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Anticancer and Cytotoxic Potential of Aqueous Extract of *Triticum aestivum* on Colorectal Carcinoma

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ABSTRACT

Introduction: Cancer is caused by abnormalities in genetic material of the transformed cells. Cancer may also be initiated by carcinogens, tobacco, smoke, radiation, chemicals or infectious agents, especially some viruses. Cancers cause annually more than 13% of all human deaths. More than 70% of all cancer deaths occurred in low and middle income countries. Deaths from cancer worldwide are projected to continue rising, with an estimated 12 million deaths in 2030 (WHO estimate). Natural products have been used as traditional medicines in many parts of the world like Egypt, China, Greece, and India from ancient times. It is from these medicinal plants, the modern drugs been developed known to be free of the deleterious effects, are inexpensive and effective. One of these herbs is Wheatgrass, the young grass of *Triticum aestivum Linn.*, family: Poeaceae. **Objectives:** Objective of the study was to analyze anticancer property of leaves of *Triticum aestivum* on HCT-15 cells. **Materials and methods:** The young grass of *Triticum aestivum* is was collected. The aqueous extract was prepared by using standard protocols. The antiproliferative effect of the aqueous extract was evaluated *in vitro* by employing MTT assay. The potency of plant extract was calculated in terms of percent decrease in viable HCT-15 cells as compared to the control. **Result and conclusion:** The extract showed dose dependent antitumor activity. The MTT assay showed an anti proliferative activity (IC50) at 258.8 µg/ml of crude extract.

Keywords: *Triticum aestivum*, CRC, HCT-15, IC50, MTT Assay.

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INTRODUCTION

Cancer is one of the major human diseases and causes large suffering and economic loss worldwide. Colorectal carcinoma is the third most commonly diagnosed cancer worldwide with a prevalence of 1.4 million (9.7%) preceded by lung cancer (13.0%) and breast cancer (11.9%), despite all the advanced therapeutic and surgical interventions available.[1,2] Given the temporal profiles and demographic projections, the global burden of CRC is expected to increase by 60% to more than 2.2 million new cases and 1.1 million cancer deaths by 2030.[3] Hence, it is the need of the hour to plunge our fingers into the field of research to find out safer and more effective therapeutic modalities which not only would limit the spread of colorectal carcinoma but also would ensure patient's wellbeing by having minimal adverse effects unlike the current day chemotherapeutic regimens. Various new strategies are being developed to control and treat several human cancers[4]. Over 60% of anticancer drugs available in the market are of natural origin. Natural products are also the lead molecules for many of the drugs that are in use. [5] Therefore, the phytochemicals present in several herbal products and plants may have the potential to act as preventive or therapeutic agents against various human cancers. [4] The increased popularity of herbal remedies for cancer therapy perhaps can be attributed to the belief that herbal drugs provide benefit over that of allopathy medicines while being less toxic. [6] Since the conventional therapies have devastating side effects, there is a continuous need for search of new herbal cures of cancer. [7]

Triticum aestivum

Wheat, (*Triticum* species) a cereal grass of the *Gramineae* (*Poaceae*) family, is the world's largest edible grain cereal-grass crop. It is commonly 60-150 cm. in height, but may be as short as 30 cm. Stem is tufted, erect or semi-erect to

prostrate, generally hollow with thin walls, in stem nodes are present generally 5-7 at 3-4 cm. Leaves are long and narrow having glabrous or hairy on one or both surface.[8,9]

Scientific reports on nutritional analysis of wheatgrass have been published frequently in various

Journals. [10,11,12] These reports and the chemical analyses undertaken reveal that wheatgrass is rich in chlorophyll, minerals and trace elements including calcium, iodine, magnesium, selenium, zinc, chromium, antioxidants like beta carotene (pro-vitamin A), vitamin B1, vitamin E, vitamin C, anti-anemic factors like vitamin B12, iron, folic acid, pyridoxine and many other minerals, amino acids and enzymes, which have significant nutritious and medicinal value. [8]

Wheatgrass is known to contain antioxidant enzymes superoxide dismutase (SOD) and cytochrome oxidase that have the potential to convert reactive oxygen species (ROS) to a hydrogen peroxide and an oxygen molecule. [13] Chlorophyll, one of the primary components in the wheatgrass, was found to augment blood formation and strengthen the immune system through inhibition of metabolic activation of carcinogens. [14, 15] It also possesses the ability to inhibit oxidative DNA damage. [16]

Dr. Ann Wigmore, founder director of the Hippocrates Health Institute, Boston, U.S.A., she claimed that wheatgrass is a safe and effective treatment for ailments such as high blood pressure, some cancers, obesity, diabetes, gastritis, ulcers, anemia, asthma and eczema. [17] Few clinical trials have been accomplished that have shown on consumption of wheatgrass juice, the number of transfusions in patients with thalassemia major is decreased. [18] Reduction in the overall disease activity index and the severity of rectal bleeding in cases of distal ulcerative colitis on consumption of wheatgrass juice has also been observed. [19]

VERO Cell line

The *Vero* lineage was isolated from kidney epithelial cells extracted from an African green monkey. The lineage was developed on 27 March 1962, by Yasumura and Kawakita at the Chiba University in Chiba, Japan. [20] The original cell line was named "Vero" after an abbreviation of "Verda Reno", which means "green kidney" in esperanto, while "Vero" itself means "truth" also in Esperanto. [21] Vero cells are one of the most common mammalian continuous cell lines used in research. [22]

HCT-15 Cell Line:

HCT-15 is a human colorectal adeno-carcinoma that has a mutated p53 tumor suppressor gene and over expresses P-glycoprotein. P-glycoprotein acts as an efflux pump resulting in decreased intracellular accumulation of certain drugs contributing to drug resistance.

MATERIALS AND METHODS

Plant Material

Certified sample of *Triticum aestivum* (Wheatgrass), was acquired from Anand Agricultural University, Gujarat. The authenticity of this certified sample was also confirmed by comparing its morphological characters with the description mentioned in different standard texts and floras.[23] Voucher specimen of the plant has been deposited at Department of Pharmacognosy, B. Pharmacy College, Rampura, Kakanpura, Godhra, Dist. Panchmahal, Gujarat, India for future reference. This wheat variety was grown in plastic tray as per the standard procedure described below. [17]

Procedure for growing wheatgrass

- Adequate quantities of unpolished wheat grain were soaked overnight in water in a container.
- On the next day, the soaked wheat-grain were spread on the surface of the soil filled in plastic trays. Care was taken so that the grains did not touch one another.
- A thin layer of soil was sprinkled on the wheat grains and then tray was covered with a newspaper to provide darkness, which helps the sprouting.
- The tray was kept in a covered balcony. Next day the tray was uncovered to spray on some water and was covered again with the newspaper.
- Previous step was repeated every day until sprouting took place, after which the tray was left uncovered and watered everyday for 8 days.
- On 9th day the wheatgrass was harvested by cutting it with a clean pair of scissors about 1/2" above the surface of the soil.

Preparation of aqueous extract:

For preparation of aqueous extract, 100 g of fresh wheatgrass was crushed thoroughly, using mortar and pestle. The crushed wheatgrass was completely exhausted by adding small quantities of water several times followed by filtration, to yield final volume of 1 liter. The extract was filtered and concentrated to dryness under reduced pressure and controlled temperature (40 ºC to 50 ºC) in a rotary evaporator. The dried extract was dissolved in Dimethyl sulphoxide (DMSO) to prepare the stock solution 100 mg/ml.

Determination of cell viability by trypan blue dye exclusion method:

The viability of cells was determined by the Trypan Blue dye exclusion method. It takes advantage of the ability of healthy cells with uncompromised cytoplasmic membrane integrity to exclude dyes such as trypan blue. [24]

1. Hemocytometer cell counts:

- ✓ Hemocytometer & cover slip were cleaned and wipe with 70% alcohol. Cover slip was placed on hemocytometer. In the separate 2ml centrifuge tube, cell suspension (cells in culture media) was added. Two fold dilution of reaction mixture was prepared by mixing aliquot of 0.1 ml cell suspensions with 0.1 ml trypan blue.
- ✓ Afterwards 0.1ml of Cell suspension was placed in to the chamber of hemocytometer.
- ✓ By using a Lieca inverted microscope, numbers of cells were counted in $1mm^2$ area under 10X objective.
- ✓ Viable & non-viable cells were counted in both halves of the chamber.

2. Calculations:

1) **Total number of viable cells** = A XBX C X 10^4
2) **Total dead cell count** = A X B X D X 10^4

Where,

A = Vol. Of cell solution (ml)

B = Dilution factor in trypan blue

C = Mean number of unstained cells

D = Mean number of dead/stained cells

10^4 = Conversion of 0.1 mm^3 to ml

3) Total cell count = Viable cell count + dead cell count

% viability = (Viable cell count/Total cell count) X 100

(Vol.: Volume, CS: Cell Solution TB: Trypan blue)

***In-vitro* evaluation of anticancer activity by MTT assay**

Cell culture

The human colon cancer cell line (HCT- 15) & Vero (normal kidney cells) was provided by National Centre for Cell Science (NCCS), Pune. Stock cells of these cell lines were cultured in DMEM (high glucose) or Eagle's Minimum Essential Medium (EMEM) with 10% fetal bovine serum (FBS). All cells were maintained at 37°C, 5% CO2, 95% air. Cells were used in experiments during the linear phase of growth.

Preparation of working herbal extracts

0.5ml of stock (100 mg/ml) herbal extract was dissolved in 4.5 ml of DMSO giving a concentration of 10mg/ml. 10 µl of 10 mg conc. of test compound was added in to 900 µl of complete media and as a result 100 µg conc. of test sample was obtained. Than 1:3 dilution of test sample was done as shown in Table 1. It was done by mixing 50 µl of test compound with 100 µl of complete media. For this, initially 100 µl of complete media was added in to well no. 1 – 9. Well 10 contained 150 µl test substance only, from that 50 µl was pipetted out and added into well no. 9 which already contain 100 µl of complete media, which lead to 1:3 dilution of test sample. Same procedure was repeated 9 times in order to get final conc. of test Sample up to 0.005 µm. (Table 1). Colchicine was used as a reference standard for the comparison of activity of the extract and dilutions were prepared as that of sample extract.

Table 1: (1:3) dilution of test compound used in the assay

well no:1-9 contain complete media 100 µl + Test Compound 30 µl										
Well no:	1	2	3	4	5	6	7	8	9	10
Compound dilution	50 µl T.C from well 2	50 µl T.C from well 3	50 µl T.C from well 4	50 µl T.C from well 5	50µl T.C from well6	50µl T.C from well 7	50µl T.C from well 8	50µl T.C from well 9	50µl T.C from well 10	150µl T.C
Final conc. (µm)	0.05 µg	0.15 µg	0.46 µg	1.37 µg	4.12 µg	12.35 µg	37.04 µg	111.1 µg	333.3 µg	1000 µg

Where, T.C= Test Compound; C.M= Culture Media

Cytotoxicity assay

Principle

This Colorimetric assay is based on the capacity of Mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3- (4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into an insoluble, purple colored formazan product which is measured spectrophotometrically. [25-27] This formazan production is directly proportional to the viable cell number and inversely proportional to the degree of cytotoxicity. [28-29]

MTT assay [23,27,30-32]

Protocol:

- Cells were preincubated at a concentration of 1 × 1 0^6 cells / ml in culture medium for 3 hrs at 37°C and 6.5 % CO2, 75 % Relative Humidity.
- Cells were seeded at a concentration of 5 × 10^4 cells / well in 100 µl culture medium and various amount of compound and standard (final concentration, e.g. 1000 µg/ml – 0.05µg/ml) were added into microplates (tissue culture grade, 96 wells, flat bottom).
- Cell cultures were incubated for 24 hrs at 37 °C and 6.5% CO2.
- 10 µl MTT labeling mixture was added and incubate for 4 hrs at 37 °C and 6.5 % CO2, 75 % Relative Humidity.
- 100 µl of solubilization solution was added to each well and incubate for overnight.
- Absorbance of the samples was measured using a microplate (ELISA) reader. The wavelength to measure absorbance of the formazan product is between 540 and 600 nm according to the filters available for the ELISA reader, used. (The reference wavelength should be more than 650 nm).

Data interpretation:

Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely a higher absorbance rate indicates an increase in cell proliferation. Rarely, an increase in proliferation may be offset by cell death; evidence of cell death may be inferred from morphological changes.

After 24 hrs, the cytotoxicity data were evaluated by determining absorbance and calculating the correspondent chemical concentrations. Linear regression analysis with 95 % confidence limit and R^2 were used to define dose-response curves and to compute the concentration of chemical agents needed to reduce absorbance of the formazan by 50 % (IC_{50}).

Percentage cell growth inhibition or percentage cytotoxicity was calculated by following formula:

% viability = $(A_T - A_B) / (A_C - A_B) \times 100$…… (1)

Where,

A_T = Absorbance of treated cells (drug)

A_B = Absorbance of blank (only media)

A_C = Absorbance of control (untreated)

There by,

cytotoxicity = 100 – % cell survival … … … (2)

Cell viability % = Mean OD of wells receiving each plant extract dilution/ Mean OD of control wells x 100… …. …(3)

Cell death % = 1 – (OD of sample/OD of control) x 100… …(4)

Determination of IC_{50} value:

According to FDA, IC_{50} represents the concentration of a drug that is required for 50 % inhibition *in-vitro*. In the present study, IC_{50} is a concentration of drug at which 50 % of cell population die.

For primary screening, a threshold of 50 % cell growth inhibition as a cut off for compound toxicity against cell lines. IC_{50} determined from plot of Dose Response curve between log of compound concentration and percentage growth inhibition.

Graph was plotted by keeping log concentration of drug on X axis and % cell growth inhibition or % cytotoxicity Y axis. IC_{50} was estimated as a concentration of the drug at 50 % position on the Y axis.

RESULTS AND DISCUSSION

In order to evaluate the cytotoxic effect of aqueous extract of *Triticum aestivum*, a MTT assay with HCT-15 (human colorectal adenocarcinoma) & Vero (normal kidney cells) cell line was performed. The extract was screened for its cytotoxicity at different concentrations to determine the IC_{50} (50% growth inhibition) value. The cytotoxic effect of extract was compared with colchicine as the reference standard.

A chart was plotted using the % cell inhibition in Y-axis and concentration of the plant extract in X-axis. The results are tabulated in (Table 2) and graphically represented (Fig. 1).

Table 2: % Cell inhibition on VERO & HCT-15 cell lines by aqueous extract of leaves of *Triticum aestivum.*

Sr. no	Conc. µg/ml	Log conc	VERO	HCT-15
1	0.05	-1.29	-93.85	-23.85
2	0.15	-0.82	-116.4	-32.35
3	0.46	-0.34	-115	-23.03
4	1.37	0.14	-115.6	-34.67
5	4.12	0.61	-115.4	-28.4
6	12.35	1.09	-114	-22.46
7	37.04	1.57	-91.66	-12.81
8	111.11	2.05	-60.82	9.27
9	333.33	2.52	1.28	30.12
10	1000	3	37.49	63.14

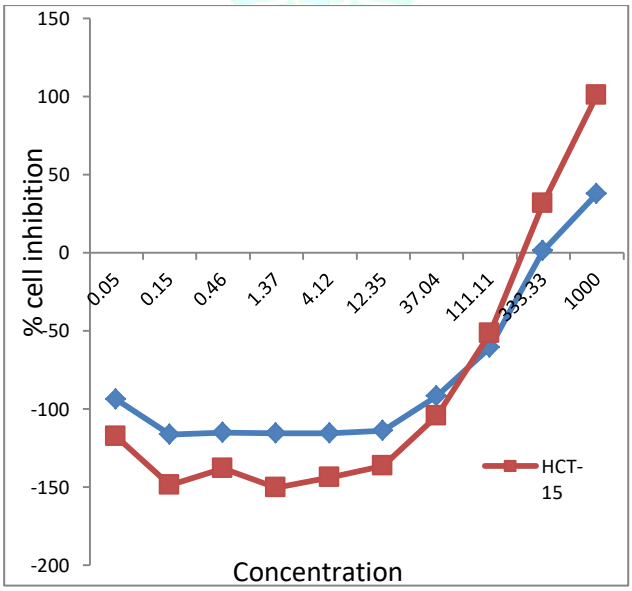

Fig. 1: Dose response curve of aqueous extract of leaves of *Triticum aestivum* on HCT-15 and VERO-cell-lines.

When HCT-15 and VERO cells were treated with the aqueous extract of the leaves of *Triticum aestivum* and colchicine, there was a concentration dependent cytotoxic effect. As the concentration increased from 0.05 – 1,000 µg/ml, percentage of inhibition increases from -93.85% to 37.49 for VERO cell line and -23.85 % to 63.14 % for HCT-15 cell line. The IC50 value was found to be 1000 µg/ml for VERO cell line and 258.8 µg/ml for HCT-15 cell line from the graph and R^2 values 0.9783 for VERO cell line and 0.9832 for HCT-15 cell line (Table 3).

Colchicine was used as a reference standard for the comparison of activity of aqueous extract. Colchicine was evaluated in the same cellular system VERO & HCT-15 to obtain cytotoxicity parameters. As the concentration increased from 0.05 – 1,000 µg/ml, percentage of inhibition increases from 0.85 % to 37.49% for VERO cell line and 4.37 % to 67.99 % for HCT-15 cell line (Table 4) and graphically represented (Fig 2).

Table 3: IC_{50} and R^2 values of aqueous extract of leaves of *Triticum aestivum.*

Parameters	VERO	HCT-15
IC_{50} Value	1000	258.8
R^2	0.9783	0.9832

Table 4: % Cell inhibition on VERO & HCT-15 cell lines by colchicine.

Sr. no	Conc. µg/ml	Log conc	VERO	HCT-15
1	0.05	-1.29	0.85	4.37
2	0.15	-0.82	0.35	7.34
3	0.46	-0.34	1.02	7.98
4	1.37	0.14	1.63	9.04
5	4.12	0.61	2.44	13.31
6	12.35	1.09	14.97	19.7
7	37.04	1.57	19.66	23.88
8	111.11	2.05	20.82	38.95
9	333.33	2.52	29.28	41.88
10	1000	3	37.49	67.99

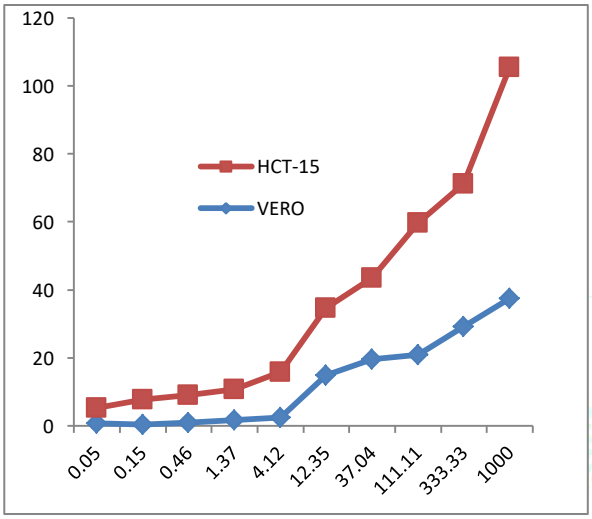

Fig. 2: Dose response curve of colchicine on HCT-15 and VERO-cell-lines.

The IC50 value was found to be 1000 µg/ml for VERO cell line and 139.1 µg/ml for HCT-15 cell line from the graph and R^2 values 0.9471 for VERO cell line and 0.9444 for HCT-15 cell line (Table 5).

Table 5: IC_{50} and R^2 values of Colchicine.

Parameters	VERO	HCT-15
IC_{50} Value	1000	139.1
R^2	0.9471	0.9444

Traditionally many medicinal plants, which possess the ability to prevent and even to stall the progress of cancer, were in use. Plants possess certain chemicals, which have the ability to modify the physiological function of cells and hence act as anti-cancer drugs to arrest the proliferation of cancer cells. The mode of action of the drugs is unknown but successfully integrating our documented knowledge of plant properties and modern technological tools, effective anti-cancer drugs can be derived from plant sources and their mechanism can be elucidated.[33-35]

The present need is to develop drugs that can potentially target cancer cells by means of their inherent difference to normal cells. The development of such drugs with differential action will be very valuable in cancer chemotherapy without the observed side effects. The methodology involves use of cancer cell lines to test the efficacy of the plant extracts in vitro.

The potential use of *Triticum aestivum* as therapeutic agent holds great promise as the isolation of one or more cytotoxic chemicals from crude extract and the judicious use of such chemicals can control the progression of cancer and also can prevent the formation of tumour in individuals who are highly susceptible to developing a tumour.

CONCLUSION

The results obtained from the *in-vitro* studies performed using the HCT-15 cell lines reveals that the aqueous extract of *Triticum aestivum* has a moderate anticancer activity. The extract did not show any cytotoxic potential to the normal cell line i.e. Vero cell line. Even though there was increase in the cell growth inhibition when concentration of sample was increased, the IC50 value was 258.8 µg/ml for the HCT-15 cell line as shown by the MTT assay method. This holds great promise for future research in human beings. The anticancer property of *Triticum aestivum* will provide useful information in the possible application in the prevention and treatment of cancer.

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