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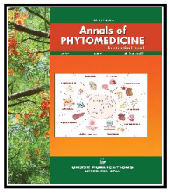

Original Article : Open Access

Anticancer activity of *Diplocyclos palmatus* (L.) C. Jeffrey fruit on HCT116 cell line

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Abstract

India is the richest biodiversity country, having various varieties of medicinal plants, also connected to resulting medicines, are used in traditional medicine. *Diplocyclos palmatus* (L.) C. Jeffrey is a wild medicinally significant plant, a member of Cucurbitaceae. The current examination was to observe and record the result of the anticancer study of wild *D. palmatus* fruit, was subjected to a drying method (tray drying) using ethanolic solvent for extraction. MTT assay and apoptosis study of ethanolic extract of the fruit *D. palmatus* was tested against HCT116 cell line at various concentrations at three varied period, *viz*., 24, 48, and 72 h. The outcomes exhibited that ethanolic solvent extract of the fruit of *D. palmatus* constrained the growth of HCT116 cells of 69.58 µg/ml at IC_{50} concentration, respectively. Alternation in the cell morphology also revealed that *D. palmatus* comprises a few valuable chemical elements extracted using ethanol that can be used further in the management of cancer treatment.

1. Introduction

The family Cucurbitaceae is a special group of tritriterpenoids, having cucurbitane skeleton. Whole plant of cucurbits is supposed to have medicinal values, including fruits, seeds, leaf, *etc*., and the compounds isolated from the plant parts, have hepatoprotective, cytotoxic, and cardiovascular effects. Cucurbitacins are acknowledged for their toxicity and unpleasantness by triterpenoid (Dhiman *et al.,* 2012). The secondary metabolites isolated from the plants are supportive in preliminary screening and cytotoxicity studies; it is growing the healing efficiency of plant-based drugs. Disease is caused by internal and external factors, and cancer is a malignant disease, and one of the leading occurrences and reasons for worldwide death; even though temporary treatment is to cure the disease using chemotherapeutic drugs, maximum of them have toxic reactions. Plants are upright source for expansion of both actual and safe drugs in contradiction of cancer. Human colon cancer is the fourth common incidence recorded around 9 per cent of the worldwide proportion, and antitumor activity of the Cucurbitaceae family due to triterpenoid compound (Mesal *et al*., 2022). Low fat in fruits and vegetables and high animal fat in meat used in Westernized diets are the key roots of colorectal cancer (Guon and Chung, 2017).

D. palmatus is generally recognized as a native bryony or striped cucumber that goes to the family Cucurbitaceae, also called Shivalingi expected to the shape of the seed of the fruit. The genus constitutes four or five species, except *Diplocyclos,* remaining four species are restricted to Africa. The plant is broadly distributed in sub-tropical and tropical dry broad leaf besides rainy forests. The leaves and fruits of the plants are eaten in small quantities as a vegetable in some parts of the world. The species belongs to the tropical and South Africa, the South Arabian Peninsula, and tropical and subtropical Asia. It is found in India including the Himalayas, at an altitude of 200-1500 m. The wild plants hit a leading act in the contest with the nourishing recruitments of the tribal population as they afford diverse nutrients beneficial for humans (Andola and Purohit, 2010). Compared with the commercial fruits harvested using chemicals, a favorable amount of nutrients is present in the wild edible fruits for the rural population. In rural populations, wild edible plants are favourable authority of nutrients, well proportionate with profitable fruits (Sundriyal and Sundriyal, 2001). Despite possessing medicinal and nutritional values of *D. palmatus,* the fruits of this plant have been neglected due to a lack of awareness and mistreatment has not been accurately used (Alexpandi *et al*., 2019). The tribal people are dependent mainly on the native plant for food security and medicine in everyday living, the fruits have appeared globose, smooth, and green to orange when they ripen (Tripathy *et al*., 2014).

MTT assesses to regulate the cytotoxicity and cell proliferation, since it is a colorimetry assay, it works upon the contraction of tetrazolium dye to formazan crystals. The mitochondrial enzyme intensity is proportionate to the number of cells, spectrophotometrically recorded at 570 nm (Alley *et al.,* 1986; Mosamann, 1983; Raynal and Pollard,1994).

Apoptosis is a regular physiological process that happens through evolving progress along with the protection of tissue homeostasis. One of the earliest features is injury of plasmalemma irregularity and connection, compression of the nucleus, protoplasm, and nucleosome

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division of DNA is the characteristic feature of apoptosis (Homburg *et al*.,1995).

In apoptotic cells, the phospholipid phosphatidylserine (PS) is translocated from the inside to the external plasmalemma, Thus opposing it towards the outdoor atmosphere. Annexin V is a calcium-reliant phospholipid-binding protein that has a huge affection for PS and binds to cells with uncovered PS. It may be associated with fluorochromes comprising FITC (Andree *et al.,*1990).

Loss of membrane integrity with FITC Annexin V staining which appears in the present-day point of cell death succeeding from one of necrotic orapoptotic processes. Hence, the FITC stain was conjugated with propidium iodide (PI) to recognize the primary apoptotic cells. Whereas, the layers of unalive and injured cells are penetrable to PI dye, cells with complete membranes exclude PI (Koopman *et al*., 1994).

When apoptosis is consists of completed time, cells can be frequently followed from FTIC Annexin V and PI negative to FTIC Annexin V positive and PI negative and finally to FTIC Annexin V and PI positive. Hence, this assay does not discriminate among cells that have knowledgeable apoptotic damage against those that have expired because of a necrotic pathway because in one of two cases, the lifeless cells will stain with the two FITC Annexin V and PI (Zhang and Webster, 2012; O'Brienand Bolton, 1995).

2. Materials and Methods

2.1 Plant specimen

The *Diplocyclos palmatus* (L.) C. Jeffrey plant wildly distributed and it is collected locally, transferred to the lab, and separated the green and red fruit.The plant specimen is identified and Botanically Authenticated (UOMBOT19DP1) by Professor K.N. Amruthesh, Department of Studies in Botany, Mansagagngotri, Mysore. The herbarium is deposited in the Department.

2.2 Preparation of the extract

D. palmatus is an extremely branched periodic climber, with a smooth stem, it blossoms from August to September, and the fruits are green with white bands that gradually shift to orange/red when it is fully ripe. The fruits were separated into unripe (green) and ripe (red), then washed with tap water to discharge the debris, and rubbed with muslin cloth. The fruits were cut open to remove seeds and make uniform-sized slices, then spread onto a clean tray wrapped with a polythene sheet for drying (Sun *et al.,* 2015). The samples were removed from the dryer, ground with a mixer, to make coarse powder and stored in a Ziplock cover until further use. The ground red fruit sample was named TR (tray dry). Around 5-10 g of desiccated sample was weighed and transferred into apre-weighed conical flask, approximately 100 ml of respective solvent then kept in a rotary shaker at 37ºC for 24 h of 150-200 rpm for extraction. Filtered extracts were desiccated and shifted to the Eppendorf tube and stored at 4ºC for further analysis.

2.3 Cell culture and treatment

The (colorectal adenocarcinoma cell line) HCT-116 was procured from NCCS, Pune, India. The HCT-116 cells were maintained according to Bohlooli *et al*. (2012), and sub-cultured every 2-3 days. 53 passage number was used.

2.4 MTT assay

MTT assay was achieved according to Mosamann (1983) with the eight different fruit samples, *viz*., S1-AqFG (aqueous freeze green), S2-EATG (ethyl acetate tray green), S3-EtOHTG (ethanol tray green), S4-MTG (ethanol tray green), S5-AqFR (aqueous freeze red), S6-EATR (ethyl acetate tray red), S7-EtOHTR (ethanol tray red), S8-MTR (methanol tray red) of maceration extraction method at three different periods (24 h, 48 h and 72 h) at three con. (125, 250, and 500 µg/ml).

In a 96-well microplate seed, an appropriate amount of cell suspension as per the compulsory cell compactness (20,000 cells per well), without adding a crude reagent. Allow the cells for 24 h for incubation, then add suitable con. of crude agent and incubate at 37ºC for 72 h in a 5% CO_2 atmosphere. Once, the gestation is completed, remove the media from the plates and add MTT reagent (0.5 mg/ml) of a final con. Cover the plate with aluminium foil, to evade the sunlight, and incubate again for about 3 h (Note: For each cellline, the incubation period is different), remove the MTT agent from the plate and then 100 of DMSO is a solubilizing solution. Mild agitation in a gradatory shaker helps in more dissolution. Read the abs at 570 nm wave length with an ELISA reader to calculate the percentage cell feasibility. Here, toxic control was used, 5 µg/ml of curcumin, negative control-absence of the compound, and control-medium without cells:

Percentage of cell viability = (mean abs of treated cells/mean abs of untreated cells) × 100

2.5 Apoptosis study

An apoptosis study was conducted. Sample details were marked in (Table 3), approximately 0.5 × 10^6 cells/2 ml of cell density was seeded in a microplate (96-well) and nurtured for 37ºC at 24 h. Later, the incubation spent media will be removed, using 1 × PBS buffer, the cells and test the cells with crude compound with anticipated concentration and control incubation done with 72 h. One well should be kept empty for untreated ones without any treatment. After the treatment, try-psinize and harvest the cells move them straight into 12 × 75 mm polystyrene tubes. Centrifuge the polystyrene tube at 300 × g at 25ºC for 5 min, remove the supernatant, treat the cells with PBS buffer double, again decant, and add 5 µl Annexin V binding buffer. Vortex and incubate the cells for15 min in a dark at 25ºC, with the addition of 5 µl of PI and 400 µl of IX Annexin binding buffer and vortex properly. Analyse by flow cytometry.

2.6 Statistical analysis

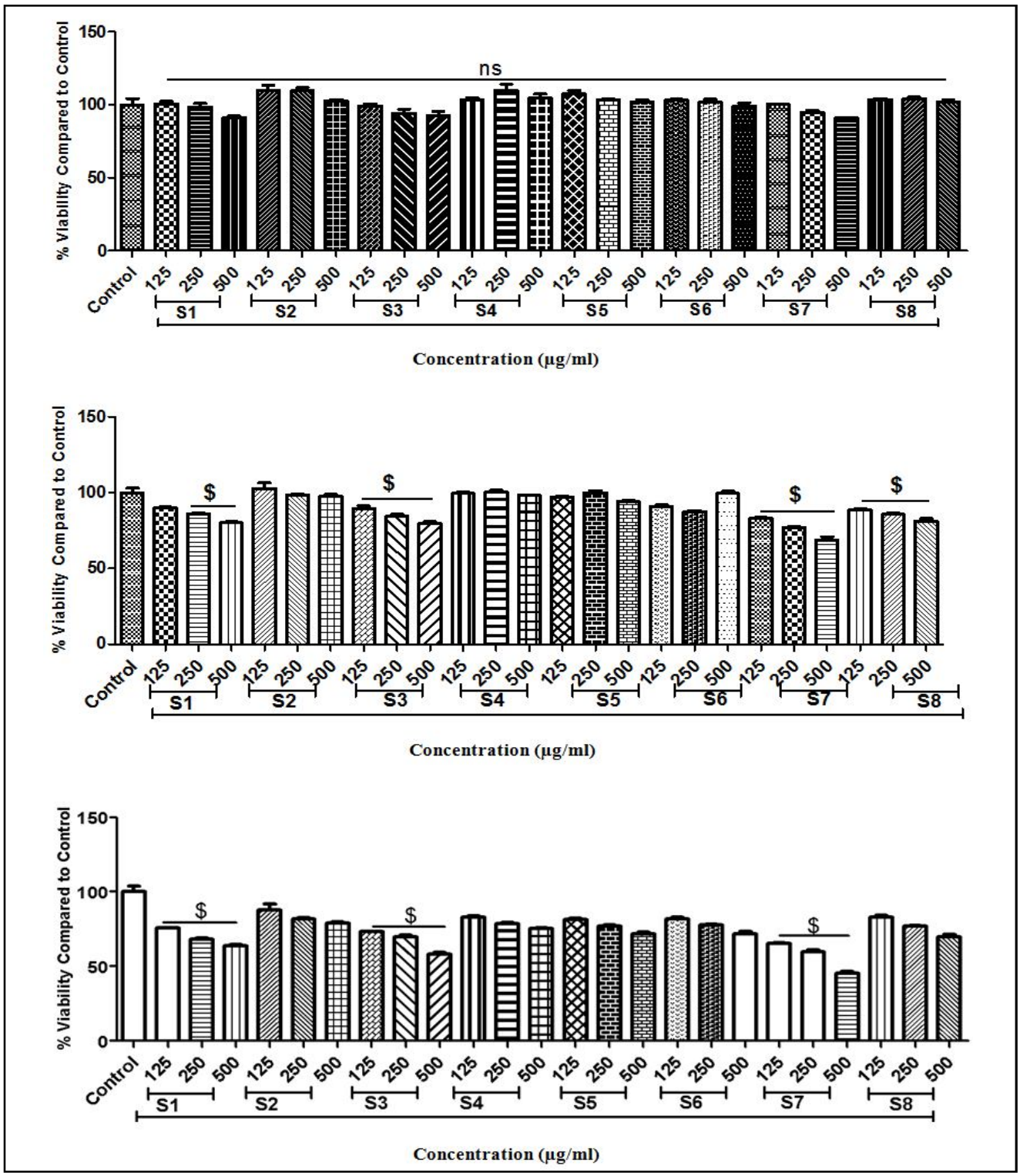
The outcomes were assessed by the students' test tracked by the Newman-Keuls test for numerous assessments. The significance level was calculated at $p<0.05$. Data values for 50% growth inhibition were represented as mean ± SD and calculated with Sigma Plot 11 software (Bohlooli *et al*., 2012).

3. Results

3.1 Cytotoxicity

The result of the cytotoxicity effect of crude extract of the above *D. palmatus* fruit employed a cytotoxic result on HCT-116 cancer cell lines at 3 time periods (24, 48, and 72 h) of incubation (Figures 1, 2,

3) at three different concentrations of (125,250, and 500 µg/ml). In all the time intervals of 72 h with 500 µg/ml of EtOHTR-C sample showed less than 50% of viable cells, so the activity was confirmed by using less concentration of sample extracts.



Figures 1, 2, 3: **Percentage viability of fruit extracts on HCT-116 cancer cell line at three different concentrations. Extracts; S1-AqFG (aqueous freeze green), S2-EATG (ethyl acetate tray green), S3-EtOHTG (ethanol tray green), S4-MTG (methanol tray green), S5-AqFR (aqueous freeze red), S6-EATR (ethyl acetate tray red), S7-EtOHTR (ethanol tray red), S8-MTR (methanol tray red).**

Based on the above investigation, further the EtOHTR-C (ethanol tray dried red fruit with maceration) sample is considered a crude sample and remains estimated to investigate the cytotoxicity results on HCT-116 cells. There are five different concentrations given below (Table 1).

Table 1: Details of drug treatment

S.No.	Culture condition	Cell line	Concentrations
1	Untreated	HCT-116	-
2	Blank	-	Media without cells
3	5-fluorouracil	HCT-116	15 µM/ml
4	EtOHTR-C	HCT-116	5 (6.25, 12.5, 5, 25, 50, 100 µg/ml)

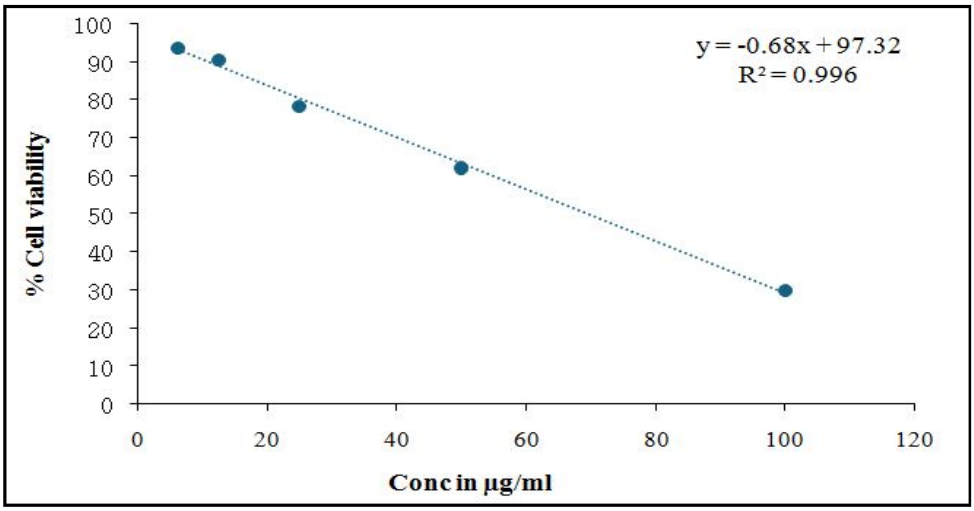


Figure 4: Standard graph of EtOHTR-C vs HCT-116.

Table 2: Percentage cell viability values of EtOHTR-C treated on HCT-116 cells

Condition	Percentage cell viability	IC_{50} conc (µg/ml)
Untreated	100.00	69.58
5-fluorouracil -15 µM	25.81	
EtOHTR-C 6.25 µg	93.60	
EtOHTR-C 12.5 µg	90.29	
EtOHTR-C 25 µg	78.37	
EtOHTR-C 50 µg	62.23	
EtOHTR-C 100 µg	30.12	

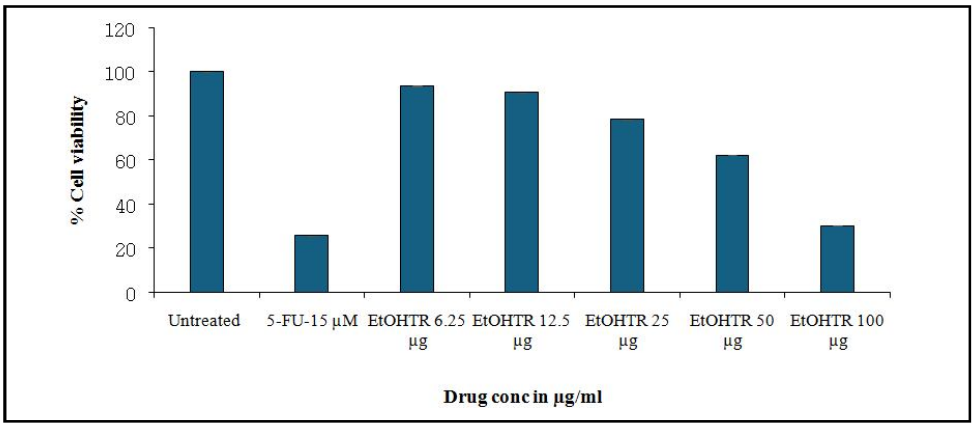


Figure 5: Percentage of cell viability after the incubation period of 72 h

The MTT assay of cytotoxicity result suggests that the crude test compound, *viz*., EtOHTR-C was anticolon and cytotoxic with the IC_{50} value of 69.58 ug/ml (Table 2), and the standard graph of EtOHTR-C vs HCT-116 and percentage viability cells was showed in Figures 4 and 5 against the human colorectal cancer (HCT-116) cells after the 72 h of incubation period. Representative photomicrographs are revealed in Figure 6. However, additional investigationis required to regulate the molecular system behind the anticancer activity of the crude sample, *viz*. (EtOHTR-C) *in vitro* level with studies like apoptosis/necrosis study, cell cycle study, ROS study and apoptotic proteins expression studies, *etc.,* of a compound to find the molecular mechanism of action behind the anticancer result on human colorectal cancer cells after the 72h of incubation.

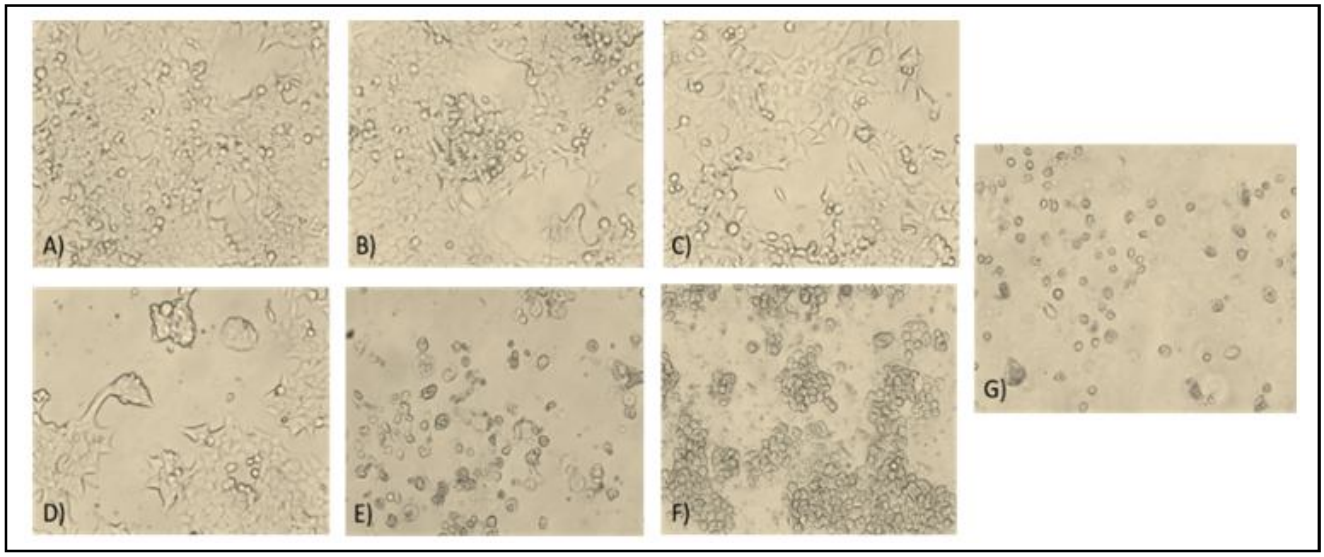


Figure 6: Cytotoxicity assay: (A) Control, (B) 6.25 µg, (C) 12.5 µg, (D) 25µg, (E) 50 µg, (F) 100 µg and (G) standard.

3.2 Apoptosis study

The ethanolic extract of tray-dried red fruit with cold maceration (EtOHTR-C) significantly enhanced the 50.34% and 6.22% (Table 4) of apoptosis and necrosis in human colon cancer cells. The reference standard control used here is 5-fluorouracil with 15 µM 5-fluorouracil meant for the study which showed 76.81% and 0.33% (Figure 8) of apoptosis and necrosis after 72 h of incubation. Annexin V/PI expression of the HCT-116 cells in the occurrence and nonappearance of EtoHTR-C was represented in quadrangular plots (Figure 7). Further studies like ROS study and cell cycle study requirement to be achieved to authorize the mechanism of action behind the apoptotic effect of these compounds on colon cancer cells *in vitro*.

Table 3: Sample details for apoptosis

S. No.	Sample ID	Concentration	Cell line used
1.	EtOHTR-C	1(70 µg/ml)	HCT-116
2.	5-fluorouracil	1(15 µM)	HCT-116

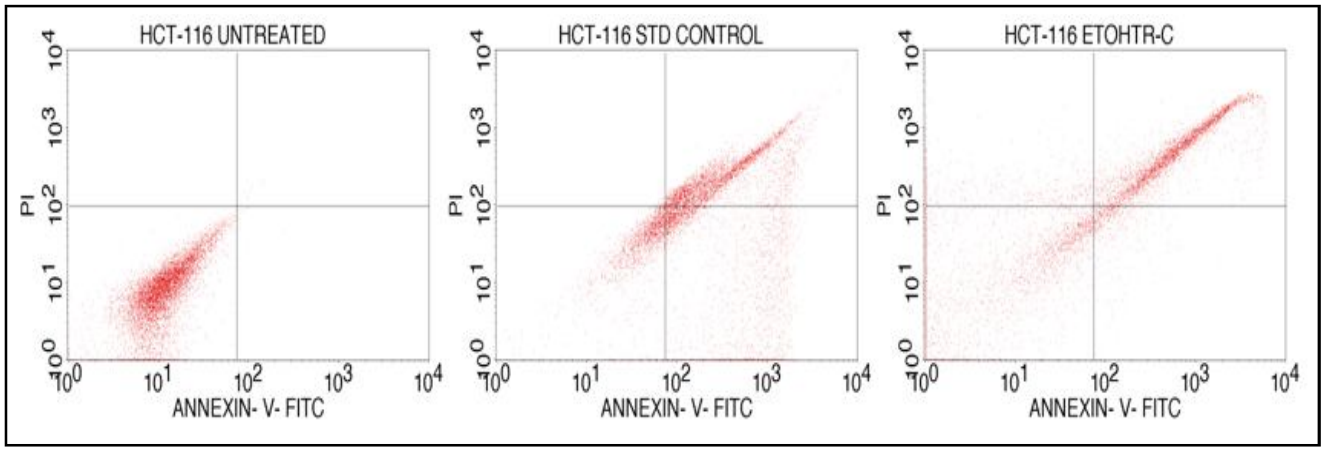


Figure 7: Annexin V/PI expression of the HCT-116 cells was shown in quadrangular plots analysed by flow cytometry. The acquisition was completed by adopting BD FACS calibre and data was analysed by BD Cell Quest Pro Software (Version:6.0).

Table 4: Percentage of cells that undergone apoptosis and necrosis

Quadrant	% Necrotic cells	% Late apoptotic cells	% Viable cells	% Early apoptotic cells
Lavel	UL-Upper left	UR-Upper right	LL-Lower left	LR-Lower right
Untreated	0.01	0.12	99.74	0.13
Std. control	0.33	46.29	22.86	30.52
EtOHTR-C	6.22	44.85	43.44	5.49

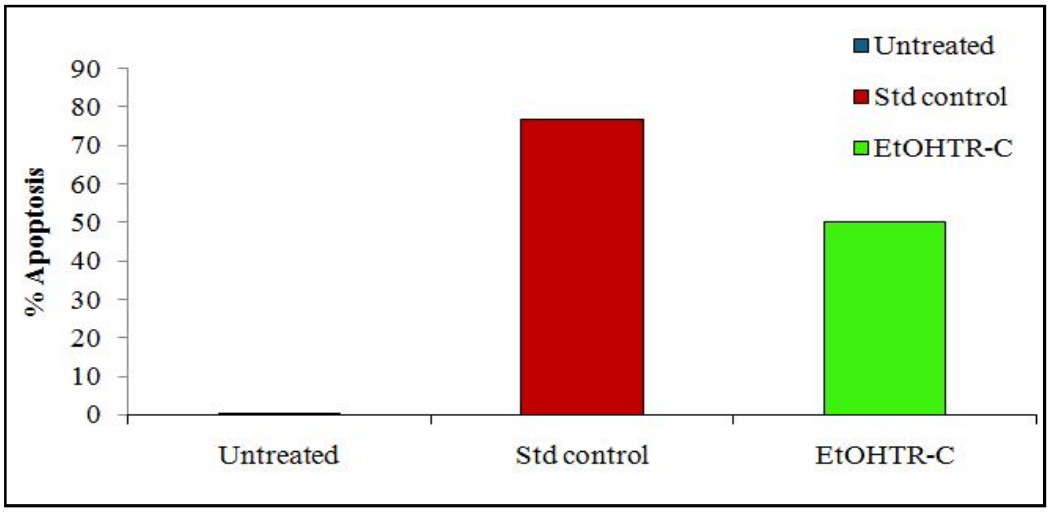


Figure 8: Percentage of apoptotic cells showed in a bar graph.

4. Discussion

ROS (reactive oxygen species) is the foremost origin for oxidative stress, resulting in the damaging of essential molecules, deoxyribonucleic acid, proteins, and unsaturated lipids; it displays an important part in the pathogenesis and ageing of chronic disease, *viz*., arteriosclerosis and cancer (Sies, 1997). The qualitative phytochemical analysis and antioxidant analysis of fruit extracts showed secondary metabolites and a good number of antioxidants, as per our knowledge. There are no reports available about the drying methods and HCT-116 cell lines studies, particularly in our plant *D. palmatus* fruits. An effort was made to check the cytotoxicity effects of individual solvents with tray drying with red fruit out of the four solvents. EtOHTR (Ethanol tray dried red fruit) showed 30.12 of viability and cytotoxicity of 69.58 ug/ml IC_{50} value.

In vitro cytotoxicity effects of *Bryonia laciniosa* (Linn.) Naud leaves on human cancer cell lines showed around 18 µg/ml of the IC_{50} values. Of the diverse extracts, the aqueous extract established extreme cytotoxicity to cancer cells. *Lagenaria siceraria* fruit was evaluated by Attar and Ghane (2019) (MCF-7 and HCT-29) on two human cancer cell lines. The plant displayed significant anticancer action due to the occurrence of bitter principle.

The related investigation was done by Gunes *et al*. (2019) using breast cancer (MCF-7), A549 (lung cancer), K562 (chronic myeloid leukemia and T cell leukemia (Jurkat); 24 h of ethanol fruit extract showed 87-90 % of cytotoxicity. *Luffa echinate* Roxb. is a neglected plant, but rich in bioactive metabolites used in Ayurveda. Patel and Ghane (2021) reported that the fruits of this plant have excellent antitumor activity in contradiction of colon (HT-29) and breast (MCF-7) LC_{50} 385.17µg/ml and 239.36 µg/ml. Presence of phenolics acts in bioactivities of the extracts. The cell viability assay of four extracts reported by Talib and Mahasneh (2010) also showed similar results under the MCF-7 cell line with the 27.96 µg/ml of IC_{50} value. MTT assay was displayed with colon cancer cells (HT-29 and HCT-15) of *Lagenaria siceraria*, *Luffa cylindrica*, and *Cucurbita pepo* wild fruits (Sharma *et al*., 2015) compared with cucurbitacin B and results discovered in a concentration-dependent method. The cucurbitacin B of the cucurbit extracts decreased the viability of HCT-15 and HCT-29 cells substantially. Cucurbitacins widely exist in cucumber, pumpkin, melon, and watermelon fruits (Jing *et al*., 2020).

The ethanolic extract from the leaf extract of *Pidium guajava* L. shows a high level of anticancer and antioxidant activities (Sriram *et al*., 2023). The MTT assay of *D. palmtaus* plant hexane extract was prepared for the cytotoxicity at various doses, *i.e.* (62.5, 125, 250, 500 and 1000) µg/ml. The maximal cytotoxicity was assessed with an IC_{50} value of 453.33 ± 1.6 µg/ml of 75.25 ± 2.4 % at 1000 µg/ml (Nalinaksh and Anand, 2024). The observation from the assay reveals that the cytotoxic effect is increased with increasing concentration of the hexane extract of the plant in a dose-dependent manner.

5 Conclusion

The outcome of the investigation showed that colon cancer may be prevented with the use of wild fruit extract. The crude compounds with antioxidant activity may be useful in suppressing tumour growth and antiproliferative effects, making this wild fruit a potential source of anticancer drug development and thus well-meaning for upcoming investigation. An essential future study would be to examine the mechanism of response of isolated compounds.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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