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ORIGINAL ARTICLE

AMELIORATING REACTIVE OXYGEN SPECIES-INDUCED *IN VITRO* LIPID PEROXIDATION IN BRAIN, LIVER, MITOCHONDRIA AND DNA DAMAGE BY *ZINGIBER OFFICINALE* ROSCOE

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ABSTRACT

Iron is an essential nutrient for a number of cellular activities. However, excess cellular iron can be toxic by producing reactive oxygen species (ROS) such as superoxide anion (O_2^-) and hydroxyl radical ($HO^\bullet$) that damage proteins, lipids and DNA. Mutagenic and genotoxic end products of lipid peroxidation can induce the decline of mitochondrial respiration and are associated with various human ailments including aging, neurodegenerative disorders, cancer etc. Zingiber officinale Roscoe (ginger) is a widely used spice around the world. The protective effect of aqueous ethanol extract of Z. officinale against ROS-induced in vitro lipid peroxidation and DNA damage was evaluated in this study. The lipid peroxidation was induced by hydroxyl radical generated from Fenton's reaction in rat liver and brain homogenates and mitochondrial fraction (isolated from rat liver). The DNA protection was evaluated using H_2O_2-induced changes in pBR-322 plasmid and Fenton reaction-induced DNA fragmentation in rat liver. The results indicated that Z. officinale significantly ($P<0.001$) protected the lipid peroxidation in all the tissue homogenate/mitochondria. The extract at 2 and 0.5 mg/ml could protect 92 % of the lipid peroxidation in brain homogenate and liver mitochondria respectively. The percent inhibition of lipid peroxidation at 1mg/ml of Z. officinale in the liver homogenate was 94 %. However, the extract could partially alleviate the DNA damage. The protective mechanism can be correlated to the radical scavenging property of Z. officinale. The results of the study suggest the possible nutraceutical role of Z. officinale against the oxidative stress induced human ailments.

KEY WORDS

Antioxidants, Nutraceutics, Reactive oxygen species, Zingiber officinale.

INTRODUCTION

Over the last few decades, the science of nutrition has been progressing based on the greater understanding of the physiological and genetic mechanisms by which diet and individual food components influence health and disease. Some of the natural products find their use not as pharmaceuticals (real medicine) but as a novel class of dietary supplements or nutraceuticals that fall well into the concept of functional food. According to glossary produced by American Diabetics and Association, nutriceutics are substances considered as food or a part of it that offers health or medicinal benefits, including prevention and treatment of diseases (1). Medicinal uses of spices/herbs have been gradually increasing in developed countries. *Zingiber officinale* Roscoe, commonly known as ginger, is one of the commonly used spices in India and around the world. It is an indispensable component of curry and it belongs to Zingiberaceae family. Rhizome of ginger has been recommended for use as carminative, diaphoretic, antispasmodic, expectorant, peripheral circulatory stimulant, astringent, appetite stimulant, anti-inflammatory agent, diuretic and digestive aid (2-8).

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Free radicals such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced during the normal metabolism can damage the cells resulting in lipid peroxidation, alteration of protein and nucleic acid structures. The declined activities of antioxidant enzymes with concomitant increased levels of oxidative DNA damage have been reported in many cancerous and non-cancerous conditions (9). The carcinogenic effect of dietary fat occurs through the activation of pro-carcinogen to ultimate carcinogen by fat oxidation product such as lipid hydroperoxides (10). Further, most of the end products of lipid peroxidation can react with cysteine, histidine and lysine residues of protein that result in the disruption of proteins and inactivation of enzymes (11, 12). One of the major products of lipid peroxidation, 4-hydroxy-2-nonenal (HNE), is most reactive and cytotoxic which can induce the decline of *in vitro* mitochondrial respiration (13). Therefore, preservation of mitochondrial function is important for maintaining overall health during aging. The MDA, another end product of lipid peroxidation, was also demonstrated to be a mutagenic and genotoxic agent that can contribute to the development of human cancers (14). Hence, agents that can inhibit lipid peroxidation in organs with variations in the level of poly unsaturated fatty acids (PUFA) and antioxidants status will be an addition to the concept of functional food. Smith et al. (15) reported that daily supplementation of fruits and vegetable extract may reduce the levels of DNA damage in human. Several experimental studies have been demonstrated that extract of ginger can ameliorate the organ toxicity through enhancing the antioxidant status (16-18). However, its protective effect against Fe^{2+}- H_2O_2-EDTA (Fenton reaction)-induced lipid peroxidation in various tissues especially brain and mitochondria or protection against DNA damages has not yet been established. Iron, the most abundant transition metal ion in our body, may work as a catalyst for the generation of ROS in pathological conditions. Hence, the current study was designed to evaluate the protective effect of *Z. officinale* against Fenton reaction induced lipid peroxidation in rat brain, liver homogenates and mitochondrial fraction as well as the H_2O_2-induced pBR plasmid changes and hydroxyl radical induced DNA fragmentation in rat liver.

MATERIALS AND METHODS

Chemicals: Hydrogen peroxide (H_2O_2), ferrous sulphate ($FeSO_4.7H_2O$), pyridine (C_5H_5N), ethanol (C_2H_5OH), disodium hydrogen phosphate (Na_2HPO_4), bromophenol blue, glycerol (C_3H_8O), boric acid and thiobarbituric acid were purchased from Merck, India Ltd., Mumbai. Agarose, pBR 322 plasmid DNA were purchased from the Genie Bangalore, India. All other chemicals and reagents used were analytical reagent grade.

Preparation of the extract: Aqueous ethanol extract of rhizome of *Z. officinale* was prepared by the method described in Ajith et al (16). Briefly, rhizome of *Z. officinale* (500 g) (purchased from local market, Amala Nagar, Thrissur, Kerala, South India) were cut into small pieces and homogenized with 50 % ethanol (v/v). The homogenate was centrifuged at 2500 g for 10 min to collect the supernatant. Solvent in the supernatant was completely evaporated at low temperature (50-60°C). The residue designated as ethanol extract (6.5 g, w/w) was solubilised in double-distilled sterile water for the study.

Inhibition of lipid peroxidation-induced in rat whole liver homogenate: Lipid peroxidation was induced by Fe^{2+}-EDTA system in the rat liver homogenate (normal rat liver/brain received from Amala Cancer Research Centre, Amala Nagar, Thrissur, Kerala, India) in the presence and absence of *Z. officinale* extracts to form thiobarbituric acid reacting substance (TBARS). The TBARS thus formed was determined by the method of Ohkawa et al (19). Briefly, the reaction mixture contained 0.2 ml of rat liver homogenate (25 %, w/v in 20 mmol/l KH_2PO_4-KOH buffer pH 7.4); EDTA (0.1 mmol/l); $FeSO_4.7H_2O$ (0.1 mmol/l); H_2O_2 (0.44 mol/l) and various concentrations of the extracts of *Z. officinale* (0.25, 0.5, and 1 mg) in a final volume of 1 ml. The reaction mixture was incubated for 1 h at 37°C. After the incubation, 0.5 ml of the reaction mixture was treated with 0.2 ml SDS (8.1 %); 1.5 ml thiobarbituric acid (0.8 %); and 1.5 ml acetic acid (20 %, v/v, pH 3.5). The total volume was made up to 4 ml by distilled water and kept in water bath at 95-100°C for 1 h. One ml of distilled water and 5.0 ml of n-butanol and pyridine mixture (15:1,v/v) were added to the reaction mixture, vortexed and centrifuged at 2750 g for 10 min. The organic layer was removed and its optical density (O.D) was measured at 532 nm in a double beam spectrophotometer (SL 164, double-beam spectrophotometer; Elico Ltd, Hyderabad, India). Tubes with out Fenton's reaction mixture or extract of *Z. officinale* but with liver homogenate were included as normal. O.D of the normal tube was subtracted from that of Fenton treated and Fenton plus *Z. officinale* treated tubes for calculating the percent inhibition of lipid peroxidation which was determined by comparing the mean O.D of the extract treated tubes with that of Fenton reaction alone performed tubes. The experiments were repeated twice with each concentration in triplicate.

Inhibition of lipid peroxidation in mitochondrial fraction/ brain homogenate: The experiment was conducted as described above expect 0.2 ml of mitochondrial suspension (isolated from 25 % rat liver homogenate in 0.25 mol/l sucrose containing 0.1 mmol/l EDTA) or 0.2 ml of rat brain homogenate (10% in phosphate buffer) and H_2O_2 (176 mmol/l) were incubated with various concentrations of *Z. officinale* (as given in the figures) in a final volume of 1 ml. The experiments were repeated twice with each concentration in triplicate.

Effect of extract of Z. officinale against Fenton reaction induced DNA fragmentation in rat liver: The DNA fragmentation in rat liver was induced by Fenton reaction and was assessed as laddering of DNA in agarose gel. Fresh rat liver (400-500 mg) was thoroughly washed with chilled normal saline-EDTA solution. The minced tissues were incubated with $FeSO_4.7H_2O$ (0.1mmol/l); EDTA (0.1 mmol/l); H_2O_2 (0.44 mol/l); various concentrations of extract of *Z. officinale* (0.5, 1 and 2 mg) and phosphate buffer (20 mmol/l KH_2PO_4-KOH; pH 7.4) in a final volume of 1 ml. The reaction mixture was incubated for 1 hr at 37°C. After the incubation, the reaction mixture was washed 3-4 times in cold saline-EDTA solution and centrifuged. DNA was isolated in the pellet (20). Briefly, 1 ml of lysis buffer (10 mmol/l Tris-HCl, 10 mmol/l EDTA, and 10% SDS, pH 8) was added to pellet and incubated at 37°C for 1hr. The lysate was treated with cold saturated NaCl and kept on ice for half an hour. The supernatant was mixed with RNAse and incubated further for 30 min. The contents were centrifuged at 3000 g for 10 min at 4°C and the supernatant was collected. To the supernatant, double volumes of chilled ethanol (95%, v/v) was added and kept on ice over night. The tubes were centrifuged at 7000 g at 4°C for 20 min to separate the DNA. The DNA was washed with 70 % alcohol and suspended in TE buffer (1mol/l Tris-HCl, 0.5 mol/l EDTA, pH 8). The concentration of DNA in the sample was determined spectrophotometrically.

Agarose gel electrophoresis: Sample (10µg DNA) was mixed with loading buffer (10 mM EDTA, 0.25% bromophenol blue and 50% glycerol) in a 1:5 ratio and loaded in the wells of 1.8% agarose (contained 0.5 µg/ml of ethidium bromide). The electrophoresis was carried out at 50 V using 1x TBE buffer (2 mmol/l EDTA, 89 mmol/l Tris and 89 mmol/l boric acid; pH 8.6) for 2-3 hr. After the electrophoresis, the gel was observed for fragmentation of DNA under an UV lamp. The experiments were repeated twice.

Effect of extract of Z. officinale against H_2O_2 induced changes of pBR 322 plasmid DNA: pBR 322 plasmid DNA (100 ng in TE buffer) was incubated with H_2O_2 (88 µmol); various concentrations of *Z. officinale* (10, 50 and 100 µg) and TE buffer in a net volume of 20 µl (21). The reaction mixture was incubated for 30 min at 37°C. After the incubation, the reaction mixture was mixed with loading buffer and subject to electrophoresis in a 1% agarose gel system using TBE buffer as described above. The electrophoresis was carried out at 50 v for 2-3 hrs. The gel was stained with ethidium bromide and observed under UV lamp. *Z. officinale* (50 and 100 µg) was also incubated with plasmid DNA to detect its effect on the super coiled plasmid. The experiments were repeated twice.

Statistical analysis: All data were represented as mean ± SEM. Significance between the O.D values were statistically analyzed using one-way analysis of variance (ANOVA) followed by the post test Bonferroni's t-test (using Graph Pad Instat software demo package).

RESULTS

Z. officinale significantly (P<0.001) and dose dependently inhibited Fenton reaction induced lipid peroxidation in rat liver homogenate (Fig 1). The O.D of Fenton plus *Z. officinale* treated (1 mg/ml) liver homogenate was non-significantly (P> 0.05) different from that of normal. The percentage inhibition of lipid peroxidation in Fenton plus *Z. officinale* (1 mg/ml) treated tube was 93.5 % with respect to the Fenton alone performed tube. Effect of *Z. officinale* against Fenton reaction induced lipid peroxidation in rat brain homogenate is given in

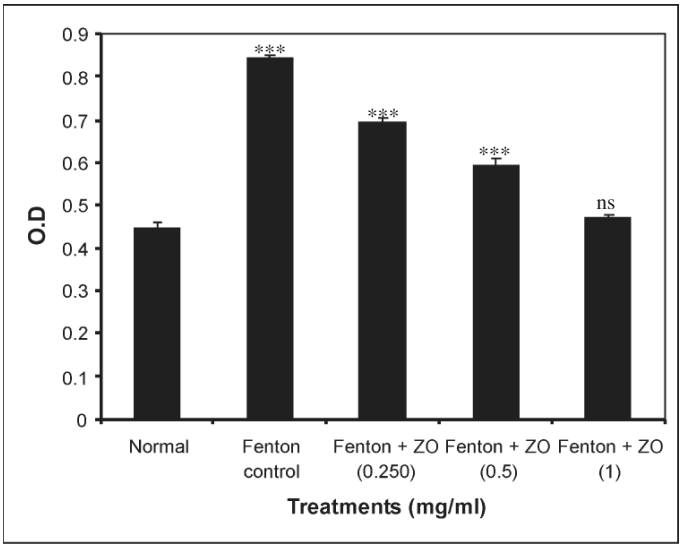

Fig 1: Effect of *Z. officinale* (ZO) against Fenton reaction-induced lipid peroxidation in rat liver homogenate. Values are mean ± SEM, (n = 3). ***P<0.001 significantly and ns P>0.05 non-significantly (Bonferroni test) different from the normal OD

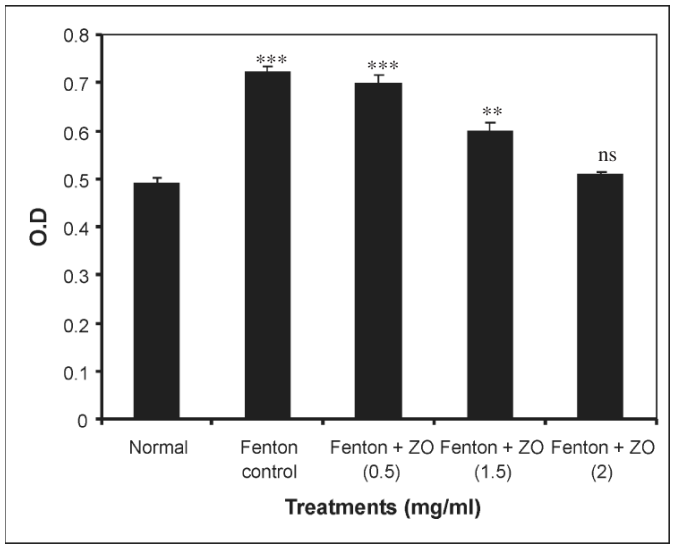

Fig 2: Effect of *Z. officinale* (ZO) against Fenton reaction-induced lipid peroxidation in rat brain homogenate. Values are mean ± SEM, (n=3). ***P<0.001, **P<0.01 significantly and ns P>0.05 non-significantly (Bonferroni test) different from the normal OD

Figure 2. *Z. officinale* has given a significant ($p < 0.001$) and dose dependent inhibition of lipid peroxidation except in the 0.5 mg/ml treated tube, which was non-significantly ($p > 0.05$) different from that of Fenton alone treated tube Further, the O.D of Fenton reaction plus *Z. officinale* (2 mg/ml) treated brain homogenate was non-significantly different from that of normal. The percentage inhibition (92 %) of lipid peroxidation

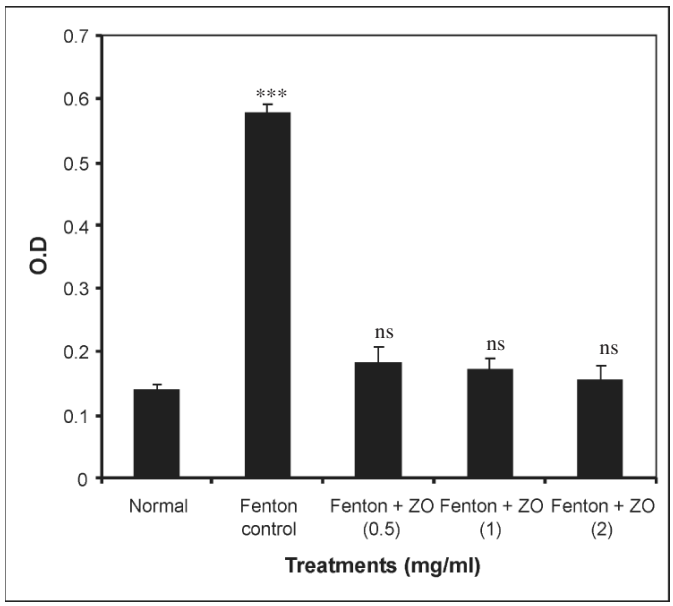

Fig 3: Effect of *Z. officinale* (ZO) against Fenton reaction-induced lipid peroxidation in rat liver mitochondria. Values are mean ± SEM, (n=3). *** p < 0.001 significantly and ns p > 0.05 non-significantly (Bonferroni test) different from the normal OD

was found to be highest in the 2 mg/ml *Z. officinale* treated homogenate.

Similarly, *Z. officinale* significantly (P<0.001) inhibited the lipid peroxidation induced by the Fenton reaction in rat liver mitochondria (Fig 3). However, the O.Ds of Fenton plus *Z. officinale* treated liver mitochondria were statistically non-significant from each other, indicated the dose-independent protection. Moreover, the O.Ds of Fenton plus *Z. officinale* (0.5, 1 and 2 mg/ml) treated tubes were not significantly (P>0.05) different from that of normal. The highest percent inhibition (97%) of lipid peroxidation was obtained in the Fenton plus *Z. officinale* (2 mg/ml) treated mitochondria.

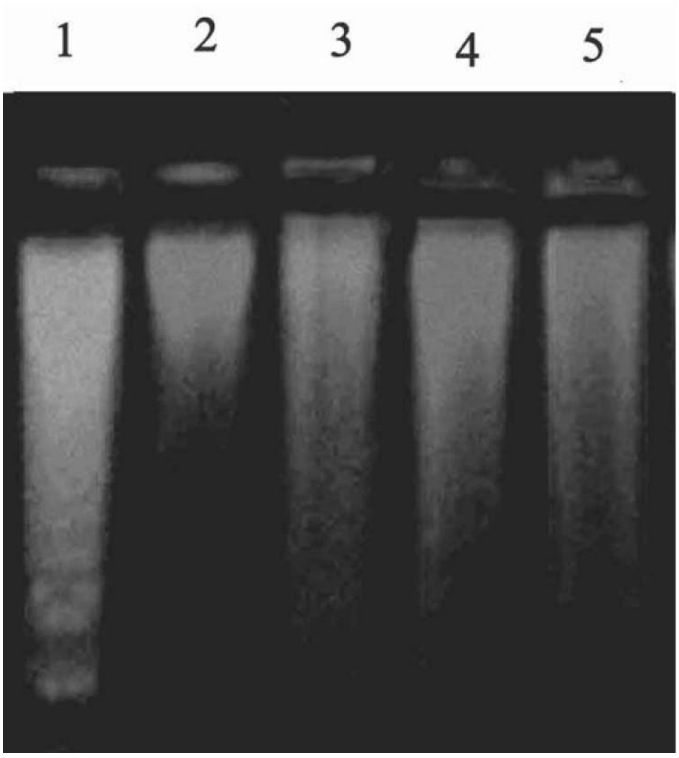

Fig 4: Effect of *Z. officinale* (ZO) against Fenton reaction (FR)-induced fragmentation of DNA in liver. Lane 1: FR; Lane 2: Normal; Lane 3: ZO (0.5 mg) + FR; Lane 4: ZO (1 mg) + FR; Lane 5: ZO (2 mg) + FR

Incubation of liver with Fenton reaction plus *Z. officinale* led to only partial protection of the fragmentation of DNA (Fig 4; lane 3, 4 and 5) when compared to that of Fenton reaction alone treated DNA. Similarly, incubation of plasmid pBR322 with H_2O_2 produced an open circular form from its normal super coiled form (Fig 5b; lane 2). However, incubation of pBR322 with *Z. officinale* at 50 or 100 μg alone did not convert the pBR322 to open circular form (Fig 5a; lane 2 and 3). Further, extract at all the tested concentrations (10, 50 and

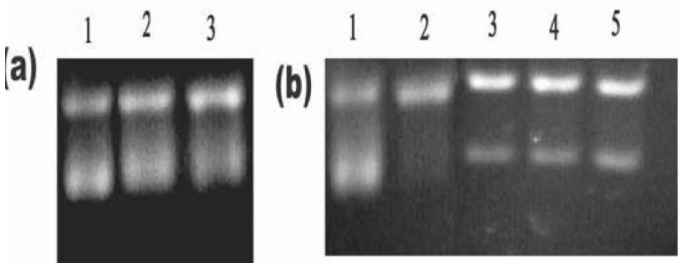

Fig 5: Effect of *Z. officinale* (ZO) against H_2O_2 induced changes of pBR 322 DNA. a) Lane 1: pBR 322 DNA; Lane 2 : pBR 322 + ZO (50 mg); Lane 3: pBR 322 + ZO (100 mg); b) Lane 1: pBR 322 DNA; Lane 2 : pBR 322 + H_2O_2; Lane 3: pBR 322 + H_2O_2 + ZO (10 mg); Lane 4: pBR 322 + H_2O_2 + ZO (50 mg); Lane 5: pBR 322 + H_2O_2 + ZO (100 mg)

100 μg) could partially protect H_2O_2 induced conversion of super coiled pBR 322 (Fig 5b; lane 3, 4 and 5).

DISCUSSION

Results of the study revealed that extract of *Z. officinale* effectively protected the *in vitro* ROS-induced lipid peroxidation and DNA damage. A high concentration of H_2O_2 used to induce the lipid peroxidation in liver (0.44 mmol/l) homogenate to over come the possible higher antioxidant status of the liver cells. The same dose of H_2O_2 was effective to execute the DNA fragmentation in rat liver tissue has been previously reported (21). H_2O_2 is thought to be an important precursor of highly reactive free radicals, such as superoxide anion (O_2-•) and hydroxyl radicals (•OH) and it has been reported to induce apoptosis in the central nervous system (CNS) (22). Brain cells are highly vulnerable to oxidative damage due to the high rate of oxygen consumption, the presence of high PUFA and iron (23). In addition the levels of lipid peroxidation products are found to be increased during the progression of degenerative processes (11). Extract of *Z. officinale* effectively and dose dependently protected the lipid peroxidation in the brain homogenate. Extract at 2 mg/ml could completely inhibit the lipid peroxidation in brain homogenate as evident from the non-significant OD value with respect to that of the normal. The highest concentration of *Z. officinale* (2 mg/ml) that required to inhibiting the lipid peroxidation in brain homogenate can be ascribed to the high level of PUFA in the brain.

The mitochondrial respiratory chain is the major sub-cellular source of free radicals. ROS are produced *in vivo* by electron leakage from electron transport chain (ETC) complexes during normal respiration, in particular complex I and complex III, which leads to decreased electron transportation, oxidative phosphorylations, decreased energy production or loss of calcium homeostasis (24-26). Experimental studies have demonstrated that mutative and oxidative damage to mitochondrial DNA and decline of mitochondrial respiratory function are important contributors to human aging (27) and a number of diseases particularly associated with muscle and nerve (28). *Z. officinale* extract at lowest tested concentration (0.5 mg/ml) offered effective protection against Fenton reaction induced lipid peroxidation in mitochondria. Further, the ODs of all the Fenton plus *Z. officinale* treated tubes were non-significantly different from that of the normal indicated its efficacy in protecting the lipid peroxidation. This can be explained with the lowest PUFA level in the liver mitochondria.

H_2O_2 is a prominent ROS that causes lipid peroxidation and DNA damage in cells (29). 1-10 mmol of H_2O_2 was reported to induce DNA double strand break in cell after 20 mints of incubation (30). Further, the generated ·OH from H_2O_2 by Fenton's reaction can also induce the DNA fragmentation effectively (31). Hence, in this study we had selected both H_2O_2 and ·OH for inducing the DNA damages. The results of this study clearly demonstrated the fragmentation of rat liver DNA by ·OH or conversion of super coiled pBR 322 to open circular form by H_2O_2. Incubation of liver samples with extract of *Z. officinale* partially protected the ·OH-induced DNA fragmentation. Similarly the extract of *Z. officinale* could partially protect H_2O_2 -induced conversion of super coiled pBR 322 to open circular form. The *Z. officinale* render protection either by neutralizing the H_2O_2 or by scavenging the ·OH generated from the Fenton's reaction.

The experiments using different homogenates/mitochondria were selected in this study were to demonstrate the protective efficiency of *Z. officinale* against Fe^{2+}- H_2O_2-EDTA (Fenton reaction)-induced lipid peroxidation in tissues/sub-cellular fraction with variations in the antioxidant status. Moreover, they are closely related to the incidence of various human diseases. Though iron is an essential nutrient for a number of cellular activities including gene regulation, iron can work as a double-edged sword. The liver, which is the primary site for iron storage is at particular risk for iron-induced tissue damage. Excess iron deposited chronically in hepatic parenchymal cells in hereditary hemochromatosis and secondary hemochromatosis causes peroxidative damage to the lipid membranes of cellular organelles resulting in hepatic injury, fibrosis, and ultimately cirrhosis (32). Both experimental and epidemiologic studies have shown the associations of iron with several human cancers. Pathological conditions such as hemochromatosis, chronic viral hepatitis B and C, exposure to asbestos fibers, as well as endometriosis have been recognized as iron overload-associated risks for human cancer (33). The carcinogenic effect of metals including iron has been

either related to activation of mainly redox-sensitive transcription factors, involving NF-kappaB, AP-1 and p53 or catalyst for the generation of ROS in Fenton reaction to target the genes (34, 35).

Recent unresolved hypothesis indicate that ·OH generated from Fenton reaction can promote lipid peroxidation, mutagenesis, DNA strand breaks, oncogene activation, and tumor suppressor inhibition, increasing the risk of breast cancer (35). Peroxidative mechanisms have been implicated in chronic neurodegenerative such as Alzheimer's and Parkinson's diseases and demyelinating (e.g., multiple sclerosis) disorders (36). Iron-dependent free radical reactions are involved in the neurotoxicity of aluminium and in damage to the substantia nigra in Parkinson's disease (37). Iron-dependent oxidative stress in the penumbra can also lead to necrosis and further neurological deterioration following ischemic stroke (38). Therefore, an antioxidant treatment should be considered when there is excessive iron. Extract of *Z. officinale* through the exhibited activity may suggest its effectiveness against the lipid peroxidation involved pathological conditions.

The results of the present study concluded that *Z. officinale* could completely protect the lipid peroxidation but was partially effective to alleviate the DNA damages. Ginger has been listed in "Generally Recognized as Safe" (GRAS) document of the US FDA. A dose of 0.5 – 1.0 g of ginger powder ingested 2-3 times for periods ranging from 3 months to 2.5 years did not cause any adverse effects in human (39). The previous studies have demonstrated the protective effect of *Z. officinale* against anticancer drugs induced organ damages in experimental animals (16-18). Therefore, these results further suggest the possible nutraceutical role of *Z. officinale* against the lipid peroxidation involved human aliments. However, the concentrations of *Z. officinale* selected in this *in vitro* experiment can not be correlated with that required to render protection *in vivo.* Further detailed *in vivo* experimental models loaded with excess of iron are required to establish the nutraceutical role of ginger.

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