Research Article

In vitro antioxidative and cytotoxic effect of protein fraction from *Tabernaemontana divaricata* leaves against DLA cells

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Abstract

Background: The rising burden of cancer worldwide calls for an alternative treatment solution. Herbal medicine provides a very feasible alternative to western medicine against cancer. **Objectives:** The present study assessed the *in vitro* antioxidative potential and cytotoxicity of *Tabernaemontana divaricata* leaves protein fraction. **Materials and Methods:** The antioxidant activity of protein fraction of *T. divaricata* was performed by several antioxidative assays including 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay, ABTS, superoxide radical (SO) scavenging assay and Hydrogen peroxide (H_2O_2) with standard protocol, followed by Cytotoxicity test with tryphan blue assay. **Results:** From the results, *T.divaricata* has been found to have the significant antioxidant activity in a dose-dependent manner and IC_{50} value was 59.1µg/ml for DPPH, 48 µg/ml for ABTS, 60 µg/ml for SO and 38.5 µg/ml for H_2O_2. Further, the cytotoxicity analysis was determined against Dalton Lymphoma Ascites (DLA) cell line and the IC_{50} value was found to be 62µg/ml for protein fraction of *T.divaricata.* **Conclusion:** Hence, the current study attests that *T. divaricata* is a fine source of natural antioxidants with anticancer agents and can be used in pharmaceutical preparations for the treatment of diseases induced by oxidative stress.

***Keywords*:** *T. divaricata,* antioxidants, dalton lymphoma ascites, trypan blue assay

Introduction

Medicinal plants are considered as potential source for drug development and many novel products have reached clinical trials. Scientists are investigating properties of medicinal plants in order to develop novel drugs against disease like cancer, from natural products. Medicinal herbs have profound scope and have been used to find potential anticancer compounds in them (Riaz *et al.,* 2016) Oxidative stress can damage cells as well as tissues, which therefore leads to various dreadful degenerative diseases like cancer (Wolf and Dean, 1987). About 127 lakhs of new cancer cases was estimated according to the International Agency for Research on Cancer in 2008. The global burden is may rise to 21.4 million by 2030 (Jacques *et al.,* 2010). Hence, supplementation of herbal antioxidants is necessary to suppress the oxidative stress in a healthier way. Use of man-made anti-oxidants like butylated hydroxy toluene (BHT) and butylated hydroxy anisole (BHA) are restricted due to their side effects (Madhavi and Salunkhe, 1995). Recently, various antioxidants are acquired from naturally available plants that have the capacity to scavenge free radical or active oxygen (Knekt *et al.,* 1996). Free radicals are a class of highly reactive, chemical species which contains one or more unpaired electrons due to which they are highly unstable and cause damage to other molecules by extracting electrons from them in order to attain stability. Reactive oxygen species (ROS) and free radicals, such as superoxide anion, DPPH and hydroxyl radical, are constantly formed in the human body by normal metabolic action. Their action is opposed by a balanced system of antioxidant defenses, including antioxidant compounds and enzymes. Excess production of these free radicals and reactive oxygen species leads to a number of oxidative degenerative disorders in the body. In order to neutralize the free radical damage, the biological system acts synergistically to deactivate the free radicals before they attack the cell. Recently, much attention has been given to naturally occurring antioxidants, which may play an important role in inhibiting both free radicals and oxidative chain reactions. Ascetic fluid is the direct nutritional source for tumor cells, and therefore, a rapid

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increase in ascetic fluid with tumor growth would be a means to meet the nutritional requirement of tumor cells (Prasad and Giri, 1994); (Haldar *et al.,* 2010).Therefore, searching for safer and effective antioxidants from natural plants is of great interest among researchers. Based on that phytomolecule from plant sources have been widely reported to possess antioxidant activity (Srivastava *et al.,* 2013). In addition, plant proteins have also been demonstrated to be a good source of antioxidants for instance, active peptides from walnuts (Chen *et al.,* 2012) pulses (Lopez-Girona *et al.,* 2012) and corn gluten meal have been assessed for their antioxidant capacities (Zhuang *et al.,* 2013).

Materials and Methods

Collection and authentication of Plant

The fresh leaves of ***Tabernaemontana divaricata*** (Apocynacea) were collected from Coimbatore district, India. Taxonomic authentication was done by Dr.G.V.S.Murthy Taxonomist, TNAU, Coimbatore and Tamilnadu. India and the authentication number BSI/SRC/5/23/2015/Tech/2083.

Preparation of Protein extract of *Tabernaemontana divaricata* leaf (TdPf)

Protein was extracted by recrystallization of ammonium sulphate. Fresh leaves of *Tabernaemontana divaricata* leaves 20% were taken and homogenized with PBS buffer pH 7.2 and were centrifuged for 5000 rpm for 10minutes. Pellets were discarded and supernatant were saved. To the supernatant add known volume of ammonium sulphate 10-100% and was centrifuged at 10,000 rpm, 4°C for 10minutes. The supernatant were discarded and the pellet was suspended with Dialysis membrane for salting out. The crude extract was kept at -20°C (Kiba *et al.,* 2003).

Chemicals

The chemicals and solvents used in the study were of highest purity and analytical reagents grade. The Chemicals were purchased from SD Fine Chem., Himedia and Sigma, India.

In vitro Antioxidants assays

DPPH (Diphenyl- 1-Picryl-Hydrazyl) Radical Scavenging Activity of TdPf

The ability of the leaf protein fraction to scavenge the DPPH radical was quantified using spectrophotometric assay. The scavenging ability of leaves extract towards the stable free radicals was determined through DPPH assay by the method of Pathiranan and Shahidi (2005). Ascorbic acid was used as standard. The reactants were vortexed and incubated in dark at room temperature for half an hour. The absorbance was measured at 518 nm in a spectrophotometer (Pathiranan and Shahidi, 2005).. The assay was carried out in triplicates. DPPH radical scavenging activity was calculated as a percentage using the formula:

$$\text{Scavenging activity (Percent)} = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times 100$$

ABTS Assay

ABTS radical scavenging activity assay was performed according to (Arnao *et al.,* 2001) with some modifications. The ABTS stock solution was prepared by mixing ABTS solution (7 mM, 3 mL) and ammonium per sulphate (2.45 mM, 15 mL) in distilled water. The mixture was left in the dark at room temperature for 16 h before use. Fresh ABTS working solution was prepared by mixing ABTS stock solution in 0.2 M sodium phosphate buffered saline (pH 7.4) to an absorbance of 0.7 ± 0.02 at 734 nm. Then 50 µL of the sample (0.3-1.5 µM) was added to 5 mL of fresh ABTS working solution. The reaction mixture was kept in the dark for 6 min and absorbance was monitored at 734 nm (Long and Halliwell, 2001). BHA was used as a standard. The control was conducted in the same manner, except that distilled water was used instead of sample. The percentage inhibition was calculated using the formula:

$$\% \text{ Inhibition} = [(A_0 - A)/A_0] \times 100$$

Where A_0 = absorbance of control; A = absorbance of sample.

Superoxide Assay

The superoxide radical generated from the photo reduction of riboflavin was detected by NBT (Nitro blue tetrazolium) reduction by the method of McCord and Fridovich, 1969).The reaction mixture contained EDTA (0.1 M), 0.0015% NaCN, riboflavin (0.12 mM), NBT (1.5 mM) and protein fraction of *T.divaricata* at various concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg/ml)and phosphate buffer (67 mM, pH 7.8) in a total volume of 3 ml. The tubes were uniformly illuminated for 15 minutes and the optical density was measured at 530 nm before and after the illumination (McCord and Fridovich, 1969). The percent scavenged was calculated by the following equation.

$$\text{Percent scavenged} = \frac{Abs_{Control} - Abs_{Sample}}{Abs_{Control}} \times 100$$

The IC_{50} value was calculated, which is the effective concentration at which the antioxidant activity is 50%.

Hydrogen Peroxide Assay

The ability of the plant extract to scavenge H_2O_2 was determined according to the method of (Ruch *et al.,* 1989). A solution of H_2O_2 (4mM) was prepared in phosphate buffer (pH 7.2). H_2O_2 concentration was determined spectrophotometrically from its absorption at 230nm was determined after 10minutes against a blank solution containing phosphate buffer without H_2O_2. The scavenging activity of H_2O_2 by plant extract and the standard compounds was calculated using the formula:

$$\text{Scavenging } H_2O_2 \text{ (percent)} = \frac{A_0 - A_1}{A_0} \times 100$$

In vitro cytotoxicity analysis

Maintenance of DLA tumor cells Cell line

Dalton's Lymphoma Ascites (DLA) tumor cells were procured from Amala Cancer Research Centre, Thirussur, Kerala and the cells were propagated by intraperitoneal transplantation of $1x10^6$ cells in 100µl of PBS. After 10-15 days, the cells were drawn from the intraperitoneal cavity and used for the *in vitro*.

Trypan Blue exclusion assay

The cytotoxic effect of protein fraction was evaluated by using DLA tumor cells by intra peritoneal propagated DLA cells. *In vitro* cytotoxicity studies were carried out to find out the 50 per cent effective concentration (EC_{50}) of TdPf by trypan blue exclusion method[17]. The Dalton's Lymphoma Ascites cells were propagated in the peritoneal cavity of mice were taken and washed with saline thrice by centrifuging at low speed. 0.1 ml of PBS containing $1x10^6$ cells was used for the in vitro assay. Various concentrations (10 to 100 g/0.1ml of PBS) of protein fraction containing $1x10^6$ DLA tumor cells were incubated at 37^0 C for three hours. At the end of the incubation period 0.1 ml of trypan blue was added and layered the cells on the haemocytometer for counting. The dead cells were blue in color and counted to calculate the percentage of dead cells (per cent cytotoxicity) using the formula:

$$\text{Percent cytotoxicity} = \frac{\text{Dead cell count}}{\text{Dead cell count + Viable cell count}} \times 100$$

Results

Antioxidants have the capacity to protect the body from oxidative stress damage. Epidemiological studies indicate that intake of fruits; vegetables as well as indigenous herbal products have the capacity to prevent the free radicals in the human body. In this study, the antioxidant properties and cytotoxic effect of tumor cells of the protein fraction of *T. divaricata* was assessed.

In vitro Antioxidants assays

DPPH Assay

The results showed the antioxidant activity of the protein fraction by scavenging the DPPH free radicals when compared to the scavenging the standard ascorbic acid. The IC_{50} concentration of Ascorbic acid (49µg/ml) was found to be less than that of TdPf (59µg/ml). It means that the protein extract of plant at higher concentration captured more DPPH radicals resulting into decrease in absorbance and increase in IC_{50} value are shown in the Figure 1.

ABTS Assay

The ABTS is a stable blue colour radical reduced by antioxidants to green coloured ABTS radical. ABTS has the ability to donate hydrogen atoms to free radicals slowing the process of lipid peroxidation (Desire *et al.,* 2016). The IC_{50} concentration of Ascorbic acid (35µg/ml) was found to be

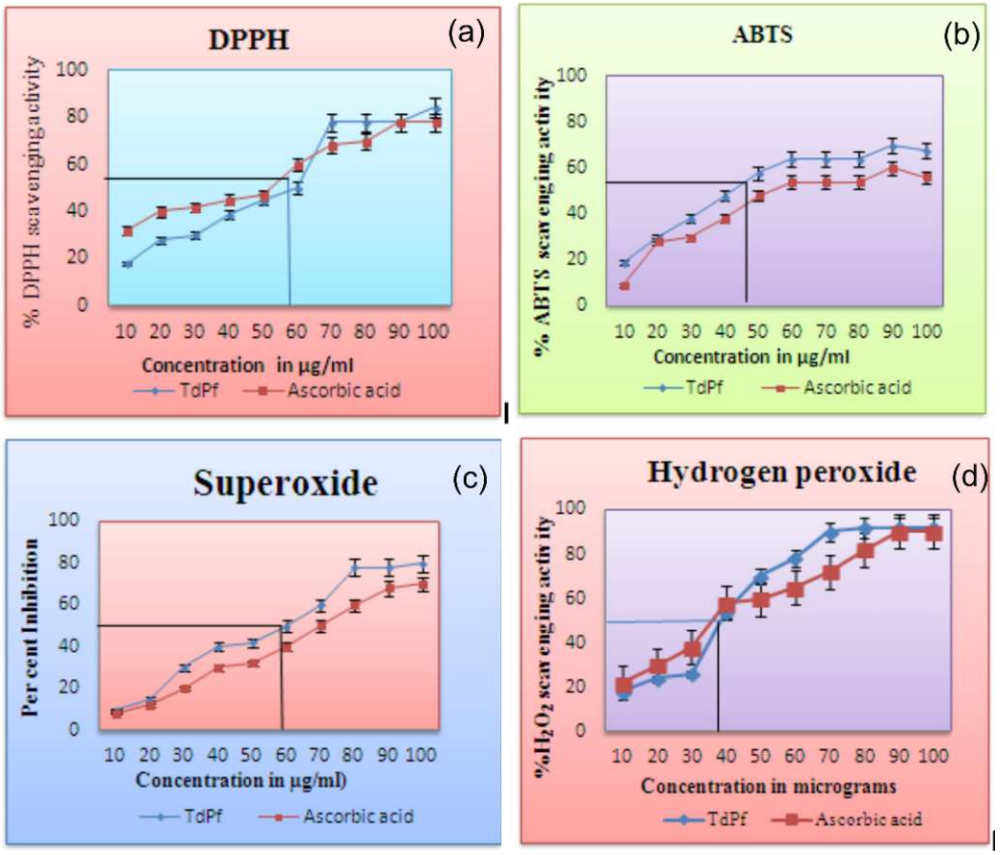

Figure 1. Observation of different assay: **(a) DPPH, (b) ABTS, (c) SO, (d) H_2O_2**

less than that of TdPf (48µg/ml). The scavenging activities of Phyto protein fraction of Td and the standard ascorbic acid are shown in the Figure 2.

Superoxide Assay

Protein fractions showed free radical scavenging effect on Superoxide in a concentration dependent manner. The results clearly indicated that proteins isolated from *T. divaricata* leaves have higher superoxide scavenging potential activity with IC_{50} value 60 µg/ml. It was also observed that Low molecular weight proteins exhibited stronger superoxide activity as compared with the standard Ascorbic acid as shown in the Figure 3.

Hydrogen Peroxide Assay

The *T. divaricata* protein fraction was capable of scavenging H_2O_2 in a concentration dependent manner (Figure 4). Scavenging of H_2O_2 *T. divaricata* might be due to the donation of electrons to H_2O_2 thus neutralizing it to water. The IC_{50} concentration of Ascorbic acid (42µg/ml) was found to be less than that of TdPf (38.5µg/ml).This dose dependent trend was also observed in the aqueous extract of *P. boissieriana* and showed moderate nitric oxide and H_2O_2 scavenging activity (Ebrahimzageh *et al.,* 2009).

Effect of *T.divaricata* Protein fraction on antitumorigenic activity to DLA tumor Cells

In vitro antitumorigenic effect of TdPf was assessed by cytotoxic studies against intraperitoneally propagated DLA tumor cells using trypan blue exclusion method. Figure 5 and 6 shows the antitumorigenic effect and dose dependent *in vitro* cytotoxic effect of TdPf to DLA tumor cells.

Fifty percent effective concentration (EC_{50}) was found to be 62 µg/ml of *T.divaricata* Protein fraction. This cytotoxic effect showed the antiproliferative role of *T.divaricata* against DLA tumor cells.

Discussion

Free radicals, especially ROS can react with substances in the body resulting in cellular damage and human disorders. According to this, the elimination of ROS and free radicals is considering as one of the most important defense mechanisms of a living body against different disorders.

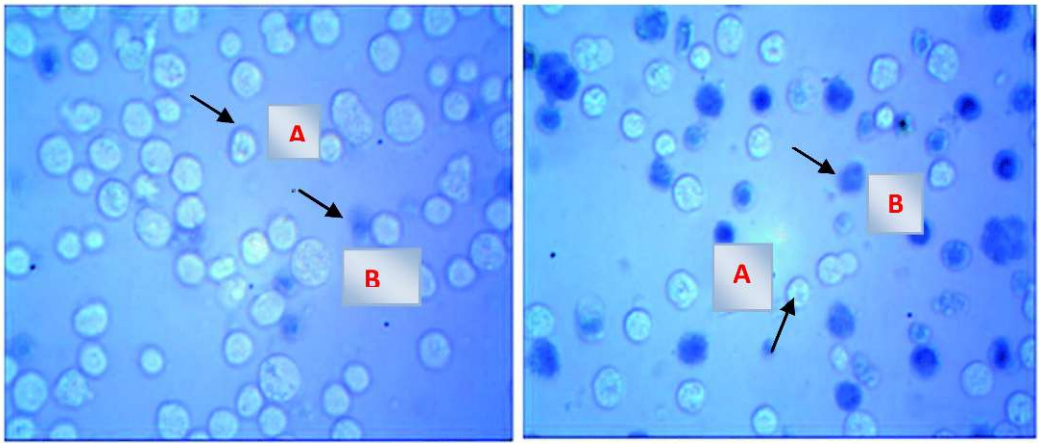

Figure 2. Antitumorigenic effect of *T. divaricata* to DLA tumor cells: (a) DLA tumor cells without *T.divaricata* Pf, (b) DLA tumor cells with *T.divaricata* Pf. A-Live cell B-Dead cell

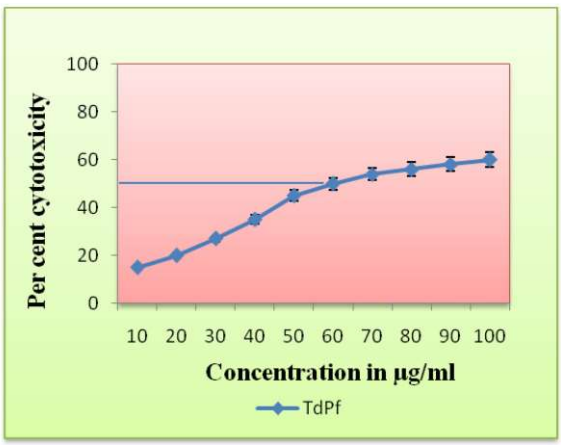

Figure 3. *In vitro* cytotoxic effect of *T. divaricata* protein fraction to DLA tumor cells in trypan blue assay

Antioxidant abilities of the peptides are thought to be due to their amino acid composition and hydrophobicity (Sarmadi and Ismail, 2010). Some amino acids, such as Tyr, Lys, Arg, Gly, Leu and His had been reported to exhibit antioxidant ability (Xie *et al.,* 2008). Antioxidant compounds can inhibit oxidative reactions using both free radicals scavenging and interrupting the radical chain reaction of lipid peroxidation (Kim *et al.,* 2007). The DPPH free radical scavenging assay was frequently used to evaluate the antioxidative role of plant extracts. The scavenging of DPPH by the protein fraction was found to be increased sharply with increasing concentration of the protein fraction when compared to standard ascorbic acid reflected its antioxidative potential. The free radical scavenging activity of the protein extract was examined in *in vitro* using DPPH radical from the leaves of *Leucas linifolia* (Ramakrishna *et al.,* 2012). The formation of the ABTS radical cation takes place almost instantaneously after adding ammonium per sulphate to an ABTS solution. The scavenging ability of peroxides against ABTS radicals was concentration dependent. A more appropriate format for the assay is decolourization technique in that the radical is generated directly in stable form prior to reaction with putative antioxidants (Ilhami Gulcin, 2006). Similar to our results cacao protein fractions also showed dose dependent ABTS scavenging effect which was comparable to that of standard antioxidant ascorbic acid (Huang *et al.,* 2005) Bean seed protein (Comfort et al., 2011).), low molecular weight fraction of chickpea protein hydrolysate (Li *et al.,* 2008), also reported strong superoxide radical scavenging activity. The protein fraction from the leaves of *Cynodon dactylon* showed a dose dependent radical scavenging activity for hydrogen peroxide which was found to be comparable with standard ascorbic acid (Santhi and Annapoorani, 2009). The results obtained in the present study revealed that TdPf can be effectively scavenged H_2O_2 and prevented the inhibition of enzymes and oxidation of SH thiol groups. The dose dependent cytotoxic activity of the leaf extract of *Hymenodictyon* against different cell lines were reported (Kamuhabwa *et al.,* 2010). The antitumorigenic effect of *Solanum nigrum* against HeLa cell line and Vero cell line (Patel *et al.,* 2009).

Similar observations was showed maximum cytotoxicity to DLA tumor cells by four ayurvedic herbs such as *Curcuma longa* L., *Ocimum sanctum* L., *Tinospora cordifolia* (Wild) and *Zizypus mauritiana* (Adhvaryu *et al,* 2010). The potent antitumorigenic effect of aqueous extract of *Areca catechu* was also reported (Chetan *et al.,* 2010). The EC_{50} of *Dendrobium formosum* on DLA cells was found to be 350µg/ml (Prasad and Koch, 2014). Similar results were also reported by Gopika showed significant *in vitro* cytotoxicity activity against DLA cell lines (Gopika *et al.,* 2015).

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Conflicts of interest

We declare that we have no conflicts of interests

Ethical clearance

Not necessary for this work.

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