Protective role of *Withania somnifera* root extract on lipid peroxidation of Erythrocytes

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Abstract – *Withania Somnifera* (Ashwagandha) is an important medicinal plant that has been used in Ayurvedic and indigenous medicine. It is also known as Indian ginseng. It exerts antioxidant, anti-inflammatory and immune modulatory activities. In the present study, we studied the free radical scavenging activity of *Withania Somnifera* root extract. The radical scavenging effect of the extract was studied by its effect on hydroxy radicals generated by Fe-ascorbate-H_2O_2 system. Our results showed that *Withania Somnifera* root extract (1-10,000 µg/ ml) resulted in a significant dose dependent increase in percent inhibition of hydroxylation. Further, we studied the *in vitro* effect of *Withania Somnifera* root extract on Lipid peroxidation in terms of MDA level of human erythrocytes. Our results showed a significant dose dependent decrease in lipid peroxidation at a concentration of (1-1000 µg/ ml) as evident by decrease in level of MDA. Maximum effect was observed at 100-1000 µg/ ml. *Withania Somnifera* root extract thus can act as a potential antioxidant containing compound and protects erythrocytes from oxidative damage by either directly scavenging the free radical, sparing it or activating other enzymatic processes. At higher concentration (10,000 µg/ ml), *Withania Somnifera* extract increased the oxidative stress as evident from erythrocyte MDA level which became significantly more than the maximum effect shown at 1000 µg/ ml of extract.

Introduction

Changing lifestyle and environment conditions have predisposed common man towards numerous diseases. Nowadays the major threat for the survival among others include chemicals, toxic metals and the stress of modern living which are the major cause of various type of diseases. "One of the paradoxes of life on this planet is that molecules that sustain aerobic life i.e. oxygen, is not only fundamentally essential for energy metabolism and respiration, but it has been implicated as major cause in many disease and degenerative disorders. The importance of ROS and free radicals in pathogenesis of various diseased conditions has attracted increasing attention over the past decades.(Valko et al., 2007) ROS are generated in specific organelles of cells under normal physiological conditions and these can easily initiate the peroxidation of membrane lipid leading to the

accumulation of lipid peroxides (Farmer and Mueller, 2013). Antioxidant helps our body in protecting us from oxidative damage by neutralizing the free radicals directly or scavenging them through a series of reaction coupled with anti-oxidative enzymes (Baek and Lee, 2016). In addition to endogenous there are number of exogenous antioxidants that include lots of natural and synthetic antioxidants.

Overproduction of ROS results in oxidative stress. On a global scale free radical combined induced oxidative damage has claimed more lives than all of the wars and plaques thought-out human history. Of all deaths, nine out of ten people die from cancer, heart attack, rheumatic diseases, skin wrinkling, diabetes, alzheimer's disease, parkinson disease, AIDS and stroke and in these conditions, oxidative damage caused by free radicals has been implicated as one of the major causes (Rizvi and Zaid, 2001).

Oxidative damage generates reactive oxygen species (ROS) and reactive nitrogen species (RNS) which mainly include free radicals which are chemical entities possessing a single electron. Free radical can be formed either by losing an electron or accepting an electron. These free radicals are actually responsible for cellular damage and cause various diseases (ligouri et al., 2018). Reactive species include free radical such as superoxide anion radicals ($O_2^{-}\cdot$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$) and hydroperoxyl radical ($\cdot HO_2$). These free radicals can influence each and every molecule of the body, such as DNA, proteins, lipids and carbohydrates (Rowe, et al., 2008 and Griffiths, et al., 2014).

The most important source of free radical production are the auto-oxidation of molecule such as hydroquinone, catechol and ferredoxin, the activity of enzyme such as xanthine oxidase, aldehyde oxidase and NADPH-cytochrome P_{450} reductase, the electron transfer reaction of the inner mitochondrial membrane and the metabolism of xenobiotics or drugs (Mohora, 2001). Our immune system also produces free radicals to destroy bacteria etc (Knight, 2000). Most often source of free radical is the environment to which our body is exposed such as air, tobacco smoke, radiation, toxic wastes and various chemicals (Aseervatham et al., 2013).

Nature did not leave us defenseless against this onslaught by free radicals. Antioxidants play a crucial role in preventing or delaying the damage caused by these oxidants. Antioxidants trap the lone pair of electron from free radicals and neutralize them. Thus various antioxidants are free radical scavenger and may be of enzymatic and non-enzymatic nature. This antioxidant can be any substance or constituent of molecule, synthetic or natural origin which delays or inhibits propagation of reactive species and prevent the oxidative damage of a target molecule (Zaid et al., 2007)

Our body actually has its own army of antioxidants and antioxidant enzyme which are able to neutralize free radicals and render them harmless. But due to increasing exposure of our body to high levels of these reactive species the administration of exogenous antioxidant as food constituent or therapeutic agents is beneficial. Most exogenous natural antioxidant comes from raw vegetable, fruits, spices, herbs and various medicinal plants and include molecule such as α-tocopherol, vitamin A, vitamin B, vitamin C and various polyphenolic compounds. These are known as natural antioxidant. In

addition to these there are synthetic antioxidants also like dimethyl sulphoxide, BHT, and BHA etc. Natural antioxidants are always appreciated over synthetic one, because they lack toxic side effects and show effect by multiple mechanisms. (Sharma et al., 2017)

Long-established systems of traditional medicine have evolved from systematic recordings of human experience over several millennia. Although not strictly based on concepts of modern science, they nevertheless are founded on a corpus of organized knowledge written in documents, and the evident conclusion is that the alleged "trial and error" methodology has provided useful drugs for humans. Medicinal plants have been gaining increasing importance over the past decades due to their increased use as a source of herbal drugs (Huang. et al., 1992).

Nature is the treasure house of unexplored medicinal plants. In search of effective antioxidant from natural source, we have studied the antioxidant property of *Withania Somnifera* on human erythrocyte *in vitro*. *Withania Somnifera* has been reported to have anti-inflammatory, anti-tumor, anti-stress, immuno modulatory properties, hemopoetic effect, rejuvenating effect on nervous system (Speed et al., 2021 and Dhar et al., 2015)

In the present study, we studied the free radical scavenging activity of Aqueous-alcoholic (50% alcohol and 50% distilled water) *Withania Somnifera* root extract. The radical scavenging effect of the extract was studied by its effect on hydroxyl radical generation by Fe-ascorbate-H_2O_2 system and its effect on lipid peroxidation (MDA levels) in red blood cell.

Material and method

Preparation of extracts

Dry powder of *Withania Somnifera* roots was used for extract formation *Withania Somnifera* extract was prepared in soxhlet assembly (Anand and Zaid 2020). In this extraction process the raw material was placed in a thimble made of cotton and inserted into a wide central tube of the soxhlet extractor. Aqueous-alcoholic (50% distilled water and 50% alcohol) extraction was done for 72 hours at temperature between 60°C to 80°C. After incubation the residual solvent was dried at room temperature in vacuum dedicators under reduced pressure. The dried extract was finally dissolved in DMSO and diluted to appropriate concentration by distilled water.

Effect of *Withania Somnifera* extract on Hydroxyl ($OH^{\cdot}$) radical generation

Hydroxyl radicals were generated by Fe^{3+}-ascorbate-H_2O_2 system (Rowley and Halli well, 1983). Formation of hydroxyl radicals appears to occur by the following reaction:

$$Fe^{3+}\text{- EDTA} + AH^{-} \longrightarrow Fe^{2+}\text{- EDTA} + A^{-}$$
$$Fe^{2+}\text{- EDTA} + H_2O_2 \rightarrow Fe^{3+}\text{-EDTA+ OH} + OH^{-}$$

$$AH + H_2O_2 \longrightarrow O_2 + {}^{\cdot}OH + OH^{-}$$

Hydroxyl radicals so formed were quantitated by their ability to attack aromatic compounds with the formation of hydroxylated product. In this study salicylate (2-hydroxy benzene) at a concentration of 2.5mM is included in the reaction mixture, and the hydroxylated product (2, 3-dihydroxy benzoate) is extracted with ether and assayed spectrophotometrically at 510 nm.

The reaction mixture of 2 ml contains 2.5mM salicylate, 0.3mM EDTA, 0.1mM $FeCl_3$ (fresh solution just before use), 2mM ascorbate (fresh), 150mM KH_2PO_4-KOH buffer (pH 7.4), and 10 µl of *Withania Somnifera* extract ratio (0-10,000 µg/ml final concentration), reaction was started by the addition of 1mM H_2O_2. The reaction mixture was incubated for 90 minutes at 37^0C. At the end of incubation, reaction was stopped by adding 80µl (11.6 N) HCl and 0.5g NaCl.

Hydroxylated product (2, 3-dihydroxy benzoate) was then extracted with 4 ml chilled diethyl ether, 3 ml of upper layer was taken and evaporated to dryness on sand bath. Residue so obtained was dissolved in 0.5 ml cold double distilled water. To this was added in order 0.25 ml 10 % (w/v) TCA dissolved in 0.5M HCl, 0.5 ml 10 % (w/v) sodium tungstate, and 0.5 ml 0.5 % (w/v) sodium nitrite (freshly prepared). Reaction mixture was made to stand at room temperature for 5 minutes and then added 1 ml 0.5M KOH and absorbance read at 510 nm and compared with the extinction coefficient of hydroxylated product (2, 3-dihydroxy benzoate).

Effect of *Withania Somnifera* extract on Lipid peroxidation (MDA) of erythrocytes

MDA is the most abundant individual aldehyde resulting from lipid peroxidation and its determination by TBA is the most common method of estimating lipid peroxidation. (Esterbauer and Cheeseman, 1990)

Isolation and treatment of erythrocytes with *Withania Somnifera* extract

Blood was washed 2-3 times with Krebs ringer phosphate buffer with 5mM glucose, pH-7.4 (KRPG). Packed erythrocytes were suspended in 4 volume of KRPG. Suspended erythrocytes were incubated with different concentrations of *Withania Somnifera* root extract (1-10,000 µg/ml) for 30 mints. After incubation erythrocytes were washed 2-3 times with KRP buffer and then packed RBC were obtained. Packed RBC was used for MDA estimation (Rizvi et al., 2007)

Estimation of MDA content of erythrocytes

Packed erythrocytes 0.2 ml was suspended in 3 ml of KRP buffer. To 1 ml lysate was added 1 ml of 10 % TCA (for ppt. of protein) and centrifuged for 5 minute at 1000g.1 ml of supernatant was added to 1 ml of 0.6 % TBA in 0.05 M/l of NaOH and boil for 20 minute at temperature greater than 90^0C. Cooled and absorbance taken at 532 nm and 630 nm as OD1 and OD2. For MDA level, final OD was calculated as OD2 – OD1. Results were calculated from the reading of known MDA standards (Rizvi et al., 2007)

Results and Discussion

Effect of *Withania Somnifera* root extract on Hydroxylation

The effect of different concentration (1-10,000 µg/ ml) of *Withania Somnifera* root extract on Hydroxyl radicals generated by Fe^{3+}-ascorbate-H_2O_2 system is shown in table-1. *Withania Somnifera* root extract(1-10,000 µg/ ml) showed a dose dependent decrease in hydroxyl radical generation as evident by a decrease generation of hydroxylated product. *Withania Somnifera* extract significantly inhibited formation of hydroxylated products generated by Fe-ascorbate-H_2O_2 system (Table-1) which may be due to blocked generation of OH radical primarily either by chelating Fe^{3+}

or by decomposing H_2O_2 in the reaction system and/ or secondary due to the scavenging of OH radicals (Gulcin, 2020).

Table-1 Effect of *Withania Somnifera* root extract on hydroxy radical generation by Fe^{3+}-ascorbate-H_2O_2 system. Each value is the mean of at least 5-6 independent experiment. Values are expressed as mean ± S.D.

Conc. of *Withania Somnifera* root extract. (µg/ml)	n mole of hydroxylated product	% inhibition of hydroxylation
0	92.3 ± 4.2	00
1	61.6 ± 3.8	33.26
10	55.2 ± 2.2	40.19
100	51.5 ± 2.9	44.74
1000	43.3 ± 3.0	50.08
10,000	40.4 ± 3.2	56.29

Effect of *Withania Somnifera* extract on Malondialdehyde (MDA) content of erythrocytes

The effect of different concentration (1-10,000 µg/ ml) of *Withania Somnifera* root extract on Malondialdehyde (MDA) content of erythrocytes is shown in figure-1.MDA is one of the end products in lipid peroxidation. *Withania Somnifera* root extract (10-1000 µg/ ml) showed a significant dose dependent decrease in MDA level of RBC as compared to normal control. Maximum effect was observed at a concentration of 1000 µg/ ml) of *Withania Somnifera* root extract.

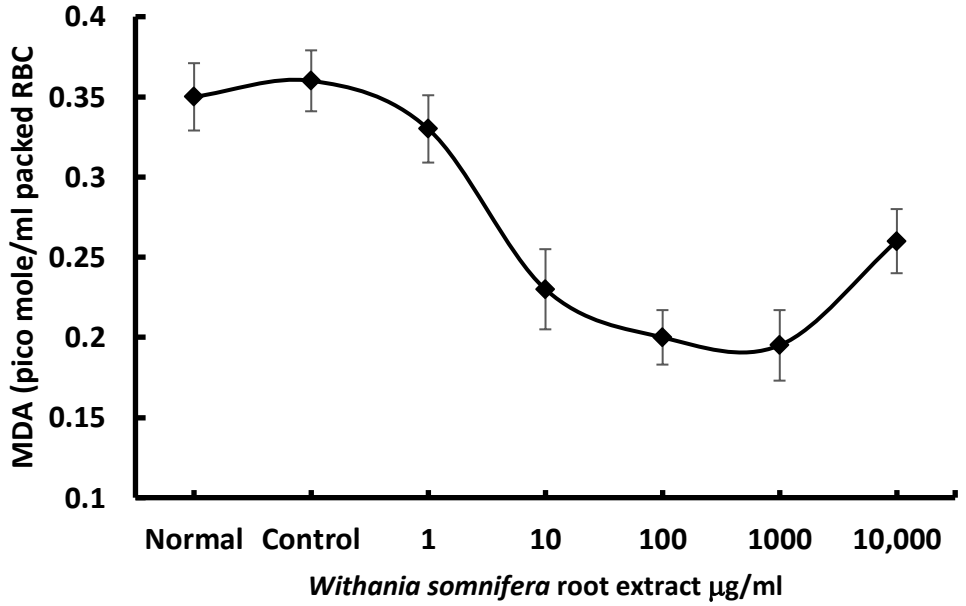

Figure-1 Effect of *Withania Somnifera* extract on MDA level of erythrocyte results are expressed in pico mole/ml of packed RBC. Each value is the mean of at least 5-6 independent experiment. Values are expressed as mean ± S.D.

Our results showed that erythrocyte MDA level decreases with increasing concentration of *Withania Somnifera* extract upto concentration of 1000 µg/ml but further increase in extract concentration 10,000 µg/ ml resulted in a significant increase in the MDA level as compared to the maximum effect shown at 1000 µg/ ml.

An increased MDA concentration of erythrocytes is indicator for prooxidant status in erythrocytes that can result in oxidative stress. Our results are similar with previous reports on MDA (Rizvi et al., 2005). Erythrocytes can be highly susceptible to oxidative damage due to their high content of Polyunsaturated fatty acids present in their membranes along with high content of oxygen due to hemoglobin makes them prone to oxidative processes. An increase in MDA is known to cause a decrease in fluidity of membrane and can make erythrocytes fragile (Ozturk et al., 2003). Thus the oxidative process and accumulation of MDA can result in clinical conditions. Thus treatment of erythrocytes with *Withania Somnifera* extract may thus protect erythrocytes from stress induced oxidative damages by either directly scavenging the free radical or activating other processes. *Withania Somnifera* contains many antioxidant compounds including (Somniferine, Somnine, Withanaine, Somniferinine, Withanolides etc.) which are known antioxidants. (Khan et al., 2021). Our results also show that at higher *Withania Somnifera* concentration 10000 µg/ ml the extract may itself can cause generation of free radicals as high levels of antioxidants can itself cause generation of free radical (Iwasaki et al., 2014) Further studies are needed to elucidate its mechanism of action and its cytotoxic properties.

Conclusion

The present study was undertaken to evaluate the antioxidant effect of Withania Somnifera. Antioxidant and free radicals scavenging activities in extract of *Withania Somnifera* on RBC was studied *in-vitro*.

Our study shows:

1. *Withania Somnifera* extract shows dose dependent inhibition of hydroxylation (OH formation). This indicates that *Withania Somnifera* extract posses an effective antioxidant and hydroxyl radical scavenging properties.
2. A decrease in lipid peroxidation (MDA content) of erythrocyte was observed at *Withania Somnifera* extract concentration of 10-1000 µg/ml as compared to normal control.
3. Our result also showed that, much higher concentration of *Withania Somnifera* extract (10,000 µg/ml) started to show adverse effect on erythrocytes lipid peroxidation as evident from increase in MDA levels as compared to maximum effect shown at 1000 µg/ml.

From our study, we can conclude that the *Withania Somnifera* extract can act as a potential antioxidant and protect the erythrocytes from oxidative damage by either directly scavenging the free radical or activating other processes. This property of *Withania Somnifera* may be due to its antioxidant features because *Withania Somnifera* contains many antioxidant compounds including (Somniferine, Somnine, Withanaine, Somniferinine, Withanolides etc.) which

are known antioxidants. But its indiscriminate use especially at higher concentrations should be avoided. As *Withania Somnifera* at higher concentration itself can cause generation of free radicals. Further studies are needed to elucidate its mechanism of action and its cytotoxic properties.

Disclaimer Statement

Authors declare that no competing interest exists. The products used for this research are commonly used products in research. There is no conflict of interest between authors and producers of the products.

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