

**ADDIS ABABA UNIVERSITY**  
**COLLEGE OF HEALTH SCIENCE**  
**SCHOOL OF GRADUATE STUDIES**  
**DEPARTMENT PHARMACOLOGY**



**CHEMO PREVENTIVE POTENTIAL OF *COFFEE ARABICA* IN  
COLORECTAL CANCER INITIATED AND PROMOTED BY DMH IN  
RAT MODEL.**

**BY SHEWATATEK GEDAMU**

**A THESIS PAPER SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES,  
DEPARTEMENT OF PHARMACOLOGY, ADDIS ABABA UNIVERSITY IN PARTIAL  
FULFILLMENT OF THE REQUIRMERNT FOR THE DEGREE OF MASTERS IN  
PHARMACOLOGY**

**April, 2015**

**Addis Ababa, Ethiopia**

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**April, 2015**

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## **Dedication**

I dedicate this work with deepest love and affection to my Queen, Eliam Shewatatek. Her radiant love continues to bring out the best in me. My cute, never I satisfy hugging you but do not forget that I have not yet got enough chance to do so; time is coming very soon so that we may remain in close love. Thank you for being just the way you are!!!

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## Abstract

Colorectal cancer (CRC) is a malignant tumor recognized as a major cause of morbidity and mortality throughout the world. Epidemiological and experimental studies unveiled the importance of compounds derived from plants in reducing the risk of CRC. Recent meta-analyses demonstrate inverse associations between coffee intake and CRC. The aim of the present study was to evaluate chemopreventive potential of *Coffea arabica* in DMH-induced colorectal carcinogenesis in the rat model through a two-phase study (initiation and post-initiation). Thirty five female wistar rats were divided into seven equal groups. Rats in group I-VII except group VI (received normal saline alone) were given freshly prepared DMH (20 mg/kg body weight, ip) in normal saline and pH adjusted (7.0) in 1mM NaOH, once a week for 5 weeks. Groups II and III were received additional oral dose of *Coffea arabica* (20 mg/kg and 40 mg/kg body weight, respectively) in the initiation phase. Group IV and V were received the same dose of *Coffea arabica* given in group II and III, respectively) in the post-initiation phase. Group VII was received low dose aspirin orally. After 10 weeks of treatment period, blood was withdrawn for serum biochemical analysis, then animals were scarified and their colons were subjected to macroscopic and microscopic studies. Taken together, the result of this study showed that well characterized preneoplastic features such as multiple plaque lesions, aberrant crypts and aberrant crypt foci were significantly found in the DMH alone treated group. The numbers were significantly reduced in DMH followed by *Coffea arabica* or Aspirin treated groups. Histologically different degree of dysplasia and hyperplasia observed in DMH treated group. The simultaneous administration of DMH and *Coffea arabica* or Aspirin reduced these features. In addition, our results showed that an appreciable counteracts effect of *Coffea arabica* on serum biomarkers and body weights observed in DMH alone treated rats. The results of this study surmise that the effects of *Coffea arabica* may be due to the presence of phenolic compounds which have antioxidant activities. It brings suppression of cellular proliferation, inhibits lipid profile elevation and interferes with serum protein and body weight declination. Furthermore, this study was provided that *Coffea arabica* treatment, in both phases, tended to considerably suppress tumor progression and invasion. The elucidation of the inhibitory effects of *Coffea arabica* on the progressions and invasion of colorectal preneoplastic lesions is thus of great importance and will provide promising targets for preventive and therapeutic interventions of CRC.

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### **List of acronyms**

- AOM:- Azoxymethane
- ACs:- Aberrant crypts
- ACF:- Aberrant crypt foci
- AP-1:- Activator protein-1
- CaA:- Caffeic acid
- ChA:- Chlorogenic acid
- CAT:- catalase
- COX:-Cyclooxygenase
- CRC:- Colorectal cancer
- DFUR:- 5'-deoxy-5-fluorouridine 5'
- DMH:- 1, 2 Dimethyl hydrazine
- DNA:- Deoxyribonucleic acid
- H&E:- hematoxylin and eosin
- 5-FU:- Fluorouracil
- IARC:- International Agency for Research on Cancer
- LD<sub>50</sub>:- Lethal dose of 50%
- LP:- lamina propria
- MPLs:- Multiple Plaque Lesions
- MM:-muscularis mucosa layer
- NaCl:-Sodium chloride
- NaOH:- Sodium hydroxide
- NF:- Nuclear factor
- NSAIDs:- Non-steroidal anti-inflammatory drugs
- PGE<sub>2</sub>:-Prostaglandin E<sub>2</sub>
- P<0.05:-Significance of prevalence<0.05
- ROS:-Reactive oxygen species
- SEM:-Standard error mean
- SM:-submucosa layer
- SOD:-Superoxide dismutase
- SPSS:- Statistical software Package for social science

## Operational definition

- **Aberrant crypts (ACs):-** Crypts that were 2 to 3 times larger than the surrounding normal crypts.
- **Aberrant crypt foci:-**Aggregations of abnormal crypts composed of one or multiple enlarged crypts elevated above the surrounding mucosa.
- **Multiple Plaque Lesions:-** Lesions of the mucosal surface recognized as either raised or non-raised lesions with identifiable tissue growth
- **MPL incidence:** - the percentage of animals having MPLs.
- **MPL burden:** - the total number of MPLs counted/total number of rats.
- **MPL multiplicity:** - the total number of MPLs counted/number of MPL bearing rats.
- Initiation:** - Exposure of normal cells to carcinogenic agent, which result in change(s) at the genomic level that imparts selective growth advantage to the cells.
- **Promotion (post-initiation):** - clonal expansion of the initiated cells, which is generally associated with altered morphologic and/or phenotypic changes.



## **1. Introduction**

### **1.1. Background**

Colorectal cancer (CRC) is a malignant tumor recognized as a major cause of morbidity and mortality throughout the world (1). It is the third most common cancer worldwide and the fourth most common cause of death (2). According to the International Agency for Research on Cancer (IARC), the most recent from 2008, estimates that CRC is the fifth most common cancer in Sub-Saharan Africa (3).

Colorectal cancer, however, is not uniformly common throughout the world. It is mainly a disease of developed countries with a western culture(4). In fact, the developed world accounts for over 63% of all cases (5). Colorectal adenomas/polyps are extremely common in the western world, occurring for approximately 20% of people over the age of sixty(6).The incidence rate of CRC ranges from more than 40 per 100,000 people in the United States, Australia, New Zealand, and western Europe to less than 5 per 100,000 in Africa and some parts of Asia (2) . In Syria, the incidence and mortality rates of CRC are increasing at an alarming rate in recent decade, being the second most prevalent type of cancer in males and females (7). Historically, CRC death rates were higher in individuals with higher rather than lower socioeconomic status and in whites than in blacks (8–10).CRC is the third leading cause of cancer death in both men and women in the United States (11). According to the International Agency for Research on Cancer (IARC), it is estimated that by the year 2020 the global cancer burden will reach 16.8 million cases and CRC becomes one of the leading burden. These data suggest that CRC prevalence is on the rise and that it is rapidly becoming a global pandemic (12). Cancer is an emerging public health problem in Africa (3).

According to the International Agency for Research on Cancer, about 715,000 new cancer cases and 542,000 cancer deaths occurred in 2008 in Africa (3).These numbers are projected to nearly double (1.28 million new cancer cases and 970,000 cancer deaths) by 2030 (13). Incidence rates remain highest in more developed regions, but mortality is relatively much higher in less developed countries due to a lack of early detection and access to treatment facilities (14). Factors that contribute to CRC disparity are complex and multifactorial (15). Differences in

income, education and geographic residence between socio demographic groups result in inequalities in the prevalence of CRC risk factors and in access to screening and treatment services(16).Screening for CRC is known to not only help detect the cancer in its early stages, and therefore improve chances of a curative treatment, but also to detect precancerous lesions which, if removed successfully, can avoid more expensive and radical treatments (17). Racial disparities in CRC death rates may be explained by differences in the use, availability, and quality of screening and treatment services (18,19). There are several factors considered to be causally associated with the development of colorectal cancer. For instance, the risk of colorectal cancer is clearly increased by a Western diet and some inherited genes responsible for colorectal cancer have also been identified (6). Most colon carcinomas, besides their multiple assaults by various exogenous/endogenous carcinogenic compounds, develop as a result of genetic familial background. It is well established that colon cancer may be ameliorated when risk factors such as life-style and environmental factors are modified (20).

CRC has been postulated as a complex and multistage process well established in both humans and experimental models. Development of rodent and human colon cancer includes a series of pathological alterations ranging from discrete microscopic mucosal lesions like aberrant crypt foci (ACF) to malignant tumors (21). It is believed that there are two pathogenetically distinct pathways for the development of colon cancer (22). The first pathway is characterized by chromosomal instability resulting in stepwise accumulation of mutations in a series of oncogenes and tumor suppressor genes. The molecular evolution of colon cancer in this pathway can be seen through a series of morphologically identifiable stages: initial localized colonic epithelial hyperplasia, followed by formation of adenomas that progressively enlarge and ultimately develop into invasive cancers i.e. the adenoma-carcinoma sequence. The second pathway is characterized by genetic lesions in DNA mismatch repair genes (microsatellite instability). All the above mentioned series of genetic alterations destroy normal mechanisms controlling cellular growth, leading to abnormal cellular proliferation which is one of crucial mechanisms in carcinogenesis (23).

1, 2 Dimethyl hydrazine (DMH), methylnitrosurea, N-methyl-N'-nitro-N-nitrosoguanidine are a potent colon carcinogen, inducing colorectal tumors in experimental animals and is the most widely used model of chemically induced colon carcinogenesis (24,25). DMH is metabolically activated to the active carcinogen in the liver to form azoxymethane and methylazoxymethanol, which is further transported to colon to generate its carcinogenic metabolite, diazonium ion, which produces free radicals that induce oxidative DNA damage in the colon. The diazonium ion elicits an oxidative stress by methylating biomolecules of colonic epithelial cells and leads to promutagenic events as a result of inflammation and tumor promotion(26). Colon carcinogenesis models using DMH or the related azoxymethane, with putative preneoplastic aberrant crypt foci as end-point marker lesions have been used to assess the influence of modulatory factors(27).

DMH induced CRC is a multistep process involving a series of pathological alterations (28). Experimental Colon carcinogenesis involving three distinct stages, initiation, that alters the molecular message of a normal cell, followed by promotion and progression that ultimately ends up with a phenotypically altered “transformed cell”(29). ACF are recognized preneoplastic morphological putative lesions of colonic neoplasia in rodents and humans. During the process of colon carcinogenesis, ACF appears in the early stages and subsequently develop into polyps, adenomas and eventually carcinomas (30). Aberration in epithelial colonic crypt cell proliferation leads to hyperplasia is higher risk of colon cancers both in humans and experimental animal models (31). Hence ACF development is commonly used as a parameter for assessment of colorectal cancer even in animals (32). Epithelial cells affected by the abnormal proliferation under genetic influence, leading to the creation of new clones, unrecognized by suppressor genes that probably are so damaged that they are unable to recognize the changes at the level of DNA, so that now new, different cells produce new cells that will be used to form tumor. Histological analysis of the tissue sample from the upper third of the crypt, see enhanced proliferation activity in neoplastic lesions (33). Colonic adenoma is a particular type of lesion that is prone to develop into adenocarcinoma, a malignant tumor that constitutes the majority of colorectal cancer cases (34).

Some molecules have been recommended as CRC markers, such as serum total protein and lipid profile. Some studies showed that, treatment with DMH reduced the serum total protein concentration (35). In most cohort studies also, serum triglyceride (36,37) and cholesterol (38) levels are positively related to an increased risk of colorectal adenoma, while several investigators report an insignificant or even inverse relationship between serum lipids and colorectal adenoma (39). The occurrence of CRC and dyslipidemia is rising dramatically in both developed and developing countries (40).

Besides the DMH-target tissue colon, the liver was preferentially selected for assaying the biotransformation and detoxification pattern (41,42). Many chemical changes of the liver are detectable prior to the onset of secondary pathological and nutritional changes associated with conditions such as neoplasia. The pathological alterations in the liver often act as an indicator of overall damage caused by a carcinogen since liver enzymes provide more sensitive indicators of pathogenesis (43).

## **1.2. Treatments of Colorectal cancer**

The vast majority of cases and deaths from colorectal cancer can be prevented by applying existing knowledge about cancer prevention. Appropriate dietary changes, regular physical activity, and maintenance of healthy weight, together with targeted screening programs and early therapeutic intervention could, in time, substantially reduce the morbidity and mortality associated with colorectal cancer (6).

Although complete removal of causative agents of cancer may not always be possible, chemoprevention of cancer using antimutagens and anticarcinogens present in foods (44,45) or natural products (herbs and spices) has been suggested by various studies and much effort has been dedicated to a search for natural preventive agents, which would block or attenuate colorectal carcinogenesis process (46–48). The formation and growth of ACF are associated with the induction of colon tumors in rats and are influenced by exposure to chemopreventive agents (49–51).

Current medical treatment in colon cancer consists of chemotherapy, hormonal treatment and targeted therapies (like surgery and radiation therapy). They are sophisticated, expensive and not widely available. Therefore, a search for novel anticancer agents from natural products may provide an alternative and cost-effective treatment modality (52). At present, the treatment for colon cancer remains primarily surgical. Colon cancer is resected either through an open or minimally invasive approach following standard oncological principles that have withstood the test of time (53,54).

The most widely used agent in the treatment of metastatic colorectal cancer is fluorouracil (5-FU), which was developed more than 40 years ago and is included in most regimens of palliative chemotherapy for colorectal cancer (55–57).Capecitabine an oral fluoropyrimidine carbamate, was rationally designed to generate 5-FU predominantly within tumor cells (58,59). After rapid and extensive absorption as an intact molecule, capecitabine is converted to 5-FU predominantly in tumor tissue by exploiting the high activity of thymidine phosphorylase in malignant tissue (59). The enzymatic conversion of capecitabine occurs in three steps. In the first stage, capecitabine is hydrolyzed by hepatic carboxylesterase to 5'-deoxy-5-fluorocytidine. This intermediate is then converted to 5'-deoxy-5-fluorouridine (5'-DFUR) by cytidine deaminase in tumor cells and the liver. The third and final step involves the conversion of 5'-DFUR to 5-FU by thymidine phosphorylase and occurs predominantly in tumor tissue as result of the high activity of thymidine phosphorylase (60).The increasing specificity for tumor cells occurring with each successive conversion step potentially reduces systemic 5-FU exposure while increasing the 5 FU dose within tumor tissue. The tumor selectivity of capecitabine has been confirmed in patients with colorectal cancer (61).

### **1.3. The need of anti colorectal cancers drug development**

The need of anti cancers drug development might be a root cause for chemoprevention stems from epidemiological evidence that some factors in the diet may play important roles in its development, where others may reduce the risk (30,62). Epidemiological and experimental studies unveiled therapeutic potential of many culinary herbs and demonstrated the importance of compounds derived from plants in reducing the risk of colon cancer (63).

Most chemopreventive agents known until today are plant extracts which used to inhibit; the initiation step by preventing carcinogen activation and malignant cell proliferation during promotion and progression steps of carcinogenesis (64). Natural compounds that inhibit ACF induced colon carcinogenesis have proved to be protective against colon cancer in rodents (65). Plants have been used as an age old remedy of cancer history of use in the treatment of cancer (66).

#### **1.4. Effect of *Coffea arabica* on colorectal cancer**

Coffee, one of the most important beverage crops in the world and a valuable agricultural export commodity, is cultivated in over 80 countries (67). About 7.2 billion tons of coffee was harvested from 10.4 million hectare land in 2003 (68). Arabica coffee accounts for about 70% of the world coffee production and known for the preparation of high quality beverage (69). It is the only tetraploid ( $2n = 4x = 44$ ) and self-fertile (over 95%) species in the genus *Coffea* (70) as well as the most widely cultivated and the longest known coffee species (71). *Coffea arabica* is native to the highlands of Southwest Ethiopia (72). It is cultivated almost throughout the country (73). Several researchers reported the presence of phenotypic variation among *Coffea arabica* genotypes grown in Ethiopia (74,75).

Coffee is among the most widely consumed beverages in the world. More than 50% of Americans drink coffee daily. Epidemiological studies suggest that coffee consumption might lower the risk of type 2 diabetic (76). Recent meta-analyses demonstrate inverse associations between coffee intake and colon, liver, breast and endometrial cancer risks (77–80). Results of epidemiological studies have not resolved whether coffee consumption is related to colorectal cancer risk. A report by the World Cancer Research Fund concluded that the available evidence was not sufficient to draw any firm conclusions about a decreased risk of colorectal cancer associated with coffee (81). However, some researchers contend that a link between consumption of coffee and a low incidence of colorectal cancer has been firmly established (82,83). In a prospective population-based cohort study, consumption of at least one cup of coffee per day, compared with no coffee, was associated with a 49% lower risk for upper gastrointestinal cancers (84).

A number of case control studies have demonstrated reduced risk of colorectal cancer with coffee consumption (85). In a review, Tavani and La Vecchia showed that not only was there no risk of colon or colorectal cancer with caffeinated beverages, but there may even be a protective effect (86). A study by Michels et al. also showed that there is no association between development of rectal cancer and consumption of caffeinated beverages (87).

Coffee is a leading source of methylxanthines, such as caffeine (88); thus, caffeine can be found in micromolar concentrations in the human circulation as a result of dietary intake or pharmacological use (89). Coffee also is a rich source of dietary phenolic phytochemicals, including caffeic acid (CaA; 3,4-dihydroxycinnamic acid) and chlorogenic acid (ChA; 5-O-caffeoylquinic acid). The ChA content of a 200 ml cup of coffee ranges from 70 to 350 mg, which would provide about 35–175 mg of CaA. Previous studies suggested that ~33% of ingested ChA and 95% of CaA are absorbed intestinally (90). Thus, about two thirds of ingested ChA reaches the colon where it is probably metabolized to CaA (91). Bioavailability data suggest that the biological effects of ChA would become apparent after its metabolism to CaA, and hence studying the effects of CaA is necessary. ChA was reported to reduce chemical carcinogenesis in animals (92,93). It was shown to inhibit lipid peroxidation-induced DNA adduct formation (93) and to suppress reactive oxygen species-mediated nuclear factor (NF)- $\kappa$ B, activator protein-1 (AP-1) and mitogen-activated protein kinase activation by upregulating antioxidant enzymes (94).

The development of novel plant derived natural products and their analogs for anticancer activity details efforts to synthesize new derivatives based on bioactivity and mechanism of action directed isolation and characterization coupled with rational drug design – based modification. Based on the above facts the purpose of the present study was to evaluate chemopreventive effects of *Coffea arabica* (in vivo) in the rat model of DMH-induced colonrectal cancer and to evaluate its roles in suppressing cellular proliferation, inhibiting tumor growth and in obstructing tumor progression and invasion through a two-phase-study: an initiation phase and promotion phase.

## **2. Objective of study**

### **2.1. General objective**

To evaluate chemopreventive effects of *Coffea arabica* in the rat model of colorectal precancerous lesions induced and promoted by DMH.

### **2.2. Specific objectives**

- ✓ To assess the inhibitory effect of coffee on grossly visible variations (weight loss and MPLs) produced by DMH in different treatment groups.
- ✓ To identify the aberrant crypt foci cells produced by DMH in different treatment groups
- ✓ To identify the histological variation produced by DMH among treatment groups of rats.
- ✓ To determine the blood markers (total protein and lipid profile) variation among the treatment groups of rats.

### **3. Materials and methods**

#### **3.1. Chemicals and Reagents**

1,2- Dimethylhydrazine dihydrochloride (ACROS organics), sodium chloride (Merck KGaA), sodium hydroxide (NaOH) (Lobachemie PVT.LTD), methylene blue (MERCK), formalin(10%, Reagent chemical services limited), sodium di-hydrogen phosphate, monohydrate (NaH<sub>2</sub>PO<sub>4</sub>. H<sub>2</sub>O) (BDH chemical Ltd), and anhydrous di-sodium hydrogen phosphate, anhydrous (Na<sub>2</sub>HPO<sub>4</sub>) (BDH chemical Ltd), filter paper (Whatman No 3, Whatman Ltd., England) were used. All chemicals and reagents used in the present study were analytical grade available and were obtained commercially.

#### **3.2. Collection and preparation of plant materials**

The fine powder of roasted Coffee seeds (*Coffea arabica*) were obtained from local commercial Supplier (Tomoka coffee) in Addis Ababa, Ethiopia. The successive aqueous extract of Coffee was prepared by adding 70 g of powder to 1000 ml of boiling water under reflux for 10 minutes, allowed to stand at room temperature for 30 minutes. After the extraction, the solution was filtered using filter paper (Whatman No 3, Whatman Ltd., England) to collect the extract and the solvent was lyophilized by lyophilizes machine. The resultant extract was dehydrated in an oven at 50°C for 24 h (95). The dose was obtained on dry matter and the percentage yield was found to be 20.3% (w/w).

#### **3.3. Dosing of extract**

The dose selection was calculated based on the corresponding amount consumes by an adult person and the dose used produce anti diabetic effect. The adult person who weigh 70kg consumes on average 2 small cups of coffee daily (about 100ml/day), this amount contain about 6-8gm of *coffee arabica* powder. Therefore, the average dose for human would be 6000-8000mg/ 70 kg of body weight (96). The dose used to produce anti oxidant effect in alloxan induced diabetic rat was 43mg/kg, as it may be linked with the anti- anti oxidant activity of coffee against cancer (97).Accordingly, doses of 20mg/Kg of body weight and 40mg/kg of body weight were considered. The rats were expected to tolerate the doses, because a preliminary

study of 15 days found no acute toxicity and the LD50 was greater than 1000 mg/kg. Dose was calculated for individual rats based on their weekly weight which was taken at the beginning of each week. The dried extract was re-constituted in normal saline (0.9% NaCl). A 50 ml stock solution was freshly prepared every day for the entire experiment. The rats were given 1ml of coffee powder orally every day for ten weeks at a dose of 20 mg/Kg and 40mg/Kg of body weight accordingly.

### **3.4.Preparation of Carcinogen**

1, 2-Dimethylhydrazine dihydrochloride (DMH) was dissolved in normal saline solution (0.9% NaCl); the pH was adjusted to 7.0 with 1 Mol/L NaOH to ensure the pH suitability and the stability of the chemical. The final solution of the carcinogen was used immediately after preparation. DMH was injected interaperitoneal (i.p.) at a dose of 20 mg/kg/body weight once a week for five consecutive weeks on the ventral front of the animal (98).

### **3.5.Animals**

Female Wistar rats of body weight between 180-270g were obtained from the Animal House of Ethiopian public health institute (EPHI) and maintained in pharmacology department of Addis Ababa University animal house. They were acclimatized with standard laboratory diet and water *ad libitum* for one week. Animals were maintained as per the principles and guidelines of the Ethical Committee of the Animal Care of the department in accordance with the Helinskiy II law on animal care and use. The protocol was approved by the institutional ethics committee. The animals were housed 5 per cage in polypropylene cages with a wire mesh top and a hygienic bed of husk (regularly changed every 3 days) in a well ventilated animal room till the end of the experimental period, and the room temperature was maintained at  $25 \pm 1^{\circ}\text{C}$  with  $55 \pm 5\%$  humidity. The animals were also maintained under a 12hr photoperiod of light and darkness, respectively. Body weights were recorded every week.

### **3.6.Experimental design**

After one week of acclimatization the thirty five rats were randomly assigned into seven groups. All animals (group I-VII) except (group VI) were received intraperitoneal injections of DMH (a highly specific colorectal carcinogen in rats) at a dose of 20 mg/kg body weight once a week for 5 consecutive weeks. Animals in Group I: (n =5) served as carcinogen controls or positive controls. They were given intraperitoneal injections of DMH once a week at a dose of 20 mg/kg body weight for 5 consecutive weeks (DMH alone group). Group II (n = 5) served as pre initiation low dose coffee treatment group or DMH and low dose coffee initiation group. They were received the same DMH treatment as in group I and simultaneously gavaged coffee at a daily dose of 20 mg/kg body weight( starting two weeks before DMH injection) for the whole 10 weeks of the experiment period . Group III (n = 5) served as pre initiation high dose coffee treatment group or DMH and high dose coffee initiation group. They were subjected to the same protocol as in group II except a daily dose of coffee in this group was 40 mg/kg body weight (starting two weeks before DMH injection) for the whole 10 weeks period of the experiment. Group IV (n = 5) served as promotion low dose coffee treatment group or DMH and low dose coffee post initiation group. They were subjected to the same carcinogen protocol as in group III and subsequently gavaged low dose coffee at a daily dose of 20 mg/kg/body weight (starting two weeks after DMH injection) during the last post-initiation weeks of the experimental period. Group V (n = 5) served as promotion high dose coffee treatment group or DMH and high dose coffee post initiation group. They were subjected to the same protocol as in group IV except a daily dose of coffee in this group was 40 mg/kg body weight (starting two weeks after DMH injection) during the last post-initiation weeks of the experimental period. Group VI animals: (n = 5) served as negative control group or normal control group. Animals in this group represent the normal untreated controls and received 0.5 ml of normal saline vehicle instead of DMH intraperitoneally, once a week and the same dose of vehicle orally, throughout the entire course of experimental study. Animals in Group VII (n = 5) served as Aspirin group or standard drug group. They were gavaged aspirin at a daily dose of (6 mg/kg body weight) for the whole period of the experiment. Animals were also weighed weekly till the termination of the treatment period.

### **3.7.Experimental protocol**

#### **3.7.1. Biochemical Analysis**

At the end of the experimental period, the rats were fasted overnight and subjected to diethyl ether anesthesia and blood samples were collected by hurt puncture. Blood samples were allowed to clot for 30 minutes at room temperature before centrifuging for 10 minutes at 4000 rpm (according to the instructions of the ELISA kit). Serums were removed, aliquoted and stored at  $-80^{\circ}\text{C}$  until they were assayed. Serum levels of total Cholesterols, total triglyceride and total proteins were determined using different enzyme-linked immunosorbent assay (ELISA) kit accordingly. Total protein (gm/dl) was estimated according to the method of Bradford, (99) using Bio diagnostic kits, Egypt. Total lipid (mg /dl) was estimated according to the method of Knight, *et al.*, (100) using Bio diagnostic kits, Egypt.

#### **3.7.2. MPLs, ACF and Histopathological analysis**

After blood samples were withdrawn, the rats were sacrificed by cervical dislocation. Then, the colon were excised, flushed with saline, cut open longitudinally along the main axis, and then again washed with saline. The inner surface was examined for visible macroscopic lesions. Tumors/polyps were easily discernible in the inflamed section of the colon. Colon sections of equal length and tumors were examined grossly for the location and number (101). These colonic sections fixed in 10% formalin for 24 h. Topographic analysis of the colonic mucosa according to Bird's procedure aberrant (102) was done after 24 h fixation. Colons were stained with 2% methylene blue solution for 10 min, placed mucosal side up on a microscopic slide, and examined microscopically using a low power objective at 10x magnifications. The total number of ACF in the entire colon was determined starting from the distal to the proximal end of the colons. Aberrant crypts were distinguished from the surrounding normal crypts by their increased size, increased distance from lamina to basal surfaces of cells, and easily discernible pericryptal zone. The variables used to assess the aberrant crypts were mucosal plaques or nodular lesions incidence, burden and multiplicity (103). Crypts or distinct foci of crypts were counted as an ACF if they displayed at least two of the following characteristics-(i) occupy a greater area than surrounding crypts; (ii) have a thickened epithelial lining; (iii) have elongated or altered shape of luminal opening; and (iv) have an increased pericryptal zone separating the

crypt or foci from surrounding crypts (104). Histopathological examination of colorectal tissue was done after the colonic sections fixed in 10% buffered formalin for at least 24 h. After fixation the specimens washed in tap water, and then serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56° in hot air oven for 24 h. Paraffin bees wax tissue blocks were prepared for sectioning transversely at 4 µm by sledge microtome. The obtained tissue sections were collected on glass slides, deparaffinized and stained by eosin and hematoxylin stain and examined through the microscope (105).

### **3.8. Ethical considerations**

All the animals' procedures were performed in accordance with the standard guidelines for care and use of laboratory animals. The protocol was approved by Addis Ababa University, college of health sciences, school of medicine, and department of pharmacology Ethical Committee on the use of the experimental animals for biomedical research.

### **3.9. Statistical analysis**

All statistical analyses were performed using the statistical software for social science (SPSS), version 21 for windows (SPSS inc., Chicago, Illinois, USA). The results were expressed as mean ± standard error mean (SEM) for each group. Statistical differences between groups analyzed by One-way analysis of variance (ANOVA) followed by the Tukey post hoc test. Statistical significance was accepted when  $P < 0.05$

## 4. Results

### 4.1. Effect on body weight

In this study, it was found that the body weight of the rats in DMH alone treatment groups did not appreciably decrease when compared with other groups, for the first two weeks but by the end of the experiment period, final mean body weight of the rats in DMH alone treatment group was significantly lower compared with the other groups ( $P < 0.05$ ). In animals undergoing treatment with DMH and different dose of *coffee arabica* (both DMH with *coffee arabica* initiation and DMH with *coffee arabica* post-initiation group), maintained near normal body weights when compared to each other. When compared to the normal control group body weights of the rats in these groups were decreased; however, the reductions were not statistically significant. On the contrary, when compared with the DMH group, average body weight of *coffee arabica* treated groups was significantly higher. In this study no significant change in the body weight were observed between the normal Control and the *coffee arabica* treated groups. Animals in Aspirin control group showed body weights close to those observed in normal control group (Table 1).

**Table 1. Comparison of Body weight in *Coffee arabica* and Aspirin treated rats after DMH-induced colon carcinogenesis for ten weeks**

Group	Body Weigh at week 2 (gm)		Final body weight(gm) <sup>a</sup>	
	Mean	SEM	Mean	SEM
DMH	188.00	3.00	204.67 <sup>b</sup>	.97
Post initiation low dose	198.00	3.39	224.00 <sup>c</sup>	4.17
Post initiation high dose	196.00	5.79	222.00 <sup>c</sup>	5.25
Pre initiation low dose	198.00	5.61	223.00 <sup>c</sup>	6.16
Pre initiation high dose	197.00	7.35	221.33 <sup>c</sup>	6.96
Normal Saline	199.00	1.87	229.67 <sup>c</sup>	1.62
Asprin	193.00	4.64	224.00 <sup>c</sup>	4.61

Data is expressed as Mean  $\pm$  SEM (n=5). a:  $p < 0.05$  ( $p = .033$ ) when compared the significance between groups ; b:  $p < 0.05$  when compared to the Controls; c:  $p < 0.05$  when compared to DMH by one way ANOVA.

## 4.2. Morphological and histopathological studies

### 4.2.1. Multiple Plaque Lesions

In the ten weeks study, the gross anatomy of the colorectal mucosal surface was depicting the occurrence of multiple plaque lesions (MPLs). MPLs were recognized as either raised or non-raised lesions with identifiable tissue growth in carcinogen treated animals, often appearing singly or in multiple forms throughout the length of the colon. The proportion of rats that develop MPLs in the colon was 100% in DMH treatment group, while co-administration with different concentration of *Coffee arabica* in initiation phase and promotion phase reduced the incidence to 40% and 60% respectively. Incidence of MPLs in Aspirin treated group was the same to that of animals treated *Coffee arabica* in initiation phase. Colons from control only groups didn't show any such feature. This incidence of MPLs in DMH treatment group was significantly elevated ( $P < 0.05$ ) when compared with the normal control and other treatment groups. Differences in MPLs incidence were observed in different concentration *Coffee arabica* treated groups in initiation and promotion phase: however, the difference was not statistically significant ( $P > 0.05$ ). Maximum numbers of MPLs proportion (6.0) were observed in DMH alone treated group. A decreased in MPLs burdens (1.6, 1.4, 1.8, and 2.0) however, were observed in different *Coffee arabica* (Low and high dose initiation and the same dose promotion group) treated animals respectively, but the number was found much less (1.2) in Aspirin treated group. The differences in MPLs burdens were statistically significant ( $P < 0.05$ ) in DMH treated animals when compared with *Coffee arabica* or Aspirin treated groups. But the differences among *Coffee arabica* or Aspirin treatment groups were not statistically significant ( $P > 0.05$ ) (Table 2).

**Table 2. Chemopreventive response of *Coffea arabica* and Aspirin in terms of total multiple plaque lesions (MPLs), MPL incidence, MPLs burden and MPLs multiplicity in DMH-induced colon carcinogenesis for ten weeks**

Animal groups	Total <sup>a1</sup> MPLs	Rats with MPL	MPLs incidence (%)	MPLs <sup>a1</sup> burden	MPLs <sup>a2</sup> multiplicity
DMH	30 <sup>b</sup>	5	100 <sup>b</sup>	6 <sup>b</sup>	6
Post initiation low dose	9 <sup>c</sup>	3	60	1.8 <sup>c</sup>	3 <sup>c</sup>
Post initiation high dose	10 <sup>c</sup>	3	60	2 <sup>c</sup>	3.3 <sup>c</sup>
Pre initiation low dose	8 <sup>c</sup>	2	40	1.6 <sup>c</sup>	4 <sup>c</sup>
Pre initiation high dose	7 <sup>c</sup>	2	40	1.4 <sup>c</sup>	3.5 <sup>c</sup>
Normal Saline	0 <sup>c</sup>	0	0 <sup>c</sup>	0 <sup>c</sup>	0 <sup>c</sup>
Aspirin	6 <sup>c</sup>	2	40	1.2 <sup>c</sup>	3 <sup>c</sup>

Data is expressed as Mean  $\pm$  SEM (n=5). a:  $p < 0.05$  ( $a_1 = 0.000, a_2 = 0.018$ ) when compared the significance between groups ; b:  $p < 0.05$  when compared to the Controls; c:  $p < 0.05$  when compared to DMH by one way ANOVA.

**Calculation of MPLs (Multiple Plaque Lesions).**

MPL incidence= the percentage of animals having MPLs.

MPL burden= the total number of MPLs counted/total number of rats.

MPL multiplicity= the total number of MPLs counted/number of MPL bearing rats (106).

#### 4.2.2. Aberrant crypts foci

Aberrant crypts (ACs) were identified as the crypts that were larger than the surrounding normal crypts; with increased methylene blue staining due to a thickened layer of epithelial cells; slit-shaped lumina and microscopically elevated above the plane of normal crypts. ACFs were discrete aggregations of abnormal crypts. It was characterized as lesions composed of one or multiple enlarged crypts elevated above the surrounding mucosa and are considered as putative preneoplastic lesions of the colon.

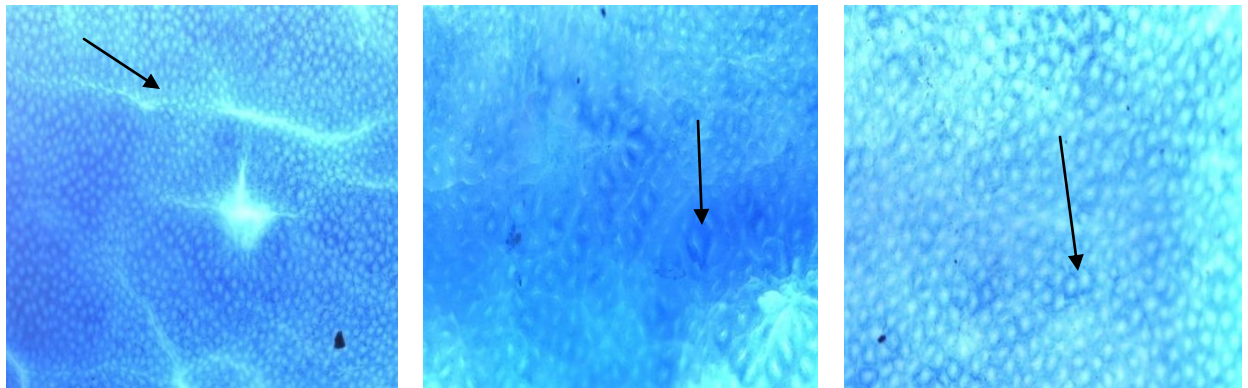
**Table 3. Chemopreventive response of *Coffea arabica* and Aspirin in terms of aberrant crypts (ACs) and aberrant crypt foci (ACF) in DMH-induced colon carcinogenesis for ten weeks**

Animal groups	AC/colon <sup>a</sup>		ACF/colon <sup>a</sup>	
	Mean	SEM	Mean	SEM
DMH	407.6 <sup>b</sup>	40.0	150.2 <sup>b</sup>	13.8
Post initiation low dose	148.6 <sup>c/b</sup>	13.4	57.0 <sup>c/b</sup>	9.4
Post initiation high dose	131.2 <sup>c/b</sup>	16.6	50.8 <sup>c/b</sup>	10.3
Pre initiation low dose	105.8 <sup>c/b</sup>	17.5	41.4 <sup>c/b</sup>	9.7
Pre initiation high dose	70.8 <sup>c/b</sup>	14.2	28.4 <sup>c/b</sup>	8.5
Normal Saline	0.0 <sup>c</sup>	0.0	0.0 <sup>c</sup>	0.0
Asprin	71.8 <sup>c/b</sup>	11.6	28.2 <sup>c/b</sup>	7.1

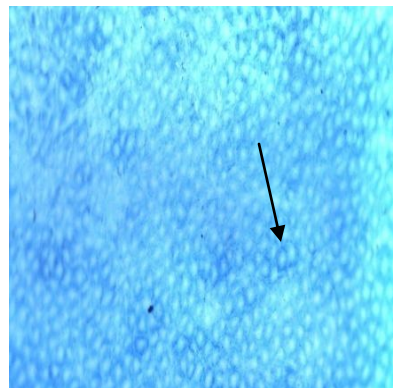
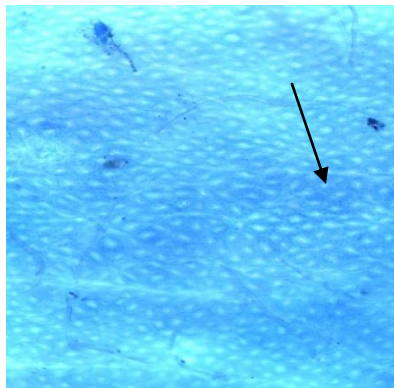
Data is expressed as Mean  $\pm$  SEM (n=5). a:  $p < 0.05$  ( $p = .000$ ) when compared the significance between groups ; b:  $p < 0.05$  when compared to the Controls; c:  $p < 0.05$  when compared to DMH by one way ANOVA.

The average numbers of ACs and ACF per colon were observed at the end of the experimental period. The ACs and ACF of each animal were recorded. The results are depicted as mean  $\pm$  SEM of five rats from each group and one way ANOVA was done to compare the means between the different treatments using Post-Hoc comparison by Tukey test method. In the present study ACs and ACF frequencies were significantly higher in the DMH treated animals. Colonic mucosa from control only group was free from aberrant crypts. Simultaneous administration of *Coffea arabica* with DMH reduced these features.

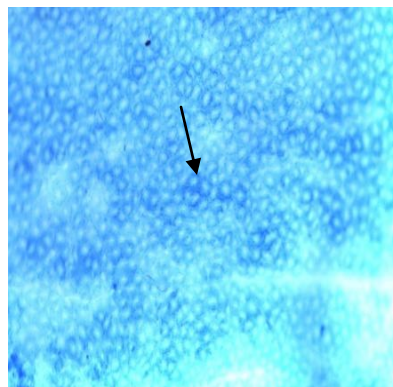
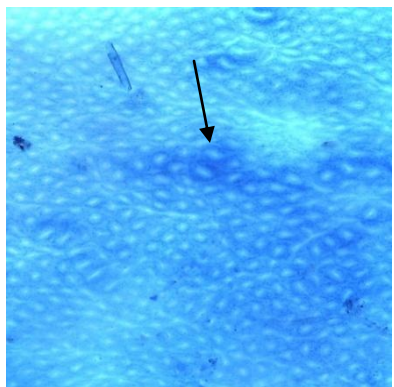
The effect of *Coffee arabica* on the development of ACF, induced by DMH was identified during the study period. There was a remarkable decrease in the frequency of ACs and ACF from  $407.6 \pm 40$  and  $150.2 \pm 13.8$  in DMH alone group to  $70.8 \pm 14.2$  and  $28.4 \pm 8.5$  (ACs and ACF respectively) in *Coffee arabica* treated animals. In Aspirin treated rats also, the same regularity of ACs and ACF ( $71.8 \pm 11.6$  and  $28.2 \pm 7.1$ ), which was shown in *Coffee arabica* treated groups was observed, with the crypts assuming normal features (Table 3). An increase in the mean number of ACs and ACF for the DMH group was significant ( $P < 0.05$ ) when compared with *Coffee arabica* or Aspirin treated rats. There were no observable foci witnessed in rats of negative control group which had administered normal saline alone *ad libitum* throughout the experimental periods. There was difference in crypt distribution among different groups of *Coffee arabica* treated rats. But the difference was not affected significantly with each other groups ( $P > 0.05$ ). Fig. 1 also showed that the development of colon AC and ACF in different treatment groups during the study period. The distribution of normal colonic epithelial cells and the majority of abrupt crypts in the foci were shown diverse in different treatment groups.



(A) Normal crypt (B) many, very larger crypts & 3-4ACs/ACF (C) few slightly raised crypts



(D) Few slightly raised crypts (E) few slightly raised 2ACs/ ACF



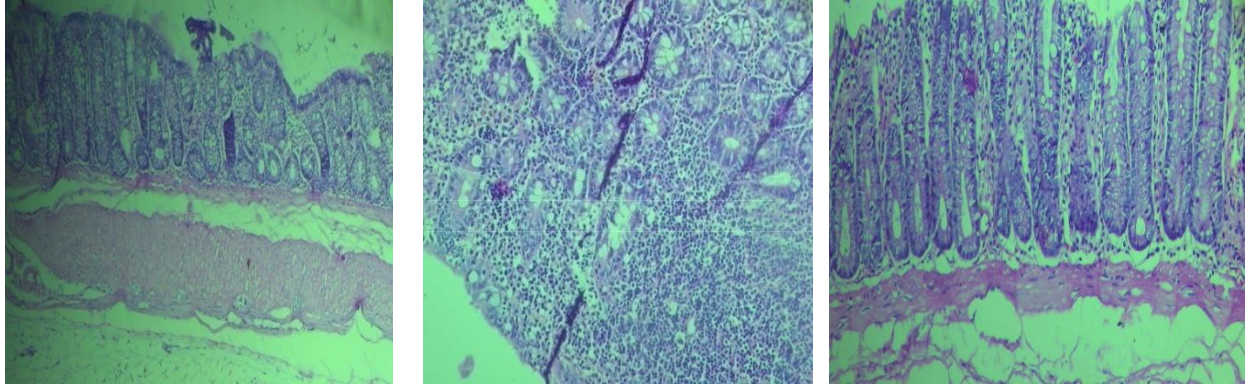
(F) Many raised 2-3ACs/ ACF. (G) few slightly raised 2-3ACs/ ACF

**Figure 1.** Longitudinal section of colonic mucosa stained with 2% methylene blue in low power (10 X) light microscope which includes normal looking colonic mucosa and abrupt crypts with one or multiple foci in different treatment groups; (A) Normal Saline group, (B) DMH only treated groups, (C) Aspirin treated group, (D) Pre Inhibition High Dose, (E) Pre Inhibition low Dose, (F) Post Inhibition low Dose and (G) Post Inhibition high Dose

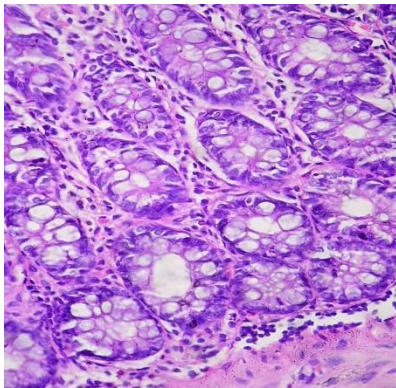
### 4.2.3. Histopathological examination

Histopathological differences among the colon of different treatment groups were examined under light microscope in high power (40X) magnification. The photomicrographs of the present study subject's colon section histology displayed different histological architectures among different experimental groups (Fig. 2a-g).

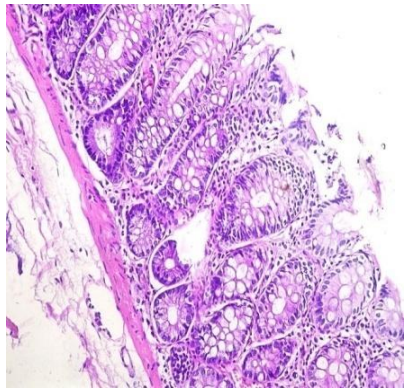
Histological investigation of colon sections of normal control group showed customary histological structure of the mucosal layers (Fig. 2a). While colon sections histology of DMH alone treated rats showed different degrees of cell dysplasia with more crowded glands which are irregular in shape and size. Furthermore some glands were filled with necrotic debris (Fig. 2b). Regional destruction of the mucosa with severe loss of crypts, increased inflammatory cells infiltration in mucosal and submucosal layers were also showed. Colons of prolonged reactivation rat showed large lymphoid follicles invading the lamina propria and causing loss of mucosal architecture. In pre initiation low dose coffee and pre initiation high dose coffee treated groups, histological sections showed that *coffee arabica* protected the mucosal from damage and there was notable decrease in the inflammatory cells permeation in the lamina propria of the mucosa, muscularis mucosa and submucosa, space between crypts and submucosal edema. The mucosal glands showed mild dysplasia within normal limits surrounded by lymphoid aggregates when compared with DMH alone treated group. (Fig. 2d and 2e). In post initiation low dose and post initiation high dose *coffee arabica* treatment group, histological section showed slight decrease in mucosal damage, inflammatory cells permeation in the lamina propria of the mucosa, muscularis mucosa and submucosa, relative decreased in the space between crypts, and submucosal edema with lymphoid aggregates when compared with DMH alone treated group. The mucosal glands showed mild hyperplasia and mild degree of dysplastic changes, inflammatory cells filling the submucosa, but the protection was not as effective as in pre initiation group (Fig. 2f and 2g). Moreover, microscopic investigation of colon section of rats treated with Aspirin showed few inflammatory cells infiltration in the lamina propria of the mucosa, muscularis mucosa and submucosa (Fig. 2c).



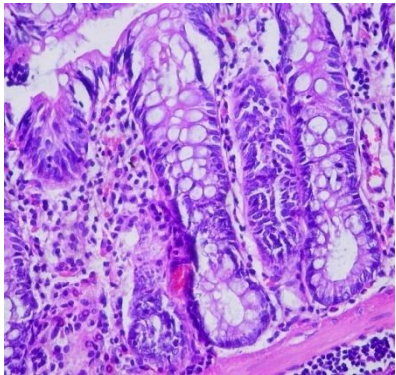
(A) Normal architecture (B) flat and loss of crypts (C) few inflammatory cells in LP&MM



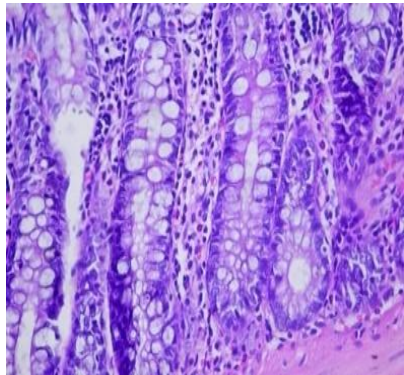
(D) Inflammatory cells in LP, MM, SM



(E) inflammatory cells in LP, MM & few in SM



(F) Many inflammatory cells in LP, MM, SM & lymphoid aggregates



(G) Many inflammatory cells in LP,MM, SM

**Figure 2.** Photomicrographs showing H & E stained colon histopathological sections in DMH-induced colorectal tumorigenesis in rats followed by treatment with *coffee arabica* and Aspirin; (A) Normal Saline, (B) DMH only, (C) Aspirin, (D) Pre inhibition low dose (E) Pre inhibition high dose, (F) Post inhibition low dose and (G). Post inhibition high dose

### 4.3. Biochemical studies

The biochemical variables among the different treatment groups were identified at the end of the study period and the sequential results were shown in table 4. Serum total cholesterol and triglyceride levels were higher ( $116.40 \pm 2.73$  &  $96.00 \pm 3.36$ ) respectively, in DMH alone treated group. A reduction in the levels of Serum total cholesterol and triglyceride were observed in sera of rats administered *coffee arabica* in; pre initiation low dose ( $107.80 \pm 4.28$  &  $85.20 \pm 4.43$ ), pre initiation high dose ( $105.80 \pm 3.52$  &  $84.60 \pm 3.600$ ), post initiation low dose ( $111.80 \pm 2.59$  &  $89.40 \pm 2.22$ ), post initiation high dose ( $108.60 \pm 4.26$  &  $86.20 \pm 2.15$ ), Aspirin ( $101.80 \pm 3.51$  &  $82.80 \pm 3.33$ ) and normal control groups ( $100.40 \pm 2.80$  &  $79.20 \pm 3.80$ ) respectively. An elevated serum total triglyceride and serum total cholesterol levels in DMH alone treated groups were significantly higher ( $P < 0.05$ ) than in normal control groups. Although decreased in serum total triglyceride and serum total cholesterol levels were observed in different dose of coffee or aspirin treated groups when compared with DMH alone group, still the reduction was not significant. There was a difference in serum total cholesterol and triglyceride levels among different dose of coffee treated and normal control groups but the difference failed to reach the statistical significance.

The present study also showed decreases in the levels of Serum total protein in sera of rats administered DMH alone ( $6.24 \pm .14$ ) compared to normal control group ( $7.38 \pm .17$ ). This down regulation was prevented by treatment with different dose coffee and Aspirin. The levels of Serum total protein in sera of rats administered pre initiation low dose coffee was ( $7.12 \pm .12$ ), pre initiation high dose coffee was ( $7.14 \pm .08$ ), post initiation low dose coffee was ( $6.98 \pm .14$ ), post initiation high dose coffee was ( $7.10 \pm .16$ ) and Aspirin low dose was ( $7.2 \pm .18$ ). A marked reduction in serum total protein value was observed in some treatment groups than those of normal control group as shown in (table 4). The results were exhibited significantly decreased ( $P < 0.05$ ) in the levels of serum total protein in sera of rats administered DMH alone when compared to normal saline (normal control group). Serum total protein values were also higher among different dose of coffee and Aspirin treated groups when compared with the positive control (DMH alone) group and the difference also statistically significance ( $P < 0.05$ ).

**Table 4. Chemopreventive response of *Coffea arabica* and Aspirin in terms of Serum total proteins, total Cholesterol and total triglycerides in DMH-induced colon carcinogenesis for ten weeks**

Animal groups	Serum total proteins <sup>a1</sup>		total Cholesterol <sup>a2</sup>		total triglycerides <sup>a3</sup>	
	Mean	S.E	Mean	S.E	Mean	S.E
DMH	6.24	.14	116.40 <sup>b</sup>	2.73	96.00 <sup>b</sup>	3.36
Post initiation low dose	6.98	.14	111.80 <sup>b</sup>	2.59	89.40 <sup>c/b</sup>	2.22
Post initiation high dose	7.10	.16	108.60	4.26	86.20 <sup>c</sup>	2.15
Pre initiation low dose	7.12	.12	107.80	4.28	85.20 <sup>c</sup>	4.43
Pre initiation high dose	7.14	.08	105.80 <sup>c</sup>	3.52	84.60 <sup>c</sup>	3.60
Normal Saline	7.38	.17	100.40 <sup>c</sup>	2.80	79.20 <sup>c</sup>	3.80
Asprin	7.26	.18	101.80 <sup>c</sup>	3.51	82.80 <sup>c</sup>	3.33

Data is expressed as Mean ± SEM (n=5). a: p<0.05(a1=.000, a2 =0.040,a3=0.045) when compared the significance between groups ; b: p<0.05 when compared to the Controls; c: p< 0.05 (p=) when compared to DMH by one way ANOVA.

## 5. Discussion

In the present study, rats treated with DMH alone were found to have a significant loss of body weight as compared to the normal control group, which may be due to induction of cancer by DMH. These results are in agreement with the result of studies done by Mohania et al., and Wasfi *et al.*, (12,107) which showed that average body weight of animals treated with DMH alone was profoundly decreased as compared to other group of animals. DMH induced weight loss in animals showed good prognosis in colon cancers. Our findings are in agreement with those studies reported earlier(32,108). Weight loss is an important prognostic factor in cancer; the higher the extent of weight loss, the shorter the survival time. The prognostic effect of weight loss is greatest in patients with a more favorable prognosis (109). Treatment with *coffee arabica* or Aspirin on the other hand significantly reduces DMH induced weight loss. This might be showed that the protective role *coffee arabica* or Aspirin against the development of colon tumors otherwise *coffee arabica* or Aspirin did not alter the metabolic status of the animals in a significant way or both. Similar finding was reported by Olfat *et al.* However, there was a slight increase in body weight in rats fed single or double dose of coffee (96). Another study done by chinthalapally *et al* also reported that the inhibitory effect of coffee fiber against the development of colon cancer without affecting the body weight of the rat (110). A Reduction in DMH induced weight loss observed in Aspirin treated group during this study period was comparable with similar study done by Mittal *et al*, and Barnes and Lee (111,112).

Morphologically, there was seen marked increased in the grossly visible neoplastic growth on MPLs in DMH alone treated rats during the experimental period. The morphological analysis mentioned above revealed that marked occurrence of preneoplastic features such as MPLs in DMH alone treated group indicated the contributory factor of DMH in tumor development. In consistency with this finding, Kaur and Sanyal in one study and Burali and Kulkarni in other study reported that animals in DMH alone treated group had the highest MPL incidence and burden (106,113)

The result of the present study clearly demonstrates that regular administration of *coffee arabica* or Aspirin brings lesser MPLs incidences and burdens in the colon of rats at the initial stages of carcinogenesis. This result showed that oral administrations of different dose of *coffee arabica* or

Aspirin were able to weaken these features prominently. The observed differences might be indicated its efficiency as a chemopreventive agent at the present dose for a period of ten weeks. The anti preneoplastic lesion properties of Aspirin may be through the blockage of COX enzyme, which in turn suppresses the eicosanoid production of prostaglandins that affect the cell proliferation, tumor growth and immune responsiveness (114). The result of the present study and the given plausible mechanism has been supported by the study done earlier. It has been reported that the PGE<sub>2</sub> concentration was higher in human colorectal tumor than in the surrounding normal tissue (115,116) and NSAIDs are known to prevent the formation of PGE<sub>2</sub> and inflammatory changes in the large intestinal epithelium (107).

The decrease in MPLs incidences and burdens in the colon of rats treated with *coffee arabica* may be due to anti-carcinogenic potential effect for the presences of phenolic acids present in coffee. The result of this study was in line with the studies done on the effect of ginger on lipidperoxidation and antioxidant status in DMH induced experimental colon carcinogenesis, showed that the reactive phenolic group helping to scavenge reactive oxygen species that initiate lipidperoxidation and preventing cell proliferation by increase the susceptibility and decrease the resistance of tumor cells to free radical attack leading to decreased cell proliferation (117). This idea further supported by finding of other study, which suggested that Phenolic compounds has the ability to decrease cellular proliferative activity (106).

Aberrant crypt foci (ACF) are putative preneoplastic lesions of colonic neoplasia in rodents and humans. During the process of colon carcinogenesis, ACF appears in the early stages and subsequently develop into polyps, adenomas and eventually carcinomas (118). As proliferation is a key event in the development and normal functioning of intestine, several reports from animal studies showed that experimental colonic tumors induced by DMH are of epithelial origin and results in increased colonic crypt cell proliferation (119–121).

In the present study rats treated with DMH alone has shown the presence of noticeable increased ACF, AC and severity of dysplasia. This might be indicates that the development of carcinomatous changes in the DHM alone treated group. These ideas were also supported in other studies. ACF are indeed the earliest manifestations of colon cancer, and then ACF could

provide insight into the earliest events of colon tumorigenesis. The genesis of colon tumor is a multistep process, and ACF have been proposed to precede the earliest adenoma (122–124).

In this study ACF and AC were found significantly reduced in colon of rats simultaneously treated with both DMH and *Coffea arabica* when given during the initiation and promotion phase, thus suggesting that *Coffea arabica* possesses activity against DMH induced colon cancer. This is further strengthened by observation that there was significant prevention of the loss of body weight in rats treated with *Coffea arabica* or Aspirin in addition to DMH. Although the mechanisms underlying the protective effect of *Coffea arabica* against ACF formation are not clearly understood, the inhibitory action of *Coffea arabica* might be explained in part, by their putative antioxidant activity. The inhibitory effects of antioxidants on ACF were also observed in other studies (125), which showed that *Peucedanum japonicum*, a traditional herb in the Ryukyu Islands and an antioxidant, inhibited ACF formation induced by azoxymethane carcinogen. The other study on the antioxidant activity of resveratrol as an inhibitor of colon carcinogenesis showed that, resveratrol markedly reduced ACF incidence in Wistar rats, and oxidative imbalance in DMH-treatment was significantly modulated with the supplementation of this compound (118).

Our present study showed that a greater reduction in ACF and AC density in the colons of rats treated with *Coffea arabica* during the initiation phase compared with the promotion phase. The cause of this differential suppression of ACF and AC development in different phase treatment with *Coffea arabica* is unclear. The differences observed in the initiation and promotion groups might be the exposure to coffee or its constituents to suppress ACF and AC development more commonly effective during initiation phase of cancer development; therefore, the protective effect of coffee on early stage of colon cancer development may be high relevant than later stage of colon cancer through its inhibition on cell proliferation (85,126).

The result of the present study also clearly demonstrated that regular administration of Aspirin at low dose brings lesser ACF and dysplastic changes in the colon of treated rats at the initial stages of carcinogenesis. The mechanism of Aspirin to carry out the above effect may be through the blockage of COX enzyme, which in turn suppresses the eicosanoid production of prostaglandins

that affect the cell proliferation, tumor growth and immune responsiveness (114). These results were in agreement with the result of the study done on the involvement of long-term administration of aspirin to inhibit AOM-induced colonic pre-neoplastic lesions and tumour development; Which reported that the anti-carcinogenic effects of aspirin were associated with a 50% reduction in prostaglandin PGE<sub>2</sub> concentration and to changes in the expression of key genes involved in the control of inflammatory, host defense and apoptotic responses (127). The aspirin-triggered down regulation of inflammatory genes and up-regulation of host defense mediators may favor a rebalanced regulation between proliferation and death of colonic epithelial cells (128,129). The other study by Ruffin et al also suggested that a single 81 mg dose of aspirin taken daily should be sufficient to significantly reduce colorectal mucosal prostaglandins E<sub>2</sub> (130,131).

In view of our experimental findings, colons of rats treated with DMH alone showed different degrees of cell dysplasia with more crowded glands which were irregular in shape and size, some glands showed large size and were filled with necrotic debris. Regional destruction of the mucosa with severe loss of crypts, increased inflammatory cells infiltration in mucosal and submucosal layers was also showed. Colons of prolonged reactivation rat showed large lymphoid follicles invading the lamina propria and causing loss of mucosal architecture. These results were in agreement with the study done by Sengottuvelan *et al*, (118) which reported that DMH is a colon-specific carcinogen, which is metabolically activated in the liver and then delivered to the colon via the blood stream or via bile as glucuronide conjugates. After further activation, it methylates DNA mainly at the N<sup>1</sup> and O<sup>6</sup>-positions of guanine. DNA adduct formation is considered to be the initiating step in the formation of tumorigenesis. Rajeshkumar and Kuttan also stated that cell proliferation plays an important role in multistage carcinogenesis with multiple genetic changes (132). Mechanisms by which certain carcinogens which cause carcinogenesis are believed to be mediated by free radicals. Hydrazines and its derivatives, DMH and isoniazid, which can produce active oxygen species, have been shown to induce DNA damage process and carcinogenesis which can be abolished by free radical scavengers, also provide indirect evidence of the involvement of free radicals in carcinogenesis. The above mentioned evidence and results of our finding also supported by the study of Mohamed *et al*, (133) which stated that the observed adenocarcinomas in DMH alone treated group were those in

which neoplastic cells had penetrated the muscularis mucosa to invade the submucosa or deeper layers. Therefore, control of cell proliferation is important for cancer prevention.

In pre initiation low dose coffee and pre initiation high dose coffee treated groups, histological sections showed that *coffee arabica* protected the mucosal from damage and there was notable. The photomicrographs of colons in DMH followed by *coffee arabica* treated rats showed different degrees of cell dysplasia with more crowded disorganized glands than normal group, the cell proliferation generating tubular shapes more finger like projection lined by dysplastic lies directly on the muscularis mucosa and submucosa. There was decreased in the inflammatory cells permeation in the lamina propria of the mucosa, muscularis mucosa and submucosa, space between crypts and submucosal edema. The mucosal glands showed mild dysplasia within normal limits surrounded by lymphoid aggregates when compared with DMH alone treated group. But the histopathological architecture was by far better than the positive (DMH alone treated) group. Histopathological observations of colons of treated rats during the experiment period clearly imply that, treatments with *coffee arabica* greatly inhibit colon carcinogenesis by altering the efficacy of DMH in initiating and promoting neoplastic changes. The ability of *coffee arabica* in altering the histological changes observed in DMH alone group was may be due to anti-carcinogenic effect for the presences of phenolic acids in coffee. In line with the above mentioned results, previous researches showed that the reactive phenolic group helping to scavenge reactive oxygen species that initiate lipidperoxidation and preventing cell proliferation by increasing the susceptibility and decreasing the resistance of tumor cells to free radical attack leading to decreased cell proliferation (117).

From the present study, histopathological microscopic examinations of colon section of rats treated with Aspirin showed, few inflammatory cells infiltration in the lamina propria of the mucosa and also reduce dysplastic neoplasia. It may be through the blockage of COX enzyme. Different studies reported that blockage of COX enzyme suppresses the eicosanoid production of prostaglandins that affect the cell proliferation, tumor growth and immune responsiveness (114). Free arachidonic acids are known to promote apoptosis in cancer cells (134) and apoptosis plays an important role in the regulation of normal and cancer cells (135).In agreement with our

finding previous research has shown that the anti inflammatory efficacy of NSAID had the potential to reduce the dysplastic neoplasia of crypt cells and hyperplasia (121).

Biochemical measurements were carried out in sera of different treatment groups, the result was indicated that serum levels of Cholesterol and triglyceride in animals treated with DMH alone were significantly elevated ( $P<0.05$ ) in comparison with the normal control animals. The above findings were maintained by previous studies; a case-control study in Korea on the association of serum lipid with the risk of colorectal adenomatous polyp in men has been suggested that the serum triglyceride concentration is positively associated with bile acids synthesis (136). Furthermore, serum triglyceride and fecal bile acids may be biologically related to each other (137). Both may promote carcinogenesis in large intestine (136,138). The other study carried out in old Chinese concludes that colorectal polyps were significantly associated with increased total cholesterol and triglycerides levels (40). The possible mechanisms of relationship between colorectal polyps and serum lipids might be explaining our findings. First, hypercholesterolemia is associated with hyperinsulinemia and insulin resistance (139,140). Hyperinsulinemia and insulin resistance also can induce colorectal cancer risks in several European cohorts (141,142). Second, serum triglyceride concentration may be positively associated with bile acid synthesis and fecal bile acids. An increase in synthesized and secreted bile acids may provide abundant substrates for the formation of secondary bile acids and promote carcinogenesis in the large bowel (40,137).

Our results also showed that serum levels of Cholesterol and triglyceride in *coffee arabica* treated groups were lower than those measured in the positive control (DMH alone treated) group. This suggested that *coffee arabica* had the potential to inhibit serum Cholesterol and triglyceride production and thereby can exert a negative impact on cell proliferation. Consequently, it could be the suppressive effect of *coffee arabica* on serum Cholesterol and triglyceride production, which may contributes to the chemopreventive and tumor growth inhibitory effects in this animal model of colon carcinogenesis.

Several mechanisms have been proposed to explain the inverse association between coffee consumption and colorectal cancer risk observed in case-control studies. The mechanisms underlying the association between decreasing serum lipids level by administering *coffee arabica*

for its role in colorectal polyp inhibition in experimental rats are unclear. There are two possible mechanisms that might enlighten our findings. First, coffee has phenolic compounds, mostly chlorogenic acids and the Maillard reaction products, which are formed during the roasting of coffee, may contribute at least in part, to the antioxidant activity and inhibition of free radical generation. In addition these compounds can reduce the initiation and propagation of lipid peroxidation (143). Secondly phenolic chlorogenic acid has been shown to reduce glucose concentrations (144,145) and intake of quinides, degradation products of chlorogenic acids, increase insulin sensitivity (146) and may reduce hepatic glucose output through inhibition of glucose-6-phosphatase (147). This may contribute to the antioxidant effects of coffee.

Our findings are in agreement with those of Kempf *et al* (148) who reported that coffee consumption led to an increase in coffee derived compounds, mainly serum chlorogenic acid and decrease in serum cholesterol level. These indicate that coffee consumption appears to have beneficial effects on serum cholesterol. It has been hypothesized that one of the principal causes of colon cell proliferation is the formation of lipid peroxides by free radical derivatives. Thus, the antioxidant activity or the inhibition of the generation of free radicals is important (149). The body has an effective defense mechanism to prevent and neutralize the free radical-induced damage. This is proficient by a set of endogenous antioxidant enzymes such as SOD and CAT. These enzymes constitute a mutually supportive team of defense against ROS (150,151). The other case-control studies have been suggested that compounds in coffee could decrease the synthesis and secretion of bile acids, which may promote colon carcinogenesis (86). Coffee may promote the elimination of carcinogens and improve antioxidant status by enhancing phase II enzyme activity and glutathione synthesis (152–154).

This study also showed that, in comparison with positive control group given DMH alone, rats of negative control group given normal saline and rats treated with *coffee arabica* or Aspirin had increased levels of serum total protein. Which suggest that DMH (precursor of colorectal cancer) may have more apparent metabolic changes and early treatments with antioxidants (like coffee) decreases these metabolic changes. DMH induced decreased in serum total protein is in agreement with protein loss usually associated with most cancer and the ability of *Coffee arabica* to reverse it. Weight loss, which is important prognostic factor in cancer (109,155), and it is also

observed in DMH alone group of this study, has been attributed to increased protein degradation and /or decreased protein synthesis (156). In line with our findings in recent years, numbers of epidemiological studies had shown that, a variety of metabolic disorders were related to the occurrence and development of different kinds of malignant tumor and colon cancer (157–159)

## 6. Conclusion

Based on this study, the results of the findings conclude that *coffee arabica* has chemopreventive effects in 1, 2- DMH induced and promoted colorectal precancerous lesions in rat model. *Coffee arabica* changed the morphological identity of the crypts in the mucosa and histological alterations that varied from hyperplasia to dysplasia. *Coffee arabica* also contributed on the formation of less AC or ACF, which indicates one of the earlier abnormalities that occur during the colorectal cancer induction. Furthermore, *coffee arabica* interfered with the intermediate biomarker like serum total cholesterol, triglycerides and proteins for colon cancer development and metastasizes as evidenced by the various levels of certain oxidative stress markers. This may be due to the antioxidative properties of the powder as free radical scavengers.

## 7. Recommendation

In light of this finding and other studies done at different levels the following recommendations are forwarded.

- Since short term studies are insufficient to fully answer questions of long term effects of *coffee arabica* on the inhibitory effect on colorectal cancer initiation and promotion, long-term studies are warranted to examine such effects. Further experiments, including pre-clinical efficacy and mechanistic studies are necessary to fully evaluate this natural compound for its cancer preventive properties and to understand its mode of action.
- Further studies, however, including the isolation and chemical characterization of the major compounds that contribute to the promotion of the immune system and to the inhibition of carcinogenesis, are needed and may generate new targets for therapy.
- Moreover, the present study establishes that *coffee arabica* have appreciable anti-cancer activity and may be improve health.
- So *coffee arabica* daily uses as beverage may significantly reverse the development of colorectal cancer and also may interfere with the multi-step progression to carcinoma.

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## **Annex I**

### **DECLARATION**

I, the undersigned, declare that this thesis is my original work, has never been presented in any other University and that all resources of Materials have been duly acknowledged.

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**Signature** \_\_\_\_\_ **Date of submission**    April 20, 2015

This thesis has been submitted for examination with my approval as a University advisor.

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The result of this thesis has been submitted for the department of pharmacology with my approval as a University internal examiner.

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