WITHASTEROID METAL ION CONJUGATES: THEIR NATURAL OCCURRENCE IN *WITHANIA SOMNIFERA* AND EFFECTS ON COLD-RESTRAINT STRESS IN MICE

Partha Ganguly[1*], Amartya K Gupta[1], Upal K Majumder[1], Shibnath Ghosal[2]

[1] Department of Pharmaceutical Technology,

Jadavpur University, Kolkata-700032.

[2] R & D Centre, Indian Herbs Ltd., Saharanpur-247001.

*Corresponding Author's contact id: partha.1p@gmail.com

Summary

The role of metal ions in conjugation with the withasteroids of *Withania somnifera*, on anti-stress activity in a cold restraint stress (CRS) model was investigated. A standardized *Withania somnifera* extract (WSE, 100 mg/kg, p.o. x 7 days, prior to stress, and 5 days treatment during stress), administered to Swiss albino mice**,** of either sex, produced significant anti-stress activity when the animals were subjected to CRS. WSE beneficently altered stress-induced changes in plasma corticosterone level in a significant way. WSE also altered the stress induced changes in the levels of blood glucose, WBC count, weight of the organs (liver and spleen), MDA levels, plasma antioxidant profile (ABTS and FRAP), and lipid profile. WSE/excluded-I, (100 mg/kg, p.o.), comprising of strongly metal ion conjugated withasteroids and oligosaccharides, exhibited trends of further improvement, compared to those of WSE in these parameters. In contrast, metal ions-depleted bioactives of WSE, viz., WSE/excluded-II, showed only marginal anti-stress and antioxidant effects. These bioactivities were more pronounced at higher dose levels (200 mg/kg, p.o.) of the two extracts, WSE and WSE/excluded-I, indicating a dose-dependant relationship. Comprehensive chromatographic, spectroscopic and chemical analyses established the nature of metal ion-conjugation of withasteroids, comprising the withanolide aglycones, withanolide-indolealkylamines, withanolide glycosides, sitoindosides, and oligosaccharides.

Keywords: *Withania somnifera*, Withasteroid-metal ion conjugates, Cold restraint stress, Corticosterone, Antioxidant.

Introduction

Withania somnifera (WS) Dunal. (family Solanaceae), known as *Ashwagandha* in Ayurveda, is used as an adaptogen. It is claimed as a *rasayana* (connoting *super vitalizer*), that produces adaptive resistance against diverse forms of stress (predictable and unpredictable), by augmenting SNIR (State of Non-specifically Increased Resistance) in the human body. Extensive pharmacological research has shown that *Ashwagandha* is effective for a variety of ailments and the collective bioactive principles, viz., the free and conjugated withasteroids, have also been identified and quantitated [1-4].

Isolation of bioactives of WS by Lobar chromatography has now yielded appreciable amounts of metal ion-conjugated withasteroids which had earlier eluded the attention of investigators. The superior role of metal ion-conjugated withasteroids has been revealed by their enhanced capacity to prevent *ex vivo* copper induced swelling of mitochondria, following an established procedure [5], than that of the metal ion depleted corresponding withasteroids (isolated after passing through strong cation exchange resin).

Stress is a biological response to aversive conditions that tend to threaten or perturb the homeostasis of the organisms [6]. Corticotropin releasing hormone is released during stress and stimulates the release of corticosterone from the adrenal cortex. Elevation of corticosterone level accelerates the generation of free radicals [7] and suppresses the immune function [8]. Stress particularly in the form of restraint or immobilization has been associated with a variety of pathological conditions in rodents [9-10]. These include increase in blood glucose level [11], decrease in the total number of WBC [12], increase in the liver weight and decrease in the spleen weight [13].

A stressful condition leads to increased production of free radicals, which results in oxidative stress, an imbalance in the systemic oxidant/antioxidant levels. During stressful situations, the energy requirement of the organism is increased, resulting in enhanced generation of free radicals [14-16]. Free radicals, aside from other adverse effects [17], cause oxidation of nucleic acids and proteins. The free radical activity and the extent of tissue damage are related quantitatively to the amount of lipid peroxide level in the blood [18] and are also reflected in the plasma antioxidant profile as determined by ABTS and FRAP. Malondialdehyde (MDA) is one of the end products of lipid peroxidation and extent of lipid peroxidation is measured by estimating MDA levels. Increased plasma level of MDA has been reported in cardiovascular, neurological and other diseases [19, 20]. The biochemical feature which has attracted the most sustained and widespread attention in relation to aetiology and prevention of these diseases is plasma cholesterol or its fractions like low density lipoproteins (LDL) and high density lipoproteins (HDL) levels. Cholesterol and lipoprotein levels were correlated with the risk of cardiovascular diseases [21]. Stress was found to increase lipid peroxidation and thereby alter lipid profile [22].

In the present study, the beneficial as well as deleterious effects (if any) of metal ion-conjugated withasteroids in stress-induced changes in mice were assessed. The effects of these bioactive constituents, occurring in a standardized *Withania somnifera* extract (WSE) (containing mixtures of native withasteroid-metal ion conjugates, and metal ion-

depleted corresponding withasteroids), were evaluated using cold restraint stress in Swiss albino mice. The details constitute the subject of this paper.

Materials and Methods

Chemistry:

Test samples- Authenticated plant material of WS, cultivated in the Western Himalayas, was obtained from Indian Herbs Ltd, Saharanpur (U.P.). A specimen of the sample has been preserved in our file for further reference.

Extraction of WS- Dried and powdered plant material (root and leaf) of WS was hot extracted with water for 2 hr. The extract was concentrated under reduced pressure and then spray dried. The yield of total extractives was ca. 8% (w/w). The different categories of bioactive constituents present in WSE were determined by HPLC and HPTLC, and the undesirable constituents were removed as before [23].

Drugs and chemicals- Corticosterone, Amberlite IRC-86 H^+, Dowex 50WX8-100 H^+, 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), 2,2'-Azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt (ABTS), were obtained from Sigma-Aldrich, St. Louis, U.S.A; thiobarbituric acid (TBA), trichloroacetic acid (TCA), hydrochloric acid, iron (III) chloride, butylated hydroxy toluene (BHT), were obtained from Merck, India; sodium fluoride, ethylenediamine tetraaceticacid disodium salt (EDTA) were obtained from SRL, India.

Comprehensive techniques used for phytochemical screening:

HPLC of withasteroids of WS- WATERS assembly [equipped with a PDA detector (WATERS 2996), pump (WATERS 515), injector (Rheodyne 7725i). Column, (Merck) Hiber® RP pre-packed column, (part no. 1.50333.0001); 4 x 250 mm particle size 5µ, with a guard column] was used. The conditions were as follows: mobile phase, acetonitrile-water (1:1); flow rate, 0.6 ml/min; run time, 20 min; injection volume 20 µl with a loop injector; detection at 225 nm.

HPLC of oligosaccharides- WATERS assembly [equipped with a RI detector (WATERS 2414), Column, Carbohydrate analysis column, (WATERS, part no. 1186000496) 3.9 x 300 mm with particle size 5µ]. The conditions were as follows: mobile phase, acetonitrile-water (8:2); flow rate, 2.0 ml/min; run time, 20 min; injection volume 20 µl with a loop injector.

HPTLC of withasteroids of WS- CAMAG assembly, equipped with Linomat TLC applicator and Scanner 3. Instrument and data processing were monitored by winCATS software ver. 1.3.4. Plate, Silica gel $60F_{254}$/Merck; mobile phase, *n*-butanol-acetic acid-water (4:1:2); densitometry at 225 nm.

Spectrophotometric estimation of metal ions- Jasco V-530 dual beam spectrophotometer was used for the estimation of Iron content [24].

Titrimetric method for the estimation of metal ions- Calcium and Magnesium content were estimated using titrimetric method [25].

AAS for the estimation of metal ions [26] - GBC-AWANTA system; standard Certified Reference Material (CRM) were obtained from MERCK-India.

Separation of differently conjugated metal ions from WSE- In a typical experiment, WSE was dissolved in a minimum volume of water and passed, successively, through a weak (Amberlite IRC-86 H^+) and a strong cation exchange resin (Dowex-50 H^+) column. The excluded fractions (WSE/excluded-I&II respectively) were evaporated under reduced pressure and the residue was analysed for metal ions by spectrophotometric, titrimetric and AAS methods.

The retarded fractions (WSE/retarded-I&II obtained from weak and strong cation exchange resin columns, respectively), comprising the constituents remained in the resin columns, were eluted with 2N-HCl and each acidic solution was evaporated under reduced pressure. The residues were analysed for metal ions.

Analysis of metal ions in WSE extract- A general method for sample preparation and estimation of metal ions in WSE is given in scheme-I.

Pharmacology:

Animals- Swiss albino mice of either sex, 3-4 months old and weighing around 20-25 g, procured from Central Research Institute (Ayurveda), Govt. of India, Salt lake City, Kolkata, were used. The animals had free access to food and water and were housed in an animal room with alternating light-dark cycle of 12 hr each. The animals were acclimatized for at least 5 days to the laboratory conditions before conducting experiments. Experiments were carried out between 0900 h and 1700 h. The study was conducted in accordance with Good Laboratory Practice (GLP) Regulations of WHO (WHO Document, 1998). The "Principles of laboratory animal care" (NIH Publication # 85-23, 1985) were also followed in the study. The 'Institutional Animal Ethics Committee' (IAEC) approved the experimental protocol.

Cold restraint stress (CRS) [13]- Mice were pretreated with the test compounds orally for 7 consecutive days prior to stress treatment followed by conjoint treatment of the test compound for 5 days and application of stress. From 8^{th} to 12^{th} day, 60 min after administration of the drug, animals were individually placed in plastic containers of capacity 350 ml. They were immobilized to their normal position using adhesive tape. They containers were placed in a fridge (at 4ºC) for 5 consecutive days (3hr/day) of post treatment.

Vehicle- The test compounds were dissolved in distilled water.

Experimental protocols:

Animals were divided into 7 groups and each group comprised of 6 mice.

Normal Control (NC)- Treated with vehicle only.

Cold restraint stress (CRS)- Treated with vehicle only and the animals were subjected to cold restraint stress from 8^{th} to 12^{th} day.

WSE-100 +CRS- Treated with WSE 100 mg/kg, p.o. and the animals were subjected to cold restraint stress from 8th to 12th day.

WSE-200 +CRS- Treated with WSE 200 mg/kg, p.o. and the animals were subjected to cold restraint stress from 8th to 12th day.

WSE/excluded/I-100+CRS-Treated with WSE/excluded/I-100 mg/kg, p.o. and the animals were subjected to cold restraint stress from 8th to 12th day.

WSE/excluded/I-200+CRS-Treated with WSE/excluded/I-200 mg/kg, p.o. and the animals were subjected to cold restraint stress on from 8th to 12th day.

WSE/excluded/II-100+CRS-Treated with WSE/excluded/II-100 mg/kg, p.o. and the animals were subjected to cold restraint stress from 8th to 12th day.

On 12th day blood was collected from the venous plexus behind the eye and different parameters were estimated.

Determination of plasma corticosterone level [27]- Corticosterone levels were estimated from plasma by HPTLC using marker using simplified method of Fenske M, 1998.

HPTLC of corticosterone level of plasma- CAMAG assembly, equipped with Linomat TLC applicator and Scanner 3. Instrument and data processing were monitored by winCATS software ver. 1.3.4. Plate, Silica gel 60F_{254}/Merck; mobile phase, toluene-acetone-acetic acid-ethanol (40:30:5:2); densitometry at 254 nm.

Determination of blood glucose level [28]**:**

In the experiment the blood glucose level of the animals were estimated by Glucose Oxidase-Peroxidase Enzymatic Method (GOD-POD). Blood was collected from venous plexus. About 100 µl of blood was taken in Eppendorff's tube (1.5 ml capacity) to which anticoagulant (sodium EDTA + sodium fluoride, 2:1) was previously added. Blood was mixed properly and centrifuged at 3000 rpm for 10 min. Out of it 10 µl of plasma was further used with 1ml of enzyme solution. Thoroughly mixed plasma and enzyme were incubated at room temperature for 30 min. Then the absorbance of pink colored solution formed was noted at 505 nm. Further glucose value was calculated using 100 mg/dl standard solution of glucose.

Glucose conc. (mg/dl) = Absorbance (sample) / Absorbance (standard) X 100

Estimation of total white blood cell (WBC)- Blood was collected on 12th day of test compound treatment and blood samples were diluted in the ratio of 1:20 using WBC diluting fluid, allowed for 10 min for the erythrocytes to lyse. Thereafter, the total WBC count was made by using the Neubauer chamber.

Weighing of organs- On the 12th day, after collection of blood samples, the mouse was dissected in the abdomen to remove the spleen and liver. Fatty tissue surrounding the organs was removed and organs were then weighed.

Measurement of antioxidant parameters- Plasma samples were analysed to assess the antioxidant profile following published procedures. The different antioxidant parameters analysed were: MDA [29], ABTS [30] and FRAP [31].

Determination of lipid parameters:

Total cholesterol (TC), Triglyceride (TG), High Density Lipoprotein (HDL) in plasma was determined using enzymatic kits (Span diagnostic Ltd.). Low Density Lipoprotein (LDL) was calculated using the following Friedewald's equation:

LDL = TC- TG/5 – HDL.

Very Low Density Lipoprotein (VLDL) was calculated using the following formula:

VLDL= TC – (HDL + LDL).

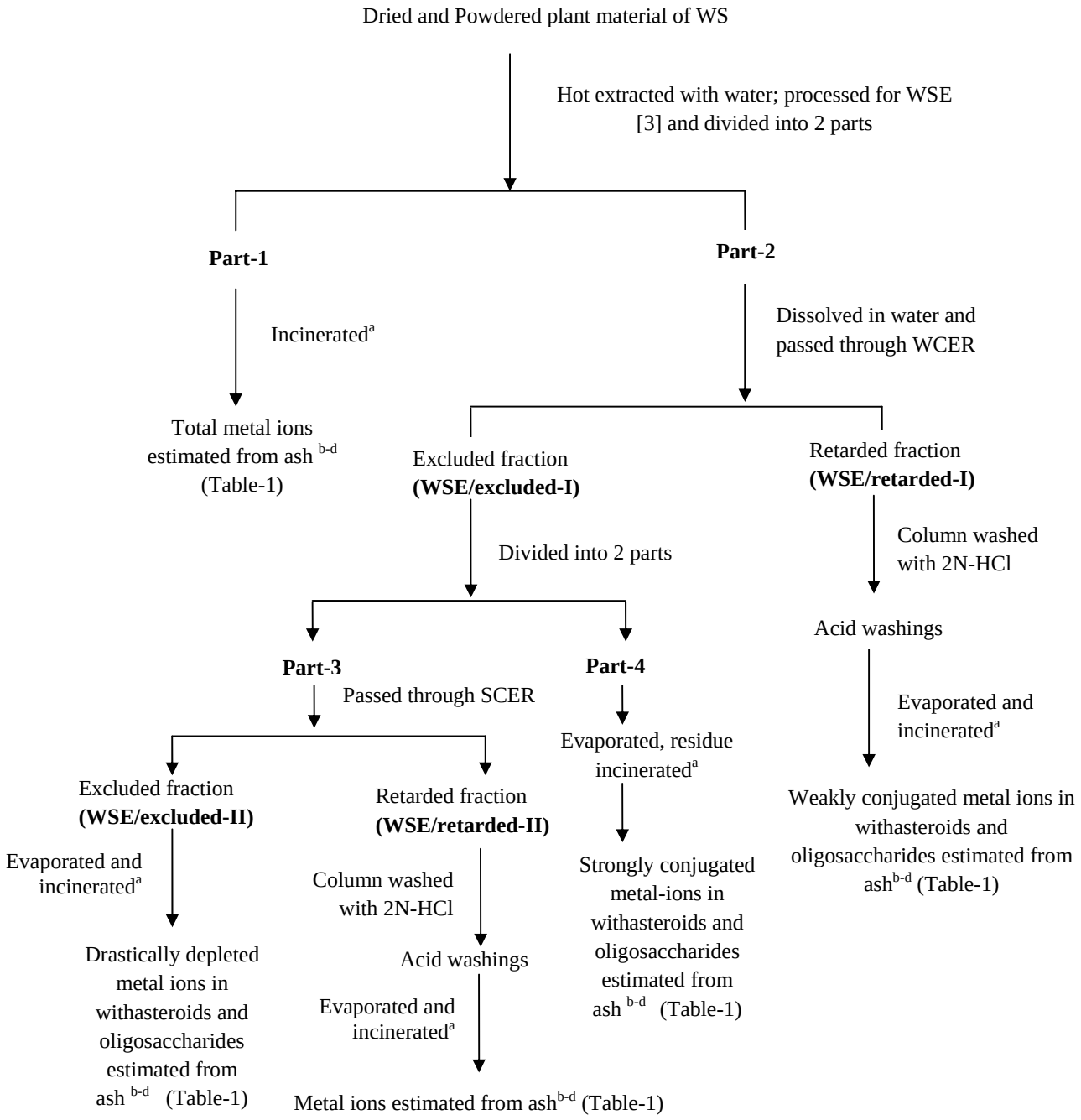
Statistical analysis- Statistical analysis was carried out using Prism software ver.4.0 statistical software (Graphpad software Inc). All the results were expressed as Mean ± standard error of the mean (SEM). Data were analyzed using one-way ANOVA followed by Tukey's test. In the entire test, the criterion for statistical significance was $p<0.05$.

Abbreviation: WS- *Withania somnifera*; WSE- *Withania somnifera* extract; MDA-malondialdehyde; LDL-low density lipoproteins, HDL-high density lipoproteins; TC-total cholesterol; TG- triglyceride; VLDL-very Low Density Lipoproteins; CRS-cold restraint stress; NC- normal control; ABTS- 2,2'-Azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) diammonium salt; FRAP- ferric reducing ability of plasma; WCER-weak cation exchange resin; SCER-strong cation exchange resin; ACTH- adreno cortico tropic hormone.; AAS-atomic absorption spectrophotometry.

Results

Chemical structures of the bioactives of WSE- The structures of the withasteroid metal ion complexes occurring in WSE (see Materials and Methods) were established by comprehensive spectroscopic, chromatographic and chemical analyses. The IR and ^{1}H-NMR spectra of these constituents, before and after treatment with cation exchange resins, suggested occurrence of differently conjugated metal ions, in the native state in WS. The chemical proof of the strength of metal ion conjugation in withasteroids was obtained by differential release of metal ions from the conjugates by passing through cation exchange resin, followed by metal ions analysis by spectrophotometry, titrimetry and AAS (Scheme I, Table 1). The nature of the ligands was established and quantitated by HPLC and HPTLC analyses using withasteroid markers (Table 2). The major bioactives identified and estimated were withanolide aglycones, withanolide-indolealkylamines, withanolide glycosides, sitoindosides, and oligosaccharides along with moisture, polysaccharides, and minor micro-molecular metabolites.

Scheme-I. A general method for sample preparation and estimation of metal ions in *Withania somnifera* extractives (WSE).



[a]500°C for 2.5 hr; [b]Fe ion estimated by 1,10-orthophenanthroline; [c]Ca and Mg ions estimated by complexometric (EDTA) titration; [d]Cu, Zn, Pb, Cd, Hg estimated by AAS.

WCER- Weak Cation Exchange Resin (Amberlite IRC-86 H^+).
SCER- Strong Cation Exchange Resin (Dowex 50 H^+).

Table 1: Amounts of weakly and strongly conjugated metal ions in WSE obtained after successively passing through weak and strong cation exchange resins.

Sample	Metal content in μg/gm of sample							
	Fe	Ca	Mg	Cu	Zn	Pb[b]	Cd[b]	Hg[b]
WSE(Total metal ions)	642.4±20.3	3193.7±60.2	1663.6±20.7	22.4±1.1	160.6±12.5	-	-	-
WSE/excluded- I	622.4±11.7	3045.6±28.4	1553.5±40.3	20.4±1.5	141.7±7.6	-	-	-
WSE/retarded-I	12.5±0.8	131.7±12.6	93.9±9.6	1.4±0.2	11.5±1.6	-	-	-
WSE/excluded-II[a]	2.2±0.3	50.4±3.2	11.1±0.9	1.2±0.1	5.1±0.4	-	-	-
WSE/retarded-II	615.7±20.1	2850.6±32.3	1540.5±33.6	18.4±1.1	122.7±5.5	-	-	-

Values are mean± SEM; n=4

[a]Chelated/complexed metal ions are largely removed by strong cation exchange resin;

[b]Note the absence of heavy metals ions in WSE;

-, indicates <0.0001

WSE: Weakly and strongly metal ions conjugated withasteroids and oligosaccharides.

WSE/excluded-I: Strongly-metal ions conjugated withasteroids and oligosaccharides.

WSE/excluded-II: Metal ions-depleted withasteroids and oligosaccharides.

Table 2: Relative abundance of chemical constituents of WSE before and after passing through cation exchange resin.

Sample	Withanolide glycosides/ Sitoindosides	Withaferin-A	Withanolide-indolealkylamines	Oligosaccharides
	Abundance in % (w/w) of extractives			
WSE	14.5±0.42	0.54±0.04	0.015±0.001	41.4±1.4
WSE/excluded-I	14.1±0.31	0.52±0.03	0.014±0.001	40.5±1.1
WSE/retarded-I	-	-	-	-
WSE/excluded-II	13.8±0.27	0.51±0.03	0.014±0.001	39.3±1.3
WSE/retarded-II	0.41±0.05	0.02±0.007	-	1.26±0.24

Values are mean± SEM; n=4

-, indicates <0.0001

Effects of WSE fractions in plasma corticosterone level, glucose and total WBC count in mice exposed to CRS:

There was a significant elevation (p<0.001) in plasma corticosterone level of mice in cold-restraint stress (CRS) group as compared to the normal control (NC) group. Administration of WSE and WSE/excluded-I (100 mg/kg, p.o.) to mice exposed to CRS prevented the augmentation in corticosterone level in a significant way. However, in case of WSE/excluded-II fraction the effect was marginal and statistically insignificant. The results are incorporated in Table 3.

The levels of blood glucose and WBC count, after CRS induced stress, were elevated in a significant way (p<0.001) as compared to NC group. However, such enhancement in blood glucose level and WBC count were significantly attenuated, with prior treatment of WSE and WSE/excluded-I (100 mg/kg, p.o.). But, in case of WSE/excluded-II fraction (depleted metal ions conjugation of withasteroids) the effects were marginal and statistically insignificant. The results are incorporated in Table 3.

Table 3: Effects of WSE and WSE/excluded fractions in plasma corticosterone level, glucose level and total WBC count in mice exposed to CRS.

Groups	Plasma corticosterone (µg/dl)	Plasma glucose(mg/dl)	Total WBC count (cell mm^{-3})
NC	13.26±0.35	84.68±1.35	5564.17±161.03
CRS	19.81±0.67$^{\#\#\#}$	133.10±1.95$^{\#\#\#}$	7636.17±142.14$^{\#\#\#}$
WSE-100+CRS	17.10±0.43*	124.74±1.05*	6615.33±264.53**
WSE/excluded/I-100+CRS	16.98±0.41*	124.69±1.48*	6687.52±216.23**
WSE/excluded/II-100+CRS	18.87±0.66	127.19±1.48	7294.17±106.26

Data represented as Mean ± SEM; for 6 mice.

$^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$; in comparison to normal control (NC) mice treated with vehicle.

$*p<0.05$; $**p<0.01$; $***p<0.001$; in comparison to Cold restraint stress (CRS) group treated with vehicle.

Effects of WSE and WSE/excluded-I fraction at higher dose in plasma corticosterone level, glucose level and total WBC count in mice exposed to CRS:

Administration of WSE and WSE/excluded-I at higher dose (200 mg/kg, p.o.) to mice exposed to CRS inhibited the increase in corticosterone, blood glucose and WBC levels, in a dose dependant manner. WSE/excluded-I fraction exhibited a better trend in effects, in these parameters, compared to those of WSE. The results are incorporated in Table 4.

Table 4: Effects of WSE and WSE/excluded-I fractions at higher dose in plasma corticosterone level, glucose level and total WBC count in mice exposed to CRS.

Groups	Plasma corticosterone (µg/dl)	Plasma glucose(mg/dl)	Total WBC count (cell mm^{-3})
WSE-200+CRS	15.03±0.49***	114.96±1.40***	5754.00±169.82***
WSE/excluded/I-200+CRS	14.96±0.58**	114.25±1.61**	5748.17±112.38***

Data represented as Mean ± SEM; for 6 mice.

*p<0.05; ** p <0.01; *** p <0.001*;* in comparison to Cold restraint stress (CRS) group treated with vehicle.

Effects of WSE fractions on liver and spleen weight in mice exposed to CRS:

Administration of WSE and WSE/excluded-I fractions (100 mg/kg, p.o.) significantly attenuated the stress induced changes in the weight of the organs and restored them to near normal level. In contrast, the effects of WSE/excluded-II fraction were insignificant. The results are incorporated in Table 5.

Table 5: Effect of WSE and WSE/excluded fractions on liver and spleen weight in mice exposed to CRS.

Groups	Liver weight (gm/100gm body weight)	Spleen weight (gm/100gm body weight)
NC	4.54±0.11	0.744±0.006
CRS	5.26±0.05$^{\#\#\#}$	0.381±0.004$^{\#\#\#}$
WSE-100+CRS	4.76±0.07**	0.581±0.009***
WSE/excluded/I -100+CRS	4.82±0.10**	0.572±0.007***
WSE/excluded/II-100+CRS	5.08±0.10	0.471±0.013

Data represented as Mean ± SEM; for 6 mice.

$^{\#}p$<0.05; $^{\#\#}p$ <0.01; $^{\#\#\#}p$ <0.001*;* in comparison to normal control (NC) mice treated with vehicle.

*p<0.05; ** p <0.01; *** p <0.001*;* in comparison to Cold restraint stress (CRS) group treated with vehicle.

Effects of WSE and WSE/excluded/I fraction at higher dose on liver and spleen weight in mice exposed to CRS:

The trend of recuperation from altered organ weights after CRS was further manifested after administration of higher dose (200 mg/kg, p.o.) of both WSE and WSE/excluded-I. The results are incorporated in Table 6.

Table 6: Effects of WSE and WSE/excluded-I at higher dose on liver and spleen weight in mice exposed to CRS.

Groups	Liver weight (gm/100gm body weight)	Spleen weight (gm/100gm body weight)
WSE-200+CRS	4.65±0.10***	0.711±0.008***
WSE/excluded/I -200+CRS	4.63±0.08****	0.705±0.009***

Data represented as Mean ± SEM; for 6 mice.

*p<0.05; ** p <0.01; *** p <0.001*;* in comparison to Cold restraint stress (CRS) group treated with vehicle.

Effect of WSE fractions on plasma antioxidant profile:

The effects of WSE fractions on lipid peroxidation and the plasma antioxidant status of mice exposed to cold restraint stress were evaluated. Significant improvement of MDA level and betterment of plasma antioxidant status was observed in the mice treated with WSE and WSE/excluded-I fractions (100 mg/kg, p.o.). Furthermore, the effects of WSE/excluded-I fraction were even better than those of WSE. In contrast, WSE/excluded-II fraction elicited antioxidant effect which was only marginally improved than that of the CRS group. The results are incorporated in Table 7.

Table 7: Effect of WSE and WSE/excluded fractions on plasma antioxidant profile in mice exposed to CRS

Antioxidant parameters after exposed to CRS					
Parameters	**Groups**				
	NC	**CRS**	**WSE-100+CRS**	**WSE/excluded/I-100+CRS**	**WSE/excluded/II-100+CRS**
MDA (nmole/ml plasma)	4.20±0.05	4.95±0.07$^{\#\#\#}$	4.55±0.08*	4.51±0.07*	4.77±0.09
ABTS (IC_{50} in µg/ml) plasma	2.03±0.03	3.96±0.25$^{\#\#\#}$	3.57±0.05	3.52±0.05	3.66±0.16
FRAP (IC_{50} in µg/ml) plasma	2.27±0.06	4.57±0.19$^{\#\#\#}$	4.12±0.08	4.10±0.09	4.24±0.06

Data represented as Mean ± SEM; for 6 mice.

$^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$; in comparison to normal control (NC) mice treated with vehicle.

*$p<0.05$; ** $p<0.01$; *** $p<0.001$; in comparison to Cold restraint stress (CRS) group treated with vehicle.

Effect of WSE and WSE/excluded-I at higher dose on plasma antioxidant profile in mice exposed to CRS:

Administration of WSE and WSE/excluded-I at higher dose (200 mg/kg, p.o.) to mice exposed to CRS prevented significantly the changes in the antioxidant profile in a dose dependant manner. However, the betterment of antioxidant profile by WSE/excluded-I fraction than those of WSE, on similar treatment, was further manifested with higher dose. The results are incorporated in Table 8.

Table 8: Effect of WSE and WSE/excluded-I at higher dose on plasma antioxidant profile in mice exposed to CRS

Antioxidant parameters after exposed to CRS		
Parameters	**Groups**	
	WSE-200+CRS	**WSE/excluded/I-200+CRS**
MDA (nmole/ml plasma)	4.32±0.10***	4.18±0.12***
ABTS IC_{50} in µg/ml plasma	3.12±0.10**	3.02±0.08**
FRAP IC_{50} in µg/ml plasma	3.89±0.08**	3.78±0.10**

Data represented as Mean ± SEM; for 6 mice.

*p<0.05; ** p <0.01; *** p <0.001*;* in comparison to Cold restraint stress (CRS) group treated with vehicle.

Effect of WSE fractions on plasma lipid parameters:

Exposure to CRS resulted in a significant increase in TC, TG, LDL and VLDL and a concomitant decrease in HDL level of mice in comparison to NC group. The LDL/HDL ratio also found to be increased significantly (p<0.001) after mice exposed to CRS. Administration of WSE and WSE/excluded-I fractions reduced TC, TG, LDL, VLDL, LDL/HDL ratio significantly with a concomitant increase in HDL level. However, in case of WSE/excluded-II fraction the effects were marginal and statistically insignificant. The results are incorporated in Tables 9.

Table 9: Effect of WSE and WSE/excluded fractions on plasma lipid parameters in mice exposed to CRS

Groups	Plasma lipid level					
	Total cholesterol (mg/dl)	**Triglyceride (mg/dl)**	**LDL (mg/dl)**	**HDL (mg/dl)**	**VLDL (mg/dl)**	**LDL/HDL**
NC	82.49±1.39	50.21±1.49	37.75±0.79	34.70±0.86	10.04±0.30	1.09±0.03
CRS	130.73±2.92$^{\#\#\#}$	66.10±2.07$^{\#\#\#}$	89.23±2.79$^{\#\#\#}$	28.28±1.17$^{\#\#\#}$	13.22±0.41$^{\#\#\#}$	3.18±0.16$^{\#\#\#}$
WSE-100+CRS	111.04±0.97***	58.45±2.31*	69.91±1.99***	29.44±1.03	11.69±0.46*	2.40±0.15**
WSE/ excluded/I -100+CRS	110.21±0.65***	59.45±1.81*	67.91±2.14***	30.41±1.27	11.89±0.53*	2.23±0.34**
WSE/excluded/II-100+CRS	124.70±1.07	64.60±1.99	82.81±1.08*	28.97±1.33	12.92±0.40	2.89±0.16

Data represented as Mean ± SEM; for 6 mice.

$^{\#}p<0.05$; $^{\#\#}p<0.01$; $^{\#\#\#}p<0.001$; in comparison to normal control (NC) mice treated with vehicle.

*$p<0.05$; ** $p<0.01$; *** $p<0.001$; in comparison to Cold restraint stress (CRS) group treated with vehicle.

Effect of WSE and WSE/excluded-I at higher dose on plasma lipid parameters in mice exposed to CRS:

Administration of WSE and WSE/excluded-I at higher dose (200 mg/kg, p.o.) to mice exposed to CRS prevented significantly the changes in the lipid profile in a dose dependant manner. The results are incorporated in Table 10.

Table 10: Effect of WSE and WSE/excluded-I at higher dose on plasma lipid parameters in mice exposed to CRS

Groups	Plasma lipid level					
	Total cholesterol (mg/dl)	**Triglyceride (mg/dl)**	**LDL (mg/dl)**	**HDL (mg/dl)**	**VLDL (mg/dl)**	**LDL/HDL**
WSE-200+CRS	102.42±1.83***	54.62±1.11***	57.98±2.36***	33.51±1.10*	10.92±0.22***	1.73±0.16***
WSE/ excluded - 200+CRS	102.21±1.43***	54.68±1.31***	58.16±1.76***	33.11±1.27*	10.94±0.22***	1.76±0.14***

Data represented as Mean ± SEM; for 6 mice.

*p<0.05; ** p <0.01; *** p <0.001*;* in comparison to Cold restraint stress (CRS) group treated with vehicle.

Discussion

Metal ions play an important role in plant biochemistry. As trace elements, they play an important role in the plant metabolism and biosynthesis of chemical constituents and as cofactors for enzymes. The trace elements are parts of important metabolic products of plant cells. They significantly contribute also to the bioactivities of plants, administered to animal organisms including humans.

WSE and WSE/excluded-I (100 mg/kg, p.o.) administered to mice produced significant anti-stress effects. In contrast, WSE/excluded-II fraction on similar treatment exhibited marginal and statistically insignificant anti-stress activity.

In response to stress, ACTH is released, which acts on the adrenal cortex to stimulate the synthesis and release of corticosterone [32]. Increased plasma corticosterone level influences the mobilization of stored fat and carbohydrate reserves [33], which in turn increases blood glucose level. Both WSE and WSE/excluded-I (100 mg/kg, p.o.) significantly inhibited the increase in corticosterone and glucose levels. The effect of WSE/excluded-II fraction, on similar treatment, was marginal and statistically insignificant. Also, the stress induced increase in total WBC count was better attenuated by WSE and WSE/excluded-I (100 mg/kg, p.o.). Furthermore, WSE/excluded-II fraction, drastically depleted of conjugated metal ions (Ca^{2+}, Mg^{2+}, Fe^{2+}/ Fe^{3+}, Zn^{2+} and Cu^{2+}), on similar treatment elicited only marginal effect indicating significant contribution of metal ions conjugation to withasteroids to confer different types of anti-stress effects.

In the present study, it was observed that the liver was hypertrophied as the animal was exposed to cold restraint stress with decreased spleen weight since glucocorticoid level leads to widespread lympholysis in the lymphoid organs [34]. WSE and WSE/excluded-I fraction (100 mg/kg, p.o.) significantly attenuated the stress induced changes in the liver and spleen weight. WSE/excluded-II, however, (100 mg/kg, p.o.) did not produce any significant effect on similar treatment.

Stress leads to increased production of reactive oxygen species (ROS), which leads to lipid peroxidation. ROS initiates a series of chain reactions *in vivo* and ultimately damages tissue and DNA [35]. The MDA content is a marker of the extent of lipid peroxidation [36]. ABTS and FRAP are the other two antioxidant assay system to assess the antioxidant status. Administration of WSE and WSE/excluded-I (100 mg/kg, p.o.) significantly inhibited the increase in MDA level, after the mice exposed to CRS, and the effects were marginally better in case of WSE/excluded-I. The plasma antioxidant status (ABTS, FRAP) was also improved significantly in case of above two groups. However, the effect was marginal in case of WSE/excluded-II fraction in the antioxidant parameters.

This study showed significant increases in plasma TC, TG, LDL and VLDL levels with concomitant decrease in HDL level after the mice were exposed to cold restraint stress. Increased plasma concentration of TC and TG is a major lipid abnormality syndrome as they are often associated with cardio-vascular disorders (CVD) and many other pathological syndromes [37]. Both LDL and VLDL have a positive role in atherogenesis [38]. In the present study, total lipid contents of plasma of animals exposed to CRS were significantly elevated compared to the normal control group. Treatment of WSE and WSE/excluded-I (100 mg/kg, p.o.) significantly reduced TC, TG, LDL, VLDL, LDL/HDL ratio with a concomitant increase in HDL level. In case of WSE/excluded-II fraction (100 mg/kg, p.o.) the effects were only marginal and statistically insignificant, suggesting the role of metal ions conjugation to withasteroids for eliciting these bioactivities.

The anti-stress activities of WSE and WSE/excluded-I fractions exhibited dose-dependant relationship. Thus, administration of the two test compounds at a higher dose (200 mg/kg, p.o.) elicited better response than that elicited by 100 mg/kg, p.o. of each drug.

Among the plant adaptogens *Withania somnifera* holds a prominent rank. The withasteroids of *Withania somnifera* are known as the major bioactives of this plant [4]. Based on the above findings it seems likely that metal ions in conjugation with the withasteroids might have a profound bearing on the true bioactive principles of the adaptogenic *Withania somnifera* standardized extract(s).

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