

Coffea arabica complies Chemo-preventive Activity against DMH-induced Colorectal Cancer in Experimental Rat Model

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Abstract

The aim of this study was to evaluate chemopreventive potential of *Coffea arabica* in DMH-induced colorectal carcinogenesis in the rat model. Totally, 35 female wistar rats were divided into seven equal groups. Groups I-VII except group VI were given freshly prepared neutral DMH in normal saline, once a week for 5 weeks. Group VI received normal saline alone. Groups II and III received oral dose of coffee every time when they are given DMH. Groups IV and V received coffee after 5 months of DMH treatment. Group VII was treated with aspirin. Multiple plaque lesions, aberrant crypts and aberrant crypt foci were observed in colorectal walls of the DMH alone treated rat groups. The numbers of preneoplastic features were significantly reduced in coffee or aspirin treated groups. Histologically, different degree of dysplasia and hyperplasia was observed in DMH alone group. The above features were significantly reduced in coffee or aspirin treated groups. Serum biomarkers and body weight of DMH-induced colorectal cancer were appreciably maintained in coffee and aspirin treated groups. Taken together, the results of this study revealed that *Coffea arabica* has significant effect on reducing the number of preneoplastic lesions and polyps, considerably suppressing tumor progression and invasion.

Keywords: *Coffea arabica*, Colorectal cancer, Preneoplastic lesions

Introduction

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]. The vast majority of cases and deaths from CRC can be prevented by investigating possible therapeutic means and applying existing knowledge [3].

The animal models mimic the mode of human exposure to environment carcinogen and intended to test the agents. The chemical 1,2-Dimethylhydrazine dihydrochloride (DMH) is a potent colorectal carcinogen in experimental animals [4,5]. DMH is metabolized into active carcinogenic compound, which produces free radicals that induce oxidative DNA damage in the colon. The free radicals elicit an oxidative stress by methylating the nucleic acids of colonic epithelial cells, which in turn leads to active inflammation and promutagenic events [6]. During the process of colon carcinogenesis, aberrant crypt foci (ACF) appear in the early stages and subsequently develop into polyps, adenomas and eventually carcinomas [7]. An ACF formation leads to hyperplasia and has higher risk of colon cancer development both in human and experimental animal models [8]. Hence, ACF development is commonly used as a parameter for assessment of potential development of colorectal cancer [9]. Some molecules have been also recommended as CRC markers, such as serum total protein and lipids [10].

A number of case control studies have demonstrated reduced risk of colorectal cancer development with coffee consumption [11], which is among the most widely consumed beverages in the world. Coffee is a

rich source of dietary phenolic phytochemicals, including caffeic acid and chlorogenic acid [12] and ChA was reported to reduce chemical carcinogenesis in animals studies [13,14]. Among different species, *Coffea arabica* is native to the highlands of Southwest Ethiopia [15].

Current colorectal cancer treatment modalities are invasive, 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 [16]. Based on the above facts the purpose of the present study was to evaluate chemo preventive effects of *Coffea arabica* against the DMH-induced colorectal cancer in rat model. This study has two phases: an initiation phase and promotion phase.

Materials and Methods

Experimental animal

Thirty five Female Wistar rats (180-270 gm) were randomly housed in group of 7 (5 rats of each group) in clean polypropylene cages with a wire mesh top with hygienic bed of sawdust (regularly changed every 3 days) and were maintained in a well ventilated room (25 ± 1°C with 55 ± 5% humidity) with a 12 h light/dark cycle and body weights were recorded every week till the end of the experiment. The rats were acclimatized with standard laboratory diet and water ad libitum for one week before commencement of the experiment. All animal procedures were conducted 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 ethics committee on the use of the experimental animals for biomedical research.

Chemicals and reagents

The 1,2-Dimethylhydrazine dihydrochloride (ACROS organics), sodium hydroxide (Lobachemie PVT.LTD), methylene blue (MERCK) were used. All other chemicals and reagents used in this study were analytical grade.

Collection and preparation of plant materials: In the present study, the roasted powder of *Coffee arabica* was collected from local market. The powder was authenticated by plant biologist of Natural and Computational Science College, Addis Ababa University, Ethiopia. Then voucher specimen (Voucher No. SG0018) was deposited at herbarium until it was used for extraction. 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. Then, the solution was filtered using filter paper (Whatman No 3, Whatman Ltd., England) to collect the extract and the solvent was lyophilized by lyophilizing machine. The resultant extract was dehydrated in an oven at 50°C for 24 h [17]. The dose was obtained on dry matter and the percentage yield was found to be 20.3% (w/w). The anti-oxidant dose of coffee had been referred from the study of Florián et al. [18] who has demonstrated the antidiabetic role of *Coffee arabica* in alloxan-induced diabetic rats.

Preparation of carcinogen

DMH was dissolved in 1mM EDTA. The pH was adjusted to 7.0 in 1mM NaOH and used immediately after preparation. DMH was injected intraperitoneal (i.p.) at a dose of 20 mg/kg/body weight once a week for five consecutive weeks on the ventral of the animal [19].

Experimental design

After one week of acclimatization thirty five rats were randomly assigned into seven groups.

All animals (except group VI that was injected with vehicle) have received i.p injections of DMH (20 mg/kg) once a week for 5 consecutive weeks. Animals in pre-initiation group were gavaged low dose (20 mg/kg) and high dose (40 mg/kg) coffee daily starting from the date of DMH injection to the end of the experimental period whereas animals in post initiation group received coffee treatment two weeks after DMH injection. The grouping and treatments are summarized in Table 1.

Group	Treatment	Group category
I	DMH	Carcinogen controls
II	DMH + <i>Coffee arabica</i> (20 mg/kg)	pre initiation low dose
III	DMH + <i>Coffee arabica</i> (40 mg/kg)	pre initiation high dose
IV	DMH + <i>Coffee arabica</i> (20 mg/kg)	post initiation low dose
V	DMH + <i>Coffee arabica</i> (40 mg/kg)	post initiation high dose
VI	0.5 ml normal saline	negative control group
VII	DMH + Aspirin (6 mg/kg)	Aspirin group

Table 1: Animal groups and their treatment.

Experimental Protocol

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 cardiac or heart puncture. Blood samples were allowed to clot for 30 minutes at room T0 then centrifuged (4000 rpm) for 10 minutes. Serum was collected, aliquot and stored at -80°C. Total protein (gm/dl) was estimated by applying the Bradford method [20]. Similarly, total lipid (mg/dl) was estimated according to Knight method [21].

Macroscopic and microscopic analysis

The rats were sacrificed by cervical dislocation and the colons were excised, flushed with saline, cut open longitudinally along the main axis, rinsed several times in saline. The inner surface was examined for visible macroscopic multiple plaque lesions (MPLs).

The colon were divided into three equal parts, fixed in 10% buffered formalin for 24 h and then stained with 2% methylene blue in Krebs Ringer solution for 10 min. The mucosal surface of the colon was evaluated for the number of AC and ACF in the stained colon under 40X magnification using a light microscope. 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. Topographic analysis of the colonic mucosa was done according to Bird's procedure aberrant [22]. For Histopathological examination, colon sections fixed in 10% buffered formalin were processed for light microscope at department of pathology, College of Health sciences, Addis Ababa University. The slides were read and interpreted blindly by a pathologist from Addis Ababa University.

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 \pm standard error mean (SEM) for each group. Statistical differences between groups were analyzed by One-way analysis of variance (ANOVA) using Tukey post hoc test. Statistical significance was accepted when $P < 0.05$.

Results

Coffee arabica complies anticancer activity by maintaining the body weight of rats with chemically induced colorectal cancer

In this study, it was found that the body weight of DMH alone treated rats did not appreciably decrease when compared with other groups in the first two weeks. However, by the end of the experimental period, final mean body weight of the rats in DMH alone treated group was significantly decreased compared with the other groups ($p < 0.05$). On the contrary, when compared with the DMH alone treated group, the average body weight of *Coffee arabica* treated group was significantly higher (Table 2).

Group	Body weigh at week 2 (gm)		Final body weight (gm)a	
	Mean	SEM	Mean	SEM
DMH	188	3	204.67b	0.97
Post initiation low dose	198	3.39	224.00c	4.17
Post initiation high dose	196	5.79	222.00c	5.25
Pre initiation low dose	198	5.61	223.00c	6.16
Pre initiation high dose	197	7.35	221.33c	6.96
Normal Saline	199	1.87	229.67c	1.62
Asprin	193	4.64	224.00c	4.61

Table 2: Comparison of body weight in *Coffee arabica* and aspirin treated rats after DMH-induced colorectal carcinogenesis for ten weeks. Data is expressed as Mean ± SEM (n=5). a: p<0.05(p=0.033) 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.

Continuous coffee consumption interferes with development of multiple plaque lesions in DMH-induced colorectal cancer rat model. In the ten weeks study, the gross anatomy of the colorectal mucosal surface was depicting the occurrence of multiple plaque lesions (MPLs). The proportion of rats that developed MPLs in colon was 100% in DMH alone treated group, while co-administration with different concentration of *Coffee arabica* in initiation phase and promotion phase reduced the incidence of MPL to 40% and 60%, respectively. Interestingly, the incidence of MPLs in aspirin treated group was similar to that of animals treated with *Coffee arabica* in initiation phase. Colorectal tissues from control groups didn't show any such features. This incidence of MPLs in DMH treatment group was significantly elevated (p>0.05) when compared with the normal

control and other treatment groups. Variations in the incidence of MPLs were observed in different concentration of *Coffee arabica* treated groups in initiation and promotion phase; however, the difference was not statistically significant (p>0.05). Maximum number of MPLs proportion (6.0) were observed in DMH alone treated group. In contrary, decreased burden of MPLs were observed in animals treated with various concentration of *Coffee arabica* (Low and high dose initiation and the same dose promotion group), but the number of MPL was extremely decreased in (1.2) aspirin treated groups. 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 3).

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

Table 3: Chemopreventive response of *Coffee arabica* and aspirin in terms of total multiple plaque lesions (MPLs), MPL incidence, MPLs burden and MPLs multiplicity in DMH-induced colorectal carcinogenesis for ten weeks. Data is expressed as Mean ± SEM (n=5). a: p<0.05 (a1=0.000, a2=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 Multiple Plaque Lesions (MPLs), 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 (23).

***Coffee arabica* inhibits aberrant crypt foci development in DMH-induced colorectal cancer rat model**

In the present study, ACs and ACF frequencies were significantly higher in the DMH alone treated animals. Colonic mucosa from negative control group was free from aberrant crypts.

An increased 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 (Table 4). There was a difference in aberrant crypt distribution among different groups of *Coffee arabica* treated rats. But

the difference in aberrant crypt distribution was not affected significantly with each other groups ($p > 0.05$). The distribution of colonic AC and ACF in different treatment groups during the study period has been displayed in Figure 1.

Animal groups	AC/colona		ACF/colona	
	Mean	SEM	Mean	SEM
DMH	407.6 ^b	40	150.2 ^b	13.8
Post initiation low dose	148.6 ^{c/b}	13.4	57.0 ^{c/b}	9.4
Post initiation high dose	131.2 ^{c/b}	16.6	50.8 ^{c/b}	10.3
Pre initiation low dose	105.8 ^{c/b}	17.5	41.4 ^{c/b}	9.7
Pre initiation high dose	70.8 ^{c/b}	14.2	28.4 ^{c/b}	8.5
Normal Saline	0.0 ^c	0	0.0 ^c	0
Asprin	71.8 ^{c/b}	11.6	28.2 ^{c/b}	7.1

Table 4: Chemopreventive response of *Coffee arabica* and aspirin in terms of aberrant crypts (ACs) and aberrant crypt foci (ACF) in DMH-induced colorectal carcinogenesis for ten weeks.

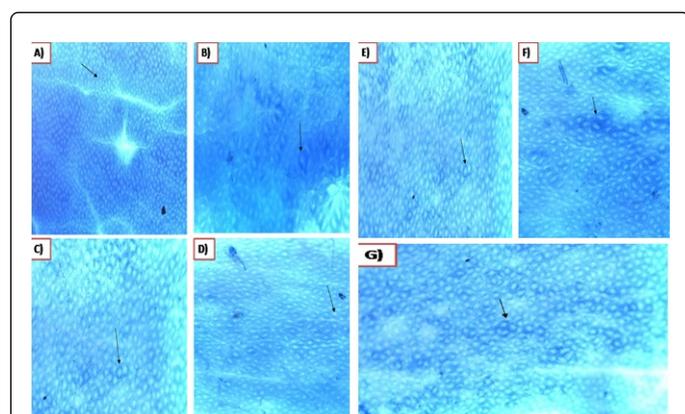


Figure 1: Longitudinal section of colonic mucosa stained with 2% methylene blue in low power (10 X) light microscope which includes normal looking colonic mucosa and abrupt crypts with one or multiple foci in different treatment groups; (A) Normal Saline group displaying normal crypt, (B) DMH only treated groups with many, very larger crypts and 3-4ACs/ACF, (C) Aspirin treated group with few slightly raised crypts, (D) Pre-inhibition high dose group displaying few slightly raised crypts, (E) Pre-inhibition low dose group displaying few slightly raised 2ACs/ ACF, (F) Post-inhibition low dose group with many raised 2-3ACs/ ACF and (G) Post-inhibition high dose group displaying few slightly raised 2-3ACs/ ACF.

Continuous coffee consumption is linked with decreased inflammatory activity of colorectal tissue in DMH-induced colorectal cancer rat model

Histological investigation of colon sections of normal control group showed customary histological structure of the mucosal layers (Figure

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 (diffused form of tissue architecture) (Figure 2b).

In pre-initiation low dose and high dose treated groups, there was notable decrease in the inflammatory cells permeation in the lamina propria of the mucosa, muscularis mucosa and submucosa.

The mucosal glands also showed mild dysplasia within normal limits surrounded by lymphoid aggregates (Figure 2d and 2e). In post-initiation low dose and high dose treated groups, 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 (Figure 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 (Figure 2c).

Coffee consumption results in maintained total protein but decreased total cholesterol and triglyceride in DMH-induced colorectal cancer rat model

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 5. Serum total cholesterol and triglyceride levels were significantly higher ($p < 0.05$) in DMH alone treated group.

Although decreased in serum total triglyceride and serum total cholesterol levels were observed in different dose of coffee or aspirin treated groups, but the reduction was not significant.

The result of the present study also showed significantly increased ($p > 0.05$) in the levels of serum total protein among different dose coffee and aspirin treated groups when compared with the DMH alone treated group (Table 5).

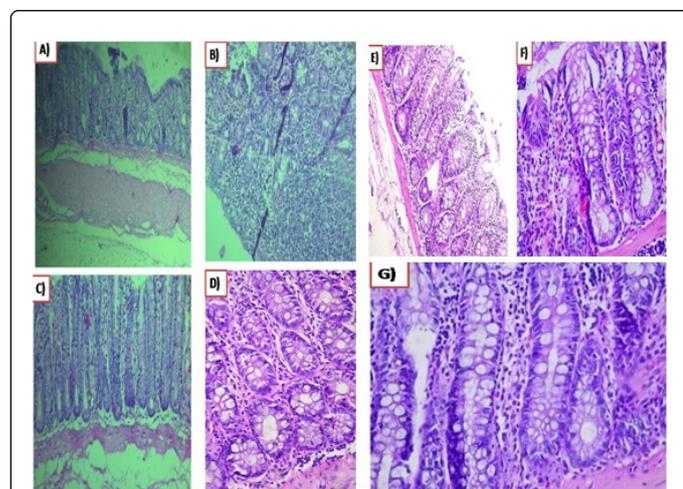


Figure 2: Photomicrographs showing H & E stained colon histopathological sections in DMH-induced colorectal tumorigenesis in rats followed by treatment with *Coffee arabica* and aspirin: (A) Normal Saline treated characterized by normal architecture (B) DMH only treated displaying flat and loss of crypts, (C) Aspirin treated colon characterized by few inflammatory cells in LP&MM, (D) Pre-inhibition low dose treated displaying inflammatory cells in LP, MM, SM (E) Pre-inhibition high dose treated colon displaying inflammatory cells in LP, MM and few in SM, (F) Post-inhibition low dose treated displaying many inflammatory cells in LP, MM, SM and (G). Post-inhibition high dose treated ones displaying many inflammatory cells in LP, MM, SM and lymphoid aggregates.

Animal groups	Serum proteins ^{a1} total		Total Cholesterol ^{a2}		Total triglycerides ^{a3}	
	Mean	S.E	Mean	S.E	Mean	S.E
DMH	6.24	0.14	116.40b	2.73	96.00 ^b	3.36
Post initiation low dose	6.98	0.14	111.80b	2.59	89.40 ^{cb}	2.22
Post initiation high dose	7.1	0.16	108.6	4.26	86.20 ^c	2.15
Pre initiation low dose	7.12	0.12	107.8	4.28	85.20 ^c	4.43
Pre initiation high dose	7.14	0.08	105.80c	3.52	84.60 ^c	3.6
Normal saline	7.38	0.17	100.40c	2.8	79.20 ^c	3.8
Aspirin	7.26	0.18	101.80c	3.51	82.80 ^c	3.33

Table 5: Chemopreventive response of *Coffee arabica* and aspirin in terms of serum total proteins, total cholesterol and total triglycerides in DMH-induced colorectal carcinogenesis for ten weeks. Data is expressed as Mean \pm SEM (n=5). a: $p < 0.05$ ($a^1 = 0.000$, $a^2 = 0.040$, $a^3 = 0.045$) 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.

Discussion

Ethiopia is home land for *Coffee arabica*, which is one of the most widely consumed daily beverage in the world. In the current study, we showed for the first time that treatment of colorectal cancer with roasted *Coffee arabica* rescues weight loss induced by DMH in wistar rats. Although the detail mechanism is not yet known, this might be due to the protective role of *Coffee arabica* against the development of colon tumors. Another study done by Chinthalapally et al also demonstrated inhibitory effect of coffee fiber against the development of colon cancer without affecting the body weight of the rat [23,24].

Morphologically, marked increment in the grossly visible neoplastic growth on MPLs in DMH alone treated rats was observed. This is attributed to contributory factor of DMH in tumor development. In consistency with this finding, Kaur and Sanyal in one study [25] and Burali and Kulkarni in other study reported that animals in DMH alone treated group had the highest MPL incidence and burden. Regular administration of *Coffee arabica* or aspirin brings lesser MPLs incidences and burdens in the colon of rats at the initial stages of carcinogenesis. The underlining mechanism how *Coffee arabica* revert MPLs incidences and burdens in the colon of rats is to be investigated in later studies. In line with our finding earlier studies showed that reactive phenolic chemicals in coffee helping to scavenge reactive oxygen species that initiate lipid peroxidation and preventing cell proliferation [26].

In this study ACF and AC were significantly reduced in colon of rats treated with *Coffee arabica* during initiation and promotion phase. This suggests that *Coffee arabica* possesses activity against DMH-induced colorectal carcinogenesis. The inhibitory effects of antioxidants on ACF were also documented by Morioka and his colleague who showed that Peucedanum japonicum (a traditional herb in the Ryukyu Islands and an antioxidant) inhibited ACF formation induced by azoxymethane carcinogen. The differences observed in the initiation and promotion groups might be the exposure to coffee or its constituents to suppress the onset of ACF and AC development. This indicates that the protective effect of coffee on early stage of colon cancer development may be more relevant than later stage of colon cancer through its inhibition on cell proliferation initiation [11,27].

In view of our experimental findings, the photomicrographs of colons in *Coffee arabica* treated rats showed different degrees of cell dysplasia with more crowded, architecturally disorganized glands and inflammatory cells infiltration than normal saline treated group. But the histopathological architecture was by far better than DMH alone treated group. Histopathological observations in the colon of coffee-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. This might be due to the active antioxidant compositions that are found in *Coffee arabica*. Supporting our findings, other researches also showed that anti-oxidant substances increase the susceptibility and decrease the resistance of tumor cells to free radical attack leading to decreased cell proliferation [28].

Biochemical measurements were carried out in sera of different treatment groups. The results indicated that serum levels of cholesterol and triglyceride in animals treated with DMH alone were significantly elevated in comparison with the normal control animals. These findings are in line with other case-control study in Korea on the association of serum lipid with the risk of colorectal adenomatous polyp in men, which has been suggested that the serum triglyceride

concentration is positively associated with bile acid synthesis [29]. Furthermore, serum triglyceride and fecal bile acids may be biologically related to each other [30]. Both may promote carcinogenesis in large intestine [29,31]. The other study carried out in China concludes that colorectal polyps were significantly associated with increased total cholesterol and triglycerides levels [32]. There are important biochemical mechanisms proposed for the link between colorectal polyps and serum lipids. First, hypercholesterolemia is associated with hyperinsulinemia and insulin resistance [33,34]. Hyperinsulinemia and insulin resistance also can induce colorectal cancer risks in several European cohorts [35,36]. 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 [30,32]. Our results indicate that serum levels of cholesterol and triglyceride in *Coffee arabica* treated groups were lower than 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. The mechanisms underlying the inverse association between serum lipids and *Coffee arabica* are unclear. There are two possible mechanisms that might enlighten our findings. First, coffee has 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 [37]. Secondly phenolic chlorogenic acid has been shown to reduce glucose concentrations [38,39] and intake of quinides, degradation products of chlorogenic acids, increase insulin sensitivity [40]. This may contribute to the antioxidant effects of coffee.

We also documented an increased level of serum total protein in rats treated with normal saline and *Coffee arabica* or aspirin compared to DMH alone treated group. This could be due to the fact that rats treated with DMH may have more apparent metabolic changes and early treatment with antioxidants (such as coffee) decreases these metabolic changes. DMH-induced reduction in serum total protein was in agreement with protein loss usually associated with most cancer and the ability of *Coffee arabica* to reverse it. Supporting our study, a number of recent 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 [41–43].

Conclusion

We show that *Coffee arabica* has chemopreventive effects in DMH induced and promoted colorectal precancerous lesions in rat model. *Coffee arabica* reduced development of 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. However, the underlying mechanisms remain to be explored in future studies.

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Authors' contributions

These authors (SG.WL and GY) contributed equally to this work. The author (AT) contributed in laboratory activities and data analysis.

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