

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

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### ABSTRACT

Our environment is being contaminated daily with varieties of 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 in lipid peroxidation in erythrocytes of goat haemic system *in-vitro* and assess the protective effect of *Withania somnifera* root extract against the changes in lipid peroxidation induced by these two environmental pollutants.

**KEY WORDS:** *Withania somnifera* L., 1,4-Dioxane, Trichloroethylene, Lipid Peroxidation

### INTRODUCTION:

Our environment is being contaminated with various 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. 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 odor 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. Exposure to trichloroethylene can potentially affect a number of organs and systems, including the nervous system, liver, kidney, blood, cardiovascular system, immune system, and reproductive 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.

### *Withania somnifera*

Order	:	Solanales
Family	:	<i>Solanaceae</i>
Genus	:	<i>Withania</i>
Species	:	<i>Withania somnifera</i> (L.) Dunal

**Common Name:** Ashwagandha

**English :** Winter cherry



Figure 1: Different Views of *Withania somnifera*

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 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.

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 protective effect of *Withania somnifera* (Ashwagandha) root extract on 1,4-dioxane and trichloroethylene-induced changes in peroxidation in erythrocytes of in-vitro goat haemic system.

#### **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.

#### **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 veinipuncture using sterile 21G needle and syringe. Heparin (2mg/ ml blood) was used as an anticoagulant.

#### **SEPARATION OF ERYTHROCYTES:**

The heparinised 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; Yagi *et al.*, 1989). The washed erythrocyte pellets were suspended in PBS; pH 7.4 and kept at 4°C until further analysis. This 33% packed RBC was used for the estimation of LPO. PBS was prepared by dissolving NaCl (8 g), KCl (0.2g), KH<sub>2</sub>PO<sub>4</sub> (0.2 g), Na<sub>2</sub>HPO<sub>4</sub> (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.

#### **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.

#### **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°C. The dried water extract (yield 1.0 g) designated as WS AQ was stored in refrigerator at 4°C 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°C in the rotary vacuum evaporator. The dried methanolic extract (yield 1.5 g) designated as WS ME was stored in refrigerator at 4°C for use in subsequent experiment. For *in-vitro* experiments, the desired concentration of WS ME was prepared by dissolving the residue in DMSO.

**EXPERIMENTAL DESIGN:****1. DETERMINATION OF EFFECTIVE CONCENTRATION OF TEST AGENTS:**

For determining the effective concentration, 1,4-Dioxane and TCE were added separately (from respective stock solutions) in test tubes containing 5 ml goat blood to get concentrations of 0.0, 0.05, 0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 5.0 and 10.0 mg/ml. For each concentration of the test agent, the samples were processed in quadruplicate. The blood samples were incubated at 37°C for 6 hr in a shaking water bath. At the end of exposure period, the blood samples were centrifuged at 2000 rpm for 15 min, plasma was separated and erythrocyte Lipid peroxidation was determined for each concentration of test agent. The detail of this procedure has been explained in subsequent section of this chapter. The concentration of each 1,4-Dioxane and TCE showing maximum LPO was employed for further in-vitro studies. The effective concentrations of 1,4-Dioxane and TCE were found to be 1.0 and 1.5 mg/ml, respectively.

**2. DETERMINATION OF EFFECTIVE CONCENTRATION OF WS ROOT EXTRACT:**

For determining the effective concentration of *Withania somnifera* root extract, 5 ml of freshly collected goat blood was taken in different test tubes to which

effective concentration of either 1,4-Dioxane or TCE were added (as determined in 3.5.1) and mixed properly. To these test tubes WS AQ and WS ME were added in such a way so as to get the final concentration of 0.0, 0.05, 0.1, 0.5, 1.0, 1.5, 2.0, 2.5 and 5.0 mg/ml. Different sets of tubes were used for each test agent with WS AQ and WS ME. For each concentration of WS extract, the samples were processed in triplicate. The blood samples were incubated at 37°C for 6 hr in a shaking water bath and then level of LPO in erythrocytes were determined as mentioned above.

The concentration of each WS AQ (2.0 mg/ml) and WS ME (1.0 mg/ml) exhibiting maximum protection against 1,4-Dioxane and TCE-induced LPO was employed for further in-vitro studies.

**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°C 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
Control (DMSO)	0.1%	6 h
II	WS ME	1 mg/ml
WS AQ	2 mg/ml	6 h
1,4-Dioxane	1 mg/ml	6 h
1,4-Dioxane + WS AQ	1 mg/ml + 2 mg/ml	6 h
1,4-Dioxane + WS ME	1 mg/ml + 1 mg/ml	6 h
TCE	1.5 mg/ml	6 h
TCE + WS AQ	1.5 mg/ml + 2 mg/ml	6 h
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

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 (nM) of MDA formed per ml packed RBC. determination of various parameters.

$$\frac{\text{OD of test}}{\text{EC}} \times \frac{\text{Total volume of reaction mixture}}{\text{Volume of sample of incubation (2 hr)}} \times 109 \times \text{DF} \times \text{time}$$

**ESTIMATION OF LIPID PEROXIDATION (LPO) IN ERYTHROCYTES:**

Membrane peroxidative damage in erythrocytes was determined in terms of malondialdehyde (MDA) production by the method of Shafiq-U-Rehman (1984).

**REAGENTS:**

- 1) Normal saline solution (NSS): Sodium chloride, 8.9 g was dissolved in one liter of distilled water.
- 2) Trichloroacetic Acid (TCA); 10% solution: TCA (10 g) was dissolved in distilled water and the volume was made up to 100 ml with distilled water.
- 3) Thiobarbituric acid (TBA); 0.67% solution: Prepared by taking 0.67 g of TBA in 100 ml of distilled water and warmed up for dissolving TBA.

**PROCEDURE:**

One ml of 33% packed erythrocytes was incubated at 37°C for 2 h. To each sample, 1 ml of 10% w/v TCA was added. After thorough mixing, the reaction mixture was centrifuged at 2000 rpm for 10 min. To 1 ml of supernatant 1 ml of 0.67 % w/v of TBA was added and kept in boiling water bath for 10 min, cooled and diluted with 1 ml of distilled water. Blank was made by adding all the reagents except the packed erythrocytes. The absorbance was read at 535 nm.

**CALCULATION:**

Calculation was done using the molar extinction coefficient (EC) of MDA-TBA complex at 535 nm, i.e., 1.56 x 108/M/cm. The amount of LPO is expressed as nanomole

DF = Dilution factor; 109 = nM

**RESULT:**

The present 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 somnifera* root extract against the changes in lipid peroxidation in erythrocytes induced by the two environmental pollutants.

**EFFECTIVE CONCENTRATION OF TEST AGENTS:**

Goat RBCs were treated in-vitro with different concentrations of 1,4-dioxane or trichloroethylene (TCE) and lipid peroxidation was used as an index to determine the effective concentration of test agents. Lipid peroxidation in terms of nM MDA formed/ ml of packed RBC at various concentrations of test agent has been shown in Table 2. It was observed that dioxane at a concentration of 1 mg/ml caused maximum increase in LPO (48.54 ± 2.04), whereas LPO levels were highest (46.44 ± 2.08) at a concentration of 1.5 mg/ml of TCE. There was no further increase in LPO even after increasing the concentration of the test agents up to 10 mg/ ml. Hence, for further in-vitro experimentation 1.0 and 1.5 mg/ml of dioxane and TCE respectively were employed.

**Table 2: Determination of effective concentration of test agents effect of 1,4-Dioxane/ Trichloroethylene on Lipid peroxidation\* of gaot RBCs in-vitro**

Test chemical concentration (mg/ml)	1,4-Dioxane	TCE
Vehicle Control	25.28 ± 1.34	25.28 ± 1.44
0.05	28.26 ± 0.86	27.26 ± 0.68
0.1	36.42 ± 1.22	34.21 ± 1.52
0.5	42.46 ± 1.36	40.62 ± 1.84
1.0	<b>48.54 ± 2.04</b>	42.34 ± 1.18
1.5	46.36 ± 2.24	<b>46.44 ± 2.08</b>
2.0	44.53 ± 2.22	45.39 ± 2.34

2.5	45.60 ± 2.33	44.57 ± 2.42
5.0	46.28 ± 2.42	43.58 ± 2.14
10.0	46.34 ± 2.07	43.45 ± 2.23

Values expressed as Mean ± SEM, n=4

\*LPO= nM of MDA formed/ ml of packed RBC

**EFFECTIVE CONCENTRATION OF WS ROOT EXTRACT:** which was increased by dioxane (27.65±1.32) and TCE (28.28±1.21), respectively. Similarly, WS AQ at 2 mg/ml produced maximum reduction in LPO. Hence, a concentration of 1 mg/ml of methanolic extract and 2 mg/ml of aqueous extract were employed for further in-vitro experimentation.

**Table 3: Determination of effective concentration of Withania somnifera root extract. Effect of different concentrations of Withania somnifera (WS) root extract on 1,4-Dioxane, Trichloroethylene- induced Lipid peroxidation\* of goat RBCs in-vitro.**

Conc. of WS root Extract (mg/ml)	1,4-Dioxane(1 mg/ml)		Trichloroethylene (1.5 mg/ml)	
	WS Methanolic	WS Aqueous	WS Methanolic	WS Aqueous
0.0	48.54± 2.14	48.54 ± 2.14	46.44± 2.08	46.44± 2.08
0.05	46.72± 2.18	47.08 ± 2.24	43.32± 2.13	46.26± 2.12
0.1	38.58± 1.88	42.20 ± 1.46	36.44± 1.23	45.34± 2.24
0.5	32.06± 2.30	44.38 ± 2.42	31.52 ± 1.62	42.18 ± 2.06
1.0	<b>27.65± 1.32</b>	36.88 ± 1.29	<b>28.28± 1.26</b>	39.35 ± 1.36
1.5	32.00± 1.86	30.50 ± 1.72	30.62± 1.34	34.46± 1.16
2.0	30.42± 1.34	<b>28.44 ± 1.21</b>	32.14± 1.40	<b>30.54± 1.09</b>
2.5	30.62± 1.43	30.46 ± 1.86	34.36± 1.33	33.41± 0.98
5.0	30.74± 1.64	32.54 ± 1.64	34.23± 1.27	34.51± 1.22

Values expressed as Mean ± SEM, n=4

\*LPO= nM of MDA formed/ ml of packed RBC

**EFFECT ON LIPID PEROXIDATION IN ERYTHROCYTES (RBCS):**

LPO was measured in terms of malondialdehyde (MDA) formed which is an end product of the LPO. There was significant increase in LPO of goat erythrocytes after *in-vitro* exposure to dioxane ( $48.64 \pm 2.34$  nM) and TCE ( $46.62 \pm 2.36$  nM) as compared to control ( $25.38 \pm 1.62$  nM). This increase was found to more with dioxane (91.64 %) as compared to TCE (83.68 %).

The data related to LPO induced by dioxane and TCE is presented in Table 4 and Figure 2, respectively. Co-exposure of goat RBCs *in-vitro* with WS ME resulted in attenuation of the increased levels due to dioxane ( $27.66 \pm$

$1.04$ nM) or TCE ( $27.74 \pm 1.06$  nM) which were comparable to control value but non-significantly higher than control i.e. by 8.98 and 9.29 %, respectively. Similarly, treatment with WS AQ also resulted in reduction in the LPO levels with dioxane ( $28.34 \pm 1.11$  nM) and TCE ( $29.43 \pm 1.32$  nM) which were 11.66 and 15.52 % more as compared to control. Thus WS ME appears to be more effective in protecting RBCs against dioxane/ TCE-induced LPO as compared to WS AQ. However, both the extracts had no effect on basal LPO (control) levels and are effective in counteracting xenobiotic induced LPO.

**Table 4: Effect of 1,4-Dioxane, Trichloroethylene, *Withania Somnifera* root extract and their combination on Lipid peroxidation (LPO) in goat RBCs *in-vitro*.**

Treatment	1,4-Dioxane (1 mg/ml) (LPO)		Trichloroethylene (1.5 mg/ml) (:PO)	
	nM of MDA formed/ ml of packed RBC	Percent Increase over control	nM of MDA formed/ ml of packed RBC	Percent decrease over control
Vehicle Control	$25.38 \pm 1.62^a$	—	$25.38 \pm 1.62^a$	—
Test compound	$48.64 \pm 2.34^b$	$91.64 \pm 6.83$	$46.62 \pm 2.36^b$	$83.68 \pm 5.78$
WS ME (1 mg/ml)	$26.10 \pm 0.80^a$	$02.83 \pm 0.26$	$26.10 \pm 0.80^a$	$02.83 \pm 0.26$
WS AQ (2 mg/ml)	$26.06 \pm 0.96^a$	$02.67 \pm 0.18$	$26.06 \pm 0.96^a$	$02.67 \pm 0.18$
Test + WS ME	$27.66 \pm 1.04^{ac}$	$08.98 \pm 0.14$	$27.74 \pm 1.06^{ac}$	$09.29 \pm 0.64$
Test + WS AQ	$28.34 \pm 1.11^c$	$11.66 \pm 0.86$	$29.43 \pm 1.32^c$	$15.52 \pm 0.86$

Values (Mean  $\pm$  SEM, n=6) bearing different superscript in the same column differ significantly (P<0.05) in Duncan multiple comparison post hoc t

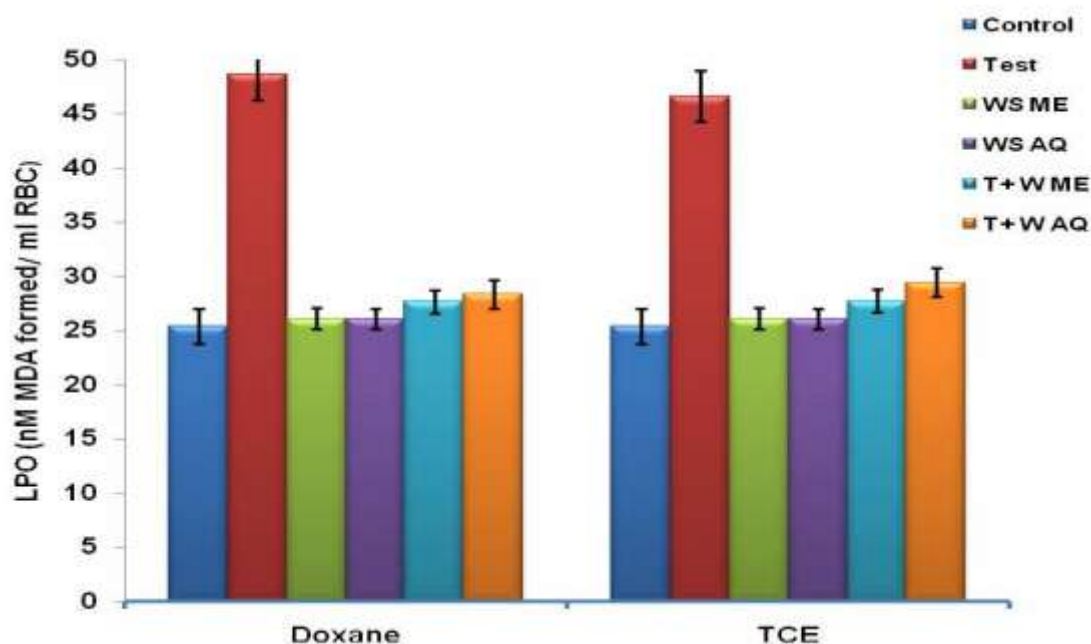


Figure 2: Effect of 1,4-Dioxane, Trichloroethylene, *Withania Somnifera* root extract and their combination on Lipid peroxidation (LPO) in goat RBCs *in-vitro*.

#### 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*.

#### LIPID PEROXIDATION:

The present study showed *in-vitro* exposure to dioxane and TCE resulted in increase in LPO in erythrocytes as evidenced by increased production of malondialdehyde (MDA). This increase was found to more with dioxane (91.64 %) as compared to TCE (83.68 %). Excessive production of free radicals or ROS is mainly responsible for peroxidation of cell membrane lipids, and malondialdehyde (MDA) is a terminal product of the lipid peroxidation process. Determination of MDA levels provides a good measure of lipid peroxidation, which is among the chief mechanisms of cell damage leading to necrosis or apoptosis. Elevated levels of MDA in dioxane and TCE treated erythrocytes indicative of oxidative damage.

Treatment of the affected RBCs with root extracts of *Withania somnifera* leads to significant protective effect in RBCs. WS ME appears to be more effective in protecting RBCs against dioxane/ TCE-induced LPO as compared to WS AQ. On the whole the potential source of active compounds present in the *W. somnifera* could have directly scavenged the free radicals, increased the

biosynthesis antioxidants, and reduced the degradation of antioxidants through the scavenging the excess amount of free radicals thereby increased bioavailability of the antioxidants under oxidative stress conditions. Thus could have potentiated the whole anti-oxidants system in red blood cells.

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 elevating MDA content (peroxidation). *Withania somnifera* root extract has a potential protective/ ameliorating effect against dioxane/ TCE-induced oxidative stress.

#### REFERENCE:

1. IRAC (1999). Monographs on the Evaluation of Carcinogenic Risk to humans, Summaries and evaluations, International Agency for Research on Cancer, Vol:71, IRAC, Lyon, France. p. 589.
2. Kokate, C.L. (1994). Practical Pharmacognosy. Vallabh Prakashan New Delhi. p. 10.
3. MSDS (1997). Material Safety Data Sheet "Hazardous Ingredients" MSDS, No,4099-1464, Bacharach Inc, Pittsburgh.
4. Patwardhan, B., Panse, G.T. and Kulkarni, P.H. (1988). Ashwagandha (*Withania somnifera*): a review. *Journal of National Integrated Medical Association* 30: 7-11.
5. Senthil Kumar M., Vinoth Kumar D., Saravana Kumar A., Aslam A. and Shajahan A. (2011). The Phytochemical Constituents of *Withania somnifera* and *Withania obtusifolia* by GC-MS Analysis. International

- Journal of Pharmacognosy and Phytochemical Research 3: 31-34.
6. Sharma, K. and Dandiya, P.C. (1991). *Withania somnifera* (Dunal): present status. *Indian Drugs* 29: 247-250.
7. U.S. EPA. (1985). U.S. Environmental Protection Agency. Assessment of trichloroethylene as a potentially toxic air pollutant; proposed rule. Federal Register; Part IV 50(246):52422-52425.