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MODULATORY ACTIVITY OF RHIZOMES OF *WITHANIA SOMNIFERA* AGAINST ENDOSULFAN INDUCED HEPATIC DEGENERATION IN FRESH WATER AIR BREATHING FISH *CLARIAS BATRACHUS*

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ABSTRACT

Effect of aqueous extract of root of *Withania somnifera* (WSR) on the biochemical alteration in few profiles of Liver Function Test (LFT) and histopathological anomalies in hepatic tissue of endosulfan induced fish was investigated. Serum Alanine Amino Transferase (ALT), Asperate Amino Transferase(AST),Alkaline Phosphatase(ALP) and Acid Phosphatase(ACP) showed a significant rise in test group (4 ppb & 8 ppb endosulfan treated). Dilation of nuclear membrane, congregation of heterochromatin on the inner nuclear membrane, broken array of cisternae of endoplamic reticulum were prominent histopathological alterations incurred in liver tissues due to endosulfan exposure at transmission electron microscope (TEM) level. The animals were subjected to aqueous WSR extract @ 50 mg/kg body weight) for 14 days by gavage method. A significant decrease in ALT, AST, ALP and ACP level in curative group (WSR treatment in endosulfan exposed group) was observed. Besides these groups showed nearly 70% restoration in the cytoarchitecture of hepatic cells. Biochemical and histopathological findings highlight the modulatory effect of aqueous WSR extract against endosulfan induced hepatic injury in fish.

KEY WORDS: Endosulfan, *Withania somnifera*, *Clarias batrachus*, ALT, AST, ALP, ACP.

INTRODUCTION

Inland water including rivers and lakes receive massive flux loaded with industrial and anthropogenic wastes that can exert a high impact on aquatic life. Physiochemical changes in

aquatic environment drastically influence fish physiology. Endosulfan is persistent organic pesticide widely used in agriculture & horticulture fields of more than 70 countries[1]. Fish are able to accumulate several fold higher concentration of pesticide residue than the surrounding water[2]. Endosulfan is toxic to liver cells [3-5]. Liver and kidney is the primary organ of degradation, detoxification and elimination. These are the most affected organ by the toxic assault. The plant based formulations have been used since ancient times as remedial measures against various human and animal ailments. Plant extracts and phyto-constituents have been found effective as radical scavengers and inhibitors of lipid peroxidations[6-7]. Besides, the chemical constituents present in the herbal medicines or plants are a part of physiological functions of living flora. Although phytoremediation have been used as natural antioxidant as human beings since time immemorial but its wide spectrum use in the aquatic organism is still in its infancy stage. A few plant extracts have been reported to have mitigating impact against various intrinsic diseases in aquatic organism[8-10]. In Ayurveda and Unani system of medicine, rhizomes of *Withania somnifera* have been used in treating various liver disorders[11.]

The species *Withania somnifera* (Dunal) (Family – Solanaceae) is commonly known as Ashwagandha (Hindi) or Indian Ginseng (English). The roots are stout, fleshy and whitish brown. Biochemically heterogenous rhizome alkaloids contain cuscohygrine, anahygrine, tropine, pseudotropine, anaferine, isopellatierine, nicotine and withasomnine, solasodine, visamine, dulcitol, glucosides, sitoindocides etc [12-18]. The withanoloids are known to be antiinflammatory[19], powerful antioxidant[20], hypocholestemic[21], hepatoprotective[22], antitumourous[23-24], antimalarial[25-26] as well as immunoprotective[27].

Most of the studies are focussed on the antioxidant properties of *Withania somnifera* on human beings and mammalian experimental model but the true modulatory impact of aqueous rhizome extract of the plant on aquatic animals have not been explored. The present study has been designed to investigate the probable mitigating impact of *Withania somnifera* root extract against endosulfan induced hepatotoxicity in freshwater air breathing fish *Clarias batrachus*, based on histopathological examination of liver tissues and biochemical assessment of few LFT (Liver Function Tests) profiles like Alanine amino transferase (ALT), Asperate amino transferase (AST), Alkaline phosphatase (ALP), and Acid phosphatase (ACP).

MATERIALS AND METHODS

Experimental Animal

Fresh water air breathing fish *Clarias batrachus* commonly known as "Mangur" of 80±10 g weight and 20±2 cm length were collected during prespawning season (March – May). The fish were brought to the laboratory, disinfected with 0.01% $KMNO_4$ solution and kept in four large cylindrical metallic aquaria (150 cm diameter X 180 cm height) having a constant flow of dechlorinated tap water. Fish were acclimated for 15 days in the laboratory condition. A water temperature of 20℃ and a constant photoperiod of 12 hrs light and 12 hrs dark were maintained. After acclimation, the fish were transferred randomly from acclimation tank to plexi-glass aquaria of 80 Litre capacities @ 20 fish each having dechlorinated aerated tap water and the experiment was conducted in accordance with established method[28]. The aerated water was changed daily during morning hours.

Experimental Design

The acclimated fish were categorized into following 6 groups consisting of 6 fish each

Group I (Control group): Normal

Group II (WSR extract treated group): The aqueous root extract was given @ 50mg/kg b.w. for 14 days.

Group III (Endosulfan test group): 4 ppb and 8 ppb of endosulfan treated for 14 days.

Group IV (Prophylactic group): 4 ppb and 8 ppb of endosulfan for 14 days with simultaneous treatment of aqueous root extract of *W. somnifera* @ 50 mg/kg body weight for 14 days.

Group V (Curative group): 4 ppb and 8 ppb of endosulfan were given for 14 days followed by administration of aqueous root extract of *W. somnifera* @ 50mg/kg body weight for 14 days.

Group VI (Self healing group): 4 ppb and 8 ppb endosulfan treated group left without further treatment for next 14 days.

Chemicals

Endocel (EC 35%) manufactured by Excel Industries Ltd, Gujarat was purchased from local market. Fresh root of *W. somnifera* were procured from Sanjay Gandhi Botanical Garden, Patna. The plant root was identified and authenticated by Late Prof. M. O. Siddiqui, Head, Dept. Of Botany, Patna University, Patna and a voucher specimen was preserved in our laboratory. The 96 hrs LC_{50} of endosulfan was calculated by standard APHA[29] method and

confirmed by pilot test as 20 ppb. The fish were exposed to non-lethal dose of 4 ppb and 8 ppb respectively. Gluteraldehyde and OSO_4 were obtained from Sigma Chemicals, USA and all kits and chemicals used for estimation of ALT, AST, ALP and ACP were of reagent grade and purchased from local Merck India distributor.

Preparation of Plant Extract

Lyophilized aqueous rhizome extract of *W. somnifera* was prepared[30]. The rhizomes were weighted, washed, thoroughly grinded to a paste in motors and then homogenized in Potter Elvehjen homogenizer. It is further dried in an incubator at 40℃ for 2 days, crushed in an electrical grinder and dissolved in hot distilled water. The suspension was filtered under suction and the filtrate was freeze dried using Labcono Freeze drier model 75018, yielding brown residue. The NOEL (No observed effective level) and MPD (Maximum permissible dose) of the aqueous extract of *W. somnifera* root (WSR) were determined and the diluted WSR aqueous extract were administered by gastric intubation method daily @ 50 mg/kg b.w. for 14 days to different experimental groups.

Serum enzymes

On the termination of exposure day blood samples were collected in a heparinised syringe from cardiac puncture. The serum was separated by centrifuging at 5000 rpm for 10 minutes at 4℃. The serum was assessed for ALT, AST, ALP and ACP using photo-colorimeter model 312E, by Standard methods[31].

Histopathology

After each schedule exposure, the fish were anesthetized with MS_{222} and liver tissues were dissected out, rinsed in NaCl (0.65%) to remove any adhering unwanted tissues. The tissues were cut into small pieces (1 – 2 mm thick) with sharp surgical blades and were fixed in 2.5% Gluteraldehyde in 0.1 M Phosphate buffer at 4℃ (pH 7.4) for 24 hours. Post fixation was done in 1% OSO_4 in 0.1 M phosphate buffer. They were dehydrated through graded series of alcohol, cleared in toluene and embedded in araldite mixture. The ultrathin sections of 60 – 90 nm were cut on Leica Ultra cut microtome of Richert Jung Supernova, stained in uranyl acetate and lead citrate and section in copper grid were viewed under “Morgaginy” TEM at SAIF-EM Unit, Dept of Anatomy AIIMS, New Delhi. The micro photographs taken were evaluated for any pathological changes in hepatic tissues.

Statistical analysis

The data obtained in each group were analyzed using students'‘t’ test. Values of $p<0.05$, $p< 0.01$ and $p<0.001$ were considered to be significant. All the statistical analysis were done using sigma plot 8.0 version.

RESULTS

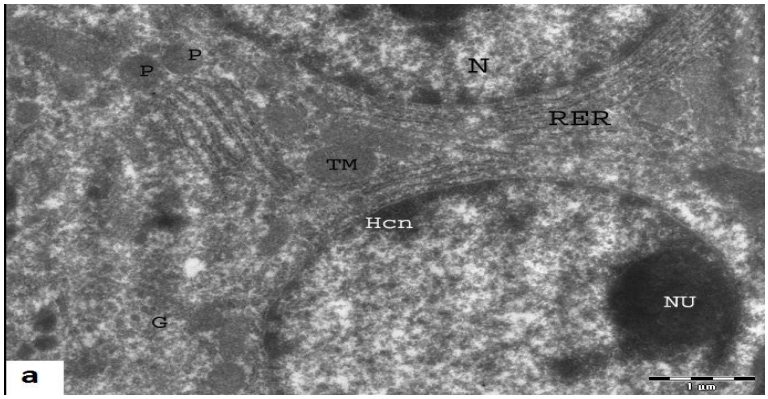
Biochemical

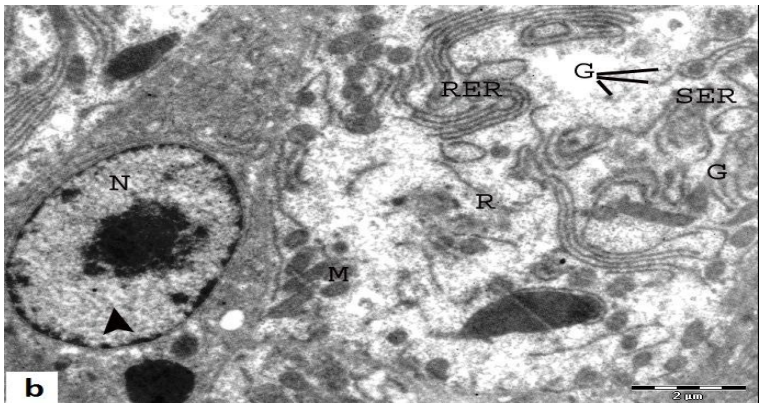
The extent of liver damage was assessed by estimating serum level of ALT, AST, ALP and ACP (Table – 1, 2). The test group (group III) showed a significant ($p<0.01$) increase in ALT, AST, ALP and ACP levels in both 4 ppb and 8 ppb endosulfan exposure. However, the percentage increase was higher in 8 ppb exposure. The prophylactic group (Group IV) showed increasing trend in all the enzymes when compared to control group (Group I) but showed a characteristic significant decline in comparison to test group. The curative group maintained for 14 days showed an improvement in the liver functioning as reflected by significant ($p<0.01$) decreased enzyme level. Only WSR extract treated group (Group II) showed a non-significant decline in serum level of ALT and AST, while a non significant increase in serum level of ALP and ACP, when compared to control. Self healing group (Group VI) showed a non-significant decline in enzyme level in contrast to test group.

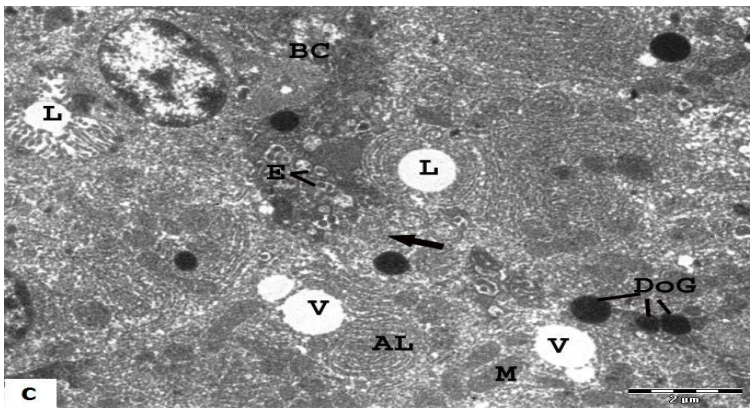
Histopathology

In the control group (Group I), the transmission electron micrograph of liver showed normal histological structure as marked by the presence of normal nucleus of hepatocytes with outer and inner nuclear membrane; later being covered by a rim of heterochromatin(Fig.2a), abundance of tubular mitochondria, peroxisomes and rough endoplasmic reticulum (Fig. 1a). Hyperactive condition of hepatocytes associated with the abundance of vacuoles, dense osmiophilic granules, lysosomes and annulated lamellae, inflamed tubular cisternae of ER with marked infiltration of lipid droplets were seen in test group (Group III) (Fig 1.c & Fig. 2.b). The liver sections of WSR treated group (Group II) showed less alteration in normal cytoarchitecture of hepatocytes with abundant glycogen granules (Fig 1.b).Lesser degree of inflammation in tubular cisternae of RER was seen in the prophylactic group (Group IV) with the appearance of fewer macrophages, glycogen rosette and normal nuclear content marking stress response in fish (Fig. 1.d and Fig. 2.c).Least abnormalities was seen in curative group (Group V) as marked by least swelling in tubular cisternae of RER and restoration of normalcy in nucleus, golgi, mitochondria and rough endoplasmic reticulum (Fig. 1.e; Fig

2.d).Self healing group (Group VI) showed lesser degree of restoration in cytoarchitecture of hepatic cells in comparison to curative group (Fig. 1.f; Fig. 2.e).







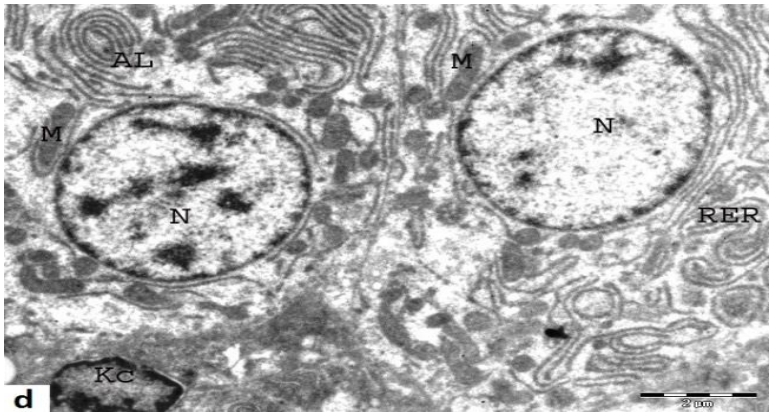


Table – 1: Assessment of liver damage in various groups at 4 ppb endosulfan exposure.

Parameters/ unit	Group – I	Group – II	Group – III	Group – IV	Group – V	Group – VI
ALT (U/ml)	30±3.033	32±2.53 [a] (+6.66)	56±5.656 [a] (+86.66)	46±2.82 [a] (-17.85)	40±3.577 (-28.57)	54±3.346 [a] (-3.57)
AST (U/ml)	16±1.673	15±3.03 (-6.25)	34±4.195 [a] (+112.15)	25±4.69 [b] (-26.47)	20±3.098 [a d] (-41.17)	33±4.242 (-2.94)
ALP (KA units)	4.44±0.60	4.92±0.866 [a d] (+10.81)	16.5±2.20 [b] (+271.62)	10.2±1.286 [b d] (-38-18)	8.92±1.21 [b d] (-45.93)	17.22±0.646 (+4.36)
ACP (KA units)	0.60±0.12	0.70±0.147 [a] (+16.66)	3.90±0.18 [c] (+5.50)	2.8±0.463 [b] (-28.20)	2.1±0.2[b](-46.15)	4.1±0.766 (+5.12)

Values are expressed in Mean±SD of six replicates in each group. Figures in parentheses are % increase (+) or decrease (-) over control and test group. P: [a] ≤0.05; [b] ≤0.01; [c] ≤0.001 (Vs Control group), [d] ≤0.05 (Vs test groups).

Table – 2: Assessment of liver damage in various groups at 8 ppb endosulfan exposure.

Parameters/ unit	Group – I	Group – II	Group – III	Group – IV	Group – V	Group – VI
ALT (U/ml)	30±3.033	32±2.53 [a] (+6.66)	72±4.733[a] (+140.0)	52±5.215[b] (-27.77)	44±6.693 (-38.88)	67±5.477 (-6.94)
AST (U/ml)	16±1.673	15±3.03 (-6.25)	42±4.0 [b] (+162.5)	33±4.242[b] (-21.42)	27±4.195 (-35.71)	38±2.828 (-9.52)
ALP (KA units)	4.44±0.60	4.92±0.866 [a d] (+10.81)	12.5±0.479 [b] (+181.53)	8.24±0.901[bd] (-34.08)	6.75±0.983[d] (-46.00)	11.5±1.226 (-8.00)
ACP (KA units)	0.60±0.12	0.70±0.147 [a] (+16.66)	4.8±0.52 [c] (+700.0)	2.6±0.36[d] (-45.83)	2.2±0.233[d] (-54.16)	4.1±0.766 (-14.58)

Values are expressed in Mean±SD of six replicates in each group. Figures in parentheses are % increase (+) or decrease (-) over control and test group. P: [a] ≤0.05; [b] ≤0.01; [c] ≤0.001 (Vs Control group), [d] ≤0.05 (Vs test groups).

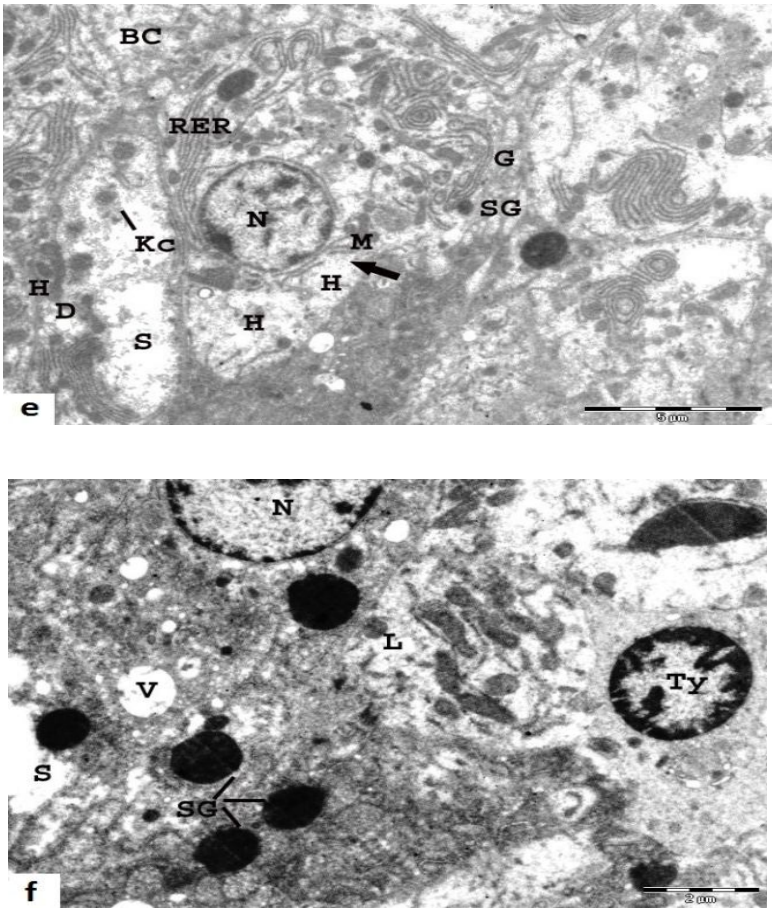
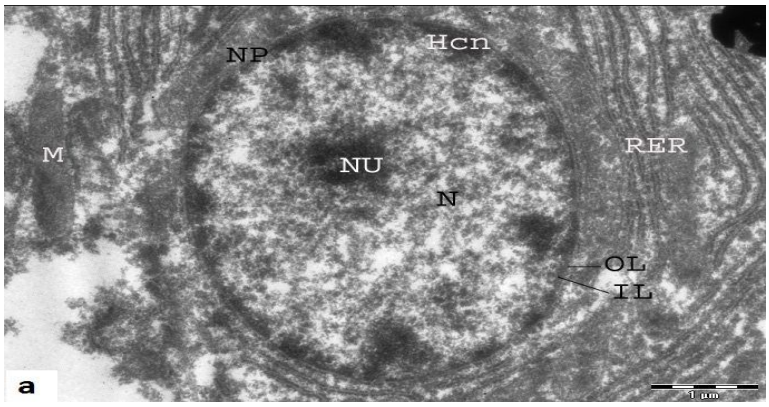


Figure –1:Transmission Electron Microphotographs of the liver. (a)Normal fish showing normal nucleus of hepatocytes with bilayered nuclear membrane, abundance of tubular mitochondria, peroxisomes and RER. (b) *W. somnifera* treated fish liver showing big nucleolus with amorphous fibrillar portion, mitochondria encircled by RER and abundance of glycogen granules. (c)marked hyperactive condition of hepatic cell associated with abundance of dense osmiophilic granules and annulated lamellae representing abnormal RER are seen in endosulfan (4ppb) treated group. (d) Increased stress response with the appearance of macrophags and abundant RER are seen in the fish treated simultaneously with endosulfan and *W. somnifera*. (e) hepatocytes of fish treated with endosulfan (4 ppb) for 14 days followed by *W. somnifera* root extract for next 14 days show restoration in cytoarchitecture of golgi, SER and RER. (f) hepatocytes of fish treated with endosulfan (4 ppb) for 14 days and left for another 14 days without any treatment show abnormalities as nuclear vacuoles, less percentage of RER and few secretory granules (stained in Uranyl acetate and Lead citrate).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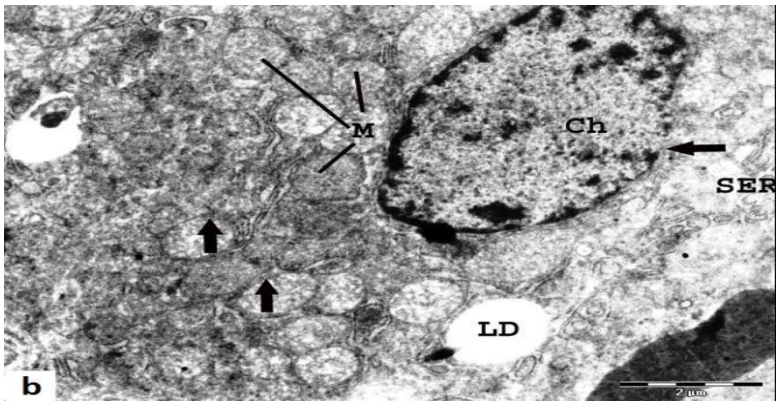
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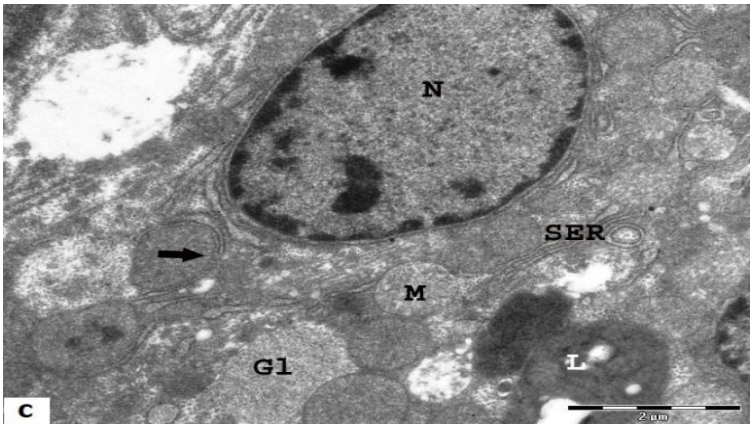
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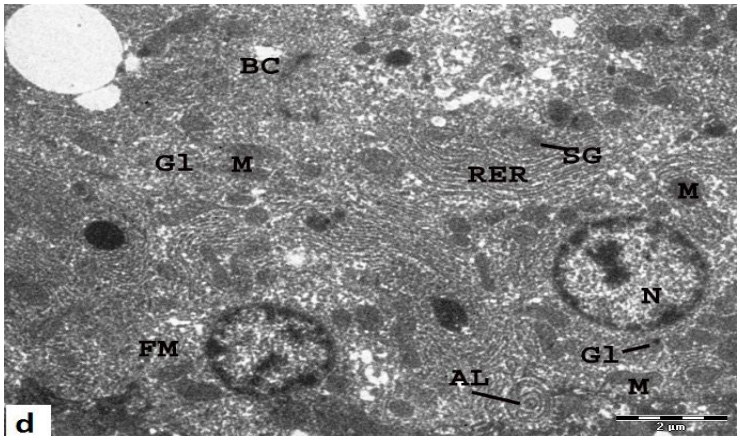
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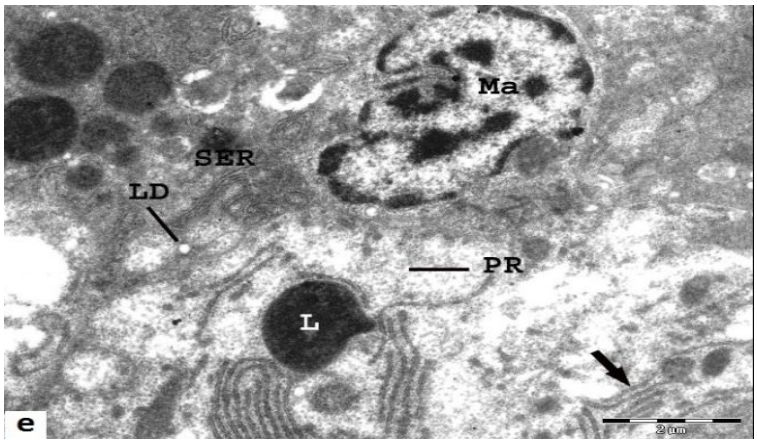

Figure – 2: Transmission Electron Microphotographs of the liver. (a) normal fish. (b) Hepatocytes of fish treated with higher dose of endosulfan(8 ppb for 14 days) showing. increased diameter of nuclear pores, shrinkage of nuclear membrane, swollen tubular cisternae as prominent anomalies. (c) Hepatocytes of the fish treated with *W. somnifera* and endosulfan simultaneously showing less dilation in nuclear pore and less swollen tubular cisternae. (d) Curative group showing significant recovery in the architecture of nucleus, mitochondria and RER. (e) Self healing group showing marked signs of abnormalities and stress response in hepatocytes. (Stained in Uranyl acetate and Lead citrate)

DISCUSSION

Hepatoprotective activity of *Withania somnifera* was explored by evaluating its effect on serum level of ALT, AST, ALP and ACP as well as histopathology of liver tissues of endosulfan treated fish. Hepatic system is the major organ system involved in the metabolism, detoxification and excretion of various endogenous and exogenously ingested substances like xenobiotics, pollutants etc. These substances alter the hepatic metabolism resulting in the generation of highly reactive free radicals which covalently bound with the membrane, alter their permeability and cause extensive tissue damage. The hepatic cells are consistently attacked by the free radicals and cell necrosis results. Although inbuilt antioxidant system protect the tissues from free radical attack but the excessive releases of ROS results in considerable organ damage. Administration of various antioxidants to strengthen the inbuilt protective mechanism may be useful in protecting the organ against various toxicants. The sensitivity of aquatic animals to endosulfan has been explored [32]. Toxicity is known to be primarily mediated by inhibition of important ion transport proteins in a variety of tissue [33] or by inducing oxidative stress [34-35]. Administration of endosulfan as both lower (4 ppb) and higher (8 ppb) non- lethal exposure enhanced the serum biochemical markers viz. ALT, AST, ALP and ACP. At 4 ppb endosulfan exposure serum ALT showed a significant ($p<0.05$) increase of 86.66% while prophylactic and curative group showed a

significant decline of 17.85% and 28.57% respectively. A non-significant recovery in serum ALT was recorded in self healing group (Table – 1).

Serum ALT followed the same trend at higher sublethal exposure of endosulfan, where it showed a significant ($p<0.01$) increase of 112.15% and 162.5% at 4 ppb and 8 ppb endosulfan exposure respectively. Serum ALP and ACP showed significant ($p<0.001$) increase when compared to control. Similar kind of elevation in ALT and AST of *Channa gachua*[36] *and Clarias batrachus*[37], *Tilapia mossambica*[38] and *Channa striatus*[39] due to different pesticide exposure has been reported. A similar increase in ALT and ALP levels was observed in CCl_4 test group [40]. Antioxidant effects of *Withania somnifera* in different mammalian group have been explored [17, 41]. *Withania somnifera* root extract (@50mg/kg b.w. orally) treatment for 14 days in curative group showed significant decline in serum ALT by 28.5% and 38.88%, in serum AST by 41% and 35.7%, in serum ALP by 46% and in serum ACP by 46% and 54% respectively, when compared to test group (4 ppb and 8 ppb endosulfan exposure).

Disruption of outer nuclear membrane, dilation of nuclear pore, fragmentation in RER, increased number of crista type mitochondria and swelling of organelles of hepatocytes were prominently marked in test group. Similar kind of hepatocellular anomalies after endosulfan & disulfoton treatment in the hepatocytes of male rainbow trout was reported. Abundance of annulated lamellae was marked in the hepatocytes in test group. They are usually considered as specialized form of SER. They are prevalent in cells with a high membrane turn over. SER proliferation is an indicator of induction of biotransformation process[42-43].

WSR extract ameliorates oxidative damages and protects against endosulfan induced hepatotoxicity in fish. Restoration of normal shape of hepatocytes might be associated with the synergistic action on suppression of over expression of PTP-S2 which accounts for leukemic cell proliferation and stimulation of MAP essential for normal cytoskeleton of cell[44]. ROS play a pivotal role in apoptosis by initiating mitochondrial damage and activating sensitive signalling pathway[45]. Endosulfan initiates mitochondrial damage by the generation of ROS. WSR extract helps in restoration of normal level of Bcl-2, suggesting its antioxidant, antiapoptotic role[41].

CONCLUSION

The histopathological & biochemical findings signify the restorative potential of WSR extract against endosulfan induced hepatotoxicity. The appropriate dose of WSR extract may be considered as an antidote to organochlorine toxicity.

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