Antioxidant Effect of *Stevia Rebaudiana* on Humanerythrocytes

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Abstract-The changing lifestyle and environment conditions have predisposed common man towards numerous diseases. Today most of the diseases are said to be caused by synthetic chemicals, toxic heavy metals, and the stress of modern living. It is also true that oxygen is essential for sustaining life but it is also dangerous to our existence. Oxygen is being viewed as playing a lead role in the generation of reactive intermediates, thereby causing cellular damage. Our body has the mechanism to handle free radicals and prevent its damaging effect, which involves the use of antioxidants as glutathione and antioxidant enzymes as superoxide dismutase, catalase, glutathione peroxidase glutathione reductase, glutathione-S-transferase to counter these free radicals. When the redox status of the body is overwhelmed by these radical species, this may result in variety of chronic diseases and even premature senility. The administration of natural antioxidant as food constitutes or therapeutic agents is looked-for to neutralize these reactive oxygen species and prevent or delay diseased condition caused by these reactive species. Most exogenous antioxidants come from raw vegetable fruits, spices, herbs and various medicinal plants.Natural antioxidants are always appreciated over synthetic ones because they lack toxic side effects.

The present study deals with the effects of *Stevia rebaudiana* leaf extract on the status antioxidant of RBC as evident by an *in vitro* dose-dependent decrease in the activity of erythrocytes superoxide dismutase and catalase as compared to the normal control whereas at much higher concentration of *stevia* leaf extract (100µg/ml) started to show a reversing trend of its protective action.
Keywords: *Stevia*, Antioxidants, Free radicals, ROS, Superoxide Dismutase (SOD), Catalase**.**

Introduction

The recent growth in the understanding of free radicals in biology has produced a medical revolution that potentials a new age of health and disease management (Aruoma, 2003). It is ironic that oxygen, which is essential for life under certain circumstances also can show damaging effects on the human body (Bagchi and Puri,1988). Most of the potentially damaging effects of oxygen are due to the formation of a number of chemically active compounds, known as reactive oxygen species (ROS), which have a tendency to contribute oxygen to other substances. Antioxidants which normally neutralize these reactive oxygen species and resulting into free radicals have been commonly used in modern clinical system to explain the mechanisms for causing disease (Aruoma, 1994).

During metabolism and exposure to external environment, a large amount of free radicals are produced in human body that can attack various biological macromolecules such as proteins, fatty acids and nucleic acids, causing oxidative damage to cells, tissues or even result in mutation of genes(*Young and Woodside, 2001)*.These radicals at high concentration cause oxidative stress, this destroys internal redox balance and can even result in a variety of chronic diseases(Gupta et al., 2014). Researches show that many diseases including cancers, arteriosclerosis, diabetes, cataract, cardiovascular diseases, Parkinson's disease, Alzheimer's disease and arthritis, correlate with cellular redox imbalance and free radical's generations(Labat-Robert and Robert, 2014). To scavenge excessive free radicals and maintain homeostasis, at the same time to prevent or delay diseases conditions, consumption of antioxidants become necessary.

Since, synthetic antioxidants to some extent have toxic effects, the uptake of natural antioxidants from natural sources becoming the first choice (*Papas, 1990*).Natural antioxidants not only play an important role in the prevention and treatment of disease conditions but also can avoid the adverse reactions caused by the ever increasing stress condition to human health (Liguori, et al., 2018)

Though oxidation reactions are essential for life, they can also be injurious. Inadequate levels of antioxidants or functioning of antioxidant enzymes, can cause oxidative stress and can result in damage to cellular components, including proteins, lipids and nucleic acids (Blokhina, et al., 2003). Under normal situations, the endogenous antioxidants in cells control the damage caused due to reactive oxygen species. However, exposure to adverse factors, abiotic or biotic causes excess generation of reactive oxygen species that cannot be efficiently controlled by the body defense system. This leads to oxidative stress where there is an imbalance between reactive species production and its elimination by antioxidants defense system. (De Gara, 2010). Antioxidants are reducing agents that include thiols, ascorbic acid, or polyphenols molecules that prevents oxidation of other molecules by getting oxidized themselves. Plants and animals maintain a systems of antioxidants such as glutathione, vitamin C, vitamin A and vitamin E as well as oxidant neutralizing enzymes such as catalase, superoxide dismutase and various peroxidases. For delay and prevention of a variety of diseases, antioxidants are widely used as dietary supplements and have been shown to be used as adjuvant in number of diseased conditions as cancer, coronary heart disease and even altitude sickness (Aune, 2019). Aging is another health threat related to stress, that affects whole populations and also can lead to premature mortality. The deleterious effects of free radicals on proteins, nucleic acids, and fats as well as enhanced glycosylation of proteins and DNA are widespread during aging processes(Maynard et al., 2015).Most exogenous antioxidant comes from vegetable, fruits, spices and herbs. They are important in food as they contain antioxidant molecules such as Alpha-tocopherol, vitamin A, vitamin B, Vitamin C and glutathione and can be called as natural antioxidants. Synthetic antioxidants like dimethyl sulphoxide, butylated hydroxytoluene and butylated hydroxyanisole, etc can also be used. But natural antioxidants are always preferred over synthetic ones, because antioxidants derived from natural sources show less toxic side effects and work by several mechanisms (Lourenço,et al., 2019). Therefore, in search of an effective antioxidant from natural source we studied the antioxidant property of *Stevia rebaudiana* leaves extract on human erythrocytes. Efforts were made to see its effect on the anti-oxidative stress related enzymes, superoxide dismutase (SOD) and catalase.

Material and Methods

Preparation of extract: Fresh leaves of *Stevia* plant were collected and dried under shade at room temperature. Dry powder of leaves was used for the extract preparation. Aqueouse thanol extract of *Stevia* leaves was prepared using the soxhlet apparatus following the methods of Da Porto et al. (Da Porto et al.,2016). In this process10 g grounded *Stevia* leaves were kept in extraction thimbles of the Soxhlet apparatus. Extraction was done for 12 hours at a maximum temperature of 70^0 C. After extraction, the residual solvent was removed at 50^0 C under reduced pressure using a vacuumdesiccator.

Isolation of erythrocytes - The blood was centrifuged at 4°C for 10 minutes at 3500 RPM to remove plasma and buffy coat, care was taken to eliminate leukocyte contamination. The isolated erythrocytes were washed with Krebs-Ringer phosphate buffer (KRPG buffer) (glucose 5mM, pH 7.4) in order to obtain completely washed packed erythrocytes, free of leukocyte contamination.

Treatment of isolated erythrocytes with extract - The packed erythrocytes were suspended in 4 volume of Krebs-Ringer phosphate buffer (KRPG buffer). Suspended erythrocytes were incubated with different concentration of *Stevia* leaves extract for 1 hour at 37°C. After incubation erythrocytes were washed with Krebs-Ringer phosphate buffer

(KRPG buffer) at 3500 RPM for 2-3 times and packed erythrocytes were obtained.

Preparation of Lysate -0.2 ml of packed RBC's was suspended in 3ml of Krebs-Ringer phosphate buffer (KRPG buffer, pH 7.4) and used for SOD whereas for catalase the above lysate was diluted 10 times with distilled water.

Estimation of Superoxide Dismutase (SOD):SOD was assayed according to the method Misra and Fridovich (1977) based on the ability to inhibit auto-oxidation of epinephrine to adrenochrome. The auto-oxidation of epinephrine in the solution at alkaline pH values, produce oxygen free radical, once formed precipitate in the oxidation of further molecules of epinephrine in a chain reaction to give rise to adrenochrome, which exhibits absorption maxima at 480 nm. Since SOD has the ability to inhibit this auto-oxidation at pH 10.2, thus inhibition can be measured spectrophotometrically at 480nm a per unit time of enzyme activity is equal to amount required to inhibit 50% of the above reaction in one min / mg protein.

To 0.25ml of lysate, 0.5ml carbonic buffer, 0.4ml EDTA (0.1mM), 0.25ml D.W. followed by addition of 0.1ml of epinephrine (0.3mM) just before taking the OD. Initial absorbance was noted at zero minute and readings were taken at every 30 seconds interval for 3 minutes at 480 nm using spectrophotometer against the blank. Protein content was estimated by Lowry's method.

SOD activity (unit/mg of protein)= ΔA_{480}/min x100xVolume of reaction mixture/mg protein.

Estimation of Catalase (CAT): Catalase activity in lysate was performed following the procedure of Beers and Sizer (1952). Catalase exerts a dual function because it catalyzes the following reaction.

$2H_2O_2 \longrightarrow 2H_2O+O_2$

$ROOH+AH_2 \longrightarrow H_2O+ROH+A$

In the UV range H_2O_2 shows a continued increase in absorbance with decreased wavelength. The decomposition of H_2O_2 can be followed directly by decrease in absorbance at 240nm. The difference in absorbance of H_2O_2 at 240nm per unit time is measure of catalase activity. To 1 ml of lysate, 1ml of phosphate buffer (50mM, pH- 7.4) followed by 1 ml of H_2O_2(30mM) just before taking the OD at 240 nm. The initial absorbance was taken at zero min followed by absorbance at every 30 second interval for 3 minutes at 480 nm. Protein concentration was estimated by Lowry's method.

Catalase (unit/mg of protein) = ΔA_{240} / minx Volume of reaction mixture x 100/ mg of protein

Results and discussion

Effect of *Stevia rebaudiana* leaf extract on SOD activity

The effect of SOD activity on different concentrations (0.01-100µg/ml) of *Stevia rebaudiana* leaves extract is shown in Figure-1. *Stevia rebaudiana* showed a significant decrease in SOD activity as compared to normal control up to a concentration of 10µg/ml of extract. *In vitro* treatment of RBC with *Stevia rebaudiana* leaves extract resulted in a dose dependent decrease in SOD activity with the increasing concentration of extract (0.01 - 10µg/ml).

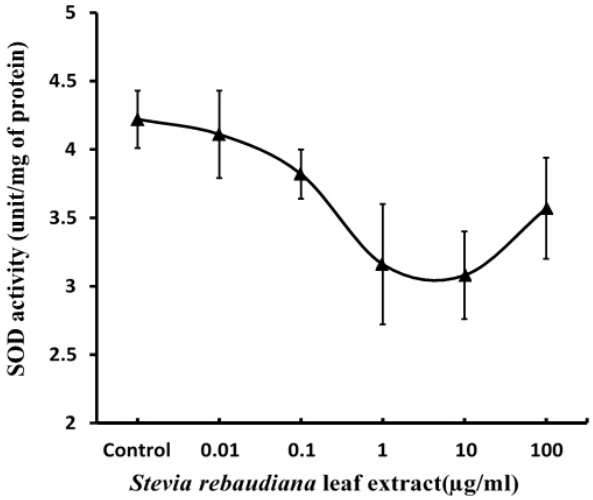

Figure -1

Figure-1 *In-vitro* effect of *Stevia rebaudiana* leaves extract on SOD activity of erythrocytes. Each value is the mean of at least 3-4 independent experiment. Values are expressed as mean ±S.D.

Our results further showed that treatment of erythrocytes with higher concentration of *Stevia*

rebaudiana leaves extract the effect of *Stevia rebaudiana* leaf extract on SOD activity started to reverse and became significantly increase at concentration of 100µg/ml, as compared to the maximum effect shown at 10 µg/ml extract.

Effect of *Stevia Rebaudiana* Leaf extract on Catalase activity

The effect of different concentrations (0.001-10µg/ml) of *Stevia rebaudiana* leaf extract on Catalase activity is shown in figure-2. *Stevia rebaudiana* leaf extract (0.01-.100µg/ml) showed a significant dose dependent decrease in Catalase activity as compared to normal control. Maximum effect was observed at a concentration of 1 µg/ ml. The effect of *Stevia rebaudiana* leaf extract at a concentration of 10µg/ml) started to decrease and catalase activity became significantly increased as compared to the maximum effect at 100 µg/ ml. Our results showed that erythrocyte catalase activity decreased with increasing concentration of *Stevia rebaudiana* extract up to a concentration of 10 µg/ml but further increase in *Stevia rebaudiana* extract at concentration **(**100µg/ ml.**)** resulted in increase in Catalase activity as compared to the maximum decrease at 1µg/ml.

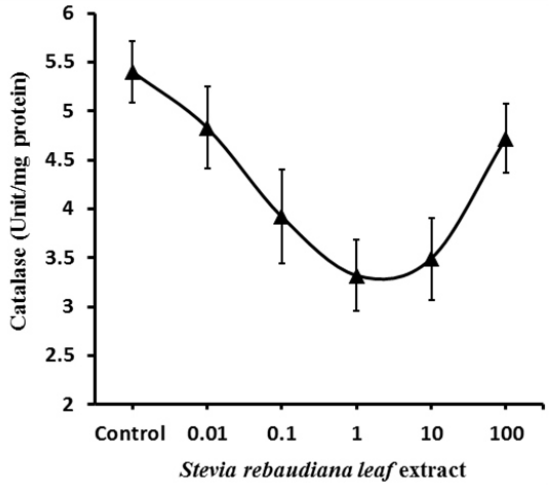

Figure-2

Figure-2 *In vitro* effect of *Stevia rebaudiana* leaves extract on erythrocytes catalase activity. Each value is the mean of at least 3-4 independent experiment. Values are expressed as mean± S.D.

ROS and other free radicals are nowadays being thought to be are involved in many disease conditions. ROS are produced in cells under normal physiological conditions and if left uncontrolled leads to formation of lipid peroxides and by subsequent chain reaction more free radicals are produced. Antioxidants help in eliminating these free radicals. Antioxidants minimize the action of ROS, compounds which act as antioxidants can trap the free radicals directly or scavenge them through a series of reaction coupled with anti-oxidative enzymes (Pizzino,*et. Al.*,2017).

We investigated the effect of Stevia leaf aqueous ethanolic extract on free radical scavenging enzymes Superoxide Dismutase and Catalase, the key component of the antioxidant defense mechanism. These scavenging enzymes play a significant role in removing of the ROS from the tissues. Superoxide Dismutase and Catalase provide a defense against the potential damaging action of superoxide and hydrogen peroxide. High Superoxide Dismutase activity in conjugation with catalase activity leads to increased level of hydrogen peroxide and hydrogen peroxide derived related species such as hydroxyl radical. Study proposes that the imbalance of Superoxide Dismutase and Catalase activity may play a role in initiating and propagating oxidative damage.(Maciejczyk,*et.al.*, 2019).Superoxide dismutase and Catalase provide a defense against the potential damaging action of superoxide and hydrogen peroxide.

Our results show a dose dependent protection of Superoxide dismutase and catalase activity upto a concentration of 10µg/ml extract. The decreased activity of CAT and SOD in our study could probably be associated with elevated exogenous antioxidant level, due to neutralization of peroxidant and free radical scavenging property of *antioxidant spresent in plants*(Prakash,*et al.*, 2007). The decreased activity of Catalase and Superoxide Dismutase observed in our study could probably be associated with elevated antioxidant status due to neutralization of peroxidant by antioxidant and free radical scavenging property of *Stevia rebaudiana* leaf (Moselhy,et al., 2016). At higher

concentration, of extract 100mg/ml the protective effect seems to be reversed towards the normal controlin the parameters studied,which could probably be associated with anti-oxidative stress and/or reduced antioxidant defense potential. This increase in activity of SOD and Catalase may be related to an increase in free radical levels due to adverse effect of high level of phytochemicals in *Stevia rebaudiana* leaf extract. Superoxide-producing lipoprotein fraction-have been reported in Stevia leaves (Isoyan,et al.,2019)

Conclusion

Indian folk medicine is replete with drugs purported to have anti-oxidative properties. The present study was undertaken to evaluate the antioxidants potential of *Stevia rebaudiana* leaf extract on red blood cells *in vitro.* Our results show a decrease in SOD and CAT activity was observed in *Stevia rebaudiana* leaf extract at concentration (0.01-10 µg/ml) as compared to the control probably due to the decrease in peroxidant mediated by antioxidant property of *Stevia rebaudiana* leaf (Milani,et al., 2017).

From our study we can conclude that the *Stevia rebaudiana* leaf extract can act as a potential antioxidant and protect the erythrocytes from oxidative damage by either directly scavenging the free radicals or activating other processes. This property of *Stevia rebaudiana* leaf may be due to various antioxidant compounds. But at higher concentration this protective effect starts to show a reversing trend probably at higher concentration. *Stevia* leaves extract itself may cause oxidative stress and /or generation of free radicals. Therefore, its indiscriminate use especially at higher concentration should be avoided. Further studies are needed to elucidate its mechanism of action and its cytotoxic property.

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