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ORIGINAL ARTICLE

Chemoprotective Effect of Crocetin Against 1,2 dimethyl Hydrazine induced Colorectal Cancer In Albino Wistar Rats through Antioxidant Pathway

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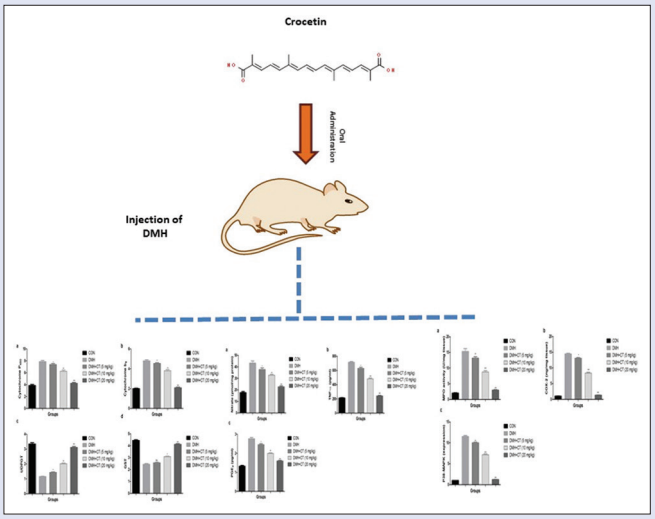
ABSTRACT

Background: Colorectal cancer is one of the chief causes of death and morbidity among all types of cancer worldwide, comprising both sexes. Crocetin (CT) is a major phytoconstituent of *Crocus sativus* L. which performed a number of pharmacological activities. The current study established CT's anticancer effect against 1,2-dimethylhydrazine (DMH)-persuaded colorectal cancer and discovered likely *in vivo* mechanisms. **Methods:** To tempt colorectal cancer in experimental albino Wistar rats, DMH was inserted subcutaneously. The rats were separated into five groups as follows: Group I – normal control rats, Group II – DMH-treated rats, Group III – DMH-treated rats receiving CT (5 mg/kg), Group IV – DMH-treated rats receiving CT (10 mg/kg), and Group V – DMH-treated rats receiving CT (20 mg/kg) for 10 weeks. At consistent intervals, the body weight and tumor weight were assessed. Biochemical, hepatic, antioxidant, Phase II antioxidant enzymes, inflammatory mediators, cytokine parameters, and apoptosis markers were projected at the end of the experimental study. **Results:** CT treatment suggestively augmented body weight ($P < 0.001$) and reduced tumor weight. CT administration also changed the level of antioxidant parameters – superoxide dismutase, glutathione peroxidase, and glutathione reductase; Phase I enzymes – cytochrome B_5 and cytochrome P450; and Phase II enzymes – glutathione-S-transferase and UDP-glucuronyltransferase, respectively. Attained results also reveal that CT treatment abridged the level of cyclooxygenase-2, prostaglandin-2, and nitric oxide and diminish the expression of p38 mitogen-activated protein kinases. CT also augmented the expression of apoptosis markers – caspase-3 and caspase-9. **Conclusion:** Thus, the complete outcomes recommended the chemoprotective role of CT against DMH-induced colorectal cancer through the inhibition of inflammation and apoptosis pathways.

Key words: Apoptosis, colorectal cancer, crocetin, cyclooxygenase-2, inflammation, mitogen-activated protein kinase-myeloperoxidase

SUMMARY

- Crocetin exhibited the chemoprotective effect against 1,2-Dimethylhydrazine induced colorectal cancer. Crocetin considerably reduced the antioxidant parameters, pro-inflammatory cytokine and inflammatory parameters. Crocetin considerably altered the caspase-3 and caspase-9 parameters. The result suggest the chemoprotective effect of crocetin against 1,2-Dimethylhydrazine induced colorectal cancer



Abbreviations used: DMH: 1,2-dimethylhydrazine; SOD: Superoxide dismutase; GR: Glutathione reductase; GSH-Px: Glutathione peroxidase; UDP-GT: UDP-glucuronyltransferase; GST: Glutathione-S-transferase; COX-2: Cyclooxygenase-2; PGE2: Prostaglandin-2; NO: Nitric oxide; p38-MAPK: p38 mitogen-activated protein kinase; WHO: World Health Organization; iNOS: Inducible nitric oxide synthase; DNA: Deoxyribonucleic acid; TNF-α: Tumor necrosis factor-α; MAM: Methylazoxymethanol; AOM: Azoxymethane; EDTA: Ethylenediaminetetraacetic acid; CT: Crocetin; PMSF: Phenylmethylsulfonyl fluoride; DCFH-DA: 2′,7′-Dichlorofluorescein diacetate; H_2O_2: Hydrogen peroxide; O_2: Superoxide.

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INTRODUCTION

The rate of incidence of colorectal cancer is greater among all types of cancer worldwide. Approximately 639,000 colorectal cancer-related deaths stated worldwide per year.[1] Report of the World Health Organization designated that colorectal cancer is the

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fourth most predominant cancer type and the third foremost cause of cancer-related death in established and developing countries.[2] Colorectal cancer changed due to the steady deposition of epigenetic and genetic variations that contribute to regular colonic epithelium transformation fallouts in colon adenocarcinoma.[3] Another deliberation in development of colorectal cancer is unnecessary feeding of high fat and red meat with low fruit, fibers, and vegetable.[4] Preceding studies confirmed that both parameters can play a key role in evolving colorectal cancer.[5,6]

It is well recognized that overexpression of COX-2 is related to colorectal cancer. An augmented level of COX-2 was detected in frequent premalignant as well as the malignant stage.[7] COX-2 has also been displayed to be rapidly caused in response to various growth factors such as hormones, tumor promoters, bacterial endotoxins, shear stress, and cytokines.[8] The continuous production of nitric oxide (NO) through inducible NO synthase can persuade deoxyribonucleic acid (DNA) injury, either directly or indirectly.[8] Earlier indication proposes that the different physiological conditions and plentiful pathological conditions play an important role in tumor development and inflammatory reactions.[9,10] Cytokines such as tumor necrosis factor-α (TNF-α) played an imperative role in the endurance and initiation of colorectal cancer. Formerly available studies showed that the activation of pro-inflammatory cytokines such as TNF-α is based on nuclear factor-kappa B (NF-kB) activation, which further hints to the inflammation and eventually inflammatory response.[9,10]

Dimethylhydrazine (DMH) is an alkylating agent, normally employed to induce colon cancer in *in vivo* studies. DMH-tempted colorectal cancer is a multistage process which involved several pathological variations comprising aberrant modification of the cryptic foci.[11] DMH metabolites are secreted in bile and are accountable for the carcinogenic colon impact after move through the digestive tract.[12] DMH formed intermediates in the hepatic tissue, such as methylazoxymethanol (MAM) and azoxymethane, which are relocated to the colon. MAM converted to methyldiazonium ion methylates cellular rudiments after decomposition process which, in turn, causes colon tumors.[12,13]

Saffron (*Crocus sativus* L.) is a food colorant and spice which is typically found in the dry stigmas of plant. Saffron has been usually employed as the traditional therapies for numerous diseases contain cancer in ancient Chinese, Arabian, and Indian cultures.[14] Crocetin (CT), a phytoconstituent of saffron, showed considerable potential as an anticancer agent in cell culture and rodent model. Earlier research recommended that CT inhibited the inflation of cancer cells through suppression of nucleic acid synthesis, increasing the anti-inflammatory and antioxidant system, hampering the growth factor and induced apoptosis pathway.[14,15] Furthermore, in this current experimental study, we observed the chemoprotective impact of CT on colorectal cancer which was persuaded by DMH and assessed the potential mechanism of action.

METHODS

Materials

Ethylenediaminetetraacetic acid, 1,2-DMH, CT, and phenylmethylsulfonyl fluoride were acquired from Sigma-Aldrich (St. Louis, MO, USA). 2′,7’-dichlorofluorescein diacetate was obtained from the Molecular Probes (Eugene, OR, USA). All the reagents and chemicals employed in the experimental study were procured from Sigma-Aldrich Co., Ltd. (St. Louis, MO, USA).

Animals

The current experimental study applied Swiss albino Wistar rats (140–170 g; 6–8 weeks). The experimental rats were obtained from the institutional animal house and kept in the standard laboratory condition (temperature: 20°C ± 5°C; relative humidity 60%–70%; 12/12-h dark/light cycle) and fed the standard food and water (*ad libitum*). The experimental protocol was permitted by the Animal Ethics Committee of the institute (SU/19/034).

Induction of colorectal cancer

Colorectal cancer was tempted in rats by injection of DMH subcutaneously at a dose of 40 mg/kg for 10 weeks.[16]

Experimental protocol

The rats were uselessly alienated into five groups after successful induction of colorectal cancer, and each group covers 15 rats. The groups of the animal are as follows:

- Group A: Sham control (received CMC)
- Group B: DMH control (40 mg/kg DMH)
- Group C: DMH control (40 mg/kg DMH) + CT (5 mg/kg)
- Group D: DMH control (40 mg/kg DMH) + CT (10 mg/kg) and
- Group E: DMH control (40 mg/kg DMH) + CT (20 mg/kg), respectively.

The blood sample of all group rats was composed at the end of the experimental study (16 weeks) by puncturing the retro-orbital plexus and centrifuged at 12000 rpm for 10 min at 4°C, and the supernatant was collected in the sample tubes and stored at −20°C for the assessment of biochemical parameters.

Biochemical parameters

Colon tissue was collected and employed for the approximation of CYP4502E1[17] and TNF-α as a previously available method with minor changes.[18]

Apoptosis markers

The expression of caspase-3 and caspase-9 was assessed in the colon tissue homogenates using the commercially accessible kits as per manufacture instructions.

Antioxidant parameters

The antioxidant parameters such as superoxide dismutase (SOD), glutathione reductase, and glutathione peroxidase (GPx) were projected with minor alterations using the formerly published method.[13,19-21]

Phase I enzymes such as cytochrome b5, cytochromeP450, and cytochrome C reductase and Phase II enzymes comprising UDP-glucuronyltransferase and glutathione-S-transferase were appraised with minor alterations using the earlier reported method.[22,23]

Deoxyribonucleic acid fragmentation

The DNA fragmentation was predictable with minor alteration using the beforehand reported method.[24]

Inflammatory parameters

Cyclooxygenase-2 (COX-2) (Catalog No: EH125RB) and myeloperoxidase (MPO) (Catalog No: BMS2038INST) activity were projected through enzyme-linked immunosorbent assay (ELISA) kits as per manufacture instruction (MyBioSource Inc., San Diego, California, USA).

Cytokine parameter

Cytokine that contains TNF-α (Catalog No: BMS223HS) was assessed by the ELISA kit (eBioscience, Inc., San Diego, USA) as per manufacture instructions.

Statistical analysis

For the statistical analysis, a one-way variance of analysis was followed through multiple tests by Dennett. The entire statistical analysis was carried out using the software GraphPad Prism 5. $P < 0.05$ values were observed as noteworthy.

RESULTS

Quantitative real-time polymerase chain reaction (qPCR)

To test qPCR, full RNA was effectively extracted and reversed depending on the treatment from the ischemic penumbras of the rats' brains. The entire experiment was carried out in accordance with the manufacturer's instructions. The normalised to β-Actin threshold process is used to quantify all data. Table 1 lists all of the primers.

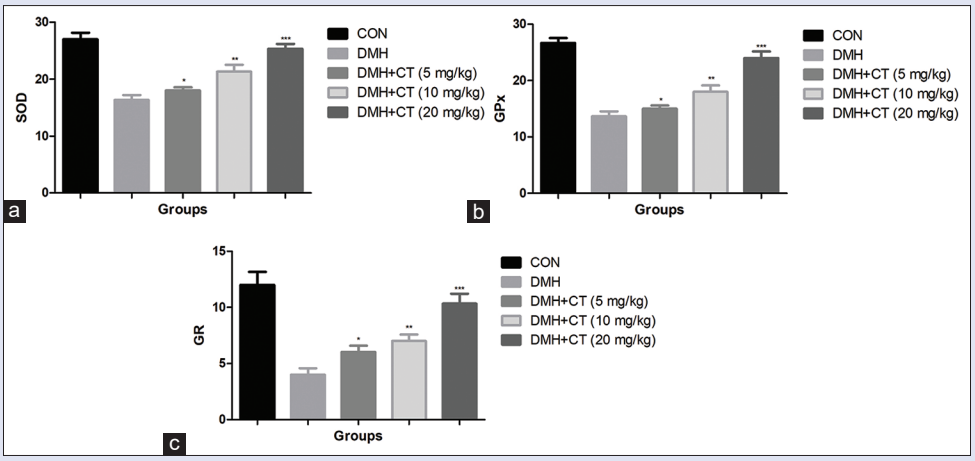

Figure 1: Effect of crocetin on the antioxidant parameter of control and experimental rats. (a) SOD, (b) GPx, and (c) GR. The data were shown as mean ± standard error of the mean. All tested groups compared with the DMH treated group, *$P < 0.05$, *$P < 0.01$, and * $P < 0.001$ considered as the significant, more significant, and extreme significant. Where CON: Control; DMH: 1,2-Dimethylhydrazine; CT: Crocetin; SOD: Superoxide dismutase; GPx: Glutathione peroxidase and GR: Glutathione reductase

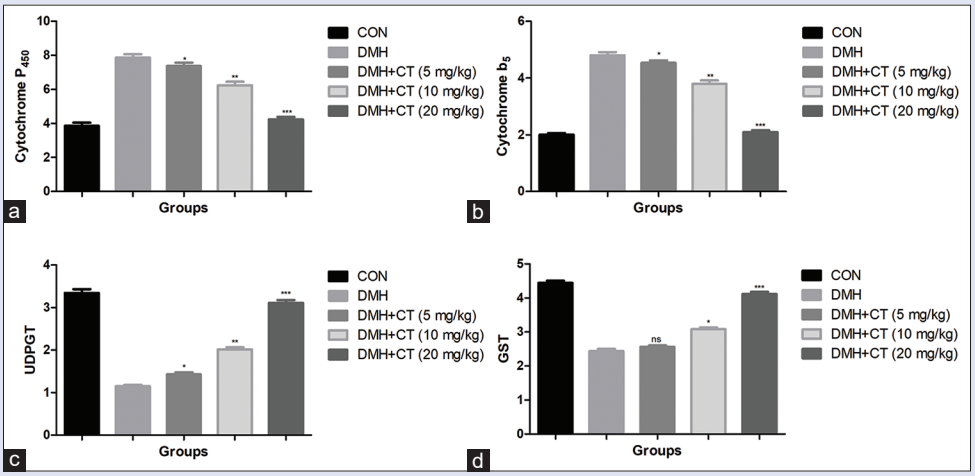

Figure 2: Effect of crocetin on Phase I and Phase II antioxidant enzymes of control and experimental rats. (a) Cytochrome P_{450}, (b) Cytochrome b_5, (c) UDP-GT, and (d) GST. The data were shown as mean ± standard error of the mean. All tested groups compared with the DMH treated group, *$P < 0.05$, *$P < 0.01$, and *$P < 0.001$ considered as the significant, more significant, and extreme significant. Where CON: Control; DMH: 1,2-dimethylhydrazine; CT: Crocetin; UDP-GT: UDP-glucuronyltransferase; GST: Glutathione-S-transferase

Table 1: List of primers

Genes	Primers	
	Forwarded	Reverse
P53	CCTCTTGCTTCTCTTT TCCTATCC	CTTGGTCTCCTCCACCGCTTCTTG
β-actin	CGGAGTCAACGGATTTGGTCGTAT	AGCCTTCTCCATGGTGGTGAAGAC

Bodyweight and tumor weight

In the current experimental study, all the rats of each group showed augmented body weight. In the DMH control group, rats displayed enlarged body weight compared to the initial body weight, but the DMH control group showed condensed body weight compared to the other group [Table 2]. In the group receiving CT (5 and 10 mg/kg), rats exhibited augmented body weight compared to the DMH group. The CT (20 mg/kg)-received group exhibited an increased body weight and followed nearly a similar design as the normal control group.

The normal control group presented no sign of tumor. The DMH-treated group showed that the tumor (287.5 ± 1.34 mg) and treatment with CT displayed the tumor weight 102.4 ± 2.03, 34.9 ± 1.02, and 2.2 ± 0.01 at a dose of 5, 10, and 20 mg/kg, respectively [Table 2].

Antioxidant parameters

Figure 1 exemplifies the antioxidant parameters of all groups. DMH receiving rats displayed an augmented level of antioxidant parameters such as SOD [Figure 1a], GPx [Figure 1b] and GR [Figure 1c] as compared to normal control rats. Treatment of CT in DMH receiving rats pointedly (P<0.001) diminished the level of the antioxidant parameter in concentration dose-dependent manner.

DMH-treated rats presented the augmented level of Phase II enzymes such as cytochrome P450 [Figure 2a], cytochrome b5 [Figure 2b], and abridged level of UDP-GT [Figure 2c], and administration of CT significantly (P < 0.001) changed the level of Phase II enzymes [Figure 2].

The DMH group also displayed the declined level of glutathione-S-transferase (GST) [Figure 2d], and CT treatment expressively (P < 0.001) augmented the level of GST [Figure 2d], and CT treatment expressively (P < 0.001) augmented the level of GST.

Nitrate level

In colorectal cancer, nitrate levels augmented and similar results were found in DMH-treated rats. DMH rats treated with the CT pointedly (P < 0.001) reduced the level of nitrate at a concentration-dependent manner [Figure 3a].

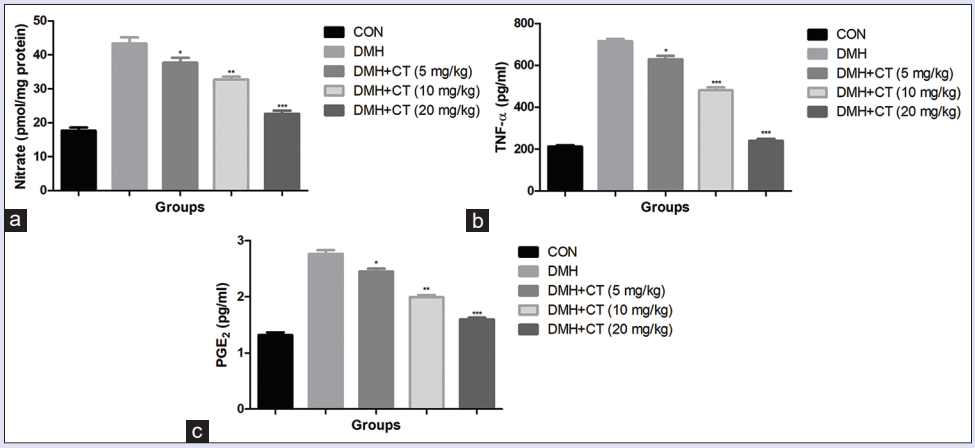

Figure 3: Effect of crocetin on the cytokines and inflammatory parameters of control and experimental rats in the blood sample. (a) Nitrate, (b) TNF-α, and (c) PGE_2. The data were shown as mean ± standard error of the mean. All tested groups compared with the DMH-treated group, *P < 0.05, *P < 0.01, and *P < 0.001 considered as the significant, more significant, and extreme significant. Where CON: Control; DMH: 1,2-dimethylhydrazine; CT: Crocetin; TNF-α: Tumor necrosis factor-α; PGE_2: Prostaglandin E_2

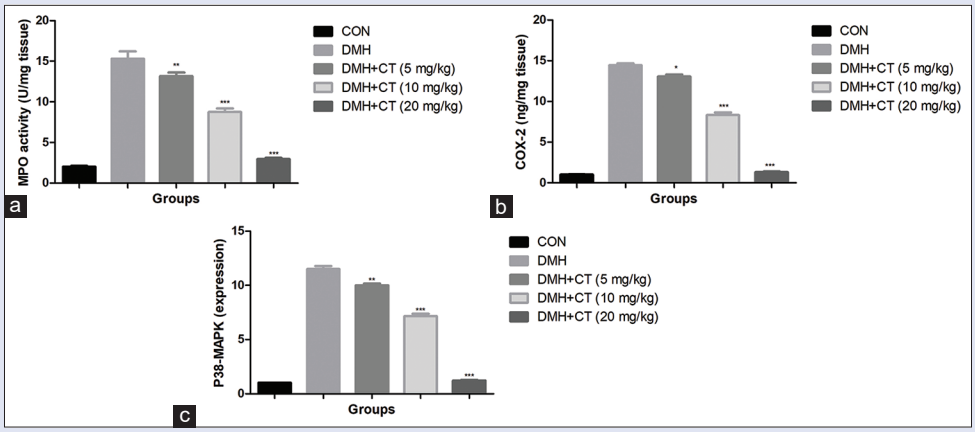

Figure 4: Effect of crocetin on the inflammatory parameters of control and experimental rats in the blood sample. (a) MPO, (b) COX-2, and (c) P38-MAPK. All tested groups compared with the DMH-treated group, *P < 0.05, *P < 0.01, and * P < 0.001 considered as the significant, more significant, and extreme significant. Where CON: Control; DMH: 1,2-dimethylhydrazine; CT: Crocetin and MPO: Myeloperoxidase; COX-2: Cyclooxygenase-2, and p38-MAPK: p38 mitogen-activated protein kinase

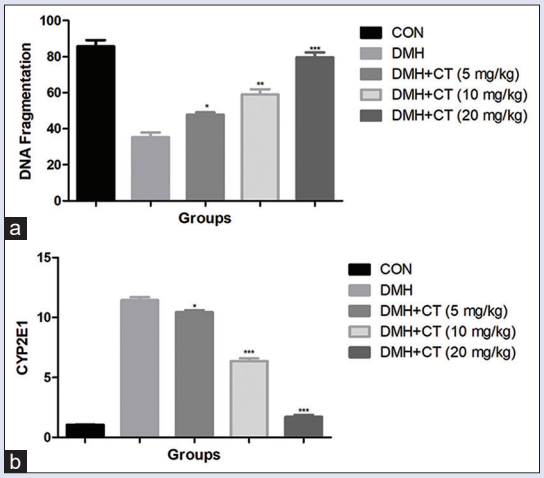

Figure 5: Effect of crocetin on the DNA fragmentation and CYP2E1 of control and experimental rats. (a) DNA fragmentation and (b) CYP2E1. All tested groups compared with the DMH-treated group, *$P < 0.05$, *$P < 0.01$, and *$P < 0.001$ considered as the significant, more significant, and extreme significant. Where CON: Control; DMH: 1,2-dimethylhydrazine; CT: Crocetin

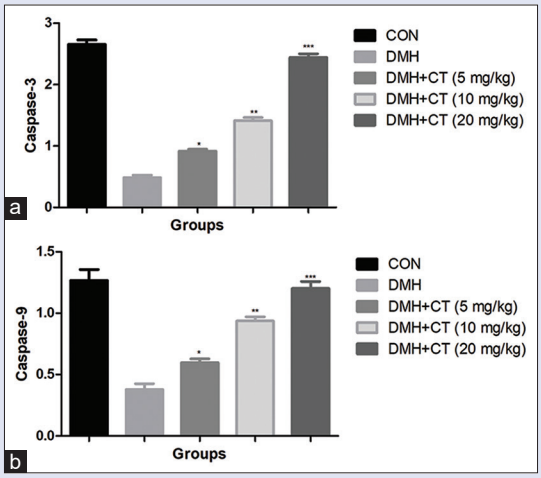

Figure 6: Effect of crocetin on the caspase parameter of control and experimental rats. (a) Caspase-3 and (b) Caspase-9. All tested groups compared with the DMH-treated group, *$P < 0.05$, *$P < 0.01$, and *$P < 0.001$ considered as the significant, more significant, and extreme significant

Table 2: Effect of tumor and body weight of control and experimental rats

Group	Body weight		Tumor weight
	Initial	Final	
Control	146±4.34	222.3±6.03	-
DMH	152±5.93	194.6±5.34	287.5±1.34
DMH + CT (5 mg/kg)	155±4.82 (NS)	205.3±6.53*	102.4±2.03**
DMH + CT (10 mg/kg)	148±3.98 (NS)	208.4±5.94**	34.9±1.02***
DMH + CT (20 mg/kg)	152±4.89 (NS)	221.6±4.83***	2.2±0.1***

Values present mean±SD of 15 rats in each group. Experimental group rats treated with the DMH control group rats. *P<0.05; **P<0.01, and ***P<0.001 consider as significant. NS: Not significant; SD: Standard deviation; DMH: Dimethylhydrazine; CT: Computerized tomography

Cytokines

Cytokines parameter, namely TNF-α, improved in colorectal cancer. DMH-treated rats displayed augmented TNF-α levels and CT treatment knowingly ($P < 0.001$) condensed TNF-α levels in the serum [Figure 3b].

Inflammatory mediator

Inflammatory mediators in colorectal cancer were augmented. DMH-treated rats presented a higher level of prostaglandin-2 (PGE_2) than normal control rats. The DHM group established that the CT ominously ($P < 0.001$) suppressed the level of PGE_2 in a dose-dependent manner in the serum [Figure 3c].

The level of MPO and COX-2 was improved in the DMH-treated rats. Treatment with CT significantly ($P < 0.001$) condensed the level of MPO and COX-2 in a dose-dependent manner in the serum [Figure 4a and b].

In DMH-treated rats, augmented expression of p38 mitogen-activated protein kinase (p38-MAPK) was detected which abridged after CT treatment meaningfully ($P < 0.001$) [Figure 4c].

Deoxyribonucleic acid fragmentation

In DMH-treated rats, DNA fragmentation condensed in comparison to normal control rats DMH group rats treated with the CT expressively ($P < 0.001$) augmented the DNA fragmentation [Figure 5a].

CYP2E1

In DMH-induced rats, an augmented level of CYP2E1 was detected which meaningfully ($P < 0.001$) diminished after treatment of CT [Figure 5b].

Caspase parameter

In colorectal cancer, abridged level of caspase proteins perceived and a similar result was gotten in the DMH-treated rats and CT pointedly ($P < 0.001$) increased the level of caspase-3 and caspase-9 [Figure 6].

DISCUSSION

Chemoprevention is the best tactic among all kinds of currently treating choice for the treatment of cancer and phytoconstituents isolated from plant having the benefit over the conventional therapy with more protective effects and less side effects[25] and lessens the side effects and cost.[26] Plant-based phytoconstituents are nontoxic substances and are usually found in various dietary agents, fruits, and vegetables. Earlier research recommended that the various plant-based phytoconstituents were used as the chemopreventive agents against numerous cancers.[27,28] Phytoconstituents are frequently naturally happening antioxidant in flowers and fruits which have been planned as primary chemopreventive agents[29] against the various types of cancers such as hepatic cancer, renal cancer, and colon cancer.[30,31] We employed the plant-based phytoconstituent CT and examined against the DMH-induced colorectal cancer and discovered the possible mechanism of action.

DMH is a powerful carcinogen, which excites reactive oxygen species (ROS) production direct to stabilize colon cell metabolism,[32] and eventually caused colon cancer which can be expected by various tumor marker variations.[8]

The activation of cellular metabolism results in ROS generation in both animal and plant cells with intracellular and extracellular environmental circumstances.[33] The increase level of ROS in the cells start the deterioration of endogenous oxidant and increase the production of free radical inside the body and start to deteriorate the tissue. The similar result was osbeerved in the DMH induced colorectal rats, that confirmed the expansion of oxidative stress induced cancer in rats.[16] Earlier studies recommended that ROS-induced oxidative stress is considered for both

malignancy and fibrosis, leading to cancer-related fibroblasts.[19,20,33] Accordingly, the researcher primarily targeted to preserve the oxidative stress-related pathological condition and homeostasis in the cells through keeping the excess ROS production. Antioxidant therapy is found to be the finest therapy to remove the ROS and showed a preventive effect against numerous diseases.[19,20] Endogenous antioxidants eradicate or minimize the free radical generation. During the malignancy condition, the amount of free radicals is in excess due to the development of cancerous cells and endogenous antioxidants incapable to abolish the free radicals.[20] A similar impetus was detected in DMH-induced colorectal cancer. DHM metabolizes into the hepatic tissue and induces the secretion of additional free radicals.[33] To uphold homeostasis, the cells twitch producing antioxidants such as SOD (family members of Zn, Cu, and Mn SOD), which catalyze the dismutation of hydrogen peroxide and superoxide (O_2), to water through GPx.[12,20,33] Other endogenous antioxidant parameters such as CAT played a noteworthy role in the lessening of free radicals. It produced a balance between ROS destruction and production in the organisms.[19,20] Superoxide radical incapacitated the CAT and GPx, and a similar outcome was detected in the DMH-induced colorectal cancer in rats. Hence, entire cell safety could only be proficient if an acceptable balance is conserved between the activities of those enzymes.[20] Other enzymes comprise GST, which catalyzes glutathione conjugation with dissimilar electrophilic compound species to defend and detoxify cells against reactive oxygen metabolites. Non-enzymatic antioxidants such as Vitamin C and Vitamin E play an important part to diminish the deleterious effect generate through cellular toxicants. Studies have exposed that a non-enzymatic antioxidant has a momentous role in the reduction of oxidative stress. The oxidative degradation of lipids moderates the membrane fluidity, causes cellular redox imbalance, and abolishes immune functions resulting in lipid peroxidation that reflects as a significant carcinogenesis indicator of persuaded cytotoxic and mutagenic cell-related death.[9,12,20] The lipid peroxidation reaction augmented during cancer as a result of the disease feast and similar verdicts in colorectal cancer of rats induced by DMH were detected. Pro-inflammatory cytokines contain TNF-α, and numerous studies have shown that cytokine expression (TNF-α) is based on the activation of the NF-kB and is observed as the significant inflammatory mediators. In fact, the treatment of colorectal cancer with cytokines is an optimistic tactic.[12] In the current experimental study, DMH-induced colorectal cancer in rats displayed the augmented expression of TNF-α and treatment with CT significantly ($P < 0.001$) diminished the expression of cytokines and suggesting the preventive effect against colorectal cancer.

There are frequent upstream kinase pathways accountable for COX-2 transcriptional regulation including protein kinases (MAPKs) caused by mitogen.[8] Prior studies recommended that MAPKs are made of p38 kinase and c-jun HN2 terminal and extracellular receptor kinase.[34,35] It is well shown that the p38 mitogen-activated protein kinase is not only regulated the inflammatory reaction but also preserved the inflammatory reaction tissue homeostasis comprising survival, cell proliferation, and differentiation.[33,35] In the DMH-induced colorectal cancer group rats exhibited the increased expression of p38-MAPKs and dose dependently treatment of crocetin significantly ($P < 0.001$) abridged the expression of p38-MAPKs. It is well shown that anticarcinogenic effects of various drugs occur through reduction of COX-2 and prostaglandin synthesis (inhibitor of COX-2), thus lessening the inflammatory reaction and development of cancer.[8] The abridged COX-2 level may occur due to the induction of protein p21 from the cell cycle regulation, which is concerned as a possible mechanism of colorectal carcinogenesis chemoprevention.[36,37] Remarkably, CT has been revealed to have an anti-inflammatory effect by plummeting the pro-inflammatory responses by reducing the p-38-MAPK signaling. Inflammatory reaction and oxidative stress play a substantial role in affecting the development and initiation of tumors. COX-2 is an inflammatory mediator and plays a weighty role in inflammation and augmented rates during colorectal cancer.[36,38] COX-2 plays a significant role in the production of polyps, induce the toxicity to the colon and inhibition of COX-2 is a vital tool for the treatment of colon cancer. The COX-2 augmented level replicated the elevation of prostaglandin output which was verified by p38-MAPK level prior to the elevation of the antiapoptotic protein such as Bcl-2 and reduced the apoptotic marker counting p53.[39,40]

CONCLUSION

Based on the outcome such as biochemical, antioxidant, cytokine, and inflammatory parameters, CT meaningfully changed all the parameters in the DMH-induced colorectal rats. CT displayed that the protective effect on colorectal cancer may be due to inhibition of inflammation, oxidative stress, and modification of apoptosis markers. Cumulatively, the current experimental study proposes that oral administration of CT has preventable and protective effects toward colorectal cancer. The study also specifies that CT having anticancer potential, and therefore, it could be employed as possible therapeutic agents for the treatment of colorectal cancer. The current study powerfully quantified that CT holds the potential for an anticancer agent and can, therefore, might be valuable clinically after a more molecular anticancer examination.

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Conflicts of interest

There are no conflicts of interest.

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