ISSN: 2321-3760

Research Article

International Journal of Current Trends in Pharmaceutical Research

IJCTPR, 2014: Vol. 2(1): 298-303

www.pharmaresearchlibrary.com/ijctpr

Protective effect of *withania somnifera* on 1, 4-dioxane and trichloro ethylene-induced changes in catalase activity in erythrocytes of *in-vitro* goat haemic system

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Abstract: Today, numbers of chemicals have been registered for use in the USA and new are being introduced as agricultural chemicals, personal care products, industrial chemicals etc. The atmosphere is being polluted frequently with varieties of chemical substances daily during their manufacture, distribution, use and disposal. The chronic exposure of animals and humans to toxic environmental pollutants is a global health concern. 1,4-dioxane and trichloroethylene are the two of such pollutants which affects can vital functions of human and animal body. The present study reveals the effects of 1,4-Dioxane and Trichloroethylene on catalase present in erythrocytes and assess the protective effect of *Withania somnifera* root extract on catalase level and activity of erythrocytes of in-vitro goat haemic system.

Key words: *Withania somnifera*, 1,4- Dioxane, Trichloroethylene, Catalse

Contents

Received 12 December 2013
Accepted 5 January 2014
Available Online 9 January 2014

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Manuscript ID: PRL2014-IJCTPR1923

PAPER-QR CODE

1. Introduction

Our atmosphere is being polluted frequently with varieties of chemical substances daily during their manufacture, distribution, use and disposal. Today more than 80,000 chemicals are registered for use in the USA and new are being introduced as agricultural chemicals, personal care products, industrial chemicals etc. Thus chronic exposure of animals and humans to toxic environmental pollutants is a global health concern. 1,4-dioxane and trichloroethylene are the two of such pollutants which affects can vital functions of human and animal body[1,2]. 1,4-

dioxane is a colorless liquid with a mild ether-like odor, used as a solvent and in textile processing, detergent preparations. 1-4-Dioxane is also found in ordinary household products like shampoos, soap, baby lotion, hair lotions, manufactured food additives and some condiments.[3] Barber described dioxane exposed factory workers, some of them exhibited signs of liver changes, increased urinary protein and increased white blood cell counts, and some of them died from apparent acute exposures.[4] Due to accidental or voluntary ingestion of Roundup (Glyphosate) contaminated with dioxane were induced red blood cell destruction, low blood pressure and kidney failure or damage[5,6]. Trichloroethylene, C_2HCl_3, is a man-made, colorless liquid with a sweet odor, widely used as a solvent in the industrial degreasing of metals, with secondary solvent uses in adhesive paint and polyvinyl chloride production.[1,7] Trichloroethylene is used as a solvent in the textile industry, as a solvent for adhesives and lubricants, and as a low-temperature heat transfer fluid.[8] Exposure to trichloroethylene can potentially affect a number of organs and systems. Inhalation or ingestion of very high concentrations can lead to loss of consciousness and death.

***Withenia somnifera*[2]**

Family	:	Solanaceae
Genus	:	Withania
Species	:	Withania somnifera (L.) Dunal
Common name	:	Ashwagandha, English : Winter cherry

Figure 1: (a) Ashwaghandha plant; (b) Ashwaghandha root

Ashwagandha, that is *Withania somnifera* L. (Solanaceae), is an Ayurvedic medicinal plant which is popular as a home remedy for several diseases and human requirements.[9,10] The major biochemical constituents of ashwaganda root are steroidal alkaloids and steroidal lactones in a class of constituents called withanolides. At present, 12 alkaloids, 35 withanolides, and several sitoindosides from this plant have been isolated and studied. Much of ashwaganda's pharmacological activity has been attributed to two main withanolides, withaferin A and withanolide D.[1,2] It is mentioned in Vedas as a herbal tonic and health food. Different investigators reported antiserotogenic, adaptogenic anticancer and anabolic activity, and beneficial effects in the treatment of arthritis, geriatric problems[11] and stress.[12]

Objectives

1. To evaluate the effects of 1,4-dioxane on erythrocytes' catalase level of *in-vitro* goat haemic system.
2. To evaluate the effects of Trichloroethylene on erythrocytes' catalase level of *in-vitro* goat haemic system.
3. To assess the protective effect of root extracts of *Withania somnifera* on 1,4-dioxane and Trichloroethylene-induced changes in erythrocytes' catalase level and activity of *in-vitro* goat haemic system.

2. Material and Method

The present study was carried to investigate the protective effect of *Withania somnifera* (Ashwagandha) root extract on 1,4-dioxane and trichloroethylene-induced changes in erythrocytes' catalase of in-vitro goat haemic system.

Chemicals and Reagents

All the chemicals of analytical grade, kits and enzymes used in the present study were obtained from reputed firms such as Sigma, Merck, Qualigens Fine Chemicals and Span Diagnostics.

Test Agents

Following agents were used for *in-vitro* experiments.

1. 1,4-Dioxane: (MW 88.11), 1.032 g/ml, Qualigens Fine Chemicals
2. Trichloroethylene (TCE): (MW 131.40), 1.462 g/ml, Qualigens Fine Chemicals.

The desired dilutions of 1,4-Dioxane were prepared in normal saline as dioxane is soluble in water and TCE was dissolved in dimethylsulphoxide (DMSO) and were used for *in-vitro* studies.

Plant Material and Preparation of Extracts[1,2]

The roots of *Withania somnifera* grown in natural habitat and purchased from an authorized local Ayurvedic medical shop, Bareilly and was authenticated from a botanist. The roots were cut into 1~2 cm pieces and shade dried inside the laboratory for 24 h at room temperature (28^0C -30^0C). These were finely powdered using an electrical grinder.

a) Aqueous extract:

The aqueous extract was prepared by cold maceration of 15 g of powdered root in 100 ml of distilled water for 7 days with intermittent shaking. The supernatant was decanted, filtered, evaporated and dried in rotary vacuum evaporator at 40^0C. The dried water extract (yield 1.0 g) designated as WS AQ was stored in refrigerator at 4^0C for use in subsequent experiment. For *in-vitro* experiments, the desired concentration of WS AQ was prepared by dissolving the residue in normal saline.

b) Methanolic extract:

The root powder 15 g was exhaustively extracted with methanol by soxhlet extraction. The methanolic extract was filtered and concentrated under negative pressure at 40^0C in the rotary vacuum evaporator. The dried methanolic extract (yield 1.5 g) designated as WS ME was stored in refrigerator at 4^0C for use in subsequent experiment. For *in-vitro* experiments, the desired concentration of WS ME was prepared by dissolving the residue in DMSO.

Collection of Blood samples

Six male healthy goats weighing about 17 – 21 kg were used in the study. Fresh blood samples were collected aseptically by jugular veinipuncturte using sterile 21G needle and syringe. Heparin (2mg/ ml blood) was used as an anticoagulant.

Separation of erythrocytes

The heparinized blood samples were centrifuged at 2000 rpm for 15 min. Plasma and buffy coat were removed. The resulting erythrocyte pellet was washed thrice with 0.15 M NaCl. Dilution of the packed RBC (33%) was made in phosphate buffer saline (PBS; pH 7.4). The washed erythrocyte pellets were suspended in PBS; pH 7.4 and kept at 4°C until further analysis. The 1:10 dilution of packed erythrocytes in PBS was used for the estimation of catalase. PBS was prepared by dissolving NaCl (8 g), KCl (0.2g), KH_2PO_4 (0.2 g) , Na_2HPO_4 (0.94 g) in distilled water of about 800 ml. The pH was adjusted to 7.4 and the final volume was made to 1 liter with distilled water.[2]

Effective concentration of test agents and WS root extracts

The effective concentrations of 1,4-Dioxane and TCE were found to be 1.0 and 1.5 mg/ml, respectively. The effective concentrations of WS AQ and WS ME were found to be 2.0 mg/ml and 1.0 mg/ml respectively and were employed for further *in-vitro* studies.

Experimental Protocol [1]

Fresh blood in anticoagulant was collected from six goats and distributed in different test tubes. The blood samples were incubated for 6 hr at 37^0C with test agents i.e. 1,4-Dioxane, TCE and WS root extract i.e. WS AQ, WS ME as mentioned below. At the end of exposure period, the blood samples were centrifuged at 2000 rpm for 15 min, plasma and erythrocyte pellet were separated and use for determination of various parameters.

Table 1: Experimental protocol for *in vitro* study in goat blood

Group	Treatment	Concentration in blood (5 ml)	Exposure Period
I	Control (DMSO)	0.1%	6 h
II	WS ME	1 mg/ml	6 h
III	WS AQ	2 mg/ml	6 h
IV	1,4-Dioxane	1 mg/ml	6 h
V	1,4-Dioxane + WS AQ	1 mg/ml + 2 mg/ml	6 h
VI	1,4-Dioxane + WS ME	1 mg/ml + 1 mg/ml	6 h
VII	TCE	1.5 mg/ml	6 h
VIII	TCE + WS AQ	1.5 mg/ml + 2 mg/ml	6 h
IX	TCE + WS ME	1.5 mg/ml + 1 mg/ml	6 h

n=6,

WS AQ: Aqueous extract, WS ME: Methanolic extract of *Withania somnifera* root

TCE: Trichloroethylene

Determination of changes in erythocytes's catalase level and activity [13]

Catalase was assayed in erythrocytes by spectrophotometric method as described by Bergmeyer (1983). Diluted

(1:10) of haemolysate was used for estimation of catalase.

Reagents

1) Phosphate buffer (50 mM; pH 7.0)
   (a) 50mM KH_2PO_4 -1.37 g/200 ml
   (b) 50 mM Na_2HPO_4 -1.42 g/200 ml
   The solutions (a) and (b) were mixed in 1: 1.5 (v/v) and the pH adjusted to 7.
2) H_2O_2 (10mM): 0.1 ml of 30% H_2O_2 was diluted to 100 ml in water. The solution was checked at 230 nm and the concentration was adjusted using the molar extinction coefficient of H_2O_2 (0.081/ mM/cm).

Procedure

In a test tube 2 ml phosphate buffer and 10 µl haemolysate (1:10 dilution) were added, and the contents were transferred to the cuvette. Adding 1ml of H_2O_2 directly into the cuvette started the reaction and the optical density was recorded at every 10 sec for 1 min at 240 nm against water bank.

Calculation

The activity of catalase is expressed as µM H_2O_2 utilized/ min/ mg of haemoglobin and calculated using the following formula.

$$= \frac{\text{OD / time}}{0.067} \times \frac{\text{Total volume of reaction mixture}}{\text{Volume of sample taken}} \times \frac{1}{\text{mg of haemoglobin}}$$

OD = Mean of difference between ODs at 10 sec intervals.

3. Results and Discussion

Result

The present *in-vitro* study was undertaken with the objective to assess the protective effect of *Withania somnifer*a root extract against the changes in level of catalse enzyme in erythrocytes induced by these two environmental contaminants.

A significant decline in levels of catalytic enzyme, catalase were observed in goat RBCs after *in-vitro* exposure to dioxane (40.65 ± 2.74 µM) or TCE (42.74 ± 2.56 µM) as compared to control (68.83±4.31 µM). The reduction in the values was about 40.95 and 37.91% respectively (Table 2 & Figure 2). WS ME and WS AQ did not alter the basal levels of catalase in goat RBCs. Dioxane-induced decreased catalse levels were significantly restored after simultaneous treatment with WS ME (66.08 ± 3.52 µM) and WS AQ (65.10 ± 3.52 µM) and the values were comparable to control and the decrease was by 4.0 and 5.42 % over control.

Table 2: Effect of 1,4-Dioxane, Trichloroethylene, *Withania Somnifera* root extract and their combination on Catalase in goat RBCs *in-vitro*.

Treatment	1,4-Dioxane (1 mg/ml) Catalase		Trichloroethylene (1.5 mg/ml) Catalase	
	µM H_2O_2 utilized/min/ mg Hb	Percent decrease over control	µM H_2O_2 utilized/min/ mg Hb	Percent decrease over control
Vehicle Control	68.83 ± 4.31[a]	—	68.83 ± 4.31[a]	—
Test compound	40.65 ± 2.74[b]	40.95 ± 2.46	42.74 ± 2.56[b]	37.91 ± 2.34
WS ME (1 mg/ml)	67.78 ± 4.58[a]	1.53 ± 0.06	67.78 ± 4.58[a]	1.53 ± 0.06
WS AQ (2 mg/ml)	67.49 ± 4.37[a]	1.94 ± 0.08	67.49 ± 4.37[a]	1.94 ± 0.08
Test + WS ME	66.08 ± 3.52[a]	4.00 ± 0.03	64.38 ± 4.12[ac]	6.46 ± 0.04
Test + WS AQ	65.10 ± 3.52[a]	5.42 ± 0.04	62.84 ± 4.21[c]	8.70 ± 0.06

Values (Mean ± SEM, n=6) bearing different superscript in the same column differ significantly (P<0.05) in Duncan multiple comparison post hock test.

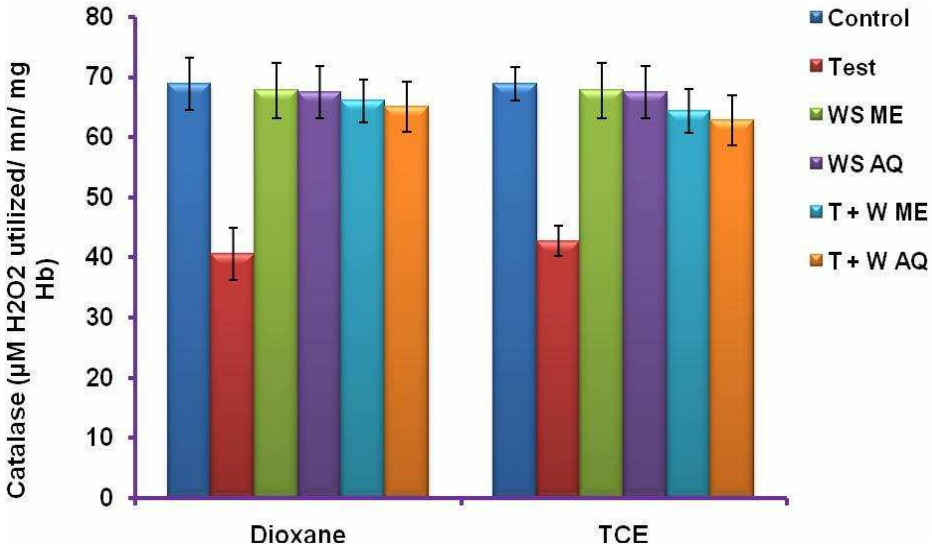

Figure 2: Effect of 1,4-Dioxane, Trichloroethylene, *Withania Somnifera* root extract and their combination on Catalse in goat RBCs *in-vitro*.

TCE-induced decrease in catalase levels were significantly increased and comparable to control value after co-exposure with WS ME (64.38 ± 4.12 µM) and WS AQ (62.84 ± 4.21 µM). However, the levels were still lower than control by 6.46 and 8.70 %, respectively. These results suggest that protective effect of WS ME is greater than as produced by WS AQ against dioxane or TCE-induced reduction in catalase activity of goat RBCs *in-vitro*.

Discussion

Catalase is ubiquitously present in a wide range of aerobic cell types, with the highest activities in mammals being found in liver, kidney and red blood cells.[14] It is found as a soluble protein in erythrocytes, where it play a crucial role in protecting red blood cells against oxidative damage. In the present study, the red blood cells incubated with either dioaxne or TCE resulted in significant decrease in catalase *in vitro*, reflecting impaired catalase activity in erythrocytes which implies Fenton-reaction-mediated conversion of more H_2O_2 to the ultimate toxicant, the OH•. The introduction of WS extracts protects from the changes in catalase. The anti-peroxidative and antioxidant action of *W. somnifera* as observed in the present investigation could be attributed to Withanolides (Sitoindosides VII-X) and Withaferin A (Glycowithanolides) present in WS extract.[15] Besides, the presence of other potential sources of antioxidant compounds such as polyphenols, flavonoids and alkaloids, vitamin- C can attribute to the antioxidant efficacy of *W. somnifera*.[16]. From the results of the present study it could be concluded that *in vitro* exposure of goat blood to 1,4-dioxane and TCE can alter the biochemical parameters, induce oxidative imbalance by reducing antioxidant enzymes' activities. *Withania somnifera* root extract has a potential protective/ ameliorating effect against dioxane/ TCE-induced oxidative stress.

4. Acknowledgement

The Authors of this research articles are heartily thankful to their guide and principal to provide all necessary help to construct such a fruitful work. The authors are greatly thankful to management and staff of reputed firms such as Sigma, Merck, Qualigens Fine Chemicals and Span Diagnostics for providing necessary chemicals, reagents, kits etc.

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