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PROTECTIVE EFFECT OF ROOT EXTRACT OF *WITHANIA SOMNIFERA* ON 1,4-DIOXANE AND TRICHLOROETHYLENE-INDUCED CHANGES ON *IN-VITRO* GOAT HAEMIC SYSTEM

Neetu Bharti[1], Poonam Garg[1], D.P.Singh*[2], Dilip K.Singh[3], Alok Upadhyay[3], Ankit Saini[4]

[1]Bareilly College, Bareilly, UP, India.
[2]Bhagwant Institute of Pharmacy Muzaffarnagar, UP, India.
[3]Ashoka Institute Technology and management, Varanasi, UP, India.
[4]Krishna College of Pharmacy, Bijnor, UP, India.

ABSTRACT

The environment is being contaminated/polluted daily with various chemicals from several industries during their manufacture, distribution, use and disposal. Thus chronic exposure of animals and humans to toxic environmental pollutants is a global health concern. Upon exposure to these chemicals like 1,4-Dioxane and Trichloroethylene, the vital organs and functions of the body are being affected. The Ashwagandha (*Withania somnifera L.*) is an Ayurvedic medicinal plant which is popular as a home remedy for several diseases would have some protective effect against these chemicals. The present study reveals the effects of 1,4-Dioxane and Trichloroethylene on goat haemic system *in-vitro* and assess the protective effect of *Withania somnifera* root extract against the biochemical changes induced by these two environmental pollutants.

Key words: *Withania somnifera* L., 1,4-Dioxane, Trichloroethylene, Goat haemic system.

INTRODUCTION

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. Our environment is being contaminated with such chemicals during their manufacture, distribution, use and disposal. Thus chronic exposure of animals and humans to toxic environmental pollutants is a global health concern.

1,4-dioxane is a colorless liquid with a mild ether-like odor. It is used as a solvent and in textile processing, printing processes and detergent preparations. 1,4-Dioxane is also present in ordinary household products, cosmetics and manufactured food additives (Sack and Steele,1989). Dioxane is absorbed by all routes of administration (HSDB, 1995). 1-4,dioxane has been reported to cause reproductive toxicity, teratogenic and mutagenic effect in animals or humans. Inhalation exposure of higher dose level (>5000 ppm), can cause irregular heartbeat, kidney and liver damage; fall in blood pressure and even death. Ingestion of 1,4-dioxane may cause moderate decrease in Hemoglobin and red blood cell counts (MSDS, 1997). Also IRAC (1999) has classified 1,4-Dioxane in group 2B(possibly carcinogenic to humans).

Trichloroethylene, C_2HCl_3, is a man-made, colorless liquid with a sweet odour that most people can detect at levels of about 100 parts per million (ppm). Also known as trichloroethene and often called TCE, this compound is moderately soluble in water. It is converted to phosgene gas and hydrogen chloride. Currently, it is widely used as a solvent in the industrial degreasing of metals, with secondary solvent uses in adhesive paint and polyvinyl chloride production (U.S. EPA, 1985). Trichloroethylene is present in many household products, such as spot removers, carpet cleaning fluids, typewriter correction fluids, and paint removers. Exposure to trichloroethylene can potentially affect a number of

Corresponding Author

Devendra Pratap Singh
Email: mdps1341619@gmail.com

organs and systems, including the nervous system, liver, kidney, blood, cardiovascular system, immune system, and reproductive system. Skin contact with trichloroethylene can cause rashes, while inhalation or ingestion of high concentrations for a short time primarily affects the central nervous system. The EPA is currently reviewing its carcinogenicity, and in a 2001 draft health assessment, characterized trichloroethylene as "highly likely to produce cancer in humans" based on the 1999 proposed (and now accepted) cancer guidelines and as a probable human carcinogen, based on the former 1986 cancer guidelines.

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 (Patwardhan *et al*., 1988; Sharma and Dandiya, 1991). It is mentioned in Vedas as a herbal tonic and health food. It is an official drug and is mentioned in the Indian Pharmacopoeia (1985). It is in use for a very long time for all age groups and both sexes and even during pregnancy without any side effects (Sharma *et al*., 1985).

Long term animal studies determining the toxicological risk of various compounds are time consuming and expensive to perform, ethical factor also play an important role. The development of simple, reproducible and reliable *in-vitro* test system for the quick screening of environmental is necessary. To our knowledge, the effects of 1,4-Dioxane and Trichloroethylene on haemic system and the oxidative stress of erythrocytes are yet to be evaluated. Hence the present study has been designed with the objective to assess the protective effect of root extract of *Withania somnifera* on 1,4-dioxane and Trichloroethylene-induced changes on *in-vitro* goat haemic system.

Withania somnifera

Order	**:** Solanales
Family	**:** *Solanaceae*
Genus	**:** *Withania*
Species	**:** *Withania somnifera* (L.) Dunal

Common name: Ashwagandha **English:** Winter cherry

Withania somnifera L. belongs to the family solanaceae. It is a xerophytic plant, found in the drier parts of India, Sri Lanka, Afghanistan, Baluchistan and Sind and is distributed in the Mediterranean regions, the Canaries and Cape of Good Hope. It is found to possess antiperoxidative, anti-inflammatory, antitumour, antistress, cardioprotective, antioxidant, analgesic, immunomodulatory, haemopoietic, and rejuvenating properties. It is also one of the members of GRAS (generally regarded as safe) category of plants that can be used for therapeutic purposes.

Withania somnifera is an evergreen, erect, branching, tomentose shrub, 30-150 cm in height. Leaves are simple, ovate, glabrous, and up to 10 cm long. Flowers are greenish or lurid yellow, small about 1 cm long; few flowers (usually about 5) born together in axillary, umbellate cymes (short axillary clusters). Fruits are globose berries, 6 mm in diameter, orange red when mature, enclosed in the inflated and membranous persistent calyx. Seeds are yellow, reniform and 2.5 mm in diameter (Anonymous, 2007).

Chemical composition

The roots are reported to contain alkaloids, amino acids, steroids, volatile oil, starch, reducing sugars, glycosides, hentriacontane, dulcitol, withaniol, an acid (m.p. 280-283^{0}C decomp.), and a neutral compound (m.p. 294-296^{0}C).

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. A sitoindoside is a withanolide containing a glucose molecule at carbon 27. Much of ashwaganda's pharmacological activity has been attributed to two main withanolides, withaferin A and withanolide D. Withaferin A, a steroidal lactone is the most important withanolide isolated from the extract of the leaves and dried roots of *Withania somnifera*.

MATERIAL AND METHOD

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

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.

Chemical 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 Extract

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! ~30!). These were finely powdered using an electrical grinder.

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.

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.

Preliminary Photochemical Screening

The preliminary phytochemical analysis of both the extracts for presence of alkaloids, terpenoids, steroid, tannin, flavonoids was carried out using standard methods as describe by Kokate (1994) and Harborne (1998).

Experimental protocol

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.

Estimation of Parameters in Blood/ plasma

Haemoglobin

Haemoglobin (Hb) in blood/ haemolysate was estimated by cyanomethaemoglobin method spectrophotometrically as describe by Van-kampen and Ziglstra,1961.

Reagents

Ammonium hydroxide (0.007 N): 4 ml of ammonium hydroxide (Sp gr. 0.91, about 25 % purity) was added to 996 ml of distilled water and stored in amber coloured bottle.

Procedure

Five ml of 0.007N ammonium hydroxide was taken in a test tube and 0.02 ml of blood/ packed RBC was added and mixed well. 0.007 N Ammonium hydroxide was taken as blank and absorbance was measured at 578 nm.

Calculation

Hb (g/dl) = Abs_{578} X 26.3

Evaluation of erythrocyte osmotic fragility (EOF)

The erythrocyte osmotic fragility was evaluated using the method described by Faulkner and King (1970) as modified by Oyewale (1991). Briefly, freshly obtained heparinized blood samples from each rat was pipetted into the test tubes containing 0.0, 0.1, 0.3, 0.5, 0.7, 0.9 g/ dl of NaC solutionl (pH 7.4) and then followed by careful mixing and incubation for 30 minutes at room temperature, 26-28°C. The test tubes were then centrifuged at 2000 x g for 10 minutes using a centrifuge (REMI, R-8). The supernatant was transferred into a glass cuvette and the absorbance of the supernatant was measured at wavelength of 540 nm in a spectrophotometer (Beckman, DU 640, USA). The percent hemolysis for each sample was then calculated using the following formula:

$$\% \text{ Haemolysis} = \frac{\text{OD of test solution}}{\text{OD of standard solution}} \times 100$$

For evaluating the effect of dioxane and TCE on EOF, two concentrations of sodium chloride solution producing 25-50 % response (haemolysis) were selected for further experimentation.

Estimation of plasma protein

Plasma total proteins were estimated by the method of Lowry *et al.* (1951) using commercially available kit (Span Diagnostics, Ahmedabad, India).

Reagents

1. Reagent A: 2% Sodium carbonate in 0.1 N NaOH (2 gm Na_2CO_3 in 100 ml of 0.1 N NaOH).
2. Reagent B: 1% Copper sulphate (100 mg of $CuSO_4$

was dissolved in 10 ml distilled water and stored in amber colored bottle).
2. Reagent C: 2% sodium potassium tartrate (200 mg of sodium potassium tartrate was dissolved in 10 ml of distilled water).
3. Reagent D: (Alkaline copper sulphate): Prepared by mixing 49 ml reagent A with 0.5 ml of Reagent B and Reagent C just before assay.
4. Foline-Ciocalteau's phenol reagent (1N): 2 ml Foline-Ciocalteau's phenol reagent was diluted with 2 ml distilled water (prepared freshly).
5. Bovine serum albumin (BAS): 100 mg of BSA was dissolved in 100 ml of distilled water and used as standard.

Procedure

The reaction mixture consist of 20 µl tissue homogenate (10%), 980 µl distilled water and 2.5 ml alkaline copper sulphate was incubated for 10 min at room temperature. After this foline-Ciocalteau's phenol reagent was added and tubes were shaken immediately. Resultant mixture was again incubated for 30 min at room temperature and absorbance was read at 750 nm. For blank 100 µl distilled water was added instead of tissue homogenates. Ten different BSA standards were prepared by serial dilution from stock; further 100 µl was transferred from respective one into the reaction mixture and used for preparation of standard curve by plotting concentration vs. OD and values were expressed as mg/gm of wet tissue.

RESULT

The present *in-vitro* study was undertaken with the objective to evaluate the effect of Dioxane and Trichloroethylene on goat haemic system and to assess the protective effect of *Withania somnifer*a root extract against the biochemical and oxidative stress changes induced by these two environmental contaminants.

Preliminary Phytochemical Screening

Table 2 shows that the methanolic extract of Ashwagandha root was found to contain more number of phytochemicals than that of aqueous extract.

Effect on Parameters of Blood

Haemoglobin

Haemoglobin levels (gm %) after *in-vitro* exposure of goat blood to dioxane (1 mg/ml), or TCE (1.5 mg/ml) and WS root extract have been shown in Table 3A & 3B. There was significant reduction in Hb values after traetment with dioxane (07.56 ± 0.05) or TCE (8.42 ± 0.05) as compared to control (12.25). WS ME and WS AQ alone did not produce any change in haemoglobin values and were almost equal to contol. However, after co-exposure to dioxane / TCE with WS extracts, haemoglobin levels were normlized and were comparable to control values. Further, haemoglobin levels in WS ME group (with dioxane, 11.88±0.06 or TCE, 11.68±0.07 gm %) were higher than those observed in WS AQ group(with dioxane, 11.56±0.07 or TCE, 10.98± 0.06 gm%) although statistically there was no significnt difference between these two groups.

Erythrocytic Osmotic Fragility (EOF)

EOF was determined as an index of integrity of RBC membrane. EOF was first determined after incubation of blood with different concentrations of sodium chloride solution and per cent haemolysis observed are shown in Table 4.

Decreasing the hypotonicity of the NaCl solution, resulted in increase in haemolysis of the goat blood. It was observed that 25.32 ± 1.46 and 58.86 ± 4.68 per cent haemolysis was produced at concentration of 0.5 and 0.3 gm %, respectively. Hence, further EOF studies were conducted with dioxane/ TCE and WS extracts at these two concentrations.

EOF determined after exposure to either dioxane or TCE with WS ME and WS AQ has been shown in Table 5A & 5B. A significant increase in EOF was observed after *in-vitro* treatment with dioxane (42.68 ± 2.78) or TCE (38.46 ± 2.32%) as compared to control (25.42 ± 1.68%). WS ME and WS AQ alone did not produce any change in values and were almost equal to control. Co-treatment with WS ME & AQ restored the incresaed EOF values due to dioxane (26.82 ± 0.86, 27.26 ± 1.04 %) or TCE (27.62 ± 1.22, 28.35 ± 1.64 %) and were comparable to control values. Further, in combination gropus, the values with WS ME were more close to control group than WS AQ group indicating that there was more protection by WS ME extract than WS AQ extract.

Total Proteins

A significant decrease in protein levels (mg/dl) were recorded after *in-vitro* exposure of goat blood to dioxane (5.56 ± 0.03) or TCE (5.22± 0.04) alone. The values are shown in Table 6. Co-exposure with WS ME (Dioxane, 7.84±0.04, TCE 7.66±0.03 mg/dl) & WS AQ (Dioxane 7.54± 0.03, TCE 7.36±0.04 mg/dl) significantly restored the decreased values and was comparable to conrol. In case of WS ME it was observed that the increase in protein level was more as compared that in WS AQ, although statistically there was no differnce in these two groups.

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

Table 2. Phytochemicals present in *Withania somnifer*a root extract

Sr.No Class of Phytochemical	Methanolic Extract	Aqueous Extract
1.	Alkaloid	+
2.	Anthraquinone	-
3.	Coumarin	+
4.	Flavonoids	-
5.	Glycoside	+
6.	Protien	+
7.	Resins	+
8.	Reducing sugar	+
9.	Saponin	+
10.	Steroids	+
11.	Tannin	+
12.	Triterpenes	+

Further, the yeild of extract was found to be more in methanolic extract (10.0 gm %) as that of aqueous extract (6.66gm%).

Table 3(A). Effect of 1,4-Dioxane, *Withania Somnifera* root extracts and their combination on Haemoglobin *in-vitro*

Treatment	Hb (gm %)	Per Cent Control
Normal blood	12.25 ± 0.08^{a}	-
Vehicle Control	12.25 ± 0.06^{a}	-
Dioxane (1 mg/ml)	07.56 ± 0.05^{b}	61.71 ± 5.38
WS ME (1 mg/ml)	12.21 ± 0.04^{a}	99.67 ± 7.42
WS AQ (2 mg/ml)	12.20 ± 0.03^{a}	97.6 ± 7.34
Dioxane + WS ME	11.88 ± 0.06^{a}	96.97 ± 7.26
Dioxane + WS AQ	11.56 ± 0.07^{a}	94.36 ± 6.48

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

Table 3(B). Effect of Trichloroethylene, *Withania Somnifera* root extracts and their combination on Haemoglobin *in-vitro*.

Treatment	Hb (gm %)	Percent Control
Normal blood	12.25 ± 0.08^{a}	—
Vehicle Control	12.25 ± 0.06^{a}	—
TCE (1.5 mg/ml)	8.42 ± 0.05^{b}	68.73
WS ME (1 mg/ml)	12.21 ± 0.04^{a}	99.67
WS AQ (2 mg/ml)	12.20 ± 0.03^{a}	97.6 ± 7.34
TCE + WS ME	11.68 ± 0.07^{ac}	94.37
TCE + WS AQ	10.98 ± 0.06^{c}	89.63

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

Table 4. Erythrocyte Osmotic Fragility of goat blood in saline solution

Sodium Chloride solution (gm %)	Percentage Haemolysis
0.9	1.24
0.7	8.56 ± 0.68
0.5	25.32
0.3	58.86
0.1	96.88
0.0	100

Values expressed as Mean ± SEM, n=5

Table 5(A). Effect of 1,4-Dioxane, *Withania Somnifera* root extract and their combination on erythrocytic osmotic fragility determined at 0.3 and 0.5 % sodium chloride solution.

Treatment	Erythrocyte Osmotic	Fragility (% Haemolysis)
	0.5 % Nacl Solution	0.3 % Nacl Solution
Vehicle Control	25.42 ± 1.68[a]	58.48 ± 3.34[a]
Dioxane (1 mg/ml)	42.68 ± 2.78[b]	86.34
WS ME (1 mg/ml)	25.48 ± 1.11[a]	58.52
WS AQ (2 mg/ml)	24.84 ± 1.14[a]	58.66
Dioxane + WS ME	26.82 ± 0.86[a]	60.28
Dioxane + WS AQ	27.26 ± 1.04[a]	62.64

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

Table 5(B). Effect of Trichloroethylene, *Withania Somnifera* root extract and their combination on erythrocytic osmotic fragility determined at 0.3 and 0.5 % sodium chloride solution.

Treatment	Erythrocyte Osmotic	Fragility (% Haemolysis)
	0.5 % Nacl Solution	0.3 % Nacl Solution
Vehicle Control	25.42 ± 1.68[a]	58.48 ± 3.34[a]
TCE (1.5 mg/ml)	38.46 ± 2.32[b]	80.26
WS ME (1 mg/ml)	25.48 ± 1.42[a]	58.24
WS AQ (2 mg/ml)	24.84 ± 1.54[a]	58.66
TCE + WS ME	27.62 ± 1.22[ac]	62.36
TCE + WS AQ	28.35 ± 1.64[c]	64.88

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

Table 6. Effect of 1,4-Dioxane, Trichloroethylene, *Withania Somnifera* root extract and their combination on Total proteins in Blood plasma of goats *in-vitro*.

Treatment	1,4-Dioxane (1 mg/ml) Total Proteins		Trichloroethylene (1.5 mg/ml) Total proteins	
	(mg/dl)	Per cent control	(mg/dl)	Per cent control
Normal blood	8.14± 0.06[a]	—	8.14± 0.06[a]	—
Vehicle Control	8.10± 0.04[a]	—	8.10± 0.04[a]	—
Test compound	5.56± 0.03[b]	68.30 ± 5.44	5.22 ± 0.04[b]	64.12 ±5.32
WS ME (1 mg/ml)	8.09 ± 0.04[a]	99.38 ± 7.26	8.10 ± 0.04[a]	99.50 ±7.18
WS AQ (2 mg/ml)	8.10± 0.02[a]	99.50 ± 7.18	8.08± 0.02[a]	99.50 ±7.43
Test + WS ME	7.82± 0.04[a]	96.54 ± 7.65	7.66 ± 0.03[a]	94.56 ±6.86
Test + WS AQ	7.54± 0.03[a]	93.08 ± 7.36	7.36 ± 0.04[a]	90.86 ±6.78

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

Overall Summery of the Protective Effect of W. somnifera

Shown in the Table 7 & 8

Table 7. Amelioration/ Protection by *Withania somnifera* root extract against 1,4-dioxane-induced changes in various parameters

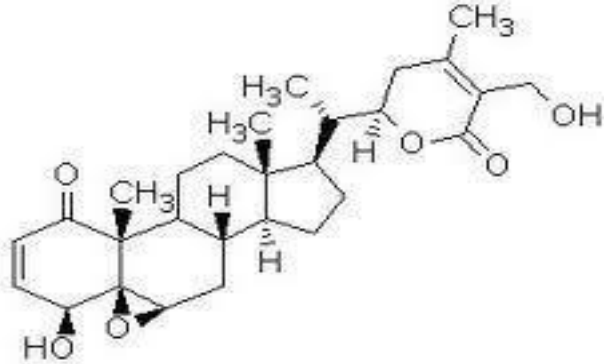
Parameter Evaluated	% Amelioration / Protection against Dioxane	
	WS Methanolic Extract	WS Aqueous Extract
Haemoglobin	92.11	80.00
Erythrocytic Osmotic Fragility		
At 0.3 % Nacl	93.54	85.06
At 0.5 % Nacl	91.88	89.33
Total Proteins	88.97	77.95

Table 8. Amelioration/ Protection by *Withania somnifera* root extract against Treichloroethylene-induced changes in various parameters

Parameter Evaluated	% Amelioration / Protection against Dioxane	
	WS Methanolic Extract	WS Aqueous Extract
Haemoglobin	85.11	66.84
Erythrocytic Osmotic Fragility		
At 0.3 % Nacl	82.18	71.53
At 0.5 % Nacl	83.12	77.53
Total Proteins	84.72	74.30

Fig 1. Different Views of *Withania somnifera*

Fig 2. Structure of Withaferin A



DISCUSSION

The present investigation study was undertaken with the objective to evaluate the effects of 1,4-Dioxane and Trichloroethylene on goat haemic system *in-vitro* and to assess the protective effect of *Withania somnifera* root extract against the biochemical changes induced by these two environmental pollutants. For this purpose goat blood/ erythrocytes were incubated with either dioxane or

trichloroethylene with or without *Withania somnifera* root extract (methanolic/ aqueous) for six hours *in-vitro*. At end of the exposure period various biochemical parameters were evaluated.

Extraction and phytochemical analysis

Withania somnifera roots were exhaustively extracted for preparation of methnolic and aqueous extracts. The yeild of methanolic extract (10.0 gm %) was found to be more as compared to aqueous extract (6.66 gm %). The phytochemical analysis (Table 2) of both the extracts revealed presence of important phytochemicals like alkaloid, steroids, triterpenes and tannins. However, presence of glycoside, saponin and resin were detected only in methanolic extract and not in aqueous extract. Thus the methanolic extract of Ashwagandha root was found to contain more number of phytochemicals than that of aqueous extract.

Withania somnifera is also rich in iron. The roots of *Withania somnifera* consist primarily of compounds known as withanolides, which are believed to account for its extra ordinary medicinal properties. Withanolides are steroidal and bear a resemblance, both in their action and appearance, to the active constituents of Asian ginseng (Panax ginseng) known as ginsenosides. Much of Ashwaganda's pharmacological activity has been attributed to two main withanolides, withaferin A and withanolide D. Senthil Kumar et al. (2011) performed the GC-MS analysis of alcoholic extract of *Withania somnifera* root and identified 21 bioactive photochemical compounds.

Parameters in blood

Hematological profile is an important diagnostic tool to assess the general health. In the present study effect of dioxane and TCE on parameters like haemoglobin, and osmotic fragility were evaluated.

Haemoglobin

A significant reduction in Hb value was observed after exposure to dioxane or TCE as compared to control. Few reports are available on the haematological effects of dioxane or TCE. According to early worker study, rats exposed to dioxane for two years either by inhalation (Torkelson *et al*., 1974) or through drinking water (Kociba, 1974) did not produce haematological changes. However, Moussa (2004) investigated effects of orally administered 1,4- Dioxane at 5.7mg/kg dose twice a week for 8 weeks on hematological parameters of the swiss albino mice and observed highly significant decrease in Hb values. The results of the present study with dioxane and TCE are in accordance with this report. The reduction in Hb as observed in the present study may be resulted from the decreased RBCs resulting from the toxicity by Dioxane/ TCE causing destructive effect on RBCs. Linman (1975) postulated that the destructive effect of the toxic substances on the erythrocytes directly increase the catabolism of hemoglobin.

In the present study it was observed that dioxane/ TCE-induced reduction in Hb was prevented after co-treatment with WS extracts and haemoglobin levels were normlized and were comparable to control values. The protective effect of WS ME and WS AQ against dioxane was 92.11 and 80.00 per cent, respectively. Similarly, against TCE the ameliorative effect was found to be 85.11 and 66.84, respectively. The protection cause by WS extracts could be due to its cytoprotective effect on RBCs preventing its destruction and catabolism of haemoglobin.

Erythrocytic Osmotic Fragility (EOF)

EOF is as an index of integrity of RBC membrane. It has been established that erythrocyte osmotic fragility (EOF) is a good indicator to evaluate stress due to transportation by road in goats (Minka and Ayo, 2010). Further, it is claimed that *in-vitro* haemolysis is a sensitive test to express degree of cytotoxicity. Therefore, EOF was evaluated in the present study at 0.3 and 0.5 % concentration of Nacl solution as 25-50 % response (haemolysis) was observed at these concentrations. The results of EOF indicated a significant increase in haemolysis (EOF) after *in-vitro* treatment with dioxane or TCE as compared to 0.3 and 0.5 % concentrations of Nacl alone.

This increase in EOF could be due to either direct toxic effect of dioxane or TCE on erythrocyte membrane causing destabilization of plama membrane leading to influx of water increasing the fragility and haemolysis. Or it could be due to formation of free radicals which in turn induce a reduction in membrane fluidity and increase the erythrocyte membrane fragility. Although free radicals were not measured in this study but the results showed that dioxane/ TCE exposure significantly increased lipid peroxidation with concurrent decrease in antioxidants.

The results of the present study indicated that the dioxane/TCE-induced increase in EOF (haemolysis) was prevented by co-exposure to WS root extracts. Against dioxane, WS ME and WS AQ caused 93.54 and 85.06 percent protection at 0.3% Nacl whereas it was 91.88 and 89.33 percent, respectively at 0.5 % Nacl solution. Similarly, in case of TCE the protection observed at 0.3% Nacl was 82.18 and 71.53, respectively and at 0.5% Nacl it was 83.12 and 77.53, respectively for WS ME and WS AQ. Further, it could be seen that the protective effect exerted by WS ME was more pronounced as compared that of WS AQ.

Total Proteins

In the present study a significant decline in the

levels of plasma protein was observed after *in-vitro* exposure of goat blood to dioxane or TCE. This effect may be a consequence of lipid peroxidation and proteolytic effect of dioxane/ TCE as discussed above. It has been shown that oxidative stress cause activation of proteolysis (Davis and Goldberg, 1987) Further, co-treatment with WS extract significantly restored the reduced levels of proteins. When incubated with dioxane the protection with WS ME was 88.97 percent and with WS AQ it was 77.95 percent. Similarly, percent amelioration against TCE-induced reduction in proteins was found to be 84.72 and 74.30 percent with WS ME and WS AQ, respectively.

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. *Withania somnifera* root extract has a potential protective/ ameliorating effect against dioxane/ TCE-induced biochemical alterations.

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REFERENCES

Davis KJ and Goldberg AL. Oxygen radical stimulate intracellular proteolysis and lipid peroxidation by independent mechanisms in erythrocytes. *J. Biol Chem.*, 262, 1987, 8220-8226.

Faulkner WR and King JW. Manual of Clinical Laboratory Procedures: Chemical Rubber Company, Cleveland, Ohio. 1970.

Harborne JB. Phytochemical Methods. A Guide to Modern Techniques of Plant Ananlysis. 3rd Edition, Thosmas Publishing IT, 1998, 5-21.

Hazardous Substances Data Bank, National Library of Medicine TOXNET network. Bethesda, MD, 1990.

Kociba RJ. Chronic toxicity study of dioxane in the drinking water of Sherman rats. Midland, MI: Dow Chemical Company, 1974.

Kokate CL. Practical Pharmacognosy. Vallabh Prakashan, New Delhi, 1994, 10.

Linman JW. Haematology, physiologic and clinical principles. Macmillan publishing Company, Inc. New York, 1975.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. Protein measurement with the folin phenol reagent. *J. Biol. Chem.*, 193, 1951, 265-75.

Minka NS and Ayo JO. Physiological responses of erythrocytes in goats to transportation and the modulatory role of ascorbic acid. *Journal of Veterinary Medical Science*, 72(7), 2010, 875–881.

Monographs on the Evaluation of Carcinogenic Risk to humans, Summaries and evaluations, International Agency for Research on Cancer, IRAC, Lyon, France. 71, 1999, 589.

Moussa EA. Effects of 1,4- Dioxane on Some Blood Parameters of the Swiss Albino Mice. *Scientific Journal of King Faisal University (Basic and Applied Sciences)*, 5, 2004, 249-261.

MSDS. Material Safety Data Sheet Hazardas Ingredients, No 4099-1464, Bacharach Inc, Pittsburgh, 1997.

Oyewale JO. Effects of temperature and pH on osmotic fragility of erythrocytes of the domestic fowl (*Gallus domesticus*) and guinea fowl (*Numida maleagris*). *Research in Veterinary Science*, 52, 1991, 1.

Patwardhan B, Panse GT and Kulkarni PH. Ashwagandha (*Withania somnifera*): a review. *Journal of National Integrated Medical Association*, 30, 1988, 7–11.

Sack T and Steele D. Indoor air pollution from household sources. EPA Contract No 68-02-4252. US. EPA, Washington, 1989.

Senthil Kumar M, Vinoth Kumar D, Saravana Kumar A, Aslam A and Shajahan A. The Phytochemical Constituents of *Withania somnifera* and *Withania obtusifolia* by GC-MS Analysis. *International Journal of Pharmacognosy and Phytochemical Research*, 3, 2011, 31-34.

Sharma K and Dandiya PC. *Withania somnifera* (Dunal): present status. *Indian Drugs*, 29, 1991, 247–250.

Sharma S, Dahanukar S and Karandikar SM. Effects of long-term administration of the roots of ashwagandha and shatavari in rats. *Indian Drugs*, 29, 1985, 133–139.

Torkelson TR, Leong BKJ, Kociba, RJ, Richter WA and Gehring PJ. 1.4-Dioxane. II. Results of a 2- year inhalation study in rats. *Toxicol. Appl. Pharmacol*., 30, 1974, 287-298.

U.S. EPA. Environmental Protection Agency. Assessment of trichloroethylene as a potentially toxic air pollutant; proposed rule. Federal Register, Part IV. 50(246), 1985, 52422-52425.

Van-kampen E and Zijlstra WG. Standardization of hemoglobinometry. II. The hemoglobin cyanide method. *Clin. Chim. Acta*, 6, 1961, 538-544.