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Research Paper

Purification of bioactive compounds from methanolic crude extracts of Zingiber Officinale and their anticancer activity against mammalian cell line

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Abstract

A few of the drugs are simple synthetic modifications of naturally obtained substances from medicinal plants. Ginger (Zingiber officinale), falls among the frequently used medicinal plant. The present study mainly focused on the anticancer activities of methanol fraction of Zingiber officinale. The active compounds were purified from methanolic crude extracts of Zingiber officinale (Ginger) by using TLC. Methanolic extracts showed four different bands with 0.4, 0.15, 0.43 and 0.60 retention factors respectively. In this study, seven fractions were reconstituted in methanol and subjected to column chromatography (300mm x 10 mm) on silica gel. The Gingerols was very interesting a biological activity was selected for the identification of compounds by HPLC. In LC-MS analysis, the purified column fraction in total ion chromatogram was identified. The ion mass $[M+H]^{+2}$ was detected in positive mode with relative abundance. The ion 180.89 and 977.21 were determined the mass of abundant when compared with other. The cytotoxicity activity of Z. officinale methanol fraction showed against HCT-116 and DU145 cell lines. The maximum percentage (75.62 %) of inhibition was showed the methanolic fraction of Z. officinale at 320µg/ml concentration against HCT 116 cell line. . In this study revealed that the maximum (61 %) and minimum percentage (1.97 %) of inhibitions were noted in the concentrations of 320 and 5 µg/ml respectively.

Key words: *Zingiber officinale Medicinal plant, Methanol, HPLC, Cell line and Cytotoxicity activity.*

Introduction

Cancer can be defined as a disease in which a group of abnormal cells grow uncontrollably by disregarding the normal rules of cell division. Normal cells are constantly subject to signals that dictate whether the cell should divide, differentiate into another cell or die. Cancer cells develop a degree of autonomy from these signals, resulting in uncontrolled growth and proliferation. If this proliferation is allowed to continue and spread, it can be fatal. In fact, almost 90% of cancer-related deaths are due to tumor spreading, a process called metastasis (Kaku et al., 2011). The foundation of modern cancer biology rests on a simple principle - virtually all mammalian cells share similar molecular networks that control cell proliferation, differentiation and cell death. The prevailing theory, which underpins research into the genesis and treatment of cancer, is that normal cells are transformed into cancers as a result of changes in these networks at the molecular, biochemical and cellular level and for each cell there is a finite number of ways this disruption can occur. Phenomenal advances in cancer research in the past 50 years have given us an insight into how cancer cells develop this autonomy. We now define cancer as a disease that involves changes or mutations in the cell genome.

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural a source that plays a vital role in treatment of diseases (Cragg and Newman, 2001). Zingiber officinale, belonging to family Zingiberaceae is one of the most important plant with several medicinal, nutritional and ethnomedical values therefore, used extensively worldwide as a spice, flavouring agent and herbal remedy. Traditionally, Z. officinale is used in Ayurveda, Siddha, Chinese, Arabian,

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Africans, Caribbean and many other medicinal systems to cure a variety of diseases viz, nausea, vomiting, asthma, cough, palpitaion, inflammation, dyspepsia, loss of appetite, constipation, indigestion and pain.

The family Zingiberaceae is the largest family of Zingiberales and is one of the ten largest monocotyledonous families in India. It occurs chiefly in the tropics with about 52 genera and 1400 species with the greatest concentration in the Indo-Malayan region of Asia and represented by 22 genera and 178 species in India. Zingiberaceae forms an important group with economic potential and many members of this family yield spices, dyes, perfumes and medicines and some are ornamental. Many of them are used in ayurvedic and other native systems of medicine (Sacchetti et al., 2005). Different constituents of ginger has been established its role in medicine to treat various ailments from time immemorial in different parts of the world. Recent years have seen an increased enthusiasm in treating various diseases with natural products. Ginger (Zingiber officinale) is a non-toxic highly promising natural antioxidant compound having a wide spectrum of biological function (antimicrobial, anti-inflammatory, immunomodulatory, antioxidant, anticarcinogenic). Safety evaluation studies indicate that Zingiber officinale are well tolerated even at a very high dose without any toxic effects (Brinker, 1998).

Ginger and many of its chemical constituents have strong anti-oxidant actions. Although many people are aware of the health benefits of ginger, few people realize that pre-clinical studies have indicated that this natural product may have value as a complementary treatment for various forms of cancer. In recent years, nutraceutical compounds have gained wide acceptance as preferred alternatives to various synthetic drugs available on the market, particularly against cancer and diabetes. The long-term use of synthetic drugs is often associated with serious side effects that can even result in death. It may be worthwhile investigating the effect of ginger on cancer chemotherapy, as the crude drug and its constituents have themselves anti- cancer actions. Gingerols are effective against various types of cancer in vitro, including colon, lung, skin and breast cancer. However, few studies have been done to confirm in vivo efficacy of these compounds. Further clinical studies are warranted to assess their efficacy and safety and, consequently, minimize any risks associated with their use. In the present study we take the task to investigate the anticancer activity of bioactive compound derived from Ginger on human prostate and colon cancer cell lines.

Materials and method

Sample collection

Tubers of Zingiber officinalis (Ginger) were collected from Thanjavur district, Tamil Nadu, they were dried under shade and powdered with a blender and freeze dried. They were identified authenticated and used for the extraction of the bioactive compounds.

Preparation of extracts

The dried samples were ground to a fine powder using a dry grinder, and then kept in an air-tight container and stored in a freezer (-20°C) before extraction. 20 grams of dried powder was used for serial extraction in a soxhlet apparatus using methanol (300 ml) for 8 hours. The extracts were filtered and evaporated to dryness in a rotary evaporator. 1mg/ml of the extract was prepared by dilution of the stock with sterile dimethylsulphoxide(Duraipandiyan et al., 2006).

Purification of crude extract by thin layer chromatography (TLC)

TLC was carried out using pre-coated TLC plates Silica gel 60 F 254 (Merck). Thin layer chromatography (TLC) is a chromatography technique used to separate compounds present in a mixture as previously described (Selvameenal et al., 2009). Thin layer chromatography is performed on a sheet of glass, plastic, or aluminum foil, which is coated with a thin layer of

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adsorbent material, usually silica gel, aluminum oxide, or cellulose. This layer of adsorbent is known as the stationary phase. After the sample has been applied on the plate, a solvent or solvent mixture (known as the mobile phase) is drawn up the plate via capillary action. The silica gel coated sheet was activated at 110 C for 15 minutes. The sheet was cut into thin strips and the plant extracts were dissolved in methanol (20μl). With the help of a capillary tube, the sample was spotted at the bottom of the silica gel coated sheet and then it was placed in a beaker containing the mobile phase, covered with the watch glass in order to prevent the evaporation of the solvents. The chromatogram was performed with the solvent systems (a) Toluene: ethylacetate: formicacid (2.5:1:1v/v); (b) chloroform:acetone (6:4 & 8:4v/v); (c) hexane: acetone (6:4 & 8:2 v/v); (d) dichloromethane: acetone (6:4 & 8:2v/v); (e) Toluene. Once the solvent reached 3/4th of the sheet, the TLC sheet was removed and chromatograms were detected under UV – light (254 and366nm). The Rf value was calculated for each of the bands observed under the UV light. Each band was marked under UV and was scraped, mixed with methanol and centrifuged at 3000 rpm for 15min. Supernatant was collected in a pre-weighed vial and kept for evaporation. After the evaporations of the solvents, the weight of the fractions was determined.

Identification of compounds by HPLC

Identification of compounds from Z. officinalis was done using the HPLC system (consisting of a vacuum degasser, an auto sampler, and a binary pump with a maximum pressure of 600 bar; Agilent 1260, Agilent technologies, Germany). Equipped with a reversed phase C18 analytical column of 4.6 100mmand 3.5 μm particle size (Zorbax Eclipse XDB C18).The DAD detector was set to a scanning range of 200–400 nm. Column temperature was maintained at 25 °C. The injected sample volume was 2 μl and the flow-rate of mobile phase was 0.4 mL/min. Mobile phase B was milli-Q water that consisted of 0.1% formic acid and mobile phase A was methanol. Total run time was 40 min. The elution program was as follows: 10 A/90 B (0–5 min); 20 A/80 B (5–10 min); 30 A/70 B (10–15 min); 50 A/50 B (15–20 min); 70 A/30 B (20–25 min); 90 A/10 B (25–30 min); 50 A/ 50 B (30–35 min); 10 A/90 B (35–40 min). Each sample was directly injected and chromatograms were monitored at 254 nm. Peak identification was obtained comparing the retention time and the UV spectra of Z. officinalis chromatogram with those of pure standards.

LC-MS analysis

The separation of phenolics was performed with a Shimadzu LC-MS 2020 system equipped with an online degasser (DGU-20A3R), a two binary pump (LC-20ADXR), an autosampler (SIL-20AXR), a column heater (CTO-20AC) and a diode array detector (SPD-M20A). Instrument control and data analysis was carried out using Shimadzu absolution V5.42 SP6 edition through Windows XP. The chromatographic separation was performed using an AQUASIL C18 analytical column (150 mm x 3 mm x 3 μm particle size). Used as a stationary phase at 40°C as temperature. The mobile phase consisted of methanol with formic Acid (0.1 mL/100 mL methanol) (solvent B) and water with formic Acid as solvent A (0.1 mL/100 mL water). The flow rate was kept at 0.4 mL/min. The gradient elution started with 90% A/10% B 0-45 min, 100% B 45-55 min, 90% A/10% B 55-55.1 min, 90% A/10% B 55.1-60 min. Photodiode array detector was set at 350 nm for acquiring chromatograms. The injection volume was 20 μL and peaks were monitored at 250 nm. Peak identification was obtained by comparing the retention time and the UV spectra of the fraction phenolic chromatogram with those of pure standards which were purchased from Sigma Aldrich and LGC standards. Mass spectrometric analysis was performed on a Shimadzu mass spectrometer. Mass spectra data were recorded on an ionization mode for a mass range of m/z 50-1500. Other mass spectrometer conditions were as follows: nebulizing gas pressure: 40 psi; drying gas flow: 12L/min; drying gas temperature: 400°C; nebulizing gas flow: 1.5 L/min. The specific negative ionization modes (m/z [M-H]-) were used to analyze the compounds.

Purification of Compound

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The methanol was reconstituted in methanol and subjected to column chromatography (300mm x 10 mm) on silica gel (60–120 mesh, Merck). The extract was eluted with n-hexane (100%), ethyl acetate–n-hexane (20:80, 50:50 and 90:10) solvent systems.

Cytotoxicity studies

The cancer cell lines ((DU145 & HCT116) procured from ATCC, USA are cultured in Tissue culture flasks and dishes by standard animal cell culture technique. The cell lines are treated with different concentrations of plant methanol fraction. The cytotoxic activity of the selected plant extracts will be evaluated against cancer cell lines using standard MTT colorimetric assay. Briefly, 10,000 cells were plated in 96 well plates and were allowed to adhere in CO_2 incubator at 37°C for 24h. Then, cells were exposed to different concentrations (10–1000 μg/ml) of Z. officinalis extract for 24h. After the exposure, 10 μl of MTT (5 mg/ml of stock) was added in each well and plates were incubated further for 4 h. The supernatant was discarded and 200 μl of DMSO was added in each well and mixed gently. The developed purple color was read at 550 nm. Untreated sets run under identical conditions served as control. The percentage viability was calculated using the following formula.

$$\text{Percentage viability} = \frac{\text{OD of treated cells (540nm)}}{\text{OD of control cells (540nm)}} \times 100$$

Results and discussion

Purification of compounds by TLC

The active compounds were purified from methanolic crude extracts of Zingiber officinale (Ginger) by using TLC. Methanolic extracts showed four different bands with 0.4, 0.15, 0.43 and 0.60 retention factors respectively (Table 1). TLC chromatogram of methanolic extracts of Zingiber officinale showed at three different wave lengths such as, visible light, 254 nm short wave length and 366 nm long wave lengths. Light grey, light pink and greenish yellow colour showed the spot in respected wave length like visible light, short and long wave length.

The methanolic fraction was reconstituted in methanol and subjected to column chromatography (300mm x 10 mm) on silica gel (60–120 mesh, Merck). The extract was eluted with n-hexane (100%), ethyl acetate–n-hexane (20:80, 50:50 and 90:10) solvent systems. Fractions (5ml each) were collected and pooled based on colour to obtain 7 fractions. The fractions 1 to 3 did not showed any spots in TLC plate but the spots showed the fraction 4, 5, 6 and 7. Fourth fraction showed one band with 0.43 retention factors at three different wave lengths (Table.2). The 5^{th} fraction showed the three different bands with respected retention factors (0, 0.21 and 0.43) (Table.3). The 6^{th} fraction showed the two different bands with 0 and 0.2 retention factors respectively (Table.4). The fraction seven showed the two different bands with respective retention factors (0.0 and 0.18) at three different wave lengths such as, visible light, 254 nm short wave length and 366 nm long wave lengths (Table 5).

Identification of Gingerols from Zingiber officinale by HPLC

The Gingerols was very interesting a biological activity was selected for the identification of compounds by HPLC (Table. 6). In the present study retention time of Zingiber officinale showed a chemical profile composed by four identified compounds, including 6-Gingerol (5.840), 8-Gingerol (11.315), 6-Shogaol (14.134) and 6-Gingerol (25.757). The attendance of these compounds in ethanol extracts approved the interesting biological activity of this species. Jolad et al., (2005) reported 51 compounds from Zingiber officinale they have identified through GC-MS analysis and the major identified compounds were gingerols, zingernone, shogaols, paradols, dihydroparadols, dihydroshogaols, and methyl ether derivatives of some of these compounds. These compounds were also identified (Hu et al., 2011) by using HPLC-MS analysis.

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LC-MS analysis

The analysis of Zingiber officinale purified column fraction in total ion chromatogram (TIC) by LC-MS. To developed a gradient solvent system of acetonitrile (A) and 0.1% formic acid (B) to separate gingerol within 10 min with flow rate at 0.150 mL/min and column temperature at 35°C and detection by electro spray ionization (ESI)-time of flight (TOF)-mass spectrometry (MS).The ion mass $[M+H]^{+2}$ was detected in positive mode with relative abundance such as 165.08, 180.89, 218.05, 297.19, 327.30, 378, 413.28, 710.60, 794.29, 808.57, 872.10, 933.75, 968.81 and 977.21 at retention time 2.50 (Fig. 1and 2). Among this the ion 180.89 and 977.21 were determined the mass of abundant when compared with other. In the previous study, mass spectra of peak 2 displayed a parent ion at m/z 341 and two fragment ions with one at m/z 179 for caffeic acid through the loss of a hexose moiety, and the other at m/z 135 for decarboxylated caffeic acid after elimination of both hexose and CO2, conclusively indicating the compound to be caffeoyl hexoside. Peak 3 was identified as caffeoyl-D-glucose based on comparison of $[M - H]^-$ of parent ion (m/z 339) with that reported by Shakya et al. (2006). Likewise, based on comparison of absorption spectra (232, 280, 310 nm) and $[M - H]^-$ value (m/z 137) with that reported by Atoui et al. (2005), peak 4 was characterized as p-hydroxybenzoic acid. Peak 6 was assigned to be caffeoyl hexoside as a similar MS pattern as that of peak 2 was obtained. By comparison of absorption data with that reported by Schütz et al. (2005)

Cytotoxic activity of methanol fraction of Z. officinale against cell lines

The compounds of Z. officinale exhibited promising cytotoxic activity towards the cell lines compared with control. The various concentrations (5 to 320 µg/ml) of methanolic fraction of Z. officinale were treated against both HCT-116 and DU145 cell lines (Fig.3).

HCT-116 cell lines

The maximum percentage (75.62 %) of inhibition was showed the methanolic fraction of Z. officinale at 320µg/ml concentration. The minimum inhibition (6.33 %) was also observed in the 5 µg/ml concentration of methanolic fraction. 50 % of Inhibition concentration was showed the 52.16 µg/ml. Colorectal cancer, also known as bowel cancer and colon cancer, is the development of cancer from the colon or rectum. Globally, colorectal cancer is the third most common type of cancer, making up about 10% of all cases. It is more common in developed countries, where more than 65% of cases are found. The effect of ginger on the initiation and post-initiation stages of DMH-induced colon carcinogenesis in male Wistar rats was studied by Manju and Nalini (2005).

DU145 cell lines

DU145 cells were treated with various concentration of the methanol fraction for 24 hrs. In this study revealed that the maximum (61 %) and minimum percentage (1.97 %) of inhibitions were noted in the concentrations of 320 and 5 µg/ml respectively. The IC50 value of 165.4 µg/ml was noted. DU145 is a human prostate cancer cell line. The DU145 cell line was derived from a central nervous system metastasis of primary prostate adenocarcinoma origin, removed during a parieto occipital craniotomy. Masuda et al. (2004) determined the structures of more than 50 antioxidants isolated from the rhizomes of ginger. [6]- Gingerol, the major pungent principle constituent of ginger, has been found to possess substantial antioxidant activity. Ginger has been found to be anticarcinogenic via multiple pathways. Although chemopreventive activities of ginger have been examined (Katiyar et al., 1996), very little information is available in the literature with regard to the effects of individual constituents of ginger on experimental carcinogenesis.

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Table 1. TLC Characteristics of methanol extracts of Zingiber officinale

TLC Band	Retention Factor	TLC Profile characteristics		
		Visible light	Shortwave UV 254 nm	Longwave UV 366 nm
1	0.4	Light Grey	Light pink	Greenish yellow
2	0.15	NV	Light pink	Greenish yellow
3	0.43	Light Grey	Light pink	Greenish yellow
4	0.60	NV	Light pink	Greenish yellow

Table 2. TLC characteristics of 4th fraction of methanol extracts of Zingiber officinale

TLC Band	Retention Factor	TLC Profile characteristics		
		Visible light	Shortwave UV 254 nm	Longwave UV 366 nm
1	0.43	Greenish Grey	Brick Red	Greyish Yellow

Table 3. TLC characteristics of 5th fraction of methanol extracts of Zingiber officinale

TLC Band	Retention Factor	TLC Profile characteristics		
		Visible light	Shortwave UV 254 nm	Longwave UV 366 nm
1	0.0	Greenish Grey	Brick Red	Greyish Yellow
2	0.21	Greenish Grey	Brick Red	Greyish Yellow
3	0.43	Greenish Grey	Brick Red	Greyish Yellow

Table 4. TLC characteristics of 6th fraction of methanol extracts of Zingiber officinale

TLC Band	Retention Factor	TLC Profile characteristics		
		Visible light	Shortwave UV 254 nm	Longwave UV 366 nm
1	0.0	Greenish Grey	Brick Red	Greyish Yellow
2	0.2	Greenish Grey	Brick Red	Greyish Yellow

Table.5 TLC characteristics of 7th fraction of methanol extracts of Zingiber officinale

TLC Band	Retention Factor	TLC Profile characteristics		
		Visible light (Figure 1)	Shortwave UV 254 nm (Figure 2)	Longwave UV 366 nm (Figure 3)
1	0.0	Greenish Grey	Brick Red	Greyish Yellow
2	0.18	Greenish Grey	Brick Red	Greyish Yellow

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Table.6 Identification of Gingerols from Zingiber officinale by HPLC

Detector A (278nm)				
Pk #	**Retention Time**	**Area**	**Area %**	**Name***
1	5.633	3201	0.302	-
2	5.840	559375	52.824	6-Gingerol
3	6.033	63098	5.959	-
4	11.315	67167	6.343	8-Gingerol
5	14.134	230973	21.812	6-Shogaol
6	25.757	135132	12.761	10-Gingerol

*Parameters tested are not covered under the scope of NABL accreditatio

Fig. 1 Total ion chromatogram of purified column fraction

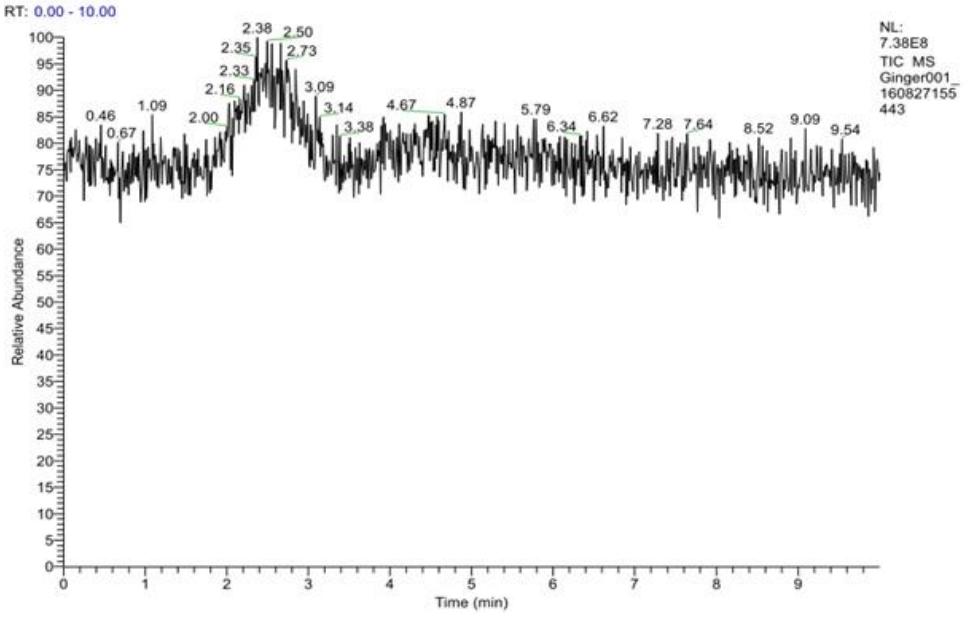

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Fig. 2 Extracted ion chromatogram of Zingiber officinale methanol fraction by LC-MS

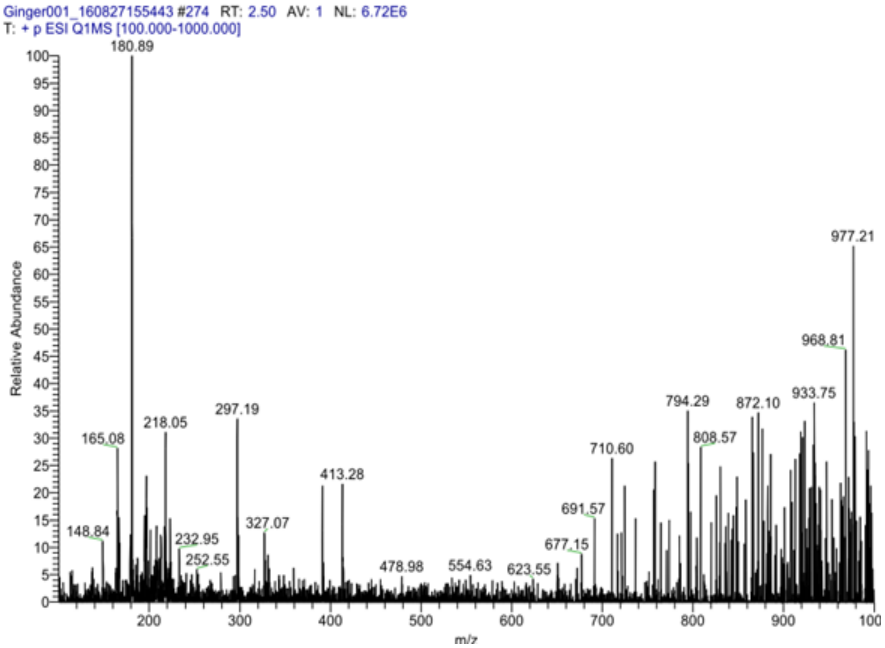
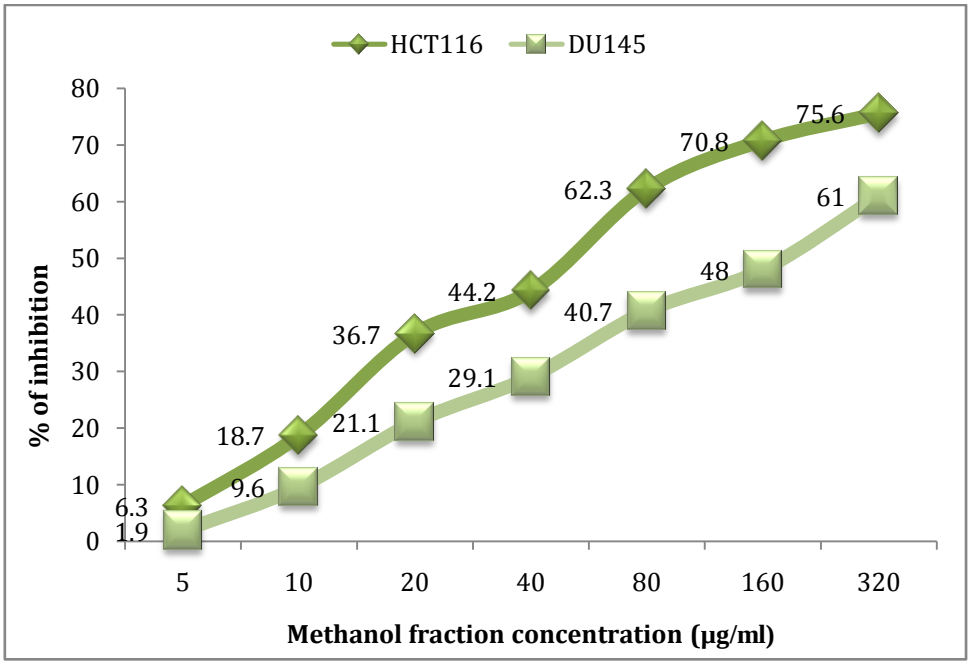


Fig.3 Cytotoxic effect of methanol fraction of Zingiber officinale against HCT116 and DU145 cell lines



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Conclusion

Ginger (Zingiber officinale) has been used since time immemorial for treating different ailments and as dietary condiment. . Thus our studies showed that ginger has potent anticancer activity and should be used as a remedy for disorders arising due to oxidative stress like neurodegeneration and cancer. From present study, it can be concluded that previously reported anticancer activity of the abovementioned plant extract was due to its marked anticancer activity. The outcomes of the aforementioned study demonstrated that the crude extract of Z. officinale has potent cytotoxic effects on Colelateral and Prostate cancer cell lines. Therefore, Z. officinale extract could be a promising phytotherapeutic drug for the effective treatment of cancer. In the present in vitro studies showed that the methanolic fraction of ginger induces cytotoxic effect in cancer cell models with dose dependent manner.

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