Protective activity of *Tinospora cordifolia* (Willd.) Hook. f. and Thoms. and *Withania somnifera* (L.) Dunal against lipid peroxidation, protein oxidation and deoxyribose oxidation

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Abstract

Withania somnifera L. (family - Solanaceae) and *Tinospora cordifolia* (family - Menispermaceae), medicinal herbs have different biological properties such as anticancer, immunomodulatory, hypoglycemic, anti-hepatotoxic, anti-inflammatory, gastroprotective, antioxidant, radioprotective effects. The present study was carried out to evaluate the protective effects of aqueous extracts of *T. cordifolia* (TC) and *W. somnifera* (WS) against lipid peroxidation, protein oxidation, and deoxyribose oxidation. The potential of inhibition of lipid peroxidation, protein oxidation, and deoxyribose oxidation by different concentrations of TC and WS aqueous extracts was tested with standard protocols. The EC_{50} values for inhibition of lipid peroxidation of *T. cordifolia* (TC) and *W. somnifera* (WS) were 146.2±1.2 µg/mL and 37.1±1.6 µg/mL, respectively. The EC_{50} value obtained for ascorbic acid as a positive control was 47.1±.1.1 µg/mL. Both extracts of TC (EC_{50} 8.0±1.4 µg/mL) and WS (EC_{50} 7.2±1.1 µg/mL) showed higher protective activities against the inhibition of deoxyribose oxidation compared with the positive control Gallic acid (EC_{50} 8.6±1.0 µg/mL). *W somnifera* (EC_{50} 75.5±1.0 µg/mL) and *T. cordifolia* (EC_{50} 112.4±1.7 µg/mL) showed less potential for inhibition of protein oxidation compared to positive control ascorbic acid, which was EC_{50} 57.0±1.3 µg/mL. The potential for inhibition of protein oxidation of both WS (EC_{50} 75.5±1.0 µg/mL) and TC (EC_{50} 112.4±1.7 µg/mL) was less than the positive control ascorbic acid (EC_{50} 57.0±1.3 µg/mL). *W somnifera* exhibited a more potent protective activity against lipid peroxidation, protein oxidation, and deoxyribose oxidation than TC. TC showed moderate activity compared with positive controls. Hence WS and TC may serve as potential sources of natural antioxidants for pharmaceutical applications.

Keywords: *Withania somnifera, Tinospora cordifolia,* inhibition of lipid peroxidation, protein oxidation, deoxyribose oxidation

Introduction

The reactive oxygen species (ROS) are generated in the human body from exogenous chemicals, physical sources, and endogenous metabolic processes. In addition to non-radical species like hydrogen peroxide (H_2O_2), ROS comprise free radicals including superoxide (O2•-), hydroxyl radical (•OH), and peroxyl radical (RO2•)[1,2]. The production of excess uncontrolled ROS steers oxidative stress activating free radicals causing cellular injury and the ageing process. The major target of ROS is cellular components, including lipids, DNA, and proteins in the body[3]. ROS cause catastrophic and irreversible damage to proteins, lipids, and DNA due to their high chemical reactivity.

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Lipids are attacked and oxidised by ROS to produce peroxides and aldehydes. Peroxidation of membrane lipids can inactivate cellular components leading to diseased conditions[4,5]. Proteins are also vulnerable to ROS attacks, which can modify their function through nitrosylation, carbonylation, glutathio nylation and the formation of disulfide bonds[6]. Furthermore, site-specific amino acid modification, fragmentation of the peptide chain, aggregation of cross-linked reaction products, altered electric charge, and increased susceptibility of proteins to proteolysis happen due to excessive ROS production[7]. In recent years, much attention has been focused on ROS, especially in clinical medicine, due to its cause of many degenerative diseases such as atherosclerosis, ischemia-reperfusion, heart failure, Alzheimer's disease, rheumatic arthritis, cancer, and other immunological disorders[8]. Therefore, developing and utilising more effective antioxidants is a timely requirement.

The bioavailability of antioxidants derived from natural sources is higher. It is therefore preferred that natural antioxidants have more protective activity. In general, major health-beneficial substances are natural antioxidants from medicinal plants. β-carotene and other natural antioxidants are essential for avoiding cancer and numerous cardiovascular problems[3]. It is crucial to comprehend these plants' potential toxicity and health advantages. A wide variety of substances, including phenolic compounds, flavonoids, and carotenoids, are natural antioxidants. Recently, various plant materials' antioxidant capacities have been characterized[9]. In the present study, two medicinal plants (*Withania somnifera* L and *Tinospora cordifolia*) were selected to investigate their antioxidant capacities against lipid peroxidation, protein oxidation, and deoxyribose oxidation.

Withania somnifera L (family-Solanaceae), commonly known as Ashwagandha. It is widely distributed in India, the Middle East, and parts of Africa. It is a short, delicate, evergreen shrub that is wild-grown and cultivated for medicinal purposes. For more than 2500 years, it has been used as a home remedy for many diseases. The root of *W. somnifera* has long been thought to have the most significant medicinal potential[10]. The roots of *W. somnifera* are rich with several alkaloids, withanolides, a few flavonoids, and reducing sugars[11]. Bishayi *et al* 2002 reported that there are more active compounds in *W. somnifera*, including withaferin A, sitoindosides VII–X, 5-dehydroxy withanolide-R, withasomniferin-A, 1-oxo-5b, 6b-epoxy-witha-2-ene-27-ethoxy-olide, 2,3-dihydro-withaferin A, 24,25-dihydro-27-desoxy withaferin A, 27-O-b-D-glucopyranosylphysagulin D, physagulin D, withanoside I–VII,27-O-b-D-glucopyranosyl-viscosalactone B, 4,16-dihydroxy-5b, 6b-epoxyphy-sagulin D, viscosalactone B and diacetylwithaferin A[12]. Previous research showed that *W. somnifera* has beneficial effects in treating arthritis, geriatric issues, and stress, as well as anticancer, anti-inflammatory, and anabolic activity[13].

Ayurvedic and folk medicine both extensively use the well-known medicinal herb *Tinospora cordifolia*. It is a large, glabrous, succulent climbing shrub from the Menispermaceae family. It has been demonstrated that this plant's roots, stems, and leaves have various therapeutic uses. There have been reports of various pharmacological characteristics, including immunomodulatory,[14,15] hypoglycemic,[10], anti-hepatotoxic, [11,12], anti-inflammatory,[16] antioxidant[17]. Therefore, in the present study, the protective effects of aqueous extracts of *T. cordifolia* and *W. somnifera* against lipid peroxidation, protein oxidation, and deoxyribose oxidation were evaluated under *in vitro* conditions.

Materials and Methods

Preparation of extracts

The plants were obtained from "Weda Waththa" (6.801746, 79.977027) located in Maththegoda, Colombo district, Sri Lanka from August to September 2021. The plants were identified by a Senior Lecturer at the Institute of indigenous medicine, University of Colombo. The stem (*T. cordifolia*) and root (*W. somnifera*) of the plants were used for the study. Plant parts were cleaned and subjected to freeze drying to avoid oxidation of endogenous substances. After that, plant parts were

ground to a fine powder, 60 g was extracted with 1920 mL of deionised water, and the volume was reduced to 240 mL under low heat. Extracts were freeze-dried, and samples were stored at -20^0 C.

Inhibition of protein oxidation

The effect of TC and WS aqueous extracts on protein oxidation was carried out using a modified method of Wang and co-workers 2006[18]. A Fenton-type reaction oxidised bovine serum albumin (BSA). Different concentrations (7.81-250 µg mL^{-1}) of TC and WS extracts (0.5 mL) were mixed with a reagent mixture (1.5 mL) containing potassium phosphate buffer (PBS) (20 mM, pH 7.4, 300 µL), BSA (4 mg mL^{-1}), $FeSO_4$ (2 mM, 300 µL), H_2O_2 (30%, 400 µL) and was incubated for 30 min at 37º C. After that 2,4-Dinitrophenylhydrazine (DNPH) (1.0 mL of 10 mM) in 2 M HCl was added to the mixture to determine the protein carbonyl content of the samples. Then, 1.0 mL of cold trichloroacetic acid (TCA) (10%, w/v) was added followed by 30 min incubation at room temperature for 30 min. Then the mixture was subjected to centrifugation at 3000 rpm for 10 min. The resulting protein pellet was washed with ethanol/ethyl acetate (1:1, v/v, 2.0 mL) and the pellet was resuspended in guanidine hydrochloride (6 M, pH 2.3, 1.0 mL). The absorbances of the samples were read at 370 nm wavelength. L- Ascorbic acid was used as the positive control. The following equation calculated the percentage inhibition of protein oxidation.

% inhibition = $(A_{control} - A_{sample})/A_{control} \times 100$

EC_{50} value was calculated using a standard graph constructed with different concentrations vs % inhibition.

Inhibition of lipid peroxidation

The inhibition of lipid peroxidation was evaluated by the method of Dhar et al. 2013[19]. The egg yolk was used as the lipid-rich source to form lipid peroxides. Briefly, 1.15% w/v KCl was added to the fresh egg yolk emulsion to prepare a 10% v/v solution. Then different concentrations (7.81-250 µg/mL) of TC and WS extracts were mixed with egg yolk emulsion solution (50µL), and trichloroacetic acid (20% aqueous, 150 µL) and thiobarbituric acid (150 µL, 0.67% w/v) added respectively. The reaction mixture was incubated at 95° C in the water bath for 1 hour, followed by the vortex. The mixture was subjected to centrifugation at 3000 rpm for 10 min. The absorbance of the upper layer was measured at 532 nm wavelength, and percentage inhibition was calculated with the following formula.

% Inhibition = $(A_{control} - A_{sample})/A_{control} \times 100$

EC_{50} value was calculated using a graph constructed with different concentrations vs % inhibition. Results were compared with positive control L- Ascorbic acid

Inhibition of deoxyribose oxidation

The inhibition of deoxyribose oxidation was measured according to the modified method of Halliwell 1987[20]. The absorbance was obtained at 532 nm and compared with the positive control, Gallic acid. The percentage inhibition of deoxyribose oxidation was calculated with the following formula.

% inhibition= $(A_{control} - A_{sample})/A_{control} \times 100$

EC_{50} value was calculated using a graph constructed with different concentrations vs % inhibition.

Statistical analysis

All the results were expressed as the mean ± standard deviation (Mean ± SD) of at least three independent experiments. Calibration curves were considered linear if $R^2 > 0.99$. The EC_{50} values were calculated from linear dose-response curves where $R^2 > 0.95$. The paired t-test was used for the statistical analysis, and all analyses were done using graph pad prism (2010) statistical software.

Results

Inhibition of lipid peroxidation by aqueous extracts of *Tinospora cordifolia* and *Withania somnifera* and standard (Ascorbic acid) is shown in Figure 1.

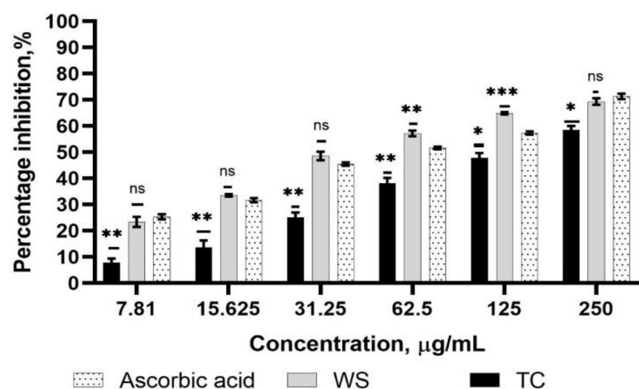

Figure 01: **Inhibition of lipid peroxidation by aqueous extracts of *Tinospora cordifolia* and *Withania somnifera* and standard (Ascorbic acid)**
P values are represented as $^{*}< .05$, $** < .01$ and $*** < .001$ in comparison to the control (Ascorbic acid).

The aqueous extracts of WS showed significant inhibition of lipid peroxidation compared with the positive control ascorbic acid ($p < .001$) (Figure 01) and, WS exhibited a 50 % inhibition of lipid peroxidation at 37.1±1.6 µg/mL. The TC exerted moderate protective activity against lipid peroxidation compared with ascorbic acid. The EC50 value for the TC was recorded as 146.2±1.2 µg/mL (Table 01).

Table 01: EC_{50} values of inhibition of lipid peroxidation by *Tinospora cordifolia*, *Withania somnifera* and Ascorbic acid

Positive control/Plant extracts	EC_{50}, µg/mL
T. cordifolia	146.2±1.2 (***)
W. somnifera	37.1±1.6 (***)
Ascorbic acid	47.1±.1.1

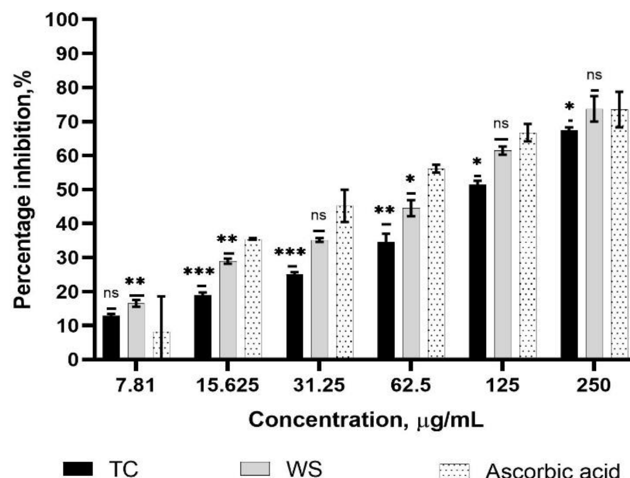

Figure 02: Inhibition of protein oxidation by aqueous extracts of *Tinospora cordifolia* and *Withania somnifera* and standard (Ascorbic acid)
P values are represented as $*< .05$, $**< .01$ and $*** < .001$ in comparison to the control (Ascorbic acid).

The effect of aqueous extracts of TC, WS, and ascorbic acid against protein oxidation are shown in Figure 2. TC and WS exhibited a dose-dependent reduction of albumin oxidation, induced by the H_2O_2/Fe^{3+}system, which resulted in the formation of a carbonyl group. Fifty percent of protein oxidation inhibit by TC and WS at 112.4±1.7 µg/mL and 75.5±1.0 µg/mL, respectively (Table 2). The effect of ascorbic acid at 57.0±1.3 µg/mL concentration exhibited 50% inhibition.

Table 02: EC_{50} values of inhibition of protein oxidation by *Tinospora cordifolia*, *Withania somnifera* and Ascorbic acid

Positive control/Plant extracts	EC_{50}, µg/mL
T. cordifolia	112.4±1.7 (***)
W. somnifera	75.5±1.0 (***)
Ascorbic acid	57.0±1.3

Figure 3 shows the Inhibition of deoxyribose oxidation by an aqueous extract of TC and WS and standard (Ascorbic acid). TC and WS inhibit the oxidation of deoxyribose in a dose-dependent manner. Interestingly WS and TS exhibit potent protective activity compare with the standard. The EC_{50} value of TC and WS is 8.0±1.4 µg/mL and 7.1±1.1 µg/mL, respectively (Table 03).

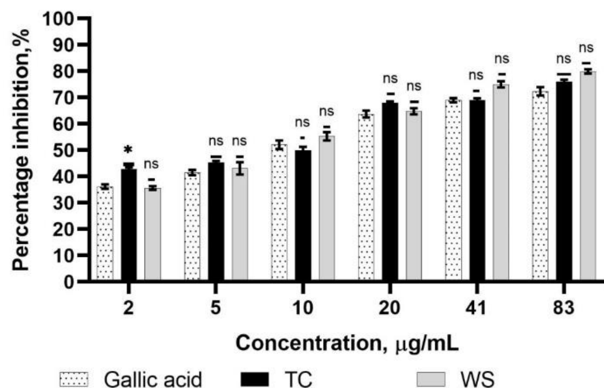

Figure 03: Inhibition of deoxyribose oxidation by aqueous extract of *Tinospora cordifolia*, *Withania somnifera* and standard (Ascorbic acid).

P values are represented as *< .05, **< .01 and *** < .001 in comparison to the control (Ascorbic acid).

Table 03: EC_{50} values of Inhibition of deoxyribose oxidation by *Tinospora cordifolia*, *Withania somnifera* and Ascorbic acid.

Positive control/Plant extracts	EC_{50}, µg/mL
T. cordifolia	8.0±1.4 (**)
W. somnifera	7.1±1.1 (**)
Ascorbic acid	8.6±1.0

Discussion

Antioxidants are synthetic or natural chemical substances capable of reducing or preventing cellular damage. Utilising natural antioxidants can reduce oxidative damage via direct scavenging of intra- or extra-cellular reactive molecules and activation of cellular antioxidant mechanisms leading to prevent diseases with minimum side effects. The Most natural non-enzymatic antioxidants from natural sources such as diet, plants, fungus, other microbes, animals, etc. Plants are the primary source of dietary antioxidants. Natural products are used directly as medication in traditional and Ayurveda medicine. One of the major bioactivities of antioxidants are the inhibition of lipid peroxidation, protein oxidation and deoxyribose oxidation. The present study evaluated the protective activities of aqueous extracts of TC and WS against lipid peroxidation, protein oxidation, and deoxyribose oxidation.

Lipid peroxidation is the reaction between unsaturated lipids and reactive oxygen species[21]. Malondialdehyde (MDA) is one of the final products of polyunsaturated acids peroxidation form in the cells[22]. MDA level is commonly recognised as a marker of oxidative stress and overproduction of MDA due to increased free radicals[23]. Thiobarbituric acid (TBA) is a common method used to determine the degree of malondialdehyde (MDA) compound in a biological solution [24].

The mechanism of the assay is that MDA reacts with TBA and produces a pink colour which reads at 532 nm. A higher reduction of lipid peroxidation is observed in WS extract (EC_{50}, 37.1±1.6 µg/mL) in comparison to the positive control (Ascorbic acid EC_{50} 47.1±.1.1 µg/mL) (Table 01). Molecules present in the WS extract may have a higher contribution to the inhibition of lipid peroxides. The results of the present study suggest that plant extracts can reduce cell membrane damage by scavenging lipid peroxides. IC50 values for the extract and standard trolox were 284.13±146.66 g/ml and 13.52±0.33 g/ml, respectively, in the earlier study by Chaudhuri et al. (2012)[25], which demonstrated that methanol-water extract exhibited effective suppression of lipid peroxidation. According to Gupta et al. 2003[26], Ashwagandha (*Withania somnifera*) had a concentration-dependent rise in the inhibitory ratio on lecithin peroxidation that reached as high as 77.2± 4.4% at a concentration of 45 g/ml (p 0.05).

Direct oxidant damage to a protein's backbone results in fragmentation and conformational changes in the protein's secondary and tertiary structures. For oxidised proteins, the most frequent harm is the

formation of carbonyls[27]. Protein oxidation levels in the food system may now be measured easily and often using the DNPH derivation approach[28]. In this procedure, DNPH combines with protein carbonyl groups to produce hydrazones, and the absorbance is measured at a wavelength of 370 nm[29].

In the present study, inhibition of protein oxidation by WS and TC was tested. The results revealed that WS contains a moderate potential to prevent protein oxidation (EC_{50}, 75.5±1.0 µg/mL, Table 02). The positive control (Ascorbic acid) was EC_{50} 51.2±0.1 µg/mL. The *W. somnifera* extract demonstrated more than 50% suppression of protein oxidation at 10 g/mL in a prior study by Gupta et al. 2003[26]. Therefore, WS is highly applicable for a disease that arises due to increased levels of protein carbonyls, such as neurodegenerative diseases (amyotrophic lateral sclerosis, Alzheimer's, Parkinson's, and Huntington's diseases), cataractogenesis, systemic amyloidosis, muscular dystrophy, progeria, Werner's syndrome, rheumatoid arthritis, and respiratory distress syndrome[30].

DNA damage is one of the major effects of ROS[31]. DNA is the cell's genetic material, and OH- radicals react with all purine and pyrimidine bases. The deoxyribose backbone changes the encoded proteins, which may lead to malfunctions or complete inactivation of the encoded proteins. Further, changes in the nucleotides of one strand can result in mismatches with the nucleotides in the other strand, yielding subsequent mutations[32]. Accordingly, the inhibition of the DNA oxidation power of WS and TC was evaluated in the present study. The hydroxyl radical resulted from the interaction of iron (III)-EDTA and H_2O_2 with the ascorbic acid present. Thiobarbituric acid is heated with the attacked pentose sugar 2-deoxyribose at a low pH, producing a pink chromogen whose absorbance can be measured at 532 nm wavelength[33]. Interestingly higher inhibition of deoxyribose oxidation was observed in Both plant extracts (The EC50 value of TC and WS is 8.0±1.4 µg/mL and 7.1±1.1 µg/mL, respectively (Table 03)) when compared with the positive control (Ascorbic acid, EC_{50}, 8.7±0.6 µg/mL).

Conclusion

Tinospora cordifolia (TC) and *Withania somnifera* (WS) extracts exhibit a good, conferred protection against biomolecule oxidative damage. Therefore, TC and WS extracts could be a promising antioxidant source for the prevention and/or treatment of oxidative stress-related diseases as it could retard oxidative degradation of protein, lipids, and deoxyribose.

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