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Estimation of antigenotoxic properties of Ginger (*Zingibe officinale*) for DNA Damage after Exposure to Patulin in rats

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ABSTRACT. Patulin, a known fungal poison, is an important pollutant in apples and products derived from apples and fodder. Ginger improved the liver function and prevents hepatotoxicity against many toxins and used in treatment of various diseases. The genetic toxicity of patulin in the liver of rats using the comet assay in vivo was investigated and effect of ginger (*Zingibe officinale*) on DNA damage induced by patulin. Five groups of adult male Sprague Dawley rats were used. The rats were given 3.75 mg kg⁻¹ intraperitoneal (i.p) patulin. The rats were treated with ginger at a dose (100 mg kg⁻¹) for four weeks and eight weeks. DNA damage was measured in the liver of rats using a standard genetic toxicity tests comet assay. Tail length and percentage of tail DNA within the comet increased significantly ($p < 0.05$) in patulin group ($p = 0.01$), ($p = 0.000$) respectively, compared with control group a time-dependent manner, indicating an increase in DNA damage. The liver tissue showed signs of improvement in DNA damage after treating rats with ginger. In conclusion, these results indicate that patulin is genotoxic at the injected dose and that liver is an important target organ. Instead, ginger therapy can reduce DNA damage in liver cells from the toxicity caused by patulin.

Keywords: patulin; ginger; genotoxicity; antioxidant DNA damage; alkaline comet assay.

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Introduction

Patulin (4-hydroxy-4H-furo [3,2c] pyran-2[6H]-one), is a mycotoxin caused by several species of fungi of the genus *Penicillium* and *Aspergillus*, found in different species of fruit, and significant in apples. It is a very reactive compound that easily comes into combination with proteins and nucleic acids. This property is the main reason of harmfulness for living organisms, on the other hand it also permits quick inactivation of toxins in food and feed. Patulin has been declared the cause of gastrointestinal disorders with ulceration and bleeding, as well as genotoxicity, neurotoxicity, hepatotoxicity and immunotoxicity (Vidal et al., 2019). On account of the toxicological effects of patulin, different regulatory institutions worldwide have estimated maximum levels of patulin, chiefly in the apple-based products, ranging from 25 to 50 µgkg⁻¹. The Codex Alimentarius (Codex Alimentarius Commission, 1995) set the maximum allowable patulin levels in fruits and their juices at 50 µgkg⁻¹.

Zingiber officinale commonly known as ginger is one of the great widespread used spice worldwide with medicinal value. It has been used commonly in different conventional and folk medicinal systems around the world. Ginger oil is a very good antibacterial, antifungal compound and prevents food borne diseases when used in food preparation. The phytochemicals in ginger oil also have free radical removing, antioxidant and antiperoxidative effects. These characteristics are attributed to several biologically active constituents present in the fresh as well dried ginger oils. The antioxidant and lipid peroxidation inhibition features of ginger prevent peroxidative damage, referring the benefits of ginger in prohibition of microbial food spoilage, free radical-induced damage and rancidity (Akoachere et al., 2002; Mahboubi, 2019). Ginger has the antioxidant ability to ameliorate lead-induced hepatic injury by increase of superoxide dismutase (SOD) activity (Attia, Ibrahim, Nabil, & Aziz, 2013; Reddy, Chalamaiah, Ramesh, Balaji, & Indira, 2014). Ginger is an example of botanicals that play a remarkable role in pharmacology and treatment of various diseases (Zhou, Deng, & Xie, 2006). Ginger improved the liver function and prevents hepatotoxicity against lamotrigine (Poorrostami, Farokhi, & Heidari, 2014). Ginger has an antigenotoxic effect against Benzo(a)pyrene induced DNA damage (Nirmala, Prasanna, & Polasa, 2007).

The comet assay is a susceptible and fast technique for DNA damage revelation in individual cells and the first description of this method in 1984. Moreover, this method has been developed but is still not completely standardized, and differences are currently in widespread use with emphasis on applications in research and genetic toxicology (Liao, McNutt, & Zhu, 2009). The comet assay, which is also referred to as the individual cell gel electrophoresis assay, is a quantitative technique by which clearly evidence of DNA strand break in eukaryotic cells may be measured. It is depending on quantification of the denatured DNA fragments migrating out of the cell nucleus through electrophoresis. This assay has commonly been used in various areas including human biomonitoring, genetic toxicology, environmental monitoring and as a tool for research into DNA damage or repair in various cell types in response to a range of DNA-damaging agents (Collins, 2004). Quantitative analysis for DNA damage has given several parameters, including tailed nuclei, tail length, percentage of DNA in the tail and tail moment in the comet assay (Olive & Banáth, 2006; Hovhannisyan, 2010).

The target of our study was to assess if the toxicity of patulin could be attributed to its genotoxic effect in the target tissue. For this purpose, we examine the DNA damage induction by patulin in rat liver, as discovered using an in vivo comet assay. The intraperitoneal route of administration was selected, although not being the natural route of exposure, in order to permit rapid and direct delivery of the drug to the purpose organ, avoiding loss of activity and potential interspecies differences in the absorption rate of the drug. The results of this study may be beneficial in detecting patulin genotoxicity of liver and in evaluating the effects of ginger as a possible treatment against these genotoxic effects.

Material and methods

Chemicals

Patulin has chemical formula $C_7H_6O_4$ was purchased from SIGMA-ALDRICH Germany. Ginger powdered was obtained from local market.

Experimental animals

Adult male rats (Sprague Dawley) were used. Animals were 6-8 weeks of age at the beginning of the treatment and weighed about 100 g for each rat. They were kept under good conditions of moisture temperature and photoperiod (12 h light and 12 h dark). In general, five animals were used for each treatment group and for the positive control groups. Lodging and nutrition of animals were completed according to the conditions in place in the laboratory. Generally, rats had ability to access commercial food pellets and water through the study period.

Study design and treatment

Animals were divided into five groups of five rats each related to the treatments: NC group (Negative control), has no patulin nor ginger. PAT group (positive control), rats were injected with patulin at a single dose of 3.75 mg kg^{-1} , GIN group; rats were given food including ginger 100 mg kg^{-1} for eight weeks and PAT+GIN4 group; animals injected with a single dose of patulin as in PAT group (positive group) and given diet containing ginger as in GIN group for four weeks. Additionally, PAT+GIN8 group; rats injected with a single dose of patulin and ginger as in group PAT+GIN4 but, for eight weeks.

Comet assay

Liver samples were collected at necropsy. A middle part of the right lateral liver lobe was minced in cold mincing solution (20 mM EDTA in HBSS [Ca^{++} and Mg^{++} -free], pH 7.5 with 10% DMSO added immediately before using) to generate a single-cell suspension. Processing of tissues was completed as quickly as possible and was carried out within 60 min. from the beginning of necropsy. The alkaline comet assay, which detects strand breaks, alkali-labile sites, crosslinking and incomplete excision repair sites in DNA, was performed as described by Singh, McCoy, Tice, and Schneider (1988), with minor modifications (Tice et al., 2000; Collins, 2004).

Briefly, 20 μL of the cell suspension was mixed with 0.75% low-melting point agarose and instantly spread onto a glass microscope slide pre-coated with a layer of 1% normal-melting point agarose. The agarose was allowed to set at 4°C for 5 min. followed by incubating the slides in ice-cold lysis solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 1% Triton X-100 and 10% DMSO, pH 10.0) at 4°C for at least 1h to remove cellular proteins

and membranes, gaining the DNA as “nucleoids”. Then, the slides were placed in a horizontal electrophoresis unit (Pharmacia Biotech – EPS 200). The unit was contained a buffer (300 mM NaOH, 1 mM EDTA, pH >13.0), which was cover the slides for 20 min. at 4°C to allow for DNA unwinding and the expression of alkali-labile sites. Electrophoresis was carried out for 20 min. at 25 V (78 V/cm) and 300 mA. All the above methods were done under yellow light or in the dark to stop further DNA damage. Slides were neutralized (0.4 M Tris, pH 7.5), washed in double distilled water and stained using a silver staining procedure as reported by Nadin, Vargas-Roig, and Ciocca (2001). After the staining protocol, the gels were let dry at room temperature overnight and were analyzed using an optical microscope. To ensure suitable electrophoresis conditions and efficiency, negative and positive internal controls were included in each experiment. Test slides were scored only when internal controls showed clearly positive or negative results (Franke,Prá, Erdtmann, Henriques, & da Silva, 2005). One hundred cells were chosen and analyzed for DNA migration. When choosing cells, cells around the edges or air bubbles were excluded (Collins, 2004).

Statistical analysis

All the results were subjected to statistical analysis using IBM SPSS software version 21 utilizing one-way ANOVA. All results are presented as mean $\pm$ standard deviation at $p < 0.05$ level of significance. The diagram was made using GraphPad Prism software version 5.

Results

The findings obtained by the comet assay after injection of patulin and treatment with ginger are shown in Table 1. Comet length and percent tail DNA are the two essential parameters used to estimate the DNA damage. Animals treated with ginger after injection by patulin showed a significant ($p < 0.05$) decrease in the tail length and percent tail DNA in time-dependent manner (Table 1). Figures from 1 to 5 showed comet morphology of the various studied groups.

Table 1. Variation in % damage, tail length, % DNA in tail and tail moment after injection of patulin and treatment with Ginger.

Groups	%Damage Mean $\pm$ SD	Tail length Mean $\pm$ SD	%DNA intail Mean $\pm$ SD	Tail moment Mean $\pm$ SD	Treatment group
Control	1.5 $\pm$ 0.46	0.29 $\pm$ 0.057	1.75 $\pm$ 0.10	0.45 $\pm$ 0.086	8 days
Ginger	2.5 $\pm$ 0.56	0.78 $\pm$ 0.093*	4.5 $\pm$ 0.62*	0.61 $\pm$ 0.88	60 days
	p=0.11	p=0.03	p=0.01	p=0.25	
Patulin	19.6 $\pm$ 1.32*	5.77 $\pm$ 1.08*	32.8 $\pm$ 1.13*	2.24 $\pm$ 0.29*	24 h
	p=0.003	p=0.01	p=0.000	p=0.01	
PAT & Ginger (4weeks)	11 $\pm$ 0.20*	2.38 $\pm$ 0.35*	16.6 $\pm$ 0.56*	2.19 $\pm$ 0.27*	30 days
	p=0.002	p=0.01	p=0.001	p=0.01	
PAT &Ginger (8weeks)	7.67 $\pm$ 0.30	1.76 $\pm$ 0.23*	11.4 $\pm$ 0.52*	1.25 $\pm$ 0.25*	60 days
	p=0.001	p=0.01	p=0.001	p=0.05	

Mean $\pm$ SD; * denotes significant at $p < 0.05$ against control group.

In the current study, different comet assay parameters were used to detect DNA damage of liver cell. They were provided by using the image analysis software including tail length, percentage of DNA in the tail, percentage tailed cells and tail moment (Figure 1). The obtained results revealed that patulin induced a statistically significant increase in the frequency of tailed nuclei, tail length, percentage of DNA in the tail, and tail moment in rat liver cells compared to the control group (which showed some degree of DNA damage).

After treatment with ginger, liver DNA damage decreased and all comet assay parameters showed a statistically significant decrease compared to patulin injected group (Figure 1 A). Tail length statistically significant increased ($p < 0.05$) after injection by patulin compared to negative control group as shown in (Figure 1 B). The mean of tail length was about 2.38 in the group treated with ginger for four weeks (PAT+GIN4 group) and was about 1.76 in the ginger group treated for eight weeks, which means some repair but did not reach the normal level.

The mean percentage of tail DNA revealing the proportion of DNA that migrated from the head and was calculated as an average for the 50-100 cells selected for measurement (Figure1 C). The mean percentage of tail DNA in negative control group was 1.75% then significantly increased in the other studied group ($p < 0.05$). Comparing with injected positive control group (PAT group), the percentage tail DNA for the group treated with ginger was significantly decreased ($p < 0.05$).

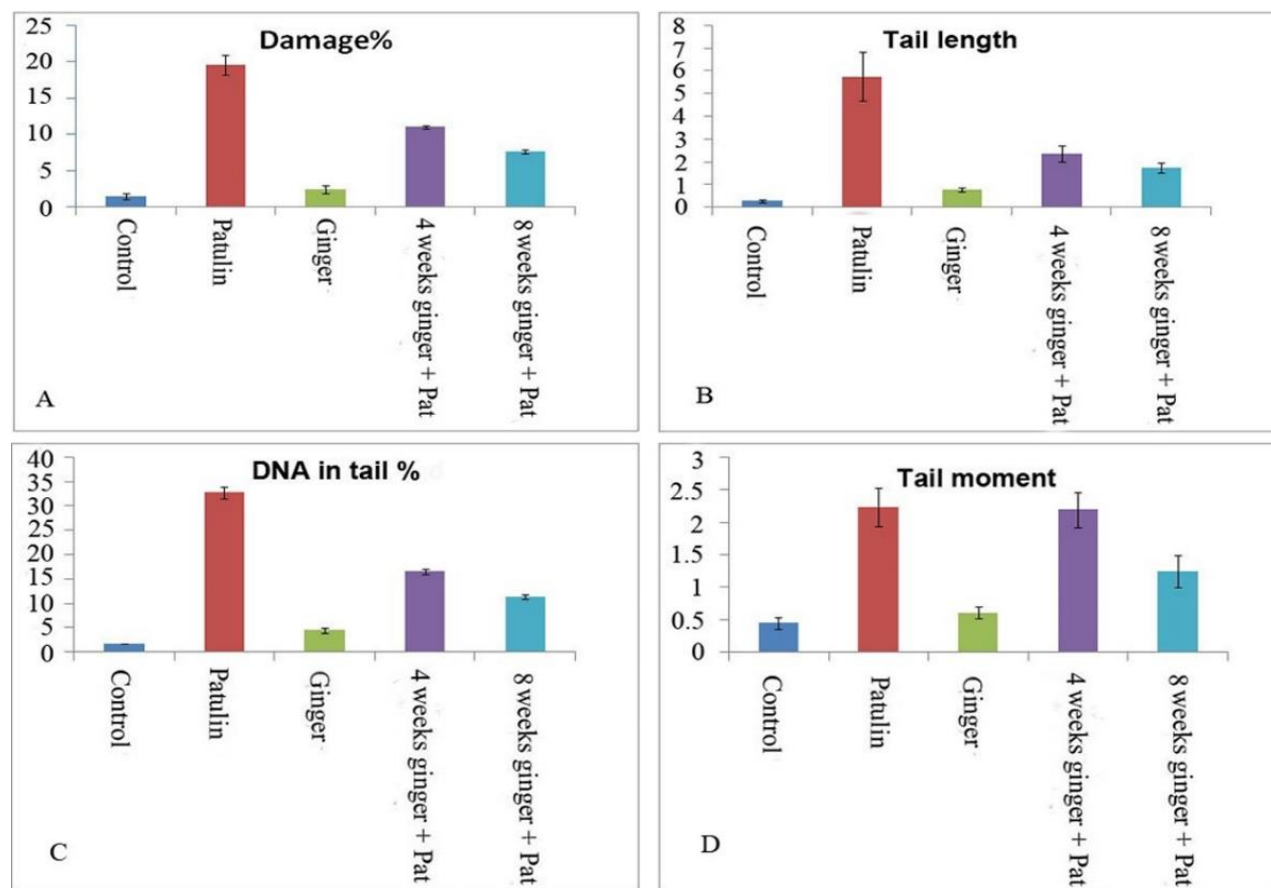


Figure 1. Histograms showing the DNA damage in liver of rats among studied groups as revealed by various comet assay parameters. A: Percentage of tail DNA; B: Tail length; C: Percentage of DNA in tail; D: Tail moment.

On the other hand, the mean tail moment (whose magnitude shows the frequency of DNA strand breaks per nucleus) for all groups compared to negative control group in Figure (1 D). These parameters show the same phenomena for percentage of tail DNA that ginger reduced the degree of damage induced by patulin but did not reach the control limit.

In this study, it was cleared that no significant DNA damage was established in the negative control group. In contrast, patulin induces significant DNA damage in the liver of rats (Positive control). These results show that patulin is genotoxic at the injected dose and liver is its important target organ. Cells in the Negative control animals appeared mostly with no comet. DNA was tightly compressed and maintained the circular disposition of the normal nucleus (Figure2).



Figure 2. Representative photograph of Comet morphology showing DNA migration pattern in liver cells of rats stained with ethidium bromide. Comet morphology of the control group of rats showing Grade 0 represents no damage (%tail DNA <5%).

The nuclear DNA in the positive control group has different profile. It was altered with the appearance of a fluorescent streak extending from the nucleus and the number of damaged DNA increases in liver cells (Figure3).

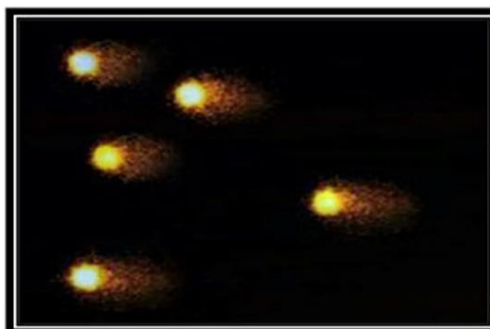


Figure 3. Representative photograph of Comet morphology of patulin group of rats showing grade 2 denotes medium damage (%tail DNA 20-40%)

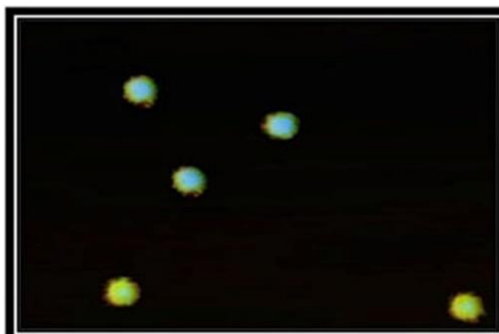


Figure 4. Representative photograph of Comet morphology of the Ginger group of rats showing Grade 0 represents no damage (%tail DNA <5%).

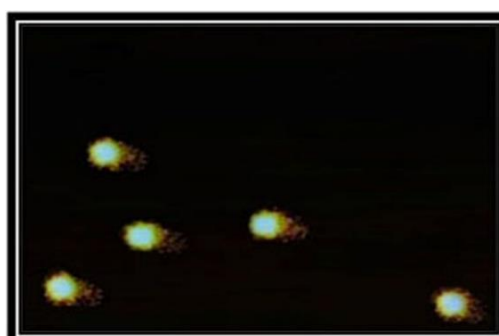


Figure 5. Representative photograph of Comet morphology of the patulin and Ginger group of rats treated for 4 weeks showing grade 1 signifies slight damage (%tail DNA 5-20%).

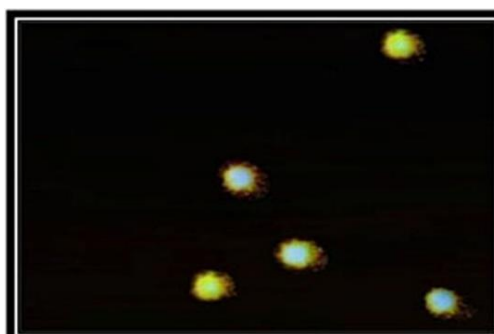


Figure 6. Representative photograph of Comet morphology of patulin group of rats treated for 8 weeks showing grade 1 signifies slight damage (%tail DNA 5-20%).

Ginger treatment induced significant decrease in DNA damage in the liver (Figure4). Ginger also showed protective role on patulin - induced DNA damage (Figure5 and 6).

Discussion

There is a rising attention to dependable biological markers of exposure to genotoxins. The harmful effects of ecologically occurring genotoxins including diet and other lifestyle factors result in the

formation of reactive oxygen species which have detrimental effects on DNA leading to genomic instabilities that are involved in the etiology of cancer (Limoli, Kaplan, Phillips, Adair, & Morgan, 1997).

The comet assay has been used widely spread in estimating genotoxicity. It has been used to measure DNA by breakage of strands and alkali-labile sites. The comet assay is applied to all kinds of animals tissues and is able to measure organ-specific toxic effects (Azqueta & Dusinska, 2015). It was commonly used for measuring the repair activity of cells, and in the past decade also of tissues.

The degree of DNA damage illustrated in the control group might be demonstrated by the fact that about 10 000 oxidation hits to DNA per cell have been evaluated to occur per day within the human body, and more than 35 different forms of oxidized bases are found in DNA (Ames, Shigenaga, & Hagen, 1993; Halliwell, 2000). Most damage is repaired by active DNA-repair enzymes, but some damage escapes repair, causing permanent damage (Jackson & Loeb, 2001).

The damage observed in liver in the present study could be attributed to increased cellular accumulation of patulin in these tissues, mediated by specific membrane transport of this hydrophilic compound. Interaction of human and rat organic anion transporters and human organic cation transporters with mycotoxins has been recognized (Tachampa et al., 2008). Transport characteristics and localization of these transporters have been elucidated mainly in the kidneys and liver, and recently in the blood-brain barrier (Alebouyeh et al., 2003). It has been observed that these organic ion transporters are the potential entrance pathway for mycotoxins mainly in kidneys and liver, resulting in the induction of adverse effects in humans and rats (Tachampa et al., 2008).

By means of the comet assay, the present study has illustrated some of the DNA changes in liver cells of rats subsequent to patulin. In undamaged liver cells, the DNA was tightly compressed and preserved the circular disposition of the normal nucleus. After patulin injection the profile of the nuclear DNA was changed with the presence of a comet that had a bright head and tail, thus allowing the cells' discrimination from those that had normal DNA. Comet assay was used to reveal DNA damage such as strand breaks and DNA-protein cross-links. Cross-links may stabilize chromosomal DNA and inhibit DNA migration (Merk & Speit, 1999; Yousef, Omar, El-Guendi, & Abdelmegid, 2010).

In the current study, the observed increased DNA migration in patulin group may be attributed to the induction of DNA strand breaks. Reduced DNA migration in the control group may be due to the induction of crosslinks, which are relevant lesions with regard to mutagenesis. While in the ginger treated groups, it was observed a repair of DNA damage, the reason may be that ginger contains gingerol, zingerone, phenolic, 3-diketones and shogaol. They act as removers of oxygen radicals resulting in the amelioration of anti-oxidants levels (Ramesh , Viswanathan, & Pugalendi., 2007) and protecting against DNA and cellular spoilage (Mohan & Nagini, 2003). Our study revealed that treatment with ginger improved and decreased the frequency of chromosomal aberration, increased mitotic index and enhanced DNA fragmentation. This is in parallel with the finding of (Yadamma & Devi, 2014) who observed the protective effect of ginger against genotoxicity (Salah, Abdouh, Booles, & Rahim, 2012).

Conclusion

The present study was carried out to evaluate the modification of antioxidant and hepatotoxicity by ginger against patulin induced damage in rats. Overall findings of the present work prove the protective effect of ginger and its enforcement to treat genotoxicity associated maladies.

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