Inhibition of Pro-oxidant induced DNA Damage in isolated human peripheral lymphocytes by methanolic extract of *Guduchi* (*Tinospora cordifolia*) leaves.

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ABSTRACT

The present preliminary investigation reveals the anti-oxidant effects of *Guduchi* (*Tinospora cordifolia*) leaves. Dried and powdered leaves of *Tinospora cordifolia* was subjected to methanol extraction, and total phenols, sugars and proteins were determined. DNA fragmentation was carried out using isolated human peripheral lymphocytes and Calf thymus DNA as a model system. The results showed that the methanol extract of *Tinospora cordifolia* is rich in total polyphenols (>3 mg/g) in comparison to sugars (<2 mg/g) and protein (<1 mg/g). DNA fragmentation was measured by diphenylamine method. The polyphenols rich fraction of *Tinospora cordifolia* leaves were found to offer 64% protection on Fe:As (10:100µmole) induced DNA fragmentation, where as BHA offered protection by 88% and was found to have direct correlation between the total polyphenols and its protective against pro-oxidant induced DNA fragmentation. The *in-vitro* anti-oxidant activity of *Tinospora cordifolia* justifies the medicinal use of this plant.

KEYWORDS: *Tinospora cordifolia,* DNA fragmentation, Lymphocytes, Free radicals, pro-oxidant DNA protectant Activity. Reactive oxygen species

INTRODUCTION

Anti-oxidant are compounds that inhibit or delay the oxidation of 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 [1]. The main role of antioxidants is to help the body protect itself against damage caused by reactive oxygen species and degenerative diseases [2].

Recent studies in humans have shown that supplementation with antioxidant compounds such as vitamins E and C, lycopene and β-carotene can help to reduce levels of free-radical damage [3]. 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 [4]. Spices and condiments, which are a part of the Indian diet, have chemical constituents, which have anti-bacterial, anti-oxidant, anti-mutagenic, and anti-carcinogenic properties [5]. 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 [6]. 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 [7].

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 neutraceuticals. 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 [8].

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-leprotic, anti-malarial, hepatoprotective, immunomodulatory and anti-neoplastic activities [9].

Normal metabolism of cell results in a continuous generation of pro-oxidants, such as superoxide radical or hydroxyl radical or the non-radical hydrogen peroxide [10]. Lipid peroxides and reactive oxygen species are involved in numerous pathological events, including inflammation, radiation damage, metabolic disorders, cellular aging and reperfusion damage [11]**.**

Polyphenolic compounds occur ubiquitously in foods of plant origin and have several hydroxyl (OH^-) groups; it was expected to express radical scavenging effect, and polyphenols or multiphenolic complexes, have an even wider range of biological activities such as antioxidant, antibacterial, anticancer, antitumor and cardio protective effects [12].

Bioflavonoids comprise of a diverse class of polyphenolic compounds with antioxidant activity, which are present in most foods that we eat, and their antioxidant potency lies in their ability to function as reducing agents, singlet oxygen quenchers and terminators of free radicals [13].

Therefore, the present study is focused on studying the protective role against pro-oxidant induced DNA damage in isolated human peripheral lymphocytes by methanolic extract of *Tinospora cordifolia* (*T. cordifolia*) leaves. The *T. cordifolia leaves* were tested for the presence of total proteins, total polyphenols and total sugars and its preventive role on pro-oxidant induced DNA damage in isolated human peripheral lymphocytes.

MATERIALS AND METHODS:

Preparation of methanolic extract of *T. cordifolia leaves*

Fresh and healthy leaves of *T. cordifolia* 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 40^0C for 48 h, powdered and used for extraction. 2g of powdered leaves of *T. cordifolia* was macerated with 50ml sterile distilled water in a blender for 10 min. The macerate was first filtered through double layered muslin cloth and centrifuged at 10000g for 15 min. The supernatant was filtered through Whatman No.1 filter paper and heat sterilized at 120^0 C for 30 min and reconstituted in small volume of methanol, the extract was preserved aseptically in a brown bottle at 4^0C until further use.

Estimation of total proteins, sugars and phenolic compounds

Total content of phenolics was determined according to the method of folin-ciocalteau reaction [14], 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 10 min followed by the addition of 1.0ml of Na_2CO_3. After 10 min incubation at ambient temperature, the absorbance of the supernatant was measured at 730 nm. The total phenolics content was expressed as gallic acid equivalents (GAE) in milligrams per gram of protein sample**.**

The total sugar concentration was estimated by Dubois method [15]. 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.

The protein content of the extract was measured by Bradford's method [16] 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 at 595nm. The total protein content was calculated according to the standard calibration curve.

Isolation of human peripheral lymphocytes

Human peripheral lymphocytes were isolated from 10ml of venous blood drawn from young, healthy, non-smoking donors as described by [17] with minor modifications. Blood was collected in anticoagulant 8.5mM Acid Citrate Dextrose (ACD). Hemolysing buffer was added, mixed well, incubated at 4^0C 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 tryphan dye blue exclusion method [18]. To 10µl of lymphocyte sample added 10µl of tryphan blue dye and the cells were charged to Neubars

chamber and the cell number was counted. The dead cells being permeable to tryphan blue appear blue against white color of the viable cells.

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 by diphenylamine method

100µl Cells (~98% viable) were pretreated with or without extracts of *T. cordifolia* (100µg/ml) in 0.5ml HBSS, for 20 min, then added ferrous Sulphate: Ascorbate (radical generator–DNA damaging factor) in total volume of 1ml of HBSS and incubated for 60minutes. Centrifuged 10000rpm for 20 min, 4^0C 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 (Tris-triton-X-100-EDTA) 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^{2+} chelation by EDTA in TTE solution. The DNA was separated from chromatin by centrifugation 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 90^0C. A blank was also prepared with TCA alone, and 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 blue color [19] 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.

Effect of methanolic extract of *T. cordifolia* on calf thymus DNA

DNA protectant activity was carried out using BHA (400µM) as standard synthetic antioxidant. Calf thymus DNA (10µg/lane) with or without *T. cordifolia* leaves (100µg/lane) was used. Hydrogen peroxide at 1mM/lane was used as source of pro-oxidant.

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 shown to exert Reactive oxygen species (ROS) inhibitory effect against ROS induced DNA damage. Green tea and *Ginkgo biloba* extracts are rich in antioxidants such as vitamin C, flavonoids and polyphenols [20] and such plant derived compounds and extracts are inexpensive with no toxicity. Medicinal plants are important source natural anti-oxidants which increase the anti-oxidant capacity of the plasma and reduce the risk of certain diseases [21]. Polyphenols are the major plant compounds with anti-oxidant activity. Typical phenolics that possess anti-oxidant activity are known to be mainly phenolic acids and flavonoids. It is reported that the phenolics are responsible for the variation in the anti-oxidant activity of the plant [22]. They exhibit anti-oxidant activity by inactivating lipid free radicals or preventing decomposition of hydro peroxides into free radicals [23].

T. cordifolia is mentioned as *vishaghni, vishahara* and *tridoshashamaka* in various texts of Ayurveda. A significant increase in the concentration of thiobarbituric acid reactive substances (TBARS) in brain, along with its decrease in heart, was observed in diabetic rats. *Tinospora cordifolia treatment* decreased the concentrations of glutathione reductase (GSH) and decreased activities of superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) in the tissues of diabetics rats. Alcoholic extract of the root of *T. cordifolia* administered at a dose of 100 mg/kg orally to diabetic rats for 6 weeks normalized the antioxidant status of heart and brain. The effect of *T. cordifolia* root extract was better than glibenclamide (600 µ/kg) although Insulin (6 units/kg) restored all the

parameters to normal status [24]. *T. cordifolia* has also been reported to elevate GSH levels, expression of the gammaglutamylcysteine ligase and Cu-Zn SOD genes. The herb also exhibited strong free radical–scavenging properties against reactive oxygen and nitrogen species as studied by electron paramagnetic resonance spectroscopy [25].

Among the various free radicals, hydroxyl radicals ($OH^{\cdot}$) 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 that polyphenols rich compound from the methonolic extract of *T.cordifolia* exhibit significant DNA protectant activity on oxidative DNA damage in human peripheral lymphocytes.

Flavonoids, (a large group of naturally occurring plant phenolic compounds including flavones, flavonols, isoflavones, flavonones and chalcones), also known as nature's tender drugs, possess numerous biological/ pharmacological activities. Recent reports of antiviral, anti-fungal, antioxidant, anti-inflammatory, antiallergenic, antithrombic, anticarcenogenic, hepatoprotective, and cytotoxic activities of flavonoids have generated interest in studies of flavonoid-containing plants. Of these biological activities, the anti-inflammatory capacity of flavonoids has long been utilized in Chinese medicine and the cosmetic industry as a form of crude plant extracts [26]**.** The test conducted for total polyphenols, total sugar and the protein present in the methonolic crude extract of *T.cordifolia* leaves showed that the methonolic extract of *T.cordifolia* was rich in polyphenols *i.e.,* Comparison between control and treated groups were performed with a Student's *t*-test [27] and a *p*-value of less than 0.05 was considered significant. In which Methanol extract of T. cordifolia is rich in total polyphenols (>3 mg/g) in comparison to sugars (<2 mg/g) and protein (<1 mg/g).

Normal metabolism of cell results in a continuous generation of pro-oxidants, such as superoxide radical or hydroxyl radical or the non-radical hydrogen peroxide [28]. Oxidative DNA damage has been implicated to be involved in various degenerative diseases, DNA damage is a crucial event in metazoan life cycle since it poses a situation where the cells have to decide between repair and cell death [29]**.** The preventive effect of plant polyphenols on hydrogen peroxide mediated DNA damage was analyzed in agarose gel electrophoresis. In the present study when calf thymus DNA upon treatment with hydrogen peroxide showed extensive DNA damage (Figure .1; Lane 2). The efficient DNA Protectant activity of plant polyphenols (Figure1; Lane 4) was comparable to known antioxidant BHA (Figure 1; 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.2 shows the inhibitory effects of antioxidants on Fe: As induced DNA fragmentation 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. Similar study was conducted by [30], where they have extracted plant polyphenols and flavonoid from *T.cordifolia* leaves from different solvent system *viz.,* hexane, choloform, methanol, ethanol and water out of these solvents, polyphenols and flavonoid are rich in ethanol extract different anti-oxidant assays were carried out to determined the efficiency of the plant extract in which ethanol extract showed the highest anti-oxidant activity when compared to other solvent extracts. Where as in our study we have been able to get the comparably rich anti-oxidant activity using methanol extract of *T.cordifolia.*

Thus, on the studies we could conclude that the *T.cordifolia* 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 pro-oxidants may lead to normal DNA and cellular functions. The above studies have shown that methanolic extract of *Tinospora cordifolia* acts against pro-oxidant 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 cordifolia*.

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FIGURES

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 1: Agarose gel electrophoresis of Calf thymus DNA + hydrogen peroxide ± 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.

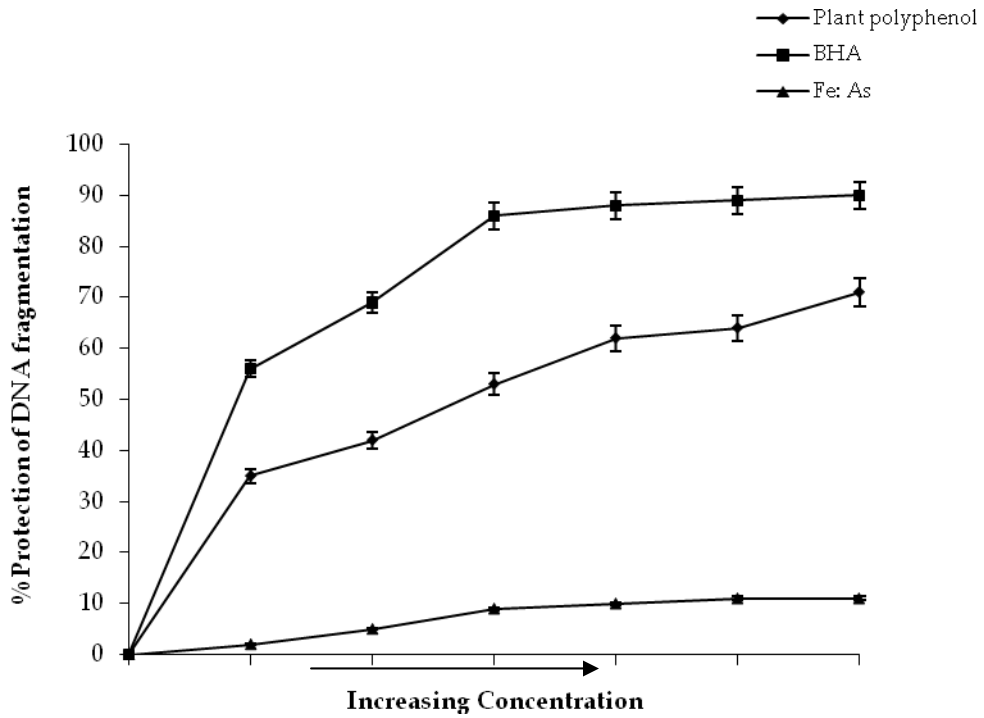

Figure 2: Inhibitory effect Plant polyphenol on Fe: As induced DNA fragmentation in Calf thymus DNA- Calf thymus DNA pre treated with or without BHA, plant polyphenol at various concentrations, for 20min in 0.5ml HBSS. Then Fe: As was added and final volume was made to 1ml with HBSS, and incubated for 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 ± SD.