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Anticancer and Antioxidant activity of *Zingiber officinale* Roscoe rhizome

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Dietary components present in foods, spices and herbs are source of natural compounds *viz*. phenols, flavonoids, tocopherols, ascorbic acid and carotenoids with potential benefits. Ginger is one such herb commonly used throughout the world as a spice for dietary as well as medicinal purpose since ancient period. Here, we investigated the methanolic extract of *Zingiber officinale* rhizome (ZOME) for anticancer activity against human cervical cancer HeLa cells and breast cancer MDA-MB-231 cells and antioxidant activity using 1,1-diphenyl-2-picryl hydroxyl (DPPH) scavenging assay, 2,2'-azinobis-3-ethylbenzothiozoline-6-sulfonic acid (ABTS) cation decolorization test. Antiproliferative activity was substantiated by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and colony formation assay for cell viability and cell proliferation, Hoechst staining was performed to examine apoptosis. Our results demonstrated that ZOME inhibited the proliferation and colony formation in HeLa and MDA-MB-231 cells, in a dose- and time-dependent manner and induced typical changes in nuclear morphology, chromatin condensation and fragmentation, membrane shrinkage and blebbing in both cells indicated apoptotic property of *Z. officinale*. ZOME exhibited potent antiradical activity against DPPH and ABTS. On the basis of the results of the present study, it may be suggested that *Z. officinale* has promising anticancer and antioxidant properties. Since, *Z. officinale* has been commonly used throughout the world as a spice for dietary as well as for medicinal purposes since prehistoric times. Therefore, enriched use of *Z. officinale* as dietary material could be recommended in ethno-medicine for the management of cervical and breast cancers. Moreover, further studies are needed to isolate and characterize the potent compounds for further adjuvant therapy against such malignancies.

Keywords: ABTS, Antiproliferative activity, Breast cancer, Cervical cancer, Ginger, DPPH

Cancer is the leading cause of morbidity and mortality worldwide. Currently, over a hundred types of cancers are known differentiated by etiology and natural history. Among different cancers, breast and ovarian cancers are the most frequent and highly prevalent malignant diseases in women. The low selectivity of chemotherapeutic agents and limited success of clinical therapies including radiation, immunomodulation and surgery, as evidenced by the high morbidity and mortality rates, it is justified and timely that there is an urgent need for new and better therapeutic resources, including new chemotherapeutic agents for cancer management[1,2]. Recently, Jayashree *et al*. summarized various approaches in anticancer research, their target mechanisms and the candidate drugs developed against specific targets[3].

Generation of free radicals or reactive oxygen species (ROS) during even normal metabolism can cause extensive damage to cells and tissues. These reactive species plays a role in oxidative stress related diseases like heart diseases, neurodegenerative diseases, cancer and in the aging process[4]. It is reported that active oxygen may be involved in carcinogenesis through induction of gene mutations that result from cell injury and the effects on signal transduction and transcription factors[3,5]. Cellular targets affected by oxidative stress include all biomolecules *viz*. DNA, phospholipids, proteins, and carbohydrates. Antioxidants are the compounds that act as radical scavengers, prevent the radical chain reactions of oxidation and are recognized for their good potential in promoting health and lowering the

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Abbreviations: ABTS, (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); DPPH, (2,2-diphenyl-1-picrylhydrazyl); FBS, Fetal Bovine Serum; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; ZOME, Methanolic extract of *Zingiber officinale* rhizome

risk for cancer, hypertension and heart diseases[6]. Spices and herbs have been recognized as an excellent source of antioxidant compounds such as phenols, flavonoids, tocopherols, ascorbic acid and carotenoids which have been reported to show good antioxidant activity[7]. Dietary components present in foods, spices and herbs do contribute to the decreased incidence of colon, prostate, breast and other cancers in South East Asian countries[8]. Therapeutic potential of dietary cucurbits protecting against cancer and inflammatory cytokine have been demonstrated by Sharma *et al*.[9] while Krishnamurthy *et al*.[10] reported a potent anticancer fraction from the leaf extracts of *Moringa oleifera*.

Zingiber officinale (Ginger, Zingiberaceae) is a rhizomatous perennial plant which has been reported primarily a remedy for digestive disorders, dyspepsia, nausea, vomiting, gastritis, diarrhoea and also used for treatment of asthma, common cold disorders, nervous disease, inflammation, hepatotoxicity, diabetes, migraine, hypercholesterolaemia, helminthiasis and schistosomiasis[11-18]. Ginger has been commonly used throughout the world as a spice for dietary as well as medicinal purpose since prehistoric time. Consistent with this belief, we investigated the antiproliferative and antioxidant activity of *Z. officinale* rhizome methanolic extract against Human Cervix HeLa cancer cells and Human Breast MDA-MB-231 cancer cells.

Materials and Methods

Chemicals

Solvents (Analytical grade) used in this study were purchased from Thermo Fischer, USA. Fetal Bovine Serum (FBS), phosphate buffered saline (PBS) and RPMI-1640 medium were purchased from Gibco BRL, USA. The penicillin/streptomycin mixture was procured from Lonza, USA. 3-[4, 5-Dimethyl-2-thiazolyl]-2, 5-diphenyltetrazolium bromide (MTT), Trypsin-EDTA and Hoechst 33342 was obtained from Sigma Aldrich, USA. Dimethyl Sulfoxide (DMSO) was purchased from Calbiochem. DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bi's (3-ethyl-benzothiazoline-6-sulphonic acid) were procured from Sigma Aldrich, USA. Ascorbic acid, rutin, cisplatin and other chemicals used in the study were obtained from Merck.

Plant material and extraction

Rhizomes of *Zingiber officinale* were procured from Lucknow vegetable market. Collected rhizome was identified at the Department of Botany, Shia PG College, Lucknow, India. Rhizomes were air dried and ground to fine powder for the study. About 3.2 kg of the rhizome was soaked in methanol for 72 h and filtered. This process was repeated thrice for complete extraction. The filtrate combined and concentrated under reduced pressure with controlled temperature, final residue obtained was named as ZOME and used for bioassay.

Antiproliferation activity

Antiproliferative activity was substantiated by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay and colony formation assay for cell viability and cell proliferation, Hoechst staining was performed to examine the apoptosis.

Cell line and culture

Human Cervix carcinoma HeLa cell line was obtained from cell repository-National Centre for Cell Sciences, Pune, India. HeLa cells were cultured in Minimum Essential Medium Eagle (MEM) supplemented with 2.0 mM L-glutamine,1.5 g/L $NaHCO_3$, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate and supplemented to contain 10% (v/v) Fetal Calf Serum. Human Breast adenocarcinoma (MDA-MB231, triple negative, invasive) cell line was obtained from ATCC, USA. Cells were cultured with medium (RPMI) and penicillin gentamicin containing 5% FBS and grown at 37°C, 5% CO_2 in a humidified air.

In vitro MTT assay for antiproliferation activity

The antiproliferative activity of extract was evaluated by MTT reduction assay[19]. This assay is based on the enzymatic reduction phenomenon of MTT dye. The assay provides a direct relationship between the viable cells and color formation (absorbance). In brief, the cells (1×10^4) were seeded in 100 µL complete medium in each well of 96-well culture plate for 24 h at 37°C and 5% CO_2. Stock extract (ZOME) was prepared in DMSO of 100 mg/mL and working dilutions of desired concentrations (400, 200, 100, 50, 25 and 12.5 µg/mL) in medium and cisplatin (1.25-40 µg/mL) which is used as standard drug, added to the wells in triplicate as per experimental design. The final concentration of DMSO in any of the wells did not exceed 0.05% (v/v). After 21 h of treatment, 10 µL of MTT (5 mg/mL of media without phenol red and serum) solution was added to each well and the plates were further incubated for 3 h at 37°C until the formazan blue crystal developed. Then the supernatant was

discarded from each well and 100 µL of DMSO was added to solubilize formazan crystals for 10 min at 37° he optical density as recorded at nm by a microplate reader he percentage viability as calc l ated by s ing the form l a ell viability D of treated OD of control)] × 100

Colony formation assay

The colony formation assay was performed to evaluate the efficacy of the extract on the survival and proliferation of cells. Briefly, cells at the initial density of $1×10^3$ in 2 mL medium were seeded in 6-well plates. Cells were incubated for 16-18 h. Next day, media were changed and cells were treated with different concentrations (12.5, 25, 50 and 100 µg/mL) of ZOME. After 24 h, media were removed and cells were grown in complete growth media for next 7-10 days until distinct colonies were formed. Thereafter, colonies were washed with PBS and fixed with a chilled 100% methanol for 10 min. Fixed cells were stained with 0.05% Coomassie blue stain for 10 min and washed with tap water. Plates were air dried and the number of colonies was determined by counting them under an inverted phase-contrast microscope at X400 magnification and a group of ~50 cells were counted as a colony.

Morphological apoptosis by Hoechst 33342 Staining

To determine morphological apoptosis in cells, we performed Hoechst 33342 staining and fl or escence microscopy. Cells were grown to 50% confluence on cover slip and left for 18-20 h in the incubator until full morphology is attained. Next day, old media was replaced with fresh media and cells were treated with different concentrations (12.5, 25 and 50 µg/mL) of ZOME. After 24 h, cells were fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton-X100 in PBS. Cells were then stained with Hoechst 33342 at a final concentration of (0.5 µg/mL) in PBS and incubated for 5 min at 25°C. Brightly stained, condensed nuclei with characteristic features of apoptotic cells were counted using a fluorescence microscope. A minimum of 5 fields of at least 100 cells per field will be counted. Images were captured at 10-20X magnification.

Antioxidant activity

The antioxidant activity of ZOME was determined by DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity[20] and ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium) assay[21].

Statistical analysis

All experiments were performed in triplicates and repeated at least three times and the data were presented as mean ± SD and analyzed using one-way ANOVA. The results were considered statistically significant if the *P* values were 0.05 or less.

Results

Effect of ZOME on proliferation of HeLa and MDA-MB-231 cells

To determine the effect of ZOME on cervical and breast cancer cell growth, we performed an MTT assay. HeLa and MDA-MB-231 cancer cells were treated with different concentrations of the ZOME for 24 and 48 h. Our results demonstrated that ZOME exhibited significant growth inhibitory effect on both the cell lines as compared to untreated cells (Fig. 1A). ZOME induced growth inhibition was dose and time dependent as depicted in Fig. 1A. Comparison of cell viability between control and treated cells revealed that the significant viability decline was initiated at 12.5 µg/mL concentration in both HeLa and MDA-MB-231 cells after 24 hour treatment with ZOME. The standard drug cisplatin in our study showed excellent indication of cancer cell inhibition on HeLa and MDA-MB-231 cancer cells. These results altogether indicate the growth inhibitory activity of ZOME on cervical and breast cancer cells. IC_{50} values (Table 1) indicated that ZOME inhibited cervical cancer cell growth at lower concentrations as compared to breast cancer cell.

Effect of ZOME on colony formation of HeLa and MDA-MB-231 cells

In continuation with cell viability assay, we evaluated the effect of ZOME on proliferation and ability to colony formation of cervical and breast cancer cell. Cancer cells proliferate and form colonies as such clonogenicity provides an indirect assessment of the tendency of tumor cells to undergo neoplastic transformation. As shown in Fig. 2, during the 7-10 day culture period, ZOME treatment reduced the

Table 1—IC_{50} value obtained for ZOME and standard cisplatin for HeLa and MDA-MB-231 cells after exposure of 24 and 48 h. [Data represent the means±SD of the three experiments conducted in triplicate]

Extract/standard	IC_{50} Value (µg/mL)			
	HeLa		MDA-MB-231	
	24h	48h	24h	48h
ZOME	46.5±4.08	37.5±4.72	86.7±5.35	57.5±4.8
Cisplatin	5.1±0.61	<1.25	43.92±3.13	29.84±2.2

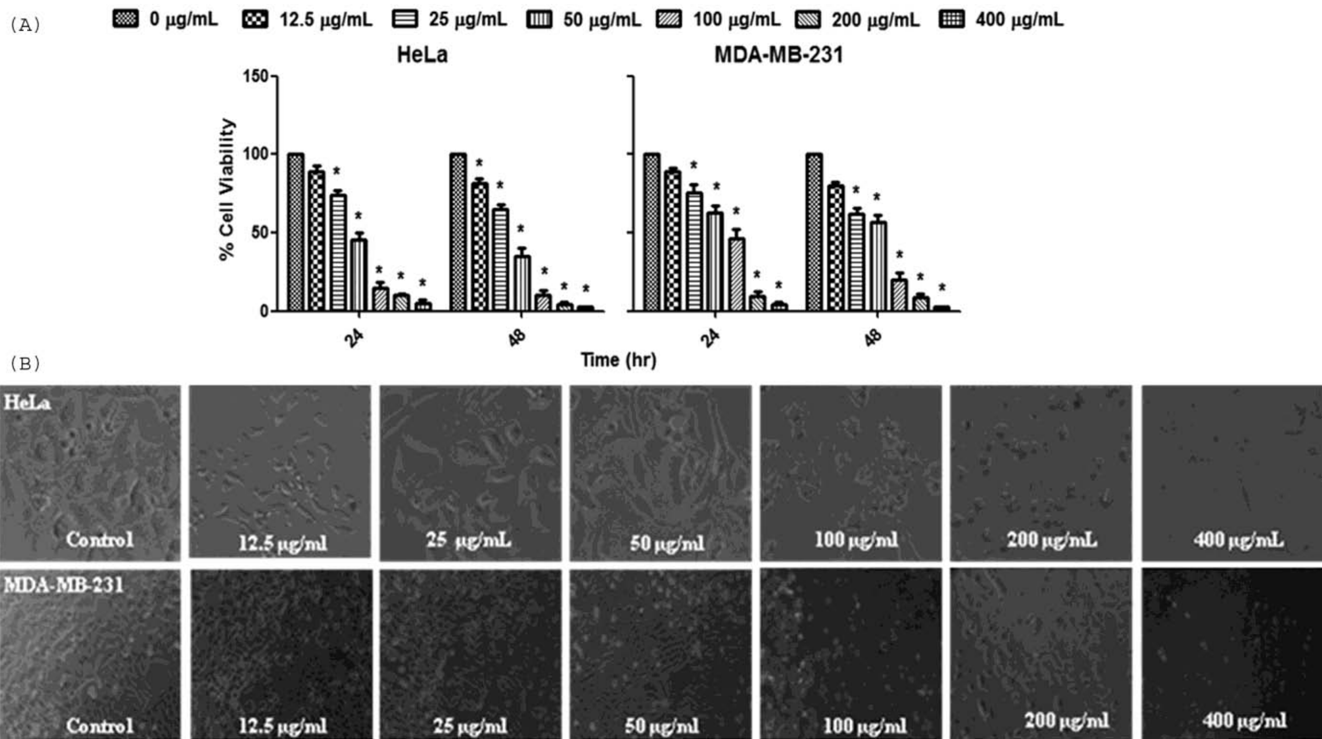

Fig. 1—(A) Effect of ZOME on percentage viability of HeLa and MDA-MB-231 cancer cells after exposure to 24 and 48 h. [Cell viabilities are shown as percentages, and the untreated cells were regarded as 100% viable. Data represent the means±SD of the three experiments conducted in triplicate and were significant (*$P \leq$]; and (B) Microscopic examination of Human cervix carcinoma (HeLa) and Human Breast Cancer (MDA-MB-231) cells after treatment with ZOME. [The cells were treated with different concentrations for 24 h. Photographs were taken using an Nikon Eclipse Ti-S at a magnification of 10-20X]

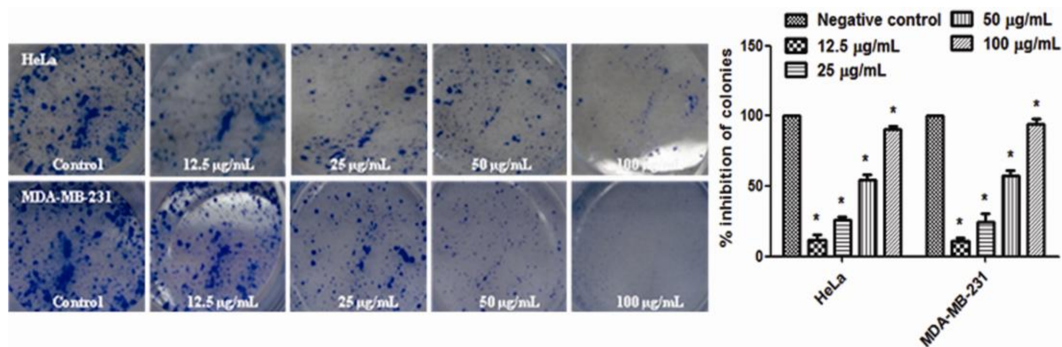

Fig. 2—ZOME inhibits clonogenicity of HeLa and MDA-MB-231 cells. [A survival of 100% corresponds to the number of colonies obtained with cells that were not treated with ginger extract (control). Dose-dependent colony formation inhibition was clearly evident. Data represent the means±SD of the three experiments conducted in triplicate and were significant (*$P \leq$]

number of growing colonies and visibly modulated the size of growing colonies. These observations suggested that ZOME showed antiproliferative activity against cervical and breast cancer cells.

ZOME induced changes in the nuclear morphology in HeLa and MDA-MB-231 cells

Furthermore, to determine whether ZOME induces apoptosis in HeLa and MDA-MB-231 cancer cells, the cells were stained with Hoechst 33342. Morphological changes in the nucleus were observed under a fluorescence microscope. Results showed that there were significant changes in nucleus morphology of ZOME treated HeLa and MDA-MB-231 cells as compared with untreated HeLa and MDA-MB-231 cells. After exposure of ZOME, the intensity of blue fluorescence was bright due to changes in the morphology of nucleus, condensation and fragmentation. However, the untreated cells showed

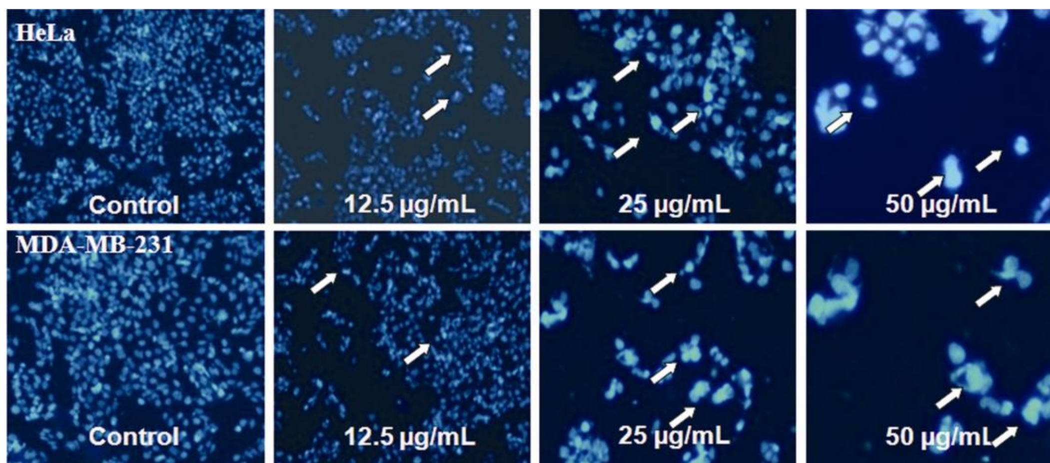

Fig. 3—Control and ZOME treated HeLa and MDA-MB-231 cells. [Cells stained with Hoechst 33342, and arrow marks indicate apoptotic cells. Fluorescence was observed at fluorescent microscope]

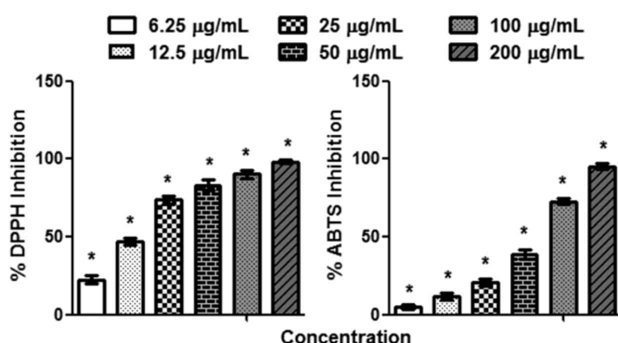

Fig. 4—% Inhibition of DPPH and ABTS radicals by ZOME at different concentrations. [Data represent the means±SD of the three experiments conducted in triplicate and were significant (*$P \leq$]

normal morphology of nuclei such as round, homogenous and intact chromatin (Fig. 3). These results suggested that ZOME inhibited cervical and breast cancer cell proliferation through induction of apoptosis, which is a potent feature of chemotherapeutic agents.

ZOME inhibits DPPH and ABTS free radicals

The results obtained from the preliminary analysis of antioxidant activity are shown in (Fig. 4) According to the data obtained, significant inhibition of DPPH radical by different concentrations of ZOME was observed. From the results, the % inhibition of DPPH radicals ranged from 47.24-93.07 % at a concentration of 6.25-200 µg/mL. Highest percentage of DPPH inhibition was observed at a concentration of 100 µg/mL of ZOME. The IC_{50} value was calculated as 14.0 µg/mL. Our results showed ABTS radical inhibition ranging from 20.65-99.54 % with a concentration from 25-400 µg/mL of the ZOME. Moreover, the IC_{50} value obtained was 67.5 µg/mL for ZOME (Fig. 4). The efficiency of radical inhibition was compared with standard ascorbic acid with an IC_{50} value of 2.05±0.2 µg/mL and with rutin IC_{50} value was found to be 4.25±0.46 µg/mL. These results suggested that ZOME has significant antiradical property.

Discussion

The results of the present study demonstrated that the methanol extract of ginger potently inhibited, in a dose- and time-dependent manner, the proliferation of HeLa and MDA-MB-231. The results of our study suggested that ZOME possess the strongest cytotoxic and apoptotic effects on HeLa cancer cells at a concentration of 100 µg/mL with 84.88% growth inhibition, however, at 200 and 400 µg/mL concentrations none of the cells were viable. Nalbantosy *et al.*[22] reported that the ethanol extract of ginger exhibited cytotoxic effect on HeLa cancer cells with IC_{50} values of 33.78 µg/mL for 48 h treatment. However, Choudhary and co-workers[23] reported aqueous extract of ginger inducing apoptosis in HeLa cancer cells and the IC_{50} dose was 253.4±8.9 µg/mL. In our study, we found an IC_{50} value of 46.5±4.08µg/mL for 24 h and 37.5±4.08 µg/mL for 48 h treatment for HeLa cells. Our study is in concordance with Nalbantosy *et al.*[22] while contradicting the results of Choudhary and co-workers[23].

Similarly, in case of MDA-MB 231 cancer cells, 90.56% growth inhibition were marked at 200 µg/mL concentration of extract, however, at 400 µg/mL no cell viability was observed. Rahman *et al.*[24] reported that ginger grown in elevated CO_2 concentration exhibited the highest anticancer activity on MDA-

MB-231 cancer cells with IC_{50} values of 32.53 and 30.20 µg/mL for two different regions. Our result corresponds with Rahman *et al*.[24] for MDA-MB-231 cells with a little difference in IC_{50} values. The growth inhibitory effect of ZOME was compared with standard drug cisplatin and we found that there was little difference in IC_{50} values in case of ZOME treated MDA-MB-231 cancer cells and cisplatin treated MDA-MB-231 cancer cells. However, in case of ZOME treated HeLa cells and cisplatin treated HeLa cells the difference in IC_{50} values was relatively more. These findings indicate the competency of ZOME with a standard anticancer drug. Cojocaru *et al*.[25] reported that fresh ginger extract induced no changes in normal skin, fibroblast viability, but caused profound cytotoxic effects on amelanotic melanoma. Moreover, the low cytotoxic effect on non-cancerous cell line Vero indicates that ginger essential oil exhibits cytotoxicity specifically to cancer cells only but not in normal cells[26]. The National Cancer Institute (NCI), USA specified that extract with IC_{50} values <30-40 µg/mL considered as a potent chemotherapeutic agent and granted for active principles isolation and identification. We found that ZOME demonstrated IC_{50} values against human cervical cancer and breast cancer cells, an in accordance with the NCI guidelines one can recommend ginger extract and its active constituents for treating cancers.

Consequently, with antiproliferative and cytotoxicity activity ZOME potentially inhibited the formation of colonies of HeLa and MDA-MB-231 cancer cells in a dose dependent manner as revealed by colony formation assay. This assay can be also correlated with antineoplastic activity of ZOME as evident by tumor cell colony inhibition undergoing neoplastic transformation. Morphological features of apoptotic cells involved condensation and fragmentation of nucleus, cytoplasm and chromatin, membrane shrinkage and blebbing. The Hoechst 33342 staining assay was employed to observe the effect of ZOME on morphological alterations of the HeLa and MDA-MB-231 cancer cells. This assay showed that ZOME induced morphological changes such as cell shrinkage and nuclear condensation demonstrating apoptotic property of *Zingiber officinale*. Moreover, apoptosis of HeLa and MDA-MB-231 cells were gradually raised with increasing order of concentration of extract which revealed dose dependent apoptosis. These assays hold assurance for further *in vitro* and *in vivo* molecular target-based studies to scrutinize the mechanism of chemoprotective efficacy of ZOME. Our findings correlates with previous literature reported *Zingiber officinale* has significant anticancer and apoptotic properties[22-24,27].

We assessed the antioxidant potential of ZOME via free radical radicals scavenging activity. The DPPH and ABTS assays demonstrated that ZOME inhibits in a dose-dependent manner scavenging of the free radicals. There are many reports demonstrating that the most natural products with anticancer activity act as strong antioxidants and modify the proteins involved in cell cycle control as well as alter the regulation of cancer-related genes which leads to apoptosis in cancer cells[28-31]. In our results, we observed that ZOME not only induces apoptosis in breast and cervical cancer cells but also possesses strong antioxidant activity. ZOME, therefore, could prevent or assuage the free radical induced peroxidation of cellular biomolecules such as lipids, proteins and nucleic acids. It can be stated from the above results that *Z. officinale* rhizome has excellent potential to inhibit free radicals, and thus have antiradical property. ZOME is believed to include a combination of entire non-polar to polar compounds such as essential oils, phenolics, hydroxy-phenolic compounds with acids, alcohols, sugars or glycosides which may be involved in therapeutic properties of *Z. officinale*[32].

Conclusion

In conclusion, our results demonstrated that methanol extract of *Zingiber officinale* inhibits, in a dose- and time-dependent manner, the proliferation and colony formation in the cervical cancer HeLa cells and breast cancer cell lines, MDA-MB-231. The anticancer potentiality of *Z. officinale* could be attributed to its induction of apoptosis. Also, the results demonstrated the free radical inhibitory properties of *Z. officinale*. Thus, enriched use of *Z. officinale* as dietary material could be recommended in ethnomedicine for the management of cervical and breast cancers. However, more focused clinical studies are needed along with the isolation and characterization of its active components, having anticancer properties.

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