 570 nm and 630 nm, and the IC_{50} value was calculated to determine the inhibitory concentration of the ginger extract

Statistical Analysis:

Descriptive statistics were performed for the study and cell viability % was evaluated. Data entry was done in MS excel and data analysis was done in IBM SPSS 26. Data distribution was assessed using the Shapiro–Wilk test. Comparison of proliferation between untreated DOK and B(a)P/DMBA-treated T-DOK cell lines was performed using an independent samples t test.

For evaluation of cytotoxicity of *Zingiber officinale* extract at different concentrations, one-way analysis of variance was applied separately for each incubation period 24, 48 and 72 hours, followed by Tukey post hoc test for multiple comparisons. To assess the combined effect of concentration and incubation time on cell viability, two-way analysis of variance was performed.

Percentage cell viability was calculated as:

Cell viability (%) = (Absorbance of treated cells / Absorbance of control cells) × 100

Results:

Phytochemical Analysis of Zingiber officiale:

The studies showed the presence of phytochemicals such as alkaloids, phenolic compounds terpenoids, flavonoids steroids and phlobatannins in varying percentages of 11.2mg/ml, 0.12mg/ml, 0.052mg/ml, 10.6mg/ml respectively in the methanol extract of the ginger sample. Alkaloids, Phenols, Terpenoids were present significantly. Steroids and phlobatannins were negligibly present.

Transformation of DOK cell lines to T-DOK cell lines: MTT assays showed B(a)P/DMBA-treated DOK cells had a 1.3- to 1.98-fold growth increase over untreated cells from day 1 to 5, resulting in highly proliferative dysplastic T-DOK cell lines. Similar cell growth was observed for next sub cultures also (Upto 6 subcultures) [Figure :1].

Zingiber extract showed strong cytotoxicity against the T-DOK cell line after 24, 48, and 72 hours of incubation [Table:2]. The half maximal inhibitory concentration (IC50), a key metric for comparing inhibitors, was measured at 207.35 µg/mL, 181.82 µg/mL, and 145.53 µg/mL, respectively [Figure:1]. Furthermore, the study findings indicate that the tested compound effectively induced apoptosis in T-DOK cells after 72 hours of incubation at its IC50 concentration [Table :3].

Table 1: Depicting the presence of Quantitative compounds on Zingiber extract using methanol

Sl. No	Phytochemical constituents	Methanol extract	Quantitative (mg/ml)
1.	Alkaloids: Hager's test	Present	11.2mg/ml
2.	Phenolic compound: $Fecl_3$ test	Present	0.12mg/ml
3.	Flavonoids: Lead acetate test	Present	0.052mg/ml
4.	Terpenoids: Horizon test	Present	10.6mg/ml
5.	Steroids: Salkowski's test	Present	-
6	Phlobatannins: Acidic test	Present	-

Table 2: Zingiber Extract is evaluated to analyse the cytotoxicity effect on T - DOK cell line. By MTT assay

SL. No	Test Compounds	Cell Line	Concentration treated to cells
1	Untreated	T – DOK	No treatment
2	Standard (Cisplatin)	T – DOK	7.5 µM for 72 hrs, 11.8µM for 48 hrs and 26.5µM for 24 hrs
3	Blank	T – DOK	Only Media without cells
4	Ginger extract	T – DOK	5(31.25, 62.5,125, 250,500 µg/ml)

Table 3: Determination of cytotoxicity and IC50 value of Zingiber extracts on carcinogen-transformed DOK cell line using MTT assay

INCUBATION TIME OF EXTRACT	IC 50 VALUE
24hrs	**207.335 µg/ml**
48hrs	**181.815 µg/ml**
72hrs	**145.53 µg/ml**

Figure 1: Graph comparing cell growth between B(a)P/DMBA-treated (T-DOK) and untreated DOK cell lines. The graph depicts how treating with these carcinogens affects the growth of DOK cells compared to controls.

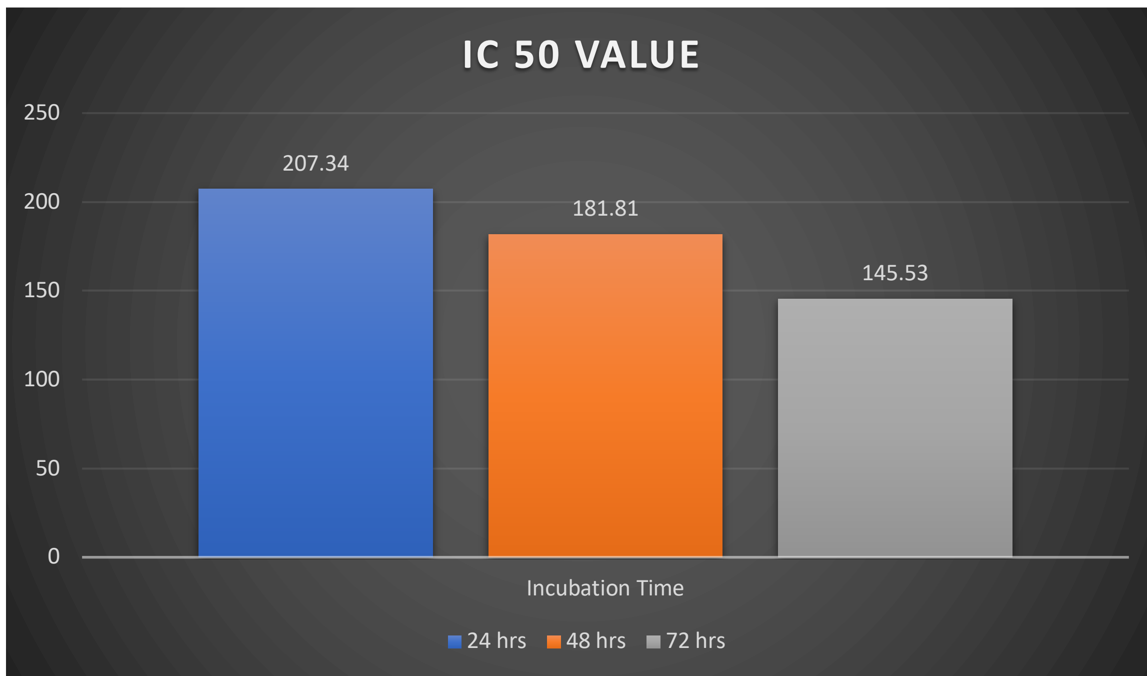
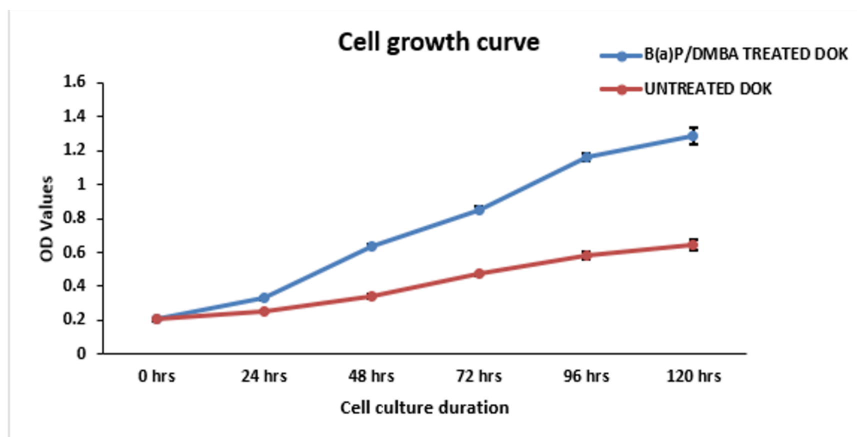


Figure 2: Graph comparing cell growth between B(a)P/DMBA-treated (T-DOK) and untreated DOK cell lines. The graph depicts how treating with these carcinogens affects the growth of DOK cells compared to controls.



Discussion

Ginger (*Zingiber officinale Roscoe*) is commonly used as a spice and has been studied for its possible effects on various diseases.[10] Zingiber extract is proven to have, antioxidant, anti-inflammatory, and antinociceptive effects thus making it an effective tool in the management of premalignancy and cancer and our study aligns with the same.[11] Previous studies exposed DOK cell lines to the carcinogens B(a)P, a polycyclic aromatic hydrocarbon that induces DNA adducts, and DMBA, which causes DNA damage in animal models. B(a)P and DMBA a potent carcinogen was used. A 70 µM mixture was applied for 3 months to transform DOK cell lines into oral cancer cell lines.[12] In this study, DOK cell lines were treated with a combination of B(a)P and DMBA at 70 µM for 6 weeks to induce transformation into T-DOK cell lines, resulting in a pre-malignant state rather than full malignancy. Therefore, the potential chemopreventive effects of ginger extract can be evaluated in this context which is a novelty not published so far. Various studies have been done on varying type of cancer and cell lines like breast, prostate etc.[13] but to the best of our

knowledge this is the first study using Zingiber extract with Transformed T-DOK premalignant cell line.

The half-maximal inhibitory concentration (IC50) is an important indicator of drug potency, where lower IC_{50} values reflect greater effectiveness and cytotoxic activity. In the present study, the in vitro effects of a methanolic extract of locally sourced ginger (*Zingiber officinale*) were evaluated on T-DOK cell lines. The extract demonstrated a dose- and time-dependent cytotoxic effect, with IC50 values of 207.35 µg/mL, 181.82 µg/mL, and 145.53 µg/mL at 24, 48, and 72 hours, respectively. These findings support the cytotoxic and antiproliferative potential of ginger extract. Similarly, Chen KL et al.[14] reported antiproliferative and pro-apoptotic effects of ginger-derived compounds in human OSCC cell lines, where apoptosis was assessed through oxidative stress–mediated mechanisms. However, studies evaluating the effects of ginger extract on transformed premalignant cell lines remain limited, highlighting the novelty of the present investigation.

This study has certain limitations. Firstly, as the study was conducted in vitro, it lacks physiological complexity and cannot fully replicate the intercellular signaling, hormonal influences, and immune responses present in a living organism. Secondly, the T-DOK cell culture lacks the tumor microenvironment, including the extracellular matrix (ECM) and vascular supply that play important roles in the development of premalignant lesions. Finally, the use of a single T-DOK cell line represents only one genetic profile, which may not reflect the genetic variability seen in the broader patient population.

Conclusion

To conclude, the methanolic extract of *Zingiber officinale* demonstrated the presence of significant phytochemicals, including alkaloids, phenols, terpenoids, and flavonoids. Transformation of DOK cells into highly proliferative T-DOK cells was successfully achieved using B(a)P/DMBA. The ginger extract exhibited notable cytotoxic and apoptotic effects on T-DOK cells, with decreasing IC_{50} values over time, indicating its potential chemopreventive activity in premalignant oral lesions.

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