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MODULATORY ROLE OF *WITHENIA SOMNIFERA* ROOT EXTRACT MIXED PELLETED FEED ON PESTICIDE INDUCED HEPATIC ANOMALIES' IN FRESH WATER CATFISH *CLARIAS BATRACHUS* (LINN.)

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ABSTRACT: The pharmacological activity of *Withenia somnifera* commonly called Ashwagandha, a herb having multi-medicinal properties, was investigated against carbofuran exposed hepatic anomalies in the air breathing fish *Clarias batrachus*. Fishes were exposed to 1 mg/L carbofuran for three weeks to induce the toxic effects. These fishes were then fed with *Withenia somnifera* root extract mixed pelleted feed @ 10% of the total feed ingredients for the next 6th and 8th week respectively. The histopathological examination of hepatic tissues was done on light microscopy and transmission electron microscopy. Major changes attributed to the hepatic cells after carbofuran treatment showed irregular shape of hepatic cells, increased sinusoidal spaces, rough endoplasmic reticulum, secretary granules, dilated mitochondrial cristae and presence of kupffer cells. Amoeboid nucleus with increased heterochromatin material, granular nucleoplasm and enlargement of nucleolus, were some of the prominent histopathological alteration in the liver tissue after carbofuran toxicity at transmission electron microscope (TEM) level. The histopathological examination of liver cell of carbofuran exposed fish followed by administration of *W.somnifera* root extract mixed pelleted feed showed marked restoration of hepatic cell towards normalcy, maintaining nearly uniform shape with decrease in vacuolization, sinusoidal spaces and increased granulations. Aggregation of polyribosome, endoplasmic reticulum, mitochondria and Golgi body were distinctly visible in the hepatoplasm. Nucleus with uniform nuclear membrane, nucleolus and increased euchromatin material showed reliable sign of retrieving towards the normalacy. It can be concluded that WSR extracts promote the modulatory activity against carbofuran induced hepatic anomalies in fish.

INTRODUCTION: Inappropriate and indiscriminate use of pesticides leads to degradation of aquatic environmental, which in turn harms aquatic biota including fish. The interaction of various chemical pollutants in the aquatic system may be synergistic, antagonistic or additive.

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Impact of carbofuran on morphological and behavioral responses at different time intervals in freshwater cat fish, *H.fossilis* (Bloch.) has been studied [1]. Carbaryl, a carbamate pesticide induces perturbations in the level of certain biochemical component, including activities of some enzymes in the blood and liver of the freshwater catfish *Clarias batrachus* [2]. Toxic effect of carbaryl on the gonads of *C. batrachus* has also reviewed.[3] Carbofuran altered the lipid and free fatty acid level in all the tissue viz. liver, kidney, muscles and ovary of *C. batrachus*.[4] The pesticides also creates atrophy in ovarian cells [5, 6, 7] hepatic cells [8, 9] alteration in phospholipid in female fish.[10] Though,

various literature have been accomplished regarding the harmful effect of pesticides on different organs of different animals but very few data are available on the systematic approach to mitigate pesticide impact by the use of herbal extracts viz. on mammalian systems [11, 12, 13], negligible on fish e.g. in ovarian cell [14, 15], in testicular cells [16], but yet unverified in hepatic cells of fish. Thus the present paper deals with the possible healing effects of the herbal extracts on hepatic cells of carbofuran exposed *C.batrachus.*

MATERIAL AND METHODS:

Materials:

Test animal selected -The live specimens of *Clarias batrachus* (60±10) gm. average weight were collected from the wetlands of rural areas of Patna. Test fish were disinfected for 1-2 minutes with 0.01% $KMnO_4$ solution and kept in plastic pool and large Plexiglas aquaria under standard laboratory condition. After 48 hours, fish were fed formulated pelleted feed @ 5% of their body weight. After two weeks of properly acclimatization, fishes were separated into three groups of 12 each. Group A - control (fed with normal feed), Group B- pesticide exposed group.
Group C - pesticide exposed fish followed by medicinal plant extract formulated feed.

Pesticide used:

Analytical grade carbofuran (2-3 dihydro 2,2 dimethyl, 7 benzo furanyl methyl carbamate) EC 3% manufactured by TATA Rallis India Ltd. Mumbai was selected. It is a widely used pesticide known to produce hypo cholinergic activity of central as well as peripheral organs by inhibiting the cholinesterase enzyme at synapse in the brain and neuromuscular junction at sub acute concentration[17].

The 96 hours LC_{50} value of carbofuran for *Clarias batrachus* was calculated by standard graphical interpolation method[18] as 2.5 mg/l. Then fishes of group –B were exposed to 1 mg/l Carbofuran for 3 week respectively, aquaria water and pesticide solution were changed every day and fishes were fed in the morning. Every week test fishes were sacrificed; liver tissues were taken out and fixed for ultra structural studies.

Medicinal Plant Used:

In the present investigation medicinal plant used is *Withenia somnifera* belongs to family Solanaceae. It is an erect herbaceous perennial under shrub and widely distributed in drier parts of India. The root are stout, fleshy and whitish in colour. The leaves are simple, ovate, glabrous. The flowers are inconspicuous, greenish or lurid-yellow and umbellate cyme. Phyto chemical investigations of roots and leaves have shown the presence of alkaloids, flavinoids and steroidal lactones, which are grouped under a general name" Withanolides" [19]. Characterized by ergostane type steroid of C28 basic skeleton having a side chain of C9 units of which six members lactones ring is a characteristic feature, Withaferin A, Withanolides -D, E have been identified as active Withanolides. Presence of various Amino acids e.g.-Aspartic acid, Glycine, Glutamic acid, Alanine, Proline, Cystine and Tyrosine along with Tryptophan and high content of iron has also reported. Pharmacological studies and medicinal evaluation in different sets of studies revealed vast range of applications of the herb in treatment of physiological disorders[20, 21, 22, 23, 24] and anti-oxidative role [25].

Preparation of *W. somnifera* mixed feed:

W. somnifera roots were collected from Botanical Garden of Patna and from Garden of Department of Zoology, Patna University Patna. Aqueous extract of *W. somnifera* roots was prepared by using the protocol of Prabhu *et al* [26]. The freshly collected roots of *W. somnifera* were weighed and grinded in the mortar pastel. The powder was then cold macerated in the 1.5 liter of water for 24 hours with continuous shaking using GMB shaker. The extract was then filtered and dried over water bath to obtain thick residue. Then it is further diluted. Maximum permissible dose and NOEL (No Observed Effective Level of plant extract to fishes was calculated. The thick paste was mixed in the feed ingredient @ 10% of total feed (wheat flour + egg + starch). After 3 weeks of carbofuran exposure, group - C fishes were administered formulated feed with *W. somnifera* root extract @ 10% of total feed for next 6^{th} & 8^{th} weeks. Sampling was done in 6^{th} and 8^{th} week respectively.

Methods: After each exposure of pesticide and *Withenia somnifera* (WSR) root extracts mixed

pelleted feed administration, fishes (group-A,B and C) were sacrificed, liver tissues were fixed for light & electron microscopy. For Electron Microscopy, small pieces of liver of controlled, pesticide treated and herbal feed fed, fish were fixed in 2.5% gluteraldehyde (chilled; 4ºC) phosphate buffered (pH- 7.2) gluteraldehyde . Tissues were processed as per the routine method. Ultra thin sections were stained in the Uranyl acetate and lead citrate and viewed under Philip's CM-10, Transmission Electron Microscope at SAIF-EM facility Unit, Department of Anatomy AIIMS, New Delhi.

Observations:

Liver cells of normal fish:

In the control *Clarias batrachus* (Group-A), the transmission electron micrograph of liver showed normal histological structure as marked by the indistinct hepatic lobular boundary membrane and normal hepatoplasm (Plate I, Fig.1, 2). Hepatic parenchyma with prominent cell organelles like endoplasmic reticulum (Plate I, Fig. 2, 3), mitochondria elongated or oval shape with distinct cristae (Plate I, Fig.1). Ribosome was observed at the outer periphery of rough endoplasmic reticulum (Plate I, Fig.2, 3), hepatoplasm largely occupied by glycogen granules (Plate I, Fig.4). Kuffer cells seems to be absent in the normal hepatic cells.

Nucleus with well-defined nuclear membrane, perinuclear spaces, homogeneous chromatin material and nucleolus (Plate I, Fig.1 &2)

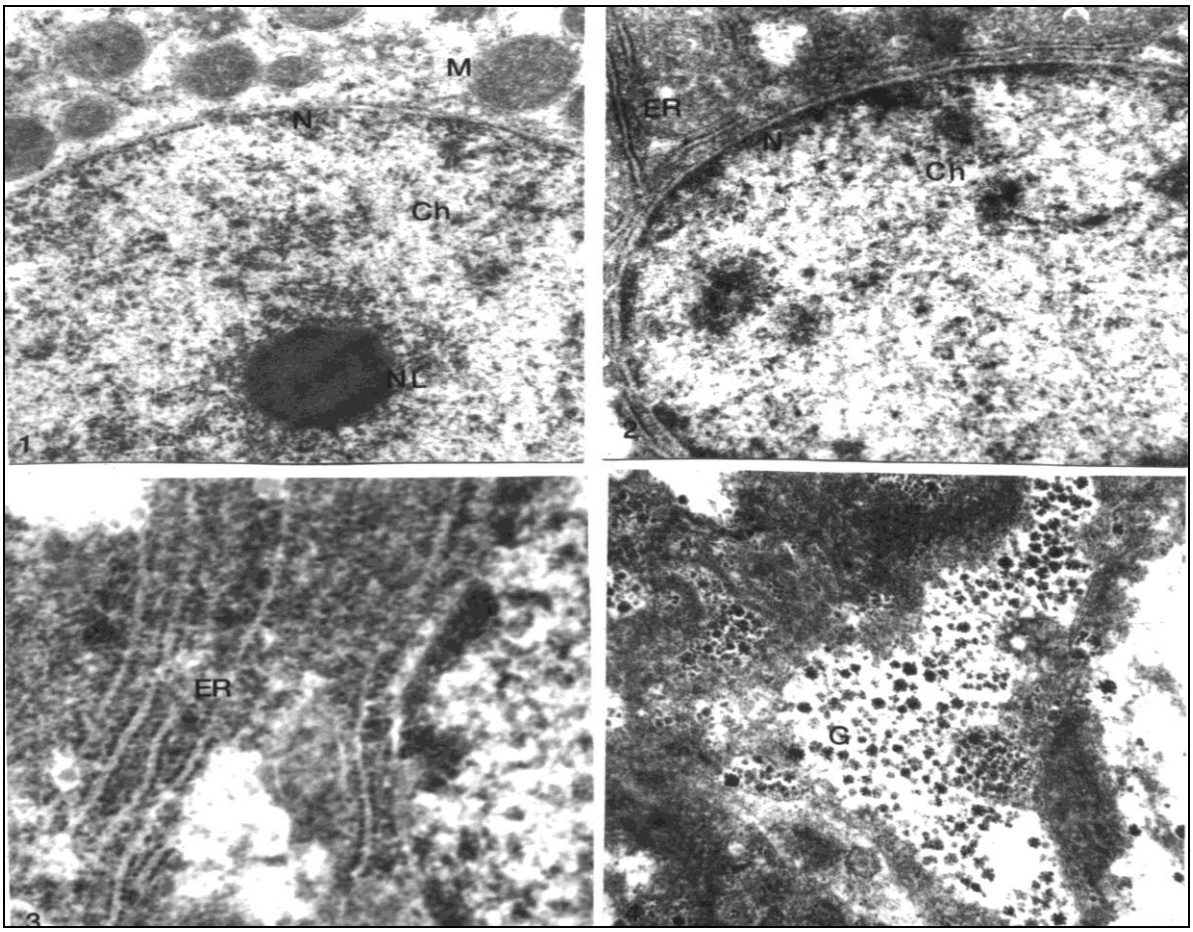

PLATE: I

TRANSMISSION ELECTRON MICROGRAPH OF SECTION OF NORMAL *C. BATRACHUS* LIVER

Fig 1 & 2: Showing the normal liver cell with clear hepatoplasm, containing mitochondria(M) and Endoplasmic reticulum (ER). Intact nucleus (N) with well defined nuclear membrane and perinuclear spaces. Chromatin material (Ch) and nucleolus (NL) are also prominent. X34000

Fig 3 & 4: Magnified portion at the side of nucleus showing parallel stacks of Endoplasmic reticulum (ER) studded with ribosomes (Fig 3) and glycogen rosettes (Fig 4). X46200

Liver cell of pesticide exposed fish:

The liver section of carbofuran exposed fish (group-B) showed enormous infiltration of cytoplasm with the appearance of large vacuoles (Plate II, Fig.1, 2, 3, 4). Hyperactive condition of hepatocyte with few mitochondria and dilated cristae were seen after three week exposure in test (group B) fishes (Plate-II, Fig.1). Rupture of lobular boundary membrane and cell autolysis Rupturing of nuclear membrane with dilation of nuclear pore, enlargement of nucleolus and highly condensed chromatin material (heterochromatin) were scattered throughout the nucleoplasm. (Plate II, Fig.1). Rough endoplasmic reticulum studded with abundance of ribosome (Plate II, Fig.2) associated with newly formed proteins at the side of the lumen of E.R were the significant features, showed inflamed condition due to toxicity (Plate II, Fig.2). Proliferation of glycogen rosette. (Plate II, Fig 3) and highly active functional Kupffer cells (Plate II, Fig.4,) indicated the pathological condition of hepatic cell.

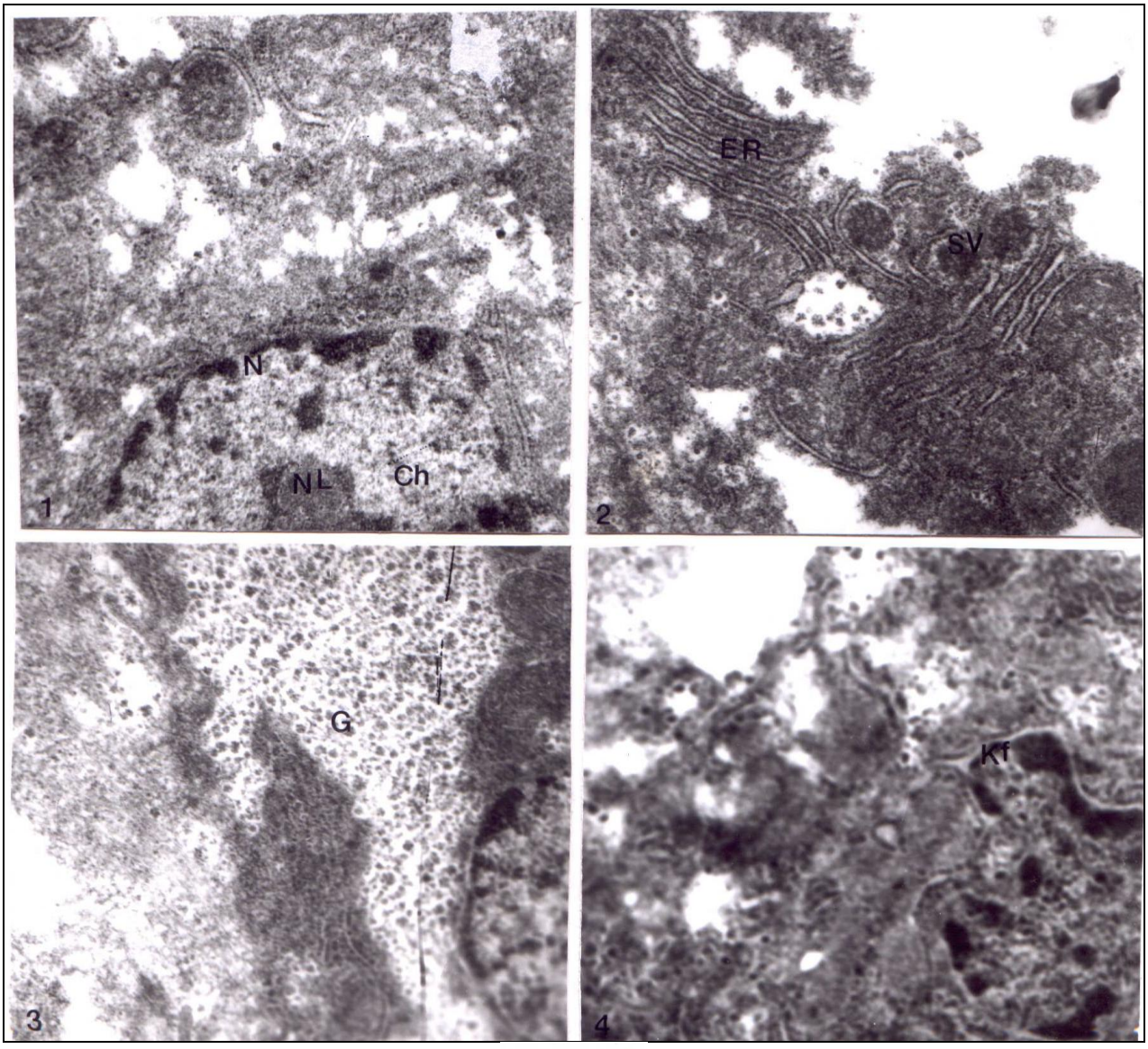

PLATE- II

TRANSMISSION ELECTRON MICROGRAPH OF SECTION OF 1 mg/L CARBAFURAN FOR THREE WEEK TREATED *C. BATRACHUS* LIVER.

Fig 1 & 2: Rupturing of hepatic lobular boundary membrane and cell autolysis. Decrease in hepatoplasm (Fig 1) and degeneration of outer nuclear membrane (Fig 1), increased heterochromatin (Fig 1) and increased stacks of RER and secretory vesicles (Fig 2).X13500

Fig 3: Magnified portion at the side of nucleus showing increased glycogen rosette and degenerated mitochondria. X46 00

Fig 4: showing sinusoidal region guarded by phagocyted Kupffer cell. X13500

Liver cells of pesticide exposed fish followed by *W. somnifera* root extract mixed feed:

After administration of *W somnifera* root (WSR) extracts mixed pelleted feed for six weeks showed marked restoration of cytoplasmic material (Plate- III, Fig.1, 2). Increased stacks of smooth endoplasmic reticulum, mitochondria (Plate- III, Fig.1, 2, 3). Golgi vesicle, round nucleus with double membrane, distinct nuclear pore, more euchromatin than heterochromatin and granular nucleolus were also clearly seen (Plate- III, Fig.3). Cellular vacuolization reduced to a great extent. (Plate- III, Fig, 1, 2, 3 & 4). After 8th week administration of *W. somnifera* root (WSR) extract mixed pellated treatment extrusion of granular materials through nuclear pore were prominent.

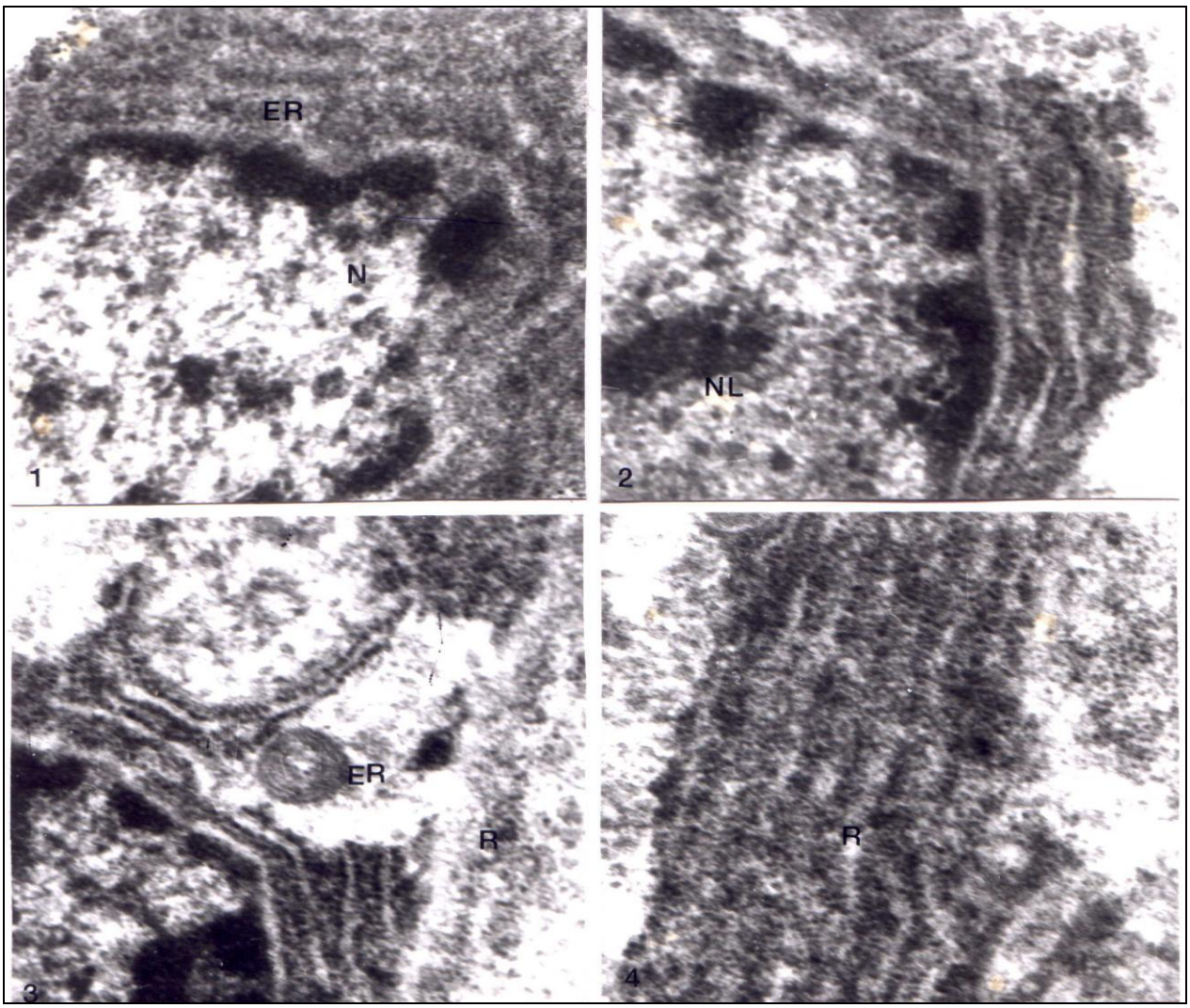

PLATE- III

TRANSMISSION ELECTRON MICROGRAPH OF SECTION OF LIVER CELL OF *C. BATRACHUS* TREATED WITH 1 mg/L CARBOFURAN FOR THREE WEEKS FOLLOWED BY *WITHENIA SOMNIFERA* ROOT EXTRACT MIXED PELLETED FEED FOR SIX WEEK.

Fig 1 & 2: Enlarged view at the side of nucleus showing hyperactivity of cell, abundance of newly formed ribosome, arranged at parallel stacks of Endoplasmic Reticulum (ER), decreased granules. Nucleus with granular nucleoplasm. (X 34000) and nucleus showing amyloid region (RER membrane encircling around foreign particle). X46200

Fig 4: Numerous ribosome arranged in parallel stacks of RER. X 15500

(Plate IV, Fig, 1, 2,). They were probably r-RNA's and m-RNA's granules. Rough Endoplasmic reticulum in association with round and elliptical mitochondria, and many secretary vesicles were prominent. (Plate IV, Fig, 1, 2). Nucleus present in its normal shape with intact nuclear membrane, granular nucleolus with normal distribution of chromatin material. (Plate IV. Fig. 1, 2, 3). Functional kuffer cell present showed phagocytosis (Plate IV. Fig. 4)

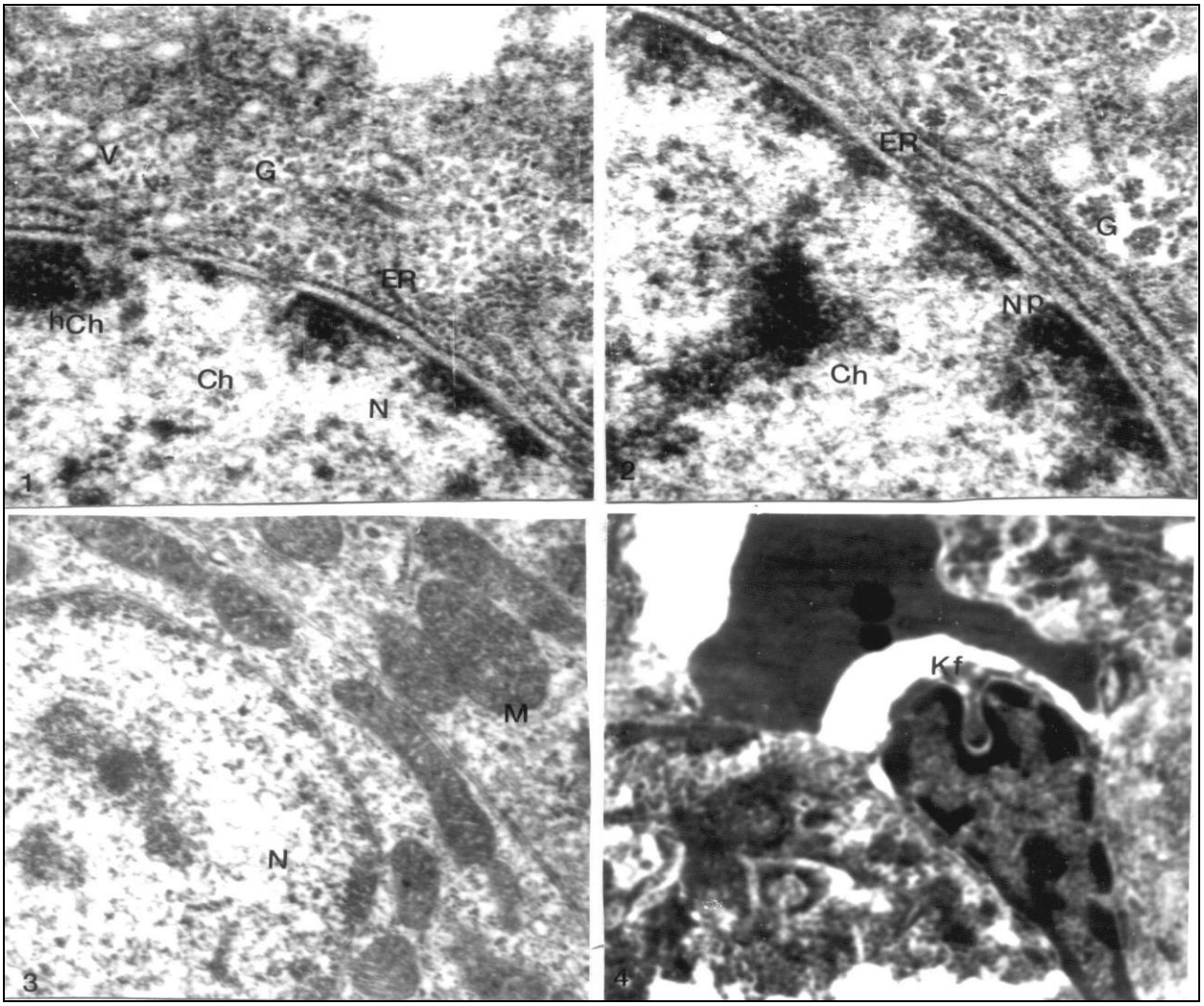

PLATE- IV

TRANSMISSION ELECTRON MICROGRAPH OF SECTION OF LIVER CELL OF *C. BATRACHUS* TREATED WITH1 mg/L CARBOFURAN FOR THREE WEEKS FOLLOWED BY *WITHENIA SOMNIFERA* ROOT EXTRACT MIXED PELLETED FEED FOR EIGHT WEEK.

Fig 1 & 2: Showing decrease in hyperactivity of cell involving normal endocytotic secretory pathway. Secretory vesicles normal glycogen rosettes. Nucleus with well defined double nuclear membrane, nuclear pore and perinuclear spaces, chromatin material normal with some heterochromatin at inner periphery of the nuclear membrane and some nucleolus associated chromatin material. X13500

Fig 3: Magnified portion at the side of the nucleus showing rounded and elliptical mitochondria. X46200

Fig 4: Magnified portion showing Kupffer cells involve in phagocytosis to reduce toxicity. X46200.

DISCUSSION: The present study demonstrates the modulatory activity of WSR extract mixed pelleted feed on pesticide induced liver anomalies on *C.batrachus* based on ultrastructural study. Liver is a bilobed gland and relatively larger organ, brown or pink in colour as per the physiological condition of the fish. Light microscope studies of the liver cells of the normal *Clarias batrachus* showed polygonal cells unlike other teleosts. But, no distinct division of the hepatic lobule was observed [27]. Hepatocytes bear more or less centrally placed nucleus containing a nucleolus with almost homogenous cytoplasm. Between two neighboring sinusoids, the hepatocytes were arranged as cords. Ramification of portal veins, hepatic artery and biliary duct were also observed in the liver of *Clarias batrachus*. The hepatic veins and central vein were found randomly throughout the liver. Kupffer cells were not observed in the liver of normal *Clarias batrachus*. At the transmission electron microscope level, the hepatocyte appears to have endoplasmic reticulum in close connection with mitochondria. Golgi apparatus were present throughout the cytoplasm aggregated into smooth surface vesicles and flattened sac-like structure. Cytoplasm was occupied by glycogen granules in clusters and may

be referred to as rosettes. Ribosomes were also observed at the outer periphery of rough endoplasmic reticulum [28].

Marked disruptions in the hepatic cells after the administration of carbofuran 1 mg/l for three weeks showed acellularity and immense infiltration of the cytoplasm with the appearance of large vacuoles. Sinusoids were irregularly arranged between the hepatocytes. Similar observations were also reported in the malathion exposed *Channa punctatus* [29]. Increased rough endoplasmic reticulum, mitochondria and ribosome showed after carbofuran exposure in the present investigation, probably due to increased energy requirement of the cell and also the pathological condition which reflects the process of uncoupling of oxidative phosphorylation [30]. Administration of Carbofuran led to formation of large lipid vacuoles, increased sinusoidal spaces and proliferation of glycogen granule into glycogen rosettes. This clearly showed the high rate of protein synthesis responsible for immuno -defensive system [31].

The liver cells of fish treated with Carbofuran three weeks followed by (WSR) extract mixed formulated pelleted feed, for six and eight weeks showed mark restoration of normal cytoplasmic structures. Decreased sinusoidal spaces, increased mitochondria with uniform cristae and parallel stacks of rough and smooth endoplasmic reticulum showed high cellular activity. A similar increase in mitochondria and endoplasmic reticulum has been reported [32].

In the present investigation typical interconnection between cisternae of RER indicated that it may facilitates the movement of membranous and luminal protein from their site of synthesis to site of that faces of central region of cells [33]. The antioxidant and hepatoprotective role of *Punilalagin* and *Puncatin* against acetaminophen induced damages in the liver cells of rats has also been explored [34]. The effect of *Sweritia* extracts reduces toxicity induced in rat hepatocyte by CCl_4 and paracetamol as *W. somnifera* reduces the toxicity of hepatic abnormalities [35].

Nucleus with granular nucleoplasm and distinct nucleolus in association with chromatin and peripheral heterochromatin were observed. Dense fibrillar portion and distinct granular portion of nucleolus clearly indicated the well synthesize and assembly of rRNAs [36].

Any alteration in hepatic cells reflects a variety of reactions of the whole organism to intoxication leading to a pathological condition. Such responses allow the fish liver to be considered as a good indicator of fish health status.[37, 38] Similar antioxidant and hepatoprotective role of *Acanthus ilicifolius* has also been reported [39].

CONCLUSION: Thus the present study demonstrates the non toxic extracts of WSR protects the liver cells from Carbofuran toxicity and may enhance the healing of self defense capacity of fish due to modulatory and antioxidative properties and significant recoupment in the histoarchitecture of hepatic cells. 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.

The preliminary studies of WSR reveals the presence of flavinoids in the root which may be support to the hepatoprotective activities and maintain the good metabolic activity. Such findings indicate WSR root extract probably channelizes the biochemical endocytotic pathway and other biochemical Para cellular pathway. Among the three pathways endocytotic, secretary and biochemical; WSR seems to modulate the secretary pathway and thereby retrieve altered cellular integrity.

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