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## Studies on pro-oxidant induced DNA damage: Inhibition by methanolic extract of *Tinospora cordifolia*

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### ABSTRACT

Isolation of polyphenols from the plant source *Tinospora cordifolia*, Guduchi is a large, glabrous, deciduous climbing shrub belonging to the family Menispermaceae. The knowledge of absorption, biodistribution and metabolism of polyphenols is partial and incomplete, yet it is sufficient to state that in general, some polyphenols are bioactive compounds that are absorbed from the gut in their native or modified form. Polyphenols exhibit a wide range of biological effects as a consequence of their antioxidant properties. They inhibit LDL oxidation in vitro. Thus, polyphenols probably protect LDL oxidation in vivo with significant consequences in atherosclerosis and also protect DNA from oxidative damage with important consequences in the age-related development of some cancers. We know that the free radicals are formed in the body constantly. Surely the body has mechanisms to take care of the oxidative stress. All cells have defenses against the free radicals and these defenses are antioxidants. Therefore proximate analysis and a study has performed to find out the preventive role of the extract on pro-oxidant induced DNA damage using calf thymus DNA as model system. © 2011 Trade Science Inc. - INDIA

### KEYWORDS

*Tinospora cordifolia*;  
DNA damage;  
Antioxidant activity;  
Polyphenolic compound;  
DNA protectant activity;  
Kujala method.

### INTRODUCTION

Polyphenolic compounds occur ubiquitously in foods of plant origin and have several hydroxyl (OH) groups; it was expected to express radical scavenging effect<sup>[30]</sup>. Polyphenolic compounds may have beneficial health effects because of their antioxidant properties and their inhibitory role in various disorders. Polyphenols or multiphenolic complexes, have an even wider range of biological activities such as antioxidant,

antibacterial, anticancer, antitumor and cardio protective effects<sup>[7,22]</sup>.

Bioflavonoids comprise of a diverse class of polyphenolic compounds with antioxidant activity, which are present in most foods that we eat<sup>[8]</sup>. Their antioxidant potency lies in their ability to function as reducing agents, singlet oxygen quenchers and terminators of free radicals<sup>[31]</sup>.

Normal metabolism of cell results in a continuous generation of pro-oxidants, such as superoxide radical

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or hydroxyl radical or the non-radical hydrogen peroxide<sup>[4,10]</sup>. Lipid peroxides and reactive oxygen species are involved in numerous pathological events, including inflammation, radiation damage, metabolic disorders, cellular aging and reperfusion damage<sup>[1,29]</sup>. DNA damage is a crucial event in metazoan lifecycle since it poses a situation where the cells have to decide between repair and cell death<sup>[13]</sup>.

Antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions. Natural antioxidants constitute a broad range of compounds including Phenolic compound, nitrogen compounds and carotenoids<sup>[28]</sup> the main role of antioxidants is to help the body protect itself against damage caused by reactive oxygen species and degenerative diseases<sup>[23]</sup>.

Recent studies in humans have shown that supplementation with antioxidant compounds such as vitamins E and C, lycopene and beta-carotene can help to reduce levels of free-radical damage<sup>[3,12,21]</sup>. This lends support to the hypothesis that dietary products high in antioxidants potentially exert a protective effect against degenerative disorders, such as cancer by a decrease in DNA damage<sup>[1]</sup>. Spices and condiments, which are a part of the Indian diet, have chemical constituents, which have antibacterial, antioxidant, anti-mutagenic, and anticarcinogenic properties<sup>[16]</sup>. Plants grow on different nature of soils, which are extremely rich in microorganisms, and infection remains a rare event. To keep out potential invaders, plants produce a wide range of selective antibacterial compounds either in a constitutive or an inducible manner<sup>[6]</sup>. Currently there is growing interest to use natural antibacterial compounds, like plant extracts of herbs and spices for the preservation of foods as these possess a characteristic flavor and sometimes show antioxidant activity as well as antibacterial activity<sup>[25]</sup>.

*Tinospora cordifolia* (*Guduchi*) is a widely used shrub in folk and ayurvedic systems of medicine. The chemical constituents reported from this shrub belong to different classes such as alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compounds and polysaccharides. The notable medicinal properties reported are anti-diabetic, anti-periodic, anti-spasmodic, anti-inflammatory, anti-arthritic, anti-oxidant, anti-allergic, anti-stress, anti-lep-

rotic, anti-malarial, hepatoprotective, immunomodulatory and anti-neoplastic activities<sup>[24]</sup>.

The WHO estimates that up to 80% of people still rely mainly on traditional remedies such as herbs for their medicine<sup>[27]</sup>. India has been identified as a major resourceful area in the traditional and alternative medicines globally. Multi-factorial health beneficial activity of these plant extracts has been attributed to multi-potent anti-oxidant, anti-microbial, anti-cancer, anti-ulcerative and anti-diabetic properties. Generally, anti-oxidants have been identified as major health beneficial compounds reported from varieties of medicinal plants and are sources for alternative medicines<sup>[9]</sup>.

Medicinal plants constitute one of the main sources of new pharmaceuticals and health care supplements, phytochemicals and pro-vitamins that assist in maintaining good health and combating disease are now being described as functional ingredients and nutraceuticals<sup>[20]</sup>.

Therefore, the present study is focused on studying the protective role in pro-oxidants induced DNA damage of the given plant source (*Tinospora cordifolia*).

## EXPERIMENTAL

Calf thymus DNA, Ascorbic acid, ferrous sulphate, Ferric chloride, Gallic acid and Hydrogen peroxide procured from laboratory. All other chemicals and reagents were of analytical grade. Solvents were distilled prior to uses.

### Extraction of crude extract (*Tinospora cordifolia*)

Fresh and healthy leaves of were collected from local growers. The leaves were washed thoroughly in distilled water and the surface water was removed by air drying under shade. The leaves were subsequently dried in a hot air oven at 40°C for 48h, powdered and used for extraction. 2 grams of powdered leaves of *T. cordifolia* was macerated with 50 ml sterile distilled water in a blender for 10 min. The macerate was first filtered through double layered muslin cloth and centrifuged at 10000 g for 15 min. The supernatant was filtered through Whatman No.1 filter paper and heat sterilized at 120°C for 30 min and reconstituted in small volume of methanol, the extract was preserved aseptically in a brown bottle at 4°C until further use.

### Determination of total phenolic compounds

Total content of phenolics was determined according to the method of folin-ciocalteau reaction<sup>[17]</sup>, using gallic acid as standard. Various concentrations of sample, and gallic acid were dissolved in 0.5ml of water and were mixed with 500µl of folin-ciocalteau reagent. The standard calibration curve was prepared using gallic acid solution. The mixture was then allowed to stand for 10min followed by the addition of 1.0ml of Na<sub>2</sub>CO<sub>3</sub>. After 10min incubation at ambient temperature, the absorbance of the supernatant was measured at 730nm. The total phenolics content was expressed as gallic acid equivalents (GAE) in milligrams per gram of protein sample (Figure 1 and TABLE 1).

Estimation of total polyphenols by Kujala method

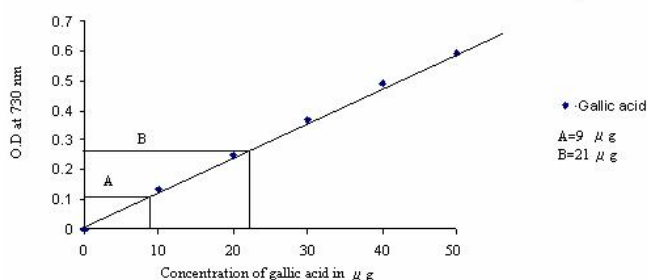


Figure 1 : The concentration of total phenolics in 15µl solution of crude extract is 9µg and in 30µl solution is 21µg.

TABLE 1 : Determination of total phenolic compounds

| Sl.no    | Vol. in µl | Conc. in µg | Distilled water in µl | Folin's reagent in µl | Sodium carbonate in ml | Incubation                                  |
|----------|------------|-------------|-----------------------|-----------------------|------------------------|---|
| 1        | Blank      | -           | 100                   |                       |                        |   |
| 2        | Standard   | -           |                       |                       |                        |   |
| a        | 10         | 1           | 90                    |                       |                        |   |
| b        | 20         | 2           | 80                    |                       |                        |   |
| c        | 30         | 3           | 70                    | 500                   | 1.0                    | Incubate at room temperature for 10 minutes |
| d        | 40         | 4           | 60                    |                       |                        |   |
| e        | 50         | 5           | 50                    |                       |                        |   |
| Sample 1 | 15         |             | 85                    |                       |                        |   |
| Sample 2 | 30         |             | 70                    |                       |                        |   |

### Estimation of total sugar

The total sugar concentration was estimated by Dubois method<sup>[11]</sup>. Different aliquots of extract were made up to 1ml with distilled water. To this 1ml of phenol and 5ml of concentrated Sulphuric acid were added. Orange colour developed was read at 520nm immediately. The

sugar concentration was calculated according to the standard glucose calibration curve (Figure 2 and TABLE 2).

Estimation of Total Sugars by Dubois Method

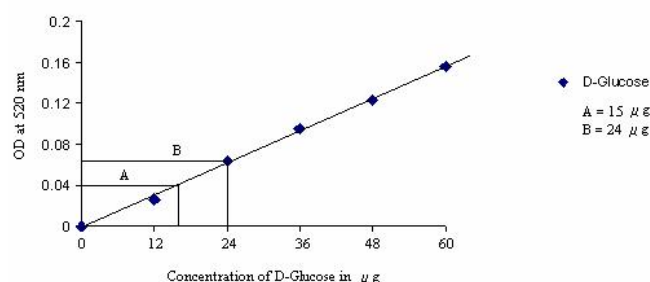


Figure 2 : The concentration of total sugars in 15µl solution of crude extract is 15µg and in 30µl solution is 24µg.

TABLE 2 : Estimation of total sugar

| Sl.no    | Vol. in µl | Conc. in µg | Distilled water in µl | 5% phenol in ml | Conc. H <sub>2</sub> SO <sub>4</sub> in ml                                  |
|----------|------------|-------------|-----------------------|-----------------|---|
| 1        | Blank      | -           | 1000                  |                 |   |
| 2        | Standard   | -           |                       |                 |   |
| a        | 20         | 12          | 980                   | 1.0             | Keep in cold water bath then add conc. H <sub>2</sub> SO <sub>4</sub> 5.0ml |
| b        | 40         | 24          | 960                   |                 |   |
| c        | 60         | 36          | 940                   |                 |   |
| d        | 80         | 48          | 920                   |                 |   |
| e        | 100        | 60          | 900                   |                 |   |
| Sample 1 | 15         | -           | 985                   |                 |   |
| Sample 2 | 30         | -           | 970                   |                 |   |

### Estimation of total protein content

The protein content of the extract was measured by Bradford's method<sup>[2]</sup> using bovine serum albumin as standard. Different aliquots of extract were made upto 0.1ml with distilled water. To this 0.9ml of Bradford's reagent was added. The blue color developed was read

Estimation of Total Proteins by Bradford's Method

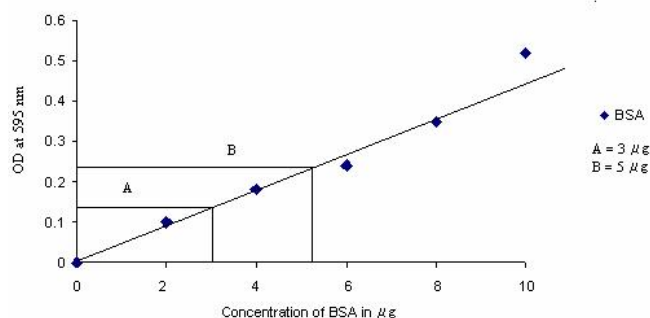


Figure 3 : The concentration of total proteins in 10µl solution of crude extract is 3µg and in 20µl solution is 5µg.

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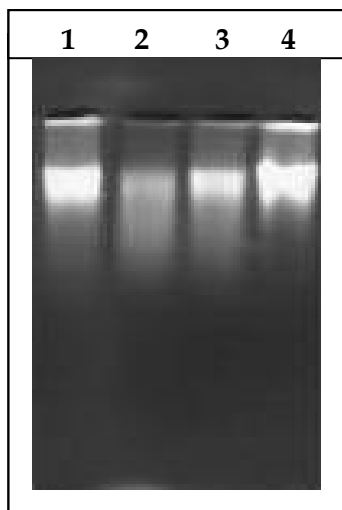
at 595nm. The total protein content was calculated according to the standard calibration curve (Figure 3 and TABLE 3).

**TABLE 3 : Estimation of total protein content**

| Sl.no    | Vol. in $\mu$ l | Conc. in $\mu$ g | Distilled water in $\mu$ l | Bradford's reagent in $\mu$ l | Incubation   |
|----------|-----------------|------------------|----------------------------|-------------------------------|--|
| 1        | Blank           | -                | 100                        | ↓<br>900<br>↑                 | Incubate<br>for 5<br>minutes at<br>room<br>temperature |
| 2        | Standard        | -                |                            |                               |  |
| a        | 10              | 2                | 90                         |                               |  |
| b        | 20              | 4                | 80                         |                               |  |
| c        | 30              | 6                | 70                         |                               |  |
| d        | 40              | 8                | 60                         |                               |  |
| e        | 50              | 10               | 50                         |                               |  |
| Sample 1 | 10              |                  | 90                         |                               |  |
| Sample 2 | 20              |                  | 70                         |                               |  |

### DNA protectant activity

Agarose (0.8%) is melted, added ethidium bromide (EtBr-1 $\mu$ g/ $\mu$ l), swirled the flask to mix EtBr and poured the entire solution into gel platform slowly without trapping any air bubble. After gel formation, carefully removed the comb and the adhesive tapes out. Placed the gel platform on the bed of the electrophoresis tank



- Lane 1 : Calf thymus DNA  
 Lane 2 : Calf thymus DNA + Hydrogen peroxide  
 Lane 3 : Calf thymus DNA + Hydrogen peroxide + Tinospora cordifolia extract  
 Lane 4 : Calf thymus DNA + Hydrogen peroxide + BHA

Figure 4: Agarose gel electrophoresis of Calf thymus DNA + hydrogen peroxide  $\pm$  plant polyphenol, BHA in 0.1ml potassium phosphate buffer incubated at 37°C for 30minute. The assay and electrophoresis was carried out as described in methods.

and poured buffer to cover the gel surface. Injected the samples into different wells using a micropipette and closed the lid. Connected the apparatus to power pack and turned power on setting it at constant voltage (100V). Continued the run until the marker dye reached the end of gel. The gel was examined under U.V Transilluminator (Figure 4).

### Lymphocyte isolation

Human peripheral lymphocytes were isolated from 10ml of venous blood drawn from young, healthy, non-smoking donors. Blood was collected in anticoagulant 8.5mM Acid Citrate Dextrose (ACD). Hemolysing buffer was added, mixed well, incubated at 4°C for 30 min. centrifuged at 1200rpm for 12 min, the supernatant (hemolysate) was discarded, 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) solution and suspended in same solution (~1ml). The cell viability was determined by trypan dye blue exclusion method<sup>[19]</sup>. To 10 $\mu$ l of lymphocyte sample added 10 $\mu$ l of trypan blue dye and the cells were charged to Neubars chamber and the cell number was counted. The dead cells being permeable to trypan blue appear blue against white color of the viable cells (Figure 5).

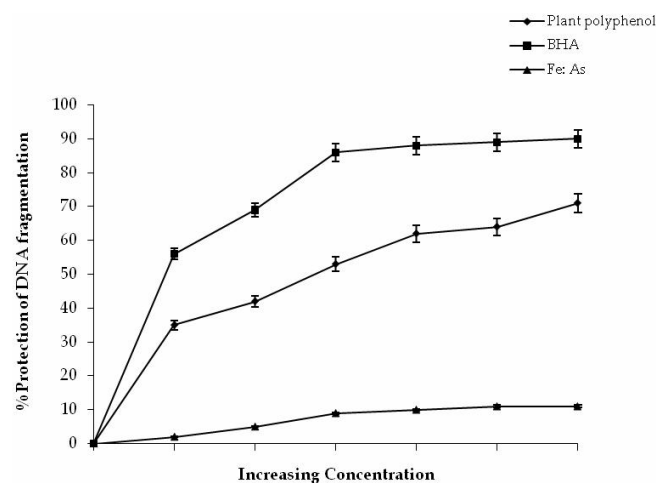


Figure 5 : Calf thymus DNA pre treated with or without BHA, plant polyphenol at various concentrations, for 20min in 0.5ml HBSS. Then Fe: As was added and final volume was made to 1ml with HBSS, and incubated for 1 hr. Fragmented double stranded DNA was quantitated colorimetrically at 600nm with diphenylamine reaction as described in methods. The control was without test compound and the % inhibition was calculated accordingly. The values are mean  $\pm$  SD.



The percentage viability was calculated by the formula

$$\% \text{ Viability} = \frac{\text{Number of viable cells}}{\text{Total number of cells (dead + viable)}} \times 100$$

### Analysis of oxidative DNA damage in lymphocytes

Quantitative analysis of DNA fragmentation by diphenylamine method

100µl Cells (~98% viable) were pretreated with or without extracts at various concentrations in 0.5ml HBSS, for 20minutes, then added ferrous Sulphate: Ascorbate (radical generator–DNA damaging factor) in total volume of 1ml of HBSS and incubated for 60minutes. Centrifuged 10000rpm for 20min, 4°C and processed for the estimation of DNA fragmentation by diphenylamine (DPA) reaction method as follows  
Supernatants were transferred carefully into new tubes (labeled S) and the pellets (labeled B) were lysed in 1ml of TTE solution and vortexed vigorously that allows the release of fragmented chromatin from nuclei, after lysis due to triton X100 and disruption of nuclear structure following Mg<sup>++</sup> chelation by EDTA in TTE solution. The DNA was separated from chromatin by centrifugation (ependorf) at 4°C, 10min, the supernatant was carefully transferred to the tubes (labeled S) suspended in 1ml of TTE solution (lysis buffer) and 1 ml of TCA was added to the pellets (B) and supernatants (T and S) and vortexed vigorously and incubated for 24h for overnight precipitation at 4°C. The samples were centrifuged for 20min at 4°C. Supernatants were discarded by aspiration (using pipette) and the DNA was then hydrolyzed by adding 160µl of TCA to each pellet and heated for 15min at 90°C. A blank was also prepared with TCA alone, then to each sample (tube), 1000µl of freshly prepared DPA solution was added and mixed by vortexing. Incubated at ambient temperature for 24hour to allow for the development of color (blue colour)<sup>[5]</sup> and the absorbance was read at 600nm. The control was read without test compound and the proportion of fragmented DNA and the %inhibition was calculated (Figure 5).

### Statistical analysis

Comparison between control and treated groups were performed with a Student's t-test<sup>[26]</sup> and a p-value of less than 0.05 was considered significant (TABLE 4).

**TABLE 4 : Proximate analysis of plant extract (*Tinospora cordifolia*). The total protein, total sugar and the polyphenol content of the crude extract were determined as described in methods. The results are mean ± SD of five experiments.**

| Test sample   | Total polyphenols (g %) | Total Sugar (g %) | Protein (g %) |
|---------------|-------------------------|-------------------|---------------|
| Crude extract | >3                      | <2                | <1            |

## RESULTS AND DISCUSSION

Numerous studies have been carried out to extract various natural products for screening antioxidant activity and only a few species have been screened for biological activity. Green tea and ginkgo biloba extracts are rich in antioxidants such as vitamin c, Flavonoids and Polyphenols<sup>[18]</sup> and such plant derived compounds and extracts will be inexpensive and no toxicity.

Among the various free radicals, hydroxyl radicals (OH<sup>•</sup>) are well studied for their effect on the genetic material DNA. Hydroxyl radicals have very short life span than any other free radicals and these radicals attack the DNA double strands and break down into single strand. Therefore antioxidants play significant role in inhibiting the DNA damage. Polyphenols have a role in quenching the hydroxyl radicals and thus inhibit the DNA damage.

In the present study, we have shown here a novel factor from the given plant source exhibit significant DNA protectant property on oxidative lymphocyte cell damage and oxidative DNA damage.

TABLE 4 shows the amount of total Polyphenols, total Flavonoids, total sugar and the protein present in the crude extract of the given plant source and it shows that the plant source was rich in Polyphenols.

Oxidative DNA damage has been implicated to be involved in various degenerative diseases<sup>[14,15]</sup>. The preventive effect of plant polyphenol on hydrogen peroxide mediated DNA damage was analyzed in agarose gel electrophoresis. As shown in Figure 4, when calf thymus DNA was treated with hydrogen peroxide extensive DNA damage was observed (Lane 2). The efficient DNA Protectant activity of Plant polyphenol (Lane 4) was comparable to known antioxidant BHA (Lane 3).

In the present study, we investigated the potency of inhibitory effect of antioxidants on oxidative damage in lymphocytes.

The DNA fragmentation was measured by diphenylamine method. Figure 5 shows the inhibitory effects of antioxidants on Fe: As induced DNA fragmentation

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in calf thymus DNA. As shown in the figure the plant polyphenol was found to offer 64% protection on Fe: As induced DNA fragmentation, where as BHA offered protection by 88%.

The viability of lymphocytes on simultaneous, post and pre treatment of hydrogen peroxide, and a time course study. It shows that the protection offered in the post treatment of polyphenol was less when compared to simultaneous and pre treatment. The protection of the plant protein was compared to the standard BHA.

Thus, on the studies we could conclude that the plant polyphenol is potent pro-oxidants induced lymphocyte cell damage and oxidative DNA damage in lymphocytes.

## CONCLUSION

In recent years, many studies on various pathological conditions reveal that free radicals play a significant role in enhancing the disease conditions. Free radicals are well known to damage proteins, lipids and DNA. Hydroxyl radicals are mainly involved in DNA damage and hence leading to abnormalities in DNA functions. Hence quenching of these prooxidants may lead to normal DNA and cellular functions. The above studies have shown that methanolic extract of *Tinospora cardifolia* acts against prooxidants induced DNA damage. Since preliminary studies have shown promising results an extensive investigation is required for understanding the modus operandi of the inhibition of DNA damage by *Tinospora cardifolia*.

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