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Zingerone Protects Against Stannous Chloride-Induced and Hydrogen Peroxide-Induced Oxidative DNA Damage In Vitro

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Abstract In this paper, we report the dose-dependent antioxidant activity and DNA protective effects of zingerone. At 500 μg/mL, the DPPH radical scavenging activity of zingerone and ascorbic acid as a standard was found to be 86.7 and 94.2 % respectively. At the same concentration, zingerone also showed significant reducing power (absorbance 0.471) compared to that of ascorbic acid (absorbance 0.394). The in vitro toxicity of stannous chloride ($SnCl_2$) was evaluated using genomic and plasmid DNA. $SnCl_2$-induced degradation of genomic DNA was found to occur at a concentration of 0.8 mM onwards with complete degradation at 1.02 mM and above. In the case of plasmid DNA, conversion of supercoiled DNA into the open circular form indicative of DNA nicking activity was observed at a concentration of 0.2 mM onwards; complete conversion was observed at a concentration of 1.02 mM and above. Zingerone was found to confer protection against $SnCl_2$-induced oxidative damage to genomic and plasmid DNA at concentrations of 500 and 750 μg/mL onwards, respectively. This protective effect was further confirmed in the presence of UV/H_2O_2-a known reactive oxygen species (ROS) generating system-wherein protection by zingerone against ROS-mediated DNA damage was observed at a concentration of 250 μg/mL onwards in a dose-dependent manner. This study clearly indicated the in vitro DNA protective property of zingerone against $SnCl_2$-induced, ROS-mediated DNA damage.

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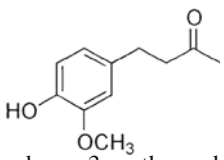
Introduction

The use of tin and tin compounds continues unabated in food industry and health sciences, despite proof of their toxic effects [1–6]. Genotoxic effects include high frequency of base substitutions involving guanines resulting in G:C to T:A and G:C to C:G transversions [7]. The inhibitory effects of Sn^{2+} on repair of methyl methane sulfonate-induced DNA damage leading to increased mutation has also been reported [8]. Stannous chloride ($SnCl_2$), alone or in its associated form, is able to produce a formation of lesion on DNA [9], which has been attributed to oxidative stress via generation of reactive oxygen species (ROS) by Fenton-like reaction [10] or by direct action of Sn^{2+} on DNA [6, 8, 11]. Free radicals are known to cause several types of damage to biomolecules such as proteins, lipids, and DNA. Damage to DNA includes—modification of DNA bases, loss of purines, damage to deoxyribose sugar, single and double-strand breaks, and DNA-protein cross-linkage [12, 13]. Modifications on DNA at the molecular level lead to cellular changes such as arrest or induction of transcription and signal transduction pathways, replication errors, and genomic instability associated with carcinogenesis. $SnCl_2$ can deplete SH-groups in proteins, glutathione, and glutathione-dependent enzymatic systems, leading to enhanced lipid peroxidation and changes in calcium and sulfhydryl homeostasis [14].

The generation of excess ROS is deleterious to cells. Hence, agents that scavenge or prevent their production become relevant in ensuring cellular health. Though cells possess antioxidant enzymes and other nonenzymatic antioxidant molecules,

external supply of such agents, synthetic or natural, should prove beneficial under oxidative stress. Artificial antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary butylhydroquinone (TBHQ), widely used in food industry to prevent free radical generation, are suspected to be toxic and carcinogenic[15–17]. Natural antioxidant secondary metabolites, especially polyphenols, found in dietary plants thus assume significance.

Ginger is heavily consumed not only as a condiment in food and beverages but also as part of traditional systems of medicine worldwide from ancient times [18]. The constituents responsible for its "hot" pungent sensation include gingerols, shogaols, paradols, and zingerone (ZO). It is known that cooking transforms some gingerols and shogaols into ZO which is the least pungent with a sweet spicy aroma [19]. Unlike the other constituents mentioned, ZO, a phenolic alkanone, containing a vanilloid (3-methoxy-4-hydroxy benzene) group in its structure, exhibits antigenotoxic, antiapoptotic, and antilipid peroxidative ability; antimutagenic, anticarcinogenic, antioxidative, and anti-inflammatory activities [20]. It has also been found to inhibit radiation-induced genetic damage [21], decrease catalase activity [22], and increase levels of endogenous antioxidants [20]. In this study we have evaluated the antioxidant property and in vitro DNA protective effects of ZO against stannous chloride-induced free radicals generated via Fenton's reaction as well as those produced directly by the action of UV on H_2O_2. The following is the structure of zingerone [4-(4-hydroxy-3-methoxyphenyl)butan-2-one]:



Zingerone [4-(4-hydroxy-3-methoxyphenyl)-2-butan-2-one]

Materials and Methods

Materials

Stannous chloride was purchased from Merck specialities Pvt. Ltd., Germany; ZO from Sigma, St. Louis, MO, USA; DPPH (1,1-diphenyl-2-2-pricylhydrazyl) from HiMedia Laboratories Pvt. Ltd., Mumbai, India. pUC18 and agarose were purchased from Genei, Bangalore, India. All other chemicals and solvents used were of analytical grade.

Antioxidant Activity

DPPH free radical scavenging activity of ZO was evaluated by the method of Chen et al. [23] using ascorbic acid as positive control. In short 1 mL of 100 mM methanolic DPPH was mixed with an equal volume of ZO at different concentrations (0–750 μg/mL). The mixture was incubated in the dark for 30 min at 25 °C. The absorbance was read at 517 nm, and the activity was determined using the formula DPPH scavenging activity (%)=[(absorbance of control−absorbance of sample)/absorbance of control]×100.

The reducing power of ZO was assessed using the method of Chen et al. [23]. Briefly, 1 mL of ZO (0–750 μg/mL) mixed with 0.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium hexacyanoferrate (1 %*w*/*v*) was incubated at 50 °C in water bath for 20 min. After cooling to room temperature, 0.5 mL of trichloroacetic acid (10 %*w*/*v*) was added followed by centrifugation at 3,000×*g* for 10 min. The supernatant (1 mL) was mixed with an equal volume of distilled water and freshly prepared 0.1 % ferric chloride solution (0.1 mL). Absorbance was recorded at 700 nm. Ascorbic acid at various concentrations served as the standard.

DNA Damage Protection Potential

DNA protective activity of ZO against oxidative stress was evaluated using genomic DNA, isolated from *Physarum polycephalum*, and pUC18 plasmid DNA. To determine the effective concentration of $SnCl_2$ for inducing DNA damage, a 15 μL reaction containing genomic or plasmid DNA (500 ng per reaction) was taken in microcentrifuge tubes and incubated with different concentrations (0.4 to 1.04 mM) of $SnCl_2$ at 37 °C for 1 h in 10 mM of Tris–HCl buffer at pH 7.4 or 7.5 [10]. The reaction mixture was then mixed with the gel loading dye (6×) and electrophoresis was done on 1 % agarose gel. The gels were analyzed on a AlphaImager 2200 gel-documentation system using AlphaEaseFC software. The fluorescence intensity of plasmid DNA, supercoiled (form I, sc-DNA), and relaxed open circular (form II, oc-DNA) generated due to the nicking of DNA was quantified to assess DNA damage as described previously [24] based on integrated density value (IDV). Likewise, decrease in the IDV of the genomic DNA band in the $SnCl_2$-treated sample, compared to the untreated control, was used to assess DNA damage. The DNA protection potential of ZO was evaluated by incubating reaction mixtures containing the highest DNA damage-inducing concentration of $SnCl_2$ (see results) in the presence of varying concentrations (50 μg/mL–1 mg/mL) of ZO.

In addition to $SnCl_2$, DNA was also subjected to oxidative damage employing UV/H_2O_2 as hydroxyl free radical generating system which was described by Russo et al. [25] in the presence of ZO and then checked on 1 % agarose gels. Briefly, a 15 μL reaction was set up in a microcentrifuge tube containing 10 mM of NaCl, phosphate buffer at pH 7.4, 500 ng of DNA, 2 μL of 50 mM H_2O_2, and 5 μL of varying concentrations of ZO ranging from 50 μg/mL to 1 mg/mL. The reactions were initiated by a total dose of UV–C irradiation (1,400 J m^{-2} s^{-1}; dose rate 5.1 J m^{-2} s^{-1}) at a distance of 11 cm using a Philips germicidal lamp (30 W) at room temperature.

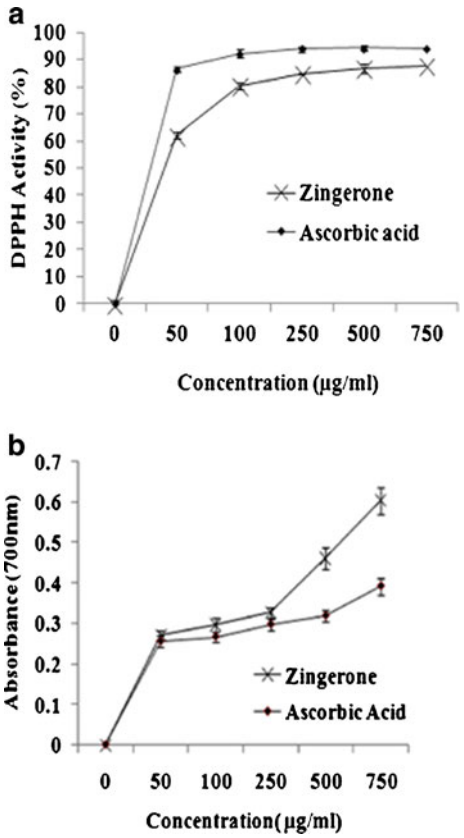


Fig. 1 Antioxidant activity of ZO. **a** DPPH radical scavenging activity. **b** Total reducing power of ZO. Ascorbic acid was used as a standard. Data are presented as mean±SD ($n=5$)

Results and Discussion

Antioxidant Activity

The antioxidant activity of ZO, determined by the DPPH free radical scavenging ability, was found to increase in a dose-dependent manner (Fig. 1a). The radical scavenging activity at 500 µg/mL of ascorbic acid (standard) and ZO was found to be 94.2 and 86.7 % respectively. The reducing power of ZO also showed a dose-dependent increase with an OD_{700} value of 0.394 and 0.471 at 500 µg/mL of ascorbic acid and ZO, respectively (Fig. 1b). It has been well established that the prophylactic and therapeutic properties of increased intake of antioxidants is attributable to phenolic compounds since they donate electrons and scavenge free radicals by the formation of stable phenoxyl radicals [26, 27]. Our results show that the presence of phenolic alkanone ZO in and as a dietary supplement should, therefore, confer health-protective benefits as has already been shown for ascorbic acid [3] and other antioxidant blends [28].

$SnCl_2$ Induces DNA Damage

The toxicity of $SnCl_2$ on genomic and plasmid DNA was evaluated at concentrations ranging from 0.4 to 1.04 mM under in vitro conditions. In the case of plasmid DNA, conversion of sc-DNA into oc-DNA in the positive controls was used to assess DNA strand breaks. Likewise, any smearing of the genomic DNA resulting from $SnCl_2$-induced DNA nicking in the positive controls was taken as an indication of DNA damage. This was evident in samples treated with $SnCl_2$ at concentrations of 0.8 mM onwards. The complete disappearance of high-molecular weight genomic DNA band was observed at a concentration of 1.02 mM and above. In other words, these results demonstrate the concentration range and the extent of DNA degradation possible with $SnCl_2$. Genomic DNA samples showed a distinct trail of degraded DNA (~50 %) (Fig. 2a), while in plasmid DNA, conversion of sc-DNA to oc-DNA (~70 %) was observed (Fig. 2b). However, in comparison to genomic DNA, the sensitivity of plasmid DNA to nicking was apparent at a relatively lower concentration of 0.2 mM onwards up to a level of complete conversion to oc-DNA (form II) at 1.02 mM. Denaturation of double-stranded DNA by stannous ions as a result of direct interaction with the polyanionic regions of DNA or via ROS generation in the vicinity of bases has been suggested to be the cause of

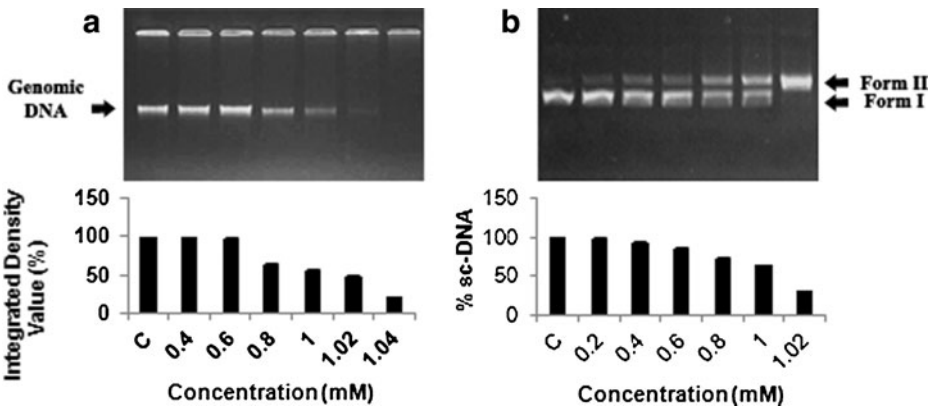


Fig. 2 $SnCl_2$-induced DNA damage. Agarose gel (1 %) and its densitometric data showing the DNA damaging effects of different concentration of $SnCl_2$ (**a**). Smearing of genomic DNA was observed at 0.8 mM onwards (**b**). Conversion of sc (form I) to oc (form II) form of plasmid—indication of nicks in plasmid DNA—was observed from 0.2 mM onwards with complete conversion by 1.02 mM. Data are presented as mean±SD ($n=5$)

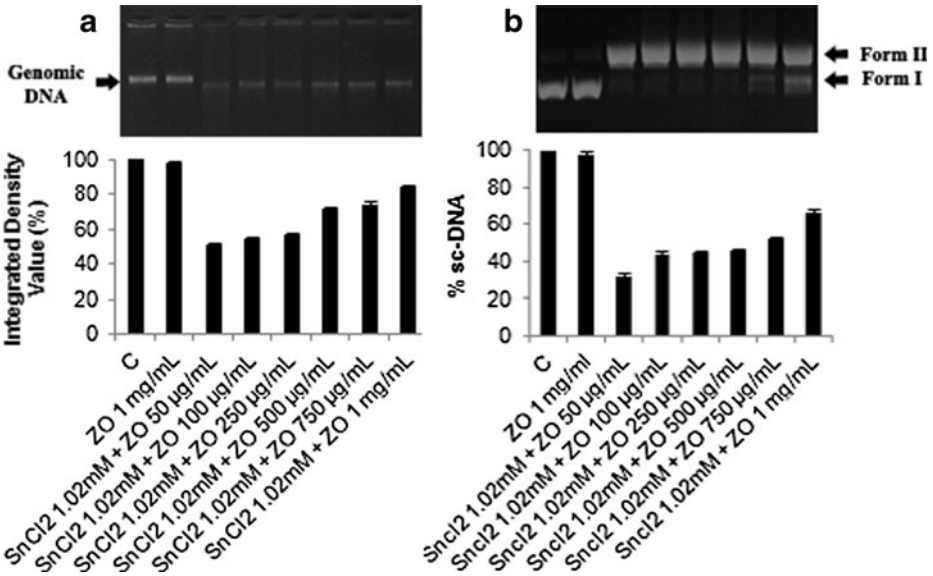


Fig. 3 Zingerone protection against $SnCl_2$. Agarose gel and its densitometric data showing DNA protection property of ZO against $SnCl_2$-induced DNA damage (1.02 mM) in **a** genomic DNA and **b** plasmid DNA. Data are presented as mean±SD ($n=5$)

DNA lesions such as single and double-stranded nicks, in addition to depurination and base and sugar modifications [6, 10, 12, 13].

Zingerone Protects Against $SnCl_2$-Induced DNA Damage

ZO was found to protect DNA against $SnCl_2$-induced DNA damage (1.02 mM). The concentration at which protection was conferred to genomic DNA was found to be at 500 μg/mL and above (Fig. 3a) while that for plasmid DNA was observed to be at 750 μg/mL and above (Fig. 3b). It was also observed that this protection was better in the Tris–Cl reaction buffer at pH 7.5 compared to that in pH 7.4. The DNA protective activity observed by us is most likely ascribable to the free radical scavenging activity by the hydroxyl groups present on ZO. The antigenotoxic, antiapoptotic, and antiperoxidative effects of ZO mediated by reduction of ROS have also been reported in Chinese hamster fibroblast cells and human lymphocytes [21, 29, 30].

Zingerone Protects Against UV/H_2O_2-Induced DNA Damage

The protective effect of ZO was also investigated against DNA damage induced by free radicals generated by a known hydroxyl radical generating system based on UV photolysis of hydrogen peroxide. Genomic DNA from *P. polycephalum* was found to be completely protected from cleavage in the presence of ZO at a concentration of 250 μg/mL onwards in comparison to the DNA smear observed in the absence of ZO (Fig. 4a). In the case of plasmid DNA, the amount of sc-DNA in the UV/H_2O_2-treated positive controls was found to be 33.63 %. Increasing amounts of sc-DNA ranging from 35 to

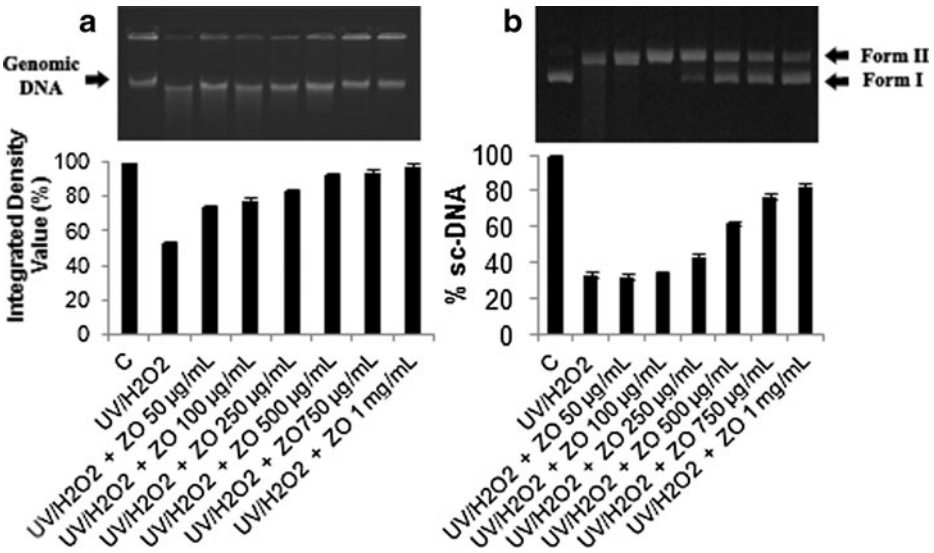


Fig. 4 Zingerone protection against ROS generated by UV/H_2O_2. Agarose gel and its densitometric data showing DNA protection property of ZO against UV/H_2O_2-induced DNA damage in **a** genomic DNA and **b** plasmid DNA. Data are presented as mean ±SD ($n=5$)

84 % were observed following treatment with ZO (50 μg/mL–1 mg/mL) in a dose-dependent manner (Fig. 4b). This clearly shows that ZO can protect DNA against damage induced by free radicals other than those produced by $SnCl_2$.

In conclusion, the results clearly show that the phenolic alkanone ZO has demonstrable protective effects against free radical-induced DNA damage as evidenced by inhibition of DNA strand breaks in plasmid and genomic DNA. Given the fact that ginger is used as a food additive and as traditional medicine globally and that the process of cooking converts some of the constituents in ginger into ZO, our results on its DNA protective action against oxidative damage attests to its potential beneficial use as a natural antioxidant food additive.

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