



Raw Chicory Roots Powder has Impact on Various Biomarkers of Colorectal Cancer in Male Wistar Rats

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ABSTRACT

Probiotics modulate metabolism and ensure a healthy gastrointestinal tract that may prevent colorectal cancer (CRC) development. The present study was designed to explore the effects of chicory roots derived fructans, in raw form, against CRC in rats. After acclimatization period, forty male Wistar rats were randomly and equally divided into four groups. Chicory powder treatments were started to group G4 before administering carcinogenic injections. After one-month, groups G2-G4 received four subcutaneous injections of carcinogen 1,2-dimethylhydrazine dihydrochloride (DMH) at dose of 40 mg/kg body weight, twice a week for 2 weeks. After DHM injections, chicory powder treatments were started to group G3 as well and group G2 was DHM control group receiving only basal diet while G1 was normal control group. The amount of chicory roots powder given was 5% w/w mixed in basal diet. It was observed that group G4 showed better results overall. Enzymatic activities of pathogenic bacteria were lowest in this group and were significant ($p \leq 0.05$) when compared to group G2. Percentage of total aberrant crypt foci (ACF) inhibition was also higher (30.14%) in group G4 as compared to group G3 (18.55%). Elevated levels of short chain fatty acids were observed in chicory root powder groups particularly in G4 which having significant higher levels of acetate and butyrate compared to group G2. Group G4 also hindered body weight loss due to carcinogenesis effect. It can be concluded that chicory root powder has protective impact against CRC biomarkers particularly if it is taken as preventive measure.

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Authors' Contribution

TRQ completed animal trial, performed analysis and prepared initial manuscript. SI conceptualized the idea, supervised trial and analysis, provided raw material supplies and edited the manuscript. MN helped in data analysis and edited the manuscript. HUR improved discussion and finalized for submission.

Key words

Colorectal cancer, Probiotics, Chicory, Aberrant crypt foci, SCFA's

INTRODUCTION

Chicory (*Cichorium intybus* L.) is an herbaceous plant belonging to the family Asteraceae. It is normally grown as fodder for livestock, however, it is extensively cultivated in Belgium, France, Netherlands and Germany as coffee replacer due to its natural bitter taste (Janda *et al.*, 2021) and its extracts are used in alcoholic and non-alcoholic beverages (Kaur and Gupta, 2002). Furthermore, it is a Mediterranean plant and its roots are used as additive in wide range of food products after processing along with sugar beet and rye especially during economic crises (Luzina, 2013). The fresh chicory roots

contain 15-20% inulin, the dried roots have 68% inulin, 14% sucrose, 6% protein, 5% cellulose, 4% ash and 3% other compounds (Nwafor *et al.*, 2017) while the extracts have up to 98% inulin and other fructans (Milala *et al.*, 2009). This composition makes chicory a functional food which gives some extra health benefits other than nutrients.

The major active ingredient in chicory roots is inulin categorized as prebiotic and is considered to be very effective against various diseases. While minor bioactive ingredients include several phenolic compounds such as chicoric acid in leaves, and chlorogenic acid and other antioxidant compounds in roots (Janda *et al.*, 2021). These phytochemicals can be extracted from different parts of chicory and have proven health benefits (Cunningham, 2010; Janda *et al.*, 2021). Inulin is a fructose-based polysaccharide having glucosyl moieties at terminal and are linked with β -(2,1) bonds (Petrovsky, 2010). This glycosidic linkage makes it non-digestible by mammalian

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Abbreviations

ACF, Aberrant crypt foci; CRC, Colorectal Cancer; DMH, 1,2-Dimethylhydrazine; FOS, Fructo-oligosaccharides; GOS, Galacto-oligosaccharides; SCFA, Short chain fatty acids.

gastrointestinal digestive enzymes and inulin exhibit reduced calorie, dietary fiber and functional properties (Roberfroid, 1999). The degree of polymerization ranges from 2-60 units of fructosyl moieties. Hydrolysis of inulin resulted in fructans, short and long-chain fructo-oligosaccharides (FOS). The inulin and FOS are selectively fermented by beneficial bacteria in the colon and produce short chain fatty acids (SCFA) mainly acetate, butyrate and propionate which reduce the pH of colon (Gibson *et al.*, 2004). These probiotics facilitate host metabolism, improve minerals absorption, directly boost immunity, increase mucin secretion, competitively remove pathogenic microbes and ultimately improve health of the host (Gibson *et al.*, 2004; Saulnier *et al.*, 2009; Slavin, 2013). Some of the confirmed and postulated health benefits of inulin and FOS (Slavin, 2013) are anti-inflammatory (Seifert and Watzl, 2007). They are known to reduce stress, prevent and/or delay the onset of diabetes, aid gut health, have bifidogenic effect, relieve constipation (Linetzky *et al.*, 2012), prevent or manage of various types of cancers especially colorectal cancer (Qamar *et al.*, 2017).

Colorectal cancer (CRC) is the 3rd most common cancer among men and women after breast and prostate cancers and is responsible for large number of deaths annually around the globe (Siegel *et al.*, 2017). It is the second cause of death from tumors developed in gastrointestinal tract especially colon and rectum. CRC prevalence is more in elder age group as more than 58% of new cases diagnosed after the age 65 years in USA. Intake of red meat especially processed and smoked meat, smoking, higher BMI, sedentary life style, elder age, genetic factors, increased alcohol intake, less intake of fresh fruits and vegetables, pulses, dietary fibers, deficiency of important vitamins/ minerals especially calcium and vitamin D (Chan and Giovannucci, 2010) and exposure to environmental pollutants are the major reasons for high incidence of CRC (Johnson *et al.*, 2013; Siegel *et al.*, 2017). Almost all factors are prevailing in developing countries like Pakistan with increased prevalence of CRC. The CRC is preventable as well as curable if diagnosed at early stages. As CRC does not always cause symptoms, it is highly recommended for its screening in susceptible individuals on the basis of risk factors, previously mentioned. The most important biomarkers for initial screening of CRC are blood and stool genetic and protein biomarkers, colonic microflora and their enzymatic activities like SCFA's (acetate, butyrate, propionate and others) (Lupton 2004), enzymes (β -Glucosidase, β -Glucuronidase, Nitroreductase, Azoreductase) (Qamar *et al.*, 2016, 2017) and on later stages aberrant crypt foci (ACF) (Akiko *et al.*, 2015) and pedunculated colon polyp (Lech *et al.*, 2016).

It is strongly recommended by nutritionists and dietitians to adopt healthy life style along with balanced and healthy diet having functional foods especially prebiotics and probiotics in routine life to prevent CRC. Several laboratory animal trials (Cunningham, 2010; Slavin, 2013) and meta-analysis (Johnson *et al.*, 2013) concluded that inclusion of dietary fibers, prebiotics especially inulin, FOS and galacto-oligosaccharides (Qamar *et al.*, 2016, 2017) and probiotics mainly lactobacilli and bifidobacteria in routine diet are strongly associated with reduction of CRC biomarkers and colonic tumor inhibition. However, previously used prebiotics and functional dietary fibers especially inulin and FOS, were in purified form extracted after extensive and costly purifications steps, so it was direly needed to evaluate the potential of these fibers directly from their natural sources like chicory roots.

In the present study, raw, dried and powdered chicory roots as source of inulin were evaluated against CRC in Wistar rats induced by 1, 2-Dimethylhydrazine (DMH). The chicory roots were given before and after DMH treatment and weight gain, feed intake, SCFA's, ACF and bacterial enzymes were analyzed in various groups as CRC biomarkers. The main results of the study showed that chicory roots powder have more preventive effect rather than curative.

MATERIALS AND METHODS

Chemicals, reagents and enzymes

Analytical grade chemicals and reagents were procured from Merck Chemicals (Darmstadt, Germany) unless otherwise explained. Sigma-Aldrich (St. Louis, MO, USA) was the supplier for DMH used for CRC induction in rats, m-nitrobenzoic acid, phenolphthalein- β -D-glucuronide, and nitrophenyl- β -D-glucoside used as substrate for enzymatic activity. Feed ingredients for rats were purchased from local market and were of highest quality and purity.

Preparation of chicory roots powder

Fresh chicory roots were obtained from local market. After washing, roots were dried at 70 °C for 24 h in oven (Mommert GmbH+Co. KG, Schwalbach, Germany; Modell 100-800), powdered in electric blender and stored at room temperature for further analysis and use in animal trial.

Animal housing and experimental design

Forty male Wistar rats having weight 130 \pm 5 g and age 6-7 weeks were procured from National Institute of Health, Islamabad and were housed in animal room of the University of Veterinary and Animal Sciences, Lahore at

25±2°C and 55±10% humidity under light and dark cycles of 12 h. The adaptation period was one week during which rats were fed on basal diet i.e. AIN-93G/M (Reeves *et al.*, 1993). All protocols were approved from Institutional Ethical Board for care of Lab Animals of the Institute.

After acclimatization, the rats were randomly divided into four groups each containing 10 rats: Group G1 (control group) was fed on basal diet, for 16 weeks; Group G2 (DMH control group treated with DMH) fed on basal diet, Group G3 (treated with DMH and fed on diet having 5% chicory roots powder in basal diet), G4 fed on basal diet with added chicory roots powder (5%) for first 4 weeks. After 4 weeks rats were treated with DMH and diet for continued for further 12 weeks.

Application of DMH

The groups G2 and G3 were treated with four subcutaneous injections, in two weeks twice a week, of carcinogenic chemical i.e., DMH, after adaptation period of one week while G4 was treated after 4 weeks. The DMH dose was 40mg Kg⁻¹ body weight, dissolved in ethylenediaminetetraacetic acid (EDTA) (Dias *et al.*, 2010; Qamar *et al.*, 2016, 2017). G1 was treated with EDTA in same pattern to provide similar conditions to all groups.

Body weight, feed and water intake

Body weight changes and feed intake were measured weekly using weighing scale (UX420H, Shimadzu, Japan) however water intake was taken on daily basis using graduated bottles.

Aberrant crypt foci (ACF)

To observe ACF in three parts of colon i.e., proximal, middle and distal (near rectum), the colon was opened longitudinally and using normal saline was rinsed to remove bowel contents. After that it was fixed in formalin buffer (10%) for 24 h at 25°C. All parts of the colon were stained with 0.2% methylene blue and ACF were counted under light microscope (Bird, 1995). The small, medium and large ACF were counted to calculate total number of ACF/colon.

Enzyme activities

To determine bacterial enzymatic activities in fecal samples, fresh fecal specimens were collected by squeezing the rectal region of rat and fresh pellets were collected. The samples were analyzed for various enzyme activities following procedure explained by Goldin and Gorbach (1976) with little modifications. The β-glucosidase and β-glucuronidase were measured as μg/min/mg of protein while nitroreductase, azoreductase were expressed as μg/h/mg cecal of protein. These modifications have been

previously explained in our studies (Qamar *et al.*, 2016, 2017). The total fecal protein contents were determined using Bradford method (Bradford, 1976).

Short chain fatty acids (SCFA)

The fresh pellets of fecal samples were stored at -80 °C for further analysis of SCFA's using gas chromatography (Agilent 6890 Plus, CA, USA). After thawing, 1g fecal sample was weighed and suspended in 5 mL of distilled and purified water. A 20% (w/v) suspension of fecal contents was prepared through homogenization (Ultra Turrax T 25, Staufen, Germany) for 3 min. The pH of samples was adjusted to 2-3 using hydrochloric acid followed by shaking for 10 min. The contents were centrifuged at 5000g for 15 min and supernatant was separated. The final, 1 mM concentration of 2-ethylbutyric acid was adjusted in supernatant as an external standard and concentration of various SCFA's was measured and expressed in μ mol/g of fecal contents (Zhao *et al.*, 2006; Qamar *et al.*, 2016).

Statistical analysis

All results were expressed as means ± standard error (SE). The difference between various groups was determined using one-way analysis of variance (ANOVA) on SPSS ver. 22. The differences were considered significant at p<0.05. Duncan Multiple Range Test as post-hoc was performed to observe level of significance among various groups.

RESULTS

Body weight and feed intake

The body weight gain and feed intake by different groups is given in Table I. The results showed that DMH induction significantly (p<0.05) reduced the weight gain in treated groups as G2 to G4 have significantly less weight as compared to G1, control group. The weight gain in G4, fed on chicory roots before DMH treatment, was significantly higher as compared to other groups followed by G3, fed on chicory after DMH induction and G2 (DMH control). The feed intake in all groups was significantly same.

Aberrant crypt foci (ACF)

The presence of ACF in different parts of colon of rats in various groups is presented in Table II. First of all, it is confirmed from the results that four subcutaneous injections of DMH @ 40mg Kg⁻¹ body weight was successful for induction of CRC in all treated groups. Group 2 (DMH control) had significantly higher number of ACF in all parts of colon and in total (169.2±6.98) as well when compared with G3 (137.8±6.07) and G4 (118.2±7.58). Interestingly, maximum number of ACF were observed in

Table I. Weight gain and feed intake by rats fed on various diets.

	G1 (Basal diet control group)	G2 (DMH control group)	G3 (Chicory after DMH)	G4 (Chicory before DMH)
Body weight gain (g)	279.8±3.98 ^a	161.8±4.22 ^d	181.5±4.51 ^c	209.7±3.69 ^b
Feed intake (g/ rat/day)	21.2±0.52	20.3±0.61	20.6±0.57	21.5±0.57

distal part of colon (near rectum) as compared to middle and proximal parts of colon in all groups. It is clear from the results that inclusion of chicory roots powder before DMH treatment (G4) significantly ($p<0.05$) inhibited the ACF formation in all parts of colon as compared to group G3 fed on chicory roots powder after induction of CRC. Furthermore, ACF inhibition was 30.14% in group G4 while 18.55% in G3 as compared to DMH control group. The ACF were present in the form of single, double, triple and clusters as well as in small and medium form as shown in Figure 1.

Table II. Aberrant crypt foci (ACF) in different parts of colon in different groups.

ACF	G1	G2	G3	G4
ACF/proximal colon	ND	22.08±2.39 ^a	19.38±1.90 ^b	19.23±1.74 ^b
ACF/middle colon	ND	39.23±2.92 ^a	32.53±2.93 ^b	28.1±2.89 ^c
ACF/distal colon	ND	107.9±6.45 ^a	85.8±4.33 ^b	70.9±4.13 ^c
Total ACF/colon	ND	169.2±6.98 ^a	137.8±6.07 ^b	118.2±7.58 ^c
% inhibition of total ACF	-	-	18.55%	30.14%

G1, control group (fed on basal diet); G2, DMH control group treated with DMH (fed on basal diet); G3, treated with DMH (fed on diet having 5% chicory roots powder in basal diet); G4 fed on basal diet with added chicory roots powder (5%).

Groups having same superscript letters in a row are statistically similar.

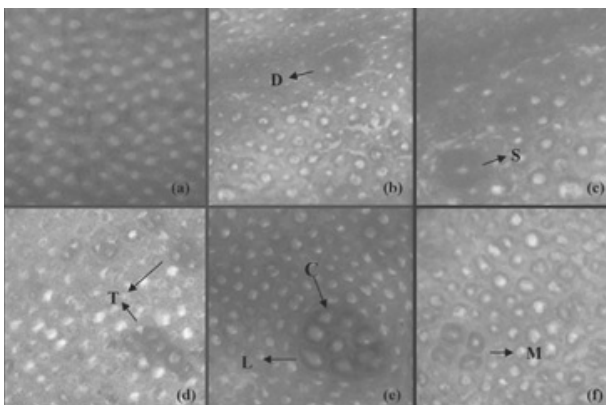


Fig. 1. Topographic view of colon showing: (a) normal crypts in colon of rats of normal basal group, (b–f) DMH treated rats showing ACF after staining with 0.2% methylene blue (40x). Arrows indicate ACF: S, single; D, double; T, triple; C, cluster; L, large; M, medium.

Bacterial enzyme activities

The activities of bacterial enzymes β -glucosidase, β -glucuronidase, nitroreductase and azoreductase in fecal specimens of various groups are mentioned in Table III. The results showed that enzymatic activities were significantly higher ($p<0.05$) in basal control and DMH control groups as compared to groups provided chicory roots powder in addition to basal diet either before or after DMH treatment. It can also be observed from Table III that activities of all enzymes were significantly lower in group 4 as compared to all other groups especially basal control and DMH control groups. In other words, it can be concluded that provision of chicory roots powder before induction of colon cancer was significantly better to reduce these enzymes activities as compared to chicory given after occurrence of CRC.

Short chain fatty acids (SCFA)

The amount of SCFA (acetate, butyrate and propionate) in fecal contents of rats of various groups fed on different diets are presented in Table III. The results showed that chicory roots powder significantly ($p<0.05$) enhanced the amount of all SCFA in fecal contents in group G3 and G4 as compared to control groups. Interestingly like ACF inhibition and enzyme activities, the impact of chicory powder was significantly better in group G4 provided prebiotic before CRC induction as compared to G3 fed on chicory after DMH treatment. It showed that chicory powder impact was preventive rather than curative. The amount of butyrate was significantly higher only in group 4 followed by G3 and G2. Furthermore, propionate activity was significantly higher in both chicory treated groups as compared to both control groups.

DISCUSSION

Colon cancer is one of the leading cause of deaths in the world especially in the countries where food supply chain is contaminated with aflatoxins, industrialized processed foods are consumed, meat products prepared directly on flame and malnutrition problems are prevailing (Lim *et al.*, 2005; Anand *et al.*, 2008; Chan and Giovannucci, 2010). In this scenario, it is major responsibility of nutritionists

and food scientists to explore natural indigenous sources which have potential to combat CRC. Furthermore, community nutritionists have to advocate the people to incorporate these functional foods in their routine diet. The main objective of the present study was to evaluate the preventive as well as curative role of chicory roots powder against various biomarkers of CRC.

The results showed that feed intake in all groups, irrespective of normal, DMH control and treatment groups, is statistically same however body weight gain is different (Table I). The carcinoma conditions due to DMH treatment in colon may restrict the minerals and vitamins absorption, resulting change in gastrointestinal metabolism, require more energy for cell division, change in bowel movement and loss of energy and ultimately reduction in weight (Jucá *et al.*, 2014). However, dietary fibers and prebiotics reverse the conditions and help in weight gain as observed in G4 as compared to G3 which received chicory roots after induction of CRC.

Table II shows that DMH induced dysplasia to carcinoma conditions in all parts of colon in groups G2-G4 as compared to control group. The DMH is more effective in induction of CRC, development of ACF in lab animals as compared to azoxymethane (Jucá *et al.*, 2014). The ACF are preneoplastic lesions initially developed in colon in case of exposure to any carcinogen and developed to adenomas or carcinomas if untreated and are normally used as early biomarker for CRC studies in animal models (Bird, 1995; Qamar *et al.*, 2016, 2017). It can be observed that maximum ACF were present in distal part of the colon near rectum, more than 60% in all groups as compared to proximal and middle parts of colon, even though the distal part is the small part of colon. It might be that, DMH has special impact near rectum as higher number of ACF observed in same area by other studies (Qamar *et al.*, 2016, 2017) conducted using DMH 20 mg/kg body weight for 15 weeks in rats (Sangeetha *et al.*, 2010) or two weeks in mice (Moulahoum *et al.*, 2018). It has been observed in multiple studies that incorporation of bioactive compounds (Dias *et al.*, 2010; DeLuca *et al.*, 2018), minerals (Moulahoum *et al.*, 2018), dietary fibers, prebiotics, probiotics and or synbiotics (Dias *et al.*, 2010; Gavresea *et al.*, 2018) significantly inhibit the development of ACF, not only their intensity as well as their distribution in the colon. Among the prebiotics inulin, GOS (Qamar *et al.*, 2016, 2017) lactulose and FOS have widely used in raw and pure form however, raw chicory roots powder has not been utilized in any cancer prevention study. It is also evident from table 3 that incorporation of chicory roots powder before DMH treatment was significantly ($p < 0.05$) effective as compared to after or during CRC induction. The impact of prebiotic inulin in inhibition of ACF is more

effective if given before DMH induction i.e. 51% (Qamar *et al.*, 2016) in comparison to 27% (Qamar *et al.*, 2017) if provided after DMH treatment showing better preventive role than curative. These findings are further supported by present study (Table II) however the percentage of ACF inhibition is less due to impure form of inulin in chicory roots as compared to our previous studies (Qamar *et al.*, 2016, 2017). It shows that prebiotic inulin in chicory roots and other similar foods like unrefined grains, fruits and vegetables in comparison to so called “western diet” enhance immune system, improve the balance of beneficial gut bacteria and can prevent or delay CRC (Anand *et al.*, 2008; Chan and Giovannucci, 2010).

The increased enzymatic activity of β -glucosidase, β -Glucuronidase, nitroreductase and azoreductase is considered as biochemical marker for colon cancer. It has been observed in several animal (Goldin and Gorbach, 1976; Qamar *et al.*, 2016, 2017) and human studies (Molan *et al.*, 2014) that activities of these enzymes significantly increase in CRC as compared to healthy individuals. These enzymes produce toxic compounds in the colon, convert inactive to active metabolites as well as convert procarcinogens into carcinogens which damage the normal mucosal cells (Goldin and Gorbach, 1976). While induction of dietary fibers, various functional foods like beet carrot juice, prebiotics, probiotics and synbiotic in routine diet is responsible for considerable decrease in gut enzymatic activities. It might be the reason that in present study, group G4 fed on chicory roots powder with normal diet before CRC induction has significantly reduced activity of all enzymes as compared to all other groups (Table III). Once the CRC is induced, dietary impact to treat disease is reduced as in the case of group G3 proving their more preventive role as compared to curative.

Table III. Enzymatic activities and short chain fatty acids (SCFA) in fecal contents of rats of various groups fed on different diets.

	G1	G2	G3	G4
Enzymes				
β -Glucosidase	0.991 \pm 0.11 ^a	0.992 \pm 0.11 ^a	0.915 \pm 0.99 ^a	0.884 \pm 0.10 ^b
β -Glucuronidase	3.33 \pm 0.19 ^a	3.32 \pm 0.18 ^a	2.95 \pm 0.16 ^{ab}	2.71 \pm 0.19 ^b
Nitroreductase	4.31 \pm 0.29 ^a	4.38 \pm 0.24 ^a	3.77 \pm 0.28 ^{ab}	3.38 \pm 0.29 ^b
Azoreductase	12.23 \pm 1.06 ^a	12.15 \pm 1.08 ^a	9.31 \pm 0.90 ^{ab}	8.92 \pm 0.99 ^b
SCFA				
Acetate	77.6 \pm 3.13 ^b	76.2 \pm 3.17 ^b	83.5 \pm 3.88 ^{ab}	87.2 \pm 3.35 ^a
Propionate	24.2 \pm 1.73 ^b	23.8 \pm 1.78 ^b	26.2 \pm 1.71 ^a	27.8 \pm 1.48 ^a
Butyrate	16.2 \pm 0.78 ^c	16.1 \pm 0.87 ^c	18.2 \pm 1.08 ^b	20.7 \pm 0.99 ^a

Groups having same superscript letters in a row are statistically similar. For details of experimental groups, see Table II.

The production of SCFA mainly acetic acid (C2:0), propionic acid (C3:0) and butyric acid (C4:0) in the colon is directly related to good health of colon while their decreased production is an indication of some underlying gut disturbance like imbalance of gastrointestinal microbiota, small intestinal bacterial overgrowth, colon cancer, etc. (Lupton, 2004; Zeng *et al.*, 2014). These SCFA are the metabolites of saccharolytic microbial activity, therefore, presence of non-digestible oligosaccharides like GOS, FOS and inulin based fructans in the colon is very much important (Saulnier *et al.*, 2009). This is evident from present study where all SCFA in groups G3 and G4 are significantly higher than basal and DMH controls (Table III). The mechanism of these dietary fibers is much more complicated as their fermentation by beneficial bacteria in the gut not only improves the growth of selective bacteria but also produce SCFA's mainly acetate, butyrate, propionate and in minor quantities iso-butyric acid, valeric acid and iso-valeric acid. The major SCFA's perform several physiological functions including energy to colonocytes, proliferate normal crypt cell especially acetate and help in colonic muscle contraction and blood flow (Zeng *et al.*, 2014). These SCFA's not only reduce the pH of colon to provide favorable conditions to probiotics but also work as ligands for expression of various anti-carcinogenic genes especially *FFAR2*, *FFAR3* and *GPR109A* through increased histone deacetylase activity (HDAC) and suppress the inflammatory genes (Pan *et al.*, 2018). Furthermore, DMH treatment decrease the defensive mechanism of the body against various free radicals which disturb the DNA and polyunsaturated fatty acids in the cellular membranes and is also involved in p53 mediated apoptotic pathway through change in expression of different genes (Walia *et al.*, 2018). The increased production of butyrate is also responsible for decrease in expression of *MUC4*, a gene directly related with CRC (Algamas-Dimantov *et al.*, 2013) using HNF-4 α as nuclear receptor. All these physiological and genetic factors affected by SCFA's ultimately help in reduction in CRC as observed in treatment groups (G3-G4) in current study as compared to DMH and basal control.

It can be concluded from the study that chicory roots powder as source of inulin is effective against various biomarkers of colon carcinogenesis i.e., ACF formation, SCFA's production and bacterial enzymatic activity. Furthermore, incorporation of chicory roots in routine diet before induction of cancer is more effective for all markers showing its more preventive role than curative. On the basis of final results, it can be advised that dietary fibers and their sources must be included in routine diet.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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