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RESEARCH ARTICLE

Anti-Colorectal Cancer Properties of Hill Banana (cv.Virupakshi AAB) fruits: An *in vitro* assay

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ABSTRACT

Banana fruits are known for nutrients, antioxidants and other phytonutrients which provides immense disease protection through providing many nutrients, dietary fibers and vitamins.The ripened banana fruits are having higher anti-cancer properties than unripened fruits. Colorectal cancer is the fourth most common cancer in the world and in India, it is the fifth most common cancer. The *in vitro* assay of hill banana (Virupakshi) fruit juice revealed that, it has higher potential to inhibit growth the HT-29 cells and causes mortality at very low concentration of fruit juice. We have illustrated that the banana fruit juice has inhibited the proliferation of the colon cancer cell line HT-29 at low concentration through *in vitro* assay.

Keywords: Hill Banana Fruit; Anti-Colorectal Cancer; MTT Assay ; ETBr Assay

INTRODUCTION

In India, banana fruits are called as poor man's apple and “kalpatharu" which means a "virtuous plant. As a whole plant, roots, stem and flowers of banana plants have been widely used in traditional system of medicine for various ailments [1, 2]. Bananas are loaded with antioxidant compounds, thereby helping to reduce premature aging of the body’s cells. Alpha linolenic acid, one of these compounds, demonstrates significant antioxidant, anti-inflammatory and anti-cancer activity**.** Alpha linolenic acid also boosts immune function and enhances blood circulation. foods contain not only nutrients but also large amounts of compounds called phytochemicals. About 10,000 types of phytochemicals are considered to be present in nature. As banana ripening process progresses, its antioxidant levels rises. The well ripened banana fruit has better anti-cancer property and the dark spots on ripe yellow bananas

produce a substance called Tumor Necrosis Factor (TNF) that destroys cancerous tumors [3]. In terms of cancer fighting potential, the ripened bananas are eight times as effective as in their un-ripened state of green banana [3]. Colorectal cancer is the fourth most common cancer in the world with 1.3 million new cases each year and a 5-year prevalence rate of 3.2 million [4, 5]. There were an estimated 693,333 deaths due to colorectal cancer (CRC) in 2012. In India, it is the fifth most common cancer following breast, cervix/uteri, lip/oral cavity, and lung cancer [6]. In India, it is predicted to rise approximately by 80% in 2035, with an incidence of 114,986 new cases and a mortality of 87,502 [3].

There are many types of cancer treatments, it includes surgery, chemotherapy, radiation therapy, hormonal therapy or combined therapy. However, these kinds of therapies known to have different side-effects. Hence, identification and development of new chemotherapeutic agents from plants "phytochemicals" have gained significant recognition in the field of cancer therapy and become a major area of experimental cancer research [7].Recently, scientists all over the world are concentrating on the herbal medicines to fight against cancer. By understanding the complex synergistic interaction of various constituents of anticancer herbs, new novel herbal anticancer agents can be discovered and designed to attack the cancerous cells without affecting normal cells of the body [8]. With this background, the objective of the work is to analyze the anti-colon cancer properties of hill banana fruits through *in vitro* assay.

MATERIALS AND METHODS

The experiment was carried out ICAR-National Research Center for Banana, Trichy. The hill banana fruits (Virupakshi) are purchased from the local market and allowed to ripen fully at 19 - 21^{0}C. After full ripening the pulp was homogenized and pectinase enzyme (5 ml / Kg of pulp) was added and incubated for two hours. Then the clear banana juice was extracted and subjected to MTT and ETBr AO (Ethidium Bromide Acridine Orange dye). All this operation was done under sterile to ensure free from contamination. The hill banana juice (BJ) was assayed for anti-colorectal cancer activity through *in vitro* MTT and EtBr assays. These assays were done at Trichy Research Institute of Biotechnology (P) Ltd (TRI Biotech), Trichy. The procedure is described briefly as follows.

Cell culture

HT - 29 cells (Human Colon Carcinoma) cell line were cultured in liquid medium (DMEM) supplemented 10% Fetal Bovine Serum (FBS), 100 u/ml penicillin and 100 µg/ml streptomycin, and maintained under an atmosphere of 5% CO_2 at 37^{o}C.

MTT Assay

The BJ Sample was tested for *in vitro* cytotoxicity, using HT - 29 cells (Human colon carcinoma) cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [9,10,11]. Briefly, the cultured HT - 29 cells (Human colon carcinoma) cells were harvested by trypsinization, pooled in a 15 ml tube. Then, the cells were plated at a density of 1×10^5 cells/ml cells/well (200 µL) into 96-well tissue culture plate in DMEM medium containing 10 % FBS and 1% antibiotic solution for 24-48 hour at 37°C. The wells were washed with sterile PBS and treated with various concentrations of the BJ sample in a serum free DMEM medium. Each BJ sample was replicated three times and the cells were incubated at 37°C in a humidified 5% CO2 incubator for 24 h. After the incubation period, MTT (20 µL of 5 mg/ml) was added into each well and the cells incubated for another 2-4 h until purple precipitates were clearly visible under an inverted microscope. Finally, the medium together with MTT (220 µL) were aspirated off the wells and washed with 1X PBS (200 µl). Furthermore, to dissolve formazan crystals, DMSO (100 µL) was added and the plate was shaken for 5 min. The absorbance for each well was measured at 570 nm using a micro plate reader

(Thermo Fisher Scientific, USA) and the percentage cell viability and IC50 value was calculated using GraphPad Prism 6.0 software (USA).

ETBr and AO Staining

This protocol was followed on line with [16,17].Briefly, 5 x 10^5 cells/ml of HT-29 (human colon carcinoma) cells were seeded into the 24 well tissue culture plate and treated with 92.09 µg/ml of BJ sample in a serum free DMEM medium. The plate was incubated at 37°C in 5% Co2 incubator for 24 hours. After incubation, 50 µl of 1 mg/ml acridine orange (AO) and ethidium bromide (ETBr) were added to the wells and mixed gently. Finally, the plate was centrifuged at 800 rpm for 2 minutes and evaluated immediately within an hour and examined at least 100 cells by fluorescence microscope using a fluorescent filter.

RESULTS AND DISSCUSSION

The intensity of dark blue colour developed by the MTT reaction is directly proportional to the number of viable cancer cells. In this test, we used different concentrations (Table 1) of samples to evaluate its effect on the viability of cancer cells. In the control reaction without any sample didn't showed any mortality on the cell population (Sl. No.1). In the reaction with the lowest sample concentration of 20µg / ml kept most of the cancer cells viable without showing any notable effect on cancer cells. In the reaction with the highest sample concentration of 200 µg/ml showed nearly 55% of mortality on cancer cells. This trend is visible in the dose response curve (Fig.1). The anticancer activity of the samples increases as the concentration of the sample increases. When the cells are photographed under the microscope the formazan crystals formed in the viable cell. The number of cells are more in HT-29 untreated cells than hill banana juice (BJ) treated samples (Fig. 3).

The cell viability reaction is directly proportional to the number of viable cancer cells. In this test, we used different concentrations (Table 2) of samples to evaluate its effect on the viability of cancer cells. In the control reaction without any sample didn't showed any mortality on the cell population. In the reaction with the lowest sample concentration of 20 µg/ml kept most of the cancer cells viable without showing any notable effect on cancer cells. In the reaction with the highest sample concentration of 200 µg/ml showed nearly 55% of mortality on cancer cells. The anticancer activity of the samples increases as the concentration of the sample increases is also well depicted in the dose response curve (Fig2).

The half maximal inhibitory concentration (IC50) is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function. This quantitative measure indicates how much of a banana juice substance (inhibitor) is needed to inhibit a given biological process. In this analysis the hill banana juice (BJ) recorded IC50 as 92.05 µg/ml (Table. 3). The Fig 4a (Control) showed, the normal tumor cells (control). The Fig 4b exhibited early & late apoptotic cells, and necrotic cells under fluorescent microscopy (Fig 4b). Early-stage apoptotic cells were marked by a crescent-shaped or granular yellow-green acridine orange nuclear staining. Late-stage apoptotic cells were marked with concentrated and asymmetrically localized orange nuclear ethidium bromide staining. Necrotic cells increased in volume and showed uneven, orange-red fluorescence at their periphery.

Many fruits such as guava, banana, papaya, orange, lemon, apple, litchi possess proven medicinal activities as whole fruit, seeds, leaves, and as peels and many of them are reported to have anticancer potential such as lemon, orange, papaya, guava [10]. The well ripened banana fruit has better anti-cancer property and the dark spots on ripe yellow bananas produce a substance called Tumor Necrosis Factor (TNF) that destroys cancerous tumors [3]. The anticancer activity was carried out by MTT assay in banana, guava, orange and papaya showed good anticancer activity with IC_{50} values 31.7, 27, 95.5, and 18.5 µg /ml, respectively [17, 18]. The similarity in our present study results and the previous study data on banana fruits anti cancer effect in human cancer cells (Ht-29) confirms the efficiency of the

extract in inhibition the cancer cells growth. Further more study and careful experiments are needed to be done in the future to explore more pathways and mechanisms induced by this fruit extract.

CONCLUSION

Nowadays, finding a balance in cell proliferation expected to be a master key in homeostatic maintenance by inhibiting uncontrolled cells proliferation. Through arresting the cell cycle and induction of apoptosis in progressed cancer cells. An increasing number of evidences focused on the importance of phytochemicals and their effect in the metastasis of different cancers including colon cancer. At this study, we have illustrated that the banana juice extract inhibited the proliferation of the colon cancer cell line HT-29. We have investigated a cell death mechanism which inhibits the cell cancer growth.

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Table: 1. The OD values of the cell concentration at 570 nm

S. No.	Hill Banana Juice Concentration (µg/ml)	OD at 570 nm Mean
1.	Control	0.467
2.	200 µg/ml	0.177
3.	180 µg/ml	0.213
4.	160 µg/ml	0.240
5.	140 µg/ml	0.293
6.	120 µg/ml	0.300
7.	100 µg/ml	0.318
8.	80 µg/ml	0.337
9.	60 µg/ml	0.372
10.	40 µg/ml	0.417
11.	20 µg/ml	0.466

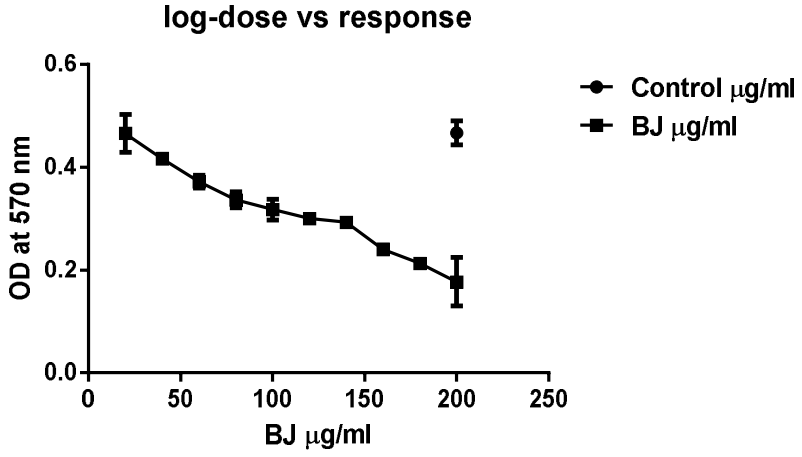


Fig.1: The dose response curve of bill banana juice against HT—29 cells

Table 2: Cell (HT-29) Viability (%) against the Hill banana juice treatment

S. No.	Hill Banana Juice Concentration (µg/ml)	Mean Value (%)
1.	Control	100
2.	200 µg/ml	37.89
3.	180 µg/ml	45.60
4.	160 µg/ml	51.45
5.	140 µg/ml	62.66
6.	120 µg/ml	64.30
7.	100 µg/ml	68.01
8.	80 µg/ml	72.16
9.	60 µg/ml	79.65
10.	40 µg/ml	89.21
11.	20 µg/ml	99.78

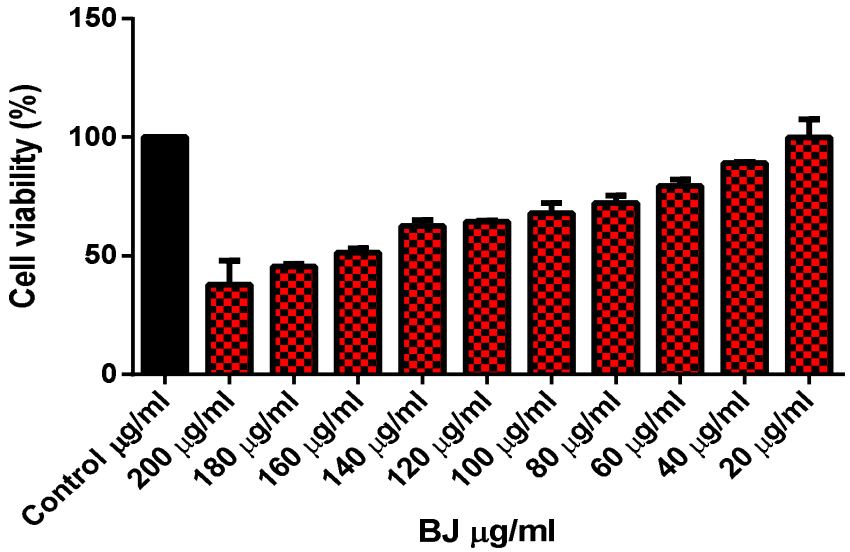
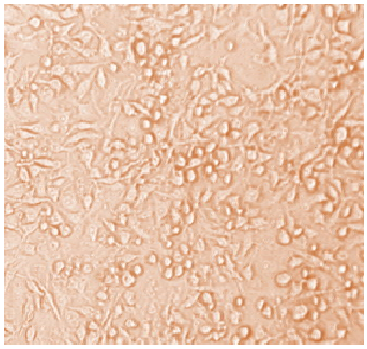


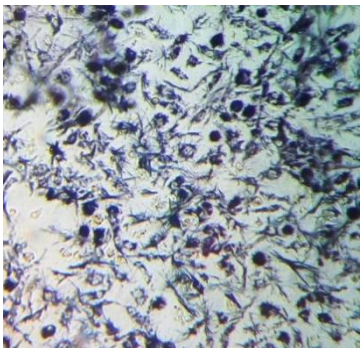
Fig-2: The cell (HT-29) viability percentage versus the hill Banana Juice (BJ)

Table 3: IC50 Value of tested BJ Sample: 92.09 µg/ml

log(inhibitor) vs. normalized response -- Variable slope	
Best-fit values	
LogIC50	1.964
HillSlope	-2.225
IC50	92.09
Std. Error	
LogIC50	0.01962
HillSlope	0.2370
95% Confidence Intervals	
LogIC50	1.924 to 2.004
HillSlope	-2.710 to -1.740
IC50	83.95 to 101.0
Goodness of Fit	
Degrees of Freedom	28
R square	0.9032
Absolute Sum of Squares	2630
Sy.x	9.691
Number of points	
Analyzed	30

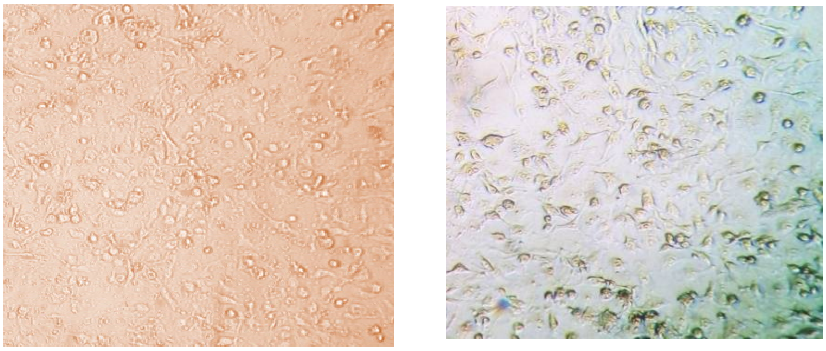


Before MTT treatment



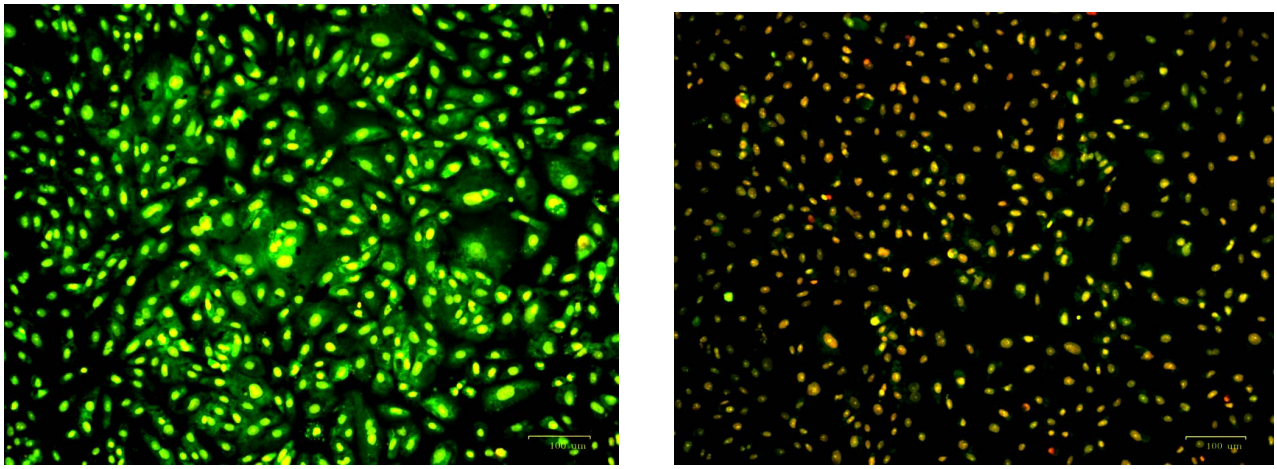
After MTT treatment

1. Control Cells (HT- 29)



BJ (200 μg/ml Treated Cells (HT-29)

Fig 3. Formation of formazan crystals in control cells and BJ Sample treated cells



4a. Control **4 b. Treated with 92.09 μg/ml of BJ sample**

Fig.4.ETBr assay with Hill Banana Juice (BJ)