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ANTIPROLIFERATIVE ACTIVITY OF CHLOROFORM EXTRACT OF *Z.nimmonii* (J. GRAHAM) DALZELL ON HCT-15 CELL LINES

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ABSTRACT

Aim: Colorectal cancer being a leading cause of death,an invitro investigation has been performed so as to check the antiproliferative potential of Zingiber nimmonii (J. Graham) Dalzell a major plant of Zingiberaceae family. Materials and Methods: Antiproliferactive activity, JC1 and DAPI stainings were the major assays conducted. Results: MTT Assay on HCT-15 colorectal cancer cell lines revealed significant activity when compared to the most commonly used chemotherapeutic drug 5 Flurouracil.JC-1 staining was performed in the cells so as to detect the mitochondrial potential changes. Apoptosis induction was also assessed using DAPI staining technique and was documented. Conclusion: All the results indicated that the chloroform extracts possess significant anticancer potential and can be a promising drug in colorectal cancer therapy.

KEY WORDS

Antiproliferative, colorectal, chemotherapeutic, apoptosis.

INTRODUCTION

Usage of plants in treatment is the basis of modern medical science and great source of new drugs[1-5].Medicinal plants which include both herbs and spices, constitute many phytochemicals which are useful as natural antioxidants[6-8]. Phenolics, flavonoids, alkaloids, tannins and terpenoids are a few classes of bioactive constituents. Natural products are having great therapeutic value as they contain complex chemicals possessing a variety of biological activities[9, 10]. Chemical components isolated from different plants are important source to be used as apoptotic inducers. Mechanisms of action of each of these compounds are useful in cancer therapy as well as cancer prevention[11, 12].

Colorectal cancer is one of the leading causes of cancer deaths worldwide[13, 14].Usually western type of diet is rich in meat, animal fat, alcohol and carbohydrates (refined) which elevates the level of colorectal cancer[15]. Colon cancer development is a multistep process, which ranges from discrete microscopic mucosal lesions to malignant tumors[16].

Zingiber nimmonii (J. Graham) Dalzell belongs to the family Zingiberaceae (Ginger species). Zingiberaceae plants are having significant medicinal properties[17-19].They are having insecticidal, repellant[20-25], anti-inflammatory and chemopreventive activities[26-29].*Zingiber nimmonii* (J. Graham) Dalzell, an endemic species from the Western Ghats in South India, grows both at low and high altitudes, in moist areas under the shades of trees[30,31]. The oil from *Z.nimmonii*

rhizome showed significant activities against the human pathogenic fungi, *Candida glabrata, C. albicans and Aspergillus niger,* but no activity against the plant pathogen, *Fusarium oxysporum*[32].Antiproliferative studies in the plant will reveal the presence of some major chemical constituents which upon isolation can be a significant achievement in colon cancer therapy.

MATERIALS AND METHODS

Plant material

Zingiber nimmonii (J. Graham) Dalzell, was collected from Calicut University Campus, Kerala, India and taxonomically identified by Dr. A. K Pradeep, Herbarium Curator, University of Calicut, Kerala, India. The voucher specimen (95954) has been retained in Department of Botany, University of Calicut, Kerala, India.

Plant extraction

The collected rhizomes were dried under shade and then powdered with mechanical grinder. 25gms of plant material was further weighed and extracted with 250ml Chloroform using a soxhlet apparatus. Excess solvent was removed using a rotary flash evaporator,dried and stored at 4°C.This was further used for the studies.

Chemicals

Cell titre 96® non-radioactive cell proliferation Assay plates (MTT) was purchased from Tarsons, Kolkata.5-Flurouracil and Mitochondria staining kit for mitochondrial potential changes detection was purchased from Biochem sigma chemical Co., St.Louis, MO,USA. Chloroform and DMSO were purchased from Merck, Mumbai. Cell culture flasks were obtained from Griener, Frickhausen, Germany. Roswell Park Memorial Institute medium (RPMI) and Antibiotic - Antimycotic solution were purchased from Gibco, Grand Island, N.Y, USA. All the other chemicals used were of high purity grade.

Maintenance of cell lines

Human colon cancer cell lines (HCT-15) was purchased from National Centre for cell Sciences, Pune, India. Cells were maintained in culture flasks with complete media i.e RPMI, 10%FBS and antibiotic-antimycotic solution. After the cells attained confluency the medium was removed and washed with PBS(Phoshate Buffered Saline).Trypsin-EDTA solution was added and incubated for 3 to 5 min at 37°C. Fresh medium along with serum was added and cells were gently dispersed using a pipette. A known volume of cells were further dispersed into new flasks for further analysis. The cells were incubated at 37°C at 5% CO_2 atmosphere.

1. MTT assay

Cell growth inhibition was determined by MTT assay[33]. MTT [3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay is a simple nonradioactive colorimetric assay. Determination of cell viability is based on reduction of the yellow colored water soluble tetrazolium dye to formazan crystals. Four different concentrations of the chloroform extract of *Z.nimmonii* (12.5-100µg/ml) of the extract and 100µl of cell suspension was used for the analysis. After 24 h appropriate concentration of the test agent was added and incubated for 24, 48 and 72 h respectively at 37°C at 5% CO_2 atmosphere. After the respective time period each plate was taken out. MTT reagent was added to a final concentration of10% of the total volume. After incubation of 2-4 h the culture medium was aspirated and solubilization solution was added. After gentle stirring absorbance was read on a spectrophotometer or ELISA reader at 570nm.Percentage inhibition was calculated according to the formula. 5- Flurouracil in the same concentrations was used as the positive control.

$$\text{Percentage growth Inhibition} = \left[\frac{\text{Mean OD of the Test group}}{\text{Mean OD of the Control}} \times 100\right] - 100$$

2. Mitochondrial potential changes detection assay (JC1 staining)

The dissipation of mitochondrial electrochemical potential gradient is known as an early event in apoptosis. Uptake of JC-1 stain (JC1-5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethyl benzimidazolo carbocyanin iodide) may be utilized as an effective distinction between apoptotic and healthy cells.Any event that dissipates the mitochondrial membrane potential prevents the accumulation of JC-1 dye in the mitochondria and leads to a shift from red (J-aggregates) to green fluroscence(JC-1 monomers). Three different concentration of the chloroform extract 25- 100µg/ml and 50 µg/ml concentration of 5 flurouracil as the positive control was also added to the adhered HCT-15 cells.After 18h of incubation media was removed and washed with PBS buffer. JC-1 stain was further added to the wells followed by incubation in dark for 30 min and consequently viewed under a microscope.

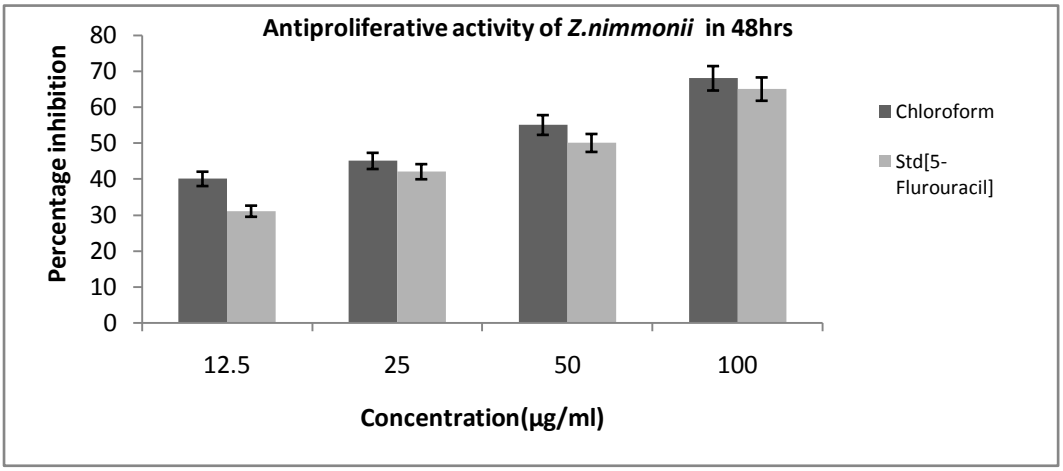
3. DAPI staining

DAPI (4, 6 – Diamidino - 2- phenylindole, dihydrochloride**)** is a fluorescent stain that binds strongly to DNA. When bound to double-stranded DNA, its absorption maximum is at 358 nm and its emission maximum is at 461 nm. Since DAPI passes through an intact cell membrane, it can be used to stain live cells besides fixed cells. Extracts were seeded in three different concentrations ranging from 25-100 µg/ml and 5-flurouracil as the positive control in a concentration of 50µg/ml. After 24 h incubation the cells were fixed with 2% paraformaldehyde for 15 min and were treated with 0.2% Triton X-100 in TBS for 15 min at room temperature. Cells after washing with TBS were stained with DAPI (1g/mL) and incubated in dark for 30 min. The cells were examined under fluorescence microscope and documented.

RESULTS AND DISCUSSION

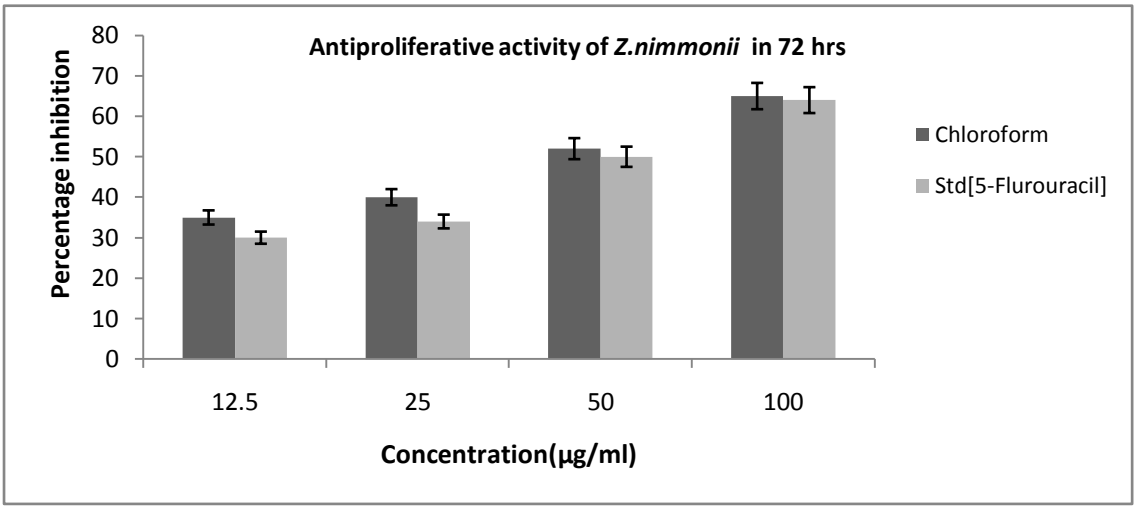
1. Antiproliferative assay

Percentage inhibition of *Z.nimmonii* in 48 h.



Graph 1: It shows the percentage inhibition of the chloroform extract of *Z.nimmonii* on HCT-15 Colon cancer cell lines for a time period of 48 hours;compared with standard drug 5 Flurouracil.

Percentage inhibition of *Z.nimmonii* in 72 h.



Graph 2: It shows the percentage inhibition of the chloroform extract of *Z.nimmonii* on HCT-15 Colon cancer cell lines for a time period of 72 hours; compared with standard drug 5 Flurouracil.

2. JC1 Staining

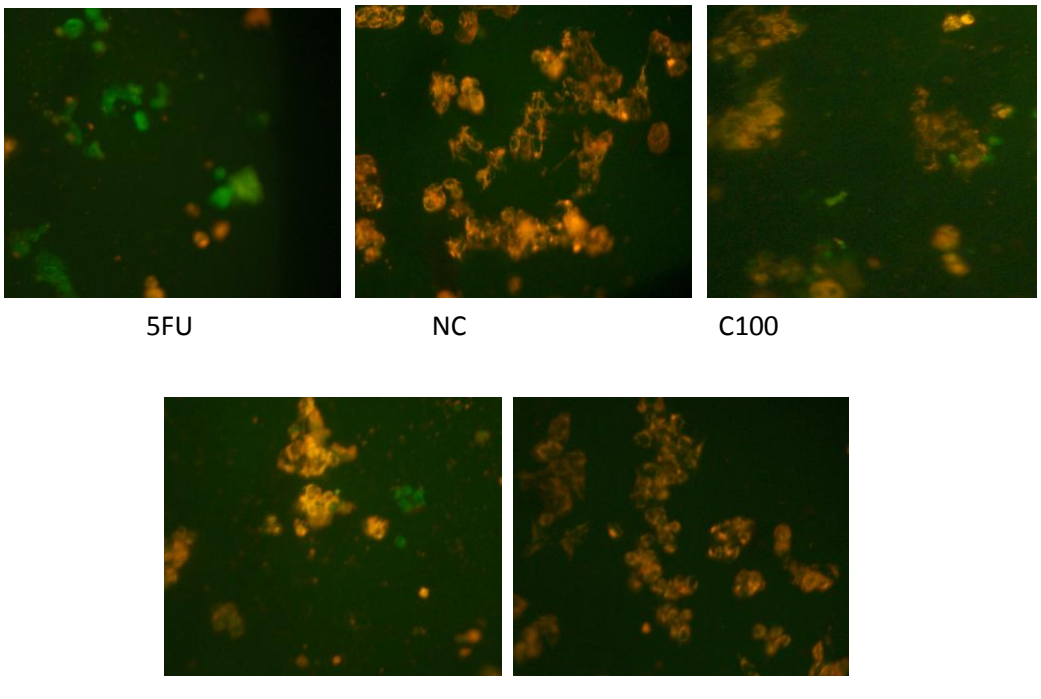


Figure 1: Microscopic view of the HCT-15 cell lines after staining with JC1. Green fluorescence represents the dead cells and orange fluorescence represents the live cells.[5FU:5 Flurouracil [Std], NC: Negative Control [without extract],C100:Extract [Conc.100 µg/ml], Extract [Conc.50 µg/ml], C 25: Extract (Conc.25 µg/ml)].

3. DAPI Staining

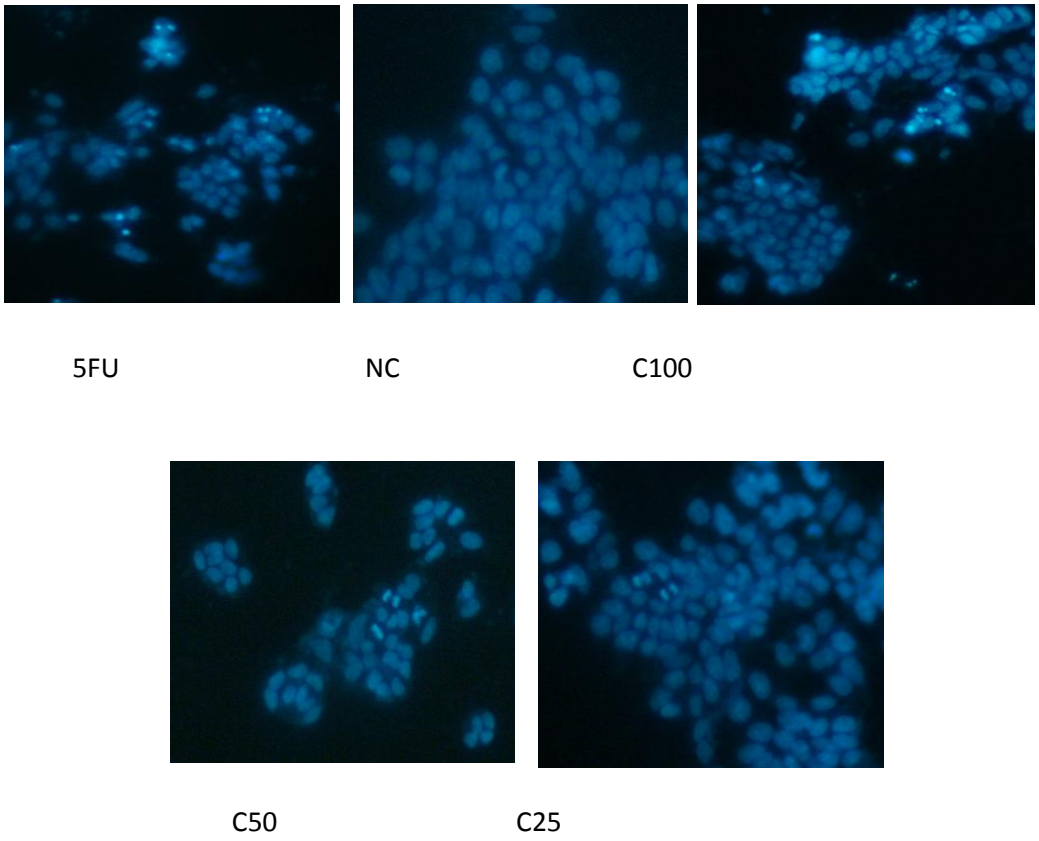


Figure 2: Microscopic view of the HCT-15 cell lines after staining with DAPI. Bright fluorescence represents the dead cells along with chromatin condensation and normal fluorescence represents the live cells [5FU:5 Flurouracil(Std), NC: Negative Control(without extract),C100:Extract (Conc.100 µg/ml), C 50 Extract (Conc.50 µg/ml), C 25 Extract (Conc.25 µg/ml)].

MTT Assay is a reliable assay used to detect the percentage inhibition of the sample drugs. Initially the extract didn't show any activity in the cells when incubated for 24h. Chloroform extracts of *Z.nimmonii* rhizomes at 48 h gave percentage inhibition of 40, 45, 55 and 68 respectively for concentrations 12.5-100 µg/ml which was significantly greater than that of the standard drug 5 flurouracil which showed an activity of 31, 42, 50 and 65 percent at 48 h [Graph 1].At 72 h also the drug showed greater activity than that of the standard. Plant showed a percentage inhibition of 35, 40, 52 and 65 for concentrations 12.5-100 µg/ml compared to 30, 34, 50 and 64 of the standard [Graph 2].

JC1 staining gave promising results for the three different concentrations 25,50 and 100 µg/ml and all the extract concentrations were documented for mitochondrial potential changes detection. Live cells were initially orange in color which upon the addition of the drug turned into dead cells which gave green fluorescence [Figure 1].DAPI staining revealed the dead and live cells from the culture. DAPI binds to the double stranded DNA and helps to detect the apoptosis along with chromatin condensation [Figure 2]. It

was also performed in three different concentrations (25-100 µg/ml).

CONCLUSION

One third of human cancers are associated with dietary intake and also due to lifestyle and lack of physical exercise[34]. Since bowel cancers are attributable to environmental factors,it is potentially preventable. *Z.nimmonii* previously has significant activity against human pathogens and being a member of zingiberaceae family, it is supposed to have significant chemopreventive potential.MTT cytotoxicity assay of the chloroform extract of *Z.nimmonii* on HCT-15 cell liones revealed that the plant was cytotoxic to the cells in a dose dependent manner.These findings provide an insight into implementation of traditional usage of *Z.nimmonii* which is an endemic medicinal plant which is seen in the western ghats of India. After 18h of treatment of HCT-15 cells with chloroform extract of *Z.nimmonii* result a green fluorescence of the majority of HCT-15 cells stained with JC-1 stain. Normal cells without the extract gave red fluorescence. This is an indication of the loss of mitochondrial membrane potential of the cells which is an earlier event in apoptosis. Therefore green fluorescence showed that the mitochondrial membrane potential has been lost by the HCT-15 cell lines and apoptosis been commenced on the addition of the extract.DAPI staining of the HCT-15 cells with the addition of the chloroform extract showed bright fluorescence along with the presence of apoptotic bodies near cells.Chromatin condensation was visible in a few cells which signifies the potential of the extract.Further isolation of bioactive compounds from the extract will be a significant achievement in the colorectal cancer therapy and drug development.

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CONFLICT OF INTEREST

Conflict of interest declared none.

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