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EVALUATION OF ANTIOXIDANT POTENTIAL OF *TINOSPORA CORDIFOLIA* AND *TINOSPORA SINENSIS*

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ABSTRACT

A study was undertaken to evaluate the antioxidant potential of two species of *Tinospora* viz *Tinospora cordifolia* and *Tinospora sinensis* on a comparative basis. Aqueous and ethanolic extracts of both the species were subjected to *in vitro* antioxidant activity screening models such as DPPH, ABTS, nitric oxide and superoxide radical scavenging activity, inhibition of lipid peroxidation, reduction of ferric ions and total antioxidant capacity. Ascorbic acid was used as the standard. In all the models studied, both the plants showed nearly equal activities thereby justifying the claims of *Tinospora sinensis* which has not been studied extensively, and augmenting it into the present day system of medicine.

INTRODUCTION: Normal physiological processes involve utilization of oxygen in which approximately 5% of the oxygen gets reduced univalently to oxygen-derived free radicals [1]. These radicals, known as reactive oxygen species (ROS), exert oxidative stress towards the cells of human body rendering each cell to face about 10000 oxidative hits per second [2]. When generation of ROS overtakes the antioxidant defense of the cells, the free radicals start attacking the cell proteins, lipids and carbohydrates and this leads to a number of physiological disorders such as glycated protein oxidation in diabetes mellitus, low-density lipoprotein oxidation in atherosclerosis, red blood cell hemolysis in glucose- 6- phosphate dehydrogenase deficiency, etc [3]. These reactive species are capable of reversibly or irreversibly damaging compounds of all biochemical classes including nucleic acids, proteins and free amino acids, lipids and lipoproteins, carbohydrates and connective tissue macromolecules [4].

Natural products have been used to prevent such types of damage since long. Many plants contain substantial amounts of antioxidants like vitamin C and E, carotenoids, flavonoids, tannins, etc. that can be used to scavenge the excess free radicals from human body [5]. The intake in the human diet of antioxidant compounds, or compounds that ameliorate or enhance the biological antioxidant mechanism can prevent and in some cases, help in the treatment of some oxidative related disorders [6]. In Indian system of medicine, different species of the genus *Tinospora* (Menispermaceae) such as *Tinospora cordifolia, Tinospora fragosa, Tinospora sinensis*, *Tinospora smilacina, Tinospora tuberculata,* etc. have been used for the treatment of liver, kidney and bladder problems and diabetes [7]. Among all these species, *Tinospora cordifolia* and *Tinospora sinensis* are climbing shrubs widely distributed through out India, China, North West South Africa, Myanmar and Sri Lanka. *Tinospora cordifolia*, commonly known as Guduchi, Giloya, and Choti Gulvel, is a large, glabrous, deciduous climbing shrub and is reported to have antistress, anti-neoplastic, antimicrobial, anti-ulcer, antioxidant, anti-malarial, anti-allergic, anti-diabetic and immunomodulator activities. *Tinospora sinensis,* commonly called as Badi gulvel is a climbing shrub used for treating piles, ulcerated wounds, as a muscle relaxant and in liver complaints [8]. Various parts of these two species are reported to have therapeutic actions such as stem as anti-periodic, diuretic and tonic [9].

The roots and stem of *Tinospora cordifolia* are prescribed in combination with other drugs as an antidote to snake bite and scorpion sting [10]. Aqueous extract of stem and roots of *Tinospora cordifolia* have shown immunomodulatory, antidiabetic, anti-malarial and anti-allergic properties [11, 12]. Tinosporine and tinosponone, identified in the stem and roots of *Tinospora cordifolia* are reported as hepatoprotective agents and have exhibited *in vitro* inactivating property against Hepatitis-B and E [13]. Although Tinospora *cordifolia* has been studied extensively, and various pharmacological activities reported, other species have been studied to a limited extent. Hence the present study is an attempt to study the antioxidant activities of *Tinospora sinensis* and compare it with that of *Tinospora cordifolia* in order to find out which species is a better one.

MATERIALS AND METHODS: All chemicals used were of analytical grade. DPPH (1, 1 di phenyl 2 picryl hydrazyl) and ABTS i.e., 2, 2 azino bis (3 ethyl benzo thiazoline 6 sulphonic acid) were obtained from Sigma Chemicals, USA. Sodium do decyl sulphate , nitro blue tetrazolium chloride, phenanthroline, naphthyl ethylene di amine di hydrochloride, potassium per sulphate, dimethyl

sulphoxide, hydroxylamine hydrochloride, ammonium molybdate, sulphanilamide, ortho phosphoric acid, sodium nitroprusside, riboflavin, EDTA and sodium phosphate were obtained from Loba Chemie Private. Limited, Mumbai, India

Plant material: The dried stems of *Tinospora cordifolia* were collected from the local market, Indore (M.P.) and *Tinospora sinensis* from Shaman shop Herb Pvt. Ltd. (New York, USA).They were identified in Government Agriculture College, Indore, India. Herbarium specimen (SCOPE/PHCOG/06-08/01) has been maintained in our department for further reference.

Preparation of Extracts: The collected stems were dried and. These were extracted successively with 95% ethanol using soxhlet apparatus and aqueous extract was prepared by cold maceration method [14]. The alcoholic and aqueous extracts was concentrated *in vacuo* and kept in a vaccum dessicator for complete removal of solvent. Both the extracts were used for the antioxidant study.

DPPH Radical Scavenging Activity [15]: 15 mg of DPPH was dissolved in 10 ml of methanol. 75µl of this solution was taken and the final volume was adjusted to 3 ml with methanol, absorbance was taken immediately at 517 nm for control reading. 75µl of DPPH was added to a mixture of methanol and 50 µl of extract. The final volume was adjusted to 3 ml. Decrease in absorbance of the DPPH was measured 517 nm

ABTS Radical Scavenging Activity [16]: ABTS 2mM and Potassium per sulphate 70mM were prepared in distilled water (0.0548g in 50 ml and 0.0189g in 1ml respectively). 200ml of Potassium per sulphate and 50 ml of ABTS were mixed and used after 2 hrs. To 0.5 ml of various concentrations of the extracts, 0.3 ml of ABTS radical cation and 1.7 ml of Phosphate buffer, pH 7.4 was added. For control, instead of extract, methanol for alcoholic extract and water for aqueous extract were taken. The absorbance was measured at 734 nm.

DMSO Radical Scavenging Activity [17]: To 0.5 ml of different concentration of the extracts, 1 ml alkaline DMSO and 0.2 ml NBT 20mM (50 mg in 10ml phosphate buffer pH 7.4) were added. The absorbance was measured at 560 nm.

Nitric oxide Radical Scavenging Activity [18]: Griess reagent was prepared accordingly: Solution A: 1% Sulphanilamide in 5% ortho Phosphoric acid or 25% v/v Hydrochloric acid. Solution B: 0.01% Napthyl ethylene diamine in distilled water. Solution A and Solution B were in mixed equal volumes within 12hrs of use. Sodium nitroprusside 5mM was prepared in phosphate buffer PH 7.4 (0.0373g in 25 ml). To 1ml of various concentrations of the extract, 0.3 ml of sodium nitroprusside was added in the test tubes. The test tubes were incubated at 25°C for 5hr. 0.5ml of Griess reagent was added. The absorbance was measured at 546 nm.

Superoxide dismutase Scavenging Activity [15]: To 1.3 ml of different concentrations of the extract was added a mixture containing 0.2 ml EDTA 60mM (4.47 mg in 10ml water), 0.25 ml Riboflavin 53µm (31.92 mg in 100ml distilled water), 0.25 ml Hydroxylamine HCl 10mM (0.114g in 100ml distilled water) and 2 ml phosphate buffer pH 7.4. Riboflavin was added at the end after the tubes had been brought to a standard temperature of 20-22°C. The above solutions were incubated for 30 min in room temperature. Then, 1ml of Griess reagent was added to all test tubes. After 20 minutes, the absorbance was measured at 540 nm.

Reduction of Ferric Ions by Ortho-Phenanthroline Color Method [19]: A reaction mixture containing 1ml ortho-Phenanthroline (0.005g in 10 ml methanol), 2 ml ferric chloride 200 M (3.24 mg in 100 ml distilled water) and 2 ml of various concentrations of the extracts was incubated at ambient temperature for 10 min, then the absorbance was measured at 510 nm.

Total Antioxidant Capacity [20]: To 0.1 ml of the extract, 1ml of reagent solution (0.6M sulphuric acid, 28mM sodium phosphate, 4mM ammonium molybdate combined in eppendorf tube) was added .The tubes were capped and incubated at 35^0 C for 90 min. After cooling to room temperature the absorbance was measured at 695 nm against blank. Ascorbic acid was used as the standard and the total antioxidant capacity is expressed as equivalents of ascorbic acid.

Lipid Peroxidation Assay [21]: 15% w/v Trichloroacetic acid, 0.375%w/v thiobarbituric acid and 0.25N Hydrochloric acid were mixed to form the stock Thiobarbituric acid (TBA)- Trichloro acetic acid (TCA)- HCl reagent. This solution was mildly heated to assist the dissolution of TBA]. Albino rats (180-200g) of either sex were used for the study. After decapitation, the brain was removed carefully. The tissue was immediately weighed and homogenated with cold 1.15%w/v KCl to make 10%v/v homogenate. The homogenate (0.5ml) was added to 1 ml of various concentrations of the extracts. Then the mixture was incubated for 30 min. The per-oxidation was terminated by the addition of 2 ml of TBA-TCA –HCl reagent. The solution was heated for 15 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 rpm for 10 min. The absorbance of the supernatant was measured at 535 nm. The % inhibition of various radicals was calculated by comparing the results of the test with those of control using the formula.

$$\% \text{ inhibition} = \frac{\text{Control - Test}}{\text{Control}} \times 100$$

The institutional animal ethical committee approved the use of animals for lipid peroxidation assay (IAEC/SCOPE/07-08/01). All experiments were performed in triplicate and the results averaged. Linear regression analysis was used to calculate the IC_{50} values [22].

RESULTS AND DISCUSSION: Several concentrations ranging from 50-150 µg/ml of the ethanolic and aqueous extracts of both these species were compared for their antioxidant activity in different *in vitro* models. It was observed that free radicals were scavenged by the extracts in a concentration dependent manner up to the given concentration in all the models. On a comparative basis, both the extracts of both species showed almost near values (**Tables 1** and **2**).

TABLE 1: IC_{50} VALUES OF *IN VITRO* ANTIOXIDANT STUDY OF AQUEOUS AND ETHANOLIC EXTRACTS OF *TINOSPORA CORDIFOLIA* AND STANDARD (ASCORBIC ACID); (Values are mean ± SEM of 3 replicates)

Models studied	IC_{50} (µg/ml)		
	Aqueous extract	**Ethanolic extract**	**Standard**
DPPH activity	97.68 ± 1.08	106.86 ± 0.80	91.53 ± 0.31
ABTS activity	94.25 ± 0.84	85.48 ± 0.52	31.01 ± 0.62
DMSO activity	102.45 ± 1.28	103.40 ± 0.39	88.72 ± 0.47
Nitric oxide scavenging	85.71 ± 2.10	86.43 ± 1.31	82.69 ± 0.49
Superoxide dismutase activity	91.84 ± 0.71	90.20 ± 2.20	84.73 ± 1.08
Reduction of ferric ions	96.65 ± 1.97	96.32 ± 1.59	91.78 ± 1.75
Total antioxidant activity	100.71 ± 0.67	86.49 ± 0.82	84.28 ± 0.20
Lipid peroxidation activity	93.15 ± 1.03	95.05 ± 0.12	85.12 ± 2.61

TABLE 2: IC_{50} VALUES OF *IN VITRO* ANTIOXIDANT STUDY OF AQUEOUS AND ETHANOLIC EXTRACTS OF *TINOSPORA SINENSIS* AND STANDARD (ASCORBIC ACID); (Values are mean ± SEM of 3 replicates)

Models studied	IC_{50} (µg/ml)		
	Aqueous extract	Ethanolic extract	Standard
DPPH activity	100.46 ± 0.36	94.66 ± 0.049	91.53 ± 0.31
ABTS activity	104.75 ± 0.64	90.44 ± 0.36	82.22 ± 0.065
DMSO activity	97.98 ± 1.63	97.99 ± 0.15	88.18 ± 0.32
Nitric oxide scavenging	90.55 ± 3.47	87.25 ± 2.72	82.69 ± 0.49
Superoxide dismutase activity	88.55 ± 2.99	93.72 ± 0.91	84.73 ± 1.08
Reduction of ferric ions	97.71 ± 1.44	95.69 ± 1.47	91.78 ± 1.75
Total antioxidant activity	94.53 ± 0.19	91.63 ± 0.46	84.28 ± 0.20
Lipid peroxidation activity	90.27 ± 2.63	92.14 ± 0.91	85.12 ± 2.61

Free radicals are chemical entities that can exist separately with one or more unpaired electrons [23]. The propagation of free radicals brings about a myriad of reactions and thus may cause extensive tissue damage. Lipids, proteins and DNA are all susceptible to attack by these free radicals. The oxidative stress exerted due to these free radicals has been implicated in the pathology of various diseases like diabetes, inflammations, cardiovascular complications, cancer and ageing [24]. Antioxidants offer resistance against the oxidative stress by scavenging the free radicals, inhibiting lipid peroxidation and by many other mechanisms and thus prevent disease [25]. In our study, both the extracts of both species showed scavenging of free radicals in all the *in vitro* models studied.

DPPH is a stable free radical. The *in vitro* study carried out on this radical is based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH [26]. This radical reacts with suitable reducing agents, the electrons become paired off and the solution loses color stoichiometrically depending on the number of electrons taken up [27]. From the present results, it may be concluded that both *Tinospora cordifolia* and *Tinospora sinensis* reduce the radical to the corresponding hydrazine when they react with the hydrogen donors in the antioxidant principles.

ABTS assay is based on the inhibition of the absorbance of the radical cation $ABTS^+$ which has a long wavelength absorption spectrum [28]. The ABTS chemistry involves direct generation of ABTS radical mono cation with no involvement of any intermediary radical [29]. It is a decolorization assay, thus the radical cation is performed prior to the addition of antioxidant test system, rather than the generation of the radical taking place continuously in presence of the antioxidant [30]. The results obtained imply the activity of the extracts either by inhibiting or scavenging the $ABTS^+$ radical as reported in earlier works.

Nitric oxide is a free radical produced in mammalian cells, involved in the regulation of various physiological processes [31]. However, its excess production is associated with several diseases [32]. It is a very unstable species that reacts with free radicals thereby producing the highly damaging peroxy-nitrite [33]. In the present study, the nitrite produced by the incubation of solution of sodium nitrprusside in phosphate buffer was reduced by both the extracts of both species. This may be due to the antioxidant principles in the extracts which compete with oxygen to react with nitric oxide and thus inhibit the generation of nitrite [34]. Superoxide dismutase catalyses the dismutation of highly reactive superoxide anion to oxygen and hydrogen peroxide [35]. Superoxide anion is the first

reduction product of oxygen which is measured in terms of inhibition of generation of O_2 [36].

Ortho- substituted phenolic compounds may exert pro-oxidant effects by interacting with iron [37]. O- phenanthroline quantitatively forms complexes with ferric ion which get disrupted in the presence of chelating agents [38]. The extracts interfered with the formation of ferrous- O-phenanthroline complex, thereby suggesting that the extract has metal chelating activity [39].

The total antioxidant activity of the extracts was calculated based on the formation of phosphomolybdenum complex which was measured spectrophotometrically [40]. Free radicals induce lipid peroxidation in polyunsaturated lipid rich areas like liver and brain [41]. In this study, lipid peroxidation was induced *in vitro* and the extracts showed concentration dependent prevention towards generation of lipid peroxides.

Preliminary phytochemical screening revealed the presence of alkaloids, phenolic compounds, tannins and flavonoids in both the species [42]. Phenolics, flavonoids and tannins have been proved to be responsible for the antioxidant activity of various medicinal plants reported earlier [43 - 45]. Hence, these may be responsible for the observed activity in both these species. The present study proved *Tinospora sinensis* to be nearly potent as *Tinospora cordifolia* thereby justifying its traditional claims and its use in the present day system of medicine.

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