



**HYDROGEN PEROXIDE INDUCED DNA DAMAGE: PROTECTION
BY PIPPALI (*PIPER LONGUM*) AQUEOUS EXTRACT**

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ABSTRACT

Reactive oxygen species causes oxidative damage to biological molecules like DNA which leads the formation of cancer and other chronic diseases. Dietary antioxidants are easily available, inexpensive, nontoxic in nature and well known to prevent the oxidative damage. The aqueous extract of Pippali (*Piper longum*) was investigated for its protective activity against Hydrogen peroxide

induced DNA damage by using a sensitive DNA submarine gel electrophoresis method. The antioxidants present in the extract prevent the DNA damage very effectively. The water extract at 15µg concentration was used against H₂O₂ hydrogen peroxide induced DNA fragmentation in agarose gel electrophoresis which provides same protection like other standard antioxidant BHA (400µM). The cytotoxicity studies showed that, the extract and BHA provides a protection of 75% and 71% respectively. In other words, the dietary spice source Pippali (*Piper longum*) extract showed a better DNA protectant activity against Hydrogen peroxide induced DNA damage.

KEYWORDS: Reactive oxygen species, DNA damage, Dietary antioxidants, lymphocytes, Hydrogen peroxide.

INTRODUCTION

Reactive Oxygen Species (ROS) induced oxidative damage plays a key role in DNA damage (Ray et al., 2012). The term ROS is often used by biologists to include oxygen radicals and certain non-radicals that are either oxidizing agents and /or converted into radicals, like HOCl, ozone, peroxy nitrite, singlet oxygen and Hydrogen peroxide (Wiseman H and

Halliwell B, 1996). ROS have been implicated in the pathophysiology of various states, including ischemia reperfusion injury, atherosclerosis, heart failure, acute hypertension, cancer etc. The free radicals, nitric oxide (NO) and $O_2^{\cdot-}$ react to form peroxynitrite (ONOO⁻) known as potent cytotoxic oxidant (Hemnani T and Parihar MS, 1998). During oxidative stress, the over produced Hydrogen peroxide can damage proteins, nucleic acids, cell membranes.^[4] It is reported that, Hydrogen peroxide cause oxidative DNA damage primarily through the hydroxyl radical that results from the Fenton reaction (Siddique et al., 2009, Imlay et al., 1988, Halliwell B and Gutteridge JM., 1992, Josephson et al., 1991), reported that, Hydrogen peroxide associated with the induction of cancer in animals, mutagenic and carcinogenic (Andreoli et al., 1999, Pryor WA, 1986). It has been reported that, which may cause DNA damage in the form of chromosomal aberrations, sister chromatid exchanges, single strand breaks and double strand breaks (Sofni T, and Ishidate M, 1984, Tsuda H., 1981, Prise et al., 1989, Thibodeau PA and Paquette B, 1999, Namrah anwar et al., 2014). In the present study, we have examined the DNA damage protectant activity of aqueous extract of *Piper longum*.

MATERIALS AND METHODS

Calf thymus DNA (CT DNA), BHA, Agarose, Ethidium bromide, SDS, H_2O_2 , Thiobarbutaric acid, Diphenylamine (DPA) was from Sigma Chemical company USA. Ascorbic acid was from HIMEDIA, India. EDTA, H_2O_2 were from s.d. fine Chem. Ltd. India. All the other chemicals were of Anal. R grade. All organic solvents were distilled prior to use.

The fresh *Piper longum seeds* were collected from local market, homogenized to fine powder and stored in air tight glass container. The extraction was done according to the method of Dinesha and Chikkanna (Dinesha R and Chikkanna D, 2014) using 10g of powder in 100ml of double distilled water. The mixture was vortexed for 2 hours and centrifuged at 10000 rpm. The obtained supernatant was concentrated to require volume using hot water bath and the preliminary qualitative phytochemical studies was done as explained by Dinesha and Chikkanna, 2014.

Isolation of lymphocytes

The lymphocytes were isolated from human blood according to Phillips HJ, 1973 method. Human peripheral lymphocytes were isolated from 10 ml of venous blood drawn from healthy donors. Blood was collected in ACD (85mM citric acid, 71mM trisodium citrate, 165mM D-glucose) in the ratio of 5:1. Four volumes of hemolyzing buffer (0.85%

NH₄Cl in 10mM tris buffer, pH 7.4) were added, mixed well, incubated at 40°C for 30 min. Centrifuged at 1200rpm for 12 min, pellet was washed again with 5ml of hemolysing buffer and the pellet containing cells were washed thrice with 10ml of Hank's Balanced salt solution (HBSS- 250mM m-insositol in 10mM phosphate buffer, pH 7.4) and suspended in same solution. The cell viability test was determined by tryphan blue exclusion method (Kotzé, 1997). To 10µl of lymphocyte sample 10µl of tryphan blue (0.02%) added and the cells were charged to Neuber's chamber and the cell number was counted. The survival rate lymphocytes were determined at 60 minutes of incubation. Viability was tested by Tryphan blue exclusion method. The percentage viability was calculated by using the following formula

$$\% \text{ viability} = \frac{\text{Total no. of viable cells}}{\text{Total no. of viable cells} + \text{dead cells}} \times 100$$

Time course study of the effect of H₂O₂ on the viability of lymphocytes. Lymphocytes cells (1X 10⁶) were treated with H₂O₂ (144µM) in the presence or absence of antioxidants in 1ml of HBSS, pH 7.4 at 37°C. The simultaneous, post and pre treatment of antioxidants were carried out and after the desired incubation time up to 6 hours, the viability of the cells was determined by tryphan blue exclusion analysis and the percentage of the viable cells was calculated.

DNA damage and its protection by Agarose gel electrophoresis

The DNA damage studies using agarose gel electrophoresis was done according the method of Sultan et al., 1995. 10µg of Calf thymus DNA was pretreated with Pippali (*Piper longum*) extract (10µg), or BHA (400µM) in 0.5 ml HBSS, pH 7. At 37°C for 20 minutes, then H₂O₂ (144µM) was added and the final volume was made to 1ml with HBSS, pH 7.4 and incubated at 37°C for 60 min, then centrifuged at 1200 rpm, 20 minutes at 40°C. Then DNA samples were run on 1% Agarose prepared in TBE buffer and the Ethidium bromide was incorporated into the gel at a concentration of 1µg/ml, 2µg of DNA was loaded on to the wells and run in TBE buffer (10mM Tris, Boric acid, EDTA, pH 8.0) at 60 volts. The bands were visualized and photographed under transilluminator.

Statistical analysis

The data were expressed as means \pm standard deviations (SD). All the experiments were repeated at least six times and the values are expressed as Mean \pm SD. The significance of the experimental observation was checked by student's test and the value of p value

RESULTS

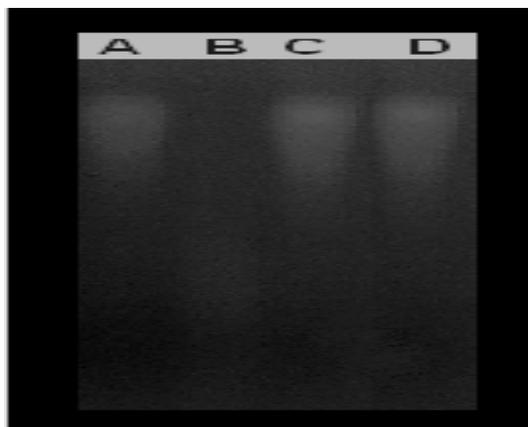


Fig.1: H₂O₂ (144 μ M) induced DNA damage and its prevention by Pippali (*Piper longum*) aqueous extract.

Lane A: Calf thymus DNA (10 μ g)

Lane B: Calf thymus DNA (10 μ g) + H₂O₂ (144 μ M)

Lane C: H₂O₂ (144 μ M) + Pippali (*Piper longum*) aqueous extract (25 μ g)

Lane D: H₂O₂ (144 μ M) + BHA (400 μ M)

in 100 μ l TBE (10 mM Tris–boric acid–EDTA, pH 7.4), incubated at 37°C for 30min.

Electrophoresis was carried out at 80 V. Bands were visualized using UV transilluminator.

Table -1: Study of cell toxicity induced by H₂O₂ and protection by antioxidants

Lymphocytes	% viability
Lymphocytes alone (10 μ l)	88 \pm 3
Lymphocytes (10 μ l) + H ₂ O ₂ (144 μ M)	43 \pm 1
Lymphocytes + H ₂ O ₂ (144 μ M) + BHA (400 μ M)	71 \pm 4
Lymphocytes + H ₂ O ₂ (144 μ M) + Pippali (<i>Piper longum</i>) (25 μ g)	75 \pm 3

Lymphocytes (10⁶ cells) pretreated with or without antioxidants at indicated concentrations in 0.5ml HBSS pH 7.4, incubated at 37°C for 20min., then H₂O₂ (144 μ M) was added, incubated at 37°C for 60 minutes in final volume of 1ml HBSS, pH 7.4. After the desired incubation time (60 minutes), viability of the cells was determined by Tryphan blue exclusion and the percentage of viable cells was calculated as mentioned in methods.

DISCUSSION

The extent of H₂O₂ (144μM) induced DNA damage was considerable in the lymphocytes as measured by diphenylamine method. As shown in Fig.1., the *Piper longum* extract offered effective protection at 25μg concentration against H₂O₂ (144μM) induced DNA fragmentation in lymphocytes where as *Piper longum* aqueous extract at 25μg and BHA at 400 μM dosage inhibited DNA fragmentation respectively. The above result indicated that *Piper longum* aqueous extract is effective in preventing DNA fragmentation like standard antioxidant BHA. We also investigated the protective effects of *Piper longum* extract against H₂O₂ induced lymphocyte cell death. The viability of lymphocytes on simultaneous pre treatment of H₂O₂, a time course study was done. As shown in the Table-1, the decrease in viability brought about by H₂O₂ after 60 mins of incubation and the viability was found to be 43 ± 1% from 88 ± 3% and it was vastly improved by the presence of BHA at 400 μM and *Piper longum* (25μg) which showed 71 ± 4 and 75 ± 3% viability, respectively. These results indicate that the efficiency of the each antioxidant tested exhibits efficient protection against H₂O₂. Thus the protective mechanism against oxidative DNA damage by *Piper longum* extract is probably due to quenching the free radicals mainly hydroxyl radical or ROS. In summary, the protective effect of aqueous extract of *Piper longum* was equally efficient to BHA and more efficient on pro-oxidants induced lymphocyte cell damage and oxidative DNA damage in lymphocytes at a lower dose of 25μg concentration.

CONCLUSION

This preliminary results showed that, the protective effect of an aqueous extract of *Piper longum* against pro-oxidant induced DNA damage and proved that, it is non toxic to cells.

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