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IN-VITRO EVALUATION OF HERBS ON HUMAN EPITHELIAL COLON CARCINOMA (CaCo2) AND ITS PERMIABILITY STUDIES

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ABSTRACT

Cancer is a fatal disease taking many lives across the world and colon cancer stands third among all cancer after lung and breast cancer. Many conventional drugs have been used to treat cancer. Studies have shown that many compounds extracted from plants exhibit anticancer properties and also show negligible side effects. Few plants that have proven to exhibit antioxidant and anticancerous properties according to the literature survey and the phytochemical assays conducted by us, was chosen and tested on human epithelial colon carcinoma (CaCo2 cell lines) in vitro and the drug permeability studies was performed using FACS (fluorescent Assorted Cell Sorting). In this article we have discussed few related researches and our present work as a review.

KEYWORDS: Colon cancer, Plants, anticancer properties, CaCo2 cell line, permeability studies.

I INTRODUCTION

WHO also released a world cancer report 2014 indicating the occurred death rates due to cancer to be 8.2 million worldwide from 2012.[1] It has defined cancer as the uncontrolled growth and spread of cells which can affect almost any part of the body. Cancer is caused by multiple changes in gene expression leading to dysregulated balance of cell proliferation and cell death and ultimately evolving into a population of cells that can invade tissues and metastasize to distant sites, causing significant morbidity and, if untreated, death of the host. Alterations in gene often are associated with different forms of cancer. The main genes can

be broadly classified into three groups. The first group, called proto-oncogenes, produces protein products that normally enhance cell division or inhibit normal cell death. The mutated forms of these genes are called oncogenes. The second group, called tumor suppressors, makes proteins that normally prevent cell division or cause cell death. The third group contains DNA repair genes, which help prevent mutations that lead to cancer. Any mutation in DNA repair gene also causes cancer. The major factors that trigger cancer are smoking dietary imbalances, hormones and chronic infections leading to chronic inflammation.[2] Cancer does not develop overnight, instead often evolves over many years with detectable premalignant lesions presaging the development of full-blown malignancy. Malignant tumours not only invade surrounding tissue, but are able to colonize other, often vital, organs, a process known as metastasis. Widespread metastatic disease is usually a harbinger of imminent patient death. Whereas benign tumours are generally slow-growing expansive masses that compress rather than invade surrounding tissue. As such they generally pose little threat, except when growing in a confined space like the skull, and can usually be readily excised.[3]

Colon cancer is the fourth most frequently diagnosed cancer and the second leading cause of cancer death.[4] It is a type of Adenocarcinoma that arises in colon cells. Adenocarcinoma is a type of cancer that forms in mucus-secreting glands throughout the body. Risk factors of colon cancer include high fat consumption, alcohol consumption, smoking, genetic factors, and viral infections. It is also said that diabetes can be a triggering factor for colon cancer [29]. The symptoms of colon cancer usually includes bleeding from the rectum, blood in the stool or in the toilet after having a bowel movement, dark- or black-colour stools, change in the shape of the stool, cramping pain in the lower stomach, feeling of discomfort or an urge to have a bowel movement when there is no need to have one, new onset of constipation or diarrhoea that lasts for more than a few days, unintentional weight loss.[5]

Colon cancer shows a survival rate of 5 years and the disease can be easily treated when found in early stages. Hence the screening step becomes very necessary.[6] The conventional drugs that are used to treat colon cancer are 5-Fluorouracil (5-FU), Capecitabine (Xeloda), leucovorin, and oxaliplatin. Most of the times these drugs are used in combination. But the treatment is associated with many side effects like hair loss, mouth sores, loss of appetite, nausea and vomiting, low blood counts.[7]

Rout *et al.*, have worked on the importance of plant based drugs. Their article reviews some of the past successes of the natural products approach and also explores some of the reasons why it has fallen out of favor among major pharmaceutical companies and the challenges in drug discovery from Natural Products especially Higher Plants. In this review we consider the past, present, and future value of employing information from plants used in traditional medical practices (ethnomedicine) for the discovery of new bioactive compounds. Herbal medicines are relatively simple, although they are quite distinct from conventional medicine. India is a rich source of medicinal plants and a number of plant extracts are used against diseases in various systems of medicine such as ayurveda, unani and siddha. Plant derived natural products such as flavonoids, terpenes, and alkaloids and soon has received considerable attention in recent years, due to their diverse pharmacological properties including cytotoxic and cancer chemo preventive effects.[8]

Sushma Kainsa *et al.*, and Sumitra Chanda *et al.*,in their short reviews have mentioned few of the medicinal plants and listed out the compounds in them that have been studied to exhibit anticancerous properties. Since plants are natural agents they show negligible side effects and are easily available.

Based on a few previous researches we selected a few plants that exhibit fair antioxidant and anticancerous properties namely *Curcumin longa*(Turmeric)[9,10], *Zingiber officinale*(Ginger)[10], *Tinospora cordifolia*(Guduchi) [10], *Cymbopogon citratus*(Lemon grass) [11] and *oxalis articulate*(Sour grass).[12] *Curcuma longa* (turmeric) has curcumin (diferulovl methane) and curcuminoids, isolated from its rhizome suppress cancer at initiation, growth and metastasis. It also inhibits angiogenesis. It arrests the cancer cells proliferation in G2/S phase and induces apoptosis.[14] *Cymbopogon citratus* (lemon grass) has got compounds such as hydrocarbon terpenes, aldehydes, alcohols, ketones and esters. The methanolic extracts of the leaf have potential contents of flavonoids. These show antioxidant activity, antimutagenic activity, anti-inflammatory activity used for the treatment of the gastro intestinal tract [15]. *Oxalis articulate* (sour grass) is an ornamental herb upon which not much literature is found, but has shown considerable antioxidant property. *Tinospora cordifolia* (amruthballi) stem, bark, fruit of *T.cordifolia* contains berberine, tinosporine, giloin and giloinin. Sesqueterpenes and diterpenes isolated from this herb inhibit growth and spread of various cancers. It also possesses anticancer and antiulcer properties.[13] *Zingiber officinale* (ginger) is a commonly used medicinal herb which has natural dietary components with antioxidant and

anticarcinogenic properties. Active phenolic compounds in ginger such as shagol and gingerol exhibits antitumorous and cancerous properties.[16]

The above mentioned plant extracts were tested on CaCo2 cell line in vitro. CaCo 2 cell is a continuous cell of heterogeneous human epithelial colorectal adenocarcinoma cells, developed by the Sloan-Kettering Institute for Cancer Research through research conducted by Dr. Jorgen Fogh.[17] Caco-2 cells express tight junctions, microvillus, and a number of enzymes and transporters making them a suitable in vitro model of the human small intestinal mucosa to predict the absorption of orally administered drugs.[18] Based on the review of Hayeshi *et al.*,the CaCo2 is a adherent cell line that grow well in MEM(Modified Eagle's Media) and has a doubling time of 48 hrs.

II MATERIALS AND METHODS

Plant materials

All plant materials -*Curcumin longa*(Turmeric)[rhizome], *Zingiber officinale*(Ginger)[rhizome], *Tinospora cordifolia*(Guduchi)[leaves], *Cymbopogon citratus*(Lemon grass)[leaves] and *oxalis articulate*(Sour grass)[whole plant] were collected from within Bangalore area like GKVK, nursery. All plant materials were shade dried and finely powdered for further extract preparation. We chose to prepare methanolic extracts for our studies after a good amount of literature survey.

Preparation of crude extract

Extraction was carried out for all the five plants by using overnight maceration techniques according to the method described by Harbone.[19] About 10 g were macerated in 50ml of methanol for 3-4 hrs at 50°C with occasional shaking on boiling water bath. The supernatant was decanted and clarity field by filtration through a filter paper, after filtration, the solvent was then removed by evaporation at 70-80°C on boiling water bath. Each residue was weighed and the yield percentage was calculated then stored at room temperature in tightly sealed vial ready for use.

Different stocks for different assays were prepared.

Stock 1) 100mg extract+ 1ml methanol,

Stock 2) 32mg extract + 1ml DMSO,

Stock 3)120mg extract + 1.2 ml dH2O.

Cell line used

CaCo 2 cells (human epithelial colon carcinoma) an adherent cell line is cultured in DMEM(Dulbecco's Modified Eagle's Medium) and subcultured twice a week.

Culturing Adherent Cells (CaCo2)

Remove and discard the spent cell culture media from the culture vessel. Wash cells using a balanced salt solution (1XPBS), rock the vessel back and forth several times.Remove and discard the wash solution from the culture vessel. Add the pre-warmed dissociation reagent such as trypsin-EDTA (2ml) to the side of the flask. Gently rock the container to get complete coverage of the cell layer.

Incubate the culture vessel at room temperature or 37°**C** incubator for approximately 2 minutes. (incubation time varies with the cell line used).Observe the cells under the microscope for detachment. If cells are less than 90% detached, increase the incubation time a few more minutes, checking for dissociation every 30 seconds. You may also tap the vessel to expedite cell detachment.

When ≥ 90% of the cells have detached, tilt the vessel for a minimal length of time to allow the cells to drain. Add the equivalent of 2 volumes (twice the volume used for the dissociation reagent) of pre-warmed complete growth medium. Disperse the medium by pipetting over the cell layer surface several times.

Transfer the cells to a 15-mL conical tube and centrifuge then at 1200 × g for 5 to 10 minutes. (Centrifuge speed and time vary based on the cell type). Resuspend the cell pellet in a minimal volume of pre-warmed complete growth medium and remove a sample for counting. Determine the total number of cells and percent viability using a hemocytometer, cell counter and Trypan Blue exclusion, or the Countess® Automated Cell Counter.

Dilute cell suspensions to the seeding density recommended for the cell line and pipette the appropriate volume into new cell culture vessels, and return the cells to the incubator.

Phytochemical Analysis (Qualitative)

According to Mehta *et al.,*

Test for Alkaloids: 3 ml aqueous extract was stirred with 3 ml of 1% HCl on steam bath. Mayer and/or Dragondroff's reagent was then added to mixture. Turbidity of the resulting precipitate was taken as an evidence for the presence of alkaloid.

Test for Tannins: $FeCl_3$ Test: About 2 ml of the aqueous extract was stirred with 2 ml of distilled water and few drops of FeCl3 Solution were added. Formation of green precipitate was indication of presence of tannins.

Test for Saponins: 5 ml of aqueous extract was shaken vigorously with 5 ml of distilled water in a test tube and warmed. The formation of stable foam was taken as an indication of the presence of saponins.

Test for Phlobatannins: About 2 ml of aqueous extract was added to 2 ml of 1% HCl and the mixture was boiled. Deposition of a red precipitate was taken as an evidence for the presence of phlobatannins.

Test for Flavonoids: Alkaline reagent test: To 1 ml of aqueous extract, 1 ml of 10% lead acetate solution was added. The formation of a yellow precipitate was taken as a positive test for flavonoids.

Test for Terpenoids: The extract is treated with chloroform with few drops of concentrated sulphuric acid, shaken well and allowed to stand for some time, formation of yellow coloured lower layer indicated the presence of terpenoids.

Tests for glycosides: Liebermann's test: 2 ml of the organic extract was dissolved in 2 ml of chloroform and then 2 ml of acetic acid was added in it. The solution was cooled well in ice. Sulphuric acid was then added carefully. A colour change from violet to blue to green indicates the presence of a steroidal nucleus (that is, a glycone portion of glycoside).

Tests for steroids: A red colour produced in the upper layer when 2 ml of organic extract was dissolved in 2 ml of chloroform and 2 ml concentrated sulphuric acid was added in it, indicates the presence of steroids.[20]

Antioxidant activity of plant extracts

DPPH assay is carried out as per the method of Rajakumar *et al.*[21] In brief, 90 µl of DPPH solution is treated with 180µl of various concentration of test solution & standard. The different concentrations tested for reference standard are 0.5, 1.0, 1.5, 2.0, 2.5 mcg/ml. The reaction mixture is mixed and incubated at 25°C for 15 minutes. The absorbance is measured at 510 nm using Plate reader. A control reaction is carried out without the test sample.

Determination of % Inhibition

$$\% \text{ Inhibition} = \frac{(\text{Control} - \text{Sample})}{(\text{Control})} \times 100$$

Haemolysis

Principle

This assay is to test the extract for its haemolytic activity. The RBC's were collected and washed with 1X PBS(phosphate buffer saline) and diluted. 100µl of different concentrations of extracts were treated with 50µl of erythrocytes and incubated for 1 hour with test material during rotation at 37°C. After incubation, samples are collected and centrifuged to obtain supernatant, containing free hemoglobin. The hemoglobin concentration is measured by means of a spectrophotometer (540 nm). Test samples are compared to reference materials (1% TritonX-100 and 1% SDS).[22, 23]

MTT Assay

Principle

Ouedraogo, Geoffroy G *et al.*, 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, blue colour formazan product which is measured spectrophotometrically. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells.

FACS Studies

Researchers have also used FACS in apoptotic studies to know the stage of cell cycle in which the drug is seizing the proliferation of the cell.[25] FACS uses fluorescent dyes like Propidium Iodide (PI), phycoerythrin, allophycocyanin and many more. These dye molecules closely associate themselves with the DNA strands and when illuminated they will fluoresce. FACS data is a dot plot. As a cell intersects the laser beam, the instrument detects it as a point on an x-y graph. This form of data presentation looks at two parameters of the sample at the same time. The parameters can be any combination of scatter and fluorescence. Three common modes of use for the dot plot are the scatter plot: 1) forward scatter (FSC) vs. side scatter (SSC); 2) single color vs. side scatter; and 3) two-color fluorescence plot.[26]

III RESULTS

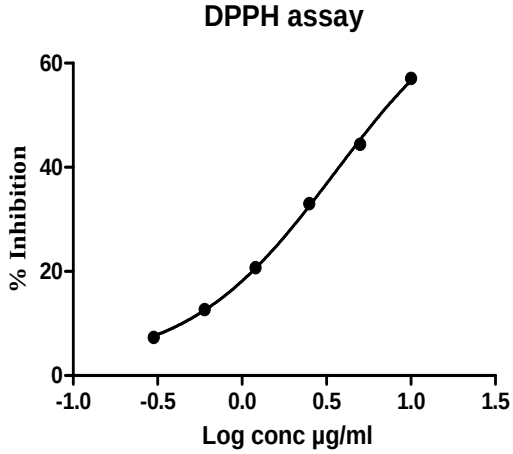
Phytochemical assay

Table 1: Results showing the presence of different phytochemicals in the respective extracts

SL No	TESTS	Oxalis articulata	Cymbopogon citratus	Zingiber officinale	Curcumin longa	Tinospora cordifolia
1	Alkaloids	+	+	+	+	+
2	Tanins	+	+	-	-	+
3	Saponins	-	+	-	-	-
4	Phlobatanins	-	-	-	+	-
5	Terpenoids	+	+	+	-	+
6	Glycosides	+	+	+	-	+
7	Steroids	+	+	+	+	+
8	Flavanoids	+	+	+	+	+
9	Molisch's test	+	+	+	+	+

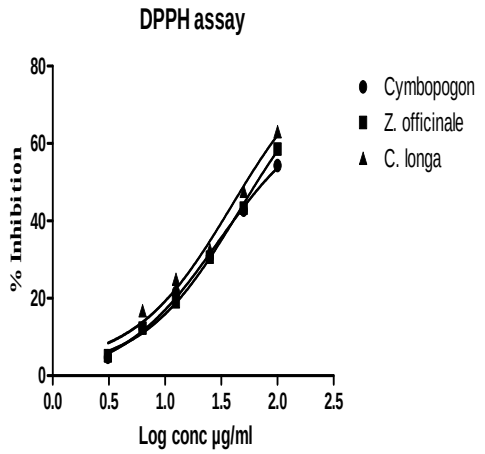
ANTIOXIDANT ASSAY

DPPH assay: Only three extracts showed antioxidant activity and cymbopogon citratus showed the highest antioxidant capacity.



	Quercitin
log(inhibitor) vs. response	
Best-fit values	
BOTTOM	76.09
TOP	1.767
LOGIC50	0.5487
IC50	3.538

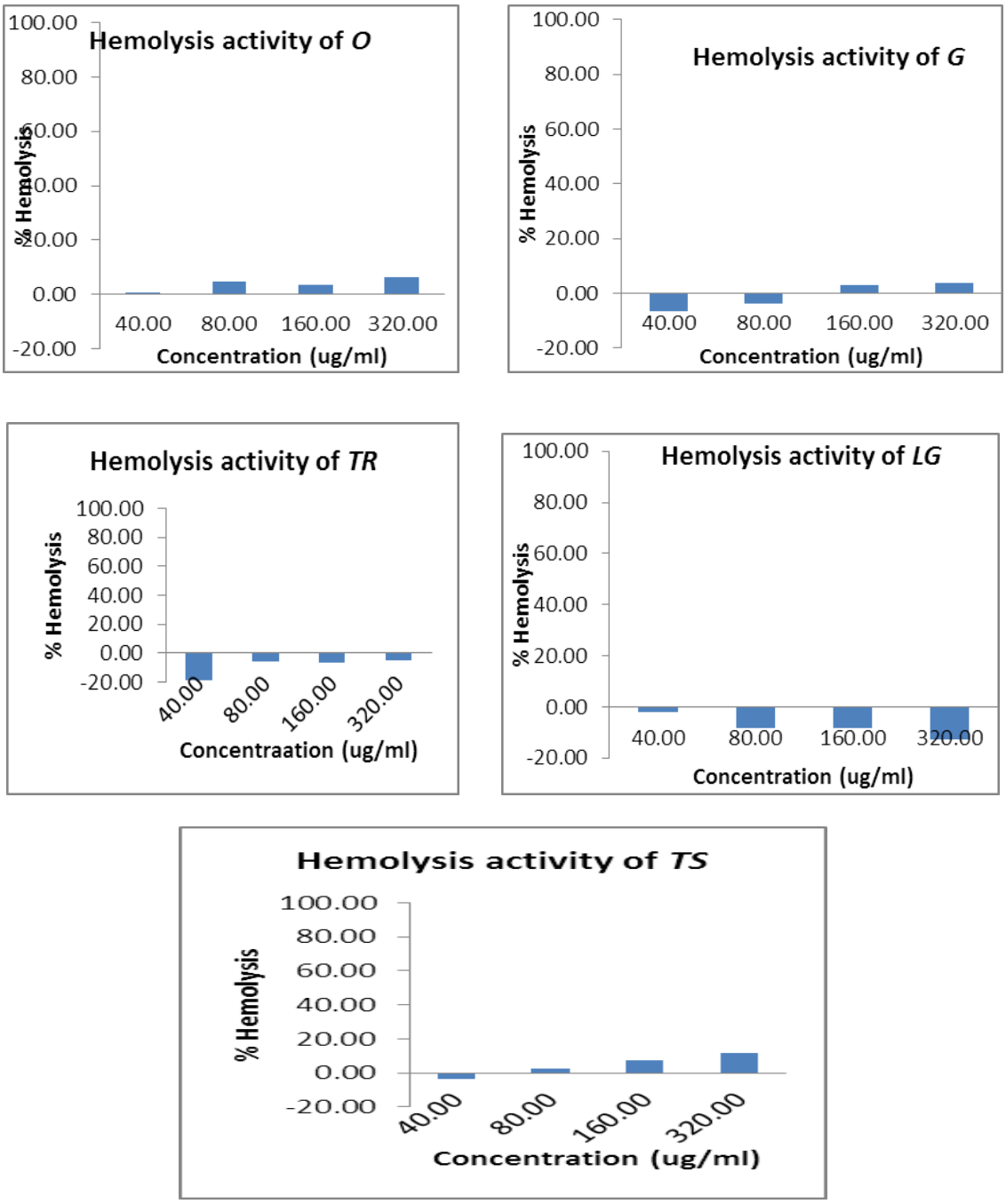
Graph 1: Standard (Quercitin)



	Cymbopogon	Z. officinale	C. longa
log(inhibitor) vs. response			
Best-fit values			
Bottom	69.71	84.22	85.47
Top	-0.9630	1.022	2.299
LogIC50	1.468	1.662	1.592
IC50	29.36	45.88	39.09

Graph 2: % inhibition of extracts

Haemolysis results

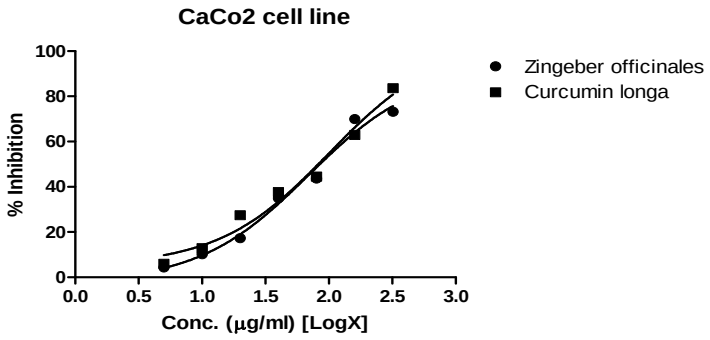


O-Oxalis articulata
G-Zingiber officinale
LG-Cymbopogon citratus
TR-Curcumin longa
TS-Tinospora cordifolia

Graph 3: Effect of *O. articulata, Z. officinale, C. citratus, C. longa, T. cordifolia* extracts on hemolysis of human erythrocytes. Human erythrocytes were incubated with various concentrations (0, 40, 80, 160 and 320 □g/ml) of above mentioned plant extracts at 37^0C water bath for 60 min. The absorbance of the resulting supernatant was measured at 540 nm

by spectrophotometer to determine the extent of hemolysis. The percentage of hemolysis was calculated by the equation *% Hemolysis = [(^{A}Control– ^{A}Sample) / ^{A}Control] x 100*. All analyses are the mean of two independent experiments.

Cytotoxicity assay (MTT) results

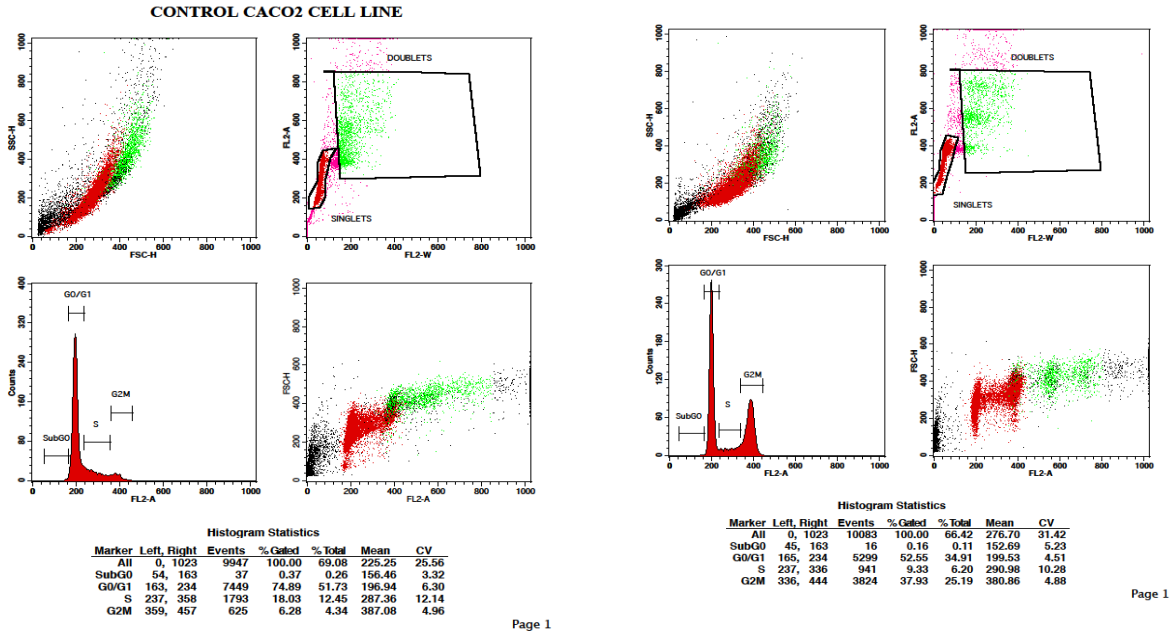


	Zingeber officinales	Curcumin longa
log(inhibitor) vs. response		
Best-fit values		
BOTTOM	92.60	104.4
TOP	-2.309	5.020
LOGIC50	1.839	2.000
IC50	69.08	99.91

Graph 4.Cytotoxic effect of *Z. officinale, C.longa on colon carcinoma (CaCo 2 cell line)*

Cells were treated with various concentrations (0, 10, 20, 40, 80, 160 and 320 µg/ml) of above mentioned plant extracts for 24 hrs grown in a serum free media. The percentage of cell death induced was determined using MTT.

FACS RESULTS



***Zingeber officinales* effect at 50 µg/ml on CaCo2 cell line**

There are not many reviews of apoptotic studies of plant extracts on CaCo2 cell lines using FACS. Susewind *et al.*, wrote a review on 3D co-culture model of the intestinal mucosa. They had worked on CaCo2 cells and nanoparticles and performed FACS. S Su *et al.*, worked on a Chinese medicinal plant called *Arctium lappa to* isolate lignans and study the underlying mechanism of action on CaCo2 and also performed FACS.

According to the cytotoxicity assay ginger showed good results. So, we performed FACS studies of the action of ginger on CaCo2 cells invitro in the concentration of 50µg/ml and 100µg/ml and by taking colchicin as control. In spite of CaCo2 being a very adherent cell and cells that form tight junction colchicin should around 90% inhibition of cancer cells at G2 phase and Ginger (50µg/ml) showed good results of 42% inhibition in G2 phase. We suspect the above results are because of the absorption capabilities of colon cells. Hence it gave us fair information about the permeability status of CaCo2 cells for our plant extract.

IV CONCLUSION

Cancer, even after extensive research is not fully understood. It is the second reason for the death after heart diseases and colon cancer is one of the cancers that contribute to around 5-8% of deaths. So, there is a need to search for an appropriate medication for it. The idea behind selecting plant based drug is the fact that it is natural and causes negligible side effects and is inexpensive making it affordable to everyone. Ginger has come out as a better compound to use against colon cancer when compared to the other four plants that were selected. Further studies about this plant extract can reveal the compounds responsible for anticancer property and the mechanism of action of the compound on colon cancer cell.

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