# INHIBITORY EFFECTS OF GALLIC ACID IN COLON CANCER CELLS

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*Dedicated to the resting souls of my Grandparents*
*Mr. & Mrs. Yazh. Sundaram*

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# ABSTRACT

Colorectal cancer is a malignant tumor arising from the inner wall of the large intestine, which is the third most common type of cancer. American Cancer Society estimates about 93,090 new cases and 49,700 deaths due to colorectal cancer in the United States since 2015. Diet is thought to have a major role in the etiology of colorectal cancer. Scientists explore the phenolic phytochemicals found in various food substances for colorectal cancer treatment and prevention. In this research, a diet-derived phenolic compound Gallic Acid (GA) was explored for its antiproliferative action against the colon cancer cell line HCT-15. MTT assay results illustrated that GA has an inhibitory effect on HCT-15 with $IC_{50}$ value of 740 μmol/L. A time-dependent inhibition of colony formation was evident with GA treatment as the maximum number of colonies formed was about 110 after 48 h. Cell cycle arrest was evident from the accumulation of GA treated HCT-15 cells at sub-G1 phase (0.98±1.03 *vs* 58.01±2.05) with increasing exposure time. Flow cytometric analysis of GA treated HCT-15 cells depicted various events associated with apoptosis like lipid layer breakage and reduction in mitochondrial membrane potential apart from an increase in the generation of ROS, which were in a time dependent manner. SEM and photomicrograph images of the GA-treated cells displayed membrane blebbing and cell shrinking characteristics of apoptosis. Further, the Yo-Pro-1 staining of GA treated cells confirmed apoptosis in a time dependent manner. These results propel the role of GA as a putative agent in colon cancer treatment. However, further experiments in preclinical and clinical settings are required to promote GA as a likely candidate for chemotherapy of colon cancer.

# ABSTRAK

Kanser kolorektal adalah tumor malignan yang timbul daripada dinding dalam usus besar, yang merupakan jenis yang ketiga paling umum kanser. Anggaran Persatuan Kanser Amerika tentang 93,090 kes baru dan 49,700 kematian akibat kanser kolorektal di Amerika Syarikat sejak tahun 2015. Diet dianggap sebagai mempunyai peranan utama dalam etiologi kanser kolorektal. Para saintis meneroka fitokimia fenolik didapati dalam pelbagai bahan makanan untuk rawatan kanser kolorektal dan pencegahan. Dalam kajian ini, diet yang diperolehi sebatian fenolik Gallic Acid (GA) telah diterokai untuk tindakan antiproliferative terhadap garis sel kanser usus besar HCT-15. Keputusan MTT assay digambarkan bahawa GA mempunyai kesan yg melarang pada HCT-15 dengan nilai $IC_{50}$ 740 μmol / L. A perencatan masa yang bergantung kepada pembentukan koloni terbukti dengan rawatan GA sebagai bilangan maksimum jajahan dibentuk adalah kira-kira 110 selepas 48 h. Kitaran sel penangkapan terbukti daripada pengumpulan GA dirawat HCT-15 sel-sel pada fasa sub-G1 (0.98 ± 1.03 vs 58.01 ± 2.05) dengan meningkatkan masa pendedahan. Aliran analisis cytometric GA dirawat HCT-15 sel-sel yang digambarkan pelbagai acara yang berkaitan dengan apoptosis seperti kerosakan lapisan lipid dan pengurangan potensi membran mitokondria selain peningkatan dalam penjanaan ROS, yang dengan cara yang bergantung kepada masa. SEM dan photomicrograph imej sel GA dirawat dipaparkan blebbing membran dan ciri-ciri sel pengecutan apoptosis. Di samping itu, Yo-Pro-1 mengotorkan GA dirawat sel-sel yang disahkan apoptosis dengan cara yang bergantung kepada masa. Keputusan ini melonjakkan peranan GA sebagai ejen diduga dalam rawatan kanser kolon. Walau bagaimanapun, eksperimen lanjut dalam tetapan pra-klinikal dan klinikal diperlukan untuk menggalakkan GA sebagai calon mungkin untuk kemoterapi kanser kolon.

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| | | |
|---|---|---|
| 4OMGA | - | 4-O-methyl Gallic acid |
| 5-FU | - | Fluorouracil |
| A375S2 | - | Human melanoma cells |
| A459 | - | Human melanoma cells |
| ABC family | - | ATP-binding cassette family |
| ADC | - | Analog to digital converter |
| ADI | - | Acceptable daily intake |
| AGS | - | Gastric adenocarcinoma cells |
| AIF | - | Apoptosis-inducing factor |
| ANOVA | - | Analysis of variance |
| APC | - | Adenomatous polyposis coli |
| ATP | - | Adenosine triphosphate |
| BRCA1 | - | Breast cancer 1 onset gene |
| BRCA2 | - | Breast cancer 2 onset gene |
| $C_6H_2(OH)_3COOH$ | - | 3, 4, 5-trihydroxybenzoic acid |
| CDK | - | Cyclin-dependent kinases |
| $CO_2$ | - | Carbon dioxide |
| COLO 205 | - | Dukes' type D, colorectal adenocarcinoma cells |
| DCFH-DA | - | Dichlorofluorescein-diacetate |
| DNA | - | Deoxyribonuclic acid |
| EDTA | - | Ethylenediaminetetraacetic acid |
| EGCG | - | Epigallocatechin gallate |
| Endo G | - | Endonuclease G |
| Erk | - | Extracellular signal-regulated kinases |
| F344 | - | Fischer inbred rat |

| | | |
|---|---|---|
| FACS | - | Fluorescence-activated cell sorting |
| FBS | - | Fetal bovine serum |
| $G_0/G_1$ phase | - | Gap phase |
| GA | - | Gallic acid |
| GSH | - | Glutathione |
| HCLE | - | Human corneal limbal epithelial cells |
| HCT-116 | - | Colorectal carcinoma cells |
| HCT-15 | - | Colorectal adenocarcinoma cells |
| HeLa | - | Human epithelial cells |
| HL 60 | - | Human promyelocytic leukemia cells |
| HSC-2 | - | Human oral carcinoma cells |
| $IC_{50}$ | - | Half maximal inhibitory concentration |
| ICAM-1 | - | Intercellular adhesion molecule 1 |
| I-kB | - | Ikappab kinase |
| JECFA | - | FAO/WHO Joint Expert Committee on Food Additives |
| JNK | - | C-Jun N-terminal kinases |
| LLB | - | Lipid layer break |
| M phase | - | Mitosis phase |
| MDR | - | Multidrug Resistance |
| MCF-7 | - | Breast cancer cells |
| MiaPaCa-2 | - | Human pancreatic cancer cells |
| MMP | - | Matrix metalloproteinase |
| MPAK | - | Mitogen-activated protein kinase |
| MSI | - | Microsatellite instability |
| MTT | - | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tertazolium-bromide |
| NAD(P)H | - | Nicotinamide adenine dinucleotide phosphate |
| NF-kB | - | Nuclear factor kappa B |
| NOAEL | - | No observed-adverse-effect level |
| p53 | - | Phosphoprotein p53 |
| PBS | - | Phosphate-buffered saline |
| PC3 | - | Prostate cancer cells |
| RB1 | - | Epigenetic retinoblastoma gene |
| RhoA | - | Ras homolog gene family, member A |

| | | |
|---|---|---|
| RhoB | - | Ras homolog gene family, member B |
| ROS | - | Reactive oxygen species |
| RPMI-1640 | - | Roswell Park Memorial Institute medium |
| S phase | - | Synthesis phase |
| SD | - | Standard deviation |
| SEM | - | Scanning electron Microscope |
| siRNA | - | Small interfering RNAs |
| SMMC-7721 | - | Human hepatoma cells |
| TE-2 | - | Esophageal cancer cells |
| Trk | - | Tyrosine kinases |
| U-2 OS | - | Human osteosarcoma cells |
| U937 | - | Human monocytic lymphoma cells |
| VCAM-1 | - | Vascular cell adhesion protein 1 |
| VEGF | - | Vascular endothelial growth factor |
| WHO | - | World health organization |
| MnSOD | - | Manganese superoxide dismutase |
| SOD | - | Superoxide dismutase 2, mitochondrial enzyme |

## LIST OF SYMBOLS

| | | |
|---|---|---|
| H | - | Hour |
| μL | - | Microliter |
| μg/mL | - | Microgram per milliliter |
| mmol/ L and mM $L^{-1}$ | - | Milimolar per liter |
| mg $kg^{-1}$ | - | Milligram per kilogram |
| mg/mL | - | Milligram per milliliter |
| mM | - | Millimolar |
| Min | - | Minutes |
| $\Delta\Psi_m$ | - | Mitochondrial membrane potential |

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# CHAPTER 1

# INTRODUCTION

## 1.1 Background

Cancer has become a major concern around the globe. Cancer is the second major cause of death worldwide (Bernard *et al*., 2014), which is a class of diseases characterized by uncontrolled cell proliferation. At present, cancer is the cause of 1 in every 7 deaths worldwide. The burden is mounting at a rapid level, around 21.7 million new cancer cases and 13.0 million cancer deaths are expected in the year 2030 due to varying daily habits. The change in lifestyle (such as smoking, unbalanced diet, physical inactivity, reproductive patterns) associated in the developing countries are other factors that add fuel to this problem. As diet plays a crucial role in causing as well as preventing cancer, the field of investigations on the role of nutrition and cancer is ruthlessly broad. It has been estimated that about 30-40 % of all types of cancer can be prevented by proper diet consumption and physical activity in a project funded by the American Institute for Cancer Research and the World Cancer Research (WCRF/AICR, 1997).

In the year 2015 alone, it is estimated that about 1,658,370 new cancer cases and 589,430 Americans will die from cancer, corresponding to about 1,640 deaths per day (American cancer society. Cancer Facts & Fig.s 2015). According to the survey of the world health organization, global cancer rates could increase by 50% in the year 2020, which is approximately to 15 million. Cancers of the lung and bronchus, prostate, and colorectal continue to be the most common causes of cancer death. In particular, colon cancer claims to be the third most common type of cancer

(World Cancer Report 2014.WHO). The American Cancer Society's estimates 93,090 new cases and 49,700 deaths due to colorectal cancer in the United States for 2015 (American cancer society. Cancer Facts & Fig.s 2015). The survival of this chronic disease is very dependent primarily on the stage of diagnosis, type of cancer diagnosed, choice of treatment and comorbidities, as opposed to differences in cancer biology. Cancer statistics often focuses on the Five-year survival rate, yet there is no clear information that the cancer survivors are still undergoing treatment for these five years or cancer free.

However, the technological development offers countless choices of treatments for cancer patients diagnosed with cancer. The prime ones include surgery, chemotherapy and radiotherapy (Rebecca *et al*., 2014). The selection of treatment depends upon the type, location and stage of the cancer as well as the person's health and wishes. Chemotherapy is one of the standardized treatments that employ chemotherapeutic agents to kill cells that divide rapidly (Lind, 2008). Chemotherapeutic drugs induce apoptosis, which is a programmed cell death involving biochemical events leading to morphological and molecular changes leading to death, in the cancer cells. The basic flow of chemotherapy is shown in Figure 1.1.

Although there are plenty of drugs, which can retard the cancer, it cannot cure cancer completely when detected at the latter stages. Apart from this, there are other side effects due to the administration of anticancer drugs. Hence, there is a prolonged search for novel anticancer drugs. Scientists not only focus on the synthetic drugs, but also the natural compounds from our diets are widely experimented.

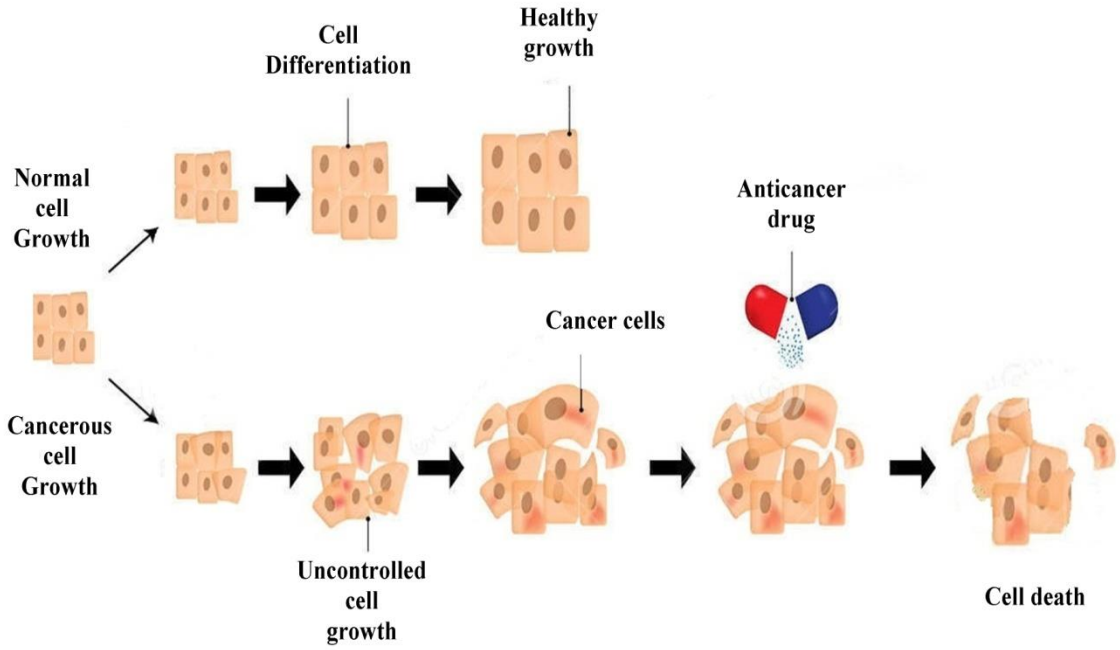


**Figure 1.1** Basics of cancerous growth and chemotherapy

The colon cancer commonly develops from the epithelial cells that line the colon and rectum regions of the gastrointestinal tract. It is due to the conversion of the normal functioning colonic epithelium to adenomatous polyps. Its etiology is known to be a combination of hereditary, environmental, dietary factors and lack of physical activity (Mordechai *et al., 1995*). There are several anticancer agents such as 5-fluorouracil, folinic acid, oxaliplatin available for treating colon cancer. These agents exert their effect by inducing apoptosis of the colon cancer cells. Various lines of evidence suggest that apoptosis provides a protective mechanism against neoplasia by removing genetically damaged stem cells from the epithelium before they can undergo clonal expansion.

Some compounds present in our diet were found to have this anticancer property. These compounds are naturally occurring and can be easily consumed. These dietary compounds include flavonoids and phenolic compounds (Johnson., 2002). Moreover, the locality of colon cancer in the gastrointestinal track makes it more susceptible to these dietary compounds. In this scenario, research communities

explore more diet-derived compounds to treat colon cancer as the lining epithelial cells are chronically exposed to these dietary agents (Reynolds *et al.,* 1991).

Gallic acid (GA) is one such diet-derived phenolic substance being constantly surveyed. GA is a type of phenolic organic compound found in many plants and food substances. GA is found both free as well as part of hydrolyzable tannins and can be easily freed from gallotannins by oxidation (Stein *et al.,* 2011). Literatures have demonstrated a range of biological activities of GA. These biological properties include anti-fungal, anti-viral, anti-oxidant protecting the cells from oxidative damage, cytotoxicity against colon cancer cells without harming the healthy cells (Locatelli *et al.,* 2011). Besides, GA is used as a remote astringent for internal haemorrhage, albuminuria and diabetes. With respect to the anticancer property, numerous *in vitro* assays have shown that the GA and its derivatives are active against several types of tumor cells (Subramanian *et al.,* 2015).

Particularly, these studies show that GA induced cell death in colon cancer lines COLO 205, HCT 15, HCT 116 (Inoue *et al.,* 1995). However, the mechanism induced by GA against colon cancer is not yet elucidated. Thus, this research proposes to study the activity of GA against HCT-15 colon cancer cells as well as, intends to find the different events associated with apoptotic effect of GA in HCT-15 colon cancer cells.

## 1.2 Statement of the Problem

According to the statistics, colon cancer is one of the leading causes of death in the world. Anyhow, the United States is not left alone as this problem extends its tentacles even in Asian countries. Hence, historically low-incidence Asian countries are embarking themselves as one of the leaders in this cause. Malaysia is not an exceptional case and there is a growing statistics of people affected with colon cancer. According to the National Cancer Registry of Malaysia (NCR) records shows 21,773 Malaysians being diagnosed with cancer but estimates that almost 10,000

cases are unregistered every year. Further, colorectal cancer claims to be the second most common cancer affecting about 2,900 Malaysians each year, mostly above the age of 50 (Quick facts of National Cancer Registry of Malaysia, http://www.cancer.org.my/quick-facts/types-cancer/). Chemotherapy is one of the common choices of treatment followed by the patients all around the world. Even though several anticancer drugs are available for colon cancer, there is no single drug, which can effectively cure colon cancer at all stages. Apart from this, they also exhibit some other side effects after administration. These factors persuade scientists to continuously search for a novel anticancer drug.

All types of cancers are said to have link between the diets we consume. In case of colon cancer, diet plays a major role as the colonic epithelial cells are exposed to diets directly. Because of this reason, scientists explore various natural compounds present in food substances to treat colon cancer. Some studies have already shown that these natural compounds are absorbed by the body and have potential to reduce the risk of colon cancer. The current study deals with examining the growth inhibitory effect of the dietary phenolic phytochemical GA against HCT-15 colon cancer cells as well as on promoting it as a promising candidate in colon cancer treatment.

## 1.3 Objectives

The objectives of the research may be enlisted as follows:

1. To determine the effect of GA against HCT-15 colon cancer cells.
2. To evaluate the events associated with GA induced apoptosis in HCT-15 colon cancer cells.

## 1.4 Scope of Study

The first part of the study deals with evaluating the effect of GA against colon cancer cell line HCT-15. The HCT-15 colon cancer cells are cultured and treated with GA. The reaction induced by the GA treatment in HCT-15 cells is assessed by MTT assay and cell cycle analysis. Along with this, the ability of GA to interrupt the colony formation of HCT-15 is also tested.

The final part of the study deals with determining the various events induced by GA in HCT-15 cells. This is achieved by mentoring the mitochondrial membrane potential fluctuation, estimation of lipid layer organization and determination of reactive oxygen species (ROS) level generated. Finally, the occurrence of apoptosis is confirmed by Yo-Pro-1 staining. The Figure 1.2 shows the scope of the study.

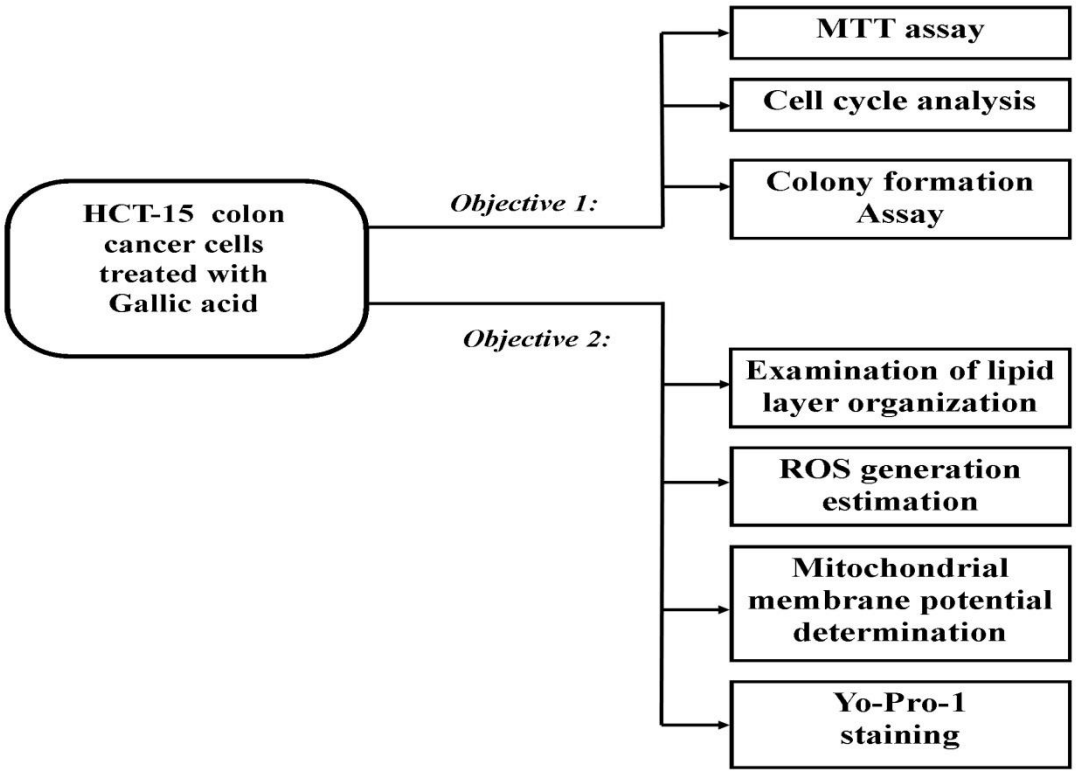


**Figure 1.2** Scope of the study

## 1.5 Significance of Study

The study gives a possibility for the phenolic compound GA to be employed in the treatment of colon cancer cells. The outcome may suggest that GA inhibits the growth of colon cancer cells by inducing apoptosis through ROS generation. Hence, this research will afford a new drug to be used in treating colon cancer. As GA is a compound available in the diets that we consume, the various toxic effects exhibited by the currently available anticancer drugs may be avoided. However, further experiments in preclinical and clinical settings might promote this for medication to cure of colon cancer.

Apart from this, GA may be tested for its effect in combination with other chemotherapeutic drugs available. More molecular level studies in this field would provide ways to combine GA with other drugs already known to be active against colorectal cancer, such as irinotecan and oxaliplatin, (Wheate *et al*., 2010) and improve their effectiveness. Further, it may also lead a way to utilize GA as a potential candidate in drug resistance reversal of colon cancer cells.

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