Current Trends in Biotechnology and Pharmacy
Vol. 16 (2) 114 - 129, April 2022, ISSN 0973-8916 (Print), 2230-7303 (Online)
10.5530/ctbp.2022.2.12

Evaluation of the Chemo-Preventive Effects of *Camellia sinensis* Silver Nanoparticles and Synergistic Effects with 5-fluorouracil in Colorectal Cancer Induced Rats

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Abstract

In the present study, the chemopreventive effect of green tea nanoparticles (GTNP) and synergistic effect between GTNP and 5-Fluorouracil (5FU) on 1, 2-Dimethylhydrazine (DMH) induced colorectal carcinogenesis was studied in Wistar albino rats. GTNP synthesized and characterized by transmission electron microscope (TEM) and Fourier-transform infrared spectroscopy (FTIR) and tested for their acute toxicity. All groups except for normal group received subcutaneous injections of DMH once a week for 5 weeks. GT group was treated daily with GTNP alone, GTFU group received both GTNP and intraperitoneal injection of 5FU once a week and 5FU group was treated with 5FU once a week for 16 weeks. In the GT group, a significant reduction in the number of aberrant crypt foci (ACF) was observed suggesting the inhibitory effect of green tea in attenuating colorectal cancer progression. By comparing GTNP and GTFU treatment, there was a significant decrease in total ACF number in combined treatment of GT and 5FU compared to GT alone (13.33 ± 0.88 vs 25.00 ± 2.31, $p < 0.05$). The total number of ACF in 5FU treatment alone was significantly reduced when compared to combined GTFU treatment (5.67 ± 2.19 vs 13.33 ± 0.88, $p < 0.05$). These results suggested that green tea nanoparticles significantly inhibited ACF formation on DMH-induced colorectal cancer which has led to no tumor formation. The large ACF only significantly reduced by this combined therapy compared to small ACF. In overall, it does not exert the synergistic effect between the combination therapy of GTNP and 5FU on colorectal cancer.

Keywords: *Camellia Sinensis*, Silver nanoparticles, Colorectal cancer, Aberrant crypt foci, pre-neoplastic lesion

Introduction

Colorectal cancer (CRC) is the most frequent malignant disease of gastrointestinal tract (GIT) which is responsible for the major causes of cancer related morbidity and mortality throughout the world and accounts for over 9% of all cancer incidence (1,2). The disease is known to be the third most common diagnosed cancer and ranking as the fourth of most common cancer death globally (1,3). In 2018, it is estimated that over 1.8 million of new CRC cases and approximately 881,000 deaths occurs worldwide which accounting for 1 in 10 cancer cases and deaths (4). Carcinogenesis is a multistep processes that involve three phases; initiation, promotion and progression (5,6). The drug, 5-Fluorouracil (5FU) is a chemotherapeutic drug that is widely used to treat various cancers such as breast, gastrointestinal, pancreatic and particularly colorectal cancer (7,8) Tea drinking is widely practiced in the world and has recent-

ly increased among cancer patients. However, the effects of concurrent consumption of tea on the bioavailability and the net therapeutic potential of co-administered chemical drugs are not clear. In this study, the effects of green tea on the pharmacokinetics of 5-fluorouracil (5-FU. In treating colorectal cancer, the 5FU medication can be given as a single agent or in combination with other cancer drugs such as leucovorin [9]. Similar to other chemotherapeutic drugs, 5FU has many side effects as it is not only targeting cancerous cells but also has harmful effects on healthy body cells [9]. Some of the common side effects that occur in more than 30% of patients using 5FU drug are diarrhea, nausea, vomiting, mouth sores, poor appetite, neutropenia and thrombocytopenia [9]. It was also reported that 5FU is associated with the long term side effects of cognitive impairment as commonly seen as memory problems known as 'chemo brain' which is the dead of healthy brain cells [10]it primarily inhibits the enzyme thymidylate synthase blocking the thymidine formation required for DNA synthesis. Although having a relatively short half-life (< 30 mins. Examples of memory problems include memory lapses, slower thinking and remembering words [9].

Tea is one the most popular beverages that is widely consumed globally [11,12]. Many studies on green tea have reported that green tea have various beneficial remedial effects on human health such as lowering blood pressure, lowering the risk of cardiovascular disease, improving the function of brain, promoting weight loss and combating cancer and type II diabetes [11,13,14]. Green tea mainly contains high level of natural tea catechins, proteins, amino acids, carbohydrates, minerals and trace elements, trace amounts of lipids, sterols, vitamins, xanthic bases, plant pigments and volatile compounds [15]. The major green tea polyphenols present are flavanols commonly known as catechins [15]. There are five major catechins found in green tea; (+)-catechin (C), (-)-epicatechin (EC) (6.4%), (-)-epigallocatechin (EGC) (19%), epicatechin gallate (ECG) (13.6%) and epigallocatechin-3-gallate (EGCG) [16]. Among all catechins, EGCG is the most abundant which accounts for 59% of the total catechins [16]. The amount of catechins present also varies in the original tea leaves because of the differences in variety, origin and growing conditions of tea [15]. Several epidemiological studies have demonstrated that the consumption of green tea polyphenols particularly EGCG can lower the risk of developing several cancer types [17]. Some of the reported anti-cancer effects of green tea that are modulated by EGCG include the induction of apoptosis, inhibition of tumorigenesis, tumor proliferation and angiogenesis, suppression of tumor migration and invasion [13,18]. These anti-cancer activities were believed to be associated with the modulation of ROS production, inhibition of NF-kB, down- or up- regulation of MAPKs activation and regulation of epigenetic alteration [19].

In a conventional anti-cancer drug delivering system, the chemotherapeutic agents are mostly distributed non-specifically throughout the body where they affect both cancerous and non-cancerous cells. This unspecific distribution of drug has led to insufficient dose achieved within the tumor and also resulted in excessive toxicities and unwanted side effects [20]. Since the conventional drug delivery system leads to rapid elimination of drug, administration of drug at a higher dosage is required to reach the cancerous targeted site and this usually exhibits even more high toxicities [21]. The ability to differentiate between malignant cells and non-malignant cells and to selectively eliminate malignant cells is main objective of nanotechnology based cancer treatment [22]. There are two important processes involve in discriminating malignant from non-malignant cells; passive and active targeting strategies [22]. Nanoparticles use these strategies to enhance the intracellular concentration of drugs in cancer cells while avoiding the toxicity to normal cells [20]. Passive targeting strategy takes advantages of the enhanced permeability and retention (EFR) effects with an aim to increase the con-

centration of nanoparticles in the tumor. Active targeting strategy may involve in the selection of molecular biomarkers that are expressed on the surface of cancer cells to localize nanoparticles to only malignant cells. These targeting strategies benefited from surface modifications of nanoparticles that minimize the uptake by macrophages and thus maximizing the drug circulation time [23]. The main aims of this present study are to evaluate the chemo-preventive effects of green tea nanoparticles and investigate the synergistic inhibitory effects between green tea nanoparticles and 5-fluorouracil chemotherapeutic drug in DMH-induced rats. The study is specifically assessing the effects of the green tea nanoparticle and its combination with 5FU.

Materials and Methods

Chemicals

Silver nitrate (Merck, Darmstadt, Germany), Green Tea (*Camellia sinensis)* procured from (Doddabetta Tea Factory, Coonoor, India) were procured for the study. Polyvinyl Pyrrolidone (PVP) and D (+) glucose anhydrous were acquired from Sigma-Aldrich (Steinheim, Germany). 1,2 dimethylhydrazine dihydrochloride (DMH), 5-fluorouracil (5FU), were procured from Pi chemicals, (Shanghai, China). For reliability and accuracy of the result, sterility conditions with minimum contamination were maintained throughout the experiment. Formalin (Sigma-Aldrich, Steinheim, Germany), methylene blue, Merck, Germany and Histochoice clearing agent (Sigma, Germany) were purchased.

Preparation of green tea extract

The dried green tea leaves were firstly ground until the consistency of a fine powder. About 10g of finely ground green tea leaves was added into a beaker containing 100ml of boiling distilled water. The green tea powder was then boiled at 100^{o}C for 10 minutes with occasional stirring. The extract was then allowed to cool down at room temperature and filtered through a tea filter or muslin cloth. The filtered tea extract was then passed through Whatman filter paper No.1 for getting a much clearer filtrate. The green tea extract was stored at 4^{o}C until further use.

Green synthesis of green tea silver nanoparticles

The protocol described below is a standard protocol for 50ml of green tea extract. Two solutions; A and B were needed to be prepared for the synthesis of green tea nanoparticles. Preparation of solution A (Green tea solution): In a conical flask containing 50ml of green tea extract, 0.7206g of glucose and 0.3300g of PVP were added and allowed to dissolve by constant stirring on a magnetic stirrer. Preparation of solution B (0.1M silver nitrate ($AgNO_3$) solution): To prepare 0.1M of silver nitrate ($AgNO_3$) aqueous solution, 0.3398g of silver nitrate salt was dissolved in a beaker containing 20ml of distilled water by constant stirring on a magnetic stirrer. 1M of sodium hydroxide was then added drop by drop into the solution until pH 8 to 10 was reached [24].

For the synthesis of silver nanoparticles, $AgNO_3$ solution (solution B) was added into solution A containing green tea extract with continuous stirring on a magnetic stirrer. The mixed solution was left for 1 to 2 hours until there was a colour change from yellow to black or dark grey. The solution was then centrifuged at 11,000 rpm for 20 minutes. The supernatant was discarded and anhydrous (99%) ethanol was then added to the resulting pellet. The pellet was then ultrasonicated in a sonicator for 5 minutes. The solution was then recentrifuged at same previous settings, supernatant was discarded, anhydrous ethanol was added and these steps were repeated 2 to 3 times. The resulting pellet was then oven dried overnight. The dried pellet was scraped, weighed and stored in an amber coloured glass bottle covered with aluminium foil at room temperature [25,26] The purpose of the study was to develop a transdermal tolterodine tartrate (TT.

Characterisation of synthesized silver nanoparticles

The dried green tea nanoparticles were characterized for determination of their morphology and properties. The synthesized silver nanoparticles were characterized by using Fourier Transform Infrared spectroscopy (FTIR) and Transmission Electron Microscopy (TEM).

Fourier transform infrared spectroscopy (ftir)

The synthesized green tea AgNPs were analysed using FTIR Spectroscopy. FTIR (Perkin Elmer, Germany) was used to characterize the functional groups and composition components of synthesized silver nanoparticles.

Transmission electron microscopy (tem)

The surface morphology and particle size of synthesized green tea AgNPs were analysed using JEM-3100F transmission electron microscope (TEM) (JEOL, Tokyo, Japan).

Animals studies

A total of twenty-four Wistar albino rats (male and female) weighing between 70g to 130g were used for the purpose of this study. Animals were kept at 25 ± 2°C of under a 12 hour light and dark cycle. Throughout the study period, the male and female rats were kept in separate cages to avoid breeding. The animals were given free access to standard diet and water *ad libitum*. The food, water and cages were changed daily, and bedding materials were changed every once a week.

Acute toxicity study

A total of six Wistar albino male rats, 2 – 3 months old, were used in this present acute toxicity study to evaluate the acute toxicity of oral administration of the green tea nanoparticles. All rats were maintained under constant condition at a temperature of 25 ± 2°C with 12 hours light and dark cycles. All animals were allowed free access to standard diet and clean tap water *ad libitum* except when fasting was needed. All rats were fasted overnight prior to drug (green tea nanoparticles) administration, they were weighed, and doses were calculated. Single oral dose of 2000mg/kg body weight of the nanoparticles was administered orally to each rat respectively according to body weight. Food and drinks were given after 3 - 4 hours of dosing. The animals were closely observed for the first 30 minutes and then were kept under observation for a period of 2 hours, 4 hours for any behavioral changes such as restlessness, tremors, and gait. All rats were further observed for 72 hours period for any mortality[27]. Further, the animals were kept under observation daily for one month to observe for occurrence of any abnormalities.

Study design for colon cancer

The animals were randomly divided into five groups. Firstly, all rats were given subcutaneous injection of 1,2-Dimethylhydrazine (DMH) once weekly for a total of 5 weeks at a dose of 65mg/kg body weight except for rats in Group 3 (Negative control). Rats in Group 1, designated as the Disease control (n = 6) were not given any treatment and only received DMH, a cancer inducing agent. Rats in Group 2 (Positive control, n = 6) received an intraperitoneal (*i.p.*) injection of 5-FU at a dose of 50mg/kg body weight once weekly for 16 weeks. Rats in Group 3 (Negative control, n = 6) only received normal diet and clean tap water. Rats in Group in 4 (GTNP, n = 6) were treated daily with freshly prepared green tea extract nanoparticles that were administered orally by oral gavage. Rats in Group 5 (GTNP + 5FU, n = 6) were treated with green tea nanoparticles (*p.o.*) once daily and 5FU (*i.p.*) once a week. Green tea nanoparticle treatments, for Groups 4 and 5, were given at a dose of 50mg/kg body weight. All treatments were started a week after the first DMH injection and given daily for a total of 16 weeks. The body weights of all rats were measured at the beginning of the study and once weekly until all the animals were sacrificed.

Organ collection

At the end of the study, all animals were sacrificed by cervical dislocation method. The rats were dissected, and their abdomens were cut open. The liver, kidney, spleen, and colon were removed from the body. Any external connective and adipose tissues were trimmed and washed with 0.9% saline (NaCl). All organs were weighed, recorded, and stored until further use.

Tumor analysis

The colons were excised and cut opened longitudinally exposing the mucosal surfaces and washed with 0.9% saline solution. The colons were carefully inspected for any visible tumors with a characteristic raised lump were recorded. The three dimensions of the tumors namely, length, width and depth were measured using a digital Vernier caliper for calculation of each tumor volume using the formula: Tumor volume (mm^3) = length x width x depth x π/6 [28]. The colon was then extended between two filter papers and fixed in 10% neutral buffered formalin for 24 hours at room temperature.

Aberrant crypt foci (ACF) analysis

After 24 hours of fixation in 10% formalin, the colon tissue sections were stained in 0.2% methylene blue solution for 5 to 10 minutes followed by one rinsing wash with distilled water. The colon section was then placed on a microscope glass slide with the mucosal surface uppermost and viewed under light microscope (Olympus BX41) at a magnification of x40. The number of ACF in each section were counted and the number of Aberrant Crypts (ACs) in each ACF were also evaluated and classified as 1AC, 2ACs, 3ACs and ≥4ACs. 1AC, 2ACs and 3ACs were categorized as small ACF and ≥4ACs classified as large ACF.

Colon length to weight ratio

The isolated colons were measured for their length in centimeters and weight in grams and the colon length to weight ratio was calculated as follows [29].

$$\text{Length to weight of colon} = \frac{\text{Colon length (cm)}}{\text{Colon weight (g)}}$$

Relative organs weight index

Organs such as spleen, kidney, liver and colon were collected, and their weights were measured. The relative organs weights index were calculated as follows [29].

$$\text{Spleen, kidney, liver or colon index} = \frac{\text{Weight of spleen, kidney, liver or colon}}{\text{Final body weight (g)}}$$

Histopathological analysis of colon

After fixation, the colon samples were dehydrated in ascending concentrations (50%, 70%, 95%, and 100%) of absolute ethanol with incubation time of 1 hour each. The sections were then cleared in xylene or histoclearing agent and embedded in paraffin wax. The embedded colonic tissue was then cut into 5µm thick and placed on 3-aminopropyltriethoxysilane (APES) coated slides for hematoxylin and eosin (H&E) staining. The slides were then deparaffinized by incubating in xylene and ethanol, followed by H&E staining, and were observed under light microscope at 40X magnification to evaluate the histoarchitecture of colonic mucosa.

Statistical analysis

All data were expressed as mean ± SEM and the statistical analysis was carried out using one-way Analysis of Variance (ANOVA) and T-test for comparison between groups, by Graph Pad Prism software version 8.0.2. $p<0.05$ was statistically significant different.

Visual observation

A colour change was observed when silver nitrate solution was added into a flask containing green tea extract (Figure 1). The colour of the

solution changed to dark grey or black after 1-2 hours on constant stirring. The colour changes indicate the formation of silver nanoparticles in the solution. The formation of silver nanoparticles was further confirmed by FTIR and TEM analysis.

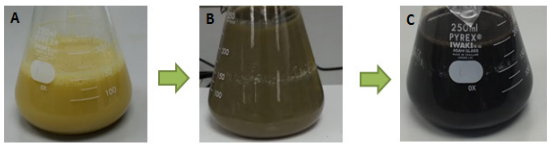


Fig. 1. Colour changes observed during the preparation of green tea nanoparticles. (A) Green tea extract; (B) Mixture of green tea extract + 0.1M $AgNO_3$ after 1 hour of stirring; (C) Mixture of green tea extract + 0.1M $AgNO_3$ after 2 hours of stirring.

FTIR analysis

The spectra analysis for green tea silver nanoparticles was obtained using an FTIR spectrophotometer as shown in Figure 2. Several peaks were observed which indicated the presence of various functional groups in the synthesized silver nanoparticles.

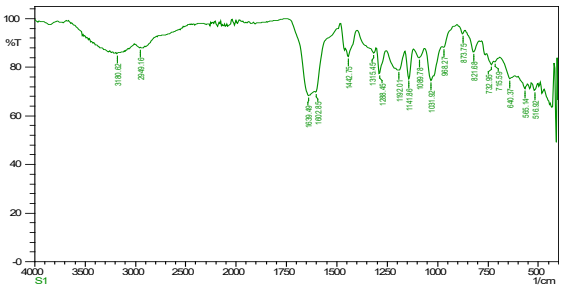


Fig. 2. FTIR spectra of synthesized green tea silver nanoparticle

TEM analysis

Transmission electron microscopy (TEM) has been used to identify the morphological characteristics like size and shape of synthesized silver nanoparticle. The TEM images of silver nanoparticles are shown in Figure 3. It showed that the synthesized silver nanoparticles have different shape such as spherical, hexagonal, pseudo spherical and square shape.

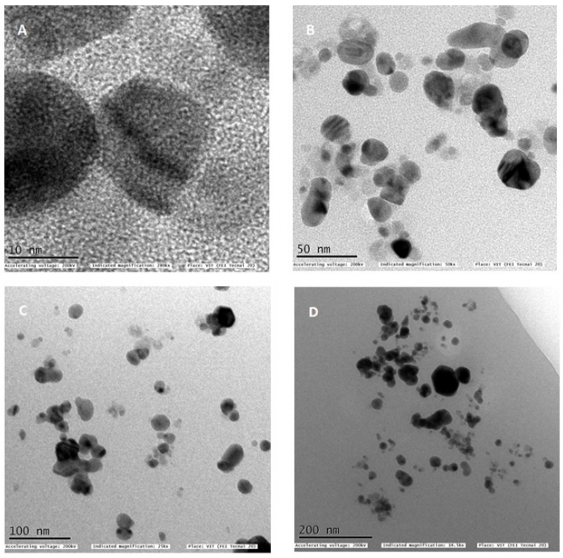


Fig. 3. TEM images of synthesized green tea silver nanoparticles at different magnification. (A) 10nm; (B) 50nm; (C) 100nm and (D) 200nm

Acute toxicity study

In the acute toxicity study, at a dose of 2000mg/kg body weight of green tea nanoparticles, there was no abnormal behavioral changes observed in all rats at a period of 2 and 4 hours. No mortality was observed during the experimental period.

Colon cancer by DMH induction

In this study, 1, 2-dimethylhydrazine (DMH) induced colorectal cancer was used as experimental animal models in evaluating the chemo-preventive effects of green tea nanoparticles and the synergistic inhibitory effects of green tea nanoparticle on 5-fluorouracil chemotherapeutic drug with a focus on the examination of ACF formation and tumor analysis in DMH induced rats. The use of DMH induced animal models is an ideal model for chemoprevention studies as it mimics most of the clinical, histopathological and molecular features of human colorectal cancer such as pre-neoplastic lesion, aberrant

crypt foci (ACF) formation, *K-RAS* mutation and inflammation [30]. DMH induces DNA damages and affects the liver, colon and ileum. It is metabolized in the liver by a series of reactions forming intermediates carcinogenic substances namely, methylazomethanol and azoxymethane. Methylazomethanol can be transported to colon via bile or blood to produce its carcinogenic metabolite, methydiazonium ion.

Colon length to weight ratio

Table 1 shows the initial and final body weights of each group of experimental animals. Throughout the 16 weeks of study, all rats in each group showed a gain in body weight. However, when compared to negative control group, the final body weights of all DMH-treated groups, except 5FU group (DMH, GT and GTFU groups) decreased. Nevertheless, statistical analysis revealed that there were no significant differences between both DMH and GT groups versus negative control group (p = 0.065). Meanwhile, there was significant decrease in body weight of GTFU group in comparison to negative control group (213.67 ± 0.88 *vs* 431.00 ± 16.60, p < 0.001). 5FU group has the highest final body weight compared to both DMH and negative control group, however there were no significant difference between them (p = 0.796).

DMH: 1,2-Dimethylhydrazine; 5FU: 5-Fluorouracil; GT: Green tea nanoparticles; GTFU: Green tea nanoparticles + 5FU; SEM: Standard errors of mean. Data are represented as

Table 1 Initial and final body weights in grams (g), relative organ index uracil; GT: Green tea nanoparticles; GTFU: Green tea nanoparticles + 5FU; SEM: Standard errors of mean. Data are represented as mean ± SEM mean ± SEM [a]p < 0.001: weight of negative control *vs* GTFU, weight of GT *vs* GTFU, weight of DMH *vs* GTFU, spleen index of negative control *vs* GTFU; [b]p < 0.05 weight of DMH vs GT

Group	Weight in (g)		Weight of organs (g)			
	Week 0 (Initial)	Week 16 (Final)	Spleen index	Liver index	Kidney index	Colon L/W ratio (cm/g)
DMH	133.33 ± 0.88	430.67 ± 11.78[a]	0.129 ± 0.029	3.198 ± 0.121	0.296 ± 0.016	10.38 ± 1.951
5FU	94.00 ± 1.00	433.00 ± 8.74	0.174 ± 0.011	3.365 ± 0.133	0.307 ± 0.012	7.33 ± 1.371
GT	75.67 ± 1.76	381.67 ± 4.84[b]	0.181 ± 0.013	3.120 ± 0.182	0.253 ± 0.036	9.11 ± 0.407
GTFU	70.67 ± 5.61	213.67 ± 0.88[a]	0.241 ± 0.016[a]	3.249 ± 0.099	0.440 ± 0.094	12.61 ± 0.379
Negative control	103.67 ± 7.03	431.00 ± 16.60	0.181 ± 0.001	3.63 ± 0.174	0.280 ± 0.027	8.30 ± 1.486

Relative organ index and colon length to weight ratio

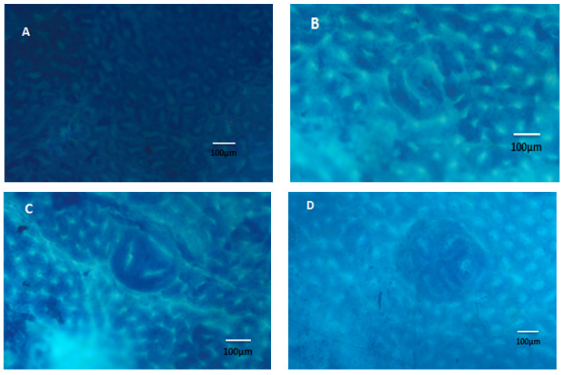
The relative organ indices for spleen, liver and kidney of the green tea nanoparticles treated animals were determined at the end of the study to evaluate whether green tea nanoparticles administration resulted in any side effect or toxicity to the experimental animals. During the entire experiment, there was no sign of toxicity or condition suggesting adverse reaction caused by administration of green tea nanoparticles. Based on the gross examination of spleen, liver and kidney, there were no abnormalities observed in all groups. Table 4 showed that spleen, liver and kidney indices in the GT treated animals (GT group) showed no significant differences compared to negative control (GT *vs* negative control; Spleen, p = 0.990; Liver, p = 0.103; Kidney, p = 0.567).

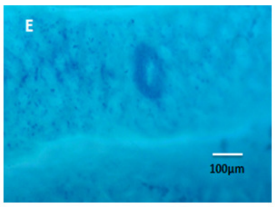
ACFs formation and ACs analysis

Aberrant crypt foci (ACF) were identified and differentiated from the surrounding normal crypts by their enlarged size with dark-

er and thicker epithelial lining than normal crypt and large luminal opening (**Figure 4**). In this study, the ACF formation in the colonic mucosa of experimental animal models was assessed by recording two ACF parameters; number of ACF and number of ACs in each ACF which were classified as small ACF containing 1AC, 2ACs, 3ACs and large ACF with ≥ 4ACs. After daily treatments with 5FU, GT and GTFU, ACFs were identified in all rats in DMH group (disease control), 5FU group (positive control) and treated (GT and GTFU groups). No ACF were identified in normal group (negative control).

Fig. 4. Topographical view of aberrant crypt foci (ACF) stained with methylene blue in the control and treated rats (x40). (A) Normal colon tissue; (B) ACF with 2 crypts; (C) ACF with 3 crypts; (D)





Large ACF containing ≥4 crypts; (D) ACF with a single crypt. Scale bars = 100µm.

The results showed that in Table 2 and Figure 5A, the total number of ACF was highest in the DMH group (37.33 ± 3.18) compared to all treated groups (5FU, GT and GTFU). GT treatment alone has markedly reduced the total number of ACF as compared to DMH group (25.00 ± 2.31 *vs* 37.33 ± 3.18, $p < 0.05$). In a combined treatment (GTFU) group, the total number of ACFs was significantly reduced as compared to DMH group (13.33 ± 0.88 vs 37.33 ± 3.18, $p = 0.001$). In addition, there was a significant decreased in total ACF number in combined treatment of GT and 5FU compared to GT alone (13.33 ± 0.88 vs 25.00 ± 2.31, $p < 0.05$). The total number of ACF in 5FU treatment alone was significantly reduced when compared to combined GTFU treatment (5.67 ± 2.19 *vs* 13.33 ± 0.88, $p < 0.05$).

DMH: 1,2-Dimethylhydrazine; 5FU: 5-Fluorouracil; GT: Green tea nanoparticles; GTFU:

Table 2 Effects of 5-Fluorouracil (5FU), Green tea nanoparticles (GT) and their combined treatment (GTFU) on ACF formation and number of ACs in each ACF

Group	Total ACF *	Small ACF**	Large ACF***
DMH	37.33 ± 3.18	24.67 ± 1.20	12.67 ± 2.40
5FU	5.67 ± 2.19[c]	4.00 ± 1.00[c,d]	1.67 ± 1.20[a]
GT	25.00 ± 2.31[a,c]	13.33 ± 2.19[d]	11.67 ± 0.88
GTFU	13.33 ± 0.88[b]	10.33 ± 0.33[d]	3.00 ± 5.20[a,e]
Negative control	0	0	0

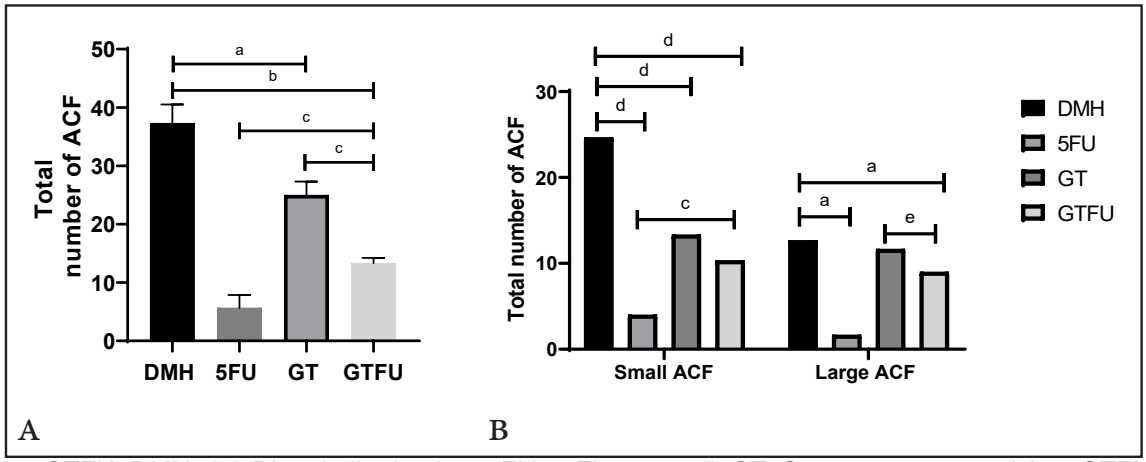
Data were represented as mean ± SEM.
[a]$P < 0.05$ *vs* DMH; [b]P = 0.001 *vs* DMH; [c]$P < 0.05$ *vs* GTFU; [d]$P < 0.001$ *vs* DMH; [e]$P = 0.001$ *vs* GT.
[a]$p < 0.05$ GT *vs* DMH; [b]$p = 0.001$ GTFU *vs* DMH; [c]$p < 0.05$ GT *vs* GTFU, 5FU vs GTFU; [d]$p < 0.001$ For small ACF 5FU *vs* DMH, GT *vs* DMH, GTFU *vs* DMH; [e]$p = 0.001$ *vs* GT. For small
*Total ACF was calculated as the sum of 1AC, 2ACs, 3ACs and ≥ 4ACs.
**Small ACF was classified as the sum of ACF with 1AC, 2ACs and 3ACs.
***Large ACF was classified as the sum of ACF with ≥ 4ACs.

Green tea nanoparticles + 5-Fluorouracil; ACs: Aberrant crypts; ACF: Aberrant crypt foci; SEM: Standard errors of mean.

The number of small ACF was significantly reduced in all treatment groups, 5FU (4.00 ± 1.00 *vs* 24.67 ± 1.20, $p < 0.001$), GT alone (13.33 ± 2.19 *vs* 24.67 ± 1.20, $p < 0.001$) and GTFU (10.33 ± 0.33 *vs* 24.67 ± 1.20, $p < 0.001$) when compared to the DMH group as shown in Table 2 and Figure 5B. The number of small ACF was reduced in GTFU compared to GT treatment alone but not significantly different ($p = 0.2302$), however the number of small ACF in 5FU group was significantly decreased compared to GTFU ($p < 0.05$). The large ACF were significantly reduced in all treated groups in comparison to DMH group (5FU *vs* DMH; 1.67 ± 1.20 *vs* 12.67 ± 2.40, $p < 0.05$ and GTFU *vs* DMH, 3.00 ± 5.20 *vs* 12.67 ± 2.40, $p < 0.05$), however the reduction in large ACF number between GT and DMH groups was not statistically different (11.67 ± 0.88 *vs* 12.67 ± 2.40, $p = 0.7160$). By comparing GT and GTFU, there was a significant decreased in large ACF number in GTFU compared to GT ($p = 0.001$). In comparison to 5FU, 5FU was the lowest in large ACF number than GTFU however the reduction was not significantly different ($p = 0.3739$).

Fig. 5. A Effects of different treatment on the number of ACF formation.

Each value is represented by mean ± SEM. [a]$p < 0.05$ *vs* DMH; [b]$p = 0.001$ *vs* DMH; [c]$p < 0.05$ *vs* GTFU. DMH: 1,2-Dimethylhydrazine; 5FU: 5-Fluorouracil; GT: Green tea nanoparticles; GTFU: Green tea nanoparticles + 5-Fluorouracil; ACF: Aberrant crypt foci.



B Effects of different treatment on the number of small and large ACF.

[a]$p < 0.05$ *vs* DMH; [c]$p < 0.05$ *vs* GTFU; [d]$p < 0.001$ *vs* DMH; [e]$p = 0.001$ *vs* GT. DMH: 1,2-Dimethylhydrazine; 5FU: 5-Fluorouracil; GT: Green tea nanoparticles; GTFU: Green tea nanoparticles + 5-Fluorouracil; ACF: Aberrant crypt foci.

In addition, many experimental studies have been conducted and showed that green tea and its tea polyphenols have chemo-preventive and inhibitory effects on colorectal carcinogenesis in rodent induced colorectal cancer [31]. A study conducted by Xu et al., shown that green tea treated rats had significantly inhibited the mean number of ACF [32]. Another similar study which was done by Xiao et al., demonstrated that Polyphenon E (a standardized green tea preparation with 65% of EGCG and 22% other catechins) treated rats has significantly decreased in the total number of ACF [33]. The chemopreventive effect of green tea is mainly attributable to its most abundant and active tea polyphenol catechins, (-)-epigallocatechin-3-gallate (EGCG).

Various studies have shown that EGCG play an important role in cancer prevention by inhibiting anti-cancer molecular targets and cancer related cellular processes such as downregulation of NF-kB, targeting the mitogen-activate protein kinase signaling pathway and c-jun N-terminal kinase pathway, triggering programmed cell death through upregulation Bcl-2 expressions, inhibition of angiogenic molecules such as VEGF, induction of cell cycle arrest and modulation of intracellular signaling pathways [13,34,35]. People who drink adequate amount of green tea would have less risk of developing colorectal cancer because of the scavenging effects of green tea in inhibiting cancer progression. The concept of nanochemoprevention involves the use of nanotechnology to improve the pharmacokinetic and pharmacodynamics of chemopreventive agents in order to prevent or slow-down cancer progression [36].

The aims of nano-carrier or nanoparticle in chemo prevention treatment are mainly to increase the bioavailability and for efficient delivery of chemo-preventive agents by protecting them from premature degradation, prolonged their circulation time and induce higher levels of specificity to targeted site [36]. The results of this study suggest that the effect of 5FU alone is more effective than combination of green tea nanoparticles and 5FU treatment. This indicates that EGCG present in the green tea nanoparticles does not enhance the chemotherapeutic potential of 5FU. The reasons for no synergism effect might be due to incompatible combination between silver nanoparticles and 5FU and possible interaction that occurs between 5FU and green tea nanoparticles which has led to no synergistic effects. However, an in vitro study conducted by Sun et al., on investigating the effect of the combination of EGCG and 5FU on human mammary gland adenocarcinoma cell line MCF7 have shown that EGCG enhanced the anti-tumor activity of 5FU through regulation of Bcl-xL expression [7]. The results in Table 4 showed that there was a significant increase in the spleen index of GTFU group compared to negative control group. An acute toxicity study performed by Wen et al., showed that the spleen of AgNP treated rats has the second highest level of silver detected and mild irritation was also observed in the spleen of animals treated with AgNPs [37]. This is in line with the current findings in which we assumed that there is no synergism which is due to the accumulation of AgNPs in the spleen that has led to low bioavailability of EGCG in the colon, thus less anti-cancer effects were observed which were shown by significantly increased ACF number in GTFU compared to 5FU alone. However, the nanoparticles toxicity effect needs to be confirmed by further studies.

Histopathological examination of colon

There were no macroscopic lesions or colorectal tumors identified in all DMH-induced groups. The histopathological examinations of colonic sections are shown in **Figure 6** at 40X magnification. The colon section of negative control groups showed normal histological features of the mucosa, submucosa and muscularis layer with mucosa gland lined with epithelial cells. All DMH-induced groups showed a normal colonic structure with no any abnormal

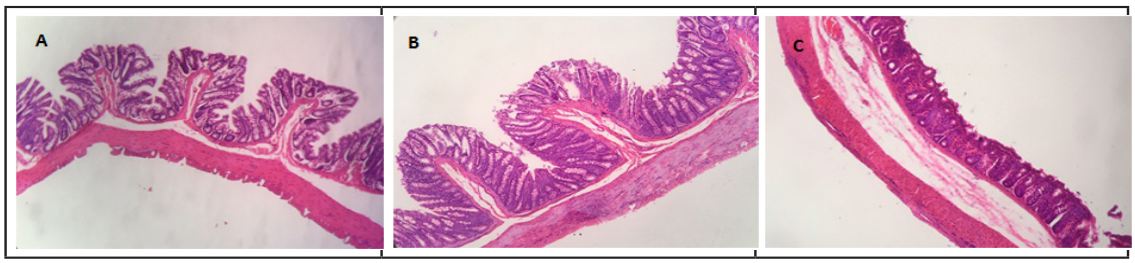


Fig. 6. Histopathological examination of H&E-stained colonic section at 40X magnification. (A) Negative control group; (B) DMH group; (C) GTFU group

sign of tumor progression particularly mild dysplasia[38,39]the mode of cell death by MM has not been investigated. Objective: We investigated the cytotoxic effects of MM in both human breast and lung cancer cell lines, MCF-7 and A549, respectively, and defined the mode of cell death. Materials and Methods: Cell viability was measured using the 3-(4-, 5-dimethylthiazol-2-yl.

Results and Discussion

FTIR and TEM analysis

The highest broad band was observed at 3180.62 cm^{-1} which indicated the presence of -OH group. The second highest band at 2949.16 cm^{-1} was seen which contributed to -CH group. The peaks at 1639.49 cm^{-1} and 1602.85 cm^{-1} corresponded to primary amine I group and secondary amine group respectively [27,40]. From the image, it was also observed that some particles were bound together which might be due to the presence of reducing agent. In the magnification of 10nm, spherical and square shaped nanoparticles were clearly seen. The size of the nanoparticles measured from the TEM image are between 3 -12 nm.

Colon Length to Weight Ratio

This metabolite elicits oxidative stress by methylating the biomolecules of epithelial cells in the proliferative compartment of the colonic crypts resulting in a great loss of colonic cells by apoptosis and increase in proliferation as well as mutation of the colonic epithelial cells [41]. There was significant increase in body weight between GT and DMH groups (381.67 ± 4.84 *vs* 430.67 ± 11.78, p <0.05). GTFU has the lowest final body weight compared to all of the other groups. There were significant decreased between GTFU with both GT and DMH groups (p < 0.001).

Relative organ index and colon length to weight ratio

These results indicate that the administration of green tea nanoparticles did not cause any increase in relative organ index and therefore indicated that there were no toxicity effects due to green tea nanoparticles to the rats. In addition, there were no significant differences in the relative organ indices between negative control and all the groups (**Table 1**). However, the spleen index of GTFU group compared to negative control group was statistically significant (0.241 ± 0.016 *vs* 0.181 ± 0.001, p < 0.01). In DMH, GT and GTFU groups, the colon length to weight ratio increased but were not significant when compared to negative control. In 5FU group, the ratio decreased however it was not significantly different as compared to negative control group (**Table 1**). The formation of adenomas and adenocarcinoma causes a decrease in colon length to weight ratio [29].

ACFs formation and ACs analysis

The results showed that in Table 2 and Figure 5A, the total number of ACF was highest in the DMH group (37.33 ± 3.18) compared to all treated groups (5FU, GT and GTFU). GT treatment alone has markedly reduced the total number of ACF as compared to DMH group (25.00 ± 2.31 *vs* 37.33 ± 3.18, p < 0.05). In a combined treatment (GTFU) group, the total number of ACFs was significantly reduced as compared to DMH group (13.33 ± 0.88 vs 37.33 ± 3.18, p = 0.001). In addition, there was a significant decreased in total ACF number in combined treatment of GT and 5FU compared to GT alone (13.33 ± 0.88 vs 25.00 ± 2.31, p < 0.05). The total number of ACF in 5FU treatment alone was significantly reduced when compared to combined GTFU treatment (5.67 ± 2.19 *vs* 13.33 ± 0.88, p < 0.05).

Aberrant crypt foci (ACF) are the pre-cancerous lesion or pre-neoplastic lesion of the colon. It has been accepted and regarded as an ideal biological index in evaluating the development of colorectal cancer [42]. ACF are present in the colon of carcinogen-treated rodents as well as in humans with high risk for colorectal cancer development and in those patients with colorectal cancer [31]. It is well known that car-

cinogen-induced ACF are the early indicators for the initiation of colorectal cancer in animal models (43). In addition crypt multiplicity may represent a step in promoting colorectal carcinogenesis (44).

The results showed that the administration of 50mg/kg body weight of green tea nanoparticles alone significantly inhibit the formation of ACF which were characterized by the reduction in the number of total ACF, small and large ACFs as compared to DMH group. Our results suggested that, the green tea silver nanoparticles have chemo-preventive inhibitory effects on colorectal carcinogenesis by inhibiting the ACF formation. Several epidemiologic studies have examined the association between green tea consumption and the risk of colorectal cancer. Many of the studies have found that the regular consumption of green tea was inversely associated with the risk of developing CRC particularly if such habit were maintained over a long duration [45,46]. It was also stated that the risk of CRC decreased as the amount of tea consumption increased (47).

In addition, many experimental studies have been conducted and showed that green tea and its tea polyphenols have chemo-preventive and inhibitory effects on colorectal carcinogenesis in rodent induced colorectal cancer [31]. A study conducted by Xu et al., shown that green tea treated rats had significantly inhibited the mean number of ACF [32]. Another similar study which was done by Xiao et al., demonstrated that Polyphenon E (a standardized green tea preparation with 65% of EGCG and 22% other catechins) treated rats has significantly decreased in the total number of ACF (33). The chemopreventive effect of green tea is mainly attributable to its most abundant and active tea polyphenol catechins, (-)-epigallocatechin-3-gallate (EGCG). Various studies have shown that EGCG play an important role in cancer prevention by inhibiting anti-cancer molecular targets and cancer related cellular processes such as downregulation of NF-kB, targeting the mitogen-activate protein kinase signaling pathway and c-jun N-terminal kinase pathway, triggering programmed cell death through up-regulation Bcl-2 expressions, inhibition of angiogenic molecules such as VEGF, induction of cell cycle arrest and modulation of intracellular signaling pathways (13,34,35). People who drink adequate amount of green tea would have less risk of developing colorectal cancer because of the scavenging effects of green tea in inhibiting cancer progression. The concept of nanochemoprevention involves the use of nanotechnology to improve the pharmacokinetic and pharmacodynamics of chemopreventive agents in order to prevent or slow-down cancer progression (36).

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current findings in which we assumed that there is no synergism which is due to the accumulation of AgNPs in the spleen that has led to low bioavailability of EGCG in the colon, thus less anti-cancer effects were observed which were shown by significantly increased ACF number in GTFU compared to 5FU alone. However, the nanoparticles toxicity effect needs to be confirmed by further studies.

Histopathological examination of colon

The colon tissue sections obtained from all the DMH-induced groups rats were subjected to histopathological investigation and were not found to have abnormal changes in the colonic histo-architecture. In this study, DMH at a dose of 65mg/kg/week for a total of 5 weeks was used for induction following the protocol by Juca et al., [48]. In their study, at the end of 15 weeks it was found that the DMH induced group had developed areas of hyperplasia, mild dysplasia, severe dysplasia and carcinoma [48]. The number of ACF in DMH disease control group has been the highest amongst all the groups indicating that the DMH had been effective in inducing the ACF. Based on the ACF results (Table 6), the ACFs formed were significantly inhibited. This suggests that the given drug concentration (50mg/kg body weight) of green tea nanoparticles was sufficient to inhibit the ACF formation. The green tea nanoparticles were able to reduce the formation of ACF and thus led to no progression of ACF forming into tumor.

Conclusion

In conclusion, this study showed that green tea nanoparticles significantly inhibited ACF formation on DMH-induced colorectal cancer which has led to no tumor formation. The inhibitory effects are mainly attributed to its most active and abundant catechin, EGCG which are associated in modulating multiple signaling pathways involved in cancer carcinogenesis. This study also demonstrated that a combination of green tea nanoparticles and 5-fluorouracil drug had no synergistic inhibitory effect on the formation of ACF. However, Large ACF only significantly reduced by this combined therapy compared to small ACF. However, further studies should be carried out to determine the anti-cancer effects and the synergism between green tea nanoparticles and 5-fluorouracil for different stages of colon cancer. It also suggests that further studies need to be done on evaluating the effectiveness of green tea nanoparticles in drug delivery and examine the level of biomarkers changes that are involved in cancer growth and progression.

Acknowledgements

We sincerely thank PAPRSB Institute of Health Science, Universiti Brunei Darussalam for providing laboratory and animal facilities.

Competing interests

The authors declare that they have no competing interests.

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