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Amelioration potential of *Withania somnifera* root extract on hexavalent chromium induced micronucleus in *Channa punctatus* (Bloch, 1793)

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Abstract

Aim: This study aimed to infer the ameliorative potential of *Withania somnifera* ('Ashwagandha') against hexavalent chromium induced micronuclei in *Channa punctatus*.

Methodology: After laboratory acclimatization of 15 days, *C. punctatus* (12.20 cm, 42 g) were maintained in six groups. Group I, served as control. Fishes of groups II and III were separately exposed to root extract of *W. somnifera* (3 mg l⁻¹) and 96 hr-LC₅₀/10 of Cr (VI), 7.89 mg l⁻¹, respectively, for 24, 48, 72 and 96 hr. Contrarily, the fish of groups IV, V and VI were exposed to 7.89 mg l⁻¹ of Cr (VI) along with increasing concentrations of root extract of *W. somnifera* (1, 2, 3 mg l⁻¹), respectively. Induction of micronuclei was assessed in fishes of all the six groups after stipulated exposure periods.

Results: A significant induction ($p < 0.05$) in micronuclei frequency was observed in Group-III as compared to the control. On contrary, there was a significant ($p < 0.05$) decrease in frequency of micronuclei induction with increasing concentrations of root extract of *W. somnifera*, as compared to Group-III, after stipulated exposure periods in a dose and time-dependent manner.

Interpretation: Preliminary investigations evinced that the root extract of *W. somnifera* has enough ameliorative potential against short term sub-lethal exposure to Cr (VI) induced genomic instability, i.e., micronuclei induction in *C. punctatus*.

Key words: *Channa punctatus*, Chromium trioxide, Genotoxicity, Micronucleus, *Withania somnifera*

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Introduction

Hexavalent chromium, prevalent due to the anthropogenic activities, is a toxic industrial pollutant that possesses mutagenic and teratogenic properties. On contrary, occurrence of Cr (III) is mostly due to the natural activities. However, there have been a spurt in naturally occurring Cr (VI) in ground and surface waters at values surpassing the limits of 50 $\mu\text{g l}^{-1}$ as prescribed by World Health Organization (2008). Hexavalent chromium, a known pollutant in aquatic habitats, got prominence due to its mutagenic abilities. It has been classified as Group 1 carcinogen by International Agency for Research on Cancer owing to its established carcinogenic effect (Banerjee *et al.*, 2017). Blatant use of hexavalent chromium in tanning, paints, varnishes, electroplating, wood processing, dyes, paper making and mining industries has resulted in its accumulation to toxic levels that adversely affect both flora and fauna (Gu *et al.*, 2015).

Chromium, the seventh most abundant element on earth has earned notoriety due to its variable oxidation states (Fendorf, 1995). In nature, Cr (III) and Cr (VI) found in abundant states are capable of harming animals, humans and plants as well (Mohanty and Patra, 2013). Cr (III) is not as efficient as Cr (VI) in entering the body of the organism as former is insoluble whereas the latter one is soluble in water making it dangerous (Gürkan *et al.*, 2017; Wolińska *et al.*, 2013). Chromium, particularly hexavalent chromium (Zayed and Terry, 2003) finds its way into the water bodies and becomes a threat for aquatic species (Ghani, 2011). India occupies third place in the world in chromite production followed by South Africa and Kazakhstan (Koleli and Demir, 2016). Cr (VI) has also been placed at 17th position by the Agency for Toxic Substances and Disease Registry in its Substance Priority List (ATSDR, 2017). The U.S. Environmental Protection Agency (2018) has recommended 100 $\mu\text{g l}^{-1}$ of chromium consumption in all forms, as safe in drinking water. It has been documented that even at very low concentrations chromium is not only cytotoxic but also carcinogenic and mutagenic in nature (Hanaa *et al.*, 2000). Cr (VI) may react with biological reductants like thiols and ascorbate to give rise to different reactive oxygen species (ROS) like superoxide ions (O_2^-), hydrogen peroxide (H_2O_2) molecules and hydroxyl radicals (OH^-) that lead to oxidative stress in the cells (Stohs and Bagchi, 1995).

Under oxidative stress, H_2O_2 is transported into the nucleus and directly damages the genomic DNA. Oxidative stress mediated DNA damage and oxidation of proteins and lipid peroxidation have been documented to implicate in carcinogenesis (Trachootham *et al.*, 2009), neurodegeneration (Andersen, 2004; Shukla *et al.*, 2011), atherosclerosis, diabetes (Paravicini and Touyz, 2006) and aging (Haigis and Yankner, 2010). Cr (VI), capable of damaging different cellular molecules due to its ability to produce reactive oxygen species (Feng *et al.*, 2017), leads to molecular damages like DNA double-stranded breaks (DSBs) with greater micronuclei induction in blood cells of fish (Yadav and Trivedi, 2009a). Access of Cr (VI) to human beings

through water-fish-human- continuum cannot be ruled out. Further, presence of Cr (VI) in aquatic habitats is liable to influence fish metabolism after its absorption through their gills, skin and gastro-intestinal tract. This adversely affects fish survival.

Thus, to safeguard fish biodiversity and productivity suitable measures to mitigate Cr (VI) contamination is the need of the hour. Conventional methods are energy intensive and cost expensive (Ohtake and Silver, 1994). The use of chromate reducing bacterial strains is although effective but has limited scope. For aquaculture purposes, where larger water bodies are involved, dietary inclusion (nutrigenomic approach) of plant metabolites, after preliminary biological screening of their therapeutic potential in laboratory microcosm seems to be an appropriate solution (Prasad and Trivedi, 2018). Ample studies emphasize biological screening of plant metabolites for their therapeutic/ameliorative potential against water borne toxicants in fishes (Sevgiler *et al.*, 2011; Kumar and Trivedi, 2015; Dwivedi *et al.*, 2015; Tiwari *et al.*, 2016; Tiwari *et al.*, 2017; Tiwari *et al.*, 2019; Prasad *et al.*, 2017). *Withania somnifera* (Solanaceae), vernacularly known as 'Ashwagandha' possesses enough therapeutic potential. Therapeutically significant metabolites, viz., alkaloids, steroidal flavonoids, saponins, lactones, withanolides, sitoindosides, and tannins etc., have already been identified, extracted and isolated from ashwagandha (Kapoor, 2001; Atta-ur-rahman *et al.*, 1991; Bandyopadhyay *et al.*, 2007; Mirjalili *et al.*, 2009). Different parts of *W. somnifera* have been found with varied medicinal applications viz., anti-inflammatory, anti-oxidative, anticancer, anti-stress and immune-modulator, adaptogenic, besides being promoters for central nervous system, endocrine and cardiovascular activities (Alam *et al.*, 2011). *W. somnifera* also has the ability to decrease oxidative stress as it decreases lipid peroxidation (Ahmad *et al.*, 2010) and protein carbonyl content (Datta *et al.*, 2011).

Thus, eco-toxicological manifestations in fishes can be effectively employed to find out early responses against environmental toxicants and, therefore, can also be adequately applied for routine environmental monitoring programs. Every Environmental Management Plan (EMP) emphasizes for routine bio-monitoring of aquatic regimes contaminated with noxious xenobiotics. Cytotoxic end markers, viz., Chromosomal Aberration Test and Micronucleus Assay etc., are preferred tools to monitor the extent of genotoxicity among aquatic animals. For assessing mutagenic, aneugenic and clastogenic effects of toxicants, the micronucleus assay can be relevant for both *in-vitro* and *in-vivo* studies (Bolognesi and Hayashi, 2011). Interestingly, the assay can be used in different target cells and tissues (Fenech *et al.*, 2011; Laingam *et al.*, 2008). Micronucleus assay is an important tool for uncovering the genotoxicity of an array of compounds, including nanoparticles. It has now become a preferred method for quantifying chromosome breakage, impaired DNA repair, chromosome loss, non-disjunction, necrosis, apoptosis and cytostasis (Fenech, 2007). Further, the Micronucleus assay is easy to score giving it an edge over other

cytogenetic tests such as sister chromatid exchanges and chromosomal aberration test. Keeping above mentioned facts into consideration, the present study was conducted to explore the ameliorative potential of *W. somnifera* root extract against water borne hexavalent chromium induced genomic instability in *C. punctatus* in terms of Micronucleus induction.

Materials and Methods

Fish and fish maintenance: Live and apparently healthy specimens of freshwater murrel, *C. punctatus* (12.20 ± 1.29 cm, 42 ± 2.01 g) were procured from the local freshwater habitats around Lucknow (U.P.) and brought to the laboratory in plastic buckets and subjected to dip treatment of 0.05% potassium permanganate (KMnO_4) for 2 min to avoid any external dermal infection. Fish were washed repeatedly and transferred to a glass aquarium ($100 \times 40 \times 40 \text{ cm}^3$) and acclimatized for 15 days under laboratory conditions in 100 l glass aquarium having 40 l of 10 d (days) aged tap water is used for acclimatization of fish according to APHA, 2012. They were fed with pellets of commercial fish food, 'OPTIMUM', Bangsaothong, Thailand at fixed time intervals. In this study, the selection of exposure concentrations was based on 96 hr- LC_{50} value and No Observed Effect Concentration (NOEC) for Cr (VI) and *Withania somnifera*, respectively (Hutchinson et al., 2009).

Preparation of plant extract : The plant extract was prepared by weighing 25 g of root powder (Batch No. 0068, Jamna Herbal Research Ltd., Mandideep, Madhya Pradesh) of *W. somnifera* and wrapped in a thimble made of strong filter paper. Four thimbles (100 g) soaked in 250 ml of 50% ethanol was extracted with the help of Soxhlet apparatus for 72 hr. The extract was further filtered, concentrated on rotavapor (Zhengzhou Great Wall, R-1050); dried in lyophilizer (Labconco, USA) under low pressure.

Experimental Design: *C. punctatus* being a bottom feeder has greater chances of accumulating higher concentration of metallic toxicants (Kumar et al., 2019). Fishes were subjected to short term static bioassays where they were exposed to sub-lethal concentration of Cr (VI) (96 hr- $\text{LC}_{50}/10$) separately and simultaneously with varying concentrations of ethanolic root extract of *W. somnifera*. A 72 well-acclimatized fish were divided into 6 groups; each containing 12 specimens. Group-I served as a control (without any treatment), fish of group-II were given ethanolic extract of *W. somnifera* root powder (3 mg l^{-1}) and that of group-III were subjected to chromium trioxide (96 hr- $\text{LC}_{50}/10$). Fish of groups IV, V and VI were subjected to chromium trioxide (7.89 mg l^{-1}) along with increasing concentrations, of *W. somnifera* root extract (1, 2 and 3 mg l^{-1}). After termination of stipulated exposure period, 24, 48, 72 and 96 hr, three fish (for reproducibility of the results) from each treated group along with control were anaesthetized with 0.1% diethyl ether. Blood samples were collected from their caudal vein by using a heparinized syringe and stored in Ethylenediamine-tetraacetic acid coated vials for estimation of micronucleus.

Micronucleus (MN) Assay: Micronuclei, induced by multiple molecular mechanisms, are potential biomarkers of genotoxicity, applicable in both *in-vitro* and *in-vivo* systems (Fenech et al., 2011). Further, the MN assay, capable of detecting both aneugenic and clastogenic defects, is highly sensitive and simple in scoring, widely applicable in different cell types. It is internationally validated, and has potential for automation (Kirsch-Volders et al., 2011). The blood samples of fish from each groups were smeared on the slides. After overnight drying of slides at room temperature in a dust-free environment, they were fixed in methanol for 5 min and stained with May-Grunwald's solution I and II sequentially for 3 and 5 min; washed with distilled water and dried. Finally, slides were stained with 5% Giemsa (Sigma Aldrich, the USA) in phosphate buffer (pH 6.8) for 30 min and subsequently washed for removing all the unbound Giemsa stain. The slides were mounted with DPX (a mixture of distyrene, plasticizer and xylene) for microscopic examination (Schmid, 1975) using an oil immersion microscope (Nikon Corporation, K-12432). Approximately, 2000 erythrocytes with well-defined cytoplasm on each slide were scored for the presence of MN (Fenech et al., 2011). Micronucleus frequency was calculated by the following formula (Fenech, 2000):

$$\text{MN}\% = \frac{(\text{Number of cells containing micronuclei} \times 100)}{(\text{Total number of cells counted})}$$

Statistical Analyses: Each treatment was performed with three replicates and the results were represented as mean $\pm$ S.E. Statistical analyses were performed with the help of SPSS (Version-20.0) software. For the calculation of significance, one-way analysis of variance (ANOVA) with Tukey's post-hoc test was applied to test the significance ($p < 0.05$) of each result in fish erythrocytes.

Results and Discussion

The status of genomic instability was observed in terms of induction of MN frequency in erythrocytes of fish, *C. punctatus*. After exposure to sub-lethal test concentrations of Cr-trioxide (96 hr- $\text{LC}_{50}/10$; 7.89 mg l^{-1}) MN induction was found in an increasing trend with a steep increase at an interval of 24 and 48 hr of exposure period (Fig. 1). In comparison to control, the frequency of micronuclei induction was significantly ($p < 0.05$) increased in Group-III with an increasing fold change of $2.12 < 4.40 < 5.16 < 5.34$ after 24, 48, 72 and 96 hr respectively. The highest fold change of 5.34 was observed after 96 hr of exposure period. Interestingly, there was a reduction in the frequency of micronuclei induction in groups- IV, V and VI exposed to 96 hr- $\text{LC}_{50}/10$ of Cr (VI) along with increasing concentrations (1, 2 and 3 mg l^{-1}) of *W. somnifera* root extract, after 24, 48, 72 and 96 hr of exposure periods; the highest reduction in the frequency of MN induction was observed after 96 hr of exposure period in group VI, as evident by a fold change of 3.97. After 24 hr of exposure period, in groups IV, V, and VI, the fold changes of MN induction were

Fig. 1: Variations in induction of micronuclei frequencies in erythrocytes of *Channa punctatus* at different exposure periods. (*represent the significant values ($p < 0.05$). Values are expressed as mean $\pm$ S.E.

Fig. 2: Representative images showing Cr (VI) induced micronuclei (B) and decreased micronuclei induction after exposure to *W. somnifera* root extracts as compared to control (A) in erythrocytes of *C. punctatus*.

recorded in the following decreasing order: $1.96 > 1.54 > 1.38$ in comparison to group III (2.12) with respect to control. Further, a declining trend was again recorded in frequency of MN induction in terms of observed fold changes $4.00 > 3.64 > 3.32$ after 48 hr of exposure period, in groups IV, V and VI in comparison to group III (4.40) with respect to control. After 72 hr, a declining trend in the fold changes of MN induction in groups IV, V and VI were observed to be as $4.72 > 4.40 > 3.92$, when fold changes in MN was recorded as 5.16 with respect to control (Table. 1). Microphotographs showed higher formation of micronuclei in group III as compared to control and significant reduction in group

VI simultaneously exposed to 3 mg l^{-1} of *W. somnifera* root extract and 7.89 mg l^{-1} of Cr (VI) (Fig.2).

A similar decreasing trend in fold changes of MN induction was also observed after 96 hr of exposure in groups IV, V and VI ($5.00 > 4.48 > 3.97$), in comparison to group III (5.34), with respect to control. These observations suggestive of the fact that a fold change difference of 1.37 between group-III and group-VI exists, depicting a decrement in the frequency of MN induction after 96 hr of exposure, wherein fish are simultaneously exposed with sub-lethal fraction of Cr (VI) (7.89 mg l^{-1}) and root extract of *W.*

Table 1: Relative fold change in micronuclei induction in different exposure periods

MN induction	Exposure groups	Exposure period (hr)			
		24	48	72	96
Relative Fold change	Group-I (Control)	1.00	1.00	1.00	1.00
	Group-II (3 mg l ⁻¹ of WS)	1.00	1.00	1.00	1.00
	Group-III (96hr-LC ₅₀ of Cr (VI))	2.12	4.40	5.16	5.34
	Group-IV (96hr-LC ₅₀ of Cr (VI) + 1 mg l ⁻¹ of WS)	1.96	4.00	4.72	5.00
	Group-V (96 hr-LC ₅₀ of Cr (VI) + 2 mg l ⁻¹ of WS)	1.54	3.64	4.40	4.48
	Group-VI (96 hr-LC ₅₀ of Cr (VI) + 3 mg l ⁻¹ of WS)	1.38	3.32	3.92	3.97

somnifera @ 3 mg l⁻¹. Environmental stressors are known to induce oxidative stress by over producing free radicals in fish and damaging cellular macromolecules-proteins, DNA etc. (Dwivedi et al., 2017; Pandey et al., 2011). A strong correlation between oxidative stress and impairment in cellular macromolecules like DNA, RNA, proteins etc. has also been reported by Chittezhath and Kuttan (2006). DNA damage or fragmentation involves double strand breaks (DSBs) leading to induction of MN (Xu et al., 2011). Further, induction of MN in a cell reflects structural or numerical chromosomal aberrations appearing during mitosis (Hedde et al., 1991; Fenech et al., 2011). Aziz et al. (2012) also suggested genomic instability due to oxidative stress mediated DNA damage. A correlation between DNA damage and increased MN induction is amply reported in fishes (Matsumoto et al., 2006; Ratn et al., 2017; Yadav and Trivedi, 2009b). Thus, this can be inferred that MN assay can efficiently serve as an endpoint marker for assessment of genomic instability in fish and other aquatic organisms exposed to toxicants.

Plant metabolites are being increasingly used to safeguard aquatic biodiversity against contamination by noxious xenobiotics. Some plant products have enough potential to counteract genotoxic manifestations in animals after their exposure to genotoxicants. Moreover, they are eco-friendly and of medicinal importance and applied only for cure of mammalian models against physiological, environmental and ecotoxicological manifestations. Only a handful of information is available depicting application of plant based products for amelioration of genotoxicity/ genomic instability against a few remarkable toxicants in fishes. The plants amply used for the purpose in reference include, *Azadirachta indica* (neem) against pentachlorophenol and 2, 4-dichlorophenoxyacetic acid (2,4-D) (Farah et al., 2006); *Lawsonia inermis* ('henna') against copper sulphate (Kumar and Trivedi, 2015); *Acacia catechu* ('cutch') against arsenic tri-oxide (Dwivedi et al., 2015); *Rauvolfia serpentina* ('sarpagandha') against carbofuran (Tiwari et al., 2016); *Curcuma longa* (Turmeric powder) against chromium tri-oxide (Prasad et al., 2017); *Melissa officinale* (lemon balm) against arsenite (Dwivedi et al., 2017); *Melissa officinalis* (lemon balm) against Furadan 3G (Tiwari et al., 2017); *Withania somnifera* ('ashwagandha') against chromium tri-oxide (Prasad and Trivedi, 2018); *Spirulina platensis* against lead nitrate (Hamed et al., 2019); *Curcuma longa* (curcumin) against

chromium tri-oxide (Awasthi et al., 2019). *W. somnifera* has gained therapeutic prominence and is widely used in Unani and Ayurvedic systems of medicine due to its ability to reduce reactive oxygen species, attune mitochondrial function, monitor apoptosis, reduce inflammation and boost endothelial function (Singh et al., 2011; Uddin et al., 2012). The ameliorative potential of *W. somnifera* against a variety of environmental stressors is well documented in non-piscean species (Alam et al., 2011). Withanolides and withaferin obtained from *W. somnifera* are promising therapeutic agents and are considered to possess anti-inflammatory, anti-arthritis and anti-oxidative properties by reducing lipid peroxidation in rats (Bhattacharya et al., 2001; Misra et al., 2008; Khan et al., 2015; Kushwaha, 2019). Panjamurthy et al. (2009) demonstrated the protective effect of Withaferin-A on 7, 12-dimethyl benz[a]anthracene (DMBA)-induced micronucleus frequency in the bone marrow of golden Syrian hamsters by decreasing the frequency of micronucleus eventually to evade DNA damage. Several researches have been carried out to study the efficacy of plant-based metabolites to uncover their ameliorative potential against aquatic toxicants in fishes (Dwivedi et al., 2017; Awasthi et al., 2019).

The protective effects of *W. somnifera* against genotoxicity are probably due its strong reactive oxygen species scavenging ability that may have reduced the frequency of MN induction in fishes exposed to increasing concentrations of *W. somnifera* along with hexavalent chromium. This establishes its genoprotective role in *C. punctatus*. Priyandoko et al. (2011) opined that the cells treated with *Withania somnifera* could be protected against toxicity by multiple mechanisms, including subsequent damage at DNA and mitochondrial level, and induction of cellular defense machinery. In another study Mansour and Hafez (2012) also illustrated its protective role against radiation toxicity. Additionally, Sharma et al. (2011) documented the ameliorative effect of *Withania somnifera* on regulatory of lead nitrate induced nephrotoxicity in Swiss albino mice. Further, protective roles of *Withania somnifera* are mediated via its antioxidant activity (Bharavi et al., 2010; Rajasankar et al., 2009 and Udayakumar et al., 2010). The ameliorative effect of *W. somnifera* against hexavalent chromium was also observed in this study. Cr (VI) treatment significantly enhanced Micronuclei in blood cells however, there was a decline in micronuclei induction after treatment with root extract of *W. somnifera* in a close dependent

manner. Therefore, it can be inferred that this decline in the frequency of MN induction with increasing concentrations of *W. somnifera* along with chromium trioxide (96 hr-LC₅₀/10, 7.89 mg l⁻¹) can be ascribed to genoprotective potential of this herb.

In the present study, a duration dependent increment in MN induction in erythrocytes of fish exposed to sub-lethal concentration of Cr (VI) in group III was observed. The frequency of micronuclei induction subsequently increased significantly ($p < 0.05$) with the highest fold change of 5.34 after 96 hr of exposure. Our findings are, thus, in concurrence with that of Awasthi et al. (2018) who also documented a significant ($p < 0.05$) increase in frequencies of MN induction in *C. punctatus* exposed to Cr (VI) in a concentration and exposure dependent manner. However, findings of groups IV, V and VI wherein fishes were subjected to sub-lethal concentration (96 hr-LC₅₀/10; 7.89 mg l⁻¹) of Cr (VI) along with 1, 2 and 3 mg l⁻¹ of *W. somnifera* root extract, respectively, indicate a significant ($p < 0.05$) ameliorative potential of *W. somnifera* in *C. punctatus* (Fig. 1). For all the three aforementioned groups, genomic instability registered a significant ($p < 0.05$) declining trend as evident by a gradual reduction in the frequency of micronuclei induction with corresponding fold changes in a decreasing order: 1.96 > 1.54 > 1.38 in comparison to group III (2.12) with respect to control, after 24 hr of exposure. Similar declining trend in induction of MN frequency in terms of fold changes were also obtained for 48, 72 and 96 hr of exposure. These are 4.00 > 3.64 > 3.32 after 48 hr of exposure period, in comparison to group III (4.40) with respect to control; 4.72 > 4.40 > 3.92 after 72 hr of exposure with respect to Group-III (5.16) when compared to the control and 5.00 > 4.48 > 3.97 after 96 hr of exposure, in comparison to group III (5.34), with respect to control, respectively in groups IV, V, and VI (Table 1). Laboratory microcosm based studies involving preliminary biological screening of plants and their metabolites with promising therapeutic potential finds ample scope in substantially counteracting several environmental toxicants. Besides, their role as geno-protective and ameliorative agents against anomalies induced by waterborne toxicants, after proper standardization, they can be successfully applied as dietary inclusions, based on nutrigenomic approach, for aquaculture activities in water bodies contaminated with metals like hexavalent chromium.

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