RESEARCH ARTICLE

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The Carcinogenic Agent Diethylnitrosamine Induces Early Oxidative Stress, Inflammation and Proliferation in Rat Liver, Stomach and Colon: Protective Effect of Ginger Extract

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Abstract

Background: Diethylnitrosamine (DENA), a well-known dietary carcinogen, related to cancer initiation of various organs. The present study investigated the deleterious mechanisms involved in the early destructive changes of DENA in different organs namely, liver, stomach and colon and the potential protective effect of GE against these mechanisms. **Methods:** Adult male albino rats were assigned into four groups. A normal control group received the vehicle, another group was injected with a single necrogenic dose of DENA (200 mg/kg, i.p) on day 21. Two groups received oral GE (108 or 216 mg/kg) daily for 28 days. Sera, liver, stomach and colon were obtained 7 days after DENA injection. Serum aspartate transaminase and alanine transaminase were detected as well as reduced glutathione (GSH), malondialdehyde, nitric oxide metabolites, interleukin 1β, tumor necrosis factor (TNF-α), alpha-fetoprotein (AFP) and nuclear factor-erythroid 2-related factor2 (Nrf2) in liver, stomach and colon. Histopathological studies and immunohistochemical examination of cyclooxygenase-2 (COX2) were conducted. **Results:** DENA induced elevation in liver function enzymes with significant increase in oxidation and inflammation biomarkers and AFP while decreased levels of Nrf2 in liver, stomach and colon were detected. Histologically, DENA showed degenerative changes in hepatocytes and inflammatory foci. Inflammatory foci displayed increased expression of COX2 in immunohistochemical staining. GE-pretreatment improved liver function and restored normal GSH with significant mitigation of oxidative stress and inflammatory biomarkers compared to DENA-treated group. AFP was reduced by GE in both doses, while Nrf2 increased significantly. Histology and immunostaining of hepatic COX-2 were remarkably improved in GE-treated groups in a dose dependent manner. **Conclusion:** GE exerted a potential anti-proliferative activity against DENA in liver, stomach and colon via Nrf2 activation, whilst suppression of oxidation and inflammation.

Keywords: Diethylnitrosamine- ginger extract- oxidative stress- inflammation- proliferation- liver- stomach-colon- Rat

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Introduction

Ginger (*Zingiber officinale*) is a medicinal herb in Southeast Asia that consequently spread through the world as natural remedy for many diseases such as gastrointestinal, and rheumatic disorders (Afzal et al., 2001). Ginger extract and its active constituents stand behind the antioxidant, anti-inflammatory, and antimutagenic properties with accumulated evidence from in vitro, in vivo, and epidemiological studies (Rani et al., 2011; Prasad and Tyagi, 2015b). Gingerols, among the bioactive compounds in ginger that have prominent cancer preventive effects against gastric and colon cancer in vitro (Jeong et al., 2006; Brown et al., 2009; Prasad and Tyagi, 2015a) and skin cancer in vivo (Kim et al., 2005; Mashhadi et al., 2013).

Nitrosamines embrace a wide category of environmental carcinogens present in smoked pickled fish, cheese, nitrite-cured meats, dried milk and alcoholic beverages or tobacco smoke. They are also formed in the acidic conditions of the stomach from nitrite precursors and amines present in food constituents or additives, residues of agricultural chemicals and pharmaceutical drugs (Mittal et al., 2006).

Nitrosamines are able to induce tumors in the majority of organs and systems (Bartsch and Montesano, 1984). Diethylnitrosamine (DENA) is one of the most significant hepatotoxicants and hepatocarcinogens. Amines are activated by reaction with nitrate, leading to N-nitrosamines under the acidic medium of stomach

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(Sadik et al., 2008; Janani et al., 2010).

Induction of hepatocyte injury with enhanced cell proliferation accompanied by hepatocellular necrosis is a well-established mechanism of carcinogenesis by several hepatocarcinogens such as DENA (Glauert et al., 2010; Qiu et al., 2011). Since DENA needs to be bio-activated by hepatic cytochrome P450 enzymes (CYP450), specifically CYP2E1, resulting in DNA-adducts, through an alkylation mechanism (Kang et al., 2007), inducing genetically altered hepatocytes during initiation (Vasquez-Garzon et al., 2013). This bio-activation process is a crucial step in initiation of carcinogenesis through subsequent oxidative stress and cellular injury (Kang et al., 2007; Sadik et al., 2008; He et al., 2012; Jayakumar et al., 2012). Moreover, the development of esophageal squamous-cell cancer (SCC) and gastric adenocarcinoma have been attributed to nitrosamines (Binato et al., 2008).

The potential chemopreventive role of different antioxidants on oxidative stress-induced tissue damage by wide range of carcinogens including DENA has been substantially investigated (Janani et al., 2010; He et al., 2012; Jayakumar et al., 2012).

The present study investigated the deleterious mechanisms that are involved in the early destructive changes induced by DENA in different organs namely, liver, stomach and colon. The study extended to test the potential protective effect of GE against these mechanisms.

Materials and Methods

Animals

Forty adult male Wistar albino rats weighing 180-200 g were utilized in the present study. Standard food pellets and tap water were supplied ad libitum. Animals and food pellets were obtained from the animal house colony of the National Research Centre (NRC, Egypt). Animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (1996, published by National Academy Press, 2101 Constitution Ave. NW, Washington, DC 20055, USA) and the experiment was conducted in accordance with ethical rules for standard experimental animal studies and the Medical Research Ethics Committee (MREC) of the National Research Centre under approval number: 15130.

Drugs and chemicals

Diethylnitrosamine (DENA) was purchased from Sigma-Aldrich (Germany). DENA was injected intraperitoneally in a single necrogenic dose of 200 mg/kg (Tessitore, 1998; Bansal et al., 2005). Ginger pure powder (Sigma), was suspended in 0.5% carboxymethylcellulose (CMC) in distilled water (vehicle). All other chemicals were of highest analytical grade available. All mandatory laboratory health and safety procedures have been complied with in the course of conducting the experimental work in this study.

Experimental design and treatment protocol

Animals were randomly allocated into four groups (10 rats each). Rats of the 1st group received 0.5% CMC and intraperitoneal injections of saline and served as normal control group. Group 2 received a single necrogenic dose of DENA (200 mg/kg, i.p)(Tessitore et al., 1996) on day 21 of the study. Groups 3 and 4 received oral GE (108 or 216 mg/kg/day), respectively, for 28 days during which a single necrogenic dose of DENA (200 mg/kg, i.p) on day 21 was injected. All animals were sacrificed 24 h after last GE treatment.

Serum biochemical analysis

Rats were anaesthetized with diethyl ether and blood samples were withdrawn from the retro-orbital venous plexus. Collected blood samples were allowed to stand for 10 min at room temperature then centrifuged at 4^{o}C using cooling centrifuge (Laborezentrifugen, 2k15, Sigma, Germany) at 3,000 r.p.m for 10 min. Sera were separated for assessment of levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) using commercially available colorimetric assay kits (Biodiagnostic, Egypt) as previously described (Reitman and Frankel, 1957).

Hepatic, stomach and colon tissue biochemical analysis

Directly after blood sampling, rats were sacrificed by cervical dislocation under ether anesthesia. Liver, stomach and colon tissues were collected, washed in normal saline. The tissue was homogenized using MPW–120 homogenizer (Med instruments, Poland); the homogenate was centrifuged using a cooling centrifuge (Laborezentrifugen, 2k15, Sigma, Germany) at 4,000 r.p.m for 10 min. and the supernatant was assessed for hepatic, stomach and colon levels of reduced glutathione (GSH) (Beutler et al., 1963), lipid peroxides as malondialdehyde (MDA) (Mihara and Uchiyama, 1978) and nitric oxide (NOx) metabolites (Miranda et al., 2001). Moreover, inflammatory markers such as interleukin-1β (IL-1β) and tumor necrosis factor-alpha (TNF-α) were assessed using ELISA kits (Hycult Biotech, Netherlands) and (RayBio, USA), respectively, according to the manufacturer's instructions. Finally, alpha-fetoprotein (AFP) and nuclear factor-erythroid 2-related factor 2 (Nrf 2) were assessed using ELISA kits (KAMIYA BIOMEDICAL, USA, Cat. No. KT-59172) and (CUSABIO, China, Cat. No CSB-EQ027869RA), respectively, according to the manufacturer's instructions.

Immunohistochemical analysis of cyclooxygenase-2 (COX-2)

For immunohistochemistry, 4 μm thick deparaffinized liver tissue sections were used. Briefly, deparaffinized liver slices were incubated overnight with the antibodies against COX-2 diluted 1:100, Endogenous peroxidase activity was blocked by incubation in 0.075% hydrogen peroxide in PBS. For antibody detection DAKO EnVision+ System, Peroxidase/DAB kit was employed. The sections were then counterstained with hematoxylin, dehydrated using graded alcohols and xylene, and mounted with Entelan. The immunostaining intensity and cellular localization of COX-2 was analyzed by light microscopy.

Histopathological examination

The other parts of liver tissues were fixed in 10%

neutral buffered formalin and embedded in paraffin wax. Sections of 4 µm thick were stained with Hematoxylin and Eosin (H and E) and examined using binocular Olympus CX31 microscope (Bancroft et al., 1996).

Statistical analysis

All values are presented as means ± standard error of the means (SEM) of eight experiments. Comparisons between different groups were carried out using one way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test. Difference was considered significant when $p<0.05$. GraphPad prism® software version 6 for Windows (USA) was used to carry out these statistical tests.

Results

Effect of ginger extract on liver function enzymes

Administration of single intraperitoneal dose of DENA (200 mg/kg) resulted in a significant elevation of liver function biomarkers. Liver function enzymes, AST and ALT, were elevated significantly in DENA-treated rats compared to their normal counterparts. Pre-treatment of rats with ginger extract at 108 mg/kg/day, showed insignificant effect on either AST or ALT serum levels. While Pre-treatment of rats with ginger extract at 216 mg/kg/day, showed significant reduction in AST level reporting normal levels of AST in DENA- treated rats with insignificant effect on ALT serum levels (Figure 1).

Effect of ginger extract on liver, stomach, and colon tissues oxidative stress parameters

The GSH content in liver, stomach, and colon was significantly reduced following DENA administration in rats. Treatment of rats with ginger restored the normal GSH content in the liver and colon. However, normal level of stomach GSH was only observed in the group treated with the lower dose of ginger; in the rats treated with the high dose, the stomach GSH content was significantly lower than the normal and non-significantly different from that

Table 1. Effect of Ginger Extract on Interleukin 1-Beta (IL-1β) Level in Liver, Stomach and Colon of DENA-Treated Rats

	IL-1beta (IL-1β,pg/ml)		
	Liver	Stomach	Colon
Normal	316.5^{b}+ 22.7	144.2^{b} + 5.7	184.6^{b} + 15.47
DENA	552.2^{a} + 18.8	442.6^{a} + 39.7	416.0^{a} + 20.36
DENA-Ginger (108mg/kg)	$390.2^{a,b}$ + 3.7	$249.8^{a,b}$ + 18.1	$269.0^{a,b}$ + 11.46
DENA-Ginger (216mg/kg)	364.9^{b} + 12.0	$295.7^{a,b}$ + 11.4	228.6^{b} + 16.11

Control, rats treated with the vehicle and represented the normal group; DENA, rats treated with diethyl nitrosamine; DENA-Ginger (108 mg/kg), rats treated with diethyl nitrosamine and ginger (108 mg/kg/day); DENA-Ginger (216 mg/kg), rats treated with diethyl nitrosamine and ginger (216 mg/kg/day). Each value represents the mean ± S.E.M (n=6).Statistical analysis was carried out using one-way ANOVA test followed by Tukey post hoc test. [a] Significantly different from normal group at $p<0.05$. [b] Significantly different from DENA group at $p<0.05$. [c] Significantly different from the other DENA-Ginger group at $p<0.05$.

Table 2. Effect of Ginger Extract on Tumor Necrosis Factor-Alpha (TNF-α) Level in Liver, Stomach and Colon of DENA-Treated Rats

	Tumor necrosis Factor-alpha (TNF-α,pg/ml)		
	Liver	Stomach	Colon
Normal	556.5^{b} + 30.9	461.4^{b} + 11.8	147.4^{b} + 12.6
DENA	1245.0^{a} + 93.0	946.3^{a} + 32.4	495.9^{a} + 24.7
DENA-Ginger (108mg/kg)	$879.3^{a,b}$ + 29.3	$699.7^{a,b}$ + 46.8	$279.6^{a,b}$ + 23.7
DENA-Ginger (216mg/kg)	$831.1^{a,b}$ + 21.1	$515.1^{b,c}$ + 12.2	$264.5^{a,b}$ + 22.4

Control, rats treated with the vehicle and represented the normal group; DENA, rats treated with diethyl nitrosamine; DENA-Ginger (108 mg/kg), rats treated with diethyl nitrosamine and ginger (108 mg/kg/day); DENA-Ginger (216 mg/kg), rats treated with diethyl nitrosamine and ginger (216 mg/kg/day). Each value represents the mean ± S.E.M (n=6).Statistical analysis was carried out using one-way ANOVA test followed by Tukey post hoc test. [a] Significantly different from normal group at $p<0.05$. [b] Significantly different from DENA group at $p<0.05$. [c] Significantly different from the other DENA-Ginger group at $p<0.05$.

Table 3. Effect of Ginger Extract on Alpha-Fetoprotein (AFP) Level in Liver, Stomach and Colon of DENA-Treated Rats

	Alpha-fetoprotein (AFP, ng/g tissue)		
	Liver	Stomach	Colon
Control	13.30^{b}± 0.49	2.00^{b}±0.13	3.59^{b}±0.20
DENA	154.57^{a}±5.26	11.61^{a}±0.60	29.45^{a}±1.92
DENA-Ginger (108 mg/kg)	$70.14^{a,b}$±1.89	$6.22^{a,b}$±0.21	$10.28^{a,b}$±0.42
DENA-Ginger (216 mg/kg)	$49.67^{a,b,c}$ ±1.58	$3.72^{a,b,c}$ ±0.21	6.78^{b}±0.21

Control, rats treated with the vehicle and represented the normal group; DENA, rats treated with diethyl nitrosamine; DENA-Ginger (108 mg/kg), rats treated with diethyl nitrosamine and ginger (108 mg/kg/day); DENA-Ginger (216 mg/kg), rats treated with diethyl nitrosamine and ginger (216 mg/kg/day). Each value represents the mean ± S.E.M (n=6).Statistical analysis was carried out using one-way ANOVA test followed by Tukey post hoc test. [a] Significantly different from normal group at $p<0.05$. [b] Significantly different from DENA group at $p<0.05$. [c] Significantly different from the other DENA-Ginger group at $p<0.05$.

Table 4. Effect of Ginger Extract on Nuclear Factor-Erythroid 2-Related Factor 2 (Nrf2) Level in Liver, Stomach and Colon of DENA-Treated Rats

	Nuclear factor-erythroid 2-related factor 2 (Nrf2, pg/g tissue)		
	Liver	Stomach	Colon
Control	29.37^{b} ±1.29	7.99^{b} ±0.32	18.92^{b} ±0.37
DENA	6.52^{a} ±0.34	1.33^{a} ±0.05	3.5^{a} ±0.14
DENA-Ginger (108 mg/kg)	$13.98^{a,b}$ ±0.39	$3.19^{a,b}$ ±0.20	$9.25^{a,b}$ ±0.26
DENA-Ginger (216 mg/kg)	$20.13^{a,b,c}$ ±0.46	$4.52^{a,b,c}$ ±0.21	$11.98^{a,b,c}$ ±0.36

Control, rats treated with the vehicle and represented the normal group; DENA, rats treated with diethyl nitrosamine; DENA-Ginger (108 mg/kg), rats treated with diethyl nitrosamine and ginger (108 mg/kg/day); DENA-Ginger (216 mg/kg), rats treated with diethyl nitrosamine and ginger (216 mg/kg/day). Each value represents the mean ± S.E.M (n=6).Statistical analysis was carried out using one-way ANOVA test followed by Tukey post hoc test. [a] Significantly different from normal group at $p<0.05$. [b] Significantly different from DENA group at $p<0.05$. [c] Significantly different from the other DENA-Ginger group at $p<0.05$.

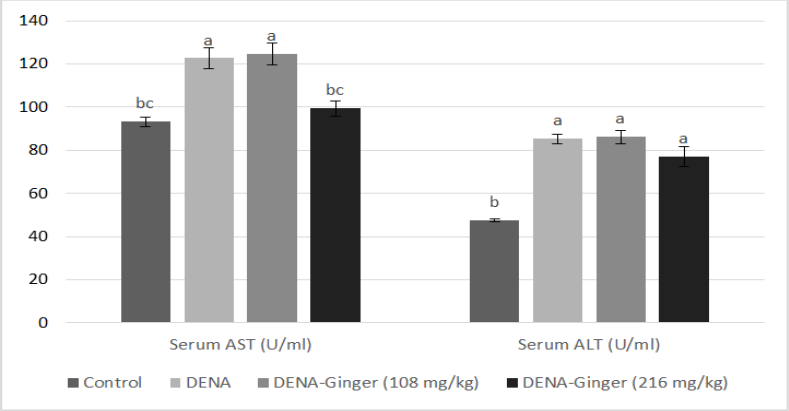

Figure 1. Effect of Ginger Extract on Liver Function Enzymes of DENA-Treated Rats. Control, rats treated with the vehicle and represented the normal group; DENA, rats treated with diethyl nitrosamine; DENA-Ginger (108 mg/kg), rats treated with diethyl nitrosamine and ginger (108 mg/kg/day); DENA-Ginger (216 mg/kg), rats treated with diethyl nitrosamine and ginger (216 mg/kg/day). a Significantly different from normal group at $p < 0.05$; b Significantly different from DENA group at $p < 0.05$; c Significantly different from the other DENA-Ginger group at $p < 0.05$.

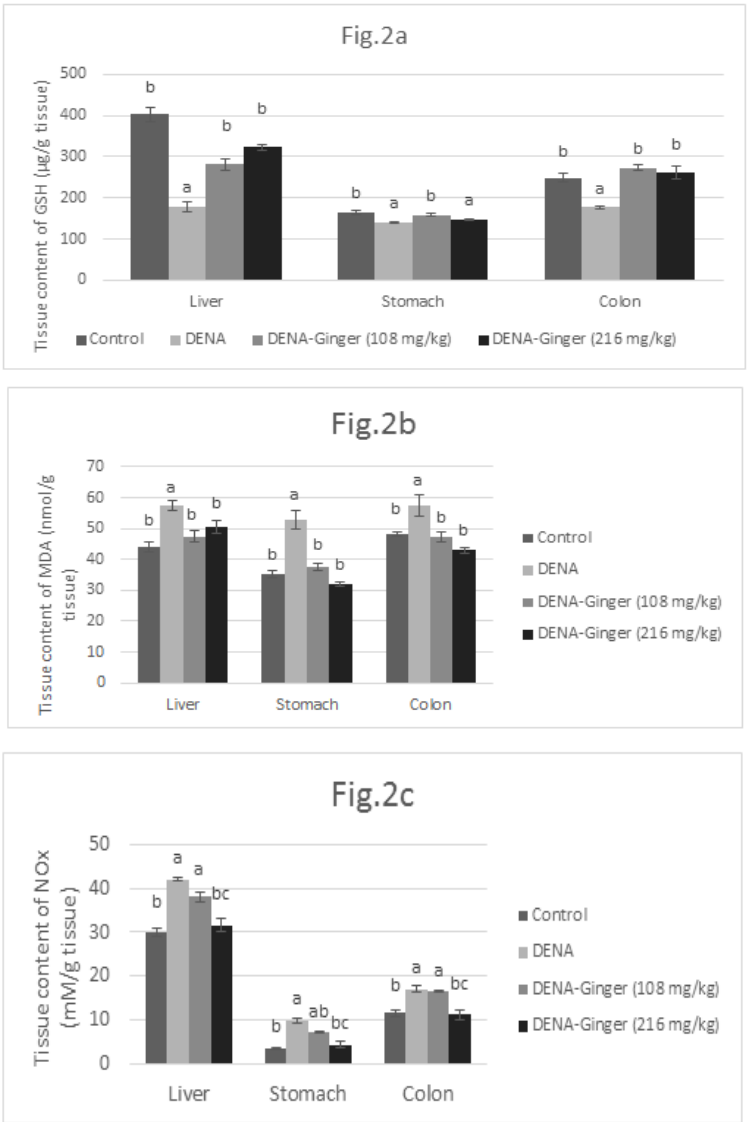

Figure 2. Effect of Ginger Extract Liver, Stomach and Colon contents of GSH (a), MDA (b), and NOx(c) of DENA-treated Rats. Control, rats treated with the vehicle and represented the normal group; DENA, rats treated with diethyl nitrosamine; DENA-Ginger (108 mg/kg), rats treated with diethyl nitrosamine and ginger (108 mg/kg/day); DENA-Ginger (216 mg/kg), rats treated with diethyl nitrosamine and ginger (216 mg/kg/day). a Significantly different from normal group at $p < 0.05$; b Significantly different from DENA group at $p < 0.05$; c Significantly different from the other DENA-Ginger group at $p < 0.05$.

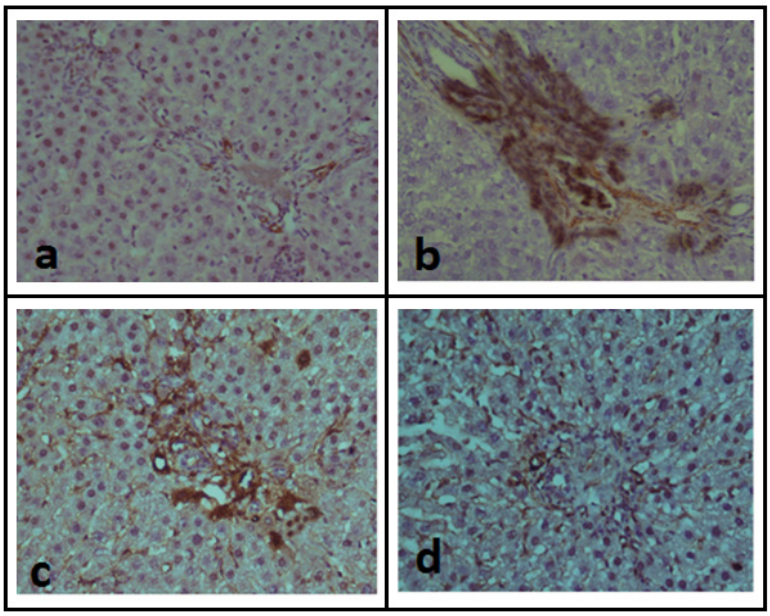

Figure 3. Effect of GE on COX2 Immunoreactivity in Hepatic Tissues in DENA-Induced Injury. (a) Control group: showed no positive inflammatory foci and negative COX2 immunoreactivity. (b) DENA group: showed strong positive stain in the area of inflammation exhibited sever COX2 immunoreactivity. (c) GE (108 mg/kg) group: moderate immunoreactivity. (d) GE (216 mg/kg) group: mild COX2 immunoreactivity. (COX2, x400)

of DENA-treated group. On the other hand, a significant elevation of the liver, stomach, and colon MDA content was detected following DENA administration. Treatment of rats with the both doses of ginger significantly retrieved the altered level of MDA in those organs' tissues.

Moreover, a significant increase in liver, stomach, and colon content of NOx was observed in rats treated with DENA. Treatment of rats with the higher dose of ginger has been found to restore the normal levels of NOx and its effect was significantly better than that of the lower dose (Figure 2 a, b, and c).

Effect of ginger extract on liver, stomach, and colon tissues pro-inflammatory cytokines; interleukin-1

Liver, stomach, and colon content of the pro-inflammatory cytokine IL-1β was significantly reduced by DENA administration in rats. Treatment of rats with ginger significantly reduced IL-1β content in

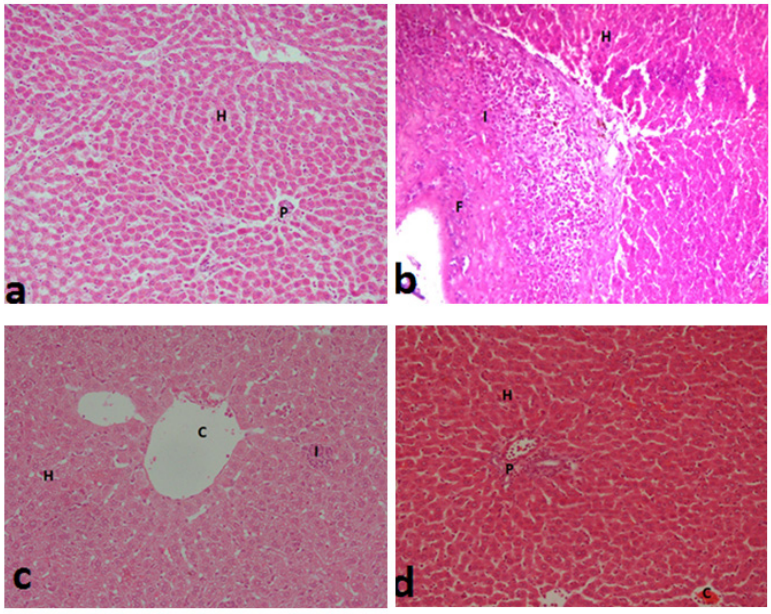

Figure 4. Effect of GE on the Histopathological Changes in the Hepatic Tissue in DENA-Induced Injury. (a) Normal control: showed normal architecture with the hepatocytes are normal run in thin plates (H), the portal areas showed normal structures with no fibrosis or inflammation (P). (b) DENA group: dense inflammatory cell infiltrate (I), areas of fibrosis (F), the hepatocytes are degenerated (H). (c) GE (108 mg/kg) group: degeneration of some of the hepatocytes (H), the cytoplasm appear foamy. Few foci of inflammation are still seen (I), some vessels are still dilated (C). (D) GE (216 mg/kg) group; showed better cytomorphology of the hepatocytes (H); with almost normal appearance of their cytoplasm. No foci of inflammation. No portal inflammation (P). The blood vessels showed mild dilatation and congestion (C). (H&E) (a and d x200; b and cx400).

the liver, stomach and colon by the two applicable doses. While the high dose of ginger significantly reduced both liver and colon IL-1β with no significant difference from normal group (Table 1). In addition, DENA administration significantly increased liver, stomach, and colon content of TNF-α, while pretreatment with ginger extract for four weeks significantly reduced TNF-α compared to DENA-treated rats. Ginger extract, at 216mg/kg, significantly reduced TNF-α in stomach of DENA-treated animals compared to the other dose of ginger (108mg/kg) (Table 2).

Effect of ginger extract on liver, stomach and colon tissues alpha-fetoprotein (AFP)

Alpha-fetoprotein (AFP) was significantly increased after DENA single intraperitoneal injection in liver, stomach and colon of rats compared to normal rats. Pretreatment with ginger extract significantly inhibited the dramatic elevation in AFP after DENA injection in liver, stomach and colon. Moreover, ginger extract at, 216 mg/kg, exerted significant reduction in AFP compared to the other dose of the extract in both liver and stomach, while restored normal levels of AFP in the colon of DENA-treated rats (Table 3).

Effect of ginger extract on liver, stomach and colon nuclear factor-erythroid 2-related factor 2 (Nrf2)

Diethylnitrosamine (DENA) in a single intraperitoneal injection (200 mg /kg) significantly decreased nuclear factor-erythroid 2-related factor-2 (Nrf-2) in liver, stomach and colon of rats compared to normal rats. Pretreatment with ginger extract significantly inhibited the reduction in Nrf-2 level of liver, stomach and colon compared DENA group. Moreover, ginger extract, at 216 mg/kg, exerted significant elevation in Nrf-2 level of liver, stomach and colon tissues compared to the other dose of the extract (Table 4).

Immunohistochemical and histopathological assessment of hepatic tissues

Immunohistochemical assessment of hepatic cyclooxygenase-2 (COX2) revealed strong immunoreactivity of COX2 in hepatic tissues on week after 200 mg/kg of DENA intraperitoneal injection. Meanwhile, administration of GE at 108 mg/kg showed moderate COX2 immunoreactivity compared to GE at 216 which displayed mild COX2 immunoreactivity (Figure 3).

Histopathological examination of liver tissues revealed dense inflammatory cell infiltrate areas of fibrosis and degeneration of hepatocytes after DENA injection. GE (108 mg/kg) exerted slight improvement while degeneration of some of the hepatocytes, foamy cytoplasm and inflammation are still seen. GE (216 mg/kg) showed better cytomorphology of the hepatocytes with almost normal appearance of their cytoplasm, no foci of inflammation, No portal inflammation, and blood vessels showed mild dilatation and congestion with significant improvement in the overall hepatic histopathological picture (Figure 4).

Discussion

Being one of the most frequently and heavily consumed natural dietary component, ginger and its polyphenolic compounds; zingerone, [6]-gingerol, and shogaol; have been reported for their chemoprotective and antioxidant effects in carcinogenesis (Chung et al., 2001; Mohd-Yusof et al., 2002; Manju and Nalini, 2005). Having more profound effect than gingerols, its active components, (Mukkavilli et al., 2014); researchers highlight the importance of utilizing entire ginger extract over its active components (Prasad and Tyagi, 2015b) with recently reported anti-oxidant, anti-inflammatory and anti-apoptotic effects of whole ginger rhizome extract in rat model of diabetic nephropathy (Al Hroob et al., 2018)

Nitrosamines as dietary carcinogens are associated with the etiology of HCC and contribute to the development of oxidative stress, chronic inflammation, and cellular proliferation in response to tissue injury, leading to hepatocarcinogenesis (Darvesh and Bishayee, 2013).

Both environmental and N-nitrosamines born-food hold a health hazard for human and animals. Experimentally, DENA is used to investigate its cytotoxic mechanisms on different tissues and organs. Moreover, DENA causes alterations in serum and tissue enzyme markers (Atakisi et al., 2013). The International Agency for Research on Cancer (IARC) categorized DENA as a "probable carcinogenic to humans" (category A2) (IARC, 1987). The catalysis of DENA by cytochrome P-450-dependent enzymes of monooxygenase system yields active metabolite ethyl radical that covalently binds to DNA leading to cellular necrosis, mutation and cancer (Skog, 2002). Oxidative stress-induced cell injury plays a crucial role in DENA-induced carcinogenesis (Bansal et al., 2000).

Liver-specific enzyme markers, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and γ-glutamyltransferase (GGT), are released during hepatocytes' injury (Galle et al., 2014). AST, ALT and ALP are considered more sensitive parameters to assess liver injury in rodent species (Galle et al., 2014). Previous studies indicated that DENA-induced liver injury is accompanied by elevated activities of these enzymes (Sadik et al., 2008; Sayed-Ahmed et al., 2010; Jin et al., 2013; Galle et al., 2014). In accordance with present data that clearly stated a significant elevation in ALT and AST enzyme activities after a single necrogenic dose of DENA, indicating established liver injury. Taking into consideration the hepatoprotective activity of ginger extract (GE) against several hepatotoxic agents (Atta et al., 2010; Abdel-Azeem et al., 2013; Shivashankara et al., 2013; Vasquez-Garzon et al., 2013), ginger treatment significantly ameliorated the elevation in AST at high dose level while no significant reduction in ALT activity was reported.

Oxidative stress and nitrosative stress, through generation of reactive oxygen species (ROS) and reactive nitrogenous species, act as an important influencing factor to carcinogenesis and both are substantial key factors in cancer as an end-stage of chronic diseases (Kawanishi et al., 2006). Current results demonstrated significant

decrease in GSH with increased levels of MDA and total nitrate/nitrite (NOx) in liver, stomach and colon of DENA-treated rats. DENA, in a single necrogenic dose (200 mg/kg), produced significant increase in hepatic thiobarbituric acid reactive substances (TBARs) and total nitrate/nitrite (NOx) and decreased reduced glutathione (GSH), glutathione peroxidase (GPx), glutathione-s-transferase (GsT) and catalase (CAT) activity (Gayathri et al., 2009; Sayed-Ahmed et al., 2010; Atakisi et al., 2013). Ginger attenuated the perturbation in oxidative stress markers as evidenced by increase in liver, stomach and colon reduced GSH, and alleviation of MDA and NOx levels in DENA-treated rats. The antioxidant activities of ginger in liver (Yusof et al., 2008), stomach (al-Yahya et al., 1989; Yoshikawa et al., 1994; Nanjundaiah et al., 2011) and colon (Manju and Nalini, 2005) have been well-established. Nevertheless, modulation of oxidative damage and subsequently inflammation is considered to be of advantage for cancer prevention (Bishayee et al., 2010). On the other hand, DNA- oxidative and nitrosative damage occurs at the sites of carcinogenesis is a potential risk of inflammation-mediated carcinogenesis in humans and animal models, regardless of etiology (Kawanishi et al., 2006; Laothong et al., 2010). As a consequence of DENA-induced oxidative and nitrosative DNA damage (Klaunig and Kamendulis, 2004; Unsal and Belge-Kurutas, 2017), inflammatory markers such as interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) are significantly elevated in DENA-treated liver, stomach and colon, in the present study. Further, the release of pro-inflammatory cytokines and chemokines (IL-6, IL-8, IL-1β, TNF-α, CCL2, CCL20) as a result of macrophages and neutrophils activation, is thought to play an important role in the pathogenesis of cancer in human liver (Uehara et al., 2014). Others reported a mixed inflammatory cell infiltration (lymphocytes, neutrophils, eosinophils, and Kupffer cells) by DENA administration (Duan et al., 2014; Ding et al., 2017).

Ginger pretreatment exerted a significant anti-inflammatory effect on DENA-treated liver, stomach and colon contents of IL-1β and TNF-α, in a dose dependent pattern. Ginger extract (100 mg/kg) acts as an anti-cancerous and anti-inflammatory agent by abrogating the activation of NFκB via the suppression of pro-inflammatory cytokine, TNF-α (Hudson et al., 2006; Habib et al., 2008). The active components of ginger, 6-gingerol and 6-paradol, possess a strong anti-inflammatory activity through suppression of TNF-α production in TPA-treated female ICR-mice and rats (Park et al., 1998; Surh, 2003).

On the other hand, being overexpressed in many types of malignant tumors, cycloxygenase-2 (COX-2) is one of the inflammatory mediators associated with carcinogenesis in colorectal, prostate, breast and hepatocellular cancers. Its frequent aberrant expression has been observed in human and animal HCCs. Purportedly, COX-2 expression increased in well-differentiated HCCs than normal or less differentiated HCCs suggesting its involvement in early stages of hepatocarcinogenesis (Cui et al., 2005; Wu, 2006; Giannitrapani et al., 2009). Obviously, DENA induced COX-2 over-expression as evidenced by immunohistochemical study of rat liver after single intraperitoneal dose of DENA. Nevertheless, an evident cross-talk between NO and COX-2 expression in cancer cells is reported where NO donor induced COX-1 and COX-2 expression in colon cancer cell lines in a dose and time dependent fashion (Liu et al., 2003) with correlation between iNOS expression and COX-2 overexpression in enhanced tumor growth, angiogenesis and inflammation in various tumors (Rahman et al., 2001; Ohta et al., 2006). Similarly, a well-established association between NO, inflammatory mediators; MPO, IL-1β and COX-2 overexpression was reported in other models of hepatic and renal injuries (Mansour et al., 2017a; Mansour et al., 2017b).

Animal models of liver cancer have shown that non-steroidal anti-inflammatory drugs (NSAIDs) exert chemopreventive and therapeutic effects (Cervello and Montalto, 2006), with significant reduction in tumor size (Galant et al., 2013). Hence, the utilization of natural agents with an anti-inflammatory activity is noteworthy. Ginger extract pretreatment showed lower COX-2 immunohistochemical staining in DENA-treated liver tissues in a dose dependent manner. The observation is in accordance with other studies reported potent anti-inflammatory effect of ginger preparations and isolated compounds via inhibition of COX-2 (Tjendraputra et al., 2001; Rani et al., 2011), nuclear factor κB (Grzanna et al., 2005), along with three fold inhibition of COX-2 more than COX-1 (van Breemen et al., 2011).

Alpha-fetoprotein (AFP) is a well-known tumor marker indicator of HCC (Sell et al., 1983). Being a growth regulatory cell-signaling factor, it has been reported to promote cell proliferation, suppress apoptosis, and act as an immunosuppressive agent (Mizejewski, 2013). More than 70% of HCC patients displayed high serum concentration of AFP, therefore, AFP is strongly suggestive of HCC (Endo et al., 1975). Moreover, elevated serum levels of AFP indicates the growth of malignant lung and bladder tumors as well as gastrointestinal cancers of stomach, pancreas, and colon (Mizejewski, 2014). High mortality and morbidity rates were reported to patients with AFP-positive-gastrointestinal cancer (AFP(+)GC) than AFP(-)GC-patients due to active cell proliferation, high mitotic rate, amplified cell invasion and migration, rapid tumor progression and advanced tumor stage (He et al., 2016).

Elevated serum levels of AFP have been detected in animals bearing liver tumors after treatment with certain hepatocarcinogens including DENA (Kroes et al., 1975). The increased level of AFP observed in DENA-induced animals is indicative of HCC (Jagan et al., 2008). In the present study, DENA induced significant elevation of AFP in liver, stomach and colon by 92, 82 and 86%, respectively, compared to normal groups. DENA-induced elevation in AFP was reported earlier (Das et al., 2016).

It was observed that stimulation of the nuclear oncogenes (c-fos, c-jun, c-myc) and the two gene transcripts of the AFP gene are triggered after 4-12 hrs and after 4-24 hrs, respectively, following turpentine-induced acute inflammation in the rat (Koj et al., 1983). Addition of AFP to skin cultures of human keratinocytes with

T-lymphocytes resulted in boosted baseline expression of cytokines, chemokines, and growth factors (Potapovich et al., 2009). AFP exhibits a vital role in the regulation of tumor growth, cell differentiation and proliferation of human hepatoma cells through the AFP receptors (Li et al., 2002). Accordingly, elevated levels of inflammatory cytokines and NOx along with AFP, following DENA administration, support the notion that AFP serves as both an acute and a chronic phase reactant depending on its stage of ontogeny (Mizejewski, 2015). The majority of AFP-producing cancers originate from the stomach, bile duct, and pancreas. Clinically, eleven cases of colorectal cancer with only one case with early rectal cancer have been diagnosed as an AFP-producing tumor by immunohistochemistry (Anzai et al., 2015). Hepatic, gastric and colonic contents of AFP were markedly decreased by ginger extract pretreatment by approximately 50% compared to DENA group in a dose dependent manner. The chemopreventive activity of ginger extract and its constituents has been reported previously against myriad models of liver cancer (Mansour et al., 2010; Taha et al., 2010), gastric cancer rat models (Ko and Leung, 2010; Prasad and Tyagi, 2015b) and experimental colon carcinogenesis (Yoshimi et al., 1992; Manju and Nalini, 2006). Clinically, two grams daily of ginger supplement, to patients with increased risk for colorectal cancer, reduce proliferation in the crypts of normal-appearing colorectal epithelium and increase apoptosis and differentiation relative to proliferation (Citronberg et al., 2013).

The redox-sensitive transcription factor, nuclear factor-erythroid 2−related factor 2 (Nrf2), plays a central role in the inducible expression of genes encoding detoxifying systems, including phase II drug-metabolizing enzymes; NADPH, NAD(P)H quinone oxidoreductase 1, glutathione peroxidase, ferritin, heme oxygenase-1 (HO-1) (Jaiswal, 2004). These defense enzymes are coordinately induced through the antioxidant responsive element (ARE) and are tightly regulated by Nrf2 (Nguyen et al., 2003). The attenuated expression of these enzymes in Nrf2-deficient mice has verified the role of Nrf2 in the regulation of many detoxifying and antioxidant enzymes under oxidative stress conditions; rendering Nrf2-deficient mice more vulnerable to carcinogen-induced toxicity and carcinogenesis (Enomoto et al., 2001; Ramos-Gomez et al., 2001). Diethylnitrosamine administration significantly decreased hepatic, gastric and colonic Nrf2 by 78, 83 and 82%, respectively, after 7 days of administration. This result was reflected by the dramatic decrease in reduced GSH content of the investigated tissues besides the increase in oxidative stress marker (MDA) and inflammatory markers (IL-1β and TNF-α). Similarly, a recent study reported that DENA down-regulates Nrf2 in the liver along with induction of oxidative stress, inflammation and angiogenesis (Mahmoud et al., 2017). Both doses of ginger extract protected liver, stomach and colon from DENA-induced decrease in Nrf2 with significant difference between low and high dose of GE. Previous studies showed increased antioxidant enzymes including GSH, SOD, and GPx by GE (Jeena et al., 2013). Zerumbone, component of Asian ginger oil, elevates phase II detoxification enzymes as well as nuclear localization of Nrf2/ARE (Nakamura et al., 2004). The upregulation of Nrf2 by ginger extract could exert an anti-inflammatory effect through elevation of HO-1 expression leading to the inhibition of NFκB signaling (Chi et al., 2015) giving a new insight in cancer prevention through upregulation of Nrf2/ARE pathway by ginger consumption.

Ginger inhibits transcription factor NF-κB, inflammatory cytokine TNF-α and targets several cellular molecules that contribute to tumorigenesis, cell survival, cell proliferation, invasion, and angiogenesis in different forms of GI cancers. Those molecular targets of ginger indicate that it may have the potential for preventing and treating GI cancer (Prasad and Tyagi, 2015b). Though the notion that Nrf2 inducers and/or Keap-1 suppressors may serve as promoters of cancer cell proliferation with increased resistance to ferroptosis cell death (Fan et al., 2017); ginger extract exerted an Nrf-2-inducing activity with concurrent inhibition of alpha-feto protein, proliferation marker, in all examined tissues and decline in oxidative and inflammatory markers, thus contributing to its chemoprevention activity probably via mechanism involving Nrf2/Keap1/ARE pathway. Therefore, further molecular investigation is warranted to outline ginger antioxidant/anti-inflammatory/anti-proliferative crosstalk mechanism.

Taking together current observation and previous supporting literature, GE supplementation ameliorated the distortion in liver architecture induced by DENA through hepatoprotective; antioxidative, anti-inflammatory, anti-proliferative and chemopreventive properties as evident by current histopathologic examination of liver tissues.

In conclusion, Ginger Extract alleviated DENA-induced decrease in reduced GSH, increase in MDA and NOx, elevations of IL-1β, TNF-α, and hepatic COX-2 expression, increase in AFP and decrease in Nrf2 of liver, stomach and colon of male Wistar albino rats via antioxidative, anti-inflammatory, and eventually chemopreventive properties with proposed anti-proliferative effect by inhibition of AFP-producing tumor pathway.

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Conflict of Interests

The authors declare none.

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