International Journal of Current Pharmaceutical Research

ISSN- 0975-7066 Vol 3, Issue 1, 2011

Research Article

ANTIOXIDANT ACTIVITY OF *SPATHODEA CAMPANULATA* IN PREVENTION OF T-BOOH AND H_2O_2 INDUCED DNA DAMAGE

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Received: 22 Aug 2010, Revised and Accepted: 24 Sep 2010

ABSTRACT

Reactive oxygen species (ROS) causes the oxidative damage to biological macromolecules like DNA which leads to the generation of cancer and other chronic diseases. Dietary antioxidants are known to prevent the oxidative damage. In this study, antioxidant rich dietary sources in leaves and flowers of *Spathodea campanulata* are used to prevent the DNA damage induced by t-Butyl hydroperoxide and hydrogen peroxide. The antioxidants present in the ethanol extract of leaves and flowers of *Spathodea campanulata* prevent the DNA damage very effectively. The both ethanol extracts offered protection up to 95% at 50µg concentration against t-BOOH and H_2O_2 induced DNA fragmentation in agarose gel electrophoresis. Whereas the standard antioxidants like BHA showed 90% protection at 400µM concentration. Hence the combined effect of antioxidants present in the both ethanol extracts are very potent in prevention of ROS induced DNA damage.

Keywords: Reactive oxygen species, DNA damage, Dietary antioxidants, lymphocytes, t-Butyl hydroperoxide

INTRODUCTION

Excessive production of Reactive Oxygen species is very detrimental to the DNA is the key macromolecule of human cell. ROS at physiological concentration is required for normal cell function. The excessive production of ROS is very hazardous to cells and it can cause oxidative damage to lipids, proteins and DNA[1, 2]. ROS induced DNA damage leads to the ageing, atherosclerosis, cancer, cardiovascular diseases and other neurodegenerative diseases in human beings[3, 4]. The oxidative damage results in DNA bases modifications such as single and double strand breaks formation of apurine/apyrimidine lesions and many of which are toxic and mutagenic[5]. Oxidative DNA damage can also increases the frequency of strand exchange intermediates during DNA replication which leads to genomic instability. Finally, this slipped or exchange intermediates have the way for the modification of base or strand breaks[6, 7, 8].

It has been shown that hydrogen peroxide induced oxidative DNA damage cause microsatellite instability, which is associated with colorectal cancer[9]. Protection of DNA damage by using antioxidants is a new strategy. Hence, it is important to maintain a balanced antioxidant status and reducing the levels of DNA damage and causes of other diseases. The use of dietary antioxidants which play very important role in preventing the ROS induced DNA damage.

In this view, we have selected an herb called as *Spathodea campanulata* which has been used as the folk medicine. *SpathodeacampanulataP.Beauv* species belonging to the family of Bignoniaceae. The bark is reported to contain sterols, triterpenoids, tranorin, vannillic acid, ferulic acid, verminoside, pelargonidin diglycoside, maldivin and tannins. Leaves contain polyhydroxy sterol spathodol, quercetin and chlorogenic acid[10]. The flowers are employed as a diuretic and anti-inflammatory, while the leaves are used against kidney diseases, urethral inflammations and as an antidote against animal poisons. The stem bark preparations are employed against enemas, fungus skin diseases, herpes, stomachaches and diarrhea [11].

Several medicinal property have been reported on it including, S. *campanulata* an experimental evaluation of the analgesic and anti-inflammatory properties[12], Hypoglycemic, anti-HIV and antmalarial activity were also observed in stem bark extracts[13,14] and the presence of anthocyanins in flowers of *S. campanulata*[15]. The present study we focused to evaluate the antioxidant activity of *Spathodea campanulata* are very effective in preventing the DNA damage which is mediated by H_2O_2 and t-BOOH.

MATERIALS AND METHODS

Plant collection and extraction

The fresh leaves and flowers of *Spathodea campanulata* were collected from B.G. Nagar, Mandya (District), Karnataka, India. Fresh plant material were washed with tap water, air dried, homogenized to a fine powder and stored in air-tight containers. Same procedure was followed for both leaf and flower extraction. The air dried flowers of *Spathodea campanulata* coarsely powdered and extracted with ethanol in a Soxhlet extractor for 18-20 hours and solution was evaporated to dryness under reduced pressure and controlled temperature by using rotavapor. The extract was stored in a refrigerator at 4°C until further use. The extract was subjected for preliminary qualitative phytochemical studies.

Preliminary phytochemical screening[16]

Phytochemical studies of ethanol extract of *S. campanulata* leaves and flowers showed the presence of alkaloids, saponins, steroids, glycosides, flavonoids, triterpenoids and tannins.

Isolation of lymphocytes

Human peripheral lymphocytes were isolated from 10 ml of venous blood drawn from young healthy, non-smoking donors. Blood was collected in ACD (85mM citric acid. 71mM trisodium citrate, 165mM D-glucose) in the ration of 5:1. Four volumes of hemolysing buffer (0.85% NH_4Cl in 10mM tris buffer, pH 7.4) were added, mixed well, incubated at 40^0C 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 was determined by tryphan blue exclusion method[17]. To 10ul 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 time intervals 20^{th}, 40^{th} and 60^{th} minutes of incubation. Viability was tested by tryphan blue exclusion and exceeded 96% in each isolation.

The percentage viability was calculated by using the formula

$$\% \text{ cell viability} = \frac{\text{Number of viable cells}}{} \times 100$$

Total number of cells

Time course study of the effect of H_2O_2 and t-BOOH on the viability of lymphocytes

Lymphocytes cells (1X 10^6) were treated with ferrous sulphate: ascorbate (2:20µmole) in the presence or absence of antioxidants in 1ml of HBSS, pH 7.4 at 37^0C. 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.

Analysis of Calf thymus DNA damage and its protection by Agarose gel electrophoresis

10µg of Calf thymus DNA was pretreated with *S.Campanulata* leaves and *flowers* extracts (50µg), or BHA (400µM) in 0.5 ml HBSS, pH 7. At 37^0C for 20 minutes, then t-BOOH (125µM)), or H_2O_2 (144µM) was added and the final volume was made to 1ml with HBSS, pH 7.4 and incubated at 37^0C for 60 min, then centrifuged at 1200 rpm, 15-20 minutes at 40^0C. 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 UV light transilluminator.

Statistical analysis

The data were expressed as means ± standard deviations (SD). All the experiments were repeated at least six times. 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 < 0.05 was considered significant. The significance was indicated by a star mark (*).

RESULTS AND DISCUSSION

Excessive production of ROS follows cell aging, apoptosis or DNA mutant mediated carcinoma. For elucidating the oncogenetic process and mechanism, it is important to quantify the DNA oxidative damage. The extent of t-BOOH (125µM) and H_2O_2(144 µM)induced DNA damage was considerable in the lymphocytes as measured by diphenylamine method. As shown in Fig.1. *S.Campanulata* leaves and *flowers* extracts offered effective protection about 95% & 90% at 50µg concentration against t-BOOH (125µM) induced DNA fragmentation in lymphocytes where as BHA at 400 µM inhibited DNA fragmentation by 61% and 85%, respectively. The above results indicate that *S.Campanulata* leaves and *flowers* extracts are effective in preventing DNA fragmentation than standard antioxidant BHA.

We also investigated the protective effects of *S.Campanulata*leaves and *flowers* extracts against H_2O_2 induced lymphocyte cell death. The viability of lymphocytes on simultaneous, post and pre treatment of ferrous sulphate: ascorbate (2: 20 µmole), a time course study was done. As shown in the Table1, the decrease in viability brought about by H_2O_2 after 60 mins of incubation and the viability was found to be 36 ± 2% and it was vastly improved by the presence of *S.Campanulata* leaves and *flowers* extracts at 50µg and BHA at 400 µM which showed 85 ± 3, 84 ± 7, and 64 ± 4% viability, respectively. These results indicate that the efficiency of the each individual antioxidant tested exhibits efficient protection against Fe: As. Thus the protective mechanism against oxidative DNA damage by *S.Campanulata* leaves and *flowers* extracts is probably due to quenching the free radicals mainly hydroxyl radical or ROS.

Further, the protective effect of *S.Campanulata*leaves and *flowers*extracts on t-BOOH and H_2O_2 induced DNA damage in calf thymus DNA was established by agarose gel electrophoresis. As shown in Fig 1, the treatment of t-BOOH (125µM) (Lane 2) concentration revealed as more indicating DNA damage. The above DNA damage was protected by *S.Campanulata* leaves and *flowers* extracts at 50ug concentration as seen in Lane3 & 4, which is comparable to the untreated calf thymus DNA (Lane 1). Similarly Lane 3 shows that the protection of DNA damage exhibited by BHA at 400 µM concentration. This study may well present an evidence for modulatory effect of natural antioxidants on oxidative DNA damage induced by t-BOOH.

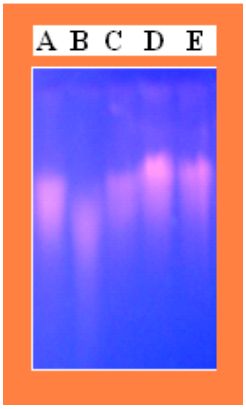

Fig. 1: t-BOOH (125µM) induced DNA damage and its prevention by *S.Campanulata*leaves and *flowers* extracts

Lane A: Calf thymus DNA (10µg)

Lane B: Calf thymus DNA (10µg) + t-BOOH (125µM)

Lane C: As 2+ BHA (400µM)

Lane D: As 2+leaves extract (50µg)

Lane E: As 2+flowers extract (50µg)

Fig.1. DNA protectant activity of the leaves and flowers extracts against t-BOOH *(125µM)* induced calf thymus DNA damage. Lane A: Calf thymus DNA (10 µg), Lane B: As 1 +t-BOOH *(125µM)*, Lane C: As 2 + BHA (400 µM), Lane D: As 2 + leaves (50µg), Lane E: As 2 + flowers (50µg) in 100 µl TBE (10 mM Tris–boric acid–EDTA, pH 7.4), incubated at 37 ^{0}C for 30 min. Electrophoresis was carried out at 80 V. Bands were visualized in a UV transilluminator.

Fig. 2: H_2O_2 (144µM) induced DNA damage and its prevention by *S.Campanulata* leaves and *flowers* extracts

Lane A: Calf thymus DNA (10µg)

Lane B: Calf thymus DNA (10µg) +H_2O_2 (144µM)

Lane C: As 2+ BHA (400µM)

Lane D: As 2+ leaves extract (50µg)

Lane E: As 2+ flowers extract (50µg)

Fig.2. DNA protectant activity of the leaves and flowers extracts against H_2O_2(144µM) induced calf thymus DNA damage. Lane A: Calf thymus DNA (10 µg), Lane B: As 1 + H_2O_2(144µM), Lane C: As 2 + BHA (400 µM), LaneD: As 2 + leaves (50µg), Lane E: As 2 + flowers (50µg) in 100 µl TBE (10 mM Tris–boric acid–EDTA, pH 7.4), incubated at 37 ^{0}C for 30 min. Electrophoresis was carried out at 80 V. Bands were visualized in a UV transilluminator.

Thus the protective effect of *S.Campanulata* leaves and *flowers*extracts 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 50µg concentration. This is the first study that reports the protective effect of an antioxidant protein against prooxidant induced DNA damage.

ACKNOWLEDGEMENT

We are thankful to Principal, SAC college of Pharmacy for providing the facilities for research work. Authors are also thankful to Adichunchanagiri Biotechnology and Cancer Research Institute, B.G. Nagar for their kind support.

Table 1: Percentage Viability of lymphocytes against H_2O_2 and t-BOOH induced damage on lymphocytes

S. No.		Experiments	% of Viability of lymphocytes at				
			0 mins	30 mins	40 mins	50 mins	60mins
1.	01	Fresh lymphocytes alone	98	97	93	90	88
2.	02	Lymphocytes + H_2O_2(144µM)	-	60	55	40	35
3.		Lymphocytes + t-BOOH(125µM)	-	56	51	38	30
4.	03	Lymphocytes + H_2O_2+ leaves extract(50µg)	-	64	57	43	31
5.	04	Lymphocystes + t-BOOH+ leaves extract(50µg)	-	92	85	80	85
6.	05	Lymphocytes + H_2O_2+ flowers extract(50µg)	-	93	91	89	84
7.	06	Lymphocytes + t-BOOH+ flowers extract (50µg)	-	91	89	85	83

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