ABSTRACT

Objective: The main objective of the study is to find a natural resource towards preventing oxidative DNA damage induced by t-BOOH.

Materials and methods: By using standard antioxidant Butylated Hydroxy Anisole, Coleus aromatic extract, Tertiary butyl hydroperoxide, calf thymus DNA. The sheared DNA was used in submarine agarose gel electrophoresis where DNA damage was done by using tertiary butyl hydroperoxide. Similarly, the cytotoxicity study was also done to know the natural antioxidant itself is not toxic to cells by using lymphocytes and these lymphocytes were isolated from fresh human blood. Results and conclusions: The agarose gel electrophoresis bands were visualized in Transilluminator where, it was confirmed that, tertiary butyl hydroperoxide induced DNA damaged was successfully prevented by BHA (400µM) and Coleus aromatic (25µg) extract. In cell cytotoxicity studies, BHA and Coleus aromatic extract inhibits the cell damage 71% and 75% respectively. The results confirms that, Coleus aromatic extract can inhibit tertiary butyl hydroperoxide induced DNA damage and is nontoxic to cells.

KEYWORDS: Reactive oxygen species, DNA damage, Dietary antioxidant, lymphocytes, t-BOOH.
INTRODUCTION
ROS are widely found in living organisms as well as in the environment. ROS is though having function in living things, if it is excess, toxic to cells (Graves and David, 2012). Excessive production of Reactive Oxygen species is very detrimental to the DNA is the key macromolecule of human cell (Circu, 2010). When ROS were uncovered in the environment, they were found to cause the same types of harm as they do in living organisms (Huang et al., 2005). In nature, ROS can be generated from number of sources like combustion and photochemical reactions (Shiraiwa et al., 2012). There are different types of DNA damage takes place due to ROS like OH radical attack on DNA causes base modification particularly of guanine to 8 hydroxy-guanine (Gredilla, 2010). ROS cause a spectrum of DNA lesions, including single-strand breaks, double-strand breaks, crosslinking of DNA and damage to bases and the deoxyribose moiety, because of the moderate reactivity of these species in aqueous solutions (Johnson et al., 2014). It has been proposed that their conversion to the highly reactive hydroxyl radical is responsible for DNA damage and the organic hydroperoxide, tertiary-butylhydroperoxide (t-BOOH), causes oxidative damage in a number of cell types (Jirásek, 2014). Though there are number of standard synthetic antioxidants are available, one should also see the side effects, hence, there is lot of interest and need to find alternate natural dietary antioxidants which we are already using as dietary source or component. The earlier studies by the same authors showed that, the Coleus aromatic extract is an good antioxidant and also prevents Hydrogen peroxide induced DNA damage (Dinesha et al., 2014).

MATERIALS AND METHODS
Calf thymus DNA (CT DNA), BHA, Agarose, Ethidium bromide, SDS, t-BOOH, Thiobarbutaric acid, Diphenylamine (DPA) was from Sigma Chemical company USA. Ascorbic acid was from HIMEDIA, India. EDTA, t-BOOH 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 extraction was done according to the method of Subhas CM et al. (2010). In brief, fresh leaves of Coleus aromaticus were collected from authentic source. The plant material was washed with double distilled water, shadow dried, homogenized to a fine powder and stored in air tight glass container. The extraction was done using 10g of plant leaves 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 required volume using hot water bath. The extract was subjected for preliminary qualitative phytochemical studies.

**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 ealhy 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 trypan blue exclusion method. 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 and exceeded 96% in each isolation. 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 t-BOOH on the viability of lymphocytes. Lymphocytes cells (1X 10⁶) were treated with t-BOOH (10μg) 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 Coleus aromaticus leaf
extract (10μg), or BHA (400μM) in 0.5 ml HBSS, pH 7. At 37°C for 20 minutes, then t-BOOH (125μM) 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 ± standard deviations (SD). All the experiments were repeated at least six times and the values are expressed as Mean ± SD. The significance of the experimental observation was checked by student’s test and the value of p value.

**RESULTS AND DISCUSSION**

![Fig1](image)

**Fig.1: t-BOOH (125μM) induced DNA damage and its prevention by Coleus aromatic* aqueous extract.**

Lane A: Calf thymus DNA (10μg)
Lane B: Calf thymus DNA (10μg) + t-BOOH (125μM)
Lane C: t-BOOH + Coleus aromatic* aqueous extract (25μg)
Lane D: t-BOOH + BHA (400μM)

In 100 μl 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 t-BOOH and protection by antioxidants.

<table>
<thead>
<tr>
<th>Lymphocytes</th>
<th>% viability</th>
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<tbody>
<tr>
<td>Lymphocytes alone (10µl)</td>
<td>88± 3</td>
</tr>
<tr>
<td>Lymphocytes (10µl) + t-BOOH (125µM)</td>
<td>43± 1</td>
</tr>
<tr>
<td>Lymphocytes + t-BOOH + BHA (400µM)</td>
<td>71± 4</td>
</tr>
<tr>
<td>Lymphocytes + t-BOOH + <em>Coleus aromatica</em> (25µg)</td>
<td>75± 3</td>
</tr>
</tbody>
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Lymphocytes (10^6 cells) pretreated with or without antioxidants at indicated concentrations in 0.5ml HBSS pH 7.4, incubated at 37°C for 20min., then t-BOOH (125µM) 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.

Uncontrolled production of ROS follows cell aging, apoptosis – a programmed cell death or DNA mutant mediated carcinoma (Van Remmen et al., 2003). For elucidating the Oncogenic process and mechanism, it is necessary to quantify the DNA oxidative damage (Mangerick et al.,2012) The extent of t- BOOH (125µM) induced DNA damage was considerable in the lymphocytes as measured by diphenylamine method. As shown in Fig.1. *Coleus aromatic* leaves extract offered effective protection at 25µg concentration against t-BOOH (125µM) induced DNA fragmentation in lymphocytes where as Coleus aromatic aquaeous extract at 25µg and BHA at 400 µM dosage inhibited DNA fragmentation respectively. The above result indicated that *Coleus aromatic* aqueous extract is effective in preventing DNA fragmentation like standard antioxidant BHA. We also investigated the protective effects of Coleus aromatic aqueous extract against t-BOOH induced lymphocyte cell death. The viability of lymphocytes on simultaneous pre treatment of t-BOOH, a time course study was done. As shown in the Table-1, the decrease in viability brought about by t-BOOH after 60 mins of incubation and the viability was found to be 43 ± 1% from 83 ± 1% and it was vastly improved by the presence of BHA at 400 µM and *Coleus aromatic* aqueaeus extract (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 t-BOOH. Thus the protective mechanism against oxidative DNA damage by *Coleus aromatic* extract is probably due to quenching the free radicals mainly hydroxyl radical or ROS.
SUMMARY
The protective effect of aqueous extract of *Coleus aromatica* 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. This is the preliminary study that reports the protective effect of an aqueous extract of *Coleus aromatica* against prooxidant induced DNA damage.

REFERENCE

