Withania Somnifera L Root Extract Ameliorates Toxin Induced Cytotoxicity

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Abstract

In this study the use of plants for medicinal purpose used locally in the treatment of various diseases and we examined for their antioxidant activity. Therefore, the present investigation is part of continuing programme related to the biochemical screening of local plants used in Ancient Indian Medicine, Ayurveda, Siddha and Yunani. An Aqueous root extract of *Withania Somnifera* (L.) Dunal (Ashwagandha) was evaluated for its protective effect (antioxidant effect) against Cypermethrin (CM) toxin induced oxidation in male albino rats. Cypermethrin, (CM) toxin [(RS)-3-phenoxybenzyl (IRS)-cis-trans-3-(2,2-dichlorovinyl)-2,2-dimethyl cyclopropane carboxylate] a Type II pyrethroid pesticide is commonly used in agriculture and many other domestic applications for pest control. Studies have shown that cypermethrin (toxin) induces lipid peroxidation and alters the antioxidant status in non-target organisms. Aqueous root extract of *Withania Somnifera* (L.) Dunal (Ashwagandha), a source of several flavonoids and steroids is a potent antioxidant. In our present study, an attempt has been made to study the effect of Cypermethrin (CM) toxin on biochemical parameters and ameliorating effect of *Withania Somnifera* (L.) Dunal (Ashwagandha) in male wistar rats. Adult male wistar rats were divided into four different groups. Group I Served as vehicle treated normal saline (Control), Group II Rats received intragastric tubing (Ig) injection of cypermethrin (CM) toxin1/10 LD50 (LD50 of CM is 250mg) (25mg/kgbw/day in corn oil), Group III received *Withania Somnifera* (10%) aqueous root extract (5ml of 10% extract per day, for 60 days) Group IV Rats received *Withania Somnifera* aqueous root extract alone (5ml of 10% per day for 60 days) treated. Cypermethirn (CM) toxin induction leads to reduction in the levels of Enzymic and Non-Enzymic antioxidants levels. However, on treatment with *Withania Somnifera* aqueous root extract normalized the levels of all the biochemical and hematological parameters. These findings highlight the efficacy of *Withania Somnifera* aqueous root extract as protective effects Cypermethirn (CM) toxin induced oxidative stress.

Key words: *Withania Somnifera* (L.) dunal aqueous root extract, SOD, CAT, LPO, GPx, GPT.

Introduction

Withania Somnifera (L.) Dunal is a well known and important medicinal plant widely used in several indigenous systems of medicine for the treatment of various ailments, viz. asthma, bronchitis, inflammatory diseases, ulcer and stomach problems. Steroidal lactones have been reported as the major phytoconstituents of this species. Different pharmacological experiments in a number of *in vitro* and *in vivo* models have convincingly demonstrated the ability of *W.somnifera* to exhibit anti-inflammatory, anti-oxidative, antimicrobial, anti-anxiety aphrodisiac, immunomodulation, anti-diabetic, anti-ulcer, anticancer, central nervous system depressant and hepatoprotective activities, lending support to the rationale behind several of its traditional uses.

Cypermethrin, a synthetic pyretheroid has become one of the most important insecticides in wide-scale use. In 1988, pyrethroids amounted to 40% of the sales for insecticides for cotton treatment in the world (cypermethrin 8%). Pyrethroids are synthetic analogs of pyrethrins belonging to non-systemic chemical group of insecticides. This group can be classified into two categories-Type I and II, depending on their structure, properties and mechanism of toxicity. Pyrethroids generally affect central and peripheral nervous system. Cypermethrin is a class II-moderately toxic, highly active and broad spectrum, non accumulative pyrethroid insecticide, which is effective in public health and animal husbandry, and targets a wide range of pests in agriculture. Pyrethroid pesticides are used preferably over organochlorine, organophosphates and carbamates due to their greater field stability, rapid metabolism and elimination from mammalian system, limited persistence in soil and greater potency [1]. Pyrethroids are divided into two types according to their chemical structure. Type I pyrethroid do not contain an alpha cyano group and cause mainly tremors (T syndrome). Type II pyrethroid contains an alpha cyano group and causes choreoathetosis and salivation (CS syndrome) [2]. Studies in rats show that CM is rapidly metabolized and over 99% eliminated within hours, but accumulation of cypermethrin and its fatty acid conjugates in adipose tissue, brain and liver of rats was reported [3]. Several studies have indicated that pyrethroids induce oxidative stress [4]. Antioxidants can ameliorate the oxidative stress induced by pyrethroids [5,6,7].

Oxidative Stress

The term oxidative stress is a state of unbalanced tissue oxidation refers to a condition in which cells are subjected to excessive levels of molecular oxygen or its chemical derivatives called reactive oxygen species (ROS). Under physiological conditions, the molecular oxygen undergoes a series of reactions that ultimately lead to the generation of superoxide anion (O_2-), hydrogen peroxide (H_2O_2) and H_2O. Peroxynitrite (OONO-), hypochlorus acid (HOCl), the hydroxyl radical (OH^-), reactive aldehydes, lipid peroxides and nitrogen oxides are considered among the other oxidants that have relevance to vascular biology. Mild, chronic oxidative stress may alter the anti-oxidant systems by inducing or repressing proteins that participate in these systems, and by depleting cellular stores of anti-oxidant materials such as glutathione and vitamin E [8]. Free radicals and other reactive species are thought to play an important role oxidative stress resulting in many human diseases. Establishing their precise role requires the ability to measure them and the oxidative damage that they cause [9]. Oxidative stress is involved in the process of aging [10] and various chronic diseases such as atherosclerosis [11], diabetes [12] and eye disease, whereas fruit and vegetable diets rich in antioxidants such as polyphenols, vitamin C, and carotenoids are correlated with a reduced risk of such chronic diseases [13]. An excessive amount of reactive oxygen/nitrogen species (ROS/RNS) leading to an imbalance between antioxidants and oxidants can cause oxidative damage in vulnerable targets such as unsaturated fatty acyl chains in membranes, thiol groups in proteins, and nucleic acid bases in DNA [14]. Revelation of the mechanism of action of antioxidants and their true antioxidant potential can lead to identifying proper strategies to optimize the antioxidant defense systems in the body.

Formation of Free Radicals

Normally, bonds don't split in a way that leaves a molecule with an odd, unpaired electron. But when weak bonds split, free radicals are formed. Free radicals are very unstable and react quickly with other compounds, trying to capture the needed electron to gain stability. Generally, free radicals attack the nearest stable molecule, gaining its electron. When the "attacked" molecule loses its electron, it becomes a free radical itself, beginning a chain reaction. Once the process is started, it can cascade, finally resulting in the disruption of a living cell. Some free radicals arise normally during metabolism. Sometimes the body's immune system's cells purposefully create them to neutralize viruses and bacteria. However, environmental factors such as pollution, radiation, and toxins can also spawn free radicals. Normally, the body can handle free radicals, but if antioxidants are unavailable, or if the free-radical production becomes excessive, damage can occur of particular importance is that free radical damage accumulates with age [15].

Antioxidants

To minimize the negative effects of ROS generated by any pro-oxidant, endogenous defensive mechanisms called antioxidant defense (AD) system, which utilizes enzymatic and non-enzymatic mechanisms. Antioxidants are naturally occurring substances that combat oxidative damage in biological entities. An antioxidant achieves this by slowing or preventing the oxidation process that can damage cells in the body. The antioxidant nutrients themselves don't become free radicals by donating an electron because they are stable in either form. They act as scavengers, helping to prevent cell and tissue damage that could lead to cellular damage and disease [16,17.18,19]. The body produces several enzymes, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSHPX), and glutathione reductase (GR) that neutralize many types of free radicals. Melatonin is a hormone secreted by pineal gland and proves to be powerful antioxidant and free radical scavenger [20].

Antioxidants are substances that neutralize free radicals or their actions [21].The effectors include various antioxidants like Vitamin C, Vitamin E glutathione, other thiol compounds, carotenoids flavonoids, etc., at the repair and reconstitution level, mainly repair enzymes are involved [21].

Materials and Methods

Chemicals

Cypermethrin (CM) toxin [(RS)-3-phenoxybenzyl (IRS)-cis-trans-3-(2,2-dichlorovinyl)-2,2- dimethyl cyclopropane carboxylate] of greater than 95% purity was obtained as a gift from IIBAT , Padappai, Chennai. W*ithania Somnifera* (Ashwagandha) root powder was procured as a gift from Siddhamaruthuva Salai, Vellore. All other chemicals used were of good quality and analytical grade.

W*ithania Somnifera L.* Root (Ashwagandha) Extract Preparation

Withania Somnifera L. (Ashwagandha) aqueous extract was prepared by weighing accurately 10grams of roots powder dissolved in 100ml of double distilled water (1/10 w/v). It was centrifuged at 4°C for 20 min at 4000 g the solution was stirred on a magnetic stirrer for one hour. It was then centrifuged and supernatant stored at -20 °C until use. We selected an aqueous extract because most of the antioxidant components of *Withania* root are extracted in water. During the experience, the aqueous *Withania* root extract was daily prepared and

administrated to rats. 10% *Withania* aqueous root extract was prepared by weighing 1g of root powder in 10ml of double distilled water.

Animal Model

Male albino rats of Wistar strain (200±10g) procured from Tamil Nadu University for Veterinary and Animal Sciences, (TANUVAS) Chennai, India were used for the study. Animals were fed with commercially available standard rat pelleted feed (M/s Pranav Agro Industries Ltd., India) under the trade name Amrut rat/mice feed and water was provided ad libitum. The rats were housed under conditions of controlled temperature (25±2°C) and acclimatized to 12-h light, 12-h dark cycle. Animal experiments were conducted according to the guidelines of institutional animal ethical committee.

Experimental Design

Seggregation of Groups

Experimental animals were divided into four groups of three rats each as follows.

Group I : Served as vehicle treated normal saline (Control).

Group II : Rats received intragastric tubing (Ig) injection of Cypermethrin toxin (25mg/kg bw) for 60 days.

Group III : Rats received Cypermethrin (CM) (25mg/kg bw) as in group II along with 10% W*ithania Somnifera* aqueous root extract (5ml/day) for 60 days by intragastric tubing.

Group IV : Rats received alone W*ithania Somnifera* aqueous root extract (5ml/day).

Collection of Samples for Biochemical Analysis

After the experimental period, the animals were anaesthetized by intra-peritoneal injection of phenobarbital sodium (30mg/kg body weight) and were sacrificed. Blood was collected in sterile tubes by sinocular puncture into heparinised tubes and plasma was separated by centrifugation at 2000 x g for 10 minutes.

Preparation of Haemolysate

After the separation of plasma, the buffy coat was removed and the packed cells (RBCs) were washed thrice with cold physiological saline. To determine the activity of RBC antioxidant enzymes, RBC lysate was prepared by lysing a known volume of RBCs with cold hypotonic phosphate buffer, pH 7.4. The haemolysate was separated by centrifuging at 3000 x g for 10 minutes at 2°C.

Biochemical Investigations

Determination of LPO, Enzymatic and Non Enzymatic Antioxidants

Lipid Peroxidation TBARS Species used to study lipid peroxidation (LPO) due to its sensitivity and simplicity. Plasma and RBC LPO were measured according to the method of Okhawa (1979). Superoxide dismutase (SOD) (EC 1.15.1.1) was determined by the method of Kakkar (1984). Catalase (CAT) (EC 1.11.1.6) was assayed colorimetrically by the method of Sinha (1972). Glutathione peroxidase (GPx) (EC 1.11.1.9) was measured by the method of Rotruck (1973). Glutathione S-Transferase (GST) (EC 2.5.1.18) activity was measured by the method of Habig (1974).

Statistical Analysis

Results from biochemical investigations were analysed using analysis of variance (ANOVA) and the group means were compared by Duncan's Multiple Range Test (DMRT). The results were considered statistically significant if "p" value was 0.05 or less.

Results and Discussions

Effect on Lipid Peroxidation (LPO) on Plasma and Erythrocytes

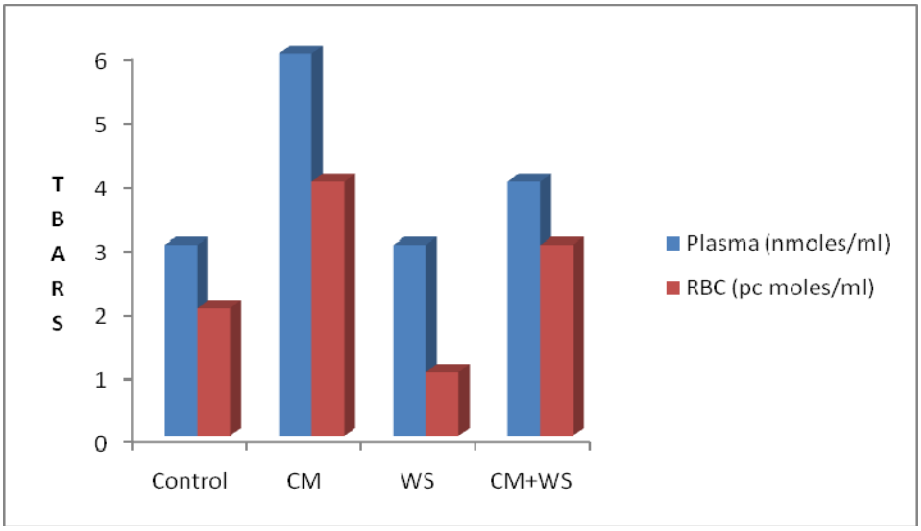
The TBARS levels in circulation and in tissues in different groups of our present study is shown in (Graph 1). Toxin treatment enhanced lipid peroxidation in circulation. Significantly higher TBARS ($p < 0.05$) was noticed in the Toxin treated rats when compared with the normal control. The LPO significantly reduced ($p < 0.05$) in the Toxin treated (CM) and *Withania Somnifera* (L.) treated rats when compared with the Toxin (CM) treated group.

Effect on Antioxidant Enzymes

The activities of antioxidant enzymes (SOD, CAT, GPx, GST) in control and experimental animals are shown in (Graph 2 & 3). The activities of all the enzymatic antioxidants, SOD, CAT, GPx and GST in erythrocytes was significantly lower in the ($p < 0.05$) Toxin (CM) treated rats when compared with the normal control. The activities of these enzymes were found be significantly higher ($p < 0.05$) in the Toxin (CM) and *Withania Somnifera* (L.) aqueous root extract treated rats when compared with the Toxin (CM) treated rats.

Our results of the present work clearly indicate that Toxin (CM) induced oxidative stress in circulation. Previous workers have shown the generation of ROS such as O_2^{o} and ^{o}OH and H_2O_2 resulting in oxidative stress with altered antioxidant status in pyrethroid treated rats [22]. Studies on toxin (CM) have indicated the generation of ROS, LPO and an increased oxidative stress [23]. The toxin (CM), a Type II pyrethroid pesticide containing an α cyano group [24], can decompose to cyanide and aldehydes, and forms lipophilic conjugates and aldehydes, which may also produce oxidative stress in pyrethroid toxicity [25]. Cypermethrin (CM) treated rats showed an increased plasma and RBC TBARS when compared with the normal control. Lipid hydroperoxides produced as a result of LPO, break down in biological systems, producing a great variety of aldehydes like malondialdehyde (MDA) and 4-hydroxynonenal (HNE) which may alter the structure, fluidity and permeability of erythrocytes increasing their sensitivity products are also reactive and highly cytotoxic [26]. Increased LPO may cause disintegration of biomembranes and subcellular organelles, decreases membrane fluidity leading to gross disturbance in cellular architecture [27]. Cpermethrin (CM) and toxin (CM) metabolites could be responsible for the increased LPO. The decreased TBARS in the plasma and RBC of CM + *Withania Somnifera* (L.) treated rats when compared to that of toxin (CM) treated group suggests that the *Withania* root extract counteracted the oxidative stress induced by toxin (CM) and reduced the LPO. The endogenous enzymatic and non enzymatic antioxidants scavenge the ROS and counteract the pesticide-induced oxidative stress. In the present study a significant decrease in SOD, CAT, GPx and GST in circulation in toxin (CM) treated rats when compared with the untreated group. SODs scavenge $O2^{o}$ by a rapid dismutation reaction and Catalase dismutates H_2O_2 to water and oxygen [27]. GPx catalyses the reduction of organic peroxides (ROOH) and transforms lipid hydroperoxides produced at the membrane level into less reactive species, hence it plays a major role in protecting the plasma membrane from LPO [28]. A significant reduction in these enzymes in toxin (CM) treated rats show the depletion of enzymatic antioxidants in scavenging toxin (CM) induced reactive oxygen species. Treatment with deltamethrin at 150 mg/kg body weight (1/10 of LD50) for 30 days in Wistar rats showed altered hepatic antioxidant status with activities of SOD, CAT (Fig 2) and reduced GSH content [29]. Thus, the study shows that in toxin (CM) treated rats the oxidative status of the system is imbalanced as indicated by enhanced LPO and depleted antioxidant status. In toxin (CM) Cypermethrin+*Withania Somnifera* (L.) aqueous root extract treated rats the LPO and the antioxidant status were reverted to near normal in circulation. The levels of TBARS decreased significantly and the activities of SOD, catalase, GPx and GST were close to the normal control. In the earlier studies have shown that antioxidants play a protective role in toxin (CM) induced toxicity by reducing the LPO and oxidative stress [30]. Pretreatment of rats with antioxidant like Vitamin E or allopurinol provided significant protection against the elevation of TBARS levels in cerebral and hepatic tissues, induced by cypermethrin [31]. Pretreatment with Vitamin E reduced the LPO in cypermethrin and fenvalerate treated rats [32]. Studies show that *Withania Somnifera* (L.) aqueous root extract have antioxidant properties. Treatment with *Withania Somnifera* (L.) aqueous root extract normalized the enhanced lipid peroxidation and increased susceptibility to oxidative stress due to the depletion of antioxidants in diabetic rats, while normal rats showed increased antioxidant status with decreased lipid peroxidation [33,34] have shown that the *Withania Somnifera* (L.) aqueous root extract are rich in phytochemicals and bioactive compounds.

In particular phytochemicals viz., withaferin A, withanolide A and withanolide D, Withanine, somniferine, somnine, somniferinine, withananine, pseudo-withanine tropane, pseudo-tropine, choline, anaferine, anahydrine, isopelletierine could be potent ^{o}OH radical scavengers due to the presence of active alkaloids and steroidal lactones groups. Our study clearly suggests that *Withania Somnifera* (L.) aqueous root extract has potent antiradical and antioxidant properties, attributed to the bioactive compounds like alkaloids and steroidal present in aqueous root fraction of *Withania Somnifera* (*L*.), which is responsible for amelioration of toxin (CM) induced toxicity and oxidative stress in circulation.



Graph 1. Levels of TBARS in plasma (n moles/ml) and RBC (p moles/ml) in rats treated with CM (toxin) (25 mg/kg body weight) and 10% WS for 60 days. Values are mean±S.D, n=6. (a) As compared with Group I (Normal control), (b) As compared with Group II (CM Treated), (*p < 0.05).

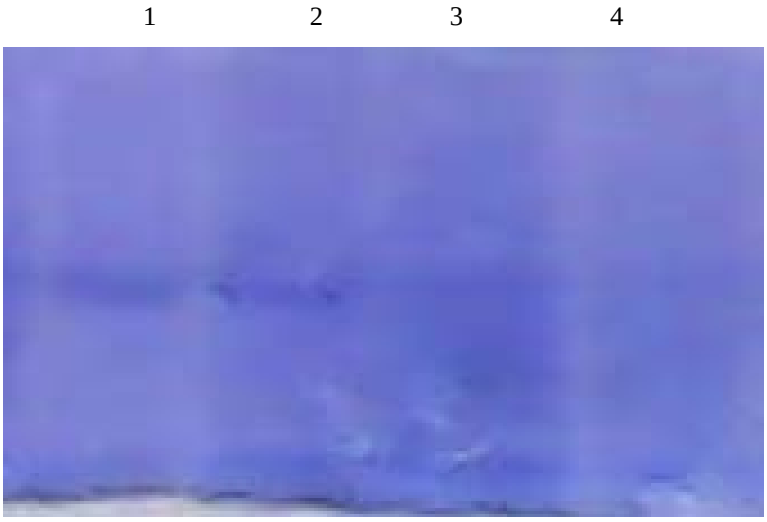
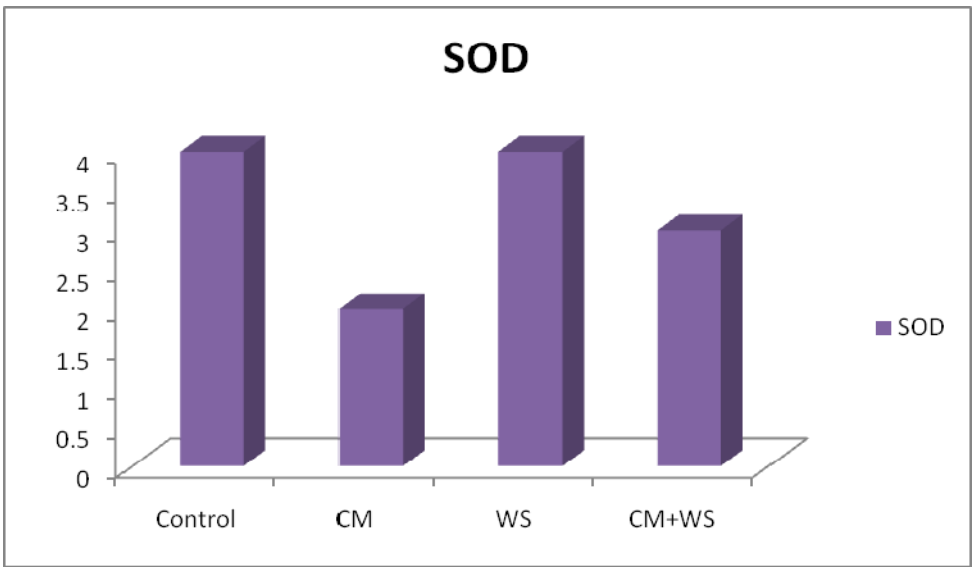
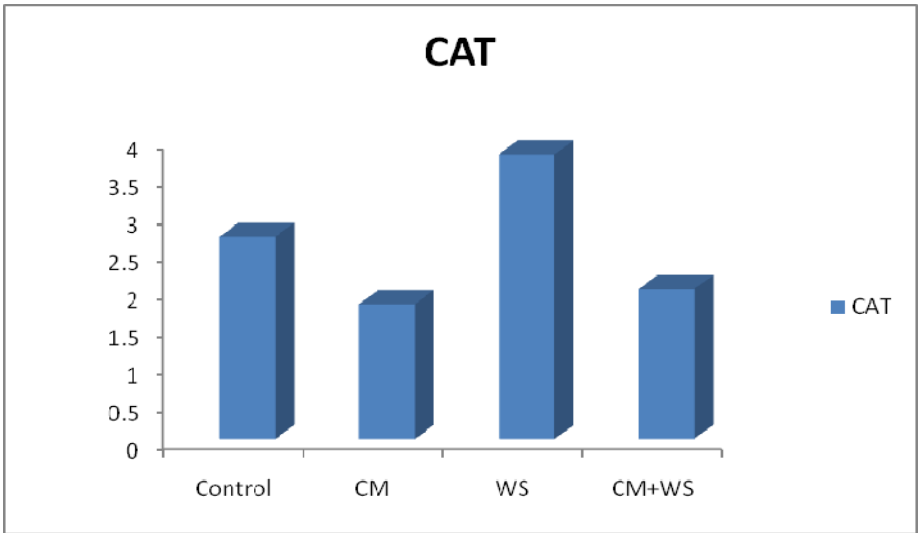
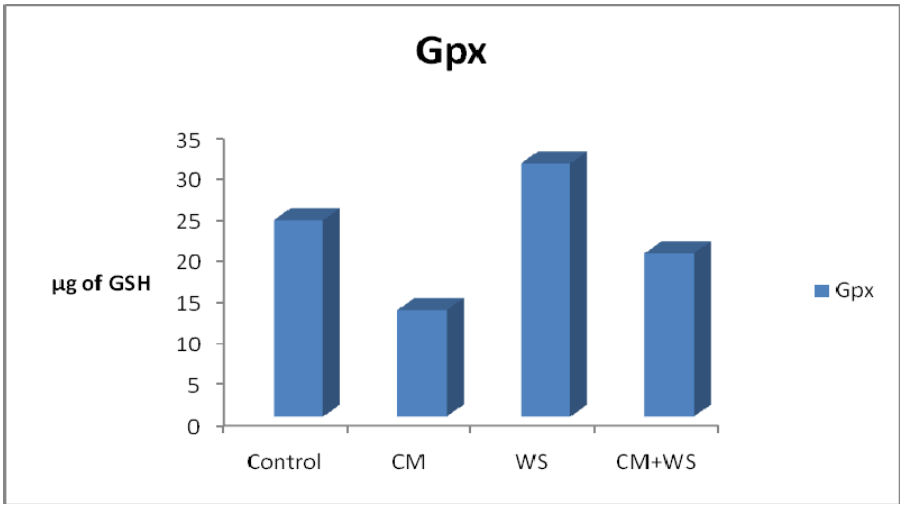


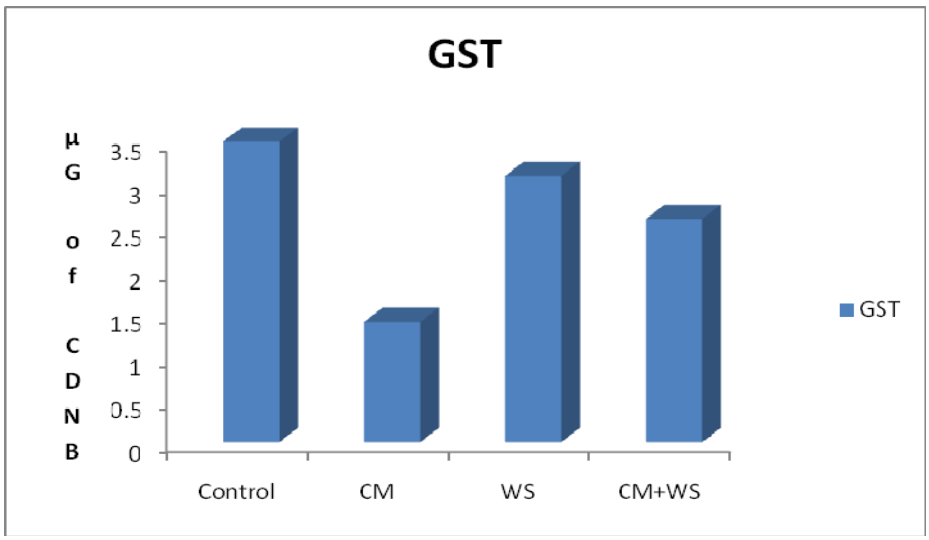
Fig 1. Effect of Cypermethrin and *Withania Somnifera L* Dunal aqueous root extract on the SDS PAGE pattern of the blood plasma of control and experimental groups (Lane 1,2,3 & 4).





Graph 2. Enzymatic activity of SOD [enzyme required for 50% inhibition of NBT (Nitro blue tetrazolium formazan) reduction], CAT (µM of H_2O_2 utilised/mg of protein) in RBC in rats treated with CM (25 mg/kg body weight) and 10% WS for 60 days. Values are mean±S.D, n=6. (a)Compared with Group I (Normal control) (b) Compared with Group II (CM Treated); (*p < 0.05).





Graph 3. Enzymatic activity of Gpx (µg of GSH utilized/min/mg of protein), GST [µM of CDNB (1-chloro-2,4-dinitrobenzene) conjugate formed/min/mg of protein] in RBC in rats treated with CM (25 mg/kg body weight) and 10% WS for 60 days. Values are mean±S.D, n=6. a as compared with Group I (Normal control); b as compared with Group II (CM Treated); (*p < 0.05).

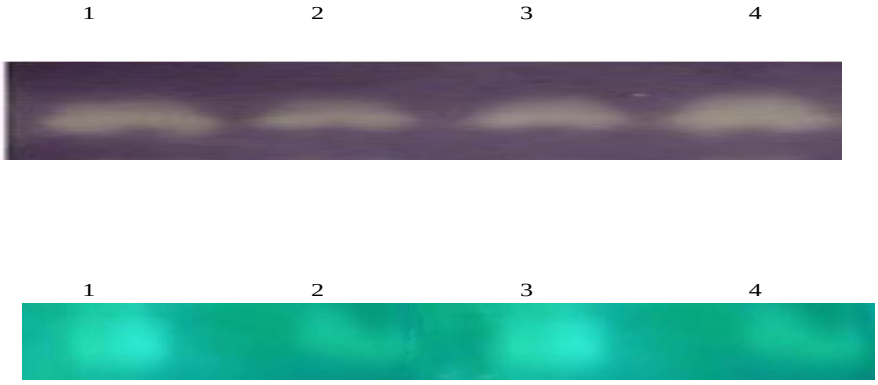


Fig 2. Effect of Toxin and *Withania* on the Native gel electrophoresis (SOD & CAT) pattern of the blood control and experimental groups

Summary

The protective effect of *Withania Somnifera* (L.) dunal aqueous root extract on (CM) Cypermethrin (toxin) induced pesticide poisoning in male wistar rats model was studied. Oxidative stress was proposed to be an important/vital reason for the pesticide induced activity. Hence our work was designed to biochemically evaluate the protective effect of *Withania Somnifera* (L.) dunal (Ashwagandha) aqueous root extract against Cypermethrin (toxin) induced pesticide toxicity by assessing the Biochemical parameters, Antioxidant Enzymes status, Non-Enzymatic antioxidant Enzymes, Lipid peroxidation, proteins etc., Our results of the study are summarized as follows: Exposure to cypermethrin (toxin) significantly decreased the serum and plasma antioxidant enzymes like superoxide dismutase, catalase, glutathione peroxidase, glutathione-Stransferases & glutathione reductase and non enzymic antioxidants like reduced glutathione, vitamin C & vitamin E, and the activities of *Withania Somnifera* (L.) dunal aqueous root extract treatment improved the antioxidant status by increasing the activities/levels of these enzymic and non-enzymic antioxidants. Exposure to cypermethrin (toxin) showed significant increase in the activities of marker enzymes such as SOD, CAT, (Fig 2), LPO (TBARS) species GPx, GPT in serum and a subsequent decrease in these enzyme activities in serum and plasma after supplementation of *Withania Somnifera* (L.) dunal aqueous root extract. The electrophoretic pattern of proteins by SDS-PAGE also showed the protective role of *Withania Somnifera* (L.) dunal aqueous root extract on cypermethrin (toxin) induced protein fragmentation. Along with biochemical and molecular alterations. Biochemical studies revealed alterations of the serum, plasma, Biochemical parameters, Antioxidant Enzymes status, Non-Enzymatic antioxidant Enzymes, Lipid peroxidation, proteins etc., caused by cypermethrin (toxin) induced were prevented by *Withania Somnifera* (L.) dunal aqueous root extract administration.

Conclusion

Withania Somnifera (L.) aqueous root extract ameliorates cypermethrin (toxin) induced toxicity by counteracting LPO and restoring antioxidant status. This may be due to the presence of various flavonoids and bioactive components which could function as antioxidant and antitoxic components. In conclusion that toxin, cypermethrin (CM) mediates circulatory toxicity by enhancing the oxidative stress. *Withania Somnifera* (L.) aqueous root extract ameliorates cypermethrin induced toxicity by counteracting LPO and restoring antioxidant status. This may be due to the presence of various flavonoids which could function as antioxidant and antitoxic components being easily affordable, on regular consumption can impart significant protection from pesticide induced toxicity. The emphasis of the future studies will be characterization and identification of the active constituents of the aqueous extract of *Withania Somnifera* (L.) for drug formulation in near future.

Our studies showed that lipid peroxidation, an index of oxidative stress, significantly increased in circulation in cypermethrin (toxin) treated rats. The activities of SOD, CAT, (Fig.2), GPx, GST in RBCs also decreased concomitantly in the cypermethrin (toxin) treated rats. Treatment with *Withania Somnifera* L. Dunal (Ashwagandha) aqueous root extract brought the values to near normal showing that *Withania* ameliorates cypermethrin-induced oxidative stress. Our findings demonstrate the potent antioxidant properties of *Withania Somnifera* (L.) dunal.

Acknowldegement

We are thankful to the faculty members of the PG & Research Department of Biochemistry for their appreciativeness and making the necessary facilities available for the research. The authors would like to thank the Secretary & Correspondent, Principal of K.M.G college Gudiyattam for their encouragement, providing the necessary facilities and support in carrying out the work.

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